Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

JANUARY 1970
VOLUME 59 NUMBER 1

REVIEW ARTICLE

Recent Trends in the Synthesis of Linear Peptides

A. KAPOOR

Keyphrases ☐ Peptides, linear—synthesis trends ☐ Amino protecting groups—peptide synthesis ☐ Carboxyl protection—peptide synthesis ☐ Group protection, amino acids—peptide synthesis ☐ Bond formation—peptides

Synthetic peptides with an ordered sequence of amino acids are of great interest as biologically active substances, as test materials for probing the details of biochemical processes, and as models with which to study the chemical and physical properties of proteins. The synthesis of peptides is also of great importance to confirm structures deduced for naturally occurring peptides and fragments obtained from protein hydrolysates.

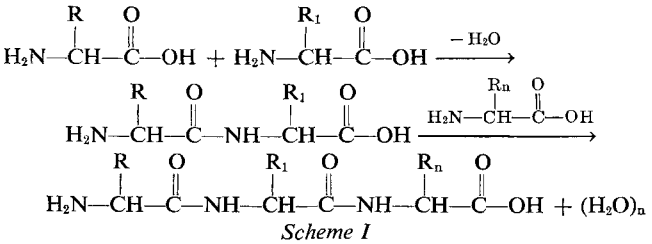
Insulin, which produces hypoglycemia, is perhaps one of the most frequently used peptide hormones in medicine. Other peptide hormones of therapeutic interest are adrenocorticotropic hormone (ACTH), oxytocin, vasopressin, melanocyte-stimulating hormones (α- and β-MSH), angiotensin, bradykinin, glucagon, *etc.* Another important class of biologically active peptides includes the antibiotics such as tyrocidins, gramicidin, bacitracin, valinomycin, polymyxin, and actinomycin-D which are produced by lower organisms such as bacteria and fungi.

Since the turn of this century, synthetic peptide chemistry has undergone periods of varying activity. After the excellent contributions of Fischer (1), who may well be considered as the father of the present day concept of step-by-step synthesis of peptides, a rather slow period of development followed. The next breakthrough came with the introduction of the carbobenzoxy protecting group in 1932 by Bergmann (2) who, with his collaborators, used this protecting group in combination with acid chloride and azide methods to synthesize a wide range of relatively small peptides which were used for the studies of the substrate specificity of enzymes. The next major triumph of synthetic peptide chemistry was the synthesis of the posterior pituitary hormone in 1953 by du Vigneaud *et al.* (3). This opened a new era. The rapid advances and continuous refinement in techniques of isolation, purification, sequence determination of the peptides and proteins, and availability of a rich choice of protecting and coupling methods led to the synthesis of a large number of pure peptides with intricate structures. The synthesis of peptide hormones such as angiotensin (4), bradykinin (5), eledoisin (6), α-MSH (7), β-MSH (8), gastrin (9), ACTH (10), insulin (11), secretin (12), glucagon (13), thyrocalcitonin (14), and many others is a remarkable achievement on the part of peptide chemists. In addition to commercial synthesis of corticotropin, angiotensin, and many other hormones, a number of hormone analogs with more potency than the natural hormones have been synthesized (15, 16).

The tremendous growth of interest in the synthesis of peptides is well reflected in ever increasing publications in this field. So rich is the literature concerning the synthesis of peptides that it is barely possible to include every development in a review article of this size. Fortunately, there have been a number of timely reviews and books in this field which have provided peptide researchers with necessary information. An excellent review article by Fruton (17) marked the begin-

ning of monographs in the field of peptides. This was followed by a number of reviews by Wieland (18–22) and many others (23–29). "Chemistry of Amino Acids" in three volumes by Greenstein and Winitz (30), published in 1961, "Peptides" in two volumes by Schroder and Lubke (31), in 1965, and "Peptide Synthesis" by Bodanszky and Ondetti (32), in 1966, provide a comprehensive literature survey of the progress in the synthesis of peptides. The scope of this review therefore will be limited to a brief survey of the existing methods frequently used in peptide synthesis. The contributions of modified and new methods will be discussed in some detail along with some of the problems associated with their use in the synthesis of peptides.

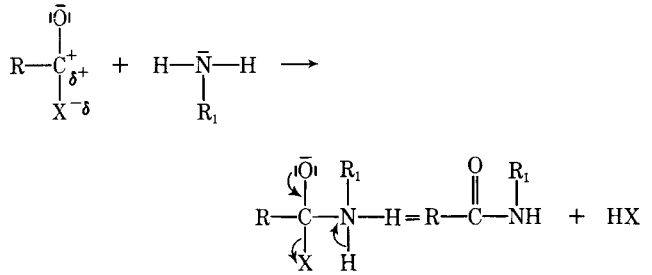
In essence, peptide and protein molecules consist of amino acids which are linked to each other by amide bonds to form what may be called "peptide chains." The amide bond formation involves the elimination of water; this can be brought about either by elevated temperatures, or by suitable activation of carboxyl or amino groups (Scheme I).



Scheme I

Elevated temperature peptide synthesis has not been the choice approach because of extensive racemization. However, the recent work of Fox, who has successfully used thermal condensation, will be briefly discussed at a later stage.

The synthesis of pure peptides requires the protection of all functional groups not expected to participate in the desired reaction. The carboxyl activation which is most frequently used in the formation of the peptide bond involves the introduction of a negative group, X, which will augment the electrophilic character of the carboxyl carbon atom. This facilitates the attack of a nucleophilic amino group to form the peptide bond (Scheme II).



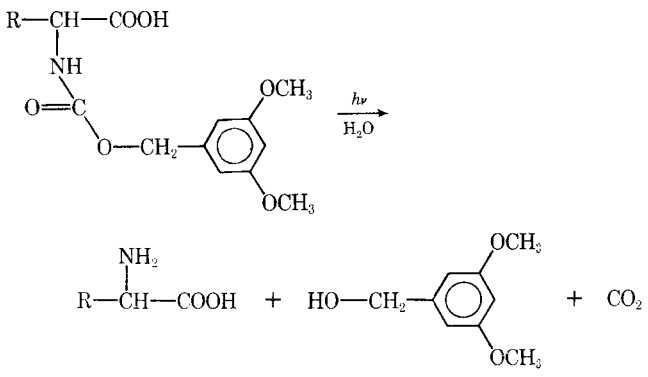
Scheme II

The peptide bond formation through the activation of the amino group has met with relatively less success; however, suitable selection of esters to block the carboxyl of amino acids may make them better reactive partners. For example, the positive inductive effect of tertiary butyl esters would make the free electron pair of amino nitrogen more reactive, when compared to methyl, ethyl, or benzyl esters. In general, a peptide synthesis can be accomplished with the proper selection of (*a*) amino or *N*-protecting groups; (*b*) carboxyl or *C*-protecting groups: and (*c*) carboxyl-activating groups. When the incorporation of polyfunctional amino acids is required in the peptide chain, the ω-groups, which may give rise to side reactions, must also be protected by suitable blocking groups. The protecting groups must be readily and selectively removed during or at the end of the synthesis.

AMINO-PROTECTING GROUPS

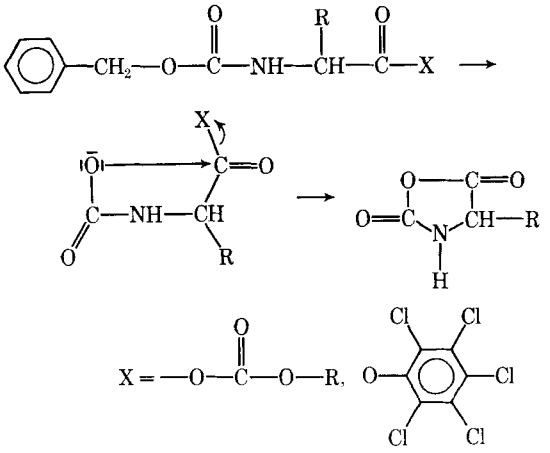
A detailed review on the most important amino protecting groups was recently published by Boissonnas (33).

Benzyloxycarbonyl and Related Groups—The carbobenzoxy or more appropriately the benzyloxycarbonyl (Z) group which was introduced in 1932 by Bergmann and Zervas (2), has been and still is responsible for the major work done in the peptide synthesis field. This versatile group can be conveniently introduced by the treatment of benzyloxycarbonyl chloride with the corresponding amino acid in aqueous alkaline solution. These derivatives are stable compounds and the protecting group can be easily removed by a variety of methods. The most frequently used conditions for the cleavage of benzyloxycarbonyl group are the hydrogenolysis (2) and the treatment of hydrobromic acid and acetic acid (34). The other deprotecting conditions which may be employed are reduction with sodium in liquid ammonia (35) and hydrogen chloride in ethanol (36). Barltrop and Schofield (37) have reported another approach for the removal of this protecting group by means of UV irradiation. Photochemical solvolysis is considerably enhanced by *m*-methoxyl substitution in the benzene ring. The use of 3,5-dimethoxybenzyloxycarbonyl as an amino-protecting group and the deprotection by irradiation of this group from several amino acids and a dipeptide derivative was reported by Chamberlin (38) (Scheme III).



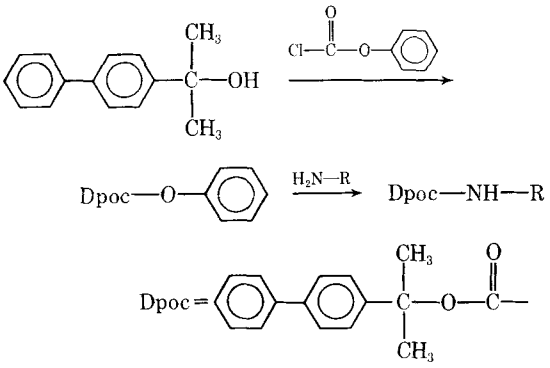
Scheme III

The removal of the benzyloxycarbonyl group by hydrobromic acid probably follows the protonation of the carbonyl oxygen as the first step followed by fission of the benzyl-oxygen bond. The carbamic acid intermediates so formed undergo decarboxylation with the liberation of the amino group (39). The benzyloxycarbonyl group is fairly stable to alkaline conditions required for the removal of methyl, ethyl, or the benzyl



Scheme IV

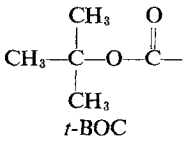
esters which are frequently used for carboxyl protection. However, the formation of urea or hydantoin derivatives may be encountered when the *N*-benzyloxycarbonyl peptides, particularly in the peptides where glycine is next to the amino acid which is protected by the benzyloxycarbonyl group, are treated with alkali (40). The formation of *N*-carboxy anhydrides (Leuch's anhydride) is another serious limitation with the use of benzyloxycarbonyl groups particularly when they are used in combination with acid chlorides (41). The formation of Leuch's anhydride *via* the carbamic acid derivatives was recently confirmed when the *C*-activated *N*-benzyloxycarbonyl amino acids were subjected to catalytic hydrogenation under anhydrous conditions (42) (Scheme IV).



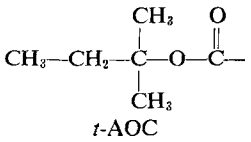
Scheme V

A number of substituted benzyloxycarbonyl and other urethan-type protecting groups have been used frequently for amino protection. The electron-releasing substituents in the benzyl group normally increase the rate of cleavage while the electron-attracting groups increase their stability towards acids. The substituted benzyloxycarbonyl groups which have been successfully used in the synthesis of peptides include *p*-bromo (43), *p*-chloro (44), *p*-nitro (45), and *p*-methoxy (46) derivatives. In a comparative study of different *p*-substituted benzyloxycarbonyl glycine ethyl esters, it was observed that the *p*-methoxy derivatives were cleaved five to ten times faster than the other derivatives on treatment with hydrogen bromide in acetic acid (47). β-Phenylazo and *p*-(*p'*-methoxyphenylazo)benzyloxycarbonyl derivatives (48) which afford the colored compounds have been used relatively less frequently. Sieber and Iselin (49) suggested the use of 2-(*p*-diphenyl)-isopropyloxycarbonyl (Dpoc) group for amino protection. This group has an interesting feature; it can be removed under mild acid condition. The Dpoc group can be easily introduced into amino acids by reacting either the mixed carbonate or the azide of Dpoc with esters or salts of amino acids (Scheme V).

Among the modified urethan derivatives, *tert*-butyloxycarbonyl (*t*-BOC) has come to be a considerably important amino-protecting group (46, 50). The removal of this protecting group is accomplished with considerable ease with 90% trifluoroacetic acid. While this group is reasonably sensitive to acidic conditions, it can still be used in combination with even more acid-sensitive groups such as trityl group. The advantage of this fact has been applied in planning the synthesis of longer peptide chains. *tert*-BOC amino acids can be prepared by using *tert*-butylazidoformate (51), *tert*-butyl-*p*-nitrophenyl carbonate (52), *tert*-butyl-pentachlorophenyl carbonate (53), or *tert*-butyl fluoroformate (54) as condensing agents.



t-BOC

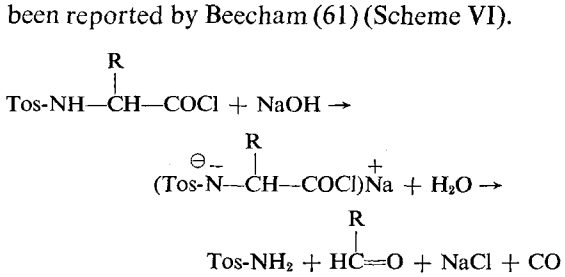


t-AOC

The use of *tert*-amyloxycarbonyl (*t*-AOC) group for amino protection has been suggested by Sakakibara (55). This protecting group has no particular advantage over the *t*-BOC group with the exception that *t*-AOC-protected derivatives are relatively less soluble in water and can be easily extracted with organic solvents from the aqueous reaction media. Deprotection of the *t*-AOC group can be achieved under the same conditions used for the removal of the *t*-BOC group.

p-Toluenesulfonyl (Tosyl) Group 

For a period of time this group enjoyed considerable popularity and even today affords a good protection for ω-amino functions, particularly in the case of lysine (56). The main limitations for the tosyl-protecting group is that α-tosyl amino acids generally cannot be coupled through the mixed anhydride method or through their active esters (57). Usually the acid chloride (58), azide (59), and tetraethyl pyrophosphite (60) methods have been used for the synthesis of peptides with α-tosyl protection. The ready decomposition of tosyl amino acid chlorides in alkaline solutions to toluenesulfonamide, probably because of the high reactivity of amide nitrogen, has been reported by Beecham (61) (Scheme VI).

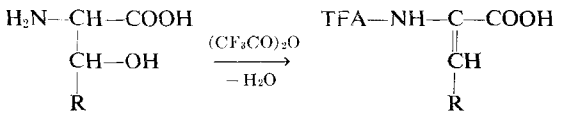


Scheme VI

The tosyl group is remarkably stable to the commonly

used methods for the cleavage of other protecting groups. The method of choice for the removal of the tosyl group is reduction with sodium in liquid ammonia, which was suggested by du Vigneaud (62). The mechanism of deprotection which was reinvestigated by Kovacs (63) appears to proceed by the formation of sulfite and toluene instead of thiocresol as previously reported. There are recent conflicting reports which indicate that during reduction, in addition to the removal of tosyl group, cleavage of the peptide chain between certain amino acids is also possible (64, 65). In the case of carboxyl-activated δ-tosyl ornithine derivatives, the easy six-membered lactam formation poses an additional problem in the use of tosyl group (66).

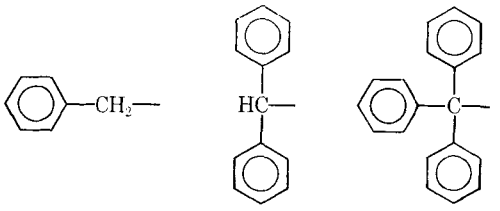
Trifluoroacetyl (TFA) Group (CF_3CO—)—This group which was introduced by Weygand (67) has a remarkable advantage in that it can be cleaved by very mild alkali hydrolysis; however, a number of problems are associated with the use of this group. Some of the serious factors which have contributed to the relative lack of interest in this protecting group are (*a*) possibilities of racemization both at the amino protection and at the peptide bond formation stage; (*b*) problems in the removal of this group from larger peptides; and (*c*) difficulties encountered in the introduction of TFA group, particularly in serine and threonine (Scheme VII).



Scheme VII

Because of high vapor pressures, TFA-protected amino acid and peptide esters can be easily sublimed in high vacuum (68). This offers new applications for the quantitative determination of amino acids in protein hydrolysates. The gas chromatographic separation of diastereomeric TFA dipeptide esters provides a useful tool in determining the degree of racemization during peptide synthesis (69).

Triphenylmethyl (Trityl) and Related Groups—

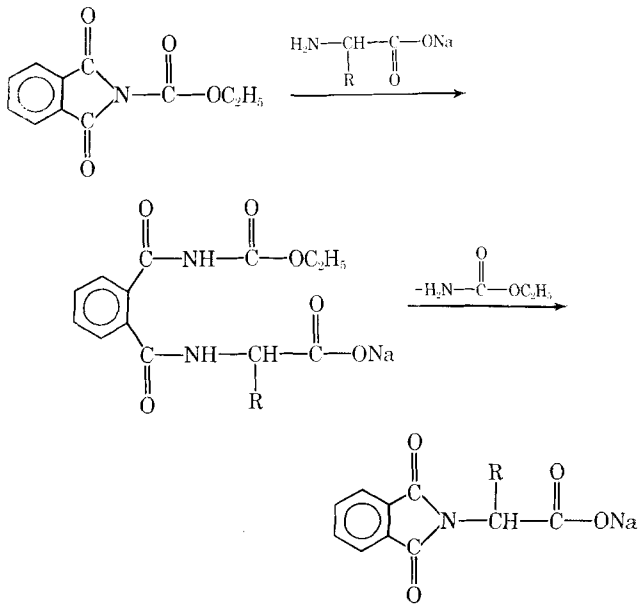


benzyl benzhydryl trityl

The trityl group is peculiar, in that while it is very easily removed from a peptide, it is quite difficult to introduce this group to the amino acids. Tritylation which is normally carried out with trityl chloride proceeds in rather low yields; however, the yields may be improved to a certain extent by using diethylamine instead of triethylamine as an acid acceptor in the reaction (70). The easiest tritylation is that of amino acid esters. However, the saponification of the ester which necessarily follows offers serious problems. The lack of hydrolysis may well be attributed to the bulky trityl group. The tritylglycine ethyl ester and the tritylalanine methyl ester are perhaps the only esters which can be saponified at room temperature under normal conditions (70). This may be explained by the lack of an elaborate side chain in these amino acids which would permit the nucleophilic attack by the hydroxyl group. The trityl group can be removed with acetic acid even in the presence of *tert*-butyloxycarbonyl group and this selective detritylation was used by Schwyzer for the synthesis of an ACTH sequence (71). The trityl group can also be removed by catalytic hydrogenation. Under controlled conditions of hydrogenation, benzyl esters may be removed in preference to trityl groups as in the case of trityl amino acid benzyl esters (72). This procedure may find use in the preparation of trityl amino acids.

Benzyl and dibenzyl protections for the amino group have met with little success because of their stability in acids and considerable resistance to catalytic hydrogenation. The benzhydryl group, which has been so far used only for the protection of sulfhydryl and carboxyl groups, holds good promise for amino protection as the properties of this group are between those of benzyl and trityl groups.

Phthalyl Group—This group which was introduced into peptide synthesis in the late forties (73, 74) until recently did not enjoy popularity because of difficulties encountered during phthalylation. However with the elegant conditions introduced by Nefkens (75), this protecting group is gaining considerable attention. Phthalylation, according to Nefken's procedure, is carried out using *N*-ethoxycarbonylphthalimide in dilute sodium carbonate solution. The mechanism suggested for the reaction assumes the opening of the five-membered ring and subsequent formation of the phthalylamino acid with the elimination of urethan (Scheme VIII).

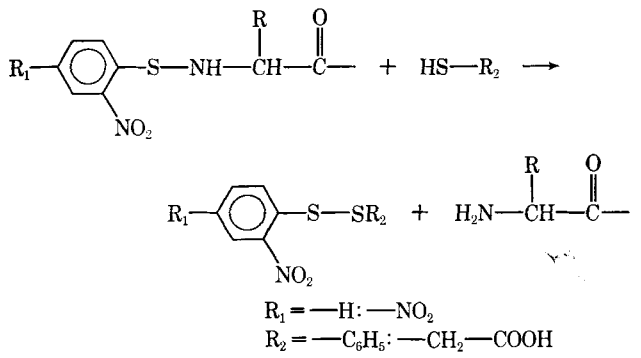


Scheme VIII

The phthalyl group has recently been used for the selective protection of the ω-amino group of lysine (72) and of ornithine (76). The deprotection of this group is achieved only by hydrazinolysis. Since the phthalyl group is not stable to alkali, the ester group used for

carboxyl protection in the synthesis of peptides with phthalyl protection must be hydrolyzed under acidic conditions. A combination of phthalyl and *tert*-butyl groups is quite suitable. A useful method for the introduction of phthalyl protection with ethoxyacetylene and phthalic acid was reported recently (77). *N*-protected derivatives were prepared from amino acids or their esters, both in anhydrous and in aqueous solution.

Arylsulfenyl and Related Groups—These groups were introduced by Zervas *et al.* (78) and provide an excellent method for amino protection. Tritylsulfenyl and arylsulfenyl amino acids, with electron-withdrawing substituents in the ring are rapidly cleaved by mild acid treatment. The *o*-nitrophenylsulfenyl (NPS) group, which has been the group of choice, is selectively removed when the *tert*-butyl ester is present. This group can be also removed by Raney nickel (79) and this provides a useful approach to avoid the partial cleavage of the other acid-labile groups present. However, the benzyloxycarbonyl group is removed under the same conditions. Raney-nickel cleavage of NPS group fails when applied to sulfur-containing peptides. Other useful deprotecting agents for the NPS group which were suggested by the Scoffone laboratories are thioglycolic acid and thiophenol (80). A disulfide is formed in addition to the free amino acid or peptide (Scheme IX).

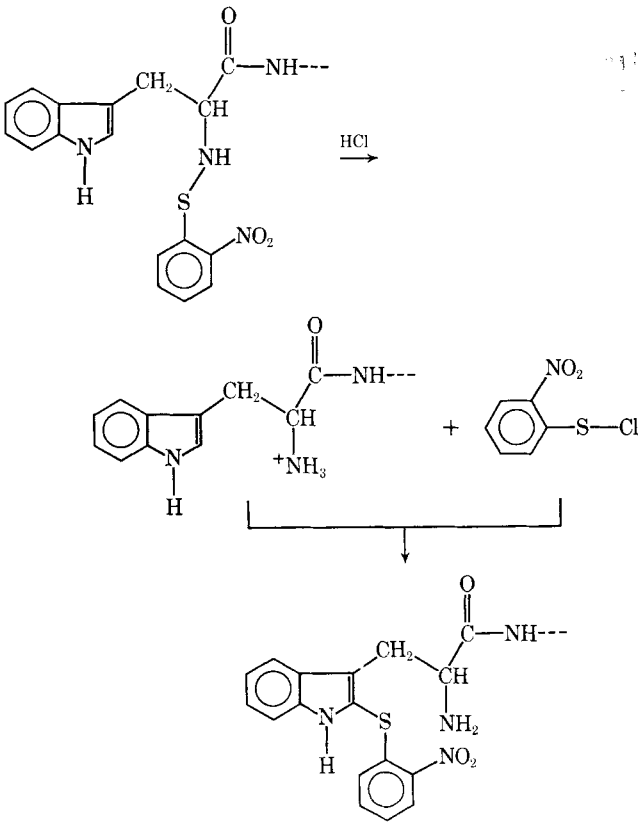


R_1 = —H: —NO_2
R_2 = —C_6H_5: —CH_2—COOH

Scheme IX

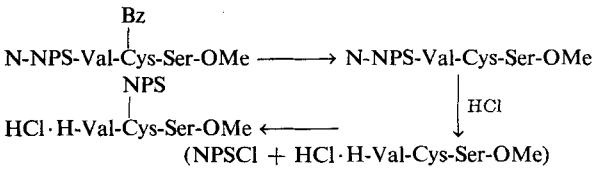
Various other reagents have been reported for the cleavage of the NPS group (81). The NPS derivatives of amino acids generally are not easily obtainable in a crystalline form, and their purification and characterization is often carried out by preparing the corresponding dicyclohexylammonium salts. Fontana *et al.* (82) suggested the use of the 2,4-dinitrophenylsulfenyl (DNPS) group which gave easily crystallizable compounds with a higher melting point when compared with the corresponding NPS derivatives. The DNPS group can be cleaved with the same conditions as those used for the NPS group. When the NPS or DNPS are cleaved with acid from tryptophan peptides, the sulfenyl halide formed during the removal step reacts quantitatively at the 2-position of the indole ring (80). This reaction mechanism has been discussed by Wunsch (83) (Scheme X).

The use of sulfenyl halides has been successfully extended as modifying reagents for polypeptides and proteins. Tryptophan was converted to 2-hydroxytryptophan through an intermediate 2-thio-aryl-tryptophan derivative (84). When the *N*-*o*-nitrophenylsulfenyl



Scheme X

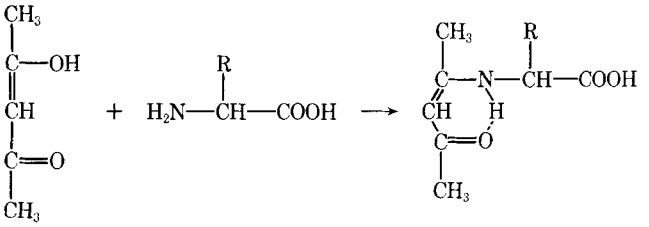
group is removed from cysteine peptides bearing free thiol groups by means of hydrogen chloride in methanol or in nonpolar solvents, an N → S transfer of the NPS group takes place to give the corresponding S-NPS derivative (85) (Scheme XI).



Scheme XI

Therefore, during the preparation of cysteine peptides using *N*-NPS derivatives of amino acids and *S*-protected cysteines, the removal of NPS-group must be done either in *S*-protected cysteine peptides or in the oxidized -*S*-*S*-peptides, *i.e.*, in the cystine peptides.

Tritylsulfenyl amino acids have a remarkable advantage over trityl amino acids in that they can be coupled conveniently by the mixed anhydride or *p*-nitrophenyl ester methods. Trityl amino acids do not



Scheme XII

undergo coupling with these methods because of steric hindrance.

Schiff Bases—Schiff bases are normally quite unstable and cannot be used as protecting groups. However, Schiff bases which can be stabilized by hydrogen bonds by the use of 1,3-dicarbonyl compounds (86) or by hydroxy derivatives (87) provide suitable protecting groups in the synthesis of peptides (Scheme XII).

CARBOXYL PROTECTION

The primary consideration for the substitution of the carboxyl group is the release of the amino acid from its zwitterion state rather than actual protection. The protecting groups most commonly used have been limited to methyl, ethyl, benzyl, *tert*-butyl, and *p*-methoxy-benzyl esters. Several new carboxyl groups have come to the limelight in recent years.

Methyl and Ethyl Esters—These esters are normally prepared by introducing dry hydrogen chloride into a suspension of the amino acid in absolute methanol or ethanol. The corresponding amino acid esters are obtained as hydrochlorides. Another convenient approach is the use of thionyl chloride (88) or acetyl chloride (89). Methyl or ethyl esters are removed during or after the synthesis of peptides by alkaline hydrolysis which generally produces a number of problems such as racemization, transpeptidation, and formation of urea or hydantoin derivatives. Alkali-labile *N*-protecting groups, such as phthaloyl, have to be excluded when *C*-protection is carried out by methyl or ethyl esters. Cleavage of sensitive amide bonds is another possibility when alkali is used for the removal of these esters. Removal of methyl or ethyl *C*-protection becomes more difficult as the number of amino acids increase in the peptide chains. Dilute hydrochloric acid in aqueous acetone (73) and lithium halides in pyridine (90) have been recommended as alternative methods for the removal of the methyl or ethyl *C*-protection.

Benzyl Esters—The main advantage of benzyl esters over methyl or ethyl esters is their easy removal by hydrogenolysis. These esters can be prepared in satisfactory yields by using other catalysts rather than hydrochloric acid. Polyphosphoric acid (91), *p*-toluenesulfonic acid (92), and benzene sulfonic acid (93) have been successfully used for benzylation. Water formed during the reaction is removed by azeotropic distillation. The catalytic hydrogenation of benzyl esters in ethanol may be accompanied by transesterification and formation of the ethyl ester. This can be prevented by the use of *tert*-butanol as solvent (94). Benzyl esters cannot be cleaved selectively by hydrogenation in the presence of a benzyloxycarbonyl group; however, they may be hydrogenated selectively in the presence of trityl groups. Benzyl esters are removable by hydrogen bromide and by sodium in liquid ammonia. Benzyloxycarbonyl groups in the presence of these esters may be selectively removed by controlled treatment with hydrogen bromide in glacial acetic acid (95) or in nitromethane (96). Catalytic hydrogenation of benzyl esters in large peptides is generally quite difficult.

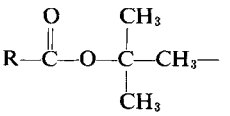
***p*-Nitrobenzyl Esters**—These esters which can be prepared by the modification of esterification methods (97) used for the preparation of benzyl esters have a definite advantage in that they are stable to hydrogen bromide–acetic acid reagent; therefore, these esters allow the selective removal of the benzyloxycarbonyl group. They are removed easily by hydrogenolysis. The derivatives of *p*-nitrobenzyl esters are generally higher melting compounds and can be easily crystallized. Another significant advantage is the α-esterification when the benzyloxycarbonyl-aspartic anhydride is treated with *p*-nitro benzyl alcohol under anhydrous conditions (98).

***p*-Methoxybenzyl Esters**—*p*-Methoxybenzyl esters which were first reported by Weygand and Hunger (99) have not gained great popularity mainly because of the difficulties encountered during their preparation. However, Stewart (100) has suggested a convenient method for the preparation of *p*-methoxybenzyl esters by imidazole-promoted condensation of the corresponding *p*-nitrophenyl esters with *p*-methoxybenzyl alcohol. Since these esters are more sensitive to acid reagents, their future use as carboxyl protecting agents seems to be imminent.

Recently, Weygand *et al.* (101) reported an extensive study on the kinetics of different methoxy-substituted benzyl esters and found 2,4-dimethoxy and 2,4,6-trimethoxy benzyl esters as suitable protecting groups for amides such as asparagine and glutamine. These esters are stable to catalytic hydrogenation and methanolic hydrochloric acid, but can be conveniently removed by trifluoroacetic acid.

Methyl-Substituted Benzyl Esters—The use of 2,4,6-trimethylbenzyl and pentamethylbenzyl groups for carboxy protection was recently reported by Stewart (102). The advantage of these esters over benzyl esters is their rapid cleavage by hydrogen bromide in acetic acid under conditions which result in complete removal of the benzyloxycarbonyl group.

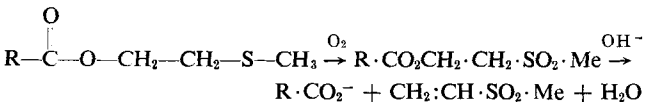
Tertiary Butyl Esters



The introduction of these esters has been a significant contribution in the synthesis of peptides (103). They are cleaved with extreme ease by acid reagents probably by the formation of the carbonium ion. The free *tert*-butyl esters of most amino acids are stable and distillable liquids, and normally do not undergo self-condensation to form diketopiperazine derivatives. They are very stable to hydrogenation and less sensitive to alkali treatment. These esters have been particularly useful when the incorporation of monoamino dicarboxylic acids is desired in the peptide chains. Among the methods for their removal, the treatment with trifluoroacetic acid is perhaps most suitable (104).

β-Methylthioethyl Esters—The use of β-methylthioethyl esters for the protection of carboxyl group is a novel approach which was suggested by Rydon (105). Their utility in the synthesis of peptides is based on the observations that the meththiodides and sulfones derived from β-methylthioethyl esters of amino acids and peptides are split to the parent carboxylic acids under extremely mild alkaline conditions. In the initial reports, their removal was suggested by first converting

these esters into the meththiodides by the action of methyliodide, and subsequent treatment of the meththiodides with aqueous alkali solution at room temperature. The free carboxylic acid is regenerated along with the formation of dimethylvinylsulfoniumiodide. The above method for deprotection failed with a series of *N*-*tert*-butoxycarbonylglutamic acid peptides in which the side chain carboxyl group was protected as benzyl ester because of contamination with considerable amounts of side products, which led to difficulties during purification. In a recent communication, Rydon (106) has proposed a new method for the removal of these esters. The new approach involves the oxidation of methylthioethyl esters with ammonium molybdate to the corresponding sulfones which, in turn, can be readily split at room temperature in mild alkaline solutions (Scheme XIII).

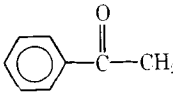


Scheme XIII

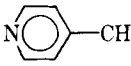
Amides and Substituted Hydrazides—The hydrazide group itself is not suitable for carboxyl protection because an amino group cannot be acylated selectively in its presence. The hydrazides used are therefore blocked with suitable amino protecting groups in order to prevent the side reactions (107, 108). These include benzyloxycarbonyl, *tert*-butyloxycarbonyl, and trityl hydrazides. The cleavage of hydrazide bond to form the free acid is not possible; therefore, this type of carboxyl protection is suitable only for the synthesis of intermediates which can be converted into reactive derivatives such as azides.

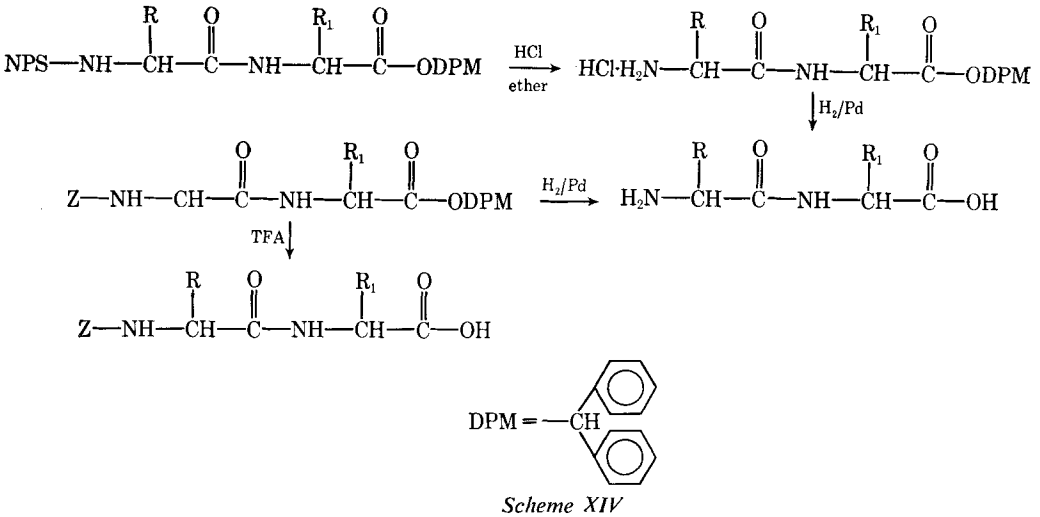
Phthalimidomethyl Esters—This carboxyl-protecting group introduced by Nefkens (109) has an attractive feature in that it is stable to catalytic hydrogenation, but can be removed under a number of conditions such as the following: hydrogen chloride in organic solvents, diethylamine in ethanol, dilute aqueous sodium hydroxide, and hydrazine.

Diphenylmethyl Esters (Benzhydryl)—Significant progress was made in the laboratories of Zervas (110), Fruton (111), and Hiskey (112) by the introduction of benzhydryl esters. This group can be conveniently removed by hydrogen chloride (or hydrogen bromide) in nonpolar solvents or by hydrogenolysis. With the introduction of NPS amino-protecting groups, the use of these esters is guaranteed a secure place for carboxyl protection in the synthesis of peptides. The NPS group can be selectively removed in the presence of the benzhydryl group (113) (Scheme XIV).

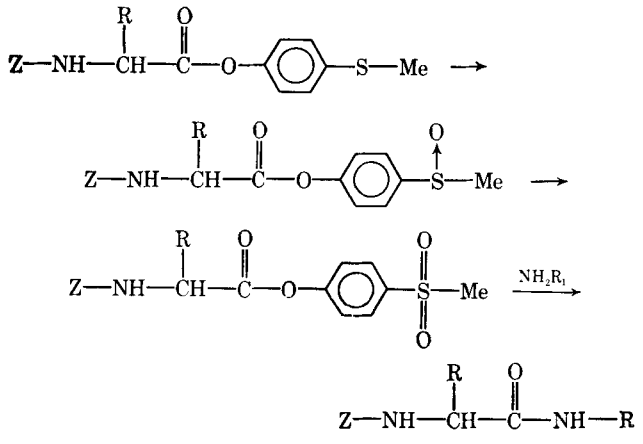
Phenacyl Esters (PAC)  —The phenacyl group differs from the tertiary butyl and diphenylmethyl ester groups in that it is stable to hydrogen chloride or hydrogen bromide even when high concentrations of these reagents are used. This group can be removed by catalytic hydrogenation. A combination of diphenylmethyl and phenacyl ester protection as outlined by Zervas (114) offers a convenient approach for the incorporation of monoamino dicarboxylic acid in the peptide chains.

4-(Methylthio)phenyl Esters (MTP)—This ester for carboxyl protection (115) may be considered as an extension of Rydon's approach (105). The interesting feature of this ester is that it can be directly converted by oxidation to the sulfoxide, 4-(methylsulfinyl)phenyl ester, and further oxidation of the sulfoxide leads to the formation of the sulfone, 4-(methylsulfonyl)phenyl active ester, which can be used for coupling in the synthesis of peptides (Scheme XV).

4-Picolyl Esters  —An ingenious method of blocking the carboxyl group with 4-picolyl esters was recently suggested from the laboratories of Young (116). These esters, in addition to being good protecting groups, also provide a basic "handle" when incorporated into the *C*-terminal residue and facilitate the isolation of the growing peptide after each coupling step. The 4-picolyl esters at the termination of the synthesis can be removed by catalytic hydrogenation or by electrolytic reduction. The usefulness of this procedure for the lengthening of the peptide chain from *C*-



Scheme XIV



Scheme XV

terminal residues of amino acid was demonstrated by the synthesis of Val[5]-Angiotensin II.

A related method using *p*-dimethylazobenzyl esters has also been reported (117).

Salt Formation—The protection of the carboxyl group by salt formation represents the least elaborate approach. In order to avoid protonation of the amino group in amino acids, a suitably strong base such as sodium hydroxide or organic bases such as triethylamine, tributylamine, or dicyclohexylamine may be used. The relative lack of success of this approach is due to low yields and the difficulties encountered during the purification of the reaction products. However, recently a number of active esters of *N*-protected amino acids have been used successfully for coupling with amino acids and peptides that were *C*-protected by suitable salt formation (118, 119).

PROTECTION OF ω-GROUPS IN THE AMINO ACIDS

Excellent surveys enumerating the masking of ω-groups during the synthesis of peptides have been reported (31, 32).

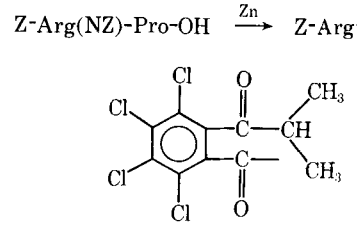
The selective formation of α-esters by the ring opening of the *N*-benzyloxycarbonyl glutamic anhydride by the corresponding alcohols was indeed a big contribution by Gibian and Klieger (120). The selective ring opening to form α-esters in the case of *N*-protected aspartic anhydride is relatively more difficult; however, the reaction of *p*-nitrobenzyl alcohol with *N*-benzyloxycarbonyl aspartic anhydride usually results in the formation of α-*p*-nitrobenzyl ester (121). β-Carboxyl can then be protected by a tertiary butyl ester. Another very useful approach for selective protection of carboxyl groups of glutamic acid by diphenylmethyl and phenacyl esters has been suggested by Zervas (114).

Serine and threonine can be incorporated in the peptide chain with their free hydroxy groups; however, in order to limit any possibility of side reactions it is advantageous to suitably mask the hydroxyl group.

A number of derivatives with suitable protection for the hydroxyl groups of serine and threonine have been reported (32). Among the most promising of the masking groups for the hydroxy function of serine and threonine is the 2,2,2-trifluoro-1-benzyloxycarbonylaminoethyl (Z-TF) group suggested by Weygand. This group can be cleaved by catalytic hydrogenation or with hydrogen bromide in acetic acid. A very useful combination of this hydroxyl-protecting group with *p*-methoxybenzyloxycarbonyl and tertiary butyloxycarbonyl as amino protection has been suggested (122).

The weakly basic imidazole ring in the side chain of histidine poses a number of problems and it is desirable to provide suitable protection for the imidazole ring in the synthesis of peptides incorporating histidine. Benzyl (123), benzyloxycarbonyl (124), and trityl (125) groups for imidazole protection have been successfully used in combination with a variety of amino-protecting groups for the synthesis of histidine peptides. Two very attractive protecting groups, 2,2,2-trifluoro-1-benzyloxycarbonylaminoethyl (Z-TF) and 2,2,2-trifluoro-1-*tert*-butyloxycarbonylaminoethyl (BOC-TF) for the imino group of histidine, appear to be of particular interest (126). The first-mentioned protecting group (Z-TF) can be removed by catalytic hydrogenation while the latter (BOC-TF), which is stable to hydrogenation, can be conveniently cleaved with trifluoroacetic acid. A combination of these groups with pentachlorophenyl active esters for lengthening the peptide chains and for the synthesis of sequential polypeptides incorporating histidine has shown promising results in the author's laboratories. The use of adamantyloxycarbonyl (AdOC) group was suggested for amino protection (127) and this group has been successfully employed by Wunsch (13) for histidine protection during the synthesis of glucagon.

Arginine, which contains a guanidine group of an unusually basic nature, can be incorporated if suitable protonation is available. In order to reduce the basic character of the guanidine group, the classical approach is to convert this group into the nitroguanidine derivatives. Zervas has used the tribenzyloxycarbonyl and tritrityl derivatives of arginine (128) in the synthesis of peptides. It is interesting to observe that one acyl group, the ω-benzyloxycarbonyl group, is removable by hydrogen bromide in acetic acid. However, all three benzyloxycarbonyl groups are labile to hydrogenolysis. The α-trityl group forms tritrityl arginine which can also be selectively removed, thereby offering a useful ditrityl arginine derivative for lengthening the peptide chain from *C*-terminal residue. The trityl group in the guanido moiety is exceptionally stable to hydrogenolysis and can be cleaved only by hydrogen bromide in acetic acid. The idea of using derivatives of ornithine throughout the synthesis and to convert ornithine to arginine by guanylation at a suitable stage was proposed by Fruton (129) and was successfully applied by Bodanszky (130) for the synthesis of biologically active peptides. The tosyl group has also been used for the protection of



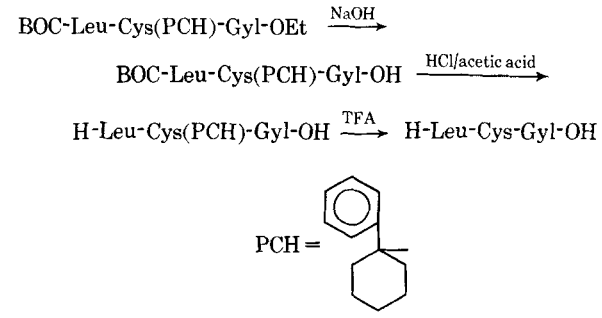
(TIP)

Scheme XVI

the guanido group in arginine. While the α-amino tosyl group can be conveniently removed by electrolytic reduction, the removal of tosyl protection from ω-tosyl arginine under the same conditions is relatively difficult, particularly in longer peptides. In addition to nitration, protonation, tosylation, and other methods, the use of the tetrachloroisopropyloxyphthaloyl (TIP) group and the *p*-nitrobenzyloxycarbonyl (NZ) group for the protection the of guanido group in arginine has been suggested (131, 132). An interesting method for the removal of the nitro or *p*-nitrobenzyloxycarbonyl guanido-protecting groups by acidic zinc reduction has been suggested by Pless and Guttmann (133) even in larger peptides. This method leaves the α-amino benzyloxycarbonyl group intact (Scheme XVI).

The sulfhydryl group in cysteine is highly reactive and must be afforded suitable protection. The *S*-benzyl group which is cleaved by sodium in liquid ammonia was suggested by du Vigneaud (134) and has remained the group of choice. However, in order to provide the sulfhydryl-protecting groups which can be removed under relatively milder conditions, an extensive search was undertaken in various laboratories. A number of *SH*-protecting groups such as acyl, trityl, benzhydryl, benzyloxycarbonyl, and *p*-methoxybenzyloxycarbonyl have been suggested. All these groups can be selectively removed under mild conditions without affecting sensitive parts of the molecule and especially the already existing -*S*-*S*-bridge. Another approach is the incorporation of *o*-tosyl serine (135) or β-chloroalanine (136) residues into a peptide chain and their subsequent conversion into *S*-protected cysteine residues by nucleophilic displacement reactions. The use of the acetamidomethyl group for the protection of the *SH* group of cysteine has been suggested recently (137). This protecting group is stable under conditions which cause the removal of commonly employed acid-labile substituents, but can be easily cleaved with two equivalents of Hg at pH 4.

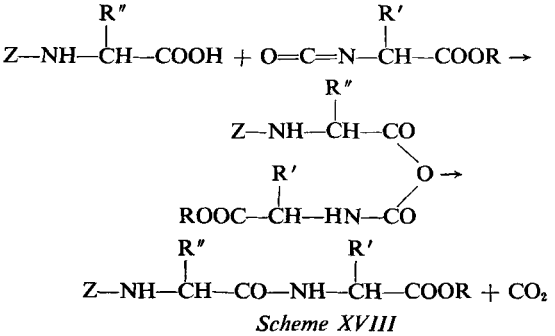
A series of *tert*-arylalkyl groups such as 1-phenylcyclohexyl (PCH), for *SH* protection of cysteine were reported by Konig (138). Because of the stability of these groups toward HCl/acetic acid, HBr/acetic acid and hydrazide, they allow the selective removal of *N*-benzyloxycarbonyl, *N*-*tert*-butyloxycarbonyl, and *N*-phthalyl groups. Trifluoroacetic acid is the reagent of choice for the cleavage of PCH and related protecting groups (Scheme XVII).



Scheme XVII

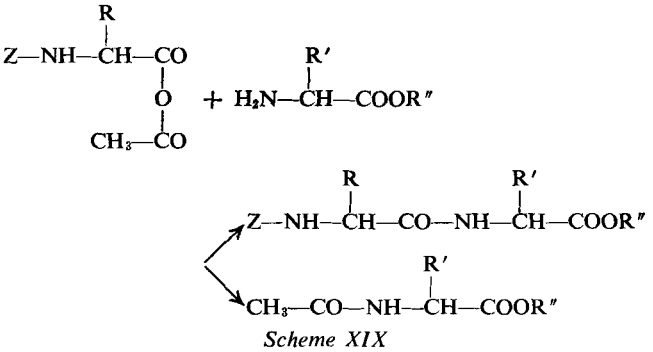
FORMATION OF THE PEPTIDE BOND

Since the turn of this century, a number of classical methods joining the amino acids through amide bonds have been developed for the formation of well defined peptides. In a broad sense, a peptide bond may be formed either by the activation of an amino group or by the activation of a carboxyl group. The amino group is capable of amide formation by way of its free electron pair. The nucleophilic character of the amino group would be expected to increase with the substitution of electron-releasing groups, thereby allowing easy formation of the amide bond. This approach has not been fully explored. However, a number of reactive derivatives of the amines have been reported for the formation of peptide bonds. The actual coupling may be visualized to proceed through the activated carboxyl component. For example, isocyanates, which are prepared from amino ester hydrochlorides and phosgene as reactive amino derivatives (139), form a peptide bond with *N*-protected amino acids or peptides through a mixed anhydride of a carbamic acid (Scheme XVIII).



Scheme XVIII

Other methods based on reactive amine derivatives which have been used in the synthesis of peptides include phosphazo (140), phosphorus acid esters (141), arsenious acid esters (142), and phosphorus pentoxide (143). While these methods have a definite place in peptide chemistry, the bulk of peptide synthesis involves the peptide bond formation through the activation of the carboxyl group.

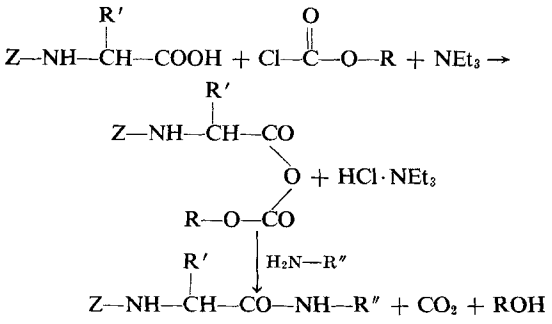


Scheme XIX

Peptide Synthesis by Activation of the Carboxyl Group—*Mixed Anhydride Methods*—The actual application of the mixed anhydride method in the synthesis of peptides was made in the laboratories of Wieland (144). One of the main considerations in the selection of anhydride-forming acid, or the "partner" acid as more appropriately termed by Bodanszky and Ondetti (32), is the presence of an electron-releasing structure.

This will limit the formation of *N*-acetyl amino acids or peptides during coupling (Scheme XIX).

The acids with low dissocation constants and long, branched aliphatic chains are more useful and lead to selective formation of peptide bonds. Introduction of carbonic acid–half ester–half chlorides, where the alkoxy group provides the electrons, is a tremendous improvement in mixed anhydride method. Ethylchloroformate (145) and isobutylchloroformate (146) are the reagents of choice for the formation of mixed anhydrides of *N*-protected amino acids and peptides (Scheme XX).



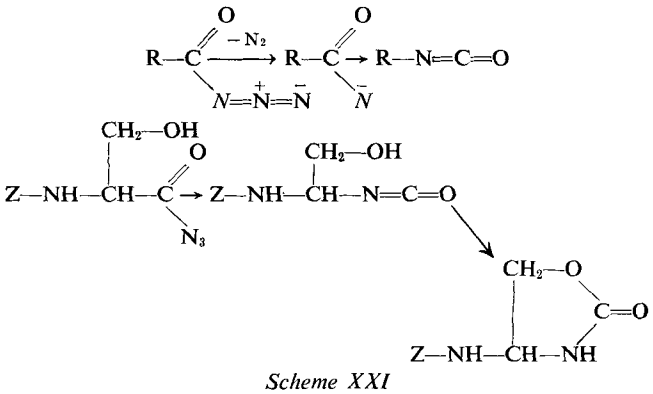
Scheme XX

Easy removal of the by-products greatly facilitates the isolation of the desired peptide from the mixed anhydride reaction mixture. The scope and limitations of this method have been discussed by Albertson (147). One of the most serious limitations is the danger of racemization when the *N*-protection for peptides used in mixed anhydride coupling is other than benzyloxycarbonyl or *tert*-butyloxycarbonyl. Anderson *et al.* (148) have reported an extensive reevaluation of the conditions used for mixed anhydride coupling. The tertiary base used for mixed anhydride formation is not merely a hydrogen chloride acceptor, but it first reacts with the chloroformate to form a quaternary compound which then reacts with the carboxyl group. The degree of racemization is dependent on the basicity of the amine used. The use of weaker bases such as *N*-methylmorpholine has been recommended. The best results are obtained with isobutylchloroformate, and the solvents of choice for mixed anhydride coupling are ethyl acetate and tetrahydrofuran.

N-Acyl amino acids, after activation with isobutylchloroformate, are used for the coupling reaction without the isolation of the active intermediate; however Anderson (148) has reported the isolation of *N*-phthalyl alanine mixed anhydride with isobutyl carbonic acid. This is perhaps the first time that a reactive derivative of a mixed anhydride has been isolated. In the author's experience, a combination of modified mixed anhydride and pentachlorophenyl active ester methods provides a useful approach for lengthening the peptide chains of *N*-protected, *C*-activated peptide units from *C*-terminal residues of amino acids (149). The synthesis of these peptide units without any significant racemization is of considerable importance for the preparation of sequential polypeptides. Another approach for the preparation of mixed anhydrides is by the use of diphenylphosphoric acid (150) which is particularly useful for the activation of hindered trityl amino acids. A large variety of "partner acids" such as derivatives of phosphoric, phosphorous, and sulfuric acids, and aliphatic or aromatic sulfonic acids which are employed for the formation of mixed anhydrides have been reviewed (32).

Azide Method—Despite a number of disadvantages, this method, which was introduced by Curtius (151), is very highly regarded in the synthesis of peptides, mainly because racemization during the coupling reaction is not observed. The azides are unstable compounds which are used without isolation and are prepared *via* the crystalline hydrazides of *N*-protected amino acid or peptide esters.

The formation of isocyanates due to the rearrangement of azides may lead to the formation of urea derivatives, and cyclic urethans in serine-containing peptides (152) (Scheme XXI).

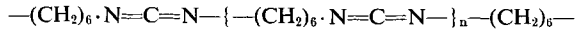


Scheme XXI

The preparation of hydrazides *via* hydrazinolysis is not possible with phthalyl or trifluoroacetyl *N*-protected amino acid or peptide esters. Hofmann (153) has suggested the use of protected hydrazides, and this approach has been used by Weygand (154) for the synthesis of very useful intermediates for lengthening the peptide chains both from the amino and carboxyl terminal residues of amino acids.

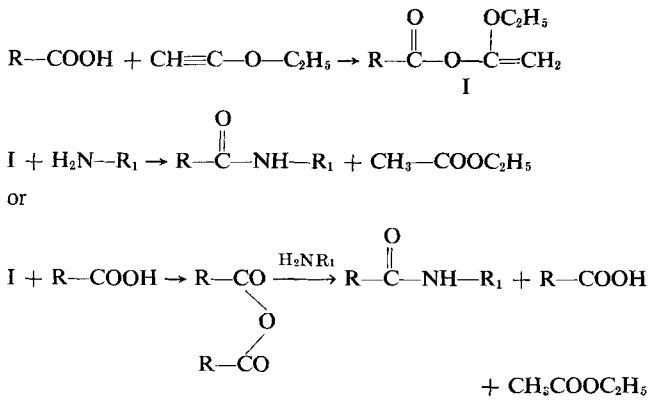
Carbodiimides—Among the most frequently used carbodiimides is the *N,N'*-dicyclohexylcarbodiimide which was introduced by Sheehan and Hess (155). This method is perhaps the most popular method of peptide synthesis and the mechanism of possible routes of acylation with the aid of carbodiimides has been discussed in detail by Khorana (156). When a dialkylcarbodiimide is allowed to react with a carboxyl group, an *o*-acylurea is first formed which in turn reacts readily with nucleophiles. One of the disadvantages of this method is the rearrangement of the reactive intermediate *o*-acylurea to a more stable *N*-acylurea which is only slightly susceptible to reactions with nucleophiles. Another disadvantage is the difficulty of the removal of *N,N'*-dicyclohexylurea which is formed as a by-product. To meet this difficulty, dialkylcarbodiimides such as *N*-ethyl, *N'*-(γ-dimethylaminopropyl) carbodiimide which yield water soluble ureas have been devised (157). The use of insoluble polycarbodiimides such as polyhexamethylene carbodiimide, which allows easy purification of reaction products, has been suggested by Frankel (158) (Scheme XXII).

The use of an interesting coupling reagent, ethoxyacetylene, was reported by Arens (159). The coupling



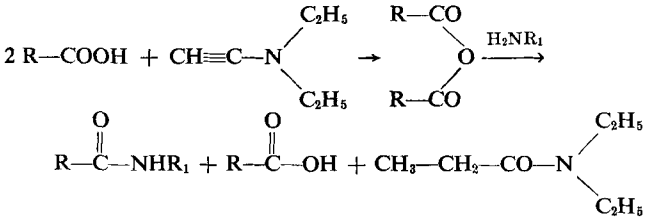
Scheme XXII

with this reagent has an attractive feature in that the by-product ethyl acetate does not interfere with the isolation of the desired product. The peptide bond formation may proceed either directly, by the reaction to amino component to the addition product of carboxylic acid and ethoxyacetylene, or through the formation of symmetrical anhydride (Scheme XXIII).



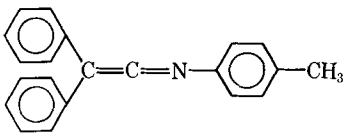
Scheme XXIII

Buyle and Viehe (160) introduced inamines such as dimethylamino *tert*-butylacetylene or dimethylaminophenyl acetylene as coupling reagents. These types of inamines are also capable of forming symmetrical anhydrides; and, Weygand (161) has used symmetrical anhydrides formed by diethylaminomethyl acetylene for the synthesis from *C*-terminal residue of amino acids of a protected nonpeptide. The by-product, propionic acid diethyl amide, is soluble in petroleum ether and can be easily removed (Scheme XXIV).



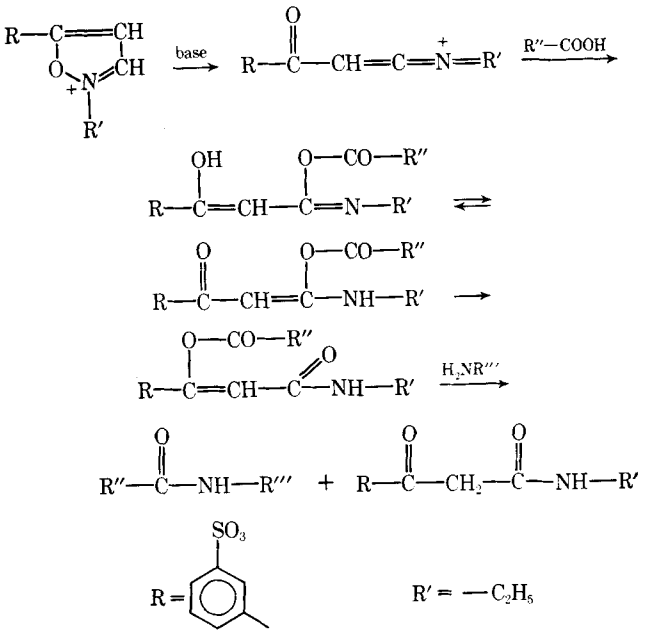
Scheme XXIV

Ketenamines such as diphenyl ketene *p*-tolylimine (Scheme XXV) have been used in the synthesis of peptides (162) and in a sense, the mechanism of their reaction closely resembles that of isoxazoliums salts which were introduced by Woodward (163, 164) a few years later.



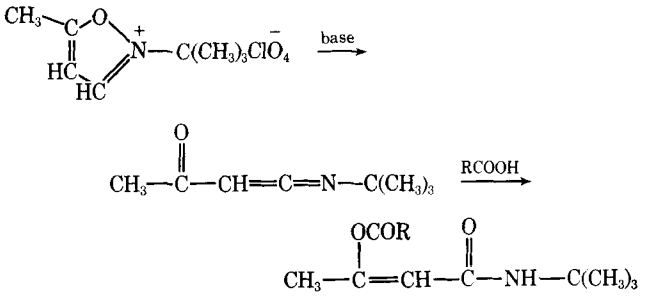
Scheme XXV

Woodward's reagent, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate, reacts with a base to form a ketimine which adds to the carboxyl group and a tautomeric enol ester is formed. This is followed by a rearrangement involving an acyl migration to the actual activated enol ester derivative (Scheme XXVI).



Scheme XXVI

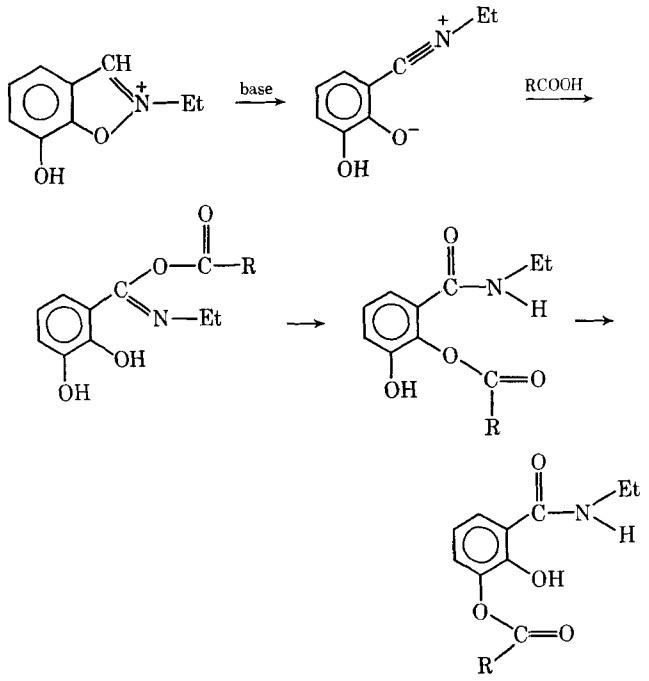
Although high yields are obtained during coupling with Woodward's reagent under optimum conditions, lower yields result if the addition of amine component to active enol ester is delayed. This is perhaps due to intramolecular O → N acyl migration. Woodward and Woodman (165) have proposed the solution to this problem by using bulky *N-tert*-butyl-substituent which gives a stable enol ester. A new coupling reagent, *N-tert*-butyl-5-methylisoxazolium perchlorate, has been suggested, which in addition to forming stable activated enol esters, also makes possible the isolation of the intermediate ketoketenimes (Scheme XXVII).



Scheme XXVII

Another isoxazolium cation, 2-ethyl-7-hydroxybenzisoxazolium fluoroborate, which seems to be quite promising was proposed by Kemp (119). The interesting feature of this coupling reagent is the remarkable base stability of the activated 3-acyloxy-2-hydroxy-*N*-ethylbenzamide derivatives, and this should make them useful for coupling with salts of amino acids (Scheme XXVIII). Attachment of electron-withdrawing substituents in the benzene ring of benzisoxazolium cation such as in the case of 5,7-dichloro-2-ethyl benzisoxazolium salt, has been suggested for a possible increase in the activity of the intermediate active esters (166) (Scheme XXIX).

Belleau and Malek (167) have used *N*-ethoxycarbonyl-2-ethoxy 1,2-dihydroquinoline (EEDQ) as a

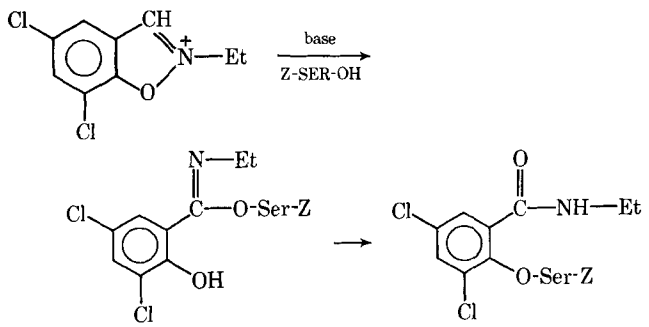


Scheme XXVIII

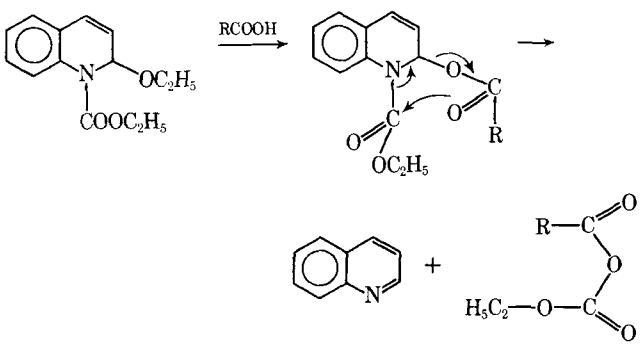
coupling reagent. The actual activation proceeds *via* the formation of the mixed anhydride which is preceded by the displacement of the 2-ethoxy substituent by carboxylic acids. Slow formation but rapid consumption of the active derivative should minimize the side reactions (Scheme XXX).

Reactive *N*-acyl Derivatives—The transfer of acyl residues from derivatives of N^{im}-acylhistidine to amino groups, and thus the possibility of carboxyl activation by imidazolide formation, has been described by several authors (168). Sheehan found that the intramolecular amide (active lactam) of *N*-α-*p*-nitrobenzyloxycarbonyl histidine reacts with benzylamine to form *N*-α-*p*-nitrobenzyloxycarbonyl histidine benzylamide (169). This, along with the contributions from Staab (170), Anderson (171), and Beyerman (172), have led to a number of useful coupling reagents such as *N,N'*-carbonyldiimidazole, thionyldiimidazole, and carbonyl dipyrazole. The reaction proceeds through the formation of acyl derivatives which couple with the amino group to form the peptide bond. The liberated imidazole has a beneficial catalytic effect on the rate of the reaction (Scheme XXXI).

Active Esters—The synthesis of peptides *via* the aminolysis of activated esters was pioneered with the

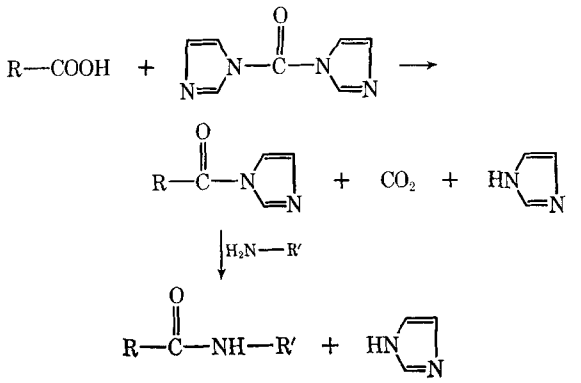


Scheme XXIX

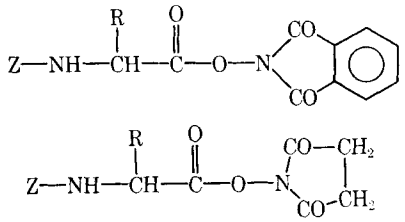


Scheme XXX

use of phenylthiol esters by Wieland (173). One of the main advantages with the use of active esters is the possibility of isolation and purification of the reactive intermediates. These esters have relatively less energy when compared to other activating methods such as mixed anhydride or DCC, and therefore normally do not acylate weaker nucleophiles such as the OH group in hydroxy or phenolic amino acids. A large number of methyl esters with different negative substituents were examined by Schwyzer, and as a result, cyanomethyl esters were introduced in the synthesis of peptides (174). Bodanszky embarked on an elaborate study of phenyl esters, in which the already pronounced electron attraction of the phenyl group was further enhanced by appropriate substituents, and recommended the use of *p*-nitrophenyl esters (175), which indeed was a valuable service to the peptide chemists. Schwyzer's group, shortly afterwards, described the preparation of a series of negatively substituted aryl esters, among them the very interesting *p*-methanesulfonylphenyl ester (176). The use of halogen-substituted phenyl esters such as 2,4,5-trichlorophenyl and pentachlorophenyl ester was suggested by Kupryszewski (177). A detailed kinetic study of various active esters was reported by Stick and Leemann (178). The use of pentachlorophenyl esters has been reinvestigated by Kovacs and this author as a versatile tool for the synthesis of peptides and sequential polypeptides (179). The pentachlorophenyl active esters have the following advantages: (*a*) they are one of the most active esters; (*b*) they are generally higher melting compounds than other active esters, which leads to their easy crystallization and purification; (*c*) they are conveniently prepared without any significant racemization by *N,N'*-dicyclo-



Scheme XXXI

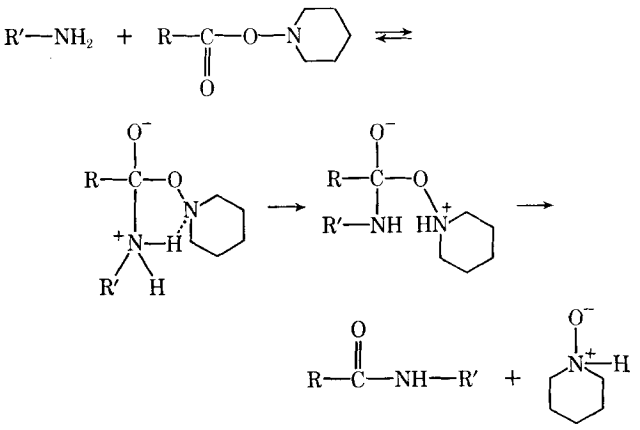


Scheme XXXII

hexylcarbodiimide (DCC) method; (*d*) they are stable to controlled hydrogenation conditions and make an excellent combination with *N*-benzyloxycarbonyl and *tert*-butyl protecting groups when the incorporation of trifunctional amino acids in the peptides is desired. Pentachlorophenyl esters of *N*-protected amino acids also couple in satisfactory yields with amino acids and peptides *C*-protected by suitable salt formation (118).

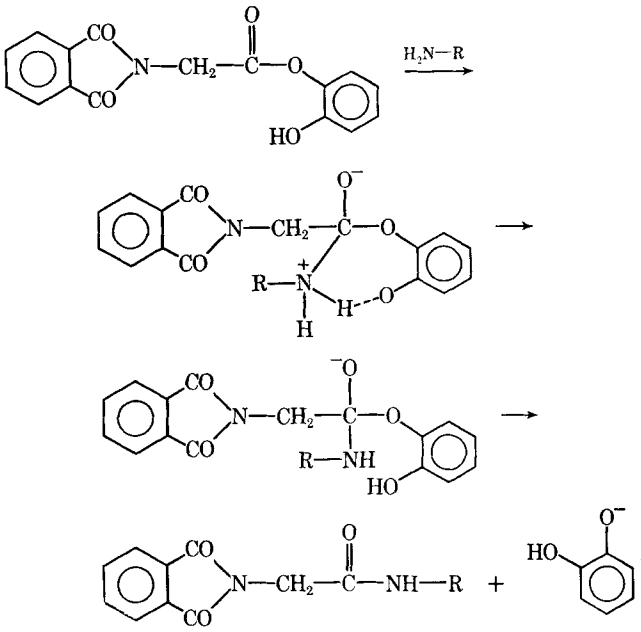
Further search for active esters led Nefkens (180) to the use of *N*-hydroxyphthalimide esters which were shortly followed by the introduction of analogous esters namely the *N*-hydroxysuccinimide esters from the laboratories of Anderson (181) (Scheme XXXII). During the past few years, the latter esters have received considerable attention mainly due to the easy removal of the by-product of acylation, *N*-hydroxysuccinimide, by water.

Another type of active esters which have already proven their worth in the synthesis of peptides, and which hold a bright future, are the reactive derivatives of *o*-acylhydroxylamine such as the esters of *N*-hydroxypiperidine introduced by Young (182). The coupling through these esters proceeds without any significant racemization. This may be attributed to less chances for oxazolone formation because of the ability of the heterocyclic nitrogen atom to share and then accept the proton from the incoming amine. The condensation is accelerated on account of the stability through hydrogen bonding of the transition complex of the adduct; and further, the leaving group will be a tautomer of 1-hydroxypiperidine rather than the unstable anion (183) (Scheme XXXIII).



Scheme XXXIII

Closely related to *N*-hydroxypiperidine esters are the oxine esters which also undergo coupling without racemization, and their reaction mechanism seems to be analogous to 1-piperidyl esters (184). The idea of stabilization of the transition complex through hydrogen bonding and therefore limiting the possibility of oxazolone formation during coupling has been extended in the use of *o*-hydroxyphenyl esters. Their initial success in the synthesis of peptides without any racemization (183) will certainly lead to the development of more versatile active esters (Scheme XXXIV).



Scheme XXXIV

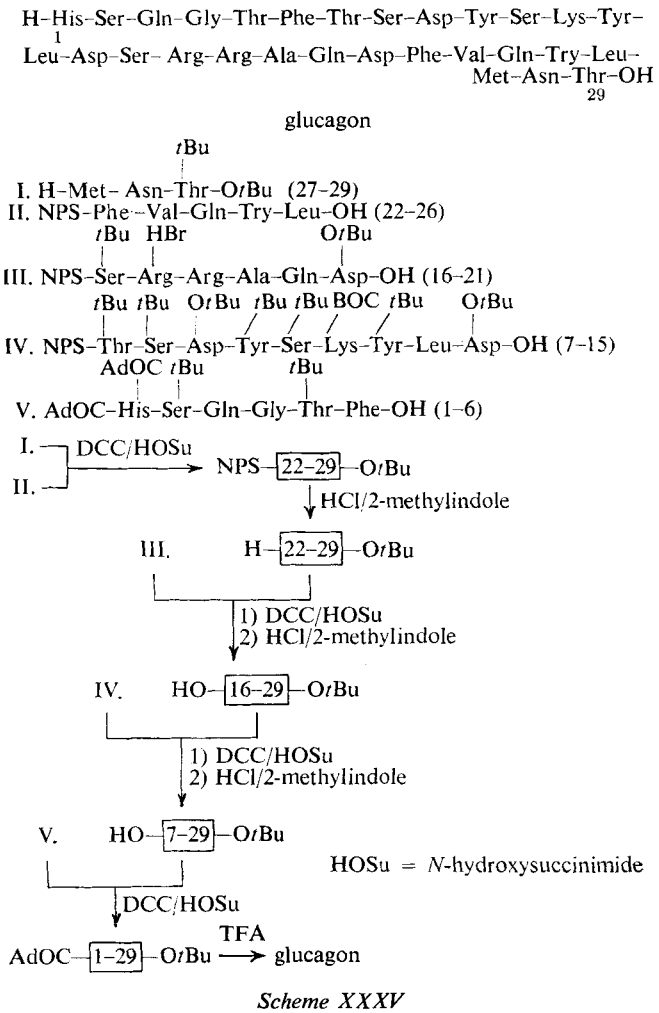
Young (185) prepared the acyl amino acid esters of 2-hydroxypyridine and of 2-mercaptopyridine (SPyr). Esters of the latter (2-pyridylthiolesters) react very rapidly and exothermically with amino esters, giving high yields of protected peptides. A notable feature of these esters (SPyr) is the easy coupling with strongly hindered amino acids such as valine, isoleucine, and α-aminoisobutyric acid (186).

Active esters can be prepared by the condensation of the phenols or corresponding components with *N*-protected amino acids and peptides by a number of coupling methods. Most frequently used are the dicyclohexyl carbodiimide and mixed anhydride methods. A number of active esters of hydroxyamino acids with *N*-α-NPS and *o*-*tert*-butyl protection have been recently reported (187). These intermediates should be quite useful in lengthening the peptide chains. By using isopropyl alcohol, Walter *et al.* (188) were able to prepare previously unreported NPS-protected glutamine and asparagine *N*-hydroxysuccinimide esters. In certain cases, the rate of aminolysis by active esters may be accelerated by the use of suitable catalysts such as imidazoles and 2-hydroxypyridine.

During the preparation of active esters, the *C*-terminal amino acid residue of the peptide chain may undergo racemization; however, with the use of controlled experimental conditions, this problem can be overcome. Formation of the peptide bond is normally associated with the partial loss of optical purity of the amino acid whose carboxyl is activated. This problem may be avoided by lengthening the peptide chains from *C*-terminal residues of amino acids, in which case the

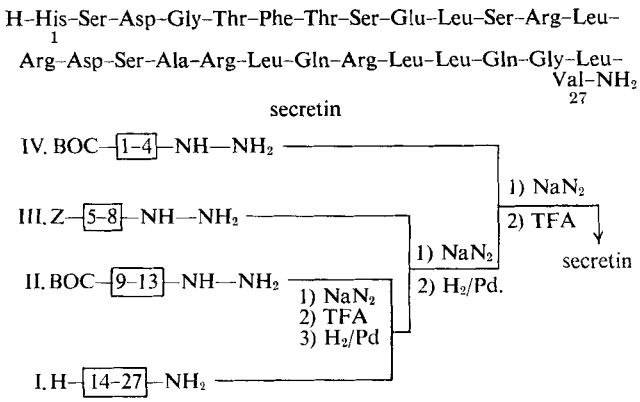
activated component would always be a monomer and, hence, less prone to oxazolone formation. The procedure of fragment condensation, with suitable activation methods and selection of the fragments in which the *C*-terminal residue is glycine or proline, has been very rewarding to limit the degree of racemization during the peptide synthesis. To cite only a few examples, glucagon, secretin, and thyrocalcitonin were recently synthesized by fragment condensation approach.

Glucagon—The synthesis of the pancreatic hormone, glucagon, was achieved by the condensation of five fragments (13). A combination of NPS, and *tert*-butyl and benzyloxycarbonyl groups were used throughout the synthesis. *N*-Terminal histidine was used as *Nα,N*-(im)-di-adamantyloxycarbonyl histidine and the peptide bond formation and condensation of the fragments was carried out mainly with DCC/HOSu method. The following fragments were synthesized and then condensed stepwise (Scheme XXXV).



Scheme XXXV

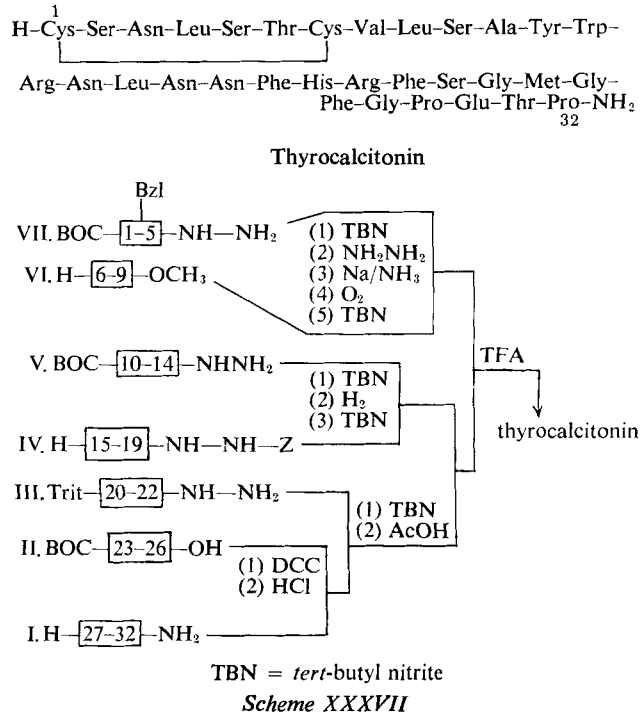
Secretin—The structure of secretin resembles glucagon a greal deal. The presence of four arginine residues in secretin imparts a strong basic character to the molecule. The *C*-terminal amino acid valine is present in the amide form. Synthesis of secretin was accomplished in a stepwise manner, starting from *C*-terminal amino acid



Scheme XXXVI

(189). The other approach was the stepwise synthesis of *C*-terminal tetradecapeptide, and this was attached to three fragments of the *N*-terminal half of the molecule, using the stepwise condensation approach (12). Benzyloxycarbonyl and BOC groups were predominantly used for amino protection. Serine and arginine residues were incorporated as *o*-benzyl and nitro derivatives respectively. Both of these protections were removed by hydrogenation after each fragment condensation. The peptide bonds were generally formed with *p*-nitrophenyl esters and 2,4-dinitrophenyl esters (for arginine and threonine). The fragments were condensed *via* the azide method (Scheme XXXVI).

Thyrocalcitonin—In thyrocalcitonin which is a calcium-regulating hormone, the *C*-terminal amino acid proline is present in the amide form, and cysteines at positions one and seven are joined by a disulfide bridge. The synthesis of thyrocalcitonin was achieved by stepwise condensation of seven fragments (14). Benzyl protection was used for sulfhydral groups and the disulfide bridge was formed at the nonapeptide stage. Serine residues were incorporated without OH



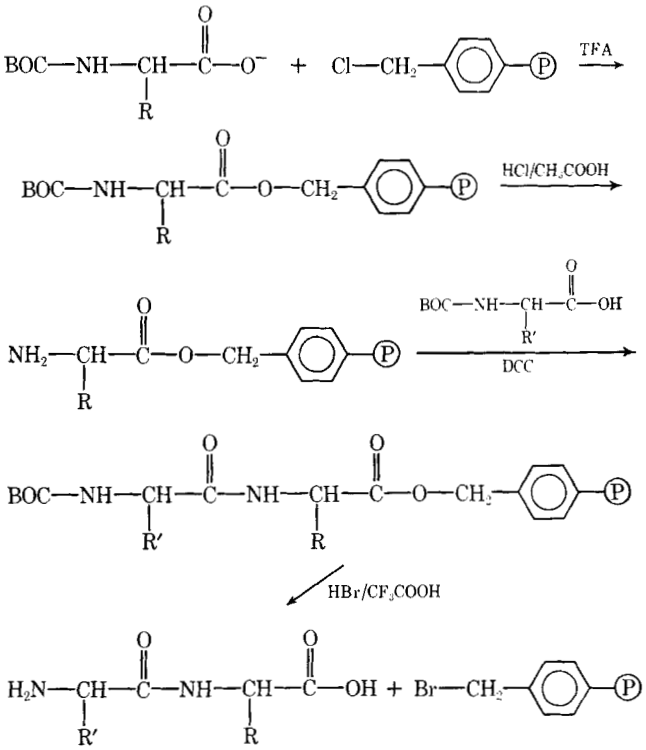
TBN = *tert*-butyl nitrite

Scheme XXXVII

protection. BOC protection was predominantly used for amino groups and the coupling reactions were mainly carried out by azide methods. Di-Z-histidine was used for the preparation of the tripeptide III and was converted to the dityrosyl derivative before coupling for the formation of the tridecapeptide amide (Scheme XXXVII).

Peptide Synthesis with the Help of Polymeric Support—The isolation and purification of each intermediate during the building of a peptide chain is of utmost importance to limit the extent of racemization in the final product. However, the development of newer techniques and the modification of conventional methods has inspired the synthesis of the peptides without the isolation of intermediates. An apparent advantage of the approach is the tremendous saving of time which is normally required for the stepwise purification. Knorre and Shubina (190) have elongated the peptide chain at the carboxyl end by coupling an aqueous solution of formyl amino acid with an amino acid ester by means of water-soluble carbodiimides. The reaction mixture upon passing through resin columns is freed from reactant and reagents. The purified product, thus obtained, is saponified and is used directly for the next step in the synthesis. A protected hexapeptide was synthesized with less than 5% racemization. Sheehan (191) used a somewhat similar approach but extended the peptide chain from the amino end. The technique proved quite successful for the synthesis of a protected heptapeptide.

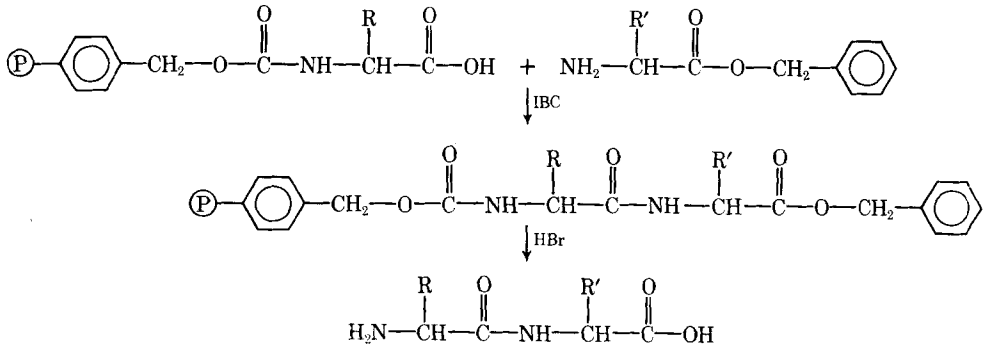
The above two approaches were only the beginning of the new ventures into less conventional approaches in the synthesis of peptides. In 1963, Merrifield (192) proposed an ingenious idea for lengthening the peptide chains by providing an insoluble polymeric support to the *C*-terminal residue of amino acids and extending the peptide chains by stepwise addition of succeeding amino acids. In this approach, which is called solid phase method for peptide synthesis, the growing peptide chain is at all times during the synthesis firmly bound by a covalent linkage to a solid particle and is, therefore, completely insoluble in all the reaction solvents. This allows separation of the peptide chain from soluble starting materials and by-products by filtration and thorough washings. Thus the intermediate peptides are purified, not by the usual recrystallization procedures, but by dissolving away the impurities. This greatly simplifies the manipulations and shortens the time required for the synthesis of peptides. After the desired sequence is assembled, the peptide is removed from the solid support. For a supporting solid, a copolymer of 98% styrene and 2% divinyl benzene in the form of small (20–80μ) beads is chloromethylated and the product is attached to the *N*-protected amino acid to give a substituted benzyl ester derivative. The amino group was originally protected by the benzyloxycarbonyl group but it was replaced by the *tert*-butyloxycarbonyl group and recently by *o*-nitrophenylsulfenyl group (193). The advantage of the last two groups is the easy removal by relatively mild acid conditions which minimizes the danger of cleavage of the peptide chain from the resin during the synthesis. After deprotection of the amino group, the next protected amino acid is coupled by DCC or active ester methods



Scheme XXXVIII

in solvents having high dielectric constants, such as methylene chloride or dimethylformamide (Scheme XXXVIII).

The selection of benzyl ester linkage can be justified on the basis of its stability to various synthetic reactions and easy cleavage by anhydrous hydrogen bromide at the end of the synthesis. The reactions outlined in Eq. 38 constitute one cycle in which a dipeptide is formed. However, additional cycles may be carried out in the same way, one at a time, until the desired sequence of amino acids in the peptide is completed. One of the most critical requirements for a successful synthesis, using a polymeric support is the quantitative coupling reaction. Unless this can be achieved at each step of the synthesis, a chain which does not react at one step will have a chance to react at each of the later stages and will lead therefore to undesired peptides with different amino acid composition. An excess of activated component, a longer period of reaction time, and the use of low cross-linked, high swelling resin generally leads to complete coupling. Of course the potential difficulty in the final purification after the peptide is removed from the resin must be accepted in return for convenience and speed in building up the peptide chain. In case DCC is used for activation, the possibilities for the loss of excess reagent due to the formation of *N*-acylurea must be considered. A suitable selection of active esters (194) for coupling to resin attached amino acids or peptides would eliminate this side reaction, and would also permit the incorporation of hydroxy amino acids without any protection on hydroxyl group. Active ester coupling has been of particular use in the case of glutamine or asparagine incorporation on account of the dehydration of carboxamide groups in the side chains of these amino acids when DCC is used. In the case of peptides incorporating aspartyl



IBC = isobutylchloroformate

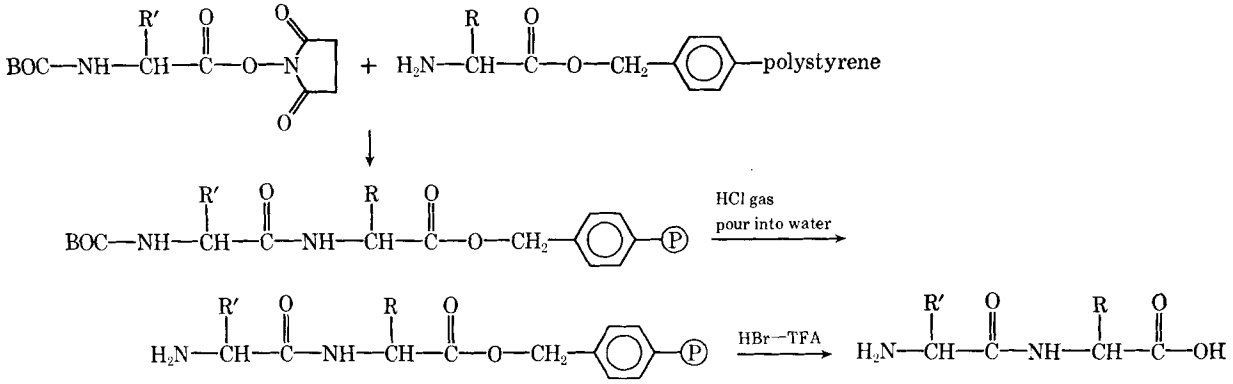
Scheme XXXIX

or glutamyl residues with side chain carboxyl esterified, transpeptidation normally does not occur under the conditions used during synthesis by solid phase method. However, the treatment of HBr-trifluoroacetic acid, which is used to liberate the peptide from the resin support, may lead to the formation of amino succinyl derivatives in the case of peptides containing β-benzyl aspartyl-seryl residues. The opening of the imide derivatives may produce undesirable peptides with β-aspartyl linkages. Bumpus has reported the difficulties encountered during the formation of arginylproline bond under the conditions of the solid phase method using DCC (195). For the synthesis of peptides where the *C*-terminal amino acid occurs as an amide such as in oxytocin, the desired peptides, after the synthesis, are cleaved from the resin by ammonolysis in methanol. Although this approach has been quite successful with smaller peptides, particularly those with glycine as *C*-terminal amino acid, a considerable degree of difficulty was encountered during the ammonolytic cleavage of secretin where the *C*-terminal valine could not be removed from the resin as an amide (196).

Letsinger and Kornet (197) suggested as a modification to Merrifield's approach the use of "popcorn polymer" of polystyrene with a very low degree of cross-linking by divinylbenzene. In addition, the *N*-terminal amino acid was the anchoring group with the resin and the chain was extended by stepwise incorporation of subsequent amino acids. This approach has not gained much popularity mainly because of the possibility of racemization which is normally associated with the lengthening of the peptide chains from the *N*-terminal residues of amino acids (Scheme XXXIX).

A very interesting idea was proposed by Shemyakin (198) in which the insoluble cross-linked polystyrene was substituted by a soluble polymeric support. This offers the apparent advantage of having all reactions carried out in solution, thereby eliminating the problem of permeability or diffusion. The polymeric support was an emulsion polystyrene of average molecular weight of 200,000. The coupling of amino acid and peptide derivatives of oxymethyl-polystyrene was carried out with hydroxysuccinimide esters of BOC-protected amino acids. This approach may prove to be a very effective synthetic tool if certain problems can be overcome, such as formation of viscous gum when the linear polystyrene is thrown out of solution by pouring it into water (Scheme XL).

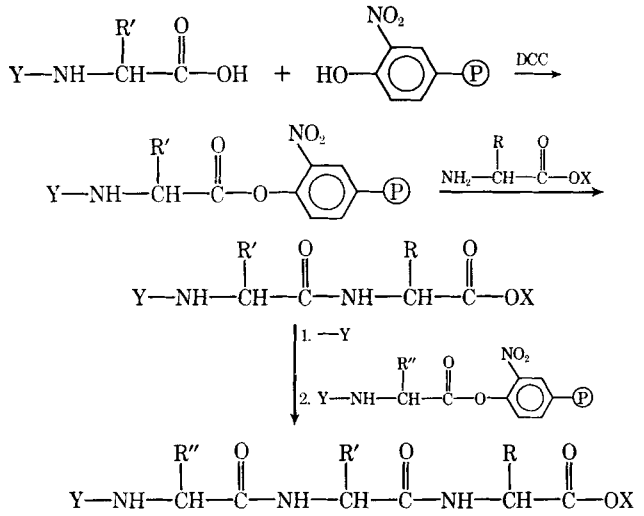
One of the main concerns in the solid phase peptide synthesis is that the purification of intermediates cannot be effected. At about the same time, a new approach was suggested from the laboratories of Katchalski (199) and Wieland (200). Where in Merrifield's method, it is the peptide which is bound to the insoluble carrier and the *N*-blocked amino acid-activated derivative is added while in solution, in the new approach a solution of free peptide ester is added to an insoluble polymer-supported, *N*-blocked amino acid-active ester. The reaction between peptide ester and active amino acid ester can be carried out in the presence of a large excess of insoluble active ester, and the unreacted reagent (active ester) can be readily removed by filtration or centrifugation and washing. The new peptide remains in solution and can be suitably purified before it is reacted with the next insoluble active ester



Scheme XL

component. Katchalski proposed a number of insoluble polymer-active esters. However, best use was made of DCC coupling of *N*-protected amino acids or peptides with a polymer made from 4-hydroxy-3-nitrostyrene cross-linked with 4% divinylbenzene (Scheme XLI). Another very interesting feature of this approach is the application of these insoluble polymer-active esters in the synthesis of cyclic peptides. The use of insoluble polymer-active esters attached to peptides limits the intermolecular condensation and facilitates intramolecular aminolysis, thereby avoiding the formation of linear polymers; cyclic peptides of high purity are isolated in good yields (201) (Scheme XLII).

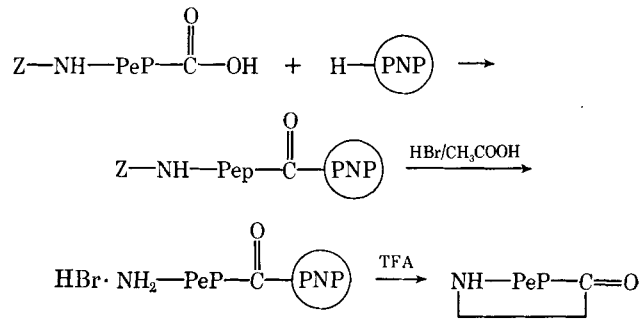
Wieland utilized formaldehyde cross-linked *p,p'*-dihydroxydiphenylsulfone as a starting material for the synthesis of the corresponding insoluble *N*-blocked amino acid active esters (200) (Scheme XLIII).



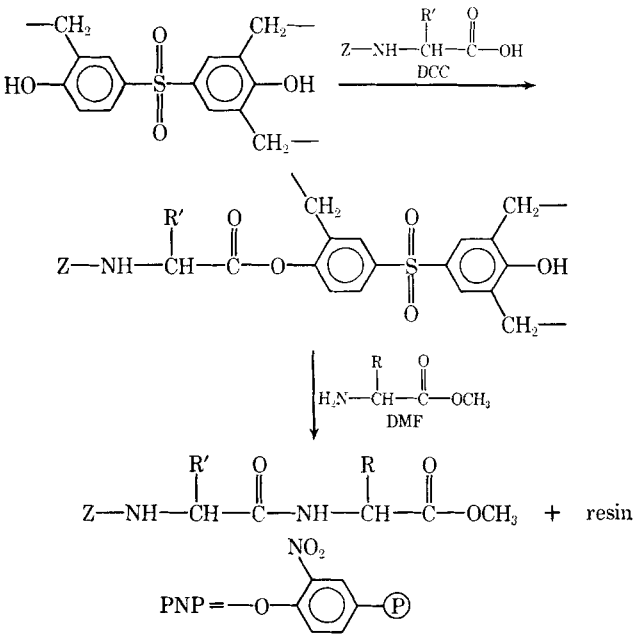
Y = amino protection X = carboxyl protection

Scheme XLI

The synthesis of peptides with polymeric active esters was also investigated by Blout (202). BOC-amino acid *N*-hydroxysuccinimide active ester derivatives of copoly(ethylene-*N*-hydroxymaleimide) were used as insoluble active ester reagents. For the preparation of these esters, copoly (ethylenemaleic anhydride) was condensed with hydroxylamine hydrochloride, and the resulting *N*-hydroxy polymer derivative was coupled either by mixed anhydride or DCC methods with BOC-amino acids (Scheme XLIV).



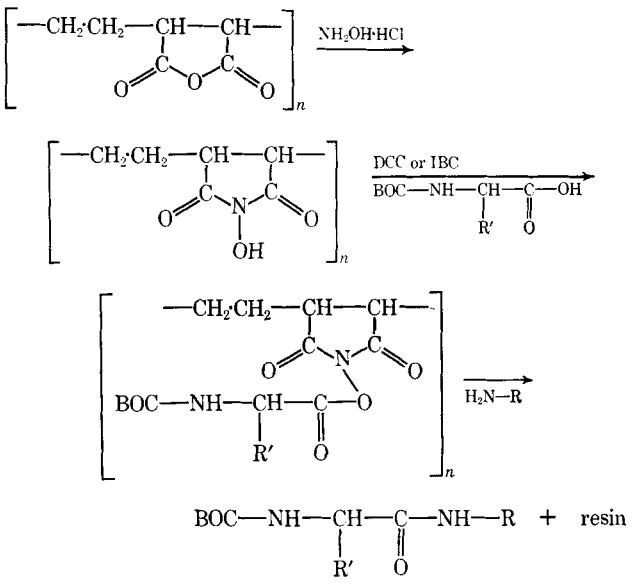
Scheme XLII



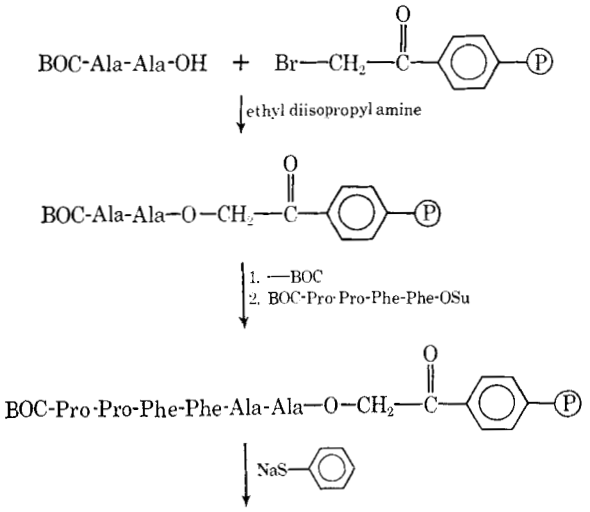
Scheme XLIII

The substitution of activated amino acids in the polymers previously mentioned can be varied by appropriate adjustment of the *N*-protected amino acid : polymer molar ratio. Satisfactory results are obtained when 1–2 mmole of activated amino acid is bound to each gram of the resin.

Weygand (203, 204) investigated the possibilities for the coupling of the *N*-protected peptides with amino acids or peptides anchored to polymers. The advantage of this approach is the availability of a relatively larger number of peptide intermediates, which can be properly characterized before incorporation in the peptide chain. Dipeptides, *N*-protected by BOC, were reacted with the bromoacetyl derivative of polystyrene; and, after the removal of *N*-protection from the resulting dipeptide polymer derivatives, coupling was carried



Scheme XLIV



Scheme XLV

out with *N*-protected di- or tetrapeptide *N*-hydroxysuccinimide esters. Selective removal of the peptides from the polymer was achieved by sodium thiophenolate, and the *N*-protected peptides so obtained were free from racemization (Scheme XLV).

While the above approach which has also been used by Anfinsen (205) is indeed a very useful modification in solid phase synthesis of peptides, the proper evaluation of this will depend on the successful synthesis of larger peptides.

The success of the solid phase method and new approaches using various forms of solid supports is apparent from the synthesis of a large number of biologically active peptides such as bradykinin, oxytocin, angiotensin, insulin, *etc.*, and their analogs. However, the most frequently used approach is still the one which was originally suggested by Merrifield, and in order to accelerate the synthesis of peptides, a simple automated apparatus has been designed (206). It consists of a reaction vessel where the actual synthesis takes place and devices which transfer reagents and solvents into and out of the vessel. The entire series of events is controlled by a programmer which can be arranged in advance to direct the various operations of the instrument. The automated process produces a protected peptide chain attached to the solid polymer support, and after cleavage from the resin and deprotection, the peptide is suitably purified.

It was apparent, from the onset of the introduction of the solid-phase method, that the synthesis of larger peptides and proteins would become feasible, and this was indeed demonstrated by the synthesis of a protein. A linear polypeptide of 124 amino acid residues, with the sequence of bovine pancreatic ribonuclease A (RNase A), was synthesized by Merrifield using 369 chemical reactions and 11,931 steps of the automated peptide synthesis machine without any intermediate isolation steps (207). Anhydrous hydrogen fluoride was used for the single step cleavage of the peptide from resin and simultaneous removal of all protecting groups.

The properties of anhydrous hydrogen fluoride (HF) as a reagent for the acidolysis of various protecting groups have been studied by Sakakibara (208). Amino acids with various protecting groups, particularly the nitro group in nitroarginine, *tert*-butyl group in *S-tert*-butyl cysteine and many other protecting groups can be conveniently removed by HF at low temperatures in the presence of anisole. When carefully handled, HF should prove to be an effective tool for planning the strategy in the synthesis of peptides.

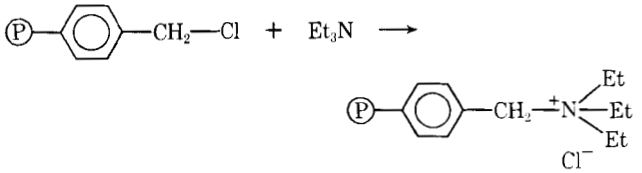
Iselin suggested the use of hydrazine for the cleavage of peptides from polymer support (209). Hydrazinolysis leaves the *N*-protection intact, and the cleaved peptide is isolated as hydrazide which can be directly coupled to another peptide after conversion into the azide. The same approach has been used by Anfinsen for the synthesis of water-insoluble *N*-protected peptide hydrazides which do not contain ω-protected aspartic or glutamic acid residues or other hydrazine-labile groups (210).

The possibility of a side reaction during esterification of the *C*-terminal amino acid with chloromethylated resin has been reported by Beyerman (211). This side reaction probably occurs between the free triethylamine and chloromethyl groups producing a quaternary ammonium compound (Scheme XLVI).

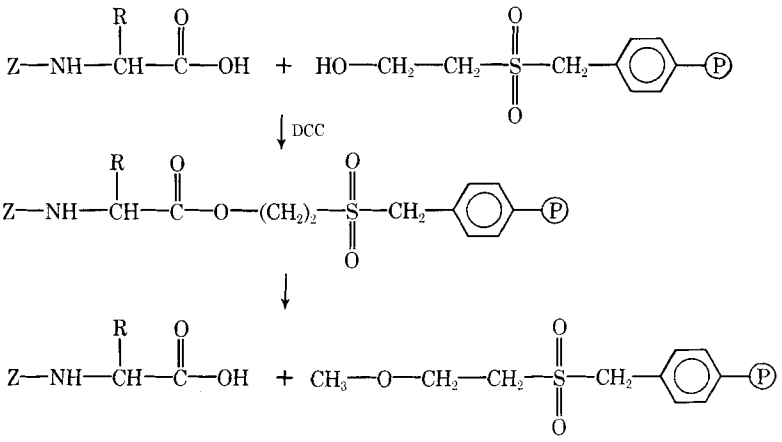
The *N*-protected *C*-terminal amino acid may be attached to the polymeric support by DCC method, if the chloromethylated polymer is first reacted with thioglycol and the resulting thioether is oxidized with perbenzoic acid. The *N*-protected peptides attached to the modified solid support can be selectively cleaved from the resin without affecting the *N*-protected group (212) (Scheme XLVII).

Solid phase method of peptide synthesis has been extended in the synthesis of polymers with different sequence and size. Different applications in the synthesis of oligo-nucleotides have been reported (213). Oligomers of nylon (214) and polypeptides with an ordered sequence of amino acids, such as H(prolyl-glycyl-glycyl)-OH (215), and H(prolyl-prolyl-glycyl)-OH (216) have been successfully synthesized with solid support. Recently, Rothe (217) reported the synthesis of different-sized oligomers of epsilon amino caproic acid by using dimeric and tetrameric units for the chain growth. Coupling of these units with the polymer-supported chain was carried out with the aid of diester chlorides of phosphorus acid instead of DCC.

The reverse of the peptide synthesis by solid phase, *i.e.*, the degradation of the peptide on a polymeric support using the Edmann method, has been suggested by two different groups. Stark's (218) procedure involves attaching the peptide by its *N*-terminal residue to a support of polystyryl isothiocyanate, cyclizing, isolating and analyzing the residual peptide, and reattaching the peptide to the resin. Repetition of these



Scheme XLVI

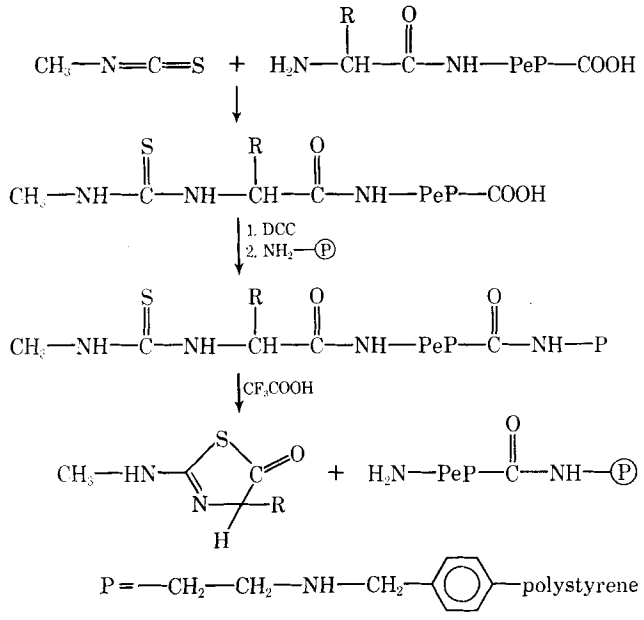


Scheme XLVII

steps leads to the establishment of the sequence of amino acid residues in the peptide chain. In the method proposed by Laursen (219), the peptide is attached by its *C*-terminal amino acid and the degradation is performed in the usual manner. The liberated thiazolinone is removed in each cycle simply by filtration (Scheme XLVIII).

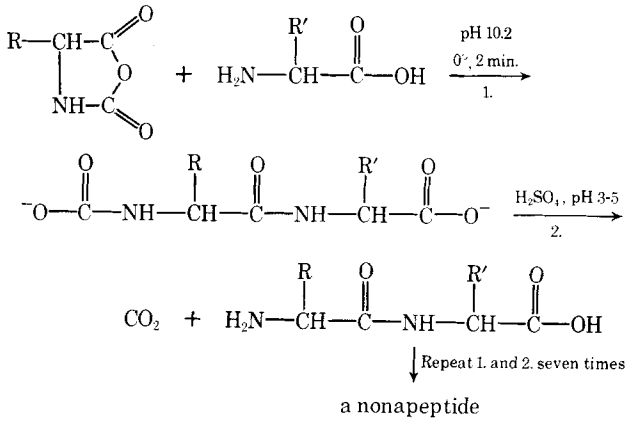
A closely related approach, using a combination of Edmann degradation on polymeric support and mass-spectrometric methods, has been suggested by Weygand (220) for the determination of the extent of amino acid residues incorporated in the growing peptide chains attached to the resin supports.

Leuch's Anhydrides—*N*-Carboxy-α-amino acid anhydrides (Leuch's anhydrides, NCA's) have been widely used in polymerization reactions (221) in anhydrous media for the synthesis of homopeptides and the random synthesis of heteropeptides. While the controlled synthesis of heteropeptides *via* Leuch's anhydrides has been attempted by many laboratories, relatively little success has been achieved in the use of this method. Bartlett (222), on the basis of his studies with NCA's of various amino acids, reported that the reaction cannot be



Scheme XLVIII

controlled adequately to provide a useful general method for peptide synthesis in water. Grant (223) proposed the limited use of Leuch's anhydrides for the formation of dipeptides in water at pH 5, particularly when the reacting amine is a much weaker base than the amine resulting from the synthesis. The real breakthrough in the utilization of NCA's in peptide synthesis came with the development of suitable reaction condition by Denkewalter *et al.* (224). They proposed the addition of solid NCA directly to an amino acid at 0–2°, generally at pH 10.2, with rapid mixing of the reactants, thus minimizing side reactions. Rapid mixing, in the case of relatively insoluble NCA's, may enhance the rate of dissolution of the NCA thereby reducing the possibility for carbamate exchange which leads to the formation by overactivation of tripeptides, and also to inactivation of the starting amino acids. Overactivation of more soluble NCA's *via* their anions is also limited by rapid mixing, thus preventing the formation of oligomers. Carbamate stability increases with pH, but at a pH above 10.5, hydrolysis of NCA becomes an important side reaction leading to the formation of hydantoic acid, most likely through NCA anion. The peptide carbamate is decarboxylated at pH 3–5 to produce the free peptide which can be immediately extended in length by repetition of the process with a new *N*-carboxyanhydride, or can be first isolated and purified before continuing with the synthesis. Racemization was not detected during the synthesis of peptides *via* NCA's; this method, used under careful



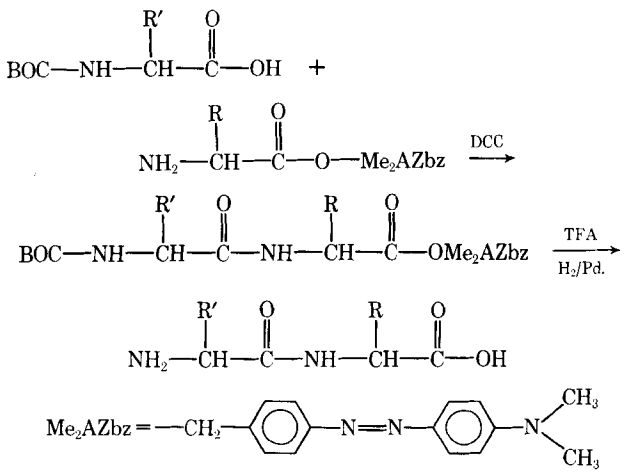
Scheme XLIX

conditions and in combination with other more conventional methods, should prove to be of great value in the synthesis of larger peptides (Scheme XLIX).

The extraordinary rapidity of the NCA method, which is particularly useful in the sequential preparation of higher peptides without isolation of intermediates, is indeed a very interesting feature of this method. Another significant advantage of the NCA method is that the reactions are carried out in aqueous medium and with the exception of the ϵ-amino group of lysine and sulfhydryl group of cysteine, polyfunctional amino acids used as a nucleophile can be used without any protection of the third functionality. NCA's with the exception of those of proline and glycine are quite stable when stored at low temperatures with protection from moisture.

Since the thiocarbamate salts proved to be more stable than the corresponding carbamate salts, the use of sulfur analogs of NCA's, the 2,5-thiazolidinediones (*N*-thiocarboxyanhydrides, NTA's), in the peptide bond formation has been suggested (225). NTA's usually give higher yields in the peptide synthesis. The optimal pH for their reaction is lower, *i.e.*, 9–9.5. Low pH usually favors the desired aminolysis of an anhydride over hydrolysis. NTA of glycine does not form the isocyanate which is characteristic of glycine NCA; therefore, the chances of hydantoic acid formation as an undesirable side reaction are eliminated with the use of the NTA of glycine. There is another interesting difference between the NCA and the NTA of unprotected histidine. While the NCA of histidine undergoes intramolecular imidazole catalyzed isocyanate formation, this reaction is relatively less favored in the case of the NTA of histidine; therefore NTA of histidine is more suitable for the incorporation of histidine in histidyl peptides.

The tremendous scope of the NCA and NTA methods in the synthesis of larger peptides was demonstrated by a group of workers at Merck (226) when they applied these approaches, in combination with the *N*-hydroxysuccinimide and azide methods, toward the successful synthesis of a protein (*S*-protein) composed of 104 amino acids. The fragment condensation approach was used to attach 19 fragments. About 40% of the bonds



Scheme L

in the peptide fragments were formed through the use of NCA's and NTA's; the remainder with *N*-hydroxysuccinimide active esters. The azide method was used throughout for the condensation of fragments which contained 6-17 amino acids. The acetamido methyl group which is resistant to HF at 0° was used for the protection of sulfhydryls of eight cysteines, and acid labile BOC-protecting group was employed as a temporary blocking group of the growing peptide chain. All the protecting groups at the end of the synthesis were removed by HF still leaving the cysteines protected by the acetamido methyl group. After removal of sulfhydryl protections by mercuric acetate, the deprotected tetrahectapeptide was oxidized at pH 6.5 in the presence of mercaptoethanol to form four disulfide bridges. The resulting *S*-protein combined with the *S*-peptide to produce the enzyme activity.

Synthesis of Peptides with the Aid of Synthetic Polysaccharide[1]—Wieland (227, 228) has suggested the use of cationic benzyl ester derivatives for stepwise lengthening of the peptide chains from *C*-terminal residues of amino acids. These derivatives are strongly adsorbed on columns of sulfoethyl-(SE)-polysaccharide, and can be purified by rinsing of the colored zones with methanol. The azoesters of the peptide derivatives can be easily removed at the end of the synthesis by catalytic hydrogenation (Scheme L).

Synthesis of Polypeptides—Practically all the methods which are used for the stepwise synthesis of oligopeptides have been tried for the synthesis of polypeptides with an ordered sequence of amino acids. High molecular-weight polypeptides with known repeating sequences of amino acids, especially those which are water-soluble and contain polyfunctional amino acids, are of special interest. These polypeptides would be expected to resemble proteins more closely than those consisting of only difunctional amino acids. Polymerization of the selected sequences of amino acids in proteins can be of considerable help in the conformational studies and also to explore possible biological activities. The polymerization with the help of active esters such as pentachlorophenyl esters (179) has been quite useful and recently, in the author's laboratories, a number of sequential polypeptides incorporating histidine and serine at suitable distances have been synthesized *via* pentachlorophenyl esters. These polymers possess a definite pattern of activity comparable to the proteolytic hydrolysis of chymotrypsin. Further studies with polypeptides incorporating amino acids around the active site of enzymes are under progress and may provide interesting data for the catalytic activity of certain enzymes.

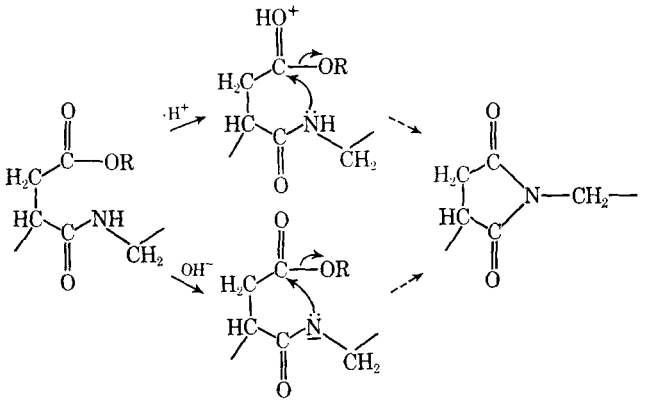
Fox (229) has introduced a rather unconventional but extremely interesting approach for the synthesis of polymers by thermal condensation. In this type of polymerization instead of "prespecified" sequences, the polymers obtained are of "predetermined" sequences. The thermal method of polymerization would lead to the polymers in which reacted α-amino acids are largely racemized. For the synthesis of thermal poly-

[1] Sephadex.

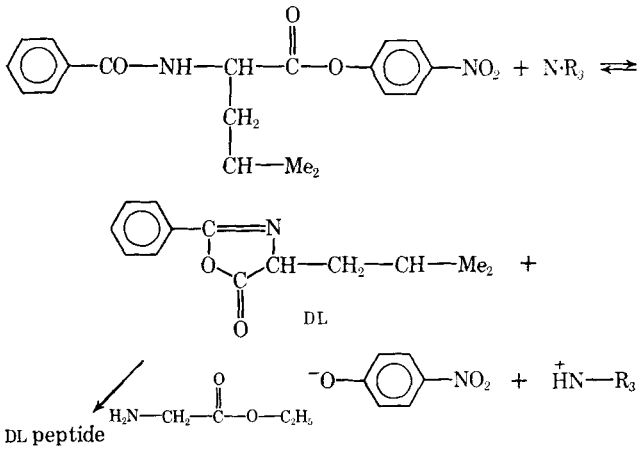
mers, the best results are obtained when sufficient proportions of aspartic acid, glutamic acid, or lysine are heated to 170° for 6 hr. with 16 other amino acids common to proteins. The molecular weights of these polymers are in the range of 4000 to 10,000. Some proportion of each of the amino acids common to protein is found in the polymers synthesized by thermal condensation, and the term "octadecatonic anhydropolymers" has been suggested to designate these polymers. It is of great interest that when six amino acids, that is glutamic acid, glycine, arginine, histidine, phenylalanine, and tryptophan which are considered as a part of the center of the active site in melanocyte stimulating hormone, were subjected to thermal polymerization, the resulting polymer exhibited MSH-activity (230). This kind of study may provide a new type of approach to identification of necessary or sufficient amino acid residues in active sites of protein hormones.

Cyclic Peptides—Most peptide antibiotics are cyclic in character and in contrast to proteins, contain both D and L amino acid residues. Certain amino acids common to proteins, *e.g.*, arginine, methionine, and histidine, occur rarely in peptide antibiotics. The cyclic peptide antibiotics include both homodetic and heterodetic structures. The synthesis of homodetic cyclic peptides is, in principle, the same as that of linear peptides. However, it is essential to work at the right dilution to suppress polycondensation as much as possible in favor of the formation of the intramolecular peptide bond. An interesting phenomenon, "doubling reaction," was reported by Schwyzer (231). If a linear cyclizable peptide with an odd number of amino acid residues, *e.g.*, a pentapeptide, is subjected to cyclization, the corresponding cyclic pentapeptide is not obtained, but rather, through dimerization, a cyclic decapeptide is obtained. A detailed classification of different types of cyclic peptides and specific synthetic methods for their preparation have been reviewed in detail during recent years (29, 31, 32).

The Problem of Transpeptidation Reactions—Intramolecular chemical transpeptidation of peptides and polypeptides with amino dicarboxylic acid residues has been observed in two different cases: (*a*) on peptides and polypeptides containing aspartyl or glutamyl residues in the peptide chains with free ω-carboxyl group; (*b*) on peptides and polypeptides with esterified



Scheme LI

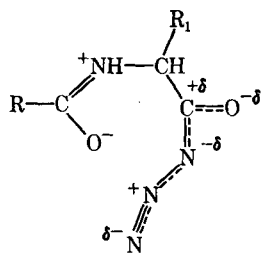


Scheme LII

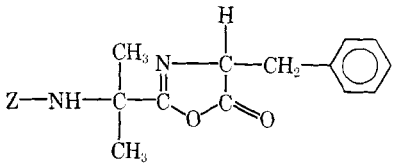
aspartyl and glutamyl residues under basic conditions. The formation of glutarimide derivatives from *N*-protected γ-glutamyl dipeptides and subsequent ring opening to yield α-peptides has been reviewed (179). In the case of the transpeptidation reactions with esterified glutamyl and aspartyl peptides, the imide ring formation is a well established base catalyzed reaction; and, to prevent this type of transpeptidation reaction, *tert*-butyl esters, which can be removed by acid treatment, provide a special advantage.

The formation of cyclic imide was observed as a side reaction in the synthesis of peptides containing the aspartyl-glycyl sequence when the β-carboxyl group of aspartic acid was esterified. The intermediates where the β-carboxyl group of the aspartyl residue is in its free unesterified form show no tendency to undergo cyclization either in the acid or alkaline conditions. This is perhaps due to the fact that alkoxy groups are better leaving groups than hydroxyls, and are therefore more easily displaced in the nucleophilic attack of the glycine nitrogen (232) (Scheme LI).

Racemization—Synthesis of biologically active peptides must proceed without affecting the configuration about any of the asymmetric centers of the components. A continuously increasing amount of attention is being devoted to the problem of racemization, and many laboratories are actively engaged in developing new approaches which would limit the degree of racemization during the synthesis of peptides. While there are many instances where racemization has been observed during the introduction or removal of protecting groups from amino acids or peptides, the most critical aspect, which has received the maximum attention, is the possibility of racemization during the peptide bond formation. Racemization may occur



Scheme LIII



Scheme LIV

either because of the formation of oxazolone (azalactone) intermediates or by the direct proton abstraction from the asymmetric alpha carbon. A recent discussion by Bodanszky and Ondetti (32) summarizes generally accepted beliefs about the loss of optical purity during the synthesis of peptides.

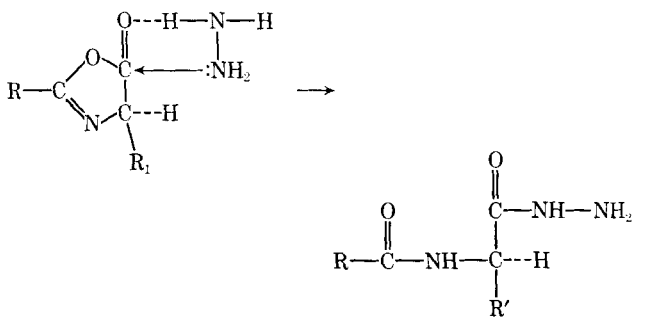
Mechanistic investigations of racemization began when it was shown by Young (233) that the reaction between benzoyl-L-leucine *p*-nitrophenyl ester and glycine ethyl ester, in the presence of a tertiary base, proceeds through the reversible formation, racemization, and coupling of the oxazolone (Scheme LII).

Independent of the above work, Goodman (234) reported the preparation of the optically active crystalline oxazolone from benzoyl-L-phenylalanine. On the basis of equilibrium studies of this oxazolone with various nucleophiles, it was concluded that during the coupling process a small steady state concentration of oxazolone can form which racemizes much faster than its ring opens. The reason for the racemization-free coupling of azide method may be attributed to the inhibition of bond movements (essential for proper conformation for oxazolone formation) due to the attraction between the negative charge developing on the amide oxygen and the positive charge on the central nitrogen atom of the azide group (235) (Scheme LVIII).

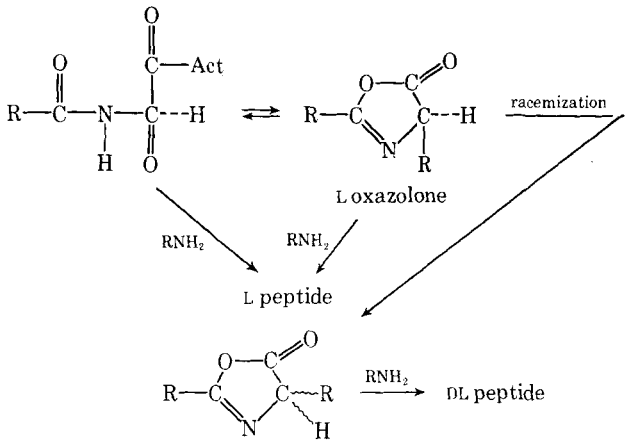
There is a clear dependence of oxazolone formation in the presence of base; Kemp (236) recently reported kinetic evidence which supports the presence of equilibrated amide anions as reactive intermediates leading to azalactone formation in the case of peptide activated esters.

Goodman's group (237) synthesized optically active 2-(1-benzyloxycarbonyl-amino-1′-methyl)ethyl-4-benzyloxazolone from benzyloxycarbonylaminoisobutyl-L-phenylalanine, and reported a number of interesting observations about racemization and ring opening reactions of this oxazolone with several amino acid esters (Scheme LIV).

Racemization of oxazolones may follow pseudo-first-order or second-order kinetics, depending on the attacking amino ester and the nature of the solvent. One of the most important factors which controls the



Scheme LV

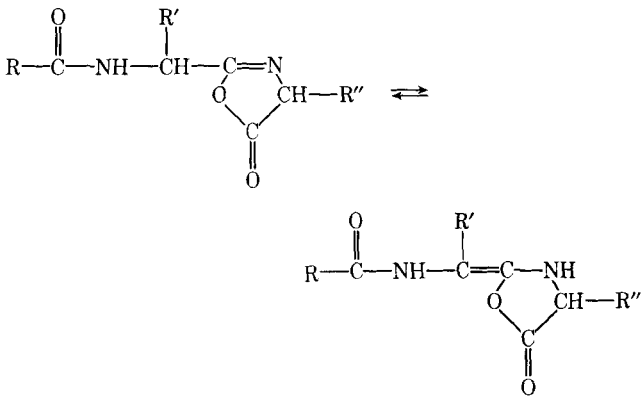


Scheme LVI

racemization process is the nucleophilicity-basicity ratio of the attacking nucleophile. Racemization rate can be accelerated by the presence of chlorides or phosphates which increase the ionic strength of the solution and facilitate the charge separation necessary for racemization. The coupling or ring-opening reaction is always a second-order reaction which is governed by the nucleophilicity of amino components in different solvents. The rate at which a reagent opens an oxazolone ring is a measure of the reagent's nucleophilicity, and the rate at which a reagent racemizes an oxazolone is a measure of reagent's basicity. Certain 1,2-dinucleophiles such as hydrazine which have high nucleophilicity-basicity ratio open the oxazolone ring without racemization (Scheme LV).

Elegant studies carried out by Young (238) on base-catalyzed racemization of various acyldipeptide *p*-nitrophenyl esters have, in fact, established that racemization of many peptide activated species proceeds through oxazolone during the coupling reaction (Scheme LVI).

The degree of racemization of peptide product will no doubt depend on the relative rates of each reaction. For example, coupling of acylpeptides by means of DCC can lead to racemization since this is an excellent reagent for the preparation of oxazolones. In the coupling reactions which proceed through oxazolone formation, the chances of racemization can be limited by the use of nonpolar solvents and by proper selection of the tertiary base. When an oxazolone is formed from the *C*-terminal residue of a peptide chain, the possi-



Scheme LVII

bility of proton abstraction from the alpha carbon of penultimate amino acid residue may lead to its partial racemization, due to rearrangement of the oxazolone (239) (Scheme LVII).

In the case of acetyl and benzoyl amino acids, oxazolones are formed very readily. This, perhaps, is the reason for the racemization in the case of benzoyl and formyl-L-leucine *p*-nitrophenyl esters. The tendency toward oxazolone formation decreases from benzoyl to acetyl to formyl, and this is indeed paralleled by a decrease in racemization during the coupling reactions. The oxazolone formation, in the case of urethan-protecting groups such as benzyloxycarbonyl-protected amino acids, is not favored, and this may be due to resonance stabilization due to the polarization of the carbonyl bond of benzyloxycarbonyl amino acids. The phthalyl-protected amino acids which do not undergo oxazolone formation also should be safe from racemization. However, racemization in the case of activated acyl amino acids such as benzyloxycarbonyl esters of β-cyanoalanine, derivatives of cysteine, and phthalyl-D-valine, which cannot form oxazolones, has been recorded. The mechanism of this type of racemization may be postulated mainly as a result of excellent studies by Liberek (240). Racemization in these cases most likely occurs by direct exchange of hydrogen at the asymmetric center, particularly when the activation is strong and when the acyl groups and the side chains are powerfully electrophilic.

It has been observed that *N*-benzyloxycarbonyl-*S*-benzyl cysteine esters racemize easily in the presence of base. This racemization may proceed either by resonance stabilization of the anion formed by α-hydrogen abstraction or by reversible β-elimination of the benzyl thiol moiety. The racemization of *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine-*p*-nitrophenyl ester was attributed to base-catalyzed reversible β-elimination of good leaving benzyl thiol group (241, 242). However, in a recent study using benzyl (35S) thiol, Kovacs *et al.* were able to demonstrate that racemization in the case of *p*-nitrophenyl, as well as of the pentachlorophenyl esters of *N*-benzyloxycarbonyl-*S*-benzylcysteine, does not follow the β-elimination readdition mechanism (243).

On the basis of conformational studies of the amide bond, it has been suggested that oxazolone formation is possible only in the case of *trans*-conformation of the amide, since in the case of *cis* amides, the distance of oxygen atom of amide bond and carbon atom in carboxyl group is too far to facilitate the formation of oxazolones (244).

Detection of Racemization—There are several methods available for determining the optical purity of a peptide once it has been synthesized. However, these methods must be distinguished from different approaches in which synthetic model peptides are used to detect the degree of racemization during peptide bond formation. These approaches are based on the isolation of enantiomers by employing such techniques as countercurrent distribution, fractional crystallization and gas-liquid chromatography (GLC), paper and thin-layer chromatography. Most frequently used model peptides, in the fractional crystallization method, are Young's benzoyl-L-leucylglycine ethyl ester (245) and Anderson's benzyloxycarbonylglycyl-L-phenylalanylglycine ethyl ester (246). The formation of D-alloisoleucine from L-isoleucine has been observed by many authors, and the amounts of alloisoleucine and leucine can be quantitatively determined. This affords a good method to determine the extent of racemization during coupling reaction (247). The GLC separation of diastereoisomeric *N*-trifluoroacetyl dipeptide esters provides a good method for the detection of racemization (248); however, it entails a two-step chemical modification of the sample before analysis (replacement of *N*-protecting groups such as benzyloxycarbonyl or *tert*-butyloxycarbonyl by trifluoroacetyl residues). Halpern's findings that *tert*-butyloxycarbonyl amino acid amides can also be separated by GLC (249), and the separation of trifluoroacetyl tripeptide esters by Weygand by GLC (250) will provide further versatility to this method. Recent work on the preparation of diketopiperazines and their steric analysis by different analytical methods (251) may be useful for the determination of degree of racemization, particularly of dipeptides which do not yield volatile trifluoroacetyl derivatives suitable for gas chromatographic analysis.

A relatively new, though extremely promising approach, is the application of nuclear magnetic resonance (NMR) spectroscopy for the detection of racemization in peptides (252). A series of diastereoisomeric *N*-acylalanylphenylalanine methyl esters and *N*-acylphenylalanylalanine methyl esters have been shown to possess different NMR spectra. The methyl doublet signal in an L-L (or D-D) is at lower field than the equivalent signal for the D-L (or L-D) analog. This is most probably due to deshielding. The application of this property has been extended in determining the degree of racemization during the synthesis of a number of peptides with different *N*-acyl-protecting groups. The sensitivity of the NMR method was improved by the use of ^{13}C-H satellite peak of predominant L-L isomer as an internal standard (225).

In many cases, the biological activity of a natural peptide depends, to a large extent, on its optical purity, and the comparison of the biological activities of the natural and synthetic compounds can be used as a reasonable criteria for the optical purity of the synthetic compound. However, the exactitude of biological testing methods poses a serious limitation to this approach. The comparison of optical rotations of synthetic and natural peptides is useful to some extent for determining the optical purity; however, in the compounds with several asymmetric centers, identical values of optical rotation do not necessarily imply optical homogeneity. The most satisfactory method for the determination of steric homogeneity is the enzymatic hydrolysis (253). Renal amino peptidase can be used for total hydrolysis, and the analysis of the hydrolysate can be performed with L or D amino acid oxidase. The hydrolysis with stereo specific enzymes such as leucine amino peptidase, carboxyl peptidase A and B, trypsin, and chymotrypsin has been successfully used for the determination of optical purity. The complete disappearance of the original peptide established by electrophoresis or paper chromatography is evidence for the optical

purity of the peptide bond for which the enzyme is selective. Another method for the determination of the optical purity of the synthetic peptides is the total hydrolysis by acid; the comparison of the hydrolysate with a control containing the expected amino acid residues treated under the identical conditions, provides a guideline for optical purity. A serious drawback of the acid hydrolysis is that some free amino acids, when exposed to acid, may behave differently from the same moieties built in a peptide sequence.

CONCLUSION

A proper selection of protecting groups and coupling methods is one of the most important tasks during the synthesis of larger peptides. Both the classical and relatively less conventional methods have their advantages and disadvantages. It is hard to rationalize the scope and usefulness of one particular method without considering the utilities and refinements of many other techniques which are so important to the successful completion of the synthesis of peptides and polypeptides. The considerations must be given to the control of racemization, the maximum yields during coupling, and the product purification.

Wherever possible, the amino component should be first liberated and then added to the activated component. In addition to the proper selection of the solvents, the choice of hindered amines, such as tribenzylamine or the amines with suitable basicity such as *N*-methylmorpholine, further limit the degree of racemization in the reactions which require the use of a tertiary amine. The use of bifunctional catalysts which were developed by Beyerman is often helpful in accelerating the aminolysis of active esters without accelerating oxazolone formation.

The remarkable progress during the past few years in the techniques used in peptide synthesis strongly confirms that the aims which were once visualized for peptide synthesis, are indeed attainable. This has been demonstrated by the total synthesis of ribonuclease A. Although the synthetic peptide was relatively less active when compared with pure natural bovine pancreatic ribonuclease A, the complete synthesis of a protein molecule with true enzyme activity towards its natural substrate must be considered as one of the landmarks in the progress toward the continuous efforts for better understanding of biochemical processes. These present indications of the progress and refinement of the techniques used in the synthesis of peptides hold excellent promise for the future. The synthesis of larger quantities of biologically active peptides for medicine and the synthesis of intricate enzymes and complex proteins appear to be only a few steps away.

REFERENCES

(1) E. Fischer, *Chem. Ber.*, **39,** 530(1906).

(2) M. Bergmann and L. Zervas, *ibid.*, **65,** 1192(1932).

(3) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Am. Chem. Soc.*, **75,** 4879 (1953).

(4) H. Schwarz, F. M. Bumpus, and I. H. Page, *ibid.*, **79,** 5697(1957); R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *Helv. Chim. Acta*, **41,** 1273(1958).

(5) R. A. Boissonnas, S. Guttmann, P. A. Jaquenoud, H. Konzett, and E. Sturmer, *Experientia*, **16,** 326(1960).

(6) E. Sandrin and R. A. Boissonnas, *ibid.*, **18,** 59(1962).

(7) R. Schwyzer, A. Costopanagiotis, and P. Sieber, *Helv. Chim. Acta*, **46,** 870(1963).

(8) R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *ibid.*, **46,** 1975(1963).

(9) J. C. Anderson, G. W. Kenner, J. K. MacLeod, and R. C. Sheppard, *Tetrahedron Suppl.*, **8,** 39(1966).

(10) R. Schwyzer and P. Sieber, *Nature*, **199,** 172(1963).

(11) J. Meienhofer, E. Schnabel, H. Bremer, O. Brinkhoff, R. Zabel, W. Sroka, H. Klostermeyer, D. Brandenburg, T. Okuda, and H. Zahn, *Z. Naturforsch.*, **18b,** 1130(1963); P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Susuki, and M. Tilak, *J. Am. Chem. Soc.*, **86,** 930(1964).

(12) M. A. Ondetti, V. L. Narayanan, M. von Saltza, J. T. Sheehan, E. F. Sabo, and M. Bodanszky, *J. Am. Chem. Soc.*, **90,** 4711(1968).

(13) E. Wunsch, *Z. Naturforsch.*, **22b,** 1269(1967).

(14) S. Guttmann, J. Pless, E. Sandrin, P. A. Jaquenoud, H. Bossert, and H. Willems, *Helv. Chim. Acta*, **51,** 1155(1968).

(15) V. du Vigneaud, G. Winestock, V. V. S. Murti, D. B. Hope, and R. D. Kimbrough, Jr., *J. Biol. Chem.*, **235,** RC 64(1960).

(16) B. Berde, H. Weidman, and A. Cerletti, *Helv. Physiol. Pharmacol. Acta*, **19,** 285(1961).

(17) J. S. Fruton, "Advances in Protein Chemistry," vol. 5, Academic, New York, N. Y., 1949, p. 1.

(18) T. Wieland, *Angew. Chem.*, **63,** 7(1951).

(19) *Ibid.*, **66,** 507(1954).

(20) T. Wieland, and B. Heinke, *ibid.*, **69,** 362(1957).

(21) T. Wieland, *ibid.*, **71,** 417(1959).

(22) T. Wieland and H. Determann, *Angew. Chem.*, **75,** 539 (1963).

(23) W. Grassmann and E. Wunsch, "Progress in the Chemistry of Organic Natural Products," vol. 13, Springer Verlag, Vienna, Austria, 1956, p. 444.

(24) M. Goodman and G. W. Kenner, "Advances in Protein Chemistry," vol. 12, Academic, New York, N. Y., 1959, p. 465.

(25) M. Bodanszky, *Records Chem. Progr.*, **18,** 187(1957).

(26) E. Bricas, *Bull. Soc. Chim. France*, **1961,** 2001.

(27) K. Hofmann and P. G. Katsoyannis, "The Proteins," 2nd ed., Vol. 1, H. Neurath, Ed., Academic, New York, N. Y., 1963, p. 54.

(28) J. Meienhofer, *Chimia (Aarau)*, **16,** 385(1962).

(29) E. Schroder and K. Lubke, "Progress in the Chemistry of Organic Natural Products," vol. 26, Springer Verlag, Vienna, Austria, 1958, p. 48.

(30) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Wiley, New York, N. Y., 1961.

(31) E. Schroder and K. Lubke, "The Peptides," vol. 2, Academic, New York, N. Y., 1965.

(32) M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience, New York, N. Y., 1966.

(33) R. A. Boissonnas, *Advan. Org. Chem.*, **3,** 159(1963).

(34) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17,** 1564(1952).

(35) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108,** 753(1935).

(36) A. E. Barkdoll and W. F. Ross, *J. Am. Chem. Soc.*, **66,** 951(1944).

(37) J. A. Barltrop and P. Schofield, *Tetrahedron Letters*, **1962,** 697.

(38) J. W. Chamberlin, *J. Org. Chem.*, **31,** 1658(1966).

(39) K. Blaha and J. Rudinger, *Collection Czech. Chem. Commun.*, **30,** 585(1965).

(40) J. A. MacLaren, *Australian J. Chem.*, **11,** 360(1958).

(41) M. Bergmann, L. Zervas, and W. F. Ross, *J. Biol. Chem.*, **111,** 245(1935).

(42) J. Kovacs, N. H. Kovacs, J. K. Chakrabarti, and A. Kapoor, *Experientia*, **21,** 20(1965).

(43) D. M. Channing, P. B. Turner, and G. T. Young, *Nature*, **167,** 487(1951).

(44) R. A. Boissonnas and G. Preitner, *Helv. Chim. Acta*, **36,** 875(1953).

(45) F. H. Carpenter and D. T. Gish, *J. Am. Chem. Soc.*, **74,** 3818(1952).
(46) F. C. McKay and N. F. Albertson, *ibid.*, **79,** 1680(1957).
(47) J. Meienhofer, in "Proceedings of the Sixth European Peptide Symposium, Athens, 1963," L. Zervas, Ed., Pergamon Press, Oxford, England, 1966, p. 55.
(48) R. Schwyzer, P. Sieber, and K. Zatsko, *Helv. Chim. Acta*, **41,** 491(1958).
(49) P. Sieber and B. Iselin, *ibid.*, **51,** 622(1968).
(50) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79,** 6180(1957).
(51) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42,** 2622(1959).
(52) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79,** 6180(1957).
(53) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull. (Tokyo)*, **15,** 2015(1967).
(54) E. Schnabel, H. Herzog, P. Hoffmann, E. Klauke, and I. Ugi, *Angew. Chem.*, **7,** 380(1968).
(55) S. Sakakibara and M. Fujino, *Bull. Chem. Soc. Japan*, **39,** 947(1966).
(56) N. Izumiya, *ibid.*, **26,** 53(1953).
(57) M. Zaoral, *Collection Czech. Chem. Commun.*, **27,** 1273 (1962).
(58) M. T. Leplawy, D. S. Jones, G. W. Kenner, and R. C. Sheppard, *Tetrahedron*, **11,** 39(1960).
(59) R. Schonheimer, *Z. Physiol. Chem.*, **154,** 203(1926).
(60) B. C. Barras and D. T. Elmore, *J. Chem. Soc.*, **1957,** 3134.
(61) A. F. Beecham, *Chem. Ind. (London)*, **1955,** 1120.
(62) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117,** 27(1937).
(63) J. Kovacs and U. R. Ghatak, *Chem. Ind. (London)*, **1963,** 913.
(64) S. Guttmann, in "Proceedings of the Fifth European Peptide Symposium, Oxford, 1962," G. T. Young, Ed., Pergamon Press, Oxford, 1963, p. 41.
(65) S. Bajusz and K. Medzihradszky, *ibid.*, p. 49.
(66) M. Bodanszky and C. A. Birkhimer, *Chimia (Aarau)*, **14,** 368(1960).
(67) F. Weygand and E. Csendes, *Angew. Chem.*, **64,** 136(1952).
(68) F. Weygand, H. Geiger, and W. Swodenk, *ibid.*, **68,** 307 (1956).
(69) F. Weygand, *Chimia (Aarau)*, **14,** 378(1960).
(70) L. Zervas and D. M. Theodoropoulos, *J. Am. Chem. Soc.*, **78,** 1359(1956).
(71) R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, **44,** 159 (1961).
(72) R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *ibid.*, **46,** 1975(1963).
(73) D. A. A. Kidd and F. E. King, *Nature*, **162,** 776(1948).
(74) J. C. Sheehan and V. S. Frank, *J. Am. Chem. Soc.*, **71,** 1856(1949).
(75) G. H. L. Nefkens, *Nature*, **185,** 309(1960).
(76) M. Bodanszky, M. A. Ondetti, C. A. Birkhimer, and P. L. Thomas, *J. Am. Chem. Soc.*, **86,** 4452(1964).
(77) G. R. Banks, D. Cohen, G. E. Pattenden, and J. A. G. Thomas, *J. Chem. Soc.*, **1967,** 126.
(78) L. Zervas, D. Borovas, and E. Gazis, *J. Am. Chem. Soc.*, **85,** 3660(1963).
(79) J. Meienhofer, *Nature*, **205,** 73(1965).
(80) A. Fontana, F. Marchiori, L. Moroder, and E. Scoffone, *Tetrahedron Letters*, **1966,** 2985.
(81) W. Kessler and B. Iselin, *Helv. Chim. Acta*, **49,** 1330 (1966).
(82) A. Fontana, F. Marchiori, and L. Moroder, *Ric. Sci.*, **36,** 261(1966).
(83) E. Wunsch, A. Fontana, and F. Drees, *Z. Naturforsch.*, **22b,** 607(1967).
(84) F. M. Veronese, A. Fontana, E. Boccu, and C. A. Benassi, *ibid.*, **23b,** 1319(1968).
(85) I. Phocas, C. Yovanidis, I. Photaki, and L. Zervas, *J. Chem. Soc.*, **1967,** 1506.
(86) E. Dane, F. Drees, P. Konrad, and T. Dockner, *Angew. Chem.*, **74,** 873(1962).
(87) J. C. Sheehan and V. J. Grenda, *J. Am. Chem. Soc.*, **84,** 2417(1962).
(88) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36,** 1109 (1953).
(89) W. E. Hanby, S. G. Waley, and J. Watson, *J. Chem. Soc.*, **1950,** 3239.
(90) E. Taschner and B. Liberek, *Roczniki Chem.*, **30,** 323 (1956).
(91) B. F. Erlanger and R. M. Hall, *J. Am. Chem. Soc.*, **76,** 5781(1954).
(92) E. Brand, B. F. Erlanger, H. Sachs, and J. Polatnick, *ibid.*, **73,** 3510(1951).
(93) H. K. Miller and H. Waelsch, *ibid.*, **74,** 1092(1952).
(94) L. Benoiton and H. N. Rydon, *J. Chem. Soc.*, **1960,** 3328.
(95) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17,** 1564(1952).
(96) N. F. Albertson and C. F. McKay, *J. Am. Chem. Soc.*, **75,** 5325(1953).
(97) H. Schwarz and K. Arakawa, *ibid.*, **81,** 5691(1959).
(98) E. Schroder and E. Klieger, *Ann. Chem.*, **673,** 196(1964).
(99) F. Weygand and K. Hunger, *Chem. Ber.*, **95,** 1(1962).
(100) F. H. C. Stewart, *Australian J. Chem.*, **21,** 2543(1968).
(101) F. Weygand, W. Steglich, J. Bjarnason, R. Akhtar, and N. Chytil, *Chem. Ber.*, **101,** 3623(1968).
(102) F. H. C. Stewart, *Australian J. Chem.*, **20,** 2243(1967).
(103) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82,** 3359(1960).
(104) H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, **44,** 1136 (1961).
(105) M. J. S. A. Amaral, G. C. Barrett, H. N. Rydon, and J. E. Willett, *J. Chem. Soc.*, **1966,** 807.
(106) P. M. Hardy, H. N. Rydon, and R. C. Thompson, *Tetrahedron Letters*, **1968,** 2525.
(107) K. Hofmann and A. Johl, *J. Am. Chem. Soc.*, **77,** 2914 (1955).
(108) R. A. Boissonnas, S. Guttmann, and P. A. Jaquenoud, *Helv. Chim. Acta*, **43,** 1349(1960).
(109) G. H. L. Nefkens, *Nature*, **193,** 974(1962).
(110) G. C. Stelakatos, A. Paganou, and L. Zervas, *J. Chem. Soc.*, **1966,** 1191.
(111) A. A. Aboderrin, G. R. Delpierre, and J. S. Fruton, *J. Am. Chem. Soc.*, **87,** 5469(1965).
(112) R. G. Hiskey and J. B. Adams, *ibid.*, **87,** 3969(1965).
(113) G. C. Stelakatos, A. Paganou, and L. Zervas, *J. Chem. Soc.*, **1966,** 1191.
(114) J. Taylor-Papadimitriou, C. Yovanidis, A. Paganou, and L. Zervas, *ibid.*, **1967,** 1830.
(115) B. J. Johnson and P. M. Jacobs, *Chem. Commun.*, **1968,** 73.
(116) R. Garner and G. T. Young, *Nature*, in press; R. Garner, D. J. Schaffer, W. B. Watkins, and G. T. Young, *Reference 204*, p. 145.
(117) T. Wieland and W. Racky, *Chimia (Aarau)*, **22,** 375(1968).
(118) A. Kapoor, E. J. Davis, and M. J. Graetzer, *J. Pharm. Sci.*, **57,** 1514(1968).
(119) D. S. Kemp and S. W. Chien, *J. Am. Chem. Soc.*, **89,** 2743 (1967).
(120) E. Klieger, E. Schroder, and H. Gibian, *Ann. Chem.*, **640,** 157(1961).
(121) E. Schroder and E. Klieger, *ibid.*, **673,** 208(1964).
(122) F. Weygand, W. Steglich, F. Fraunberger, P. Pietta, and J. Schmid, *Chem. Ber.*, **101,** 923(1968).
(123) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117,** 27(1937).
(124) A. Patchornik, A. Berger, and E. Katchalski, *J. Am. Chem. Soc.*, **79,** 6416(1957).
(125) L. Zervas and D. M. Theodoropoulos, *ibid.*, **78,** 1359 (1956).
(126) F. Weygand, W. Steglich, and P. Pietta, *Chem. Ber.*, **100,** 3841(1967).
(127) W. L. Haas, E. V. Krumkalns, and K. Gerson, *J. Am. Chem. Soc.*, **88,** 1988(1966).
(128) E. Gazis, D. Borovas, C. Hamalidis, G. C. Stelakatos, and L. Zervas, in "Proceedings of the Sixth European Peptide Symposium, Athens, 1963," L. Zervas, Ed., Pergamon Press, Oxford, England, 1966.
(129) J. Fruton, "Advances in Protein Chemistry," vol. 5, Academic, New York, N. Y., 1949, p. 64.
(130) M. Bodanszky, M. A. Ondetti, C. A. Birkhimer, and P. L. Thomas, *J. Am. Chem. Soc.*, **86,** 4452(1964).

(131) S. Guttmann and J. Pless, *Acta Chim. Acad. Sci. Hung.*, **44,** 23(1964).
(132) S. Guttmann and J. Pless, *Chimia (Aarau)*, **18,** 185(1964).
(133) J. Pless and S. Guttmann, in "Proceedings of the Eighth European Peptide Symposium," H. C. Beyerman, A. Van DeLinde, and W. Massen van der Brink, Eds., Wiley, New York, N. Y., 1967, p. 50.
(134) V. du Vigneaud, L. F. Audrieth, and H. S. Loring, *J. Am. Chem. Soc.*, **52,** 4500(1930).
(135) I. Photaki and V. Bardakos, *Experientia*, **21,** 371(1965).
(136) M. Wilchek, C. Zioudrou, and A. Patchornik, *J. Org. Chem.*, **31,** 2865(1966).
(137) D. F. Veber, J. D. Milkowski, R. G. Denkewalter, and R. Hirschmann, *Tetrahedron Letters*, **26,** 3057(1968).
(138) W. Konig, R. Geiger, and W. Siedel, *Chem. Ber.*, **101,** 681(1968).
(139) S. Goldschmidt, *Angew. Chem.*, **62,** 538(1950).
(140) S. Goldschmidt and H. Lautenschlager, *Ann. Chem.*, **580,** 68(1953).
(141) G. W. Anderson, A. D. Welcher, and R. W. Young, *J. Am. Chem. Soc.*, **73,** 501(1951).
(142) J. R. Vaughan, Jr., *ibid.*, **73,** 1389(1951).
(143) B. F. Erlanger and N. Kokowsky, *J. Org. Chem.*, **26,** 2534 (1961).
(144) T. Wieland, W. Kern, and R. Sehring, *Ann. Chem.*, **569,** 117(1950).
(145) T. Wieland and H. Bernhard, *ibid.*, **572,** 190(1951).
(146) J. R. Vaughan, Jr., and R. L. Osato, *J. Am. Chem. Soc.*, **74,** 676(1952).
(147) N. F. Albertson, "Organic Reactions," vol. 12, Wiley, New York, N. Y., 1962, p. 157.
(148) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **89,** 5012(1967).
(149) A. Kapoor, L. W. Gerencser, and W. R. Koutnik, *J. Pharm. Sci.*, **58,** 281(1969).
(150) A. Cosmatos, I. Photaki, and L. Zervas, *Chem. Ber.*, **94,** 2644(1961).
(151) T. Curtius, *ibid.*, **35,** 3226(1902).
(152) J. S. Fruton, *J. Biol. Chem.*, **146,** 463(1942).
(153) K. Hofmann, M. Z. Magee, and A. Lindenmann, *J. Am. Chem. Soc.*, **72,** 2814(1950).
(154) F. Weygand and W. Steglich, *Chem. Ber.*, **92,** 313(1959).
(155) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77,** 1067 (1955).
(156) H. G. Khorana, *Chem. Ind. (London)*, **1955,** 1087.
(157) J. C. Sheehan, P. A. Cruickshank, and G. L. Boshart, *J. Org. Chem.*, **26,** 2525(1961).
(158) Y. Wolman, S. Kivity, and M. Frankel, *Chem. Commun.*, **1967,** 629.
(159) J. F. Arens, *Rec. Trav. Chim.*, **74,** 769(1955).
(160) R. Buyle and G. H. Viehe, *Angew. Chem.*, **76,** 572(1964).
(161) F. Weygand, P. Huber, and K. Weiss, *Z. Naturforsch.*, **22b,** 1084(1967).
(162) C. L. Stevens and M. E. Munk, *J. Am. Chem. Soc.*, **80,** 4069(1958).
(163) R. B. Woodward and R. A. Olofson, *ibid.*, **83,** 1010(1961).
(164) R. B. Woodward, R. A. Olofson, and H. Mayer, *Tetrahedron*, **1966,** 321.
(165) R. B. Woodward and D. J. Woodman, *J. Am. Chem. Soc.*, **90,** 1371(1968).
(166) S. Rajappa and A. S. Akerkar, *Chem. Commun.*, **1966,** 826.
(167) B. Belleau and G. Malek, *J. Am. Chem. Soc.*, **90,** 1651 (1968).
(168) K. D. Kopple and D. E. Nitecki, *ibid.*, **83,** 4103(1961); A. Patchorik, A. Berger, and E. Katchalski, *ibid.*, **79,** 185(1957).
(169) J. C. Sheehan, K. Hasspacher, and Y. L. Yeh, *ibid.*, **81,** 6086(1959).
(170) H. A. Staab and K. Wendel, *Chem. Ber.*, **96,** 3374(1963).
(171) G. W. Anderson and R. Paul, *J. Am. Chem. Soc.*, **80,** 4423(1958).
(172) H. C. Beyerman and W. Maassen van der Brink, *Rec. Trav. Chim.*, **80,** 1372(1961).
(173) T. Wieland, W. Schafer, and E. Bokelmann, *Ann. Chem.*, **573,** 99(1951).
(174) R. Schwyzer, *Helv. Chim. Acta*, **37,** 674(1954).
(175) M. Bodanszky, *Nature*, **175,** 685(1955).
(176) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, **40,** 373(1957).
(177) G. Kupryszewski and M. Formela, *Roczniki Chem.*, **35,** 1933(1961).
(178) K. Stick and G. H. Leemann, *Helv. Chim. Acta*, **46,** 1887 (1963).
(179) J. Kovacs, R. Gianotti, and A. Kapoor, *J. Am. Chem. Soc.*, **88,** 2282(1966).
(180) G. H. L. Nefkens and G. I. Tesser, *ibid.*, **83,** 1263(1961).
(181) G. W. Anderson, J. E. Zimmerman, and F. Callahan, *ibid.*, **85,** 3039(1963).
(182) S. M. Beaumont, B. O. Hanford, J. H. Jones, and G. T. Young, *Chem. Commun.*, **1965,** 53.
(183) G. T. Young, in "Proceedings of the Eighth European Peptide Symposium," H. C. Beyerman, A. Van DeLinde, and W. Massen van der Brink, Eds., Wiley, New York, N.Y., 1967, p. 55.
(184) H. D. Jakubke and A. Voigt, *Chem. Ber.*, **99,** 2419(1966).
(185) J. H. Jones and G. T. Young, *J. Chem. Soc.*, **1968,** 436.
(186) K. Lloyd and G. T. Young, *Chem. Commun.*, **1968,** 1400.
(187) E. Wunsch and A. Fontana, *Chem. Ber.*, **101,** 323(1968).
(188) C. Meyers, R. T. Havran, I. L. Schwartz, and R. Walter, *Chem. Ind.*, in press.
(189) M. Bodansky, M. A. Ondetti, S. D. Levine, and N. J. Williams, *J. Am. Chem. Soc.*, **89,** 6753(1967).
(190) D. G. Knorre and T. N. Shubina, *Acta Chim. Acad. Sci. Hung.*, **44,** 77(1965).
(191) J. C. Sheehan, J. Preston, and P. A. Cruickshank, *J. Am. Chem. Soc.*, **87,** 2492(1965).
(192) R. B. Merrifield, *ibid.*, **85,** 2149(1963).
(193) V. A. Najjar and R. B. Merrifield, *Biochemistry*, **5,** 3765 (1966).
(194) M. Bodanszky and J. T. Sheehan, *Chem. Ind.*, **1964,** 1423.
(195) M. C. Khosla, N. C. Chaturvedi, R. R. Smeby, and F. M. Bumpus, *Biochemistry*, **7,** 3417(1968).
(196) M. Bodanszky and J. T. Sheehan, *Chem. Ind.*, **1966,** 1597.
(197) R. L. Letsinger and M. Kornet, *J. Am. Chem. Soc.*, **85,** 3045(1963).
(198) M. M. Shemyakin, Y. A. Ovchinnikov, A. A. Kiryushkin, and I. V. Kozhevnikova, *Tetrahedron Letters*, **27,** 2323(1965).
(199) M. Fridkin, A. Patchornik, and E. Katchalski, *J. Am. Chem. Soc.*, **87,** 4646(1965).
(200) T. Wieland and C. Birr, *Angew. Chem.*, **78,** 303(1966).
(201) A. Patchornik, M. Fridkin, and E. Katchalski, in "Proceedings of the Eighth European Peptide Symposium," H. C. Beyerman, A. Van DeLinde, and W. Massen van der Brink, Eds., Wiley, New York, N. Y., 1967, p. 91.
(202) D. A. Laufer, T. M. Chapman, D. I. Marlborough, V. M. Vaidya, and E. R. Blout, *J. Am. Chem. Soc.*, **90,** 2696(1968).
(203) F. Weygand and U. Ragnarsson, *Z. Naturforsch.*, **21b,** 1141(1966).
(204) F. Weygand, in "Proceedings of the Ninth European Peptide Symposium," E. Bricus, Ed., North-Holland Publishing Co., Amsterdam, The Netherlands, 1968, p. 183.
(205) G. S. Omenn and C. B. Anfinsen, *J. Am. Chem. Soc.*, **90,** 6571(1968).
(206) R. B. Merrifield, J. M. Stewart, and N. Jernberg, *Anal. Chem.*, **38,** 1905(1966).
(207) B. Gutte and R. B. Merrifield, *J. Am. Chem. Soc.*, **91,** 501(1969).
(208) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40,** 2164(1967).
(209) W. Kessler and B. Iselin, *Helv. Chim. Acta*, **49,** 1330 (1966).
(210) M. Ohno and C. B. Anfinsen, *J. Am. Chem. Soc.*, **89,** 5994 (1967).
(211) H. C. Beyerman, C. A. M. Boers-Boonekamp, W. J. Van Zoest, and D. Van Den Berg, in "Proceedings of the Eighth European Peptide Symposium," H. C. Beyerman, A. Van DeLinde, and W. Massen van der Brink, Eds., Wiley, New York, N. Y., 1967, p. 117.
(212) G. I. Tesser and B. W. J. Ellenbroek, *ibid.*, p. 124.
(213) H. Hayatsu and H. G. Khorana, *J. Am. Chem. Soc.*, **88,** 3182(1966).
(214) P. Kusch, *Angew. Chem.*, **78,** 611(1966).
(215) M. Rothe, W. Dunkel, K.-D. Steffen, and Hj. Schneider, *ibid.*, **7,** 399(1968).

(216) S. Sakakibara, Y. Kishida, Y. Kikuchi, R. Sakai, and K. Kakiuchi, *Bull. Chem. Soc. Japan*, **41,** 1273(1968).
(217) M. Rothe and W. Dunkel, *Polymer Letters*, **5,** 589(1967).
(218) G. R. Stark, *Federation Proc.*, **24,** 225(1965).
(219) R. A. Laursen, *J. Am. Chem. Soc.*, **88,** 5344(1966).
(220) F. Weygand and R. Obermeier, *Z. Naturforsch.*, **23b,** 1390(1968).
(221) M. Szwarc, *Advan. Polymer Sci.*, **4,** 1(1965).
(222) P. D. Bartlett and R. H. Jones, *J. Am. Chem. Soc.*, **79,** 2153(1957).
(223) N. H. Grant and H. E. Alburn, *ibid.*, **86,** 3870(1964).
(224) R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Veber, E. F. Schoenewaldt, H. Barkemeyer, W. J. Paleveda, Jr., T. A. Jacob, and H. Hirschmann, *ibid.*, **88,** 3163 (1966).
(225) R. S. Dewey, E. F. Schoenewaldt, H. Joshua, W. J. Paleveda, Jr., H. Schwam, H. Barkemeyer, B. H. Arison, D. F. Veber, R. G. Denkewalter, and R. Hirschmann, *ibid.*, **90,** 3254(1968).
(226) R. Hirschmann, R. F. Nutt, D. F. Veber, R. A. Vitali, S. L. Varga, T. A. Jacob, F. W. Holly, and R. G. Denkewalter, *ibid.*, **91,** 507(1969).
(227) T. Wieland and C. Birr, *Chimia (Aarau)*, **21,** 581(1967).
(228) T. Wieland and W. Racky, *ibid.*, **22,** 375(1968).
(229) S. W. Fox, *Nature*, **205,** 328(1965).
(230) S. W. Fox and C. Wang, *Science*, **160,** 547(1968).
(231) R. Schwyzer, J. P. Carrion, B. Group, H. Nolting, and T. K. Aung, *Helv. Chim. Acta*, **47,** 441(1964).
(232) M. A. Ondetti, A. Deer, J. T. Sheehan, J. Pluscec, and O. Kocy, *Biochemistry*, **7,** 4069(1968).
(233) M. W. Williams and G. T. Young, *J. Chem. Soc.*, **1964,** 3701.
(234) M. Goodman and L. Levine, *J. Am. Chem. Soc.*, **86,** 2918 (1964).
(235) S. M. Beaumont, B. O. Handford, and G. T. Young, *Acta Chim. Acad. Sci. Hung.*, **44,** 37(1965).
(236) D. S. Kemp and S. W. Chien, *J. Am. Chem. Soc.*, **89,** 2745 (1967).
(237) M. Goodman and W. J. McGahren, *ibid.*, **88,** 3887(1966); *Tetrahedron*, **23,** 2031(1967).
(238) I. Antonovics and G. T. Young, *Chem. Commun.*, **1965,** 398.
(239) F. Weygand, A. Prox, and W. Konig, *Chem. Ber.*, **99,** 1446(1966).
(240) B. Liberek and A. Michalik, *Roczniki Chem.*, **40,** 597 (1966).
(241) J. A. Maclaren, W. E. Savige, and J. M. Swan, *Australian J. Chem.*, **11,** 345(1958).
(242) M. Bodanszky and A. Bodanszky, *Chem. Commun.*, **1967,** 591.
(243) J. Kovacs, G. L. Mayers, R. H. Johnson, and U. R. Ghatak, *ibid.*, **1968,** 1066.
(244) I. S. Siemion and D. Konopinska, in "Proceedings of the Eighth European Peptide Symposium," H. C. Beyerman, A. Van DeLinde, and W. Massen van der Brink, Eds., Wiley, New York, N. Y., 1967, p. 78.
(245) N. A. Smart, G. T. Young, and M. W. Williams, *J. Chem. Soc.*, **1960,** 3902.
(246) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **80,** 2902(1958).
(247) M. Bodanszky and L. E. Conklin, *Chem. Commun.*, **1967,** 773.
(248) F. Weygand, A. Prox, L. Schmidhammer, and W. Konig, *Angew. Chem. Intern. Ed.*, **2,** 183(1963).
(249) B. Halpern, L. F. Chew, and J. W. Westley, *Anal. Chem.*, **39,** 399(1967).
(250) F. Weygand, D. Hoffmann, and A. Prox, *Z. Naturforsch.*, **23b,** 379(1968).
(251) D. E. Nitecki, B. Halpern, and J. W. Westley, *J. Org. Chem.*, **33,** 864(1968).
(252) B. Halpern, L. F. Chew, and B. Weinstein, *J. Am. Chem. Soc.*, **89,** 5051(1967).
(253) K. Hofmann and E. T. Schwartz, *ibid.*, **83,** 2289(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Pharmaceutical Chemistry, College of Pharmacy, St. John's University, Jamaica, NY 11432*

The author sincerely appreciates the valuable assistance of Dr. H. Eisen, Dr. C. Ward, Mrs. M. Colgan, and Miss C. Czech during the preparation of this manuscript.

The abbreviations used in this paper for amino acids and peptides are mainly those recommended in (*a*) "Peptides," *Proceedings of the Fifth, European Peptide Symposium Oxford, 1962*, G. T. Young, Ed., Pergamon Press, Oxford, 1963, p. 262, and (*b*) IUPAC-IUB Commission on Biochemical Nomenclature, abbreviated designation of amino acids derivatives and peptides. Tentative rules: *J. Biol. Chem.*, **241,** 527, 2491(1966); *Biochim. Biophys. Acta*, **121,** 1(1966).

Intermolecular and Intramolecular Catalysis in Deamination of Cytosine Nucleosides

ROBERT E. NOTARI, MARILYN LUE CHIN, and ALEX CARDONI

Abstract □ The rates of deamination of arabinosylcytosine (Ara-C), cytidine (Cyd) and cytosine (C) are compared in the presence and in the absence of catalytic buffers. Ratios of the rate constants in the absence of intermolecular catalysis by buffers indicate intramolecular catalysis by the 2′-hydroxyl in Ara-C. For example, the ratio of the deamination rate constants at pH 4.7, 70° is 53/1.4/1 for Ara-C/Cyd/C. In contrast to Cyd and C which show catalysis by both the acidic and basic components of the buffers, Ara-C exhibits only general-acid catalysis. These data suggest that intramolecular participation by the 2′-hydroxyl in Ara-C replaces the general-base catalytic role which the buffers perform in the deamination of C and Cyd. Mechanisms for inter- and intramolecular catalysis in the deamination of Ara-C, Cyd, and C are discussed.

Keyphrases □ Cytosine nucleosides—deamination □ Catalysis, inter- and intramolecular—cytosine nucleosides □ Kinetics—cytosine nucleosides deamination □ UV spectrophotometry—analysis

Cytosine (C), cytidine (Cyd) and 1-β-D-arabinosylcytosine (Ara-C)[1] are known to undergo hydrolytic deamination in aqueous buffered solutions to yield the corresponding uracil derivatives as shown in Scheme I.

The authors have previously reported on the kinetics of Ara-C deamination in phosphate buffers at several temperatures in the presence and absence of sodium bisulfite (1). The major catalytic species in aqueous phosphate buffers at 70°, pH 6–8, was shown to be $H_2PO_4^-$. Other workers have demonstrated that the deamination of C and Cyd is subject to buffer catalysis by carboxylate and pyridine buffers at pH < 6, 95° (2, 3). The catalytic constants for the individual buffer species could not be calculated from previous data for C and Cyd (2, 3) and the catalytic constant for $H_2PO_4^-$ was not reported (1).

In the authors' initial publication the fact was emphasized that insufficient data was available to quantitatively compare the catalytic effects of buffers on the deamination of Ara-C, Cyd, and C. The authors have now demonstrated that the kinetics of deamination of the arabinosyl nucleoside is dramatically different from either the ribosyl nucleoside or cytosine itself and that C and Cyd behave relatively similarly by comparison. This report provides the first data allowing: (*a*) comparison of the catalytic constants for several buffer components in the deamination kinetics of C, Cyd, and Ara-C and (*b*) a direct comparison of their deamination rates in the absence of buffer catalysis under a variety of experimental conditions. The results clearly indicate a significant contribution due to intramolecular participation by the 2′-hydroxyl in the hydrolytic deamination of Ara-C. There is evidence that C and Cyd are subject to both general-acid and general-base catalysis while general-acid catalysis predominates in the case of Ara-C at 70 and 80°. Under certain conditions the apparent first-order rate constants for Ara-C deamination are shown to increase rapidly as a function of acetic acid concentration and then become relatively constant and independent of the buffer concentration. This apparent change in the rate determining step is not evident in the buffer data for the deamination of C or Cyd under the conditions of this report.

[1] USAN approved name is cytarabine; common name is cytosine arabinoside.

EXPERIMENTAL

Materials—Arabinosylcytosine and arabinosyluridine (Upjohn Co., Kalamazoo, Mich.), and cytosine, uracil, cytidine, and uridine (Mann Research Laboratories, New York, N. Y. and Nutritional Biochemicals Co., Cleveland, Ohio) were used.

Analytical Methods—The wavelengths of maximum absorption as a function of pH were determined using a Cary model 15 spectrophotometer. Beer's law plots for cytosine and uracil in 0.1 *N* HCl were constructed using a Beckman DU spectrophotometer and the absorptivities, *a*, were calculated to be 1.0×10^4 (275 mμ), 5.9×10^3 (259 mμ) for C, and 3.8×10^3 (275 mμ), 8.2×10^3 (259 mμ) for U. Solution of the simultaneous equations for total absorption, *A*, from a mixture of C and U yields:

$$C = (1.37\,A_{275} - 0.625\,A_{259}) \times 10^{-4} \quad \text{(Eq. 1)}$$

$$U = (1.67\,A_{259} - 0.990\,A_{275}) \times 10^{-4} \quad \text{(Eq. 2)}$$

The concentration of each component in mixtures containing C and U were calculated from the A_{275} and A_{259} in 0.1 *N* HCl using Eqs. 1 and 2.

A similar treatment for arabinosylcytosine and arabinosyluracil results in Eqs. 3 and 4.

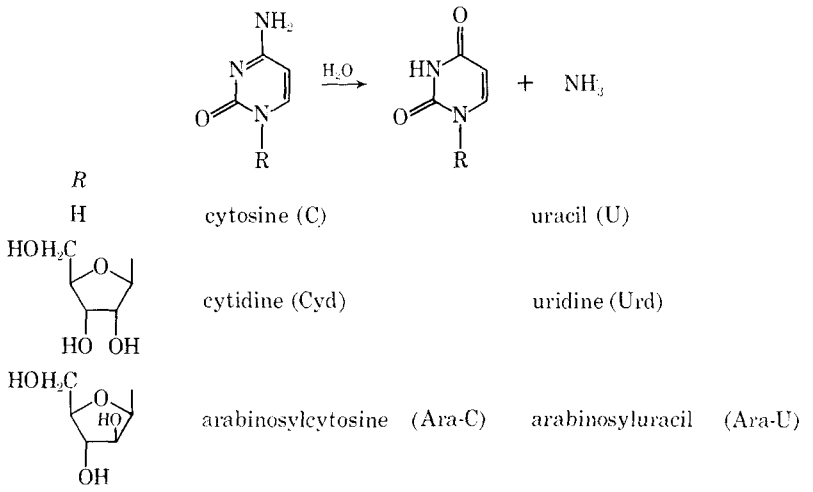
$$\text{Ara-C} = (0.927\,A_{280} - 0.389\,A_{260}) \times 10^{-4} \quad \text{(Eq. 3)}$$

$$\text{Ara-U} = (1.21\,A_{260} - 0.546\,A_{280}) \times 10^{-4} \quad \text{(Eq. 4)}$$

The concentration of Ara-C or Ara-U in a mixture was calculated from Eq. 3 or 4 using the A_{280} and A_{260} in 0.1 *N* HCl where the absorptivities were determined as 1.3×10^4 (280 mμ), 6.0×10^3 (260 mμ) for Ara-C, and 4.3×10^3 (280 mμ), 1.0×10^4 (260 mμ) for Ara-U.

The method of Loring and Ploeser (4) was employed in the analysis of cytidine and uridine mixtures.

Kinetics of Deamination—The kinetics of hydrolytic deamination of C, Cyd, and Ara-C, were determined under pseudo first-order conditions by maintaining constant pH and a sufficient excess of buffer. The temperature and ionic strength were held constant within each study. The pH was measured at the temperature of the



Scheme I

kinetic run using a digital readout pH meter (Sargent model DR) and combination electrode pH 7–14, 0–80° before and after the reactions were carried out. All reactions were followed for one to seven half-lives in the case of Cyd, for more than 40% of the reaction in the Case of C, and until complete loss of substrate in the case of Ara-C. The concentrations of the reactants and products were determined as described in the previous section. Details of the experimental conditions are given in Tables I, II, and III.

RESULTS

Kinetics of Deamination—Good first-order plots were obtained under all experimental conditions when graphed according to the equation

$$\ln X = \ln X_0 - k_1 t \qquad \text{(Eq. 5)}$$

where X is the molar concentration of unreacted C, Cyd, or Ara-C and X_0 is the corresponding initial concentration. The apparent first-order rate constants, k_1, calculated from the slopes of these plots are listed in Tables I, II, and III. The effect of buffer concentration on the apparent first-order rate constants is discussed in the following sections.

Table I—Experimental Conditions and Apparent First-Order Rate Constants for Deamination of 5.0×10^{-4} *M* Arabinosylcytosine

°C.	Observed pH	Buffer Compn. [CH_3–COOH]	Buffer Compn. [CH_3–COONa]	Buffer Compn. [NaCl]	$10^3 k_1$, hr.$^{-1}$
70	3.66 ± 0.05	3.60	0.36	0.00	53.8
		0.50	0.050	0.31	51.2
		0.30	0.030	0.33	49.1
		0.10	0.010	0.35	43.4
		0.05	0.005	0.36	38.8
	4.71 ± 0.07	0.36	0.36	0.00	25.9
		0.10	0.10	0.26	17.7
		0.050	0.050	0.31	15.3
		0.005	0.005	0.35	10.4
	5.66 ± 0.08	0.035	0.35	0.01	3.24
		0.020	0.20	0.16	2.79
		0.010	0.10	0.26	2.29
		0.005	0.05	0.31	1.84
80	3.66 ± 0.05	3.50	0.35	0.00	154
		0.50	0.050	0.30	107
		0.30	0.030	0.32	103
		0.10	0.010	0.34	93.4
	4.72 ± 0.05	0.32	0.32	0.00	49.7
		0.090	0.090	0.23	30.6
		0.045	0.045	0.27	25.2
		0.005	0.005	0.31	19.0
	5.66 ± 0.08	0.035	0.35	0.00	7.15
		0.020	0.20	0.15	5.52
		0.010	0.10	0.25	4.60
		0.005	0.05	0.30	3.95

General Acid-General Base Catalysis in Deamination of C and Cyd—Plots of the apparent first-order rate constants, k_1, *versus* total buffer concentration were linear for both C and Cyd under the conditions given in Tables II and III. The apparent first-order rate constants were shown to be defined by the equation:

$$k_1 = k_{HA}[HA] + k_{A^-}[A^-] + k_i \qquad \text{(Eq. 6)}$$

where HA is the acidic component and k_i is the rate constant in the absence of HA or A^-. At any given pH a plot of k_1 *versus* $[HA]$ will be linear with intercept, k_i, and slope defined as $S = (k_{HA} + k_{A^-}R)$ where R is the ratio $[A^-]/[HA]$. Thus a knowledge of S and R at two or more pH values will allow calculation of both catalytic constants. The calculation can also be done by a plot of k_1 *versus* $[A^-]$ where $S = (k_{HA}R + k_{A^-})$ and $R = [HA]/[A^-]$. Both of these methods were applied to the data in Tables II and III with consistent results. The catalytic constants calculated in this way are listed in Table IV. It is apparent that both the acidic and basic components of the buffers are catalytic. The effects of a number of buffer systems on the deamination of C have been established as part of another study which will examine the possibility of a Brönsted correlation of the catalytic constants for various buffers. In all cases general-acid and general-base catalysis is indicated. As evidenced in Table IV neither species appears to predominate as the catalyst.

The reaction mixtures were examined for material balance by calculating the sum of the concentrations of C and U or Cyd and Urd as a function of time. Under all conditions reported in Tables II and III there was no indication of the presence of any reaction product other than the U or Urd.

General-Acid Catalysis in Deamination of Ara-C—The method described in the previous section was applied to data for the deamination of Ara-C in phosphate buffers at pH 6.15 and 6.90 (1) by

Table II—Experimental Conditions and Apparent First-Order Rate Constants for Deamination of 5.0×10^{-4} *M* Cytidine

°C.	Observed pH	Buffer Compn. [CH_3–COOH]	Buffer Compn. [CH_3–COONa]	Buffer Compn. [NaCl]	$10^3 k_1$, hr.$^{-1}$
70	4.71 ± 0.04	0.360	0.360	0.000	0.767
		0.100	0.100	0.260	0.390
		0.050	0.050	0.310	0.328
		0.005	0.005	0.355	0.266
80	3.63 ± 0.05	1.350	0.135	0.180	4.40
		0.450	0.045	0.270	3.12
		0.270	0.027	0.288	2.93
		0.090	0.009	0.306	2.41
	4.72 ± 0.04	0.315	0.315	0.000	1.80
		0.090	0.090	0.225	0.992
		0.045	0.045	0.270	0.817
		0.0045	0.0045	0.311	0.646

Table III—Experimental Conditions and Apparent First-Order Rate Constants for Deamination of 1.8×10^{-3} *M* Cytosine at 70°

Observed pH	Buffer Compn. [CH_3COOH]	[CH_3COONa]	[NaCl][a]	$10^4 k_1$, $hr.^{-1}$
3.75 ± 0.13	0.20	0.020	0.18	3.50
	0.15	0.015	0.19	3.11
	0.082	0.008	0.19	2.77
	0.020	0.002	0.20	2.48
4.72 ± 0.02	0.20	0.20	0.00	3.59
	0.15	0.15	0.05	3.16
	0.082	0.082	0.12	2.54
	0.020	0.020	0.18	2.07
5.64 ± 0.02	0.020	0.20	0.00	1.29
	0.015	0.15	0.05	1.12
	0.0082	0.082	0.12	0.865
	[$NaH_2PO_4 \cdot H_2O$]	[Na_2HPO_4]	[NaCl][b]	
5.76 ± 0.03	0.20	0.020	0.00	1.94
	0.10	0.010	0.065	1.48
	0.05	0.005	0.13	1.18
6.70 ± 0.03	0.060	0.060	0.02	1.29
	0.040	0.040	0.10	1.10
	0.020	0.020	0.18	0.949
7.69 ± 0.04	0.0060	0.060	0.074	0.875
	0.0040	0.040	0.14	0.795
	0.0020	0.020	0.20	0.723

[a] Ionic strength adjusted with NaCl to 0.020. [b] Ionic strength adjusted with NaCl to 0.26.

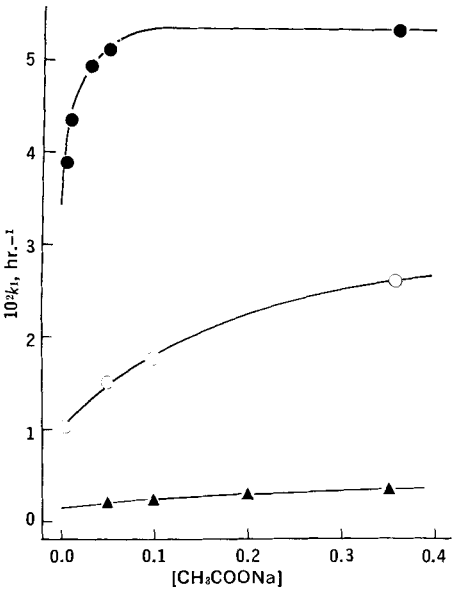


Figure 1—*Apparent first-order rate constants for deamination of Ara-C at 70° in acetate buffers at pH 3.7,* ●*; 4.7,* ○*; and 5.7,* ▲*; versus sodium acetate concentration.*

determining the slopes of the plots k_1 *versus* [$HPO_4^=$]. Solving the simultaneous equations for the catalytic constants yields a value of 2.0×10^{-2} (l./mole/hr.) for $k_{H_2PO_4^-}$ and a negligible value for $k_{HPO_4^=}$ (estimated to be 3×10^{-4} l./mole/hr.).

Catalysis in the presence of the acetic acid-acetate buffer system did not exhibit the usual linear dependency of rate constant on buffer concentration. Plots of k_1 *versus* total buffer concentration (or the concentration of either component) showed negative deviation from linearity. Typical examples are presented in Figs. 1 and 2. Figure 2 also shows a plot for the deamination of Cyd to allow a direct comparison.

The plot k_1 *versus* CH_3COONa at pH 3.7 shows the greatest degree of deviation of all cases reported in this paper. This plot shows essentially two regions. In the region of low buffer concentration there exists a sharp increase in k_1 with increasing concentration of buffer. Examination of Fig. 1 will reveal that this increase can be attributed to CH_3COOH rather than [CH_3COO^-] since the most significant buffer catalysis is seen to occur at pH 3.7 where there is primarily CH_3COOH (see Table I). As the pH is increased to 4.7 and 5.7 the slopes of the plots can be seen to decrease. Indeed the nearly linear plot of k_1 *versus* CH_3COONa at pH 5.7 represents the case where the buffer is composed primarily of the acetate with relatively little acetic acid present. This plot shows an increase in k_1 of only 1.4×10^{-3} ($hr.^{-1}$) when total buffer concentration is increased from 0.055 to 0.39 *M*. The plot at pH 3.7 shows an increase of more than 10×10^{-3} ($hr.^{-1}$) over the same range. If one assumes that all of the increase in k_1 at pH 5.7 is due to the acetate the maximum catalytic constant for CH_3COO^- would be $1.4 \times 10^{-3}/0.3$ or 4.7×10^{-3} (l./mole/hr.). (See Table I for sodium acetate and acetic acid concentrations.) This would allow calculation of the acetic acid catalytic constant at pH 3.7 from the initial slope of k_1 *versus* CH_3COOH which is 94×10^{-3} (Fig. 2) yielding a value of $k_{CH_3COOH} = 93 \times 10^{-3}$ (l./mole/hr.). Thus the maximum possible contribution due to the acetate component of the buffer at 70° is negligible in comparison to that of acetic acid.

Since the plots in Figs. 1 and 2 show decreasing buffer dependency with increasing buffer concentration, a more accurate assessment of the catalytic constants can be performed using the initial slopes. The initial slopes of k_1 *versus* CH_3COOH were obtained at pH 3.7 and 4.7 by regraphing the data in Fig. 2 on an expanded scale. Applying the technique discussed in the previous section results in the following:

$$0.094 = k_{HA} + 0.1\ k_{A^-} \qquad \text{(Eq. 7)}$$

$$0.103 = k_{HA} + k_{A^-} \qquad \text{(Eq. 8)}$$

Solving for the constants yields $k_{HA} = 9.3 \times 10^{-2}$ and $k_{A^-} = 1 \times 10^{-2}$ (l./mole/hr.). However, the initial slope of k_1 *versus* CH_3COONa at pH 5.7 is 0.0090 which would indicate a negative value for acetate since $S = k_{A^-} + 0.1\ k_{HA}$ at this pH. If it is assumed that k_{A^-} is actually zero and that the small positive value calculated from Eqs. 7 and 8 is due to the approximation in determining the initial slopes, the catalytic constant $k_{HA} = 0.093$ can be used to calculate the initial k_1 values at pH 5.7 according to Eq. 6 where k_i is 1.39×10^{-3} ($hr.^{-1}$). The calculated values for 10^3 k_1 ($hr.^{-1}$) are 2.32 at 0.01 *M* CH_3COOH and 1.84 at 0.005 *M* CH_3COOH which agree with the experimental values 2.29 and 1.84. Since the most pronounced acetate effect would be exhibited at pH 5.7 it is evident that its catalytic constant is negligible as compared to that for acetic acid.

The same generalities can be made for the acetic acid-acetate buffer at 80°. The slope of k_1 *versus* [CH_3COO^-] at pH 5.7 was again minimal in comparison to that of pH 4.7 and 3.7. Attempts to calculate the catalytic constants from the initial slopes using Eq. 6 resulted only in approximate values for k_{HA} (15×10^{-2} l./mole/hr.) and k_{A^-} (0.1×10^{-2} l./mole/hr.). However, the data are unambiguous in exhibiting the predominance of acetic acid as the catalytic species.

It is apparent that acetic acid is also responsible for the negative deviation in the k_1 *versus* buffer concentration plot since this is most pronounced at pH 3.7. When the acetic acid is increased from 0.05 to 0.10 there is a corresponding increase in k_1 of 4.6×10^{-3} for a slope value, $S_1 = 92 \times 10^{-3}$. However, when the concentration is increased from 0.5 to 3.6 *M* there is an increase of only 2.6×10^{-3} in k_1 for a value of $S_2 = 0.8 \times 10^{-3}$. Thus the ratio S_1/S_2 is 115/1. The rate constant, k_1, has become relatively independent of buffer concentration in the region of 0.5 to 3.6 *M* acetic acid, pH 3.7, 70°. This type of plot is evidence for a change in the rate determining step of the reaction (5). In the buffer region up to 0.5 *M* acetic acid the reaction was significantly catalyzed by acetic acid. At concentrations greater than 0.5 *M*, acetic acid has supplied sufficient catalysis

Table IV—Catalytic Constants for Acetate and Phosphate Buffers in Deamination of Arabinosylcytosine, Cytidine, and Cytosine

°C.	Substrate	$10^4 k$, l./mole/hr. HAc	Ac^-	$H_2PO_4^-$	$HPO_4^=$
70	C	5.43	2.99	4.75	3.45
	Ara-C	~930	~0	200	~3
80	Cyd	11.5	26.3	—	—
	Ara-C	~1500	~0	—	—

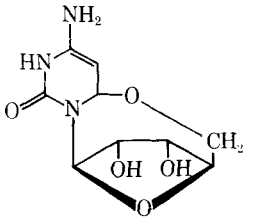
to result in a new rate determining step which is apparently independent of buffer.

The deamination of Ara-C differs from that of C and Cyd in one other aspect. Under certain experimental conditions, particularly in dilute carboxylate buffers at pH 2–4, 60–80°, material balance data provide evidence for the formation of an intermediate in the deamination of Ara-C to Ara-U. By proper control of conditions we can demonstrate formation of as much as 40% of the intermediate which subsequently reacts to form Ara-U. The isolation and identification of this intermediate is currently in progress and will be the subject of a future report.

The presence of the intermediate does not affect the validity of the rate constants reported in the present paper. It has been shown that rate constants determined from Ara-C concentration are the same as those calculated directly from the UV absorption data. In addition, the intermediate shows no effect on the spectra of the reaction mixtures from 250–300 mμ and would not therefore be expected to interfere with the spectrophotometric assays.

DISCUSSION

Intramolecular Participation in the Hydrolytic Deamination of Ara-C—The rate constants for deamination of Ara-C, Cyd, and C in the absence of buffer at a variety of pH values, 70 and 80°, are given in Table V. The data at 70° show a slight rate enhancement when the ribosyl nucleoside, Cyd, is compared to cytosine alone, C. The ratio of the rate constants is 2.2/1 at pH 4.0 and 1.4/1 at pH 4.7. This increase in deamination rate may be due to participation by the 5′-hydroxyl to form the 6,5′-anhydro cyclonucleoside intermediate, I. The formation of similar 6,5′-cyclic intermediates has



I

been proposed in the 5-H exchange of ribofuranosyl nucleosides in MeONa-MeOD (6). In the present case, however, this increase in deamination rate, which may be attributed to the ribosyl 5′-hydroxyl, is insignificant in comparison to the rate increase of the arabinosyl nucleoside which is 142 times faster than C at pH 3.7. At pH 4.7 the ratio of Ara-C/Cyd/C is 53/1.4/1. (Other ratios are listed in Table V.) It is obvious from these data that the configuration of the 2′-hydroxyl in Ara-C (Scheme I) is responsible for a 30- to 40-fold rate increase as compared to the deamination of Cyd where the 2′-hydroxyl is *trans* to the pyrimidine base. It should also be mentioned here that Fig. 2 illustrates why the uncatalyzed rate constants rather than those in buffer solutions must be compared for a true indication of the degree of intramolecular catalysis. As the buffer concentration is increased the ratio of reactivity of Ara-C to Cyd or C would be found to first increase and then decrease. Figure 1 shows the most dramatic example of this fact in that Ara-C deamination becomes practically

Table V—Comparison of First-Order Rate Constants for Hydrolytic Deamination of Arabinosylcytosine, Cytidine, and Cytosine in Absence of Buffer[a]

°C.	pH	10^4 k, hr.$^{-1}$ Ara-C	Cyd	C	Ratio Ara-C/Cyd/C
70	3.7	340	—	2.4	142/–/1
	4.0	260[b]	6.4[c]	2.9[c]	90/2.2/1
	4.7	100	2.6	1.9	53/1.4/1
	5.7	14	—	1	14/–/1
80	3.7	900	24	—	40/1
	4.7	180	6.4	—	28/1
	5.7	35	—	—	—

[a] Determined from intercepts of plots of k_1 *versus* buffer concentration. See Tables I, II, and III for experimental details. [b] Estimated from plot of k_i *versus* pH where k_1 is intercept of k_i *versus* buffer concentration for data in Table I. [c] Taken from *Reference 9*.

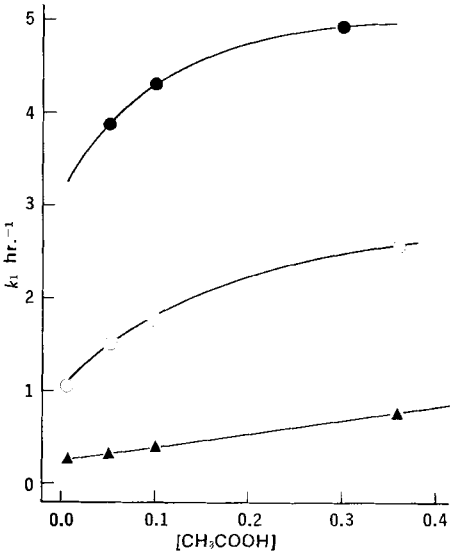


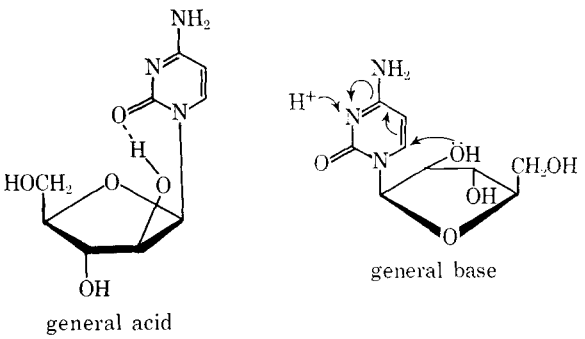
Figure 2—*Apparent first-order rate constants at 70° for deamination of Ara-C in acetate buffers at pH 3.7,* ● *and 4.7,* ○ *(10^2k$_1$ in hr.$^{-1}$) and Cyd at pH 4.7,* ▲ *(10^3k$_1$ in hr.$^{-1}$) versus acetic acid concentration.*

independent of buffer at concentrations above 0.5 *M* acetic acid. Since the rate constants in Table V do not reflect the differences in intermolecular catalysis by buffer, it is apparent that the 2′-hydroxyl in the arabinosyl nucleoside is capable of intramolecular catalysis while the 2′-hydroxyl in cytidine does not exhibit effective intramolecular participation.

Buffer Catalysis of Ara-C, Cyd, and C Deamination—Table IV summarizes the values of the catalytic constants in the acetic acid-acetate and phosphate buffer systems. It is readily apparent that catalysis of the deamination of Cyd and C differs from that of Ara-C in both magnitude and type. Cyd and C are catalyzed by both the acidic and basic components of the buffers. Thus general-acid and general-base catalysis are evident.

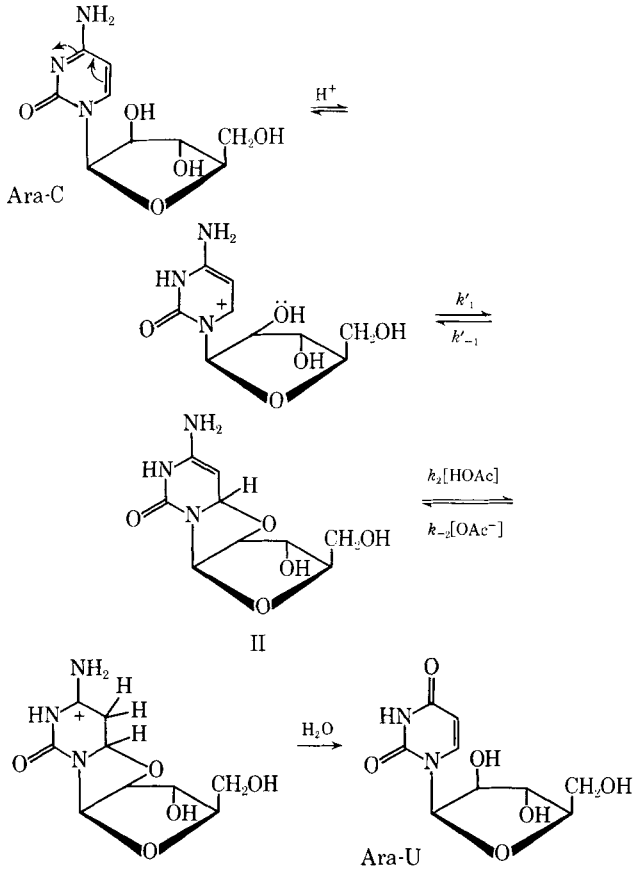
The catalytic constants for deamination of Ara-C were calculated only in dilute buffer solutions in the case of the acetic acid-acetate system because of the decreasing effect of buffer at higher concentrations (Fig. 1). It is obvious that Ara-C is subject primarily to general-acid catalysis under the conditions of this study. Furthermore, the catalytic constants for acetic acid and $H_2PO_4^-$ are significantly larger than those for the corresponding constants in C or Cyd deamination (Table IV).

In a preliminary communication we have discussed two possibilities for participation by the 2′-hydroxyl (7). One case is that of intramolecular general-acid catalysis and the other that of general-base (Scheme II).



Scheme II

Consideration of the buffer data suggests that the general base mechanism is the more likely of the two. This is evident from the fact that Ara-C deamination exhibits buffer catalysis primarily by general acids whereas C and Cyd are susceptible to catalysis by both acidic and basic buffer components. Thus the Ara-C appears to be supplying its own basic catalyst and to require only the general-acid catalyst to undergo relatively rapid deamination.



Scheme III

Intramolecular catalysis by nucleophilic attack of the 2′-oxygen on C-6 would lead to the formation of the 2′,6-cyclic intermediate, II, shown in Scheme III. This mechanism is basically similar to that previously proposed for Cyd (2) and Ara-C (1) except that the arabinosyl 2′-hydroxyl now serves as the general base.

The formation of 2′,6-cyclonucleosides has also been implicated in the 5-H exchange reactions of Ara-U and 5′-deoxy Ara-U in MeONa-MeOD (6) and Ara-U and Ara-C in aqueous acid-base (8). This mechanism accounts for the increased deamination rates of Ara-C over Cyd and C in the absence of buffer since only H_3O^+ is required for the reaction to proceed.

Scheme III is also consistent with the observed behavior of the first-order rate constants for Ara-C deamination, k_1, as a function of acetic acid concentration at pH 3.7, 70° (Figs. 1 and 2). The dependency of k_1 on acetic acid at low buffer concentrations can be explained on the basis of reversible proton addition to the C-5 of II as shown in the scheme. The maximum concentration of II would thus be a function of the rate of ring formation and the relative rate of proton addition to the cyclic intermediate. Thus at low buffer concentrations and sufficient H-ion to catalyze the 2′-hydroxyl attack, one would expect to accumulate II. This is in agreement with the authors' observation that Ara-C deamination involves formation of an intermediate under such conditions (see *Experimental*). Increasing the concentration of acetic acid would result in a more rapid loss of II which would approach a steady state in high buffer concentrations. The observed deamination rate constant in more concentrated buffer (0.5 to 3.6 *M* acetic acid) appears to approach a constant value which would represent the rate constant for the formation of II under steady state conditions at pH 3.7. This would be expected to be a constant at a given pH since it represents a process which is first-order in Ara-C protonated at N-3.

Scheme III is therefore consistent with the current data for inter- and intramolecular catalysis in Ara-C deamination. The deamination of C and Cyd presumably proceeds through a similar mechanism with the intermolecular general-acid and general-base catalysis being supplied by buffers. It is possible that the 5′-hydroxyl in Cyd participates to some extent as discussed earlier under the formation of I. This effect is minimal as compared to the 2′-hydroxyl in Ara-C under the present experimental conditions.

SUMMARY

1. At pH 3.7–4.7 70–80°, in the absence of buffers arabinosylcytosine undergoes hydrolytic deamination 30 to 40 times faster than cytidine which deaminates slightly faster than cytosine. This rate enhancement is attributed to intramolecular participation by the 2′-hydroxyl in the arabinosyl nucleoside.

2. First-order deamination rate constants, k_1, for cytidine and cytosine showed a linear dependency on buffer concentration. Catalytic constants could be calculated from slopes of appropriate plots in the usual fashion. Both compounds were susceptible to general-acid and general-base catalysis by the buffer components.

3. Deamination rate constants for arabinosylcytosine did not show the usual linear dependency on acetic acid-acetate buffer concentration. Curves were obtained with negative deviation from linearity. In the most extreme case studied, a region where k_1 is nearly independent of buffer concentration is reported.

4. Catalytic constants were estimated from the initial slopes of k_1 *versus* acetic acid concentration in the arabinosyl case. Although the values for the catalytic constants are estimates it is unequivocal that general-acid catalysis predominates in Ara-C deamination. General-acid catalysis was also shown in phosphate buffer which showed no curvature.

5. It is hypothesized that Ara-C is subject to general-base type of intramolecular catalysis by the 2′-hydroxyl group. Thus only intermolecular general-acid catalysis is observed whereas both general-acid and general-base catalysis are required in the deamination of cytosine and cytidine.

REFERENCES

(1) R. Notari, *J. Pharm. Sci.*, **56**, 804(1967).

(2) R. Shapiro and R. S. Klein, *Biochem. J.*, **5**, 2358(1966).

(3) Ibid., **6**, 3576(1967).

(4) H. S. Loring and J. M. Ploeser, *J. Biol. Chem.*, **178**, 439 (1949).

(5) W. P. Jencks, *Progr. Phys. Org. Chem.*, **2**, 63(1964).

(6) D. V. Santi and C. F. Brewer, *J. Am. Chem. Soc.*, **90**, 6236 (1968).

(7) R. E. Notari, M. Lue Chin, and A. Cardoni, *Tetrahedron Letters*, No. 40, **1969**, 3499.

(8) W. J. Wechter and K. C. Smith, *Biochem. J.*, **7**, 4064(1968).

(9) A. Cardoni, "Buffer Catalysis in the Hydrolytic Deamination of Cytosine," M.S. thesis, The Ohio State University (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 11, 1969 from *College of Pharmacy, The Ohio State University, Columbus, OH 43210*

Accepted for publication August 28, 1969.

This investigation was supported in part by funds from the American Cancer Society Institutional grant IN-16J.

The authors wish to thank Dr. Jack Hine for helpful discussions in the preparation of this manuscript. They also thank Drs. Gordon Flynn and Louis Schroeter, and The Upjohn Company for samples of Ara-C and Ara-U.

Dynamic Dialysis as a Method for Studying Protein Binding I: Factors Affecting the Kinetics of Dialysis Through a Cellophane Membrane

MARVIN C. MEYER* and DAVID E. GUTTMAN†

Abstract □ A potentially useful method for determining the extent to which binding occurs in a protein-small molecule system consists of studying the kinetics of dialysis of the small molecule in the absence and presence of protein. The kinetics of dialysis of a number of compounds, in the absence of protein, was studied in some detail in order to characterize the nature of the dialytic process and its dependency on experimental variables. It was shown that the rate of escape of the small molecule from a dialysis cell was a first-order process provided that sink conditions were maintained. The influence of such variables as cell size, solution volume, stirring rates, temperature, pH, ionic strength, and viscosity on the rate process was considered and investigated. It was found that stirring rate (above a minimum), ionic strength, and viscosity did not markedly affect the kinetic picture. Temperature, pH (for one compound), the size of the cell, and the volume of solution contained by the cell did affect the rate of escape. Binding by the cellophane membrane was not encountered as a problem with the compounds studied. Experimental variables could be readily controlled to yield reproducible and dependable results.

Keyphrases □ Dialysis, dynamic—protein binding □ Protein binding determination—cellophane membrane, dialysis □ Permeation half-lives, apparent—small molecules □ Temperature, stirring, pH-buffer concentration effect—dialysis rate □ Viscosity effect—dialytic rate □ Ultrafiltration—phenol red-methylcellulose interaction determination □ UV spectrophotometry—analysis

A previous report (1) described a potentially useful method for studying protein-small molecule interactions. The technique consisted of determining, as a function of time, the escape of the small molecule from a dialysis cell, in the presence and absence of protein. The report indicated the promise of this approach for the rapid and convenient determination of fundamental binding parameters.

The present communication describes studies which were conducted to evaluate the influences of experimental variables which might be encountered in utilizing this technique. The effect of temperature, pH, viscosity, stirring rate, membrane area, liquid volume of the system, and binding of the small molecule by the dialysis membrane were considered.

EXPERIMENTAL

Materials—Bovine serum albumin (BSA), Fraction V (Calbiochem Laboratories) was used in this study. The 8-nitrotheophylline was synthesized according to the procedure reported by Morozowich (2). Other xanthine derivatives, phenol red, and methyl orange were obtained from commercial sources. Regenerated cellulose dialysis tubing [Union Carbide No. 20, 2.50 cm. (0.984 in.) flat width, with an average pore size of 24 Å] was conditioned prior to use by rapidly running distilled water through the tubing for several hours, with the tubing immersed in distilled water. The tubing was stored in distilled water at 2°.

Dynamic Dialysis Studies—The experimental system, the general protocol, and the treatment of data were described previously(1). Generally the systems were prepared to contain 7 ml. of a solution of small molecule in 0.04 *M*, pH 7.3, phosphate buffer, inside a 7-cm. long sac prepared from the dialysis tubing. The sac was immersed in 200 ml. of 0.04 *M*, pH 7.3, phosphate buffer contained in a water-jacketed beaker, in the manner previously described (1). The systems were maintained at 25 ± 0.2°. The contents of the sac were stirred with a twisted glass rod rotated at 125 r.p.m. Stirring of the external solution was achieved with a magnetic stirring bar. At time intervals, 100 ml. of the external solution was removed and immediately replaced with 100 ml. of fresh buffer. The concentration of small molecule in the removed sample was determined spectrophotometrically and the concentration of small molecule in the protein compartment was calculated from a knowledge of the initial concentration and the total amount of small molecule which had appeared in the external solution. During the investigation of a particular experimental variable, the remaining variables were held constant, as described in the conditions for the general system. Whenever possible, the order of experiments concerned with a particular variable, was selected to evaluate any progressive or irreversible effect.

The influence of temperature on the rate of dialysis of phenol red was studied at 25, 10, and 40 ± 0.2°, in that order. Excellent temperature stability was attained through the use of the water-jacketed beakers in combination with a constant-temperature water bath and circulator.

In order to test the effect of stirring the system, the dialysis of phenol red was followed under four different conditions of stirring. The same dialysis sac was used for the four experiments. At the termination of each run the dialysis sac was thoroughly rinsed with distilled water. In the initial study, a dialysis sac containing the dye was suspended in the external buffer solution. The twisted glass rod used to agitate the sac contents, and the magnetic stir bar used to stir the external buffer bathing solution, were in place, but motionless. In a second study, only the magnetic stir bar was rotated. The third and fourth studies had external stirring *via* the magnetic stir bar, and internal stirring at either 125 or 500 r.p.m. The internal rate of stirring was controlled with a Servodyne rotator.

The influence of viscosity on the rate of dialysis was studied using methylcellulose (Fisher-100 cps.) as the viscosity inducing agent. Phenol red solutions were prepared at pH 7.3, to contain 0, 0.032 or 0.064% methylcellulose. The relative viscosities of the various phenol red-methylcellulose solutions or phenol red-BSA solutions, at pH 7.3 and 25°, were determined using a Gilmont falling-ball viscometer.

The effect of pH on dialytic rate was evaluated over a pH range of 3–11 for 8-nitrotheophylline, caffeine, 8-chlorotheophylline, and phenol red, in 0.06 *M* phosphate buffer. In addition, the dialysis of 8-chlorotheophylline was studied at pH 3 in 0.006 *M* phosphate buffer. The same dialysis sac was used for each experimental series involving a given small molecule. The pH of each system was maintained by preparing the internal and external solutions at the same pH and buffer concentration. The pH values were measured before and after each dialysis run and no variation in pH was noted during the course of an experiment.

The influence of the size of the dialysis sac, *i.e.*, the membrane area exposed to the external buffer solution, and the influence of the volume of the solution both inside the sac and external to the sac, were studied in a series of experiments conducted with phenol red in 0.04 *M*, pH 7.3, phosphate buffer, at 25°. First, three control runs were made in which 7 ml. of phenol red solution was placed into each of three, 7-cm. long, dialysis sacs. The sacs were placed into 200 ml. of pH 7.3 phosphate buffer in the usual manner. The loss of phenol red from each of these sacs was followed through about three half-lives. For the second portion of this study the sacs were rinsed and 6, 5, or 4 ml. of dye solution, of the same concentration as the initial control run, was placed into the three sacs and dialyzed. The third experimental series involved rinsing the sacs from the previous study, placing 7 ml. of phenol red solution into two of the sacs, and placing the two sacs into 350 or 500 ml. of pH 7.3 buffer

Table I—Summary of Apparent Permeation Half-Lives for Various Small Molecules

Compound	Mol. Wt.	Apparent Permeation Half-Life, min.[a]	No. of Trials
Trypan blue	960.83	No diffusion after 2 hr.	(2)
Phenol red	354.37	77.1 ± 5.6	(9)
Methyl orange	327.34	51.0 ± 4.0	(5)
Warfarin	308.32	67.5 ± 6.9	(5)
8-Nitro-theophylline	225.17	33.2 ± 2.7	(15)
8-Chloro-theophylline	214.62	38.2 ± 2.1	(6)
Caffeine	194.19	31.9 ± 3.1	(15)
Salicylic acid	138.13	24.0	(1)

[a] Values reported are average values ±1 *SD*.

Table II—Summary of Viscosity Determinations for Various BSA and Methylcellulose Solutions

Methylcellulose, %	BSA, %	Relative Viscosity[a]	Literature (8) Rel. Viscosity for BSA[b]
0.0	0.0	1.000	—
—	1.52	1.090	1.06
0.032	—	1.127	—
—	3.07	1.170	1.13
0.064	5.18	1.290	1.23

[a] BSA and/or methylcellulose solutions in pH 7.3 phosphate buffer at 25°. [b] BSA in pH 6.9 phosphate buffer at 25°.

solution. The loss of phenol red was again followed. The fourth experimental series was conducted to ensure that the sacs had not suffered any damage or alteration in dialytic characteristics during the course of the first three studies. This study involved repeating the control runs in which 7 ml. of phenol red solution was placed into the dialysis sacs and the sacs were immersed in 200 ml. of buffer. Finally, a portion of each sac was cut off so that instead of each being 7 cm. long they were reduced to 6, 5, or 4 cm. The sacs were then placed into the three jacketed beakers containing 200 ml. of pH 7.3 buffer. A sufficient volume of the phenol red solution was added to the sacs to equalize the internal and external liquid levels. The final volumes inside the sacs were 5.7, 3.6, and 2.1 ml., respectively, for the 6-, 5-, and 4-cm. sacs. In addition to these studies, a system was also run in which the level of the liquid inside the sac exceeded the external liquid level. For this system 10 ml. of the phenol red solution was placed into a 10-cm. long dialysis sac and the sac was immersed in 425 ml. of buffer. The height of the internal liquid extended about 1.5 cm. above the height of the external liquid level. Data from this system were compared with data obtained with the same sac containing only 7 ml. of phenol red solution. In the latter system the internal and external liquid levels were the same. A final study determined the effect of variation in internal liquid volume under conditions of a progressively decreasing internal volume during the course of the dialysis. Initially 10 ml. of 0.44 mg./ml. phenol red-buffer solution, at pH 7.3, was placed into a 9-cm. long dialysis sac, and the sac was placed into 500 ml. of pH 7.3 buffer. The dialysis was allowed to proceed with sampling of the external solution as usual. After approximately 2 hr., 2 ml. of the internal solution was withdrawn without replacement. Sampling of the external solution proceeded for an additional 2 hr., at which time 2 ml. was again withdrawn from the internal solution. After an additional 2 hr. the sac was removed and washed with distilled water. Ten milliliters of a 0.28 mg./ml. phenol red solution was then placed into the sac, the sac was immersed in 500 ml. of fresh buffer, and the rate of phenol red dialysis was again determined for the 10-ml. internal volume system. Thus in these studies the internal volume was decreased from 10 to 8 to 6 ml. and then restored to 10 ml.

The influence of the volume of solution in the sac on the rate of dialysis of 8-chlorotheophylline was also studied. The investigation was conducted by initially placing 10 ml. of 8-chlorotheophylline in pH 11.0 buffer solution into a 9-cm. sac, and suspending the sac in 500 ml. of pH 11.0 buffer. After the dialysis had been followed for a period of 1–1.5 hr., 4 ml. of the 8-chlorotheophylline solution inside the sac was withdrawn without replacement. The dialysis was allowed to proceed for an additional period of time. Thus the positioning of the membrane remained undisturbed throughout the course of the dialysis. This procedure was repeated at pH 3.0 using the same membrane.

The potential effect of membrane binding of the small molecule on the dialytic rate was illustrated by simulating strong membrane binding using activated charcoal (Merck, NF Powder). In these studies the dialysis of methyl orange in pH 7.3, 0.04 *M*, phosphate buffer, at 25°, was followed in the presence of 7.45 mg. of charcoal and in the absence of charcoal. The same dialysis sac was used for both experiments.

Ultrafiltration Studies—Ultrafiltration was employed to measure the interaction between phenol red and methylcellulose. Phenol red solutions were prepared, in pH 7.3, 0.04 *M*, phosphate buffer, to contain from 0.0–0.09% methylcellulose. Ten milliliters of each

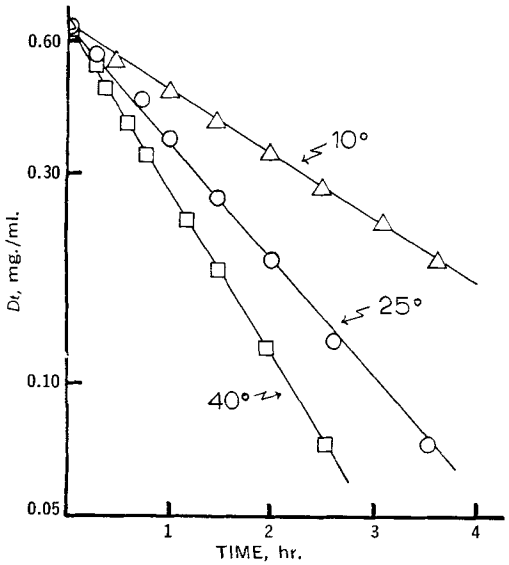


Figure 1—*The influence of temperature on the apparent first-order escape of phenol red from the dialysis cell, at pH 7.3.*

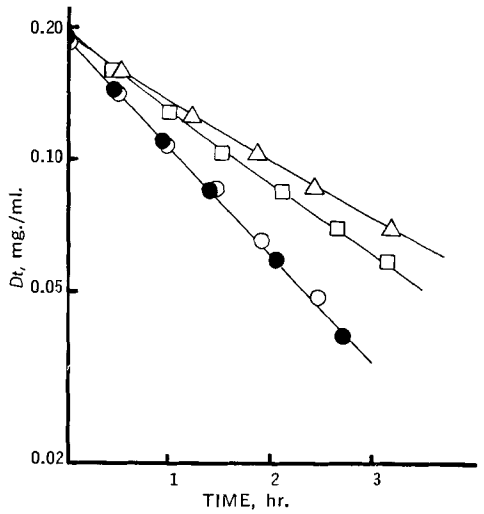


Figure 2—*The influence of stirring on the apparent first-order escape of phenol red from the dialysis cell, at 25°. △, no stirring; □, external stirring only; ○, internal stirring at 125 r.p.m.; ●, internal stirring at 500 r.p.m.*

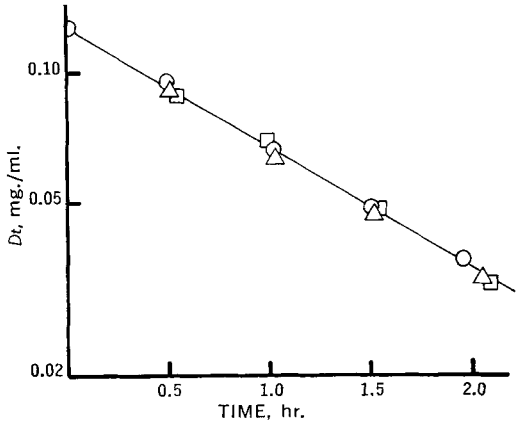


Figure 3—*The effect of viscosity on the apparent first-order escape of phenol red in the presence and absence of methylcellulose at pH 7.3 and 25°. Key: △, 0% methylcellulose; ○, 0.032% methylcellulose; □, 0.064% methylcellulose.*

solution was placed into 12.5-cm. long, double-walled dialysis sacs. The sacs were prepared by partially everting a 25-cm. segment of hydrated cellulose dialysis tubing. The sacs were secured in glass stoppered centrifuge tubes by means of the excess tubing extending out of the mouth of the centrifuge tube. The tubes were centrifuged at 2000 r.p.m. for about 5 min. The filtrate, approximately 0.2 ml., was discarded to minimize error due to moisture initially associated with the membrane. The centrifugation was then continued for approximately 25 min. to collect an additional 0.5 ml. The filtrate and solution inside the sac were analyzed spectrophotometrically to obtain the free and total dye concentration, respectively. There was no evidence of leakage of the methylcellulose from the sac, as determined by the colorimetric test of Kanzaki and Berger (3), or of significant membrane binding of the phenol red.

Analytical Methods—The compounds studied were assayed spectrophotometrically with a spectrophotometer (Beckman DU) equipped with a power source and digital, absorbance read-out (Gilford). Concentrations were determined from Beer's law plots prepared at appropriate wavelengths and pH's.

RESULTS AND DISCUSSION

There are at least three mechanisms which have been suggested to explain the selective permeability of dialysis membranes (4).

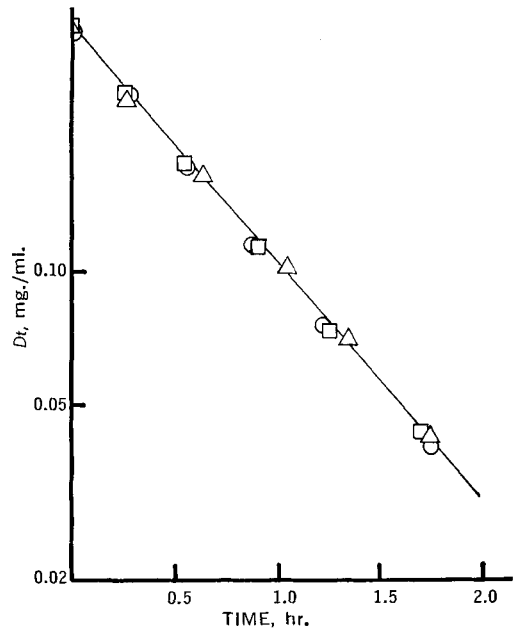


Figure 4—*The effect of pH on the apparent first-order escape of 8-nitrotheophylline at 25°. Key: □, pH 3.0; ○, pH 7.0; △, pH 11.0.*

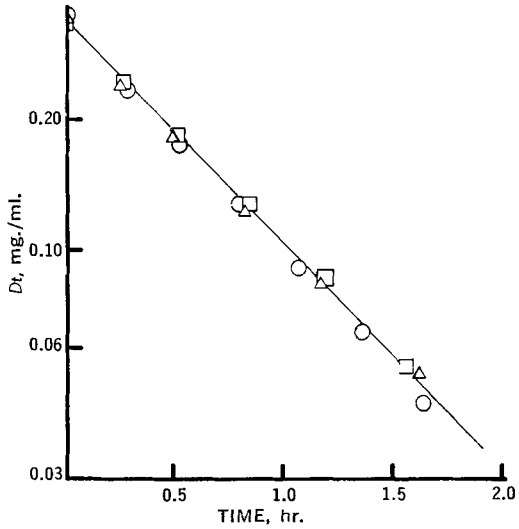


Figure 5—*The effect of pH and buffer strength on the apparent first-order escape of 8-chlorotheophylline at 25°. Key: ○, pH 3.0, 0.06 M buffer; △, pH 3.0, 0.006 M buffer; □, pH 11.0, 0.06 M buffer.*

Cellophane membranes are generally thought to function on the basis of diffusion through pores (5), rather than by dissolution in the membranes, or some other mechanism.

Table I indicates the relationships observed between molecular weight and the apparent permeation half-lives for the dialysis of a variety of small molecules, as determined in the system employed at 25°. The permeation half-life is defined as the time required for the total small molecule concentration within the dialysis sac to decrease by 50%. The half-lives were determined from studies conducted at pH 7.0–7.3, with the exception of studies involving the xanthines. The dialytic rates of the xanthines studied were determined, in separate experiments, to be independent of pH. As a result, average half-lives for these compounds include data for studies conducted over a pH range of 3–11.

The data in Table I show that the bulky, high molecular weight compounds diffuse through the membrane with the most difficulty, and the smaller, lower molecular weight xanthines diffuse more rapidly, and at similar rates. Some of the data in Table I conformed quite well to the direct relationship between the permeability of small molecules through membrane pores and the square root of the molecular weight, as suggested by Danielli (6). Trypan blue, phenol red, and warfarin, however, permeated the membrane at rates which were considerably slower than would be predicted on this basis. These relatively bulky compounds illustrate the)in-

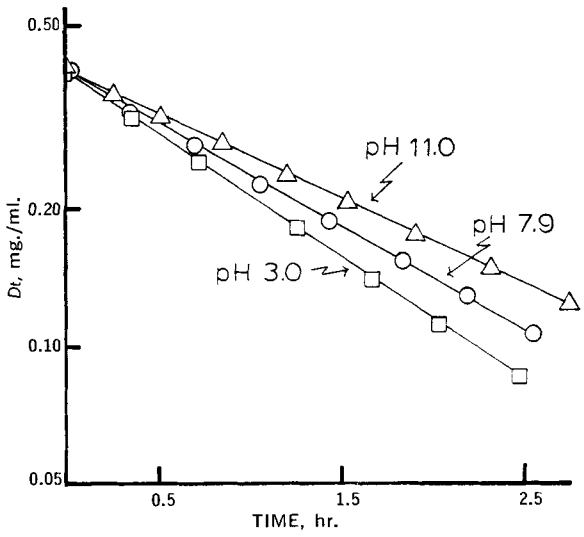


Figure 6—*The effect of pH on the apparent first-order escape of phenol red at 25°.*

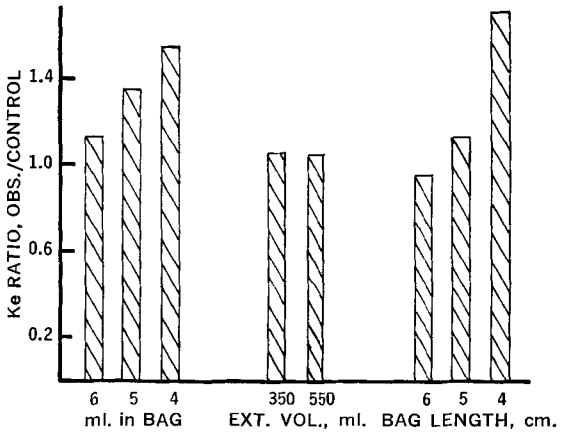


Figure 7—*Effect of liquid volume and membrane area on the apparent permeability constant for the dialysis of phenol red at pH 7.3 and 25°.*

fluence of molecular volume on membrane permeability, which has also been discussed by Danielli (6).

Osmotic Effect of the Protein—It was not anticipated that there would be any dilution of the contents of the dialysis sac due to the presence of protein because the protein concentrations were at least 100 times less than the buffer concentration of 0.04 *M* which was present both inside the dialysis sac and external to it. The lack of dilution was verified by determining that solutions of 1.7% BSA prepared in pH 7.3, 0.04 *M*, phosphate buffer, and placed into dialysis sacs, showed no change in BSA concentration after several hours, when the sacs were placed into the external buffer bathing solution.

The Effect of Temperature on Rate of Dialysis—Figure 1 illustrates the effect of temperature on the rate of dialysis of phenol red, at pH 7.3. The apparent half-life for escape of phenol red from the dialysis cell was calculated to be 122.4, 67.8, and 48.6 min. for the 10, 25, and 40° systems, respectively. The data were further treated utilizing the Arrhenius expression, and the energy of activation for the dialysis of phenol red through the cellophane membrane was graphically determined to be 5.5 kcal./mole/deg. The results of these studies clearly indicate the importance of adequate temperature control, in order to preclude any temperature effect on the intrinsic dialytic rate of the small molecule through the membrane.

Influence of Stirring on Rate of Dialysis—The effect of agitation on the rate of dialysis through a membrane has been discussed by Carr (7). At the initiation of dialysis a concentration gradient for the diffusing species exists across the membrane, from a region of uniform high diffusate concentration on one side, to a region of zero concentration on the other. As the dialysis proceeds there is an accumulation of diffusate on one side and a depletion on the other, with a resulting marked diminution of the concentration gradient across the membrane. Therefore agitation of the solution on each side of the membrane tends to homogenize the concentrations of the solutions and results in an increase in the rate of dialysis, which is proportional to the concentration gradient.

The dynamic dialysis system allows stirring, both internally *via* a twisted glass rod attached to a variable speed motor, and externally by means of a magnetic stir bar. Figure 2 illustrates the effect of stirring on the rate of dialysis of phenol red. There was no apparent difference between an internal stirring rate of 125 and 500 r.p.m., indicating that no advantage is to be gained by stirring the internal solution at other than a moderate sitrring rate. It was interesting to observe that for the unstirred systems, there was an initial rapid rate of dialysis, followed by a significantly slower rate which was apparent after approximately 1 hr. This was undoubtedly a result of the accumulation and depletion phenomena discusssed with respect to the diffusion gradient existing across the membrane. In the totally unstirred system it was observed that a visible diffusion layer developed on the outside of the membrane.

In view of these results, subsequent dynamic dialysis studies were conducted under stirred conditions. The external solution was agitated at a relatively constant rate with a magnetic stir bar. The contents of the dialysis sac were stirred with a twisted glass rod rotated at approximately 125 r.p.m.

Influence of Viscosity on Rates of Dialysis—The dynamic dialysis method for measuring protein-small molecule interactions is dependent on the assumption that the free, unbound diffusing species will dialyze with an identical rate in the presence or absence of protein. It was therefore necessary to establish that increased viscosity of the internal solution, due to the presence of protein, did not affect the rate of dialysis. In other words, it was necessary to show that in the presence of protein, diffusion through the membrane rather than diffusion to the membrane was still the rate-limiting step. To study the potential effect of viscosity, an agent was required which would simulate the viscosity of a protein solution, would not bind the diffusing species, and would not diffuse through the dialysis membrane. Phenol red was utilized as the small molecule and methylcellulose was employed in these studies to simulate the viscosity of solutions containing protein.

The relative viscosities of a series of protein-phenol red solutions of various BSA concentrations were measured with a Gilmont falling-ball viscometer. Other experiments showed that phenol red solutions, at 25°, containing 0.01–0.07% methylcellulose, in pH 7.3 buffer, had relative viscosities comparable to those of 1–5% BSA-phenol red solutions, also at pH 7.3 and 25°. The relative viscosities were calculated as the observed viscosity divided by the viscosity of water at the experimental temperature. Table II illustrates the relative viscosities obtained for solutions of BSA and methylcellulose at various concentrations. In addition, data obtained by Shikama (8), using an Ostwald-type coil viscometer, are given for comparison.

It was also necessary to show that methylcellulose did not interact, to a significant degree, with phenol red. The lack of interaction was established through ultrafiltration experiments. The results of these studies indicated that a maximum of approximately 4 % of the phenol red was bound to the methylcellulose, over the concentration range studied. This amount was too small to significantly affect the rate of phenol red dialysis in the dynamic dialysis system.

The results of the dialysis studies of phenol red at pH 7.3 and 25°, in the presence and absence of methylcellulose, is given in Fig. 3. It is apparent that the increased viscosity did not affect the rate of dialysis. Thus it may be concluded that increases in viscosity due to the presence of at least 5.2 % BSA, will not influence the rate of dialysis.

Influence of pH and Buffer Concentration on Rates of Dialysis—The effect of pH on dialytic rate was studied to observe whether or not the degree of ionization of a diffusing species affected the observed rate of dialysis. In addition, it was of interest to investigate the possibility that ionizable groups in the cellophane membrane could influence the rate of dialysis of a charged species. It should be noted, however, that there is some disagreement in the literature (5, 9) as to whether charged groups, primarily carboxyl groups, are actually present in cellophane.

The dialysis of 8-nitrotheophylline, which has a pKa of 2.11, was studied at pH 7.0, 3.0, and 11.0, in that order, with the same dialysis sac utilized for all three experiments. The results are illustrated

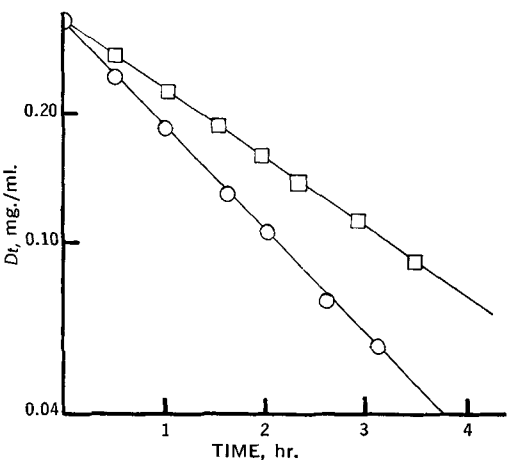


Figure 8—*The effect of a pressure head on the apparent first-order escape of phenol red, at pH 7.3 and 25°. Key: □, 10 ml. of solution inside sac; ○, 7 ml. of solution inside sac.*

in Fig. 4. It is apparent that the rate of dialysis was constant over the wide pH range. In addition to the lack of effect of pH, Fig. 4 indicates the excellent reproducibility for various experiments conducted with the same dialysis sac.

As a further test of the effect of pH on the rate of dialysis, the behavior of 8-chlorotheophylline, which has a pKa of 5.28, was studied. The investigations were conducted at pH 3.0 and 11.0 in 0.06 *M* phosphate buffer, and at pH 3.0 in 0.006 *M* phosphate buffer, using the same dialysis sac. The results of these studies are shown in Fig. 5 and indicated that the state of ionization of the 8-chlorotheophylline and the buffer concentration, within the concentration range utilized, did not influence the rate of dialysis.

The dialysis of caffeine, which is nonionized over the pH range of interest, was studied at pH 3.0 and 11.0. The results were identical to those obtained for 8-nitrotheophylline and 8-chlorotheophylline and showed that the rate of caffeine dialysis was independent of pH and highly reproducible for a given membrane.

Finally, the dialysis of phenol red, at several pH's, was studied using the same sac for each pH. This dye was selected because it is a dibasic acid with a $pK_1 < 1$ and a $pK_2 = 7.9$. The dialysis studies were conducted at pH 11.0, 3.0, and 7.9. The order of the experiments was again chosen to enable the determination of reversibility of any observed effect of pH. The results of these studies are shown in Fig. 6, and illustrate a definite pH effect. The observed results may be rationalized on the basis of an increased effective molecular size due to an increased state of hydration of the phenol red as it changed from a singly charged to a doubly charged anion. This increase in effective size may retard passage of the ion either through the tortuous pores of the membrane or through a diffusion layer. The possibility also exists that the pH affected the ionization of groups of the membrane and that these groups are only important in retarding the dialysis of a relatively large anionic species such as phenol red.

The primary conclusion to be obtained from these experiments is that if protein binding studies are to be conducted at several pH's, using the dynamic dialysis technique, it may be necessary to run control experiments in the absence of protein at each pH, to establish the value for the apparent permeability constant for the small molecule.

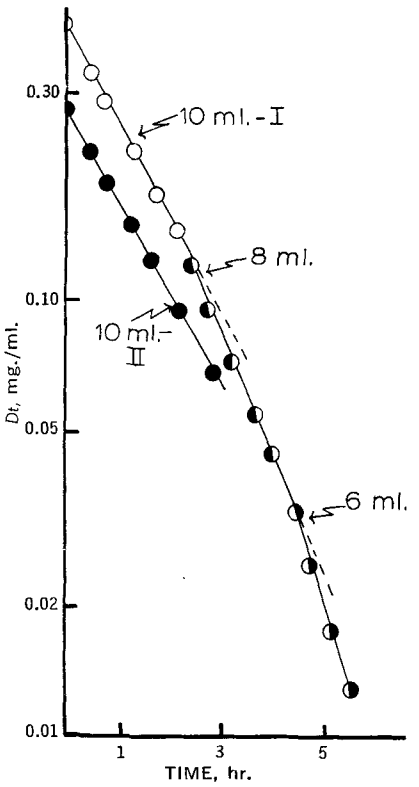


Figure 9—*The influence of changing solution volume, within the dialysis sac, during the course of phenol red dialysis at pH 7.3 and 25°. (I), initial 10-ml. volume of the dialysis cell decreased by 2 ml. at approximately 2-hr. intervals; (II), volume of the dialysis cell restored to 10 ml.*

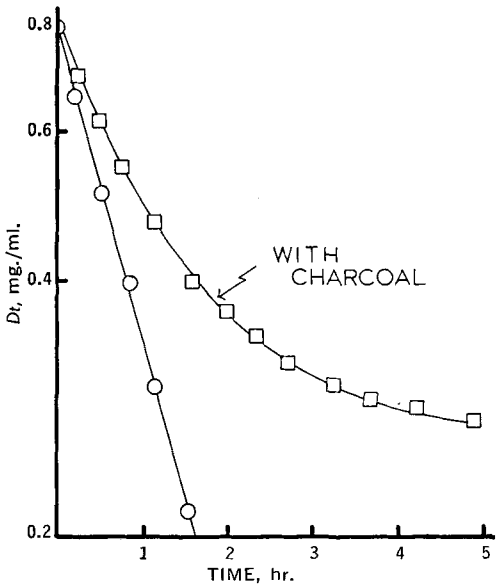


Figure 10—*The effect of 7.45 mg. of charcoal on the apparent first-order escape of methyl orange at pH 7.3 and 25°.*

Influence of Dialysis Sac Size and Solution Volume on Rates of Dialysis—The rate of dialysis would be expected to depend on the area of the membrane exposed to the solution of diffusing species. It was, therefore, considered important to evaluate the extent to which sac size influenced rate of dialysis. In addition, experiments were conducted to determine if the volume of external bathing solution could affect the observed rate of dialysis.

The first experimental series followed phenol red dialysis from systems in which the internal volume was 6, 5, or 4 ml. The next series utilized an external volume of 350 or 500 ml. of buffer bathing solution. Finally, the length of the sacs was decreased and the phenol red dialysis was followed from sacs which were 6, 5, or 4 cm. long. In order to evaluate the effects of volume and sac size variation, the apparent permeability rate constants obtained from the various experiments were divided by the appropriate apparent rate constant obtained with the same sac under control conditions. This was done to normalize the data so as to correct for variation in the intrinsic permeability of each of the three dialysis sacs employed in these studies. Control runs were conducted with 7-cm. long sacs containing 7 ml. of phenol red solution. The control sacs were suspended in 200 ml. of external buffer bathing solution.

The results of these investigations are illustrated in Fig. 7, where the Ke ratio is the apparent permeability constant obtained for a given experimental condition, divided by the constant obtained for the given membrane in the control study. It is apparent from this figure that a decrease in the volume of the liquid inside the sac, or decreasing the length of the sac, with a concomitant decrease in volume of the internal solution, caused a progressive increase in the apparent permeability rate constant, and therefore a progressive decrease in the apparent half-life for dialysis. An analysis of these results showed that as the ratio of membrane surface area to the volume of solution in the sac increased, the apparent permeability constant also increased.

In addition to these studies, a system was also run in which the level of the liquid inside the sac exceeded the external liquid level, *i.e.*, a pressure head was present inside the sac. The results of this study are shown in Fig. 8 and indicate an increase in the apparent half-life of dialysis of approximately 50% for the system with the smaller surface area-to-liquid volume ratio.

As a final illustration of the effect of varying the internal liquid volume, a system was investigated which involved progressively decreasing the internal volume of phenol red solution throughout the course of the run. After sampling of the internal solution, without replacement, the internal and external liquid levels immediately equalized. Thus the area of the membrane exposed to both solutions remained relatively constant. The results of this study are illus-

trated in Fig. 9 and clearly demonstrate the previously observed increase in rate of dialysis with decrease in internal liquid volume. It should be noted that the influence of volume of internal liquid was entirely reversible as evidenced by the close agreement between the two runs, with 10 ml. of liquid inside the sac, conducted before and after the systems involving 8 and 6 ml. of internal liquid. Results, analogous to these, were obtained when 8-chlorotheophylline was employed as the diffusing species at both pH 3 and 11.

Several explanations may be offered to account for the observed behavior. It was noted that as the volume of the liquid inside the sac was decreased the sac assumed a semicollapsed state. It is possible, therefore, that as the size of the sac was increased for a given internal volume, or the volume was decreased for a given size sac, the mean distance from the center of the sac to the membrane decreased, and this decrease resulted in an increased rate. However, this would suggest that diffusion to the membrane was the rate limiting step, and since the solutions were stirred inside and outside, this explanation seemed unlikely in view of the stirring rate experiments. It is possible, however that as the distance between the stirring rod and the membrane decreased, more efficient stirring resulted and the thickness of the diffusion layer, which may still be present under moderate stirring conditions, was reduced and led to an enhancement of the diffusion rate. Alternatively, it is possible that as the surface-to-volume ratio increased, *i.e.*, as the bag was able to assume a somewhat collapsed state, the decrease in tension on the sac caused an alteration in the pore structure to one more favorable for the passage of the phenol red molecule. This may be visualized as either a decrease in the tortuosity of the pore, or perhaps a realignment of charged groups, which may be present in the membrane, in the vicinity of the pores. Other workers (5) have observed that changes in the tension on a membrane can have an effect on the permeability characteristics of a membrane. While the changes in the tension of the membrane, due to the changes in volume of the internal solution, are admittedly relatively small, it is conceivable that they are sufficiently great to cause an alteration in the tortuosity or size of the pore. This is more convincing when one examines the schematic concept which Mosse (10) has presented of the complex and tortuous nature of the pore structure of a cellulose derivative membrane. As further evidence for this hypothesis of variable pore structure, the work of Craig (5) may be cited. He studied the rate of diffusion of ribonuclease through cellophane casing under various conditions of membrane stretching. A threefold increase, in the apparent half-life observed for ribonuclease diffusion with linear stretching of the membrane, was attributed to deformation of the pores. The degree of stretching was much greater in these studies than in the present investigation. However, the results do give some insight into the potential effect of alteration of pore structure.

As a result of these experiments it was concluded that the volume of the solution external to the dialysis sac does not affect the rate of dialysis of a small molecule from within the sac. However the size of the sac and the volume of the diffusate solution within the sac can have a significant influence on the rate of dialysis. Fortunately, when sac size and volume are maintained constant, excellent run to run reproducibility can be achieved.

Binding of Small Molecules by the Dialysis Membrane—Some degree of membrane binding of a compound is a frequently observed occurrence in dialysis studies. If it is extensive, then a correction must be applied to account for the amount of small molecule which has been bound in this manner.

If this type of interaction is essentially an adsorption phenomenon, then it should be reversible. Ideally the membrane may be considered as another binding species and for systems containing protein, the membrane may be treated mathematically as though a second protein species were present. If the preceding concepts of membrane binding are correct, then in the absence of protein, a small molecule which strongly interacts with the membrane should yield curvature in the semilog plot of total drug remaining in a dialysis sac *versus* time. Thus the possibility of sac binding must be recognized in using the dynamic dialysis technique or erroneous characterization of protein binding behavior may result.

If membrane binding is acknowledged to be essentially an adsorption phenomenon, then very strong sac binding should be simulated by the presence of an adsorbent such as charcoal. Experiments were, therefore, conducted to measure the diffusion of methyl orange from a dialysis sac, in the presence and absence of 7.45 mg. of charcoal. The results of these studies are shown in Fig. 10 and illustrate the expected result that the presence of the adsorptive agent resulted in a nonexponential escape of the methyl orange from the dialysis sac.

Most studies in the literature, involving diffusion of small molecules through cellulose dialysis tubing, have reported less than 10%, and usually negligible membrane binding. Since a reasonably strong association between the small molecule and membrane would be necessary for any significant effect on the kinetics of dialysis, it is likely that only in the event of very strong sac binding would the dynamic dialysis method be affected. One means of detecting any effect of sac binding would be to observe for curvature in the semilog plot of total drug concentration remaining in the sac *versus* time, in the absence of protein. Such curvature was never observed for any of the small molecules employed during the course of these investigations.

REFERENCES

(1) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57,** 1627 (1968).

(2) W. Morozowich, Master's thesis, The Ohio State University (1956).

(3) G. Kanzaki and E. Y. Berger, *Anal. Chem.*, **31,** 1383(1959).

(4) R. E. Stauffer, in "Technique of Organic Chemistry," vol. 3, A. Weissberger, Ed., Interscience, New York, N. Y., 1950, pp. 323–326.

(5) L. C. Craig, *Advan. Anal. Chem. Instr.*, **4,** 35(1966).

(6) J. F. Danielli, in "Permeability of Natural Membranes," H. Davson and J. F. Danielli, Eds., Macmillan, New York, N. Y., 1943, p. 78.

(7) C. W. Carr, in "Physical Methods in Chemical Analysis," vol. 4, W. G. Berl, Ed., Academic, New York, N. Y., 1961, pp. 10–12.

(8) K. Shikama, *J. Biochem. Tokyo*, **64,** 55(1968).

(9) S. M. Neale, *Trans. Faraday Soc.*, **44,** 1027(1948).

(10) M. J. Mosse, *Chim. Anal. Paris*, **39,** 10(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication September 2, 1969.

* Predoctoral Fellow, Public Health Service. Present address: College of Pharmacy, Department of Pharmaceutics, University of Tennessee.

† Present address: Research & Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101

Dynamic Dialysis as a Method for Studying Protein Binding II: Evaluation of the Method with a Number of Binding Systems

MARVIN C. MEYER* and DAVID E. GUTTMAN†

Abstract □ A method for determining the concentrations of unbound and bound small molecule in a protein solution is described, based on the fact that the rate of disappearance of small molecule from a dialysis cell is proportional to the concentration of the unbound species. Curves describing the kinetics of dialysis for 8-nitrotheophylline, 8-chlorotheophylline, caffeine, warfarin, methyl orange, and phenol red in the presence and absence of albumin were obtained and analyzed to yield estimates of unbound and bound forms of the compounds. The data were treated by means of Scatchard plots to provide values for the binding parameters, n and K. The values were found to be in agreement with those reported in the literature and/or determined by independent methods. The ease of application and merits of the method were demonstrated by a comprehensive study of the influence of pH on the binding of 8-nitrotheophylline by bovine serum albumin. Utilization of the method for the study of competitive inhibition of protein binding was also illustrated.

Keyphrases □ Dialysis, dynamic—protein binding determination □ Small molecule binding—protein □ Protein binding—competitive inhibition, drugs □ Kinetic equations—dialysis, small molecules □ pH binding profile, small molecule—kinetics □ UV spectrophotometry—analysis

Previous reports in this series (1, 2) have described in detail a new method for the determination of the degree of interaction between proteins and small molecules. The method is based on the fact that the rate of disappearance of small molecule from a dialysis cell is proportional to the concentration of the unbound species. The influence of experimental variables such as pH, temperature, stirring rate, viscosity, size of the membrane, volumes of liquid within the system, and membrane binding of the small molecule have been previously considered (2).

The present communication describes a series of studies designed to further evaluate the dynamic dialysis method as a technique to quantitate protein binding systems, and to obtain values for the fundamental binding parameters of n (the number of binding sites involved in the interaction) and K (the intrinsic association constant characterizing the interaction). The method was also applied to the comprehensive determination of the effect of pH on the interaction between 8-nitrotheophylline and bovine serum albumin over a wide range of pH. Studies were also conducted to demonstrate the potential of the dynamic dialysis method for the study of competitive inhibition of protein binding.

EXPERIMENTAL

Materials—Human serum albumin, (HSA) Fraction V, (Calbiochem), was used in this study. Warfarin sodium (Endo Laboratories) was used, and the remainder of the materials utilized in the study have been previously described (2).

Dynamic Dialysis Studies of Protein Binding—The protocol for the dynamic dialysis system and the treatment and interpretation of data have been discussed previously (1, 2). The method was employed to assess the binding of phenol red, warfarin, caffeine, 8-chlorotheophylline, 8-nitrotheophylline, salicylate, and methyl orange by bovine serum albumin (BSA). Initially, 7 ml. of a control solution, prepared to contain the given small molecule in 0.04 M phosphate buffer at an appropriate pH, was placed into a 7-cm. long dialysis sac. The sac was then immersed in the buffer bathing solution of 0.04 M, phosphate buffer. The system was maintained at 25 ± 0.2°. Each control dialysis was followed for about 2 hr., and the results were used to calculate the apparent permeability rate constant characterizing the escape of small molecule from each sac. The sac was then rinsed thoroughly with distilled water and blotted with tissue to remove excess moisture. Seven milliliters of a small molecule-protein solution, prepared in 0.04 M buffer at the same pH as the control, was then introduced into the sac. The sac was placed into fresh buffer bathing solution and the dialysis of the small molecule from the protein compartment was followed for 5 to 9 hr. The frequency at which 100-ml. samples were removed from the external solution and replaced with fresh buffer solution was dictated by the need to maintain sink conditions, and by the sensitivity of the assay method for the particular small molecule. It was observed from preliminary experiments that sink conditions were approximated if the concentration of small molecule in the external solution was not allowed to exceed one-tenth of that unbound in the internal solution. At the termination of a run, the internal solution was sampled and analyzed for small molecule content. The binding parameters obtained from the dynamic dialysis studies were then compared with values obtained by alternate approaches such as equilibrium dialysis or ultrafiltration, and/or with literature values.

For the study of the influence of pH on the interaction between BSA and 8-nitrotheophylline, three separate experimental systems could be conveniently run at one time, each maintained at a different pH. For these determinations, a series of solutions were prepared containing 8-nitrotheophylline in the presence and absence of BSA. The solutions covered a pH range of 3.0–10.9, in 0.04 M phosphate buffer. The protein systems contained approximately 0.75% BSA. The external buffer bathing solution was also prepared at the same pH and buffer concentration as the internal solution. The dialysis was followed for a sufficient period of time to fully characterize the kinetics of each system. The pH's of the internal and external solutions were redetermined at the termination of each experimental run and were found not to have changed.

Data Treatment—Kinetic curves for each system investigated were analyzed by the previously reported method (1). The data treatment yielded values for the concentrations of unbound (D_f) and bound (D_b) species, for a number of different total small molecule concentrations (D_t). Results were displayed in the form of Scatchard plots where $\bar{\nu}/(D_f)$ ($\bar{\nu}$ = moles of small molecule bound per mole of protein) was plotted as a function of $\bar{\nu}$. Values of the binding parameters (n's and K's) were estimated by fitting the data, with the aid of a nonlinear regression computer program,[1] to the generalized binding expression given in Eq. 1.

$$\bar{\nu}_d = \sum_{i=1}^{i} \frac{n_i K_i (D_f)}{1 + K_i (D_f)} \qquad \text{(Eq. 1)}$$

Two classes of binding sites were assumed if the experimentally determined Scatchard plot exhibited curvature (3). In this report experimental data are indicated in the Scatchard plots by points while solid lines represent behavior predicted on the basis of computed n and K values.

[1] Revised Share Distribution No. 1428, S.U.N.Y.A.B. Library Subroutine-NLIN.

Table I—Summary of the Binding Constants for the Interaction of Various Small Molecules with BSA at pH 7–7.3 and 25°

Compound	Source of Constants	n_1[a]	n_2	K_1[b]	K_2
Phenol red	Experimental: ultrafiltration and dynamic dialysis	1	6	1.74×10^5	1.97×10^3
	Literature: ultrafiltration (15)	1	6	1.10×10^5	1.20×10^3
Methyl orange	Experimental: dynamic dialysis	22	—	2.08×10^3	—
	Literature: equil. dialysis (5)	22	—	2.27×10^3	—
	Literature: equil. dialysis (16)	16	—	3.80×10^3	—
Warfarin	Experimental: ultrafiltration and dynamic dialysis	1	6	6.24×10^6	2.61×10^3
Caffeine	Experimental: dynamic dialysis	1	—	1.02×10^3	—
	Literature: equil. dialysis-9° (7)	1	—	1.68×10^3	—
8-Nitrotheophylline	Experimental: dynamic dialysis	1	4	2.34×10^5	2.53×10^3
	Literature: equil. dialysis (pH 6.85 and 9°) (7)	1	2	2.64×10^5	1.27×10^3
8-Chlorotheophylline	Experimental: dynamic dialysis	1	4	2.16×10^5	1.47×10^3
	Literature: equil. dialysis (pH 6.85 and 9°) (7)	1	2	3.07×10^5	1.10×10^3
Salicylic acid	Experimental: dynamic dialysis	1	5	2.00×10^5	1.75×10^3

[a] n_i = the number of binding sites in the i'th class. [b] K_i = the intrinsic association constant for the i'th class of sites (l./M).

Ultrafiltration—Ultrafiltration was utilized to study the binding of warfarin by BSA and HSA and the procedure used was that previously described (2). These studies were performed with solutions containing warfarin in a concentration range of 0.032–0.380 mg./ml. in 0.04 M, pH 7.3, phosphate buffer. The initial protein concentration was 0.37% for the BSA studies, and 0.39 or 0.78% for the HSA studies. After centrifugation the concentration of small molecule in the filtrate and the total small molecule concentration of the solution remaining in the sacs was determined. From a knowledge of these concentrations and the total protein concentration in the system, the values for $\bar{\nu}/(D_f)$ and $\bar{\nu}$ were calculated. Preliminary experiments indicated that approximately 4% of the warfarin introduced into the system was bound to the membrane.

Equilibrium Dialysis Studies—The binding of warfarin by BSA was also studied by the technique of equilibrium dialysis. These experiments were conducted using 10-ml. capacity, Plexiglas dialysis cells. The warfarin concentrations employed in these studies ranged from 0.002–0.6 mg./ml., in 0.04 M, pH 7.3, phosphate buffer. The BSA concentration was 0.4%. Preliminary experiments indicated that about 3% of the warfarin was bound to the membrane separating the two compartments of the dialysis cell, and that equilibrium was attained in the cells within 12 hr. The systems were prepared by placing 5 ml. of warfarin solution on one side of the membrane, and 5 ml. of warfarin and protein solution on the other. The cells were agitated, on a platform shaker, for at least 14 hr. at room temperature, 25 ± 2°. After equilibration, both sides of the cell were sampled, and from a knowledge of the total warfarin in the system, the free warfarin concentration, the degree of membrane binding, and the total protein concentration, the values for $\bar{\nu}/(D_f)$ and $\bar{\nu}$ were calculated.

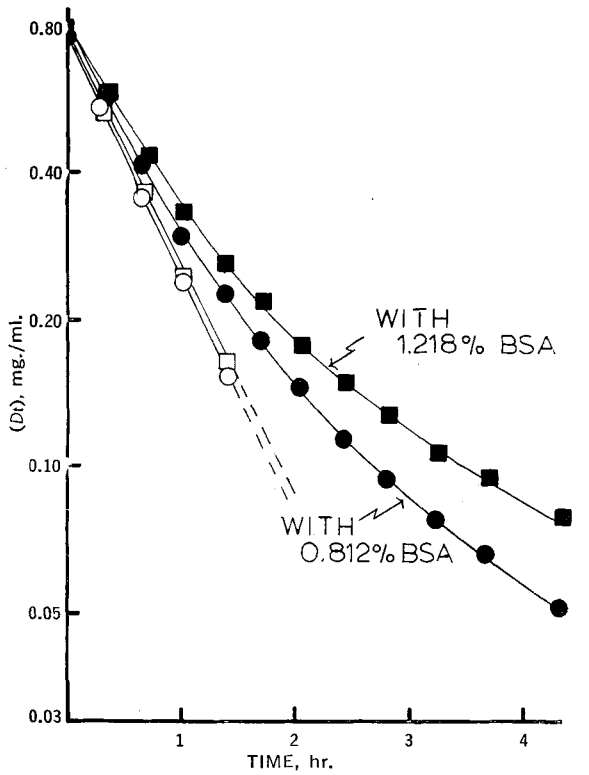


Figure 1—*The loss of 8-chlorotheophylline from inside a dialysis sac, in the presence and absence of BSA. The studies were run at pH 7.0 and 25°.*

Dynamic Dialysis Studies of Competitive Inhibition of Binding—The preliminary step in these studies was to determine if the presence of the competitor in the system exerted an effect on the intrinsic rate of dialysis of the small molecule under consideration. 8-Nitrotheophylline was utilized as the test species, and 8-chlorotheophylline or salicylic acid were employed as the competitors. A control run with 8-nitrotheophylline was first conducted, and then using the same membrane sac, the dialysis of 8-nitrotheophylline was followed in the presence of the competitor. Then an 8-nitrotheophylline and BSA system was dialyzed to obtain the kinetic and equilibria parameters operant in the absence of competitor. Finally, still using the same membrane, 8-nitrotheophylline, BSA, and competitor were dialyzed. Two approaches were used in the competitive studies. In the first, a known competitor concentration was incorporated inside the dialysis sac. The decline of both 8-nitrotheophylline and competitor concentration inside the sac was followed by determining the amounts of 8-nitrotheophylline and competitor appearing in the external solution. The second approach was such that the concentration of competitor remained essentially constant throughout the dialysis. Here a known competitor concentration was placed into the dialysis sac containing the 8-nitrotheophylline. In addition, the external buffer bathing solution was prepared to contain an equivalent concentration of competitor. Further, the samples which were withdrawn from the external solution were replaced with a solution which contained the same concentration of the competitor as originally present. In this manner the concentration of the competitor was held constant during the course of the dialysis. All studies involving 8-nitrotheophylline and/or competitor species were conducted at 25° in pH 7.0, 0.04 M, phosphate buffer.

Analytical Methods—The compounds studied were assayed spectrophotometrically with a Beckman DU spectrophotometer equipped with a Gilford power source and digital, absorbance read-out. Concentrations were determined from Beer's law plots constructed at appropriate wavelengths and pH values.

The concentration of 8-nitrotheophylline in the presence of BSA was determined spectrophotometrically at pH 11 *versus* an appropriate BSA blank. The absorbance due to protein was negligible in the visible region of the spectrum, and at pH 11 there were no spectral manifestations of interactions between the BSA and 8-nitrotheophylline.

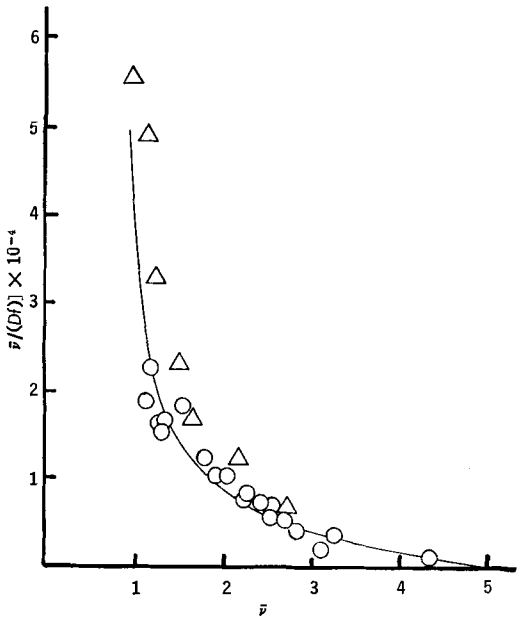


Figure 2—*The binding of 8-chlorotheophylline by BSA at pH 7.0 and 25°. Key: ○, 0.812 and 1.218% BSA, dynamic dialysis; △, literature values; (6), equilibrium dialysis at pH 6.85 and 9°.*

Because BSA interfered with the direct spectrophotometric determination of caffeine, warfarin, and 8-chlorotheophylline, extraction procedures were utilized. Two milliliters of sample containing caffeine and BSA were quantitatively extracted with 25 ml. of chloroform, and the organic layer was examined spectrophotometrically at 272 mμ *versus* an appropriate chloroform blank. Samples containing 8-chlorotheophylline and BSA were assayed for 8-chlorotheophylline by diluting 2 ml. of sample to 3 ml. with diluted phosphoric acid (1:5) and extracting with 25 ml. of a chloroform–isopropyl alcohol (19:1) solution. The organic phase was assayed spectrophotometrically at 276.5 mμ *versus* an appropriate solvent blank. Solutions containing both warfarin and BSA were assayed by the method of O'Reilly *et al.* (4). Centrifugation was utilized to break any emulsions formed during the extraction procedures.

Samples containing 8-nitrotheophylline in combination with 8-chlorotheophylline, caffeine, or salicylic acid were assayed for both components by a differential spectrophotometric assay conducted at 386 mμ, and 276.5, 272, or 302 mμ, respectively. Samples containing 8-nitrotheophylline, 8-chlorotheophylline, and BSA were adjusted to pH 11 and assayed for 8-nitrotheophylline at 386 mμ. The 8-chlorotheophylline concentration was determined by extracting an acidified sample and measuring the absorbance of the organic phase at 276.5 mμ. The latter absorbance was corrected for the small contribution of the 8-nitrotheophylline to the reading at this wavelength.

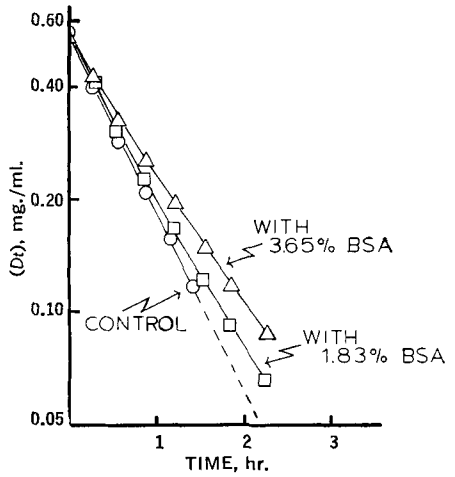


Figure 3—*The loss of caffeine from inside a dialysis sac, in the presence and absence of BSA. The studies were run at pH 7.0 and 25°.*

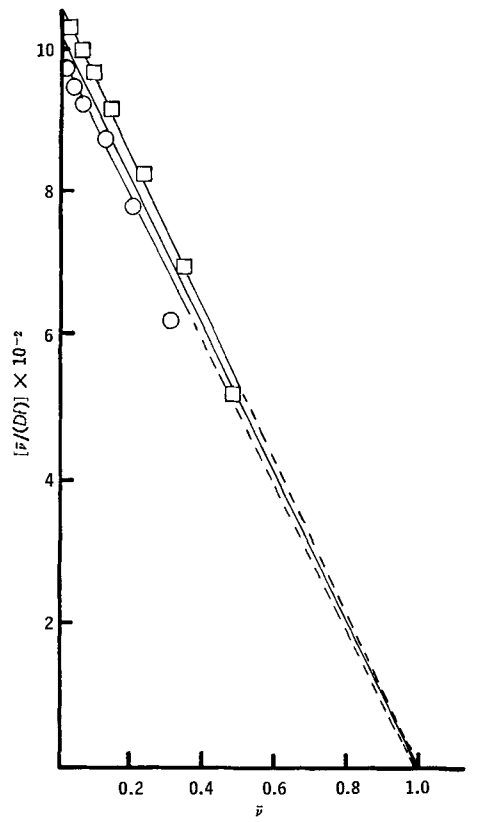


Figure 4—*The binding of caffeine by BSA at pH 7.0 and 25°, as depicted by a Scatchard plot. Key: ○, 1.83% BSA, □, 3.65% BSA.*

RESULTS AND DISCUSSION

Determination of the Binding Constants for the Interaction of Various Small Molecules with BSA—A previous report (1) described studies employing this technique with phenol red and methyl orange. For comparative purposes, binding parameters for these two compounds are presented in Table I, along with values for a number of other compounds which were investigated. It should be noted that parameters characterizing the binding of methyl orange (1, 5) were reported incorrectly and have been corrected in Table I.

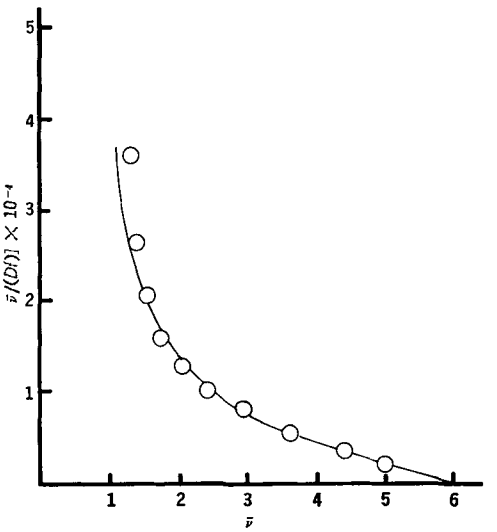


Figure 5—*The binding of salicylic acid by BSA at pH 7.0 and 25°.*

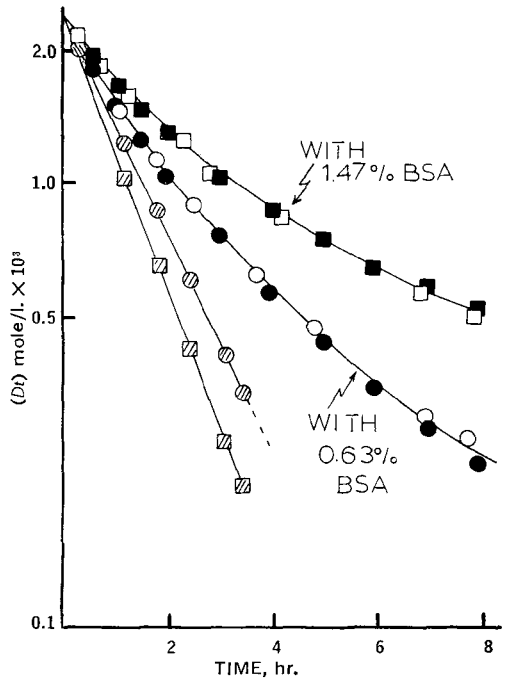


Figure 6—*The loss of warfarin from inside a dialysis sac, in the presence and absence of BSA. The studies were run at pH 7.3 and 25°. Key: ○, 0.63% control; □, 1.465% control; ●, 0.63% BSA, experimental; ■, 1.465% BSA, experimental; ○, 0.63% BSA, theoretical; □, 1.456% BSA, theoretical.*

Binding of 8-Chlorotheophylline—Figure 1 illustrates the results of dynamic dialysis experiments with 8-chlorotheophylline in the presence and absence of BSA at 25° and pH 7.0. Transformation of the data, in the manner described, yielded the Scatchard plot of Fig. 2. Data obtained by Eichman *et al.* (6) for this system at pH 6.85 and 9°, using equilibrium dialysis, is also shown. The small differences between Eichman's data and those of the present study can be attributed to a difference in the temperatures employed.

Binding of Caffeine—Caffeine has been shown to interact rather weakly with BSA (6, 7). It was, therefore, of interest to attempt to quantitate its binding behavior by the dynamic dialysis approach.

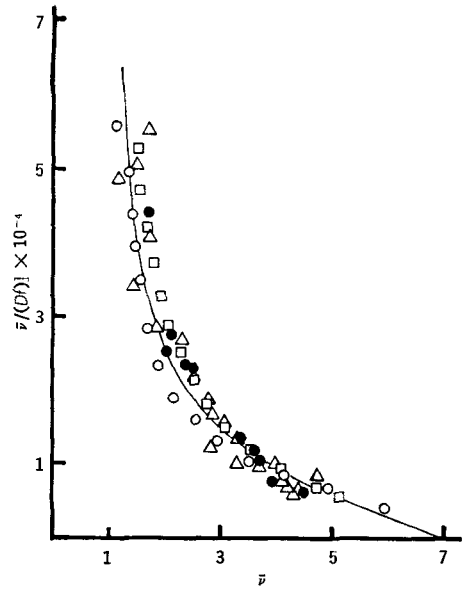


Figure 7—*The binding of warfarin by BSA and HSA at pH 7.3 and 25°. Key: △, BSA, ultrafiltration and equilibrium dialysis; ●, HSA, ultrafiltration; ○, 0.63% BSA, dynamic dialysis; □, 1.465% BSA, dynamic dialysis.*

Results obtained at 25° in 0.04 *M* pH 7 buffer are shown in Fig. 3. It can be seen that in spite of relatively high protein concentrations, the difference in kinetic behavior in the absence and presence of protein was small and reflects the weak nature of the interaction. Figure 4 shows the corresponding Scatchard plot. From a least-squares fit of the Scatchard plot, it was determined that the interaction between BSA and caffeine could be appropriately characterized by the parameters $n = 1$, and $K = 1.02 \times 10^3$ l./mole. These values are in reasonable agreement with the parameters reported by Eichman *et al.* (6) from equilibrium dialysis studies at pH 6.85 and 9°. The somewhat lower association constant obtained in the present study, which was conducted at a higher temperature than the work of Eichman *et al.* is consistent with a previous observation (8) of decreased xanthine binding with increased temperature.

It should be noted that the binding of a weakly interacting compound is difficult to quantitate accurately by any experimental technique due to magnification of experimental error by subsequent data treatment. The caffeine studies demonstrate that the dynamic dialysis method can be useful in characterizing even weak interactions.

Binding of Salicylic Acid—Figure 5 illustrates a Scatchard plot derived from the kinetics of dialysis of salicylic acid at pH 7.0 and 25° in the presence of 1.616% BSA and in the absence of BSA. The data were found to be consistent with the binding parameters given in Table I.

The binding observed in this study was somewhat stronger than that observed by Davison and Smith (9) in their studies conducted at 4° and pH 5.4, in acetate buffer. They reported binding constants of $n_1 = 0.37$, $n_2 = 3.5$, $K_1 = 3 \times 10^4$ l./mole, and $K_2 = 1 \times 10^2$ l./mole, and observed little difference in binding behavior between pH 5.4 and 7.0. They also reported that acetate can competitively inhibit salicylate binding and this might be the reason for the difference in strengths of binding between the two studies.

Binding of Warfarin—The binding of warfarin by albumin has been well studied and characterized (10) and thus, it was felt that warfarin would serve admirably as an additional compound with which to assess this new approach for quantitating binding behaviors. The results of the dynamic dialysis examination of warfarin binding are summarized in Fig. 6 which depicts the kinetics of dis-

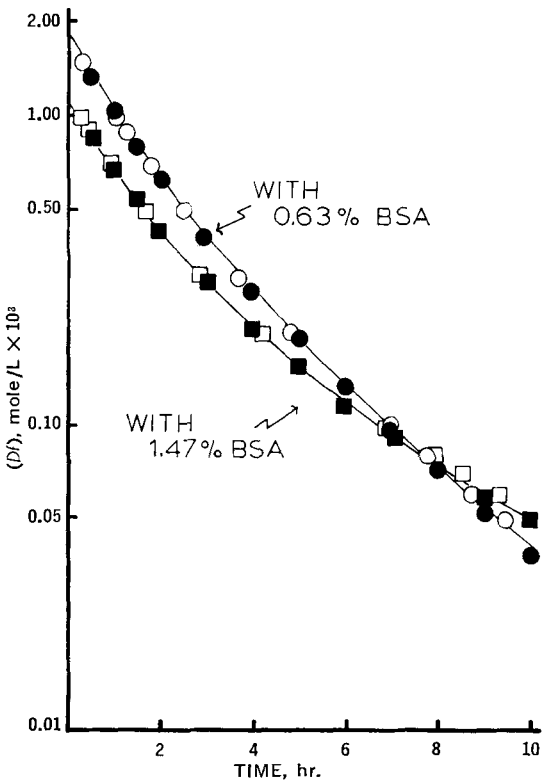


Figure 8—*The loss of free warfarin from inside a dialysis sac, in the presence of BSA, at pH 7.3 and 25°. Key: ●, ■, dynamic dialysis data; ○, □, theoretical data.*

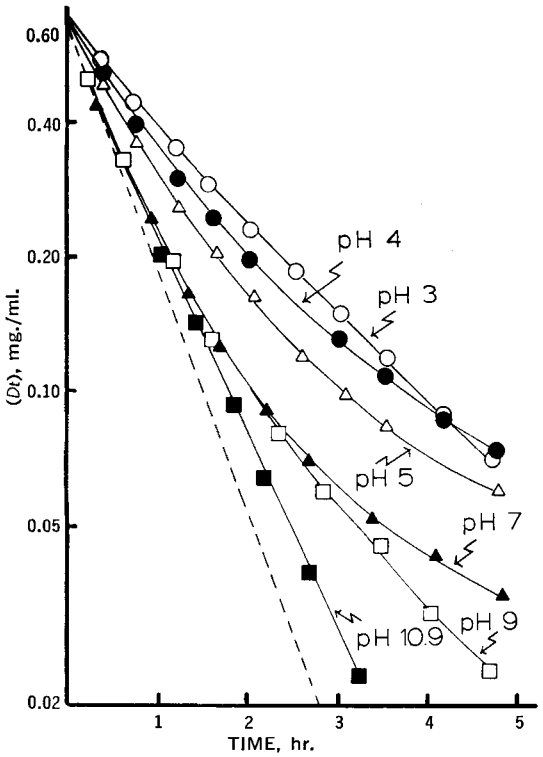


Figure 9—*The loss of 8-nitrotheophylline from inside a dialysis sac in the presence and absence of 0.75% BSA. The studies were conducted at a variety of pH's and 25°. Key: - - -, average control run.*

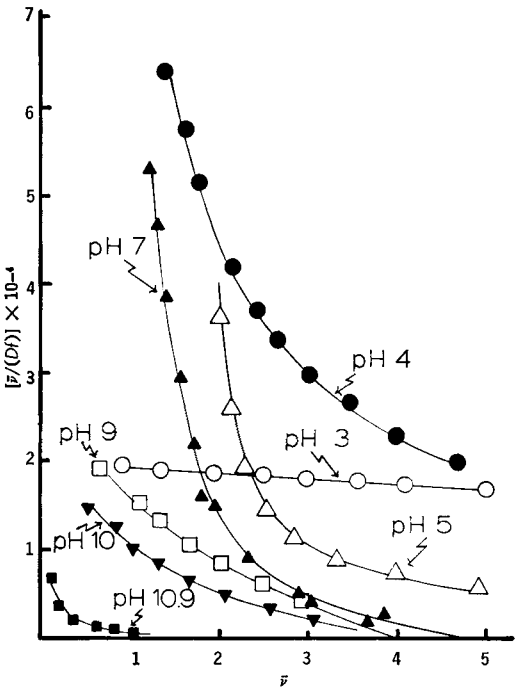


Figure 10—*The binding of 8-nitrotheophylline by BSA at various pH's and 25°.*

appearance of warfarin from a 0.630% solution of BSA and from a 1.465% solution of BSA, as well as for corresponding control systems. The difference between control runs is significant and reflects the fact that a different membrane was used for the two systems. The control runs were repeated and found to be entirely reproducible.

The Scatchard plot is shown in Fig. 7. It was disturbing to compare this plot with that presented by O'Reilly (10) who studied, by equilibrium dialysis, the interaction of warfarin with human serum albumin (HSA). O'Reilly's data indicated a much weaker degree of interaction and the marked lack of agreement prompted further studies on the binding of warfarin. To assess whether or not HSA possessed a significantly different affinity for warfarin than did BSA, the binding of warfarin by the two proteins was investigated using ultrafiltration. The results are also shown in Fig. 7 and demonstrate that the nature and extent of binding were the same for both proteins. The interaction was also investigated by equilibrium dialysis and the results are presented in Fig. 7. It is obvious that the three different experimental methods provided data which were in excellent agreement and, therefore, indicated that neither the source of the albumin nor the experimental approach could explain the lack of agreement with the results of O'Reilly. In view of this, O'Reilly's data were examined more critically to reveal two computational errors. First, a correction for sac binding was made which was based on the total concentration of warfarin rather than on the concentration of free warfarin in the system. This, however, resulted in only a minor error since the sac binding was only of the order of 3.6%. A more serious computational error was involved in the calculation of $\bar{\nu}$. When a correction was made the $\bar{\nu}$ values increased and were quite consistent with those of the present study. The combined data of Fig. 7 were used to generate the n and K values presented in Table I.

Theoretical Treatment Describing the Escape of a Small Molecule from a Protein-Containing Compartment—It has been demonstrated that kinetic data obtained in the dialysis of a small molecule in the absence and presence of protein can be treated to gain estimates of (D_f) and (D_b) and subsequently n's and K's. The validity of the approach has been confirmed by the close agreement of binding parameters, estimated by using this method, with those determined by classical methods. It did seem desirable, however, as a further check, to compare experimentally observed kinetic behavior with that theoretically expected on the basis of initial concentration conditions and binding parameters. Theoretical expressions for such a system have been considered, in a pharmacokinetic context, by Kruger-Thiemer (11) who derived equations which describe the rates of loss of unbound and total small molecule capable of being bound from a protein compartment. His derivations were restricted to situations where binding occurred to one class of sites on the protein but can be readily modified to account for the existence of two classes of sites. Such a modification results in Eq. 2 which describes dynamic dialysis behavior, *i.e.*, the rate of disappearance of total concentration of small molecule (D_t) from a protein compartment as a function of an apparent first-order elimination constant, K_e, the concentration of unbound small molecule (D_f), the total concentration of protein (P_t), the number of binding sites

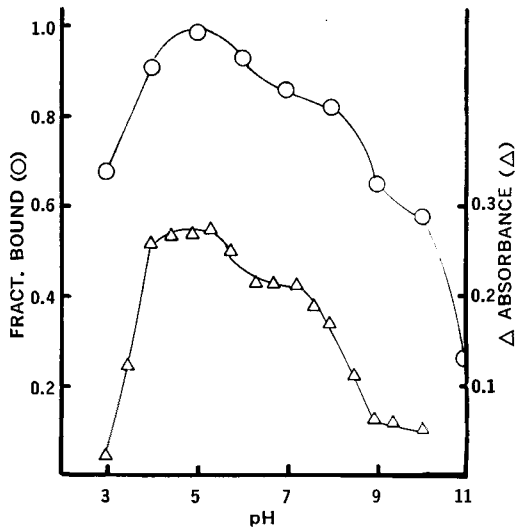


Figure 11—*The influence of pH on the binding of 8-nitrotheophylline at 25°. Key: △, delta absorbance values determined from spectrophotometric studies; ○, fraction bound values calculated from dynamic dialysis studies.*

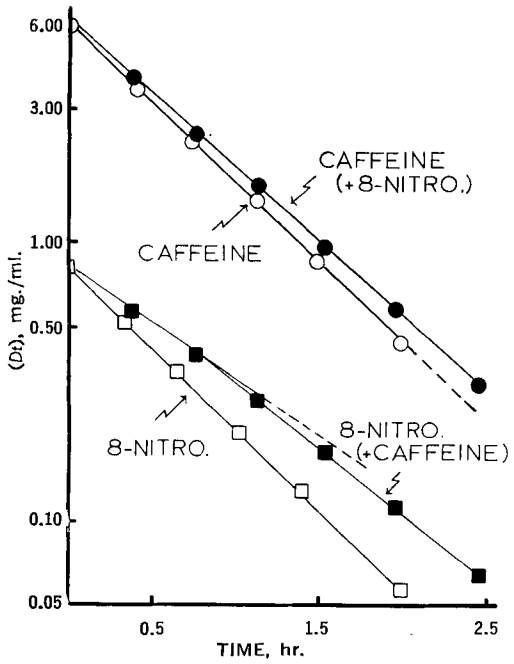


Figure 12—*The loss of 8-nitrotheophylline in the presence and absence of caffeine, and the loss of caffeine in the presence and absence of 8-nitrotheophylline, from inside a dialysis sac. The studies were conducted at pH 7.0 and 25°.*

in each of two classes, n_1 and n_2, and the corresponding dissociation constants, k_1 and k_2.

$$\frac{-d(D_t)}{dt} = \frac{K_e(D_t)}{1 + [n_1(P_t)]/[(D_f) + k_1] + [n_2(P_t)]/[(D_f) + k_2]} \quad \text{(Eq. 2)}$$

Equation 3 gives the rate of loss of free drug (D_f) from the protein compartment.

$$\frac{-d(D_f)}{dt} = \frac{K_e(D_f)}{1 + [n_1k_1(P_t)]/[(D_f + k_1)^2] + [n_2k_2(P_t)]/[(D_f + k_2)^2]} \quad \text{(Eq. 3)}$$

Finally, Eq. 4 gives the relationship between the total and unbound drug concentration.

$$(D_t) = (D_f)\left[1 + \frac{n_1(P_t)}{(D_f) + k_1} + \frac{n_2(P_t)}{(D_f) + k_2}\right] \quad \text{(Eq. 4)}$$

An expression for (D_t) as a function of time cannot be obtained directly by integration of Eq. 2 and, therefore, a more circuitous approach must be utilized to generate theoretical kinetic data. First, the value for the initial free drug concentration (D_f^0) in the protein compartment is calculated as follows: Eq. 4 may be written in terms of initial total (D_t^0) and initial unbound (D_f^0) drug concentrations. The resulting equation may then be rearranged to yield a cubic expression in (D_f^0):

$$(D_f^0)^3 + (D_f^0)^2[k_1 + k_2 + (P_t)(n_1 + n_2) - (D_t^0)] + (D_f^0)[k_1k_2 + (P_t)(n_1k_2 + n_2k_1) - (D_t^0)(k_1 + k_2)] - (D_t^0)(k_1k_2) = 0 \quad \text{(Eq. 5)}$$

It may be noted that when one class of binding sites is involved, a quadratic expression in (D_f^0) results. By integration of Eq. 3 between the limits of $t = 0$ to t, and $(D_f) = (D_f^0)$ to (D_f), an expression for time may be obtained:

$$t = \frac{1}{K_e}\left\{\ln\frac{(D_f^0)}{(D_f)} + \frac{n_1(P_t)}{k_1}\left[\ln\frac{(D_f^0)(k_1 + D_f)}{(D_f)(k_1 + D_f^0)}\right] + \frac{n_2(P_t)}{k_2}\ln\left[\frac{(D_f^0)(k_2 + D_f)}{(D_f)(k_2 + D_f^0)}\right] - (P_t)(D_f^\circ - D_f) \times \left[\frac{n_1}{(k_1 + D_f^0)(k_1 + D_f)} + \frac{n_2}{(k_2 + D_f^0)(k_2 + D_f)}\right]\right\} \quad \text{(Eq. 6)}$$

Thus, for values of n_1, n_2, k_1, k_2, (D_t^0), and (P_t), a value of (D_f^0) may be calculated from Eq. 5. Then assuming various values for (D_f), the time, t, at which the assumed (D_f) concentrations will be present in the protein compartment can be calculated from Eq. 6. Finally, using Eq. 4, the values for (D_t), corresponding to the times which were determined from Eq. 6, are calculated. These computations were carried out with the aid of a Fortran IV computer program and a CDC 6400 computer.

Comparisons between dialytic behaviors which were theoretically expected and experimentally determined for warfarin are shown in Figs. 6 and 8. Theoretical points were obtained using the outlined treatment with (D_t^0) and (P_t) values selected to correspond to the experimental system. The values of n and K used were those tabu-

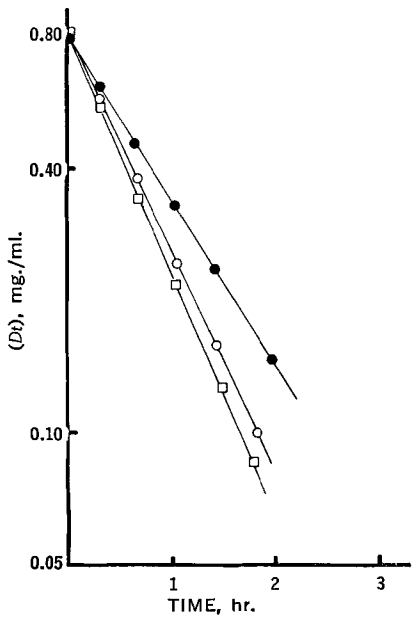


Figure 13—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of 8-chlorotheophylline. The studies were conducted at pH 7.0 and 25°. Key: □, control; ○, with 0.75 mg./ml. 8-chlorotheophylline; ●, with 1.74 mg./ml. 8-chlorotheophylline.*

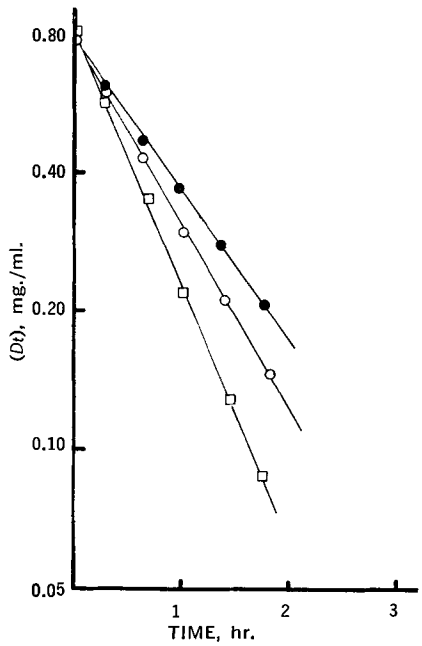


Figure 14—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of salicylate. The studies were conducted at pH 7.0 and 25°. Key: □, control; ○, with 9.8 mg./ml. salicylic acid; ●, with 22.6 mg./ml. salicylic acid.*

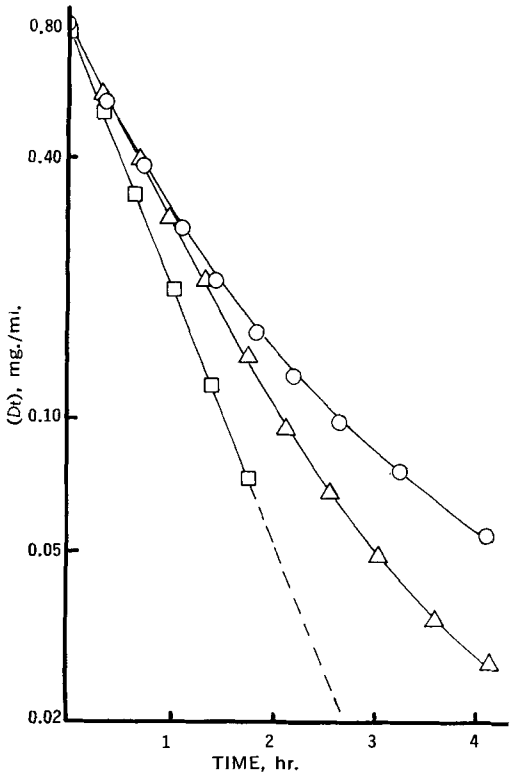


Figure 15—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of BSA, and BSA and 8-chlorotheophylline. The studies were conducted at pH 7.0 and 25°. Key: □, control; ○, with 0.818% BSA; △, with 0.818% BSA and an initial 8-chlorotheophylline concentration of 1.6 mg./ml.*

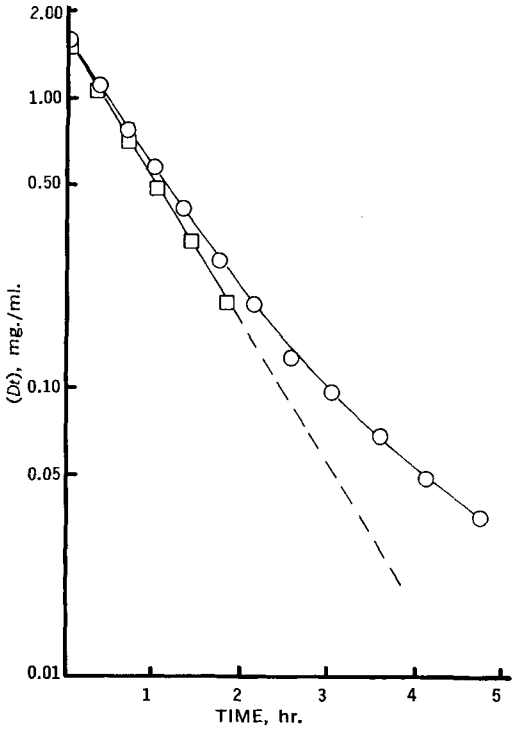


Figure 16—*The loss of 8-chlorotheophylline from inside a dialysis sac, in the presence and absence of BSA and 8-nitrotheophylline. These data were obtained in the same experimental run as was illustrated in Fig. 15. Key: □, control; ○, with 0.818% BSA and 0.82 mg./ml. 8-nitrotheophylline.*

lated in Table I. The K_e values employed corresponded to the experimentally determined apparent permeability constants for the membranes. The close agreements between experimental results and theoretical predictions illustrate the applicability of the equations and supports the validity of this experimental approach for studying binding behavior.

Kinetic Determination of the pH-Binding Profile for the Interaction of 8-Nitrotheophylline with BSA—The influence of pH on the binding of 8-nitrotheophylline by BSA, as determined by spectrophotometric investigations, has been previously reported (12). It seemed appropriate, in view of the interesting pH effects observed, to study more quantitatively the effect of pH on the interaction. In addition, it was thought that such a study would serve to evaluate further the dynamic dialysis method and demonstrate its applicability and convenience. From kinetic data obtained with the dynamic dialysis procedure, it was possible to generate Scatchard plots characterizing the interaction at each pH. Thus, binding behavior over a wide range of pH could be conveniently obtained in a relatively short period of time. Accumulation of such data by conventional techniques of equilibrium dialysis or ultrafiltration would require considerably more effort and a much greater expenditure of time.

The results of these studies are summarized in Fig. 9 where the kinetic behavior of 8-nitrotheophylline in the presence of BSA under different conditions of pH is shown. An average control run is also depicted. Data for pH 6, 8, and 10 were also obtained, but were omitted to reduce the complexity of the figure. From examination of the data of Fig. 9, it would appear that as the pH was increased from pH 3 to 10.9, the degree of interaction between the 8-nitrotheophylline and the BSA steadily decreased. The data were further treated by computer analysis, in the usual manner, to obtain the values for $\bar{\nu}$ and $\bar{\nu}/(D_f)$. In addition, the fraction of 8-nitrotheophylline bound to the BSA at each total 8-nitrotheophylline concentration was determined. The resulting Scatchard plots are shown in Fig. 10. Data for studies conducted at pH 6 and 8, which were similar to that obtained for pH 7, have been omitted to permit the figure to be more readily interpreted. This figure dramatically demonstrates differences in binding behavior as the pH was varied. At pH 3 the interaction was somewhat unique. The apparent linearity of the plot indicates the participation of one class of binding sites, containing a relatively large number of sites. The Scatchard plots representing other pH values are curved, indicating that at these pH's more than one class of sites were involved. The unusual behavior apparent at pH 3.0 is likely related to a pH dependent alteration of the protein configuration. Here the interaction is relatively weak as apparent from the shallow slope of the Scatchard plot. Thus the high degree of binding, suggested by the kinetic data

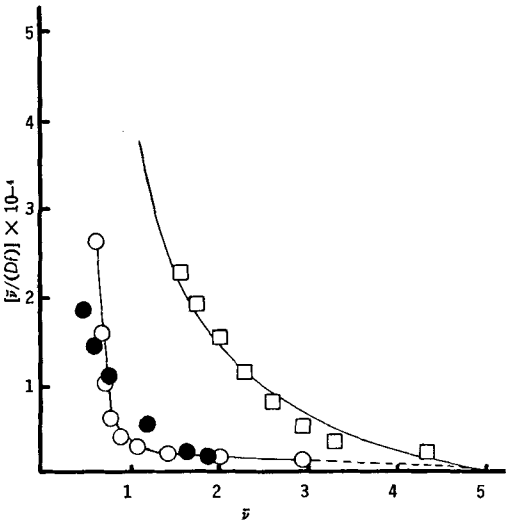


Figure 17—*The binding of 8-nitrotheophylline by BSA in the presence and absence of a variable 8-chlorotheophylline concentration. The studies were conducted at pH 7.0 and 25°. Key: □, 8-nitrotheophylline and 0.818% BSA; ○, 8-nitrotheophylline, 0.818% BSA and 8-chlorotheophylline; ●, theoretical data for the binding of 8-nitrotheophylline in the presence of 8-chlorotheophylline.*

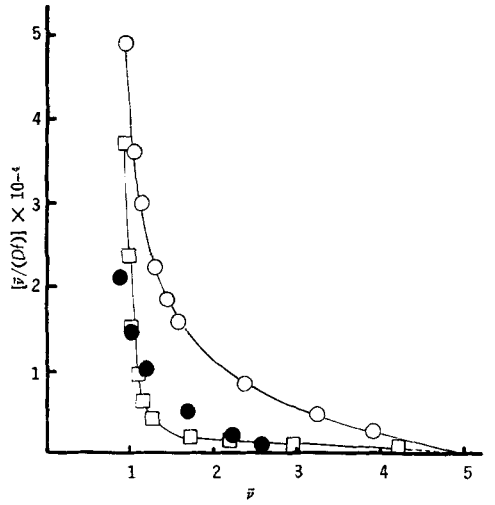


Figure 18—*The binding of 8-chlorotheophylline by BSA in the presence and absence of 8-nitrotheophylline. The studies were conducted at pH 7.0 and 25°. Key: ○, 8-chlorotheophylline and 0.818% BSA; □, 8-chlorotheophylline, 0.818% BSA and 8-nitrotheophylline; ●, theoretical data for the binding of 8-chlorotheophylline by BSA in the presence of 8-nitrotheophylline.*

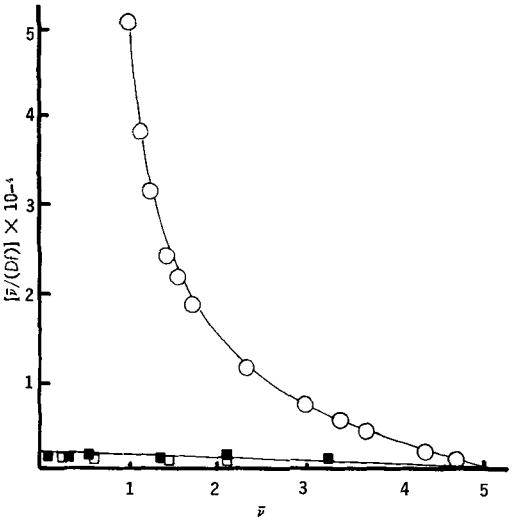


Figure 20—*The binding of 8-nitrotheophylline by BSA in the presence and absence of a constant 8-chlorotheophylline concentration of 0.76 mg./ml. Key: ○, 8-nitrotheophylline and BSA; ■, 8-nitrotheophylline, BSA, and 0.76 mg./ml. 8-chlorotheophylline; □, theoretical data for the binding of 8-nitrotheophylline in the presence of 0.76 mg./ml. 8-chlorotheophylline.*

obtained at pH 3, was a reflection of the involvement of a relatively large number of binding sites rather than a high intrinsic affinity. The pH 4 Scatchard plot also suggests the participation of a large number of binding sites with the added involvement of a second class of sites with a significantly higher affinity. Two or more classes of sites were apparently involved throughout the remainder of the pH range studied. An increase in pH from 4 to 6 resulted in a decrease in the total number of sites available for binding and an increase in the strength of association. As the pH was increased from 7 to 10.9 the extent of interaction diminished, probably as a result of changes in the degree of ionization of participating groups on the protein and/or pH-induced conformation changes (13). In order to compare more readily the results of this study to those obtained from the spectrophotometric studies previously reported (12), a plot of fraction bound *versus* pH was constructed and is shown in Fig. 11. The fraction bound, which is plotted, was that obtained at a total 8-nitrotheophylline concentration of 1.4×10^{-4} mole/l. in the presence of 0.75% BSA. The pH profile previously reported, for the interaction between 1.42×10^{-4} *M* 8-nitrotheophylline and 0.4% BSA, is illustrated for comparative purposes. There is good qualitative agreement between the two methods. However, at the extremes of pH, *i.e.*, at pH 3 and 10.9, the two techniques yielded significantly different results. The spectrophotometric studies indicated a relatively small degree of interaction at these pH extremes, whereas the dynamic dialysis method detected appreciable binding, with about 68% bound at pH 3 and 23% bound at pH 10.9. Some of this difference can, of course, be attributed to the higher BSA concentration used in the dynamic dialysis systems. However, the data may also reflect a shortcoming of the spectrophotometric method in that if interaction does not alter spectral properties of the small molecule, then the interaction cannot be detected from spectral studies. It is important to note that both studies yielded pH profiles having the same general shape and both studies, therefore, indicated that three or more classes of sites on BSA are involved in the binding of 8-nitrotheophylline, as previously discussed (12).

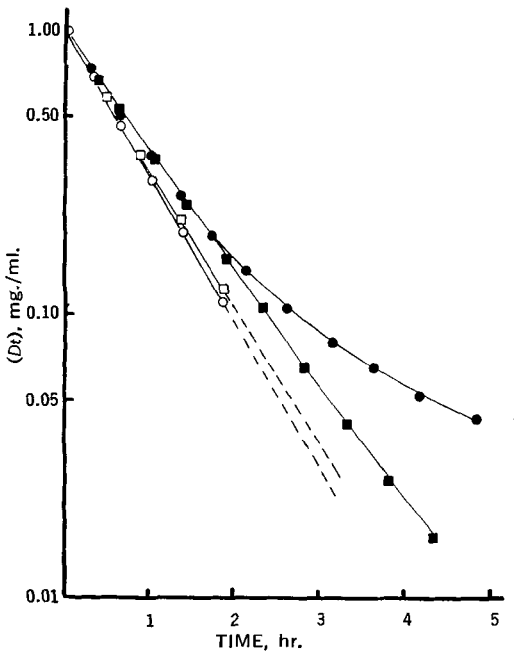


Figure 19—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of BSA, and BSA and a constant 8-chlorotheophylline concentration. The studies were conducted at pH 7.0 and 25°. Key: ○, 8-nitrotheophylline control; □, 8-nitrotheophylline control in the presence of 0.76 mg./ml. 8-chlorotheophylline; ●, 8-nitrotheophylline and 0.778% BSA; ■, 8-nitrotheophylline, 0.778% BSA and 0.76 mg./ml. 8-chlorotheophylline.*

The binding of 8-nitrotheophylline by BSA, at pH 7.0, was further evaluated to determine the binding parameters characterizing this interaction. The data appeared to be adequately described by an interaction involving two classes of binding sites, where $n_1 = 1$, $n_2 = 4$, $K_1 = 2.34 \times 10^5$ l./mole, and $K_2 = 2.53 \times 10^3$ l./mole.

Competitive Inhibition of Protein Binding—Inhibition of protein-small molecule interactions due to the presence of a species capable of competing for binding sites is a well recognized phenomenon and has recently been reviewed (14). Equation 7 describing competitive binding may be easily derived from mass law considerations and defines the dependency of binding of a species "*D*," in the presence of a competitor "*C*," on the unbound "*D*" concentration (D_f), and unbound competitor concentration (C_f), the number of binding sites on the protein (n) which interact with the drug and the competitor, and the corresponding intrinsic association constants for "*D*" and "*C*" (K_d and K_c).

$$\bar{\nu}_d = \frac{n_1 K_{d1}(D_f)}{1 + K_{d1}(D_f) + K_{c1}(C_f)} + \frac{n_2 K_{d2}(D_f)}{1 + K_{d2}(D_f) + K_{c2}(C_f)} \quad \text{(Eq. 7)}$$

Here, $\bar{\nu}_d$ = moles of Species "D" bound per total mole of protein. This equation may be rearranged in the form of a Scatchard equation, as given in Eq. 8.

$$\frac{\bar{\nu}_d}{(D_f)} = \frac{\Sigma nK_d + (D_f)K_{d1}K_{d2}\,\Sigma n + (C_f)(n_1K_{c2}K_{d1} + n_2K_{c1}K_{d2})}{1 + (C_f)[\Sigma K_c + K_{c1}K_{c2}(C_f)]} - \bar{\nu}_d\left[\frac{\Sigma K_d + K_{d1}K_{d2}(D_f) + (C_f)(K_{c2}K_{d1} + K_{c1}K_{d2})}{1 + (C_f)[\Sigma K_c + K_{c1}K_{c2}(C_f)]}\right] \quad \text{(Eq. 8)}$$

It may be shown that while Eq. 8 is derived in terms of $\bar{\nu}_d$, a completely analogous equation can similarly be derived in terms of $\bar{\nu}_c$ (moles of Species "C" bound per mole of protein).

An expression describing the rate of loss of small molecule "D" from a protein compartment containing a competitor "C," can be derived from the consideration that the rate of loss of "D" is proportional to the concentration of unbound species (D_f):

$$-\frac{d(D_t)}{dt} = K_e(D_f) \quad \text{(Eq. 9)}$$

From mass balance:

$$(D_t) = (D_b) + (D_f), \quad \text{(Eq. 10)}$$

where (D_b) is the concentration of "D" which is bound. Further, in the presence of competitor, it can be shown that,

$$(D_b) = \frac{n_1K_{d1}(D_f)(P_t)}{1 + K_{d1}(D_f) + K_{c1}(C_f)} + \frac{n_2K_{d2}(D_f)(P_t)}{1 + K_{d2}(D_f) + K_{c2}(C_f)} \quad \text{(Eq. 11)}$$

Combining Eqs. 10 and 11 and solving for (D_f) an expression for (D_f) is obtained which may then be substituted into Eq. 9 to yield:

$$\frac{-d(D_t)}{dt} = \frac{K_e(D_t)}{1 + [n_1K_{d1}(P_t)]/[1 + K_{d1}(D_f) + K_{c1}(C_f)] + [n_2K_{d2}(P_t)]/[1 + K_{d2}(D_f) + K_{c2}(C_f)]} \quad \text{(Eq. 12)}$$

The intuitive expectation that the presence of a competitor will result in a faster decline of (D_t) is verified by inspection of Eq. 12, which predicts that the rate of loss of (D_t) will increase with increasing (C_f). Further, the equation indicates that as (D_f) and (C_f) approach zero, the slope of a semilog plot of (D_t) *versus* time will approach a constant value which is a function of K_e, n, (P_t), and the association constants (K_d, K_c) for the given system. Finally, Eq. 12 shows that if the values of K_{d1} (D_f), and K_{d2} (D_f) are significantly smaller than the values of K_{c1} (C_f), and K_{c2} (C_f), then the slope of a semilog plot of (D_t) *versus* time will be constant, if (C_f) is constant.

A series of studies were conducted to evaluate dynamic dialysis as a tool for studying the phenomenon of competitive inhibition of protein binding. The studies were carried out by using 8-chlorotheophylline and salicylic acid as the small molecules "C" competing with 8-nitrotheophylline "D" for binding sites on the BSA molecule.

As a prerequisite to these studies it was necessary to determine if the presence of "C" exerted an effect on the rate of dialysis of "D," in the absence of BSA. Thus, a control run with 8-nitrotheophylline was first conducted, and then using the same membrane sac, the dialysis of 8-nitrotheophylline was followed in the presence of competitor, with the competitive concentration either maintained constant or allowed to decline. The loss of both "D" and "C," from inside the sac, was followed in the latter case.

The results of such studies where caffeine was employed as the inhibitor are shown in Fig. 12. Here the initial caffeine concentration was approximately eight times that of 8-nitrotheophylline. Only a slight reduction in the permeation rate of the caffeine caused by the presence of 8-nitrotheophylline was noted. However, the dialytic rate of 8-nitrotheophylline was significantly reduced by the presence of caffeine. In addition, the semilog plot for such a system was not linear. A reasonable explanation for the observed effect is that complex formation occurred between 8-nitrotheophylline and caffeine and that the complex diffused through the membrane at a slower rate than did 8-nitrotheophylline alone.

The effect of a constant 8-chlorotheophylline concentration on the dialysis of 8-nitrotheophylline was also measured and the results are shown in Fig. 13. It is apparent that the presence of 8-chlorotheophylline resulted in a decrease in the rate of loss of 8-nitrotheophylline.

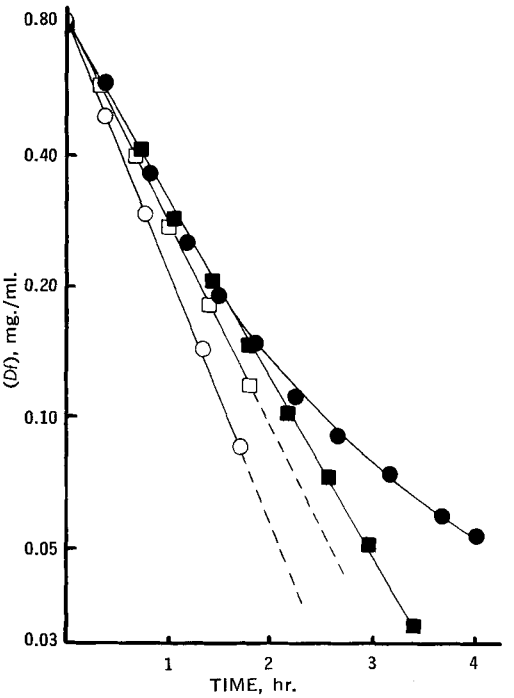


Figure 21—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of BSA, and BSA and 9.8 mg./ml. salicylic acid. The studies were conducted at pH 7.0 and 25°. Key: ○, 8-nitrotheophylline control; □, 8-nitrotheophylline control in the presence of 9.8 mg./ml. salicylic acid; ●, 8-nitrotheophylline and 0.802% BSA; ■, 8-nitrotheophylline, 0.802% BSA, and 9.8 mg./ml. salicylic acid.*

Finally, the influence of the presence of salicylate on the dialytic behavior of 8-nitrotheophylline was studied using a constant salicylate concentration. The results are illustrated in Fig. 14 and demonstrate the previously observed effect; the permeation rate of the 8-nitrotheophylline was decreased in the presence of salicylate ion.

This unexpected effect is currently being rigorously investigated. Preliminary results indicate that complex formation is indeed

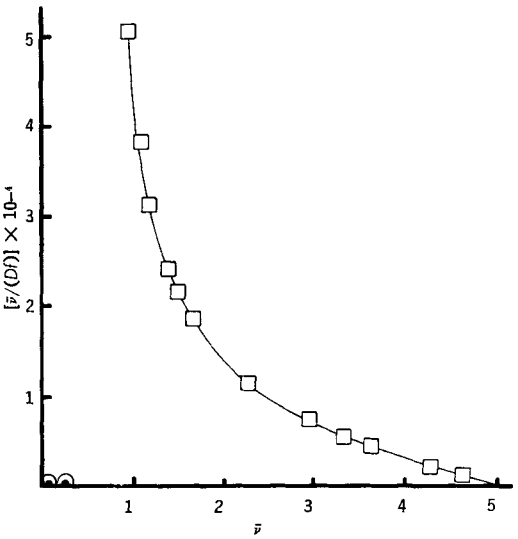


Figure 22—*The binding of 8-nitrotheophylline by BSA in the presence and absence of a constant salicylic acid concentration of 9.8 mg./ml. The studies were conducted at pH 7.0 and 25°. Key: □, 8-nitrotheophylline and BSA; ○, 8-nitrotheophylline, BSA and 9.8 mg./ml. salicylic acid; ●, theoretical data for the binding of 8-nitrotheophylline by BSA in the presence of 9.8 mg./mg. salicylic acid.*

responsible for the observed behavior. Studies are in progress to evaluate the possibility that dynamic dialysis might be of utility in investigating and quantitating small molecule-small molecule interactions as well as small molecule-macromolecule binding.

Relative to protein binding studies, these results emphasize the importance of considering small molecule-small molecule interactions before attempting to study the phenomenon of competitive inhibition. Thus, if the degree of interaction between "*D*" and "*C*" is moderately large, results of competitive binding studies might well be uninterpretable since one does not know *a priori* whether inhibition is due to competition for binding sites or the inability of "*D:C*" complexes to be bound by the protein. In the present studies, for example, the interaction between 8-nitrotheophylline and caffeine was sufficiently large to preclude a meaningful study of the inhibition of 8-nitrotheophylline binding by caffeine. The ability of 8-chlorotheophylline and salicylate to inhibit the binding of 8-nitrotheophylline could, however, be studied since in the former case relatively low concentrations could be employed and in the latter, the apparent degree of complexation was relatively low.

The binding of 8-nitrotheophylline by BSA in the presence of a variable concentration of 8-chlorotheophylline was studied at pH 7.0 and 25° and the results are depicted by Fig. 15. Figure 16 summarizes results which characterize the dialysis of 8-chlorotheophylline from the competitive system. It should be noted that preliminary experiments demonstrated that 8-chlorotheophylline, in the concentration used in this study, did not significantly affect the intrinsic rate of 8-nitrotheophylline dialysis. Figure 15 clearly illustrates the displacing effect caused by competition and which resulted in an increase in the rate of loss of 8-nitrotheophylline from the protein compartment. The data of Figs. 15 and 16 were analyzed in the manner previously described to obtain the concentrations of unbound 8-nitrotheophylline and 8-chlorotheophylline at various times during the course of the dialysis. Then, knowing unbound and total concentrations, the bound concentrations could be calculated. This permitted subsequent computations of $\bar{\nu}_c/(C_f)$, $\bar{\nu}_d/(D_f)$, $\bar{\nu}_c$, and $\bar{\nu}_d$. The results of these computations are illustrated in Figs. 17 and 18 in the form of Scatchard plots. The competitive effect is clearly evident. In addition the plots suggest that both of the theophylline derivatives were bound to the same sites on the protein since the ordinate intercept, which is equal to Σn, appears to be the same for all systems.

Also depicted in Figs. 17 and 18 are points obtained by independent considerations of competitive binding. Here, Eq. 8 was utilized to solve for the concentration of bound species by using n and K values determined from independent studies and experimental values of (D_f) and (C_f). The concentration of protein used corresponded to that in the experimental systems. Thus, theoretical points for a Scatchard plot were generated and are represented by the closed circles in the figures. The agreement of theory and experiment is reasonable, particularly in view of the fact that small variations in the values of the four association constants can have rather marked influences on the shape and position of a theoretically generated plot.

Figure 19 compares results obtained in the dialysis of various 8-nitrotheophylline systems with and without a constant concentration of 8-chlorotheophylline. The constancy of the latter was ensured by formulating internal solution, external solution, and replacement solution to contain the same concentration of 8-chlorotheophylline. It is apparent from the figure that the rate of dialysis of 8-nitrotheophylline was markedly increased by incorporation of 8-chlorotheophylline in the system. In addition, it can be observed that for the competitive study, the semilog plot appeared essentially constant after about 1 hr. which is indicative of the binding of a constant fraction of the 8-nitrotheophylline. Figure 20 illustrates the Scatchard plot corresponding to this system. The permeability constant utilized in analyzing the kinetic data to obtain the Scatchard plot was obtained from the study of 8-nitrotheophylline dialysis in the presence of 0.763 mg./ml. of 8-chlorotheophylline. The competitive effect is dramatically reflected by this figure. Theoretical points are also shown in Fig. 20. These were calculated in the manner previously described using the assumption that here (C_f) could be approximated by the analytical concentration of competitor in the system. The agreement between theoretical and experimental data is excellent and serves to confirm the utility of the dynamic dialysis method in studying competitive binding.

The results of similar studies, employing salicylate as a competing species, are depicted in Figs. 21 and 22. These similarly demonstrate the kinetic manifestations of competitive inhibition and the ability to quantitate competitive binding behavior by the dynamic dialysis approach.

In summary, this investigation has demonstrated the utility of the dynamic dialysis approach by application to a variety of protein-binding systems. The method was shown to be applicable to the quantitation of binding covering a spectrum of binding strengths. In addition it was demonstrated that competitive inhibition of binding can be readily detected and quantitatively evaluated by the technique. Some idea of the relative rapidity and convenience of the method was evidenced by the ability to comprehensively study the influence of pH, over a wide range, on the interaction of 8-nitrotheophylline with BSA. Although the present study was limited to protein-containing systems, there is no reason why the method could not be used in studies of binding by other natural macromolecules, synthetic polymers, adsorbents, and micelle-forming materials.

REFERENCES

(1) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 1627 (1968).

(2) *Ibid.*, **58**, 33(1969).

(3) G. Scatchard, J. S. Coleman, and A. L. Shen, *J. Am. Chem. Soc.*, **79**, 12 (1957).

(4) R. A. O'Reilly, P. M. Aggeler, M. S. Hoag, and L. Leong, *Thromb. Diath. Haemorrhag.*, **8**, 82(1962).

(5) I. M. Klotz, F. M. Walker, and R. B. Pivan, *ibid.*, **68**, 1486 (1946).

(6) M. L. Eichman, D. E. Guttman, Q. Van Winkle, and E. P. Guth, *J. Pharm. Sci.*, **51**, 66(1962).

(7) D. E. Guttman, and A. E. Gadzala, *ibid.*, **54**, 742(1965).

(8) M. L. Eichman, Ph.D. thesis, The Ohio State University, Columbus, Ohio (1964).

(9) C. Davison and P. K. Smith, *J. Pharm. Exptl. Therap.*, **133**, 161(1961).

(10) R. A. O'Reilly, *J. Clin. Invest.*, **46**, 829(1967).

(11) E. Kruger-Thiemer, *Arzneimittel-Forsch.*, **16**, 1431(1966).

(12) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 245 (1968).

(13) J. F. Foster, in F. Putnam, "The Plasma Proteins," vol. 1, Academic, New York, N. Y., 1960.

(14) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968).

(15) F. L. Rodkey, *Arch. Biochem. Biophys.*, **94**, 38(1961).

(16) K. Shikama, *J. Biochem. Tokyo*, **64**, 55 (1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from *the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication August 5, 1969.

* Present address: Department of Pharmaceutics, College of Pharmacy, University of Tennessee, Memphis, TN 38103

† Present address: Smith Kline & French Laboratories, 1500 Spring Garden Street, Philadelphia, PA 19101

Effect of Tablet Processing and Formulation Factors on Dissolution Rate of the Active Ingredient in Human Gastric Juice

SISSEL SOLVANG and PER FINHOLT

Abstract □ *The effect of the granulation and tableting processes* on the dissolution rate of phenobarbital, sodium phenobarbital, phenacetin, and prednisone in human gastric juice has been investigated. A study has also been made on the influence of the particle size of the active ingredient and the effect of different binders on the dissolution rate of phenobarbital.

Keyphrases □ Tablet dissolution rates—human gastric juice □ Gastric juice, human—phenobarbital, phenacetin, prednisone tablets, dissolution □ Formulation, processing effects—dissolution rates □ Particle size effect—phenobarbital dissolution □ Binders effect—tablet dissolution

The effect of formulation and processing factors on the dissolution rate of the active ingredients of compressed tablets has been the subject of a number of reports (1–14). Various dissolution media such as water, hydrochloric acid, buffer solutions, simulated gastric juice, and simulated intestinal fluid, have been used, but, surprisingly enough, not that solvent, in which the dissolution process takes place *in vivo*—that is, human gastric juice.

The scope of the present investigation was to study *the effect of the granulation and tableting processes* and the influence of certain formulation factors on the dissolution rate of the active ingredient in human gastric juice.

EXPERIMENTAL

Materials—All drugs were of Pharmacopoea Nordica grade. The phenobarbital and phenacetin powders were separated into the desired size fractions by sieving. The prednisone used was a micronized product, having a particle size between 1 and 8 μ determined with a Celloscope, an apparatus that works on the Coulter counter principle.

The gastric juice was obtained as described in an earlier paper (15). A mixture of equal parts of gastric juice and water was used as dissolution medium. This mixture is named "diluted gastric juice" in the present paper.

Preparation of Granules and Tablets—The desired size fraction of the drug powder was mixed with potato starch (or potato starch + lactose) and gently moistened with the granulating solution. The moistened mass was sieved and dried. The dry granules were separated into different size fractions by sieving. The 0.71–1.00 mm. size fraction of the granules[1] was mixed with a lubricant and compressed into tablets on a single punch tablet machine. Specifications for the experimental granules and tablets are given in Table I.

Determination of Hardness—The hardness of the tablets was determined with a Pfizer tablet hardness tester.

Determination of Disintegration Time—The disintegration time of the tablets was determined according to *The Nordic Pharmacopeia:* three tablets were placed in a conical flask, mixed with 30 ml. of water at a temperature between 36 and 40° and kept at this temperature with frequent swinging of the flask.

Determination of Dissolution Rate—The dissolution rate was determined by the beaker method of Levy (16) with minor changes (15). For each test the drugs, granules, and tablets were—if not otherwise stated—used in amounts that were more than sufficient for saturation of the dissolution medium with the drug. The drug powders and the granules were gently spread over the surface of the fluid, and settled down through the fluid during the test. The tablets were dropped into the dissolution medium. At appropriate intervals samples of the dissolution medium were withdrawn, filtered and analyzed.

Analytical Methods—*Determination of Prednisone*—The samples withdrawn were diluted with 0.1 *N* HCl and absorbances were

[1] Granules passing through standard sieve U. S. No. 18 (sieve opening 1.00 mm.), but not through sieve No. 25 (sieve opening 0.71 mm.).

Table I—Specifications for Experimental Granules and Tablets

	Phenobarbital Granules and Tablets[a] I	II	III
Phenobarbital[b]	100 g.	100 g.	100 g.
Potato starch	70 g.	70 g.	70 g.
Gelatin[c]	A sufficient quantity		
Carboxymethylcellulose[d]		A sufficient quantity	
Polyethylene glycol 6000[e]			A sufficient quantity
Magnesium stearate and talc (1+9)	10 g.	10 g.	10 g.
Tablet hardness	4.4 kg.cm.$^{-2}$	2.1 kg.cm.$^{-2}$	0.9 kg.cm.$^{-2}$
Disintegration time	30 sec.	5 min.	20 sec.

Sodium Phenobarbital Granules and Tablets I[f]	
Sodium phenobarbital[g]	125 g.
Potato starch	70 g.
Gelatin[h]	A sufficient quantity
Magnesium stearate and talc (1+9)	10 g.
Tablet hardness: 6.4 kg.cm.$^{-2}$	
Disintegration time: 3 min.	

Phenacetin Granules and Tablets[i]	
Phenacetin (0.21–0.30 mm.)	500 g.
Potato starch	115 g.
Gelatin[c]	A sufficient quantity
Magnesium stearate and talc (1+9)	30 g.
Tablet hardness: 4.5 kg.cm.$^{-2}$	
Disintegration time: 1 min.	

Prednisone Granules and Tablets[j]	
Prednisone (<8 μ)	5 g.
Lactose	80 g.
Potato starch	85 g.
Gelatin[c]	A sufficient quantity
Magnesium stearate and talc (1+9)	10 g.
Tablet hardness: 6.6 kg.cm.$^{-2}$	
Disintegration time: 1.5 min.	

[a] 1000 tablets, diameter 8 mm. [b] Particle size: 0.21–0.30 mm., if not otherwise stated. [c] 4% solution of gelatin in water as granulating agent. [d] 2% solution of sodium carboxymethylcellulose in water as granulating agent. [e] 50% solution of Carbowax 6000 in alcohol (95%) as granulating agent. [f] 1000 tablets, diameter 8 mm. [g] A crystalline powder with most particles within the 0.11–0.30 mm. range was used. [h] 10% solution of gelatin in alcohol as granulating agent. [i] 1000 tablets, diameter 13.5 mm. [j] 1000 tablets, diameter 8 mm.

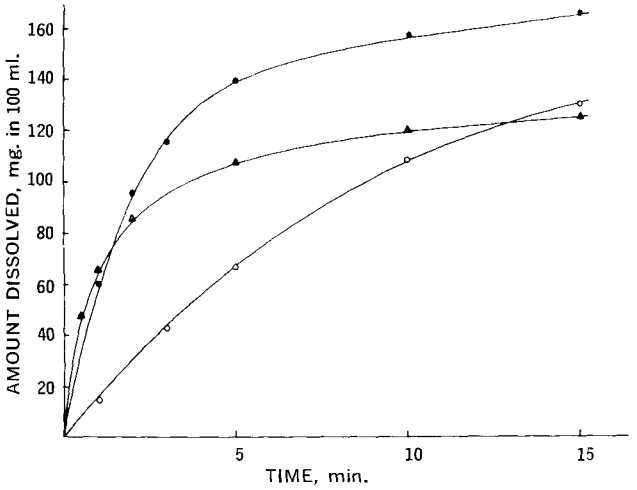


Figure 1—*Rate of dissolution of phenobarbital from powder, granules I, and tablets I in diluted gastric juice (surface tension 42.5 dynes cm.$^{-1}$, pH 1.70). Key: ○, phenobarbital powder; ▲, phenobarbital granules I; ●, phenobarbital tablets I.*

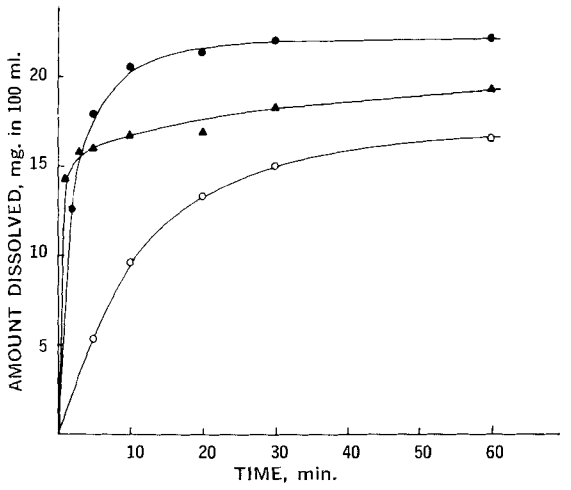


Figure 3—*Rate of dissolution of prednisone from powder, granules, and tablets in diluted gastric juice (surface tension 41.1 dynes cm.$^{-1}$, pH 1.50). Key: ○, prednisone powder; ▲, prednisone granules; ●, prednisone tablets.*

measured at 243 mμ with a Beckman DK 2A spectrophotometer.

Phenobarbital and phenacetin were determined as described earlier (15).

RESULTS AND DISCUSSION

Effect of the Granulation and Tableting Processes—The rate of dissolution of phenobarbital, phenacetin, and prednisone was determined before and after granulation and compression into tablets.

It will be seen from Figs. 1–3 that the rate of release of all three drugs from the granules is higher than the rate of dissolution of the pure drugs. The granulation process has increased the dissolution rate, probably by making the originally hydrophobic drug particles hydrophilic.

Figs. 1 and 3 show that phenobarbital and prednisone are released at a higher speed from the tablets than from the granules. The reason for this is probably that the drugs—during the dissolution test—are more rapidly wetted when contained in tablets, because the tablets are dropped into the dissolution medium and disintegrate completely within 1–2 min., whereas the granules are spread over the surface of the fluid and settle more slowly down through the fluid. It is also possible that the compression process leads to deformation or crushing of the granule particles, thus increasing the specific surface area of the granules and their rate of dissolution. In this connection it may be noted that Higuchi *et al.* (17) have shown that the specific surface area of tablet granules becomes greater with increasing compression pressure. These authors assumed that this was due to the crushing of the granule particles. In a later paper Higuchi *et al.* (18) have shown that with normal tableting pressures, deformation of the granules takes place but without the particles breaking up. The particles are changed from being almost spherical to being nearly disk-shaped and hence their surface area is increased.

The experiments, the results of which are given in Figs. 1–3, were so scaled that there would always be undissolved drug remaining at the end of each test. Figure 4 shows the results of an experiment where such amounts of phenobarbital powder, granules and tablets were used for each test that a complete dissolution might occur (one 100-mg. tablet or an equivalent amount of granules or drug powder). It will be seen that this procedure leads in principle to the same result as the other procedure, *i.e.*, the tablets showing a faster dissolution rate than the granules and the drug powder.

Effect of Particle Size of the Active Ingredient—Phenobarbital tablets were prepared according to Formula I. Different size fractions of the drug powder were used. Figure 5 shows that the rate of dissolution of phenobarbital from the tablets increases with decreasing particle size of the drug. This result was expected since the granulation process has made the surface of the drug particles hy-

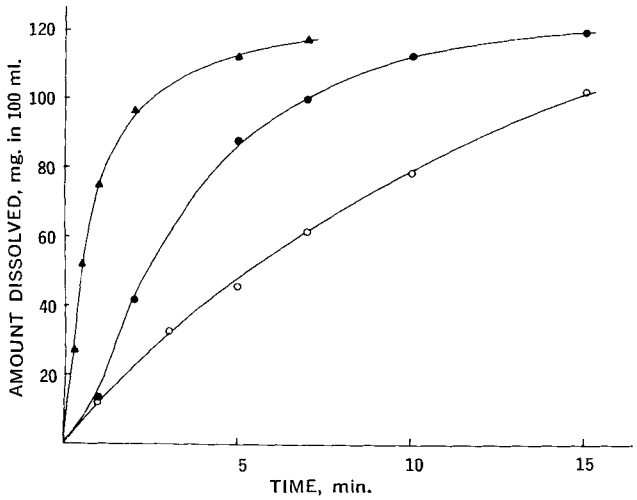


Figure 2—*Rate of dissolution of phenacetin from powder, granules, and tablets in diluted gastric juice (surface tension 42.7 dynes cm.$^{-1}$, pH 1.85). Key: ○, phenacetin powder; ▲, phenacetin granules; ●, phenacetin tablets.*

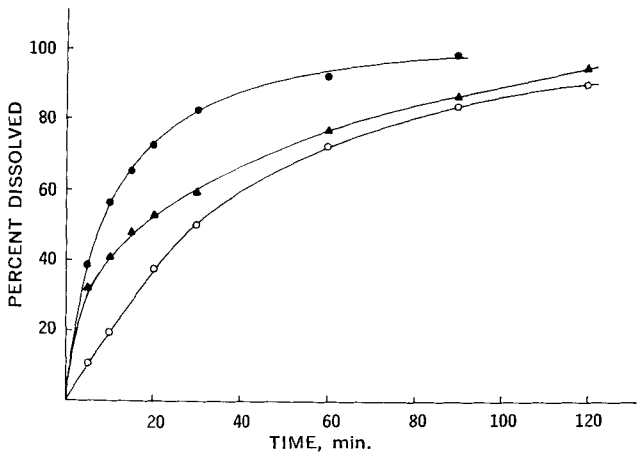


Figure 4—*Rate of dissolution of phenobarbital from powder, granules I, and tablets I in diluted gastric juice (surface tension 46.4 dynes cm.$^{-1}$, pH 1.95). Key: ○, phenobarbital powder; ▲, phenobarbital granules; ●, phenobarbital tablets.*

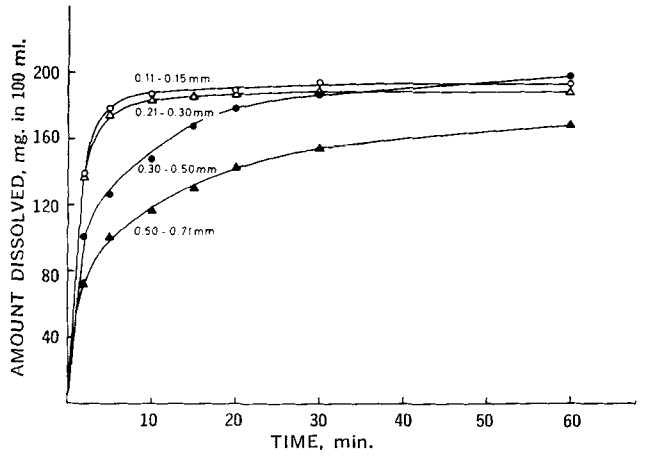


Figure 5—*Effect of particle size of phenobarbital on dissolution rate of the drug from tablets I in diluted gastric juice (surface tension 40.5 dynes cm.$^{-1}$, pH 1.50).*

drophilic. In addition the dissolution medium has a low surface tension.

Effect of Different Binders—Phenobarbital tablets were prepared according to Formulas I, II, and III. The same pressure was, as far as possible, used for the compression of the three batches of tablets.

Figure 6 shows that there are great differences in dissolution rates. These differences could not be attributed to differences in disintegration times during the dissolution test. Tablets I disintegrated within 45 sec., tablets II within 6 min., and tablets III within 20 sec. This means—as an example—that even though tablets III had a slightly shorter disintegration time in diluted gastric juice than tablets I, the rate of dissolution was much lower.

It will be seen from Fig. 6 that the tablets prepared with gelatin as granulating agent have a much higher dissolution rate than the tablets prepared with CMC or polyethylene glycol 6000 as binders. This result is in accordance with earlier findings (8) from this laboratory that the use of gelatin as binder leads to granules and tablets with a very high dissolution rate in 0.1 *N* HCl. The high dissolution rate of these granules and tablets is believed to be due to the ability of gelatin to make originally hydrophobic surfaces of drug particles hydrophilic. This theory is supported by earlier experiments with phenacetin as model substance, showing that this hydrophobic drug after mixing with potato starch and granulation with gelatin dissolved at the same high rate in 0.1 *N* HCl as the ungranulated drug did in 0.1 *N* HCl containing 0.2% polysorbate 80 as wetting agent (8).

The reason why phenobarbital dissolves so slowly from tablets II is probably that the binder, sodium carboxymethylcellulose, at

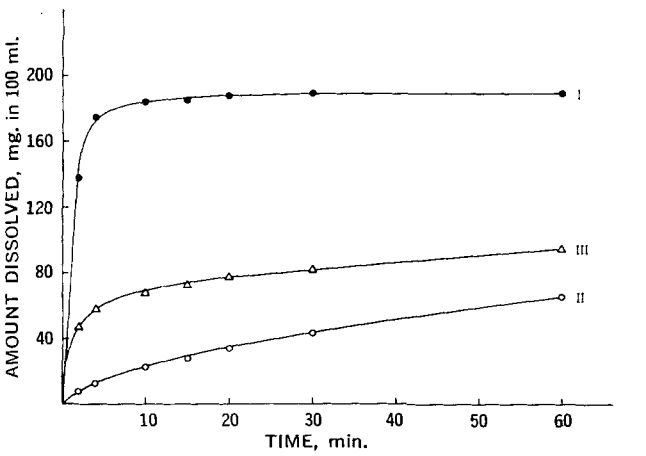


Figure 6—*Rate of dissolution of phenobarbital from tablets I, II, and III in diluted gastric juice (surface tension 39.4 dynes cm.$^{-1}$, pH 1.50).*

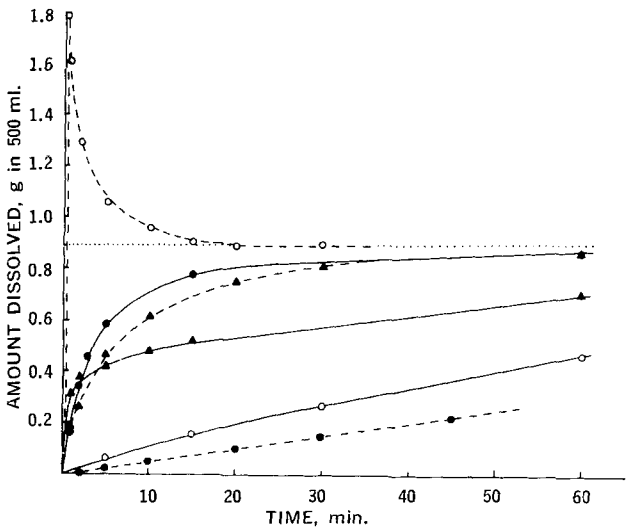


Figure 7—*Rate of dissolution of phenobarbital and sodium phenobarbital from powder, granules I, and tablets I in 0.1 N HCl. The dotted line parallel with the x-axis indicates the concentration of a saturated solution of phenobarbital in 0.1 N HCl at 37°. Key: ——, phenobarbital; - - -, phenobarbital sodium; ○, powder; ▲, granules I; ●, tablets I.*

the pH of the dissolution medium (pH 1.5) is converted into the less hydrophilic free acid.

As far as tablets III is concerned the slow dissolution is assumed to be due to a complex formation between the drug and the granulating agent. Each tablet III contained 100 mg. of phenobarbital and about 35 mg. of polyethylene glycol 6000. Higuchi and Lach (19) and later Singh *et al.* (20) have shown that phenobarbital forms stable compounds of reduced solubility with polyethylene glycols. Singh *et al.* found the ratio to be 45.5 mg. of polyethylene glycol 4000 per 100 mg. of phenobarbital at 37° and pH 5.3. The following experiment seems to indicate that a complex of reduced solubility is formed between the drug and the granulating agent of tablets III.

Two tablets were placed in 40 ml. of hydrochloric acid solution pH 1.5 (the pH of the diluted gastric juice) and the mixture was equilibrated at 37° for 30 hr. A phenobarbital concentration of only 65 mg.% was found, whereas tablets I and II gave a phenobarbital concentration of 180 mg.% under the same experimental conditions. The complex between the drug and the granulating agent of tablets III is probably formed during the dissolution test and not

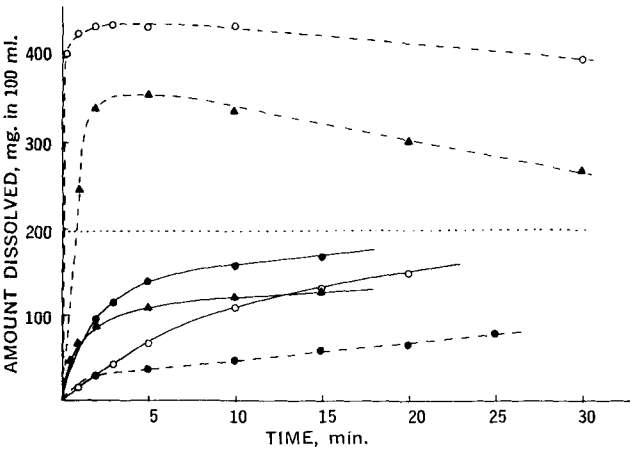


Figure 8—*Rate of dissolution of phenobarbital and sodium phenobarbital from powder, granules I, and tablets I in diluted gastric juice (surface tension 40.5 dynes cm.$^{-1}$, pH 1.70). The dotted line parallel with the x-axis indicates the concentration of a saturated solution of phenobarbital in diluted gastric juice at 37°. Key: —, phenobarbital; - - -, phenobarbital sodium; ○, powder; ▲, granules I; ●, tablets I.*

during the preparation of the tablets, since the amount of alcohol used for granulation (about 35 g. per 100 g. of phenobarbital) can not dissolve more than 4% of the total amount of drug present.

It may be mentioned that complexation in some cases may *increase* dissolution rates. Wurster and Kildsig (21) found that addition of creatinine, tartaric acid, malic acid, or succinic acid to the dissolution medium increased the solubility and the dissolution rate of *m*-aminobenzoic acid.

Comparative Studies of Release Rate of a Drug and Its Sodium Salt from Granules and Tablets—In a previous study (15) the rate of dissolution of sodium phenobarbital in diluted gastric juice was determined and compared with the rate of dissolution of the salt in a hydrochloric acid solution having the same pH as the diluted gastric juice. It was found that initially nearly the same high drug concentration was obtained in both dissolution media. However, the precipitation of the free acid in excess of solubility took place at a much slower rate in gastric juice than in hydrochloric acid.

In the present study the rate of dissolution of phenobarbital and sodium phenobarbital from granules and tablets has been determined using 0.1 *N* HCl and diluted gastric juice as dissolution media. Comparisons with the rate of dissolution of the pure drug have been made.

Figures 7 and 8 show that the rate of dissolution of the sodium salt in both dissolution media is lowered by granulating a mixture of the drug and potato starch with gelatin. Compression of the granules into tablets results in a further reduction of the dissolution rate. If the free acid is used instead of the sodium salt, the granulation procedure and the tableting have—as mentioned earlier—the opposite effect: the dissolution rate is increased by granulation of the drug and further increased by compression of the granules.

By comparing the curves for sodium phenobarbital powder in Figs. 7 and 8 it will be seen that the precipitation of the free acid is much faster in 0.1 *N* HCl than in diluted gastric juice. It is also evident from these figures that granulated sodium phenobarbital forms a supersaturated solution of the free acid in diluted gastric juice but not in 0.1 *N* HCl. The rate of precipitation of the free acid in diluted gastric juice is obviously so low compared with the rate of dissolution of the granules that a supersaturated solution may be formed, whereas in 0.1 *N* HCl this is not the case.

Figures 7 and 8 also show that granulation of the free acid leads to a greater increase of the dissolution rate in 0.1 *N* HCl than in diluted gastric juice. This was expected since 0.1 *N* HCl has a much higher surface tension than diluted gastric juice.

Tablets prepared from the free acid had a much higher dissolution rate in both media than tablets prepared from the sodium salt. This was due to the fact that tablets containing the free acid disintegrated rapidly in 0.1 *N* HCl and diluted gastric juice, whereas tablets containing the sodium salt, although disintegrating fast in water, did not disintegrate in the acidic media, but swelled and dissolved slowly from the surface.

Some experiments were done to prepare sodium phenobarbital tablets with better dissolving properties in acidic media. Dry granulation of a mixture of the drug and potato starch instead of wet granulation of the same mixture with gelatin did not cause any improvement. Better results were obtained by direct compression of a mixture of the drug with microcrystalline cellulose. But even these tablets exhibited much slower dissolution rate than phenobarbital tablets prepared according to Formula I.

SUMMARY AND CONCLUSIONS

1. The rate of release of phenobarbital, phenacetin, and prednisone from granules and tablets prepared with gelatin as granulating agent was faster than the rate of dissolution of the pure drugs. Phenobarbital and prednisone were released at a higher speed from the tablets than from the granules. It is thus evident that both the granulation process and the compression of the granules may increase the rate of dissolution of drugs in human gastric juice.

2. The rate of dissolution of phenobarbital from tablets prepared with gelatin as granulating agent increased with decreasing particle size of the drug.

3. Phenobarbital tablets prepared with gelatin as granulating agent were found to dissolve much faster in human gastric juice than tablets prepared with sodium carboxymethylcellulose or polyethylene glycol 6000 as binders, probably because gelatin makes the originally hydrophobic surface of the drug particles hydrophilic, whereas sodium carboxymethylcellulose at the pH of the dissolution medium is converted into the less hydrophilic free acid and polyethylene glycol 6000 forms a complex of reduced solubility with phenobarbital.

4. The rate of dissolution of sodium phenobarbital in human gastric juice and in 0.1 *N* HCl was lowered by granulating a mixture of the drug and potato starch with gelatin. Compression of the granules into tablets resulted in a further reduction of the dissolution rate. These tablets dissolved much slower in both media than tablets prepared in a similar way from phenobarbital. The reason for this was that the sodium phenobarbital tablets, although disintegrating rapidly in water, did not disintegrate in the acidic media, but swelled and dissolved slowly from the surface. In contrast to this the phenobarbital tablets disintegrated very rapidly in acidic media as well as in water. Attempts to use dry granulation or direct compression in order to obtain sodium phenobarbital tablets dissolving as fast as the phenobarbital tablets in acidic media were not successful. It appears thus to be more difficult to prepare fast dissolving tablets from the highly soluble sodium salt of phenobarbital than from the corresponding poorly soluble free acid. It has often been claimed that rapid dissolution of the active ingredient of tablets in the stomach represents a problem only as far as poorly soluble drugs are concerned, but it may certainly be a problem also when the drug in question is very soluble.

REFERENCES

(1) J. R. Harris, Ph.D. dissertation, Purdue University, 1961.

(2) E. Marlow, Ph.D. dissertation, University of Maryland, 1962.

(3) G. Levy, *J. Pharm. Sci.*, **52,** 1039(1963).

(4) G. Levy, J. M. Antkowiak, J. A. Procknal, and D. C. White, *ibid.*, **52,** 1047(1963).

(5) G. Levy and R. H. Gumtow, *ibid.*, **52,** 1139(1963).

(6) K. J. Randeri, Ph.D. dissertation, University of Texas, 1964.

(7) J. K. C. Yen, *Can. Pharm. J.*, **97,** 493(1964).

(8) P. Finholt, H. Kristiansen, O. C. Schmidt, and K. Wold, *Medd. Norsk Farm. Selskap*, **28,** 17(1966).

(9) P. Finholt, R. Højlund Pedersen, S. Solvang, and K. Wold, *ibid.*, **28,** 238(1966).

(10) J. Thekkattil, Ph.D. dissertation, University of Washington, 1966.

(11) H. E. Huber, L. B. Dale, and G. L. Christenson, *J. Pharm. Sci.*, **55,** 974(1966).

(12) E. Marlowe and R. F. Shangraw, *ibid.*, **56,** 498(1967).

(13) H. E. Huber and G. L. Christenson, *ibid.*, **57,** 164(1968).

(14) T. J. Jacob and E. M. Plein, *ibid.*, **57,** 798, 802(1968).

(15) P. Finholt and S. Solvang, *ibid.*, **57,** 1322(1968).

(16) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262,** 1053 (1960).

(17) T. Higuchi, A. N. Rao, L. W. Busse, and J. V. Swintosky, *J. Am. Pharm. Assoc., Sci. Ed.*, **42,** 194(1953).

(18) W. A. Strickland, E. Nelson, L. W. Busse, and T. Higuchi, *ibid.*, **45,** 51(1956).

(19) T. Higuchi and J. L. Lach, *ibid.*, **43,** 465(1954).

(20) P. Singh, J. K. Guillory, T. D. Sokoloski, L. Z. Benet, and V. N. Bhatia, *J. Pharm. Sci.*, **55,** 63(1966).

(21) D. E. Wurster and D. O. Kildsig, *ibid.*, **54,** 1491(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 24, 1969 from the *Institute of Pharmacy, University of Oslo, Norway.*

Accepted for publication August 28, 1969.

This study was supported by grants from the Royal Norwegian Council for Scientific and Industrial Research, Oslo, and from Norsk Medisinaldepot, Oslo.

Assessment of Pharmacokinetic Constants from Postinfusion Blood Curves Obtained after I.V. Infusion

J. C. K. LOO and S. RIEGELMAN

Abstract □ Pharmacokinetic constants have usually been derived from the parameters of the exponential equation which describe the blood curve after a single bolus i.v. injection. Occasionally, due to potential toxicity, irritation, or limited solubility, it is not possible to inject a drug by a single bolus; it may be feasible to circumvent these difficulties by administering the drug intravenously at a slower rate of infusion. A mathematical equation is presented which enables one to determine the parameters identical to an i.v. bolus injection curve by utilizing the postinfusion blood curve. The equation is applicable to all compartmental models that may be described by linear first-order differential equations with constant coefficients. Experimental data are presented whereby drugs were administered by very rapid and prolonged i.v. infusion with blood sampling at appropriate intervals. The experimentally estimated parameters calculated from the postinfusion blood curves were in agreement with those obtained from the rapid infusion blood curves.

Keyphrases □ Pharmacokinetic constants determination—postinfusion curves □ Kinetic equations—slow i.v. infusion □ Griseofulvin—slow, rapid i.v. infusion □ Sulfisoxazole—slow, rapid i.v. infusion

Pharmacokinetic parameters are usually derived from the exponential equations which describe the blood curve after a single bolus i.v. injection. Occasionally, due to potential toxicity, irritation, or limited solubility, it is not possible to inject a drug by a single bolus. It may be feasible to circumvent these difficulties by administering the drug intravenously at a slower rate of infusion. An equation is derived below which enables one to determine the parameters identical to those obtained from an i.v. bolus injection curve by utilizing the postinfusion blood level *versus* time curve. The equation is applicable to all compartment models describable by linear first-order differential equations with constant coefficients. Experimental data will be presented from studies in which drugs were administered by very rapid and prolonged i.v. infusion into man and rabbit.

THEORETICAL

If drug elimination and distribution processes obey first-order kinetics then the blood curve, after a single bolus i.v. injection, is describable by a summation of exponential terms.

$$(Cp)_{\text{i.v.}} = \sum_{i=1}^{n} A_i e^{-k_i t} \qquad \text{(Eq. 1)}$$

where $(Cp)_{\text{i.v.}}$ represents the plasma concentration[1] at time t, after administration of the i.v. dose, A_i is the intercept at zero time, and k_i is the corresponding first-order rate constant as defined in Eq. 1. The Laplace transform of Eq. 1 is

$$\bar{C}p(s) = \sum_{i=1}^{n} \frac{A_i}{(s + k_i)} = f_1(s) \qquad \text{(Eq. 2)}$$

where $\bar{C}p$ represents the Laplace transform of Cp. If the same dose of the drug is infused continuously at a constant rate (k_0) (see Footnote 2) until time τ, then ceased abruptly, the Laplace transform for this input function is:

$$f_2(s) = \frac{k_0}{s}(1 - e^{-\tau s}) \qquad \text{(Eq. 3)}$$

where $f_2(s)$ is the step function of height k_0 starting at zero-time and stopping at $t = \tau$. The Laplace transform (1) of the postinfusion curve is the product of the input function $f_2(s)$ and the unit impulse function $f_1(s)$.

$$(\bar{C}p)_{\text{post}}(s) = f_1(s) \cdot f_2(s) \qquad \text{(Eq. 4)}$$

$(\bar{C}p)_{\text{post}}$ denotes the Laplace transform of the plasma concentration when $t > \tau$. Substituting Eqs. 2 and 3 for $f_1(s)$ and $f_2(s)$, respectively, one obtains:

$$(\bar{C}p)_{\text{post}}(s) = \frac{k_0}{s}(1 - e^{-\tau s})\left[\sum_{i=1}^{n} \frac{A_i}{s + k_i}\right] \qquad \text{(Eq. 5)}$$

Separating terms we arrive at:

$$(\bar{C}p)_{\text{post}}(s) = \frac{k_0}{s}\left[\sum_{i=1}^{n} \frac{A_i}{s + k_i}\right] - \frac{k_0}{s} e^{-\tau s}\left[\sum_{i=1}^{n} \frac{A_i}{s + k_i}\right] \qquad \text{(Eq. 6)}$$

The antitransform of Eq. 6 at times greater than τ is

$$(Cp)_{\text{post}} = k_0\left[\sum_{i=1}^{n} \frac{A_i}{k_i} - \sum_{i=1}^{n} \frac{A_i}{k_i} e^{-k_i t} - \sum_{i=1}^{n} \frac{A_i}{k_i} + \sum_{i=1}^{n} \frac{A_i}{k_i} e^{-k_i(t-\tau)}\right] \qquad \text{(Eq. 7)}$$

Upon simplification of Eq. 7, we have:

$$(Cp)_{\text{post}} = k_0\left[\sum_{i=1}^{n} \frac{A_i}{k_i} e^{-k_i(t-\tau)} - \sum_{i=1}^{n} \frac{A_i}{k_i} e^{-k_i t}\right] \qquad \text{(Eq. 8)}$$

Substituting τ for k_0 and setting $t' = (t - \tau)$ upon simplifying Eq. 8, one gets[3]

$$(Cp)_{\text{post}} = \sum_{i=1}^{n} \frac{A_i(1 - e^{-k_i\tau})}{k_i\tau} e^{-k_i t'} \qquad \text{(Eq. 9)}$$

When the infusion time is sufficiently short such that $\sum_{i=1}^{n}(1 - e^{-k_i\tau})$ approaches $\sum_{i=1}^{n} k_i\tau$, Eq. 9 reduces to:

$$(Cp)_{\text{post}} = \sum_{i=1}^{n} A_i e^{-k_i t'} \qquad \text{(Eq. 10)}$$

This equation is equivalent to the equation defining an i.v. bolus injection blood curve. However, when the infusion time is sufficiently

[1] The plasma concentration could be expressed as the concentration of the free drug and/or its metabolite.

[2] k_0 is expressed as the fractional infusion rate and is equal to amount infused per unit time/amount of dose administered = (infusion time)$^{-1}$.

[3] This is a general solution for the postinfusion curves pertaining to all multicompartment model systems. However, an equation for the special two-compartment model case has been derived previously by Rescigno and Segre (7).

Table I—The Biexponential Equations[a] Describing the Postinfusion Curves Obtained after Rapid and Prolonged Administration of Sulfisoxazole Intravenously into a Male Rabbit

Infusion Time	A_1'	k_1	A_2'	k_2	Error Mean Square
10 sec.					
Experimental[b] values	57.3 ± 2.6[c]	0.1244 ± 0.0099	42.7 ± 2.5	0.01115 ± 0.00091	1.5485
30 min.					
Experimental values	33.0 ± 2.3	0.1286 ± 0.0171	67.3 ± 2.2	0.00982 ± 0.00047	1.2027
Calculated[d] values	29.3	0.1244 ± 0.0099	70.7	0.01115 ± 0.00091	
60 min.					
Experimental values	22.8 ± 2.6	0.1338 ± 0.0278	77.2 ± 2.5	0.01018 ± 0.00056	1.5563
Calculated values	19.8	0.1244 ± 0.0099	80.2	0.01115 ± 0.00091	

[a] The equation is : $P\% = A_1e^{-k_1t} + A_2e^{-k_2t}$. A_1, A_2, and $P\%$, are expressed in percentage of $(A_1 + A_2)$. k_1, k_2 are expressed in reciprocal minutes. t is the time after infusion in minutes. [b] These values were determined from the experimental data by using an IBM 360/50 digital computer programmed according to a nonlinear regression subroutine of the B.M.D. X85 series. [c] Standard deviation units. [d] These values were generated from Eq. 9 by using the parameters of the postinfusion curves after the fastest infusion experiment.

long such that $\sum_{i=1}^{n} (1 - e^{-k_i\tau'})$ approaches n, Eq. 9 becomes:

$$(Cp)_{post} = \sum_{i=1}^{n} \frac{A_i}{k_i\tau} e^{-k_it'} \qquad \text{(Eq. 11)}$$

It is evident from these considerations that k_i is the same whether it is obtained from a postinfusion curve or a single i.v. bolus curve. However, the intercepts of the exponential terms (*i.e.*, A_i and $A_i[(1 - e^{-k_it})/(k_i\tau)]$) are different and the differences increase as the infusion time lengthens. Since these intercepts are used to calculate kinetic constants intrinsic to multicompartmental models, an appropriate correction must therefore be made. The intercepts can be calculated from the following equation:

$$A_i = \frac{k_i\tau}{1 - e^{-k_i\tau}} A_i' \qquad \text{(Eq. 12)}$$

where A_i' is the intercept, extrapolated to $t' = 0$, obtained from the postinfusion curve. Incorporating Eq. 12 into Eq. 1, we arrive at the following equation:

$$(Cp)_{i.v.} = \sum_{i=1}^{n} \frac{k_i\tau}{1 - e^{-k_i\tau}} A_1' e^{-k_it} \qquad \text{(Eq. 13)}$$

Thus, an equation has been derived which will lead to the same parameters as obtained after a single i.v. bolus injection. If the parameters were obtained from free drug plasma concentration *versus* time curve, then the kinetic and volume constants intrinsic to the compartmental model can be calculated by procedures previously described[4] (2, 3). However if the parameters were de-

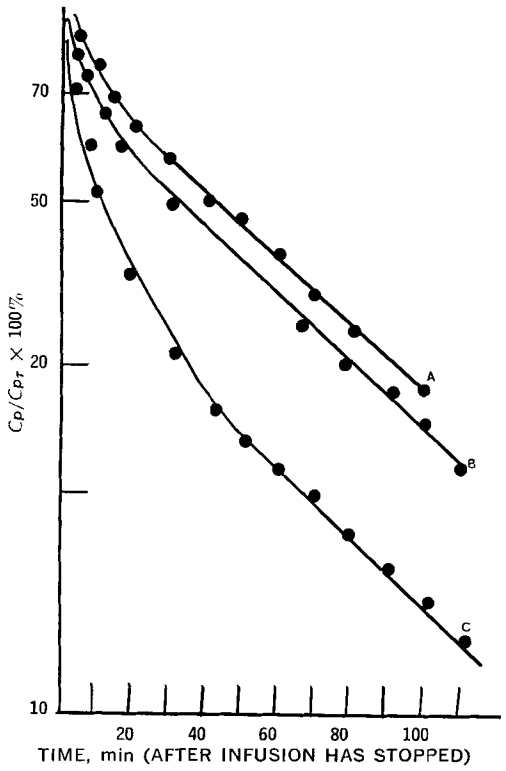


Figure 1—*Sulfisoxazole plasma level after intravenous infusion into rabbit. Key:* A, *after an infusion time period,* τ, *of 60 min.;* B, *after 30-min. infusion;* C, *after 10-sec. infusion;* Cp, *plasma concentration* after *infusion has stopped;* Cp_τ, *plasma concentration at the end of the infusion. The use of* Cp/Cp_τ *normalizes the data allowing more convenient comparison of data among the experiments.*

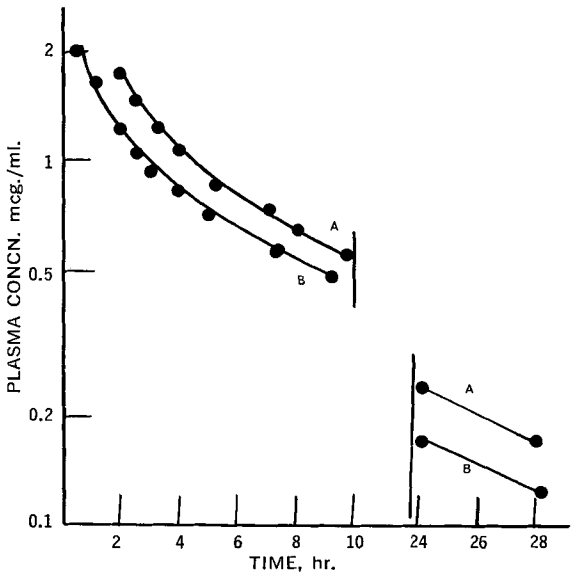


Figure 2—*Griseofulvin plasma level after intravenous infusion into man. Key:* A, *plasma level after 3-min. infusion;* B, *plasma level after 2-hr. infusion.*

[4] In *Reference* 3 the equation for the $Vd°$ is in error. The correct equation should be $Vd° = Vd_{ss} = Vp\ (k_{21} + k_{12}/(k_{21}) = Vd$ infusion. This error has been pointed out to the authors by Gibaldi (4).

Table II—The Biexponential Equations[a] Describing the Postinfusion Curves Obtained after Rapid and Prolonged Intravenous Administration of Griseofulvin into a Male Human Subject

Dose	Infusion Time		A_1'	k_1	A_2'	k_2	Error Mean Square
142 mg.	3 min.	Experimental values	1.492 ± 0.046	0.7032 ± 0.0510	0.9854 ± 0.0486	0.07252 ± 0.00499	0.0039
153.6 mg.	2 hr.	Experimental values	0.7101 ± 0.0877	0.7677 ± 0.1825	1.0631 ± 0.0884	0.06377 ± 0.00761	0.0170
		Calculated values	0.8664	0.7032 ± 0.0510	0.9921	0.07252 ± 0.00499	

[a] All terms and values as denoted in Table I except A_1', A_2', are expressed in micrograms per milliliters. k_1, k_2 are expressed in reciprocal hours.

rived from the metabolite or total drug concentration *versus* time curve then different procedures must be developed to calculate these constants.

EXPERIMENTAL PROCEDURE

Griseofulvin—Griseofulvin was injected into a healthy male adult in a specially prepared solution of griseofulvin dissolved in polyethylene glycol 300. The solution was not injected directly. It was diluted into a rapidly flowing normal saline solution, which was administered by intravenous drip over a 3-min. period. Several weeks later the drug was administered over a 2-hr. interval at a constant rate of infusion into the same test subject. The plasma samples were assayed for the intact drug by a modification of the method of Bedford *et al.* (5).

Sulfisoxazole—The left and right marginal ear veins of a male rabbit (4.5 kg.) were cannulated by small catheters (Bardic Intracath Cat. No. 1619 small catheters 20.32 cm. (8 in.), i.d. 0.015 mm.). Sulfisoxazole injection (Roche) was diluted to an appropriate volume with sterile normal saline solution and then infused into the left vein by means of a constant infusion pump. Blood samples were then withdrawn from the right cannula. The catheters were removed from the ear veins after the experiments. The rabbits were utilized for additional experiments after their ear veins had properly healed. The plasma samples were assayed for sulfonamide (unconjugated at the N_4 position) using the Bratton and Marshall procedure (6).

RESULTS AND DISCUSSION

The rapid infusion or the single bolus i.v. blood curves are describable by the following biexponential equation:

$$Cp_{\text{i.v.}} = A_1e^{-k_1t} + A_2e^{-k_2t} \qquad \text{(Eq. 14)}$$

By applying Eq. 9 to Eq. 14, one gets

$$(Cp)_{\text{post}} = \frac{A_1(1 - e^{-k_1\tau})}{k_1\tau} e^{-k_1t'} + \frac{A_2(1 - e^{-k_2\tau})}{k_2\tau} e^{k_2t'} \qquad \text{(Eq. 15)}$$

or

$$(Cp)_{\text{post}} = A_1'e^{-k_1t'} + A_2'e^{-k_2t'} \qquad \text{(Eq. 16)}$$

where A_1' and A_2' are definable from Eq. 15; Figs. 1 and 2 represent the experimental data obtained on griseofulvin and sulfisoxazole. The experimentally determined A_1', A_2', k_1', k_2', and the corresponding values as calculated from Eq. 9 are summarized in Tables I and II.

The experimental data points were initially fitted to biexponential equations by the use of an E.A.I. T.R. 48 analog computer. The experimental data together with the initial estimated parameters were then refitted or reassessed with an IBM 360/50 computer programmed in accordance with a modified nonlinear least-squares regression subroutine of the B.M.D. X85 series.

The theoretical A_1' and A_2' were calculated using A_1, A_2, k_1, and k_2 values obtained from the single i.v. bolus or rapid infusion curves.

The satisfactory correlation between the experimental values and the calculated values are evident. Most of the experimental values are within one standard deviation of the calculated values. All of the experimental values are within two standard deviations of the calculated values. From Tables I and II it is seen that the ratio A_1'/A_2' decreases as the infusion time increases. This observation is in accordance with Eq. 9. Since $k_1 > k_2$, the value $A_1(1 - e^{-k_1\tau})/(k_1\tau)$ will decrease more rapidly with increasing τ than the value for $A_2(-e^{k_2\tau})/(k_2\tau)$ thus accounting for the above observation.

From Fig. 1 it is seen that the changes in the intercept ratio are also reflected by the decreasing degree of the initial curvature of the blood curve with increasing infusion time. Since this curved region is critical to the definition of the multicompartmental pharmacokinetic parameters, sampling must be sufficiently frequent to characterize adequately this region of the blood curve, especially if the infusion is prolonged. It is, therefore, advisable to minimize the infusion time not only for the convenience of the subject, but also to reduce the necessity for frequent sampling.

In Fig. 2 the data points lie close to the theoretical line as calculated from the single i.v. bolus parameters. The change in the initial curvature is not so apparent visually from the *Cp versus t* curve. However, the computer-analyzed data given in Table II show a drastic change in the intercept A_1 and very little change in the intercept A_2. This is quite in accord with Eq. 15. The graphical representations given in Figs. 1 and 2 are for illustrative purposes only and should not be used for final estimation of the parameters of the equation in lieu of a computer solution.

The findings reported in this study suggest that in multicompartmental pharmacokinetic studies the post infusion curve can be utilized to obtain pharmacokinetic parameters in lieu of the more conventional single i.v. bolus injection. This infusion procedure is especially useful when one encounters potential difficulties, such as solubility and toxicity.

REFERENCES

(1) H. T. Milhorn, Jr., "The Application of Control Theory to Physiological Systems," W. B. Saunders, Philadelphia, Pa. and London, England, 1966.

(2) S. Riegelman, J. C. K. Loo, and M. Rowland, *J. Pharm. Sci.*, **57**, 117(1968).

(3) *Ibid.*, **57**, 128(1968).

(4) M. Gibaldi, *J. Pharm. Sci.*, **58**, 327(1969).

(5) C. Bedford, D. Bushfield, K. J. Child, J. MacGregor, P. Sutherland, and E. G. Tomich, *Arch. Dermatol.*, **81**, 137(1960).

(6) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

(7) A. Rescigno and G. Segre, "Drug & Tracer Kinetics," Blaisdell, New York, N. Y., 1966, pp. 86, 87.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122*

Accepted for publication August 26, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This research was supported in part by a grant-in-aid from the research funds of the Academic Senate, San Francisco Division, University of California.

Inhibited Dissolution of Drug Crystals by a Certified Water-Soluble Dye

J. PICCOLO and R. TAWASHI

Abstract □ Quantitative experiments to investigate the effect of FD&C Blue No. 1 on the dissolution of single crystals of sulfathiazole, phenobarbital, thymol, and sulfaguanidine under controlled conditions, are described. At a very low concentration FD&C Blue No. 1 exerted a remarkable inhibition on dissolution. Dissolution experiments on compressed disks and crystalline powder, gave a significantly lower dissolution rate than the pure drug alone. The dependence of the dissolution rate on the inhibitor concentration was studied in sulfaguanidine crystals, and a concentration of 100 mcg./ml. reduced the dissolution rate by 55%. The data presented is in agreement with the current theories concerning dissolution inhibition by small concentrations of impurities and suggests the dye molecules are preferentially adsorbed at the primary dissolution sources in the crystals investigated.

Keyphrases □ Drug crystal dissolution—inhibition □ Crystal dissolution inhibition—water-soluble dye □ Dissolution rates, behavior—single crystals, compressed disks □ FD&C Blue No. 1—crystal dissolution inhibition

The effect of small quantities of dissolved impurities on the dissolution, growth, and habit modification of crystalline materials has been a subject of different papers (1–3). The development of the dislocation theory, supported by the fact that many organic and inorganic crystals grow by dislocation (4), offered a new basis for examining the influence of impurities on dissolution and growth of crystals.

Albon and Dunning (5) found that a low concentration of raffinose (1 part in 10,000 parts) lowered the rate of step movement of sucrose crystals. The inhibitory action of raffinose and the retardation of step advancement was explained as being governed by the adsorption of raffinose on the steps of sucrose crystals. Ives *et al.* (6, 7) studied the dissolution kinetics in single crystals of lithium fluoride. The dissolution rate of lithium fluoride was inhibited by small concentrations of ferric ion (1 p.p.m.). The concept of impurity adsorption on the kinks—the primary dissolution sources—was discussed.

Crystal poisoning and habit modification by dyes were reported by Buckley (2), Whetstone (8), and Engelhardt (9). At the present, certified water-soluble dyes are used extensively as colorants in drug formulations, e.g., in tablets, tablet-coating, suspensions, *etc.* The possible effect of these colorants on drug dissolution and eventually drug availability needs a careful study. The purpose of this report is to investigate quantitatively the effect of FD&C Blue No. 1 (as an example of certified dyes) on the dissolution behavior of some drug crystals.

EXPERIMENTAL

Materials—Conditions have been selected to grow nearly perfect drug crystals, suitable for single-crystal work. Crystals of sulfaguanidine, thymol, and phenobarbital monohydrate were grown by slow evaporation at room temperature from a saturated solution of the appropriate solvent. The solvents used were acetone for sulfaguanidine, carbon tetrachloride for thymol (10), and a mixture of 50% acetone in water for phenobarbital monohydrate (11). Sulfathiazole Form I crystals were prepared from a saturated solution of sulfathiazole in 95% ethanol using the method described by Grove and Keenan (12). The materials used in this study are USP grade and solvents were purified before use.

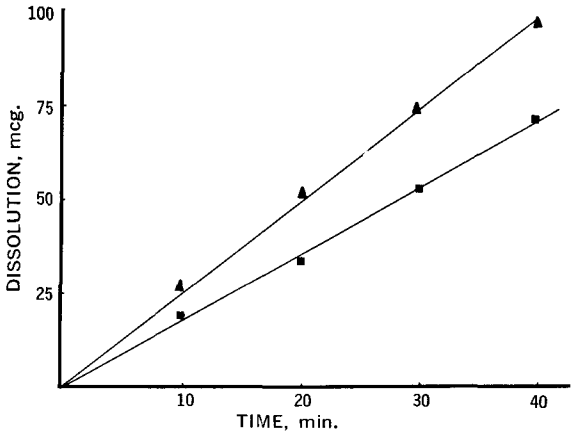


Figure 1—*Single-crystal dissolution of phenobarbital monohydrate as a function of time. Key: ▲, in 0.1 N HCl; ■, in 0.1 N HCl containing 10 mcg./ml. FD & C Blue No. 1.*

Dissolution Rate Studies—The linear dissolution rate was measured by a direct optical method which has been previously described (13, 14). A single crystal was fixed in a rubber slit and placed in a jacketed dissolution cell. The cell was then filled with 200 ml. of 0.1 *N* HCl at 30°, stirring was maintained at 150 r.p.m. by synchronized motor (Hurst Corp., Princeton, Ind.). The distance between the two parallel faces and boundary movement were measured as function of time. Measurements were done with a microscope fitted with a special filar micrometer (Zeiss). Each point represents the average of ten determinations. The rate of dissolution was determined in absence and in presence of low concentrations of FD&C Blue No. 1 (5–100 mcg./ml.).

To study the dissolution behavior from compressed disks, the same apparatus was used after a slight modification. Tablets from the drug crystals were prepared, having the same diameter (1.88 cm.) and compressed under the same pressure. The tablets were

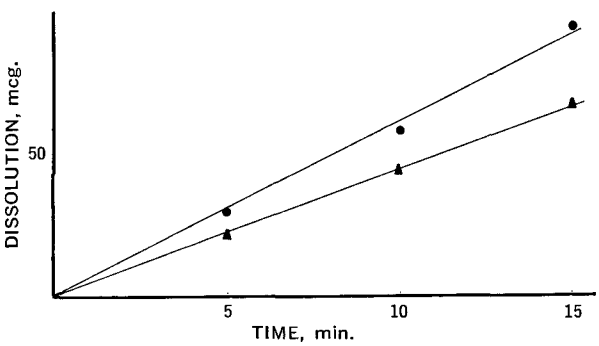


Figure 2—*Single-crystal dissolution of sulfathiazole as a function of time. Key: ●, in 0.1 N HCl; ▲, in 0.1 N HCl containing 5 mcg./ml. FD&C Blue No. 1.*

Table I—Single-Crystal Dissolution Rate in cm./sec.

Material	Dissolution Rate in 0.1 *N* HCl	Dissolution Rate in 0.1 *N* HCl Containing 10 mcg./ml. Dye
Phenobarbital monohydrate	4.2×10^{-6}	2.9×10^{-6}
Sulfathiazole	8.8×10^{-6}	5.1×10^{-6}
Sulfaguanidine	6.8×10^{-5}	4.6×10^{-5}

prepared without the use of any fillers, antiadhesives, or lubricants. The tablet was placed in one end of a short glass tube having the same diameter as the tablet, the other end of the tube was filled with molten white bees wax and left to set. At time zero, the tube was introduced into the dissolution cell containing 200 ml. of 0.1 *N* HCl at 30°, stirring at 150 r.p.m. Samples were pipeted out at specified time and assayed spectrophotometrically for drug content at the appropriate wavelength in the UV region of the spectrum. The dissolution behavior was studied in absence and in presence of FD&C Blue No. 1. A blank having the same concentration of FD&C Blue No. 1 as the dissolution media was used as a reference in determining the drug release in presence of the dye.

Dissolution behavior of the crystalline powder was determined in absence and in presence of FD&C Blue No. 1 using the same dissolution cell. The crystalline powder was introduced into the dissolution cell at time zero; samples were pipeted out at specified time and assayed for drug content. To exclude the effect of particle size on the dissolution rate, the crystals used in the dissolution study came from the same batch.

RESULTS AND DISCUSSION

The results in Figs. 1–3 show that small concentrations of FD&C Blue No. 1 exerted a remarkable effect on the dissolution behavior of phenobarbital monohydrate, sulfathiazole, and sulfaguanidine. The linear dissolution rates in 0.1 *N* HCl and in 0.1 *N* HCl containing 10 mcg./ml. Blue No. 1 are given in Table I.

The dissolution behavior of sulfaguanidine single crystals was studied in presence of variable concentrations of FD&C Blue No. 1 ranging from 0–100 mcg./ml. The data presented in Fig. 3 shows inhibiting effect of the dye on dissolution and Fig. 4 demonstrates

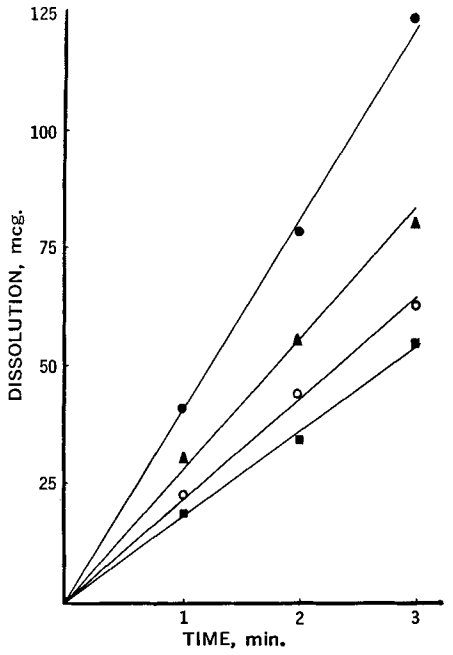


Figure 3—*Single-crystal dissolution of sulfaguanidine as a function of time. Key:* ●, *in 0.1* N *HCl;* ▲, *in 0.1* N *HCl containing 10 mcg./ml. FD&C Blue No. 1;* ○, *in 0.1* N *HCl containing 50 mcg./ml. FD&C Blue No. 1;* ■, *in 0.1* N *HCl containing 100 mcg./ml. FD&C Blue No. 1.*

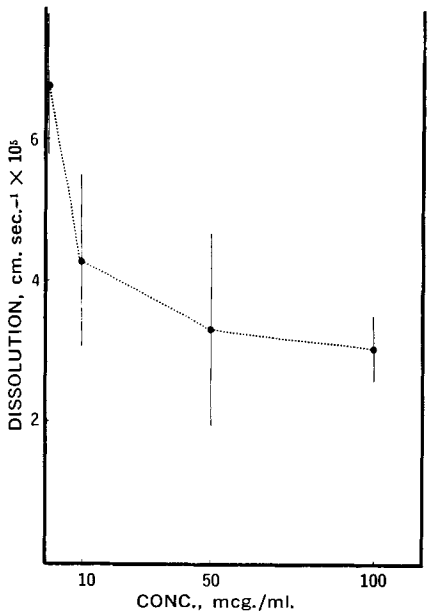


Figure 4—*Effect of dye concentration on the dissolution rate of sulfaguanidine single crystals.*

the dissolution rate dependence on the inhibitor concentration. It is obvious that this curve tends to stabilize after 50 mcg./ml.

Dissolution from the planar surface of a compressed disk was carried out to substantiate the results obtained from single crystals. Figure 5 shows the dissolution behavior of sulfathiazole compressed disks in 0.1 *N* HCl and in 0.1 *N* HCl containing 5 mcg./ml. Blue No. 1. The inhibition obtained is in close agreement with that obtained from a sulfathiazole single crystal.

Figure 6 represents the same phenomenon in compressed disks of thymol. The thymol crystals used in preparing the tablet were grown from CCl_4. These crystals exhibited spiral steps as shown in Fig. 7. Because of the fragile nature and shape irregularities of these crystals, it was difficult, in practice, to measure the dissolution rate of single crystals.

The effect of 50 mcg/ml. of dye on 1.5 g. of sulfathiazole crystalline powder is shown in Fig. 8. Although the dye concentration is high, the dissolution rate was reduced by a value smaller than those obtained from studies on single crystals and tablets. This can be explained by the relatively large surface area provided by 1.5 g. of the crystalline powder.

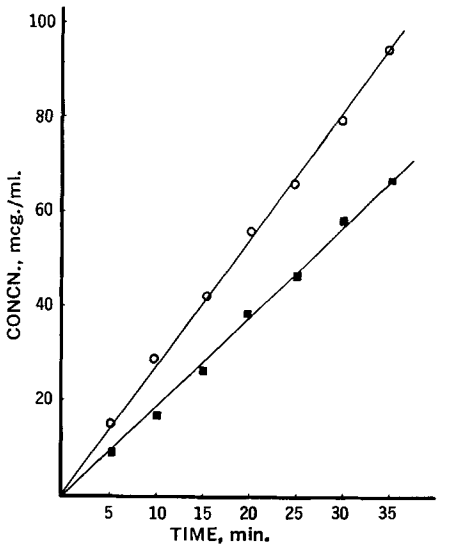


Figure 5—*Dissolution behavior of sulfathiazole compressed disks. Key:* ○, *in 0.1* N *HCl;* ■, *in 0.1* N *HCl containing 5 mcg/ml. FD&C Blue No. 1.*

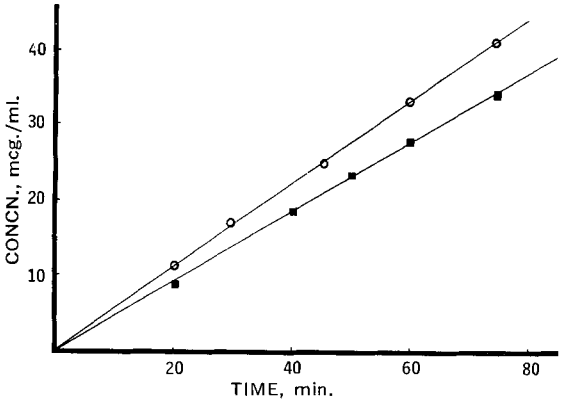


Figure 6—*Dissolution behavior of thymol compressed disks. Key:* ○, *in 0.1* N *HCl;* ■, *in 0.1* N *HCl containing 5 mcg./ml. FD&C Blue No. 1.*

The inhibitory effect of Blue No. 1 on the dissolution of the investigated drug crystals, is in agreement with the work of Saad and Higuchi (1), representing a situation where a relatively large amount of sodium cholate retarded the growth and dissolution rates of cholestrol crystals. The inhibition of the surface dissolution rate by the dye is in agreement with the studies of Ives *et al.* dealing with the effect of traces of ferric ion on the dissolution kinetics of a single crystal of lithium fluoride.

The data obtained are consistent with the kink mechanism proposed by Gilman *et al.* (15) and Ives (16). The mechanism involves preferred adsorption of the dye molecules at the primary dissolution sources of the crystal surface. The kinks in crystal ledges are the primary dissolution sources, where individual drug molecules can deposit or escape readily.

In absence of dye, the drug molecules will be lost preferentially from the kinks where binding is weakest and effect a motion of the kinks along the ledges. The mean time for stripping will always be a simple factor of the ledge length. Continuous dissolution will require successive nucleation of the kinks. In presence of dye inhibition, the dye molecules tend to deposit on the kinks and reduce the kink nucleation rate.

The fact that the curve in Fig. 4 tends to stabilize after a certain concentration of dye is in agreement with previous observations reported by Ives and Plewes (7). It is in accord with the postulate that the observed optimum concentration in the plot of rate of dissolution *versus* inhibitor concentration represents a situation where mono-kink adsorption is attained.

A further evidence was found in investigating the morphological changes produced by the dye on the dissolving faces of sulfaguanidine and sulfathiazole crystals. Figures 9 and 10 show the dissolution features produced by 0.1 *N* HCl and 0.1 *N* HCl containing 50

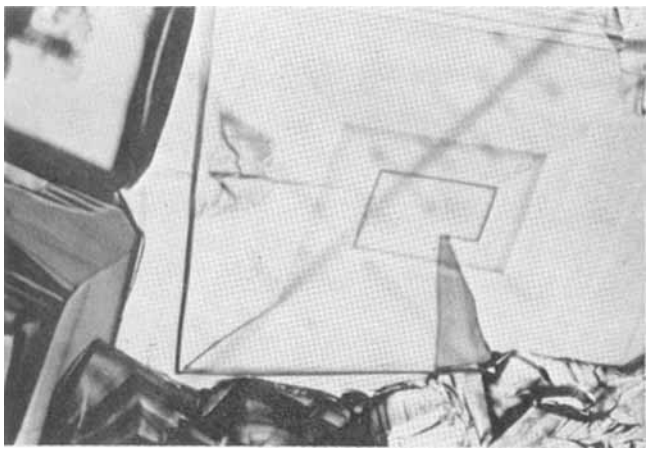


Figure 7—*Thymol crystal exhibiting spiral steps. Magnification: 125×.*

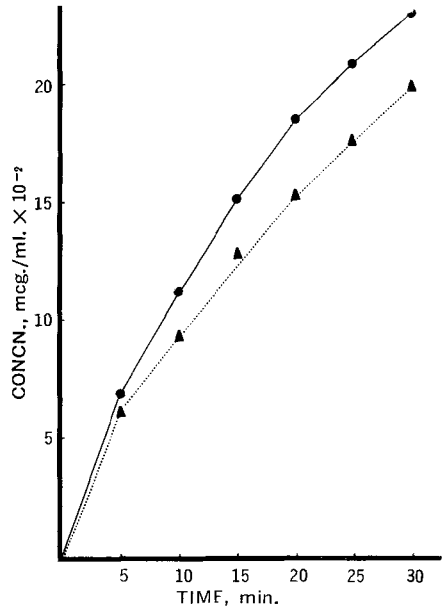


Figure 8—*Dissolution behavior of sulfathiazole crystalline powder. Key:* ●, *in 0.1* N *HCl;* ▲, *in 0.1* N *HCl containing 50 mcg./ml. FD&C Blue No. 1.*

μ/ml. of the dye for a limited period of time. FD&C Blue No. 1 seems to act as an etchant producing etch pits on these dissolving faces. The observations are similar to those obtained by Gilman *et al.* (15) in their study of dislocation etch pit formation in lithium fluoride in presence of ferric ion as dissolution inhibitor.

The major emphasis in this study is on surface dissolution of single crystals and of compressed disks of the drug crystals. For the present, the inhibited dissolution in presence of Blue No. 1, is

a

b

Figure 9—*Effect of FD&C Blue No. 1 on the dissolving face of sulfaguanidine single crystal after 15 min. Key:* a, *in 0.1* N *HCl,* b *in 0.1* N *HCl containing 50 mcg./ml. FD&C Blue No. 1. Magnification: 125×.*

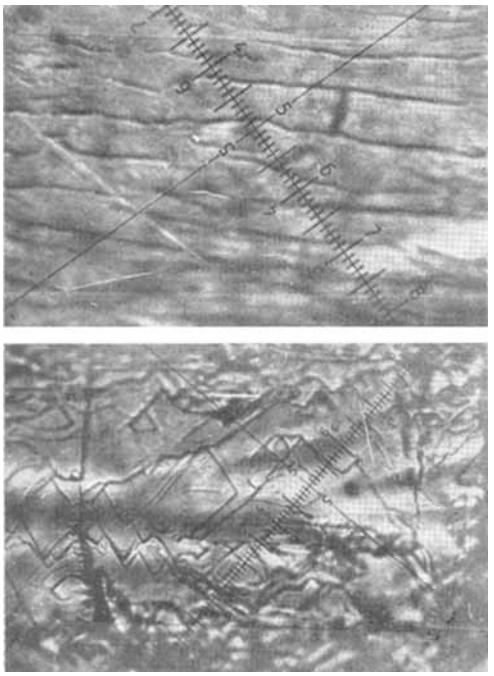


Figure 10—*Effect of FD&C Blue No. 1 on the dissolving face of sulfathiazole single crystal after 15 min. Key:* a, *in 0.1* N *HCl*, b, *in 0.1* N *HCl containing 50 mcg./ml. FD&C Blue No. 1. Magnification: 500×.*

limited to the systems that have been studied, and to the experimental conditions described. The significance of dissolution inhibition in powder systems in presence of a low concentration of the dye is not clear yet. The extension of this study to other powder systems and other water-soluble dyes will contribute to better understanding of the role of water-soluble colorants in drug formulations.

REFERENCES

(1) H. Y. Saad and W. I. Higuchi, *J. Pharm. Sci.*, **54,** 1303 (1965).

(2) H. E. Buckley, "Crystal Growth," Chapman and Hall, London, England, 1951.

(3) M. B. Ives, *Ind. Eng. Chem.*, **57,** No. 6, 34(1965).

(4) D. Fox, B. H. Labes, and A. Weisberger, "Physics and Chemistry of Organic Solids State," vol. I, Interscience, New York, N. Y., 1963.

(5) N. Albon and W. J. Dunning, *Acta Cryst.*, **15,** 479(1962).

(6) M. B. Ives and J. P. Hirth, *J. Chem. Phys.*, **33,** 517(1960).

(7) M. B. Ives and J. T. Plewes, *ibid.*, **42,** 292(1965).

(8) J. Whetstone, *J. Chem. Soc.*, **1957,** 4289.

(9) W. V. Engelhardt, "International Congress of Surface Activity," vol. 2, 1961, pp. 202–217.

(10) E. Votava, S. Amelinckx, and W. Dekeyser, *Naturwissenschaften*, **40,** 143(1953).

(11) S. Chopra and R. Tawashi, *Pharm. Ind.*, **31,** 489(1969).

(12) D. C. Grove and G. L. Keenan, *J. Am. Chem. Soc.*, **63,** 97(1941).

(13) P. D. Bernardo, Ph.D. thesis, University of Michigan, 1966.

(14) R. Tawashi, *Science*, **160,** 76(1968).

(15) J. J. Gilman, W. G. Johnston, and G. W. Sears, *J. Appl. Phys.*, **29,** 747(1968).

(16) M. B. Ives, *J. Phys. Chem. Solids*, **24,** 275(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 5, 1969 from the *Faculty of Pharmacy, University of Montreal, Montreal, Canada 101*

Accepted for publication September 2, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported by the Medical Research Council of Canada.

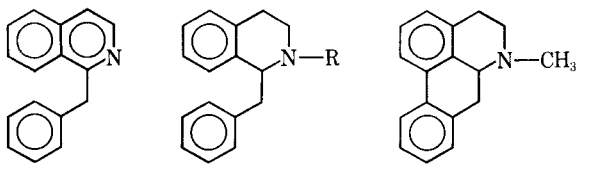
Phenylisoquinolines and Hydroisoquinolines

J. SAM, R. M. SHAFIK*, and K. APARAJITHAN

Abstract □ The synthesis of some derivatives of 5-, 6-, and 7-phenylisoquinoline, 3,4-dihydroisoquinoline, and 1,2,3,4-tetrahydroisoquinoline are described. Results of preliminary pharmacological tests are reported.

Keyphrases □ Phenylisoquinolines—synthesis □ Hydroisoquinolines—synthesis □ Pharmacological screening—phenylisoquinolines, hydroisoquinolines □ IR spectrophotometry—identity

Several alkaloids which incorporate the 1-benzylisoquinoline (I) or tetrahydroisoquinoline (II) moiety in their structures possess interesting biological properties (1, 2). The latter structural feature has been proposed (3) as a precursor in the biosynthesis of the aporphine alkaloids (III). Whereas, extensive investigations (4, 5) have been directed toward the synthesis of 1-benzylisoquinolines, little effort has been expended in studies of arylisoquinolines (VII) and the corresponding hydrogenated derivatives (VIII, X, and XI) (6). Consequently,



I II III

it was of interest to determine whether compounds such as VII and hydrogenated VII possess CNS properties similar to known aporphines (7) (bulbocapnine) and/or cardiovascular properties.

The synthetic approach followed is shown in Scheme I. The nitration of isoquinoline yielded 5-nitroisoquinoline (V) (8). The reduction of the latter compound to 5-aminoisoquinoline (VI) (9, 10) followed by diazotization and coupling with benzene, according to a procedure similar to that described by Cadogan (11) for the preparation of biphenyls, provided 5-phenylisoquinoline. Hydrogenation of VII*a* hydrochloride using plati-

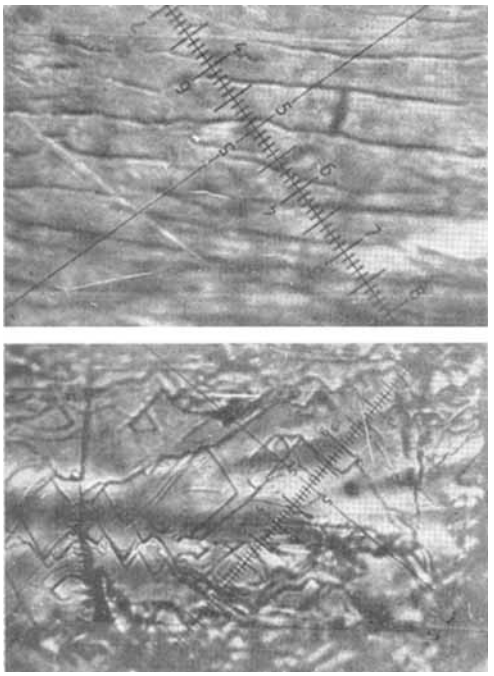


Figure 10—*Effect of FD&C Blue No. 1 on the dissolving face of sulfathiazole single crystal after 15 min. Key:* a, *in 0.1* N *HCl*, b, *in 0.1* N *HCl containing 50 mcg./ml. FD&C Blue No. 1. Magnification: 500×.*

limited to the systems that have been studied, and to the experimental conditions described. The significance of dissolution inhibition in powder systems in presence of a low concentration of the dye is not clear yet. The extension of this study to other powder systems and other water-soluble dyes will contribute to better understanding of the role of water-soluble colorants in drug formulations.

REFERENCES

(1) H. Y. Saad and W. I. Higuchi, *J. Pharm. Sci.*, **54,** 1303 (1965).

(2) H. E. Buckley, "Crystal Growth," Chapman and Hall, London, England, 1951.

(3) M. B. Ives, *Ind. Eng. Chem.*, **57,** No. 6, 34(1965).

(4) D. Fox, B. H. Labes, and A. Weisberger, "Physics and Chemistry of Organic Solids State," vol. I, Interscience, New York, N. Y., 1963.

(5) N. Albon and W. J. Dunning, *Acta Cryst.*, **15,** 479(1962).

(6) M. B. Ives and J. P. Hirth, *J. Chem. Phys.*, **33,** 517(1960).

(7) M. B. Ives and J. T. Plewes, *ibid.*, **42,** 292(1965).

(8) J. Whetstone, *J. Chem. Soc.*, **1957,** 4289.

(9) W. V. Engelhardt, "International Congress of Surface Activity," vol. 2, 1961, pp. 202–217.

(10) E. Votava, S. Amelinckx, and W. Dekeyser, *Naturwissenschaften*, **40,** 143(1953).

(11) S. Chopra and R. Tawashi, *Pharm. Ind.*, **31,** 489(1969).

(12) D. C. Grove and G. L. Keenan, *J. Am. Chem. Soc.*, **63,** 97(1941).

(13) P. D. Bernardo, Ph.D. thesis, University of Michigan, 1966.

(14) R. Tawashi, *Science*, **160,** 76(1968).

(15) J. J. Gilman, W. G. Johnston, and G. W. Sears, *J. Appl. Phys.*, **29,** 747(1968).

(16) M. B. Ives, *J. Phys. Chem. Solids*, **24,** 275(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 5, 1969 from the *Faculty of Pharmacy, University of Montreal, Montreal, Canada 101*

Accepted for publication September 2, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported by the Medical Research Council of Canada.

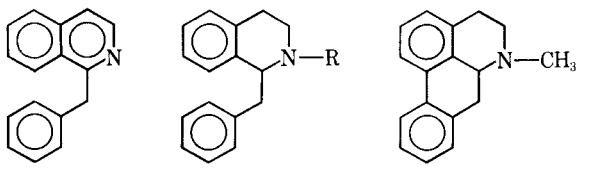
Phenylisoquinolines and Hydroisoquinolines

J. SAM, R. M. SHAFIK*, and K. APARAJITHAN

Abstract □ The synthesis of some derivatives of 5-, 6-, and 7-phenylisoquinoline, 3,4-dihydroisoquinoline, and 1,2,3,4-tetrahydroisoquinoline are described. Results of preliminary pharmacological tests are reported.

Keyphrases □ Phenylisoquinolines—synthesis □ Hydroisoquinolines—synthesis □ Pharmacological screening—phenylisoquinolines, hydroisoquinolines □ IR spectrophotometry—identity

Several alkaloids which incorporate the 1-benzylisoquinoline (I) or tetrahydroisoquinoline (II) moiety in their structures possess interesting biological properties (1, 2). The latter structural feature has been proposed (3) as a precursor in the biosynthesis of the aporphine alkaloids (III). Whereas, extensive investigations (4, 5) have been directed toward the synthesis of 1-benzylisoquinolines, little effort has been expended in studies of arylisoquinolines (VII) and the corresponding hydrogenated derivatives (VIII, X, and XI) (6). Consequently,



I II III

it was of interest to determine whether compounds such as VII and hydrogenated VII possess CNS properties similar to known aporphines (7) (bulbocapnine) and/or cardiovascular properties.

The synthetic approach followed is shown in Scheme I. The nitration of isoquinoline yielded 5-nitroisoquinoline (V) (8). The reduction of the latter compound to 5-aminoisoquinoline (VI) (9, 10) followed by diazotization and coupling with benzene, according to a procedure similar to that described by Cadogan (11) for the preparation of biphenyls, provided 5-phenylisoquinoline. Hydrogenation of VII*a* hydrochloride using plati-

Table I—Phenylisoquinolines and Hydroisoquinolines

No.	R	R′	Method	C_6H_5 Position	Yield, %	M p. °C. (Recrystn. Solvent)[a]	Molecular Formula	Anal., % Calcd.	Found
VII*a*	H	—	A	5	26	185–186(WAc)	$C_{21}H_{14}N_4O_7$[b,c]	C, 58.1 H, 3.3 N, 12.9	C, 57.6 H, 3.7 N, 13.5
VII*b*	H	—	A	5	—	237–239(W)	$C_{16}H_{14}NI$[d,e]	C, 55.4 H, 4.1 N, 4.0	C, 55.5 H, 4.2 N, 4.0
VII*c*	CH_3	—	E	7	60	233–235(WAc)	$C_{22}H_{16}N_4O_7$[c,f,g,h]	C, 58.9 H, 3.6 N, 12.5	C, 58.6 H, 4.0 N, 12.2
VIII*a*	H	H	B	5	98	290–292(W)	$C_{15}H_{16}NCl$[i,j]	C, 73.3 H, 6.6 N, 5.7	C, 73.8 H, 6.6 N, 5.4
VIII*b*	H	H	D	6	76	235–237(W)	$C_{15}H_{16}NCl$[i]	C, 73.3 H, 6.6 N, 5.7	C, 73.4 H, 6.5 N, —
VIII*c*	CH_3	H	D	6	70	231–233(ME)	$C_{16}H_{18}NCl$[i,k]	C, 74.0 H, 7.0 N, 5.4	C, 73.6 H, 7.1 N, 5.3
VIII*d*	H	H	D	7	51	249–251(ME)	$C_{15}H_{16}NCl$[i,l]	C, 73.3 H, 6.6 N, 5.7	C, 73.0 H, 6.9 N, 5.8
VIII*e*	CH_3	H	D	7	70	185(WAc)	$C_{22}H_{20}N_4O_7$[c,m,n]	C, 58.4 H, 4.7 N, 12.4	C, 58.5 H, 4.6 N, 12.8
X*a*	H	—	C	6	45	176–178(E)	$C_{21}H_{16}N_4O_7$[c]	C, 57.8 H, 3.7 N, 12.8	C, 58.8 H, 3.9 N, 13.0
X*b*	CH_3	—	C	6	77	254–256(E)	$C_{22}H_{18}N_4O_7$[c,o]	C, 58.7 H, 4.0 N, 12.4	C, 59.0 H, 4.4 N, 12.3
X*c*	H	—	C	7	40	190–192(E)	$C_{21}H_{16}N_4O_7$[c]	C, 57.8 H, 3.7 N, 12.8	C, 58.0 H, 3.9 N, 12.9
X*d*	CH_3	—	C	7	44	216–218(EW)	$C_{22}H_{18}N_4O_7$[c,p]	C, 58.7 H, 4.0 N, 12.4	C, 59.0 H, 4.0 N, 12.8
XI*a*	H	$CNHNH_2$	F	5	73	222–224(ME)	$C_{16}H_{15}N_3Cl$[i,q]	C, 66.8 H, 6.3 N, 14.6	C, 66.7 H, 6.3 N, 14.0
XI*b*	H	CH_3	G	5	80	228–230(W)	$C_{16}H_{18}NI$[r,s,t]	C, 54.7 H, 5.2 N, 4.0	C, 54.9 H, 5.2 N, 4.1
XI*c*	CH_3	CH_3	H	7	92	237–238(EW)	$C_{17}H_{20}NI$[s,u,v]	C, 55.9 H, 5.5 N, 3.8	C, 56.0 H, 5.4 N, 4.0
XI*d*	CH_3	CH_3	H	7	—	202–204(E)	$C_{18}H_{22}NI$[d,w]	C, 57.0 H, 5.9 N, 3.7	C, 56.8 H, 5.9 N, 3.9
XI*e*	CH_3	$C_{10}H_7ClNO$[x]	I	7	80	140–141(AW)	$C_{26}H_{23}N_2ClO$[i,y]	C, 75.3 H, 5.6 N, 6.8	C, 75.4 H, 5.6 N, 7.0

[a] WAc = water-acetic acid; W = water; ME = methanol-ether; E = ethanol; EW = ethanol-water; P = petroleum ether (30–60°). [b] Free base, b.p. 146–148° (0.6 mm.). [c] Picrate. [d] Methiodide. [e] Calcd. for I: 36.6; Found: 36.8. [f] Free base, b.p. 160° (0.2 mm.). [g] Hydrochloride, m.p. 261–263°(E). [h] Methiodide, m.p. 297–300°(W). [i] Hydrochloride. [j] Calcd. for Cl: 14.4; Found: 14.1. [k] Calcd. for Cl: 13.7; Found 13.9. [l] Calcd. for Cl: 14.4; Found: 14.5. [m] Free base, b.p. 164° (0.65 mm.). [n] Hydrochloride, m.p. 261–263°(E). [o] Hydrochloride, m.p. 263–264° dec.(ME). [p] Free base, m.p. 83–85°(P). [q] Calcd. for Cl: 12.3; Found: 12.6. [r] Free base, m.p. 73–74°(EW). [s] Hydriodide. [t] Calcd. for I: 36.1; Found: 36.0. [u] Free base, b.p. 137° (0.2 mm.). [v] Calcd. for I: 34.8; Found: 34.6. [w] Calcd. for I: 33.5; Found: 33.5. [x] 5-Chloro-8-hydroxy-7-quinolylmethyl. [y] Calcd. for Cl: 8.6; Found: 8.4.

num oxide catalyst yielded 5-phenyl-1,2,3,4-tetrahydroisoquinoline (VIII*a*) whereas VII*b* gave the 2-methyl derivative (XI*b*).

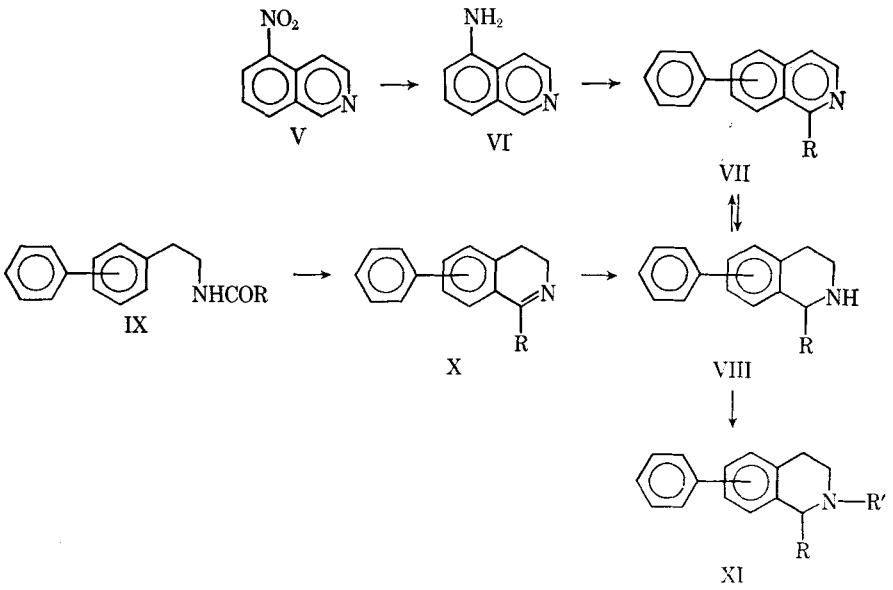
The 6-, and 7-phenyl-2,3-dihydroisoquinolines (X) were prepared *via* the Bischer-Napieralski reaction by heating *N*-acylbiphenylethylamines (IX) with polyphosphoric acid (12). Reduction of X with sodium borohydride provided the 1,2,3,4-tetrahydroisoquinolines (VIII). Dehydrogenation of VIII*e* in *p*-cymene with 10% palladium on carbon (13) provided the corresponding unsaturated isoquinoline (VII*c*).

Heating VIII*a* with cyanamide (14) gave the guanidine derivative (XI*a*). Methylation of VIII*e* yielded the corresponding *N*-methyl derivative (XI*c*). Treating VIII*e* with 5-chloro-8-hydroxyquinoline and formaldehyde provided the Mannich product (XI*e*). The compounds that were prepared are listed in Table I.

PHARMACOLOGICAL RESULTS[1]

Acute effects (Table II) after intraperitoneal injection were observed in male albino mice (20–30 per compound) weighing 25–35 g. Estimated 48-hr. LD_{50} and 95% confidence limits were determined by the method of Horn (15). The method of Irwin (16) was utilized in noting the effects produced.

[1] The authors are grateful to Dr. W. Marvin Davis, Department of Pharmacology, for the pharmacological data.



Scheme I

EXPERIMENTAL[2]

5-Phenylisoquinoline (VIIa)—*Method A*—A solution of 14.4 g. (0.1 mole) of 5-aminoisoquinoline (VI) in 300 ml. of dry benzene was treated with 25 ml. of freshly prepared isoamyl nitrite. The reaction mixture, which turned brown, was heated under reflux on a steam bath for 1 hr. and then allowed to remain at room temperature for 18 hr. The solvent and unreacted isoamyl nitrite were removed by distillation. The dark-colored residue was extracted with hot petroleum ether (30–60°). The pale yellow liquid obtained after the removal of the solvent was distilled under reduced pressure. The picrate (VII*a*) and the methiodide (VII*b*) were prepared in the usual manner and recrystallized.

5-Phenyl-1,2,3,4-tetrahydroisoquinoline (VIIIa)—*Method B*—A solution of 7 g. (0.029 mole) of 5-phenylisoquinoline (VII*a*) in 50 ml. of 1 *N* hydrochloric acid was hydrogenated at 47 p.s.i. with 0.1 g. of PtO_2 catalyst for 12 hr. The mixture was heated to dissolve the solid that had separated and then filtered to remove the catalyst. The hydrogenated product was separated from the cooled filtrate and recrystallized.

N-Acylbiphenylylethylamines (IX)—*N*-[2-(3-Biphenylyl)ethyl]-formamide (IX*a*) and *N*-[2-(4-biphenylyl)ethyl]formamide (IX*c*) were prepared using a procedure similar to that described by Cannon and Webster (12) for the preparation of *N*-acylphenylethylamines. To 0.014 mole of the appropriate 2-biphenylylethylamine was added 22.5 ml. of formic acid and 18 ml. of acetic anhydride. The solution was refluxed for 7 hr. and thereafter diluted with water and extracted with ether. The ether layer was washed subsequently with 10% sodium hydroxide, 10% hydrochloric acid, and water, respectively, and then dried over anhydrous sodium carbonate. The ether was removed under reduced pressure; the crude products (65% of IX*a* and 55% of IX*c*) were used without further purification in Method C.

N-[2-(3-Biphenylyl)ethyl]acetamide (IX*b*), and *N*-[2-(4-biphenylyl)-ethyl]acetamide (IX*d*) were prepared according to the procedure described by Sam *et al.* (17, 18).

6-, and 7-Phenyl-3,4-dihydroisoquinolines (X)—*Method C*—To 61 g. of commercial polyphosphoric acid, heated to 150°, was added with stirring 0.015 mole of the appropriate, dried, *N*-acylbiphenylylethylamine (IX). The mixture was kept at 180–190° for 3 hr. and then set aside for 9 hr. at room temperature. Thereafter, the mixture was diluted with 500 ml. of water and allowed to remain at room temperature for an additional 12 hr. The solution was filtered, rendered alkaline with a solution of 70 g. of sodium hydroxide in 140 ml. of water and then extracted with ether. The ether extract was washed with water and dried over anhydrous sodium sulfate. The amine was distilled under reduced pressure and/or converted to a salt in the usual manner and recrystallized.

6-, and 7-Phenyl-1,2,3,4-tetrahydroisoquinolines (VIII)—*Method D*—A solution of the proper phenyl-3,4-dihydroisoquinoline (X) in 80 ml. of ethanol was treated dropwise, while stirring, with a solution of 8 g. of sodium borohydride in 80 ml. of ethanol. The reaction mixture was allowed to remain at room temperature for 18 hr. and then heated under reflux for 1 hr. The ethanol was distilled under reduced pressure and the residue diluted with water and extracted with ether. The ether extract was washed with water and dried over anhydrous sodium sulfate. The ether was evaporated; the residue was distilled under reduced pressure and/or isolated as a salt and recrystallized.

1-Methyl-7-phenylisoquinoline (VIIc)—*Method E*—A mixture of 5.2 g. (0.023 mole) of 1-methyl-7-phenyl-1,2,3,4-tetrahydroisoquinoline (VIII*e*), 5 g. of 10% Pd-C and 350 ml. of dry *p*-cymene was heated under reflux with stirring for 1 hr. The catalyst was removed by filtration and washed with two 50-ml. portions of hot benzene. The combined filtrate was extracted with dilute hydrochloric acid. The acid extract was rendered alkaline with dilute sodium hydroxide, and then extracted with ether. The ether extract was washed with water and dried over anhydrous magnesium sulfate. The residue obtained on evaporation of the ether was distilled. A picrate was prepared in the usual manner and recrystallized.

5-Phenyl-3,4-dihydro-2(1H)-isoquinolinocarboxamidine hydrochloride (IXa)—*Method F*—A mixture of 1.23 g. (0.005 mole) of 5-phenyl-1,2,3,4-tetrahydroisoquinoline (VIII*a*), 0.25 g. (0.006 mole) of cyanamide and 3 ml. of water was heated in an oil bath at 180° for 1.5 hr. The product was isolated from the resulting mixture by recrystallization.

2-Methyl-5-phenyl-1,2,3,4-tetrahydroisoquinoline (XIb)—*Method G*—A suspension of 5.05 g. (0.014 mole) of 5-phenyl-2-methylisoquinolinium iodide (VII*b*) in 50 ml. of water was hydrogenated at 47.5 p.s.i. with 0.1 g. of PtO_2 for 48 hr. The mixture was heated and filtered to remove the catalyst and then cooled. The product was removed by filtration and recrystallized.

1,2-Dimethyl-7-phenyl-1,2,3,4-tetrahydroisoquinoline (XIc)—*Method H*—Methylation of 1.5 g. (0.0067 mole) of 1-methyl-7-phenyl-1,2,3,4-tetrahydroisoquinoline (VIII*e*) by the Eschwiler-Clarke method (17) gave a liquid which was distilled under reduced pressure. A hydriodide and a methiodide (XI*d*) were prepared in the usual manner and recrystallized.

2-[5-Chloro-8-hydroxy-7-quinolylmethyl]-1-methyl-7-phenyl-1,2,3,4-tetrahydroisoquinoline (XIe)—*Method I*—To a solution of 0.49 g. (0.0022 mole) of 1-methyl-7-phenyl-1,2,3,4-tetrahydroisoquinoline (VIII*e*) in 10 ml. of ethanol was added 0.4 g. (0.0022 mole)

[2] All melting points were taken in open glass capillaries using a Thomas-Hoover melting point apparatus and are uncorrected. IR spectra of all compounds were determined on a Perkin-Elmer model 137 infracord spectrophotometer using KBr pellets. The spectra were consistent with the assigned structures.

Table II—Acute Interperitoneal Toxicity and Symptomatic Observations

No.[a]	LD_{50},mg./kg.	Observations
VIIa[b,c]	242(208–282)	Sedation, ataxia, tremors
VIIb[d]	141(117–170)	Sedation, ataxia, tremors
VIIc[b,c]	521(447–607)	Sedation, ataxia, loss of righting reflex
VIIe[e,c]	187(165–210)	Sedation, tremor, vasodilation
VIIIa[b,c]	96(76–123)	Sedation, convulsion at toxic doses
VIIIb[b,c]	104(86–125)	Slight motor excitation; convulsions at lethal doses
VIIIc[b,c]	141(117–170)	Slight analgesia early followed by long-continuing clonic convulsions
VIIId[b,c]	153(126–184)	Slight analgesia early, clonic convulsions, tremors, and ataxia
VIIIe[b,c]	77(66–89)	Sedation, convulsion at toxic doses
Xb[b,c]	52(45–61)	Long-continuing clonic convulsions
Xd[d]	95(74–123)	Mixed CNS sedation, excitation, slight analgesia
XIa[b,c]	59(44–79)	Depression of motor activity and respiration; ataxia and respiratory failure with lethal doses
XIb[f,c]	383(308–476)	Sedation at sublethal doses; mild clonic convulsions at lethal doses
XIc[f,c]	261(210–325)	Sedation, ataxia; mild clonic convulsions at lethal doses
XIe[d]	328(258–419)	Sedation, ataxia

[a] Refers to compound numbers found in Table I. [b] Hydrochloride. [c] Administered as an aqueous solution. [d] Administered as an aqueous suspension in acacia. [e] Methiodide. [f] Hydrodide.

of 5-chloro-8-hydroxyquinoline and 0.2 ml. of 38% formaldehyde. The mixture was refluxed for 1 hr. and then allowed to remain at room temperature for 18 hr. The product was removed from the cooled solution by filtration and recrystallized.

REFERENCES

(1) A. Burger, "The Alkaloids," vol. IV, Academic, New York, N. Y., 1954, p. 29.

(2) T. A. Henry, "The Plant Alkaloids," Blakiston, Philadelphia, Pa., 1949, p. 154.

(3) D. H. R. Barton, *Proc. Chem. Soc.*, **1963,** 293.

(4) W. M. Whaley and T. R. Govindachari, "Organic Reactions," vol. VI, Wiley, New York, N. Y., 1951, pp. 74, 151.

(5) W. J. Gensler, "Heterocyclic Compounds," vol. IV, Wiley, New York, N. Y., 1952, p. 344.

(6) Y. Ahmad and D. H. Hey, *J. Chem. Soc.*, **1961,** 3882.

(7) R. H. F. Manske, "The Alkaloids," vol. IV, Academic, New York, N. Y., 1954, p. 119.

(8) C. G. LeFevre and R. J. W. LeFevre, *J. Chem. Soc.*, **1935,** 1475.

(9) J. J. Craig and W. E. Cass, *J. Am. Chem. Soc.*, **64,** 783(1942).

(10) F. W. Bergstrom and R. E. Paterson, *J. Org. Chem.*, **10,** 480(1945).

(11) J. I. G. Cadogan, *J. Chem. Soc.*, **1962,** 4257.

(12) J. G. Cannon and G. L. Webster, *J. Am. Pharm. Assoc., Sci. Ed.*, **47,** 353(1958).

(13) J. M. Bobbit, J. M. Kiely, K. L. Khanna, and R. E. Ebermann, *J. Org. Chem.*, **30,** 2247(1965).

(14) J. H. Short and T. D. Darby, *J. Med. Chem.*, **10,** 833(1967).

(15) H. J. Horn, *Biometrics*, **12,** 311(1956).

(16) S. Irwin, "Animal and Clinical Techniques in Drug Evaluation," J. H. Nodine and P. E. Siegles, Eds., Year Book Publishers, Chicago, Ill., 1964.

(17) J. Sam and R. M. Shafik, *J. Pharm. Sci.*, to be published.

(18) J. Sam, K. Aparajithan, and R. Shafik, *ibid.*, **57,** 564(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 9, 1969 from the *Department of Pharmaceutical Chemistry, The University of Mississippi, University, MS 38677*

Accepted for publication August 28, 1969.

This investigation was supported in part by grant MH 05292 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md. and during a tenure (R.M.S. and K.A.) of a Mississippi Heart Association Fellowship.

* Present address: Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt, U.A.R.

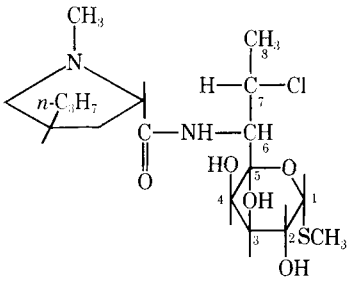
Aqueous Stability of Clindamycin

T. O. OESTERLING

Abstract □ The kinetics and mechanism of degradation of the antibiotic clindamycin were studied in buffered aqueous solution in the pH range 0.4–12. Clindamycin showed maximum stability at pH 3–5; however, high temperature studies indicated that not more than 10% degradation will occur in the pH range 1–6.5 after two years at 25°. In the pH range 0.4–4 the major degradative pathway was hydrolysis of the thioglycoside linkage to form *1*-dethiomethyl-*1*-hydroxy clindamycin and methyl mercaptan. The major degradative pathway in the pH range 5–10 was scission of the 7-(S)-Cl of clindamycin to form the 7-(R)-OH analog, lincomycin. Evidence was obtained that supports the hypothesis that this conversion to lincomycin proceeds through an oxazolonium intermediate and that the extent of conversion depends on the degree of protonation of the amine function of clindamycin. The activation energy for clindamycin degradation in 0.1 *M* HCl, where thioglycoside hydrolysis is predominant, is 38.0 ± 1.2 kcal./mole, and the activation energy in 0.2 *M* citrate buffer adjusted to pH 5, where conversion to lincomycin is predominant, is 29.1 ± 0.6 kcal./mole.

Keyphrases □ Clindamycin—stability, aqueous solution □ Stability—clindamycin aqueous solutions □ Degradation, clindamycin—kinetics, mechanism, product identity □ pH effect—clindamycin stability □ Temperature effect—clindamycin stability □ GLC—analysis □ Mass spectroscopy—identity

The synthesis and biological properties of the antibiotic clindamycin (I) were first reported by Magerlein *et al.* (1, 2). Clindamycin possesses marked antiplasmodial activity (3) and is highly effective in the treatment of infections caused by Gram-positive organisms (2).



I

The purpose of this study was to investigate the degradation of clindamycin in aqueous solution in order to supply basic information necessary for the successful formulation of the drug in liquid dosage forms. The effects of pH and temperature on the rate of clindamycin degradation were studied and the major products of degradation in the pH range 0.4–12 were identified. From these data, the conditions of maximum stability of clindamycin in aqueous solution were established and the stability of clindamycin in pharmaceutical formulations could be predicted.

EXPERIMENTAL

Materials—Clindamycin, lincomycin, lincomycin tetraacetate, 7-epilincomycin, and 7-epiclindamycin (The Upjohn Co.) were used and all other chemicals were reagent grade.

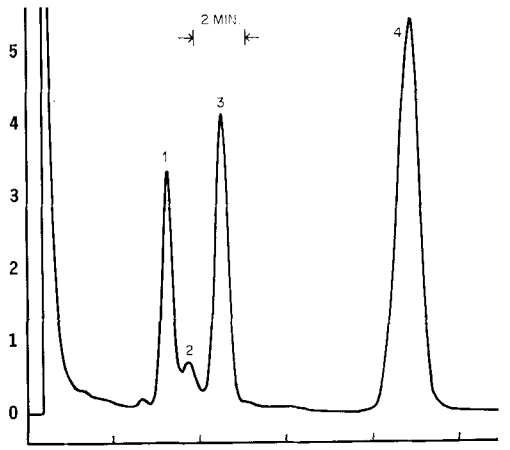


Figure 1—*Typical gas-liquid chromatogram of acetylated sample from low pH (<4) reaction mixtures. Key: Peak 1, 1-dethiomethyl-1-hydroxy clindamycin tetraacetate; Peak 2, suspected anomer of Peak 1; Peak 3, clindamycin triacetate; Peak 4, cholesteryl acetate.*

Kinetic Studies—*pH-Rate Studies*—Reaction mixtures containing clindamycin hydrochloride and buffers were prepared to cover the pH range 0.40–12 using the buffers shown in Table I. Individual buffer systems were prepared from citric acid, tartaric acid, acetic acid, succinic acid, disodium phosphate, boric acid, and sodium carbonate, and adjusted to the desired pH at 70° by the addition of hydrochloric acid or sodium hydroxide.

The concentration of clindamycin hydrochloride was 1% (0.02 *M*) in the pH range 1–6, 0.2% at pH 7, and 0.1% at pH values greater than 7. Reaction mixtures at higher pH contained less clindamycin due to the relatively low solubility of the undissociated species (about 2 mg./ml.). After preparation, volumes of each reaction mixture equivalent to 12 mg. of clindamycin hydrochloride were filled into ampuls, sealed, and placed into a 70° constant-temperature bath. At appropriate times two samples were withdrawn, the pH of one was measured, and an aliquot of the second equivalent to 10 mg. of clindamycin hydrochloride was freeze-dried. The amount of intact clindamycin present in the freeze-dried samples was determined by the gas-liquid chromatographic assay described below.

Temperature-Rate Studies—The effect of temperature on the rate of clindamycin degradation was studied in 0.1 *M* hydrochloric acid and in 0.2 *M* citrate buffer adjusted to pH 5.

Ampuls containing 10 mg./ml. of clindamycin hydrochloride in 0.1 *M* hydrochloric acid were placed into constant-temperature baths set at 47, 53, 70, 79, and 93°. At appropriate times 1.0-ml. samples were withdrawn, freeze-dried, and the amount of intact clindamycin present in the freeze-dried samples was determined by the gas-liquid chromatographic assay described below.

Ampuls containing 10 mg./ml. of clindamycin hydrochloride in the citrate buffer were placed into 47, 53, 60, 70, 80, and 90° constant-temperature baths. At appropriate times two ampuls were withdrawn, the pH of one was measured, and 1.0 ml. of the other was freeze-dried. The pH did not vary by more than 0.2 pH units from the initial pH during the reaction in all of the buffered reaction mixtures including the pH-rate studies described above. The amount of intact clindamycin present in the freeze-dried samples was determined by the gas-liquid chromatographic assay described below.

Gas-Liquid Chromatographic Assay—One milliliter of deionized water was added to the freeze-dried sample, the pH was adjusted to 11 with concentrated ammonium hydroxide, and the solution was extracted with two 1-ml. portions of ethyl acetate. The ethyl acetate extracts were combined and the solvent was removed with a stream

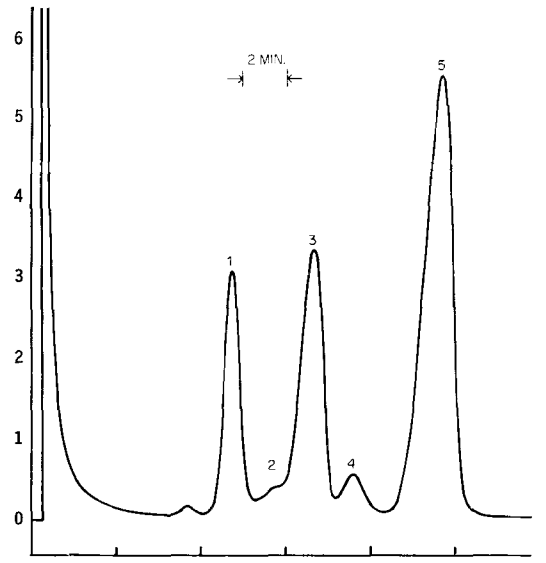


Figure 2—*Typical gas-liquid chromatogram of acetylated sample from high pH (5–8) reaction mixture. Key: Peak 1, clindamycin triacetate; Peak 2, suspected 7-epilincomycin tetraacetate or 7-epiclindamycin triacetate; Peak 3, lincomycin tetraacetate; Peak 4, unknown; Peak 5, cholesteryl acetate.*

of nitrogen. To the residue 1.0 ml. of a mixture of pyridine (dried over KOH)–acetic anhydride (2:1) containing 10 mg./ml. cholesteryl acetate as internal standard was added. After acetylating for 1 hr. at 100°, the solution was cooled to room temperature and 1 μl. was injected into an F & M model 402 gas chromatograph adjusted to the following conditions. Samples were partitioned between helium carrier gas flowing at 40 ml./min. and 3% OV-1 on diatomaceous earth (Gas Chrom Q) 60–80 mesh in a U-shaped glass column 120 cm. long × 3 mm. i.d. Columns were preconditioned by heating at 300° for at least 6 hr. with low helium flow and then for 1 hr. under no flow conditions. The column temperature was 220°, flash heater temperature 230°, and flame ionization detector 240°. Air and hydrogen flow rates were adjusted to give maximum response.

The concentration of clindamycin per sample was obtained by comparing clindamycin–internal standard peak height ratios to the peak height ratios of a standard curve prepared from known amounts of clindamycin. Standard curves were obtained daily.

Determination of pKa of Clindamycin at 70°—Ten milliliters of a 2.0 mg./ml. solution of clindamycin hydrochloride were titrated with 0.05 *N* NaOH in a jacketed vessel at 70° with a Radiometer TTT1C titrator and SBR2C titragraph. The average pKa of six replicate titrations was 6.90 ± 0.10.

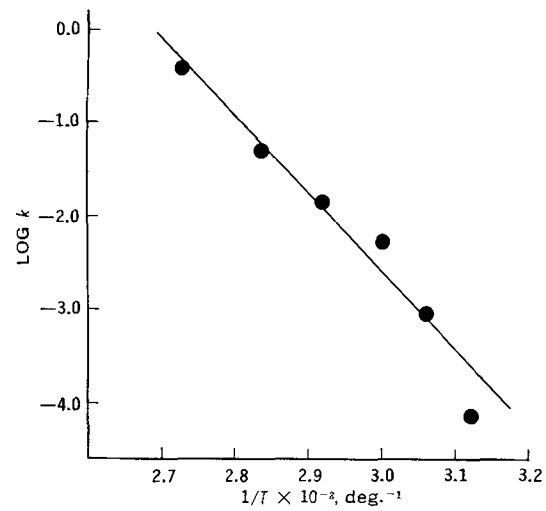


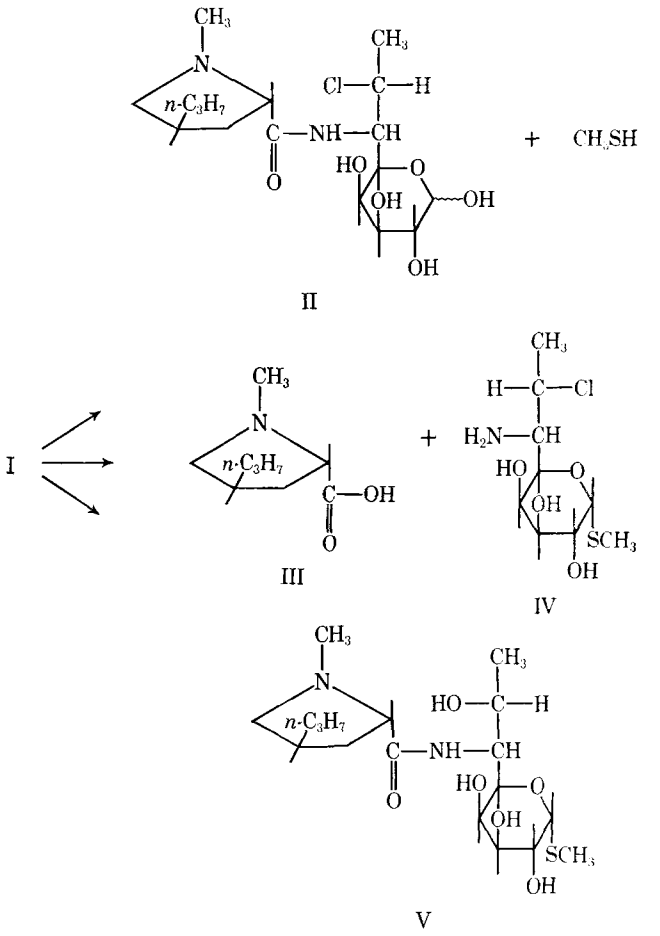
Figure 3—*Arrhenius plot for degradation of clindamycin in 0.1 M HCl.*

Identification of Degradation Products—Degradation products were identified by mass spectral studies and by comparison of gas-liquid chromatographic retention times and thin layer chromatographic R_f values with authentic samples when available. Mass spectra of acetylated clindamycin and degradation products were obtained by processing acetylated reaction mixture samples through an LKB 9000 gas chromatograph-mass spectrometer and recording mass spectra of peaks of interest as they exited from the gas chromatograph column. Thin layer chromatographic studies were carried out by spotting about 50 μl. of clindamycin reaction mixtures on Silica Gel G and developing with chloroform–methanol 90:10. Spots were visualized with iodine vapor.

RESULTS AND DISCUSSION

Identification of Products—Gas-liquid chromatographic data indicated that the mechanism of clindamycin degradation is pH-dependent with a change in mechanism occurring in the vicinity of pH 4–5. For example, chromatograms similar to Fig. 1 were obtained from reaction mixtures whose pH was less than 4, whereas samples from reaction mixtures in the pH range 5–8 yielded chromatograms similar to the one shown in Fig. 2. The major product of degradation in the pH range 0.4–4, represented by Peak 1 in Fig. 1, had a shorter retention time than clindamycin while the major degradation product at higher pH, represented by Peak 3 of Fig. 2, had a longer retention time than clindamycin. Chromatograms from reaction mixtures at pH greater than 8 which were buffered with borate, carbonate, or NaOH showed only disappearance of clindamycin and no other peaks were observed.

The identity of the major degradation product of clindamycin at pH less than 4 was established by mass spectral studies and with a knowledge of the reactivity of lincomycin (V), the 7(R)-OH analog of clindamycin, in acid. Prescott (5) and Herr and Slomp (6) have



Scheme I

Table I—Apparent First-Order Rate Constants of Degradation of Clindamycin

pH	Buffer	Temperature, °C.	k, sec.$^{-1}$ × 10^{-6}
0.44[a]	0.5 *M* HCl	70	19.0
0.54[a]	0.4 *M* HCl	70	19.3
0.66[a]	0.3 *M* HCl	70	14.3
0.83[a]	0.2 *M* HCl	70	8.25
1.10[a]	0.1 *M* HCl	70	4.50
1.10[a]	0.1 *M* HCl	70	4.66
1.10[a]	0.1 *M* HCl	47	0.0199
1.10[a]	0.1 *M* HCl	53	0.252
1.10[a]	0.1 *M* HCl	59	1.43
1.11[a]	0.1 *M* HCl	79	13.0
1.11[a]	0.1 *M* HCl	93	66.5
1.94	0.2 *M* Citrate	70	0.831
1.95	0.2 *M* Citrate	70	0.805
2.90	0.2 *M* Citrate	70	0.295
2.95	0.2 *M* Citrate	70	0.318
3.92	0.2 *M* Citrate	70	0.243
4.00	0.2 *M* Citrate	70	0.249
4.07	0.2 *M* Citrate	70	0.242
4.00	0.2 *M* Citrate	47	0.0124
4.00	0.2 *M* Citrate	53	0.0279
4.00	0.2 *M* Citrate	59	0.0831
4.00	0.2 *M* Citrate	80	0.969
4.00	0.2 *M* Citrate	92	3.62
4.80	0.2 *M* Tartrate	70	0.276
4.85	0.2 *M* Acetate	70	0.278
5.00	0.2 *M* Succinate	70	0.280
5.00	0.2 *M* Citrate	70	0.276
5.95	0.2 *M* Citrate	70	0.665
6.00	0.2 *M* Phosphate	70	0.620
6.00	0.2 *M* Citrate	70	0.946
6.90	0.2 *M* Citrate	70	2.07
6.95	0.2 *M* Citrate	70	2.89
7.25	0.2 *M* Phosphate	70	4.00
7.85	0.2 *M* Phosphate	70	5.91
8.20	0.2 *M* Phosphate	70	4.08
8.30	0.2 *M* Phosphate	70	7.11
8.50	0.2 *M* Borate	70	15.2
9.30	0.2 *M* Borate	70	15.5
9.50	0.2 *M* Borate	70	16.7
9.70	0.2 *M* Borate	70	17.7
9.75	0.2 *M* Borate	70	14.9
10.00	0.2 *M* Borate	70	29.9
11.00	0.2 *M* Carbonate	70	149
11.66[b]	0.1 *M* NaOH	70	204

[a] Calculated from pH = − Log f(HCl): where (HCl) is the experimental molarity and f is the mean activity coefficient for HCl at 70° extrapolated from the literature (4). [b] Calculated from pH = pKw − pOH: where pKw = 12.77 at 70° (4), pOH = − Log f(NaOH), (NaOH) is the experimental molarity, and f is the mean activity coefficient for NaOH extrapolated from the literature (4).

reported that lincomycin (V) degrades in acid *via* thioglycoside hydrolysis to form 1-dethiomethyl-1-hydroxylincomycin and methyl mercaptan. Clindamycin might be expected to react in acid in a manner similar to lincomycin and mass spectral data indicate that the molecular weight of the compound represented by Peak 1 of Fig. 1 is 562. This molecular weight corresponds to acetylated 1-dethiomethyl-1-hydroxyclindamycin which indicates that II in Scheme I is the major product of clindamycin degradation at pH less than 4.

II can be detected gas chromatographically as the predominant reaction product for about three clindamycin half-lives after which it begins to disappear. The decrease in magnitude of the gas-liquid chromatographic peaks of II and clindamycin at extended reaction times is accompanied by the appearance of several small peaks of shorter retention time. This further degradation may result from breakdown of the sugar moiety or amide hydrolysis (6).

Peak 2 of Fig. 1 represents another product of clindamycin degradation at pH less than 4 which is present in the early stages of the reaction. As the reaction proceeds, Peak 2 behaves similarly to Peak 1 and decreases in magnitude after about three clindamycin half-lives. Since thioglycoside hydrolysis proceeds through a carbonium ion intermediate (7), the products of clindamycin would be methyl mercaptan and an anomeric mixture of II. Mass spectral studies indicated that Peak 1 represents one of the anomers and

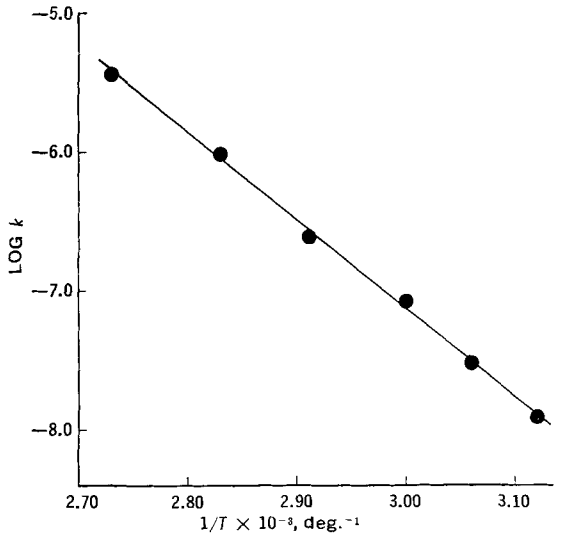


Figure 4—*Arrhenius plot of clindamycin degradation at pH 4 in 0.2* M *citrate buffer.*

Peak 2 may represent the other. The identification of Peak 2 was not attempted.

Lincomycin (V) was identified as the major degradation product of clindamycin at pH greater than 5. Mass spectral studies of Peak 3 of Fig. 2 showed that the molecular weight of this compound was 574, the molecular weight of acetylated lincomycin. Further, the gas-liquid chromatographic retention time of Peak 3 of Fig. 2 is identical to authentic lincomycin tetraacetate. Lincomycin, as well as clindamycin, is unstable in the pH range 5–8 as both Peaks 1 and 3 of Fig. 2 disappear at long reaction times. One possible route of degradation of lincomycin and clindamycin at extended reaction times is amide hydrolysis.

It is obvious from Fig. 2 that lincomycin is not the sole product of clindamycin degradation at pH 5–8. At least two other reaction products are represented by Peaks 2 and 4 of Fig. 2. Both Peaks 2 and 4 represent minor products and neither was ever present in

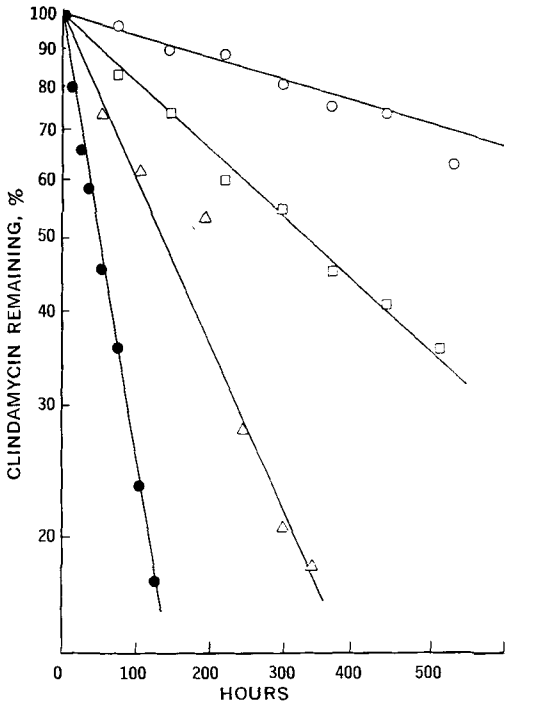


Figure 5—*Apparent first-order disappearance of clindamycin in 0.1* M *HCl at various temperatures. Key:* ○ *47°,* □ *53°.* △ *59°,* ● *69°.*

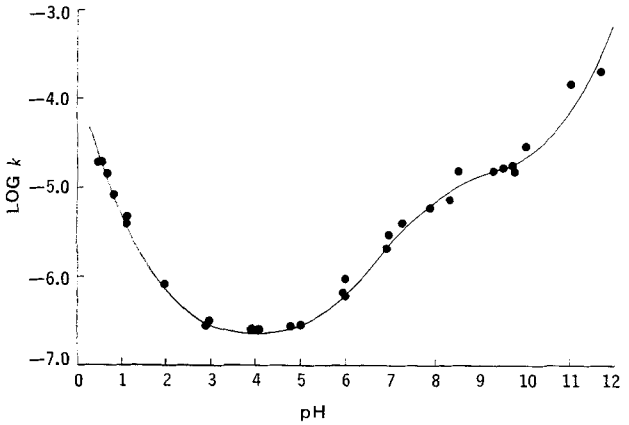


Figure 6—*pH-Rate profile of clindamycin degradation at 70°.*

amounts greater than that shown in Fig. 2. Peak 2 may represent acetylated 7-epilincomycin or acetylated 7-epiclindamycin since its retention time was identical to authentic samples of each. Magerlein has reported the formation of a trace of 7-epilincomycin after refluxing an aqueous solution of clindamycin at pH 7.8 (8). Peak 4 of Fig. 2 was not identified.

Although lincomycin (V) and Peaks 2 and 4 of Fig. 2 could not be detected above pH 8 by GLC, TLC of these reaction mixtures indicated that some of these degradation products were present but were not being extracted for gas chromatographic assay. Thin layer chromatograms of the reaction mixtures above pH 8 showed clindamycin and lincomycin by comparison with authentic samples and three other spots which were not identified. Gas chromatograms of reaction mixtures below pH 8 showed that the major degradation products were extracted by ethyl acetate. The sum of the moles of acetylated IV (Scheme I) and clindamycin between pH 5 and 8 and of acetylated II and clindamycin below pH 5 was greater than 90% of the initial clindamycin concentration in the early stages of the reaction.

Temperature-Rate Studies—Arrhenius plots of clindamycin degradation in 0.1 *M* HCl and at pH 4 are shown in Figs. 3 and 4. The activation energy for clindamycin degradation in 0.1 *M* HCl was calculated to be 38.0 ± 1.2 kcal./mole. The principal degradative pathway in 0.1 *M* HCl is hydrolysis of the *1*-thio-α-D-galactopyranoside moiety of clindamycin at Position 1 to form *1*-dethio-*1*-hydroxy-clindamycin (II) and methyl mercaptan (Scheme I). The activation energy for clindamycin degradation in 0.1 *M* HCl falls in the 30–38 kcal./mole range reported by other investigators for acid degradation of compounds of similar structure such as alkyl *1*-thio-β-D-glucopyranosides (9), alkyl β-D-xylopyranosides (10), and alkyl α- and β-D-glucopyranosides (11).

The activation energy for clindamycin degradation at pH 5, the pH of maximum stability, was calculated to be 29.1 ± 0.6 kcal./mole. Using the rate constants in Table I and the appropriate activation energy, the prediction can be made that clindamycin in pharmaceutical formulations adjusted to pH 1–6.5 will not degrade by more than 10% after 2 years at 25°.

pH-Rate Studies—Clindamycin disappeared from reaction mixtures by an apparent first-order process under all of the conditions studied. Some typical log clindamycin concentration-time curves are shown in Fig. 5.

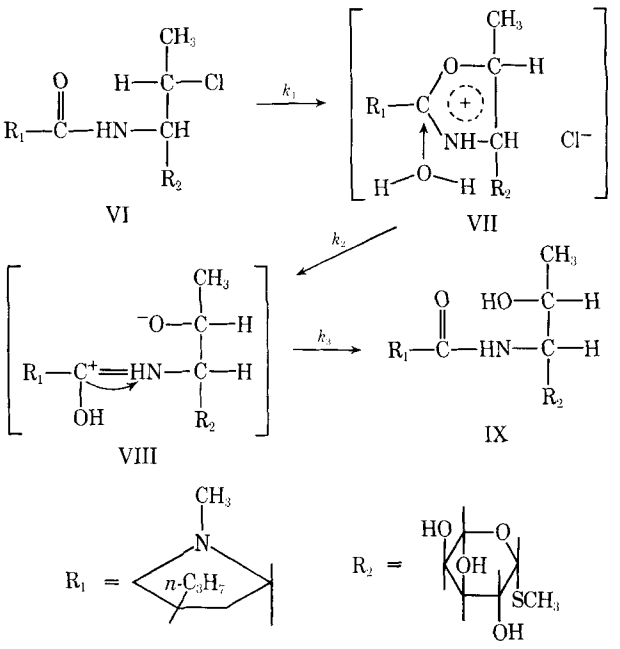
The effect of pH on the rate of clindamycin degradation in the pH range 0.40–12 at 70° is shown in Fig. 6.

Different buffer species did not appear to significantly influence the rate of degradation since a continuous curve was obtained for the pH profile and since the rate constant did not vary with different buffers at the same pH (Table I).

The pH profile of clindamycin (Fig. 6) shows that the rate of degradation increases with decreasing pH in the pH range 0.4–4. This is expected since both major degradative routes in this pH region, thioglycoside and amide hydrolysis, are susceptible to hydrogen ion catalysis. Being of similar structure, lincomycin (V) should also degrade by these two routes in acid media and the rate constant should agree with that of clindamycin when reacted under similar conditions. Forist, *et al.* (12) studied the degradation of V in 0.1 *N* HCl at 70° and the rate constant of 4.85×10^{-6} sec.$^{-1}$ calculated from their data agrees quite favorably with the clindamycin values in Table I.

In the pH range 5–12 the degradative rate increases with increasing pH with an inflection point in the pH 9–10 region. GLC and TLC of reaction mixtures in this pH region show that at least four products are formed by clindamycin degradation. The times of appearance of the various products and their relative concentrations indicate parallel reactions. Assuming there are parallel reactions occurring, the shape of the pH-rate profile in Fig. 6 in the pH 4–12 region might be interpreted as follows. In the pH 4–9 region all degradative rates are increasing with increasing pH with one of the reactions predominating. Above pH 9 the rate constant of the reaction which was predominating at pH 5–9 becomes constant but the rates of the other reactions continue to increase with increasing pH.

Gas-liquid and thin layer chromatographic data of this study show that the predominant degradative reaction at pH 5–9 is conversion to lincomycin (V). Results of studies by Magerlein (8, 13) show how the rate of lincomycin conversion could be predominant in this pH range and then become constant above pH 9. Magerlein has postulated that conversion to lincomycin occurs through the oxazolonium ion intermediate (VII) shown in Scheme II and that the extent of conversion is highly dependent on the participating ability of the substituent on the amide carbonyl (13). For example, solvolysis is anchimerically assisted by the neighboring amide carbonyl if the carbonyl substituent is methyl, whereas when R_1 is a strong electron withdrawing group such as trifluoromethyl, participation does not occur. Magerlein also reports that the 7(S)-Cl position of clindamycin is resistant to direct S_N2 nucleophilic displacement (8).



Scheme II

In the present study no conversion to lincomycin was observed at pH values less than 5, whereas lincomycin was detected by GLC or TLC in all reaction mixtures buffered to pH values greater than 5. Assuming that the protonated form of the *N*-methyl-4-propylpyrrolidine portion of clindamycin (R_1) is analogous to trifluoromethyl as a nonparticipating substituent and $k_1 \ll k_2$, no conversion to lincomycin would be expected in the pH range where R_1 is fully protonated, *i.e.*, at pH values less than 5. On the other hand, it is possible that lincomycin can be detected in the pH 5 reaction mixtures because the small amount of nonprotonated R_1 has facilitated conversion to lincomycin and the increase in rate of clindamycin degradation is partly due to this reaction. As the pH increases the rate of conversion to lincomycin increases due to the presence of more nonprotonated R_1. The leveling trend in the pH profile at pH 9, two pH units above the pKa of 6.90, might be due to a constant rate of lincomycin conversion since at pH 9 and above all of R_1 exists in the nonprotonated form. The further increase in overall rate above pH 10 might then be due to an increase in the rate of other reactions such as amide hydrolysis.

The relationship between pH and the mechanism of clindamycin degradation could be summed as follows. Below pH 4 clindamycin degrades *via* thioglycoside and amide hydrolysis with thioglycoside hydrolysis predominant in the pH range 0.4–4. Above pH 5 clindamycin degrades by conversion to lincomycin and by other reactions such as amide hydrolysis. The extent of lincomycin conversion is dependent on the degree of protonation of the *N*-methyl-4-propylpyrrolidine moiety. At pH less than 5 where the amine is fully protonated no conversion to lincomycin occurs. Then this process can be detected in the vicinity of pH 5 and its rate increases as pH increases to pH 9 and then becomes constant since the amine function is completely nonprotonated. The overall rate of clindamycin degradation continues to increase with increasing pH above pH 9, however, due to the hydroxide ion dependency of the other degradative routes.

REFERENCES

(1) R. D. Birkenmeyer, Abstracts of Papers, Fifth Interscience Conference on Antimicrobial Agents and Chemotherapy and IV International Congress of Chemotherapy, Washington, D. C., October 17–21, 1965.

(2) B. J. Magerlein, R. D. Birkenmeyer, and F. Kagan, *Antimicrobial Agents Chemotherapy*, **1966**, 727.

(3) C. Lewis, *J. Parasitol.*, **54**, 169(1968).

(4) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolyte Solutions," Reinhold, New York, N. Y., 1958.

(5) G. C. Prescott, *J. Pharm. Sci.*, **55**, 423(1966).

(6) R. R. Herr and G. Slomp, *J. Am. Chem. Soc.*, **89**, 2444(1967).

(7) J. N. Be Miller, in "Advances in Carbohydrate Chemistry," vol. 22, M. L. Wolfrom and R. S. Tipson, Eds., Academic, New York, N. Y., 1967, p. 25 ff.

(8) B. J. Magerlein, "Chemical Modifications of Lincomycin," Medicinal Chemistry Symposium, Quebec, Canada, June 23–26, 1968.

(9) M. D. Saunders and T. E. Timell, *Carbohydrate Res.*, **6**, 121(1968).

(10) C. K. De Bruyne and F. Van Wijnendaele, *ibid.*, **6**, 367 (1968).

(11) T. E. Timell, *Can. J. Chem.*, **42**, 1456(1964).

(12) A. A. Forist, L. W. Brown, and M. E. Royer, *J. Pharm. Sci.*, **54**, 476(1965).

(13) B. J. Magerlein and F. Kagan, *J. Med. Chem.* (in press).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication August 28, 1969.

Presented to the Basic Pharmaceutics Section of the APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The author wishes to thank Dr. P. B. Bowman for obtaining and aiding in the interpretation of mass spectra, and Mr. G. R. Munting for excellent technical assistance in this work.

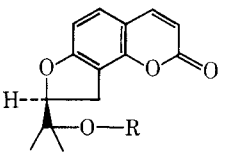
Coumarins XI: A Total Synthesis of (±)-Columbianetin

M. SHIPCHANDLER*, T. O. SOINE†, and P. K. GUPTA‡

Abstract □ (±)-Columbianetin [(±)-I] has been synthesized by a ten-step sequence starting with 2,6-dihydroxybenzoic acid which was converted to the methyl ester, benzylated, and reduced to the benzyl alcohol which was oxidized to the aldehyde and monodebenzylated to provide 2-hydroxy-6-benzyloxybenzaldehyde (VI). Treatment of VI with methyl bromoacetate converted it to methyl 3-benzyloxy-2-formylphenoxyacetate which was cyclized to methyl 4-benzyloxybenzofuran-2-carboxylate, the latter being converted to 2-(α-hydroxyisopropyl)-4-benzyloxybenzofuran (XIV) by the action of CH_3MgI. Reduction and debenzylation of XIV to the corresponding dihydrobenzofuran followed by acid-catalyzed condensation with ethyl propiolate provided (±)-I.

Keyphrases □ (±)-Columbianetin—total synthesis □ TLC—separation identity □ Mass spectroscopy—identity □ UV spectrophotometry—identity □ IR spectrophotometry—identity □ NMR spectroscopy—identity

The isolation of two new coumarins from the umbellifer, *Lomatium columbianum* Math. and Const., was reported in 1964 (1). One of these coumarins, a glycoside assigned the name columbianin, has since been shown to occur in *L. dissectum* var. *multifidum* (Nutt.) Math. and Const. (2) and *L. nuttallii* (A. Gray) Macbr. (3). Acid hydrolysis of columbianin yielded D-glucose and a tertiary coumarinic aglycone, columbianetin (I) and led to the postulation of II as the structure for the glycoside. More recent studies (4) have revised the structure to III, *i.e.*, the β-D-gentiobioside of I (III). The other coumarin, columbianadin, was assigned Structure IV, *i.e.*, the angelate ester of I, and has been found in *Peucedanum palustre* (5) as well as in *Zosimia absinthifolia* (Vent.) Link (6, 7). The absolute configuration of I has been shown to be 8(S) (8).

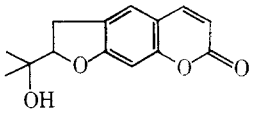


I, R = H

II, R = D-Glucosyl

III, R = β-D-Gentiobiosyl

IV, R = Angeloyl



V

The recent total synthesis of marmesin (V) and its optical antipode, nodakenetin, by Nakajima *et al.* (9) and confirmed by Harada *et al.* (10) in a study of the absolute configuration suggested that a similar synthesis could be applied to the preparation of I by utilizing 2-hydroxy-6-benzyloxybenzaldehyde (VI) as starting material in place of the isomeric 2-hydroxy-4-benzyloxybenzaldehyde employed by these workers. The preparation of VI from 2,6-dihydroxybenzaldehyde (*i.e.*, γ-resorcylaldehyde) was the obvious route but a survey of the literature pertaining to the preparation of the latter (11–15) indicated that all of the published methods were characterized by poor overall yields as well as lengthy synthetic sequences. Thus, a synthesis of

The relationship between pH and the mechanism of clindamycin degradation could be summed as follows. Below pH 4 clindamycin degrades *via* thioglycoside and amide hydrolysis with thioglycoside hydrolysis predominant in the pH range 0.4–4. Above pH 5 clindamycin degrades by conversion to lincomycin and by other reactions such as amide hydrolysis. The extent of lincomycin conversion is dependent on the degree of protonation of the *N*-methyl-4-propylpyrrolidine moiety. At pH less than 5 where the amine is fully protonated no conversion to lincomycin occurs. Then this process can be detected in the vicinity of pH 5 and its rate increases as pH increases to pH 9 and then becomes constant since the amine function is completely nonprotonated. The overall rate of clindamycin degradation continues to increase with increasing pH above pH 9, however, due to the hydroxide ion dependency of the other degradative routes.

REFERENCES

(1) R. D. Birkenmeyer, Abstracts of Papers, Fifth Interscience Conference on Antimicrobial Agents and Chemotherapy and IV International Congress of Chemotherapy, Washington, D. C., October 17–21, 1965.

(2) B. J. Magerlein, R. D. Birkenmeyer, and F. Kagan, *Antimicrobial Agents Chemotherapy*, **1966**, 727.

(3) C. Lewis, *J. Parasitol.*, **54**, 169(1968).

(4) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolyte Solutions," Reinhold, New York, N. Y., 1958.

(5) G. C. Prescott, *J. Pharm. Sci.*, **55**, 423(1966).

(6) R. R. Herr and G. Slomp, *J. Am. Chem. Soc.*, **89**, 2444(1967).

(7) J. N. Be Miller, in "Advances in Carbohydrate Chemistry," vol. 22, M. L. Wolfrom and R. S. Tipson, Eds., Academic, New York, N. Y., 1967, p. 25 ff.

(8) B. J. Magerlein, "Chemical Modifications of Lincomycin," Medicinal Chemistry Symposium, Quebec, Canada, June 23–26, 1968.

(9) M. D. Saunders and T. E. Timell, *Carbohydrate Res.*, **6**, 121(1968).

(10) C. K. De Bruyne and F. Van Wijnendaele, *ibid.*, **6**, 367 (1968).

(11) T. E. Timell, *Can. J. Chem.*, **42**, 1456(1964).

(12) A. A. Forist, L. W. Brown, and M. E. Royer, *J. Pharm. Sci.*, **54**, 476(1965).

(13) B. J. Magerlein and F. Kagan, *J. Med. Chem.* (in press).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication August 28, 1969.

Presented to the Basic Pharmaceutics Section of the APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The author wishes to thank Dr. P. B. Bowman for obtaining and aiding in the interpretation of mass spectra, and Mr. G. R. Munting for excellent technical assistance in this work.

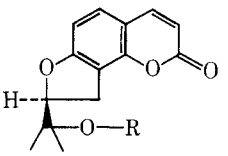
Coumarins XI: A Total Synthesis of (±)-Columbianetin

M. SHIPCHANDLER*, T. O. SOINE†, and P. K. GUPTA‡

Abstract □ (±)-Columbianetin [(±)-I] has been synthesized by a ten-step sequence starting with 2,6-dihydroxybenzoic acid which was converted to the methyl ester, benzylated, and reduced to the benzyl alcohol which was oxidized to the aldehyde and monodebenzylated to provide 2-hydroxy-6-benzyloxybenzaldehyde (VI). Treatment of VI with methyl bromoacetate converted it to methyl 3-benzyloxy-2-formylphenoxyacetate which was cyclized to methyl 4-benzyloxybenzofuran-2-carboxylate, the latter being converted to 2-(α-hydroxyisopropyl)-4-benzyloxybenzofuran (XIV) by the action of CH_3MgI. Reduction and debenzylation of XIV to the corresponding dihydrobenzofuran followed by acid-catalyzed condensation with ethyl propiolate provided (±)-I.

Keyphrases □ (±)-Columbianetin—total synthesis □ TLC—separation identity □ Mass spectroscopy—identity □ UV spectrophotometry—identity □ IR spectrophotometry—identity □ NMR spectroscopy—identity

The isolation of two new coumarins from the umbellifer, *Lomatium columbianum* Math. and Const., was reported in 1964 (1). One of these coumarins, a glycoside assigned the name columbianin, has since been shown to occur in *L. dissectum* var. *multifidum* (Nutt.) Math. and Const. (2) and *L. nuttallii* (A. Gray) Macbr. (3). Acid hydrolysis of columbianin yielded D-glucose and a tertiary coumarinic aglycone, columbianetin (I) and led to the postulation of II as the structure for the glycoside. More recent studies (4) have revised the structure to III, *i.e.*, the β-D-gentiobioside of I (III). The other coumarin, columbianadin, was assigned Structure IV, *i.e.*, the angelate ester of I, and has been found in *Peucedanum palustre* (5) as well as in *Zosimia absinthifolia* (Vent.) Link (6, 7). The absolute configuration of I has been shown to be 8(S) (8).

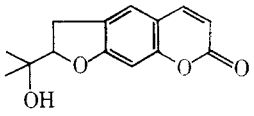


I, R = H

II, R = D-Glucosyl

III, R = β-D-Gentiobiosyl

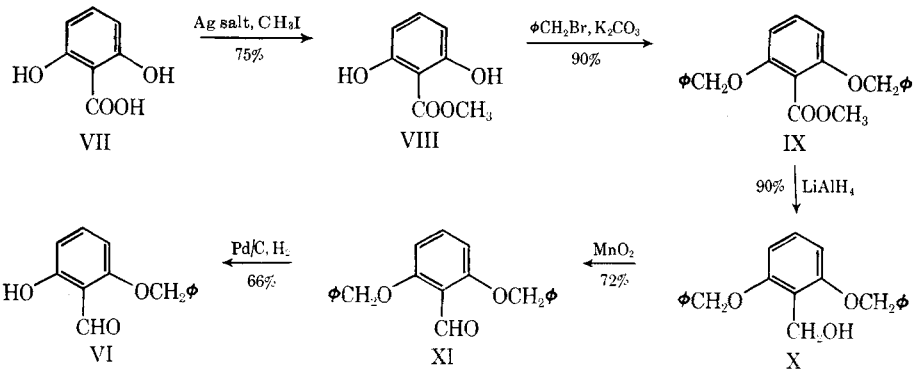
IV, R = Angeloyl



V

The recent total synthesis of marmesin (V) and its optical antipode, nodakenetin, by Nakajima *et al.* (9) and confirmed by Harada *et al.* (10) in a study of the absolute configuration suggested that a similar synthesis could be applied to the preparation of I by utilizing 2-hydroxy-6-benzyloxybenzaldehyde (VI) as starting material in place of the isomeric 2-hydroxy-4-benzyloxybenzaldehyde employed by these workers. The preparation of VI from 2,6-dihydroxybenzaldehyde (*i.e.*, γ-resorcylaldehyde) was the obvious route but a survey of the literature pertaining to the preparation of the latter (11–15) indicated that all of the published methods were characterized by poor overall yields as well as lengthy synthetic sequences. Thus, a synthesis of

VI starting from readily obtainable 2,6-dihydroxybenzoic acid (VII) appeared attractive (see Scheme I). The usual Fischer method of acid-catalyzed esterification of VII is reported (16) to give low yields and the authors found it also gave a poor yield of the methyl ester (VIII).



Scheme I

Employment of the method of Tomino (17), however, reacting the silver salt of VII with methyl iodide provided a 75% yield of VIII. Benzylation of VIII was carried out by the method of Doyle *et al.* (16) who prepared it without characterization and simply hydrolyzed it for further synthetic procedures. In the present study IX was obtained in 90% yield and, upon saponification, yielded the corresponding acid already described by Doyle *et al.* (16). Lithium aluminum hydride reduction of IX yielded the corresponding alcohol (X) in 90% yield. Oxidation of X with active manganese dioxide provided the aldehyde (XI) in 72% yield (18). Removal of a single benzyl group to obtain the desired monobenzyl ether (VI) in 66% yield was effected by catalytic hydrogenolysis with palladium-on-carbon.

The sequence of reactions to provide (±)-I from VI is shown in Scheme II and begins with alkylation of VI in 90% yield with methyl bromoacetate to provide XII followed by cyclization in the presence of magnesium methoxide by the method of Davies *et al.* (19) resulting in XIII in 63% yield.[1] Treatment of XIII with methyl magnesium iodide resulted in an oily tertiary alcohol (XIV) reminiscent of the similarly oily isomeric product obtained by Nakajima *et al.* (9) who proceeded directly to its hydrogenation without further characterization. Utilizing the alkaline palladium-on-carbon (20) of the above authors in the present study to minimize hydrogenolysis of the allylic tertiary alcohol function, the resulting product appeared to be only that (XV) resulting from simple debenzylation of XIV. This was illustrated by its mass spectrum which showed a strong molecular ion peak at *m/e* 192 accompanied by the base peak at *m/e* 177 (doubly charged ion at *m/e* 88.5), undoubtedly due to a loss of methyl radical (see Scheme III).[2] The *m/e* 177 ion does not fragment further to any appreciable extent. Compound XV, when hydrogenated further in the presence of commercially available palladium-on-carbon, provided the dihydrobenzofuran compound (XVI) which was subsequently obtained also by direct hydrogenation of XIV in the presence of the same catalyst. TLC examination in each case revealed a spot with a higher R_f value than that of XVI, presumably the hydrogenolyzed desoxy compound. Compound XVI gave a molecular ion at *m/e* 194 in the mass spectrum and its fragmentation behavior had a marked resemblance to that of columbianetin (I) as evidenced by the loss of H_2O from the molecular ion followed by methyl expulsion to give the benzopyrilium ion at *m/e* 161 (21) (see Scheme IV). Further, a strong peak at *m/e* 59 is due to the protonated form of acetone arising from the side chain of the molecular ion. Loss of acetone from the molecular ion provides an ion of *m/e* 136 (base peak) which fragments further as illustrated.

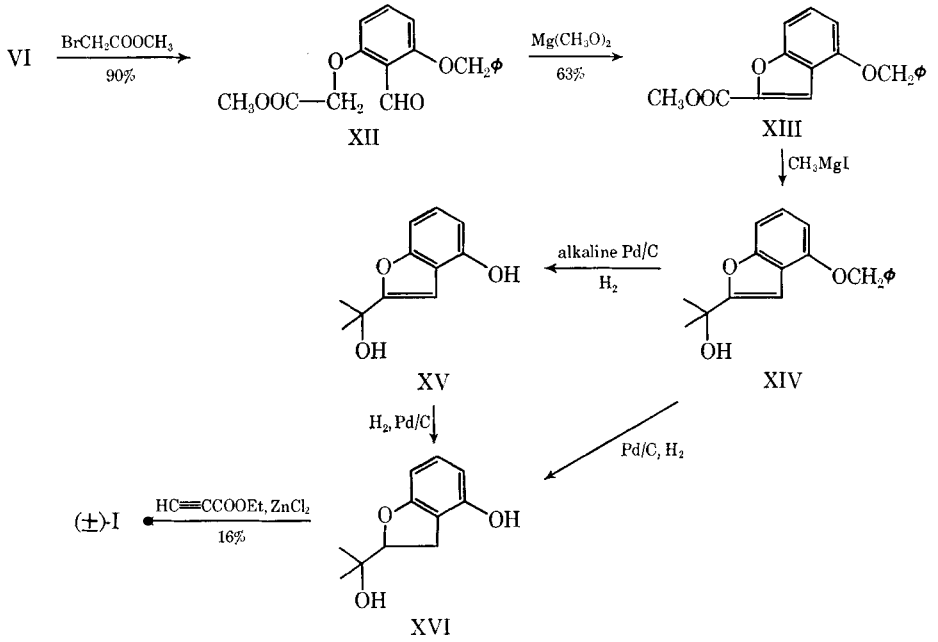
The final conversion of XVI to (±)-I was accomplished through the method of Kaufman and Kelly (22) who employed ethyl propiolate in the presence of an acid to form the coumarinic lactone ring. This method has also been used successfully by Ganguly *et al.* (23). In the present study, condensation of XVI with ethyl propiolate in the presence of zinc chloride provided (±)-I in low yield. The identity of the racemic product was established by TLC, UV, IR, and mass spectral comparison with natural (+)-columbianetin.

EXPERIMENTAL

Melting points were determined in a Thomas-Hoover melting point apparatus and are uncorrected. IR spectra were determined with a Perkin-Elmer 237B grating infrared spectrophotometer and, unless otherwise specified, were determined in potassium bromide pellets. NMR spectra were determined with Varian Associates A-60 and A-60D instruments using tetramethylsilane (TMS) as the internal standard. Unless otherwise specified, all spectra were obtained in $CDCl_3$ in approximately 15% concentration and s. refers to singlet, d. to doublet, t. to triplet, and m. to multiplet. Mass spectra were determined with a Hitachi Perkin-Elmer RMU-6D mass spectrometer (courtesy of Mr. A. R. Swanson and Mr. D. L. Hobbs, School of Chemistry, University of Minnesota). The instrument was operated with a source temperature of 250° and an ionizing voltage of 50 ev. The samples were introduced by the direct inlet technique. UV spectra were determined on a Cary-14 recording spectrophotometer in 95% ethanol. Silica gel for column chromatography (Baker Analyst No. 3405) was activated at 110° and impregnated with 5% of water. Alumina for column chromatography (Woelm neutral alumina purchased from Brinkmann Instruments, Inc., Great Neck, N. J.), silica gel and alumina for TLC (Chroma-

[1] Attempts to reduce XIII catalytically under varying conditions prior to treatment with the Grignard reagent were uniformly unsuccessful and accompanied only by debenzylation and/or reduction of the aromatic ring.

[2] Fragmentation supported by the appearance of corresponding metastable ions has been indicated by heavily-lined arrows here and in Scheme IV. Numbers in parentheses correspond to abundance ratios.



Scheme II

gram sheets with fluorescent indicator supplied by Distillation Products Industries, Rochester, N. Y.), and preparative silica gel-coated thin-layer plates (Brinkmann Instruments, Inc.) were also used. Microanalyses were performed by the Microanalytical Laboratory, School of Chemistry, University of Minnesota or by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

Methyl 2,6-Dihydroxybenzoate (VIII)—*Method A*—Using the method of Doyle *et al.* (16) which employs methanolic hydrogen chloride provided a 32% yield of white crystals, m.p. 68–70°. Lit (16) reported m.p. 69–71°.

Method B—The method of Tomino *et al.* (17) employing the silver salt of 2,6-dihydroxybenzoic acid[3] with methyl iodide provided a 75% yield of white crystals, m.p. 68–70° which on repeated crystallization gave m.p. 69–70°.

Methyl 2,6-Dibenzyloxybenzoate (IX)—Methyl 2,6-dihydroxybenzoate (VIII) (16.8 g., 0.1 mole), benzyl bromide (34.2 g., 0.2 mole) and anhydrous potassium carbonate (36.0 g., 0.26 mole) were refluxed together in 100 ml. of dry acetone for 4 hr. with magnetic stirring (16). At the end of the reflux period the solvent was removed under vacuum and the residual mass was extracted with ether several times. The extract was washed with aqueous sodium hydroxide solution (10%), followed by water, and was finally dried over anhydrous sodium sulfate. Removal of the solvent under vacuum gave an oily mass which solidified upon standing. The product was crystallized from ethanol to yield 31.2 g. (90% of theory) of white crystals, m.p. 71–73°. Repeated crystallization from aliphatic naphtha[4] gave an analytical sample, m.p. 73–74°.

Anal.—Calcd. for $C_{22}H_{20}O_4$: C, 75.84; H, 5.79. Found: C, 76. 12; H, 6.08.

The above ester was hydrolyzed by the method of Doyle *et al.* (16) and 2,6-dibenzyloxybenzoic acid was crystallized twice from ethyl acetate-naphtha, m.p. 123–124°. Lit. (16) gives m.p. 124–126°.

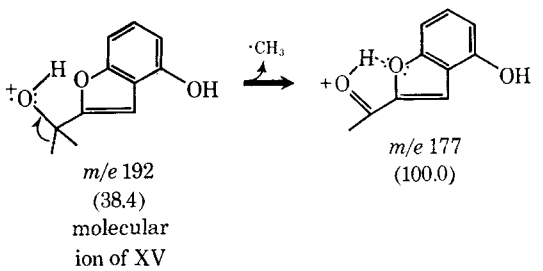
2,6-Dibenzyloxybenzyl Alcohol (X)—Methyl 2,6-dibenzyloxybenzoate (IX) (17.4 g., 0.05 mole) was dissolved in 150 ml. of anhydrous ether and the solution was added dropwise to a magnetically stirred slurry of 3.8 g. (0.1 mole) of lithium aluminum hydride in 100 ml. of dry ether kept in an ice bath. The reaction mixture was stirred for 30 min. after the addition was complete and the excess of lithium aluminum hydride was then decomposed by the cautious addition of the required amount of water. After stirring for 15 min. following the decomposition, the reaction mixture was filtered through a sintered-glass funnel and the residual mass was thoroughly washed with ether. The filtrate and washings were combined, dried over anhydrous sodium sulfate, and the solvent removed under vacuum. The product was crystallized from cyclohexane, to provide 14.4 g. (90% of theory) of white needles, m.p. 78–79°. Several recrystallizations gave an analytical sample, m.p. 79–80°.

Anal.—Calcd. for $C_{21}H_{20}O_4$: C, 78.72; H, 6.29. Found: C, 79.01; H, 6.31.

2,6-Dibenzyloxybenzaldehyde (XI)—Active manganese dioxide[5] (16 g.) was added to 200 ml. of toluene and stirred magnetically for a few minutes before addition of 8 g. (0.024 mole) of 2,6-dibenzyloxybenzyl alcohol (X). The mixture was then refluxed for 4 hr. with continued stirring. The reaction mixture was filtered through a diatomaceous earth[6] bed while hot and the residue was washed well with hot and cold benzene. The combined filtrate and washings were stripped of solvent under reduced pressure and the resulting oil which had an odor reminiscent of benzaldehyde solidified on standing. The product was crystallized from cyclohexane to yield 5.7 g. (72% of theory) of white needles, m.p. 80–81.5°. Repeated crystallization provided an analytical sample, m.p. 81.5–82.5°. The compound gave a positive test with 2,4-dinitrophenylhydrazine reagent.

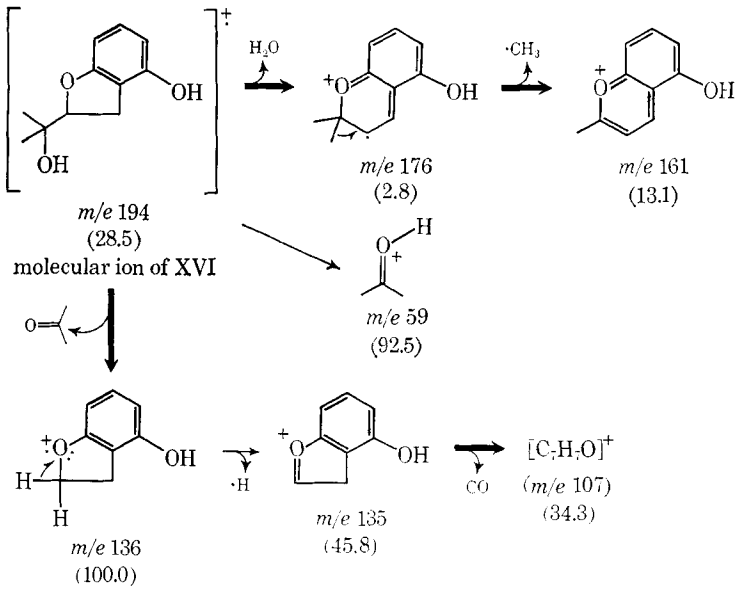
Anal.—Calcd. for $C_{21}H_{20}O_3$: C, 79.22; H, 5.70. Found: C, 79.51; H, 5.66.

2-Hydroxy-6-benzyloxybenzaldehyde (VI)—Palladium-on carbon (5%, 2 g.)[7] was shaken in an atmosphere of hydrogen for 30 min. in 50 ml. of ethanol. 2,6-Dibenzyloxybenzaldehyde (XI) (3.18 g., 0.01 mole) was dissolved in 100 ml. of ethanol with the aid of heat. After cooling, this was added to the prereduced catalyst and hydrogenated at atmospheric pressure until the uptake of hydrogen amounted to 270 ml. (1.1 mole equivalent). The catalyst was removed by filtration and washed well with hot and cold ethanol. The



Scheme III

[3] The acid is a product of Aldrich Chemicals, Milwaukee, Wis.
[4] Skellysolve-B, Skelly Oil Co., Kansas City, Mo.
[5] Winthrop Laboratories, New York, N. Y.
[6] Celite, Johns-Manville Products Corp., New York, N. Y.
[7] Matheson, Coleman & Bell, Cincinnati, Ohio.



Scheme IV

filtrate, on alumina TLC examination, showed very little of the starting material (a blue spot under UV light having the greatest mobility when developed with C_6H_6:$CHCl_3$, 1:1) while the monobenzyl compound appeared as a yellowish-green fluorescent spot. A third spot, having the least polarity and showing a dark spot under UV light, was assumed to be 2,6-dihydroxybenzaldehyde. The filtrate and washings were combined and concentrated under reduced pressure to 70 ml. On standing and cooling, the product crystallized out as yellow needles weighing 1.35 g., m.p. 72–73°. The mother liquor was evaporated to dryness and chromatographed through a silica gel column 25 g., 1.92 × 25.40 cm. (0.75 × 10 in.). The product was eluted with cyclohexane: benzene (3:1) and on evaporation of the eluant solvent under reduced pressure followed by crystallization provided 0.16 g. more of the product for a total yield of 1.51 g. (66% of theory). Repeated crystallization from naphtha gave an analytical sample as lemon-yellow needles, m.p. 73–74°.

Anal.—Calcd. for $C_{13}H_{12}O_3$: C, 73.67; H, 5.30. Found: C, 73.46; H, 5.16.

The compound gave a violet coloration with ferric chloride solution but did not show a hydroxyl peak in the IR. It showed, however, a split C=O band at 1625 and 1650 $cm.^{-1}$ (probably due to hydrogen bonding).

Methyl 3-Benzyloxy-2-formylphenoxyacetate (XII)—2-Hydroxy-6-benzyloxybenzaldehyde (VI) (2 g., 0.009 mole), anhydrous potassium carbonate (10 g., 0.073 mole) and methyl bromoacetate (10 g., 0.073 mole) were refluxed together in 60 ml. of dry acetone for 72 hr. The precipitated potassium bromide and the unreacted potassium carbonate were filtered off and washed well with solvent. The filtrate and washings were combined and most of the unreacted methyl bromoacetate was removed under vacuum with the final traces being removed by passing a stream of air over the residue for a few hours. The product was crystallized from ethanol to give 2.38 g. (90% of theory), m.p. 99–101°. Several recrystallizations gave an analytical sample as white needles, m.p. 100–101°.

Anal.—Calcd. for $C_{17}H_{16}O_5$: C, 67.99; H, 5.37. Found: C, 67.91; H, 5.59.

Methyl 4-Benzyloxybenzofuran-2-carboxylate (XIII)—Magnesium methoxide was prepared from magnesium metal powder (20 g., 0.825 mole, 70–80 mesh) and 600 ml. of dry methanol. Methyl 3-benzyloxy-2-formylphenoxyacetate (XII) (4 g., 0.013 mole) was added and the mixture refluxed for 12 hr. (19). After removal of most of the methanol, crushed ice and water were added and the excess of methoxide was decomposed by portionwise addition of 130 ml. of concentrated hydrochloric acid while cooling the mixture continually in an ice bath combined with simultaneous addition of crushed ice to the reaction flask. After decomposition of all of the methoxide, the aqueous layer was extracted several times with ether and the extract dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure and crystallization from ethanol gave 2.27 g. of the product, m.p. 128–129°. The mother liquor was chromatographed through a silica gel column (25 g., 1.92 × 25.40 cm.) (0.75 × 10 in.). The product was eluted with benzene and the column monitored with a hand UV lamp, the desired compound showing a blue fluorescence. Evaporation of the eluant and crystallization provided an additional 0.1 g. of the product for a total yield of 2.37 g. (63% of theory). Repeated crystallizations provided an analytical sample as shiny white plates, m.p. 129–130°.

Anal.—Calcd. for $C_{17}H_{14}O_4$: C, 72.33; H, 5.00. Found: C, 71.99; H, 5.22.

2-(α-Hydroxyisopropyl)-4-benzyloxybenzofuran (XIV)—Grignard reagent was prepared from 0.4 g. (0.017 mole) of magnesium metal powder (70–80 mesh) and 1.4 ml. (0.022 mole) of methyl iodide in 20 ml. of dry ether under nitrogen. Methyl 4-benzyloxybenzofuran-2-carboxylate (XIII) (0.4 g., 0.0014 mole) in 8 ml. of dry tetrahydrofuran was added dropwise with magnetic stirring at 0°. The reaction mixture was then stirred at room temperature for 15 min. and allowed to stand for 18 hr. The Grignard complex was then decomposed with saturated aqueous solution of ammonium chloride (10 ml.) and the ethereal layer separated. The aqueous layer was extracted with ether (4 × 20 ml.) and the combined ethereal solutions were dried over anhydrous sodium sulfate. Silica gel TLC examination ($CHCl_3$:EtOAc, 1:1 as developing solvent) showed a blue spot under UV light with slightly less mobility than the starting material which was present only in traces. Removal of the ether under reduced pressure provided a yellow oil which could not be induced to crystallize. The IR spectrum (neat) showed the complete disappearance of the C=O band and the appearance of a hydroxyl absorption at 3370 $cm.^{-1}$. In the NMR spectrum the gem-dimethyl protons appeared at 1.62 (s., 6H), the *t*-hydroxyl proton at 2.30 (broad s., 1H), the benzylic protons at 5.15 (s., 2H), and the aromatic protons including the protons of the benzofuran ring appeared between 6.50 and 7.50 δ (m., 9H). The peak at 2.30 δ disappeared completely upon addition of D_2O. This oily compound (XIV) was subjected to hydrogenation without further characterization.

2-(α-Hydroxyisopropyl)-4-hydroxybenzofuran (XV)—The oily compound (XIV) prepared as above was stirred with 0.2 g. of alkali treated 5% palladium-on-carbon (20) in 20 ml. of ethanol in an atmosphere of hydrogen at atmospheric pressure for 24 hr. As the reaction proceeded a spot having less polarity than the starting material appeared on silica gel TLC examination ($CHCl_3$:EtOAc, 1:1 as the developing solvent using UV light for spot detections). At the end of 24 hr. the TLC examination showed very little of the starting material. The catalyst was removed by filtration and washed well with hot and cold ethanol. The filtrate and washings were combined and the solvent removed under reduced pressure. The product was crystallized from ethyl acetate-cyclohexane to yield 0.18 g. (66% of theory in two steps), m.p. 158–161°. The product

was further purified by passing it through a silica gel column 10 g., 1.60 × 20.32 cm. (0.63 × 8 in.) using chloroform as the eluant. An analytical sample was prepared by repeated crystallization, m.p. 163–164°. Compound XV gave a violet coloration with alcoholic ferric chloride solution.

Anal.—Calcd. for $C_{11}H_{12}O_3$: C, 68.73; H, 6.29. Found: C, 68.58; H, 6.51.

2-(α-Hydroxyisopropyl)-4-hydroxy-2,3-dihydrobenzofuran (XVI)—The oily compound (XIV) prepared as above was hydrogenated in a Parr apparatus at 10 p.s.i. pressure in the presence of 0.4 g. of palladium-on-carbon (5%) in 20 ml. of ethanol for 24 hr. At the end of this period the catalyst was removed by filtration and washed well with hot and cold ethanol. The filtrate and washings were combined and the solvent removed under reduced pressure. Silica gel TLC examination ($CHCl_3$:EtOAc, 1:1 as developing solvent) showed two spots when treated with iodine vapors. The spot with the higher R_f value was presumably the corresponding desoxy compound which was eluted out along with other impurities using benzene on a silica gel 10 g., 1.60 × 20.32 cm. (0.63 × 8 in.) column. Elution with a large volume of chloroform, evaporation under reduced pressure and crystallization from ethyl acetate-cyclohexane gave 0.18 g. (65% of theory in two steps) of the product, m.p. 150–152°. An analytical sample was obtained by several recrystallizations, m.p. 153–154°.

Anal.—Calcd. for $C_{11}H_{14}O_3$: C, 68.02; H, 7.27. Found: C, 68.12; H, 7.26.

The same product was obtained from the corresponding benzofuran compound (XV) when 0.1 g. was hydrogenated for 24 hr. in a Parr apparatus at 10 p.s.i. pressure in the presence of the same catalyst (0.1 g.) in 10 ml. of ethanol. The isolation was effected in the same manner to yield 0.055 g. of the product.

(±)-Columbianetin [(±)-I]—2-(α-Hydroxyisopropyl)-4-hydroxy-2,3-dihydrobenzofuran (XVI) (0.2 g., 0.001 mole), zinc chloride (0.2 g., 0.0015 mole, fused before use) and ethyl propiolate (1.0 g., 0.0058 mole) were heated at 90° for 1 hr. under nitrogen (22). After cooling, the yellow mass was treated with dilute hydrochloric acid (4 ml.) and the acid layer extracted exhaustively with chloroform. The chloroformic extract was dried over anhydrous sodium sulfate and the solvent removed under reduced pressure. Silica gel TLC examination ($CHCl_3$:EtOAc, 1:1 as developing agent) showed a fluorescent spot under UV light with the same R_f value as authentic (+)-columbianetin[8] along with several other spots. The oily residue was chromatographed through neutral alumina 20 g., 1.92 × 25.40 cm. (0.75 × 3.5 in.) and eluted with chloroform. The fractions were examined by TLC and those containing (±)-I were mixed together and the solvent removed under reduced pressure. The product, weighing 0.028 g., m.p. 167–168.5° was obtained after three crystallizations from chloroform-*n*-hexane. All the mother liquors were combined, concentrated, and streaked on a preparative silica gel plate and developed with the same solvent system. The zone corresponding to (±)-I was scraped off and eluted with methanol. The solvent was then removed and the product was passed through a short neutral alumina 5 g., 1.27 × 5.08 cm. (0.5 × 2 in.) column using chloroform as the eluant. Removal of the solvent and two crystallizations from chloroform-*n*-hexane provided 0.013 g. of white crystals, m.p. 165.5–168.5°, providing a total yield of 0.041 g. (16% of theory). Several crystallizations gave an analytical sample, m.p. 170–171°.

Anal.—Calcd. for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73. Found: C, 68.24; H, 5.84.

The product had an identical R_f value to that of (+)-columbianetin on a silica TLC sheet using the $CHCl_3$:EtOAc, 1:1 solvent system. It also had an identical UV spectrum with the natural product. The IR spectra in KBr pellets of the two samples showed minor differences which were removed when solution spectra (2.5% in $CHCl_3$) were compared. In the mass spectrum the molecular ion appeared at *m/e* 244. When the mass spectra of the natural I and the synthetic (±)-I were run under identical conditions it was found that the base peaks appeared at *m/e* 59 and all of the major peaks above *m/e* 75 were in excellent agreement insofar as abundance ratios were concerned.

[8] Obtained during the studies of Gupta and Soine (2).

REFERENCES

(1) R. E. Willette and T. O. Soine, *J. Pharm. Sci.*, **53,** 275(1964).
(2) P. K. Gupta, and T. O. Soine, *ibid.*, **53,** 1543(1964).
(3) K. H. Lee and T. O. Soine, *ibid.*, **57,** 865(1968).
(4) M. Shipchandler and T. O. Soine, *ibid.*, **57,** 747(1968).
(5) B. E. Nielsen and J. Lemmich, *Acta Chem. Scand.*, **18,** 1379(1964).
(6) G. K. Nikonov and D. I. Baranauskaite, *Chem. Natl. Prod.*, **1,** 169(1966).
(7) T. O. Soine and K. H. Lee, *J. Pharm. Sci.*, **57,** 655(1967).
(8) B. E. Nielsen and J. Lemmich, *Acta Chem. Scand.*, **18,** 2111(1964).
(9) M. Nakajima, J. Oda, and H. Fukami, *Agr. Biol. Chem. Tokyo*, **27,** 695(1963).
(10) I. Harada, Y. Hirose, and M. Nakazaki, *Tetrahedron Letters*, **1968,** 5463.
(11) R. C. Shah and M. C. Laiwalla, *J. Chem. Soc.*, **1938,** 1828.
(12) D. B. Limaye, *Rasayanam.* **1,** 8(1936); through *Brit. Chem. Abstr.* **A,** ii, 258(1937).
(13) K. Nakazawa, *J. Pharm. Soc. Japan*, **59,** 169(1939).
(14) A. A. Shamshurin, *J. Gen. Chem. USSR*, **14,** 211(1944); through *Chem. Abstr.*, **39,** 2286(1945).
(15) S. M. Parikh and V. N. Thakor, *J. Univ. Bombay*, **23,** 37 (1954); through *Chem. Abstr.*, **49,** 10276(1955).
(16) F. P. Doyle, K. Hardy, J. H. C. Nayler, M. J. Soulal, E. R. Stove, and H. R. J. Waddington, *J. Chem. Soc.*, **1962,** 1453.
(17) K. Tomino, *Yakugaku Zasshi*, **78,** 1425(1958); through *Chem. Abstr.*, **53,** 8018(1959).
(18) L. F. Fieser and M. Fieser, "Reagents for Organic Synthesis," Wiley, New York, N. Y., 1967, p. 637.
(19) J. S. H. Davies, P. A. McCrea, W. L. Norris, and G. R. Ramage, *J. Chem. Soc.*, **1950,** 3206.
(20) R. Mozingo, *Org. Syn.*, **26,** 77(1946).
(21) M. Shipchandler and T. O. Soine, *J. Pharm. Sci.*, **57,** 741 (1968).
(22) K. D. Kaufman and R. C. Kelly, *J. Heterocyclic Chem.*, **2,** 91(1965).
(23) A. K. Ganguly, B. S. Joshi, V. N. Kamat, and A. H. Manmade, *Tetrahedron*, **23,** 4777(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 5, 1969 from the *Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455*

Accepted for publication August 28, 1969.

This work was supported by N.I.H. grant No. HE-7101 from the United States Public Health Service.

* Present address: Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, KS 66044

† To whom all requests for reprints and information should be directed.

‡ Present address: Custom House, Madras-1, India.

Drug Absorption III: Effect of Membrane Storage on the Kinetics of Drug Absorption

J. T. DOLUISIO, W. G. CROUTHAMEL, G. H. TAN, J. V. SWINTOSKY, and L. W. DITTERT

Abstract □ Monoexponential and biexponential disappearance of drug was observed both from the simulated gut phase of a three-phase *in vitro* model for drug absorption and from the lumen of an *in situ* rat gut. Monoexponential disappearance occurred when accumulation of drug in the membrane phase was low or absent, whereas biexponential disappearance occurred when membrane accumulation was appreciable. In all cases, overall transfer of drug to blood was essentially irreversible. Kinetic analysis of biexponential lumen phase data, in terms of a two-compartment open model, was accomplished by applying the technique of "feathering." An analog computer was programmed with the calculated rate constants and the computer generated curves were compared with experimental absorption data from the three phases of the *in vitro* model. Good agreement between the computer curves and the experimental data confirmed the validity of the computational technique and the accuracy of the individual rate constants. An analog computer was also used to simulate the fraction of the dose of drug in the membrane for the *in situ* experiments since the membrane was not available for analysis.

Keyphrases □ Drug absorption—membrane storage effect □ Membrane storage effect—drug absorption kinetics □ Intestinal drug transfer—*in vitro*, *in situ* preparations □ Model, three-phase—drug absorption kinetics □ Analog computer generated curves, experimental data curves—comparison

This paper reports on two kinetic cases of drug transfer commonly observed in an *in vitro* three-phase model for drug absorption (1), and in an *in situ* rat intestinal preparation (2). The objectives of this investigation were (*a*) to correlate kinetic models observed in the *in vitro* system with the kinetics of drug disappearance from the lumen in the *in situ* system; (*b*) to evaluate the role of drug accumulation in a "membrane compartment" on the observed kinetics; and (*c*) to compare various computational methods for estimating the rate constants involved.

Many investigators have attempted to correlate o/w partitioning of drugs in various solvent systems with their gastrointestinal absorption rates. Although rank ordering of a homologous series of drugs in terms of their pH-partition behavior and apparent gastrointestinal absorption rates may be possible, a direct correlation between these two factors is often difficult to demonstrate (3). Possible reasons for the lack of correlation are: (*a*) there is no solvent system that mimics all the physicochemical properties of the absorbing membrane; (*b*) o/w partition coefficients are equilibrium parameters whereas gastrointestinal drug absorption is a dynamic process; and (*c*) no experimental procedure was available which would allow determination of reproducible realistic drug absorption rates in a living system under closely controlled conditions.

Previous reports from these laboratories (1) have dealt with the use of an *in vitro* model for the drug absorption process which employs an artificial lipid-like barrier (organic solvent) separating two aqueous buffered phases representing gut lumen fluid and blood. This technique allows a study of the o/w partition behavior of drugs in a dynamic system which mimics the *in vivo* absorption process and in which all phases can be analyzed so that each rate constant can be evaluated individually. In this model several kinetic mechanisms involving reversible and irreversible transfer among the phases were observed (4). A frequently observed mechanism was one involving reversible transfer of drug from "gut lumen" (Phase A) to "absorbing membrane" (Phase B) followed by irreversible transfer from "absorbing membrane" to "blood" (Phase C); that is, $A \rightleftharpoons B \rightarrow C$. This mechanism fits the expectations of the pH-partition hypothesis because it suggests partitioning of drugs into the membrane. The *in vivo* gut to blood transfer of many drugs may be described by the $A \rightleftharpoons B \rightarrow C$ mechanism because factors such as the volume, pH, and protein-binding capacity of the blood as well as the large tissue distribution and rapid metabolism and excretion of these drugs often tend to make the rate constant for the $C \rightarrow B$ process very small. Consequently, the transport from gut to blood may take on some characteristics of a unidirectional process.

The development of a method for determining drug absorption rates from segments of the gastrointestinal tracts of rats, *in situ*, which yields rates which are closely reproducible from animal to animal and are comparable to those calculated from blood concentration data following oral drug administration to humans and intact animals has also been reported (2). By following the concentration of drug in the lumen of the intestine at closely spaced intervals and analyzing the data using methods for estimating appropriate rate constants in multicompartmental systems when only one phase is available for analysis (5, 6), it is possible to quantitate the kinetic model which describes the absorption of drugs showing biexponential disappearance from the gut lumen.

EXPERIMENTAL

In Vitro Three-Phase Model—*Apparatus and Reagents*—All chemicals were reagent grade unless otherwise specified. A Leeds & Northrup 7401 pH meter, a Beckman model DB spectrophotometer, a Hitachi-Perkin-Elmer spectrophotometer, and an Electronic Associates, Inc. TR-48 analog computer were utilized.

The glass tubes, rocking apparatus, and aqueous buffers used in these studies were described in previous communications (1, 4).

Procedure—The procedure for studying drug transfer kinetics in the *in vitro* three-phase model has been described in previous communications (1, 4).

In Situ Rat Gut—*Apparatus and Reagents*—All chemicals were reagent grade unless otherwise specified. Chlorpromazine hydrochloride,[1] prochlorperazine edisylate,[1] trimeprazine tartrate,[1] trifluoperazine hydrochloride,[1] and haloperidol were used.[2] All solutions were prepared with distilled, deionized, boiled water. A Beckman

[1] Smith Kline & French Laboratories, Philadelphia, Pa.
[2] McNeil Laboratories, Fort Washington, Pa.

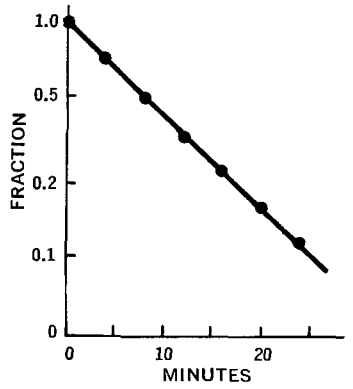


Figure 1—*Semilogarithmic plot of fraction of dose of salicylic acid remaining in rat intestinal lumen,* in situ, versus *time. Dose = 22 mg., half-life = 8 min., lumen solution pH = 6.0.*

Zeromatic II pH meter, a Haake type FBE constant-temperature water bath, and a Cary model 15 spectrophotometer were utilized.

The perfusion and drug solutions used in these studies were described in a previous communication (2).

Test Animals—Male Sprague Dawley albino rats weighing 220–260 g. were fasted 15–20 hr. prior to use.

Procedure—The procedure for studying drug disappearance from the *in situ* rat gut lumen and the analytical methods employed for these drugs have been described in previous communications (2, 7).

RESULTS AND DISCUSSION

Figure 1 illustrates the disappearance of salicylic acid (SA) from the *in situ* rat gut lumen when the pH of the lumen solution is 6.0 (2). The figure shows that the disappearance follows first-order kinetics (monoexponential) for the entire experiment (three half-lives). In another experiment, only trace amounts of salicylic acid could be demonstrated in the lumen solution when 75 mg. of drug was administered intravenously and drug-free buffer was placed in the gut lumen. These results suggest that the kinetics of oral salicylic acid absorption under these conditions may be described in terms of the following model:

$$SA_{lumen} \underset{(\leftarrow)}{\rightarrow} SA_{blood} \qquad \text{(Eq. 1)}$$

that is, overall drug absorption follows apparent irreversible first-order kinetics.

The disappearance of prochlorperazine (PCZ) from the *in situ* rat gut lumen (pH 6.0) is shown in Fig. 2. In this case, the disappearance follows a biexponential profile in contrast to the monoexponential profile observed with salicylic acid. These results suggest that the kinetics of prochlorperazine absorption may be described in terms of the following model:

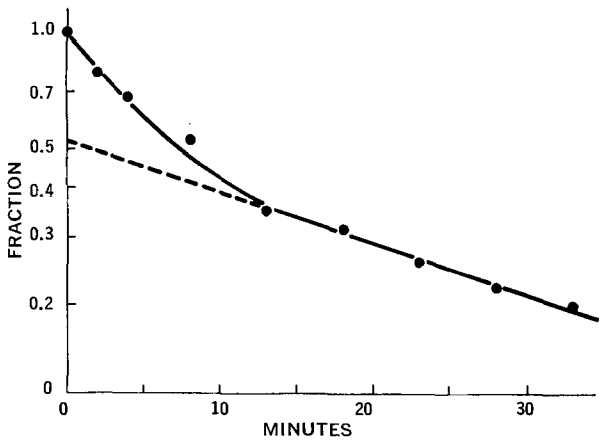


Figure 2—*Semilogarithmic plot of fraction of dose of prochlorperazine hydrochloride in rat intestinal lumen,* in situ, versus *time. Dose = 10 mg., half-life (of straight line) = 23 min., lumen solution pH = 6.0.*

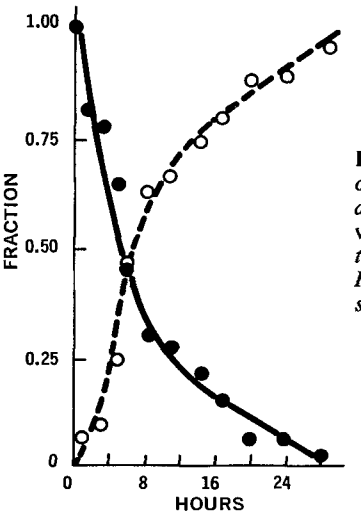


Figure 3—*Plot of fraction of salicylic acid in Phases A and C of a three-phase* in vitro *model for drug absorption. pH: Phase A, 2.0; Phase C, 7.4. Phase B consisted of cyclohexane.*

$$PCZ_{lumen} \underset{(\leftarrow)}{\rightleftharpoons} PCZ_{membrane} \rightarrow PCZ_{blood} \qquad \text{(Eq. 2)}$$

that is, rapid distribution of prochlorperazine occurs between the gut lumen and a compartment representing the membrane followed by slower, essentially irreversible, transfer of the drug from the membrane compartment into the blood. As with salicylic acid, when prochlorperazine was administered intravenously, only a negligible amount of drug could be detected in the gut lumen, confirming that at least one step in the transfer process is essentially irreversible. It can be assumed that the initial transfer was reversible since the disappearance of drug from the gut lumen was not simple first-order (monoexponential).

Current concepts of passive drug absorption suggest that drugs pass through the absorbing membrane after first dissolving or partitioning into it. In the *in vitro* model, drug partitioning into the "Membrane" phase is the only way drug can transfer from Phase A (gut lumen) to Phase C (blood); and the membrane could be considered a discrete compartment in any absorption model. Based on this premise, Eq. 2 is the explicit equation describing the absorption of many passively absorbed drugs. However, if accumulation of drug in the membrane compartment is negligible, Eq. 1 may adequately fit the data. Since both salicylic acid and prochlorperazine are essentially irreversibly transferred from lumen to blood, it would appear that these drugs differ greatly in the degree to which they accumulate in the membrane. This phenomenon can be easily illustrated by means of data obtained in the three-phase *in vitro* model for drug absorption which have previously been described (1, 4). Figure 3 shows plots of the fractions of salicylic acid in Phases A and C of the model system when the simulated gut Phase A is buffered at pH 2.0 and the simulated membrane Phase B is cyclohexane. Figure 4 shows a semilogarithmic plot of the Phase A data, and the plot suggests that the disappearance of salicylic

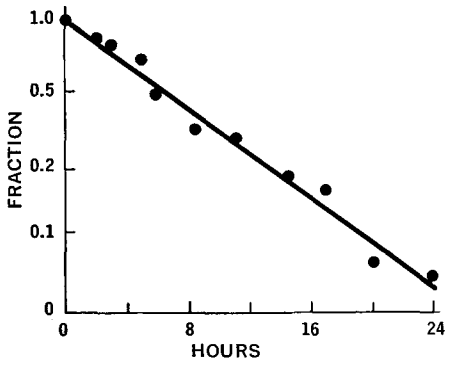


Figure 4—*Semilogarithmic plot of Phase A data from Fig. 3 showing first-order nature of drug transfer.*

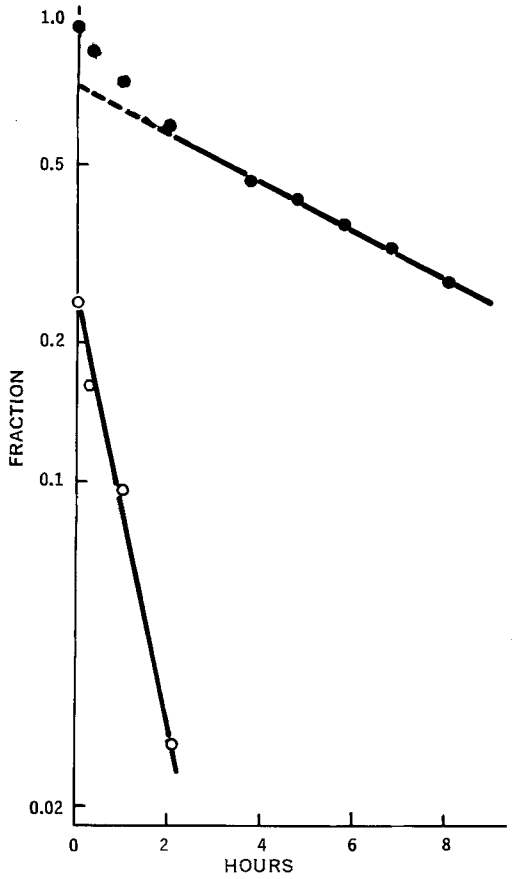


Figure 5—*Semilogarithmic plot of fraction of salicylic acid in Phase A of a three-phase* in vitro *model for drug absorption. The pH of Phase A = 2.0. The pH of Phase C = 7.4. Phase B consisted of 0.1% n-octanol in cyclohexane. Values of the intercepts and slopes were determined graphically: $X_1 = 0.27$; $X_2 = 0.73$; a = 1.08; and b = 0.12. The following values for the rate constants in the model (Eq. 5) were calculated using Eqs. 6–8: $k_{12} = 0.37$; $k_{21} = 0.48$; and $k_{23} = 0.35\ hr.^{-1}$.*

acid from Phase A may be described in terms of an irreversible first-order transfer of drug between Phases A and C:

$$F_A \xrightarrow{b} F_C \qquad \text{(Eq. 3)}$$

where F_A and F_C are the fractions of total drug in the respective phases, and b is the apparent first-order rate constant describing the overall transfer process. A semilogarithmic plot of $(F_C^{\infty} - F_C)$ *versus* time has the same slope as the plot in Fig. 4. Consequently, Eq. 3 can be used to describe this system even though the equation ignores the existence of Phase B. This simplification is justifiable because F_B is extremely low, therefore essentially constant, throughout the experiment. (The concentrations of salicylic acid in cyclohexane were so low that they could not be measured experimentally.) Thus, F_B may be considered to be at steady state; and, under these circumstances, b is a complex function of the rate constants controlling transfer of drug to and from Phase B (4). Because F_B cannot be measured experimentally, and because the overall transfer is monoexponential, the individual rate constants

Table I—Kinetic Data for the Disappearance of Drug from Rat Intestinal Lumen, *In Situ* (Lumen pH = 6.0)

	X_1[a]	X_2[a]	a[a]	b[a]	k_{12}[b]	k_{21}[b]	k_{23}[b]
Prochlorperazine	0.47	0.53	0.20	0.030	0.11	0.065	0.055
Chlorpromazine	0.40	0.60	0.70	0.036	0.30	0.35	0.084
Trifluoperazine	0.41	0.59	0.28	0.026	0.13	0.12	0.056
Trimeperazine	0.18	0.82	0.36	0.031	0.091	0.18	0.12
Haloperidol	0.16	0.84	0.35	0.030	0.082	0.17	0.13

[a] Intercepts (X_1 and X_2 as fraction of dose) and Slopes (a and b in $hr.^{-1}$) were determined as illustrated in Fig. 5 from semilogarithmic plots of fraction of dose in the lumen *versus* time. [b] Rate constants (in $hr.^{-1}$) were calculated by means of Eqs. 6–8.

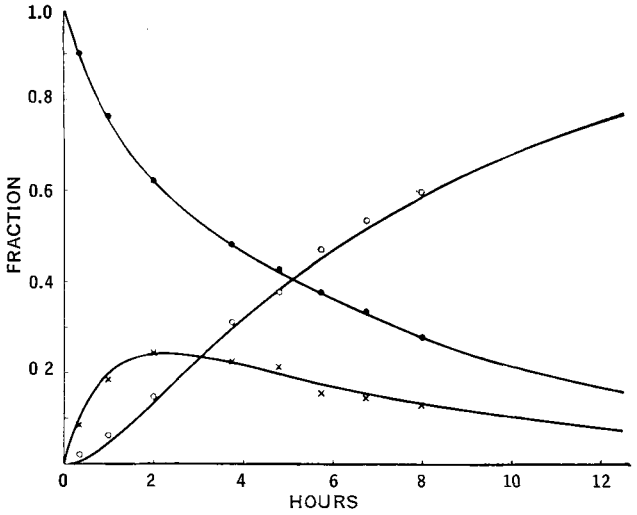


Figure 6—*Plots of the fractions of salicylic acid in Phases A (•), B (×), and C (○) for the experiment shown in Fig. 5. The points were obtained experimentally, and the lines were drawn by an analog computer programmed with Eq. 5 and the following values of the rate constants:* $k_{12} = 0.37$; $k_{21} = 0.50$; *and* $k_{23} = 0.40\ hr.^{-1}$.

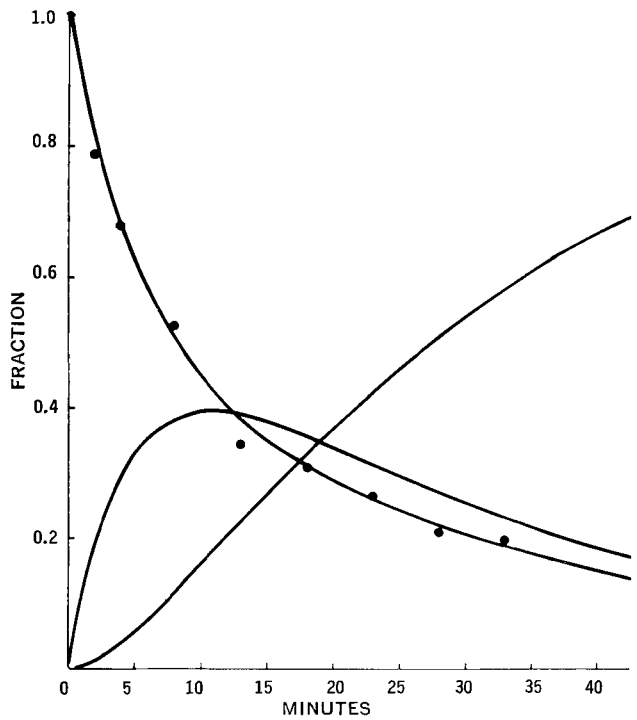


Figure 7—*Plots of the fractions of prochlorperazine hydrochloride in the lumen, the membrane compartment, and the blood compartment of the* in situ *rat intestinal preparation* versus *time. The points were obtained experimentally from the lumen solution (pH = 6.0). Initially, these data were plotted on semilogarithmic paper and values of the intercepts and slopes for the biexponential equation were determined as shown in Fig. 2. Values for the rate constants (Eq. 4) were then calculated using Eqs. 6–8; and these values* ($k_{12} = 0.11$, $k_{21} = 0.065$, $k_{23} = 0.055\ hr.^{-1}$) *were used to program an analog computer which generated the membrane compartment and blood compartment curves.*

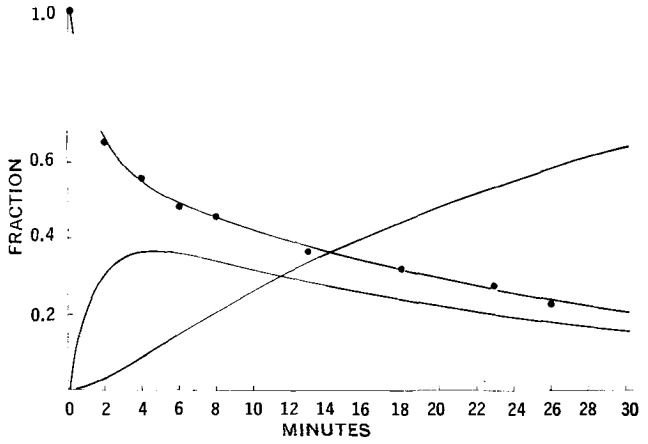


Figure 8—*Plots for chlorpromazine similar to those shown in Fig. 7;* ($k_{12} = 0.30, k_{21} = 0.35, k_{23} = 0.084\ hr.^{-1}$).

cannot be determined easily. It might be concluded that when simple first-order disappearance of drug from the lumen is observed in the *in situ* preparation it can be presumed that membrane accumulation of drug is not great. Inherent in this conclusion is the assumption that transfer of drug from gut lumen to membrane is reversible. If drug transfer from gut to membrane were irreversible, disappearance from the gut lumen would always be monoexponential.

Figure 5 shows a semilogarithmic plot of fraction of salicylic acid in Phase A of the three-phase model when Phase A is buffered at pH 2.0 and Phase B is 0.1% *n*-octanol in cyclohexane. In this case, disappearance of salicylic acid from Phase A follows a biexponential profile, suggesting that there is appreciable accumulation of salicylic acid in Phase B. The presence of significant amounts of drug in Phase B was confirmed experimentally (see Fig. 6). The kinetic model may be depicted as follows:

$$F_A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} F_B \xrightarrow{k_{23}} F_C \qquad \text{(Eq. 4)}$$

Mathematically, F_A is given by the following biexponential equation:

$$F_A = X_1 e^{-at} + X_2 e^{-bt} \qquad \text{(Eq. 5)}$$

Table II—Data Illustrating the Accuracy of the Feathering Technique for Determining Rate Constants from Curves Generated by an Analog Computer

Value	k_{12}	k_{21}	k_{23}
Analog[a]	0.25	1.00	0.20
Feathering[b]	0.25	0.98	0.19
Analog	0.25	1.00	0.080
Feathering	0.23	0.87	0.065
Analog	0.25	0.35	0.050
Feathering	0.25	0.35	0.048

[a] Values used to program an analog computer which generated curves. [b] Values obtained by feathering the computer generated curves.

The values of X_1, X_2, a, and b can be determined graphically from the semilogarithmic plot as shown in Fig. 5; and k_{12}, k_{21}, and k_{23} can be calculated from these values using the following equations (5, 6):

$$k_{12} = \frac{X_1 a + X_2 b}{X_1 + X_2} \qquad \text{(Eq. 6)}$$

$$k_{23} = \frac{ab}{k_{12}} \qquad \text{(Eq. 7)}$$

$$k_{21} = a + b - k_{12} - k_{23} \qquad \text{(Eq. 8)}$$

The results of these calculations are summarized in the legend of Fig. 5.

A distinct advantage of the three-phase model over the *in situ* gut preparation is that all phases are easily accessible for analysis. In cases such as the one under discussion, it is possible to confirm values estimated for the individual rate constants by using them to predict data for Phases B and C and then comparing the predicted data with experimental data for these phases. Such a comparison is shown in Fig. 6 for the salicylic acid data. In this figure, the lines were drawn by an analog computer programmed with Eq. 4, and the points are actual experimental data for the three phases. The values of the computer constants used to generate the lines are shown in the legend of Fig. 6. These values are slightly different from those calculated using Eqs. 6–8 (see Fig. 5) but the agreement is close and the discrepancies can be attributed to experimental error and to error in feathering the semilogarithmic plot (see Fig. 5).

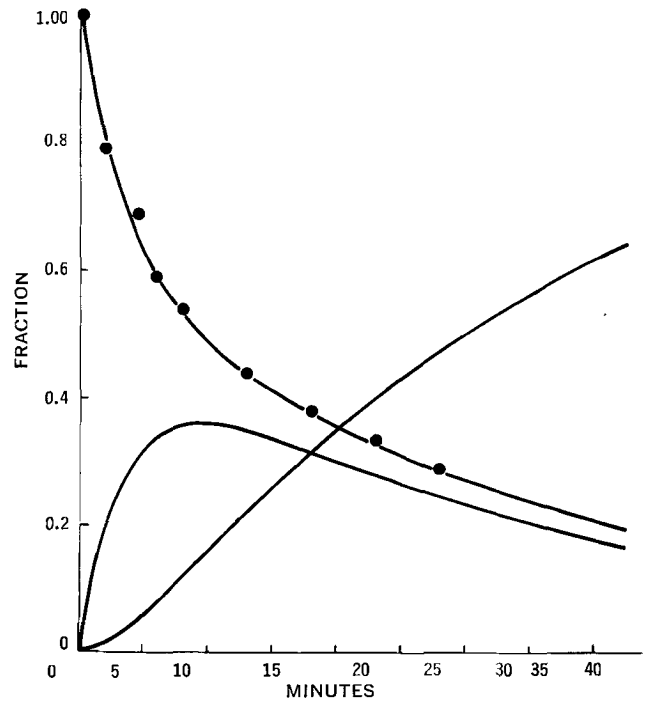


Figure 9—*Plots for trifluoperazine similar to those shown in Fig. 7;* ($k_{12} = 0.13, k_{21} = 0.12, k_{23} = 0.056\ hr.^{-1}$).

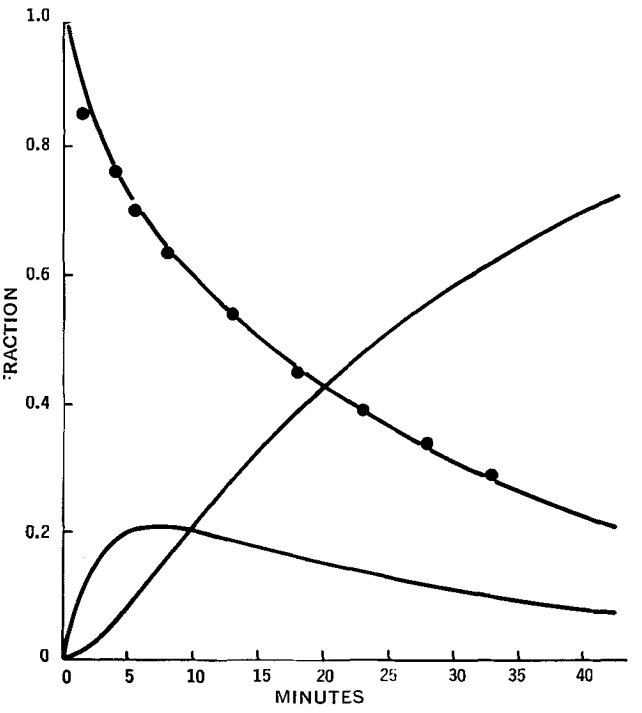


Figure 10—*Plots for trimeperazine similar to those shown in Fig. 7;* ($k_{12} = 0.091, k_{21} = 0.18, k_{23} = 0.12\ hr.^{-1}$).

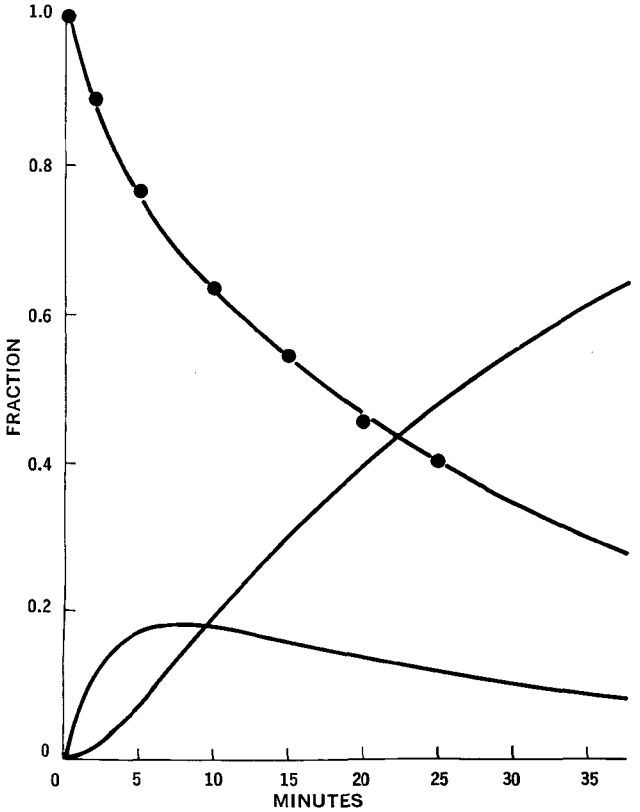


Figure 11—*Plots for haloperidol similar to those shown in Fig. 7;* ($k_{12} = 0.082, k_{21} = 0.17, k_{23} = 0.13\ hr.^{-1}$).

When the absorption of highly lipid soluble drugs such as the phenothiazine or butyrophenone tranquilizers was studied in the *in situ* rat gut preparation, biexponential loss of drug from the lumen solution was always observed. Such experiments were carried out with prochlorperazine, chlorpromazine, trifluoperazine, trimeperazine, and haloperidol. In each case the data were plotted on semilogarithmic paper, and values for X_1, X_2, a, and b (Eq. 5) were determined by feathering as illustrated in Fig. 5. These values along with values for the three rate constants k_{12}, k_{21}, and k_{23} (calculated by means of Eqs. 6–8) are shown in Table I. The three rate constants were used as input to an analog computer programmed with Eq. 4, and curves for each of the three compartments were generated by the computer. The results for the four phenothiazines and haloperidol are shown in Figs. 7–11. The lines are computer generated curves and the points are experimental data from the gut lumen. These figures show that, as might be expected, there is good agreement between the theoretical curve and the experimental data for the gut lumen compartment. Also with these highly lipid soluble drugs, the three-compartment model predicts that the amount of drug in the membrane compartment reaches a maximum of between 20 and 40% of the dose. Thus, there is appreciable membrane storage of these drugs during gastrointestinal absorption.

One might question whether analog computer simulations such as those described truly reflect drug behavior in the inaccessible phases of the model. The accuracy of these simulations depends in turn upon the accuracy of the rate constants calculated by means of Eqs. 6–8. The results of an experiment based on ideal data generated by an analog computer suggest that the accuracy of the rate constants depends directly upon the accuracy of the data obtained in the gut lumen. An analog computer programmed with the three-compartment model and with known values of k_{12}, k_{21}, and k_{23} (see Table II) was used to generate an ideal plot of the fraction of the dose in the "gut lumen" compartment *versus* time. Points were picked off the curve and plotted on semilogarithmic paper. The curve was feathered to obtain X_1, X_2, a, and b and values for k_{12}, k_{21}, and k_{23} were calculated using Eqs. 6–8. The results in Table II show excellent agreement between the calculated and known values of the rate constants suggesting that rate constants calculated from experimental data in this manner and analog simulations based on these rate constants are as accurate, but no more accurate, than the experimental data itself. The *in situ* rat gut preparation yields such precise and reproducible data that the analog simulations in Figs. 7–11 might be expected to truly represent distribution of these drugs among the gut lumen, absorbing membrane, and blood compartments of the experimental animals.

SUMMARY

The disappearance of salicylic acid and certain other drugs (2) from the *in situ* rat gut lumen was found to be monoexponential, whereas certain highly lipid soluble drugs exhibited biexponential disappearance in the same preparation. In each case, the overall transfer of drug from gut lumen to blood was essentially irreversible. Experiments with an *in vitro* three-phase model for drug absorption showed that monoexponential disappearance from the lumen can be expected when accumulation of the drugs in the membrane is negligible. Conversely, biexponential disappearance from the lumen can be expected when accumulation of the drugs in the membrane is appreciable.

By feathering the lumen data which exhibited biexponential drug loss and applying standard mathematical techniques, specific rate constants were determined for drug transfer into and out of the membrane. The validity of these derived rate constants was confirmed by means of an analog computer for data obtained with the three-phase *in vitro* model in which the amounts of drug in all three phases were determined experimentally. The validity of the computational method was confirmed using ideal data generated by an analog computer programmed with known rate constants.

Resolution of the absorption process into its individual components should allow closer and more meaningful study of the factors influencing it. For example, fasting of animals has been reported to slow drug disappearance from the gut lumen (7); and studies are presently being conducted to determine which rate constants are affected by this fasting. Experiments are also being carried out to determine the effect of pH on the individual rate constants, and simultaneous sampling of serum and lumen contents are also being conducted to further elucidate the overall gut to blood transfer process.

REFERENCES

(1) J. T. Doluisio and J. V. Swintosky, *J. Pharm. Sci.*, **53**, 597 (1964).

(2) J. T. Doluisio, N. F. Billups, L. W. Dittert, E. T. Sugita, and J. V. Swintosky, *ibid.*, in press.

(3) C. A. M. Hogben, D. J. Tocco, B. B. Brodie, and L. S. Schanker, *J. Pharmacol. Exptl. Therap.*, **125**, 275(1959).

(4) J. T. Doluisio and J. V. Swintosky, *J. Pharm. Sci.*, **54**, 1594 (1965).

(5) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Waltham, Mass., 1966.

(6) J. S. Robertson, D. C. Tosteson, and J. L. Gamble, Jr., *J. Lab. Clin. Med.*, **49**, 497(1957).

(7) J. T. Doluisio, G. H. Tan, N. F. Billups, and L. Diamond, *J. Pharm. Sci.*, **58**, 1200(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 8, 1969 from the *College of Pharmacy, University of Kentucky, Lexington, KY 40506*

Accepted for Publication September 2, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This research was supported, in part by a grant from McNeil Laboratories, Inc., Fort Washington, PA 19034

Effect of Molecular Interaction on Permeation of Organic Molecules Through Dimethyl Polysiloxane Membrane

MASAHIRO NAKANO* and NAGIN K. PATEL†

Abstract □ In order to investigate the nature of permeation of organic molecules through a nonpolar membrane in the presence of other molecular species, some model studies have been made with *p*-nitrophenol as an ionizable diffusate and *N,N*-dialkylamides and alkylxanthines as complexing agents. Effect of diethylpropionamide on permeation of salicylic acid was also examined. 8-Methoxycaffeine and caffeine were used as model compounds for nonionizable molecules and permeability constants were determined with and without complexing agents. The permeability of a diffusate increased in the presence of complexing agents which interact strongly with the diffusate in nonpolar environments, while it decreased in the case of the agents which complex with the diffusate mainly in aqueous solution.

Keyphrases □ Molecules, organic—dimethyl polysiloxane membrane permeation □ Membrane permeation, organic molecules—molecular interaction effect □ Ionizable diffusates-complexing agents—membrane permeation □ Colorimetric analysis—spectrophotometer □ UV spectrophotometry—analysis □ GLC—analysis

Extensive studies by Garrett and Chemburkar on diffusion of drug molecules through a silicone rubber membrane (1–3) have contributed much to knowledge on drug diffusion through a polymeric membrane. Because of the nonpolar nature of silicone rubber materials (4), a dimethyl polysiloxane membrane would be useful for investigation of effects of molecular interaction on drug permeation through a membrane. The first objective of the present investigation was to examine the nature of permeation of molecules through a nonpolar membrane in the presence of some other molecular species. This would provide additional information concerning the effect of complex formation on drug absorption which has been studied by Levy and his associates (5, 6).

The second objective was to explore some possibilities of controlling rate of permeation of organic molecules through a membrane by physicochemical means. In view of possible application of silicone rubber materials to some dosage forms (7, 8), it would be worthwhile to examine how the rate of permeation of molecules through a membrane can be modified.

EXPERIMENTAL

Materials—Dimethyl polysiloxane[1] sheeting in a labeled thickness of 5 mil was used throughout this study. *p*-Nitrophenol[2], salicylic acid[3], 8-methoxycaffeine[4] (recrystallized from methanol-carbon tetrachloride), and caffeine[5] (recrystallized from water) were used as model diffusates. Other compounds employed as complexing agents are also commercially available chemicals and were purified whenever necessary (9).

Diffusion Studies—The quasi steady-state diffusion cell described by Garrett and Chemburkar (1) was used with the following modifications. One polytetrafluoroethylene[6] O-ring was used instead of two silicone rubber gaskets and the diameter of the area available for diffusion was 32 mm. The cell was initially equilibrated overnight in a shaker bath maintained at 30° with 100 ml. of distilled water in both arms. Subsequently water was removed by suction and 50 ml. of 0.005 *N* sodium hydroxide (for *p*-nitrophenol and salicylic acid) or water (for 8-methoxycaffeine and caffeine) was added to one arm and an equal volume of test solution was placed in the other arm.[7] All the solutions were prewarmed to 30°. In order to suppress the dissociation of *p*-nitrophenol and salicylic acid, their solutions were prepared in 0.001 *N* and 0.004 *N* hydrochloric acid, respectively. The cell was mechanically shaken horizontally at a rate of 148 ± 2 strokes/min.

Analytical Methods—*p-Nitrophenol*—A 0.5-ml. aliquot of a desorbing solution was pipeted into a 10-ml. volumetric flask, then 5 ml. of 0.01 *N* sodium hydroxide was added to it, and the mixture was made up to volume with water. The absorbance of this solution was recorded on a spectrophotometer[8] at the wavelength of maximum absorbance, 400 mμ. No complexing agent was found to interfere with the assay at this wavelength.

Salicylic Acid—A 0.5-ml. aliquot from the desorbing solution was acidified with 2 ml. of 0.1 *N* hydrochloric acid to suppress the dissociation and made up to volume (10 ml.) with water. The concentration of the resultant solution was spectrophotometrically determined at 302 mμ. Diethylpropionamide did not interfere with the assay.

8-Methoxycaffeine—A 1-ml aliquot was pipeted out of both desorbing and diffusing solutions. When methyl nicotinate was used as a complexing agent, absorbance at both 262 and 281 mμ was recorded and the content of each component was calculated by the standard method of simultaneous spectrophotometric analysis (10). When tryptophan was used as a complexing agent, 8-methoxycaffeine was extracted into carbon tetrachloride. A 1-ml. portion of carbon tetrachloride solution was then appropriately diluted with methanol and the xanthine was spectrophotometrically determined at 281 mμ. In the presence of phenols and *p*-hydroxybenzoic acid, the sample solutions were made alkaline with sodium hydroxide (0.005 *N* with respect to the final concentration) and 8-methoxycaffeine was then extracted into carbon tetrachloride.

Caffeine—A 1-ml. aliquot was pipeted out of both desorbing and diffusing solutions. The concentration of caffeine in the presence of salicylamide was determined in the following way. The sample solution was made alkaline with sodium hydroxide (0.005 *N* with respect to the final concentration) and the concentrations of salicylamide were determined at 328 mμ. The concentration of caffeine was then calculated from absorbance at 272 mμ and correction was made for absorbance due to salicylamide at the same wavelength. The concentration of caffeine in the presence of *p*-bromophenol was determined in the following way. To a 1-ml. aliquot was added 9 ml. of 0.1 *N* sodium hydroxide and the resultant solution was shaken with 2 ml. of chloroform. One milliliter of the organic layer was pipeted out and absorbance at 272 mμ was measured after appropriate dilution with methanol.

Determination of Partition Coefficient—Five-milliliter portions of sample solutions were shaken with a 5-ml. portion of carbon

[1] Silastic, Medical Products Div., Dow Corning Corp., Midland, Mich.
[2] Fisher reagent grade, Chemical Manufacturing Div., Fisher Scientific Co., Fair Lane, N. J.
[3] Baker & Adamson reagent, General Chemical Div., Allied Chemical, New York, N. Y.
[4] Eastman Organic Chemicals, Div. of Eastman Kodak Co., Rochester, N. Y.
[5] British Drug Houses, Toronto, Canada.
[6] Teflon, E. I. du Pont de Nemours & Co., Wilmington, Del.
[7] In this paper a solution containing diffusate at time zero is referred to as a diffusing solution while the other side of the membrane is designated as a desorbing solution.
[8] Beckman Model DB, Beckman Instruments, Fullerton, Calif.

tetrachloride by means of an aliquot shaker[9] in a low temperature incubator[10] (25°) for about 2 hr. The concentrations of aromatic compounds remaining in the water layer were determined as described in the *Analytical Methods*. The concentrations in carbon tetrachloride were assumed to be equal to the difference between the initial concentration in water and the concentration in water after extraction. The concentrations of aliphatic molecules remaining in the water layer were determined by GLC. A gas chromatograph[11] equipped with a dual flame ionization detector and a 0–10 mv. recorder[12] was employed. The chromatographic column was a 0.31-cm. (0.125-in.) o.d. stainless steel column, 1.83 m. (6 ft.) in length and packed with 10% silicone elastomer[13] on a diatomite[14] (80–100 mesh). The column was silanized *in situ* with hexamethyldisilazane and equilibrated overnight at the operating conditions; oven temperature 140°; injection port temperature, 301°; detector temperature, 283°; hydrogen pressure 15 lb./sq. in.; air pressure, 25 lb./sq. in., helium pressure, 40 lb./sq. in. (80 ml./min.). Either dimethylacetamide or diethylpropionamide was used as an internal standard.

RESULTS AND DISCUSSION

Ionizable Diffusates—*p*-Nitrophenol and salicylic acid, with pKa values (11) of 7.1 and 3.0 were selected as model ionizable compounds. The concentration of diffusible species in the desorbing solution can be kept to a zero value by maintaining diffused molecules in a dissociated form. During the first 4 to 5-hr. period of the permeation experiment, the drug concentration of the diffusing solution is not altered significantly; and it may be assumed that the concentration of a diffusate during this period is equal to its initial concentration, C_0, in the diffusing solution. For such a system the following equation modified from that derived by Garrett and Chemburkar (2) for a steady-state diffusion can be employed

$$C_2 = k_p \frac{AC_0t}{V_2} \qquad \text{(Eq. 1)}$$

where C_2 = concentration of diffusate in desorbing solution, A = area of the membrane, V_2 = volume of the desorbing solution, and k_p = permeability constant, which is the product of diffusivity of the drug in the membrane and its membrane/water partition coefficient divided by thickness of the membrane. Plots of C_2 *versus* t will give a straight line with slope = $k_p \times AC_0/V_2$ from which k_p can be computed.

Effect of Complexing Agents on Permeation of p-Nitrophenol—A typical set of data for the permeation of *p*-nitrophenol in the presence and absence of complexing agents are plotted in Fig. 1 in accordance with Eq. 1. Depending on the nature of the complexing agent, two opposing effects on the rate of permeation were observed. Accordingly, the rate of permeation increased in the presence of diethylpropionamide while it decreased in the presence of caffeine. In order to explore the observed effects, the rate of permeation of *p*-nitrophenol was determined in the presence of a number of complexing agents. The data for this study were plotted in the same way as illustrated in Fig. 1. All the results gave linear relationships of C_2 *versus* t. The apparent permeability constants, k_p, were computed from the slopes of the lines and they are summarized in Table I. If one compares the effect of amides on the k_p values, it can be observed from the table that effect on the permeation varies among the *N,N*-dialkylamides and that it is concentration dependent, k_p value increasing with increase in concentration of diethylpropionamide.

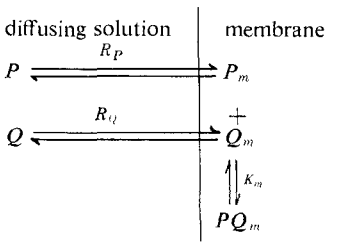
In order to explain the difference in the drug permeability in the presence of the amides, apparent partition coefficients (CCl_4/H_2O) of the amides as well as of *p*-nitrophenol in the presence of the amides were determined and these results are summarized in the third and fifth columns of Table I. In view of approximately the same hydrogen bonding ability of the amides with *p*-nitrophenol in a nonpolar solvent (12), the observed difference in the apparent partition coefficients of *p*-nitrophenol can be related to the difference

Table I—Apparent Permeability Constants, k_p, and Apparent Partition Coefficients, R, of *p*-Nitrophenol in the Presence of Complexing Agents

Complexing Agent	Concn., m*M*	R[a]	*p*-Nitrophenol— $k_p \times 10^2$ cm./hr.	*p*-Nitrophenol— $R_{app.}$[b]
None	—	—	6.55	0.086
Dimethylacetamide	40	0.00	6.55	0.18
Dimethylpropionamide	40	0.15	7.27	0.73
Diethylacetamide	40	0.35	8.45	1.9
Diethylpropionamide	40	2.1	12.8	4.2
	20		9.98	
	10		8.51	
Dioxane	40	0.74	6.52	0.13
n-Amyl alcohol	40	2.3	7.63	0.20
Dimethyl sulfoxide	40	0.031	6.55	0.086
Theophylline	10	0.002	5.74	0.086
Caffeine	10	0.21	5.59	0.097
8-Methoxycaffeine	10	5.5	5.48	0.26

[a] CCl_4/H_2O at 25°. [b] CCl_4/0.001 *N* HCl at 25°.

in the partition coefficients of the amides themselves. This point is elaborated by the following scheme and equation:



$$R_{app.P} = \frac{P_m + PQ_m}{P} = \frac{P_m(1 + K_mQ_m)}{P} = R_P(1 + K_mQ_m) \qquad \text{(Eq. 2)}$$

where P and Q represent equilibrium concentrations of *p*-nitrophenol and an amide, respectively. Rx is a partition coefficient of species X, K is a stability constant, and the subscript m stands for "in the membrane." According to Eq. 2, the apparent partition

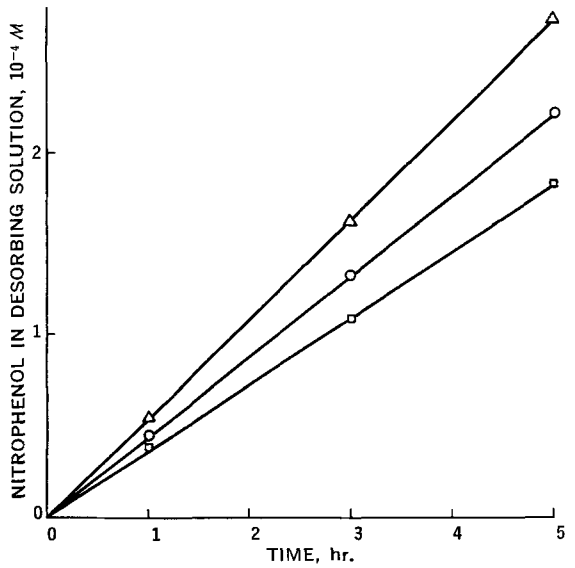


Figure 1—*Permeation of* p*-nitrophenol in the presence of 10 m*M *caffeine* (□) *or 10 m*M *diethylpropionamide* (△) *and in the absence of complexing agent* (○) *at 30°. The diffusing solution was 50 ml. of 4 m*M p*-nitrophenol in 0.001* N *hydrochloric acid with or without complexing agent and the desorbing solution was 50 ml. of 0.005* N *sodium hydroxide solution.*

[9] Lab-Tek, Ames Lab-Tek, Inc., Westmont, Ill.
[10] Model 82, Fisher Scientific Co.
[11] F & M Model 700, F & M Scientific Div., Hewlett Packard, Palo Alto, Calif.
[12] Speedomax W, Leeds & Northrup Co., Philadelphia, Pa.
[13] W98, F & M Scientific Div., Hewlett Packard, Palo Alto, Calif.
[14] Diatoport S, F & M Scientific Div., Hewlett Packard, Palo Alto, Calif.

Table II—Effect of Diethylpropionamide (DEP) on Permeability Constant, k_p, of *p*-Nitrophenol

Location of DEP	$k_p \times 10^2$, cm./hr.
(Absent)	6.55
Diffusing solution[a]	12.8
Desorbing solution[a]	7.37
Both diffusing and desorbing solutions[a]	13.8
Membrane[b]	6.91

[a] 40 m*M* DEP. [b] The membrane was presoaked with 50 ml. each of 100 m*M* DEP in both arms for 16 hr.

Table IV—Apparent Permeability Constants, k_p, and Apparent Partition Coefficients, *R*, of Caffeine and Salicylamide

Diffusate[a]	Complexing Agent	$k_p \times 10^2$, cm./hr.	R^c
Caffeine	None	0.216	0.21
Caffeine	*p*-Bromophenol[b]	0.228	0.72
Caffeine	Salicylamide[a]	0.148	0.13
Salicylamide	None	2.57	0.14
Salicylamide	Caffeine[a]	2.17	0.098

[a] 16 m*M*. [b] 32 m*M*. [c] CCl_4/H_2O at 25°.

coefficient of the phenol depends on the values of R_P, K_m, and Q_m. Since the partition coefficient of uncomplexed phenol, R_P, is constant, and the stability constants of hydrogen bonded complex, K_m, are about the same for the *N,N*-dialkylamides (12), the apparent partition coefficient is primarily a function of the amide concentration in the membrane. Thus amides with greater partition coefficients gave greater apparent partition coefficients of *p*-nitrophenol as seen in Table I.

In order to evaluate the role of diethylpropionamide in the transfer of the phenol across the membrane, diethylpropionamide was placed unilaterally in the diffusing solution, in the desorbing solution, and in the membrane; and bilaterally in both the diffusing and desorbing solutions. These data, as given in Table II, showed that the amide substantially increased the permeability constant of the phenol only when it was placed in the diffusing solution. From these results it is highly improbable that the amide merely changes the permeation characteristic of the membrane (diffusivity of a diffusate in the membrane). The increased permeation of *p*-nitrophenol may be attributed to the increase in concentration of *p*-nitrophenol in the membrane at its interface with diffusing solution or in other words, to the increase in apparent partition coefficient. The greater apparent partition coefficient of *p*-nitrophenol in the presence of diethylpropionamide may be rationalized by the greater partition coefficient of the amide itself and the presence of hydrogen bonding interaction in the nonpolar medium between the phenol and the amide (13). Dimethylacetamide, on the other hand, though it is as good a proton acceptor as diethylpropionamide, does not partition into a nonpolar solvent, and consequently has insignificant effect on the partition coefficient of the *p*-nitrophenol, thus supporting the importance of partitioning prior to diffusion through the membrane.

A possible alternative mechanism includes faster diffusion of the *p*-nitrophenol-amide complex through the membrane than uncomplexed *p*-nitrophenol. This is highly unlikely since the stability constant of such a complex in a bulk aqueous solution was found to be very small (12).

The results showing the influence of dioxane, *n*-amyl alcohol, dimethyl sulfoxide, and xanthines on rate of permeation of *p*-nitrophenol are also included in Table I. Although dioxane and *n*-amyl alcohol partiton into the nonpolar solvent to an appreciable extent, they failed to modify the permeability constant of *p*-nitrophenol in any substantial way. This is in accord with the small increase in partition coefficients of *p*-nitrophenol in the presence of these molecules. The weak proton acceptor property of ethers and alcohols (14) may explain the present observation; namely, K_m in Eq. 2 is so small that $R_{app.P}$ does not differ significantly from R_P. Dimethyl sulfoxide, on the other hand, is reported to be a strong proton acceptor (15). The failure of the sulfoxide to modify the permeability can be attributed to its small partition coefficient. Thus a large partition coefficient, together with the strong hydrogen acceptor property of the complexing agent appear to be the factors responsible for accelerated permeation of *p*-nitrophenol.

Table III—Apparent Permeability Constants, k_p, and Apparent Partition Coefficients, *R*, of 8-Methoxycaffeine in the Presence of Complexing Agents

Complexing Agent	Concn., m*M*	8-Methoxycaffeine: *R*[a]	8-Methoxycaffeine: $k_p \times 10^2$, cm./hr.	8-Methoxycaffeine: $R_{app.}$[a]
None	—	—	3.73	5.4
p-Chlorophenol	60	2.1	4.11	49
p-Nitrophenol	10	0.097	3.51	5.2
Methyl nicotinate	60	5.3	2.17	5.2
Tryptophan	24	0.0	2.29	2.7
p-Hydroxybenzoic acid	30	0.018	2.02	1.8

[a] CCl_4/H_2O at 25°.

Alkylxanthines in general reduced the apparent permeability coefficients of *p*-nitrophenol. *p*-Nitrophenol interacts with alkylxanthines both in aqueous and nonpolar solutions (12). In the case of theophylline with a very minor partitioning into the nonpolar solvent, its complexation with *p*-nitrophenol in aqueous solution might be the sole cause of the reduction in the permeability. The retarding effect of 8-methoxycaffeine was not found to be as pronounced as expected from its great complexing tendency (16) since the xanthine partitions into a nonpolar solvent to an appreciable extent.

In spite of an increase in the apparent partition constant of *p*-nitrophenol in the presence of alkylxanthines, its permeability decreased. For example, the permeability constants of caffeine and 8-methoxycaffeine are 0.216×10^{-2} cm./hr. and 3.73×10^{-2} cm./hr., while their partition coefficients are relatively large, namely, 0.21 and 5.4 (see Tables III and IV). This discrepancy may have resulted from the fairly small diffusivity of alkylxanthines. It should be noted here that permeation is a kinetic phenomenon whereas partition coefficients are equilibrium constants.

Effect of Diethylpropionamide on Permeation of Salicylic Acid—The permeation behavior of salicylic acid in the presence and absence of diethylpropionamide is depicted in Fig. 2. The partition coefficients of salicylic acid at a 4 m*M* concentration in the absence and presence of the amide (40 m*M*) are 0.59 and 5.7, respectively. Thus, the increased permeation of salicylic acid in the presence of diethylpropionamide may be attributed to its increase in the partitioning property.

Nonionizable Diffusates—8-Methoxycaffeine and caffeine were chosen as model nonionizable molecules. For these drugs the following equation developed by Garrett and Chemburkar for quasi steady-state diffusion is applicable.

$$\log \frac{C_0}{C_1 - C_2} = \frac{0.869 k_p A}{V_2} t \qquad \text{(Eq. 3)}$$

wherein C_1 is the concentration of diffusate in a diffusing solution and all other terms are as previously defined in Eq. 1. A plot of log $C_0/(C_1 - C_2)$ *versus* t will yield a straight line and the permeability constant can be calculated from the slope of the line.

Effect of Complexing Agents on Permeation of 8-Methoxycaffeine—The permeation data for 8-methoxycaffeine in the presence and absence of tryptophan or *p*-chlorophenol are plotted in Fig. 3. The apparent permeability constants obtained from these plots and from similar plots with other complexing agents are compiled in Table III together with pertinent partitioning data. In the case of phenols, *p*-nitrophenol reduced the permeability constant of 8-methoxycaffeine, while *p*-chlorophenol increased it. Since both of these phenols interact with the xanthine in aqueous as well as in non-

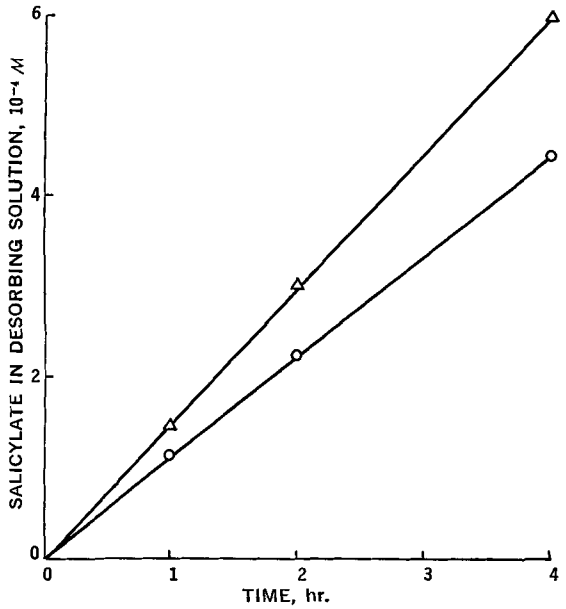


Figure 2—*Permeation of salicylic acid in the presence* (△) *and absence* (○) *of diethylpropionamide at 30°. The diffusing solution was 50 ml. of 4 mM salicylic acid in 0.004* N *hydrochloric acid solution with or without 40 mM diethylpropionamide and the desorbing solution was 50 ml. of 0.005* N *sodium hydroxide solution.*

polar environment (12), two counteracting effects may take place for the present systems. Complexation in aqueous solution would reduce the permeability constant of 8-methoxycaffeine while interaction at the membrane interface with the diffusing solution would increase it. With *p*-nitrophenol, whose partition coefficient is small, complexation with 8-methoxycaffeine in aqueous solution is a dominant factor, while for *p*-chlorophenol, with a large partition coefficient, hydrogen bonding interaction at the interface outweighs the complexation effect in an aqueous solution, resulting in a net increase in the permeabiliy constant.

Methyl nicotinate (Table III) was observed to lower the permeation of 8-methoxycaffeine. Although methyl nicotinate has a large partition coefficient, it has no hydrogen bonding affinity for the xanthine in a nonpolar environment (12). Therefore complexation in the aqueous solution may account for the reduction in the

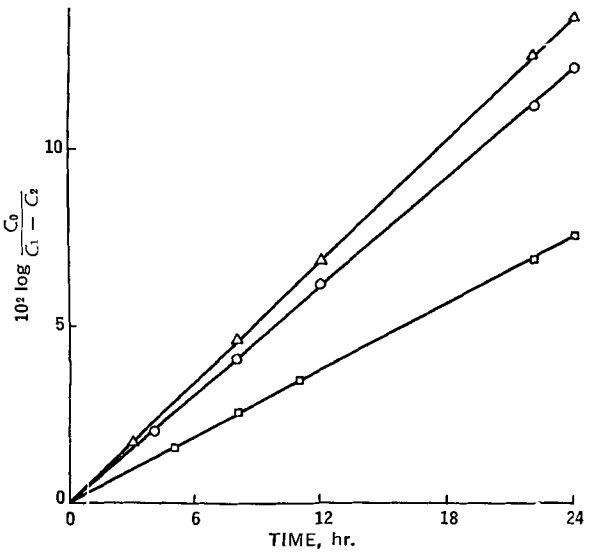


Figure 3—*Permeation of 8-methoxycaffeine (4 mM) in the presence of 24 mM tryptophan* (□) *or 60 mM p-chlorophenol* (△) *and in the absence of complexing agent* (○) *at 30°.*

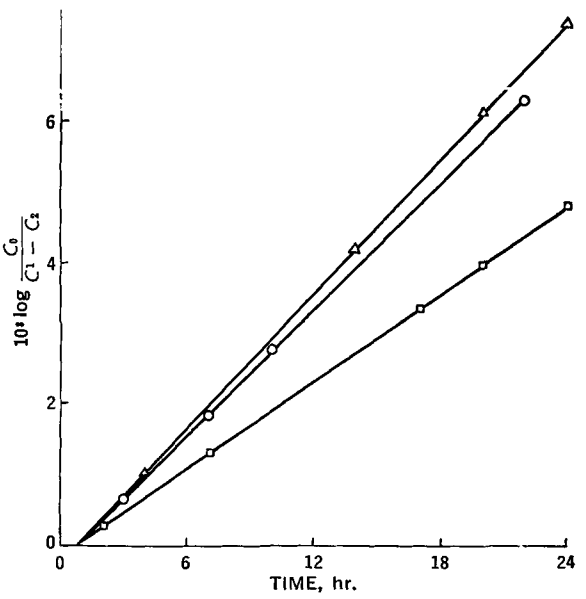


Figure 4—*Permeation of caffeine (16 mM) in the presence of 16 mM salicylamide* (□) *or 32 mM p-bromophenol* (△) *and in the absence of complexing agent* (○) *at 30°.*

permeation of the xanthine. The apparent partition coefficients of 8-methoxycaffeine are reduced due to the presence of tryptophan and *p*-hydroxybenzoic acid. These complexing agents are not partitioned into the nonpolar solvent as shown in Table III. Thus the decrease of the permeability constants of 8-methoxycaffeine by tryptophan and *p*-hydroxybenzoic acid can be inferred as being due to the complexation in the aqueous solution.

Effect of Salicylamide and p-Bromophenol on Permeation of Caffeine—Data for the permeation of caffeine with and without complexing agents are presented in Fig. 4. The permeation and partitioning results for caffeine and salicylamide are given in Table IV. It should be noted in Fig. 4 that the permeation rate plots yielded an intercept on the abscissa, indicating an apparent time lag. Comparing the k_p value of caffeine with those of other drugs this would be expected, since caffeine gave the lowest value. This figure also illustrates that the rate of permeation of caffeine was accelerated by *p*-bromophenol and it was decelerated by salicylamide. The results may be rationalized in the same way as for 8-methoxycaffeine. For example, with salicylamide whose partition coefficient is small (R = 0.14), complexation in an aqueous solution may be considered to have a dominant effect, while with *p*-bromophenol whose partition coefficient is large (R = 3.14), interaction at the interface would outweigh the complexation in an aqueous solution. The permeability constant of salicylamide was also found to decrease in the presence of caffeine. These observations can be substantiated by the measured values of partition coefficient of the diffusate in the presence of the complexing agents. Experimentally obtained values are summarized in Table IV.

CONCLUSIONS

This study illustrates that the rate of permeation of a drug can be increased or decreased in the presence of a complexing agent, and the magnitude of this effect is dependent upon the physicochemical nature of the agent. From the results it may be concluded that the agents which complex with a diffusate (*a*) only in aqueous solution would reduce the permeability constant; (*b*) both in aqueous and nonpolar environments would either decrease or increase it depending upon the partitioning behavior of the complexing agents and the relative stability constants of the complex in both environments; and (*c*) only in nonpolar environment would increase it.

If a drug in question is an acidic molecule, a complexing agent of an aliphatic proton acceptor type, with a fairly large partition coefficient and favorable toxicological characteristics, would be a candidate for an agent which may be used to enhance the permeation of the drug across biological membranes.

REFERENCES

(1) E. R. Garrett and P. B. Chemburkar, *J. Pharm. Sci.*, **57**, 944(1968).

(2) *Ibid.*, **57**, 949(1968).

(3) *Ibid.*, **57**, 1401(1968).

(4) R. West, *Proc. Intern. Symp. Mol. Struct. Spectry., Tokyo*, **1962**, through *Chem. Abstr.*, **61**, 4193c(1964).

(5) G. Levy and E. J. Mroszczak, *J. Pharm. Sci.*, **57**, 235 (1968).

(6) R. H. Reuning and G. Levy, *ibid.*, **58**, 79(1969); and earlier works cited therein.

(7) D. M. Long, Jr. and M. J. Folkman, U. S. pat. 3,279,996 (1966).

(8) K. Sundaram and F. A. Kincl, *Steroids*, **12**, 517(1968).

(9) D. D. Perrin, W. L. F. Armarego, and D. R. Perrin, "Purification of Laboratory Chemicals," Pergamon Press, Oxford, England, 1966.

(10) H. H. Willard, L. L. Merritt, Jr., and J. A. Dean, "Instrumental Method of Analysis," 4th ed., D. Van Nostrand, Princeton, N. J., 1965, pp. 94–95.

(11) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen & Co., London, England, 1962, pp. 130, 134.

(12) M. Nakano, unpublished work.

(13) M. Nakano, N. I. Nakano, and T. Higuchi, *J. Phys. Chem.*, **71**, 3954(1967).

(14) T. Higuchi and S. Chulkaratana, cited in T. Higuchi and and K. A. Connors, *Advan. Anal. Chem. Instr.*, **4**, 117, (1965).

(15) R. S. Drago, B. Wayland, and R. L. Carlson, *J. Am. Chem. Soc.*, **85**, 3125(1963).

(16) K. Kakemi, H. Sezaki, T. Mitsunaga, and M. Nakano, *J. Pharm. Sci.*, in press.

ACKNOWLEDGMENTS AND ADDRESSES

Received May 20, 1969 from *Faculty of Pharmacy and Pharmaceutical Sciences, The University of Alberta, Edmonton, Alberta, Canada.*

Accepted for publication September 2, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported by the University of Alberta Postdoctoral Fellowship (to M.N.) and by a grant from Smith Kline & French Inter-American Corporation (Canada).

* Present address: Pharmaceutical Chemistry Division, Food and Drug Directorate, Ottawa 3, Ontario, Canada.

† Present address: Frank W. Horner Ltd. Research Laboratories, Montreal 307, Quebec, Canada. To whom reprint requests should be addressed.

Factors Affecting the Synthesis of Penicillinase by Staphylococcus Aureus

R. F. BOYD and JOSEPH JUDIS

Abstract □ The effects of aeration, ferric ions, porphyrins, and disodium versenate were studied on the production of penicillinase (ps) by *Staphylococcus aureus* 55-C-1 in synthetic medium. The production of ps was twice as high in shaken compared to static cultures although growth ultimately was the same. Ferric ions in a concentration of 2.5×10^{-4} *M* had some stimulatory effect on ps production in static cultures but caused much greater ps stimulation in shaken cultures. This stimulatory effect could be demonstrated only if ferric ions were added prior to 3 hr. of incubation. Hemin in a concentration of 2.5×10^{-5} *M* depressed ps production in the presence of ferric ions but one-tenth the concentration of hemin did not. Hematin in the same concentration was without effect. Protoporphyrin in a concentration of 2.5×10^{-5} *M* inhibited growth in the absence of ferric ions and depressed ps production in the presence of ferric ions. One-tenth the concentration of protoporphyrin had no effect on growth but depressed ps production and only slightly depressed ps production in the presence of ferric ions. A concentration of 2.5×10^{-6} *M* hematoporphyrin inhibited ps production in the presence of ferric ions when the former was added early in the growth curve. Disodium versenate in a concentration of 1.32×10^{-5} *M* in the presence of ferric ions permitted good growth but no ps production.

Keyphrases □ Penicillinase production—*Staphylococcus aureus* □ Aeration, shaking effect—penicillinase production □ Porphyrins, ferric ions, disodium versenate, effect—penicillinase production □ Optical density—*S. aureus* growth determination □ Manometric determination—penicillinase

Abraham and Chain (1) were the first to show that certain bacteria produce an enzyme capable of hydrolyzing penicillin. Since the latter paper was published in 1940, a number of workers have demonstrated that the resistance of strains of *Staphylococcus aureus* to penicillin was related to the production of penicillinase, the enzyme which was shown to hydrolyze penicillin. Although in some studies penicillin resistance was demonstrable in the absence of penicillinase production (2, 3), there seems to be good correlation between the ability of staphylococci to produce penicillinase and to be resistant to the action of penicillin both *in vitro* and *in vivo* (4–6). Penicillinase production has been similarly implicated in penicillin resistance of *Bacteroides* sp. (7).

In some microorganisms, penicillinase has been found to be an inducible enzyme and many studies (8–12) have concentrated on the nature of the induction of penicillinase elaboration. Some strains of *S. aureus* produced penicillinase constitutively (13, 14) and a number of workers have been concerned with the conditions and factors affecting constitutive production of penicillinase. It was felt that if the factors which control or affect penicillinase production in a strain of *S. aureus* producing penicillinase constitutively could be identified, it was possible that this knowledge could be applied ultimately in the treatment of an infection caused by a penicillinase-producing strain of *S. aureus*. That is, a strain which otherwise would be resistant to penicillin could be made susceptible to the action of penicillin if penicillinase production could be inhibited by the concurrent administration of a nontoxic

REFERENCES

(1) E. R. Garrett and P. B. Chemburkar, *J. Pharm. Sci.*, **57**, 944(1968).

(2) *Ibid.*, **57**, 949(1968).

(3) *Ibid.*, **57**, 1401(1968).

(4) R. West, *Proc. Intern. Symp. Mol. Struct. Spectry., Tokyo*, **1962**, through *Chem. Abstr.*, **61**, 4193c(1964).

(5) G. Levy and E. J. Mroszczak, *J. Pharm. Sci.*, **57**, 235 (1968).

(6) R. H. Reuning and G. Levy, *ibid.*, **58**, 79(1969); and earlier works cited therein.

(7) D. M. Long, Jr. and M. J. Folkman, U. S. pat. 3,279,996 (1966).

(8) K. Sundaram and F. A. Kincl, *Steroids*, **12**, 517(1968).

(9) D. D. Perrin, W. L. F. Armarego, and D. R. Perrin, "Purification of Laboratory Chemicals," Pergamon Press, Oxford, England, 1966.

(10) H. H. Willard, L. L. Merritt, Jr., and J. A. Dean, "Instrumental Method of Analysis," 4th ed., D. Van Nostrand, Princeton, N. J., 1965, pp. 94–95.

(11) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen & Co., London, England, 1962, pp. 130, 134.

(12) M. Nakano, unpublished work.

(13) M. Nakano, N. I. Nakano, and T. Higuchi, *J. Phys. Chem.*, **71**, 3954(1967).

(14) T. Higuchi and S. Chulkaratana, cited in T. Higuchi and and K. A. Connors, *Advan. Anal. Chem. Instr.*, **4**, 117, (1965).

(15) R. S. Drago, B. Wayland, and R. L. Carlson, *J. Am. Chem. Soc.*, **85**, 3125(1963).

(16) K. Kakemi, H. Sezaki, T. Mitsunaga, and M. Nakano, *J. Pharm. Sci.*, in press.

ACKNOWLEDGMENTS AND ADDRESSES

Received May 20, 1969 from *Faculty of Pharmacy and Pharmaceutical Sciences, The University of Alberta, Edmonton, Alberta, Canada.*

Accepted for publication September 2, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported by the University of Alberta Postdoctoral Fellowship (to M.N.) and by a grant from Smith Kline & French Inter-American Corporation (Canada).

* Present address: Pharmaceutical Chemistry Division, Food and Drug Directorate, Ottawa 3, Ontario, Canada.

† Present address: Frank W. Horner Ltd. Research Laboratories, Montreal 307, Quebec, Canada. To whom reprint requests should be addressed.

Factors Affecting the Synthesis of Penicillinase by Staphylococcus Aureus

R. F. BOYD and JOSEPH JUDIS

Abstract □ The effects of aeration, ferric ions, porphyrins, and disodium versenate were studied on the production of penicillinase (ps) by *Staphylococcus aureus* 55-C-1 in synthetic medium. The production of ps was twice as high in shaken compared to static cultures although growth ultimately was the same. Ferric ions in a concentration of 2.5×10^{-4} *M* had some stimulatory effect on ps production in static cultures but caused much greater ps stimulation in shaken cultures. This stimulatory effect could be demonstrated only if ferric ions were added prior to 3 hr. of incubation. Hemin in a concentration of 2.5×10^{-5} *M* depressed ps production in the presence of ferric ions but one-tenth the concentration of hemin did not. Hematin in the same concentration was without effect. Protoporphyrin in a concentration of 2.5×10^{-5} *M* inhibited growth in the absence of ferric ions and depressed ps production in the presence of ferric ions. One-tenth the concentration of protoporphyrin had no effect on growth but depressed ps production and only slightly depressed ps production in the presence of ferric ions. A concentration of 2.5×10^{-6} *M* hematoporphyrin inhibited ps production in the presence of ferric ions when the former was added early in the growth curve. Disodium versenate in a concentration of 1.32×10^{-5} *M* in the presence of ferric ions permitted good growth but no ps production.

Keyphrases □ Penicillinase production—*Staphylococcus aureus* □ Aeration, shaking effect—penicillinase production □ Porphyrins, ferric ions, disodium versenate, effect—penicillinase production □ Optical density—*S. aureus* growth determination □ Manometric determination—penicillinase

Abraham and Chain (1) were the first to show that certain bacteria produce an enzyme capable of hydrolyzing penicillin. Since the latter paper was published in 1940, a number of workers have demonstrated that the resistance of strains of *Staphylococcus aureus* to penicillin was related to the production of penicillinase, the enzyme which was shown to hydrolyze penicillin. Although in some studies penicillin resistance was demonstrable in the absence of penicillinase production (2, 3), there seems to be good correlation between the ability of staphylococci to produce penicillinase and to be resistant to the action of penicillin both *in vitro* and *in vivo* (4–6). Penicillinase production has been similarly implicated in penicillin resistance of *Bacteroides* sp. (7).

In some microorganisms, penicillinase has been found to be an inducible enzyme and many studies (8–12) have concentrated on the nature of the induction of penicillinase elaboration. Some strains of *S. aureus* produced penicillinase constitutively (13, 14) and a number of workers have been concerned with the conditions and factors affecting constitutive production of penicillinase. It was felt that if the factors which control or affect penicillinase production in a strain of *S. aureus* producing penicillinase constitutively could be identified, it was possible that this knowledge could be applied ultimately in the treatment of an infection caused by a penicillinase-producing strain of *S. aureus*. That is, a strain which otherwise would be resistant to penicillin could be made susceptible to the action of penicillin if penicillinase production could be inhibited by the concurrent administration of a nontoxic

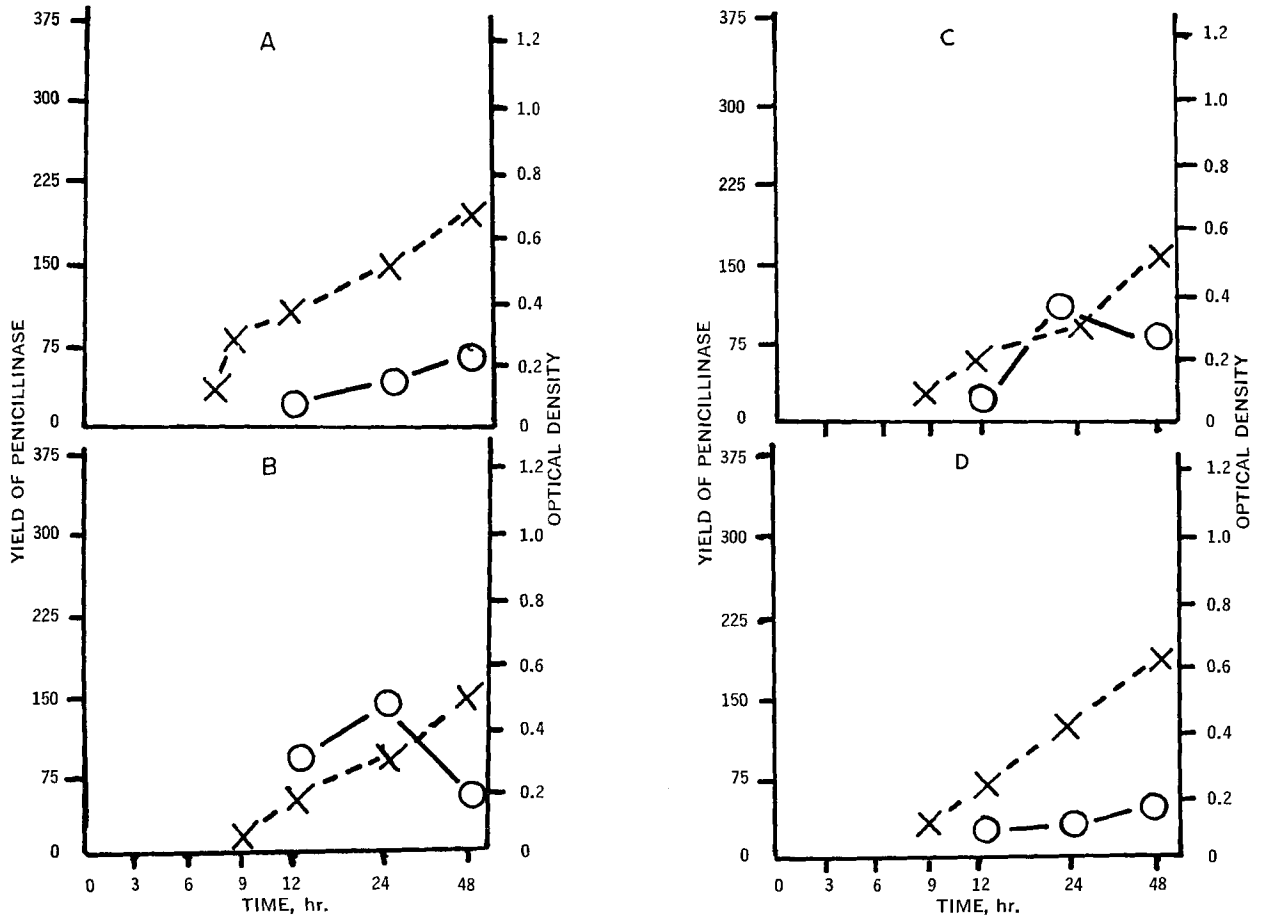


Figure 1—*The effect of ferric ions on the production of penicillinase in static cultures of* S. aureus. (A) *no added ferric ions.* (B) *2.5 × 10^{-4} M final concentration of ferric ions.* (C) *2.5 × 10^{-5} M final concentration of ferric ions.* (D) *2.5 × 10^{-6} M final concentration of ferric ions. Key:* ○, *penicillinase;* ×, *growth.*

substance inhibiting the production of penicillinase by these microorganisms. A study was, therefore, undertaken to examine the effects of several conditions and substances on the production of penicillinase by a strain of *S. aureus* producing this enzyme constitutively.

MATERIALS AND METHODS[1]

Materials—The culture media used in all experiments was that used by Wright and Mundy (15) with certain modifications made by Leitner and Cohen (14). The medium was made up in two separate portions. Solution 1 contained the following: L-leucine, 0.8 g.; L-tryptophan, 0.025 g.; L-proline, 0.05 g.; DL-phenylalanine, 0.26 g.; DL-threonine, 0.5 g.; L-histidine monohydrochloride, 0.15 g.; L-tyrosine, 0.21 g.; DL-valine, 1.00 g.; L-arginine monohydrochloride, 0.4 g.; L-glutamic acid, 0.65 g.; DL-serine, 0.61 g.; glycine, 0.06 g.; DL-methionine, 0.05 g.; DL-alanine, 0.43 g.; DL-lysine monohydrochloride, 1.70 g.; L-aspartic acid, 0.45 g.; DL-isoleucine, 0.44 g.; L-cystine, 0.05 g. The above amino acids were dissolved in a solution consisting of 482 ml. of distilled water and 18 ml. of 4% NaOH. Solution 2 contained the following minerals and vitamins; KCl, 0.2 g.; $MgSO_4.7H_2O$, 0.1 g.; thiamine hydrochloride, 0.01 g., and nicotinamide, 0.01 g. These chemicals were dissolved in 500 ml. of distilled water and then mixed with Solution 1, cleared by filtration, and the pH adjusted to 7.2 using 1 *N* HCl. The medium was autoclaved for 15 min. at 15 pounds of pressure. The culture was maintained on nutrient agar and transferred monthly.

The experimental cultures were grown in side-arm, 300-ml. flasks (Bellco No. 10-514, 14 × 130 mm.) containing a total volume of 40 ml. of medium including all other additions. The flasks were inoculated with 0.3 ml. of a 24-hr. statically grown culture which had reached an optical density of 0.30 (measured in 16 × 150-mm. screw-cap test tubes at 625 mμ in a Bausch & Lomb Spectronic 20 spectrophotometer). Also added was 0.2 ml. of 50% dextrose. Any pH adjustments were made with 1 *N* HCl. During typical growth experiments, samples were removed at various time intervals and frozen until assayed for penicillinase activity. The period of storage at −10° never exceeded 24 hr. and in preliminary experiments, it was found that the penicillinase activity of samples stored for various periods of time up to 24 hr. under these frozen conditions did not vary by more than 10% from the values of the samples prior to freezing. Thawing of the samples up to three times within a 24-hr. period of storage in the frozen state yielded similar results, that is, penicillinase activities varying by no more than 10% from that of the fresh sample prior to freezing. Optical density measurements of cell suspensions were made at 625 mμ in a Bausch & Lomb Spectronic 20 spectrophotometer in all cases and optical density measurements were related to viable cell counts as had been determined by Steinman (16) and confirmed by the present authors. An optical density of 0.102 in a 14 × 130-mm. side-arm flask was equivalent to 1.7 × 10^8 cells/ml.

Methods—Penicillinase was measured manometrically at 37° and pH 7.46 by the method of Henry and Housewright (17) because this has been the most commonly used method by other investigators especially with whole cells. The main compartment of the Warburg flasks contained 2.0 ml. of chloramphenicol succinate

[1] The culture used in all experiments was *Staphylococcus aureus* strain 55-C-1 obtained from Drs. Felix Leitner and Sidney Cohen, Department of Microbiology, Michael Reese Hospital and Medical Center, Chicago, Ill. (14).

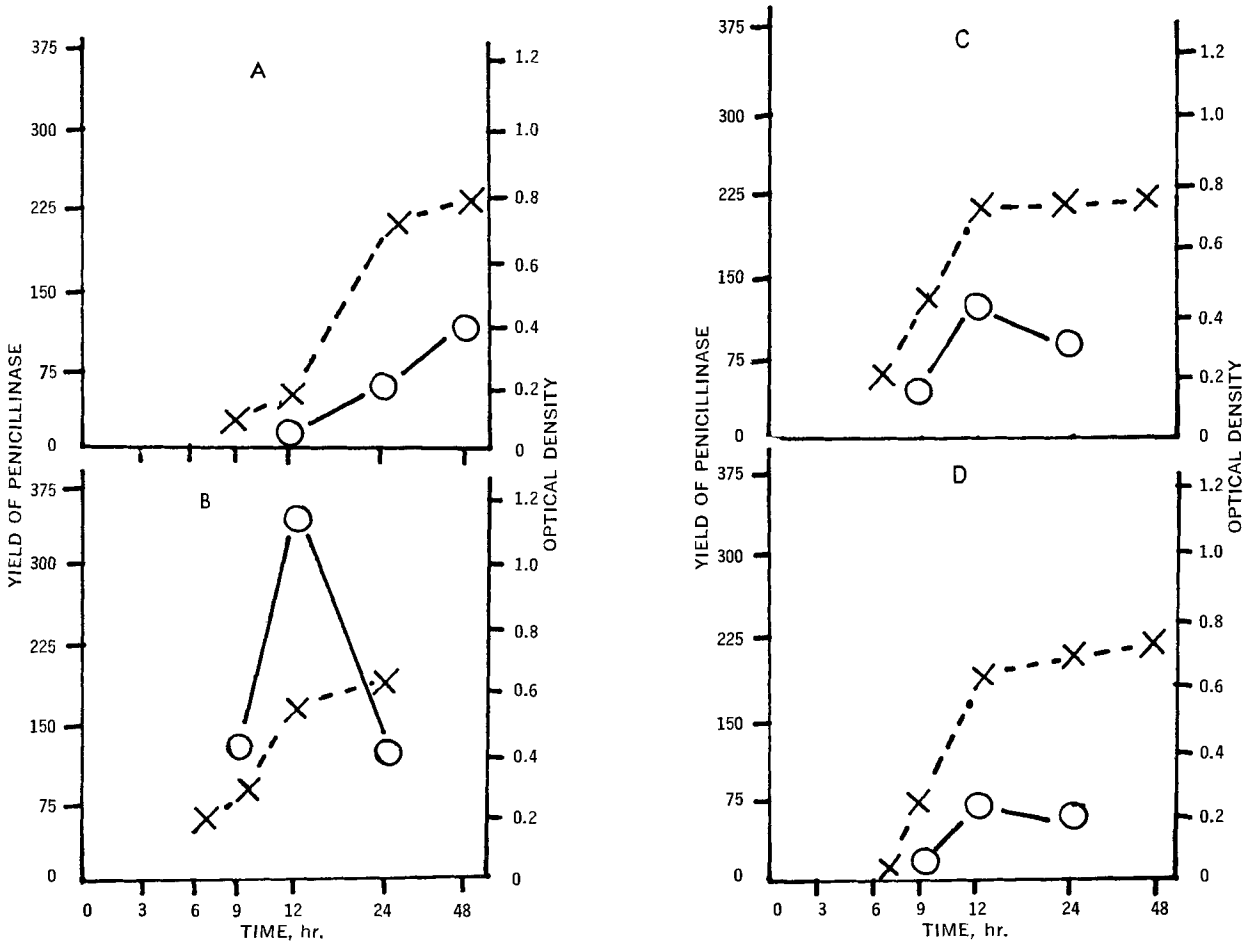


Figure 2—*The effect of ferric ions on the production of penicillinase in cultures of* S. aureus *shaken at an amplitude of 0.8 cm.* (a) *no added ferric ions.* (b) *2.5* × *10^{-4}* M *final concentration of ferric ions.* (c) *2.5* × *10^{-5}* M *final concentration of ferric ions.* (d) *2.5* × *10^{-6}* M *final concentration of ferric ions. Key:* ○, *penicillinase;* ×, *growth.*

solution (200 mcg./2 ml. of 0.017 *M* $NaHCO_3$) and 0.5 ml. of distilled water. One side-arm contained 0.5 ml. of bacterial suspension. Bacterial cultures were centrifuged and the cells resuspended in the same volume of distilled water. The other side-arm of the two side-arm Warburg flasks contains 0.2 ml. sodium penicillin G (12 mg./0.2 ml. of 0.017 *M* $NaHCO_3$). The flasks were flushed with 5% carbon dioxide and 95% nitrogen for 15 min. Following equilibration, the contents of the side-arms were tipped into the main compartments and measurements taken every 5 min. Occasional assays of the supernatant were made, and it was found that 95% of the enzyme was associated with the bacterial cells. Activity was expressed as microliters of carbon dioxide per hour per 1.7 × 10^8 cells/ml.

Culture flasks were shaken on a Burrel Wrist Action shaker to effect aeration. Ferric chloride ($6H_2O$) was dissolved in distilled water and autoclaved. The porphyrins were dissolved in distilled water, the pH adjusted to 7.6, and the solution sterilized by Seitz filtration. Adjustment of pH was necessary because the porphyrins tend to precipitate out of solution at lower pH's.

Biochemicals (Nutritional Biochemicals Corp. or the California Corporation for Biochemical Research) and chloramphenicol succinate (Parke, Davis & Co.) were used and all other chemicals were of reagent grade.

Table I—Penicillinase Production in Shaken and Static Cultures

Cultural Conditions	Time of Incubation, hr.	Optical Density	Yield of Penicillinase[a]
Shaken, 0.8 cm. stroke amplitude	24	0.677	50
	30	0.745	109
	48	0.796	147
Shaken, 1.5 cm. stroke amplitude	12	0.221	0
	24	0.770	100
	48	0.854	123
Static	12	0.337	20
	24	0.523	37
	48	0.658	50
	72	0.745	50
	96	0.824	65

[a] The units of penicillinase activity are microliters of CO_2/hr/1.7 × 10^8 cells/ml.

RESULTS AND DISCUSSION

The effect of the degree of aeration of the cultures on penicillinase production can be seen from the data presented in Table I. Although more vigorous aeration as shown by a 1.5-cm. stroke did not give greater yield than more moderate aeration, penicillinase production in aerated cultures was clearly greater than in static cultures. This effect was observed even though the final cell density reached in static cultures was equivalent to that reached in shaken cultures.

The addition of ferric ions was studied on penicillinase production in both static and shaken cultures (Figs. 1–3). Penicillinase production in static cultures was only slightly affected by the addition of ferric ions. However, these ions were clearly stimulatory to penicillinase production in shaken cultures at either of the two stroke amplitudes used. In order to gain some clue as to the mechanism by which ferric ions may be stimulatory to penicillinase production, the time of addition of the ferric ion and the age of the cultures were studied. From Fig. 4, it can be seen that if ferric ions

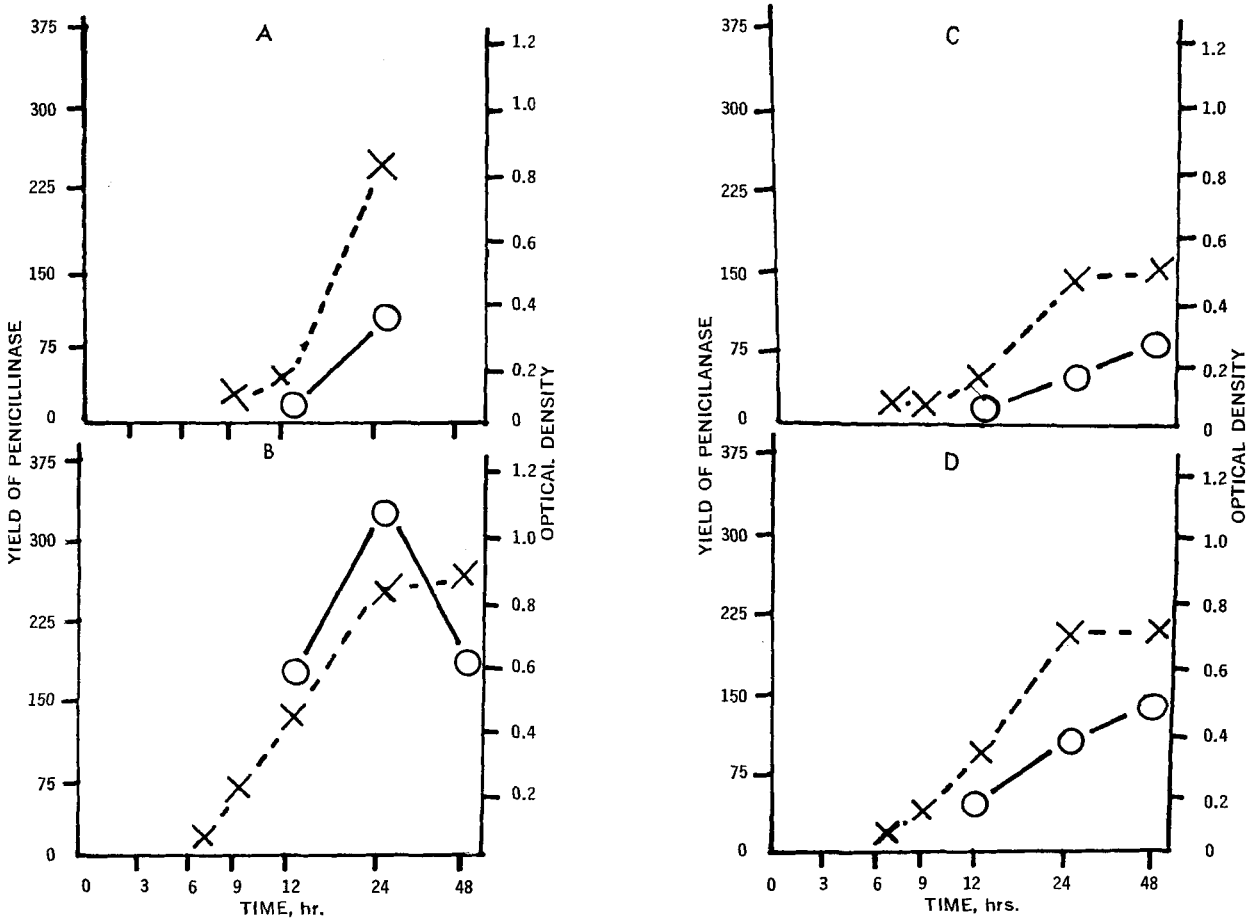


Figure 3—*The effect of ferric ions on the production of penicillinase in cultures of* S. aureus *shaken at an amplitude of 1.5 cm.* (a) *no added ferric ions.* (b) *2.5 × 10^{-4} M final concentration of ferric ions.* (c) *2.5 × 10^{-5} M final concentration of ferric ions.* (d) *2.5 × 10^{-6} M final concentration of ferric ions. Key:* ○, *penicillinase;* ×, *growth.*

are added at 0 or 3 hr. of incubation, the stimulatory effects are seen but if added after that time, there is a decreasing effect on penicillinase production.

Sack (18) and Sack and Judis (19) showed that certain porphyrin compounds affected the production of coagulase by *S. aureus* and it was of interest to determine whether such compounds would affect penicillinase production by this organism. Hemin (Fig. 5) in a concentration of 2.5 × 10^{-5} *M* depressed penicillinase production in the presence of ferric ions, but in a concentration of 2.5 × 10^{-6} *M* this depression did not appear. Hematin, in the presence of ferric ions, had no effect on penicillinase production as stimulated by ferric ions (Fig. 6). Protoporphyrin, in a concentration of 2.5 ×

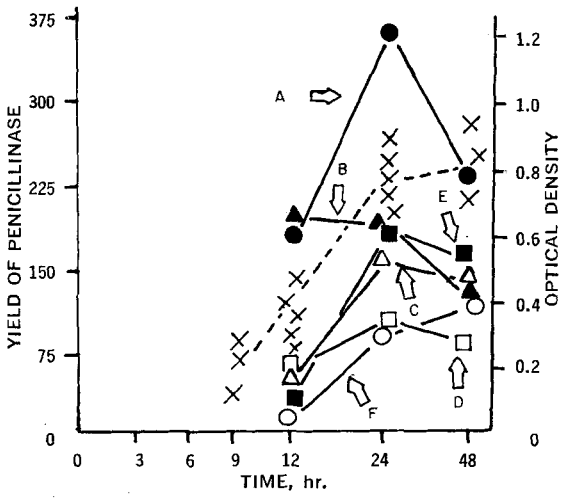


Figure 4—*Influence of time of addition of ferric ions on the stimulation of penicillinase production in shaken cultures of* S. aureus. *Ferric ions, in a final concentration of 2.5 × 10^{-4} M were added at the following times of incubation:* (a) *0 hr.;* (b) *3 hr.;* (c) *6 hr.;* (d) *9 hr.;* (e) *12 hr.;* (f) *no added ferric ions. The growth curve shown is a composite of the individual growth curves for* (a) *through* (f). *Key:* ●, A; ▲, B; △, C; □, D; ■, E; ○, F; ×, *growth.*

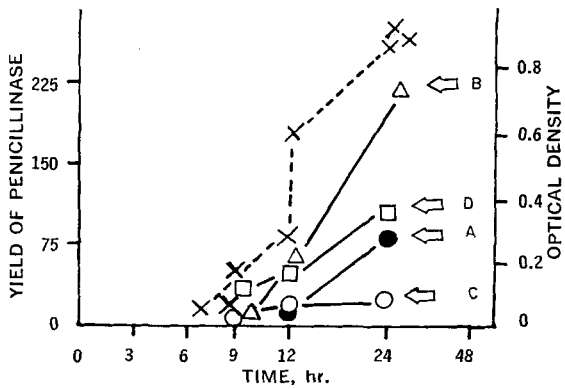


Figure 5—*Effect of hemin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added was 2.5 × 10^{-4} M.* (a) *No added hemin or ferric ions.* (b) *Ferric ions added to a final concentration of 2.5 × 10^{-4} M.* (c) *hemin added at a final concentration of 2.5 × 10^{-5} M.* (d) *Both hemin and ferric ions added. The growth curve shown is a composite of the individual curve for* (a) *through* (d). *Key:* ●, *neither hemin or ferric ions added;* △, *ferric ions added;* ○, *hemin added;* □, *both hemin and ferric ions added;* ×, *growth.*

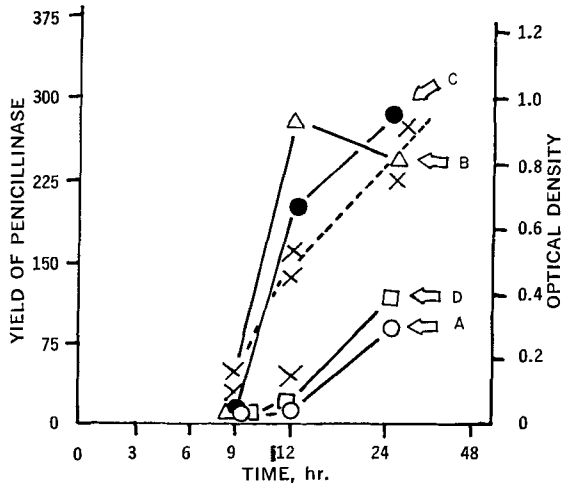


Figure 6—*Effect of hematin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Hematin added at a concentration of 2.5 × 10⁻⁵* M. (b) *Ferric ions added only.* (c) *Hematin (at same concentration as in* a) *and ferric ions added.* (d) *Neither hematin nor ferric ions added. Key:* ○, *hematin added;* △, *ferric ions added only;* ●, *hematin and ferric ions added;* □, *neither hematin nor ferric ions added;* ×, *growth.*

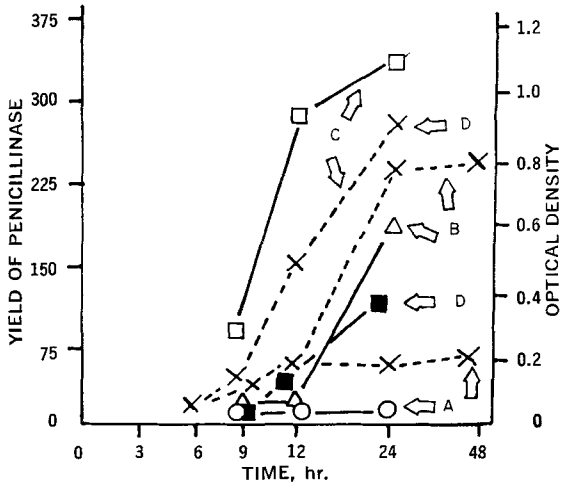


Figure 8—*Effect of protoporphyrin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Protoporphyrin added at a concentration of 2.5 × 10⁻⁵* M. (b) *Protoporphyrin (same concentration as in* a) *and ferric ions added.* (c) *Ferric ions only added.* (d) *Neither protoporphyrin nor ferric ions added. Key:* ○, *protoporphyrin added;* △, *protoporphyrin and ferric ions added;* □, *ferric ions only added;* ■, *neither protoporphyrin nor ferric ions added;* ×, *growth.*

10^{-6} *M* inhibited the production of penicillinase but had no effect on growth (Fig. 7). In the presence of ferric ions, there was observed a slight decrease in penicillinase production when added at 0, 3, or 6 hr. of incubation but had no effect after 6 hr. of incubation. At a concentration of 2.5×10^{-5} *M*, protoprophyrin inhibited both growth and penicillinase production but not in the presence of ferric ions (Fig. 8).

Hematoporphyrin, at a concentration of 2.5×10^{-5} *M*, inhibited both growth and penicillinase production in the absence of ferric ions and was capable of inhibiting growth in the presence of ferric ions to a considerable extent. When the concentration of hematoporphyrin was reduced to 2.5×10^{-6} *M*, growth was not affected although penicillinase production was reduced (Fig. 9). The addition of ferric ions overcame the inhibition of growth and penicillinase production if the hematoporphyrin was added as late as after 9 hr. of incubation but penicillinase production was inhibited if the porphyrin was added at 0, 3, or 6 hr. of incubation. The data obtained in the studies on the effects of disodium versenate are represented in Fig. 10. A concentration of disodium versenate of 1.32×10^{-5} *M* permitted good growth but no penicillinase production. Reduction in concentration of the disodium versenate to 6.7×10^{-6} *M* also permitted good growth and only moderate production of penicillinase and a further reduction of the concentration of disodium versenate to 3.35×10^{-6} *M* permitted both good growth and good penicillinase production. In all of these studies, ferric ions were present and whether the disodium versenate was added at 0 hr. of incubation or 12 hr., the results obtained were the same.

It is interesting to speculate on the mechanism by which ferric ions stimulate the production of penicillinase. In experiments in

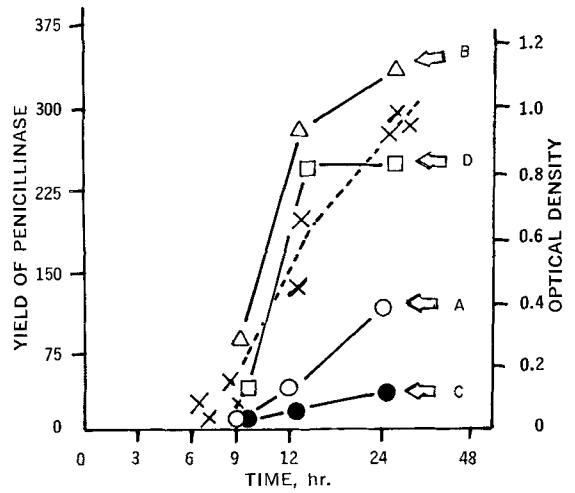


Figure 7—*Effect of protoporphyrin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Neither protoporphyrin nor ferric ions added.* (b) *Ferric ions only added.* (c) *Protoporphyrin added at a concentration of 2.5 × 10⁻⁶* M. (d) *Protoporphyrin (same concentration as in* c) *and ferric ions added. Key:* ○, *neither protoporphyrin nor ferric ions added;* △, *ferric ions only added;* ●, *protoporphyrin added;* □, *protoporphyrin and ferric ions added;* ×, *growth (composite of individual growth curves).*

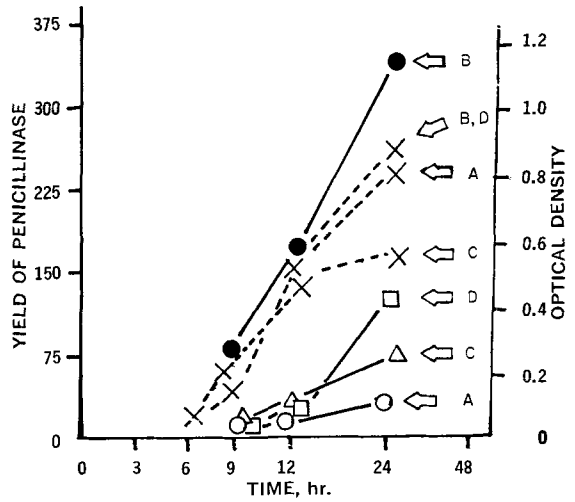


Figure 9—*Effect of hematoporphyrin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Hematoporphyrin added at a concentration of 2.5 × 10⁻⁶* M. (b) *Ferric ions only added.* (c) *Both ferric ions and hematoporphyrin added.* (d) *Neither ferric ions nor hematoporphyrin added. Key:* ○, *hematoporphyrin added;* ●, *ferric ions only added;* △, *both ferric ions and hematoporphyrin added;* □, *neither ferric ions nor hematoporphyrin added;* ×, *growth.*

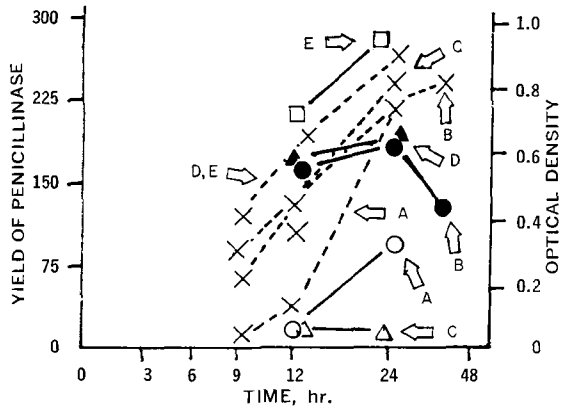


Figure 10—*Effect of disodium versenate on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Neither ferric ions nor disodium versenate added.* (b) *Ferric ions only added.* (c) *Disodium versenate added in a concentration of 1.32 × 10⁻⁵* M *and ferric ions.* (d) *Disodium versenate added in a concentration of 6.7 × 10⁻⁶* M *and ferric ions.* (e) *Disodium versenate added in a concentration of 3.35 × 10⁻⁶* M *and ferric ions. Key:* ○, *neither ferric ions nor disodium versenate added;* ●, *ferric ions only added;* △, *disodium versenate added—1.32 × 10⁻⁵* M *and ferric ions;* ▲, *disodium versenate added—6.7 × 10⁻⁶* M *and ferric ions;* □, *disodium versenate added—3.35 × 10⁻⁶* M *and ferric ions;* ×, *growth.*

which the time of addition of ferric ions to the culture was varied, it was seen that maximal stimulation of penicillinase production by ferric ions occurred if the latter were added early in the growth period. It is during this time that RNA synthesis and protein synthesis are most active and possibly some inhibitor either in the medium or produced by the microorganism is present and it is the latter which ferric ions are inactivating. Also, perhaps ferric ions may be inhibiting the synthesis of a repressor substance as suggested by Cohen *et al.* (20). For example, Rogers (21) who studied the relationship between growth of *S. aureus* and hyaluronidase production found that early in the growth curve, cellular growth was more rapid than enzyme production and at a certain time, the reverse was true. The initial lag in the formation of the enzyme was found to be primarily due to accumulation of α-amino butyric acid, which was subsequently (22) found to inhibit the formation of the enzyme. In the authors' studies with penicillinase, it was found that in the presence of ferric ions, penicillinase is produced rapidly and subsequently drops. This might lead one to believe that the penicillinase formed in the presence of ferric ions is an unstable one but further studies comparing the stability of the staphylococcal penicillinase and commercially purchased penicillinase indicated no difference in stability. It is possible that the role of ferric ions is to inhibit the production of something analogous to the α-amino butyric acid found by Rogers to affect the production of hyaluronidase.

Perhaps the inhibitory effects shown by porphyrins can be related to their ability to coordinate with inorganic ions (23). Disodium versenate is known to form a strong metal complex with ferric ions and perhaps inhibit the production of penicillinase by making the stimulatory effect of ferric ions impossible. It should be of interest to explore the effect of disodium versenate on the course of staphylococcal infections in experimental animals, especially the ability of penicillin to eradicate infections caused by penicillin resistant staphylococci. If, indeed, disodium versenate is able to inhibit the production of penicillinase by a penicillin resistant staphylococcus, the latter should become amenable to penicillin therapy.

REFERENCES

(1) F. P. Abraham and E. Chain, *Nature*, **146,** 837(1940).

(2) A. Bondi and C. C. Dietz, *Proc. Soc. Exptl. Biol. Med.*, **56,** 135(1944).

(3) R. Knox, *Proc. Intern. Congr. Chemotherapy, 3rd Stuttgart*, **2,** 1257(1963).

(4) N. W. Coles and R. Gross, *Australian. J. Exptl. Biol. Med. Sci.*, **43,** 725(1965).

(5) L. Eyckmans and R. Tompsett, *J. Bacteriol.*, **90,** 589(1965).

(6) Y. A. Chapbert and J. G. Baudens, *Proc. Intern. Congr. Chemotherapy, 3rd, Stuttgart*, **2,** 1401(1963).

(7) G. Pinkus, G. Veto, and A. I. Braude, *J. Bacteriol.*, **96,** 1437(1968).

(8) M. R. Pollock, *Brit. J. Exptl. Pathol.*, **31,** 739(1950).

(9) *Ibid.*, **33,** 587(1952).

(10) M. R. Pollock and C. J. Perret, *ibid.*, **32,** 387(1951).

(11) M. R. Pollock, *Biochem. J.*, **66,** 419(1957).

(12) J. Imsande and M. J. Gerber, *J. Bacteriol.*, **94,** 1672(1967).

(13) A. Bondi, M. De St. Phalle, J. Kornblum, and A. G. Moat, *Arch. Biochem. Biophys.*, **53,** 348(1954).

(14) R. Leitner and S. Cohen, *J. Bacteriol.*, **83,** 314(1962).

(15) E. S. Wright and R. A. Mundy, *ibid.*, **80,** 279(1960).

(16) H. G. Steinman, *ibid.*, **81,** 895(1961).

(17) R. J. Henry and R. D. Housewright, *J. Biol. Chem.*, **67,** 559(1947).

(18) G. Sack, M.S. thesis, University of Toledo, Toledo, Ohio (1962).

(19) G. Sack and J. Judis, *Ohio J. Sci.*, **63,** 232(1963).

(20) S. Cohen, H. M. Sweeney, and F. Leitner, *J. Bacteriol.*, **93,** 1277(1967).

(21) H. J. Rogers, *J. Gen. Microbiol.*, **10,** 209(1954).

(22) *Ibid.*, **16,** 22(1957).

(23) S. Granick and O. Mauzerall, *J. Biol. Chem.*, **232,** 1119 (1958).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Department of Biology and College of Pharmacy, The University of Toledo, Toledo, OH 43606*

Accepted for publication September 2, 1969.

Abstracted in part from a thesis submitted by R. F. Boyd to The University of Toledo, Toledo, Ohio in partial fulfillment of Master of Science degree requirements.

Assay of Hyoscyamine, Atropine, Scopolamine, and Phenobarbital in Unit Doses of Tablets and Elixirs

RUPERT O. ZIMMERER, Jr. and LEE T. GRADY

Abstract □ A gas chromatographic assay for unit dose quantities of scopolamine and atropine-hyoscyamine was developed for tablets and elixirs containing phenobarbital. The method allows the complete assay of unit dose forms. Standards assayed with precisions of 4.8% for scopolamine, 6 mcg./unit dose, 2.5% for atropine-hyoscyamine at 100 mcg./dose, and 1.0% for phenobarbital (spectrophotometric) at 16 mg./dose. Homatropine served as extracted internal standard. Derivatives of the alkaloids were unnecessary using properly cured columns.

Keyphrases □ Hyoscyamine, atropine, scopolamine, phenobarbital tablets, elixirs—analysis □ Internal standard, "extracted"—homatropine □ GLC—analysis □ UV spectrophotometry—analysis

Quantitative methods for small amounts of belladonna alkaloids are few. No method has been reported which is suitable for the complete assay of hyoscyamine sulfate, atropine sulfate, scopolamine hydrobromide, and phenobarbital tablets and elixir.[1] This paper reports the development of a procedure which allows the complete assay of these formulations on unit dose forms.

Das Gupta and Ferguson (1) noted recently the need for determination of small amounts of atropine in elixirs and tablets and applied a dye-complex method. A separation of phenobarbital by partition column and subsequent colorimetric determination of total belladonna alkaloids was reported by Koch *et al.* (2). This sequence was adapted to kaolin-pectin suspensions by Bracey and Selzer (3). None of these methods determined scopolamine.

Sterescŭ and Popovici (4) determined atropine and scopolamine individually by paper chromatography in the presence of papaverine and sparteine. Schill and Agren separated scopolamine from hyoscyamine by partition chromatography on kieselguhr columns (5). Countercurrent distribution (6) also separates these alkaloids. Paper chromatographic separation of scopolamine from atropine-hyoscyamine was effective, and Reichelt (7) was able to separate as much as 50-fold ratios. Thin-layer chromatographic separation of scopolamine from the other belladonna alkaloids has been particularly successful, for example Wartman-Hafner's system (8).

Some workers have explored the gas chromatography of belladonna alkaloids. Kazyak and Knoblock (9) used silicone gums on silanized supports to separate atropine and scopolamine, but reported no satisfactory system. Brochmann-Hanssen and Fontan (10) reported separation of these from morphine and homatropine, most usably with a cyanosilicone liquid phase. The identification of alkaloids, including atropine, in blood was studied by Jain and Kirk (11) who recommended a polyester for general use. Most recently, Solomon *et al.* (12) used methylsilicone gum on silanized diatomite in assaying atropine and scopolamine in plant extracts; however, their system decomposed the alkaloids.

[1] Provisionally admitted to NF XIII.

MATERIALS AND METHODS

Chromatographic grade methylene chloride (Matheson, Coleman & Bell). Borate buffer, 0.05 *M*, was prepared from boric acid and sodium hydroxide. Dibasic potassium phosphate and sodium hydroxide were used to prepare 0.2 *M* phosphate pH 10.5 buffer. Both buffers were standardized against the glass electrode. Homatropine hydrobromide, atropine sulfate, and scopolamine hydrobromide (Merck & Co., Inc.) and hyoscyamine sulfate and the tablet and elixir (A. H. Robins & Co., Inc., Richmond, Va.) were also used.

Internal Standard Solution—Dissolve 20 mg. homatropine hydrobromide in 250 ml. 0.005 *N* sulfuric acid and mix well.

Alkaloids Standard Solution—Dissolve 16.2 mg. scopolamine hydrobromide in 100 ml. 0.005 *N* sulfuric acid. Dissolve 30.8 mg. atropine sulfate in 200 ml. 0.005 *N* sulfuric acid, add 10.0 ml. of the scopolamine solution, and make to 250 ml. with acid. Dilute 20.0 ml. of this solution to 100 ml. with the acid. All solutions were prepared fresh daily.

Gas Chromatography[2]—Analyses were performed using an 0.6-m. × 3-mm. i.d. glass column packed with 3% methylphenylsilicone gum on 80/100 mesh silanized, acid-washed, flux-calcined diatomite.[3] Column temperature was 210°, with the injection port at 235°. Helium flow was about 60 ml./min. Samples, 1–2 μl., were injected on-column. Flame ionization detectors were used.

Column Preparation—Maintain the column at 250° for 1 hr. with helium flowing to remove oxygen and solvents, stop the flow of helium and heat at about 340° 4 hr., lower temperature to 250°, and condition with helium flowing until stable. A suitable initial test for support inertness, necessary with these low polarity liquid phases, is the delivery of a single, symmetric peak for injected cholesterol with no evidence of decomposition.

PROCEDURE

Standards—Weigh 16.2 mg. phenobarbital USP reference standard into a separator and add 5.0 ml. of the alkaloids standard Solution. Add 1.00 ml. of the internal standard solution and

[2] F & M model 810, Avondale, Pa.
[3] OV-17 on Gas Chrom Q, Applied Science Laboratories, State College, Pa.

Table I—Assay of Commercial Preparations, Percent of Labeled Amounts

Sample[a]		Hyoscyamine-Atropine	Scopolamine	Phenobarbital
Elixir		103 Av. = 103%	94 Av. = 96%	102 Av. = 102
		105	90	101
		102	103	103
Tablet	Lot A	101 Av. = 101%	93 Av. = 97%	100 Av. = 101%
		101	100	102
	Lot B	102	98	101
		95 Av. = 96%	109 Av. = 111%	100 Av. = 99%
	Lot C	97	112	98
		95 Av. = 96%	112 Av. = 114%	101 Av. = 100%
		96	116	99

[a] 103.7 mcg. Hyoscyamine sulfate and 19.4 mcg. atropine sulfate, 6.5 mcg. scopolamine hydrobromide, 16.2 and mg. phenobarbital in each tablet or 5 ml. of elixir.

extract with two 25-ml. portions of methylene chloride, filtering through anhydrous sodium sulfate into a 100-ml. volumetric flask. Wash the sodium sulfate with 25 ml. methylene chloride, dilute to volume, and mix well. Pipet 10 ml. of the solution into a 150-ml. beaker and evaporate to dryness on a steam bath. Add 1 ml. alcohol and, using pH 9.5, 0.05 *M* borate buffer, quantitatively transfer the residue to a 100-ml. volumetric flask. Dilute to volume with buffer and determine the absorbance at 240 mμ using 1-cm. silica cells.

To the aqueous phase in the separator remaining after extraction of phenobarbital add 4.0 ml. of pH 10.5, 0.2 *M* phosphate buffer and extract twice with 10 ml. of methylene chloride, filtering through anhydrous sodium sulfate. Wash the sodium sulfate with 5 ml. of the methylene chloride. Evaporate[4] the combined extracts to about 0.1 ml. at reduced pressure and inject an appropriate volume into the chromatograph. Record the chromatogram, decreasing the amplifier attenuation by a factor of about 16 about halfway between the hyoscyamine-atropine peak and the scopolamine peak to increase the measured peak height of scopolamine. Obtain the peak heights, *H*, of scopolamine, hyoscyamine-atropine, and homatropine and determine *Rs* for each drug, where *Rs* is = *H* alkaloid/*H* homatropine. *Ru* values are determined similarly for the assay preparations. The amount of each alkaloid in each dosage form may be calculated[5] from the formula $Wu = Ws\,(Ru/Rs)$, where *Wu* = weight of unknown, *Ws* = exact weight of standard.

When using a column for the first time prepare additional standards by adding 4.0 and 6.0 ml. of alkaloid solution and carrying out the assay. A plot of *Ru versus* amount yields a straight line. Assay values should be read directly from this calibration curve if the line does not pass through zero.

The amount of phenobarbital in each dosage form may be calculated from the formula $Wu = Ws\,(Au/As)$, where *Wu* = weight of unknown, *Ws* = exact weight of standard, *Au* = absorbance of unknown, and *As* = absorbance of standard.

Tablet Composite—Weigh and finely powder not less than 20 tablets. Transfer the equivalent of one tablet to a 60-ml. separator, add 1.0 ml. of the internal standard solution, 5 ml. water, mix, and adjust the pH to a value not greater than 3 with 1 *N* H_2SO_4 (about 0.5 ml. for those used in this report). Proceed as directed in the standard assay beginning with "Extract with two 25-ml. portions..."

If necessary, the aqueous phase in the separator, after adding 4 ml. pH 10.5 buffer, should have the pH adjusted to greater than 9 using 1 *N* NaOH (about 0.5 ml. for those used in this report).

Tablet Content Uniformity—Finely powder one tablet and transfer to a 60-ml. separator and proceed as in tablet assay above. Discard the phenobarbital extracts. Determine hyoscyamine-atropine only after evaporation of the extract to about 1 ml.

Elixir—Pipet 5.0 ml. of the elixir into a 60-ml. separator and add 1.0 ml. of the internal standard solution. Proceed as in the tablet assay, beginning with "Add 1.00 ml. internal standard solution...."

RESULTS AND DISCUSSION

The small amounts and similar structures of the alkaloids in these formulations recommended gas chromatographic analysis. Moreover, a method was desired that would allow the entire assay to be performed on individual dose units.

Ten complete standards were taken through the procedure. The coefficients of variation in the hyoscyamine-atropine and scopolamine assay ratios were 2.5 and 4.8%, respectively. Phenobarbital precision was 1.0%. Applicability of the procedures to commercial formulations was checked (See Table I). The elixir was assayed in triplicate. Tablet composites of three lots were assayed. Placebos were not available.

In summary, this procedure allows assay of individual dose units of hyoscyamine sulfate, atropine sulfate, scopolamine hydrobromide, and phenobarbital tablets and elixir. The method is specific, rapid, and highly sensitive. Precisions are typical for gas chromatography, and using duplicates where possible, particularly for scopolamine, is recommended for accuracy.

This procedure also applies to other, simpler official tablets and solutions of belladonna alkaloids after appropriate change in the amount of internal standard and, usually, elimination of the acid extraction step. This has been done with hyoscyamine sulfate tablets NF and morphine sulfate and atropine sulfate tablets NF.

Applicability to content uniformity determination is inherent in single dose procedures, so only an estimate was prepared in a single lot. Content uniformity is based on hyoscyamine-atropine rather than scopolamine (the least abundant active ingredient) because of the better precision for that peak and to eliminate some of the time for concentration. This decision assumes that the ratio of scopolamine to the other alkaloids is fixed prior to manufacture of the dosage forms.

Comments on Procedure—Homatropine was chosen as the "extracted" internal standard prior to selection and acceptance of the column. It differs from these alkaloids only in a methylene group and the nature of substitution of the carbinol so chromatographic characteristics are nearly identical. Because of functional group similarities, minor alkaline ester cleavage, or amine degradation are controlled. Mechanical losses of various layers and volumetric considerations along the way are rendered insignificant as the partition behaviors are the same. Finally, purified material is readily available.

Phenobarbital must be eliminated in any event prior to the gas chromatographic work-up, so the acid extraction conveniently was adopted to determine phenobarbital. Methylene chloride was chosen because it is easily evaporated and distribution data indicated that the double extraction removes all but 0.2% of the phenobarbital. Spectrophotometric assay was chosen for speed and precision and to avoid introduction of a second column. The spectra of extracted standard and those from elixir and tablets showed no differences and all were identical to directly prepared standards. pH limits for the two extractions are specified in the procedure so that different product formulations will not interfere with alkaloid, particularly scopolamine, recovery. Phosphate buffer was added so that the final pH of the aqueous phase was 9.1 and was used instead of alkali to minimize ester cleavage. At this pH distribution data indicate that the two extractions remove 99.6% of the atropine and all scopolamine. Heat and air during the subsequent concentration should be avoided to prevent cleavage or oxidation of the small amounts of the alkaloids. The additional problem of racemization is not considered in this report.

The final volume, 0.1 ml., was chosen so that almost any flame ionization-equipped gas chromatograph would have the necessary sensitivity. The chromatograph in this laboratory was used at one-

[4] Rotary Evapo-mix, Büchler Instruments, Fort Lee, N. J.
[5] For the case of a linear curve "passing through zero."

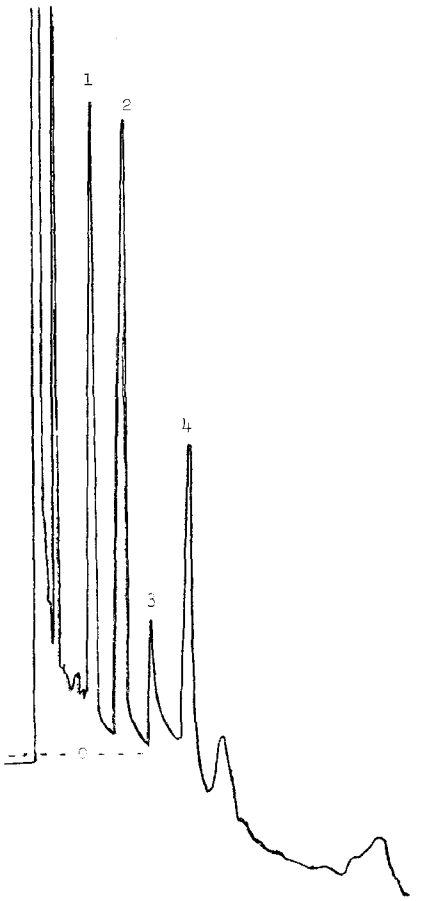


Figure 1—*Typical chromatogram (tablet). Key: 1, homatropine; 2, hyoscyamine-atropine; 3, attenuation change; 4, scopolamine.*

tenth of its maximum usable sensitivity range for scopolamine. The reagents must be pure as high boiling impurities are concentrated 200 times. Gas chromatographic grade methylene chloride is essential.

Reagent blanks were run to locate the origin of the additional peaks appearing in the chromatogram and see if there were interferences. Ten small peaks corresponding to those seen on the extracted chromatogram appeared. There were no peaks with retentions the same as or close enough to any of the drugs to interfere with the analysis (see Fig. 1). All these additional peaks were attributable to the reagent blank, mostly from methylene chloride. Of these, six emerged prior to homatropine. The others had relative (atropine = 1.0) retentions of 0.88, 1.18, 2.05, and 2.75. However, a less efficient column could allow minor species to bias the homatropine measurement. Additional standards, in triplicate, showed the analysis to be linear in the region of interest for both hyoscyamine-atropine and scopolamine. For this particular column the two lines obtained graphically terminated along the abscissa, so the assay data reported herein were taken from the calibration curves. The formula presented in the procedure would be accurate only where calibration curves have been demonstrated to "pass through zero." The column was rechecked (cholesterol, alkaloids, standard curves) at the end of the study and found intact.

Chromatography—The difficulties associated with the GLC of polar amines are well known. Improperly or partially-cured and conditioned columns often cause extensive tailing of such compounds and nonlinear recoveries of sample sizes. An additional problem is partial on-column dehydration of atropine and scopolamine as was reported by Solomon *et al.* (12). Although the preparation of less polar derivatives often allows successful chromatography, the additional steps and problems are appreciable. Current phases, supports, and column treatments have extended the range of molecules which can be chromatographed directly, without prior formation of less polar derivatives.

The peaks were symmetric and tailing was negligible (see Table II). Examination of the chromatograms of pure compounds gave peaks

Table II—Gas Chromatographic Data

Parameter	Homatropine	Hyoscyamine-Atropine	Scopolamine
Retention[a]	0.65	(1.00)	1.76
Theor. plates (15)	1000	1100	1200
Asymmetry, *As* (15)	1.10	1.02	1.08
Tailing[b]	1.06	1.06	1.04
Response[c]	0.97	(1.00)	0.99

[a] Actual retentions = × 2.95 min., see Fig. 1. [b] T. F. = $a + b/2a$ at 5% of peak height. [c] Equimolar amounts, ratio of peak areas.

of equal area (± 1% by planimeter). A mixture was prepared containing atropine and scopolamine in the same proportion (20:1) as that found in the commercial preparation: the scopolamine peak area was 97.4% of the value calculated based on equimolar amounts, showing that nonlinear adsorption losses are minor. Repetitive injections, $n = 12$, of this mixture in assay quantities gave coefficients of variation in the assay ratios of 1.0 and 4.0% for atropine and scopolamine, respectively. As seen from these results, most of the overall assay precision is attributable to the GLC step. Accuracy for scopolamine relative to atropine thus would be improved by duplicate injection of the same sample. Peaks in the procedural blank also were separated completely, so additional length of column was superfluous. Efficiencies of the order of a thousand plates appear sufficient for this assay.

The column preparation detailed in this procedure was essential to successful chromatography. Tailing values prior to curing were 1.4–1.7 with asymmetry factors of greater than 1.2. Resolution and recoveries were increased. Similar effects have been found with columns prepared from various samples of low (*W*) and medium (*G*) density silanized supports, both self-coated and supplier-coated. Street (13) reported recently his procedures for support treatment prior to coating and noted the importance of this for GLC of amines and alkaloids. Vessman (14) found that 315° conditioning of SE 30 on a silanized support did not eliminate tailing of high boiling amines, including scopolamine, and that alkali treatment of the support decreased tailing but decomposed the silicone.

REFERENCES

(1) V. das Gupta and N. M. Ferguson, *Am. J. Hosp. Pharm.*, **26**, 168(1969).

(2) S. A. Koch, J. Levine, and N. Zenker, *J. Pharm. Sci.*, **54**, 1046(1965).

(3) A. Bracey and G. Selzer, *ibid.*, **57**, 464(1968).

(4) M. Sterescŭ and N. Popovici, *Pharmazie*, **14**, 313(1959).

(5) G. Schill and A. Agren, *Svensk Farm. Tidskr.*, **56**, 55(1962); through *Chem. Abstr.*, **46**, 6324b(1952).

(6) H. Brauniger and G. Borgwordt, *Pharmazie*, **10**, 591(1955).

(7) J. Reichelt, *ibid.*, **9**, 968(1954).

(8) F. Wartman-Hafner, *Pharm. Acta Helv.*, **41**, 406(1966); through *Chem. Abstr.*, **65**, 19925g(1966).

(9) L. Kazyak and E. C. Knoblock, *Anal. Chem.*, **35**, 1448 (1963).

(10) E. Brochmann-Hanssen and C. R. Fontan, *J. Chromatog.*, **19**, 296(1965).

(11) N. C. Jain and P. L. Kirk, *Microchem. J.*, **12**, 229(1967).

(12) M. J. Solomon, F. A. Crane, B. L. Wu Chu, and E. S. Mika, *J. Pharm. Sci.*, **58**, 264(1969).

(13) H. V. Street, *J. Chromatog.*, **37**, 162(1968).

(14) J. Vessman, *Acta Pharm. Suecica Suppl.*, **1**, 183(1964).

(15) O. E. Schupp, "Technique of Organic Chemistry, Gas Chromatography," vol. 13, Interscience, New York, N. Y., 1968, p. 43 and p. 22.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 20, 1969 from the *Drug Standards Laboratory of the American Pharmaceutical Association Foundation, 2215 Constitution Ave., N. W., Washington, DC 20037*

Accepted for publication August 26, 1969.

The authors express gratitude to the A. H. Robins Co. for generous supplies of tablets, elixir, and alkaloids.

Differential Nonaqueous Titration of Isoniazid and Sodium *p*-Aminosalicylate Mixtures

M. B. DEVANI and C. J. SHISHOO

Abstract □ Isoniazid and sodium *p*-aminosalicylate are estimated potentiometrically in nonaqueous media. Their mixtures are amenable to differential nonaqueous titrimetry. The titration solvent consists of a mixture of equal volumes of acetonitrile and acetic anhydride, the titrant is acetous perchloric acid, and electrode system is the glass-calomel electrode pair. Satisfactory resolution of end points is obtained with the ratio of sodium *p*-aminosalicylate-to-isoniazid as high as 4:1. Both of these components in mixtures can be determined in a single titration by the proposed method without resorting to preliminary separation.

Keyphrases □ Isoniazid, Na *p*-aminosalicylate mixture—analysis □ Perchloric acid, acetous—titrant □ Differential nonaqueous titration—analysis

A variety of chromatographic, volumetric, colorimetric, spectrophotometric, and nonaqueous titrimetric procedures have been described for the analysis of isoniazid as well as sodium *p*-aminosalicylate (1–3).

The individual components in mixtures are generally determined after initial separation by one of these methods (4–6). Although satisfactory results can be obtained by these methods, they suffer from several drawbacks, particularly that of time-consuming preliminary separation processes. While isoniazid and sodium *p*-aminosalicylate can be readily determined in nonaqueous media individually, their differential titration in a mixture has not been reported.

The present study has been directed to find a simple differential nonaqueous titration procedure for the analysis of isoniazid–sodium *p*-aminosalicylate combinations. The mixtures having sodium *p*-aminosalicylate-to-isoniazid ratio up to 4:1 are resolved in a solvent system of acetonitrile–acetic anhydride (1:1). When the disproportion ratio of the components is higher, only one end point corresponding to total base is realized.

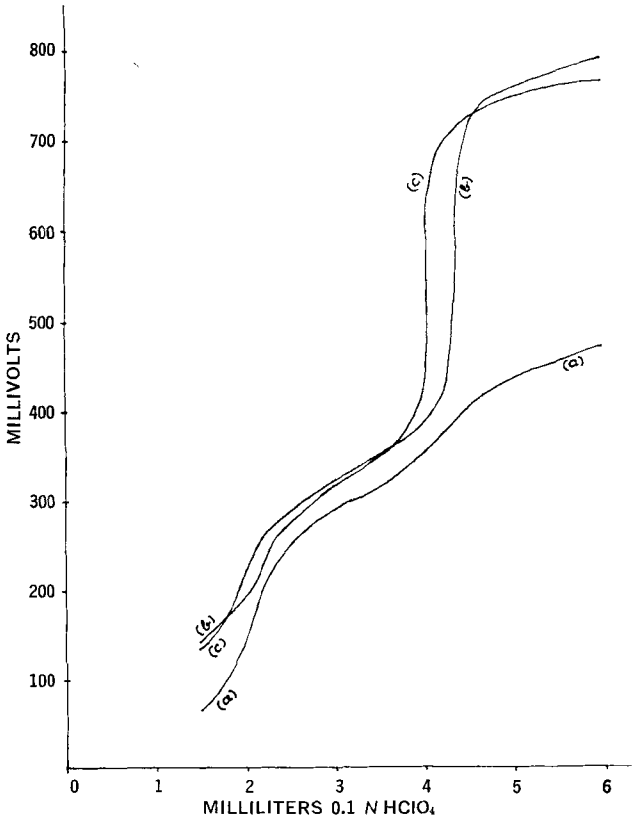


Figure 1—*Titration curves of isoniazid-sodium* p-*aminosalicylate (1:1) mixture in various solvents against 0.1* N *acetous perchloric acid. Key:* a, *acetonitrile;* b, *acetic anhydride;* c, *acetonitrile-acetic anhydride (1:1) mixture.*

PROCEDURE

Apparatus—All titrations were performed potentiometrically. A pH meter (Polymetron) employing a glass electrode and a calomel electrode with a salt bridge was used for the purpose. A saturated solution of lithium chloride in glacial acetic acid served as the salt bridge (7). Electrodes were dipped in acetic anhydride for at least 24 hr. before use.

Reagents and Solutions—Isoniazid BP was recrystallized from alcohol and dried at 105°. Sodium *p*-aminosalicylate USP was taken for analysis. Acetonitrile was purified by shaking it with two batches of silica gel (50 g./l.), two batches of alumina (20 g./l.), and phosphorus pentoxide (20 g./l.). Finally it was distilled over phosphorus pentoxide (5 g./l.) (8). Acetic anhydride reagent grade was distilled before use.

A 0.1 *N* solution of perchloric acid in glacial acetic acid was prepared and standardized potentiometrically against primary standard potassium acid phthalate dissolved in acetic acid. All other chemicals and solvents used in the study were reagent grade and were employed without further purification.

Analysis of Isoniazid—In a 100-ml. beaker about 60 mg. isoniazid, accurately weighed, was dissolved in 25 ml. of acetonitrile and stirred magnetically. An equal volume of acetic anhydride was added to it. The solution was further stirred for 5 min. and titrated potentiometrically against 0.1 *N* acetous perchloric acid. Near the

Table I—Effect of Isoniazid-to-Sodium *p*-Aminosalicylate Ratio on Sensitivity of Differentiating Titration

Amt. Weighed, meq.		Recovery, %	
Isoniazid	Sodium *p*-Aminosalicylate	Isoniazid	Sodium *p*-Aminosalicylate
0.3	—	99.9 ± 0.42[a]	—
—	0.3	—	99.8 ± 0.37
0.2	0.1	99.9 ± 0.67	99.9 ± 0.48
0.2	0.15	98.9 ± 0.47	100.6 ± 0.56
0.1	0.1	99.6 ± 0.52	99.0 ± 0.87
0.1	0.2	99.7 ± 0.60	99.8 ± 0.72
0.1	0.3	100.0 ± 0.53	99.5 ± 0.64
0.1	0.4	100.0 ± 0.45	100.2 ± 0.76
0.1	0.5	One end point[b]	

[a] Standard deviation based on at least five determinations. [b] Corresponds to isoniazid plus sodium *p*-aminosalicylate.

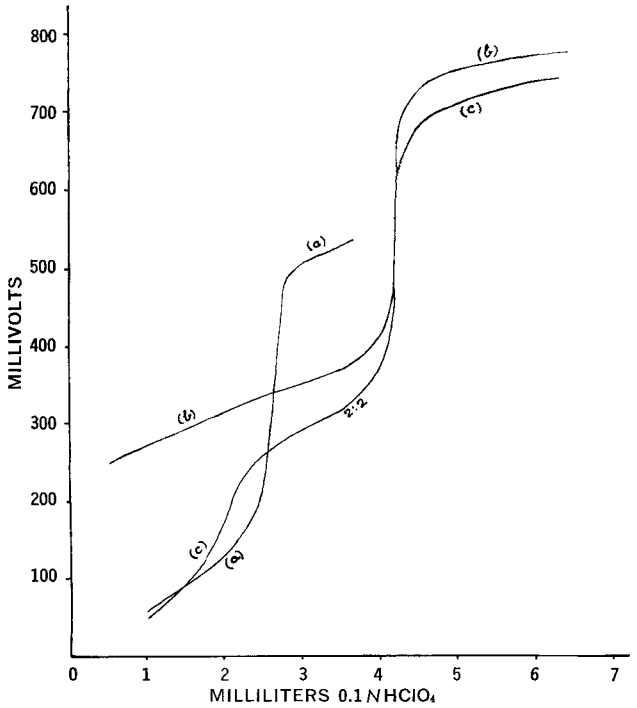


Figure 2—*Titration curves using acetonitrile–acetic anhydride (1:1) mixture as solvent system. Key:* a, *sodium* p-*aminosalicylate 0.25 meq.;* b, *isoniazid 0.4 meq.;* c, *isoniazid–sodium* p-*aminosalicylate mixture containing 0.2 meq. of each component.*

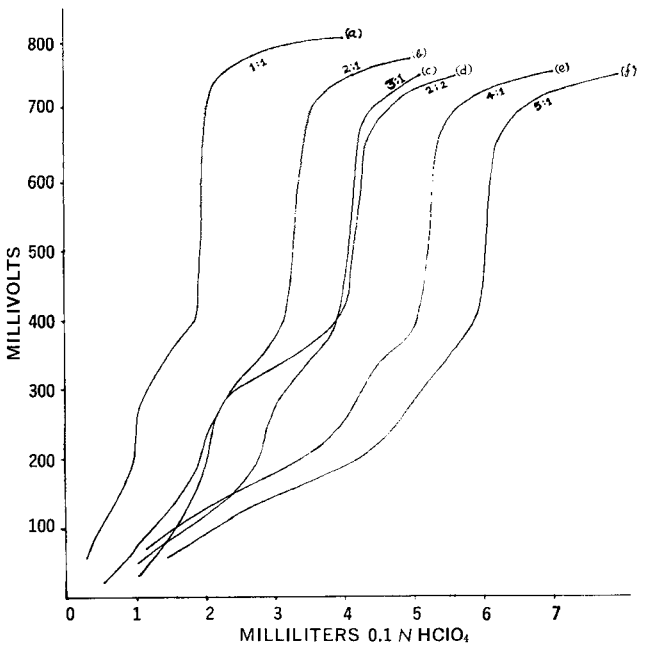


Figure 3—*Effect of sodium* p-*aminosalicylate-to-isoniazid ratio on differential titration. The ratios above the curves indicate milliequivalents of sodium* p-*aminosalicylate–isoniazid.*

end point, titration was continued adding 0.05 ml. of titrant at intervals of 1 min. and the solution kept stirred vigorously. A blank titration was performed.

Titration curves were traced, plotting potential reading (mv.) *versus* volume of titrant (ml.).

Analysis of Sodium p-Aminosalicylate—About 60 mg. of sodium *p*-aminosalicylate, accurately weighed, was mixed with 25 ml. of acetonitrile in a 100-ml. beaker and stirred for about 15 min. An equal volume of acetic anhydride was then added and stirring continued further for 5 min. before the solution was titrated potentiometrically with 0.1 *N* acetous perchloric acid. The end point was determined from the titration curve as described earlier.

Differential Titration of Isoniazid–Sodium *p*-Aminosalicylate Mixture—About 0.1 meq. of isoniazid and 0.1 meq. of sodium *p*-aminosalicylate, accurately weighed, were mixed with 25 ml. acetonitrile and stirred for about 15 min. An equal volume of acetic anhydride (25 ml.) was added to it and the stirring was continued for about 5 min. The solution was titrated rapidly, stirring all the time, until the first end point was obtained. The titration was carried out slowly near the second end point. The exact end points were determined from the titration curve as before.

Effect of Isoniazid Concentration on Differential Titration—Isoniazid and sodium *p*-aminosalicylate, in varying proportions corresponding to desired ratios (Table I), were accurately weighed and titrated as above.

RESULTS AND DISCUSSION

Isoniazid and sodium *p*-aminosalicylate, individually, have been titrated with perchloric acid as monoacidic and diacidic bases, using different solvents (9–12). Acetonitrile and acetic anhydride have been found suitable solvents for the titration of bases (13, 14). In the preliminary experiments, both these solvents were tried. The titration curves using acetonitrile as well as acetic anhydride as solvents (Curves *a* and *b* of Fig. 1) indicate two inflections: the first corresponds to sodium *p*-aminosalicylate content and the second indicates the isoniazid end point. It is seen that with acetonitrile, the first inflection is sharply defined while the second inflection point is indistinct (Fig. 1, Curve *a*). On the other hand, the first inflection is somewhat vague but the second inflection stands out quite distinctly in acetic anhydride medium (Fig. 1, Curve *b*).

On the basis of these results, mixtures of the two solvents, in varying proportions, were tried as solvent systems. It was seen that while isoniazid–sodium *p*-aminosalicylate (1:1) mixture could be successfully resolved in the solvent mixtures having acetonitrile–acetic anhydride in ratios of 4:1, 2:1, 1:1, 1:2, and 1:3, the solvent mixture in 1:1 was most ideally suited.

Typical titration curves of isoniazid, sodium *p*-aminosalicylate, and isoniazid–sodium *p*-aminosalicylate mixture are shown in Fig. 2. Both the compounds, individually as well as in a mixture, are determined with fair degree of accuracy. Both isoniazid and sodium *p*-aminosalicylate are titrated as monoacidic bases in acetonitrile–acetic anhydride (1:1) mixture.

Since in the usual dosage forms, the amount of sodium *p*-aminosalicylate considerably predominates over the content of isoniazid, the effect of varying the ratio of concentrations of the components on the sensitivity of the differentiating titration was studied. Typical titration curves are shown in Fig. 3 and the recovery of components is recorded in Table I.

Two distinct inflections in the titration curves are obtained when sodium *p*-aminosalicylate-to-isoniazid ratio does not exceed 4 (Fig. 3, Curves *a* to *e*). In the titration of mixtures with ratio of disproportion exceeding 4, only one end point corresponding to the total base is obtained (Fig. 3, Curve *f*). The differential titration of mixtures having sodium *p*-aminosalicylate-to-isoniazid ratio higher than 4:1 was tried employing solvent mixture acetonitrile–acetic anhydride in proportion other than 1:1. The resolution of the end points was not satisfactory.

The proposed procedure makes possible a simple and accurate determination of isoniazid–sodium *p*-aminosalicylate combinations, without preliminary separation of the components. The technique is applicable to those mixtures in which sodium *p*-aminosalicylate-to-isoniazid ratio does not exceed 4.

REFERENCES

(1) B. W. Mitchell, E. A. Haugas, and C. S. McRoe, *J. Pharm. Pharmacol.*, **9**, 42(1957).

(2) D. C. Garrat, "The Quantitative Analysis of Drugs," 3rd ed., Chapman & Hall, Ltd., London, England, 1964, pp. 360, 561.

(3) J. Kucharsky and L. Safarik, "Titrations in Nonaqueous Solvents," Elsevier, Amsterdam-London-New York, 1965, pp. 182, 199.

(4) "Official Methods of Analysis," 9th ed., Assoc. Offic. Agr. Chemists, Washington, D. C., 1960, sec. 32.292.

(5) M. C. Fan and W. G. Wald, *J. Assoc. Offic. Agr. Chemists*, **48**, 1148(1965).
(6) M. C. Dutt and T. H. Chua, *J. Pharm. Pharmacol.*, **16**, 696(1964).
(7) M. B. Devani and C. J. Shishoo, *J. Pharm. Sci.*, **57**, 1051 (1968).
(8) J. F. Coetzee, G. P. Gunninghan, D. K. McGuire, and G. R. Padmanabhan, *Anal. Chem.*, **34**, 1139(1962).
(9) E. Kühni, M. Jacob, and H. Grossglauser, *Pharm. Acta Helv.*, **29**, 233(1954).
(10) A. Q. Butler and J. C. Ramsey, *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 338(1953).
(11) J. R. Stockton and R. Zuckerman, *ibid.*, **43**, 273(1954).
(12) L. G. Chatten, *ibid.*, **45**, 556(1956).
(13) J. S. Fritz, *Anal. Chem.*, **25**, 407(1953).
(14) J. S. Fritz and M. O. Fulda, *ibid.*, **25**, 1837(1953).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 18, 1969 from the *Department of Pharmaceutical Chemistry, L.M. College of Pharmacy, Ahmedabad-9/India.*
Accepted for publication August 28, 1969.
The authors are thankful to Dr. C. S. Shah for his interest in this work.

Nonaqueous Titrimetric Determination of Isoniazid in Presence of Excess of Sodium *p*-Aminosalicylate in Dosage Forms

C. J. SHISHOO and M. B. DEVANI

Abstract □ Isoniazid in the presence of excess of sodium *p*-aminosalicylate is estimated by differential titration of the acetonitrile extract of the mixture against perchloric acid. Sodium *p*-aminosalicylate content is then calculated from the total basicity obtained by direct titration of the mixture with the same titrant. The procedure is applied to determine the isoniazid and sodium *p*-aminosalicylate content of tablets, granules, and cachets. The results obtained are comparable to those obtained by the official method.

Keyphrases □ Isoniazid, Na *p*-aminosalicylate mixture—analysis □ Differential titration, nonaqueous—analysis □ Perchloric acid—titrant

Commercial dosage forms of isoniazid–sodium *p*-aminosalicylate combinations usually contain the components in molar proportions of 1:33. The conventional methods for the analysis of these dosage forms give erroneous and inconsistent results (1, 2).

Differential titration of isoniazid–sodium *p*-aminosalicylate mixture has been described earlier (3). The method is restricted only to the analysis of the mixtures having sodium *p*-aminosalicylate-to-isoniazid ratio up to 4:1.

In the present study, mixtures having large disproportion of the components were extracted with acetonitrile to remove the bulk of sodium *p*-aminosalicylate. The molar proportion of sodium *p*-aminosalicylate-to-isoniazid in the acetonitrile extract was favorable for the differential nonaqueous titration of these compounds. The nonaqueous titrimetric procedure described earlier (3) was applied to estimate isoniazid in the presence of sodium *p*-aminosalicylate in the acetonitrile extract and found to give satisfactory recovery of isoniazid. Sodium *p*-aminosalicylate content of the mixture was determined from total basicity obtained by direct titration of the mixture.

The procedure was applied to the analysis of tablets, granules, and cachets and the results were found comparable to those obtained by the official method (4).

Table I—Recovery of Isoniazid in the Presence of Excess Sodium *p*-Aminosalicylate

Amt. Weighed, meq.		Recovery, %	
Isoniazid	Sodium *p*-Aminosalicylate	Isoniazid[a]	Sodium *p*-Aminosalicylate[b]
0.1	0.5	100.42 ± 0.42[c]	99.90 ± 0.50
0.1	1.0	100.10 ± 0.39	100.00 ± 0.41
0.1	2.0	99.75 ± 0.52	100.10 ± 0.39
0.1	3.0	99.85 ± 0.47	99.73 ± 0.52
0.1	4.0	99.50 ± 0.37	99.85 ± 0.40
0.1	5.0	100.52 ± 0.53	99.95 ± 0.61

[a] Isoniazid content was determined from the titration of acetonitrile extract of the mixture. [b] Amount of sodium *p*-aminosalicylate was estimated from the total basicity of the mixture determined by direct titration, as described, after subtraction of the volume of titrant corresponding to isoniazid content of the mixture. [c] Standard deviation based on at least five determinations.

EXPERIMENTAL

Apparatus and Reagents—The reagents and apparatus were employed as described previously (3). The electrodes were dipped in acetic anhydride for 24 hr. before use.

Analysis of Mixtures Containing Isoniazid and Excess of Sodium p-Aminosalicylate—*Isoniazid*—Milliequivalent quantities of finely powdered isoniazid and sodium *p*-aminosalicylate as given in Table I were accurately weighed and transferred to a 100-ml. beaker. After adding 25 ml. of acetonitrile, the mixture was stirred vigorously for 20 min. and filtered. The residue in the beaker was treated with 10 ml. acetonitrile and stirred for 5 min. and filtered. It was further washed twice with 5 ml. of acetonitrile. An equal volume of acetic anhydride was added to the combined filtrates and washings. The mixture was stirred for 5 min. It was then titrated against 0.1 *N* acetous perchloric acid. Near the second end point, 0.05 ml. of titrant was added at an interval of 1 min. A blank titration was performed. Titration curves were obtained by plotting potential reading (mv.) *versus* volume (ml.) of the titrant.

Total Basicity—An aliquot of the mixture equivalent to 0.25 meq. of sodium *p*-aminosalicylate was accurately weighed and transferred to a 100-ml. beaker. Acetonitrile (25 ml.) was added to it and stirred. After addition of an equal volume of acetic anhydride, it was titrated as described previously.

Analysis of Dosage Forms—The procedure was applied to analyze the powder mass obtained from tablets, granules and cachets.

(5) M. C. Fan and W. G. Wald, *J. Assoc. Offic. Agr. Chemists*, **48**, 1148(1965).
(6) M. C. Dutt and T. H. Chua, *J. Pharm. Pharmacol.*, **16**, 696(1964).
(7) M. B. Devani and C. J. Shishoo, *J. Pharm. Sci.*, **57**, 1051 (1968).
(8) J. F. Coetzee, G. P. Gunninghan, D. K. McGuire, and G. R. Padmanabhan, *Anal. Chem.*, **34**, 1139(1962).
(9) E. Kühni, M. Jacob, and H. Grossglauser, *Pharm. Acta Helv.*, **29**, 233(1954).
(10) A. Q. Butler and J. C. Ramsey, *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 338(1953).
(11) J. R. Stockton and R. Zuckerman, *ibid.*, **43**, 273(1954).
(12) L. G. Chatten, *ibid.*, **45**, 556(1956).
(13) J. S. Fritz, *Anal. Chem.*, **25**, 407(1953).
(14) J. S. Fritz and M. O. Fulda, *ibid.*, **25**, 1837(1953).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 18, 1969 from the *Department of Pharmaceutical Chemistry, L.M. College of Pharmacy, Ahmedabad-9/India.*
Accepted for publication August 28, 1969.
The authors are thankful to Dr. C. S. Shah for his interest in this work.

Nonaqueous Titrimetric Determination of Isoniazid in Presence of Excess of Sodium *p*-Aminosalicylate in Dosage Forms

C. J. SHISHOO and M. B. DEVANI

Abstract □ Isoniazid in the presence of excess of sodium *p*-aminosalicylate is estimated by differential titration of the acetonitrile extract of the mixture against perchloric acid. Sodium *p*-aminosalicylate content is then calculated from the total basicity obtained by direct titration of the mixture with the same titrant. The procedure is applied to determine the isoniazid and sodium *p*-aminosalicylate content of tablets, granules, and cachets. The results obtained are comparable to those obtained by the official method.

Keyphrases □ Isoniazid, Na *p*-aminosalicylate mixture—analysis □ Differential titration, nonaqueous—analysis □ Perchloric acid—titrant

Commercial dosage forms of isoniazid–sodium *p*-aminosalicylate combinations usually contain the components in molar proportions of 1:33. The conventional methods for the analysis of these dosage forms give erroneous and inconsistent results (1, 2).

Differential titration of isoniazid–sodium *p*-aminosalicylate mixture has been described earlier (3). The method is restricted only to the analysis of the mixtures having sodium *p*-aminosalicylate-to-isoniazid ratio up to 4:1.

In the present study, mixtures having large disproportion of the components were extracted with acetonitrile to remove the bulk of sodium *p*-aminosalicylate. The molar proportion of sodium *p*-aminosalicylate-to-isoniazid in the acetonitrile extract was favorable for the differential nonaqueous titration of these compounds. The nonaqueous titrimetric procedure described earlier (3) was applied to estimate isoniazid in the presence of sodium *p*-aminosalicylate in the acetonitrile extract and found to give satisfactory recovery of isoniazid. Sodium *p*-aminosalicylate content of the mixture was determined from total basicity obtained by direct titration of the mixture.

The procedure was applied to the analysis of tablets, granules, and cachets and the results were found comparable to those obtained by the official method (4).

Table I—Recovery of Isoniazid in the Presence of Excess Sodium *p*-Aminosalicylate

Amt. Weighed, meq.		Recovery, %	
Isoniazid	Sodium *p*-Aminosalicylate	Isoniazid[a]	Sodium *p*-Aminosalicylate[b]
0.1	0.5	100.42 ± 0.42[c]	99.90 ± 0.50
0.1	1.0	100.10 ± 0.39	100.00 ± 0.41
0.1	2.0	99.75 ± 0.52	100.10 ± 0.39
0.1	3.0	99.85 ± 0.47	99.73 ± 0.52
0.1	4.0	99.50 ± 0.37	99.85 ± 0.40
0.1	5.0	100.52 ± 0.53	99.95 ± 0.61

[a] Isoniazid content was determined from the titration of acetonitrile extract of the mixture. [b] Amount of sodium *p*-aminosalicylate was estimated from the total basicity of the mixture determined by direct titration, as described, after subtraction of the volume of titrant corresponding to isoniazid content of the mixture. [c] Standard deviation based on at least five determinations.

EXPERIMENTAL

Apparatus and Reagents—The reagents and apparatus were employed as described previously (3). The electrodes were dipped in acetic anhydride for 24 hr. before use.

Analysis of Mixtures Containing Isoniazid and Excess of Sodium p-Aminosalicylate—*Isoniazid*—Milliequivalent quantities of finely powdered isoniazid and sodium *p*-aminosalicylate as given in Table I were accurately weighed and transferred to a 100-ml. beaker. After adding 25 ml. of acetonitrile, the mixture was stirred vigorously for 20 min. and filtered. The residue in the beaker was treated with 10 ml. acetonitrile and stirred for 5 min. and filtered. It was further washed twice with 5 ml. of acetonitrile. An equal volume of acetic anhydride was added to the combined filtrates and washings. The mixture was stirred for 5 min. It was then titrated against 0.1 *N* acetous perchloric acid. Near the second end point, 0.05 ml. of titrant was added at an interval of 1 min. A blank titration was performed. Titration curves were obtained by plotting potential reading (mv.) *versus* volume (ml.) of the titrant.

Total Basicity—An aliquot of the mixture equivalent to 0.25 meq. of sodium *p*-aminosalicylate was accurately weighed and transferred to a 100-ml. beaker. Acetonitrile (25 ml.) was added to it and stirred. After addition of an equal volume of acetic anhydride, it was titrated as described previously.

Analysis of Dosage Forms—The procedure was applied to analyze the powder mass obtained from tablets, granules and cachets.

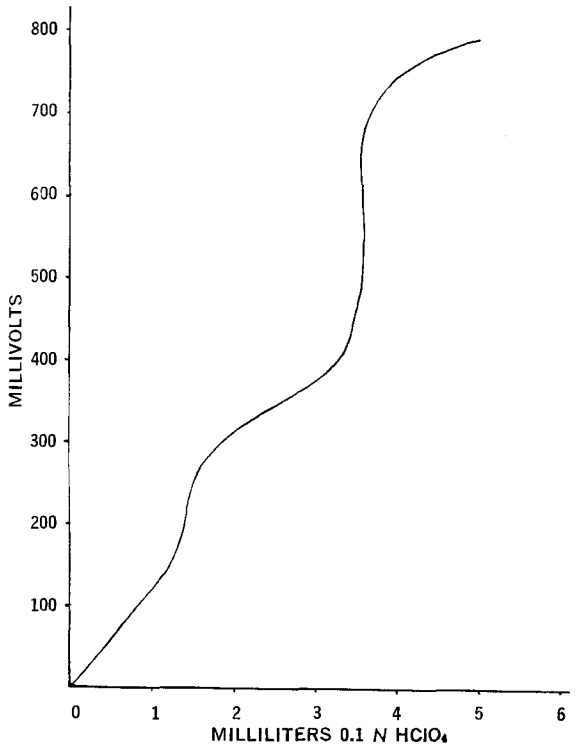


Figure 1—*Typical titration curve of acetonitrile extract of isoniazid–sodium* p*-aminosalicylate (1:50) mixture after diluting it with equal volume of acetic anhydride.*

Where magnesium stearate was used as a lubricant in the preparation of tablets, the aliquot of tablet mass equivalent to 0.25 meq. of sodium *p*-aminosalicylate was mixed with salicylaldehyde (3.0 ml.) and treated three times with 10-ml. quantities of acetonitrile and filtered. The combined filtrates were then diluted with equal volume of acetic anhydride and titrated as described to obtain reading for total basicity.

RESULTS AND DISCUSSION

At the very outset, it was evident that because of the large excess of sodium *p*-aminosalicylate associated with isoniazid in commercial dosage forms, a direct differential nonaqueous titrimetry would be of no avail (3).

The quantitative extraction of isoniazid–sodium *p*-aminosalicylate mixture has been attempted by several workers (5–7). However, either the separation of the components was not efficient or the procedure adopted was time consuming.

In the present work, advantage was taken of the difference in solubilities of these compounds in acetonitrile to obtain molar ratio of the components in the extract favorable for differential titration. Thus, the removal of the bulk of sodium *p*-aminosalicylate was effected by extracting the mixture with acetonitrile. The acetonitrile extract contained only a part of sodium *p*-aminosalicylate but the whole of isoniazid. The end points for both the components could be resolved in the titration of acetonitrile extract (after diluting it with equal volume of acetic anhydride) against acetous perchloric acid.

A typical titration curve thus obtained is reproduced in Fig. 1. The first inflection in the curve (see Fig. 1) corresponds to the sodium *p*-aminosalicylate while the second inflection denotes the end point corresponding to isoniazid present in the extract.

Acetonitrile extracts of a number of mixtures were analyzed by the proposed procedure for their isoniazid content (see Table I).

Table II—Analysis of Dosage Forms Containing Isoniazid and Sodium *p*-Aminosalicylate by Various Procedures

Dosage Form		Active Ingredients Labeled	Amt.,[a] mg.	Recovery in mg. By—Known Method Isoniazid	Known Method Na-PAS	Proposed Method Isoniazid	Proposed Method Na-PAS
Tablet	A	Isoniazid	25.0	24.3	840.0	24.0	845.0
		Na-PAS	834.0				
Tablet	B	Isoniazid	25.0	20.0	835.0	20.2	839.0
		Na-PAS	850.0				
Tablet	C	Isoniazid	30.0	29.0	995.0	29.3	1000.0
		Na-PAS	1000.0				
Tablet	D	Isoniazid	26.7	26.6	851.0	26.2	846.0
		Na-PAS	850.0				
Granules	A	Isoniazid	25.0[b]	25.2	782.0	25.5	788.0
		Na-PAS	780.0[b]				
Granules	B	Isoniazid	23.3[b]	23.5	779.1	23.1	776.0
		Na-PAS	777.0[b]				
Cachet	A	Isoniazid	33.0	33.0	1509.0	32.7	1500.0
		Na-PAS	1500.0				

[a] Per unit dosage. [b] Per gram of the granules.

The recovery of isoniazid from synthetic mixtures having sodium *p*-aminosalicylate-to-isoniazid ratio as high as 50:1 was found to be satisfactory.

For the estimation of sodium *p*-aminosalicylate, an aliquot of the mixture dissolved in acetonitrile–acetic anhydride (1:1) mixture is titrated against acetous perchloric acid. Only one inflection point in the titration curve is obtained which corresponds to total basicity of the mixture. The amount of sodium *p*-aminosalicylate present in the mixture can be calculated from the reading for the total basicity of the mixture after subtracting the volume of the titrant required for isoniazid.

The procedure was applied to analyze commercial dosage forms. The results agreed closely with those obtained by the official method (4) (see Table II). The usual diluents, lubricants, and colors used in the preparation of dosage forms did not appear to interfere with the titration. However, the presence of magnesium stearate in tablets as lubricant induces high recoveries of sodium *p*-aminosalicylate. In such a case, the sample of the powdered tablet was treated with salicylaldehyde and extracted with acetonitrile. The solubility of sodium *p*-aminosalicylate increases in the presence of salicylaldehyde, so that its complete extraction is effected with a relatively small volume of solvent. The extract after diluting with equal volume of acetic anhydride is titrated as usual. The method gave satisfactory recovery of sodium *p*-aminosalicylate.

REFERENCES

(1) B. W. Mitchell, E. A. Haugas, and C. S. McRoe, *J. Pharm. Pharmacol.*, **9**, 42(1957).

(2) K. Lee and Y. Ho, *ibid.*, **14**, 123(1962).

(3) M. B. Devani and C. J. Shishoo, *J. Pharm. Sci.*, **59**, 90(1970).

(4) "British Pharmaceutical Codex 1963," Supplement, The Pharmaceutical Press, London, England, 1966, p. 67.

(5) R. Biffoli, *Boll. Lab. Chim. Provinciali Bologna*, **4**, 83(1953).

(6) M. C. Dutt and T. H. Chua, *J. Pharm. Pharmacol.*, **16**, 69 (1964).

(7) L. H. Welsh, *J. Assoc. Offic. Agr. Chemists*, **40**, 807(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 18, 1969 from the *Department of Pharmaceutical Chemistry, L.M. College of Pharmacy, Ahmedabad-9/India.*

Accepted for publication August 28, 1969.

The authors are thankful to Dr. C. S. Shah for his interest in this work.

Sampling and Analysis of Oxygen in the Void Volume of Ampuls and Vials

L. F. CULLEN and G. J. PAPARIELLO

Abstract □ A technique recognized as a considerable aid in stabilizing parenteral formulations sensitive to oxidative decomposition consists of replacing air in the container's headspace with an inert atmosphere. To monitor and investigate inert gas flushing techniques and to assist in evaluating antioxidant suitability, a procedure is described for the sampling and analysis of gaseous oxygen content in the void volume headspace of ampuls and vials. The procedure utilizes a paramagnetic oxygen analyzer in conjunction with a unique sampling cell and flow system which permit the collection of the headspace gas without atmospheric contamination. The sampling device and flow system were designed and evaluated to provide the necessary sensitivity, accuracy, and precision for the quantitative analysis of low air contaminant levels in the sample gas. With this method it is possible to analyze nine samples per hour with a relative standard deviation of 3.9% at a 19-μl. air level (*i.e.*, 5 mcg. of oxygen), which corresponds to approximately 2% of the theoretical gaseous void volume of a typical 1-ml. ampul product. Accuracy of the technique is demonstrated by experimentally confirming the relationship of headspace oxygen consumption to intact bisulfite and metabisulfite antioxidant content, following accelerated storage, in sealed ampuls.

Keyphrases □ Oxygen sampling, analysis—void volumes, ampuls, vials □ Sampling device, void volume oxygen—diagram □ Ampuls—oxygen determination □ Vials—oxygen determination

The adverse influence of atmospheric oxygen on both the physical and chemical stability of parenteral formulations and the numerous problems arising from the oxidative decomposition of drugs in the development of new products has been emphasized repeatedly in the past (1–6). The inclusion of a suitable antioxidant system to inhibit the oxidative process and the replacement of air in the headspace of a product's container with an inert atmosphere are generally recognized as considerable aids in stabilizing an injectable formulation. However, prior to the recent development of a method for measuring the oxygen level in the headspace of vial products (7), a survey of the literature failed to disclose any other analytical procedures specifically designed to monitor the efficiency of the inert gas flushing technique on the various parenteral containers. Thus, the pharmaceutical manufacturer has been in the dark with regard to the effectiveness of his oxygen exclusion process employed during the filling operation.

Clearly, a need existed for a sensitive, accurate, and rapid procedure for sampling and analysis of low air contamination levels in the void volume of inert gas flushed parenteral products, including ampuls. Such a technique could be employed to monitor and investigate the inert gas flushing processes and to assist in evaluating antioxidant suitability.

The accurate sampling of the void volume from a sealed, glass ampul within a controlled oxygen-free atmosphere and subsequent analysis, under similarly controlled conditions, for low oxygen levels is indeed difficult. Existing methods of oxygen analysis could not be readily modified to handle this problem. Several of the wide variety of methods which have been employed to determine gaseous oxygen vary from a highly sensitive luminescent-bacterial method (8), to colorimetric methods (9–12), microvolumetric (13, 14) and manometric (15) techniques, galvanic cell-type systems (16, 17), and coulometric procedures (18, 19). Many of these methods have the necessary accuracy and sensitivity at the oxygen level considered; however, they are either time-consuming and/or require meticulous care for satisfactory results. Furthermore, none of the procedures offered a convenient system for the sampling of the headspace gas within a sealed, glass ampul. Rapid and precise polarographic (7, 20) and gas chromatographic procedures (21, 22) which have found extensive use in oxygen analysis are also restricted by not affording systems readily adaptable to the collection and analysis of the headspace gas under anaerobic conditions.

Several commercial instruments are available which utilize the paramagnetic property of oxygen for its determination. This instrumentation is primarily employed for the continuous monitoring of the oxygen levels of expired air in respiratory physiology (23–25). The reported accuracy and sensitivity of the Beckman E2 Oxygen Analyzer (26), which has available a small volume internal analysis system equipped to measure nonflowing samples and an adaptable sample inlet port, suggested a promising approach. A consideration of the capabilities and limitations of this instrument led to the design of a unique sampling cell and flow system, of definite and critical specifications, which were evaluated to provide an accurate and rapid sampling technique for the analysis of the headspace gas of ampul products. The design of the sampling device permitted an extension of the procedure to the analysis of low air contamination levels in the void volume of inert gas-flushed injectable vial products. With this method it is possible to analyze nine ampuls and/or vials per hour.

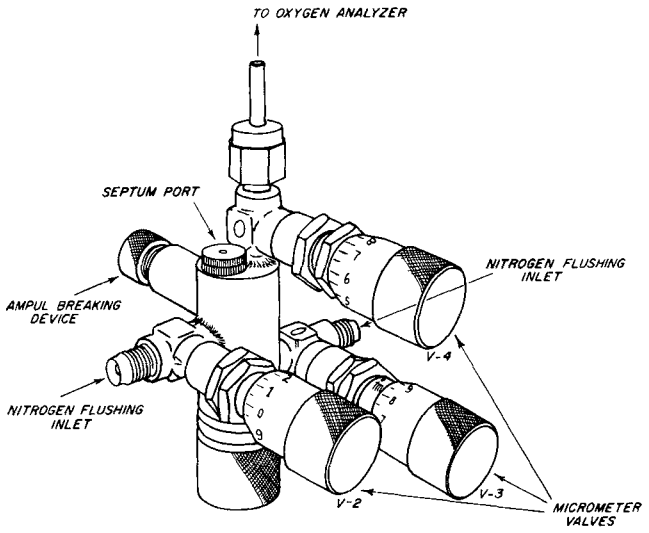


Figure 1—*Headspace gas sampling device.*

EXPERIMENTAL

Sampler Construction—The sampling device, designed to collect the headspace gas in an ampul without atmospheric contamination, is illustrated in Fig. 1. Dimensional details of this cell are given in Fig. 2. The O-ring-sealed components of the sampler, the sleeve adapter and chamber plug were machined from brass rods. For fine control of the flushing gas during deaeration of the sampling cell and to prevent damage to the detector of the analyzer from pressure surges, minimum void volume metering valves[1] were fitted to the sample chamber. Following the removal of a port connector on each of the metering valves, the valves were soldered directly onto openings in the cell, as shown in Fig. 1. This reduced the increase in total void space of the sampler introduced by the micrometer valves and eliminated the need for additional fittings. The septum inlet assembly is used for calibration purposes in the ampul procedure and as the injection port for a gas-tight syringe containing headspace gas sampled from vial products.

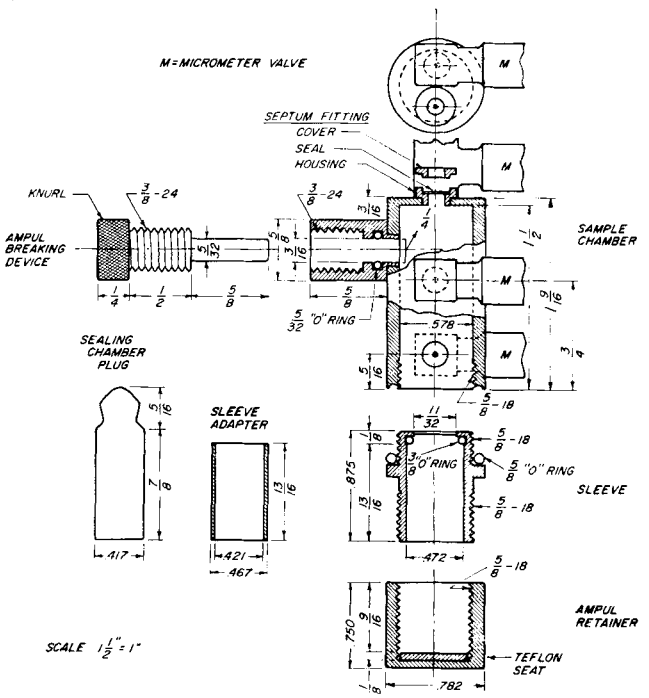


Figure 2—*Dimensional details of sampling device.*

[1] Nupro model B-2S, Nupro Co., Cleveland, Ohio.

Apparatus—A Beckman E2 Oxygen Analyzer[2] equipped with 0–1 and 0–10% oxygen percentage ranges was employed in all experiments. The sensitive detection system of the analyzer utilizes the magnetic susceptibility of oxygen to provide the basis for a quantitative evaluation of the oxygen content in the sample gas. Detailed discussions of this characteristically strong property of oxygen and how it is applied to the paramagnetic oxygen analyzers were described by Catton (27) and Johnson (28).

Accurate flow control of nitrogen during deaeration of the sampling device was provided by a flowmeter unit.[3]

A vacuum pump[4] in conjunction with a fine metering valve[5] permitted a complete and graduated evacuation of the flow system and analysis cell of the oxygen analyzer. A McLeod-type vacuum gauge[6] was inserted into the manifold to monitor the efficiency of the evacuation process.

Rubber vacuum tubing (0.32 cm. [0.125 in.] i.d. × 0.32 cm. [0.125 in.] wall thickness)[7] was connected to each of the threaded fittings of the metering valves and sample ports of the oxygen analyzer with 0.95 cm. (0.375 in.) o.d. tubing clamps. To minimize the total void volume of the actual analysis system, only 3.81-cm. (1.5-in.) lengths of this vacuum tubing were employed.

Pressure-Lok gas-tight syringes of 0.5-ml. and 1.0-ml.[8] capacity were used to calibrate the system in the analysis of both ampul and vial products and to sample the headspace gas in the analysis of vial products.

Analytical Procedures—*Ampul Products*—A diagram of the flow system indicating the equipment arrangement for the analytical method is shown in Fig. 3. The collection of an uncontaminated sample of headspace gas followed by conveyance of the gaseous sample from the sampling device to the sensitive detector of the oxygen analyzer consists of the following steps: (*a*) deaeration, with nitrogen,[9] of the sampling device containing the sealed ampul; (*b*) complete evacuation of the analysis cell to purge it of air; and (*c*) the introduction of the sample or calibration gas into the cell. Exact flow rate conditions are described to avoid sudden pressure changes which can damage the magnetic unit.

In operation, the sealed ampul is placed into the sleeve component of the sampler and pressed into firm contact with the 0.95 cm. (0.375 in.) O-ring seal with the ampul retainer. This assembly is inserted into the sample chamber and tightened. In preparation for deaeration of the sampling device and flow system, as illustrated in Fig. 3, the flowmeter needle valve (V-1) is closed, and the second stage of a two-stage regulator on the nitrogen cylinder is set to deliver 10 psig Needle valves V-2 and V-3 are opened to provide a nitrogen gas flow rate of approximately 125 ml./min. Valves V-4 and V-5 are set to permit a flow rate of 250 ml./min., with the T-bore stopcock (T-6) vented to the atmosphere. To nitrogen-flush the sampling device, slowly open flowmeter needle valve (V-1) until a flow rate of 220 ml./min. is established as indicated by a 105 mm. metering-tube reading with the Pyrex float. After a 2-min. flush close valve V-1, followed by valves V-2, V-3, and V-4 in successive order. Break ampul, then evacuate flow system and detector by closing valve V-5 to produce a flow rate of 70 ml./min., setting stopcock (T-6) for system evacuation, and drawing a vacuum for 1.5 min. To ensure a relatively complete deaeration process, a vacuum reading after approximately 1.25 min. of this evacuation period should indicate a system pressure of less than 0.4 mm. Hg. To convey sample gas mixture to the evacuated detector of the analyzer for compositional analysis, close valve V-5 and slowly open valve V-4 to a flow rate of 100 ml./min. Following sample readout, the magnetic unit is gradually adjusted to atmospheric pressure by venting stopcock (T-6) to the atmosphere and slowly opening valve V-5 to deliver a flow rate of 70 ml./min. The sampling cell pressure

[2] Beckman catalog No. 118521, Beckman Instruments, Inc., Fullerton, Calif.
[3] Matheson model 620BBV with No. 602 metering tube, Matheson Co., Inc., East Rutherford, N. J.
[4] Welch model 1400B, W. M. Welch Scientific Co., Skokie, Ill.
[5] Nupro model B-2S.
[6] Nester/Faust model 68, Nester/Faust Manufacturing Corp., Newark, Del.
[7] Thomas catalog No. 8844, Arthur H. Thomas Co., Philadelphia, Pa.
[8] Models 306000-A and 306001-A, Precision Sampling Corp., Baton Rouge, La.
[9] Prepurified grade, Matheson Co., Inc.

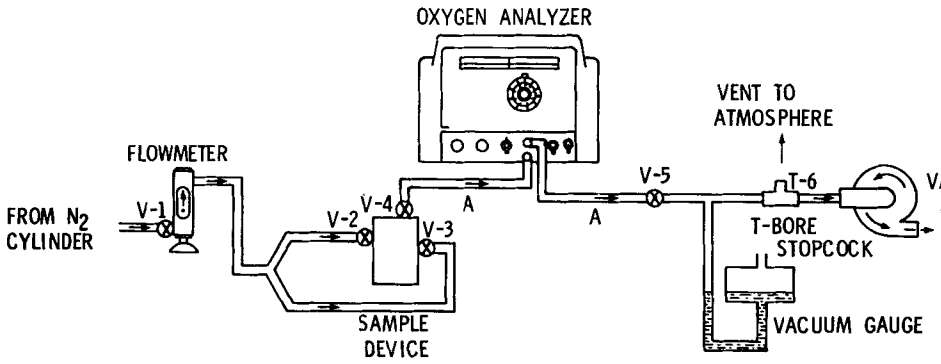


Figure 3—*Flow system for oxygen analysis.*

is brought to atmospheric by opening valve V-4 to provide a flow rate of 250 ml./min.

The exact flow system conditions described above for the analysis of the headspace gas are used in calibration of the system. Calibration is effected by injecting, in duplicate, known volumes of air at the appropriate level into the nitrogen-purged sampling device, which contains an ampul. Standardization is repeated at the end of a series of 20 samples in order to minimize the effects of instrumental variations. Calculations are made using corresponding instrument responses of standards and headspace gas of ampul products.

Vial Products—The flow system, as illustrated in Fig. 3, and exact analytical conditions described under *Ampul Products* for conveyance of the sample gas from the sampling device to the detector were employed in the analysis of vial products. The chamber plug, dimensionally detailed in Fig. 2, is inserted to seal the sampling chamber. In sampling, an accurately measured portion of the headspace gas is drawn into a gas-tight syringe and locked in the syringe prior to withdrawal from the vial. This is followed by injection into the nitrogen-flushed sampling cell after penetration of the septum and opening the actuation valve of the syringe. The volume of the headspace gas mixture sampled should, preferably, contain the equivalent of 0.02–2.5 ml. of air. It should be noted that during the vial sampling process a partial vacuum is created. Consequently, a correction must be made for this sampling effect in order to determine the actual sample volume.

The system is calibrated by injecting appropriate levels of air, sampled directly from the atmosphere, into the nitrogen-purged sampling device. Using this standardization technique and the Eq. 1 which corrects for the previously described sampling effect, the percent air in the sample can be calculated.

$$\% \text{ air (v/v)} = \frac{(R_V)(A_S)(V_T)100}{(R_S)(V_V)} \qquad \text{(Eq. 1)}$$

where R_V = response of sample; R_S = response of standard; A_S = volume of air injected for calibration; V_V = measured void volume of vial; V_T = measured void volume of vial (V_V) plus the indicated sample volume of the gas-tight syringe.

As an alternate technique in standardization of the system, known volumes of either air or air-inert gas calibration mixtures are sampled from vial packages filled with water to yield a headspace volume equivalent to the vial products. Employing the gas-tight syringe to collect, store, and transport the headspace gas as described previously, the withdrawal of identical volumes of sample and reference gas from equal voids eliminates a consideration of the dilution factor introduced in the sampling process, as required in Eq. 1. Calculations are made using the corresponding instrument responses of standards and samples of the headspace gas of vial products.

RESULTS AND DISCUSSION

Design of Sampling Device—The major consideration in the development of the sampling system was to design a sampling cell of a sufficiently low void volume to provide the necessary sensitivity for accurately monitoring inert gas flushing efficiency at levels greater than 98%, *i.e.*, less than 2% air contamination. Two percent air contamination corresponded to a 20-μl. air level (*i.e.*, 5 mcg. of oxygen) in the typical 1-ml. ampul product investigated. At such levels of oxygen, the analyzer has a reported accuracy of ±1.7 μl. air (±0.4 μl. oxygen) and a limit of detection of 0.2 μl. air in the sample gas with its 3.5-ml. internal analysis system at 760 mm. pressure (28). However, with a gaseous void volume of approximately 1 ml. for most ampul products, insufficient sample gas is available to satisfy the detector at atmospheric pressure. Thus, it was realized that a special flow system, as described under *Analytical Procedure*, would be required which results in the analysis of the sample gas at a reduced pressure. Under these operating conditions, the accuracy and sensitivity characteristics of the detector are reduced, necessitating an evaluation of pressure effects in establishing the optimum sample chamber volume for the sampling device.

In designing the cell for optimum accuracy and sensitivity conditions, a balance had to be considered between: (*a*) satisfying the pressure requirements of the detector by increasing the void space of the sample chamber in order to approach atmospheric conditions in the analysis cell; (*b*) reducing the volume of the sample chamber to prevent a loss in sensitivity from an unnecessary high dilution with the inert gas. Employing the ideal gas laws to calculate the effect of sample concentration and pressure on instrument readout (28), the dimensional parameters of the sampling system were derived. A graphic representation of the effect of sample cell volume on the obtainable theoretical accuracy is shown in Fig. 4.

It was calculated that a sample chamber and associated metering valves with a 5.2-ml. void volume provided the optimum analysis conditions. A sampling device of these dimensions has an ultimate, theoretical accuracy of ±7 μl. air and a limit of detection of 0.7 μl. air. The sampling device and flow system have been fabricated to these specifications (see Figs. 2 and 3). Thus, the indicated accuracy and sensitivity to monitor inert gas flushing efficiency at levels greater than 98% has been built into the system.

Linearity and Sensitivity—A typical calibration curve obtained by injecting known volumes of air into the sampling device in the

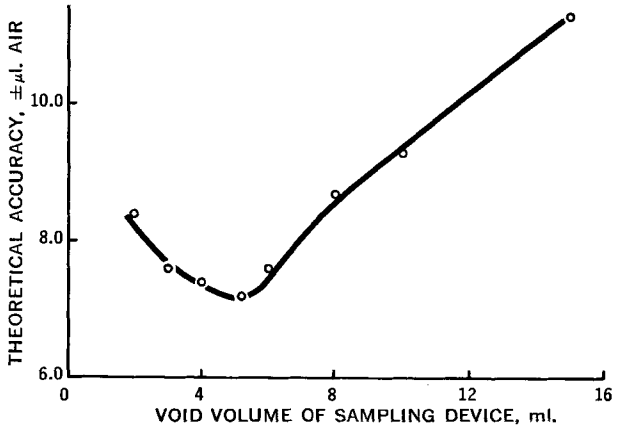


Figure 4—*Effect of void volume of sample device on the theoretical accuracy of system.*

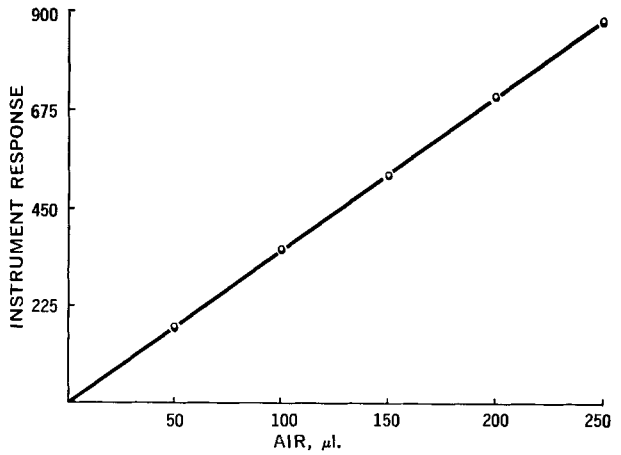


Figure 5—*Relationship of instrument response to air level.*

50–250 μl. range, equivalent to 14–68 mcg. of oxygen, demonstrates the linearity of the instrument response-air level relationship (Fig. 5). These data had been collected with the oxygen analyzer set at its most sensitive operating range, *i.e.*, the 0–1% oxygen percentage range. The 0–10% range on the instrument extends the applicability of the sampling and flow system (Fig. 3) to include the analysis of most ampul and vial parenteral products at any level of inert gas flush efficiency by permitting an evaluation of headspace gas which contains 0.25 to 2.5 ml. of air. A linear relationship exists between instrument response and air level at these higher concentrations.

The actual measured lower limit of sensitivity of both the ampul and vial procedures is 2 μl. of air in the sample gas mixture. This air level represents 0.2% of the total void volume of the typical 1-ml. and 2-ml. ampul products. The determined sensitivity of 2 μl. of air approaches the theoretical sensitivity of 0.7 μl. of air.

Ampul Procedure—*Precision*—Repeatability of the procedure was determined by assaying the headspace gas of 20 individual ampuls flushed with a 0.4% (v/v) oxygen-nitrogen calibration gas mixture.[10] Experimentally, 1.0-ml. aliquots of water were introduced into 1-ml. ampuls under an atmosphere of the oxygen-nitrogen gas mixture within an inflatable polyethylene glove chamber. Using the gas mixture the ampuls were flushed for exactly 20 min., then immediately sealed under the oxygen-nitrogen atmosphere with approximately 1 ml. of headspace above the water. Following the analysis of the headspace gas, the void volumes of the individual ampuls were measured and calculated into the results. This eliminated any differences in the void volumes of the ampuls produced by the sealing process. Reported in terms of air at 25° and 760 mm. Hg, a relative standard deviation of 3.9% was determined at the 19-μl. air level. The 19-μl. air level, which corresponds to 98.1% nitrogen flushing efficiency, demonstrates the precision of the procedure at a high level of inert gas layering.

Accuracy—Accuracy of the ampul procedure was established by experimentally determining the relationship of oxygen consumption to intact bisulfite and metabisulfite content after interaction in sealed ampuls. In this study, 1.0 ml. of an 0.1% solution of the antioxidant (sodium bisulfite or sodium metabisulfite) was introduced into 1-ml. ampuls and flushed with air-nitrogen gas mixtures in order to obtain either 60 or 500 μl. air per 1 ml. of ampul void volume. A gas proportioning unit[11] provided accurate metering of air and nitrogen to the desired ratio during this flushing process. The ampuls were sealed to contain a void volume of about 1 ml. under the air-nitrogen atmosphere, then immediately assayed for the initial antioxidant content (29) and headspace air. The ampuls were stored at temperatures from 35 to 60° to accelerate oxygen consumption and assayed for antioxidant and headspace air content at periodic time intervals. Typical data collected in this study are summarized in Table I.

The theoretical consumptions of sodium bisulfite and sodium metabisulfite by the oxygen content of 1.00 ml. of air at 25° and 760 mm. are 1.77 and 1.62 mg., respectively (6). The results indicate the experimental values are in excellent agreement with theory. Data obtained on ampuls containing less than 10 μl. of air in the headspace gas, *i.e.*, less than 1% air in the typical 1-ml. and 2-ml. ampul products, demonstrate that the technique quite adequately meets the accuracy requirements for monitoring inert gas flushing efficiency at levels greater than 98%.

Table I—Relationship of Consumed Air to Intact Antioxidant Level

Reaction Condition	Air Remaining per Ampul, μl[a]	Antioxidant Remaining per Ampul, mg.[a]	Antioxidant Reacted per Ampul, mg.	Air Consumed per Ampul, μl. Exptl.	Theoret.
Sodium Metabisulfite					
30 hr., 35°	22	0.89	0.07	41	43[b]
48 hr., 45°	9	0.87	0.09	58	56[b]
48 hr., 60°	4	0.86	0.10	61	62[b]
24 hr., 35°	161	0.43	0.53	332	329[b]
48 hr., 60°	35	0.22	0.74	455	459[b]
Sodium Bisulfite					
30 hr., 35°	22	0.85	0.07	39	37[c]
48 hr., 45°	6	0.83	0.09	53	51[c]
24 hr., 35°	192	0.39	0.53	297	300[c]
48 hr., 60°	48	0.14	0.78	443	442[c]

[a] Average assay value on five individual ampuls (air analyses $\sigma_{av.} = \pm 3.2\%$; antioxidant analyses $\sigma_{av.} = \pm 1.9\%$). [b] Calculated from the relationship that 1.62 mg. of $Na_2S_2O_5$ is equivalent to 1.00 ml. air at 25°, 760 mm. [c] Calculated from the relationship that 1.77 mg. of $NaHSO_3$ is equivalent to 1.00 ml. air at 25°, 760 mm.

Vial Procedure—*Precision and Accuracy*—Both the precision and accuracy of this technique were obtained by performing replicate assays on known volumes of air injected into nitrogen-flushed vials with a butyl rubber stopper-aluminum cap closure and measuring the percentage recovery. This was accomplished by flushing and crimping empty vials, with accurately measured void volumes of approximately 15 ml., under an absolute nitrogen atmosphere. Following the removal and analysis of a portion of the headspace gas from the individual vials to correct for residual air in the flushing process, 0.15 and 1.50-ml. levels of air were injected into the vials, producing synthetic air contamination levels at 1.0 and 10%, respectively. Data collected on replicate assays of 15 individual vials at the 1.0% contamination level indicated recoveries of 97 to 103% of the theoretical amount present with a relative standard deviation of 4.2%. Recoveries of 99 to 101% of theory were obtained on 15 individual vials at the 10% air contamination level with a relative standard deviation of 2.1%. In this phase of the study, the data were calculated as described by Eq. 1.

REFERENCES

(1) F. S. Hom and J. Autian, *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 608(1956).

(2) S. A. Schou, *Am. J. Hosp. Pharm.*, **17**, 153(1960).

(3) S. Yeh and J. L. Lach, *J. Pharm Sci.*, **50**, 35(1961).

(4) L. C. Schroeter, *ibid.*, **50**, 891(1961).

(5) E. W. Martin, "Remington's Practice of Pharmacy," 13th ed., Mack Publishing Co., Easton, Pa., 1965.

(6) L. C. Schroeter, "Sulfur Dioxide," Pergamon Press, New York, N. Y., 1966.

(7) M. Brown and L. J. Leeson, *J. Pharm. Sci.*, **58**, 242(1969).

(8) K. P. Meyer, *Helv. Phys. Acta*, **15**, 3(1942).

(9) I. Mellan, "Organic Reagents in Inorganic Analysis," Blakiston, Philadelphia, Pa., 1941.

(10) J. A. Shaw, *Ind. Eng. Chem., Anal. Ed.*, **14**, 891(1942).

(11) D. D. Williams, C. H. Blachly, and R. R. Miller, *Anal. Chem.*, **24**, 1819(1952).

(12) P. B. Sweetser, *ibid.*, **39**, 979(1967).

(13) V. M. Lewis, *ibid.*, **21**, 635(1949).

(14) D. M. Updegraff and W. B. Huckabay, *Anal. Biochem.*, **5**, 28(1963).

[10] Matheson Co., Inc.
[11] Matheson model 665, Matheson Co., Inc.

(15) J. N. Pitts, D. D. DeFord, and G. W. Recktenwald, *Anal. Chem.*, **24,** 1566(1952).
(16) P. Hersch, *Nature*, **169,** 792(1952).
(17) J. M. Ives, E. E. Hughes, and J. K. Taylor, *Anal. Chem.*, **40,** 1853(1968).
(18) F. A. Keidel, *Ind. Eng. Chem.*, **52,** 490(1960).
(19) W. G. Knapp, *Anal. Chem.*, **31,** 1463(1959).
(20) I. M. Kolthoff and J. J. Lingane, "Polarography," vol. II, 2nd ed., Interscience, New York, N. Y., 1952.
(21) P. G. Jeffery and P. J. Kipping, "Gas Analysis by Gas Chromatography," Macmillan, New York, N. Y., 1964.
(22) I. Lysyj and P. R. Newton, *J. Chromatog.*, **11,** 173(1963).
(23) R. J. Shephard, *Intern. Z. Angew. Physiol.*, **22,** 279(1966).
(24) S. Barold, F. Burkart, and E. Sowton, *Brit. Heart J.*, **28,** 776(1966).
(25) K. Winkler and N. Tygstrup, *Scand. J. Clin. Lab. Invest.*, **10,** 221(1958).
(26) Technical Bulletin No. 7081, Beckman Instruments, Inc., Scientific and Process Instruments Division, Fullerton, Calif.
(27) W. T. Catton, "Physical Methods in Physiology," Pitman and Sons, Ltd., London, England, 1957.
(28) D. Johnson, Instruction Manual 1023-B, Beckman Instruments, Inc., Scientific and Process Instruments Division, Fullerton, Calif.
(29) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from *Wyeth Laboratories, Pharmacy Research and Development Division, Radnor, PA 19101*
Accepted for publication August 8, 1969.
Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May, 1969.
The authors acknowledge assistance of E. J. Adams and J. J. Peterson in the fabrication of the sampling device.

NOTES

Muscarinic Agents: The Isomeric 6-Acetoxy-2-methylisoquinuclidine Methiodides

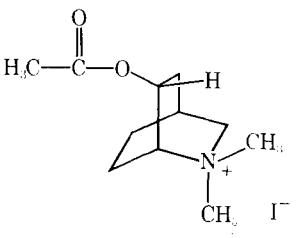
WENDEL L. NELSON and RAYMOND S. WILSON*

Abstract □ Preparation of 6-*endo* and 6-*exo*-acetoxy-2-methylisoquinuclidine methiodides are described. Muscarinic assay data are reported. Neither of the compounds showed activity when compared to acetylcholine and 3-acetoxyquinuclidine methiodide.

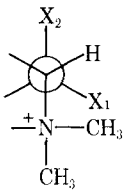
Keyphrases □ 6-Acetoxy-2-methylisoquinuclidine methiodides—synthesis □ Pharmacological screening—6-acetoxy-2-methylisoquinuclidine methiodides □ IR spectrophotometry—identity, structure □ NMR spectroscopy—structure

Hypotheses delineating the architectural features of cholinergic receptors have been based on observations of pharmacological activity of various substituted derivatives of the neurohormone, acetylcholine. Differences in the activity of these analogs of acetylcholine have long been explained on the basis of molecular steric and electronic effects in the drug-receptor interaction (1, 2). Spectral data concerned with conformational aspects of acetylcholine have also been studied and developed in recent attempts to describe receptor site architecture (3–7).

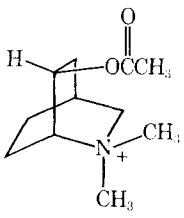
In further studies to determine the conformational requirements of the drug-receptor complex, in which the authors' assume a large degree of complementarity of the drug and receptor in this interaction, a number of conformationally rigid or semirigid analogs of acetylcholine have been prepared (8–13). Each, although incorporating the essential features for cholinergic activity, also inherently must be constructed of an additional number of carbons to maintain the desired conformational rigidity. Comparison of activities of agents structurally similar to each other seems valid.



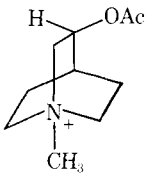
I



IA, $X_1 = H, X_2 = OAc$
IIA, $X_1 = OAc, X_2 = H$



II



VIII

PREPARATION OF ANALOGS

In a program of preparation of agents to further determine the geometric requirements of the muscarinic agents, it was decided to prepare cholinergic analogs in the isoquinuclidine system in which

(15) J. N. Pitts, D. D. DeFord, and G. W. Recktenwald, *Anal. Chem.*, **24,** 1566(1952).
(16) P. Hersch, *Nature*, **169,** 792(1952).
(17) J. M. Ives, E. E. Hughes, and J. K. Taylor, *Anal. Chem.*, **40,** 1853(1968).
(18) F. A. Keidel, *Ind. Eng. Chem.*, **52,** 490(1960).
(19) W. G. Knapp, *Anal. Chem.*, **31,** 1463(1959).
(20) I. M. Kolthoff and J. J. Lingane, "Polarography," vol. II, 2nd ed., Interscience, New York, N. Y., 1952.
(21) P. G. Jeffery and P. J. Kipping, "Gas Analysis by Gas Chromatography," Macmillan, New York, N. Y., 1964.
(22) I. Lysyj and P. R. Newton, *J. Chromatog.*, **11,** 173(1963).
(23) R. J. Shephard, *Intern. Z. Angew. Physiol.*, **22,** 279(1966).
(24) S. Barold, F. Burkart, and E. Sowton, *Brit. Heart J.*, **28,** 776(1966).
(25) K. Winkler and N. Tygstrup, *Scand. J. Clin. Lab. Invest.*, **10,** 221(1958).
(26) Technical Bulletin No. 7081, Beckman Instruments, Inc., Scientific and Process Instruments Division, Fullerton, Calif.
(27) W. T. Catton, "Physical Methods in Physiology," Pitman and Sons, Ltd., London, England, 1957.
(28) D. Johnson, Instruction Manual 1023-B, Beckman Instruments, Inc., Scientific and Process Instruments Division, Fullerton, Calif.
(29) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from *Wyeth Laboratories, Pharmacy Research and Development Division, Radnor, PA 19101*
Accepted for publication August 8, 1969.
Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May, 1969.
The authors acknowledge assistance of E. J. Adams and J. J. Peterson in the fabrication of the sampling device.

NOTES

Muscarinic Agents: The Isomeric 6-Acetoxy-2-methylisoquinuclidine Methiodides

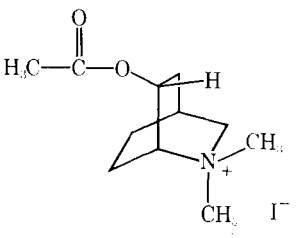
WENDEL L. NELSON and RAYMOND S. WILSON*

Abstract □ Preparation of 6-*endo* and 6-*exo*-acetoxy-2-methylisoquinuclidine methiodides are described. Muscarinic assay data are reported. Neither of the compounds showed activity when compared to acetylcholine and 3-acetoxyquinuclidine methiodide.

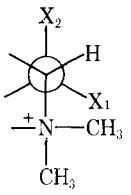
Keyphrases □ 6-Acetoxy-2-methylisoquinuclidine methiodides—synthesis □ Pharmacological screening—6-acetoxy-2-methylisoquinuclidine methiodides □ IR spectrophotometry—identity, structure □ NMR spectroscopy—structure

Hypotheses delineating the architectural features of cholinergic receptors have been based on observations of pharmacological activity of various substituted derivatives of the neurohormone, acetylcholine. Differences in the activity of these analogs of acetylcholine have long been explained on the basis of molecular steric and electronic effects in the drug-receptor interaction (1, 2). Spectral data concerned with conformational aspects of acetylcholine have also been studied and developed in recent attempts to describe receptor site architecture (3–7).

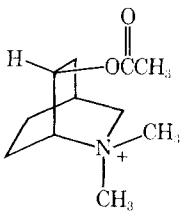
In further studies to determine the conformational requirements of the drug-receptor complex, in which the authors' assume a large degree of complementarity of the drug and receptor in this interaction, a number of conformationally rigid or semirigid analogs of acetylcholine have been prepared (8–13). Each, although incorporating the essential features for cholinergic activity, also inherently must be constructed of an additional number of carbons to maintain the desired conformational rigidity. Comparison of activities of agents structurally similar to each other seems valid.



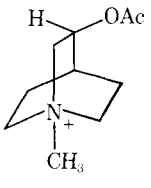
I



IA, $X_1 = H, X_2 = OAc$
IIA, $X_1 = OAc, X_2 = H$



II



VIII

PREPARATION OF ANALOGS

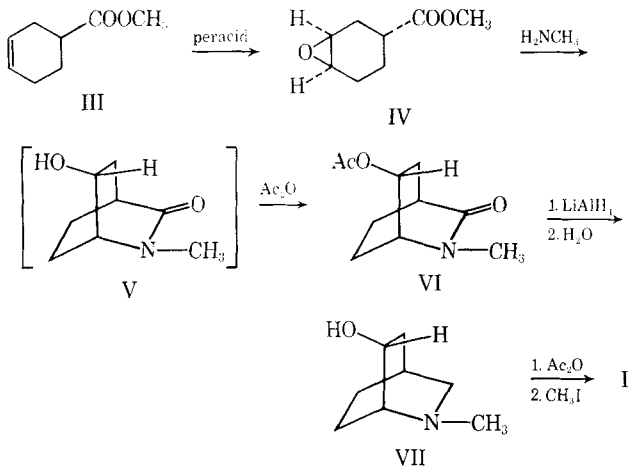
In a program of preparation of agents to further determine the geometric requirements of the muscarinic agents, it was decided to prepare cholinergic analogs in the isoquinuclidine system in which

the quaternary ammonium head is held in a fixed position by the carbocyclic skeleton. The compounds, 6-*endo*- and 6-*exo*-acetoxy-2-methylisoquinuclidine methiodides,[1] I and II, represent *anti* and *gauche* conformations of acetylcholine, IA and IIA, respectively, in a fused system.

Preparation of I was accomplished beginning with methyl 3-cyclohexencarboxylate (III) (Scheme I). Epoxidation of III with *m*-chloroperbenzoic acid produced epoxide IV. The authors could not detect the presence of more than a single epoxide, which has been assigned the *trans* stereochemistry by Henbest (14). It would not be unusual to expect both epoxides, which may be both dependent on solvent and oxidizing agent (15).

The epoxide (IV) was allowed to react with methylamine, with the expected *trans*-diaxial opening by this nucleophile. The reaction mixture was heated at reflux to effect ring closure to the isoquinuclidone, V, as well as dimeric and polymeric products. Similar opening of this epoxide has been reported by Huffman (15), who readily sublimed a quinuclidone from the mixture when benzylamine was used. In this case, only a sticky glass was obtained, and the sublimation failed. Crude V was acetylated to produce VI, 6-*endo*-acetoxy-2-methyl-3-isoquinuclidone.

Lithium aluminum hydride reduction of VI provided VII. Considerable losses were encountered in this reduction, probably due to the water solubility of the product. The crude amino alcohol was acetylated and then was allowed to react with methyl iodide to afford I.



Scheme I

During the early part of the work on I, a method appeared for the preparation of II from the Diels-Alder adduct of 1,3-cyclohexadiene and *N*-methylidene-urethan (16). This method obviated the intended conversion of VII to an intermediate ketone useful to reach II.

Of interest is the difference in NMR spectra of I and II. Whereas the spectrum of I shows magnetic equivalence of the *N*-methyl groups at 3.28 δ, two different signals are observed for the methyl groups in II at 3.26 and 3.35 δ, respectively. This difference reflects the long range effect of the acetoxy group (*vide infra*), although it is not possible to assign it a shielding or deshielding effect in this case, although the latter is most probable.

The best models available are 2β-substituted steroids (effects on the C-19 methyl group) and 17β-substituted compounds (effects on the C-18 methyl groups) (17, 18). Deshielding effects of the acetoxy group are noted in each case. However, the geometry of the isoquinuclidine ring is not completely analogous.

PHARMACOLOGICAL TESTING

The isoquinuclidines, I and II, as racemates, were tested for muscarinic activity in rabbit ileum. Neither of the compounds showed activity at concentrations up to 10^{-2} *M*. Acetylcholine showed half maximal activity at 1×10^{-7} *M* ($pD_2 = 7$, $\alpha = 1.0$).

For comparison, a structurally related compound, 3-acetoxyquinuclidine methiodide VIII was also tested in this system. This compound showed a dose-response curve parallel to that of acetylcholine, and a pD_2 of 4.2, $\alpha = 1.0$. Similar results have been reported (19).

Two rational explanations may be advanced for the lack of activity in the isoquinuclidines. The conformations of acetylcholine, which these compounds represent, may not be those in the drug-receptor complex at the muscarinic site. A more valid possibility is that the carbocyclic skeleton present in these compounds may prevent either of the compounds from forming a drug-receptor complex leading to pharmacological action. The observed lack of activity may be due to this hydrocarbon portion of the molecules, which may interact with hydrophobic regions outside the site, or prevent interaction of the quaternary ammonium head and acetoxy moieties.

Examination of Dreiding models of II shows a great deal of steric hindrance about the acetoxy group, to one of the *N*-methyl groups. The NMR spectrum supports some type of interaction between these groups, at least an electronic effect. Additionally, each of these compounds can be analyzed as an α,β-di-alkyl-substituted acetylcholine, with both an isopropyl and neopentyl group attached to nitrogen. One of these factors, or both, may be responsible for the lack of pharmacological activity.

EXPERIMENTAL

Melting points were determined on a calibrated Thomas-Hoover Unimelt and are corrected. IR spectra were recorded on Beckman IR-8 and IR-20 spectrophotometers. NMR spectra were determined with Varian Associates A-60 and T-60 spectrometers using tetramethylsilane as an internal standard in organic solvents and dimethylsilapentanoic acid sodium salt in aqueous solution. Elemental analyses were conducted by Drs. G. Weiler and F. B. Strauss, Oxford, England.

3-Carbomethoxy-7-oxabicyclo[4.1.0]heptane (IV)—To a cooled solution (0°) of 137 g. (0.98 mole) of methyl 3-cyclohexencarboxylate in 200 ml. of $CHCl_3$ was slowly added a $CHCl_3$ solution of 180 g. (1.02 moles) of *m*-chloroperbenzoic acid. After the addition, the mixture was stirred at room temperature for 2 hr. then worked with 5% aqueous sodium sulfite solution, 5% aqueous sodium hydroxide solution, water, and dried ($MgSO_4$). Evaporation of the $CHCl_3$ afforded a colorless oil, identical in IR spectral qualities with those reported by Henbest (14).

6-*endo*-Acetoxy-2-methyl-3-isoquinuclidine (VI)—To a cooled (0°) solution of 93.0 g. (0.60 mole) of crude epoxide, IV, in 200 ml. of CH_3OH was slowly added 77.5 g. (1.00 mole) of 40% aqueous methylamine. The mixture was allowed to stand at room temperature for 3 days, heated to reflux for 1 hr., then evaporated *in vacuo* to remove solvents and excess methylamine. The crude amino alcohol was refluxed for 3 hr. to effect ring closure to lactam VI. The residue, 87.0 g. of a viscous glass, resisted all attempts at sublimation, a technique that had been successful in similar compounds (15). The IR spectrum showed both ester (5.80 μ) and amide (6.10 μ) carbonyl bands.

The crude mixture (87.0 g., 0.40 mole) was dissolved in 86.4 g. (1.10 moles) of pyridine and 110.7 g. (1.10 moles) of acetic anhydride was added. The solution was refluxed for 2 hr.; excess pyridine, acetic anhydride, and acetic acid were removed utilizing a water aspirator. Aqueous 2% HCl was added and after 1 hr. the mixture was extracted with EtOAc. The EtOAc layer was dried ($MgSO_4$) and solvent evaporated. The residue oil, was distilled, b.p. 110° (1.0 mm.) affording 31.5 g. (17% of theory) of VI; IR (neat), 3.02, 3.41, 5.82, 6.05, 6.96, 7.19, 7.33, 8.12, 8.55, 8.79, 9.53, 9.80, 11.0, 12.15 μ; NMR δ ($CDCl_3$), 5.10 (sextet, H_6, $J_{6,1} \sim J_{6,5} \sim 4$ c.p.s., $J_{6,5'} = 10$ c.p.s.), 3.75 (multiplet, H_1 proton, $W_h = 8$ c.p.s.), 3.05 (singlet, $N—CH_3$ proton), 2.18 (singlet, $CH_3—\overset{O}{\overset{\|}{C}}—$), 1.6–2.7 (multiplet, seven protons, methylene-methine envelope).

6-*endo*-Acetoxy-2-methylisoquinuclidine Methiodide (I)—A solution of isoquinuclidone VI, 29.9 g. (0.165 mole) in 100 ml. of anhydrous ether was added dropwise to a slurry of 12.0 g. (0.316 mole) of lithium aluminum hydride in 150 ml. of ether. After the addition, the mixture was refluxed for 4 hr. Excess lithium aluminum hydride was destroyed with a 40% aqueous solution of Rochelle salt. The mixture was filtered (Celite), dried, and the solvent removed. The crude product was partitioned between ethyl acetate

[1] An *exo* substituent is defined as one which is *cis* to the nitrogen containing bridge to the isoquinuclidine ring, and an *endo* substituent is *trans* to the bridge.

and 5% aqueous hydrochloric acid. The acidic solution was made alkaline with 20% aqueous NaOH and extracted with EtOAc, which was dried, and solvent removed affording crude VII, 6.5 g. (22%) as a dark orange oil; IR (neat), 3.05 (broad), 3.47, 6.12, 6.58, 6.98, 8.65, 8.83, 9.38, 9.88, 10.55, 11.05, and 12.21 μ.

A mixture of crude VII, 1.5 g. (11 mmoles), and 1.0 g. (10 mmoles) of acetic anhydride was refluxed for 1 hr. and then allowed to stand overnight at room temperature. Sufficient 10% aqueous NaOH was added to make the mixture alkaline, and the ester extracted with ether. The crude ester was partitioned between ether and aqueous acid again, the acidic solution neutralized, and the basic ester isolated, 500 mg (30%); IR (neat), 2.91, 3.42, 5.78, 6.13, 6.96, 7.10, 7.32, 8.09, 8.60, 8.79, 9.29, and 9.65 μ.

To a solution of 500 mg. (3.0 mmoles) of the crude acetate ester of amino alcohol VII in 10 ml. of methanol was added 10 ml. of methyl iodide and the mixture allowed to stand overnight. The solvent and excess methyl iodide were removed, the solution was decolorized (Norite) in methanol, and crystallized twice from methanol–ethyl acetate affording 300 mg. (53%) of colorless crystals, m.p. 165–165.5°; IR (KBr) 3.29, 3.36, 5.73, 6.79, 6.88, 7.69, 8.02, 8.64, 9.30, 9.55, 9.63, 10.49, 10.77, and 11.03 μ; NMR (D_2O) δ, 5.48 (sextet, H_6 proton, $J_{6,1} \sim J_{6,5} = 4$ c.p.s., $J_{6,5'} = 10$ c.p.s.); 3.60 (broadened quartet, H_1, $J_{1,7} \sim 3$ c.p.s.), 4.45 (broadened singlet, CH_3 protons), 3.28 [singlet, $\overset{+}{N}(CH_3)_2$], 2.12 (singlet, $CH_3-\overset{O}{\overset{\|}{C}}-$), 1.26–3.0 (multiplet, seven protons, methylene-methine envelope).

Anal.—Calcd. for $C_{11}H_{20}INO$: C, 40.60; H, 6.15; N, 4.31. Found: C, 40.67; H, 6.02; N, 4.21.

6-*exo*-Acetoxy-2-methylisoquinuclidine Methiodide (II)—This compound was prepared by the method of DeGraw and Kennedy (16).

3-Acetoxyquinuclidine Methiodide (VIII)—This compound was prepared by the method of Grob (20, 21) from 3-hydroxyquinuclidine.

REFERENCES

(1) R. B. Barlow, "Introduction to Chemical Pharmacology," 2nd ed., Wiley, New York, N. Y., 1964.

(2) M. Friedman, in "Drugs Affecting the Peripheral Nervous System," A. Burges, Ed., Marcel-Dekker, New York, N. Y., 1967, pp. 79–132.

(3) C. C. J. Culvenor and N. S. Ham, *Chem. Commun.*, **1966**, 537.

(4) H. Sorum, *Acta Chem. Scand.*, **13**, 345(1959).

(5) J. F. Dunitz, *ibid.*, **17**, 1471(1963).

(6) F. G. Canepa, *Nature*, **207**, 1152(1965).

(7) F. G. Canepa, P. Pauling, and H. Sorum, *ibid.*, **210**, 970 (1966).

(8) S. Archer, A. M. Lands, and T. R. Lewis, *J. Med. Pharm. Chem.*, **5**, 423(1962).

(9) M. May and D. J. Triggle, *J. Pharm. Sci.*, **57**, 511(1968).

(10) E. E. Smissman, W. L. Nelson, J. B. LaPidus, and J. L. Day, *J. Med. Chem.*, **9**, 458(1966).

(11) M. Martin-Smith, G. A. Smail, and J. B. Stenlake, *J. Pharm. Pharmacol.*, **19**, 565(1968).

(12) C. Y. Chiou, J. P. Long, J. G. Cannon, and P. D. Armstrong, *J. Pharmacol. Exptl. Therap.*, **166**, 243(1969).

(13) P. D. Armstrong, J. G. Cannon, and J. P. Long, *Nature*, **220**, 65(1968).

(14) H. B. Henbest and B. Nicholls, *J. Chem. Soc.*, **1959**, 221.

(15) J. W. Huffman, C. B. S. Rao, and T. Kamiya, *J. Org. Chem.*, **32**, 697(1967).

(16) J. I. DeGraw and J. G. Kennedy, *J. Heterocyclic Chem.*, **4**, 251(1967).

(17) N. S. Bhacca and D. H. Williams, "Applications of NMR Spectra in Organic Chemistry," Holden-Day, San Francisco, Calif., 1964, pp. 19–23.

(18) K. Tori and E. Kondo, *Steroids*, **4**, 713(1964).

(19) J. B. Robinson, B. Belleau, and B. Cox, *J. Med. Chem.*, **12**, 848(1969).

(20) C. A. Grob, A. Kaiser, and F. Renk, *Helv. Chim. Acta*, **40**, 2170(1957).

(21) A. W. Solter, *J. Pharm. Sci.*, **54**, 1755(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 15, 1969 from the *College of Pharmacy, University of Washington, Seattle, WA 98105*

Accepted for publication September 22, 1969.

This investigation was supported in part by grant MH-13,514 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

* Recipient of the Lunsford Richardson Pharmacy Award, Regional Honorable Mention, 1969, for a manuscript based in part on this work.

The authors wish to thank Dr. Frank F. Vincenzi and Mr. Peter H. Blake for the muscarinic assays.

Potential Antitumor Agents: Derivatives of 2-Hydrazino-5-nitropyridine

BENJAMIN PRESCOTT and GEORGE CALDES

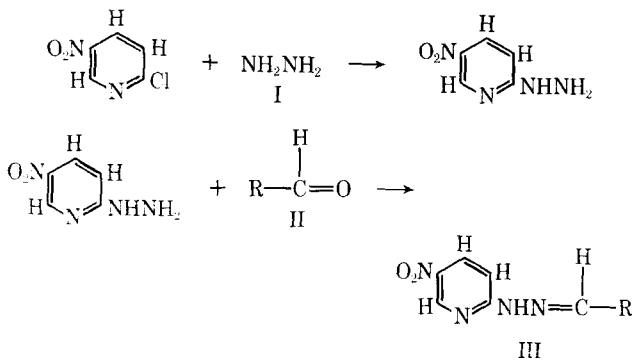
Abstract □ Fifty-one hydrazones of 2-hydrazino-5-nitropyridine have been synthesized for tolerance in DBA mice and for study as potential antitumor agents against the three mouse tumor systems (sarcoma 180, adenocarcinoma 755, and leukemia 1210). Of four compounds with activity against the sarcoma 180 tumor, two derivatives—*p*-acetaminobenzaldehyde and *p*-nitrobenzaldehyde—showed good inhibition and confirmed activity. The salicylaldehyde derivative showed slight activity against the adenocarcinoma 755 tumor. None of the compounds were active in the leukemia 1210 mouse tumor system. The highest tolerated dose of the active compounds in mice by intraperitoneal injection was 2 g./kg. The compounds were thus of low toxicity.

Keyphrases □ 2-Hydrazino-5-nitropyridines—synthesis □ Acute toxicity, mice—2-hydrazino-5-nitropyridines □ Biological activity —2-hydrazino-5-nitropyridines

The synthesis of thiosemicarbazones and hydrazones has received considerable attention since Domagk's (1) report on the antituberculous activity of tibione (*p*-acetylaminobenzaldehyde thiosemicarbazone). Numerous reports followed which indicated that a number of different hydrazones possessed potential antimicrobial, antifungal, and antitumor activity. Freedlander and Furst (2) studied the effects of substituted hydrazines and related compounds on myeloid mouse leukemia C-1498. Petersen and Domagk (3) synthesized the guanylhydrazone of *p*-benzoquinone-monothiosemicarbazone and found the product to possess strong bacteriostatic action on *Mycobacterium tuberculosis* and various other organisms. In addition Chang (4) reported that the isonicotinylhydrazones of 2-carboxymethoxy-3-methoxybenzaldehyde and 2-carboxymethoxybenzaldehyde were effective against mouse leprosy. Following these reports Jucker (5) published a review of hydrazone derivatives used as medicinals.

A few hydrazone derivatives of 2-hydrazino-5-nitropyridine were prepared by Mangini and Frenguelli (6). However, no biological properties were reported concerning these compounds. In extensive investigations from this laboratory into the bacteriostatic, fungistatic, and carcinostatic effects of hydrazones (7, 8) particularly with a view to their possible application as potential chemotherapeutic agents, results indicated that certain hydrazones may possess significant biological activity.

The present report also describes the synthesis of 51 5-nitro-2-pyridylhydrazones with analyses, tests for acute toxicity in mice, and for their effectiveness as potential antimicrobial and antitumor agents. Studies with this series of hydrazones reported herein have shown that certain derivatives have some effect as potential anticancer agents.



Scheme I—5-Nitro-2-pyridylhydrazone; I, *preparation of 2-hydrazino-5-nitropyridine;* II, *aldehyde where R = aliphatic, aromatic, or heterocyclic group;* III, *hydrazone derivative.*

MATERIALS AND METHODS

The desired 2-hydrazino-5-nitropyridine was synthesized from 2-chloro-5-nitropyridine by a modification of the method of Mangini and Frenguelli (6). Seventy-nine grams (0.5 mole) of 2-chloro-5-nitropyridine was warmed to solution in 2 l. of 95% EtOH, in a 3-l. three-necked flask equipped with a stirrer, thermometer, and dropping funnel. Twenty-five grams (0.5 mole) of 95% hydrazine hydrate were added dropwise to the stirred solution and the mixture cooled in an ice bath. The alcoholic solution turned orange colored immediately after the hydrazine was added and a voluminous yellowish-green precipitate began to separate with the evolution of a gas. The mixture was stirred for an additional 2 hr. at room temperature after all the hydrazine had been added and held at room temperature overnight. The heavy precipitate that formed was filtered, washed with petroleum ether, and air-dried. The crude greenish insoluble condensation product was crystallized from a large volume of 70% EtOH. The yellow fine crystals melted at 203–205° with decomposition.

Anal.—Calcd for $C_5H_6N_4O_2$: C, 38.96%; H, 3.92%; N, 36.25%. Found: C, 38.87%; H, 3.87%; N, 36.5%. The 2-hydrazino-5-nitropyridine readily reacted with aldehydes to form crystalline hydrazones which exhibited intense colors ranging from bright yellow, orange, through shades of red. All the aldehydes employed were commercial preparations. The chemical structures of the hydrazones are shown in Scheme I.

The general procedure for the synthesis of the hydrazones was as follows: a solution of the aldehyde (0.1 mole) in 95% ethanol (50 ml.) was added to a warm solution of the 2-hydrazino-5-nitropyridine (0.1 mole) in 95% ethanol (200 ml.) and refluxed gently for 30 min. on a hot plate. In numerous instances immediate reaction took place as seen by the formation of a solid. The solutions were cooled to room temperature and the insoluble products filtered, washed with cold water and petroleum ether, and air-dried. The products were purified by recrystallization from 70% ethanol in presence of decolorizing carbon. The compounds appeared as colorful, shiny crystals with yields from 90–95%. Table I gives a summary of the aldehydes used and the analysis of the compounds.

Biological Studies—Acute toxicity studies were performed in the DBA strain of mice as maintained at the National Institutes of Health, Bethesda, Md. The chemicals were suspended in 0.25% methocel (Dow methylcellulose) so that the dose per 20-g. mouse was contained in 0.25-ml. volume for intraperitoneal injection and the results judged by 72-hr. survival. The tolerated dose of each of the fifty-one compounds is 2 g./kg. The compounds are of low toxicity and may be administered in larger doses than are commonly used in the administration of various anticancer drugs.

Antitumor Studies—The compounds were tested for antitumor activity in the three tumor (sarcoma 180, adenocarcinoma 755, and leukemia 1210) mouse screening program by screeners under contract to the Cancer Chemotherapy National Service Center. The

Table I—5-Nitro-2-pyridylhydrazones—Chemical and Physical Properties

Derivative of Aldehyde	M.p. °C.[a]	Empirical Formula	Anal., % Calcd.	Found
Formaldehyde	220–225	$C_{11}H_{12}N_8O_4$	C, 41.25 H, 3.78 N, 34.99	C, 41.42 H, 4.10 N, 34.19
2-Ethylbutyraldehyde	152–155	$C_{11}H_{16}N_4O_2$	C, 55.91 H, 6.82 N, 23.71	C, 55.49 H, 6.79 N, 23.69
Heptaldehyde	191–194	$C_{12}H_{18}N_4O_2$	C, 57.58 H, 7.25 N, 22.38	C, 57.71 H, 7.30 N, 22.53
2-Ethylhexanal	136–138	$C_{13}H_{20}N_4O_2$	C, 59.07 H, 7.63 N, 21.19	C, 59.14 H, 7.81 N, 21.38
Nonal	108–111	$C_{14}H_{22}N_4O_2$	C, 60.41 H, 7.96 N, 20.13	C, 60.68 H, 7.24 N, 20.34
Decanal	88	$C_{15}H_{24}N_4O_2$	C, 61.62 H, 8.28 N, 19.16	C, 61.38 H, 8.77 N, 19.47
Lauraldehyde	78–80	$C_{17}H_{28}N_4O_2$	C, 63.72 H, 8.81 N, 17.49	C, 63.49 H, 8.36 N, 17.34
Crotonaldehyde	174–177	$C_9H_{10}N_4O_2$	C, 52.42 H, 4.88 N, 27.17	C, 52.69 H, 4.84 N, 27.13
Tiglaldehyde	216	$C_{10}H_{12}N_4O_2$	C, 54.53 H, 5.49 N, 25.44	C, 54.39 H, 5.22 N, 25.43
Methyl glyoxal	>300	$C_{13}H_{12}N_8O_4$	C, 45.35 H, 3.51 N, 32.55	C, 45.32 H, 3.86 N, 32.41
Benzaldehyde[b]	227–229	$C_{12}H_{10}N_4O_2$	C, 59.47 H, 4.20 N, 23.12	C, 59.72 H, 4.76 N, 23.17
4-Fluorobenzaldehyde	228	$C_{12}H_9FN_4O_2$	C, 55.38 H, 3.49 N, 21.53	C, 55.48 H, 3.70 N, 21.27
2-Chlorobenzaldehyde	210	$C_{12}H_9ClN_4O_2$	C, 52.09 H, 3.28 N, 20.25	C, 52.41 H, 3.50 N, 29.05
4-Chlorobenzaldehyde	218–219	$C_{12}H_9ClN_4O_2$	C, 52.09 H, 3.28 N, 20.25	C, 52.37 H, 3.50 N, 20.29
2,4-Dichlorobenzaldehyde	211	$C_{12}H_8Cl_2N_4O_2$	C, 46.32 H, 2.59 N, 18.00	C, 46.10 H, 2.52 N, 18.50
3,4-Dichlorobenzaldehyde	168–170	$C_{12}H_8Cl_2N_4O_2$	C, 46.32 H, 2.59 N, 18.00	C, 46.01 H, 2.41 N, 18.21
2-Nitrobenzaldehyde	217–222	$C_{12}H_9N_5O_4$	C, 50.18 H, 3.16 N, 24.39	C, 50.83 H, 3.40 N, 24.42
3-Nitrobenzaldehyde	248–250	$C_{12}H_9N_5O_4$	C, 50.18 H, 3.16 N, 24.39	C, 50.42 H, 3.62 N, 24.76
4-Nitrobenzaldehyde	278	$C_{12}H_9N_5O_4$	C, 50.18 H, 3.16 N, 24.39	C, 50.38 H, 3.85 N, 24.72
Phenylacetaldehyde	137–138	$C_{13}H_{12}N_4O_2$	C, 60.93 H, 4.72 N, 21.87	C, 60.07 H, 4.50 N, 21.59
4-Tolylaldehyde	212–216	$C_{13}H_{12}N_4O_2$	C, 60.93 H, 4.72 N, 21.87	C, 60.72 H, 4.45 N, 21.39
Hydrocinnamaldehyde	126–128	$C_{14}H_{14}N_4O_2$	C, 62.21 H, 5.22 N, 20.73	C, 62.32 H, 5.40 N, 20.68
4-Isopropylbenzaldehyde	160	$C_{15}H_{16}N_4O_2$	C, 63.36 H, 5.67 N, 19.71	C, 64.05 H, 5.51 N, 19.53

(*continued on next page*)

Table I—(*continued*)

Derivative of Aldehyde	M.p. °C.[a]	Empirical Formula	Anal., % Calcd.	Found
Cinnamaldehyde	237	$C_{14}H_{12}N_4O_2$	C, 62.68 H, 4.51 N, 20.88	C, 62.31 H, 4.64 N, 20.92
α-Methylcinnamaldehyde	230–232	$C_{15}H_{16}N_4O_2$	C, 63.36 H, 5.67 N, 19.71	C, 63.70 H, 5.90 N, 19.64
1-Naphthaldehyde	195–198	$C_{16}H_{12}N_4O_2$	C, 65.74 H, 4.14 N, 19.17	C, 65.06 H, 3.78 N, 19.70
Salicylaldehyde	217	$C_{12}H_{10}N_4O_3$	C, 55.81 H, 3.90 N, 21.69	C, 55.16 H, 4.03 N, 21.38
2-Methoxybenzaldehyde	275–276	$C_{13}H_{12}N_4O_3$	C, 57.35 H, 4.44 N, 20.58	C, 57.22 H, 4.74 N, 20.35
2-Ethoxybenzaldehyde	228	$C_{14}H_{14}N_4O_3$	C, 58.73 H, 4.93 N, 19.57	C, 58.32 H, 4.44 N, 19.25
5-Chlorosalicylaldehyde	290–292	$C_{12}H_9ClN_4O_3$	C, 49.24 H, 3.10 N, 19.14	C, 49.43 H, 3.27 N, 19.64
5-Bromo-2-hydroxybenzaldehyde	298	$C_{12}H_9BrN_4O_3$	C, 42.75 H, 2.69 N, 16.62	C, 42.86 H, 3.01 N, 16.90
3-Hydroxybenzaldehyde	248–250	$C_{12}H_{10}N_4O_3$	C, 55.82 H, 3.90 N, 21.69	C, 55.69 H, 4.21 N, 21.66
4-Hydroxybenzaldehyde	>300	$C_{12}H_{10}N_4O_3$	C, 55.82 H, 3.90 N, 21.69	C, 56.46 H, 3.68 N, 21.33
4-Methoxybenzaldehyde	195–197	$C_{13}H_{12}N_4O_3$	C, 57.35 H, 4.44 N, 20.58	C, 57.68 H, 4.33 N, 20.82
2-Methoxy-5-*tert*-butylbenzaldehyde	199–203	$C_{17}H_{20}N_4O_3$	C, 62.17 H, 6.14 N, 17.07	C, 62.08 H, 6.00 N, 17.21
2-Hydroxy-1-naphthaldehyde	277–279	$C_{16}H_{12}N_4O_3$	C, 62.33 H, 3.92 N, 18.18	C, 62.48 H, 4.14 N, 18.09
2-Hydroxy-3-methoxybenzaldehyde	190–195	$C_{13}H_{12}N_4O_4$	C, 54.12 H, 4.20 N, 19.44	C, 54.33 H, 4.31 N, 19.11
2,3-Dimethoxybenzaldehyde	244	$C_{14}H_{14}N_4O_4$	C, 55.62 H, 4.67 N, 18.54	C, 55.85 H, 4.66 N, 18.54
3,4-Dimethoxybenzaldehyde	238–240	$C_{14}H_{14}N_4O_4$	C, 55.62 H, 4.67 N, 18.54	C, 55.69 H, 4.45 N, 18.07
2,4-Dihydroxybenzaldehyde	>300	$C_{12}H_{10}N_4O_4$	C, 52.55 H, 3.68 N, 20.43	C, 52.66 H, 3.85 N, 20.46
3-Ethoxy-4-hydroxybenzaldehyde	197–198	$C_{14}H_{14}N_4O_4$	C, 55.62 H, 4.67 N, 18.54	C, 55.32 H, 4.77 N, 18.86
3,4-Diethoxybenzaldehyde	190–191	$C_{16}H_{18}N_4O_4$	C, 58.17 H, 5.49 N, 16.96	C, 58.02 H, 5.53 N, 16.67
Phthalaldehydic acid	273–275	$C_{13}H_{10}N_4O_4$	C, 54.54 H, 3.52 N, 19.57	C, 54.18 H, 3.64 N, 19.40
2-Benzaldehyde sulfonic acid (Na)	>300	$C_{12}H_9N_4NaO_5S$	C, 41.85 H, 2.64 N, 16.25	C, 41.18 H, 2.88 N, 15.97
4-Dimethylaminobenzaldehyde	263–264	$C_{14}H_{15}N_5O_2$	C, 58.93 H, 5.30 N, 24.55	C, 58.53 H, 5.22 N, 24.53

(*Continued on next page*)

Table I—*(continued)*

Derivative of Aldehyde	M.p. °C.[a]	Empirical Formula	Anal., % Calcd.	Anal., % Found
4-Diethylaminobenzaldehyde	150–151	$C_{16}H_{19}N_5O_2$	C, 61.32 H, 6.11 N, 22.35	C, 61.56 H, 5.96 N, 22.47
4-Acetaminobenzaldehyde	273–275	$C_{14}H_{13}N_5O_3$	C, 56.25 H, 4.38 N, 23.41	C, 56.42 H, 4.58 N, 23.51
Furfural	213–215	$C_{10}H_8N_4O_3$	C, 51.72 H, 3.47 N, 24.13	C, 51.94 H, 3.43 N, 23.83
Piperonal	195–200	$C_{13}H_{10}N_4O_4$	C, 54.54 H, 3.52 N, 19.57	C, 54.20 H, 3.47 N, 19.20
Ribose	155 dec.	$C_{10}H_{14}N_4O_6$	C, 41.96 H, 4.93 N, 19.58	C, 41.66 H, 4.71 N, 19.09
Glucose	185–188	$C_{11}H_{16}N_4O_7$	C, 41.77 H, 5.10 N, 17.72	C, 41.70 H, 4.92 H, 17.35

[a] All melting points are uncorrected and were determined on a Fisher-Johns melting point apparatus. [b] Degussa reported m.p. 226–228° (Beilstein, 4th ed., 2nd Suppl. vol. 22, p. 488).

testing procedures employed have been described previously (9). Among the fifty-one compounds, four derivatives, salicyaldehyde, 4-chlorobenzaldehyde, 4-nitrobenzaldehyde, and 4-acetaminobenzaldehyde were found to exhibit significant inhibition against the S-180 mouse tumor system with the latter two compounds showing confirmed activity. The salicylaldehyde derivative showed slight activity against the adenocarcinoma 755 tumor in a dose of 450 mg./kg. None of the compounds in the series showed any significant or reproducible antitumor effects in the L-1210 mouse tumor system. The results of the biological tests supplied by the Cancer Chemotherapy National Service Center are summarized in Table II.

Table II—Antitumor Activity of Certain 5-Nitro-2-pyridylhydrazones against Sarcoma 180

Derivative	Dose, mg./kg.	Survivors, Mice	Animal wt. Difference, T/C	Tumor wt., mg., Test/Control	T/C, %
4-Nitrobenzaldehyde	250	4/6	−0.1	488/1730	28
	185	6/6	−1.3	317/1014	31
	82.2	5/6	−1.6	371/784	47
	41.1	5/6	−0.8	508/784	64
	20.5	6/6	−0.4	481/784	61
	10.2	6/6	0	696/784	88
4-Acetaminobenzaldehyde	562	4/6	−3.8	335/710	47
	500	6/6	−2.8	333/1371	24
	375	6/6	−4.4	342/710	48
	250	6/6	−3.4	159/710	22
	166	6/6	−3.1	547/710	77
4-Chlorobenzaldehyde	500	6/6	−2.2	442/1371	32
	500	6/6	−2.7	450/1064	42
Salicylaldehyde	350	6/6	−6.2	675/1852	36
	350	6/6	−5.7	362/1063	34

Antibacterial Activity—*In vitro* bacteriostatic studies were performed on the fifty-one compounds with *Staphylococcus aureus* FD 209 and with *Mycobacterium tuberculosis* B 103. None of the compounds exhibited any appreciable antibacterial activity. The highly active *p*-acetaminobenzaldehyde derivative was also studied by Y. T. Chang of the Laboratory of Pharmacology and Toxicology, National Institutes of Health, for its effect on rat leprosy in mice and found to be inactive. In the light of the results with the *p*-acetaminobenzaldehyde and *p*-nitrobenzaldehyde derivatives as antitumor agents a test of their effectiveness in clinical tests is indicated.

REFERENCES

(1) G. Domagk, R. Behnisch, F. Mietzsch, and H. Schmidt, *Naturwissenschaften*, **10,** 315(1946).

(2) B. S. Freedlander and A. Furst, *Proc. Soc. Exptl. Biol. Med.*, **81,** 638(1952).

(3) S. Petersen and G. Domagk, *Naturwissenschaften*, **41,** 10 (1954).

(4) Y. T. Chang, *Leprosy*, **25,** 130(1957).

(5) E. Jucker, *Angew. Chem.*, **71,** 317(1959).

(6) A. Mangini and B. Frenguelli, *Gazz. Chim. Ital.*, **69,** 97 (1939).

(7) B. Prescott, G. Caldes, W. R. Piggott, and W. B. Hill, *Chimica Therap.*, **2,** 228(1967).

(8) B. Prescott, G. Lones, and G. Caldes, *Intern. J. Clin. Pharmacol.*, **1,** 529(1968).

(9) J. Leiter, A. R. Bourke, S. A. Schepartz, and I. Wodinsky, *Cancer Res.*, **20,** 734(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 1, 1969 from the *Laboratory of Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20014*

Accepted for publication September 24, 1969.

Synthesis of New Hydrazidines with Vasoconstrictor Activity

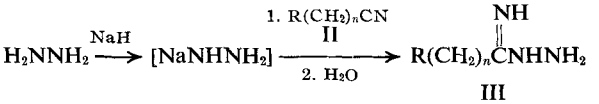
GEORGE C. WRIGHT*, ROBERT P. HALLIDAY, and CHARLES S. DAVIS†

Abstract □ A series of hydrazidines with a structural similarity to the guanidines of the guanethidine series was synthesized. The observation that the hydrazidines raised blood pressure led to their screening as nasal decongestants. The most active compound, 3-(*hexahydro-1H-azepino*)*propionimidic* acid hydrazide dihydrochloride, was less potent than phenylephrine hydrochloride.

Keyphrases □ Hydrazidines—synthesis □ Vasoconstrictive activity—hydrazidines □ Hypertensive activity—hydrazidines

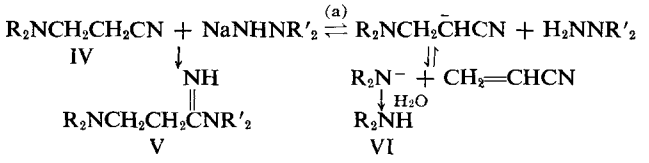
A series of hydrazidines with a structural similarity to the guanidines of the guanethidine series (1) was prepared for screening as antihypertensive compounds. The observation that the amino-substituted imidic acid hydrazides (III) raised blood pressure led to the screening of these compounds as nasal decongestants. In this paper the name hydrazidine refers to the general class of compounds, $-C(=N)N-N=$; specific types are named according to the system of *Chemical Abstracts*.

Several disubstituted aminoacetimidic acid hydrazides (III*a–c*, Table I) were prepared by a modification (Method *A*) of Kauffmann's procedure (2) for the synthesis of aliphatic hydrazidines.

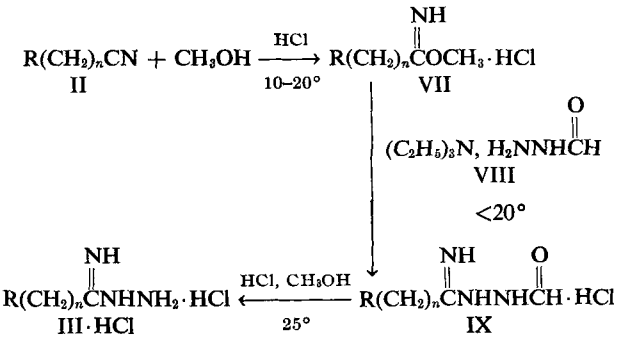


Method A

Certain limitations to Method *A* have been observed. The reaction did not occur with acetonitriles possessing a labile α-hydrogen atom which could yield a resonance stabilized anion, as in the case of 2-phenyl-2-(2-pyridyl)-acetonitrile. In the case of 3-aminopropionitriles (IV), the competitive retrograde Michael reaction (a) was dependent upon the nature of the amino substituent (R_2N^-). When R_2N^- was incapable of resonance stabilization, competition was nil, and the desired hydrazidine V was formed. With R_2N^- capable of resonance stabilization, *e.g.* $C_6H_5N^-Et$, however, the amine VI was a primary product of the reaction.



A variety of substituted imidic acid hydrazides (III*d–i*, Table I) has been prepared *via* a modified procedure (Method *B*) of Westermann *et al.* (3), through the intermediate *N*-formamidoalkylamidines (IX*a–f*, Table II). Method *B* affords a safer preparation of the hydrazides (III) than Method *A*, where metastable sodium hydrazides are formed as intermediates.



Method B

PHARMACOLOGIC RESULTS

All of the hydrazidines that were tested (Table II) for their effect on nasal volume were less active than phenylephrine hydrochloride. The most active compound was III*g* which caused marked changes in nasal volume with relatively little effect on systemic blood pressure. Although these studies are preliminary in nature and involve few animals, a tentative order of decreasing potency of the compounds may be listed as III*g* > III*f* ≥ III*h* ≥ III*c* ≥ III*i* > III*b* > III*d*. Thus, the 3-disubstituted aminopropionimidic acid hydrazides appear to be the most potent compounds prepared.

EXPERIMENTAL

Chemistry

2-(Hexahydro-1H-azepino)acetimidic Acid Hydrazide Dihydrochloride, IIIc—(*Method A*)—The reaction was conducted with special equipment and precautions as described by Kauffmann *et al.* (2). The main modification was the substitution of sodium hydride for sodamide in the generation of sodium hydrazide (I), affording a more rapid and reliable reaction with acetonitrile (II, $n = 1$).

Thus, to a mixture of 50% NaH (14.4 g., 0.30 mole) dispersed in mineral oil and dry ether (300 ml.) was added dropwise a solution of anhydrous hydrazine (9.6 g., 0.30 mole) in dry ether at 0–5°; this then was warmed to 10–15° over a 2-hr. period. The gray suspension was cooled to 0–5°, and treated dropwise with a solution of azepinoacetonitrile (41.4 g., 0.30 mole) in dry ether. The resultant creamy white suspension was warmed to 15° for 1.5 hr., cooled to 0–5° and treated with H_2O (14 ml.). The reaction mixture was stirred for 1 hr., and then filtered. The ethereal filtrate was dried (Na_2SO_4), concentrated, and distilled to give 17.4 g. (29%) of the free base; b.p. 114–126° (0.3–0.5 mm.).

The dihydrochloride (III*c*) was prepared by treating the free base in acetonitrile at 60° with a saturated solution of hydrogen chloride in acetonitrile. The salt suspension was heated to boiling, cooled, and filtered. Recrystallization from ethanol gave white crystals.

Method B—The method of Westermann *et al.* (3) was employed with several modifications. The methyl imidates VII rather than ethyl imidates were used since the intermediate nitrile hydrochlorides encountered in the preparation of the imidates were readily soluble in methanol. To ensure anhydrous conditions the semistable imidates were stored in a vacuum desiccator overnight. The commercially available formic acid hydrazide (VIII), which contained water, was unacceptable for the preparation of Compounds IX. Anhydrous VIII was prepared as follows.

Formic Acid Hydrazide (VIII)—To a solution of anhydrous hydrazine (960 g., 30 moles) in anhydrous ethanol (3.7 l.) was added ethyl formate (2220 g., 30 moles) at 20–25° during 2 hr. with mechanical stirring. The stirring was continued an additional

Table I—Amino-Substituted Hydrazidines

No.	R	*n*	Method	Yield, %	M.p., °C.[a]	Formula	Anal., % Calcd.	Found
III								
$R(CH_2)_nC(=NH)NHNH_2$								
a	$(C_2H_5)_2N$	1	A	46[b]	175–177	$C_6H_{16}N_4 \cdot 2HCl$	C, 33.19	33.26
							H, 8.36	8.46
							N, 25.80	25.84
b	$(C_5H_{10})N$	1	A	20[b]	200–201	$C_7H_{16}N_4 \cdot 2HCl$	C, 36.73	36.65
							H, 7.98	7.92
							N, 24.52	24.38
c	$(C_6H_{12})N$	1	A	29[b]	195–196	$C_8H_{18}N_4 \cdot 2HCl$	C, 39.51	39.51
							H, 8.29	8.55
							N, 23.04	22.99
d	$3,4\text{-}(CH_3O)_2C_6H_3$	1	B	68[c]	199–201	$C_{10}H_{15}N_3O_2 \cdot HCl$	C, 48.90	49.07
							H, 6.52	6.49
							N, 17.12	17.13
e	$(CH_3)_2N$	2	B	47[c]	158–159	$C_5H_{14}N_4 \cdot 2HCl$	C, 29.56	29.82
							H, 7.94	7.99
							N, 27.58	27.58
	$(C_5H_{10})N$	2	B	90[c]	179–180	$C_8H_{18}N_4 \cdot 2HCl$	C, 39.63	39.74
							H, 8.31	8.29
							N, 23.11	23.00
g	$(C_6H_{12})N$	2	B	73[c]	182–184	$C_9H_{20}N_4 \cdot 2HCl$	C, 42.02	42.32
							H, 8.62	8.55
							N, 21.78	21.83
h	$(C_7H_{14})N$	2	B	36[c]	164–166	$C_{10}H_{22}N_4 \cdot 2HCl$	C, 44.30	44.32
							H, 8.92	8.84
							N, 20.65	20.92
i		2	B	72[c]	180–181	$C_7H_{16}N_4O \cdot 2HCl$	C, 34.30	34.33
							H, 7.40	7.40
							N, 22.82	22.60
IX								
$R(CH_2)_nC(=NH)NHNHCH(=O)$								
a	$(CH_3)_2N$	2		32[d]	118–120	$C_6H_{14}N_4O \cdot HCl$	C, 37.02	36.90
							H, 7.77	7.83
							N, 28.78	28.81
b	$(C_5H_{10})N$	2		29[d]	157–159	$C_9H_{18}N_4O \cdot 2HCl$	C, 39.86	39.90
							H, 7.43	7.42
							N, 20.66	20.53
c	$(C_6H_{12})N$	2		45[d]	159–160	$C_{10}H_{20}N_4O \cdot 2HCl$	C, 42.10	42.27
							H, 7.78	7.62
							N, 19.64	19.48
d	$(C_7H_{14})N$	2		35[d]	165–167	$C_{11}H_{22}N_4O \cdot 2HCl$	C, 44.15	44.23
							H, 8.08	8.11
							N, 18.72	18.75
e		2		40[d]	166–167	$C_8H_{16}N_4O_2 \cdot 2HCl$	C, 35.18	35.15
							H, 6.64	6.79
							N, 20.51	20.35
	$3,4\text{-}(CH_3O)_2C_6H_3$	1		42[b]	126–128	$C_{11}H_{15}N_3O_3$	C, 55.68	55.54
							H, 6.37	6.15
							N, 17.71	17.77

[a] Melting points were determined on a Fisher-Johns (hot stage) apparatus and are uncorrected. [b] Yield of free base; based on nitrile (II). [c] Yield based on *N*-formamidoamidine (IX). [d] Yield of hydrochloride; based on nitrile (II).

0.5 hr. The product was filtered on cloth (S/26 Jaybeeco Chain Weave); and the cake was kept wet with cold, anhydrous ethanol until all of the slurry was on the filter, after which it was protected from air with a rubber dam. The product was dried in a vacuum desiccator over $CaCl_2$ and finally over P_2O_5; m.p. 55–56°, yield: 1150–1330 g. (64–74%). A second crop was obtained by concentrating the mother liquor under N_2 to about 500 ml.; m.p. 54–56° [lit. (4) m.p. 54°], yield: 305–470 g. (17–26%).

Methyl 3-Dimethylaminopropionimidate Dihydrochloride—A solution of 3-dimethylaminopropionitrile (88.0 g., 1.05 moles) in absolute methanol (700 ml.) was treated with dry hydrogen chloride at 10–20° over 3 hr. until saturated. The reaction was diluted with dry ether, and the resultant white crystalline product was collected by filtration and washed with dry ether (1 l.); m.p. 99–100° [lit. (5) m.p. 100° dec.], yield: 118 g. (55%). Both dilution and filtration operations were conducted in a dry box.

N-Formamido-3-dimethylaminopropionamidine Hydrochloride (IXa)—To a mixture of methyl 3-dimethylaminopropionimidate dihydrochloride (105.0 g., 0.52 mole) and absolute ethanol was added dropwise triethylamine (78 ml., 0.57 mole) at 2–4° with rapid stirring, under an atmosphere of dry N_2. Then formic acid hydrazide (35.0 g., 0.58 mole) was added at 4–7° during 1 min. The temperature was held at 5–7° for 15 min., and allowed to rise gradually to 13° over 25 min., and finally to 23° over 2.5 hr. The mixture was cooled to 10°, and the solid product was collected and washed with ethanol and ether. Recrystallization from 95% ethanol (300 ml.) gave white crystals.

3-Dimethylaminopropionimidic Acid Hydrazide Dihydrochloride, IIIe—To *N*-formamido-3-dimethylaminopropionamidine hydrochloride (90.0 g., 0.46 mole) was added 270 ml. of a cold solution of dry hydrogen chloride in methanol. The reaction mixture was allowed to warm to room temperature over 40 min. The solid product was collected, and washed with ethanol and ether. Recrystallization from methanol (300 ml.) gave white crystals.

Pharmacology

Volume changes in the nasal cavity were measured utilizing a modification of the method of Jackson (6). Mongrel dogs were anesthetized with sodium pentobarbital, 35 mg./kg., i.v., and a

Table II—Vasoconstrictor-Hypertensive Activity

No.[a]	Dose, mg./kg. i.v.	Nasal Volume: Δ mm.[3]	Nasal Volume: Duration, min.	Blood Pressure: Δ mm. Hg	Blood Pressure: Duration, min.	Blood Pressure: Dose, mg./kg. i.v.	Blood Pressure: Change, %	Blood Pressure: Duration, min.
III								
a			[b]			10	+25	5
b	1.0	+300	6	+15	6			
c	1.0	+500	14	+45	5			
d	5.0	+80	14	−25[c]	>14			
e	1.0	+240	10	+5	5	10	+22	45
f	1.0	+580	17	+50	>17			
g	0.5	+680	9	+20	9			
h	1.0	+600	13	+20	13	10	+100	45
i	5.0	+530	36	+37	20			
Phenylephrine hydrochloride	0.01	733	9	+30	9			

[a] Refers to compound numbers in Table I and in the text. [b] Nasal volume changes were not measured for this compound. [c] Doses of 10 to 100 mg./kg. did not produce consistent blood pressure effects.

tracheotomy performed. A femoral artery was cannulated for blood pressure recording. A 22.9-cm. (9-in.) metal rod fitted with a small rubber ball was passed into the dog's mouth to the soft palate so that the ball pressed against the wall of the nasopharynx. A glass nose cannula was then inserted into a nostril, and a clamp was applied to hold the cannula in place and to seal the other nostril. The cannula was connected to a Grass PT-5A volumetric low pressure transducer, and the volume (pressure) changes of this cavity were measured. In some experiments only blood pressure was monitored. All compounds were administered intravenously

REFERENCES

(1) R. P. Mull, M. E. Egbert, and M. R. Dapero, *J. Org. Chem.*, **25**, 1953(1960).

(2) T. Kauffmann, C. Kosel, and D. Wolf, *Chem. Ber.*, **95**, 1540 (1962); through *Chem. Abstr.*, **57**, 7140h(1962).

(3) P. Westermann, H. Paul, and G. Hilgetag, *ibid.*, **97**, 528 (1964); through *Chem. Abstr.*, **61**, 655h(1964).

(4) B. Helferich and H. Schirp, *ibid.*, **84**, 469(1951); through *Chem. Abstr.*, **46**, 3503*a*(1952).

(5) A. N. Baksheev and N. I. Gavrilov, *Zh. Obshch. Khim.*, **22**, 2021(1952); through *Chem. Abstr.*, **47**, 8641*e*(1953).

(6) D. E. Jackson, "Experimental Pharmacology and Materia Media," 2nd ed., C. V. Mosby, St. Louis, Mo., 1939, pp. 94–104.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 9, 1969 from the *Research and Development Department, The Norwich Pharmacal Company, Norwich, NY 13815*

Accepted for publication September 10, 1969.

The authors wish to thank Mr. Benjamin F. Stevenson and Mr Raymond C. Finch for the preparation of anhydrous formic acid hydrazide, Mr. Grant Gustin and Mr. Marvin Tefft for the elemental analysis, Mr. Paul S. Bowes for the pharmacologic data, and Mr. Guy S. Lougheed and Mr. Nelson J. Miles for assistance in the preparation of the compounds.

* To whom inquiries should be addressed.

† Present address: Upstate Medical Center, Syracuse, N. Y.

Gas Chromatographic Determination of Chlorphenesin in Plasma

J. F. DOUGLAS, J. A. STOCKAGE*, and N. B. SMITH

Abstract □ A procedure is described for the determination of chlorphenesin in plasma. Chlorphenesin is extracted from plasma with chloroform, reacted with bis-(trimethylsilyl)-acetamide, and the silylated derivative is measured quantitatively by gas chromatography. The technique is reproducible and accurate in the range of 1–10 mcg./ml. Dibutyl phthalate is used as internal standard for quantitation by the relative peak height method.

Keyphrases □ Chlorphenesin in plasma—determination □ Plasma, analysis—chlorphenesin □ GLC—analysis □ Dibutyl phthalate—internal standard

Chlorphenesin (3-*p*-chlorophenoxy-1,2-propanediol) has been shown to suppress immunological response in a number of animal systems (1–3) and to inhibit the release of histamine from sensitized human leucocytes by ragweed antigen (4). Although chlorphenesin has been known since 1949 and a number of procedures have been described in the literature for the analysis of chlorphenesin (5, 6) or chlorphenesin carbamate (7), none are sufficiently sensitive to measure the presence of chlorphenesin in biological fluids after therapeutic doses. Procedures for the quantitative analysis in plasma of a closely related compound, mephenesin [3-(*o*-tolyloxy)-1,2-propanediol], have been reported by Titus *et al.* (8) and by Wyngaarden *et al.* (9). However, these analyses depend upon diazotization or nitration of mephenesin at the *para* position of the phenol ring and are not applicable to chlorphenesin which contains a chlorine atom at this location.

Table II—Vasoconstrictor-Hypertensive Activity

No.[a]	Dose, mg./kg. i.v.	Nasal Volume: Δ mm.[3]	Nasal Volume: Duration, min.	Blood Pressure: Δ mm. Hg	Blood Pressure: Duration, min.	Blood Pressure: Dose, mg./kg. i.v.	Blood Pressure: Change, %	Blood Pressure: Duration, min.
III								
a			[b]			10	+25	5
b	1.0	+300	6	+15	6			
c	1.0	+500	14	+45	5			
d	5.0	+80	14	−25[c]	>14			
e	1.0	+240	10	+5	5	10	+22	45
f	1.0	+580	17	+50	>17			
g	0.5	+680	9	+20	9			
h	1.0	+600	13	+20	13	10	+100	45
i	5.0	+530	36	+37	20			
Phenylephrine hydrochloride	0.01	733	9	+30	9			

[a] Refers to compound numbers in Table I and in the text. [b] Nasal volume changes were not measured for this compound. [c] Doses of 10 to 100 mg./kg. did not produce consistent blood pressure effects.

tracheotomy performed. A femoral artery was cannulated for blood pressure recording. A 22.9-cm. (9-in.) metal rod fitted with a small rubber ball was passed into the dog's mouth to the soft palate so that the ball pressed against the wall of the nasopharynx. A glass nose cannula was then inserted into a nostril, and a clamp was applied to hold the cannula in place and to seal the other nostril. The cannula was connected to a Grass PT-5A volumetric low pressure transducer, and the volume (pressure) changes of this cavity were measured. In some experiments only blood pressure was monitored. All compounds were administered intravenously

REFERENCES

(1) R. P. Mull, M. E. Egbert, and M. R. Dapero, *J. Org. Chem.*, **25**, 1953(1960).

(2) T. Kauffmann, C. Kosel, and D. Wolf, *Chem. Ber.*, **95**, 1540 (1962); through *Chem. Abstr.*, **57**, 7140h(1962).

(3) P. Westermann, H. Paul, and G. Hilgetag, *ibid.*, **97**, 528 (1964); through *Chem. Abstr.*, **61**, 655h(1964).

(4) B. Helferich and H. Schirp, *ibid.*, **84**, 469(1951); through *Chem. Abstr.*, **46**, 3503*a*(1952).

(5) A. N. Baksheev and N. I. Gavrilov, *Zh. Obshch. Khim.*, **22**, 2021(1952); through *Chem. Abstr.*, **47**, 8641*e*(1953).

(6) D. E. Jackson, "Experimental Pharmacology and Materia Media," 2nd ed., C. V. Mosby, St. Louis, Mo., 1939, pp. 94–104.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 9, 1969 from the *Research and Development Department, The Norwich Pharmacal Company, Norwich, NY 13815*

Accepted for publication September 10, 1969.

The authors wish to thank Mr. Benjamin F. Stevenson and Mr Raymond C. Finch for the preparation of anhydrous formic acid hydrazide, Mr. Grant Gustin and Mr. Marvin Tefft for the elemental analysis, Mr. Paul S. Bowes for the pharmacologic data, and Mr. Guy S. Lougheed and Mr. Nelson J. Miles for assistance in the preparation of the compounds.

* To whom inquiries should be addressed.

† Present address: Upstate Medical Center, Syracuse, N. Y.

Gas Chromatographic Determination of Chlorphenesin in Plasma

J. F. DOUGLAS, J. A. STOCKAGE*, and N. B. SMITH

Abstract □ A procedure is described for the determination of chlorphenesin in plasma. Chlorphenesin is extracted from plasma with chloroform, reacted with bis-(trimethylsilyl)-acetamide, and the silylated derivative is measured quantitatively by gas chromatography. The technique is reproducible and accurate in the range of 1–10 mcg./ml. Dibutyl phthalate is used as internal standard for quantitation by the relative peak height method.

Keyphrases □ Chlorphenesin in plasma—determination □ Plasma, analysis—chlorphenesin □ GLC—analysis □ Dibutyl phthalate—internal standard

Chlorphenesin (3-*p*-chlorophenoxy-1,2-propanediol) has been shown to suppress immunological response in a number of animal systems (1–3) and to inhibit the release of histamine from sensitized human leucocytes by ragweed antigen (4). Although chlorphenesin has been known since 1949 and a number of procedures have been described in the literature for the analysis of chlorphenesin (5, 6) or chlorphenesin carbamate (7), none are sufficiently sensitive to measure the presence of chlorphenesin in biological fluids after therapeutic doses. Procedures for the quantitative analysis in plasma of a closely related compound, mephenesin [3-(*o*-tolyloxy)-1,2-propanediol], have been reported by Titus *et al.* (8) and by Wyngaarden *et al.* (9). However, these analyses depend upon diazotization or nitration of mephenesin at the *para* position of the phenol ring and are not applicable to chlorphenesin which contains a chlorine atom at this location.

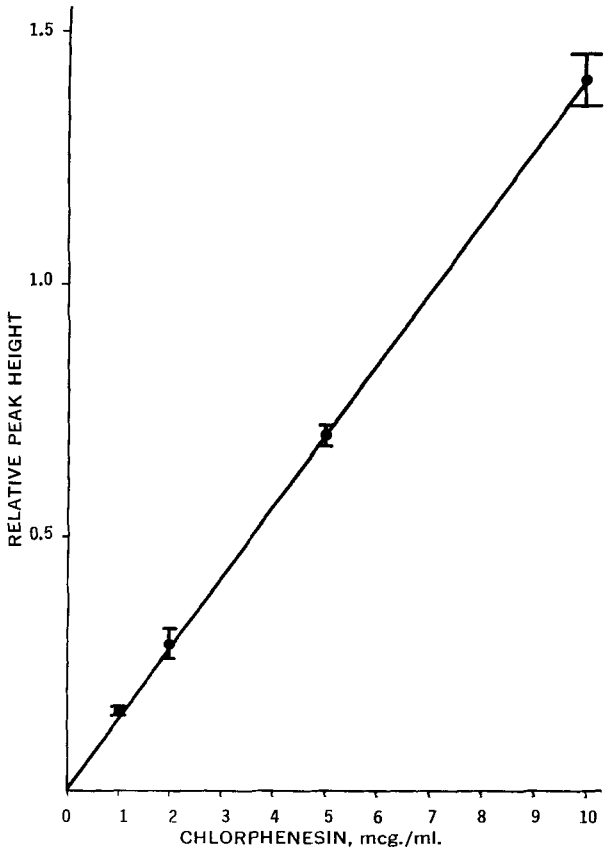


Figure 1—*The relationship between relative peak height and concentration of chlorphenesin in plasma.*

This manuscript describes a procedure for the quantitative determination of chlorphenesin in plasma.

EXPERIMENTAL

Equipment and Reagents—A F and M model 402 dual column gas chromatograph equipped with a hydrogen flame ionization detector and a 1-mv. Minneapolis-Honeywell recorder were employed. The chromatographic columns used were 91.4-cm. × 0.635-cm. (3-ft. × 0.25-in.) glass tubes packed with 5.0% DC200 on 80–100 mesh diatomaceous earth[1]. The instrument settings were as follows: temperature-column, 170°; injection port, 275°; detector block, 230°. Gas flow rates—hydrogen, 60 ml./min; helium (carrier gas), 38 ml./min; oxygen, 70 ml./min. Sensitivity settings were range 10 with an attenuation factor of 8×. The reagents were redistilled chloroform, dibutyl phthalate (Eastman), and bis-(trimethylsilyl)-acetamide (Applied Science).

Procedure—Plasma, 0.5 ml., was diluted with 0.5 ml. of saline and the fluid was thoroughly shaken with 0.5 ml. of redistilled chloroform. Following centrifugation, 0.2 ml. of the chloroform layer was transferred to a small centrifuge tube and treated with 20 μl. of bis-(trimethylsilyl)acetamide for 1 hr. at room temperature. The reaction mixture was evaporated to dryness in a stream of nitrogen, and the residue was taken up in 20 μl. of redistilled chloroform containing 100 mcg./ml. of dibutyl phthalate. This chloroform solution (2.6 μl.) was injected into the gas chromatograph, and the concentration of chlorphenesin was determined by the relative peak height method using dibutyl phthalate as internal standard.

[1] Chromosorb WAW, Johns Manville Products Corp., New York N. Y.

RESULTS AND DISCUSSION

It was necessary to convert chlorphenesin to a suitable derivative for gas chromatographic analysis since its chromatogram showed two peaks. Chlorphenesin can be quantitated gas chromatographically when relative peak height is used as an index of concentration since a linear relationship exists between relative peak height and drug concentration (trimethylsilyl ether derivative of chlorphenesin) in the range of 1–10 mcg./ml. of plasma (Fig. 1). The reproducibility of the procedure is indicated by the standard error of the triplicate determinations shown in Fig. 1.

The extraction procedure effectively separates chlorphenesin from normal interfering plasma constituents since determinations in normal human plasma give little or no blank (<0.5 mcg./ml.). It was also established that the known major metabolites of chlorphenesin, *p*-chlorophenoxylactic acid and *p*-chlorophenoxyacetic acid (10), do not interfere in the assay procedure.

REFERENCES

(1) F. M. Berger, G. Fukui, B. J. Ludwig, and S. Margolin, *Proc. Soc. Exptl. Biol. Med.*, **124**, 303(1967).

(2) G. M. Fukui, F. M. Berger, G. C. Chandlee, and E. G. Goldenbaum, *J. Bacteriol.*, **96**, 1133(1968).

(3) F. M. Berger, G. M. Fukui, E. G. Goldenbaum, M. DeAngelo, and G. C. Chandlee, *J. Immunol.*, **102**, 1024(1969).

(4) L. M. Lichtenstein and N. F. Adkinson, *ibid.*, to be published.

(5) P. Stross and R. E. Stuckey, *J. Pharm. Pharmacol.*, **2**, 549(1950).

(6) A. A. Forist and R. W. Judy, *J. Pharm. Sci.*, **53**, 1244(1964).

(7) E. F. Salim and R. E. Booth, *ibid.*, **56**, 623(1967).

(8) E. Titus, S. Ulich, and A. P. Richardson, *J. Pharmacol. Exptl. Therap.*, **93**, 129(1948).

(9) J. B. Wyngaarden, L. A. Woods, and M. H. Seevers, *Proc. Soc. Exptl. Biol. Med.*, **66**, 256(1947).

(10) J. Edelson, J. F. Douglas, and B. J. Ludwig, *Biochem. Pharmacol.*, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 7, 1969 from *Wallace Laboratories, Division of Carter-Wallace, Inc., Cranbury, NJ 08512*

Accepted for publication September 5, 1969.

* Present address: Wallace and Tiernan, Inc., Cedar Knolls, New Jersey.

Determination of Trace Amounts of Water in Glycerides by Near-Infrared Spectroscopy

R. J. WARREN, J. E. ZAREMBO, C. W. CHONG, and M. J. ROBINSON

Abstract □ A rapid accurate method for determining trace amounts of water in glycerides is presented. The method is based on the measurement of the combination band for water which occurs at 1.896 μ in chloroform solution. In addition to the advantage of making it possible to work at low levels, the method is specific for water and free of interferences due to other OH groups. Several commercial samples of glyceryl mono- and distearates were analyzed by this method, and showed water contents of 0.4 to 1.2%.

Keyphrases □ Glycerides—water determination □ Water determination—trace amounts □ Near IR spectrophotometry—analysis

During the authors' investigation of methods for the analysis of mono-, di-, and triglycerides, it became necessary to develop a rapid and accurate method for the analysis of trace amounts of water. Chemical methods, such as the Karl Fischer technique, were tedious, time-consuming, and/or unsatisfactory for these particular needs. Solution spectra on conventional IR instruments were rejected because of the many overlapping absorption bands from the various —OH groups in the glyceride molecule itself. Near-IR spectroscopy was investigated to determine the feasibility of using this technique to determine the OH absorption of water in the presence of OH groups on the glyceride molecule. It was found that OH groups in different molecular environment, such as water or glyceride —OH's, do behave differently, and are amenable to investigation by near-IR spectrophotometry. This has also been shown by others (1). The absorption of water OH, attributable to a combination band of the OH group (2, 3), occurs at 1.896 μ in chloroform solution. The various OH groups of the glyceride molecule are found in the vicinity of 1.430 and 2.100 μ (4). This leaves the area of water absorption free from OH absorptions related to the materials under investigation, and presents us with a method specific for water and free from interfering substances.

EXPERIMENTAL

Glycerides were obtained from the Kessler Chemical Company. The glycerides are commercial samples consisting of a mixture of mono-, di- and tripalmitate, stearate and glyceryl esters. There are also traces of free glycerol, stearic, palmitic, myristic, and other unknown acids in trace amounts. Duplicate analyses of these samples, made using the proposed method, showed 1.2, 1.4, 0.4, 0.5, and 0.8% water.

The near-IR spectra were obtained on a Cary model 14 recording spectrophotometer, using matched fused silica cells of infrared quality having a light path of 5 cm. Chloroform was used as a solvent and as solvent compensator in the reference beam.

RESULTS AND DISCUSSION

The method for the determination of water in glycerides is as follows: transfer about 2.5 g. of sample, accurately weighed, to a 50-ml. volumetric flask; dissolve in, and dilute to volume with chloroform. Record the absorption spectrum of this solution *versus* chloroform in 5-cm. cells from 1.930 to 1.850 μ. Measure the difference (ΔA) between the absorbances at 1.896 and 1.920 μ. From a calibration chart of standards, determine the percent of water present.

$$\text{calculation: } \frac{\text{wt. of water present (from chart)} \times 100}{\text{wt. of glyceride sample}} = \% \text{ water}$$

The calibration or standard curve is prepared as follows: dissolve known amounts of water in 50 ml. of chloroform, and run the absorption spectrum on the Cary Model 14 in 5-cm. cells from 1.930 to 1.850 μ. Measure the difference (ΔA) between the absorbances at 1.896 and 1.920 μ. Plot the absorbance *versus* concentration values to prepare a calibration curve.

The spectra obtained from the calibration curve are shown in Fig. 1. Plotting these values against the concentration or percent water produces the calibration or working plot shown in Fig. 2.

This method has the advantages of being rapid, accurate for small

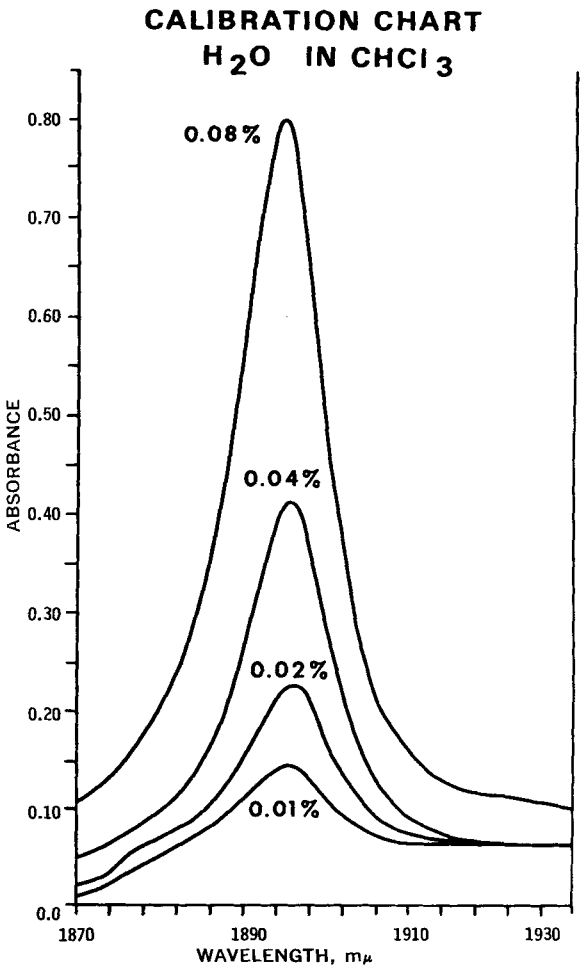


Figure 1—*Spectra obtained from calibration curve (see text for calculation).*

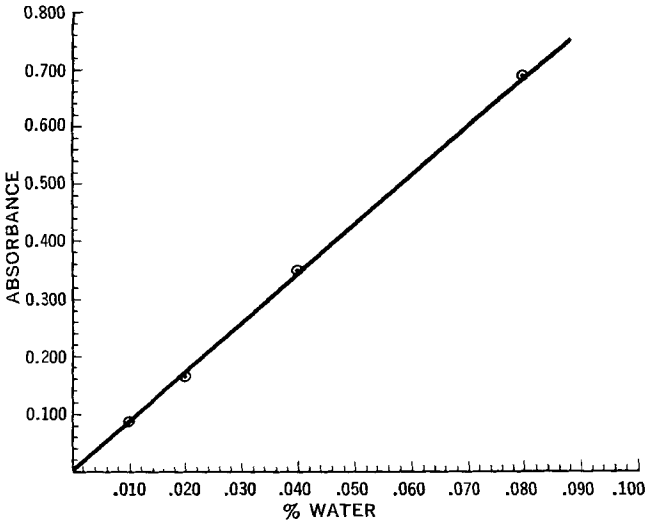


Figure 2—*Calibration or working plot obtained from the values in Fig. 1 plotted against concentration.*

amounts of water, and free from interferences. In addition, it makes it possible to determine water down to the level of 0.05% based on an initial sample weight of 2.5 g.

REFERENCES

(1) D. Chapman and J. F. Nacey, *Analyst*, **83**, 377(1958).

(2) R. F. Goddu, in "Advances in Analytical Chemistry and Instrumentation," Vol I, C. N. Reilley, Ed., Interscience, New York, N. Y., 1960, p. 347.

(3) J. E. Sinsheimer and N. M. Poswalk, *J. Pharm. Sci.*, **57**, 2007(1968).

(4) H. Susi, S. G. Morris, and W. E. Scott, *J. Am. Oil Chemists' Soc.*, **38**, 199(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 10, 1969 from *Smith Kline & French Laboratories, Philadelphia, PA 19101*

Accepted for publication August 13, 1969.

The authors are grateful to Mrs. Andrea Morgan for technical assistance in obtaining the spectra presented here.

Aminosteroids

ARVIN P. SHROFF

Abstract □ Synthesis of 17β-acetamido-6α,16α-dimethylandrost-4-en-3-one and 3-aza-17β-acetamido-6α,16α-dimethylandrost-4a-en-4-one *via* Beckmann and Schmidt rearrangement has been described. Both of these compounds were judged inactive when subjected to Herschberger androgen-anabolic assay.

Keyphrases □ 17β-Acetamido-6α, 16α-dimethylandrost-4-en-3-one—synthesis □ 3-Aza-17β-acetamido-6α, 16α-dimethylandrost-4a-en-4-one—synthesis □ UV spectrophotometry—identity □ IR spectrophotometry—identity

Torizuka *et al.* (1) while investigating the dynamics of protein metabolism in man, found that anabolic steroids such as 19-nor-testosterone phenylpropionate and 4-chlorotestosterone acetate, did not inhibit degradation of protein but exclusively stimulated its synthesis. The primary site of action is possibly at the nuclear level for the production of RNA's essential for protein biosynthesis (2). There are a large number of theoretical possibilities by which an androgen molecule can stimulate this RNA production. One of them suggested by Hübener (3) would be to inhibit a repressor aimed at an operator gene and controlled by a regulator gene. The interaction between androgen molecule and protein has been reported by Westphal (4) and that this interaction is on the β-face of the steroid molecule was suggested by Wolff *et al.* (5).

This work was initiated to investigate this hypothesis and to see if creation of high electron densities in Ring A and D would impart or enhance biological response. 6α,16α-Dimethylprogesterone was chosen for these molecular modifications. Treatment of I with pyrrolidine gave the enamine II which could be converted into 20-oximino Compound III with hydroxylamine hydrochloride. Beckman rearrangement of III using thionyl chloride, followed by acid hydrolysis gave the desired 17β-acetamido-6α,16α-dimethylandrost-4-en-3-one (IV). Compound I when subjected to Schmidt rearrangement gave 3-aza-17β-acetamido-6α,16α-dimethylandrost-4a-en-4-one (V). The spectral data confirmed all the structural assignments. Compounds IV and V were subjected to Herschberger androgen-anabolic assay (6) and judged inactive (see Scheme I).

EXPERIMENTAL

All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. The UV and IR data were obtained on Cary Model 11 and Beckman IR-5 spectrophotometers, respectively. Elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

3-[1-Pyrrolidinyl]-6-16α-dimethylpregn-3,5-dien-20-one (II)—Two grams of I was dissolved in 10 ml. of MeOH with heat and treated with 10 drops of pyrrolidine. Heating was continued for an additional 5 min. when copious precipitates of enamine separated. The precipitates were collected by filtration, washed several times with MeOH and dried to give 1.8 g. (77%) of II, m.p. 137–139°; $\lambda_{max.}^{EtOH}$ 278 mμ; $\lambda_{max.}^{KBr}$ 5.88, 6.1, and 6.23μ.

Anal.—Calcd. for $C_{27}H_{41}NO$: N, 3.54. Found: N, 3.23.

3-[1-Pyrrolidinyl]-6,16α-dimethylpregn-3,5-dien-20-one oxime (III)—A mixture containing 1.7 g of II, 500 mg. of $NH_2OH \cdot HCl$ in 5 ml. of pyridine was heated on a steam bath for 1.5 hr. and poured

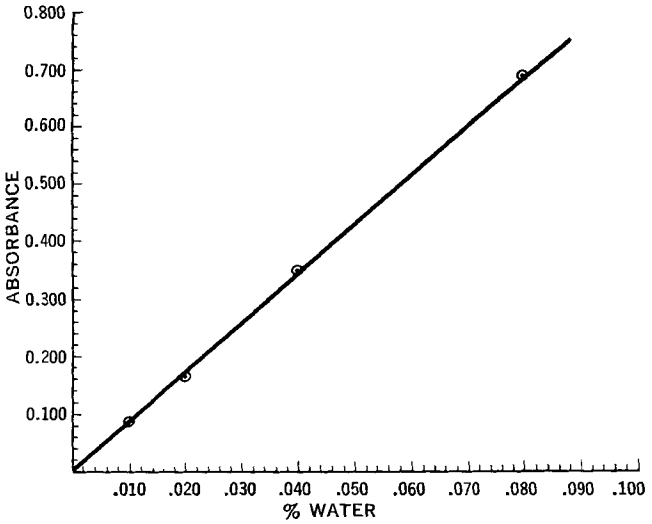


Figure 2—*Calibration or working plot obtained from the values in Fig. 1 plotted against concentration.*

amounts of water, and free from interferences. In addition, it makes it possible to determine water down to the level of 0.05% based on an initial sample weight of 2.5 g.

REFERENCES

(1) D. Chapman and J. F. Nacey, *Analyst*, **83**, 377(1958).

(2) R. F. Goddu, in "Advances in Analytical Chemistry and Instrumentation," Vol I, C. N. Reilley, Ed., Interscience, New York, N. Y., 1960, p. 347.

(3) J. E. Sinsheimer and N. M. Poswalk, *J. Pharm. Sci.*, **57**, 2007(1968).

(4) H. Susi, S. G. Morris, and W. E. Scott, *J. Am. Oil Chemists' Soc.*, **38**, 199(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 10, 1969 from *Smith Kline & French Laboratories, Philadelphia, PA 19101*

Accepted for publication August 13, 1969.

The authors are grateful to Mrs. Andrea Morgan for technical assistance in obtaining the spectra presented here.

Aminosteroids

ARVIN P. SHROFF

Abstract □ Synthesis of 17β-acetamido-6α,16α-dimethylandrost-4-en-3-one and 3-aza-17β-acetamido-6α,16α-dimethylandrost-4a-en-4-one *via* Beckmann and Schmidt rearrangement has been described. Both of these compounds were judged inactive when subjected to Herschberger androgen-anabolic assay.

Keyphrases □ 17β-Acetamido-6α, 16α-dimethylandrost-4-en-3-one—synthesis □ 3-Aza-17β-acetamido-6α, 16α-dimethylandrost-4a-en-4-one—synthesis □ UV spectrophotometry—identity □ IR spectrophotometry—identity

Torizuka *et al.* (1) while investigating the dynamics of protein metabolism in man, found that anabolic steroids such as 19-nor-testosterone phenylpropionate and 4-chlorotestosterone acetate, did not inhibit degradation of protein but exclusively stimulated its synthesis. The primary site of action is possibly at the nuclear level for the production of RNA's essential for protein biosynthesis (2). There are a large number of theoretical possibilities by which an androgen molecule can stimulate this RNA production. One of them suggested by Hübener (3) would be to inhibit a repressor aimed at an operator gene and controlled by a regulator gene. The interaction between androgen molecule and protein has been reported by Westphal (4) and that this interaction is on the β-face of the steroid molecule was suggested by Wolff *et al.* (5).

This work was initiated to investigate this hypothesis and to see if creation of high electron densities in Ring A and D would impart or enhance biological response. 6α,16α-Dimethylprogesterone was chosen for these molecular modifications. Treatment of I with pyrrolidine gave the enamine II which could be converted into 20-oximino Compound III with hydroxylamine hydrochloride. Beckman rearrangement of III using thionyl chloride, followed by acid hydrolysis gave the desired 17β-acetamido-6α,16α-dimethylandrost-4-en-3-one (IV). Compound I when subjected to Schmidt rearrangement gave 3-aza-17β-acetamido-6α,16α-dimethylandrost-4a-en-4-one (V). The spectral data confirmed all the structural assignments. Compounds IV and V were subjected to Herschberger androgen-anabolic assay (6) and judged inactive (see Scheme I).

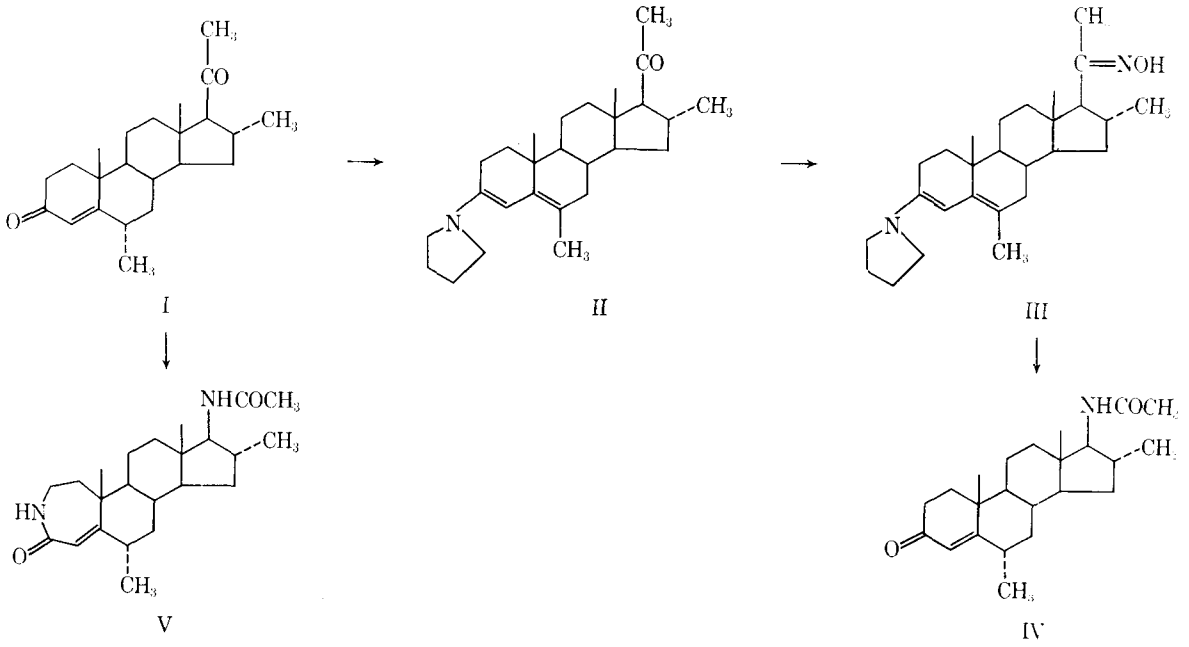
EXPERIMENTAL

All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. The UV and IR data were obtained on Cary Model 11 and Beckman IR-5 spectrophotometers, respectively. Elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

3-[1-Pyrrolidinyl]-6-16α-dimethylpregn-3,5-dien-20-one (II)—Two grams of I was dissolved in 10 ml. of MeOH with heat and treated with 10 drops of pyrrolidine. Heating was continued for an additional 5 min. when copious precipitates of enamine separated. The precipitates were collected by filtration, washed several times with MeOH and dried to give 1.8 g. (77%) of II, m.p. 137–139°; $\lambda_{max.}^{EtOH}$ 278 mμ; $\lambda_{max.}^{KBr}$ 5.88, 6.1, and 6.23μ.

Anal.—Calcd. for $C_{27}H_{41}NO$: N, 3.54. Found: N, 3.23.

3-[1-Pyrrolidinyl]-6,16α-dimethylpregn-3,5-dien-20-one oxime (III)—A mixture containing 1.7 g of II, 500 mg. of $NH_2OH \cdot HCl$ in 5 ml. of pyridine was heated on a steam bath for 1.5 hr. and poured



Scheme I

over a large amount of ice water. The solid thus separated was collected by filtration and recrystallized from MeOH-H_2O to yield 1.5 g. (88%) of III, m.p. 171–173°. $\lambda_{max.}^{EtOH}$ 279mμ; $\lambda_{max.}^{KBr}$ 3.01 and 6.11μ.

Anal.—Calcd. for $C_{27}H_{42}N_2O$: N, 6.82. Found: N, 6.87.

17β-Acetamido-6α,16α-dimethylandrost-4-en-3-one (IV)—A dioxane solution of 3.0 g. of III was treated with 1.0 ml. of $SOCl_2$ and the mixture stirred for 0.5 hr. It was poured over a large amount of ice water and neutralized with NaOH solution. The neutral solution was extracted with CH_2Cl_2 and the organic layer was thoroughly washed with H_2O, dried (Na_2SO_4), and evaporated to give an oil. $\lambda_{max.}^{EtOH}$ 278 mμ; $\lambda_{max.}^{NaCl}$ 3.0 and 6.0μ.

The oil was hydrolyzed with 25 ml. of 5% methanolic H_2SO_4 by refluxing the solution for 2 hr. Evaporation of excess alcohol and pouring the concentrate into a large amount of ice water gave solid residue. It was collected by filtration and subjected to column chromatography on neutral alumina. Elution with 50% CH_2Cl_2–Et_2O gave IV m.p. 205–207°. $\lambda_{max.}^{EtOH}$ 240 mμ; $\lambda_{max.}^{KBr}$ 2.99, 5.99 and 6.05μ.

Anal.—Calcd. for $C_{23}H_{35}NO_2$: C, 77.26; H, 9.87; N, 3.92. Found: C, 77.55; H, 9.78; N, 3.36.

3-Aza-17β-acetamido-6α,16α-dimethylandrost-4a-en-4-one (V)—Compound I (5.0 g.) was added by 150 g. of polyphosphoric acid (PPA) and was maintained at 55°. To this 5.0 g. of NaN_3 was added in small portions over a period of 1–5 hr. with intermittent stirring. The mixture was allowed to remain at about 55° for 7 hr. and the PPA then decomposed with H_2O. The solution was neutralized with 25% NaOH solution and extracted with CH_2Cl_2. The organic phase was washed several times with H_2O, dried (Na_2SO_4), and evaporated. The residue was recrystallized from CH_2Cl_2–Et_2O to give 2.5 g. (50%) of V, m.p. 167–170°. $\lambda_{max.}^{EtOH}$ 218 mμ; $\lambda_{max.}^{KBr}$ 3.0; 6.02μ.

Anal.—Calcd. for $C_{23}H_{36}N_2O_2$: C, 74.15; H, 9.74; N, 7.52. Found: C, 74.18; H, 10.01; N, 7.31.

REFERENCES

(1) K. Torizuka, K. Hamamoto, K. Koshiyama, K. Iwai, H. Takayama, and T. Miyalke, *Metabolism*, **12**, 11(1963).

(2) C. D. Kochakian, "Mechanism of Hormone Action," Academic, New York, N. Y., 1965, p. 192.

(3) H. J. Hübener, *Deut. Med. Wochschr.*, **87**, 438(1962).

(4) U. Westphal, "Mechanism of Action of Steroid Hormones," C. A. Villee and L. L. Engel, Eds., Pergamon, New York, N. Y., 1961, p. 33.

(5) M. E. Wolff, W. Ho, and P. Kwok, *J. Med. Chem.*, **7**, 577 (1964).

(6) L. G. Herschberger, E. G. Shipley, and R. K. Meyer, *Proc. Soc. Exptl. Biol. Med.*, **83**, 175(1953).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 1, 1969 from the *Division of Organic Chemistry, Ortho Research Foundation, Raritan, NJ 08869*

Accepted for publication August 11, 1969.

The author thanks Dr. R. P. Blye of the Division of Pharmacology for the biological evaluation and Dr. Irving Scheer of the Division of Organic Chemistry for his encouragement.

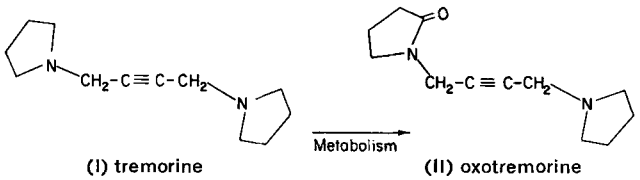
Molecular Orbital Conformation of Oxotremorine and a Comparison With the Muscarinic Pattern

LEMONT B. KIER

Abstract □ The conformational preference of oxotremorine has been calculated using extended Hückel molecular orbital theory. The molecule was found to be comparatively free in regard to the relationship of the two rings and the rotation of the lactone ring. The energy-permitted stereochemistry allows the quaternary nitrogen, the triple bond, and the carbonyl oxygen to assume the same relationship as previously calculated for the muscarinic pharmacophore, if the triple bond in oxotremorine is assumed to mimic the electronic character of the ether oxygen in the muscarinics.

Keyphrases □ Oxotremorine—molecular orbital conformation □ Molecular orbital conformation, oxotremorine—muscarinic activity □ Conformational molecular preference—oxotremorine

A current hypothesis on the peripheral and central actions of tremorine (I) and its metabolite oxotremorine (II) (Scheme I) is that the latter molecule is a muscarinic



Scheme I

agonist. The peripheral action of oxotremorine, studied by Cho *et al.* (1) leaves little doubt that this compound is a peripheral muscarinic agent equal in potency to acetylcholine. The effects can be blocked by pretreatment with anticholinergic drugs like methanthelinium or atropine bromides. In animals so pretreated, a tremor, ataxia, and spasticity still persist. Atropine sulfate blocks these effects and so it is presumed that the tremor effect is due to activation of muscarinic receptors in the central nervous system (*2*).

Studies by Lundgren and Malmberg (3) suggest that oxotremorine-induced tremors are not due to direct action in the CNS, but are due to the ability of the drug to induce an increased biosynthesis of acetylcholine in the CNS. Oxotremorine, however, fails to stimulate *in vitro* systems containing choline acetylase. It has been observed that there is a rise in acetylcholine level which roughly parallels the oxotremorine-induced

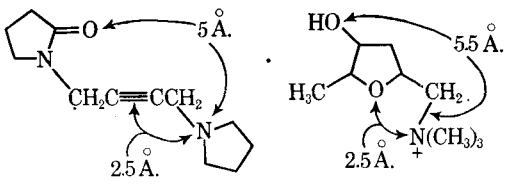


Figure 1—*Proposed steric relationship of essential features in oxotremorine and muscarine (6).*

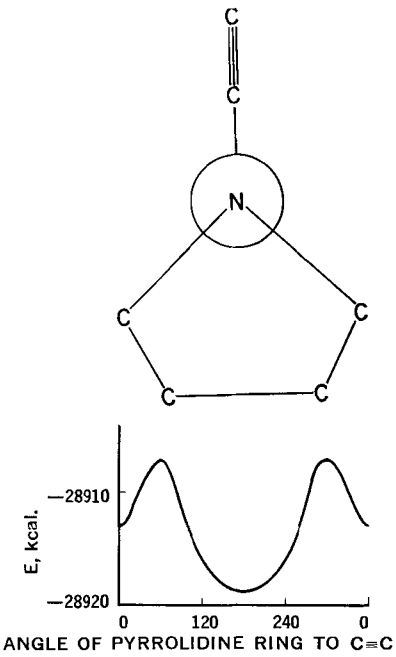


Figure 2—*Calculated relationship of pyrrolidine ring and triple bond in oxotremorine.*

tremor duration. Recent detailed studies by Cox and Potkanjak (4) on this comparative time course show a lack of parallel behavior between tremor manifestation and acetylcholine increase.

Since oxotremorine may be a CNS muscarinic agonist, it is of interest to contemplate how the structure of this molecule compares with known muscarinic agents. Cho *et al.* (1) have commented on the structural dissimilarity of oxotremorine to known muscarinic agents and suggested that oxotremorine was highly active owing to a process at the receptor related to Koshland's (5) induced-fit theory. However, Bebbington *et al.* (6) have compared molecular models of oxotremorine with muscarine and noted that the two could conceivably assume a common geometry, in which the same pattern of charged atoms would prevail. This required the invoking of the acetylenic bond as a negative site of binding in the oxotremorine molecule, mimicking the ether oxygen in muscarine. A plausible explanation for the possible direct receptor action of oxotremorine then centers on the ability of the molecule to assume a favorable conformation, mimicking the muscarinic pharmacophore, Fig. 1.

In a previous study, the author calculated the conformations of three potent muscarinic agents using a molecular orbital theory in which overlap integrals and nonbonded interactions are treated for all valence

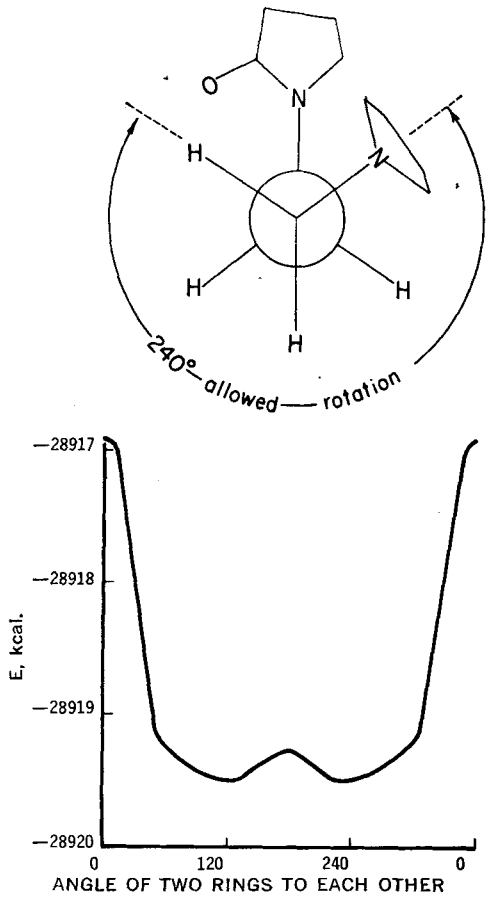


Figure 3—*Calculated relationship of the two rings in oxotremorine.*

electrons (7). The method, known as extended Hückel theory, generates a total energy as a function of geometry; hence, conformation preference can be assessed by energy minimization (8). A common pattern of charged atoms or groups was calculated for acetylcholine, muscarine, and muscarone. The author has predicted that this pattern is a reasonable working model of the muscarinic pharmacophore.

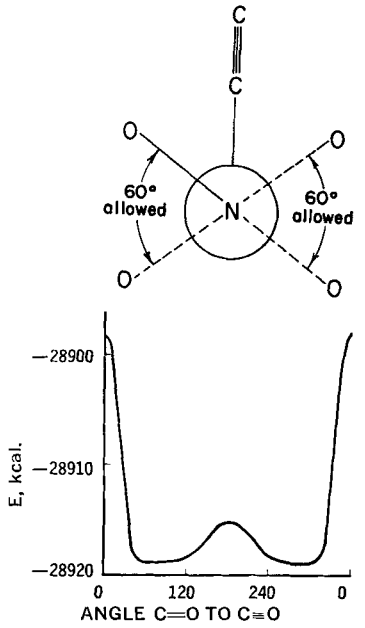


Figure 4—*Calculated relationship of the lactone ring and the triple bond in oxotremorine.*

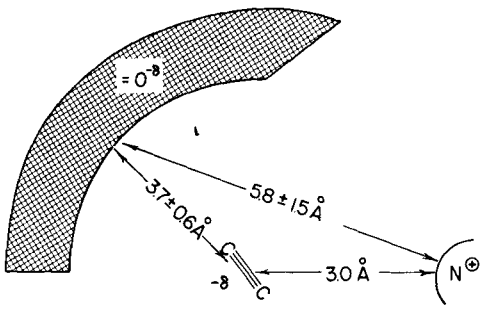


Figure 5—*Calculated energy-permitted relationship of essential features in oxotremorine. Shaded area shows region which oxygen atom may occupy relative to the other molecular features.*

In the present study, the author undertook molecular orbital calculations on the oxotremorine molecule, to determine its preferred conformation and to see whether a pattern of charged atoms or groups corresponded to the previously calculated muscarinic pharmacophore.

EXPERIMENTAL

The molecule was calculated as the protonated salt. Standard bond lengths and angles were used according to Pople and Gordon (9). The Coulomb integrals and Slater exponents were those previously employed by Hoffmann (8) and the author (7). In comparing the energy *versus* geometry profiles, the preferred conformation was taken to be the geometry of lowest energy. All geometries within 1 kcal. or less of this energy minimum were considered to be equally preferred.

RESULTS AND DISCUSSION

The results of the calculations show that the molecule can exist in a variety of preferred structures. The two rings exhibit considerable independence with respect to their conformational preferences. When the distance between any two first-row atoms on opposite rings exceeds 4 Å., the two rings are not influential in respect to their preferred conformations. We have justified the assumption that distant atoms do not influence conformation in a recent quantum mechanical discussion (10). This assumption has been used by others (11).

The pyrrolidine ring is symmetrically disposed relative to the triple bond, Fig. 2. The two rings can assume any conformation except that which eclipses their methylene connecting groups, Fig. 3. The lactone ring can assume two zones of conformational preference to within 60° of coplanarity with the acetylenic bond, Fig. 4. The distance separating the pyrrolidine protonated nitrogen from the center of the triple bond is 3.0 Å. The distance between the carbonyl oxygen and the protonated nitrogen ranges from 4.3–7.2

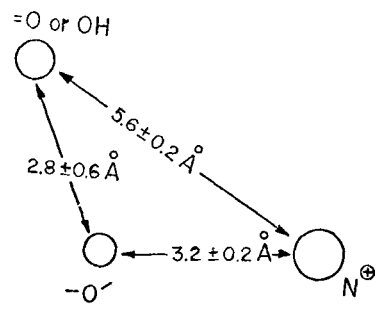


Figure 6—*Calculated muscarinic pharmacophore based on conformations calculated for acetylcholine, muscarine, and muscarone (7).*

Å., owing to the variation possible in conformational preference. The carbonyl oxygen distance from the center of the triple bond ranges from 3.1–4.2 Å.

An allowed pattern of atoms can thus be drawn for oxotremorine, Fig. 5. This calculated energy-allowed pattern clearly mirrors the muscarinic pattern, previously proposed, Fig. 6.

It can be concluded that oxotremorine can assume a muscarinic pharmacophore, within its structure, on the basis of theoretical calculations of its preferred conformations. Although conclusive pharmacological evidence that would brand oxotremorine as a central muscarinic agonist is still lacking, these studies implicate the feasibility of this mechanism.

REFERENCES

(1) A. K. Cho, W. L. Haslett, and D. J. Jenden, *J. Pharmacol. Exptl. Therap.*, **138**, 249(1962).

(2) R. George, W. L. Haslett, and D. J. Jenden, *Life Sci.*, **2**, 361(1962).

(3) G. Lundgren and J. Malmberg, *Biochem. Pharmacol.*, **17**, 2051(1968).

(4) B. Cox and D. Potkanjak, *Brit. J. Pharmacol.*, **35**, 295 (1969).

(5) E. E. Koshland, in "The Enzymes," Academic, New York, N. Y., 1959.

(6) A. Bebbington, R. W. Brimblecomb, and D. Shakeshaft, *Advan. Drug Res.*, **2**, 143(1965).

(7) L. B. Kier, *Mol. Pharmacol.*, **3**, 487(1967).

(8) R. Hoffmann, *J. Chem. Phys.*, **39**, 1397(1963).

(9) J. A. Pople and M. Gordon, *J. Am. Chem. Soc.*, **89**, 4253 (1967).

(10) L. B. Kier, *J. Med. Chem.*, **11**, 915(1968).

(11) J. A. Kapecki and J. E. Baldwin, *J. Am. Chem. Soc.*, **91**, 1120(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Biochemistry Division, Columbus Laboratories, Battelle Memorial Institute, Columbus, OH 43201*

Accepted for publication September 11, 1969.

This research was supported by grant No. GM-16312 from the National Institutes of Health, Bethesda, Md.

Antiradiation Compounds XIII: 1-(Dithioacetic Acid)-Pyridinium Betaines

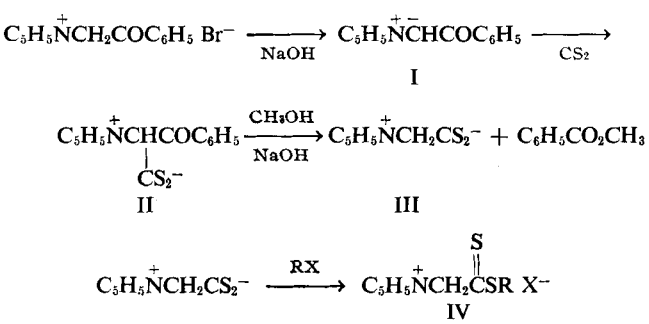
WILLIAM O. FOYE, YOUNG JA CHO, and KYUNG HEE OH

Abstract □ Active mono-*S*-alkyl esters prepared from 1-(dithioacetic acid)-pyridinium betaine (III) were found to be sufficiently stable for screening as radiation-protective agents, and *e*-withdrawing substituents in the pyridine ring gave stable betaines. Reaction of the methyl ester of III with phenacyl bromide and alkali resulted in *S*-alkylation to give a ketene mercaptal betaine (VIII). Both the allyl and *p*-nitrobenzyl esters of 1-(dithioacetic acid)-pyridinium halides were radiation-protective in mice, and betaines with substituents in the pyridine ring were radiation-protective in a bacterial test.

Keyphrases □ Antiradiation compounds—synthesis □ 1-(Dithioacetic acid)-pyridinium betaines—synthesis □ Pharmacological screening—antiradiation compounds □ IR spectrophotometry—structure

Amino and guanidino zwitterions containing the thiosulfate (1), phosphorothioate (2), and trithiocarbonate (3) groups, as well as other zwitterionic structures (4) which contain the β-mercaptoethylamine moiety have shown appreciable radiation-protective abilities in mice. α-Acetamidinium thiosulfate zwitterions (5) have also shown good radiation-protective properties. It appeared likely, therefore, that other zwitterions containing, or giving rise to, a thiolate anion should be radiation-protective. Dithioacetic acid pyridinium betaines, obtained from the reaction of carbon disulfide and pyridinium ylids, appeared to have the necessary structural requirements for protective activity in a charged nitrogen and a thiolate anion, and were therefore investigated for possible radiation-protective properties.

Pyridinium betaines are compounds, termed by Kröhnke (6), which contain a negatively charged carbon, oxygen, or sulfur adjacent to, or at greater distance from, the positively charged nitrogen; the term ylid is now generally used where the charges are on adjacent atoms. When phenacylpyridinium bromide is treated with alkali, a red solution of the ylid (I) results. Reaction with carbon disulfide gives the α-dithiocarboxylate zwitterion (II) which decomposes to the dithioacetic acid betaine (III) in methanolic alkali (6). This compound, although unstable, can be isolated, and remains stable long enough for derivatives to be prepared. Conversion to the active allyl and *p*-nitrobenzyl esters (IV) was carried out, and compounds of sufficient stability for antiradiation screening were obtained.



Reaction of the dithioacetic acid betaine (III) with bromoethylamine hydrobromide did not give the desired ester; also, no reaction with phenyl halides or

Å., owing to the variation possible in conformational preference. The carbonyl oxygen distance from the center of the triple bond ranges from 3.1–4.2 Å.

An allowed pattern of atoms can thus be drawn for oxotremorine, Fig. 5. This calculated energy-allowed pattern clearly mirrors the muscarinic pattern, previously proposed, Fig. 6.

It can be concluded that oxotremorine can assume a muscarinic pharmacophore, within its structure, on the basis of theoretical calculations of its preferred conformations. Although conclusive pharmacological evidence that would brand oxotremorine as a central muscarinic agonist is still lacking, these studies implicate the feasibility of this mechanism.

REFERENCES

(1) A. K. Cho, W. L. Haslett, and D. J. Jenden, *J. Pharmacol. Exptl. Therap.*, **138**, 249(1962).

(2) R. George, W. L. Haslett, and D. J. Jenden, *Life Sci.*, **2**, 361(1962).

(3) G. Lundgren and J. Malmberg, *Biochem. Pharmacol.*, **17**, 2051(1968).

(4) B. Cox and D. Potkanjak, *Brit. J. Pharmacol.*, **35**, 295 (1969).

(5) E. E. Koshland, in "The Enzymes," Academic, New York, N. Y., 1959.

(6) A. Bebbington, R. W. Brimblecomb, and D. Shakeshaft, *Advan. Drug Res.*, **2**, 143(1965).

(7) L. B. Kier, *Mol. Pharmacol.*, **3**, 487(1967).

(8) R. Hoffmann, *J. Chem. Phys.*, **39**, 1397(1963).

(9) J. A. Pople and M. Gordon, *J. Am. Chem. Soc.*, **89**, 4253 (1967).

(10) L. B. Kier, *J. Med. Chem.*, **11**, 915(1968).

(11) J. A. Kapecki and J. E. Baldwin, *J. Am. Chem. Soc.*, **91**, 1120(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Biochemistry Division, Columbus Laboratories, Battelle Memorial Institute, Columbus, OH 43201*

Accepted for publication September 11, 1969.

This research was supported by grant No. GM-16312 from the National Institutes of Health, Bethesda, Md.

Antiradiation Compounds XIII: 1-(Dithioacetic Acid)-Pyridinium Betaines

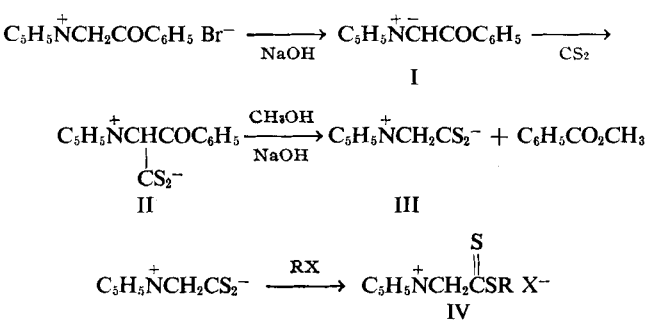
WILLIAM O. FOYE, YOUNG JA CHO, and KYUNG HEE OH

Abstract □ Active mono-*S*-alkyl esters prepared from 1-(dithioacetic acid)-pyridinium betaine (III) were found to be sufficiently stable for screening as radiation-protective agents, and *e*-withdrawing substituents in the pyridine ring gave stable betaines. Reaction of the methyl ester of III with phenacyl bromide and alkali resulted in *S*-alkylation to give a ketene mercaptal betaine (VIII). Both the allyl and *p*-nitrobenzyl esters of 1-(dithioacetic acid)-pyridinium halides were radiation-protective in mice, and betaines with substituents in the pyridine ring were radiation-protective in a bacterial test.

Keyphrases □ Antiradiation compounds—synthesis □ 1-(Dithioacetic acid)-pyridinium betaines—synthesis □ Pharmacological screening—antiradiation compounds □ IR spectrophotometry—structure

Amino and guanidino zwitterions containing the thiosulfate (1), phosphorothioate (2), and trithiocarbonate (3) groups, as well as other zwitterionic structures (4) which contain the β-mercaptoethylamine moiety have shown appreciable radiation-protective abilities in mice. α-Acetamidinium thiosulfate zwitterions (5) have also shown good radiation-protective properties. It appeared likely, therefore, that other zwitterions containing, or giving rise to, a thiolate anion should be radiation-protective. Dithioacetic acid pyridinium betaines, obtained from the reaction of carbon disulfide and pyridinium ylids, appeared to have the necessary structural requirements for protective activity in a charged nitrogen and a thiolate anion, and were therefore investigated for possible radiation-protective properties.

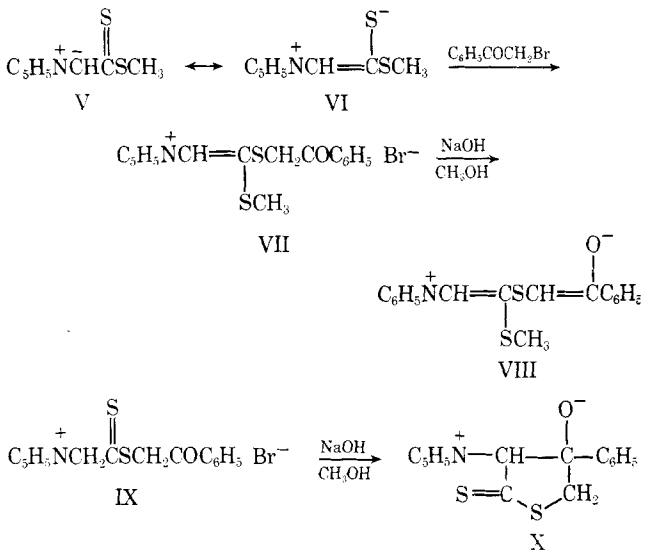
Pyridinium betaines are compounds, termed by Kröhnke (6), which contain a negatively charged carbon, oxygen, or sulfur adjacent to, or at greater distance from, the positively charged nitrogen; the term ylid is now generally used where the charges are on adjacent atoms. When phenacylpyridinium bromide is treated with alkali, a red solution of the ylid (I) results. Reaction with carbon disulfide gives the α-dithiocarboxylate zwitterion (II) which decomposes to the dithioacetic acid betaine (III) in methanolic alkali (6). This compound, although unstable, can be isolated, and remains stable long enough for derivatives to be prepared. Conversion to the active allyl and *p*-nitrobenzyl esters (IV) was carried out, and compounds of sufficient stability for antiradiation screening were obtained.



Reaction of the dithioacetic acid betaine (III) with bromoethylamine hydrobromide did not give the desired ester; also, no reaction with phenyl halides or

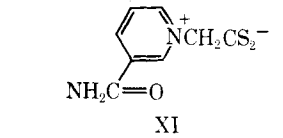
2,4-dinitrochlorobenzene took place. Kröhnke *et al.* (7) have found that the methyl ester of dithioacetic acid pyridinium betaine exists in methanolic alkali as both the carbon ylid (V) and the sulfur ylid (VI), and that alkyl halides react preferentially with the thiolate anion. To determine whether a di-*S*-alkyl derivative would form preferentially to an *S*-alkyl-C-alkyl derivative, reaction of the methyl ester (IV) with phenacyl bromide was carried out. The product, according to the IR spectrum, was the ketene mercaptal, 2-methylthio-2-phenacylthio-1-vinylpyridinium bromide (VII). In the IR, both C=O and C=C absorption were found; no absorption due to C=C would be expected in the C-alkylation product of IV.

Treatment of VII with methanolic alkali gave yellow crystals of a low-melting, unstable compound from which HBr had been lost. This compound still revealed IR absorption due to both C=O and C=C, as well as enol, and was therefore concluded to be the betaine (VIII). Similar treatment of the phenacyl ester of 1-(dithioacetic acid)-pyridinium bromide (IX) by Kröhnke (6) led to ring-closure to give a stable, high-melting dihydrothiophene betaine (X), which was ultimately converted to a thiophene. Compound X was prepared, and its IR spectrum showed no evidence of either C=O or C=C. Similar attempts to convert VIII to a dihydrothiophene resulted in decomposition.

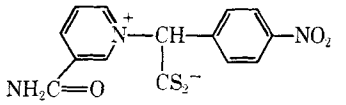


Besides making modifications in the dithioacetic acid portion of the molecule, the authors also desired to learn the effects that *e*-withdrawing substituents in the pyridine ring might have both on stability of the betaines and their radiation-protective ability. Accordingly, 1-phenacyl and 1-*p*-nitrobenzyl derivatives of nicotinamide, ethyl nicotinate, and ethyl isonicotinate were prepared. Reaction of 1-phenacyl-3-carbamylpyridinium bromide with carbon disulfide and alkali gave at once a red solution from which the dithioacetic acid betaine (XI) was isolated. The compound could not be recrystallized without decomposition, but it was stable after being dried.

Treatment of 1-*p*-nitrobenzyl-3-carbamylpyridinium chloride with carbon disulfide in alkali gave a red solution only slowly. Isolation of the product and elemental analysis showed that the *p*-nitrophenyl group had not been removed; the α-dithiocarboxylic acid betaine XII was obtained.



XI



XII

Similarly, reaction of both 1-phenacyl- and 1-*p*-nitrobenzyl-3-carbethoxypyridinium halides with carbon disulfide and alkali gave the α-dithiocarboxylic acid betaines without loss of the benzoyl or *p*-nitrophenyl groups. The same result was obtained with the 1-phenacyl- and 1-*p*-nitrobenzyl-4-carbethoxypyridinium halides; in these cases, very poor analyses were obtained for the products. None of these compounds could be recrystallized without decomposition, but they were relatively stable after drying.

RADIATION-PROTECTIVE PROPERTIES[1]

Tests were carried out in mice *versus* 825r (X-rays) with an observation period of 30 days. The *p*-nitrobenzyl ester (IV) of dithioacetic acid pyridinium chloride gave fair protection (33%), and the allyl ester (IV) provided slight protection (17%) to mice. In antiradiation testing on bacteria, 1-(dithioacetic acid)-3-carbamylpyridinium betaine (XI) gave good protection (>45%), and the α-*p*-nitrophenyl-3-carbamyl (XII), α-benzoyl-3-carbethoxy (XIII), and α-*p*-nitrophenyl-4-carbethoxy pyridinium dithioacetic acid betaines gave poor protection (1–24%).

EXPERIMENTAL

Analyses for carbon, hydrogen, and nitrogen were done by Weiler and Strauss, Oxford, England. Sulfur analyses were done by Parr bomb peroxide fusion. Melting points were taken on a Mel-Temp apparatus and are corrected. IR absorption spectra were obtained with a Perkin-Elmer model 137B spectrometer.

1-(Dithioacetic acid)-pyridinium Betaine (III)—Phenacylpyridinium bromide (6) (28 g., 0.10 mole) was dissolved in 120 ml. of methanol, and 8 ml. of carbon disulfide (0.12 mole) was added at room temperature, followed by 60 ml. of 2 *N* methanolic sodium hydroxide. After 2 hr., the orange crystals were filtered and washed with water. Recrystallization was accomplished from water not over 60°, giving 15.3 g. (90%), m.p. 110–112° (lit. m.p. 110°) (6). The product decomposed after standing in a desiccator for a day.

Anal.—Calcd. for $C_7H_7NS_2$: S, 37.87. Found: S, 38.05.

1-(Dithioacetic acid p-nitrobenzyl ester)-pyridinium Chloride (IV)—*p*-Nitrobenzyl chloride (1.71 g., 0.01 mole) was dissolved in dimethylformamide (20 ml.), and 1-(dithioacetic acid)-pyridinium betaine (1.57 g., 0.009 mole) was added gradually with stirring. A red solution resulted from which yellow crystals were separated and washed with ether. The yield was 1.03 g. (37%); m.p. 167–168°.

Anal.—Calcd. for $C_{14}H_{13}ClN_2O_2S_2$: C, 49.34; H, 3.80; N, 8.25; S, 18.82. Found: C, 48.66; H, 3.89; N, 7.99; S, 19.25.

1-(Dithioacetic acid allyl ester)-pyridinium Bromide (IV)—1-(Dithioacetic acid)-pyridinium betaine (0.85 g., 0.005 mole) was added to a solution of chloroform (5 ml.) and allyl bromide (2 ml., 0.01 mole). Warming on a water bath gave a red solution, and addition of ether

[1] Antiradiation screening of some of the compounds described has been carried out at the Walter Reed Army Institute of Research, and results have been reported through the courtesy of Drs. D. P. Jacobus and T. R. Sweeney.

produced yellow crystals, which were filtered and washed with ether. The yield was 1.0 g. (71%); m.p. 107–111°.

Anal.—Calcd. for $C_{10}H_{12}BrNS_2$: C, 41.38; H, 4.40; N, 4.82; S, 22.10. Found: C, 40.92; H, 4.46; N, 4.67; S, 22.68.

1 - (2 - Methylthio - 2 - phenacylthio - 1 - vinyl) - pyridinium Bromide (VII)—1-(Dithioacetic acid methyl ester)-pyridinium betaine (6) (1.83 g., 0.01 mole) was dissolved in 5 ml. of chloroform, and phenacyl bromide (2.0 g., 0.01 mole) was added. A yellow solution resulted, and addition of ether and refrigeration for a few days produced yellow crystals; 3.08 g. (81%), m.p. 90–95°; IR (KBr) 1675 (C=O), 1630 (C=C), 1600 (doublet, ring stretch) cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{16}BrNOS_2$: C, 50.26; H, 4.19; N, 3.66; S, 16.77. Found: C, 49.71; H, 4.26; N, 3.58; S, 16.20.

1 - (2 - Methylthio - 2 - phenacylthio - 1 - vinyl) - pyridinium Betaine (VIII)—To a solution of the previous product (3.82 g., 0.01 mole) in 5 ml. of methanol cooled by an ice bath was added 2 *N* methanolic sodium hydroxide. After the solution was refrigerated for 1 hr., yellow crystals appeared and were filtered and washed with water. Recrystallization from methanol gave 2.35 g. (75%), m.p. 80–83°; IR (mineral oil) 1675 (C=O), 1615 (C=C), 1575 (enol) cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{15}NOS_2$: C, 63.78; H, 4.19; N, 4.64; S, 21.28. Found: C, 63.32; H, 4.67; N, 4.24; S, 21.03.

1-Phenacyl-3-carbethoxypyridinium Bromide—A solution of ethyl nicotinate (8), (6.53 g., 0.044 mole), phenacyl bromide (7.86 g., 0.044 mole), and methanol (60 ml.) was refluxed for 16 hr. The solvent was removed by a stream of air, and the residue was washed with ether. Recrystallization from ether–methanol gave 14 g. (93%); m.p. 181–182°.

Anal.—Calcd. for $C_{16}H_{16}BrNO_3$: C, 54.87; H, 4.61; N, 4.00. Found: C, 54.61; H, 4.65; N, 3.62.

1-(p-Nitrobenzyl)-3- carbethoxypyridinium Chloride—A solution of ethyl nicotinate (8) (3.02 g., 0.02 mole), *p*-nitrobenzyl chloride (3.24 g., 0.02 mole), and methanol (30 ml.) was refluxed for 35 hr. Addition of ether produced yellow crystals which were collected and recrystallized from ether–methanol, giving 2.15 g. (41%); m.p. 96–97°.

Anal.—Calcd. for $C_{15}H_{15}ClN_2O_4$: C, 55.82; H, 4.68; N, 8.68. Found: C, 55.66; H, 4.95; N, 8.85. . .

1-Phenacyl-4-carbethoxypyridinium Bromide—A solution of ethyl isonicotinate (8) (7.55 g., 0.05 mole), phenacyl bromide (9.95 g., 0.05 mole), and methanol (95 ml.) was refluxed for 5 hr. The solvent was evaporated, and the residue was washed with ether. The product was recrystallized from methanol, giving 15.1 g. (85%) of yellow crystals, m.p. 183.5–185°.

Anal.—Calcd. for $C_{16}H_{16}BrNO_3$: C, 54.87; H, 4.61; N, 4.00. Found: C, 54.60; H, 4.51; N, 4.12.

1-(p-Nitrobenzyl)-4-carbethoxypyridnium Chloride—A solution of ethyl isonicotinate (8) (7.55 g., 0.05 mole), *p*-nitrobenzyl chloride (8.56 g., 0.05 mole), and methanol (25 ml.) was refluxed for 24 hr. The solvent was removed by a stream of air, and the residue was washed with ether. Recrystallization from ether–methanol gave 8.0 g. (49%); m.p. 173.5–174°.

Anal.—Calcd. for $C_{15}H_{15}ClN_2O_4$: C, 55.82; H, 4.68; N, 8.68. Found: C, 55.32; H, 4.75; N, 8.82.

1-(Dithioacetic acid)-3-carbamylpyridinium Betaine (XI)—1-Phenacyl-3-carbamylpyridinium bromide (9) (11 g., 0.034 mole) dissolved in 1 l. of methanol was treated with carbon disulfide (2.6 g., 0.34 mole) dissolved in 4 ml. of methanol, and the solution was stirred at room temperature for 25 min. It was then treated with 20 ml. of 2 *N* NaOH in methanol, stirred at 5–10° for 25 min., and stored over dry ice overnight. The orange precipitate was filtered, washed with cold ether, and dried *in vacuo*. The yield was 2.53 g. (35%); m.p. 104.5–107°.

Anal.—Calcd. for $C_8H_8N_2OS_2$: C, 45.26; H, 3.79; N, 13.19. Found: C, 45.36; H, 3.92; N, 12.95.

1-[α-(p-Nitrophenyl)dithioacetic acid]-3-carbamylpyridinium Betaine (XII)—1-(*p*-Nitrobenzyl)-3-carbamylpyridinium chloride (9) (2.93 g., 0.01 mole) dissolved in 250 ml. of methanol was treated with carbon disulfide (1 g., 0.013 mole) dissolved in 5 ml. of methanol. Addition of 6 ml. of 2 *N* NaOH in methanol gave a red solution which deposited greenish crystals when stored over dry ice. A yield of 2.19 g. (67%) was obtained; m.p. 115–117°.

Anal.—Calcd. for $C_{14}H_{11}N_3O_3S_2$: C, 50.43; H, 3.32; N, 12.63. Found: C, 49.97; H, 3.44; N, 12.39.

1-(α-Benzoyldithioacetic acid)-3-carbethoxypyridinium Betaine (XIII)—To 1-phenacyl-3-carbethoxypyridinium bromide (3.5 g., 0.01 mole) in 32 ml. of methanol was added carbon disulfide (1 g., 0.013 mole) in 5 ml. of methanol, and the solution was stirred for 15 min. Addition of 6 ml. of 2 *N* NaOH in methanol gave a red solution from which a black precipitate appeared after several hours at room temperature. The product was collected and washed with cold ether, giving 0.77 g. (29%) of red-black solid; m.p. 120–124°.

Anal.—Calcd. for $C_{17}H_{15}NO_3S_2$: C, 59.11; H, 4.37; N, 4.06. Found: C, 59.84; H, 3.99; N, 4.23.

1-[α-(p-Nitrophenyl)dithioacetic acid]-3-carbethoxypyridinium Betaine—1-*p*-Nitrobenzyl-3-carbethoxypyridinium chloride (2 g., 0.006 mole) in 10 ml. of methanol was treated with carbon disulfide (0.5 g., 0.006 mole) in 2 ml. of methanol, and the solution was refluxed 3 hr. After addition of 2 *N* NaOH in methanol (3.6 ml.), a red precipitate appeared, which was filtered and dried. The yield was 1.7 g. (75%); m.p. 125–126°.

Anal.—Calcd. for $C_{16}H_{14}N_2O_4S_2$: C, 53.02; H, 3.95; N, 7.74. Found: C, 53.17; H, 4.53; N, 8.37.

REFERENCES

(1) B. Holmberg and B. Sorbo, *Nature*, **183,** 832(1959).

(2) B. Hansen and B. Sorbo, *Acta Radiol.*, **56,** 141(1961).

(3) W. O. Foye, J. Mickles, R. N. Duvall, and J. R. Marshall, *J. Med. Chem.*, **6,** 509(1963).

(4) L. Field and H. K. Kim, *ibid.*, **9,** 397(1966).

(5) L. Bauer and K. R. Sandberg, *ibid.*, **7,** 766(1964).

(6) F. Kröhnke and K. Gerlach, *Chem. Ber.*, **95,** 1108(1962).

(7) F. Kröhnke, K. Gerlach, and K. Schnalke, *ibid.*, **95,** 1118 (1962).

(8) H. O. Burrus and G. Powell, *J. Am. Chem. Soc.*, **67,** 1468 (1946).

(9) J. L. Hartwell and S. R. L. Korberg, *ibid.*, **68,** 868(1947).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115*

Accepted for publication September 5, 1969.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported by research grant RH00297 from the National Center for Radiological Health, U. S. Public Health Service, Bethesda, Md.

Trace Solvent Contamination as Determined by Surface Pressure Measurement

JOEL L. ZATZ

Abstract □ Amphipathic contaminants, present in organic solvents, were detected by spreading the solvent on water and measuring its surface pressure. The method is quite sensitive and, in many cases, is superior to conventional techniques.

Keyphrases □ Solvent contamination, trace—determination □ Contaminants in solvents—surface pressure effect □ Surface pressure measurements—solvent contamination determination

A number of instances have been reported in which the presence of small quantities of impurities caused a significant change in the properties of the system. For example, the stability of suspensions of channel black in toluene was increased considerably by the presence of traces of moisture in the solvent (1). Contact angle hysteresis in experiments on wetting of solids by liquids has been ascribed to the presence of impurities (2). In studies of adsorption by solids from the liquid phase a high order of solvent purity is necessary. Traces of contaminants may be preferentially adsorbed, leading to spurious results (3). Similar considerations apply to work on monolayers. Surface-active impurities will be concentrated at the interface resulting in a considerable error. Grease from the fingers may represent an important source of contamination (4).

Spectroscopy and chromatography are often used to check the purity of solvents. Both of these methods are capable of detecting small concentrations of foreign substances, under favorable conditions. However, spectroscopy is useful only at wavelengths at which the pure solvent does not itself strongly absorb. Of the various chromatographic techniques, gas chromatography is probably the most sensitive. Contaminants of very low volatility (polymers for example) may be sorbed so strongly by the column that they are not eluted for many hours.

In this communication, another method for detecting solvent contamination is described. A small quantity of the liquid to be tested is spread on an aqueous substrate. Water-insoluble molecules and a number of water-soluble amphipathic molecules and polymers will be concentrated at the air-water interface, causing changes in surface tension which are easily detectable.

EXPERIMENTAL

Water employed as the substrate was deionized and then distilled in an all-glass still. All organic liquids were reagent or spectro grade. Glassware was cleaned in chromic acid cleaning solution prior to use. Near IR spectra were obtained using the Beckman DK-2 spectrophotometer. All gas chromatography was performed using a Perkin-Elmer vapor fractometer, model 154. A column of propylene glycol was employed; helium was the carrier gas.

The Teflon surface balance has been previously described (5). Surface tension was determined by the Wilhelmy plate method (6), using a roughened platinum plate. The apparatus employed had a sensitivity of 0.1 dynes/cm. The water surface was swept with the Teflon barrier. Then 1 ml. of the organic liquid to be tested was placed dropwise on the water surface and allowed to spread. After a waiting time of 10 to 15 min. the barrier of the surface balance was moved so as to reduce the available surface area to 80 cm.^{2} and the surface tension was determined.

Table I—Surface Pressure of Pure Organic Liquids Spread on Water

Liquid	Surface Pressure, dynes/cm.
Hexane	0.2
Benzene	0.2
Isopropanol	1.2
Acetone	0.7

RESULTS AND DISCUSSION

The spreading experiments are summarized in Table I. Results are expressed in terms of surface pressure, which is the decrease in surface tension using a clean water surface as reference. All of the liquids tested had positive spreading coefficients (7), and spreading was observed visually in all cases. Volatile, poorly soluble liquids, such as benzene and hexane, would be expected to disappear very rapidly from the surface, producing a negligible surface pressure. However, even liquids as water-insoluble as these may diffuse through the surface layers of water and require some time for back diffusion to the surface and subsequent evaporation (8). For this reason, it is necessary to wait for about 10 min. after spreading before measuring surface pressure. Pure, insoluble liquids will then indeed yield no significant surface pressure (Table I).

Molecules of water-soluble substances, such as isopropanol and acetone, are distributed between the surface and bulk regions, resulting in a low surface pressure (Table I). The surface pressure values obtained for these solvents were independent of surface area and persisted after the surface was swept clean. The observed surface pressures were therefore due to the presence of solvent molecules in the interface and not to impurities. Regardless of the solubility of the solvent, impurities concentrated at the surface cause large changes in surface pressure, which indicate their presence.

The sensitivity of the spreading test was recently demonstrated in this laboratory. A sample of benzene spectrograde, taken from an old bottle which had been opened many times, gave a surface pressure of 11.3 dynes/cm. when spread on water. Near IR spectra of the contaminated benzene and of benzene known to be pure were identical. Gas chromatography at low attenuation of the impure sample yielded a large peak and a much smaller one (whose area was about $^1/_{2000}$ that of the large peak). The pure sample of benzene produced the same two peaks of the same relative size.

The impure sample of benzene was passed through a silica gel-alumina column. The purified material, spread on water, gave a surface pressure of 0.2 dynes, showing that the contaminant had been removed by the column.

Assume, for purposes of calculation, that contamination occurred as a result of careless handling and that the contaminant was a skin lipid. Skin phospholipids spread on water occupy an area of about 80 to 100 Å.^{2} per molecule at moderate surface pressures (9). Under the conditions of the author's spreading test one could therefore be capable of detecting less than 10^{16} molecules, or about 10^{-8} moles of contaminant.

The spreading test may be extended to nonspreading liquids which are volatile or water-soluble. Such liquids may be deposited on the surface by first dissolving them in a solvent (*e.g.*, hexane)

which is capable of spreading. The spreading test may also be applied in certain cases to the detection of solid contaminants, such as detergents which are leached by solvents. The recent report of silicones present in disposable syringes (10) suggests another possible application. Silicones are surface active; if spread on water, as little as 0.01 mg. can easily be determined (11).

Although the spreading method is very sensitive, it is limited to those impurities which are not very volatile and which have some affinity for the surface. Included in this category are surfactants, lipids, most polymers, and some other materials such as steroids and antibiotics. The method is incapable of detecting, for example, the presence of low boiling homologs or isomers in hexane. Therefore it should not be considered a substitute for other methods of checking purity. Nevertheless, if it is necessary to determine trace quantities of surface-active impurities, the spreading method represents a convenient and efficient tool.

REFERENCES

(1) F. W. Meadus, I. E. Puddington, A. F. Sirianni, and B. D. Sparks, *J. Colloid Interface Sci.*, **30,** 46(1969).

(2) L. I. Osipow, "Surface Chemistry," Reinhold, New York, N. Y., 1962, p. 271.

(3) J. J. Kipling, "Adsorption from Solutions of Non-Electrolytes," Academic, New York, N. Y., 1965, p. 17.

(4) J. T. Davies and E. K. Rideal, "Interfacial Phenomena," 2nd ed., Academic, New York, N. Y. 1963, p. 219.

(5) A. Felmeister, M. Amanat, and N. Weiner, *Environ. Sci. Technol.*, **2,** 40(1968).

(6) A. W. Adamson, "Physical Chemistry of Surfaces," Interscience, New York, N. Y., 1960, p. 110.

(7) *Ibid.*, p. 107.

(8) I. R. Miller and L. Nanis, *J. Colloid Sci.*, **17,** 699(1962).

(9) R. A. Demel, *J. Am. Oil Chemists' Soc.*, **45,** 305(1968).

(10) J. R. Miller, J. J. Helprin, and J. S. Finlayson, *J. Pharm. Sci.*, **58,** 455(1969).

(11) H. W. Fox, P. W. Taylor, and W. A. Zisman, *Ind. Eng. Chem.*, **39,** 1401(1947).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 31, 1969 from the *Department of Pharmacy, College of Pharmacy, Rutgers, The State University, Newark, NJ 07104*

Accepted for publication September 17, 1969.

Anticholinesterase Activity of Some Degradation Products of Physostigmine

B. A. HEMSWORTH and G. B. WEST

Abstract ☐ The anticholinesterase activities of four degradation products of physostigmine have been determined *in vitro* using biochemical and biological assay techniques and *in vivo* by means of the rat chromodacryorrhea response. The degradation products are at least 100 times less active than the parent compound as anticholinesterase agents. Eseroline, rubreserine, and eserine blue are effective in high concentrations against the cholinesterase of horse and rabbit plasma but are much less active against the red blood cell cholinesterase; they exert no inhibitory action on the two human cholinesterase enzymes. *In vivo*, the degradation products are at least 1000 times less active than physostigmine. Lack of coloration of solutions of physostigmine does not necessarily indicate full anticholinesterase activity because a colorless product of hydrolysis, eseroline, possesses little or no anticholinesterase activity.

Keyphrases ☐ Physostigmine—degradation products ☐ Anticholinesterase activity—physostigmine degradation products ☐ Biological assay—physostigmine degradation products ☐ *In vitro* assay—physostigmine degradation products activity

When physostigmine in solution undergoes decomposition, a red-colored compound is formed which turns blue on further decomposition (1). Hydrolysis which initially removes the urethane grouping and produces eseroline, a colorless compound (Scheme 1), is followed by oxidation to yield rubreserine, a red material, which is converted into eserine blue or eserine brown. The molecular weight of eserine blue exceeds that of rubreserine and it has been suggested that condensation of rubreserine with other physostigmine degradation products occurs at this stage in the reaction. The end product of the degradation by heat or by exposure to oxygen and alkali is eserine brown.

The anticholinesterase activity of these degradation products has been investigated. Their biological activity has particular relevance as solutions of physostigmine are used in ophthalmology. Furthermore, in the preparation of eye drops sterilization is effected by filtration or by autoclaving the solution at 98 to 100° for 30 min. (BPC, Supplement, 1966) or by steam sterilization at 121°. (USP XVII, 1965). This heating process may result in decomposition of the physostigmine, with possible loss of activity. A preliminary note concerning this work has been published (2).

METHODS

Warburg Manometric Technique—The anticholinesterase activity of the different compounds was compared by this technique using horse serum (0.5 ml.) as the source of cholinesterase and acetylcholine (0.5 ml.) at a final concentration of 0.0138 *M* (3) as the substrate. The pH value of the incubation mixture was controlled by adding 1.5 ml. of 0.04 *M* sodium bicarbonate (adjusted to pH 7.6 by the addition of hydrochloric acid) and each compound under test was added to 0.5 ml. of this bicarbonate solution before addition to the incubation flask. The total volume of fluid in each flask was 3 ml. After gassing with a mixture of 95% nitrogen and 5% carbon dioxide for 10 min., the flasks were incubated at 37° and the manometers read at 10-min. intervals for 30 min. Values in the text are the mean of three experiments. Differences in percentage inhibition greater than 15% are significant ($p = 0.05$).

Biological Assay Technique—Horse serum (0.1 ml.) was used as the source of cholinesterase, with acetylcholine as the substrate (2 ml. of 5 mcg./ml.). Each compound under test was dissolved in 2

which is capable of spreading. The spreading test may also be applied in certain cases to the detection of solid contaminants, such as detergents which are leached by solvents. The recent report of silicones present in disposable syringes (10) suggests another possible application. Silicones are surface active; if spread on water, as little as 0.01 mg. can easily be determined (11).

Although the spreading method is very sensitive, it is limited to those impurities which are not very volatile and which have some affinity for the surface. Included in this category are surfactants, lipids, most polymers, and some other materials such as steroids and antibiotics. The method is incapable of detecting, for example, the presence of low boiling homologs or isomers in hexane. Therefore it should not be considered a substitute for other methods of checking purity. Nevertheless, if it is necessary to determine trace quantities of surface-active impurities, the spreading method represents a convenient and efficient tool.

REFERENCES

(1) F. W. Meadus, I. E. Puddington, A. F. Sirianni, and B. D. Sparks, *J. Colloid Interface Sci.*, **30,** 46(1969).

(2) L. I. Osipow, "Surface Chemistry," Reinhold, New York, N. Y., 1962, p. 271.

(3) J. J. Kipling, "Adsorption from Solutions of Non-Electrolytes," Academic, New York, N. Y., 1965, p. 17.

(4) J. T. Davies and E. K. Rideal, "Interfacial Phenomena," 2nd ed., Academic, New York, N. Y. 1963, p. 219.

(5) A. Felmeister, M. Amanat, and N. Weiner, *Environ. Sci. Technol.*, **2,** 40(1968).

(6) A. W. Adamson, "Physical Chemistry of Surfaces," Interscience, New York, N. Y., 1960, p. 110.

(7) *Ibid.*, p. 107.

(8) I. R. Miller and L. Nanis, *J. Colloid Sci.*, **17,** 699(1962).

(9) R. A. Demel, *J. Am. Oil Chemists' Soc.*, **45,** 305(1968).

(10) J. R. Miller, J. J. Helprin, and J. S. Finlayson, *J. Pharm. Sci.*, **58,** 455(1969).

(11) H. W. Fox, P. W. Taylor, and W. A. Zisman, *Ind. Eng. Chem.*, **39,** 1401(1947).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 31, 1969 from the *Department of Pharmacy, College of Pharmacy, Rutgers, The State University, Newark, NJ 07104*

Accepted for publication September 17, 1969.

Anticholinesterase Activity of Some Degradation Products of Physostigmine

B. A. HEMSWORTH and G. B. WEST

Abstract ☐ The anticholinesterase activities of four degradation products of physostigmine have been determined *in vitro* using biochemical and biological assay techniques and *in vivo* by means of the rat chromodacryorrhea response. The degradation products are at least 100 times less active than the parent compound as anticholinesterase agents. Eseroline, rubreserine, and eserine blue are effective in high concentrations against the cholinesterase of horse and rabbit plasma but are much less active against the red blood cell cholinesterase; they exert no inhibitory action on the two human cholinesterase enzymes. *In vivo*, the degradation products are at least 1000 times less active than physostigmine. Lack of coloration of solutions of physostigmine does not necessarily indicate full anticholinesterase activity because a colorless product of hydrolysis, eseroline, possesses little or no anticholinesterase activity.

Keyphrases ☐ Physostigmine—degradation products ☐ Anticholinesterase activity—physostigmine degradation products ☐ Biological assay—physostigmine degradation products ☐ *In vitro* assay—physostigmine degradation products activity

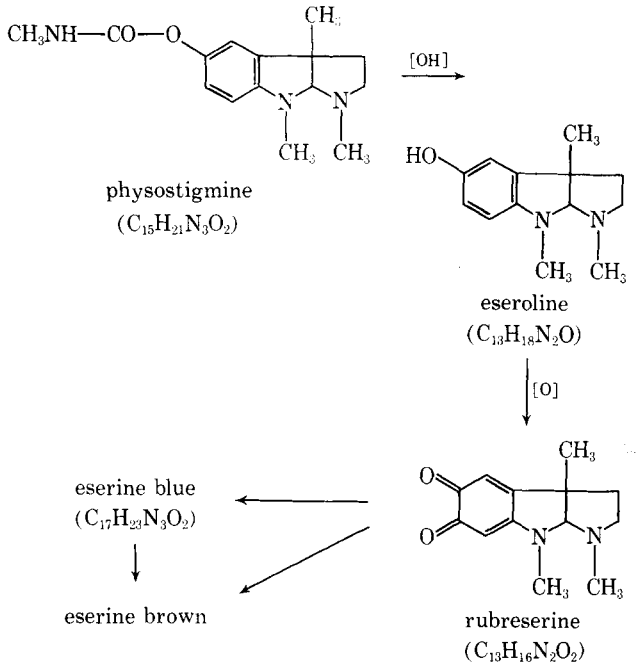
When physostigmine in solution undergoes decomposition, a red-colored compound is formed which turns blue on further decomposition (1). Hydrolysis which initially removes the urethane grouping and produces eseroline, a colorless compound (Scheme 1), is followed by oxidation to yield rubreserine, a red material, which is converted into eserine blue or eserine brown. The molecular weight of eserine blue exceeds that of rubreserine and it has been suggested that condensation of rubreserine with other physostigmine degradation products occurs at this stage in the reaction. The end product of the degradation by heat or by exposure to oxygen and alkali is eserine brown.

The anticholinesterase activity of these degradation products has been investigated. Their biological activity has particular relevance as solutions of physostigmine are used in ophthalmology. Furthermore, in the preparation of eye drops sterilization is effected by filtration or by autoclaving the solution at 98 to 100° for 30 min. (BPC, Supplement, 1966) or by steam sterilization at 121°. (USP XVII, 1965). This heating process may result in decomposition of the physostigmine, with possible loss of activity. A preliminary note concerning this work has been published (2).

METHODS

Warburg Manometric Technique—The anticholinesterase activity of the different compounds was compared by this technique using horse serum (0.5 ml.) as the source of cholinesterase and acetylcholine (0.5 ml.) at a final concentration of 0.0138 *M* (3) as the substrate. The pH value of the incubation mixture was controlled by adding 1.5 ml. of 0.04 *M* sodium bicarbonate (adjusted to pH 7.6 by the addition of hydrochloric acid) and each compound under test was added to 0.5 ml. of this bicarbonate solution before addition to the incubation flask. The total volume of fluid in each flask was 3 ml. After gassing with a mixture of 95% nitrogen and 5% carbon dioxide for 10 min., the flasks were incubated at 37° and the manometers read at 10-min. intervals for 30 min. Values in the text are the mean of three experiments. Differences in percentage inhibition greater than 15% are significant ($p = 0.05$).

Biological Assay Technique—Horse serum (0.1 ml.) was used as the source of cholinesterase, with acetylcholine as the substrate (2 ml. of 5 mcg./ml.). Each compound under test was dissolved in 2



Scheme I—*The relationship between physostigmine and some of its degradation products.*

ml. of 0.04 *M* sodium bicarbonate and added to the incubation mixture. Incubation was carried out at 37° for 30 min., during which time all of the acetylcholine was hydrolyzed when no anticholinesterase was present. In the presence of an inhibitor, the residual acetylcholine was assayed biologically on three different preparations. The isolated colon and uterus of a rat were set up in de Jalon's solution at 32° while the ileum of a guinea pig was suspended in Tyrode's solution also at 32°. Contractions were recorded isotonically on a Grass polygraph. Assays were carried out using the 2 × 2 dose schedule. The amounts of anticholinesterase and horse serum used had no effect on any of these assay preparations. Values in the text are the mean results from the three preparations. Differences in percentage inhibition greater than 15% are significant ($p = 0.05$).

Anticholinesterase Activity Using Plasma and Red Blood Cells—Blood was collected from rabbits, man, and horses into heparinized syringes. After centrifugation to obtain the plasma, the red cells were washed three times with 0.9% (w/v) saline and then haemolyzed in a volume of distilled water corresponding to the initial volume of plasma. The anticholinesterase activities of the compounds under test were then compared using the biological assay techniques, as described previously.

Chromodacryorrhea Test in Rats—Male rats (100–200 g.) were first injected subcutaneously with acetylcholine (200 mcg./100 g. rat) and those producing red tears within 2 min. were used (4). The anticholinesterase activities of the test compounds were then evaluated by injecting them intraperitoneally 30 min. before the subcutaneous injection of a smaller dose of acetylcholine (20 mcg./100 g. rat), and examining for the presence of red tears at 2-min. intervals for 14 min.

Degradation Products of Physostigmine—Samples of eseroline, rubreserine, eserine blue, and eserine brown were supplied by Dr. G. A. Smith, Department of Pharmacy, Heriot-Watt University, Edinburgh, Scotland. They were dissolved in freshly distilled water and the solutions were stored at 4° until required.

RESULTS

Anticholinesterase Activity Determined Manometrically—Concentrations of physostigmine as low as 10^{-8} *M* were effective in inhibiting the cholinesterase of horse serum and higher concentrations had correspondingly greater activities (Fig. 2). All the degradation products tested were less active than the parent compound and activities of these products at concentrations of 5×10^{-5} *M* only

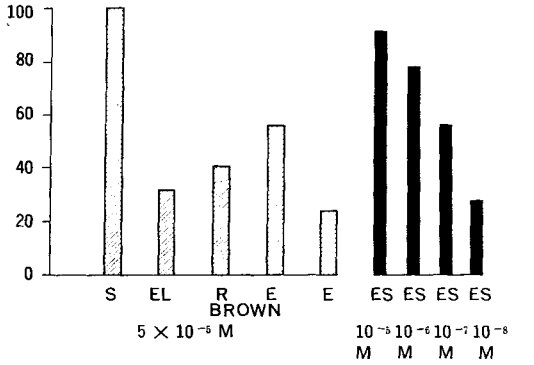


Figure 1—*The anticholinesterase activities of physostigmine (ES), eseroline (EL), rubreserine (R), eserine blue (E Blue), and eserine brown (E Brown), determined manometrically using horse serum as the source of cholinesterase. The ordinate shows percentage inhibition of cholinesterase with reference to 5 × 10⁻⁵ M physostigmine as 100%. The effects of graded concentrations of physostigmine are also shown for comparison.*

are recorded. Eserine blue was the most active of these products and at this concentration produced 52% inhibition of the cholinesterase, comparable with that produced by physostigmine at 10^{-7} *M* (Fig. 1). Thus, eserine blue is at least 500 times less active as an anticholinesterase agent, and eseroline is about 5000 times less active in this test.

Anticholinesterase Activity Determined Biologically—Concentrations of physostigmine as low as 10^{-7} *M* were effective in reducing the cholinesterase activity of horse serum and higher concentrations exerted greater activity (Fig. 2). All the degradation products were less active than the parent compound which reduced cholinesterase activity to 54% of control at 10^{-7} *M*. The most active of these products, eserine blue, is about 100 times less active than physostigmine in this test.

Inhibition of Cholinesterase of Plasma and Red Blood Cells—Concentrations of physostigmine greater than 10^{-5} *M* completely inhibited both plasma and red cell cholinesterase of rabbit, horse, and man. Comparisons of the activities of the degradation products of physostigmine are shown in Fig. 3 where only concentrations of 5×10^{-5} *M* are recorded. Eserine blue, the most active of the degradation products, inhibited the pseudo-cholinesterase of rabbit plasma by about 50%, but it was almost inactive against the true cholinesterase of the red cells; against the cholinesterase of horse plasma, it exerted about 60% inhibition (as it did against horse serum, Figs. 1 and 2) whereas it was only slightly less active against the cholinesterase of horse red cells; however, it was inactive against the cholinesterase enzyme of both human plasma and red cells. This difference

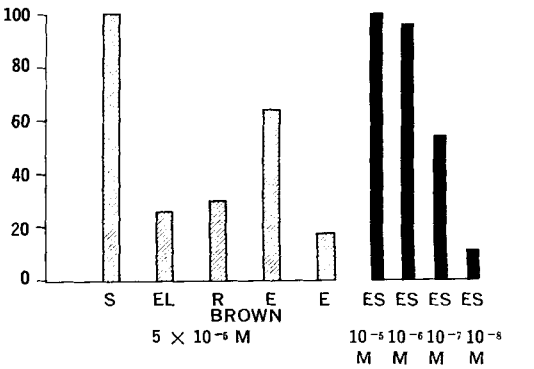


Figure 2—*The anticholinesterase activities of physostigmine (ES), eseroline (EL), rubreserine (R), eserine blue (E Blue), and esrine brown (E Brown), determined biologically using horse serum as the source of cholinesterase. The ordinate shows percentage inhibition of cholinesterase with reference to 5 × 10⁻⁵ M physostigmine as 100%. The effects of graded concentrations of physostigmine are also shown for comparison.*

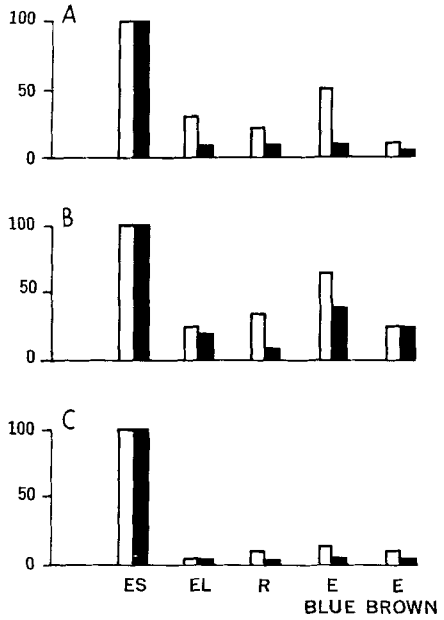


Figure 3—*The anticholinesterase activities of physostigmine (ES), eseroline (EL), rubreserine (R), eserine blue (E Blue), and eserine brown (E Brown) determined biologically using plasma (open columns) and red blood cells (closed columns) as sources of cholinesterase. Activities recorded on the ordinate are percentages of those obtained with 5 × 10⁻⁵ M physostigmine. All compounds were tested at concentrations of 5 × 10⁻⁵ M. A = rabbit, B = horse, C = man.*

in activity of eserine blue against the cholinesterases of all three species contrasts markedly with that of physostigmine, which was equally active against the true and pseudocholinesterases, even at concentrations of 10^{-8} *M*. The other degradation products of physostigmine were less active than eserine blue; with the rabbit and horse enzymes, they had greater effects on the plasma cholinesterase, but with the human enzymes, all were without significant effect at concentrations of 5×10^{-5} *M*.

Rat Chromodacryorrhea Response—Physostigmine was active at doses of 0.1 mg./kg., red tears being produced within 2 min. of the injection of acetylcholine. However, eserine blue, rubreserine, and eseroline were about 1000 times less active than physostigmine in this test. Eserine brown was inactive at all dose levels tested.

DISCUSSION

The results show that the degradation products of physostigmine used in the present work are all much less active as anticholinesterase agents than the parent compound. These products lack the urethane grouping, but it is of interest that when oxidation occurs the anticholinesterase activity first increases and then decreases. Thus, eserine blue is more active than rubreserine or eseroline and under certain circumstances may exert an activity equivalent to about 1% of the parent compound. This result agrees with that of Ellis *et al.* (5) who showed that eserine blue was more active than rubreserine as an anticholinesterase agent. However, these workers were unable to find any anticholinesterase activity in eseroline or eserine brown.

The results using the standard Warburg technique agree well with those involving biological assay of the residual acetylcholine, when a standard enzyme preparation such as horse serum is used. When the two cholinesterases of horse blood are used as the source of the enzyme and comparisons are made of the anticholinesterase activities of the degradation products, rubreserine and eserine blue have a greater inhibiting effect on the plasma cholinesterase. With rabbit blood, almost no activity has been found at the high concentrations tested on the red cell enzyme although results with the plasma enzyme have corresponded well with those obtained using the horse enzyme. Both human plasma enzyme and red cell cholinesterase are not inhibited to any great extent by these degradation products when used at concentrations of 5×10^{-5} *M;* however, physostigmine is equally active against both enzymes in much lower concentrations (6, 7). It is most likely that species differences of plasma cholinesterase (8–10) play a role in the different degrees of inhibition of cholinesterase produced by the degradation compounds.

From the clinical point of view, only physostigmine therefore exerts an anticholinesterase activity, and this is supported by the results of the *in vivo* test where the red tear response involving protection of the injected acetylcholine from both true and pseudocholinesterases shows that all the degradation compounds (including eserine blue) are 1000 times less active than physostigmine. The similar activities of the degradation compounds *in vivo* may be due to the fact that male rats were used in this test and male rat serum has been shown to contain much lower levels of pseudo-cholinesterase than does the serum of female rats (11, 12).

Since all ophthalmic solutions (BPC and USP) are now required to be sterile and physostigmine eye drops may be sterilized by heat, the formation of relatively inactive decomposition products (one of which is colorless) must be taken into account. These intermediate products have no therapeutic value and so the appearance of a pink color is not the only guide to loss of activity of the solution because a colorless solution may also be inactive.

REFERENCES

(1) S. Ellis, *J. Pharmacol. Exptl. Therap.*, **79,** 364(1943).

(2) B. A. Hemsworth and G. B. West, *J. Pharm. Pharmacol.*, **20,** 406(1968).

(3) W. N. Aldridge, *Biochem. J.*, **46,** 451(1950).

(4) A. S. V. Burgen, *Brit. J. Pharmacol.*, **4,** 185(1949).

(5) S. Ellis, O. Krayer, and F. L. Plachte, *J. Pharmacol. Exptl. Therap.*, **79,** 309(1943).

(6) D. Richter and P. G. Croft, *Biochem. J.*, **36,** 746(1942).

(7) K-B. Augustinsson, *Acta Physiol. Scand.*, **15,** *Suppl.* **52,** 1(1948).

(8) K-B. Augustinsson, *Acta Chem. Scand.*, **13,** 571(1959).

(9) *Ibid.*, **13,** 1081(1959).

(10) *Ibid.*, **13,** 1097(1959).

(11) R. W. Brauer and M. A. Root, *J. Pharmacol. Exptl. Therap.*, **88,** 109(1946).

(12) C. H. Sawyer and J. W. Everett, *Am. J. Physiol.*, **148,** 675 (1947).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 10, 1969 from the *Department of Pharmacology and Toxicology, University of Rochester School of Medicine, Rochester, NY 14620 and British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England.*

Accepted for publication August 13, 1969.

Supported in part by grant 1-P11-GM-15190 from the National Institutes of Health, Bethesda, Md.

Sterically Hindered Esters of Vitamin A II: Vitamin A α,α-Dimethylpalmitate

A. J. FORLANO*, C. I. JAROWSKI, H. F. HAMMER, and E. G. MERRITT

Abstract □ A previous study showed that short-chain sterically hindered esters of vitamin A demonstrated better stability in solution than the commercial plamitate. The oxidative stability of the short-chain sterically hindered esters, however, was poor. This work indicated that a long aliphatic chain with steric hindrance in the α-position was necessary for maximal stability. A compound of this type, vitamin A α,α-dimethylpalmitate, was prepared and evaluated.

Keyphrases □ Sterically hindered vitamin A esters □ Vitamin A α,α-dimethylpalmitate—synthesis □ Stability—vitamin A α,α-dimethylpalmitate □ UV spectrophotometry—analysis □ IR spectrophotometry—analysis

The possibility of developing a vitamin A ester of optimum stability was suggested by the observation that steric hindrance in the α-position of the acid portion was required for good solution stability and that a long aliphatic chain was required for maximal oxidative stability (1, 3). Previously, Forlano and Harris (2) prepared a series of vitamin A esters containing an electronegative group such as chlorine in the α-position of the acid portion. These esters demonstrated increased stability in anhydrous ethanolic HCl compared to commercial vitamin A palmitate. Biologically inactive anhydrovitamin A, formed by the elimination of a molecule of water, was the main degradative pathway in acidic solutions. Since it also was observed that the resultant polarization of the ester linkage by the electronegative groups rendered these esters more sensitive to solvent-catalyzed elimination, the increased stability in anhydrous acidic media was somewhat academic.

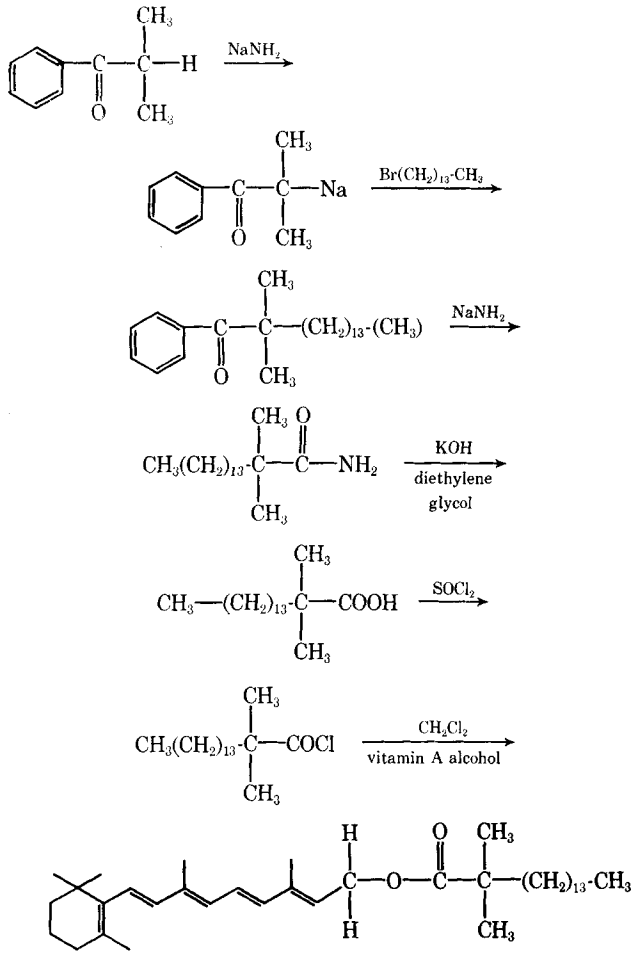
A careful re-examination of the Forlano and Harris data (2) indicated that the replacement of the electronegative groups with electropositive groups should yield esters having greater stability towards solvent- and base-catalyzed attack. A series of compounds incorporating electropositive groups in the α-position of the esters (1, 3) were demonstrated to resist such attack. The excellent stability pattern of these esters in alcoholic acid was quite unexpected, since it was assumed that the introduction of an electropositive group would increase the basicity of the carbonyl group and consequently favor proton-catalyzed attack. It became evident that steric factors as well as electronic effects were important parameters in the stability of vitamin A esters. These data indicated that reactions which involved either hydrolysis of the ester or lysis of the alkyl oxygen bond as the initiating step of the degradation were considerably decreased by the introduction of steric hindrance in the α-position of the acid. The increase in steric hindrance from a methyl to an ethyl group produced a slight but significant increase in solution stability. These short-chain (C_3–C_6) esters, however, lacked good oxidative stability, suggesting the need for a long aliphatic chain.

From previous studies, therefore, it was determined that the features needed for optimum stability in a vitamin A ester are:

Table I—Time Required for Complete Oxidative Degradation of Vitamin A Esters at 64°

	Vitamin A Palmitate Commercial	Vitamin A α,α-Dimethyl-palmitate
Additives	BHA,[a] BHT[b]	BHA, BHT
Degradation time, hr.	64	184
Stability factor (ratio to vitamin A palmitate)	1.0	2.86

[a] Butylated hydroxyanisole. [b] Butylated hydroxytoluene.



Vitamin A α,α-dimethylpalmitate

Scheme I

Preparation of Vitamin A α,α-Dimethylpalmitate

Table II—Stability of Vitamin A Esters at 20° in the Presence of Diacetyl Tartaric Acid Mono- and Diglycerides With and Without Corn Oil

Time, days	DMP[a] units/g.	DMP[a] % Retained	DMP + Corn Oil units/g.	DMP + Corn Oil % Retained	VAP[b] units/g.	VAP[b] % Retained	VAP + Corn Oil units/g.	VAP + Corn Oil % Retained
0 (est.)	324,000	—	324,000	—	324,000	—	324,000	—
11	278,000	86	329,000	100	182,000	56	263,000	81
21	227,000	70	303,000	94	122,000	38	215,000	66
42	208,000	64	292,000	90	No UV[c]	0	182,000	56
90	158,000[d]	49	255,000[d]	79	No UV[c]	0	No UV[c]	0

[a] Vitamin A α,α-dimethylpalmitate. [b] Vitamin A palmitate. [c] No detectable vitamin A by UV method; samples had solidified indicating that considerable oxidation and polymerization had occurred. [d] Samples still fluid at this time.

1. Steric hindrance in the α-position of the acid portion, provided by electropositive alkyl groups, which protect the carbonyl and alkyl oxygen groups from reactive species through physical blockage and electronic effects.

2. A long aliphatic chain to protect the conjugated double bond system from oxygen.

Vitamin A α,α-dimethylpalmitate, Scheme I, which was not prepared previously theoretically appeared to satisfy all these requirements, and this report, describing its preparation and properties, shows that it does. The biological availability of vitamin A from the α,α-dimethylpalmitate and α,α-methylethylcaproate will be discussed in a future report.

EXPERIMENTAL

The α,α-dimethylpalmitic acid was prepared by the method of Bui-Hoi *et al.* (4). The acid chloride, prepared by reacting the acid with thionyl chloride, was subsequently condensed with vitamin A alcohol to form the ester.

Materials—Sodamide 90%,[1] isobutyrophenone,[2] 1-bromotetradecane,[3] and diethylene glycol.[1]

Procedure—Eighty-eight grams sodamide (2.03 moles) was added to 600 ml. boiling dry toluene in a three-neck flask equipped with a reflux condenser, dropping funnel, and thermometer; 300 g. isobutyrophenone (2.03 moles) was added dropwise to the stirring mixture and the system was refluxed for 1 hr. after the addition was completed. The reaction mixture was cooled to room temperature and 400 g. 1-bromotetradecane (1.45 moles) was added and stirring was initiated. After the initial evolution of heat subsided, the mixture was refluxed for 4 hr. with stirring. The cooled mixture was slowly poured into 5 l. 5% acetic acid solution cautiously, since some unreacted sodamide may be present.

The separated organic solution was washed with water until free of base, dried with anhydrous Na_2SO_4, and the toluene was removed by atmospheric distillation. The isobutyrophenone and 1-bromotetradecane were subsequently removed by vacuum distillation at 85–95° $_{15\ mm.}$ and 151–168° $_{8\ mm.}$, respectively. There were 486 g. α,α-dimethylpalmitophenone (1.41 moles) collected at 213–217° $_{6\ mm.}$ (87% yield) and this was added slowly to a suspension of 72 g. sodamide in 600 ml. boiling anhydrous toluene with stirring. The suspension was refluxed for 8 hr. and the cooled mixture was carefully washed with water to destroy any excess sodamide. Emulsification at this point can be reversed by distilling the mixture, since water forms an azeotrope with toluene which distils below the boiling point of both solvents. The residue was vacuum distilled using an air condenser, and 344 g. α,α-dimethylpalmitamide was collected by distilling between 229–235° $_{10\ mm.}$. Then 344 g. (1.21 moles) of the amide was hydrolyzed in 1250 ml. diethylene glycol containing 265 g. KOH by heating the mixture at 210–220° for 16 hr. with constant agitation. The cooled solution was diluted with three times its volume of water and made strongly acidic with concentrated HCl. The free fatty acid, which rose to the top of the solution, was removed with chloroform. The chloroform layer was washed with water until neutral and the solvent was removed. The residue was distilled under vacuum and 200 g. of a material corresponding to α,α-dimethylpalmitic acid was collected at 225–236° $_{20\ mm.}$. The boiling point was the same as that reported by Bui-Hoi *et al.* (4).

The acid was converted to 142 g. of the acid chloride, b.p. 148–150° $_{1\ mm.}$ by refluxing 197 g. of the former compound with 166 g. thionyl chloride in $CHCl_3$. Subsequently, 81 g. of the acid chloride was reacted with 80 g. vitamin A alcohol to yield 53 g. of vitamin A ester, purified by the alumina-column chromatographic procedure of Forlano and Harris (2). This material had an absorption maximum at 327 mμ, which is typical of vitamin A esters, and an a = 84.71 in isopropyl alcohol corresponding to 1.52×10^6 units of vitamin A/g. The IR spectrum was typical of a vitamin A ester showing no alcohol peaks. The heavy metal content was insignificant.

Vitamin A esters are not crystalline and because of their high molecular weight and lack of thermal stability they do not lend themselves to melting point or boiling point determinations. Consequently, the major determination of purity for vitamin A esters is the a determination (a measure of the concentration of vitamin A chromophore) at the vitamin A ester's UV maximum. The absence of shoulders on either side of the maximum indicated the absence of oxidative, isomerization and eliminative degradation products in the sample. The IR spectrum showed an absence of starting materials such as alcohols, carboxylic acids, or acid chlorides. The combination of column chromatography, proper UV maximum, and a value with an absence of shoulders and a good IR spectrum indicated that the product was chemically pure.

Testing Under Use Conditions—The new ester and commercial vitamin A palmitate were studied in a series of practical use tests. The first was a thin-film oxidation test at 64° by the previous method (1). The time required for the complete oxidation of the ester (Table I) indicated that the new ester was more resistant to oxidation than commercial vitamin A palmitate. All vitamin A assays were performed by the USP method (5).

Since vitamin A palmitate is the most stable commercial ester of vitamin A in resisting acid degradation, the relative stability of the α,α-dimethylpalmitate was determined in an acidic medium known to cause rapid degradation of the palmitate. The agent used was an acidic emulsifying agent, diacetyl tartaric acid mono- and diglycerides.[4] The tests were conducted by dissolving enough vitamin A ester in the emulsifying agent to produce an initial concentration of 325,000 units/g. In the second part of this experiment the esters were dissolved in a mixture of equal parts of the emulsifying agent and corn oil (Wesson Oil). The samples were stored at 20° and were periodically assayed by the USP method (5). Table II shows that commercial vitamin A palmitate was completely inactivated in 42 days while the α,α-dimethylpalmitate ester retained half its original potency for twice as long. Corn oil prolonged the stability of the samples possibly due to a reduction in concentration of the acidic emulsifying agent.

The stability of the esters in the emulsifying agent was also determined at 28°. Table III shows that vitamin A palmitate was completely inactivated in half the time at 28° than at 20°. The slower rate of decomposition of the α,α-dimethylpalmitate was approximately the same at both temperatures.

[1] Matheson Coleman & Bell.
[2] Eastman No. 6272.
[3] Eastman No. 3558.

[4] Marketed as TEM-4C by Hachmeister Division, H. J. Heinz Co.

Table III—Stability of Vitamin A Palmitate and Vitamin A α,α-Dimethylpalmitate in Diacetyl Tartaric Acid Mono- and Diglycerides at 28°

Time, days	Vitamin A Palmitate, units/g.	Vitamin A α,α-Dimethylpalmitate, units/g.
1	301,000	283,000
10	202,000	—
15	—	243,000
21	No UV curve[a]	216,000
31	No UV curve[a]	177,000
41	No UV curve[a]	179,000
53	No UV curve[a]	158,000
73	No UV curve[a]	115,000

[a] At the time of assay, there was no UV absorption peak corresponding to vitamin A indicating complete loss of vitamin A potency.

The tests in Tables II and III were quite rigorous because the dispersing agent contained noticeable quantities of free acetic acid. The results clearly indicate the new ester's superior ability to withstand strong acidic conditions and acid-catalyzed degradation.

Further evidence of resistance to acid degradation may be found in the incidence of isomerization occurring in the diacetyl tartaric acid mono- and diglyceride mixtures. Isomerization, no doubt a pathway in acid-catalyzed degradation, is not readily detected because the molecule remains intact although there is a loss of biopotency. The degree of isomerization in terms of maleic values, as determined by reaction with maleic anhydride and colorimetric measurement (6), is given in Table IV. A value of approximately 33% represents the percentage of 13-*cis* isomers present and approaches isomeric equilibrium. The degree of isomerization of the α,α-dimethylpalmitate ester is smaller than that for the palmitate ester in the presence of the acidic emulsifier. Corn oil retarded isomerization and prolonged potency of the vitamin A esters probably by a reduction in concentration of the acidic emulsifying agent.

SUMMARY AND CONCLUSIONS

When the stability of a new ester of vitamin A was compared with commercial vitamin A palmitate, the following observations were made:

1. Vitamin A α,α-dimethylpalmitate was considerably more resistant to auto-oxidation than commercial vitamin A palmitate.

Table IV—Degree of Isomerization of Vitamin A/Esters (in Terms of Maleic Values[a]) in the Presence of the Acidic Emulsifier, Diacetyl Tartaric Acid Mono- and Diglycerides at 25°

Time, days	DMP[b]	DMP + Corn Oil	VAP[c]	VAP + Corn Oil
0	<5	<5	<5	<5
11	8.2	8.6	9.3	6.3
21	10.8	9.6	19.0	9.7
42	12.6	9.7	22.4	11.8
90	16.4	12.7	36.3	21.6

[a] Expressed as % of 13-*cis* isomers present; value of 33% indicates approximate isomeric equilibrium. [b] Vitamin A α,α-dimethylpalmitate. [c] Commercial vitamin A palmitate.

2. The new ester was more stable than vitamin A palmitate in the presence of the acidic emulsifier, diacetyl tartaric acid mono- and diglycerides.

3. Corn oil appears to retard the loss in vitamin A ester potency due to both acid-catalyzed degradation and isomerization probably by a reduction of the concentration of the acidic emulsifying agent.

4. Isomerization of the new ester is somewhat slower in the presence of the acidic emulsifier than the commercial palmitate.

REFERENCES

(1) A. J. Forlano, C. I. Jarowski, and H. F. Hammer, *J. Pharm. Sci.*, **57**, 1184(1968).

(2) A. J. Forlano and L. E. Harris, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 458(1960).

(3) A. J. Forlano, U. S. pat. 3,092,657, June 4, 1963.

(4) P. Bui-Hoi and P. Cagniant, *Z. Physiol. Chem.*, **279**, 76 (1943).

(5) "The United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa. (1960).

(6) C. D. Robeson and J G. Baxter, *J. Am. Chem. Soc.*, **69**, 136 (1947).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Chas. Pfizer & Co., Inc., Brooklyn, NY 11206*

Accepted for publication September 10, 1969.

*Present address: School of Pharmacy, Ferris State College, Big Rapids, MI 49307

Thin-Layer Chromatographic Method for the Determination of Flurandrenolone Acetonide and Some Closely Related Foreign Steroids

RAFIK H. BISHARA* and IVAN M. JAKOVLJEVIC

Abstract □ A method is presented for the separation and determination of flurandrenolone acetonide (6α-fluoro-11β,16α,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione-16,17-acetonide) and some closely related steroids. This is achieved by TLC on silica gel using a one-dimensional multiple development technique. The related foreign steroids are determined semiquantitatively while flurandrenolone acetonide is determined quantitatively by extracting its spot from the plate, and comparing it to a standard treated similarly.

Keyphrases □ Flurandrenolone acetonide, related steroids—separation, determination □ TLC—separation, identification □ Colorimetric analysis—spectrophotometer

Interest in this laboratory for the determination of steroid purity using TLC has made necessary the development of TLC solvent systems for the separation and quantitation of closely related steroids. A system for the separation of some estrogens (1) and another for the separation of a mixture of closely related 6-fluoro-16α-hydroxycorticosteroids (2) have been reported recently. These solvent systems proved to be very useful for the separation of related foreign steroids, intermediates, or decomposition products from the therapeutically active steroid.

This paper describes a TLC method for the semiquantitation of the related foreign steroids that may possibly exist, or the steroids which may be formed under artificial decomposition of flurandrenolone acetonide. These related foreign steroids were arbitrarily chosen after examining the different methods of flurandenolone acetonide syntheses (3, 4) and it was concluded that their presence was a possibility. Also included are some of the products resulting from severe hydrolytic deacetonation (5) and chromic acid oxidation (6) of flurandrenolone acetonide. The closely related foreign steroids were determined semiquantitatively by visualizing the developed plate under short wavelength UV light and comparing their intensities to known amounts, while flurandrenolone acetonide was determined quantitatively by extracting the silica gel, color development with tetrazolium blue reagent, and measuring the absorbance at 520 mμ.

EXPERIMENTAL

Reagents—All chemicals, if not otherwise mentioned, were of the highest grade commercially available.

Solvent System—Benzene–ethylacetate (1:1) (2).

Flurandrenolone Acetonide Reference Standard Solution—A solution containing exactly 10 mg. of flurandrenolone acetonide per milliliter of chloroform–methanol (1:1).

Related Foreign Steroids Solution—A mixture containing 1 mg. of each of the following steroids[1]/5 ml. of chloroform–methanol (1:1): (*a*) 6α-fluoro-16α,17α,21-trihydroxy-pregn-4-ene,3,11,20-trione-16,17-acetonide; (*b*) 6α-fluoro-11β,16α,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione-16,17-acetonide-21-acetate; (*c*) 6α-fluoro-11β,16α,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione-16,17-acetonide-11-acetate; (*d*) 6α-fluoro-11β,16α,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione (flurandrenolone).

Specially treated ethanol, tetrazolium blue reagent, and tetramethylammonium hydroxide solution were prepared according to the method of Jakovljevic (7).

METHOD

The TLC plate[2] (20 × 20 cm.) was activated before use by heating at 110° for 10–15 min. The developing chamber was lined with blotting paper and allowed to stand with the solvent for 30 min. before use.

Ten milligrams of the sample was weighed accurately and dissolved in 1 ml. of chloroform–methanol (1:1). The plate was divided into seven equal sections and the following amounts were applied 2.5 cm. from the bottom edge of the plate using micropipets.[3]

Section 1, 5 μl. of the related foreign steroids solution; Section 2, 10 μl. of the ample solution; Section 3, 10 μl. of flurandrenolone acetonide standard solution; Section 4, was used as the plate blank; Section 5, 15 μl. of the related foreign steroids solution; Section 6, 10 μl. of the sample solution; Section 7, 10 μl. of flurandrenolone acetonide standard solution.

The plate was developed in the chamber allowing the solvent front to travel 15 cm. after passing through the point of application. It was then removed from the chamber and the solvent allowed to evaporate for about 3 min. Developing and evaporation of the solvent were done at room temperature (25°). The plate was redeveloped twice more in the same direction as the first time. After the third removal of the solvent the spots were detected using a short wavelength UV light,[4] and marked.

Evaluation of the Plate—In order to determine the percentage of related foreign steroids in flurandrenolone acetonide, each extra spot, other than the main compound, appearing in the sample sections was compared with the spot having the same mobility (R_f value) in the sections containing the related foreign steroids. Sections 1 and 5, equivalent to 1 and 3% of related foreign steroids, respectively, were used for the semiquantitation of possible impurities.

For the quantitative determination of flurandrenolone acetonide, the marked areas of the main spot in Sections 2 and 6 representing the sample, and Sections 3 and 7 representing the standard, as well as an equivalent area from the blank, Section 4, were removed and quantitatively transferred to separate glass-stoppered centrifuge tubes. Ten milliliters of absolute ethanol were added to each tube followed by vigorous shaking for 3 min. using a Vortex mixer.[5] The tubes were centrifuged for about 10 min. at 2000 r.pm. until the supernatant was clear. A 4-ml. aliquot from each tube (equivalent to 40 mcg. of flurandrenolone acetonide) was pipeted into separate 10-ml. volumetric flasks. The contents of all flasks were evaporated to dryness using mild heat and an air stream, then the color was developed with the tetrazolium blue reagent (7). The plate blank absorbance was subtracted from both the sample and the standard absorbances, and the corrected values were used for the calculation.

[1] The steroids used were obtained from Syntex Corp., Palo Alto, Calif.

[2] Precoated 250-μ thin-layer plates (Silica Gel F_{254}) supplied by Brinkmann Instruments, Inc., Westbury, N. Y.

[3] Micropipets, Microcaps, Drummond Scientific Co., Broomall, Pa.

[4] Chromato-Vue equipped with a short wavelength lamp (about 254 mμ), Ultraviolet Products, Inc., Calif.

[5] Vortex Jr. Mixer, Scientific Industries, Inc., Queens Village, N. Y.

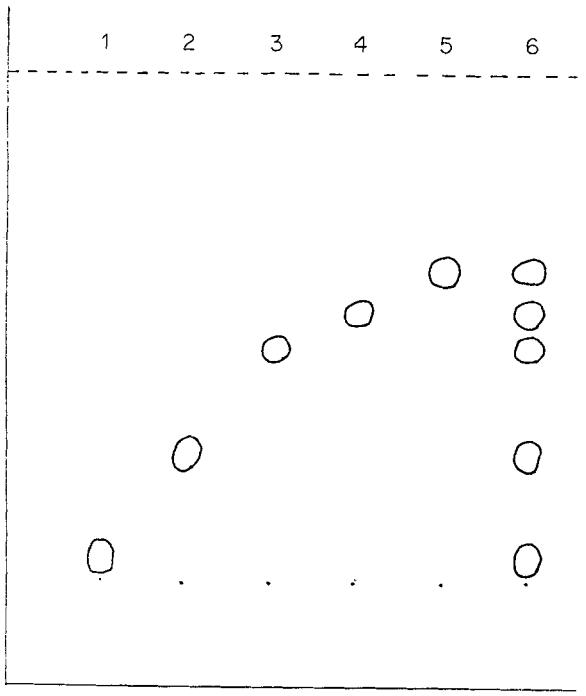


Figure 1—*1. Flurandrenolone; 2. flurandrenolone acetonide; 3. 6α - fluoro - 11β,16α,17α,21 - tetrahydroxy-pregn - 4 - ene - 3,20-dione - 16,17 - acetonide - 11 - acetate; 4. 6α - fluoro - 16α,17α,21-trihydroxy - pregn - 4 - ene - 3,11,20 - trione - 16,17 - acetonide; 5. 6α - fluoro - 11β,16α,17α,21 - tetrahydroxy - pregn - 4 - ene - 3,20-dione - 16,17 - acetonide - 21 - acetate; 6. mixture. Each spot represents 2 mcg.*

DISCUSSION

In recent years, TLC has become recognized as a valuable method for the separation and identification of closely related compounds. Many solvent systems for the TLC of steroids (1, 2, 8, 9) have been reported for the separation of closely related steroids and the resolution (2, 10) of α- and β- steroid pairs.

The related foreign steroids test (11–13) is now becoming one of the most important tests of steroidal drugs. This test uses TLC as a tool to detect any structurally related compounds that may exist in the therapeutically active steroid.

The method described here has been used satisfactorily in this laboratory for the semiquantitative determination of the related foreign steroids that may be present in flurandrenolone acetonide. It was also useful in stability studies where flurandrenolone was detected after severe hydrolytic deacetonation (5) of flurandrenolone acetonide. This reaction was found (7, 14) to occur in some formulations containing flurandrenolone acetonide that were aged by accelerated conditions.

The oxdiation product formed upon exposing flurandrenolone acetonide to chromic acid (6) was isolated and identified by IR, TLC, and melting point, and found to be 6α-fluoro- 6α,17α,21-trihydroxy-pregn-4-ene-3,11,20-trione-16,17-acetonide. This compound separated by the TLC solvent system used in this method, was incorporated in the related foreign steroids mixture.

Although several solvent systems have been reported for the TLC of corticosteroids, it was found that none of them could resolve the mixture used in this work. The development of a new TLC system (2) in this laboratory made it possible to have complete resolution of this particular mixture. This was achieved after developing the plate three times in the same direction. Figure 1 shows the thin-layer chromatogram of the separated steroids individually and as a mixture. Their R_f values were reported previously (2). To overcome any unevenly distributed plate layers, the sample and the standard were applied in duplicates. It was found that the results were reproducible.

Table I—Variability Data[a]

Average Absorbance of the Duplicate Samples	Plate Blank Absorbance	Corrected Absorbance
0.290	0.010	0.280
0.299	0.005	0.294
0.290	0.010	0.280
0.315	0.010	0.305
0.310	0.008	0.302
0.307	0.012	0.295
0.294	0.014	0.280
0.320	0.012	0.308

[a] Numbers represent spectrophotometric readings at 520 mμ. Average $\bar{x}$ = 0.293; range R = 0.028; variance S^2 = 0.00013742; SD = 0.01172262; RSD = ±4.000894.

The order of the plate sections makes it possible to have the sample close to two levels of the related foreign steroids; namely 1 and 3%, thus facilitating the visual semiquantitation under short wavelength UV light.

For the quantitative elution of flurandrenolone acetonide from the scraped silica gel, absolute ethanol was used instead of 95% ethanol since a clearer separation was obtained.

In order to evaluate the precision of this method, 100 mcg. of a flurandrenolone acetonide standard material was spotted in duplicate on eight TLC plates, which were then developed, eluted, and assayed as mentioned. It was found that the plate blank absorbances vary from 1.67–4.76% of the sample absorbances. The quantitative determination of flurandrenolone acetonide by this method showed (Table I) a relative SD of ±4.0% for eight independent assays.

REFERENCES

(1) R. H. Bishara and I. M. Jakovljevic, *J. Chromatog.*, **41,** 136(1969).

(2) R. H. Bishara, *ibid.*, in press.

(3) J. S. Mills, A. Bowers, C. Casas Campillo, C. Djerassi, and H. J. Ringold, *J. Am. Chem. Soc.*, **81,** 1264(1959).

(4) J. S. Mills, A. Bowers, C. Djerassi and H. J. Ringold, *ibid.*, **82,** 3390(1960).

(5) G. R. Allen, Jr. and M. J. Weiss, *ibid.*, **82,** 2840(1960).

(6) G. I. Poos, G. E. Arth, R. E. Beyler, and L. H. Sarett, *ibid.*, **75,** 422(1953).

(7) I. M. Jakovljevic, P. E. Hartsaw, and G. E. Drummond, *J. Pharm. Sci.*, **54,** 1771(1965).

(8) R. D. Bennett and E. Heftmann, *J. Chromatog.*, **21,** 488 (1966).

(9) B. L. Hamman and M. M. Martin, *Anal. Biochem.*, **20,** 423(1967).

(10) R. S. Wright, *J. Chromatog.*, **37,** 363(1968).

(11) "The United States Pharmacopeia," 17th. rev., Mack Publishing Co., Easton, Pa., 1965, p. 874.

(12) "British Pharmacopoeia," The Pharmaceutical Press, London, England, 1968, p. 1287.

(13) "The National Formulary," 12th. ed., Mack Publishing Co., Easton, Pa., 1965, p. 453.

(14) J. P. Comer and P. E. Hartsaw, *J. Pharm. Sci.*, **54,** 524 (1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 7, 1969 from the *Analytical Development Laboratory, Eli Lilly and Company, Indianapolis, IN 46206*

Accepted for publication September 3, 1969.

The authors acknowledge the stimulating discussions and helpful criticism of Dr. R. T. Rapala while preparing this paper.

* Present address: Bionucleonics Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907

Effect of Acute and Chronic Stress on Amobarbital Metabolism in the Rat

JAMES EDWARD HUFF,* STANLEY M. SHAW, and JOHN E. CHRISTIAN

Abstract □ Labeled amobarbital was employed in an investigation of the utilization of urinary excretion of a labeled compound as an index for indicating altered amobarbital metabolism due to a stress. A study of the effect of an external acute unilateral hindleg ligation stress showed significant differences in amobarbital and/or metabolite urinary excretion patterns between stress and control groups. Adrenal ascorbic acid as an index of acute stress showed a significant difference between groups. Repeated injection of amobarbital as an internal chronic stress resulted in a significant difference between stress and control rat urinary excretion of amobarbital and/or metabolites.

Keyphrases □ Amobarbital-^{14}C metabolism—acute, chronic stress effect □ Adrenal ascorbic acid—acute stress index □ Stress, hindleg ligation—amobarbital-^{14}C metabolism □ Excretion, fecal, urinary—amobarbital-^{14}C □ UV spectrophotometry—analysis □ Scintillometry—analysis

The effect of various stress conditions or stressors on living organisms has been an area of increasing concern and activity in the scientific community. A recent review (1) listing 326 references defined stress "as the response of an organism to a variety of challenging and threatening events which inevitably occur and recur throughout life." These conditions of stress may be initiated by a variety of physical and/or mental stimuli which may include infections, wounds, burns, accidental injuries, exposure to toxic chemicals, noise, vibrations, extremes of temperature, prolonged or extreme physical activity, ionizing radiations, sleeplessness, pain, fear, anxiety, and other emotional strains (2). It is generally accepted that most stressors, if sufficiently intense and prolonged, initiate the general adaptation syndrome (3), with the successive stages of alarm reaction, adaptation, and exhaustion.

Information relating to the interaction between stress and drug effect is only in the embryonic stage. There exists a void in scientific knowledge, specifically pertaining to "what does happen" when a stressed individual receives a drug, the dose of which was titrated on normal subjects at the time of the drug's inception. Barry and Buckley (1) stated that the effect of stress may potentiate, counteract, or have no effect on the action of a drug, but that the variation in drug action, if any, should be known. Levy (4) recently stated that most pharmacokinetic studies are conducted on healthy ambulatory subjects. Levy suggested that many of these investigations should be carried out also on debilitated patients in order to assess the effect of various pathological conditions on the kinetics of drug absorption, distribution, and elimination.

Since barbiturates are centrally acting nervous system depressants used in conditions of mental and physical stress, an investigation of the effects of experimental stress on the pharmacology of barbiturates would seem to be of paramount concern. Therefore, the overall objective of this investigation was to follow the urinary and fecal excretion of amobarbital and metabolites as observed in stressed and nonstressed rats, using tracer techniques, in order to determine if an alteration in the process of drug metabolism occurs from stressor effects; and to observe if the alteration can be detected using urinary excretion as a quantitative index of stress. The selected methods of stress were short term unilateral hindleg ligation, designated as an external acute stress, and repeated intraperitoneal administration of the barbiturate, designated as an internal chronic stress.

EXPERIMENTAL

Animals—Male rats of the Sprague-Dawley strain[1] were used throughout this investigation. They were maintained on commercial laboratory chow[2] and water *ad libitum*. The animal quarters were air conditioned and kept at 21.1–22.1° (70–72°F.) at all times. As closely as possible, lighting was regulated to allow 12 hr. of uniform illumination and 12 hr. of darkness each day.

The animals were initially placed in large community cages in groups of five for a period of 7–14 days. Gentling and petting were performed twice daily to individual rats. After this adjustment period the animals were randomly transferred to individual metabolism cages. For 7–10 days, the animals were individually weighed and handled each morning. Moreover, a conditioning regimen (5) was followed for the rats used in the acute stress studies. In the conditioning program the animals received 0.5 ml. of physiologic saline intraperitoneally for 5 days prior to measurement of experimental parameters.

Liquid Scintillation Counting—A liquid scintillation spectrometer[3] was used for radioactivity determinations. An XDC scintillator (6) was used for counting urine samples since the scintillator solution can contain almost 30% water and still allow good counting efficiency for ^{14}C. The TC scintillator counting solution of Mahin and Lofberg (7) was modified[4] and used for ^{14}C fecal counting. Absolute disintegration rates were determined by internal standardization (8).

Amobarbital-^{14}C Assay—The radiochemical purity of amobarbital-2-^{14}C[5] was determined by TLC and autoradiography. A series of spots ranging from 1–100 mcg. of labeled amobarbital were spotted on commercial adsorbent (Adsorbosil-P-1) plates prepared in the usual manner and each developed in one of four solvent systems.[6] Autoradiograms of the developed plates were obtained with Kodak No-Screen X-ray film. In all cases only one spot was visible and R_f values for the series of spots from each of four solvent systems were identical. No radiochemical impurities were present. No differences in R_f value of radiochemical purity were observed between the acid form and a sodium salt of the amobarbital-2-^{14}C when spotted using alcohol, water, or urine as the dissolution vehicle.

[1] Sprague-Dawley, Inc., Madison, Wis.
[2] Wayne Lab-Blox, Allied Mills, Inc., Chicago, Illinois.
[3] Packard Model 3003 Tri Carb with Packard Model 574 Automatic Control, Packard Instrument Co., Inc., Downers Grove, Illinois.
[4] The TC scintillator consisted of 5 parts toluene, 4 parts 2-ethoxyethanol, and 0.03% 2,5-diphenyloxazole (6 g./l. toluene).
[5] Supplied by Smith Kline & French Laboratories, Philadelphia, Pa., as 5-ethyl-5-isopentylbarbituric acid-2-^{14}C.
[6] Chloroform–acetone (9:1), benzene–acetic acid (9:1), dioxane–benzene–aqueous ammonia (20:75:5), acetone–*n*-butyl alcohol–ammonium hydroxide (9:9:2).

Chemical purity of the labeled compound was determined spectrophotometrically.[7] A series of five scan curves were attained from 350 to 190 mμ using standard and labeled amobarbital in concentrations of 25 to 1.6 mcg./ml. in 0.5 *N* sodium hydroxide solution. The Lambert-Beer law was obeyed and the resulting straight lines were similar. The scan curves were in agreement and exhibited identical maximum and minimum inflections at 253 and 232 mμ, respectively. Melting point values were attained using a Buchi melting point apparatus for standard amobarbital (157–159°) and amobarbital-2-^{14}C (158–159°). The amobarbital melting points were in good agreement with the official compendia (9).

Statistics—The Student's *t* test of significance between two sample means was used throughout the investigation. An analysis of variance using a two-factor experimental design with repeated measures on one factor was used with an *F* test to determine overall differences in each study. The level of significance used for all determinations between stress groups and control groups was $p < 0.05$.

Determination of Ascorbic Acid—Eight animals were subjected to unilateral hindleg ligation for 1.5 hr. while eight animals served as controls. Animals were sacrificed by cervical dislocation. The adrenals were rapidly removed, trimmed of fat, and frozen until assay. Less than 5 min. elapsed between removal of the rat from the metabolism cage until the adrenals were frozen. The method of Maickel (10) was employed for the determination of adrenal ascorbic acid. The study was conducted during the winter season.

External Acute Stress Program—An excretion study was undertaken during the winter season using unilateral hindleg ligation as the stress. Rupe (11, 12) showed that rats subjected to such stress exhibited a shortened duration of pharmacologic response to various drugs. Driever (13, 14) further demonstrated that stressed rats clear various drugs from the blood at an increased rate compared to controls. The stress was accomplished by wrapping a small unbroken rubber band (2 cm. in diameter) three times around the upper portion of the hindleg of the rat. A rapid swelling and purple discoloration of the leg took place. Hindleg ligation was applied to the stress group of 5 rats 1.5 hr. prior to intraperitoneal injection of amobarbital-2-^{14}C and removed upon injection. A group of five animals served as controls. A dose of 10.18 μc. (17.0 to 17.9 mg./kg.) of amobarbital-2-^{14}C was administered to each 380–400-g. stress and control rat in the form of a freshly prepared sodium salt. Urine was collected at numerous intervals for a period of 144 hr. Fecal samples were taken at intervals of 0–24, 24–48, 48–72, and 72–96 hr.

Internal Chronic Stress Program—During the winter season, 30 rats weighing between 205–234 g. were randomly divided into two groups; the stress group received an anesthetic dose of 35 to 40 mg./kg. of unlabeled sodium amobarbital intraperitoneally twice daily while the control group received normal saline twice a day. Following the 14 days of pretreatment with unlabeled amobarbital or normal saline, all stressed and control rats received 4.98 μc. (11.3 to 13.6 mg./kg.) of amobarbital-2-^{14}C. The intervals of urine collection were 0–2, 2–4, 4–6, 6–8, 8–10, and 10–12 hr. while feces were collected only for the 0–24 hr. interval. The rats were sacrificed 24 hr. after the injection of labeled drug by cervical dislocation. The entire liver from each rat was rapidly removed, rinsed in normal saline, blotted on a paper towel, and accurately weighed.

RESULTS AND DISCUSSION

Adrenal Ascorbic Acid Depletion—Mean values of 445 ± 46 and 605 ± 22 mg.[8] adrenal ascorbic acid per 100 g. (mg. %) of adrenal tissue weight for the stress and control groups, respectively, were obtained from this phase of the investigation. A greater depletion of adrenal ascorbic acid was observed in the ligated group than in the control group ($p < 0.01$), which indicated that a stress condition existed. The adrenal tissue weight of the stress and control groups was 35.38 ± 3.56 and 38.04 ± 3.54 mg., respectively. Although not statistically significant the adrenal tissue weight of the stress group was consistently lower than the control group.

External Acute Stress Urinary Excretion—Comparison between stress and control groups of the level of amobarbital and/or metabolites excreted at each urine collection interval showed that no statistical difference could be assigned to any specific time period. The cumulative mean percent of the administered dose of labeled amobarbital recovered from the urine as amobarbital and/or metabolites during the 0–144 hr. collection interval was 82.7% for the control group and 90.2% for the stressed group. The urinary excretion rate reached a plateau at the 0–8 hr. interval (Fig. 1) with 74.8% excreted for the nonstressed group and 81.8% for the stressed group. Since the two curves were relatively straight lines after the 10–12 hr. collection period, only the 0–12 hr. cumulative interval is shown in Fig. 1. The control and stress urinary excretion data for the 0–12 hr. interval were 78.8 and 86.5%, respectively. Statistically significant differences ($p < 0.05$) were observed between the two curves for the 0–12 hr. cumulative excretion period. During the 0–12 hr. collection interval the urine volume of the stress animals (7.9 ml./animal) did not differ significantly from the controls (7.2 ml./animal).

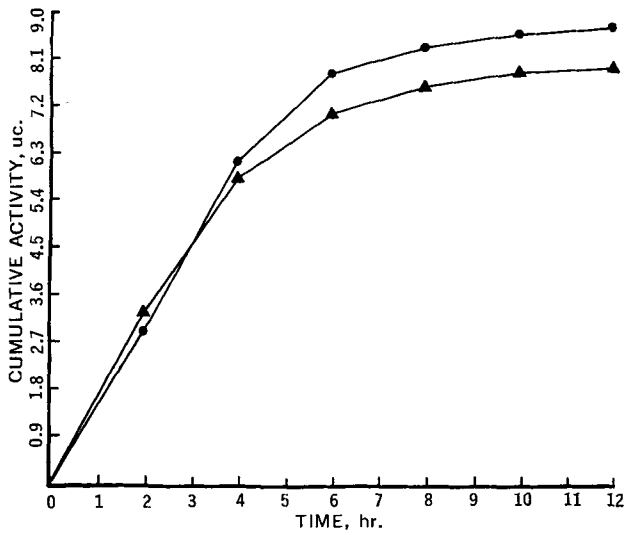


Figure 1—*The effect of an external acute stress on the cumulative urinary excretion of amobarbital and/or metabolites. Key: ●, stress group; ▲, control group.*

Internal Chronic Stress Excretion—Since most drugs are prescribed in multiple doses at regular intervals, an internal chronic dosage program was designed to test the more subtle type stress effect of repeated drug administration. Repeated administration of amobarbital was selected as the chronic stress even though the use of repeated amobarbital injections may confound the development of enzyme induction and tolerance to the particular drug in measuring effects on metabolism of the same drug.

The mean urine, fecal, and total excretion of amobarbital and/or metabolites for the stress group were 88.3, 10.2, and 98.5% of the

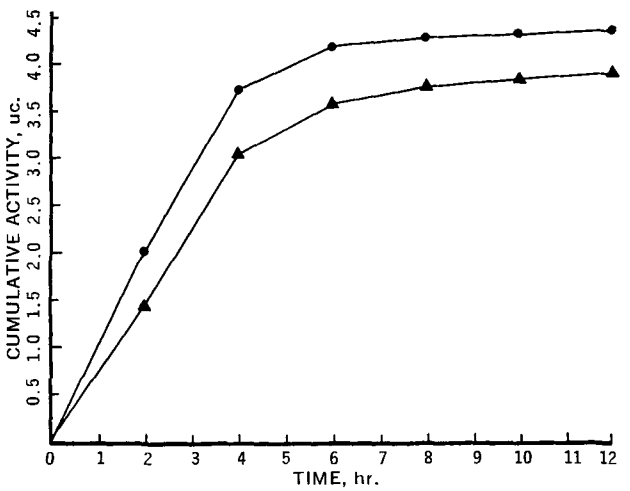


Figure 2—*The effect of an internal chronic stress on the cumulative urinary excretion of amobarbital and/or metabolities. Key: ● stress group; ▲, control group.*

[7] Beckman DK-2A Ratio Recording Spectrophotometer, Beckman Instruments, Inc., Fullerton, California.

[8] Results expressed as the mean ± standard error.

administered dose, while the values for the control group were 79.1, 11.1, and 90.2%. The mean cumulative urinary excretion plots for the stress and control groups (Fig. 2) indicate a higher cumulative urinary excretion level of amobarbital and/or metabolites for the stress group. An analysis of the stress and control data show a definite statistical difference ($p < 0.01$) between groups for the cumulative urinary excretion of amobarbital and/or metabolites. A statistical comparison of the individual time period urinary excretion data showed a significant difference ($p < 0.05$) between groups for the 6–8, 8–10, and 10–12 hr. collection intervals. During the 0–12 hr. collection interval the urine volume of the stress animals (7.0 ml./animal) did not differ significantly from the controls (6.5 ml./animal).

During the 2-week pretreatment period it was noticed that the stress rats exhibited a shorter and shorter sleep time response to the same dosage of amobarbital sodium, while the control rats were not noticeably affected by the saline injections. The sleep time observations were recorded only visually. Therefore, the following observations were only qualitative; but the trend of decreased sleep time was definitely noticeable. The injections of amobarbital caused immediate induction of sleep ($\leq$ 5 min.) during the first three days of treatment. Sleep induction time was initially (3 to 6 days) decreased, also noted by Aston and Hibbeln (15), in that the rats exhibited narcosis almost as soon as the needle was withdrawn. Subsequently, however, the induction time increased until the 9–11 day period in which more than one-half the rats only exhibited ataxia and not anesthesia. The final injection of the central nervous system depressant resulted in the majority of the rats remaining awake, while the ones which lost the righting reflex did so for only a brief period of time ($\leq$ 10 min.). Decreased sleeping time has been demonstrated by others (12, 16) after barbiturate administration to rats subjected to unilateral hindleg ligation. It is interesting to note that mean liver weights were recorded as 4.571 $\pm$ 0.087 and 4.478 $\pm$ 0.085 g./100 g. body weight for the internal chronic stress and control rats, respectively. The results indicated no differences between groups.

SUMMARY AND CONCLUSIONS

Tracer techniques were utilized in an investigation of the urinary excretion of amobarbital in stressed and nonstressed rats. Adrenal ascorbic acid depletion was used as an index of external acute stress induction. The adrenal ascorbic acid level of the hindleg ligation stress group was 445 $\pm$ 46 mg.% while that of the control group was 605 $\pm$ 22 mg.%. The significant difference of adrenal ascorbic acid levels between groups showed that a stress condition existed and that hindleg ligation as a stress was therefore applicable to the study of the utilization of urinary excretion as an index for indicating a stress condition. A study of the effect of an external acute unilateral hindleg ligation stress on the urinary excretion of amobarbital and/or metabolites showed significant differences in urinary excretion patterns between stress and control groups. An internal chronic stress study consisting of twice daily repeated injections of amobarbital for 14 days showed a significant difference in urinary excretion of amobarbital and/or metabolites between treated and nontreated rats. Changes in urinary excretion phenomena as a quantitative index of a stress-altered metabolic rate response in rats were shown to be applicable for the detection of the effects of external acute ligation stress and internal chronic repeated injection stress.

REFERENCES

(1) H. Barry, III and J. P. Buckley, *J. Pharm. Sci.*, **55**, 1159 (1966).

(2) W. H. Griffith, *Federation Am. Soc. Exptl. Biol. Conf.*, 1967, p. 3.

(3) H. Selye, *J. Clin. Endocrinol.*, **6**, 117(1946).

(4) G. Levy, *J. Pharm. Sci.*, **56**, 928(1967).

(5) C. Sanchez, T. S. Miya, and W. F. Bousquet, *Proc. Soc. Exptl. Biol. Med.*, **123**, 615(1966).

(6) G. A. Bruno and J. E. Christian, *Anal. Chem.*, **33**, 1216 (1961).

(7) D. T. Mahin and R. T. Lofberg, *Anal. Biochem.*, **16**, 500 (1966).

(8) C. H. Wang and D. L. Willis, "Radiotracer Methodology in Biological Sciences," Prentice-Hall, Englewood Cliffs, N. J., 1965, p. 131.

(9) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 40.

(10) R. P. Maickel, *Anal. Biochem.*, **1**, 498(1960).

(11) B. D. Rupe, Ph.D. thesis, Purdue University, 1963.

(12) B. D. Rupe, W. F. Bousquet, and T. S. Miya, *Science*, **141**, 1186(1963).

(13) C. W. Driever, Ph.D. thesis, Purdue University, 1965.

(14) C. W. Driever, W. F. Bousquet, and T. S. Miya, *Intern. J. Neuropharmacol.*, **5**, 199(1966).

(15) R. Aston and P. Hibbeln, *Science*, **157**, 1463(1967).

(16) W. F. Bousquet, B. D. Rupe, and T. S. Miya, *J. Pharmacol. Exptl. Therap.*, **147**, 376(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 12, 1969 from the *Department of Bionucleonics, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907*

Accepted for publication September 29, 1969.

This investigation was supported in part by the National Center for Radiological Health, U. S. Public Health Service and conducted under the auspices of the Institute for Environmental Health, Purdue University.

* Present address: The University of Rochester, School of Medicine and Dentistry, Rochester, NY 14620

Studies of Enzyme Active Sites: Synthesis and Catalytic Properties of L-Histidyl-glycyl-L-aspartyl-L-seryl-L-phenylalanine

Keyphrases ☐ Enzyme active sites—catalytic peptide ☐ L-Histidyl-glycyl-L-aspartyl-L-seryl-L-phenylalanine—synthesis, catalytic properties ☐ Paper chromatography, electrophoresis—identity

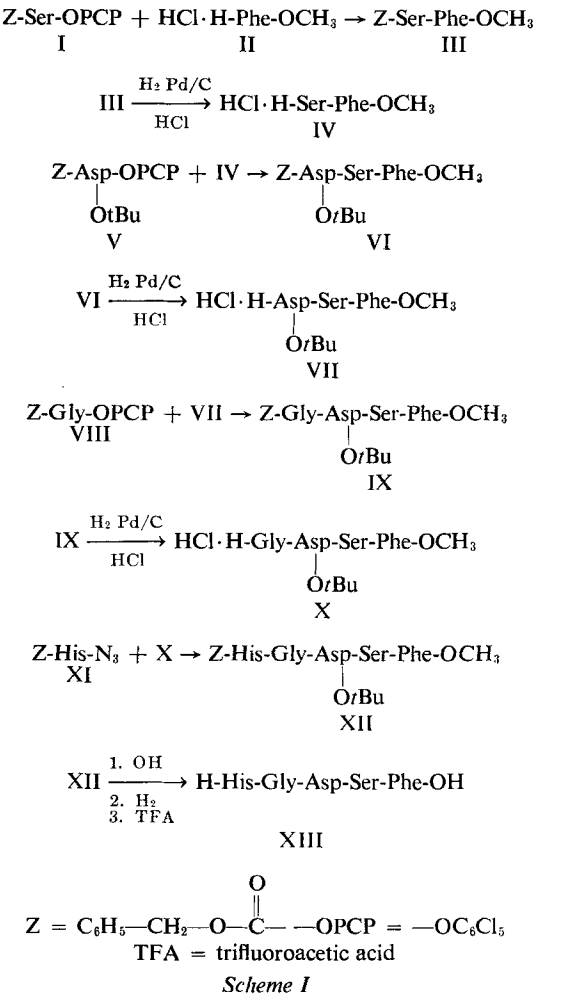
Sir:

Considerable attention has been focused recently on the role played by L-histidine and L-serine in the active site of chymotrypsin and other proteolytic enzymes towards the hydrolysis of various esters such as *p*-nitrophenyl acetate (1–4). One of the approaches for studying the active site of enzymes is the synthesis and evaluation of the catalytic activity of relatively simple peptides embodying as many as possible of the known features of active sites of proteolytic enzymes (5, 6). Katchalski *et al.* reported the synthesis of poly-L-histidine and copolymers of L-histidine and L-serine as model compounds to investigate the catalytic effect on the hydrolysis of *p*-nitrophenyl acetate (7). Sheehan and Cruickshank reported an interesting pentapeptide, L-threonyl-L-alanyl-L-seryl-L-histidyl-L-aspartic acid, possessing considerable catalytic activity (8). In order to provide increased flexibility in the molecule, recently Sheehan *et al.* reported the synthesis of L-seryl-γ-aminobutyryl-L-histidyl-γ-aminobutyryl-L-aspartic acid. This pentapeptide exhibited about 50% more catalytic activity towards the hydrolysis of *p*-nitrophenyl acetate, than that of previously reported synthetic peptides (9). As the separation of serine and histidine in a peptide chain led to relatively more catalytic activity, it was considered worthwhile to investigate the catalytic activity in simple peptides incorporating serine and histidine at different distances in the peptide chains. We now wish to report the preparation of a relatively more potent esterase model, L-histidyl-glycyl-L-aspartyl-L-seryl-L-phenylalanine (XIII).

The catalytic activity of the pentapeptide XIII was determined by the liberation of *p*-nitrophenol from *p*-nitrophenylacetate following the procedure used by Sheehan *et al.* (8, 9). The catalytic coefficient (7) for XIII was 179 l. mole^{-1} min.$^{-1}$ compared with 92 l. mole^{-1} min.$^{-1}$ for L-threonyl-L-alanyl-L-seryl-L-histidyl-L-aspartic acid (8), 147 l. mole^{-1} min.$^{-1}$ for L-seryl-γ-aminobutyryl-L-histidyl-γ-aminobutyryl-L-aspartic acid (9) and 10^4 l. mole^{-1} min.$^{-1}$ for α-chymotrypsin (4).

The pentapeptide was synthesized as outlined in Scheme I. In order to limit the degree of racemization, the peptide chain was extended from the *C*-terminal residue phenylalanine and all peptide bonds were formed using the pentachlorophenyl active ester method (10, 11). *N*-Benzyloxycarbonyl-L-serine pentachlorophenyl ester (I) was condensed with L-phenylalanine methyl ester hydrochloride (II) and the resulting dipeptide, *N*-benzyloxycarbonyl-L-seryl-L-phenylalanine methyl ester (III) was hydrogenated in the presence of hydrogen chloride in anhydrous methanol to afford L-seryl-L-phenylalanine methyl ester hydrochloride (IV). Coupling of IV with *N*-benzyloxycarbonyl-β-*t*-butyl-L-aspartic acid pentachlorophenyl ester (V) afforded *N*-benzyloxycarbonyl - β - *t* - butyl - L - aspartyl - L - seryl - L - phenylalanine methyl ester (VI). Hydrogenation of VI by the usual method afforded β-*t*-butyl-L-aspartyl-L-seryl-L-phenylalanine methyl ester hydrochloride (VII). Coupling of VII with *N*-benzyloxycarbonyl glycine pentachlorophenyl ester (VIII) afforded *N*-benzyloxycarbonyl glycyl - β - *t* - butyl - L - aspartyl - L - seryl - L - phenylalanine methyl ester (IX). Glycyl-β-*t*-butyl-L-aspartyl-L-seryl-L-phenylalanine methyl ester hydrochloride (X) was isolated by the hydrogenation of IX. Coupling of *N*-benzyloxycarbonyl-L-histidine azide (XI) with X led to the formation of *N*-benzyloxycarbonyl-L-histidyl-glycyl - β - *t* - butyl - L - aspartyl - L - seryl - L - phenylalanine methyl ester (XII), m.p. 158–159°.

Anal.—Calcd. for $C_{37}H_{47}N_7O_{11}$: C, 58.04; H, 6.14; N, 12.81. Found: C, 58.31; H, 5.97; N, 12.62.



Scheme I

Complete deprotection of the pentapeptide XII was achieved by saponification, hydrogenation and treatment with trifluoroacetic acid affording L-histidyl-glycyl-L-aspartyl-L-seryl-L-phenylalanine (XIII), m.p. 215–218° dec.

Anal.—Calcd. for $C_{24}H_{31}N_7O_9$: C, 51.34; H. 5.53; N, 17.47. Found: C, 51.12; H, 5.68; N, 17.19.

The free pentapeptide XIII was homogeneous to paper chromatography and paper electrophoresis under a variety of conditions.

Further studies on the catalytic activity and the chemistry of the pentapeptide XIII and other peptides incorporating histidine and serine separated by different distances of amino acids are under investigation.

REFERENCES

(1) H. Neurath, *Advan. Protein Chem.*, **12**, 319(1957).

(2) E. A. Barnard and W. D. Stein, *Advan. Enzymol.*, **20**, 51 (1958).

(3) M. L. Bender and F. Kezdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964).

(4) H. Neurath, "Bio-Organic Chemistry," W. H. Freeman, San Francisco, Calif., 1968, p. 249.

(5) J. C. Sheehan and D. N. McGregor, *J. Am. Chem. Soc.*, **84**, 3000(1962).

(6) I. Photaki, V. Bardakos, A. W. Lake, and G. Lowe, *J. Chem. Soc.*, **1968**, 1860.

(7) E. Katchalski, G. D. Fasman, E. Simons, E. R. Blout, F. R. N. Gurd, and W. L. Koltun, *Arch. Biochem. Biophys.*, **88**, 361 (1960).

(8) P. A. Cruickshank and J. C. Sheehan, *J. Am. Chem. Soc.*, **86**, 2070(1964).

(9) J. C. Sheehan, G. B. Bennett, and J. A. Schneider, *ibid.*, **88**, 3455(1966).

(10) J. Kovacs, R. Giannotti, and A. Kapoor, *ibid.*, **88**, 2282 (1966).

(11) A. Kapoor, E. J. Davis, and M. J. Graetzer, *J. Pharm. Sci.*, **57**, 1514(1968).

A. KAPOOR
SOON M. KANG
MARY ANN TRIMBOLI
College of Pharmacy
St. John's University
Jamaica, NY 11432

Received September 2, 1969.
Accepted for publication October 2, 1969.

Interference of Polycarboxylic Acids in the Determination of Ester Degradation by the Hydroxamic Acid Procedure

Keyphases □ Ester degradation determination—polycarboxylic acid interference □ Hydroxamate assay procedure—polycarboxylic buffer interference □ Colorimetric analysis—spectrophotometer

Sir:

In recent studies dealing with the kinetics of degradation of polyethylene glycol-600-mono-oleate in hydro-

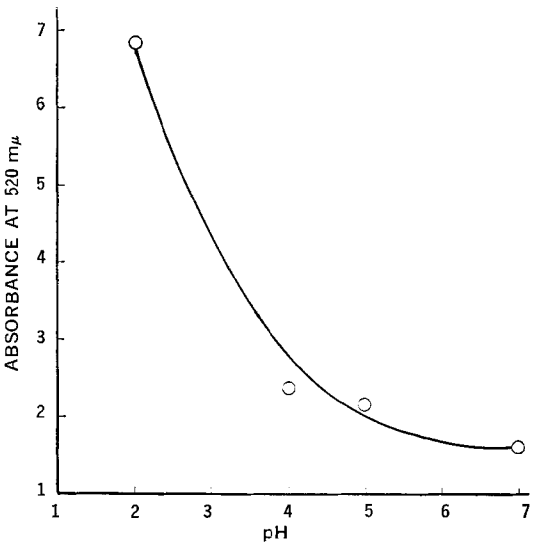


Figure 1—*Absorbance of ferric hydroxamate after heating hydroalcoholic solution buffered to various pH values with succinic acid for 24 hr.*

alcoholic solution at 80°, inconsistent results were observed. The degradation of the ester was determined by the ferric hydroxamate procedure (1), which indicated an apparent increase in ester concentration as a function of time. Investigation of the system leads us to believe that the succinate buffer system employed appears to react with the ethanol present in the system to form esters.

Blank solutions were prepared containing 0.1 *M* succinic acid in 50% ethanol and adjusted to pH 2, 4, 5, and 7 with 0.1 *N* sodium hydroxide and then subjected to refluxing at 80° for 24 hr. Samples were then taken and assayed by the hydroxamate procedure (1) with the results shown in Fig. 1. It is readily apparent that even in the absence of the test compound there is an increase in absorbance at 520 mμ which indicates the formation of an ester species. The reaction probably proceeds *via* an intermediate anhydride formation as reported earlier (2, 3), which would show an identical pH dependency. As expected, similar results were noted in our experiments using other polycarboxylic buffer systems such as citrate and tartrate. The interference was essentially eliminated by the use of phosphate systems.

These findings clearly indicate again the potential side reactions induced by buffers which may give rise to significant interference with analytical procedures. To test the validity of the assumption that the reaction involved esterification of the ethanol, identical solutions were prepared but substituting acetone for ethanol. Acetone was chosen since, if the proposed mechanism were correct, it would not react with the proposed anhydride intermediates. Solutions, after heating under the identical conditions as cited previously, showed no change in absorbance at 520 mμ. These findings would further support the hypothesis that esterification was taking place during the heating process.

The above-cited interference could cause significant errors in the interpretation of high-temperature kinetic

Complete deprotection of the pentapeptide XII was achieved by saponification, hydrogenation and treatment with trifluoroacetic acid affording L-histidyl-glycyl-L-aspartyl-L-seryl-L-phenylalanine (XIII), m.p. 215–218° dec.

Anal.—Calcd. for $C_{24}H_{31}N_7O_9$: C, 51.34; H. 5.53; N, 17.47. Found: C, 51.12; H, 5.68; N, 17.19.

The free pentapeptide XIII was homogeneous to paper chromatography and paper electrophoresis under a variety of conditions.

Further studies on the catalytic activity and the chemistry of the pentapeptide XIII and other peptides incorporating histidine and serine separated by different distances of amino acids are under investigation.

REFERENCES

(1) H. Neurath, *Advan. Protein Chem.*, **12**, 319(1957).

(2) E. A. Barnard and W. D. Stein, *Advan. Enzymol.*, **20**, 51 (1958).

(3) M. L. Bender and F. Kezdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964).

(4) H. Neurath, "Bio-Organic Chemistry," W. H. Freeman, San Francisco, Calif., 1968, p. 249.

(5) J. C. Sheehan and D. N. McGregor, *J. Am. Chem. Soc.*, **84**, 3000(1962).

(6) I. Photaki, V. Bardakos, A. W. Lake, and G. Lowe, *J. Chem. Soc.*, **1968**, 1860.

(7) E. Katchalski, G. D. Fasman, E. Simons, E. R. Blout, F. R. N. Gurd, and W. L. Koltun, *Arch. Biochem. Biophys.*, **88**, 361 (1960).

(8) P. A. Cruickshank and J. C. Sheehan, *J. Am. Chem. Soc.*, **86**, 2070(1964).

(9) J. C. Sheehan, G. B. Bennett, and J. A. Schneider, *ibid.*, **88**, 3455(1966).

(10) J. Kovacs, R. Giannotti, and A. Kapoor, *ibid.*, **88**, 2282 (1966).

(11) A. Kapoor, E. J. Davis, and M. J. Graetzer, *J. Pharm. Sci.*, **57**, 1514(1968).

A. KAPOOR
SOON M. KANG
MARY ANN TRIMBOLI
College of Pharmacy
St. John's University
Jamaica, NY 11432

Received September 2, 1969.
Accepted for publication October 2, 1969.

Interference of Polycarboxylic Acids in the Determination of Ester Degradation by the Hydroxamic Acid Procedure

Keyphases □ Ester degradation determination—polycarboxylic acid interference □ Hydroxamate assay procedure—polycarboxylic buffer interference □ Colorimetric analysis—spectrophotometer

Sir:

In recent studies dealing with the kinetics of degradation of polyethylene glycol-600-mono-oleate in hydro-

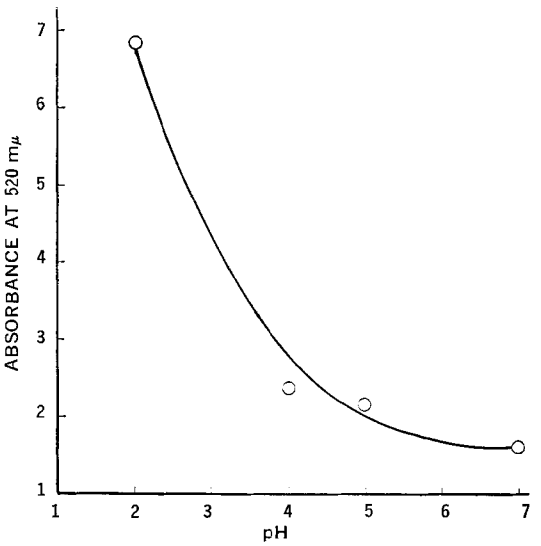


Figure 1—*Absorbance of ferric hydroxamate after heating hydroalcoholic solution buffered to various pH values with succinic acid for 24 hr.*

alcoholic solution at 80°, inconsistent results were observed. The degradation of the ester was determined by the ferric hydroxamate procedure (1), which indicated an apparent increase in ester concentration as a function of time. Investigation of the system leads us to believe that the succinate buffer system employed appears to react with the ethanol present in the system to form esters.

Blank solutions were prepared containing 0.1 *M* succinic acid in 50% ethanol and adjusted to pH 2, 4, 5, and 7 with 0.1 *N* sodium hydroxide and then subjected to refluxing at 80° for 24 hr. Samples were then taken and assayed by the hydroxamate procedure (1) with the results shown in Fig. 1. It is readily apparent that even in the absence of the test compound there is an increase in absorbance at 520 mμ which indicates the formation of an ester species. The reaction probably proceeds *via* an intermediate anhydride formation as reported earlier (2, 3), which would show an identical pH dependency. As expected, similar results were noted in our experiments using other polycarboxylic buffer systems such as citrate and tartrate. The interference was essentially eliminated by the use of phosphate systems.

These findings clearly indicate again the potential side reactions induced by buffers which may give rise to significant interference with analytical procedures. To test the validity of the assumption that the reaction involved esterification of the ethanol, identical solutions were prepared but substituting acetone for ethanol. Acetone was chosen since, if the proposed mechanism were correct, it would not react with the proposed anhydride intermediates. Solutions, after heating under the identical conditions as cited previously, showed no change in absorbance at 520 mμ. These findings would further support the hypothesis that esterification was taking place during the heating process.

The above-cited interference could cause significant errors in the interpretation of high-temperature kinetic

data involving hydroalcoholic solutions such as elixirs which have been buffered with polyfunctional acids.

(1) S. Siggia, "Quantitative Organic Analysis via Functional Groups," 3rd ed., Wiley, New York, N. Y., 1963, p. 140.
(2) T. Higuchi, S. O. Eriksson, H. Uno, and J. J. Windheuser, *J. Pharm. Sci.*, **53**, 280(1964).
(3) T. Higuchi, A. Shah, T. Miki, and A. Herd, *J. Am. Chem. Soc.*, **85**, 3655(1963).

DIAN CHU
J. J. WINDHEUSER
School of Pharmacy
University of Wisconsin
Madison, WI 53706

Received July 22, 1969.
Accepted for publication September 19, 1969.

Books

REVIEWS

Amphetamine Abuse. Edited by J. ROBERT RUSSO. Charles C Thomas, Springfield, IL 62703, 1968. xii + 159 pp. 16 × 23.5 cm. Price 7.50.

This 159-page book is made up of the edited papers of the first National Institute on Amphetamine Abuse which resulted from A Delinquency Study and Youth Development Project of Southern Illinois University. It was funded from a grant awarded by the Office of Juvenile Delinquency and Youth Development, United States Department of Health, Education, and Welfare and held during February, 1966. One finds contained within this volume the various viewpoints of law enforcement, the drug industry, the sociologist, the psychiatrist, and medical researcher, and finally the interesting experience of a young amphetamine abuser himself.

Although no attempt was made to correlate the contradictions of opinions expressed, the book stands as a good example of the complex spectrum of this social problem. It covers the topics of use, misuse and abuse; psychiatric implications; violations; law enforcement problems; and the important role of education.

The reviewer can only reiterate the cover leaf description of the book which states that an educator predictively recommends education as a preventative, a member of the Narcotics Bureau pleads for stronger laws and greater police freedom in enforcement and so on. Hence, this book represents a forum for viewpoints from the various establishments affected by the amphetamine abuse problem, but does not cogently and coherently express ways to solve the problem. However, the book does clarify much confusion in this area, it provides some lines of communication and it will help provide some basis for future legal, medical, and moral decisions about amphetamine abuse.

Reviewed by Ara H. Der Marderosian
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104 ■

Non-Specific Factors in Drug Therapy. Edited by KARL RICKELS. Charles C. Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1968, xii + 149 pp. 16 × 23.5 cm. Price $7.75.

This symposium volume is derived from a session of the 4th World Congress of Psychiatry held in 1966. Nonspecific (*i.e.*, nondrug) factors are considered particularly as they relate both to pharmacologic treatment of depressions, neuroses, and schizophrenia, and to the psychopharmacologic effects of various drugs in normal subjects. Major nonspecific variables affecting drug therapy are grouped as characteristics of patient, physician, treatment milieu, and nontreatment milieu. Examples of many nonspecific factors considered are attitudes of patient and physician (optimistic or pessimistic) toward drug treatment, personality characteristics of patients (or normals), duration as well as intensity of emotional disturbance being treated, and prior drug experiences. The relationship of such factors to side effects and paradoxical responses as well as to therapeutic success of drug treatment is examined.

Various nonspecific influences of psychological and sociocultural characteristics are discussed at length by several contributors in relation to the phenomena associated with placebo treatment including the controversial concept of "placebo reactors." While one chapter emphasizes the significance or "power" of the placebo and our knowledge of its relative effectiveness according to the varied circumstances, another chapter emphasizes the lack of knowledge on the true incidence of the placebo reactions and on the longitudinal consistency of the supposed "placebo reactor" characteristic. Certainly all agree to the need for further clarification of placebo phenomena and for enlarged and enlightened use of placebos in clinical drug studies.

Designed "to provide the reader with some of the latest findings and thoughts" in an interesting and significant area of psychopharmacology, the volume accomplishes this objective reasonably well. It will be most useful to those with some previous awareness of the subject and to those who will make use of the good selection of bibliographic citations included to pursue their interest among important earlier works.

Reviewed by W. Marvin Davis
School of Pharmacy
Department of Pharmacology
The University of Mississippi
University, MS 38677 ■

Autoxidation of Hydrocarbons and Polyolefins: Kinetics and Mechanisms. By LEO REICH and SALVATORE S. STIVALA, Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xi + 527 pp. 15.5 cm. × 23.5 cm. Price $29.75.

This book deals primarily with the kinetics and mechanisms of hydrocarbon and polyolefin autoxidation. Following a brief account of some early historical developments, Chapter 1 gives a clear, concise treatment of the general kinetics of free radical reactions. Chapter 2 covers the autoxidation of hydrocarbons in the absence of inhibitors and accelerators while Chapters 3 and 4 deal with simple hydrocarbon autoxidation in the presence of antioxidants and metal catalysts, respectively. The topic of weak chemiluminescence during hydrocarbon autoxidation is presented in Chapter 5,

data involving hydroalcoholic solutions such as elixirs which have been buffered with polyfunctional acids.

(1) S. Siggia, "Quantitative Organic Analysis via Functional Groups," 3rd ed., Wiley, New York, N. Y., 1963, p. 140.
(2) T. Higuchi, S. O. Eriksson, H. Uno, and J. J. Windheuser, *J. Pharm. Sci.*, **53**, 280(1964).
(3) T. Higuchi, A. Shah, T. Miki, and A. Herd, *J. Am. Chem. Soc.*, **85**, 3655(1963).

DIAN CHU
J. J. WINDHEUSER
School of Pharmacy
University of Wisconsin
Madison, WI 53706

Received July 22, 1969.
Accepted for publication September 19, 1969.

Books

REVIEWS

Amphetamine Abuse. Edited by J. ROBERT RUSSO. Charles C Thomas, Springfield, IL 62703, 1968. xii + 159 pp. 16 × 23.5 cm. Price 7.50.

This 159-page book is made up of the edited papers of the first National Institute on Amphetamine Abuse which resulted from A Delinquency Study and Youth Development Project of Southern Illinois University. It was funded from a grant awarded by the Office of Juvenile Delinquency and Youth Development, United States Department of Health, Education, and Welfare and held during February, 1966. One finds contained within this volume the various viewpoints of law enforcement, the drug industry, the sociologist, the psychiatrist, and medical researcher, and finally the interesting experience of a young amphetamine abuser himself.

Although no attempt was made to correlate the contradictions of opinions expressed, the book stands as a good example of the complex spectrum of this social problem. It covers the topics of use, misuse and abuse; psychiatric implications; violations; law enforcement problems; and the important role of education.

The reviewer can only reiterate the cover leaf description of the book which states that an educator predictively recommends education as a preventative, a member of the Narcotics Bureau pleads for stronger laws and greater police freedom in enforcement and so on. Hence, this book represents a forum for viewpoints from the various establishments affected by the amphetamine abuse problem, but does not cogently and coherently express ways to solve the problem. However, the book does clarify much confusion in this area, it provides some lines of communication and it will help provide some basis for future legal, medical, and moral decisions about amphetamine abuse.

Reviewed by Ara H. Der Marderosian
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104 ■

Non-Specific Factors in Drug Therapy. Edited by KARL RICKELS. Charles C. Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1968, xii + 149 pp. 16 × 23.5 cm. Price $7.75.

This symposium volume is derived from a session of the 4th World Congress of Psychiatry held in 1966. Nonspecific (*i.e.*, nondrug) factors are considered particularly as they relate both to pharmacologic treatment of depressions, neuroses, and schizophrenia, and to the psychopharmacologic effects of various drugs in normal subjects. Major nonspecific variables affecting drug therapy are grouped as characteristics of patient, physician, treatment milieu, and nontreatment milieu. Examples of many nonspecific factors considered are attitudes of patient and physician (optimistic or pessimistic) toward drug treatment, personality characteristics of patients (or normals), duration as well as intensity of emotional disturbance being treated, and prior drug experiences. The relationship of such factors to side effects and paradoxical responses as well as to therapeutic success of drug treatment is examined.

Various nonspecific influences of psychological and sociocultural characteristics are discussed at length by several contributors in relation to the phenomena associated with placebo treatment including the controversial concept of "placebo reactors." While one chapter emphasizes the significance or "power" of the placebo and our knowledge of its relative effectiveness according to the varied circumstances, another chapter emphasizes the lack of knowledge on the true incidence of the placebo reactions and on the longitudinal consistency of the supposed "placebo reactor" characteristic. Certainly all agree to the need for further clarification of placebo phenomena and for enlarged and enlightened use of placebos in clinical drug studies.

Designed "to provide the reader with some of the latest findings and thoughts" in an interesting and significant area of psychopharmacology, the volume accomplishes this objective reasonably well. It will be most useful to those with some previous awareness of the subject and to those who will make use of the good selection of bibliographic citations included to pursue their interest among important earlier works.

Reviewed by W. Marvin Davis
School of Pharmacy
Department of Pharmacology
The University of Mississippi
University, MS 38677 ■

Autoxidation of Hydrocarbons and Polyolefins: Kinetics and Mechanisms. By LEO REICH and SALVATORE S. STIVALA, Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xi + 527 pp. 15.5 cm. × 23.5 cm. Price $29.75.

This book deals primarily with the kinetics and mechanisms of hydrocarbon and polyolefin autoxidation. Following a brief account of some early historical developments, Chapter 1 gives a clear, concise treatment of the general kinetics of free radical reactions. Chapter 2 covers the autoxidation of hydrocarbons in the absence of inhibitors and accelerators while Chapters 3 and 4 deal with simple hydrocarbon autoxidation in the presence of antioxidants and metal catalysts, respectively. The topic of weak chemiluminescence during hydrocarbon autoxidation is presented in Chapter 5,

data involving hydroalcoholic solutions such as elixirs which have been buffered with polyfunctional acids.

(1) S. Siggia, "Quantitative Organic Analysis via Functional Groups," 3rd ed., Wiley, New York, N. Y., 1963, p. 140.
(2) T. Higuchi, S. O. Eriksson, H. Uno, and J. J. Windheuser, *J. Pharm. Sci.*, **53**, 280(1964).
(3) T. Higuchi, A. Shah, T. Miki, and A. Herd, *J. Am. Chem. Soc.*, **85**, 3655(1963).

DIAN CHU
J. J. WINDHEUSER
School of Pharmacy
University of Wisconsin
Madison, WI 53706

Received July 22, 1969.
Accepted for publication September 19, 1969.

Books

REVIEWS

Amphetamine Abuse. Edited by J. ROBERT RUSSO. Charles C Thomas, Springfield, IL 62703, 1968. xii + 159 pp. 16 × 23.5 cm. Price 7.50.

This 159-page book is made up of the edited papers of the first National Institute on Amphetamine Abuse which resulted from A Delinquency Study and Youth Development Project of Southern Illinois University. It was funded from a grant awarded by the Office of Juvenile Delinquency and Youth Development, United States Department of Health, Education, and Welfare and held during February, 1966. One finds contained within this volume the various viewpoints of law enforcement, the drug industry, the sociologist, the psychiatrist, and medical researcher, and finally the interesting experience of a young amphetamine abuser himself.

Although no attempt was made to correlate the contradictions of opinions expressed, the book stands as a good example of the complex spectrum of this social problem. It covers the topics of use, misuse and abuse; psychiatric implications; violations; law enforcement problems; and the important role of education.

The reviewer can only reiterate the cover leaf description of the book which states that an educator predictively recommends education as a preventative, a member of the Narcotics Bureau pleads for stronger laws and greater police freedom in enforcement and so on. Hence, this book represents a forum for viewpoints from the various establishments affected by the amphetamine abuse problem, but does not cogently and coherently express ways to solve the problem. However, the book does clarify much confusion in this area, it provides some lines of communication and it will help provide some basis for future legal, medical, and moral decisions about amphetamine abuse.

Reviewed by Ara H. Der Marderosian
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104 ■

Non-Specific Factors in Drug Therapy. Edited by KARL RICKELS. Charles C. Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1968, xii + 149 pp. 16 × 23.5 cm. Price $7.75.

This symposium volume is derived from a session of the 4th World Congress of Psychiatry held in 1966. Nonspecific (*i.e.*, nondrug) factors are considered particularly as they relate both to pharmacologic treatment of depressions, neuroses, and schizophrenia, and to the psychopharmacologic effects of various drugs in normal subjects. Major nonspecific variables affecting drug therapy are grouped as characteristics of patient, physician, treatment milieu, and nontreatment milieu. Examples of many nonspecific factors considered are attitudes of patient and physician (optimistic or pessimistic) toward drug treatment, personality characteristics of patients (or normals), duration as well as intensity of emotional disturbance being treated, and prior drug experiences. The relationship of such factors to side effects and paradoxical responses as well as to therapeutic success of drug treatment is examined.

Various nonspecific influences of psychological and sociocultural characteristics are discussed at length by several contributors in relation to the phenomena associated with placebo treatment including the controversial concept of "placebo reactors." While one chapter emphasizes the significance or "power" of the placebo and our knowledge of its relative effectiveness according to the varied circumstances, another chapter emphasizes the lack of knowledge on the true incidence of the placebo reactions and on the longitudinal consistency of the supposed "placebo reactor" characteristic. Certainly all agree to the need for further clarification of placebo phenomena and for enlarged and enlightened use of placebos in clinical drug studies.

Designed "to provide the reader with some of the latest findings and thoughts" in an interesting and significant area of psychopharmacology, the volume accomplishes this objective reasonably well. It will be most useful to those with some previous awareness of the subject and to those who will make use of the good selection of bibliographic citations included to pursue their interest among important earlier works.

Reviewed by W. Marvin Davis
School of Pharmacy
Department of Pharmacology
The University of Mississippi
University, MS 38677 ■

Autoxidation of Hydrocarbons and Polyolefins: Kinetics and Mechanisms. By LEO REICH and SALVATORE S. STIVALA, Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xi + 527 pp. 15.5 cm. × 23.5 cm. Price $29.75.

This book deals primarily with the kinetics and mechanisms of hydrocarbon and polyolefin autoxidation. Following a brief account of some early historical developments, Chapter 1 gives a clear, concise treatment of the general kinetics of free radical reactions. Chapter 2 covers the autoxidation of hydrocarbons in the absence of inhibitors and accelerators while Chapters 3 and 4 deal with simple hydrocarbon autoxidation in the presence of antioxidants and metal catalysts, respectively. The topic of weak chemiluminescence during hydrocarbon autoxidation is presented in Chapter 5,

data involving hydroalcoholic solutions such as elixirs which have been buffered with polyfunctional acids.

(1) S. Siggia, "Quantitative Organic Analysis via Functional Groups," 3rd ed., Wiley, New York, N. Y., 1963, p. 140.
(2) T. Higuchi, S. O. Eriksson, H. Uno, and J. J. Windheuser, *J. Pharm. Sci.*, **53**, 280(1964).
(3) T. Higuchi, A. Shah, T. Miki, and A. Herd, *J. Am. Chem. Soc.*, **85**, 3655(1963).

DIAN CHU
J. J. WINDHEUSER
School of Pharmacy
University of Wisconsin
Madison, WI 53706

Received July 22, 1969.
Accepted for publication September 19, 1969.

Books

REVIEWS

Amphetamine Abuse. Edited by J. ROBERT RUSSO. Charles C Thomas, Springfield, IL 62703, 1968. xii + 159 pp. 16 × 23.5 cm. Price 7.50.

This 159-page book is made up of the edited papers of the first National Institute on Amphetamine Abuse which resulted from A Delinquency Study and Youth Development Project of Southern Illinois University. It was funded from a grant awarded by the Office of Juvenile Delinquency and Youth Development, United States Department of Health, Education, and Welfare and held during February, 1966. One finds contained within this volume the various viewpoints of law enforcement, the drug industry, the sociologist, the psychiatrist, and medical researcher, and finally the interesting experience of a young amphetamine abuser himself.

Although no attempt was made to correlate the contradictions of opinions expressed, the book stands as a good example of the complex spectrum of this social problem. It covers the topics of use, misuse and abuse; psychiatric implications; violations; law enforcement problems; and the important role of education.

The reviewer can only reiterate the cover leaf description of the book which states that an educator predictively recommends education as a preventative, a member of the Narcotics Bureau pleads for stronger laws and greater police freedom in enforcement and so on. Hence, this book represents a forum for viewpoints from the various establishments affected by the amphetamine abuse problem, but does not cogently and coherently express ways to solve the problem. However, the book does clarify much confusion in this area, it provides some lines of communication and it will help provide some basis for future legal, medical, and moral decisions about amphetamine abuse.

Reviewed by Ara H. Der Marderosian
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104 ■

Non-Specific Factors in Drug Therapy. Edited by KARL RICKELS. Charles C. Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1968, xii + 149 pp. 16 × 23.5 cm. Price $7.75.

This symposium volume is derived from a session of the 4th World Congress of Psychiatry held in 1966. Nonspecific (*i.e.*, nondrug) factors are considered particularly as they relate both to pharmacologic treatment of depressions, neuroses, and schizophrenia, and to the psychopharmacologic effects of various drugs in normal subjects. Major nonspecific variables affecting drug therapy are grouped as characteristics of patient, physician, treatment milieu, and nontreatment milieu. Examples of many nonspecific factors considered are attitudes of patient and physician (optimistic or pessimistic) toward drug treatment, personality characteristics of patients (or normals), duration as well as intensity of emotional disturbance being treated, and prior drug experiences. The relationship of such factors to side effects and paradoxical responses as well as to therapeutic success of drug treatment is examined.

Various nonspecific influences of psychological and sociocultural characteristics are discussed at length by several contributors in relation to the phenomena associated with placebo treatment including the controversial concept of "placebo reactors." While one chapter emphasizes the significance or "power" of the placebo and our knowledge of its relative effectiveness according to the varied circumstances, another chapter emphasizes the lack of knowledge on the true incidence of the placebo reactions and on the longitudinal consistency of the supposed "placebo reactor" characteristic. Certainly all agree to the need for further clarification of placebo phenomena and for enlarged and enlightened use of placebos in clinical drug studies.

Designed "to provide the reader with some of the latest findings and thoughts" in an interesting and significant area of psychopharmacology, the volume accomplishes this objective reasonably well. It will be most useful to those with some previous awareness of the subject and to those who will make use of the good selection of bibliographic citations included to pursue their interest among important earlier works.

Reviewed by W. Marvin Davis
School of Pharmacy
Department of Pharmacology
The University of Mississippi
University, MS 38677 ■

Autoxidation of Hydrocarbons and Polyolefins: Kinetics and Mechanisms. By LEO REICH and SALVATORE S. STIVALA, Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xi + 527 pp. 15.5 cm. × 23.5 cm. Price $29.75.

This book deals primarily with the kinetics and mechanisms of hydrocarbon and polyolefin autoxidation. Following a brief account of some early historical developments, Chapter 1 gives a clear, concise treatment of the general kinetics of free radical reactions. Chapter 2 covers the autoxidation of hydrocarbons in the absence of inhibitors and accelerators while Chapters 3 and 4 deal with simple hydrocarbon autoxidation in the presence of antioxidants and metal catalysts, respectively. The topic of weak chemiluminescence during hydrocarbon autoxidation is presented in Chapter 5,

and the qualitative and quantitative aspects of autoxidation of saturated polyolefins are dealt with in Chapters 6 and 7, respectively. The final chapter is devoted to the investigation of polyolefin autoxidation by various techniques.

The arrangement and sequence of the subject matter is well organized and allows even the nonexpert to follow the kinetics without too much difficulty. There is an abundance of reaction rate equations which although essential for kinetic studies would appear to be overexcessive. There are also numerous chemical equations for the various autoxidation schemes. The problems given at the end of some chapters are adequate, being fairly difficult and of good variety and these should prove stimulating and challenging to the graduate student or research worker specializing in this field. However, I feel that answers to the numerical problems should have been provided and these would be most helpful to the student. The rate constants compiled in the various tables will prove to be a most useful feature to research workers in this field. The bibliography is up-to-date, covering the literature up to 1967. However, the inclusion of reference to private communication and unpublished work in a textbook is undesirable and meaningless. Several of the figures are very poorly drawn and detract from the quality of the book. The authors should have been able to reproduce the figures from the original papers. To sum up, the book can be highly recommended to the advanced graduate student and the specialist in this field who will find a wealth of material gathered together in a single book.

Reviewed by Lucy S. C. Wan
School of Pharmacy
University of Singapore
Sepoy Lines, Singapore 3 ■

Purification of Inorganic and Organic Materials: Techniques of Fractional Solidification. Edited by MORRIS ZIEF. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xvii + 318 pp. 15×23 cm. Price $17.75.

An earlier volume edited by Zief and Wilcox, entitled "Fractional Solidification," brought together for the first time the basic principles, apparatus, and applications of all types of solid-liquid processes associated with ultra-purification of chemical compounds. The volume here reviewed is a logical sequel inasmuch as it presents reliable methods for the preparation and analysis of such compounds. The choice of the methods presented admittedly reflects the interests of the author, however, they are described by experts in the respective areas and were chosen on the basis of originality, reproducibility, and adaptability to problems normally encountered in the laboratory. Contributing authors represent a broad distribution of research laboratories, both foreign and domestic.

Part I of the book concerns the description of analytical methods for ultra-pure compounds, including a variety of spectral techniques, thermal methods, electrochemical and gas-liquid chromatographic procedures. Part II describes in detail the most useful methods available for zone melting, progressive freezing and column crystallization. Particularly unique are the chapters describing rapid purification of organic compounds, purification in kilogram quantities, zone melting chromatography, and the concentration of flavors at low temperature.

As though in answer to the question of how one maintains the integrity of an ultrapure compound, Part III presents, in a single chapter, a discussion and tabulation of data concerning containers for pure substances. Quite interesting is the fact that polyfluorocarbons compare most favorably with quartz and borosilicate as acceptable container materials.

While they ought to be, at least for pharmacological evaluation and for reference standards, medicinal compounds are not subjected to ultrapurification. The book under review presents considerations which are applicable to some organic medicinal compounds, if and when it should be desirable to render them ultrapure.

Reviewed by Paul J. Jannke
College of Pharmacy
University of Connecticut
Storrs, CT 06268 ■

NOTICES

Acetylenes and Allenes: Addition, Cyclization, and Polymerization Reactions. By THOMAS F. RUTLEDGE. Van Nostrand Reinhold Company, 450 West 33rd St., New York, NY 10001, 1969. xvi + 432 pp. 23.4 × 15.6 cm. Price $21.50.

Adrenergic Neurotransmission. (Study Group #33), Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1969. x + 123 pp. 12.5 × 18.9 cm. Price $4.50.

Amino Acides Peptides Proteines. Cahier No. 6. Edited by MAURICE VIGNERON. AEC Societe de Chimie Organique et Biologique, 24, Avenue de l'Opera, Paris 1er, France, 1966. 457 pp. 21 × 26.7 cm.

Antibiotike-Fibel—Antibiotika und Chemotherapie. By M. PLEMPEL and H. OTTEN. Georg Thieme Verlag, 7 Stuttgart 1, P.O. Box 732, Gerdweg 63, Germany, 1969. xl + 994 pp. 15.5 × 23 cm. Price DM 98,-.

Behavior of Enzyme Systems. 2nd Edition. By JOHN M. REINER. Van Nostrand Reinhold Company, 450 West 33rd St., New York, NY 10001, 1969. xviii + 345 pp. 15.5 × 23.5 cm.

British National Formulary 1968. Published jointly by the British Medical Association and the Pharmaceutical Society of Great Britain, The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England, 1968. 384 pp. 10.7 × 17.2 cm. Price 13s.6d plus postage.

British Pharmaceutical Codex 1968. Edited by S. C. JOLLY. The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England, 1968. U. S. Distribution: Rittenhouse Book Store, Philadelphia, Pa. xxxvii + 1513 pp. 15 × 22.3 cm. Price $19.50.

Degradation of Herbicides. Edited by P. C. KEARNEY and D. D. KAUFMAN. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xi + 394 pp. 15.7 × 23.6 cm. Price $18.75.

Die Interkantonale Vereinbarung uber die Kontrolle der Heilmittel vom 16. Juni 1954. By FELIX WUST. Kommissionsverlag Herbert Lang & Cie AG Bern, Switzerland, 1969. 368 pp. 15.5 × 22.5 cm. Price 3 fr./Dm 52.—.

Index Nominum 1966, Supplementum 1968. Edited by Laboratory of the Swiss Pharmaceutical Society. Swiss Pharmaceutical Society, Marktgasse 52, 3011 Bern, Switzerland 1969. 120 pp. 17 × 24 cm. Price Sw. fr. 35.—plus postage.

Medicaments Organiques de Synthese. Vol. I. Edited by GUILLAUME VALETTE. Masson Et Cie, Editeurs, 120, Boulevard Saint-Germain, Paris VIe, France, 1969. 226 pp. 15.4 × 22 cm. Price 88 fr.

Methods of Biochemical Analysis. Vol. 17. Edited by DAVID GLICK. Interscience Publishers. Div. of John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10016, 1969. vi + 428 pp. 15.5 × 23.4 cm. Price $16.50.

Pharmaceutical Chemistry Part II. Inorganic Vol. 1. By M. L. SCHROFF. National Book Centre P-76, Dr. Sundari Mohan Ave., Calcutta, 14, India, 1968. v + 700 + xvi pp. 14.5 × 22.2 cm. Price $10.00.

Pharmacological Research Communications. Vol. I. No. I. The Italian Pharmacological Society. Academic Press Inc. (London) Ltd., Berkeley Square House, London WIX 6 BA, 1969. 126 pp. 16.8 × 24 cm. Price 84/-, $10.00 per 4 issues per annum.

Pharmacology for the Preoperative Visit. Vol. 6, No. 1. Edited by J. S. GRAVENSTEIN. Little, Brown and Company, 34 Beacon St., Boston, MA, 1969. xi + 396 pp. 15.5 × 23.5 cm. Price $22.00/yr. subscription rate.

Precis de Microbiologie. Vol. I. By S. LAMIN and A. GERMAN. Masson & Cie, Editeurs, 120, Boulevard Saint-Germain, Paris VIe, France, 1969. viii + 669 pp. 16.5 × 21.5 cm. Price 75 fr.

The Psychopharmacology of the Normal Human. Edited by WAYNE O. EVANS and NATHAN S. KLINE. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xi + 252 pp. 15.6 × 23.3 cm. Price $11.50.

Psychotropic Drug Response: Advances in Prediction. Edited by PHILIP R. A. MAY and J. R. WITTENBORN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xii + 292 pp. 16 × 23.4 cm. Price $12.75.

The Role of Learning in Psychotherapy. Edited by RUTH PORTER. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1968. xii + 340 pp. 15.5 × 23.2 cm. Price $12.00.

Steroid Reaction Mechanisms. By D. N. KIRK and M. P. HARTSHORN. American Elsevier Publishing Co., Inc., 52 Vanderbilt Ave., New York, NY 10017, 1968. xi + 476 pp. 14 × 22 cm. Price $30.00.

and the qualitative and quantitative aspects of autoxidation of saturated polyolefins are dealt with in Chapters 6 and 7, respectively. The final chapter is devoted to the investigation of polyolefin autoxidation by various techniques.

The arrangement and sequence of the subject matter is well organized and allows even the nonexpert to follow the kinetics without too much difficulty. There is an abundance of reaction rate equations which although essential for kinetic studies would appear to be overexcessive. There are also numerous chemical equations for the various autoxidation schemes. The problems given at the end of some chapters are adequate, being fairly difficult and of good variety and these should prove stimulating and challenging to the graduate student or research worker specializing in this field. However, I feel that answers to the numerical problems should have been provided and these would be most helpful to the student. The rate constants compiled in the various tables will prove to be a most useful feature to research workers in this field. The bibliography is up-to-date, covering the literature up to 1967. However, the inclusion of reference to private communication and unpublished work in a textbook is undesirable and meaningless. Several of the figures are very poorly drawn and detract from the quality of the book. The authors should have been able to reproduce the figures from the original papers. To sum up, the book can be highly recommended to the advanced graduate student and the specialist in this field who will find a wealth of material gathered together in a single book.

Reviewed by Lucy S. C. Wan
School of Pharmacy
University of Singapore
Sepoy Lines, Singapore 3 ■

Purification of Inorganic and Organic Materials: Techniques of Fractional Solidification. Edited by MORRIS ZIEF. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xvii + 318 pp. 15×23 cm. Price $17.75.

An earlier volume edited by Zief and Wilcox, entitled "Fractional Solidification," brought together for the first time the basic principles, apparatus, and applications of all types of solid-liquid processes associated with ultra-purification of chemical compounds. The volume here reviewed is a logical sequel inasmuch as it presents reliable methods for the preparation and analysis of such compounds. The choice of the methods presented admittedly reflects the interests of the author, however, they are described by experts in the respective areas and were chosen on the basis of originality, reproducibility, and adaptability to problems normally encountered in the laboratory. Contributing authors represent a broad distribution of research laboratories, both foreign and domestic.

Part I of the book concerns the description of analytical methods for ultra-pure compounds, including a variety of spectral techniques, thermal methods, electrochemical and gas-liquid chromatographic procedures. Part II describes in detail the most useful methods available for zone melting, progressive freezing and column crystallization. Particularly unique are the chapters describing rapid purification of organic compounds, purification in kilogram quantities, zone melting chromatography, and the concentration of flavors at low temperature.

As though in answer to the question of how one maintains the integrity of an ultrapure compound, Part III presents, in a single chapter, a discussion and tabulation of data concerning containers for pure substances. Quite interesting is the fact that polyfluorocarbons compare most favorably with quartz and borosilicate as acceptable container materials.

While they ought to be, at least for pharmacological evaluation and for reference standards, medicinal compounds are not subjected to ultrapurification. The book under review presents considerations which are applicable to some organic medicinal compounds, if and when it should be desirable to render them ultrapure.

Reviewed by Paul J. Jannke
College of Pharmacy
University of Connecticut
Storrs, CT 06268 ■

NOTICES

Acetylenes and Allenes: Addition, Cyclization, and Polymerization Reactions. By THOMAS F. RUTLEDGE. Van Nostrand Reinhold Company, 450 West 33rd St., New York, NY 10001, 1969. xvi + 432 pp. 23.4 × 15.6 cm. Price $21.50.

Adrenergic Neurotransmission. (Study Group #33), Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1969. x + 123 pp. 12.5 × 18.9 cm. Price $4.50.

Amino Acides Peptides Proteines. Cahier No. 6. Edited by MAURICE VIGNERON. AEC Societe de Chimie Organique et Biologique, 24, Avenue de l'Opera, Paris 1er, France, 1966. 457 pp. 21 × 26.7 cm.

Antibiotike-Fibel—Antibiotika und Chemotherapie. By M. PLEMPEL and H. OTTEN. Georg Thieme Verlag, 7 Stuttgart 1, P.O. Box 732, Gerdweg 63, Germany, 1969. xl + 994 pp. 15.5 × 23 cm. Price DM 98,-.

Behavior of Enzyme Systems. 2nd Edition. By JOHN M. REINER. Van Nostrand Reinhold Company, 450 West 33rd St., New York, NY 10001, 1969. xviii + 345 pp. 15.5 × 23.5 cm.

British National Formulary 1968. Published jointly by the British Medical Association and the Pharmaceutical Society of Great Britain, The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England, 1968. 384 pp. 10.7 × 17.2 cm. Price 13s.6d plus postage.

British Pharmaceutical Codex 1968. Edited by S. C. JOLLY. The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England, 1968. U. S. Distribution: Rittenhouse Book Store, Philadelphia, Pa. xxxvii + 1513 pp. 15 × 22.3 cm. Price $19.50.

Degradation of Herbicides. Edited by P. C. KEARNEY and D. D. KAUFMAN. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xi + 394 pp. 15.7 × 23.6 cm. Price $18.75.

Die Interkantonale Vereinbarung uber die Kontrolle der Heilmittel vom 16. Juni 1954. By FELIX WUST. Kommissionsverlag Herbert Lang & Cie AG Bern, Switzerland, 1969. 368 pp. 15.5 × 22.5 cm. Price 3 fr./Dm 52.—.

Index Nominum 1966, Supplementum 1968. Edited by Laboratory of the Swiss Pharmaceutical Society. Swiss Pharmaceutical Society, Marktgasse 52, 3011 Bern, Switzerland 1969. 120 pp. 17 × 24 cm. Price Sw. fr. 35.—plus postage.

Medicaments Organiques de Synthese. Vol. I. Edited by GUILLAUME VALETTE. Masson Et Cie, Editeurs, 120, Boulevard Saint-Germain, Paris VIe, France, 1969. 226 pp. 15.4 × 22 cm. Price 88 fr.

Methods of Biochemical Analysis. Vol. 17. Edited by DAVID GLICK. Interscience Publishers. Div. of John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10016, 1969. vi + 428 pp. 15.5 × 23.4 cm. Price $16.50.

Pharmaceutical Chemistry Part II. Inorganic Vol. 1. By M. L. SCHROFF. National Book Centre P-76, Dr. Sundari Mohan Ave., Calcutta, 14, India, 1968. v + 700 + xvi pp. 14.5 × 22.2 cm. Price $10.00.

Pharmacological Research Communications. Vol. I. No. I. The Italian Pharmacological Society. Academic Press Inc. (London) Ltd., Berkeley Square House, London WIX 6 BA, 1969. 126 pp. 16.8 × 24 cm. Price 84/-, $10.00 per 4 issues per annum.

Pharmacology for the Preoperative Visit. Vol. 6, No. 1. Edited by J. S. GRAVENSTEIN. Little, Brown and Company, 34 Beacon St., Boston, MA, 1969. xi + 396 pp. 15.5 × 23.5 cm. Price $22.00/yr. subscription rate.

Precis de Microbiologie. Vol. I. By S. LAMIN and A. GERMAN. Masson & Cie, Editeurs, 120, Boulevard Saint-Germain, Paris VIe, France, 1969. viii + 669 pp. 16.5 × 21.5 cm. Price 75 fr.

The Psychopharmacology of the Normal Human. Edited by WAYNE O. EVANS and NATHAN S. KLINE. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xi + 252 pp. 15.6 × 23.3 cm. Price $11.50.

Psychotropic Drug Response: Advances in Prediction. Edited by PHILIP R. A. MAY and J. R. WITTENBORN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xii + 292 pp. 16 × 23.4 cm. Price $12.75.

The Role of Learning in Psychotherapy. Edited by RUTH PORTER. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1968. xii + 340 pp. 15.5 × 23.2 cm. Price $12.00.

Steroid Reaction Mechanisms. By D. N. KIRK and M. P. HARTSHORN. American Elsevier Publishing Co., Inc., 52 Vanderbilt Ave., New York, NY 10017, 1968. xi + 476 pp. 14 × 22 cm. Price $30.00.

and the qualitative and quantitative aspects of autoxidation of saturated polyolefins are dealt with in Chapters 6 and 7, respectively. The final chapter is devoted to the investigation of polyolefin autoxidation by various techniques.

The arrangement and sequence of the subject matter is well organized and allows even the nonexpert to follow the kinetics without too much difficulty. There is an abundance of reaction rate equations which although essential for kinetic studies would appear to be overexcessive. There are also numerous chemical equations for the various autoxidation schemes. The problems given at the end of some chapters are adequate, being fairly difficult and of good variety and these should prove stimulating and challenging to the graduate student or research worker specializing in this field. However, I feel that answers to the numerical problems should have been provided and these would be most helpful to the student. The rate constants compiled in the various tables will prove to be a most useful feature to research workers in this field. The bibliography is up-to-date, covering the literature up to 1967. However, the inclusion of reference to private communication and unpublished work in a textbook is undesirable and meaningless. Several of the figures are very poorly drawn and detract from the quality of the book. The authors should have been able to reproduce the figures from the original papers. To sum up, the book can be highly recommended to the advanced graduate student and the specialist in this field who will find a wealth of material gathered together in a single book.

Reviewed by Lucy S. C. Wan
School of Pharmacy
University of Singapore
Sepoy Lines, Singapore 3 ■

Purification of Inorganic and Organic Materials: Techniques of Fractional Solidification. Edited by MORRIS ZIEF. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xvii + 318 pp. 15×23 cm. Price $17.75.

An earlier volume edited by Zief and Wilcox, entitled "Fractional Solidification," brought together for the first time the basic principles, apparatus, and applications of all types of solid-liquid processes associated with ultra-purification of chemical compounds. The volume here reviewed is a logical sequel inasmuch as it presents reliable methods for the preparation and analysis of such compounds. The choice of the methods presented admittedly reflects the interests of the author, however, they are described by experts in the respective areas and were chosen on the basis of originality, reproducibility, and adaptability to problems normally encountered in the laboratory. Contributing authors represent a broad distribution of research laboratories, both foreign and domestic.

Part I of the book concerns the description of analytical methods for ultra-pure compounds, including a variety of spectral techniques, thermal methods, electrochemical and gas-liquid chromatographic procedures. Part II describes in detail the most useful methods available for zone melting, progressive freezing and column crystallization. Particularly unique are the chapters describing rapid purification of organic compounds, purification in kilogram quantities, zone melting chromatography, and the concentration of flavors at low temperature.

As though in answer to the question of how one maintains the integrity of an ultrapure compound, Part III presents, in a single chapter, a discussion and tabulation of data concerning containers for pure substances. Quite interesting is the fact that polyfluorocarbons compare most favorably with quartz and borosilicate as acceptable container materials.

While they ought to be, at least for pharmacological evaluation and for reference standards, medicinal compounds are not subjected to ultrapurification. The book under review presents considerations which are applicable to some organic medicinal compounds, if and when it should be desirable to render them ultrapure.

Reviewed by Paul J. Jannke
College of Pharmacy
University of Connecticut
Storrs, CT 06268 ■

NOTICES

Acetylenes and Allenes: Addition, Cyclization, and Polymerization Reactions. By THOMAS F. RUTLEDGE. Van Nostrand Reinhold Company, 450 West 33rd St., New York, NY 10001, 1969. xvi + 432 pp. 23.4 × 15.6 cm. Price $21.50.

Adrenergic Neurotransmission. (Study Group #33), Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1969. x + 123 pp. 12.5 × 18.9 cm. Price $4.50.

Amino Acides Peptides Proteines. Cahier No. 6. Edited by MAURICE VIGNERON. AEC Societe de Chimie Organique et Biologique, 24, Avenue de l'Opera, Paris 1er, France, 1966. 457 pp. 21 × 26.7 cm.

Antibiotike-Fibel—Antibiotika und Chemotherapie. By M. PLEMPEL and H. OTTEN. Georg Thieme Verlag, 7 Stuttgart 1, P.O. Box 732, Gerdweg 63, Germany, 1969. xl + 994 pp. 15.5 × 23 cm. Price DM 98,-.

Behavior of Enzyme Systems. 2nd Edition. By JOHN M. REINER. Van Nostrand Reinhold Company, 450 West 33rd St., New York, NY 10001, 1969. xviii + 345 pp. 15.5 × 23.5 cm.

British National Formulary 1968. Published jointly by the British Medical Association and the Pharmaceutical Society of Great Britain, The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England, 1968. 384 pp. 10.7 × 17.2 cm. Price 13s.6d plus postage.

British Pharmaceutical Codex 1968. Edited by S. C. JOLLY. The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England, 1968. U. S. Distribution: Rittenhouse Book Store, Philadelphia, Pa. xxxvii + 1513 pp. 15 × 22.3 cm. Price $19.50.

Degradation of Herbicides. Edited by P. C. KEARNEY and D. D. KAUFMAN. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xi + 394 pp. 15.7 × 23.6 cm. Price $18.75.

Die Interkantonale Vereinbarung uber die Kontrolle der Heilmittel vom 16. Juni 1954. By FELIX WUST. Kommissionsverlag Herbert Lang & Cie AG Bern, Switzerland, 1969. 368 pp. 15.5 × 22.5 cm. Price 3 fr./Dm 52.—.

Index Nominum 1966, Supplementum 1968. Edited by Laboratory of the Swiss Pharmaceutical Society. Swiss Pharmaceutical Society, Marktgasse 52, 3011 Bern, Switzerland 1969. 120 pp. 17 × 24 cm. Price Sw. fr. 35.—plus postage.

Medicaments Organiques de Synthese. Vol. I. Edited by GUILLAUME VALETTE. Masson Et Cie, Editeurs, 120, Boulevard Saint-Germain, Paris VIe, France, 1969. 226 pp. 15.4 × 22 cm. Price 88 fr.

Methods of Biochemical Analysis. Vol. 17. Edited by DAVID GLICK. Interscience Publishers. Div. of John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10016, 1969. vi + 428 pp. 15.5 × 23.4 cm. Price $16.50.

Pharmaceutical Chemistry Part II. Inorganic Vol. 1. By M. L. SCHROFF. National Book Centre P-76, Dr. Sundari Mohan Ave., Calcutta, 14, India, 1968. v + 700 + xvi pp. 14.5 × 22.2 cm. Price $10.00.

Pharmacological Research Communications. Vol. I. No. I. The Italian Pharmacological Society. Academic Press Inc. (London) Ltd., Berkeley Square House, London WIX 6 BA, 1969. 126 pp. 16.8 × 24 cm. Price 84/-, $10.00 per 4 issues per annum.

Pharmacology for the Preoperative Visit. Vol. 6, No. 1. Edited by J. S. GRAVENSTEIN. Little, Brown and Company, 34 Beacon St., Boston, MA, 1969. xi + 396 pp. 15.5 × 23.5 cm. Price $22.00/yr. subscription rate.

Precis de Microbiologie. Vol. I. By S. LAMIN and A. GERMAN. Masson & Cie, Editeurs, 120, Boulevard Saint-Germain, Paris VIe, France, 1969. viii + 669 pp. 16.5 × 21.5 cm. Price 75 fr.

The Psychopharmacology of the Normal Human. Edited by WAYNE O. EVANS and NATHAN S. KLINE. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xi + 252 pp. 15.6 × 23.3 cm. Price $11.50.

Psychotropic Drug Response: Advances in Prediction. Edited by PHILIP R. A. MAY and J. R. WITTENBORN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xii + 292 pp. 16 × 23.4 cm. Price $12.75.

The Role of Learning in Psychotherapy. Edited by RUTH PORTER. Little, Brown and Company, 34 Beacon St., Boston, MA 02106, 1968. xii + 340 pp. 15.5 × 23.2 cm. Price $12.00.

Steroid Reaction Mechanisms. By D. N. KIRK and M. P. HARTSHORN. American Elsevier Publishing Co., Inc., 52 Vanderbilt Ave., New York, NY 10017, 1968. xi + 476 pp. 14 × 22 cm. Price $30.00.

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

FEBRUARY 1970
VOLUME 59 NUMBER 2

REVIEW ARTICLE

Pharmacological Testing Methods for Drugs Acting on the Peripheral Nervous System

J. P. LONG and C. Y. CHIOU*

Keyphrases ☐ Pharmacological test methods—drug activity, peripheral nervous system ☐ Nervous system, peripheral—test methods, drug effects ☐ Observational studies, animals—drug effect, peripheral nervous system ☐ *In vivo* studies—drug effects, peripheral nervous system ☐ *In vitro* studies—drug effects, peripheral nervous system

CONTENTS

The goal of pharmacological testing of chemical agents is (*a*) to detect any biological activity that may be present. Following demonstration of activity the next steps are (*b*) to identify the type of activity and then (*c*) to compare the activity with the activity of a reference compound. To progress through steps *a* and *b* requires much experience and selective judgment. For comparison of activity, data from the experiments must be quantified. There is now a large number of excellent statistical procedures and a biologist must design his experiments so that one of the standard statistical procedures can be followed—otherwise an experimenter will end up with an experience and not an experiment. No reference to structure-activity relationships or even whether biological activity is present can be made until dose-response curves have been constructed and evaluated.

The hazards of pharmacological evaluation are many. The biologist must be aware and attempt constantly to eliminate as many variables as possible. He will always have fundamental questions that may be difficult to answer. Some of the points that must be considered are:

1. Selection of type of preparation is important. Preparations that are used widely are usually prudent choices.

2. The problem of choice of species is always present. To evaluate a particular type of pharmacological activity, choose a species that is highly sensitive to the particular agent. For example, one would not use the blood pressure of the rat to evaluate histamine-like compounds, *etc.*

3. It is necessary to determine the time-effect curves before attempting to compare the activity of two chemical agents. Agents will vary in time required for onset and duration of action. The agent's activity must be compared at time of peak effect.

4. The absorption, distribution, and excretion of agents in a given series may vary greatly. These factors

may be extremely important in determining the intensity and duration of biological activity.

5. In structure-activity relationship comparisons, the above variables make it highly desirable to evaluate compounds both *in vivo* and *in vitro*. If the relative activity of compounds evaluated in the two types of preparations remains constant, this is suggestive evidence that the difference in biological activity is related to differences in chemical structure and not related to factors such as plasma protein binding, metabolism, excretion, *etc.*

6. Perhaps an area where erroneous assumptions are sometimes made is in assuming that two compounds have the same mechanism of action. Unless two compounds have the same mechanism of action, one cannot compare statistically their biological activity. For example, it has recently been shown that choline is apparently devoid of muscarinic activity.[1] The literature contains many citations of the relative muscarinic activity of choline and acetylcholine and these comparisons are probably invalid. As for references to "mechanisms of action," this term means different things to different investigators. To a physiologist this term may refer to interactions at the organ level. A pathologist may be interested in cell damage or structural changes. A biochemist may be concerned with enzyme inhibition or activation. A molecular biologist may be interested in drug-receptor interactions, *etc.* All of these investigators using the techniques of their respective disciplines feel that they are determining the "mechanism of action." Each is for his particular biological level of integration, but one should remember that the "mechanism of action" at the molecular level awaits explanation for probably all drugs.

7. In comparing drugs one should also remember that although the end responses may look similar, the sites of action may be different. For example there could be a sequence of events as follows:

$$\underset{\text{drug}_1}{\overset{E_1}{S_1 \rightarrow}} \; \underset{\text{drug}_2}{\overset{E_2}{S_2 \rightarrow}} \; \text{response } (R)$$

where S = substrate, E = enzyme. Drug 1 may act on E_1 and drug 2 on E_2. Alteration of R may appear to be the same but the sites of action that altered the response are different.

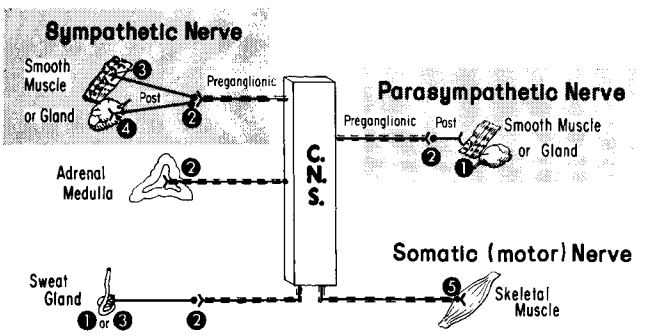


Figure 1—*A simplified diagram illustrating the sites of action of some standard drugs. The reference drugs that stimulate or inhibit at the various sites are listed in Table I.*

[1] When assayed using superfused guinea pig ilea, the intestinal stimulating action of choline is antagonized markedly by: (1) cooling the segment for 24 hr. at 2°, (2) treatment with triethylcholine, or (3) hexamethonium. None of these treatments alters the responsiveness of acetylcholine.

ANATOMY OF THE PERIPHERAL NERVOUS SYSTEM

Detailed outlines of the nervous system may be found in any standard physiology or pharmacology textbook. A simplified diagram illustrating the sites of action of some of the standard drugs is shown in Fig. 1 and Table I. Other anatomical points that should be considered in evaluating drug action are listed as follows:

1. Only postganglionic fibers are nonmyelinated.

2. Postganglionic sympathetic nerve fibers are usually long fibers with the ganglia outside the organs innervated. The ganglia and postganglionic parasympathetic nerve fibers usually lie within the organ innervated (the exceptions being the organs of the head and neck where the parasympathetic ganglia are on the surface or slightly removed from the organ that is innervated).

3. Various sympathetic fibers tend to be activated in unison. The activity of the parasympathetic nervous system tends to be much more localized—often serving as the efferent component of a reflex. For example, in a bright light miosis will occur, but the heart rate will not be slowed.

4. Acetylcholinesterase is found in high concentration where acetylcholine is the neurotransmitter. Inhibition of this enzyme with neostigmine or analogs or the organic phosphates will prolong the integrity of the acetylcholine molecule and increase its concentration at the transmitter site. The adrenergic enzymes, catechol-*O*-methyltransferase and monoamine oxidase, probably play a minor role in terminating the action of norepinephrine that has been liberated from the nerve terminal. Diffusion of norepinephrine from the receptor site appears to be the major terminating mechanism.

5. During the past 20 years evolution of the alpha and beta receptor theory has been seen along with the development of the respective stimulating and inhibiting agents. The major organs with their predominant type of receptor innervation are shown below:

Alpha Receptor	*Beta Receptor*
Vasoconstriction (cutaneous, renal, *etc.*)	Vasodilation (sk. muscle, *etc.*)
Myocardial ectopic excitation	Cardioacceleration
Splenic-capsule contraction	Myocardial augmentation
Myometrial contraction (rabbit, dog, human, *etc.*)	Myometrial relaxation (rat, nonpregnant cat, human)
Iris dilator contraction (mydriasis)	Bronchial relaxation
Nictitating membrane contraction	Intestinal relaxation
Intestinal relaxation	
Pilomotor contraction	
Glycogenolysis (?)	

6. The influence of drugs on the cholinergic nerve terminal has not been widely explored, but the future will probably see a marked increase of research activity. Evidence is appearing that many "nicotinic" agents may be acting by releasing acetylcholine from preganglionic or motor nerve terminals. These would be agents such as nicotine or choline. A number of agents have been reported that will inhibit or block the cholinergic nerve

Table I—Stimulants and Inhibitors at Various Sites in the Peripheral Nervous System

Site	Name	Neurotransmitter	Stimulants	Inhibitors
1	Postganglionic parasympathetic	Acetylcholine	Acetylcholine Methacholine Carbachol Neostigmine DFP	Atropine Scopolamine
2	Ganglionic	Acetylcholine	Acetylcholine Nicotine Tetramethylammonium Neostigmine	Hexamethonium (C_6) Nicotine (large concn.)
3	Postganglionic sympathetic	Norepinephrine	Norepinephrine (α) Epinephrine (α or β) Isoproterenol (β)	Phentolamine (α) Propranolol (β)
4	Sympathetic nerve terminal		Tyramine Ephedrine Cocaine	Reserpine Guanethidine Brethylium
5	Neuromuscular junction	Acetylcholine	Acetylcholine Nicotine	Curare Succinylcholine

terminal. These agents would include high Mg^{++} concentrations, hemicholinium (HC-3), triethylcholine, hexamethonium, or tetrodotoxin (puffer fish poison).

Since a drug or experimental chemical can only alter (stimulate or inhibit) the basal activity of the peripheral nervous system, one must understand the physiology of transmission. With this information one should have no difficulty in classifying autonomic agents.

The major influence of the autonomic nervous system innervation on the major organ system is outlined in Table II.

TERMINOLOGY

Often the same site of drug action is referred to by different names. For a large surplus of descriptive names, the autonomic drugs probably have a longer list than other classes of agents. The long list of synonyms used to describe autonomic drugs make the literature difficult to read for a nonpharmacologist. Table III lists those terms used commonly in the literature to describe actions of agents on the peripheral nervous system.

METHODS

The following portion of the manuscript discusses some of the procedures used to study the activity of chemical agents on the peripheral nervous system. The authors have tried to select preparations that are used quite widely and those with which they have had personal experience. Page space makes it necessary for the user of these preparations to refer to the original manuscripts for detailed procedures. All individuals interested in drug development must be familiar with a wide variety of preparations. One must clearly understand the uses and limitations of each preparation. In order to evaluate and explore new mechanisms, a biologist may need to develop his own special experimental procedures. The science of methodology is probably the pharmacologist's major tool that will lead to the discovery of new types of biologically active agents.

Observational Studies of Animals

Available methods for detecting drug actions in the intact unanesthetized animal are convenient and valuable but are less reliable and nonspecific in the analysis of individual drug effects. They can be used only for preliminary confirmation of drugs affecting the peripheral nervous system. Therefore, no attempt will be made to describe or to discuss these methods in detail. For definitive studies on the sites of drug actions, the *in vivo* and *in vitro* methods described in this paper should be employed.

Pupil Size—Mice are widely used in this experiment but dogs, cats, and rabbits can also be utilized. The pupil size is controlled primarily by tonic activity of the cholinergic nervous system. An increase in cholinergic activity or a blockade of adrenergic activity produces

Table II—Usual Responses Seen with Autonomic Function Alteration (Stimulation or Inhibition)[a]

Organ	Sympathetic: Stimulation	Sympathetic: Inhibition	Parasympathetic: Stimulation	Parasympathetic: Inhibition
Heart				
Rate	↑	↓ or 0	↓[b]	↑ or 0
Force	↑	↓ or 0	↓[b]	↑ or 0
Arterioles	↑	↓	—[c]	—
Veins	↑	↓	—[d]	—
Blood pressure	↑	↓ or 0	↓[e]	↑ or 0
Eye				
Miosis (constriction)	↓	↑	↑	↓
Mydriasis (dilatation)	↑	↓	↓	↑
Intraocular pressure	↓	0	↓	↑
Bronchi	↓	↑ or 0	↑	↓ or 0
Salivary glands	↑[f]	0	↑[g]	↓
Gastrointestinal tone	↓	↑ or 0	↑	↓ or 0
Sweat glands	↑	↓ or 0	↑	↓

[a] ↑, increased, stimulated; ↓, decreased, inhibited; 0, no change; —, not relevant. [b] Acetylcholine in the presence of atropine will produce a positive inotropic and chronotropic action. [c] Though there are no parasympathetic nerve fibers to arterioles, acetylcholine may be a powerful dilator (for example the limbs or the vascular beds of skeletal muscle). In some vascular beds ACh will induce constriction—aortic strips, rabbit ear, renal artery. [d] The cephalic vein is constricted by large doses of ACh. [e] Large doses of ACh administered intravenously will produce a pressor response. This response will be enhanced by prior treatment with atropine. [f] Small volume, high viscosity. [g] Large volume, low viscosity.

Table III—Synonymous or Related Terms Used in Autonomics

Site	Stimulant	Blocking Agent
Ganglia (sympathetic & parasympathetic)	Ganglionic stimulant Nicotinic Preganglionic stimulant	Ganglionic blocking agent Ganglionic depressant Preganglionic depressant
Postganglionic parasympathetic neuroeffector junction	Postganglionic parasympathetic stimulant Cholinergic Muscarinic Parasympathetic stimulant Parasympathomimetic Cholinomimetic	Postganglionic parasympathetic blocking agent Anticholinergic Antimuscarinic Parasympathetic blocking agent Parasympatholytic Cholinolytic
Postganglionic sympathetic neuroeffector junction	Postganglionic sympathetic stimulant Adrenergic stimulant Sympathetic stimulant Sympathomimetic Releasing agents	Postganglionic sympathetic blocking agent Adrenergic blocking agent Sympathetic blocking agent Sympatholytic Antirelease agents Depleting agents "False transmitters" Myoneural blocking agents
Myoneural junction neuromuscular junction	Myoneural stimulants Nicotinics	Myoneural depressants Neuromuscular blocking agents Relaxants Muscle relaxants

miosis, whereas an increase in adrenergic activity or a blockade of cholinergic activity produces mydriasis. The pupil size of mice can be measured with a dissecting microscope *via* a calibrated eyepiece (1–3). The drugs are administered parenterally (3) or topically on the cornea (1, 2).

Tone of the Nictitating Membrane—The position of the nictitating membrane is controlled primarily by the tonic activity of the adrenergic nervous system. Adrenergic agents cause contraction of this membrane while adrenolytic agents produce relaxation. Although cats are most satisfactory in this test, dogs and rabbits may also be used (4).

Respiratory Arrest—Subsequent to drug administration, respiratory arrest prior to cardiac arrest indicates that either the respiratory center is inhibited or that the neuromuscular junction of the phrenic nerve diaphragm is blocked. Mice can be used satisfactorily for this test (5). Central and peripheral effects can be differentiated by using isolated preparations.

Blood Pressure and Heart Rate—The overall peripheral vascular resistance is regulated by the adrenergic nervous system which can be blocked by adrenolytic agents or physiologically antagonized by cholinergic agents to produce hypotension. The heart rate is controlled primarily by the cholinergic mechanism which can be blocked by cholinolytic agents or physiologically antagonized by adrenergic agents to induce tachycardia. The indirect methods for blood pressure determination in dogs are described by Prioli and Winbury (6) and those in rats are reviewed by Van Proosdij-Hartzema (7) and Boura and Green (8). The advantages and disadvantages of these methods are discussed by Fregly (9).

In Vivo Study

Dog Blood Pressure Preparations—Mongrel dogs are anesthetized with 15 mg./kg. of thiopental sodium and 250 mg./kg. of barbital sodium administered intravenously. The trachea is cannulated and the vagi are sectioned to eliminate the vagal reflexes. The arterial pressure is measured with a pressure transducer (10–13) or mercury manometer (11, 14). The blood pressure can be measured at the common carotid artery, the femoral artery, or any other large artery. All compounds are injected *via* a polyethylene catheter inserted into the femoral vein. The injection is followed immediately by a wash of isotonic saline. In all cases the total volume of the injected solutions is kept constant. However, drugs can be injected directly into the femoral vein without using the polyethylene catheter. In this case, the isotonic saline wash is not necessary. The species of animal, the routes of administration, the procedures of cannulation, and the methods of recording may all be varied to meet the needs of the investigator. This preparation can also be done with rats, cats, and rabbits. Depressor responses that are blocked by atropine sulfate are regarded as muscarinic and pressor reponses after 1–2 mg./kg. of atropine sulfate are regarded as nicotinic. However, due to the multiple factors affecting the mean systemic pressure, interpretation of the pressure change is difficult. If the blood flow in the large vessels is recorded by use of an electromagnetic flowmeter (4) simultaneously with the pressure change, then the interpretation of the experimental results is facilitated.

The catecholamines released by ganglionic stimulants can be estimated by collecting blood samples at the time of peak pressor response. Blood cells and protein in the blood samples are removed (15) and the catecholamines in the final samples are assayed fluorometrically using the method described by Chang (16). The sites of catecholamine release, either from the adrenal medulla (mainly epinephrine release) or from sympathetic nerve terminals (mainly norepinephrine release), have thus been differentiated (14).

The blood pressure preparation is widely used to determine dose-response curves and activities of drugs (10–12, 14). The eviscerated cat blood pressure preparation is quite sensitive to acetylcholine and can readily detect 0.002 mcg. ACh/kg. (17). The rat preparation is also widely used for bioassay of minute amounts of acetylcholine and the analogs from tissue extracts (11, 18, 19).

Recently more attention has been given to the direct measurement of blood pressure using unanesthetized animals. This preparation enables investigators to study the drugs with a very long duration, to repeat measurements of blood pressure over a long period of time, and to avoid the untoward interaction between anesthetics and drugs to be studied. The advantages and disadvantages of both preparations have been discussed by Freyburger (20). For dogs, the polyethylene catheters are implanted chronically in various parts of the circulation (21), mostly into thoracic aorta (22, 23) and the femoral artery (20). The transcutaneous needle puncture of a femoral artery immediately before experimenting is also used and is the most simple method to be performed with satisfactory results. Hypodermic needles (19–21 gauge) are widely used to obtain full pulse pressure measurements. However, smaller needles are recommended to avoid vessel hardening and hematomas (20, 24). The polyethylene catheters or hypodermic needles are connected to the pressure transducer for recording the blood pressure described previously. The direct measurements of blood pressure on unanesthetized rats are described by Weeks and Jones (25) and Fujita and Tedeschi (26).

Cat Superior Cervical Ganglia Preparation—Cats are anesthetized with 30–35 mg./kg. of pentobarbital sodium administered intrathoracicly. The trachea is cannulated as usual and the carotid artery is exposed. The lymphatic gland is removed and all the arterial branches, except those running to the tissue around the superior cervical ganglion, are tied and divided. The small internal carotid artery and veins are divided, leaving only the vein from which perfusion fluid is to be collected, usually the internal jugular vein. A length of cervical sympathetic nerve, sufficient for placement of the electrodes, is freed and the cat's head is then fixed in position so that the contraction of the nictitating membrane can be recorded. The common carotid artery is then ligated and perfused. A small cannula is then tied into the internal jugular vein to collect the perfusion fluid. The electrodes are placed on pre- and postganglionic fibers (27–29). In order to prevent blood coagulation during manipulation, 0.5 ml./kg. of 8% solution of chlorazal fast pink or 5 mg./kg. of heparin sodium is injected intravenously (30). The nerve is stimulated with monophasic pulses, 6 msec. in duration, with maximal voltage of 10–15 v. and frequencies of 10–25 c.p.s. (31). A viable preparation will respond to single shock stimulation. This is an extremely useful preparation for detecting the release of acetylcholine from ganglia, either by preganglionic nerve stimulation (32, 33) or by close arterial injection of the drugs (34, 35). The effects of drug and nerve stimulation can be detected from the responses of nictitating membrane. An ingenious experiment was devised by Collier *et al.* (36) in which the ACh in the ganglia was replaced with ^{3}H-ACh through perfusion of the ganglia with Locke solution containing ^{3}H-choline during continuous preganglionic stimulation. Both preganglionic stimulation and close arterial injection of carbachol released radioactive materials and contracted the nictitating membrane, indicating that endogenous ACh was released during these treatments. The close arterial injection of ACh, however, contracted the nictitating membrane without releasing appreciable amounts of radioactive substance. This suggests that ACh might not be involved in the release of endogenous ACh from the preganglionic site.

Dog Chorda Tympani–Wharton's Duct Preparation—Dogs weighing 12 kg. or more are anesthetized with 15 mg./kg. of thiopental sodium and 250 mg./kg. of the barbital sodium administered intravenously. The trachea is cannulated. Wharton's ducts are located directly beneath the mylohyoideus and extend across the lingual nerve between the chorda tympani nerve and the submandibular ganglion. Both Wharton's ducts and the chorda tympani nerve are ligated. The chorda tympani nerve is cut free from the lingual nerve with a thread attached to the nerve end. One of the Wharton's ducts is then cannulated with polyethylene tubing (PE-60) which is attached to a reservoir filled with physiological saline. The outflow of the reservoir is connected to a dropping tube and a Becker signal magnet is connected to a relay which is activated by each drop. A shielded silver electrode is attached to the chorda tympani nerve for stimulation. The nerve is stimulated supramaximally with 15 v. for a duration of 2 msec. with a single biphasic impulse and with a frequency of 20 c.p.s. for 1 sec. every 10 sec. (37). After 10–20 min. the saliva flow will remain quite constant for at least 90 min.

Salivation from the Wharton's duct can be stimulated either by drugs such as pilocarpine, acetyl-β-methylcholine, and acetylcholine injected intravenously into the femoral vein or by nerve stimulation. The salivation is effectively blocked by atropine. This preparation is useful for studying drugs which act on or block the cholinergic system causing salivation. Hemicholinium has been shown to block gradually the salivation induced by chorda tympani nerve stimulation, suggesting that its blocking effect is due to the inhibition of ACh synthesis in the nerve tissues (37).

Rabbit Sciatic Nerve–Gastrocnemius Muscle Preparation—Dutch rabbits, weighing 1–2 kg., are anesthetized with 200 mg./kg. of phenobarbital sodium administered slowly (at least 2 min.) into the marginal ear vein. The sciatic nerve is ligated and cut, and a shielded electrode is placed on the peripheral portion of the nerve. The gastrocnemius muscle is freed as completely as possible from surrounding muscles and a thread is attached to the tendon of the muscle. The twitches of the muscle are elicited by supramaximal stimulation and are recorded through a force transducer. The parameters for interrupted tetanic stimulation are 250 c.p.s. with pulse duration of 1 msec. at 15 v. applied for 0.2 sec. every 10 sec. Single shock stimulation is performed with pulse duration of 5 msec. and a supramaximal voltage of 15 v. is applied every 10 sec. The drugs are administered intravenously into the marginal ear vein. This is a convenient and simple preparation for studying drugs acting at the neuromuscular junction *in vivo* (37–41). Although there are some *in vitro* neuromuscular preparations available, this preparation has the advantage of studying the drug effects under physiological conditions. For example, hemicholinium and its derivatives have been shown to block neuromuscular transmission in all preparations. In this preparation, blockade can be efficiently reversed by choline (39–41)

but this is difficult using *in vitro* preparations. Animals other than rabbits, such as cats and rats, can be used satisfactorily.

There are two other preparations, the cat soleus muscle preparation and the cat tibialis anticus muscle preparation which are very useful and similar to the gastrocnemius muscle preparation. These can be prepared in a method similar to that described for gastrocnemius muscle except that close arterial injection can be made using the tibialis anticus preparation. Also, soleus muscle consists primarily of slow muscle, whereas tibialis anticus is fast muscle (42). The experimental procedures for these two preparations are described by some investigators in detail (42–44). The characteristics of slow and fast muscles are analyzed anatomically, histochemically and biochemically by some investigators. The fast muscle is composed of three distinct fibers (45) whereas the slow fiber is relatively homogeneous, consisting primarily of Type B fibers (46). Also, the fast muscle contains higher activities of glycolytic enzymes and lower activities of oxidative enzymes, while the reverse is true for the slow muscle (47, 48). The effects of drugs on slow and fast muscles will be discussed further in the following sections.

In Vitro Study

The organ bath method is the most widely used technique for studying isolated organs. The equipment is rather simple and is available commercially. Usually, the organ bath is made of glass and placed inside a water bath so that the temperature can be well controlled. The physiological salt solution is warmed before it is added to the organ bath, so that there will be no temperature change when the tissue is washed. The physiological salt solution can be warmed either by putting the reservoir bottle in a water bath or by passing the physiological solution through coils connected to the organ bath within the same water bath. The isolated tissue is mounted in such a way that one end of the tissue is fixed at the bottom of the organ bath while the other is fixed to the recording lever or a force transducer for isotonic or isometric recording. The organ bath varies both in size and volume depending on the specific purposes and the size of tissues. The volume of the drug solutions to be added to the organ bath should be less than 10% of the total volume of the bath fluid to avoid possible decrease of fluid temperature in the organ bath.

The superfusion method was first described by Finkleman (49) and modified by Gaddum (50). It is more rapid and more sensitive than the organ bath method for bioassay. This technique reduces the volume of solution to an absolute minimum by replacing the organ bath with a slow flow of bathing fluid over the external surface of the tissue. Consequently, the total amount of drug required to induce a response in the tissue is very small. Recently, this technique has been used successfully to study the fate and release of vasoactive hormones in the circulation (51–53). In this application the heparinized blood was continuously removed from the anesthetized animal, assayed for its hormone content by superfusion over a series of isolated muscle preparations, and then returned to the animal body intravenously.

For superfusion, the isolated tissue is mounted in air in the middle of a wide glass tube immersed in a warm water bath. The physiological salt solution is oxygenated and kept in a bottle immersed in a warm water bath. It is then pumped through a Holter motor pump to the top of the isolated tissue. The rate of flow of the superfusion fluid is adjusted to 1–5 ml./min. depending on the size of the tissues; the drug solutions are injected into the stream of the superfusion fluid in volumes of not more than 0.1 ml. Of course drugs may be added directly to the perfusing solution. Isometric recordings are preferred to isotonic recordings because the changes in the length of the tissue may dislocate the superfusion fluid in some cases and cause dryness of part of the tissue.

Skeletal Muscle Preparations—*Frog Rectus Abdominis Muscle Preparation*—The frog rectus abdominis muscle is usually obtained from *Rana pipiens* weighing approximately 20 g. The frog is stunned and decapitated. The spinal cord is destroyed with a long needle. The rectus abdominis muscles are dissected from the pelvic girdle to their insertion in the cartilage of the pectoral girdle. The threads are attached to both ends of the muscle before they are dissected from the body. An initial tension of 0.5–1.0 g. is placed on the rectus muscle. The preparation is kept at room temperature (25°) in frog Ringer solution (NaCl, 6.5; KCl, 0.14; NaH_2PO_4, 0.005; glucose, 2.0; $NaHCO_3$, 0.4; and $CaCl_2$, 0.12 g./l.) and oxygenated with 95% O_2 and 5% CO_2. At least 30 min. is allowed for the preparation to stabilize. This preparation was orginally described by Burn (54). Both the organ bath method (5–10 ml.) (54–56) and the superfusion technique (10, 39) can be used to perform experiments. The frog Ringer solution can also be made by diluting 1 l. of Ringer solution to 1.4 l. with distilled water.

The rectus abdominis muscle preparation is extremely useful because it develops slow contracture in responding to acetylcholine. This contracture is blocked by curare and its derivatives. Depolarizing agents, such as decamethonium or succinylcholine, will induce contracture that is similar to acetylcholine. Although the semispinalis muscle of the chick has the same useful properties, the simplicity and economy of the rectus preparation are widely appreciated by many investigators.

After contraction, the rectus muscle does not relax rapidly. It is, therefore, difficult to obtain the straight base line which is essential for bioassay. Thus, in order to obtain this straight base line, the muscle must be stretched gently by increasing the tension. This disadvantage can be eliminated by using isometric recording instead of isotonic recording. Also since the rectus muscle does not relax rapidly, it takes at least 6-min. intervals between doses in the organ bath method to assay acetylcholine. The superfusion technique is considerably faster, since it requires only 1–2-min. intervals between doses.

The rectus muscle preparation can also be used for assaying acetylcholine and its derivatives, although its sensitivity is less than that of the guinea pig ileum, chick semispinalis muscle, and leech dorsal muscle treated with physostigmine. The sensitivity of

the preparation can be increased markedly by using the superfusion technique instead of the organ bath method and by treating the rectus muscle with physostigmine or neostigmine. The frog rectus muscle preparation, treated with 1.5×10^{-6} mole of physostigmine, can detect 0.01 mcg./ml. of acetylcholine chloride (1×10^{-7} mole) in the organ bath method (55) or 0.01 mcg. of acetylcholine chloride per injection in the superfusion method (10, 39).

Leech Dorsal Muscle Preparation—The Leech, *Hirudo medicinalis*, is pinned on its back through the mouth and the tail sucker and a cut is made along the two pale lateral lines to get two parallel strips from the dorsal body wall. The threads are attached at both ends of each piece. The muscle is suspended at room temperature (25°) in frog Ringer solution, or in leech Locke solution. These solutions are made by diluting 1 l. of Ringer solution or Locke solution (NaCl, 9.0; KCl, 0.42; glucose, 1.0; $NaHCO_3$, 0.5; and $CaCl_2$, 0.12 g./l.) to 1.4 l. with distilled water. The solution is oxygenated with 95% O_2 and 5% CO_2. An organ bath with a capacity of 5–10 ml. is used. The preparation must be stabilized for about 3 hr. before an experiment is begun. The contraction responses are very slow. However, unlike the frog rectus muscle, it should not be stretched by increasing the tension. Consequently, the responses are slow and it is necessary to allow at least 20 min. between doses (56). Physostigmine (3×10^{-5} mole) is preferred to neostigmine (3×10^{-4} mole) in order to sensitize this preparation (57). The muscle can be sensitized about 1000-fold to acetylcholine responses by physostigmine. This preparation is highly sensitive and is suitable for detection of acetylcholine (less than 10^{-8} mole or 0.001 mcg./ml.) released from nerve tissue (58–60). However it is not suitable for studying the pharmacology of drugs because of the length of response time involved. It is much better to choose the guinea pig ileum, chick semispinalis muscle, or frog rectus abdominis muscle for studying the pharmacology of the drugs since a larger quantity of results can be obtained in a shorter period of time.

Chick Semispinalis Cervicis Muscle Preparation—A chick, 2–3 weeks old, is sacrificed with chloroform. A longitudinal incision is made in the skin at the back of the neck from the base of the skull to the region of the thoracic vertebrae. The two biventer cervicis muscles can be seen on either side of the midline just underneath the surface skin. Those located beneath the biventer cervicis muscles are the two semispinalis cervicis muscles. The semispinalis cervicis muscle is dissected away from the surrounding muscles and the threads are tied around each end of the preparation. The muscle can be mounted either in an organ bath (5–10 ml.) (61–63) or on a superfusion apparatus (64). An initial tension of 1 g. is placed on the tissue. Tyrode solution (NaCl, 8.0; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.26; NaH_2PO_4, 0.05; glucose, 1.0; $NaHCO_3$, 1.0; and $CaCl_2$, 0.2 g./l.) at 40°, oxygenated with 95% O_2 and 5% CO_2, is used for the bathing fluid.

Like the frog rectus abdominis muscle, this preparation produces a slow contracture in response to acetylcholine and acetylcholine-like substances. Because it is more sensitive to drugs mimicking acetylcholine and because the muscle contracts and relaxes more rapidly this preparation is more advantageous than the frog rectus abdominis preparation (61, 62). Consequently, artificial stretching of the muscle is not required. This preparation is capable of detecting 0.004 mcg./ml. of acetylcholine chloride (2.4×10^{-8} mole). Edrophonium chloride is preferred to physostigmine, neostigmine, diisopropylfluorophosphate, and tetraethylpyrophosphate for inhibition of the cholinesterases in the tissue because these anticholinesterases either produce too little increase in sensitivity or cause a partial contracture of the muscle. Edrophonium chloride (final concentration of 8 mcg./ml. or 4×10^{-5} mole) is injected into the organ bath before each addition of acetylcholine. It is not included in the Tyrode solution, since, if used in this way, it will cause long lasting contracture even at extremely low concentrations (62).

Rat Phrenic Nerve–Diaphragm Preparation—A rat is decapitated and the blood is drained. The skin is removed from the middle of the chest. The thorax is opened and the front part of the left thoracic wall is removed. The phrenic nerve can be seen quite distinctly. The nerve is cut just below the thymus and a thread is attached to the cut end. The nerve is then freed carefully from the attached tissue. However, no attempt is made to clean the nerve completely from the tissues attached to it. An incision is made in the left abdominal wall just below the diaphragm. Two converging cuts are made through the diaphragm and the ribs towards the tendinous part of the diaphragm with the phrenic nerve attached to the center of the diaphragm. The fan-like preparation is about 3 mm. wide at the tendinous end and is about 15 mm. wide at the costal margin. A thread is attached to the tendinous part of the diaphragm. The preparation is fixed by a stainless steel rod with a pair of pins hooked on the rib. It is lowered into the organ bath and the thread from the muscle is attached to the writing lever or the force transducer. The nerve is stimulated with a pair of electrodes with a hole about 1 mm. wide. The right phrenic nerve–diaphragm preparation is isolated in the same manner. The organ bath should be 30–40 mm. in diameter and 30–40 ml. in capacity. Tyrode solution at 37° containing double the amount of dextrose or Krebs solution (NaCl, 6.9; KCl, 0.35; $MgSO_4 \cdot 7H_2O$, 0.14; KH_2PO_4, 0.16; glucose, 2.0; $NaHCO_3$, 2.1; and $CaCl_2$, 0.28 g./l.) is used as bathing fluid. The solution should be well oxygenated with 95% O_2 and 5% CO_2. The nerve is usually stimulated about 12 times per minute by rectangular-wave pulses of about 0.5-msec. duration at 3–5 v.

This preparation was originally described by Bülbring (65) and was modified and widely used thereafter by many investigators for studying drugs affecting the neuromuscular transmission (55, 66–68). The drugs are left in the organ bath either for quite short periods of time (3–8 min.) or for as long as the maximum effect can be observed. This preparation is composed mainly of fast muscle and is excellent for determining the drug potency to block or facilitate neuromuscular transmission. However, it is not a good preparation for differentiating between depolarizing and nondepolarizing neuromuscular blocking agents because, in many cases, depolarizing blocking agents fail to demonstrate initial facilitation and fail to reverse the effect of nondepolarizing blocking agents. For differentiating between depolarizing and nondepolarizing blocking agents the

chick biventer cervicis nerve muscle preparation and the chick sciatic nerve–tibialis anticus muscle preparations are preferred.

Chick Biventer Cervicis Nerve Muscle Preparation—This preparation is isolated by the same method described in the section on the chick semispinalis cervicis muscle. A loop is tied around the caudal belly of the muscle and hooked on the bottom of the electrode assembly. The oral end of the tendon is passed through the electrode and attached to a lever or an isometric force transducer. The whole assembly is placed in the organ bath (63). Superfusion technique has been applied to this preparation by Chiou and Long (64). The electrode assembly is similar to the one used in the organ bath method described by Ginsborg and Warriner (63) except that a small cup with a hole for superfusion is attached above the electrode. The small cup over the electrode is essential for superfusion of the biventer cervicis nerve muscle preparation because it eliminates the mechanical stimuli of the drops of superfusion fluid on the motor nerve, which cause irregular twitching of the muscle (64).

Tyrode or Krebs solution, at 37°, is used as bathing fluid and oxygenated with 95% O_2 and 5% CO_2. The initial tension placed on the muscle is 1 g. The organ bath is similar to that used for the rat phrenic nerve–diaphragm preparation. The nerve is stimulated about 6–12 times per minute by rectangular-wave pulses of about 0.5-msec. duration at 100 v. Supramaximal interrupted tetanic stimulation can also be applied (64). This preparation is extremely useful for easily differentiating between depolarizing and nondepolarizing neuromuscular blocking agents because the former agents contract slow fiber while the responses of fast fiber to the nerve stimuli are blocked, whereas the latter agents block the responses of fast fiber to the nerve stimuli without disturbing the tonus of the slow fiber. In addition, this preparation has been used successfully to demonstrate the acetylcholine releasing effects of some nicotinic agents which were originally believed to act directly at the acetylcholine receptors on the muscle membrane (64). The sensitivity of this preparation to acetylcholine and nicotinic agents can be increased markedly with 7×10^{-7} mole of physostigmine. Neostigmine is not a good agent to be used since it causes long-lasting contracture with concentrations at 1.5×10^{-6} mole or higher.

Chick Sciatic Nerve–Tibialis Anticus Muscle Preparation—Chicks, 3–8 days old, are decapitated and the skin of the legs is rapidly removed. The leg is removed from the body by cutting through the hip joint, and is suspended in a Petri dish containing Krebs-Henseleit solution equilibrated with 95% O_2 and 5% CO_2 at 4°. The muscles of the thigh are dissected and the sciatic nerve with the superficial peroneal branch is freed from the upper leg tissue. The fascia is removed from the lower leg and the tibialis anticus tendon identified. A thread is attached to the tendon of the muscle and the tibialis anticus muscle is freed towards but not up to the knee joint attachment where the nerve enters into this region. The upper and lower leg bones are then cut off leaving the muscle with its nerve attached to the knee joint. The tendon is fastened by the hook to a ring in the bottom of the organ bath. A thin steel rod is attached to the knee joint by means of a pointed clamp, partially driven into the bone. The contraction of the muscle is recorded isometrically. The nerve is passed through an electrode similar to the one used in the rat phrenic nerve–diaphragm or the chick biventer cervicis nerve muscle preparations. The nerve is stimulated six times per minute for 0.5-msec. duration at supramaximal voltage. The organ bath is filled with 20 ml. Krebs-Henseleit fluid, at 37°, and is oxygenated with 95% O_2 and 5% CO_2.

This preparation was originally described by Van Riezen (69, 70) and is similar to the chick biventer cervicis nerve muscle preparation. It is a simple and useful preparation for measuring drug potency and for classifying the neuromuscular blocking agents into depolarizing and nondepolarizing agents. The curare-like drugs produce a neuromuscular blockade whereas the decamethonium-like drugs induce a contracture of the slow fiber when the neuromuscular transmission is blocked.

Smooth Muscle Preparations—*Guinea Pig Ileum Preparation*—Guinea pigs, weighing 300–500 g., are stunned by a blow on the head. The abdomen is opened, the cecum is lifted forward and the ileum which joins to the back portion of the cecum is identified. The terminal portion of the ileum is excised and placed in a dish containing Tyrode soluton. The mesentery is trimmed away and pieces are cut from the length of ileum as required. Threads are tied around both ends of the ileum. Both the organ bath method (5–10 ml. capacity) (55, 56, 71–73) and the superfusion technique can be used satisfactorily (10, 49, 74, 75). Tyrode solution at 37°, oxygenated with 95% O_2 and 5% CO_2, is used. This is a simple, sensitive, and accurate preparation for the detection of acetylcholine (0.0025 mcg./ml. or 1.5×10^{-8} mole with organ bath method and 0.0025 mcg./injection with superfusion technique). Occasionally the spontaneous contraction of the ileum makes it difficult to establish a straight base line which is essential for bioassay. Antihistamines (71) or morphine (72, 73) may be added to the Tyrode solution to eliminate these spontaneous contractions. The longitudinal smooth muscle of the guinea pig ileum is preferred to the whole ileum in this respect because it produces very little if any spontaneous contractions (55). Three minutes should be allowed between doses in the organ bath method and 1 min. between doses in the superfusion technique.

A superfused cooled guinea pig ileum preparation has been developed particularly for biosassay of endogenous acetylcholine released by acetylcholine releasers (74, 75).

Longitudinal Smooth Muscle of Guinea Pig Ileum—The guinea pig ileum, up to 15.24 cm. (6 in.) in length, is excised according to the method described in the section on the guinea pig ileum preparation. The ileum is gently freed of excess mesentery and pulled over a glass rod immersed in Tyrode solution in a large shallow container at room temperature (25°). Dissection of the longitudinal muscle layer is performed by making the initial incision along the site of attachment of the mesentery. The mesenteric remnant is held with forceps to minimize crush damage and the longitudinal muscle is separated from the underlying structure by gentle tension with forceps in a direction parallel to the underlying circular muscle fiber (55, 76–80). A piece of longitudinal smooth muscle, about 2 cm. in length, tied at both ends,

is placed in an organ bath with a capacity of 1–5 ml. or in a superfusion assembly. The Tyrode solution, at 32°, is oxygenated with 95% O_2 and 5% CO_2. The preparation shows minimum spontaneous contractions when the Tyrode solution is kept at 32° (55, 81).

This preparation is preferred to the whole ileum for the following reasons (55): (*a*) The preparation consists of fairly pure longitudinal smooth muscle (about 82%) and the amount of noncontractile tissues such as nervous elements, serosa, *etc.*, is small compared to that of the whole ileum. (*b*) The preparation is very thin so that the drugs can easily contact the cell membrane and can easily be washed away. Therefore, the preparation lasts longer and gives more consistent responses to the drugs. (*c*) The preparation shows little, if any, spontaneous contraction, whereas spontaneous contraction of the whole ileum makes it difficult to establish a uniform base line. (*d*) The preparation is much smaller than the whole ileum so that a much smaller organ bath can be used to perform the experiment. The capacity of 1 ml. has been used in this preparation, whereas 5 ml. is the minimum capacity for whole ileum preparation. The sensitivity of this preparation to acetylcholine is about the same as that of whole ileum preparation.

Peristalsis Movement of Guinea Pig Ileum—A piece of guinea pig ileum, about 4–6 cm. in length, is suspended in an organ bath with a capacity of 50 ml. The oral end of the ileum is tied to the short end of a "J" tube while the caudal end is attached to an inverted "U" tube which is fitted with a valve made from flat drainage tubing. The inverted U tube is suspended from a lever or a force transducer which records the longitudinal contractions. The long limb of the J tube is used to transfer the pressure changes in the intestinal lumen by air transmission to a float recorder. Tyrode solution from a Marriotte bottle enters the intestinal lumen through a polyethylene tube connected to the short end of the J tube. The peristaltic reflex is initiated by raising the pressure in the lumen by 2–3 cm. of Tyrode solution, at 37°, which is oxygenated with 95% O_2 and 5% CO_2.

This preparation was described by Bülbring *et al.* in detail (82). It can be used to study the intestinal peristalsis, longitudinal contractions, intraluminal pressure, and volume of fluid expelled. The peristaltic movements can be inhibited effectively by catecholamines. The doses of catecholamines required for complete inhibition of peristalsis are: 0.1 mcg./ml. of epinephrine HCl, 0.5 mcg./ml. of norepinephrine HCl, and 30 mcg./ml. of *dl*-isoproterenol sulfate. The inhibitory effect of the catecholamines on the peristaltic movement can be reversed by the adrenergic blocking agents (83). The drugs can be applied to the inside of the intestine as well as to the outside. This preparation can also be used to test local anesthetics, ganglionic blocking agents, and atropine-like compounds (84–86). However, the sensitivity of the tissue fluctuates considerably. Thus, it is more suitable for qualitative than quantitative work. This method is modified from those described by Bülbring and Lin (87), and Trendelenburg (88).

Coaxial Stimulation of Guinea Pig Ileum—A loop of guinea pig ileum is suspended in Krebs solution, at 37°, and oxygenated with 95% O_2 and 5% CO_2. The lower end of the intestine is tied to glass tubing into which an electrode protrudes so that the electrode can move freely with the movements of the intestine. The upper end of the ileum is tied to a fine polyethylene tubing which encloses the upper part of the platinum wire from its emergence from the intestine, up above the surface of the bath fluid. A second platinum electrode is dipped into the bath fluid and makes the whole bath a diffuse external electrode. The arrangement permits a uniform excitation over the whole ileum and ensures that all the stimulation current applied traverses the intestinal wall. The preparation is stimulated with shocks of 0.5-msec. duration. The threshold voltage is about 1 v. and the maximal voltage is about 5–25 v. (89).

This preparation is more suitable for qualitative than for quantitative study and was originaly described by Paton (89). It has been suggested that the postganglionic cholinergic fibers are selectively stimulated through coaxial electrodes in this preparation, which has been used to differentiate between direct and indirect drug actions on the guinea pig ileum (86, 90–92).

Nerve–Jejunum Preparation of Rabbit—The rabbit is stunned by a blow on the head. The abdomen is opened and the jejunum which has the most mobile mesenteric attachment is taken. The nerve which lies in the mesentery along with the arterial blood supply is identified. Great care is taken not to stretch or otherwise damage the nerve in the mesentery. The intestine, about 2–3 cm. in length, is mounted in the organ bath of 30-ml. capacity or on a superfusion assembly. The mesentery is threaded and passed through an electrode similar to that used in the rat phrenic nerve–diaphragm preparation. The nerve is stimulated with rectangular-wave pulses of 0.5-msec. duration and about 10 v. The slow rate stimulation (2–4 pulses/min.) may produce parasympathetic effects, whereas a high rate of stimulation (30–50 pulses/min.) produces sympathetic effects. To avoid fatigue, the stimulus should not be applied for more than 30 sec. and at least 1.5–2 min. should be allowed for recovery. Tyrode solution at 37°, oxygenated with 95% O_2 and 5% CO_2, is used to perform the experiment.

This preparation was originally described by Finkleman (49) and is suitable for qualitative rather than quantitative studies of adrenergic blocking agents, cholinergic blocking agents, and local anesthetics. The preparation is especially useful for evaluating agents acting on the adrenergic nerve terminals. Physiological antagonism between catecholamines and parasympathetic effects can also be shown in this preparation (56).

Rat Fundus Strip Preparation—A rat is decapitated and is left to bleed. The abdomen is opened. The fundal part of the stomach is dissected, opened out longitudinally and placed in a dish containing Krebs solution. Suitable transverse cuts are made to obtain a strip about 4–5 cm. long. Threads are attached at both ends and the strip is mounted in an organ bath of 5–10-ml. capacity. An initial tension of 1 g. is placed on the preparation. Krebs solution, at 37°, is oxygenated with 95% O_2 and 5% CO_2. Since the muscle does not relax spontaneously after contraction by drugs, it must be stretched to assist its recovery to the original length. Isometric recording is better than isotonic recording since the muscle length

can be kept constant. The preparation should be left in the organ bath at least 30 min. before use and 6 min. or longer should be allowed between doses.

This method was originally described by Vane (93). It is extremely sensitive to 5-hydroxytryptamine which contracts the muscle at dose levels as low as 0.4–0.8 ng./ml. For bioassaying 5-hydroxytryptamine in the tissue extracts, atropine or hyoscine must be added to the Krebs solution. The sensitivity of this preparation to acetylcholine is lower than that of the leech dorsal muscle treated with physostigmine and is about the same as that of the guinea pig ileum. This muscle is rather insensitive to nicotine and histamine.

Rat Uterus Preparation—Young female rats, weighing 150–200 g., are decapitated and the abdomen opened. The animal should be in oestrus which can be induced by injecting subcutaneously 0.1 mg./kg. of stilbestrol 24 hr. before the animal is sacrificed. The two horns of uteri are transferred to a dish containing warm DeJalon solution (NaCl, 9.0; KCl, 0.42; glucose, 0.5; $NaHCO_3$, 0.5; and $CaCl_2$, 0.03 g./l.) or Krebs bicarbonate solution. Each horn is cut open and divided longitudinally so that four pieces can be obtained from one animal. The threads are tied to each end of each piece (about 2 cm. in length *in situ*), which is then mounted in an organ bath of 10 ml. capacity. The DeJalon solution, at 30–32°, is oxygenated with 95% O_2 and 5% CO_2, which maintains the pH at about 7.4. An initial tension of 0.5 g. is applied to the muscle and the contraction is measured isometrically. The preparation should be left for 30 min. before use (94).

The responses obtained with this tissue are quicker than those obtained with the rat fundus strip preparation but are slower than those produced by the guinea pig ileum preparation. Spontaneous contractions are quite obvious in this preparation when usual physiological salt solutions are used as bathing fluid. The calcium concentration in DeJalon solution is therefore lower than in other physiological salt solutions. Three minutes or longer should be allowed between doses.

This preparation is extremely useful for studying the cholinergic effects of acetylcholine and its derivatives for the following reasons (56): (*a*) The slope of the log dose-response curve obtained with acetylcholine and its derivatives is very steep and thus is convenient for analysis of the results. (*b*) It is remarkably insensitive to histamine. However, it is not suitable for bioassay of acetylcholine and its derivatives obtained from tissues because the sensitivity of this preparation is lower than those of the guinea pig ileum and the leech dorsal muscle treated with physostigmine. Though the preparation is less sensitive than the rat fundus strip for 5-hydroxytryptamine assays, it has the definite advantage of readily returning to basal tension following exposure.

This preparation is historically very important because it has β-adrenergic receptors. Therefore, it is very sensitive to epinephrine (10^{-7} mole) and *dl*-isoproterenol (2×10^{-8} mole) but is relatively insensitive to norepinephrine (2×10^{-6} mole) and ephedrine (2×10^{-5} mole) (94–96). The bioassay of catecholamines has been largely replaced by the sensitive fluorometric method. However, this preparation is still very useful for pharmacologic studies on catecholamines. For pharmacologic study of α-adrenergic agents, the rabbit aortic strip preparation can be used.

Rabbit Aortic Strip Preparation—A rabbit is sacrificed by a blow on the head and cutting the throat for bleeding. The descending aorta is removed and is placed in a dish containing Krebs solution at room temperature. The excised aorta is cut spirally with a small sharp-pointed scissors to produce a strip about 3 mm. wide and 2–4 cm. long. The uncut portion of the aorta is held gently between thumb and fingers of the free hand and is moved gradually forward to the scissors to permit a continuous spiral incision. It is not recommended to pull the aorta over a glass rod or a wooden rod for faster cutting because the aorta strip does not respond well to drugs because of injury by stretching on the rod. Threads are attached to both ends of the preparation which is then mounted in an organ bath of 10-ml. capacity. The Krebs solution, at 37°, is oxygenated with 95% O_2 and 5% CO_2. An initial tension of 3–4 g. is placed on the muscle. The preparation should be allowed to equilibrate for 30 min. or more before use (97, 98).

Since drug response is slow and recovery is long, 30 min. or more is necessary between doses. However, if isometric recording is used, a much shorter period of time is required for recovery and more accurate results can be obtained, due to the small changes in length during muscle contraction. In contrast to the rat uterus preparation, this preparation contains α-adrenergic receptors. Therefore, the effects of catecholamines on this preparation are not blocked by β-adrenergic blockers such as propranolol (99).

This preparation is extremely sensitive to epinephrine (5×10^{-9} mole) and norepinephrine (5×10^{-9} mole) but is less sensitive to *dl*-isoproterenol (2×10^{-7} mole), histamine (5×10^{-8} mole), 5-hydroxytryptamine (5×10^{-8} mole), and acetylcholine (2×10^{-6} mole) (98, 100, 101). Because of the long duration of the experiments, it is important to check the solutions of catecholamines for deterioration. If necessary, a freshly made solution should replace the original.

Dog Mesenteric Artery Preparation—Mongrel dogs are anesthetized with 15 mg./kg. of thiopental sodium and 250 mg./kg. of barbital sodium. The bundles of nerves which follow the vessels through the mesentery are freed at the central end of the arteries. The superior mesenteric artery is freed of mesenteric attachment and cannulated with polyethylene tubing (PE 100). Distally, the branching fans of smaller arteries are cut at the sites of entrance into the small intestine. The isolated preparation is quickly mounted in an organ bath with 100 ml. of Krebs bicarbonate solution at pH 7.3. It is oxygenated with 95% O_2 and 5% CO_2 and is maintained at 37°. The mesenteric arteries are perfused with a constant flow of Krebs bicarbonate solution (30–50 ml./min.). An arterial pressure transducer is attached to the cannula which leads from the pump to the artery. Changes of perfusion pressure are then proportional to changes in arterial resistance. The drugs are injected into the rubber tubing close to the mesenteric artery with a volume of not more than 0.1 ml. The nerve is pulled through the electrodes and is held in place by a ligature. The nerve is stimulated with 1-msec. duration, 50 c.p.s. frequency and 10–15 v. supramaximal voltage (102, 103).

This preparation gives reproducible pressor responses to injected catecholamines and to nerve stimulation for periods of several hours (102). The responses to nerve stimulation are postganglionic α-adrenergic in nature (103). Various adrenergic and adrenolytic agents have been studied with this preparation (103). This is a good preparation for qualitative study of the pharmacology of α-adrenergic and adrenolytic agents on the blood vessels innervated with sympathetic nerves.

Guinea Pig Tracheal Chain Preparation—Adult guinea pigs are sacrificed by a blow on the head. The trachea is dissected and transferred to a dish containing Krebs solution. The trachea is then sectioned into rings by cutting transversely between the segments of cartilage. The rings are tied together to form a chain with the muscular parts of the rings arranged alternatively at two sides of the tracheal chain. The Krebs solution, at 37°, is oxygenated with 95% O_2 and 5% CO_2. The capacity of the organ bath is about 10–15 ml. An initial tension of 0.2–0.5 g. is placed on the tracheal chain.

This preparation was originally described by Castillo and DeBeer (104). The tracheal muscle of the calf can also be used (105, 106). It is a slow responding preparation. Thus at least 15 min. should be allowed between doses for responses induced by small doses and 30 min. or longer for responses induced by large doses of agonists such as acetylcholine and histamine. This preparation can be used to study the pharmacologic effects and dose-response curves of catecholamines. In this case the preparation is contracted with acetyl-β-methylcholine first and then relaxed with various doses of catecholamines. Ariens added cumulative doses of catecholamines to the organ bath to get satisfactory cumulative dose-response curves (105, 106). The threshold doses of epinephrine, norepinephrine, and *dl*-isoproterenol are about 10^{-7} mole, 10^{-7} mole, and 5×10^{-8} mole, respectively.

Guinea Pig Vas Deferens Preparation—Male guinea pigs, weighing 400–800 g., are sacrificed by a blow on the head. The abdomen is opened in the midline and the intestines displaced to the right. The vas deferens is freed from connective tissue and cut from the epididymis. The vas deferens is held with small forceps near the cut end and is freed from the adjacent tissue. The right and left hypogastric nerves can be seen in the middle of the mesentery of the colon. One nerve is tied, and cut about 5 cm. from the vas deferens; this is cleaned to within 0.5 cm. of the vas deferens. The remainder of the nerve which is fine and diffuse is preserved by isolating the piece of peritoneum which contained it. The vas deferens is then cut from the urethra and removed together with its nerve and small piece of peritoneum and is placed in a dish containing Krebs solution. An organ bath containing 20 ml. of Krebs solution at 32°, oxygenated with 95% O_2 and 5% CO_2, is used. The preparation is set up as described below and an initial tension of 0.75–1.0 g. is applied.

For stimulating the hypogastric nerve, the vas deferens is set up in a method similar to that of the rabbit nerve jejunum preparation (Finkleman preparation) (107, 108). The vas deferens is tied by its proximal end to the bottom of the organ bath and the distal end to a lever or a force transducer. The hypogastric nerve is stimulated submaximally with rectangular pulses of 2–3 v. and of 2 msec. duration for 2–3 sec. every minute at a frequency of 80 c.p.s.

For transmural stimulation one end of the vas deferens is tied to a supporting hook and the other (upper end) to the recording lever. The electrodes are two parallel lengths of platinum wire, 0.05-cm. (0.02-in.) diameter, cemented to the edges of a Perspec gutter so that the vas deferens can be suspended between them. Stimulation is performed for periods of 15 sec. at 3-min. intervals at a frequency of 25 c.p.s. with a pulse duration of 0.1 msec. at supramaximal voltage, usually 90–120 v. (109, 110). The transmural stimulation can also be performed by Paton's method (89) described in the section of the coaxial stimulation of the guinea pig ileum.

The vas deferens can also be stimulated alternatively through the hypogastric nerve and transmurally. For both stimulations, a frequency of 25 c.p.s. and a pulse width of 0.1 msec. with supramaximal voltage (90–120 v. for transmural and 30–60 v. for hypogastric nerve stimulation) are used (110).

This preparation was originally described by Huković (111) and modified by other investigators (107–110, 112). It is extremely useful for stimulating separately, preganglionic and postganglionic fibers; the former through the hypogastric nerve and the latter through transmural stimulation. The electrodes must be placed within 2 mm. of the tissue to obtain primarily postganglionic hypogastric nerve activation. The usual 3–5-cm. distance on the hypogastric nerve from the tissue will activate primarily preganglionic fibers. The preparation can also be done successfully using rats, rabbits, and mice (112, 113).

For studying the pharmacologic mechanism of adrenergic systems, this is an extremely useful preparation because when the vas deferens is removed without the hypogastric nerve and stimulated transmurally, the contractions produced are primarily due to the excitation of postganglionic adrenergic nerves (109, 110). It is also useful for studying the pharmacology of cholinergic agents and local anesthetics. This preparation can be contracted both by cholinergic and adrenergic agents. The greatest advantage of this preparation for studying adrenergic agents, as compared with the guinea pig tracheal chain preparation and rabbit aortic strip preparation, is that it responds and relaxes to the drugs rapidly. Consequently, much data can be obtained within a short period of time. However, the sensitivity of this preparation to most of the drugs in comparison with the other preparations is rather low and thus is not suitable for quantitative bioassay.

Heart Muscle Preparations—*Rabbit Heart Preparation*—A rabbit is sacrificed by a blow on the head. The heart is removed as quickly as possible. It is placed in a bath containing Ringer-Locke solution (NaCl, 9.0; KCl, 0.42; glucose, 1.0; $NaHCO_3$, 0.5; and $CaCl_2$, 0.12 g./l.) at room temperature and is squeezed gently to remove the blood. The aorta is identified, freed, and cut just below the point where it divides. The aorta is then tied onto the glass cannula of the perfusion apparatus. The Ringer-Locke solution, oxygenated with 95% O_2 and 5% CO_2, at 37° is perfused through the heart with constant pressure (30 mm. Hg). The fluid passes only through the coronary vessels and escapes from the in-

Table IV—Methods for Bioassay of Cholinergic Agents

Preparation	Speed of Assay	Technique	Threshold Dose of Acetylcholine	References
Leech muscle	Slow	Organ bath	6×10^{-9} mole (0.001 mcg./ml.)	17
Guinea pig ileum	Rapid	Organ bath	1.5×10^{-8} mole (0.0025 mcg./ml.)	55
Chick semispinalis	More rapid	Organ bath	2.4×10^{-8} mole (0.004 mcg./ml.)	62
Frog rectus muscle	Adequate	Organ bath	6×10^{-8} mole (0.01 mcg./ml.)	55
Cat blood pressure	Rapid	*in vivo*	0.002 mcg./kg.	17
Guinea pig ileum	Very rapid	Superfusion	0.0025 mg./injection	10, 75
Frog rectus muscle	More rapid	Superfusion	0.01 mcg./injection	10, 39

ferior vena cava since the aortic valve is closed by the pressure of the perfusion fluid. The perfusate is collected by a funnel and the rate of flow can be measured with a graduated cylinder and a stopwatch. A thread is attached to the ventricle by a hook and connected to spring levers or a force transducer to record the amplitudes of the heart contractions. Readings of the heart rate and of the coronary flow are usually taken over a period of 30 sec. Drugs are injected through the rubber cap into the perfusion fluid. The heart rate and the flow rate of the fluid should be taken approximately every 3 min.

This preparation is well known as the Langendorff preparation (114, 115) and can be used to study coronary dilators, coronary constrictors, and drugs affecting the inotropic and chronotropic effects of the heart (116, 117). For example, coronary vessels are constricted by vasopressin (8×10^{-11} mole/injection) and dilated by amyl nitrite (2×10^{-5} mole/injection). The rate and force of the heart are consistently increased by epinephrine (2.5×10^{-9} mole/injection), norepinephrine (3×10^{-9} mole/injection) or *dl*-isoproterenol (7.5×10^{-10} mole/injection) which are blocked effectively by β-adrenergic blocking agents such as propranolol (7.5×10^{-9} mole/injection). Ventricular fibrillation may be observed when chloroform (1×10^{-5} mole/injection) is given and followed by catecholamines. This preparation is used mainly for qualitative pharmacologic studies.

Guinea Pig Auricle Preparation—Guinea pigs are killed by a blow on the head. The heart is removed as quickly as possible and placed in Feigan solution (NaCl, 9; KCl, 0.42; $CaCl_2$, 0.62; glucose, 1.0; and $NaHCO_3$, 0.6 g./l.) at room temperature. All other tissue is cut away, leaving only the auricles. Threads are tied to the tips of each auricle and the preparation is mounted on a superfusion assembly or in an organ bath with a capacity of 25 ml. The auricle is maintained with a resting tension of 1 g. and is allowed to stabilize for at least 30 min. before use. Feigan's solution is oxygenated with 95% O_2 and 5% CO_2 at 30°.

For transmural stimulation in the organ bath, platinum electrodes are placed on each side of the auricle at a distance of 1 cm. from the auricle (118, 119). The duration of the pulse is held constant at 20 msec., the voltage at 150 v., and the period of the stimulus at 15 sec. A frequency of 2 c.p.s. is used to elicit a parasympathetic response and a frequency of 15 c.p.s. is used to induce both sympathetic and parasympathetic response (119). The preparation can have the vagus nerve attached, which can be stimulated with biphasic stimulation of 35 c.p.s., 6-msec. duration, at 3 v. (120).

For transmural stimulation of the auricle in the superfusion assembly, platinum wire electrodes are placed above and below the auricle, so that they are in continuous contact with the bathing fluid, but not with the tissue. The stimulus parameters are the same as in the organ bath method (121).

Both Feigan solution and Locke-Ringer solution (NaCl, 9.0; KCl, 0.42; $CaCl_2 \cdot 2H_2O$, 0.24; $NaHCO_3$, 0.3; and glucose, 2.0 g./l.) can be used as bathing fluid. However, the former solution is preferred due to the higher concentration of $CaCl_2$ (0.62 g./l.) which produces a heart beat that is stronger, more consistent, and longer lasting. The pH of the solution should be kept at 7.4. This preparation can be done using the frog, turtle, chicken, rat, and rabbit as well (119).

The normal preparation is used primarily for qualitative studies of drugs affecting heart function, such as acetylcholine and its derivatives, catecholamines, atropine-like compounds, and adrenergic blocking agents. However, it can also be used to determine the affinity constants of competitive antagonists of acetylcholine. The transmurally stimulated and the vagus nerve

Table V—Methods for Bioassay of Adrenergic Agents

Preparation	Speed of Assay	Technique	Threshold Dose of Catecholamines[a]	References
Rat uterus	Adequate	Organ bath	2×10^{-8} mole Iso 1×10^{-7} mole Epi 2×10^{-6} mole N.E.	94, 95, 96
Rabbit aorta strip	Slow	Organ bath	2×10^{-7} mole Iso 5×10^{-9} mole Epi 5×10^{-9} mole N.E.	97, 98
Guinea pig tracheal chain	Slow	Organ bath	5×10^{-8} mole Iso 1×10^{-7} mole Epi 1×10^{-7} mole N.E.	104
Guinea pig vas deferens	Adequate	Organ bath	3×10^{-5} mole N.E.	111

[a] Iso, isoproterenol; Epi, epinephrine; and N.E., norepinephrine.

Table VI—Methods for Bioassay of Histaminergic Agents

Preparation	Speed of Assay	Technique	Threshold Dose of Histamine	References
Rabbit aorta strip	Slow	Organ bath	5×10^{-8} mole	97, 98
Guinea pig tracheal chain	Slow	Organ bath	5×10^{-7} mole	104

stimulated preparations are used primarily for the qualitative studies of the mechanisms of heart functions and drugs affecting heart functions (119, 120).

Kitten Papillary Muscle Preparation—Kittens are killed by a blow on the head and the heart is dissected as quickly as possible. The papillary muscles which are usually 0.5–0.7 mm. wide and 5–8 mm. long are dissected. The preparation is mounted on the apparatus described by Blinks (122, 123). The tension of the muscle is adjusted initially to approximately half the level associated with maximally developed tension and the length kept constant thereafter. The papillary muscle can also be obtained from the guinea pig.

This preparation is used primarily for qualitative studies of the drugs affecting heart function. The field stimuli can affect the heart function *via* sympathetic effects and parasympathetic effects. The heart function is also affected by synchronization of the contractions of the various parts of the preparation when the conduction in the muscle is slow. Field pulses of 50-ma. strength and 2-msec. duration are found to be nearly maximally effective in eliciting both sympathetic and parasympathetic effects (123).

Subcellular Preparations—*Synaptic Vesicles*—Rats, weighing about 300 g., are decapitated and the cerebral cortex or any other portion of the brain tissue is isolated as quickly as possible. All procedures described here should be performed at 0–4°. The brain tissue is weighed and homogenized in 0.32 *M* sucrose solution at 1200 r.p.m. for four strokes in a glass homogenizer with a Teflon plunger. The homogenate is diluted to 10% with 0.32 *M* sucrose and centrifuged in the cold for 10 min. at 900 $\times g$. The supernate is saved and the sediment is washed once by rapid rehomogenization in sucrose solution and centrifuged as above. The two supernates are combined and centrifuged for 20 min. at 11,500 $\times g$. The pellet is treated with distilled water (10 ml. per 1 g. tissue) containing 1×10^{-6} mole of Ca^{++} and 1.5×10^{-5} mole of physostigmine. The material is rehomogenized and centrifuged at 11,500 $\times g$ for 20 min. The supernatant is recentrifuged at 100,000 $\times g$ for 30 min. The synaptic vesicle is localized mainly at the pellet portion. This procedure is described by DeRobertis *et al.* (124–126). The procedure described by Whittaker *et al.* is essentially the same as that described by DeRobertis *et al.*, except that the final supernatant is subjected to density gradient purification in order to obtain a purer preparation (127, 128).

This is a convenient preparation to be used to study the drugs which release acetylcholine from the nerve terminals (129). Recently, it has been found that most of the nicotinic agents act through release of acetycholine from the nerve terminals (64, 75). These nicotinic agents can also release acetylcholine effectively from the synaptic vesicle preparation. The acetylcholine-releasing effects of these nicotinic agents are markedly blocked by triethylcholine on the isolated guinea pig ileum and baby chick biventer cervicis nerve muscle preparations (64, 75), but are not blocked on the synaptic vesicle preparation (129). This indicates that the nicotinic agents are taken up by the nerve terminals before they can release acetylcholine from the synaptic vesicles. The uptake of nicotinic agents by the nerve terminals is possibly blocked by triethylcholine at the membrane site of the nerve terminals. This hypothesis can be further illustrated using the synaptosome preparation described in the following section. The acetylcholine released from the synaptic vesicles or synaptosomes can be bioassayed with superfused, cooled guinea pig ileum described in the section on the guinea pig ileum preparation. This preparation can also be obtained from the brains of animals such as guinea pig, rabbit, cat, mouse, and others.

Synaptosome Preparation—Rats, weighing about 300 g., are sacrificed by decapitation and the brain tissue is removed as quickly as possible. All manipulations are performed at 0–4°. A 10% homogenate of brain tissue is prepared in 0.25 *M* sucrose using a glass and Perspex homogenizer rotating at 1200 r.p.m. with a difference in diameter of 0.025 mm. between pestle and mortar. The homogenate is centrifuged at 1000 $\times g$ for 15 min. The supernatant is recentrifuged at 12,500 $\times g$ for 15 min., and the sediment is resuspended in 0.25 *M* sucrose (2 ml./g. of original tissue). Five milliliters of this crude mitochondrial fraction is carefully layered on the top of the synthetic polymer (Ficoll) gradient and the contents are centrifuged at 90,000 $\times g$ for 60 min. The synaptosomes are obtained mainly from the second and third fractions of the six distinct layers. These fractions are diluted with 0.25 *M* sucrose and centrifuged at 120,000 $\times g$ for 20 min. to remove the polymer gradient. The pellets are suspended in 0.25 *M* sucrose and aliquots are used for assay or incubation. The polymer gradients are made by successive layering of 5 ml. each of 20, 16, 12, 8, and 2% polymer solutions made with 0.25 *M* sucrose into a 2.54 × 7.62-cm. (1 × 3-in.) cellulose tube. The

Table VII—Methods for Bioassay of Serotonin

Preparation	Speed of Assay	Technique	Threshold Dose of Serotonin	References
Rat fundus	Adequate	Organ bath	1×10^{-9} mole	93
Rabbit aorta strip	Slow	Organ bath	5×10^{-8} mole	97, 98

Table VIII—Methods for Study of the Sites of Action of Drugs on the Peripheral Nervous System

Cholinergic Nervous System			Adrenergic Nervous System	
Muscarinic or Postganglionic Site	Nicotinic or Preganglionic Site	Neuromuscular Junction	Postganglionic Site	Preganglionic Site
Dog blood pressure	Dog blood pressure after atropine	Rabbit sciatic nerve–gastrocnemius (fast muscle)	Cat nictitating membrane	Cat superior cervical ganglia
Dog chorda tympani–Wharton's duct	Dog chorda tympani-Wharton's duct	Cat soleus muscle (slow muscle)	Peristalsis movement of guinea pig ileum	Guinea pig vas deferens–hypogastric nerve
Guinea pig ileum	Frog rectus abdominus muscle	Cat tibialis anticus muscle (fast muscle)	Rat fundus (for β-adrenergic receptor)	Rabbit nerve–jejunum (fast stimulation)
Longitudinal smooth muscle of guinea pig ileum	Leech dorsal muscle	Rat phrenic nerve diaphragm (fast muscle)	Rabbit aortic strip (for α-adrenergic receptor)	
Coaxial stimulation of guinea pig ileum	Chick semispinalis cervicis muscle	Chick biventer cervicis (fast and slow muscles)	Guinea pig tracheal chain	
Rat fundus	Rabbit nerve–jejunum (slow stimulation)	Chick sciatic-tibialis anticus (fast and slow muscles)	Guinea pig vas deferens (transmural stimulation)	
Rabbit aortic strip			Dog mesenteric artery	

tubes are allowed to stand at room temperature for 60 min. and at 4° for 30 min. (130, 131).

The synaptosomes can also be obtained by methods described by Whittaker *et al.* (127, 132, 133) or De-Robertis *et al.* (124). However, since these methods are performed with the density gradient in hypertonic sucrose solution, the synaptosomes show characteristic morphological changes (134), decrease in oxidative phosphorylation (135), and spontaneous release of bound acetylcholine (131).

The synaptosome preparation can be used to study the drugs which release acetylcholine from the nerve terminals and the drugs which block the release of acetylcholine. It is a convenient method and gives direct evidence to demonstrate the turnover, kinetics, and pharmacology of cholinergic mechanisms at the nerve terminals. It is hoped that combined use of the synaptic vesicle and synaptosome preparations may facilitate the clarification of cholinergic mechanisms which are much less understood than are adrenergic mechanisms. Methods for determining the quantity of drugs taken-up into synaptosomes and the volume of synaptosomes are described by Marchbanks (136, 137).

CONCLUSION

Bioassay is a technique developed entirely by the pharmacologists (138). A good bioassay technique must be sensitive, accurate, and rapid in responding to the drugs administered. For years the leech dorsal muscle and the frog rectus abdominis muscle preparations were the only skeletal muscle preparations widely used for bioassay of nicotinic agents (54, 57). The introduction of the chick semispinalis cervicis muscle preparation furnishes a more rapid technique for the bioassay of these agents (62).

The superfusion technique was first described by Finkleman (49) and later modified by Gaddum (50). This is an extremely useful technique for bioassay because it can be performed with more speed, greater accuracy, and higher sensitivity than the organ bath technique (Table IV). It is highly recommended for bioassay of drugs of trace quantity. It is also a good technique for obtaining ample amounts of data within a short period of time. The methods for bioassay of drugs acting at the peripheral systems are summarized in Tables IV–VII.

Besides being used for bioassay, the biological preparations described are also widely used to study the sites of drug effects on the peripheral nervous system. The methods for studying the specific sites of the peripheral nervous systems are classified in Tables VIII and IX. The rat phrenic nerve–diaphragm muscle preparation has long been used as the sole preparation for *in vitro* study of neuromuscular transmission (65). The chick biventer cervicis nerve muscle preparation (63, 64) and chick sciatic–tibialis anticus preparation (69, 70) were introduced later. They have the advantages of consisting of both fast and slow muscles. They are easy to prepare and can survive with interrupted tetanic stimulation (74). The dog mesenteric artery preparation is an interesting one with sympathetic nerve attached to the artery. It is extremely useful for studying the pharmacology of drugs and nerve regulations on the blood vessels (102, 103).

The synaptic vesicle and synaptosomes are newly developed preparations with high potential for solving

Table IX—Methods for Study of the Sites of Drug Actions at Heart and Nerve Terminals

Heart	Nerve Terminals
Rabbit heart	Synaptic vesicles
Guinea pig auricle	Synaptosome
Kitten papillary muscle	

the mechanism of drug actions at the nerve terminals. For example, nicotinic agents such as nicotine, trimethylamino alcohols, decamethonium, and others have been reported to release acetylcholine from the nerve terminals, which can be blocked by triethylcholine (64, 75). The action mechanism of these findings are explored by using synaptosome and synaptic vesicle preparations. Nicotine and choline have been found to be taken into the intracellular site of superior cervical ganglia (139) and synaptosomes (137), which can be blocked by hexamethonium and hemicholinium, respectively. It has also been found that nicotinic agents are capable of releasing acetylcholine from the synaptic vesicles, which is not blocked by triethylcholine nor by hemicholinium (129). It is thus concluded that the nicotinic agents are probably taken into nerve terminals for releasing endogenous acetylcholines whereas the uptake of nicotinic agents is blocked by triethylcholine or hexamethonium at the membrane site of the nerve terminals (64, 75, 129).

REFERENCES

(1) J. P. Long and M. F. Armaly, *J. Pharmacol. Exptl. Therap.*, **150,** 389(1965).

(2) M. F. Armaly and J. P. Long, *Arch. Intern. Pharmacodyn.*, **161,** 423(1966).

(3) N. D. Edge, *Brit. J. Pharmacol.*, **8,** 10(1953).

(4) J. A. Bevan, *Med. Res. Series*, **3,** 235(1968).

(5) F. W. Schueler, *J. Pharmacol. Exptl. Therap.*, **115,** 127 (1955).

(6) N. A. Prioli and M. M. Winbury, *J. Appl. Physiol.*, **115,** 323(1960).

(7) E. G. Van Proosdij-Hartzema, *Acta Physiol. Pharmacol. Neerl.*, **3,** 472(1954).

(8) A. L. A. Boura and A. F. Green, in "Evaluation of Drug Activities: Pharmacometrics," D. R. Laurence and A. L. Bacharach, Eds., Academic, New York, N. Y., 1964, p. 432.

(9) M. J. Fregly, *J. Lab. Clin. Med.*, **62,** 223(1963).

(10) C. Y. Chiou, J. P. Long, J. G. Cannon, and P. D. Armstrong, *J. Pharmacol. Exptl. Therap.*, **166,** 243(1969).

(11) F. Shibayama, H. Sokabe, and F. Sakai, *Arch. Intern. Pharmacodyn.*, **153,** 334(1965).

(12) L. L. Darko, J. G. Cannon, J. P. Long, and T. F. Burks, *J. Med. Chem.*, **8,** 841(1965).

(13) F. M. Knapp, W. K. Mitchell, and W. A. Himwich, *Life Sci.*, **4,** 251(1965).

(14) C. Y. Chiou and B. V. R. Sastry, *Arch. Intern. Pharmacodyn.*, to be published.

(15) A. Vandsalu, *Acta Physiol. Scand. Suppl.*, **49,** 173(1960).

(16) C. C. Chang, *J. Intern. Neuropharmacol.*, **3,** 363(1964).

(17) F. C. MacIntosh and W. L. M. Perry, *Methods Med. Res.*, **3,** 78(1950).

(18) M. Chrusciel, *Brit. J. Pharmacol.*, **26,** 295(1966).

(19) G. Dauda, M. Szokol, and I. Dévényi, *Acta Physiol. Hung.*, **28,** 273(1965).

(20) W. A. Freyburger, in "Medicinal Research Series," vol. 3, Marcel Dekker, New York, N. Y., 1968, p. 169.

(21) A. M. Rudolph and M. H. Paul, *J. Appl. Physiol.*, **10,** 327(1957).

(22) M. F. Wilson, N. P. Clarke, O. A. Smith, and R. F. Rushmer, *Circulation Res.*, **9,** 491(1961).

(23) I. H. Page and F. Olmsted, *Am. J. Physiol.*, **201,** 92(1961).

(24) N. G. Heatley and J. R. Weeks, *J. Appl. Physiol.*, **19,** 542 (1964).

(25) J. R. Weeks and J. A. Jones, *Proc. Soc. Exptl. Biol. Med.*, **104,** 646(1960).

(26) T. Fujita and D. H. Tedeschi, *Life Sci.*, **7,** 673(1968).

(27) A. W. Kibjakow, *Pfluegers Arch. Ges. Physiol.*, **232,** 432 (1933).

(28) W. Feldberg and J. H. Gaddum, *J. Physiol.*, **81,** 305(1934).

(29) D. Chungcharoen, M. DeB. Daly, and A. Schweitzer, *ibid.*, **118,** 528(1952).

(30) A. St. G. Huggett and F. M. Rowe, *ibid.*, **78,** 25P(1933).

(31) S. Wong and J. P. Long, *J. Pharmacol. Exptl. Therap.*, **156,** 469(1967).

(32) W. L. M. Perry, *J. Physiol.*, **119,** 439(1953).

(33) K. D. Cairncross, M. W. McCulloch, D. F. Story, and F. Trinker, *J. Intern. Neuropharmacol.*, **6,** 293(1967).

(34) T. Desiraju, *J. Neurochem.*, **13,** 323(1966).

(35) D. N. McKinstry, E. Koenig, W. A. Koelle, and G. B. Koelle, *Can. J. Biochem. Physiol.*, **41,** 2599(1963).

(36) B. Collier, F. H. L. Vickerson, and D. R. Varma, *Federation Proc.*, **28,** 670(1969).

(37) H. Wilson and J. P. Long, *Arch. Intern. Pharmacodyn.*, **120,** 343(1959).

(38) J. P. Long, C. T. Evans, and S. Wong, *J. Pharmacol. Exptl. Therap.*, **155,** 223(1967).

(39) C. Y. Chiou and J. P. Long, *ibid.*, **167,** 344(1969).

(40) F. W. Benz and J. P. Long, *ibid.*, **166,** 225(1969).

(41) N. L. Reitzel and J. P. Long, *Arch. Intern. Pharmacodyn.*, **119,** 20(1959).

(42) B. Salafsky, *ibid.*, **174,** 294(1968).

(43) G. L. Brown, *J. Physiol.*, **92,** 22P(1938).

(44) W. C. Bowman, A. A. J. Goldberg, and C. Raper, *Brit. J. Pharmacol.*, **19,** 464(1962).

(45) R. Wuerker, A. McPhedran, and E. Henneman, *J. Neurophysiol.*, **28,** 85(1965).

(46) A. McPhedran, R. Wuerker, and E. Henneman, *ibid.*, **28,** 71(1965).

(47) F. C. A. Romanul, *Arch. Neurol.*, **2,** 355(1964).

(48) D. M. Dawson and F. C. A. Romanul, *ibid.*, **2,** 368(1964).

(49) B. Finkleman, *J. Physiol.*, **70,** 145(1930).

(50) J. H. Gaddum, *Brit. J. Pharmacol.*, **8,** 321(1953).

(51) J. R. Vane, *ibid.*, **35,** 209(1969).

(52) J. R. Vane, *J. Physiol.*, **143,** 75P(1958).

(53) J. R. Vane, *Brit. J. Pharmacol.*, **23,** 360(1964).

(54) J. H. Burn, "Practical Pharmacology," Oxford Blackwell, 1952, p. 347.

(55) C. Y. Chiou and B. V. R. Sastry, *J. Pharmacol. Exptl. Therap.*, to be published.

(56) Department of Pharmacology, University of Edinburgh, "Pharmacological Experiments on Isolated Preparations," E. & S. Livingstone Ltd., Edinburgh and London, England, 1968.

(57) B. K. Battercharya and W. Feldberg, *Brit. J. Pharmacol.*, **13,** 151(1958).

(58) J. Szerb, *J. Physiol.*, **158,** 8P(1962).

(59) A. K. Armitage, G. H. Hall, and C. M. Sellers, *Brit. J. Pharmacol.*, **35,** 152(1969).

(60) B. Collier and J. F. Mitchell, *J. Physiol.*, **184,** 239(1966).

(61) K. J. Child and E. Zaimis, *Brit. J. Pharmacol.*, **15,** 412 (1960).

(62) C. Tyler, *Analyst*, **85,** 8(1960).

(63) B. L. Ginsborg and J. Warriner, *Brit. J. Pharmacol.*, **15,** 410(1960).

(64) C. Y. Chiou and J. P. Long, *Proc. Soc. Exptl. Biol. Med.*, to be published.

(65) E. Bülbring, *Brit. J. Pharmacol.*, **1,** 38(1946).

(66) L. Beani, C. Bianchi, and F. Ledda, *J. Physiol.*, **174,** 172 (1964).

(67) M. Randic and D. M. Straughan, *ibid.*, **173,** 130(1964).

(68) M. Kojima and H. Takagi, *European Pharmacol.*, **5,** 161 (1969).

(69) H. Van Riezen, *ibid.*, **5,** 29(1968).

(70) *Ibid.*, **5,** 37(1968).

(71) R. P. Stephenson, *Brit. J. Pharmacol.*, **11,** 379(1956).

(72) A. Bartolini and G. PePeu, *ibid.*, **31,** 66(1967).

(73) L. A. Barker, J. Amaro, and P. S. Guth, *Biochem. Pharmacol.*, **16,** 2181(1967).

(74) C. Y. Chiou and J. P. Long, *J. Pharm. Sci.*, **58,** 1168(1969).

(75) C. Y. Chiou and J. P. Long, to be published.

(76) N. Ambache, *J. Physiol.*, **125,** 53P(1954).

(77) G. B. Weiss, R. E. Coalson, and L. Hurwitz, *Am. J. Physiol.*, **200,** 789(1961).

(78) H. P. Rang, *Brit. J. Pharmacol.*, **22,** 356(1964).

(79) W. D. M. Paton and A. Zar, *J. Physiol.*, **179,** 85P(1965).

(80) *Ibid.*, **194,** 13(1968).

(81) H. O. Shield, *J. Physiol.*, **101,** 115(1942).
(82) E. Bülbring, A. Crema, and O. B. Saxby, *Brit. J. Pharmacol.*, **13,** 440(1958).
(83) C. Y. Chiou and C. Y. Lee, *J. Formosan Med. Assoc.*, **60,** 1128(1961).
(84) M. L. Sharma and M. V. Rajapurkar, *Ind. J. Med. Res.*, **54,** 765(1966).
(85) D. B. Beleslin, S. B. Bogdanović, and M. M. Rakić, *Arch. Intern. Pharmacodyn.*, **164,** 345(1966).
(86) V. Varagić and T. Kazić, *ibid.*, **165,** 181(1967).
(87) E. Bülbring and R. C. Y. Lin, *J. Physiol.*, **140,** 381(1958).
(88) P. Trendelenburg, *Arch. Exptl. Pathol. Pharmakol.*, **81,** 55(1917).
(89) W. D. M. Paton, *J. Physiol.*, **127,** 40P(1955).
(90) M. Day and J. R. Vane, *Brit. J. Pharmacol.*, **20,** 150(1963).
(91) V. Varagić and T. Kazić, *J. Pharm. Pharmacol.*, **18,** 513 (1966).
(92) K. Takagi, I. Takayanagi, T. Irikura, and K. Nishino, *Japan. J. Pharmacol.*, **17,** 115(1967).
(93) J. R. Vane, *Brit. J. Pharmacol.*, **12,** 344(1957).
(94) J. Diamond and T. M. Brody, *J. Pharmacol. Exptl. Therap.*, **152,** 202(1966).
(95) *Ibid.*, **152,** 212(1966).
(96) T. M. Brody and J. Diamond, *N. Y. Acad. Sci.*, **139,** 772(1967).
(97) R. F. Furchgott and S. Bhadrakom, *J. Pharmacol. Exptl. Therap.*, **108,** 129(1953).
(98) R. F. Furchgott, *Methods Med. Res.*, **8,** 177(1960).
(99) R. F. Furchgott, *N. Y. Acad. Sci.*, **139,** 553(1967).
(100) G. D. Maengwyn-Davies and T. Koppanyi, *J. Pharmacol. Exptl. Therap.*, **154,** 481(1966).
(101) J. J. Krzanowski, Jr. and R. A. Woodbury, *ibid.*, **154,** 472 (1966).
(102) L. A. Rogers, R. A. Atkinson, and J. P. Long, *ibid.*, **151,** 313(1966).
(103) L. A. Rogers, R. A. Atkinson, and J. P. Long, *J. Physiol.*, **209,** 376(1965).
(104) J. C. Castillo and E. J. DeBeer, *J. Pharmacol. Exptl. Therap.*, **90,** 104(1947).
(105) E. J. Ariens, *N. Y. Acad. Sci.*, **139,** 606(1967).
(106) E. J. Ariens, in "Ciba Foundation Symposium," G. E. W. Wolstenholme and M. O'Connor, Eds., J & A Churchill Ltd., London, England, 1960, p. 264.
(107) G. A. Bentley, *Brit. J. Pharmacol.*, **19,** 85(1962).
(108) G. A. Bentley and J. R. Sabine, *ibid.*, **21,** 190(1963).
(109) A. T. Birmingham, *ibid.*, **27,** 145(1966).
(110) A. T. Birmingham and A. B. Wilson, *ibid.*, **21,** 569(1963).
(111) S. Huković, *ibid.*, **16,** 188(1961).
(112) N. O. Sjöstrand, *Acta Physiol. Scand. Suppl.*, **65,** 257(1965).
(113) J. D. P. Graham and H. AlKatib, *Brit. J. Pharmacol.*, **31,** 42(1967).
(114) O. Langendorff, *Pfluegers Arch. Ges. Physiol.*, **61,** 291 (1895).
(115) J. A. Gunn, *J. Physiol.*, **46,** 506(1913).
(116) A. Juhasz-Nagy, M. Szentivanyi, S. Racz, and G. Deak, *Arch. Intern. Pharmacodyn.*, **157,** 360(1965).
(117) A. G. Roy, *Can. J. Pharm. Sci.*, **1,** 41(1966).
(118) T. S. Whitacre, J. P. Long, and W. J. Whalen, *Am. J. Physiol.*, **210,** 557(1966).
(119) E. G. Gross, T. S. Whitacre, and J. P. Long, *Arch. Intern. Pharmacodyn.*, **166,** 273(1967).
(120) T. S. Chiang and F. E. Leaders, *J. Pharmacol. Exptl. Therap.*, **149,** 225(1965).
(121) D. A. Shriver, M.S. thesis, University of Iowa, 1968.
(122) J. R. Blinks, *J. Appl. Physiol.*, **20,** 755(1965).
(123) J. R. Blinks, *J. Pharmacol. Exptl. Therap.*, **151,** 221(1966).
(124) E. DeRobertis, A. P. DeIraldi, G. R. DeL. Arnaiz, and L. Salganicoff, *J. Neurochem.*, **9,** 23(1962).
(125) E. DeRobertis, R. DeL. Arnaiz, L. Salganicoff, A. P. DeIraldi, and L. M. Zieher, *ibid.*, **10,** 225(1963).
(126) K. Kataoka and E. DeRobertis, *J. Pharmacol. Exptl. Therap.*, **156,** 114(1967).
(127) V. P. Whittaker, I. A. Michaelson, and R. J. Kirkland, *Biochem. J.*, **90,** 293(1964).
(128) V. P. Whittaker and M. N. Sheridan, *J. Neurochem.*, **12,** 363(1965).
(129) C. Y. Chiou, J. P. Long, J. L. Spratt, and R. F. Potrepka, to be published.
(130) A. A. Abdel-Latif, *Biochem. Biophys. Acta*, **121,** 403(1966).
(131) M. Kurokawa, T. Sakamoto, and M. Kato, *Biochem. J.*, **97,** 833(1965).
(132) E. C. Gray and V. P. Whittaker, *J. Anat.*, **96,** 79(1962).
(133) V. P. Whittaker, *Biochem. J.*, **72,** 694(1959).
(134) V. P. Whittaker, *Progr. Biophys. Mol. Biol.*, **15,** 41(1965).
(135) W. L. Stahl, J. C. Smith, L. M. Napolitano, and R. E. Basford, *J. Cell. Biol.*, **19,** 293(1963).
(136) R. M. Marchbanks, *Biochem. J.*, **106,** 87(1968).
(137) *Ibid.*, **110,** 533(1968).
(138) J. H. Gaddum, in "Drugs in Our Society," Johns Hopkins Press, Baltimore, Md., 1964, p. 18.
(139) D. A. Brown, P. C. Hoffmann, and L. J. Ruth, *Brit. J. Pharmacol.*, **35,** 406(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, IA 52240*

This work was supported in part by USPHS Research Grants NB-1396 and NB-4431.

* Present address: Department of Pharmacology and Therapeutics, University of Florida, College of Medicine, Gainesville, Fla.

Preliminary Model for Methotrexate Pharmacokinetics

K. B. BISCHOFF*, R. L. DEDRICK, and D. S. ZAHARKO

Abstract □ A pharmacokinetic model is presented to describe the distribution of methotrexate in mice, and the required physicochemical, anatomical, and physiological data are discussed. The model is used to simulate methotrexate concentrations in plasma, lean tissue, liver, kidneys, and gastrointestinal tract following a single intravenous injection. Methotrexate is excreted in the urine and bile; partial reabsorption occurs in the gastrointestinal tract. Tissue-to-plasma distribution ratios were: muscle (0.15:1); kidney (3:1); liver (10:1). Observed bile concentrations were 300 times those of plasma.

Keyphrases □ Methotrexate pharmacokinetics—model □ Distribution, methotrexate—mice □ Tissue/plasma ratios—methotrexate □ Plasma protein binding—methotrexate □ Pharmacokinetic equations—methotrexate absorption, distribution, excretion □ Model, methotrexate distribution—one, two compartments plus gut lumen

This paper presents the results of some first attempts to formulate mathematical models for the distribution of the cancer chemotherapeutic agent methotrexate (MTX) in mammals. It begins with a short discussion of why and how such pharmacokinetic models can be of practical use as well as enhancing insight and appreciation of relevant physiological phenomena.

One important application of the models can be to aid in the evaluation of screening tests. Most cancer chemotherapeutic agents (as well as many other drugs) are initially screened by a series of experimental evaluations using mice. Normally, only the gross success or failure of the drug to combat tumors is considered. The actual sequence of events, however, can be divided into two major aspects: first, the spatial and temporal physical distribution of the drug within the body and, second, the physiological (therapeutic and toxic) effects at discrete tissue loci. The latter, of course, is the crucial issue, but until local concentrations for known time intervals throughout the body are defined, reliable correlations between drug distribution and drug effect cannot be established. Thus the distribution information is required to more fully appreciate the problems of drug evaluation.

If initial screening tests are successful, trials in other animal species, eventually leading to man, are performed. Many relevant interspecies differences are associated with chemical phenomena—such as metabolism, binding, and membrane transport. Physical aspects, such as organ sizes and intracorporeal fluid flow rates, also vary among species. These facts suggest a scale-up approach based on maximum use of physiological, anatomical, and physicochemical information. A reliable quantitative model based on physiology can serve as a useful framework for designing experiments and evaluating results.

Such a model with appropriate scale-up information may significantly enhance success in the ultimate application in the clinic. Concentration history may be predictable at any site as a function of dose schedule and route of administration. This information, coupled with knowledge of drug concentration and exposure time necessary for maximum tumor toxicity, should provide a rational basis for optimization of dosage regimens. This can be critical for cancer chemotherapeutic agents because of the rather narrow range between effective and toxic doses.

RATIONALE AND GENERAL FEATURES OF MODELS

Before discussing the details of the experiments and models for MTX, a few brief statements will be made concerning pharmacokinetic models. The approach used here has been extensively discussed and illustrated elsewhere: Bischoff and Brown (1), Bischoff (2), Dedrick and Bischoff (3), and Bischoff and Dedrick (4). Mass transfer models are based on "lumping" body regions, with similar physicochemical properties, into discrete compartments. Volumes, flows, and other properties will all have independently verifiable anatomical significance. The use of parameters obtained from experiments other than the one being modeled is important, since it can lead to generally applicable *a priori* predictions. Further, the model leads to insight concerning some of the physiological mechanisms.

One broad assumption used in developing the model, that of flow-limited conditions, deserves mention here. This assumption, commonly used in pharmacokinetics, implies that the blood leaving a tissue is in diffusion equilibrium with the tissue, *i.e.*, the free (not protein bound) concentrations of blood and tissue are the same. This assumption requires that membrane permeability is sufficiently larger than the perfusion rate so that the latter is rate controlling. A mathematical criterion for this was given by Dedrick and Bischoff (3) and it was shown that the assumption was valid for certain barbiturates. MTX is not particularly lipid soluble and has a molecular weight of 454; thus, its permeability is probably somewhat lower than the barbiturates. Since there seem to be no numerical values available, the work will be done with flow-limited models, bearing in mind, however, that modifications may be necessary to account for finite permeabilities.

EXPERIMENTAL INVESTIGATIONS

The data discussed here are preliminary in nature from the first of a series of experiments. Only one dose level, 3 mg./kg., is considered. This dose is in the range of clinical therapeutic values [Henderson, Adamson, Denham, and Oliverio (5, 6)]. Other levels will be used in future work. Figure 1 shows the data points from several experiments. The points at any one time represent the distribution of methotrexate (MTX), 3′,5′-tritium labeled in one mouse (CDF-1 male) which is injected i.v. and then sacrificed for tritium analysis of the various tissues. Thus these data represent many mice, which would tend to add variability.

Several features of Fig. 1 will be briefly described as an aid in developing models. The implied curves contain certain typical

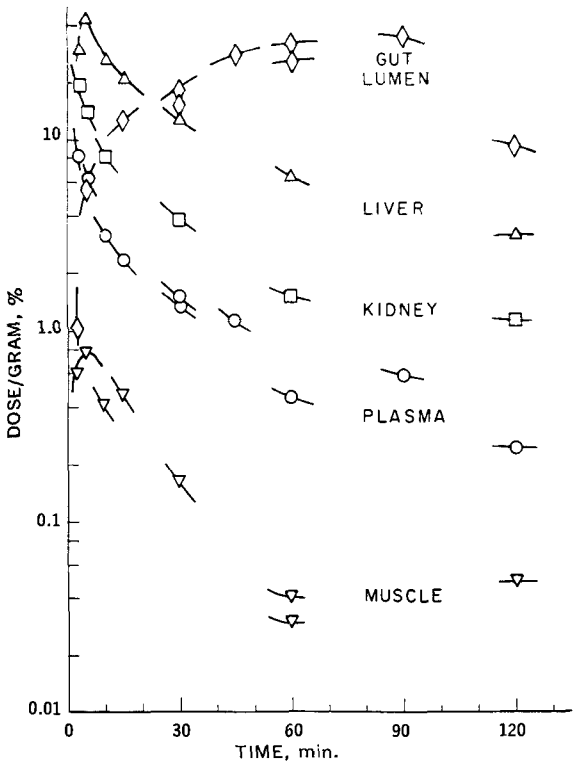


Figure 1—*Distribution of MTX in mice following pulse injection in tail vein.*

features, *e.g.*, immediately following injection there is a short time period of rapid drop in plasma concentration followed by a period of slower rate of decrease. One difference from many drugs, however, is the magnitude of the initial drop. Since Henderson *et al.* (5, 6) have shown that MTX is not appreciably metabolized in mice, this concentration drop results from rapid localization in tissues and excretion. The data points for the small intestine rapidly increase at the outset, which indicates the significant role of the biliary route, as has also been indicated by Henderson *et al.* (5, 6). In fact, 1–2 hr. postinjection, the gut concentration is about 100 times the plasma concentration. From chemical analysis in addition to the radioactivity counting, it appears that the MTX is not conjugated or metabolized in the enterohepatic cycling (5) in contrast to most drugs. The liver here acts differently than with many drugs in that even with its high perfusion rate, several minutes are required to attain maximum concentration. This effect is probably also influenced by biliary clearance and bile formation in the liver.

After a sufficient length of time, all of the curves level asymptotically, suggesting (but not proving) a zero-order absorption from the gut. The tissue/plasma ratios also vary substantially: muscle/plasma ~0.15:1; liver/plasma ~10:1, kidney/plasma ~3:1. These are some of the major characteristics that a successful model must predict.

An essential step in the synthesis of meaningful models is the definition of reliable physical parameters. One obvious set includes the organ weights and flows. The former were obtained from the previously described experiments. The relevant flows are more difficult to obtain, but the model developed here does not require an extensive set. Renal clearance was obtained by comparing integrated plasma concentration data with cumulative urinary excretion. The value was found to be somewhat less than an estimate of inulin clearance based on the literature (7). Little is known about quantitative biliary clearances. These were estimated by separate experiments with anesthetized mice in which the bile duct was cannulated and the flow rate and concentrations directly measured. An estimate of the time delay between injection and appearance of MTX in the bile was also noted.

The data in Fig. 1, together with results published by Henderson *et al.* (5, 6), indicate that gut absorption of MTX may be a zero-order process, in some concentration ranges, presumably caused by saturation of a transport mechanism. The saturation point in humans seems to lie between doses of 0.1–1.0 mg./kg., which is less than our experimental level. An experiment was performed where a known amount of MTX was injected directly into the duodenum and determinations made of the fraction remaining after specific time intervals. Much more experimental work will be required to describe the gut absorption adequately. For the present, zero-order kinetics will be used. Finally, the binding of MTX was evaluated. Plasma protein binding was about 25% and could be correlated with a linear isotherm. Tissue binding is much more difficult to obtain. Separate steady infusion experiments were performed, and the results for the muscle/plasma distribution ratio agreed with that obtained during the kinetic study.

Such separate experiments serve independently to determine or verify most of the model parameters so that generalized *a priori* predictions can be attempted.

MODEL EQUATIONS

Based on the above considerations, the general equations as presented in Dedrick and Bischoff (3) can be considerably simplified. The muscle compartment will be considered to illustrate details. Equations for other compartments are derived similarly. The mass balance accounting for both free and bound drug is:

$$(f_B V_{MB} + f_{MT} V_{MT}) \frac{dC_M}{dt} + (1 - f_B) V_{MB} \frac{dx_{MB}}{dt} + (1 - f_{MT}) V_{MT} \frac{dx_{MT}}{dt} = Q_M[f_B C_P + (1 - f_B) x_B] - Q_M[f_B C_M + (1 - f_B) x_{MB}] \quad \text{(Eq. 1)}$$

where the terms are defined in the *Nomenclature* Section at the end of the paper. The right-hand side of Eq. 1 represents inflow and outflow of free and bound drug. The left-hand side gives a complete accounting for the accumulation of (*a*) free drug in actual tissue and equilibrium blood in the total tissue [as defined by Mapleson (8)]; (*b*) bound drug in the equilibrium blood; and (*c*) bound drug in the actual tissue.

Now with the assumption of linear binding and using effective protein fractions to account for the different levels of binding (see *References 3, 4*),

$$x_p = BC_p,\ x_{MB} = BC_M,\ x_{MT} = BC_M \quad \text{(Eq. 2)}$$

Eq. (1) becomes

$$\{[f_B + B(1 - f_B)]V_{MB} + [f_{MT} + B(1 - f_{MT})]V_{MT}\} \frac{dC_M}{dt} = Q_M[f_B + B(1 - f_B)](C_p - C_M) \quad \text{(Eq. 3)}$$

To simplify the equations, define

$$\phi_B \equiv [f_B + B(1 - f_B)],\ \phi_{MT} \equiv [f_{MT} + B(1 - f_{MT})]$$

With a similar treatment for the other compartments, the full set of equations becomes (refer to Fig. 2):

$$\phi_B V_p \frac{dC_p}{dt} = Mg(t) + Q_L \phi_B C_L + Q_K \phi_B C_K + Q_M \phi_B C_M - Q_p \phi_B C_p \quad \text{(Eq. 4)}$$

$$(\phi_B V_{MB} + \phi_{MT} V_{MT}) \frac{dC_M}{dt} = Q_M \phi_B (C_p - C_M) \quad \text{(Eq. 5)}$$

$$(\phi_B V_{KB} + \phi_{KT} V_{KT}) \frac{dC_K}{dt} = Q_K \phi_B (C_p - C_K) - k_K C_K \quad \text{(Eq. 6)}$$

$$(\phi_B V_{LB} + \phi_{LT} V_{LT}) \frac{dC_L}{dt} = (Q_L - Q_G)\phi_B C_p + Q_G \phi_B C_G - Q_L \phi_B C_L - k_L C_L U(t - D_L) \quad \text{(Eq. 7)}$$

$$(\phi_B V_{GB} + \phi_{GT} V_{GT}) \frac{dC_G}{dt} = Q_G \phi_B (C_p - C_G) + k_0 U(t - D_L) \quad \text{(Eq. 8)}$$

$$Q_p = Q_M + Q_K + (Q_L - Q_G) + Q_G \quad \text{(Eq. 9)}$$

Also, a balance on the gut lumen, assuming again a lumped compartment gives,

$$\frac{dM_{GL}}{dt} = k_L C_L U(t - D_L) - k_0 U(t - D_L) - k_F M_{GL} U(t - D_F) \quad \text{(Eq. 10)}$$

The bile excretion term in Eq. 7 contains a time delay term, $U(t - D_L)$, to approximate the bile formation and holding time. A continuous *s*-shaped function would be more realistic but would introduce additional mathematical complexity not considered important to this preliminary work. The same time delay appears in the zero-order gut absorption term in Eq. 8 since absorption cannot occur before the bile appears from the liver. These time delays appear also in Eq. 10 for the gut lumen with an additional time delay in the feces removal term, $U(t - D_F)$, representing the travel time down the small intestine.

As discussed in detail in Bischoff and Dedrick (9), with linear binding the above equations can be written in terms of total (free and bound) concentration in a simple form, with each parameter maintaining a well-defined physiological meaning. The concentrations actually measured in the laboratory were total wet tissue values, indicated by:

$$C_p' = \phi_B C_p \qquad \text{(Eq. 11)}$$

$$C_M' = \frac{\phi_B V_{MB} + \phi_{MT} V_{MT}}{V_{MB} + V_{MT}} C_M \qquad \text{(Eq. 12)}$$

$$C_K' = \frac{\phi_B V_{KB} + \phi_{KT} V_{KT}}{V_{KB} + V_{KT}} C_K \qquad \text{(Eq. 13)}$$

$$C_L' = \frac{\phi_B V_{LB} + \phi_{LT} V_{LT}}{V_{LB} + V_{LT}} C_L \qquad \text{(Eq. 14)}$$

$$C_G' = \frac{\phi_B V_{GB} + \phi_{GT} V_{GT}}{V_{GB} + V_{GT}} C_G \qquad \text{(Eq. 15)}$$

The basis of these equations can be stated in words:

$$C_M' = \frac{\left\{\left[\begin{array}{c}\text{amount in}\\ \text{equilibrium blood}\end{array}\right] + \left[\begin{array}{c}\text{amount in}\\ \text{actual tissue}\end{array}\right]\right\}}{\text{(total volume of equilibrium blood and tissue)}}$$

$$= \left\{\left[\begin{array}{l}\text{(free + bound concentration in equilibrium}\\ \text{blood)(volume of equilibrium blood)}\end{array}\right]\right.$$

$$\left. + \left[\begin{array}{l}\text{(free + bound concentration in actual}\\ \text{tissue)(volume of actual tissue)}\end{array}\right]\right\} \Big/ \text{(total volume)}$$

$$= \frac{[f_B + B(1 - f_B)]C_M V_{MB} + [f_{MT} + B(1 - f_{MT})]C_M V_{MT}}{V_{MB} + V_{MT}}$$

This then leads to Eq. 12. Similar reasoning was used for the other tissues.

The equilibrium (tissue/plasma) ratios are indicated by:

$$R_M \equiv \left(\frac{C_M'}{C_p'}\right)_{eq.} = \frac{\phi_B V_{MB} + \phi_{MT} V_{MT}}{\phi_B (V_{MB} + V_{MT})} \qquad \text{(Eq. 16)}$$

$$R_K \equiv \left(\frac{C_K'}{C_p'}\right)_{eq.} = \frac{\phi_B V_{KB} + \phi_{KT} V_{KT}}{\phi_B (V_{KB} + V_{KT})} \qquad \text{(Eq. 17)}$$

and similarly for R_G and R_I. These distribution ratios permit the relation between total and free concentrations to be written in a simpler form:

$$C_M' = \phi_B R_M C_M \qquad \text{(Eq. 18)}$$

and similarly for the others. If these relations are substituted into the mass balances, Eqs. 4–10, the final form is:

$$V_p \frac{dC_p'}{dt} = Mg(t) + Q_L \frac{C_L'}{R_L} + Q_K \frac{C_K'}{R_K} + Q_M \frac{C_M'}{R_M} - Q_p C_p' \qquad \text{(Eq. 19)}$$

$$(V_{MB} + V_{MT}) \frac{dC_M'}{dt} = Q_M \left(C_p' - \frac{C_M'}{R_M}\right) \qquad \text{(Eq. 20)}$$

$$(V_{KB} + V_{KT}) \frac{dC_K'}{dt} = Q_K \left(C_p' - \frac{C_K'}{R_K}\right) - \frac{k_K}{\phi_B R_K} C_K' \qquad \text{(Eq. 21)}$$

$$(V_{GB} + V_{GT}) \frac{dC_G'}{dt} = Q_G \left(C_p' - \frac{C_G'}{R_G}\right) + k_0 U(t - D_L) \qquad \text{(Eq. 22)}$$

$$(V_{LB} + V_{LT}) \frac{dC_L'}{dt} = (Q_L - Q_G)\left(C_p' - \frac{C_L'}{R_L}\right) + Q_G \left(\frac{C_G'}{R_G} - \frac{C_L'}{R_L}\right) - \left(\frac{k_L}{\phi_B R_L}\right) C_L' U(t - D_L) \qquad \text{(Eq. 23)}$$

$$\left(\frac{dM_{GL}}{dt}\right) = -k_0 U(t - D_L) - k_F M_{GL} U(t - D_F) + \left(\frac{k_L}{\phi_B R_L}\right) C_L' U(t - D_L) \qquad \text{(Eq. 24)}$$

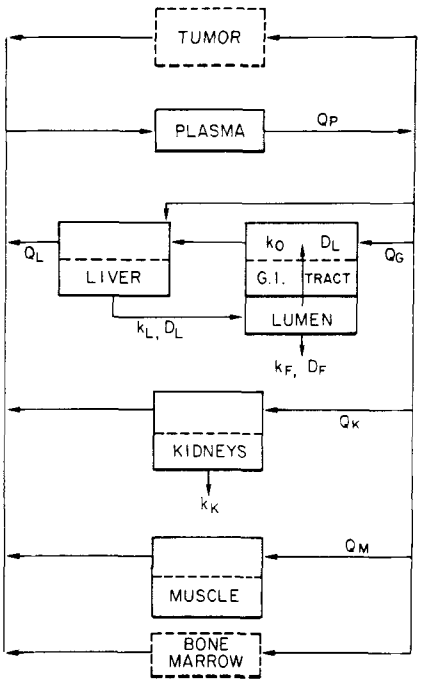


Figure 2—*Compartmental model for MTX distribution. The bone marrow and a hypothetical tumor are indicated because these are regions of interest even though they would normally exert little influence on systemic drug distribution kinetics.*

It should be again emphasized that all of the volume, flow, and other parameters in these equations have well defined physiological meanings and are not derived by "curve fitting" to an arbitrary set of functions.

This set of simultaneous linear differential equations could be solved analytically albeit with considerable algebraic complexity. Figure 1 shows that several of the regions are characterized by similarly shaped curves with different levels. These different levels can be accounted for by the distribution ratios, and thus it would appear that some of the equations could be combined by assuming that these tissues are essentially in physical distribution equilibrium. In particular, this is true of the highly perfused viscera, except for the liver which is complicated by the bile formation at short times. Similar behavior is found for other drugs—see Bischoff and Dedrick (9). The poorly perfused muscle tissue does not exactly follow the same pattern, particularly at short times, but since the muscle tissue is not of primary importance and also does not contain a large fraction of the drug, the simplifying equilibrium assumption will also be used. Using Eqs. 16–17, this means

$$C_M' = R_M C_p' \qquad \text{(Eq. 25a)}$$

$$C_K' = R_K C_p' \qquad \text{(Eq. 25b)}$$

$$C_G' = R_G C_p' \qquad \text{(Eq. 25c)}$$

and Eqs. 19–23 condense to:

$$V_p' \frac{dC_p'}{dt} = Q_L \left(\frac{C_L'}{R_L} - C_p'\right) - k_K' Cp' + Mg(t) \qquad \text{(Eq. 26a)}$$

where

$$V_p' \equiv V_p + R_M V_M + R_K V_K + R_G V_G \qquad \text{(Eq. 26b)}$$

and

$$V_L \left(\frac{dC_L'}{dt}\right) = Q_L \left(C_p' - \frac{C_L'}{R_L}\right) - k_L' \frac{C_L'}{R_L} \times U(t - D_L) + k_0 U(t - D_L) \qquad \text{(Eq. 27)}$$

Figure 3 is a diagram of the simplified model. A slight modification of the transport network, shifting the gut absorption term from the main body compartment to the liver compartment, is made to more closely correspond to actual anatomy.

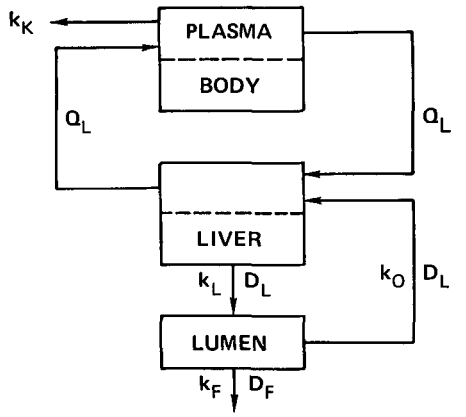


Figure 3—*Two-compartment model plus gut lumen.*

These equations can be readily solved for all times, but for these initial studies, further simplification is useful. Figure 1 shows that for all but short times, the liver behavior is similar to that of plasma and other tissues. Thus, for the longer-time regime:

$$C_L' = R_L C_p' \qquad \text{(Eq. 28)}$$

Eqs. 26, 27, and 24 condense to

$$V_p''\left(\frac{dC_p'}{dt}\right) = Mg(t) - k_K' C_p' - k_L' C_p' U(t - D_L) + k_0 U(t - D_L) \qquad \text{(Eq. 29}a\text{)}$$

where

$$V_p'' \equiv V_p' + R_L V_L \qquad \text{(Eq. 29}b\text{)}$$

$$\left(\frac{dM_{GL}}{dt}\right) = -k_0 U(t - D_L) - k_F M_{GL} U(t - D_F) + k_L' C_p' U(t - D_L) \qquad \text{(Eq. 30)}$$

Figure 4 is a diagram of this model.

PARAMETER VALUES

The parameter values for the models are summarized in Table I. Those directly measured are averages from several mice, and those from correlations are adjusted to the mean-size standard mouse of 22 g.

EQUATION SOLUTIONS AND RESULTS

The various model equations are solved for sequential time steps. For $0 \leq t_1 \leq D_L$, the solutions to Eqs. 26 and 27 with an impulse i.v. injection, $g(t) = \delta(t)$, and with zero initial conditions, $C_{p_1}'(0) = 0$, $C_{L_1}'(0) = 0$, $M_{GL_1}(0) = 0$, are:

$$\frac{C_{p_1}' V_p'}{M} = \frac{\left(r_2 + \frac{Q_L}{R_L V_L}\right) e^{r_2 t} - \left(r_1 + \frac{Q_L}{R_L V_L}\right) e^{r_1 t}}{r_2 - r_1} \qquad \text{(Eq. 31)}$$

$$\frac{C_{L_1}' V_p'}{M} = \frac{Q_L}{R_L V_L} \frac{e^{r_2 t} - e^{r_1 t}}{r_2 - r_1} \qquad \text{(Eq. 32)}$$

$$M_{GL_1} \equiv 0 \qquad \text{(Eq. 33)}$$

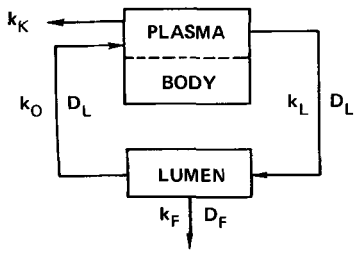


Figure 4—*Single-compartment model plus gut lumen.*

Table I—Model Parameters for 22-g. Mouse

Parameter	Value
V_p	1 ml.
V_G, Gut wall and contents	1.5 g.
V_K	0.34 g.
V_M	~10 g.
V_L	1.3 g.
R_G, Intestine tissue	0.15, Estimate
R_K	3
R_M	0.15
R_L	10
R_{bile}	300
k_K'	0.26 ml./min.[a]
k_L'	0.75–1.1 ml./min.[b]
k_0 For 3 mg./kg.	0.39 mcg./min. = 0.64%/min.
k_F, Mean residence time^{-1}	$\frac{1}{120}-\frac{1}{180}$ min.$^{-1}$ = 7.5×10^{-3} min.$^{-1}$
D_L	~2.5–5 min.
D_F	~120–180 min.

[a] Inulin clearance reported by Selkurt (13) per gram of rat kidney: (0.75 ml./min. g. kidney) (0.34 g. kidney) = 0.26 ml./min. [b] Biliary clearance bounds estimate by: (*a*) maximum clearance = hepatic plasma, flow rate = (1.4 ml./min. ml. liver)* (1.3 g. liver) × (0.6 ml. plasma/ml. blood) = 1.1 ml./min.; (*b*) minimum clearance = (bile flow rate)(MTX concentration ratio) = (2.5 μl./min.)(300) = 0.75 ml./min. * Bradley, *Reference 13.*

where

$$r_{(\frac{1}{2})} = -\tfrac{1}{2}\left[\frac{k_M'}{V_p'} + \frac{Q_L}{V_p'}\left(1 + \frac{V_p'}{R_L V_L}\right)\right] \pm R \qquad \text{(Eq. 34}a\text{)}$$

$$R \equiv \sqrt{\tfrac{1}{4}\left[\frac{k_K'}{V_p'} + \frac{Q_L}{V_p'}\left(1 + \frac{V_p'}{R_L V_L}\right)\right]^2 - \frac{Q_L k_K'}{V_p' R_L V_L}} \qquad \text{(Eq. 34}b\text{)}$$

With the parameter values for a dose of 3 mg./kg.,

$$C_{p_1}' = 4.57e^{-0.014 t_1} + 26.6e^{-0.496 t_1} \qquad \text{(Eq. 35)}$$

$$C_{L_1}' = 54.9(e^{-0.014 t_1} - e^{-0.496 t_1}) \qquad \text{(Eq. 36)}$$

For the next time period, $D_L \leq t_2 \leq D_F$ and with initial conditions $C_{p_2}'(0) = C_{p_1}'(D_L)$, $C_{L_2}'(0) = C_{L_1}'(D_L)$, $M_{GL}(0) \simeq 0$ and $g(t) \equiv 0$ the solution to Eqs. 29 and 30 are:

$$C_{p_2}' = \frac{k_0}{\sigma} + \left(C_{p_1}'(D_L) - \frac{k_0}{\sigma}\right) e^{-(\sigma/V_p'')t_2} \qquad \text{(Eq. 37)}$$

where

$$\sigma = k_K' + k_L'$$

$$M_{GL_2} = -\frac{k_0 k_K'}{\sigma} t_2 + \frac{K_L' V_p''}{\sigma}\left(C_{p_1}'(D_L) - \frac{k_0}{\sigma}\right)(1 - e^{-(\sigma/V_p'')t_2}) \qquad \text{(Eq. 38)}$$

Again with the parameters for 3 mg./kg.

$$C_{p_2}' = 0.508 + 6.0e^{-0.0777 t_2} \qquad \text{(Eq. 39)}$$

and

$$\left(\frac{M_{GL}}{V_G}\right) = -0.088 t_2 + 51.3(1 - e^{-0.0777 t_2}) \qquad \text{(Eq. 40)}$$

Finally, for long times, $t_3 > D_F$, the data indicate in Fig. 5 that the plasma and tissue concentrations are essentially constant. These data are not highly accurate, however, since, as can be seen in the plots, they represent less than 1% of the dose. Thus the radioactivity measured could result from substances other than MTX, from irreversible binding, or other small deviations. Nevertheless, in this regime

$$C_{p_3}' \simeq \frac{k_0}{\sigma} \qquad \text{(Eq. 41}$$

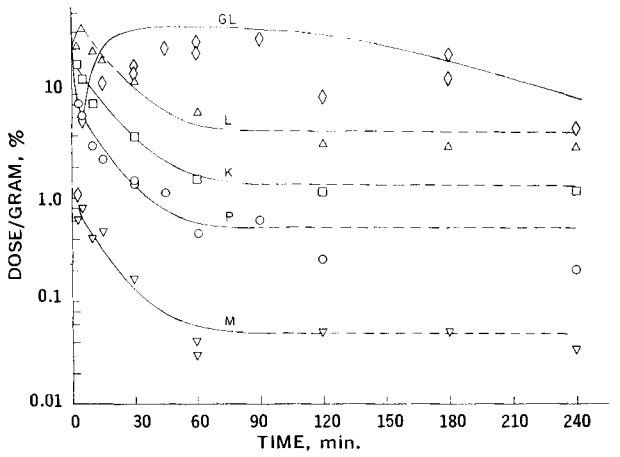


Figure 5—*Comparison between curves predicted by equations and observed data.*

and

$$M_{GL_3} = \left(M_{GL_2}(D_F) + \frac{k_0 k_K'}{\sigma k_F}\right) e^{-k_F t_3} - \frac{k_0 k_K'}{\sigma k_F} \quad \text{(Eq. 42)}$$

For 3 mg./kg.

$$C_{p_3}' = 0.508 \quad \text{(Eq. 43)}$$

$$\left(\frac{M_{GL_3}}{V_G}\right) = 52e^{-0.0075t_3} - 12 \quad \text{(Eq. 44)}$$

Comparison of the *a priori* predicted results with the data of Fig. 1 is shown in Fig. 5. Eqs. 35 and 36 are used up to $D_L \sim 5$ min., Eqs. 39 and 40 to $D_F \sim$ 120–180 min., and Eqs. 43 and 44 for the remaining time. On the whole, excellent agreement is obtained, but several comments are in order. For very short times, Eqs. 26 and 27 depend upon essentially instantaneous equilibration between all parts of the body except for the liver. This results in the nearly vertical line from 100% dose/1 ml. plasma to 31% dose/3.2 ml. body, which is $C_{p_1}'(0)$.

The major discrepancy is the gut lumen predictions for $t < 60$ min., where the predicted value is too high. Probably the main reason for this is the assumption of pure transport time delays which implies abrupt infusion of concentrated bile into the gut lumen at $t = D_L$. As more data are obtained, a better model for biliary clearance should be possible. Another modification would be to use a plug flow model of the small intestine rather than the simple lumped compartment. This approach is now under consideration, and the authors also have some data on drug passage through the intestines. The liver curve between 5 and 30 min. does not completely parallel the plasma curve, and so Eq. 28 is not exactly correct early in the second time region. The full two-compartment equations will be used eventually.

FUTURE WORK

Based on these encouraging initial results, work is in progress to extend and verify the model in several directions. One is to consider other dose levels. Because of the linear nature of the mathematical model, the relative concentrations should remain the same except for possible changes in absorption or excretion mechanisms. For smaller doses, the saturated zero-order gut absorption should become first order if a simple Michaelis-Menten form is followed. Some work by Burgen and Goldberg (10) using direct perfusion methods indicate that this happens for folic acid, which is close in chemical structure to MTX. Both their data and results of Henderson *et al.* (6) suggest that such behavior might occur at dose levels of ~0.1 mg./kg., which is much smaller than the 3 mg./kg. used here. This phenomenon merits careful investigation. Evidence by Guarino and Schanker (11) suggests that the bile excretion mechanism becomes saturated at high doses. Additional information in this regard is required. Data by Borsa *et al.* (12) indicate that this may be true with MTX in mice.

For some of these future studies, the simple form of the differential equations will not suffice and analytical solutions may no longer be possible. The two-compartment model for short times will be utilized using numerical computation techniques to describe more accurately the very important action of the liver and biliary excretion. With more data concerning the latter process, the abrupt time-delay forms will probably also be modified to use a continuous sigmoid rather than a step function—this could also be readily handled with the computer solutions.

Finally, the fundamental issues of scale-up will be emphasized. Some data on rats are now available, and an attempt will also be made to directly apply the model to these data as well as to published human clinical data.

NOMENCLATURE

B = binding constant, dimensionless
C = free concentration, mcg./ml.
C' = total concentration, mcg./ml., Eqs. 11–15
D = delay time, min.
f = fraction water, dimensionless
$g(t)$ = injection function, min.$^{-1}$
k = clearance, ml./min.
k' = clearance based on total concentration in plasma, ml./min.
k_0 = gut absorption rate, mcg./min.
M = amount of drug, mcg.
Q = flow rate, ml./min.
$r_{(\frac{1}{2})}$ = defined in Eqs. 34*a* and *b*
R = equilibrium tissue/plasma ratio, dimensionless
t = time, min.
U = time delay function, dimensionless
V = volume of compartment, ml.
V' = defined by Eq. 26*b*, ml.
V'' = defined by Eq. 29*b*, ml.
x = bound concentration, mcg./ml.
ϕ = defined below Eq. 3, dimensionless
σ = defined below Eq. 37, ml./min. $= k_K' + k_L'$

SUBSCRIPTS

B = blood
F = feces
G = gut
GB = gut equilibrium blood
GL = gut lumen
GT = gut equilibrium tissue
K = kidney
KB = kidney equilibrium blood
KT = kidney equilibrium tissue
L = liver
LB = liver equilibrium blood
LT = liver equilibrium tissue
M = muscle
MB = muscle equilibrium blood
MT = muscle equilibrium tissue
P = plasma
1, 2, 3 = three time periods of integration: $0 \leq t_1 \leq D_L$; $D_L \leq t_2 \leq D_F$; $t_3 > D_F$

REFERENCES

(1) K. B. Bischoff and R. G. Brown, *Chem. Eng. Progr. Symp. Ser. 66*, **62**, 32(1966).

(2) K. B. Bischoff, in "Chemical Engineering in Medicine and Biology," D. Hershey, Ed., Plenum, New York, N. Y., 1967, p. 417.

(3) R. L. Dedrick and K. B. Bischoff, *Chem. Eng. Progr. Symp. Ser. 84*, **64**, 32(1968).

(4) K. B. Bischoff and R. L. Dedrick, *J. Pharm. Sci.*, **57**, 1346 (1968).

(5) E. S. Henderson, R. H. Adamson, C. Denham, and V. T. Oliverio, *Cancer Res.*, **25**, 1008(1965).

(6) E. S. Henderson, R. H. Adamson, and V. T. Oliverio, *ibid.*, **25**, 1018(1965).

(7) E. E. Selkurt, in "Handbook of Physiology," Section 2, Circulation, vol. 2, Am. Physiol. Soc., 1963, p. 1483.

(8) W. W. Mapleson, *J. Appl. Physiol.*, **18**, 197(1963).
(9) K. B. Bischoff and R. L. Dedrick, unpublished data, 1969.
(10) A. S. V. Burgen and N. J. Goldberg, *Brit. J. Pharmacol.*, **19**, 313(1962).
(11) A. M. Guarino and L. S. Schanker, *J. Pharmacol. Exptl. Therap.*, **164**, 387(1968).
(12) J. Borsa, A. F. Whitmore, F. A. Valeriote, D. Collins, and W. R. Bruce, *J. Natl. Cancer Inst.*, **42**, 235(1969).
(13) S. E. Bradley, in "Handbook of Physiology," Section 2, 1963, Circulation, vol. 2, *Am. Physiol. Soc.*, p. 1405.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 14, 1969, from the *National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, MD 20014*

Accepted for publication September 10, 1969.

This investigation was supported in part by a National Institutes of Health fellowship 1-F3-GM39 700-01 from the National Institute of General Medical Sciences.

* Permanent address: Department of Chemical Engineering, University of Maryland, College Park, Md.

Intestinal Drug Absorption and Metabolism I: Comparison of Methods and Models to Study Physiological Factors of *In Vitro* and *In Vivo* Intestinal Absorption

WILLIAM H. BARR* and SIDNEY RIEGELMAN†

Abstract □ Two experimental methods for use in kinetic studies on a compartment model for intestinal metabolism and absorption were evaluated. The *in vitro* cannulated everted intestinal sac and the *in vivo* intestinal loop with complete mesenteric venous collection were compared in the same region of rabbit intestine. These experimental methods were used to study the effects of metabolism, tissue accumulation, and blood flow on the transport of salicylamide across the basal barrier and provide experimental evidence to support the cell compartment model. At lower initial mucosal concentration (10^{-3} *M*), over 60% of the drug appearing in mesenteric blood is conjugated with glucuronic acid. At higher initial mucosal fluid concentrations, glucuronide conjugation appears to be capacity limited and the disappearance from the lumen-curve shows a distinct distributive phase characteristic of a cell compartment model. The rate of transport of free drug across the basal barrier is blood flow rate-limited while the transport of glucuronide is essentially independent of blood flow. Appearance of free salicylamide into mesenteric blood, *in vivo*, shows a lag time of 4 min. compared to a lag time of about 10 min. for the appearance of free drug into serosal fluid *in vitro*. The steady state rate of appearance of free drug into the plasma (*in vivo*) is five to ten times greater than the rate of appearance of free drug into the serosal fluid (*in vitro*) at similar mucosal concentrations. The *in vivo* intestinal loop with complete venous collection was found to have many advantages in studying physiological factors of intestinal drug absorption.

Keyphrases □ Drug absorption—intestinal □ Everted intestine—drug absorption, *in vitro* □ Intestinal loop, cannulated—drug absorption, *in vivo* □ Salicylamide—absorption, accumulation, metabolism □ Glucuronic acid-salicylamide conjugation—concentration effect □ Fluorometry—analysis

From the standpoint of a drug administered orally to produce a systemic effect, intestinal absorption can be considered as the amount of unchanged drug absorbed from the intestinal lumen which appears in the portal circulation or intestinal lymph. Using this definition, the amount of drug disappearing from the lumen contents is sufficient to characterize the absorption process only if it reflects the rate of appearance of unaltered drug in the blood. If one accepts the above definition, then it becomes quite important to evaluate critically the widely used (1) practice of assessing absorption by monitoring only the rate of disappearance of drug from the lumen. The condition that the rate of disappearance of free (unaltered) drug from the lumen contents is identical to the rate of appearance of free drug in the mesenteric blood is implicit in the classical lipoid barrier model of intestinal absorption which assumes that the intestinal tissue is a single barrier which does not contribute to the material balance of the system.[1] When accumulation or metabolism of drug occurs in the intestinal tissue, these rates may not be identical and a tissue compartment model for absorption is considered here as a more appropriate model to describe the entire absorption sequence.

The studies described in this report were designed to compare *in vivo* and *in vitro* experimental methods and mathematical models that might be useful in studying the effect of physiological factors, such as accumulation and metabolism of drug in the intestinal tissue and intestinal blood flow, on the appearance of free drug into mesenteric blood.

The general multiple barrier tissue compartment model that will be used to describe these physiological variables in *in vivo* and *in vitro* preparations is shown in Fig. 1. This catenary three-compartment model assumes that the intestinal tissue can be described as a homogeneous compartment, which is separated from the lumen compartment by an apical barrier (α) and from the terminal blood or serosal fluid compartment by a

[1] In compartment theory, a barrier may be defined as that rate-limiting step between two compartments which distinguishes these compartments by the fact that the rate of distribution within each compartment is sufficiently rapid, compared to the rate of transport across the barrier, that each compartment may be described by a separate volume of distribution. The barrier is considered to be only a rate-limiting step of negligible volume which does not contribute to or alter the material balance of the system. See, for example, the discussion on the concept of a barrier by Riggs (*Reference 32*, p. 188) and the concept of a compartment being represented as a volume by Resigno and Segre (*Reference 31*, p. 16).

(8) W. W. Mapleson, *J. Appl. Physiol.*, **18**, 197(1963).
(9) K. B. Bischoff and R. L. Dedrick, unpublished data, 1969.
(10) A. S. V. Burgen and N. J. Goldberg, *Brit. J. Pharmacol.*, **19**, 313(1962).
(11) A. M. Guarino and L. S. Schanker, *J. Pharmacol. Exptl. Therap.*, **164**, 387(1968).
(12) J. Borsa, A. F. Whitmore, F. A. Valeriote, D. Collins, and W. R. Bruce, *J. Natl. Cancer Inst.*, **42**, 235(1969).
(13) S. E. Bradley, in "Handbook of Physiology," Section 2, 1963, Circulation, vol. 2, *Am. Physiol. Soc.*, p. 1405.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 14, 1969, from the *National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, MD 20014*

Accepted for publication September 10, 1969.

This investigation was supported in part by a National Institutes of Health fellowship 1-F3-GM39 700-01 from the National Institute of General Medical Sciences.

* Permanent address: Department of Chemical Engineering, University of Maryland, College Park, Md.

Intestinal Drug Absorption and Metabolism I: Comparison of Methods and Models to Study Physiological Factors of *In Vitro* and *In Vivo* Intestinal Absorption

WILLIAM H. BARR* and SIDNEY RIEGELMAN†

Abstract □ Two experimental methods for use in kinetic studies on a compartment model for intestinal metabolism and absorption were evaluated. The *in vitro* cannulated everted intestinal sac and the *in vivo* intestinal loop with complete mesenteric venous collection were compared in the same region of rabbit intestine. These experimental methods were used to study the effects of metabolism, tissue accumulation, and blood flow on the transport of salicylamide across the basal barrier and provide experimental evidence to support the cell compartment model. At lower initial mucosal concentration (10^{-3} *M*), over 60% of the drug appearing in mesenteric blood is conjugated with glucuronic acid. At higher initial mucosal fluid concentrations, glucuronide conjugation appears to be capacity limited and the disappearance from the lumen-curve shows a distinct distributive phase characteristic of a cell compartment model. The rate of transport of free drug across the basal barrier is blood flow rate-limited while the transport of glucuronide is essentially independent of blood flow. Appearance of free salicylamide into mesenteric blood, *in vivo*, shows a lag time of 4 min. compared to a lag time of about 10 min. for the appearance of free drug into serosal fluid *in vitro*. The steady state rate of appearance of free drug into the plasma (*in vivo*) is five to ten times greater than the rate of appearance of free drug into the serosal fluid (*in vitro*) at similar mucosal concentrations. The *in vivo* intestinal loop with complete venous collection was found to have many advantages in studying physiological factors of intestinal drug absorption.

Keyphrases □ Drug absorption—intestinal □ Everted intestine—drug absorption, *in vitro* □ Intestinal loop, cannulated—drug absorption, *in vivo* □ Salicylamide—absorption, accumulation, metabolism □ Glucuronic acid-salicylamide conjugation—concentration effect □ Fluorometry—analysis

From the standpoint of a drug administered orally to produce a systemic effect, intestinal absorption can be considered as the amount of unchanged drug absorbed from the intestinal lumen which appears in the portal circulation or intestinal lymph. Using this definition, the amount of drug disappearing from the lumen contents is sufficient to characterize the absorption process only if it reflects the rate of appearance of unaltered drug in the blood. If one accepts the above definition, then it becomes quite important to evaluate critically the widely used (1) practice of assessing absorption by monitoring only the rate of disappearance of drug from the lumen. The condition that the rate of disappearance of free (unaltered) drug from the lumen contents is identical to the rate of appearance of free drug in the mesenteric blood is implicit in the classical lipoid barrier model of intestinal absorption which assumes that the intestinal tissue is a single barrier which does not contribute to the material balance of the system.[1] When accumulation or metabolism of drug occurs in the intestinal tissue, these rates may not be identical and a tissue compartment model for absorption is considered here as a more appropriate model to describe the entire absorption sequence.

The studies described in this report were designed to compare *in vivo* and *in vitro* experimental methods and mathematical models that might be useful in studying the effect of physiological factors, such as accumulation and metabolism of drug in the intestinal tissue and intestinal blood flow, on the appearance of free drug into mesenteric blood.

The general multiple barrier tissue compartment model that will be used to describe these physiological variables in *in vivo* and *in vitro* preparations is shown in Fig. 1. This catenary three-compartment model assumes that the intestinal tissue can be described as a homogeneous compartment, which is separated from the lumen compartment by an apical barrier (α) and from the terminal blood or serosal fluid compartment by a

[1] In compartment theory, a barrier may be defined as that rate-limiting step between two compartments which distinguishes these compartments by the fact that the rate of distribution within each compartment is sufficiently rapid, compared to the rate of transport across the barrier, that each compartment may be described by a separate volume of distribution. The barrier is considered to be only a rate-limiting step of negligible volume which does not contribute to or alter the material balance of the system. See, for example, the discussion on the concept of a barrier by Riggs (*Reference 32*, p. 188) and the concept of a compartment being represented as a volume by Resigno and Segre (*Reference 31*, p. 16).

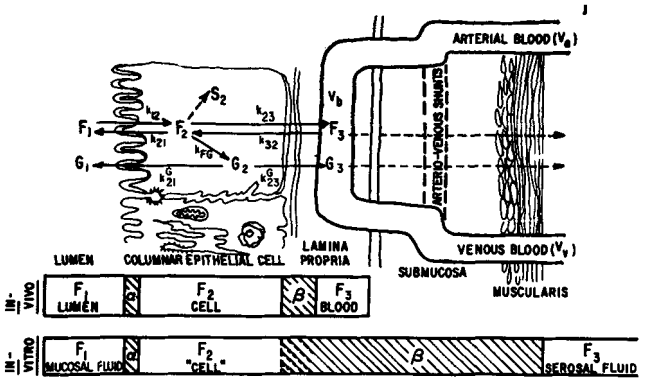


Figure 1—*General compartment model for intestinal absorption including metabolism in the epithelial cell and transport across the apical* (α) *and basal* (β) *barrier into mesenteric blood* (in vivo) *or serosal fluid* (in vitro). *Key:* F_1 *is the amount of free (unmetabolized) drug in the lumen contents;* F_2 *is the amount of free drug in the tissue;* F_3 *is the amount of free drug in the blood in* in vivo *preparations and in the serosal fluid of isolated* in vitro *intestinal preparations;* G_2 *is the amount of metabolite formed in the intestinal cell* (e.g., *glucuronide*). *For some cases, more than one metabolite may be formed as illustrated by* S_2; k_{mn} *is the rate constant for the transfer of amount from Compartment* m *to Compartment* n, e.g., k_{21} *is the rate constant for the transfer of free drug from Compartment 2 to Compartment 1;* k_{mn}^g *is the rate constant for the transfer of amount of metabolite from Compartment* m *to Compartment* n; k_{fg} *is the rate constant for the formation of metabolite.*

basal (β) barrier and that metabolism of the drug may occur in the tissue.

The anatomical entities corresponding to the α and β barriers cannot, at the present state of knowledge, be defined. There are many potential barriers that a drug may encounter during gastrointestinal absorption. It is probable that the barriers corresponding to the rate-limiting steps may be different for different preparations and drugs and possibly for different ionic species of the same drug. It is also clear that the present three-compartment model may be inadequate and additional compartments or a stochastic model may be required to describe the absorption mechanisms of some drugs.

It can be shown that the tissue compartment model will reduce to a simple apparent barrier model when metabolism is negligible and the rate constant for transfer of drug across the basal barrier is much greater than the rate constant for transfer of drug across the apical barrier. The important differences between the compartment and barrier models are summarized in Table I.

It is likely that no single experimental method will be ideal to study physiological factors of absorption and that the maximum information will often require corroborative evidence from more than one method. It was of interest, therefore, to compare different methods in the same intestinal region of the same animal. Two preparations were selected to compare *in vivo* and *in vitro* methods using the same intestinal region.

The *in vitro* preparation selected was a cannulated everted intestine which would eliminate possible variables due to blood flow and permit serial sampling from both the mucosal and serosal contents. The everted intestinal sac which was introduced by Wilson and Wiseman (2) has the advantage of simplicity and flexibility and is beginning to find use in studies on drug absorption (3–8). Everted intestinal sacs have also been used to show intestinal glucuronide formation (6) and

Table I—Comparison of Apparent Barrier and Tissue Compartment Models of Intestinal Absorption

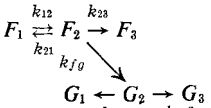
Apparent Barrier Model	Tissue Compartment Model
The intestinal tissue does not contribute to or alter the material balance of free drug in the lumen, blood, or serosal compartments.	The intestinal tissue must be considered in the material balance of free drug in the system.
No appreciable accumulation of free drug in tissue.	Accumulation of free drug in tissue may occur.
Apical barrier is rate-limiting step for appearance of free drug in plasma ($k_{12} < k_{23}$).	Basal barrier is rate-limiting step for appearance of free drug in plasma ($k_{12} > k_{23}$).
No metabolism of drug in tissue.	Metabolism of drug in the tissue may occur.
Rate of appearance of free drug in plasma can be predicted by measurements on the disappearance of drug from the lumen fluid.	Measurements on lumen fluid alone may not be sufficient to characterize appearance of free drug in plasma. Tissue or blood may also have to be sampled.
Semilog plot of amount of drug in the lumen *versus* time will appear linear with intercept near to the initial amount in lumen.	Semilog plot of amount of drug in the lumen *versus* time is biexponential showing distributive phase.

to study the mechanisms involved in glucuronide transport (7, 8).

The *in vivo* method selected for this study was a cannulated intestinal preparation with complete mesenteric venous blood collection. By cannulating the mesenteric vein and leaving the arterial supply intact, the rate of appearance of drug in the blood is measured directly without the complicating factors of tissue distribution in the body, hepatic metabolism, and urinary excretion. This type of preparation has not been generally applied to studies of drug absorption, but has been used to study intestinal metabolic processes of sugars and amino acids in the cat (9), dog (10), guinea pig and rat (11), and steroid conjugation in humans (12).

These two preparations have the advantage that the terminal compartment is closed, permitting direct evaluation and comparison of transfer across the *in vivo* and *in vitro* basal barrier.

Through the use of experimental procedures such as the "*in vivo* intestinal loop with complete venous collection," the assumptions of a closed system and irreversible transfer of drug from the tissue to the blood compartment are valid and permit considerable simplification of the general model (Model I) to the form,



Model II

which is described by the following differential equations:

$$-dF_1/dt = k_{12}F_1 - k_{21}F_2 \quad \text{(Eq. 1)}$$

$$dF_2/dt = k_{12}F_1 - k_{21}F_2 - k_{23}F_2 - k_{fg}F_2^* \quad \text{(Eq. 2)}$$

$$dF_3/dt = k_{23}F_2 \quad \text{(Eq. 3)}$$

$$dG_2/dt = k_{fg}F_2^* - k_{23}^gG_2 - k_{21}^gG_2 \quad \text{(Eq. 4)}$$

The quantity, F_2*, will vary and be equal to F_2 in the case of first-order metabolism or be fixed at a certain value in the case of zero-order metabolism. The fixed amount may be considered that amount which saturates the enzyme system so that $V_{max.}$ is reached and is equal to $k_{f_0}F_2$* which is an apparent zero-order rate constant.

Rabbits were selected as the experimental animal for several reasons. Cannulation of the mesenteric vein in smaller animals is difficult. A larger animal such as a dog would require the replacement of large amounts of blood to maintain blood flow. Furthermore, as the size of the animal increases, the thickness of the intestine precludes use for *in vitro* methods such as everted intestinal sacs or *in vitro* intestinal perfusions so that *in vivo* and *in vitro* methods cannot be easily compared in the same animal. It is stated that the general intestinal vasculature of the rabbit is very similar to that of man (13).

Salicylamide (SAM) was chosen as a suitable drug to use for the following studies on accumulation and metabolism in the cell compartment for two reasons. First, it has sufficient lipid solubility and is largely undissociated at physiological pH (pKa 8.9) (14). Second, Schachter (6) has shown that salicylamide is appreciably conjugated with glucuronic acid in *in vitro* everted intestinal sacs. Salicylamide therefore provides a model substrate to study the effect of intestinal metabolism on the *in vivo* availability of a drug.

EXPERIMENTAL METHODS

Everted Sacs for Kinetic Studies—The apparatus used, a modification of that used by Crane and Wilson (15), is shown in Fig. 2. An intestinal segment (about 10 cm.) was quickly removed from a male New Zealand rabbit under pentobarbital anesthesia, washed gently with Krebs-Ringer bicarbonate solution (16) (pH 7.4), and everted by means of a glass rod inserted through the lumen. A disposable polyethylene centrifuge tube was inserted in one end of the intestine and tied securely with umbilical tape. The opposite end was tied and 7 to 10 ml. of Krebs-Ringer bicarbonate solution introduced through the hypodermic needle inserted in the polyethylene tube. The intestine was then placed in the mucosal fluid containing 75 ml. of 10^{-4} to 10^{-2} M drug in Krebs-Ringer bicarbonate solution maintained at 37° and saturated with 95% O_2 and 5% CO_2 by directly bubbling the O_2 and CO_2 into the mucosal fluid. Serial samples of known volume were taken from both mucosal and serosal fluids by means of 1-ml. disposable plastic syringes.

Fixed-time studies used everted sacs closed at both ends which were incubated either under the conditions used by Herz *et al.* (7) for rat intestine, which was oxygenated by an O_2 and CO_2 (95% and 5%) atmosphere flowing at 122–152.5 cm.³/hr. (4–5 ft.³/hr.), or by bubbling the O_2–CO_2 mixture directly into the mucosal fluid.

***In Situ* Isolated Intestinal Preparation with Intact Arterial Supply and Complete Venous Collection**—Male rabbits weighing between 2 and 3 kg. were anesthetized with pentobarbital or urethan–pentobarbital mixture. A small midline incision allows the gentle exposure of a 20–60-cm. midileal portion of the intestine. This portion was selected because of its accessibility and suitable vasculature to facilitate cannulation. The mesenteric arcades to adjacent portions were carefully tied off. The intestine was cut and cannulated with Tygon tubing for either perfusion (Fig. 3*A*) or a closed loop (Fig. 3*B*). The mesenteric vein was cannulated with an appropriate size of Silastic or polyethylene tubing and all venous blood collected in heparinized, calibrated centrifuge tubes. The mean-flow rate was determined directly by recording the quantity of blood collected in successive intervals, usually 10 min. The blood lost from the mesenteric vein was continuously replaced with an equal volume by an intravenous infusion, *via* the jugular vein, of heparinized blood–saline mixture or whole blood previously collected from donor animals. The carotid artery was also cannulated for systemic arterial samples.

The isolated intestine was kept warm and moist by frequent application of warm (37°) saline to a gauze pad covering the intestine

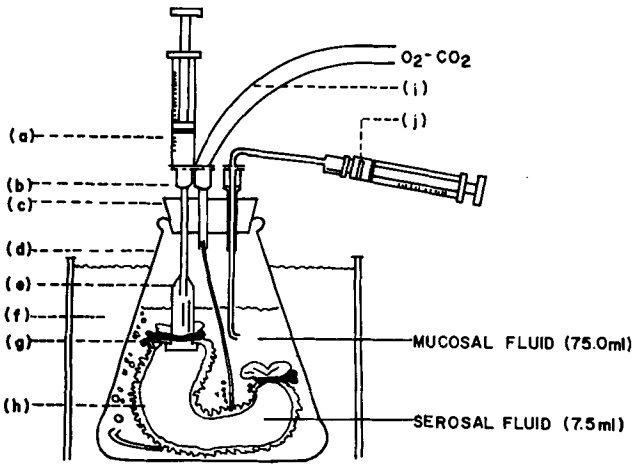


Figure 2—*Diagramatic illustration of apparatus used in the* in vitro *cannulated everted intestine preparations. Key:* (a) *1-ml. disposable plastic syringe used to collect serosal fluid;* (b) *large hypodermic needle (14 gauge);* (c) *rubber stopper;* (d) *125-ml. conical flask;* (e) *disposable polyethylene centrifuge tube;* (f) *water bath (37°);* (g) *umbilical tape used to fasten intestine to centrifuge tube;* (*h*) *everted intestine;* (i) *inlet for gas mixture (O_2–CO_2, 95%–5%);* (j) *1-ml. disposable plastic syringe used to collect mucosal fluid.*

SCHEMATIC DIAGRAM ILLUSTRATING
THE IN VIVO INTESTINAL PREPARATION WITH COMPLETE
VENOUS COLLECTION

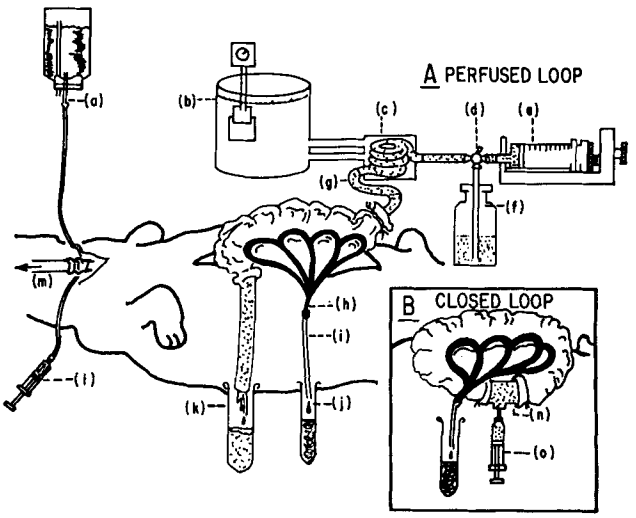


Figure 3—In vivo *intestinal preparation with complete venous collection.* A, *perfused intestine;* B, *closed intestinal loop. Key:* (a) *blood (or blood-saline mixture) infused into jugular vein;* (b) *constant temperature water bath;* (c) *heat exchange coil for perfusate;* (d) *three-way stopcock to refill infusion pump* (e) *with perfusate* (f); (g) *Tygon tubing connecting the perfusion system to the isolated intestinal loop;* (h) *mesenteric vein draining ileal segment;* (i) *polyethylene cannula draining mesenteric venous blood;* (j) *calibrated centrifuge tube (15 ml.);* (k) *centrifuge tube to collect perfusion effluent (50 ml.);* (l) *carotid cannulation for arterial sample;* (m) *attachment to animal respirator;* (n) *Tygon tubing attaching both ends of intestine to form closed loop;* (o) *1-ml. syringe used to collect samples of lumen fluid and also periodically to mix lumen fluid.*

which in turn was covered by a dental rubber dam. A small lamp placed over the area was sufficient to maintain the preparation at 37°.

Spectrophotofluorometric Assay of Salicylamide (SAM) and Its Metabolites in Plasma and Biological Fluids—The usual colorimetric method of assaying salicylamide (SAM) by a ferric ion complex proved too insensitive to be applicable to these studies. Salicylamide (SAM) is fluorescent in basic solution. This property was used to develop a fluorescence assay in which free SAM (pKa 8.9) (14) was extracted from an acidified or buffered (pH 4.3) biological fluid into an ethylene dichloride–cyclohexane (65:35) mixture and reextracted into an aqueous 0.2 *N* sodium hydroxide solution. The fluorescence of SAM in the aqueous basic solution was determined directly by an Aminco-Bowman spectrophotofluorometer at the maximum activation and emission wavelengths of 350 and 430 mμ, respectively (both uncorrected). This procedure provided a sensitive and reproducible assay capable of estimating concentration of free SAM as low as 0.5 mcg./ml. Concentrations of 1 to 50 mcg./ml. were easily assayed with an average standard error of the mean usually less than ±3%. SAM glucuronide is expressed as the free SAM released after hydrolysis with bacterial β-glucuronidase. Total SAM (free, glucuronide, and sulfate) can be determined as free SAM after acid hydrolysis.

Analog Computer Simulation of Model—An analog computer[2] program corresponding to Eqs. 1–4 is shown in standard analog computer symbols in Fig. 4. The steps for subsequent metabolite transport, k_{21}^{g}, k_{23}^{g}, have been omitted in this program as they will not affect transport of free drug which is of interest here.

When the effect of metabolism in the cell is evaluated ($k_{fg} > 0$), metabolism in the cell proceeds by a first-order process at the variable rate, $k_{fg}F_2^*$, where F_2^* is equal to F_2. When the variable input into the electronic comparator (F_2) equals the selected fixed reference voltage of the electronic comparator (F_2^* *ref.*), the comparator operates through a digital output to an electronic switch which changes the rate of metabolism to a fixed (zero-order) rate[3] equal to k_{fg} (F_2^* *ref.*). When metabolism is negligible, the potentiometer representing k_{fg} is set to zero.

RESULTS AND OBSERVATIONS

Influence of Intestinal Region and Method of Oxygenating on *In Vitro* Glucuronidation—In order to compare intestinal metabolism in *in vitro* everted intestines and *in vivo* perfused intestines in the same region, preliminary studies were made to determine the region of intestine which produced maximal glucuronide formation and experimental factors influencing final glucuronide concentrations.

Closed rabbit everted sacs were incubated with 10^{-4} *M* salicylamide under a closed O_2 and CO_2 (95%–5%) atmosphere flowing at 122–152.5 cm.³/hr. (4–5 ft.³/hr.) similar to the conditions used by Herz *et al.* (7) to study conjugation of thyroxine analogs in rat everted intestines. All regions of the intestine had essentially the same final salicylamide glucuronide concentrations and glucuronide-to-free drug ratios in the serosal fluid as shown in Table II, Rows 1–8. The ileum appeared to have slightly greater amounts of glucuronide and was selected as the standard region for this and other reasons. It is the most convenient region for cannulation in the *in vivo* preparation and is the region usually used when minimal fluid flux is desired (17).

When the O_2 and CO_2 mixture was bubbled *directly* into the mucosal fluid, two effects were noted. First, peristaltic contractions of the intestine were increased, leading to variable amounts of fluid remaining in the sac. The final serosal concentrations in the bubbled preparation of both glucuronide and free drug showed greater variation due to the fluid loss. Second, both glucuronide and free drug serosal concentrations were generally higher than those found in the O_2 and CO_2 atmosphere conditions. The increase in serosal glucuronide was greater than the increase of serosal free drug, however, as is reflected by increased serosal glucuronide-to-free drug ratios. These data are shown in Table II where Rows 1–8 are under O_2–CO_2 atmosphere conditions and Rows 9–14 are with bubbled O_2–CO_2 conditions.

[2] The analog computer used was a TR-20 with a series 1133 Variplotter recorder, manufactured by Electronics Associated Inc., Long Branch, N. J.

[3] Although this scheme has the disadvantage that the rate of metabolism shifts from first-order to zero-order instantaneously rather than following the more complex true enzyme kinetics, it is adequate to illustrate the points for which it is intended in this paper.

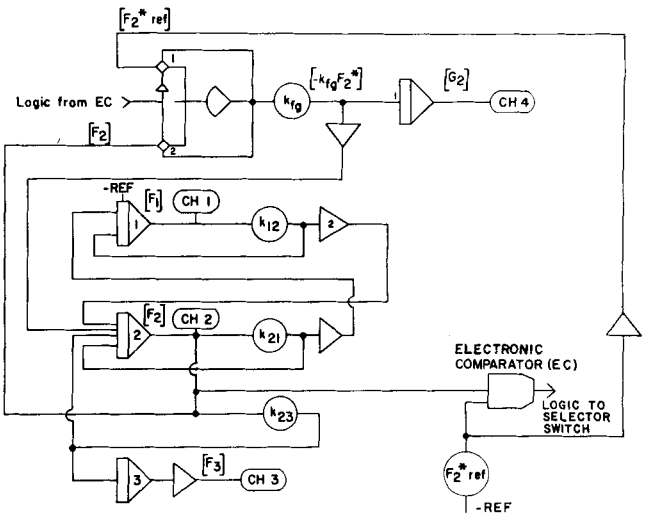


Figure 4—*Analog computer program for the model* $F_1 \rightleftharpoons F_2 \rightarrow F_3$, $F_2 \rightarrow G_2$ *where metabolite formation may be first-order or zero-order when* F_2 *reaches the reference voltage of the comparator* (F_2^* ref.). (*See text for discussion.*)

It was subsequently found that the loss of fluid due to peristaltic squeezing can be satisfactorily prevented by leaving an open syringe in the cannulating needle to act as a reservoir for serosal fluid pushed up during contraction. This procedure improves the reproducibility and usefulness of this preparation and permits the use of directly bubbled oxygen in kinetic studies.

Figure 5 shows the typical linear plot obtained in the cannulated everted sac when appropriate volume corrections are made and the amount of drug reaching serosal fluid is plotted against time. The volume corrections are as follows.

Samples of equal volume are removed from the serosal fluid every 10 min., so that the volume decreases linearly with time. Since the mucosal concentration is essentially constant, a constant amount is delivered to the serosal fluid per unit time while the serosal concentration increases inordinately, due to the decrease of

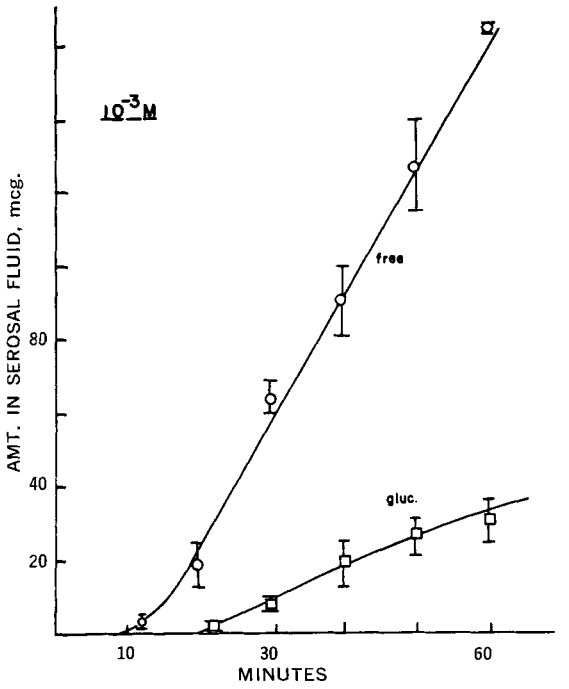


Figure 5—*The amount of free drug* (○) *and glucuronide in the serosal fluid* (□) *of the cannulated* in vitro *everted intestine as a function of time (initial concentration of* 10^{-3} M *SAM in mucosal fluid).*

Table II—Effect of Intestinal Region and Method of Oxygenation on Glucuronide Formation[a]

Row	Region of Intestine	Rabbit[b]	Method	Serosal Volume—Initial	Serosal Volume—Final	Glucuronide, Final Serosal Concn., mcg./ml.	Free Drug, Final Serosal Concn., mcg./ml.	G/F[c]
1	Duodenum	D	O_2 Atmosphere	8	8	2.10	1.20	1.75
2	Duodenum	D	O_2 Atmosphere	8	8	2.16	1.29	1.67
3	Jejunum	D	O_2 Atmosphere	8	8	2.10	.90	2.33
4	Jejunum	D	O_2 Atmosphere	8	8	1.72	1.35	1.27
5	Ileum	D	O_2 Atmosphere	8	8	1.42	1.42	1.00
6	Ileum	D	O_2 Atmosphere	8	8	3.32	1.12	2.96
7	Ileum	B	O_2 Atmosphere	10	10	1.83	1.40	1.31
8	Ileum	B	O_2 Atmosphere	8	8	2.09	1.49	1.40
9	Ileum	C	O_2 Bubbled	8.0	7.4[d]	5.48	2.92	1.88
10	Ileum	C	O_2 Bubbled	7.5	7.4[d]	9.74	3.29	2.96
11	Ileum	D	O_2 Bubbled	8.0	4.7[e]	2.44	.52	4.69
12	Ileum	D	O_2 Bubbled	8.0	3.0[e]	10.21	2.25	5.54
13	Ileum	K	O_2 Bubbled	8.0	7.5	3.98	.82	4.85

[a] Comparison of concentrations of SAM (free) and SAM glucuronide in the serosal fluid after 1-hr. incubation at 37° with an initial mucosal concentration of 10^{-4} *M* SAM. Comparisons are made of different regions of the intestine (duodenum, jejunum, ileum) with the same oxygenating system or the same region (ileum) with different oxygenating systems [95% O_2–5% CO_2 bubbled in directly or in an atmosphere flowing through a Dubnoff shaker at 122–152.5 cm.^{3} (4–5 ft.^{3})/hr.]. [b] Each different letter indicates a particular experiment using a different rabbit. Intestinal segments come from the same animal if they have the same letter (*e.g., D*). [c] The ratio of the concentrations of glucuronide and free in serosal fluids after 1 hr. [d] Fluid loss due to one sample taken at 30 min. [e] Fluid loss due to peristalsis.

serosal volume, resulting in a convex concentration-time curve requiring the following correction.

When each sample aliquot of equal volume (v) is removed from the initial volume (V^0), the amount of drug in the serosal fluid (F_t), at a given time (t), corresponding to sample number (n), of concentration (C_{Fn}), is given by

$$F_t = C_{Fn}[V^0 - v(n-1)]$$

and the cumulative amount of free drug, $\sum F_t$, which has reached the serosal fluid at time t, including the amount which has been removed during previous sampling is

$$\sum F_t = C_{Fn}[V^0 - v(n-1)] + v\sum_{1}^{n-1} C_{Fn}$$

It is possible that even corrected-amount plots will be curved upwards if metabolism is significant and becomes capacity limited within the range of mucosal cell concentrations obtained during the course of the experiment.

Evaluation of *In Vivo* Intestinal Loop with Complete Venous Collection—The technique of collecting all venous blood draining from the region of absorption was developed to provide an *in vivo* preparation with intact circulation where the mesenteric venous blood could be treated as a closed compartment. The most important criterion for the usefulness of this preparation, therefore, is that all free drug and drug metabolites which are absorbed into the capillary blood must be collected in the venous effluent and not reach the general circulation. Since arteriovenous anastomoses in the gastrointestinal circulation are present (18, 19), the following study was performed to establish that the amount of drug which reaches the systemic circulation is negligible. A closed intestinal loop with complete venous collection was filled with 10 ml. of 10^{-3} *M* SAM (1.37 mg.). After 2 hr. the intestine was rinsed and 10 ml. of a 10^{-2} *M* solution (13.7 mg.) was placed in the lumen. The venous blood was collected and assayed for free drug and glucuronide. The carotid artery was sampled periodically during the absorption process. The resulting concentrations of free drug and glucuronide in carotid arterial plasma and the mesenteric venous effluent as a function of time are shown in Fig. 6.

The concentrations in arterial blood were only very slightly above the arterial blood blank and were insignificant compared to venous effluent concentrations. Arterial blood concentrations were checked periodically throughout the experiments and never found to be significant. In some cases, total urine collection was also assayed at the end of the experiment and found to contain negligible amounts of free drug or glucuronide. These results show that the primary criterion for the usefulness of this preparation—*viz.*, complete collection of all absorbed drug and drug metabolites in the mesenteric venous effluent, is quite adequately satisfied.

Effect of Metabolism, Accumulation, and Blood Flow on the Disappearance of Free Drug from the Lumen and Appearance of Free Drug in the Plasma—In the study previously described and illustrated by the plasma concentration curves in Fig. 6, the amounts of free drug in the lumen remaining to be absorbed were also determined and are shown in Fig. 7. These curves illustrate the difficulty of deducing mechanisms of absorption from measurements only on the disappearance of drug from the lumen. Curve *b*, obtained with the lower dose (1.37 mg.), appears linear throughout with an intercept equal to the initial amount in the intestinal lumen. This is exactly the type of curve predicted by a true irreversible barrier model, $F_1 \rightarrow F_3$. Curve *a*, which was obtained in the same intestinal preparation with a higher dose (13.7 mg.), shows a distinct distributive phase, however, and follows the shape predicted by a compartment model, $F_1 \rightleftharpoons F_2 \rightarrow F_3$. If the rate of appearance of free drug in the plasma (dF_3/dt) is examined for the lower concentration (1.37 mg.), it is apparent from Fig. 8 that the slope of the semilog-time-plot is not the same as the slope of the semilog-time-plot of amount remaining in the lumen. The barrier model would require that these slopes both be equal to $-k_{13}/2.303$, since $F_1 = F_1^0 e^{-k_{13}t}$, and $dF_3/dt = k_{13}F_1^0 e^{-k_{13}t}$. Thus, although the data on the disappearance of drug from the lumen suggests that the barrier model is applicable for the lower dose, the rate of appearance of drug in the plasma at this dose does not correspond to the barrier model. These apparently anomalous results can be readily reconciled by considering the effects of cellular metabolism on the amounts of free drug in the intestinal tissue.

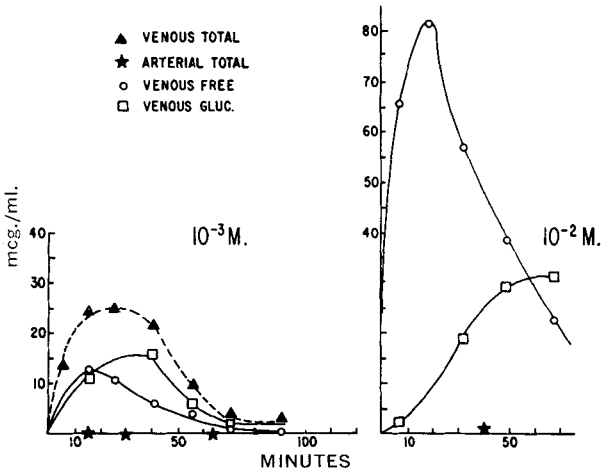


Figure 6—*Comparison of the concentrations of free drug, glucuronide, and total drug in the closed sac,* in vivo *preparation, containing* (a) *10 ml. of* 10^{-3} M *(1.37 mg.) SAM, or* (b) *10 ml. of* 10^{-2} M *(13.7 mg.) SAM.*

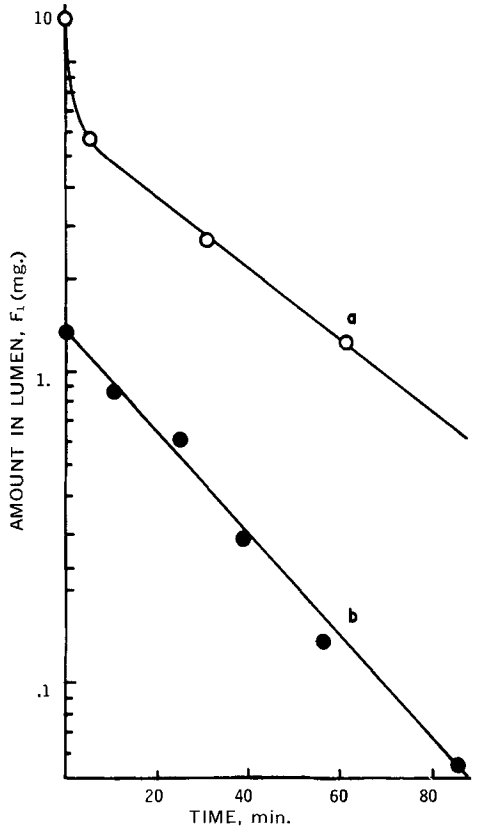


Figure 7—*Comparison of the amount of free drug in the lumen remaining to be absorbed (log scale) in the closed loop* in vivo *preparation containing 10 ml. of 10^{-3}* M *SAM* (●) *and 10 ml. of 10^{-2}* M *SAM* (○).

If it is assumed that at lower doses the free drug in the tissue is rapidly metabolized by a first-order process, the amount of free drug in the cell then remains very low and the cell compartment approximates a barrier containing negligible amounts of free drug.

At higher doses, metabolism in the cell is capacity-limited (*i.e.*, is approaching zero-order metabolism kinetics) and a smaller fraction is metabolized. This allows accumulation of the free drug in the tissue and requires distribution equilibrium to be established between the free drug in the lumen compartment and the cell compartment before monoexponential disappearance is observed. Indeed, when one examines the appearance of glucuronide in the plasma given in Fig. 6, this explanation appears quite reasonable. At 10^{-3} *M*, the fraction which appears as glucuronide in the plasma exceeds that of the free drug. At the higher (10^{-2} *M*) concentration, the fraction of drug which appears in the plasma as glucuronide is much less, particularly in the initial phase. The experimental results shown in Fig. 7 are in reasonable agreement with the types of lumen disappearance curves predicted by the analog computer program when the above assumptions on capacity-limited metabolism were made as shown in Fig. 9.

These *in vivo* studies provide experimental evidence for four important concepts: (*a*) an appreciable amount of drug absorbed from the lumen may appear in the plasma as metabolized drug; (*b*) the intestinal glucuronide conjugating system can apparently be saturated; (*c*) the intestine behaves like a compartment when the fraction of salicylamide metabolized is negligible. Approximate calculations[4] show that the apparent volume of distribution in the cell compartment is almost equal to the volume of the mucosal fluid (about 10 ml.) and (*d*) measurements on the disappearance of drug from the lumen alone are not adequate to characterize the absorption process when metabolism in the cell is significant.

[4] Present difficulties in assaying drug in the tissue allow only indirect evidence of tissue accumulation of free drug. The volume of distribution was estimated by the analog computer program. There are difficulties in curve fitting, however, as (*a*) the fraction of drug metabolized is changing and (*b*) some hydrolysis of glucuronide in the lumen fluid appears operative.

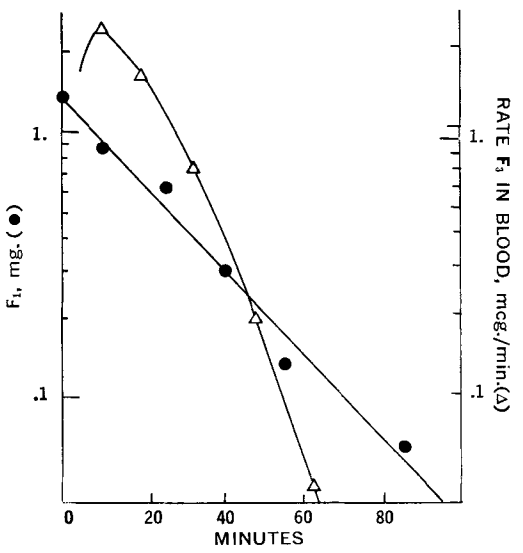


Figure 8—*Comparison of the amount of free drug* (F_1) *in the lumen* (●) *and the rate of appearance of free drug* (F_3) *in the venous plasma* (△) *obtained in an* in vivo *closed loop preparation containing 10 ml. of a 10^{-3}* M *solution of SAM.*

Effect of Blood Flow on the Rate of Appearance of Drug in Plasma—The effect of blood flow on the rate of appearance of drug in the plasma is shown by the fluctuations observed in the rates of appearance of free drug in the *in vivo* perfused intestine long after steady state should have been established. An example is given in Fig. 10 which shows the rate of appearance of free drug and glucuronide in plasma in an *in vivo* loop perfused with 10^{-3} *M* SAM. It is evident that there are considerable variations in the rate of appearance of free drug. When the rates of blood flow at the same time points are plotted, the peaks coincide. The rate of appearance of glucuronide, however, appears to be relatively constant, and independent of blood flow.

The rate of appearance of free salicylamide and glucuronide in the mesenteric plasma as a function of blood flow when the mucosal concentration is maintained constant by perfusion with 10^{-3} *M* SAM is shown in Fig. 11.

It is apparent that transport of free drug across the basal barrier is rate limited by blood flow while the more polar glucuronide is diffusion rate limited except at very low flow rates.

The role of intestinal blood flow in intestinal absorption has been controversial. Some reviewers have stated that although very little work has been done on the effect of blood flow, it may be a rate-limiting factor (20, 21). Others believe it to be unimportant because of the large rate of flow through the splanchnic region (13, 22), and that diffusion will always be rate limiting.

One must of course be cautious in extrapolating the results obtained in an anesthetized surgical preparation to absorption under normal physiological conditions. In general, the splanchnic circulation receives about 30% of the cardiac output of which about 15% goes to the intestines. For a rabbit with a cardiac output of 0.35 l./min. (0.26–0.48 by the direct Fick method), the flow through the entire intestine should be about 55 ml./min. (23). In the cannulated preparation about one-tenth of the intestine is used. Therefore, the flow rate through this segment should be about 4.0 to 7.0 ml./min. The actual flow rates achieved were somewhat less, ranging between 0.6 and 6 ml./min., but sufficiently close to indicate that similar effects might be observed in the intact animal.

Comparison of Lag Times in *In Vitro* and *In Vivo* Preparations—The existence of a lag time in the *in vitro* rabbit preparation is clearly evident from Fig. 5 and has also been demonstrated in the rat by other workers (4, 24, 25). Figure 5 shows a significant lag time of almost 10 min. for free drug and over 15 min. for the linear appearance of glucuronide into serosal fluid. The physiological significance of the lag time for transport of drug into the serosal fluid in the *in vitro* preparations has been questioned, however, because blood circulation is nonfunctional and the drug must additionally traverse the submucosa and muscularis layers which may account for a sizable drug reservoir or barrier.

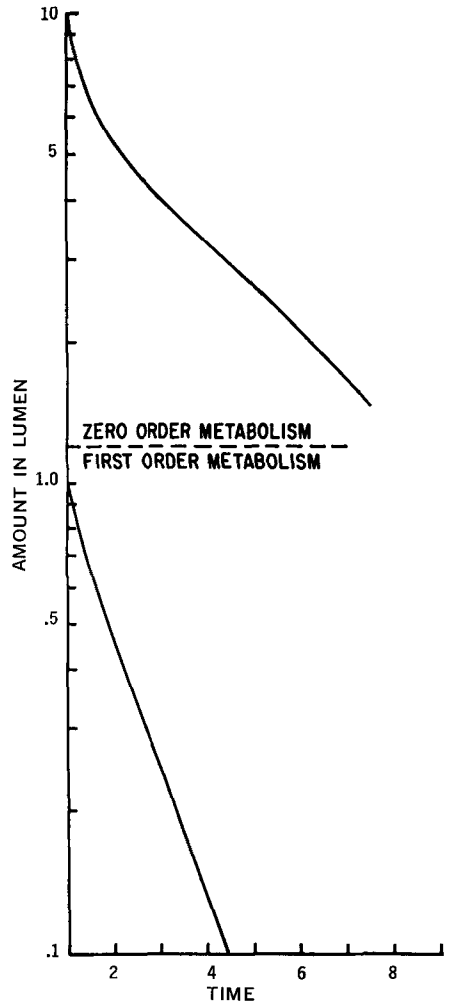


Figure 9—*Analog computor simulation of amounts of free drug remaining in the lumen when metabolism in the tissue compartment proceeds by first-order (bottom curve) at low initial dose, or zero-order at a higher initial dose (top curve). The rate constants for the analog program shown in Fig. 4 (corresponding to the symbolism in Fig. 1) were the same in both cases;* k_{12} *(0.5),* k_{21} *(0.5),* k_{23} *(0.2), and* k_{fg} *(1.2) in units of reciprocal time.* F_2* ref. *was set at 0.5 v. in the case of zero-order metabolism corresponding to a zero-order rate of 0.6 v., unit time*$^{-1}$.

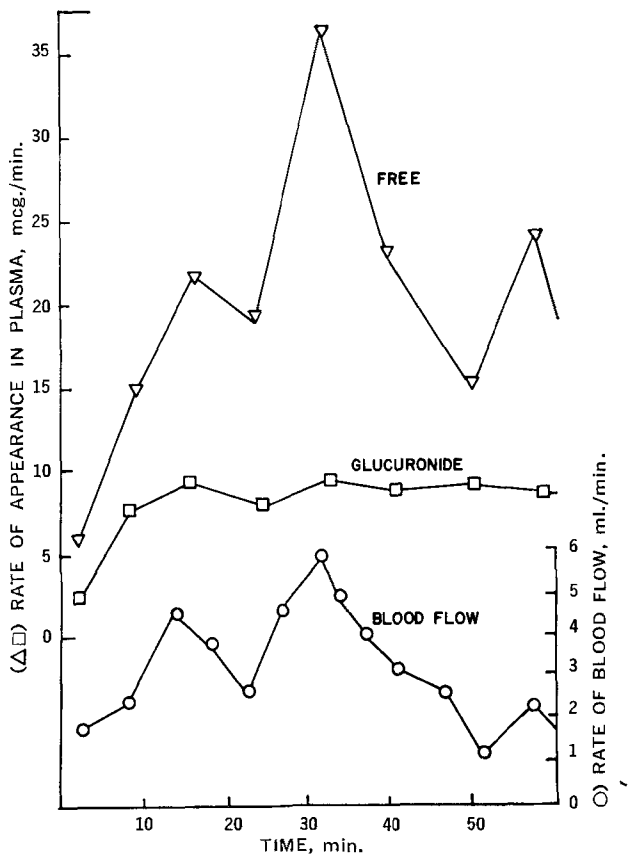


Figure 10—*The rate of appearance of free drug (▽) and glucuronide (□) in the venous plasma correlated with the rate of blood flow (○) in an* in vivo *preparation with complete mesenteric venous collection perfused with a* 10^{-3} M *solution of SAM.*

Several investigators have found it necessary to include a lag time in kinetic evaluations of drug absorption in man (26, 27). It is not possible to directly associate this lag time with the intestinal tissue, however, since in these cases other factors such as dosage form release, gastric emptying, intestinal transit times, and distribution in other tissues, *e.g.*, hepatic (27), may also be involved. Conclusive evidence for an *in vivo* lag phase due to the intestinal tissue should be evident from the *in vivo* perfused intestinal loop with complete venous collection which would not be subject to the above criticisms.

To simulate the conditions of the *in vitro* everted intestinal preparations where the mucosal concentration is essentially constant, the loops were perfused with constant concentrations of 10^{-4}, 10^{-3}, or 10^{-2} *M* SAM in Krebs-Ringer bicarbonate solution. Periodic samples of the lumen perfusate showed that the perfusion rate used (1.9 ml./min.) was sufficient to maintain a constant mucosal concentration. Since all drug is collected in the venous effluent, the plasma can be treated as a closed compartment, and the cumulative amount absorbed is easily obtained. Examples of cumulative amounts of free drug absorbed at different perfusion concentrations are given in Fig. 12. The absolute slopes vary slightly for a given concentration since no attempt was made to keep blood flow conditions and length of the loop constant. The *x*-axis intercept of the extrapolated linear portion of the curve represents the lag time. This lag time is quite consistent for all perfusion concentrations, ranging from 4 to 6 min. with an average of 4.5 ($SE \pm 0.4$) min.

These results show that intestinal lag time can be expected in a physiologically realistic preparation with intact mesenteric circulation even when dosage form delays and diffusion of drug in the lumen fluid are obviated by using a well-stirred solution of the drug. The *in vivo* lag time is, therefore, a real phenomenon attributable to the intestinal tissue.

DISCUSSION

Interpretation of Lag Time in Barrier and Compartment Models—The lag time may be interpreted either as the approach to diffusion steady state in a barrier model or the approach to apparent distribution equilibrium in a tissue compartment. The usual (29) equation describing steady-state diffusion across the intestinal "barrier" is the application of Fick's law to a plane sheet

$$-dF_1/dt = dF_3/dt = P_m A_m (C_{F1} - C_{F3}) \qquad \text{(Eq. 5)}$$

where F_1 is the amount of free drug in the mucosal fluid, P_m is the permeability constant of the intestinal barrier equal to the diffusion constant D, divided by the thickness of the barrier, x; A_m is the effective absorbing area, C_{F1} and C_{F3} are the concentrations of free drug in the mucosal fluid and serosal fluid, respectively. In the noncompartmental diffusion model for diffusion of drug through a simple[5] cylindrical barrier of inner surface area, A_1, and outer surface area, A_3, the lag time, T, is given by

$$T = (A_1 - A_3)^2/6D \qquad \text{(Eq. 6)}$$

It is difficult to assign values to the parameters of Eqs. 5 and 6 for several reasons (29). The intestine is not a simple[5] barrier but

[5] That is, a single isotropic medium.

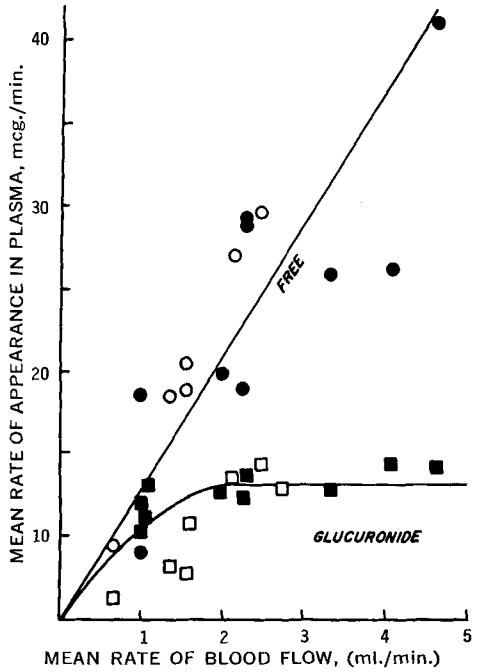


Figure 11—*The rate of appearance of free drug* (○ ●) *and glucuronide* (□ ■) *in the venous plasma as a function of the mean rate of blood flow in two* in vivo *preparations perfused with* 10^{-3} M *SAM.*

a composite barrier in which D may vary.[6] The area of the mucosal surface is much greater than the area of the serosal surface due to villi and microvilli and is difficult to estimate. Metabolism in the tissue may alter the material balance of free drug in the barrier.

When the rate out of the cell across the basal barrier is the rate-limiting step ($k_{23} \ll k_{12}$), accumulation of drug in the tissue occurs. A steady-state approximation based on negligible quantities in the intestine, implicit in a barrier model, will not be valid. Following a single dose, disappearance of the drug from the lumen will be biexponential of the form[7]

$$F_1 = A_1e^{-\alpha t} - A_2e^{-\beta t} \qquad \text{(Eq. 7)}$$

and

$$F_2 = A_3e^{-\alpha t} - A_4e^{-\beta t} \qquad \text{(Eq. 8)}$$

The rapid initial rate of disappearance from the lumen which represents the distributive phase between the lumen compartment and tissue compartment is not paralleled by a rapid rate of appearance of drug in the plasma. In fact, the opposite is true. An initial slow rate of appearance will be observed, resulting in an apparent lag time. During the distributive phase, measurements on the lumen alone will not be sufficient to characterize the rate of appearance of the drug in the blood. Accordingly, the lag time can be considered as the time to reach distribution equilibrium in the tissue compartment corresponding to the distributive phase. Apparent distribution equilibrium can be defined as follows: Apparent distribution equilibrium between two connected compartments is established during the time that the rate of change of the amount of drug in one compartment is proportional to the rate of change of the amount of drug in the other compartment. Following this definition, which will be termed "the proportional rates assumption," the following relationship exists

$$dF_2/dt = R(dF_1/dt) \qquad \text{(Eq. 9)}$$

where R is a proportionality constant.

[6] The difficulties of describing the intestine by diffusion kinetics are apparent when one considers that the simplest compartment model consisting of two barriers (α and β) would be equivalent to a diffusion model for a composite cylinder of two coaxial cylinders having different permeability constants. Crank (30) states that the extra parameters involved make numerical analysis quite formidable.

[7] A complete solution to these equations is given in *Reference 31.*

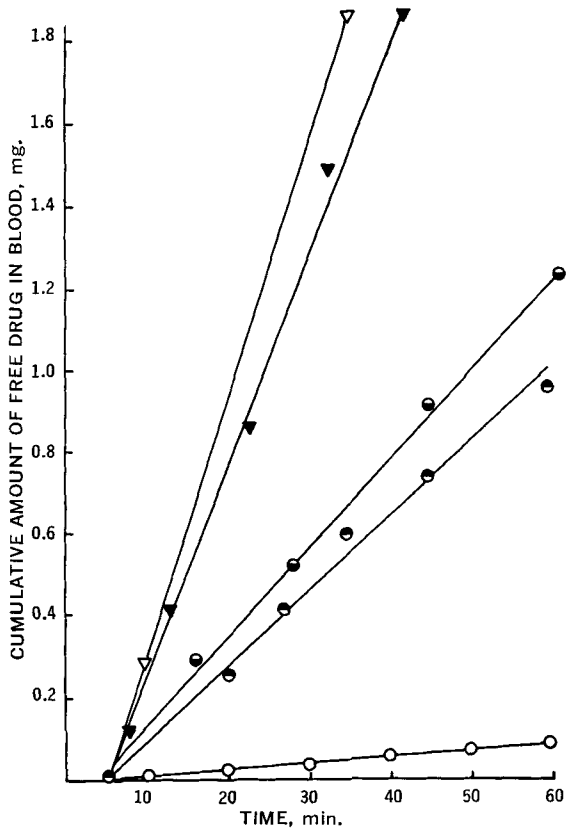


Figure 12—*The cumulative amount of free drug appearing in the blood in* vivo *intestinal loops with complete venous collection perfused* with 10^{-2} M (▽,▼, *two trials*), 10^{-3} M (◒,◓, *two trials*), *and* 10^{-4} M (○, *one trial*) *salicylamide in Krebs-Ringer bicarbonate solution.*

It might be noted that other definitions of distribution equilibrium have been proposed which are not applicable to the intestinal compartment model. Riggs (32) states that distribution equilibrium is achieved when the rate of transfer from Compartment 1 to Compartment 2 equals the rate of transfer from Compartment 2 to Compartment 1. The condition given by Riggs, $dF_{1\to2}/dt = dF_{2\to1}/dt$ for distribution equilibrium is applicable to the intestinal compartment model only when $k_{23} = 0$. Thus, if one accepts the definition of absorption used in this paper, the condition of distribution equilibrium given by Riggs would be valid only when there is no absorption. The basic relationship between the concentration in the reference region (C_1), total amount of drug in the two compartments which are in apparent equilibrium, and the volume of distribution (V_{i2}) is the same for both the condition given by Riggs and the proportional rates condition—*viz.*, $V_{i2} = (F_1 + F_2)/C_1$.

Implicit in Eq. 9, the slopes of the amount-time curves must be proportional at each point in time during apparent distribution equilibrium.

When k_{23} is less than k_{12}, the $e^{-\alpha t}$ term in Eqs. 7 and 8 will approach zero and the linear segments of the semilog plots of amount *versus* time in Compartments 1 and 2 will be parallel in the monoexponential phase. Thus,

$$R = \frac{dF_2/dt}{dF_1/dt} = \frac{\beta F_2}{\beta F_1} = \frac{\beta F_2{}^\circ e^{-\beta t}}{\beta F_1{}^\circ e^{-\beta t}} = \frac{F_2}{F_1} \qquad \text{(Eq. 10)}$$

where β is the apparent hybrid rate constant obtained from the terminal linear segment of the semilog plot of amounts *versus* time in each compartment and $F_1{}^\circ$ and $F_2{}^\circ$ are the extrapolated intercepts of these segments.

This shows that the proportionality constant, R, is equal to the constant ratio of amounts of drug in Compartments 2 and 1, after apparent distribution equilibrium is achieved.

These relationships are illustrated and verified by the analog computer plot shown in Fig. 13. Using these relationships, the identity

can be written

$$F_1 + F_2 = C_1V_1 + RF_1 \qquad \text{(Eq. 11)}$$

where C_1 is the concentration of free drug in Compartment 1 and V_1 is the true volume of Compartment 1. Rearranging Eq. 11, it is seen that the term $V_1(R + 1)$ is equal to $V_{\bar{12}}$, the apparent volume of distribution

$$V_{\bar{12}} = \frac{F_1 + F_2}{C_1} = V_1(R + 1) \qquad \text{(Eq. 12)}$$

When $V_{\bar{12}}$ is constant, it is easily shown that only measurements on the concentration of drug in the first compartment (lumen contents) are necessary to describe the rate of appearance of drug in the third compartment since

$$dF_3/dt = V_{\bar{12}}\, dC_1/dt \qquad \text{(Eq. 13)}$$

After apparent distribution equilibrium is achieved, Compartments 1 and 2 may be considered one open functional compartment with a single volume of distribution ($V_{\bar{12}}$). Thus, the rate of appearance of drug in the blood can be followed after apparent distribution equilibrium by monitoring the lumen concentration only, providing metabolism does not occur. It is important to realize, however, that the apparent volume of distribution, $V_{\bar{12}}$, must be used rather than the actual lumen volume, V_1, or the amount in the tissue will be ignored.

Interpretation of Blood Flow Effects on Drug Transference—When the intestine cannot be considered as a simple barrier, the terms P_m and A_m lose physical significance and can be replaced by an intestinal transference term (3). Transference can be defined for a given unit of tissue as the rate of transport of unaltered substance from the initial compartment to the terminal compartment per unit concentration difference between the initial and terminal compartment. For *in vitro* intestinal transfer, the terminal compartment will be the serosal fluid and transference will be identical to the tissue permeability coefficient as defined by Pappenheimer (33) in units of ml./min. per unit tissue. For *in vivo* intestinal absorption, intestinal transference will be dependent on both the tissue permeability and rate of mesenteric blood flow and is similar to the dialysis term used by Wolf to describe hemodialysis (34).

When the rate of transport of free drug is diffusion rate-limited by the basal barrier and accumulation in the tissue occurs, the transference term will include the apparent volume of distribution term $V_{\bar{12}}$ as discussed in the previous section.

The relationship between intestinal transference (3), tissue permeability (P), and mesenteric blood flow ($\dot{V}$) is given by the following equation (35):

$$\mathfrak{Z} = \dot{V}(1 - e^{-P/\dot{V}}) = kV_{\bar{12}} \qquad \text{(Eq. 14)}$$

where 3 is the transference constant in units of ml./min., k is the apparent first-order rate constant (time^{-1}) for the transfer of amount of drug $(F)_{\bar{12}}$ from the combined mucosal fluid-tissue compartment to the serosal compartment. When the mucosal or tissue concentration is constant ($\bar{C}_{F1}$) and much greater than the plasma concentration and transfer is essentially unidirectional (transference is then a clearance term), the rate of appearance (dQ/dt) of the amount of free drug (Q) in plasma will be given by

$$\frac{dQ}{dt} = V_{\bar{12}}\frac{dC_{F1}}{dt} = \bar{C}_{F1}\,\mathfrak{Z} = \bar{C}_{F1}\,\dot{V}(1 - e^{-P/\dot{V}}) \qquad \text{(Eq. 15)}$$

Thus, when P is very large relative to $\dot{V}$, the exponential term approaches zero and dQ/dt is proportional to blood flow as in the case of free drug. When $\dot{V}$ becomes large relative to P, dQ/dt will become independent of blood flow as illustrated by the glucuronide.

In view of these results, it might be assumed that intestinal blood flow will most likely influence intestinal absorption when: (*a*) the absorbed substance has a high permeability coefficient such as very lipoid-soluble drugs or pore-diffusible substances or (*b*) the blood flow through the absorbing area is low. The blood flow through the absorbing area could be altered by changes in cardiac output, redistribution, or local arterio-venous shunting. These processes may be affected by pathological conditions, emotional state, food, or in fact, the drug itself. The intestinal vasculature is governed by sympathetic tone and many drugs such as sympathomimetics, ergot derivatives, and ganglionic blocking agents could conceivably reduce the effective flow through the absorbing region and reduce their own or the absorption of other drugs given concomitantly. It is clear that the role of intestinal blood flow on intestinal absorption may be quite important and requires much further study.

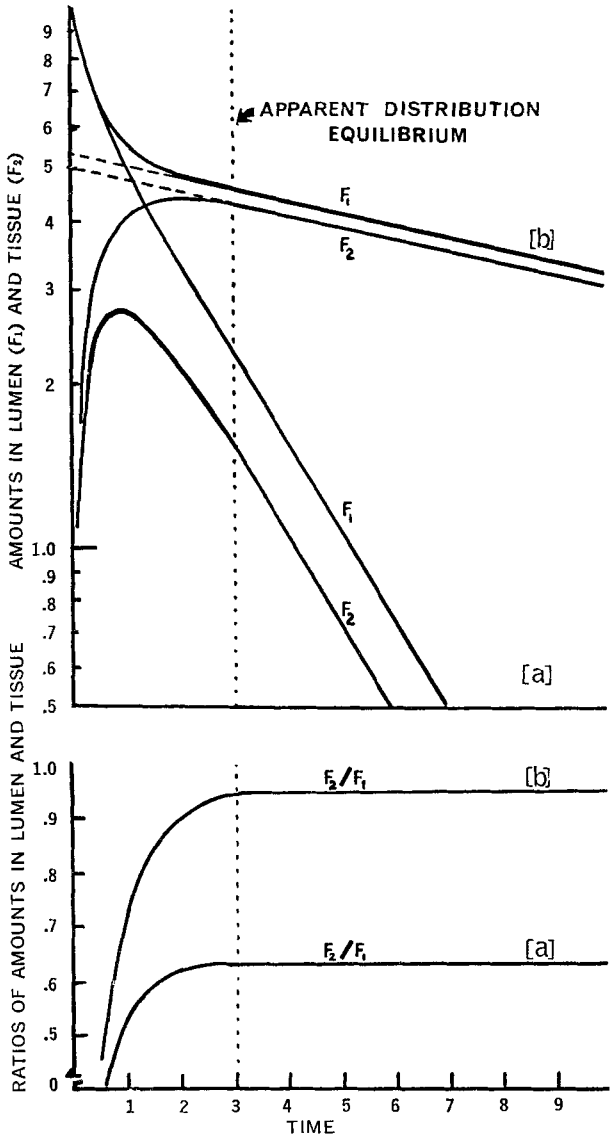


Figure 13—*Illustration of apparent distribution equilibrium by analog computer model when tissue accumulation occurs. The top curves illustrate the parallel slopes for lumen and tissue compartments in the monoexponential phase in which distribution equilibrium, defined by* $dF_2/dt = R(dF_1/dt)$, *exists where* R, *the ratio between* F_2 *and* F_1 *becomes constant as shown in the bottom curves for these cases. Rate constants (unit time*$^{-1}$*) for Case* (a) *were* k_{12} (*0.5*), k_{21} (*0.5*), k_{23} (*0.1*), *and Case* (b) k_{12} (*0.5*), k_{21} (*0.5*), k_{23} (*0.05*). *Metabolism is considered negligible* ($k_{fg} = 0$).

SUMMARY AND CONCLUSIONS

Most previous studies on mechanisms of drug absorption have used *in vivo* methods which sample only disappearance of drug from the lumen rather than appearance of drug across the basal barrier into the blood. When transport across the basal barrier has been followed, it has been with *in vitro* preparations with nonfunctional blood circulation such as the isolated perfused intestine or everted intestinal sacs.

In the present study, both the rate of appearance of free salicylamide and salicylamide glucuronide into the mesenteric blood were measured directly by cannulating the mesenteric vein of exposed rabbit intestine and collecting all venous blood draining from the absorbing region. Using this technique, it was shown that the usual *in vivo* measurements on the disappearance of drug from the lumen alone would be inadequate to characterize the appearance of free drug in the mesenteric blood due to accumulation and metabolism of drug in the tissues and the effect of mesenteric blood flow.

The effect of intestinal glucuronide conjugation was found in this study to be quite significant, *in vivo*. As much as 60% of the drug appeared in the blood as the biologically inactive glucuronide.

When significant metabolism of the drug in the tissue occurs, measurements on the lumen alone will not be sufficient to predict the rate of appearance of free drug in the blood. When metabolism is capacity limited (*i.e.*, shifts from first-order to zero-order) the apparent mechanism of absorption of free drug might erroneously appear to proceed by two different mechanisms if only disappearance from the lumen is followed. The disappearance from the lumen curves, at low doses, indicated that an apparent barrier model was applicable with no accumulation of free drug in the tissue. At larger initial doses, the glucuronide-forming system appeared to be saturated and kinetic evidence indicated accumulation of free drug, characteristic of the compartment model for absorption. Studies on tissue levels are now in progress to further evaluate this conclusion.

The mesenteric blood flow plays a significant role in the *in vivo* preparation. Comparison of the *in vitro* and *in vivo* lag times shows that the presence of an intact mesenteric blood circulation in the *in vivo* preparation reduces the lag time for appearance in the terminal compartment from about 10 min. to about 4 min. presumably by obviating the extra diffusional barriers or reservoirs beyond the lamina propria which are present in the *in vitro* preparation. In addition to circumventing the submucosa and muscularis barriers, the rate of blood flow is also shown to play a role in the rate of transport of free drug across the basal barrier. The rate of transport of free drug across the *in vivo* basal barrier was clearly blood flow rate-limited. The rate of transport of the more polar glucuronide across the basal barrier appeared to be diffusion rate-limited and independent of the rate of blood flow. The *in vivo* rates of transport of free drug was five to ten times as great as those observed at the same initial mucosal concentration in the *in vitro* preparation.

In vitro methods such as the everted intestinal sac may provide an indication of relative permeabilities. However, permeability constants obtained with *in vitro* methods will not necessarily reflect the permeabilities of the tissues *in vivo* because of the extra barriers involved. *In vitro* procedures may also give useful information on capacity of the intestinal mucosal enzymes to metabolize drugs providing attention is given to the effect of experimental variables associated with this type of preparation. For example, the method of oxygenation, serosal sampling, and fluid loss due to peristalsis were found to greatly affect serosal glucuronide concentration.

In conclusion, the barrier model, which assumes that the intestinal tissue barrier does not alter or contribute to the material balance of the system, should not be assumed in contemplating experimental design. Sampling from the lumen alone may be unsatisfactory to predict the rate of free drug in the mesenteric blood which is the most significant criterion for assessment of absorptive capacity. Methods which measure the rate of unaltered drug across the basal barrier, such as the intestinal loop with complete mesenteric blood collection, provide a useful experimental approach in studying the physiologic factors of absorption such as tissue accumulation, metabolism, and blood flow.

REFERENCES

(1) "Methods in Medical Research," vol. 9, J. H. Quastel, Ed., Year Book Medical Publishers, Chicago, Ill., pp. 255, 273.

(2) T. H. Wilson and G. Wiseman, *J. Physiol.*, **123,** 116(1954).

(3) G. Levy and W. J. Jusko, *J. Pharm. Sci.*, **54,** 216(1965).

(4) G. Levy and T. Matsuzawa, *ibid.*, **54,** 1003(1965).

(5) F. J. Dea, S. A. Maurer, and J. Biles, presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, 1967.

(6) D. Schachter, D. J. Kass, and T. J. Lamnon, *J. Biol. Chem.*, **234,** 201(1959).

(7) R. Herz, Jr., D. Tapley, and J. R. Ross, *Biochim. Biophys. Acta*, **53,** 273(1961).

(8) M. J. Kreck, F. G. Guggenheim, J. E. Ross, and D. F. Tapley, *ibid.*, **74,** 418(1963).

(9) D. M. Matthews and D. H. Smyth, *J. Physiol. (London)*, **126,** 96(1954).

(10) R. M. Atkinson, B. J. Parsons, and D. M. Smyth, *J. Physiol.*, **135,** 581(1957).

(11) J. Y. Kiyasu and I. L. Chaikoff, *J. Biol. Chem.*, **224,** 935 (1957).

(12) E. Diczfalusy, C. Franksson, and B. Martinsen, *Acta Endocrinol.*, **38,** 59(1961).

(13) H. W. Davenport, "Physiology of the Digestive Tract," 2nd ed., Year Book Medical Publishers, Chicago, Ill., 1966.

(14) A. Agren, *Acta Chim. Scand.*, **9,** 49(1955).

(15) R. K. Crane and T. H. Wilson, *J. Appl. Physiol.*, **12,** 145 (1958).

(16) W. W. Umbreit, R. H. Burris, and J. F. Stauffer, "Manometric Techniques," 4th ed., Burges, Minneapolis, Minn., 1964.

(17) L. E. Jervis and D. H. Smyth, *J. Physiol.*, **144,** 21(1958).

(18) R. Chambers and B. W. Zweifack, *Am. J. Anat.*, **75,** 173 (1944).

(19) B. W. Zweifack, "Functional Behavior of the Microcirculation," Charles C Thomas, Springfield, Ill., 1961.

(20) L. S. Schanker, *J. Med. Pharm. Chem.*, **2,** 343(1960).

(21) D. H. Smyth, "Absorption and Distribution of Drugs," T. B. Binns, Ed., Williams & Wilkins, Baltimore, Md. 1964, p. 1.

(22) C. A. M. Hogben, *Federation Proc.*, **19,** 864(1960).

(23) "Methods of Animal Experimentation," vol. 1, W. I. Gay, Ed., Academic, New York, N. Y., 1965.

(24) R. F. Crampton and D. M. Mathews, in "Absorption and Distribution of Drugs," T. B. Binns, Ed., Williams & Wilkins, Baltimore, Md., 1964, p. 49.

(25) Y. Gressel, A. Tye, and J. W. Nelson, "An *In Vitro* Study of Intestinal Transfer," presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, March 1962, reprint B-1.

(26) G. Levy, *Arch. Intern. Pharmacodyn.*, **152,** 59(1964).

(27) J. G. Wagner, "Design and Analysis of Biopharmaceutical Studies in Man," Proceedings of the Industrial Pharmacy Section, APhA Academy of Pharmaceutical Sciences, Dallas meeting, April 1966, p. 20.

(28) V. M. Whitehead and B. A. Cooper, *Brit. J. Haematol.*, **13,** 679(1967).

(29) T. H. Wilson, "Intestinal Absorption," W. B. Saunders Philadelphia, Pa., 1962.

(30) J. Crank, "The Mathematics of Diffusion," Oxford University Press, London, England, 1956.

(31) A. Resigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Waltham, Mass., 1966.

(32) D. S. Riggs, "The Mathematical Approach to Physiological Problems," Williams & Wilkins, Baltimore, Md., 1963.

(33) J. R. Pappenheimer, E. M. Renkin, and L. M. Borrero, *Am. J. Physiol.*, **167,** 13(1951).

(34) A. V. Wolf, D. G. Romp, J. E. Kiley, and G. D. Currie, *J. Clin. Invest.*, **30,** 1062(1951).

(35) S. S. Kety, *Pharmacol. Rev.*, **3,** 1(1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 31, 1968, from the *Schools of Pharmacy at the State University of New York at Buffalo* and the University of California Medical Center at San Francisco†.*

Accepted for publication August 28, 1969.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported in part by grant number T51-CM 728-C5 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md. and grant C-R-68-UB-7 from the United Health Foundation of Western New York.

The authors wish to thank Dr. Frederick Meyers for assistance in developing the *in vivo* cannulated intestinal loop preparation and Mrs. Darlene Tisdale for technical assistance.

Intestinal Drug Absorption and Metabolism II: Kinetic Aspects of Intestinal Glucuronide Conjugation

WILLIAM H. BARR* and S. RIEGELMAN†

Abstract □ Mechanisms of intestinal salicylamide glucuronide formation and transport were studied in the rabbit with *in vitro* cannulated everted intestines and *in vivo* perfused and closed loops with complete mesenteric venous blood collection. Both experimental techniques indicate that glucuronide formation is capacity limited when the lumen concentration exceeds 10^{-3} *M*. The glucuronide-to-free drug ratio appearing in mesenteric blood (*in vivo*) and serosal fluid (*in vitro*) decreases with increasing lumen concentrations. Appearance of glucuronide across the basal barrier is limited by the transport step rather than the rate of glucuronide synthesis leading to accumulation of glucuronide in the tissue compartment. Transport of glucuronide appears to be a simple first-order diffusion process into the lumen contents as well as the mesenteric blood. Some implications of these findings in pharmacokinetic studies and dosage form design are discussed.

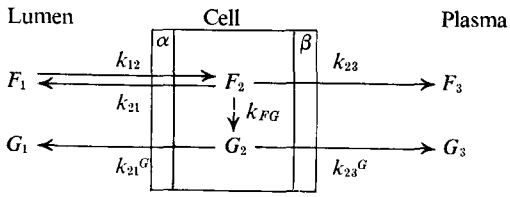
Keyphrases □ Drug absorption, metabolism—intestinal □ Salicylamide glucuronide formation, transport—intestinal □ Everted intestinal sacs—experimental technique □ Intestinal loop—*in vivo* experimental technique □ Conjugation, intestinal—salicylamide, glucuronic acid

The absorbing columnar cell of the intestine appears to be equipped with a wide variety of enzyme systems capable of metabolizing drugs. It has been shown as early as 1952 that *in vitro* intestinal slices can conjugate phenolic drugs with glucuronic acid (1). Glucuronide formation in the intestine has been shown *in vitro* for a large number of compounds including salicylic acid, salicylamide, anthranilic acid, thyroxine analogs, *p*-nitrophenol, *o*-aminophenol, testosterone derivatives, and estrogens (1–6). Sulfate conjugation also occurs in intestinal slices (8, 9). Recently, Hartiala *et al.* have shown that intestinal acetylation of sulfonamides can occur *in vitro* (10) and the work of Tapley *et al.* (6, 7, 11) indicates that reduction, hydroxylation, and oxidation of steroids can also take place in the intestinal mucosa.

The extent to which these metabolic transformations can affect the physiologic availability of an orally administered drug *in vivo* is almost completely unknown. Some information on this point has been recently obtained by use of a technique which allows complete collection of all mesenteric blood draining the absorption site of an intestinal loop. It has been shown by this method that an appreciable amount of salicylamide is absorbed *in vivo* as the glucuronide (12). That work also indicated that the appearance of the salicylamide glucuronide in the plasma was diffusion limited rather than blood flow limited and that the system could be saturated, giving capacity-limited (pseudo-zero-order) kinetics for the appearance of glucuronide in the plasma.

It is the purpose of this report to present further information on the manner in which intestinal metabolism may affect the *in vivo* absorption process using glucuronic acid conjugation of salicylamide as a model.

The mechanism of glucuronide synthesis and transport may be considered from the standpoint of the previously proposed cell compartment model (12):



where F_1,F_2,F_3 represent the amount of free drug in the lumen, tissue, and plasma (or serosal fluid *in vitro*), respectively; G_1,G_2,G_3 represent the amounts of glucuronide in the lumen, cell, and plasma (or serosal fluid *in vitro*), respectively; k_{mn} represents the rate constant for amount of free drug transferred from Compartment *m* to Compartment *n*. For example, k_{23} is the rate constant for transfer from Compartment 2 (the tissue) to the plasma or serosal fluid (Compartment 3); $k_{mn}{}^G$ represents the rate constant for the transfer of amount of glucuronide from Compartment *m* to Compartment *n*; k_{FG} represents the rate constant for the metabolism of amount of free drug to glucuronide; α and β refer to the apical and basal barriers, respectively.

EXPERIMENTAL

The methods used in this study are the same as used in the previous report in this series (12). Free salicylamide was determined by extraction into a 1,2-dichloroethane–cyclohexane mixture (65:35) from which it is reextracted into 0.2 *N* sodium hydroxide solution. The basic solution is read directly in an Aminco-Bowman spectrophotofluorometer at excitation and emission wavelengths of 350 and 430 mμ, respectively (uncorrected). Salicylamide glucuronide is determined as the total salicylamide after hydrolysis with bacterial β-glucuronidase less the free salicylamide.

All intestinal studies were done with the midileal portion of the intestine from male New Zealand rabbits weighing between 2.0 and 2.5 kg. Everted intestinal sacs for fixed time and kinetic studies were prepared as before (12) and oxygenated by an O_2–CO_2 (95:5) gas mixture bubbled directly into Krebs-Ringer bicarbonate mucosal fluid.

The *in vivo* preparation with complete venous collection was prepared by cannulating the mesenteric vein serving the intestinal loop which was either open and perfused or closed containing solutions of salicylamide in Krebs-Ringer bicarbonate solution (12).

RESULTS

Capacity-Limited Glucuronide Appearance—If metabolism in the cell follows first-order kinetics, the ratio of free drug to metabolized drug appearing in the plasma (*in vivo*) or serosal fluid (*in vitro*) should be constant and independent of the initial dose or mucosal concentration. It can be shown by both *in vitro* and *in vivo* experimental techniques that the glucuronide-to-free drug ratio is not constant and that the appearance of glucuronide in plasma is capacity limited.

Figures 1*a* and 1*b* illustrate the cumulative amounts of glucuronide and free drug appearing in the serosal fluid in everted intes-

tines containing mucosal concentrations of 10^{-4} and 10^{-3} *M* of salicylamide, respectively. When the mucosal concentration is low (10^{-4} *M*), the amount and rate of glucuronide appearing in the serosal fluid exceed the free drug. When a higher mucosal concentration is used (10^{-3} *M*), the glucuronide exhibits the same prolonged lag time but the rate and amount of glucuronide appearing in the serosal fluid are always less than that of the free drug.

Figure 2 illustrates the cumulative amounts of glucuronide appearing in the plasma in the *in vivo* intestinal loops with complete venous collection perfused with 10^{-4}, 10^{-3}, and 10^{-2} *M* salicylamide. The rates of appearance of glucuronide in the plasma as measured by the slope of the cumulative amount-time curve (or the cumulative amounts of glucuronide after 60 min.) do not differ greatly[1] even though the mucosal concentration varies over a hundredfold

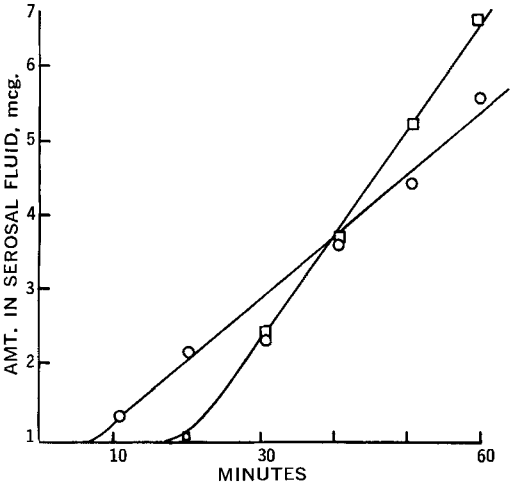


Figure 1a—*The cumulative amount of free drug* (○) *and glucuronide* (□) *in the serosal fluid of the cannulated* in vitro *everted intestine with an initial mucosal concentration of* 10^{-4} M *SAM.*

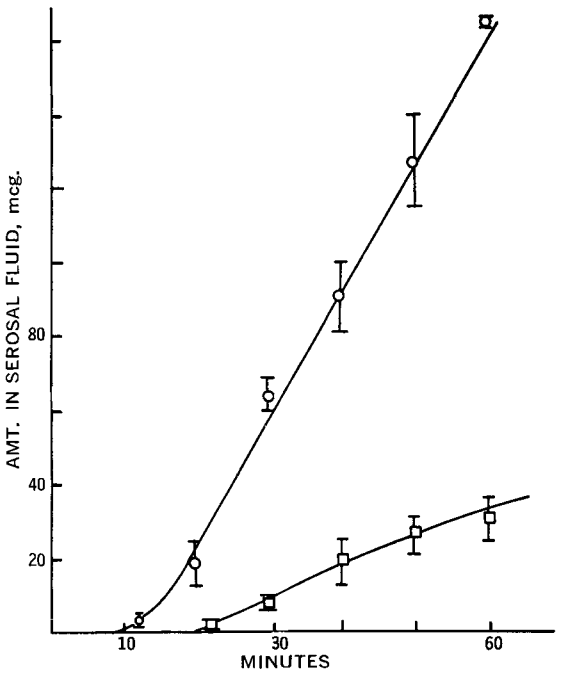


Figure 1b—*The cumulative amount of free drug* (○) *and glucuronide* (□) *in the serosal fluid of the cannulated* in vitro *everted intestine with an initial mucosal concentration of* 10^{-3} M *SAM. Bars indicate the range of two experiments.*

[1] It is difficult to exactly standardize the length of intestine and control the mean rate of blood flow in different experiments which causes some variation in the total amount of metabolite recovered in a set time. This is the probable explanation for the lower amounts of glucuronide obtained with 10^{-2} *M* perfusion shown in Fig. 2 rather than inhibition of glucuronide formation at higher concentrations of salicylamide.

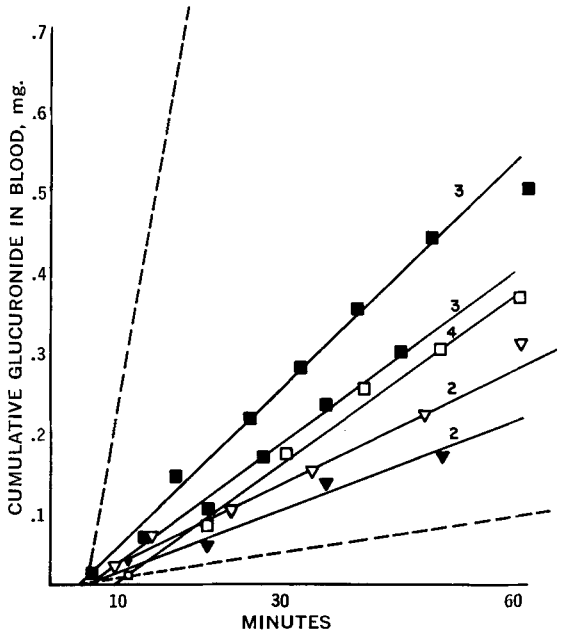


Figure 2—*Cumulative amounts of glucuronide appearing in the mesenteric blood in the* in vivo *preparation perfused with* 10^{-4} M (□), 10^{-3} M (■), *and* 10^{-2} M (△) *SAM. Broken lines show the range of amounts of free drug appearing in mesenteric blood perfused with* 10^{-4} M (*lower broken line*) *and* 10^{-2} M (*upper broken line*) *solutions of SAM.*

range. The amounts of free drug appearing in the plasma, shown by the dotted lines for comparison, do show great variations in the slope as would be expected. These points are also illustrated by Fig. 3 which shows the mean rates of appearance in plasma for free drug and glucuronide as a function of the perfusion concentration. These data provide evidence that appearance of glucuronide in the plasma or serosal fluid is capacity limited in both *in vitro* and *in vivo* experimental situations. Figure 4 compares the glucuronide-to-free drug ratio after 1 hr. in the plasma for the *in vivo* procedure and the serosal fluid for the *in vitro* procedure. The parallel lines suggest that the *in vitro* everted intestines serve well to characterize the glucuronide-to-free drug ratio that might be observed *in vivo*.

Implications of a Capacity-Limited System—The data obtained from different experimental procedures, both *in vitro* and *in vivo*, clearly indicate that the appearance of glucuronide in serosal fluid

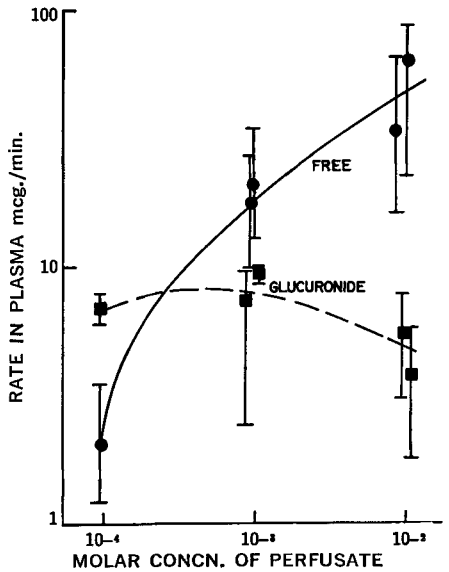


Figure 3—*Comparison of mean rates of appearance in mesenteric blood of free drug* (●) *and glucuronide* (■) *obtained with* in vivo *preparations perfused with* 10^{-4}, 10^{-3}, *and* 10^{-2} M *solutions of SAM. Each point represents the mean rate of a separate experiment. Bars represent the range of rates for each separate experiment.*

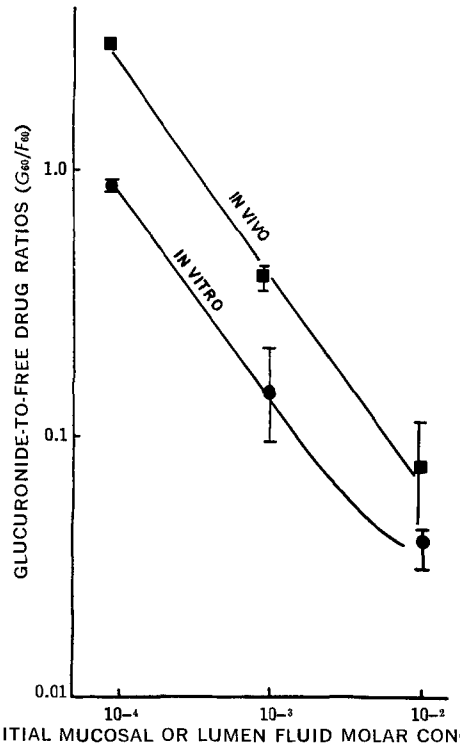


Figure 4—*Illustrates the ratios of the cumulative amounts of glucuronide to the cumulative amounts of free drug* (G_{60}/F_{60}) *appearing in the plasma after 1 hr. in the* in vivo *preparation perfused with* 10^{-2}, 10^{-3}, *and* 10^{-4} M *SAM* (■) *and the glucuronide-to-free drug ratio in the serosal fluid of the* in vitro *everted intestine with initial mucosal fluid concentrations of* 10^{-2}, 10^{-3}, *and* 10^{-4} M *SAM* (○). *Bars indicate the range of two experiments.*

or plasma is independent of the concentration of free drug in the lumen compartment at lumen concentrations greater than 10^{-3} *M*.

There are several possible physiologic mechanisms that might be postulated to account for this zero-order kinetic behavior. The two most likely possibilities are either capacity-limited glucuronide formation or capacity-limited transport of glucuronide out of the cell. The formation of glucuronide could be rate limiting if the amount of enzyme, glucuronyl transferase, responsible for coupling UDP-glucuronic acid and the phenol is limited. It is also possible that the amount of the cofactor, UDP-glucuronic acid, is limited due to small amounts present in the cell because of a slow rate of synthesis by the DPN-dependent oxidation of UDP-glucose or rapid breakdown by UDP-glucuronic acid pyrophosphatase (4).

The alternate possibility is that the transport of the polar glucuronide out of the cell is capacity limited. This would imply that some sort of carrier transport mechanism or active transport is swamped at high cellular concentrations of the glucuronide.

Appearance of Glucuronide in Lumen—Because of the extra diffusion barriers of the submucosal and muscularis layers on the basal side of the *in vitro* everted intestine, it might be expected that greater amounts of glucuronide would diffuse into the mucosal fluid than the serosal fluid if passive diffusion was the limiting factor of glucuronide transport. Representative data presented in Table I, which compares the amounts of glucuronide in mucosal fluid and serosal fluid after 1-hr. incubation, show that this is indeed the case. The amount of glucuronide in mucosal fluid is from 2 to 5 times greater than that in the serosal fluid. The relative permeabilities of the mucosal barrier and serosal barrier obtained from the *in vitro* everted intestine are probably not representative of the relative permeabilities of the mucosal and plasma barriers in the normal *in vivo* absorption process where the drug does not traverse the submucosal and muscularis layers. The fact that significant amounts of glucuronide formed in the cell actually appear in the lumen may be indicative of the *in vivo* process, which would be of considerable importance. If this phenomenon occurs in the normal absorption process, a reduction in the net amount of drug absorbed would occur since the drug present as the polar glucuronide is not

Table I—Comparison of Amounts of SAM Glucuronide Appearing in the Mucosal and Serosal Fluid of Everted Intestines after 1-hr. Incubation at 37°

No.	Initial Mucosal Concentration	Amount (mcg.) in Serosal Fluid	Amount (mcg.) in Mucosal Fluid	Ratio (S/M)
1	10^{-4} *M*	36.3	151.0	0.24
2	10^{-4} *M*	29.8	50.3	0.59
3	10^{-4} *M*	28.5	168.0	0.17
4	10^{-3} *M*	49.1	156.0	0.31
5	10^{-3} *M*	25.1	133.0	0.19

absorbed unless hydrolyzed (3, 13, 14). These considerations prompted the following studies to determine the relative amounts of glucuronide which appear in the lumen fluid *in vivo*.

***In Vivo* Lumen-Plasma Glucuronide Ratios**—To obtain an indication of the amount of lumen glucuronide and the relative permeabilities of the apical and basal barriers *in vivo*, the cannulated preparation with complete venous collection was employed. There are certain difficulties in obtaining true *in vivo* glucuronide ratios even with this preparation. First, hydrolysis of the glucuronide in mucosal fluid and possibly in the plasma complicates quantitation of the true total amount of glucuronide delivered to each fluid. Second, the concentration of glucuronide in the blood depends on the distribution ratio between plasma and red blood cells which is not known. Salicylamide (free) is equally distributed between the erythrocytes and plasma. It is assumed, on the basis of its low permeability to other cells, that the glucuronide does not enter the erythrocyte.

The concentration of the glucuronide in the lumen fluid cannot be accurately determined by the difference assay when the ratio of free drug to glucuronide is greater than 100. For this reason, it proved impractical to use a perfused intestine which maintains high concentrations of free drug. Closed loops were therefore used, and the cumulative amount of glucuronide in the mucosal fluid was obtained by multiplying the concentration by the fixed fluid volume. The cumulative amount of glucuronide in the blood was estimated by multiplying the volume of plasma collected by the concentration of glucuronide in the plasma, assuming that no glucuronide was present in the erythrocytes. The amount of free drug in the blood was obtained by multiplying the plasma concentration by the total blood volume as the free drug was found to be equally distributed between the plasma and erythrocytes. The cumulative amounts of glucuronide appearing in lumen fluid and plasma are shown in Fig. 5. It is seen that the amount of glucuronide in the

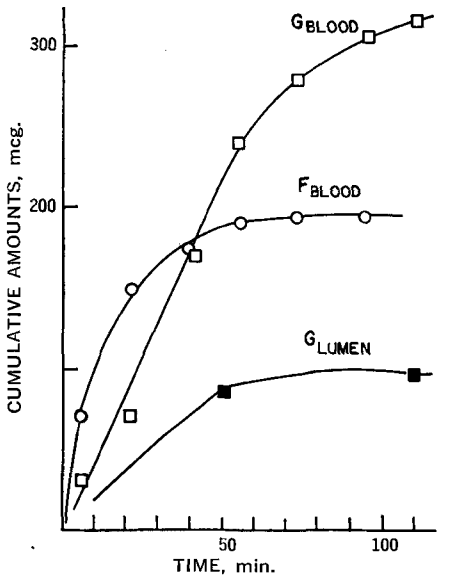


Figure 5—*Cumulative amounts of free drug in blood* (○), *glucuronide in blood* (□), *and glucuronide in lumen fluid* (■) *of a closed loop in* in vivo *preparation containing 10 ml. of* 10^{-3} M *salicylamide in Krebs-Ringer bicarbonate solution.*

mucosal fluid is significant but does not exceed the amount in the plasma. The ratio of plasma glucuronide to lumen glucuronide is from 3 to 5.

The occurrence of glucuronide in the lumen in the *in vivo* cannulated loop gives reasonable evidence that significant amounts of drug metabolites may be expected to be found in lumen during the normal absorption process in the intact animal. This may be an important consideration in explaining the presence of some drug metabolites in the lumen which has previously been assumed to be a consequence of enterohepatic cycling and biliary secretion.

It must be realized, however, that some mucosal glucuronide may be a result of mucosal cell disruption either as a result of experimental manipulation or the normal sloughing of intestinal mucosa. The latter process occurs to a high degree in normal intestinal function. The normal turnover time of intestinal epithelium is quite rapid (15).

Relative Rates of Glucuronide Synthesis and Transport—The cumulative amounts appearing in the plasma of free drug and glucuronide shown in Fig. 5 show that glucuronide is still appearing in the plasma while free drug is approaching an asymptote. This suggests that accumulation of glucuronide in the cell is occurring, which would indicate that the rate of synthesis of the glucuronide is greater than subsequent transport out of the cell ($k_{FG} \gg k_{21}^{G}$, k_{23}^{G}). Accumulation of glucuronide in the cell compartment is also suggested for *in vitro* intestinal preparations by the cumulative amounts appearing in the serosal fluid of the *in vitro* everted sacs shown in Fig. 1 and the data given by Herz *et al.* (5).

Further evidence for accumulation of metabolite in the cell, *in vivo*, can be obtained by comparing the rates of appearance in plasma of free drug and glucuronide as shown in Fig. 6. Several facts from this curve support the conclusion that the rate of glucuronide transport out of the cell rather than the rate of metabolism in the cell is the rate-limiting step for the appearance of glucuronide in the plasma. First, the linear terminal portions of the curve for both free drug and glucuronide indicate that exit from the cell is first-order and therefore proportional to the concentration in the cell. At time 60 min., the amount of free drug in the cell has dropped to 1/100th of its original concentration. The concentration of glucuronide in the cell at this time, however, is still quite high even though most of the free drug in the cell is gone. Second, if metabolism was the only rate-limiting step, the precursor-successor relationship (16) would predict that the precursor free drug curve should intersect the peak of the successor glucuronide curve and both curves would then decline with parallel slopes. The delay in the glucuronide peak requires an additional rate-limiting step in the model. The increase in the slope of the glucuronide curve compared to the slope of the free drug then reflects the slower diffusion step out of the cell for the glucuronide.

Active or Passive Transport of Glucuronide—The data presented in previous sections do not require an active transport process for the glucuronide. All data seem adequately explicable by rapid first-order or zero-order cellular glucuronide formation followed by a slow diffusion process proportional to the amount of glucuronide in the cell. This does not rule out an active transport system entirely. If such a process was present but saturated at low levels, the result would be a simultaneous mixed zero-order–first-order process, which at high concentrations of glucuronide in the cell would give the appearance of an apparent first-order process. Further work is necessary to determine the mechanisms involved in the transport of glucuronide out of the cell.

DISCUSSION

If the results of these studies on intestinal glucuronide formation and absorption of salicylamide in rabbits apply to man, there would be several important pharmaceutical and clinical implications to be considered. There are reasons to believe that these results may, in fact, be applicable to man. It has been shown that *in vitro* human intestinal slices produce comparable amounts of salicylic acid glucuronide to that formed by rabbit intestine (3). It has also been shown *in vivo* that human intestine forms steroid glucuronides (17). Analysis of the kinetics of glucuronide formation following oral doses of salicylamide in man suggests that intestinal metabolism may be a factor in the low plasma levels of free drug obtained after oral administration, even though absorption appears to be complete. Furthermore, as in rabbits, the process appears to be capacity limited at higher doses (18).

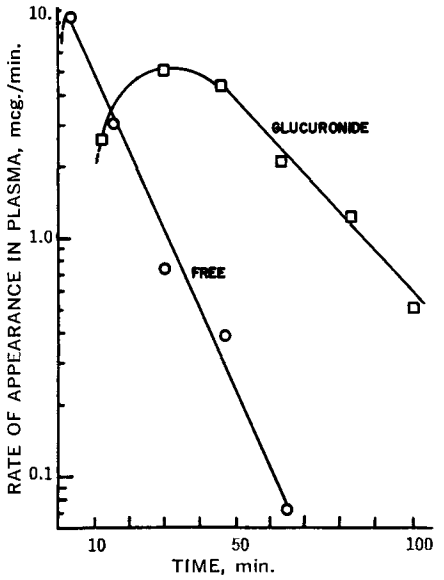


Figure 6—*Comparison of the rates of appearance in plasma of free drug* (○) *and glucuronide* (□) *in a closed loop* in vivo *preparation. These data are from the same experiment shown in Fig. 5.*

The extrapolation of this work to man offers many interesting implications.

(1) Intestinal metabolism may offer an alternate explanation for many drugs which are said to be poorly absorbed. Drug assays involving extraction into organic solvents measure only free drug and may not detect the amount of drug absorbed as the polar metabolite. Several phenolic compounds are said to be poorly absorbed orally while their *o*-methylated or dehydroxy congeners are well absorbed, *e.g.*, morphine, phenylephrine, and isoproterenol. Decreased or erratic therapeutic effects following oral doses may be a consequence of intestinal metabolic inactivation rather than decreased absorption.

(2) Drug assays which measure only total drug in the plasma may also be inadequate to characterize the absorption process of free drug when intestinal metabolism is significant. The apparent rate constant of absorption as measured by total drug in the plasma will be decreased and the peak time delayed due to the slower diffusion of the metabolite (*i.e.*, Fig. 5).

(3) The fact that appreciable amounts of metabolite are released from the cell back into the intestine may also affect the rate and extent of absorption. A cycling process may occur in which the drug is absorbed and partially metabolized in the cell. A fraction of the metabolite is released back into the lumen where it is nonabsorbable unless it is hydrolyzed to the free drug by bacterial and endogenous enzymes. The free drug would be reabsorbed and the cycle repeated until all drug is absorbed or eliminated in the feces. If the hydrolyzing enzymes are localized in a particular portion of the intestine, hydrolysis of the metabolite may be delayed and a discontinuous absorption process observed.

(4) The total amount of drug absorbed may be decreased if the metabolite released back into the intestine is not completely hydrolyzed. Furthermore, if the metabolic process is capacity limited, one might expect that the fraction of the total dose which is absorbed into the systemic circulation, and subsequently recovered in the urine, would be less with small doses than larger doses.

(5) Several factors in formulation might be considered in light of the effect of lumen concentration on the glucuronide-to-free drug (G/F) ratio absorbed when intestinal metabolism is capacity limited. The best dosage form would be that which would give maximum availability in the shortest time such as a solution or a form with a rapid dissolution rate. A sustained-release form that delivered a low concentration over a long period of time could possibly be worthless because of the high glucuronide-to-free drug ratio seen at lower concentrations. Competitive inhibition of metabolism may occur and one drug of a combination formulation may modify the absorption of another (*e.g.*, aspirin, *N*-acetyl *para*-aminophenol and salicylamide).

(6) There are also important clinical consequences which deserve attention. The use of urinary excretion of drug glucuronides as an index of hepatic function is a common clinical procedure (19). It has recently been suggested that salicylamide would be an excellent choice for this purpose (20, 21). It is possibly unwise, however, to use salicylamide, or any other drug which is largely glucuronylated in the intestine, as a measure of the ability of the liver to form glucuronides. A further consideration is that intestinal glucuronide formation may be well developed in the intestine of the fetus and newborn before appreciable hepatic glucuronide formation is established (22). The role of the intestine in bilirubin glucuronide formation may be important but has not been clearly defined (22, 23). In cases of neonatal hyperbilirubinemia, caution should be exercised in determining hepatic conjugating ability by oral tolerance curves of compounds also metabolized in the intestine. Further information is necessary on the relative importance of intestinal and hepatic metabolism for both bilirubin and any proposed test drug.

(7) It is interesting that one of the first reports of intestinal glucuronide formation was by Hartiala (24) who found increased glucuronide in the portal blood after giving cinchophen. He used this finding to support his theory that cinchophen caused ulcers by forming glucuronides in the intestine and thus depleting glucuronic acid from the mucopolysaccharide protective layer. Actually the opposite may be true, and glucuronide formation may operate as a protective mechanism. Salicylamide forms large amounts of glucuronide in the intestine but produces no mucosal damage or ulceration (25). Aspirin forms relatively small amounts of glucuronide (3) and is well known to cause mucosal damage (26, 27).

SUMMARY

At concentrations below 10^{-3} *M*, a considerable fraction of salicylamide is rapidly conjugated with glucuronic acid in the intestinal mucosal cell and subsequently appears in the mesenteric blood in the *in vivo* preparation with complete mesenteric blood collection or the serosal fluid in the *in vitro* everted intestinal preparation. Appearance of salicylamide glucuronide in the plasma is rate limited by transport across the basal barrier rather than metabolism. Exit of glucuronide from the cell is bidirectional, appearing in the lumen contents as well as in the plasma by what appears to be first-order diffusion process.

The relative order of magnitude of the rate constants in the general compartment model of intestinal absorption for the *in vivo* preparation is $k_{FG} > k_{23} \gg k_{23^G} > k_{21^G}$. In the *in vitro* preparation greater amounts of glucuronide appear in the mucosal fluid than the serosal fluid due to the extra diffusional barriers of the submucosal and muscularis layers and $k_{23^G} < k_{21^G}$.

When lumen or mucosal fluid concentrations are greater than 10^{-3} *M*, the conjugating system is capacity limited leading to zero-order appearance of glucuronide. Some implications of these findings in dosage form design, pharmacokinetic studies, and clinical studies are discussed.

REFERENCES

(1) F. Zini, *Sperimentale*, **102,** 40(1952).

(2) K. J. W. Hartiala, *Acta Physiol. Scand.*, **31,** Suppl. **114,** 20(1954).

(3) D. Schachter, D. J. Kass, and T. J. Lannon, *J. Biol. Chem.*, **234,** 201(1959).

(4) T. A. Miettinen and E. Leskinen, *Biochem. Pharmacol.*, **12,** 565(1963).

(5) R. Herz, Jr., D. F. Tapley, and J. E. Ross, *Biochim. Biophys. Acta*, **53,** 273(1961).

(6) M. J. Kreek, F. G. Guggenheim, J. E. Ross, and D. F. Tapley, *ibid.*, **54,** 418(1963).

(7) F. R. Smith, D. F. Tapley, and J. E. Ross, *ibid.*, **69,** 68 (1963).

(8) W. D. Conway, H. Minatoya, M. Lands, and J. Shekowski, *J. Pharm. Sci.*, **57,** 1135(1968).

(9) H. Bostrom, *Scand. J. Clin. Invest.*, **17,** 33(1966).

(10) K. J. W. Hartiala and T. Terho, *Nature*, **205,** 809(1965).

(11) T. J. Stahl and D. F. Tapley, *Endocrinology*, **73,** 271(1963).

(12) W. H. Barr and S. Riegelman, *J. Pharm. Sci.*, **59,** 154(1970).

(13) G. Levy, L. Weintraub, T. Matsuzawa, and S. R. Oles, *ibid.*, **55,** 1319(1966).

(14) W. Vogt, G. Schmidt, and T. Dakhil, *Arch. Exptl. Pathol. Pharmacol.*, **250,** 488(1965).

(15) L. Laster and F. J. Ingelfinger, *New Engl. J. Med.*, **264,** 1138(1961).

(16) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Waltham, Mass., 1966.

(17) E. Diczfalusy, C. Franksson, and B. Martinsen, *Acta Endocrinol.*, **38,** 59(1961).

(18) W. H. Barr and S. Riegelman, to be published.

(19) I. Snapper and A. Saltzman, *Am. J. Med.*, **2,** 327(1947).

(20) H. T. F. Barniville and R. Misk, *Brit. Med. J.*, **1,** 337(1959).

(21) K. Tareta, *Acta Med. Okayama*, **16,** 129(1962).

(22) I. H. Stevenson and G. J. Dutton, *Biochem. J.*, **82,** 330 (1962).

(23) J. L. Bollman, *Gastroenterology*, **36,** 1313(1959).

(24) K. J. W. Hartiala, P. Leikkda, and P. Savola, *Acta Physiol. Scand.*, **42,** 36(1958).

(25) "Salicylamide," S. B. Penick & Co., New York, N. Y., 1959.

(26) P. Wood, E. Harvey-Smith, and A. Dixon, *Brit. Med. J.*, **1,** 669(1962).

(27) H. F. Lange, *Gastroenterology*, **33,** 778(1959).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 31, 1969, from the *Schools of Pharmacy at the State University of New York at Buffalo* and the University of California Medical Center at San Francisco†.*

Accepted for publication August 28, 1969.

In Vitro Dissolution from Several Experimental Capsule Formulations

JOSEPH C. SAMYN and WAN YIN JUNG*

Abstract □ The *in vitro* dissolution behavior of several experimental capsule formulations is presented. The first series of powder blends clearly demonstrated that the capsule lubricant, magnesium stearate, and the filler, lactose or dibasic calcium phosphate dihydrate, had the greatest influence on the disintegrations of the capsules. Additional blends of these ingredients with an added drug were studied. In those instances where a slow dissolution was observed, the capsule contents characteristically remained as a wet powder pack long after the gelatin wall had dissolved. The drug dissolution was thus limited by the rate of erosion, diffusion, and/or solution of the drug and/or filler from this wet powder mass. Auxiliary measurements of liquid penetration rates of the packed powders and water content and viscosities of the wet powder packs were performed. These data suggested that the high viscosities of several of these powder packs were due to their restricted water content arising from the added hydrophobic lubricant.

Keyphrases □ Capsules—*in vitro* dissolution □ Formulation components, effect—capsule dissolution □ Powder beds—liquid penetration rate □ Disintegration, capsules—lubricant, filler effects

Different dosage forms containing various drugs have been compared by several investigators (1–3). Reviews of the physical factors affecting drug availability have been published by Wagner (4) and Nelson (5). Wood (6) has considered the *in vitro* evaluation of the tablet in some detail. The influence of granulation, granule size, compressional load, particle size, and the presence of surfactants, lubricants, and other tablet additions on *in vitro* drug availability have been studied (7–13).

Capsule formulations have received considerably less attention than tablets, presumably because of their apparent simplicity and consideration as loose powders. Wood (14) pointed out that although the hard gelatin capsule is widely used in preliminary studies of a new drug, very little literature on drug availability, even *in vitro*, exists for this type of dosage form. He quoted the National Formulary XII (15) as follows: "Disintegration time limits are not specified for capsules, since the shell dissolves rapidly in the gastrointestinal tract."

He further reported the release of aspirin from hard gelatin capsules to be irregular and showed a mean delay time of about 15 min. The importance of particle dispersion from tablets and capsules has been reported (16, 17). The failure of the capsule contents to disperse during *in vitro* testing has also been reported. Calesnick *et al.* (2) stated that during their study, "the contents of the hard gelatin capsule remained as a pasty mass for some time after the gelatin capsule had dissolved." Aguiar *et al.* (18) reported a similar observation in the *in vitro* testing of several generic chloramphenicol preparations.

The method of assessing drug availability has also been the subject of much concern. The disintegration requirements of various official compendia have recently been reviewed and compared (19). Levy (20) and Steinberg (21) reviewed the medical literature and suggested that low agitation conditions should prevail in the *in vitro* dissolution studies. In addition, higher pH values than that of the USP simulated gastric fluid, pH 1.2, have also been reported in the stomach. Kuna (22) stated that the pH of the gastric juice of the human resting stomach is near neutral, pH 7.3.

The present study is concerned with the influence of commonly used pharmaceutical adjuvants on disintegration and drug dissolution from experimental capsule formulations. Capsule fillers employed were lactose and dibasic calcium phosphate dihydrate. The latter is water insoluble and acid soluble. Preliminary mixtures were made with different concentrations of talc, starch, and magnesium stearate. These preparations clearly indicated that the filler, lactose or dibasic calcium phosphate dihydrate, and the capsule lubricant, magnesium stearate, had the greatest influence on the disintegration and dissolution characteristics. A low concentration of a water-soluble dye was incorporated in these preparations to permit dissolution measurements.

More typically, capsules are formulated to contain a relatively high proportion of the drug moiety. Therefore, a second series of preparations were prepared in which a drug of moderate solubility and no dominant hydrophobic or hydrophilic characteristics was used as a test drug. It was incorporated at approximately two-thirds of the total capsule weight. The two capsule fillers, lactose and $CaHPO_4 \cdot 2H_2O$, and the lubricant, magnesium stearate, which were the dominant factors in the first study were also added.

As with the first series of powder blends, dissolution rates of capsules were measured. They were found to vary over a wide range. In those cases where the dissolution rates were slow, the capsule contents characteristically remained as a "slug" of wet powder; *i.e.*, after dissolution of the gelatin capsule walls, the powder, although wet, remained intact as one or a few large masses. Additional measurements of liquid penetration rates, water contents of the wet powder beds, and the viscosities of the wet powder beds were performed to aid in explaining this behavior. The data suggested that the wet powder beds remained essentially intact because of their high viscosities. It will be shown that these high viscosities resulted from a reduced amount of water in the wet bed due to the presence of hydrophobic magnesium stearate.

MATERIALS AND PROCEDURES

The capsule fillers used in this study were lactose USP No. 80-mesh powder and dibasic calcium phosphate dihydrate. The magnesium stearate, corn starch, and talc were each USP grade powders.

The dye tracer used at 0.1% level in the first portion of the study was F. D. & C. Red No. 1. The drug employed in the second

Table I—Powder Blends Studied Showing Disintegration Times for No. 2 Capsules Packed with These Powders

	Disintegration Time in H_2O, min. (6 Capsules) —% Magnesium Stearate—			
	0	1	2	5
Lactose	5–6	8–12[a]	10–19[a]	21–35[a]
Lactose–3% talc	6–11			
Lactose–10% talc	7–10		13–18[a]	29–31[a]
Lactose–10% starch	10–13		10–15[a]	
$CaHPO_4 \cdot 2H_2O$	8–12	>60[c]	>60[c]	>60[b]
$CaHPO_4 \cdot 2H_2O$–10% talc	10–15			
$CaHPO_4 \cdot 2H_2O$–5% starch	10–12		>60[c]	

[a] Capsule contents break up into small pieces which dissolve. [b] Capsule contents remain in capsule shape—powder does not wet. [c] Capsule contents remain in one or a few pieces which slowly dissolve or erode.

portion of the study was approximately 100-mesh chemical (90% through a No. 100-mesh screen and 50% through a 200-mesh). Dissolution test media easily wetted the drug. A powder blend of the drug with lactose yielded a 90% dissolution value in less than 5 min. under the test conditions employed. All of the powder mixtures were prepared by blending and screening the various ingredients. The low levels of magnesium stearate were added by serial dilutions. The No. 2 capsules were hand-filled to the desired weights.

Drug dissolution was measured in the USP disintegration apparatus without the use of disks. This is in keeping with the reduced agitation conditions suggested above and is essentially a return to the USP XV method (23). Test fluids employed were simulated gastric fluid (low pH) and water (neutral pH). All tests were run at 37 ± 1°. One capsule was added to 800 ml. of the selected media at zero time. Five-milliliter samples were withdrawn as a function of time and rapidly filtered through a type HA 0.45-μ Millipore[1] membrane held in a Swinney adapter.

All dilutions were made with distilled water and the amount of drug in solution was determined by visible or UV measurement on a Beckman DU spectrophotometer. The data were converted to percent drug dissolved.

The liquid penetration rate measurements and water contents of the various powder blends were determined by the method of Studebaker and Snow (24). A straight Plexiglas tube (1.25 cm. i.d., 12 cm. length) open at both ends was filled with 4.0 g. of the individual powder blends packed to a bulk density of 0.94 g./cm.[3] with the aid of the Fisher Sub-Sieve Sizer[2] compaction apparatus. This represented the same bulk density as in a No. 2 capsule. The packed tube, free of the packing plugs, was mounted in a vertical position. Eight milliliters of distilled water was added to the upper surface of the bed at zero time. The rate of movement of the water interface through the packed bed was measured with the aid of a Gaerdner cathotometer[3] except for the dibasic calcium phosphate powder blends at high magnesium stearate levels. These readings were made with a scale held up to the powder bed because insufficient visual contrast existed between the wet and unwet portions of the powder when viewed through the cathotometer. The additions of the 0.1% of a water-soluble dye to the first series of powder blends provided a clearer measure of the liquid interface since the dissolved dye migrated with the interface. Dye was not added to the second series of drug-filler-lubricant mixtures. These measurements were carried out at room temperature (no temperature controls). The data have been plotted as the square of the distance of penetration *versus* the elapsed time in agreement with the Washburn equation (25).

The water contents of the wet powder beds were determined on the samples after the completion of the liquid penetration rate measurements. The wet powder beds were removed from the Plexiglas tubes and segmented. Portions were immediately weighed and reweighed after overnight drying to constant weight. The water content was calculated based on the difference.

The comparative viscosities of the powder blends with varying amounts of water were measured with a Universal penetrometer.[4] Samples were prepared as follows: 40 g. of powder blend was mixed with 20 ml. of distilled water in a 250-ml. beaker. The wet mass was transferred to a glass conical container and packed to give a smooth surface. The penetrometer consisted of an inverted cone (30° and 90° double cone) and a rod with a combined weight of 150 g. The apex of the cone was adjusted so as to just touch the surface of the wet powder bed prior to release. The combined cone and rod assembly was allowed to free fall for a 5-sec. period into the wet mass. The depth of penetration of the cone was read in 0.1-mm. units on the attached scale. Several readings were made on each bed, remixing the wet mass and reforming the smooth surface each time. A portion of the wet powder bed was weighed, dried to constant weight, and reweighed to determine the water content; the remainder of the wet mass was mixed with additional water and the measurements repeated. By this procedure, a series of penetration measurements were obtained at various water contents.

[1] Marketed by Millipore Filter Corp., Bedford, Mass.
[2] Fisher Scientific Co., Pittsburgh, Pa.
[3] Gaerdner Scientific Co., Chicago, Ill.
[4] Precision Scientific Co., Chicago, Ill.

RESULTS AND DISCUSSION

The first series of powder blends using lactose or $CaHPO_4 \cdot 2H_2O$ as the filler with varying quantities of talc, starch, and magnesium stearate are listed in Table I. The lactose blends were filled to 350 mg.; the $CaHPO_4 \cdot 2H_2O$ to 400 mg. In addition, disintegration times—range values for six capsules—are shown. The values varied from the rapid, 5–6 min., to the very slow or nondisintegrating capsule, greater than 60 min. Magnesium stearate has the greatest influence on the disintegration times. The filler, lactose or $CaHPO_4 \cdot 2H_2O$, also appears to have a measurable effect on disintegration. The starch and the talc at the levels investigated in the blends with and without magnesium stearate did not markedly affect the disintegration times.

Dissolution data shown in Figs. 1 and 2 for several of these same blends confirm this interpretation. The physical nature of the wet capsule contents for several of these powder blends has been noted in Table I. The extended disintegration coincides with the observations that the capsule contents either remain intact or break up into a limited number of wet powder masses. In one instance the compacted powder mass did not wet. This occurred with the $CaHPO_4 \cdot 2H_2O$ plus 5% magnesium stearate blend.

Additional liquid penetration rate measurements were performed on these same powder blends. Figure 3 shows the decreased penetration rates measured for the lactose-magnesium stearate systems. Figure 4 shows the relatively slight decrease in liquid penetration caused by the addition of starch up to the 10% level. Similar data were obtained with the lactose–talc blends. In blends containing magnesium stearate, the influence of starch or talc on the liquid penetration rate was not detectable. This was true for both the lactose and the $CaHPO_4 \cdot 2H_2O$ systems. The data in Fig. 5 are for the $CaHPO_4 \cdot 2H_2O$–2% magnesium stearate blends with starch.

This first series of experiments has shown that magnesium stearate has the greatest influence on the disintegration/dissolution behavior of the various capsule blends. The nature of the filler has also been shown to be of importance.

The second portion of this study consisted of capsules prepared with a drug at a relatively high concentration (approximately

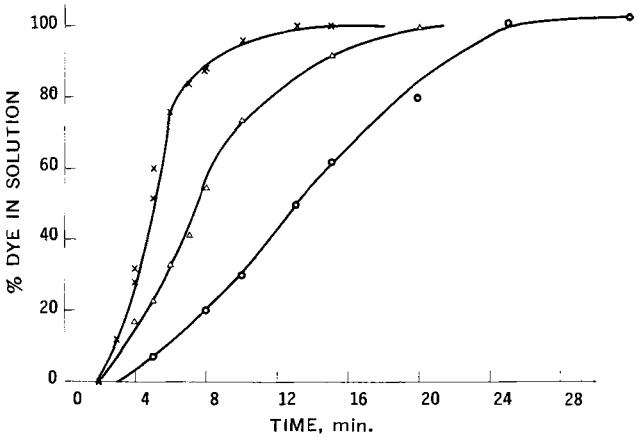


Figure 1—*Dissolution of dye from capsules of lactose–magnesium stearate. Key: ×, 0% magnesium stearate; △, 2% magnesium stearate; ○, 5% magnesium stearate.*

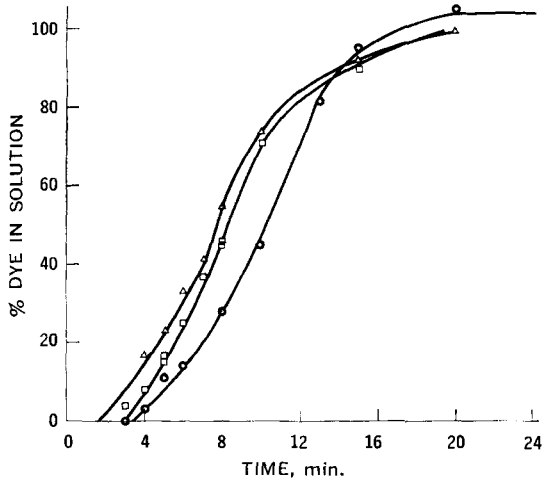


Figure 2—*Dissolution of dye from capsules (lactose + 2% magnesium stearate). Key: △, 0% corn starch; ○, 3% corn starch; □, 10% corn starch.*

two-thirds of total weight). The two fillers and the various levels of magnesium stearate found to be of importance in the first portion of the study were studied. The talc and starch were not included in these studies because of their apparently limited role. Dissolution behavior studies in both water and simulated gastric fluid were carried out. Disintegration times were noted in some of the figures.

The dissolution behavior of No. 2 capsules filled with the drug-lactose and drug-dibasic calcium phosphate dihydrate blends in water and in simulated gastric fluids are shown in Fig. 6. The dissolutions were rapid in all cases and essentially the same for the two different dissolution media and the two capsule fillers. The influence of 2% magnesium stearate on these two blends is shown in Fig. 7.

The slowest dissolution was observed for the drug-dibasic calcium phosphate–2% magnesium stearate system tested in water. This was due to the hydrophobic nature of the magnesium stearate and the insolubility of the calcium phosphate in the water. In simulated gastric fluid, this combination dissolved as fast as the comparable lactose system. In acid the dibasic calcium phosphate is soluble and the magnesium stearate is decomposed (26). The drug–lactose–2%

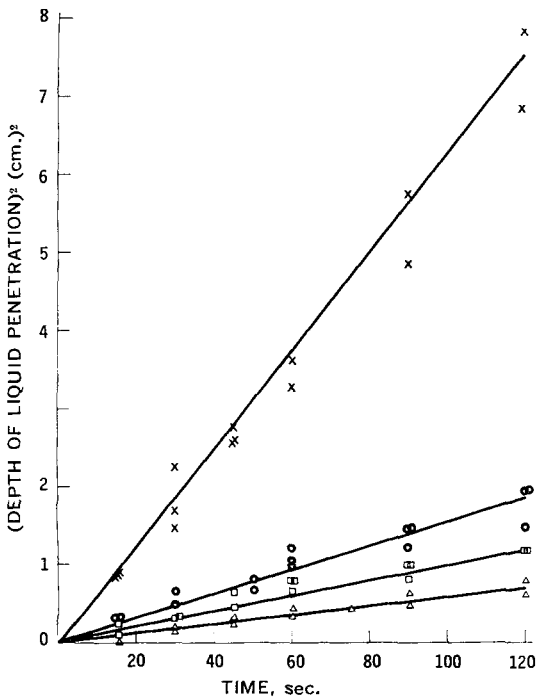


Figure 3—*Liquid penetration of packed powder beds. Key: ×, lactose; ○, lactose + 1% magnesium stearate; □, lactose + 2% magnesium stearate; △, lactose + 5% magnesium stearate.*

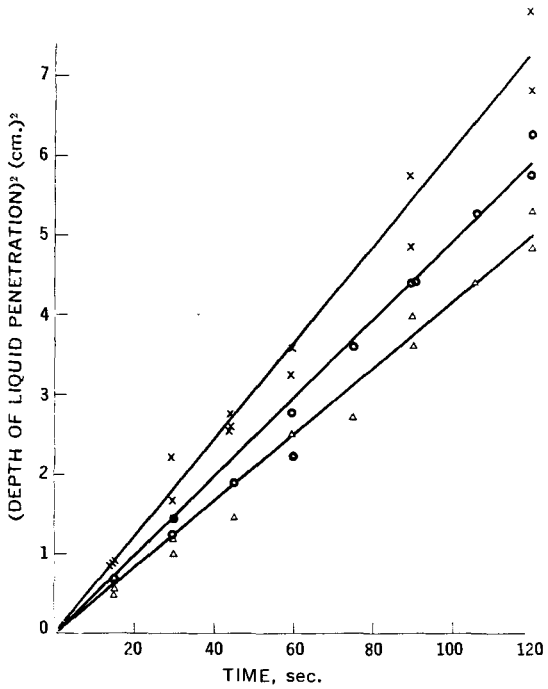


Figure 4—*Liquid penetration of packed powder beds. Key: ×, lactose; ○, lactose + 3% corn starch; △, lactose + 10% corn starch.*

magnesium stearate system also showed a difference in dissolution rate when tested in gastric fluid and water. The release in water, although slowed, was faster than the comparable dibasic calcium phosphate system. This probably was due to the water solubility of the lactose which permitted a faster erosion of the wet powder mass.

The magnesium stearate was further increased to 5% in both the lactose and dibasic calcium phosphate blends. In water these systems failed to wet after dissolution of the capsule walls. The powder remained as an essentially dry powder pack. Therefore, under these conditions, 5% magnesium stearate prevented dissolution when tested in water. In simulated gastric fluid, the powder beds did wet but remained as a wet mass during the dissolution process with erosion and dissolution slowly occurring. Figures 8 and 9 show the pronounced effect of this higher level of magnesium stearate with both fillers, even in simulated gastric fluid.

Figure 10 gives the dissolution data obtained in simulated gastric fluid by increasing the packing density in the No. 2 capsule with 0 and 5% magnesium stearate formulations (356 mg. *versus* 400 mg.). The data indicate that the packing density did not influence the dissolution rate in the absence of magnesium stearate. For the 5%

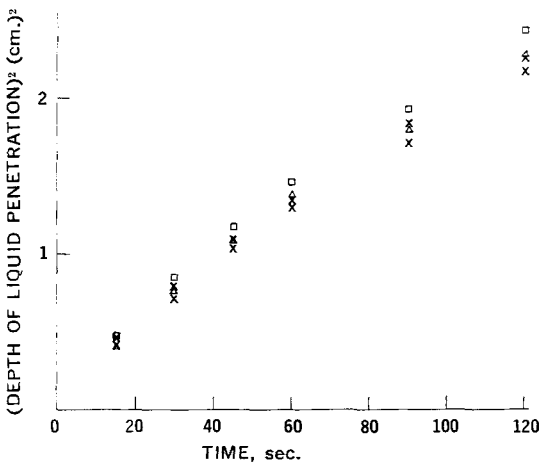


Figure 5—*Liquid penetration of packed powder beds. Key: ×, $CaHPO_4 \cdot 2H_2O$ + 2% magnesium stearate; △, $CaHPO_4 \cdot 2H_2O$ + 5% corn starch; □, $CaHPO_4 \cdot 2H_2O$ + 10% corn starch.*

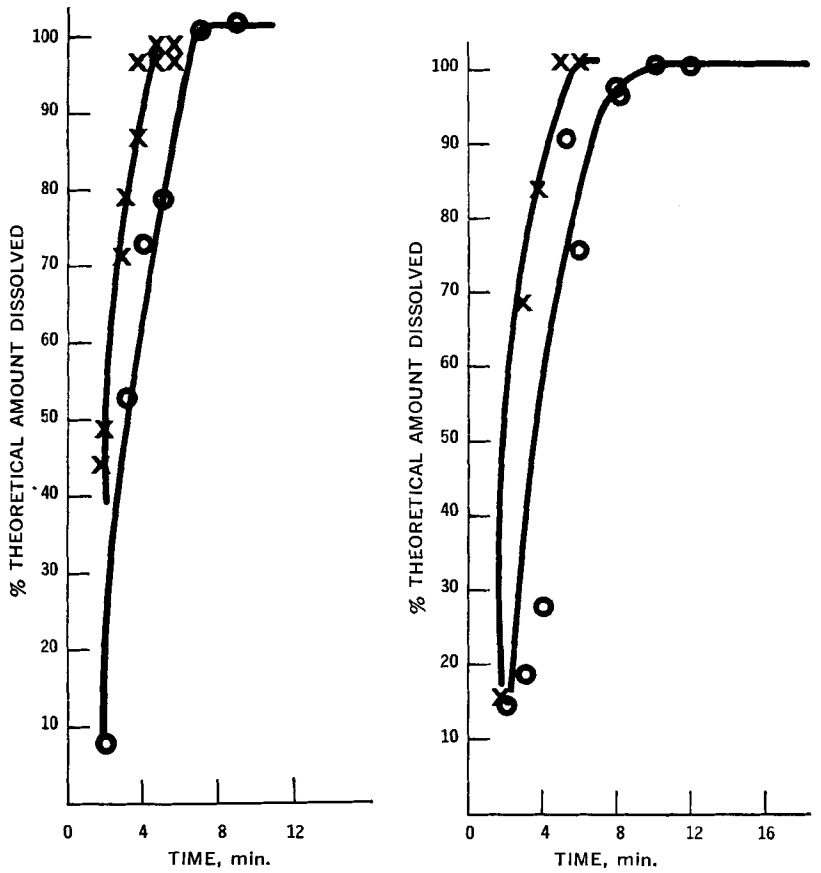


Figure 6—*Dissolution of drug from capsules; left, drug–lactose, no magnesium stearate; right, drug–$CaHPO_4 \cdot 2H_2O$, no magnesium stearate. Key: ×, simulated gastric fluid; ○, water.*

magnesium stearate samples, the 50% dissolution value did increase for both systems with increasing packing density; the dibasic calcium phosphate system showed the greatest increase of the 50% dissolution time, changing from about 7 min. to 23 min.

The behavior of the capsule blends shown in Fig. 6 indicates that the wettability and solubility of the drug used were adequate for rapid dissolution under these test conditions. In the majority of the systems in which the dissolution rate was slowed, the capsule contents—even after solution of the gelatin capsule walls—remained as an intact wet powder mass. Occasionally, this wet powder mass would break into more than one fragment. However, the dissolution was still essentially occurring from a very limited surface area. Because of these observations on the wet powder mass, further examination of the liquid penetration rates of the powder blends and water contents and viscosities of the wet powder beds were undertaken.

Due to the very limited size of the packed powder beds in an actual capsule, the measurements of liquid penetration, water content, and viscosities could more readily be carried out on larger samples of these powder blends. Using the method of Studebaker and Snow (24), the powder blends were therefore packed into open-ended cylindrical tubes for measurements. This method provided

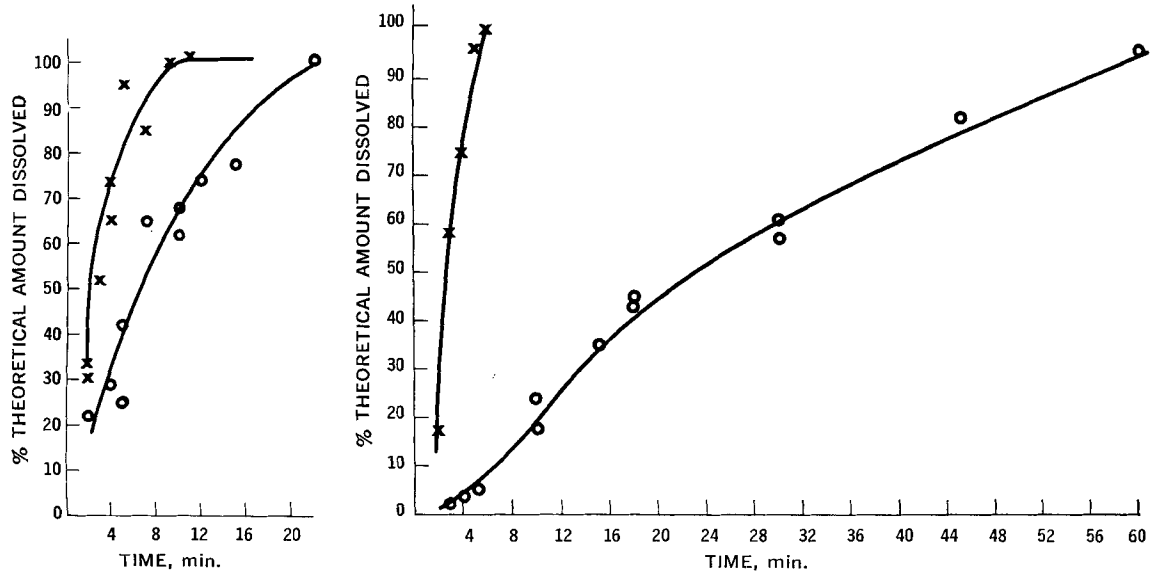


Figure 7—*Dissolution of drug from capsules: left, drug–lactose–2% magnesium stearate; right, drug–$CaHPO_4 \cdot 2H_2O$–2% magnesium stearate. Key: ×, simulated gastric fluid; ○, water.*

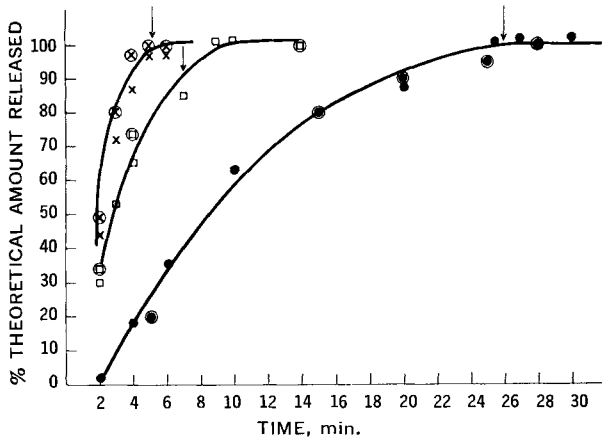


Figure 8—*Dissolution of drug from capsules in simulated gastric fluid (drug–lactose–magnesium stearate). Key: ×,⊗, 0% magnesium stearate; □,▣, 2% magnesium stearate; ●,◉, 5% magnesium stearate.*

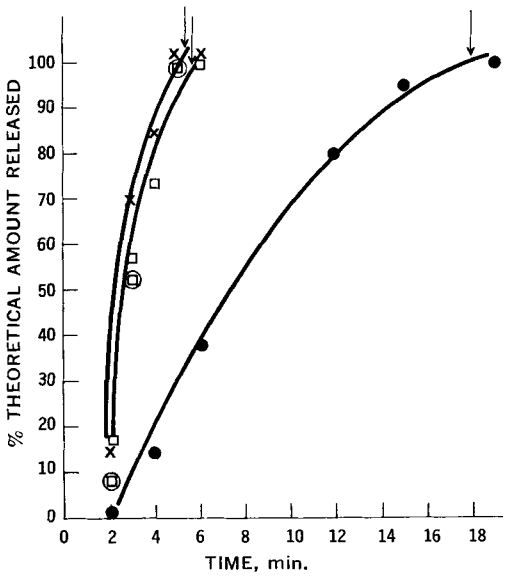


Figure 9—*Dissolution of drug from capsules in simulated gastric fluid (drug–$CaHPO_4 \cdot 2H_2O$–magnesium stearate). Key: ×, 0% magnesium stearate; □, ▣, 2% magnesium stearate; ●, 5% magnesium stearate.*

large samples, controlled packing density, and permitted measurements of penetration rates and water content of the wet powder beds.

The liquid penetration rates of the various drug–lactose and drug–dibasic calcium phosphate with magnesium stearate blends are shown in Figs. 11 and 12, respectively. The data were plotted as the square of the depth of liquid penetration *versus* time (25). From these figures, decreased liquid penetration rates are seen with increasing magnesium stearate levels. The dissolution data on some of these systems were shown in Figs. 6–9. It is clear that extended dissolution rates are obtained with powder blends that show reduced liquid penetration rates.

During these liquid penetration measurements, it was observed that the wet powder beds varied quite widely in viscosity when extruded from the Plexiglas tubes. Some of the wet compacted powders were thick pastes while others were quite fluid, almost to the point of flowing. Since many of the capsule blends remained intact long after the gelatin shells had dissolved, it would appear that these observations might also contribute to the prolonged dissolution rate measured.

Therefore, after completion of the liquid penetration experiments, the wet powder plugs were extruded from the tubes and segmented into two portions. The quantities of water present were

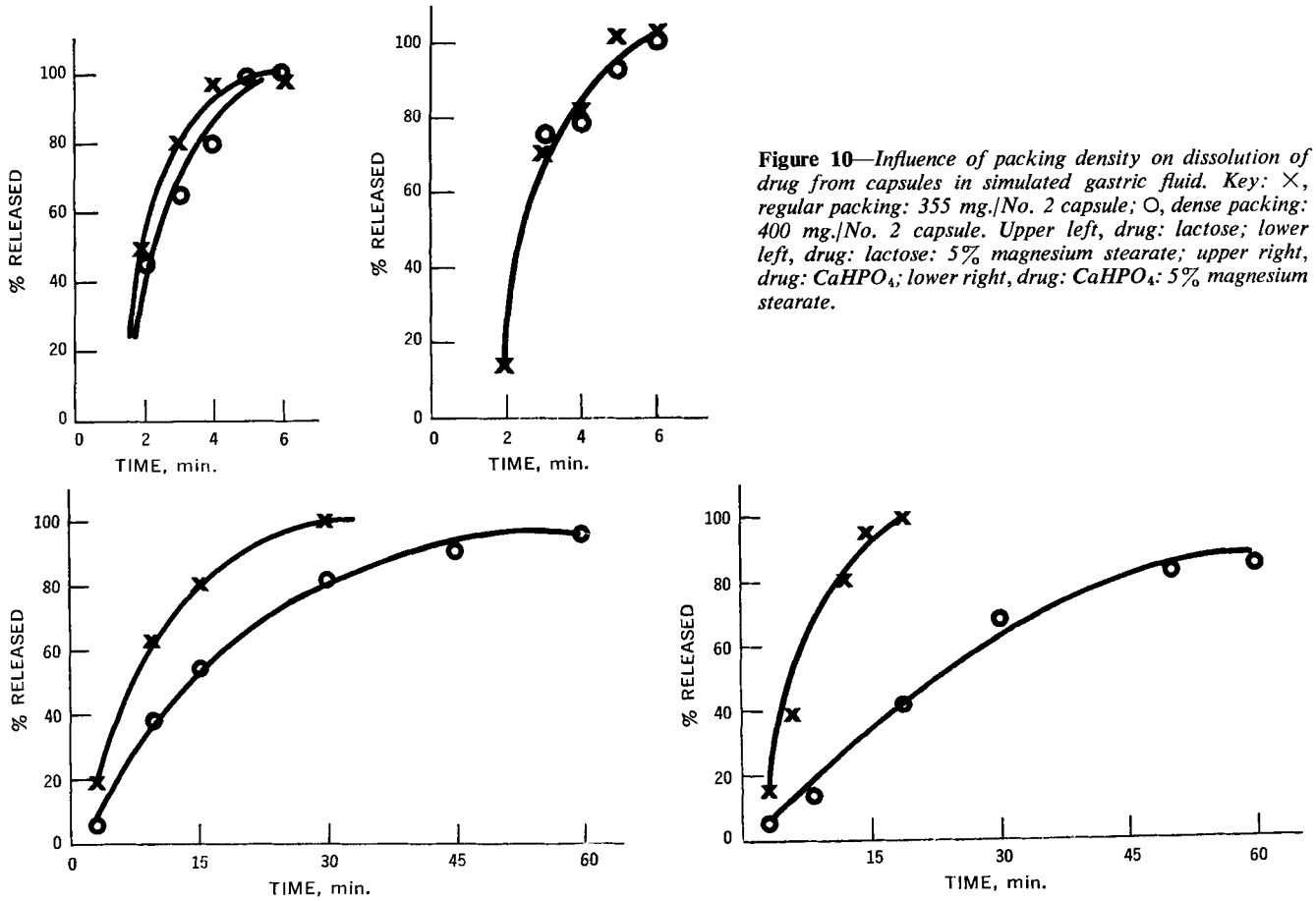


Figure 10—*Influence of packing density on dissolution of drug from capsules in simulated gastric fluid. Key: ×, regular packing: 355 mg./No. 2 capsule; ○, dense packing: 400 mg./No. 2 capsule. Upper left, drug: lactose; lower left, drug: lactose: 5% magnesium stearate; upper right, drug: $CaHPO_4$; lower right, drug: $CaHPO_4$: 5% magnesium stearate.*

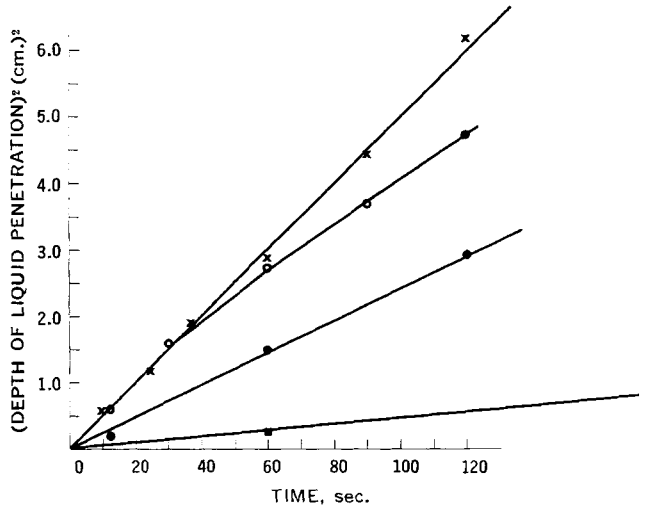


Figure 11—*Liquid penetration of packed powder beds (drug–lactose–magnesium stearate blends). Key: ×, 0% magnesium stearate; ○, 1% magnesium stearate; ●, 2% magnesium stearate; ■, 5% magnesium stearate.*

cetermined and are shown in Fig. 13. It can be seen that the water dontent did decrease with the added magnesium stearate level. Qualitatively, the physical appearance of the wet powder beds varied from a semifluid mass (no magnesium stearate) to a stiff paste (5% magnesium stearate) for both the lactose and dibasic calcium phosphate systems.

Comparative measurement of these viscosity changes with variable water content in the powder blends was carried out with a penetrometer. Three systems were checked: (*a*) drug–lactose–2% magnesium stearate; (*b*) drug–$CaHPO_4 \cdot 2H_2O$; and (*c*) drug–$CaHPO_4 \cdot 2H_2O$–2% magnesium stearate. Figure 14 shows the depth of cone penetration *versus* the amount of water in the powder. The samples low in water were more rigid and the depth of penetration was less than in the more fluid samples. The two $CaHPO_4 \cdot 2H_2O$ samples tested (with and without 2% magnesium stearate) described the same curve. This would suggest that the magnesium stearate affected the viscosity of the wet powder bed solely by limiting the water content in the bed.

At any given water content, the lactose-based capsule blend would appear to be more fluid than the $CaHPO_4 \cdot 2H_2O$ blend.

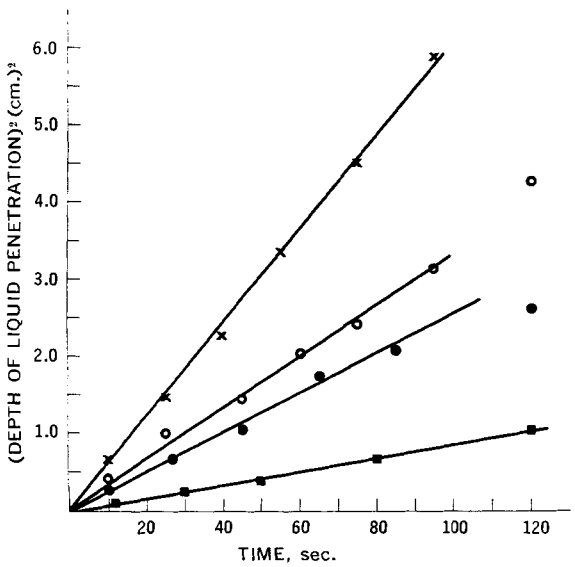


Figure 12—*Liquid penetration of packed powder beds (drug–$CaHPO_4 \cdot 2H_2O$–magnesium stearate blends). Key: ×, 0% magnesium stearate; ○, 1% magnesium stearate; ●, 2% magnesium stearate; ■, 5% magnesium stearate.*

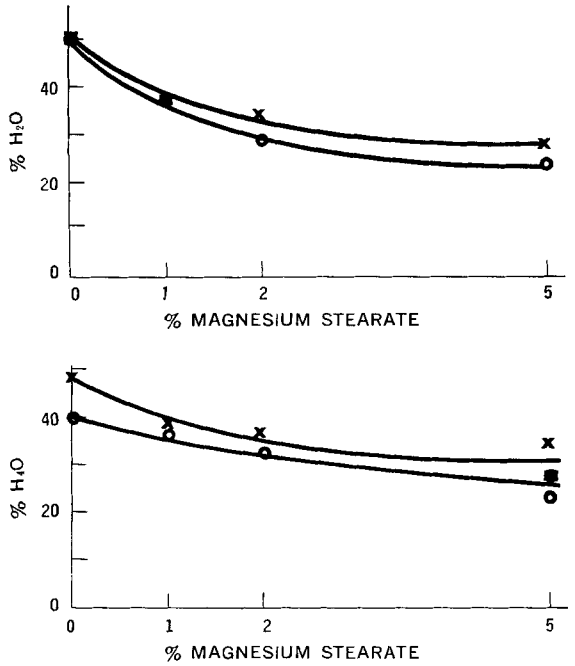


Figure 13—*Water contents of the wet powder beds; top, drug–$CaHPO_4 \cdot 2H_2O$–magnesium stearate blends; bottom, drug–lactose–magnesium stearate blends. Key: ×, first segment of bed; ○, second segment of bed.*

This may be due to partial solution of the lactose or other factors such as particle size and shape.

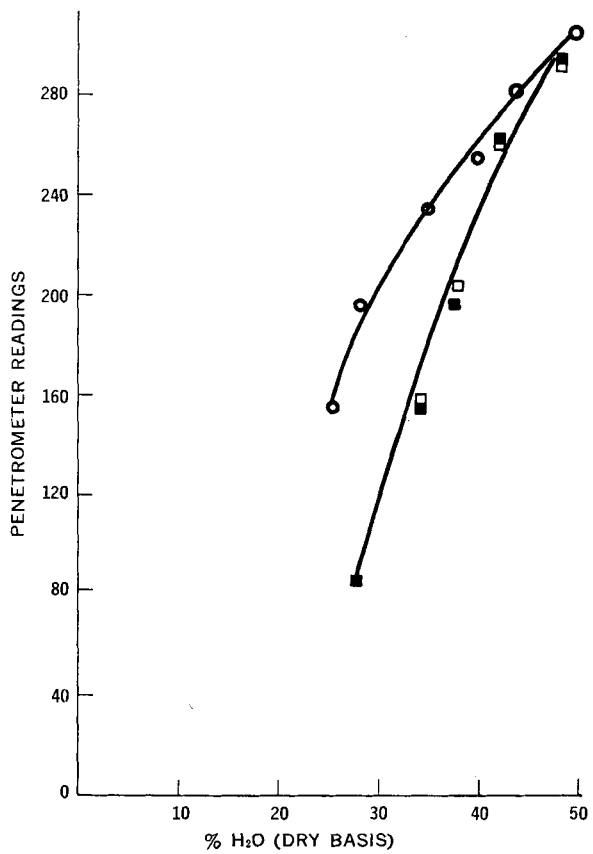


Figure 14—*Penetrometer measurements of powder beds containing varying quantities of water. Key: ○, drug–lactose–2% magnesium stearate; □, drug-$CaHPO_4 \cdot 2H_2O$-0% magnesium stearate; ■, drug–$CaHPO_4 \cdot 2H_2O$–2% magnesium stearate.*

Although these data clearly showed that the comparative viscosity decreased with added water as would be expected, the actual differences in viscosity between the various systems were felt to be greater than the numerical differences in the penetrometer readings. This is because the probe used for the measurements was an inverted cone and at greater penetrations, a greater cross section of the cone would contact the sample. Therefore, for greater penetration, a larger portion of the sample would support the load and prevent further penetration. A better estimate of the difference in relative viscosity of these samples would take into account this change in effective contact area of the probe with the sample. Considering this aspect, it would then be estimated that the sample with 27.5% water would be over 200 times as viscous as the sample with 48.5% water. From Fig. 14, the 5% magnesium stearate sample would therefore approximately correspond to the 27% water sample while the drug–$CaHPO_4 \cdot 2H_2O$ behaved like the 48% water sample.

The combined interpretation of Figs. 13 and 14 would suggest that magnesium stearate did reduce the water content in the powder bed and that this, in turn, yielded a wet powder blend of high viscosity. Dissolution of the drug would then be slowed due to the limited area of contact between the wet powder mass and the fluid. Dissolution would proceed by mechanical erosion, diffusion, and/or solution of drug and filler. *In vivo* it would seem these powder masses might remain intact for a considerable time if the agitation conditions are indeed low. In addition, an *in vivo* pH near neutral would favor stability of the wet powder mass.

REFERENCES

(1) R. Boswell and J. T. McGinn, *N. Y. State J. Med.*, **64**, 887 (1964).

(2) B. Calesnick, B. Katchen, and J. Black, *J. Pharm. Sci.*, **54**, 1277(1965).

(3) J. G. Wagner, E. S. Gerard, and D. G. Kaiser, *Clin. Pharmacol. Therap.*, **7**, 610(1966).

(4) J. G. Wagner, *J. Pharm. Sci.*, **50**, 359(1961).

(5) E. Nelson, *Clin. Pharmacol. Therap.*, **3**, 673(1962).

(6) J. H. Wood, *Pharm. Acta Helv.*, **42**, 129(1967).

(7) W. C. Sunkes, *Dissertation Abstr.*, **23**, 2496(1962).

(8) G. Levy, J. H. Antkowiak, J. A. Procknal, and D. C. White, *J. Pharm. Sci.*, **52**, 1047(1963).

(9) J. K. C. Yen, *Can. Pharm. J.*, **97**, 493(1964).

(10) K. Muenzel, *Pharm. Acta Helv.*, **40**, 65(1965).

(11) P. Finholt, H. Kristiansen, O. C. Schmidt, and K. Wold, *Medd. Norsk Farm. Selskap.*, **28**, 17(1966); through *Chem. Abstr.*, **65**, 8679(1966).

(12) P. Finholt, R. H. Pedersen, S. Solvang, and K. Wold, *ibid.*, **28**, 238(1966); through *Intern. Pharm. Abstr.*, **4**, 627(1967).

(13) P. Fuchs, J. Riemann, H. Richter, H. Roepke, and H. Gibian, *Arzneimittel-Forsch.*, **18**, 112(1968).

(14) J. H. Wood, *J. Pharm. Sci.*, **54**, 1207(1965).

(15) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 473.

(16) M. Roland, *J. Pharm. Belg.*, **22**, 67(1967); through *Intern. Pharm. Abstr.*, **4**, 951(1967).

(17) A. J. Aguiar, J. E. Zelmer, and A. W. Kinkel, *J. Pharm. Sci.*, **56**, 1243(1967).

(18) A. J. Aguiar, L. M. Wheeler, and S. Fusari, to be published.

(19) W. N. French, F. Matsui, D. Cook, and L. Levi, *J. Pharm. Sci.*, **56**, 1622(1967).

(20) G. Levy, *ibid.*, **52**, 1039(1963).

(21) W. H. Steinberg, G. H. Frey, J. N. Masci, and H. Hutchins, *ibid.*, **54**, 747(1965).

(22) S. Kuna, *Arch. Intern. Pharmacodyn.*, **152**, 79(1964).

(23) "The United States Pharmacopeia," 15 rev., Mack Publishing Co., Easton, Pa., 1955, p. 936.

(24) M. L. Studebaker and C. W. Snow, *J. Phys. Chem.*, **59**, 973(1955).

(25) E. W. Washburn, *Phys. Rev.*, **17**, 273(1921).

(26) "The Merck Index," 8th ed., Merck & Co., Inc., Rahway, N. J., 1968, p. 638.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Product Development Department, Division of Medical and Scientific Affairs, Parke Davis & Company, Detroit, MI 48232

Accepted for publication September 11, 1969.

The authors wish to acknowledge the assistance of Dr. L. M. Wheeler in the publication of this paper.

Hydrolysis of Lincomycin-2-phosphate and Clindamycin-2-phosphate

T. O. OESTERLING and E. L. ROWE

Abstract □ The aqueous stabilities of lincomycin-2-phosphate and its 7-deoxy-7(S)-chloro analog, clindamycin-2-phosphate, were studied at a variety of temperatures and pH values. The predominant degradative routes of lincomycin-2-phosphate in the pH range 1–10 are thioglycoside and phosphate ester hydrolysis, and pH-rate studies show that it is most stable at pH 6–10. Clindamycin-2-phosphate degrades by three major routes from pH 1 to 10 with phosphate ester and thioglycoside hydrolysis predominating at pH less than 6 and scission of 7(S)-Cl to form the 7(R)-OH analog predominating at pH greater than 6. The rate of 7(S)-Cl to 7(R)-OH conversion was obtained using gas chromatography to measure the disappearance of clindamycin in reaction mixtures prepared under identical conditions as clindamycin-2-phosphate reaction mixtures. Rates of phosphate ester hydrolysis of the two compounds were measured by spectrophotometric determination of the amount of inorganic phosphate formed in reaction mixtures as a function of time. Apparent first-order rate constants of phosphate ester hydrolysis as well as activation energies (32.1 kcal./mole for lincomycin-2-phosphate and 32.9 kcal./mole for clindamycin-2-phosphate) agree favorably. The rate of lincomycin-2-phosphate hydrolysis in a formulated pediatric syrup at room temperature agrees favorably with extrapolated rates from high temperature hydrolysis in simple solutions.

Keyphrases □ Lincomycin-2-PO_4—hydrolysis kinetics □ Clindamycin-2-PO_4—hydrolysis kinetics □ Stability—lincomycin-, clindamycin-2-PO_4 aqueous solutions □ pH effect—lincomycin-, clindamycin-2-PO_4 hydrolysis rates □ Colorimetric analysis—spectrophotometer

The antibiotics lincomycin and clindamycin are highly effective in the treatment of infections caused by Gram-positive organisms (1, 2) and clindamycin possesses marked antiplasmodial activity as well (3). At times it is desirable to make derivatives from compounds such as lincomycin and clindamycin in order to circumvent disadvantages such as bitter taste or poor absorption inherent in the parent molecule. The derivatives must possess the same activity as the parent compound or be rapidly reverted to parent *in vivo*. Lincomycin has a bitter taste which is difficult to mask in pediatric liquid formulations. The C_2 phosphate ester of lincomycin

Although these data clearly showed that the comparative viscosity decreased with added water as would be expected, the actual differences in viscosity between the various systems were felt to be greater than the numerical differences in the penetrometer readings. This is because the probe used for the measurements was an inverted cone and at greater penetrations, a greater cross section of the cone would contact the sample. Therefore, for greater penetration, a larger portion of the sample would support the load and prevent further penetration. A better estimate of the difference in relative viscosity of these samples would take into account this change in effective contact area of the probe with the sample. Considering this aspect, it would then be estimated that the sample with 27.5% water would be over 200 times as viscous as the sample with 48.5% water. From Fig. 14, the 5% magnesium stearate sample would therefore approximately correspond to the 27% water sample while the drug–$CaHPO_4 \cdot 2H_2O$ behaved like the 48% water sample.

The combined interpretation of Figs. 13 and 14 would suggest that magnesium stearate did reduce the water content in the powder bed and that this, in turn, yielded a wet powder blend of high viscosity. Dissolution of the drug would then be slowed due to the limited area of contact between the wet powder mass and the fluid. Dissolution would proceed by mechanical erosion, diffusion, and/or solution of drug and filler. *In vivo* it would seem these powder masses might remain intact for a considerable time if the agitation conditions are indeed low. In addition, an *in vivo* pH near neutral would favor stability of the wet powder mass.

REFERENCES

(1) R. Boswell and J. T. McGinn, *N. Y. State J. Med.*, **64**, 887 (1964).

(2) B. Calesnick, B. Katchen, and J. Black, *J. Pharm. Sci.*, **54**, 1277(1965).

(3) J. G. Wagner, E. S. Gerard, and D. G. Kaiser, *Clin. Pharmacol. Therap.*, **7**, 610(1966).

(4) J. G. Wagner, *J. Pharm. Sci.*, **50**, 359(1961).

(5) E. Nelson, *Clin. Pharmacol. Therap.*, **3**, 673(1962).

(6) J. H. Wood, *Pharm. Acta Helv.*, **42**, 129(1967).

(7) W. C. Sunkes, *Dissertation Abstr.*, **23**, 2496(1962).

(8) G. Levy, J. H. Antkowiak, J. A. Procknal, and D. C. White, *J. Pharm. Sci.*, **52**, 1047(1963).

(9) J. K. C. Yen, *Can. Pharm. J.*, **97**, 493(1964).

(10) K. Muenzel, *Pharm. Acta Helv.*, **40**, 65(1965).

(11) P. Finholt, H. Kristiansen, O. C. Schmidt, and K. Wold, *Medd. Norsk Farm. Selskap.*, **28**, 17(1966); through *Chem. Abstr.*, **65**, 8679(1966).

(12) P. Finholt, R. H. Pedersen, S. Solvang, and K. Wold, *ibid.*, **28**, 238(1966); through *Intern. Pharm. Abstr.*, **4**, 627(1967).

(13) P. Fuchs, J. Riemann, H. Richter, H. Roepke, and H. Gibian, *Arzneimittel-Forsch.*, **18**, 112(1968).

(14) J. H. Wood, *J. Pharm. Sci.*, **54**, 1207(1965).

(15) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 473.

(16) M. Roland, *J. Pharm. Belg.*, **22**, 67(1967); through *Intern. Pharm. Abstr.*, **4**, 951(1967).

(17) A. J. Aguiar, J. E. Zelmer, and A. W. Kinkel, *J. Pharm. Sci.*, **56**, 1243(1967).

(18) A. J. Aguiar, L. M. Wheeler, and S. Fusari, to be published.

(19) W. N. French, F. Matsui, D. Cook, and L. Levi, *J. Pharm. Sci.*, **56**, 1622(1967).

(20) G. Levy, *ibid.*, **52**, 1039(1963).

(21) W. H. Steinberg, G. H. Frey, J. N. Masci, and H. Hutchins, *ibid.*, **54**, 747(1965).

(22) S. Kuna, *Arch. Intern. Pharmacodyn.*, **152**, 79(1964).

(23) "The United States Pharmacopeia," 15 rev., Mack Publishing Co., Easton, Pa., 1955, p. 936.

(24) M. L. Studebaker and C. W. Snow, *J. Phys. Chem.*, **59**, 973(1955).

(25) E. W. Washburn, *Phys. Rev.*, **17**, 273(1921).

(26) "The Merck Index," 8th ed., Merck & Co., Inc., Rahway, N. J., 1968, p. 638.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Product Development Department, Division of Medical and Scientific Affairs, Parke Davis & Company, Detroit, MI 48232

Accepted for publication September 11, 1969.

The authors wish to acknowledge the assistance of Dr. L. M. Wheeler in the publication of this paper.

Hydrolysis of Lincomycin-2-phosphate and Clindamycin-2-phosphate

T. O. OESTERLING and E. L. ROWE

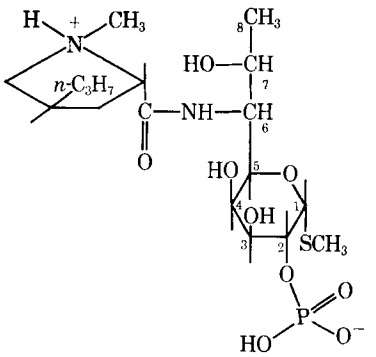
Abstract □ The aqueous stabilities of lincomycin-2-phosphate and its 7-deoxy-7(S)-chloro analog, clindamycin-2-phosphate, were studied at a variety of temperatures and pH values. The predominant degradative routes of lincomycin-2-phosphate in the pH range 1–10 are thioglycoside and phosphate ester hydrolysis, and pH-rate studies show that it is most stable at pH 6–10. Clindamycin-2-phosphate degrades by three major routes from pH 1 to 10 with phosphate ester and thioglycoside hydrolysis predominating at pH less than 6 and scission of 7(S)-Cl to form the 7(R)-OH analog predominating at pH greater than 6. The rate of 7(S)-Cl to 7(R)-OH conversion was obtained using gas chromatography to measure the disappearance of clindamycin in reaction mixtures prepared under identical conditions as clindamycin-2-phosphate reaction mixtures. Rates of phosphate ester hydrolysis of the two compounds were measured by spectrophotometric determination of the amount of inorganic phosphate formed in reaction mixtures as a function of time. Apparent first-order rate constants of phosphate ester hydrolysis as well as activation energies (32.1 kcal./mole for lincomycin-2-phosphate and 32.9 kcal./mole for clindamycin-2-phosphate) agree favorably. The rate of lincomycin-2-phosphate hydrolysis in a formulated pediatric syrup at room temperature agrees favorably with extrapolated rates from high temperature hydrolysis in simple solutions.

Keyphrases □ Lincomycin-2-PO_4—hydrolysis kinetics □ Clindamycin-2-PO_4—hydrolysis kinetics □ Stability—lincomycin-, clindamycin-2-PO_4 aqueous solutions □ pH effect—lincomycin-, clindamycin-2-PO_4 hydrolysis rates □ Colorimetric analysis—spectrophotometer

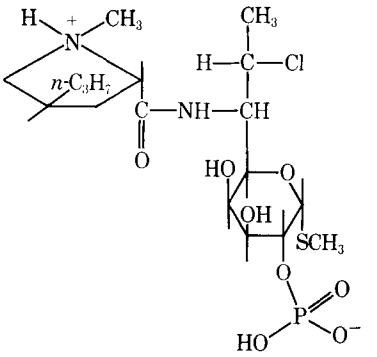
The antibiotics lincomycin and clindamycin are highly effective in the treatment of infections caused by Gram-positive organisms (1, 2) and clindamycin possesses marked antiplasmodial activity as well (3). At times it is desirable to make derivatives from compounds such as lincomycin and clindamycin in order to circumvent disadvantages such as bitter taste or poor absorption inherent in the parent molecule. The derivatives must possess the same activity as the parent compound or be rapidly reverted to parent *in vivo*. Lincomycin has a bitter taste which is difficult to mask in pediatric liquid formulations. The C_2 phosphate ester of lincomycin

was synthesized by Morozowich *et al.* (4) as a potential nonbitter derivative with the same activity as the parent compound. Clindamycin hydrochloride is poorly soluble at pH values above 6 and its C_2 phosphate ester was synthesized (5) to obtain a derivative of high water solubility near neutral pH.

The purpose of this study was to investigate the stability of lincomycin-2-phosphate (I) and clindamycin-2-phosphate (II) in aqueous solutions in order to supply basic information necessary for the successful formulation of these drugs in liquid dosage forms. The effects of pH and temperature on the rate of phosphate ester hydrolysis of lincomycin-2-phosphate and clindamycin-2-phosphate were determined. These data allowed the establishment of maximum stability conditions and the prediction of room temperature stability.



I



II

EXPERIMENTAL

Materials—Lincomycin-2-phosphate, clindamycin-2-phosphate, and clindamycin hydrochloride containing less than 2% impurities were supplied by the research laboratories of the Upjohn Company. All other chemicals were reagent grade.

Kinetic Studies—Lincomycin-2-phosphate—The rate of hydrolysis of lincomycin-2-phosphate was studied in the pH range 6–9 at 59.4, 69.6, 79.6, and 93.5° and in the extended pH range 0.1–10 at 90.0°. All reaction mixtures except those of less than pH 2 at 90° were prepared at the temperature of the run by adjusting the pH of 90 ml. of solution containing lincomycin-2-phosphate and sodium chloride to the desired pH by the addition of 1 *M* HCl or NaOH. After the volume was adjusted to 100 ml., the final concentration of lincomycin-2-phosphate was 0.02 *M* and that of sodium chloride was 0.1 *M*. The sodium chloride was added to minimize ionic strength effects. Reaction mixtures adjusted to pH less than 2 at 90° were prepared to contain 0.02 *M* lincomycin-2-phosphate and 0.1, 0.5, and 1 *M* HCl, respectively. These reaction mixtures did not contain sodium chloride. After preparation, 5-ml. portions of reaction mixtures were filled into ampuls, sealed, and immersed into the appropriate constant-temperature baths. Ampuls were withdrawn at appropriate times and frozen until assayed for inorganic phosphate by the procedure described below.

Clindamycin-2-phosphate—The effect of pH on the rate of phosphate ester hydrolysis of clindamycin-2-phosphate was studied in the pH range 6–9 at 90°. It was necessary to buffer clindamycin-2-phosphate reaction mixtures because, unlike lincomycin-2-phosphate, the pH drifted sharply downward as the reaction proceeded. This decrease in pH may be caused by degradation at C_7 of clindamycin-2-phosphate (6). Reaction mixtures were prepared to contain 0.02 *M* clindamycin-2-phosphate, 0.2 *M* ethylenediamine, or tris (hydroxymethyl) aminomethane[1] (tromethamine) buffers adjusted to the desired pH at the temperature of the run, and sufficient potassium chloride to adjust the ionic strength to 0.3. Aliquots of the reaction mixtures were filled into ampuls which were sealed and immersed in constant temperature baths. Ampuls were withdrawn at appropriate times and frozen until assayed for inorganic phosphate by the procedure described below.

All reaction mixtures in which phosphate ester hydrolysis was measured were prepared with freshly boiled deionized water. The pH's of the samples from all kinetic runs were measured, and a run was discarded if the pH of the final sample varied by more than 0.2 pH units from the initial sample.

Rates of phosphate ester hydrolysis were determined by least-squares analysis of log $(P_{i,\infty} - P_{i,t})$-time data, where $P_{i,\infty}$ is the molar concentration of inorganic phosphate present at completion of hydrolysis which is equivalent to the initial molar concentration of lincomycin-2-phosphate or clindamycin-2-phosphate, and $P_{i,t}$ is the molar concentration of inorganic phosphate present at any time.

A previous study (6) has indicated that in addition to phosphate ester hydrolysis at Position 2, clindamycin-2-phosphate will undergo degradation at C_7 to form the 7-(R)-OH analog in the pH range 6–9. To measure the rate of degradation at Position 7, reaction mixtures containing clindamycin hydrochloride were prepared identical to those containing clindamycin-2-phosphate, and the amount of intact clindamycin remaining with time was determined by GLC (6).

Inorganic Phosphate Assay—The method is essentially that developed for assay of free inorganic phosphate in methylprednisolone phosphate (7). The reagents used were ammonium molybdate, 2.0% solution in double-distilled water, acetate buffer prepared by dissolving 310 ml. glacial acetic acid, 49 g. anhydrous potassium acetate, and 48 mg. anhydrous cupric sulfate in double-distilled water to a total volume of 1000 ml., 2.0% ascorbic acid solution in double-distilled water, and 20% stannous chloride stock solution in concentrated hydrochloric acid. One milliliter of the stock stannous chloride solution was diluted to 100 ml. with double-distilled water just prior to use.

Stability samples were assayed for inorganic phosphate by adding 1.0-ml. aliquots of the reaction mixtures to 50 ml. of acetate buffer in 100-ml. volumetric flasks. After thorough mixing, 5.0 ml. ammonium molybdate solution, 5.0 ml. ascorbic acid solution, and 5.0 ml. of the diluted stannous chloride solution were added with mixing after each addition. The solution was adjusted to 100 ml., mixed again, and allowed to stand 30 min. The absorbance was determined at 740 mμ against a water blank using 1.0-cm. cells within a period of 3 hr. After confirming that Beer's law applied in the concentration range of interest, the concentration of inorganic phosphate was determined by comparing the absorbance of the unknown solutions with that of a standard solution.

RESULTS AND DISCUSSION

The phosphate ester moieties of lincomycin-2-phosphate and clindamycin-2-phosphate hydrolyzed by an apparent first-order process under all experimental conditions. Some typical first-order curves of lincomycin-2-phosphate hydrolysis are shown in Fig. 1.

The effects of pH and temperature on the rate of phosphate ester hydrolysis of lincomycin-2-phosphate are shown in Table I. In general the hydrolysis rate constants from reaction mixtures of similar pH were in good agreement. The discrepancy in the data from the pH 1.1 reaction mixtures at 90° may be due to the high dependency of rate constant on pH in this region. The rates of phosphate ester hydrolysis of clindamycin-2-phosphate as a function of pH and temperature are shown in Table II. Although ionic strengths are different and the clindamycin-2-phosphate reaction mixtures contain buffers, there is good agreement between its rate constants and those of lincomycin-2-phosphate at a given temperature and pH.

[1] TRIS

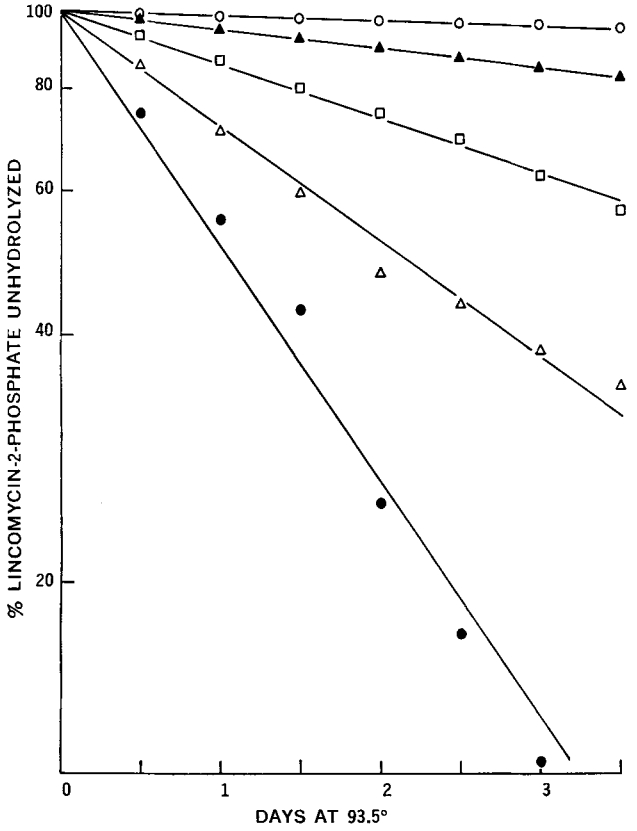


Figure 1—*Hydrolysis of lincomycin-2-phosphate at 93.5°. Key:* ○, *pH 8.4;* ▲, *pH 8.0;* □, *pH 7.5;* △, *pH 7.0;* ●, *pH 6.2.*

The pH-rate relationship for lincomycin-2-phosphate is shown in Fig. 2 for 59.4, 69.6, and 79.6° and in Fig. 3 for 90°. Above pH 5 the dependency of rate on pH is quite similar to that of monoalkyl phosphates such as methyl phosphate (9) and carbohydrate monophosphates such as α-D-glucose-1-phosphate (10). For monoalkyl phosphates and carbohydrate monophosphates the decrease in rate with increasing pH in this pH range is attributed to the decreasing concentration of the monoanionic species, the dianionic species being much less reactive. Since the pKa values of the phosphate moiety of lincomycin-2-phosphate are roughly 2 and 6 (4), the monoanionic species is present at maximum concentration at about pH 4, and the decrease in rate above pH 5 in Fig. 3 is probably due to decreasing concentration of monoanion.

Below pH 5 the rate of lincomycin-2-phosphate hydrolysis in Fig. 3 appears to be constant to about pH 1. In this pH range the two predominant species are the monoanion and the undissociated form of the phosphate moiety. The monoanion actually exists as a zwitterion and the undissociated form carries a net charge of +1 due to protonation of the amino group. (The pKa of lincomycin hydrochloride is 7.6.) The monoanion is present at maximum concentration at about pH 4 and decreases with decreasing pH, whereas the undissociated form increases in concentration as pH decreases in this range. The rate of hydrolysis in this pH range is the sum of the rates of hydrolysis of the monoanionic and undissociated form and this sum is a constant between pH 1 and 5. The pH profile of methyl phosphate shows a decrease in rate from pH 4 to pH 1 and this decrease is explained as corresponding to a decrease in the monoanion concentration, the undissociated form being relatively unreactive (9). On the other hand, the rate of glucose-1-phosphate hydrolysis increases rapidly between pH 5 and 1 and this increase is explained as due to the increase in concentration of undissociated glucose-1-phosphate (10) which is much more reactive than the corresponding form of methyl phosphate. Since the reactivities of the monoanionic species of lincomycin-2-phosphate (Table I), methyl phosphate (9), and glucose-1-phosphate (10) as well as hydroxyalkyl and alkyl monophosphates of similar structure (11) are of the same order, the plateau region of the lincomycin-2-phosphate pH profile between pH 1 and 5 indicates that the undissociated form of lincomycin-2-phosphate is more reactive than that of methyl phosphate but less reactive than the undissociated form of glucose-1-phosphate.

Table I—Apparent First-Order Rate Constants of Phosphate Ester Hydrolysis of Lincomycin-2-phosphate

pH	Temperature, °C.	$k \times 10^8$ sec.$^{-1}$
6.2	59.4	9.06
6.6	59.4	4.05
7.0	59.4	1.68
7.5	59.4	0.613
8.0	59.4	0.289
8.4	59.4	0.243
8.6	59.4	0.162
6.2	69.6	28.6
6.6	69.6	14.9
7.0	69.6	6.69
7.5	69.6	2.50
8.0	69.6	0.903
8.4	69.6	0.532
8.6	69.6	0.556
6.2	79.6	119
6.6	79.6	59.1
7.0	79.6	26.0
7.5	79.6	10.7
8.0	79.6	4.83
8.4	79.6	2.88
8.6	79.6	2.11
0.15[a]	90.0	5010
0.46[a]	90.0	2040
1.1[a]	90.0	1770
1.1[a]	90.0	765
2.0	90.0	735
2.1	90.0	875
3.0	90.0	749
3.1	90.0	719
4.0	90.0	755
4.2	90.0	725
5.0	90.0	668
5.1	90.0	708
6.6	90.0	309
7.4	90.0	66.4
8.0	90.0	12.2
8.5	90.0	8.89
9.6	90.0	4.23
6.2	93.5	941
6.6	93.5	356
7.0	93.5	184
7.5	93.5	61.5
8.0	93.5	23.8
8.4	93.5	14.3
8.6	93.5	11.3

[a] Calculated from pH = log f(HCl) where (HCl) is the experimental molarity and f is the mean activity coefficient for HCl at 90°, extrapolated from the literature (8).

There are at least two possible explanations for the sharp increase in rate of lincomycin-2-phosphate hydrolysis below pH 1 shown in Fig. 3. Both are based on the formation of new species which are more reactive than the undissociated form of lincomycin-2-phosphate. The first of these species may be the conjugated acid form of lincomycin-2-phosphate where conjugate acid is defined as the structure(s) resulting from protonation of the uncharged phosphate moiety. The other reactive species which may be formed at pH less than 1 is lincomycin-1-phosphate. Acid-catalyzed phosphoryl group

Table II—Apparent First-Order Rate Constants of Phosphate Ester Hydrolysis of Clindamycin-2-phosphate

pH	Buffer	Temperature, °C.	$k \times 10^8$ sec.$^{-1}$
7.50	0.2 *M* Tromethamine	59.5	0.757
7.50	0.2 *M* Tromethamine	70.0	3.61
7.50	0.2 *M* Tromethamine	80.0	14.6
5.95	0.2 *M* Ethylenediamine	90.4	278
6.40	0.2 *M* Ethylenediamine	90.4	185
6.80	0.2 *M* Tromethamine	90.4	72.7
7.30	0.2 *M* Ethylenediamine	90.4	69.5
7.40	0.2 *M* Tromethamine	90.4	41.3
7.50	0.2 *M* Tromethamine	90.4	46.8
7.75	0.2 *M* Tromethamine	90.4	24.4

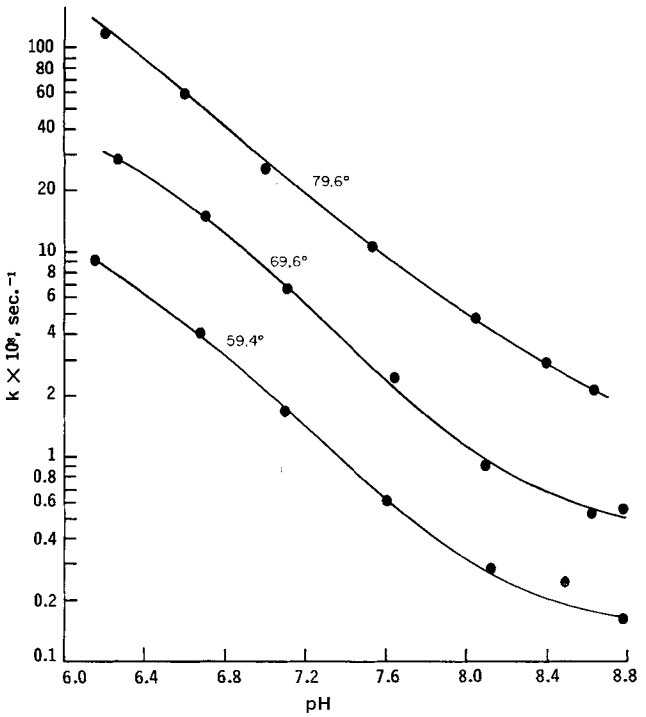


Figure 2—*pH-rate profile of phosphate ester hydrolysis of lincomycin-2-phosphate.*

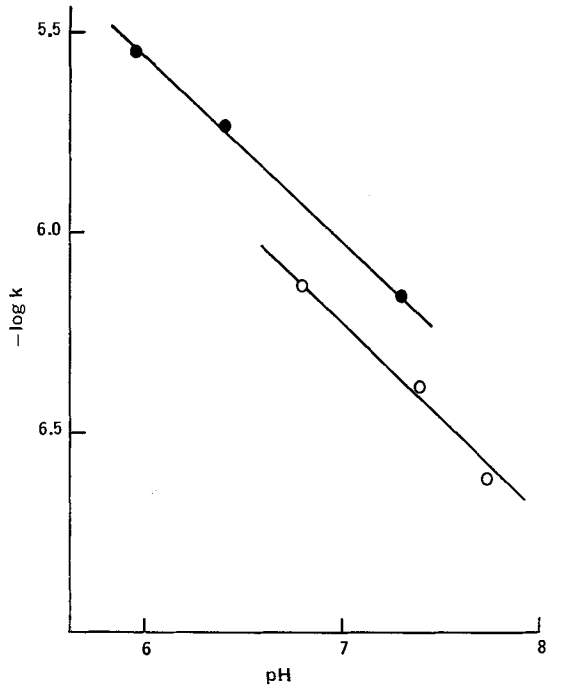


Figure 4—*Rate-pH profile of phosphate ester hydrolysis of clindamycin-2-phosphate at 90° and μ = 0.3. Key: ●, Tromethamine buffer; ○, ethylenediamine buffer.*

migration in monoesters containing suitably placed hydroxyl groups, such as lincomyin-2-phosphate, has been extensively reported (12, 13), and the new reactive species, lincomycin-1-phosphate, may be formed by phosphoryl group migration from C_2 to C_1. These migrations occur through formation of a five-membered cyclic intermediate (13). Glucose-1-phosphate is much more reactive than glucose-2-phosphate in this pH region (14) and by analogy lincomycin-1-phosphate would be expected to be more reactive than lincomycin-2-phosphate, thus causing the increase in observed hydrolysis rate below pH 1. A prerequisite to migration of the phosphoryl group from C_2 to C_1 in lincomycin-2-phosphate is cleavage of the thioglycoside bond to form the C_1 hydroxy compound. Thioglycoside hydrolysis has been reported as the major route of degradation of clindamycin in the pH range 0.4 to 4 (6). Extrapolation of these reported rate constants of thioglycoside hydrolysis to 90° reveals that the rate of thioglycoside hydrolysis is about five times greater than phosphate ester hydrolysis at pH 1 and falls to about two times greater at pH 4. Thus migration of the phosphoryl group from C_2 to C_1 is possible, and the increase in rate

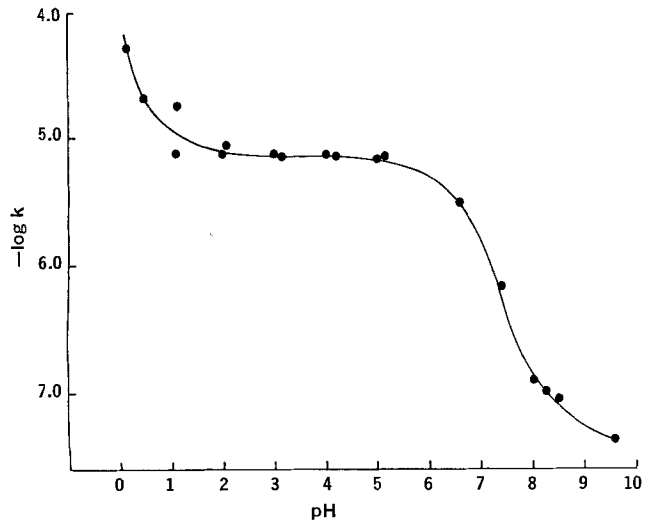


Figure 3—*pH-rate profile of lincomycin-2-phosphate hydrolysis at 90°.*

of lincomycin-2-phosphate hydrolysis below pH 1 may be due to the formation of the more reactive species, lincomycin-1-phosphate.

Migration of the C_2 phosphate group to the other adjacent hydroxyl at C_3 probably also occurs at pH < 1 (12), forming another species of the same order of reactivity as the 2-phosphate. However, it is unlikely that any phosphate migration occurs at higher pH since none was detected when lincomycin-2-phosphate was heated in 80% acetic acid (4), and alkaline migration does not occur in monoesters of this type (13).

The pH-rate relationship for phosphate ester hydrolysis of clindamycin-2-phosphate in ethylenediamine and tromethamine buffer in pH range 6–8 is shown in Fig. 4. Similar slopes of the two curves indicate that the rate of hydrolysis changes with pH to approximately the same extent in each buffer; however, nonoverlapping curves indicate that there is a buffer effect since at constant pH the rate is faster in ethylenediamine buffers. In both buffers, however, the pH-rate relationship is similar to that observed with lincomycin-2-phosphate between pH 6–8, *i.e.*, increasing hydrolysis rate as pH decreases.

The apparent first-order rate constants of degradation of clindamycin under conditions identical to clindamycin-2-phosphate are shown in Table III. According to a previous report (6), there are several products of clindamycin degradation in this pH range but the major degradative route is conversion to lincomycin. The influence of the phosphate ester moiety on the rate of lincomycin conversion is unknown when clindamycin alone is reacted; however, the relatively large distance between Positions 2 and 7 and the absence of conjugation minimize any substituent effects. The data in Table III indicate that the sum of the rate constants of lincomycin conversion and the other minor reactions is about 100 times greater than the rate of phosphate ester hydrolysis between pH 6 and 7.85. Thus, the phosphate ester moiety of clindamycin-2-phosphate is not the least stable portion of the molecule in this pH range. Extrapolation of rates of

Table III—Apparent First-order Rate Constants of Clindamycin Degradation at 90°C.

pH	Buffer	$k \times 10^6$ sec.$^{-1}$
6.05	0.2 *M* Ethylenediamine	1.05
6.90	0.2 *M* Tromethamine	2.58
7.40	0.2 *M* Tromethamine	4.80
7.85	0.1 *M* Tromethamine	6.32

Table IV—Activation Energy of Phosphate Ester Hydrolysis of Lincomycin-2-phosphate and Clindamycin-2-phosphate

Compound	pH	E_A, kcal./mole
Lincomycin-2-phosphate	6.20	33.3
Lincomycin-2-phosphate	6.65	32.1
Lincomycin-2-phosphate	7.05	33.5
Lincomycin-2-phosphate	7.55	33.1
Lincomycin-2-phosphate	8.05	32.2
Lincomycin-2-phosphate	8.40	30.1
Lincomycin-2-phosphate	8.65	30.4
Clindamycin-2-phosphate	7.50	32.9

phosphate ester hydrolysis and rates of degradation of other areas of the clindamycin-2-phosphate molecule to 25° shows that a liquid formulation adjusted to pH 7.4 would not meet stability criteria of less than 10% degradation after 2 years. However, these criteria are satisfied in the pH range 3.5 to about 6.5. Preliminary studies of lincomycin degradation alone indicate that the phosphate ester moiety is the least stable area of lincomycin-2-phosphate in the pH range 1–10. Calculations, using Tables I and IV data, show that lincomycin-2-phosphate meets the above stability criteria in the pH range 3.5–10.

Arrhenius plots of phosphate ester hydrolysis of lincomycin-2-phosphate at a variety of pH values and of clindamycin-2-phosphate at pH 7.5 in tromethamine buffer are shown in Fig. 5. Since clindamycin-2-phosphate was a parenteral product candidate, its activation energy was determined only at optimum physiological pH. Activation energies calculated from these curves are shown in Table IV. Good agreement between the activation energies of the two compounds was observed as expected since conversion of clindamycin to lincomycin is about 100 times faster than phosphate ester hydrolysis at this pH. Assuming that the monoanion is the predominant reactive species in the pH range reported in Table IV, the activation energies are in good agreement with values reported for phosphate esters of similar structure (11).

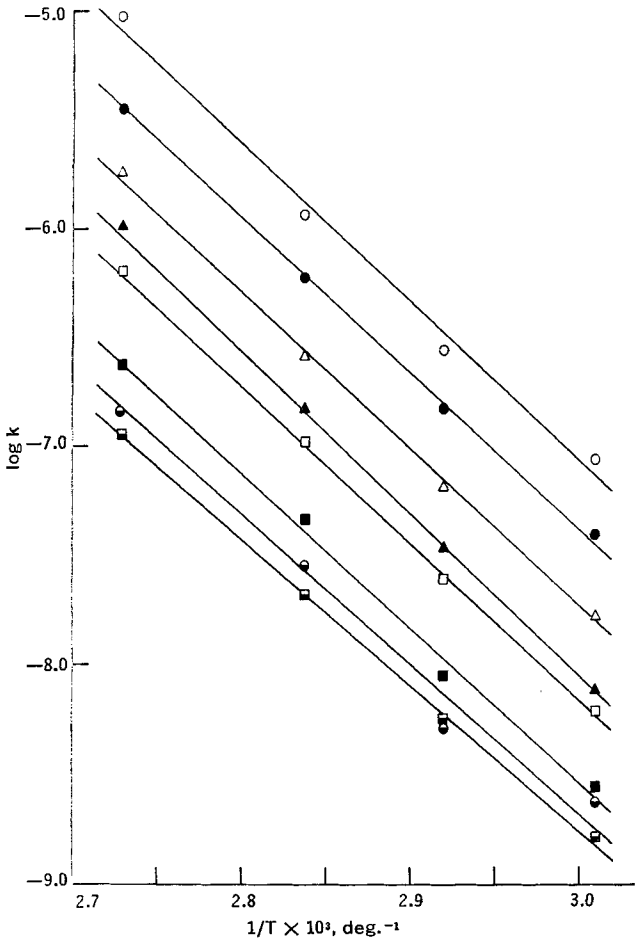


Figure 5—*Arrhenius plot of phosphate hydrolysis of lincomycin-2-phosphate and clindamycin-2-phosphate. Key:* ○, *lincomycin-2-phosphate, pH 6.20;* ●, *lincomycin-2-phosphate, pH 6.65;* △, *lincomycin-2-phosphate, pH 7.05;* ▲, *clindamycin-2-phosphate, pH 7.50;* □, *lincomycin-2-phosphate, pH 7.55;* ■, *lincomycin-2-phosphate, pH 8.05;* ◒, *lincomycin-2-phosphate, pH 8.4;* ◘, *lincomycin-2-phosphate, pH 8.65.*

Table V—Apparent First-order Rate Constants for Lincomycin-2-phosphate Hydrolysis in Pediatric Formulation

Temperature, °C.	$k \times 10^{10}$ sec.$^{-1}$ Predicted[a]	$k \times 10^{10}$ sec.$^{-1}$ Found
25	0.239	0.441
40	2.68	5.01
47	9.77	7.54
56	35.9	35.4

[a] Calculated from E_A = 32.1 kcal./mole and $k = 2.50 \times 10^{-8}$ sec.$^{-1}$ at 70°.

Accelerated stability studies of the type described in this study are useful in the prediction of product stability and determination of optimum formulation parameters. These studies in simple buffered solution provide baseline rate data for comparison with rates of degradation in actual formulations. Any influence of additives in a liquid formulation such as preservative, flavor, color, or solubilizing agent on the rate of drug degradation can be detected and corrected if necessary. Some early data on the hydrolysis of lincomycin-2-phosphate in a pediatric formulation containing sucrose, sorbitol, glycerin, preservatives, alcohol, saccharin, flavor, and color at a pH of 7.5 are shown in Table V along with rate constants predicted from 70° data in simple solution. Although hydrolysis had not occurred to greater than 3%, agreement between simple solution and formulation hydrolysis is reasonable and indicates that none of the formulation ingredients significantly influences lincomycin-2-phosphate hydrolysis.

REFERENCES

(1) D. J. Mason, A. Dietz, and C. DeBoer, *Antimicrobial Agents Chemotherapy*, **1964**, 554.

(2) B. J. Magerlein, R. D. Birkenmeyer, and F. Kagan, *ibid.*, **1966**, 727.

(3) C. Lewis, *J. Parasitol.*, **54**, 169(1968).

(4) W. Morozowich, D. J. Lamb, H. A. Karnes, F. A. MacKellar, C. Lewis, K. F. Stern, and E. L. Rowe, *J. Pharm. Sci.*, to be published.

(5) W. Morozowich, personal communication.

(6) T. O. Oesterling, *J. Pharm. Sci.*, **59**, 63(1970).

(7) W. F. Beyer, personal communication.

(8) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolyte Solutions," Reinhold, New York, N. Y., 1958.

(9) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, **1958**, 3574.

(10) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *ibid.*, **1958**, 3588.

(11) E. M. Kosower, "Molecular Biochemistry," McGraw-Hill, New York, N. Y., 1962, p. 239.

(12) A. Thompson and M. L. Wolfrom in "The Carbohydrates," W. Pigman, Ed., Academic, New York, N. Y., 1957, p. 175.

(13) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," Wiley, New York. N. Y., 1961, p 63.

(14) K. R. Farrar, *J. Chem. Soc.*, **1949**, 3131.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication September 5, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, 1969.

The authors wish to thank Mr. R. W. Smith and Mr. G. R. Munting for excellent technical assistance in the work and Dr. G. L. Flynn for helpful discussions.

Influence of Ionic and Nonionic Materials on Thermally-induced Ribonucleic Acid Degradation and Leakage in *Staphylococcus aureus*

M. C. ALLWOOD* and A. D. RUSSELL

Abstract □ Suspensions of *Staphylococcus aureus* were held at 50 or 60° in water, 1 *M* sucrose, or 0.5 *M* sodium chloride, and analyses were made of cellular ribonucleic acid (RNA), the content of RNA-like material in the acid-soluble (metabolic) pool and the amount of RNA-like material leaked. In sucrose and sodium chloride, as compared to water, RNA degradation was reduced at 50° and increased at 60°. In comparison to water, Mg^{++} ions reduced RNA-like material leaked at 37 and 50°, but not at 60°. The results are discussed in relation to the effect of thermal damage and loss of viability of the organism.

Keyphrases □ Ribonucleic acid (RNA) degradation, leakage—thermally induced □ Degradation, *Staphylococcus aureus* RNA—temperature effect □ Ionic, nonionic materials effect—RNA degradation □ UV spectrophotometry—analysis

Previous studies (1–4) have been concerned with the reasons for thermally-induced death in *Staphylococcus aureus* strain NCTC 3761. Several changes took place in heated suspensions of this organism: protein coagulation, a decrease in cell volume, a breakdown of ribonucleic acid (RNA), an increase in the content of RNA-like material in the acid-soluble (metabolic) pool and leakage of RNA-like material, damage to the cytoplasmic membrane, and leakage of amino acids. Deoxyribonucleic acid (DNA) did not leak from the cells and did not appear to be degraded. RNA degradation may be responsible for thermally-induced death in *Aerobacter aerogenes* (5), *Staphylococcus aureus* (3, 6, 7), and *Streptococcus faecalis* (8).

Some effects of sucrose on thermal death of *Staph. aureus* have also been described (3). These earlier findings are here considered in greater detail, and the effects of sodium chloride and Mg^{++} on RNA degradation or leakage are also presented.

MATERIALS AND METHODS

Materials—Magnesium sulfate, $MgSO_4 \cdot 7H_2O$, sucrose, and sodium chloride were of analytical reagent quality. Solutions were sterilized by heating in an autoclave.

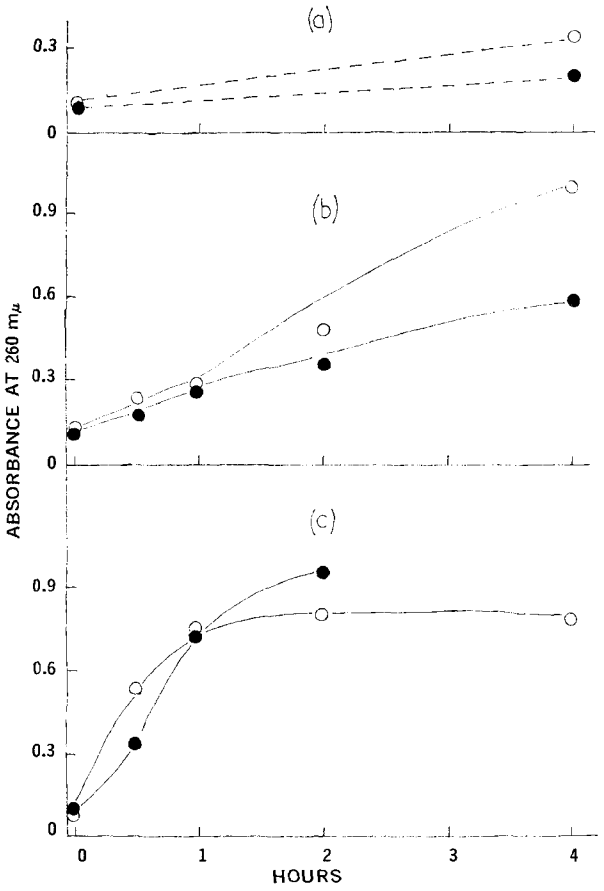


Figure 1—*Leakage of 260 mμ-absorbing material from suspensions of* Staph. aureus *stored in water* O—O *and 2* × 10^{-3} M *magnesium sulfate solution* ●—● *at* a *37°*, b *50°*, *and* c *60°*.

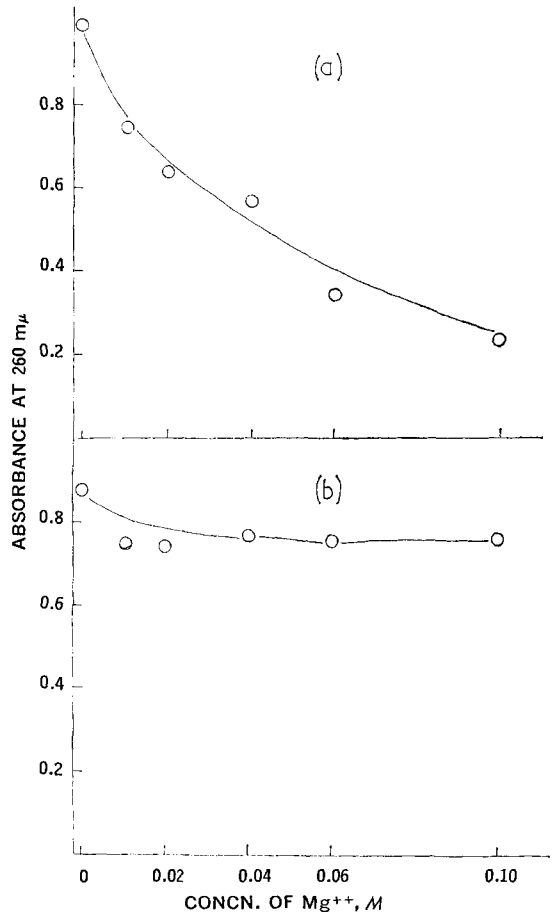


Figure 2—*Effect of magnesium sulfate concentration on the amount of 260 mμ-absorbing material from suspensions of* Staph. aureus *held for 4 hr. at* a *50°*, b *60°*.

Organism—*Staph. aureus* was grown for 18 hr. in nutrient broth (Oxoid Laboratories, Ltd., London, England), the culture centrifuged, the pellet washed twice with sterile water, and finally adjusted with sterile water to contain approximately 10^{10} viable cells/ml.

Heating Procedure—One part of a washed suspension was added to nine parts of the desired suspending medium, already at the required temperature (±0.1°), in a thermostatically-controlled water bath.

Viable Counts—Samples were removed at intervals, and viable counts made as described previously (3) using the pour-plate method, a plating medium consisting of nutrient agar containing 1% w/v yeast extract (Difco Laboratories, Ltd., Detroit, Mich.) and an incubation period of 48 hr. at 37°.

Analytical Procedure—The breakdown and release of RNA with *Staph. aureus* cells held at 50 or 60° in water, sucrose or sodium chloride were examined. An increase in the content of RNA-like material in the cold perchloric acid-soluble metabolic pool is indicative of breakdown of RNA. Details of the methods of obtaining the cold acid-soluble fraction, and of the RNA fraction, consisting of cellular RNA, *i.e.*, the RNA in the cell except for the small molecular weight RNA material in the metabolic pool, have been considered previously (3). Cellular RNA, RNA-like material in the pool, the RNA-like material leaked were determined by the orcinol technique (9) and by absorbance at 260 mμ, employing yeast RNA (British Drug Houses, Ltd., London, England) as standard. It has previously been found (1, 10) that closely related results were obtained in assaying RNA (or RNA-like material) by these two methods. Moreover, when sucrose was used as suspending medium, the orcinol technique could not be employed. When *Staph. aureus* cells were suspended in magnesium sulfate solutions, leakage of 260 mμ-absorbing material was measured.

RESULTS AND DISCUSSION

Mg^{++} ions are known to play an important part in membrane stability (11) and Strange (12) suggested that Mg^{++} stabilized bacterial membranes and reduced the lethal influences of chilling. It can be seen from Fig. 1 that, Mg^{++} reduced the amount of leakage at 37 or 50°. There appears to be no influence of Mg^{++} on leakage during storage at 60°, although the rate of loss of 260 mμ absorbing material may be reduced during the early stages of storage. It is apparent that Mg^{++} ions may influence the loss of intracellular material. The apparent lack of effect of Mg^{++} on leakage from cells stored at 60° was confirmed by storing cell suspensions in suspending fluids containing different concentrations of Mg^{++} for 4 hr. at 50 or 60°. The results are shown in Fig. 2. The total amount of 260 mμ-absorbing material lost from the cells is directly reduced at 50° as the concentration of Mg^{++} present is increased, but such a reduction is not observed at increasing Mg^{++} concentrations during storage at 60°. It seems unlikely that magnesium sulfate at the highest concentration employed could exert any osmotic influences to reduce the loss of intracellular material.

Mg^{++} ions are known to increase the stability of cellular ribosomes (13) and it was found that magnesium sulfate reduced the leakage of 260 mμ-absorbing material from heated *Aerobacter aerogenes* (5) and from starved *Sarcina lutea* (14), possibly due to stabilization of ribosomes. Thus, the apparent protective influences of Mg^{++} may be a result of the stabilization of RNA-containing units within the cell as well as the cell membrane. However, the variation in response of *Staph. aureus* stored in the presence of Mg^{++} at 50 and 60° is emphasized by the results presented here.

The changes in RNA in *Staph. aureus* cells suspended in water, 1 *M* sucrose, or 0.5 *M* sodium chloride are shown in Figs. 3–5. Cells in water at 50° (Fig. 3*a*) show a small but rapid initial decrease of cellular RNA, paralleled by a rise in the RNA-like content of the metabolic pool; this is followed by a slower loss of RNA-like

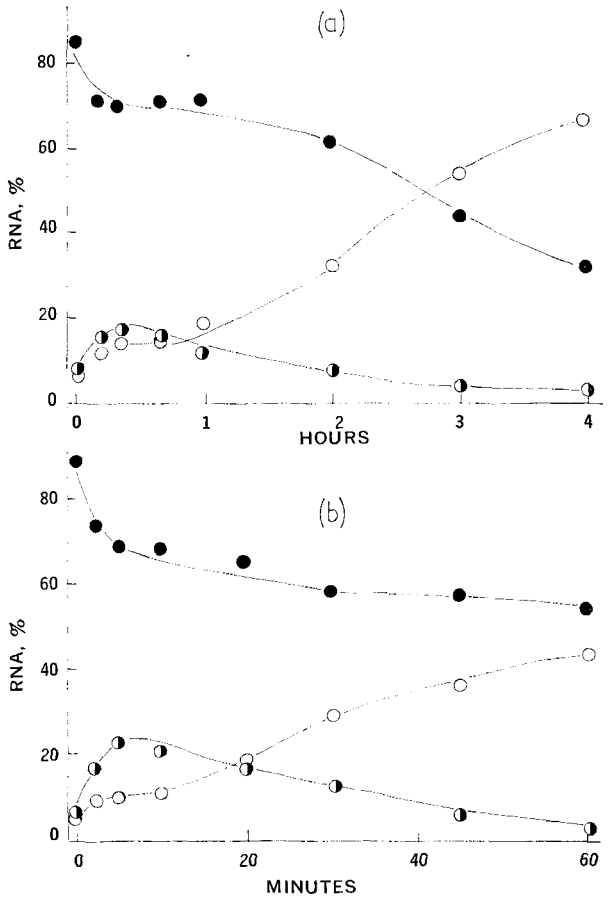


Figure 3—*Changes in the composition of RNA-like material in the metabolic pool* ◑—◑, *cellular RNA* ●—●, *and of RNA-like material leaked* ○—○, *with a suspension of* Staph. aureus *held in water at* a *50°*, b *60°*.

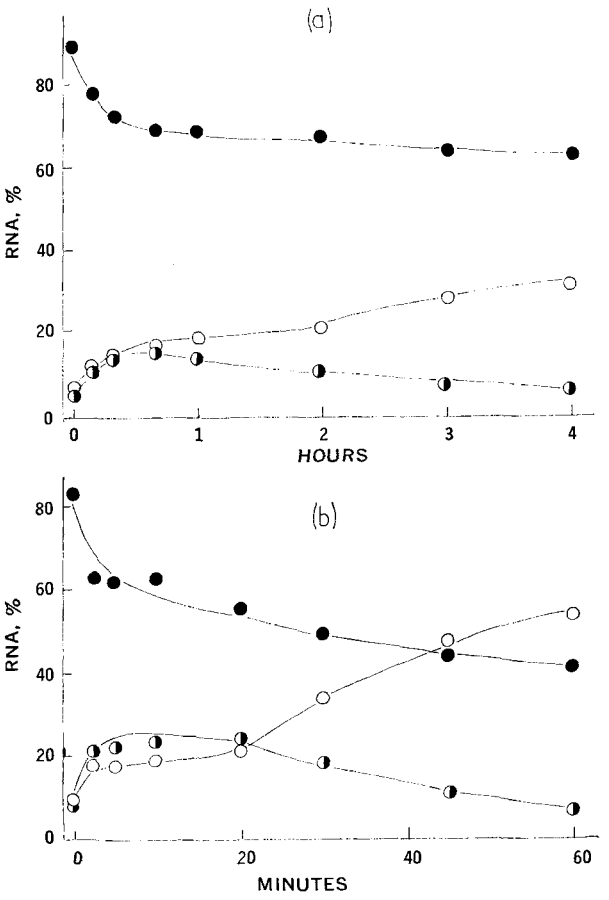


Figure 4—*Changes in the composition of RNA-like material in the metabolic pool* ◑—◑, *cellular RNA* ●—●, *and of RNA-like material leaked* ○—○, *with a suspension of* Staph. aureus *held in 1* M *sucrose at* a *50°*, b *60°*.

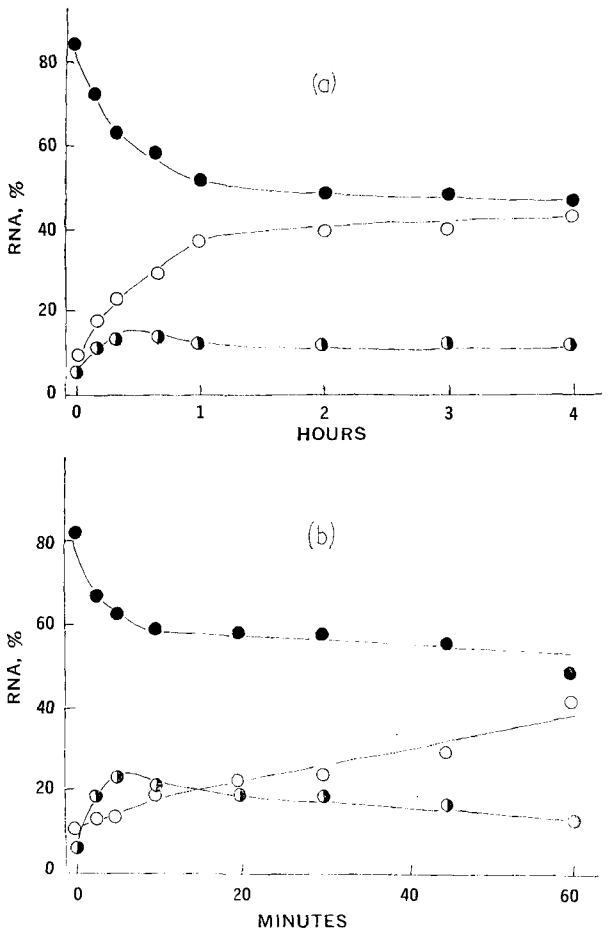


Figure 5—*Changes in the composition of RNA-like material in the metabolic pool* ◑—◑, *cellular RNA* ●—●, *and of RNA-like material leaked* ○—○, *with a suspension of* Staph. aureus *held in 0.5 M sodium chloride at* a *50°*, b *60°*.

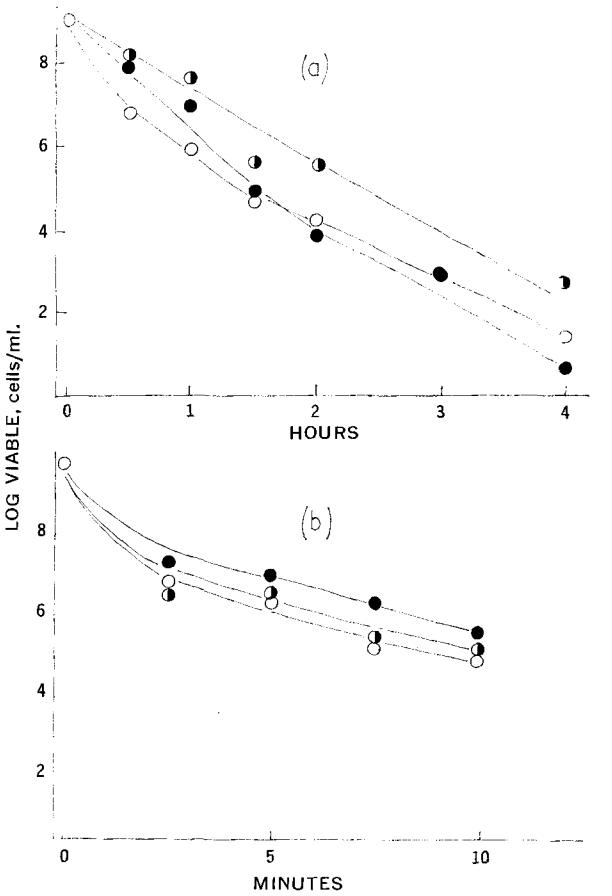


Figure 6—*Loss of viability of suspensions of* Staph. aureus *held in water* ○—○, *1 M sucrose* ●—●, *and 0.5 M sodium chloride* ◑—◑ *at* a *50°*, b *60°*.

material into the supernatant fluid, this loss clearly being derived from the degradation of cellular RNA and not principally from the cell pool. Subsequently, there is a decrease in the content of RNA-like material in the pool, and an increased leakage of RNA-like material into the surrounding environment; this diphasic loss of RNA-like material could be the result of enzyme action, as previously discussed (3), although there is no evidence as yet to substantiate this, or could be caused by leakage of large molecular weight RNA through the damaged cycloplasmic membrane. More delicate techniques are needed, however, since the reported techniques do not permit the authors to determine this.

Sodium chloride (Fig. 5*a*), like sucrose (Fig. 4*a*) protects *Staph. aureus* at 50° against the leakage of intracellular RNA-like material. It also appears that the loss of RNA-like material from the metabolic pool occurs more slowly in the presence of an osmotic stabilizer than when cells are held in water at 50°. The presence of 1 *M* sucrose as suspending medium appears to prevent the secondary breakdown (or leakage) of RNA; RNA degradation, or leakage, is higher when cells are held in saline, but again there is no diphasic pattern in the depletion of cellular RNA or in the leakage of this material.

At 60°, with *Staph. aureus* cells held in water (Fig. 3*b*), the pool content of RNA-like material increases rapidly, the content of cellular RNA falls, but leakage of RNA-like material occurs to a considerably slower extent. Subsequently, there is a decrease in the amount of RNA-like material in the pool, presumably as the membrane control over the pool is lost due to thermal damage, and this material leaks into the environment. Sucrose (Fig. 4*b*) or sodium chloride (Fig. 5*b*), at 60°, apparently caused a more rapid degradation of RNA, although a longer period elapses before there is any appreciable loss of material from the metabolic pool. After 1 hr. at 60°, the greatest loss of RNA-like material occurs from cells held in sucrose, which confirms previous findings (1, 3); the loss from cells in water and saline appears to be of a similar, but lower, order.

A comparison has been made of the loss of viability of cells stored in water, 1 *M* sucrose or 0.5 *M* sodium chloride at 50 or 60°. The results are present in Fig. 6. A statistical analysis of the results was made, using a common regression line calculation, to test if the three regressions could be represented by a common line. During storage at 50° (Fig. 6*a*) sodium chloride had a significant protective influence on the loss of viability of the *Staph. aureus* suspension, but there was no significant difference in loss of viability of cells held in water or sucrose. During storage at 60° (Fig. 6*b*), the three regression lines could be represented by one common line, and any differences are thus not significant.

As evidenced by a rise in the content of RNA-like material in the metabolic pool, RNA degradation obviously occurs in heated *Staph. aureus* cells. The rate of degradation increases with temperature, and occurs before leakage takes place (particularly at 60°). Such a degradation may be responsible for thermally-induced death in this strain (1, 3) and in other strains of *Staph. aureus* (6, 7). It is, however, difficult to link this degradation with loss of viability of *Staph. aureus* held in different suspending media (Fig. 6). Moreover, in assessing the primary site of damage in heated cells, other changes in the organism must be considered, including changes in appearance under the electron microscope, in size, and in light-scattering properties (4). Thus, whereas RNA degradation may be the cause of death in heated cells (3, 6, 7) it is apparent that further research is needed to substantiate this contention.

REFERENCES

(1) M. C. Allwood and A. D. Russell, *Appl. Microbiol.*, **15**, 1266 (1967).

(2) M. C. Allwood and A. D. Russell, *Experientia*, **23**, 878(1967).
(3) M. C. Allwood and A. D. Russell, *J. Bacteriol.*, **95**, 345 (1968).
(4) M. C. Allwood and A. D. Russell, *J. Appl. Bacteriol.*, **32**, 68(1969).
(5) R. E. Strange and M. Shon, *J. Gen. Microbiol.*, **34**, 99(1964).
(6) J. J. Iandolo and Z. J. Ordal, *J. Bacteriol.*, **91**, 134(1966).
(7) S. J. Sogin and Z. J. Ordal, *ibid.*, **94**, 1082(1967).
(8) L. R. Beuchat and R. V. Lechowich, *Appl. Microbiol.*, **16**, 772(1968).
(9) G. Ceriotti, *J. Biol. Chem.*, **214**, 54(1955).
(10) M. C. Allwood, Ph.D. thesis, University of Wales (1968).
(11) K. McQuillen, in "The Bacteria," vol. 1, I. C. Gunsalus and R. Y. Stanier, Eds., Academic, New York N. Y., 1960.
(12) R. E. Strange, *Nature*, **203**, 1304(1964).
(13) D. Kennell and A. Kotoulos, *J. Bacteriol.*, **93**, 334(1967).
(14) I. G. Burleigh and E. A. Dawes, *Biochem. J.*, **102**, 236(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 16, 1969, from the *Welsh School of Pharmacy, University of Wales Institute of Science and Technology, Cardiff, Great Britain.*

Accepted for publication September 5, 1969.

* Present address: Department of Pharmacy, The University, Nottingham, England.

Renal Effects of a Vasodilator: 2-[*N*-methyl-piperidyl-(4)]-3-amino-5-(4′-pyridyl)-pyrazole HCl (Ciba 31-531 Ba)

RONALD L. WILLIAMS and JAMES E. PEARSON

Abstract □ Acetylcholine (Ach), when infused directly into the renal artery of a kidney, causes unilateral vasodilatation and increased salt excretion. Ciba 31-531 Ba, a nonspecific vasodilator, was infused both directly into the renal artery and given systemically, and the effects were compared to Ach. Infusion of Ach at 0.1 μg./kg./min. into the left renal artery (LRA) of an anesthetized dog effected a unilaterally increased salt excretion and increased effective renal plasma flow (ERPF) and urine volume, but no demonstrable change in glomerular filtration rate (GFR) or blood pressure. Infusion of Ciba 31-531 Ba (LRA) at 0.1 mg./kg./min. resulted in a similar unilateral saluresis of approximately 50% increase over Ach with an increase in ERPF and urine volume but no change in GFR or blood pressure. The right kidney served as an internal control. When Ach and Ciba 31-531 Ba were given systemically, at higher doses there was a drop in blood pressure, decreased solute excretion, and decreased ERPF and GFR for both drugs. The effects of Ach were reversed with atropine. The effects of Ciba 31-531 Ba were not reversed by atropine or propranolol. The authors believe that the direct renal effects of these drugs are the results of direct tubular effects associated in some way with hemodynamic alterations.

Keyphrases □ 2-[*N*-methyl-piperidyl-(4)]-3-amino-5-(4′-pyridyl)-pyrazole HCl (Ciba 31-531 Ba)—renal effects □ Renal infusion—Ciba 31-531 Ba □ Acetylcholine, Ciba 31-531 Ba renal effects—comparison □ IV infusion, Ciba 31-531 Ba—renal effects

A new vasodilator, recently synthesized by Ciba (1), given systemically appeared to have contradictory effects on the kidney (2). In some cases it acted as a vasodilator and increased renal blood flow. In other experiments the various systemic effects appeared to predominate and no change or decrease in renal blood flow was observed.

Ciba compound 31-531 Ba, 2-[*N*-methyl-piperidyl-(4)]-3-amino-5-(4′-pyridyl)-pyrazole HCl, antagonized the effects of epinephrine, norepinephrine, angiotensin II-amide, and histamine on smooth muscle of the guinea pig and the cat. It did not antagonize the effects of $BaCl_2$, acetylcholine, or bradykinin (1).

When compared to papaverine, nitroglycerin, and isoproterenol in an open-chest cat preparation where it is possible to measure both systemic arterial and venous pressure, isoproterenol and Ciba 31-531 Ba administration caused both arterial and venous pressure to fall.

The direct renal effects of adrenergic and cholinergic agents have recently been reported in the literature (3). It was felt that a study of this rather nonspecific muscle-relaxing drug on renal function as compared to acetylcholine would be of interest. The following experiments were designed to study the effect on sodium excretion and renal hemodynamics when Ciba 31-531 Ba is given directly into the renal artery and when given systemically.

MATERIALS AND METHODS

Eight mongrel dogs were anesthetized with intravenous injections of pentobarbital sodium, 30 mg./kg. Both ureters were cannulated through an abdominal midline incision, and the cannulae were positioned approximately 1.27 cm. (0.5 in.) below the ureteral pelvic junction. A femoral vein and artery were cannulated and the arterial cannula was connected to an E & M linear transducer (E & M Co., Inc.) with a three-way stopcock for recording blood pressure with an E & M polygraph. Arterial blood samples were obtained through the three-way stopcock. The sustaining solution containing 1.8 mg./ml. of creatinine, 0.5 mg./ml. of *p*-aminohippurate (PAH) in normal saline was infused at a rate of 5 ml./min. through the venous system by means of a dual-syringe constant-flow pump. After exposing the left renal artery by the retroperitoneal approach, a 27-gauge hypodermic needle, attached to No. 10 polyethylene tubing, was placed into the left renal artery in the direction of blood flow. Through this renal arterial system, a solution of isotonic sodium chloride was continuously infused at a rate of 0.1 ml./min. Solutions of drugs were also infused at the same rate through the system by changing the renal arterial infusate to one containing test drugs dissolved in normal saline. One to two hours were allowed for equilibration and then collections of 10-min. urine samples from each kidney were begun. Blood samples, drawn every 20 min., were heparinized, centrifuged, and the plasma immediately removed. At

(2) M. C. Allwood and A. D. Russell, *Experientia*, **23**, 878(1967).
(3) M. C. Allwood and A. D. Russell, *J. Bacteriol.*, **95**, 345 (1968).
(4) M. C. Allwood and A. D. Russell, *J. Appl. Bacteriol.*, **32**, 68(1969).
(5) R. E. Strange and M. Shon, *J. Gen. Microbiol.*, **34**, 99(1964).
(6) J. J. Iandolo and Z. J. Ordal, *J. Bacteriol.*, **91**, 134(1966).
(7) S. J. Sogin and Z. J. Ordal, *ibid.*, **94**, 1082(1967).
(8) L. R. Beuchat and R. V. Lechowich, *Appl. Microbiol.*, **16**, 772(1968).
(9) G. Ceriotti, *J. Biol. Chem.*, **214**, 54(1955).
(10) M. C. Allwood, Ph.D. thesis, University of Wales (1968).
(11) K. McQuillen, in "The Bacteria," vol. 1, I. C. Gunsalus and R. Y. Stanier, Eds., Academic, New York N. Y., 1960.
(12) R. E. Strange, *Nature*, **203**, 1304(1964).
(13) D. Kennell and A. Kotoulos, *J. Bacteriol.*, **93**, 334(1967).
(14) I. G. Burleigh and E. A. Dawes, *Biochem. J.*, **102**, 236(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 16, 1969, from the *Welsh School of Pharmacy, University of Wales Institute of Science and Technology, Cardiff, Great Britain.*

Accepted for publication September 5, 1969.

* Present address: Department of Pharmacy, The University, Nottingham, England.

Renal Effects of a Vasodilator: 2-[*N*-methyl-piperidyl-(4)]-3-amino-5-(4′-pyridyl)-pyrazole HCl (Ciba 31-531 Ba)

RONALD L. WILLIAMS and JAMES E. PEARSON

Abstract □ Acetylcholine (Ach), when infused directly into the renal artery of a kidney, causes unilateral vasodilatation and increased salt excretion. Ciba 31-531 Ba, a nonspecific vasodilator, was infused both directly into the renal artery and given systemically, and the effects were compared to Ach. Infusion of Ach at 0.1 μg./kg./min. into the left renal artery (LRA) of an anesthetized dog effected a unilaterally increased salt excretion and increased effective renal plasma flow (ERPF) and urine volume, but no demonstrable change in glomerular filtration rate (GFR) or blood pressure. Infusion of Ciba 31-531 Ba (LRA) at 0.1 mg./kg./min. resulted in a similar unilateral saluresis of approximately 50% increase over Ach with an increase in ERPF and urine volume but no change in GFR or blood pressure. The right kidney served as an internal control. When Ach and Ciba 31-531 Ba were given systemically, at higher doses there was a drop in blood pressure, decreased solute excretion, and decreased ERPF and GFR for both drugs. The effects of Ach were reversed with atropine. The effects of Ciba 31-531 Ba were not reversed by atropine or propranolol. The authors believe that the direct renal effects of these drugs are the results of direct tubular effects associated in some way with hemodynamic alterations.

Keyphrases □ 2-[*N*-methyl-piperidyl-(4)]-3-amino-5-(4′-pyridyl)-pyrazole HCl (Ciba 31-531 Ba)—renal effects □ Renal infusion—Ciba 31-531 Ba □ Acetylcholine, Ciba 31-531 Ba renal effects—comparison □ IV infusion, Ciba 31-531 Ba—renal effects

A new vasodilator, recently synthesized by Ciba (1), given systemically appeared to have contradictory effects on the kidney (2). In some cases it acted as a vasodilator and increased renal blood flow. In other experiments the various systemic effects appeared to predominate and no change or decrease in renal blood flow was observed.

Ciba compound 31-531 Ba, 2-[*N*-methyl-piperidyl-(4)]-3-amino-5-(4′-pyridyl)-pyrazole HCl, antagonized the effects of epinephrine, norepinephrine, angiotensin II-amide, and histamine on smooth muscle of the guinea pig and the cat. It did not antagonize the effects of $BaCl_2$, acetylcholine, or bradykinin (1).

When compared to papaverine, nitroglycerin, and isoproterenol in an open-chest cat preparation where it is possible to measure both systemic arterial and venous pressure, isoproterenol and Ciba 31-531 Ba administration caused both arterial and venous pressure to fall.

The direct renal effects of adrenergic and cholinergic agents have recently been reported in the literature (3). It was felt that a study of this rather nonspecific muscle-relaxing drug on renal function as compared to acetylcholine would be of interest. The following experiments were designed to study the effect on sodium excretion and renal hemodynamics when Ciba 31-531 Ba is given directly into the renal artery and when given systemically.

MATERIALS AND METHODS

Eight mongrel dogs were anesthetized with intravenous injections of pentobarbital sodium, 30 mg./kg. Both ureters were cannulated through an abdominal midline incision, and the cannulae were positioned approximately 1.27 cm. (0.5 in.) below the ureteral pelvic junction. A femoral vein and artery were cannulated and the arterial cannula was connected to an E & M linear transducer (E & M Co., Inc.) with a three-way stopcock for recording blood pressure with an E & M polygraph. Arterial blood samples were obtained through the three-way stopcock. The sustaining solution containing 1.8 mg./ml. of creatinine, 0.5 mg./ml. of *p*-aminohippurate (PAH) in normal saline was infused at a rate of 5 ml./min. through the venous system by means of a dual-syringe constant-flow pump. After exposing the left renal artery by the retroperitoneal approach, a 27-gauge hypodermic needle, attached to No. 10 polyethylene tubing, was placed into the left renal artery in the direction of blood flow. Through this renal arterial system, a solution of isotonic sodium chloride was continuously infused at a rate of 0.1 ml./min. Solutions of drugs were also infused at the same rate through the system by changing the renal arterial infusate to one containing test drugs dissolved in normal saline. One to two hours were allowed for equilibration and then collections of 10-min. urine samples from each kidney were begun. Blood samples, drawn every 20 min., were heparinized, centrifuged, and the plasma immediately removed. At

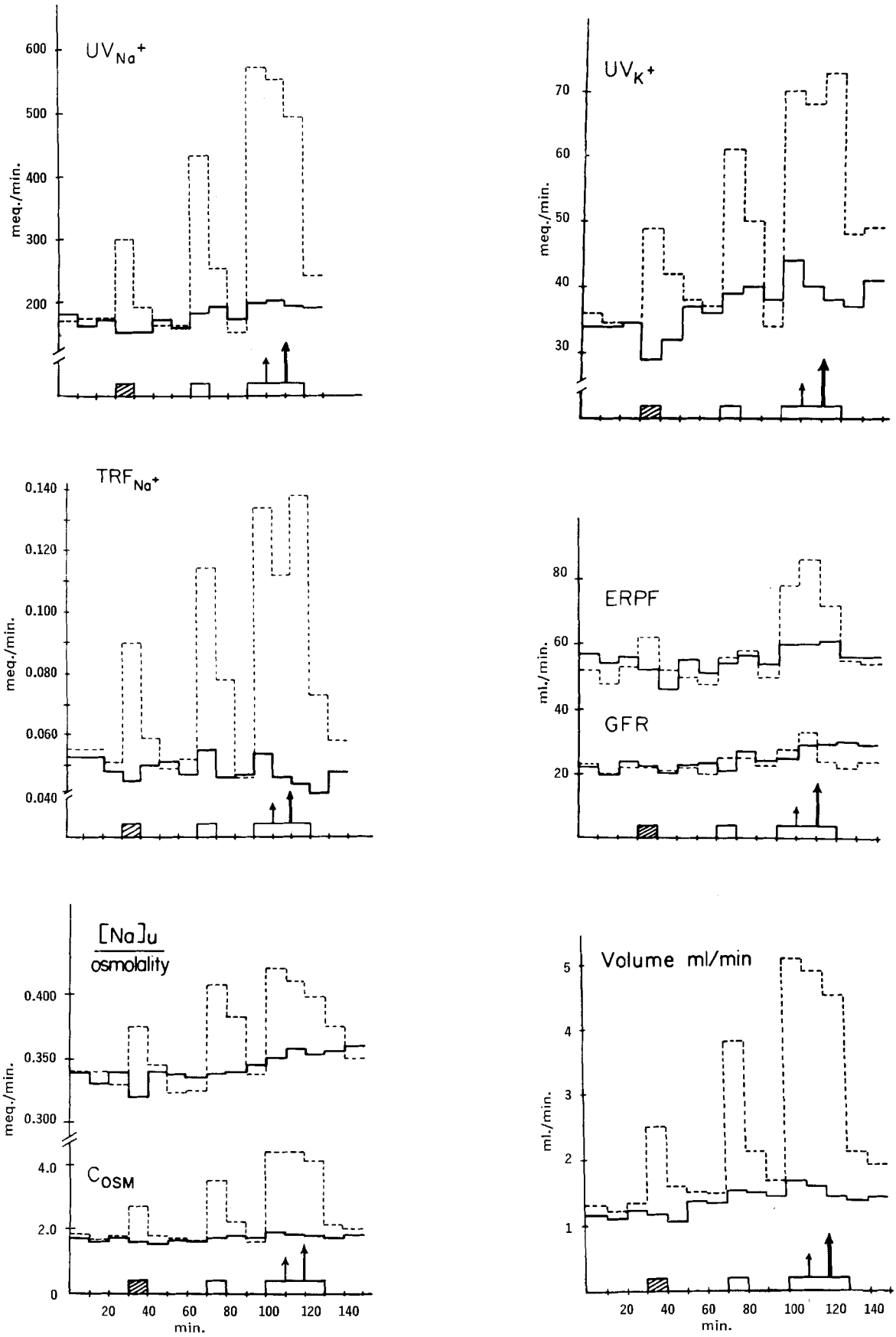


Figure 1.—*Comparison of the direct renal effects of Ciba 31-531 Ba and acetylcholine during infusion into the left renal artery of a 15.5-kg. female dog. Note that the infusion dosage of Ciba 31-531 Ba is 1000 times greater than acetylcholine. Key: experimental (left kidney), - - -; control (right kidney) ——; ▨ acetylcholine (0.1 mcg./kg./min.); □, Ciba 31-531 Ba (0.1 mg./kg./min.); →, atropine (0.1 mg./kg. i.v.); →, propanolol (0.33 mg./kg. i.v.).*

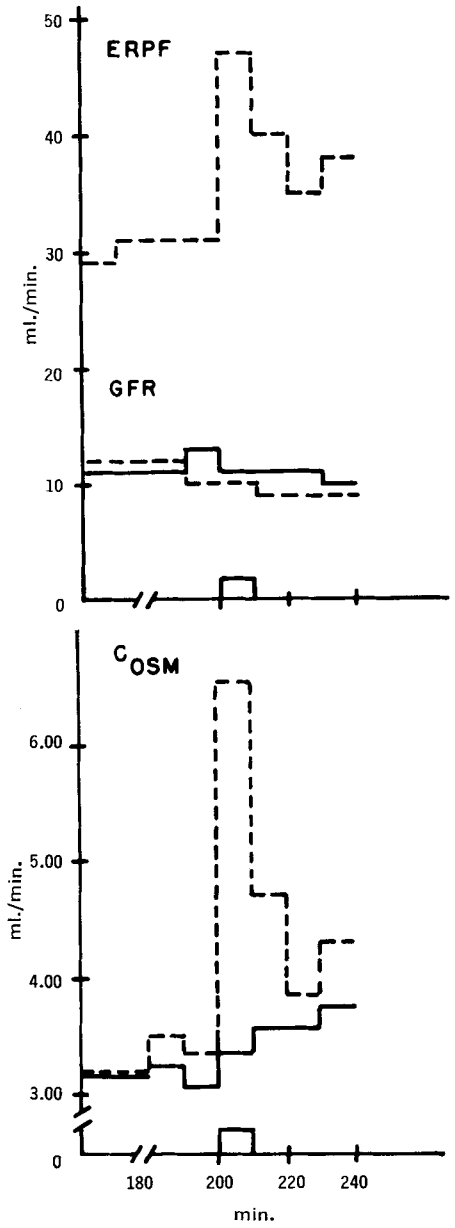
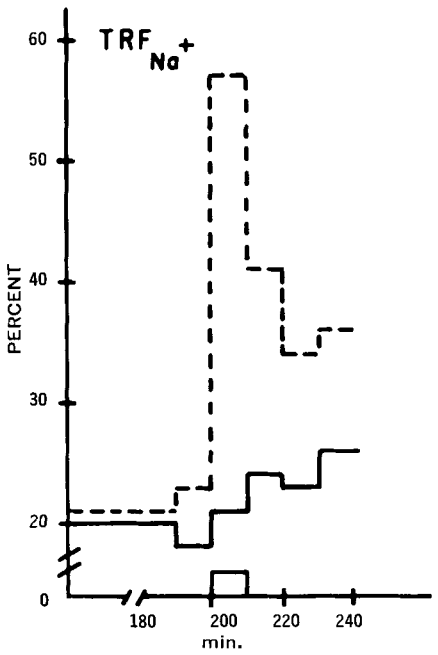


Figure 2—*Effect of Ciba 31-531 Ba during infusion into the left renal artery of a dog with oliguria which had to be given 500 ml. of a solution of 6% mannitol and 2% sodium chloride. The PAH clearances on the right kidney were not determined. Key: experimental (left kidney), - - -; control (right kidney), ——; Ciba 31-531 Ba (0.1 mg./kg./min.), □.*



least three control urine samples were collected before test agents were infused into the kidney. Intravenous experiments used the same technique with sham retroperitoneal exposure.

ANALYTICAL

Chloride concentrations in urine and plasma were determined using a Buchler-Cotlove chloridometer, sodium and potassium concentrations in urine and plasma were determined using a Baird-Atomic flame photometer with an internal lithium standard, a modification of the Bonsnes and Taussky (4) method was used in the determination of plasma and urine creatinine, and PAH determinations were made using a modification of the method of Bratton and Marshall (5). The osmolalities were determined with a Fiske osmometer. In addition to the electrolyte excretion rates, the glomerular filtration rates (GFR), the effective renal plasma flow (ERPF), and the tubular rejection fraction (TRF) of sodium were calculated. The tubular rejection fraction is the ratio of the rate of sodium excretion to the filtered load of sodium. The TRF can vary independently of changes in filtered load (6). Because only concentrations are used in the calculation of TRF, its magnitude is independent of the size of the animal and facilitates comparisons between dogs. The absolute rate of tubular reabsorption (T_{Na}) is less useful because it is markedly affected by filtered load, which in turn is a product of extrarenal as well as renal factors:

$$TRF_{Na} = \frac{U_{Na}V}{P_{Na}GFR} = \frac{U_{Na}P \text{ creatinine}}{P_{Na}U \text{ creatinine}}$$

where U is the urine concentration, P is the plasma concentration, V is the urine flow in ml./min., and GFR is the clearance of creatinine.

All drug doses are based on the respective salts: acetylcholine bromide and atropine sulfate. Ciba 31-531 Ba is 2-[*N*-methylpiperidyl-(4)]-3-amino-5-(4′-pyridyl)-pyrazole HCl (obtained from Ciba Pharmaceutical Co., Summit, N. J.), propranolol hydrochloride (obtained from Ayerst Lab., Inc., New York, N. Y.).

RESULTS

Direct Renal Effect of Ciba 31-531 Ba Compared to Acetylcholine—The administration of Ciba 31-531 Ba (0.1 mg./kg./min.) by infusion into the left renal artery (LRA) resulted in a diuresis and saluresis similar to acetylcholine (0.1 mcg./kg./min.) (Fig. 1). The response to direct renal infusion of 0.1 mg./kg./min. of Ciba 31-531 Ba was about 50% greater than that to the renal infusion of 0.1 mcg./kg./min. acetylcholine and was the result of an increased tubular rejection of sodium (TRF Na^+). This direct renal response to

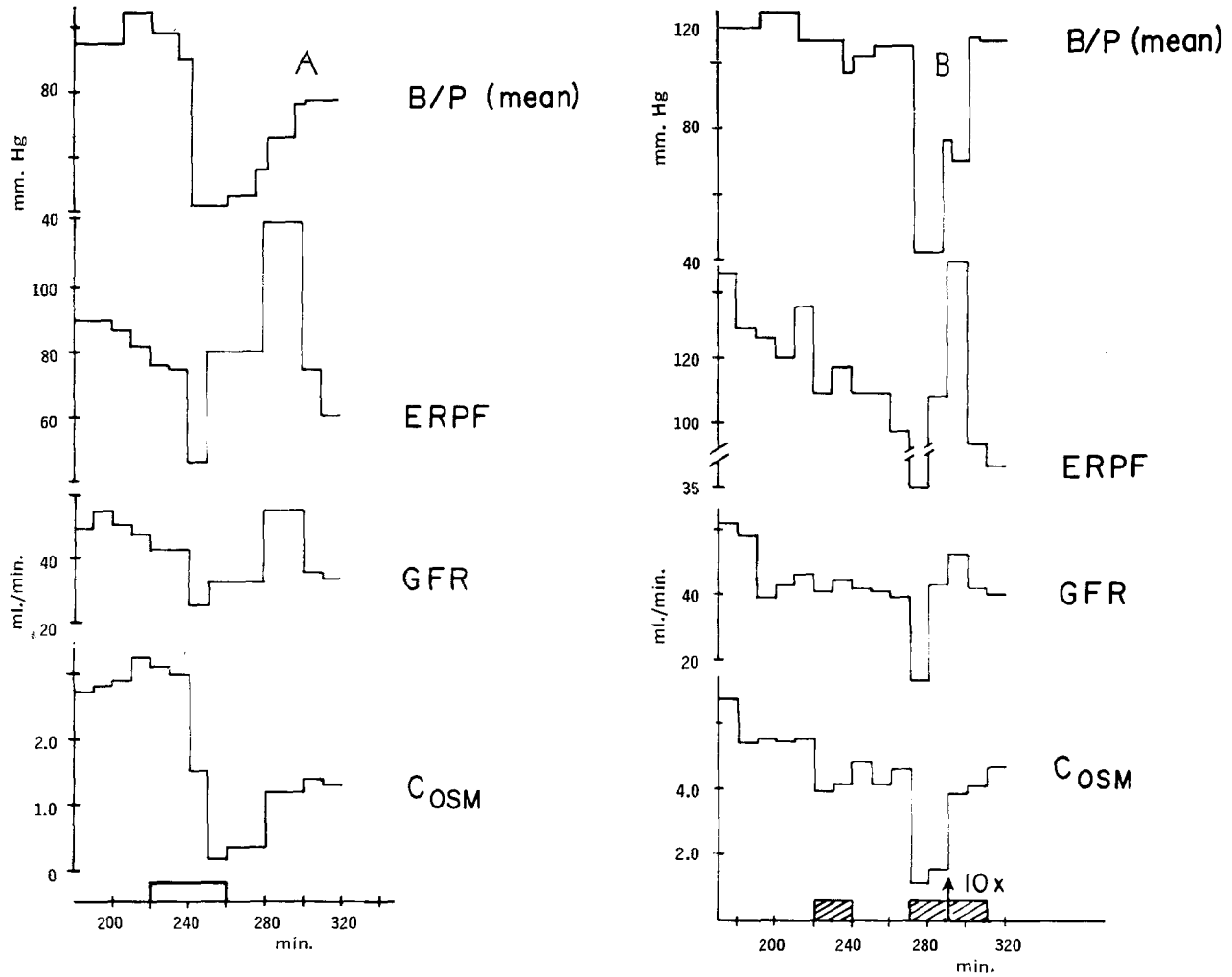


Figure 3—*Comparison of the indirect renal effects of Ciba 31-531 Ba given intravenously to a 10-kg. female dog and acetylcholine given intravenously to a 14-kg. female dog. Key:* A, □, *Ciba 31-531 Ba (20 mg./ml. i.v.);* B, ▨, *acetylcholine (1 mcg./kg./min.);* →, *atropine (1 mg./kg. i.v.).*

Ciba 31-531 Ba was not blocked by the systemic administration of atropine or propranolol (Fig. 1). The effective renal plasma flow (ERPF) was not changed during the first infusion, but it was markedly increased on the experimental side during the second 30-min. infusion. There was no change in glomerular filtration rate (GFR) during the acetylcholine infusion or during the first 10-min. infusion of Ciba 31-531 Ba. There was, however, a unilateral increase in the excretion of Na^+, K^+, Cl^-, and water. The osmolar clearance (Cosm) increased with both acetylcholine and Ciba 31-531 Ba, and the sodium contribution to the osmolality also increased with both agents.

There was no significant change in systemic blood pressure during this entire experiment.

Effect in a Dog with Marked Sodium Retention—The administration of Ciba 31-531 Ba (0.1 mg./kg./min.) LRA to a dog with plasma sodium of 168 meq./l. and oliguria resulted in a large unilateral saluresis. This animal had failed to respond with diuresis even when given 6% mannitol in a 2% NaCl solution (Fig. 2). The TRF Na^+ was 58% of filtered load. During this 10-min. infusion period the ERPF increased from a depressed 32 ml./min. to only 47 ml./min. with no change in GFR. The osmolar clearance was increased from 3.4 to 6.6 ml./min.

Indirect Renal Effects—Intravenous infusion of Ciba 31-531 Ba (20 mg./ml.) at increasing rates (total infused 400 mg.) until there was a substantial decrease in blood pressure resulted in depressed ERPF, GFR, Cosm, Na^+UV, $TRFNa^+$, and K^+UV. The infusion was discontinued and the blood pressure returned to 75% of control value with an unusual increase in ERPF and GFR during the period from 280–300 min. The osmolar clearance, however, remained depressed during the same exaggerated increase in ERPF and GFR (Fig. 3A).

The intravenous infusion of acetylcholine for two experimental periods is shown in Fig. 3B. The first period of 20 min. at 1 mcg./kg./min. i.v. did not result in a blood pressure depression. The second period of 40 min. at 10 mcg./kg./min. caused a substantial decrease in blood pressure with a decrease in GFR, ERPF, Cosm, Na^+UV, $TRFNa^+$, and K^+UV. This effect of Ach i.v. was reversed with an injection of atropine, 14 mg. i.v. (1 mg./kg.).

DISCUSSION

Evidence has accumulated that acute increases in GFR *per se* have little effect on sodium excretion (7, 8). Therefore, natriuresis requires changes in fractional tubular reabsorption. However, the performance of the renal tubules are dependent for their several clearance functions upon the blood supply to the kidney. The exact quantitative influences of renal blood flow (9), intrarenal hydrostatic pressure (10), and intrarenal distribution of blood flow (11) on sodium excretion are not understood. Some diuretics such as furosemide and ethacrynic acid, which have no marked vascular effects elsewhere other than on renal vasculature, do increase the renal blood flow in parallel with natriuresis. However, for these diuretics the increased blood flow is not a requirement for the natriuretic response (12, 13). Autonomic drugs such as acetylcholine, which have powerful vascular effects throughout the body, produce natriuresis only when they are infused into a renal artery (14). Such is the case with Ciba 31-531 Ba in these experiments. Ciba 31-531 Ba must be infused into a renal artery to obtain the saluretic

response. However, this does not mean that the saluresis produced by infusing acetylcholine, or other autonomic agents, is simply the result of changes in hemodynamics (15). McGiff *et al.* (16) have shown that the natriuresis produced by angiotensin given intravenously was independent of the magnitude of increases in blood pressure, increases of GFR, and reduction in renal blood flow. Also the degrees of renal concentration and of rates of biotransformation will determine the magnitude of systemic vascular effects. The magnitude of the systemic vascular effects will bring into play various reflex autonomic nervous activities which may enhance or antagonize direct renal effects of autonomic drugs (17). This reflex antagonism of direct renal effects is especially demonstrated by diazoxide which produces salt retention when given systemically but produces salt excretion when infused directly into the renal artery (18).

It appears from the data presented here that Ciba 31-531 Ba has both tubular and hemodynamic effects. Changes in GFR do not seem to be concerned in these phenomena. In the case of the animal with extremely high plasma sodium (Fig. 2) the GFR remained unchanged in spite of 300% increase in TRFNa^+ and 300% increase in osmolal clearance. During this 300% increase in osmolal clearance, the ERPF increased only by a factor of 47%. In this experiment the animal was retaining sodium in the face of an elevated plasma sodium. This response suggests a proximal site of action since it is improbable that a TRF Na^+ of 58% could be accounted for by a distal mechanism.

The interpretation of the renal mechanism of action remains complicated even though changes in GFR may be ignored. Saluresis was produced by increased tubular rejection of sodium accompanied by increased renal plasma flow. What the interrelationship is between these two parameters remains unknown. Neither were they always congruent (Fig. 1). Also, Harvey (19) showed that acetylcholine produced a generalized vasodilatation in the kidney along with reduced plasma extraction ratios of PAH and inulin. However, there was also an increased tubular secretion of PAH. Something more is involved than a mere shunting of the blood supply. Williams *et al.* (20) believe that acetylcholine produces its effect by an action mainly upon the renal tubules. Martino and Earley (21) postulate that the hemodynamic alterations are the chief causes for the saluresis. However, they obtained no quantitative correlation between changes in sodium excretion and changes in intrarenal venous pressure. May and Carter (15) infused arecoline into the renal portal system of hens and obtained a marked unilateral saluresis. They postulated a direct effect upon the permeability of the renal tubule since it is unlikely that the slight increases in plasma flowing through the portal system in these preparations could be the cause of the natriuresis. The authors of this article also hesitate to describe the direct renal action of Ciba 31-531 Ba as mere vasodilatation since this agent also antagonizes the effects of norepinephrine which the authors believe to cause salt retention by a tubular action (3).

Perhaps the evidence from the direct renal effects of different pharmacological agents will help in the future understanding of the relationship between tubular rejection of sodium and peritubular capillary blood flow, distribution, pressure, and permeability.

REFERENCES

(1) H. Brunner, K. Eichenberger, M. Meier, M. Wilhelm, and P. Schmidt, *Experientia*, **22,** 208(1966).

(2) Ciba Limited, Basle, Orientation on Ciba 31, 531-Ba, Basle, 1964.

(3) J. E. Pearson and R. L. Williams, *Brit. J. Pharmacol.*, **33,** 223(1968).

(4) R. W. Bonsnes and H. H. Taussky, *J. Biol. Chem.*, **158,** 581(1945).

(5) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128,** 537(1939).

(6) D. H. Simmons, R. B. Harvey, and T. Hoshiko, *Am. J. Physiol.*, **178,** 182(1954).

(7) M. D. Lindheimer, R. C. LaLone, and N. G. Levinsky, *J. Clin. Invest.*, **46,** 256(1967).

(8) N. G. Levinsky, *Ann. N. Y. Acad. Sci.*, **139,** 295(1966).

(9) A. Shuster, E. A. Alexander, R. C. LaLone, and N. G. Levinsky, *Am. J. Physiol.*, **211,** 1181(1966).

(10) R. E. Shipley and R. S. Study, *ibid.*, **167,** 676(1951).

(11) S. Carriere, *Can. J. Physiol. Pharmacol.*, **47,** 199(1969).

(12) J. B. Hook, A. H. Blatt, M. J. Brady, and H. E. Williamson, *J. Pharmacol. Exptl. Therap.*, **154,** 667(1966).

(13) A. G. Birtch, R. M. Zakheim, L. G. Jones, and A. C. Barger, *Circulation Res.*, **21,** 869(1967).

(14) A. R. Lavender, I. Aho, and T. N. Pullman, *Proc. Soc. Exptl. Biol. Med.*, **119,** 887(1965).

(15) D. G. May and M. K. Carter, *Am. J. Physiol.*, **212,** 1351 (1967).

(16) J. C. McGiff, J. R. Lynch, J. A. Leinicke, J. C. Strand, and A. Aboosi, *J. Clin. Invest.*, **48,** 146(1969).

(17) R. L. Williams, *Perspectives Biol. Med.*, **10,** 251(1967).

(18) J. A. Greene, Jr., *Proc. Soc. Exptl. Biol. Med.*, **125,** 375 (1967).

(19) R. B. Harvey, *Am. J. Physiol.*, **211,** 487(1966).

(20) R. L. Williams, J. E. Pearson, Jr., and M. K. Carter, *J. Pharmacol.*, **147,** 32(1965).

(21) J. A. Martino and L. E. Earley, *Circulation Res.*, **23,** 371 (1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 15, 1969, from the *Department of Pharmacology, LSU Medical Center, New Orleans, LA 70112*

Accepted for publication September 10, 1969.

N-(1,1-dihydroperfluorooctyl)pyridinium Trifluoromethanesulfonate, a New Quaternary Ammonium Antiseptic

D. M. UPDEGRAFF*, D. C. KVAM, and J. E. ROBERTSON

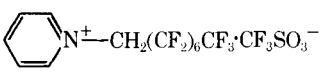
Abstract □ The synthesis and biological evaluation of *N*-(1,1-dihydroperfluorooctyl)pyridinium trifluoromethanesulfonate (MBR 3092-42), a highly fluorinated quaternary ammonium antiseptic, are described. The material is structurally related to cetylpyridinium chloride. It shows approximately equivalent bacteriostatic activity to cetylpyridinium chloride but somewhat less fungistatic activity. The bactericidal activity of MBR 3092-42 is slightly less than that of cetylpyridinium chloride in equal concentration in distilled water or in 10% blood serum, but is slightly greater in 80% human blood. MBR 3092-42 is also antibacterially equivalent to cetylpyridinium chloride on the resident bacterial flora of human skin. Although this new compound slowly decomposes in water at pH 7 and above, it is stable in weakly acidic aqueous or hydroalcoholic solutions. Solutions of MBR 3092-42 yield much lower surface tension values than equivalent concentrations of cetylpyridinium chloride.

Keyphrases □ *N*-(1,1-dihydroperfluorooctyl)pyridinium trifluoromethanesulfonate (MBR 3092-42)—synthesis □ Antimicrobial activity—MBR 3092-42 □ Dermal toxicity—MBR 3092-42 □ Acute toxicity—MBR 3092-42 □ Irritation, sensitivity testing—MBR 3092-42

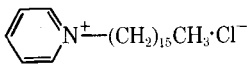
The quaternary ammonium-type germicides, introduced by Domagk (1), are highly effective broad spectrum germicides and fungicides. However, this class of compound suffers from the disadvantage of being inactivated by lecithin, long-chain fatty acids, and other anionic materials. In spite of this, they have been successfully used as antiseptics for general and surgical applications for many years.

In general, quaternary ammonium germicides are nontoxic and nonirritating to skin and mucous membranes at the concentrations used for antiseptic effects (2). At higher concentrations, however, these compounds are toxic and irritating to skin and mucous membranes. Most commercially available compounds of this class show similar toxicological and pharmacological properties (3–6).

This report concerns itself with the synthesis and biological properties of MBR 3092-42, and compares these properties, in most cases, with those of cetylpyridinium chloride, one of the oldest and most active of the quaternary ammonium germicides currently in use.



MBR 3092–42



cetylpyridinium chloride

EXPERIMENTAL

Synthesis—*N*-(1,1-dihydroperfluorooctyl)pyridinium trifluoromethanesulfonate. 1,1-Dihydroperfluorooctyl trifluoromethanesulfonate (7) (1.30 kg., 2.44 moles) was added over 2 hr. to a solution of 2.59 kg. (3.06 moles) of pyridine in 2.1 l. of acetone. After the solution was warmed at 40° for 24 hr., the product was precipitated by cooling and addition of excess isopropyl ether. The collected solid was purified by reprecipitation from acetone–ether to afford 870 g. (58%) of product, m.p. 112.5–114.5°.

Antimicrobial Activity—Bacteriostatic and fungistatic activities were compared using the filter paper disk-agar plate diffusion assay method of Vincent and Vincent (8). Activity was determined both in the presence and absence of blood or serum. Experiments were carried out at three dosage levels: 40, 4, and 0.4 mcg. per paper disk on PGY agar:

PGY Agar	Concentration, g./l.
NaCl	8.0
KCl	0.4
$MgSO_4 \cdot 7H_2O$	0.154
$CaCl_2 \cdot 7H_2O$	0.016
$Na_2HPO_4 \cdot 7H_2O$	0.29
KH_2PO_4	0.15
Phenol Red	0.6 ml. of 0.2% soln.
Yeast extract	1.0
Glucose	2.0
Ionagar	8.5
Deionized water to 1 l.	

Bactericidal properties were assayed using a quantitative kill-rate method similar to that originally described by Chick (9). The test was carried out in three media: deionized water, 10% horse serum, or 80% human blood in deionized water. The test medium was inoculated with the test microorganisms at a rate of approximately 10^5 cells per ml., and the test chemical was added. Solutions were prepared of the test chemicals at 1% in 20% alcohol (necessary to aid in solubilizing MBR 3092-42), and serial dilutions were prepared in deionized water out to extinction of activity. Dilutions of 1–10 or higher were found not to contain sufficient alcohol to have significant antibacterial activity. At intervals of 1, 5, 30, and 120 min., portions were removed and diluted serially in a dilution medium containing lecithin and polysorbate 80[1] to neutralize the germicide.

	Concentration, g./l.
KH_2PO_4	6.80
$K_2HPO_4 \cdot 3H_2O$	11.4
$Na_2S_2O_3 \cdot 5H_2O$	5.0
Lecithin	2.5
Polysorbate 80	15.0
Deionized water to 1 l.	

Plate counts were then made using standard methods agar.

The ability of the compounds to kill the resident bacteria on human skin was compared using a serial skin-stripping method as described by Updegraff (10). For these studies, MBR 3092-42 and cetylpyridinium chloride[2] were dissolved in water at 1.0%. Solution of MBR 3092-42 was accomplished by warming in a water bath to 40 to 50°, and the solution was kept warm in this bath during the test to prevent precipitation of the compound. Dilutions were made in deionized water to 0.1 and 0.01%. These were not warmed

[1] Tween-80, Atlas Chemical Industries, Wilmington, Del.

[2] The cetylpyridinium chloride used in this and other studies was purchased from Calbiochem.

in the case of MBR 3092-42 as there was no tendency for the compound to precipitate at the low concentrations. Test solutions were applied to areas of the skin on the plantar surface of the forearm of three experimental human subjects and allowed to remain for 30 min. Residual germicide was washed off with sterile deionized water and the skin was gently patted dry with sterile gauze. Cultural counts were then made for bacteria in sequential layers of skin.

Effects of MBR 3092-42 in Anesthetized Dogs—Mongrel dogs were anesthetized with intravenous pentobarbital sodium, 30 mg./kg., and both vagus nerves were sectioned in the midcervical region. Blood pressure was recorded directly from the femoral artery by means of a P23AA Statham pressure transducer. Respiration was recorded *via* a bellows pneumograph fastened around the chest and connected to a P23BB Statham pressure transducer. A Lead II electrocardiogram was recorded to monitor heart rate. All parameters were recorded on a Type R Beckman Dynagraph. Drugs were administered intravenously in the indicated doses directly into the femoral vein.

Acute Toxicity—Determinations of the acute LD_{50} were carried out in rats and mice by both the oral and intraperitoneal routes. Calculations of the LD_{50} values were accomplished by the method of Miller and Tainter (11).

Mice—Simonsen Swiss Webster male mice weighing 18–30 g. were used in all studies. The compounds were administered either orally or intraperitoneally suspended in 4% acacia. Ten mice were used at each dosage level and were group-housed by dosage level in solid bottom plastic cages with food and water available *ad libitum*. The mice were not fasted prior to compound administration. Animals were observed for pharmacodynamic signs and mortality for several hours postdosage and daily thereafter for 14 days.

Rats—Simonsen Sprague Dawley female rats (150–200 g.) were used for all studies. The compounds were administered as a suspension in 4% acacia to fasted animals by either the oral or intraperitoneal route. Eight rats were used for each dosage level and were group-housed by dosage level in suspended screen bottom cages with food and water available *ad libitum*. The rats were observed for pharmacodynamic signs and mortality for several hours after dosing and daily for 14 days thereafter.

Other Species—Oral lethality studies were carried out in dogs and cats by administration of the compound in gelatin capsules to fasted animals of unspecified sex.

Acute Dermal Toxicity—Albino rabbits weighing initially 1630 to 2314 g. were used in this study. The animals were divided into seven groups of four rabbits (two males and two females) each. An area of the back corresponding to approximately 10% of the body surface was prepared by close clipping with an electric clipper. Additionally, one male and one female rabbit in each group were abraded over this area by producing shallow incisions with a scalpel blade. A 10% concentration of the test compound in 70% ethanol was applied to the backs of the rabbits by repeated applications of 4–5 ml. until the animals had received the proper dosage. The area was then covered with surgical gauze which was held in place by wrapping the body with an elastic bandage. Application of compounds was carried out once only at dosage levels of 0.5, 1.0, and 2.0 g./kg. The seventh group of four rabbits was treated with 70% ethanol only in a volume equivalent to that applied to the 2.0 g./kg. group for control purposes.

All of the animals were immobilized for 24 hr. in stocks. After this restraint period the compound was washed off with tap water and animals were observed for dermal irritation. The animals were the nhoused individually for a 14-day observation period in suspended metal cages. Hematologic examinations and urinalysis were conducted once during the control period and at 24 hr. and 2 weeks after compound administration. Daily observations were made for mortality, food consumption, systemic toxicity, and dermal irritation. Dermal irritation was scored as outlined by Draize (12).

Irritation Tests—Rabbits were utilized for eye irritation assay as follows. Albino rabbits of unspecified sex were placed in a restraining stock until quiet. The test materials in varying concentrations (0.5, 1.0, and 2.0%) were then instilled into the right eye in 0.1-ml. amounts. Isotonic saline was instilled into the left eye which served as control. The lids were gently held together for 1 sec. and then released. The eyes were not washed following instillation of the test material. Two rabbits were utilized for each concentration of material tested. The eyes were examined for degree of irritation and scored at intervals as described under Section 191.12 of the Federal Hazardous Substances Labeling Act Regulations (13). The lowest concentration producing signs of irritation considered positive under these regulations (ulceration or opacity of cornea, inflammation of iris, or swelling or redness of the conjunctiva) was considered to be the threshold irritation concentration.

Table I—Surface Tension of Aqueous Solutions Determined Using a DuNoüy Ring and Instron Tester

Compound	Concentration, % w/v	Surface Tension, dynes/cm.
MBR 3092-42	0.05	34.0
MBR 3092-42	0.10	26.0
Cetylpyridinium chloride	0.05	42.0
Cetylpyridinium chloride	0.10	45.5
Water	—	76.0

Primary dermal irritation was determined utilizing guinea pigs. Twelve male albino guinea pigs weighing from 340 to 441 g. were used in this study. The dorsal hair was removed with an electric clipper over an area of approximately 5.08 cm. (2 in.) square. The animals were separated into two groups and the skin of one-half the guinea pigs in each group was further prepared by abrading the shaved area with a scalpel blade.

The prepared skin was dampened with normal saline, and 0.1 g. of the test compound was then placed on the prepared area of six guinea pigs (three with intact and three with abraded skin). The area was covered with gauze and the body of each animal wrapped with elastic bandage. Twenty-four hours later the bandages were removed and the areas of application washed with tepid tap water and examined for dermal irritation. Seventy-two hours later the areas were again examined. Scoring of irritation was as outlined by Draize (12).

Sensitization Testing—Testing for dermal sensitization was performed on male albino guinea pigs. Twenty guinea pigs weighing from 260 to 336 g. were used for this study. The animals were individually housed in suspended metal cages with food and water available *ad libitum*. The laboratory food[3] diet was supplemented with fresh cabbage three times weekly.

The test compounds and the positive control (2,4-dinitro-1-chlorobenzene) were dissolved in sterile distilled water at 0.1% concentration just prior to injection. The back of each guinea pig was prepared by close shaving with an electric clipper. This was repeated as necessary throughout the study.

The positive control and test compounds were injected intradermally (using a 26-gauge needle) into the prepared area every other day, until a total of 10 such sensitizing doses had been given. The volume of the first dose was 0.05 ml. and that for the remaining doses was 0.10 ml. No injection site was used more than once.

Two weeks after the final sensitizing dose had been administered, a challenge dosage of 0.05 ml. of the respective test and positive control compounds was administered intradermally.

The injection sites were examined for diameter, height, and intensity of erythema and edema 24 and 48 hr. following each individual injection. Results were scored as follows: 0.0, normal; 0.5, trace (faint pink); 1.0, pink; 2.0, red; 3.0, yellow-pink; 4.0, yellow (necrosis).

The 24- and 48-hr. readings were averaged and means calculated. Similar measurements were made 24 and 48 hr. after the challenge dosages and averaged.

RESULTS

Analysis of the synthetic product obtained by the procedure described was as follows:

Anal.—Calcd. for $C_{14}H_7F_{18}NO_3S$: C, 27.53; H, 1.15; F, 55.92; N, 2.30. Found: C, 27.58; H, 1.29; F, 55.87; N, 2.37.

Physical and Chemical Properties—MBR 3092-42 is a white, odorless, crystalline compound, with a bitter taste and somewhat less aftertaste than most quaternaries. In comparative taste tests with cetylpyridinium chloride, it was somewhat less bitter and was judged to have less aftertaste.

It is soluble in water to 0.1% (w/v); ethanol, 24.0%; acetone, 53.0%; hexane, 0.7%; and polyethylene glycol 400, 3.0%. MBR 3092-42 is very effective at lowering the surface tension of water

[3] Purina Laboratory Chow.

Table II—Antimicrobial Activity of MBR 3092-42 and Cetylpyridinium Chloride as Measured by Agar Plate Diffusion

Test Organism	MBR 3092-42 — Diameter of Zone of Inhibition, mm. — Serum Free: 40 mcg.	4 mcg.	0.4 mcg.	MBR 3092-42 — With 10% Horse Serum: 40 mcg.	4 mcg.	0.4 mcg.	Cetylpyridinium Chloride — Serum Free: 40 mcg.	4 mcg.	0.4 mcg.	Cetylpyridinium Chloride — With 10% Horse Serum: 40 mcg.	4 mcg.	0.4 mcg.
Staphylococcus aureus (FDA 209)	29	16	x[a]	25	13	x	18	11	x	15	11	7
Streptococcus sp. 104[b]	18	10p[c]	x	24	14	x	16	12	x	14	12	9
Escherichia coli (Gratia)	25	x	x	21	x	x	16	10	x	10	x	x
Pseudomonas aeruginosa	x	x	x	x	x	x	8	x	x	x	x	x
Bacillus subtilis	51	35	17	32	21p	10p	26	16	8	17	12	8
Aspergillus niger	13p	x	x	15p	x	x	18	11	2	14	9p	x
Candida albicans	13p	x	x	10	x	x	19	11	x	12	x	x

[a] x = no activity. [b] A species isolated from human dental caries. [c] p = partial inhibition in the zone.

Table III—Bactericidal Activity of MBR 3092-42 and Cetylpyridinium Chloride as Determined in Deionized Water or 10% Horse Serum

Germicide	Test Medium	Kill After 2 hr., % — Dilution of Test Compound — *S. aureus* 1/10	1/100	1/1000	1/10000	1/100000	*E. coli* 1/10	1/100	1/1000	1/10000	1/100000	*P. aeruginosa* 1/10	1/100	1/1000	1/10000	1/100000
MBR 3092-42, 1% in 20% alcohol	Water	100	100	100	83	82	100	100	100	58	0	100	100	99	93	0
MBR 3092-42, 1% in 20% alcohol	10% Horse serum	100	100	0	0	0	100	90	23	0	0	99	84	0	0	0
Cetylpyridinium chloride, 1% in 20% alcohol	Water	100	100	100	100	92	100	100	100	100	51	100	100	100	97	0
Cetylpyridinium chloride, 1% in 20% alcohol	10% Horse serum	100	100	0	0	0	100	100	30	0	0	100	100	36	0	0

(Table I) and it is unique in that the concomitant foaming property frequently seen with surfactants is lacking.

Like cetylpyridinium chloride, MBR 3092-42 is decomposed by alkali, producing a brown or yellow solution or precipitate devoid of antibacterial activity, as shown by complete lack of any inhibition zones at the highest test dose, 40 mcg., against any of the test organisms listed in Table II.

Antimicrobial Activity—The bacteriostatic and fungistatic activities of MBR 3092-42 and cetylpyridinium chloride are compared in Table II. From these data, we can conclude that both agents are powerful bacteriostatic agents and moderately potent fungistatic agents.

The bactericidal activities of these agents toward *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* are presented in Table III and Figs. 1 and 2. It is clear that both agents are powerfully bactericidal. Cetylpyridinium chloride appears to be slightly more active in water and 10% horse serum as indicated by Table III, but when tested in 80% human blood, MBR 3092-42 showed superior activity against both *S. aureus* and *E. coli*, as shown in Figs. 1 and 2.

The ability of MBR 3092-42 to kill the resident flora of the human skin, as compared with cetylpyridinium chloride, is presented in Table IV. The first two experiments, carried out on subjects DMU and RMW, showed complete kills in several cases with 1% solutions of the test materials; therefore, lower concentrations were used in subsequent tests. Control counts were well within the range reported for normal human subjects (10). Both agents are highly effective and comparable in activity within experimental error reported for this procedure (10).

Effects in Anesthetized Dogs—When administered intravenously to anesthetized dogs as single doses, neither MBR 3092-42 nor cetylpyridinium chloride produced marked effects on heart rate, blood pressure, and respiration until the toxic dose level was reached (Table V). High doses of either agent produced bradycardia and a fall in blood pressure. The fatal single doses of MBR 3092-42 and cetylpyridinium chloride were found to be 20 mg./kg. and 40 mg./kg., respectively. Death in both cases was apparently due to res-

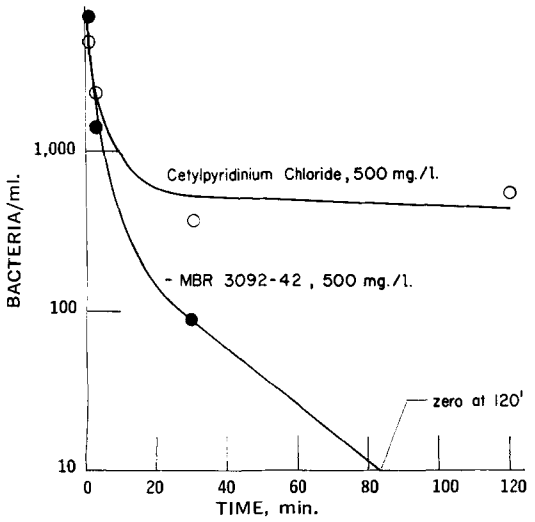


Figure 1—*Kill-rate curves of* Escherichia coli *by MBR 3092-42 and cetylpyridinium chloride in 80% human blood.*

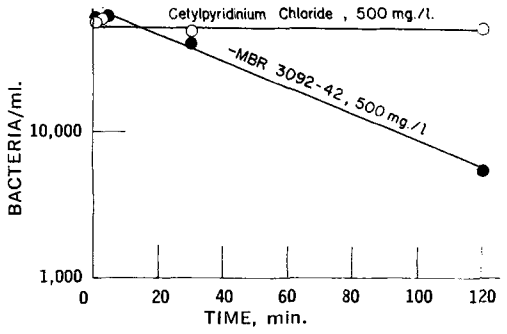


Figure 2—*Kill-rate curves of* Staphylococcus aureus *by MBR 3092-42 and cetylpyridinium chloride in 80% human blood.*

Table IV—The Germicidal Activity of MBR 3092-42 and Cetylpyridinium Chloride Against Human Skin Bacteria Determined by a Serial Skin-Stripping Method

Treatment	Concn.	Test Subject	Bacterial Count per 16 cm.² of Skin[a] 1	2	3	4	5	6	Total	Kill, %
Control		DMU	650	560	350	400	192	116	2268	—
C.P. Cl[b]	1%	DMU	5	2	0	0	0	0	7	99.7
3092-42	1%	DMU	3	0	0	0	0	3	6	99.7
Control		RMW	135	56	45	27	20	8	291	—
C.P. Cl	1%	RMW	7	1	0	0	0	1	9	97.0
3092-42	1%	RMW	1	0	3	0	0	1	5	98.3
Control		RMW	100	150	—	56	43	31	380	—
C.P. Cl	0.1%	RMW	10	5	7	7	2	10	41	89.0
C.P. Cl	0.01%	RMW	7	7	6	7	7	8	41	89.0
3092-42	0.1%	RMW	8	6	3	1	2	3	23	94.0
3092-42	0.01%	RMW	11	15	12	6	8	5	57	85.0
Control		VSP	150	46	107	111	95	105	624	—
C.P. Cl	0.1%	VSP	0	0	0	1	1	0	2	99.7
C.P. Cl	0.01%	VSP	14	19	8	13	5	8	67	89.0
3092-42	0.1%	VSP	2	2	1	4	3	1	13	97.9
3092-42	0.01%	VSP	30	28	31	12	35	25	161	74.0

[a] Each value represents the bacterial count obtained from each 16 cm.² layer of skin, the higher numbers representing serially deeper layers. [b] Cetylpyridinium chloride.

piratory failure. Continuous intravenous infusion of these agents at constant rates produced death at an average cumulative dose of 88 mg./kg. with cetylpyridinium chloride and at an average cumulative dose of 97 mg./kg. with MBR 3092-42 (Table VI). Death in both cases was due to respiratory failure preceded by marked bradycardia and hypotension. MBR 3092-42 was found to cause greater depression of heart rate and blood pressure than cetylpyridinium chloride at doses below the toxic level.

Table V—Effect of Single Intravenous Injections of MBR 3092-42 or Cetylpyridinium Chloride on Heart Rate, Blood Pressure, and Respiration in Anesthetized Dogs[a]

Compound	Dose, mg./kg.[b]	Maximum Change, % Heart Rate	Blood Pressure	Respiration
Cetylpyridinium chloride	1	−5	0	0
	3	0	−5	0
	10	0	+5	0
	20	+5	−30	0
	40		Fatal[c]	
MBR 3092-42	1	0	−5	+5
	3	0	−10	0
	10	−15	−25	0
	20		Fatal[c]	

[a] MBR 3092-42 was administered as a 1% solution in distilled water acidified with 0.1 *N* HCl sufficient to solubilize the compound. Cetylpyridinium chloride was administered as a 4% solution in distilled water. [b] One dog was used for each dosage level administered. [c] Respiration ceased shortly after drug administration with death following from respiratory failure. Bradycardia and hypotension were noted immediately preceding respiratory arrest.

The mechanism for the toxic effect of cetylpyridinium chloride has been found to be a neuromuscular blockade with subsequent paralysis of the muscles of respiration (3). From these experiments it would appear that a similar mechanism is also responsible for the toxic effect of MBR 3092-42.

Acute Toxicity—The acute LD_{50} values obtained are summarized in Table VII. MBR 3092-42 proved to be less toxic than cetylpyridinium chloride in rats and mice by most routes, with the exception of the intraperitoneal route in rats. Administration of either compound to dogs or cats produced emesis of such severity as to preclude determination of lethal potential in these species by the oral route.

Results of the acute dermal toxicity studies in rabbits are reported in Table VIII. The degree of dermal irritation produced was much more prominent in those rabbits receiving cetylpyridinium chloride than in those receiving MBR 3092-42. Rabbits receiving cetylpyridinium chloride exhibited erythema, edema, atonia, desquamation, leathery texture, fissuring, necrosis, ulceration, and sloughing of the skin. These signs bore a dose relationship, particularly in relation to the necrosis, sloughing, and ulceration. Rabbits administered MBR 3092-42 exhibited erythema, edema, and atonia of the skin over a 24–48-hr. period following application. However, the skin appeared essentially normal thereafter.

Control rabbits and rabbits administered MBR 3092-42 exhibited essentially normal values in hematology and urinalysis during this study. Rabbits receiving cetylpyridinium chloride exhibited an elevation in nonsegmented neutrophils at the 24-hr. period after treatment which was dose related in severity.

No unusual alterations in body weight or food consumption were noted in animals receiving MBR 3092-42. Rabbits administered cetylpyridinium chloride exhibited partial anorexia for the first week after treatment. This appeared to be dose related and was accompanied by loss of body weight, particularly at the 2.0 g./kg. dose level.

Irritation Testing—In ocular irritation studies in rabbits, the threshold irritation concentrations were found to be 0.5% for cetylpyridinium chloride and 1.0% for MBR 3092-42.

Dermal irritation studies in guinea pigs over a 72-hr. period produced no evidence of skin irritation under the conditions of the

Table VI—Effect of Intravenous Infusion of MBR 3092-42 and Cetylpyridinium Chloride on Heart Rate, Blood Pressure, and Respiration in Anesthetized Dogs

Dog No.	Compound	Infusion Rate, mg./kg./min.[a]	Fatal Dose, mg./kg.	Maximum Change, % Heart Rate	Blood Pressure	Respiration
1	Cetylpyridinium chloride	1.0	81	−67	−73	Progressive depression to failure
2	MBR 3092-42	1.0	90	−23	−38	Progressive depression to failure
3	Cetylpyridinium chloride	2.0	98	−24	−50	Progressive depression to failure
4	MBR 3092-42	2.0	104	−69	−91	Progressive depression to failure

[a] Drugs were infused as solutions in distilled water at a concentration of 3 mg./ml.

Table VII—Acute Toxicity of MBR 3092-42 and Cetylpyridinium Chloride

Test Species	Route	LD_{50}, mg./kg. and *SE* MBR 3092-42	Cetylpyridinium Chloride
Mouse, male	Oral	925 ± 45.2	175 ± 14.6
Mouse, male	i.p.[a]	30.0 ± 1.4	7.0 ± 0.6
Rat, female	Oral	1159 ± 141.3	538 ± 49
Rat, female	i.p.[a]	18.0 ± 1.0	26.5 ± 1.3
Cat	Oral	Emetic[b]	Emetic[b]
Dog	Oral	Emetic[b]	Emetic[b]

[a] Intraperitoneal. [b] Oral doses of 250 and 500 mg./kg. (two animals/dose level) to dogs or cats produced emesis and no observable toxic signs. The emesis prevented establishment of a minimal lethal dose.

Table VIII—Acute Dermal Toxicity of MBR 3092-42 and Cetylpyridinium Chloride in Albino Rabbits

Compound	Dermal Dose, g./kg.	Mortality No. Dead/ No. Tested	Irritation No. Positive[a]/ No. Tested
70% Ethanol	—	1/4[b]	0/4
Cetylpyridinium chloride	0.5	0/4	4/4
	1.0	0/4	4/4
	2.0	2/4[c]	4/4
MBR 3092-42	0.5	0/4	2/4
	1.0	0/4	4/4
	2.0	0/4	4/4

[a] Positive animals were those showing any signs of irritation. [b] One rabbit died from a broken neck while restrained in the stock. [c] One rabbit (abraded) died on Day 4 and one (intact) on Day 5.

experiment with either MBR 3092-42 or cetylpyridinium chloride. Due to the negative scores obtained, no table of results is presented.

Sensitization Testing—The results of a dermal sensitization study conducted in guinea pigs are presented in Table IX. These indicate that the test compounds, cetylpyridinium chloride and MBR 3092-42, produced irritation since wheal and flare formation was evident throughout all 10 sensitizing injections. The values obtained from the challenge dosage were similar to the average values obtained with the sensitizing injections. Dinitrochlorobenzene as the positive control also produced evidence of irritation. However, the values obtained from the challenge dosage exceeded those obtained from the sensitizing injections. Thus, it is concluded that MBR 3092-42 and cetylpyridinium chloride produced irritation but not sensitization and that dinitrochlorobenzene produced sensitization as well as irritation.

DISCUSSION

The antibacterial activity of quaternary compounds is believed to be correlated with their surfactant properties (2). Fluorine substitution markedly increases the surface activity of many surfactants, as measured by surface tension reduction (14). This property could account for the high bactericidal activity of MBR 3092-42 as contrasted with its nonfluorinated homolog *n*-octylpyridinium chloride, which has very slight activity (15).

On the other hand, the introduction of fluorine decreases water solubility and increases formula weight to a value of 611 for MBR 3092-42 *versus* 358 for cetylpyridinium chloride. Thus, on a molar basis, MBR 3092-42 is proportionately more active than cetylpyridinium chloride.

The relative lack of toxicity and irritation to the skin and mucous membranes with MBR 3092-42 and its effectiveness in the presence of blood would appear to be advantageous for its use as an antiseptic for the skin and surgical wounds. Further, its high surface activity and lack of foaming properties as well as its somewhat less unpleasant taste should prove advantageous for other uses, *e.g.*, as an oral antiseptic. For this purpose, dilute hydroalcoholic solutions could conceivably be preferable to weakly acidic solutions, since acidic solutions might aggrevate the formation of dental caries.

REFERENCES

(1) G. Domagk, *Deut. Med. Wochschr.*, **61**, 829(1935).

(2) C. A. Lawrence, "Surface-active Quaternary Ammonium Germicides," Academic, Inc., New York, N. Y., 1950, pp. 68–71.

(3) M. R. Warren, L. J. Becker, D. G. Marsh, and R. S. Shelton, *J. Pharmacol. Exptl. Therap.*, **74**, 401(1942).

(4) J. W. Nelson and S. C. Lyster, *J. Am. Pharm. Assoc., Sci. Ed.*, **35**, 89(1946).

(5) G. Woodward and H. O. Calvery, *Proc. Sci. Sec. Toilet Goods Assoc.*, **3**, 1(1945).

(6) B. V. Alfredson, J. R. Steiffel, F. Throp, W. D. Baten, and M. L. Gray, *J. Am. Pharm. Assoc., Sci. Ed.*, **40**, 263(1951).

(7) R. L. Hansen, French pat. 1,470,669 (1967).

(8) J. G. Vincent and H. W. Vincent, *Proc. Soc. Exptl. Biol. Med.*, **55**, 162(1944).

(9) H. Chick, *J. Hyg.*, **8**, 92(1908).

(10) D. M. Updegraff, *J. Invest. Dermatol.*, **43**, 129(1964).

(11) L. C. Miller and M. L. Tainter, *Proc. Soc. Exptl. Biol. Med.*, **57**, 261(1944).

(12) J. H. Draize, in "Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics," Association of Food and Drug Officials of the United States, Austin, Tex., 1959, pp. 46–59.

(13) *Federal Register*, **29**, 13009 (September 17, 1964).

(14) R. A. Guenthner and M. L. Vietor, *Product. Res. Devel.*, **3**, 165(1962).

(15) R. S. Shelton, M. G. Vam Campen, C. H. Tilford, H. C. Lang, L. Nisonger, F. J. Bandelin, and H. L. Rubenkoenig, *J. Am. Chem. Soc.*, **68**, 757(1946).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 5, 1969, from the *Biochemical Research Laboratory, Central Research Laboratory, 3M Company, Saint Paul, MN 55101*

Accepted for publication October 8, 1969.

The authors express appreciation to J. A. Seitz for determining the physical properties of MBR 3092-42, to J. R. Schmid for the pharmacodynamic studies in dogs, and to R. A. Nelson and R. R. Hamilton for certain toxicology work.

* Present address: Microbiology Section, Denver Research Institute, Denver, Colo.

Table IX—Studies on Dermal Sensitization in the Guinea Pig with Cetylpyridinium Chloride, MBR 3092-42 and 2,4-Dinitro-1-chlorobenzene

Test Compound	No. Guinea Pigs	Reaction Scores, Mean ± *SE*: Sensitizing Doses, Diameter, mm.	Sensitizing Doses, Height mm.	Sensitizing Doses, Color[a]	Challenge Dose, Diameter, mm.	Challenge Dose, Height, mm.	Challenge Dose, Color[a]
2,4-Dinitro-1-chlorobenzene	4	9.2 ± 0.3	1.2 ± 0.1	2.1 ± 0.1	14.2 ± 0.1	1.4 ± 0.2	2.3 ± 0.2
Cetylpyridinium chloride	7[b]	8.9 ± 0.2	0.8 ± 0.04	2.7 ± 0.05	9.7 ± 0.03	0.4 ± 0.1	3.0 ± 0.1
MBR 3092-42	8	8.6 ± 0.1	1.1 ± 0.05	2.4 ± 0.1	9.7 ± 0.4	0.9 ± 0.1	2.3 ± 0.1

[a] 0 = normal, 0.5 = trace, 1 = pink, 2 = red, 3 = yellow-pink, 4 = yellow. [b] One animal died during the final week of the study.

Reactions of Acetaminophen in Pharmaceutical Dosage Forms: Its Proposed Acetylation by Acetylsalicylic Acid

E. KALATZIS

Abstract □ Acetylation of acetaminophen (paracetamol) by acetylsalicylic acid to *O,N*-diacetyl-*p*-aminophenol (DAPAP) was not evident in experimental pharmaceutical dosage forms. DAPAP, which was found to be present in small quantities in commercial grade acetaminophen, is unstable in pharmaceutical preparations. The destruction of DAPAP increases rapidly with the temperature and moisture content of the system. Solid DAPAP seems to exist in at least two forms, as shown by their IR spectra.

The observed color darkening of suspensions containing acetaminophen is mainly due to the oxidative degradation of the liberated *p*-aminophenol. The presence of codeine phosphate and caffeine seems to enhance the color deterioration of solid preparations containing acetaminophen kept at 45° and in a humid atmosphere. It is recommended that moisture be excluded, as much as possible, from such preparations.

Keyphrases □ Acetaminophen reactions—dosage forms □ Aspirin—acetaminophen acetylation □ Codeine PO_4, caffeine effect—acetaminophen stability □ Humidity effect—acetaminophen stability □ TLC—analysis □ IR spectrophotometry—identity □ UV spectrophotometry—identity

Previous publications have indicated that drugs containing amino- or phenolic-groups, such as phenylephrine (1) or codeine (2), as well as proteins (3) can be acetylated by acetylsalicylic acid in tablet formulations.

In 1967, it was reported (4) that acetaminophen (*N*-acetyl-*p*-aminophenol) can be acetylated by acetylsalicylic acid in tablet formulations, with the formation of *O,N*-diacetyl-*p*-aminophenol and salicylic acid. The same authors (4) proposed a linear relationship between the rate of formation of salicylic acid and the rate of formation of *O,N*-diacetyl-*p*-aminophenol and indicated the presence of *p*-aminophenol in some tablets, but they did not discuss its formation. However, a study of the kinetics of the formation of *p*-aminophenol by the hydrolysis of acetaminophen in aqueous solutions has been reported earlier (5).

Because there was a strong move in 1967 to have phenacetin removed from analgesic preparations being sold in Australia, many manufacturers replaced the phenacetin in their formulations by the same weight of acetaminophen. In this laboratory, however, it was found that in aspirin–acetaminophen preparations held at 45° under humid conditions or aspirin–acetaminophen suspensions held at 45°, *p*-aminophenol, together with its colored oxidation products, was being produced in a relatively short time.[1] In a number of these aspirin–acetaminophen preparations or suspensions the presence of *O,N*-diacetyl-*p*-aminophenol has also been established.

The formation of *p*-aminophenol in preparations containing acetaminophen is of some concern, therefore, and the presence of *O,N*-diacetyl-*p*-aminophenol attracted the author's attention, because any acetylation of acetaminophen (APAP) by acetylsalicylic acid (ASA) to the *O,N*-diacetyl-*p*-aminophenol (DAPAP) could, in addition to the direct hydrolysis of acetaminophen, be responsible for an alternative reaction path affecting the rate of the formation of *p*-aminophenol (PAP). However, the results of this paper show also that, contrary to what has been assumed earlier (4), acetylation of APAP by ASA was not evident under the conditions examined.

EXPERIMENTAL

Thin-layer Chromatography (TLC)—Analyses by TLC were carried out on 0.25 mm. Silica Gel GF_{254} (Merck reagent). Solvent systems (4) used in all cases were as follows: (*a*) chloroform–acetone–acetic acid (80:18:2), and (*b*) chloroform–ethanol–acetic acid (88:10:2).

TLC plates were usually eluted only once but, when necessary, a second elution (placing the plate in the solvent tank for a second time) improved the separation of spots. The various spots separated on the TLC plate could be easily seen against the green fluorescence of the plate by viewing in the UV light of short wavelength (254 mμ).

Materials—Acetaminophen, phenacetin, and acetylsalicylic acid were commercial (pharmaceutical) grade reagents. Acetaminophen (Winthrop), which showed more than one spot by TLC examination, was recrystallized four times from methyl isobutyl ketone before the spot corresponding to DAPAP disappeared. Figure 1 shows the results of the TLC examination of the residues and of the final product obtained from the recrystallization of acetaminophen; also, it shows that when 2% DAPAP was added before or after purification, only one DAPAP spot was obtained by TLC. The spots which correspond to pure DAPAP have UV and IR spectra identical with those of DAPAP. The UV spectra in ethanol have $\lambda_{max.}$ at 246 mμ and log $\epsilon \cong 4.20$. Solid DAPAP seems to be polymorphic and the form obtained depends on the solvent from which it is recovered. Thus, when it was recrystallized from water, the IR spectrum of the solid showed two carbonyl frequencies at

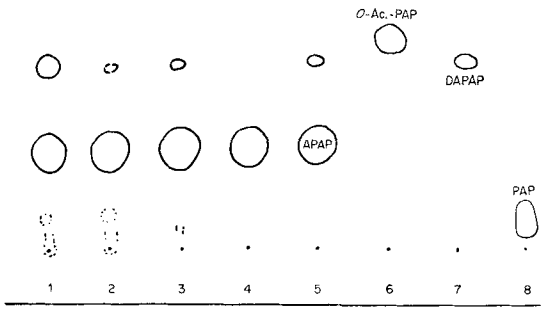


Figure 1—*Thin-layer chromatogram of APAP (Solvent* a) *Key:* 1, *residue from the filtrate of the first recrystallization of commercial APAP;* 2, *same as* 1 *but from second and third recrystallizations combined;* 3, *commercial APAP before recrystallization;* 4, *pure recrystallized APAP;* 5, *commercial APAP containing 2% DAPAP;* 6, *O-acetyl-p-aminophenol;* 7, *DAPAP; and* 8, *PAP.*

[1] Part of this work was presented at the I.U.P.A.C. 2nd International Congress on Pharmaceutical Chemistry, held at Münster/Westf. on July 22–26, 1968, in a paper on "Reactions of Acetylsalicylic Acid and Paracetamol in Dosage Forms," by B. G. Boggiano, E. Kalatzis, and F. E. Peters.

Table I—Uncompressed Mixtures of Powders

Mixture	APAP, g.	Phenacetin, g.	ASA, g.	SA, g.
1[a]	1.0	—	1.0	—
2[a]	1.0	—	—	1.0
3	—	1.0	1.0	—
4	—	1.0	—	1.0
5[b]	0.5	—	0.5	—
6[b]	0.5	—	—	0.5
7[b]	—	0.15	0.15	—

[a] Commercial (pharmaceutical) grade acetaminophen, which was shown by TLC to contain DAPAP. [b] Recrystallized acetaminophen, acetylsalicylic acid, and phenacetin.

1688 and 1738 cm.$^{-1}$ and an N—H frequency at 3364 cm.$^{-1}$. There were a shift and an increase in the complexity of these frequencies when the solid was recovered from other solvents. For example, the solid recovered from alcohol or benzene showed the two carbonyl frequencies at 1665 and 1752 cm.$^{-1}$ and what appeared as a number of N—H frequencies in the range of 3074–3292 cm.$^{-1}$. Furthermore, the solid recovered from acetone seemed to be a mixture of the two forms, because its spectrum contained all these frequencies. These changes, which may be due to the presence of the amide group, are reversible and have been observed with commercial DAPAP as well as with that recovered by TLC from commercial APAP. This problem is under investigation.

Phenacetin and acetylsalicylic acid were recrystallized three times from ethylacetate; they were shown by TLC to be pure (one spot). Salicylic acid (AnalaR), acetylsalicylic acid (May & Baker), codeine phosphate (May & Baker), and caffeine (D.H.A.) were used without further purification.

Commercial APAP containing 2% DAPAP was prepared as follows: 0.2 g. of DAPAP (Tokyo Kasei & Co.) together with 10 g. of APAP (commercial grade D.H.A. shown by TLC to contain no DAPAP) were dissolved in alcohol (20 ml.) and the clear solution obtained was then evaporated slowly to dryness under vacuum.

Preparations—*Suspensions*—Typical suspensions were prepared as follows: 10 g. acetylsalicylic acid, 10 g. acetaminophen, 200 ml. demineralized water, with or without 0.4 g. codeine phosphate or 1.0 g. caffeine, and with or without the following mixture: 4 g. compound powder of tragacanth BP and 20 ml. syrup of orange and 5 ml. concentrate chloroform–water (60% chloroform in 60% ethanol).

Similar suspensions containing phenacetin instead of acetaminophen were also prepared. All suspensions were kept at room temperature (about 25°) or at 45° for 14 weeks.

Table II—Uncompressed Mixtures of Powders

Mixture	APAP, g.	ASA, g.	SA, g.	Codeine Phosphate, g.	Caffeine, g.
1[a]	1.0	1.0	—	—	—
2	1.0	—	1.0	—	—
3	1.0	1.0	—	0.043	—
4	1.0	—	1.0	0.041	—
5	0.75	1.23	—	—	—
6	0.75	—	1.23	—	—
7	0.75	1.23	—	—	0.15
8	0.75	—	1.23	—	0.15
9	2.0	—	—	—	—
I[c]	1.0	1.0	—	—	—
II	1.0	—	1.0	—	—
III	1.0	1.0	—	0.042	—
IV	1.0	—	1.0	0.041	—
V	0.75	1.23	—	—	—
VI	0.75	—	1.23	—	—
VII	0.75	1.23	—	—	0.15
VIII	0.75	—	1.23	—	0.15
IX	2.0	—	—	—	—

[a] Mixtures 1–9 contain APAP (commercial grade) which was shown by TLC to be free from DAPAP. [b] Mixtures I–IX contain APAP (commercial grade as in Mixtures 1–8) to which 2% DAPAP was added.

Powders—Uncompressed mixtures of powders were prepared by grinding the weighed quantities of the appropriate materials and mixing thoroughly. No excipients were included in these mixtures, as is seen in Tables I–III.

Each uncompressed mixture was divided into six equal parts which were kept at room temperature (about 25°) and at 45° under the following conditions: (*a*) in sealed vials (dry mixtures), (*b*) in a desiccator over water (humid mixtures), and (*c*) in sealed vials in which 1–2 ml. of water was added (moist mixtures).

Samples (25–50 mg.) were taken out at regular intervals of time, dissolved in alcohol (about 2 ml.), and examined by TLC. Satisfactory results were obtained when many applications (about eight each of about 5 μl.) were used for each sample, thus ensuring that the minor components, if present, would appear as recognizable spots on the thin-layer chromatograms. The limits of detection lie between 0.2 and 0.4 mcg. of DAPAP spots on the TLC plate by the method described.

Tablets—The tablets examined were commercially manufactured products.

RESULTS AND DISCUSSION

Suspensions—Suspensions containing phenacetin and held at 45° for 14 weeks showed no color change and, as expected, neither DAPAP nor PAP could be detected by TLC.

Suspensions containing acetaminophen (commercial grade, which was shown to contain DAPAP) rapidly turned brown and deposited a black precipitate. Spots indicating the presence of both PAP and DAPAP were observed. While the amount of PAP and its colored oxidation products increased with time, the amount of DAPAP decreased and eventually could not be detected.

The level of PAP in the aqueous phase of these suspensions rose to about 10^{-2} *M*, as determined spectrophotometrically by using 4-nitrobenzaldehyde and removing the colored oxidation products (6).

Similar, but much slower, changes were observed in suspensions kept at room temperature. For example, at the end of 14 weeks the level of PAP was less than 10^{-4} *M* and 24 weeks later these suspensions, although discolored, were not as dark as the suspensions kept at 45° for 14 weeks.

Powders—The results obtained after examining the powder mixtures containing recrystallized APAP (Table I) indicate that no DAPAP is formed in the dry mixtures when kept at room temperature for 6 months. Also, no DAPAP is formed in all the other mixtures held at room temperature or at 45° for 2 months.

On the other hand, humid and moist mixtures containing commercial acetaminophen (Table I) and kept at room temperature indicated that the original DAPAP remained almost completely unaffected for 2 months. Similar results were obtained with the dry mixtures kept at room temperature for 6 months and at 45° for 2 months. However, the DAPAP present in the humid and moist mixtures held at 45°, for 6 and 2 weeks, respectively, decreased steadily and at the end it could not be detected, presumably because of hydrolysis to APAP. This view is supported by the fact that no additional spots were revealed by TLC (*O*-acetyl-*p*-aminophenol appears as a different spot than that of APAP, as shown in Fig. 1).

Uncompressed powder mixtures based on two-tablet formulations of the Australian Pharmaceutical Formulary were also prepared. No excipients were added to these mixtures as seen in Table II.

Table III—Uncompressed Mixtures of Powders

Mixture	APAP, g.	ASA, g.	SA, g.	Codeine Phosphate, g.	PAP, g.
1[a]	0.5	0.5	—	—	—
2[a]	0.5	0.5	—	0.05	—
3[b]	0.5	0.5	—	—	—
4[b]	0.5	0.5	—	0.10	—
5	—	1.0	—	—	1.0
6	—	—	1.0	—	1.0
7	—	—	—	—	2.0

[a] Commercial (pharmaceutical) grade acetaminophen, which was shown by TLC to contain DAPAP. [b] Recrystallized acetaminophen and acetylsalicylic acid.

The formulations are: (*a*) acetylsalicylic acid, 250 mg.; acetaminophen, 250 mg.; and codeine phosphate, 8 mg.; and (*b*) acetylsalicylic acid, 225 mg.; acetaminophen, 150 mg.; and caffeine, 30 mg.

The APAP used was a commercial grade, but TLC examination showed that it contained no DAPAP. Accordingly, DAPAP was added and thus two series of powder mixtures were prepared: one with APAP free from DAPAP and the other with APAP containing 2% DAPAP (Table II).

All the mixtures were exposed to the same conditions as indicated above. The results have shown that again no DAPAP was formed under any of the conditions examined and that the DAPAP added had a fate similar to that of the DAPAP present in the previous powder mixtures (Table I). These findings hold true for all the mixtures including those containing codeine phosphate or caffeine. Increased quantities of codeine phosphate added in a number of uncompressed powder mixtures (Table III) gave similar results.

No appreciable decrease in the DAPAP was observed in the dry mixtures held at room temperature and at 45° for 2 months (Fig. 2*a*). The DAPAP present in the humid mixtures which were held at 45° for 2 months decreased steadily to traces and disappeared completely when codeine phosphate was present. This acceleration in the destruction of DAPAP is possibly due to the liberation of phosphoric acid in the presence of moisture.

Only a slow decrease in the DAPAP present in the moist mixture held at room temperature was observed and DAPAP was still present after 2 months. On the other hand, the decrease in the DAPAP in the moist mixtures kept at 45° was rapid and no DAPAP could be seen after about 2 weeks (Figure 2*b*).

These observations seem to be consistent whether ASA or SA is mixed with APAP. Of course, ASA hydrolyzes to SA and acetic acid, and this can be easily seen by TLC, because ASA appears as a green spot in the UV light (254 mμ) against the fluorescent background of the plate while SA appears as a blue spot. Adequate separation of the ASA from SA can be effected in Solvent *b* referred to in the *Experimental* section.

Powder mixtures containing phenacetin (Table I) have shown no recognizable changes except that, as in the case of those containing acetaminophen, humid and moist mixtures develop SA rapidly at 45° and more slowly at room temperature.

Because the results of the present work have shown that DAPAP is not stable and that its destruction, like the hydrolysis of ASA, increases with temperature and moisture content of the systems under consideration, the work reported by the earlier authors (4) has been reexamined. Figure 3 shows a plot of the number of moles of free SA against the number of moles of DAPAP based on their data (4).

Such a plot, however, does not seem to support satisfactorily the suggestion made by the above authors (4) that there is a relationship between the rate of formation of SA and the rate of formation of DAPAP. For example, in the case of their results with Product C,

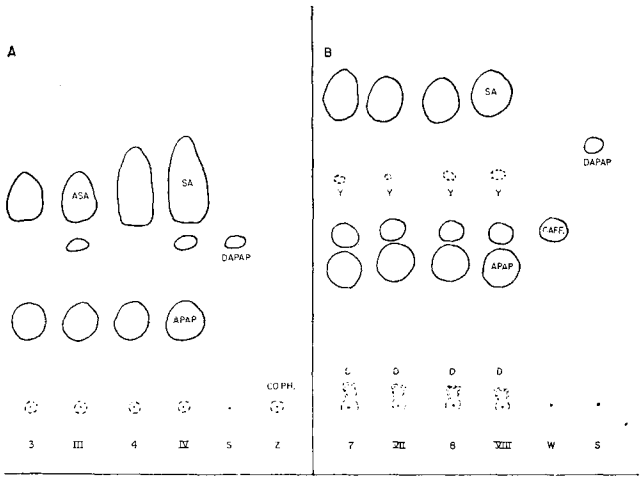


Figure 2—*Thin-layer chromatogram of uncompressed powder mixtures. (Solvent* a) *Key: for* 3 *to* IV *and* 7 *to* VIII, *see Table II;* S, *DAPAP;* Z, *codeine phosphate;* W, *caffeine;* Y, *yellow spots (seen in ordinary light); and* D, *rather dark brown spots. Chromatogram* A *was eluted once and Chromatogram* B *was eluted twice. (Dry* A *and moist* B *mixtures at 45°).*

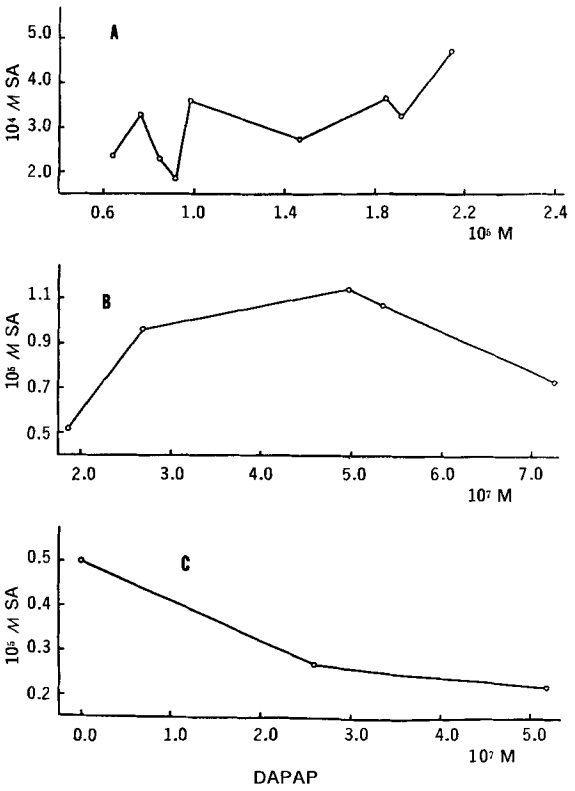


Figure 3—*Plot of the number of moles of free SA against number of moles of DAPAP in the samples of products* A, B, *and* C, *from the data of* Reference 4.

a fivefold increase in the number of moles of DAPAP corresponds to an approximate twofold decrease in that of the free SA.

These results rather suggest a random distribution of DAPAP in the various products. Furthermore, their (4) proposed linear relationship between the amount of DAPAP and that of SA formed with time in tablets from the same batch is not in agreement with the results of the present work, which indicates that the DAPAP is unstable especially under accelerated storage conditions. Since the storage conditions for the different products were probably not identical, the amount of DAPAP determined at a certain time would be expected to vary according to the history of the products concerned and the amount, if any, of DAPAP initially present. Moreover, all the figures quoted by the above authors (4) indicate that the number of moles of SA formed as a result of the proposed acetylation is far greater than that of DAPAP. For example, their (4) proposed linear relationship states that about 6 moles of SA are formed as a direct result of the formation of 1 mole of DAPAP. These results, however, are not in agreement with the proposed acetylation of APAP by ASA when it is considered that the formation of 1 mole of DAPAP is expected to be accompanied by the formation of 1 mole of SA.

An approximate estimation (semiquantitative TLC examination) of the DAPAP in commercial APAP (Table I) indicated that the level of DAPAP is within the range of 1.1–1.3%.

It is interesting to note that when APAP, with or without 2% DAPAP (Table II), was subjected to the same treatment as the previous mixtures, the destruction of DAPAP present in the moist sample held at 45° was complete within 6 weeks. Moreover, no color deterioration was observed.

Tablets—*p*-Aminophenol was detected (6) in very small amounts (less than 0.005%) in tablets containing acetaminophen, but was not detected in tablets containing phenacetin. Tablets containing acetaminophen (codeine phosphate or caffeine present) held at 45° in a humid atmosphere discolored or developed a mottled appearance after about 4 days.

In view of these discolorations, an examination of the color changes taking place in the powder mixtures discussed above in the *Powders* section of *Results and Discussion* is of interest.

None of the mixtures containing phenacetin (Table I) and held at room temperature showed any color deterioration, while at 45°

Table IV—Summary of Main Observations

	DAPAP[a]			Color Deterioration[a]		ASA Hydrolysis, APAP and Phenacetin Mixture
	Formation		Hydrolysis, Complete			
	APAP Mixture, DAPAP Absent	Phenacetin Mixture	APAP Mixture, DAPAP Present	APAP Mixture	Phenacetin Mixture	
At room temperature, about 25°						
Suspensions	No	No	No	Darkening, 6 months	No	Greatly increased
Mixtures						
dry	No, 6 months	No, 6 months	No, 6 months	No	No	Negligible
humid	No	No	No	No	No	Slight
moist	No	No	No	Slight	No	Greatly increased
Tablets humid	—	No	No	Negligible, more than 2 months	No	Slight, more than 2 months
At 45°						
Suspensions	No	No	Yes	Excessive darkening	Slight	Complete
Mixtures						
dry	No	No	No	No	No	Very slight
humid	No	No	Yes	Noticeable mainly in the presence of codeine phosphate or caffeine	No	Greatly increased
moist	No	No	Yes, 2 weeks	Excessive darkening of all mixtures	Slight	Almost complete
Tablets humid	—	No	No	Rather dark and mottled appearance	Slight	Greatly increased

[a] Observations carried out for a period of 2 months, unless otherwise stated.

only the moist ones showed a very slight deterioration. These results are different in many respects from those obtained from mixtures containing APAP.

At room temperature all APAP mixtures showed no color deterioration except for the moist mixtures containing caffeine, which turned light yellow.

At 45° the dry mixtures showed no color deterioration. Of the humid mixtures, those containing codeine phosphate or caffeine became slightly red initially with subsequent darkening of the color. This deterioration was more pronounced in mixtures containing codeine phosphate and SA (Table II). On the other hand, all the moist mixtures turned dark brown and eventually black due mainly to the oxidative degradation of *p*-aminophenol produced from the hydrolysis of APAP, as was observed in the case of the suspensions already discussed.

For comparative purposes, therefore, mixtures containing *p*-aminophenol instead of APAP (Table III) were prepared and examined in the same way as was done above. Color deterioration of these mixtures took place within a day or two, even at room temperature. They were examined periodically by TLC and compared with the moist acetaminophen mixtures, because the darkening of the color was similar in both these groups. The results showed that the darkening of the color of the samples of both groups was due to yellowish, dark brownish, and to some extent reddish products (Fig. 2*b*). They also showed that PAP can be acetylated by ASA with the formation of APAP and that its oxidation was very pronounced in the presence of SA. However, no DAPAP could be observed under any conditions.

Deterioration in the color of these mixtures greatly increases with temperature and moisture content, and sealed samples kept at room temperature did not show as many dark spots by TLC. It is worth noting, however, that acetylation of PAP to APAP by ASA takes place readily at room temperature also in the dry, humid, or moist mixtures.

In view of the above findings, APAP (1 g.), ASA (1 g.), and SA (1 g.), each containing codeine phosphate (40 mg.) or each containing caffeine (150 mg.), were placed at 45° in a humid atmosphere (above water in a desiccator) for 12 weeks. The color of the mixtures containing APAP deteriorated faster than those containing ASA or SA. Even codeine phosphate or caffeine kept at 45° and in humid atmosphere on their own showed a color deterioration. On the other hand, when these mixtures were heated at 45° for the same period of time in sealed vials, *i.e.*, in a rather dry atmosphere, no color deterioration could be observed.

It is, therefore, suggested that color deterioration in APAP tablets, kept under accelerated storage conditions, may be due partly to interactions between the codeine phosphate or caffeine, if present, with the other components or breakdown products in the tablets, and partly to the oxidative degradation of *p*-aminophenol formed from APAP.

SUMMARY AND CONCLUSION

A summary of the main observations is presented in Table IV.

From the results it can be seen that acetylation of acetaminophen to *O*,*N*-diacetyl-*p*-aminophenol by acetylsalicylic acid is not evident under the conditions examined and that the stability of the pharmaceutical preparations containing acetaminophen decreases rapidly with increase in their moisture content and temperature. Furthermore, when the moisture of these preparations is kept to the minimum possible level (without any special drying of the ingredients), their stability is greatly improved. It is, therefore, recommended that such preparations be kept in a dry atmosphere in order to increase their stability during storage.

REFERENCES

(1) A. E. Troup and H. Mitchner, *J. Pharm. Sci.*, **53**, 375(1964).

(2) A. L. Jacobs, A. E. Dilatush, S. Weinstein, and J. J. Windheuser, *ibid.*, **55**, 893(1966).

(3) M. A. Schwartz and G. L. Amidon, *ibid.*, **55**, 1464(1966).

(4) K. T. Koshy, A. E. Troup, R. N. Duvall, R. C. Conwell, and L. L. Shankle, *ibid.*, **56**, 1117(1967).

(5) K. T. Koshy and J. L. Lach, *ibid.*, **50**, 113(1961).

(6) E. Kalatzis, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 14, 1969, from the *National Biological Standards Laboratory, Canberra City, A.C.T. 2601 Australia.*

Accepted for publication September 11, 1969.

Partition of Alkylsulfates of Quaternary Ammonium Compounds: Structure Dependence and Transport Study

FOTIOS M. PLAKOGIANNIS*, E. J. LIEN, CHARLES HARRIS†, and JOHN A. BILES

Abstract □ The apparent partition coefficients (log $K_{app.}$, chloroform–water) of 48 alkylsulfates of substituted quinolinium compounds and 19 alkylsulfates of substituted pyridinium derivatives were correlated with Bondi's group contribution to the surface area ($A_w \times 10^9$), Hansch's π constant, and Hammett's σ constant. The surface area of the ring substituent and the alkyl group appears to be the most important factor in governing the apparent partition coefficient. The kinetic study of the dialysis of nicotinic acid *N*-methyl iodide through Visking cellulose membrane showed an effective rate constant of 1.6×10^{-2} min.$^{-1}$ in the first hour and a rate constant of 0.3×10^{-2} min.$^{-1}$ after the first hour. The *in vitro* intestinal absorption of four quaternary ammonium iodides was studied. It was found that the presence of equimolar concentration of sodium decylsulfate inhibited the transfer of the quaternary ammonium compounds.

Keyphrases □ Quaternary ammonium alkylsulfates—apparent partition coefficients □ Partition coefficient correlation—Hansch's π-, Hammett's constants, Bondi's group contribution surface area □ Intestinal absorption, *in vitro*—quaternary ammonium compounds □ Dialysis, kinetics—nicotinic acid *N*-methyl iodide

In 1957–1959, Hogben *et al.* (1, 2) postulated that the gastric mucosa and intestinal blood barrier are essentially lipoid in nature and permit the passive diffusion of the unionized lipid-soluble form of a drug. Schanker (3) suggested that the absorption of organic ions might occur by a specialized transport process analogous to those which operate in transport of certain inorganic cations. He further suggested that the organic ions might penetrate the gastrointestinal blood barrier by the diffusion of the ions in the form of a less polar complex formed with some material normally present in the lumen.

Levine *et al.* have studied the transport of quaternary ammonium compounds extensively (4–9). They attributed the poor absorption to the formation of nonabsorbable complexes with mucin. They also proposed that a phosphatidopeptide fraction allowed a more efficient absorption of certain quaternary ammonium compounds. Other mechanisms in addition to passive diffusion were proposed by Levine (9).

Although hundreds of partition coefficients have been reported for unionized molecules by Hansch *et al.* (10–13), relatively few data on the charged molecules are available in the literature (11, 14). Biles *et al.* showed that partitioning of organic ions into the organic layer from the aqueous layer could be increased by the addition of organic ions of opposite charge and by the addition of water-insoluble proton donor-type molecules to the organic layer (15–17).

The purposes of this report are to analyze quantitatively which physicochemical parameter is most important in governing the partition of the alkylsulfates of pyridinium and quinolinium derivatives, and to explore whether alkylsulfates would enhance the absorption of quaternary ammonium compounds by the small intestine.

EXPERIMENTAL

Synthesis of Quaternary Ammonium Iodides—The appropriate tertiary arylamine was reacted with an excess of methyl iodide to form the quaternary ammonium iodide according to the published methods (18). The precipitated crystals were isolated by filtration and recrystallized from methanol or ethanol. Ethyl ether was used as a washing agent. The melting points were checked for all the compounds synthesized and agreed with those recorded in the literature.

Synthesis of the Alkylsulfates—The alkylsulfates were synthesized either by the method previously described (17) or by the method of Dreger *et al.* (19).

Analysis of the Quaternary Ammonium Compounds—The analysis of the quaternary ammonium compounds was performed either by the method previously described (17) or by UV absorption ($\lambda_{max.}$ 236–322 mμ).

Determination of the Apparent Partition Coefficients—The procedure used was identical with that used for the anticholergic compounds (17). The log $K_{app.}$ values (Tables I and II) were obtained from the following expression (16) using the chloroform–water system:

$$\underset{c_1}{B\text{-}\overset{+}{M}e_{(aq.)}} + \underset{c_2}{SO_3\text{-}\overset{-}{O}R_{(aq.)}} \overset{K_{app.}}{\rightleftharpoons} \underset{c_3}{Me\text{-}B\text{-}SO_3\text{-}OR_{(CHCl_3)}}$$

$$K_{app.} = \frac{c_3}{c_1 c_2} \text{ (l./mole)} \qquad \text{(Eq. 1)}$$

Since the dielectric constant of water is 80.4 and that of chloroform is only 4.8, the ion-pair formation in aqueous phase should be negligible as compared to that in the chloroform phase. It is known that highly polar solvents favor ionization. For example, dilute aqueous solutions of sodium chloride and sodium acetate will have practically 100% ionization. It was felt that the same assumption would be valid in the case of an alkylsulfate of quaternary ammonium compound (a salt of a strong acid and a strong base).

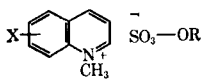
Regression Analysis—The method of least squares was employed to derive the equations using a Honeywell-800 computer. The

Table 1—Equations Correlating log $K_{app.}$ with the Substituent Constants

	n	r	s	Eq.
For *X*-quinolinium-$\overset{+}{\underset{-}{S}}O_3$-OR				
$\log K_{app.} = 0.496\,(A_w \cdot 10^9)_X + 0.345\,(A_w \cdot 10^9)_R - 2.341$	48	0.924	0.272	2
$\log K_{app.} = 0.494\,(A_w \cdot 10^9)_X + 0.345\,(A_w \cdot 10^9)_R + 0.420\,\sigma_X - 2.320$	48	0.931	0.263	3[a]
$\log K_{app.} = 0.536\,\pi_X + 0.931\,\pi_R - 0.582\,\sigma_X - 1.227$	48	0.831	0.401	4
For *X*-pyridinium-$\overset{+}{\underset{-}{S}}O_3$-OR				
$\log K_{app.} = 0.169\,(A_w \cdot 10^9)_X + 0.253\,(A_w \cdot 10^9)_R - 1.366$	19	0.981	0.130	5[a]
$\log K_{app.} = 0.479\,\pi_X + 0.639\,\pi_R + 0.934\,\sigma_X - 0.561$	19	0.967	0.175	6

[a] Equation which is statistically most significant.

Table II—The Apparent Partition Coefficients of the Alkylsulfates of the Quinolinium Derivatives and the Physicochemical Constants Used in the Correlation



Log $K_{app.}$ Obs.	Log $K_{app.}$ Calc.[a]	$A_w \cdot 10^9$ of X	π_X	σ_X	$A_w \cdot 10^9$ of R	π_R	X	R
2.79	2.52	0.78	0.00	0.00[b]	12.92	4.50	H	C_9H_{19}
3.24	2.99	0.78	0.00	0.00	14.27	5.00	H	$C_{10}H_{21}$
3.78	3.45	0.78	0.00	0.00	15.62	5.50	H	$C_{11}H_{23}$
4.20	3.92	0.78	0.00	0.00	16.97	6.00	H	$C_{12}H_{25}$
2.98	3.12	2.12	0.50	−0.14[b]	12.92	4.50	2-CH_3	C_9H_{19}
3.49	3.59	2.12	0.50	−0.14	14.27	5.00	2-CH_3	$C_{10}H_{21}$
3.93	4.05	2.12	0.50	−0.14	15.62	5.50	2-CH_3	$C_{11}H_{23}$
4.35	4.52	2.12	0.50	−0.14	16.97	6.00	2-CH_3	$C_{12}H_{25}$
3.05	3.11	2.12	0.50	−0.17[b]	12.92	4.50	4-CH_3	C_9H_{19}
3.49	3.58	2.12	0.50	−0.17	14.27	5.00	4-CH_3	$C_{10}H_{21}$
3.95	4.04	2.12	0.50	−0.17	15.62	5.50	4-CH_3	$C_{11}H_{23}$
4.42	4.51	2.12	0.50	−0.17	16.97	6.00	4-CH_3	$C_{12}H_{25}$
3.13	3.15	2.12	0.50	−0.08[c]	12.92	4.50	6-CH_3	C_9H_{19}
3.62	3.61	2.12	0.50	−0.08	14.27	5.00	6-CH_3	$C_{10}H_{21}$
4.06	4.18	2.12	0.50	−0.08	15.62	5.50	6-CH_3	$C_{11}H_{23}$
4.58	4.54	2.12	0.50	−0.08	16.97	6.00	6-CH_3	$C_{12}H_{25}$
3.79	3.75	3.46	1.00	−0.22[c]	12.92	4.50	2,6-$(CH_3)_2$	C_9H_{19}
4.20	4.22	3.46	1.00	−0.22	14.27	5.00	2,6-$(CH_3)_2$	$C_{10}H_{21}$
4.66	4.68	3.46	1.00	−0.22	15.62	5.50	2,6-$(CH_3)_2$	$C_{11}H_{23}$
5.13	5.15	3.46	1.00	−0.22	16.97	6.00	2,6-$(CH_3)_2$	$C_{12}H_{25}$
2.98	3.21	2.09	0.86	0.11[c]	12.92	4.50	6-Br	C_9H_{19}
3.48	3.68	2.09	0.86	0.11	14.27	5.00	6-Br	$C_{10}H_{21}$
3.99	4.14	2.09	0.86	0.11	15.62	5.50	6-Br	$C_{11}H_{23}$
4.40	4.61	2.09	0.86	0.11	16.97	6.00	6-Br	$C_{12}H_{25}$
2.86	3.08	1.82	0.71	0.11[c]	12.92	4.50	6-Cl	C_9H_{19}
3.30	3.55	1.82	0.71	0.11	14.27	5.00	6-Cl	$C_{10}H_{21}$
3.78	4.01	1.82	0.71	0.11	15.62	5.50	6-Cl	$C_{11}H_{23}$
4.26	4.48	1.82	0.71	0.11	16.97	6.00	6-Cl	$C_{12}H_{25}$
3.02	3.35	2.66	−0.02	−0.24[c]	12.92	4.50	6-OCH_3	C_9H_{19}
3.56	3.81	2.66	−0.02	−0.24	14.27	5.00	6-OCH_3	$C_{10}H_{21}$
3.98	4.28	2.66	−0.02	−0.24	15.62	5.50	6-OCH_3	$C_{11}H_{23}$
4.52	4.74	2.66	−0.02	−0.24	16.97	6.00	6-OCH_3	$C_{12}H_{25}$
3.31	3.37	2.66	−0.02	−0.20[c]	12.92	4.50	8-OCH_3	C_9H_{19}
3.82	3.83	2.66	−0.02	−0.20	14.27	5.00	8-OCH_3	$C_{10}H_{21}$
4.29	4.30	2.66	−0.02	−0.20	15.62	5.50	8-OCH_3	$C_{11}H_{23}$
4.77	4.76	2.66	−0.02	−0.20	16.97	6.00	8-OCH_3	$C_{12}H_{25}$
3.13	2.77	1.46	−0.67	−0.20[d]	12.92	4.50	8-OH	C_9H_{19}
3.56	3.24	1.46	−0.67	−0.20	14.27	5.00	8-OH	$C_{10}H_{21}$
4.04	3.70	1.46	−0.67	−0.20	15.62	5.50	8-OH	$C_{11}H_{23}$
4.50	4.17	1.46	−0.67	−0.20	16.97	6.00	8-OH	$C_{12}H_{24}$
3.29	3.52	2.51	1.15	0.35[b]	12.92	4.50	2-I	C_9H_{19}
3.79	3.99	2.51	1.15	0.35	14.27	5.00	2-I	$C_{10}H_{21}$
4.21	4.45	2.51	1.15	0.35	15.62	5.50	2-I	$C_{11}H_{23}$
4.65	4.92	2.51	1.15	0.35	16.97	6.00	2-I	$C_{12}H_{25}$
4.65	3.96	3.54[e]	1.24	0.17[b]	12.92	4.50	2,3-$(CH)_4$	C_9H_{19}
4.96	4.42	3.54	1.24	0.17	14.27	5.00	2,3-$(CH)_4$	$C_{10}H_{21}$
5.45	4.89	3.54	1.24	0.17	15.62	5.50	2,3-$(CH)_4$	$C_{11}H_{23}$
5.86	5.35	3.54	1.24	0.17	16.97	6.00	2,3-$(CH)_4$	$C_{12}H_{25}$

[a] Calculated value using Eq. 3. [b] Using Hammett's σ constant from H. H. Jaffé, *Chem. Rev.*, **53**, 191(1953). [c] Estimated value. [d] Calculated from the pKa values of quinoline and 8-hydroxyquinoline, from A. Albert and J. N. Phillips, *J. Chem. Soc.*, **1956**, 1294. [e] Calculated from 4 × 1.08 − 0.78 for $(CH)_4$-H.

log $K_{app.}$ values, Hansch's π constant (10–14), Bondi's group contribution to the surface area (A_w cm.²/mole × 10^9) (20), and Hammett's σ constant used in the analysis are assembled in Tables II and III.

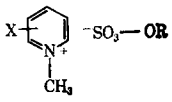
Protein-binding Studies and Kinetic Study of the Dialysis Through Artificial Membrane—The static dialysis method of Matsumoto *et al.* (27) was adapted for these studies. Visking cellulose tubing (Visking Corp., Chicago, average pore size of 24 Å.) was used to carry out the equilibrium dialysis for the binding of *N*-methylacridinium iodide and *N*-methyl-2,6-dimethylquinolinium iodide by bovine serum albumin (2.5×10^{-5} *M*) for a period of 24 hr. Before the binding studies were performed, the Visking tubing was hydrated by soaking it in refrigerated phosphate buffer (pH 6.7) overnight. After soaking, the bag was immediately refilled with 10 ml. of buffer or buffered protein solution and then placed in the bottle which contained 25 ml. of a 6.67×10^{-4} *M* solution of a quaternary ammonium salt in the same buffer. About a dozen assemblies were kept in the refrigerator during the period of dialysis. At specified time intervals, one of the bottles was removed from the refrigerator and the solutions were assayed for the quaternary ammonium compound, so no replacement or correction of the volume was necessary.

Equilibrium time was established by subjecting four compounds (nicotinic acid *N*-methyl iodide, *N*-methyl-2,6-dimethylquinolinium iodide, *N*-methyl-2-chloroquinolinium iodide, and *N*-methylacridinium iodide) to dialysis. It was found that 24 hr. were sufficient for equilibrium to be established.

A kinetic study was done on the dialysis of nicotinic acid *N*-methyl iodide and *N*-methylacridinium iodide for the first 6 hr.

***In Vitro* Intestinal Transport of Some Quaternary Ammonium Compounds**—Preliminary studies were performed on a limited number of *N*-methylquinolinium salts. Fasted, male Sprague Dawley rats weighing 215–307 g. were used. Each rat was anesthesized with ethyl ether. A midline incision was made and the intestine was removed and washed with normal saline solution. The intestine was divided into four segments. The first two segments were considered as the jejunum and the other two as ileal segments. Each segment was everted and attached to the modified Wiseman apparatus previously described by Saltman (21). To the luminal side was added 100 ml. of a 0.001 *M* solution of the quaternary ammonium

Table III—The Apparent Partition Coefficients of the Alkylsulfates of Pyridinium Derivatives and the Physicochemical Constants Used in the Correlation



Log $K_{app.}$ Obs.	Log $K_{app.}$ Calcd.[a]	$A_w \cdot 10^9$ of X	π_X	σ_X[b]	$A_w \cdot 10^9$ of R	π_R	X	R
3.55	3.75	0.78	0.00	0.00	19.67	7.00	H	$C_{14}H_{29}$
3.95	3.97	2.12	0.50	−0.14	19.67	7.00	2-CH_3	$C_{14}H_{29}$
4.46	4.20	3.46	1.00	−0.21	19.67	7.00	2,5-$(CH_3)_2$	$C_{14}H_{29}$
2.70	2.64	4.32	−0.01	0.32	12.92	4.50	3-$COOCH_3$	C_9H_{19}
3.06	2.98	4.32	−0.01	0.32	14.27	5.00	3-$COOCH_3$	$C_{10}H_{21}$
3.36	3.32	4.32	−0.01	0.32	15.62	5.50	3-$COOCH_3$	$C_{11}H_{23}$
3.72	3.66	4.32	−0.01	0.32	16.97	6.00	3-$COOCH_3$	$C_{12}H_{25}$
4.30	4.35	4.32	−0.01	0.32	19.67	7.00	3-$COOCH_3$	$C_{14}H_{29}$
2.97	2.86	5.67	0.49	0.40	12.92	4.50	3-COOEt	C_9H_{19}
3.27	3.21	5.67	0.49	0.40	14.27	5.00	3-COOEt	$C_{10}H_{21}$
3.60	3.55	5.67	0.49	0.40	15.62	5.50	3-COOEt	$C_{11}H_{23}$
3.96	3.89	5.67	0.49	0.40	16.97	6.00	3-COOEt	$C_{12}H_{25}$
4.60	4.57	5.67	0.49	0.40	19.67	7.00	3-COOEt	$C_{14}H_{29}$
3.19	3.32	8.37	1.49	0.40	12.92	4.50	3-COOBu	C_9H_{19}
3.56	3.66	8.37	1.49	0.40	14.27	5.00	3-COOBu	$C_{10}H_{21}$
3.91	4.00	8.37	1.49	0.40	15.62	5.50	3-COOBu	$C_{11}H_{23}$
4.27	4.34	8.37	1.49	0.40	16.97	6.00	3-COOBu	$C_{12}H_{25}$
5.12	5.03	8.37	1.49	0.40	19.67	7.00	3-COOBu	$C_{14}H_{29}$
3.23	3.50	3.34	−1.49	0.28	19.67	7.00	3-$CONH_2$	$C_{14}H_{29}$

[a] Calculated value using Eq. 5. [b] Using Hammett's σ constant in benzene ring system, from H. H. Jaffé, *Chem. Rev.*, **53**, 191(1953).

compound in normal saline; to the serosal side was added 50 ml. of normal saline solution. The system was immersed in a 37° bath, and all solutions were adjusted to 37° before using. The solutions were circulated by bubbling oxygen through the serosal and luminal solutions. Samples were removed at 15, 30, 60, 90, and 120-min. intervals. Two milliliters was removed from the serosal solution and 4 ml. was removed from the luminal solution for each assay. The following quaternary ammonium compounds were studied in this phase of the research: *N*-methylquinolinium iodide, *N*-methyl-6-methoxyquinolinium iodide, *N*-methyl-2-iodoquinolinium iodide, *N*-methyl-6-bromoquinolinium iodide, and *N*-methyl-6-methylquinolinium iodide.

RESULTS AND DISCUSSION

The equations obtained by the method of least squares are listed in Table I. The data used to derive the equations are listed in Tables II and III. In Table I, n is the number of data points used in the regression analysis, r is the correlation coefficient, and s is the standard deviation. From Eqs. 2 and 5, it appears that the surface areas of the substituent on the quaternary ammonium compound as well as that of the alkyl group on the sulfate moiety are the most important factors in governing the apparent partition coefficient (log $K_{app.}$). More than 86 and 96% of the variance in the data (r^2 = 0.86 and 0.96) can be explained by Eqs. 3 and 5, respectively. This reflects the importance of the van der Waals forces (dipole-induced dipole and induced dipole-induced dipole) of the quaternary ammonium–alkylsulfate in the chloroform phase, and the hydrophobic interactions of the alkyl group with the aromatic ring in the aqueous phase. This agrees with the previous finding that the apparent distribution constant of alkylamine salts of tropeolin 00 was governed by the molecular weight and branching of the aliphatic amine, the relative concentration of the amine and dye, and the dielectric constant of the solvent (15). In fact the log $K_{app.}$ of six amine salts of tropeolin 00 reported previously (15) can be correlated with the surface area or the π of the alkyl groups of the amine. This is illustrated by deriving the mathematical expressions by the method of least squares and the equations obtained are expressed (Eqs. 7 and 8).

	n	r	s	
$\log K_{app.} = 0.448\,(A_{wR} \cdot 10^9) - 4.782$	6	0.992	0.150	(Eq. 7)
$\log K_{app.} = 1.177\,(\pi_R) - 2.232$	6	0.994	0.137	(Eq. 8)

Addition of the electronic parameter σ in Eq. 3 gives a better correlation (r = 0.931, s = 0.263). This term is significant at the 95-percentile level ($F_{1,44}$ = 4.02). The positive dependence on σ in Eq. 3 suggests that electron withdrawing groups would enhance the log $K_{app.}$; this is explainable since an electron withdrawer makes the quaternary ammonium ion more positive and attracts the alkylsulfate ion more strongly.

Addition of a σ term to Eq. 5 does not result in an improved correlation. This is probably due to the fact that only two substituents studied have minus σ values and 16 molecules have very close σ values (0.28–0.40). A better selection of the substituents may reveal the role of the electronic effect.

The log $K_{app.}$ values calculated from Eqs. 3 and 5 are in good agreement with the observed values. The calculated and observed values are listed in Tables II and III.

The use of Hansch's π constant has been explored in view of the fact that log P_1 obtained from one solvent system can be related to log P_2 obtained from another system by the linear equation (22): $\log P_1 = a \log P_2 + b$, where a and b are constants. For the quinolinium compounds (Eqs. 2–4) the correlation obtained by using π is not as good as what is obtained by using A_w (r = 0.83 *versus* 0.93), although for the pyridinium compounds (Eqs. 5 and 6) both A_w and π give good correlations (r = 0.98 and 0.97, respectively). This may be due to somewhat different π values of the substituents in the quinolinium system from what were obtained from phenoxyacetic acid system (10).

Equilibrium dialysis studies showed that 24 hr. is sufficient for equilibrium to be established for the compounds studied. Since both solutions were assayed at the same time, it appears that no protein binding occurred with the quaternary compounds investigated. The Student t test revealed that no binding occurred to *N*-methyl-2,6-dimethylquinolinium iodide at P = 0.01 level. The kinetics of the dialysis of nicotinic acid *N*-methyl iodide through Visking cellulose membrane is shown in Fig. 1, using the reversible model (28). In Fig. 1, A_0 is the initial concentration of the drug (5.0×10^{-4} M), A is the outside drug concentration at time t, A_e is the theoretical equilibrium concentration (3.57×10^{-4} M). It is most interesting to note in Fig. 1 that within the 1st hr. the effective rate constant, $k + k'$ (the slope of the regression line) is $(1.62 \pm 0.9) \times 10^{-2}$ min.$^{-1}$, and that following the 1st hr. is $(0.3 \pm 0.15) \times 10^{-2}$ min.$^{-1}$, with the 95% confidence interval

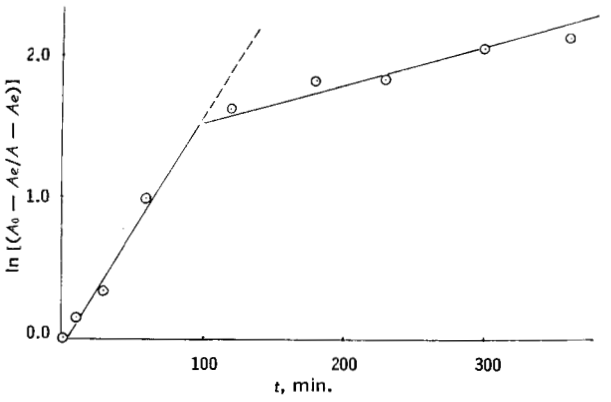


Figure 1—*The kinetic study of the dialysis of nicotinic acid* N-*methyl iodide through Visking cellulose membrane.*

given. The change in the effective rate constant may be due to plugging of some of the smaller pores of the cellulose membrane by the quaternary ammonium compound leaving fewer pores available for transport, and this process appears to be completed within the first hour.

The authors' results on the transport of *N*-methylacridinium iodide showed that this compound might bind on the dialysis membrane since the assay, after a given period of time, resulted in an overall value less than the original value.

The regression lines for the *in vitro* intestinal absorption of some quaternary ammonium salts are given as Eqs. 9–12, where *C* (micromoles) is the amount of quaternary ammonium salt transferred from the mucosal to the serosal side of the intestinal segment, and *t* is time in minutes. Each data point represents the average of four determinations. No transference of *N*-methyl-2-iodoquinolinium iodide was detected. This may be due to steric inhibition because of the presence of the fairly bulky iodo group next to the quaternary ammonium head. The equations expressing the intestinal absorption of the compounds are shown in Table IV.

From the slopes of Eqs. 9–12, it is evident that 6-methylquinoline *N*-methyl iodide and quinoline *N*-methyl iodide were transferred more rapidly. The rate of transfer of 6-bromo-quinoline *N*-methyl iodide and 6-methoxyquinoline *N*-methyl iodide was lower.

The transference of the quaternary ammonium salts was then investigated with the addition of an equimolar concentration of sodium decylsulfate to determine if the ion-ion pair, which is more nonpolar soluble than the iodide salt, occurred at a greater rate. The results showed that no transfer of any of the alkyl salts occurred. This may be due to steric inhibition and/or hydrophobic interactions of the decyl group and the aryl group of the quaternary ammonium moiety with the lipoprotein membrane since it is known that the strength of hydrophobic interaction is about 0.37–1.00 kcal./CH_2 (23–25) and an alkyl group of 10 or more carbon atoms will bind to another hydrophobic counterpart as strongly as an ion pair in the biological system (5 kcal./mole) (26).

Although the apparent partition coefficients of some alkylsulfates of quaternary ammonium compounds were correlated with their molecular structure, it was found that the rate of transfer of these same compounds across the intestinal wall could not be predicted on the basis of their partition coefficients since the presence of equimolar concentration of alkylsulfate inhibited the transfer of the quaternary ammonium compounds. The possible explanations for the failure of absorption of the alkylsulfates of the quaternary ammonium compounds by intestine may be due to: (*a*) steric hindrance because of the attachment of the long alkylsulfate moiety, (*b*) the increased hydrophobic interactions of the alkyl group with the lipoprotein membrane, or (*c*) the absence of the positive charge which is necessary for binding with a carrier.

Table IV—Regression Line Equations Expressing the *In Vitro* Absorption of Some Quaternary Ammonium Salts

Compound	Equation	*n*	*r*	*s*	Eq.
6-Me-quinoline MeI	$\text{Log } C = 6.5 \cdot 10^{-3} t + 0.186$	4	0.99	0.03	9
Quinoline MeI	$\text{Log } C = 5.3 \cdot 10^{-3} t + 1.190$	3	1.00	0.00	10
6-MeO-quinoline MeI	$\text{Log } C = 2.7 \cdot 10^{-3} t + 0.907$	4	0.91	0.05	11
6-Br-quinoline MeI	$\text{Log } C = 2.5 \cdot 10^{-3} t + 1.467$	3	0.98	0.02	12

REFERENCES

(1) C. A. Hogben, L. S. Schanker, D. T. Tocco, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.*, **120,** 540(1957).

(2) C. A. Hogben, D. T. Tocco, B. B. Brodie, and L. S. Schanker, *ibid.*, **125,** 275(1959).

(3) L. S. Schanker, *J. Med. Pharm. Chem.*, **2,** 343(1960).

(4) R. R. Levine, M. R. Blair, and B. B. Clark, *J. Pharmacol. Exptl. Therap.*, **114,** 78(1955).

(5) R. R. Levine and W. G. Pelikan, *ibid.*, **131,** 319(1961).

(6) R. R. Levine and A. F. Spencer, *Biochem. Pharmacol.*, **8,** 248(1961).

(7) R. R. Levine, *Federation Proc.*, **22,** 310(1963).

(8) R. R. Levine and G. M. Steinberg, *Nature*, **209,** 269(1966).

(9) R. R. Levine, *Arzneimittel-Forsch.*, **16,** 1373(1966).

(10) T. Fujita, J. Iwasa, and C. Hansch, *J. Am. Chem. Soc.*, **86,** 5175(1964).

(11) J. Iwasa, T. Fujita, and C. Hansch, *J. Med. Chem.*, **8,** 150 (1965).

(12) C. Hansch and S. M. Anderson, *J. Org. Chem.*, **32,** 2583 (1967).

(13) C. Hansch, J. E. Quinlan, and G. L. Lawrence, *ibid.*, **33,** 347(1968).

(14) E. J. Lien, C. Hansch, and S. M. Anderson, *J. Med. Chem.*, **11,** 430(1968).

(15) G. J. Divatia and J. A. Biles, *J. Pharm. Sci.*, **50,** 916(1961).

(16) R. L. Hall and J. A. Biles, *ibid.*, **53,** 869(1964).

(17) J. A. Biles, F. M. Plakogiannis, B. J. Wong, and P. M. Biles, *ibid.*, **55,** 909(1966).

(18) I. A. Vogel, "Practical Organic Chemistry," 3rd ed., Wiley, New York, N. Y., 1962, p. 662.

(19) E. E. Dreger, I. G. Keing, D. G. Miller, L. Shedlovsky, and J. Rose, *Ind. Eng. Chem.*, **36,** 610(1944).

(20) A. Bondi, *J. Phys. Chem.*, **68,** 441(1964).

(21) P. Saltman, *J. Chem. Educ.*, **42,** 682(1965).

(22) R. Collander, *Acta Chem. Scand.*, **5,** 774(1951).

(23) B. M. Anderson, M. L. Reynolds, and C. D. Anderson, *Biochem. Biophys. Acta*, **99,** 46(1965).

(24) G. Némethy, *Angew. Chem. Intern. Ed. Engl.*, **6,** 195(1967).

(25) W. Kauzmann, *Advances Protein Chem.*, **14,** 1(1959).

(26) A. Albert, "Selective Toxicity," Wiley, New York, N. Y., 1964, p. 129.

(27) H. Matsumoto, H. Matsumura, and S. Iguchi, *Chem. Pharm. Bull. Tokyo*, **14,** 391(1966).

(28) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed., Wiley, New York, N. Y., 1961, p. 186.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 9, 1969, from the *Pharmaceutical Chemistry Laboratories, School of Pharmacy, University of Southern California, University Park, Los Angeles, CA 90007*

Accepted for publication September 12, 1969.

This investigation was supported by grant AM-08652 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

* Present address: Brooklyn College of Pharmacy, Long Island University, Brooklyn, NY 11216

† National Science Foundation Undergraduate Research Participation Awardee, GY-327.

Absorptiometric, Polarographic, and Gas Chromatographic Assays for the Determination of *N*-1-substituted Nitroimidazoles in Blood and Urine

J. A. F. de SILVA, N. MUNNO, and N. STROJNY

Abstract □ This report describes an absorptiometric, a polarographic, and a gas-liquid chromatographic assay procedure for the quantitative determination of an *N*-1-substituted 2- and 5-nitroimidazole in blood and urine. Using the same selective extraction procedure and thin-layer chromatographic separation of the ethyl acetate extracts of blood and urine, the compounds are eluted from the silica gel into methanol. A suitable aliquot of this methanolic extract can be assayed sequentially using the absorptiometric (Bratton-Marshall) assay which has a sensitivity limit of 0.5–1.0 mcg./ml., or the pulsed polarographic assay (sensitivity limit 0.2–0.3 mcg./ml.) or the electron capture–GLC assay using the trimethyl silyl (TMS) derivatives of these compounds (sensitivity limit 0.01–0.02 mcg./ml.) for quantitation. Thus the sequential analysis of a specimen of blood or urine covers a sensitivity range of three orders of magnitude (*i.e.*, from 0.01 to 10 mcg./ml. of sample). The absorptiometric assay was used in the determination of blood level fall-off curves and urinary excretion of each compound in a dog following the administration of single 10-mg./kg. doses by oral and i.v. routes, while the GLC assay was used to extend the limits of detection of the absorptiometric assay and to determine the blood level fall-off curve in man following a single oral 50-mg. dose of Compound I.

Keyphrases □ Nitroimidazoles, *N*-1-substituted—determination in blood, urine □ Blood, urine analysis—*N*-1-substituted nitroimidazoles □ Polarography—analysis □ GLC—analysis □ TLC—analysis □ UV spectrophotometry—analysis

The nitroimidazole class of compounds has yielded several antimicrobial agents that are currently in use as trichomonacides (metronidazole)[1] and amebicides (dimetridazole).[2] The above agents are 5-nitroimidazoles with antimicrobial activity. The synthesis of 2-nitroimidazole by Beaman *et al.* (1) and by Lancini and Lazzari (2) represents the first total chemical synthesis of this antibiotic agent hitherto isolated from a microbial strain resembling *Nocardia mesenterica*. This led to the synthesis of several *N*-1-substituted 2- and 5-nitroimidazoles which were screened for antimicrobial properties.

From among these 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol (hereafter referred to as Compound I) was synthesized by Beaman (3) and is under clinical investigation as a trichomonacidal agent, whereas α-chloromethyl-2-methyl-5-nitro-1-imidazoleethanol (hereafter referred to as Compound II) was synthesized by Hoffer (4) and is under clinical investigation as an amebicide. The chemical reactions of I and II are shown in Scheme I, and the analytical parameters used in the sequential analysis of the compounds by absorptiometric, polarographic, and gas chromatographic (GLC) methods are shown in Fig. 1.

[1] 1-(2-Hydroxyethyl)-2-methyl-5-nitroimidazole (Flagyl, G. D. Searle & Co).

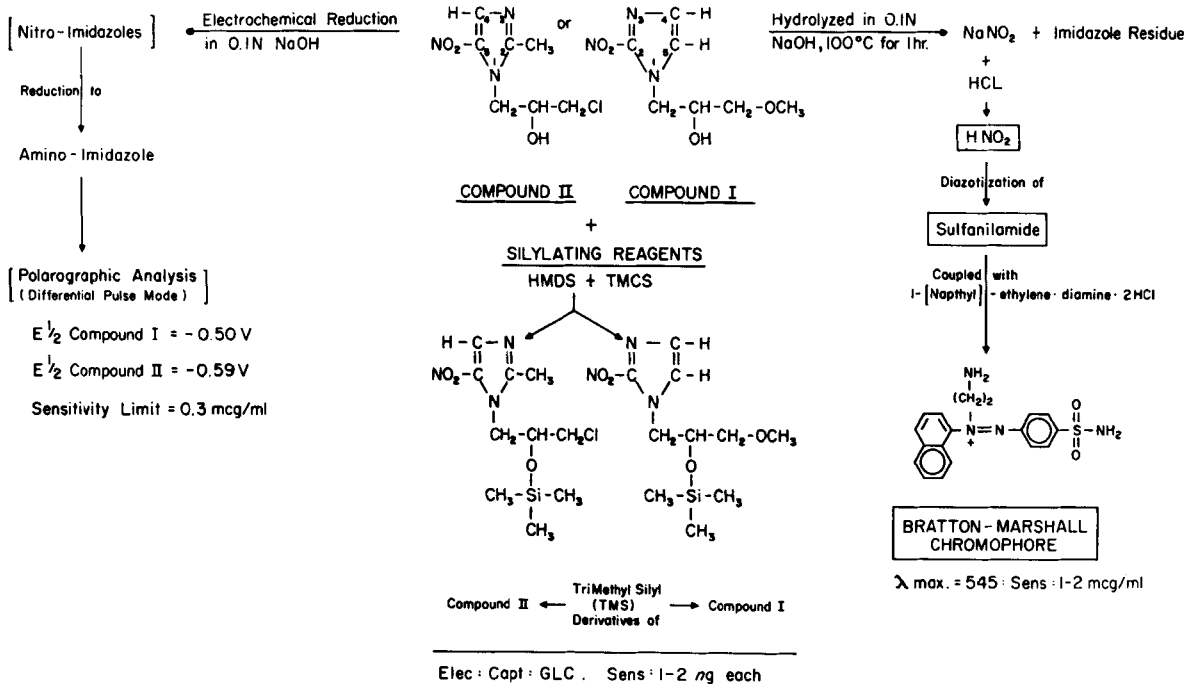
[2] 1,2-Dimethyl-5-nitroimidazole (Emtryl).

The Ehrlich diazo coupling reaction between diazobenzene sulfonic acid and imidazole or phenol derivatives in acidic media to produce colored azo dye-chromophores has been widely used in biological chemistry for the detection of these physiologically important compounds. However, the reaction is inhibited by *N*-substitution or the presence of strong electronegative substituents such as the nitro group on the imidazole ring. Nitroimidazoles can be detected by the Bratton-Marshall method (5) following reduction of the nitro group to the corresponding amine. Stambaugh and Manthei (6) used this principle of reducing nitroimidazoles to their amino derivatives, followed by coupling with either diazotized sulfanillic acid, *p*-dimethylaminobenzaldehyde, or ninhydrin to produce a chromophore characteristic of the coupling agent used as a means of differentiating nitroimidazoles. These reactions were found to be unsatisfactory for use in solvent extracts of blood or urine due to the presence of many interfering substances of biological origin.

The most widely used analytical procedures for the analysis of nitroimidazoles in pharmaceutical or agricultural products has been by the use of polarographic techniques (7–10), which are sufficiently sensitive and specific but complicated because of the need of specialized instrumentation. The analytical parameters for the polarographic assay were developed by MacDonald *et al.* (11), and applied directly in the analysis of Compounds I and II in blood. The method was, however, not used for the analysis of biological specimens because of the superiority of the GLC assay.

A rapid and sensitive absorptiometric assay was reported by Lau *et al.* (12). It is based on the alkaline hydrolysis of *N*-1-substituted 5-nitroimidazoles to yield stoichiometric amounts of the nitrite ($-NO_2$) ion which after acidification is used to diazotize sulfanilamide and couple it to *N*-(1-naphthyl)ethylenediamine to produce the characteristic Bratton-Marshall azo dye chromophore (5). The authors found, however, that *N*-1-substituted 2-nitroimidazoles also undergo a similar reaction, and this principle was used to develop the absorptiometric assay described for the determination of both I and II in blood and urine with a sensitivity limit of 0.5–1.0 mcg. of compound/ml. of blood or urine and an overall recovery of about 70%. The TLC separation of the ethyl acetate extract was required to ensure the specificity of the assay.

The highly sensitive and specific polarographic and GLC assays described use the same extraction, thin-layer chromatographic separation, and methanolic elution steps described in the absorptiometric assay. Suitable aliquots of the methanolic solution are evaporated to dryness and analyzed sequentially by the polaro-



Scheme I

graphic and/or GLC assays as shown in Fig. 1. The GLC assay requires that both I and II be reacted with the silylating reagents at room temperature for 15 min. to produce their respective trimethyl silyl (TMS) derivatives (Scheme I). These derivatives are very sensitive to detection by electron-capture GLC and can be quantitated in the nanogram (10^{-9} g.) to picogram (10^{-12} g.) range. The GLC method has an overall recovery of 83% and a sensitivity limit of 0.01–0.02 mcg. of Compound I/ml. of blood. The method was applied to the determination of a blood level fall-off curve of Compound I in man, following a single oral 50-mg. dose and in extending the limits of detection of the absorptiometric assay.

EXPERIMENTAL

Absorptiometric Assay in Blood and Urine

Standard Solutions of Compounds I and II—*Compound I*—

$C_7H_{11}N_3O_4$, mol. wt. 201.18, m.p. 111–114° (3)

Compound II—

$C_7H_{10}N_3O_3Cl$, mol. wt. 219.5, m.p. 77–78° (4)

A. Stock Solution—Dissolve 10 mg. of Compound I in 100 ml. of methanol (100 mcg./ml.) in an actinic flask, store refrigerated, and make fresh weekly.

B. Working Solution—Dilute 1 ml. of A to 10.0 ml. with methanol (concentration 10 mcg./ml.). Make fresh daily. Aliquots of this solution (B) are evaporated to dryness and the residues are redissolved in blood and used as internal standards. Stock solution A′ and working solution B′ for Compound II are also prepared as described above.

Reagents—All reagents used were of analytical reagent grade purity (>98%) and all inorganic reagents were made up in distilled deionized water (d.d.w.).

1.0 *M* (pH 9.0) H_3BO_3–Na_2CO_3–KCl buffer solution: dissolve 62.8 g. of orthoboric acid (H_3BO_3) and 74.6 g. of KCl/l. of distilled deionized water. Dissolve 106 g. of Na_2CO_3/l. of distilled water. To 630 ml. of the boric acid–KCl solution, add 370 ml. of the Na_2CO_3 solution to make 1 l. of buffer solution. Shake well, check the pH, and adjust it to pH 9.0 if necessary with the Na_2CO_3 solution. The final solution is 1 *M* with respect to H_3BO_3–Na_2CO_3–KCl and should be stored at 37° to prevent the salts from crystallizing out of solution. The following are also used: methanol, absolute, reagent grade; NaCl, reagent grade crystalline salt; 0.1 *N* NaOH in distilled deionized water; and 4.0 *N* HCl in distilled deionized water. To make the 0.1% sulfanilamide solution, use *p*-aminobenzene-sulfonamide, mol. wt. 172.21, m.p. 163° (Matheson). Dissolve 100 mg. sulfanilamide in 5 ml. of glacial acetic acid and bring to 100 ml. with distilled deionized water. Bratton-Marshall coupling reagent *N*-(1-naphthyl)ethylenediamine·2HCl (Eastman), mol. wt. 259.25, m.p. 190°. Dissolve 100 mg. in 100 ml. of distilled deionized water. Ethyl acetate, reagent grade (Mallinckrodt) was the extracting solvent.

Procedure—Into a 40-ml. glass-stoppered centrifuge tube, add 2 ml. of blood or urine specimen, 4.0 ml. of pH 9.0 buffer solution, 5 g. of NaCl, and 15 ml. of ethyl acetate.

With each series of samples, run a 2.0-ml. specimen of control blood or urine (preferably taken from the subject prior to medication or from a pooled control source) and a set of internal standards consisting of 2.0-ml. specimens of control blood or urine added to 2.5, 5.0, and 10.0 mcg. each of Compound I or II [0.25, 0.50, and 1.0 ml. of the methanol working solution (B or B′), respectively, is evaporated to dryness under a stream of nitrogen at 35–40°]. Stopper the tubes (seal with a drop of distilled water), shake for 10 min. on a reciprocating shaker, centrifuge (0–5°) for 10 min. at 2000 r.p.m., and transfer the clear supernatant into a 15-ml. centrifuge tube. Repeat the extraction with another 10-ml. aliquot of ethyl acetate and centrifuge. While this step is in progress, evaporate the first ethyl acetate extraction in a 50° water bath under nitrogen to a small volume ≃ 1 ml. Combine the second extraction with the first and evaporate to dryness at 50° under nitrogen. Dissolve the residue in 100 μl. of ethyl acetate and transfer quantitatively onto a 20 × 20-cm. precoated (Silica Gel F_{254}) Brinkmann TLC plate. The TLC plate should be ruled into strips 2.54 cm. (1 in.) in width prior to the application of the samples. This is done to prevent the samples from diffusing into each other and also for a more uniform development of the chromatogram. Rinse the tube with 50 μl. of methanol and combine the wash on the TLC plate. External standards of 50 mcg. of Compounds I and II [0.5 ml. of stock solution (A or A′), respectively, are evaporated to dryness under nitrogen, the residue dissolved in 100 μl. of ethyl acetate, and transferred onto the TLC plate] are run alongside the sample extracts. The plate is developed in a vapor-saturated tank in chloroform–acetone–glacial acetic acid (85:10:7.5) until the solvent front has ascended 12–15 cm. The area on the TLC plate corresponding in R_f to that of the external standards of Compound I (R_f = 0.25–0.30) or Compound II (R_f = 0.35–0.40), respectively, is delineated using a sharp stylus.

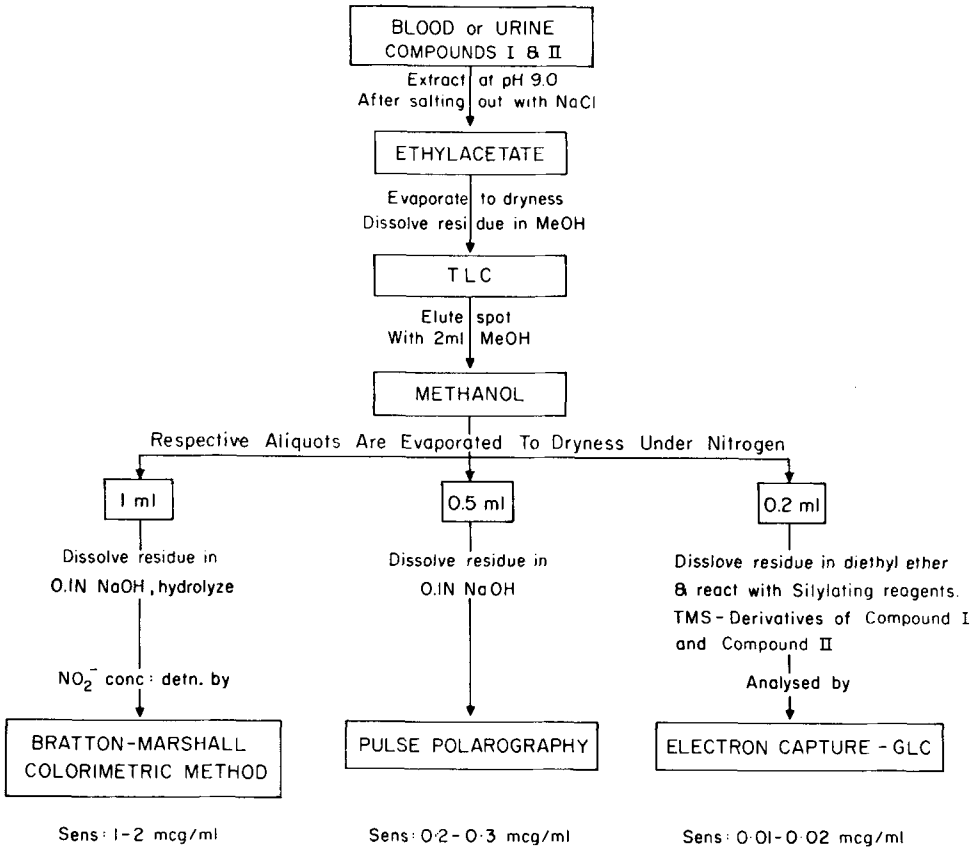


Figure 1—*Flow diagram of the sequential analysis of blood and urine for the determination of Compounds I and II.*

It must be noted that the visibly detectable limit of either Compound I or II on the TLC plate under shortwave UV is about 5–15 mcg. Amounts below this cannot be visibly defined; hence an area on the TLC plate 6.451 cm.^{2} (1 in.^{2}) corresponding to the R_f of the authentic standard is delineated, the silica gel is carefully scraped off, and transferred quantitatively into a 15-ml. centrifuge tube. The silica gel is eluted with 2 ml. of absolute methanol by slurrying for 1 min. on a vortex high-speed supermixer. The sample is centrifuged and the supernatant is decanted off carefully into another 15-ml. tube. The silica gel is reeluted with another 2-ml. aliquot of methanol as before, centrifuged, and the supernatants are combined. A 1.0-ml. aliquot of the methanolic solution is set aside for polarographic or GLC assay (if desired). The remaining supernatant (≃3.0 ml.) is evaporated to dryness under nitrogen in a 50° water bath and the residue is dissolved in 2.0 ml. of 0.1 *N* NaOH. However, if the sequential analysis of the sample using the polarographic and/or GLC assays is not required, then the entire sample is used for the absorptiometric assay.

At this stage external standards of Compound I or II in amounts of 2.5, 5.0, and 10.0 mcg. (0.25, 0.50, and 1.0 ml. of standard solution B or B′, respectively, are evaporated to dryness under nitrogen in a 50° water bath and the residues dissolved in 2.0 ml. of 0.1 *N* NaOH) are included for the determination of percent recovery and for establishing an analytical standard curve, Fig. 2A and B.

In the assay for Compound I *only*, transfer all the sample tubes and the external standards to a 100° water bath, allow to equilibrate for 5 min., stopper the tubes by sealing them with a drop of distilled water, and hydrolyze for 45 min. at 100°. In the assay for Compound II, however, all the sample tubes and external standards are hydrolyzed photolytically by irradiating them for 60 min. with UV energy from a Pyro Lux R-57 lamp (Luxor Corp., N. Y.). The samples are placed in a single row 30.48 cm. (12 in.) in front of the lamp which is housed in a aluminum foil-lined reflector box.

After hydrolysis, the samples are cooled in ice and acidified by adding 1.0 ml. of 4 *N* HCl. The samples are swirled on the vortex supermixer for 1 min. while cold, 0.5 ml. of 0.1% sulfanilamide solution is added, and the samples swirled again for 1 min. on the supermixer. Finally, 0.5 ml. of the coupling agent is added, the samples are swirled again on the supermixer for 1 min., and then allowed to stand at room temperature in the dark for 15 min. (minimum) to 30 min. (maximum) for full color development.

The absorbance of the Bratton-Marshall chromophore produced in each solution (final volume = 4.0 ml.) is measured in a 1-cm. path cell at 545 mμ in a Beckman DU or equivalent spectrophotometer.

Calculations—All biological samples are corrected for control (blank) values, and the external standards are corrected for reagent blank values.

Determination of Unknowns—The concentration of Compound I or II in the unknowns is determined by direct comparison of the absorbance of the unknowns to the mean absorptivity value of the three concentrations of the respective recovered internal standards thus:

$$\frac{A_{545}\ \text{unknowns}}{[a/\text{mcg./ml.}]\ \text{int. std.}} \times \frac{1}{\text{ml. of sample}} = \text{mcg. of Compound I or II/ml. of blood or urine}$$

Determine the *A*/mcg./ml. value of each of the three concentrations of recovered internal standards and calculate the mean *A*/mcg./ml. value of those standards. Use the mean value for determining the concentration in the unknowns.

Determination of Percent Recovery—The percent recovery of each internal standard is determined by direct comparison of the absorbance of the recovered internal standard against that of the corresponding external standards thus:

$$\frac{[A/\text{mcg./ml.}]\ \text{of int. std.}}{[A/\text{mcg./ml.}]\ \text{of ext. std.}} \times 100 = \text{percent recovery}$$

Polarographic Analysis

Procedure—The sample preparation was carried out as described for the absorptiometric assay up to the point where the compound is eluted off the silica gel successively with 2 × 2 ml. of methanol, the sample is centrifuged, and the supernatants are combined in a 15-ml. tube and a 1.0-ml. aliquot is set aside for polarographic or GLC assay (if desired). A 0.2-ml. aliquot of this sample is set aside for GLC analysis and the rest is evaporated to dryness under nitrogen. The residue is dissolved in 5 ml. of 0.1 *N* NaOH and then transferred into the polarographic cell. The three-electrode assembly is placed in the sample solution and the sample is deoxygenated with nitrogen for 2 min. The solution was analyzed in the differential pulse

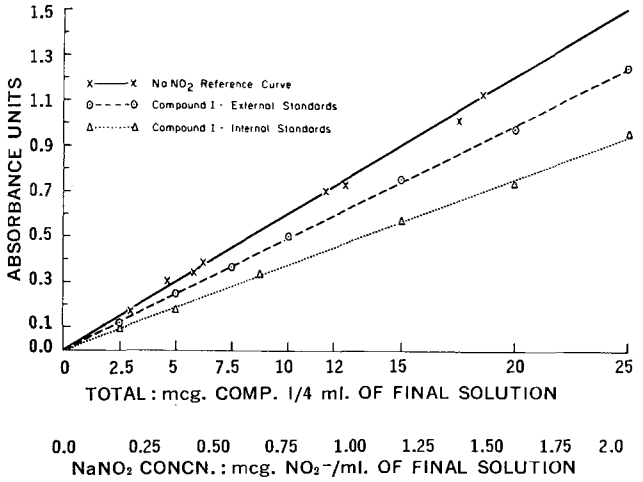


Figure 2A—*Standard curves of Bratton-Marshall chromophores of nitrite ion released by the alkaline hydrolysis of Compound I (external standards and internal standards recovered from blood including TLC) compared against an equivalent $NaNO_2$ reference curve.*

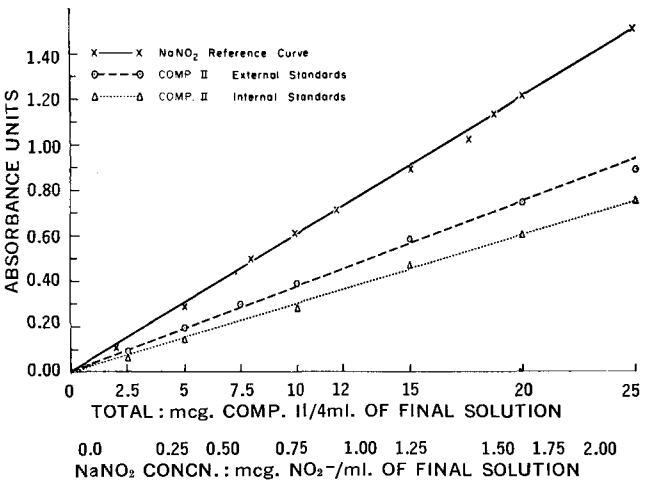


Figure 2B—*Standard curves of Bratton-Marshall chromophores of nitrite ion released by the photolysis in alkali of Compound II (external standards and internal standards recovered from blood including TLC) compared against an equivalent $NaNO_2$ reference curve.*

mode using instrumental parameters which will be published elsewhere (11).

The halfwave potential $E_{1/2} = -0.50$ v. for the nitro group reduction of Compound I and -0.59 v. for the nitro group reduction of Compound II, respectively. The peak area was calculated using height × width at half-height (cm.) and the sensitivity limit was 0.30 mcg. of I or II/ml. of final solution. A typical polarogram of the reference standard *versus* that of an internal standard of 5 mcg. each of Compound I or II recovered from blood is shown in Fig. 3.

Gas Chromatographic Assay in Blood and Urine

Principle—The GLC assay uses the same extraction procedure from blood or urine and the same TLC separation step as the absorptiometric assay. The silica gel covering a 6.45 cm.^{2} (1 in.^{2}) area corresponding in R_f to that of an external standard of Compound I or II is scraped off and eluted with methanol; the eluate is transferred to a fresh tube, evaporated to dryness, and the residue is vacuum dried. The residue is reacted with the silylating reagents to yield the trimethyl silyl (TMS) derivatives of I and II, respectively (Scheme I) and is quantitated by electron-capture GLC. The sensitivity limit of detection is of the order of 10–20 ng. of compound/ml. of blood or urine.

Reference Standard for GLC Assay—Since the assay involves several steps at which losses of compound are unavoidable, such as a TLC separation and a TMS derivatization step prior to quantitation, the use of a reference standard as a means of correcting for individual variations in recovery is good analytical practice. For this

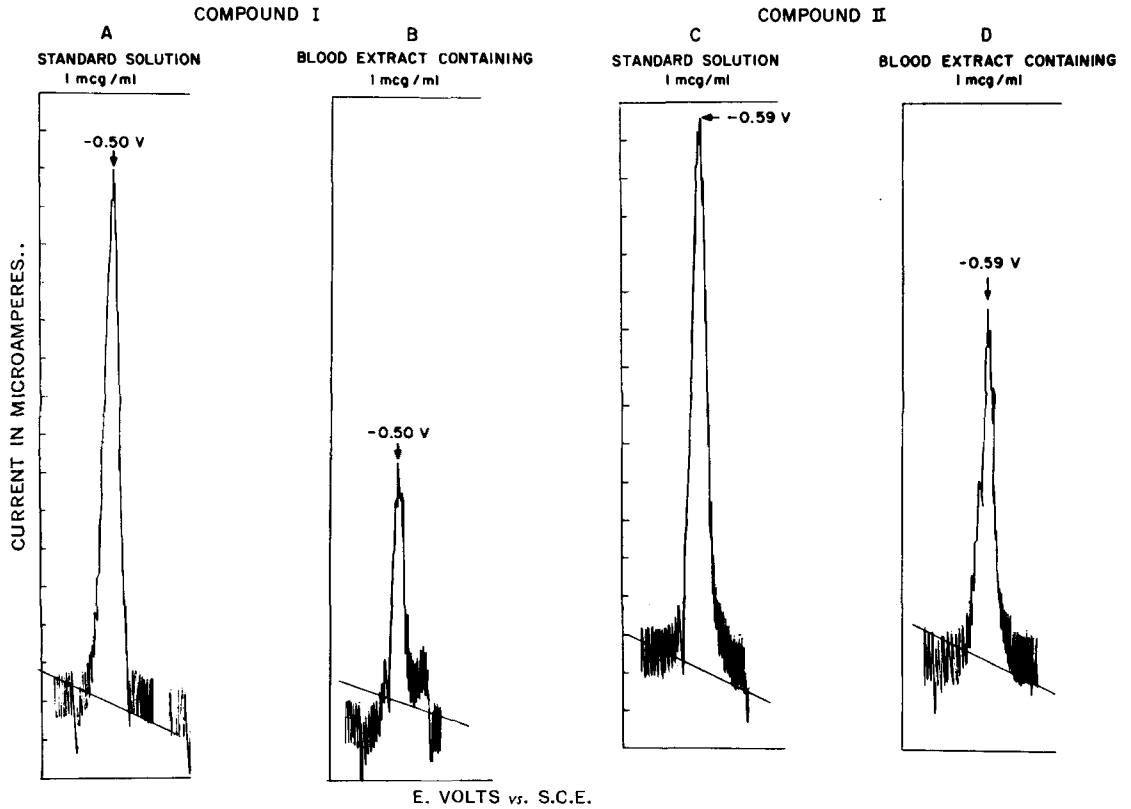


Figure 3—*Polarograms of standard solutions* (A *and* C) *and blood extracts* (B *and* D) *containing an added amount of Compounds I and II, respectively.*

purpose in the analysis of Compound I from biological media, an analogous nitroimidazole Compound II is used as the reference analytical standard and vice versa.

Standard Solutions—*Compound I*—Use working solution B from the absorptiometric assay (10 mcg./ml.). Suitable aliquots of solution B are evaporated to dryness and used as internal standards added to blood or urine for the determination of percent recovery. Aliquots of the same solution B are evaporated to dryness and used as external standards for determining the GLC standard curve.

Compound II Reference Standard—1-(3-Chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole, empirical formula $C_7H_{10}N_3O_3Cl$, mol. wt. 219.5, m.p. 77–78°. The stock solution (A′) and the working solution (B′) are made up as described for Compound I (see *Absorptiometric Assay*).

Parameters for GLC Analysis—*Column*—The column packing was a preconditioned phase containing 3% OV-17 on a 60/80 mesh Gas Chrom Q (Applied Sciences, State Park, Pa.) packed in a U-shaped 1.83-m. (6-ft.), 4-mm. i.d. borosilicate glass column.

Instrumental Parameters for GLC—A Micro-Tek gas chromatograph, model MT-220 (biomedical), was used equipped with a ^{63}Ni electron-capture detector containing a 10 mc. ^{63}Ni β-ionization source. Argon–methane (90:10) (oil pumped and dry) was used as the carrier gas, the column head pressure being adjusted to 40 psig. and the flow rate to 115–120 ml./min. The temperature settings were as follows: oven, 180°; injection port, 220°; detector, 290°. The conditions of column head pressure, flow rate, and oven temperature must be adjusted so as to obtain retention times of 5 and 6.5 min., respectively, for the TMS derivatives of Compounds I and II. A typical chromatogram is shown in Fig. 4. The solid-state electrometer (model No. 8169) input was set at 10^2 and the output attenuation was 16, giving a response of about 1.0×10^{-9} amp. for full-scale deflection (fsd); the chart speed was 76.2 cm./hr. (30 in.); and the time constant on the 1.0 mv. Honeywell recorder (model No. 194) was 1 sec. (fsd). The response of the ^{63}Ni electron-capture detector (operated in the pulsed d.c. mode) to the TMS derivatives of Compounds I and II showed maximum sensitivity at 30 v., at a 270-μsec. pulse rate and a 3-μsec. pulse width. Under these conditions a concentration of the TMS derivatives of 1 ng. of I:1.25 ng. of II gives nearly full-scale pen response on the 1.0-mv. recorder.

Calibration of Compound I by GLC—A calibration (external standard) curve of the peak area ratio of TMS–Compound I/TMS–Compound II *versus* concentration of Compound I/ml. of *n*-hexane (final solution) is constructed as shown in Fig. 5A. Determine the electron-capture detector response ratio of the TMS derivatives of Compounds I and II using the formula:

$$\frac{\text{response of Compound I}}{\text{response of Compound II}} = \frac{\text{peak area [height (cm.)} \times \text{width at half-height (cm.)] of Compound I}}{\text{peak area [height (cm.)} \times \text{width at half-height (cm.)] of Compound II}}$$

A fresh calibration curve is prepared for each day of analysis to establish the reproducibility of the GLC system. A similar curve of the added internal standards recovered from blood is also constructed. The amount of Compound I per aliquot of the unknown sample injected is calculated from the internal standard curve. In the analysis of Compound II, an external standard curve of the compound is constructed as shown in Fig. 5C using Compound I as the reference standard.

Since the peak area response ratio of Compound I:Compound II in any given sample is constant irrespective of the actual volume of sample injected, the concentration in the unknowns is obtained directly from the internal standard curve. Since the internal standards and unknowns are all dissolved in 1 ml. of *n*-hexane, there is no dilution or aliquot factor to be considered, unless the sequential analysis of the sample is employed (Fig. 1). The recovery factor for both internal and reference standards remains constant throughout and is not needed for the calculation of the unknowns.

Calculation of Unknowns—

$$\frac{\text{concn. (ng.) in unknown (extrapolated from int. std. curve)}}{\text{ml. of sample assayed}} \times \frac{\text{dilution factor}}{10^{-3}} = \text{mcg. compound/ml. of blood or urine}$$

where ng. compound = amount in the aliquot of sample injected extrapolated from the internal standard curve; dilution (aliquot) factor = sample aliquot (from sequential analysis if employed); and 10^{-3} = conversion factor for nanograms to micrograms.

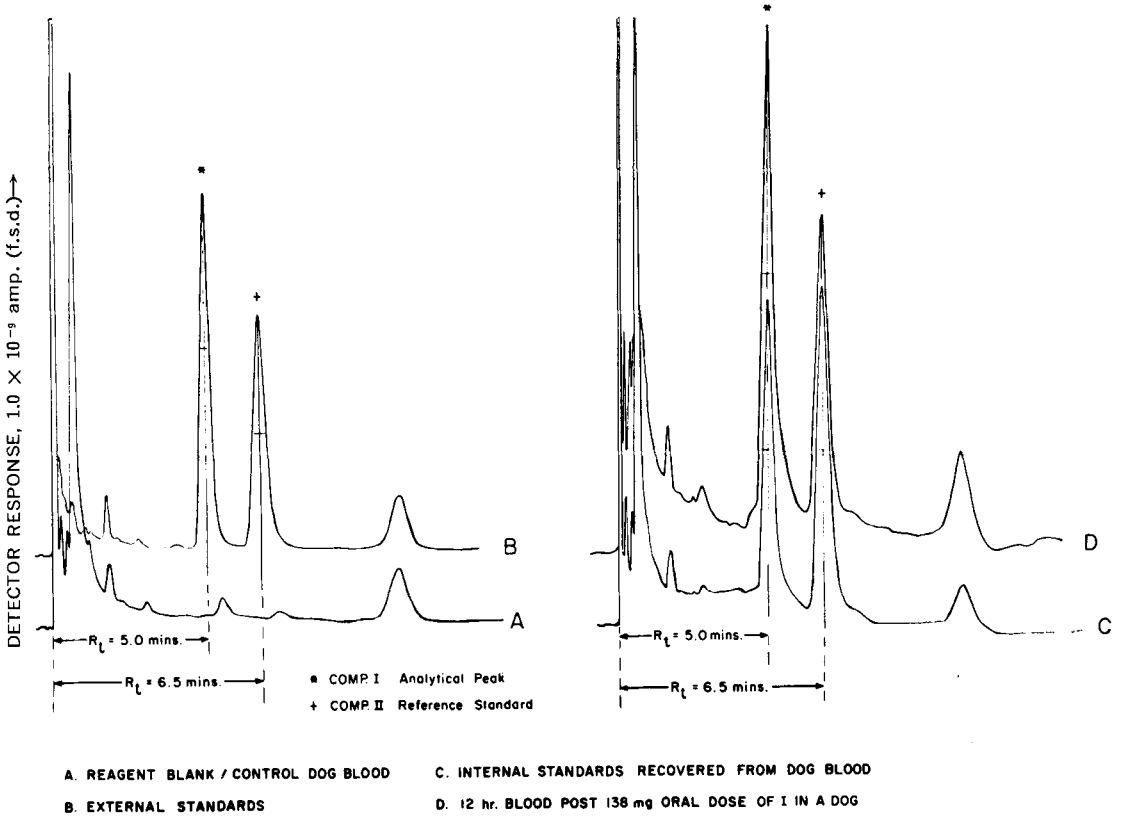


Figure 4—*Chromatograms showing the electron-capture GLC analysis of the TMS derivatives of:* (A *and* B) *the authentic standards of I and II and* (C *and* D) *Compounds I and II recovered from blood* in vitro *and* in vivo.

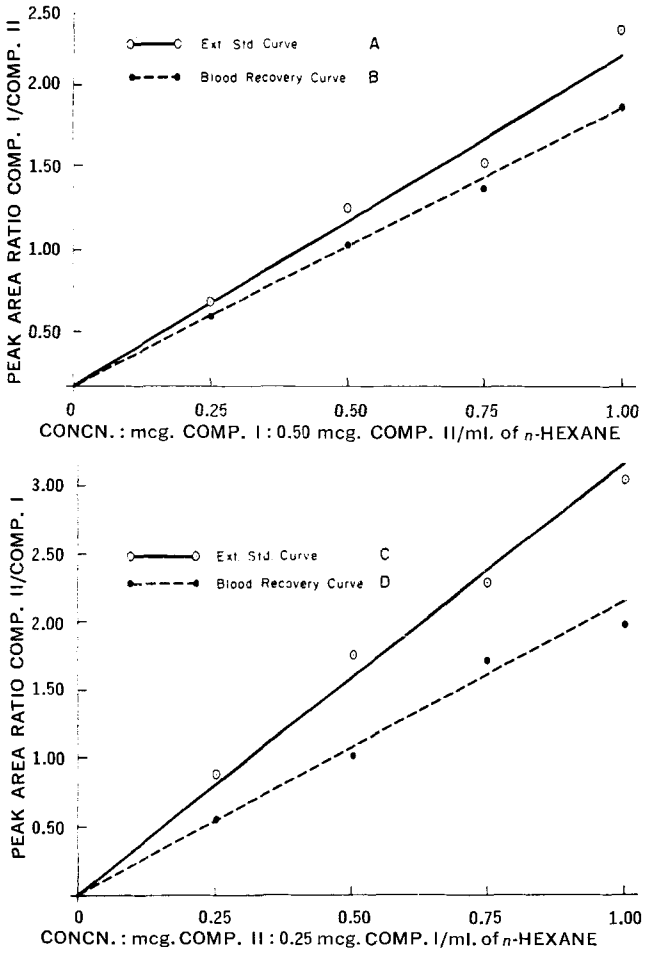


Figure 5—*Standard curves of the electron-capture detector response to the TMS derivatives of Compound I using Compound II as the reference standard* (A/B) *and vice versa* (C/D).

Sample Preparation for GLC Analysis of Blood and Urine—*Reagents*—Same as for the *Absorptiometric Assay*. In addition the following silylating reagents are required: hexamethyldisilazane (HMDS); trimethylchlorosilane (TMCS, Applied Science Labs, State College, Pa.); *n*-hexane, Fisher Spectrograde reagent (anhydrous).

Procedure for Blood and Urine—Into a 40-ml. glass-stoppered centrifuge tube, add 50 μl. of reference standard solution B′ of Compound II equivalent to 500 ng. and evaporate off the ethanol under nitrogen. Add a 2-ml. specimen of blood or urine and mix sample for 1 min. on a vortex high-speed mixer. Then add 4.0 ml. of pH 9.0 borate buffer, 5.0 g. of NaCl, and 15 ml. of ethyl acetate to the sample tube. Stopper the tube (sealing it with a drop of distilled water). Along with the unknowns, run a 2-ml. control specimen of blood or urine (taken prior to medication) and triplicate control specimen added to the residues of 250, 500, and 750 ng. of Compound I (25, 50, and 75 μl. of solution B) as the internal standard and 500 ng. of Compound II (50 μl. solution B′) as the reference standard. If the sequential analysis of the samples is used (Fig. 1), then 500 ng. of Compound II must be added to the aliquots taken of both internal standards and unknowns, the methanol evaporated to dryness, and the residues silylated as described. Follow the entire extraction procedure, TLC separation of the ethyl acetate residue, and methanol elution of the respective silica gel areas corresponding to Compound I (R_f = 0.3) and Compound II (R_f = 0.4) as described in the absorptiometric assay. However, in the GLC assay *only*, the silica gel areas of Compounds I and II are combined in a 15-ml. centrifuge tube prior to elution into methanol. The samples are then eluted successively with 2-ml. portions of methanol by slurrying for 60 sec. on a vortex high-speed supermixer. The samples are centrifuged, the clear supernatant transferred quantitatively (by decanting) into another 15-ml. centrifuge tube, evaporated to dryness at 50° under nitrogen, and vacuum-dried for 15 min. (under mild vacuum) to remove all traces of moisture.

At this point in the assay a series of external standard solutions of Compound I covering the concentration range of 100 to 750 ng. in suitable increments are prepared for the determination of the external standard curve. Suitable aliquots of standard solution B, Compound I, are pipeted into 15-ml. centrifuge tubes into which 50 μl. of solution B′ containing 500 ng. of Compound II (the reference standard) are added. The methanol is evaporated to dryness and vacuum-dried to remove all traces of moisture. These standards are processed along with the sample extracts to standardize the silylation process.

The residues are dissolved in 250 μl. of diethyl ether followed immediately by the successive addition of 40 μl. of HMDS and by 20 μl. of TMCS (silylating reagents) into the ether solutions. The tubes are stoppered immediately and mixed vigorously on a vortex supermixer for 60 sec. and then allowed to stand at room temperature (25°) for 15 min. to effect complete silylation of the two compounds. During this time period the samples are mixed again on the vortex mixer about 5–6 min. after the addition of the reagents. The sample tubes will develop a white precipitate of NH_4Cl due to the silylation reaction. This precipitate does not affect the reaction since the silylating reagents are present in a large excess relative to the amount of compound to be silylated.

After the 15-min. reaction period, the tubes are unstoppered and the solvent is evaporated to dryness under nitrogen at room temperature to remove the ether and all of the silylating reagents. The TMS derivatives are dissolved in 1 ml. of *n*-hexane and suitable aliquots of these standards are injected into the GLC instrument for preparing the external standard curve. This standard curve is required to establish the reproducibility of the GLC system and also for recovery determination. All the sample residues are also dissolved in 1 ml. of *n*-hexane (anhydrous) added to each tube, stoppered, and mixed well for 60 sec. on a vortex supermixer to dissolve the TMS derivatives. A suitable aliquot of this *n*-hexane extract (1–10 μl.) is injected into the gas chromatograph for the quantitation of Compound I. The large dilution is necessary because of the high sensitivity of the electron-capture detector to the TMS derivatives of these compounds and also to reduce the sample blank.

Determination of Percent Recovery—The use of a reference standard (Compound II) in the analysis, together with the internal standard (Compound I) added to blood or urine, eliminates the need to determine the percent recovery obtained with each run. Once the actual recovery factor has been experimentally determined by GLC (*viz.*, 83 ± 2.5% for Compound I and 65 ± 5.0% for Compound II), this factor can be assumed to be constant throughout. Since the relative recovery of each compound is assumed to remain constant, any change in the overall recovery of one would be automatically reflected in the recovery of the other to the same degree. Hence, irrespective of the actual recovery of each, the response ratio of the electron-capture detector would be constant and thus compensate for any variations incurred in sample processing. Consequently the response ratio of the TMS derivatives of Compound I/Compound II in the recovered internal standards and in the unknowns would reflect the relative amounts of each compound present irrespective of the actual recovery obtained.

If the determination of the actual recovery is desired, then proceed as follows: prepare a series of standards of either Compound I or II (100 to 1000 ng.) added to blood or urine as before but *without* the addition of the appropriate reference standard. Prepare the samples as described up to the silylation reaction step. At this point, add 250 ng. of Compound I or 500 ng. of Compound II (reference standard) to the appropriate series of samples and vacuum dry the residues. Continue with the silylation reaction as described previously, dissolving the final residue containing the respective TMS derivatives in 1 ml. of *n*-hexane. A suitable aliquot of this solution (1–10 μl.) is analyzed by GLC. The peak area response ratio of the recovered standards is directly compared against that of the external standards to determine the percent recovery of Compound I or II, respectively.

Calculation of Percent Recovery of Added Standards—The peak area response ratio of the recovered standards is plotted against concentration of the added standards of Compound I (Fig. 5B) to construct a blood-recovery curve. The slope of this curve is compared directly with that of the external standard curve of Compound I (Fig. 5A) to obtain the overall percent recovery. The same procedure is applied in the determination of the recovery of Compound II using Compound I as the reference standard. The slope of the blood-recovery curve of II (Fig. 5D) is compared directly with that

of the external standard curve (Fig. 5C) to determine the overall recovery. Alternatively, the response ratio of any one recovered standard is compared directly with that of the same concentration of the external standard, thus:

$$\frac{\text{[peak area response ratio] rec. std.}}{\text{[peak area response ratio] ext. std.}} \times 100 = \% \text{ recovery}$$

The sensitivity of the GLC method is of the order of 0.01–0.02 mcg. of either Compound I or Compound II/ml. of blood or urine, but the overall recovery from blood of Compound I is of the order of 83 ± 2.5%, whereas that for Compound II is 65 ± 5.0% (Table I).

RESULTS AND DISCUSSION

A systematic investigation of suitable analytical parameters for use in the quantitation of *N*-1-substituted nitroimidazoles was undertaken in the development of suitable analytical procedures.

The UV absorption spectra of the two compounds determined in 0.1 *N* HCl showed absorption maxima at 325 mμ (*A*/mcg./ml. = 0.038) for Compound I and at 278 mμ (*A*/mcg./ml. = 0.029) for Compound II, respectively. The specific absorptivity was sufficient to quantitate 10–100 mcg. of each compound/ml. of final solution. The determination of the extractability of these compounds into organic solvents from blood buffered at different pH values was done initially using the UV absorption of these compounds in 0.1 *N* HCl for quantitation. It was determined that using ethyl acetate as the extracting solvent gave better than 90% recovery of these nitroimidazoles from blood buffered to pH 9.0. The recovery declined significantly at pH values greater or less than 9.0.

The ethyl acetate extract was evaporated to dryness, the residue dissolved in 0.1 *N* HCl, and backwashed with diethyl ether as a cleanup step prior to quantitation in the UV. Not withstanding the cleanup of the sample, high UV absorption values from control blood suggested the incorporation of a TLC separation step for specificity and additional cleanup of the sample prior to quantitation. The sensitivity limits of the UV assay were unsatisfactory for use as a routine method due to high blank values from control blood and urine.

The principle of the sensitive absorptiometric assay reported by Lau *et al.* (12) was used to advantage in the absorptiometric assay described for the quantitation of these nitroimidazoles in blood and urine. The kinetics of the hydrolysis (at 100°) of Compound I to the $-NO_2$ ion indicated that the reaction was complete in 45 min., giving a >80% yield of nitrite ion. The yield was linear with concentration (2.5–25 mcg./4 ml.) measured against an external standard curve of $NaNO_2$ in concentrations equivalent to a theoretical 100% yield of $-NO_2$ from Compound I (Fig. 2A). Following diazotization of sulfanilamide with the released $-NO_2$ ions, coupling of the diazonium salt with *N*-(1-naphthyl) ethylenediamine·2 HCl to form the characteristic Bratton-Marshall azo dye chromophore was complete in 30 min. The hydrolysis of Compound II (at 100°) to the $-NO_2$ ion however was erratic. Although the overall yield was >70% it was not very reproducible. The compound was noted to be unstable to light exposure, the investigation of which led to the finding that the compound undergoes photolysis to the $-NO_2$ ion in alkali when irradiated with UV energy from a Pyro Lux R-57 lamp. The kinetics of the photolysis of Compound II in alkali to yield $-NO_2$ indicated that the reaction was complete in 60 min., giving a >80% yield of nitrite ion. The yield was also linear with concentration (2.5–25 mcg./4 ml.) (Fig. 2B).

Due to the intense color of the chromophore and its high absorption, the useful range for quantitation was in the concentration range of 0.25–5.00 mcg./ml. of final solution, Fig. 2A and 2B, respectively. Concentrations of either compound greater than 5.0 mcg./ml. should be diluted.

Initial recovery experiments were conducted from blood buffered to pH 9.0, extracted into ethyl acetate, the residue of which was hydrolyzed directly in alkali and quantitated by the Bratton-Marshall procedure. The erratic recoveries and high blank values obtained indicated the need for extensive cleanup of the sample prior to quantitation. The incorporation of a TLC separation step to ensure specificity and sample cleanup, the use of deionized distilled water to reduce possible contamination with nitrite or nitrate ions in preparing the reagents, and the use of NaCl to salt out the lipoproteins after buffering the blood or urine to pH 9.0 with 1.0 *M* borate buffer achieved the necessary cleanup of the sample. The residues of the ethyl acetate extracts on TLC analysis were free of interfering substances, especially in the areas on the chromatoplate corresponding in R_f to that of Compounds I and II.

Table I—Recovery of Compounds I and II from Blood and Urine Determined by the Electron-capture GLC Assay

ng. Compd. Added/2 ml.	ng. Compd. Recovered	% Recovery
A—Recovery of Compound I from Blood		
250	210	84.0
500	425	85.0
750	600	80.0
1000	845	84.5
Mean 83.4 ± 2.5		
B—Recovery of Compound II from Blood		
250	170	68.0
500	315	63.0
750	530	71.0
1000	600	60.0
Mean 65.5 ± 4.9		
C—Recovery of Compound II from Urine		
250	221	88.2
500	397	79.4
750	588	78.4
Mean 82.0 ± 5.4		

The final procedure yielded an overall recovery for Compounds I and II of 72 ± 5.7% and 70 ± 7.2% from blood, respectively (Table II, A and C). The recovery from blood was linear in the concentration range of 2.5–25 mcg./4 ml. of final solution (Fig. 2A and 2B). The recovery from urine was of the order of 59 and 53%, respectively (Table II, B and D). The sensitivity limit of the assay is 0.50–0.60 mcg. of Compound I or 1–2 mcg. of Compound II/ml. of blood or urine using a 2-ml. specimen per assay and a 2:1 sample–blank absorbance ratio as the limit of detectability.

The sensitivity limits of the absorptiometric assay can be extended into the submicrogram range using the polarographic assay and further extended into the nanogram range using the GLC assay. Therefore, if the sequential analysis of a specimen of blood or urine is undertaken as shown in Fig. 1, the ultimate sensitivity limit of quantitation obtained is of the order of 10–20 ng. of compound /ml. of blood or urine by electron-capture GLC. The polarographic assay was not investigated in any great detail since its usefulness in the analysis of nitroimidazoles in general has already been demonstrated (7–11). However the recovery of 5–15 mcg. of Compounds I and II was determined to be of the order of 47 and 60%, respectively, with a limit of detectability of 0.3 mcg. of compound/ml. of blood.

The relatively low melting point and the presence of the $-NO_2$ group in the imidazole ring of these compounds suggested that they could be readily quantitated by electron-capture GLC with potential sensitivities in the nanogram range. However, initial attempts at GLC analysis revealed very poor response characteristics with minimum detectability in the 1–5-mcg. range together with tailing peaks. It was apparent that the polar hydroxyl groups present in the side chain of the molecules (Scheme I) were interacting with the polar liquid phase to cause tailing and also possibly reduce the electron-capture response of the $-NO_2$ group due to inductive effects on the imidazole ring. The silylating of hydroxyl groups on aliphatic side chains and in sterically unhindered aromatic rings is known to proceed readily at room temperature (13). The preparation of suitable silyl derivatives of these compounds was effected using diethyl ether as the solvent and was found to proceed almost instantaneously at room temperature when reacted with hexamethyldisilazane (HMDS) synergized by trimethylchlorosilane (TMCS). The reaction products were analyzed by TLC using the solvent system heptane–$CHCl_3$–ethanol, 50:50:10 (v/v). The chromatoplate showed the presence of a single UV absorbing spot (R_f = 0.50) for the trimethyl silyl (TMS) derivatives of Compounds I and II. No residual I (R_f = 0.20) or II (R_f = 0.25) was seen on the plate, indicating complete reaction. The TMS derivatives when eluted off the silica gel with diethyl ether and analyzed by GLC gave excellent response to the electron-capture detector with nanogram range sensitivity with well-resolved peaks demonstrating the feasibility of the method. An aliquot of the *n*-hexane solution of the reaction mixtures when analyzed directly by GLC also gave a single sharp well-resolved peak with the same retention time and sensitivity as the respective TLC-

Table II—Recovery of Compounds I and II[a] from Blood and Urine Determined by the Bratton-Marshall Absorptiometric Assay

mcg. Added/ 2 ml. Blood	Internal Standard, E/mcg./ml.	External Standard, E/mcg./ml.	% Recovery
A—Recovery of Compound I from Blood			
2.5	0.158	0.195	81.0
5.0	0.169	0.246	68.7
5.0	0.134	0.205	65.2
5.0	0.139	0.200	69.5
7.5	0.123	0.198	63.0
10.0	0.174	0.236	73.7
10.0	0.168	0.231	72.7
10.0	0.122	0.176	69.3
10.0	0.118	0.176	67.0
10.0	0.110	0.176	63.0
15.0	0.151	0.201	75.0
15.0	0.144	0.201	71.6
20.0	0.139	0.176	79.0
20.0	0.137	0.172	79.7
20.0	0.146	0.194	75.3
25.0	0.152	0.198	76.8
Mean 71.91 ± 5.74			
B—Recovery of Compound I from Urine			
10.0	0.107	0.178	60.1
10.0	0.102	0.178	57.0
Mean 59.0 ± 2.3			
C—Recovery of Compound II from Blood			
2.5	0.100	0.174	58.0
2.5	0.104	0.174	59.8
5.0	0.116	0.174	66.7
5.0	0.114	0.174	65.8
10.0	0.132	0.166	79.5
10.0	0.124	0.166	74.7
10.0	0.118	0.164	72.2
10.0	0.082	0.128	64.1
10.0	0.083	0.128	64.8
10.0	0.096	0.136	70.6
20.0	0.137	0.166	82.5
20.0	0.101	0.140	72.1
20.0	0.102	0.140	73.1
Mean 69.53 ± 7.21			
D—Recovery of Compound II from Urine			
10.0	0.068	0.128	53.1
10.0	0.066	0.128	51.6
Mean 52.4 ± 2.4			

[a] Compound I is 1-(3-methoxy-2-hydroxypropyl)-2-nitroimidazole; Compound II is 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole.

isolated TMS derivatives, indicating that the reaction products were homogeneous and free from contaminating impurities. The silica gel area on the TLC plate corresponding in R_f to that of any unreacted residual parent compound that may have been present in the reaction mixture but undetectable by UV was eluted with methanol, evaporated to dryness, resilylated using the HMDS-TMCS mixture, and reanalyzed directly by electron-capture GLC. The chromatogram did not show any residual TMS derivatives, indicating that the initial silylation reaction was quantitative at room temperature.

Optimum conditions for the concomitant silylation of both compounds were obtained in diethyl ether as solvent using a 2:1 ratio of HMDS:TMCS and a total reaction time of 15 min. at room temperature for quantitative reaction. It is essential that the two silylating agents be added successively into the reaction mixture rather than from a synthetic mixture of the two reagents and that they also be vigorously mixed immediately after addition. The reaction mixture is also mixed vigorously at intervals of 5 and 10 min. after the addition of the reagents during the 15-min. reaction period. The reaction mixture is evaporated to dryness under nitrogen and the residue is dissolved immediately in anhydrous *n*-hexane without any vacuum drying. Although the TMS derivatives are stable in anhydrous *n*-hexane, they should nevertheless be analyzed by GLC as soon as possible. These derivatives are easily hydrolyzed by traces of moisture; hence it is of the utmost importance to maintain an anhydrous environment during their preparation and subsequent analysis by electron-capture GLC. The response of the electron-capture detector operated in the pulsed d.c. mode to the TMS derivatives of I and II is linear with concentration in the range of 0.1 to 1.0 mcg. of compound/ml. of *n*-hexane equivalent to 1.0 to 10 ng./10 μl. of sample injected. The sensitivity of the detector was such that 1.00 ng. of TMS-I and 1.25 ng. of TMS-II gave peak heights equivalent to nearly full-scale pen response for each compound on the recorder at the electrometer settings described. Since the response of the electron-capture detector to nearly equal (nanogram) amounts of the TMS derivatives of both compounds was comparable, *i.e.*,

$$\left[\frac{\text{peak area of TMS-I}}{\text{peak area of TMS-II}} \simeq \frac{1.0}{0.8} \simeq 1.25\right]$$

the peak area ratio of the two was used as the index of detector performance and overall efficiency of the analytical procedure. Thus the external standard curve and the blood- or urine-recovered internal standard curves were constructed by plotting the peak area response ratios of TMS-I/TMS-II *versus* concentration of I containing a constant amount of II as the reference standard/ml. of *n*-hexane or vice versa when Compound II is the analytical peak and Compound I the reference standard. The overall recovery of 250–1000 ng. of Compound I or II from blood was of the order of 83 ± 2.5% and 65 ± 5.0%, respectively (Table I, A and B), whereas that of Compound II from urine was 82 ± 5.4% (Table I, C). The GLC method was used to extend the range of sensitivity of detection of the absorptiometric assay in the determination of blood level fall-off curves in the dog and in man.

Application of the Absorptiometric Assay in Biological Specimens—The Bratton-Marshall absorptiometric assay was applied to the determination of blood and urine levels of I and II in a dog following the administration of a single 138-mg. dose of I and a 122-mg. dose of II (equivalent to 10 mg./kg.) by i.v. and oral routes, respectively.

The blood level fall-off curves of Compounds I and II are shown in Figs. 6 and 7, respectively. Following administration by both oral and i.v. routes, blood levels were measurable for up to 12 hr. by the absorptiometric assay, beyond which time period they were nonmeasurable by this method (*i.e.*, <0.5–1.0 mcg./ml.). Both compounds showed a typical biphasic i.v. blood level fall-off curve with a rapid initial distribution phase (half-life ≃ 20–30 min.) followed by a linear elimination phase with a half-life of 2.51 and 6.10 hr. for Compounds I and II, respectively.

The oral blood level fall-off curves of both compounds indicate good absorbability, Compound II showing a rapid absorption pattern with a blood level peak at 1 hr. of 11.5 mcg./ml. while Compound I showed a slower absorption pattern with a blood level peak at 2 hr. of 9.0 mcg./ml. Both compounds showed a linear elimination phase, Compound I having a faster elimination (half-life ≃ 2.55 hr.) running almost parallel with its i.v. elimination, while Compound II showed a slower elimination pattern (half-life ≃ 5.30 hr.). The blood levels in the 12–72-hr. postmedication period, where measurable, could only be determined by the electron-capture GLC assay with a sensitivity limit of the order of 0.01–0.02 mcg./ml. of blood.

The urinary excretion data following i.v. and oral administration of Compounds I and II are given in Table III, A and B, respectively. The data indicate that approximately equal amounts of both Compound I or II are recovered in the 0–72-hr. collection period following either i.v. or oral administration. This amounts to a recovery of 6.2 and 6.9% of Compound I and 7.1 and 6.6% of Compound II following i.v. and oral administration, respectively. The low percent recovery of the administered dose indicates either extensive biotransformation, tissue distribution, and/or alternate routes of elimination.

Application of the GLC Assay to Biological Samples—A blood level fall-off study on Compound I was conducted in a human subject following the administration of a single 50-mg. oral dose (0.67 mg./kg.). This study was primarily designed to test the capabilities of the GLC assay in the determination of blood levels following therapeutic doses.

The blood level fall-off curve, Fig. 8, indicates rapid absorption of the drug with a blood level peak of 1.0 mcg./ml. at 30 min. postdosing. The levels declined to 0.5 mcg./ml. at 1 hr. followed by a plateau in the levels up to 11.5 hr. Between 11.5 and 48 hr. the levels declined progressively, with a half-life of about 8 to 9 hr. Because of

Table III—Urinary Excretion Data on Compounds I and II in a Dog (Absorptiometric Assay)

Route	Time Period, hr.	mcg./ml.	Total mcg.	Cumulative Total mcg.	Excretion Rate, mcg./hr.	% of Dose Recovered
		A—Compound I[a]				
i.v.	0–23	34.0	8500	8500	370.0	6.2
	23–47	n.m.[b]	—	—	—	—
	47–71	n.m.	—	—	—	—
Oral	0–24	38.7	9288	9288	387.0	6.7
	24–48	1.0	340	9628	14.2	6.9
	48–72	n.m.	—	—	—	—
		B—Compound II[c]				
i.v.	0–2	1.10	340	340	170.0	0.3
	2–24	36.8	8290	8630	376.8	7.07
	24–48	1.10	80	8710	3.3	7.14
	48–72	n.m.[d]	—	—	—	—
Oral	0–24	18.1	4440	4440	185.0	3.64
	24–48	15.1	3650	8090	152.1	6.63
	48–72	n.m.	—	—	—	—

[a] Following single 138-mg. doses by i.v. and oral routes. [b] n.m. = not measurable, *i.e.*, <0.5–0.6 mcg. I/ml. urine using a 2-ml. specimen per assay. [c] Following single 122-mg. doses by i.v. and oral routes. [d] n.m. = not measurable, *i.e.*, <1.0 mcg. II/ml.

its inherent sensitivity the GLC assay thus can be used to determine blood levels in man up to 48 hr. postoral dosing following a single 50-mg. dose, and also in extending the limits of detection of the absorptiometric assay in determining blood levels in the nanogram (10^{-9} g.) range of sensitivity. This was demonstrated in the blood level fall-off curve of Compound II in a dog (Fig. 7) which indicates the range of detectability of the two assays.

Specificity of the Assay—Studies on the metabolism of the 5-nitroimidazole, metronidazole (14), have indicated that the -NO_2 group is stable to metabolic reduction *in vivo* and that metabolism occurs preferentially on the alkyl substituents resulting mostly in the excretion of the resulting alcohol, as such, and as the glucuronide and sulfate esters.

Since the -NO_2 group is left intact the presence of any metabolites in blood or urine would interfere with the specificity of the absorptiometric assay since they too would yield -NO_2 ion upon alkaline hydrolysis. The specificity of the three assays described for Compounds I and II is ensured by the inclusion of the TLC-separation step which effectively resolves the intact drug from other possible metabolites and from extracted impurities. Two-dimensional TLC examination of the silica gel eluates of the spots corresponding in R_f to the intact compound (I and II) migrate as one component when cochromatographed with the respective authentic standard. Thus the spot assayed following one-dimensional TLC represents the respective intact compound only. This was further verified using the GLC assay of the compounds as their respective TMS derivatives following elution from the silica gel.

No metabolites were seen in either the blood or urine specimens in a dog following the administration of Compound I. However, several metabolites were seen in blood and urine in a dog following the administration of Compound II. Since they were all well resolved from the intact compound by TLC analysis, they do not interfere with the specificity of the three assays for Compound II.

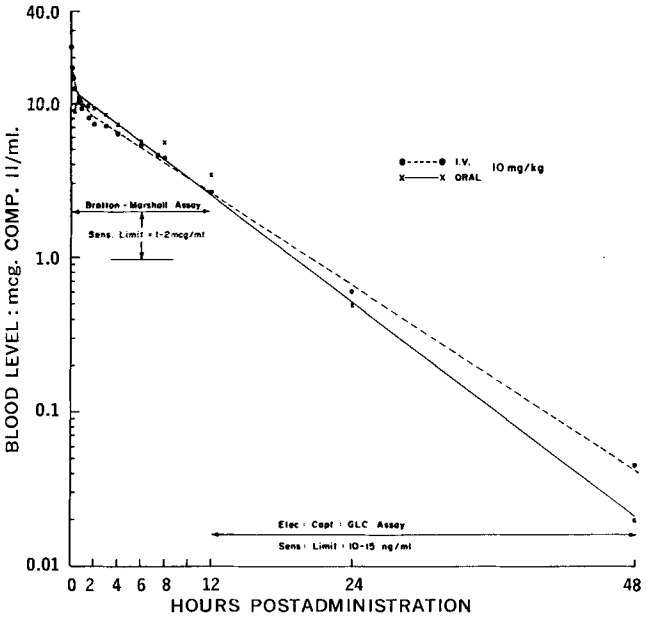


Figure 7—*Blood level fall-off curves of Compound II in a dog following the administration of a single 122-mg. dose by i.v. and oral routes.*

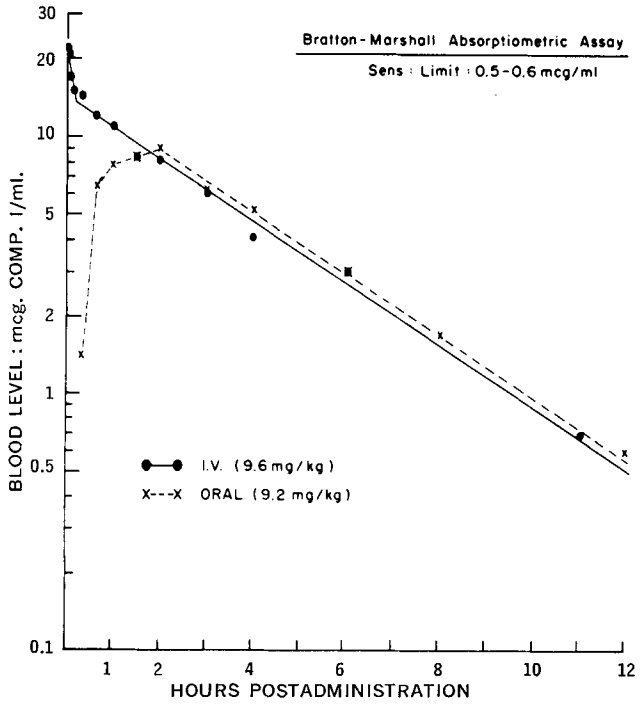


Figure 6—*Blood level fall-off curves of Compound I in a dog following the administration of a single 138-mg. dose by i.v. and oral routes.*

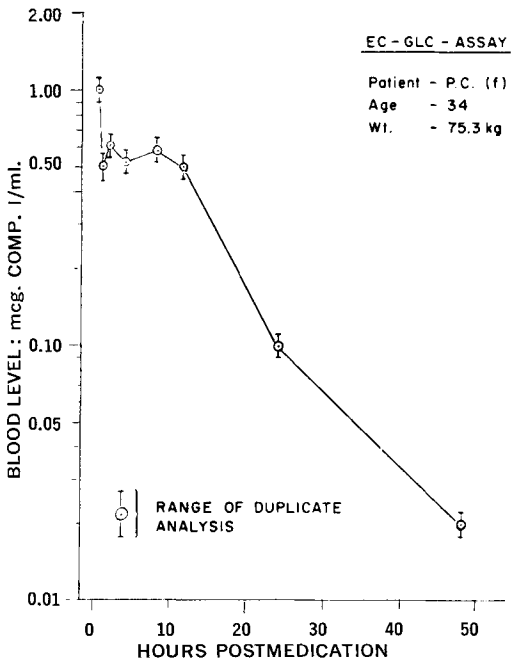


Figure 8—*Blood level fall-off curve of Compound I in man following the administration of a single oral 50-mg. dose.*

REFERENCES

(1) A. Beaman, W. Tautz, T. Gabriel, O. Keller, V. Toome, and R. Duschinsky, *Antimicrobial Agents Chemotherapy*, **1965**, 469.

(2) G. C. Lancini and E. Lazzari, *Experientia*, **21**, 83(1965).

(3) A. Beaman, W. Tautz, and R. Duschinsky, *Antimicrobial Agents Chemotherapy*, **1967**, 520.

(4) M. Hoffer, unpublished report, Hoffmann-La Roche Inc., Nutley, N. J., 1968.

(5) A. C. Bratton, E. K. Marshall, D. Babbitt, and A. R. Hendrikson, *J. Biol. Chem.*, **128**, 537(1939).

(6) J. E. Stambaugh and R. W. Manthei, *J. Chromatog.*, **31**, 128(1967).

(7) E. Laviron, *Bull. Soc. Chimie France*, **1963**, 2840.

(8) A. C. Daftsios and E. D. Schall, *J. Assoc. Agr. Chemists*, **45**, 278(1962).

(9) A. C. Daftsios, *ibid.*, **47**, 231(1964).

(10) P. J. Cooper and R. A. Hoodless, *Analyst*, **92**, 520(1967).

(11) A. MacDonald, E. Castro, G. Chen, and P. Hopkins, *Ann. Meeting Poultry Sci. Assoc.*, 1969.

(12) E. Lau, C. Yao, M. Lewis, and B. Senkowski, *J. Pharm. Sci.*, **58**, 55(1969).

(13) A. E. Pierce, "Silylation of Organic Compounds," Pierce Chemical Co., Rockford, Ill., 1968, pp. 18–21.

(14) J. E. Stambaugh, L. G. Feo, and R. W. Manthei, *J. Pharmacol. Exptl. Therap.*, **161**, 373(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1969, from *Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication September 15, 1969.

The authors thank Drs. B. A. Koechlin and W. B. Abrams for their interest in this study and for their critical review of the manuscript. Thanks are also due to Dr. S. A. Kaplan and Mr. S. Cotler for conducting the animal experiments, to Dr. A. S. Leon for the clinical specimens from his patient, to Dr. A. MacDonald for conducting the polarographic investigations, and to T. Danielian and R. McGlynn for the drawings of the figures.

Estimation of Mean Potency and Content Uniformity of Tablets: A New Approach

J. P. COMER, H. L. BREUNIG, D. E. BROADLICK, and C. B. SAMPSON

Abstract □ Current official procedures for the estimation of mean drug level and of content uniformity in individual tablets do not efficiently utilize all information available to the analyst, nor can confidence statements be made concerning the reported results. The authors present here a plan for combining readily available weight data with that from assays to generate distributions of potency per tablet. Tolerance limits on these distributions reflect not only the proportion of tablets bracketed, but also the degree of confidence to be placed in the finding. Reference is made to Monte Carlo studies on theoretical distributions as well as to examples from production lots of certain tablet items. The efficient utilization of this combined information leads to an improved method for estimating mean potency and content uniformity.

Keyphrases □ Tablets—mean potency, content uniformity □ Drug content uniformity, mean potency estimations—tablets □ Equations—tablet drug uniformity, mean potency □ Computer simulation—drug distribution, tablets

When considering drug dosage forms, the primary concern of the ethical pharmaceutical industry is that the patient receive in his individual dose an amount of medicament close to that claimed on the label. If this is so, the physician may prescribe the product with confidence that the desired drug substance will be available to perform its intended function. There are many facets to pharmaceutical quality assurance but all lead toward ensuring the identity, safety, and bioavailability of the drug of interest. This paper is solely concerned with the amount of drug substance in the unit dose. Such considerations as particle size, rates of dissolution and of absorption, freedom from impurities, and numerous others, while understood to be parts of pharmaceutical quality assurance, are not directly considered here.

Although this paper refers to compressed tablets, the techniques presented could also apply to filled capsules, ampuls, and other dry product dosage forms. Because of variation in homogeneity of granulation and in individual tablet weights, it is obviously unrealistic to expect every unit of product to possess *exactly* the same amount of physiologically active drug, but with good manufacturing practice these variations may be controlled. The subject of drug substance variability has been considered by a number of authors. Olson and Lee (1) have summarized much of the discussion and present an extensive list of references. A more recent paper is that by French *et al.* (2). Breunig (3) has emphasized the importance of weight control for individual units of product. Roberts (4) points out how easily many tablets (and filled capsules and sterile solids) may fail the USP weight variation test when based upon a sample of 20. He develops four rules for acceptance based upon the coefficient of variation of unit weights and provides charts which may be used for evaluation.

PRODUCT SPECIFICATIONS

The existence of variability in pharmaceutical products is recognized by USP XVII (5) and NF XII (6). These official compendia include at least three types of product specifications:

Rubric Limits—Referred to in the separate monographs, they are the bounds within which the mean response of samples of N units of product must fall based upon physiological, biological, or chemical assay. This response is in terms of the weight of drug substance per unit of product as determined upon individual units or as drug substance weight per average unit where test methods applicable to single units are not available. The bounds and the

REFERENCES

(1) A. Beaman, W. Tautz, T. Gabriel, O. Keller, V. Toome, and R. Duschinsky, *Antimicrobial Agents Chemotherapy*, **1965**, 469.

(2) G. C. Lancini and E. Lazzari, *Experientia*, **21**, 83(1965).

(3) A. Beaman, W. Tautz, and R. Duschinsky, *Antimicrobial Agents Chemotherapy*, **1967**, 520.

(4) M. Hoffer, unpublished report, Hoffmann-La Roche Inc., Nutley, N. J., 1968.

(5) A. C. Bratton, E. K. Marshall, D. Babbitt, and A. R. Hendrikson, *J. Biol. Chem.*, **128**, 537(1939).

(6) J. E. Stambaugh and R. W. Manthei, *J. Chromatog.*, **31**, 128(1967).

(7) E. Laviron, *Bull. Soc. Chimie France*, **1963**, 2840.

(8) A. C. Daftsios and E. D. Schall, *J. Assoc. Agr. Chemists*, **45**, 278(1962).

(9) A. C. Daftsios, *ibid.*, **47**, 231(1964).

(10) P. J. Cooper and R. A. Hoodless, *Analyst*, **92**, 520(1967).

(11) A. MacDonald, E. Castro, G. Chen, and P. Hopkins, *Ann. Meeting Poultry Sci. Assoc.*, 1969.

(12) E. Lau, C. Yao, M. Lewis, and B. Senkowski, *J. Pharm. Sci.*, **58**, 55(1969).

(13) A. E. Pierce, "Silylation of Organic Compounds," Pierce Chemical Co., Rockford, Ill., 1968, pp. 18–21.

(14) J. E. Stambaugh, L. G. Feo, and R. W. Manthei, *J. Pharmacol. Exptl. Therap.*, **161**, 373(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1969, from *Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication September 15, 1969.

The authors thank Drs. B. A. Koechlin and W. B. Abrams for their interest in this study and for their critical review of the manuscript. Thanks are also due to Dr. S. A. Kaplan and Mr. S. Cotler for conducting the animal experiments, to Dr. A. S. Leon for the clinical specimens from his patient, to Dr. A. MacDonald for conducting the polarographic investigations, and to T. Danielian and R. McGlynn for the drawings of the figures.

Estimation of Mean Potency and Content Uniformity of Tablets: A New Approach

J. P. COMER, H. L. BREUNIG, D. E. BROADLICK, and C. B. SAMPSON

Abstract □ Current official procedures for the estimation of mean drug level and of content uniformity in individual tablets do not efficiently utilize all information available to the analyst, nor can confidence statements be made concerning the reported results. The authors present here a plan for combining readily available weight data with that from assays to generate distributions of potency per tablet. Tolerance limits on these distributions reflect not only the proportion of tablets bracketed, but also the degree of confidence to be placed in the finding. Reference is made to Monte Carlo studies on theoretical distributions as well as to examples from production lots of certain tablet items. The efficient utilization of this combined information leads to an improved method for estimating mean potency and content uniformity.

Keyphrases □ Tablets—mean potency, content uniformity □ Drug content uniformity, mean potency estimations—tablets □ Equations—tablet drug uniformity, mean potency □ Computer simulation—drug distribution, tablets

When considering drug dosage forms, the primary concern of the ethical pharmaceutical industry is that the patient receive in his individual dose an amount of medicament close to that claimed on the label. If this is so, the physician may prescribe the product with confidence that the desired drug substance will be available to perform its intended function. There are many facets to pharmaceutical quality assurance but all lead toward ensuring the identity, safety, and bioavailability of the drug of interest. This paper is solely concerned with the amount of drug substance in the unit dose. Such considerations as particle size, rates of dissolution and of absorption, freedom from impurities, and numerous others, while understood to be parts of pharmaceutical quality assurance, are not directly considered here.

Although this paper refers to compressed tablets, the techniques presented could also apply to filled capsules, ampuls, and other dry product dosage forms. Because of variation in homogeneity of granulation and in individual tablet weights, it is obviously unrealistic to expect every unit of product to possess *exactly* the same amount of physiologically active drug, but with good manufacturing practice these variations may be controlled. The subject of drug substance variability has been considered by a number of authors. Olson and Lee (1) have summarized much of the discussion and present an extensive list of references. A more recent paper is that by French *et al.* (2). Breunig (3) has emphasized the importance of weight control for individual units of product. Roberts (4) points out how easily many tablets (and filled capsules and sterile solids) may fail the USP weight variation test when based upon a sample of 20. He develops four rules for acceptance based upon the coefficient of variation of unit weights and provides charts which may be used for evaluation.

PRODUCT SPECIFICATIONS

The existence of variability in pharmaceutical products is recognized by USP XVII (5) and NF XII (6). These official compendia include at least three types of product specifications:

Rubric Limits—Referred to in the separate monographs, they are the bounds within which the mean response of samples of N units of product must fall based upon physiological, biological, or chemical assay. This response is in terms of the weight of drug substance per unit of product as determined upon individual units or as drug substance weight per average unit where test methods applicable to single units are not available. The bounds and the

mean response are generally given in terms of percentage of the labelled amount of drug.

Weight Variation Requirements—These are sampling plans for controlling the variation in weight of units of product about their sample mean. A certain proportion of the sample is expected to fall within various percentages of the mean sample weight, according to the weight class of the item. In the case of dry products, no statement is made relating the mean sample weight to the expected weight for the batch.

Content Uniformity Limits—Official at this time for certain tablet items only, these limits are specifications on drug substance weight present in individual units of product such that at least 96.7% of the assayed units are expected to fall between 85 and 115% of the mean of the rubric limits "average of the tolerances" (see *Reference 5*, p. 906).

None of the above specifications contains any provision for stating the degree of confidence to be placed on the experimental finding. Depending on how a sample is taken, the requirements listed may or may not characterize the population, *i.e.*, the batch or lot from which it is drawn.

PRESENT PROPOSAL

A major unexplored area in this field has been the effective utilization of information from two sources: (*a*) the distribution statistics obtained from large (100–200) numbers of tablets weighed on automatic recording balances, and (*b*) the information recorded for single unit assays on smaller numbers (3–10) of units from the same lot.

A plan is presented for combining the information from these two sources to place approximate tolerance limits on the drug substance weight in individual dosage units of the product from a lot. Several computer simulation studies have been completed. The results of these studies suggest that the proposed method for setting tolerance limits is reasonable. Since this paper is directed towards the pharmaceutical scientist, it contains no mathematical derivations. These may be the subject of a subsequent paper.

THEORY AND REFERENCES

Tolerance limits for normal distributions are discussed by several authors, including Hald (7) and Dixon and Massey (8). The assumption of normality was made for both types of populations under consideration. This assumption should be checked for a particular item by any of the usual tests for normality that the reader may judge pertinent. The following populations are considered: (*a*) tablet weights, say Y, and (*b*) drug substance in formulation material, say P.

Y is measured in milligrams per tablet (mg./tablet) and P in milligrams of drug substance per milligram of formulation material. It is also assumed that the concentration of drug, P, is independent of the weight of the tablet, Y. The relevant distribution statistics are the sample means, $\bar{Y}$ and $\bar{P}$, and the sample variances, S_Y^2 and S_P^2. These estimates are based on N_Y observations of the Y-distribution and N_P observations of the P-distribution.

The variable of interest is YP, the weight of drug substance per tablet. The unbiased estimates of the population mean and variance of YP are easily shown to be

$$\bar{Y}\bar{P} \qquad \text{(Eq. 1)}$$

and

$$S_{YP}^2 = \bar{Y}^2S_P^2 + \bar{P}^2S_Y^2 + \left(1 - \frac{1}{N_Y} - \frac{1}{N_P}\right)S_Y^2S_P^2 \qquad \text{(Eq. 2)}$$

respectively, *e.g.*, Goodman (9).

The distribution of YP (even when P and Y are both normally distributed) is, in general, somewhat unmanageable, but Aroian (10) has shown that as the population coefficients of variation, of which $S_Y/\bar{Y}$ and $S_P/\bar{P}$ are estimates either singly or together, become small, then the distribution of YP is approximately normal. This being so, normal theory can be utilized and approximations thereof to set tolerance limits on the distribution of YP by constructing the interval

$$\bar{Y}\bar{P} \pm K(\gamma_1, \gamma_2, F)S_{YP} \qquad \text{(Eq. 3)}$$

where $K(\gamma_1, \gamma_2, F)$ is the multiplicative factor indexed by the degree of confidence, γ_1, the proportion of distribution covered, γ_2, and the degrees of freedom, F. For values of K see *References 7*, p. 315, and *8*, p. 436. In other words,

$$\bar{Y}\bar{P} \pm KS_{YP} \qquad \text{(Eq. 3}a\text{)}$$

gives approximate γ_1/γ_2 tolerance limits on the distribution of values of YP, based on F degrees of freedom. For instance, if $\gamma_1 = 0.95$ and $\gamma_2 = 0.99$, the expression 95/99 indicates that one can expect, with 95% confidence, that 99% of the individual values of YP are bounded by $\bar{Y}\bar{P} \pm KS_{YP}$ utilizing the amount of information provided by F. In this case, F is not simply some linear function of N_Y and N_P, but is approximately given by

$$F = \frac{(\bar{P}^2S_Y^2 + \bar{Y}^2S_P^2)^2}{[(\bar{P}^2S_Y^2)^2/(N_Y - 1)] + [(\bar{Y}^2S_P^2)^2/(N_P - 1)]} \qquad \text{(Eq. 4)}$$

Equation 4 may be rewritten

$$F = \frac{(C_P^2 + C_Y^2)^2}{[(C_P^2)^2/(N_P - 1)] + [(C_Y^2)^2/(N_Y - 1)]} \qquad \text{(Eq. 5)}$$

where C_Y and C_P are sample coefficients of variation (also called relative standard deviation), derived from either past data, current data, or both. This formula for F was adapted from the results of Welch (11).

Additional Monte Carlo studies suggest that F, as computed in Eq. 5, will be, on the average, too large in some parametric situations. A more comprehensive treatment of the theory, as well as some suggested improvements, will be the subject of a future paper as previously mentioned.

In the next sections some of the Monte Carlo results and the application of the above equations are reported, using actual control laboratory data.

COMPUTER SIMULATIONS

As mentioned in the previous section, Aroian has shown that the distribution of YP is approximately normal if the coefficients of variation of Y and P are "small." To examine this empirically, several Monte Carlo simulations were carried out on an IBM 360-30 computer with various population coefficients of variation. As an illustrative example, the following are presented.

Initially, the histogram (Y-distribution) of 2000 hypothetical tablet weight observations shown in Fig. 1 was generated from a normal distribution with a population mean of 122.58 and variance of 4.0466. The sample mean and variance from these data were calculated to be 122.55 and 4.1299, respectively. The population coefficient of variation is 1.64%.

Secondly, the histogram (P-distribution) of 2000 hypothetical assays shown in Fig. 2 was generated from a normal distribution with a population mean of 0.7106×10^{-3} and a population variance of 0.2760×10^{-10}. The sample mean and sample variance from these data were calculated to be 0.7104×10^{-3} and 0.2730×10^{-10}, respectively. The population coefficient of variation is 0.757%.

Finally, the histogram shown in Fig. 3 is that of the products of the two random variables, Y and P, *i.e.*, YP-distribution. From the data thus generated, the sample mean and variance are 87.065×10^{-3} and 2.4610×10^{-6}, respectively. The theoretical mean and variance obtained by the proposed calculation are 87.105×10^{-3} and 2.490×10^{-6}, respectively. The population coefficient of variation is 1.81%.

It should be noted that the sample estimates of skewness and kurtosis for the YP-histogram are of the same magnitude as those for the Y- and P-histograms, thus supporting Aroian's work. Neither of these two latter sample statistics is significantly different from zero in any of the cases illustrated by Figs. 1–3.

These sampling results, in addition to Aroian's work, provide justification for using normal theory approximations for the YP-distribution.

APPLICATIONS

To test the calculations further, sufficient data from a tablet product were needed to obtain reliable estimates of sample mean potency and tolerance intervals. Fortunately, such data were available on a steroid tablet assayed by a method described in the

Lower Limit of Cell × 10^{-3}	No. of Observations	Histogram (Each X = 5 Observations)
<116.55048	4	X
116.55048	9	XX
117.47868	24	XXXXX
118.40691	72	XXXXXXXXXXXXXX
119.33513	154	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
120.26334	252	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
121.19156	338	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
122.11978	336	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
123.04799	333	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
123.97621	235	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
124.90443	140	XXXXXXXXXXXXXXXXXXXXXXXXXX
125.83264	64	XXXXXXXXXXXX
126.76086	27	XXXXX
127.68909	9	XX
>128.61729	3	X
	2000	

	Mean	Variance
Population	122.58	4.0466
Computed	122.55	4.1299
Skewness	0.02538	
Kurtosis	0.006658	

Figure 1—*Histogram and distribution statistics for variable* Y.

Analytical Methods section under *Mestranol Assay*. The assay has been found to be very precise and accurate. Routine control computer computations for S_Y and S_P had been made on 200 lots of this product. A summary of the information from these lots is presented in Table I. There were 103 lots with a target dose of 84 mcg./tablet and 97 lots with a target dose of 86 mcg./tablet. Nine tablets per lot were assayed and this information was used to calculate drug dosage. The results of this method are reported under column heading A. The results under B are from the calculations recommended in this paper; *i.e.*, there is information from 100 tablet weighings per lot which were ignored in the A calculations but were utilized in the B calculations. When this information was combined with assay information, the 95/99 tolerance limits (in percentages of sample means) were reduced considerably by the additional degrees of freedom gained from including the tablet weight data. The mean potencies found by the two methods were not significantly different from each other.

Table II summarizes data taken from routine computer printouts for production lots of several official and proprietary items. These printouts display results computed for the conventional assay procedure as well as for the composite procedure, described in this paper. In the conventional procedure, N_x tablets are weighed and the mean weight $\bar{Y}_{N_x}$ is obtained. These N_x tablets are assayed and the mean drug weight per tablet, $\bar{x}$, and the standard deviation, S_x, reported. From these are computed the 95/99 tolerance limits

$$\bar{x} \pm KS_x$$

with $F_x = N_x - 1$ degrees of freedom. It should be noted that these N_x assays are the same ones which give N_P values of P, the proportion of drug in the tablet substance, so that $N_x = N_P$. Table II thus compares the several statistics calculated from both the conventional procedure and the authors' composite procedure. It is noted that, as a rule, the composite procedure results in shortened tolerance intervals. In the isoniazid examples, this represents the difference between rejecting and passing a lot.

Table III gives some guidance to the average number of assays and tablet weights required in order to set 95/99 tolerance limits to ±15% of label claim if the target is label claim and if the process is running at label claim with the population coefficients of variation, λ_Y and λ_P, listed in the first two columns of the table. This table should be used only as a rough guideline. Equations 1 through 5 may be used to prepare similar tables for various confidence levels, portions of population distribution, and postulated tolerance limits.

ANALYTICAL METHODS

Mestranol Assay—A direct reference standard tablet method previously published (12) for the determination of a 17α-ethynylestradiol 3-methyl ether (mestranol) was selected for this study because of its good precision and accuracy as used in these laboratories. In the course of development and production, both domestic and foreign, more than 17,000 determinations have been made by this procedure. Nine test tablets plus three reference tablets may be assayed in 1 hr. of analyst's time. The average difference in the means of single tablet assays comparing this direct method with a manual extraction method on composite samples for a series of 260 assays was 0.56%. During an 18-month period the absorbance values per gram of the reference tablets were found to change no more than absorbance values from color reactions on freshly prepared standard solutions of mestranol. The average assay relative standard deviation for the 1800 determinations on the 200 lots

Lower Limit of Cell × 10^{-3}	No. of Observations	Histogram (Each X = 5 Observations)
<0.69487	2	X
0.69487	8	XX
0.69729	42	XXXXXXXX
0.69972	72	XXXXXXXXXXXXXX
0.70214	121	XXXXXXXXXXXXXXXXXXXXXXXX
0.70456	251	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
0.70698	335	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
0.70941	372	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
0.71183	333	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
0.71425	226	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
0.71668	145	XXXXXXXXXXXXXXXXXXXXXXXXXX
0.71910	60	XXXXXXXXXXX
0.72152	24	XXXXX
0.72395	4	X
>0.72637	5	X
	2000	

	Mean	Variance
Population	0.7106×10^{-3}	0.2760×10^{-10}
Computed	0.7104×10^{-3}	0.2730×10^{-10}
Skewness	−0.0263	
Kurtosis	−0.0969	

Figure 2—*Histogram and distribution statistics for variable* P.

Lower Limit of Cell × 10^{-3}	No. of Observations	Histogram (Each X = 5 Observations)
<82.44057	4	X
82.44057	10	XX
83.15900	36	XXXXXXXX
83.87743	69	XXXXXXXXXXXXX
84.59586	148	XXXXXXXXXXXXXXXXXXXXXXXXXXXX
85.31429	252	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
86.03271	316	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
86.75114	350	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
87.46957	320	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
88.18800	260	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
88.90643	145	XXXXXXXXXXXXXXXXXXXXXXXXXXX
89.62486	58	XXXXXXXXXXX
90.34329	25	XXXXX
91.06172	5	X
>91.78015	2	X
	2000	

	Mean	Variance
From Data	87.065×10^{-3}	2.4610×10^{-6}
From Calcd.	87.110×10^{-3}	2.4900×10^{-6}
Skewness	−0.09520	
Kurtosis	−0.05849	

Figure 3—*Histogram and distribution statistics for variable* YP.

Table I—Data from 200 Lots of a Steroid Tablet

Lots →	103		97	
Determinations →	927		873	
Target →	84 mcg./Tablet		86 mcg./Tablet	
	A[a]	B[b]	A[a]	B[b]
Mean potency mcg./tablet	85.1	85.2	86.2	86.3
Mean *SD*	1.5	1.6	1.4	1.5
Av. tolerance limits (95/99) (in percent of mean)	±7.5	±5.7	±7.5	±5.5

[a] Average results from estimating mean and content uniformity by weighing and assaying nine tablets. [b] Average estimates from the use of the procedure recommended in this paper.

of Table I was less than 0.7%. The direct method cannot be used with tablets whose excipients react with sulfuric acid to form colored components. For such tablets, a selective method such as that reported by Tsilifonis and Chafetz (13) may be used.

Isoniazid Assay—The isoniazid from a single tablet was dissolved in water and filtered. An aliquot of filtrate was diluted with 0.003 *N* HCl. The absorbance of the sample solution at 265 mμ was compared to that of a standard solution similarly prepared using 0.003 *N* HCl as the blank. The relative standard deviation of the method was found to be about 0.6%.

Diethylstilbestrol Assay—The diethylstilbestrol tablets were assayed by the USP procedure (5).

Atropine Sulfate—The atropine sulfate tablets were assayed by a modification (14) of an automated procedure (15) using a bromocresol purple dye complex. The tablets were dissolved in 10 ml. water and placed in the liquid sampler. Ethylene dichloride was used as the extraction solvent.

Methyltestosterone Assay—The methyltestosterone tablets were assayed by the NF procedure (6).

Phenobarbital Assay—A single tablet was disintegrated in water, made alkaline, and filtered. An aliquot of the filtered solution was diluted to a concentration of 8 mcg. of phenobarbital per ml. of 0.04% sodium hydroxide soution. The absorbance at 241 mμ of the sample solution was compared to that of a standard solution similarly prepared using 0.04% sodium hydroxide as the blank. The relative standard deviation of the method was found to be about 1.2%.

Table II—Comparison of Data from Computer Printouts on Production Lots

	Isoniazid USP (Lot A)	Isoniazid USP (Lot B)	Diethylstilbestrol USP	Atropine Sulfate USP
Label claim, mg./tablet	100	100	0.250	0.40
No. tablets weighed (N_Y)	199	199	201	198
No. determinations (N_x, N_P)	9	3	10	10
$\bar{Y}$ (N_x), mg.	320.2	312.9	70.28	35.5
$\bar{Y}$ (N_Y), mg.	311.8	315.1	69.87	36.7
Mean assay ($\bar{x}$), mg.	99.3	97.8	0.252	0.395
$F_{(x)}$	8	2	9	9
$\bar{Y}\bar{P}$ (calcd.), mg.	96.7	98.5	0.253	0.409
$F_{(YP)}$	22	200	27	163
Tol. Int. (x), %	81.1–117.5	42.1–153.0	90.7–110.6	83.3–114.1
Tol. Int. (YP), %	85.5–107.9	93.4–103.0	91.1–111.4	91.5–112.8
Percent of S^2_{YP} due to weight of tablets	40.6	99.1	43.3	85.2

	Methyltestosterone NF	Phenobarbital USP	Aspirin, Phenacetin, and Caffeine NF (Caffeine Assay)	Paramethasone Acetate NF XIII (Tentative)
Label claim, mg./tablet	10.0	30.0	32.0	1.0
No. tablets weighed (N_Y)	200	199	199	200
No. determinations (N_x, N_P)	10	4	9	10
$\bar{Y}$ (N_x), mg.	129.26	121.9	528.5	303.6
$\bar{Y}$ (N_Y), mg.	129.50	120.7	516.1	303.5
Mean assay ($\bar{x}$), mg.	10.05	29.8	33.1	1.015
$F_{(x)}$	9	3	8	9
$\bar{Y}\bar{P}$ (calcd.), mg.	10.07	29.5	32.3	1.014
$F_{(YP)}$	39	38	17	18
Tol. Int. (x), %	89.1–112.0	86.6–111.9	88.0–116.4	96.4–106.5
Tol. Int. (YP), %	91.8–109.7	93.9–102.6	91.3–108.2	95.8–107.0
Percent of S^2_{YP} due to weight of tablets	53.0	73.3	31.4	30.2

Table III—Minimum Estimated Number of Determinations Needed for 95% Confidence That 99% of the Tablets Will Be Within ±15% of Label Claim

λ_Y, %	λ_P, %	K	F	Number of Tablets Weighed 10	20	30	100	∞
				Number of Assays Needed				
1.0	1.0	10.61	2	2	2	2	2	2
1.0	2.5	5.57	6	6	6	6	6	6
1.0	3.5	4.12	12	12	12	12	12	12
2.0	1.0	6.71	4	2	2	2	2	2
2.0	2.5	4.69	8	5	5	5	5	4
2.0	3.5	3.75	17	12	12	12	11	11
3.0	1.0	4.74	8	2	2	2	2	2
3.0	2.5	3.80	16	9	5	5	4	4
3.0	3.5	3.25	30	26	15	14	12	11
3.5	3.5	3.03	67	—	143	41	22	18
4.0	1.0	3.64	20	—	3	2	2	2
4.0	2.5	3.18	40	—	—	13	5	5
4.0	3.0	3.00	75	—	—	—	15	11

Caffeine Assay—The caffeine from a single tablet of aspirin, phenacetin, and caffeine tablets (NF XII) was dissolved in chloroform and determined by an IR spectrophotometric procedure previously published (16). The relative standard deviation of the method was found to be about 1.1%.

Paramethasone Acetate—The paramethasone acetate tablets were dissolved in 50 ml. of 50% methanol and determined by the semiautomated procedure using only the steroid manifold in the previously published method for propoxyphene hydrochloride and paramethasone acetate (17).

CONCLUSIONS

Formulas have been proposed for combining tablet weight and assay data into estimates of mean potency and content uniformity. Based on Monte Carlo simulations and also 20,000 tablet weighings and 1800 assays on 200 lots of a steroid tablet, this approach was found to be reasonable. More specific information on tolerance intervals is obtained by the proposed method than by existing or suggested tests for content uniformity in the official compendia.

REFERENCES

(1) T. N. T. Olson and I. Lee, *J. Pharm. Sci.*, **55,** 1(1966).

(2) W. N. French, F. Matsui, D. Cook, and L. Levi, *ibid.*, **56,** 1622(1967).

(3) H. L. Breunig, "Annual Technical Conference Transactions," American Society for Quality Control, Milwaukee, Wis. (1968).

(4) C. Roberts, *Technometrics*, **11,** 161(1969).

(5) "United States Pharmacopeia," 17th rev., Mack Publishing Company, Easton, Pa., 1965.

(6) "National Formulary," 12th ed., Mack Publishing Company, Easton, Pa., 1965.

(7) A. Hald, "Statistical Theory with Engineering Applications," Wiley, New York, N. Y., 1955.

(8) W. J. Dixon and F. J. Massey, Jr., "Introduction to Statistical Analysis," McGraw-Hill, New York, N. Y., 1957.

(9) L. A. Goodman, *J. Acoust. Soc. Am.*, **55,** 708(1960).

(10) L. A. Aroian, *Annals Math. Statist.*, **18,** 265(1947).

(11) B. L. Welch, *J. Acoust. Soc. Am.*, **51,** 132(1956).

(12) J. P. Comer, P. Hartsaw, and C. E. Stevenson, *J. Pharm. Sci.*, **57,** 147(1968).

(13) P. C. Tsilifonis and L. Chafetz, *ibid.*, **56,** 625(1967).

(14) I. Comer, personal communication.

(15) N. R. Kuzel, "Automation in Analytical Chemistry Technicon Symposia 1966," vol. 1, Medaid, Inc., White Plains, N. Y., 1967.

(16) T. V. Parke, A. M. Ribley, E. E. Kennedy, and W. W. Hilty, *Anal. Chem.*, **23,** 953(1951).

(17) C. E. Stevenson and I. Comer, *J. Pharm. Sci.*, **57,** 1227(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from *Eli Lilly and Company, Indianapolis, IN 46206*

Accepted for publication September 10, 1969.

Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Complexes of Ergot Alkaloids and Derivatives II: Interaction of Dihydroergotoxine with Certain Xanthines

MICHAEL A. ZOGLIO and HAWKINS V. MAULDING

Abstract □ Intermolecular complexation between a mixture of the comparatively water-insoluble alkaloids comprising 9,10-dihydroergotoxine and several xanthines was investigated. Substantial elevations of aqueous solubility of dihydroergotoxine in 0.1 *N* HCl and in pH 6.65 phosphate buffer was observed in most of the cases examined. This incongruity in the normally expected solubility data may be attributed to a mutual influence between the ergot derivative and the xanthine under consideration. Dissolution studies indicated a generally enhanced first-order rate of solution in the presence of xanthine which seems to evidence some "driving force" pulling the drug into solution. Partitioning rates (aqueous to chloroform) are usually increased when xanthine is added to the 9,10-dihydroergotoxine at pH 6.65 and the reverse is true in 0.1 *N* HCl. Biological data in cats and humans are in good agreement with physicochemical work.

Keyphrases □ Ergot alkaloids, derivatives—complex formation □ Dihydroergotoxine-xanthines—interactions □ Xanthines effect—dihydroergotoxine dissolution □ Partitioning rate, dihydroergotoxine—xanthine effect □ Biological activity correlation—physicochemical data

Caffeine and other naturally occurring xanthines have previously been shown to exhibit exceptional ability for formation of stable complexes with a wide variety of substances (1–3).

Mixtures of ergot alkaloids themselves have been stated to possess a tendency toward facile intermolecular complexation (4).

The interaction of caffeine and ergotamine tartrate in aqueous solution was observed in this laboratory and is exemplified by: (*a*) an increased solubility of the alkaloid in the presence of caffeine; (*b*) an increased dissolution rate constant for the ergot alkaloid in the combination over pure ergotamine; and (*c*) increased or decreased partitioning rates (aqueous to chloroform) of the alkaloid in the presence of caffeine relative to ergotamine tartrate alone depending on the pH studied (5).

This attraction between ergotamine tartrate and caffeine appeared to be an extremely weak one. The isolation of the complex eluded the authors (5). These ill-defined forces may be on the order of those found in dipole–dipole, dipole–induced dipole, and induced dipole–induced dipole systems, but have not been delineated. The two component solubility curves do not lend themselves to stoichiometric analysis (5).

It was speculated at the time that these *in vitro* results might correlate to some extent with the stated clinical effectiveness (6, 7) of orally administered caffeine, ergotamine tablets,[1] and the caffeine present was functioning by holding the ergotamine in solution in the gut. Consequently, this solubilization of the complex in enteral media was thought probably to provide increased drug activity through ease of absorption (5). If the above hypothesis were correct, it was hoped that judicious selection of new potential complexing agents and their *in vitro* study and comparison might lead to more potent and efficacious substances. In the light of presently available animal and human data, this theory has been corroborated.

An investigation was carried out on the naturally occurring xanthines, caffeine, theophylline, and theobromine, as well as several of their closely related and commercially available analogs in an endeavor to discover a more suitable solubilizing agent for ergotamine tartrate and other medicinally active bases derived from ergot. The ergot alkaloid under consideration in this work was 9,10-dihydroergotoxine.[2] This substance is an approximately equimolar mixture of the methanesulfonate salt of three alkaloids: dihydroergocristine, dihydroergokryptine, and dihydroergocornine. These three compounds are difficult to separate and the salts thereof display only slight water solubility, although they are not as insoluble as ergotamine. The three are closely related structurally, the only deviation being in amino acid composition of the peptide side chain attached to the lysergic acid nucleus.

The simplest basic structural requirements for this specific type of complexing agent were thought to be: (*a*) a planar ring system or single ring; (*b*) absence of acid or basic functions (the comparative electrical neutrality and lack of acidic hydrogen); and (*c*) some degree of water solubility. This premise has shown itself to be generally correct. Pharmacologically speaking, an innocuous substance is demanded. Caffeine, theophylline, and theobromine present major drawbacks as is evidenced by their own pharmacology represented by their important clinical applications.

In most of the compounds surveyed in this project the basic xanthine prototype was maintained, and it was believed that specific structural modification of this moiety might lead to more appropriate compounds. Commercially available materials were examined with the exception of trimethyl-isocyanurate ester which was prepared (8). This is not to imply that many other molecules should not possess the ability of complexation with ergot alkaloids as many systems fit the stipulations presented in the previous paragraph.

BIOLOGICAL

The onset and duration of action upon enteral administration of dihydroergotoxine and other ergot alkaloids were recently reported to be enhanced by several of the xanthine derivatives mentioned in this study. The pharmacological parameter employed was α-adrenergic blocking activity of ergot alkaloids in cats. The data clearly show the increased activity of some ergot alkaloids when used together with suitable xanthine derivatives. This appears

[1] Cafergot, Sandoz Pharmaceuticals, Hanover, N. J.

[2] Hydergine, Sandoz Pharmaceuticals, Hanover, N. J.

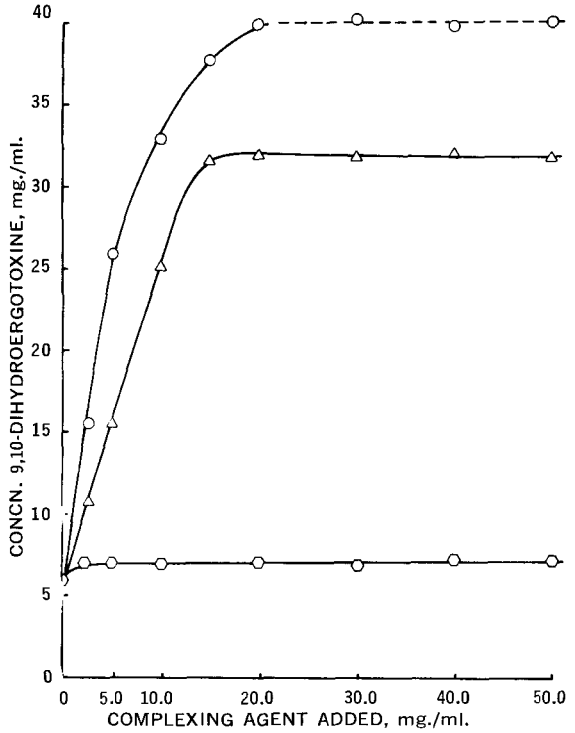


Figure 1—*Solubilizing action of xanthines on 9,10-dihydroergotoxine in 0.1* N *HCl (pH 1.25) at 30° for 24 hr. Key:* ○, *caffeine;* ○- - -, *complete solution;* △, *theophylline;* ○, *theobromine.*

to point toward the fact that this phenomenon is due to an improved absorption picture for these compounds in cats (9).

In humans the oral absorption of tritiated dihydroergocristine—one of the three alkaloidal components of dihydroergotoxine—was both faster and more complete for a 24-hr. period on administration with 7-β-hydroxypropyltheophylline. Upon the utilization in combination with the theophylline analog this was evidenced by appreciably higher and longer lasting blood levels when the xanthine

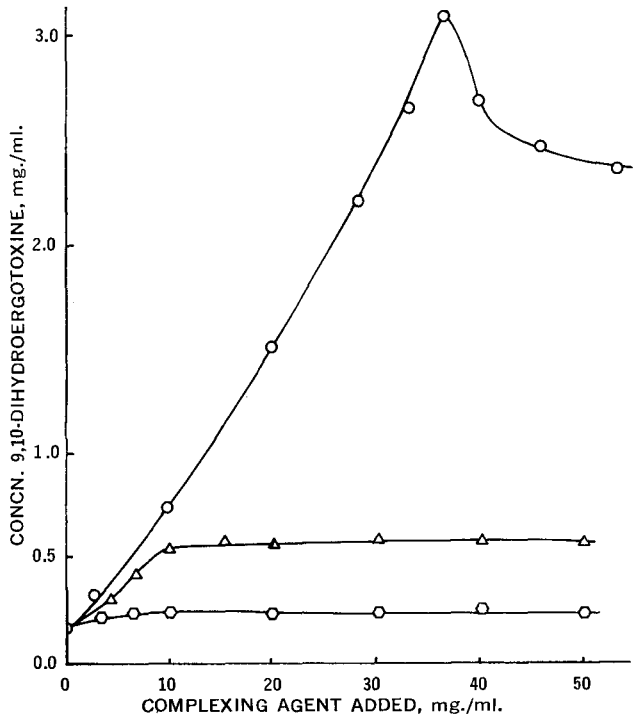


Figure 2—*Solubilizing action of xanthines on 9,10-dihydroergotoxine in phosphate buffer (pH 6.65.; ionic strength 0.2) at 30° for 24 hr. Key:* ○, *caffeine;* △, *theophylline;* ○, *theobromine.*

was present compared to when the drug alone was given. It was concomitantly noted that tritiated dihydroergocristine exhibited a significant increase in the cumulative urinary excretion of tritium when the alkaloid was administered in combination with 7-β-hydroxypropyltheophylline relative to dihydroergocristine itself (9).

EXPERIMENTAL

Materials—Dihydroergotoxine substance[2] is a mixture of equal parts by weight of dihydroergocristine methanesulfonate, dihydroergokryptine methanesulfonate, and dihydroergocornine methanesulfonate, which melted with decomposition at 196–206°. The various xanthines employed were obtained from the following sources: theophylline, m.p. 272–274° (Matheson, Coleman & Bell); theobromine (Mallinckrodt Chemical Works); caffeine anhydrous powder USP, m.p. 238° (Pfizer); 7-β-hydroxypropyltheophylline, m.p. 135–138° (Gane's Chemical Works, Inc.); 7-(2,3-dihydroxypropyl)-theophylline, m.p. 163–166° (Aldrich Chemical Co.). Melting points are uncorrected. Reagent grade chloroform (Mallinckrodt) was employed in the partitioning studies, and 0.1 *N* HCl and pH 6.65 phosphate buffer were prepared in the usual manner. All pH values were measured on a Metrhom pH meter and spectrophotometric data obtained from a Cary model 14 spectrophotometer.

Solubility Studies—Watertight screw-capped vials (18 ml.) containing exactly 10 ml. of solvent, dihydroergotoxine (100 mg. with pH 6.65 buffer and 400 mg. with 0.1 *N* HCl) and varying amounts of the xanthine, were clamped onto the edge of metal disks (15-cm. diameter) mounted on a motor-driven shaft and rotated vertically at 6 r.p.m. in a constant-temperature bath at 30 ± 0.1°. After exactly 24 hr., samples were withdrawn using pipet with filters attached and analyzed for dihydroergotoxine by the Van Urk method (10).

Dissolution Rates—Either a 25 or 60 r.p.m. stirrer motor fitted with a 2.54-cm. propeller blade placed 4 cm. from the bottom of an 800-ml. beaker containing 500 ml. 0.1 *N* HCl solution was used in determining these rates. The ergot alkaloid (50 mg.) or alkaloid in combination with xanthine (50 mg.:5 g.) was placed into the stirred solution from a height of about 1.5 cm. The temperature of

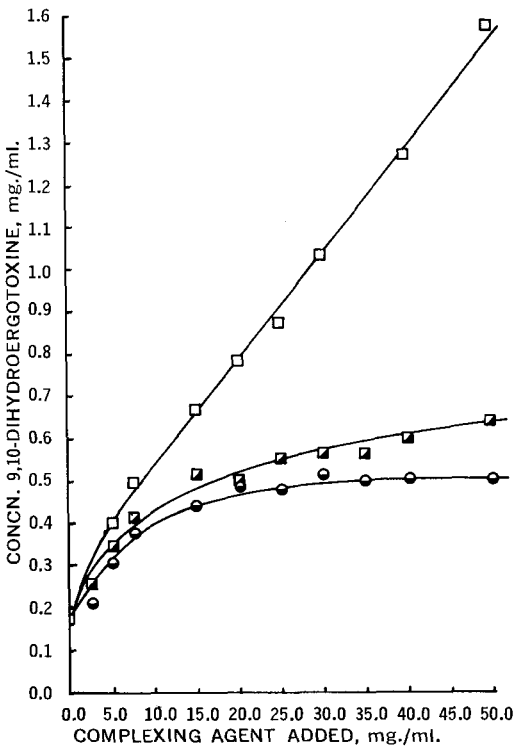


Figure 3—*Solubilizing action of compounds on 9,10-dihydroergotoxine (100 mg.) in phosphate buffer (10 ml.; pH 6.75; ionic strength 0.2) at 30° for 24 hr. Key:* ◒, *trimethyl-isocyanurate ester;* ◪, *7-β-hydroxypropyltheophylline;* □, *7-(2,3-dihydroxypropyl)-theophylline.*

the dissolution rate media was kept at 37 ± 0.1° by immersing the beaker in a constant-temperature bath. Samples were withdrawn for analysis for dihydroergotoxine content by the Van Urk method (10).

Partitioning Studies—Fifteen milliliters of an aqueous phase (0.1 *N* HCl or phosphate buffer, pH 6.65 ionic strength of 0.20) containing alkaloid or alkaloid and xanthine (1:100) made by dissolution of dihydroergotoxine, Van Urk analysis, and addition of xanthine was added carefully to 15 ml. chloroform in screw-capped vials. The vials were sealed and rotated at 6 r.p.m. in a 30 ± 0.1° constant-temperature bath using the same apparatus as described for solubility studies. Five-milliliter samples were taken at 1, 3, 5, 7, and 9-min. intervals from the aqueous phase and analyzed for alkaloidal content by the Van Urk method (10).

RESULTS AND DISCUSSION

The solubility of 9,10-dihydroergotoxine methanesulfonate varies with hydrogen ion concentration as can be seen from Figs. 1 and 2. Changes in apparent solubility of the alkaloid when caffeine is added are also a function of pH up to the point of complete solution in 0.1 *N* HCl and the downward trend evidenced in Fig. 2 (pH 6.65, phosphate buffer) after addition of 35 mg./ml. caffeine. This latter phenomenon is possibly the result of saturation of the solution with the solubilized species. It is evident from Fig. 2 that solubility of the ergot derivative is raised twentyfold through interaction with caffeine at near neutral pH and 30°. The ratio of caffeine to alkaloid which can accomplish this increase is about 10:1 on a weight basis and the molar ratio is 35:1 which brings about maximum solubilization at pH 6.65. Theophylline shows a great deal less complexation in the phosphate buffer. The plateau in Fig. 2 is conceivably caused by solubility limitations of theophylline; however, the picture in 0.1 *N* HCl generally parallels caffeine. Theobromine does not appear to form a complex as may be noted in Figs. 1 and 2. This is likely a result of the poor solubility of theobromine, about 1/40 that of caffeine.

Figure 3 illustrates the interaction of three synthetic compounds and caffeine with 9,10-dihydroergotoxine methanesulfonate in phosphate buffer. Two theophylline derivatives, 7-(2,3-dihydroxypropyl)-theophylline and 7-β-hydroxypropyltheophylline, were employed, with each exhibiting comparable solubilizing properties to caffeine. Both of these compounds have good water solubilities relative to the other xanthines. The dihydroxy derivative elevates the 9,10-dihydroergotoxine methanesulfonate solubility up to a maximum of 1.5 mg./ml. under the conditions employed, while the 7-β-hydroxy derivative gives somewhat less solubilization. Trimethyl-isocyanurate ester (1,3,5-trimethyl-2,4,6-trioxo-1,3,5-triazine) (8) showed the least interaction. This may be because of its low water solubility in addition to absence of the second ring as is found in all xanthines followed by the consequent lowering of its affinity for the ergot alkaloid.

From Fig. 2 it seems the presence of less xanthine molecules is needed to solubilize each molecule of the ergot compound than was the situation with ergotamine tartrate (5).

The question as to the species comprising the complex as well as its stoichiometry is in doubt, but protonation of one or both species is likely in 0.1 *N* HCl, while at pH 6.65 almost all the caffeine and a good percentage of the dihydroergotoxine (pKa around 6.5) is present as the neutral molecule.

The exact nature of the complex between these ergot alkaloids possessing the unusual cyclic tripeptide attached to the lysergic acid portion has not been elucidated, but work is continuing along these lines.

Complete solubility studies were not carried out in 0.1 *N* HCl as most of the alkaloid and some of the xanthine would exist as the hydrochloride salt under these conditions, and even though the amount in solution is considerable, absorption of these ionic species is doubtful (11).

The dissolution rate of poorly water-soluble drugs is considered by some to be the rate-limiting step in absorption (12–14). Dissolution rates were run on 9,10-dihydroergotoxine methanesulfonate with and without xanthine present to determine if there were any differences in rate constants. These tests were done in a relatively large volume of 0.1 *N* HCl (500 ml.) to effect "sink" conditions and allow approximation of a first-order process. A 100:1 ratio of xanthine to alkaloid was employed to correspond to the quantities found in the commercial product (6). Figure 4 gives an example of a

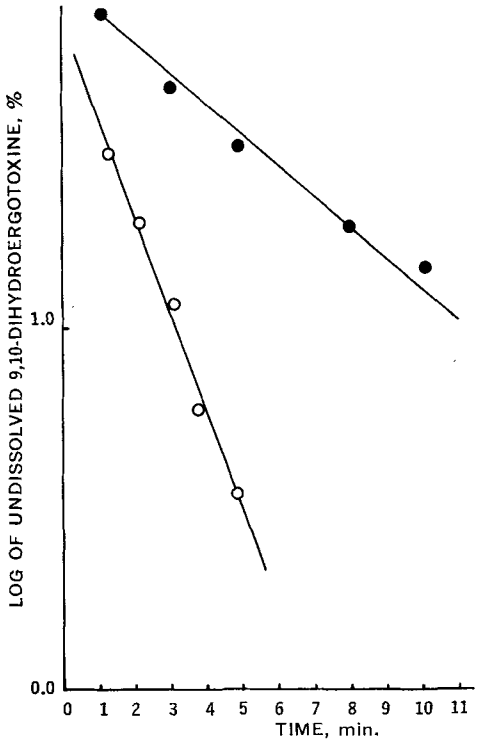


Figure 4—*Effect of caffeine on the dissolution rate of 9,10-dihydroergotoxine in 0.1* N *HCl (500 ml.) at 37° and 60 r.p.m. Key:* ●, *dissolution of 9,10-dihydroergotoxine, 50 mg.;* ○, *dissolution of 9,10-dihydroergotoxine, 50 mg., and caffeine, 5.0 g.*

system of this kind and substantial variation may be noted in the two rate constants (a factor of three). The sizable variances in the relative rate constants are again evidenced in Fig. 5 with the ex-

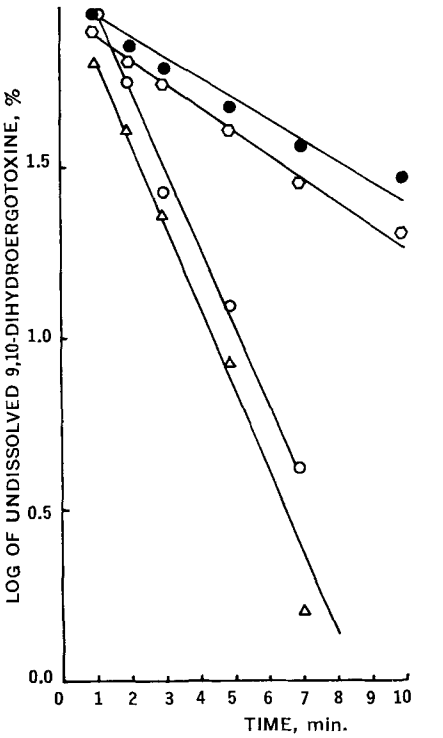


Figure 5—*Effect of naturally occurring xanthines on the dissolution rate of 9,10-dihydroergotoxine in 0.1* N *HCl (500 ml.) at 37°, stirring speed 25 r.p.m. Key:* ●, *50 mg. dihydroergotoxine;* ○, *50 mg. dihydroergotoxine and caffeine, 5.0 g.;* △, *50 mg. dihydroergotoxine and theophylline, 5.0 g.;* ○, *50 mg. dihydroergotoxine and theobromine, 5.0 g.*

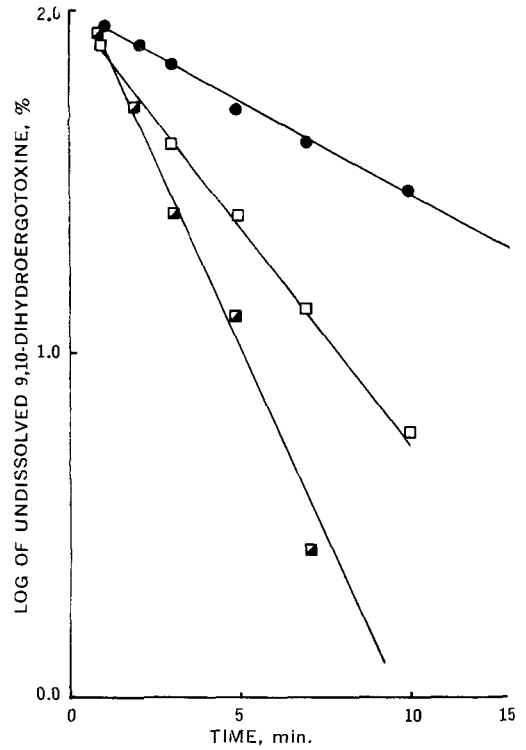


Figure 6—*Effect of synthetic xanthines on the dissolution rate of 9,10-dihydroergotoxine in 0.1 N HCl (500 ml.) at 37°, stirring speed 25 r.p.m. Key: ●, 50 mg. dihydroergotoxine; □, 50 mg. dihydroergotoxine and 5.0 g. 7-(2,3-dihydroxypropyl)-theophylline; ◪, 50 mg. dihydroergotoxine and 5.0 g. 7-β-hydroxypropyltheophylline.*

ception of theobromine where the deviation from the 9,10-dihydroergotoxine standard is negligible. It is obvious that caffeine and theophylline have a large influence on the dissolution rate of the ergot alkaloid. Figure 6 shows the same phenomena of increase in dissolution rate on addition of the two theophylline derivatives to the alkaloid, with the extremely water-soluble 7-β-hydroxypropyltheophylline showing the largest rate increase as expected.

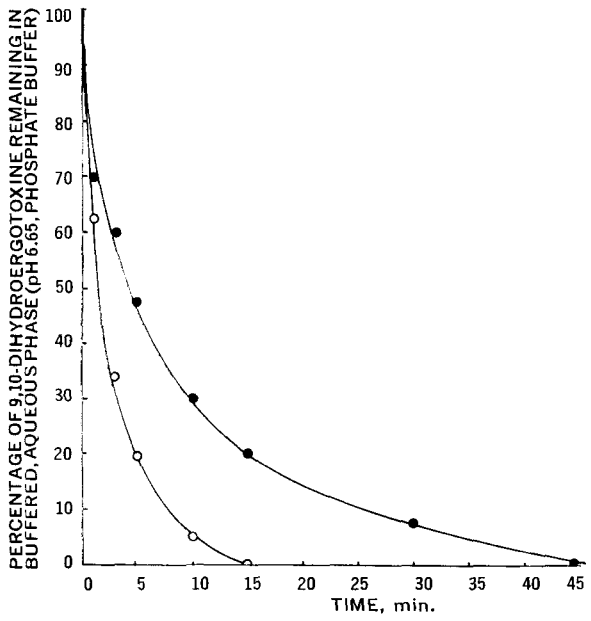


Figure 7—*Plot of amount of 9,10-dihydroergotoxine remaining on partitioning of 10 ml. pH 6.65 buffer with 10 ml. chloroform in a water bath at 6 r.p.m. and 30°. Key: ●, 0.15 mg./ml. dihydroergotoxine; ○, 0.15 mg./ml. dihydroergotoxine plus 15 mg./ml. caffeine.*

Figures 4–6 were derived from a typical experimental run with the only variation being a stirrer speed of 60 r.p.m. in Fig. 4 compared to 25 r.p.m. for the latter two.

Overemphasis should rarely be placed on dissolution rate data alone, but it is plain in this instance that there are significant changes in the dissolution rates on addition of xanthines, excepting theobromine. This information complements the solubility data.

During the course of investigation of the equilibrium distribution between an aqueous phase and chloroform of 9,10-dihydroergotoxine methanesulfonate with and without caffeine, it was noted that the time required for attaining this equilibrium was 15 min. in the former instance and 45 min. in the latter (Fig. 7). These data suggested perhaps the way to relate *in vitro* results to drug absorption was to study the rate of migration from one phase to another rather than look at the equilibrium distribution. The rate at which a drug distributes itself between phases does not necessarily parallel its equilibrium distribution coefficient.

A representative partitioning experiment involving the alkaloid and caffeine is illustrated in Fig. 8. The log of the 9,10-dihydroxyergotoxine methanesulfonate percentage remaining in the aqueous layer (phosphate buffer, pH 6.65) is plotted *versus* time. The graph shows that the rate for the first-order transfer of drug from aqueous phase to chloroform is substantially larger in the presence of caffeine than in its absence. The rate of caffeine partitioning from aqueous to organic phase is apparently the same with and without alkaloid. A 100:1 ratio of caffeine to 9,10-dihydroergotoxine was utilized.

In all instances of partitioning rates determined at pH 1.25 (0.1 *N* HCl), a retardation of transfer of 9,10-dihydroergotoxine from aqueous to organic phase was noted upon inclusion of xanthine (Fig. 9). This phenomenon is unexplained but may be due to some combination of protonation of both species coupled with interaction in acidic media. As drug absorption at this pH is rather unlikely, it was not examined further.

Partitioning rates were run in duplicate—ergotoxine *versus* ergotoxine plus xanthine—and each repeated several times. The experimental conditions approach a first-order process (see *Experimental* section) and give good results for this system. The results paralleled those found with caffeine–ergotamine with the exception of the combination 7-(2,3-dihydroxypropyl)-theophylline:dihydroergotoxine at pH 6.65. This combination exhibited no difference in partitioning rate when compared to dihydroergotoxine. This particular xanthine is insoluble in chloroform which in some way

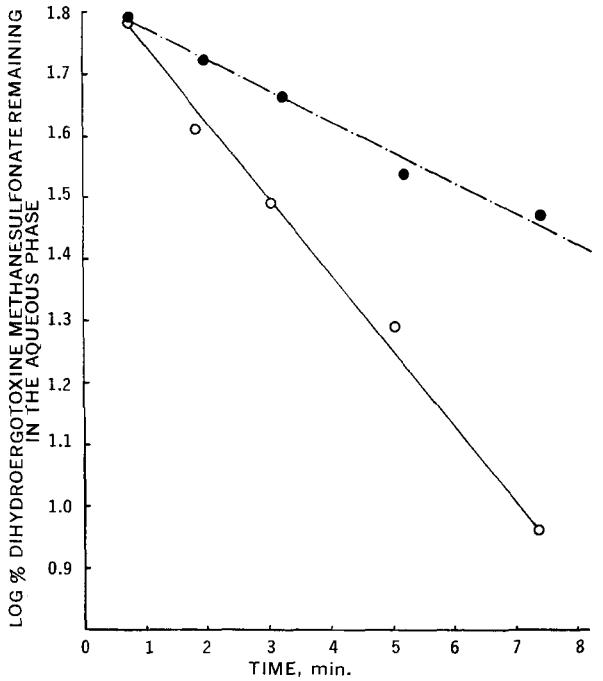


Figure 8—*Effect of caffeine on the partitioning rate of dihydroergotoxine methanesulfonate from an aqueous (phosphate buffer, pH 6.65) to an organic phase (chloroform). Key: ●, dihydroergotoxine, 0.1 mg./ml.; ○, dihydroergotoxine, 0.1 mg./ml.; and caffeine, 10.0 mg./ml.*

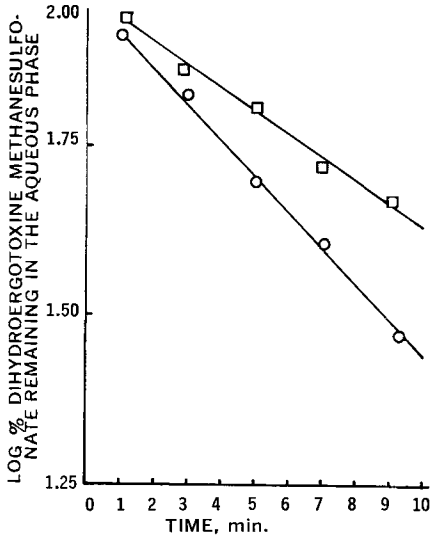


Figure 9—*Effect of 7-(2,3-dihydroxypropyl)-theophylline on the partitioning rate of dihydroergotoxine methanesulfonate from an aqueous phase (0.1 N HCl) to an organic phase (chloroform). Key: ○, dihydroergotoxine methanesulfonate, 0.1 mg./ml.; □, dihydroergotoxine methanesulfonate, 0.1 mg./ml.; and 7-(2,3-dihydroxypropyl)-theophylline, 10.0 mg./ml.*

relates to its inability to affect partitioning at this pH. Solubility problems precluded use of theobromine at this almost neutral pH. The 7-β-hydroxypropyltheophylline was tested in humans with dihydroergocristine, and the same relative results were obtained and are listed in the *Biological* section.

It has been reported by the authors (5) that when a complexing agent is present in partitioning studies, the alkaloid is transferred directly to the organic phase, while in cases of the alkaloid alone a precipitation step precedes this transfer. The increased partitioning at enteral pH (6.65) was probably not due to transfer of complex between phases, but rather to partial prevention of a precipitation step which occurred when the alkaloid itself was present in the aqueous phase.

CONCLUSIONS

The utilization of simple physicochemical studies as a rapid ranking or screening device of known or suspected biologically active agents preceding the tedious and expensive animal and human work is an ultimate goal of these investigations.

In general, the *in vitro* partitioning rate data obtained thus far indicate absorption potentiation at neutral pH should be observed with ergot alkaloids having the polypeptide moiety intact upon addition of certain xanthines such as caffeine. This phenomenon has been examined both for 9,10-dihydroergotoxine methanesulfonate and ergotamine tartrate (5). The rate effect, however, is reversed at gastric pH. Dissolution rate constants are usually increased in 0.1 *N* HCl solutions upon inclusion of xanthine while solubility of the alkaloid at enteral and gastric pH is elevated by most xanthines discussed.

An exceptional degree of success has been achieved in correlation of *in vitro* data with the increased clinical effectiveness of ergot alkaloids in the presence of complexing agents (6, 7). Recent clinical and animal evidence has substantiated the previously obtained *in vitro* results stated in this communication (9).

This ergot alkaloid:xanthine interaction is somewhat unusual as it is an exceptional example where complexation in the gastrointestinal tract leads to enhanced rather than decreased absorption of a medicinal agent.

REFERENCES

(1) W. O. Emery and C. D. Wright, *J. Am. Chem. Soc.*, **43**, 2323(1921).

(2) L. Lachman, D. Guttman, and T. Higuchi, *J. Am. Pharm Assoc., Sci. Ed.*, **46**, 36(1957).

(3) S. Bolton, D. Guttman, and T. Higuchi, *ibid.*, **46**, 38(1957).

(4) A. Burger, "Medicinal Chemistry," 2nd ed., Interscience, New York, N. Y., 1960, p. 583.

(5) M. A. Zoglio, H. V. Maulding, and J. J. Windheuser, *J. Pharm. Sci.*, **58**, 222(1969).

(6) N. A. Bercel, *Calif. Med.*, **72**, 234(1950).

(7) A. P. Friedman, *New Engl. J. Med.*, **250**, 600(1954).

(8) J. J. Windheuser, M.S. thesis, University of Wisconsin, 1959.

(9) B. Berde, A. Cerletti, M. J. Dengler, and M. A. Zoglio, presented at Third Migraine Trust Symposium, London, 1969, vol. III, Background to Migraine, in press.

(10) N. L. Alport and T. T. Cocking, *Quart. J. Pharm. Pharmacol.*, **5**, 341(1932).

(11) B. Brodie and C. Hogben, *J. Pharmacol.*, **9**, 345(1959).

(12) L. J. Edwards, *Trans. Faraday Soc.*, **47**, 1191(1951).

(13) E. Nelson, *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 96(1959).

(14) E. Nelson and I. Schaldemose, *ibid.*, **48**, 489(1959).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 31, 1969, from *Pharmacy Research & Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication September 12, 1969.

The technical assistance of David Murdock, Jane Pearson, Mathilda Wagner, and William Vincek is gratefully acknowledged.

Micellar Solubilization of Barbiturates I: Solubilities of Certain Barbiturates in Polysorbates of Varying Hydrophobic Chain Length

A. A. ISMAIL*, M. WAFIK GOUDA†, and M. M. MOTAWI*

Abstract □ The effect of the hydrophobic chain length of the nonionic surfactants, polysorbates, on the degree of solubilization of a series of 5,5-disubstituted barbituric acid derivatives was studied. The solubilities were found to increase as the hydrophobic chain length increases. A pseudo two-phase model, according to which the drug molecule is partitioned between an aqueous phase and a micellar phase, was selected to determine the effect of the chemical structure of the solubilizate on the degree of solubilization. The number of carbon atoms of the substituents on the 5-position, as well as their inductive effects, was found to determine the extent of solubilization.

Keyphrases □ Barbiturates—solubilization □ Micellar solubilization—5,5-disubstituted barbituric acid derivatives □ Polysorbates, barbiturate solubilization—hydrophobic chain length effect □ UV spectrophotometry—analysis

Molecules of materials classed as surface-active agents possess polar and nonpolar characteristics. They are able to remain in solution at higher concentrations by orienting themselves in aggregates known as micelles (1). Surface-active agents, at a concentration above the CMC, are widely used as a means of producing aqueous solutions of insoluble or poorly soluble drugs (2). This phenomenon is known as micellar solubilization. Nonionic agents are by far the most popular in pharmaceutical formulations. This is due mainly to their low toxicity combined with their good solvent action. Although a wide range of nonionic surfactants are available, those most frequently employed are the polyoxyethylene sorbitan fatty acid esters and the polyoxyethylene monoalkyl ethers. Numerous studies on the solubilities of various drugs in these nonionic surfactants were reported (2).

The barbiturates are among the most frequently employed hypnotic and sedative drugs. They are used both as the free acids and as salts (usually sodium or calcium). Because of the instabilities and incompatibilities of the salts, the free acids are recommended to be used with other soluble drugs. These acids are slightly soluble in cold water. This limited solubility represents a problem in formulating aqueous preparations and elixirs (3). Polysorbate 80 was used to prepare aqueous solutions of phenobarbital (4). Gusiakov *et al.* found that the solubility of barbital increases in 2% solutions of polysorbates 20, 40, 60, and 80[1] (5). Küttel (6, 7) reported the solubilities of phenobarbital, barbital, and butabarbital in solutions of polysorbates 20, 60, and 80 at room temperature (16–24°). None of these reports have presented quantitative information concerning the effect of the chemical structure of the solubilizer or solubilizate on the degree of solubilization. This paper deals with the study of the solubilization of certain 5,5-substituted barbituric acids, which differ from one another only in carbon content of their substituents, in aqueous solutions of polysorbates of varying hydrophobic chain lengths. The data presented might help in gaining some insight into the mechanism of solubilization of such semipolar pharmaceuticals.

EXPERIMENTAL

Materials—The following surfactants were used as received: polysorbate 20, polyoxyethylene 20 sorbitan monolaurate; polysorbate 40, polyoxyethylene 20 sorbitan monopalmitate; polysorbate 60, polyoxyethylene 20 sorbitan monostearate; polysorbate 80, polyoxyethylene 20 sorbitan monooleate.

The barbiturates used and their melting points[2] were: phenobarbital, 5-ethyl-5-phenylbarbituric acid, m.p. 173–174°; barbital, 5,5-diethylbarbituric acid, m.p. 185–187°; amobarbital, 5-ethyl-5-isoamylbarbituric acid, m.p. 153–155°; diallylbarbituric, 5,5-diallylbarbituric acid, m.p. 169–170°; cyclobarbital, 5-(1-cyclohexenyl)-5-ethylbarbituric acid, m.p. 170–171°; butethal, 5-ethyl-5-butylbarbituric acid, m.p. 120–122°; secobarbital, 5-allyl-5-(1-methylbutyl)barbituric acid, m.p. 89–91°.

Assay Procedure—All barbiturates were assayed by the differential UV spectrophotometric procedure of Walker *et al.* (8) using a Unicam SP 500 spectrophotometer. The presence of surfactants did not interfere with this method.

Solubility Determinations—Excess quantities of the solid barbiturate were placed in 50-ml. rubber-stoppered bottles together with varying concentrations of 25-ml. solutions of the polysorbates in 0.003 *N* sulfuric acid. The weakly acidic solution was employed as the solvent to suppress any dissociation of the barbituric acid derivatives. The bottles were rotated in a constant temperature water bath at 30 ± 0.2° for 24 hr. This time was found to be sufficient for equilibrium to be attained. After equilibrium, aliquot portions of the supernatant liquid were removed and assayed for their barbiturate contents.

RESULTS AND DISCUSSION

Solubilization of the Barbiturates—The solubility curves for the barbituric acid derivatives are shown in Figs. 1–7. The curves show the effect of concentrations of polysorbates, well beyond the reported CMC (9–11), on the apparent solubilities of the drugs. All barbiturates studied showed increased solubilities in the presence of the polysorbates. This increase in solubility is looked upon here as due to true or micellar solubilization. Other workers (12–14) have found increased solubilities of similar semipolar drugs in solutions of various nonionic surfactants due to the same phenomenon. The apparent solubility increased linearly with the concentration of the surfactant as would be expected of micellar solubilization of such polar solubilizates (11, 12, 15, 16, 18).

Effect of Hydrophobic Chain of Solubilizer—In comparing the solubilizing power of different homologs of solubilizers, the ratio of moles of solubilizate:moles of micelles is the sensible criterion for comparison (17). This comparison involves the assumption that all the surfactant in excess of the CMC is in the form of micelles and

[1] Marketed as Tween 20, 40, 60, and 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

[2] Uncorrected melting points determined with a Thomas-Hoover Unimelt.

that the number of units in the micelle remains constant. Although this is an approximation, it still has practical merit (17). Polysorbates 20, 40, 60, and 80 have the same hydrophilic portion in their molecule but differ in the length of the carbon atom chain of their lipophilic portion. The solubilizing capacity of the different polysorbates could be compared by calculating the slopes of the solubilization isotherms beyond the CMC. These slopes were calculated using the method of least squares. Table I shows the solubilizing capacities expressed as milligram drug per gram polysorbate and as mole drug per mole micellar solubilizer. The weight of each barbiturate solubilized per gram of polysorbate has its practical usefulness. The molar concentrations of polysorbates were calculated from the following molecular weights: polysorbate 20, 1226; polysorbate 40, 1282; polysorbate 60, 1310; polysorbate 80, 1308. Since polysorbates are heterogeneous, the significance of the molar values has its limitation.

On examining the slopes of the isotherms expressed on molar basis, it is found that all barbiturates showed a slight but gradual increase in solubility as the hydrophobic chain length of the solubilizer is increased. This increase in solubility is considered to be due to increase in the volume of the hydrocarbon in the micelle interior. In an idealized picture of spherical micelles the alkyl portions may be visualized as being directed inward. An increase in their length would result in micelles of larger size, and larger micelles will accommodate more solubilizate (16). Similar results were obtained by other workers when examining the solubilities of other semipolar pharmaceuticals (11, 14).

Effect of Chemical Structure of Solubilizate—In order to compare the effect of the chemical structure of the solubilizate on the degree of solubilization, a pseudo two-phase model was selected. According to this model, the solubilizate molecule is partitioned between an aqueous phase and a micellar phase. McBain and Hutchinson (17) suggested that a truer view is seen if solubilization is regarded as a partition between the micelle and water. The formation of hydrocarbon regions in micelles is a good jusification for treating the micelles as a separate phase. The partition coefficient K, associated with this process, was determined according to the following equation:

$$K = \frac{[D_M]}{[D_{NM}]}$$

where $[D_M]$ and $[D_{NM}]$ are the concentrations of drug, expressed as moles per moles, in the micellar and nonmicellar phases, respectively. The slopes of the solubilization isotherms expressed on molar basis will give the values for $[D_M]$. The number of moles of drug solubilized per mole of acidified water at 30° will give the values for $[D_{NM}]$. Table II lists the values of K for the various barbiturates in the different polysorbates studied. In a series of compounds such as the 5,5-substituted barbituric acids, which differ from one another only in carbon content of their substituents, the distribution coefficient would be expected to change with the alteration of the lipophilic character of the substituents as well as the influence these groups exert on the rest of the molecule. This influence will be mainly in the form of polar or inductive effects (19). The partition coefficients of the barbiturates between the micellar pseudophase and the aqueous phase in decreasing order are: secobarbital > amobarbital > phenobarbital > cyclobarbital > butethal > diallylbarbituric acid > barbital. As seen, the value of K increased with increasing the

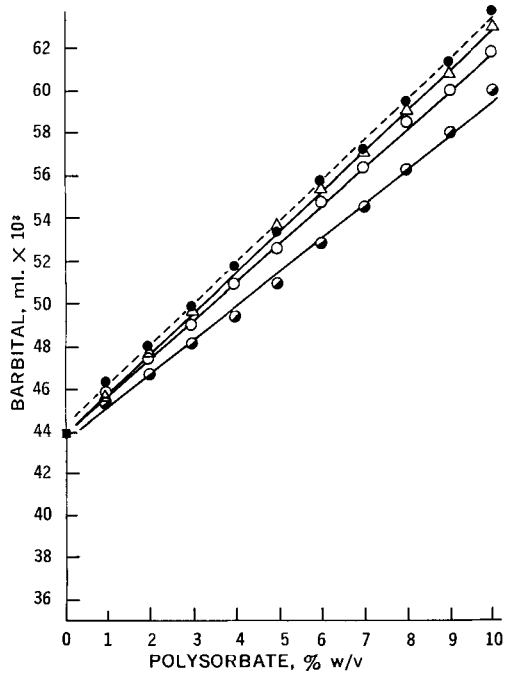


Figure 1—*Solubility of barbital in polysorbate solutions at 30°. Key: ■, solubility in water; ◒—, polysorbate 20; ○—, polysorbate 40; ●- - -, polysorbate 60; △—, polysorbate 80.*

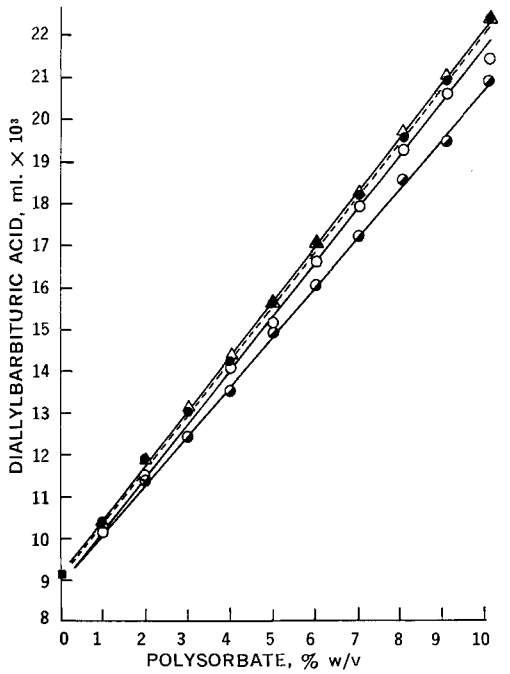


Figure 2—*Solubility of diallylbarbituric acid in polysorbate solutions at 30°. Key: ■, solubility in water; ◒—, polysorbate 20; ○—polysorbate 40; ●- - -; polysorbate 60; △—, polysorbate 80.*

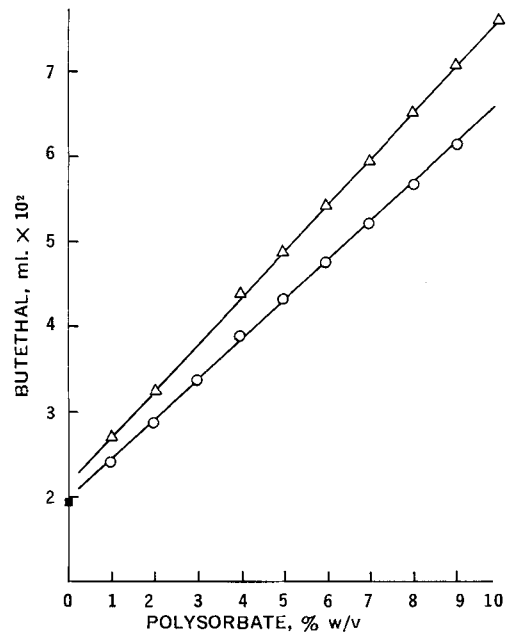


Figure 3—*Solubility of butethal in polysorbate solutions at 30°. Key: ■, solubility in water; ○—, polysorbate 20; △—, polysorbate 80.*

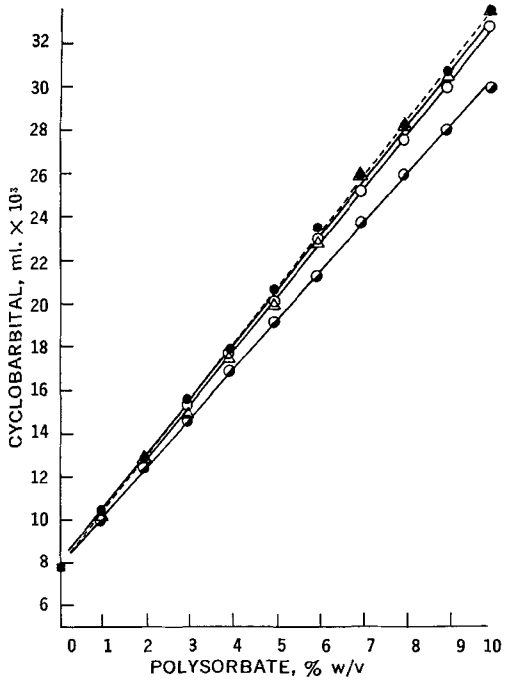


Figure 4—*Solubility of cyclobarbital in polysorbate solutons at 30°. Key:* ■, *solubiliy in water;* ◒—, *polysorbate 20;* ○—, *polysorbate 40;* ●- - -, *polysorbate 60;* △—, *polysorbate 80.*

number of carbon atoms in the substituents on the 5-position. These results compare favorably with the distribution coefficients of the same compounds between 1-octanol and water as determined by Hansch and Anderson (20). In their report the values of the distribution coefficients of the barbiturates between 1-octanol and water were in the following order: secobarbital > amobarbital > butethal > phenobarbital > cyclobarbital > diallylbarbituric acid > barbital. In comparing their results to the degree of solubilization of the barbiturates, as expressed by K, the authors found that phenobarbital and cyclobarbital show a higher degree of solubilization than what would be expected from their distribution between 1-octanol and water. As a result, phenobarbital and cyclobarbital showed higher K values than butethal which is reported to have a higher distribution coefficient. The increase in the extent of solubilization of these two barbiturates is attributed to the polar effect the phenyl and cyclohexenyl group exert on the hydrophilic portion of the barbiturate molecule. This polar effect will act in such a way as to increase the interaction between the hydrophilic portion of the barbiturate molecule and the hydrophilic portion of the polysorbate molecule. Such interaction may enhance the solubilization process. Further evidence for the effect of the phenyl group in enhancing the extent of interaction of the hydrophilic portion of the barbiturate molecule was obtained when the authors carried out an investigation of the solubilities of the same barbiturates in solutions of polyoxyethylene stearates of varying hydrophilic portion in their molecule. Phenobarbital was the

Table I—Solubilizing Capacity of Polysorbates for the Barbiturates at 30°

Drug	Surfactant	Solubility: mg. Drug/g. Surfactant	Solubility: mole Drug/mole Surfactant × 10²
Barbital	Polysorbate 20	30.0	19.9
	Polysorbate 40	33.0	23.0
	Polysorbate 60	35.3	25.1
	Polysorbate 80	35.0	24.6
Diallylbarbituric acid	Polysorbate 20	24.0	14.4
	Polysorbate 40	27.0	16.4
	Polysorbate 60	28.0	17.3
	Polysorbate 80	28.0	17.4
Butethal	Polysorbate 20	100.0	57.5
	Polysorbate 40[a]	—	—
	Polysorbate 60[a]	—	—
	Polysorbate 80	115.0	71.1
Cyclobarbital	Polysorbate 20	52.4	27.2
	Polysorbate 40	58.0	31.6
	Polysorbate 60	61.0	33.8
	Polysorbate 80	61.0	34.0
Phenobarbital	Polysorbate 20	55.1	29.1
	Polysorbate 40	61.0	33.7
	Polysorbate 60	63.0	35.5
	Polysorbate 80	66.0	37.2
Amobarbital	Polysorbate 20	32.0	17.2
	Polysorbate 40	38.0	21.7
	Polysorbate 60[a]	—	—
	Polysorbate 80	40.0	22.9
Secobarbital	Polysorbate 20	111.0	57.0
	Polysorbate 40[a]	—	—
	Polysorbate 60[a]	—	—
	Polysorbate 80	144.0	78.8

[a] Limited supply of the drug necessitated the use of a lesser number of surfactants.

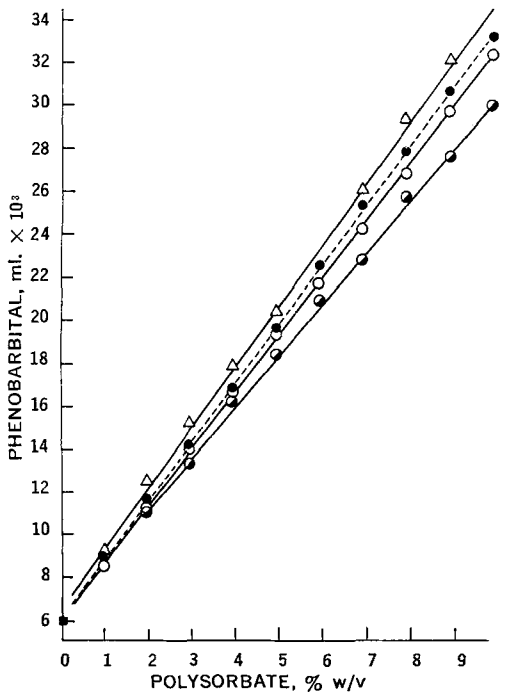


Figure 5—*Solubility of phenobarbital in polysorbate solutions at 30°. Key:* ■, *solubility in water;* ◒—, *polysorbate 20;* ○—, *polysorbate 40;* ●- - -, *polysorbate 60;* △—, *polysorbate 80.*

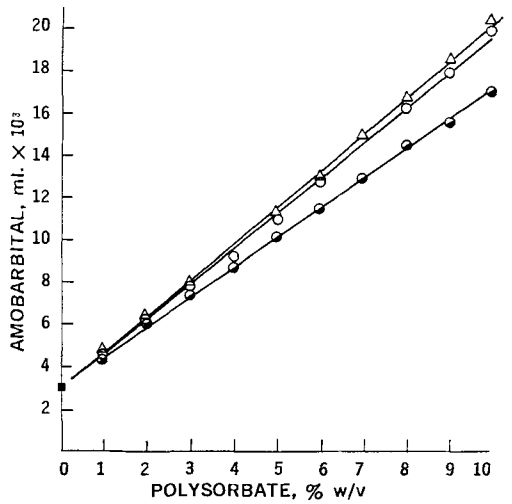


Figure 6—*Solubility of amobarbital in polysorbate solutions at 30°. Key:* ■, *solubility in water;* ◒—, *polysorbate 20;* ○—, *polysorbate 40;* △—, *polysorbate 80.*

Table II—Partition Coefficients for 5,5-Substituted Barbituric Acids Between the Micellar and Nonmicellar Phase at 30°

Drug	R_1	R_2	Surfactant	Partition Coefficient, $K \times 10^{-2}$
Barbital	C_2H_5	C_2H_5	Polysorbate 20	2.56
			Polysorbate 40	2.91
			Polysorbate 60	3.17
			Polysorbate 80	3.11
Diallylbarbituric	CH_2=CH—CH_2	CH_2=CH—CH_2	Polysorbate 20	8.73
			Polysorbate 40	9.94
			Polysorbate 60	10.48
			Polysorbate 80	10.55
Butethal	C_2H_5	n-C_4H_9	Polysorbate 20	16.43
			Polysorbate 40[a]	—
			Polysorbate 60[a]	—
			Polysorbate 80	20.31
Cyclobarbital	C_2H_5	1-cyclohexenyl	Polysorbate 20	19.29
			Polysorbate 40	22.41
			Polysorbate 60	23.97
			Polysorbate 80	24.11
Phenobarbital	C_2H_5	C_6H_5	Polysorbate 20	27.20
			Polysorbate 40	31.50
			Polysorbate 60	33.18
			Polysorbate 80	34.77
Amobarbital	C_2H_5	iso-C_5H_{11}	Polysorbate 20	31.79
			Polysorbate 40	40.11
			Polysorbate 60[a]	—
			Polysorbate 80	42.33
Secobarbital	CH_2=CH—CH_2	$C_3H_7(CH_3)_3OH$	Polysorbate 20	46.42
			Polysorbate 40[a]	—
			Polysorbate 60[a]	—
			Polysorbate 80	64.07

[a] Limited supply of the drug necessitated the use of a lesser number of surfactants.

only barbiturate to form an insoluble precipitate complex indicative of a greater extent of interaction. The results of this investigation will be the subject of a future communication.

Solubilization Type—Micellar solubilization was broadly classified into three types (16): first, adsorption on the surface of the micelle; second, incorporation in the hydrocarbon center of the micelle, a form of solution; and third, incorporation by penetration into the palisade layer of the micelle with the solubilizate oriented in approximately the same manner as is the surfactant molecule in the micelle. Mulley (1) suggests that solubilization even of polar materials in nonionic surfactants is probably due mainly to a solution process within the micelles rather than due to specific factors such as complex formation or adsorption on the surface of the micelles. He states that other factors obviously play a part but a solution process still predominates. The results obtained in this work support the assumption that the solubilization of the semipolar molecules, the barbiturates, in polysorbate solutions is essentially a micellar solubilization.

The order of the partition coefficient K, calculated from the solubility data of the various barbiturates studied, lends support to the suggestion of Mulley and leads the authors to believe that here also the type of solubilization is mainly a solution within the micelles together with some other factors playing a minor role. In the case of the barbiturates these factors are probably interactions between the hydrophilic portion of the solubilizer and that of the solubilizate.

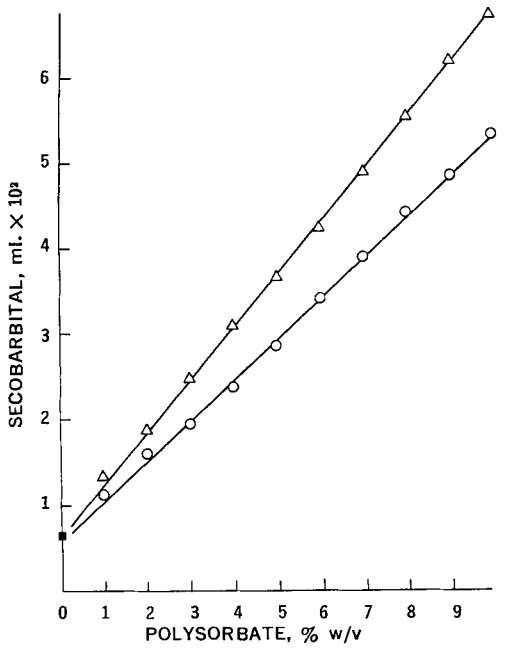


Figure 7—*Solubility of secobarbital in polysorbate solutions at 30°. Key: ■, solubility in water; O—, polysorbate 20; △—, polysorbate 80.*

SUMMARY

1. All barbiturates studied showed increased solubilities in the presence of the nonionic surfactants, polysorbates.

2. The amount of barbiturate solubilized was a linear function of the concentration of the polysorbates, characteristic of micellar solubilization of semipolar molecules.

3. The degree of solubilization increased with increase of the hydrophobic chain length of the solubilizer due to formation of larger micelles.

4. The increase in the number of carbon atoms in the substituents on the 5-position of the barbituric acid molecule, together with the polar or inductive effects of such substituents, determined the extent of solubilization.

5. The order of solubilization of the drugs compared favorably with the order of their distribution coefficients between 1-octanol and water.

6. Phenobarbital and cyclobarbital showed a greater extent of solubilization due to the inductive effect of the phenyl and cyclohexenyl groups resulting in a greater interaction between the hydrophilic portion of the solubilizer and that of the solubilizate.

7. The mechanism of solubilization is thought to be mainly a solution within the micelles together with other factors playing a role in the solubilization.

REFERENCES

(1) B. M. Mulley, in "Advances in Pharmaceutical Sciences," M. S. Bean, A. M. Beckett, and J. E. Carless, Eds., Academic, New York, N. Y., 1964.

(2) J. Swarbrick, *J. Pharm. Sci.*, **54**, 1229(1965).
(3) E. A. Swinyard and S. C. Harvey, "Remington Pharmaceutical Sciences," E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965.
(4) R. W. Applewhite, A. P. Buckley, and L. W. Nobles, *J. Am. Pharm. Assoc., Pract. Pharm. Ed.*, **15**, 164(1954).
(5) V. P. Gusiakov, N. M. Likholet, and I. M. Kutnaya, *Farm. Zh.*, **22**, 34(1967); through *Intern. Pharm. Abstr.*, **4**, 1074(1967).
(6) D. Küttel, *Pharm. Zentralhalle*, **102**, 115(1963).
(7) *Ibid.*, **103**, 10(1964).
(8) J. J. Walker, R. S. Fischer, and J. J. McHugh, *Am. J. Clin. Pathol.*, **18**, 451(1948).
(9) M. R. V. Paruta and L. D. King, *J. Pharm. Sci.*, **53**, 1217 (1964).
(10) S. G. Bjaastad, N. A. Hall, and A. L. Thakkar, *ibid.*, **54**, 1529(1965).
(11) A. L. Thakkar and N. A. Hall, *ibid.*, **56**, 1121(1967).
(12) F. W. Goodhart and A. N. Martin, *ibid.*, **51**, 52(1962).
(13) C. K. Bahl and H. B. Kostenbauder, *ibid.*, **53**, 1027(1964).
(14) A. G. Mitchell and L. S. C. Wan, *ibid.*, **53**, 1467(1964).
(15) H. B. Klevens, *J. Am. Oil Chemists Soc.*, **26**, 456(1949).
(16) H. B. Klevens, *Chem. Rev.*, **47**, 9(1950).
(17) M. E. L. McBain and E. Hutchinson, "Solubilization and Related Phenomena," Academic, New York, N. Y., 1955.
(18) T. R. Bates, M. Gibaldi, and J. L. Kanig, *J. Pharm. Sci.*, **55**, 191(1966).
(19) D. J. Lamb and L. E. Harris, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 583(1960).
(20) C. Hansch and S. M. Anderson, *J. Med. Chem.*, **10**, 745 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 17, 1969, from the *College of Pharmacy, University of Alexandria, Alexandria, U.A.R.*

Accepted for publication September 19, 1969.

* From the Department of Pharmaceutics, College of Pharmacy, University of Alexandria, Alexandria, U.A.R.

† Present address: School of Pharmacy, University of Montana, Missoula, MT 59801. To whom requests for reprints should be directed.

Improved Thin-layer Chromatographic Identification of Tetracyclines and Their Degradation Products: Application to an Epimerization Study

N. D. GYANCHANDANI*, I. J. McGILVERAY, and D. W. HUGHES

Abstract □ An improved thin-layer chromatographic method for the identification of pharmaceutically important tetracyclines and their degradation products is presented. A process is used which conditions the plates with optimum moisture content so that they may be stored without special precautions for as long as 4 weeks before use. Two solvent systems and four spray reagents for the separation and detection of tetracyclines and their degradation products are also presented. Tetracycline hydrochloride and chlortetracycline hydrochloride were allowed to epimerize in phosphate and acetate solutions, respectively. With the aid of UV spectroscopy and TLC analysis, it is demonstrated that epimerization is accompanied by extensive degradation.

Keyphrases □ Tetracyclines, degradation products—identification □ Epimerization, tetracyclines—reaction products separation, identification □ TLC—separation, identification □ UV light—TLC spot visualization

Thin-layer chromatography (TLC) has been used by a number of workers to separate and characterize certain tetracyclines of pharmaceutical importance (Table I) using adsorbent layers of silica gel (1, 2), kieselguhr (3–5), and microcrystalline cellulose (6). Difficulties encountered in these separations have been attributed to the property of tetracyclines to form chelate complexes with metallic ions and to lack of moisture in the support. Hence, sequestering agents (1–5) and glycerin (7) or mixtures of glycerin with polyethylene glycol 400 (PEG 400) (4) have been added to the support.

Of the various methods reported, two (3, 4) are most useful since they can resolve two or more tetracyclines in a single system on one chromatogram. Of these, the one employing the coating of kieselguhr containing EDTA and a developing solvent consisting of methyl ethyl ketone saturated with McIlvaine's buffer (pH 4.7) (3) was especially good for the separation of various degradation products of the tetracyclines. The other method (4), which employs acid-washed diatomaceous earth impregnated with EDTA at pH 7.0 and a glycerin–PEG 400 mixture, and which uses ethyl acetate as the developing solvent, was found satisfactory for the resolution of tetracyclines, though often it was necessary to chromatograph two to four times. In both cases, however, problems in their application were encountered. The most serious difficulty with the former method (3) was the frequent and erratic splitting and streaking of the tetracycline spots. The latter method (4) involves rigid adherance to a lengthy procedure in which the diatomaceous earth is repeatedly washed to remove binder and other acid-soluble materials. This was found not to be reproducible without considerable experience. Furthermore, the plates must be freshly prepared and used immediately. The method is, therefore, particularly inappropriate in situations where a large number of chromatograms must be run in a short time.

The tetracyclines have most commonly been detected on chromatograms by their fluorescence under long-wave UV light, either with or without exposure of the chromatograms to ammonia vapor (1, 3–5, 8). A few chromogenic spray reagents were described in the earlier literature (1) but have been largely abandoned in recent publications.

This communication describes a simple, rapid, and reproducible method for the separation of six tetracyclines presently marketed in numerous dosage forms.

(2) J. Swarbrick, *J. Pharm. Sci.*, **54**, 1229(1965).
(3) E. A. Swinyard and S. C. Harvey, "Remington Pharmaceutical Sciences," E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965.
(4) R. W. Applewhite, A. P. Buckley, and L. W. Nobles, *J. Am. Pharm. Assoc., Pract. Pharm. Ed.*, **15**, 164(1954).
(5) V. P. Gusiakov, N. M. Likholet, and I. M. Kutnaya, *Farm. Zh.*, **22**, 34(1967); through *Intern. Pharm. Abstr.*, **4**, 1074(1967).
(6) D. Küttel, *Pharm. Zentralhalle*, **102**, 115(1963).
(7) *Ibid.*, **103**, 10(1964).
(8) J. J. Walker, R. S. Fischer, and J. J. McHugh, *Am. J. Clin. Pathol.*, **18**, 451(1948).
(9) M. R. V. Paruta and L. D. King, *J. Pharm. Sci.*, **53**, 1217 (1964).
(10) S. G. Bjaastad, N. A. Hall, and A. L. Thakkar, *ibid.*, **54**, 1529(1965).
(11) A. L. Thakkar and N. A. Hall, *ibid.*, **56**, 1121(1967).
(12) F. W. Goodhart and A. N. Martin, *ibid.*, **51**, 52(1962).
(13) C. K. Bahl and H. B. Kostenbauder, *ibid.*, **53**, 1027(1964).
(14) A. G. Mitchell and L. S. C. Wan, *ibid.*, **53**, 1467(1964).
(15) H. B. Klevens, *J. Am. Oil Chemists Soc.*, **26**, 456(1949).
(16) H. B. Klevens, *Chem. Rev.*, **47**, 9(1950).
(17) M. E. L. McBain and E. Hutchinson, "Solubilization and Related Phenomena," Academic, New York, N. Y., 1955.
(18) T. R. Bates, M. Gibaldi, and J. L. Kanig, *J. Pharm. Sci.*, **55**, 191(1966).
(19) D. J. Lamb and L. E. Harris, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 583(1960).
(20) C. Hansch and S. M. Anderson, *J. Med. Chem.*, **10**, 745 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 17, 1969, from the *College of Pharmacy, University of Alexandria, Alexandria, U.A.R.*

Accepted for publication September 19, 1969.

* From the Department of Pharmaceutics, College of Pharmacy, University of Alexandria, Alexandria, U.A.R.

† Present address: School of Pharmacy, University of Montana, Missoula, MT 59801. To whom requests for reprints should be directed.

Improved Thin-layer Chromatographic Identification of Tetracyclines and Their Degradation Products: Application to an Epimerization Study

N. D. GYANCHANDANI*, I. J. McGILVERAY, and D. W. HUGHES

Abstract □ An improved thin-layer chromatographic method for the identification of pharmaceutically important tetracyclines and their degradation products is presented. A process is used which conditions the plates with optimum moisture content so that they may be stored without special precautions for as long as 4 weeks before use. Two solvent systems and four spray reagents for the separation and detection of tetracyclines and their degradation products are also presented. Tetracycline hydrochloride and chlortetracycline hydrochloride were allowed to epimerize in phosphate and acetate solutions, respectively. With the aid of UV spectroscopy and TLC analysis, it is demonstrated that epimerization is accompanied by extensive degradation.

Keyphrases □ Tetracyclines, degradation products—identification □ Epimerization, tetracyclines—reaction products separation, identification □ TLC—separation, identification □ UV light—TLC spot visualization

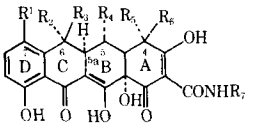
Thin-layer chromatography (TLC) has been used by a number of workers to separate and characterize certain tetracyclines of pharmaceutical importance (Table I) using adsorbent layers of silica gel (1, 2), kieselguhr (3–5), and microcrystalline cellulose (6). Difficulties encountered in these separations have been attributed to the property of tetracyclines to form chelate complexes with metallic ions and to lack of moisture in the support. Hence, sequestering agents (1–5) and glycerin (7) or mixtures of glycerin with polyethylene glycol 400 (PEG 400) (4) have been added to the support.

Of the various methods reported, two (3, 4) are most useful since they can resolve two or more tetracyclines in a single system on one chromatogram. Of these, the one employing the coating of kieselguhr containing EDTA and a developing solvent consisting of methyl ethyl ketone saturated with McIlvaine's buffer (pH 4.7) (3) was especially good for the separation of various degradation products of the tetracyclines. The other method (4), which employs acid-washed diatomaceous earth impregnated with EDTA at pH 7.0 and a glycerin–PEG 400 mixture, and which uses ethyl acetate as the developing solvent, was found satisfactory for the resolution of tetracyclines, though often it was necessary to chromatograph two to four times. In both cases, however, problems in their application were encountered. The most serious difficulty with the former method (3) was the frequent and erratic splitting and streaking of the tetracycline spots. The latter method (4) involves rigid adherance to a lengthy procedure in which the diatomaceous earth is repeatedly washed to remove binder and other acid-soluble materials. This was found not to be reproducible without considerable experience. Furthermore, the plates must be freshly prepared and used immediately. The method is, therefore, particularly inappropriate in situations where a large number of chromatograms must be run in a short time.

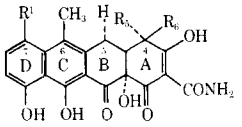
The tetracyclines have most commonly been detected on chromatograms by their fluorescence under long-wave UV light, either with or without exposure of the chromatograms to ammonia vapor (1, 3–5, 8). A few chromogenic spray reagents were described in the earlier literature (1) but have been largely abandoned in recent publications.

This communication describes a simple, rapid, and reproducible method for the separation of six tetracyclines presently marketed in numerous dosage forms.

Table I—Structures of Tetracyclines and Some of Their Degradation Products



(I)



(II)

Compound	Structure	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Tetracycline antibiotics								
Tetracycline (TC)	(I)	H	CH_3	OH	H	$N(CH_3)_2$	H	H
Chlortetracycline (CTC)	(I)	Cl	CH_3	OH	H	$N(CH_3)_2$	H	H
Oxytetracycline (OTC)	(I)	H	CH_3	OH	OH	$N(CH_3)_2$	H	H
Demethylchlortetracycline (DMCTC)	(I)	Cl	H	OH	H	$N(CH_3)_2$	H	H
Methacycline (MC)	(I)	H	CH_2		OH	$N(CH_3)_2$	H	H
Doxycycline (DOXY)	(I)	H	CH_3	H	OH	$N(CH_3)_2$	H	H
Rolitetracycline (RTC)	(I)	H	CH_3	OH	H	$N(CH_3)_2$	H	CH_2N
Degradation products								
4-Epitetracycline (4 epi-TC)	(I)	H	CH_3	OH	H	H	$N(CH_3)_2$	H
4-Epichlortetracycline (4 epi-CTC)	(I)	Cl	CH_3	OH	H	H	$N(CH_3)_3$	H
Anhydrotetracycline (ATC)	(II)	H	—	—	—	$N(CH_3)_2$	H	—
Epianhydrotetracycline (EATC)	(II)	H	—	—	—	H	$N(CH_3)_2$	—
Anhydrochlortetracycline (ACTC)	(II)	Cl	—	—	—	$N(CH_3)_2$	H	—

Various degradation and reaction products are also well separated, even when chromatographed on 4-week old plates. Four spray reagents for the detection of these antibiotics are presented. This TLC method has been used in a preliminary qualitative study of degradation products, other than epimers, formed when tetracycline hydrochloride (TC·HCl) and chlortetracycline hydrochloride (CTC·HCl) (see Table I) were allowed to epimerize at three different pH values at room temperature.

EXPERIMENTAL

Reagents—EDTA disodium salt, glycerin, PEG 400, methyl ethyl ketone, dichloromethane, ethyl formate, sodium phosphate monobasic, sodium acetate, all analytical grade reagents.

Preparation of Plates—With the aid of an applicator, five glass plates (20 × 20 cm.) were coated with 0.25-mm. layers of a slurry of 50 g. kieselguhr G (Merck) in a homogeneous mixture consisting of 95 ml. 0.1 *M* EDTA in water and 5 ml. 20% v/v PEG 400 in glycerin. The plates were allowed to dry at room temperature for at least 4 hr. (preferably overnight), then developed in the Solvent System I (see below) up to the top of the plate. They were then dried and stored in a dust-free atmosphere with no drying agent.

Spotting the Solution—Two microliters of 2 mg./ml. methanolic solutions of the tetracyclines [aqueous solutions in case of rolitetracycline(RTC)] were spotted as usual, 2 cm. from the edge of the plate.

Solvent Systems—*I*—Methyl ethyl ketone saturated with McIlvaine's buffer (pH 4.7) (3).

II—Dichloromethane–ethyl formate–ethanol (9:9:2) saturated with McIlvaine's buffer (pH 4.7).

Spray Reagents—*I—Fast Blue B(Diazo-Reagent)*—Spray Solution A: 0.5% aqueous, freshly prepared solution of fast blue B; Spray Solution B: 0.1 *N* NaOH (aqueous) (1*a*).

II—Diazotized p-Nitroaniline (1*b*)—Spray Solution A: just before spraying, 5% aqueous sodium nitrite solution (1.5 ml.) is added to 0.3% *p*-nitroaniline in 8% HCl (25 ml.); Spray Solution B: 20% aqueous sodium carbonate solution. After spraying with Solution A, the developed chromatogram is sprayed with Solution B, taking care not to make the plate transparent with an excess of the sprays.

III—Modified Sakaguchi Reagent (9)—Boric acid (5 g.) is dissolved in water (150 ml.) and concentrated sulfuric acid (350 ml.). The reagent is stored in a glass-stoppered bottle in a refrigerator and is used cold.

IV—Diphenylpicrylhydrazyl (DPPH) Reagent (10)—Solution A: methanolic solution of DPPH (~1 mg./2 ml.); Solution B: 25% aqueous NaOH solution.

Detection—All the tetracyclines and many of their degradation products could be detected by their fluorescence in longwave UV light (Black Ray UVL-22). For the visualization of the spots in daylight the dried chromatogram was sprayed with one of the spray reagents I–IV. With the first three reagents, colored spots are observed on a nearly white background but with spray reagent IV yellow spots on a bluish white background are obtained (Table II).

Epimerization Experiments—Five milliliters each of 1% aqueous solutions of TC·HCl and CTC·HCl were separately diluted to 25 ml. with distilled water (reference solution), 0.1 *M* sodium dihydrogen phosphate (aqueous) (pH 4.1), and 0.1 *M* sodium acetate (aqueous) (pH 7.3). The solutions were kept at room temperature in 25-ml. volumetric flasks and wrapped in aluminum foil for protection from light. At convenient time intervals, 2 μl. of each solution was examined by TLC using Solvent Systems I and II. Simultaneously, 0.2 ml. of each solution was diluted to 25 ml. with 0.1 *N* H_2SO_4 and immediately scanned between 210 and 400 mμ,[1] noting the absorbance at 254, 267, 298, and 355 mμ. The UV spectra (210–400 mμ) of the three TC·HCl solutions at 0 and 620 hr. are shown in Fig. 1. The corresponding spectra for CTC·HCl are presented in Fig. 2. The TLC of these solutions at 0, 24, and 620 hr. are schematically represented in Fig. 3.

[1] Beckman DB spectrophotometer.

Table II—TLC of Tetracyclines and Some Degradation Products

Compound	$R_f \times 100$[a] System I	$R_f \times 100$[a] System II	Color[b] (and Limit of Detection, mcg.) Reagent I	Reagent II	Reagent III[c]	Reagent IV	Limit of Detection (mcg.) under Longwave UV With Ammonia	Without Ammonia
Tetracycline antibiotics								
TC	53	36	Pk(0.1)	Y(1.2)	Y(0.8)	Pk(1.2)	0.005	0.05
CTC	76	60	Pk(0.16)	Y(1.2)	Y(0.8)	Y-Pk(1.2)	0.0032	0.064
DMCTC	73	44	Pk(0.16)	Y(1.2)	Y(0.8)	Pk-Y(1.2)	0.0032	0.064
OTC	60	20	Y-Br(0.16)	Pk-Y(1.2)	Y(0.8)	Br-Y(1.2)	0.0024	0.064
MC	44	29	Pk-Br(0.16)	Y(1.2)	Y(0.8)	Y(1.2)	0.0024	0.064
DOXY	53	57	Pk-Br(0.16)	Y(1.2)	Y(0.8)	Pk(1.2)	0.0032	0.064
Some Degradation Products of Tetracyclines[d]								
4 epi-TC	20	12	Y-Pk	Y	Y	Pk-Y	—	—
ATC	93	83	Pk	Y	Y	Y-Pk	—	—
EATC	47	50	Y-Pk	Y	Y	Y-Pk	—	—
4 epi-CTC	33	21	Y-Pk	Y	Y	Pk-Y	—	—
ACTC	83	57	Pk	Y	Y	Y	—	—

[a] R_f values vary considerably with tank temperature, especially in case of stored plates. If very low R_f values are obtained, the chromatograms, after brief drying may be rechromatographed in the same solvent system. [b] Pk = pink, Y = yellow, Br = brown. [c] Colors change with excess of spray reagent and with time. [d] Limit of detection is not given since very pure samples were not available.

RESULTS AND DISCUSSION

In the procedure of Ascione *et al.* (4) the support of diatomaceous earth is washed with hot 6 *N* HCl until the washings no longer show the presence of calcium or iron. It is then washed with water until a neutral pH is obtained and dried at 105°. This somewhat tedious process required considerable experience to obtain an acid-washed neutral support, and some batches, perhaps less well washed or dried, gave chromatograms with streaks and tails. Thus, it was decided to use as support the commercially available kieselguhr G (Merck) without treatment other than impregnation with EDTA and glycerin–PEG 400 mixture. Though the tetracylcines with R_f 0.4 and lower gave well-shaped spots, those with higher R_f values showed irregular zig-zag patterns, which appeared to be due to the glycerin–PEG mixture which was also moving with the solvent. The same results were obtained when the amount of glycerin–PEG mixture in the preparation of the slurry was greatly reduced. This defect was, however, completely remedied when the plates were first developed in Solvent System I and allowed to dry before spotting. This process also conditioned the plates with optimum moisture content and allowed a satisfactory resolution of the tetracycline spots, even after the plates were stored up to 4 weeks. In this system, one run with the solvent gave satisfactory resolution of the various spots, in contrast to the published method (4) where it is almost always necessary to chromatograph two to four times. It was ascertained by two-dimensional TLC that no degradation of the tetracyclines occurred on the plates.

Though a few spray reagents for the detection of tetracyclines have been reported by earlier authors (1), they proved unsatisfactory under chromatographic conditions in this laboratory. Recent

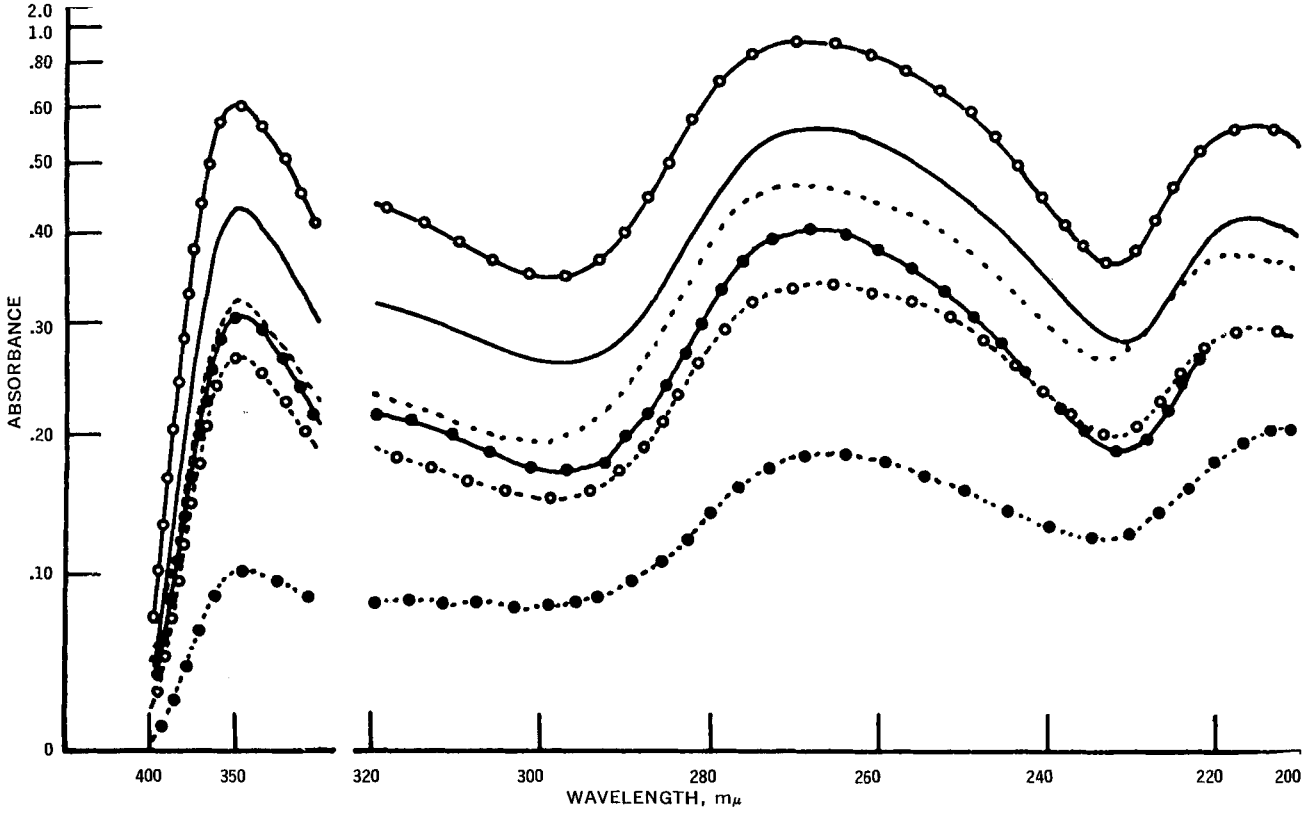


Figure 1—*UV spectra of tetracycline hydrochloride in three solutions (aqueous, phosphate, and acetate). Key: ——, T-0 hr. = aqueous solution at 0 hr.; - - -, T-620 hr. = aqueous solution after 620 hr.;* O—O, *TP-0 hr. = phosphate solution at 0 hr.;* O· · ·O, *TP-620 hr. = phosphate solution after 620 hr.;* ●—●, *TA-0 hr. = acetate solution at 0 hr.;* ●· · ·●, *TA-620 hr. = acetate solution after 620 hr.*

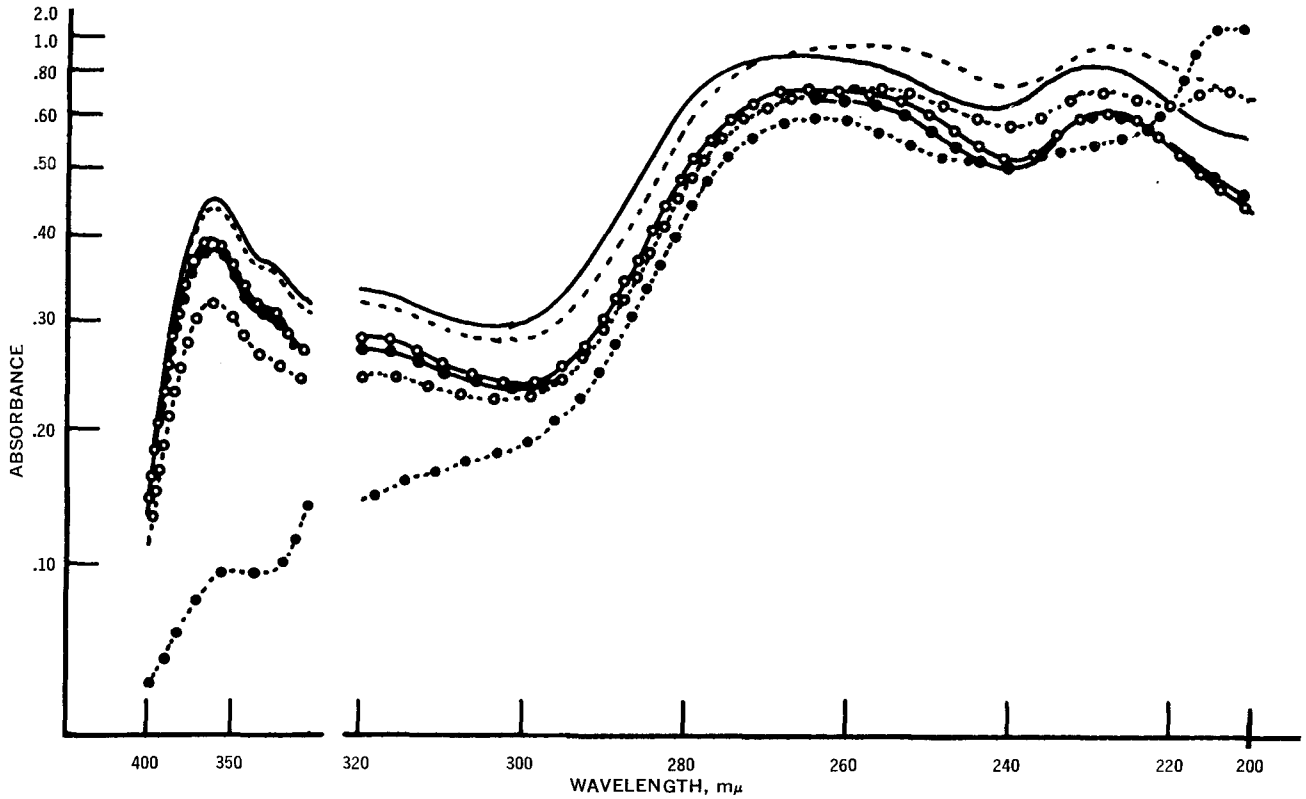


Figure 2—*UV spectra of chlortetracycline hydrochloride in three solutions (aqueous, phosphate, and acetate). Key: ——, C-0 hr. = aqueous solution at 0 hr.; - - -, C-620 hr. = aqueous solution after 620 hr.; O—O, CP-0 hr. = phosphate solution at 0 hr.; O···O, CP-620 hr. = phosphate solution after 620 hr.; ●—●, CA-0 hr. = acetate solution at 0 hr.; ●···●, CA-620 hr. = acetate solution after 620 hr.*

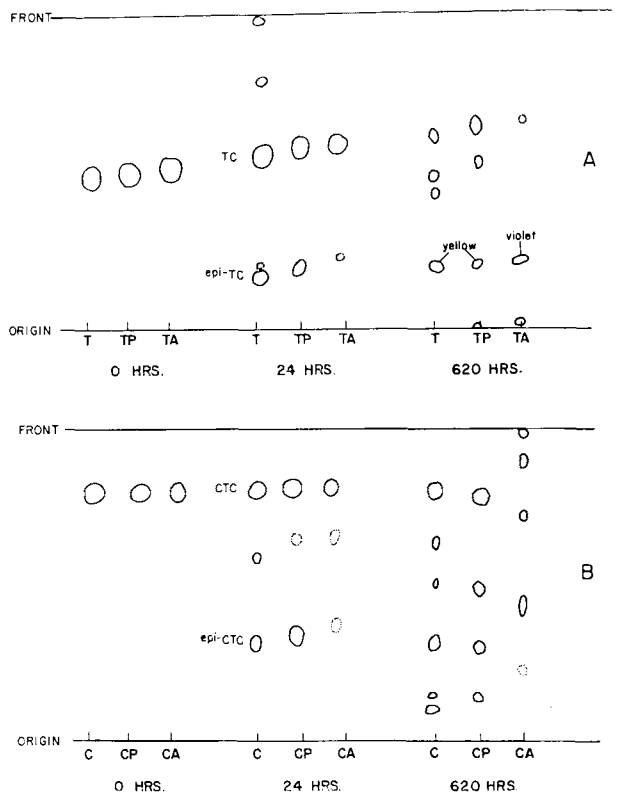


Figure 3—*Schematic TLC of the degradation products of* (A) *tetracycline hydrochloride and* (B) *chlortetracycline hydrochloride in three solutions (aqueous, phosphate, and acetate) at 0, 24, and 620 hr.; Solvent System I. T = TC·HCl in aqueous solution; TP = TC·HCl in phosphate solution; TA = TC·HCl in acetate solution; C = CTC·HCl in aqueous solution; CP = CTC·HCl in phosphate solution; CA = CTC·HCl in acetate solution.*

authors have exclusively made use of longwave UV light for the detection of these compounds (2–8). In an attempt to find a spray reagent which might be useful for the quantitation of tetracyclines by densitometric methods, several sprays used for the detection of phenols and amines (1*c*) were tried. Of these, the four described in the experimental section were found to give colored spots with various tetracyclines and their degradation products (Table II). Though these spray reagents are not as sensitive as the use of UV light, they are satisfactory for most detection purposes.

TLC of Tetracyclines—The chromatography of tetracyclines in Solvent Systems I and II is schematically represented in Fig. 4 and a compilation of approximate R_f values obtained with both the solvent systems is shown in Table II. With the exception of CTC and doxycycline (DOXY) all other tetracyclines are well separated

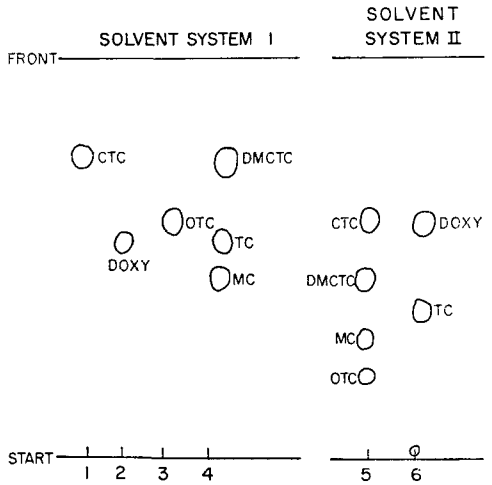


Figure 4—*Schematic chromatogram of tetracyclines in Solvent Systems I (left) and II (right). Samples: 1, CTC; 2, DOXY; 3, OTC; 4, DMCTC, TC, and MC; 5, CTC, DMCTC, MC, and OTC; 6, DOXY and TC.*

from one another in Solvent System II. CTC and DOXY can also be separated from each other in a system composed of dichloromethane–ethyl alcohol (18:1) saturated with the above-mentioned McIlvaine buffer. Though oxytetracycline (OTC) moves higher than TC or methacycline (MC) in System I, it has an R_f value lower than these compounds in System II. Rolitetracycline (RTC), which carries the pyrrolidinomethyl moiety in Position 2 (Table I), did not move in these systems but did so when the alcohol content in System II was increased [dichloromethane–ethylformate–ethanol (2:2:1), R_f = 0.15]. Furthermore, any tetracyclines formed by hydrolysis of RTC can be easily detected by employing System I or II.

TLC of Degradation Products—Following the reports of a Fanconi-like syndrome that developed in patients after the ingestion of outdated or degraded tetracycline capsules (6, 10, and references contained therein), concern was expressed over impurities and degradation products in tetracyclines. 4-Epi-, anhydro-, and 4-epianhydro-derivatives of tetracycline (Table I) have been implicated as the most toxic degradation products accompanying tetracycline (11). 4-Epianhydrotetracycline in relatively large doses was the causative agent of renal tubular damage in the rat and the dog, producing urinary findings suggestive of Fanconi-type syndrome (11). Furthermore, the epimers have been found to possess less than 5% of the antibiotic activity of the parent tetracycline (11).

Solvent Systems I and II are suitable for the separation of epitetracyclines from their parents and of epianhydrotetracycline from tetracycline. Anhydrotetracycline and anhydrochlortetracycline both move to the solvent front in System I but have reasonable mobilities in System II (see Table II).

Epimerization Experiments—The TLC method was successfully applied to a qualitative study of degradation products other than the epimers that may be formed in solutions of tetracyclines. Previously, the process of epimerization has been studied spectroscopically (12–14) and use has been made of 254 mμ/267 mμ and 355 mμ/298 mμ absorbance ratios (14) to follow the epimerization. The decrease in absorbance in the 300–380 mμ region in the spectra of tetracyclines has been regarded as an indicator for degradation other than epimerization.

In this study, TC·HCl and CTC·HCl were separately allowed to epimerize in phosphate and acetate solutions side by side with the corresponding aqueous solutions at room temperature, and the reaction products were studied at suitable time intervals both spectroscopically and by TLC.

The results obtained by spectrophotometric study were in accordance with the earlier epimerization studies (12–14). The UV spectra of TC·HCl and CTC·HCl in the three systems (aqueous, phosphate, and acetate) are shown in Figs. 1 and 2, respectively, and the corresponding TLC results are represented schematically in Figs. 3A and 3B, respectively. It was evident from both the chromatographic and spectroscopic evidence that there was considerable degradation other than epimerization occurring in each system. TLC analysis, however, appears capable of giving more detailed information on the excessive degradation than does the absorbance ratio analysis or the UV absorbance in the 300–380-mμ region.

In addition to the gradual change in color of the phosphate solutions, yellowish-gray deposits also formed and were collected at the end of about 620 hr. They were dissolved in mixtures of ethyl acetate–methanol (~1:3) and on TLC each showed at least four minor spots, beside the one major spot corresponding in R_f values (Systems I and II) to that of the parent tetracycline. Though no further study of these spots was undertaken, it seemed probable that during the process of epimerization, some of the antibiotic was precipitated out of the solution perhaps in the form of phosphate complex. In the acetate system the corresponding deposit was gray in each case and was dissolved in formic acid–methanol (~1:3). On TLC it showed at least seven spots, none of them corresponding to those reported earlier.

The TLC methods described here thus indicated the extensive degradation which occurs in solutions of tetracyclines and may be of value in studying the stability of liquid pharmaceutical preparations containing tetracyclines. If products similar to those detected in the epimerization study are found, it will be important to determine their nature and toxicity.

REFERENCES

(1) E. Stahl, "Dünnschicht-Chromatography: ein Laboratoriumshandbuch," 2nd ed., Springer-Verlag, Berlin, Heidelberg, New York, 1967, p. 547; (*a*), *ibid.*, p. 829; (*b*), *ibid.*, p. 842; (*c*), *ibid.*, p. 815.

(2) Y. Nishimoto, E. Tsuchida, and S. Toyoshima, *Yakugaku Zasshi*, **87,** 516(1967).

(3) J. Keiner, R. Huttenrauch, and W. Poethke, *Pharm. Zentralhalle*, **105,** 705(1966).

(4) P. P. Ascione, J. B. Zagar, and G. P. Chekian, *J. Pharm. Sci.*, **56,** 1393(1967).

(5) I. C. Dijkhius, *Pharm. Weekblad*, **102,** 907(1967).

(6) D. L. Simmons, C. K. Koorengevel, and R. Kubelka, *J. Pharm. Sci.*, **55,** 219(1966).

(7) D. Sonanini and L. Anker, *Pharm. Acta Helv.*, **39,** 518(1964).

(8) "British Pharmacopoeia," 1968, pp. 215, 697, and 1001.

(9) C. T. Smith, *J. Assoc. Offic. Anal. Chemists*, **51,** 750 (1968).

(10) S. R. Sulkowski and J. R. Haserick, *J. Am. Med. Assoc.*, **1964,** 152.

(11) K. F. Benitz and H. F. Diermeier, *Proc. Soc. Exptl. Biol. Med.*, **115,** 930(1964).

(12) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. A. Muller, R. Winterbottom, and A. P. Doerschuck, *J. Am. Chem. Soc.*, **79,** 2849(1957).

(13) E. G. Remmers, G. M. Seiger, and A. P. Doerschuck, *J. Pharm. Sci.*, **52,** 752(1963).

(14) D. A. Hussar, P. J. Niebergall, E. T. Sugita, and J. T. Doluisio, *J. Pharm. Pharmacol.*, **20,** 539(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 14, 1969, from the *Research Laboratories, Food and Drug Directorate, Ottawa, Canada.*

Accepted for publication September 17, 1969.

The authors are indebted to Dr. D. L. Simmons (Frank W. Horner Ltd., Montreal, P. Q.), Dr. R. G. Kelly (Lederle Laboratories, New York), and Mr. R. B. Taylor (Pfizer Company Ltd., Montreal) for gifts of certain reference substances used in this study, and to Mr. J. R. Fooks for technical assistance.

* National Research Council of Canada Postdoctoral Fellow, 1968–1969.

Effect of Silica Gel on Stability and Biological Availability of Ascorbic Acid

E. De RITTER, L. MAGID, M. OSADCA, and S. H. RUBIN

Abstract □ The alleged interaction of silica gel and ascorbic acid has been investigated in model experiments and in practical tablet trials, using wet granulation procedures. In simple mixtures stored for 3 weeks at 45° in closed tubes, losses of ascorbic acid increase progressively with increasing moisture content, whether or not silica gel is present, although losses are higher in the presence of silica gel. At an equivalent percentage of water in such mixtures, the amount of silica gel or the prior adsorption of 1½ times its weight of vitamin E on the silica gel, did not influence the loss of ascorbic acid. The data show that silica gel binds a certain fraction of the water present and that the loss of ascorbic acid is directly proportional to the amount of unbound water in the system. Sodium ascorbate is more sensitive than ascorbic acid to aerobic oxidation in the presence of moisture. Other commonly used tablet excipients, as well as silica gel, enhance losses of ascorbate. However, proper technology applied to wet granulation procedures yields excellent recoveries and stability of ascorbic acid or sodium ascorbate in dried granulations and in finished multivitamin tablets. The human bioassay technique, in which extra urinary excretion of ascorbic acid after tablet dosage is compared to that after dosage of ascorbic acid in water, has been used to demonstrate the full physiological availability of ascorbic acid in the presence of silica gel. Storage of such tablets for 3 months at 45° did not alter the complete bioavailability of the ascorbic acid.

Keyphrases □ Ascorbic acid—stability, biological availability □ Stability, ascorbic acid—humidity, excipient effects □ Silica gel effect—ascorbic acid stability, bioavailability □ Moisture concentration—ascorbic acid stability □ Biological availability, ascorbic acid—silica gel effect

Diffuse reflectance spectroscopy has been used by Lach and Bornstein (1–3) to study interactions of a number of drugs with various adjuvants after treatment of the mixtures by equilibration in aqueous or nonaqueous media, by compression, and by exposure to controlled humidity conditions. Such an interaction of ascorbic acid and silica gel has been claimed by Lach (4). Since silica gel is a useful adsorbent for converting liquid vitamins such as vitamin E and panthenol into free-flowing, dry powders, it became important to evaluate this alleged interaction of silica gel with ascorbic acid. This has been done in model experiments with simple mixtures and under practical conditions of formulating multivitamin dosage forms. In addition, physiological availability tests in humans have been utilized to check for possible influence of silica gel contained in multivitamin tablets on the biochemical behavior of ascorbic acid.

EXPERIMENTAL

Model Experiments—(*a*) *Effect of Graded Moisture Levels*—Experimental mixtures of ascorbic acid with silica gel[1] and with 60% adsorbate of *d,l*-α-tocopheryl acetate on silica gel were prepared both at normal use ratios and at an eightfold higher than normal adsorbent/vitamin ratio. Ascorbic acid alone and the various mixtures were adjusted with distilled water to graded moisture levels up to 40% and stored in closed tubes for 21 days at 45°. The percentage of water added was based in each case on the total weight of the tube contents, except for the vitamin E adsorbate mixtures where the weight of the oil phase was not included. The compositions of the mixtures before addition of water are shown in the legend of Fig. 1. Ascorbic acid was determined after storage by titration with about 0.1 *N* standard iodine solution and starch indicator.

(*b*) *Rate of Loss of Ascorbic Acid and Sodium Ascorbate at 45° with 11.6% Water*—The stress conditions used in *Experiment* (*a*), namely 3 weeks at 45° at high moisture levels, are obviously much more strenuous than those normally encountered in pharmaceutical manufacturing operations, such as wet granulation procedures, in which drying is completed in a much shorter period. It was of interest, therefore, to check the rate of decomposition of ascorbic acid in similar mixtures stored for 1, 2, and 3 days at 45° at one of the lower levels of moisture, namely 11.6%.

Ascorbic acid and the ascorbic acid plus silica gel mixture used in this test, when shaken with water at a concentration of about 20 mg. of vitamin C per ml., yielded a pH of 2.3 and 2.4, respectively. Sodium ascorbate with and without silica gel, at similar dilutions in water, gave a pH of 6.7 with silica gel and 7.2 without. The same stability tests were set up with sodium ascorbate with and without silica gel at 11.6% water.

(*c*) *Effect of Other Tablet Excipients*—The relative effect of other excipients commonly used in tablets has been compared to that of silica gel in a test similar to that in (*b*). Three hundred milligrams of sodium ascorbate were mixed with 80 mg. of the particular excipient and water added to give 11.6% by weight. The mixtures were stored in closed tubes for 3 days at 45° and ascorbate determined by iodine titration.

Granulation and Tablet Trials—Multivitamin mixtures containing ascorbic acid or sodium ascorbate and silica gel adsorbates of vitamin E were made by wet granulation procedures with and without iron. To minimize exposure to moisture stress, the granulations were milled through a No. 6, round-hole screen to the minimum practical particle size and dried in layers of 1.27 cm. (0.5 in.) or less with rapidly moving, 45° air. Vitamin C recoveries were determined for the granulations and finished tablets made from these granulations, using iodometric titrations. Stability of vitamin C was determined similarly after accelerated and room temperature storage.

Availability Studies in Men—It has been pointed out (1–3) that drug-adjuvant interactions possibly may result in significant altera-

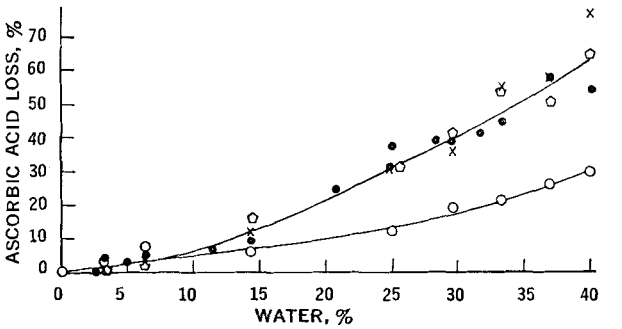


Figure 1—*Effect of silica gel on stability of ascorbic acid at graded percent moisture levels; mixtures stored in closed tubes for 3 weeks at 45°. Key:* ○, *300 mg. ascorbic acid alone;* ●, *300 mg. ascorbic acid + 80 mg. silica gel;* ⌓, *300 mg. ascorbic acid + vitamin E adsorbate (80 mg. silica gel + 120 mg.* d,l-α-*tocopheryl acetate);* ×, *300 mg. ascorbic acid + 640 mg. silica gel.*

[1] Syloid 244, W. R. Grace & Co., Davison Chemical Div., Baltimore, Md.

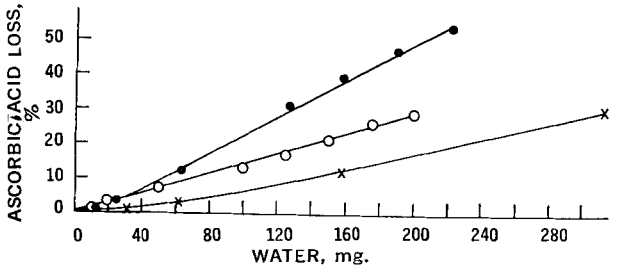


Figure 2—*Effect of silica gel on stability of ascorbic acid with graded weights of water added; mixtures stored in closed tubes for 3 weeks at 45°. Key: ○, 300 mg. ascorbic acid alone; ●, 300 mg. ascorbic acid + 80 mg. silica gel; ×, 300 mg. ascorbic acid + 640 mg. silica gel.*

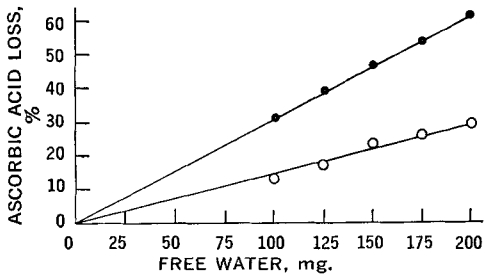


Figure 3—*Effect of free water level on stability of ascorbic acid in mixtures with or without silica gel; storage in closed tubes for 3 weeks at 45°. Key: ○, 300 mg. ascorbic acid alone; ●, 300 mg. ascorbic acid + 80 or 640 mg. silica gel.*

tions of the biochemical behavior of a medicament. Although such effects are more likely to occur at low drug to adjuvant ratios, the possible existence of the excipient as a chemisorbed layer on the drug has been mentioned as a factor that might cause similar effects at high drug to adjuvant ratios (2). To determine whether silica gel in a tablet would influence the physiological availability of ascorbic acid, the human bioassay technique described by Melnick *et al.* (9) was applied to several tablet formulations. In this test, comparison is made between the extra urinary excretion of the vitamin following dosage with the test sample and that following administration of the vitamin in pure form.

Five male subjects were saturated with ascorbic acid by daily dosing with 500 mg. for 3 weeks. Dosing with test samples and standard was not initiated until a stable plateau had been obtained for the 24-hr. urinary excretions following a 500-mg. dose, given after 2 days without dosing. Two different tablet formulations as listed in Table VI were tested, both containing vitamin E at a level of 30 mg. per tablet in the form of silica gel adsorbate. The standard dose of pure ascorbic acid taken in water was 450 mg. For the initial test, the dose taken was six tablets (453 and 466 mg. ascorbic acid for the Lot Nos. 73-69/1 and 73-69/3, respectively). For the aged sample, seven tablets (430 mg. ascorbic acid) were given.

One test was performed each week with basal urine collected on the day prior to taking each test dose. After dosage with ascorbic acid alone or in tablets, respectively, urine was collected for the periods 0–6 hr. and 6–24 hr., except for Dose 2 of ascorbic acid where only total 24-hr. collections were made. Basal urines were collected in each case according to the same schedule. Ascorbic acid in urine was determined by the dichlorophenol–indophenol–xylene extraction method, as previously described (10). In each case the extra excretion due to dose was calculated by subtracting the corresponding basal excretion value from the value after dose.

RESULTS AND DISCUSSION

Model Experiments—*Effect of Graded Moisture Levels*—The results of storage tests on the ascorbic acid plus silica gel mixtures at graded moisture levels for 3 weeks at 45° are shown in Fig. 1. Ascorbic acid alone shows progressively increasing storage losses with increasing moisture content. At low moisture levels (below 6%), no significant difference could be determined between samples with or without silica gel. At higher moisture levels, the losses increase with increasing moisture content in mixtures containing silica gel and are higher than those found with ascorbic acid alone. It is noteworthy, however, that at any percentage moisture level, an eightfold increase in the ratio of silica gel to ascorbic acid caused no further increase in the ascorbic acid loss above that observed at the lower silica gel level in the 3 weeks at 45° storage tests. This lack of concentration effect of silica gel suggests strongly that the degradation of ascorbic acid is not due to surface interaction. Further, when 60% vitamin E oil was adsorbed on the silica gel, no change in the stability of ascorbic acid was observed. Thus is due undoubtedly to the strongly hydrophilic nature of this adsorbent.

It is of interest to examine the data in Fig. 1 in terms of the losses of ascorbic acid found at equivalent weights of water in the mixtures, rather than at equivalent percentages of water. This type of plot is given in Fig. 2. The circumstance that the higher proportion of silica gel exerts a protective effect indicates that some binding of water by the silica gel is taking place and suggests that the ascorbic acid losses are related to the amount of unbound or free water in the various mixtures. If this is true, then the equivalence of the ascorbic acid losses at the high and low silica gel levels at any particular percentage of moisture would indicate an equivalent amount of free water in these two mixtures.

Assuming that the silica gel binds water as a fixed fraction of its own weight at any given percentage of water, calculation has been made of the fraction that must be bound at both the 80 and 640-mg. silica gel levels in order to yield equal weights of free water at these two levels. A typical calculation is given below for the 25% water level in both mixtures, the compositions of which are as follows: (*a*) 80 mg. silica gel, 300 mg. ascorbic acid, and 127 mg. water; (*b*) 640 mg. silica gel, 300 mg. ascorbic acid, and 313 mg. water. If X = bound water level (expressed as percent of silica gel weight) which will yield the same weight of free water for both mixtures, then in both cases the total water minus bound water = free water, and

$$127 - 80 \times \frac{X}{100} = 313 - 604 \times \frac{X}{100}$$

from which $X = 33.2$. Then milligrams of bound water are: (*a*) $80 \times 0.332 = 26.6$ and (*b*) $640 \times 0.332 = 212.6$, and milligrams of free water are: (*a*) $127 - 26.6 = 100.4$ and (*b*) $313 - 212.6 = 100.4$.

These percentages of bound water and the corresponding weights of free water at the various total water levels are listed in Table I, together with the respective losses of ascorbic acid taken from the curve in Fig. 1. Values at the lower moisture levels are not included in Table I since the magnitude of the ascorbic acid losses in this range of water content is not sharply defined, due to the difficulty of mixing the small quantities of water uniformly, the greater error inherent in the small differences in titrations before and after storage, and the possible effect in some cases of a moisture loss during storage.

Table I—Effect of Moisture Content on Stability of Ascorbic Acid in Presence of Silica Gel[a]

H_2O Added, %	H_2O Added, mg. 80 mg. S.G.	H_2O Added, mg. 640 mg. S.G.	80 or 640 mg. S.G. Bound H_2O S.G. wt., %	80 or 640 mg. S.G. Free H_2O, mg.	Loss A. A., %
25.0	127	313	33.2	100.4	31.3
29.4	159	392	41.6	125.7	39.7
33.3	190	470	50.0	150.0	47.0
36.9	222	550	58.6	175.0	54.6
40.0	254	627	66.6	200.7	62.3

[a] Ascorbic acid (A.A.), 300 mg. + 80 or 640 mg. silica gel (S.G.) + indicated percent H_2O—storage in closed tubes for 3 weeks at 45°.

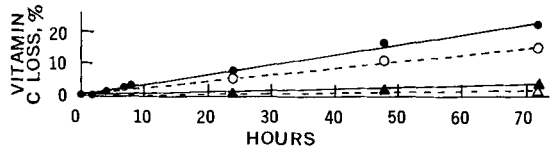


Figure 4—*Rate of loss of vitamin C in presence of silica gel; storage in closed tubes at 45° with 11.6% water. Key: △, 300 mg. ascorbic acid alone; ▲, 300 mg. ascorbic acid + 80 mg. silica gel; ○, 300 mg. sodium ascorbate alone; ●, 300 mg. sodium ascorbate + 80 mg. silica gel.*

The excellent correlation between weights of free water in the mixtures and losses of ascorbic acid is shown by the plot of Fig. 3. The losses of ascorbic acid are directly proportional to the calculated amounts of free water in the mixtures. For the sake of comparison, the data on the ascorbic acid plus water mixtures without silica gel are plotted in Fig. 3. The slope of the latter line is smaller than that of the ascorbic acid + silica gel + water mixtures. Again, in view of the fact that equal losses are found with a given weight of ascorbic acid over an eightfold range of silica gel plus bound water weights, it appears highly unlikely that surface reaction is a significant factor responsible for the higher losses of ascorbic acid in the presence of silica gel. It is believed more likely that trace metals such as iron and copper, which are present in silica gel to the extent of 110 and 1 p.p.m., respectively, are dissolved by the water and exert their well-known catalytic effect on ascorbic acid decomposition in solution (5, 6). Trace metals in the ascorbic acid (less than 10 p.p.m.) are present to the same extent in the tubes with or without silica gel. This mode of decomposition of ascorbic acid is in contrast to that reported by Carstensen *et al.* (7) for thiamine in solid dosage forms, where losses occur in an adsorbed surface monolayer of thiamine dissolved in water.

Rate of Loss of Ascorbic Acid and Sodium Ascorbate at 45° with 11.6% Water—These data are shown in Fig. 4. The loss of ascorbic acid alone in 3 days at 45° is only about 1%; in the mixture with silica gel the loss in this period is 3.6%. This small effect of silica gel in the 3-day test shows the same trend as described previously for the 3-week test.

The losses of sodium ascorbate, both with and without silica gel, are considerably higher than those found with ascorbic acid. This is to be expected in view of the fact that aerobic oxidation of ascorbic acid in the presence of metallic catalysts proceeds more slowly in acid solution than in neutral solution (8).

Effect of Other Tablet Excipients—The losses of ascorbate in the presence of the various excipients at 11.6% water are listed in Table II. Like silica gel, all these other excipients also increase the loss of ascorbate. The magnitude of the effect is undoubtedly dependent on the factors discussed above, including pH, water-binding capacity of the adjuvant, and trace metal content.

Granulation and Tablet Trials—Table III shows the excellent recoveries of ascorbic acid in dried granulations properly formulated with high levels of vitamin E in the form of silica gel adsorbates. Table IV similarly shows the excellent stability of sodium ascorbate in two dried granulations.

Finished tablets prepared by suitable techniques also show good stability of vitamin C in the presence of silica gel. This is demonstrated by the data in Table V on ascorbic acid stability in tablets prepared with 33 and 60% adsorbates of *d,l*-α-tocopheryl acetate on silica gel.

It has long been known that the sensitivity of vitamin C to oxidation in the presence of moisture is a factor that must be considered in the preparation of multivitamin tablets by wet granulation procedures. This is true whether or not silica gel is present in the granulation. In granulations containing appreciable quantities of silica gel and/or excipients, which are also potential contributors to vitamin C breakdown, it is possible to obtain excellent recovery of either ascorbic acid or sodium ascorbate by suitable wet granulation procedures. However, it is essential that the moisture level be held to the minimum level for effective granulation and that drying be carried out promptly and efficiently.

Table II—Influence of Various Excipients Plus Water on Stability of Sodium Ascorbate[a]

Excipient	Loss of Ascorbate, %
None	14.5
Cornstarch	16.4
Dicalcium phosphate anhydrous	17.7
Dicalcium phosphate dihydrate (milled)	19.0
Avicel	18.3
Silica gel	22.0
Tricalcium phosphate	25.3

[a] Excipient 80 mg. + 300 mg. sodium ascorbate + 11.6% water; storage for 3 days at 45°.

Table III—Recovery of Ascorbic Acid in Granulation and Tablets[a]

Lot No.	Vitamin E,[b] %	Excipient Added	Recovery Theoretical, % Granulation	Tablets
73-69/1	33	Dicalcium phosphate dihydrate	98	98
73-69/3	60	Same	100	100
73-69/5	60	Dicalcium phosphate anhydrous	100	100
73-69/6	60	Tricalcium phosphate	100	99

[a] Theoretical content: vitamin C = 77 mg.; vitamin E = 30 mg. [b] Adsorbate of *d,l*-α-tocopheryl acetate on silica gel.

Table IV—Stability of Na Ascorbate in Granulation with Vitamin E Containing Silica Gel

Storage Test	Na Ascorbate, Theoretical, % Lot 811-58	Lot 811-59
Initial	97	98
3 weeks at 25°	98	96
3 weeks at 45°	96	96
3 weeks at 55°	96	96

Table V—Stability of Ascorbic Acid in Coated Multivitamin Plus Iron Tablets

Storage Test	Ascorbic Acid, Theoretical, % Maintenance Formula[a]	Therapeutic Formula[b]
1 month at 55°	100	91
3 months at 45°	100	94
6 months at 37°	97	88
12 months at 25°	100	94

[a] Contains 30 mg. of *d,l*-α-tocopheryl acetate per tablet as a 33% adsorbate on silica gel. [b] Contains 30 mg. of *d,l*-α-tocopheryl acetate per tablet as a 60% adsorbate on silica gel; both formulas have a theoretical ascorbic acid content of 77 mg. per tablet.

Table VI—Physiological Availability of Ascorbic Acid from Tablets Containing Silica Gel—24-hr. Test

	Dose (about 450 mg.) Excreted, %				
			Multivitamin Tablets, Vitamin E, 30 mg.		
Subject	Standard Ascorbic Acid Dose 1	Dose 2	Lot No. 73-69/1 (33% E Adsorbate) Initial	3 mo./45°	Lot No. 73-69/3 (60% E Adsorbate) Initial
BM	45	28	33	32	25
MO	52	57	36	37	46
RG	24	34	41	36	51
JS	39	40	41	53	41
ED	38	36	43	46	39
Average	39.3		38.3	40.8	40.4
Availability ±*SE*, %			99 ± 9	103 ± 14	104 ± 13

Table VII—Physiological Availability of Ascorbic Acid from Tablets Containing Silica Gel—6-hr. Test

Subject	Dose (about 450 mg.) Excreted, %: Standard Ascorbic Acid	Lot No. 73-69/1 (33% E Adsorbate) Initial	Lot No. 73-69/1 (33% E Adsorbate) 3 mo./45°	Lot No. 73-69/3 (60% E Adsorbate) Initial
BM	28	27	27	17
MO	35	25	27	28
RG	18	26	26	37
JS	23	33	28	29
ED	26	29	28	24
Average	26.0	28.0	27.2	26.8
Availability ±*SE*, %		108 ± 13	105 ± 11	103 ± 17

Experiences with wet granulation formulations containing ascorbic acid or sodium ascorbate indicate that excellent stability can be achieved if the wet granulation is dried to about 10% moisture within a few hours and to the final, low moisture content within 24 hr.

Order of mixing of ingredients and especially the mode of addition of water can be important. The vitamin C stability may be influenced by the presence of adsorbents that bind water or soluble ingredients that serve as emulsifiers or influence the solubility or reactivity of vitamin C.

Availability Studies in Men—The results of bioavailability tests in men of ascorbic acid in tablets containing silica gel have been calculated on the basis of 24-hr. excretions of test doses. These results are summarized in Table VI. Both lots of tablets show complete bioavailability of the ascorbic acid, and this was not changed by storage for 3 months at 45°. In order to provide information on the question of whether or not silica gel reduces the rate of absorption of ascorbic acid *in vivo*, calculations of physiological availability also were made on the basis of urinary excretions in the first 6 hr. after dose. These data are given in Table VII. Again, the results show complete availability of ascorbic acid in all three tablet trials, indicating that the ascorbic acid is absorbed normally in the presence of silica gel.

REFERENCES

(1) J. L. Lach and M. Bornstein, *J. Pharm. Sci.*, **54**, 1730 (1965).

(2) M. Bornstein and J. L. Lach, *ibid.*, **55**, 1033(1966).

(3) J. L. Lach and M. Bornstein, *ibid.*, **55**, 1040(1966).

(4) J. L. Lach, personal communication.

(5) C. M. Lyman, M. O. Schultze, and C. G. King, *J. Biol. Chem.*, **118**, 757(1937).

(6) C. F. Timberlake, *J. Sci. Food Agr.*, **11**, 258(1960).

(7) J. T. Carstensen, M. Osadca, and S. H. Rubin, *J. Pharm. Sci.*, in press.

(8) A. E. Kellie and S. S. Zilva, *Biochem. J.*, **32**, 1561(1938).

(9) D. Melnick, M. Hochberg, and B. L. Oser, *J. Nutr.*, **30**, 67(1945).

(10) S. H. Rubin, F. W. Jahns, and J. C. Bauernfeind, *Fruit Prod. J. Am. Food Mfg.*, **24**, 327(1945).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 18, 1969, from the *Product Development Department, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication September 23, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors wish to express thanks to Mrs. Nancy Ladas and Miss Sandra Economos for assistance in conducting these experiments.

Interfacial Barriers in Interphase Transport II: Influence of Additives upon the Transport of Diethylphthalate Across the Hexadecane-Gelatin-Water Interface

ABDEL-HALIM GHANEM, W. I. HIGUCHI, and A. P. SIMONELLI

Abstract □ The authors recently described a novel method for investigating the effects of an interfacial barrier in interphase transport. The procedures, both theoretical and experimental, were applied to the study of the effects of an adsorbed gelatin at the hexadecane-water interface upon the transport of diethylphthalate between the two phases. The present paper describes the influences of surfactants, electrolyte type, and concentration upon the permeability coefficient for the interfacial barrier. Experiments were conducted as before, employing diethylphthalate as the solute. The transport data were analyzed by the physical model described earlier. The results showed that the two ionic surfactants, sodium lauryl sulfate and dodecylpyridinium chloride, markedly decreased (2 to 12 times) the interfacial barrier even at low concentration (0.001–0.10% in the stock emulsion). Furthermore, the analysis showed that neither the electrolyte type nor concentration influenced the permeability coefficients, although they significantly altered the interphase transport rates themselves by changing the partition coefficients. These findings are particularly interesting as they may represent types of nonspecific situations that give rise to important barriers in *in vivo* drug transport.

Keyphrases □ Transport, interphase—interfacial barriers □ Diethylphthalate transport—hexadecane-gelatin-water interface □ Electrolyte effect—diethylphthalate transport, hexadecane-gelatin-water interface □ Surfactant effect—diethylphthalate transport, hexadecane-gelatin-water interface □ Permeability coefficients, interfacial barriers—surfactant, electrolyte type, concentration effect.

Recent studies from these laboratories (1, 2) involving the use of a novel method for investigating interfacial barriers in interphase transport have shown that substances adsorbed at the oil-water interface may control the interphase transport rates of solutes. Gelatin adsorbed at the hexadecane-water interface has been shown (1) to give an interphase transport rate for diethylphthalate that is about 1×10^4 times slower than diffusion controlled. A significant reduction in the aqueous to lipid transport rate of cholesterol by an

Table VII—Physiological Availability of Ascorbic Acid from Tablets Containing Silica Gel—6-hr. Test

Subject	Dose (about 450 mg.) Excreted, %: Standard Ascorbic Acid	Lot No. 73-69/1 (33% E Adsorbate) Initial	Lot No. 73-69/1 (33% E Adsorbate) 3 mo./45°	Lot No. 73-69/3 (60% E Adsorbate) Initial
BM	28	27	27	17
MO	35	25	27	28
RG	18	26	26	37
JS	23	33	28	29
ED	26	29	28	24
Average	26.0	28.0	27.2	26.8
Availability ±*SE*, %		108 ± 13	105 ± 11	103 ± 17

Experiences with wet granulation formulations containing ascorbic acid or sodium ascorbate indicate that excellent stability can be achieved if the wet granulation is dried to about 10% moisture within a few hours and to the final, low moisture content within 24 hr.

Order of mixing of ingredients and especially the mode of addition of water can be important. The vitamin C stability may be influenced by the presence of adsorbents that bind water or soluble ingredients that serve as emulsifiers or influence the solubility or reactivity of vitamin C.

Availability Studies in Men—The results of bioavailability tests in men of ascorbic acid in tablets containing silica gel have been calculated on the basis of 24-hr. excretions of test doses. These results are summarized in Table VI. Both lots of tablets show complete bioavailability of the ascorbic acid, and this was not changed by storage for 3 months at 45°. In order to provide information on the question of whether or not silica gel reduces the rate of absorption of ascorbic acid *in vivo*, calculations of physiological availability also were made on the basis of urinary excretions in the first 6 hr. after dose. These data are given in Table VII. Again, the results show complete availability of ascorbic acid in all three tablet trials, indicating that the ascorbic acid is absorbed normally in the presence of silica gel.

REFERENCES

(1) J. L. Lach and M. Bornstein, *J. Pharm. Sci.*, **54**, 1730 (1965).

(2) M. Bornstein and J. L. Lach, *ibid.*, **55**, 1033(1966).

(3) J. L. Lach and M. Bornstein, *ibid.*, **55**, 1040(1966).

(4) J. L. Lach, personal communication.

(5) C. M. Lyman, M. O. Schultze, and C. G. King, *J. Biol. Chem.*, **118**, 757(1937).

(6) C. F. Timberlake, *J. Sci. Food Agr.*, **11**, 258(1960).

(7) J. T. Carstensen, M. Osadca, and S. H. Rubin, *J. Pharm. Sci.*, in press.

(8) A. E. Kellie and S. S. Zilva, *Biochem. J.*, **32**, 1561(1938).

(9) D. Melnick, M. Hochberg, and B. L. Oser, *J. Nutr.*, **30**, 67(1945).

(10) S. H. Rubin, F. W. Jahns, and J. C. Bauernfeind, *Fruit Prod. J. Am. Food Mfg.*, **24**, 327(1945).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 18, 1969, from the *Product Development Department, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication September 23, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors wish to express thanks to Mrs. Nancy Ladas and Miss Sandra Economos for assistance in conducting these experiments.

Interfacial Barriers in Interphase Transport II: Influence of Additives upon the Transport of Diethylphthalate Across the Hexadecane-Gelatin-Water Interface

ABDEL-HALIM GHANEM, W. I. HIGUCHI, and A. P. SIMONELLI

Abstract □ The authors recently described a novel method for investigating the effects of an interfacial barrier in interphase transport. The procedures, both theoretical and experimental, were applied to the study of the effects of an adsorbed gelatin at the hexadecane-water interface upon the transport of diethylphthalate between the two phases. The present paper describes the influences of surfactants, electrolyte type, and concentration upon the permeability coefficient for the interfacial barrier. Experiments were conducted as before, employing diethylphthalate as the solute. The transport data were analyzed by the physical model described earlier. The results showed that the two ionic surfactants, sodium lauryl sulfate and dodecylpyridinium chloride, markedly decreased (2 to 12 times) the interfacial barrier even at low concentration (0.001–0.10% in the stock emulsion). Furthermore, the analysis showed that neither the electrolyte type nor concentration influenced the permeability coefficients, although they significantly altered the interphase transport rates themselves by changing the partition coefficients. These findings are particularly interesting as they may represent types of nonspecific situations that give rise to important barriers in *in vivo* drug transport.

Keyphrases □ Transport, interphase—interfacial barriers □ Diethylphthalate transport—hexadecane-gelatin-water interface □ Electrolyte effect—diethylphthalate transport, hexadecane-gelatin-water interface □ Surfactant effect—diethylphthalate transport, hexadecane-gelatin-water interface □ Permeability coefficients, interfacial barriers—surfactant, electrolyte type, concentration effect.

Recent studies from these laboratories (1, 2) involving the use of a novel method for investigating interfacial barriers in interphase transport have shown that substances adsorbed at the oil-water interface may control the interphase transport rates of solutes. Gelatin adsorbed at the hexadecane-water interface has been shown (1) to give an interphase transport rate for diethylphthalate that is about 1×10^4 times slower than diffusion controlled. A significant reduction in the aqueous to lipid transport rate of cholesterol by an

Table I—Oil Droplet Size Distribution Taken from Fig. 2 Photograph (for 3 ml. Stock Emulsion)

i	Channels	Mean Radius, μ	Total No. of Particles $\times 10^{-8}$	Total Volume $\times 10^{-10}$, μ^3
1	0–40	1.03	27.19	1.22
2	40–80	1.48	4.12	0.56
3	80–120	1.75	23.07	5.21
4	120–160	1.96	35.43	11.20
5	160–200	2.13	23.89	9.71
6	200–240	2.28	15.66	7.77
7	240–280	2.41	11.53	6.77
8	280–320	2.52	9.06	6.14
9	320–360	2.64	8.24	6.32
10	360–400	2.74	6.59	5.65
11	400–440[a]	2.83	4.12	3.91
12	440–480[a]	2.92	2.47	2.56

[a] Obtained by extrapolation.

adsorbed polysorbate 80 film has also been observed (2). These findings have suggested that such barriers, which are probably nonspecific in nature from the biopharmaceutical standpoint, may play important rate-determining roles in the transport of drug molecules across biological membranes and into tissues.

In order to better understand the nature of these barriers, the present studies were undertaken. This article describes the results of the experiment on the effects of the different gelatin fractions of Pharmagel A,[1] the influences of electrolyte types and concentration, and the effect of surfactants on the transport rate of diethylphthalate. The data have been mechanistically analyzed and reported in terms of the permeability coefficients for the interfacial barriers.

EXPERIMENTAL

Consideration in the Design of the Experiment—A discussion o. the basic design and the advantages of the multiparticulate dispersion technique has already been given (1, 3). In the present study the same general approach was essentially employed.

The previous procedure for preparing the emulsion system was slightly modified in the direction of greater flexibility. Instead of preparing stock hexadecane-in-water emulsions near the interfacial coacervation point (1, 4) of gelatin sodium sulfate, lower electrolyte concentrations were used and the temperature was kept at 40° for the whole period of equilibration of the emulsion systems. This provided the means for varying the concentrations of the additives used in these studies without droplet-droplet aggregation taking place.

Materials—The gelatin used was Pharmagel A. Fractionation of the gelatin was accomplished (5) by adding successive amounts of ethanol to the gelatin solution adjusted to pH 7 at 40°. The hexadecane[2] was purified (6) and the diethylphthalate[3] was used without further purification. Sodium sulfate,[4] sodium chloride,[5] calcium chloride dihydrate,[4] magnesium chloride hexahydrate,[6] and magnesium sulfate[4] were used without further purification. Pure samples of sodium lauryl sulfate[7] and polysorbate 80[8] were used. Dodecylpyridinium chloride[3] was purified by recrystallizing three times from acetone. Spectroquality cyclohexane[9] was used in the UV analysis.

[1] Wilson's U-Cop-Co., Calumet City, Ill.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] "Eastman grade," Eastman Kodak Co., Rochester, N. Y.
[4] Allied Chemicals, Industrial Chemical Division, Morristown, N. J.
[5] Mallinckrodt Chemical Works, New York, N. Y.
[6] Baker Chemical Co., Phillipsburg, N. J.
[7] Supplied by Dr. K. J. Mysels, Reynolds Tobacco Co., North Carolina.
[8] Supplied by Dr. P. Becher, Atlas Chemical Industries, Delaware.
[9] Matheson, Coleman & Bell, East Rutherford, N. J.

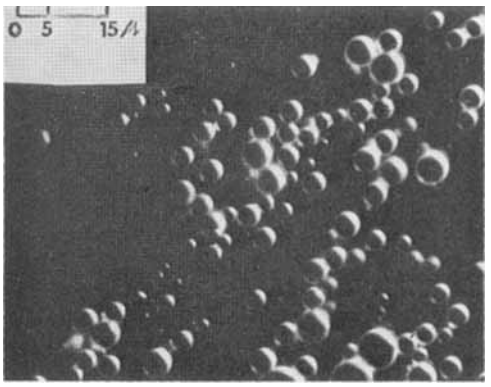


Figure 1—*Dark field photomicrograph of a typical emulsion system used in the release experiments.*

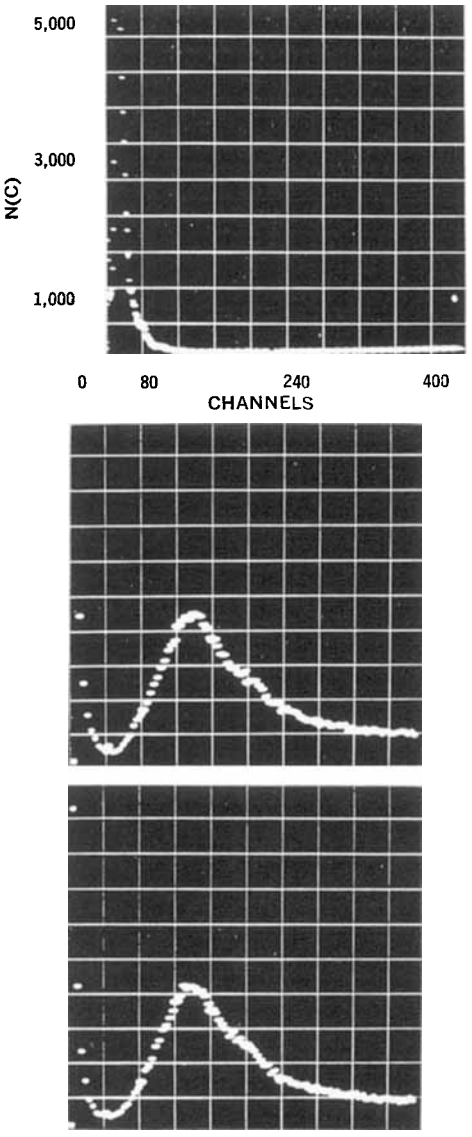


Figure 2—*Droplet size distribution data obtained with a Coulter counter and multichannel analyzer. First photograph shows calibration data obtained with 2.051-μ diameter polyvinyltoluene latex.* N(C) *is the concentration of particles in Channel* C. *Second photograph shows a typical droplet distribution in the release medium taken 1 min. after the beginning of a release experiment. Third photograph shows the droplet distribution after 15 min. in the release medium.*

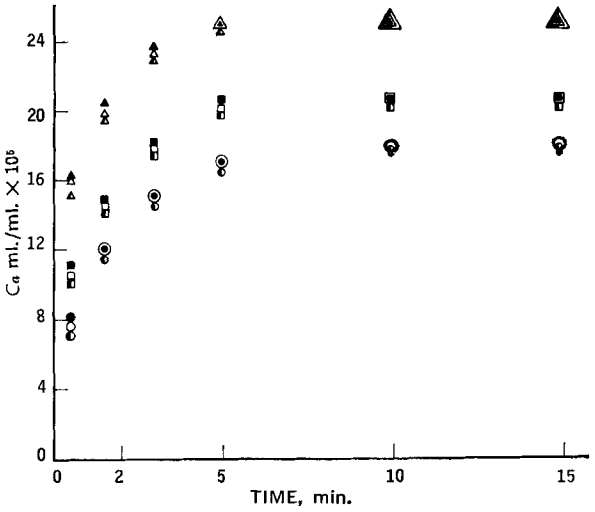


Figure 3—*Experimental results of diethylphthalate release obtained with three fractions of gelatin.* C_a *is amount of diethylphthalate released, ml./ml.,* versus *time in minutes. At zero time 3.0 ml. of emulsion system pretreated with 0.352* M *sodium sulfate was added to 100 ml. of aqueous medium at 23°. Using whole gelatin the aqueous media are: Key: ◐, 0.352* M *sodium sulfate; ◧, 0.176* M *sodium sulfate; ◭, water. Using the second gelatin fraction: ○, 0.352* M *sodium sulfate; □, 0.176* M *sodium sulfate; △, water. Using the third gelatin fraction: ●, 0.352* M *sodium sulfate; ■, 0.176* M *sodium sulfate; ▲, water.*

Experimental Procedure—The procedure for preparing the initial emulsion was exactly the same as before (1). The emulsion system was then treated with specified additives for 24 hr. Temperature during the treatment period was kept at 40°. Figure 1 shows a dark-field microphotograph of the oil droplets of a typical emulsion system used in these studies. Figure 2 gives a typical particle size distribution display obtained with a Coulter counter[10] and a multichannel analyzer.[11] Table I presents the results of the analysis of the particle size distribution taken from the Fig. 2 photograph. This information was used in the calculation of the permeability coefficients.

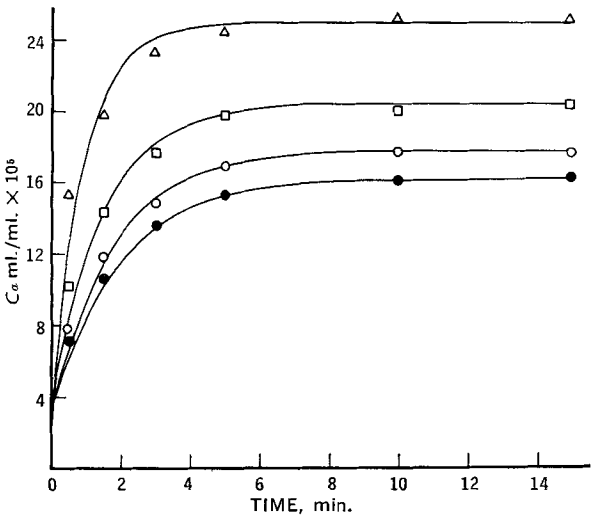


Figure 4—*Influence of electrolyte and the comparison of experimental release data with the theoretically computed ones.* C_a *is the amount of diethylphthalate released, ml./ml.,* versus *time in minutes. A 3.0-ml. aliquot of the emulsion system pretreated with 0.352* M *sodium sulfate was added to 100 ml. of aqueous medium at 23°. Key: experimental points, ●, 0.493* M *sodium sulfate; ○, 0.352* M *sodium sulfate; □, 0.176* M *sodium sulfate; △, water. Curves are theoretical values computed using Eqs. 1 and 2 with* $P = 5 \times 10^{-5}$ *cm./sec.*

[10] Coulter Electronics, Chicago, Ill.
[11] RIDL 400, Radiation Instrument Development Laboratory, Inc., Melrose Park, Ill.

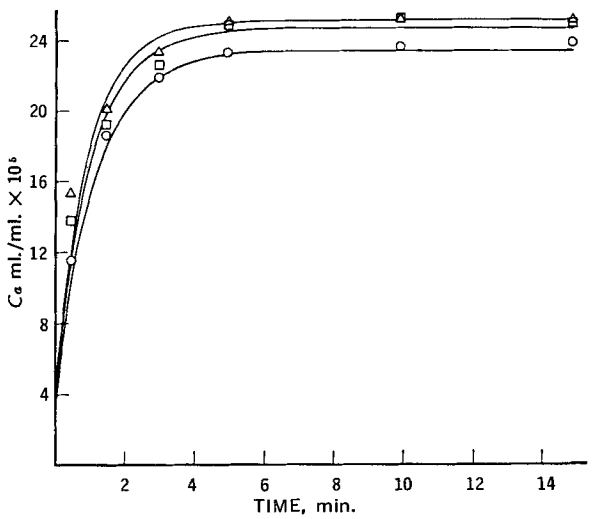


Figure 5—*Influence of electrolyte and the comparison of experimental release data with the theoretically computed ones.* C_a *is the amount of diethylphthalate released, ml./ml.,* versus *time in minutes. A 3.0-ml. aliquot of the emulsion system pretreated with 0.352* M *sodium chloride was added to 100 ml. of aqueous medium at 23°. Key: experimental points, ○, 0.352* M *sodium chloride; □, 0.176* M *sodium chloride; △, water. Curves are theoretical values computed using Eqs. 1 and 2 with* $P = 5 \times 10^{-5}$ *cm./sec.*

The hexadecane-water partition coefficients for diethylphthalate were determined in two ways as previously discussed (1) with and without gelatin present. Table II gives the partition coefficients obtained in different electrolyte media. It is clear that the partition coefficients obtained with and without gelatin at the interface are the same within the experimental error. However, the partition coefficients were found to be dependent on the nature and the concentration of the electrolytes used. This can be attributed to the salting-out effect (7).

The interphase transport experiments were carried out as described previously (1). A 3-ml. aliquot of the stock emulsion system containing the diethylphthalate was pipeted into 100 ml. of the aqueous release medium at zero time, and the amounts released to the aqueous phase were determined as a function of time.

EXPERIMENTAL RESULTS

Effect of Different Gelatin Fractions—Figure 3 gives the experimental results of diethylphthalate release obtained using whole

Table II—Partition Coefficients of Diethylphthalate[a] for Hexadecane-Aqueous Electrolyte Solutions at 23°C.

Media	Without Gelatin: Water	Without Gelatin: 0.176 *M*	Without Gelatin: 0.352 *M*	With Gelatin: Water	With Gelatin: 0.176 *M*	With Gelatin: 0.352 *M*
Water	54 ± 6[b]	—	—	—	—	—
Sodium sulfate	—	94 ± 6	130 ± 4	48 ± 7	96 ± 5	126 ± 7
Sodium chloride	—	55 ± 3	66 ± 3	50 ± 4	53 ± 3	63 ± 5
Calcium chloride	—	68 ± 3	92 ± 4	54 ± 6	67 ± 5	89 ± 4
Magnesium chloride	—	69 ± 2	92 ± 3	52 ± 3	66 ± 4	91 ± 4
Magnesium sulfate	—	89 ± 3	122 ± 5	49 ± 4	91 ± 5	118 ± 8

[a] The concentration of diethylphthalate was 0.05096 ml./ml. and in all experiments, 1.0 ml. of oil phase or equivalent amount of emulsion system was added to 100 ml. of the aqueous phase in a 250-ml. volumetric flask and shaken for 24 hr. [b] Standard deviations of triplicate or quadruplicate determinations.

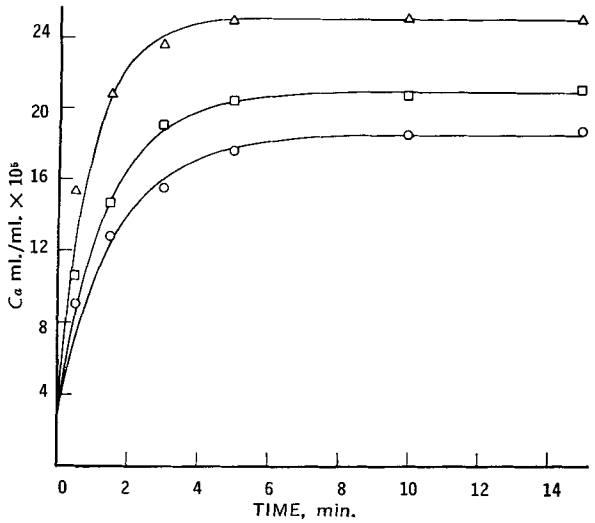


Figure 6—*Influence of electrolyte and the comparison of experimental release data with theoretically computed ones.* C_a *is the amount of diethylphthalate released, ml./ml.,* versus *time in minutes. A 3.0-ml. aliquot of the emulsion system pretreated with 0.352* M *calcium chloride was added to 100 ml. of aqueous medium at 23°. Key: experimental points,* ○, *0.352* M *calcium chloride;* □, *0.176* M *calcium chloride;* △, *water. Curves are theoretical values computed using Eqs. 1 and 2 with* $P = 5 \times 10^{-5}$ *cm./sec.*

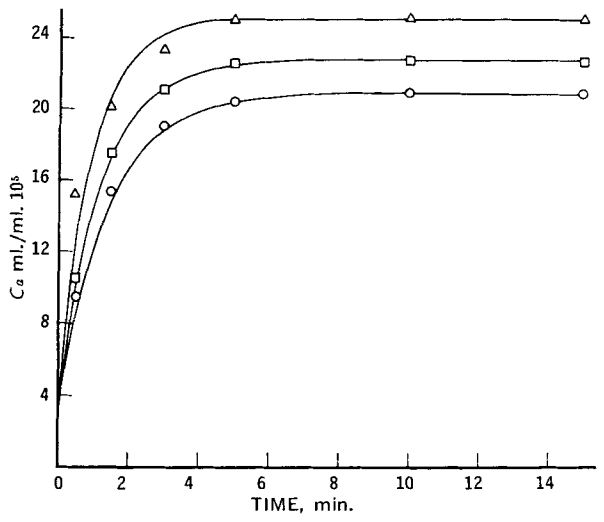


Figure 8—*Influence of electrolyte and the comparison of experimental release data with the theoretically computed ones.* C_a *is the amount of diethylphthalate released, ml./ml.,* versus *time in minutes. A 3.0-ml. aliquot of the emulsion system pretreated with 0.352* M *magnesium sulfate was added to 100 ml. of aqueous medium at 23°. Key: experimental points,* ○, *0.352* M *magnesium sulfate;* □, *0.176* M *magnesium sulfate;* △, *water. Curves are theoretical values computed using Eqs. 1 and 2 with* $P = 5 \times 10^{-5}$ *cm./sec.*

gelatin and two fractions of gelatin (second and third fraction) under similar conditions. These stock emulsions were pretreated with 0.352 *M* sodium sulfate for 24 hr. prior to the release experiments in the various electrolyte concentrations. The differences in the release-rate data among the three fractions of gelatin used were within experimental error.

Effect of Electrolytes—The experimental results obtained with five electrolytes—sodium sulfate, sodium chloride, calcium chloride, magnesium chloride, and magnesium sulfate—are shown in Figs. 4–8. In these runs an emulsion system of essentially the same particle size distribution was pretreated with a 0.352 *M* solution of the particular electrolyte for 24 hr. at 40°. The release medium was either 0.352 *M*, 0.176 *M* electrolyte, or water at 23°.

These experiments showed that both electrolyte type and concentration have significant influences upon the transport rate.

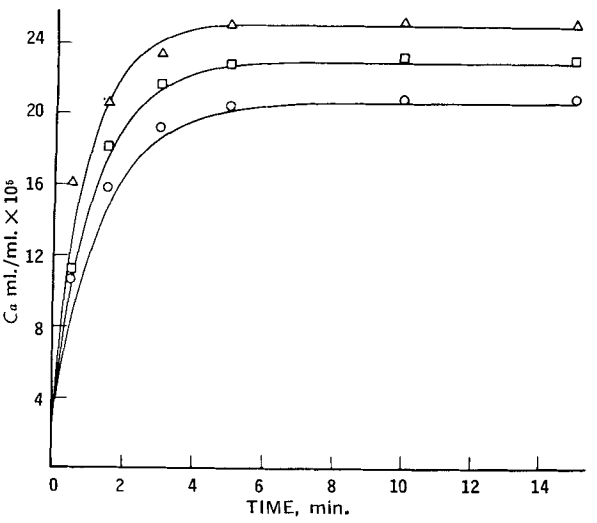


Figure 7—*Influence of electrolyte and the comparison of experimental release data with the theoretically computed ones.* C_a *is the amount of diethylphthalate released, ml./ml.,* versus *time in minutes. A 3.0-ml. aliquot of the emulsion system pretreated with 0.352* M *magnesium chloride was added to 100 ml. of aqueous medium at 23°. Key: experimental points,* ○, *0.352* M *magnesium chloride;* □, *0.176* M *magnesium chloride;* △, *water. Curves are theoretical values computed using Eqs. 1 and 2 with* $P = 5 \times 10^{-5}$ *cm./sec.*

However, as will be seen, the effects may be attributed entirely to the influences of salts upon the partition coefficient (see Table II) and not the permeability coefficients.

Figure 9 gives the results obtained employing different electrolyte concentrations in the pretreatment media prior to the release runs. As can be seen, little or no influence of the pretreatment was found. This suggested that, as far as salt interactions were concerned, essentially equilibrium conditions were present during the release period.

Effect of Surfactants—The influences of two ionic surfactants, sodium lauryl sulfate and dodecylpyridinium chloride, upon the release rate in sodium sulfate solutions are shown in Figs. 10–13. The effects of both of these surfactants were found to be similar

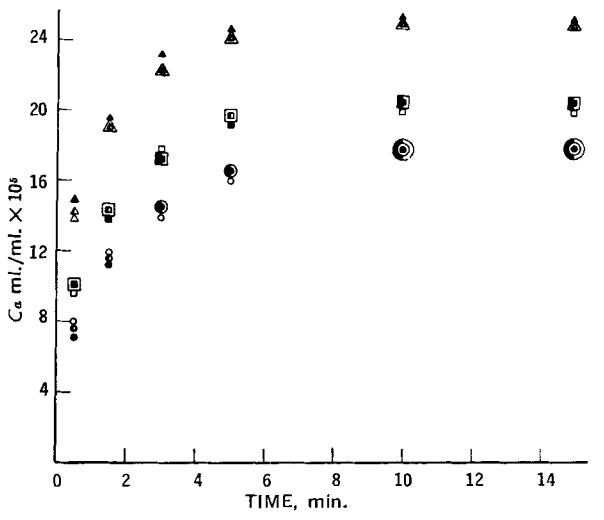


Figure 9—*Comparison of the experimental release of diethylphthalate from emulsion systems pretreated with different electrolyte concentrations. Three milliliters of emulsion system was added to 100 ml. of aqueous medium at 23°. Emulsion system was pretreated with 0.352* M *sodium sulfate and released in:* ●, *0.352* M *sodium sulfate;* ■, *0.176* M *sodium sulfate;* ▲, *water. Emulsion system was pretreated with 0.176* M *sodium sulfate and released in:* ◐, *0.352* M *sodium sulfate;* ◧, *0.176* M *sodium sulfate;* ◭, *water. Emulsion system was without electrolyte pretreatment and released in:* ○, *0.352* M *sodium sulfate;* □, *0.176* M *sodium sulfate;* △, *water.*

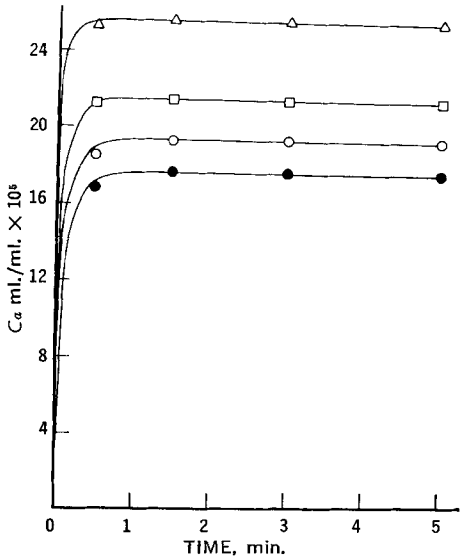


Figure 10—*Influence of sodium lauryl sulfate and comparison of the experimental release data with theory.* C_a *is amount of diethylphthalate released, ml./ml.,* versus *time in minutes. Three milliliters of emulsion system pretreated with 0.352* M *sodium sulfate and 0.1% sodium lauryl sulfate was added to 100 ml. of aqueous medium at 23°. Key: experimental points,* ●, *0.493* M *sodium sulfate;* ○, *0.352* M *sodium sulfate;* □, *0.176* M *sodium sulfate;* △, *water. The curves are theoretical values computed using Eqs. 1 and 2 and* P = 6 × 10^{-4} *cm./sec.*

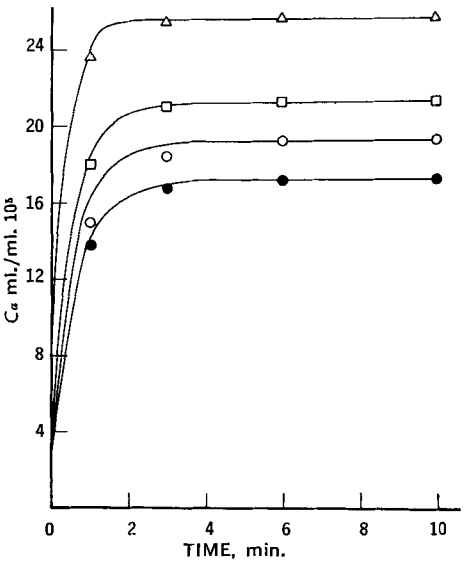


Figure 11—*Influence of sodium lauryl sulfate and comparison of the experimental release data with theory.* C_a *is amount of diethylphthalate released, ml./ml.,* versus *time in minutes. Three milliliters of emulsion system pretreated with 0.352* M *sodium sulfate and 0.01% sodium lauryl sulfate was added to 100 ml. of aqueous medium at 23°. Key: experimental points,* ●, *0.493* M *sodium sulfate;* ○, *0.352* M *sodium sulfate;* □, *0.176* M *sodium sulfate;* △, *water. The curves are theoretical values computed using Eqs. 1 and 2 and* P = 2.5 × 10^{-4} *cm./sec.*

and showed marked enhancement of the release rates at relatively low concentrations.

Experiments with polysorbate 80 were also attempted. However, the emulsions containing this material were found to be unstable with respect to coalescence. During the pretreatment stage, extensive cracking of the emulsion containing polysorbate 80 was observed.

TREATMENT OF THE DATA AND DISCUSSION

The experimental results presented in Figs. 4–13 may be analyzed by the theoretical methods discussed previously (1). Equations 1 and 2,

$$\frac{-dC_{0i}}{dt} = \frac{3DP(C_{0i}/K - C_a)}{a_i(D + a_iP)} \qquad \text{(Eq. 1)}$$

$$(C_a - E)\,V_a = \sum_{i=1}^{L} 4/3\pi\, a_i^3\, n_i\,(C_0^* - C_{0i}) \qquad \text{(Eq. 2)}$$

give the two basic relations that can be used in the treatment of the data obtained in these studies. Here C_{0i} is the solute concentration in the droplet of radius a_i, C_a is the aqueous concentration, D is the diffusion coefficient of the solute in water, P is the interfacial barrier permeability coefficient, C_0^* and E are the solute concentrations in the oil and aqueous phases at zero time, V_a is the volume of the aqueous phase, K is the oil-water partition coefficient for the solute, n_i is the number of droplets of sizes between a_i and a_{i+1}, and t is time. Equation 2 assumes (1) that no binding of the solute occurs at the oil-water interface. This assumption should be reasonable in view of the partition coefficient data in Table II which shows that the presence of gelatin at the interface does not significantly alter the experimental partition coefficients. In the previous study (1), because of the higher electrolyte concentrations used, adsorption of diethylphthalate was found to be significant, and a modified form of Eq. 2 was found to be necessary for explaining the experimental data.

Influence of Electrolytes—The results of theoretical computations employing Eqs. 1 and 2 and the computer program previously given (1) are presented as the curves in Figs. 4–8. The partition coefficients used were taken from Table II and the particle size distribution function, n_i (a_i), was taken from Table I. A value of 6 × 10^{-6} cm.^{2}/sec. was used for the diffusion coefficient. The theoretical release-time profiles were fitted to the experimental data by varying the parameter, P, the permeability coefficient for the interfacial barrier.

First it should be noted that the time-dependence agreement between experiment and theory is generally good in all cases (Figs. 4–8). The slight deviations are always in the direction of somewhat faster experimental release rates at early times.

The most significant aspects of the comparison of experiments with theory is that a single P value of 5 × 10^{-5} cm./sec. quantita-

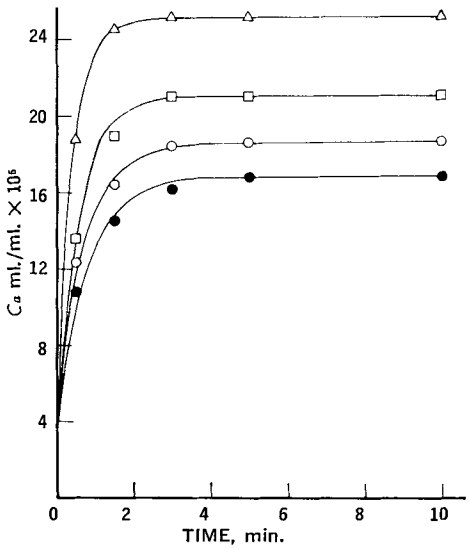


Figure 12—*Influence of sodium lauryl sulfate and comparison of the experimental release data with theory.* C_a *is amount of diethylphthalate released, ml./ml.,* versus *time in minutes. Three milliliters of emulsion system pretreated with 0.352* M *sodium sulfate and 0.001% sodium lauryl sulfate was added to 100 ml. of aqueous medium at 23°. Key: experimental points,* ●, *0.493* M *sodium sulfate;* ○, *0.352* M *sodium sulfate;* □, *0.176* M *sodium sulfate;* △, *water. The curves are theoretical values computed using Eqs. 1 and 2 and* P = 1.2 × 10^{-4} *cm./sec.*

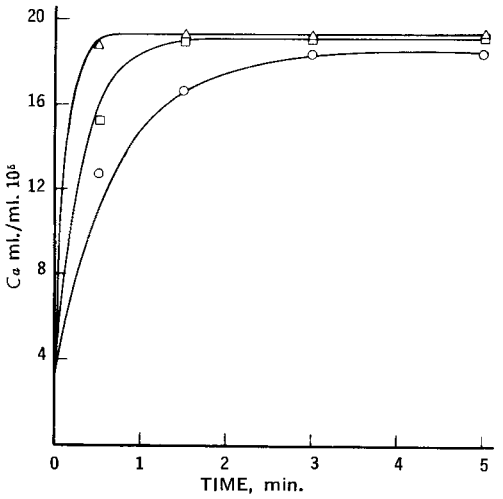


Figure 13—*Influence of dodecylpyridinium chloride (DDPCl) and the comparison of the experimental release with theory.* C_a *is amount of diethylphthalate released, ml./ml.,* versus *time in minutes. Three milliliters of emulsion system pretreated with 0.352* M *sodium sulfate and different concentrations of DDPCl was added to 100 ml. 0.352* M *sodium sulfate at 23°. Key:* △, *pretreated with 0.1%;* □, *pretreated with 0.01%;* ○, *pretreated with 0.001% DDPCl. The curves are theoretical values computed using Eqs. 1 and 2 and* P *values of* 6×10^{-4}, 2.5×10^{-4}, *and* 1.2×10^{-4} *cm./sec. for the three concentrations of DDPCl, respectively.*

tively describes the data. This was unexpected in view of the large differences in the rates found in the different electrolyte media. This can only mean that the observed differences in the rate may be attributed entirely to the effect of salts upon K, the partition coefficient. The K values given in Table II show that a significant salt effect upon K exists which may be explained on the basis of a salting-out effect (7).

The absence of the salt effect upon the permeability coefficient is somewhat surprising in view of the polyelectrolytic nature of gelatin. This effect may, however, be related in part to Veis' interpretation (8) of acid-precursor gelatin having a relatively compact structure and its configuration being less readily altered by environmental conditions. The absence of the salt effect may also be related to the concept that the main contribution to the interfacial barrier should arise from those interactions between gelatin, hexadecane, and water at the hexadecane side of the gelatin film. This effect may be enhanced by the possibility of surface denaturation (9) of the polymer at the oil interface.

Influence of Surfactants—The results of theoretical computations employing the same procedure previously mentioned are shown as the curves in Figs. 10–13. For these calculations the partition coefficients used were obtained from the terminal aqueous concentrations of the rate runs themselves. These values were generally slightly lower than those given in Table II.

First it should be noted that the shapes of the curves compare very well with the experimental results. The theoretical analysis of the surfactant effects was quite significant (Figs. 10–13). The P values obtained were 6×10^{-4}, 2.5×10^{-4}, and 1.2×10^{-4} cm./sec. for emulsion systems pretreated with 0.1, 0.01, and 0.001% surfactants, respectively. These P values were found to be independent of the electrolyte concentration.

The permeability coefficients obtained with sodium lauryl sulfate and dodecylpyridinium chloride were found to be about the same at the same percent surfactant concentration levels. Thus, it appears that both anionic and cationic surfactants operate by a similar general mechanism in influencing the permeability of diethylphthalate. These findings might be related to Pearson's studies (10, 11) which showed that cationic and anionic surfactants affect the surface viscosity of adsorbed protein films in a similar manner.

Biopharmaceutical Significance—It is noteworthy that these values for interfacial resistance are of the same order of magnitude as those observed (12) in lipid bilayer transfer studies and therefore generate the idea that biological membrane barriers might be largely of interfacial nature. Also, as pointed out by Ghanem *et al.* (1), the low apparent diffusion coefficient (10^{-9}–10^{-16} cm.^{2} sec.$^{-1}$) observed by Blank *et al.* (13) for the stratum corneum is of the same order of magnitude expected from the data obtained in these experiments. Other noteworthy results of this study are that the role of ionic surfactants in enhancing interfacial transport may closely parallel the influence of ionic surfactant in, for example, erythrocyte membrane permeability (14) or in enhancing transfer of drugs through the stratum corneum (15).

REFERENCES

(1) A. H. Ghanem, W. I. Higuchi, and A. P. Simonelli, *J. Pharm. Sci.*, **58**, 165(1969).

(2) A. Bikhazi and W. I. Higuchi, to be published.

(3) A. H. Goldberg, W. I. Higuchi, N. F. H. Ho, and G. Zografi, *J. Pharm. Sci.*, **56**, 1432(1967).

(4) A. H. Ghanem, W. I. Higuchi, and A. P. Simonelli, to be published.

(5) G. Stainsby, *Discussions Faraday Soc.*, **18**, 288(1954).

(6) S. G. Frank, "Micellar Solubilization of Water in Hydrocarbon Solvents," Ph.D. dissertation, University of Michigan, 1968.

(7) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold, New York, N. Y., 1958, p. 536.

(8) A Veis and J. Cohen, *J. Polymer Sci.*, **26**, 113(1957).

(9) C. W. N. Cumper and A. E. Alexander, *Trans. Faraday Soc.*, **46**, 235(1950).

(10) J. T. Pearson and A. E. Alexander, *J. Colloid Interface Sci.*, **27**, 53(1968).

(11) J. T. Pearson, *ibid.*, **27**, 64(1968).

(12) R. C. Bean, W. C. Shepherd, and H. Chan, *J. Gen. Physiol.*, **52**, 495(1968).

(13) I. H. Blank, R. J. Scheuplein, and D. J. Macfarlane, *J. Invest. Dermatol.*, **49**, 582(1967).

(14) M. R. J. Salton, *J. Gen. Physiol.*, **52**, 227(1968).

(15) A. J. Aguiar and M. A. Weiner, *J. Pharm. Sci.*, **58**, 210 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 16, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication September 24, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported in part by grant GM-13368 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md., and by contract PH43-68-1284 with Chemotherapy, National Cancer Institute, National Institutes of Health.

Gas Chromatographic Determination of Glycerol Guaiacolate in Pharmaceutical Preparations

JOSEPH HUDANICK

Abstract □ A sensitive gas chromatographic method was developed for the assay of glycerol guaiacolate contained in various pharmaceutical preparations. The method, based upon the chromatography of the trimethylsilyl derivative, has good precision and the desired specificity. A choice of columns and operating conditions is given in order to facilitate assaying a variety of formulations.

Keyphrases □ Glycerol guaiacolate dosage forms—analysis □ Salicylate-glycerol guaiacolate dosage forms—analysis □ GLC—analysis

Glycerol guaiacolate, 3-(*o*-methoxyphenoxy)-1,2-propanediol, is used in various asthma, cough-preventive, and similar preparations as the expectorant-antitussive ingredient.

Its determination is difficult since it is usually found in formulations containing several ingredients and a satisfactory specific color reaction has not been reported. A separation of the compound from the other ingredients is generally required prior to assay. Among the assay methods reported in the literature are: a thin-layer method (1), a paper chromatographic method (2, 3), and a gas chromatographic (4) method *via* the acetate derivative, with all of the methods being time consuming even though they do have a measure of specificity. The gas chromatographic method requires a 90-min. esterification step in addition to extraction.

A simpler and more rapid method was needed in the author's laboratory for the assay of the compound in a variety of formulations, and it was found that the most suitable one was a gas chromatographic determination *via* the trimethylsilyl derivative. Using this approach, it was found that ingredients commonly found associated with glycerol guaiacolate such as ephedrine sulfate, theophylline, chlorpheniramine maleate, and phenobarbital did not interfere. Interference from salicylates is eliminated by extracting glycerol guaiacolate from an alkaline solution with chloroform prior to formation of the derivative.

For the chromatography of the trimethylsilyl derivative, either a 45.72-cm. (1.5-ft.) column packed with 5% SE-52 on diatomaceous earth[1] or a 152.4-cm. (5-ft.) column packed with 5% SE-30 on diatomaceous earth[1] is satisfactory for use with a flame ionization-equipped gas chromatograph. The trimethylsilyl derivative of *n*-butyl-*p*-hydroxybenzoate is used as an internal standard when assaying for glycerol guaiacolate. Typical chromatograms are shown in Figs. 1 and 2. The retention times for the glycerol guaiacolate trimethylsilyl derivative are 8.5 min. on the SE-30 column at 150° and 7.5 min. on the SE-52 column at 130°. The *n*-butyl-*p*-hydroxybenzoate derivative has a retention time of 5.5 min. on the SE-30 column at 150° and 5.5 min. on the SE-52 column at 130°.

[1] Gas Chrom Q, Applied Science Laboratories, Inc., State College, Pa.

EXPERIMENTAL

Instrumentation—For the chromatography of the derivative, a gas chromatograph equipped with flame ionization detectors (Aerograph 1520, Varian) and operated in the isothermal mode is suitable. Either a 152.71-cm. (5-ft.) long, 0.38-cm. (0.125-in.) internal diameter stainless steel column packed with 5% SE-30 on diatomaceous earth or a 46.03-cm. (1.5-ft.) long, 0.38-cm. (0.125-in.) internal diameter stainless steel column packed with 5% SE-52 on diatomaceous earth is used. The SE-30 column is used with an oven temperature of 150° and the SE-52 column is operated at 130°. In either case, the injector temperature is 225° and the detector temperature is 250° with helium as the carrier gas at a flow rate of about 50 ml./min. The detector response is recorded on a strip-chart recorder (Sargent S.R.) having a 1.0-mv. span and a chart speed of 0.2 in./min.

Reagents—Commercial catalyst reagent (Tri-Syl, Pierce Chemical Co., Rockford, Ill.), acetone, chloroform, saturated aqueous sodium chloride solution, sodium hydroxide, *n*-butyl-*p*-hydroxybenzoate (Eastman Kodak), and anhydrous sodium sulfate. All chemicals are reagent grade and the solvents are redistilled.

Relative Responses of the Trimethylsilyl Derivatives of Glycerol Guaiacolate and *n*-Butyl-*p*-hydroxybenzoate—From a chloroform solution of glycerol guaiacolate (concentration of 10 mg./ml.), transfer aliquots representing 30, 40, and 50 mg. of glycerol guaiacolate to separate 15-ml. centrifuge tubes. To each add 40 mg. of *n*-butyl-*p*-hydroxybenzoate (from a chloroform solution) and evaporate to dryness on a steam bath under a gentle current of air. Add to each tube 0.5 ml. of Tri-Syl reagent, stopper, mix thoroughly, and allow to stand for about 2–3 min. Dilute each to 10 ml. with acetone and mix thoroughly.

Inject a 2.0-μl. aliquot of each solution into the chromatograph, using either the SE-30 or SE-52 column under the conditions previously described. Record the detector response using a chart speed

Table I—Recovery of Glycerol Guaiacolate from Prepared Mixtures

Batch No.	Glycerol Guaiacolate Added, mg.	Recovery, %
1	100	98.5
1	100	100.0
2	100	98.1
2	100	101.0
3	100	98.0
3	100	99.0

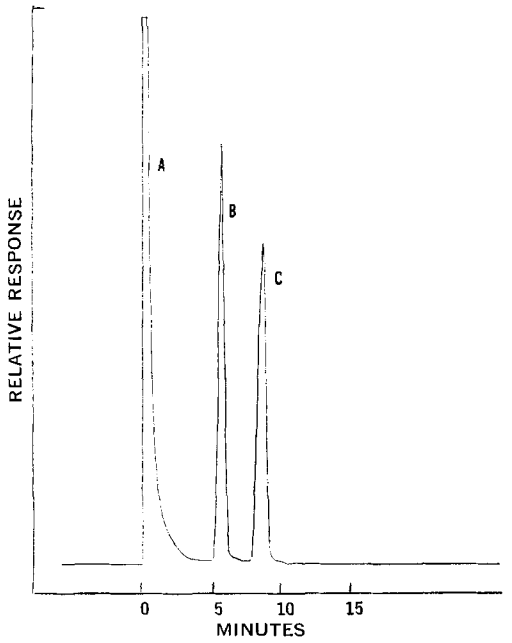


Figure 1—*Gas chromatogram of TMS derivatives of glycerol guaiacolate* (C) *and* n-*butyl*-p-*hydroxybenzoate* (B) *on 5% SE-30 at 150°. Peak* A *is the solvent.*

of 0.2 in./min. and a span voltage of 1.0 mv. Attenuation is about 32×. Calculate the relative response factor F using the equation:

$$F = (P_g/P_i) \times (W_i/W_g)$$

where P_i is the peak height of the *n*-butyl-*p*-hydroxybenzoate derivative, P_g is the peak height of the glycerol guaiacolate derivative, W_i is the weight of the *n*-butyl-*p*-hydroxybenzoate injected, and W_g is the weight of the glycerol guaiacolate injected.

The F factor used in subsequent analytical calculations is the average of the F's found.

Determination in Tablets Not Containing Salicylates—Thoroughly grind the tablets to a fine powder, weigh accurately a portion equivalent to about 100 mg. of glycerol guaiacolate, and transfer to a glass-stoppered conical flask containing 100 ml. of chloroform. Shake thoroughly for about 30 min. and then filter or centrifuge to obtain a clear solution. Transfer an aliquot containing the equivalent of about 40 mg. of glycerol guaiacolate to a glass-stoppered conical flask, add 40 mg. of the internal standard, and evaporate to dryness. Then add 0.5 ml. of Tri-Syl, stopper, and allow to stand for 2–3 min. Dilute the mixture to 10 ml. with acetone and mix thoroughly. Inject a 2.0-μl. aliquot into the chromatograph and record the response as previously described. Calculate the amount of glycerol guaiacolate per tablet as follows: glycerol guaiacolate mg./tablet $= (P_g/P_i) \times (W_b/F) \times (D/S)$, where P_g is the peak height of the glycerol guaiacolate, P_i is the peak height of the internal standard, W_b is the weight of the internal standard, D is the dilution factor, S is the sample weight, and F is the average response factor.

Determination in Tablets and Fluids Containing Salicylates—Transfer an accurately measured sample equivalent to about 200 mg. of glycerol guaiacolate to a separator, dilute to about 25 ml. with water, add 25 ml. of saturated sodium chloride, and make alkaline with sodium hydroxide. Extract with five 45-ml. portions of chloroform, filter through anhydrous sodium sulfate, and combine the chloroform extracts in a 250-ml. volumetric flask. Dilute to volume with chloroform. Withdraw an aliquot containing about 40 mg. of glycerol guaiacolate, add 40 mg. of internal standard, and proceed as previously described.

Recovery of Glycerol Guaiacolate from Mixtures—In order to test the recovery of glycerol guaiacolate when compounded into various mixtures, three batches of tablets were made containing the following ingredients: Batch 1, glycerol guaiacolate 100 mg., phenobarbital 16.25 mg. (0.25 grain), theophylline 162.5 mg. (2.5 grains), ephedrine sulfate 25 mg., and excipient. Batch 2, glycerol guaiacolate 100 mg., theophylline 162.5 mg. (2.5 grains), ephedrine sulfate 25 mg., chlorpheniramine maleate 2.0 mg., and excipient. Batch 3, glycerol guaiacolate 100 mg., sodium salicylate 130 mg. (2.0 grains), ephedrine sulfate 25 mg., theophylline 130 mg. (2.0 grains), and excipient.

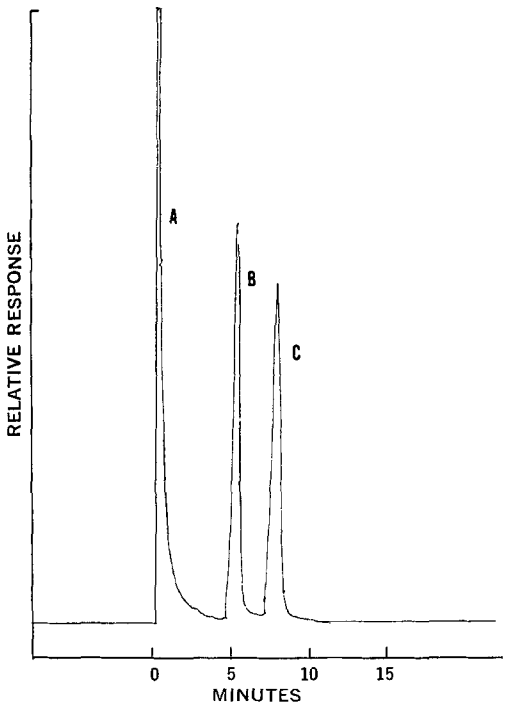


Figure 2—*Gas chromatogram of TMS derivatives of glycerol guaiacolate* (C) *and* n-*butyl*-p-*hydroxybenzoate* (B) *on 5% SE-52 at 130°. Peak* A *is the solvent.*

The results of the assays are shown in Table I.

CONCLUSIONS

An average recovery of 99.1% (SD = 1.1) may be achieved by the gas chromatographic method of assay of fluids and tablets. The method is straightforward, rapid, and has the desired specificity. A choice of columns and operating conditions is given for assaying various formulations. The amount of glycerol guaiacolate that can be estimated may be quite small by using the gas chromatograph to the limit of its sensitivity and by manipulation of the sample size.

REFERENCES

(1) J. Večerkova and E. Novotna, *Cesk. Farm.*, **14,** 462(1965).
(2) I. Hynie, K. Hatle, and J. König, *ibid.*, **14,** 481(1965).
(3) S. Ahuja, *J. Pharm. Sci.*, **57,** 313 (1968).
(4) T. Hattori, S. Kawai, and M. Nishiumi, *Japan Analyst*, **14,** 586(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 11, 1969, from the *Rexall Drug Co., The St. Louis Laboratories, St. Louis, Mo.*
Accepted for publication September 3, 1969.

A Column Chromatographic Method for the Determination of Sulfanilamide in Pharmaceutical Preparations Containing Sulfacetamide or Its Sodium Salt

EDWARD J. WOJTOWICZ

Abstract □ A simple and rapid method, using column-adsorption chromatography on alumina and UV spectrophotometry, has been developed for the separation and determination of sulfanilamide in ophthalmic solutions and tablets containing sulfacetamide or its sodium salt.

Keyphrases □ Sulfacetamide formulation—sulfanilamide determination □ Sulfanilamide—sulfacetamide degradation product □ Column chromatography—separation □ UV spectrophotometry—identity

The presence of undeclared sulfanilamide in preparations containing sulfacetamide has been reported (1–4). Hayden (1) found 17% sulfanilamide in a commercial sample of sulfacetamide powder, using paper chromatography and IR spectroscopy. Klein and Kho (2) reported undeclared sulfanilamide in suspensions of mixed sulfas containing sulfacetamide, using TLC. Previous work has shown that sulfacetamide preparations form sulfanilamide as a degradation product when exposed to light, extremes of temperature, or prolonged storage (3, 4). Gruber and Klein (4) used TLC on silica gel to separate the degradation product from the parent compound; the amount of sulfanilamide present was determined colorimetrically with the Bratton-Marshall reagent. Ophthalmic solutions contained contamination of 13% sulfanilamide, but tablets showed no detectable degradation over a period of 4 months.

Alexander and Stanley (5) used alumina for qualitative thin-layer chromatographic determination of sulfa drugs in feeds. The separation of sulfanilamide from sulfacetamide or its sodium salt was much greater in this system using alumina than in the silica gel system used by Gruber and Klein (4). (In both systems sulfanilamide has the greater R_f.) This large separation was the basis for adapting the qualitative thin-layer chromatographic procedure to a quantitative column chromatographic determination for sulfanilamide.

Sulfanilamide was easily separated and collected from a neutral alumina column, using the thin-layer developing solvent system of Alexander and Stanley (5). The sulfanilamide in the column eluate was quantified by UV absorption. The residue from the column eluate was also identified qualitatively as sulfanilamide by IR spectroscopy.

EXPERIMENTAL

Apparatus and Reagents—The following apparatus and reagents were used: 1-cm. (i.d.) glass columns fitted with Teflon stopcocks and sintered-glass frits (Kontes); aluminum oxide, neutral, suitable for chromatographic adsorption (Merck)[1]; spectrophotometric grade methanol and chloroform (Burdick and Jackson); USP sulfanilamide reference standard solution, 5 mcg./ml. in chloroform–methanol (70:30); a suitable recording UV spectrophotometer.

Preparation of Chromatographic Column—Heat the aluminum oxide for 30 min. at 100° before use. With the stopcock open, transfer 5.0 g. of aluminum oxide to the column in a slurry with chloroform–methanol (70:30). The solvent should drain quickly as the adsorbent settles. Wash the column with 20–25 ml. of the solvent mixture, and cover the top of the column with a pledget of glass wool. Drain the solvent to a level of 1 cm. above the glass wool.

If a flow rate of 1–2 drops per 10 sec. is encountered due to an excess of fine particles in the alumina, the column can be packed by the following procedure: place 10–15 g. of the alumina into a 250-ml. separator. Add 50 ml. of the solvent mixture and shake. The fine particles will stay suspended in the solvent. Open the stopcock of the column and add the alumina slurry from the separator to the predetermined height for 5 g. (about 7 cm.). Maintain a level of solvent above the alumina and wash with 20–25 ml. of the solvent mixture as in the previous column-packing procedure.

Sample Preparation—Transfer an accurately measured volume of ophthalmic solution or an accurately weighed portion of ground solid sample equivalent to about 200 mg. of sulfacetamide to a 100-ml. volumetric flask. Add 30 ml. of methanol and mix thoroughly. Dilute to volume with chloroform and mix again.

Procedure and Analysis—Pipet a 5-ml. aliquot of the sample solution onto the column; collect the eluate in a 50-ml. volumetric flask. After all the sample aliquot has entered the column, elute to volume with chloroform–methanol (70:30). Maintain a liquid level about 5 cm. above the alumina so that the time for collecting the 50 ml. is about 30 min. Obtain the absorbance of the sample at

Table I—Results of Determinations of Recoveries of Sulfanilamide

Sample	Sulfanilamide Found, %	Sulfanilamide, mcg. Added per Column Aliquot	Sulfanilamide, mcg. Recovered	Recovery, %
Standard 1[a] (sulfacetamide)	0.04	211	209	99.0
	0.02		210	99.5
	0.03		210	99.5
	0.05		212	100.4
	0.05		212	100.4
Standard 2[a] (sodium sulfacetamide)	0.54			
	0.52			
	0.52			
	0.52			
	0.53			
Standard 3 (recrystallized form of standard 2)	0.30	422	421	99.8
	0.28		420	99.6
	0.27		420	99.6
	0.29		418	99.1
	0.28		422	100.0
10% Ophthalmic solution of sodium sulfacetamide	2.31	211	208	98.6
	2.33		209	99.1
	2.32		210	99.5
	2.29		215	101.8
	2.29		213	100.9
30% Ophthalmic solution of sodium sulfacetamide	2.35	169	168	99.4
	2.36		168	99.4
	2.33		169	100.0
	2.33		168	99.4
	2.34		167	98.8
500-mg. Sulfacetamide tablets	0.08	252	253	100.4
	0.04		256	101.6
	0.05		252	100.0
	0.05		254	100.8
	0.05		253	100.4

[a] K & K Laboratories, Plainview, N. Y.

[1] Merck Reagent 71707 and Fisher Cat. No. A-950 were both found suitable.

262 mμ against the elution solvent blank. Compare the absorbance of the sample to that of the sulfanilamide reference standard solution.

RESULTS AND DISCUSSION

Proprietary standards of sulfacetamide and sodium sulfacetamide were analyzed by the column chromatographic procedure. A portion of the sodium sulfacetamide was recrystallized and also analyzed. All three standards were eluted with an additional 30 ml. of elution solvent after the first 50 ml. was collected. The 50-ml. fraction exhibited absorbance maxima at 262 mμ; the subsequent eluates did not show UV absorbance. Therefore, the sulfanilamide was completely eluted and no additional sulfanilamide or any sulfacetamide was present in subsequent 30-ml. eluates. However, each batch of alumina should be tested to ensure that the desired separation occurs in the first 50 ml. of eluting solvent.

Sulfanilamide was added to the free sulfacetamide and to the recrystallized sodium salt, and recoveries were obtained. Data from analyses using these standards are reported in Table I.

Two buffered ophthalmic solutions of 10 and 30% sodium sulfacetamide and a tablet form of sulfacetamide, 500-mg. label declaration, were assayed for their sulfanilamide content. Known amounts of sulfanilamide were added and percent recoveries were determined. Data from these analyses showed that the ophthalmic solutions contained a larger percentage of sulfanilamide than the tablets. This agrees with the quantitative findings of Gruber and Klein (4).

The USP XVII (6) or NF XII (7) procedure failed to detect any degradation of sulfacetamide preparations since it is a general titrimetric procedure for sulfonamides.

The residue left after evaporation of the column eluate was confirmed as sulfanilamide by IR spectroscopy, employing micro-KBr disk techniques.

All standards and samples used in this study were analyzed by the screening procedure of Gruber and Klein (4) on silica gel. Amounts were spotted to yield about 0.1 mcg. of sulfanilamide as determined by the column chromatographic procedure. Sulfanilamide was detected in all cases except in the sulfacetamide standard and the tablet form of sulfacetamide. These also gave the lowest results by the column chromatographic procedure.

The method described in this paper can also be used to determine purity of reference standards of the free sulfa or its sodium salt to meet compendial requirements. Levels of sulfanilamide below 1% can be quantitated.

REFERENCES

(1) A. Hayden, *J. Pharm. Sci.*, **51**, 617(1962).

(2) S. Klein and B. T. Kho, *ibid.*, **51**, 1966(1962).

(3) G. Fletcher and D. A. Norton, *Pharm. J.*, **191**, 145(1963).

(4) M. P. Gruber and R. W. Klein, *J. Pharm. Sci.*, **57**, 1212(1968).

(5) L. R. Alexander and E. R. Stanley, *J. Assoc. Offic. Agr. Chemists*, **48**, 278(1965).

(6) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

(7) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 29, 1969, from *Food and Drug Administration, Buffalo, NY 14202*

Accepted for publication September 17, 1969.

Selective Determination of Isoproterenol and Isoproterenol Sulfonic Acid in Pharmaceutical Dosage Forms

K. K. KAISTHA

Abstract □ Two simple, precise, and specific methods for the determination of isoproterenol in decomposed formulations are described. DEHP shakeout procedure, which depends upon ion-pair formation with the unchanged drug at a suitable pH, enables the quantitative determination of isoproterenol from the DEHP–ether phase and of its sulfonic acid from the aqueous buffer phase. Isoproterenol sulfonic acid can then be determined selectively by treating the aqueous buffer phase with Doty's reagents. The sodium metaperiodate method has been developed as an alternate checking procedure to validate the results obtained by the DEHP method and involves the quantitative formation of an aryl aldehyde of the unchanged drug. The merits of both the procedures over the conventional UV and visible spectrophotometric procedures are shown by their application to the analysis of simulated decomposed formulation, aged simulated inhalations and injections, and commercial formulations. A thin-layer chromatographic procedure for the separation and detection of isoproterenol and its sulfonic acid and other artifacts is described.

Keyphrases □ Isoproterenol dosage forms—analysis □ Isoproterenol sulfonic acid formed in products—analysis □ Degradation products presence—isoproterenol determination □ Di-(2-ethylhexyl)phosphoric acid extraction method—isoproterenol determination □ Metaperiodate sodium method—isoproterenol determination □ UV spectrophotometry—analysis □ TLC—analysis

The selective determination of isoproterenol (3,4-dihydroxy-α-[(isopropylamino)methyl]-benzyl alcohol hydrochloride), in the presence of isoproterenol sulfonic acid and other decomposition products or vice versa, presents an unusually difficult analytical problem. Recently it became evident that the composition of pharmaceutical dosage forms, especially inhalations and injections containing isoproterenol hydrochloride, can change with aging. The change is attributed to the interaction of isoproterenol with the bisulfite antioxidant, by which the alcoholic hydroxyl group of the drug is replaced by a sulfonic acid group (1) as shown in

262 mμ against the elution solvent blank. Compare the absorbance of the sample to that of the sulfanilamide reference standard solution.

RESULTS AND DISCUSSION

Proprietary standards of sulfacetamide and sodium sulfacetamide were analyzed by the column chromatographic procedure. A portion of the sodium sulfacetamide was recrystallized and also analyzed. All three standards were eluted with an additional 30 ml. of elution solvent after the first 50 ml. was collected. The 50-ml. fraction exhibited absorbance maxima at 262 mμ; the subsequent eluates did not show UV absorbance. Therefore, the sulfanilamide was completely eluted and no additional sulfanilamide or any sulfacetamide was present in subsequent 30-ml. eluates. However, each batch of alumina should be tested to ensure that the desired separation occurs in the first 50 ml. of eluting solvent.

Sulfanilamide was added to the free sulfacetamide and to the recrystallized sodium salt, and recoveries were obtained. Data from analyses using these standards are reported in Table I.

Two buffered ophthalmic solutions of 10 and 30% sodium sulfacetamide and a tablet form of sulfacetamide, 500-mg. label declaration, were assayed for their sulfanilamide content. Known amounts of sulfanilamide were added and percent recoveries were determined. Data from these analyses showed that the ophthalmic solutions contained a larger percentage of sulfanilamide than the tablets. This agrees with the quantitative findings of Gruber and Klein (4).

The USP XVII (6) or NF XII (7) procedure failed to detect any degradation of sulfacetamide preparations since it is a general titrimetric procedure for sulfonamides.

The residue left after evaporation of the column eluate was confirmed as sulfanilamide by IR spectroscopy, employing micro-KBr disk techniques.

All standards and samples used in this study were analyzed by the screening procedure of Gruber and Klein (4) on silica gel. Amounts were spotted to yield about 0.1 mcg. of sulfanilamide as determined by the column chromatographic procedure. Sulfanilamide was detected in all cases except in the sulfacetamide standard and the tablet form of sulfacetamide. These also gave the lowest results by the column chromatographic procedure.

The method described in this paper can also be used to determine purity of reference standards of the free sulfa or its sodium salt to meet compendial requirements. Levels of sulfanilamide below 1% can be quantitated.

REFERENCES

(1) A. Hayden, *J. Pharm. Sci.*, **51**, 617(1962).

(2) S. Klein and B. T. Kho, *ibid.*, **51**, 1966(1962).

(3) G. Fletcher and D. A. Norton, *Pharm. J.*, **191**, 145(1963).

(4) M. P. Gruber and R. W. Klein, *J. Pharm. Sci.*, **57**, 1212(1968).

(5) L. R. Alexander and E. R. Stanley, *J. Assoc. Offic. Agr. Chemists*, **48**, 278(1965).

(6) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

(7) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 29, 1969, from *Food and Drug Administration, Buffalo, NY 14202*

Accepted for publication September 17, 1969.

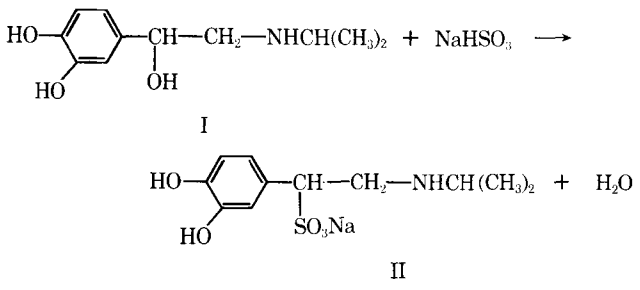
Selective Determination of Isoproterenol and Isoproterenol Sulfonic Acid in Pharmaceutical Dosage Forms

K. K. KAISTHA

Abstract □ Two simple, precise, and specific methods for the determination of isoproterenol in decomposed formulations are described. DEHP shakeout procedure, which depends upon ion-pair formation with the unchanged drug at a suitable pH, enables the quantitative determination of isoproterenol from the DEHP–ether phase and of its sulfonic acid from the aqueous buffer phase. Isoproterenol sulfonic acid can then be determined selectively by treating the aqueous buffer phase with Doty's reagents. The sodium metaperiodate method has been developed as an alternate checking procedure to validate the results obtained by the DEHP method and involves the quantitative formation of an aryl aldehyde of the unchanged drug. The merits of both the procedures over the conventional UV and visible spectrophotometric procedures are shown by their application to the analysis of simulated decomposed formulation, aged simulated inhalations and injections, and commercial formulations. A thin-layer chromatographic procedure for the separation and detection of isoproterenol and its sulfonic acid and other artifacts is described.

Keyphrases □ Isoproterenol dosage forms—analysis □ Isoproterenol sulfonic acid formed in products—analysis □ Degradation products presence—isoproterenol determination □ Di-(2-ethylhexyl)phosphoric acid extraction method—isoproterenol determination □ Metaperiodate sodium method—isoproterenol determination □ UV spectrophotometry—analysis □ TLC—analysis

The selective determination of isoproterenol (3,4-dihydroxy-α-[(isopropylamino)methyl]-benzyl alcohol hydrochloride), in the presence of isoproterenol sulfonic acid and other decomposition products or vice versa, presents an unusually difficult analytical problem. Recently it became evident that the composition of pharmaceutical dosage forms, especially inhalations and injections containing isoproterenol hydrochloride, can change with aging. The change is attributed to the interaction of isoproterenol with the bisulfite antioxidant, by which the alcoholic hydroxyl group of the drug is replaced by a sulfonic acid group (1) as shown in



Scheme I

Scheme I. Higuchi *et al.* (2, 3) have reported a similar type of reaction between bisulfite and epinephrine and other suitably substituted benzyl alcohol derivatives. The UV spectrum of the physiologically inactive product has the same absorption maximum as that of isoproterenol. Thus the current USP XVII (4) assay procedure based on spectrophotometric measurement of the active drug at 279 mμ will not yield true values for the unchanged drug in decomposed formulations. Similarly the colorimetric procedure, as described in the BP 1968 (5) for tablets is not specific for isoproterenol, as any impurity such as isoproterenol sulfonic acid possessing at least two phenolic groups attached to adjacent carbon atoms will undergo this color reaction (6).

Recently, a notable improvement in the determination of unchanged isoproterenol in pharmaceutical dosage forms was achieved by Welsh and Sammul (1), who used the principle of ion-pair formation of the drug with di-(2-ethylhexyl)phosphoric acid (DEHP) at a suitable pH. This idea of DEHP–organic base ion-pair formation was originally contributed by Temple (7) and Temple and Gillespie (8), who reported that several phenolic phenethylamines, including isoproterenol, show partition coefficients favoring their extraction into a chloroform phase containing DEHP from an aqueous phase adjusted to a suitable pH. Levine and Doyle (9) extracted phenylephrine quantitatively as its DEHP ion-pair, using partition chromatography and an ether solution of DEHP as a mobile phase. In the method proposed by Welsh and Sammul (1), a portion of the sample buffered to pH 5.8 is used as a stationary phase supported on a diatomaceous earth[1] column. The passage of an ether solution of DEHP through the column extracts the DEHP–isoproterenol ion-pair while the sulfonic acid is retained in the stationary phase. This method, when applied to simulated preparations, gave higher values for isoproterenol. The results could not be reproduced unless a step to wash the DEHP–ether effluent (from the diatomaceous earth column) with buffer was introduced prior to the extraction with 0.1 *N* sulfuric acid. Although the method as modified was found specific in the presence of Compound II (Scheme I), the recoveries for unchanged drug in the aged simulated inhalations were found higher due to some additionally encountered decomposition products.

By modification of Hellberg's fluorimetric assay for epinephrine (10), Pratt *et al.* (referred to in *Reference 1*) were able to analyze aged isoproterenol solutions in the presence of Compound II. No published data are yet available about the variability or about the inherent instability of this lutin method when applied to aged pharmaceutical dosage forms of isoproterenol.

The purpose of this communication is to present two simple, rapid, accurate, and selective assay procedures for the quantitative determination of unchanged isoproterenol in aged formulations. The chromatographic method of Welsh and Sammul (1) based on ion-pair formation with DEHP has been modified to a simple shakeout procedure. An aliquot of the formulation (without dilution) is transferred to a separator containing potassium phosphate buffer and extracted with DEHP–ether solution. The DEHP–ether solution after washing with buffer and water, respectively, is extracted with dilute sulfuric acid to partition the unchanged isoproterenol as its sulfate into the aqueous phase. The UV absorbance of the sulfate is then determined. The aqueous-buffer phase left after DEHP–ether extraction is diluted to a definite volume. The UV absorbance is measured in order to calculate the amount of total water-soluble decomposition products. Subsequently, an aliquot of this is treated with aminoacetate buffer and ferrous sulfate–citrate reagent (Doty reaction) (6) to determine quantitatively the amount of isoproterenol sulfonic acid (Compound II) present in the formulation. The second method developed in these laboratories consists of treating a suitably diluted aliquot of the preparation with 2% aqueous solution of sodium metaperiodate, extracting the resulting aryl aldehyde of the unchanged drug with chloroform, and then measuring its UV absorption at 270 mμ. This reaction is specific to those compounds having two vicinal hydroxyl groups; thus Compound II or aminochrome-type oxidation products analogous to adrenaline (11) do not yield this aldehyde. This reagent was first used by Heimlich *et al.* for the determination of phenylpropanolamine in the urine (12) and later on by Chafetz (13) for the determination of some phenethanolamine drugs having one or no phenolic group. However, as far as the author is aware, there is no reference in the literature which describes an assay procedure for the determination of catecholamines using this reagent.

EXPERIMENTAL

Preparation of Isoproterenol Sulfonic Acid—This was prepared by the modification of the Schroeter and Higuchi method described for the preparation of 1-(3,4-dihydroxylphenyl)-2-methylaminoethane sulfonic acid from epinephrine (14). Isoproterenol sulfate, 0.018 mole (5.0 g.), and sodium bisulfite, 0.018 mole (1.869 g.), were dissolved in about 25 ml. of water (pH of the reaction solution was 5.0). The solution was heated on a steam bath while keeping it flushed with nitrogen. After 1.50 hr., the flask was removed from the steam bath (pH of the yellowish solution was 6.4) and stored in the dark after flushing with nitrogen. At the end of 3 days, the excess water (about 15 ml.) was removed with a rotary evaporator and the residual solution was stored in the dark at room temperature for a further period of 6 days. The white crystalline precipitate that separated during storage was collected on a Büchner funnel, was washed with water to remove unchanged isoproterenol sulfate, and was dried under vacuum at 60° for 2 hr. Yield: 4.90 g. (70%). The product was recrystallized once from 1 *N* HCl and twice from water to give prisms, m.p. 191–194° with decomposition (Fisher-John hot stage).

[1] Celite, Johns-Manville Products Corp., New York, N. Y.

Anal.—Calcd. for $C_{11}H_{17}NO_5S \cdot H_2O$: C, 45.1; H, 6.18; N, 4.78; O, 32.76; S, 10.92. Found: C, 45.24; H, 6.12; N, 4.75; O, 32.75; S, 11.13. (Analysis was performed after drying the sample for 7 hr. under vacuum at 100°.)

ANALYTICAL METHODS

Reagents—*DEHP–Ether Solution*—Five milliliters DEHP in 140 ml. ether, washed with four 10-ml. portions of water (water washings discarded).

Potassium Phosphate Buffer—Two volumes of 1 *M* K_2HPO_4 (dibasic potassium phosphate) were mixed with eight volumes of 1 *M* KH_2PO_4 (monobasic potassium phosphate), and the pH was adjusted to 5.8 ± 0.05 with a pH meter.

Iron Reagents—(*a*) Ferrous sulfate heptahydrate (1.5 g.) in 200 ml. of distilled water to which 1 ml. of 1 *N* HCl had been added. (*b*) Ferrous sulfate–citrate solution prepared by adding 0.5 g. of sodium citrate to 10 ml. of ferrous solution (*a*) (freshly prepared before use).

Aminoacetate Buffer—Solution of sodium bicarbonate (42 g.) and potassium bicarbonate (50 g.) in 180 ml. of water was mixed with solution of aminoacetic acid (37.5 g.) and strong ammonia (17 ml.) in 180 ml. of water, and volume was adjusted to 500 ml. with water (6).

Sodium Metaperiodate Solution—Sodium metaperiodate (2%) (reagent grade) in water (freshly prepared).

Sodium Bisulfite Solution—Sodium bisulfite (0.024%) (reagent grade) in water.

Standard Isoproterenol HCl Solution (*A*)—Isoproterenol HCl USP reference standard (125 mg.) in water (25 ml.) (freshly prepared). For inhalations a 2-ml. aliquot was carried through the DEHP assay procedure along with the sample solution. For tablets a 2-ml. aliquot diluted with 3 ml. of water was carried through the DEHP assay procedure along with the sample solution.

Dilute Solution (*B*)—A 4-ml. aliquot of Solution A diluted to 100 ml. with water. For injections a 10-ml. aliquot of this solution was carried through the DEHP assay procedure along with the sample solution. For the $NaIO_4$ method a 4–6-ml. aliquot of this solution was carried through the assay procedure along with the sample solution.

Standard Isoproterenol Sulfonic Acid Solution—Isoproterenol sulfonic acid (30 mg.) in 30 ml. of water was shaken mechanically for 20 min. Then 120 mg. $NaHSO_3$ was added to the clear solution, which was made to 50 ml. with water and mixed.

Sample Preparation—*Inhalations* (*0.5% Isoproterenol HCl*)—A 2-ml. aliquot of the undiluted sample (equivalent to about 10 mg. of isoproterenol HCl) was used for the DEHP method (Procedure A). A 4-ml. aliquot was diluted to 100 ml. with water and a 4–6-ml. aliquot of this diluted solution (equivalent to 0.8–1.2 mg. of isoproterenol HCl) was used for the $NaIO_4$ method (Procedure B).

Injections (*0.02% Isoproterenol HCl*)—A 10-ml. aliquot of the undiluted sample (equivalent to 2.0 mg. of isoproterenol HCl) was used for the DEHP method and a 4-ml. aliquot (equivalent to 0.8 mg. of isoproterenol HCl) was used for the $NaIO_4$ method.

Tablets (*10 mg. Isoproterenol HCl per Dosage Unit*)—Twenty tablets were weighed and finely powdered. An accurately weighed aliquot containing about 50 mg. of isoproterenol HCl was quantitatively transferred to a 25-ml. volumetric flask with the aid of water (20 ml.). The mixture was shaken on a mechanical shaker for 20 min., made to volume with water, mixed, and filtered. A 5-ml. aliquot of the filtrate (equivalent to 10 mg. of isoproterenol HCl) was used for the DEHP method. A 10-ml. aliquot of the filtrate was diluted to 100 ml. with water and a 4-ml. aliquot of the diluted solution (equivalent to 0.8 mg. of isoproterenol HCl) was used for the $NaIO_4$ method.

Determination of Unchanged Isoproterenol—*DEHP Method* (*A*)—A 2-ml. (inhalations) or 10-ml. (injections) aliquot of the undiluted sample or 5-ml. aliquot of the aqueous filtrate in the case of tablets (prepared as already described) was transferred to a 250-ml. separator containing 3 ml. of potassium phosphate buffer (pH 5.8) and extracted with 50 ml. of DEHP–ether solution. The aqueous buffer phase was transferred to a 60-ml. separator and, after extracting successively with four 20-ml. portions of DEHP–ether solution, was drained into a 25-ml. volumetric flask. The DEHP–ether solutions were combined and washed first with two 1-ml. portions of potassium phosphate buffer and then with 2 ml. of water. These washings were combined with the aqueous buffer phase in the volumetric flask, made to volume with distilled water, and mixed (reserved for the determination of total water-soluble degradation products).

The combined residual DEHP–ether solutions were then extracted with five 25-ml. portions of 0.1 *N* H_2SO_4. Acid extracts were combined in a 200-ml. volumetric flask, made to volume with 0.1 *N* H_2SO_4, and mixed. The absorbance of the acid solution of the sample and that of the standard isoproterenol HCl carried simultaneously through the assay procedure were measured at 279 mμ against 0.1 *N* H_2SO_4 blank obtained by carrying a reagent blank through the assay procedure.

Calculations—*For Inhalations and Injections* (*a*)—

$$\text{mg. isoproterenol HCl/100 ml. formulation} = \frac{Au}{As} \times Cs \times 200 \times \frac{100}{Vu}$$

where Au = absorbance of acid solution of the sample after dilution to final volume, As = absorbance of acid solution of the standard after dilution to final volume, Cs = final concentration of isoproterenol HCl standard (mg./ml.) in acid solution after dilution to volume, and Vu = milliliters of the formulation taken.

For Tablets (*b*)—

$$\text{mg. isoproterenol HCl/tablet} = \frac{Au}{As} \times \frac{Ws \times Wo}{Wu}$$

where Au = absorbance of acid solution of the sample after dilution to final volume, As = absorbance of acid solution of the standard after dilution to final volume, Ws = weight of standard isoproterenol HCl (mg.), Wu = weight of sample (mg.), and Wo = tablet average weight (mg.).

Sodium Metaperiodate Method (*B*)—A 4-ml. aliquot of the diluted sample solution (for inhalations or tablets) or 4-ml. aliquot of the undiluted sample (for injections) was transferred to a 60-ml. separator, treated with 6 ml. of 2% sodium metaperiodate, mixed, stoppered, and allowed to stand overnight at room temperature. After 18 hr., the reaction solution was extracted successively with two 3-ml. and one 3.8-ml. portions of chloroform. The chloroform solutions were combined in a 10-ml. volumetric flask, made to volume with chloroform, and mixed. A 4-ml. aliquot of the dilute solution (Solution B) of isoproterenol HCl standard, after treating with appropriate volume of $NaHSO_3$ solution (containing $NaHSO_3$ equivalent to the amount present in the sample solution, *i.e.*, 2 ml. for inhalations containing 0.3% $NaHSO_3$; 1.3 ml. for inhalations containing 0.2% $NaHSO_3$; 1.0 ml. for injection containing 0.1% $NaHSO_3$; 0.67 ml. for tablets containing 2 mg. of $NaHSO_3$ per dosage unit), and 6 ml. of sodium metaperiodate solution were simultaneously carried through the assay procedure. The absorbance of the chloroform solution of the sample and that of the standard were measured concomitantly at 270 mμ after allowing the solutions to stand in the cells for 1 min. The chloroform solution used in the reference cell was obtained by carrying the reagent blank, *i.e.*, sodium metaperiodate and sodium bisulfite solutions, through the assay procedure.

Calculations—*For Inhalations* (*a*)—

$$\text{mg. isoproterenol HCl/100 ml. of formulation} = \frac{Au}{As} \times Cs \times 10 \times \frac{100}{4} \times \frac{100}{Vu}$$

where Au = absorbance of chloroform solution of the sample after dilution to final volume, As = absorbance of chloroform solution of the standard after dilution to final volume, Cs = final concentration of isoproterenol HCl standard (mg./ml.) in $CHCl_3$ after dilution to final volume, and Vu = milliliters of the formulation taken for dilution.

For Injections (*b*)—

$$\text{mg. isoproterenol HCl/100 ml. of formulation} = \frac{Au}{As} \times Cs \times 10 \times \frac{100}{Vu}$$

where Vu = milliliters of the formulation treated with $NaIO_4$ and the rest same as above.

For Tablets (*c*)—Same as given in Procedure A for tablets.

Determination of Isoproterenol Sulfonic Acid—A 2-ml. aliquot of the isoproterenol sulfonic acid standard (for inhalations) or 2-ml. aliquot diluted with 8 ml. of water (for injections) or 2-ml. aliquot diluted with 3 ml. of water (for tablets) was carried through the DEHP assay (Method A) simultaneously with the formulation. The absorbance of the aqueous buffer phase of the standard and that of the sample solution as obtained under *Determination of Unchanged Isoproterenol* (Method A) were measured at 279 mμ. The aqueous buffer solution used in the reference cell was obtained by carrying a reagent blank (potassium phosphate buffer and the appropriate volume of the distilled water containing sodium bisulfite) through the assay procedure. These absorbance values were used to deduce the amount of total water-soluble degradation products with reference to isoproterenol sulfonic acid.

Isoproterenol sulfonic acid was then determined colorimetrically by treating a 6-ml. aliquot each of the aqueous buffer solution of the sample and that of the standard (aqueous buffer solutions left after UV determination) with 3.9 ml. of aminoacetate buffer and 0.1 ml. of ferrous sulfate–citrate reagent. The purple color formed was measured after 2.50 hr. at 530 mμ against the reagent blank. Alternatively, a 2-ml. (inhalation) or 10-ml. (injection) aliquot of the undiluted sample was carried through the DEHP assay (Method A) starting at: "A 2-ml. (inhalations) or 10-ml. (injections) aliquot of the undiluted..." and ending at "these washings were combined with aqueous buffer phase." These combined aqueous buffer solutions were treated with 10 ml. of aminoacetate buffer and 0.1 ml. of ferrous sulfate–citrate reagent, made to 25 ml. with water, and mixed. The absorbance of the sample solution and that of the isoproterenol sulfonic acid standard (a 2-ml. aliquot) carried simultaneously through the assay procedure were measured at 530 mμ against the reagent blank. The amount of isoproterenol sulfonic acid (mg./100 ml. of formulation) was calculated from the respective absorbance values.

Determination of Total Catechols as Isoproterenol—*Modified Colorimetric Method*—A 3-ml. aliquot of the diluted sample solution (for inhalations or tablets) or 3-ml. aliquot of the undiluted sample solution (for injections) containing the equivalent of 0.6 mg. of isoproterenol HCl was treated with 1.9 ml. of aminoacetate buffer and 0.1 ml. of ferrous sulfate–citrate solution in a 10-ml. volumetric flask, made to volume with distilled water, and mixed. A 3-ml. aliquot of the dilute solution of isoproterenol HCl standard (Solution B), after treating with appropriate volume of $NaHSO_3$ solution (containing $NaHSO_3$ equivalent to the amount present in the sample solution, *i.e.*, 1.5 ml. for inhalations containing 0.3% $NaHSO_3$; 1.0 ml. for inhalations and injections containing 0.2% $NaHSO_3$ or 0.1% $NaHSO_3$, respectively; 0.51 ml. for tablets containing 2 mg. of $NaHSO_3$ per dosage unit) was concurrently carried through the assay procedure. The absorbance of the colored solutions of the sample and that of the standard were measured after 2.50 hr. at 530 mμ against the reagent blank.

USP Method—An aliquot of the inhalation (equivalent to 50 mg. of isoproterenol HCl) or injection (equivalent to 5.0 mg. of isoproterenol HCl) or tablet powder (equivalent to 50 mg. of isoproterenol HCl) was carried through the assay procedure as described in USP XVII, pp. 330–332. The absorbance of the sample and that of the standard solution were measured at 279 mμ.

Thin-Layer Chromatography (TLC)—*Preparation of Layers*—The chromatoplates were prepared using standard equipment (15). Cellulose MN300 and silica gel G were used as adsorbents. To obtain five plates 20 × 20 cm., 10 g. of cellulose powder containing 20 mg. of fluorescent indicator was mixed with 70 ml. of water and 10 ml. of 1% aqueous solution of sodium carboxymethylcellulose (CMC). For silica gel G, five plates were obtained from 25 g. of powder in 55 ml. of water. A layer-thickness of 250 μ was used throughout. Cellulose plates were activated by heating at 110° for 30 min. and stored in a desiccator until use; silica gel plates were air dried and used without activation.

Solvent Systems—The following solvent systems were used: (*a*) *n*-butanol–acetic acid–water (4:1:5) (16) were shaken together in a separator and set aside for 2 hr.; the upper organic layer was separated and used as the running solvent for cellulose plates. (*b*) *n*-Butanol–acetic acid–water (60:10:25) were mixed together to form a clear solution and used as the developing solvent for silica gel plates.

Detection Reagents—(*a*) Short wavelength and long wavelength UV light. (*b*) Potassium ferricyanide and ferric chloride solution: 5 ml. of 5% aqueous solution of potassium ferricyanide and 10 ml. of 10% aqueous solution of $FeCl_3$ were mixed and made to 50 ml. with water. This was used to spray cellulose plates. (*c*) Sodium metaperiodate 2% aqueous solution. (*d*) Doty's reagent: mix immediately before use equal volumes of ferrous sulfate–citrate solution and aminoacetate buffer. (For preparation of solutions, refer to *Analytical Methods* of this paper.) (*e*) Folin-Ciocalteu reagent (FCR) (17). This reagent was used for silica gel plates; the plates were first sprayed with FCR solution, air dried, and then sprayed with 10% aqueous Na_2CO_3 solution.

Chromatographic Procedure—A solution of isoproterenol HCl (230 mg.) and isoproterenol sulfonic acid (20 mg.) in about 30 ml. of water was prepared by mechanical shaking for 20 min., adding 139 mg. of sodium bisulfite to the clear solution, and making it to 50 ml. in a volumetric flask. Quantities of this standard solution (1–5 μl.) containing equivalent of 4.6–23 mcg. of isoproterenol HCl and 0.4–2.0 mcg. of isoproterenol sulfonic acid were spotted adjacent to the aqueous solutions of the simulated formulations and/or commercial formulations (5 μl.) containing equivalent to 25 mcg. of isoproterenol HCl. The plate was inserted in a solvent jar previously equilibrated with solvent [solvent system (*a*) for cellulose plates and solvent system (*b*) for silica gel plates] which was allowed to rise to a height of 10 cm. for cellulose plates and 15 cm. for silica gel plates (time about 120 min. for cellulose plates and 135 min. for silica gel plates). The plate was then air dried and examined under short and long wavelength UV light.

Examination of cellulose plates under short wavelength UV light showed a spot at the origin having a blue fluorescence (indicative of $NaHSO_3$ presence) and long wavelength UV light showed an additional spot having a strong blue fluorescence (R_f about 0.26) both for simulated and commercial formulations but not seen for the standard solution. Spraying the plate with DR (*b*) (potassium ferricyanide and $FeCl_3$ solution) caused the immediate formation of blue spots for isoproterenol HCl (R_f about 0.53) and isoproterenol sulfonic acid (R_f about 0.33). The spot seen under long wavelength UV light also stained blue after about half a minute, and thus the whole area from about 0.33 to 0.53 R_f values was seen as a single blue spot (limiting sensitivity of this spray for isoproterenol sulfonic acid 0.4 mcg.). Citric acid and sodium citrate present in a few commercial formulations also gave a blue spot of minor intensity after 2–5 min.; glycerin, another excipient, gave a minor blue spot after 5–10 min. Thus these spots due to their late formation did not interfere with the detection of the decomposition products. Spraying the second half of the plate with DR (*c*) or (*d*) caused the formation of reddish-colored spots for isoproterenol HCl and isoproterenol sulfonic acid with sodium metaperiodate spray and mauve-colored spots with Doty's reagent. The reddish-colored spot formed with $NaIO_4$ for isoproterenol sulfonic acid disappeared within 1 min. of its formation but that of Doty's reagent (mauve-colored spot) did not disappear. (Limiting sensitivity of both of these sprays was 2.0 mcg. for isoproterenol sulfonic acid.)

Examination of silica gel G plates under short wavelength UV light did not show any spot; long wavelength UV light showed two spots having blue fluorescence for inhalations containing 0.3% $NaHSO_3$ (R_f values of 0.03 and 0.1); three spots for inhalations containing 0.2% $NaHSO_3$ (R_f values 0.03, 0.1, and 0.16) and two spots for injections (depending upon the quantity spotted) at R_f of 0.1 and 0.16. Simulated formulations stored in the dark over a period of 4 months showed almost the same pattern, while a freshly prepared standard solution did not show any of these spots. Spraying the plate with DR(*e*)–FCR reagent followed by Na_2CO_3 spray caused the formation of purple spots for isoproterenol HCl (R_f about 0.4) and isoproterenol sulfonic acid (R_f about 0.26) (limiting sensitivity 0.6 mcg. for isoproterenol sulfonic acid). Among the spots seen under long wavelength UV, spots at R_f 0.1 gave coloration with this reagent for all formulations, while the spot at 0.16 R_f gave coloration for formulations having 0.2% $NaHSO_3$. An additional bluish-purple spot at R_f 0.43 was also seen in some formulations. A freshly prepared standard solution mixture showed only two purple spots at R_f 0.4 and 0.26 for isoproterenol HCl and isoproterenol sulfonic acid, respectively.

RESULTS AND DISCUSSION

The TLC of simulated inhalations and injections stored in the dark over a period of 4 months showed that in addition to Compound II (Scheme I), the drug can give rise to two to

Table I—Analysis of Isoproterenol Formulations

Formulation	$NaIO_4$ Method: Label Claim, Av., %	$NaIO_4$ Method: Anal. Range	$NaIO_4$ Method: *SD*	$NaIO_4$ Method: No. of Detn.	DEHP Method: Label Claim, Av., %	DEHP Method: Anal. Range	DEHP Method: *SD*	DEHP Method: No. of Detn.
Simulated Formulations								
Decomposed[b]	92.6	91.9–93.2	0.55	4	92.5	91.9–93.7	0.85	4
Tablets[c]	101.8	100.9–102.2	0.54	4	100.6	100.3–100.8	—	2
Inhalation[d]	98.4	97.1–99.3	1.0	4	—	—	—	—
Inhalation[e]	98.9	98.4–99.2	0.50	4	—	—	—	—
Injection[f]	86.0	85.4–86.9	0.66	4	—	—	—	—
Commercial Formulations								
Inhalation A[g]	106.8	106.3–107.4	0.65	4	110.8	110.5–111.2	—	2
Inhalation B[h]	106.1	104.9–107.7	1.23	4	106.2	105.9–106.5	—	2
Injection[i]	100.0	—	—	1	100.1	—	—	1
Tablets[j]	100.8	—	—	2	98.9	—	—	2

Formulation	Colorimetric Procedure[a]: Label Claim, Av., %	Colorimetric Procedure[a]: Anal. Range	Colorimetric Procedure[a]: *SD*	Colorimetric Procedure[a]: No. of Detn.	USP XVII Method: Label Claim, Av., %	USP XVII Method: No. of Detn.
Simulated Formulations						
Decomposed[b]	99.7	98.9–99.9	0.45	4	99.9	2
Tablets[c]	99.2	98.7–99.8	—	2	101.1	2
Inhalation[d]	—	—	—	—	102.6	2
Inhalation[e]	—	—	—	—	100.8	2
Injection[f]	—	—	—	—	101.8	2
Commercial Formulations						
Inhalation A[g]	117.5	—	—	2	116.7	2
Inhalation B[h]	109.2	—	—	2	110.8	2
Injection[i]	115.0	—	—	1	116.0	1
Tablets[j]	101.2	—	—	2	101.9	2

[a] Modified USP XVII method for the determination of epinephrine in procaine hydrochloride injection (determines total catechols as isoproterenol). [b] Isoproterenol HCl 115 mg., isoproterenol sulfonic acid 10 mg. (8.0%), and sodium bisulfite 65 mg. (0.3% w/v) in 25 ml. water. [c] Isoproterenol HCl 10 mg., sodium bisulfite 2 mg. plus excipients such as starch, lactose, saccharin, and talcum per tablet of average weight of 100 mg. [d] Isoproterenol HCl 125 mg., sodium bisulfite 75 mg. (0.3% w/v) plus excipients such as sodium chloride, citric acid, glycerin, and chlorobutanol dissolved in water to 25 ml. The pH was adjusted to 3.75 with 1 *N* sodium hydroxide. Molar ratio of sodium bisulfite to isoproterenol HCl is 1.42:1. [e] Isoproterenol HCl 125 mg., sodium bisulfite 50 mg. (0.2% w/v) plus excipients such as chlorobutanol and sodium chloride dissolved in water to 25 ml. The pH of the formulation was 2.8 (without adjustment). Molar ratio of sodium bisulfite to isoproterenol is 0.95:1. [f] Isoproterenol HCl 20 mg., sodium bisulfite 100 mg. (0.1% w/v) plus excipients such as lactic acid, sodium lactate, and sodium chloride dissolved in water to 100 ml. The pH of the formulation was 2.8 (without adjustment). Molar ratio of sodium bisulfite to isoproterenol is 11.9:1. [g,h,i,j] Labeled to contain active ingredients and sodium bisulfite in the same amounts as in simulated formulations [d,e,f], and [c], respectively. Formulations [g], [h], and [i] were found to have the pH values of 3.75, 3.35, and 3.75, respectively.

three decomposition products. The exact chemical nature of these additionally encountered artifacts could not be established but one of them can be envisaged as an aminochrome–sodium bisulfite addition compound by analogy with the similar compound described by Oesterling and Tse (18) in their procedure for the determination of total content of catecholamines in the urine. The total percent ratio of these to Compound II was found to be about 1:4 depending upon the molar ratio of sodium bisulfite to isoproterenol used in a formulation. In addition, other factors including conditions of storage and its length and the pH of the formulation may affect the relative formation of these components.

A method of analysis, to be completely reliable, therefore, should be capable of differentiating between the parent drug and the closely related sodium bisulfite addition product. Both isoproterenol and sulfonic acid II exhibit maximum absorption at 279 mμ and have similar molar absorptivity values. Although the method of Welsh and Sammul (1) based on the DEHP ion-pair and utilizing a diatomaceous earth column represents a major step in solving the problem, the technique suffers from certain disadvantages. When applied to a synthetic preparation containing known amounts of Compound II and sodium bisulfite, this method gave higher and inconsistent values for the parent drug. This discrepancy was reconciled by introducing a step to wash the ether effluent from the column with potassium phosphate buffer prior to the acid extraction. The aged simulated formulations containing artifacts in addition to Compound II (as shown by TLC) showed higher recoveries for the unchanged drug due to carrying over some of the degradation products other than sulfonic acid to the DEHP–ether phase. The above shortcomings and the cumbersome steps of column packing and its elution were overcome by modifying the column method to a simple shakeout procedure and by washing the combined DEHP–ether solutions first with potassium phosphate buffer and then with water prior to the extraction of the unchanged drug with 0.1 *N* H_2SO_4. These modifications not only resulted in the increased specificity of the method (by removing some of the water-soluble degradation products) but also permitted the quantitative determination of isoproterenol sulfonic acid which is retained in the aqueous buffer phase. Thus the Compound II (sulfonic acid) is then determined selectively by treating the aqueous phase with Doty's reagents.

Application of the modified procedure to the simulated isoproterenol tablets and to the simulated decomposed sample containing 8% Compound II and 0.3% $NaHSO_3$ gave the results shown in Table I. Analytical range and standard deviation values demonstrate the accuracy and precision of the method. A batch of commercial isoproterenol tablets was analyzed by this method and the results (Table I) appear satisfactory in comparison with those obtained from the USP analyses and with data obtained with simulated tablets.

Data in Table II show the recoveries of isoproterenol sulfonic acid II from a simulated decomposed sample by the procedure described under *Determination of Isoproterenol Sulfonic Acid.*

Table II—Amount of Degradation Products and Isoproterenol Sulfonic Acid in Simulated and Commercial Formulations

Formulation	Assay Values for Isoproterenol,[a] % [d]	Total Water Soluble Degradation Products,[b] % [d]	Isoproterenol Sulfonic Acid,[c] % [d]	Isoproterenol HCl Undergone Degradation, % [d]
Simulated Formulations				
Inhalation[e]	89.6	12.4	11.6	11.1
Inhalation[f]	88.6	12.0	9.0	10.7
Injection[g]	84.3	19.1	15.0	17.2
Decomposed[h]	92.6	8.1	8.2	—
Commercial Formulations				
Inhalation[i]	106.8	7.5	4.7	6.8
Inhalation[j]	106.1	5.5	3.4	4.9
Injection[k]	100.0	15.6	15.5	14.0

[a] Values quoted have been obtained by $NaIO_4$ method. [b] Values quoted for total water soluble degradation products have been obtained by measuring UV absorbance of the aqueous-buffer phase, and calculated as isoproterenol sulfonic acid. [c] Values quoted for isoproterenol sulfonic acid were obtained by the spectrophotometric quantitative measurement of the purple color formed by treating the aqueous phase with ferro-citrate solution and buffer. The reaction is specific for catechol nucleus only. [d] Calculated relative to the label claim. [e,f,g] Formulations same as [d,e,f] in Table I; assay values for [e] and [f] were obtained after 83 days of storage at room temperature, and for [g] after 88 days of storage. [h] Formulation same as [b] in Table I containing 8% isoproterenol sulfonic acid. [i,j,k] Commercial formulations same as [g,h,i] in Table I.

Simulated formulations which had been stored in the dark over a period of 3 months were also analyzed by this procedure. Table II also depicts the amounts of Compound II in the aged simulated formulations and in commercial formulations. One of the commercial formulations having a molar ratio of 11.9:1 of sodium bisulfite to isoproterenol was found to contain a considerable percentage of Compound II when it was analyzed 8–9 months after its procurement from the factory premises. The logic of using such a higher molar ratio of sodium bisulfite is not clearly understood, especially when the injection is to be administered by the intravenous route.

An alternate checking procedure to validate the results obtained by the DEHP method was sought. Consequently, sodium metaperiodate which is known to cleave carbon-carbon bonds of vicglycols, α-aminoalcohols where the amine is primary or secondary, and α-hydroxyketones (19), was used for the selective determination of the unchanged drug. This treatment results in the formation of an aryl aldehyde of the parent drug, and the Compound II or aminochrome-type oxidation products do not yield this aldehyde. The aryl aldehyde is extracted into chloroform and UV-determined at 270 mμ. The typical UV spectrum of the reaction product is given in Fig. 1.

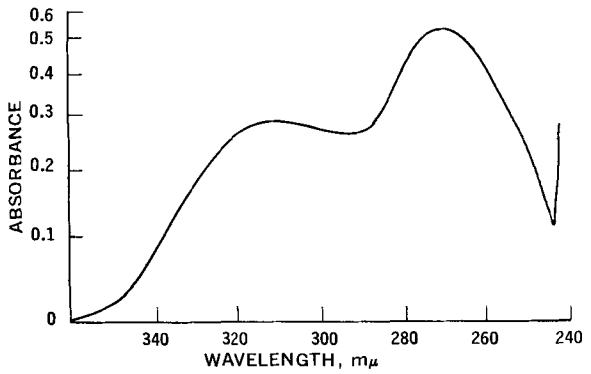


Figure 1—*Absorption spectrum of the reaction product in chloroform (concentration equivalent to 140 mg. of isoproterenol HCl per milliliter of final chloroform solution).*

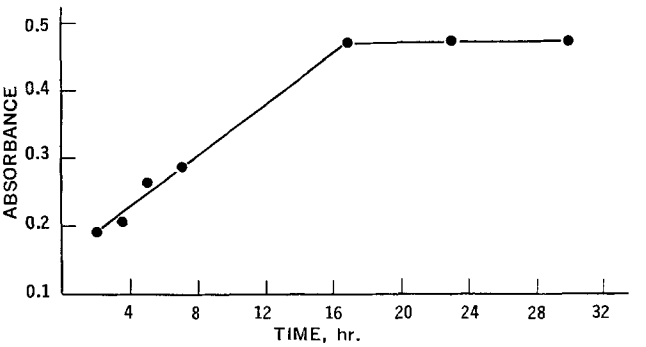


Figure 2—*Effect of time on the completion of reaction (concentration equivalent to 120 mg. of isoproterenol HCl per milliliter of final chloroform solution).*

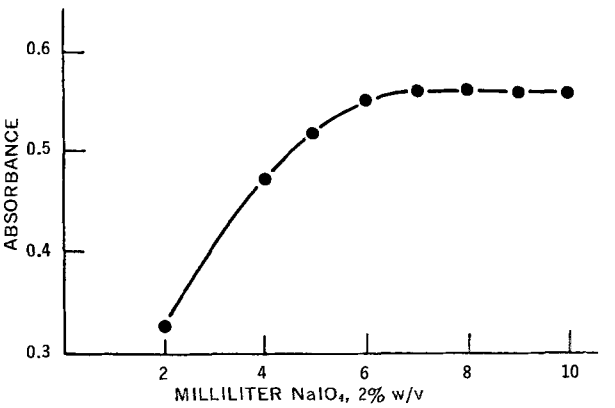


Figure 3—*Effect of varying concentration of sodium metaperiodate on the formation of reaction product.*

Experiments undertaken to obtain the quantitative yield of the reaction product indicated that (*a*) the sample and reagent be allowed to react for about 17–18 hr. (overnight) at room temperature, although periods up to 30 hr. did not adversely affect the reaction (Fig. 2); (*b*) a molar ratio, 116:1, of sodium metaperiodate to isoproterenol was satisfactory for analysis but a molar ratio of 82:1 was insufficient. On the other hand, a molar ratio as high as 165:1 did not show any untoward effect on the yield of the reaction product (Fig. 3); (*c*) the standard solution of isoproterenol HCl should contain approximately the same amount of sodium bisulfite as is present in the formulation. A 4–6-ml. aliquot of the diluted formulation used for $NaIO_4$ assay will contain about 0.48–0.72 mg. of $NaHSO_3$ and as shown in Fig. 4, the $NaHSO_3$ concentration from 0.48–0.96 mg. in the isoproterenol HCl standard did not affect the yield of the reaction product. Under these conditions, Beer's law was obeyed for a concentration of at least 140 mcg. of isoproterenol HCl per milliliter of final solution (Fig. 5). The effect of varying concentrations of Compound II on the recoveries of isoproterenol is shown in Fig. 6; the recoveries were not affected up to 28% concentration of the decomposition product.

When applied to the simulated isoproterenol tablets and to a simulated decomposed sample containing 8% Compound II and 0.3%

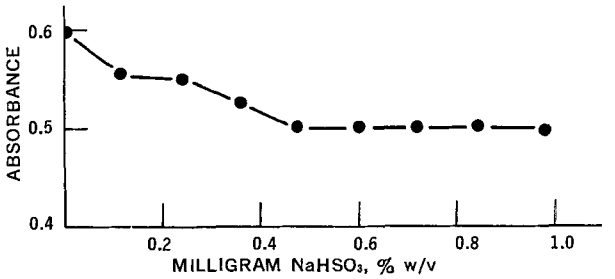


Figure 4—*Effect of varying concentration of sodium bisufilte on the formation of reaction product.*

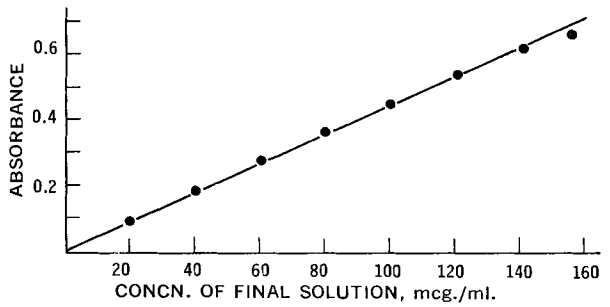


Figure 5—*Relationship between concentration and absorbance (absorbance of the reaction product in chloroform at 270 mμ).*

$NaHSO_3$, the $NaIO_4$ method gave results (Table I) virtually identical with those obtained by the DEHP method. Results obtained by applying the $NaIO_4$ method to four commercial products containing isoproterenol are shown in Table I. The comparison of the experimental data with those obtained by the DEHP method shows a good correlation between the two, with comparable precision for three formulations. Only one formulation showed higher values by the DEHP method which were explicable by additionally encountered artifacts other than Compound II. The data show that although inhalations and injections sold on the market conform to the USP limits, the formulations may contain considerably large amounts of decomposition products. The accuracy and the precision of this method are demonstrated by the analytical range and standard deviation values obtained (Table I) by its application to the freshly prepared inhalations and injections. A simulated injection containing a molar ratio of 11.9:1 of $NaHSO_3$ to isoproterenol (identical to the commercial formulation) showed only 86% of the added isoproterenol content. Virtually no further deterioration of the isoproterenol content was noticed, even when the formulation was analyzed after 88 days of storage in the dark at room temperature (Tables II and III). Lower values were ascribable to the formation of 15% isoproterenol sulfonic acid.

The isoproterenol content found by the ferro-citrate colorimetric method (6, 20) and by the USP XVII UV spectrophotometric method (4) is a measure of the isoproterenol present at the time the solutions were manufactured. Values obtained by the two methods are satisfactory and in close agreement (Table I). The colorimetric method reported in the paper is a modification of the USP XVII procedure described for the determination of epinephrine in procaine hydrochloride injection (20). It was found that the amounts of $NaHSO_3$ as specified in the USP were too high and a substantial decrease in the color intensity was noticed. The amount of $NaHSO_3$ present in the commercial formulations was found adequate for reproducible color development. The order of adding reagents was another variable considered to be important, and thus the maximum reproducibility was obtained by adding ferrous sulfate reagent to the reaction solution to which the appropriate volume of the aminoacetate buffer had already been added. Time for color development was changed from 30 min. to 2.50 hr.

Table III shows the stability data of the simulated inhalations and injections stored in the dark at room temperature over a period of 88 days. The data indicate that the inhalation having a molar ratio of 1.42:1 of $NaHSO_3$ to isoproterenol did not show further deterioration of the isoproterenol content after 39 days. The data also revealed that no consistent pattern of degradation was followed

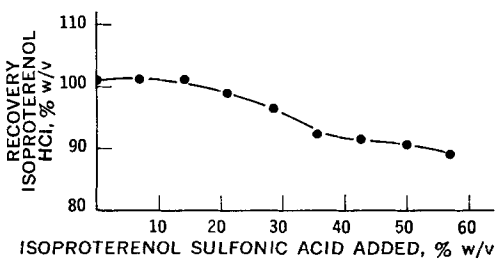


Figure 6—*Effect of varying concentration of isoproterenol sulfonic acid on the recovery of isoproterenol HCl.*

Table III—Analysis of Isoproterenol in Aged Simulated Formulations[a]

Formulations	Storage Period, days	Assay Values for Isoproterenol[b] $NaIO_4$ Method, %	DEHP Method, %	USP Method, %
Inhalation[c]	0	98.4	—	102.0
	39	89.5	—	—
	83	89.6	95.4[d]	102.6
Inhalation[e]	0	98.9	—	100.3
	39	96.7	—	—
	83	88.6	95.9[d]	100.8
Injection[f]	0	86.0	—	—
	39	86.0	—	—
	88	84.3	82.7[g]	101.8

[a] Stored in dark at room temperature. [b] Calculated relative to the label claim. [c,e,f] Formulations same as [d,e,f] in Table I. [d] Higher values for unchanged drug were obtained due to carrying over of certain decomposition products into DEHP-ether phase. [g] Values for unchanged drug in this formulation agreed very well to that of $NaIO_4$ method due to formation of isoproterenol sulfonic acid as the main degradation product. Formation of isoproterenol sulfonic acid as the main degradation product was favored due to high molar ratio of sodium bisulfite to isoproterenol, *i.e.*, 11.9:1 and DEHP method has been found to be specific if the main degradation product formed is only sulfonic acid.

in all three formulations. These findings agree well with those made by Chafetz *et al.* (21).

The investigation showed that although solvent system (*a*) on cellulose was satisfactory for resolving isoproterenol and its sulfonic acid II, the solvent system (*b*) on silica gel G was an ideal one for the complete separation and detection of isoproterenol and its artifacts. Despite the specificity of $NaIO_4$ spray (formation of reddish color for isoproterenol and transient coloration for sulfonic acid) and Doty's reagent spray (mauve coloration for isoproterenol and its sulfonic acid), these are not suitable to detect the presence of isoproterenol sulfonic acid due to their low sensitivity (2 mcg. or more is required for color formation). Potassium ferricyanide–$FeCl_3$ spray (though less specific but having a sensitivity of less than 0.4 mcg.) and FCR spray (more specific having a sensitivity of 0.6 mcg.) were found very satisfactory to detect the presence of Compound II even in minor concentrations. Under the conditions used, the problem of an apparent second front as described by Choulis (16) and Chafetz *et al.* (21) was not encountered for the standard solution containing isoproterenol and its sulfonic acid and $NaHSO_3$.

It is recommended that the DEHP shakeout method as described in the paper may be adopted as the official procedure in the USP XVIII for the analysis of isoproterenol formulations. This procedure has overcome the shortcomings of the method reported by Welsh and Sammul (1). Procedure with these modifications achieves all the essential criteria required for its inclusion in various official compendia by its simplicity, precision, accuracy, and specificity. It is further suggested that the limits (maximum and minimum) for isoproterenol sulfonic acid may be delineated for all the formulations containing sodium bisulfite as an antioxidant. TLC using silica gel G and *n*-butanol–acetic acid–water (60:10:25) as an ideal solvent may be included in the monograph to detect the presence of isoproterenol sulfonic acid and other artifacts.

The sodium metaperiodate method is also very specific and simple and can be automated very easily using the currently available Liquid Prep. Technicon Auto-Analyzer equipment. The method can be of potential value to the pharmaceutical industry to be utilized as a routine-monitoring and stability-indicating procedure for the unchanged drug in drug quality control.

REFERENCES

(1) L. H. Welsh and O. R. Sammul, *J. Assoc. Offic. Anal. Chemists*, **51**, 176(1968).

(2) L. C. Schroeter, T. Higuchi, and E. E. Schuler, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 723(1958).

(3) T. Higuchi and L. C. Schroeter, *ibid.*, **48**, 535(1959); *J. Am. Chem. Soc.*, **82**, 1904(1960).

(4) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 330.
(5) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1968, p. 536.
(6) J. R. Doty, *Anal. Chem.*, **20,** 1166(1948).
(7) D. M. Temple, *Australasian J. Pharm.*, **47,** *Suppl.*, **43,** 62 (1966).
(8) D. M. Temple and R. Gillespie, *Nature*, **209,** 714(1966).
(9) J. Levine and T. D. Doyle, *J. Pharm. Sci.*, **56,** 619(1967).
(10) H. Hellberg, *Svensk Farm. Tidskr.*, **64,** 493(1960).
(11) R. A. Heacock, G. L. Mattok, and D. L. Wilson, *Can. J. Biochem. Physiol.*, **41,** 1721(1963).
(12) K. R. Heimlich, D. R. MacDonnell, T. L. Flanagan, and P. D. O'Brien, *J. Pharm. Sci.*, **50,** 232(1961).
(13) L. Chafetz, *ibid.*, **52,** 1193(1963).
(14) L. C. Schroeter and T. Higuchi, *J. Am. Pharm. Assoc., Sci. Ed.*, **49,** 331(1960).
(15) E. Stahl, "Thin Layer Chromatography," Academic, New York, N. Y., 1965, p. 5ff.
(16) N. H. Choulis, *J. Pharm. Sci.*, **56,** 1962(1967).
(17) H. D. Beckstead, K. K. Kaistha, and S. J. Smith, *ibid.*, **57,** 1952(1968).
(18) R. L. Tse and M. J. Oesterling, *Clin. Chim. Acta*, **4,** 307 (1959); M. J. Oesterling and R. L. Tse, *Federation Proc.*, **18,** 296 (1958); *Am. J. Med. Technol.*, **27,** 112(1961).
(19) E. L. Jackson, in "Organic Reactions," vol. II, Wiley, New York, N. Y., 1944, p. 341.
(20) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 519.
(21) L. Chafetz, H. Schriftman, and A. I. Kay, *J. Pharm. Sci.*, **57,** 710(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 25, 1969, from the *Department of Psychiatry, Toxicology Laboratory, University of Chicago, Chicago, IL 60637*
Accepted for publication September 15, 1969.
Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.
The author is indebted to Drs. D. Cook and W. N. French of Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada, for allowing him to work on this project during his stay with the Directorate; to Drs. Jerome H. Jaffe and C. R. Schuster of the Department of Psychiatry, University of Chicago, for providing funds to present this work at the APhA Academy of Pharmaceutical Sciences meeting; and to Dr. B. D. Kocchar, Clinical Biochemist, Kirkland District Hospital, Ontario, Canada, for the revision of the manuscript.

TECHNICAL ARTICLES

Construction and Operation of an Automated Dispensing Analyzer for the Assay of Individual Tablets

WILLIAM F. BEYER* and EDWIN W. SMITH†

Abstract □ An automated system of modular design has been developed for the assay of single tablets. Commercially available proportioning pumps, continuous filter, tablet homogenizer, spectrophotometer, and recorder are mated to specially designed components for unattended operation. The system is constructed to analyze automatically up to 300 identifiable tablets with standards inserted prior to tablets, after any selected number of tablets, and at the end of the particular type of tablet being assayed. Up to six different concentrations or types of tablets can be automatically processed sequentially, each with its own standard insertion. Provisions are made to alter automatically the dilution of samples as tablet strengths vary. Under normal program conditions the system can operate unattended for approximately 18 hr.

Keyphrases □ Tablets, individual—automated analysis □ Automated dispensing analyzer, tablet—construction, operation □ Diagram—automated analyzer, individual tablets □ UV spectrophotometry—analysis

The assay of single tablets and single units in other dosage forms is recognized as an important feature in the quality control of manufacturing processes. Compendia requirements for content uniformity of selected tablets containing 50 mg. or less of drug substance has placed additional burdens on the quality control chemist. To make data available in the quantity needed for statistically valid results, analytical procedures have been automated to varying degrees. The majority of automated assay systems that include sample preparation have incorporated a commercially available solid sampler,[1] as evidenced by symposia of the New York Academy of Sciences and Technicon Corp. (1–5). Papers presented at the 1967 New York Academy of Sciences included a presentation by Rehm *et al.* (2) describing an automated system for the UV analysis of single tablets.[2]

An automated system constructed in these laboratories for UV analyses of individual tablets has been in routine operation for more than a year. The system is of modular design and consists of commercially available components wherever possible. Provisions have been made for the introduction of liquid standards, dilution, and

[1] SOLIDprep, Technicon, Inc., Tarrytown, N. Y.
[2] Available commercially as the Assayomat, American Instrument Company, Inc., Silver Spring, Md.

(4) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 330.
(5) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1968, p. 536.
(6) J. R. Doty, *Anal. Chem.*, **20,** 1166(1948).
(7) D. M. Temple, *Australasian J. Pharm.*, **47,** *Suppl.*, **43,** 62 (1966).
(8) D. M. Temple and R. Gillespie, *Nature*, **209,** 714(1966).
(9) J. Levine and T. D. Doyle, *J. Pharm. Sci.*, **56,** 619(1967).
(10) H. Hellberg, *Svensk Farm. Tidskr.*, **64,** 493(1960).
(11) R. A. Heacock, G. L. Mattok, and D. L. Wilson, *Can. J. Biochem. Physiol.*, **41,** 1721(1963).
(12) K. R. Heimlich, D. R. MacDonnell, T. L. Flanagan, and P. D. O'Brien, *J. Pharm. Sci.*, **50,** 232(1961).
(13) L. Chafetz, *ibid.*, **52,** 1193(1963).
(14) L. C. Schroeter and T. Higuchi, *J. Am. Pharm. Assoc., Sci. Ed.*, **49,** 331(1960).
(15) E. Stahl, "Thin Layer Chromatography," Academic, New York, N. Y., 1965, p. 5ff.
(16) N. H. Choulis, *J. Pharm. Sci.*, **56,** 1962(1967).
(17) H. D. Beckstead, K. K. Kaistha, and S. J. Smith, *ibid.*, **57,** 1952(1968).
(18) R. L. Tse and M. J. Oesterling, *Clin. Chim. Acta*, **4,** 307 (1959); M. J. Oesterling and R. L. Tse, *Federation Proc.*, **18,** 296 (1958); *Am. J. Med. Technol.*, **27,** 112(1961).
(19) E. L. Jackson, in "Organic Reactions," vol. II, Wiley, New York, N. Y., 1944, p. 341.
(20) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 519.
(21) L. Chafetz, H. Schriftman, and A. I. Kay, *J. Pharm. Sci.*, **57,** 710(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 25, 1969, from the *Department of Psychiatry, Toxicology Laboratory, University of Chicago, Chicago, IL 60637*
Accepted for publication September 15, 1969.
Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.
The author is indebted to Drs. D. Cook and W. N. French of Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada, for allowing him to work on this project during his stay with the Directorate; to Drs. Jerome H. Jaffe and C. R. Schuster of the Department of Psychiatry, University of Chicago, for providing funds to present this work at the APhA Academy of Pharmaceutical Sciences meeting; and to Dr. B. D. Kocchar, Clinical Biochemist, Kirkland District Hospital, Ontario, Canada, for the revision of the manuscript.

TECHNICAL ARTICLES

Construction and Operation of an Automated Dispensing Analyzer for the Assay of Individual Tablets

WILLIAM F. BEYER* and EDWIN W. SMITH†

Abstract □ An automated system of modular design has been developed for the assay of single tablets. Commercially available proportioning pumps, continuous filter, tablet homogenizer, spectrophotometer, and recorder are mated to specially designed components for unattended operation. The system is constructed to analyze automatically up to 300 identifiable tablets with standards inserted prior to tablets, after any selected number of tablets, and at the end of the particular type of tablet being assayed. Up to six different concentrations or types of tablets can be automatically processed sequentially, each with its own standard insertion. Provisions are made to alter automatically the dilution of samples as tablet strengths vary. Under normal program conditions the system can operate unattended for approximately 18 hr.

Keyphrases □ Tablets, individual—automated analysis □ Automated dispensing analyzer, tablet—construction, operation □ Diagram—automated analyzer, individual tablets □ UV spectrophotometry—analysis

The assay of single tablets and single units in other dosage forms is recognized as an important feature in the quality control of manufacturing processes. Compendia requirements for content uniformity of selected tablets containing 50 mg. or less of drug substance has placed additional burdens on the quality control chemist. To make data available in the quantity needed for statistically valid results, analytical procedures have been automated to varying degrees. The majority of automated assay systems that include sample preparation have incorporated a commercially available solid sampler,[1] as evidenced by symposia of the New York Academy of Sciences and Technicon Corp. (1–5). Papers presented at the 1967 New York Academy of Sciences included a presentation by Rehm *et al.* (2) describing an automated system for the UV analysis of single tablets.[2]

An automated system constructed in these laboratories for UV analyses of individual tablets has been in routine operation for more than a year. The system is of modular design and consists of commercially available components wherever possible. Provisions have been made for the introduction of liquid standards, dilution, and

[1] SOLIDprep, Technicon, Inc., Tarrytown, N. Y.
[2] Available commercially as the Assayomat, American Instrument Company, Inc., Silver Spring, Md.

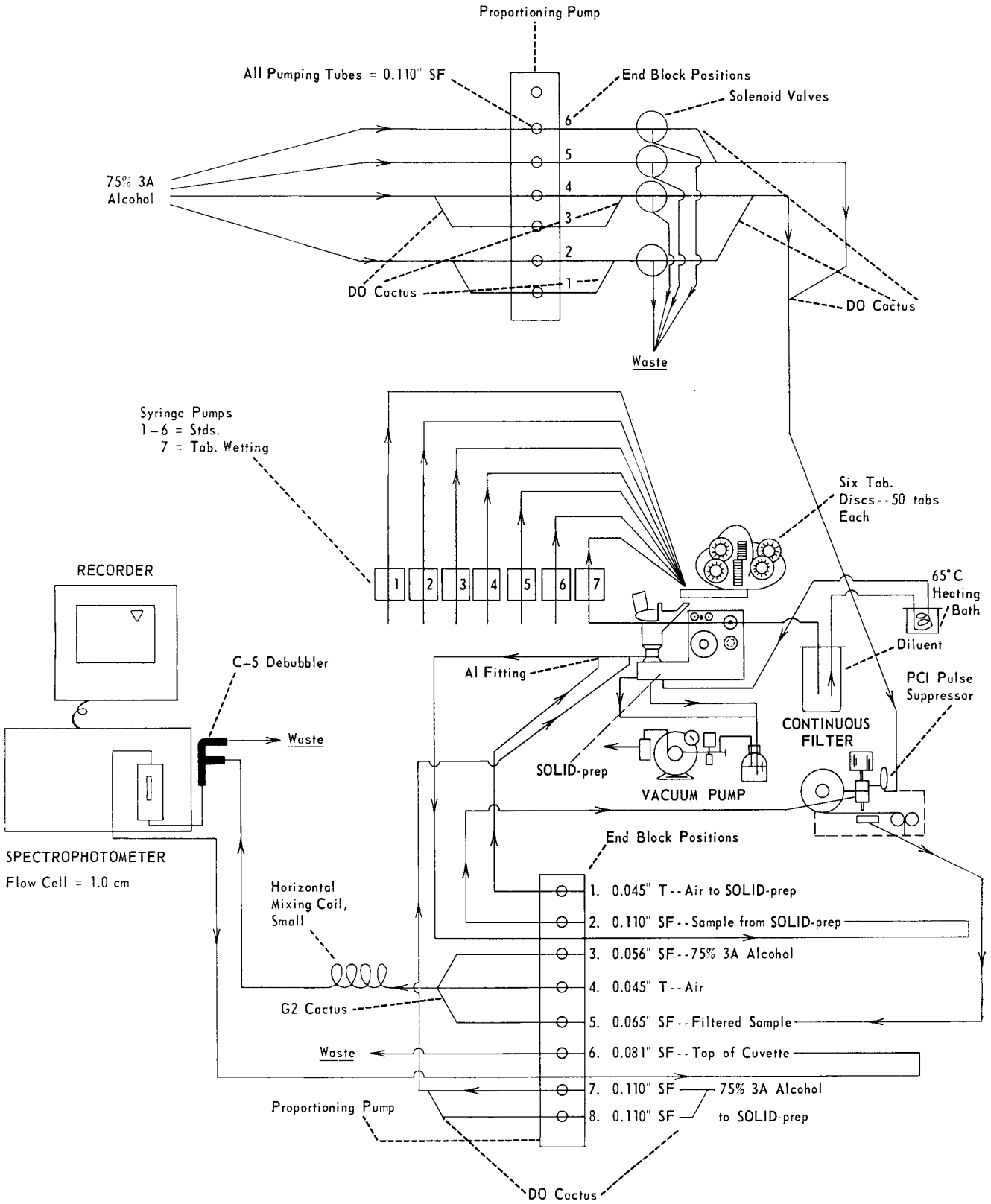


Figure 1—*Manifold flow system and associated equipment used with the automated dispensing analyzer. Solvaflex and Tygon pumping tubes are denoted as SF and T, respectively.*

filtration of tablet samples. Modifications in commercial modules, construction of new units, and the operation of the authors' automated dispensing analyzer (ADA) are the subjects of this report.

EQUIPMENT

The modules and manifold system are schematically illustrated in Fig. 1, with the modular design shown in Fig. 2. Teflon tubing is used throughout except for pumping tubes and heating bath coil.

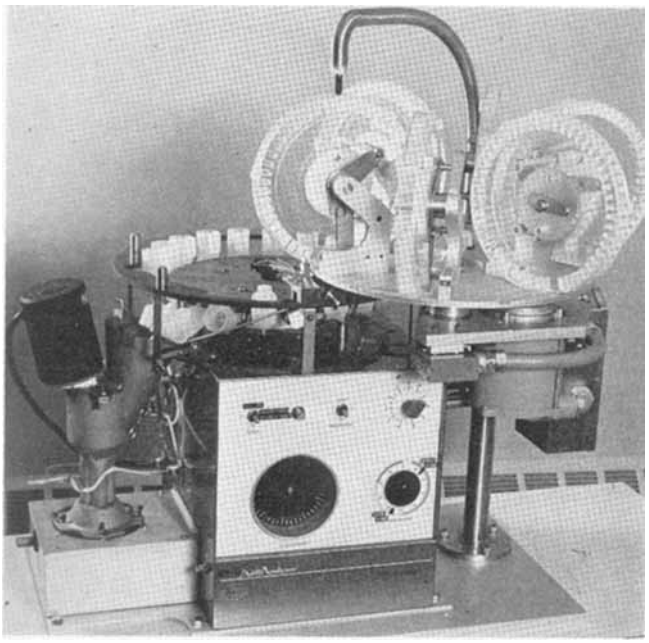


Figure 2—*Instruments comprising the automated dispensing analyzer, showing its modular design.*

the mixing block is replaced with a polyethylene tube having a capillary opening and attached to a pulse suppressor (PCl),[3] into which diluting lines are fed (Fig. 1).

Solid Sampler—An externally mounted solenoid valve[4] replaces the internal wash water distribution solenoid valve carrying three associated rinsing lines. Two check valves[5] replace the check valve for solvent introduction. Only the cup rinsing jet and the hopper jet nearest the cabinet of the solid sampler are used.

Heating Bath, 65°[6]—The diluent passes through 0.95-cm. (0.375-in.) copper tubing fashioned into a 12-turn, 11.43-cm. (4.5-in.) coil, immersed in a 65° water bath. The temperature of the diluent remains above 55° while in the blender.

UV-Visible Spectrophotometer[7] with 1.0-cm. flow cell.[8]

Absorbance Recorder with event marker.[9]

Constructed Modules—*Instream Diluting System*—Four explosion-proof three-way solenoid valves[4] are mounted in an elevated stand to match the height of the proportioning pump. Stainless steel fittings to accommodate approximately 0.32-cm. (0.125-in.) tubing were made for all ports of the solenoid valves. Entrance ports are connected to Solvaflex pumping tubes[3] and exit ports to Teflon tubing leading either to the continuous filter or to waste.

Tablet-holding Disk and Tablet-dispensing Apparatus—Six disks of 50-tablet capacity were fabricated from 0.32-cm. (0.125-in.) and 1.27-cm. (0.5-in.) Plexiglas sheet stock. Numbered slots in the tablet-holding disk accommodate nearly all tablet shapes currently produced at the authors' company. Embedded in the rim of each tablet-holding disk is a strip of brass, sensed by a proximity probe, to signal that the last tablet has been dispensed. The disks, dispensing mechanism, turret, and proximity probe are shown in Fig. 3. The

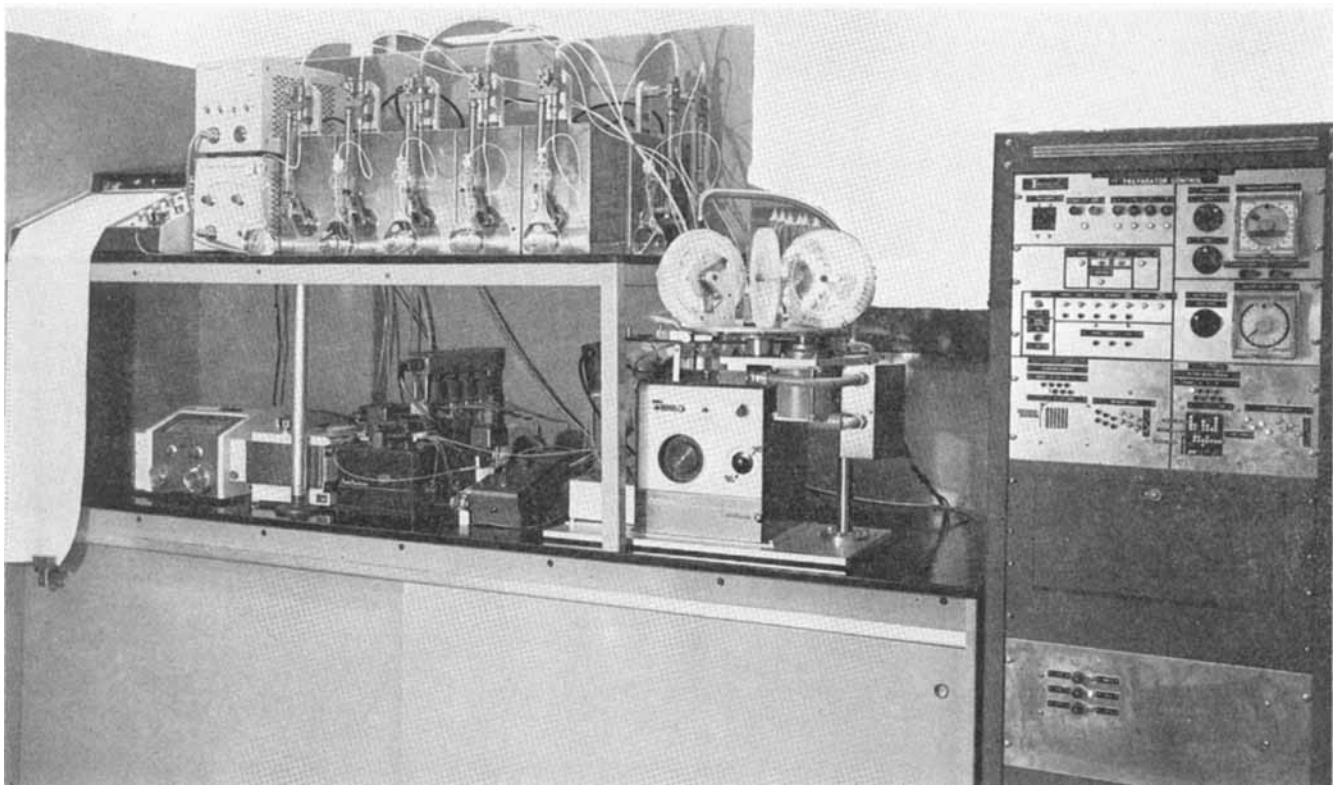


Figure 3—*Sample preparation and dispensing system: solid sampler; turret with six tablet disks, dispensing mechanism, and proximity probe; and the seven-figured tubing attachment unit for standards and tablet-wetting lines.*

Commercially Available Modules, Modified in Some Instances—*Proportioning Pumps (Two)*[3] *and Continuous Filter with Paper T-014*[3]—The chamber of the mixing block is enlarged to accommodate the large volumes of diluent required on occasion. The small nipple used to attach the sample line to the mixing block is replaced with a larger one (N-4)[3] to prevent plugging of the nipple with tablet excipients after prolonged pumping of sample. The upper nipple of tablet-dispensing mechanism is powered by a synchronous motor.[10] A pulse signal from the programmer causes the motor to rotate the

[3] Technicon Inc., Tarrytown, N. Y.

[4] Asco Red Hat, No. 832043, Automatic Switch Co., Florham Park, N. J.

[5] Circle Seal, No. 119T-lpp, Anaheim, Calif.

[6] Tamson Bath, Witt Sales, Cleveland, Ohio.

[7] Hitachi-Perkin Elmer 139, A. H. Thomas, Philadelphia, Pa.

[8] Catalog No. 9120-NO5, A. H. Thomas, Philadelphia, Pa.

[9] Model TRL, Sargent, Chicago, Ill.

[10] Hurst Manufacturing Co., Princeton, Ind.

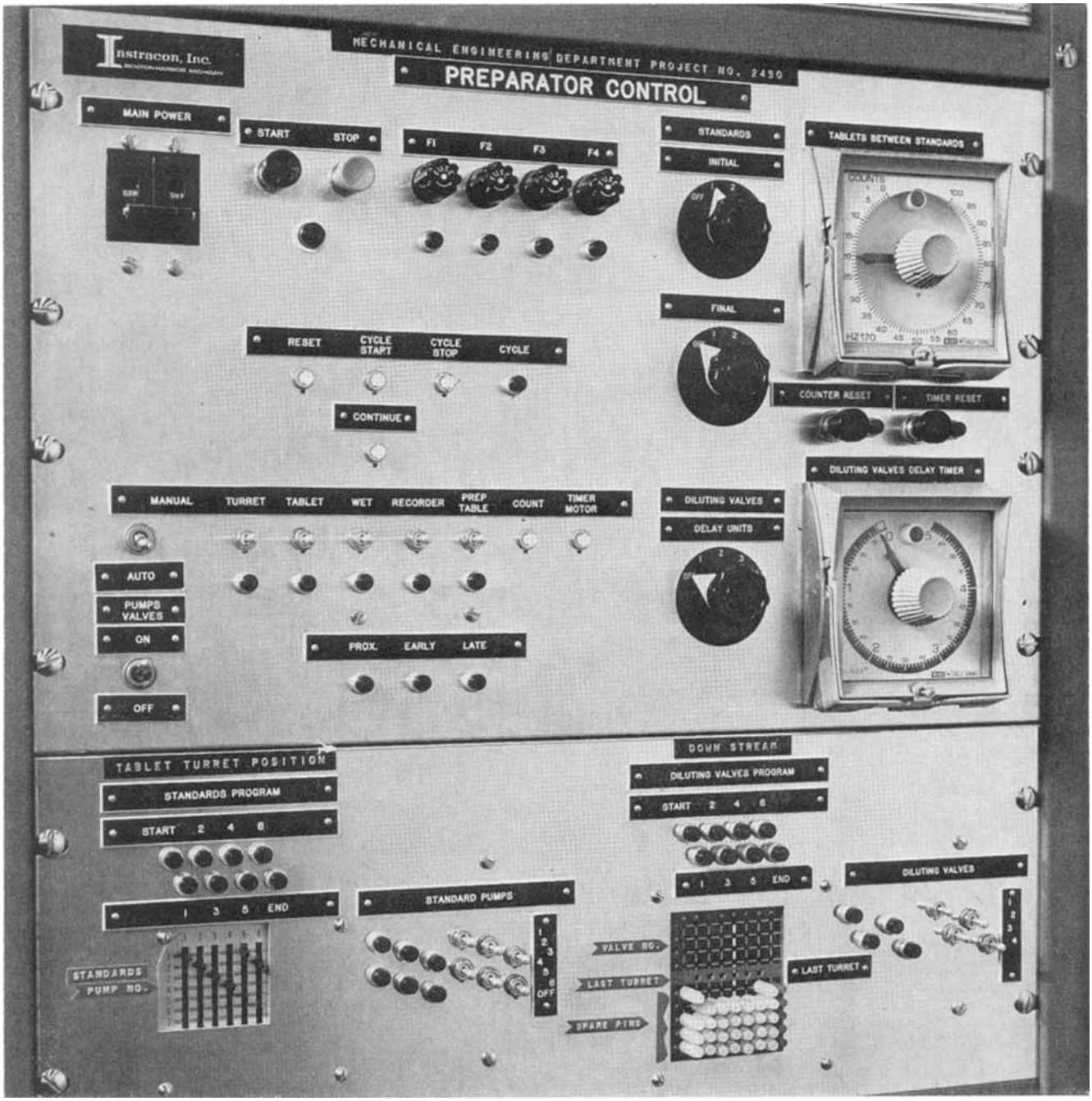


Figure 4—*Control panel of the preparator control.*

disk to the next slot and automatically brake to a stop. The turret mount for the six tablet-holding disks is indexed to the next position by a motor.[11] A pulse signal from the programmer causes this motor to move the turret and brake to a stop when the next disk is in the proper position ready to dispense tablets.

Standards and Tablet-wetting Pumps—Seven pump units consisting of stainless steel pumps, adjustable crank throws, and upper posts were purchased.[12] One-revolution drive and brake units with synchronous motors[10] were constructed to provide braking of the pumps and nonburnout (if stalled) features. Six pumps are used for the six different standards and one pump for tablet wetting (Figs. 1 and 2). The upper and lower valve casings of the pumps containing the tubing attachment nipples were replaced with valve casings having stainless steel tube fittings in order to accept approximately 0.32-cm. (0.125-in.) Teflon tubing. Standards and diluent for tablet wetting are dispensed from each particular pump to a special ring, located above the cup at the point where the tablet is dispensed from the tablet disk, as shown in Fig. 2. A pulse signal from the programmer results in only one filling and diluting cycle of the pump.

Preparator Control[13] *(6)*—This unit (Fig. 4) is an electrical hybrid, having conventional limit switches interfaced with integrated solid-state circuit logic. Output for motors and solenoids comes through contacts on conventional d.c.-driven plug-in relays. The sequence of programmed operations inherent in the solid sampler is not disturbed. Two spare switches in the solid sampler programmer (as

[11] Slo-Syn, Superior Electric Co., Bristol, Conn.
[12] Unit No. FUS-15, National Instrument Co., Baltimore, Md.

[13] Instracon, Inc., Benton Harbor, Mich.

Table I—Diluting Lines and Solenoid Valves Required for Various Steroids

Steroid	Number Diluting Lines	Solenoid Valve No.	Average Absorbance	Coefficient of Variation %
Fluprednisolone, 0.75 mg./10 ml.	None	None	0.219	0.65
Prednisolone, 2.5 mg./10 ml.	None	None	0.705	0.62
Methylprednisolone, 4.0 mg./10 ml.	1	1	0.485	0.64
Hydrocortisone, 10.0 mg./10 ml.	4	1,2,3	0.497	1.21
Methylprednisolone, 16.0 mg./10 ml.	6	1,2,3,4	0.499	1.23
Hydrocortisone, 20.0 mg./10 ml.	6	1,2,3,4	0.703	1.23

purchased) are utilized to sequence the preparator control. The latter unit coordinates the actions of five separate pieces of equipment: (*a*) the solid sampler unit; (*b*) the turret which supports six tablet-holding disks; (*c*) the pumping system for six separate standards and one tablet-wetting pump; (*d*) the instream diluting system which determines the extent of sample dilution after aspiration from the homogenizer; and (*e*) the event pen of the recorder, energized upon delivery of standard.

The preparator control automatically governs functions of the system once started, yet permits the following actions manually by the operator: (*a*) selection of the number of standards dispensed prior to any tablets, numbers of tablets dispensed between the introduction of additional standards, and whether or not a standard is dispensed after the last tablet in each tablet-holding disk; (*b*) selection of the proper times for diluting valves to open or close in order that exact correspondence may be maintained after the introduction of a new series of tablets; (*c*) rotation of the turret to any one of the six tablet-holding disks for correct startings; (*d*) dispensing of standards and tablet-wetting medium prior to initiation of a run in order to purge the lines; and (*e*) testing for malfunction of individual components by activating selected switches or observing pilot lights as the entire sequence of operational events is simulated.

Table II—Individual Tablet Assay of Corticosteroids with the Automated Dispensing Analyzer and Comparison with a Semiautomated Blue Tetrazolium Procedure

Lot No.	Automated Dispensing Analyzer, UV High %	Low %	Mean %	Semiautomated B.T.Z.,[a] %
	Prednisone, 5 mg.			
1	105	98.8	102	96.4
2	105	97.2	101	98.4
3	108	98.4	102	98.0
4	109	96.4	103	98.2
5	110	101	104	98.2
6	105	96.0	99.8	100
7	105	95.4	99.6	100
8	104	96.0	100	97.8
9	105	96.8	101	97.4
10	105	97.0	102	97.4
11	104	94.8	99.4	98.0
12	104	96.8	100	97.0
13	112	98.6	102	98.8
14	110	99.6	104	100
15	105	93.4	100	98.8
16	107	98.0	103	98.6
17	106	97.6	102	101
18	105	97.2	101	99.4
19	107	97.0	101	99.2
20	107	100	103	101
	Methylprednisolone, 4 mg.			
21	106	98.2	102	98.2
22	105	98.5	102	102
23	104	99.8	102	99.0
24	108	97.2	102	98.8
25	104	98.2	101	96.2
26	104	97.8	100	99.8
27	104	97.5	100	98.5
28	102	91.0	97.8	97.8
29	105	98.8	101	98.0
30	108	98.5	103	98.0
31	107	97.8	102	96.5
32	104	95.5	99.5	98.5
33	105	96.0	98.5	97.5
34	106	95.2	100	98.0
35	103	93.8	98.0	97.5
36	102	96.0	98.8	99.5
37	104	96.0	100	98.2
38	106	101	104	98.2
39	113	105	108	103
40	108	99.2	103	98.7

[a] Blue tetrazolium.

OPERATING PROCEDURE

A zero base line with 75% 3A alcohol is established using the manifold flow system of Fig. 1 and with all instruments operating (recorder, UV spectrophotometer at a wavelength at 242 mμ, two proportioning pumps, vacuum pump, continuous filter, 65° heating bath, solid sampler, and preparator control). Sixty milliliters of 75% 3A alcohol is used in the solid sampler. From 1–50 tablets are placed in numbered slots of each of the six disks, and the disks are mounted on the turret and secured. The tablet-wetting pump and all standard pumps used in the analysis are rinsed with solutions to be used to ensure that the lines are free of air and previous liquid. Programs for the standard pumps and associated tablet disks, diluent valves, and number of disks used in the assay are selected on the preparator control panel.

The recorded peak heights resulting from tablets and standards bracketing the tablets are used in conjunction with the concentrations of the standards to calculate the amount of active ingredient in each tablet.

RESULTS AND DISCUSSION

The manifold tubing and flow system of the automated dispensing analyzer is very similar to that reported for the automated UV assay of tolbutamide tablets (7). The durability of Solvaflex tubes during prolonged pumping with 75% 3A alcohol and the ability of this solvent to remove or minimize UV-absorbing material from the tubes were established in the tolbutamide studies (7).

A much better flow of liquid and more uniform air segmentation of the sample stream occurred when the diluent and air lines connected to the blender assembly of the solid sampler were interchanged in the manner described by Kuzel (8). The ratio of rinse to sample was increased (also suggested by Kuzel) so that during the wash cycle a portion of the rinse enters the sample line and the remainder enters the blender, blocking off seepage of sample. Directing the diluent from the solenoid valves to the mixing block of the continuous filter permitted the addition or interruption of diluent without changing sample flow rate. This was particularly important in programming the correct moment for the solenoid diluting valves to be activated. Placing a pulse suppressor in the diluent line at the mixing block of the continuous filter eliminated surging which gave poor mixing of unfiltered sample and diluent.

Standards, tablets, and tablet-wetting diluent are dispensed into the sample cups of the solid sampler 45 min. prior to homogenization. Complete dissolution of drug from the tablet is ensured by a combination of the 45-min. period and by the use of heated diluent in the homogenizing vessel of the solid sample. Delivery volumes of the six standards and one tablet-wetting pump were adjusted so that each pump filled ten 10-ml. volumetric flasks to the mark. Automatic addition of standards and manual pipeting gave equivalent results for steroids when analyzed by ADA.

Table I gives results for six levels of various steroids, four of which require the use of diluting valves. The data are the result of 10 replicate analyses with the automated system. The coefficients of variation ranged from 0.62% for prednisolone to 1.23% for hydrocortisone at concentrations of 2.5 mg./10 ml. and 20.0 mg./10.0 ml., respectively.

Table III—Statistical Results of Single Tablet Potency Assays

Product	Lot No.	No. Tablet	Steroid/Tablet, mg.	Coefficient of Variation (%)	95% Confidence Limits
Fluprednisolone, 0.75 mg.	41	50	0.78	3.74	0.72–0.84
	42	50	0.85	2.88	0.80–0.90
Fluprednisolone, 1.50 mg.	43	10	1.60	1.53	1.55–1.65
	44	10	1.58	3.01	1.49–1.68
Prednisolone, 2.5 mg.	45	50	2.50	2.40	2.38–2.62
	46	50	2.50	2.22	2.39–2.61
Prednisolone, 5.0 mg.	47	40	5.09	2.37	4.85–5.33
	48	25	5.01	2.27	4.78–5.23
Prednisone, 2.5 mg.	49	10	2.59	1.23	2.52–2.65
	50	10	2.57	1.70	2.49–2.67
Prednisone, 5.0 mg.	51	15	5.07	2.17	4.85–5.30
	52	20	5.10	1.74	4.93–5.28
Cortisone acetate, 5.0 mg.	53	10	5.13	1.21	5.00–5.25
	54	24	5.06	1.85	4.87–5.24
Cortisone acetate, 10.0 mg.	55	10	10.17	1.44	9.88–10.47
	56	10	9.41	3.13	8.82–10.00
Cortisone acetate, 25.0 mg.	57	8	24.73	2.60	23.45–26.02
	58	11	24.30	2.53	23.06–25.53
Hydrocortisone, 5.0 mg.	59	49	5.15	2.94	4.85–5.45
	60	25	5.02	1.99	4.82–5.22
Hydrocortisone, 10.0 mg.	61	50	10.0	1.64	9.67–10.33
	62	50	9.94	1.83	9.58–10.31
Hydrocortisone, 20.0 mg.	63	50	19.98	2.14	19.12–20.83
	64	41	20.32	2.63	19.25–21.39
Methylprednisolone, 2.0 mg.	65	10	2.00	1.35	1.94–2.05
	66	50	2.02	1.29	1.97–2.08
Methylprednisolone, 4.0 mg.	67	49	4.18	2.18	4.00–4.37
	68	50	4.12	2.00	3.95–4.28
Methylprednisolone, 16.0 mg.	69	19	15.98	2.15	15.29–16.67
	70	18	16.23	1.77	15.66–16.81
Fluoxymesterone, 1.0 mg.	71	10	1.03	1.59	1.00–1.07
	72	10	1.05	0.92	1.04–1.07
Fluoxymesterone, 2.0 mg.	73	15	2.04	1.70	1.97–2.10
	74	15	2.13	2.92	2.01–2.26
Fluoxymesterone, 5.0 mg.	75	10	5.19	2.38	4.95–5.44
	76	10	5.14	1.89	4.95–5.34
Fluoxymesterone, 10.0 mg.	77	10	9.79	1.10	9.57–10.00
	78	10	10.05	2.80	9.49–10.62
Medroxyprogesterone acetate, 2.5 mg.	79	15	2.54	2.22	2.43–2.65
	80	15	2.58	1.80	2.43–2.67
Medroxyprogesterone acetate, 10.0 mg.	81	10	9.93	3.00	9.34–10.53
	82	10	10.11	1.32	9.95–10.38

Numerous individual steroid tablet assays were carried out with the automated UV system. Representative data of prednisone, 5 mg., and methylprednisolone, 4 mg., are given in Table II. Approximately 50 tablets from each of 20 lots are shown with high and low tablets well within NF (9) and USP (10) limits for content uniformity (85–115% of the average of specified tolerances). Blue tetrazolium assays of composite samples of pulverized tablets are also shown in Table II.

Table III gives statistical data for individual tablet assays, with the steroid content varying from 0.75 mg. fluprednisolone to 25 mg. cortisone acetate. Steroid content, coefficients of variation, and 95% confidence limits were calculated for this series of tablets. Both production and assay precision determine the magnitude of the coefficient of variation and the spread of the 95% confidence limits. The highest coefficient of variation was 3.74% for 0.75 mg. fluprednisolone; the lowest was 0.92% for 1.0 mg. fluoxymesterone.

The tablet data of Tables II and III demonstrate the application of the automated system for unattended UV analysis of individual tablets. Changes only in manifold tubing and the addition or elimination of particular modules should permit the use of other endpoint detection methods.

REFERENCES

(1) H. E. Whipple, *Ann. N. Y. Acad. Sci.*, **130,** 1965.

(2) C. R. Rehm, T. Urbanyi, and T. J. Sloan, *ibid.*, **153,** 640 (1968).

(3) "Automation in Analytical Chemistry," Technicon 1965 Symposia, Mediad Inc., New York, N. Y., 1966.

(4) *Ibid.*, Technicon 1966 Symposia, Mediad Inc., White Plains, N. Y., 1967.

(5) *Ibid.*, Technicon 1967 Symposia, Mediad Inc., White Plains, N. Y., 1968.

(6) W. D. Mohr, "Operations and Technical Information Manual for Preparator Control," Instracon, Inc., Benton Harbor, Mich.

(7) W. F. Beyer and R. L. Houtman, *Ann. N. Y. Acad. Sci.*, **130,** 532(1965).

(8) N. R. Kuzel, "Automation in Analytical Chemistry," Technicon Symposia 1966, vol. 1, Mediad Inc., White Plains, N. Y., 1967, pp. 218–221.

(9) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 449.

(10) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 905.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 16, 1969, from *The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication September 23, 1969.

* Control Analytical Research and Development.

† Mechanical Engineering.

The data of Table II were obtained by E. Christensen, Control Division, The Upjohn Company.

Uniformity of Distribution of Cyanocobalamin in Tablet Formulations

R. J. NESSEL, H. M. APELIAN, and J. BLODINGER

Abstract □ Some of the distribution characteristics of three commercially available, protected cyanocobalamin forms were demonstrated in each of two vitamin formulations of dissimilar particle size. The incorporation of ^{57}Co cyanocobalamin into each of these protected forms permitted rapid, precise measurements of the distribution on individual unit doses. Statistical analysis of the data confirmed the visual observations of improved distribution of the 1% gelatin product.

Keyphrases □ Cyanocobalamin-^{57}Co—formulation distribution □ Distribution characteristics—cyanocobalamin-^{57}Co in tablets □ Wet granulation, direct compression—cyanocobalamin-^{57}Co distribution comparison □ Scintillometry—analysis

Solids mixing has undergone a great deal of scrutiny during the past 20 years, with particular emphasis on rates and mechanisms of the mixing process and on equipment evaluation.

Reviews on these subjects have been made by Weidenbaum (1), Jameson (2), Valentin (3), and Scarlett (4). Few workers, however, have published on the conditions, factors, and properties of the particles themselves or on the particulate system, especially those factors which influence uniform distribution of the active ingredient within a batch and thus affect accurate dosage within each unit dose.

Train (5, 6), in his review articles, considers such factors as particle bed dilation, induced forces, particulate physical properties, and surface forces of particles and their effect on the results of a mixing operation.

He states that particle bed dilation and induced forces are necessary to permit movement of the components in all directions, thus preventing (*a*) dead zones within a system, and (*b*) movement of aggregates as compound entities. In addition, such physical properties as particle size, shape, and density of the various components are key factors in obtaining a uniform mix without segregation tendencies.

The presence of surface-active forces causes aggregation of particles which are difficult to disperse evenly throughout the other components during the normal mixing time.

Rippie *et al.* (7–9) have investigated the properties which contribute to the mixing and unmixing of particulate systems.

The constantly increasing number of high-potency, low-dosage drugs being developed emphasizes the necessity for obtaining proper distribution of particles within a batch.

One of the most potent products of this type in wide use today is cyanocobalamin (vitamin B-12), which is marketed in several protected forms so as to prevent instability of the cyanocobalamin in the presence of certain other active components or their degradation products. The quantity of protected vitamin B-12 necessary to provide the required potency-per-unit dose is extremely low and is usually in the range of 0.05 to 0.15% in relation to the total batch.

Table I—Mesh Distribution of Vitamin Formulations

Mesh	Wet Granulated, %	Dry Blend, %
On 16	4.8	—
On 20	13.4	—
On 30	11.2	0.6
On 40	10.3	5.4
On 60	20.6	15.8
On 80	3.9	12.1
On 100	7.6	7.6
On 140	13.3	8.0
On 200	10.0	11.6
On 325	3.7	6.9
Passes 325	1.2	32.0
	100.0	100.0

The purpose of this investigation is to compare some of the distribution properties of three commercially available, protected forms of vitamin B-12. These properties are examined in multiple vitamin formulations prepared by wet granulation and by direct compression methods.

Presently, there are three methods of assay available for vitamin B-12 in multicomponent tablets: microbiological (10), radiotracer (11), and cyanide measurement (12).

These methods, although valid, do not present a precise picture of the distribution of the vitamin in the final dosage, since the sample required is in excess of a single dose.

The tracer method requires a sample size sufficient to contain 200 to 400 mcg. of the vitamin, thus encompassing as many as 400 dosage units. The result, therefore, is an average potency-per-unit dose.

The microbiological procedure, although not as precise as the previous method, requires a minimum of five doses for proper sampling. Thus, as with the tracer

Table II—Blender Sampling

	Counts/min./mg.[a]					
	1% in Gelatin		1% Resinate		0.1% in Gelatin	
	WG[b]	DB[c]	WG	DB	WG	DB
Top right	15.67	12.11	20.02	13.82	21.12	12.42
Top left	16.91	11.98	20.09	13.05	22.16	12.45
Bottom	16.54	11.94	19.38	13.71	20.82	11.88

[a] Conversion based on sample weight. [b] Wet granulation. [c] Dry blend.

Table III—Distribution of Vitamin B-12 Forms in Wet Granulated Formulation

Counts Per Minute Per Milligram of Tablet Weight								
Beginning[a]			Middle[a]			End[a]		
1% Gelatin	1% Resinate	0.1% in Gel.	1% Gelatin	1% Resinate	0.1% in Gel.	1% Gelatin	1% Resinate	0.1% in Gel.
15.19	18.10	17.05	13.14	16.69	14.65	12.41	15.64	13.74
14.95	17.68	17.74	13.40	15.87	15.18	12.64	15.30	13.93
14.88	17.55	17.37	13.21	16.79	14.37	12.79	15.64	13.91
14.80	17.90	16.51	13.38	16.16	14.36	13.01	14.94	14.26
14.91	17.57	16.71	13.14	17.27	14.58	12.73	15.33	14.16
15.25	18.35	16.91	13.33	16.93	14.71	12.42	15.29	14.87
15.54	17.07	16.86	13.54	17.22	14.20	12.56	15.34	14.29
14.71	17.79	17.21	12.81	15.92	14.62	12.46	16.11	13.96
14.94	18.50	17.35	12.97	16.02	14.97	12.66	15.79	14.44
15.31	17.28	16.57	13.22	16.12	14.65	12.61	15.00	14.97
15.24	17.67	16.72	13.61	16.66	14.69	12.94	15.38	14.38
15.29	17.49	17.25	13.30	16.66	14.42	13.01	15.83	14.43
15.08	18.00	17.07	13.47	16.32	14.57	12.46	14.19	13.92
14.98	17.83	16.94	13.09	17.03	14.86	12.71	14.49	14.66
15.02	18.15	16.51	13.51	16.64	14.29	12.71	14.83	14.08
15.23	16.82	15.85	13.42	16.63	14.70	12.60	15.95	13.99
15.66	18.71	16.86	13.39	16.69	14.57	12.30	15.21	14.27
14.99	17.81	17.02	13.23	16.62	14.46	12.70	15.21	14.00
15.00	17.51	16.77	13.13	16.57	14.77	12.89	16.17	14.83
15.23	18.02	17.40	13.14	16.93	15.08	12.59	15.91	13.96

[a] Portion of compression cycle.

method, only an average potency-per-dosage unit is obtained.

The cyanide measurement method, while being one of the most sensitive chemical methods for cyanocobalamin, is not specific because other nonvitamin materials may also liberate trace amounts of cyanide.

Although the last two procedures could be adapted to single tablet assays, the time required for each determination is excessive.

For this study, the protected cyanocobalamin forms were radioactively labeled, permitting rapid, precise measurements of the vitamin B-12 content on a unit dose.

EXPERIMENTAL

Preparation of Radioactive Materials—Cyanocobalamin-^{57}Co solution, USP,[1] equivalent to 50 and 500 μc., respectively, of radioactivity was used in the preparation of the 0.1%[2] and 1%[3] cyanocobalamin in gelatin products by adding it to the solution prior to the drying process.

Commercially available 1% cyanocobalamin on resin[4] was slurried for 8 hr. with USP ^{57}Co cyanocobalamin solution, equivalent to 500 μc. of the radioactive compound, and dried overnight at 40° in a circulating air oven. The dried powder was passed through an 80-mesh standard sieve to break up any soft agglomerates which formed. Mesh analysis performed before and after labeling revealed that no change in particle size was evident.

Preparation of Basic Formulations—The distributive properties of the cyanocobalamin forms were studied in each of two vitamin formulations: (*a*) a granulated formula, prepared by wet granulation procedures, representing a formulation having diversified particle size; (*b*) a dry blend, directly compressible, representing a formulation of materials in the fine-particle-size range.

The mesh distribution of each of the formulations is shown in Table I.

Mixing Evaluation—The procedure for blending the labeled compounds varied only with respect to the premix step.

In the case of the granulation, 85 g. of finer-than-80-mesh material was removed by screening. The radioactive material was added to the fines, coarsely blended, passed through a 40-mesh screen, and reblended for 5 min. in a rotating bottle.

The premix for the direct compression formulation was prepared by coarsely blending the labeled material with 95 g. of the mixture containing vitamins and diluents normally added after a milling operation, passing this blend through a 30-mesh screen, and reblending for 5 min. in a rotating bottle.

Final blending of all formulations was accomplished by adding the premix to approximately half of the material in a Twin Shell blender,[5] blending for 5 min., adding the remainder of the formula, and blending an additional 10 min.

Samples equivalent to the theoretical tablet weight were then removed for counting from the top and bottom sections of the blender to determine the efficiency of the operation.

Each formulation was compressed on a Rotary B-2 tableting machine[6] at a maximum speed of 700 tablets per minute.

Once the theoretical tablet weight was attained, samples were removed from the beginning, middle, and end of the tableting run. Twenty tablets were selected at random from each of the three samples and individually weighed on a single-pan Mettler balance.

All radioactivity counts were made on a Model SC 530 gamma spectrometer.[7]

RESULTS AND DISCUSSION

The quantities of radioactivity were utilized in the preparation of the labeled forms to provide a minimum of 5000 counts per minute when 5 mcg., plus a 10% excess of cyanocobalamin, were incorporated per tablet.

The degree of mixedness of each of the formulations prior to compression, as shown in Table II, reflects satisfactory blending in all cases.

A radioactive count was performed on tablet samples before and after disintegration in water to ascertain counting efficiency in the dry state as opposed to the wet state. Since the radioactivity count on both samples varied by less than 1%, all future counts were made on intact tablets. Each sample counted in duplicate for 1 min. yielded 6000–7500 counts, with a 10-min. background count of 1000. The average count was corrected for background interference and converted to counts per minute per milligram of tablet weight to provide an accurate determination of the distribution of the vitamin B-12 form on a unit weight basis. The results are shown in Tables III and IV.

[1] Merck & Co., Inc., Rahway, N. J.
[2] 0.1% cyanocobalamin in gelatin, Merck & Co., Inc., Rahway, N. J.
[3] Stabicote, Merck & Co., Inc., Rahway, N. J.
[4] Stablets, Type I, Chas. Pfizer & Co., Inc., New York, N. Y.
[5] Patterson-Kelly, E. Stroudsburg, Pa.
[6] Stokes Division, Pennsalt Chemical Co., Warminster, Pa.
[7] Tracer-Lab, Division of Laboratory for Electronics, Inc., Waltham, Mass.

Table IV—Distribution of Vitamin B-12 Forms in Dry Blend Formulation

Counts Per Minute Per Milligram of Tablet Weight								
Beginning[a]			Middle[a]			End[a]		
1% Gelatin	1% Resinate	0.1% in Gel.	1% Gelatin	1% Resinate	0.1% in Gel.	1% Gelatin	1% Resinate	0.1% in Gel.
11.87	13.85	13.81	12.14	14.05	13.69	12.10	13.61	13.68
11.79	14.86	13.66	12.13	13.23	13.64	12.34	13.91	13.92
12.13	13.84	13.74	12.21	13.85	13.89	12.00	13.44	13.85
11.84	13.53	13.89	11.62	14.38	13.83	12.51	13.69	14.03
12.07	13.92	13.79	11.84	14.19	13.92	12.14	13.26	13.87
11.82	13.79	13.74	11.82	13.84	13.66	12.28	13.64	13.96
11.76	14.35	13.78	11.89	13.92	13.54	12.03	13.41	13.81
12.05	14.02	13.61	12.46	14.12	14.00	11.90	13.86	13.80
11.87	13.20	13.71	11.99	13.80	13.72	12.31	14.08	14.02
12.17	13.66	13.61	12.00	13.43	13.78	12.04	14.23	13.72
12.03	14.52	13.64	12.11	13.54	13.72	12.14	13.44	14.03
12.13	13.93	13.90	11.94	13.10	13.69	12.10	14.03	13.75
12.07	13.69	13.81	12.12	13.56	13.79	12.11	13.69	13.94
12.30	13.94	13.64	12.21	14.36	13.67	12.22	13.48	13.72
11.77	13.78	13.61	11.91	14.47	13.99	11.87	14.50	13.82
12.28	13.96	13.67	11.92	14.37	13.78	12.04	13.40	13.99
12.19	14.52	13.73	12.27	13.85	13.69	11.94	13.48	13.85
12.03	13.57	13.83	11.85	14.10	13.94	11.87	14.08	13.84
12.00	13.61	13.51	11.95	14.09	13.85	12.11	13.67	13.88
11.87	13.41	13.52	11.97	13.34	13.73	12.11	13.88	13.93

[a] Portion of compression cycle.

Table V—Variances by Formulation by Position in Batch

Position	1% Gelatin	1% Resinate	0.1% Gelatin
Wet Granulated Formulation			
Beginning	0.0573	0.2129	0.1709
Middle	0.0408	0.1653	0.0655
End	0.0398	0.2737	0.1275
Dry Blend Formulation			
Beginning	0.0287	0.1640	0.0127
Middle	0.0363	0.1637	0.0158
End	0.0270	0.1060	0.0120

The variances by formulation and position in batch were calculated from the data and are shown in Table V. The analysis of variance for each formulation was made on the logarithms of the individual cell variance and is shown in Table VI. Statistically significant differences in variation were exhibited between formulations. These differences in variability were of such high levels of significance that individual F ratios were computed for the three types of formulations. In all cases, the difference in variability between the 1% gelatin products and the 1% resin adsorbate was more statistically significant than that between the 1% and the 0.1% gelatin products.

The significantly lower variability of the 0.1% product, as compared to the 1% gelatin form in the dry mix formulation, may probably be attributed to a dilution effect caused by the tenfold increase in quantity required to obtain the same potency. This may also be true for the significantly lower variability in both formulations obtained for the 0.1% gelatin product, as compared to the 1% resinate. Table VII depicts the significance levels of the F ratios. These were computed from the ratios of the variances shown in Table V.

Table VI—Analysis-of-variance Calculations on the Logarithms of the Variances

Source of Variation	*DF*	*SS*	*MS*	*F*
Wet Granulated Formulation				
Position of batch	2	0.08139	0.04069	3.30
Formulation	2	0.68376	0.34188	25.65
Pos. X form.	4	0.04932	0.01233	
Total	8	0.81447		
Dry Blend Formulation				
Position of batch	2	0.03191	0.01595	7.03
Formulation	2	1.62227	0.81113	238.22
Pos. X form.	4	0.00911	0.00227	
Total	8	1.66329		

Table VII—Significance Levels of F Ratios

Position	1% Gelatin *vs.* 1% Resinate	1% Gelatin *vs.* 0.1% Gelatin	0.1% Gelatin *vs.* 1% Resinate
Wet Granulated Formulation			
Beginning	99.5%	97.5%	<75.0%
Middle	99.5%	90.0%	97.5%
End	99.9%	99.0%	95.0%
Dry Blend Formulation			
Beginning	99.9%	95.0%	99.9%
Middle	99.9%	95.0%	99.9%
End	99.5%	97.5%	99.9%

It is interesting to note that in comparing the actual variances for both 1% products, the individual position values for the resin product were about 4 to 7 times greater than those for the gelatin product.

SUMMARY AND CONCLUSIONS

Three commercially available protected vitamin B-12 products were labeled with ^{57}Co cyanocobalamin. Each was then blended with both a granulated and a dry mix vitamin formulation and compressed into tablets.

The distribution characteristics for each form were evaluated by measuring the radioactivity of individual tablets obtained from different portions of the compression run.

Statistical analysis of the data showed that in all cases, variations in ^{57}Co labeled cyanocobalamin content throughout both formulations were significantly lower for the 1% gelatin product than for the resin product, indicating that it distributes more uniformly in tablet formulations.

For the granulated formulation, the variation of the 1% gelatin form was also less than its 0.1% counterpart. For the dry blend, however, the variation in the 0.1% product was lower, although the difference was less significant than that found when the two 1% products were compared. A significantly lower variability was also evident in both formulations for the 0.1% gelatin product, as compared to the 1% resinate.

These latter results may possibly be attributed to the fact that the quantity requirement of the 0.1% product is 10 times that of the 1% material.

REFERENCES

(1) S. S. Weidenbaum, "Advances in Chemical Engineering," vol. 2, Academic, New York, N. Y., 1958.

(2) G. J. Jameson, *Chem. Process Eng.*, **42**, 489(1961).
(3) F. H. H. Valentin, *ibid.*, **46**, 181(1965).
(4) B. Scarlett, *ibid.*, **45**, 612(1964).
(5) D. Train, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 265(1960).
(6) D. Train, *Pharm. J.*, **185**, 129(1960).
(7) J. L. Olsen and E. G. Rippie, *J. Pharm. Sci.*, **53**, 147(1964).
(8) E. G. Rippie, J. L. Olsen, and M. D. Faiman, *ibid.*, **53**, 1361 (1964).
(9) M. D. Faiman and E. G. Rippie, *ibid.*, **54**, 719(1965).
(10) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 864.
(11) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 445.
(12) G. E. Boxer and J. C. Pickards, *Arch. Biochem.*, **30**, 372 (1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 31, 1968, from the *Product Development and Service Laboratories, Merck & Co., Inc., Rahway, NJ 07065*
Accepted for publication September 11, 1969.
The authors wish to acknowledge W. Walker, B. Marcus, and E. Eggimann for their assistance in the preparation of this paper.

NOTES

Metabolic Fate of 2,3,5-Triiodobenzoic Acid in Laying Hens

SUSAN G. ROWLES, GORDON S. BORN, JOHN E. CHRISTIAN, and WAYNE V. KESSLER

Abstract □ The whole body retention, distribution, and metabolism of carboxyl-^{14}C-labeled 2,3,5-triiodobenzoic acid (TIBA*) were studied in five laying hens. A single oral dose of TIBA* showed a 22-hr. biological half-life. No organ concentration of TIBA* was noted. TIBA* and seven labeled metabolites were found in excreta collected 6 to 12 hr. after the dose, with TIBA* representing the major end product. TIBA* and four labeled metabolites were detected in excreta collected during the 78 to 90-hr. interval; the metabolites occurred in greater proportion than did TIBA* in this sample.

Keyphrases □ 2,3,5-Triiodobenzoic acid (TIBA), carboxyl-^{14}C-labeled—metabolic fate □ Distribution, metabolism, whole body retention—TIBA, carboxyl-^{14}C-labeled □ Metabolites—TIBA, carboxyl-^{14}C-labeled □ Thick-layer chromatography—separation, identification

Greer and Anderson (1) reported that treatment of soybean plants with 2,3,5-triiodobenzoic acid (TIBA) at the beginning of flowering resulted in an increased seed yield due to two major types of effects. Their studies showed that TIBA caused the plants to change from vegetative to reproductive development more rapidly and also caused morphological changes which permitted more efficient utilization of sunlight by the plants. Since a residue of TIBA remains in soybeans grown from treated plants (2), the potential environmental health hazard of TIBA should be investigated prior to the general usage of the compound in agriculture. For this reason, a study of the retention, distribution, and metabolism of TIBA in laying hens was of interest.

Ice *et al.* (3, 4) employed TIBA labeled with ^{131}I in Position 2 (TI*BA) for metabolism studies in rats and lactating animals. In both studies, a significant thyroid concentration of ^{131}I was noted, and metabolism by deiodination was indicated. In rats and in lactating animals, the whole body retention of TI*BA was characterized by a two-component system.

Ware and Barker (5) administered carboxyl-^{14}C-labeled TIBA (TIBA*) orally to rats and found TIBA* and/or its labeled metabolites in all organs analyzed. Excretion of the compound was primarily through the urine, in which both TIBA and 2,5-diiodobenzoic acid were detected.

The metabolism of TIBA by chickens was investigated by Barker *et al.* (6) who employed TIBA labeled with ^{125}I in Positions 3 and 5. They found that 90% of the orally administered radioactivity was excreted within 48 hr. and that TIBA and 2,5-diiodobenzoic acid were present in the excreta. The same investigators dosed chickens and pigs with unlabeled TIBA and detected, by using gas chromatography, the presence of TIBA, 2,5-diiodobenzoic acid, and 3,5-diiodobenzoic acid in the chicken brains and in the thyroids of both chickens and pigs.

EXPERIMENTAL

Administration of TIBA*—Carboxyl-^{14}C-labeled TIBA (TIBA*) was available from the work reported by Spitznagle (2) and was purified immediately prior to use in this study by a method similar to that reported by Jarboe (7). An ethanolic solution of the impure TIBA* was applied to thick-layer plates (1.0 mm.) of purified silica gel[1] which were then developed three times each (12 cm. per development) in petroleum ether (30 to 60° fraction)–propionic acid (10:1 v/v) (8). The silica containing the pure TIBA*, located on the chromatograms by autoradiography, was removed from the plates and extracted with anhydrous ethyl ether, using a continuous extraction apparatus. The ether was allowed to evaporate at room

[1] Adsorbosil-1 with 10% binder, Applied Science Laboratories, State College, Pa.

(2) G. J. Jameson, *Chem. Process Eng.*, **42**, 489(1961).
(3) F. H. H. Valentin, *ibid.*, **46**, 181(1965).
(4) B. Scarlett, *ibid.*, **45**, 612(1964).
(5) D. Train, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 265(1960).
(6) D. Train, *Pharm. J.*, **185**, 129(1960).
(7) J. L. Olsen and E. G. Rippie, *J. Pharm. Sci.*, **53**, 147(1964).
(8) E. G. Rippie, J. L. Olsen, and M. D. Faiman, *ibid.*, **53**, 1361 (1964).
(9) M. D. Faiman and E. G. Rippie, *ibid.*, **54**, 719(1965).
(10) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 864.
(11) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 445.
(12) G. E. Boxer and J. C. Pickards, *Arch. Biochem.*, **30**, 372 (1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 31, 1968, from the *Product Development and Service Laboratories, Merck & Co., Inc., Rahway, NJ 07065*
Accepted for publication September 11, 1969.
The authors wish to acknowledge W. Walker, B. Marcus, and E. Eggimann for their assistance in the preparation of this paper.

NOTES

Metabolic Fate of 2,3,5-Triiodobenzoic Acid in Laying Hens

SUSAN G. ROWLES, GORDON S. BORN, JOHN E. CHRISTIAN, and WAYNE V. KESSLER

Abstract □ The whole body retention, distribution, and metabolism of carboxyl-^{14}C-labeled 2,3,5-triiodobenzoic acid (TIBA*) were studied in five laying hens. A single oral dose of TIBA* showed a 22-hr. biological half-life. No organ concentration of TIBA* was noted. TIBA* and seven labeled metabolites were found in excreta collected 6 to 12 hr. after the dose, with TIBA* representing the major end product. TIBA* and four labeled metabolites were detected in excreta collected during the 78 to 90-hr. interval; the metabolites occurred in greater proportion than did TIBA* in this sample.

Keyphrases □ 2,3,5-Triiodobenzoic acid (TIBA), carboxyl-^{14}C-labeled—metabolic fate □ Distribution, metabolism, whole body retention—TIBA, carboxyl-^{14}C-labeled □ Metabolites—TIBA, carboxyl-^{14}C-labeled □ Thick-layer chromatography—separation, identification

Greer and Anderson (1) reported that treatment of soybean plants with 2,3,5-triiodobenzoic acid (TIBA) at the beginning of flowering resulted in an increased seed yield due to two major types of effects. Their studies showed that TIBA caused the plants to change from vegetative to reproductive development more rapidly and also caused morphological changes which permitted more efficient utilization of sunlight by the plants. Since a residue of TIBA remains in soybeans grown from treated plants (2), the potential environmental health hazard of TIBA should be investigated prior to the general usage of the compound in agriculture. For this reason, a study of the retention, distribution, and metabolism of TIBA in laying hens was of interest.

Ice *et al.* (3, 4) employed TIBA labeled with ^{131}I in Position 2 (TI*BA) for metabolism studies in rats and lactating animals. In both studies, a significant thyroid concentration of ^{131}I was noted, and metabolism by deiodination was indicated. In rats and in lactating animals, the whole body retention of TI*BA was characterized by a two-component system.

Ware and Barker (5) administered carboxyl-^{14}C-labeled TIBA (TIBA*) orally to rats and found TIBA* and/or its labeled metabolites in all organs analyzed. Excretion of the compound was primarily through the urine, in which both TIBA and 2,5-diiodobenzoic acid were detected.

The metabolism of TIBA by chickens was investigated by Barker *et al.* (6) who employed TIBA labeled with ^{125}I in Positions 3 and 5. They found that 90% of the orally administered radioactivity was excreted within 48 hr. and that TIBA and 2,5-diiodobenzoic acid were present in the excreta. The same investigators dosed chickens and pigs with unlabeled TIBA and detected, by using gas chromatography, the presence of TIBA, 2,5-diiodobenzoic acid, and 3,5-diiodobenzoic acid in the chicken brains and in the thyroids of both chickens and pigs.

EXPERIMENTAL

Administration of TIBA*—Carboxyl-^{14}C-labeled TIBA (TIBA*) was available from the work reported by Spitznagle (2) and was purified immediately prior to use in this study by a method similar to that reported by Jarboe (7). An ethanolic solution of the impure TIBA* was applied to thick-layer plates (1.0 mm.) of purified silica gel[1] which were then developed three times each (12 cm. per development) in petroleum ether (30 to 60° fraction)–propionic acid (10:1 v/v) (8). The silica containing the pure TIBA*, located on the chromatograms by autoradiography, was removed from the plates and extracted with anhydrous ethyl ether, using a continuous extraction apparatus. The ether was allowed to evaporate at room

[1] Adsorbosil-1 with 10% binder, Applied Science Laboratories, State College, Pa.

Table I—The Retention of Orally Administered TIBA* and/or Its Radioactive Metabolites by Laying Hens

Time, hr.	% Excretion[a]	Standard Error	% Retention[b]
6	17.3	1.9	82.7
12	20.1	3.0	62.6
18	9.3	2.1	53.3
24	14.4	0.8	38.9
30	7.5	1.0	31.4
42	11.3	2.1	20.1
54	6.5	2.7	13.6
66	2.5	0.7	11.1
78	3.2	0.9	7.9
90	1.4	0.3	6.5
96	1.8	1.2	4.7

[a] Quantity excreted since previous sample collection; mean of five animals. [b] Calculated from the accumulated mean excretion data.

Table II—The Distribution of TIBA* and/or Its Radioactive Metabolites 96 hr. after Oral Administration to Laying Hens

Tissue	% × 10^{-4} of Dose/g. of Tissue[a]	Standard Error
Brain	2.7	0.4
Breast muscle	6.0	1.3
Fat	2.8	0.3
Gizzard	7.8	2.6
Heart	11.9	3.7
Kidney	36.3	11.8
Liver	9.8	2.8
Thigh muscle	6.6	2.0
Thyroid	7.4	0.9

[a] Mean of five hens.

temperature, giving crystals of purified TIBA*. The radiochemical purity of the purified TIBA* was greater than 99%, as determined by thin-layer chromatography, autoradiography, and ^{14}C assay of the chromatogram.

Five laying single comb white leghorn hens were given, by oral intubation, a single dose of a 2.23% solution of TIBA* in ethanol and glycerin (1:1 v/v). This dose contained 7.7 mg. of TIBA* and 57.1 μc of ^{14}C per kilogram of body weight.

Sample Collection and Analysis—The animals were housed individually in metabolism cages and were supplied a breeder laying mash and tap water *ad libitum*. Total excreta samples were collected from each hen at 6, 12, 18, 24, 30, 42, 54, 66, 78, 90, and 96 hr. Eggs were collected immediately after they were laid and were hard-cooked prior to analysis. The animals were sacrificed by decapitation 96 hr. after the dosage, and samples of the brain, breast muscle, fat, gizzard, heart, kidney, liver, thigh muscle, and the thyroids were obtained. Representative aliquots of the excreta, egg albumin, egg yolk, and tissue samples were digested with a 1 *M* methanolic solution of hyamine hydroxide[2] and mixed with 15 ml. of a liquid scintillator containing: PPO, 10.0 g.; naphthalene, 80.0 g.; *p*-xylene, 143 ml.; *p*-dioxane, 429 ml.; and a sufficient quantity of 2-ethoxyethanol to make 1 l. The samples were then assayed for ^{14}C in an internal sample liquid scintillation spectrometer. Internal standardization with benzoic acid-^{14}C was employed to correct for quenching. Sample-counting efficiencies of approximately 50% were obtained.

Metabolism—Twelve-gram aliquots of the 6 to 12-hr. and 78 to 90-hr. excreta samples from the one hen whose egg-laying pattern was most regular were extracted separately with anhydrous ether using a continuous extraction apparatus. After a 48-hr. extraction period, 60% of the ^{14}C present in the samples was extracted. Following this extraction, portions of the extracted samples were then hydrolyzed separately with 85% *o*-phosphoric acid and 2 *N* nitric acid. Less than 2% of the remaining ^{14}C could be extracted from the acid-treated samples with ether.

[2] A methanolic solution of hyamine hydroxide prepared according to Bruno (9) from Hyamine 10-X crystals, Rohm & Haas, Philadelphia, Pa.

Table III—The Relative Occurrence of TIBA* and Its Radioactive Metabolites in Ether-Extracted Excreta Samples[a]

Location of Radioactive Zone (R_f)	Identity[b]	Relative % Occurrence 6 to 12-hr. Extract	78 to 90-hr. Extract
0.00		5.1	8.9
0.08–0.10		1.4	0.6
0.19		1.0	—
0.31–0.35	TIBA	52.6	10.2
0.42–0.48	2,3-DIBA	1.7	3.2
0.56		0.3	—
0.71–0.75	2,5-DIBA	29.6	69.4
0.89	3,5-DIBA	4.3	—

[a] Determined by thick-layer chromatography on purified silica gel plates (1 mm.) developed in petroleum ether (30 to 60° fraction)–propionic acid (10 to 1 v/v). [b] Based on cochromatography with reference standards.

The ether extracts were concentrated and applied to thick-layer (1.0 mm.) plates of purified silica gel. The plates were developed three times each (12 cm. per development) in petroleum ether (30 to 60° fraction)–propionic acid (10:1 v/v) (9), air-dried, and autoradiographed. Reference standards of unlabeled TIBA and six suspected metabolites were chromatographed simultaneously on the same plates and were then visualized by spraying with a 0.1% solution of bromocresol green in acetone.

The radioactive zones located by autoradiography were scraped individually from the chromatograms and added to 18 ml. of a scintillator gel,[3] mixed thoroughly, and assayed for ^{14}C to determine their relative occurrence.

RESULTS AND DISCUSSION

The retention of TIBA* and/or its labeled metabolites at various intervals following oral administration of TIBA* is given in Table I. Mathematical analysis of these data suggests that TIBA* and/or its labeled metabolites have a single component retention system with a biological half-life of approximately 22 hr. This is in contrast to the two-component systems observed in studies using ^{131}I-labeled TIBA (3, 4). At the end of the 96-hr. study, 95.3% of the administered ^{14}C was recovered in the excreta.

Very small quantities of TIBA* and/or its labeled metabolites were detected in the tissues, as shown in Table II. At the end of the 96-hr. study, the maximum ^{14}C level detected in any tissue corresponded to less than 0.004% of the administered dose per gram of tissue. Fairly wide variations of tissue ^{14}C levels were noted among the five animals, as seen by the magnitude of the mean standard errors reported in Table II. This may be due in part to the very low level of activity present in all tissues and to biological variation among the hens. No organ concentration of ^{14}C was noted. This result is significant because of its contrast to studies in rats, cows, and goats which showed that the thyroid concentrated ^{131}I-labeled TIBA and/or its labeled metabolites, most probably in the form of $^{131}I^-$ ion (3, 4).

The level of ^{14}C present in all eggs collected during the 96-hr. study was very low. The maximum amount of ^{14}C detected in any yolk or albumin fraction corresponded to less than 0.008% of the administered dose per gram of egg fraction.

The TIBA* and its labeled metabolites present in the 6 to 12-hr. excreta extract were separated by thick-layer chromatography into eight fractions corresponding to four unknowns, TIBA, 2,3-diiodobenzoic acid (2,3-DIBA), 2,5-diiodobenzoic acid (2,5-DIBA), and 3,5-diiodobenzoic acid (3,5-DIBA). The ether extract of the 78 to 90-hr. excreta sample contained two unknowns, TIBA, 2,3-DIBA, and 2,5-DIBA, as determined by thick-layer chromatography. The relative occurrence of TIBA* and its labeled metabolites in the excreta extracts is given in Table III. These results show that the major labeled compound detected in the 6 to 12-hr. excreta sample was TIBA, which accounted for 52.6% of the ether extracted radioactivity. Metabolites present in smaller quantities included

[3] A 4% w/w solution of Cab-O-Sil Thixotropic Gel Powder, Packard Instrument Co., Downers Grove, Ill., and the liquid scintillator described above.

2,5-DIBA, 3,5-DIBA, and 2,3-DIBA. TIBA was present in the 78 to 90-hr. sample in a smaller proportion than in the 6 to 12-hr. sample, and accounted for only 10.2% of the extracted ^{14}C. However, the metabolites occurred in larger proportions in the later sample. The results of the metabolite study indicate that TIBA is metabolized to a significant extent by deiodination.

SUMMARY

In the authors' opinion, TIBA has a low probability of becoming an environmental health hazard for a number of reasons. Other investigators have reported the following: TIBA is applied to soybeans in very small quantities and only a minute fraction of the amount applied can be detected in the harvested beans; the toxicity of TIBA is relatively low in humans; and the major portion of TIBA ingested orally by rats, cows, goats, and chickens is excreted rapidly. The work reported here also shows rapid excretion of TIBA in chickens, and no egg or organ concentration of the compound.

REFERENCES

(1) H. A. L. Greer and I. C. Anderson, *Crop Sci.*, **5**, 229(1965).

(2) L. A. Spitznagle, J. E. Christian, A. J. Ohlrogge, and C. E. Breckenridge, Jr., *J. Pharm. Sci.*, **57**, 764(1968).

(3) R. D. Ice, C. E. Breckenridge, Jr., and J. E. Christian, *ibid.*, **55**, 497(1966).

(4) R. D. Ice, J. E. Christian, and M. P. Plumlee, *ibid.*, **57**, 399 (1968).

(5) J. H. Ware and W. M. Barker, *Fed. Am. Soc. Exptl. Biol.*, annual meeting paper 2652, 1966.

(6) W. M. Barker, D. J. Thompson, and J. H. Ware, personal communication, International Minerals and Chemical Corp., Skokie, Ill., 1966.

(7) R. H. Jarboe, J. B. Data, and J. E. Christian, *J. Pharm. Sci.*, **57**, 323(1968).

(8) A. G. Ebert and J. H. Ware, personal communication, International Minerals and Chemical Corp., Skokie, Ill., 1965.

(9) G. A. Bruno, M.S. thesis, Purdue University, Lafayette, Ind., 1960.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *Department of Bionucleonics, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication September 10, 1969.

This research was conducted under the auspices of the Institute for Environmental Health Studies.

N-acyl Derivatives of Bis-(4-aminophenyl) Disulfide and its Thiolsulfinate

WILLIAM O. FOYE and JOSEPH P. SPERANZA

Abstract □ Bis-(4-aminophenyl)-sulfone and several *N,N'*-diacyl derivatives have shown appreciable activity as antimalarials. With the assumption that a molecule more readily cleaved to a *p*-aminophenyl sulfur or oxidized sulfur anion, as a potential anti-PAB substance, might be a more effective antimalarial, a series of *N,N'*-diacyl derivatives of bis-(4-aminophenyl) disulfide was synthesized. Oxidation with peroxide followed by acylation gave the corresponding thiolsulfinates. Antimalarial activity was found for bis-(4-*p*-acetamidobenzenesulfonamidophenyl) disulfide and *N,N'*-bis-(α-aminoacyl) derivatives of bis-(4-aminophenyl)-sulfone.

Keyphrases □ Bis-(4-aminophenyl) disulfide and thiosulfinate—*N*-acyl derivatives synthesis □ Antimalarial activity—bis-(4-aminophenyl) disulfide derivatives □ IR spectrophotometry—structure □ UV spectrophotometry—structure

Diaminodiphenylsulfone (DDS) (1) and its *N,N'*-diacetyl derivative (2) have both shown appreciable antimalarial activity, particularly against strains of *P. falciparum* resistant to chloroquine and other widely used antimalarials. Evidence has been found to suggest that these compounds are effective by interfering with the utilization of PABA by the parasites (3). If this is the case, then compounds more readily cleaved *in vivo* to a *p*-aminophenyl sulfur or oxidized sulfur moiety might interfere more effectively with PABA utilization. Accordingly, a series of *N,N'*-diacyl derivatives of bis-(4-aminophenyl) disulfide and *S*-oxidized derivatives has been prepared for antimalarial evaluation. Other disulfides, including 5,5'-diacetamido-8,8'-diquinolyl disulfide, have shown antimalarial activity (4, 5).

Bis-(4-aminophenyl) disulfide was obtained by the procedure of Price and Stacy (6), in which sodium 4-aminophenylmercaptide was oxidized by 30% hydrogen peroxide to the disulfide. The product showed the expected IR absorption bands for an aromatic amine, in addition to two sharp bands at 1175 and 1065 $cm.^{-1}$. Bredereck (7) has attributed the presence of these bands in aromatic disulfides to the disulfide linkage and a 1,4-disubstituted aromatic ring, respectively. However, aliphatic disulfides have shown similar peaks in the 1050–1250 $cm.^{-1}$ region which were believed due to CH wag on the carbon adjacent to sulfur (8). UV absorption showed a peak at 256 mμ, characteristic of disulfides (9), with a shoulder at 290–295 mμ.

Peroxide oxidation of aliphatic disulfides has led to formation of thiolsulfinates, thiosulfonates, and α-disulfones, depending on reaction conditions (10). Also, percamphoric acid oxidation of alkyl or aryl disulfides gave mixtures of disulfides, thiolsulfinates, and thiolsulfonates (11). With bis-(4-aminophenyl) disulfide, peroxide oxidation gave only the thiolsulfinate, even on long standing. Heating resulted in decomposition. IR absorption of the product showed a peak at 1050 $cm.^{-1}$, in addition to those present for the disulfide, which is characteristic of thiolsulfinates (8, 12). UV

2,5-DIBA, 3,5-DIBA, and 2,3-DIBA. TIBA was present in the 78 to 90-hr. sample in a smaller proportion than in the 6 to 12-hr. sample, and accounted for only 10.2% of the extracted ^{14}C. However, the metabolites occurred in larger proportions in the later sample. The results of the metabolite study indicate that TIBA is metabolized to a significant extent by deiodination.

SUMMARY

In the authors' opinion, TIBA has a low probability of becoming an environmental health hazard for a number of reasons. Other investigators have reported the following: TIBA is applied to soybeans in very small quantities and only a minute fraction of the amount applied can be detected in the harvested beans; the toxicity of TIBA is relatively low in humans; and the major portion of TIBA ingested orally by rats, cows, goats, and chickens is excreted rapidly. The work reported here also shows rapid excretion of TIBA in chickens, and no egg or organ concentration of the compound.

REFERENCES

(1) H. A. L. Greer and I. C. Anderson, *Crop Sci.*, **5**, 229(1965).

(2) L. A. Spitznagle, J. E. Christian, A. J. Ohlrogge, and C. E. Breckenridge, Jr., *J. Pharm. Sci.*, **57**, 764(1968).

(3) R. D. Ice, C. E. Breckenridge, Jr., and J. E. Christian, *ibid.*, **55**, 497(1966).

(4) R. D. Ice, J. E. Christian, and M. P. Plumlee, *ibid.*, **57**, 399 (1968).

(5) J. H. Ware and W. M. Barker, *Fed. Am. Soc. Exptl. Biol.*, annual meeting paper 2652, 1966.

(6) W. M. Barker, D. J. Thompson, and J. H. Ware, personal communication, International Minerals and Chemical Corp., Skokie, Ill., 1966.

(7) R. H. Jarboe, J. B. Data, and J. E. Christian, *J. Pharm. Sci.*, **57**, 323(1968).

(8) A. G. Ebert and J. H. Ware, personal communication, International Minerals and Chemical Corp., Skokie, Ill., 1965.

(9) G. A. Bruno, M.S. thesis, Purdue University, Lafayette, Ind., 1960.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *Department of Bionucleonics, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication September 10, 1969.

This research was conducted under the auspices of the Institute for Environmental Health Studies.

N-acyl Derivatives of Bis-(4-aminophenyl) Disulfide and its Thiolsulfinate

WILLIAM O. FOYE and JOSEPH P. SPERANZA

Abstract □ Bis-(4-aminophenyl)-sulfone and several *N,N'*-diacyl derivatives have shown appreciable activity as antimalarials. With the assumption that a molecule more readily cleaved to a *p*-aminophenyl sulfur or oxidized sulfur anion, as a potential anti-PAB substance, might be a more effective antimalarial, a series of *N,N'*-diacyl derivatives of bis-(4-aminophenyl) disulfide was synthesized. Oxidation with peroxide followed by acylation gave the corresponding thiolsulfinates. Antimalarial activity was found for bis-(4-*p*-acetamidobenzenesulfonamidophenyl) disulfide and *N,N'*-bis-(α-aminoacyl) derivatives of bis-(4-aminophenyl)-sulfone.

Keyphrases □ Bis-(4-aminophenyl) disulfide and thiosulfinate—*N*-acyl derivatives synthesis □ Antimalarial activity—bis-(4-aminophenyl) disulfide derivatives □ IR spectrophotometry—structure □ UV spectrophotometry—structure

Diaminodiphenylsulfone (DDS) (1) and its *N,N'*-diacetyl derivative (2) have both shown appreciable antimalarial activity, particularly against strains of *P. falciparum* resistant to chloroquine and other widely used antimalarials. Evidence has been found to suggest that these compounds are effective by interfering with the utilization of PABA by the parasites (3). If this is the case, then compounds more readily cleaved *in vivo* to a *p*-aminophenyl sulfur or oxidized sulfur moiety might interfere more effectively with PABA utilization. Accordingly, a series of *N,N'*-diacyl derivatives of bis-(4-aminophenyl) disulfide and *S*-oxidized derivatives has been prepared for antimalarial evaluation. Other disulfides, including 5,5'-diacetamido-8,8'-diquinolyl disulfide, have shown antimalarial activity (4, 5).

Bis-(4-aminophenyl) disulfide was obtained by the procedure of Price and Stacy (6), in which sodium 4-aminophenylmercaptide was oxidized by 30% hydrogen peroxide to the disulfide. The product showed the expected IR absorption bands for an aromatic amine, in addition to two sharp bands at 1175 and 1065 $cm.^{-1}$. Bredereck (7) has attributed the presence of these bands in aromatic disulfides to the disulfide linkage and a 1,4-disubstituted aromatic ring, respectively. However, aliphatic disulfides have shown similar peaks in the 1050–1250 $cm.^{-1}$ region which were believed due to CH wag on the carbon adjacent to sulfur (8). UV absorption showed a peak at 256 mμ, characteristic of disulfides (9), with a shoulder at 290–295 mμ.

Peroxide oxidation of aliphatic disulfides has led to formation of thiolsulfinates, thiosulfonates, and α-disulfones, depending on reaction conditions (10). Also, percamphoric acid oxidation of alkyl or aryl disulfides gave mixtures of disulfides, thiolsulfinates, and thiolsulfonates (11). With bis-(4-aminophenyl) disulfide, peroxide oxidation gave only the thiolsulfinate, even on long standing. Heating resulted in decomposition. IR absorption of the product showed a peak at 1050 $cm.^{-1}$, in addition to those present for the disulfide, which is characteristic of thiolsulfinates (8, 12). UV

absorption was found at 254 mμ, but lacked the shoulder at 290 mμ of the disulfide.

Oxidation of bis-(4-nitrophenyl) disulfide at room temperature with 30% peroxide returned only starting material, but in refluxing acetic acid, an oxidation product was obtained. A low yield of bis-(4-nitrophenyl) sulfone was isolated; this result agrees with previous observations on the oxidation of either *p*-nitrothiophenol (13, 14) or bis-(4-nitrophenyl) disulfide (15) where only the sulfone was isolated. This unexpected product has been attributed both to the presence of bis-(4-nitrophenyl) sulfide as an impurity (15) and to cleavage of the C—S bond (14).

Since the toxicity of bis-(4-aminophenyl) sulfone was diminished by *N*-acylation (16), various acylation products of bis-(4-aminophenyl) disulfide and its thiolsulfinate were prepared. These included the acetyl, methanesulfonyl, *p*-toluenesulfonyl, *p*-acetamidobenzenesulfonyl, glycyl, α-phthalimidoacyl, and *N*-carbodithioate. Previously, introduction of α-aminoacyl and α-phthalimidoacyl groups reduced the toxicity of bis-(4-aminophenyl) sulfone without affecting antitubercular activity appreciably (17). The dithiocarbamate of bis-(4-aminophenyl) disulfide was obtained in the presence of a large excess of triethylamine, and showed characteristic IR absorption at 990 and 1010 $cm.^{-1}$ for the dithiocarbamate group (18). Similar IR absorption was shown by the dithiocarbamate of the thiolsulfinate, but confirming analytical data were not obtained for this compound.

Antimalarial Test Results—Antimalarial screening in mice using *Plasmodium berghei* has been carried out by the Walter Reed Army Institute of Research and reported to us by Dr. D. P. Jacobus. Bis-(4-*p*-acetamidobenzenesulfonamidophenyl) disulfide effected a cure in one of five mice (60-day survival) (three dying of drug toxicity) at a dose of 640 mg./kg. No activity was found for bis-(4-aminophenyl) disulfide, bis-(4-*p*-tolylsulfonamidophenyl) disulfide or the bis-(*N*-phthalimidoacetyl), bis-[*N*-(2-phthalimido-3-phenyl)-propionyl] or bis-(*N*-carbodithioate) derivatives of bis-(4-aminophenyl) disulfide. Also, of the thiolsulfinates, the 4-acetamidophenyl, 4-methylsulfonamidophenyl, 4-phthalimidoacetamidophenyl, and 4-(2-phthalimido-3-phenyl)-propionamidophenyl derivatives were inactive.

The bis-glycyl and bis-phenylalanyl derivatives of DDS, previously prepared (17), were described as curative, however. Bis-(4-nitrophenyl) sulfone was also curative.

EXPERIMENTAL

Analyses for carbon, hydrogen, and nitrogen were done by Weiler and Strauss, Oxford, England. Sulfur analyses were done by Parr bomb peroxide fusion. Melting points were taken on a Mel-Temp block and are uncorrected. IR absorption spectra were obtained with a Perkin-Elmer model 137B spectrometer.

Bis-(4-phthalimidoacetamidophenyl) Disulfide—To a solution of 2.5 g. (0.01 mole) of bis-(4-aminophenyl) disulfide (6) in 50 ml. of dry pyridine at 0° was added with stirring 4.5 g. (0.02 mole) of phthalimidoacetyl chloride (19) during a period of 10 min. The mixture was stirred for 4 hr. at 0°, and the resulting solution was poured into 250 ml. of cold water containing 25 ml. of sulfuric acid. The solid product was collected, washed with water, and digested in a mixture of 2-ethoxyethanol and water (4:1), giving 2.8 g. (45%) of cream-colored solid, m.p. 302–305° (dec.).

Anal.—Calcd. for $C_{32}H_{22}N_4O_6S_2$: C, 61.74; H, 3.57; N, 8.99; S, 10.29. Found: C, 62.13; H, 3.44; N, 9.19; S, 10.83.

Bis-(4-aminoacetamidophenyl) Disulfide Dihydrochloride—To a suspension of 0.62 g. (0.001 mole) of bis-(4-phthalimidoacetamidophenyl) disulfide in a mixture of 20 ml. of dimethylformamide and 5 ml. of water was added with stirring 3.0 ml. (0.0026 mole) of alcoholic hydrazine hydrate (1 *M*). Stirring was continued for 1 hr. and concentrated hydrochloric acid was added dropwise to distinct acidity. Water (50 ml.) was added, the mixture was chilled, and phthalazine-1,4-dione was removed. The filtrate was distilled under reduced pressure to a volume of 10 ml., methylene chloride was added, and the precipitate was collected and recrystallized from dilute hydrochloric acid, giving 0.1 g. of yellow solid, m.p. 255–257° (dec.).

Anal.—Calcd. for $C_{16}H_{20}Cl_2N_4O_2S_2$: C, 44.14; H, 4.63; N, 12.87. Found: C, 43.51; H, 4.65; N, 13.35.

Bis-[4-(2-phthalimido-3-phenyl)-propionamidophenyl] Disulfide—To a solution of 2.5 g. (0.01 mole) of bis-(4-aminophenyl) disulfide in 50 ml. of dry pyridine at 0° was added with stirring 6.4 g. (0.02 mole) of 2-phthalimido-3-phenylpropionyl chloride (19) during 10 min. The reaction was carried out as above, and 4.5 g. (56%) of pale yellow product was obtained; m.p. 267–269° (dec.).

Anal.—Calcd. for $C_{46}H_{34}N_4O_6S_2$: C, 68.80; H, 4.27; N, 6.97; S, 7.98. Found: C, 68.89; H, 4.30; N, 7.05; S, 8.24.

Bis-(4-methanesulfonamidophenyl) Disulfide—To a solution of 0.73 g. (0.003 mole) of bis-(4-aminophenyl) disulfide in 15 ml. of dry pyridine was added with stirring 1.0 ml. (0.012 mole) of methanesulfonyl chloride. The mixture was stirred for 15 min. and poured into cold water, and the precipitate was collected and digested in hot 95% ethanol, giving 1.0 g. (83%) of pale pink solid, m.p. 212–215°.

Anal.—Calcd. for $C_{14}H_{16}N_2O_4S_4$: C, 41.57; H, 3.99; N, 6.93; S, 31.70. Found: C, 41.23; H, 3.78; N, 7.10; S, 31.90.

Bis-(4-*p*-tolylsulfonamidophenyl) Disulfide—To a solution of 1.25 g. (0.005 mole) of bis-(4-aminophenyl) disulfide in 20 ml. of dry pyridine at 0° was added with stirring 1.91 g. (0.01 mole) of *p*-toluenesulfonyl chloride during 10 min. The reaction was carried out in the same fashion as with the phthalimidoacyl chlorides. The red solid which separated from dilute sulfuric acid was purified by dissolving in 5% sodium hydroxide solution, filtering, neutralizing with 5% hydrochloric acid, and removing all material which precipitated above pH 7. The resulting cream-colored solid was collected, giving 2.2 g. (80%), m.p. 300° (dec.).

Anal.—Calcd. for $C_{26}H_{24}N_2O_4S_2$: C, 56.09; H, 4.34; N, 5.03. Found: C, 56.24; H, 4.61; N, 5.11.

Bis-(4-*p*-acetamidobenzenesulfonamidophenyl) Disulfide—To a solution of 2.5 g. (0.01 mole) of bis-(4-aminophenyl) disulfide in 40 ml. of dry pyridine at 0° was added with stirring 4.7 g. (0.02 mole) of *p*-acetamidobenzenesulfonyl chloride during 10 min. The reaction was carried out as in the previous case, and 2.5 g. (40%) of cream-colored solid was obtained, m.p. 140° (with previous softening).

Anal.—Calcd. for $C_{28}H_{26}N_4O_6S_4$: C, 52.33; H, 4.08; N, 8.72; S, 19.96. Found: C, 52.27; H, 3.99; N, 8.72; S, 20.18.

Bis-(triethylammonium) Bis-[4-amino-(*N*-carbodithioate)-phenyl] Disulfide—To a solution of 2.5 g. (0.01 mole) of bis-(4-aminophenyl) disulfide in 25 ml. of absolute ethanol was added a 15-mole excess of carbon disulfide and 10-mole excess of triethylamine at room temperature. After the mixture was stirred and refrigerated overnight, the supernatant liquid was discarded and the residual oil was mixed repeatedly with ether–acetone (3:1). The oil was spread thinly on an evaporating dish, placed under vacuum, and the resulting yellow solid was triturated with ether–acetone (3:1) and filtered, giving 3.2 g. (52%) of product, m.p. 94–96°.

Anal.—Calcd. for $C_{26}H_{42}N_4S_6$: C, 51.80; H, 7.02; N, 9.29. Found: C, 52.06; H, 6.78; N, 8.98.

4-Aminophenyl 4-Aminobenzenethiolsulfinate—To a solution of 5.0 g. (0.02 mole) of bis-(4-aminophenyl) disulfide in 50 ml. of 95% ethanol was added an excess of 30% hydrogen peroxide. The flask was stoppered and allowed to stand at room temperature for 1 week. The precipitate was collected and purified by digesting in hot 95% ethanol, giving 3.6 g. (69%) of golden-brown product, m.p. 140–142°.

Anal.—Calcd. for $C_{12}H_{12}N_2OS_2$: C, 54.50; H, 4.58; N, 10.60. Found: C, 55.04; H, 4.82; N, 10.78.

The dihydrochloride was obtained by addition of absolute ethanol to its aqueous solution; m.p. 235° (dec.).

Anal.—Calcd. for $C_{12}H_{14}Cl_2N_2OS_2$: C, 42.73; H, 4.18; N, 8.31. Found: C, 42.90; H, 3.83; N, 8.12.

4-Phthalimidoacetamidophenyl 4-Phthalimidoacetamidobenzenethiolsulfinate—To a mixture of 20 ml. of dimethylformamide and 20 ml. of pyridine at 0° were added 1.32 g. (0.005 mole) of 4-aminophenyl 4-aminobenzenethiolsulfinate and 2.24 g. (0.01 mole) of phthalimidoacetyl chloride (19) during 10 min. The reaction was carried out in the same manner as for the corresponding disulfide, and 1.0 g. (32%) of product was obtained, m.p. 258–263° (dec.).

Anal.—Calcd. for $C_{32}H_{22}N_4O_7S_2$: C, 60.19; H, 3.47; N, 8.78. Found: C, 59.95; H, 3.75; N, 8.51.

4-(2-Phthalimido-3-phenyl)-propionamidophenyl 4-(2-Phthalimido-3-phenyl)-propionamidobenzenethiolsulfinate—To a mixture of 20 ml. of dimethylformamide and 20 ml. of pyridine at 0° were added 1.32 g. (0.005 mole) of 4-aminophenyl 4-aminobenzenethiolsulfinate and 3.15 g. (0.01 mole) of 2-phthalimido-3-phenylpropionyl chloride (19) during 10 min. The reaction was carried out as in the preceding case, and 3.3 g. (78%) of pale yellow product was obtained; m.p. 268–271° (dec.).

Anal.—Calcd. for $C_{46}H_{34}N_4O_7S_2$: C, 67.45; H, 4.19; N, 6.84. Found: C, 67.52; H, 4.43; N, 6.93.

4-Acetamidophenyl 4-Acetamidobenzenethiolsulfinate—To a solution of 1.32 g. (0.005 mole) of 4-aminophenyl 4-aminobenzenethiolsulfinate in 20 ml. of dimethylformamide was added an excess of acetic anhydride. The mixture was stirred for 15 min. and poured into cold water; further stirring coagulated the product. It was collected and recrystallized from glacial acetic acid, giving 1.0 g. (60%) of yellow solid, m.p. 200° (dec.).

Anal.—Calcd. for $C_{16}H_{16}N_2O_3S_2$: C, 55.15; H, 4.63; N, 8.04. Found: C, 55.08; H, 4.59; N, 8.26.

4-Methanesulfonamidophenyl 4-Methanesulfonamidobenzenethiolsulfinate—To a solution of 0.4 g. (0.0015 mole) of 4-aminophenyl 4-aminobenzenethiolsulfinate in 30 ml. of dry pyridine was added with stirring 0.5 ml. (0.006 mole) of methanesulfonyl chloride. The mixture was stirred for 15 min. and poured into cold water; the yellow product (0.47 g., 75%) was collected; m.p. 205–208° (dec.).

Anal.—Calcd. for $C_{14}H_{16}N_2O_5S_4$: C, 39.98; H, 3.83; N, 6.66. Found: C, 40.65; H, 3.90; N, 7.20.

Bis-(4-nitrophenyl) Sulfone—To a refluxing solution of 15.4 g. (0.05 mole) of bis-(4-nitrophenyl) disulfide (Eastman Organic Chemicals) (recrystallized from glacial acetic acid) in 100 ml. of glacial acetic acid was added 6.0 ml. of 30% hydrogen peroxide. An additional 6.0 ml. of 30% hydrogen peroxide was added after 2 hr. The reaction mixture was allowed to cool after 12 hr. of refluxing, and the precipitate was isolated. The pale yellow crystals were washed several times with water and dried *in vacuo*, giving 1.5 g. (10%) of product, m.p. 251–255° (lit. 252–253°) (14, 15).

Anal.—Calcd. for $C_{12}H_8N_2O_6S$: C, 46.75; H, 2.62; N, 9.09; S, 10.40. Found: C, 47.04; H, 2.78; N, 9.17; S, 10.90.

REFERENCES

(1) L. T. Coggeshall, J. Maier, and C. A. Best, *J. Am. Med. Assoc.*, **117,** 1077(1941).

(2) E. F. Elslager and D. F. Worth, *Nature*, **206,** 630(1965).

(3) R. L. Degowin, R. B. Eppes, P. E. Carson, and R. D. Powell, *Bull. World Health Organ.*, **34,** 671(1966).

(4) J. E. Christian, G. L. Jenkins, L. C. Keagle, and J. A. Crum, *J. Am. Pharm. Assoc., Sci. Ed.*, **35,** 328(1946).

(5) I. Danielsson, J. E. Christian, and G. L. Jenkins, *ibid.*, **36,** 261(1947).

(6) C. C. Price and G. W. Stacy, *Org. Syn.*, **28,** 14(1948).

(7) H. Bredereck, A. Wagner, H. Beck, and R. J. Klein, *Chem. Ber.*, **93,** 2736(1960).

(8) W. O. Foye, A. M. Hebb, and J. Mickles, *J. Pharm. Sci.*, **56,** 292(1967).

(9) R. C. Passerini, in "Organic Sulfur Compounds," vol. 1, N. Kharasch, Ed., Pergamon Press, New York, N. Y., 1961, p. 66.

(10) P. Allen, Jr. and J. W. Brook, *J. Org. Chem.*, **27,** 1019 (1962).

(11) W. E. Savige and A. Fava, *Chem. Commun.*, **1965,** 417.

(12) L. J. Bellamy, in "Organic Sulfur Compounds," vol. 1, N. Kharasch, Ed., Pergamon Press, New York, N. Y., 1961, p. 48.

(13) E. Fromm and J. Wittmann, *Ber.*, **41,** 2264(1908).

(14) E. R. Cole, *Chem. Ind.* (London), **1957,** 1511.

(15) H. Burton and W. A. Davy, *J. Chem. Soc.*, **1946,** 542.

(16) G. A. H. Buttle, D. Stephenson, S. Smith, T. Dewing, and G. E. Foster, *Lancet*, **1,** 1331(1937).

(17) W. O. Foye, W. E. Lange, and E. G. Feldmann, *J. Am. Pharm. Assoc., Sci. Ed.*, **47,** 831(1958).

(18) L. J. Bellamy and P. E. Rogasch, *J. Chem. Soc.*, **1960,** 2218.

(19) W. O. Foye and W. E. Lange, *J. Am. Pharm. Assoc., Sci. Ed.*, **45,** 742(1956).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 14, 1969, from the *Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115*

Accepted for publication August 13, 1969.

Presented at the First Northeast Regional Meeting, ACS, Boston, Mass., October, 1968.

Abstracted from a thesis submitted by J. P. Speranza to the Massachusetts College of Pharmacy in partial fulfillment of M.S. degree requirements.

Potentiating Effect of Organomercurials on Bisphenols

FRED S. BARR, G. W. MOORE, and B. J. GRAGG

Abstract □ The activity of organomercurial–bisphenol combinations was checked by *in vitro* methods against *C. albicans*, *S. aureus*, *E. coli*, *B. subtilis*, and *S. faecalis*. The *in vivo* activity of these combinations was determined against *M. canis* and *T. mentagrophytes* in guinea pig infections. The methods for both *in vitro* and *in vivo* testings are given. A potentiating effect was observed both *in vitro* and *in vivo* when phenylmercuric acetate is added to hexachlorophene or dichlorophene.

Keyphrases □ Hexachlorophene, dichlorophene activity, potentiation—organomercurials □ Antimicrobial activity—bisphenol–organomercurial combinations □ Optical density—microbial culture concentration

Recent Food and Drug Administration regulations, which prohibit the use of most antibiotics in topical ointments, and the failure of most systemic medication in the treatment of topical fungal infections have brought about a renewed interest in some of the older antimicrobial agents. These agents, while active against specific organisms, did not inhibit a broad spectrum of infective microorganisms.

Earlier experiments in this laboratory indicated that by the combination of two of these agents the desired results could be obtained *in vitro* (1). In an earlier report the activity of phenylmercuric acetate and a bisphenol was reported. While Barker (2) reported that some cationic and some anionic agents such as surfactants tend to inactivate phenylmercuric compounds, later findings (3) show that the mercury is not inactivated by all such compounds and, in particular, the nonionic surfactants actually may potentiate the activity of the mercurials. It has been established that the surfactants potentiate the activity of the bisphenols.

This study was a continuation of earlier work at the laboratory (4) which reported on the activity of the potentiating effect of organomercurials upon the bisphenols *in vitro* and *in vivo*. In this study the active ingredients were combined in a cream base and checked for activity *in vitro* and *in vivo* in animals that were infected with *Microsporum canis* and *Trichophyton mentagrophytes*.

METHODS AND MATERIALS

In Vitro—The test organisms used in this experiment were *Staphylococcus aureus* (ATCC 9144), *Candida albicans* (ATCC 10231) *Escherichia coli* (ATCC 8330), *Bacillus subtilis* (ATCC 6633), and *Streptococcus faecalis* (ATCC 10541). The organisms were incubated in Trypticase soy broth[1] for 18 hr. at 37°. The cultures were then centrifuged and washed twice with sterile 0.85% saline and finally resuspended in sterile saline. The cultures then were diluted with sterile saline to give 75% light transmission at the 550 mμ wavelength on the Spectronic 20 colorimeter.[2] In all cases exactly 0.1 ml. of this standardized culture was used for the inoculum, and fresh suspensions were prepared daily.

The compounds tested were phenylmercuric acetate, nitromersol, dichlorophene, hexachlorophene, and combinations of the mercury salts with the bisphenols. The solutions were dissolved in ethanol and sterilized by filtration. Following the sterilization they were diluted in sterile distilled water. The agents were then serially diluted through sterile Trypticase soy broth, in 18- × 150-mm. metal cap tubes, and inoculated with 0.1 ml. of the above suspension of organisms. The tubes were incubated at 35–37° for 24 hr. and observed for growth. Control tubes using only the solvent were also tested and found to have no inhibitory properties at the levels used in these tests.

In Vivo—The test organisms in this study were *Microsporum canis* and *Trichophyton mentagrophytes*. The cultures were prepared by inoculating Sabouraud dextrose agar (Difco) with the test organism and incubating at 28° for 7 days. The mycelial mat was removed and placed in 10 ml. of sterile physiological saline in a ground-glass homogenizer and macerated until there was an even suspension of the culture. The inoculum used for each guinea pig was 0.5 ml. of this suspension.

Seventy male guinea pigs weighing 225–275 g. were infected with *Microsporum canis* and 70 were infected with *Trichophyton mentagrophytes*. The methd used to infect the animals was to remove the hair from the right side of each animal by use of electric clippers and a safety razor. The culture was then placed on the cleared area and ground into the tissue by use of No. 1 sandpaper. The animals were unmedicated for 5 days to allow the infection to become established. Following this period, cultures were made by skin scrapings onto Mycobiotic agar (Difco) and incubated at 28° for 7 days. After cultures were made, each of the 10 animals in each group were treated twice daily, with a vanishing cream[3] containing the test substance, for a period of 7 days with one of the five test substances. Ten animals were unmedicated and served as controls. The vanishing cream base was selected after it was determined that there was no antagonism between the base and the active ingredients.

Following the last day of treatment, any remaining drug was removed by washing the treated area with warm tap water. Cultures of skin scrapings were made on Mycobiotic agar immediately following the washing and again at 7 and 14 days posttreatment. At the time each culture was made, scales and newly grown hairs from the infected site of each animal were examined microscopically.

RESULTS

The *in vitro* results are recorded in Table I. It is evident from the data in this table that phenylmercuric acetate exerts an effect in combination with the bisphenols which one would not expect from an additive effect alone. Against *Candida albicans* and *Escherichia coli*, the phenylmercuric acetate–dichlorophene combination was the most active, while the most active combination against the Gram-positive organisms was phenylmercuric acetate–hexachlorophene. The combination of nitromersol with the bisphenols displays a potentiating effect; it is less than that observed with the other combinations.

The *in vivo* results are recorded in Table II. The data indicates that the combinations of phenylmercuric acetate–bisphenol is more active against *Microsporum canis* than it is against *Trichophyton mentagrophytes*. The combinations are more effective than either agent alone.

DISCUSSION

The data obtained in this study indicate that a product containing phenylmercuric acetate and a bisphenol such as dichlorophene would be of use in the treatment of superficial fungus infections such

[1] Baltimore Biological Laboratories.
[2] Bausch & Lomb, Inc., Rochester, N. Y.
[3] Contains boric acid, glycerin, cetyl alcohol, stearyl alcohol, Span 20, Tween 20, sodium lauryl sulfate, and water.

Table I—Minimum Inhibitory Concentrations, in p.p.m.

Test Organism	Hexachlorophene	Dichlorophene	Phenylmercuric Acetate	Nitromersol	Hexachlorophene + 0.1 Part Phenylmercuric Acetate[a]	Dichlorophene + 0.1 Part Phenylmercuric Acetate[b]	Hexachlorophene + 0.1 Part Nitromersol[a]
C. albicans	31.2	50.0	0.8	25.0	2.0	1.6	12.5
S. aureus	10.0	10.0	1.7	6.2	1.0	1.6	1.6
E. coli	250.0	200.0	8.2	20.0	20.0	12.6	20.0
B. subtilis	10.0	15.0	0.8	8.0	1.0	1.6	1.8
S. faecalis	15.0	20.0	1.7	4.0	1.2	1.7	2.2

[a] Expressed as p.p.m. hexachlorophene. [b] Expressed as p.p.m. dichlorophene. The results of the tests reported in this table are for 10 tests on each organism per compound or combination.

Table II—Efficacy of Products Tested; Number Animals Infected/Number Treated

Compound Tested	*Microsporum canis* Day 1 Post-Treatment	*Microsporum canis* Day 7 Post-Treatment	*Microsporum canis* Day 14 Post-Treatment	Efficacy, %	*Trichophyton mentagrophytes* Day 1 Post-Treatment	*Trichophyton mentagrophytes* Day 7 Post-Treatment	*Trichophyton mentagrophytes* Day 14 Post-Treatment	Efficacy, %
Phenylmercuric acetate (0.05%)	7/10	7/10	7/10	30	7/10	8/10	8/10	20
Hexachlorophene (0.5%)	9/10	9/10	8/10	20	10/10	10/10	9/10	10
Dichlorophene (0.5%)	8/10	8/10	8/10	20	8/10	8/10	8/10	20
Dichlorophene (0.5%) Phenylmercuric acetate (0.05%)	3/10	2/10	2/10	80	4/10	4/10	4/10	60
Hexachlorophene (0.5%) Phenylmercuric acetate (0.05%)	2/10	2/10	2/10	80	7/10	6/10	6/10	40
Vanishing cream base	10/10	10/10	9/10	10	10/10	10/10	10/10	0
Control	10/10	10/10	10/10	0	10/10	10/10	10/10	0

as ringworm and athlete's foot. This combination displayed good activity when tested *in vitro* and the potentiated activity remained when the product, in ointment form, was checked *in vivo*.

While this study indicates that excellent results were obtained with twice-daily treatment for a period of 7 days, it must be remembered that these were fresh infections and treatment of longstanding infections of *Microsporum canis* might require treatment of 14 days or even longer in some cases. The results obtained also indicate that a treatment period longer than 7 days would have increased the percentage efficacy of the tested products; however, it has been found that at 21–28 days some of the control animals do not give positive cultures from one test period to another and for this reason the test must be shortened.

The *in vitro* data obtained along with the fact that these combinations are not inactivated *in vivo* would indicate that a product for the treatment of *Candida albicans* both topically and intravaginally can be developed using the tested agents.

REFERENCES

(1) F. S. Barr, G. F. Collins, and L. G. Wyatt, *J. Pharm. Sci.*, **54**, 801(1965).

(2) H. E. R. Barker, *Australian J. Pharm.*, **29**, 801(1948).

(3) F. S. Barr, G. W. Moore, and G. F. Collins, *Vet. Med.*, **63**, 694(1968).

(4) F. S. Barr, *J. Am. Vet. Med. Assoc.*, **149**, 1295(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 5, 1969, from *the Research Division, the S. E. Massengill Company, Bristol, TN 37620*

Accepted for publication September 8, 1969.

Drug–Plastic Interactions II: Sorption of *p*-Hydroxybenzoic Acid Esters by Capran Polyamide and *In Vitro* Biologic Activity

NAGIN K. PATEL* and NAGAMANI NAGABHUSHAN

Abstract □ Sorption of methyl- and propylparaben by capran polyamide was determined by means of an equilibrium sorption method. Data are presented to show that the antimicrobial activity of the parabens for *Aspergillus niger*, *Aerobacter aerogenes*, and *Pseudomonas aeruginosa* is diminished in the presence of capran polyamide. The *in vitro* biologic activity of the parabens in the presence of the nylon was shown to be related to the concentration of "free" or the drug in equilibrium with the plastic.

Keyphrases □ *p*-Hydroxybenzoic acid ester—capran polyamide film, sorption □ Capran polyamide sorption effect—paraben antimicrobial activity □ Antimicrobial activity, parabens—capran polyamide sorption effect

It is generally recognized that the biologic activity of the sorbed drug would be reduced in proportion to the amount sorbed (1–3) and *a priori* observation by Autian *et al.* (4, 5) that preservatives such as *p*-hydroxybenzoic acid esters (parabens) are sorbed by nylon (6) from aqueous solution and hence proportionately inactivated, should be justifiable. In contrast, Myers and Lefebvre reported that nylon in concentrations up to 5% had no adverse effect on the antibacterial activity of benzalkonium chloride (7). Adsorption of drugs by insoluble fillers has been recognized, and Deutsch *et al.* noted that the biologic activity of a vitamin was reduced significantly due to its adsorption on the fillers used in capsules and tablets (8). No direct report could be found on a correlation of drug-plastic sorption data with *in vitro* antimicrobial activity of *p*-hydroxybenzoates.

This report will show that the antibacterial and antifungal activity of methyl- and propylparaben is reduced due to their sorption by capran polyamide.[1] A correlation between the sorption data and *in vitro* biologic activity will also be presented.

EXPERIMENTAL

Materials—Methyl- and propylparaben and the synthetic culture medium were the same as employed in a previous communication (3). Capran polyamide was washed with 50% aqueous ethanol and then rinsed several times with distilled water in order to remove surface contaminants. The nylon samples were dried at 65 ± 1° for 8 hr. and stored in a desiccator until used.

Equilibrium Sorption Studies—The experimental technique has been described (9) in Part I. An accurately weighed sample of capran polyamide [about 1.2 g.; one 10.2 × 15.2-cm. (4 × 6-in.) sheet] was placed in 150-ml. bottles,[2] each containing 90 ml. of varying concentrations of the paraben under study. The bottles were closed tightly using plastic (Bakelite) screw caps lined with thin polyethylene[3] film. Preliminary studies showed that under experimental conditions there was no sorption of the parabens by the polyethylene. The bottles were agitated in a general-purpose shaking bath.[4] The drug content at equilibrium was determined spectrophotometrically (5). The decrease in concentration of the drug in solution was a measure of sorption, and the equilibrium was established at the end of 24 hr. agitation.

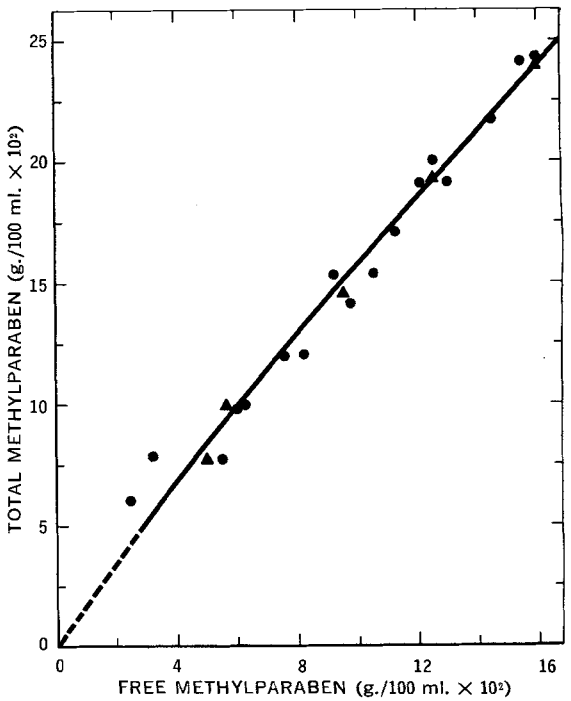


Figure 1—*Sorption of methylparaben by capran polyamide at 30°, showing the total methylparaben concentration in the system as a function of equilibrium or free methylparaben concentration. Key: ●, in culture medium; ▲, in distilled water.*

Microbiological Studies—The general procedure consisted of equilibrating varying concentrations of a paraben in culture medium with a known weight of capran polyamide and determining the inhibitory concentration for one of the organisms. A simple, chemically defined synthetic culture medium as described earlier (3) was used with the exception that 2% dextrose was employed in the place of 5% dextrose. Preliminary experimentation showed that the magnitude of growth of *A. niger* (10), *A. aerogenes* (10), and *Ps. aeruginosa*[5] in the culture medium containing 5% dextrose was about the same as in that containing 2% dextrose. This work also indicated that dextrose, lactose, or sucrose at 2% concentration supported the growth of the above organisms equally well. It was noticed that the lower concentration of sugar was less apt to caramelize at autoclaving temperature. Sterile distilled water was used to prepare all the solutions for microbiological work.

The nylon samples of a definite weight (0.600 g. methylparaben or 0.100 g. propylparaben) were placed in the bottles, each containing 45 ml. of varying known concentrations of the drug. A series of

[1] Nylon 6 film with labeled thickness of 0.0013 cm. (0.0005 in.), lot GHO1077-1-2, supplied through the courtesy of Allied Chemical Corp., Morristown, N. J.

[2] Milk dilution bottles, Fisher Scientific Co., Inc.

[3] Canadian Industries Ltd.

[4] Lab-line Instruments Inc.

[5] Mac-264. The stock culture was grown on slants of nutrient agar and it was supplied by Dr. D. W. Westlake of the Department of Microbiology.

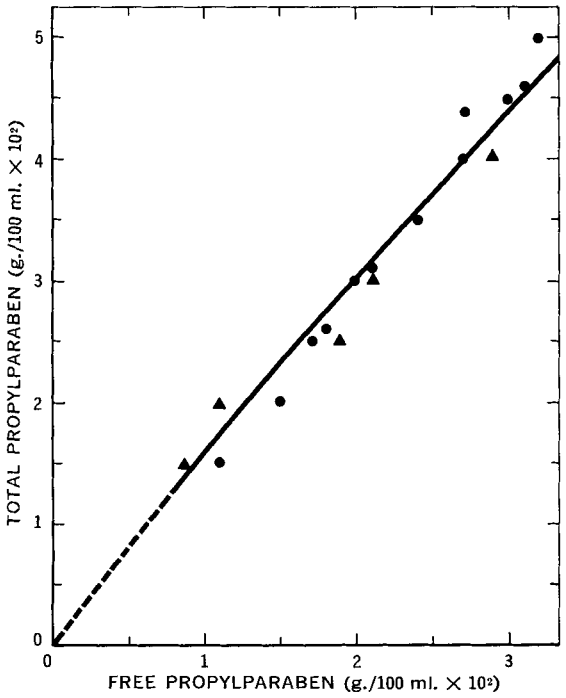


Figure 2—*Sorption of propylparaben by capran polyamide at 30°, showing the total propylparaben concentration in the system as a function of equilibrium or free methylparaben concentration. Key: ●, in culture medium; ▲, in distilled water.*

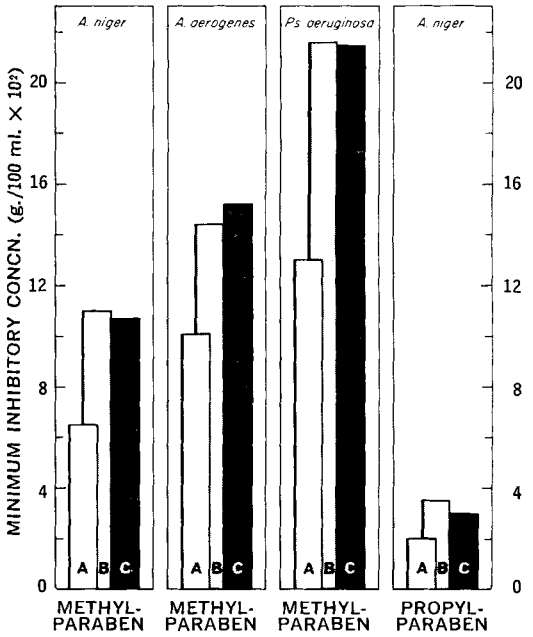


Figure 3—*Comparison of predicted and experimental minimum inhibitory concentrations (M.I.C.'s) of parabens in the presence of capran polyamide. The concentrations of the plastic were 1.33 and 0.222% for methyl- and propylparaben, respectively. Key: A, M.I.C. in absence of capran polyamide; B, experimental M.I.C. in presence of capran polyamide; and C, predicted (from Figs. 1 and 2) M.I.C. in presence of the plastic.*

concentrations of the parabens was prepared in the media, differing by 0.05%. In this manner a paraben solution was prepared containing total paraben as predicted by the procedure described under *Results and Discussion*. Four additional concentrations of the agent were prepared, two of which were lower than the computed value and the other two higher. These solutions were prepared in duplicate. The bottles were closed loosely with plastic caps and sterilized by autoclaving at 15 lb. pressure for 15 min.

The bottles were allowed to attain room temperature in an aseptic area. They were closed tightly and equilibrated by agitation in a water bath at 30° for about 24 hr.

Methyl- and propylparaben were apparently stable under experimental conditions (3). The sorption capacities of the autoclaved and unautoclaved samples of the capran polyamide were the same.

Each of the above bottles was then inoculated with two loopsful of either a spore suspension of the fungus or 48-hr. cultured suspension (3) of the bacteria. In order to maintain a constant concentration of bacteria, the optical density of the bacterial suspension was kept to a value of 0.50 by diluting the contents with sterile medium if necessary. The bottles were incubated at 30° and observed visually for growth in the form of mycelial hyphae in the case of *A. niger* and turbidity in the cases of *A. aerogenes* and *Ps. aeruginosa*.

RESULTS AND DISCUSSION

Sorption of Parabens by Capran Polyamide—The sorption of parabens by nylons has been reported in the literature (4–6). Sorption of methyl- and propylparaben is shown in Figs. 1 and 2, and it is included here for correlation with antimicrobial activity. Similar sorption data have been reported, based on the undergraduate physical pharmacy experimentation (10). Additional results are plotted in Figs. 1–3, showing that there is a fairly good correlation between sorption and microbiological data. The data of Figs. 1 and 2 also illustrate that the sorption of methyl- and propylparaben by capran polyamide in the media of distilled water and synthetic culture is essentially the same. The data are plotted to show that total (sorbed + free) paraben concentration was a function of equilibrium or free paraben concentration. This type of plot is useful for predicting the biologic activity of the drug in the presence of the nylon as explained in the following section.

Method of Prediction of Microbiological Action—If the biologic activity of a paraben can be assumed to be due to equilibrium or free drug in the presence of capran polyamide, and if the minimum inhibitory concentration (M.I.C.) of the paraben in the absence of the plastic is known, then the plots as depicted in Figs. 1 and 2 can be used to predict the total concentration of the drug required for the desired antimicrobial activity. (This assumption supports the theory that the paraben sorbed by capran polyamide is biologically unavailable.) For any desired free concentration, one can obtain the total concentration of the paraben from the ordinates of Figs. 1 and 2. The terms free concentration and M.I.C. in the absence of capran polyamide are assumed to be synonymous for the purpose of prediction.

Inactivation by Capran Polyamide Related to Sorption—The M.I.C.'s of the parabens in the culture medium in the absence of capran polyamide are as follows: methylparaben, 0.065% for *A. niger*, 0.10% for *A. aerogenes*, and 0.13% for *Ps. aeruginosa;* propylparaben, 0.020% for *A. niger*, and 0.034% for *A. aerogenes*. Propylparaben was ineffective in preventing the growth of *Ps. aeruginosa* in a concentration of 0.048%. Based on these M.I.C. values, the inhibitory paraben concentrations in the presence of definite weights of capran polyamide were predicted from Figs. 1 and 2. These predicted values together with experimental inhibitory concentrations in the presence of capran polyamide are portrayed in Fig. 3. It is to be noted that the predicted value (0.052%) of propylparaben for *A. aerogenes* exceeds solubility; therefore, it was not possible to conduct a growth study using this organism in the presence of capran polyamide.

As illustrated in Figs. 1 and 2 the components of the culture medium do not compete for sorption. Thus, any reduction in the biologic activity of the paraben in the presence of the capran polyamide can be attributed to the sorption. Figure 3 demonstrates that there is a good correlation between predicted M.I.C. and experimental antifungal and antibacterial concentrations for the parabens. It is therefore concluded that the plastic-sorbed paraben is devoid of *in vitro* biologic activity.

REFERENCES

(1) F. D. Pisano and H. B. Kostenbauder, *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 310(1959).

(2) S. M. Blaug and S. S. Ahsan, *J. Pharm. Sci.*, **50**, 138(1961).
(3) N. K. Patel, *Can. J. Pharm. Sci.*, **2**, 77(1967).
(4) A. J. Kapadia, W. L. Guess, and J. Autian, *J. Pharm. Sci.*, **53**, 28(1964).
(5) M. B. Rodell, W. L. Guess, and J. Autian, *ibid.*, **55**, 1429 (1966).
(6) N. K. Patel and H. B. Kostenbauder, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 289(1958).
(7) G. E. Myers and C. Lefebvre, *Can. Pharm. J.*, **94** (7), 55(1961).
(8) M. J. Deutsch, S. S. Schiaffino, and H. W. Loy, *J. Assoc. Offic. Agr. Chemists*, **43**, 55(1960).
(9) N. Nagabhushan, R. T. Coutts, and N. K. Patel, *Can. J. Pharm. Sci.*, in press.
(10) N. K. Patel and N. Nagabhushan, *Am. J. Pharm. Educ.*, **33**, 392(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 2, 1969, from the *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada.*
Accepted for publication October 10, 1969.
This study was supported by an operating grant MA-3023 from the Medical Research Council of Canada.
* Present Address: Frank W. Horner Limited, Research Laboratories, Montreal 307, P.Q., Canada.

Polyamide–Silica Gel Layer Chromatography of Yellow Food Dyes

HUNG-CHEH CHIANG and CHWAN-HWAI CHEN

Abstract □ The separation of five yellow food dyes and three toxic yellow dyes by mixed polyamide–silica gel thin-layer is described. The method shows good separation and sharp spots. For comparison, the thin-layer chromatography (TLC) on only polyamide and on only kieselguhr is also carried out.

Keyphrases □ Yellow dyes—analysis □ Dyes, yellow—separation, identification □ Polyamide–silica gel chromatography—analysis

The separation of synthetic food dyes on a thin-layer of cellulose (1), silica gel (2), aluminum oxide (3), starch (4), polyamide (5), and paper chromatography (6) has been reported, but none of these techniques gave entirely satisfactory results. Recently, the separation of 11 red food dyes on polyamide (12%)–silica gel G (88%) mixed thin-layers has been successfully applied by Chiang (7). Therefore, further application of this method was tried. In this note, the separation of five yellow food dyes and three toxic yellow dyes (auramine, metanil yellow, and picric acid) by mixed polyamide–silica gel TLC is described. For comparison, the TLC of only polyamide and of only silica gel is also reported.

EXPERIMENTAL

Material—The solvents and chemicals are the reagent grade of Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Preparation of Polyamide–Silica Gel Mixed Layer—Eight grams of polyamide chip (Nylon 6,[1] type 1022B of UBE Industrial Ltd., Osaka, Japan) was dissolved in 80 ml. of 90% formic acid, and then 20 ml. of distilled water was added. After gentle warming (below 40°) and stirring, a homogeneous solution was obtained; this was cooled to the room temperature and 52 g. of silica gel G (E. Merck) was added. Two hundred milliliters of the above-mentioned solution was poured into a dish (14.5 × 19.5 × 2.5 cm.) and a glass plate (12 × 14 × 0.1 cm.) was dipped into it. Both sides of the glass were covered homogeneously. The glass was placed over the dish for 2 min. to let the excess solution drain back. It was then air dried for 3 hr. and heated at 100° for 30 min.

[1] U. S. manufacturer: American Enka Corp.

Table I—Chromatographic Data

No.	Dyes	Solvent I[a] P–S[c]	Solvent I[a] S[d]	Solvent I[a] P[e]	Solvent II[b] P–S	Solvent II[b] S	Solvent II[b] P
1	Naphthol yellow S	0.48	(0.95,	0.37[f])	0.10	(0.74,[f]	0.02)
2	Yellow AB	0.14	(0.62,	0.22)	0.74	(0.97,	0.48)
3	Yellow OB	0.10	(0.52,	0.22)	0.72	(0.97,	0.45)
4	Tartrazine	0.63	(0.98,	0.85)	0.01	(0.37,[f]	0.01)
5	Sunset yellow FCF	0.56	(0.98,	0.66)	0.14	(0.56,[f]	0.08)
6	Metanil yellow	0.28	(0.80,	0.14)	0.47	(0.83,	0.23)
7	Auramine	0.37	(0.71,	0.36)	0.62	(0.71,	0.89)
8	Picric acid	0.40	(0.97,	0.22)	0.53	(0.84,	0.22)
Time required,[g] (min.)		50	30	200	90	70	600

[a] Solvent I: methanol–23% ammonium chloride solution–chloroform (30:20:1.3). [b] Solvent II: isobutanol–ethanol–0.45% sodium chloride solution (3:5:1). [c] P-S, R_f value on polyamide-silica gel mixed layer. [d] S, on silica gel layer. [e] P, on polyamide layer. [f] Tailing. [g] Time required to ascend 10 cm. from origin.

Preparation of Silica Gel Layer—Dilute slurries of silica gel G (45 g. in 100 ml. of water) were sprayed at 2 kg./cm.^{3} pressure from a distance of 20 cm. onto eight sheets of glass plates (12 × 14 cm.) in a horizontal position, then dried at 100° for 30 min. The thickness of the layers was about 250 μ.

Chromatographic Procedure—One microliter of 0.3% alcoholic solution of yellow AB, yellow OB, and auramine, and 0.3% water solution of other dyes was applied to the start line 1.5 cm. from the bottom of the layer, and the plate was developed by ascending techniques. The chamber had been equilibrated with the respective solvent for 30 min. before use.

RESULTS AND DISCUSSION

R_f values obtained with two solvent systems are given in Table I. It has been found that the results obtained by the mixed polyamide–silica gel layers show better separation and sharper spots than that obtained by polyamide and silica gel layers. Also the time required to ascend 10 cm. from origin for the mixed layers is shorter than that for the polyamide layers. Separation mechanism on the mixed layers is based on the formation of hydrogen bonds between

(2) S. M. Blaug and S. S. Ahsan, *J. Pharm. Sci.*, **50**, 138(1961).
(3) N. K. Patel, *Can. J. Pharm. Sci.*, **2**, 77(1967).
(4) A. J. Kapadia, W. L. Guess, and J. Autian, *J. Pharm. Sci.*, **53**, 28(1964).
(5) M. B. Rodell, W. L. Guess, and J. Autian, *ibid.*, **55**, 1429 (1966).
(6) N. K. Patel and H. B. Kostenbauder, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 289(1958).
(7) G. E. Myers and C. Lefebvre, *Can. Pharm. J.*, **94** (7), 55(1961).
(8) M. J. Deutsch, S. S. Schiaffino, and H. W. Loy, *J. Assoc. Offic. Agr. Chemists*, **43**, 55(1960).
(9) N. Nagabhushan, R. T. Coutts, and N. K. Patel, *Can. J. Pharm. Sci.*, in press.
(10) N. K. Patel and N. Nagabhushan, *Am. J. Pharm. Educ.*, **33**, 392(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 2, 1969, from the *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada.*
Accepted for publication October 10, 1969.
This study was supported by an operating grant MA-3023 from the Medical Research Council of Canada.
* Present Address: Frank W. Horner Limited, Research Laboratories, Montreal 307, P.Q., Canada.

Polyamide–Silica Gel Layer Chromatography of Yellow Food Dyes

HUNG-CHEH CHIANG and CHWAN-HWAI CHEN

Abstract □ The separation of five yellow food dyes and three toxic yellow dyes by mixed polyamide–silica gel thin-layer is described. The method shows good separation and sharp spots. For comparison, the thin-layer chromatography (TLC) on only polyamide and on only kieselguhr is also carried out.

Keyphrases □ Yellow dyes—analysis □ Dyes, yellow—separation, identification □ Polyamide–silica gel chromatography—analysis

The separation of synthetic food dyes on a thin-layer of cellulose (1), silica gel (2), aluminum oxide (3), starch (4), polyamide (5), and paper chromatography (6) has been reported, but none of these techniques gave entirely satisfactory results. Recently, the separation of 11 red food dyes on polyamide (12%)–silica gel G (88%) mixed thin-layers has been successfully applied by Chiang (7). Therefore, further application of this method was tried. In this note, the separation of five yellow food dyes and three toxic yellow dyes (auramine, metanil yellow, and picric acid) by mixed polyamide–silica gel TLC is described. For comparison, the TLC of only polyamide and of only silica gel is also reported.

EXPERIMENTAL

Material—The solvents and chemicals are the reagent grade of Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Preparation of Polyamide–Silica Gel Mixed Layer—Eight grams of polyamide chip (Nylon 6,[1] type 1022B of UBE Industrial Ltd., Osaka, Japan) was dissolved in 80 ml. of 90% formic acid, and then 20 ml. of distilled water was added. After gentle warming (below 40°) and stirring, a homogeneous solution was obtained; this was cooled to the room temperature and 52 g. of silica gel G (E. Merck) was added. Two hundred milliliters of the above-mentioned solution was poured into a dish (14.5 × 19.5 × 2.5 cm.) and a glass plate (12 × 14 × 0.1 cm.) was dipped into it. Both sides of the glass were covered homogeneously. The glass was placed over the dish for 2 min. to let the excess solution drain back. It was then air dried for 3 hr. and heated at 100° for 30 min.

[1] U. S. manufacturer: American Enka Corp.

Table I—Chromatographic Data

No.	Dyes	Solvent I[a] P–S[c]	Solvent I[a] S[d]	Solvent I[a] P[e]	Solvent II[b] P–S	Solvent II[b] S	Solvent II[b] P
1	Naphthol yellow S	0.48	(0.95,	0.37[f])	0.10	(0.74,[f]	0.02)
2	Yellow AB	0.14	(0.62,	0.22)	0.74	(0.97,	0.48)
3	Yellow OB	0.10	(0.52,	0.22)	0.72	(0.97,	0.45)
4	Tartrazine	0.63	(0.98,	0.85)	0.01	(0.37,[f]	0.01)
5	Sunset yellow FCF	0.56	(0.98,	0.66)	0.14	(0.56,[f]	0.08)
6	Metanil yellow	0.28	(0.80,	0.14)	0.47	(0.83,	0.23)
7	Auramine	0.37	(0.71,	0.36)	0.62	(0.71,	0.89)
8	Picric acid	0.40	(0.97,	0.22)	0.53	(0.84,	0.22)
Time required,[g] (min.)		50	30	200	90	70	600

[a] Solvent I: methanol–23% ammonium chloride solution–chloroform (30:20:1.3). [b] Solvent II: isobutanol–ethanol–0.45% sodium chloride solution (3:5:1). [c] P-S, R_f value on polyamide-silica gel mixed layer. [d] S, on silica gel layer. [e] P, on polyamide layer. [f] Tailing. [g] Time required to ascend 10 cm. from origin.

Preparation of Silica Gel Layer—Dilute slurries of silica gel G (45 g. in 100 ml. of water) were sprayed at 2 kg./cm.^{3} pressure from a distance of 20 cm. onto eight sheets of glass plates (12 × 14 cm.) in a horizontal position, then dried at 100° for 30 min. The thickness of the layers was about 250 μ.

Chromatographic Procedure—One microliter of 0.3% alcoholic solution of yellow AB, yellow OB, and auramine, and 0.3% water solution of other dyes was applied to the start line 1.5 cm. from the bottom of the layer, and the plate was developed by ascending techniques. The chamber had been equilibrated with the respective solvent for 30 min. before use.

RESULTS AND DISCUSSION

R_f values obtained with two solvent systems are given in Table I. It has been found that the results obtained by the mixed polyamide–silica gel layers show better separation and sharper spots than that obtained by polyamide and silica gel layers. Also the time required to ascend 10 cm. from origin for the mixed layers is shorter than that for the polyamide layers. Separation mechanism on the mixed layers is based on the formation of hydrogen bonds between

the CONH group of polyamide and the sample and adsorption or partition between the silica gel and the sample. In the mixed layer, polyamide also serves as a strong binder and makes the layer very durable and easy to handle. Also the layer did not crack or peel and could be stored easily. Both sides of the glass are independent of each other and chromatography can be performed simultaneously on both sides. The addition of a small amount of salt (about 0.05% sodium chloride or 0.4% ammonium chloride) in the solvent mixture is essential to break hydrogen bonding between the polyamide and the dyes. Oil-soluble dyes of yellow AB and yellow OB are rather difficult to separate because of the close similarity of their structures (different only in one methyl group). The content of polyamide (13.6%) in this mixed layer was above that of the previous report (12%) (7) in order to obtain a more durable layer.

REFERENCES

(1) P. Wollenweber, *J. Chromatog.*, **7**, 557(1962).
(2) A. Montag, *Z. Lebensm. Untersuch.-Forsch.*, **116**, 413(1962).
(3) M. Mottier and M. Potterat, *Anal. Chem. Acta*, **13**, 46(1955).
(4) J. Davídek and G. Janíček, *J. Chromatog.*, **15**, 542(1964).
(5) K. T. Wang, *Nature*, **213**, 212(1967).
(6) J. Šrámek, *J. Chromatog.*, **15**, 57(1964).
(7) H. C. Chiang, *ibid.*, **40**, 189(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 14, 1969, from the *Department of Pharmacy, Taipei Medical College, Taipei, Taiwan, China.*

Accepted for publication September 15, 1969.

Modified Granuloma Pouch Procedure for the Evaluation of Topically Applied Anti-inflammatory Steroids

G. DiPASQUALE, CHARLES L. RASSAERT, and EDWARD McDOUGALL

Abstract □ A laboratory procedure for evaluating topically applied anti-inflammatory preparations has been described. A modified croton oil-induced granuloma pouch served as the site of drug application and also as a physical barrier to avoid drug ingestion. Increasing concentrations and/or doses of test compounds are correlated with thymolysis, catabolism, and reduced exudate formation. The procedure can differentiate steroidal modifications, percutaneous absorption, and alterations of the vehicle. However, it is difficult to ascertain how much of the action of the test compound is due to its local and how much to its systemic properties. The assay also evaluated test compounds as pharmaceutical preparations. The order of increasing activity of the test substances can be listed as hydrocortisone acetate (1% cream) < methylprednisolone acetate (0.25% cream) < betamethasone 17-valerate (0.025% cream) < triamcinolone 16,17-acetonide (0.025% cream) < betamethasone (0.2% cream) < fluocinolone acetonide (0.025% alcoholic gel or cream).

Keyphrases □ Steroids, anti-inflammatory—topical activity determination □ Granuloma pouch test procedure—topical steroid activity □ Croton oil-air—granuloma pouch formation

Potential anti-inflammatory substances, topical or systemic, have been evaluated in a variety of procedures which apparently represent different modes of actions and/or different stages of the inflammatory process. Some of these procedures include: cotton pellet, granuloma pouch, paw edema, adjuvant-induced polyarthritis, cell cultures, uncoupling of mitochondrial oxidative phosphorylation, plasma protein changes, ear test, lung inflammation, and inhibition of erythema (1–4). It is evident that each test procedure has some merit; however, one cannot avoid nor explain discrepancies which occur between the various animal *in vitro* and clinical procedures. Nevertheless, most clinically efficacious drugs are active in most procedures, although in some assays a divergence may occur between steroidal and nonsteroidal drugs. Avoidance of ingestion of topically applied drugs in animals has presented some perplexing problems. Alternatives appear to introduce other problems; for example the use of occlusive dressings may alter absorption whereas the use of collars and other restraining devices produces stress which elaborates endogenous adrenocorticosteroids. The use of an air pouch which serves as the site of drug application and a physical barrier to avoid ingestion has been tried by the authors. They have also tried to observe whether or not the modified granuloma pouch procedure will distinguish steroidal modifications and/or alterations of the vehicle. It is known that these changes will affect drug absorption, retention, and biological activity (5, 6). It should also be mentioned that most animal (*in vivo* and/or *in vitro*) procedures require a solubilized test compound which requires organic solvents. The present study also evaluated drugs as pharmaceutical preparations.

MATERIALS AND METHODS

The method used was a modification of Selye's procedure (7). Male rats weighing 150–170 g. were individually housed and arranged in groups as indicated in the tables. The dorsal surface of the animal was shaved with animal clippers and the pouches were formed with the subcutaneous injection of 25 ml. of air followed by an injection of 0.5 ml. of a 1% solution of croton oil in sesame oil directly into the pouch. The test compounds included the commercial preparations: fluocinolone acetonide (Synalar cream, Syntex Laboratories Inc.); betamethasone (Celestone cream, Schering Corp.); methylprednisolone acetate (Medrol acetate cream, The Upjohn Co.); triamcinolone 16,17-acetonide (Kenalog cream, E. R. Squibb & Sons, Inc.); and also these laboratory preparations: hydrocortisone acetate in a cream base; betamethasone 17-valerate in a cream base; triamcinolone in a cream base; triamcinolone 16,17-acetonide in a cream base; and fluocinolone acetonide in an alcoholic gel base.

the CONH group of polyamide and the sample and adsorption or partition between the silica gel and the sample. In the mixed layer, polyamide also serves as a strong binder and makes the layer very durable and easy to handle. Also the layer did not crack or peel and could be stored easily. Both sides of the glass are independent of each other and chromatography can be performed simultaneously on both sides. The addition of a small amount of salt (about 0.05% sodium chloride or 0.4% ammonium chloride) in the solvent mixture is essential to break hydrogen bonding between the polyamide and the dyes. Oil-soluble dyes of yellow AB and yellow OB are rather difficult to separate because of the close similarity of their structures (different only in one methyl group). The content of polyamide (13.6%) in this mixed layer was above that of the previous report (12%) (7) in order to obtain a more durable layer.

REFERENCES

(1) P. Wollenweber, *J. Chromatog.*, **7**, 557(1962).
(2) A. Montag, *Z. Lebensm. Untersuch.-Forsch.*, **116**, 413(1962).
(3) M. Mottier and M. Potterat, *Anal. Chem. Acta*, **13**, 46(1955).
(4) J. Davídek and G. Janíček, *J. Chromatog.*, **15**, 542(1964).
(5) K. T. Wang, *Nature*, **213**, 212(1967).
(6) J. Šrámek, *J. Chromatog.*, **15**, 57(1964).
(7) H. C. Chiang, *ibid.*, **40**, 189(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 14, 1969, from the *Department of Pharmacy, Taipei Medical College, Taipei, Taiwan, China.*

Accepted for publication September 15, 1969.

Modified Granuloma Pouch Procedure for the Evaluation of Topically Applied Anti-inflammatory Steroids

G. DiPASQUALE, CHARLES L. RASSAERT, and EDWARD McDOUGALL

Abstract □ A laboratory procedure for evaluating topically applied anti-inflammatory preparations has been described. A modified croton oil-induced granuloma pouch served as the site of drug application and also as a physical barrier to avoid drug ingestion. Increasing concentrations and/or doses of test compounds are correlated with thymolysis, catabolism, and reduced exudate formation. The procedure can differentiate steroidal modifications, percutaneous absorption, and alterations of the vehicle. However, it is difficult to ascertain how much of the action of the test compound is due to its local and how much to its systemic properties. The assay also evaluated test compounds as pharmaceutical preparations. The order of increasing activity of the test substances can be listed as hydrocortisone acetate (1% cream) < methylprednisolone acetate (0.25% cream) < betamethasone 17-valerate (0.025% cream) < triamcinolone 16,17-acetonide (0.025% cream) < betamethasone (0.2% cream) < fluocinolone acetonide (0.025% alcoholic gel or cream).

Keyphrases □ Steroids, anti-inflammatory—topical activity determination □ Granuloma pouch test procedure—topical steroid activity □ Croton oil-air—granuloma pouch formation

Potential anti-inflammatory substances, topical or systemic, have been evaluated in a variety of procedures which apparently represent different modes of actions and/or different stages of the inflammatory process. Some of these procedures include: cotton pellet, granuloma pouch, paw edema, adjuvant-induced polyarthritis, cell cultures, uncoupling of mitochondrial oxidative phosphorylation, plasma protein changes, ear test, lung inflammation, and inhibition of erythema (1–4). It is evident that each test procedure has some merit; however, one cannot avoid nor explain discrepancies which occur between the various animal *in vitro* and clinical procedures. Nevertheless, most clinically efficacious drugs are active in most procedures, although in some assays a divergence may occur between steroidal and nonsteroidal drugs. Avoidance of ingestion of topically applied drugs in animals has presented some perplexing problems. Alternatives appear to introduce other problems; for example the use of occlusive dressings may alter absorption whereas the use of collars and other restraining devices produces stress which elaborates endogenous adrenocorticosteroids. The use of an air pouch which serves as the site of drug application and a physical barrier to avoid ingestion has been tried by the authors. They have also tried to observe whether or not the modified granuloma pouch procedure will distinguish steroidal modifications and/or alterations of the vehicle. It is known that these changes will affect drug absorption, retention, and biological activity (5, 6). It should also be mentioned that most animal (*in vivo* and/or *in vitro*) procedures require a solubilized test compound which requires organic solvents. The present study also evaluated drugs as pharmaceutical preparations.

MATERIALS AND METHODS

The method used was a modification of Selye's procedure (7). Male rats weighing 150–170 g. were individually housed and arranged in groups as indicated in the tables. The dorsal surface of the animal was shaved with animal clippers and the pouches were formed with the subcutaneous injection of 25 ml. of air followed by an injection of 0.5 ml. of a 1% solution of croton oil in sesame oil directly into the pouch. The test compounds included the commercial preparations: fluocinolone acetonide (Synalar cream, Syntex Laboratories Inc.); betamethasone (Celestone cream, Schering Corp.); methylprednisolone acetate (Medrol acetate cream, The Upjohn Co.); triamcinolone 16,17-acetonide (Kenalog cream, E. R. Squibb & Sons, Inc.); and also these laboratory preparations: hydrocortisone acetate in a cream base; betamethasone 17-valerate in a cream base; triamcinolone in a cream base; triamcinolone 16,17-acetonide in a cream base; and fluocinolone acetonide in an alcoholic gel base.

Table I—Effect of Several Topical Anti-inflammatory Substances in a Modified Granuloma Pouch Procedure

Test	Test Preparation, %	Daily Cream Dose, mg./ Animal	No. Animal, I/F	Body Wt. Change, g. ± *SE*	Rel. Organ Weights, mg./100 g. Body Wt. ± *SE* Adrenal	Thymus	Exudate, ml. ± *SE*	Change from Control Cream or Alc. Gel, %
1	Alc. gel control	100	20/20	63 ± 3.6	21.0 ± 0.8	205 ± 8.3	16.7 ± 2.1	—
	Fluocinolone acetonide (cream base) (0.025)	5	12/11	44 ± 4.2[a]	19.2 ± 0.6	144 ± 13.1[a]	14.9 ± 2.7	−11
	Fluocinolone acetonide (cream base) (0.025)	25	12/12	14 ± 4.1[a]	21.8 ± 1.6	54 ± 6.7[a]	5.8 ± 0.9[a]	−65[a]
	Fluocinolone acetonide (cream base) (0.025)	100	12/12	−18 ± 3.6[a]	21.6 ± 0.7	31 ± 1.7[a]	1.6 ± 0.6[a]	−90[a]
	Fluocinolone acetonide (alc. gel base) (0.025)	5	12/12	−14 ± 5.6[ab]	19.9 ± 0.9	67 ± 9.1[ab]	7.4 ± 1.2[ab]	−56[a]
	Fluocinolone acetonide (alc. gel base) (0.025)	25	12/11	− 1 ± 4.0[ab]	20.9 ± 1.1	28 ± 2.2[ab]	4.2 ± 1.0[a]	−75[a]
	Fluocinolone acetonide (alc. gel base) (0.025)	100	12/10	−29 ± 2.9[ab]	22.6 ± 1.3	30 ± 5.0[a]	0.8 ± 0.2[a]	−95[a]
2	Cream control	100	20/20	52 ± 3.9	19.3 ± 0.5	186 ± 10.3	20.0 ± 2.6	—
	Control (untreated)	—	10/10	57 ± 4.6	18.2 ± 0.5	211 ± 16.9	19.6 ± 3.3	—
	Fluocinolone acetonide (cream base) (0.001)	25	10/10	60 ± 4.1	18.6 ± 0.7	211 ± 15.4	19.0 ± 2.1	−2
	Fluocinolone acetonide (cream base) (0.0025)	25	10/10	50 ± 8.1	18.8 ± 1.2	167 ± 18.5	22.4 ± 5.0	+12
	Fluocinolone acetonide (cream base) (0.01)	25	10/9	33 ± 6.2[a]	16.4 ± 0.4[a]	106 ± 10.5[a]	15.0 ± 3.5	−25
	Fluocinolone acetonide (cream base) (0.025)	25	10/10	4 ± 4.7[a]	17.2 ± 0.6[a]	39.5 ± 4.2[a]	7.0 ± 1.8[a]	−65[a]
	Fluocinolone acetonide (cream base) (0.001)	100	10/10	43 ± 5.2	19.7 ± 1.2	186 ± 19.5	18.0 ± 2.8	−10
	Fluocinolone acetonide (cream base) (0.0025)	100	10/10	37 ± 4.4[a]	18.1 ± 0.4	130 ± 19.8	14.9 ± 1.9	−25
	Fluocinolone acetonide (cream base) (0.01)	100	10/10	−13 ± 3.4[a]	17.5 ± 0.8	31 ± 2.1[a]	2.8 ± 0.7[a]	−86[a]
	Fluocinolone acetonide (cream base) (0.025)	100	10/9	−26 ± 3.4[a]	16.3 ± 0.9[a]	30 ± 2.0[a]	0.8 ± 0.2[a]	−96[a]
3	Cream control	125	10/10	55 ± 6.7	17.7 ± 0.7	239 ± 11.9	13.0 ± 1.6	—
	Hydrocortisone acetate (cream base) (1)	125	10/9	34 ± 7.9	16.2 ± 1.1	115 ± 10.3[a]	8.1 ± 1.5[a]	−34[a]
	Hydrocortisone acetate (cream base) (1)	250	10/9	14 ± 4.3[a]	16.4 ± 0.9	72 ± 8.6[a]	4.4 ± 1.4[a]	−66[a]
	Hydrocortisone acetate (cream base) (2)	125	10/10	23 ± 4.9[a]	18.1 ± 1.0	89 ± 13.6[a]	8.0 ± 0.9[a]	−35[a]
	Hydrocortisone acetate (cream base) (2)	250	10/10	9 ± 5.8[a]	16.9 ± 1.3	52 ± 3.4[a]	3.5 ± 1.6[a]	−73[a]
	Betamethasone 17-valerate (cream base) (0.025)	125	10/8	52 ± 5.3	17.0 ± 0.7	182 ± 12.9[a]	14.2 ± 3.1	+9
	Betamethasone 17-valerate (cream base) (0.025)	250	9/9	41 ± 2.4[a]	19.2 ± 0.7	188 ± 11.7[a]	7.6 ± 0.8[a]	−42[a]
4	Cream control	100	15/15	54 ± 5.4	23.2 ± 0.8	200 ± 7.7	21.3 ± 2.5	—
	Triamcinolone (cream base) (0.001)	100	10/10	45 ± 4.9	23.6 ± 0.7	183 ± 10.9	21.0 ± 3.1	−1
	Triamcinolone (cream base) (0.01)	100	10/10	50 ± 7.3	22.0 ± 0.7	187 ± 15.6	17.2 ± 3.1	−19
	Triamcinolone (cream base) (0.025)	25	10/10	47 ± 6.2	22.8 ± 1.2	189 ± 10.6	18.6 ± 3.2	−13
	Triamcinolone (cream base) (0.025)	100	10/10	52 ± 5.4	20.8 ± 0.9	151 ± 10.6[a]	21.6 ± 3.4	+1
	Triamcinolone 16,17-acetonide (cream base) (0.001)	100	10/10	42 ± 5.5	24.0 ± 1.0	192 ± 13.8	15.0 ± 2.5	−30
	Triamcinolone 16,17-acetonide (cream base) (0.01)	100	10/9	29 ± 3.6[a]	20.5 ± 1.0	96 ± 11.1[a]	20.5 ± 1.0	−4
	Triamcinolone 16,17-acetonide (cream base) (0.025)	25	10/10	33 ± 8.1[a]	22.5 ± 1.0	128 ± 16.5[a]	17.8 ± 2.7	−16
	Triamcinolone 16,17-acetonide (cream base) (0.025)	100	10/10	4 ± 4.5[a]	21.9 ± 0.9	46 ± 4.1[a]	9.7 ± 1.6[a]	−55[a]

Table I—(*Continued*)

Test	Test Preparation, %	Daily Cream Dose, mg./ Animal	No. Animal, I/F	Body Wt. Change, g. ± *SE*	Rel. Organ Weights, mg./100 g. Body Wt. ± *SE* Adrenal	Thymus	Exudate, ml. ± *SE*	Change from Control Cream or Alc. Gel, %
	Triamcinolone acetonide[c] (0.025)	100	10/10	5 ± 6.6[a]	20.5 ± 1.6	59 ± 9.6[a]	10.3 ± 1.7[a]	−52[a]
5	Cream control	125	19/19	55 ± 2.9	20.5 ± 0.7	215 ± 10.5	18.2 ± 2.2	—
	Methylprednisolone acetate (cream base) (0.25)	125	10/10	25 ± 3.5[a]	18.3 ± 0.8	117 ± 22.7[a]	4.2 ± 1.0[a]	−53[a]
	Betamethasone (cream base) (0.2)	125	10/10	−25 ± 2.7[a]	19.3 ± 1.7	33 ± 3.0[a]	0.6 ± 0.1[a]	−100[a]

I = Initial number. F = Final number. [a] Significantly different from cream control of alcoholic gel, $p < 0.05$. [b] Significantly different from corresponding cream base preparation (Test 2). [c] Kenalog.

The control creams[1] and the test compounds in either creams or alcoholic gel[2] were applied topically to the surface of the pouch and spread evenly over a controlled area (35 mm. diameter) daily for 8 days starting on Day 2.

On Day 4, all the pouches were reshaved and slightly reinflated to original turgidity and 0.5 ml. of a 3% croton oil solution was injected directly into the pouch. All the animals were killed on Day 10 and the exudate volume was measured. The final body weights and adrenal and thymus weights were also recorded. The groups and their respective treatments are described in the tables. The significance between the control and treated groups was estimated by determining the standard error of the means and application of Student's *t* test.

RESULTS

Topically applied fluocinolone acetonide–alcoholic gel base (0.025%) at 5 mg./animal/day significantly inhibited thymus weights and the normal rate of body weight gain. On the other hand, 25 and 100 mg./animal/day significantly decreased exudate formation, thymus weights, and the normal rates of body weight gain. The comparison of fluocinolone acetonide in an alcoholic gel vehicle (0.025%) with fluocinolone acetonide in a cream vehicle (0.025%) indicates that although the latter also inhibited the same parameters, the former is more active in all the test parameters. Application of various concentrations of fluocinolone acetonide or various amounts of the cream preparations indicates that a dose-related response is obtained with fluocinolone acetonide. The biological activity is directly related to the amount of steroid and not to the quantity of the cream preparation applied (Test 2, Table I). Creams, which contained 1 or 2% hydrocortisone acetate when administered at 250 mg./animal/day significantly reduced exudate formation, thymus weights, and the body weight gain. At 125 mg./animal/day, both concentrations inhibited only the thymus weights and exudate formation whereas the 2% concentration also inhibited the body weight gain. It should be noted that the adrenal weights appeared to be the least sensitive test parameter and no dose-related response was evident.

It is also interesting to note that triamcinolone cream (0.025%) at 100 mg./animal/day significantly inhibited the thymus weight whereas triamcinolone 16,17-acetonide cream (0.025%) at all dose schedules inhibited the thymus weights and the body weight gain. Similarly, triamcinolone 16,17-acetonide (0.025%) at 100 mg./animal/day inhibited exudate formation. At equivalent concentrations the laboratory prepared triamcinolone 16,17-acetonide cream base was comparable to the commercially obtained preparation (Kenalog, 0.025%). Methylprednisolone acetate cream (0.25%) at 125 mg./animal/day had a similar activity whereas betamethasone cream (0.2%) caused a further reduction of the normal rate of body weight gain, thymus weight, and exudate formation. Betamethasone 17-valerate (0.025%) at 250 mg./animal/day significantly inhibited exudate formation, the body weight gain, and the thymus weights. At one-half the dose, only the thymus weights were inhibited.

CONCLUSIONS

The test procedure is capable of evaluating topically applied anti-inflammatory preparations and apparently percutaneous absorption. It should be mentioned that although the air pouch makes the test substances inaccessible to licking and cleaning, one cannot exclude the possibility that the rat may rub the air pouch and the test compound onto the top of the cage and then lick or ingest the steroids. However, this does not appear to be an important factor since the larger cream doses would have been more accessible for ingestion and yet one observes that the biological activity is related to the amount of active ingredient and not to the amount of the cream base applied. As with all other local/topical anti-inflammatory assays, it is difficult to ascertain how much of the action of the test compound is due to its local and how much to its systemic properties since the anti-inflammatory effect is usually associated with thymolysis and a reduced rate of body weight gain.

Katz and Shaikh (8) indicated that the relative percutaneous absorption produced by the molecular modification of corticosteroids is related to changes in solubility and partition coefficient. The physical properties of the vehicle also influence percutaneous absorption and biological activity (9, 10). It should also be kept in mind that the biological activity is also dependent upon many other factors such as plasma protein binding, receptor specificity, rate of metabolic transformation or inaction, and tissue retention subsequent to absorption to produce a more efficient local therapeutic action (8–10). The results demonstrate that an alcoholic gel vehicle for fluocinolone acetonide or the addition of 16,17-acetonide to triamcinolone enhances the biological activity when compared, respectively, to the fluocinolone acetonide cream or the triamcinolone cream. It appears that the alcoholic gel vehicle (fluocinolone acetonide) increases percutaneous absorption as indicated by the greater effect on the body weight and thymus weights. The correlation between biological activity and drug penetration was previously shown clinically where commercially available drugs were reported to be more active when applied to abraded skin or under occlusive dressing (5, 6, 11, 12).

The molecular modification of the test compound such as the formation of the acetonide apparently also leads to an enhanced percutaneous absorption. McKenzie (6) indicated that the addition of a 16,17-acetonide to specific steroids promotes a greater rat skin penetration and that the acetate is also better absorbed than the parent alcohol. The authors also observed that triamcinolone 16,17-acetonide cream base is more active in all the test parameters than triamcinolone, and the laboratory preparation is comparable to commercially obtained triamcinolone 16,17-acetonide (Kenalog). Similarly, commercially obtained methylprednisolone acetate and

[1] The laboratory creams were prepared to be similar to that employed in Synalar cream.

[2] Contains Carbopol (1.75%), diisopropanolamine (0.18%), methylparaben (0.2%), propyleneglycol (5%), alcohol (40%), and water (52.87%).

betamethasone also inhibited exudate formation, body weight gain, and the thymus weights. It is difficult to obtain a relative activity of betamethasone since the concentration used was high and may represent a maximum response.

Unlike clinical observations of topical activity in dermatologic diseases (13–15) in which betamethasone 17-valerate is generally described as comparable to fluocinolone acetonide, the authors' test procedure indicates that fluocinolone acetonide is more active. An attempt to grade the order of activity from the experimental results indicates that fluocinolone acetonide (0.025%) > betamethasone (0.2%) > triamcinolone 16,17-acetonide (0.025%) > betamethasone 17-valerate (0.025%) > methylprednisolone acetate (0.25%) > hydrocortisone acetate (1.0%). This order of decreasing activity is closely related to that described by others (16, 17). Similarly, one may also correlate steroidal modifications such as the addition of Δ^1, 6-methyl, 6-fluoro, 9-fluoro, 6,9-difluoro, 16-methyl, and 16,17-acetonide to the increasing order of biological activity (5, 16, 18, 19).

In conclusion, the modified granuloma pouch procedure not only serves as a site for drug application but is also useful for the evaluation of commercially available corticosteroids. The assay can also distinguish structural modifications and alteration of the vehicle. However, these changes did not always reflect potency differences observed in the clinic.

REFERENCES

(1) M. W. Whitehouse, "Progress in Drug Research," vol 8, Birkhauser Verlag, Basel, Switzerland, 1965, p. 321.

(2) G. Tonelli, L. Thibault, and I. Ringler, *Endocrinology*, **77**, 625(1965).

(3) C. A. Winter, "Progress in Drug Research," vol 10, Birkhauser Verlag, Basel und Stuttgart, Switzerland, 1966, p. 139.

(4) R. Domenjoz, "Advances in Pharmacology," vol 4, Academic, New York, N. Y. and London, England, 1966, p. 143.

(5) C. A. Schlagel, *J. Pharm. Sci.*, **54**, 335(1965).

(6) A. W. McKenzie, *Arch. Dermatol.*, **86**, 611(1962).

(7) H. Selye, *Proc. Soc. Exptl. Biol. Med.*, **82**, 328(1953).

(8) M. Katz and Z. I. Shaikh, *J. Pharm. Sci.*, **54**, 591(1965).

(9) B. J. Paulsen, E. Young, V. Coquilla, and M. Katz, *ibid.*, **57**, 928(1968).

(10) C. A. Schlagel, *J. Oral Therap. Pharmacol.*, **3**, 83(1967).

(11) A. W. McKenzie and R. B. Stoughton, *Arch. Dermatol.*, **86**, 608(1962).

(12) J. T. Groel, *ibid.*, **97**, 110(1968).

(13) D. M. Mitchell, S. H. Heany, and T. S. J. Eakins, *J. Irish Med. Assoc.*, **55**, 44(1965).

(14) D. B. Buckley, *ibid.*, **55**, 45(1964).

(15) V. H. Carter and R. O. Noojin, *Current Therap. Res.*, **9**, 253(1967).

(16) A. G. Ruhmann and D. L. Berliner, *Endocrinology*, **76**, 916(1965).

(17) D. R. London, *Practitioner*, **200**, 113(1968).

(18) E. W. Boland, *Am. J. Med.*, **31**, 581(1961).

(19) G. W. Liddle, *Clin. Pharmacol. Therap.*, **2**, 615(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 29, 1969, from the *Department of Physiology, Warner-Lambert Research Institute, Morris Plains, NJ 07950*

Accepted for publication September 15, 1969.

Gas-Liquid Chromatography of Salicylate Metabolites

CLARENCE H. MORRIS, JOHN E. CHRISTIAN, ROBERT R. LANDOLT, and WARREN G. HANSEN

Abstract □ A gas-liquid chromatographic separation of the methyl ester-methyl ether derivatives of acetylsalicylic acid, salicylic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and 2,3,4-trihydroxybenzoic acid is described. Separations were carried out at 155° on a column packed with 5% SE-30 on diatomaceous earth. A hydrogen-flame ionization detector was used.

Keyphrases □ Salicylate metabolites—separation, determination □ Column chromatography—separation □ GLC—analysis

It was decided to develop a gas-liquid chromatographic technique, for possible use by the authors and others, which would effect the separation and identification of acetylsalicylic acid, salicylic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and 2,3,4-trihydroxybenzoic acid. Horning *et al.* (1) report retention times for trimethylsilylether (TMS)–methyl ester derivatives of acetylsalicylic acid, salicylic acid, gentisic acid (2,5-dihydroxybenzoic acid), and salicyluric acid, but they do not mention either 2,3-dihydroxybenzoic acid, or 2,3,5-trihydroxybenzoic acid. Williams (2) reports retention times for methyl ester and methyl ester–methyl ether derivatives of several dihydroxybenzoic acids including 2,3- and 2,5-dihydroxybenzoic acids but he presents no data for acetylsalicylic acid, salicylic acid, or 2,3,5-trihydroxybenzoic acid. The following technique will effectively separate acetylsalicylic acid from its hydroxy metabolites.

Table I—Relative Retention Times

Methyl Ester–Methyl Ether Derivative of	Relative	Retention Time
Benzoic acid (internal standard)	—	1.00[a]
Salicylic acid	1.240	2.12
Acetylsalicylic acid	—	2.91
2,5-Dihydroxybenzoic acid	—	3.37
2,3-Dihydroxybenzoic acid	—	3.91
2,3,4-Trihydroxybenzoic acid	—	8.43

[a] Retention time = 1.026 min.

MATERIALS AND METHODS

Identity of the individual compounds used was established by melting point.

An ethereal alcoholic solution of diazomethane was prepared by reacting an ethereal solution of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide with ethanolic potassium hydroxide in a distilling apparatus (3). The resulting ethereal distillate contained approximately 3 g. of diazomethane.

The methyl ester–methyl ether derivatives of the respective compounds were prepared as follows. Approximately 0.25 g. of each compound was dissolved in a minimum amount of absolute ethanol. Ethereal diazomethane was added dropwise until a yellow color

betamethasone also inhibited exudate formation, body weight gain, and the thymus weights. It is difficult to obtain a relative activity of betamethasone since the concentration used was high and may represent a maximum response.

Unlike clinical observations of topical activity in dermatologic diseases (13–15) in which betamethasone 17-valerate is generally described as comparable to fluocinolone acetonide, the authors' test procedure indicates that fluocinolone acetonide is more active. An attempt to grade the order of activity from the experimental results indicates that fluocinolone acetonide (0.025%) > betamethasone (0.2%) > triamcinolone 16,17-acetonide (0.025%) > betamethasone 17-valerate (0.025%) > methylprednisolone acetate (0.25%) > hydrocortisone acetate (1.0%). This order of decreasing activity is closely related to that described by others (16, 17). Similarly, one may also correlate steroidal modifications such as the addition of Δ^1, 6-methyl, 6-fluoro, 9-fluoro, 6,9-difluoro, 16-methyl, and 16,17-acetonide to the increasing order of biological activity (5, 16, 18, 19).

In conclusion, the modified granuloma pouch procedure not only serves as a site for drug application but is also useful for the evaluation of commercially available corticosteroids. The assay can also distinguish structural modifications and alteration of the vehicle. However, these changes did not always reflect potency differences observed in the clinic.

REFERENCES

(1) M. W. Whitehouse, "Progress in Drug Research," vol 8, Birkhauser Verlag, Basel, Switzerland, 1965, p. 321.

(2) G. Tonelli, L. Thibault, and I. Ringler, *Endocrinology*, **77**, 625(1965).

(3) C. A. Winter, "Progress in Drug Research," vol 10, Birkhauser Verlag, Basel und Stuttgart, Switzerland, 1966, p. 139.

(4) R. Domenjoz, "Advances in Pharmacology," vol 4, Academic, New York, N. Y. and London, England, 1966, p. 143.

(5) C. A. Schlagel, *J. Pharm. Sci.*, **54**, 335(1965).

(6) A. W. McKenzie, *Arch. Dermatol.*, **86**, 611(1962).

(7) H. Selye, *Proc. Soc. Exptl. Biol. Med.*, **82**, 328(1953).

(8) M. Katz and Z. I. Shaikh, *J. Pharm. Sci.*, **54**, 591(1965).

(9) B. J. Paulsen, E. Young, V. Coquilla, and M. Katz, *ibid.*, **57**, 928(1968).

(10) C. A. Schlagel, *J. Oral Therap. Pharmacol.*, **3**, 83(1967).

(11) A. W. McKenzie and R. B. Stoughton, *Arch. Dermatol.*, **86**, 608(1962).

(12) J. T. Groel, *ibid.*, **97**, 110(1968).

(13) D. M. Mitchell, S. H. Heany, and T. S. J. Eakins, *J. Irish Med. Assoc.*, **55**, 44(1965).

(14) D. B. Buckley, *ibid.*, **55**, 45(1964).

(15) V. H. Carter and R. O. Noojin, *Current Therap. Res.*, **9**, 253(1967).

(16) A. G. Ruhmann and D. L. Berliner, *Endocrinology*, **76**, 916(1965).

(17) D. R. London, *Practitioner*, **200**, 113(1968).

(18) E. W. Boland, *Am. J. Med.*, **31**, 581(1961).

(19) G. W. Liddle, *Clin. Pharmacol. Therap.*, **2**, 615(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 29, 1969, from the *Department of Physiology, Warner-Lambert Research Institute, Morris Plains, NJ 07950*

Accepted for publication September 15, 1969.

Gas-Liquid Chromatography of Salicylate Metabolites

CLARENCE H. MORRIS, JOHN E. CHRISTIAN, ROBERT R. LANDOLT, and WARREN G. HANSEN

Abstract □ A gas-liquid chromatographic separation of the methyl ester-methyl ether derivatives of acetylsalicylic acid, salicylic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and 2,3,4-trihydroxybenzoic acid is described. Separations were carried out at 155° on a column packed with 5% SE-30 on diatomaceous earth. A hydrogen-flame ionization detector was used.

Keyphrases □ Salicylate metabolites—separation, determination □ Column chromatography—separation □ GLC—analysis

It was decided to develop a gas-liquid chromatographic technique, for possible use by the authors and others, which would effect the separation and identification of acetylsalicylic acid, salicylic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and 2,3,4-trihydroxybenzoic acid. Horning *et al.* (1) report retention times for trimethylsilylether (TMS)–methyl ester derivatives of acetylsalicylic acid, salicylic acid, gentisic acid (2,5-dihydroxybenzoic acid), and salicyluric acid, but they do not mention either 2,3-dihydroxybenzoic acid, or 2,3,5-trihydroxybenzoic acid. Williams (2) reports retention times for methyl ester and methyl ester–methyl ether derivatives of several dihydroxybenzoic acids including 2,3- and 2,5-dihydroxybenzoic acids but he presents no data for acetylsalicylic acid, salicylic acid, or 2,3,5-trihydroxybenzoic acid. The following technique will effectively separate acetylsalicylic acid from its hydroxy metabolites.

Table I—Relative Retention Times

Methyl Ester–Methyl Ether Derivative of	Relative	Retention Time
Benzoic acid (internal standard)	—	1.00[a]
Salicylic acid	1.240	2.12
Acetylsalicylic acid	—	2.91
2,5-Dihydroxybenzoic acid	—	3.37
2,3-Dihydroxybenzoic acid	—	3.91
2,3,4-Trihydroxybenzoic acid	—	8.43

[a] Retention time = 1.026 min.

MATERIALS AND METHODS

Identity of the individual compounds used was established by melting point.

An ethereal alcoholic solution of diazomethane was prepared by reacting an ethereal solution of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide with ethanolic potassium hydroxide in a distilling apparatus (3). The resulting ethereal distillate contained approximately 3 g. of diazomethane.

The methyl ester–methyl ether derivatives of the respective compounds were prepared as follows. Approximately 0.25 g. of each compound was dissolved in a minimum amount of absolute ethanol. Ethereal diazomethane was added dropwise until a yellow color

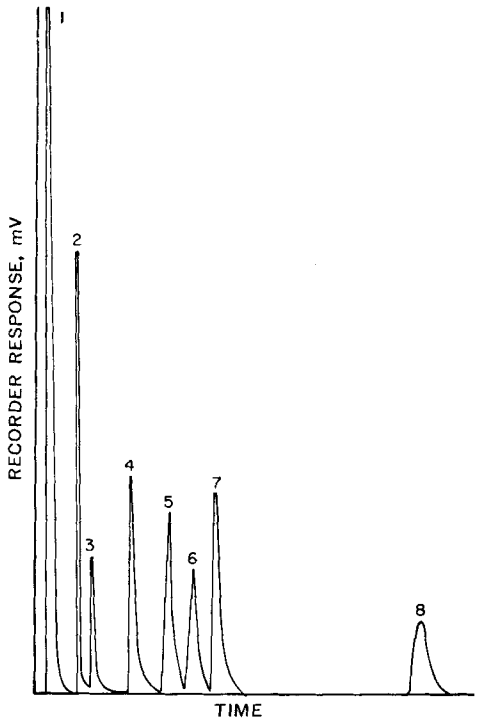


Figure 1—*Gas chromatograph showing:* (1) *solvent peak; derivatives of;* (2) *benzoic acid;* (3 *and* 4) *salicylic acid;* (5) *acetylsalicylic acid;* (6) *2,5-dihydroxybenzoic acid;* (7) *2,3-dihydroxybenzoic acid; and* (8) *2,3,4-trihydroxybenzoic acid.*

persisted, after which approximately a 20% excess by volume of ethereal diazomethane was added. The reactions were allowed to proceed at room temperature for 48 hr. in stoppered flasks. Additional ethereal diazomethane was added periodically if the resulting solutions became clear. After the reaction was completed, the solvent was evaporated at room temperature under a stream of dry nitrogen. The residual yellow viscous methyl ester–methyl ether derivatives were transferred quantitatively to 10-ml. volumetric flasks and brought to volume with pyridine.

An Aerograph 200, model 2041B (Wilkens Instrument and Research Inc., Walnut Creek, Calif.) equipped with a hydrogen-flame ionization detection system was used. Separations were carried out at 155° with a 1.524 m. (5 ft.) × 1.75 mm. i.d. stainless steel column packed with 5% SE-30 on diatomaceous earth[1] 60-80 mesh. Injector and detector temperatures were maintained at 205°. The helium carrier gas flow rate was 25 ml. per minute and the hydrogen pressure was maintained at 10 p.s.i.

RESULTS AND DISCUSSION

The relative retention times of the respective derivatives, as compared with the retention time of the methyl ester of benzoic acid, are reported in Table I. Figure 1 represents a chromatograph of a mixture of the respective compounds. Salicylic acid yielded two peaks with the same relative retention times following three separate derivative preparations. The purity of the salicylic acid was confirmed by TLC. 2,3,4-Trihydroxybenzoic acid was used as a model for the behavior of the 2,3,5-trihydroxybenzoic acid metabolite. It is not unreasonable to assume that the two compounds would demonstrate similar behavior toward the GLC system used. Since the retention time of the trihydroxy derivative is much longer than any of the other hydroxy derivatives, one would not expect the 2,3,5-trihydroxy metabolite to interfere with the analysis of the other compounds. The derivatives were found to be stable for a period of several weeks when stored at 4°.

REFERENCES

(1) M. G. Horning, K. L. Knox, C. E. Dalgliesh, and E. C. Horning, *Anal. Biochem.*, **17**, 244(1966).

(2) C. M. Williams, *ibid.*, **11**, 224(1965).

(3) Technical Information Bulletin, Product No. D 2800-0, Aldrich Chemical Co., Milwaukee, Wis., 1968.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 4, 1969, from the *Bionucleonics Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication September 29, 1969.

Conducted under the auspicies of the Institute for Environmental Health.

[1] Chromosorb W, Applied Science Laboratories Inc., State College, Pa.

New Synthesis of *rac.* Anhalonidine and *rac.* Pellotine

M. TAKIDO, K. L. KHANNA, and A. G. PAUL

Abstract □ A new synthesis of *rac.* anhalonidine and *rac.* pellotine is reported. The procedure, a modification of the method of Bobbitt *et al.* (5) for the synthesis of 1,2,3,4-tetrahydroisoquinolines, is simpler and gives better yields than those previously reported.

Keyphrases □ Anhalonidine, racemic—synthesis □ Pellotine, racemic—synthesis □ IR spectrophotometry—structure □ NMR spectroscopy—structure

The need for quantities of anhalonidine and pellotine for use in the biosynthesis of the peyote alkaloids and the inability to obtain them commercially or in sufficient quantities from the peyote cactus required that they be synthesized. Reported syntheses (1–4) are either lengthy or the yields are low. The method of Bobbitt *et al.* (5) for the synthesis of related tetrahydroisoquinolines was modified for the synthesis of these alkaloids as shown in Fig. 1.

3,4-Dimethoxygalloacetophenone (I), prepared by the method of David and Kostanecki (6), was condensed with aminoacetaldehyde diethylacetal to give Schiff's base (II) which was conveniently reduced at room temperature with sodium borohydride to yield *N*-[2-(3′,4′-dimethoxy-2′-hydroxyphenyl)ethyl]aminoacetaldehyde diethylacetal (III). Compound III was cyclized in 8 *N* HCl at room temperature to yield 4,8-dihy-

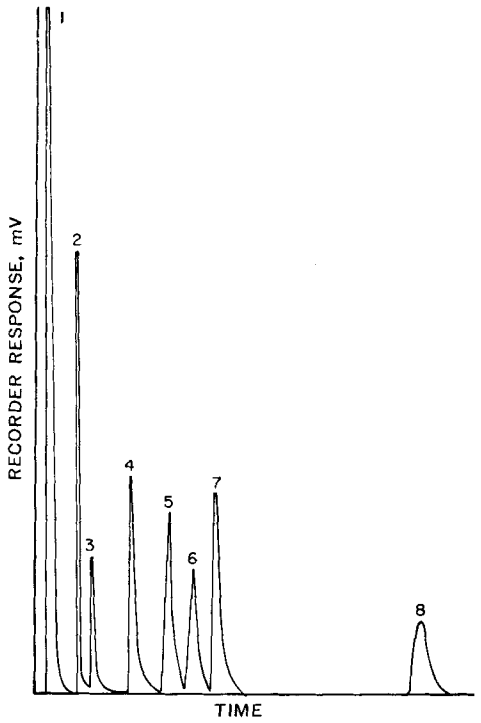


Figure 1—*Gas chromatograph showing:* (1) *solvent peak; derivatives of;* (2) *benzoic acid;* (3 *and* 4) *salicylic acid;* (5) *acetylsalicylic acid;* (6) *2,5-dihydroxybenzoic acid;* (7) *2,3-dihydroxybenzoic acid; and* (8) *2,3,4-trihydroxybenzoic acid.*

persisted, after which approximately a 20% excess by volume of ethereal diazomethane was added. The reactions were allowed to proceed at room temperature for 48 hr. in stoppered flasks. Additional ethereal diazomethane was added periodically if the resulting solutions became clear. After the reaction was completed, the solvent was evaporated at room temperature under a stream of dry nitrogen. The residual yellow viscous methyl ester–methyl ether derivatives were transferred quantitatively to 10-ml. volumetric flasks and brought to volume with pyridine.

An Aerograph 200, model 2041B (Wilkens Instrument and Research Inc., Walnut Creek, Calif.) equipped with a hydrogen-flame ionization detection system was used. Separations were carried out at 155° with a 1.524 m. (5 ft.) × 1.75 mm. i.d. stainless steel column packed with 5% SE-30 on diatomaceous earth[1] 60-80 mesh. Injector and detector temperatures were maintained at 205°. The helium carrier gas flow rate was 25 ml. per minute and the hydrogen pressure was maintained at 10 p.s.i.

RESULTS AND DISCUSSION

The relative retention times of the respective derivatives, as compared with the retention time of the methyl ester of benzoic acid, are reported in Table I. Figure 1 represents a chromatograph of a mixture of the respective compounds. Salicylic acid yielded two peaks with the same relative retention times following three separate derivative preparations. The purity of the salicylic acid was confirmed by TLC. 2,3,4-Trihydroxybenzoic acid was used as a model for the behavior of the 2,3,5-trihydroxybenzoic acid metabolite. It is not unreasonable to assume that the two compounds would demonstrate similar behavior toward the GLC system used. Since the retention time of the trihydroxy derivative is much longer than any of the other hydroxy derivatives, one would not expect the 2,3,5-trihydroxy metabolite to interfere with the analysis of the other compounds. The derivatives were found to be stable for a period of several weeks when stored at 4°.

REFERENCES

(1) M. G. Horning, K. L. Knox, C. E. Dalgliesh, and E. C. Horning, *Anal. Biochem.*, **17**, 244(1966).

(2) C. M. Williams, *ibid.*, **11**, 224(1965).

(3) Technical Information Bulletin, Product No. D 2800-0, Aldrich Chemical Co., Milwaukee, Wis., 1968.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 4, 1969, from the *Bionucleonics Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication September 29, 1969.

Conducted under the auspicies of the Institute for Environmental Health.

[1] Chromosorb W, Applied Science Laboratories Inc., State College, Pa.

New Synthesis of *rac.* Anhalonidine and *rac.* Pellotine

M. TAKIDO, K. L. KHANNA, and A. G. PAUL

Abstract □ A new synthesis of *rac.* anhalonidine and *rac.* pellotine is reported. The procedure, a modification of the method of Bobbitt *et al.* (5) for the synthesis of 1,2,3,4-tetrahydroisoquinolines, is simpler and gives better yields than those previously reported.

Keyphrases □ Anhalonidine, racemic—synthesis □ Pellotine, racemic—synthesis □ IR spectrophotometry—structure □ NMR spectroscopy—structure

The need for quantities of anhalonidine and pellotine for use in the biosynthesis of the peyote alkaloids and the inability to obtain them commercially or in sufficient quantities from the peyote cactus required that they be synthesized. Reported syntheses (1–4) are either lengthy or the yields are low. The method of Bobbitt *et al.* (5) for the synthesis of related tetrahydroisoquinolines was modified for the synthesis of these alkaloids as shown in Fig. 1.

3,4-Dimethoxygalloacetophenone (I), prepared by the method of David and Kostanecki (6), was condensed with aminoacetaldehyde diethylacetal to give Schiff's base (II) which was conveniently reduced at room temperature with sodium borohydride to yield *N*-[2-(3′,4′-dimethoxy-2′-hydroxyphenyl)ethyl]aminoacetaldehyde diethylacetal (III). Compound III was cyclized in 8 *N* HCl at room temperature to yield 4,8-dihy-

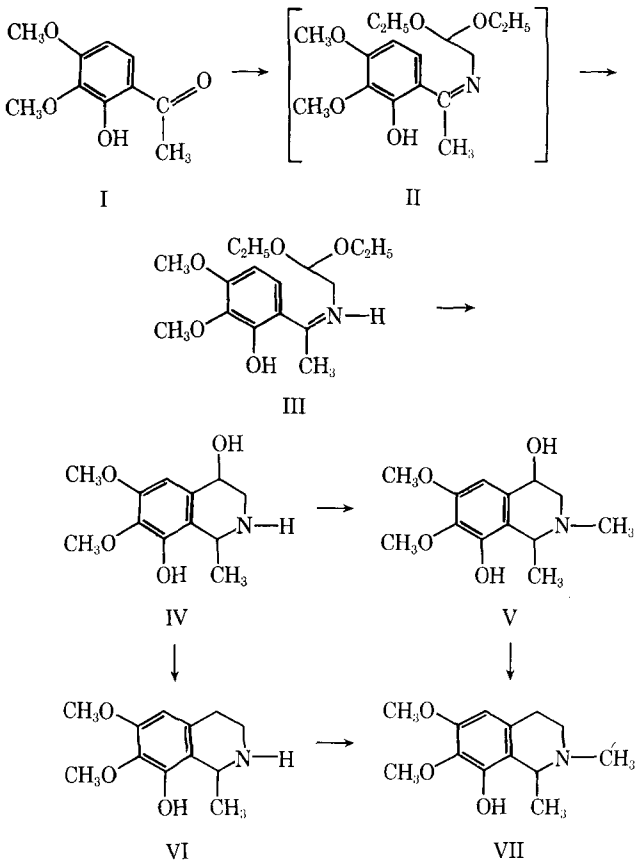


Figure 1—*Synthesis of* rac. *anhalonidine and* rac. *pellotine.*

droxy-6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline hydrochloride (IV). Reduction of IV in 6 *N* HCl with palladium-on-charcoal as the catalyst yielded anhalonidine hydrochloride (VI). Alternatively, III was mixed with 6 *N* HCl and palladium-on-charcoal and reduced to give VI directly. Yields by both methods were approximately equal.

Pellotine (VII) was synthesized by adding sodium borohydride to a mixture of IV and formaldehyde in methanol to give 4,8-dihydroxy-6,7-dimethoxy-1,2-dimethyl-1,2,3,4-tetrahydroisoquinoline (V). Reduction of V in 6 *N* HCl with palladium-on-charcoal as the catalyst yielded VII as the hydrochloride. Alternatively, VII was prepared by methylation of VI using formaldehyde in methanol in the presence of sodium borohydride.

In addition to presenting new methods for the synthesis of VI and VII in better yields then previously reported, III, IV, and V represent new compounds not previously reported in the literature. Proof of structure of these compounds is based upon elemental analyses, IR and NMR spectral data, and analogy with similar types of compounds synthesized by Bobbitt and Sih (7).

EXPERIMENTAL

***N*-[2-(3′,4′-dimethoxy-2′-hydroxyphenyl)ethyl]aminoacetaldehyde Diethylacetal (III)**—A solution of I (3.96 g.) and aminoacetaldehyde (2.66 g.) in 80 ml. of absolute ethanol was allowed to stand at room temperature for 24 hr. The solution was stirred at room temperature and sodium borohydride (1.0 g.) was added in small portions over a period of 0.5 hr. The solution was then added to 80 ml. of water and extracted with ether (3 × 200 ml.). The ether solution was concentrated and extracted with 1 *N* HCl. The acid extract was basified with 2 *N* Na_2CO_3 solution and extracted with ether. The ether was removed and the oil (4.5 g., 71%) was purified by distillation, b.p. 116–117°/0.03 mm. $\nu_{max.}^{film}$: 3275 cm.$^{-1}$ (—NH—); 3125 cm.$^{-1}$ (—OH). NMR[1] ($CDCl_3$), p.p.m.: 6.46 d [1], J = 8 c.p.s. (ar. H); 6.13 d [1], J = 8 c.p.s. (ar. H); 4.45 tr [1], J = 5 c.p.s. [—CH_2—$\underline{CH}(OC_2H_5)_2$]; 3.91 s [3], ($OCH_3$); 3.82 s [3], ($OCH_3$); 3.60 m [4], (2 × —O—$\underline{CH_2}$—$CH_3$); 2.53 d [2], J = 5 c.p.s. [—$\underline{CH_2}$—CH(OC_2H_5)$_2$]; 1.19 m [9], (2 × —OCH_2—CH_3; —NH—CH—CH_3).

Anal.—Calcd. for $C_{16}H_{27}NO_5$: C, 61.32; H, 8.68. Found: C, 61.74; H, 8.48.

4,8-Dihydroxy-6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline (IV) Hydrochloride (4-Hydroxyanhalonidine Hydrochloride)—Compound III (3.0 g.) was dissolved in 75 ml. 8 *N* HCl and the solution was permitted to stand for 10 hr. at room temperature. The acid solution was then adjusted to pH 6.5 with aqueous NaOH and washed with chloroform. The aqueous solution was filtered, freeze-dried, and the solid residue extracted with chloroform–methanol (9:1). The extract was evaporated to dryness *in vacuo* at <40° and the residue crystallized from methanol–ethylacetate as colorless plates, m.p. 217–218° (yield, 2.5 g., 87%). IR (KBr): 3420 cm.$^{-1}$ (—NH—); 3100 cm.$^{-1}$ (—OH); 1178 cm.$^{-1}$ (phenolic —OH); 1070 cm^{-1} (sec. alcohol —OH).

Anal.—Calcd. for $C_{12}H_{18}ClNO_4$: C, 52.27; H, 6.58; Cl, 12.86; N, 5.08. Found: C, 52.10; H, 6.70; Cl, 12.84; N, 5.03.

The base, prepared from the hydrochloride, crystallized from benzene–ethylacetate as colorless plates, m.p. 195–196° (dec.). IR (KBr): 3510 cm.$^{-1}$ (—NH—); 3070 cm.$^{-1}$ (—OH); 1190 cm.$^{-1}$ (phenolic —OH); 1060 cm.$^{-1}$ (sec. alcohol —OH). NMR (DMSO), p.p.m.: 6.49 s [1], (ar. H); 4.17, 4.05, 3.95 complex [2], [CH_3—$\underline{CH}$—NH—, —$\underline{CH}$(OH)—CH_2—]; 3.77 s [3], (—OCH_3); 3.68 s [3], (—OCH_3); 2.91 q [2], J = 2.5 c.p.s. [—CH(OH)—$\underline{CH_2}$]; 1.26 d [3], J = 6.5 c.p.s. ($\underline{CH_3}$—CH—NH—).

The picrate, prepared from the hydrochloride, recrystallized from benzene–ethanol as yellow plates, m.p. 206–208°.

4,8-Dihydroxy-6,7-dimethoxy-1,2-dimethyl-1,2,3,4-tetrahydroisoquinoline (V) (4-Hydroxypellotine)—Compound IV (200 mg.) and formaldehyde (120 mg., 37%) were dissolved in methanol (10 ml.). Sodium borohydride (80 mg.) was added to the stirred solution in small portions over a period of 0.5 hr. The solution was then added to water (10 ml.) and extracted with chloroform (3 × 20 ml.). The chloroform extract was evaporated to dryness and the residue washed with ether. The crystalline solid remaining was crystallized from benzene to give colorless needles, m.p. 177–178° (yield 180 mg., 86%). IR (KBr): 3130 cm.$^{-1}$ (—OH); 1185 cm.$^{-1}$ (phenolic —OH); 1080 cm.$^{-1}$ (sec. alcohol —OH). NMR ($CDCl_3$), p.p.m.: 6.54 s [1], (ar. H); 4.40 s, 4.09 s, 3.98 complex [2], [—$\underline{CH}$(OH)—CH_2—, —NH—$\underline{CH}$—CH_3]; 3.88 s [3], (—OCH_3); 3.86 s [3], (—OCH_3); 2.90 d [2], J = 13 c.p.s. [—CH(OH)—$\underline{CH_2}$]; 2.50 s [3], (>N—CH_3), 2.17 s [1], [—CH($O\underline{H}$)—CH_2—]; 1.17 d [3], J = 6.5 c.p.s. [—CH(CH_3)—N<].

Anal.—Calcd. for $C_{13}H_{19}NO_4$: C, 61.64; H, 7.56; N, 5.53. Found: C, 61.84; H, 7.66; N, 5.44.

Anhalonidine (VI) Hydrochloride—*Method A*—Compound III (1.2 g.) was dissolved in 8 *N* HCl (25 ml.) and the solution was permitted to stand at room temperature for 9 hr. The solution was then adjusted to approximately 6 *N* by the addition of water (10 ml.) and additional 6 *N* HCl (10 ml.) added. Palladium-on-charcoal (1.0 g., 5%) was added and the mixture was reduced at atmospheric pressure and room temperature for approximately 24 hr. The mixture was filtered and the filtrate concentrated *in vacuo* at <50° using a film-flash evaporator. Ethanol–benzene was added from time to time to azeotrope the water. The solid residue obtained was crystallized from methanol–ether to give colorless needles (yield 0.75 g., 73%).

Method B—Compound IV (100 mg.) was dissolved in 6 *N* HCl (20 ml.), palladium-on-charcoal (0.2 g., 5%) added, and the mixture

[1] s = singlet; d = doublet; tr = triplet; q = quartet; m = multiplet; ar. = aromatic.

was reduced and treated as described above (yield, 70 mg., 74%).

The free base, m.p. 160° [lit. m.p. (1) 160°], and the salicylate, m.p. 223–225° [lit. m.p. (4) 223.5–224.5°], prepared from VI hydrochloride showed no melting point depression when mixed with authentic samples. The IR spectra of the hydrochloride and the salicylate were identical with authentic samples.

Pellotine (VII) Hydrochloride—*Method A*—Sodium borohydride (20 mg.) was added in small portions over a period of 0.5 hr. to a stirred solution containing VI (38 mg.) and formaldehyde (2 drops, 37%) in methanol (5 ml.) at room temperature. The solution was then added to water (5 ml.) and extracted with chloroform (3 × 10 ml.). The chloroform extract was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was dissolved in a small quantity of methanol–chloroform–ether, and dry HCl gas was passed through the solution. Colorless needles crystallized (yield 35 mg., 75%).

Method B—A mixture of V (90 mg.), 6 *N* HCl (20 ml.), and palladium-on-charcoal (200 mg., 5%) was reduced at atmospheric pressure and room temperature for approximately 24 hr. The mixture was filtered and the filtrate concentrated *in vacuo* at <50° using a film-flash evaporator. Ethanol–benzene was added from time to time to azeotrope the water. The solid residue was crystallized from methanol–ether (yield 87 mg., 89%).

The free base, m.p. 116° [lit. m.p. (1) 111–112°], showed no melting point depression when mixed with authentic pellotine. The IR spectra of the base and hydrochloride were identical with that of authentic samples.

REFERENCES

(1) E. Späth, *Monatsh. Chem.*, **43**, 477(1922).

(2) E. Späth and F. Becke, *Chem. Ber.*, **67**, 266(1934).

(3) A. Brossi, F. Schenker, and W. Leimgruber, *Helv. Chim. Acta*, **47**, 2089(1964).

(4) A. Brossi, F. Schenker, R. Schmidt, R. Banziger, and W. Leimgruber, *ibid.*, **49**, 403(1966).

(5) J. M. Bobbitt, J. M. Kiely, K. L. Khanna, and R. Eberman, *J. Org. Chem.*, **30**, 2247(1965).

(6) E. David and S. V. Kostanecki, *Chem. Ber.*, **36**, 125(1903).

(7) J. M. Bobbitt and J. C. Sih, *J. Org. Chem.*, **33**, 856(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 22, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication October 10, 1969.

This investigation was supported by grant MH 13962-02 from the National Institute of Mental Health, Chevy Chase, MD 20203

The authors thank Dr. A. Brossi, Hoffmann-La Roche Inc., Nutley, N. J., for furnishing samples of anhalonidine hydrochloride and pellotine hydrochloride and to Dr. Fortüne Kohen, College of Pharmacy, University of Michigan, Ann Arbor, Mich., for the NMR spectral data.

Pentaerythritol Tetranitrate Sustained-Release Tablets: Relation of *In Vitro* Release of the Drug to Blood Pressure Changes after Administration to Anesthetized Cats

SACHCHIDANANDA BANERJEE, ARUN KUMAR MUKHERJEE, and ARUP KUMAR HALDER

Abstract □ The *in vitro* release of pentaerythritol tetranitrate (PET) from a sustained-action PET tablet was determined using USP tablet disintegration apparatus with the modification that 100-mesh stainless steel wire cloth replaced the usual 10-mesh. The immersion medium was simulated gastric fluid for the first 2 hr. and simulated intestinal fluid for the subsequent 10 hr. There was a gradual release of the drug and 80 to 100% of the drug was dissoluted at the end of 12 hr. The tablet when administered by mouth to anesthetized cats produced a 28 to 30% fall in arterial blood pressure which persisted for 12 hr. A good correlation between the rate of release of the drug *in vitro* and the *in vivo* effect in sustained lowering of the blood pressure was obtained. The *in vitro* method described is proposed for the quality control of sustained-action PET tablets.

Keyphrases □ Pentaerythritol tetranitrate sustained-release tablets—drug release □ Blood pressure changes—pentaerythritol tetranitrate release □ Dissolution rates—pentaerythritol tetranitrate tablets □ *In vivo-in vitro* release rates correlation—pentaerythritol tetranitrate tablets

Difficulties were experienced in testing the quality of sustained-release tablets of pentaerythritol tetranitrate using procedures usually adopted for the quality control of sustained-release preparations. In the dissolution test the concentration of the drug released in the bath fluid from the tubes containing tablets is estimated at various intervals. In the assay of pentaerythritol tetranitrate such a procedure could not be adopted as the analysis is based on the estimation of nitrates. To estimate nitrates in the bath fluid, large volumes of the solution had to be evaporated to dryness before extraction with glacial acetic acid (1). In the *in vivo* test the determination of the rate of absorption of the drug would involve the collection of large volumes of blood at frequent intervals and estimation of the drug in these blood samples. Both procedures were impractical.

In the dissolution test, if the remnants of the tablets in the disintegration apparatus and not the bath fluid were collected at different intervals, the estimation of pentaerythritol tetranitrate would be possible and the *in vitro* rate of release of the drug from the tablet could be calculated. The drug lowered blood pressure and a sustained fall in blood pressure could be observed when the sustained-release tablet was administered to cats. A study, therefore, was undertaken to establish a relation, if any, between the *in vitro* release of the drug and the lowering of blood pressure in cats after administration of sustained-release pentaerythritol tetranitrate tablets. A good correlation was observed between the *in vitro* release of the drug and the fall in

was reduced and treated as described above (yield, 70 mg., 74%).

The free base, m.p. 160° [lit. m.p. (1) 160°], and the salicylate, m.p. 223–225° [lit. m.p. (4) 223.5–224.5°], prepared from VI hydrochloride showed no melting point depression when mixed with authentic samples. The IR spectra of the hydrochloride and the salicylate were identical with authentic samples.

Pellotine (VII) Hydrochloride—*Method A*—Sodium borohydride (20 mg.) was added in small portions over a period of 0.5 hr. to a stirred solution containing VI (38 mg.) and formaldehyde (2 drops, 37%) in methanol (5 ml.) at room temperature. The solution was then added to water (5 ml.) and extracted with chloroform (3 × 10 ml.). The chloroform extract was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was dissolved in a small quantity of methanol–chloroform–ether, and dry HCl gas was passed through the solution. Colorless needles crystallized (yield 35 mg., 75%).

Method B—A mixture of V (90 mg.), 6 *N* HCl (20 ml.), and palladium-on-charcoal (200 mg., 5%) was reduced at atmospheric pressure and room temperature for approximately 24 hr. The mixture was filtered and the filtrate concentrated *in vacuo* at <50° using a film-flash evaporator. Ethanol–benzene was added from time to time to azeotrope the water. The solid residue was crystallized from methanol–ether (yield 87 mg., 89%).

The free base, m.p. 116° [lit. m.p. (1) 111–112°], showed no melting point depression when mixed with authentic pellotine. The IR spectra of the base and hydrochloride were identical with that of authentic samples.

REFERENCES

(1) E. Späth, *Monatsh. Chem.*, **43**, 477(1922).

(2) E. Späth and F. Becke, *Chem. Ber.*, **67**, 266(1934).

(3) A. Brossi, F. Schenker, and W. Leimgruber, *Helv. Chim. Acta*, **47**, 2089(1964).

(4) A. Brossi, F. Schenker, R. Schmidt, R. Banziger, and W. Leimgruber, *ibid.*, **49**, 403(1966).

(5) J. M. Bobbitt, J. M. Kiely, K. L. Khanna, and R. Eberman, *J. Org. Chem.*, **30**, 2247(1965).

(6) E. David and S. V. Kostanecki, *Chem. Ber.*, **36**, 125(1903).

(7) J. M. Bobbitt and J. C. Sih, *J. Org. Chem.*, **33**, 856(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 22, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication October 10, 1969.

This investigation was supported by grant MH 13962-02 from the National Institute of Mental Health, Chevy Chase, MD 20203

The authors thank Dr. A. Brossi, Hoffmann-La Roche Inc., Nutley, N. J., for furnishing samples of anhalonidine hydrochloride and pellotine hydrochloride and to Dr. Fortüne Kohen, College of Pharmacy, University of Michigan, Ann Arbor, Mich., for the NMR spectral data.

Pentaerythritol Tetranitrate Sustained-Release Tablets: Relation of *In Vitro* Release of the Drug to Blood Pressure Changes after Administration to Anesthetized Cats

SACHCHIDANANDA BANERJEE, ARUN KUMAR MUKHERJEE, and ARUP KUMAR HALDER

Abstract □ The *in vitro* release of pentaerythritol tetranitrate (PET) from a sustained-action PET tablet was determined using USP tablet disintegration apparatus with the modification that 100-mesh stainless steel wire cloth replaced the usual 10-mesh. The immersion medium was simulated gastric fluid for the first 2 hr. and simulated intestinal fluid for the subsequent 10 hr. There was a gradual release of the drug and 80 to 100% of the drug was dissoluted at the end of 12 hr. The tablet when administered by mouth to anesthetized cats produced a 28 to 30% fall in arterial blood pressure which persisted for 12 hr. A good correlation between the rate of release of the drug *in vitro* and the *in vivo* effect in sustained lowering of the blood pressure was obtained. The *in vitro* method described is proposed for the quality control of sustained-action PET tablets.

Keyphrases □ Pentaerythritol tetranitrate sustained-release tablets—drug release □ Blood pressure changes—pentaerythritol tetranitrate release □ Dissolution rates—pentaerythritol tetranitrate tablets □ *In vivo-in vitro* release rates correlation—pentaerythritol tetranitrate tablets

Difficulties were experienced in testing the quality of sustained-release tablets of pentaerythritol tetranitrate using procedures usually adopted for the quality control of sustained-release preparations. In the dissolution test the concentration of the drug released in the bath fluid from the tubes containing tablets is estimated at various intervals. In the assay of pentaerythritol tetranitrate such a procedure could not be adopted as the analysis is based on the estimation of nitrates. To estimate nitrates in the bath fluid, large volumes of the solution had to be evaporated to dryness before extraction with glacial acetic acid (1). In the *in vivo* test the determination of the rate of absorption of the drug would involve the collection of large volumes of blood at frequent intervals and estimation of the drug in these blood samples. Both procedures were impractical.

In the dissolution test, if the remnants of the tablets in the disintegration apparatus and not the bath fluid were collected at different intervals, the estimation of pentaerythritol tetranitrate would be possible and the *in vitro* rate of release of the drug from the tablet could be calculated. The drug lowered blood pressure and a sustained fall in blood pressure could be observed when the sustained-release tablet was administered to cats. A study, therefore, was undertaken to establish a relation, if any, between the *in vitro* release of the drug and the lowering of blood pressure in cats after administration of sustained-release pentaerythritol tetranitrate tablets. A good correlation was observed between the *in vitro* release of the drug and the fall in

Table I—Percent Release of Pentaerythritol Tetranitrate from a Sustained-Release Tablet Containing 80 mg. of the Drug[a]

1 hr.[b]	2 hr.[b]	4 hr.[c]	6 hr.[c]	8 hr.[c]	10 hr.[c]	12 hr.[c]
16 ± 1.5 (9)[d]	16 ± 1.5 (9)	32 ± 3.5 (9)	45 ± 3.7 (8)	56 ± 2.8 (9)	69 ± 2.9 (8)	90 ± 2.0 (9)

[a] Values are mean ± standard error. [b] Simulated gastric fluid. [c] Simulated intestinal fluid. [d] Figures in parentheses indicate the number of observations.

Table II—Relation of *In Vitro* Release of Pentaerythritol Tetranitrate (PET) to Percent Fall of Blood Pressure in Cats after Administration of a Sustained-Release Tablet

	2 hr. (10)[a]	4 hr. (10)	6 hr. (10)	8 hr. (10)	10 hr. (5)	12 hr. (5)
% fall of blood pressure[b] from basal in cats after feeding sustained-release PET tablet, 15 mg./kg.	29 ± 3.0	26 ± 2.1	31 ± 3.8	32 ± 3.0	25 ± 2.1	19 ± 3.8
Release of PET (mg.)[b] *in vitro* from a sustained-release tablet containing 80 mg. drug	14 ± 1.1	10 ± 1.4	10 ± 1.2	10 ± 1.6	14 ± 3.0	11 ± 2.9
Coefficient of correlation	0.887	0.919	0.926	0.980	0.801	0.985

[a] Figures in parentheses indicate the number of samples used. [b] Values are mean ± standard error.

blood pressure which indicates that the *in vitro* procedure described in this communication can be used for the quality control of the sustained-release dosage form of pentaerythritol tetranitrate.

EXPERIMENTAL

Dissolution Rate Determination—The sustained-action pentaerythritol tetranitrate (PET) tablets under study were two-layered compressed tablets of which one layer contained 10 mg. PET for immediate release and the second layer contained 70 mg. of the drug for sustained release.

For the determination of the dissolution rate, the tablet disintegration apparatus as described in USP XVII (2) was used with the modification that 100-mesh stainless steel wire cloth was attached to the under surface of the lower end of the disintegration tubes in place of 10-mesh usually used for determination of disintegration of tablets. One tablet was placed in each of the tubes and the machine was switched on using simulated gastric fluid with a pH of 1.2 (3). After 2 hr. the machine was switched off. One of the tubes used for disintegration was withdrawn and the remnant of the tablet transferred into a 150-ml. conical flask. The simulated gastric fluid was replaced by simulated intestinal fluid (4) adjusted to pH 7.5. The machine was switched on again and operated for another 10 hr. At intervals of 1 hr., one of the disintegration tubes was withdrawn and the remnant of the tablet was transferred into a 150-ml. flask. The remnants of the tablets were extracted with glacial acetic acid on a boiling water bath, suitably diluted with the acid to bring to a definite volume, and PET content was estimated (1). Twenty of the tablets were crushed to powder and PET content was estimated in an aliquot to obtain the contents in one tablet before disintegration. From the contents of PET in the remnants of tablets after various hours of dissolution, the rate of release of the drug from a tablet was calculated. The results are given in Table I.

Blood Pressure Studies—Cats weighing 2.5–3 kg. were anesthetized by intramuscular injection of sodium phenobarbital, 150 mg./kg., dissolved in 4 ml. of water for injection. After the animal was under the anesthesia the left common carotid artery was cannulated in the usual way to record the arterial blood pressure on a kymograph. The trachea was connected to a recording tambour for the recording of respiration. When the initial blood pressure and respiration were recorded the sustained-release PET tablet, 15 mg./kg., was fed to the animal through a stomach tube, 10 ml. water was injected through the tube so as to push the tablet into the stomach, and blood pressures were recorded at intervals. Fifteen milligrams PET per kilogram body weight was found to produce a marked fall in blood pressure without showing any undesirable effect. The relation of percent fall of blood pressure to *in vitro* release of PET with statistical analysis is shown in Table II.

When quick-release PET was administered in the same dose, there was a 40% fall in blood pressure which came to the initial level within 6 hr. The anesthesia itself did not produce any marked change in the blood pressure.

RESULTS AND DISCUSSION

The results in Table I indicate that the *in vitro* release of PET was gradual and 90% of the drug was released in the course of 12 hr. The results were found reproducible, within the limits of the assay methods, from lot to lot of the same batch and in different batches. Table II shows that the percent fall in blood pressure, after administration of PET, at different hours had a good correlation with the release of PET in the *in vitro* experiment.

With regards to PET, controlled studies in humans based on the blood concentration of the drug or its degradation product are not possible as the rate of release of the drug is only 5 to 7 mg./hr. and concentration of the drug in the blood is too low to be analyzed. The *in vitro* test, therefore, may be used as a quality control procedure for the evaluation of a sustained-release preparation of pentaerythritol tetranitrate tablets.

REFERENCES

(1) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1963, p. 567.

(2) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 919.

(3) *Ibid.*, p. 1075.

(4) *Ibid.*, p. 1076.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 24, 1968, from the *Research Division, Dey's Medical Stores (Manufacturing) Private Limited, Calcutta-19, India.*

Accepted for publication October 8, 1969.

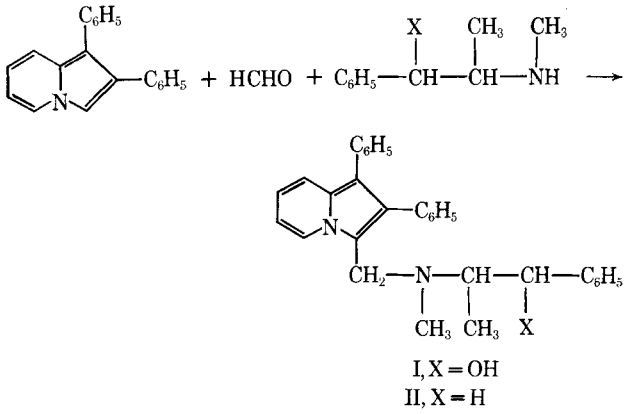
Mannich Bases from 1,2-Diphenylindolizine: Ephedrine and Methamphetamine as Amine Components

WILLIAM B. HARRELL

Abstract □ Two unique Mannich bases derived from 1,2-diphenylindolizine have been synthesized. Ephedrine and methamphetamine were employed as the secondary amine components in the syntheses. Both products showed an initial marked CNS depression. The activity of the methamphetamine derivative was reversed after 1.5 hr. to a pronounced CNS stimulation.

Keyphrases □ Mannich bases—synthesis □ 1,2-Diphenylindolizine—Mannich bases, synthesis □ Ephedrine, methamphetamine—amine components, Mannich reaction □ CNS activity—1,2-diphenylindolizine derivatives

It has been established in these laboratories, as a result of a continuing search for indolizines with useful biological activity, that certain 3-dialkylaminomethyl derivatives of 1,2-diphenylindolizine were potent CNS depressants (1). It was a natural curiousity that prompted the consideration of compounds with established CNS stimulant activity as secondary amine components in the synthesis of Mannich bases of this type. 1,2-Diphenyl-3-[*N*-(2-hydroxy-1-methyl-2-phenylethyl)-*N*-methylaminomethyl]indolizine (I) and 1,2-diphenyl-3-[*N*-(1-methyl-2-phenylethyl)-*N*-methylaminomethyl]indolizine (II) were synthesized from 1,2-diphenylindolizine using ephedrine and methamphetamine, respectively, as the secondary amine components in the Mannich reaction (2) (Scheme I).



I, X = OH
II, X = H

Scheme I

EXPERIMENTAL

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analysis of I was provided by Weiler and Strauss Microanalytical Laboratory, Oxford, England. The analysis of II was performed by Galbraith Laboratories, Knoxville, Tenn. The parent indolizine employed in the synthesis of the Mannich bases was 1,2-diphenylindolizine (3, 4).

Spontaneous Motor Activity—A preliminary evaluation of the activity of these compounds on the CNS was carried out in the following manner. The compounds were dissolved in one part *N,N*-dimethylacetamide, diluted with nine parts water, and injected intraperitoneally in mice (Swiss white, random bred, 20–30 g.) in dosages of 5 mg./kg. body weight. Spontaneous motor activity was measured in a photoelectric cell activity cage and run against controls injected with solvent only. Both I and II showed strong CNS depressant activity. However, the animals were easily aroused and did not lose their righting reflex during this period. After approximately 1.5 hr., II showed a marked reversal of activity. The spontaneous motor activity increased significantly above that of the controls. This stimulation is presumably due to the metabolic release of the methamphetamine moiety. No significant stimulation was observed with I after 4 hr. A more extensive pharmacological study of these compounds will be carried out and the results will be reported.

1,2-Diphenyl-3-[*N*-(2-hydroxy-1-methyl-2-phenylethyl)-*N*-methylaminomethyl]indolizine (I)—To a 125-ml. conical flask was added 0.75 ml. of 40% aqueous formaldehyde (0.01 mole), 2.48 g. of L-ephedrine (0.015 mole), and 30 ml. of *N,N*-dimethylformamide. The mixture was allowed to stand at −5° for 48 hr. At this point 1.35 g. of 1,2-diphenylindolizine (0.005 mole) was added to the flask and the mixture stirred at room temperature for 72 hr. The reaction solution was nearly saturated with water and stirring was continued for 24 hr., during which the product crystallized out. The yield was 1.7 g. (76%). On recrystallization from acetone–water the compound gave m.p. 157–158°. The compound gave a negative color test with *p*-dimethylaminobenzaldehyde indicating that substitution had occurred at the C-3 position (5).

Anal.—Calcd. for $C_{31}H_{30}N_2O$: C, 83.37; H, 6.77; N, 6.27. Found: C, 83.29; H, 6.61; N, 6.15.

1,2-Diphenyl-3-[*N*-(1-methyl-2-phenylethyl)-*N*-methylaminomethyl]indolizine (II)—Methamphetamine hydrochloride, 6.2 g. (0.033 mole), was converted to the free base and transferred to a 250-ml. conical flask. To the flask was added 1.5 ml. of 40% aqueous formaldehyde (0.02 mole) and 70 ml. of *N,N*-dimethylformamide. The flask was stoppered and allowed to stand at −5° for 48 hr. To the mixture was added 2.7 g. of 1,2-diphenylindolizine (0.01 mole). The reaction mixture was refrigerated for 96 hr. with occasional agitation. The product crystallized out on saturation with water. The yield was 3.6 g. (83%) of fluorescent yellow crystals. Upon recrystallization from acetone–water the product gave m.p. 141–142°. The compound gave a negative color test with *p*-dimethylaminobenzaldehyde.

Anal.—Calcd. for $C_{31}H_{30}N_2$: C, 86.47; H, 7.02; N, 6.51. Found: C, 86.20, H, 7.02; N, 6.49.

REFERENCES

(1) W. B. Harrell and R. F. Doerge, *J. Pharm. Sci.*, **56**, 1200 (1967).
(2) C. Mannich and W. Kroshe, *Arch. Pharm.*, **250**, 647(1912).
(3) A. E. Chichibabin, *Chem. Ber.*, **60**, 1607(1927).
(4) P. A. Barrett, *J. Chem. Soc.*, **1958**, 325.
(5) D. O. Holland and J. H. C. Nayler, *ibid.*, **1955**, 1657.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 17, 1969, from *The School of Pharmacy, Texas Southern University, Houston, TX 77004*

Accepted for publication September 29, 1969.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Supported by a grant from the University.

4-Thiazolylmethoxyureas as Potential Anticancer Agents

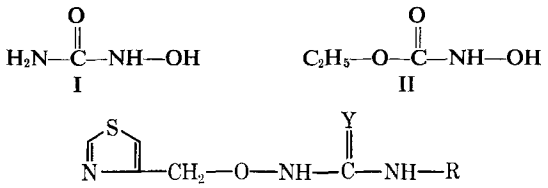
GLENN H. HAMOR and ANDON HUDAVERDI*

Abstract □ Preparation of a series of substituted 4-thiazolylmethoxyureas and 4-thiazolylmethoxythioureas is described. These compounds, which are structurally related to the antitumor agent hydroxyurea, were essentially inactive when screened against the L-1210 lymphoid leukemia test system. One compound, 4-thiazolylmethoxyurea, showed moderate anti-inflammatory activity in the carrageenin-induced rat paw edema test.

Keyphrases □ Anticancer agents, potential—synthesis □ 4-Thiazolylmethoxyureas—synthesis □ Pharmacological screening—4-thiazolylmethoxyureas □ IR spectrophotometry—identity

Recent studies have demonstrated the antineoplastic activity of hydroxyurea (I) (1, 2) and hydroxyurethan (II) (3). Hydroxyurea has been shown to inhibit several mouse tumors and has been used clinically in the treatment of cancer (4–6).

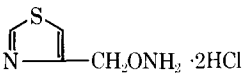
The aim of the present work is the synthesis of some 4-thiazolylmethoxyurea derivatives (III) for evaluation as possible antineoplastic agents. These 4-thiazolylmethoxyureas are structurally related to hydroxyurea, and also are related to 4-thiazolylmethoxyamine (IV) which is a potent histidine decarboxylase inhibitor (7). Histamine biosynthesis and histidine decarboxylase activity have been reported to increase in tumor tissue (8–11).



I

II

III

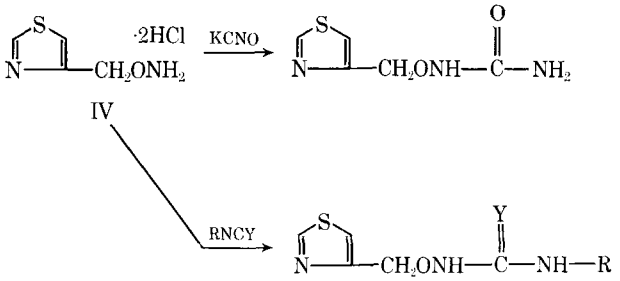


IV

Y = O; R = H, phenyl, 1-naphthyl, cyclohexyl
Y = S; R = phenyl

The five 4-thiazolylmethoxyureas synthesized are listed and their analytical data and melting points given in Table I and have the formula shown above (III). These ureas were prepared as shown in Scheme I.

The synthesis of the starting 4-thiazolylmethoxyamine dihydrochloride (IV) is described in another publication (7). The desired 4-thiazolylmethoxyurea was prepared by the reaction of potassium cyanate and 4-thiazolylmethoxyamine dihydrochloride by the method used by Bauer and Dalalian for the synthesis of aryloxyureas (12). By a modification of this method, the reaction of IV with phenyl isocyanate, with 1-naphthyl isocyanate, and with cyclohexyl isocyanate gave, respectively, 1-phenyl-3-(4-thiazolylmethoxy)urea, 1-(1-naphthyl)-3-(4-thiazolylmethoxy)urea, and 1-cyclohexyl-3-(4-thiazolylmethoxy)urea. The use of the alkyl isocyanates, methyl, ethyl, and *n*-butyl isocyanate, with IV by the above method gave no desired compounds.



Y = O; R = phenyl, 1-naphthyl, cyclohexyl
Y = S; R = phenyl

Scheme I

The reaction of phenyl isothiocyanate and IV yielded the expected 1-phenyl-3-(4-thiazolylmethoxy)thiourea. This thiourea showed apparent decomposition upon standing at room temperature for 2 months. The synthesis of 4-thiazolylmethoxythiourea by the method used in the preparation of 4-thiazolylmethoxyurea was attempted. However, the isolation and purification of the desired 4-thiazolylmethoxythiourea could not be accomplished, because the compound was very unstable, apparently decomposing in 1–2 hr.

BIOLOGICAL RESULTS

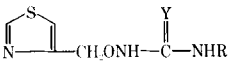
Anticancer Screening—Four of the compounds, namely 4-thiazolylmethoxyurea (Compound No. 1, Table I) (NSC 116197), 1-phenyl-3-(4-thiazolylmethoxy)urea (No. 2) (NSC 116723), 1-cyclohexyl-3-(4-thiazolylmethoxy)urea (No. 4) (NSC 119183), and 1-phenyl-3-(4-thiazolylmethoxy)thiourea (No. 5) (NSC 119182), were submitted to the Cancer Chemotherapy National Service Center, National Cancer Institute, for screening against the L-1210 lymphoid leukemia test system (13). None of the compounds possessed any significant antitumor activity. The cyclohexyl derivative (No. 4) was considerably more toxic than the other alkoxyureas, being lethal to 5/6 mice at a dose of 75 mg./kg., injected intraperitoneally. The other three compounds tested showed no lethality at 400 mg./kg.

Antimalarial Screening—Two compounds (Nos. 1 and 3) were tested for antimalarial activity against *Plasmodium berghei* in mice under the auspices of Walter Reed Army Institute of Research and were found to be inactive.

Preliminary Pharmacological Screening—Preliminary pharmacological results[1] are available for 4-thiazolylmethoxyurea (No. 1) and 1-phenyl-3-(4-thiazolylmethoxy)urea (No. 2). Compound No. 1 has no cardiovascular effect in the dog on i.v. injection. Tests performed in the rat (oral administration) showed that No. 1 has slight antipyretic and moderate anti-inflammatory (carrageenin-induced edema in the rat paw) effects, has a slight antidiuretic effect, and causes considerable anorexia. Tests in mice (i.p. injection) showed that the compound has no analgesic, antielectroshock,

[1] The authors thank Riker Laboratories, Northridge, CA 91324, for performing the pharmacological testing.

Table I—4-Thiazolylmethoxyureas



No.	Y	R	M.p., °C.	Formula	Anal., % Calcd.	Found
1	O	H	124–5	$C_5H_7N_3O_2S$	C, 34.67 H, 4.07 N, 24.26	C, 34.46 H, 4.46 N, 24.00
2	O	C_6H_5	100–1	$C_{11}H_{11}N_3O_2S$	C, 52.99 H, 4.45 N, 16.85 S, 12.86	C, 53.23 H, 4.54 N, 16.61 S, 12.51
3	O	1-Naphthyl	132–4	$C_{15}H_{13}N_3O_2S$	C, 60.18 H, 4.38 N, 14.04	C, 60.54 H, 4.46 N, 13.75
4	O	Cyclohexyl	76–7	$C_{11}H_{17}N_3O_2S$	C, 51.73 H, 6.71 N, 16.45	C, 52.12 H, 6.93 N, 16.39
5	S	C_6H_5	119–21	$C_{11}H_{11}N_3OS_2$	C, 49.78 H, 4.18 N, 15.83	C, 49.75 H, 4.21 N, 15.85

antiamphetamine, nor antireserpine activity and no effect on conditioned avoidance response. It decreases considerably the locomotor activity of the animal (i.p. or p.o.). At very high doses it causes hyperactivity. The LD_{50} of the compound in mice (i.p.) is greater than 800 mg./kg. (0/10 mice dead).

Compound No. 2 has no cardiovascular effects in the dog. In contrast to the results on No. 1, slight increases in the amount of urine and in the body temperature of the rat (p.o.) were seen. There was no effect on conditioned avoidance response. Anorexic activity was present. Tests in mice (i.p.) showed a slight antielectroshock, a slight analgesic, and no antiamphetamine nor antireserpine effect. The compound (i.p., mice) slightly increased locomotor activity, and at high doses caused ataxia. The LD_{50} of the compound is greater than 800 mg./kg., i.p. (3/10 mice dead).

EXPERIMENTAL

The syntheses of the compounds reported in Table I are described here. All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Elemental microanalyses were performed by Elek Microanalytical Laboratories, Torrance, Calif. The IR spectra of all compounds were determined on a Perkin-Elmer Infracord apparatus in mineral oil mulls, and were in agreement with the assigned structures.

4-Thiazolylmethoxyurea (No. 1)—To 20.31 g. (0.1 mole) of 4-thiazolylmethoxyamine dihydrochloride (7) dissolved in 150 ml. H_2O was added 8.9 g. (10% excess) of potassium cyanate. The solution was stirred at 60° for 1 hr. and then left overnight at 60° to evaporate to dryness. Extraction of the residue with 50 ml. boiling acetone, followed by concentration of the solution and cooling gave 6.2 g. (33.8% yield), m.p. 124–125°. An analytical sample was obtained by three recrystallizations from acetone.

1-Phenyl-3-(4-thiazolylmethoxy)urea (No. 2)—To a solution of 0.6 g. (0.003 mole) of 4-thiazolylmethoxyamine dihydrochloride in 10 ml. of water was added 100 ml. of benzene. Sufficient anhydrous K_2CO_3 (approximately 30 g.) was added not only to form the free base but also to remove all water. The mixture was filtered through a folded filter paper and the water layer (if any) discarded. To the benzene layer containing the free 4-thiazolylmethoxyamine was added 0.325 ml. (0.003 mole) phenyl isocyanate; the mixture was stirred for 30 min. at room temperature and then let evaporate to dryness overnight at room temperature to yield 0.48 g. (63%), m.p. 100°.

1-(1-Naphthyl)-3-(4-thiazolylmethoxy)urea (No. 3)—The naphthyl compound was synthesized by the same procedure used for the phenyl derivative above. From 1.02 g. (0.005 mole) 4-thiazolylmethoxyamine dihydrochloride and 0.85 g. (0.72 ml., 0.005 mole) 1-naphthyl isocyanate was obtained a white, crystalline product, which after three recrystallizations from benzene weighed 0.7 g. (49%), m.p. 132°.

1-Cyclohexyl-3-(4-thiazolylmethoxy)urea (No. 4)—The cyclohexyl derivative was prepared similarly. From 1.34 g. (0.0066 mole) 4-thiazolylmethoxyamine dihydrochloride and 0.83 g. (0.0066 mole) cyclohexyl isocyanate were obtained 0.67 g. (39%) of product, m.p. 76°. An analytical sample was obtained by three recrystallizations from anhydrous ether.

1-Phenyl-3-(4-thiazolylmethoxy)thiourea (No. 5)—In a similar manner, 1.02 g. (0.005 mole) of 4-thiazolylmethoxyamine dihydrochloride and 1.08 g. (0.6 ml., 0.005 mole) phenyl isothiocyanate gave, after recrystallization from benzene, 0.6 g. (45%) of cream-colored solid, m.p. 119°.

REFERENCES

(1) B. Stearns, K. A. Losee, and J. Bernstein, *J. Med. Chem.*, **6**, 201(1963).

(2) W. G. Thurman, C. Bloedow, C. D. Howe, W. C. Lewin, P. Davis, M. Lane, M. P. Sullivan, and K. M. Griffith, *Cancer Chemotherapy Rept.*, **29**, 103(1963).

(3) R. H. Adamson, *Proc. Soc. Exptl. Biol. Med.*, **119**, 456 (1965).

(4) B. J. Kennedy and J. W. Yarbro, *J. Am. Med. Assoc.*, **195**, 1038(1966).

(5) L. Nathanson and T. C. Hall, *Cancer Chemotherapy Rept.*, **51**, 503(1967).

(6) Anonymous, *Drug Topics*, February 19, 1968.

(7) G. H. Hamor, D. Aures, and W. G. Clark, to be published.

(8) D. Mackay, P. B. Marshall, and J. F. Riley, *J. Physiol.*, **153**, 31(1960).

(9) R. Håkanson, *Experientia*, **17**, 402(1961).

(10) G. Kahlson, *Ann. Rev. Pharmacol.*, **5**, 305(1965).

(11) D. M. Shepherd and D. Mackay, in "Progress in Medicinal Chemistry," vol. 5, G. P. Ellis and G. B. West, Eds., Plenum Press, New York, N. Y., 1967, p. 240.

(12) V. J. Bauer and H. P. Dalalian, *J. Med. Chem.*, **8**, 886(1965).

(13) *Cancer Chemotherapy Reports, No. 25*, "Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems," CCNSC, U. S. Department of Health, Education, and Welfare, Washington, D. C., December 1962.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 5, 1969, from the *School of Pharmacy, University of Southern California, Los Angeles, CA 90007*

Accepted October 8, 1969.

Abstracted from a thesis submitted by A. Hudaverdi to the Graduate School, University of Southern California, Los Angeles, in partial fulfillment of Master of Science degree requirements.

Supported in part by an Institutional General Research Cancer grant from the American Cancer Society.

* Present address: Laboratory Procedures, Division of The Upjohn Company, Woodland Hills, CA 91364

Effect of Charge Shielding by Nonpolar Groups on the Partitioning of Quaternized Amines

HOWARD L. JOHNSON, PANAYOTIS TSAKOTELLIS, and JOSEPH I. DeGRAW

Abstract □ The methiodide salts of a number of tertiary organic bases were examined for their distribution properties between chloroform and water. An attempt was made to demonstrate whether the screening of ionic charge by nonpolar groups would substantially increase the lipid solubility of the methiodides. Comparison of a series of 2,6- *versus* 3,5-methyl-substituted *N*-methylpiperidine methiodides revealed little difference in their partition coefficients. Syntheses are described for some previously unreported substituted piperidines.

Keyphrases □ Amines, quaternized—partitioning □ Charge shielding, nonpolar groups, effect—quaternized amine partitioning □ Partition coefficients—quaternized amines □ Tertiary bases—synthesis

In recent years considerable effort has been directed toward the development and study of drugs which affect the central nervous system (CNS). Both qualitative and quantitative aspects of central *versus* peripheral activity depend not only upon the "intrinsic activity" and "receptor affinity" of a given drug, but also upon its rate and extent of distribution to specific sites within the CNS. Although knowledge is still incomplete, numerous studies have served to emphasize the lipid nature of the blood-brain barrier membrane. Two physicochemical parameters become of prime importance in determining rate and degree of penetration of this barrier by a drug (1). These are the lipid/water partition coefficient (K) and the degree of ionization at physiological pH with rate of penetration being directly related to the former and inversely to the latter. Therefore it is generally assumed that there is little or no penetration by charged quaternary ammonium compounds. This is supported by pharmacological experience in that such compounds generally do not elicit effects attributable to central actions.

In the initial phase of a program designed to evaluate the membrane permeability of quaternary ammonium compounds, the effect of structural variations in some quaternized bases have been investigated with regard to the partition coefficient in chloroform–water. Specifically, an attempt has been made to assess the importance of the steric relationship of hydrophobic, charge-insulating moieties to the locus of positive coulombic charge in a series of methyl-substituted piperidine methiodides. In addition, a few other quaternary salts of similar hydrocarbon content, but of a caged nature, were evaluated. The physical data and partition coefficients for all of the methiodides are listed in Table I.

It can be seen from the data in Table I that the major factor which determined the ability of the quaternary compounds to partition more favorably into the organic phase was the amount of hydrocarbon residue. If one allows that a change of about tenfold in the K value is significant, then the compounds of similar hydrocarbon content are not sufficiently different from one another to construct a case for enhancement of partitioning due to charge shielding. Indeed, the results seem to be to the contrary when one compares the 2,6-dimethyl *versus* 3,5-dimethylpiperidines (6 *versus* 7; 10 *versus* 11) and the 2,2,6,6-tetramethyl *versus* 3,3,5,5-tetramethylpiperidines (8 *versus* 9; 12 *versus* 13). These comparisons are between compounds of equal hydrocarbon content, but with differences in proximity of the methyls to the coulombic charge. In each case the more distant 3,5-substituted methiodides have a K about equal to or greater than the corresponding 2,6-substituted compounds. The authors had originally intended to study only the acetoxy amine methiodides but were suspicious that the acetoxy groups were somehow distorting the results. However, comparison of the simple piperidine methiodides (10–13) did not alter their conclusions based on the acetoxy compounds.

It would be premature to say that these results concerning partition coefficient could be extrapolated to a biological situation involving membrane permeability. However, the speculation of Friess *et al.* (2) to explain the similarity in functional acceptance of a quaternary and tertiary amine in the tropine series would seem to be affected by the authors' data. Their hypothesis that the "tropine ring structure may obscure the classic differentiation between quaternary and tertiary amine structure in terms of preferential penetration to and adsorption at certain peripheral and central chemoreceptor surfaces" does not seem to be tenable when based on the concept of charge shielding. Such shielding, as expressed in partition coefficients, is similar in most of the compounds and is certainly not uniquely enhanced for the tropine esters. Again, one must be cautious in this interpretation when comparing chemical *versus* biological systems. The authors hope to report on the degree and rate of penetration of the quaternary compounds through the blood-brain barrier as the second phase of this work.

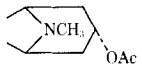
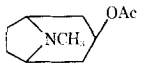
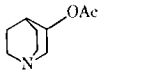
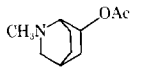
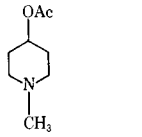
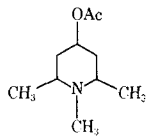
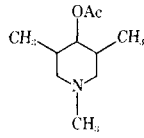
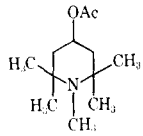
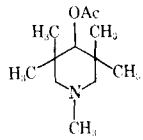
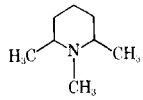
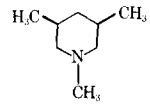
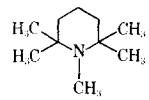
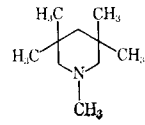
CHEMISTRY

With the exception of the 1,3,3,5,5-pentamethylpiperidine compounds, synthesis of the various tertiary bases proceeded in a straightforward manner. All of the bases were liquids and characterization was achieved *via* methiodide or picrate salts. The Reformatsky reaction of ethyl formate, amalgamated zinc, and two equivalents of ethyl 2-bromoisobutyrate afforded diethyl 3-hydroxy-2,2,4,4-tetramethylglutarate in a 75% yield. Acid hydrolysis yielded the glutaric acid (3) which was converted to the *N*-methyl-4-acetoxy-3,3,5,5-tetramethylglutarimide by the general procedure of Hall (4). Reduction of the acetoxy imide with diborane in tetrahydrofuran afforded 1,3,3,5,5-pentamethyl-4-piperidinol. 1,3,3,5,5-Pentamethylpiperidine was also prepared by Hall's procedure.

EXPERIMENTAL

Melting points are uncorrected and were taken in evacuated capillaries in the case of the methiodide salts.

Table I—Physical Data for Tertiary Amines and Methiodides

No.	Amines: Structure	Amines: B.p., °C. /mm.Hg	Methiodides: M.p., °C.	Methiodides: Formula	Methiodides: Calcd.	Methiodides: Found	$K \times 10^4$ ($CHCl_3/H_2O$)
1		64/0.65	279–280	$C_{11}H_{20}INO_2$	C, 40.6 H, 6.20 N, 4.31	C, 40.8 H, 6.28 N, 4.31	2.3, 1.8
2		60/0.65	189–190	$C_{11}H_{20}INO_2$	C, 40.6 H, 6.20 N, 4.31	C, 40.8 H, 6.22 N, 4.16	8.1
3		70/0.5	163–164	$C_{10}H_{18}INO_2$	C, 38.7 H, 5.84 N, 4.50	C, 38.7 H, 5.85 N, 4.31	2.0, 2.3
4		(Ref. 5)		$C_{11}H_{20}INO_2$			6.3
5		35/0.65	166–167	$C_9H_{18}INO_2$	C, 36.1 H, 6.07 N, 4.68	C, 36.1 H, 6.12 N, 4.66	1.5, 2.0
6		48/0.4	160–161.5	$C_{11}H_{22}INO_2$	C, 40.4 H, 6.77 N, 4.28	C, 40.5 H, 6.65 N, 4.42	5.3
7		52/0.75	210–211	$C_{11}H_{22}INO_2$	C, 40.4 H, 6.77 N, 4.28	C, 40.4 H, 6.84 N, 4.40	8.1
8		70/0.3	170–172	$C_{13}H_{26}INO_2$	C, 44.0 H, 7.37 N, 3.94	C, 43.9 H, 7.28 N, 3.98	14.0
9			260–263	$C_{13}H_{26}INO_2$	C, 44.0 H, 7.37 N, 3.94	C, 44.5 H, 7.40 N, 4.01	99.0
10		(Ref. 6, 7)		$C_9H_{20}IN$			9.0
11			280–281.5	$C_9H_{20}IN$	C, 40.2 H, 7.49 N, 5.20	C, 40.4 H, 7.61 N, 5.14	11.6
12		119/116	203–204	$C_{11}H_{24}IN$	C, 44.5 H, 8.14 N, 4.71	C, 44.6 H, 8.39 N, 4.41	20.0
13		80/65	310	$C_{11}H_{24}IN$	C, 44.5 H, 8.14 N, 4.71	C, 44.2 H, 8.05 N, 4.79	30.0

1,3,5-Trimethyl-4-piperidinol—A mixture of 0.30 g. of lithium aluminum hydride, 1.0 g. of 1,3,5-trimethyl-4-piperidone hydrochloride (8) and 20 ml. of dioxane was stirred at reflux for 5 hr. The solvent was removed *in vacuo* and the residue was suspended in ether and decomposed with ice water. The ether was decanted from the aqueous sludge and three additional ether extractions were made. The ether was dried over magnesium sulfate and evaporated to leave 0.60 g. (74%) of syrup; λ^{film} 3.0 μ (OH), no ketone remained at 5.8 μ. Vapor-phase chromatography showed a single peak. The oil was characterized as the methiodide salt, m.p. 226–230°.

Anal.—Calcd. for $C_9H_{20}INO$: C, 37.9; H, 7.08; N, 4.91. Found: C, 38.0; H, 6.99; N, 5.01.

1,2,2,6,6-Pentamethyl-4-piperidinol—To a solution of 9.2 g. (0.059 mole) of 2,2,6,6-tetramethyl-4-piperidone (free base) in 55

ml. of toluene was added 3.0 ml. (0.025 mole) of methyl chloroformate and the mixture was stirred at reflux for 45 hr. The toluene solution was washed with dilute acid, dried over magnesium sulfate, and evaporated *in vacuo* to leave 3.15 g. (59%) of the *N*-carbomethoxy compound as a syrup; λ^{film} 5.80 μ (ketone, C=O), 5.92 (urethan, C=O).

A mixture of 3.1 g. of the crude urethan, 3.1 g. of lithium aluminum hydride, and 50 ml. of dioxane was stirred at reflux for 30 hr. The mixture was cooled in ice and cautiously decomposed with ice water. The dioxane was removed *in vacuo* and the white, pasty residue was extracted with several portions of ether. The ether was dried over magnesium sulfate and evaporated to leave 2.1 g. (84%) of the pentamethyl alcohol as a viscous syrup; λ^{film} 3.0 μ (OH), no carbonyl remained. Picrate, m.p. 244–246° (from ethanol).

Anal.—Calcd. for $C_{16}H_{24}N_4O_8$: C, 48.0; H, 6.04; N, 14.0. Found: C, 48.0; H, 5.95; N, 14.0.

3-Hydroxy-2,2,4,4-tetramethylglutaric Acid—A stirred mixture of 13.3 ml. (0.165 mole) of ethyl formate, 39 g. (0.60 g. atom) of 20-mesh zinc amalgam, 30 g. of ethyl 2-bromoisobutyrate, and a catalytic amount of iodine was heated to boiling. Reaction commenced immediately and an additional 64.7 g. (total of 0.49 mole) of bromo ester was added dropwise over 15 min. The mixture was refluxed for 4 hr., cooled in ice, and decomposed by the addition of 250 ml. of 3 *N* hydrochloric acid. The benzene layer was separated and the aqueous portion extracted twice with 150-ml. portions of benzene. The combined benzene extracts were washed with water, dried over magnesium sulfate, and evaporated *in vacuo*. Distillation of the residual liquid through a short Vigreux condenser afforded 31.5 g. (75%) of diethyl 3-hydroxy-2,2,4,4-tetramethylglutarate at b.p. 86–93° (0.35 mm.).

Hydrolysis of the ester (30.4 g.) was accomplished by refluxing with 620 ml. of 6 *N* hydrochloric acid for 6 hr. The solution was chilled to afford 14.1 g. (60%) of white crystals, m.p. 170–171°; lit. (3) m.p. 169–170°.

***N*-Methyl-4-hydroxy-3,3,5,5-tetramethylglutarimide**—The hydroxy diacid above (14.1 g.) was refluxed for 16 hr. with 113 ml. of acetyl chloride, followed by evaporation of the reagent and distillation of the residue under vacuum (100° at 0.2 mm.) to afford 15.6 g. (98%) of 4-acetoxy-3,3,5,5-tetramethylglutaric anhydride. This material was immediately added to an ice-cold mixture of 60 ml. of 40% methylamine and 100 ml. of 75% acetone (4). After 20 min. the acetone was removed and the aqueous residue was acidified and extracted with chloroform to give 17.0 g. of a syrup regarded as the 4-acetoxy-3,3,5,5-tetramethylglutaric acid mono *N*-methylamide. The amino acid (16 g.) was treated with 25 ml. of acetic anhydride and 75 ml. of pyridine at reflux for 5 min. followed by standing at room temperature for 40 hr. The solvent was removed *in vacuo* and the residue treated with ice water and concentrated hydrochloric acid to precipitate *N*-methyl-4-acetoxy-3,3,5,5-tetramethylglutarimide (12.6 g., 85%). Recrystallization of a portion from water afforded white crystals, m.p. 53–55°.

Anal.—Calcd. for $C_{12}H_{19}NO_4$: C, 59.7; H, 7.94; N, 5.81. Found: C, 59.7; H, 7.82; N, 5.58.

1,3,3,5,5-Pentamethyl-4-piperidinol—A mixture of 5.0 g. of the imide, 40 ml. of tetrahydrofuran, and 60 ml. of 1 *M* diborane in tetrahydrofuran was refluxed for 3 hr. An equal charge of diborane solution was added and refluxing was continued for another 3 hr. The solution was cooled in ice and treated with 1 ml. of water and a few drops of 6 *N* hydrochloric acid. A violent reaction caused the loss of approximately one-half of the contents of the flask. The remainder was evaporated *in vacuo* and the residue was treated with 40 ml. of water and 6 *N* hydrochloric acid until strongly acidic. The resulting precipitate was extracted into chloroform, which was dried and evaporated to leave a white crystalline residue which possessed strong B-H bands in the IR at 4.2–4.4 μ. The complex was decomposed by heating with 50 ml. of 2 *N* hydrochloric acid for 15 hr. The resulting solution was made strongly alkaline with 10% sodium hydroxide and extracted with dichloromethane. The extract was dried over magnesium sulfate and evaporated *in vacuo* to leave 1.1 g. of liquid which was directly acetylated and characterized as the methiodide salt.

1,3,5-Trimethylpiperidine—A solution of 9.1 g. of 1,3,5-trimethyl-pyridinium iodide (9) in 100 ml. of 80% methanol was passed through a column of 90 g. of ion-exchange resin [Dowex 2 (chloride)]. The column was further eluted with 80% methanol and the solvent then evaporated *in vacuo* to leave 5.2 g. (91%) of the methochloride salt. The material (4.7 g.) was hydrogenated over 1.0 g. of platinum oxide in glacial acetic acid at 3 atm. After 20 hr. the calculated amount of gas was consumed. The catalyst was removed and the solvent evaporated *in vacuo*. The residue was alkalized with 10% sodium hydroxide and the base was extracted into dichloromethane. Since the amine tended to form a carbonate salt rapidly, it was directly converted to the methiodide salt.

Acetate Esters—The appropriate alcohol, as the free base, was mixed with excess acetic anhydride and the solution was heated 3–5 hr. on the steam bath. Pyridine was added in the case of hydrochloride salts. Excess reagent was removed *in vacuo* and the residue was partitioned between chloroform and 10% potassium carbonate. The chloroform extract was dried over magnesium sulfate, evaporated, and the residual liquid distilled *in vacuo* through a short path apparatus; yields were 50–70%. The boiling points of the bases are listed in Table I. Tropinol, pseudo-tropinol, 3-hydroxyquinuclidine hydrochloride, and 1-methyl-4-piperidinol were obtained from commercial sources; 1,2,6-trimethyl-4-piperidinol hydrochloride (β-epimer, m.p. 266°) was prepared by the hydrogenation of 1,2,6-trimethyl-4-pyridone (10).

Methiodide Salts—An ethereal or acetone solution of the appropriate tertiary base was treated with excess methyl iodide and allowed to stand for 24 hr. For the hindered bases, no cosolvent was used and reaction times were extended to 2 days in the case of 1,2,6-trimethyl-4-acetoxy piperidine and 7 days for 1,2,2,6,6-pentamethyl-4-acetoxypiperidine; 1,2,2,6,6-pentamethylpiperidine was refluxed 20 hr. in methyl iodide solution. The salts were recrystallized from absolute ethanol for analysis. Physical data are listed in Table I. 1,2,2,6,6-Pentamethylpiperidine was prepared by the method of Laboratorios Bonaplata, S.A. (11) and 1,3,3,5-5-pentamethylpiperidine by the procedure of Hall (4).

Determination of Partition Coefficients—Values of *K* were estimated from the distribution of quaternary salts between chloroform and water (10:1 volume ratio). Compounds were dissolved in the aqueous phase (2.0 mg./ml.) and partitioned with shaking over a period of 0.5 hr. The material in the chloroform phase was extracted into water and the quantity determined colorimetrically by the bromophenol blue method of Mitchell and Clark (12). A standard curve was determined for each compound.

REFERENCES

(1) A. Herz, H. Teschemacher, A. Hofstetter, and H. Kurz, *Intern. J. Neuropharmacol.*, **4**, 207(1965).

(2) S. L. Friess, R. C. Durant, and H. L. Martin, *Toxicol. Appl. Pharmacol.*, **9**, 240(1966).

(3) E. Blaise, *Compt. Rend.*, **126**, 1808(1898).

(4) H. K. Hall, Jr., *J. Org. Chem.*, **29**, 3135(1964).

(5) J. I. DeGraw and J. G. Kennedy, *J. Hetero. Chem.*, **4**, 251 (1967).

(6) K. Tsuda, *J. Pharm. Soc. Japan*, **56**, 359(1936).

(7) C. Mannich, *Arch. Pharm.*, **272**, 323(1934).

(8) *Ibid.*, **255**, 261(1917).

(9) J. A. Elvidge and L. M. Jackman, *J. Chem. Soc.*, **1961**, 859.

(10) I. N. Nazorov and O. I. Sorokin, *Izvest. Akad. Nauk SSSR* **1960**, 872; through *Chem. Abstr.*, **54**, 24720(1960).

(11) Spanish pat. 246,599, Laboratorios Bonaplata, S. A., Feb. 20, 1959; through *Chem. Abstr.*, **55**, 19955(1961).

(12) R. Mitchell and B. B. Clark, *Proc. Soc. Exptl. Biol. Med.*, **81**, 105(1952).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 18, 1969, from the *Stanford Research Institute, Department of Pharmaceutical Chemistry, Menlo Park, CA 94025*

Accepted for publication October 7, 1969.

Hydrophobic Self-Association of *d*-Propoxyphene Hydrochloride and the Effect of Urea: A Nuclear Magnetic Resonance Study

Keyphrases □ *d*-Propoxyphene HCl—hydrophobic self-association □ Urea effect—*d*-propoxyphene HCl self-association □ NMR spectroscopy—molecular interaction

Sir:

d-Propoxyphene is an important nonnarcotic analgesic agent. In a previous study from our laboratories, Conine has reported that aqueous solutions of *d*-propoxyphene hydrochloride[1] possess the ability to solubilize sparingly water-soluble organic acids (1). Conine's data have strong parallels to the phenomenon of micellar solubilization; he observed an apparent solute critical micelle concentration (SCMC). The amount of organic acid solubilized per mole of *d*-propoxyphene hydrochloride increased appreciably once the SCMC had been exceeded. Formation of aggregates of some type by propoxyphene ions was suggested but no direct evidence for this phenomenon was presented. Other reports (2, 3) in the literature also show that pharmacologically active amines form aggregates in aqueous solutions of their salts. It was of interest to us to develop a more definitive understanding of the aggregation phenomenon in aqueous *d*-propoxyphene hydrochloride solutions.

Nuclear magnetic resonance (NMR) spectroscopy can provide much detailed information about molecular interactions. In recent years NMR has been utilized for this purpose in such diverse areas as micelle formation (4), micellar solubilization (5), drug-protein interactions (6), and enzyme-substrate interactions (7). Applications of this versatile tool to research problems in physical pharmacy are not well documented in the literature. In this communication we wish to describe the findings of a preliminary NMR spectroscopic study of the self-association of *d*-propoxyphene hydrochloride in aqueous solutions.

The effect of concentration on the high resolution NMR spectrum of *d*-propoxyphene hydrochloride in D_2O is illustrated in Fig. 1. Assignments of the various signals, based on a first-order analysis, are also indicated in the figure.[2] As the concentration is increased, the signals of the aromatic and C-1 methylene protons are the most strongly affected. The fine structure in the aromatic region, apparent at low concentrations, is gradually lost as the solute concentration is increased.

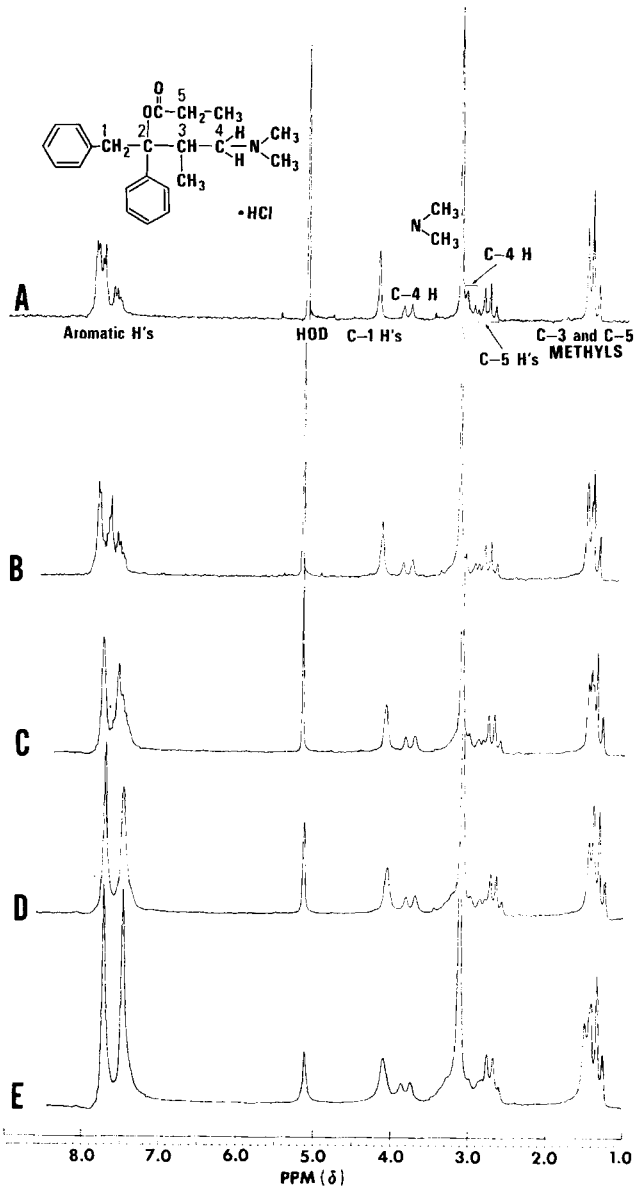


Figure 1—*Effect of concentration on 100-Mc.p.s. NMR spectrum of* d*-propoxyphene hydrochloride in D_2O at 30°. The scale is referred to external TMS which was used as the source of a lock signal. Key:* A, *0.1* M; B, *0.2* M; C, *0.3* M; D, *0.5* M; and E, *1.0* M.

In a 1 *M* solution, two rather broad singlets appear for the aromatic protons. The signal at 4.22 p.p.m. due to the C-1 methylene group is also appreciably broadened as indicated by its linewidth at half-height. The width of the sharp singlet due to the dimethylamino protons appears to be the least affected.

In addition to line broadening, all the signals move upfield as the concentration is increased; however, those due to the aromatic and the C-1 methylene protons undergo the most pronounced upfield shifts. It thus appears that aggregation in aqueous *d*-propoxyphene

[1] Marketed as Darvon by Eli Lilly and Co.
[2] The C-3 proton multiplet coincides with the *N*-methyl singlet and therefore it cannot be observed directly.

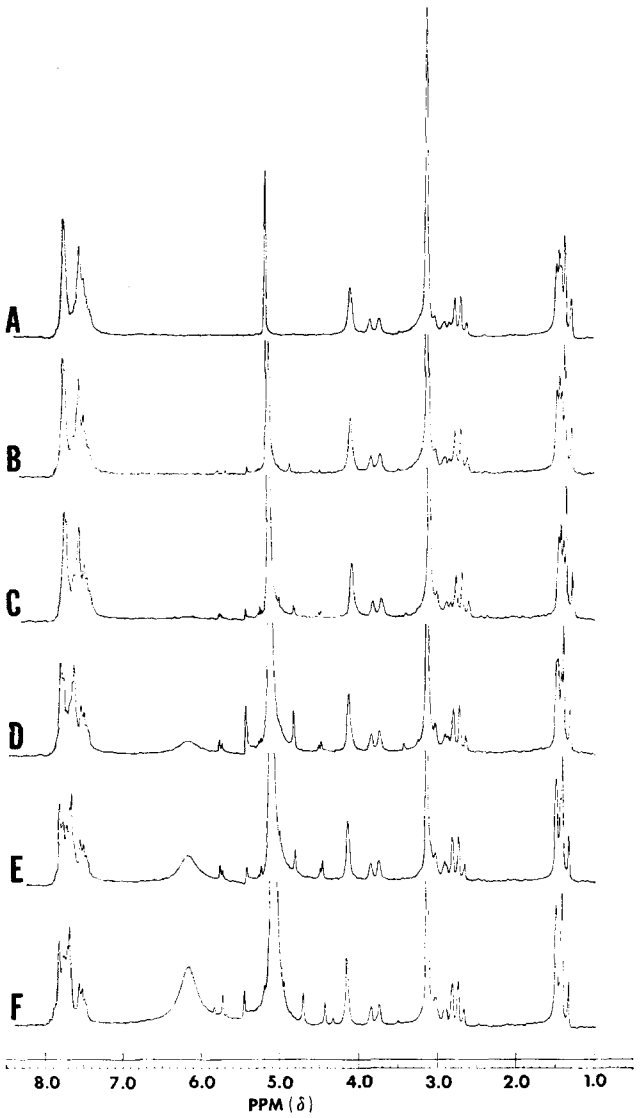


Figure 2—*Effect of urea on 100-Mc.p.s. NMR spectrum of 0.3* M d-*propoxyphene hydrochloride in D_2O at 30°. The scale is referred to external TMS which was used as the source of a lock signal. The peaks symmetrically placed on either side of the HOD peak are spinning sidebands. The rather broad peak at 6.16 p.p.m. is due to the unexchanged amide protons of urea. Key:* A, *0.0* M *urea;* B, *0.5* M *urea;* C, *1.0* M *urea;* D, *2.0* M *urea;* E, *3.0* M *urea;* F, *4.0* M *urea.*

hydrochloride solution takes place primarily through hydrophobic interactions of the aromatic systems. Further evidence supporting the proposed hydrophobic nature of this self-association comes from the fact that the concentration effects are absent in acetone and methanol solutions. It has been shown before that hydrophobic aggregation does not take place in these solvents (8, 9).

One of the factors causing such hydrophobic bonding would be the highly ordered water structure around the nonpolar moieties of the solute molecule. This ordered, cooperative, or iceberg-like structure of water may be altered by the addition of urea which has a strong potential to interact with water. There appears to be some controversy about the effect of urea on water structure as to whether it acts as a structure breaker or as a structure maker (10); however, the hydrophobic bond-weakening effect of urea is generally recognized.

Increasing the addition of urea to *d*-propoxyphene hydrochloride solutions (at a given concentration) should weaken the hydrophobic interactions of the aromatic moieties. This predicted effect was observed when the NMR spectra of a 0.3 *M d*-propoxyphene hydrochloride solution were examined (Fig. 2). This particular concentration was chosen to demonstrate the effect of urea since it appeared, from chemical shift *versus* concentration plots, to be just above the concentration at which aggregation is complete. Weakening of hydrophobic bonding can be seen by the progressive reappearance of the fine structure of aromatic multiplets and by the increasing sharpness of C-1 methylene proton singlet as the urea concentration is increased from 0.5–4 *M*. The protons which are responsible for the intermolecular hydrophobic interactions also show the most significant downfield shifts indicating that as hydrophobic bonds are weakened, solute structure becomes less ordered. To our knowledge this is the first direct NMR demonstration of the hydrophobic bond-weakening effect of urea.

We are, at present, studying in detail the effect of hydrophobic self-association on chemical shifts and spin-spin relaxation rates. We are also examining, by NMR spectroscopy, aqueous *d*-propoxyphene hydrochloride solutions containing solubilized aromatic molecules. Results of these studies will be reported in future, more complete papers.

(1) J. W. Conine, *J. Pharm. Sci.*, **54,** 1580 (1965).
(2) B. Farhadieh, N. A. Hall, and E. R. Hammarlund, *ibid.*, **56,** 18(1967).
(3) R. D. Johnson, F. M. Goyan, and L. D. Tuck, *ibid.*, **54,** 1176(1965).
(4) J. F. Yan and M. B. Palmer, *J. Colloid Sci.*, **30,** 177(1969); H. Inoue and T. Nakagawa, *J. Phys. Chem.*, **70,** 1108(1966).
(5) J. C. Eriksson and G. Gillberg, *Acta Chem. Scand.*, **20,** 2019(1966); T. Nakagawa and K. Tori, *Kolloid-Z.*, **194,** 143(1964).
(6) J. S. Cohen, *J. Clin. Pharmacol.*, **9,** 72(1969), J. J. Fischer and O. Jardetzky, *J. Am. Chem. Soc.*, **87,** 3237(1965); O. Jardetzky and N. G. Wade-Jardetzky, *Mol. Pharmacol.*, **1,** 214(1965).
(7) M. A. Raftery, F. W. Dahlquist, S. M. Parsons, and R. G. Wolcott, *Proc. Natl. Acad. Sci.*, U. S., **62,** 44(1969).
(8) D. M. Small, S. A. Penkett, and D. Chapman, *Biochim. Biophys. Acta*, **176,** 178(1969).
(9) E. S. Hand and T. Cohen, *J. Am. Chem. Soc.*, **87,** 133(1965).
(10) S. Subramanian, D. Balasubramanian, and J. C. Ahluwalia, *J. Phys. Chem.*, **73,** 266(1969); M. Abu-Hamidayyah, *ibid.*, **69,** 2720(1965), and references cited therein.

A. L. Thakkar
W. L. Wilham
P. V. DeMarco

The Lilly Research Laboratories
Eli Lilly and Company
Indianapolis, IN 46206

Received June 30, 1969.
Accepted for publication September 19, 1969.

We wish to express our appreciation to Dr. Harold E. Boaz for his interest in this work.

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

MARCH 1970

VOLUME 59 NUMBER 3

REVIEW ARTICLE

Plasmalogens and Related Derivatives: Their Chemistry and Metabolism

CLAUDE PIANTADOSI and FRED SNYDER

Keyphrases ☐ Plasmalogens, related derivatives—chemistry, metabolism, review ☐ Chemistry, plasmalogens—related derivatives ☐ Metabolism—plasmalogens, related derivatives ☐ Biosynthesis—plasmalogens ☐ Enzymatic degradation—plasmalogens

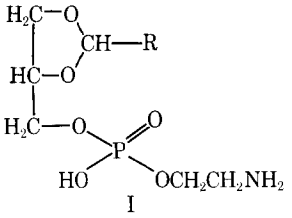
CONTENTS

Plasmalogens are a naturally occurring group of aldehydogenic phosphoglycerolipids widely distributed in plants, animals, and microorganisms but detected in highest concentration in the heart, skeletal muscle, and the myelin of the brain and nerve. Low concentrations of nonphosphatide or "neutral plasmalogens" have also been detected in mammalian tissue as well as in the tissues of different fish. A number of reviews are available on the biochemistry (1–5) and the chemistry and analyses (6–8) of plasmalogens and other ether-linked lipids, and the general reactivity of *O*-alk-1-enyl groupings in organic compounds has just recently been summarized (8).

The presence of plasmalogens in tissues was first noted in 1924 by Feulgen and Rossenbeck (9) in their histochemical studies on the cell nucleus with fuchsin-sulfurous acid. This dye was found to produce a violet stain on both the nuclear and cytoplasmic portions of the cell after mild acid hydrolysis. Because these workers were able to extract the violet color with lipid solvents, they believed that the positive reaction produced in the cytoplasm was due to the presence of lipids rather than sugar aldehydes. Further, when the staining compound was subjected to treatment with acids or mercuric chloride, it liberated a long-chain fatty aldehyde. The cytoplasmic staining was referred to as the plasmal reaction. Feulgen and Voit (10) coined the name "plasmalogen" for the lipoid precursor compound and "plasmal" for the product liberated. The

plasmals appeared to be a mixture of hexadecanal and octadecanal, although other aldehydes might have been present. To demonstrate the presence of plasmals during histochemical studies on tissues, the tissues had to be hydrolyzed in the presence of either HCl or mercuric chloride. Without hydrolysis, Feulgen found that Schiff's test for aldehydes with sulfurous acid and fuchsin dye gave a slow positive reaction with the cytoplasmic lipid-like precursor.

In 1939, Feulgen and Bersin (11) were able to isolate a pure aldehydogenic phospholipid from horse muscle after vigorous alkaline hydrolysis of the ester phosphatides. Apparently, the aliphatic aldehyde formed a cyclic acetal bond with the glycerol skeleton, and the phosphorus and nitrogen base (ethanolamine) was attached by ester bonding to the third hydroxyl group in glycerol. This crystalline ethanolamine plasmalogen (I) was designated "acetal phospholipide." In 1951,

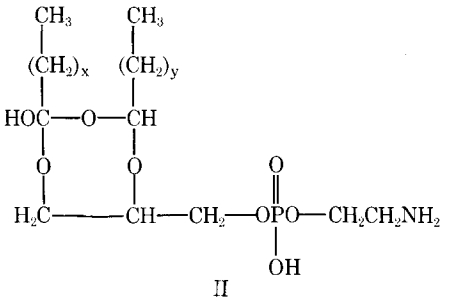


I

R = long-chain alkyl group

Thannhauser *et al.* (12) published a series of papers on the isolation of a crystalline ethanolamine plasmalogen from brain tissue. They used a more prolonged but more gentle alkaline hydrolysis than Feulgen and Bersin (11) had described earlier. The catalytic hydrolysis of their acetal phospholipid was carried out with mercuric chloride with the formation of long-chain fatty aldehydes (hexadecanal and octadecanal) and glycerylphosphorylethanolamine. By periodate titration they demonstrated that the glycerylphosphorylethanolamine derived from the brain acetal phospholipid belonged to the L-α structure.

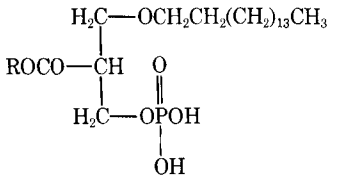
The acetal structure served as a prototype for this class of lipids in earlier years, but its chemical structure was questioned and several alternatives were proposed. Doubt was cast on the correctness of Structure I when it failed to explain the great differences in the reaction rates between synthetically prepared glyceryl acetals and lipid extracts with fuchsin-sulfurous acid. Anchel and Waelsch in 1944 (13) observed that at low temperatures their lipid extracts gave a full color development while the synthetic acetals failed to produce a color; this observation suggested that the aldehyde linkage in the native compound may be more labile than that represented by Structure I. The observations of Schmidt *et al.* (14) also suggested that the native plasmalogens probably contained an alkali-labile residue, because upon hydrolysis of crude brain lipid extracts by the fuchsin-sulfurous acid reagent, no acid-soluble phosphate (glycerylphosphorylethanolamine) was obtained as one would expect from Structure I. However, prior treatment of the crude brain lipid extract with base and then subsequent acid hydrolysis did result in the correct amount of acid-soluble glycerylphosphorylethanolamine. On the basis of these experiments, they assumed that the lipid acetals were derived from other lipid groups during analyses. It was suggested that plasmalogens normally obtained may be artifacts and that rupture of the alkali-labile linkages by which the original compound is bound to other lipid groups occurs during the isolation procedure. Assuming that the native plasmalogens were represented by Structure I, Baer and Stancer (15) proposed Structure II to explain the observations of Schmidt *et al.* (14).



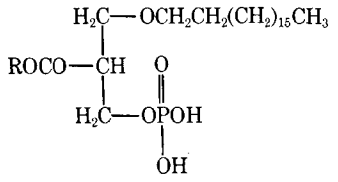
II

The removal of a fatty acid by alkali from Structure II should yield the hemiacetal structure that then could undergo ring closure to form the cyclic acetal (I), and consequently only after pretreatment with alkali would Structure I be expected to give rise to glycerylphosphorylethanolamine when subjected to mild acid hydrolysis.

Further studies have provided strong evidence that the aldehydogenic residue in the native plasmalogens is an alk-1-enyl ether that is not altered by mild alkaline hydrolysis. In 1954, Rapport *et al.* (16) fractionated "acetal" phosphatides from bovine muscle by chromatographic separation on silicic acid. Upon analysis, they showed the presence of two fatty chains per atom of phosphorus, one of which was a fatty acid and the other a fatty aldehyde. Klenk and Debuch (17) found that catalytic reduction of the ethanolamine-plasmalogen led to a complete disappearance of an aldehydic reaction. Subsequent treatment of the reduced compound with base resulted in the isolation of a mixture of *O*-hexadecyl and *O*-octadecyl analogs of phosphatidic acid (III).



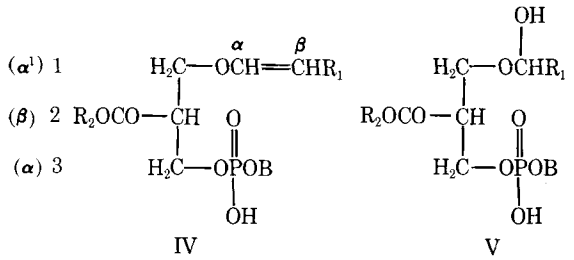
(a) Hexadecyl analog of phosphatidic acid



(b) Octadecyl analog of phosphatidic acid

III

Klenk and Debuch (17) did not investigate the linkage between the aldehyde and glycerol moieties, but they did propose three possible formulas (I, IV, and V) for the plasmalogens. Rapport *et al.* (18) firmly established the *O*-alk-1-enyl linkage in plasmalogens (IV).



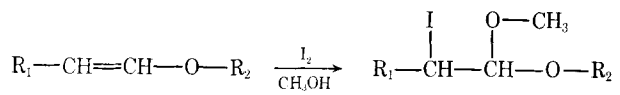
IV V

B = choline, ethanolamine, serine

CHEMISTRY

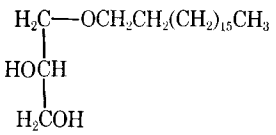
Proof of the Alk-1-enyl Structure—It is now known that the naturally occurring plasmalogens occur as glycerol derivatives of phosphorylcholine, phosphorylethanolamine, or phosphorylserine containing a fatty acid residue and an alk-1-enyl linkage (IV). The terms saturated or unsaturated (19*a*) have been used to refer to the presence or absence of an alk-1-enyl group adjacent to the *O*-ether linkage of the hydrocarbon chain attached to the 1-position of glycerol (1). The α,β-unsaturated glyceryl ethers (enol ethers and vinyl ethers) are alk-1-enyl glyceryl ethers, which are the structural components of plasmalogens; in the earlier literature, the alk-1-enyl types are often referred to as plasmalogens. "Phosphatid*al*" has been proposed to differentiate alk-1-enyl phosphoglycerides from the "phosphatid*yl*" diacyl analogs (19*b*).

The first definitive clue to the α,β-unsaturation of the aldehydogenic linkage in plasmalogens was provided by Rapport *et al.* (18) from their studies on lysophosphatid*al* ethanolamine. Alkaline hydrolysis of the ethanolamine-plasmalogen fraction in bovine muscle was used to form the "lyso" compound. The "lyso" derivative exhibited a loss of aldehyde reaction upon hydrogenation, generated one molecule of a long-chain fatty aldehyde, and contained an alk-1-enyl ether group adjacent to the oxygen. Further evidence confirming the structural analogy between α,β-unsaturated ethers and plasmalogens was obtained by Rapport and Franzl (20) on their lyso compound in 1957 by using a modification reported in 1948 by Siggia and Edsberg (21) for the quantitative determination of alk-1-enyl ethers. This method is based on the addition of iodine to alk-1-enyl ether bonds and it is specific for the α,β-unsaturated ethers in the presence of ordinary olefinic unsaturation, alcohols, aldehydes, acetals, or acetylene. An iodo acetal forms during the reaction between methanolic iodine and alk-1-enyl ethers. Other workers (22, 23) have confirmed the specificity of this iodometric procedure. The iodometric reaction was utilized by Rapport and Lerner (24) in conjunction with the procedure of



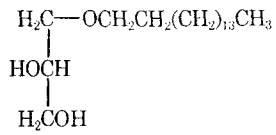
Wittenberg *et al.* (25) based on the formation of a *p*-nitrophenyl hydrazine to measure the plasmalogen content of several normal and neoplastic tissues. Further proof of the alk-1-enyl ether linkage was supplied by Blietz (26), who studied the effect of acid hydrolysis on tissue plasmalogens synthesized in the presence of tritium-labeled water; these experiments demonstrated that the original hemiacetal structure could be formed by the hydrolysis procedure itself (26). Furthermore, tritium-labeled aldehydes were isolated as the semicarbazone (26), which is not in accord with the proposed hemiacetal structure (V) but does support Structure IV.

Position of the Aldehydogenic Residue—The aldehydogenic chain in Structure IV is depicted in the α-position. The other possibility would be for the unsaturated ether chain to be attached to the β-position of the glycerol moiety. Opinions among the early investigators varied considerably with reference to the position of the aldehydogenic chain on the glycerol moiety. Initial studies determining the correct position of the aldehydogenic chain were carried out by Marinetti *et al.* (27, 28) on pig heart lipids. After catalytic reduction and alkali hydrolysis of total pig heart phosphatides, batyl alcohol (VI) (*O*-alkylglycerol) was isolated and indicated that the potential aldehyde group in the pig heart plasmalogens had to be attached to the α-carbon of glycerol. Debuch (29, 30) presented further



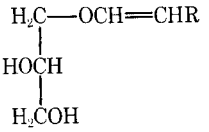
VI

chemical evidence confirming that the aldehydogenic chain is attached to the α-position in the glycerol moiety. This evidence was based on results of hydrogenation studies on brain ethanolamine plasmalogens. The mixture of batyl (VI) and chimyl alcohol (VII), isolated, provided further evidence that the chemical structure of the plasmalogen was a 1-(alk-1-enyl) ether of a 2-acyl-3-glyceryl phosphate (IV).



VII

A number of workers (31, 32) have studied the variations in the aldehydogenic chain by means of gas-liquid chromatography (GLC). A number of branched and unsaturated compounds were isolated in addition to normal C_{12}–C_{18} hydrocarbons. The aldehydogenic chain in Structure IV consists predominantly of 16:0, 18:0, and 18:1 alk-1-enyl chains; the fatty acids are almost completely unsaturated and the alk-1-enyl ether linkage possesses the *cis* configuration (29, 33–36). Norton *et al.* (35), working with pure choline plasmalogen from beef heart, and Warner and Lands (36), working with pig heart lecithin, obtained IR spectra that clearly indicated the configuration of the —OCH=CH— grouping in natural plasmalogens to be wholly *cis*. Enzymatic treatment of phosphorylcholine with phospholipase *C* and hydrolysis of the acyl groups with base resulted in alk-1-enyl glyceryl ether (VIII). There was no absorption in the 930 cm.$^{-1}$ region, which is characteristic of substituted alk-1-enyl ethers possessing a *trans* configuration (37, 38), but the IR spectrum

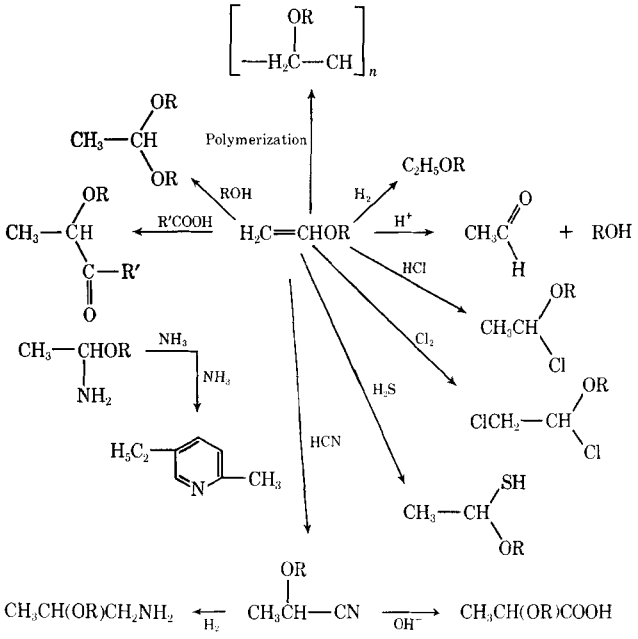


VIII

showed a maximum at 738 cm.$^{-1}$ that is characteristic of the *cis* configuration. Hydrogenation caused complete loss of absorption and resulted in the isolation of an *O*-alkylglycerol. Cymerman-Craig *et al.* (39), by means of optical rotatory dispersion, determined the absolute stereochemical configuration of *cis*-1-(alkenyl-1-enyloxy)-2,3-diacetate obtained from pig heart plasmalogen. The natural plasmalogen had the same configuration as natural chimyl (VII) and batyl alcohols (VI). These data supported the earlier experiments of Thannhauser *et al.* (12) which had established the L-α-structure for plasmalogens by the isolation of L-α-glycerylphosphorylethanolamine from their lyso compound.

Chemical Properties—Little information is available on the chemistry of long-chain α,β-unsaturated ethers, but the —OCH=CH— moiety occurs in other organic compounds on which there are more data available (8). The juxtaposition of the —OCH=CH— moiety relative to the polar glyceryl residue would probably create some chemical differences between the alk-1-enyl linkage as found in plasmalogens and other ethers. Alk-1-enyl ethers are more reactive than other olefinic compounds; they are known to undergo a number of addition reactions as is illustrated in Scheme I. Few of these reactions have been investigated with the native plasmalogens (2). It is known that plasmalogens are stable in an alkaline or neutral medium but very quickly decompose in the presence of trace amounts of acid to form long-chain fatty aldehydes and glycerol derivatives.

The double bond of alk-1-enyl ethers may add halogens, acids of halogens, halides, organic acids (40), alcohols, phenols, and naphthols, and thus gives rise to simple or mixed acetals (41). The addition of alcohols and carboxylic acids is catalyzed by anhydrous acids, such as hydrogen halides, and also by boron fluoride (42, 43). Catalytic amounts of basic compounds cause hydrogen sulfide and mercaptans to add to alk-1-enyl ethers; the addition of water produces a hemiacetal intermediate that forms the aldehyde. An alk-1-enyl transetherification, catalyzed by mercuric sulfate and reported to occur at a temperature range between -70 and $-10°$ (44), illustrates the reactivity of this ether linkage. The addition of iodine to alk-1-enyl ethers produces violent polymerization reactions as do heat and UV light (45). However, methanolic iodine produces a product that has been identified as unsymmetrical methylacetal (21) and which has been used as a sensitive and specific method for estimating plasmalogens and other alk-1-enyl ethers (2, 22). Alk-1-enyl ethers also have a characteristic absorption band in the IR at 8.3 μ (1200 cm.$^{-1}$) which does not appear in native plasmalogens (3, 46). The characteristic absorption band in the infrared of naturally occurring α,β-unsaturated ethers appears at 6.0 μ (1650 cm.$^{-1}$). The plasmalogens with their α,β-unsaturation behave in a manner similar to other alk-1-enyl ethers with reference

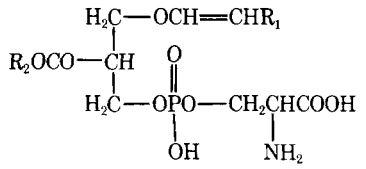


Scheme I
Reactivity of the O*-Alk-1-enyl Linkage in Organic Compounds (also see* Reference 8).

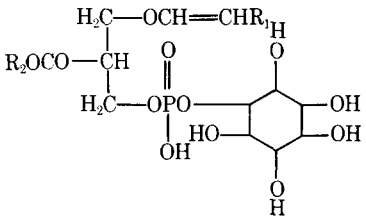
to their alkaline stability, hydrolysis with acids and mercuric ions, reaction with fuchsin-sulfurous acid, addition of hydrogen and methanolic iodine, ozonolysis, and reactions with aldehyde reagents (2, 3, 20).

Related Plasmalogen Structures—Klenk and Böhn (47) and Ansell and Norman (48) have described the isolation and characterization of alk-1-enyl serine phosphatides (IX) in brain tissue. The existence of an alk-1-enyl inositol phosphatide (X) has also been demonstrated (49). Marinetti *et al.* (28) have obtained evidence for the presence of small amounts of diether phosphatides in beef heart, and Marinetti has described reactions involving mild alkaline and acidic hydrolysis (50) of Structures XI, XII, and XIII.

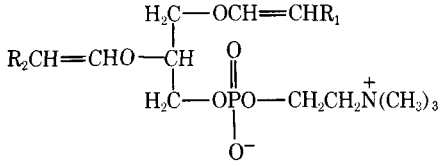
As has been pointed out in this review, the cyclic acetal phospholipid (I) isolated by Feulgen and Bersin (11) and Thannhauser *et al.* (12) from the alkaline hydrolysis of muscle and brain phospholipids is considered an artifact formed by acid hydrolysis of ethanolamine lysoplasmalogen; subsequent studies have proved that the cyclic compounds are mainly *cis*-1-(alk-1-enyl)-2-acylglycerylphosphoryl nitrogenous bases. Further evidence that the cyclic acetal is an artifact of isolation has been presented by Davenport and Dawson (51, 52), who studied the formation of cyclic acetals during the acid hydrolysis of lysoplasmalogens. Evidence was presented to indicate that the mild acid hydrolysis of alk-1-enyl ethers of glycerylphosphorylethanolamine, obtained from alkali treatment of natural plasmalogen, will cause cyclization. The IR spectra were identical with those of a synthetic 2-aminoethyl-2,3-*O*-hexadecylidene-1-glycerophosphate. Mercuric ions prevented this cyclization and liberated the fatty aldehyde. Pietruszko and Gray (53) reported similar results. Piantadosi *et al.* (54) synthesized alk-1-enyl glycerols and studied their conversion to the corresponding cyclic glycerol acetals. The conditions necessary for their



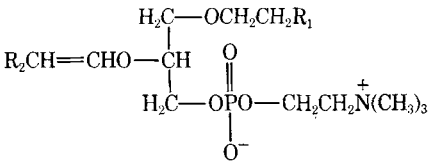
IX



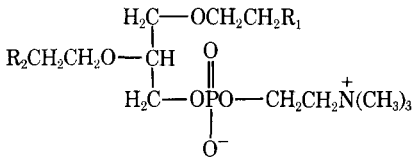
X



XI



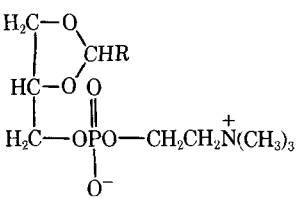
XII



XIII

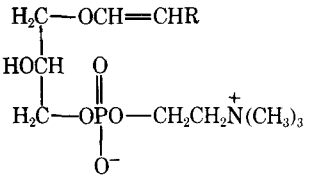
cyclization were studied and no cleavage of the alk-1-enyl linkage was observed. The compounds isolated from the cyclization reaction were identical to the synthetic cyclic glycerol acetals prepared by a different route.

Landowne and Bergmann (55) reported the isolation of a choline plasmalogen (XIV) (1,2-alkylidine glyceryl-3-phosphorylcholine) from the total lipids of the sea anemone, *Anthopleura elegantissima*. Identification of this compound was based on IR data and on the isolation of a long-chain fatty aldehyde on acid hydrolysis. The chemical synthesis of this type of compound (XIV) (R = C_{15}, *i.e.*, 1,2-*O*-hexadecylidene glyceryl-3-phosphorylcholine) has been reported (56). Cyclic acetals of the ethanolamine type (I) have also been established by synthesis (57–59*a*). Recently, Frosolono *et al.* (59*b*) reported a convenient method for the preparation of



XIV

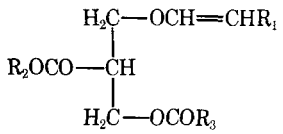
crystalline lysophosphatid*al* choline (XV) in good yields from beef heart lecithin. The plasmalogen was physically



XV

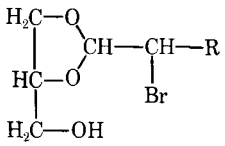
characterized and its chemical properties were compared with other α,β-unsaturated ethers of glycerol. Their (59*b*) evidence indicated that the choline plasmalogens (XIV) isolated from sea anemones were in reality Structure XV, and not a cyclic acetal of glycerol as originally proposed (55). All the evidence available indicates that the cyclic acetal structures probably do not make any significant contribution to the alk-1-enyl glyceryl ethers.

Neutral plasmalogens of nonphosphatide aldehydogenic lipids (XVI) have also been reported (60–62) and have been characterized as alk-1-enyl diacyl glycerols; this subject has been reviewed (5). Bergelson (63) has also presented an extensive review of neutral diol lipids and related compounds.



XVI

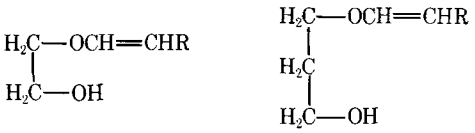
Chemical Synthesis—The synthesis of a *cis*-1-(alk-1-enyl) 2-acyl-3-glycerylphosphate constitutes a chemical problem beset with considerable practical difficulties. In 1961, Piantadosi and Hirsch (64, 65) began the first studies toward the chemical synthesis of plasmalogen. The initial step involved the synthesis of α-bromo cyclic acetal of glycerol, *i.e.*, 2-(1-bromodecyl)-4-hydroxymethyl-1,3-dioxolane (XVII) and its subsequent reaction with sodium (R = C_6–C_{11}). The method used in



XVII

preparing XVII was a transacetalation reaction (66) between glycerol and various 2-bromo-1,1-dimethoxyalkanes derived from long-chain aldehydes. The first step in the transacetalation reaction is acid-catalyzed and pictured as proceeding through a carbonium ion mechanism leading to the formation of the mixed acetal. The second step is characterized by a slow, temperature-dependent inductometric effect that leads to ring closure (67). The dehalogenation of XVII was accomplished with sodium in ether (68, 69) and produced alk-1-enyl ethers with *trans* configuration. The alk-1-enyl ethers were hydrogenated to yield the corresponding saturated ethers. Diol plasmalogens (XVIII and XIX) were prepared in an analogous manner; acylation furnished the neutral-

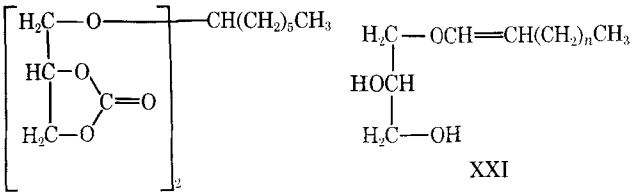
type plasmalogens (64). This synthesis resulted in isomeric products that were about 92% pure, but it was often necessary to fractionate very carefully.



XVIII XIX

Using a modification of the same reactions, Cymerman-Craig *et al.* (70) reported that the debromination of XVII by lithium in dimethoxyethane formed a mixture of four isomers. They later reported a synthesis that yielded solely the 1-(alk-1-enyl) glycerol (71, 72). To avoid the formation of the 2-alk-1-enyl glycerol, 2-benzyloxyglycerol was used in the transacetalation reaction with 2-bromo-1,1-dimethoxyhexadecane. Finally, catalytic debenzylation and debromination afforded a mixture of (±) *cis* and *trans* 3-(*n*-hexadec-1-enyloxy)-1,2-propanediols, whose diacetates could be separated by preparative GLC.

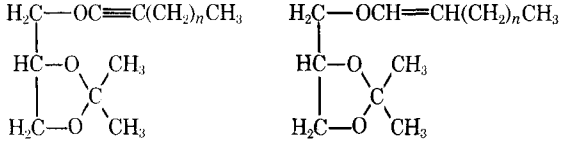
Cunningham and Gigg (73) used heptanal di(glycerol-1,2-carbonate) acetal (XX) as starting material for the synthesis of *cis* and *trans* isomers of ±1-(hept-1-enyl) glycerol (XXI), which was obtained by thermal decomposition of XX followed by alkali hydrolysis (n = 4). The acetal (XX) when treated with acetyl chloride



XX XXI

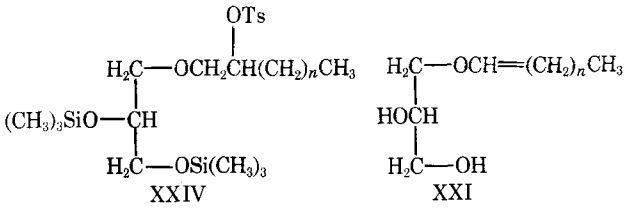
was readily converted into a chloro ether, *i.e.*, 1-chloroheptyl-glycerol-2,3-carbonate, which underwent dehalogenation by the action of triethylamine to yield 1-(hept-1-enyl) glycerol-2,3-carbonate; alkaline hydrolysis formed XXI. The base-catalyzed rearrangement of allyl ethers (74, 75) used earlier by Cunningham *et al.* (76) to prepare 1-(prop-1-enyl) glycerol did not occur with allyl ethers containing a long hydrocarbon chain.

Berezovskaya *et al.* (77, 78) proposed an interesting route leading to the formation of alk-1-enyl ethers of glycerol by the corresponding acetylenic intermediates. These workers reported the synthesis of 1-(alk-1-enyl)-2,3-isopropylideneglycerol (XXII) (n = 3, 11) by reaction of the sodium derivative of 1,2-isopropylideneglycerol with bromo alk-1-ynes. Partial reduction of XXII in the presence of Lindar catalyst yielded the *cis*-1-(alk-1-enyl)-2,3-isopropylideneglycerol (XXIII); however, doubts have been expressed (79) on the validity of this reaction sequence. The results of studies



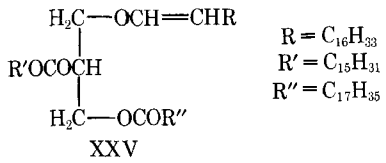
XXII XXIII

using the same method (77, 78) led Chacko *et al.* (79) to suggest that the final product in condensation to form XXII was predominantly an allenic ether rather than the expected acetylenic ether. Oswald *et al.* (80), using the same conditions (77), were unable to obtain the reported alk-1-enyl glyceryl ether. Russian workers (81–83) have further described the synthesis of various derivatives of racemic 1-(alk-1-enyl) glycerols by the elimination of a tosyloxy group from XXIV by potassium *tert*-butoxide (where n = 11, 13). Compound XXIV was prepared by reacting 1,2-epoxyhexadecane with



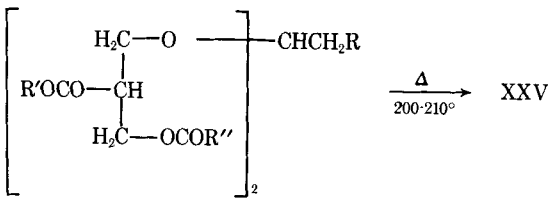
XXIV XXI

isopropylidene to form 1-(2-hydroxyalkyl)-isopropylidene(82). This alcohol was treated with *p*-toluenesulfonyl chloride to give the tosylate. The original ketal group in the isopropylidene was hydrolyzed and subsequently replaced with trimethylsilyl groups. Finally, dehydrotosylation with potassium *tert*-butoxide produced a mixture of *cis* and *trans* isomers of 1-(alk-1-enyl) glycerol (XXI) which was acylated with various long-chain acid chlorides to give a "neutral plasmalogen," 1-(alk-1-enyl) 2,3-diacylglycerol (XXV). The authors did not indicate the presence of any alk-2-enyl ethers, as one might expect. However, under the reaction conditions, the alk-2-enyl ether would decompose, whereas the alk-1-enyl ether would not (73, 85) be affected.



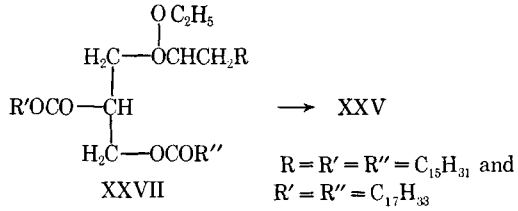
XXV

A similar procedure (82) was investigated for the synthesis of octadecyl alk-1-enyl glyceryl ethers, but results indicated that the desired compound was present in small amounts only (80). A slightly less polar impurity, the major product of the reaction, was difficult to eliminate. Analysis of the fractions obtained after resolution of the total aldehydogenic material on silicic acid columns revealed that 15% of the alk-1-enyl glyceryl ether was present. Other modifications of the dehydrotosylation route have been used to improve the preparation of XXV (85). The application of the transacetalation reaction (66, 67) has been explored and proposed as a possible route to neutral plasmalogens (86–88). The method proposed is based on pyrolytic cleavage of di(α,β-diglyceryl) acetals of long-chain fatty aldehydes, *i.e.*, reactions of diethylacetal of stearaldehyde with the appropriate α,β-diglycerides to form di(α,β-diglyceryl) acetal of stearaldehyde (XXVI) (88). Since an acid is used in the reaction, the possibility of migration of acyl groups in the starting α,β-diglycerates exists, as well as formation of other isomeric products.



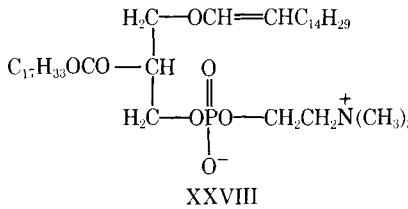
XXVI

A similar method describes the pyrolysis of di-(glycerol-1,2-carbonate) acetal of heptaldehyde (73) as a route to 1-(alk-1-enyl) ethers of glycerol, but this reaction produces a mixture of *cis* and *trans* isomers. The elimination of alcohol from 1-(1-ethoxyalkyl)-2,3-diacylglycerol (XXVII) to form plasmalogens and XXV (87) has also been proposed by other workers (84).

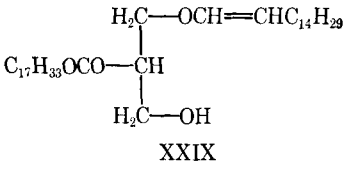


XXVII

Recently, Slotboom *et al.* (89) have described partial chemical synthesis of racemic *trans*-1-(hexadec-1-enyloxy)-2-oleoyl glycerol-3-phosphorylcholine (XXVIII). The synthesis of XXVIII required 1-(1-ethoxyhexadecyl)-2,3-dipalmitoyl glycerol (XXVII) as the intermediate; this was prepared by a modification of the method of Zvonkova *et al.* (87), using ethyl-1-hexadecyl ether and racemic 1,2-dipalmitoyl glycerol. Ethanol was eliminated from XXVII by heating it to 180° in the presence of sulfanilic acids *in vacuo;* this produced Compound XXV, *i.e.*, racemic *trans*-1-(hexadec-1-enyl)-2,3-dipalmitoyl glycerol. This compound was subjected to hydrolysis under alkaline conditions (90) and then reacylated with oleoyl chloride to yield racemic *trans*-1-(hexadec-1-enyl)-2,3-dioleoyl glycerol (XXV) (where R = $C_{15}H_{31}$ and R′ = R″ = $C_{17}H_{33}$). Conversion of Structure XXVII to the monoacyl analog (XXIX) was accomplished enzymatically with pancreatic lipase,



XXVIII



XXIX

which shows specificity for hydrolyzing fatty acyl bonds attached to the primary hydroxyl groups of glycerol. The method of Hirt and Berchtold (91) was used for the synthesis of Structure XXVIII from XXIX.

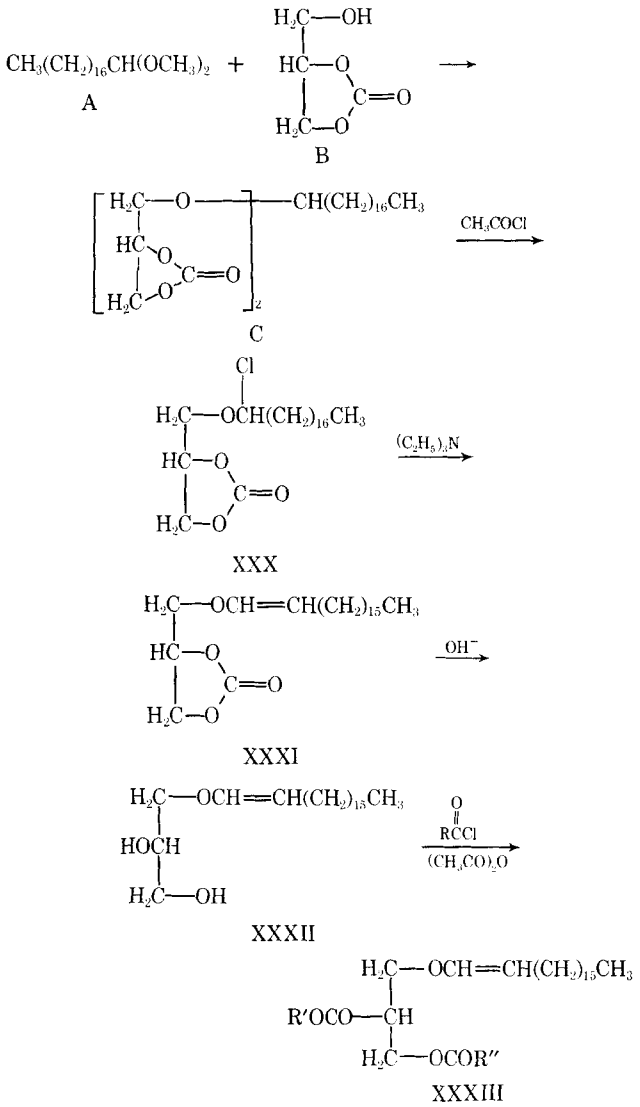
Recently, Gigg and Gigg (92), in an extension of their earlier work on plasmalogens (73), described a chloroether route for the synthesis of racemic and optically active alk-1-enyl glyceryl ethers (neutral plasmalogens) in which the *cis* isomer was the major product. Their use of the pyrolysis method (73) had given a preponderance of the *trans* isomer. Finally, the *cis* and *trans* isomers in the form of their diacetates and dipalmitates were separated by thin-layer chromatography (TLC) on silica gel layers containing silver nitrate. Other workers have also used the dehydrohalogenation reaction to prepare alk-1-enyl ethers of glycerol (93–96).

As shown in Scheme II, Gigg and Gigg (92) used a transacetalation reaction between octadecanal dimethyl acetal [A] and D-(glycerol-1,2-carbonate) [B]. The lengthy sequence required for the synthesis of [B] by degradation (97) has recently been simplified by using a stereoselective route (98). The initial reaction between [A] and [B] produced octadecanal di-D-(glycerol-1,2-carbonate) acetal [C], which was then treated with acetyl chloride to give the chloroether, 3-(1-chlorooctadecyl)-D-glycerol-1,2-carbonate (XXX). The dehydrohalogenation reaction with triethylamine produced a mixture of *cis-trans* isomers of 3-(octadec-1-enyl)-D-glycerol-1,2-carbonate (XXXI); the *cis* isomer was the major product. Alkaline hydrolysis of Structure XXXI gave a mixture of *cis-trans* isomers of 1-(octadec-1-enyl)-L-glycerol (XXXII) that was converted to the diacyl analog by means of palmitoyl chloride and acetic anhydride. The earlier pyrolysis method for preparing the alk-1-enyl ethers (84) produced mainly the *trans* isomer, and the preparation of Slotboom *et al.* (89) contained only the *trans* isomer. At the present time, the most promising route for the synthesis of possible phosphorylated and other derivatives of the naturally occurring *cis* alk-1-enyl glyceryl ethers seems to be the method described by Gigg and Gigg (92). The reaction sequences are illustrated in Scheme II.

METABOLISM

Occurrence—The occurrence of ether bonds in biological compounds is limited in comparison to that of the acetal linkage of sugars, amides of proteins, and acyl groupings of lipids. In nature, ether linkages are found in guaiacol, vanillin, methyl phenyl ethers, enolpyruvylskikimate-5-*P*, muramic acid, thyroxine, diphenyl ethers, and lipids. Lipids are known to contain alkyl and alk-1-enyl hydrocarbon moieties in ether linkage with glycerol or glycol. In general, the more prevalent ether-linked lipids are the alk-1-enyl phospholipids (plasmalogens), but phosphorus-free lipids that contain *O*-alk-1-enyl bonds have also been reported. The ether-linked lipids are found in most living cells, although most commonly in those of animal origin.

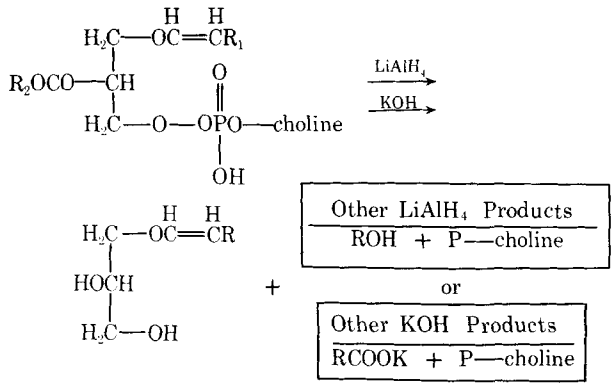
In mammals, the highest concentrations of *O*-alk-1-enyl lipids are found in brain and heart tissues, and plasmalogens appear to be an important constituent of biomembranes. The reader is referred to earlier reviews (1, 2, 5) on the occurrence of plasmalogens, but for convenience a summary of known biological sources of ether-linked lipids is provided in Table I. The table is not meant to be all inclusive but should serve to demonstrate the widespread distribution of lipids with



Scheme II

ether bonds in living cells. Although the close structural and metabolic relationships of the alkyl and alk-1-enyl ether bonds of lipids cannot be ignored (5), the discussion of their biochemistry in this paper will emphasize only the glycerolipids that contain *O*-alk-1-enyl bonds.

Methods of Detection—Methodology currently available for analyses of alk-1-enyl and alkyl ether-linked lipids has recently been reviewed (6, 7, 99). In general, plasmalogens are easily analyzed if the acyl, phosphorus, or phosphorylbase moieties are removed. Alkaline hydrolysis (100–102), enzymatic (103), or $LiAlH_4$ reduction (104–106) of these groups has been successfully used for this purpose. The *O*-alk-1-enyl glycerols formed can be measured quantitatively by photodensitometry or colorimetric reactions. During thin-layer chromatography, the *O*-alk-1-enyl glycerols migrate ahead of the *O*-alkylglycerols on silica gel G layers in solvent systems such as diethyl ether–water (100:0.5, v/v) or chloroform–methanol (98:2, v/v). This R_f behavior is probably explained by differences in the configuration of the alkyl and alk-1-enyl linkages, since the latter has a *cis* bond in the α,β-position (106).

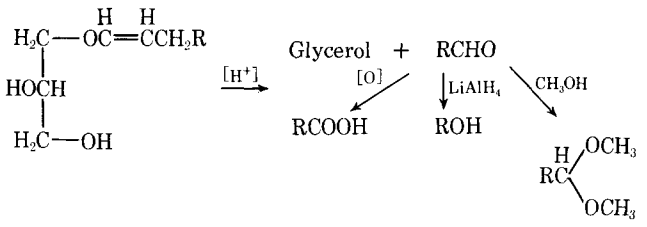


Renkonen (107) has successfully isolated intact plasmalogens from the corresponding diacyl-, alkyl-, or acyl-ethanolamine lipids by TLC after masking the polar base groups as their methylated dinitrophenyl derivatives. However, the resolution of these intact derivatives requires multiple solvent developments. The advantage of this technique is that it permits labeling of all functional groupings in the intact subclasses of *P*-ethanolamine lipids and *P*-choline lipids to be followed in tracer experiments.

The nature of the *O*-alk-1-enyl side chains is best determined by GLC after releasing the long-chain fatty aldehydes by acid treatment (108) or by forming the dimethylacetals (109). Gas-liquid chromatography of long-chain fatty aldehydes and their derivatives has recently been reviewed by Gray (110). The fatty aldehydes can also be converted to fatty alcohols by $LiAlH_4$ reduction (109) or to acids by oxidation (109). The acetate derivatives of the alcohols and the methyl esters of the fatty acids are then analyzed by GLC to determine the lengths of the carbon chains and their degree of unsaturation. Oxidation of aldehydes to acids is only practical with saturated aldehydes, since degradation of unsaturated aldehydes occurs (109).

The variety of techniques used by biological investigators to investigate the quantities and nature of plasmalogens might account for some of the discrepancies that appear in the literature. Furthermore, it is likely that the vulnerability of the *O*-alk-1-enyl linkage to oxidative and acidic alterations would further complicate the meaning of some of the quantitative findings that have been published. Nevertheless, the chemical nature of plasmalogens and their distribution in various lipid classes have been well established by numerous independent investigators.

Nature of Plasmalogens in Biological Materials—The *O*-alk-1-enyl linkage is found almost entirely in phospholipids, mostly associated with the ethanolamine-containing phosphatides (Table I). In contrast, the *O*-alkyl bonds tend to be associated with the choline-containing phosphatides which have only small quantities of the *O*-alk-1-enyl linkages (99). However, in recent years, trace quantities of *O*-alk-1-enyl glycerolipids have been detected in the neutral lipid fractions of cells (Table I), primarily as 2,3-diacyl-1-*O*-alk-1-enyl



glycerols; such compounds are often referred to as "neutral plasmalogens." The location of the *O*-alk-1-enyl linkage in the 1-position glycerolipids and its *cis* configuration have been firmly established (27–30, 36, 39, 111–114).

The *O*-alk-1-enyl moieties in all glycerolipid classes are primarily 16:0, 18:0, and 18:1 carbon chains (5). Although significant differences in the quantitative distribution of these predominant *O*-alk-1-enyl chains exist, they clearly resemble the *O*-alkyl moieties of various lipid classes. The similarities of *O*-alk-1-enyl and *O*-alkyl moieties in glycerolipids suggest that interconversions might occur. However, no enzymes have yet been found that can catalyze these interconversions.

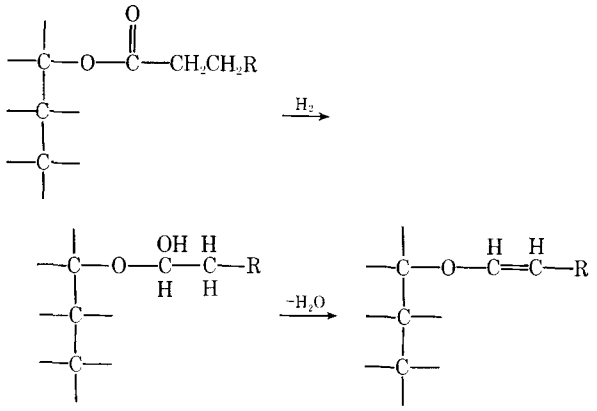
Biological Effects, Properties, and Function of Plasmalogens—Glycerolipids containing alkyl ether bonds have been thought to possess many biologically active properties, such as stimulation of growth, neurogenic activity, and hemopoiesis or inhibition of hemolysis. Bacteriostatic properties have also been associated with the ether-linked lipids. Furthermore, they have been proclaimed as therapeutic agents for radioprotection, wound healing, and bracken poisoning in cattle. These biological effects of alkyl glyceryl ethers have been thoroughly reviewed recently (5). Although many of these studies have been questioned, primarily because of the purity of the preparations or poor statistical comparisons, the fact remains that the listing of beneficial activities associated with *O*-alkyl lipids is overwhelming.

In contrast, no reports have dealt with similar type experiments carried out with plasmalogens. It is, perhaps, important to stress that the *O*-alk-1-enyl grouping in glycerolipids imparts a lower surface potential than that of the corresponding acyl analog (115). This is borne out in a biological system by the significant decrease in the hemolytic activity of lysophosphatidylethanolamine when alk-1-enyl (or alkyl) chains are substituted for an acyl chain (116).

The function of plasmalogens in living cells is not yet known. Their occurrence in biomembranes [organelles (117) and plasma membranes (118, 119)] and especially their high content in nervous tissue (120), suggest that their role is an important one in the structure and function of membranes. The orientation of protein and lipids in biomembranes could probably be a consequence of the ratio of ether- and ester-linked lipids in such residues; this proposition has been made on the basis of experimental data (117, 118). Further speculation on the function of plasmalogens is difficult since their biosynthetic pathway still remains to be elucidated.

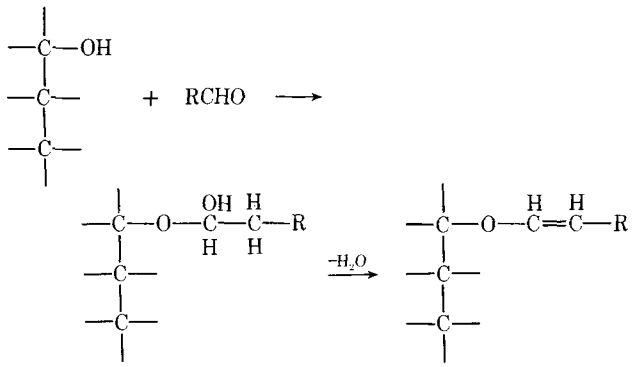
Possible Biochemical Mechanisms for the Biosynthesis of Plasmalogens—Recent experimental work has finally provided an enzymatic system that can synthesize ether-linked glycerolipids (121*a*–121*l*). However, only the alkyl linkage is formed readily in this microsomal system isolated from a number of cells. So far, the biosynthesis of *O*-alk-1-enyl bonds is unknown. In this section, a number of possible mechanisms for the biosynthesis of plasmalogens is summarized; some of these have been discussed previously (3, 5, 122–128).

A. Reduction of Acyl Groups to O*-Alk-1-enyl Groups*—



The reductive step would presumably require a hydrogen donor, such as NADH, NADPH, or FADH.

B. Reaction of a Fatty Aldehyde with a Primary Hydroxyl Group of a Derivative of Glycerol—



The glycerol precursor could be one of many glycolytic products, such as dihydroxyacetone-*P* or even a hexose-*P* that later could be split into a 3-carbon unit.

C. Direct Formation of an Alk-1-enyl Linkage by a Transfer Reaction—

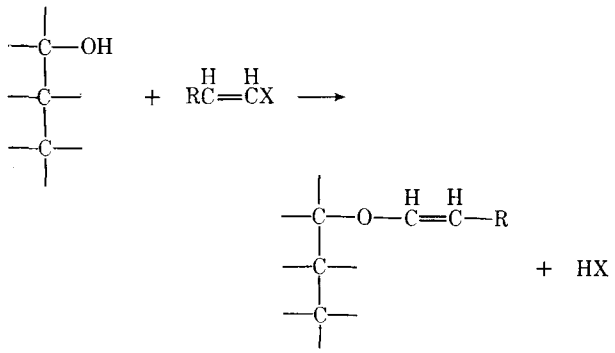
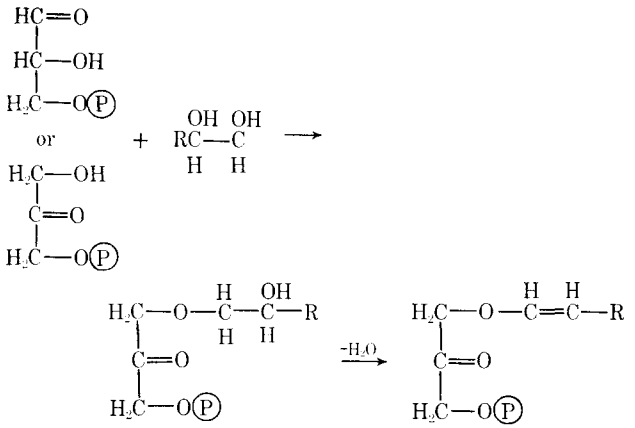


Table I—The Biological Occurrence of Plasmalogens in Phospholipids and Neutral Lipids

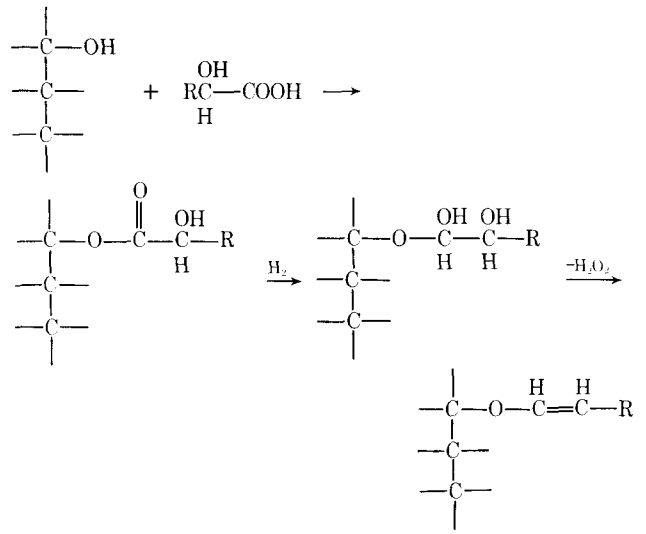
Biological Source	*O*-Alk-1-enyl Phospholipids References[a]	*O*-Alk-1-enyl Neutral Lipids References[a]
Mammals		
Normal tissues	25,106,200	61, 62,106,200,204,205
Neoplastic tissues	25,140,200	149,200
Fishes	139, 201	63,139,201
Invertebrates	61,202	61
Bacteria	149,203	149
Insects	206	

[a] Not a complete listing of references available.

*D. Reaction of an Alkane-1,2-diol with Glyceraldehyde-3-*P—

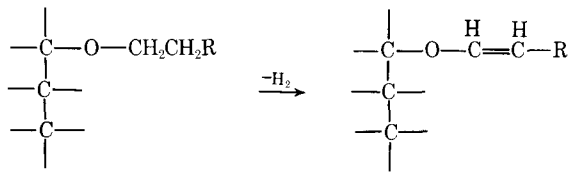


E. Reaction of an α-Hydroxy Fatty Acid and a Primary Hydroxyl Group of a Derivative of Glycerol—



This reaction would require an esterification and a reduction step before the final splitting out of hydrogen peroxide.

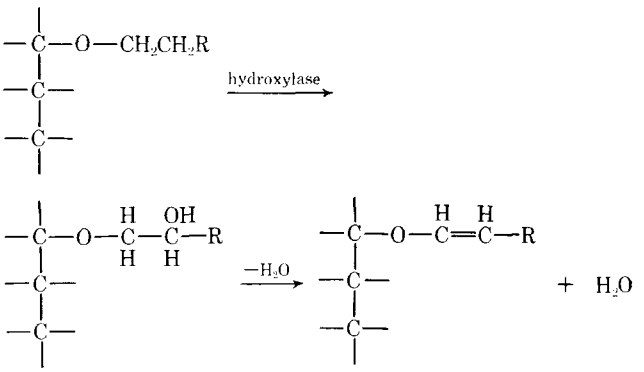
F. Conversion of an O*-Alkyl Linkage to an* O*-Alk-1-enyl Linkage*—



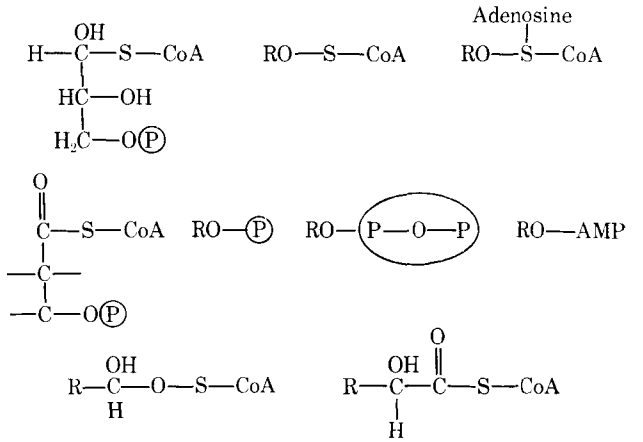
A hydrogen acceptor such as NAD, NADP, or FAD would be an essential cofactor.

Substituted groups such as $-NH_2$, $-OCH_3$, or $-OCH_2CH_3$ on the α- or β-carbons of the alkyl chain of glyceryl ethers could also produce alk-1-enyl ethers by splitting out ammonia, methanol, or ethanol, respectively.

G. Hydroxylation of an O*-Alkyl Lipid*—



H. Involvement of Cofactors—It is presumed that the reaction of functional groups listed in the preceding section would probably be facilitated through CoA or analogous intermediates, since the biosynthesis of the alkyl glyceryl ethers (121*a*) and acyl-substituted lipids (129) have an absolute requirement of coenzyme A, ATP, and Mg^{++}. Possible complexes that can be envisioned for such cofactors are shown below. All are hypothetical, since such complexes have never been isolated from biological materials.



Experimental Studies on the Biosynthesis of Plasmalogens—The reader is referred to an extensive review of this subject recently published (5).

A. Formation of the O*-Alk-1-enyl Linkage*—Cell-free systems that can form *O*-alk-1-enyl linkages in lipids have still not been isolated. Therefore, all data on the biosynthesis of these compounds available at the present time are largely circumstantial. *In vivo* studies have demonstrated that fatty acids (130–144), fatty alcohols (136, 140, 145–147*a* and *b*), fatty aldehydes (130–132, 148–150), and acetate (132, 134, 137, 138, 149, 151–155) can be incorporated into the alk-1-enyl moiety of glyceryl ethers. Experiments with doubly labeled fatty moieties have demonstrated that fatty aldehydes (132, 150) are much better precursors of the alk-1-enyl ethers than fatty acids. Some of the data obtained with fatty alde-

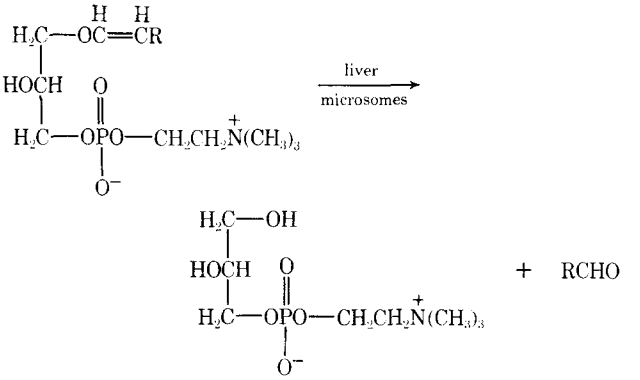
hydes have suggested that they are first oxidized and then incorporated into acyl linkages and that then the ester linkage is reduced (148). Nevertheless, experiments with the doubly labeled aldehydes strongly indicate that they are incorporated into the *O*-alk-1-enyl linkage directly instead of *via* the acyl-reductive mechanism.

Data indicating that alkyl glyceryl ethers can be desaturated in the α,β-position of the alkyl chain to form alk-1-enyl glyceryl ethers in the terrestrial slug, *Arion ater*, have been obtained by Thompson (141–143, 156*a*). Although the early evidence (141–143) for this conversion was based solely on the time course of specific activities, recent experiments with 1-^{14}C-hexadecyl-2-^{3}H-glyceryl ether (156*b*) have demonstrated that the ratio of $^{3}H/^{14}C$ in plasmalogens is identical to that of the alkyl glyceryl ether precursor. Experiments with the doubly labeled alkyl glyceryl ethers in Ehrlich ascites cells (156*b*) agree with the results obtained in slugs. Others (146, 147*a*, 157) have arrived at the same conclusion as Thompson (156*a*) by measuring specific activities of acyl, alkyl, and alk-1-enyl moieties in tracer experiments with glyceryl ether precursors. Some experiments in mammals (158–160) have not confirmed Thompson's thesis on the direct incorporation of ^{14}C-labeled alkyl glyceryl ethers into plasmalogens; radioactivity has been found in the alk-1-enyl chains of brain lipids (160), but this was attributed to the products of ether cleavage. Interconversions between neutral lipid and phospholipid classes that contain ether bonds have not been investigated; ideas about possible relationships have been based on structural studies of lipid classes (161).

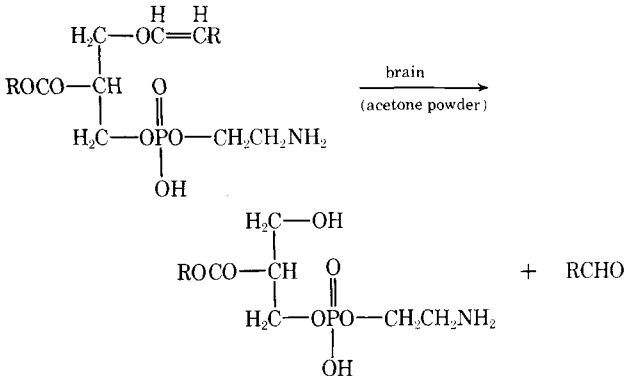
B. Phosphorylbase and Acyl Transferase Reactions Involving Alk-1-enyl Lipids as Substrates—Kiyasu and Kennedy (162) found that a particulate fraction of rat liver could catalyze the transfer of cytidine diphosphate choline or cytidine diphosphate ethanolamine to 1-*O*-alk-1-enyl-2-acyl-glycerols. The formation of the choline and ethanolamine plasmalogens by this reaction indicated that the transferases involved cannot distinguish between acyl and alk-1-enyl substituents on the substrates. Others have confirmed this reaction in homogenates (163–165) or particulate fractions (166) of brain and in a particulate fraction from ox heart (167). However, the importance of these cytidine-catalyzed reactions, at least in the brain, has been questioned by *in vivo* experiments with ^{14}C-ethanolamine (157) that involved the subsequent tracing of acyl, alkyl, and alk-1-enyl groupings in the various phospholipid classes at various times after their administration. Experiments with ^{32}P have also revealed considerable differences in the labeling of alk-1-enyl-acyl and diacyl phosphatides (130, 164, 168–172).

The acylation of alkyl glyceryl ethers occurs *in vivo* (158, 173–177) and *in vitro* (178–181). Cell-free systems isolated from numerous tissues have been shown to acylate 1-*O*-alkyl glycerols only in the 3-position (181). Presumably, acylation of the 2-position on the 1-isomer of alkyl glyceryl ethers requires the phosphorylated alkyl glyceryl ether as a substrate. Limited information on acylation reactions is available for the *O*-alk-1-enyl-linked lipids. Although rat liver microsomes (182) were unable to utilize 1-*O*-alk-1-enyl glycero-3-phosphorylcholine as a substrate for acyl-CoA–phospholipid transferase, this acylation reaction did occur in erythrocytes from humans and in sarcoplasmic reticulum from rabbits (183). Endogenous phospholipids have been shown to be important factors in the reactivation of alk-1-enyl-GPC hydrolase (184).

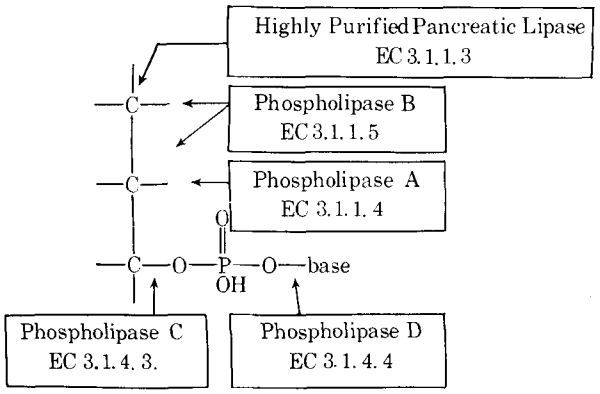
Experimental Studies on the Enzymatic Degradation of Plasmalogens—*A. Enzymatic Cleavage of the O-Alk-1-enyl Linkage*—Enzymes from liver (185) and brain (186) have been described that can catalyze the cleavage of the *O*-alk-1-enyl bond in plasmalogens. The liver enzyme, located in microsomes, can only utilize 1-*O*-alk-1-enyl glycerylphosphorylcholine as a substrate. The enzyme is specific for the lysocholine compound and no other cofactors are required.



The enzyme isolated from acetone powders of brain yields products similar to those found with the liver preparation, but it preferentially hydrolyzes the *O*-alk-1-enyl bond of 1-*O*-alk-1-enyl-2-acyl-glycerylphosphorylethanolamine, and Mg^{++} was required as a cofactor. The enzyme was also capable of cleaving the ether linkage of the lyso derivative, but the cleavage did not equal that obtained with the native plasmalogen substrate.



Other nonspecific reactions involving the enzymatic cleavage of the *O*-alk-1-enyl moieties of plasmalogens have been documented (187–190), but cleavage reactions of this type have not been reported for *O*-alk-1-enyl bonds found in neutral lipids. Considerable efforts have centered on the enzymatic cleavage of *O*-alkyl bonds in neutral lipids (191, 192). Enzymes for the cleavage of *O*-alkylglycerols have been found in rat liver that require tetrahydropteridine and NADPH as cofactors; the initial products formed during this cleavage are fatty aldehydes and glycerol. Similar systems have been found in other cells (192).



B. Removal of Acyl and Phosphorylbase Moieties from Plasmalogens by Phospholipases—Phospholipases are defined as A, B, C, or D, depending on the enzymatic specificity for the groups attached to the three positions of glycerol in phosphatides (103, 193). None of the lipase or phospholipase enzymes attacks ether bonds in lipids. Phospholipase A catalyzes the hydrolysis of fatty acids from the 2-position of phospholipids, whereas phospholipase B is thought to catalyze the hydrolysis of fatty acids from positions 1 and 2 or only position 1. Phospholipase B activity is the least understood since it is unknown whether its dual specificity is due merely to contamination with phospholipase A. Phospholipase C catalyzes the hydrolysis of the entire phosphorylbase moiety and phospholipase D catalyzes the hydrolysis of the base (choline or ethanolamine) portion only. It has also been shown that electrophoretically purified pancreatic lipase will catalyze the hydrolysis of acyl moieties in the 1-position of glycerophosphatides (194).

All of the phospholipases are capable of utilizing *O*-alk-1-enyl phosphatides as substrates under certain conditions, but generally the reaction rates are somewhat slower than with the diacyl analogs. Marinetti *et al.* (195) found that phospholipase A required several days to remove the acyl moiety from alk-1-enyl-acyl-choline phosphatides in yields equivalent to those obtained with phosphatidyl choline. The sluggishness of this reaction was successfully applied to the purification of native plasmalogens (removal of contaminating phosphatidyl choline) by Gottfried and Rapport (33). However, the source of the enzyme is thought to be important, since Hartree and Mann (196) found essentially the opposite results in similar studies of phosphatides isolated from the sperm of rams. Phospholipase C from *Clostridium welchii* has also been found to hydrolyze *O*-alk-1-enyl-acyl-choline phosphatides more slowly than the diacyl analog (197); *O*-alk-1-enyl ethanolamine phosphatides are also hydrolyzed by phospholipase C obtained from *Bacillus cereus* (198). In contrast, phospholipase D isolated from cabbage was unable to hydrolyze the choline moiety from the *O*-alk-1-enyl-acyl-choline phosphatides (182). Investigations of lipases that catalyze the removal of acyl groups in neutral lipid classes containing *O*-alk-1-enyl linkages has recently been reported by Slotboom (199).

CONCLUSIONS

Even after 40-odd years of research, the biosynthesis and function of the alk-1-enyl linkage in plasmalogens remain obscure although the structures, chemistry, and occurrence of plasmalogens in living cells are now firmly established. The striking similarities of chain lengths and degree of unsaturation between *O*-alkyl and *O*-alk-1-enyl moieties in glycerolipids suggest a close metabolic relationship and that they might perhaps originate from identical precursors. The requirements for the biosynthesis of alkyl ether bonds are known, and a clear understanding of the mechanism of this new pathway could elucidate fresh approaches to solving the riddle of plasmalogen biosynthesis. The biosynthetic problem is mirrored by the poor yields of plasmalogens synthesized in the organic laboratory. Certainly, the presence of *O*-alk-1-enyl bonds in biomembranes hints at their significance in the structural orientation of the lipid and protein in these structures. It is hoped that this review will serve to acquaint other chemists with both the past and present scope of research involving the *O*-alk-1-enyl linkages of plasmalogens.

REFERENCES

(1) M. M. Rapport and W. T. Norton, *Ann. Rev. Biochem.*, **31**, 103(1962).

(2) E. Klenk and H. Debuch, "Progress in the Chemistry of Fats and Other Lipids," vol. 6, R. T. Holman, W. O. Lundberg, and T. Malkin, Eds., Pergamon Press, Oxford, England, 1963, p. 1.

(3) E. F. Hartree, "Conference on Metabolism and Physiological Significance of Lipids," Cambridge, England, 1963; R. M. C. Dawson and D. N. Rhodes, Eds., Wiley, New York, N. Y., 1964, p. 207.

(4) O. W. Thiele, *Z. Klin. Chem.*, **2**, 33(1964).

(5) F. Snyder, "Progress in the Chemistry of Fats and Other Lipids," vol. 10, Part 3, R. T. Holman, Ed., Pergamon Press, Oxford, England, 1969, p. 287.

(6) F. Snyder, "Progress in Thin-Layer Chromatography and Related Methods," G. Pataki and A. Niederwieser, Eds., Ann Arbor Science, Ann Arbor, Mich., to be published.

(7) C. V. Viswanathan, *Chromatogr. Rev.*, **10**, 18(1968).

(8) F. Effenberger, *Angew. Chem.*, **8**, 295(1969).

(9) R. Feulgen and H. Rossenbeck, *Z. Physiol. Chem.*, **135**, 203(1924).

(10) R. Feulgen and K. Voit, *Arch. Gesamte Physiol. Menschen Tiere*, **206**, 389(1924).

(11) R. Feulgen and T. Bersin, *Z. Physiol. Chem.*, **260**, 217 (1939).

(12) S. J. Thannhauser, N. F. Boncoddo, and G. Schmidt, *J. Biol. Chem.*, **188**, 417, 423, 427(1951).

(13) M. Anchel and H. Waelsch, *ibid.*, **152**, 501(1944).

(14) G. Schmidt, B. Ottenstein, and M. J. Bessman, *Fed. Proc.*, **12**, 265(1953).

(15) E. Baer and H. C. Stancer, *J. Amer. Chem. Soc.*, **75**, 4510 (1953).

(16) M. M. Rapport, B. Lerner, and N. Alonzo, *Fed. Proc.*, **13**, 278(1954).

(17) E. Klenk and H. Debuch, *Z. Physiol. Chem.*, **296**, 179(1954).

(18) M. M. Rapport, B. Lerner, N. Alonzo, and R. E. Franzl, *J. Biol. Chem.*, **225**, 859(1957).

(19) (*a*) A. F. Hirsch, M.S. thesis, University of North Carolina, Chapel Hill, N. C., 1961; (*b*) M. M. Rapport and N. Alonzo, *J. Biol. Chem.*, **217**, 193(1955).

(20) M. M. Rapport and R. E. Franzl, *J. Neurochem.*, **1**, 303 (1957).

(21) S. Siggia and R. L. Edsberg, *Anal. Chem.*, **20**, 762(1948).

(22) J. N. Williams, Jr., C. E. Anderson, and A. D. Jasik, *J. Lipid Res.*, **3**, 378(1962).

(23) C. Piantadosi, A. F. Hirsch, C. L. Yarbro, and C. E. Anderson, *J. Org. Chem.*, **28**, 2425(1963).

(24) M. M. Rapport and B. Lerner, *Biochim. Biophys. Acta*, **33**, 319(1959).

(25) J. B. Wittenberg, S. R. Korey, and F. H. Swenson, *J. Biol. Chem.*, **219,** 39(1956).
(26) R. J. Blietz, *Z. Physiol. Chem.*, **310,** 120(1958).
(27) G. V. Marinetti, J. Erbland, and E. Stotz, *J. Amer. Chem. Soc.*, **80,** 1624(1958).
(28) *Ibid.*, **81,** 861(1959).
(29) H. Debuch, *Z. Physiol. Chem.*, **314,** 49(1959).
(30) *Ibid.*, **317,** 182(1959).
(31) G. M. Gray, *J. Chromatogr.*, **4,** 52(1960).
(32) J. C. M. Schogt, P. Haverkamp Begemann, and J. H. Recourt, *J. Lipid Res.*, **2,** 142(1961).
(33) E. L. Gottfried and M. M. Rapport, *J. Biol. Chem.*, **237,** 329(1962).
(34) G. M. Gray, *Biochem. J.*, **70,** 425(1958).
(35) W. T. Norton, E. L. Gottfried, and M. M. Rapport, *J. Lipid Res.*, **3,** 456(1962).
(36) H. R. Warner and W. E. M. Lands, *J. Amer. Chem. Soc.*, **85,** 60(1963).
(37) R. H. Hall, A. R. Philpotts, E. S. Stern, and W. Thain, *J. Chem. Soc.*, **1951,** 3341.
(38) G. J. Dege, R. L. Harris, and J. S. MacKenzie, *J. Amer. Chem. Soc.*, **81,** 3374(1959).
(39) J. Cymerman-Craig, D. P. G. Hamon, K. K. Purushothaman, S. K. Roy, and W. E. M. Lands, *Tetrahedron*, **22,** 175 (1966).
(40) M. F. Shostakovskii and N. A. Gerstein, *Zh. Obshch. Khim.*, **21,** 1452(1951).
(41) J. W. Copenhaver and M. H. Bigelow, "Acetylene and Carbon Monoxide Chemistry," Reinhold, New York, N. Y., 1949.
(42) L. Summers, *Chem. Rev.*, **55,** 317(1955).
(43) H. Bohme and H. Bentler, *Chem. Ber.*, **89,** 1468(1956).
(44) R. L. Adelman, *J. Amer. Chem. Soc.*, **77,** 1669(1955).
(45) W. Chalmers, *Can. J. Res.*, **7,** 472(1932).
(46) H. M. Teeter, E. J. Dufek, C. B. Coleman, C. A. Glass, E. H. Melvin, and J. C. Cowan, *J. Amer. Oil Chemists' Soc.*, **33,** 399(1956).
(47) E. Klenk and P. Böhm, *Z. Physiol. Chem.*, **288,** 98(1951).
(48) G. B. Ansell and J. M. Norman, *J. Neurochem.*, **1,** 32 (1956).
(49) M. H. Hack, *Fed. Proc.*, **20,** 278(1961).
(50) G. V. Marinetti, *J. Lipid Res.*, **3,** 1(1962).
(51) J. B. Davenport and R. M. C. Dawson, *Biochem. J.*, **79,** 10P(1961).
(52) *Ibid.*, **84,** 490(1962).
(53) R. Pietruszko and G. M. Gray, *Biochim. Biophys. Acta*, **56,** 232(1962).
(54) C. Piantadosi, M. F. Frosolono, C. E. Anderson, and A. F. Hirsch, *J. Pharm. Sci.*, **53,** 1024(1964).
(55) R. A. Landowne and W. Bergmann, *J. Org. Chem.*, **26,** 1257(1961); W. Bergmann and R. A. Landowne, *ibid.*, **23,** 1241(1958).
(56) G. K. Chacko and E. G. Perkins, *J. Org. Chem.*, **32,** 1623 (1967).
(57) T. Bersin, H. G. Moldtmann, H. Nafziger, B. Marchand, and W. Leopold, *Z. Physiol. Chem.*, **269,** 241(1941).
(58) M. J. Egerton and T. Malkin, *J. Chem. Soc.*, **1953,** 2800.
(59) (*a*) C. Piantadosi, C. E. Anderson, C. L. Yarbro, and E. A. Brecht, *J. Elisha Mitchell Sci. Soc.*, **81,** Suppl. 1, 34(1965); (*b*) M. F. Frosolono, A. Kisic, and M. M. Rapport, *J. Org. Chem.*, **32,** 3998(1967).
(60) J. C. M. Schogt, P. Haverkamp Begemann, and J. Koster, *J. Lipid Res.*, **1,** 446(1960).
(61) J. R. Gilbertson and M. L. Karnovsky, *J. Biol. Chem.*, **238,** 893(1963).
(62) H. H. O. Schmid and H. K. Mangold, *Biochim. Biophys. Acta*, **125,** 182(1966).
(63) L. D. Bergelson, "Progress in the Chemistry of Fats and Other Lipids," vol. 10, Part 3, R. T. Holman, Ed., Pergamon Press, Oxford, England, 1969, p. 239.
(64) C. Piantadosi and A. F. Hirsch, *J. Pharm. Sci.*, **50,** 978 (1961).
(65) C. Piantadosi and A. F. Hirsch, unpublished studies.
(66) C. Piantadosi, C. E. Anderson, E. A. Brecht, and C. L. Yarbro, *J. Amer. Chem. Soc.*, **80,** 6613(1958).
(67) C. Piantadosi, C. E. Anderson, C. L. Yarbro, and E. A. Brecht, *J. Org. Chem.*, **28,** 242(1963).
(68) H. S. Hill and L. M. Pidgeon, *J. Amer. Chem. Soc.*, **50,** 2718 (1928).
(69) H. S. Hill, *ibid.*, **50,** 2725(1928).
(70) J. Cymerman-Craig, D. P. G. Hamon, H. W. Brewer, and H. Härle, *J. Org. Chem.*, **30,** 907(1965).
(71) J. Cymerman-Craig and D. P. G. Hamon, *ibid.*, **30,** 4168 (1965).
(72) J. Cymerman-Craig and D. P. G. Hamon, *Chem. Ind.*, **1965,** 1559.
(73) J. Cunningham and R. Gigg, *J. Chem. Soc.*, **1965,** 2968.
(74) T. J. Prosser, *J. Amer. Chem. Soc.*, **83,** 1701(1961).
(75) C. C. Price and W. H. Snyder, *ibid.*, **83,** 1773(1961).
(76) J. Cunningham, R. Gigg, and C. D. Warren, *Tetrahedron Lett.* No. 19, 1191(1964).
(77) M. V. Berezovskaya, I. K. Sarycheva, and N. A. Preobrazhenskii, *Zh. Obshch. Khim.*, **34,** 543(1964).
(78) M. V. Berezovskaya, T. P. Zubkova, I. K. Sarycheva, and N. A. Preobrazhenskii, *Zh. Org. Khim.*, **2,** 1774(1966).
(79) G. K. Chacko, K. Schilling, and E. G. Perkins, *J. Org. Chem.*, **32,** 3698(1967).
(80) E. O. Oswald, C. Piantadosi, and C. E. Anderson, unpublished studies.
(81) E. A. Parfenov, G. A. Serebrennikova, and N. A. Preobrazhenskii, *Zh. Org. Khim.*, **2,** 629(1966); through *Chem. Abstr.*, **65,** 8747(1966).
(82) G. A. Serebrennikova, E. A. Parfenov, N. A. Perlova, and N. A. Preobrazhenskii, *Zh. Org. Khim.*, **2,** 1580(1966).
(83) G. A. Serebrennikova, B. A. Trubaichuk, and N. A. Preobrazhenskii, *Zh. Org. Khim.*, **4,** 755(1968).
(84) C. Piantadosi and A. F. Hirsch, unpublished studies.
(85) E. A. Parfenov, G. A. Serebrennikova, and N. A. Preobrazhenskii, *Zh. Org. Khim.*, **3,** 1566(1967).
(86) T. V. Serebryakova, G. A. Serebrennikova, and N. A. Preobrazhenskii, *ibid.*, **3,** 1412(1967).
(87) E. N. Zvonkova, I. K. Sarycheva, and N. A. Preobrazhenskii, *Dokl. Akad. Nauk SSSR*, **159,** 1079(1964); through *Chem. Abstr.*, **62,** 11, 680, 1168, 6398(1965).
(88) T. V. Serebryakova, E. N. Zvonkova, G. A. Serebrennikova, and N. A. Preobrazhenskii, *Zh. Org. Khim.*, **2,** 2004(1966); through *Chem. Abstr.*, **66,** 85405w(1967).
(89) A. J. Slotboom, G. H. de Haas, and L. L. M. van Deenen, *Chem. Phys. Lipids*, **1,** 192(1967).
(90) H. R. Warner and W. E. M. Lands, *J. Amer. Chem. Soc.*, **85,** 60(1963).
(91) R. Hirt and R. Berchtold, *Pharm. Acta Helv.*, **33,** 349 (1958).
(92) J. Gigg and R. Gigg, *J. Chem. Soc.*, **1968,** 2030.
(93) F. Sigmund and R. Vchann, *Monatsh. Chem.*, **51,** 234(1929).
(94) S. Hünig and M. Kiessel, *ibid.*, **91,** 380(1958).
(95) C. M. Hill, R. Woodberry, D. E. Simmons, M. E. Hill, and L. Haynes, *J. Amer. Chem. Soc.*, **80,** 4602(1958).
(96) O. Grummitt and J. A. Stearns, *ibid.*, **77,** 3136(1955).
(97) J. Gigg and R. Gigg, *J. Chem. Soc.*, **1967,** 1865.
(98) F. R. Pfeiffer, S. R. Cohen, K. R. Williams, and J. A. Weisbach, *Tetrahedron Lett.*, **32,** 3549(1968).
(99) F. Snyder, in "Drugs Affecting Lipid Metabolism," vol. 4, Plenum Publishing, New York, N. Y., 1969, p. 609.
(100) R. M. C. Dawson, *Biochem. J.*, **75,** 45(1960).
(101) R. M. C. Dawson, N. Hemington, and J. B. Davenport, *ibid.*, **84,** 497(1962).
(102) M. A. Wells and J. C. Dittmer, *Biochemistry*, **5,** 3405 (1966).
(103) L. L. M. van Deenen and G. H. de Haas, *Ann. Rev. Biochem.*, **35,** Part I, 157(1966).
(104) D. J. Hanahan, J. Ekholm, and C. M. Jackson, *Biochemistry*, **2,** 630(1963).
(105) G. A. Thompson, Jr., and P. Lee, *Biochim. Biophys. Acta*, **98,** 151(1965).
(106) R. Wood and F. Snyder, *Lipids*, **3,** 129(1968).
(107) O. Renkonen, *J. Lipid Res.*, **9,** 34(1968).
(108) R. Wood and R. D. Harlow, *ibid.*, **10,** 463(1969).
(109) J. W. Farquhar, *ibid.*, **3,** 21(1962).
(110) G. M. Gray, in "Lipid Chromatographic Analysis," vol. 1, G. V. Marinetti, Ed., Marcel Dekker, New York, N. Y., 1967, p. 401.
(111) G. V. Marinetti and J. Erbland, *Biochim. Biophys. Acta*, **26,** 429(1957).
(112) W. T. Norton, E. L. Gottfried, and M. M. Rapport, *J Lipid Res.*, **3,** 456(1962).

(113) H. H. O. Schmid, W. J. Baumann, and H. K. Mangold, *Biochim. Biophys. Acta*, **144,** 344(1967).
(114) H. H. O. Schmid, W. J. Baumann, and H. K. Mangold, *J. Amer. Chem. Soc.*, **89,** 4797(1967).
(115) D. O. Shah and J. H. Schulman, *J. Lipid Res.*, **6,** 341 (1965).
(116) E. L. Gottfried and M. M. Rapport, *ibid.*, **4,** 57(1963).
(117) S. Fleischer and G. Rouser, *J. Amer. Oil Chemists' Soc.*, **42,** 588(1965).
(118) R. C. Pfleger, N. G. Anderson, and F. Snyder, *Biochemistry*, **7,** 2826(1968).
(119) C. Cotman, M. L. Blank, A. Moehl, and F. Snyder, *ibid.*, **8,** 4606(1969).
(120) G. B. Ansell and J. N. Hawthorne, "Phospholipids—Chemistry, Metabolism and Function," B.B.A. Library, vol. 3, Elsevier Publishing, Amsterdam, Netherlands, 1964, p. 439.
(121) (*a*) F. Snyder, R. L. Wykle, and B. Malone, *Biochem. Biophys. Res. Commun.*, **34,** 315(1969). (*b*) S. J. Friedberg and R. C. Greene, *J. Amer. Oil Chemists' Soc.*, **46** (No. 8), Abstract No. 82 (1969). (*c*) A. K. Hajra, *Biochem. Biophys. Res. Commun.*, **37,** 486 (1969). (*d*) V. M. Kapoulas and G. A. Thompson, Jr., *Biochim. Biophys. Acta*, **187,** 594(1969). (*e*) F. Snyder, B. Malone, and M. L. Blank, *ibid.*, **187,** 302(1969). (*f*) F. Snyder, B. Malone, and R. L. Wykle, *Biochem. Biophys. Res. Commun.*, **34,** 40(1969). (*g*) F. Snyder, R. L. Wykle, and B. Malone, *ibid.*, **34,** 315(1969). (*h*) F. Snyder, M. L. Blank, B. Malone, and R. L. Wykle, *J. Biol. Chem.*, to be published. (*i*) F. Snyder, B. Malone, and M. L. Blank, *ibid.*, to be published. (*j*) F. Snyder, B. Malone, and R. B. Cumming, *Can. J. Biochem.*, to be published. (*k*) R. L. Wykle and F. Snyder, *J. Amer. Oil Chemists' Soc.*, **46** (No. 8), Abstract No. 109 (1969). (*l*) R. L. Wykle, M. L. Blank, C. Piantadosi, and F. Snyder, *Federation Proc.*, **29,** to be published.
(122) J. A. Lovern, *Biochem. J.*, **31,** 755(1937).
(123) E.Baer and H. O. L. Fischer, *J. Biol. Chem.*, **140,** 397(1941).
(124) O. W. Thiele and E. Gowin, *Z. Physiol. Chem.*, **299,** 151 (1955).
(125) O. W. Thiele, H. Schröder, and W. v. Berg, *Z. Physiol. Chem.*, **322,** 147(1960).
(126) R. M. Burton, in "The Biology of Myelin," vol. IV, S. R. Korey, Ed., Hoeber, Harper, New York, N. Y., 1959, p. 301.
(127) J. Cymerman-Craig and E. C. Horning, *J. Org. Chem.*, **25,** 2098(1960).
(128) H. Goldfine, *Ann. Rev. Biochem.*, **37,** 303(1968).
(129) E. P. Kennedy, *Fed. Proc.*, **16,** 847(1957).
(130) N. A. Baumann, P-O. Hagen, and H. Goldfine, *J. Biol. Chem.*, **240,** 1559(1965).
(131) H. G. Carr, H. Haerle, and J. J. Eiler, *Biochim. Biophys. Acta*, **70,** 205(1963).
(132) J. Ellingboe and M. L. Karnovsky, *J. Biol. Chem.*, **242,** 5693(1967).
(133) D. Gambal and K. J. Monty, *Fed. Proc.*, **18,** 232(1959).
(134) J. R. Gilbertson, J. Ellingboe, and M. L. Karnovsky, *ibid.*, **22,** 304(1963).
(135) M. L. Karnovsky and J. R. Gilbertson, in "Handbook of Physiology," A. E. Renold and G. F. Cahill, Jr., Eds., American Physiological Society, Washington, D. C., 1965, p. 177.
(136) R. W. Keenan, J. B. Brown, and B. H. Marks, *Biochim. Biophys. Acta*, **51,** 226(1961).
(137) S. R. Korey and M. Orchen, *Arch. Biochem. Biophys.*, **83,** 381(1959).
(138) S. R. Korey, in "Neurochemistry," vol. 1, S. R. Korey and J. I. Nurnberger, Eds., Harper, New York, N. Y., 1956, p. 143.
(139) D. C. Malins, *J. Lipid Res.*, **9,** 687(1968).
(140) F. Snyder and R. Wood, *Cancer Res.*, **28,** 972(1968).
(141) G. A. Thompson, Jr., *Biochemistry*, **5,** 1290(1966).
(142) G. A. Thompson, Jr., *J. Biol. Chem.*, **240,** 1912(1965).
(143) G. A. Thompson, Jr., and D. J. Hanahan, *Biochemistry*, **2,** 641(1963).
(144) G. H. Wegner and E. M. Foster, *J. Bacteriol.*, **85,** 53 (1963).
(145) S. J. Friedberg and R. C. Greene, *J. Biol. Chem.*, **242,** 5709(1967).
(146) D. C. Malins, *Biochem. J.*, **101,** 39P(1966).
(147) (*a*) *Ibid.*, **103,** 29P(1967). (*b*) R. Wood and K. Healy, *Biochem. Biophys. Res. Commun.*, to be published.
(148) R. Bickerstaffe and J. F. Mead, *Biochemistry*, **6,** 655 (1967).
(149) P-O. Hagen and H. Goldfine, *J. Biol. Chem.*, **242,** 5700 (1967).
(150) O. E. Bell, Jr., and H. B. White, Jr., *Biochim. Biophys. Acta*, **164,** 441(1968).
(151) H. Debuch, *Z. Physiol. Chem.*, **338,** 1(1964).
(152) H. Debuch, "Conference on Metabolism and Physiological Significance of Lipids," Cambridge, England, 1963; R. M. C. Dawson and D. N. Rhodes, Eds., Wiley, New York, N. Y., 1964, p. 537.
(153) J. Ellingboe and M. L. Karnovsky, *Fed. Proc.*, **24,** 477(1965).
(154) M. L. Karnovsky and A. F. Brumm, *J. Biol. Chem.*, **216,** 689 (1955).
(155) M. Kayama and Y. Tsuchiya, *Bull. Jap. Soc. Scientific Fisheries*, **34,** 640(1968).
(156) (*a*) G. A. Thompson, Jr., *Biochim. Biophys. Acta*, **152,** 409 (1968). (*b*) M. L. Blank, R. L. Wykle, C. Piantadosi, and F. Snyder, *Proc. Amer. Ass. Cancer Res.* (Abstract), to be published.
(157) L. A. Horrocks and G. B. Ansell, *Lipids*, **2,** 329(1967).
(158) F. Snyder and R. C. Pfleger, *ibid.*, **1,** 328(1966).
(159) E. O. Oswald, C. E. Anderson, C. Piantadosi, and J. Lim, *ibid.*, **3,** 51(1968).
(160) R. Bickerstaffe and J. F. Mead, *ibid.*, **3,** 317(1968).
(161) R. Wood and F. Snyder, *Arch. Biochem. Biophys.*, **131,** 478(1969).
(162) J. Y. Kiyasu and E. P. Kennedy, *J. Biol. Chem.*, **235,** 2590(1960).
(163) W. C. McMurray, *J. Neurochem.*, **11,** 287(1964).
(164) *Ibid.*, **11,** 315(1964).
(165) G. B. Ansell and R. F. Metcalfe, *Biochem. J.*, **98,** 22P (1966).
(166) *Ibid.*, **109,** 29P(1968).
(167) A. Poulos, B. P. Hughes, and J. N. Cumings, *Biochim. Biophys. Acta*, **152,** 629(1968).
(168) G. B. Ansell and S. Spanner, *Biochem. J.*, **96,** 64P(1965).
(169) Z. L. Freysz, R. Bieth, and P. Mandel, *Biochim. Biophys. Acta*, **58,** 583(1962).
(170) T. N. Ivanova, L. N. Rubel, and N. A. Semenova, *J. Neurochem.*, **14,** 653(1967).
(171) L. N. Rubel and T. N. Ivanova, *Proc. Acad. Sci. USSR, Sect. Biochem.*, **165,** 346(1965).
(172) G. Schmidt, L. H. Fingerman, H. M. Kreevoy, P. De Marco, and S. J. Thannhauser, *Amer. J. Clin. Nutr.*, **9,** 124(1961).
(173) S. Bergström and R. Blomstrand, *Acta Physiol. Scand.*, **38,** 166(1956).
(174) R. Blomstrand, *Proc. Soc. Exp. Biol. Med.*, **102,** 662(1959).
(175) R. Blomstrand and E. H. Ahrens, Jr., *ibid.*, **100,** 802(1959).
(176) L. Gallo, G. V. Valhouny, and C. R. Treadwell, *ibid.*, **127,** 156(1968).
(177) L. Swell, M. D. Law, and C. R. Treadwell, *Arch. Biochem. Biophys.*, **110,** 231(1965).
(178) S. I. Sherr, L. Swell, and C. R. Treadwell, *Biochem. Biophys. Res. Commun.*, **13,** 131(1963).
(179) S. I. Sherr and C. R. Treadwell, *Biochim. Biophys. Acta*, **98,** 539(1965).
(180) F. Kern, Jr., and B. Borgström, *ibid.*, **98,** 520(1965).
(181) F. Snyder and C. Piantadosi, *Biochim. Biophys. Acta*, to be published.
(182) W. E. M. Lands and P. Hart, *Biochim. Biophys. Acta*, **98,** 532(1965).
(183) K. Waku and W. E. M. Lands, *J. Biol. Chem.*, **243,** 2654 (1968).
(184) J. S. Ellingson and W. E. M. Lands, *Lipids*, **3,** 111(1968).
(185) H. R. Warner and W. E. M. Lands, *J. Biol. Chem.*, **236,** 2404(1961).
(186) G. B. Ansell and S. Spanner, *Biochem. J.*, **94,** 252(1965).
(187) C. E. Anderson, J. N. Williams, Jr., and C. L. Yarbro, "Proceedings of International Conference on the Biochemical Problems of Lipids," 5th, Vienna, 1958, G. Popjak, Ed., Pergamon Press, New York, N. Y., 1960, p. 105.
(188) H. Bergmann, K-E. Schneweis, and O. W. Thiele, *Naturwissenschaften*, **44,** 380(1957).
(189) O. W. Thiele, *Z. Physiol. Chem.*, **315,** 117(1959).
(190) *Ibid.*, **316,** 137(1959).
(191) A. Tietz, M. Lindberg, and E. P. Kennedy, *J. Biol. Chem.*, **239,** 4081(1964).
(192) R. C. Pfleger, C. Piantadosi, and F. Snyder, *Biochim. Biophys. Acta*, **144,** 633(1967).

(193) M. Dixon and E. C. Webb, "Enzymes," 2nd ed., Academic, New York, N. Y., 1964.
(194) G. H. de Haas, L. Sarda, and J. Roger, *Biochim. Biophys. Acta*, **106**, 638(1965).
(195) G. V. Marinetti, J. Erbland, and E. Stotz, *ibid.*, **33**, 403 (1959).
(196) E. F. Hartree and T. Mann, *Biochem. J.*, **80**, 464(1961).
(197) H. R. Warner and W. E. M. Lands, *J. Amer. Chem. Soc.*, **85**, 60(1963).
(198) G. B. Ansell and S. Spanner, *Biochem. J.*, **97**, 375(1965).
(199) A. J. Slotboom, Ph.D. thesis, State University of Utrecht, June 21, 1968.
(200) F. Snyder and R. Wood, *Cancer Res.*, **29**, 251(1969).
(201) D. C. Malins and J. C. Wekell, "Progress in the Chemistry of Fats and Other Lipids," vol. X, Pergamon Press, Oxford, England, to be published.
(202) G. A. Thompson, Jr., and D. J. Hanahan, *J. Biol. Chem.*, **238**, 2628(1963).
(203) H. Goldfine, *ibid.*, **239**, 2130(1964).
(204) H. H. O. Schmid and H. K. Mangold, *Biochim. Biophys. Acta*, **125**, 182(1966).
(205) H. H. O. Schmid and H. K. Mangold, *Biochem. Z.*, **346**, 13(1966).
(206) E. N. Lambremont and R. Wood, *Lipids*, **6**, 503(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Medicinal Chemistry, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27515*, and the *Medical Division (under contract with the U. S. Atomic Energy Commission), Oak Ridge Associated Universities, Oak Ridge, TN 37830*

This work has been supported in part by the National Institutes of Health Grant No. GM12562-06 and the American Cancer Society Grant No. P-470.

RESEARCH ARTICLES

Effect of Certain Additives on the Photochemistry of Riboflavin

CHUNG TECK SHIN*, B. J. SCIARRONE, and C. A. DISCHER

Abstract □ The quantum efficiency of riboflavin under aerobic conditions was determined by using a microirradiation method. It was found that the initial quantum efficiency was constant and independent of intensity of light, wavelength of light, and concentrations employed. The quantum efficiency of riboflavin in the presence of phenols and other compounds was also determined. Only in the presence of phenols was the quantum efficiency decreased yielding a linear relationship between the Hammett's sigma values and the rate of photodecomposition. Benzyl alcohol and benzoic acid were found to be relatively ineffective as photochemical stabilizers compared to phenols. Cinnamyl alcohol, as an electron donor, enhanced the photodecomposition of riboflavin. It appears, from the compounds tested, that the hydroxyl group should be either attached to the benzene ring or be in conjugation with the benzene ring in order to be an effective photochemical stabilizer. The effects of temperature and pH on the system were also determined. Kinetic studies were made to elucidate the reaction mechanism.

Keyphrases □ Riboflavin, photochemistry—additives, temperature, pH effects □ Microirradiation—riboflavin, quantum efficiency □ Kinetic studies—riboflavin degradation □ Quantum riboflavin, efficiency—equations derived □ Spectrophotometry—analysis

The photosensitivity of riboflavin was first observed in 1932 by Warburg and Christian (1). Since then its photochemistry has been the subject of extensive investigation, and can be followed by recent reviews (2, 3). Although the fact that riboflavin will undergo photoreduction in the absence of an electron donor has been generally accepted, there have been conflicting views concerning the actual mechanism of the reaction. However, the presently accepted theory for the photodecomposition of riboflavin proposes that the reaction proceeds from the lowest triplet state of the flavin and involves intramolecular hydrogen-transfer from the ribityl sidechain with the subsequent formation of lumichrome and/or lumiflavin depending on basicity of the solution (4–6). The photolysis of riboflavin and several other flavins in acid or neutral solution is subject to general acid and base catalysis (6).

The formation of molecular complexes between riboflavin and various compounds has also been observed (7–11). Particularly, studies of the charge-transfer complexes between riboflavin and phenol derivatives have received considerable attention, because interactions of donor-acceptor type may be quite common in biological systems (12). Thus, an understanding of the correlation of these properties with the photochemical behavior of riboflavin might provide an insight into some of the energy transfer and storage mechanisms of living organisms.

Stabilization of riboflavin to light in the presence of additives is pharmaceutically important, since certain additives were observed to have considerable influence on the light stability of the system (13–22). Despite this importance, very few photochemical kinetic studies have been published for riboflavin in the presence of complexing agents. Therefore, the present study was undertaken

(193) M. Dixon and E. C. Webb, "Enzymes," 2nd ed., Academic, New York, N. Y., 1964.
(194) G. H. de Haas, L. Sarda, and J. Roger, *Biochim. Biophys. Acta*, **106**, 638(1965).
(195) G. V. Marinetti, J. Erbland, and E. Stotz, *ibid.*, **33**, 403 (1959).
(196) E. F. Hartree and T. Mann, *Biochem. J.*, **80**, 464(1961).
(197) H. R. Warner and W. E. M. Lands, *J. Amer. Chem. Soc.*, **85**, 60(1963).
(198) G. B. Ansell and S. Spanner, *Biochem. J.*, **97**, 375(1965).
(199) A. J. Slotboom, Ph.D. thesis, State University of Utrecht, June 21, 1968.
(200) F. Snyder and R. Wood, *Cancer Res.*, **29**, 251(1969).
(201) D. C. Malins and J. C. Wekell, "Progress in the Chemistry of Fats and Other Lipids," vol. X, Pergamon Press, Oxford, England, to be published.
(202) G. A. Thompson, Jr., and D. J. Hanahan, *J. Biol. Chem.*, **238**, 2628(1963).
(203) H. Goldfine, *ibid.*, **239**, 2130(1964).
(204) H. H. O. Schmid and H. K. Mangold, *Biochim. Biophys. Acta*, **125**, 182(1966).
(205) H. H. O. Schmid and H. K. Mangold, *Biochem. Z.*, **346**, 13(1966).
(206) E. N. Lambremont and R. Wood, *Lipids*, **6**, 503(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Medicinal Chemistry, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27515*, and the *Medical Division (under contract with the U. S. Atomic Energy Commission), Oak Ridge Associated Universities, Oak Ridge, TN 37830*

This work has been supported in part by the National Institutes of Health Grant No. GM12562-06 and the American Cancer Society Grant No. P-470.

RESEARCH ARTICLES

Effect of Certain Additives on the Photochemistry of Riboflavin

CHUNG TECK SHIN*, B. J. SCIARRONE, and C. A. DISCHER

Abstract □ The quantum efficiency of riboflavin under aerobic conditions was determined by using a microirradiation method. It was found that the initial quantum efficiency was constant and independent of intensity of light, wavelength of light, and concentrations employed. The quantum efficiency of riboflavin in the presence of phenols and other compounds was also determined. Only in the presence of phenols was the quantum efficiency decreased yielding a linear relationship between the Hammett's sigma values and the rate of photodecomposition. Benzyl alcohol and benzoic acid were found to be relatively ineffective as photochemical stabilizers compared to phenols. Cinnamyl alcohol, as an electron donor, enhanced the photodecomposition of riboflavin. It appears, from the compounds tested, that the hydroxyl group should be either attached to the benzene ring or be in conjugation with the benzene ring in order to be an effective photochemical stabilizer. The effects of temperature and pH on the system were also determined. Kinetic studies were made to elucidate the reaction mechanism.

Keyphrases □ Riboflavin, photochemistry—additives, temperature, pH effects □ Microirradiation—riboflavin, quantum efficiency □ Kinetic studies—riboflavin degradation □ Quantum riboflavin, efficiency—equations derived □ Spectrophotometry—analysis

The photosensitivity of riboflavin was first observed in 1932 by Warburg and Christian (1). Since then its photochemistry has been the subject of extensive investigation, and can be followed by recent reviews (2, 3). Although the fact that riboflavin will undergo photoreduction in the absence of an electron donor has been generally accepted, there have been conflicting views concerning the actual mechanism of the reaction. However, the presently accepted theory for the photodecomposition of riboflavin proposes that the reaction proceeds from the lowest triplet state of the flavin and involves intramolecular hydrogen-transfer from the ribityl sidechain with the subsequent formation of lumichrome and/or lumiflavin depending on basicity of the solution (4–6). The photolysis of riboflavin and several other flavins in acid or neutral solution is subject to general acid and base catalysis (6).

The formation of molecular complexes between riboflavin and various compounds has also been observed (7–11). Particularly, studies of the charge-transfer complexes between riboflavin and phenol derivatives have received considerable attention, because interactions of donor-acceptor type may be quite common in biological systems (12). Thus, an understanding of the correlation of these properties with the photochemical behavior of riboflavin might provide an insight into some of the energy transfer and storage mechanisms of living organisms.

Stabilization of riboflavin to light in the presence of additives is pharmaceutically important, since certain additives were observed to have considerable influence on the light stability of the system (13–22). Despite this importance, very few photochemical kinetic studies have been published for riboflavin in the presence of complexing agents. Therefore, the present study was undertaken

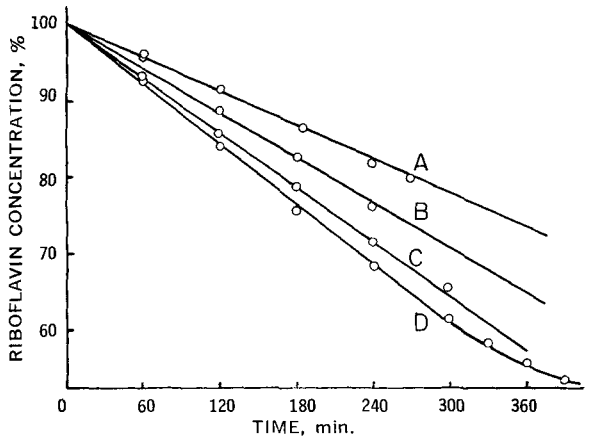


Figure 1—*The effect of light intensity (wavelength = 363 mμ) on the photodecomposition of riboflavin at 30°. Riboflavin concentration = 5.0 × 10⁻⁵ M. Key: Light intensity (photons/sec. × 10⁻¹⁴) A = 7.63; B = 10.46; C = 12.62; D = 13.66.*

to investigate the effect of certain additives, particularly, phenol derivatives and other compounds on the quantum efficiency of riboflavin. The phenol derivatives employed were phenol, *p*-chlorophenol, *p*-methoxyphenol, resorcinol, and hydroquinone. Benzyl alcohol, cinnamyl alcohol, and benzoic acid were also included to note the effect of the hydroxyl group conjugated with the benzene ring. Kinetic studies were also made to elucidate the mechanism of photodecomposition of riboflavin in the presence of additives. Temperature, pH, wavelength, and concentration were varied during these studies.

There is considerable information on the photodecomposition products and other compounds related to riboflavin using high intensity light sources. Under such conditions the photodecomposed products of riboflavin complicate the kinetic pattern and the calculation of quantum efficiency. Thus a microirradiation method developed by Dischер *et al.* (23–25) was chosen for this study.

EXPERIMENTAL

Instrumentation and Equipment—The irradiation instrumentation employed was basically that developed by Dischер *et al.* (23–25). Since the photodecomposition study was carried out at an elevated temperature, slight modifications were necessary on the reaction chamber; it was insulated with urethan foam and covered with aluminum foil to better maintain temperature at 30 ± 0.1°. Irradiation studies were performed only after 1-hr. temperature equilibration.

All solutions were analyzed in the visible region using a Beckman DU spectrophotometer, and the absorption spectra of the solutions were obtained with the Beckman DK-2 recording spectrometer.

Materials—Riboflavin[1] was used without further purification. Other materials employed were phenol, *p*-chlorophenol, *p*-methoxyphenol, resorcinol, hydroquinone, benzyl alcohol, cinnamyl alcohol, and benzoic acid. All phenol derivatives and aromatic alcohols were purified by recrystallization or distillation.

Photodecomposition Studies—These were conducted under aerobic conditions since most pharmaceutical products including riboflavin are stored in this manner. Few photochemical kinetic studies have been reported for these conditions.

Fresh solutions of riboflavin with and without additives were prepared in 0.05 *M* phosphate buffer, pH = 6.8. A 3-ml. sample of solution was pipeted into a Teflon-stoppered 1-cm. cell. The irradiation sample was analyzed by measuring its absorbance at 445 mμ on the Beckman DU spectrometer immediately before and after irradiation.

The mercury lamp employed showed two intense and sharp emission spectral lines at 363 and 433 mμ. Since these lines are close to the maximum absorbance of riboflavin, photodecomposition studies were undertaken at these wavelengths. The intensity of the light falling on the reaction cell was determined by measuring the galvanometer response as described by Dischер *et al.* (23–25). Since the intensity of the mercury lamp changed over a period of about 5 hr. by only 1.4% of the initial intensity, it was considered essentially constant for the period of irradiation. The quantum efficiency of riboflavin was calculated as described also by Dischер *et al.* (23–25). The initial rate of photodecomposition of riboflavin was determined from the linear portion of a zero-order plot of concentration *versus* time. All irradiation measurements were made two or three times.

Table I—The Effect of Intensity of Light on the Quantum Efficiency of Riboflavin at 30°. Riboflavin Concentration = 5.0 × 10⁻⁵ *M* (9.0345 × 10¹⁴ molecules/3 ml.)

Wavelength, mμ	Intensity of Light Photons/sec. ×10⁻¹⁴	Photons Absorbed/sec. ×10⁻¹⁴	Molecules Decomposed/sec. ×10⁻¹²	Average Quantum Efficiency ×10³
363	13.66	9.49	1.98	2.08
	12.62	8.67	1.78	2.05
	10.46	6.83	1.39	2.04
	7.63	5.32	1.09	2.05
			Average =	2.06
433	10.09	7.62	1.54	2.02
	8.72	6.46	1.30	2.02
	7.49	5.59	1.11	1.99
	5.34	4.03	0.79	1.95
			Average =	2.00

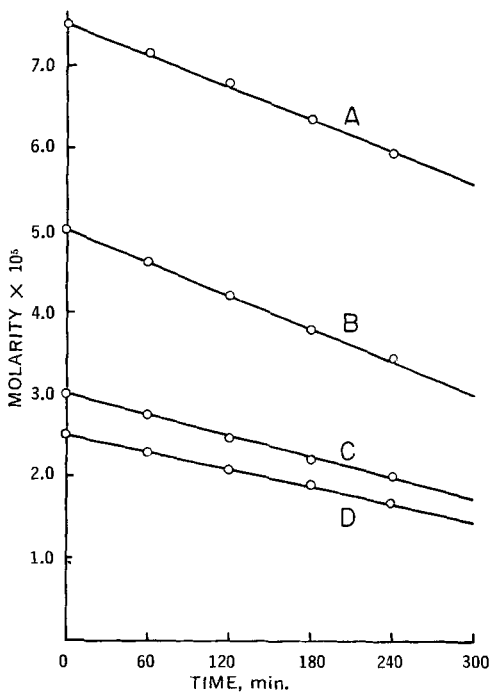


Figure 2—*Kinetic studies for the photodecomposition of riboflavin at various concentrations at 363 mμ. Key: Concentration (molarity × 10⁵) A = 7.5; B = 5.0; C = 3.0; D = 2.5.*

[1] The authors wish to thank Hoffmann-La Roche, Inc., Nutley, N. J., for supplying the necessary riboflavin.

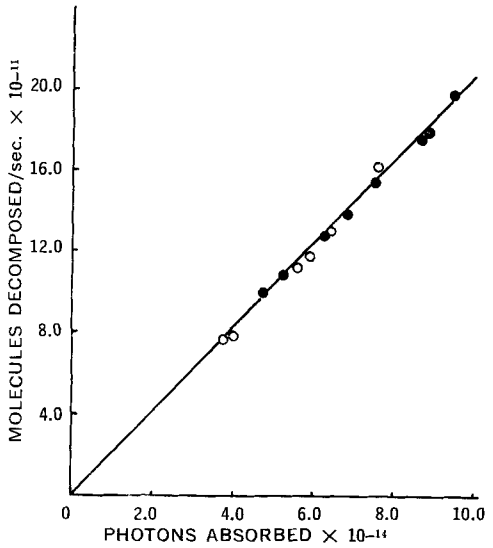


Figure 3—*Evaluation of quantum efficiency from a plot of molecules decomposed/sec. as a function of photons absorbed/sec. Key:* O, *light of wavelength 433 mμ; and* ●, *light of wavelength 363 mμ.*

RESULTS AND DISCUSSION

Kinetic Studies—The results of the kinetic studies at 363 mμ in the presence and absence of additives all fit the zero-order kinetic pattern rather than a pseudo first-order process. This is illustrated in Figs. 1 and 2. However, a linear zero-order relationship deviated after 5 hr. of irradiation, *i.e.*, when riboflavin was decomposed by more than 35% of initial concentration. This is illustrated in Fig. 1. Therefore, the length of exposure time was limited to 5 hr. so that the rate of decomposition of riboflavin was calculated from only the linear portion of the zero-order plot. When the 433 mμ wavelength of irradiating light was used a similar zero-order kinetic pattern was obtained.

Basic Quantum Efficiency Studies—The effect of the light intensity on the quantum efficiency of riboflavin was studied. The results of these studies are summarized in Table I (see Fig. 1). According to these data the quantum efficiency of riboflavin is independent of intensity of light. The number of molecules decomposed per unit time is directly proportional to the number of photons absorbed per unit time at each wavelength.

The effect of concentration of riboflavin on its quantum efficiency was also investigated. The data are summarized in Table II (see Fig. 2). These data indicate that quantum efficiency of riboflavin is constant within the concentration range tested. As shown in Table II, the higher the concentration of riboflavin solution the more photons were absorbed and subsequently more riboflavin decomposed. According to the above two series of studies the quantum efficiency of riboflavin was found to be constant at each wavelength employed.

It was deemed important to note the influence of irradiating time on the quantum efficiency of riboflavin. It was found that

Table II—The Effect of Concentration of Riboflavin on the Quantum Efficiency of Riboflavin at 30°

Wavelength, mμ	Concentration, Molarity ×10⁵	Molecules Decomposed/sec. ×10⁻¹²	Photons Absorbed/sec. ×10⁻¹⁴	Average Quantum Efficiency ×10³
363	7.5	1.80	8.88	2.03
	5.0	1.56	7.58	2.05
	3.0	1.28	6.29	2.03
	2.5	1.02	4.74	2.15
			Average =	2.07
433	7.5	1.64	7.63	2.15
	5.0	1.18	5.93	1.99
	3.0	0.77	3.75	2.04
			Average =	2.06

Table III—The Quantum Efficiency of Riboflavin in the Presence of Various Additives at 30° under Aerobic Conditions. Riboflavin Concentration = 2.5×10^{-6} *M*

Additives	Concentration of Additives, Molarity 2.5×10^{-5}	1.0×10^{-4}	5.0×10^{-4}
	Quantum Efficiency ×10³		
Phenol	1.59	1.28	0.74
p-Chlorophenol	1.46	1.13	0.70
p-Methoxyphenol	1.65	1.39	0.70
Resorcinol	1.34	0.35	—
Hydroquinone	2.94	2.75	1.56
Cinnamyl alcohol	1.86	2.03	3.22
Benzyl alcohol	1.98	1.98	1.94
Benzoic acid	1.82	1.77	1.73

quantum efficiency of riboflavin initially varied slightly in each study, but decreased after 5 hr. of irradiation. Such a result might be expected if a photodecomposition product or an intermediate quenches excited molecules or if a product of the reaction or an intermediate absorbs incident light.

The quantum efficiency of riboflavin can be evaluated from the slope of the plot of the molecules decomposed per unit time as a function of photons absorbed per unit time. All data in Tables I and II are plotted in Fig. 3. A linear relationship was observed between molecules decomposed and photons absorbed. This indicates that the riboflavin photodecomposition, under conditions used in this experiment, obeys the first law of photochemistry. The slope of the straight line was found to be 2.04×10^{-3}. Standard deviation for the measurement of quantum efficiency was 0.04×10^{-3} in this study. Theoretical and experimental rates of decomposition were compared as functions of the number of photons absorbed resulting in a superimposition of the straight line plots (not shown). Slopes of both lines were 6.76×10^{-23}.

Effect of Additives on the Quantum Efficiency—The quantum efficiency of riboflavin in the presence of various additives at

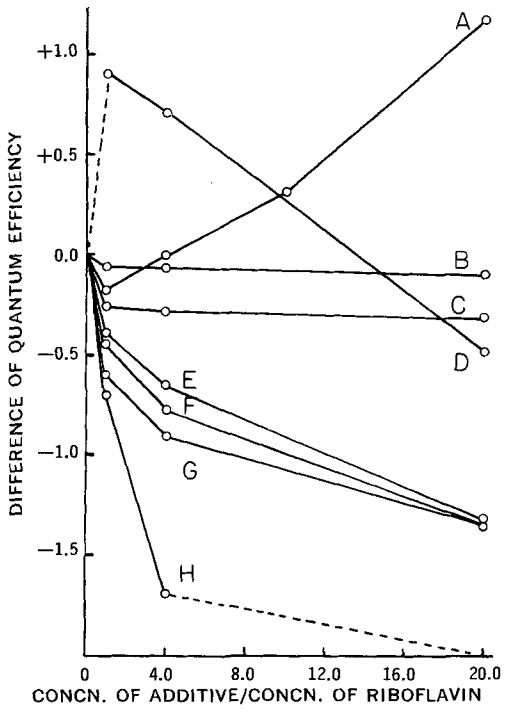


Figure 4—*Plot of difference in quantum efficiency of riboflavin in the presence and absence of additives as a function of the ratio of additive concentration to riboflavin concentration at 30°. Riboflavin concentration = 2.5 × 10⁻⁵ M. Key:* A = *cinnamyl alcohol;* B = *benzyl alcohol;* C = *benzoic acid;* D = *hydroquinone;* E = p-*methoxyphenol;* F = *phenol;* G = p-*chlorophenol;* H = *resorcinol.*

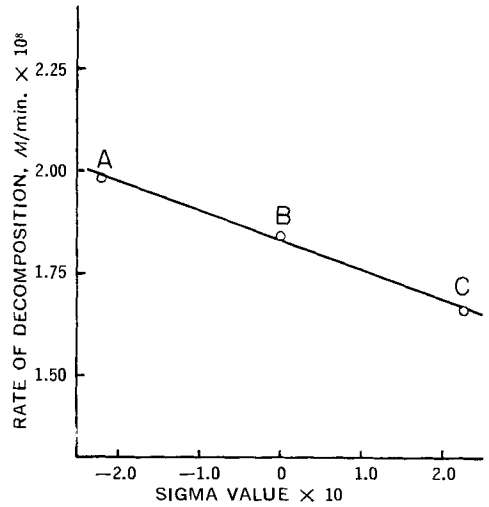


Figure 5—*A plot of rate of photodecomposition of riboflavin in the presence of phenols as a function of Hammett's sigma value for each phenol. Concentration of riboflavin = 2.5 × 10⁻⁵ M; concentration of phenols = 1.0 × 10⁻⁴ M. Key:* A = p-*methoxyphenol;* B = *phenol;* C = p-*chlorophenol.*

different concentrations are summarized in Table III. As shown in this table the quantum efficiency of riboflavin was either decreased or increased or remained constant depending on the physical and/or chemical properties of the additives. Any decrease in quantum efficiency in the presence of additives would be indicative of a photochemical stabilization process. A plot of the difference in quantum efficiencies of riboflavin in the presence and absence of additives as a function of the ratio of additive concentration to riboflavin concentration is shown in Fig. 4. The quantum efficiency of riboflavin in the presence of each phenol decreased gradually in the same fashion as the concentration of the given phenol was increased. This is probably due to the formation of the same type of complex between riboflavin and the different phenols. Therefore, the mechanism involved in the photodecomposition of riboflavin in the presence of monophenols may be considered to be similar.

The *p*-chlorophenol, which has an electron attracting chlorine atom (Hammett's sigma value = +0.227), lowered the quantum efficiency more than *p*-methoxyphenol, which has an electron donating methoxy group (Hammett's sigma value = −0.268). This indicates that the inductive and conjugative effects of substituents may influence both the stability of the complexes and the rate of photodecomposition of riboflavin. The rate of decomposition of riboflavin in the presence of phenols are plotted in Fig. 5 as a function of their respective sigma values. A linear relationship is observed in this plot and can be expressed as:

$$K_1 = 7.11 \times 10^{-9}\,\sigma + 1.83 \times 10^{-8} \qquad \text{(Eq. 1)}$$

where K_1 = the rate of decomposition of riboflavin in the presence of 1.0×10^{-4} *M* phenol (per min.) and σ = Hammett's substituent constant for phenols.

A linear relationship was observed between the pK values of phenols in the ground state and the quantum efficiency of riboflavin in the presence of the phenol. However, this linear relationship was not observed when pK* for the excited phenols was similarly plotted. In both the ground and excited states phenols which have lower pK and pK* values were found to be more effective in lowering the quantum efficiency of riboflavin. This appears to be due to the electron attracting chlorine atom in the *p*-chlorophenol which causes the molecule to be a better proton donor and subsequently giving it a greater tendency to form a stable hydrogen bond-type complex than *p*-methoxyphenol. It has been known that the excited molecules in many cases have a greater tendency to form hydrogen bonds and that a new equilibrium is reached during the life time of the excited states (26). However, none of the phenols showed absorbance at the wavelength of irradiating light used.

The quantum efficiency of riboflavin in the presence of resorcinol was significantly decreased as the concentration of resorcinol was increased. A possible explanation for this observation would be that if the excited molecules have a greater tendency to form a hydrogen bond-type complex (26) resorcinol would have a better chance to form complexes than a monohydroxyphenol since it has two hydroxyl groups. Resorcinol would then be much more effective in lowering the quantum efficiency of riboflavin. However, in the case of hydroquinone the quantum efficiency of riboflavin first increased and then decreased gradually at higher concentrations. These phenomena indicate that two different reaction mechanisms are involved depending upon the concentration of hydroquinone—*viz*, redox-reaction as well as complexation. In the presence of lower concentration of hydroquinone the redox-reaction mechanism is favored, *i.e.*, hydroquinone reduced the riboflavin, which was more easily decomposed through a triplet state. Therefore, the quantum efficiency of riboflavin was increased in the presence of lower concentration of hydroquinone. However, as the concentration of hydroquinone was increased, more of the complex(es) form(s), thereby stabilizing riboflavin.

It is interesting to note that when a riboflavin (2.5×10^{-5} *M*) solution was irradiated in the presence of a higher concentration (5.0×10^{-4} *M*) of resorcinol a red-brown colored solution resulted. The irradiation of a resorcinol solution (5.0×10^{-4} *M*) alone did not produce the colored product under the same conditions. Furthermore, in the ground state the colored products did not appear. Thus it is considered that the reaction involves interaction of riboflavin with resorcinol under irradiation. The colored solution had a maximum absorbance at 477 mμ. It was found that the photodecomposition product of resorcinol (1.0×10^{-3} *M*) formed after 78 hr. of irradiation also had this absorbance maximum. It is concluded, therefore, that the photooxidation of resorcinol was sensitized tremendously by the presence of riboflavin.

On the basis of these experiments it is clear that the photodynamic behavior of riboflavin in the presence of phenols is complicated and several other explanations are possible for the reduction of the quantum efficiency of riboflavin in addition to the complexation mechanism. It may be speculated that there might exist a direct transfer of the exicitation energy from riboflavin to phenols, probably a triplet-triplet transfer may be an acceptable explanation of the phenomena observed.

The quantum efficiency of riboflavin in the presence of 2.5×10^{-5} *M* concentration of cinnamyl alcohol was found to decrease but it then increased as the concentration of cinnamyl alcohol was increased. In the case of benzyl alcohol the quantum efficiency of riboflavin was very slightly decreased initially and remained essentially constant as the concentration of benzyl alcohol was increased. When in the presence of benzoic acid the quantum efficiency of

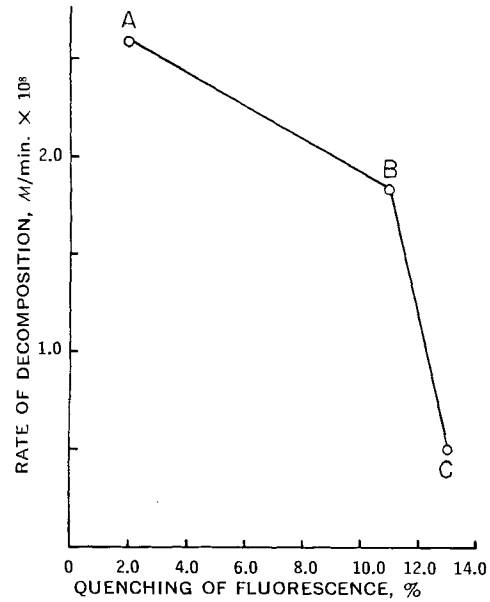


Figure 6—*A plot of rate of decomposition of riboflavin in the presence of benzoic acid, phenol, and resorcinol as a function of quenching efficiency (from* Reference 2*) of fluorescence of riboflavin. Key:* A = *benzoic acid;* B = *phenol;* C = *resorcinol.*

riboflavin was found to be sightly lower than that of riboflavin in the presence of benzyl alcohol and also remained essentially constant as the concentration of benzoic acid was increased. All three compounds were found to be less effective than the phenols in lowering the quantum efficiency. Therefore, it is considered that the hydroxyl group attached to the benzene ring must be conjugated with the benzene ring in order to be an effective stabilizer for the photodecomposition of riboflavin.

The cinnamyl alcohol, which has a conjugated double bond with the benzene ring, behaves entirely differently from benzyl alcohol and phenol in these irradiation studies. It is considered that cinnamyl alcohol is photochemically interacting with riboflavin as an electron donor. It is well known that alcohols are good electron donors and are photooxidized in the presence of riboflavin (21). However, in the case of benzyl alcohol there was no photochemical interaction between riboflavin and benzyl alcohol except possibly hydrogen bonding or a solvent effect. It has been reported that benzyl alcohol does not form complexes and does not quench the fluorescence of riboflavin (2, 9). However, benzoic acid does quench the fluorescence of riboflavin slightly (2). Therefore, it is obvious that the addition of benzoic acid can affect the excited state of riboflavin and lower its quantum efficiency more effectively than benzyl alcohol.

The rate of photodecomposition of riboflavin in the presence of resorcinol, phenol, and benzoic acid are plotted in Fig. 6 as a function of their quenching efficiency (2) of the fluorescence of riboflavin. There appears to be a direct correlation between the rate of decomposition and quenching efficiency, *i.e.*, the rate of decomposition of the riboflavin is decreased in the presence of a substance which has a higher quenching efficiency. Mataga (26) and El-Bayoumi (27) ascribed this quenching effect to the formation of the hydrogen bond type of complex.

The Effect of Temperature—The quantum efficiency of riboflavin in the absence and presence of phenol as a function of temperature is shown in Fig. 7. Separately an Arrhenius plot was constructed over the temperature range of 20 to 30°, which showed that the reaction had a greater temperature dependence in the absence of phenol. The activation energies, calculated from the slope of the Arrhenius plots, were 8.94 kcal. in the absence of phenol and 6.25 kcal. in the presence of phenol (1.0×10^{-4} *M*).

The Effect of pH—Since phosphate buffer also affects the rate of decomposition of riboflavin all samples were prepared in water by adjusting pH with hydrochloric acid and sodium hydroxide. Figure 8 presents the pH effect on the quantum efficiency of riboflavin first in the absence and then the presence of phenol as an additive. When phenol was added to a riboflavin solution at constant pH it was found that the quantum efficiency decreased. These phenomena indicate that the reduction of the quantum efficiency is not merely due to a pH effect but that the phenol itself is hindering the excitation process of riboflavin and thereby stabilizes it. Since the protonated riboflavin is essentially stable to light and does not fluoresce, the riboflavin becomes quite stable toward photodecomposition below pH 3.

Kinetic Analysis—Oster *et al.* (18) proposed a kinetic scheme for the photoreduction of riboflavin illustrating the role of the triplet state in the reaction. Such a scheme can be applied to the present study with certain modifications. On the basis of the present investigation the following reaction mechanism is proposed.

Riboflavin absorbs light to give the first electronically excited species, Rf*,

$$\mathrm{Rf} \xrightarrow[k_1]{} \mathrm{Rf}^* \qquad \text{(Eq. 2)}$$

This excited Rf* may fall to the ground state with the emission of fluorescence and the production of heat,

$$\mathrm{Rf}^* \xrightarrow[k_{2a}]{} \mathrm{Rf} + h\nu \qquad \text{(Eq. 3}a\text{)}$$

$$\mathrm{Rf}^* \xrightarrow[k_{2b}]{} \mathrm{Rf} + \text{heat} \qquad \text{(Eq. 3}b\text{)}$$

Equations 3*a* and 3*b* indicate deactivation of the first excited singlet state by fluorescence (k_{2a}) and a radiationless process (k_{2b}), respectively. For the purpose of simplification of the kinetic mechanism, these two processes can be expressed as follows:

$$\mathrm{Rf}^* \xrightarrow[k_2]{} \mathrm{Rf} + h\nu/\text{heat} \qquad \text{(Eq. 3}c\text{)}$$

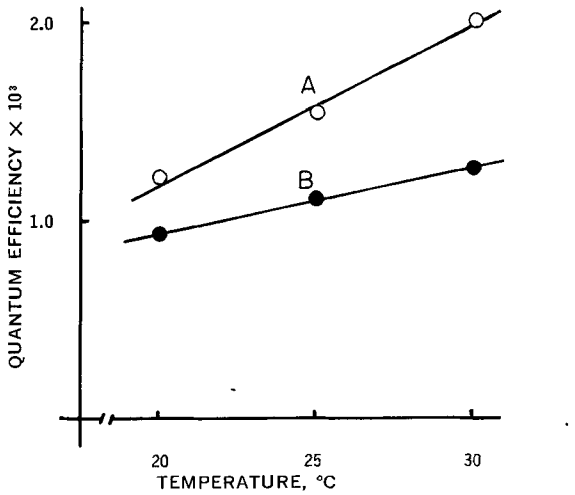


Figure 7—*Quantum efficiency as a function of temperature. Key:* A = *quantum efficiency of riboflavin in the absence of phenol;* B = *quantum efficiency in the presence of phenol* (1.0×10^{-4} M).

where $k_2 = k_{2a} + k_{2b}$

Alternatively the singlet excited species, Rf*, undergoes transition to a long-lived (triplet) state, Rf′, through electronic vibration.

$$\mathrm{Rf}^* \xrightarrow[k_3]{} \mathrm{Rf}' \qquad \text{(Eq. 4)}$$

This long-lived species may fall to the ground state, with the evolution of heat, and possibly phosphoresce,

$$\mathrm{Rf}' \xrightarrow[k_{4a}]{} \mathrm{Rf} + h\nu \qquad \text{(Eq. 5}a\text{)}$$

$$\mathrm{Rf}' \xrightarrow[k_{4b}]{} \mathrm{Rf} + \text{heat} \qquad \text{(Eq. 5}b\text{)}$$

Equation 5*b* indicates deactivation of the triplet state by a radiationless transition (k_{4b}). Again in order to simplify the kinetic mechanism the two above processes can be expressed as follows:

$$\mathrm{Rf}' \xrightarrow[k_4]{} \mathrm{Rf} + h\nu/\text{heat} \qquad \text{(Eq. 5}c\text{)}$$

where $k_4 = k_{4a} + k_{4b}$

The long-lived species may undergo transition to a more reactive species or may react directly to form various photodecomposed products. The kinetic mechanisms are simplified at this point to include all possible reactions with the triplet state in one rate constant, k_5,

$$\mathrm{Rf}' \xrightarrow[k_5]{} \text{products} \qquad \text{(Eq. 6)}$$

The addition of quencher, *Q*, reduces the rate of decomposition of riboflavin by affecting the singlet and triplet excited states. It

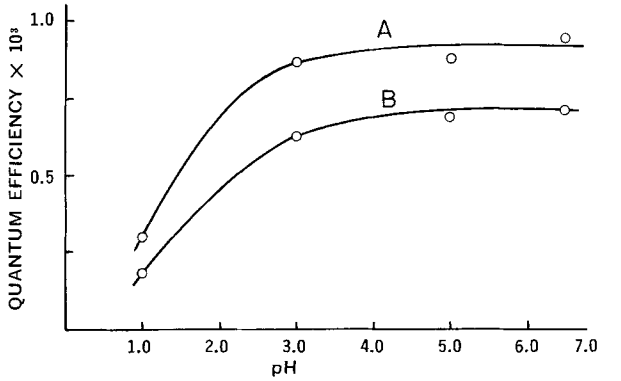


Figure 8—*The effect of pH on the quantum efficiency of riboflavin in the presence and absence of phenol. Key:* A = *riboflavin only* (2.5×10^{-5} M); B = *riboflavin with phenol* (5.0×10^{-4} M).

will probably quench the fluorescence and phosphorescence of the riboflavin and the excited species may fall to the ground state. During these processes there might exist a direct transfer of the excitation energy from the riboflavin to the quencher with the resulting formation of an excited complex. These new species may then dissociate to riboflavin and the excited quencher. The conjugative and inductive effects of substituents of a quencher (phenol) might show an influence on the stability of the excited complex. The excited quencher may finally be decomposed. The photosensitization of resorcinol in the presence of riboflavin can be explained by this mechanism.

$$Rf^* + Q \rightleftharpoons (Rf\text{-}Q)^* \rightarrow Rf + Q^* \qquad \text{(Eq. 6}a\text{)}$$

$$Rf' + Q \rightleftharpoons (Rf\text{-}Q)' \rightarrow Rf + Q' \qquad \text{(Eq. 7}a\text{)}$$

If the excited complex is assumed to be short-lived the overall reactions can be simplified as follows:

$$Rf^* + Q \xrightarrow[k_6]{} Rf + Q^* \qquad \text{(Eq. 6}b\text{)}$$

$$Rf' + Q \xrightarrow[k_7]{} Rf + Q' \qquad \text{(Eq. 7}b\text{)}$$

Using steady state assumptions regarding the transient species Rf* and Rf', the overall rate of photodecomposition of riboflavin can be expressed as follows:

$$\frac{d(Rf)}{dt} = \frac{-k_1k_3k_5(I)}{[k_2 + k_3 + k_6(Q)][k_4 + k_5 + k_7(Q)]} \qquad \text{(Eq. 8)}$$

where I = intensity of irradiating light.

The quantum efficiency of the reaction, ϕ, is equal to the overall rate divided by the initial rate. Therefore, the quantum efficiency can be expressed in terms of the rate constants:

$$\phi = \frac{k_3\,k_5}{[k_2 + k_3 + k_6(Q)][k_4 + k_5 + k_7(Q)]} \qquad \text{(Eq. 9)}$$

This equation indicates that the quantum efficiency of riboflavin is proportional to k_3 and k_5, *i.e.*, the mechanism of the photodecomposition of riboflavin involves mainly the long-lived (triplet) state. This equation also expresses the quantum efficiency of riboflavin to be inversely proportional to the concentration of quencher. This relationship is well demonstrated experimentally in Fig. 4.

REFERENCES

(1) O. Warburg and W. Christian, *Naturwissenschaften*, **20,** 980(1932).

(2) G. R. Penzer and G. K. Radda, *Quart. Rev.*, **21,** 43(1967).

(3) G. Oster, J. S. Bellin, and B. Holomström, *Experientia*, **18,** 249(1962).

(4) P. S. Song, E. C. Smith, and D. E. Metzler, *J. Amer. Chem. Soc.*, **87,** 4181(1965).

(5) W. E. Kurtin and P. S. Song, *Photochem. Photobiol.*, **9,** 127(1969).

(6) M. Halwer, *J. Amer. Chem. Soc.*, **73,** 4870(1951).

(7) D. E. Guttman and M. Y. Athalye, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 687(1960).

(8) G. Weber, *Biochem. J.*, **47,** 144(1950).

(9) K. Yagi and Y. Matsuoka, *Biochem. Z.*, **328,** 138(1956).

(10) K. Yagi and I. Ishibashi, *Vitamin*, **7,** 935(1954).

(11) D. E. Fleischman and G. Tollin, *Proc. Nat. Acad. Sci. U. S.*, **53,** 38(1965).

(12) A. Szent-Györgyi, "Introduction to Submolecular Biology," Academic, New York, N. Y., 1960, p. 67.

(13) H. M. Habermann and H. Gaffron, *Photochem. Photobiol.*, **1,** 159(1962).

(14) S. Scheindlin, A. Lee, and I. Griffith, *J. Amer. Pharm. Ass., Sci. Ed.*, **41,** 420(1952).

(15) A. W. Galston, *Proc. Nat. Acad. Sci. U. S.*, **35,** 10(1949).

(16) W. Berends, *Biochim. Biophys. Acta*, **39,** 178(1949).

(17) W. R. Frisell, C. W. Chung, and C. G. Mackenzie, *J. Biol. Chem.*, **234,** 1297(1959).

(18) B. Holomström and G. Oster, *J. Amer. Chem. Soc.*, **83,** 1867 (1961).

(19) G. K. Oster and G. Oster, *ibid.*, **81,** 5543(1959).

(20) H. B. Kostenbauder, P. P. Deluca, and C. R. Kowarski, *J. Pharm. Sci.*, **54,** 1243(1965).

(21) M. Green and G. Tollin, *Photochem. Photobiol.*, **7,** 129(1968).

(22) P. S. Song and D. E. Metzler, *ibid.*, **6,** 691(1967).

(23) C. A. Discher, P. F. Smith, I. Lippman, and R. Turse, *J. Phys. Chem.*, **67,** 2501(1963).

(24) R. Somkaite, Ph.D. thesis, Rutgers—The State University (1962).

(25) C. A. Discher and A. Felmeister, *J. Pharm. Sci.*, **53,** 756 (1964).

(26) N. Mataga, Y. Kaifu, and M. Koizumi, *Nature* (London), **175,** 731(1955); *Bull. Chem. Soc. Jap.*, **29,** 115(1956), *ibid.*, **31,** 481(1958).

(27) M. A. El-Bayoumi and M. Kasha, *J. Chem. Phys.*, **34,** 2181 (1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from *Rutgers—The State University, College of Pharmacy, Newark, NJ 07104*

Accepted for publication October 24, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Abstracted from a thesis submitted by Chung Teck Shin to the Graduate School of Rutgers—The State University in partial fulfillment of Doctor of Philosophy degree requirements.

* Present address: Bristol-Myers Products, Hillside, N. J.

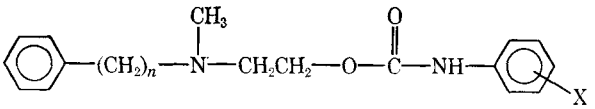
N-Aralkyl-piperidyl Carbanilates as Hypocholesteremic Agents

DARRELL E. O'BRIEN* and WILLIAM J. ROST

Abstract □ A number of *N*-aralkyl-*N*-methylaminoethyl carbanilates have been described in the literature as possessing hypocholesteremic activity. This series of compounds now has been extended to include 12 new *o*-[1-(substituted benzyl)-4-piperidyl]-*N*-(substituted phenyl) urethans and six new *o*-[1-(substituted benzyl)-4-piperidylideneimino]-*N*-(substituted phenyl) urethans. Two new derivatives of N^1-[(1-substituted benzyl)-4-piperidyl]-N^3(*p*-chlorophenyl)urea have been prepared. Eleven intermediate piperidine derivatives, that have not been previously described in the literature, have been prepared and characterized. Fifteen of these new compounds have been studied for their ability to inhibit the incorporation of mevalonate-2-^{14}C into cholesterol by homogenates of rat liver. Six of the compounds exhibited greater than 50% inhibition when tested at a concentration of 2×10^{-5} *M*. Appreciable incorporation of radioactivity into 7-dehydrocholesterol was observed with these six compounds. No significant reduction in serum cholesterol levels could be found in rats dosed at a level of 40 mg./kg. of body weight over a period of 7 days.

Keyphrases □ *N*-Aralkyl-piperidyl carbanilates, ureas—synthesis □ *N*-Aralkyl-piperidylideneimino urethans—synthesis □ Mevalonate-2-^{14}C incorporation—cholesterol □ Hypocholesteremic activity—*N*-aralkyl-piperidyl carbanilates

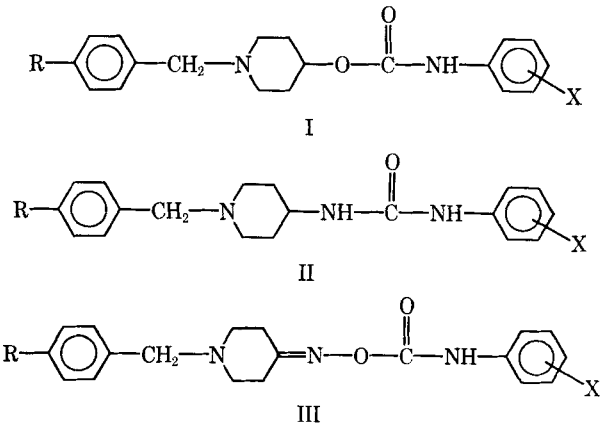
N-Aralkyl-*N*-methylaminoethyl carbanilates have been reported to possess hypocholesteremic activity (1). The potential value of compounds possessing this pharmacological activity prompted the study of this type of compound in greater detail (see Structure I).



Structure I

The benzyl, phenethyl, and phenpropylaminoalkyl carbanilates all showed significant activity. Other variations of the aralkyl group studied were detrimental to the hypocholesteremic activity. Increasing ester functions as in compounds of the dicarbanilate type did not yield useful agents. The analogous urea derivatives did not exhibit patterns similar to the carbanilate series. However, substitution in the carbanilate ring markedly affected the activity of this series. *Meta* and *para* substitutions produced compounds with a greater activity than *ortho*-substituted products. To study the effects of greater bulk in the ester portion of these carbanilates, a series of *o*-(1-aralkyl-4-piperidyl)-*N*-(substituted phenyl) urethans and related compounds have been prepared. The synthesis of compounds related to Classes I, II, and III would provide new structural modifications of this type of hypocholesteremic agent.

The various derivatives of piperidine could be studied in order to ascertain if the basic amino function of the parent compounds could be incorporated into a cyclic structure. By studying compounds of Class I, one would be able to obtain more information on the effects brought about by substitutions on the aralkyl moiety



Classes of Compounds

and the effects brought about by changing the parent compounds' primary carbinol function to a secondary carbinol function.

The compounds of Class II are the corresponding urea derivatives. The compounds of Class III are the urethan derivatives of the oximes of 1-(substituted benzyl)-4-piperidones. The pharmacological evaluation of these compounds would provide new information concerning the structure–activity relationships of this new class of hypocholesteremic agents.

Examples of the piperidine starting materials appear in the literature (2, 3). These known synthetic procedures were expanded where necessary to yield the required starting materials. Reaction of these materials with the appropriate isocyanate yielded the desired products.

It had been shown earlier that the *m*- and *p*-methyl and *m*- and *p*-chloro carbanilates were among the most active in the series. For this reason, these carbanilates of *N*-benzyl, *N*-*p*-chlorobenzyl, and *N*-*p*-methylbenzyl piperidinols were prepared. The *p*-chlorocarbanilates of *N*-benzyl and *N*-*p*-methylbenzyl piperidylamines were also synthesized. The *p*-methyl- and *p*-chlorocarbanilates of the oximes of the *N*-benzyl, *N*-*p*-methylbenzyl, and *N*-*p*-chlorobenzyl piperidones have also been synthesized.

See Tables I, II, and III for the specific compounds prepared.

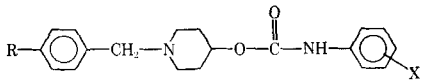
EXPERIMENTAL

Chemical—*Preparation of 1-Benzyl Piperidones*—These compounds were prepared as described in the literature by Thayer and McElvain (2).

Preparation of 1-Benzyl-4-piperidinols—1-Benzyl-4-piperidinols were prepared by the reduction of 1-benzyl-4-piperidones with diisobutyl aluminum hydride according to the method of Miller *et al.* (4).

Preparation of 1-Benzyl-4-piperidone Oximes—These compounds were prepared from the corresponding 1-benzyl-4-piperidones and

Table I—*N*-Aralkyl-piperidyl Carbanilates



No.	R	X	M.p., °C.	Formula	Anal., % Calcd.	Found
1	H	*p*-Cl	167–168	$C_{19}H_{21}ClN_2O_2$	C, 66.2 H, 6.14 N, 8.13	C, 66.4 H, 6.32 N, 8.02
2	H	*p*-CH_3	160–161	$C_{20}H_{24}N_2O_2$	C, 74.0 H, 7.45 N, 8.63	C, 73.74 H, 7.36 N, 8.55
3	H	*m*-Cl	131–132	$C_{19}H_{21}ClN_2O_2$	C, 66.2 H, 6.14 N, 8.13	C, 65.9 H, 6.06 N, 7.98
4	H	*m*-CH_3	120–122	$C_{20}H_{24}N_2O_2$	C, 74.0 H, 7.45 N, 8.63	C, 74.06 H, 7.20 N, 8.52
5	CH_3	*p*-Cl	143–145	$C_{20}H_{23}ClN_2O_2$	C, 66.9 H, 6.46 N, 7.75	C, 66.6 H, 6.46 N, 7.89
6	CH_3	*p*-CH_3	129–130	$C_{21}H_{26}N_2O_2$	C, 74.4 H, 7.72 N, 8.27	C, 74.2 H, 7.89 N, 8.28
7	CH_3	*m*-Cl	145–147	$C_{20}H_{23}ClN_2O_2$	C, 66.9 H, 6.46 N, 7.75	C, 66.9 H, 6.72 N, 7.63
8	CH_3	*m*-CH_3	131–133	$C_{21}H_{26}N_2O_2$	C, 74.4 H, 7.72 N, 8.27	C, 74.4 H, 8.02 N, 8.20
9	Cl	*p*-Cl	154–156	$C_{19}H_{20}Cl_2N_2O_2$	C, 60.3 H, 5.32 N, 7.40	C, 60.3 H, 5.18 N, 7.52
10	Cl	*p*-CH_3	143–145	$C_{20}H_{23}ClN_2O_2$	C, 66.9 H, 6.46 N, 7.75	C, 67.1 H, 6.16 N, 7.90
11	Cl	*m*-Cl	101–103	$C_{19}H_{20}Cl_2N_2O_2$	C, 60.3 H, 5.32 N, 7.40	C, 60.1 H, 5.61 N, 7.40
12	Cl	*m*-CH_3	93–95	$C_{20}H_{23}ClN_2O_2$	C, 66.9 H, 6.46 N, 7.75	C, 67.1 H, 6.16 N, 7.90

hydroxylamine as described in the literature by Brookes *et al.* (3).

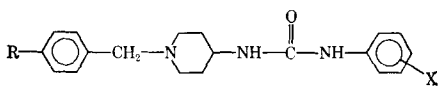
Preparation of o-[*1-(Substituted benzyl)-4-piperidyl*]-N-(*substituted phenyl*) *Urethans*—The compounds listed in Table I were prepared by reacting 0.02 mole of the appropriate alcohol with 0.022 mole of the isocyanate in 60 ml. of dry toluene at reflux temperature for 2 hr. On cooling, a white precipitate formed which was crystallized from toluene, ethanol, or hexane.

Preparation of o-(*1-Substituted benzyl-4-piperidylideneimino*)-N-(*substituted phenyl*) *Urethans*—A solution of the appropriate oxime (0.02 mole) and isocyanate (0.022 mole) in 50 ml. of anhydrous toluene was refluxed for 2 hr. At the end of this time, the clear yellow solution was allowed to cool slowly. The white crystalline product which deposited from the cooled solution was filtered, washed with toluene, and air dried. The product was recrystallized from toluene.

Preparation of 4-Amino-1-benzyl Piperidines—These compounds were prepared from the corresponding oximes by sodium metal reduction as described in the literature by Brookes *et al.* (3).

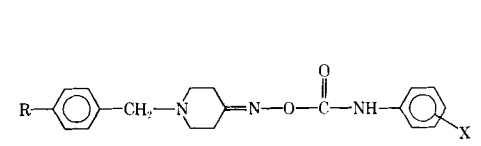
Preparation of N^1-[*1-(Substituted benzyl)-4-piperidyl*]-N^3-(*substituted phenyl*) *Ureas*—A solution of 4-amino-1-substituted benzyl piperidine dihydrochloride monohydrate (0.025 mole) in 50 ml. of water was treated with anhydrous potassium carbonate until pH 8–9 was obtained. The mixture was extracted three times with ether (25 ml.), and the combined ethereal extracts were washed two times with water (25 ml.). The ether solution was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in 100 ml. of hot anhydrous toluene, and this solution was evaporated to 50 ml. The isocyanate (0.0275 mole) was added to the hot toluene solution, and the resultant solution was refluxed for 2 hr. At the end of this time, the light yellow solution was allowed to cool slowly. The white crystalline product which deposited from the cooled solution was separated by filtration, washed with

Table II—*N*-Aralkyl-piperidyl Ureas



No.	R	X	M.p., °C.	Formula	Anal., % Calcd.	Found
13	H	*p*-Cl	203–205	$C_{19}H_{22}ClN_3O$	C, 74.3 H, 7.80 N, 13.0	C, 74.4 H, 7.52 N, 12.9
14	CH_3	*p*-Cl	210–212	$C_{20}H_{24}ClN_3O$	C, 67.2 H, 6.77 N, 11.7	C, 67.1 H, 6.83 N, 11.6

Table III—*N*-Aralkyl-piperidylideneimino Urethans



No.	R	X	M.p., °C.	Formula	Anal., % Calcd.	Found
15	H	*p*-Cl	126–127	$C_{19}H_{20}ClN_3O_2$	C, 63.9 H, 5.63 N, 11.7	C, 63.6 H, 5.41 N, 11.8
16	H	*p*-CH_3	115–117	$C_{20}H_{23}N_3O_2$	C, 71.3 H, 6.88 N, 12.4	C, 71.0 H, 7.11 N, 12.2
17	CH_3	*p*-Cl	144–146	$C_{20}H_{22}ClN_3O_2$	C, 64.7 H, 5.97 N, 11.3	C, 64.5 H, 5.95 N, 11.5
18	CH_3	*p*-CH_3	144–146	$C_{21}H_{25}N_3O_2$	C, 71.8 H, 7.13 N, 12.0	C, 72.0 H, 7.22 N, 12.1
19	Cl	*p*-Cl	126–128	$C_{19}H_{19}Cl_2N_3O_2$	C, 58.2 H, 4.88 N, 10.7	C, 58.4 H, 4.65 N, 10.5
20	Cl	*p*-CH_3	105–107	$C_{20}H_{22}ClN_3O_2$	C, 64.7 H, 5.97 N, 11.3	C, 64.4 H, 6.03 N, 11.1

Table IV—Incorporation of Mevalonate-^{14}C into Sterols[a]

Compound	Cholesterol	Desmosterol	7-Dehydrocholesterol
1	35	10	55
2	60		
3	51	11	38
4	79		
5	30	23	47
6	34	9	57
7	25	33	42
8	47	24	39
13	66	8	26
14	40	37	23
15	87	6	7
16	81	11	8
17	53	20	27
Trifluperidol McN JR 2498	31	20	49
	20	10	70
	23	35	41
Control	83	10	7
	82	8	10

[a] Values are expressed as percent of radioactivity based on activity in three sterols.

cold toluene, and air dried. The product was recrystallized from aqueous ethanol.

Pharmacological Evaluation—A number of these compounds have been evaluated for their ability to inhibit the biosynthesis of cholesterol.[1] The compounds have been evaluated by *in vitro* and *in vivo* procedures.

DISCUSSION

The hypocholesteremic activity of *N*-aralkyl-piperidyl carbanilates has been demonstrated in certain cases. Six compounds exhibited greater than 50% inhibition when tested at a concentration of 2×10^{-5} *M*. These compounds were: *o*-(1-benzyl-4-piperidyl)-*N*-(*p*-chlorophenyl) urethan I, *o*-[1-(*p*-methylbenzyl)-4-piperidyl]-*N*-(*p*-chlorophenyl) urethan V, *o*-[1-(*p*-methylbenzyl)-4-piperidyl]-*N*-(*p*-tolyl) urethan VI, *o*-[1-(*p*-methylbenzyl)-4-piperidyl]-*N*-(*m*-chlorophenyl) urethan VII, *o*-[1-(*p*-methylbenzyl)-4-piperidyl]-*N*-(*m*-tolyl) urethan VIII, and N^1-[1-(*p*-methylbenzyl)-4-piperidyl]-N^3-(*p*-chlorophenyl)urea XIV. With each of these compounds there is appreciable incorporation of radioactivity in 7-dehydrocholesterol. These compounds have also been evaluated (McNeil Laboratories) for their hypocholesteremic activity in rats. In rats receiving 40 mg./kg. of body weight for a 7-day period, it was found there was no significant reduction in serum cholesterol levels.

Some activity was seen in the urea series, but no appreciable hypocholesteremic activity was found among the piperidylideneimino urethan derivatives.

The hypocholesteremic activity of the *N*-aralkyl-piperidyl carbanilates was not superior to that activity found for the *N*-aralkylaminoethyl carbanilates. Hence, it was concluded that there was no advantage in the cyclic series of carbanilates.

In the previously reported series, substitution in the aralkyl group did not produce hypocholesteremic compounds. The *N*-*o*-chlorobenzylaminoethyl carbanilates had an unfavorable effect on cholesterol levels. However, in the present series, substitution in the aralkyl group was advantageous in certain cases. The *N*-*p*-methylbenzyl piperidyl carbanilates were among the most active in inhibiting cholesterol synthesis *in vitro*. This fact points out the need for further investigation of various substitutions on the *N*-benzyl group in this hypocholesteremic series.

[1] The evaluation of these compounds was performed by the McNeil Laboratories, Inc., under the direction of Dr. G. A. Braun.

REFERENCES

(1) W. J. Rost, B. M. Sutton, B. Blank, F. R. Pfeiffer, W. L. Holmes, N. W. DiTullio, and E. B. Ingram, *J. Pharm. Sci.*, **56**, 1598(1967).

(2) J. R. Thayer and S. M. McElvain, *J. Amer. Chem. Soc.*, **49**, 2862(1927).

(3) P. Brookes, R. J. Terry, and J. Walker, *J. Chem. Soc.*, **1957**, 3165.

(4) A. E. G. Miller, J. W. Bliss, and L. H. Schwartzman, *J. Org. Chem.*, **24**, 627(1959).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *School of Pharmacy, University of Missouri at Kansas City, Kansas City, MO 64110*

Accepted for publication October 15, 1969.

Abstracted from a thesis submitted by Darrell E. O'Brien to the Graduate School, University of Missouri at Kansas City, in partial fulfillment of the Doctor of Philosophy degree requirements.

The authors are deeply indebted to Midwest Research Institute for providing the analytical services.

* Present address: Nucleic Acid Research Institute, International Chemical and Nuclear Corp., Irvine, Calif.

Blood Levels of Chlorphentermine in Man

HUNG WON JUN and EDWARD J. TRIGGS

Abstract □ Blood level determinations of chlorphentermine (*p*-chloro-α,α-dimethylphenethylamine) in man following oral administration of the drug in solution, as a prolonged-release formulation, and as an intravenous injection have been carried out by gas-liquid chromatography (GLC). The drug had a long apparent elimination half-life in the body, and the therapeutic need for a prolonged-release formulation of a drug of this type therefore may be doubtful. The significance of blood level studies in evaluating therapeutic dosage levels was emphasized with respect to dosage form evaluation and pharmacokinetics, even though such studies may not represent pharmacological efficacy in cases of extensive drug localization.

Keyphrases □ Chlorphentermine blood levels—determination □ Pharmacokinetic parameters—chlorphentermine distribution, elimination □ Half-life, apparent—chlorphentermine □ GLC—analysis

Urinary excretion studies of amphetamine analogs have been carried out by a number of workers as a method for evaluating biopharmaceutical drug parameters in man (1, 2). This technique, however, is not completely satisfactory as a number of assumptions are implicit in the method (3, 4). To overcome some of these shortcomings, blood level studies have been developed for a number of these compounds (5, 6). Such studies require the use of specific and sensitive analytical procedures since these drugs are extensively concentrated extravascularly in the body (7). Both the use of radioisotopically labeled drugs (8) and electron-capture GLC (9) have been found satisfactory in such analysis.

A method to determine chlorphentermine blood levels in man was developed using flame ionization GLC, and a study made of the drug following oral administration of solution and prolonged-release formulations together with intravenous administration.

EXPERIMENTAL

Extraction Procedure and Analytical Method—The extraction procedure from blood was based on that used by Reynolds and Beckett (10) for a series of local anesthetics. A 3–5-ml. blood sample was diluted with an equal volume of water in a 15-ml. glass centrifuge tube, and 1 ml. of 20% w/v NaOH was added. Four milliliters of redistilled reagent grade diethyl ether was then added. The tube was stoppered tightly and shaken for 10 min., followed by centrifugation at 2000 r.p.m. for 5 min. The upper ethereal layer was transferred to a second centrifuge tube, and the procedure was repeated for two further ether extractions. The combined ethereal extracts were shaken twice for 10 min with 3 ml. 0.1 *N* HCl. The aqueous layer containing the extracted drug was transferred to a third centrifuge tube and adjusted to approximately pH 11 with 20% w/v NaOH (about 1 ml.) and extracted 3 times with 3 ml. diethyl ether as for blood. The ethereal layers were transferred to a 12-ml. glass sedimentation tube containing 1 ml. of a 2 mcg./ml. solution of azobenzene in diethyl ether (internal marker).

The sedimentation tube was placed in a water bath at 40° and the contents evaporated to near dryness. The tube was then stoppered and placed in an ice bath to produce a final volume of about 5 μl. A 2–3-μl. sample of this concentrate was then injected onto the gas chromatographic column.

The apparatus and conditions for GLC were as reported by Jun and Triggs (11) with the following modifications: (*a*) the gas chromatographic column was packed with 1% silicon gum rubber[1] on 80–100-mesh general-purpose solid support[2], and (*b*) helium flow rate 40 ml./min.

Blood Level Studies—*Oral Administration*—Chlorphentermine hydrochloride was administered in 20 ml. aqueous solution as a 100-mg. dose (5 mg. HCl/ml.) to four healthy male subjects (aged 25–30 years), approximately 30 min. after a light breakfast of coffee and toast. Blood samples (7 ml.) were taken intravenously using Vacutainer tubes[3] at 30-min. intervals for 3 hr. and then at 7, 11, 13, 25, 35, and 48 hr. after drug administration. Urine samples were also collected irregularly over the 48-hr. time period, and in some cases for a further 8 days.

The drug was also administered as a 78-mg. dose in the form of prolonged-release Preparation A[4] to three of the four subjects who had received the drug in solution. Blood samples were taken as previously at 60-min. intervals for 4 hr.; 120-min. intervals for a further 8 hr.; and at 24, 28, 32, 36, and 48 hr. after drug administration. Urine samples were taken as previously described.

Intravenous Administration—Chlorphentermine hydrochloride injections were prepared in the laboratory and sterilized by passing the solution through a bacterial filter[5] under aseptic conditions followed by autoclaving. The solution for injection contained 25 mg./ml. of the drug as the hydrochloride salt. The ampuls were tested for sterility[6] and for drug content by GLC.

The drug was infused intravenously over a 2-min. period as a 50-mg. dose to two of the four subjects. Blood samples were taken at 2, 5, 10, 20, 40, 60, and 120 min. and at 4, 7, 10, 24, 36, and 48 hr. after drug administration. Urine samples were taken as previously described.

RESULTS

Extraction Procedure and Analytical Method—Figure 1 shows a typical chromatogram of a blood extract containing chlorphentermine. Good symmetrical peaks were obtained for both chlorphentermine and the internal marker, azobenzene. Analysis of blank samples of blood from the subjects used in the studies showed no interfering peaks with the same retention times as chlorphentermine or azobenzene.

Figure 2 illustrates a calibration curve obtained for chlorphentermine in blood; the ratio peak height of chlorphentermine to peak height of azobenzene was plotted against the concentration of chlorphentermine per 5 ml. of blood. The calibration curve was a straight line passing through the origin.

Ten duplicate analyses of a blood sample containing 0.5 mcg. chlorphentermine/5 ml. were performed, and the results showed a standard deviation of ±4.92%.

The percentage recovery of chlorphentermine from blood was found to be 95 ± 5%.

Blood Level Studies—*Oral Administration*—Average blood levels for chlorphentermine following oral administration of the 100-mg. solution dose to four subjects are shown in Fig. 3. The pharmacokinetic parameters, apparent elimination half-life, apparent volume of distribution, and area under the blood level curve were estimated and appear together with the cumulative urine data in Table I.

Subjects experienced no stimulant effect from the drug at this dosage level. The only effects noticed were pupil dilation and marked anorexia.

[1] OV-1 Hewlett Packard Ltd.
[2] Chromosorb G(AW-DMCS), Hewlett Packard Ltd.
[3] Becton, Dickinson & Co., Canada, Ltd.
[4] Commercially available preparation of chlorphentermine hydrochloride.
[5] Standard Millipore filter type HA, B.D.H. (Canada) Ltd.
[6] USP XVII Sterility Test for injection.

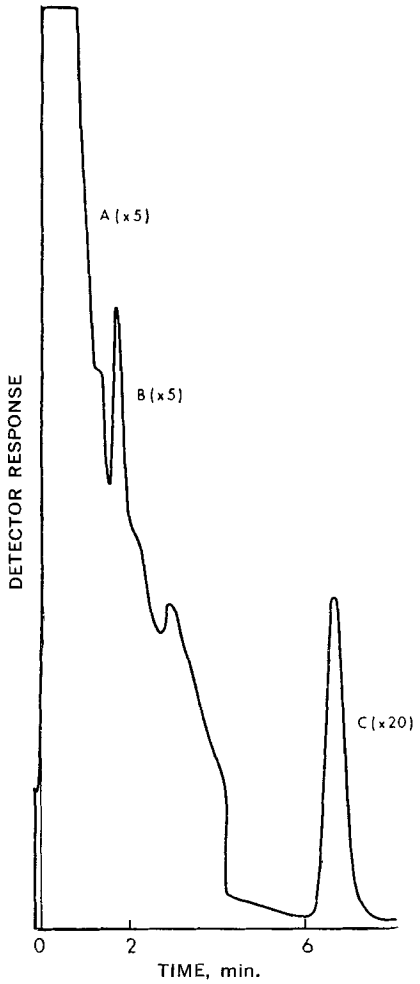


Figure 1—*Typical chromatogram of blood extract containing chlorphentermine. Key:* A = *solvent peak (diethyl ether);* B = *chlorphentermine;* C = *internal marker (azobenzene). Figures in parentheses signify attentuation.*

Average blood levels for chlorphentermine following oral administration of the 78-mg. prolonged-release formulation to three subjects are shown in Fig. 4. Areas under the blood level curves and urine data are shown in Table I. No subjective effects were noted.

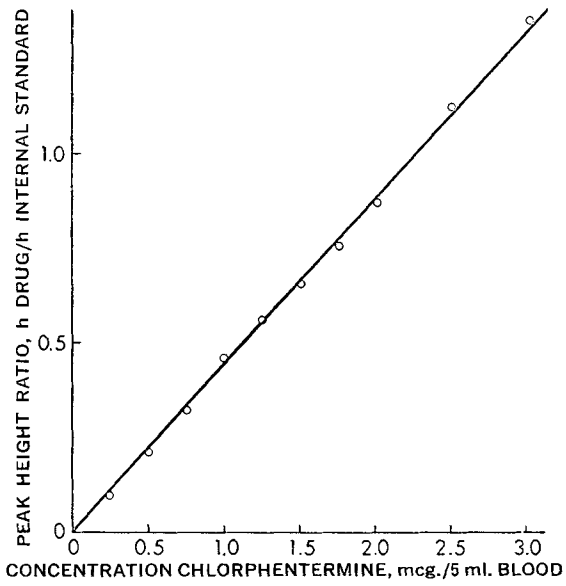


Figure 2—*Calibration curve for chlorphentermine in blood.*

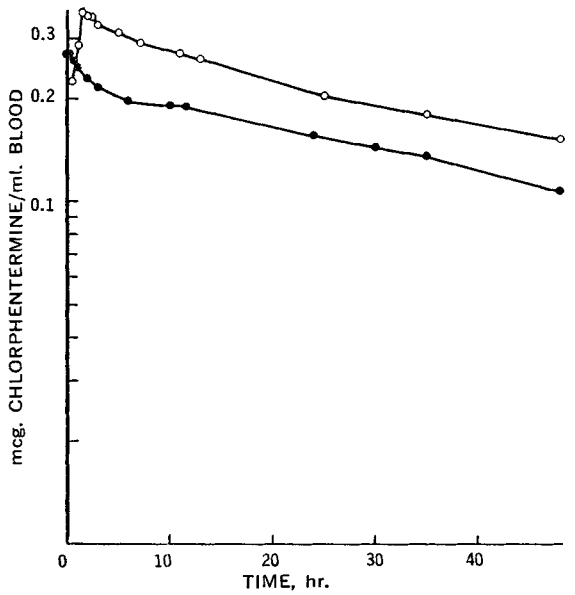


Figure 3—*Average blood level-time curves for chlorphentermine following drug administration in solution (100 mg.) and intravenous injection (50 mg.). Key:* ○, *solution; and* ●, *intravenous injection.*

Intravenous Administration—Average blood levels of chlorphentermine following intravenous administration of the drug to two subjects as a 50-mg. dose are shown in Fig. 3. The pharmacokinetic parameters and urine data appear in Table I.

Effects noted were lightheadedness and marked anorexia.

DISCUSSION

In most chromatograms the drug peak occurred on the solvent slope at the low attentuations used in these studies; however, reasonably accurate peak height measurements could be made as indicated by the size of the standard deviation. Drug concentrations as low as 125 ng./5 ml. of blood could be detected. The average blood levels for chlorphentermine (see Fig. 3) were well within the range of sensitivity of the method.

Peak blood levels for chlorphentermine in all subjects occurred at 1.5–2.5 hr. following oral administration of the drug in solution, and blood levels after the peak showed a slow exponential decline. The apparent elimination half-life for the drug ranged from 35 to 45 hr. (average 41 hr.) and was in close agreement with that obtained from predicted blood levels determined by analog computation (3). The apparent volume of distribution (average 213 l.), together with the long apparent elimination half-life, suggested extensive tissue

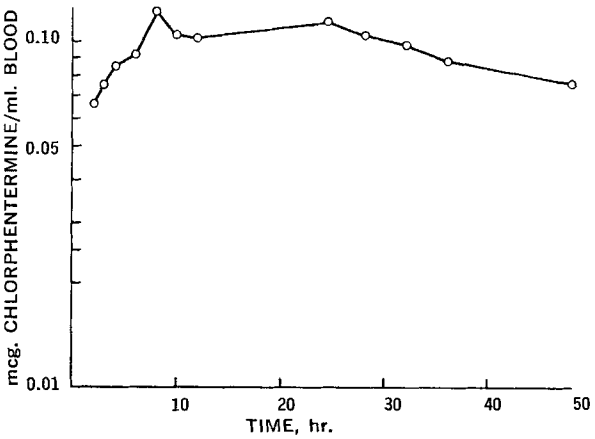


Figure 4—*Average blood level-time curve for chlorphentermine following drug administration as prolonged-release formulation A (78 mg.).*

Table I—Some Pharmacokinetic Parameters for Chlorphentermine following Solution, Prolonged-Release, and Intravenous Drug Administration in Man

Subject[a]	Mode of Administration	Dose, mg. HCl	Apparent Elimination Half-Life, hr.	Apparent Volume of Distribution[b]	Area under Blood Level Curve, mcg. hr./ml.[c]	% Dose Excreted Unchanged in Urine in 48 hr.
H.J.	Solution	100	45	185	14.5	23.3
D.S.	Solution	100	35	214	10.7	25.8
W.J.	Solution	100	45	298	8.6	19.6
K.M.	Solution	100	40	245	8.5	16.1
H.J.	i.v.	50	42	139	8.7	20.4
D.S.	i.v.	50	44	198	6.6	21.6
H.J.	Prolonged-release	78	—	—	4.5	16.4
K.M.	Prolonged-release	78	—	—	5.8	12.3
W.J.	Prolonged-release	78	—	—	3.3	14.1

[a] Incompleted crossover study. [b] See *References 1* and *21*. [c] Calculated by means of trapezoid rule (20).

distribution of the drug. These findings suggested that the drug may be localized to a large extent in the body. Previous reports (12, 13) have shown chlorphentermine to be localized in various body organs of rats and mice, notably brain and lung tissue.

Peak blood levels for chlorphentermine occurred at 6–8 hr. following oral administration of prolonged-release Preparation A, suggesting delayed release and subsequent slow absorption of the drug. The areas under the blood level curves were compared following intravenous and prolonged-release drug administrations, and an average of 62% (maximum 87%) of the drug was found to be available from the prolonged-release dosage form. However, it is possible that this average result may be an underestimate since comparisons were made only over the 48-hr. time period and ideally should be compared from zero to infinite time (22). Subject variation, which did occur (see Table I), probably also contributed to the spread of the value of the availability term.

Urinary excretion studies of chlorphentermine in this and previous studies (3) suggested almost complete drug availability from this prolonged-release preparation. Thus, the determination of drug availability from urinary excretion studies may lead to a discrepancy as the assumption is made that urine drug levels reflect tissue levels of the drug which might not be the case where some degree of localization occurs in the body.

The observations that the apparent elimination half-life of the drug was, on the average, in excess of 40 hr. and that the cumulative amount of drug in the urine in 48 hr. was less than 25% of the administered dose suggested doubts as to whether a prolonged-release drug formulation was indeed clinically necessary. It could, however, be argued that tissue levels resulting from administration of a prolonged-release formulation would perhaps follow a more clinically acceptable pattern than those levels following administration of the drug in solution. This possibility and the determination of pharmacokinetic data are the subject of further study. It has been shown previously that this particular prolonged-release formulation gives a good *in vivo/in vitro* correlation of drug availability (3).

A dose of 50 mg. of drug was chosen for the intravenous drug administration studies since it was felt that there would be less subjective effects at this dosage level. The initial phase of the blood level curve following intravenous drug administration was indicative of the extensive extravascular distribution of the drug (see Fig. 3). However, it was not possible with the blood sampling times used to extrapolate accurately this initial drug distribution phase to time zero. Reference to the area under the blood level curves (see Table I) following oral and intravenous drug administration showed that the drug was completely available when administered in solution. This confirmed results in an earlier study in which the cumulative amount of drug excreted in acid pH controlled urine over an extended period of time following drug administration in solution was about 90% (14). Also, previous reports have shown the drug to be almost completely excreted unchanged (15, 16).

It is difficult to determine the required therapeutic dose levels of a drug which is largely localized in the tissues by means of blood level studies. Ideally the concentration of drug at its site of action should be measured or a pharmacological response of the drug monitored. However, blood level studies are of importance as they enable the determination of drug pharmacokinetic parameters (17) and comparison between performance of different dosage forms to be made (18, 19).

SUMMARY

1. Blood level determinations of chlorphentermine in man following administration of the drug in solution, prolonged-release formulation, and as an intravenous injection have been carried out by GLC.

2. The apparent elimination half-life of the drug in man was on the average 41 hr.

3. Comparison of areas under the blood level–time curves for intravenous and oral solution drug administration indicated complete availability of the drug from solution. However, the area under the curve resulting from administration of the prolonged-release formulation was lower possibly due to the limited experimental time period and subject variation.

4. The shape of the blood level–time curves suggested that the drug undergoes some degree of body localization.

5. Therapeutically, prolonged-release formulations of chlorphentermine may be doubtful.

REFERENCES

(1) A. H. Beckett and G. R. Wilkinson, *J. Pharm. Pharmacol.*, **17**, 256(1965).

(2) M. Pfeffer, J. M. Schor, S. Bolton, and R. Jacobsen, *J. Pharm. Sci.*, **57**, 1375(1968).

(3) H. Jun, J. N. Hlynka, and E. J. Triggs, *Can. J. Pharm. Sci.*, to be published.

(4) G. R. Wilkinson and A. H. Beckett, *J. Pharm. Sci.*, **57**, 1933(1968).

(5) N. I. Gallagher and W. A. Knight, *J. Chronic Dis.*, **8**, 244(1958).

(6) M. Rowland, *J. Pharm. Sci.*, **58**, 508(1969).

(7) E. L. Way and T. K. Adler, *Bull. World Health Organ.*, **27**, 359(1962).

(8) E. Rosen, T. Ellison, P. Tannenbaum, S. M. Free, and A. P. Crosley, Jr., *J. Pharm. Sci.*, **56**, 365(1967).

(9) R. B. Bruce and W. R. Maynard, Jr., *Anal. Chem.*, **41**, 977 (1969).

(10) F. Reynolds and A. H. Beckett, *J. Pharm. Pharmacol.*, **20**, 704(1968).

(11) H. W. Jun and E. J. Triggs, *Can. J. Pharm. Sci.*, **3**, 73(1968).

(12) B. D. Dubnick, C. A. Towne, J. M. Hartigan, and G. E. Phillips, *Biochem. Pharmacol.*, **17**, 1243(1968).

(13) B. D. Dubnick, G. A. Leeson, R. Leverett, D. F. Morgan, and G. E. Phillips, *J. Pharmacol. Exp. Ther.*, **140**, 85(1963).

(14) H. W. Jun and E. J. Triggs, unpublished results.

(15) R. M. Portnoy, W. Weigel, E. Lambdin, I. L. Schwartz, and C. C. Smith, *Federation Proc.*, **23**, 490(1964).

(16) K. Opitz and M. L. Weischer, *Arzneimittel-Forsch.*, **16**, 1311(1966).

(17) M. Rowland and S. Riegelman, *J. Pharm. Sci.*, **57**, 1313 (1968).
(18) H. Stelmach, J. R. Robinson, and S. P. Eriksen, *ibid.*, **54**, 1453(1965).
(19) J. G. Wagner, E. S. Gerard, and D. G. Kaiser, *Clin. Pharmacol. Ther.*, **7**, 610(1966).
(20) I. S. Sokolnikoff and E. S. Sokolnikoff, "Higher Mathematics for Engineers and Physicists," 2nd. ed., McGraw-Hill, New York, N. Y., 1941, p. 555.
(21) D. S. Riggs, "The Mathematical Approach to Physiological Problems," Williams & Wilkins, Baltimore, Md., 1963, p. 213.
(22) J. G. Wagner and E. Nelson, *J. Pharm. Sci.*, **53**, 1392 (1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 15, 1969, from the *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada.*

Accepted for publication October 8, 1969.

The investigation was supported by a research grant MRC-MA-3323 from the Medical Research Council of Canada.

The authors would like to thank Warner-Chilcott Labs. (Canada) Ltd., for generously supplying them with chlorphentermine hydrochloride and for their help in obtaining volunteers for our blood level studies.

Thanks are also expressed to volunteers D. S., W. J., and K. M.

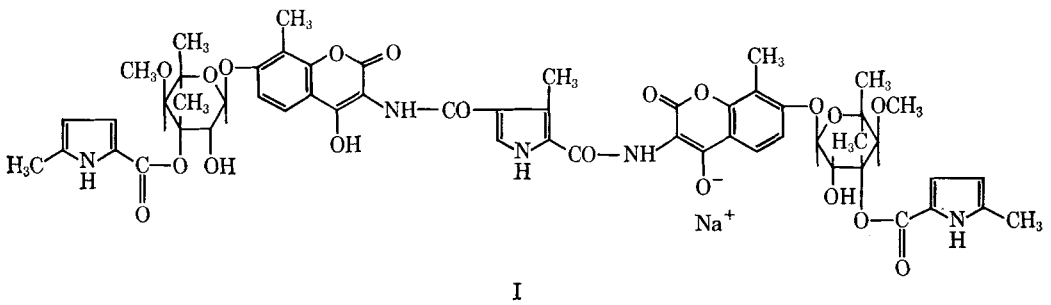
Pharmacokinetic Profile of Coumermycin A_1

STANLEY A. KAPLAN

Abstract □ The pharmacokinetic profile of coumermycin A_1 has been determined in man following intravenous and oral administration. The antibiotic is eliminated slowly from the bloodstream and appears to be highly biotransformed. The plasma level *versus* time curve after intravenous injection is consistent with a two-compartment open system containing a primary compartment with a volume equivalent to the volume of plasma water. The design of a pharmacokinetic model is discussed.

Keyphrases □ Coumermycin A_1—pharmacokinetic profile □ Absorption, elimination—coumermycin A_1 □ Model, two-compartment open—coumermycin A_1 □ Kinetic equations—coumermycin A_1 absorption, elimination

Coumermycin A_1 is an antibiotic isolated from *Streptomyces hazeliensis* var. *hazeliensis* nov. sp. which exhibits antistaphylococcal activity *in vitro* and *in vivo* (1). The compound, a monosodium salt, has a molecular weight of 1132. Coumermycin A_1 is a bishydroxy coumarin with two weakly acidic groups which are widely separated spatially in the molecule and therefore ionize simultaneously with an approximate pKa of 6. The three pyrrole groups are weakly acidic, pKa > 11, and may decrease the solubility of the compound due to hydrogen bonding. The compound is only very slightly soluble in water at 25°. The structure is given as I (2):



I

The pharmacokinetic profile of coumermycin A_1 was determined in four human subjects based on the serum level data obtained from the report of a clinical study on file (3, 4).

EXPERIMENTAL

Protocol—Four healthy human adults fasted overnight, received single intravenous and oral doses of coumermycin A_1 at the Special Treatment Unit of Martland Hospital. Subjects A and B each received single 50-mg. doses intravenously and orally, 3 weeks apart. Subjects C and D each received single 100-mg. doses intravenously and orally, 2 weeks apart. The drug was administered in the dosage forms presently used in clinical trials. Blood and urine specimens were collected periodically and the serum separated and frozen for subsequent analysis.

Microbiological Assay—Coumermycin A_1 was analyzed by the cup plate assay employing *Staphylococcus aureus* HLR No. 82. The sensitivity of the method is 0.08 mcg./ml. of biological fluid (4).

RESULTS AND DISCUSSION

The serum level data following both intravenous and oral administration of the drug are presented in Figs. 1–4. Following the intravenous injection of coumermycin A_1, a biexponential serum level curve is obtained with all four subjects. The consideration of the disposition of coumermycin A_1 in terms of a two-compartment open system model is therefore a minimal requirement in order to describe adequately the distribution of the drug into the body. The

(17) M. Rowland and S. Riegelman, *J. Pharm. Sci.*, **57**, 1313 (1968).
(18) H. Stelmach, J. R. Robinson, and S. P. Eriksen, *ibid.*, **54**, 1453(1965).
(19) J. G. Wagner, E. S. Gerard, and D. G. Kaiser, *Clin. Pharmacol. Ther.*, **7**, 610(1966).
(20) I. S. Sokolnikoff and E. S. Sokolnikoff, "Higher Mathematics for Engineers and Physicists," 2nd. ed., McGraw-Hill, New York, N. Y., 1941, p. 555.
(21) D. S. Riggs, "The Mathematical Approach to Physiological Problems," Williams & Wilkins, Baltimore, Md., 1963, p. 213.
(22) J. G. Wagner and E. Nelson, *J. Pharm. Sci.*, **53**, 1392 (1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 15, 1969, from the *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada.*

Accepted for publication October 8, 1969.

The investigation was supported by a research grant MRC-MA-3323 from the Medical Research Council of Canada.

The authors would like to thank Warner-Chilcott Labs. (Canada) Ltd., for generously supplying them with chlorphentermine hydrochloride and for their help in obtaining volunteers for our blood level studies.

Thanks are also expressed to volunteers D. S., W. J., and K. M.

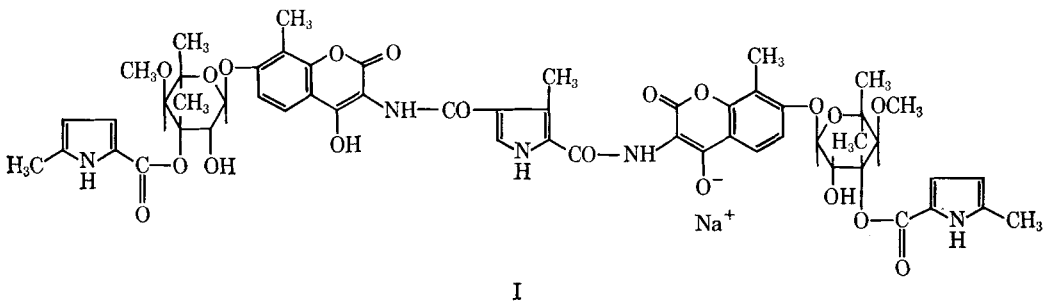
Pharmacokinetic Profile of Coumermycin A_1

STANLEY A. KAPLAN

Abstract □ The pharmacokinetic profile of coumermycin A_1 has been determined in man following intravenous and oral administration. The antibiotic is eliminated slowly from the bloodstream and appears to be highly biotransformed. The plasma level *versus* time curve after intravenous injection is consistent with a two-compartment open system containing a primary compartment with a volume equivalent to the volume of plasma water. The design of a pharmacokinetic model is discussed.

Keyphrases □ Coumermycin A_1—pharmacokinetic profile □ Absorption, elimination—coumermycin A_1 □ Model, two-compartment open—coumermycin A_1 □ Kinetic equations—coumermycin A_1 absorption, elimination

Coumermycin A_1 is an antibiotic isolated from *Streptomyces hazeliensis* var. *hazeliensis* nov. sp. which exhibits antistaphylococcal activity *in vitro* and *in vivo* (1). The compound, a monosodium salt, has a molecular weight of 1132. Coumermycin A_1 is a bishydroxy coumarin with two weakly acidic groups which are widely separated spatially in the molecule and therefore ionize simultaneously with an approximate pKa of 6. The three pyrrole groups are weakly acidic, pKa > 11, and may decrease the solubility of the compound due to hydrogen bonding. The compound is only very slightly soluble in water at 25°. The structure is given as I (2):



I

The pharmacokinetic profile of coumermycin A_1 was determined in four human subjects based on the serum level data obtained from the report of a clinical study on file (3, 4).

EXPERIMENTAL

Protocol—Four healthy human adults fasted overnight, received single intravenous and oral doses of coumermycin A_1 at the Special Treatment Unit of Martland Hospital. Subjects A and B each received single 50-mg. doses intravenously and orally, 3 weeks apart. Subjects C and D each received single 100-mg. doses intravenously and orally, 2 weeks apart. The drug was administered in the dosage forms presently used in clinical trials. Blood and urine specimens were collected periodically and the serum separated and frozen for subsequent analysis.

Microbiological Assay—Coumermycin A_1 was analyzed by the cup plate assay employing *Staphylococcus aureus* HLR No. 82. The sensitivity of the method is 0.08 mcg./ml. of biological fluid (4).

RESULTS AND DISCUSSION

The serum level data following both intravenous and oral administration of the drug are presented in Figs. 1–4. Following the intravenous injection of coumermycin A_1, a biexponential serum level curve is obtained with all four subjects. The consideration of the disposition of coumermycin A_1 in terms of a two-compartment open system model is therefore a minimal requirement in order to describe adequately the distribution of the drug into the body. The

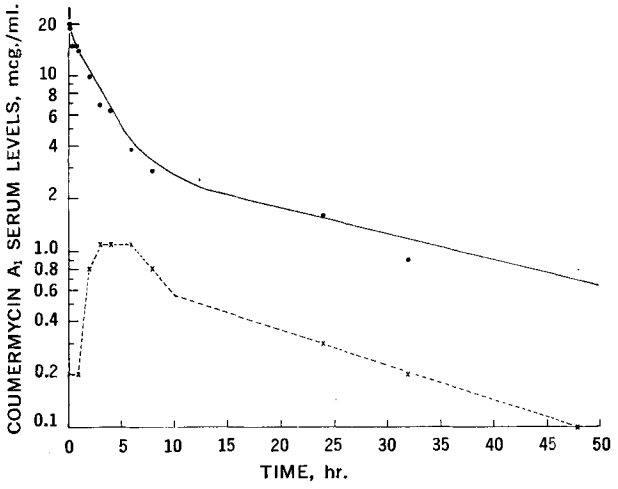


Figure 1—*Coumermycin A_1 serum levels in Subject A following the i.v. and oral administration of a single 50-mg. dose. Key:* ●, *i.v. (data point);* ×- - -, *oral (data point); and* —, *i.v. (calculated curve).*

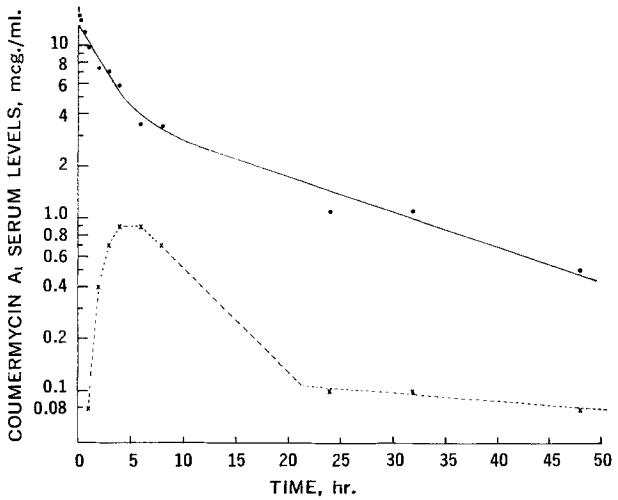


Figure 2—*Coumermycin A_1 serum levels in Subject B following the i.v. and oral administration of a single 50-mg. dose. Key:* ●, *i.v. (data point);* ×- - -, *oral (data point); and* —, *i.v. (calculated curve).*

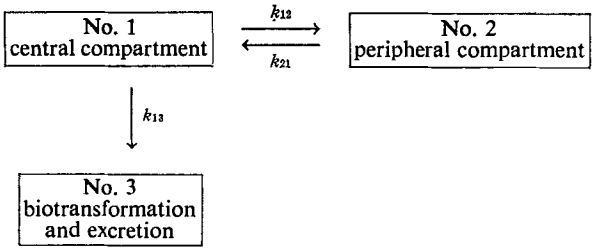
basic equations of the two-compartment open system model have been described by Riegelman *et al.* (5).

Solution of the differential equations resulting from a two-compartment open system model yields the following integrated solution describing the blood level–time curve after a single intravenous injection:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_p is the concentration of the drug in the plasma, A and B are the zero-hour ordinate axis intercepts, and α and β are both hybrid rate constants reflecting all the individual rate processes. The term $-\beta/2.303$ is the slope of the linear portion of the curve and B is its extrapolated zero-hour intercept. Resolving the curve into its two components by the method of residuals yields a second linear segment with a slope of $-\alpha/2.303$ and an extrapolated zero-hour intercept of A.

The disposition data of coumermycin A_1 were evaluated in terms of the two-compartment open system model as described by Riegelman *et al.* (5) Model I, as shown below:



where k_{12} and k_{21} are the first-order rate constants of distribution, and k_{13} is the sum of the simultaneous processes of biotransformation and excretion, all assumed to be first-order processes. The constants, A, B, α, and β, were obtained graphically. They were then used with the appropriate equations to evaluate the rate constants of the two-compartment open model shown above. In addition, the volume of the central compartment (V_p) was determined and found to range in the four subjects studied from 2.6–3.5 l. It is interesting to note that this volume corresponds almost exactly with the volume of the plasma water.

Usually when a single dose of a drug of small molecular size is injected intravenously the blood plasma does not behave as a discernible compartment. Mixing in the plasma is not instantaneous since a few recirculations of blood may be required before mixing is complete. Moreover with small molecules, filtration and diffusion out of the capillary beds is extremely rapid so that by the time the drug is uniformly distributed in the plasma, the drug has already penetrated into a much larger volume. It would be this larger volume, which includes the blood plasma, that constitutes the apparent initial volume of distribution for small drug molecules.

In contrast, coumermycin A_1 is a rather large molecule with a molecular weight of 1132 and is highly bound to plasma albumin (3). In cases where a large molecule is administered, and/or if the molecule is firmly bound, one may be able to identify a separate plasma compartment by analyzing the early part of the curve of serum level *versus* time. In fact, the V_p values of coumermycin A_1 in the four subjects studied bear this out in that the V_p range of 2.6–3.5 l. corresponds directly to the plasma water compartment *per se*. Therefore, the usual concept of a central compartment as defined by Riegelman (5) may be inappropriate for the disposition of coumermycin A_1. In the model for the disposition of coumermycin A_1 the first compartment apparently reflects solely the plasma water, while the second compartment comprises the remaining body distribution space. It is interesting to note that only one other report (6) was found with an example of a defined plasma water compartment, and this too was for a highly bound drug.

In designing a model for the disposition of coumermycin A_1 the following additional factors should be considered. The drug is not eliminated from the central or plasma compartment inasmuch as intact drug is not excreted, and biotransformation can be assumed not to occur in the plasma since the drug is not hydrolyzed or de-

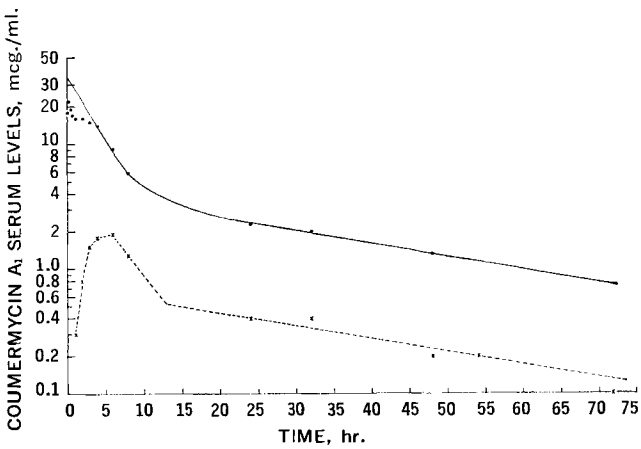
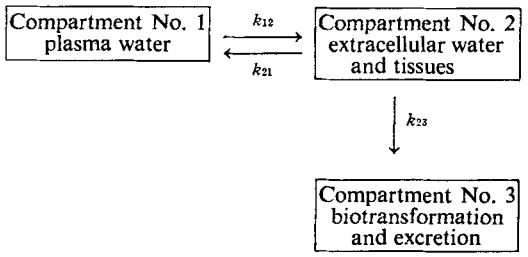


Figure 3—*Coumermycin A_1 serum levels in Subject C following the i.v. and oral administration of a single 100-mg. dose. Key:* ●, *i.v. (data points);* ×- - -, *oral (data points); and* —, *i.v. (calculated curve).*

graded in the plasma. Therefore biotransformation is occurring elsewhere in the body.

The above factors indicate that the appropriate model for the disposition of coumermycin A_1 may then be as follows (Model II):



As with the previously described model the concentration of drug in the plasma, C_p, as a function of time is given by the equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

α and β are both hybrid rate constants reflecting all the individual rate processes, while k_{12}, k_{21}, and k_{23} are individual rate constants calculable from this equation, as described in *Appendix I*.

By assigning k_{23} as the elimination rate constant in Model II we make the assumption that Compartment 2 is homogeneous, *i.e.*, the total amount of drug in the compartment is immediately accessible to the elimination mechanism. This assumption would be meaningful if the drug were distributed essentially into the liver. However, Compartment 2 in Model II represents the entire available body space minus the plasma and until such time that tissue level studies are completed the utility of Model II is governed by the accuracy of this assumption.

The rate constants associated with each of the models were calculated and are presented in Table I. In evaluating both models in terms of the disposition of coumermycin A_1, it should be noted that the elimination rate constant, k_{13} as defined in Model I, may be an overestimate of the elimination rate since the model design indicates that only the relatively small fraction of drug in the plasma compartment, Compartment No. 1, is immediately available for elimination. On the other hand the elimination rate constant, k_{23}, in Model II may be an underestimate of the elimination rate for the author is assuming that Compartment 2 is homogeneous with all the drug contained therein immediately available for elimination. The elimination rate constant, therefore could be some value other than k_{13} or k_{23}. It should also be noted that since individual compartments are not detected, the corresponding rate constants into and out of compartments are not specific in that they do not apply to any real compartments.

Although the value of the elimination rate constant is interesting from a purely pharmacokinetic point of view it is not as meaningful physiologically, in terms of the disposition of the drug as is β, the apparent elimination rate of the drug. Therefore, the inability to define the value of the elimination rate constant at this time will not alter the validity of the pharmacokinetic profile of coumermycin A_1 presented herein. In fact, one might wonder in this particular case as to the relevance of the elimination rate constant in defining the physiological disposition of a drug.

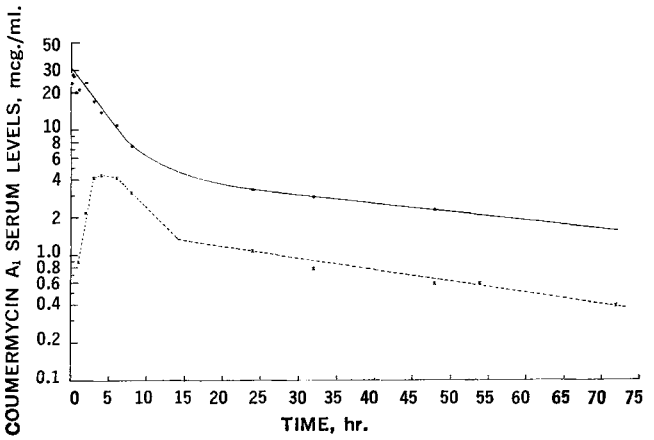


Figure 4—*Coumermycin A_1 serum levels in Subject D following the i.v. and oral administration of a single 100-mg. dose. Key: ●, i.v. (data points); ×- - -, oral (data points); and —, i.v. (calculated curve).*

Physiological Disposition Characteristics of Coumermycin A_1—In describing the physiological disposition of coumermycin A_1 many of the calculated parameters are independent of Models I and II. This model independence results from the fact that certain constants such as *A*, *B*, α, and β are the same for both models. It is these model independent parameters, therefore, which will now be discussed in terms of the pharmacokinetic profile of coumermycin A_1. They are presented in Table II. It should be noted that the simulated intravenous blood level curves in Figs. 1–4 obtained using *A*, *B*, α, and β coincide quite well with the experimentally determined data points.

Elimination Characteristics—The results in Table II indicate that coumermycin A_1 has a fast disposition rate constant, α, which ranged from 25.7–41.2%/hr., corresponding to a half-life of 2.7–1.7 hr. The slow disposition rate constant, β, derived from the slope of the linear segment of the serum level curve reflects the apparent elimination rate of the drug from the body. The slow disposition rate constant, β, ranged from 1.5–4.7%/hr., corresponding to a half-life of 46.3–14.8 hr.

It should be noted that in the four subjects studied there seems to be a trend toward a decreased elimination rate with increase of dose. However, there were too few subjects studied to make any definitive statement at this time. As seen in Table II, as the dose was increased the apparent half-life increased as follows:

Subject	Dose, mg./kg.	Half-Life of Elimination, hr.
B	0.61	14.8
A	0.78	20.6
C	1.02	28.6
D	1.85	46.3

This might be a result of saturation of plasma- or tissue-binding sites and/or saturation of the metabolizing enzymes.

Another parameter, the fraction of drug in the body in the central compartment (*fc*) during the β-phase (7) was found to be very close when calculated with the elimination rate constant from either

Table I—Rate Constants for the Disposition of Coumermycin A_1 as a Function of Model

Subject	A		B		C		D	
α, hr.$^{-1}$	0.405		0.412		0.306		0.257	
β hr.$^{-1}$	0.034		0.047		0.024		0.015	
A, mcg./ml.	15.85		9.82		31.85		28.03	
B, mcg./ml.	3.48		4.49		4.20		4.71	
	Model I	Model II	Model I	Model II	Model I	Model II	Model I	Model II
k_{12}, hr.$^{-1}$	0.201	0.338	0.178	0.297	0.145	0.273	0.144	0.222
k_{21}, hr.$^{-1}$	0.101	0.060	0.160	0.097	0.058	0.030	0.049	0.033
k_{13}, hr.$^{-1}$	0.137	—	0.121	—	0.127	—	0.079	—
k_{22}, hr.$^{-1}$	—	0.041	—	0.065	—	0.027	—	0.017

Table II—Pharmacokinetic Profile of Coumermycin A_1 in Four Human Subjects (Model Independent Parameters)

Subject	A		B		C		D	
	Model I	Model II	Model I	Model II	Model I	Model II	Model I	Model II
Dose, mg.	50		50		100		100	
Body weight, kg.	64.0		82.7		97.8		54.1	
Dose, mg./kg.	0.78		0.61		1.02		1.85	
I.V. data								
α, hr.$^{-1}$	0.405		0.412		0.306		0.257	
corresponding $t^{1/2}$, hr.	1.71		1.68		2.26		2.69	
β, hr.$^{-1}$	0.034		0.047		0.024		0.015	
corresponding $t^{1/2}$, hr.	20.6		14.8		28.6		46.3	
A, mcg./ml.	15.84		9.82		31.85		28.03	
B, mcg./ml.	3.48		4.49		4.20		4.71	
Fraction of drug in central compartment, fc								
$= \beta/k_{13}$ or	0.25	—	0.39	—	0.19	—	0.19	—
$= 1 - \beta/k_{23}$	—	0.17	—	0.28	—	0.11	—	0.2
V_p = volume of central compartment, l.	2.6		3.5		2.8		3.1	
Oral data % of dose absorbed								
Calculated from area ratio (oral/i.v.)	16.9		12.7		15.2		28.4	
Calculated from kinetic constants	16.4	14.0	13.2	13.0	14.4	13.0	30.4	28.0
Amount of drug absorbed, mg.	8.3	7.7	6.5	6.4	14.8	14.1	29.4	28.2
% of absorbed dose absorbed at:								
1 hr.	7.4	8.7	4.9	5.0	6.6	7.3	10.0	10.9
2 hr.	31.7	37.0	25.7	25.9	19.1	21.1	26.5	28.8
3 hr.	50.8	59.3	49.8	50.2	38.5	42.5	53.6	58.2
4 hr.	61.4	71.6	71.6	72.2	52.5	58.1	64.7	70.3
6 hr.	80.9	94.4	94.2	94.7	72.3	80.1	80.1	86.9
8 hr.	85.9	100	100	100	75.0	83.0	83.9	90.8
24 hr.	98.3				94.0	94.4	100	100
Absorption rate, hr.$^{-1}$	0.278	0.394	0.402	0.408	0.249	0.314	0.311	0.344

of the models in that: Model I, $fc = \beta/k_{13}$ (7) or Model II, $fc = 1 - \beta/k_{23}$.

The values reported in Table II indicated that fc ranged from 0.19–0.39 (Model I) and 0.11–0.28 (Model II).

Absorption Characteristics—Orally administered coumermycin A_1 resulted in clinically effective blood levels. Fourteen percent of the administered dose was absorbed in three of the subjects and 28% in the fourth.

The extent of absorption was determined by two different procedures. In one, the areas under the oral and intravenous serum level curves were compared to yield a measure of availability. This method would be model independent. In the other, the kinetic constants and volume of Compartment I, V_p, were used for the calculation of the percent absorbed by utilizing the equation presented by Loo *et al.* (8) for Model I, and by modifying the equation of Loo *et al.* (8) for Model II. The derivation of the modified equation is presented in *Appendix II*. The data in Table II indicate that the estimates of oral availability were practically the same when calculated with either of the models resulting in an additional model independent parameter.

The percent available drug absorbed with time has been calculated for each subject and is also reported in Table II. The mean values indicate that approximately 8% of the absorbed dose was absorbed in 1 hr., 28% in 2 hr., 53% in 3 hr. and 89% in 6 hr. In each case the serum level peaks following oral administration of the drug appear to plateau during the 3–6-hr. interval.

SUMMARY

The pharmacokinetic profile of coumermycin A_1 has been determined for four subjects following intravenous and oral administration of the drug. The apparent elimination rate from the bloodstream is slow with a half-life range of 15–46 hr. In addition, there appears to be a tendency toward a decrease in elimination rate with an increase in mg./kg. dose. The slow elimination may result in a potential for prolonged therapeutic effectiveness. The distribution characteristics are such that the drug is initially confined to the small plasma water compartment following intravenous injection. The drug appears to be highly biotransformed inasmuch as no intact drug is detectable in the urine. However, alternate routes of excretion remain to be explored.

Following the oral administration of coumermycin A_1, 14% of the administered dose was absorbed in three of the subjects and 28% was absorbed in the fourth, with a mean percent of absorbed dose absorbed with time calculated to be 8% in 1 hr., 28% in 2 hr., 53% in 3 hr., and 89% in 6 hr.

The design of a pharmacokinetic model for coumermycin A_1 is discussed with emphasis on the meaning of the elimination rate constant, as defined by each of the models.

APPENDIX I: DETERMINATION OF KINETIC CONSTANTS

Model: Compartment 1 $\underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}}$ Compartment 2 $\xrightarrow{k_{23}}$ Compartment 3

where the amount in Compartment 1 = X_p and the amount in Compartment 2 = X_t, and k_{12}, k_{21}, and k_{23} are first-order rate constants.

The differential equations for the model are:

$$\frac{dX_p}{dt} = -k_{12}X_p + k_{21}X_t \qquad \text{(Eq. 1)}$$

$$\frac{dX_t}{dt} = k_{12}X_p - (k_{21} + k_{23})X_t \qquad \text{(Eq. 2)}$$

if $X_p = X^\circ$ (administered dose) and $X_t = 0$ at time = 0, Eq. 1 and 2 can be solved by Laplace transforms so that

$$\frac{X_p}{X^\circ} = \left[\frac{k - \alpha}{\beta - \alpha}\right] e^{-\alpha t} + \left[\frac{k - \beta}{\alpha - \beta}\right] e^{-\beta t} \qquad \text{(Eq. 3)}$$

where

$$k = k_{21} + k_{23} \qquad \text{(Eq. 4)}$$

$$\alpha\beta = k_{12}k_{23} \qquad \text{(Eq. 5)}$$

$$(\alpha + \beta) = k_{12} + k_{21} + k_{23} \qquad \text{(Eq. 6)}$$

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 7)}$$

so that

$$A = \frac{C_p^\circ(k - \alpha)}{\beta - \alpha} \qquad \text{(Eq. 8)}$$

and

$$B = \frac{C_p^\circ(k - \beta)}{\alpha - \beta} \qquad \text{(Eq. 9)}$$

where

$$C_p^\circ = A + B \qquad \text{(Eq. 10)}$$

Therefore, $$k = \frac{A\beta + B\alpha}{A + B} = k_{21} + k_{23} \quad \text{(Eq. 11)}$$

from Eq. 6, $$k_{12} = \alpha + \beta - k = \alpha + \beta - \frac{A\beta + B\alpha}{A + B} \quad \text{(Eq. 12)}$$

from Eq. 5, $$k_{23} = \frac{\alpha \cdot \beta}{k_{12}} \quad \text{(Eq. 13)}$$

and therefore from Eq. 6,

$$k_{21} = \alpha + \beta - k_{12} - k_{23} \quad \text{(Eq. 14)}$$

APPENDIX II: INTRINSIC ABSORPTION RATE CALCULATION

Model: Drug at absorption site $\rightsquigarrow$ Compartment 1 $\underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}}$ Compartment 2 $\downarrow k_{23}$ Compartment 3

where the mass balance equation indicates that the amount of drug absorbed at any time, in concentration units, is defined as:

$$\left(\frac{A}{V_p}\right)_{tn} = (C_p)_{tn} + (C_t)_{tn} + (C_{me})_{tn} \quad \text{(Eq. 1)}$$

The concentration in Compartment 1 $= C_p$, and the concentration in Compartment 2 $= C_t$, and k_{12}, k_{21} and k_{23} are the first-order rate constants.

The differential equation to express the rate of change of the tissue compartment with time is

$$\frac{d(C_t)_{tn}}{dt} = -k_{21}(C_t)_{tn} + k_{12}(C_p)_{tn} - k_{23}(C_t)_{tn} \quad \text{(Eq. 2)}$$

Since the blood level data between times t_{n-1} and t_n can be approximated by a straight line segment, we write

$$(C_p)_{tn} = (C_p)_{t_{n-1}} + M\tau \quad \text{(Eq. 3)}$$

where $\tau = t_n - t_{n-1}$; $\Delta C_p = (C_p)_{tn} - (C_p)_{t_{n-1}}$ and $M = \Delta C_p/\tau$. If we take t_{n-1} as an initial time, the Eq. 2 can be written as

$$\frac{d(C_t)_{tn}}{d\tau} = -k_{21}(C_t)_{tn} + k_{12}[(C_p)_{t_{n-1}} + M\tau] - k_{23}(C_t)_{tn} \quad \text{(Eq. 4)}$$

Let $K = k_{21} + k_{23}$.

Equation 4 can be solved by Laplace transformation with initial conditions $C_t(0) = (C_t)_{t_{n-1}}$. Rewriting Eq. 4 as

$$\frac{d(C_t)_{tn}}{dt} + K(C_t)_{tn} = k_{12}[(C_p)_{t_{n-1}} + M\tau)] \quad \text{(Eq. 5)}$$

and taking the Laplace transform of both sides, we obtain

$$S(\bar{C}_t)_{tn} - (C_t)_{t_{n-1}} + K(\bar{C}_t)_{tn} = k_{12}\left(\frac{(C_p)_{t_{n-1}}}{S} + \frac{M}{S^2}\right)$$

$$(S + K)(\bar{C}_t)_{tn} = (C_t)_{t_{n-1}} + k_{12}\left(\frac{(C_p)_{t_{n-1}}}{S} + \frac{M}{S^2}\right)$$

$$(\bar{C}_t)_{tn} = \frac{(C_t)_{t_{n-1}}}{(S + K)} + \frac{k_{12}(C_p)_{t_{n-1}}}{S(S + K)} + \frac{k_{12}M}{S^2(S + K)}$$

$$= \frac{(C_t)_{t_{n-1}}}{(S + K)} + \frac{k_{12}(C_p)_{t_{n-1}}}{K}\left(\frac{1}{S} - \frac{1}{S + K}\right) + k_{12}M\left(\frac{1/K}{S^2} - \frac{1/(K)^2}{S} + \frac{1/(K)^2}{(S + K)}\right) \quad \text{(Eq. 6)}$$

taking inverse Laplace transforms on both sides of equation

$$(C_t)_{tn} = L^{-1}[(C_t)_{tn}]$$

$$= (C_t)_{t_{n-1}}e^{-K\tau} + \frac{k_{12}(C_p)_{t_{n-1}}}{K}(1 - e^{-K\tau}) + \frac{k_{12}M}{(K)^2}[\tau(K) - 1 + e^{-K\tau}] \quad \text{(Eq. 7)}$$

or

$$(C_t)_{tn} = (C_t)_{t_{n-1}}e^{-K\tau} + \frac{k_{12}(C_p)_{t_{n-1}}}{K}(1 - e^{-K\tau}) + \frac{k_{12}M}{K^2}(K\tau + e^{-K\tau} - 1) \quad \text{(Eq. 8)}$$

The third term of Eq. 8 can be expanded in terms of a two term Taylor series:

$$\frac{k_{12}}{K^2}\left(\frac{C_p}{\tau}\right)\left[1 - K\tau - \left(1 - K\tau + \frac{K^2\tau^2}{2}\right)\right] = \frac{k_{12}}{K^2}\frac{\Delta C_p}{\tau}\frac{K^2(\tau)^2}{2} = -\frac{k_{12}\tau\Delta C_p}{2}$$

Then Eq. 8 becomes

$$(C_t)_{tn} = (C_t)_{t_{n-1}}e^{-K\tau} + \frac{k_{12}(C_p)_{t_{n-1}}}{K}(1 - e^{-K\tau}) + \frac{k_{12}\Delta C_p\tau}{2}$$

i.e.,

$$(C_t)_{tn} = (C_t)_{t_{n-1}}e^{-K\tau} + \frac{k_{12}(C_p)_{t_{n-1}}}{K}(1 - e^{-K\tau}) + \frac{k_{12}\Delta C_p\tau}{2} \quad \text{(Eq. 9)}$$

Using Eq. 9, we can calculate the amount metabolized C_{me} and excreted up to time t_n in concentration units:

$$(c_{me})_{tn} = k_{23}\int_{t_0}^{t_n}(C_t)_{tn}dt \quad \text{(Eq. 10)}$$

Therefore, the amount of drug absorbed at any time in concentration units results when Eqs. 9 and 10 are substituted into Eq. 1.

$$\left(\frac{A}{V_p}\right)_{tn} = (C_p)tn' + (C_t)_{t_{n-1}}e^{-(K\tau)} + \frac{k_{12}(C_p)_{tn-1}}{K}(1 - e^{-K\tau}) + \frac{k_{12}\Delta C_p\tau}{2} + k_{23}\int_{t_0}^{t_n}(C_t)_{tn}d_t \quad \text{(Eq. 11)}$$

Eq. 11 has been programmed for computation on the GE 265 time sharing system. The slope of the percent remaining to be absorbed *versus* time plot provides the absorption rate constant of the drug.

REFERENCES

(1) E. Grunberg and M. Bennett, *Antimicrobial Agents Chemotherapy*, **1965**, p. 786.

(2) J. Berger, A. J. Schocher, A. D. Batcho, B. Pecherer, O. Keller, J. Maricq, A. E. Karr, B. P. Vaterlaus, A. Furlenmeier, and H. Spiegelberg, *ibid.*, **1965**, p. 778.

(3) H. Solomon, unpublished data on file, Hoffmann-La Roche, Nutley, N. J., 1968.

(4) J. Unowsky and M. Douglas, unpublished data on file, Hoffmann-La Roche, Nutley, N. J., 1968.

(5) S. Riegelman, J. C. K. Loo, and M. Rowland, *J. Pharm. Sci.*, **57**, 117(1968).

(6) R. Nagashima, G. Levy, and R. A. O'Reilly, *ibid.*, **57**, 1888 (1968).

(7) M. Gibaldi, R. Nagashima, and G. Levy, *ibid.*, to be published.

(8) J. C. K. Loo and S. Riegelman, *ibid.*, **57**, 918(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1969, from the *Department of Clinical Pharmacology, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication September 10, 1969.

Effect of Lipid Polarity and Cell Design on the *In Vitro* Transport of Salicylic Acid

MATTHEW A. AUGUSTINE and JAMES SWARBRICK

Abstract □ The transport kinetics of salicylic acid have been studied in a Schulman-type *in vitro* model cell containing two aqueous phases (*A* and *C*) separated by a third liquid lipid phase (*B*) simulating the biomembrane. Prime consideration was given to evaluating the effect of increasing the polarity of the lipid phase on the two forward rate constants, K_1 and K_2. While K_1 ($A \rightarrow B$) always increased with increasing polarity of the lipid phase, K_2 ($B \rightarrow C$) was found to increase or decrease depending on the stirring conditions employed in the two aqueous phases. The surface-to-volume ratio of the immiscible phases was significant in determining the magnitude of the rate constant K_1.

Keyphrases □ Salicylic acid—*in vitro* transport kinetics □ Lipid phase polarity effect—salicylic acid transport □ Surface-to-volume ratio, stirring—salicylic acid transport □ Schulman cell, evaluation—transport studies □ Partition coefficients, salicylic acid—oil-water phases

With model *in vitro* systems that employ liquid lipids to simulate the biomembrane, there is the possibility that drug concentration may build up in the lipid phase. Such an effect is in contrast to the *in vivo* situation and reduces the validity of the *in vitro* model.

The retention of drug in the lipid phase will be, in part, a function of the polarity of the lipid and this, in turn, will affect the rate constants which describe the transport of the drug through the model system. Such an effect was demonstrated by Khalil and Martin (1) who used the inverted Y-tube as an *in vitro* model. Using various single-component lipid materials as the "membrane" phase, these workers obtained forward and reverse rate constants for the transport of salicylic acid: (*a*) from an aqueous pH 2.0 phase to the lipid phase (K_1 and K_{-1}, respectively), and (*b*) from the lipid phase to a second aqueous phase buffered to pH 7.4 (K_2 and K_{-2}, respectively). It was observed, predictably, that as the polarity of the lipid phase, expressed in terms of the δ-value (2), increased, the forward rate constant K_1 increased while the forward rate constant K_2 decreased.

The *in vitro* model cell designed by Schulman (3) has been claimed to possess certain advantages over the inverted Y-tube (4). Thus, long-chain alcohols can be used as the lipid phase with less chance of emulsification; phospholipid material can also be placed at the oil-water interfaces which do not continuously expand and contract in area, as is the case with the inverted Y-tube. Accordingly, the Schulman-type cell was chosen for these *in vitro* transport studies.

As a preliminary study, the effect of polarity of the lipid phase on the transport kinetics of salicylic acid was investigated. The polarity of the oil phase was increased by the addition of isoamyl alcohol to cyclohexane, with the expectation of finding at least a qualitative correlation between these data and that of Khalil and Martin. The results obtained demonstrate, however, that the magnitude and rank order of the rate constants are dependent to a significant degree on the design of the cell and the agitation conditions used.

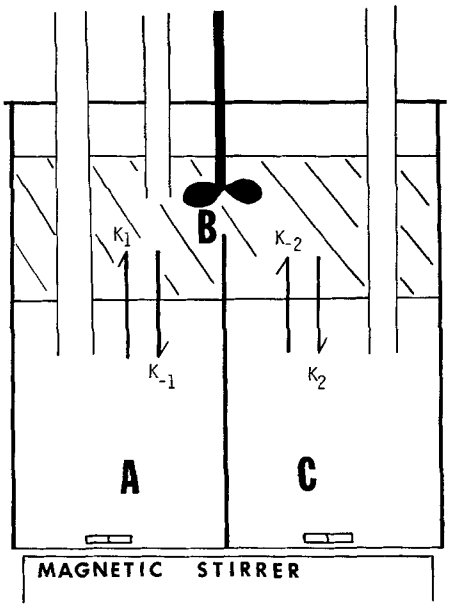


Figure 1—*Schulman-type* in vitro *model cell. See text for explanation of symbols.*

EXPERIMENTAL

***In Vitro* Model Cells**—Three Schulman-type cells, designated I, II, and III, similar to that shown in Fig. 1 were used in these preliminary studies. These model cells, constructed of Plexiglas (I, II) or glass (III), physically simulate the two membrane interfaces found *in vivo*, one between the gastrointestinal fluid (*A*) and the membrane (*B*) and one between the membrane and blood plasma (*C*). Either gastric or intestinal absorption can be studied by choosing the appropriate pH conditions for Compartment *A*, while keeping the pH of Compartment *C* constant at 7.4.

The two aqueous compartments were stirred using Teflon-coated magnetic stirring bars, and the oil phase was stirred using an overhead constant-speed motor equipped with shaft and propeller (see Tables I and II). The cells were maintained at 25 ± 0.1° in all the studies performed.

The volumes of liquid used for each of the three phases in the various cells are shown in Tables I and II. Also included in these tables are the surface-to-volume ratios of the cells, which are based on the volume of either aqueous phase and the surface area in contact with the lipid phase, *B*, in the absence of any agitation. Only slight rippling of the interfaces was noted with the various agitation conditions employed.

Materials—Salicylic acid, cyclohexane, isoamyl alcohol, and the buffer ingredients were all reagent grade. Clark and Lubs buffer solutions at pH 2.0 and pH 7.4 were prepared and their pH values checked prior to use.[1]

Procedure—In all studies, Compartment *A* was maintained at pH 2.0 while the receiving compartment, *C*, was buffered to pH 7.4.

[1] Beckman Expandomatic pH meter, Beckman Instruments, Inc., Fullerton, Calif.

Table I—Solubility, Partition Coefficients, and Forward Rate Constants for Salicylic Acid

Isoamyl Alcohol in Cyclohexane, % v/v	Solubility, g./100 ml.	Apparent Partition Coefficient[a] Oil/pH 2.0	Apparent Partition Coefficient[a] pH 7.4/Oil	Forward Rate Constants ($hr.^{-1}$)[b] K_1	Forward Rate Constants ($hr.^{-1}$)[b] K_2
5	1.74	11.0 (±0.2)	994 (±140)	0.320	0.115
10	3.13	22.1 (±0.3)	558 (±83)	0.430	0.213
20	5.58	43.7 (±1.5)	274 (±12)	0.520	0.281
30	7.96	65.9 (±6.0)	167 (±16)	0.650	0.410

[a] Average of three initial salicylic acid concentrations, 20, 40, and 50 mg. per 20 ml. (±*SD*). [b] Cell I, rectangular Plexiglas; volume of each phase is 550 ml.; surface-to-volume ratio is 0.124; aqueous phases stirred at 300 r.p.m. with 2.54-cm. (1-in.) magnetic stirring bar; oil phase stirred at 60 r.p.m.

Table II—Effect of the Surface-to-Volume Ratio and Stirring Conditions on Salicylic Acid Transport

Cell	Surface-to-Volume Ratio	Stirring Conditions	% (v/v) Isoamyl Alcohol in Cyclohexane	Forward Rate Constants ($hr.^{-1}$) K_1	Forward Rate Constants ($hr.^{-1}$) K_2
II[a]	0.148	300 r.p.m. with 2.54-cm. (1-in.) magnets	10	0.650	0.174
			20	0.752	0.202
			30	0.885	0.400
II	0.148	400 r.p.m. with 1.27-cm. (0.5-in.) circular magnets	10	0.500	0.106
			20	0.602	0.105
			30	0.652	0.089
III[b]	0.180	300 r.p.m. with 2.54-cm. (1-in.) magnets	10	0.520	0.120
			20	1.100	0.360
			30	1.250	0.410

[a] Circular glass cell; volume of each phase = 225 ml.; oil phase stirred at 60 r.p.m. [b] Rectangular Plexiglas cell; volume of each phase = 250 ml.; oil phase stirred at 60 r.p.m.

The two aqueous phases were preequilibrated with respect to the lipid phase prior to the transport studies. At various time intervals following the addition of salicylic acid into Compartment *A*, samples were removed from the aqueous and lipid phases by pipet and assayed for salicylic acid by means of previously prepared calibration curves. In all studies, Beer's law was found to be obeyed over the concentration range examined. The volume of each of the sampled phases was kept constant by replacing a quantity of either buffer or oil equal to that removed for assay. Calculations were based on the percent of salicylic acid remaining in each of the phases with corrections made for the quantity of salicylic acid removed with each sample.

Analysis of Data—The kinetics governing *in vitro* drug transport in a three-phase system have been described elsewhere (1). The solution of the differential equations involved in a consecutive reversible first-order system requires the calculation of four rate constants. These are the two forward rate constants, K_1 and K_2, which govern the transport of drug between Compartments *A* and *B* and *B* and *C*, respectively, and their corresponding reverse rate constants, K_{-1} and K_{-2}. To facilitate their determination, an analog computer[2] was employed whose output was displayed on an X-Y recorder. The analog program and the differential equations are shown in Fig. 2. The volume terms associated with the solution of these equations are absent since the volumes of all three phases were kept equal. Once goodness-of-fit of the experimental and theoretical curves was achieved with the analog computer, the resultant rate constants were then verified using a digital computer[3] programmed for the integrated forms of the differential equations (5).

Solubility of Salicylic Acid—An excess of salicylic acid was placed in 100 ml. of each of the various lipid phases contained in 125-ml. ground-glass-stoppered flasks. The flasks were shaken in a constant-temperature shaker bath for 2 weeks at 25°. Filtered, diluted samples were assayed spectrophotometrically[4] for salicylic acid, using previously determined Beer's law plots.

Determination of Partition Coefficients—Twenty milliliters of either pH 2.0 or pH 7.4 aqueous buffers containing a known amount of salicylic acid was shaken in a 60-ml. separator with an equal volume of the various lipid phases used in the transport studies. The separators were placed in a constant-temperature bath at 25° and shaken manually until equilibrium was attained. Both phases were assayed spectrophotometrically for salicylic acid and the apparent partition coefficients calculated.

RESULTS AND DISCUSSION

The percents of salicylic acid in each of the three phases at various time intervals and isoamyl alcohol concentrations are shown in Figs. 3–5 for Cell I. The disappearance of salicylic acid from the compartment buffered at pH 2.0 is plotted against time in Fig. 3. As the polarity of the lipid phase is increased by the addition of isoamyl alcohol in cyclohexane, the rate of disappearance of salicylic acid increases. Consequently, the initial rate of appearance of

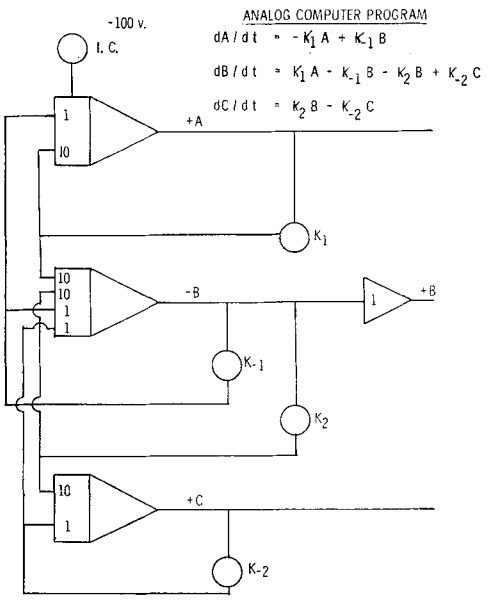


Figure 2—*Differential equations used for consecutive reversible first-order kinetics and analog computer program.*

[2] PACE 261R, EAI, Variplotter 1100, EAI, Long Island, N. Y.
[3] IBM model 360/65.
[4] Beckman model DB-G spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.

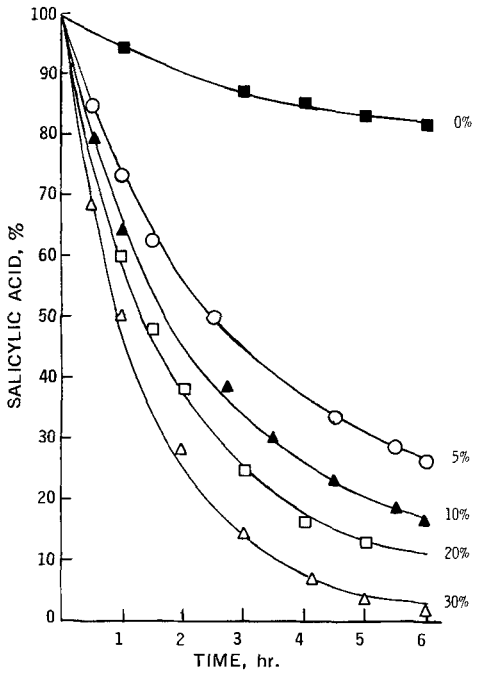


Figure 3—*Rate of disappearance of salicylic acid from Compartment* A, *buffered at pH 2.0, to Compartment* B, *composed of increasing amounts of isoamyl alcohol in cyclohexane.*

salicylic acid in the oil phase increases with increasing polarity of this phase (Fig. 4). However, while the time for the peak concentration of drug to appear in the oil phase was progressively reduced as this phase became more polar, the concentration of salicylic acid at the peak was not directly related to the polarity. The appearance of salicylic acid in the pH 7.4 buffer is shown in Fig. 5. As the polarity of the oil phase increases, so does the rate of appearance of salicylic acid into this phase.

In contrast to the results of Khalil and Martin (1), both forward rate constants, K_1 and K_2, for salicylic acid in Cell I were found to increase as the polarity of the oil phase increased. These data are presented in Table I and Fig. 6. The rank order of the K_1 values obtained with Cell I was in agreement with those previously reported. However, the reverse was apparently true in the case of the K_2 values, which increased with increased polarity of the oil phase.

In an attempt to rationalize these results, the solubility of salicylic acid in each of the various lipid phases was determined to see if saturation of this phase was occurring. While it was found that the solubility of salicylic acid increased linearly with increasing concentrations of isoamyl alcohol in cyclohexane (Table I), the concentration of salicylic acid employed in the transport studies (3.63 mg./100 ml.) was far below that needed to saturate the oil phase.

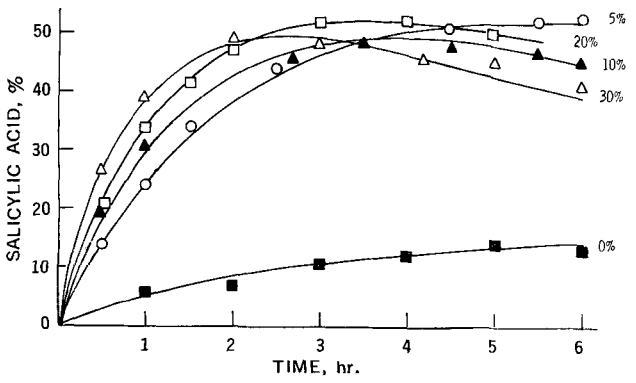


Figure 4—*Initial rate of appearance of salicylic acid in Compartment* B *as a function of the amount of isoamyl alcohol in cyclohexane.*

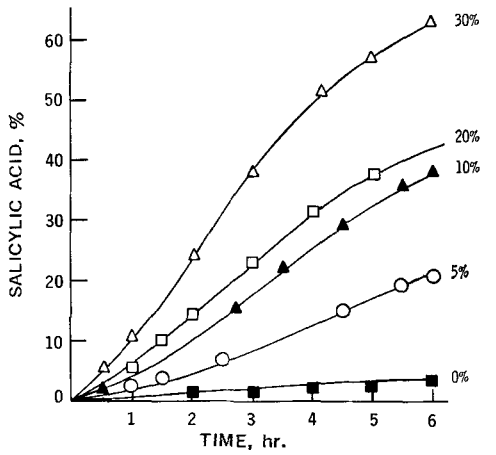


Figure 5—*Rate of appearance of salicylic acid in Compartment* C, *buffered at pH 7.4, as a function of the composition of the lipid phase.*

The next parameter investigated was the equilibrium partitioning of salicylic acid between the two aqueous phases and the various oil phases. The apparent partition coefficients were calculated based on the concentration of drug in each of the two phases at equilibrium. The possibility of dimerization of salicylic acid in the oil phase was also investigated by using initial concentrations of 20, 40, and 50 mg. of salicylic acid per 20 ml. of buffer solution. However, the data presented in Table I did not indicate the presence of a dimer in the lipid phase. As would be expected, both partition coefficients vary in a manner that is proportional to the concentration of isoamyl alcohol in the lipid phase. The relatively high standard deviations obtained for the pH 7.4/oil systems are due to the very low concentrations of salicylic acid remaining in the oil phase at equilibrium.

Although the ratio of the partition coefficient (oil/pH 2.0) to K_1 does not remain constant as the concentration of isoamyl alcohol is increased, K_1 does increase with increasing partition coefficient, as would be expected.

Predictably, the apparent partition coefficients based on the concentration of salicylic acid in the pH 7.4 buffer over that in the various lipid phases (pH 7.4/oil) were found to decrease as the polarity of the lipid phase increased. However, when these apparent partition coefficients were compared to K_2, the reverse of what would be expected, based on the partition data, occurred (Table I). Thus, the forward rate constant K_2 would be expected to decrease with increasing polarity of the lipid phase. As noted earlier, the values obtained for K_2 with Cell I increased with polarity.

Further transport studies were then carried out at 300 r.p.m. with 2.54-cm. (1-in.) magnetic stirring bars in the aqueous phases using two other model cells having surface-to-volume ratios different from that of Cell I (Tables I and II). Within any one cell, the rank order of

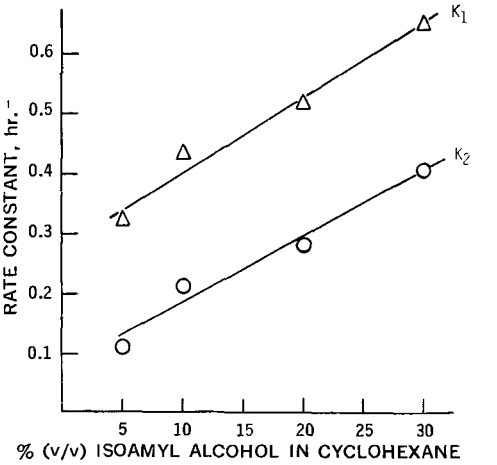


Figure 6—*The effect of increasing lipid polarity of the oil phase on the two forward rate constants,* K_1 *and* K_2, *for salicylic acid.*

K_1 with increasing polarity of the lipid phase was not changed. However, the data demonstrate a relationship between the surface-to-volume ratio and K_1. Thus, with one exception (Cell II, 10% isoamyl alcohol), the value of K_1 increased as did the surface-to-volume ratio for any one lipid phase employed. Under the same stirring conditions of the aqueous phases [namely, 300 r.p.m. with 2.54-cm. (1-in.) stirring bars] in any one cell, K_2 still showed increasing values with increasing lipid polarity. There was no rank order correlation between the value of K_2 for a particular lipid phase and the surface-to-volume ratio.

The effect of stirring conditions in the aqueous phases was then investigated. While keeping the surface-to-volume ratio constant at 0.148, the stirring conditions of the aqueous compartments were arbitrarily changed from 300 r.p.m. using 2.54-cm. (1-in.) oblong magnetic stirring bars to 400 r.p.m. using 1.27-cm. (0.5-in.) circular magnetic stirring bars. The lipid phase was stirred at the same rate of 60 r.p.m. The data, which were obtained using Cell II, are presented in Table II. It is readily apparent that the size and revolutions per minute of the stirrer did not change the order of the K_1 values, although the magnitude of this rate constant was affected. What is relevant is that, under the second set of stirring conditions, the order of K_2 now is reversed, with K_2 tending to decrease with increasing lipid polarity, as found by Khalil and Martin (1).

In light of these preliminary results, it would appear that the use of the Schulman model cell to correlate *in vitro* with *in vivo* absorption data could produce misleading results if factors such as the size and shape of the stirring bars and the rate of stirring in the various compartments are not closely monitored. The authors have also found further discrepancies between the Schulman cell and the inverted Y-tube apparatus. For example, benzene, when used as the lipid phase by Khalil and Martin, appeared to be ideal, because there was negligible retention of salicylic acid in this phase over a 24-hr. period. However, when benzene was used as the lipid phase in Cell II, appreciable retention of salicylic acid by this phase was observed over the same time period.

REFERENCES

(1) S. A. Khalil and A. N. Martin, *J. Pharm. Sci.*, **56**, 1225(1967).

(2) J. H. Hildebrand and R. L. Scott, in "The Solubility of Nonelectrolytes," 3rd ed., ACS Monograph No. 17, Reinhold, New York, N. Y., 1950.

(3) A. M. Agostini and J. H. Schulman, in "Surface Activity and the Microbial Cell," Gordon and Breach, New York, N. Y., 1965, p. 37.

(4) J. Perrin, *J. Pharm. Pharmacol.*, **19**, 25(1967).

(5) A. A. Frost and R. G. Pearson, in "Kinetics and Mechanisms," 2nd ed., Wiley, New York, N. Y., 1961, p. 176.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 14, 1969, from the *Division of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CT 06268*

Accepted for publication September 22, 1969.

Presented to the Basic Pharmaceutics Section, APhA, Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Supported by a Warner-Lambert Research Fellowship (M.A.A.) and a grant from the University of Connecticut Research Foundation.

Effect of pH of Precipitation on Antacid Properties of Hydrous Aluminum Oxide

STANLEY L. HEM*, EMANUEL J. RUSSO, SURENDRA M. BAHAL, and RALPH S. LEVI

Abstract □ The effect of the pH of precipitation on the physical and chemical properties of hydrous aluminum oxide prepared by the reaction of aluminum chloride and strong ammonia solution NF; aluminum sulfate USP, and strong ammonia solution NF; and aluminum chloride, sodium bicarbonate USP, and sodium carbonate USP, was studied. During aging, changes may occur in the hydrous aluminum oxide structure which result in a loss in acid reactivity. This loss followed apparent first-order kinetics. The rate of loss was directly dependent on the pH of precipitation and continued until a constant end-point was reached. The percentage of theoretical reactivity remaining at the end-point was inversely related to the pH of precipitation. X-ray diffraction showed no differences in form, either initially or during aging. Gel stability appears to depend on the presence of anions in the gel structure. The concentration of these anions is related to the pH of precipitation. Data are presented which demonstrate that a stable, completely acid-reactive gel would be obtained if 1 mole of a monovalent anion such as chloride or bicarbonate or 0.5 mole of a bivalent anion such as sulfate is incorporated in the gel structure per mole of aluminum.

Keyphrases □ Hydrous aluminum oxide—antacid properties, preparation □ pH precipitation effect—hydrous aluminum oxide, chemical and physical properties □ Gel stability—anion concentration data, acid reactivity kinetics □ X-ray diffraction—analysis

Alumina gel, which can be described chemically as hydrous aluminum oxide, is widely used in the management of peptic ulcer and gastric hyperacidity. Several factors, such as the precipitation temperature (1, 2), the order of addition of the reactants (2), and the concentration of the reactants (2, 3), have been shown to affect the properties of the precipitated gel. Although previous authors (4–6) have suggested that the pH of precipitation influences the crystallinity of the precipitated gel, no workers have directly examined the effect of the pH of precipitation on the acid reactivity of the gel. Therefore, the purpose of the present study was to examine the effect of the pH of precipitation on the antacid properties of hydrous aluminum oxide prepared from several reactant systems.

EXPERIMENTAL

Materials—All chemicals used were either official or reagent grade.

Method of Preparation of Hydrous Aluminum Oxide—A series of hydrous aluminum oxides, to be referred to as chloride-containing gels, were prepared at 25° by the addition of a 13% solution of strong ammonia solution NF to an aqueous 8.5% aluminum chloride heptahydrate solution. The method of Papée *et al.* (4) and the conditions described by Lewis and Taylor (2) were followed to ensure reproducible precipitations. Sufficient ammonia solution was added to control the pH of precipitation. Alumina gels were precipitated at pH 4.8, 6.1, 7.7, and 9.2 and washed with deionized water until the concentration of the chloride ion in the filtrate, as determined by the Volhard method (7), was less than 0.1%. The

K_1 with increasing polarity of the lipid phase was not changed. However, the data demonstrate a relationship between the surface-to-volume ratio and K_1. Thus, with one exception (Cell II, 10% isoamyl alcohol), the value of K_1 increased as did the surface-to-volume ratio for any one lipid phase employed. Under the same stirring conditions of the aqueous phases [namely, 300 r.p.m. with 2.54-cm. (1-in.) stirring bars] in any one cell, K_2 still showed increasing values with increasing lipid polarity. There was no rank order correlation between the value of K_2 for a particular lipid phase and the surface-to-volume ratio.

The effect of stirring conditions in the aqueous phases was then investigated. While keeping the surface-to-volume ratio constant at 0.148, the stirring conditions of the aqueous compartments were arbitrarily changed from 300 r.p.m. using 2.54-cm. (1-in.) oblong magnetic stirring bars to 400 r.p.m. using 1.27-cm. (0.5-in.) circular magnetic stirring bars. The lipid phase was stirred at the same rate of 60 r.p.m. The data, which were obtained using Cell II, are presented in Table II. It is readily apparent that the size and revolutions per minute of the stirrer did not change the order of the K_1 values, although the magnitude of this rate constant was affected. What is relevant is that, under the second set of stirring conditions, the order of K_2 now is reversed, with K_2 tending to decrease with increasing lipid polarity, as found by Khalil and Martin (1).

In light of these preliminary results, it would appear that the use of the Schulman model cell to correlate *in vitro* with *in vivo* absorption data could produce misleading results if factors such as the size and shape of the stirring bars and the rate of stirring in the various compartments are not closely monitored. The authors have also found further discrepancies between the Schulman cell and the inverted Y-tube apparatus. For example, benzene, when used as the lipid phase by Khalil and Martin, appeared to be ideal, because there was negligible retention of salicylic acid in this phase over a 24-hr. period. However, when benzene was used as the lipid phase in Cell II, appreciable retention of salicylic acid by this phase was observed over the same time period.

REFERENCES

(1) S. A. Khalil and A. N. Martin, *J. Pharm. Sci.*, **56**, 1225(1967).

(2) J. H. Hildebrand and R. L. Scott, in "The Solubility of Nonelectrolytes," 3rd ed., ACS Monograph No. 17, Reinhold, New York, N. Y., 1950.

(3) A. M. Agostini and J. H. Schulman, in "Surface Activity and the Microbial Cell," Gordon and Breach, New York, N. Y., 1965, p. 37.

(4) J. Perrin, *J. Pharm. Pharmacol.*, **19**, 25(1967).

(5) A. A. Frost and R. G. Pearson, in "Kinetics and Mechanisms," 2nd ed., Wiley, New York, N. Y., 1961, p. 176.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 14, 1969, from the *Division of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CT 06268*

Accepted for publication September 22, 1969.

Presented to the Basic Pharmaceutics Section, APhA, Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Supported by a Warner-Lambert Research Fellowship (M.A.A.) and a grant from the University of Connecticut Research Foundation.

Effect of pH of Precipitation on Antacid Properties of Hydrous Aluminum Oxide

STANLEY L. HEM*, EMANUEL J. RUSSO, SURENDRA M. BAHAL, and RALPH S. LEVI

Abstract □ The effect of the pH of precipitation on the physical and chemical properties of hydrous aluminum oxide prepared by the reaction of aluminum chloride and strong ammonia solution NF; aluminum sulfate USP, and strong ammonia solution NF; and aluminum chloride, sodium bicarbonate USP, and sodium carbonate USP, was studied. During aging, changes may occur in the hydrous aluminum oxide structure which result in a loss in acid reactivity. This loss followed apparent first-order kinetics. The rate of loss was directly dependent on the pH of precipitation and continued until a constant end-point was reached. The percentage of theoretical reactivity remaining at the end-point was inversely related to the pH of precipitation. X-ray diffraction showed no differences in form, either initially or during aging. Gel stability appears to depend on the presence of anions in the gel structure. The concentration of these anions is related to the pH of precipitation. Data are presented which demonstrate that a stable, completely acid-reactive gel would be obtained if 1 mole of a monovalent anion such as chloride or bicarbonate or 0.5 mole of a bivalent anion such as sulfate is incorporated in the gel structure per mole of aluminum.

Keyphrases □ Hydrous aluminum oxide—antacid properties, preparation □ pH precipitation effect—hydrous aluminum oxide, chemical and physical properties □ Gel stability—anion concentration data, acid reactivity kinetics □ X-ray diffraction—analysis

Alumina gel, which can be described chemically as hydrous aluminum oxide, is widely used in the management of peptic ulcer and gastric hyperacidity. Several factors, such as the precipitation temperature (1, 2), the order of addition of the reactants (2), and the concentration of the reactants (2, 3), have been shown to affect the properties of the precipitated gel. Although previous authors (4–6) have suggested that the pH of precipitation influences the crystallinity of the precipitated gel, no workers have directly examined the effect of the pH of precipitation on the acid reactivity of the gel. Therefore, the purpose of the present study was to examine the effect of the pH of precipitation on the antacid properties of hydrous aluminum oxide prepared from several reactant systems.

EXPERIMENTAL

Materials—All chemicals used were either official or reagent grade.

Method of Preparation of Hydrous Aluminum Oxide—A series of hydrous aluminum oxides, to be referred to as chloride-containing gels, were prepared at 25° by the addition of a 13% solution of strong ammonia solution NF to an aqueous 8.5% aluminum chloride heptahydrate solution. The method of Papée *et al.* (4) and the conditions described by Lewis and Taylor (2) were followed to ensure reproducible precipitations. Sufficient ammonia solution was added to control the pH of precipitation. Alumina gels were precipitated at pH 4.8, 6.1, 7.7, and 9.2 and washed with deionized water until the concentration of the chloride ion in the filtrate, as determined by the Volhard method (7), was less than 0.1%. The

Table I—Reactants for Precipitation of Chloride-Containing Alumina Gels

pH of Precipitation	Reactants: Moles $AlCl_3$/l. Washed Gel	Reactants: Moles NH_4OH/l. Washed Gel	Moles Al_2O_3/l. Washed Gel
4.8	0.294	0.80	0.144
6.1	0.294	0.84	0.158
7.7	0.294	0.88	0.129
9.2	0.294	1.68	0.129

Table II—Reactants for Precipitation of Sulfate-Containing Alumina Gels

pH of Precipitation	Reactants: Moles $Al_2(SO_4)_3$/l. Washed Gel	Reactants: Moles NH_4OH/l. Washed Gel	Moles Al_2O_3/l. Washed Gel
4.9	0.147	0.88	0.158
6.2	0.147	0.96	0.158
7.9	0.147	1.04	0.158
9.2	0.147	1.49	0.158

concentrations of the reactants required for 1 l. of washed gel and the resultant Al_2O_3 concentration for each alumina gel are listed in Table I.

A series of hydrous aluminum oxides, to be referred to as sulfate-containing gels, were prepared by the reaction of aluminum sulfate USP and strong ammonia solution NF. To ensure reproducible precipitations, the conditions described by Lewis and Taylor (2) were followed. Alumina gels were precipitated at 25° by the addition of a 13% solution of strong ammonia solution NF to an aqueous 12% aluminum sulfate USP solution. Sufficient ammonia solution was added to control the pH of precipitation. Alumina gels were precipitated at pH 4.9, 6.2, 7.9, and 9.2 and washed with deionized water until the concentration of sulfate ion in the filtrate, as determined by the USP gravimetric method (8), was less than 0.1%. The concentrations of reactants required for 1 l. of washed gel and the resultant Al_2O_3 concentration for each alumina gel are listed in Table II.

A series of hydrous aluminum oxides, to be referred to as bicarbonate-containing gels, were prepared by the reaction of aluminum chloride, sodium carbonate USP, and sodium bicarbonate USP. The alumina gels were precipitated at 25° following the conditions of Lewis and Taylor (2). An aqueous 11.4% solution of aluminum chloride heptahydrate was added to an aqueous solution containing 2.8% sodium carbonate USP and 4.5% sodium bicarbonate USP. Sufficient aluminum chloride solution or sodium carbonate USP, and sodium bicarbonate USP, solution was added to control the pH of precipitation. Alumina gels were precipitated at pH 4.8, 6.5, and 8.0 and washed with deionized water until the concentration of chloride ion in the filtrate, as determined by the Volhard method (7), was less than 0.1%. The concentrations of reactants required for 1 l. of washed gel and the resultant Al_2O_3 concentration for each alumina gel in this series are listed in Table III.

Methods of Studying Various Properties of Alumina Gels—The antacid property of each alumina gel was determined by its acid-consuming capacity. The precision of the USP acid-consuming capacity test (9) was improved by titrating to pH 3.5 rather than using bromophenol blue T.S., as given in the USP.

Table III—Reactants for Precipitation of Bicarbonate-Containing Alumina Gels

pH of Precipitation	Reactants: Moles of $AlCl_3$/l. Washed Gel	Reactants: Moles Na_2CO_3/l. Washed Gel	Reactants: Moles $NaHCO_3$/l. Washed Gel	Moles Al_2O_3/l. Washed Gel
4.8	0.864	0.538	1.260	0.386
6.5	0.755	0.570	1.336	0.376
8.0	0.755	0.856	2.012	0.376

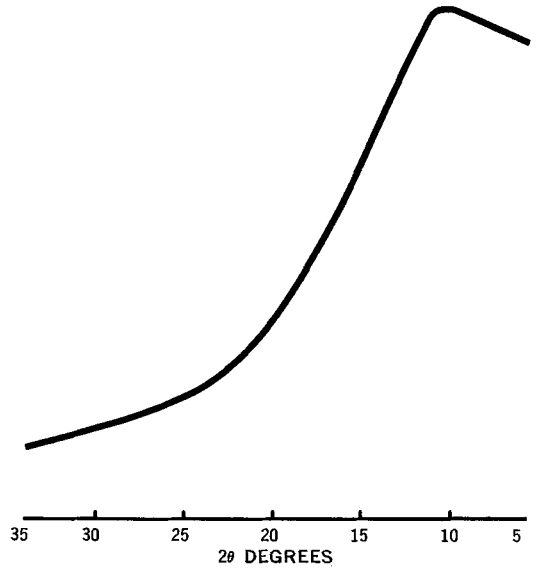


Figure 1—*Initial X-ray diffraction pattern of the chloride-containing alumina gel precipitated at pH 7.7.*

The theoretical acid-consuming capacity of each alumina gel is based on the stoichiometric reaction of the aluminum oxide in the alumina gel and 0.1 *N* HCl. The antacid property of the aged alumina gel is given as a percentage of the theoretical acid-consuming capacity.

The X-ray diffraction pattern of each alumina gel was determined in the colloidal state by irradiating a sample in dialyzer tubing.

The Al_2O_3 content of each washed alumina gel was determined by the ethylenediaminetetraacetic acid titration (10).

The concentration of chloride ion was determined by the Volhard method (7). Total chloride was determined by dissolving the gel in nitric acid and assaying for the chloride ion as described. The amount found in the filtrate and the amount which could be eluted with deionized distilled water from an equal quantity of untreated gel were determined. The difference was regarded as representing the chloride content of the gel structure.

The concentration of sulfate ion in the gel structure was determined by the USP gravimetric method (8). The total sulfate in the alumina gel was determined by dissolving the alumina gel in nitric acid prior to sulfate assay. The amount found in the filtrate and the amount which could be eluted with deionized distilled water from an equal quantity of alumina gel was also determined. The

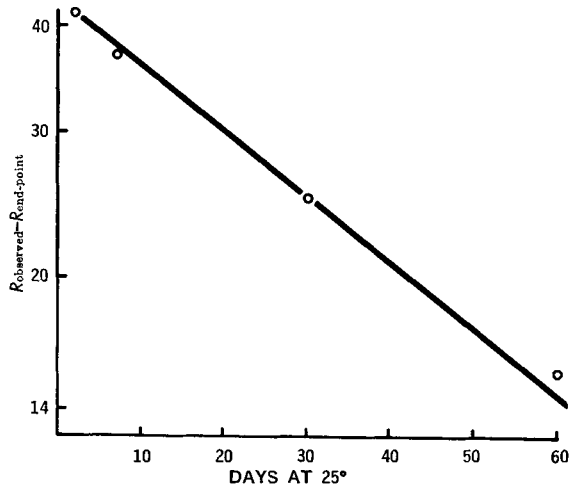


Figure 2—*Change in acid reactivity during aging of the chloride-containing alumina gel precipitated at pH 6.1.*

Table IV—Effect of Aging at 25° on the Acid Reactivity of Chloride-Containing Alumina Gels Precipitated at Various pH Conditions

pH of Precipitation	% of Theoretical Acid-Consuming Capacity after Storage at 25° for 1 Day	2 Days	7 Days	1 Month	2 Months	6 Months	1 Year	2 Years
4.8	85	—	—	82.5	—	75	70	70
6.1	—	91.5	87	75	66	50	50	50
7.7	59	—	—	37	27	25	25	25
9.2	27.5	24.3	20	10	—	10	9	10

difference was regarded as representing the sulfate content of the gel structure.

The amount of CO_2 which was evolved from the gel structure was determined by the gasometric determination of carbon dioxide (11). A volume of gel was filtered and washed with previously boiled deionized distilled water. The CO_2 which resulted from the addition of hydrochloric acid USP to the retained solids was taken as the CO_2 evolved from the gel structure.

RESULTS AND DISCUSSION

Chloride-Containing Alumina Gels—When initially precipitated, all the chloride-containing alumina gels were colloidal when examined by optical microscope and amorphous when examined by X-ray diffraction. Figure 1 shows a typical X-ray diffraction pattern. The broad, diffuse band is typical of scattering by amorphous material. The other gels in this series had similar diffraction patterns, indicating that form is independent of the pH of precipitation.

All the alumina gels studied showed a decrease in acid reactivity upon aging at 25° until they reached a constant end-point acid reactivity (Table IV). The data of Table IV were used to construct linear plots from which rate constants were calculated. Figure 2 shows the plot for the gel precipitated at pH 6.1. Each plotted point represents the logarithm of the difference between the reactivity observed at some point in aging ($R_{observed}$) and that observed at the end-point ($R_{end\text{-}point}$). The plots for all gels were linear, indicating that the loss of acid reactivity follows apparent first-order kinetics.

The rate of loss of acid reactivity is directly dependent upon the pH of precipitation (Fig. 3). Alumina gels which were precipitated at a relatively high pH showed high rates of loss of reactivity. For example, the alumina gel precipitated at pH 9.2 lost reactivity 20 times faster at 25° than the one precipitated at pH 4.8.

The end-point acid reactivity is inversely related to the pH of precipitation (Fig. 4). For example, the gel precipitated at pH 9.2 had an end-point reactivity of only 10% of theory, while the gel precipitated at pH 4.8 was 70% reactive at its end-point.

The end-point acid reactivity does not appear to be a function of the aging temperature, although the rate of loss of activity increases with increasing temperature. The alumina gel precipitated at pH 4.8 reached the same end-point reactivity of 70% of theory whether aged at 25 or 35°, but it reached it sooner at 35° than at 25°.

The losses in acid reactivity observed in these studies appear irreversible. Samples of an alumina gel, which had been precipitated at pH 7.7 and had reached its end-point reactivity of 25% of theory, did not regain any reactivity when adjusted to pH 5.5 or 4.5 with 0.1 *N* HCl and aged for 1 month at 25°.

The changes in acid reactivity on aging do not appear to be due to crystallization, since all the end point alumina gels were found to be colloidal by optical microscopy and amorphous by X-ray diffraction.

A probable explanation for the effect of pH on the acid reactivity of these gels lies in the effect of chloride ion in stabilizing the gel

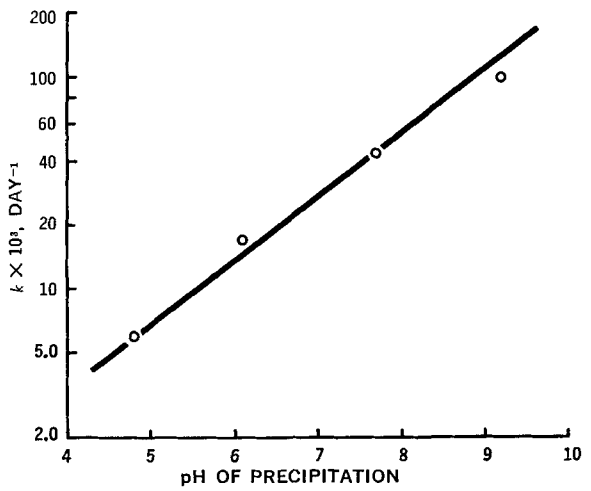


Figure 3—*Effect of pH of precipitation of chloride-containing alumina gels on the rate of change of acid reactivity at 25°.*

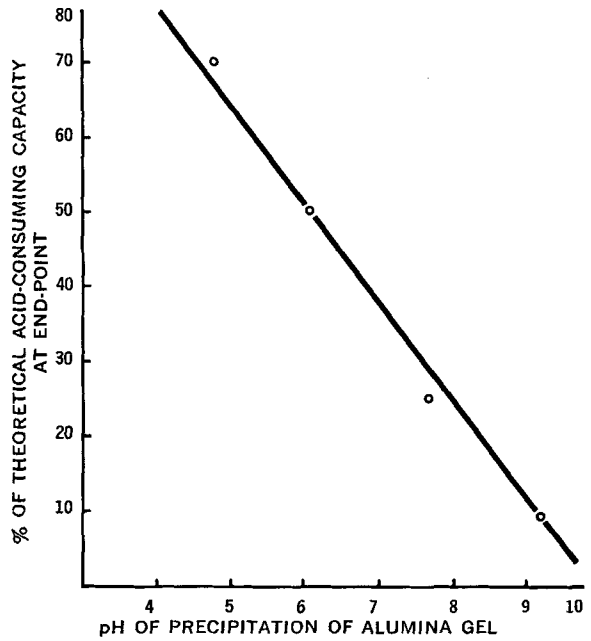


Figure 4—*Effect of pH of precipitation of chloride-containing alumina gels on the end-point acid reactivity.*

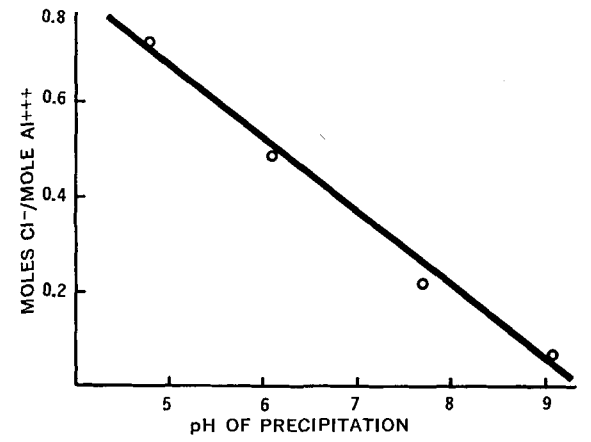


Figure 5—*Effect of pH of precipitation on the concentration of chloride ions in the chloride-containing alumina gels.*

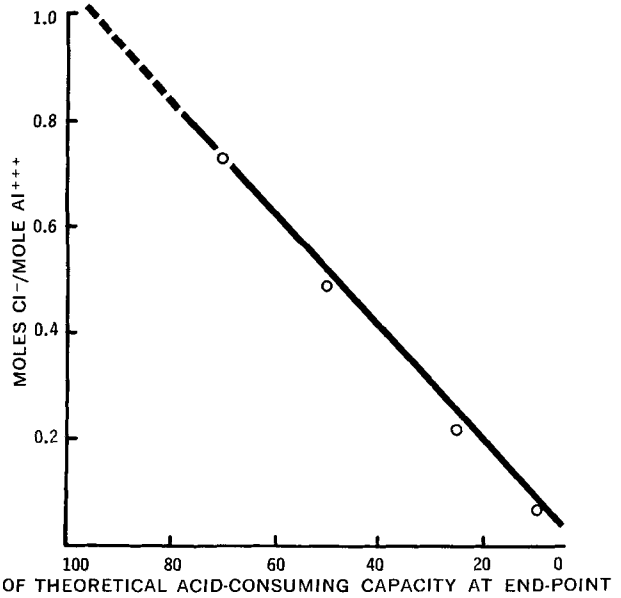


Figure 6—*Effect of chloride ion concentration on the end-point acid reactivity.*

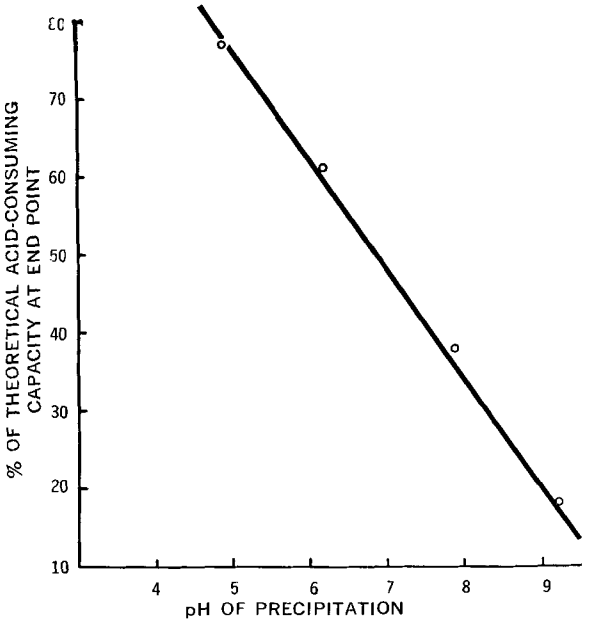


Figure 8—*Effect of pH of precipitation of sulfate-containing alumina gels on the end-point acid reactivity.*

structure. A varying concentration of chloride ion was found in the gel structure depending on the pH of precipitation (Fig. 5). The concentration did not change during the aging of these gels. A high chloride ion concentration appears to favor stability (Fig. 6). For example, the gel precipitated at pH 4.8, which retained 70% of its theoretical acid-consuming capacity at its end-point, had a high chloride ion concentration, while the alumina gel precipitated at pH 9.2, which lost virtually all its acid reactivity after 1 month at 25°, had little chloride ion in its gel structure. Figure 6 also indicates by extrapolation that a stable 100% acid-reactive gel would be obtained if 1 mole of chloride ion were present in the gel structure for every mole of aluminum. Unfortunately, this ideal gel cannot be produced from this system due to the solubility of the gel at a low pH.

Sulfate-Containing Alumina Gels—This series of alumina gels when initially precipitated were colloidal when examined by optical microscope and amorphous when examined by X-ray diffraction. The X-ray diffraction pattern was similar to that obtained for the chloride-containing gels (Fig. 1).

All the sulfate-containing gels studied showed a decrease in acid reactivity upon aging at 25° until a constant reactivity was reached. This behavior is similar to that observed for the series of chloride-containing gels. The rate of loss of acid reactivity is directly dependent on the pH of precipitation (Fig. 7).

The end-point reactivity is related to the pH of precipitation through an inverse linear relationship (Fig. 8). The changes in acid reactivity on aging do not appear to be due to crystallization, since all the sulfate-containing gels at their end point were found to be colloidal when observed by optical microscope and amorphous by X-ray diffraction.

A varying concentration of sulfate ion was found in the gel structure, depending on the pH of precipitation (Fig. 9). As was noted for the chloride-containing gels, a high concentration of sulfate ion in the gel structure favors gel stability (Fig. 10). It is predicted from Fig. 10 that a stable, 100% acid-reactive gel would be obtained if 0.5 mole of sulfate ion was present per mole of aluminum.

Bicarbonate-Containing Alumina Gels—The alumina gels precipitated at pH 4.8, 6.5, and 8.0 by the reaction of aluminum chloride, sodium carbonate USP, and sodium bicarbonate USP, were colloidal and amorphous, similar to Fig. 1, when initially precipitated.

The bicarbonate-containing gels precipitated at pH 6.5 and 8.0 retained complete acid reactivity when stored for 2 years at 25° (Table V). The gel precipitated at pH 4.8 reached an end-point reactivity of 85% of theory. All the gels remained colloidal and amorphous during aging. Less than 0.03 mole of chloride ion per mole of aluminum was found in these gels. However, 1.01 and 1.07 moles of CO_2 per mole of aluminum were evolved when the gels precipitated at pH 8.0 and 6.5 were dissolved in hydrochloric acid USP. The gel precipitated at pH 4.8 evolved 0.72 mole of CO_2 per mole of aluminum. In the pH range of 4.8 to 8.0, bicarbonate ion would be the source of the evolved CO_2. Thus a stable, 100% acid-reactive gel was obtained when 1 mole of bicarbonate ion was present in the gel structure for every mole of aluminum. In this

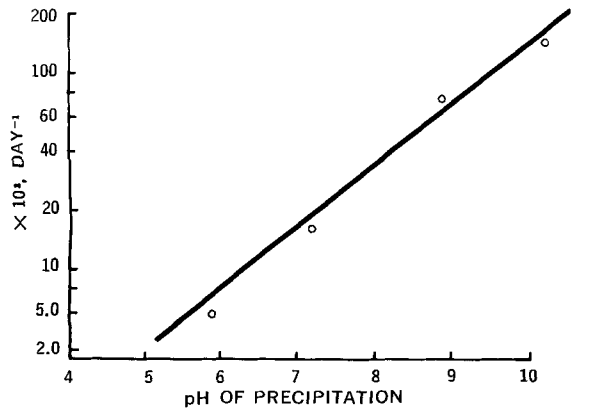


Figure 7—*Effect of pH of precipitation of sulfate-containing alumina gels on the rate of change of acid reactivity at 25°.*

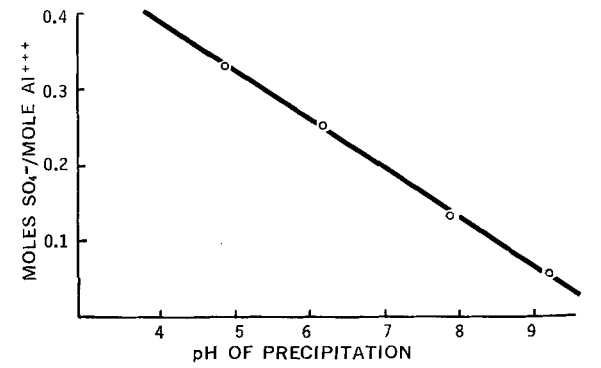


Figure 9—*Effect of pH of precipitation on the concentration of sulfate ions in the sulfate-containing alumina gels.*

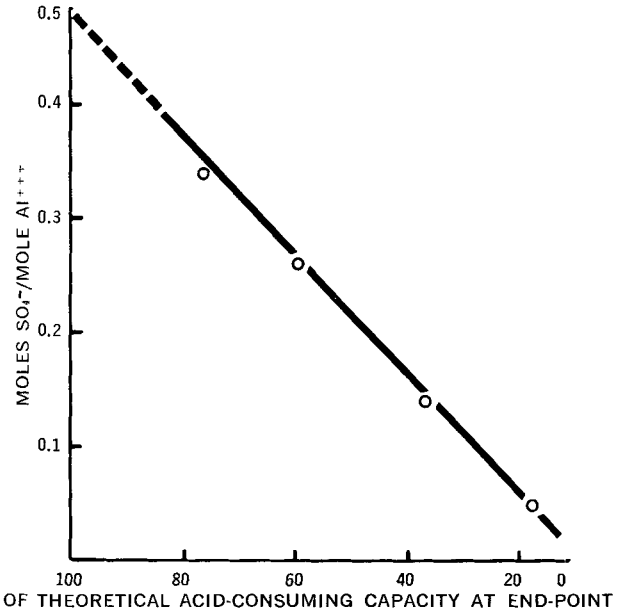


Figure 10—*Effect of sulfate ion concentration on the end-point acid reactivity.*

system the optimum concentration of bicarbonate ion was incorporated into the gel structure when the pH of the reaction mixture was 6.5 to 8.0. Effervescence occurred when the pH of the reaction mixture was below pH 5.5. Thus CO_2 was driven from the reaction mixture of the gel precipitated at pH 4.8, which resulted in a lower bicarbonate concentration in the gel structure.

It is concluded that the chloride-, sulfate-, and bicarbonate-containing gels studied may be in a highly unstable form when initially precipitated. The degree of instability is a function of the pH of precipitation. During aging, changes may occur in the gel structure which result in a loss in acid reactivity. Anions such as chloride, sulfate, and bicarbonate present in the reaction mixture are incorporated into the gel structure and act to stabilize the gel. A stable, completely reactive gel is expected if 1 mole of a monovalent anion such as chloride or bicarbonate or 0.5 mole of a bivalent anion such as sulfate is present in the gel structure per mole of aluminum.

SUMMARY

Hydrous aluminum oxide prepared by the reaction of aluminum chloride and strong ammonia solution NF; aluminum sulfate USP and strong ammonia solution NF; and aluminum chloride, sodium bicarbonate USP, and sodium carbonate USP, may exhibit an apparent first-order decrease in acid reactivity upon aging until a constant end-point is reached. The rate of loss of reactivity and the end-point reactivity are related to the pH of precipitation. The end-point reactivity is independent of storage temperature and was found to be irreversible. According to X-ray diffraction, these changes are not related to changes in form. It is concluded that anions present in the reaction mixture are incorporated into the gel structure, depending on the pH of precipitation, and act to stabilize the gel. A stable, completely acid-reactive gel is obtained if 1 mole of a monovalent anion such as chloride or bicarbonate or 0.5 mole of a bivalent anion such as sulfate is incorporated in the gel structure per mole of aluminum.

Table V—Effect of Aging at 25° on the Acid Reactivity of Bicarbonate-Containing Alumina Gels Precipitated at Various pH Conditions

pH of Precipitation	Moles CO_2 Evolved/ Mole Al^{+++}	% of Theoretical Acid-Consuming Capacity after Storage at 25° for		
		Initial	30 Days	2 Years
4.8	0.72	100	85	85
6.5	1.07	100	100	100
8.0	1.01	100	100	100

REFERENCES

(1) D. Cismaru, J. Iordachescu, G. Georgescu, and V. Cristescu, *Rev. Roum. Chim.*, **10**, 803(1965).

(2) J. A. Lewis and C. A. Taylor, *J. Appl. Chem.*, **8**, 223(1958).

(3) P. H. Hsu and T. F. Bates, *Mineral. Mag.*, **33**, 749(1964).

(4) D. Papée, R. Tertian, and R. Biais, *Bull. Soc. Chim. France*, **1958**, 1301.

(5) V. P. Souza Santos, A. Vallejo-Freire, and H. L. Souza Santos, *Kolloid Z.*, **133**, 101(1953).

(6) J. H. L. Watson, J. Parsons, A. Vallejo-Freire, and P. Souza Santos, *ibid.*, **140**, 102(1955).

(7) H. A. Laitinen, "Chemical Analysis," McGraw-Hill, New York, N. Y., 1960, pp. 214–216.

(8) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 933.

(9) *Ibid.*, p. 26.

(10) H. A. Laitinen, "Chemical Analysis," McGraw-Hill, New York, N. Y., 1960, pp. 242–243.

(11) "Official Methods of Analysis of the Association of Official Agricultural Chemists," 9th ed., Association of Official Agricultural Chemists, Washington, D. C., 1960, p. 773.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Pharmacy Research and Development Division, Wyeth Laboratories, Inc., Radnor, PA 19087*

Accepted for publication October 28, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors wish to thank Mr. J. A. Lash, Wyeth Laboratories, Inc., for chemical analyses; and Dr. T. H. Doyne, Department of Chemistry, Villanova University, Villanova, PA 19085, for analyzing the X-ray diffraction patterns.

* Present address: School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907

Prazepam Metabolites in Dog Urine

FREDERICK J. DiCARLO and JEAN-PAUL VIAU

Abstract □ Within 24 hr. after receiving a single oral dose (10 mg./kg.) of ^{14}C-labeled prazepam, two dogs excreted small quantities (4.6 and 1.8%) of the radioactivity into the urine. Thin-layer chromatograms showed the presence of at least eight radioactive compounds in these urine collections. Six of the labeled compounds were identified, and accounted for 86% of the ^{14}C in the urine from Dog 1 and 95% of the ^{14}C in the urine from Dog 2. These compounds and their contributions to the urinary radioactivity were: prazepam (1%, Dog 1; 0.2%, Dog 2); desalkylprazepam (2%, Dog 1; 0.4%, Dog 2); 3-hydroxyprazepam glucuronide (9%, Dog 1; 11%, Dog 2); oxazepam (14%, Dog 1; 3%, Dog 2); oxazepam glucuronide (52%, Dog 1; 72%, Dog 2), and 4′-hydroxyoxazepam glucuronide (8%, Dogs 1 and 2).

Keyphrases □ Prazepam-^{14}C metabolites—urinary excretion, dog □ Metabolites, prazepam—isolation, identification □ TLC—separation, identification □ Scintillometry—analysis

The identification of prazepam metabolites was of interest because it appeared that the drug might serve as the precursor to a series of tranquilizers. A previous study (1) demonstrated that prazepam is converted into one established tranquilizer, oxazepam, by the dog. Dealkylation and hydroxylation are involved in transforming prazepam into oxazepam and it was interesting to learn whether one or both oxidative sequences actually occur *in vivo*. Also in question was whether the dog hydroxylates the 5-phenyl group of benzodiazepins as does the rabbit (2). These points were investigated both qualitatively and quantitatively with the aid of ring-labeled prazepam.

EXPERIMENTAL

Reference Compounds—*N*-Phthalimidoacetyl-5-chloro-2-cyclopropylmethylamino-benzophenone-(carbonyl-^{14}C) was treated with hydrazine to form *N*-glycyl-5-chloro-2-cyclopropylmethylamino-benzophenone-(carbonyl-^{14}C) which cyclized spontaneously to yield 7-chloro-1-(cyclopropylmethyl)-5-phenyl-1*H*-1,4-benzodiazepin-2(3*H*)-one-5-^{14}C (3). The product, better known as prazepam, showed chemical and radiochemical purity in excess of 99%. Its specific activity was 1.31 mc./g.

^{14}C-2-Cyclopropylmethylamino-5-chlorobenzophenone (CACB) was prepared by hydrolyzing 1 mg. of ^{14}C-prazepam in 0.5 ml. of 6 *N* HCl for 1 hr. in a boiling water bath (4). For chromatography, 5 ml. of water was added to the reaction mixture, the solution was neutralized partially with NaOH, and the product was extracted with ethyl acetate. Upon chromatography in a variety of solvents, the reaction product yielded a single radioactive spot which was yellow and did not react as a primary aromatic amine (5).

Other available reference compounds were desalkylprazepam, 3-hydroxyprazepam, oxazepam, 2-amino-5-chlorobenzophenone (ACB), and 4′-hydroxy-2-amino-5-chlorobenzophenone (4′-hydroxy ACB). The chemical purity of these preparations was >95%.

Protocol—Two male mongrel dogs of beagle conformity were used. Their weights were 8.6 and 10.5 kg. Each animal was fed a capsule containing ^{14}C-prazepam equivalent to 10 mg./kg. body weight and placed into a separate metabolic cage. Urine was collected at 24-hr. intervals for 3 days and counted by scintillation spectrometry.

The 0–24-hr. urine collections were used to identify drug metabolites as outlined in Fig. 1. The plan was based upon: (*a*) hydrolysis of the urinary drug metabolites to ACB and its derivatives, (*b*) extraction of unconjugated urinary metabolites, and (*c*) enzymatic hydrolysis of the conjugated metabolites followed by extraction of the aglycones.

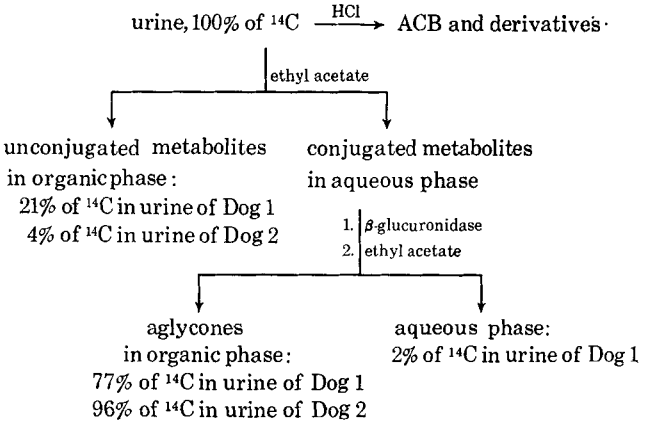


Figure 1—*Plan for identifying prazepam metabolites in dog urine. The urine, unconjugated metabolite fraction, aglycone fraction, and hydrolysate were submitted to TLC in four solvents.*

Hydrolysis of Drug Metabolites—Walkenstein *et al.* (4) described a method of quantitatively hydrolyzing diazepam, oxazepam and their analogs to their corresponding 2-amino-5-chlorobenzophenones. This procedure was used for the identification of prazepam metabolites. A 10-ml. portion of the dog urine was hydrolyzed by heating with an equal volume of concentrated HCl for 1 hr. at 100°. The hydrolysate was kept ice cold during partial neutralization by the addition of 4.4 g. of NaOH (4.8 g. required for neutralization). The acidic solution was extracted with three 5-ml. portions of ethyl acetate. All of the urinary ^{14}C was recovered in the ethyl acetate. The extracts were combined and concentrated to about 1 ml. for chromatography.

Extraction of Unconjugated Metabolites—A 50-ml. portion of the urine was adjusted to pH 7.0 and extracted with five 20-ml. portions of ethyl acetate. The combined ethyl acetate extracts were evaporated to about 1.5 ml.

Extraction of Aglycones of Conjugated Metabolites—After extracting the unconjugated metabolites, the urine was brought to pH 5.0–5.5 and incubated overnight at 37° with 50,000 units of β-glucuronidase. Then the pH was adjusted to 7.0 and the solution was extracted five times with 20-ml. portions of ethyl acetate. The combined organic phase was evaporated to about 2 ml.

Thin-Layer Chromatography—Submitted to chromatography were the reference compounds; untreated urines; hydrolyzed urines; extracts of unconjugated metabolites; extracts of the aglycones of conjugated metabolites; hydrolysates of authentic prazepam, oxazepam, 3-hydroxyprazepam, and desalkylprazepam; and hydrolysates of fractions eluted from chromatograms. All of the chromatograms were developed on glass plates coated with silica gel G. The solvent systems employed were 206: benzene–ethyl acetate (5:1), 304: chloroform–acetic acid–methanol (15:1:4), 306: chloroform–ethanol–acetone (8:1:1), and 307: chloroform–acetone (9:1).

Rechromatography—Rechromatography was conducted in order to confirm R_f data, to obtain R_f values in other solvents, and to check the purity of fractions. The technique for collecting a given radioactive fraction from TLC plates simply involved scraping the area from several plates, eluting the fraction with ethyl acetate, and concentrating the eluate.

Cochromatography—Cochromatography refers to spotting a mixture of an unknown and a reference compound and developing

Table I—TLC of Reference Compounds

Compound	R_f Values—Solvent 206	Solvent 304	Solvent 306	Solvent 307
Prazepam	0.45	0.97	0.88	0.60
Oxazepam	0.04	0.88	0.55	0.15
3-Hydroxyprazepam	0.35	0.96	0.78	0.52
Desalkylprazepam	0.11	0.95	0.72	0.30
ACB[a]	0.77	0.95	0.90	0.85
CACB[b]	0.95	0.97	0.95	0.97
4′-Hydroxy ACB	0.44	0.97	0.80	0.55

[a] 2-Amino-5-chlorobenzophenone. [b] 2-Cyclopropylmethylamino-5-chlorobenzophenone.

the chromatogram. This procedure was employed to detect any minor difference between the migration of the unknown and known compounds.

Detection Methods Applied to Chromatograms—The radioactive bands on the chromatograms were scanned with a Packard model 7201 radiochromatogram scanner to determine their R_f values. Chromatograms of the hydrolysis products, with the benzophenones (ACB or CACB) as the major components, showed distinct yellow spots for these compounds. ACB, a primary aromatic amine, was detected at low levels by running the Bratton-Marshall assay (5) directly on chromatograms. This was done by spraying chromatograms successively with 4 *N* HCl, 0.5% sodium nitrite, 0.5% ammonium sulfamate, and finally 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Prazepam, desalkylprazepam, and 3-hydroxyprazepam were sometimes located on chromatograms by enclosing the plates in a cylinder with crystalline iodine. The iodine vapor caused the compounds to become yellow. Some plates were sprayed with a phenol reagent to detect 4′-hydroxy ACB. The reagent was freshly prepared by dissolving 1.0 g. of ferric chloride and 50 mg. of potassium ferricyanide in 10 ml. of water (6).

Quantitative Methods—Scintillation spectrometry was used to determine the absolute levels of radioactivity in the various fractions. For this purpose, a dioxane cocktail was employed in conjunction with a Packard Tri-Carb model 3324 liquid scintillation spectrometer equipped with automatic external standardization. The relative quantities of the compounds resolved chromatographically were determined by using a planimeter to measure the areas under the peaks of radioscans.

RESULTS

In 24 hr., Dog 1 excreted 4.6% of the administered radioactivity into the urine and Dog 2 excreted only 1.8%. The urinary recovery values for 72 hr. were 9.2% from Dog 1 and 8.3% from Dog 2. Figure 1 shows that the distribution of radioactivity between unconjugated and conjugated metabolites also differed considerably for the 24-hr. urine collections from the two dogs. Nevertheless, there was a preponderance of conjugated labeled compounds (77 and 96%) in these specimens.

All of the benzodiazepin and benzophenone reference compounds were separable from one another by TLC (Table I). Table II illustrates the resolutions obtained by applying the same technique to the urine from Dog 1, to the same urine after hydrolysis, to extracts of the unconjugated metabolites, and to extracts of the aglycones of the labeled glucuronides. Table III shows the translation of these data into terms of specific compounds based upon the identifications described below.

Identification of Prazepam—It was known that prazepam, if present, would be in the unconjugated fraction. This fraction was submitted to chromatography in three solvents. Chromatograms developed in Solvents 206 and 307 showed 5 to 6% of the unconjugated radioactivity to be located at R_f values corresponding to prazepam (Table II). Chromatograms produced in Solvent 306 gave a higher ^{14}C value (13%) for the prazepam area and suggested that desalkylprazepam might also be present. After extracting the suspected prazepam from thin-layer chromatograms, rechromatography in different solvents yielded single radioactive bands at R_f values corresponding to prazepam. In addition, exposure of the chromatograms to iodine vapor produced yellow bands only where there was radioactivity. When the eluates of the suspected prazepam were hydrolyzed and chromatographed in four solvents, a single radioactive product was formed. This product corresponded to 2-cyclopropylmethylamino-5-chlorobenzophenone (CACB) upon chromatography in all solvents and was so identified. The identification of prazepam was based upon all of these facts (radioactivity, quantitative agreement between the quantities estimated to be present on different chromatograms, reaction with iodine, R_f values in four solvents, and conversion to tagged CACB).

Identification of Desalkylprazepam—This compound was also sought in the unconjugated fraction. Again, there was a problem with one of the solvents, this time because 72% of all of the ^{14}C in this fraction centered at R_f 0.05 and obscured the metabolite with an R_f of 0.11 (Solvent 206). However, well-resolved spots were observed at appropriate places on chromatograms developed in the other two solvents and the quantities of ^{14}C corresponded satisfactorily: 13 and 10% of the ^{14}C of the unconjugated fraction in Solvents 306 and 307, respectively (Table II). Eluates of the spots showed single radioactive bands upon rechromatography in the various solvents, and the R_f values corresponded to desalkylprazepam. Cochromatography of the metabolite with synthetic desalkylprazepam showed the presence of ^{14}C and of the iodine-reacting material at the same R_f in each solvent. Further confirmation of the identity of the metabolite came from its hydrolysis to ACB. The hydrolysis product was radioactive, yielded the same R_f values as ACB, and was inseparable from authentic ACB by cochromatography.

Identification of Oxazepam—The presence of free oxazepam was indicated by chromatography of the urine in Solvents 304 and 306 and by chromatography of the unconjugated fraction in Solvents 206, 306, and 307 (Table II). In addition, the quantities of the metabolite agreed fairly well, considering that five different experimental situations were involved. The methods used to identify oxazepam unequivocally were described earlier in detail (1).

Table II—TLC of Urine of Dog 1 and Its Major Fractions

Sample	Solvent	R_f and Amount
Urine	206	0.0 (100%)
Urine	304	0.12 (75%), 0.30 (13%), 0.89 (12%)
Urine	306	0.0 (86.5%), 0.40 (13.5%)
Urine	307	0.05 (100%)
Hydrolysate	206	0.0 (19%), 0.22 (5%), 0.35 (8%), 0.78 (62%), 0.95 (5%)
Hydrolysate	206[a]	0.0 (28%), 0.35 (7%), 0.49 (6%), 0.78 (59%)
Hydrolysate	304	0.25 (2%), 0.95 (98%)
Hydrolysate	306	0.03 (13%), 0.40 (10%), 0.58 (7%), 0.95 (70%)
Hydrolysate	307	0.03 (27%), 0.58 (13%), 0.88 (60%)
Unconjugated	206	0.05 (72%), 0.20 (11%), 0.40 (6%), 0.80 (11%)
Unconjugated	306	0.05 (12%), 0.49 (61%), 0.80 (13%), 0.92 (14%)
Unconjugated	307	0.10 (38%), 0.20 (47%), 0.35 (10%), 0.67 (5%), 0.88 (10%)
Aglycones	206	0.07 (100%)
Aglycones	304	0.89 (100%)
Aglycones	306	0.0 (9%), 0.22 (10%), 0.50 (68%), 0.75 (13%)
Aglycones	307	0.10 (91%), 0.25 (9%)

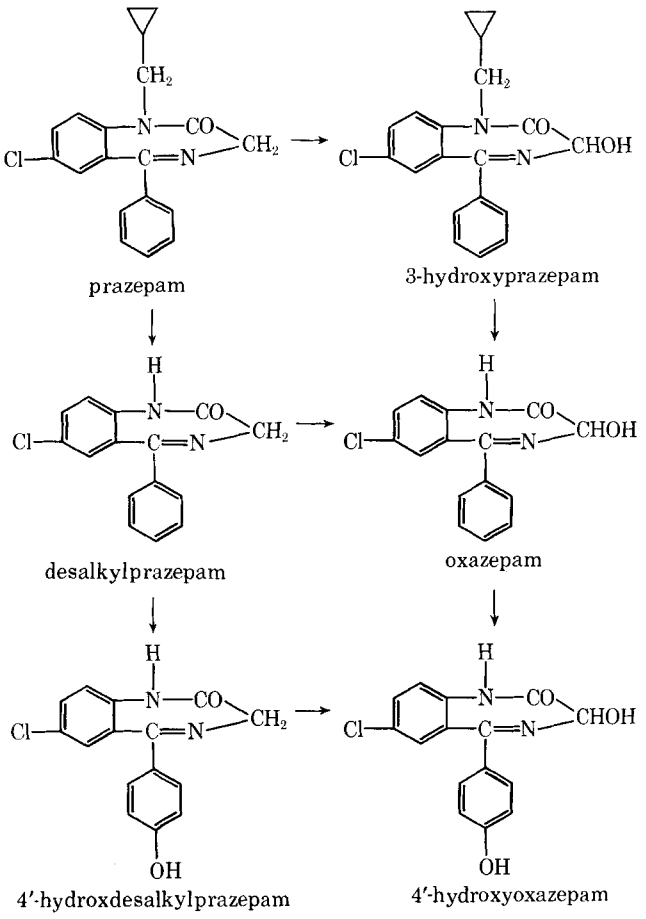
[a] Sample hydrolyzed for 18 hr. rather than the usual 1 hr.

Table III—Identification and Estimation of Metabolites in Urine of Dog 1

Compound	Urine or Fraction	Solvent	Urine, %
Prazepam	Unconjugated	206	1
Prazepam	Unconjugated	307	1
Oxazepam	Urine	304	12
Oxazepam	Urine	306	14
Oxazepam	Unconjugated	206	15
Oxazepam	Unconjugated	306	13
Oxazepam	Unconjugated	307	10
Desalkylprazepam	Unconjugated	306	3
Desalkylprazepam	Unconjugated	307	2
Oxazepam glucuronide	Aglycone	206	77
Oxazepam glucuronide	Aglycone	304	77
Oxazepam glucuronide	Aglycone	306	52[c]
Oxazepam glucuronide	Aglycone	307	70
3-Hydroxyprazepam glucuronide[a]	Aglycone	306	10
3-Hydroxyprazepam glucuronide[a]	Aglycone	307	7
4′-Hydroxyoxazepam[b]	Hydrolysate	307	13[d]

[a] N_1-Cyclopropylmethyloxazepam glucuronide. Confirmed by 4% CACB in hydrolysate, Solvent 206. [b] No model compound available; based upon detection of hydrolysis product. 4′-Hydroxyoxazepam may be the R_f 0.22 spot in Solvent 306, aglycone fraction; 8% of urinary ^{14}C. [c] Most probable value because sample was resolved into four radioactive areas whereas Solvents 206 and 304 gave one spot and Solvent 307 gave two. [d] Further chromatography in Solvents 307, 206, and 306 showed about 60% of this material to be 4′-hydroxyoxazepam.

Identification of Oxazepam Glucuronide—After treating the ethyl acetate-extracted urine with β-glucuronidase, it was possible to extract practically all of the remaining labeled material from the urine of Dog 1 into ethyl acetate (Fig. 1). Chromatograms developed in four solvents showed spots corresponding to oxazepam. The oxazepam contribution to the conjugated fraction was 68% as indicated by chromatography in Solvent 306 (Table II). The higher estimates (91 to 100%) obtained from the other solvents obviously included other aglycones.



Scheme I—Oxidative metabolism of prazepam in dogs. (The sequences involving 4′-oxidation of prazepam and of N_1-cyclopropylmethyloxazepam were omitted for clarity.)

Table IV—Prazepam and Its Metabolites in Dog Urine

Compound	—^{14}C in Urine, %— Dog 1	Dog 2
Prazepam	1	0.2
Desalkylprazepam	2	0.4
3-Hydroxyprazepam glucuronide	9	11
Oxazepam	14	3
Oxazepam glucuronide	52	72
4′-Hydroxyoxazepam glucuronide	8	8
Total	86	95

Identification of 3-Hydroxyprazepam Glucuronide—Chromatography following treatment with β-glucuronidase also suggested the presence of 3-hydroxyprazepam. The material separated from oxazepam by Solvent 306 was identified as 3-hydroxyprazepam on the basis of rechromatography data, cochromatography experiments with authentic material, and conversion to CACB by acid hydrolysis.

Identification of 4′-Hydroxyoxazepam Glucuronide—As expected from the results presented above, the direct hydrolysis of the dog urine yielded radioactive bands corresponding to ACB and CACB (Table II). Other radioactive compounds were also present. The application of spray reagents to the chromatograms showed that the radioactive band at R_f 0.58 in Solvent 307 gave positive tests for a primary aromatic amino group and for a phenolic group. Therefore, it was considered possible that the compound was 4′-hydroxy ACB which could be formed by the hydrolysis of 4′-hydroxyoxazepam or its glucuronide. Since the aglycone, 4′-hydroxyoxazepam, was not available, the authors tried to identify this metabolite through its possible hydrolysis product, 4′-hydroxy ACB, by a chromatographic application of the isotope dilution technique. To this end, radioactive material eluted from R_f 0.58 of TLC plates developed in Solvent 307 was mixed with authentic 4′-hydroxy ACB and submitted to chromatography in Solvent 307. Three radioactive bands showed up in the radioscan. The major band (R_f 0.55) corresponded exactly to the yellow area occupied by 4′-hydroxy ACB. This band was collected and eluted for chromatography in Solvent 206. The radioscan showed a single peak at the same R_f (0.49) as the yellow color. The collection and elution process was repeated for chromatography in Solvent 306. Again, a single radioactive band coincided with the yellow 4′-hydroxy ACB (R_f 0.80).

The urine of both dogs contained the same six compounds and they accounted for 86 to 95% of the radioactivity (Table IV). Although oxazepam glucuronide was the main component of both specimens, its contribution to the total radioactivity varied considerably in the two urine collections. The sums of free and conjugated oxazepam were in closer agreement. The data for the glucuronides of 3-hydroxyprazepam and 4′-hydroxyoxazepam corresponded closely. In both instances, the smallest contributions were made by prazepam and desalkylprazepam.

DISCUSSION

Three oxidative reactions characterize prazepam metabolism in the dog. They are the Phase 1 reactions: (*a*) dealkylation, probably releasing cyclopropylformaldehyde, (*b*) oxidation of the methylene at C-3 to a secondary alcohol function, and (*c*) oxidation of the phenyl group at C-5 to a phenol. Reactions 1 and 2 occur directly with prazepam, as is evident from the identification of 3-hydroxyprazepam and desalkylprazepam (Scheme I). Which of the reactions proceeds faster remains a moot question for prazepam, although the evidence indicates that diazepam is dealkylated more rapidly than it is hydroxylated *in vivo* (7–9) and *in vitro* (10). Reactions 1 and 2 are also involved in the subsequent metabolism. Thus, there are two independent but competitive routes from prazepam to oxazepam. At this time, there is no evidence for the direct hydroxylation of prazepam in the 4′-position; *i.e.*, it is not known whether prazepam simultaneously undergoes Reactions 1, 2,

and 3. Nevertheless, the authors consider that Reaction 3 is properly classified as a Phase 1 conversion (11). Although 4′-hydroxydesalkylprazepam has not been identified, this compound, as well as oxazepam, may be a precursor to 4′-hydroxyoxazepam. Other routes to 4′-hydroxyoxazepam are also possible as stated in the legend of Scheme I.

Another of the unidentified metabolites may be 2′-hydroxyoxazepam. It seems that aromatic hydroxylation in the *ortho* and *para* positions is catalyzed by different enzymes (12) and that the dog generally produces more of the *ortho* isomer (13, 14). The authors' identification of the *para* hydroxy compound (4′-hydroxyoxazepam) in dog urine implies steric hindrance of the *ortho* (2′) position. On the other hand, it is quite possible that some 2′-hydroxy ACB was present with the 4′-hydroxy ACB. Since the 2′-hydroxy compound is not available for study, the possibility cannot be excluded that the four solvent systems did not resolve 2′- and 4′-hydroxy ACB.

In the dog, a single Phase 2 reaction was observed, namely, conjugation with glucuronic acid. As far as could be determined, all of the 3-hydroxyprazepam was conjugated. Thus, a competitive situation would exist if 3-hydroxyprazepam were also converted to oxazepam, as seems likely. The data show that most of the oxazepam was conjugated. The presence of some free oxazepam is interesting and suggests intermediate ranking of the dog among species capable of forming *O*-glucuronides. Although conclusive evidence is lacking, it is surmised that 4′-hydroxyoxazepam was also excreted mainly as a glucuronide.

Unlike diazepam (7, 8) and oxazepam (4), some unaltered prazepam was excreted into the urine by dogs. Thus, dogs treated with prazepam circulate at least three compounds with tranquilizer activity, namely prazepam (15–18), oxazepam, and desalkylprazepam (19).

CONCLUSIONS

The radioactivity from ^{14}C-prazepam administered to dogs was excreted slowly into the urine. Most of the drug was transformed, apparently by oxidative enzymes of the liver microsomes. The major drug metabolite was oxazepam glucuronide. Other glucuronides were formed from 3-hydroxyprazepam and 4′-hydroxyoxazepam. The urine collections also contained unaltered prazepam, desalkylprazepam, and unconjugated oxazepam. Prazepam is considered to serve as the precursor to a series of metabolites with tranquilizer activity.

REFERENCES

(1) F. J. DiCarlo, M. C. Crew, M. D. Melgar, and L. J. Haynes, *J. Pharm. Sci.*, **58**, 960(1969).

(2) G. Jommi, P. Manitto, and M. A. Silanos, *Arch. Biochem. Biophys.*, **108**, 568(1964).

(3) E. J. Merrill, *J. Labelled Compds.*, to be published.

(4) S. S. Walkenstein, R. Wiser, C. H. Gudmundsen, H. B. Kimmel, and R. A. Corradino, *J. Pharm. Sci.*, **53**, 1181(1964).

(5) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).

(6) H. Ganshirt, "Thin Layer Chromatography," E. Stahl, Ed., Springer-Verlag, Berlin, West Germany, 1965, p. 312.

(7) M. A. Schwartz, B. A. Koechlin, E. Postma, S. Palmer, and G. Krol, *J. Pharmacol. Exp. Ther.*, **149**, 424(1965).

(8) H. W. Ruelius, J. M. Lee, and H. E. Alburn, *Arch. Biochem. Biophys.*, **111**, 376(1965).

(9) J. A. F. DeSilva, B. A. Koechlin, and G. Bader, *J. Pharm. Sci.*, **55**, 692(1966).

(10) J. Kvetina, F. Marucci, and R. Fanelli, *J. Pharm. Pharmacol.*, **20**, 807(1968).

(11) R. T. Williams, *Federation Proc.*, **26**, 1029(1967).

(12) J. R. Gillette, *Advan. Pharmacol.*, **4**, 219(1966).

(13) D. V. Parke and R. T. Williams, *Biochem. J.*, **63**, 12P (1956).

(14) D. V. Parke, *ibid.*, **77**, 493(1960).

(15) E. Dunlop and J. Weisberg, *J. Psychopharmacol.*, **1**, 75 (1966).

(16) E. Dunlop and J. Weisberg, *Psychosomatics*, **9**, 235(1968).

(17) J. W. Shaffer, M. L Yeganeh, N. H. Foxwell, and A. A. Kurland, *J. Clin. Pharmacol.*, **8**, 392(1968).

(18) E. Kingstone, A. Villeneuve, and I. Kossatz, *Current Ther. Res.*, **8**, 159(1966).

(19) L. O. Randall, C. L. Scheckel, and R. F. Banziger, *ibid.*, **7**, 590(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 28, 1969, from the *Department of Biochemistry, Warner-Lambert Research Institute, Morris Plains, NJ 07950*

Accepted for publication September 29, 1969.

The authors thank Mr. Edward J. Merrill for the synthesis of ^{14}C-prazepam.

Gastric Absorption and Distribution of Acetylsalicylic Acid and Other Acidic Compounds in the Rat

CLARENCE H. MORRIS, JOHN E. CHRISTIAN, TOM S. MIYA, and WARREN G. HANSEN

Abstract □ A comparison was made of the gastric absorption and distribution of sodium acetylsalicylate-7-^{14}C with the absorption and distribution of sodium salts of other weakly acidic compounds. Each of the other compounds observed had an absorption pattern which was characteristic for the compound. Only sodium acetylsalicylate caused gastric lesions in the rat. The observations do not rule out the possibility that absorption characteristics of acetylsalicylic acid and its salts may be associated with their ability to cause gastric ulcers.

Keyphrases □ Acetylsalicylate-7-^{14}C, Na—absorption, distribution □ Acidic compounds—acetylsalicylate-7-^{14}C, Na—absorption, distribution comparison □ Gastric lesion production—acetylsalicylate-7-^{14}C, Na, acidic compounds □ TLC—analysis □ Autoradiography—analysis

It has been known for more than 50 years that oral administration of acetylsalicylic acid is followed by erosion of the gastric mucosa and bleeding from the site of the lesion (1). These aspirin-induced lesions have been observed in humans (1), dogs (2), cats (3), guinea pigs (4), and albino rats (5). A number of hypotheses have been suggested to explain the occurrence of these lesions. Davenport (6) and Martin (7) have suggested chemical models based upon the interaction of the compound with cellular constituents following its absorption

and 3. Nevertheless, the authors consider that Reaction 3 is properly classified as a Phase 1 conversion (11). Although 4′-hydroxydesalkylprazepam has not been identified, this compound, as well as oxazepam, may be a precursor to 4′-hydroxyoxazepam. Other routes to 4′-hydroxyoxazepam are also possible as stated in the legend of Scheme I.

Another of the unidentified metabolites may be 2′-hydroxyoxazepam. It seems that aromatic hydroxylation in the *ortho* and *para* positions is catalyzed by different enzymes (12) and that the dog generally produces more of the *ortho* isomer (13, 14). The authors' identification of the *para* hydroxy compound (4′-hydroxyoxazepam) in dog urine implies steric hindrance of the *ortho* (2′) position. On the other hand, it is quite possible that some 2′-hydroxy ACB was present with the 4′-hydroxy ACB. Since the 2′-hydroxy compound is not available for study, the possibility cannot be excluded that the four solvent systems did not resolve 2′- and 4′-hydroxy ACB.

In the dog, a single Phase 2 reaction was observed, namely, conjugation with glucuronic acid. As far as could be determined, all of the 3-hydroxyprazepam was conjugated. Thus, a competitive situation would exist if 3-hydroxyprazepam were also converted to oxazepam, as seems likely. The data show that most of the oxazepam was conjugated. The presence of some free oxazepam is interesting and suggests intermediate ranking of the dog among species capable of forming *O*-glucuronides. Although conclusive evidence is lacking, it is surmised that 4′-hydroxyoxazepam was also excreted mainly as a glucuronide.

Unlike diazepam (7, 8) and oxazepam (4), some unaltered prazepam was excreted into the urine by dogs. Thus, dogs treated with prazepam circulate at least three compounds with tranquilizer activity, namely prazepam (15–18), oxazepam, and desalkylprazepam (19).

CONCLUSIONS

The radioactivity from ^{14}C-prazepam administered to dogs was excreted slowly into the urine. Most of the drug was transformed, apparently by oxidative enzymes of the liver microsomes. The major drug metabolite was oxazepam glucuronide. Other glucuronides were formed from 3-hydroxyprazepam and 4′-hydroxyoxazepam. The urine collections also contained unaltered prazepam, desalkylprazepam, and unconjugated oxazepam. Prazepam is considered to serve as the precursor to a series of metabolites with tranquilizer activity.

REFERENCES

(1) F. J. DiCarlo, M. C. Crew, M. D. Melgar, and L. J. Haynes, *J. Pharm. Sci.*, **58**, 960(1969).

(2) G. Jommi, P. Manitto, and M. A. Silanos, *Arch. Biochem. Biophys.*, **108**, 568(1964).

(3) E. J. Merrill, *J. Labelled Compds.*, to be published.

(4) S. S. Walkenstein, R. Wiser, C. H. Gudmundsen, H. B. Kimmel, and R. A. Corradino, *J. Pharm. Sci.*, **53**, 1181(1964).

(5) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).

(6) H. Ganshirt, "Thin Layer Chromatography," E. Stahl, Ed., Springer-Verlag, Berlin, West Germany, 1965, p. 312.

(7) M. A. Schwartz, B. A. Koechlin, E. Postma, S. Palmer, and G. Krol, *J. Pharmacol. Exp. Ther.*, **149**, 424(1965).

(8) H. W. Ruelius, J. M. Lee, and H. E. Alburn, *Arch. Biochem. Biophys.*, **111**, 376(1965).

(9) J. A. F. DeSilva, B. A. Koechlin, and G. Bader, *J. Pharm. Sci.*, **55**, 692(1966).

(10) J. Kvetina, F. Marucci, and R. Fanelli, *J. Pharm. Pharmacol.*, **20**, 807(1968).

(11) R. T. Williams, *Federation Proc.*, **26**, 1029(1967).

(12) J. R. Gillette, *Advan. Pharmacol.*, **4**, 219(1966).

(13) D. V. Parke and R. T. Williams, *Biochem. J.*, **63**, 12P (1956).

(14) D. V. Parke, *ibid.*, **77**, 493(1960).

(15) E. Dunlop and J. Weisberg, *J. Psychopharmacol.*, **1**, 75 (1966).

(16) E. Dunlop and J. Weisberg, *Psychosomatics*, **9**, 235(1968).

(17) J. W. Shaffer, M. L Yeganeh, N. H. Foxwell, and A. A. Kurland, *J. Clin. Pharmacol.*, **8**, 392(1968).

(18) E. Kingstone, A. Villeneuve, and I. Kossatz, *Current Ther. Res.*, **8**, 159(1966).

(19) L. O. Randall, C. L. Scheckel, and R. F. Banziger, *ibid.*, **7**, 590(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 28, 1969, from the *Department of Biochemistry, Warner-Lambert Research Institute, Morris Plains, NJ 07950*

Accepted for publication September 29, 1969.

The authors thank Mr. Edward J. Merrill for the synthesis of ^{14}C-prazepam.

Gastric Absorption and Distribution of Acetylsalicylic Acid and Other Acidic Compounds in the Rat

CLARENCE H. MORRIS, JOHN E. CHRISTIAN, TOM S. MIYA, and WARREN G. HANSEN

Abstract □ A comparison was made of the gastric absorption and distribution of sodium acetylsalicylate-7-^{14}C with the absorption and distribution of sodium salts of other weakly acidic compounds. Each of the other compounds observed had an absorption pattern which was characteristic for the compound. Only sodium acetylsalicylate caused gastric lesions in the rat. The observations do not rule out the possibility that absorption characteristics of acetylsalicylic acid and its salts may be associated with their ability to cause gastric ulcers.

Keyphrases □ Acetylsalicylate-7-^{14}C, Na—absorption, distribution □ Acidic compounds—acetylsalicylate-7-^{14}C, Na—absorption, distribution comparison □ Gastric lesion production—acetylsalicylate-7-^{14}C, Na, acidic compounds □ TLC—analysis □ Autoradiography—analysis

It has been known for more than 50 years that oral administration of acetylsalicylic acid is followed by erosion of the gastric mucosa and bleeding from the site of the lesion (1). These aspirin-induced lesions have been observed in humans (1), dogs (2), cats (3), guinea pigs (4), and albino rats (5). A number of hypotheses have been suggested to explain the occurrence of these lesions. Davenport (6) and Martin (7) have suggested chemical models based upon the interaction of the compound with cellular constituents following its absorption

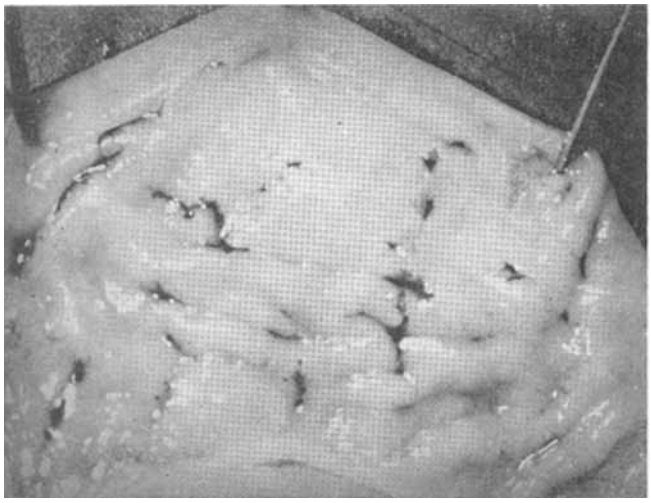


Figure 1—*Corpus mucosal surface showing lesions and areas of hemorrhage.*

into the cell. It became of interest to compare the absorption of aspirin, which causes ulceration and bleeding in the corpus portion of the rat stomach, with the absorption of other weakly acidic compounds which do not cause ulceration and bleeding.

MATERIALS AND METHODS

The radiochemical purity of sodium acetylsalicylate-7-^{14}C, sodium benzoate-7-^{14}C, sodium acetate-1-^{14}C, and sodium barbital-2-^{14}C (Tracer Labs, Waltham, Mass.) was established by TLC employing a stationary phase of silica gel G and a solvent system of low-boiling petroleum ether and 99% propionic acid. The location of the radioactive spots was determined by gross autoradiography.

All animals were fasted for 36 hr. prior to administration of the compound. Water was allowed *ad libitum* during the fast but was withdrawn after administration of the compound.

The compounds were administered orally to male Holtzman rats weighing between 150 and 275 g. (Holtzman Rat Co., Madison, Wis.) by means of a No. 16 curved steel oral catheter which had been dipped in mineral oil for lubrication. Each animal received a dose of 0.28 mmole of the selected compound per kg. of body weight. Each dose contained 30.5 μc. per kg. body weight of the ^{14}C-labeled compound diluted with the appropriate amount of carrier. The sodium acetylsalicylate was administered in 0.15 *M* citrate buffer, final pH 4.6. All other compounds were dissolved or suspended in a solution of 5% polyvinylpyrrolidone (PVP). Control animals received 0.5 ml. of the respective vehicle.

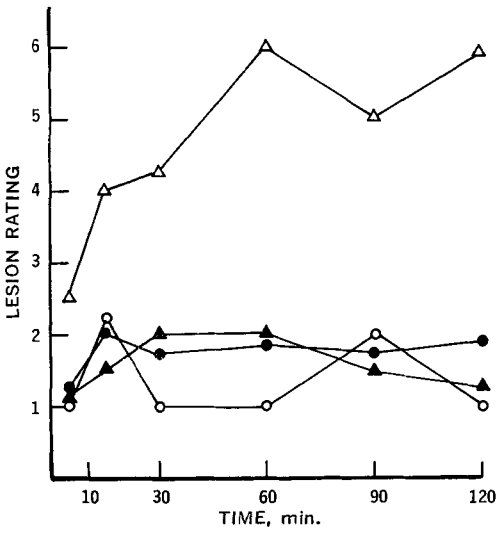


Figure 2—*Average gastric lesion rating of six animals per time interval following oral administration of: △, sodium acetylsalicylate; ○, sodium barbital; ●, sodium acetate; and ▲, sodium benzoate. All control animals had a rating of 1.*

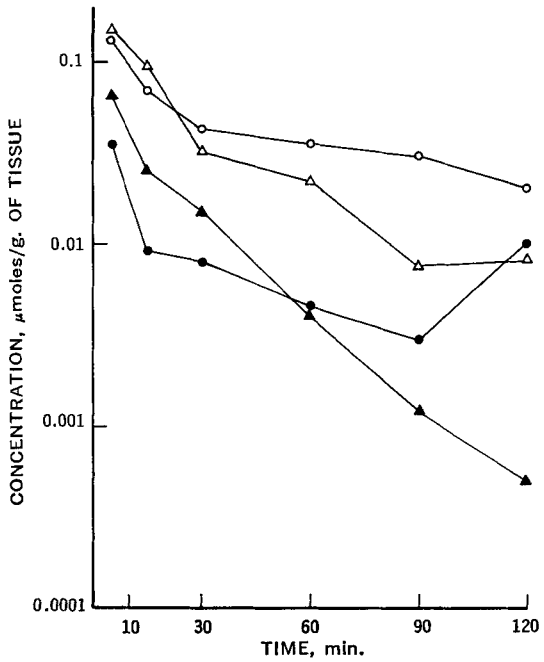


Figure 3—*Disappearance of labeled compounds from the corpus portion of the rat stomach. Key: △, sodium acetylsalicylate; ○, sodium barbital; ●, sodium acetate; and ▲, sodium benzoate. Each point represents the mean of three animals. To indicate the range of values around the averages shown in the plot, the minimum variation around any single point was 2.62 × 10^{-1}, 1.98 × 10^{-1}, and 2.24 × 10^{-1} (average 2.28 × 10^{-1}) μmoles per gram of tissue. The maximum variation around any single point was 1.94 × 10^{-3}, 1.06 × 10^{-2}, and 5.13 × 10^{-2} (average 4.95 × 10^{-2}) μmoles per gram of tissue.*

The rats were killed in groups of three by etherization at intervals of 5, 15, 30, 60, 90, and 120 min. following administration of each compound. The stomachs were removed, opened along the line of lesser curvature, stretched, and pinned on a large rubber stopper. The mucosal surface of each stomach was decontaminated with 0.9% NaCl, examined for the appearance of lesions, and rated on an 8-point scale developed by Morris *et al.* (5).

After being rated with respect to the severity of lesions, the stomachs were frozen in the stretched position with dry ice. Whole frozen stomachs were covered with plastic wrap (Saran), placed between two sheets of X-ray film (No Screen Medical, Eastman Kodak Co., Rochester, N. Y.), and stored in light, tight cassets at −20° for 60 days for gross autoradiography.

Following exposure of the film, samples of frozen rumen (nonglandular portion of the rat stomach) and corpus (glandular portion of the rat stomach) were prepared for liquid scintillation counting (Tri-Carb liquid scintillation counter model 3003, Packard Instrument Co., La Grange, Ill.) as described by Morris *et al.* (5). The amount of compound present per gram of tissue was calculated for each sample according to the following expression:

$$\mu\text{moles of compound/g. of tissue} = \frac{\text{observed d.p.m.}}{(W)\,(SA)\,(L)}$$

where: W = wet weight of the tissue in grams; SA = specific activity, d.p.m./mcg. of compound; and L = mcg. of compound/μmole.

RESULTS

Appearance of Lesions—Typical lesions as they appeared in the corpus portion of the rat stomach are shown in Fig. 1. It is of interest to note that the lesions appear in rows or furrows roughly paral-

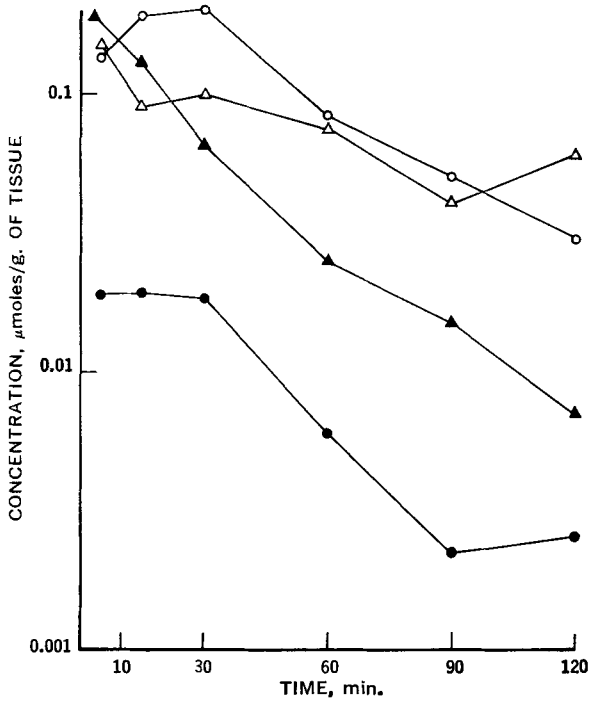


Figure 4—*Disappearance of labeled compounds from the rumen portion of the rat stomach. Key: △, sodium acetysalicylate; ○, sodium barbital; ●, sodium acetate; and ▲, sodium benzoate. Each point represents the mean of three animals. To indicate the range of values around the averages shown in the plot, the minimum variation around any single point was 4.27 × 10^{-2}, 3.94 × 10^{-2}, and 3.94 × 10^{-2} (average 4.95 × 10^{-2}) μmoles per gram of tissue. The maximum variation around any single point was 1.12 × 10^{-2}, 2.04 × 10^{-2}, and 4.6 × 10^{-3} (average 1.2 × 10^{-2}) μmoles per gram of tissue.*

lel to the longitudinal axis of the stomach. Under the conditions employed in these experiments, such lesions occurred only in the corpus portion of the rat gastric mucosa.

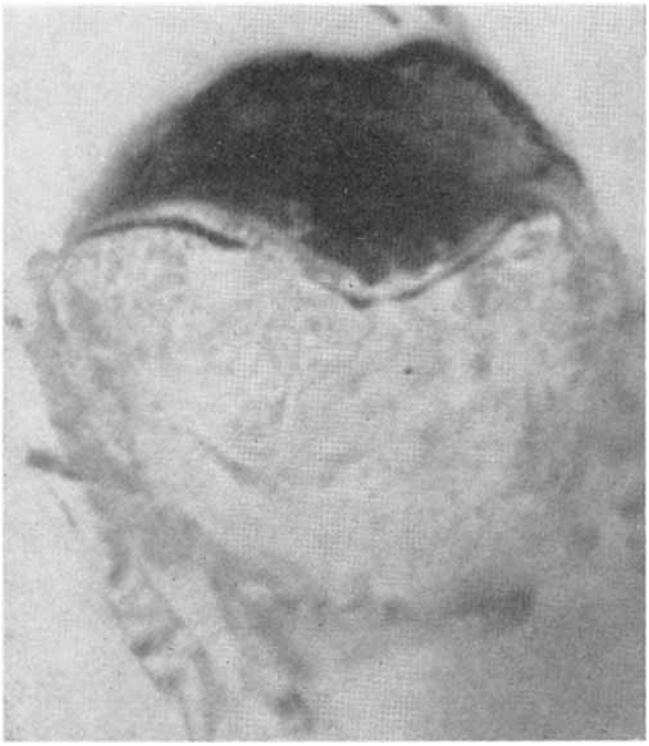


Figure 5—*Gross autoradiograph of musocal surface of rat stomach 120 min. following oral administration of ^{14}C-sodium acetylsalicylate.*

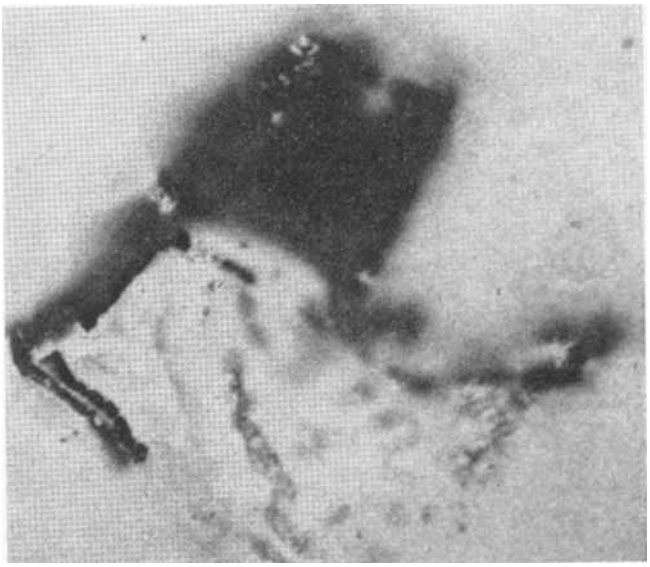


Figure 6—*Gross autoradiograph of mucosal surface of rat stomach 120 min. following oral administration of ^{14}C-sodium benzoate.*

It may be seen in Fig. 2 that, of the compounds studied, only sodium acetylsalicylate caused gastric lesions in the rat. A mild erythema, rated as 2 on the scale, occurred in some cases after the administration of the other compounds. Formation of lesions was essentially maximal 60 min. after administration of sodium acetylsalicylate.

Concentration Study—The concentration in micromoles per gram of tissue of each ^{14}C-labeled compound in gastric tissue was determined at each time interval. It is seen in Fig. 3 that the concentration of each compound in the corpus tissue diminishes rapidly with time.

The levels of these compounds in the rumen are remarkably different from the levels in the corpus (see Fig. 4). The concentration of sodium barbital in rumen tissue increases with time during the first 30 min. after its administration. Its concentration then drops

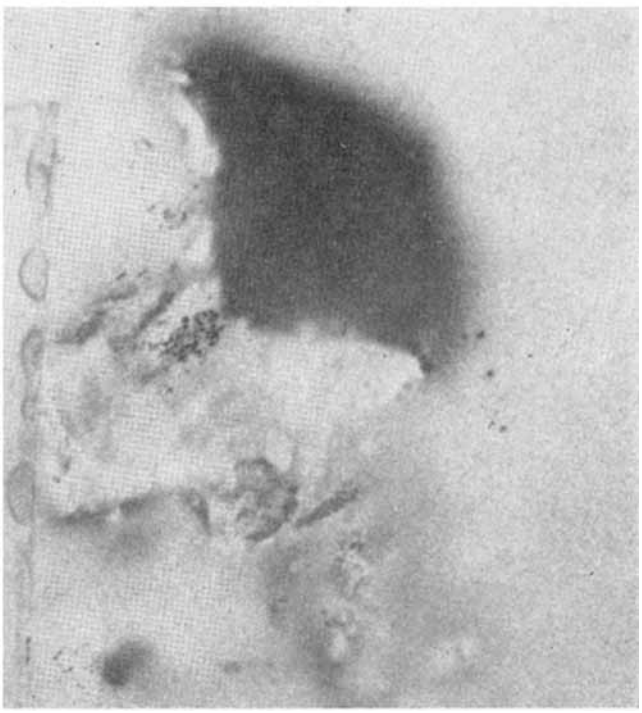


Figure 7—*Gross autoradiograph of mucosal surface of rat stomach 120 min. following oral administration of ^{14}C-sodium barbital.*

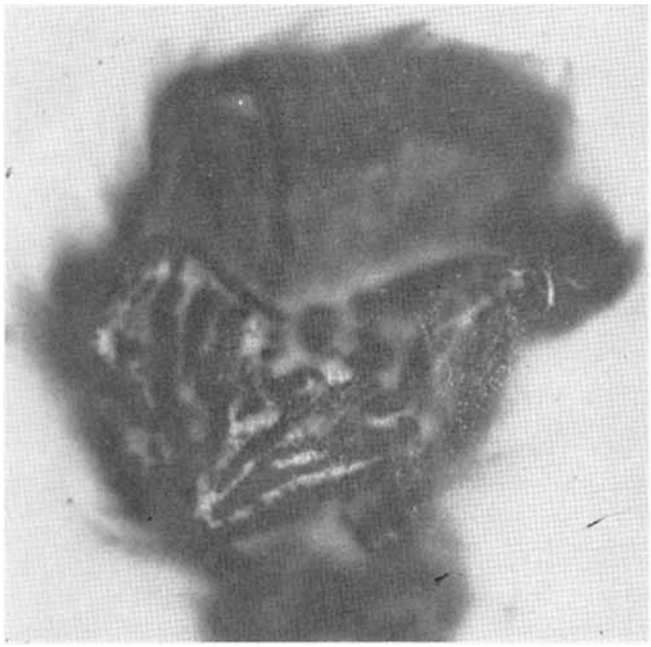


Figure 8—*Gross autoradiograph of mucosal surface of rat stomach 5 min. following oral administration of* ^{14}*C-sodium acetate.*

for the remaining time intervals. The concentration of sodium acetylsalicylate diminishes very slowly throughout the entire period of observation. The concentration of sodium acetate in rumen tissue remains essentially constant for the first 30 min. after its administration and then drops rapidly in a manner similar to that of sodium barbital. Sodium benzoate levels decrease rapidly with time.

Distribution Study—Figures 5 through 10 show selected autoradiographs of the mucosal surfaces. All stomachs are positioned such that the rumen is at the top of the figure. The compounds are localized in the rumen of all stomachs up to 120 min. following administration with the exception of sodium acetate. Sodium acetate disappears from both the rumen and the corpus within 60 min. following administration (Figs. 8–10). Sodium acetylsalicylate, sodium benzoate, and sodium barbital demonstrate gastric distribution patterns similar to the pattern o. acetylsalicylic acid reported by Morris *et al.* (5).

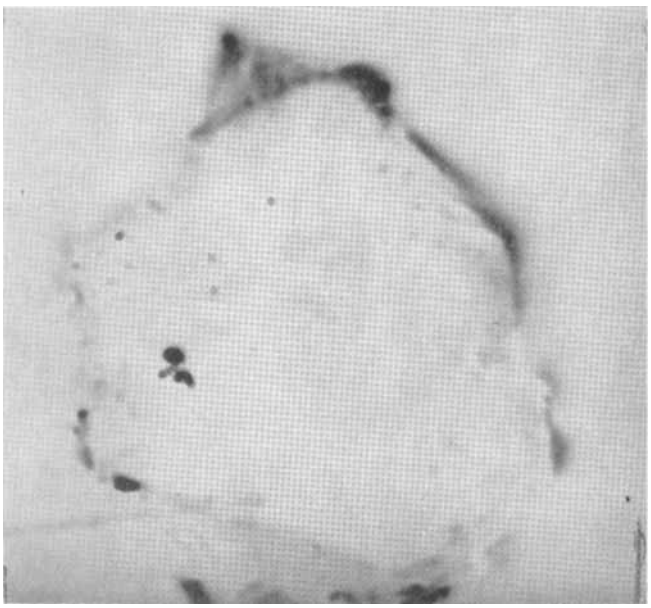


Figure 9—*Gross autoradiograph of mucosal surface of rat stomach 60 min. following oral administration of* ^{14}*C-sodium acetate.*

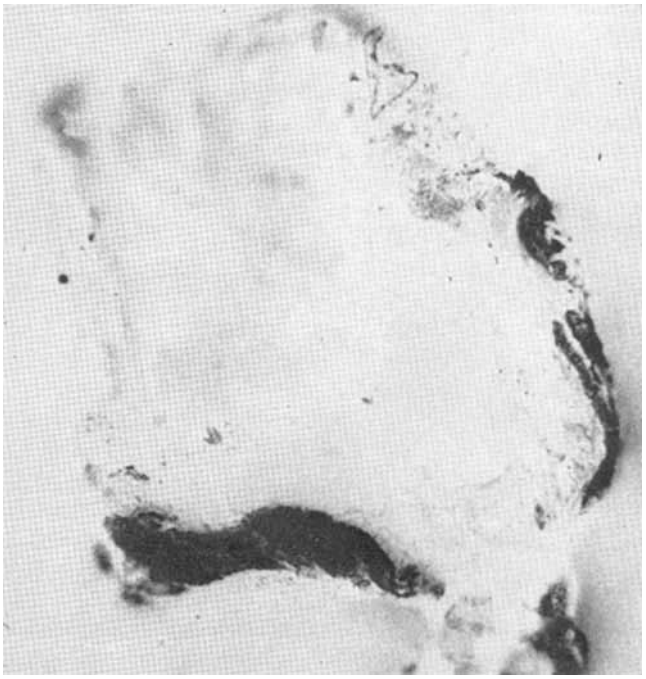


Figure 10—*Gross autoradiograph of mucosal surface of rat stomach 120 min. following oral administration of* ^{14}*C-sodium acetate.*

SUMMARY AND CONCLUSIONS

The data presented in Fig. 2 indicate a high incidence of lesions following the administration of sodium acetylsalicylate. These findings are in agreement with those reported by Morris *et al.* (5) who used acetylsalicylic acid. Sodium acetylsalicylate, sodium benzoate, and sodium barbital showed similar localization patterns. It is obvious from both the quantitative data and the gross autoradiographs that these three compounds disappear with time from the corpus but remain in relatively high concentrations at all observed time intervals in the rumen portion. By contrast, the results of the autoradiographic localization and the tissue analysis of sodium acetate indicate a relatively rapid disappearance from both the rumen and corpus.

It should be emphasized that the concentration values reported represent a specific concentration at the designated time following administration, and that this value is a function of the penetration of the compound from the lumen into the tissue, possible metabolism, binding, absorption into the bloodstream, reabsorption from the blood into the stomach tissue, and possible loss of activity from the tissue into the lumen following tissue damage. Although this study does not provide an explanation of gastric ulceration by aspirin, the observations do not rule out the possibility that absorption characteristics of acetylsalicylic acid and its salts may be associated with their ability to cause gastric ulcers. Since sodium acetylsalicylate administration results in lesion formation and administration of the other compounds studied did not result in lesion formation, it becomes difficult to escape the conclusion that absorbed sodium acetylsalicylate interacts with the corpus tissue or its secretions in some manner which is different from the interaction of the other weak acids. In addition, since lesions appeared only in the corpus, through which sodium acetylsalicylate penetrates and is absorbed, and not in the rumen, where the compound remains localized, it appears that the processes involved in penetration and absorption play a role in the mechanism of lesion formation. Investigations into the nature of the interactions between sodium acetylsalicylate and corpus and rumen tissue are presently in progress.

REFERENCES

(1) M. J. H. Smith and P. K. Smith, "The Salicylates. A Critical Bibliographic Review," Interscience, New York, N. Y., 1966, p. 236.

(2) J. W. Hurley and L. A. Crandall, *Gastroenterology*, **46**, 36(1964).
(3) L. A. Roth, "Salicylates, An International Symposium," A. St. J. Dixon *et al.*, Eds., Little, Brown, Boston, Mass., 1963, p. 224.
(4) *Ibid.*, p. 217.
(5) E. Morris, A. A. Noujaim, T. S. Miya, and J. E. Christian, *J. Pharm. Sci.*, **56**, 896(1967).
(6) H. W. Davenport, *Gastroenterology*, **46**, 245(1964).
(7) B. K. Martin, *Nature*, **198**, 896(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 25, 1969, from the *Bionucleonics and Pharmacology and Toxicology Departments, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication October 16, 1969.

Presented in part to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Research conducted under the auspices of the Institute for Environmental Health.

Quantification of the Binding Tendencies of Cholestyramine II: Mechanism of Interaction with Bile Salt and Fatty Acid Salt Anions

WILLIAM H. JOHNS and THEODORE R. BATES

Abstract □ The binding of a series of conjugated bile salt and fatty acid salt anions to cholestyramine from aqueous media was investigated and the data were plotted according to the Langmuir adsorption equation. Increases in affinity constants were noted as the number of hydroxy substituents on the bile salt-ring structure decreased. An increase in the chain length of the fatty acid salt caused a corresponding increase in the affinity constant, whereas an increase in the extent of unsaturation in the fatty acid chain produced a reduction in the affinity constants for the fatty acid–cholestyramine interaction. Apparent surface tension-lowering properties of the adsorbate molecules were found to parallel the affinities obtained for both classes of adsorbate molecules, with the exception of the fatty acid anion, linoleate. Based on the results of these studies, it is suggested that the binding mechanism involves a primary electrostatic component reinforced by a secondary nonelectrostatic interaction, the strength of the latter force being dependent on the degree of hydrophobicity of the adsorbate molecule.

Keyphrases □ Cholestyramine binding—quantification □ Bile salts, fatty acid salt anions—cholestyramine interaction mechanism □ Surface tension values–affinity constants, correlation—bile salts □ Hydrophobic character relationship, adsorbate—cholestyramine binding □ UV spectrophotometry—analysis

In previous work with the anionic exchange resin cholestyramine (1), the authors studied the effect of the physiologic electrolytes, sodium chloride and bicarbonate, on the binding process of bile salt anions to cholestyramine. The dihydroxy bile salt anions studied were noted to be insignificantly affected in their extent of interaction with the resin, while the trihydroxy bile salt anion–cholestyramine interactions were markedly reduced in the presence of an added electrolyte. These results suggested that a secondary, nonelectrostatic, type of interaction was taking place at the adsorption site.

The observation that structurally different bile salt anions exhibit varying types, as well as extents, of binding to cholestyramine is of biologic significance in that an appreciation of the possible modes of interaction could ultimately contribute to an enhancement in the efficiency of this pharmacologically important resin.

The purpose of this study was to elucidate the nature of this secondary binding mechanism and the effect of adsorbate structure thereon. In order to accomplish this, the binding tendencies of a selected series of glycine-conjugated bile salts and various physiologic fatty acid salts to cholestyramine were investigated.

EXPERIMENTAL

Materials—The sodium salts of glycocholic acid,[1] glycodeoxycholic acid,[1] glycodehydrocholic acid,[1] glycolithocholic acid,[1] lauric acid,[2] and oleic acid[3] were dried *in vacuo* for at least 48 hr. prior to use. The sodium salt of linoleic acid[3] was prepared by reacting equimolar quantities of the acid with sodium ethylate in absolute alcohol. The resulting salt was washed several times with absolute alcohol, dried at room temperature, and subjected to vacuum desiccation. The cholestyramine[4] employed in this study was of pharmaceutical grade (1). Reagent grade concentrated sulfuric acid, glacial acetic acid, hydrochloric acid, chloroform, sodium hydroxide, copper nitrate, *n*-butanol, and diethyldithiocarbamate were used as received.

Procedure for Adsorption Studies—A series of aqueous solutions of each bile salt and fatty acid salt was prepared over the concentration range of 0.75–5.0 millimolar (m*M*).[5] Twenty-five-milligram samples of cholestyramine were accurately weighed and placed into 50-ml. glass-stoppered conical flasks, together with a 25.0-ml. portion of the adsorbate solution. At each concentration, a control flask was prepared containing a similar quantity of the solution under study but no cholestyramine. These latter control solutions, which were assayed concomitantly with the solutions exposed to cholestyramine, were used to prepare the required Beer's law plots.

[1] Grade A. Obtained from Calbiochem Co., Los Angeles, Calif.
[2] Obtained from Eastman Organic Chemicals, Rochester, N. Y.
[3] Obtained from Fisher Scientific Co., Fair Lawn, N. J.
[4] Supplied by Merck and Co., Inc., Rahway, N. J.
[5] All systems exhibited complete solution over the concentration range studied, with the exception of the higher concentrations of glycolithocholate, which showed slight turbidity. Adsorption, being a dynamic process, would tend to increase the apparent solubility of a relatively insoluble material by removing molecules from solution and thereby promoting solution of any undissolved material.

(2) J. W. Hurley and L. A. Crandall, *Gastroenterology*, **46**, 36(1964).
(3) L. A. Roth, "Salicylates, An International Symposium," A. St. J. Dixon *et al.*, Eds., Little, Brown, Boston, Mass., 1963, p. 224.
(4) *Ibid.*, p. 217.
(5) E. Morris, A. A. Noujaim, T. S. Miya, and J. E. Christian, *J. Pharm. Sci.*, **56**, 896(1967).
(6) H. W. Davenport, *Gastroenterology*, **46**, 245(1964).
(7) B. K. Martin, *Nature*, **198**, 896(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 25, 1969, from the *Bionucleonics and Pharmacology and Toxicology Departments, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication October 16, 1969.

Presented in part to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Research conducted under the auspices of the Institute for Environmental Health.

Quantification of the Binding Tendencies of Cholestyramine II: Mechanism of Interaction with Bile Salt and Fatty Acid Salt Anions

WILLIAM H. JOHNS and THEODORE R. BATES

Abstract □ The binding of a series of conjugated bile salt and fatty acid salt anions to cholestyramine from aqueous media was investigated and the data were plotted according to the Langmuir adsorption equation. Increases in affinity constants were noted as the number of hydroxy substituents on the bile salt-ring structure decreased. An increase in the chain length of the fatty acid salt caused a corresponding increase in the affinity constant, whereas an increase in the extent of unsaturation in the fatty acid chain produced a reduction in the affinity constants for the fatty acid–cholestyramine interaction. Apparent surface tension-lowering properties of the adsorbate molecules were found to parallel the affinities obtained for both classes of adsorbate molecules, with the exception of the fatty acid anion, linoleate. Based on the results of these studies, it is suggested that the binding mechanism involves a primary electrostatic component reinforced by a secondary nonelectrostatic interaction, the strength of the latter force being dependent on the degree of hydrophobicity of the adsorbate molecule.

Keyphrases □ Cholestyramine binding—quantification □ Bile salts, fatty acid salt anions—cholestyramine interaction mechanism □ Surface tension values–affinity constants, correlation—bile salts □ Hydrophobic character relationship, adsorbate—cholestyramine binding □ UV spectrophotometry—analysis

In previous work with the anionic exchange resin cholestyramine (1), the authors studied the effect of the physiologic electrolytes, sodium chloride and bicarbonate, on the binding process of bile salt anions to cholestyramine. The dihydroxy bile salt anions studied were noted to be insignificantly affected in their extent of interaction with the resin, while the trihydroxy bile salt anion–cholestyramine interactions were markedly reduced in the presence of an added electrolyte. These results suggested that a secondary, nonelectrostatic, type of interaction was taking place at the adsorption site.

The observation that structurally different bile salt anions exhibit varying types, as well as extents, of binding to cholestyramine is of biologic significance in that an appreciation of the possible modes of interaction could ultimately contribute to an enhancement in the efficiency of this pharmacologically important resin.

The purpose of this study was to elucidate the nature of this secondary binding mechanism and the effect of adsorbate structure thereon. In order to accomplish this, the binding tendencies of a selected series of glycine-conjugated bile salts and various physiologic fatty acid salts to cholestyramine were investigated.

EXPERIMENTAL

Materials—The sodium salts of glycocholic acid,[1] glycodeoxycholic acid,[1] glycodehydrocholic acid,[1] glycolithocholic acid,[1] lauric acid,[2] and oleic acid[3] were dried *in vacuo* for at least 48 hr. prior to use. The sodium salt of linoleic acid[3] was prepared by reacting equimolar quantities of the acid with sodium ethylate in absolute alcohol. The resulting salt was washed several times with absolute alcohol, dried at room temperature, and subjected to vacuum desiccation. The cholestyramine[4] employed in this study was of pharmaceutical grade (1). Reagent grade concentrated sulfuric acid, glacial acetic acid, hydrochloric acid, chloroform, sodium hydroxide, copper nitrate, *n*-butanol, and diethyldithiocarbamate were used as received.

Procedure for Adsorption Studies—A series of aqueous solutions of each bile salt and fatty acid salt was prepared over the concentration range of 0.75–5.0 millimolar (m*M*).[5] Twenty-five-milligram samples of cholestyramine were accurately weighed and placed into 50-ml. glass-stoppered conical flasks, together with a 25.0-ml. portion of the adsorbate solution. At each concentration, a control flask was prepared containing a similar quantity of the solution under study but no cholestyramine. These latter control solutions, which were assayed concomitantly with the solutions exposed to cholestyramine, were used to prepare the required Beer's law plots.

[1] Grade A. Obtained from Calbiochem Co., Los Angeles, Calif.
[2] Obtained from Eastman Organic Chemicals, Rochester, N. Y.
[3] Obtained from Fisher Scientific Co., Fair Lawn, N. J.
[4] Supplied by Merck and Co., Inc., Rahway, N. J.
[5] All systems exhibited complete solution over the concentration range studied, with the exception of the higher concentrations of glycolithocholate, which showed slight turbidity. Adsorption, being a dynamic process, would tend to increase the apparent solubility of a relatively insoluble material by removing molecules from solution and thereby promoting solution of any undissolved material.

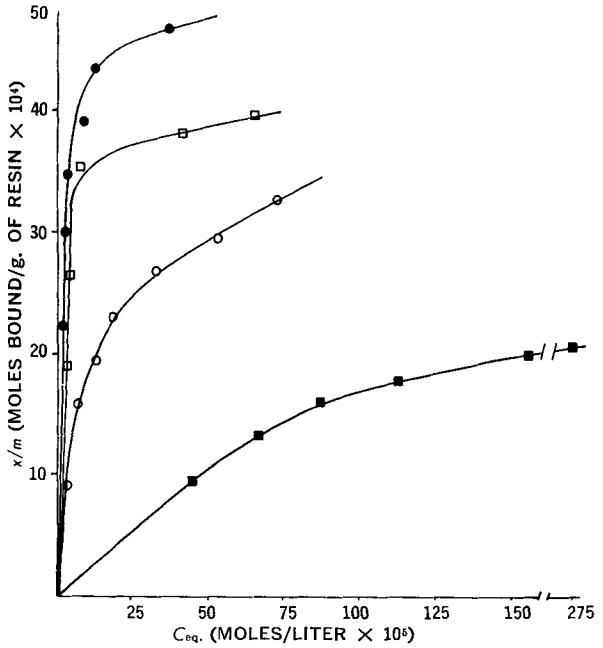


Figure 1—*Adsorption isotherms for the binding of glycine-conjugated bile salt anions to cholestyramine at 25°. Key: glycolithocholate* (●), *glycodeoxycholate* (□), *glycocholate* (○), *and glycodehydrocholate* (■).

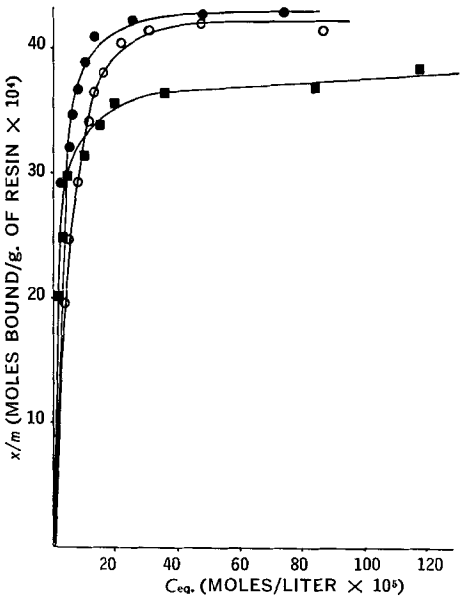


Figure 2—*Adsorption isotherms for the binding of several physiologic fatty acid salt anions to cholestyramine at 25°. Key: oleate* (●), *linoleate* (○), *and laurate* (■).

All containers were closed securely and mechanically shaken[6] at 25° until equilibrium was established; this normally occurred within 24 to 48 hr. The equilibrated samples were subjected to Millipore filtration (0.45-μ pore size), the filtrates suitably diluted or concentrated, and the equilibrium bile salt or fatty acid salt concentration determined (see *Assay Procedures*).

Procedure for Desorption Studies—In order to determine the desorption characteristics of the bound adsorbate molecules, 25.0-ml. quantities of each bile salt or fatty acid salt solution were prepared and shaken with 25.0 mg. of cholestyramine in 125-ml. glass-stoppered conical flasks. After attainment of equilibrium, the samples were diluted with 50.0-ml. portions of distilled water and agitated until equilibrium was again established. After filtration and appropriate dilution or concentration, the filtrates were assayed for free adsorbate concentration.

Surface Tension Determinations—The surface tension-lowering properties of the adsorbate molecules were studied at concentrations well below their respective critical micelle concentrations (CMC). All surface tension measurements were conducted at 25° using a Fisher ring tensiometer. At least eight determinations were performed for each solution and the pure solvent. Apparent surface tension-lowering (π) values were calculated from the difference between the mean surface tension value obtained for distilled water and the adsorbate solutions.

Assay Procedures—The equilibrium concentration of unbound or free adsorbate was determined by one of the following methods.

Bile Salts—Sodium glycocholate and glycodeoxycholate were determined spectrophotometrically in 65% sulfuric acid as described previously (1).

The spectrophotometric method of Minibeck (2) was modified for the determination of sodium glycolithocholate. The residue from an evaporated filtrate sample was dissolved in a 9:1 mixture of concentrated sulfuric and glacial acetic acids and the resultant solution heated at 60° for 30 min. A quantity of 9:1 acid mixture, treated in the same manner, served as a blank.

For the determination of sodium glycodehydrocholate, an aliquot of the equilibrated, filtered sample was evaporated to dryness and the residue dissolved in 0.1 *N* sodium hydroxide. Spectrophotometric determinations were made using the base as a blank.

Absorbance readings were determined using a Beckman model DB-G recording spectrophotometer. All of the adsorbates were found to obey the Beer-Lambert law relationship at their respective wavelengths of maximum absorbance (*i.e.*, glycodeoxycholate, 385 mμ; glycocholate, 320 mμ; glycolithocholate, 316 mμ; glycodehydrocholate, 282 mμ).

Fatty Acid Salts—The fatty acid salts were assayed by a modification of the method proposed by Duncombe (3). An aliquot of the equilibrated, filtered sample was acidified with 1.0 *N* HCl and the free fatty acid extracted with 15.0-ml. quantities of chloroform. A 5-ml. portion of the chloroform phase, diluted when necessary, was shaken with 2.5 ml. of a copper nitrate reagent for a period of not less than 2 min. The aqueous phase was removed by means of aspiration, and a 3-ml. aliquot of the chloroform solution was reacted with 0.5 ml. of a 0.1% w/v solution of diethyldithiocarbamate in *n*-butanol. The blue color which developed was read on a Bausch and Lomb Spectronic-20 colorimeter at 437 mμ using chloroform, treated in an identical manner, as the blank. All fatty acid salts under investigation followed the Beer-Lambert relationship.

The amount of bile salt or fatty acid salt bound to cholestyramine was calculated from the difference between the initial concentration of adsorbate introduced into the system and the concentration present free in solution at equilibrium.

RESULTS AND DISCUSSION

All adsorption experiments were conducted in aqueous solution. Under these conditions, the pH of the systems after equilibration with cholestyramine was essentially independent of initial adsorbate concentration. Based on reported pKa values (4) for the bile acids employed in this study,[7] at concentrations below their respective critical micelle concentrations, it was previously determined (1) that the bile salts were present essentially in the ionized form.

There is scanty information in the literature pertaining to the pKa values of long-chain fatty acids. Ralston (5) reports that small decreases in dissociation constants of fatty acids are noted with increasing molecular weight. Using a method for apparent pKa determination described by Gumtow (6), the apparent pKa's for

[6] Precision Constant Temperature Shaker Bath, Precision Scientific Co., Chicago, Ill.

[7] Ekwall *et al.* (4) report pKa values of 4.99 for lithocholic acid and 4.91 for dehydrocholic acid. Using the equilibrium pH's of 6.66 for the glycolithocholate system and 6.30 for that of the glycodehydrocholate system, percentages ionized were calculated to be 97.9 and 96.1%, respectively. Since the pKa values of glycine conjugates are normally lower than those of the unconjugated bile salts (9), the use of the reported lithocholic and dehydrocholic acid pKa's is a justifiable approximation.

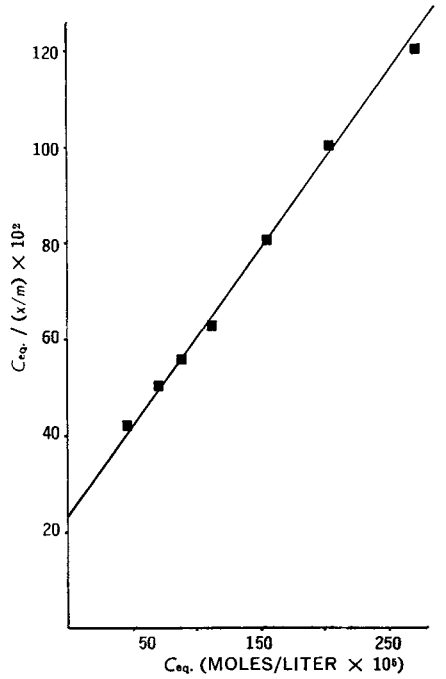


Figure 3—*Langmuir adsorption isotherm for the binding of the glycodehydrocholate anion to cholestyramine at 25°.*

lauric, oleic, and linoleic acids were found to be 4.92, 5.35, and 5.10, respectively. The value obtained for oleic acid is similar to that reported for stearic acid (7) (pKa = 5.75 at 35°), while that of lauric acid is in close agreement with the 4.96 value reported by Markley (8) for nonanoic acid. These experimental values, together with equilibrium pH's (laurate, 6.90; oleate, 6.30; linoleate, 6.10), were used in the Henderson-Hasselbach equation to obtain values of 98.9, 91.3, and 91.0% ionized, respectively. Using this

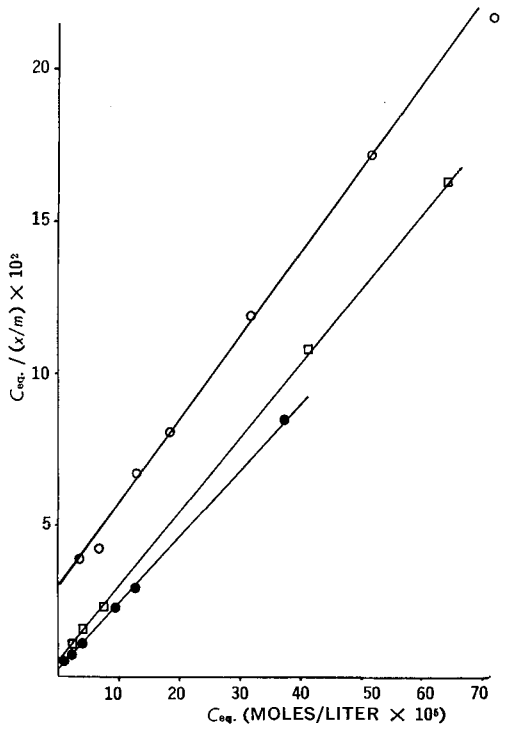


Figure 4—*Langmuir adsorption isotherms for the binding of glycine-conjugated, hydroxy-substituted bile salt anions to cholestyramine at 25°. Key: glycocholate (○), glycodeoxycholate (□), and glycolithocholate (●).*

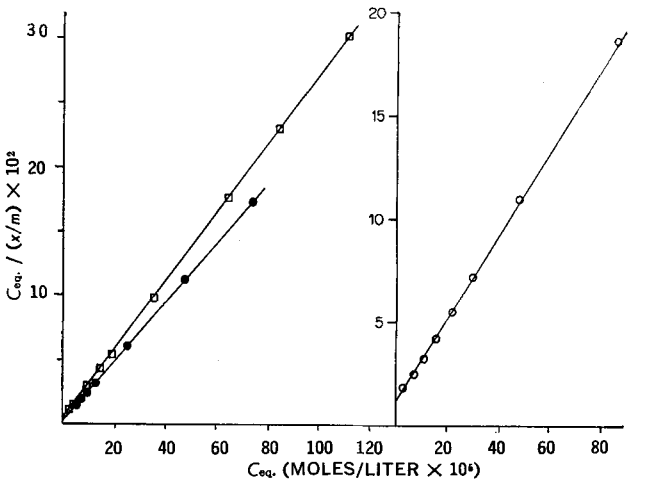


Figure 5—*Langmuir adsorption isotherms for the binding of fatty acid salt anions to cholestyramine at 25°. Key: laurate (□), oleate (●), and linoleate (○).*

as a first approximation, the fatty acids under study were considered to be completely ionized.

Adsorption Studies—The adsorption isotherms describing the binding of the investigated bile salt and fatty acid salt anions to cholestyramine were plotted according to the following Langmuir equation (10) and are shown in Figs. 1 and 2.

$$x/m = \frac{k_1 k_2 (C_{eq.})}{1 + k_1 (C_{eq.})} \qquad \text{(Eq. 1)}$$

where $C_{eq.}$ = the concentration of adsorbate molecules remaining in solution at equilibrium, x/m = the number of moles of adsorbate bound per gram of adsorbent, k_1 = the association or affinity constant, and k_2 = the capacity constant. The general shape of the x/m *versus* $C_{eq.}$ plots is representative of only monolayer adsorption (11). The curves show a tendency to plateau at high $C_{eq.}$ values, indicating that the system is approaching the limiting monomolecular-exchange capacity of cholestyramine for the particular adsorbate molecule.

Figures 3–5 represent the data plotted according to a rearranged form of Eq. 1:

$$\frac{(C_{eq.})}{x/m} = \frac{1}{k_1 k_2} + \frac{(C_{eq.})}{k_2} \qquad \text{(Eq. 2)}$$

The linearity observed with all adsorbate anions under study demonstrates the adherence of the binding process to the Langmuir-type adsorption isotherm (Eq. 2). The adsorption constants, k_1 and k_2, were obtained from the least-squares intercept and slope values of a regression line drawn through the data points and are reported in Table I.

Except for the glycodehydrocholate anion, the capacity constants, k_2 (expressed as the number of moles of anion adsorbed per mole equivalent of cholestyramine), all demonstrate a tendency toward unity. As a possible explanation of glycodehydrocholate's behavior, it is suggested that hydration, expected of the polar substituents on the phenanthrene ring system, would be more extensive with this bile salt due to the greater polarity induced by the presence of the three keto substituents (12). This could lead to enhanced steric hindrance and possible occlusion of neighboring binding positions, thus resulting in the reduced capacity observed.

An examination of the affinity constants, k_1, for the bile salt series shows an increase in the following order: glycodehydrocholate (triketo) ≪ glycocholate (trihydroxy salt) < glycodeoxycholate (dihydroxy salt) < glycolithocholate (monohydroxy salt). Since all of the bile salt anions are glycine conjugates, the observed differences cannot be related to changes in the structure of the side chain. Reduction in steric hindrance in the ring structure when going from tri- to monohydroxy salts cannot, in and of itself, be responsible for the observed affinity differences, nor is it reasonable to assume that substitution of hydroxy groups by keto functions can cause any appreciable effects on the anionic charge strength.

Table I—Langmuir Adsorption Constants for the Binding of Conjugated Bile Salt and Fatty Acid Salt Anions to Cholestyramine at 25°

Anion	k_1 (l./mole of Adsorbate, $\times 10^{-4}$)	$(k_2)^a$ (Moles of Adsorbate Bound per Mole Equivalent of Resin)	Apparent Surface Tension Lowering (dynes/cm.)
Bile salt			
Glycodehydrocholate	0.163	0.593	7.9[b]
Glycocholate	0.891	0.863	12.0
Glycodeoxycholate	4.25	0.941	23.7
Glycolithocholate	5.70	1.12	36.6
Fatty acid salt			
Laurate	5.23	0.892	10.8[c]
Oleate	7.39	1.01	38.0
Linoleate	1.92	1.03	31.8

[a] Based on a monomer equivalent weight for cholestyramine of 230. [b] Based on surface tension determinations of 2.0 m*M* bile salt solutions. [c] Based on surface tension determinations of 0.25 m*M* fatty acid salt solutions.

As stated earlier, the presence of three keto groups in the glycodehydrocholate system confers a greater hydrophilic character to this anion (12). This increased hydrophilicity would lead to a more extensive association of glycodehydrocholate with water molecules, thus producing an anion possessing larger molecular dimensions than the less hydrated hydroxy derivatives. The increased bulkiness of the glycodehydrocholate molecule could effectively prevent the close proximity of this adsorbate to the binding positions on cholestyramine and hence reduce the possibility for a significant degree of interaction. This results from the fact that the strength of the forces involved in the adsorbate-adsorbent interaction would tend to decrease with increasing separation of the two reactants. The results obtained in the present investigation are consistent with ion-exchange phenomenon in general, in that ion-exchange resin selectivity, which is related directly to the strength of adsorbate-adsorbent interaction (13), decreases as the extent of hydration of the adsorbate increases (14). In addition, the fact that the triketo-substituted bile salts exhibit negligible tendencies to undergo micelle formation as compared with hydroxy-substituted derivatives (15) would seem to indicate that their participation in nonpolar interactions, in general, is rather limited. The interrelated effects of an increase in the molecular dimensions due to the increased hydration of this adsorbate molecule and a decrease in its capability of significant nonpolar interactions with the resin matrix are probably responsible for the diminution in the affinity observed with the glycodehydrocholate–cholestyramine interaction.

The data clearly suggest that the primary electrostatic interaction (*i.e.*, between the anionic carboxyl group of the bile salt and the cationic quaternary ammonium group of cholestyramine) is being reinforced by secondary, nonelectrostatic binding forces existing between the hydrophobic portions of the bile salt and resin molecules. As the degree of polarity of the ring system, and hence the degree of hydration, increase (glycolithocholate < glycodeoxycholate < glycocholate ≪ glycodehydrocholate), the hydrophobicity, and hence the strength of the nonelectrostatic component of the interaction, diminish. These results parallel those obtained by Rudman and Kendall (16) who investigated the interaction of a series of bile salts with the protein, albumin. This phenomenon was also qualitatively alluded to in the work of Gordon *et al.* (17), in which the hydroalcoholic, chromatographic elution sequence of conjugated bile acids from a column composed of the acetate form of an anion-exchange resin (Dowex 1-X2) was studied.

The adsorption characteristics of a series of physiologic, long-chain fatty acid salts were examined to establish whether or not a similar secondary, nonelectrostatic binding mechanism was operable. The k_1 values for the laurate and oleate anions, listed in Table I, show an increase in affinity with increasing chain length. Consistent with these results, the studies of Boyer *et al.* (18) and Ballou *et al.* (19) demonstrated an intensification of fatty acid interaction with albumin as the chain length of the adsorbate molecule increased. In connection with fatty acid-albumin binding, Goldstein (20) states that "... the primary bond is presumably electrostatic, but the resulting complex is probably stabilized by van der Waals' forces through the close approximation of the nonpolar residue to similar portions of adjacent protein surfaces." The increase of six carbons, in the case of the oleate anion over that of the laurate anion, confers to the former anion a higher degree of hydrophobic character (even in the presence of the hydrophilic double bond) which results in a magnification of the strength of interaction.

The introduction of a second double bond into the adsorbate molecule, as in the linoleate system (9,12-diene), produced a marked decrease in the affinity constant relative to the oleate anion. The increase in the hydrophilicity of the carbon chain, and thus a weakening of the strength of the nonelectrostatic component of the interaction, could account for the observed reduction in the affinity. In addition, the presence of a second alkene linkage might contribute to this reduction by placing steric restrictions on the mobility of the linoleate molecule; the *cis-cis* configuration of the molecule prohibiting the proximity of adsorbate and adsorbent molecules required for significant nonionic interaction.

Desorption Studies—Under the experimental conditions employed, the studies designed to determine the dissociation or desorption characteristics of the adsorbent-adsorbate complexes showed the binding process to be essentially nonreversible.

Surface Tension Studies—The bile salt and fatty acid anions employed in this investigation all possess surface-active properties in aqueous solution and, as a result, have some facility to concentrate at interfaces. In general, the degree of surface activity elicited is dependent on the existence of a proper balance between the hydrophilic and hydrophobic regions of the molecule, normally increasing with increasing hydrophobicity (21). Since it was the objective of this investigation to establish the role of a secondary, nonelectrostatic type of interaction in the binding process, it was of interest to determine the air-water surface adsorption tendencies of the various bile salt and fatty acid anions. The purpose, therefore, of these surface tension studies was to ascertain whether a parallelism existed among the degree of hydrophobicity of the adsorbate molecules, their binding affinity, and their surface tension-lowering properties.[8]

In Table I are listed the apparent surface tension-lowering (π) values obtained for the various salts under investigation. A comparison of the bile salt values with their respective affinity constants shows that an excellent rank order correlation exists between the two parameters. Likewise, the surface activity of the laurate and oleate systems compares well with their respective k_1 values.

The deviation of the linoleate system from the parallelism found to exist with the other fatty acid salts is thought to be due to the molecular structure of that fatty acid salt. The adsorption process, being heavily dependent on proper steric alignment for maximum nonelectrostatic interaction, would be seriously hindered by the *cis-cis* configuration offered by the linoleate anion. However, the surface tension properties of this anion would not be as drastically affected, since the nonpolar portions of the molecule would be capable of assuming any packing arrangement at the air-water interface commensurate with maximum thermodynamic stability. In addition, it is possible that a minor quantity of contamination was present in the linoleate system. Such an occurrence could produce a significant change in the surface tension-lowering capabilities of the salt below the CMC (22), while at the same time having only negligible effects on the binding characteristics of the anion.

The results of this investigation indicate that the affinity with which fatty acid and bile salt anions bind to cholestyramine is, in part, dependent on the extent of hydrophobic character of the adsorbate anion. A relationship was found to exist between the surface activity of adsorbate molecules, with the exception of linoleate, and the strength of the adsorbate-cholestyramine interactions as reflected by the magnitudes of the association constants for the respective complexes.

Apparently a similar interaction mechanism is functioning in both the bile salt and fatty acid salt series. It would not be unrealistic, therefore, to imagine an *in vivo* situation where a competition existed between endogenous bile salts and exogenous fatty

[8] It should be pointed out that the authors do not intend to imply that possession of surface activity is a necessary prerequisite for significant interaction with cholestyramine.

acids, the latter being present either in food or generated in the small intestine by the action of digestive enzymes on dietary triglycerides. Such a competition, depending on the nature and relative concentration of the fatty acid, could cause a reduction in the therapeutic efficiency of cholestyramine to sequester certain bile salt anions. Studies to determine the nature and extent of any competition which may exist between bile salt anions and other physiologic substances for the binding positions on cholestyramine are in progress and will be the subject of subsequent communications.

REFERENCES

(1) W. H. Johns and T. R. Bates, *J. Pharm. Sci.*, **58,** 179(1969).

(2) H. Minibeck, *Biochem. Z.*, **297,** 29(1938).

(3) W. G. Duncombe, *Biochem. J.*, **88,** 7(1963).

(4) P. Ekwall, T. Rosendahl, and N. Lofman, *Acta Chem. Scand.*, **11,** 590(1957).

(5) A. W. Ralston, "Fatty Acids and Their Derivatives," Wiley, New York, N. Y., 1948, p. 378.

(6) R. H. Gumtow, *Amer. J. Pharm. Ed.*, **31,** 515(1967).

(7) J. K. Dale, in "Husa's Pharmaceutical Dispensing," 5th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1959, p. 423.

(8) K. S. Markley, "Fatty Acids; Their Chemistry and Physical Properties," Interscience, New York, N. Y., 1947, p. 179.

(9) A. F. Hofmann and D. M. Small, *Ann. Rev. Med.*, **18,** 333(1967).

(10) I. Langmuir, *J. Amer. Chem. Soc.*, **38,** 2221(1916).

(11) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 562.

(12) E. H. Ahrens, Jr., and L. C. Craig, *J. Biol. Chem.*, **195,** 763(1952).

(13) F. Helfferich, "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, pp. 161–164.

(14) *Ibid.*, p. 158.

(15) A. F. Hofmann, *Gastroenterology*, **48,** 484(1965).

(16) D. Rudman and F. E. Kendall, *J. Clin. Invest.*, **36,** 538 (1957).

(17) B. A. Gordon, A. Kuksis, and J. M. R. Beveridge, *Can. J. Biochem. Physiol.*, **41,** 77(1963).

(18) P. D. Boyer, G. A. Ballou, and J. M. Luck, *J. Biol. Chem.*, **167,** 407(1947).

(19) G. A. Ballou, P. D. Boyer, and J. M. Luck, *ibid.*, **159,** 111(1945).

(20) A. Goldstein, *Pharmacol. Rev.*, **1,** 102(1949).

(21) L. I. Osipow, "Surface Chemistry: Theory and Industrial Applications," Reinhold, New York, N. Y., 1962, pp. 163–166.

(22) B. A. Mulley, in "Advances in Pharmaceutical Sciences," vol. I, H. S. Bean, A. H. Beckett, and J. E. Carless, Eds., Academic, New York, N. Y., 1964, pp. 100–101.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 17, 1969, from the *Division of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CN 06268*

Accepted for publication September 25, 1969.

This investigation was supported in part by Grant 076 from the University of Connecticut Research Foundation, Storrs, CN 06268

Flexible Nonisothermal Stability Studies

H. V. MAULDING and M. A. ZOGLIO

Abstract □ A method is described which allows *ad libitum* temperature adjustment during the course of a nonisothermal kinetic study. The data obtained are compared to theoretical degradation patterns to obtain from a single experiment activation energy, reaction rates, and stability predictions at any desired temperature. The inversion of sucrose and the hydrolysis of ethyl acetate are studied to demonstrate the validity of the theory and the advantages of the method.

Keyphrases □ Stability studies—flexible, nonisothermal □ Kinetic equations—flexible nonisothermal stability studies □ Sucrose inversion—nonisothermal stability methodology □ Ethyl acetate hydrolysis—nonisothermal stability methodology □ Polarimetry—analysis

The field of nonisothermal kinetics has grown considerably in popularity since the classic treatment by Rogers (1) was published in 1963. Since then numerous publications have appeared in the literature utilizing nonisothermal techniques (2, 3). Others have introduced new ideas and techniques to the field (4, 5). The objective of this study is to eliminate the need for a fixed time-temperature profile during the course of a nonisothermal study. The advantages of such an approach lie in the freedom to change temperature at a rate consistent with analytical findings and also in minimizing experimental requirements. The method involves the subjection of a solution of the substance for study to changing temperature to provide sufficient breakdown for calculation of activation energy, reaction rates, and stability predictions. The degradation is controlled by adjusting the rate of change of temperature according to analytical findings during the experiment. The time–temperature data are fitted to a polynomial expression of sufficient degree to describe the changes. This relationship and the experimental data are then combined and utilized to synthesize a series of degradation pathways corresponding to different levels of activation energy. The curves are compared to the experimental analytical data to obtain the correct energy of activation for the reaction. Utilizing this activation energy and the analytical data, reaction rate and stability calculations can be made.

THEORETICAL

Consider a drug in solution degrading according to some unchanging reaction order as in Fig. 1. Drug concentration can then

acids, the latter being present either in food or generated in the small intestine by the action of digestive enzymes on dietary triglycerides. Such a competition, depending on the nature and relative concentration of the fatty acid, could cause a reduction in the therapeutic efficiency of cholestyramine to sequester certain bile salt anions. Studies to determine the nature and extent of any competition which may exist between bile salt anions and other physiologic substances for the binding positions on cholestyramine are in progress and will be the subject of subsequent communications.

REFERENCES

(1) W. H. Johns and T. R. Bates, *J. Pharm. Sci.*, **58,** 179(1969).

(2) H. Minibeck, *Biochem. Z.*, **297,** 29(1938).

(3) W. G. Duncombe, *Biochem. J.*, **88,** 7(1963).

(4) P. Ekwall, T. Rosendahl, and N. Lofman, *Acta Chem. Scand.*, **11,** 590(1957).

(5) A. W. Ralston, "Fatty Acids and Their Derivatives," Wiley, New York, N. Y., 1948, p. 378.

(6) R. H. Gumtow, *Amer. J. Pharm. Ed.*, **31,** 515(1967).

(7) J. K. Dale, in "Husa's Pharmaceutical Dispensing," 5th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1959, p. 423.

(8) K. S. Markley, "Fatty Acids; Their Chemistry and Physical Properties," Interscience, New York, N. Y., 1947, p. 179.

(9) A. F. Hofmann and D. M. Small, *Ann. Rev. Med.*, **18,** 333(1967).

(10) I. Langmuir, *J. Amer. Chem. Soc.*, **38,** 2221(1916).

(11) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 562.

(12) E. H. Ahrens, Jr., and L. C. Craig, *J. Biol. Chem.*, **195,** 763(1952).

(13) F. Helfferich, "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, pp. 161–164.

(14) *Ibid.*, p. 158.

(15) A. F. Hofmann, *Gastroenterology*, **48,** 484(1965).

(16) D. Rudman and F. E. Kendall, *J. Clin. Invest.*, **36,** 538 (1957).

(17) B. A. Gordon, A. Kuksis, and J. M. R. Beveridge, *Can. J. Biochem. Physiol.*, **41,** 77(1963).

(18) P. D. Boyer, G. A. Ballou, and J. M. Luck, *J. Biol. Chem.*, **167,** 407(1947).

(19) G. A. Ballou, P. D. Boyer, and J. M. Luck, *ibid.*, **159,** 111(1945).

(20) A. Goldstein, *Pharmacol. Rev.*, **1,** 102(1949).

(21) L. I. Osipow, "Surface Chemistry: Theory and Industrial Applications," Reinhold, New York, N. Y., 1962, pp. 163–166.

(22) B. A. Mulley, in "Advances in Pharmaceutical Sciences," vol. I, H. S. Bean, A. H. Beckett, and J. E. Carless, Eds., Academic, New York, N. Y., 1964, pp. 100–101.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 17, 1969, from the *Division of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CN 06268*

Accepted for publication September 25, 1969.

This investigation was supported in part by Grant 076 from the University of Connecticut Research Foundation, Storrs, CN 06268

Flexible Nonisothermal Stability Studies

H. V. MAULDING and M. A. ZOGLIO

Abstract □ A method is described which allows *ad libitum* temperature adjustment during the course of a nonisothermal kinetic study. The data obtained are compared to theoretical degradation patterns to obtain from a single experiment activation energy, reaction rates, and stability predictions at any desired temperature. The inversion of sucrose and the hydrolysis of ethyl acetate are studied to demonstrate the validity of the theory and the advantages of the method.

Keyphrases □ Stability studies—flexible, nonisothermal □ Kinetic equations—flexible nonisothermal stability studies □ Sucrose inversion—nonisothermal stability methodology □ Ethyl acetate hydrolysis—nonisothermal stability methodology □ Polarimetry—analysis

The field of nonisothermal kinetics has grown considerably in popularity since the classic treatment by Rogers (1) was published in 1963. Since then numerous publications have appeared in the literature utilizing nonisothermal techniques (2, 3). Others have introduced new ideas and techniques to the field (4, 5). The objective of this study is to eliminate the need for a fixed time-temperature profile during the course of a nonisothermal study. The advantages of such an approach lie in the freedom to change temperature at a rate consistent with analytical findings and also in minimizing experimental requirements. The method involves the subjection of a solution of the substance for study to changing temperature to provide sufficient breakdown for calculation of activation energy, reaction rates, and stability predictions. The degradation is controlled by adjusting the rate of change of temperature according to analytical findings during the experiment. The time–temperature data are fitted to a polynomial expression of sufficient degree to describe the changes. This relationship and the experimental data are then combined and utilized to synthesize a series of degradation pathways corresponding to different levels of activation energy. The curves are compared to the experimental analytical data to obtain the correct energy of activation for the reaction. Utilizing this activation energy and the analytical data, reaction rate and stability calculations can be made.

THEORETICAL

Consider a drug in solution degrading according to some unchanging reaction order as in Fig. 1. Drug concentration can then

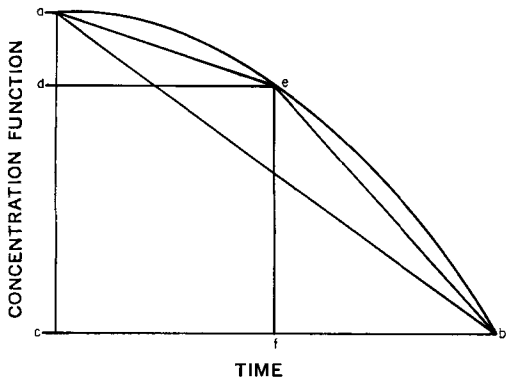


Figure 1—*Hypothetical drug degradation in solution during a flexible nonisothermal experiment.*

be considered to be a time-dependent function, where the instantaneous rate of change of drug concentration is related to the total loss in drug concentration by the following expression:

$$f(b) - f(c) = \int_c^b f'(t)dt \qquad \text{(Eq. 1)}$$

where $f'(t)$ represents drug concentration as a function of time. Dividing both sides of Eq. 1 by $(b - c)$ yields

$$\frac{f(b) - f(c)}{b - c} = \frac{\int_c^b f'(t)dt}{b - c} \qquad \text{(Eq. 2)}$$

If tc to tb is divided into two equal increments,

$$\frac{f(b) - f(c)}{b - c} = \frac{[f(b) - f(f)/(b - c)/2] + [f(f) - f(c)/(b - c)/2]}{2} \qquad \text{(Eq. 3)}$$

which is equivalent to saying that the tangent of triangle *abc* is equal to the arithmetic average of the tangents of triangles *aed* and *ebf*. If this concept is extended to include more triangles of equal base, the tangents of these triangles will approach slopes on the curve *aeb* as the number of triangles is increased. This is a somewhat intuitive way of looking at the following expression:

$$\frac{f(b) - f(c)}{b - c} = \frac{k_1 + k_2 + k_3 + \cdots k_i + \cdots k_n}{n} \qquad \text{(Eq. 4)}$$

where k_i represents the slope of the ith triangle and n equals the number of triangles being considered. Since each triangle describes some equal and individual interval Δt, the question is raised as to how large a value of n is needed for the tangent of each triangle to approach the slope on the curve *aeb* for each interval Δt.

Consider the zero-order case where

$$-k_i = \frac{C_{(i+1)} - C_i}{t_{(i+1)} - t_i} \qquad \text{(Eq. 5)}$$

C here representing concentration. If Δt is very small the slope over this interval could approximate the rate over an isothermal interval. The rate constant corresponding to k_1 of Eq. 4 may be represented by the following expression,

$$k_1 = a^* e^{-E/RT_1} \qquad \text{(Eq. 6)}$$

where E is activation energy, R is the universal gas constant, and T is temperature.

The concentration at the end of the first interval would be

$$C_1 = C_0 - a^* \Delta t e^{-E/RT_1} \qquad \text{(Eq. 7)}$$

If the temperature is changed to T_2 for the same increment of time and then to T_3 and so on, then after n increments the concentration would be

$$C_n = C_0 + \sum_{i=1}^{i=n} - \Delta t a^* e^{-E/RT_i} \qquad \text{(Eq. 8)}$$

Therefore,

$$\frac{C_n - C_0}{n\Delta t} = \frac{a^*}{n} \sum_{i=1}^{i=n} -e^{-E/RT_i} = \bar{k}_n \qquad \text{(Eq. 9)}$$

where k_n represents the average rate constant for n discrete values. For the time interval t_c to t_b of Fig. 1, temperature will most likely be related to time in a manner which can be described mathematically. The assumption is thus made that

$$T_i = G(t_i) \qquad \text{(Eq. 10)}$$

where the relationship can be obtained perhaps by some polynomial-fitting technique. Consider the function

$$-\left(\frac{a^*}{t_b - t_c}\right) e^{-E/RG(t_i)} \qquad \text{(Eq. 11)}$$

An upper Rieman sum for the interval t_c to t_b would be

$$\sum_{i=1}^{n} - \frac{(t_b - t_c)}{n} \left(\frac{a^*}{t_b - t_c}\right) e^{-E/RG(t_i)} = \frac{a^*}{n} \sum_{i=1}^{n} -e^{-E/RG(t_i)} \qquad \text{(Eq. 12)}$$

As the value of n approaches infinity, this sum will approach

$$-\frac{1}{t_b - t_c} \int_b^c a^* e^{-E/RG(t)}\, dt = \bar{k} \qquad \text{(Eq. 13)}$$

which implies through Eqs. 2 and 4 that for very large n a discrete curve over the interval t_c to t_b should approach the smooth curve, and the average value of k should not differ significantly from the quantity

$$\frac{f(b) - f(c)}{b - c}$$

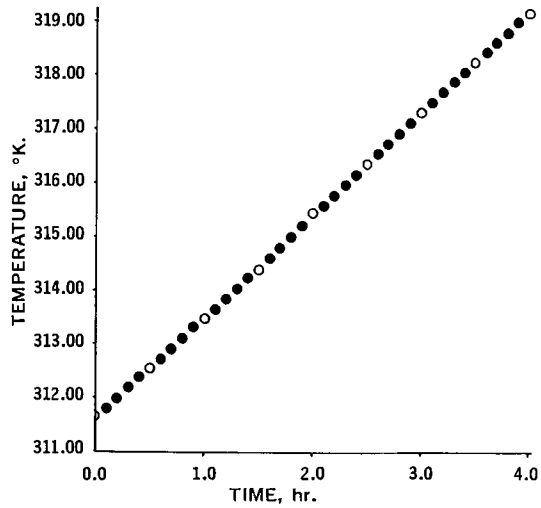


Figure 2—*Time–temperature relationship for nonisothermal acid-catalyzed inversion of sucrose (a). Key: ●, mathematical fit; and ○, experimental points.*

Table I—Convergence for the Acid-Catalyzed Inversion of Sucrose (Experiment a) for the Expression

$$\left\{1 + \sum_{i=2}^{n} \exp E/R[(T_i - T_1)/(T_iT_1)]\right\}\Big/n$$

Value of n	Value of Expression for $E = 10$ kcal.	$E = 15$ kcal.	$E = 20$ kcal.	$E = 25$ kcal.	$E = 30$ kcal.
100	1.228	1.360	1.512	1.684	1.883
200	1.222	1.355	1.504	1.676	1.874

In order to utilize Eq. 4, the magnitude of n needed for the equation to be of use must be determined. If all rates in Eq. 4 are expressed in terms of one rate (k_1), then

$$\frac{f(b) - f(c)}{b - c} = \{k_1 + k_1 \exp E/R\,[(T_2 - T_1)/(T_1T_2)] + \cdots \times k_1 \exp E/R[(T_i - T_1)/(T_iT_1)] + \cdots k_1 \exp E/R[(T_n - T_1)/(T_nT_1)]\}/n \quad \text{(Eq. 14)}$$

which may be expressed as

$$\frac{f(b) - f(c)}{b - c} = k_1\frac{\left\{1 + \sum_{i=2}^{n} \exp E/R[(T_i - T_1)/(T_iT_1)]\right\}}{n} \quad \text{(Eq. 15)}$$

The convergence of the expression

$$\left\{1 + \sum_{i=2}^{n} \exp E/R[(T_i - T_1)/(T_iT_1)]\right\}\Big/n$$

for various values of E at increasing levels of n can be used to find a value for n. Upon obtaining n, Eq. 15 can be used to calculate k_1 for a particular activation energy. The remaining rates necessary to synthesize a model degradation pathway for this energy of activation are calculated through the Arrhenius equation. The calculations are repeated for a series of activation energy values to obtain a family of degradation pathways. The experimental degradation pathway is then compared to the theoretical pathways to obtain the energy of activation for the reaction. Rates can be calculated using Eq. 15 and the Arrhenius equation. Stability predictions can then be made by integration of the proper rate equations. The arguments presented in the theory are applicable to reaction orders other than zero and can be realized through the same type of reasoning.

EXPERIMENTAL

Sucrose—A 40% w/v sucrose solution in distilled water was prepared and 400 ml. of this solution thoroughly mixed with 200 ml. 0.05 *N* HCl. Aliquots (30 ml.) of this stock solution were pipeted into 50-ml. ampuls which were flame sealed and immersed in a water bath. The water bath was fitted with a thermoregulator[1] and thermometer (0.1° graduations). The thermometer was immersed in an unsealed ampul containing the reaction mixture for determination of the temperature inside the reaction solution. Sufficient time was allowed for equilibration of the temperature in the reaction solution before the nonisothermal run was begun. Following temperature equilibration an initial sample was taken and the water bath temperature was increased by manual manipulation of the thermoregulator at an appropriate rate, with samples being removed for analysis at convenient intervals and the temperature continuously recorded.

For analysis the ampuls were cooled and opened, and the solution was diluted with 50 ml. 0.1 *N* NaOH. This solution was read on a polarimeter[2] using the sodium D line. Sucrose concentration is directly proportional to $(\alpha_t - \alpha_\infty)$ where α is the optical rotation. The rotation at time infinity, α_∞, is determined by heating a sample at 90° for 2–3 hr., diluting with 0.1 *N* NaOH, and reading in the usual manner.

[1] 115-v., 60-cycle thermoregulator, Brownell Sci. Div., Will Corp., Rochester, N. Y.

[2] Model 70 polarimeter, O. C. Rudolph and Sons Inc., Caldwell, N. J.

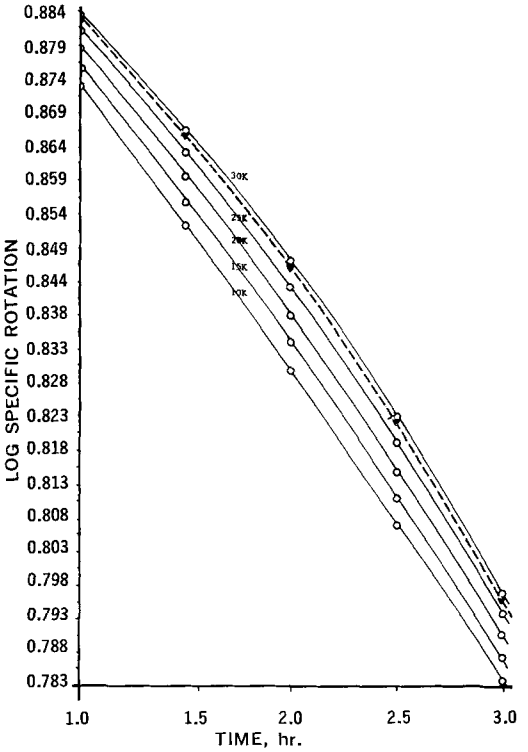


Figure 3—*Model degradation curves for nonisothermal acid-catalyzed inversion of sucrose (a). Key: - - -, experimental data.*

Ethyl Acetate—A solution of 25 ml. (22.45 g., 0.255 mole) of ethyl acetate in 500 ml. 0.1 *N* HCl was prepared (0.5096 mole/l.) and 25-ml. aliquots pipeted into 50-ml. ampuls which were heat sealed. These ampuls were immersed in a water bath fitted with a thermoregulator and thermometer (0.1° graduations). The thermometer was immersed in one of the unsealed ampuls containing the ethyl acetate solution, and the temperature of this solution was allowed to become constant. An initial sample was taken and the nonisothermal run was begun by hand manipulation of the thermoregulator at an appropriate rate of temperature increase. Samples were taken for analysis at convenient times and the temperatures continuously recorded.

The ampuls were quickly cooled under ice water. Five milliliters of solution was pipeted into a 250-ml. conical flask containing 50 ml. ice water and the flask immersed in ice water. A titration with 0.05 *N* NaOH was then performed using phenolphthalein as the indicator. Correction was made for the initial HCl content of the solution, with the remainder of NaOH consumed being proportional to the amount

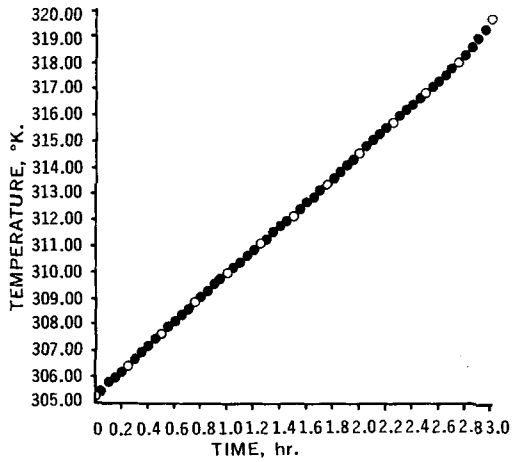


Figure 4—*Time–temperature relationship for nonisothermal acid-catalyzed hydrolysis of ethyl acetate. Key: ●, mathematical fit; and O, experimental points.*

Table II—Convergence for the Acid-Catalyzed Hydrolysis of Ethyl Acetate for the Expression

$$\left\{1 + \sum_{i=2}^{n} \exp E/R[(T_i - T_1)/(T_iT_1)]\right\}\Big/n$$

Value of n	Value of Expression for $E = 10$ kcal.	$E = 15$ kcal.	$E = 20$ kcal.	$E = 25$ kcal.	$E = 30$ kcal.
100	1.488	1.836	2.288	2.881	3.661
200	1.481	1.826	2.275	2.863	3.635

Table III—Convergence for the Acid-Catalyzed Inversion of Sucrose (Experiment b) for the Expression

$$\left\{1 + \sum_{i=2}^{n} \exp E/R[(T_i - T_1)/(T_iT_1)]\right\}\Big/n$$

Value of n	Value of the Expression for $E = 10$ kcal.	$E = 15$ kcal.	$E = 20$ kcal.	$E = 25$ kcal.	$E = 30$ kcal.
100	1.454	1.755	2.126	2.587	3.158
200	1.448	1.749	2.119	2.579	3.150

of acetic acid present. The average value of three titrations per sample was taken and the moles of acetic acid present calculated and subtracted from the ethyl acetate initially in solution for use in the first-order calculations.

RESULTS AND DISCUSSION

The time–temperature relationship for the acid-catalyzed inversion of sucrose is illustrated in Fig. 2. This relationship is described by the following polynomial expression,

$$\text{temp.} = 311.66 + 1.7482t + 0.086257t^2 - 0.013662t^3$$

When the expression was utilized to calculate the number of rates necessary to describe a degradation curve adequately for the experiment, 200 rates were found necessary. The criterion used to determine convergence was a change of less than 1% with an increase in n of 100 in the value of that portion of Eq. 15 used to test convergence. The convergence for this experiment is shown in Table I.

The model degradation pathways for the five activation energies considered in Table I were calculated and are represented in Fig. 3. Hours 1–3 of the experiment are represented. The model curves tend to lie very close to each other for the early and final stages of the experiment and do not contribute to an accurate activation energy determination. The experimental points are averages of results from six separate samples and yield an activation energy of about 28 kcal. This is slightly higher than the reported values of 25–27 kcal. (6).

The time–temperature relationship for the acid-catalyzed hydrolysis of ethyl acetate is shown in Fig. 4. The relationship can be described by the following polynomial expression,

$$\text{temp.} = 305.26 + 4.4450t + 1.6114t^2 - 2.2298t^3 + 0.98356t^4 - 0.070209t^5 + 0.011573t^6 - 0.034019t^7 + 0.0076531t^8$$

The convergence testing for this experiment is shown in Table II and resulted in a value of 200 rates to describe adequately the model degradation curves.

The model curves for this experiment are shown in Fig. 5 for hours 0.45–2.55 of the 3-hr. experiment. Segments of the model curves were eliminated for the reasons given for the sucrose experiment. Since the analytical points in this experiment were scattered, they were averaged and fitted to a second-degree polynomial to yield the smooth experimental curve shown in Fig. 5. The activation energy is approximately 17 kcal., which is in good agreement with the values of 16.5–17.3 kcal. reported in the literature (7).

The time–temperature profile of a second sucrose inversion experiment is given in Fig. 6. This experiment was essentially a test of the limits of flexibility of this method. A fair mathematical fit to this time–temperature relationship is given by the following equation,

$$\text{temp.} = 311.23 + 54.445t - 20.70t^2 - 350.95t^3 + 406.57t^4 + 187.83t^5 + 50.62t^6 - 899.25t^7 + 776.93t^8 - 193.08t^9$$

The convergence testing for this experiment showed a requirement of 200 rates to describe degradation. The results are given in Table III.

The rapid convergence is probably due to the brevity of the experiment. The model curves for the experiment are shown in Fig. 7. The experimental curve is not as smooth as in the first sucrose experiment, but the activation energy of about 27 kcal. is in good agreement with the sucrose experiment The model curves for this experiment are drawn for hours 0.45–0.9 of the experiment. The tendency of the curves to group very closely together at the beginning and second half of the experiment was the reason for their elimination. In the three experiments the assumption of first order was made. Reaction orders other than first are handled by choosing the proper concen-

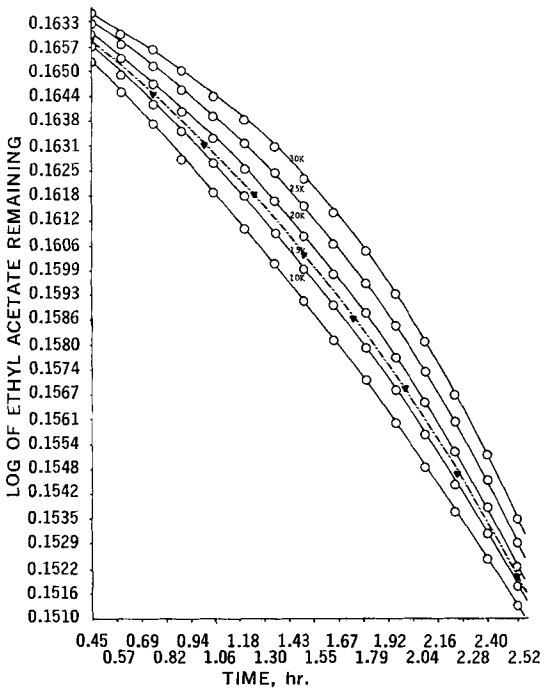


Figure 5—*Model degradation curves for nonisothermal acid-catalyzed hydrolysis of ethyl acetate. Key:* ▼, *experimental curve; and* ○, *model curves.*

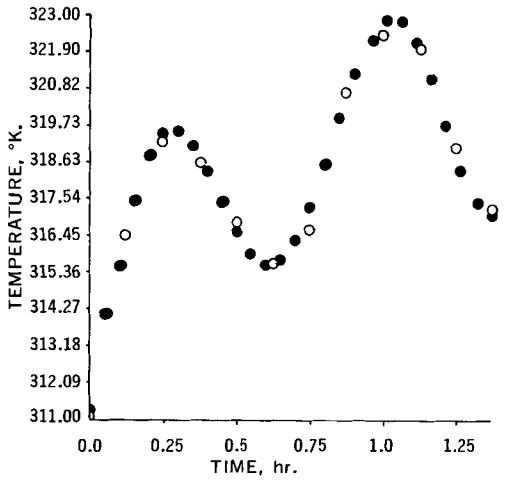


Figure 6—*Time–temperature relationship for nonisothermal acid-catalyzed inversion of sucrose (b). Key:* ●, *mathematical fit; and* ○, *experimental points.*

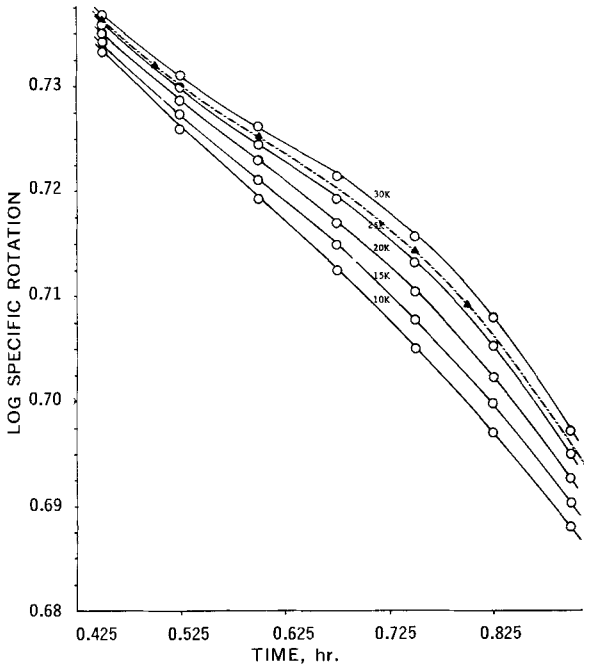


Figure 7—*Model degradation curves for nonisothermal acid-catalyzed inversion of sucrose (b). Key: ▼, experimental curve; and O—, model curves.*

tration function for calculation of model degradation pathways. The calculations in the study were performed utilizing a quiktran terminal.[3]

SUMMARY

A method which eliminates the disadvantages of fixed time–temperature relationships in nonisothermal kinetic studies has been developed. The investigator utilizing this method may change temperature during the experiment to fit analytical findings. The data obtained from the single experiment allow calculation of activation energy, reaction rate, and shelf-life prediction. To obtain this information a series of theoretical degradation pathways are synthesized by utilizing the experimental time-temperature relationship and initial and final analytical points. The concentration–time plot for degradation is then compared to the model curves to obtain the energy of activation for the reaction. Calculations using analytical data and the activation energy are then used to determine reaction rates and stability predictions at desired temperatures. In addition to the flexibility of temperature adjustment introduced, the advantages of the method lie in the use of a single experimental unit, the analysis of one set of samples, the shorter time required for completion of the experiment, and the use of readily available laboratory equipment. Disadvantages (8) lie in the need for a separate experiment to determine the order of the reaction, the nonapplicability of the technique in situations where equations cannot be made linear (as in equilibria), the need to compensate for ionic strength effect with change in temperature, and the sometimes difficult task of fitting an equation to the experimental time–temperature data. The approach is also limited by assay precision, constancy of activation energy, and applicability of the Arrhenius equation.

[3] The quiktran program used for the entire treatment will be provided to interested parties upon request.

REFERENCES

(1) A. R. Rogers, *J. Pharm. Pharmacol.*, **15,** 101T(1963).
(2) B. R. Cole and L. Leadbeater, *ibid.*, **18,** 101(1966).
(3) *Ibid.*, **20,** 48(1968).
(4) S. P. Eriksen and H. J. Stelmach, *J. Pharm. Sci.*, **54,** 1029 (1965).
(5) M. A. Zoglio, J. J. Windheuser, R. Vatti, H. V. Maulding, S. S. Kornblum, A. Jacobs, and H. Hamot, *ibid.*, **57,** 2080(1968).
(6) R. F. Jackson and C. L. Gillis, *Sci. Pap. B S*, **16,** 141(1920).
(7) K. Sadek and M. S. Z. Elamayem, *Rec. Trav. Chim.*, **76,** 785(1957).
(8) J. T. Carstensen, A. Koff, and S. H. Rubin, *J. Pharm. Pharmacol.*, **20,** 485(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Pharmacy Research and Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication September 23, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Preparation and Evaluation of the Prolonged Release Properties of Nylon Microcapsules

L. A. LUZZI, M. A. ZOGLIO*, and H. V. MAULDING*

Abstract □ A method for *in situ* preparation of nylon encapsulated sodium pentobarbital by emulsion polymerization is reported. This was followed by work-up of the microcapsules by one of two processes: (*a*) spray drying and (*b*) vacuum drying. However, somewhat different properties were noted when the free-flowing spray-dried substance was compared to that which had been vacuum dried. Nylon capsules produced in these manners containing sodium pentobarbital exhibited a considerable reduction in dissolution rates relative to the instantaneously soluble barbiturates when examined in distilled water, 0.1 *M* phosphate buffer, pH = 6.75, and 0.1 *N* HCl. When the microcapsular material was tableted, the release rate of the sodium pentobarbital was seen to be inversely proportional to tablet hardness.

Keyphrases □ Nylon microcapsules—release rate, preparation □ Sodium pentobarbital—microcapsule preparation, polymerization □ Release rate—sodium pentobarbital nylon microcapsules □ UV spectrophotometry—analysis

As a method of protecting or prolonging the release of drugs, microencapsulation has intrigued pharmaceutical scientists (1–3) for several years. The first patented use of microcapsules was made by Green and Schleicher (4, 5) to prepare "carbonless carbonpaper." They made use of a gelatin–acacia coacervate system (6) to entrap emulsified oil droplets containing dissolved dyes. In 1967, Luzzi and Gerraughty (2, 7) developed a method for the evaluation of drug-containing capsules prepared *via* complex coacervation.

Miller and Anderson (8) were granted a U. S. patent for the manufacture of microcapsules using "hydrophobic film-forming polymeric wall materials dispersed in a liquid manufacturing vehicle." This patent claims as "an unsuspected virtue ... the encapsulation of aspirin" in ethylcellulose microcapsules. The authors state that aspirin was encapsulatable since it was wetable by cyclohexane. A further, and most interesting, statement made in this patent is that the thickness of the microcapsule wall can be controlled by changing the relative quantity of shell-forming material.

Chang *et al.* (9) prepared semipermeable nylon-shell microcapsules containing an erythrocyte hemolysate and, later (10), published information concerning the use of this type of microcapsule in an extracorporeal shunt system. In this system, the authors assert that capsules prepared in such a manner will allow passage of plasma through the capsule wall, thus allowing contact with the encapsulated material.

The findings, by the above authors, indicate that encapsulation is possible by several systems. There is, however, little indication of work done on encapsulation and evaluation of pharmaceuticals, especially with nylon as the encapsulating medium. In this respect, then, this investigation was designed to encapsulate a drug in a nylon membrane and to evaluate the resultant capsular material as it may pertain to pharmaceutical dosage forms.

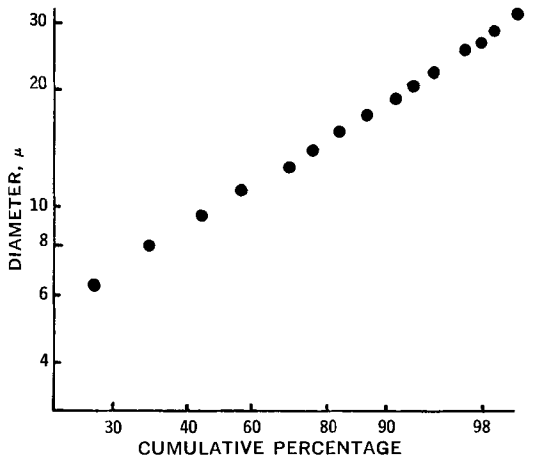


Figure 1—*Log-probability plot of a typical sample of spray-dried microcapsular material. The cumulative percentage is plotted on the X or probability axis. Distribution was determined using a Coulter Counter model B equipped with model M converter.*

EXPERIMENTAL

Preparation of Nylon Microcapsules—The method of Chang *et al.* (9), with certain modifications, was used to prepare nylon microcapsules containing sodium pentobarbital. The basic solutions included: (*a*) mixed solvent system consisting of 1 part by volume chloroform (reagent grade) and 4 parts by volume cyclohexane (reagent grade); (*b*) 0.16% sebacyl chloride (Eastman Organic Chemicals) in part of the above mixed solvent system; (*c*) 1% commercial emulsifier[1] also in part of the mixed solvent; (*d*) an aqueous solution of 2% methylcellulose[2] (50 cps.), and (*e*) an aqueous solution consisting of 2% sodium pentobarbital and 6.75% of 1,6-hexamethylenediamine (Eastman Organic Chemicals).

The procedure for the preparation of the product consisted of slowly adding at low speed[3] a mixture of equal volumes (25 ml.) of the methylcellulose and sodium pentobarbital–hexamethylenediamine solutions, then adding 165 ml. of the mixed solvent–surfactant solution, and blending for approximately 30 sec. at low speed. Sebacyl chloride solution (165 ml.) was added and blended at high speed for 10 sec. and then for 1 min. at low speed to complete the nylon-producing reaction. The capsules were allowed to settle and the supernatant was removed.

Spray-Drying Process—A Nerco-Niro portable spray dryer equipped with a special nozzle adapter atomizing assembly (Nichols Engineering and Research Corp.), a Zero-Max Sigma-motor assembly, and a vacuum exhaust system attached to the dryer were used to effect drying.

In order to prepare the mixture for the spray-drying process, approximately 15 ml. of chloroform was added to the slurry remaining after decantation. The resultant dilution was constantly agitated while liquid remained in the feed flask in order to maintain a uni-

[1] Brij 52 (HLB 5.3), Atlas Chemical Industries, Wilmington, Del.
[2] Methocel HG, The Dow Chemical Co., Midland, Mich.
[3] Waring blender, deluxe model two speed, Waring Products Co., Winstead, Conn.

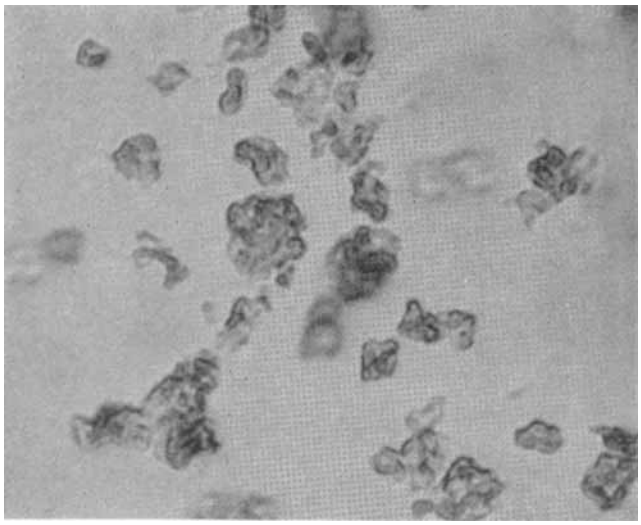


Figure 2—*A photomicrograph of dried microcapsular material (× 512).*

form flow of solids to the spray dryer. The internal temperature of the dryer was maintained at 125° throughout the drying process.

The collected powder was placed in a vacuum oven at 35° for 10–12 hr. to remove residual solvent and moisture. The resultant free-flowing powder containing 7.6% sodium pentobarbital was divided into several lots. One portion was examined as the free-flowing powder and the other portions were tableted so as to yield a range of tablet hardnesses prior to examination. Tablets were prepared by power compression on a single punch tableting machine (Stokes model E). Five hundred to one thousand tablets of each hardness were prepared with care taken to maintain a hardness variation of approximately ±0.5. Hardness was measured on a model B, Strong Cobb Arner tablet hardness tester.

Flash Evaporation Process—The wet slurry consisting of the microcapsule material and the vehicle which remained after decantation (along with the chloroform) was placed into a flash evaporator at 35° for 24 hr. The resultant dry material containing 7.6% sodium pentobarbital was held together loosely and was not dense.

Assay Methods—A Cary model 14 recording spectrophotometer was used to determine the concentration of sodium pentobarbital in each case. The wavelength of maximum absorption was found at 240 mμ, and all measurements were made at this wavelength while employing appropriate blanks. The same basic procedure was used to detect solubilized sodium pentobarbital regardless of the form of the drug being tested.

The following is the assay procedure used to study dissolution. A 200-mg. sample of free-flowing microcapsular powder or a 200-mg. tablet was placed in a 150-ml. beaker and 100 ml. of liquid (0.1 *N* HCl, 0.1 *M* KH_2PO_4–KOH buffer, pH 6.75, ionic strength 0.2, or distilled water) was added and the mixture stirred at a constant rate of 6 r.p.m., using a Bodine electric speed reducing motor (Bodine Electric Co.). The temperature was maintained at 37° and the beaker covered with aluminum foil. One-milliliter filtered samples were removed at the time intervals indicated in the various figures and appropriate dilutions were made employing 0.1 *N* NH_4OH.

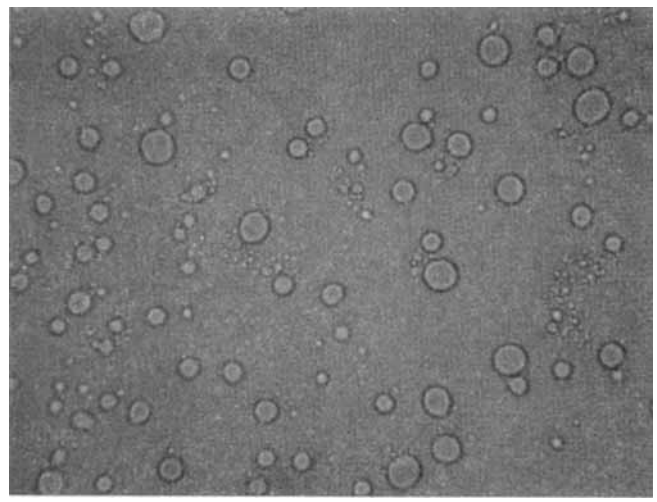


Figure 3—*A photomicrograph of nylon microcapsules in liquid medium before drying (× 400).*

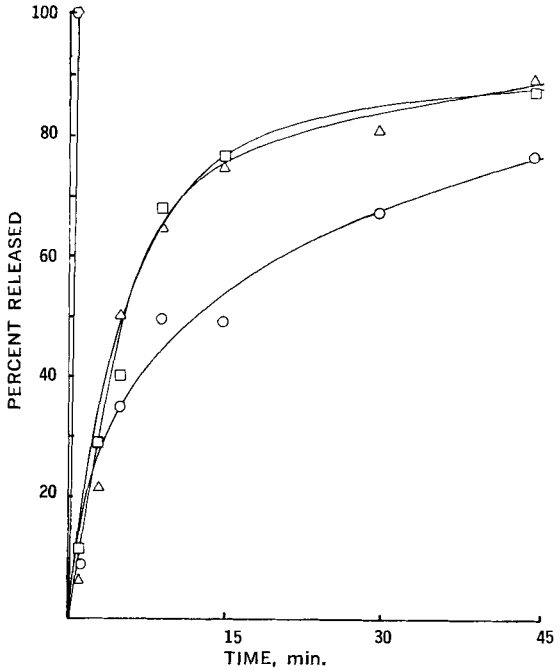


Figure 4—*Release rate of sodium pentobarbital from microcapsules prepared via evaporation and suspended in various aqueous media. Key: ○, in 0.1 N HCl; □, in H_2O; △, in 0.1 M PO_4; and ⬡, nonencapsulated sodium pentobarbital.*

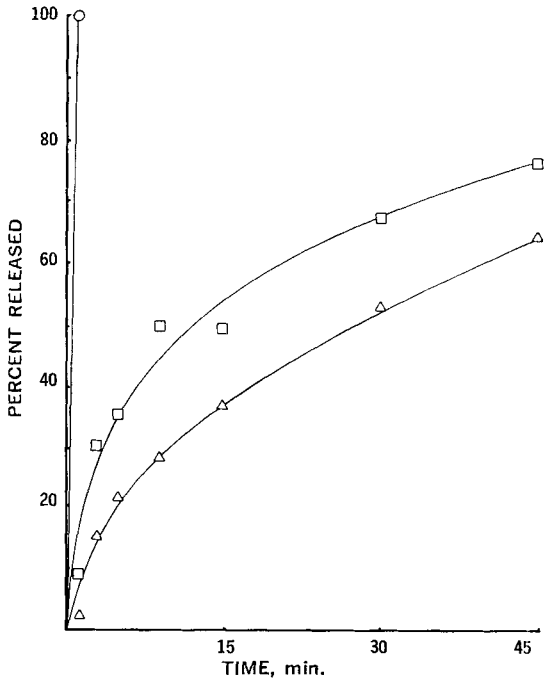


Figure 5—*A comparison of the release rate of sodium pentobarbital from microcapsules prepared by evaporation and by spray drying to 0.1 N HCl. Key: □, evaporation process; △, spray dried; and ○, nonencapsulated sodium pentobarbital.*

Table I—Rates[a] (K) of Dissolution for Tablets of Various Hardness in Three Liquid Media

Tablet Hardness	Liquid Medium	K hr.$^{-1}$	Time Measured, hr.
8	0.1 M HCl	5.01	9.0
8	0.1 M PO_4	4.12	9.0
8	H_2O	3.81	9.0
5	0.1 M HCl	7.13	8.0
5	0.1 M PO_4	4.28	8.0
5	H_2O	5.54	9.0
2	0.1 M HCl	14.37	1.5
2	0.1 M PO_4	15.22	3.0

[a] First-order rates are used for comparative analysis of results. The order of release cannot be established by the data presented in this study although there appears to be a trend toward first order.

Total Concentration—In order to be able to make a comparison of the amount of active ingredient released to that contained, 100 mg. of the microcapsular powder was placed in a blender and 500 ml. of 0.1 N NH_4OH was added. The blender was run for 5 min. at high speed and a clear liquid collected through a 0.45-μ filter. The absorbance was taken and compared to a standard.

Analysis of Data—An IBM 360 model 50 computer was used. The equations were standard and readily available (11).

Particle Size—Determination of particle distribution was carried out by using a Coulter Counter model B equipped with a model M volume converter. A 30-μ aperture tube, 2% sodium chloride, and a ragweed pollen standard (19.5 μ mean diameter) were used in this determination. No attempt was made to separate particles into definite ranges nor were other particle size distributions employed.

RESULTS AND DISCUSSION

Only one particle size range of spray-dried microcapsular material was used in this experiment. Figure 1 is a log-probability plot of the particles which shows that a statistically normal distribution was found. The geometric mean diameter was 10 μ with an arithmetic mean diameter of 12.08 μ. The vacuum-dried material did not, however, show a normal particle distribution, but contained particles as estimated microscopically ranging from about 1.0 to about 100.0 μ with 90% greater than 25.0 μ.

Figure 2 is a photomicrograph[4] of a typical grouping of spray-dried nylon-membraned microcapsules. It can be seen that the particles are not spherical and that, in most cases, several particles are clumped together. Figure 3 is a photograph of nylon-membraned microcapsules before drying. The particles here are generally spherical and clumping is minimal; no coalescence of particles was noted.

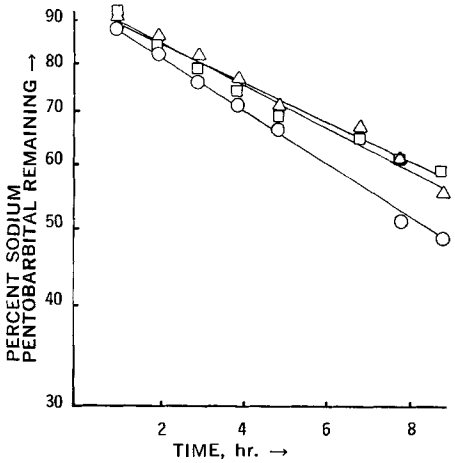


Figure 6—*A comparison of the release relationship of tablets of approximately 8 hardness in three liquid media. Key:* ○, *in 0.1* N *HCl;* △, *in 0.1* M PO_4; *and* □, *in* H_2O.

[4] Photomicrographs were taken using a Leitz Research microscope with Aristophot and Polaroid Land assembly.

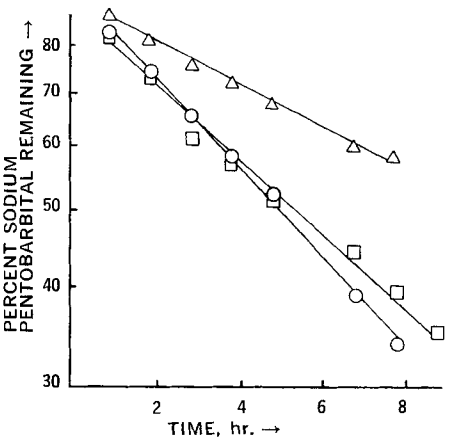


Figure 7—*A comparison of the release relationships of tablets of approximately 5 hardness in three liquid media. Key:* ○, *in 0.1* N *HCl;* △, *in 0.1* M PO_4; *and* □, *in* H_2O.

The dissolution profiles for sodium pentobarbital in a vacuum-dried microcapsular state is shown in Fig. 4. As may be expected, the results of release studies for sodium pentobarbital from the untableted powders showed a tendency to be greater at higher pH values. It may be seen that in a 45-min. period about 13.7 mg. (90%) of the encapsulated sodium pentobarbital was released to the phosphate buffer and the H_2O media while only about 11.4 mg. (75%) was released to the medium containing HCl.

Although dissolution studies for the spray-dried material were carried out in the three media previously mentioned, only those results for HCl are shown (Fig. 5). The release patterns are very similar to those obtained for other media and show that the vacuum-dried microcapsular powder initially releases sodium pentobarbital more quickly than the spray-dried material.

It was thought that since the vacuum-dried material had to be scraped from the collection vessel and then spatulated in order to obtain a powder, that some of the capsules might have been disrupted and that this led to the greater release. The spray-dried material, on the other hand, was free flowing as it was collected. For these reasons, further work was done using only the spray-dried powder.

When the microcapsular material was tableted, without lubricants or other adjuvants, both the disintegration of the tablet and the ability of the liquid medium to wet the tablet seemed to play an

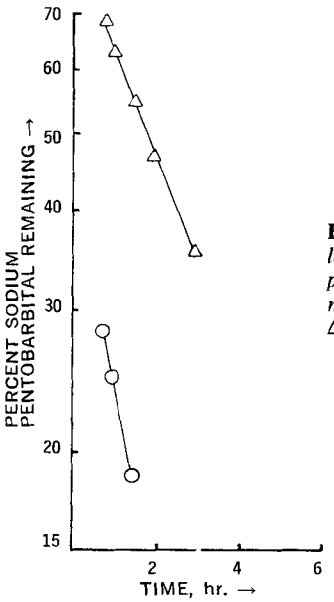


Figure 8—*A comparison of the release relationship of tablets of approximately 2 hardness in two liquid media. Key:* ○, *in 0.1* N *HCl; and* △, *in 0.1* M PO_4.

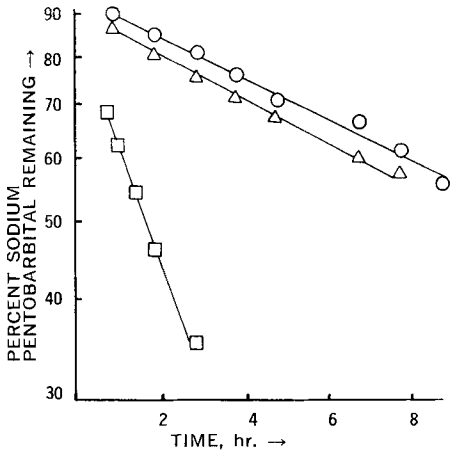


Figure 9—*A comparison of the release relationship of microcapsular tablets of three different hardnesses in 0.1* M PO_4 *liquid media. Key:* ○, *8 hardness;* △, *5 hardness; and* □, *2 hardness.*

important role in release of active ingredient. It was expected that disintegration and/or wetting would not appreciably be affected from liquid to liquid. However, as indicated in Figs. 6–8, there apparently is some disintegrating or wetting action since the release of sodium pentobarbital is slightly more rapid in acid solution; this is contrary to the trend found in Fig. 4.

Figures 6–8 demonstrate the release of sodium pentobarbital from the microcapsules. Although a first-order release is not justified except by the trend of the data, first-order rates are used for purposes of comparison. A comparison of these figures indicates that the differences in rate from one dissolution medium to the other became greater as the hardness of the tablet tested decreased. These differences in rate of release are shown more clearly in Fig. 9. Figure 9 is a comparison of the release rate of tablets of various hardness. It can be seen that the more rapid release rates were found in the softer tablets with the greater difference between tablets of hardness 5 and 2.

Table I is a compilation of slopes and significant data for the release of sodium pentobarbital from tablets of various hardnesses and the several liquid media. The mechanism of release (9, 10) probably involves leeching of the sodium pentobarbital through a network of nylon fibers constituting the microcapsule.

SUMMARY

The studies reported here demonstrate that interphasal polymerization can be used to prepare nylon-membrane microcapsules. It has also been shown that a free-flowing powder can be obtained by spray drying slurries of microcapsules.

When the free-flowing powder was tableted, it was observed that changes in release rate could be controlled by varying hardness. It was also observed that vacuum drying yielded capsule material of different appearance and release characteristics from spray-dried microcapsular material.

Although a statistical evaluation of particle size was carried out, there could be no significance attached to the range found. However, examination of the effect of size of particle on release rate is presently being investigated.

REFERENCES

(1) L. A. Luzzi and R. J. Gerraughty, *J. Pharm. Sci.*, **53**, 429 (1964).

(2) *Ibid.*, **56**, 1174(1967).

(3) R. E. Phares and G. J. Sperandio, *J. Pharm. Sci.*, **53**, 515 (1964).

(4) B. K. Green and L. Schleicher, U. S. pat. 2,703,457(1956).

(5) B. K. Green and L. Schleicher, U. S. pat. 2,703,458(1956).

(6) H. G. Bungenberg de Jong, "Colloid Science," vol. II, H. R. Kruyt, Ed., Elsevier New York, N. Y., 1949.

(7) L. A. Luzzi and R. J. Gerraughty, *J. Pharm. Sci.*, **56**, 634 (1967).

(8) R. E. Miller and J. L. Anderson, U. S. pat. 3,155,590(1964).

(9) T. M. S. Chang, F. C. MacIntosh, and S. G. Mason, *Can. J. Physiol. Pharmacol.*, **44**, 115(1966).

(10) T. M. S. Chang, *Trans. Amer. Soc. Artificial Internal Organs*, **12**, 13(1966).

(11) G. W. Snedecor and W. G. Cochran, "Statistical Methods," 6th ed., Iowa State University Press, Ames, Iowa, 1967.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *Department of Pharmacy, University of Georgia, Athens, GA 30601*, and the *Pharmacy Research and Development Department of Sandoz Pharmaceuticals,* Hanover, NJ 07936*

Accepted for publication October 8, 1969.

The authors thank Drs. J. Kotzan and T. A. Needham for their valuable help and Mr. D. C. Murdock for able technical assistance.

Pyrrolidine-Substituted Nicotine Analogs: Synthesis and Pharmacology

WARREN HANKINS* and ALFRED BURGER

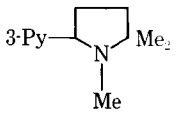
Abstract □ (—)5′,5′-Dimethylnicotine (I) was synthesized in one step from (—) cotinine; it had approximately 0.03 times the mouse toxicity of nicotine but did not cause the same qualitative toxic signs as nicotine; it stimulated and then depressed autonomic ganglia (anesthetized dog) and directly affected the respiratory center and the cardiovascular system. The total synthesis of 5,5-dimethyl-2-(3-pyridyl)-Δ^1-pyrroline (V) and its 1-oxide (VI) was performed. Compound V was hydrogenated to 2,2-dimethyl-5-(3-pyridyl)pyrrolidine (II), and this was converted to its 1-propyl homolog. Compound VI was reduced to 2,2-dimethyl-1-hydroxy-5-(3-pyridyl)pyrrolidine (VIII).

Keyphrases □ Nicotine analogs, pyrrolidine substituted—synthesis □ Pharmacology—pyrrolidine-substituted nicotine analogs □ NMR spectroscopy—structure □ Mass spectroscopy—identity □ Nitrone—synthesis, reactions

Because of the blocking action of neighboring methyl groups upon the piperidine-nitrogen in pempidine (1), the authors undertook a study of derivatives with sterically crowding methyl groups in the pyrrolidine ring of nicotine.[1] Of compounds which have been studied pharmacologically, 5′-methylnornicotine (2), and nicotine analogs with alkyl groups in the pyridine ring, including 4- and 6-methylnicotine (3, 4), and 2-(or 6-) alkyl-1,2-(or 1,6-)dihydro-1-methylnicotine compounds (5) may be mentioned; the latter paralyzed the respiratory center but lowered the dog's blood pressure.

THEORETICAL

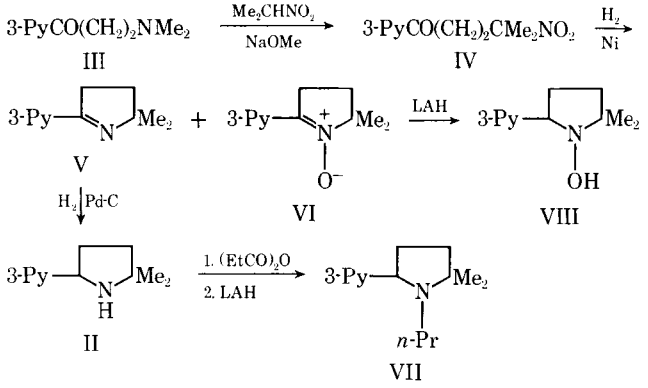
In this work, (—) cotinine was treated with excess MeMgI to give (—) 5-(3-pyridyl)-1,2,2-trimethylpyrrolidine [(—)5′,5′-dimethylnicotine (I)].



I

The corresponding 1-*nor* compound [(±)5,5-dimethyl-2-(3-pyridyl)pyrrolidine] (II) was synthesized as follows. Methoxide-induced condensation of 3-dimethylamino-1-(3-pyridyl)-1-propanone (III) with 2-nitropropane, and hydrogenation of the resulting 4-methyl-4-nitro-1-(3-pyridyl)-1-pentanone (IV) gave 5,5-dimethyl-2-(3-pyridyl)-Δ^1-pyrroline (V) together with 5,5-dimethyl-2-(3-pyridyl)-Δ^1-pyrroline-1-oxide (VI) (6). The pyrroline V was then hydrogenated to II.

Confirmation of structures V and VI rested on chemical reactions and spectra (see *Experimental*). Propionylation of II followed by reduction of the resulting amide gave 2,2-dimethyl-1-*n*-propyl-5-(3-pyridyl)pyrrolidine (VII), the propyl homolog of I. Furthermore,



the nitrone VI could be reduced to 2,2-dimethyl-1-hydroxy-5-(3-pyridyl)pyrrolidine (VIII).

The nicotine homolog I (as the dihydrochloride) was tested (Dr. Marvin J. Bleiberg of Woodard Research Corp.) and compared to nicotine (dihydrochloride and sulfate) under identical test conditions which are described in the *Experimental* section. The LD_{50} ratios in the mouse for I (I·2HCl, LD_{50} 11.0 mg./kg., 95% confidence limits) and nicotine were approximately 30.6, *i.e.*, I was about 0.03 times as toxic as nicotine. The confidence intervals of the LD_{50}'s for each compound show that the dose-mortality curves are virtually parallel. However, toxic signs such as Straub tail, tremors, eyelid ptosis, and mydriasis, which were seen at lethal doses of nicotine, were not observed with I.

The MED_{50} to obtain a pharmacodynamic effect in the mouse are (95% confidence limits) 0.018 mg./kg. for nicotine·2HCl, and 1.8 mg./kg. for I·2HCl, respectively; thus the potency ratio of nicotine/I is 100.0. There was evidence of ganglionic stimulation expressed by a greater rise in blood pressure caused by dimethylphenylpiperazinium iodide (DMPP) for I; in a dog receiving atropine before all but the initial dose of I, 0.200 mg. of I caused a marked rise in blood pressure equivalent to 0.016 mg. of nicotine sulfate. This is consistent with the potency ratio in the mouse. A ganglionic blocking action after large doses of I was suggested by the progressive depression of the blood pressure rise after AcCh following increasing doses of I. A direct effect on the respiratory center and on the cardiovascular system was indicated by the increased depth of respiration and the large blood pressure rise following injection of I.

The odor of I (free base) was very similar to, if not indistinguishable from, that of nicotine.

EXPERIMENTAL

Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer 337 spectrophotometer, NMR spectra on a Perkin-Elmer R-20 instrument (60 M c.p.s.), mass spectra with a Hitachi-Perkin-Elmer RMU-6E instrument (see Table I). IR and NMR spectra were taken on all compounds and were consistent with the proposed structures; they are listed only if not essentially identical with those tabulated by Castagnoli *et al.* (7). NMR spectra (p.p.m.) were measured in $CDCl_3$ (TMS as standard) or in D_2O (DSS as standard), respectively. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tennessee.

Pharmacological Methods—Mice of the HA/ICR strain, random breed (2 per dose level), received I at one-half log-dosage intervals i.v. Observations included the standard pharmacologic profile in

[1] After completion of both the synthetic and pharmacologic work (*vide infra*) the authors learned of an article by Castagnoli *et al.* (7) who had synthesized several of the compounds described in this paper [(±)I, II, V, VI, VIII] by a completely different route. For this reason, the authors' experimental report lists only those data that differ significantly from those of the California authors (7).

Table I—Mass Spectra (70 ev.) *m/e*

Compound	M+	Fragmentation M minus ( )
I	190	175 (CH_3)
II	176	161 (CH_3)
V	174	159 (CH_3)
		146 (CH_2=CH_2)
VI	190	175 (CH_3)
		173 (OH)
VII	218	203 (CH_3)
		189 (C_2H_5)
VIII	192	177 (CH_3)
		174 (H_2O)

dose range studies, number of mice reacting, time of onset of the pharmacologic signs, their degree and severity, and the time for recovery. LD_{50} and MED_{50} (both at 95% confidence limits) were estimated.

For tests in the dog, two mongrel dogs which were housed in temperature-controlled quarters, conditioned to the laboratory, and previously given canine distemper vaccine, were used. Food was withheld for 18–24 hr. prior to anesthesia (phenobarbital sodium, 140 mg./kg., i.v. or i.p.). The trachea was exposed and cannulated in order to maintain the respiratory airway. The right common carotid artery was exposed and cannulated to record blood pressure by means of an E and M pressure transducer, connected to an E and M physiograph. The anticoagulant was benzo fast pink (1.66 g./l. of 9% saline). Bilateral electrodes, placed on the skin next to the lower ribs, recorded the respiratory excursions by means of an impedence pneumograph; the EKG was recorded on a separate channel of the physiograph. Injections of the test material (and of nicotine dihydrochloride as a standard) were administered through a cannula inserted into a femoral vein; each injection was washed with Krebs-Ringer solution. In addition to graded increasing doses of I (dihydrochloride) and of the standard, Dog No. 1 also received dimethylphenylpiperazinium iodide, acetylcholine chloride, and atropine sulfate before a final dose of nicotine sulfate. Dog No. 2 also received epinephrine, ACh, and atropine.

Chemistry—(−)*1,2,2-Trimethyl-5-(3-pyridyl)pyrrolidine* (*I*)—To a solution of MeMgI [from 106.5 g. (0.75 mole) of MeI and 18.2 g. (0.75 mole) of Mg in dry ether (300 ml.) under N_2] was added (−) cotinine (8) (26.4 g., 0.15 mole) in dry benzene (40 ml.). Ether was removed by distillation and replaced by benzene, and the solution was refluxed at 65–75° under N_2 for 24 hr. (9). The reaction mixture was poured into ice water, the basic mixture was steam distilled, and the distillate (3–4 l.) was acidified and evaporated under vacuum. The residual oil was made basic, extracted (ether), and dried well (Na_2SO_4). Evaporation of the ether yielded 5 g. (17%) of a brown liquid. The colorless dihydrochloride, prepared in ether, was recrystallized from ethanol–ether, m.p. 258–260°.

Anal.—Calcd. for $C_{12}H_{20}Cl_2N_2$: C, 54.8; H, 7.6; N, 10.6. Found: C, 55.0; H, 7.8; N, 10.4.

The yellow dipicrate was obtained in, and recrystallized from, ethanol, m.p. 210–212°: $[\alpha]_{Hg}^{25}$ − 76.9° (c 0.13, ethanol).

Anal.—Calcd. for $C_{24}H_{24}N_8O_{14}$: C, 44.4; H, 3.7; N, 17.3. Found: C, 44.4; H, 3.8; N, 17.1.

4-Methyl-4-nitro-1-(3-pyridyl)-1-pentanone (*IV*)—A solution of sodium methoxide (3 g., 0.054 mole) in methanol (40 ml.) was added gradually to a stirred solution of 3-dimethylamino-1-(3-pyridyl)-1-propanone (III) (10.7 g., 0.05 mole) (10) and 2-nitropropane (31 g., 0.35 mole) in methanol (60 ml.) (11). After slight heating for 10 min. the stirred solution was heated to boiling, and dimethylamine was removed by distilling off about 10 ml. of methanol over a period of 20 min. Solvent and excess nitropropane were then removed under reduced pressure, the residue was taken up in water (100 ml.) containing a few drops of 10% NaOH solution, and IV crystallized on cooling. It was recrystallized from ethanol. The colorless crystals weighed 7.6 g. (68%), m.p. 73–75°.

Anal.—Calcd. for $C_{11}H_{14}N_2O_3$: C, 59.5; H, 6.3; N, 12.6. Found: C, 59.7; H, 6.4; N, 12.5.

Reduction of IV—A solution of IV (8.4 g., 38 mmoles) in absolute ethanol (40 ml.) and 2 g. of W-7 Raney nickel (12) was hydrogenated at an initial pressure of 3.6 kg./cm.[2] for 24 hr. Standard workup yielded 6.5 g. of crude reduction product which was chromatographed on magnesium silicate.[2] Elution with ether gave 5,5-dimethyl-2-(3-pyridyl)-Δ^1-pyrroline (V) (4.6 g., 69%); elution with ether–methanol (9:1) gave 5,5-dimethyl-2-(3-pyridyl)-Δ^1-pyrroline-1-oxide (VI) (0.9 g., 12%).

Compound V separated from ether as colorless crystals, m.p. 43–44°; it had previously been described as an oil (7).

Anal.—Calcd. for $C_{11}H_{14}N_2$: C, 75.8; H, 8.1; N, 16.1. Found: C, 75.8; H, 8.1; N, 16.2.

The nitrone VI had m.p. 75–76° after recrystallization from ether or hexane, and vacuum drying.

Anal.—Calcd. for $C_{11}H_{14}N_2O$: C, 69.5; H, 7.4; N, 14.7. Found: C, 69.3; H, 7.4; N, 14.6.

IR (KBr) 3400 cm.$^{-1}$ [the band at 3660 cm.$^{-1}$ reported by Castagnoli *et al.* (7) was not observed].

1-Hydroxy-2,2-dimethyl-5-(3-pyridyl)pyrrolidine (*VIII*)—A suspension of LAH (200 mg.) in dry ether (50 ml.) was added gradually over a period of 15 min. to a warm stirred solution of VI (0.5 g., 2.6 mmoles) in dry ether (150 ml.) and the mixture was refluxed for 18 hr. After dropwise decomposition with water, the ether layer was dried ($MgSO_4$) and evaporated in vacuum. VIII (0.3 g., 62%) crystallized from ether, m.p. 127–128°. IR (KBr) 3170 cm.$^{-1}$; the band at 3580 cm.$^{-1}$ (7) was not observed.

Anal.—Calcd. for $C_{11}H_{16}N_2O$: C, 68.7; H, 8.4; N, 14.6. Found: C, 68.6; H, 8.5; N, 14.3.

2,2-Dimethyl-5-(3-pyridyl)pyrrolidine (*II*)—A solution of V (3 g.) in absolute ethanol (30 ml.) was hydrogenated at 3.82 kg./cm.[2] with 1.5 g. of 10% Pd-C for 7 hr. Standard workup gave a residual oil which was converted to its dihydrochloride in ether. The salt was recrystallized from ethanol–ether, m.p. 135–137°. Drying over P_2O_5 raised the m.p. to 210–212°.

Anal.—Calcd. for $C_{11}H_{16}N_2 \cdot 2HCl \cdot H_2O$: C, 49.4; H, 7.5; N, 10.5. Found: C, 49.6; H, 7.3; N, 10.4.

Reconversion of the dihydrochloride to the base with sodium carbonate solution and ether extraction gave a clear liquid. The yellow dipicrate, formed in and recrystallized from ethanol, had m.p. 185–186°.

Anal.—Calcd. for $C_{23}H_{22}N_8O_{14}$: C, 43.5; H, 3.5; N, 17.7. Found: C, 43.7; H, 3.3; N, 17.5.

2,2-Dimethyl-1-n-propyl-5-(3-pyridyl)pyrrolidine (*VII*)—A mixture of II (1.5 g.), propionic acid (2.5 ml.), and propionic anhydride (2.5 ml.) was refluxed for 1 hr. and then cooled and poured into ice water. The solution was made strongly basic with 50% KOH solution, extracted with ether, and the extract was dried ($MgSO_4$) and evaporated. The crude 2,2-dimethyl-1-propionyl-5-(3-pyridyl)-pyrrolidine (1.4 g.), dissolved in dry ether (30 ml.), was added to LAH (0.5 g.) in dry ether (25 ml.) with stirring. After refluxing for 4 hr. the mixture was worked up [water (1.5 ml.), 10% NaOH (1.5 ml.), water (5 ml.), ether extraction, drying ($MgSO_4$), evaporation]. Yield of clear oil VII was 1.2 g. The dihydrochloride, prepared in dry ether, was recrystallized from ethanol–ether, m.p. 245–247°. NMR (D_2O) δ 0.78 (t, 3, CH_3 of propyl), 1.3 (m, 2, CH_2 of propyl), 1.52 (s, 3, CH_3), 1.66 (s, 3, CH_3), 2.6 (m, 4, protons at C-3 and C-4 of pyrrolidine), 3.2 (m, 2, N—CH_2—), 5.07 (q, 1, proton at C-2), 8.1–9.15 (4, pyridinium salt pattern).

Anal.—Calcd. for $C_{14}H_{22}N_2 \cdot 2HCl$: C, 57.7; H, 8.3; N, 9.6. Found: C, 57.4; H, 8.1; N, 9.4.

The yellow dipicrate was obtained in and recrystallized from ethanol, m.p. 205–206°.

Anal.—Calcd. for $C_{26}H_{28}N_8O_{14}$: C, 46.2; H, 4.1; N, 16.6. Found: C, 46.3; H, 4.2; N, 16.4.

REFERENCES

(1) S. J. Corne and N. D. Edge, *Brit. J. Pharmacol.*, **13,** 339 (1958); A. Spinks, E. H. P. Young, J. A. Farrington, and D. Dunlop, *ibid.*, **13,** 501(1958).

(2) I. Yamamoto and H. Kamimura, *Agr. Biol. Chem.*, **27,** 445 (1963).

(3) F. Haglid, *Acta Chem. Scand.*, **21,** 329(1967).

(4) H. Erdtman, F. Haglid, and I. Wellings, *ibid.*, **17,** 1717 (1963).

(5) P. Karrer and A. Widmer, *Helv. Chim. Acta*, **9,** 461(1926).

(6) M. C. Kloetzel, F. L. Chubb, R. Gobran, and J. L. Pinkus, *J. Amer. Chem. Soc.*, **83,** 1128(1961).

[2] Florisil, Floridin Co., Pittsburgh, PA 15222

(7) N. Castagnoli, Jr., A. P. Melikian, and V. Rosnati, *J. Pharm. Sci.*, **58**, 860(1969).
(8) H. McKennis, Jr., L. B. Turnbull, E. R. Bowman, and E. Wada, *J. Amer. Chem. Soc.*, **81**, 3951(1959).
(9) B. Lukes, *Collection Czech. Chem. Commun.*, **2**, 531(1930); through *Chem. Abstr.*, **25**, 102(1931).
(10) J. M. Snell and S. M. McElvain, *J. Amer. Chem. Soc.*, **56**, 1612(1936).
(11) M. L. Stein and A. Burger, *ibid.*, **79**, 154(1957).
(12) H. B. Billica and H. Adkins, *Org. Syn.*, vol. III, **1955**, 179.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 28, 1969, from the *Chemistry Department, University of Virginia, Charlottesville, VA 22901*

Accepted for publication October 16, 1969.

The authors are grateful to Philip Morris, Inc., for support of this study.

* Philip Morris, Inc., Postdoctoral Research Fellow. Present address: University of Evansville, Evansville, Ind.

Synthesis of Sugar Moiety Substituted Nucleosides I: 9-[3-*O*-(*n*-Hexyl)-α,β-D-xylofuranosyl]adenine and 9-[3-*O*-(*n*-Hexyl)-5-deoxy-α,β-D-xylofuranosyl]adenine

DANIEL H. MURRAY and JOHN PROKOP

Abstract □ Isopropylidene-D-xylose was coverted *via* the 5-trityl compound to a 3-*O*-(*n*-hexyl) derivative. Following detritylation, benzoylation, and acetolysis, condensation with chloromercuri-6-benzamidopurine in the presence of titanium tetrachloride gave a crude nucleoside mixture. After deacylation, the mixture of anomeric nucleosides was resolved on ion-exchange resin (Bio-Rad AGl) to give 9-[3-*O*-(*n*-hexyl)-α-D-xylofuranosyl]adenine and 9-[3-*O*-(*n*-hexyl)-β-D-xylofuranosyl]adenine. Similarly, isopropylidene-5-deoxy-D-xylose was converted to a 3-*O*-(*n*-hexyl) derivative. Acetolysis and condensation with chloromercuri-6-benzamidopurine followed by deacylation and resolution on ion-exchange resin led to isolation of 9-[3-*O*-(*n*-hexyl)-5-deoxy-α-D-xylofuranosyl]adenine and 9-[3-*O*-(*n*-hexyl)-5-deoxy-β-D-xylofuranosyl]adenine.

Keyphrases □ Nucleosides, sugar moiety substituted—synthesis, isolation, separation □ 3-*O*-(*n*-Hexyl)adenine derivatives—synthesis, isolation □ Column chromatography—separation □ IR—identification □ UV spectrophotometry—identification □ Polarimetry—identification

In a continuing series of investigations, the authors have been exploring the structural features of the sugar moiety of adenine nucleosides required for interaction with the enzyme adenosine deaminase and/or inhibition of whole cells (1, 2). Other groups as well have devoted considerable attention to this area of study, particularly the laboratories of LePage (3), Schaeffer (4), and Bloch (5), among others. Compositely, the results of many studies such as those cited suggest that the 3′-hydroxyl group is usually not an important participant in an interaction with enzymes by which an adenine nucleoside may function as an *inhibitor* rather than as a *substrate*.

Recently, Baker (6) has collated many examples of the application of a principle he enunciated earlier (7): that a group which is found not to be important in interaction with an enzyme may be an ideal candidate for further modification with even quite bulky groups, including those which may react covalently with an enzyme to yield an active site directed, irreversible inhibitor. Both Baker (8) and Schaeffer (9) have now prepared a number of such irreversible inhibitors.

To date there seems not to have been any attempt to apply the implications of the Baker principle to "unimportant" groups on the sugar moiety of nucleosides. As noted, the 3′-hydroxyl would appear to be such a group. The present and following reports describe the syntheses of a number of 3′-*O*-substituted nucleosides whose availability will allow a beginning to be made in assessing the practicality of designing an active site directed, irreversible inhibitor of a sugar moiety substituted type.

Initially, the *n*-hexyl substituent was selected since not only could it serve as the carrier of an alkylating function, but of itself might enhance binding of the nucleoside through interaction with potentially accessible hydrophobic regions on susceptible enzymes. That such a hydrophobic region exists on adenosine deaminase has been demonstrated by Schaeffer and Vogel with a series of 9-alkyl substituted adenines (10). Xylofuranosyladenine (3*c*) and 5′-deoxyxylofuranosyladenine (11), both of which show affinity toward adenosine deaminase, were slected as candidates for 3′-*O*-substitution. The present paper, therefore, reports the syntheses of the 3′-*O*-(*n*-hexyl) derivatives of these two nucleosides.

PROCEDURES

Etherification of 1,2-*O*-isopropylidene-5-*O*-triphenylmethyl-D-xylofuranose (12) (I, Scheme I) with 1-chlorohexane in the presence of potassium hydroxide gave the 3-*O*-(*n*-hexyl) derivative (II) as a noncrystallizing syrup in quantitative yield. Attempts to remove the trityl group by hydrogenolysis over palladium black or palladium-on-charcoal were unsuccessful. This group, however, was readily removed in good yield when II was refluxed in an aqueous ethanolic solution of acetic acid. The resulting distillable syrup (III) was contaminated with 8% of triphenylcarbinol which could be removed by

(7) N. Castagnoli, Jr., A. P. Melikian, and V. Rosnati, *J. Pharm. Sci.*, **58**, 860(1969).
(8) H. McKennis, Jr., L. B. Turnbull, E. R. Bowman, and E. Wada, *J. Amer. Chem. Soc.*, **81**, 3951(1959).
(9) B. Lukes, *Collection Czech. Chem. Commun.*, **2**, 531(1930); through *Chem. Abstr.*, **25**, 102(1931).
(10) J. M. Snell and S. M. McElvain, *J. Amer. Chem. Soc.*, **56**, 1612(1936).
(11) M. L. Stein and A. Burger, *ibid.*, **79**, 154(1957).
(12) H. B. Billica and H. Adkins, *Org. Syn.*, vol. III, **1955**, 179.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 28, 1969, from the *Chemistry Department, University of Virginia, Charlottesville, VA 22901*

Accepted for publication October 16, 1969.

The authors are grateful to Philip Morris, Inc., for support of this study.

* Philip Morris, Inc., Postdoctoral Research Fellow. Present address: University of Evansville, Evansville, Ind.

Synthesis of Sugar Moiety Substituted Nucleosides I: 9-[3-*O*-(*n*-Hexyl)-α,β-D-xylofuranosyl]adenine and 9-[3-*O*-(*n*-Hexyl)-5-deoxy-α,β-D-xylofuranosyl]adenine

DANIEL H. MURRAY and JOHN PROKOP

Abstract □ Isopropylidene-D-xylose was coverted *via* the 5-trityl compound to a 3-*O*-(*n*-hexyl) derivative. Following detritylation, benzoylation, and acetolysis, condensation with chloromercuri-6-benzamidopurine in the presence of titanium tetrachloride gave a crude nucleoside mixture. After deacylation, the mixture of anomeric nucleosides was resolved on ion-exchange resin (Bio-Rad AGl) to give 9-[3-*O*-(*n*-hexyl)-α-D-xylofuranosyl]adenine and 9-[3-*O*-(*n*-hexyl)-β-D-xylofuranosyl]adenine. Similarly, isopropylidene-5-deoxy-D-xylose was converted to a 3-*O*-(*n*-hexyl) derivative. Acetolysis and condensation with chloromercuri-6-benzamidopurine followed by deacylation and resolution on ion-exchange resin led to isolation of 9-[3-*O*-(*n*-hexyl)-5-deoxy-α-D-xylofuranosyl]adenine and 9-[3-*O*-(*n*-hexyl)-5-deoxy-β-D-xylofuranosyl]adenine.

Keyphrases □ Nucleosides, sugar moiety substituted—synthesis, isolation, separation □ 3-*O*-(*n*-Hexyl)adenine derivatives—synthesis, isolation □ Column chromatography—separation □ IR—identification □ UV spectrophotometry—identification □ Polarimetry—identification

In a continuing series of investigations, the authors have been exploring the structural features of the sugar moiety of adenine nucleosides required for interaction with the enzyme adenosine deaminase and/or inhibition of whole cells (1, 2). Other groups as well have devoted considerable attention to this area of study, particularly the laboratories of LePage (3), Schaeffer (4), and Bloch (5), among others. Compositely, the results of many studies such as those cited suggest that the 3′-hydroxyl group is usually not an important participant in an interaction with enzymes by which an adenine nucleoside may function as an *inhibitor* rather than as a *substrate*.

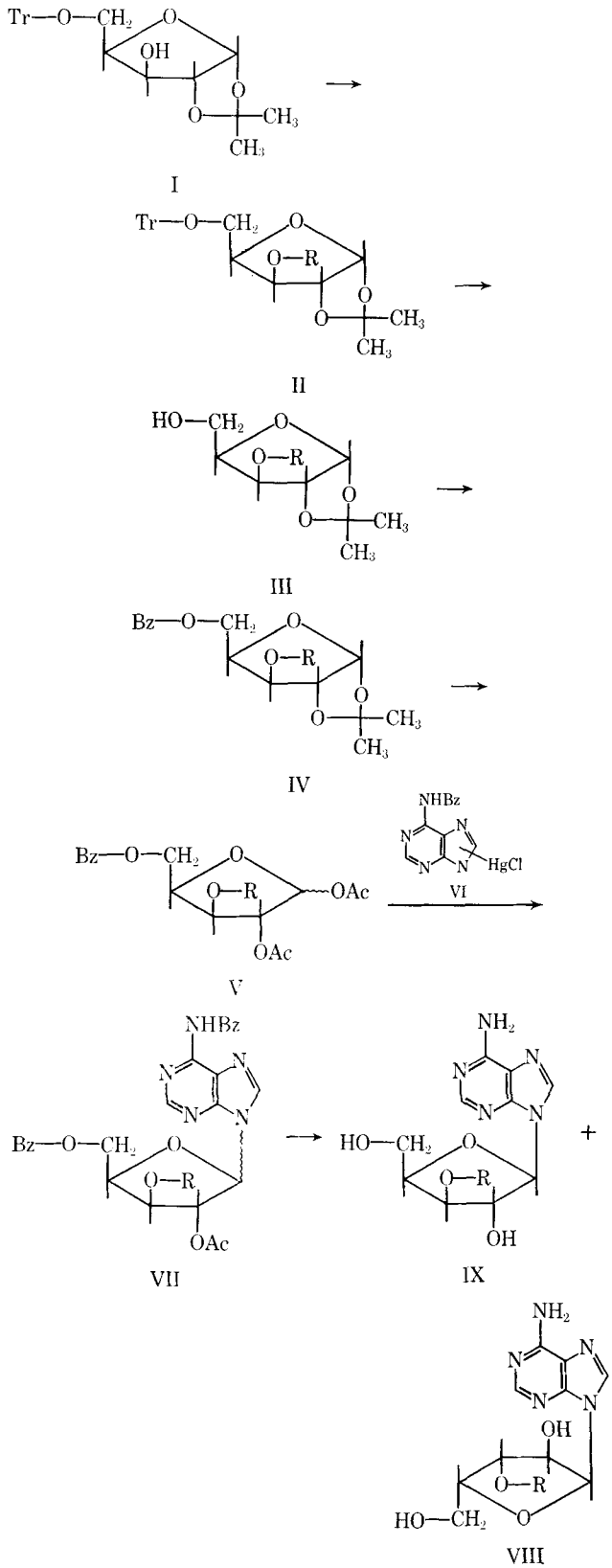
Recently, Baker (6) has collated many examples of the application of a principle he enunciated earlier (7): that a group which is found not to be important in interaction with an enzyme may be an ideal candidate for further modification with even quite bulky groups, including those which may react covalently with an enzyme to yield an active site directed, irreversible inhibitor. Both Baker (8) and Schaeffer (9) have now prepared a number of such irreversible inhibitors.

To date there seems not to have been any attempt to apply the implications of the Baker principle to "unimportant" groups on the sugar moiety of nucleosides. As noted, the 3′-hydroxyl would appear to be such a group. The present and following reports describe the syntheses of a number of 3′-*O*-substituted nucleosides whose availability will allow a beginning to be made in assessing the practicality of designing an active site directed, irreversible inhibitor of a sugar moiety substituted type.

Initially, the *n*-hexyl substituent was selected since not only could it serve as the carrier of an alkylating function, but of itself might enhance binding of the nucleoside through interaction with potentially accessible hydrophobic regions on susceptible enzymes. That such a hydrophobic region exists on adenosine deaminase has been demonstrated by Schaeffer and Vogel with a series of 9-alkyl substituted adenines (10). Xylofuranosyladenine (3*c*) and 5′-deoxyxylofuranosyladenine (11), both of which show affinity toward adenosine deaminase, were slected as candidates for 3′-*O*-substitution. The present paper, therefore, reports the syntheses of the 3′-*O*-(*n*-hexyl) derivatives of these two nucleosides.

PROCEDURES

Etherification of 1,2-*O*-isopropylidene-5-*O*-triphenylmethyl-D-xylofuranose (12) (I, Scheme I) with 1-chlorohexane in the presence of potassium hydroxide gave the 3-*O*-(*n*-hexyl) derivative (II) as a noncrystallizing syrup in quantitative yield. Attempts to remove the trityl group by hydrogenolysis over palladium black or palladium-on-charcoal were unsuccessful. This group, however, was readily removed in good yield when II was refluxed in an aqueous ethanolic solution of acetic acid. The resulting distillable syrup (III) was contaminated with 8% of triphenylcarbinol which could be removed by

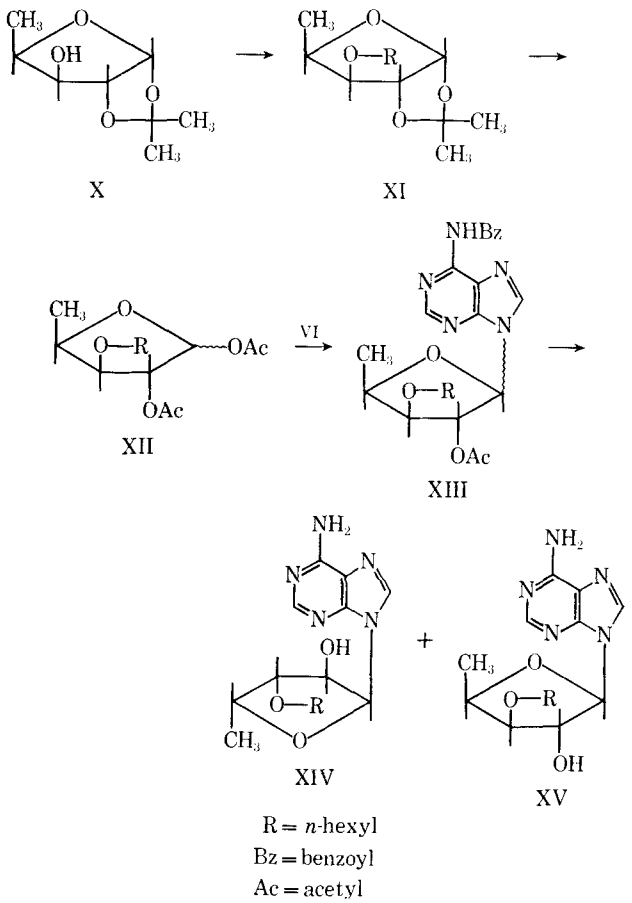


Tr = triphenylmethyl
R = *n*-hexyl
Bz = benzoyl
Ac = acetyl

Scheme I

chromatography on silica gel. Benzoylation of III in the usual manner gave a quantatiative yield of IV as a syrup. Acetolysis of IV gave the 1,2-diacetate (V) again as a syrup in quantitative yield. Condensation of V with chloromercuri-6-benzamidopurine (VI) in the presence of titanium tetrachloride resulted in a crude anomeric mixture of blocked nucleosides (VII) obtained as a glass. Without further purification, VII was deacylated with methanolic sodium methoxide, and the crude reaction products were chromatographed on ion-exchange resin[1] using 60% aqueous methanol. Rechromatographing the partially separated nucleosides gave the pure α and β anomers (VIII and IX, respectively) as crystalline solids in yields of 5.0% and 63%, respectively (from V).

In a similar manner, 5-deoxy-1,2-*O*-isopropylidene-D-xylofuranose (II) (X, Scheme II) was hexylated to give XI in 83% yield as a distillable liquid. Acetolysis of XI gave the 1,2-diacetate (XII) as a liquid which was coupled with VI in the presence of titanium tetrachloride. The resulting crude blocked nucleosides (XIII), obtained as a syrup, were deacylated and chromatographed as before. The pure crystalline α and β nucleosides (XIV and XV, respectively) were isolated, after rechromatographing the partially overlapping peaks, in yields of 5.8% and 36%, respectively (from XII).



R = *n*-hexyl
Bz = benzoyl
Ac = acetyl

Scheme II

The anomeric assignments of these nucleosides were made by application of Hudson's rules of isorotation as shown in Table I. So far as is known, no exceptions to these rules have been found for 9-glycofuranosyladenines. In addition, condensation reactions with 2-acyloxy intermediates (such as V and XII), even under titanium tetrachloride conditions, are known to obey the *trans* rule of Baker for nucleosides (1, 13); that is, the preponderant anomer will have a C1′, C2′ *trans* configuration of substituents. The yields shown in Table I confirm these assignments.

[1] Bio-Rad AGl × 8 (OH) is a more purified form of Dowex 1 supplied by Bioradlabs, New York, N. Y.

Table I—Specific Rotations and Yields of Nucleosides

Nucleoside	$[\alpha]_D^{25}$ (MeOH)	% Yield	Anomeric Assignment
VIII	$-0.6 \pm 0.2°$	5.0	α
IX	$-49.8°$	63	β
XIV	$+0.5 \pm 0.2°$	5.8	α
XV	$-61.7°$	36	β

The biological activity of the nucleosides reported in this paper is currently under investigation. The results will be the subject of a future report.

EXPERIMENTAL[2]

3-*O*-(*n*-Hexyl)-1,2-*O*-isopropylidene-5-*O*-triphenylmethyl-D-xylofuranose (II)—A mixture of 4.32 g. of 1,2-*O*-isopropylidene-5-*O*-triphenylmethyl-D-xylofuranose (I) (12), m.p. 116–118°, 7.0 ml. of 1-chlorohexane, 3.0 g. of powdered potassium hydroxide,[3] and 5 ml. of benzene were stirred and heated under reflux overnight while protected from moisture. The cooled mixture was partitioned between 70 ml. of ether and 30 ml. of water. The organic phase was separated and washed further with water (2 × 30 ml.), then dried ($MgSO_4$), filtered, and evaporated at 0.05 mm. pressure and 55° for 3 hr. to give a stiff, yellow syrup which failed to crystallize; yield, 5.17 g. (100%). IR analysis showed a substantial increase in aliphatic C—H absorption at 3000–2860 cm.$^{-1}$ (addition of hexyl) and complete loss of hydroxyl absorption at 3450 cm.$^{-1}$; $[\alpha]_D^{25}$ $-25.4°$ (c 3.25, MeOH), $[\alpha]_{546}^{25}$ $-41.7°$.

Anal.[4]—Calcd. for $C_{33}H_{40}O_5$: C, 76.71; H, 7.80. Found: C, 76.33; H, 7.39.

3-*O*-(*n*-Hexyl)-1,2-*O*-isopropylidene-D-xylofuranose (III)—A mixture of 25.8 g. of II, 400 ml. of ethanol, 280 ml. of water, and 120 ml. of glacial acetic acid was heated under reflux for 2 hr. (complete solution occurred in 30 min.). After evaporation of the solvents, the residue was dissolved in 300 ml. of chloroform and washed with 100 ml. of 1 *N* sodium hydroxide solution and two 100-ml. portions of water. The chloroform solution was evaporated to dryness and the residue diluted with 75 ml. of methanol and stored overnight at 5°. Triphenylcarbinol, 9.99 g. (77% of theory), was removed by filtration. The filtrate was evaporated to dryness and the residue distilled, giving the crude product (contaminated with triphenylcarbinol as shown by TLC) as a colorless, viscous liquid in a yield of 12.6 g. (92%), b.p. 135–145°/0.04 mm. A 3.0-g. sample of this material was chromatographed on 70 g. of silica gel using benzene and gave 0.25 g. (8%) of triphenylcarbinol. Further elution with absolute ethanol gave the desired product (2.68 g.), which was distilled to give 2.53 g. (78% overall) of III as a colorless liquid, b.p. 128–134°/0.03 mm.; $\tilde{\nu}_{max.}^{film}$ (cm.$^{-1}$) 3500 (OH), strong bands in the 3000–2850 region (aliphatic C—H of hexyl) and 1375 (doublet, isopropylidene); $[\alpha]_D^{25}$ $-33.2°$ (c 3.31, MeOH), $[\alpha]_{546}^{25}$ $-39.6°$.

Anal.—Calcd. for $C_{14}H_{26}O_5$: C, 61.29; H, 9.55. Found: C, 61.23; H, 9.70.

5-*O*-Benzoyl-3-*O*-(*n*-hexyl)-1,2-*O*-isopropylidene-D-xylofuranose (IV)—To a stirred solution of 1.74 g. (6.4 mmoles) of pure III in 10 ml. of pyridine was added dropwise 1.0 ml. (8.7 mmoles) of benzoyl chloride below room temperature. The mixture was stored overnight at 5° in a stoppered flask and then quenched by the addition of 10 drops of water. After 30 min., the reaction was diluted with 50 ml. of chloroform and washed with 50 ml. of water, 50 ml. of aqueous-saturated sodium bicarbonate, and 50 ml. of water; it was then dried ($MgSO_4$), filtered, and evaporated to dryness. Traces of pyridine were removed from the residue by codistillation *in vacuo* with two 10-ml. portions of toluene. The resulting light-amber syrup was held for several hours at <1 mm. pressure; yield, 2.39 g. (100%); $\tilde{\nu}_{max.}^{film}$ (cm.$^{-1}$) loss of 3500 absorption (OH), 1725 (benzoate C=O), 1375 doublet (isopropylidene), 1280 (benzoate C—O—C), 710 (phenyl).

Anal.—Calcd. for $C_{21}H_{30}O_6$: C, 66.64; H, 7.99. Found: C, 66.51; H, 7.80.

1,2-Di-*O*-acetyl-5-*O*-benzoyl-3-*O*-(*n*-hexyl)-D-xylofuranose (V)—To a stirred solution of 5.46 g. of IV in 58 ml. of glacial acetic acid and 6.4 ml. of acetic anhydride was added dropwise, at 15–20°, 2.8 ml. of concentrated sulfuric acid. The reaction was allowed to stand overnight in a stoppered flask and was then poured, with stirring, into 200 ml. of cold 10% aqueous sodium acetate. After 1 hr., the mixture was extracted with four 50-ml. portions of chloroform. These extracts were combined and washed with 200 ml. of water, 200 ml. of aqueous-saturated sodium bicarbonate, and 200 ml. of water; they were then dried ($MgSO_4$), filtered, and evaporated to dryness at 0.05 mm. pressure and 50° to give an almost colorless syrup; yield, 6.00 g. (99%); $\tilde{\nu}_{max.}^{film}$ (cm.$^{-1}$) 1750 (acetate C=O), 1725 (benzoate C=O), 1375 singlet (C-methyl), 1280 (benzoate C—O—C), 1220 (acetate C—O—C), 710 (phenyl).

Anal.—Calcd. for $C_{22}H_{30}O_8$: C, 62.54; H, 7.16. Found: C, 63.34; H, 7.19.

9-[3-*O*-(*n*-Hexyl)-α- and β-D-xylofuranosyl]adenine (VIII and IX, respectively)—A mixture of 2.28 g. (5.4 mmoles) of V, 2.56 g. (5.4 mmoles) of chloromercuri-6-benzamidopurine (1) (VI), 2.6 g. of diatomaceous earth,[5] and 100 ml. of ethylene dichloride was distilled until 25 ml. of distillate had been collected. After cooling to room temperature, a solution of 0.60 ml. (5.4 mmoles) of titanium tetrachloride in 6 ml. of ethylene dichloride was added dropwise and the mixture was heated under reflux overnight while protected from moisture. To the cooled reaction was added 10 ml. of aqueous-saturated sodium bicarbonate with vigorous stirring, followed by additional solid sodium bicarbonate in small portions until a neutral reaction was obtained. After filtration through a diatomaceous earth pad, the filtrate was evaporated to dryness. A solution of the residue in 50 ml. of chloroform was washed with 50 ml. of 30% aqueous potassium iodide and 50 ml. of water, then dried ($MgSO_4$), filtered, and evaporated to dryness at 0.05 mm. pressure and 50° to give the crude, blocked nucleosides (VII) as an amber glass; yield, 2.90 g. (89); $\tilde{\nu}_{max.}^{film}$ (cm.$^{-1}$) 1750 (acetate C=O), 1725 (benzoate C=O), 1700 shoulder (amide C=O), 1610, 1580 (C=C and C=N), 1280 (benzoate C—O—C), 1225 (acetate C—O—C), 710 (phenyl).

A solution of 2.57 g. of VII in 50 ml. of 0.1 *N* methanolic sodium methoxide was heated under reflux for 3 hr. The cooled reaction was neutralized with acetic acid and evaporated to dryness. Methyl benzoate was removed by codistillation *in vacuo* with three 10-ml. portions of water. The residue was dissolved in 75 ml. of 50% aqueous ethanol and chromatographed on a column (38 mm. × 50 cm.) of ion-exchange resin using 60% aqueous methanol.[6] The partially separated peaks[7] corresponding to the anomeric nucleosides were each rechromatographed under the same conditions to give the pure isomers. The α-anomer (VIII) eluted first, and evaporation of the solvents gave 84 mg. (5.6%) of a crystalline solid, m.p. 174–177°. Recrystallization from 50% aqueous methanol gave the pure compound, m.p. 180–181°; $[\alpha]_D^{25}$ $-0.6° \pm 0.2°$ (c 1.07, MeOH), $[\alpha]_{546}^{25}$ $+0.5° \pm 0.2°$; $[\alpha]_{365}^{25}$ $+34.1°$; $\lambda_{max.\ (m\mu)}^{95\%\ ETOH}$ 260 (ϵ 15,000).

Anal.—Calcd. for $C_{16}H_{25}N_5O_4$: C, 54.68; H, 7.17; N, 19.93. Found: C, 54.65; H, 7.13; N, 19.87.

The β-anomer (IX) eluted next and amounted to 1.06 g. (71%) after removal of the solvents, m.p. 159–160°. Two recrystallizations from aqueous methanol gave the analytical sample, m.p. 160–160.5°; $[\alpha]_D^{25}$ $-49.8°$ (c 1.21, MeOH), $[\alpha]_{546}^{25}$ $-60.1°$; $\lambda_{max.\ (m\mu)}^{95\%\ ETOH}$ 260 (ϵ 15,100).

Anal.—Calcd. for $C_{16}H_{25}N_5O_4$: C, 54.68; H, 7.17; N, 19.93. Found: C, 54.87; H, 6.94; N, 19.48

5-Deoxy-3-*O*-(*n*-hexyl)-1,2-*O*-isopropylidene-D-xylofuranose (XI)—A stirred mixture of 10.4 g. of 5-deoxy-1,2-*O*-isopropylidene-D-xylofuranose (11) (X), m.p. 68–70°, 42 ml. of 1-chlorohexane, 30 ml. of benzene, and 18 g. of powdered potassium hydroxide were heated under reflux overnight while protected from moisture. The cooled mixture was partitioned between 300 ml. of ether and 100 ml.

[2] All evaporations were conducted *in vacuo* in a Buchler-type evaporator at 40–45° unless specified otherwise. Melting points were determined on a Mel-Temp apparatus and are uncorrected. The IR, UV, and polarimetric determinations were made using Perkin-Elmer models 337, 202, and 141, respectively.

[3] Obtained from the Riverside Chemical Co., Inc., North Tonawanda, N. Y.

[4] Galbraith Laboratories, Knoxville, Tenn., performed the elemental analyses.

[5] Celite, Johns Manville, New York, N. Y.

[6] Practical grade methanol was entirely satisfactory.

[7] Since these chromatograms generally required 2–3 days of continuous operation, the eluate was conveniently monitored by the use of an ISCO model UA analyzer equipped with a recorder operating at 1.27 cm. (0.5 in.)/hr.

of water. The ether layer was further washed with water (2 × 100 ml.), dried ($MgSO_4$), filtered, and evaporated to dryness. The resulting liquid was vacuum distilled, giving 12.9 g. (83%) of XI as a colorless distillate, b.p. 75–80°/0.02 mm.; $\bar{\nu}_{max.}^{film}$ (cm.$^{-1}$) loss of 3500 band (OH), several strong bands at 3000–2850 (aliphatic C—H of hexyl), 1375 doublet (isopropylidene); $[\alpha]_D^{25}$ −28.3° (c 1.22, MeOH), $[\alpha]_{546}^{25}$ −33.1°.

Anal.—Calcd. for $C_{14}H_{26}O_4$: C, 65.08; H, 10.14. Found: C, 64.81; H, 10.18.

1,2-Di-*O*-acetyl-5-deoxy-3-*O*-(*n*-hexyl)-D-xylofuranose (XII)—To a stirred solution of 11.5 g. of XI in 180 ml. of glacial acetic acid and 21.3 ml. of acetic anhydride was added dropwise, at 15–20°, 8.5 ml. of concentrated sulfuric acid. The reaction was stored overnight at room temperature in a stoppered flask, then poured into 650 ml. of cold 10% aqueous sodium acetate with stirring. After 40 min., the mixture was extracted with four 125-ml. portions of chloroform. The combined extracts were washed with 300 ml. of water, 300 ml. of aqueous-saturated sodium bicarbonate, and 300 ml. of water; dried ($MgSO_4$); filtered; and evaporated to dryness at 0.1 mm. pressure and 50° to give XII as a pale-yellow liquid; yield, 13.7 g. (101%); $\bar{\nu}_{max.}^{film}$ (cm.$^{-1}$) 1750 (acetate C=O), 1375 singlet (C-methyl), 1220 (acetate C—O—C).

Anal.—Calcd. for $C_{15}H_{26}O_6$: C, 59.58; H, 8.67. Found: C, 60.11; H, 8.87.

9-[5-Deoxy-3-*O*-(*n*-hexyl)-α- and β-D-xylofuranosyl]adenine (XIV and XV, respectively)—A mixture of 4.82 g. (16 mmoles) of XII, 7.57 g. (16 mmoles) of chloromercuri-6-benzamidopurine, 8 g. of diatomaceous earth, and 290 ml. of ethylene dichloride was distilled until 55 ml. of distillate had been collected. The mixture was cooled to room temperature and a solution of 1.8 ml. (16 mmoles) of titanium tetrachloride in 9 ml. of ethylene dichloride was added dropwise. The reaction was heated under reflux 20 hr. while protected from moisture. After cooling, 20 ml. of aqueous-saturated sodium bicarbonate was added with vigorous stirring, followed by additional solid sodium bicarbonate in small portions until a neutral reaction was obtained on pH paper. The mixture was filtered through a diatomaceous earth pad, the cake washed with two 50-ml. portions of ethylene dichloride, and the combined filtrate and washings were evaporated to dryness. A solution of the residue in 100 ml. of chloroform was washed with 100 ml. of 30% aqueous potassium iodide, 100 ml. of aqueous-saturated sodium bicarbonate, and 100 ml. of water; it was then dried ($MgSO_4$), filtered, and evaporated to dryness at 0.1 mm. pressure and 55° to give the crude, blocked nucleosides (XIII) as a pale-brown syrup; yield, 6.82 g. (89%); $\bar{\nu}_{max.}^{film}$ (cm.$^{-1}$) 1750 (acetate C=O), 1700 (amide C=O), 1225 (acetate C—O—C), 710 (phenyl).

A solution of 6.81 g. of XIII in 100 ml. of 0.1 *N* methanolic sodium methoxide was heated under reflux for 2.5 hr. while protected from moisture. The cooled reaction was neutralized with acetic acid and the solvent evaporated. Methyl benzoate was removed by co-distillation of the residue with three 20-ml. portions of water *in vacuo*. A solution of the residue in 70 ml. of 80% aqueous methanol was divided into two equal portions, each of which was chromatographed on a column (58 mm. × 50 cm.) of ion-exchange resin (200–400 mesh) using 60% aqueous methanol. The resulting chromatogram showed a minor UV absorbing peak followed by a major peak with some overlapping of the two materials. These overlap regions were combined from the two runs and rechromatographed to give the cleanly separated isomers. The first peak to elute, the α-anomer (XIV), gave white needles after evaporation; yield, 0.31 g. (6.5%). Recrystallization from 30 ml. of 50% aqueous methanol gave fine white needles, m.p. 139–140°; $[\alpha]_D^{25}$ +0.5° ± 0.2° (c 1.11, MeOH), $[\alpha]_{546}^{25}$ +1.2° ± 0.2°, $[\alpha]_{365}^{25}$ +39.1°; $\lambda_{max.\ (m\mu)}^{95\%\ ETOH}$ 260 (ε 15,400).

Anal.—Calcd. for $C_{16}H_{25}N_5O_3$: C, 57.29; H, 7.51; N, 20.88. Found: C, 57.01; H, 7.53; N, 20.58.

The major peak, the β-anomer (XV), gave 1.91 g. (40%) of white crystals on evaporation, m.p. 149–151°. Recrystallization from 45 ml. of 65% aqueous methanol gave white needles, m.p. 150–151°; $[\alpha]_D^{25}$ −61.7° (c 1.29, MeOH), $[\alpha]_{546}^{25}$ −75.2°; $\lambda_{max.\ (m\mu)}^{95\%\ ETOH}$ 260 (ε 15,600).

Anal.—Calcd. for $C_{16}H_{25}N_5O_3$: C, 57.29; H, 7.51; N, 20.88. Found: C, 57.46; H, 7.67; N, 20.93.

REFERENCES

(1) J. Prokop and D. H. Murray, *J. Pharm. Sci.*, **54,** 359, 1468 (1965); **56,** 865(1967); **57,** 1697(1968).

(2) D. H. Murray, J. Prokop, and A. Bloch, *J. Pharm. Sci.*, to be published.

(3) (*a*) J. J. Brink and G. A. LePage, *Cancer Res.*, **24,** 312, 1042(1964); (b) G. A. LePage and I. G. Junga, *ibid.*, **25,** 46(1965); (*c*) J. L. York and G. A. LePage, *Can. J. Biochem.*, **44,** 331(1966).

(4) H. J. Schaeffer, S. Marathe, and V. Alks, *J. Pharm. Sci.*, **53,** 1368(1964); H. J. Schaeffer, K. K. Kaistha, and S. K. Chakraborti, *ibid.*, **53,** 1371(1964); H. J. Schaeffer, D. D. Godse, and G. Liu, *ibid.*, **53,** 1510(1964).

(5) A. Bloch, M. J. Robins, and J. R. McCarthy, Jr., *J. Med. Chem.*, **10,** 908(1967).

(6) B. R. Baker, *J. Pharm. Sci.*, **53,** 347(1964).

(7) B. R. Baker, W. W. Lee, W. H. Skinner, A. P. Martinez, and E. Long, *J. Med. Pharm. Chem.*, **2,** 633(1960); B. R. Baker, *Cancer Chemotherapy Rept.*, **4,** 1(1959).

(8) B. R. Baker, W. W. Lee, E. Long, and L. O. Ross, *J. Amer. Chem. Soc.*, **83,** 3713(1961).

(9) H. J. Schaeffer and E. Odin, *J. Med. Chem.*, **9,** 576(1966); **10,** 181 (1967); H. J. Schaeffer and R. N. Johnson, *J. Pharm. Sci.*, **55,** 929(1966).

(10) H. J. Schaeffer and D. Vogel, *J. Med. Chem.*, **8,** 507(1965).

(11) R. H. Shah, H. J. Schaeffer, and D. H. Murray, *J. Pharm. Sci.*, **54,** 15(1965).

(12) P. A. Levene and A. L. Raymond, *J. Biol. Chem.*, **102,** 331(1933).

(13) B. R. Baker, in "Ciba Foundation Symposium," G. E. W. Wolstenholme and C. M. O'Connor, Eds., Little, Brown, Boston, Mass., 1957, p. 120.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 30, 1969, from the *Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication October 10, 1969.

Potential Antineoplastics II: 1-Thiocarbamoyl-3-methyl 4-arylhydrazono-2-pyrazolin-5-ones, 2-Amino-4-phenyl-5-arylazothiazoles, and *N*-Phenyl-*N'*-2(4-phenyl-5-arylazothiazolyl)thiocarbamides

H. G. GARG and R. A. SHARMA

Abstract □ A series of 1-thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones, 2-amino-4-phenyl-5-arylazothiazoles, and *N*-phenyl-*N'*-2(4-phenyl-5-arylazothiazolyl)thiocarbamides have been prepared for evaluation as antineoplastic agents. The 1-thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones and 2-amino-4-phenyl-5-arylazothiazoles were synthesized by coupling of appropriate aryldiazonium salts with 1-thiocarbamoyl-3-methyl-2-pyrazolin-5-one and 2-amino-4-phenylthiazole, respectively. The *N*-phenyl-*N'*-2(4-phenyl-5-arylazothiazolyl)thiocarbamides were obtained by condensing phenylisothiocyanate with 2-amino-4-phenyl-5-arylazothiazoles. The hydrazone-keto structures to 1-thiocarbamoyl-3-methyl-4-arylazo-2-pyrazolin-5-ones have been based on the IR spectral data. The intermediates required in these syntheses are also described.

Keyphrases □ 1-Thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones—synthesis □ 2-Amino-4-phenyl-5-arylazothiazoles—synthesis □ *N*-Phenyl-*N'*-2(4-phenyl-5-arylazothiazolyl)thiocarbamides—synthesis □ IR spectrophotometry—structure

There has been a growing interest, during the last few years, in compounds containing the N*-N*-S* or O*-N*-S* tridentate ligand system (1–5) or arylazo grouping (6, 7). This interest stems mainly from certain interesting carcinostatic activities of heterocyclic carboxyaldehyde thiosemicarbazones and the interfering action of 5-arylazopyrimidines with nucleic acid synthesis. Moreover, various Schiff bases from benzaldehyde nitrogen mustards and thiazoleamines have been reported to possess antitumor activity (8–10). As a part of a general study[1] directed toward the development of antineoplastics (11), the above-mentioned rationale led to examination of the synthesis and properties of three series of compounds having these mixed structural features—*viz.*, 1-thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones and 2-amino-4-phenyl-5-arylazothiazoles having N*-N*-S* ligand and arylazo grouping and *N*-phenyl-*N'*-2(4-phenyl-5-arylazothiazolyl)thiocarbamides having N*-N*-S* ligand and arylazo grouping and a modified azomethine linkage. It was hoped that these series might afford compounds that would be relatively less toxic to normal cells and have a better chemotherapeutic index.[2]

THEORETICAL

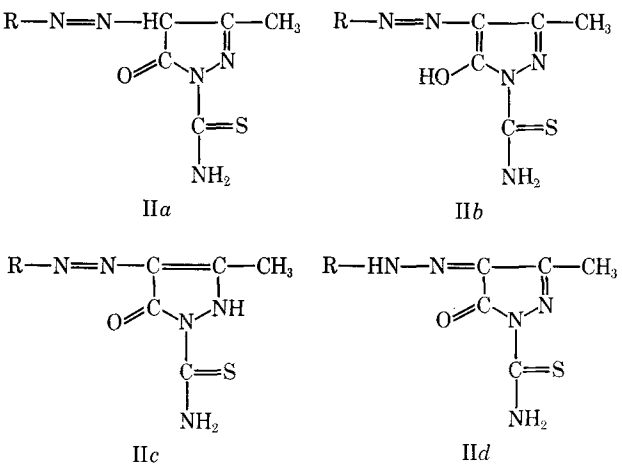
The most satisfactory route to 1-thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones (II) has been found to be the prior synthesis of 1-thiocarbamoyl-3-methyl-2-pyrazolin-5-one (I) and its subsequent coupling with diazonium salts. The required intermediate (I) is obtained in excellent yield by the cyclization of ethyl 3-oxobutyrate-β-thiosemicarbazone in liquid ammonia at room temperature. This in turn is prepared from ethyl acetoacetate and thiosemicarbazide (12) (see Scheme I). The products are all highly colored crystalline derivatives which are summarized in Table I.

The precursor for 2-amino-4-phenyl-5-arylazothiazoles, 2-amino-4-phenylthiazole (III), has been obtained by the condensation of acetophenone and thiourea in presence of iodine (13). The arylazo group at C-5 has been introduced by the condensation of the corresponding diazonium salts with III. The different 2-amino-4-phenyl-5-arylazothiazoles so obtained are crystalline substances and are summarized in Table II.

Boiling equimolar quantities of phenylisothiocyanate, prepared according to the procedure of Dains *et al.* (14), and 2-amino-4-phenyl-5-arylazothiazoles in benzene on a steam bath gives the *N*-phenyl-*N'*-2(4-phenyl-5-arylazothiazolyl)thiocarbamides in yields exceeding 60% (Table III).

It is interesting to note that the 1-thiocarbamoyl group is thermolabile in 1-thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones, and the cleavage of the thiocarbamoyl residue results in the products being the *N*-1-unsubstituted-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones (15).

The structures assigned to 1-thiocarbamoyl-3-methyl-4-arylazo-2-pyrazolin-5-ones need some comments as they can theoretically exist as one or more of the four possible structures (see structures of II).



IIa IIb IIc IId

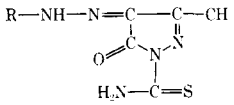
Possible Structures of Compound II

The IR spectra of all the compounds show bands characteristic of cyclic C=O frequency (16) (1660 cm.$^{-1}$ region) and C=C—NH—N=vibration (17) (1550 cm.$^{-1}$ region) (Table IV). This evidence unequivocally excludes Structures IIa, IIb, and IIc from consideration and supports hydrazone-keto Structure IId for all 1-thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones.

[1] A preliminary report of a portion of this work appeared in abstracts, Joint Convention of the Chemical Research Committee (C. S. I. R.) Institution of Chemists (India), and Society of Biological Chemists (India), Hyderabad-7 (India), 1969, p. 22.

[2] These compounds have been submitted for testing to Dr. H. B. Wood, Jr., National Institutes of Health, Bethesda, Md., the results of which will be reported elsewhere.

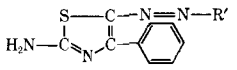
Table I—1-Thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones



Sample No.	R	Yield, %	M.p., °C.	Color	Formula	Anal., % Calcd.	Found
1	Phenyl	75	224–225	Orange needles[a]	$C_{11}H_{11}N_5OS$	C, 50.5 H, 4.2 N, 26.8 S, 12.3	C, 50.1 H, 4.0 N, 26.4 S, 12.1
2	4-MePh	80	230–231	Orange needles	$C_{12}H_{13}N_5OS$	C, 52.3 H, 4.7 N, 25.4 S, 11.6	C, 52.0 H, 4.5 N, 25.7 S, 11.5
3	2-MePh	80	210–211	Yellow plates	$C_{12}H_{13}N_5OS$	C, 52.3 H, 4.7 N, 25.4 S, 11.6	C, 52.2 H, 4.2 N, 25.2 S, 11.7
4	2,4-Me_2Ph	70	223–224	Orange needles	$C_{13}H_{15}N_5OS$	C, 53.9 H, 5.1 N, 24.2 S, 11.1	C, 53.5 H, 4.9 N, 23.9 S, 11.0
5	2,5-Me_2Ph	86	174–175	Orange needles	$C_{13}H_{15}N_5OS$	C, 53.9 H, 5.1 N, 24.2 S, 11.1	C, 53.6 H, 4.7 N, 24.0 S, 11.3
6	2,6-Me_2Ph	84	187–188	Yellow needles	$C_{13}H_{15}N_5OS$	C, 53.9 H, 5.1 N, 24.2 S, 11.1	C, 53.4 H, 5.2 N, 24.4 S, 11.2
7	2-NO_2Ph	65	202–203	Orange needles	$C_{11}H_{10}N_6O_3S$	C, 43.1 H, 3.2 N, 27.4 S, 10.5	C, 42.9 H, 3.6 N, 27.6 S, 10.3
8	3-NO_2Ph	70	247–248	Yellow needles	$C_{11}H_{10}N_6O_3S$	C, 43.1 H, 3.2 N, 27.4 S, 10.5	C, 42.7 H, 3.1 N, 27.1 S, 10.4
9	4-NO_2Ph	72	241–242	Yellow needles	$C_{11}H_{10}N_6O_3S$	C, 43.1 H, 3.2 N, 27.4 S, 10.5	C, 42.9 H, 3.2 N, 27.5 S, 10.3
10	3-MeOPh	88	196–197	Brown fibers	$C_{12}H_{13}N_5O_2S$	C, 49.4 H, 4.4 N, 24.0 S, 11.0	C, 49.1 H, 4.0 N, 23.7 S, 10.8
11	2,6-Cl_2Ph	74	171–172	Brown plates	$C_{11}H_9Cl_2N_5OS$	C, 40.0 H, 2.7 N, 21.2 S, 9.7	C, 40.2 H, 2.5 N, 21.3 S, 9.8
12	3-ClPh	82	246–247	Orange needles	$C_{11}H_{10}ClN_5OS$	C, 44.5 H, 3.3 N, 23.6 S, 10.8	C, 44.8 H, 3.8 N, 23.2 S, 10.7
13	2,5-Cl_2Ph	83	226–227	Brown needles	$C_{11}H_9Cl_2N_5OS$	C, 40.0 H, 2.7 N, 21.2 S, 9.7	C, 40.3 H, 2.9 N, 21.2 S, 9.7
14	2-Cl-6-MePh	87	194–195	Yellow needles	$C_{12}H_{12}ClN_5OS$	C, 46.6 H, 3.5 N, 22.6 S, 10.4	C, 46.4 H, 3.1 N, 22.2 S, 10.2
15	4-ClPh	67	211–212	Brown fibers	$C_{11}H_{10}ClN_5OS$	C, 44.5 H, 3.3 N, 23.6 S, 10.8	C, 44.2 H, 3.6 N, 23.5 S, 10.5
16	2,5-MeO_2Ph	88	172–173	Brown needles	$C_{13}H_{15}N_5O_3S$	C, 48.7 H, 4.6 N, 21.6 S, 10.0	C, 48.5 H, 4.2 N, 21.6 S, 9.9
17	2-Cl-4-NO_2Ph	55	108–109	Pale-yellow needles	$C_{11}H_9ClN_6O_3S$	C, 38.8 H, 2.6 N, 24.7 S, 9.4	C, 38.3 H, 2.5 N, 24.5 S, 9.1
18	4-EtOPh	60	168(d)	Orange needles	$C_{13}H_{15}N_5O_2S$	C, 51.1 H, 4.9 N, 22.9 S, 10.5	C, 51.2 H, 4.4 N, 22.8 S, 10.3
19	4-SO_2NH_2Ph	76	229–230	Yellow needles	$C_{11}H_{12}N_6O_3S_2$	C, 38.8 H, 3.5 N, 24.7 S, 9.4	C, 38.4 H, 3.2 N, 24.9 S, 9.2

[a] Lit. m.p. 217°.

Table II—2-Amino-4-phenyl-5-arylazothiazoles



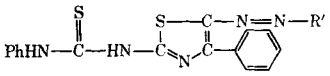
Sample No.	R′	Yield, %	M.p., °C.	Color	Formula	Anal., % Calcd.	Found
1	2-MePh	78	159–160	Orange needles	$C_{16}H_{14}N_4S$	C, 65.3 H, 4.7 N, 19.0 S, 10.9	C, 65.2 H, 4.4 N, 18.5 S, 10.7
2	2-MeOPh	85	210–211	Deep-red needles	$C_{16}H_{14}N_4OS$	C, 61.9 H, 4.5 N, 18.0 S, 10.3	C, 61.4 H, 4.2 N, 18.2 S, 10.4
3	3-MeOPh	76	201–202	Red needles	$C_{16}H_{14}N_4OS$	C, 61.9 H, 4.5 N, 18.0 S, 10.3	C, 61.7 H, 4.6 N, 17.9 S, 10.3
4	4-MeOPh	82	204–205	Orange needles	$C_{16}H_{14}N_4OS$	C, 61.9 H, 4.5 N, 18.0 S, 10.2	C, 61.5 H, 4.2 N, 17.7 S, 10.3
5	3-ClPh	64	166–167	Orange plates	$C_{15}H_{11}ClN_4S$	C, 57.3 H, 3.5 N, 17.8 S, 10.0	C, 57.5 H, 3.1 N, 17.5 S, 10.0
6	4-ClPh	70	232–233	Violet needles	$C_{15}H_{11}ClN_4S$	C, 57.3 H, 3.5 N, 17.8 S, 10.2	C, 57.1 H, 3.4 N, 17.4 S, 10.1
7	2-NO_2Ph	71	210–211	Red needles	$C_{15}H_{11}N_5O_2S$	C, 55.3 H, 3.3 N, 21.5 S, 9.8	C, 55.0 H, 3.0 N, 21.2 S, 9.6
8	3-NO_2Ph	68	233–234	Orange plates	$C_{15}H_{11}N_5O_2S$	C, 55.3 H, 3.3 N, 21.5 S, 9.8	C, 55.5 H, 3.6 N, 21.0 S, 9.7
9	4-EtOPh	78	229–230	Brown needles	$C_{17}H_{16}N_4OS$	C, 62.9 H, 4.9 N, 17.2 S, 9.9	C, 62.5 H, 4.7 N, 17.5 S, 9.8
10	3-OHPh	65	181–182	Orange needles	$C_{15}H_{12}N_4OS$	C, 60.8 H, 4.0 N, 18.9 S, 10.8	C, 60.7 H, 4.2 N, 18.4 S, 10.5
11	2-COOHPh	60	268–269	Deep-red needles	$C_{16}H_{12}N_4O_2S$	C, 59.2 H, 3.7 N, 17.2 S, 9.9	C, 59.3 H, 3.5 N, 17.3 S, 9.7
12	2,4-Me_2Ph	90	184–185	Red needles	$C_{17}H_{16}N_4S$	C, 66.2 H, 5.1 N, 18.1 S, 10.4	C, 66.4 H, 5.2 N, 18.6 S, 10.2
13	2,5-Me_2Ph	86	204–205	Orange needles	$C_{17}H_{16}N_4S$	C, 66.2 H, 5.1 N, 18.1 S, 10.4	C, 66.0 H, 4.7 N, 18.2 S, 10.3
14	2,6-Me_2Ph	85	162–163	Deep-red needles	$C_{17}H_{16}N_4S$	C, 66.2 H, 5.1 N, 18.1 S, 10.4	C, 66.2 H, 4.9 N, 18.0 S, 10.1
15	2,5-MeO_2Ph	60	124–125	Orange needles	$C_{17}H_{16}N_4O_2S$	C, 60.0 H, 4.7 N, 16.4 S, 9.4	C, 60.4 H, 4.2 N, 16.2 S, 9.2
16	2,5-Cl_2Ph	75	227–228	Orange needles	$C_{15}H_{10}Cl_2N_4S$	C, 51.5 H, 2.8 N, 16.0 S, 9.2	C, 51.1 H, 2.5 N, 16.5 S, 9.0
17	2,6-Cl_2Ph	78	134–135	Orange fibers	$C_{15}H_{10}Cl_2N_4S$	C, 51.5 H, 2.8 N, 16.0 S, 9.2	C, 51.3 H, 2.7 N, 16.3 S, 9.1
18	2-Cl-6-MePh	70	180–181	Red needles	$C_{16}H_{13}ClN_4S$	C, 58.5 H, 3.9 N, 17.0 S, 9.8	C, 58.2 H, 3.4 N, 17.2 S, 9.6
19	2-Cl-4-NO_2Ph	72	272–273	Violet needles	$C_{15}H_{10}ClN_5O_2S$	C, 50.1 H, 2.7 N, 19.4 S, 9.3	C, 49.8 H, 2.5 N, 18.9 S, 9.1

(Continued)

Table II—(*Continued*)

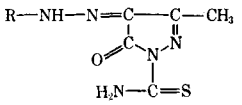
Sample No.	R′	Yield, %	M.p., °C.	Color	Formula	Anal., % Calcd.	Found
20	2,6-Cl_2-4-NO_2Ph	70	234–235	Deep-red needles	$C_{15}H_9Cl_2N_5O_2S$	C, 45.9 H, 2.2 N, 17.7 S, 8.1	C, 45.6 H, 2.0 N, 17.4 S, 8.0
21	2,4-(NO_2)$_2$Ph	65	278–279	Violet needles	$C_{15}H_{10}N_6O_4S$	C, 48.6 H, 2.7 N, 22.7 S, 8.6	C, 48.2 H, 2.8 N, 22.3 S, 8.4

Table III—*N*-Phenyl-*N*′-2(4-phenyl-5-arylazothiazolyl)thiocarbamides



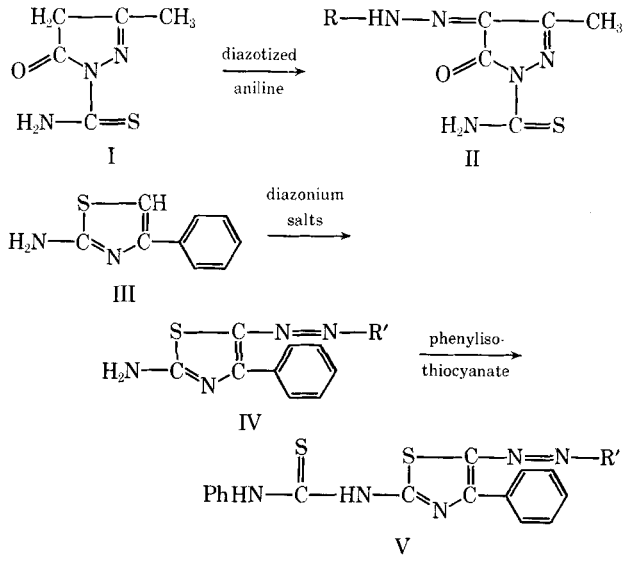
Sample No.	R′	Yield, %	M.p., °C.	Color	Formula	Anal., % Calcd.	Found
1	2,5-MeO_2Ph	65	141–142	Orange-red needles	$C_{24}H_{21}N_5O_2S_2$	C, 60.6 H, 4.4 N, 14.7 S, 13.4	C, 60.2 H, 4.0 N, 14.4 S, 13.0
2	2-MePh	69	254–255	Red needles	$C_{23}H_{19}N_5S_2$	C, 64.3 H, 4.4 N, 16.3 S, 14.9	C, 64.0 H, 4.2 N, 16.0 S, 14.5
3	2,5-Cl_2Ph	72	256–257	Deep-red plates	$C_{22}H_{15}Cl_2N_5S$	C, 54.5 H, 3.1 N, 14.4 S, 13.2	C, 54.4 H, 2.7 N, 14.6 S, 13.0
4	3-NO_2Ph	58	262–263	Yellow-orange needles	$C_{22}H_{16}N_6O_2S_2$	C, 57.4 H, 3.5 N, 18.2 S, 13.9	C, 57.6 H, 3.4 N, 17.9 S, 13.3
5	4-EtOPh	65	242–243	Red needles	$C_{24}H_{21}N_5O_2S$	C, 62.7 H, 4.6 N, 15.2 S, 13.9	C, 62.5 H, 4.3 N, 14.7 S, 13.5
6	2,6-Me_2Ph	75	185–186	Orange-red needles	$C_{24}H_{21}N_5S_2$	C, 65.0 H, 4.7 N, 15.8 S, 14.4	C, 65.3 H, 4.5 N, 15.5 S, 14.1
7	4-ClPh	60	255–256	Red plates	$C_{22}H_{16}ClN_5S_2$	C, 58.7 H, 3.5 N, 15.5 S, 14.2	C, 58.2 H, 3.2 N, 15.2 S, 13.8
8	4-MeOPh	74	239–240	Orange needles	$C_{23}H_{19}N_5OS_2$	C, 62.0 H, 4.2 N, 15.7 S, 14.3	C, 61.6 H, 4.6 N, 15.6 S, 14.0
9	2,4-Me_2Ph	71	258–259	Orange needles	$C_{24}H_{21}N_5S_2$	C, 65.0 H, 4.7 N, 15.8 S, 14.4	C, 64.7 H, 4.3 N, 15.2 S, 14.1
10	2-Cl-4-NO_2Ph	55	280–281	Violet needles	$C_{22}H_{15}ClN_6O_2S_2$	C, 53.4 H, 3.3 N, 16.9 S, 12.9	C, 53.1 H, 3.0 N, 16.4 S, 12.5
11	3-MeOPh	76	235–236	Orange plates	$C_{23}H_{19}N_5OS_2$	C, 62.0 H, 4.2 N, 15.7 S, 14.3	C, 62.4 H, 3.8 N, 15.5 S, 13.9
12	2-MeOPh	75	227–228	Orange needles	$C_{23}H_{19}N_5OS_2$	C, 62.0 H, 4.2 N, 15.7 S, 14.3	C, 62.2 H, 3.0 N, 15.2 S, 13.8
13	2-NO_2Ph	70	164–165	Brown needles	$C_{22}H_{16}N_6O_2S_2$	C, 57.4 H, 3.5 N, 18.2 S, 13.9	C, 57.5 H, 3.2 N, 18.4 S, 13.6
14	2,5-Me_2Ph	68	260–261	Orange needles	$C_{24}H_{21}N_5S_2$	C, 65.0 H, 4.7 N, 15.8 S, 14.4	C, 65.2 H, 4.5 N, 15.4 S, 14.1
15	2,4-(NO_2)$_2$Ph	56	287–288	Reddish-brown needles	$C_{22}H_{15}N_5O_4S_2$	C, 52.2 H, 2.9 N, 19.4 S, 12.6	C, 52.0 H, 2.7 N, 19.0 S, 12.2

Table IV—Spectral Properties of 1-Thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones[a]



Sample No.	R	C=O Cyclic	C=C— NH—N=	C=N	>C=S	NH, NH_2 Associated	Substituted Phenyl Ring
1	2-MeOPh	1665	1550	1615	1430	3365	750
2	3-ClPh	1680	1552	1595	1410	3360	745
3	2-NO_2Ph	1675	1550	1600	1420	3370	748
4	4-NO_2Ph	1680	1550	1600	1410	3365	755
5	4-SO_2NH_2Ph	1675	1550	1595	1410	3365	755
6	2-MePh	1670	1550	1610	1430	3370	758
7	2,5-Cl_2Ph	1675	1550	1612	1410	3375	820
8	2,5-Me_2Ph	1670	1552	1605	1435	3375	815
9	3-MeOPh	1670	1550	1600	1420	3380	745
10	2,6-Me_2Ph	1675	1552	1598	1435	3375	780
11	2-Cl-4-NO_2Ph	1680	1550	1590	1435	3370	785
12	4-EtOPh	1680	1550	1595	1425	3385	760
13	2-Cl-6-MePh	1670	1550	1605	1430	3360	755
14	4-MePh	1670	1552	1600	1410	3365	750
15	4-ClPh	1675	1550	1600	1415	3370	755

[a] IR ($cm.^{-1}$) $\nu_{max.}$ (KBr disc).



Scheme I

EXPERIMENTAL[3]

1-Thiocarbamoyl-3-methyl-2-pyrazolin-5-one—Thiosemicarbazide hydrochloride (12.7 g., 0.1 mole) was dissolved in water (30 ml.) and mixed with acetoacetic ester (13 ml., 0.1 mole). Ethyl 3-oxobutyrate-β-thiosemiarbazone separated after 15 min. and recrystallized from ethanol as colorless needles, yield 19.09 g., 90%; m.p. 92–93° (from ethanol) [lit. (12) m.p. 93°].

The latter (50 g.) was suspended in liquid ammonia (25 ml.) and thoroughly stirred until it gradually dissolved. The mixture was then made acidic with concentrated HCl. 1-Thiocarbamoyl-3-methyl-2-pyrazolin-5-one precipitated and was recrystallized as colorless needles, yield 2.9 g., 76%; m.p. 181–182° (from DMF–ethanol) [lit. (12) m.p. 180°].

1-Thiocarbamoyl-3-methyl-4-(2-methoxyphenylhydrazono)-2-pyrazolin-5-one—*o*-Anisidine (2.5 ml., 0.02 mole) was dissolved in 3 *N* HCl (2.5 ml.) and cooled to 0°. Sodium nitrite (1.4 g., 0.02 mole) dissolved in water (20 ml.) was gradually added. The diazonium salt solution was filtered into a well-cooled, stirred mixture of sodium acetate (5 g.) and 1-thiocarbamoyl-3-methyl-2-pyrazolin-5-one (3.14 g., 0.02 mole) in acetic acid (50 ml.). 1-Thiocarbamoyl-3-methyl-4-(2-methoxyphenylhydrazono)-2-pyrazolin-5-one started precipitating almost immediately. After standing for 2 hr., the precipitate was filtered, washed with water, and recrystallized as orange needles, yield 4.3 g., 82%; m.p. 210–211° (DMF–ethanol).

Anal.—Calcd. for $C_{12}H_{13}N_5O_2S$: C, 49.4; H, 4.4; N, 24.0; S, 11.0. Found: C, 49.0; H, 4.6; N, 23.6; S, 10.7.

By adopting a similar procedure as above, several 1-thiocarbamoyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones, described in Table I, were obtained.

2-Amino-4-phenylthiazole—A mixture of acetophenone (24.0 ml., 0.2 mole), thiourea (30.4 g., 0.4 mole), and iodine (50.8 g., 0.2 mole) was heated overnight on a steam bath. This was cooled and extracted with ether (2 × 25 ml.) to remove unreacted acetophenone and iodine. The residue was then dissolved in hot water and filtered to remove sulfur and other impurities. The solution was cooled somewhat (about 20°) and made alkaline with concentrated ammonia. The 2-amino-4-phenylthiazole thus precipitated was recrystallized as long colorless needles, yield 38.7 g., 65%; m.p. 145° (from H_2O–ethanol) [lit. (13) m.p. 147°].

2-Amino-4-phenyl-5-phenylazothiazole—Aniline (1.85 g., 0.02 mole) was dissolved in 3 *N* HCl (2.5 ml.) and cooled to 0°. Sodium nitrite (1.4 g., 0.02 mole) dissolved in water (25 ml.) was added. The diazonium solution was filtered to a well-cooled suspension of 2-amino-4-phenylthiazole (3.52 g., 0.02 mole) and sodium acetate (5 g.) in ethanol (50 ml.). After 2 hr., 2-amino-4-phenyl-5-phenylazothiazole was filtered and washed well with water. It was recrystallized as red needles, yield 4.5 g., 80%; m.p. 191–192° (from DMF–ethanol) [lit. (18) m.p. 195°].

Anal.—Calcd. for $C_{15}H_{12}N_4S$: C, 64.2; H, 4.2; N, 20.0; S, 11.4. Found: C, 63.7; H, 4.4; N, 19.6; S, 11.2.

Similarly prepared 2-amino-4-phenyl-5-arylazothiazoles are summarized in Table II.

***N*-Phenyl-*N'*-2(4-phenyl-5-phenylazothiazolyl)thiocarbamide**—A mixture of phenylisothiocyanate (1.35 g., 0.01 mole) and 2-amino-4-phenyl-5-phenylazothiazole (2.80 g., 0.01 mole) in benzene (15 ml.) was refluxed for 6–8 hr. on a steam bath. The solvent was removed and the residue was repeatedly triturated with petroleum ether (b.p. 40–60°) and then with ether. The crystalline thiocarbamide thus obtained was recrystallized from DMF–ethanol as deep red needles, yield 3.1 g., 75%; m.p. 241–242°.

Anal.—Calcd. for $C_{22}H_{17}N_5S_2$: C, 63.6; H, 4.1; N, 16.8; S, 15.4. Found: C, 63.2; H, 4.5; N, 16.6; S, 15.1.

Similarly a number of *N*-phenyl-*N'*-2(4-phenyl-5-arylazothiazolyl)-thiocarbamides were prepared which are summarized in Table III.

[3] Melting points were determined with a Kofler hot stage apparatus and are uncorrected. IR spectra were measured with a Beckman IR4 spectrophotometer.

REFERENCES

(1) R. W. Brockman, J. R. Thomson, M. J. Bell, and H. E. Skipper, *Cancer Res.*, **16**, 167(1956).

(2) F. A. French and B. L. Freedlander, *ibid.*, **18**, 1920(1958).

(3) *Ibid.*, **20**, 505(1960).

(4) F. A. French and E. J. Blanz, Jr., *Cancer Res.*, **23**, 9(1963).
(5) F. A. French and E. J. Blanz, Jr., *J. Med. Chem.*, **9**, 585 (1966).
(6) E. J. Modest, H. N. Schlein, and G. E. Foley, *J. Pharm. Pharmacol.*, **9**, 68(1957).
(7) R. E. Harmon, F. E. Dutton, and H. D. Warren, *J. Med. Chem.*, **11**, 627(1968).
(8) S. S. Sabnis and K. D. Kulrami, *Indian J. Chem.*, **1**, 447 (1963).
(9) F. D. Popp and W. Kirsch, *J. Org. Chem.*, **26**, 3858(1961).
(10) M. G. Dhapalapur, S. S. Sabnis, and C. V. Deliwala, *J. Med. Chem.*, **11**, 154(1968).
(11) H. G. Garg and R. A. Sharma, *J. Med. Chem.*, **12**, 1122 (1969).
(12) S. C. De and D. N. Dutt, *J. Indian Chem. Soc.*, **7**, 473(1930).
(13) R. M. Dodson and L. C. King, *J. Amer. Chem. Soc.*, **67**, 2242(1945).
(14) F. B. Dains, R. Q. Brewster, and C. P. Olander, *Org. Synth. Coll.*, **1**, 447.
(15) H. G. Garg, Ph.D. thesis, Agra University, Agra, India, 1959.
(16) H. Yasuda, *Sci. Papers Inst. Phys. Chem. Res. (Tokyo)*, **56**, 267(1962).
(17) H. Yasuda and H. Midorikawa, *J. Org. Chem.*, **31**, 1722 (1966).
(18) H. Beyer and G. Walter, *Chem. Ber.*, **85**, 1077(1952).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 8, 1969, from the *Department of Chemistry, University of Roorkee, Roorkee, India.*

Accepted for publication October 15, 1969.

The authors thank Professor W. U. Malik, Head of the Chemistry Department, for providing the necessary facilities for work, and C.S.I.R., New Delhi (India), for a junior research fellowship (held by R.A.S.).

Release of Medroxyprogesterone Acetate from a Silicone Polymer

T. J. ROSEMAN* and W. I. HIGUCHI†

Abstract □ The *in vitro* release of medroxyprogesterone acetate from a silicone rubber matrix was studied. A nonlinear dependence of release rate upon medroxyprogesterone acetate concentration within the matrix was found. Based upon a model system, equations were derived to explain this behavior and to include other parameters which may influence the release rate. Since the model, in part, is dependent upon a receding medroxyprogesterone acetate layer within the matrix, a photograph depicting depletion zones as a function of time is presented. In contrast to the T. Higuchi model for drug release, this model includes the boundary diffusion layer. Comparison of the two models suggested that when the boundary layer was considered, a better fit of experimental data to theory was found. The applicability of the model to an *in vivo* system is discussed. This study has suggested that the partition coefficient, diffusion coefficients, medroxyprogesterone acetate concentration within the polymer, and agitation conditions play important roles in the release process.

Keyphrases □ Medroxyprogesterone acetate release rate, *in vitro*—physicochemical factors □ Silicone rubber matrix—medroxyprogesterone acetate release □ Matrix boundary diffusion layer model—equations derived □ Partition coefficient—silicone, medroxyprogesterone acetate □ Vapor phase chromatography—determination

The use of a rubber material as a delivery system for various chemicals has been a subject of considerable interest. The B. F. Goodrich Co. (1) has recently incorporated toxic substances into a rubber matrix and observed effective antifouling activity for prolonged periods. Some therapeutic implications of silicone rubber as a drug delivery system have been described previously (2).

The advantage of silicone rubber as a dosage form for medroxyprogesterone acetate has been discussed by Mishell *et al.* (3). It was shown that medroxyprogesterone acetate was readily absorbed from a vaginal device in sufficient quantity to inhibit ovulation. This drug delivery system promises to be a unique approach in the field of contraception.

Although other investigators (4, 5) have studied the diffusion of drugs across silicone membranes, an *in vitro* study on the release of a drug embedded in a silicone matrix has not been presented. Therefore, the present study was designed to investigate the physicochemical factors involved in the release of medroxyprogesterone acetate from a silicone matrix system. The interdependence of various parameters can be described by mathematical relationships based upon a physical model which is an extension of concepts set forth by Higuchi (6).

EXPERIMENTAL

Medroxyprogesterone acetate[1]–silicone[2] cylinders, 4 cm. by 0.5 cm., were prepared by levigating the required amount of drug into the elastomer and polymerizing with catalyst. The mixture was then forced into prewashed vinyl tubing and allowed to cure. After the cylinders were removed from the tubing and weighed, 24 were mounted between two circular disks and secured in a 3-l. jacketed beaker. Figure 1 is a schematic diagram of the *in vitro* dissolution apparatus. Distilled water from eight 5-gal. carboys was pumped at a rate of about 60 l./day through a 37° water bath, which preheated the water, into the beaker. The effluent was discarded into a drain. This constant flow of water approximates a "perfect sink" condition, *i.e.*, there is no significant concentration build-up in the dissolution media. The same water bath provided 37° water which was continuously circulated through the walls of the beaker,

[1] The Upjohn Co.'s trademark for medroxyprogesterone acetate is Provera.
[2] Silastic Elastomer, Dow Corning Corp., Midland, Mich.

(4) F. A. French and E. J. Blanz, Jr., *Cancer Res.*, **23**, 9(1963).
(5) F. A. French and E. J. Blanz, Jr., *J. Med. Chem.*, **9**, 585 (1966).
(6) E. J. Modest, H. N. Schlein, and G. E. Foley, *J. Pharm. Pharmacol.*, **9**, 68(1957).
(7) R. E. Harmon, F. E. Dutton, and H. D. Warren, *J. Med. Chem.*, **11**, 627(1968).
(8) S. S. Sabnis and K. D. Kulrami, *Indian J. Chem.*, **1**, 447 (1963).
(9) F. D. Popp and W. Kirsch, *J. Org. Chem.*, **26**, 3858(1961).
(10) M. G. Dhapalapur, S. S. Sabnis, and C. V. Deliwala, *J. Med. Chem.*, **11**, 154(1968).
(11) H. G. Garg and R. A. Sharma, *J. Med. Chem.*, **12**, 1122 (1969).
(12) S. C. De and D. N. Dutt, *J. Indian Chem. Soc.*, **7**, 473(1930).
(13) R. M. Dodson and L. C. King, *J. Amer. Chem. Soc.*, **67**, 2242(1945).
(14) F. B. Dains, R. Q. Brewster, and C. P. Olander, *Org. Synth. Coll.*, **1**, 447.
(15) H. G. Garg, Ph.D. thesis, Agra University, Agra, India, 1959.
(16) H. Yasuda, *Sci. Papers Inst. Phys. Chem. Res. (Tokyo)*, **56**, 267(1962).
(17) H. Yasuda and H. Midorikawa, *J. Org. Chem.*, **31**, 1722 (1966).
(18) H. Beyer and G. Walter, *Chem. Ber.*, **85**, 1077(1952).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 8, 1969, from the *Department of Chemistry, University of Roorkee, Roorkee, India.*

Accepted for publication October 15, 1969.

The authors thank Professor W. U. Malik, Head of the Chemistry Department, for providing the necessary facilities for work, and C.S.I.R., New Delhi (India), for a junior research fellowship (held by R.A.S.).

Release of Medroxyprogesterone Acetate from a Silicone Polymer

T. J. ROSEMAN* and W. I. HIGUCHI†

Abstract □ The *in vitro* release of medroxyprogesterone acetate from a silicone rubber matrix was studied. A nonlinear dependence of release rate upon medroxyprogesterone acetate concentration within the matrix was found. Based upon a model system, equations were derived to explain this behavior and to include other parameters which may influence the release rate. Since the model, in part, is dependent upon a receding medroxyprogesterone acetate layer within the matrix, a photograph depicting depletion zones as a function of time is presented. In contrast to the T. Higuchi model for drug release, this model includes the boundary diffusion layer. Comparison of the two models suggested that when the boundary layer was considered, a better fit of experimental data to theory was found. The applicability of the model to an *in vivo* system is discussed. This study has suggested that the partition coefficient, diffusion coefficients, medroxyprogesterone acetate concentration within the polymer, and agitation conditions play important roles in the release process.

Keyphrases □ Medroxyprogesterone acetate release rate, *in vitro*—physicochemical factors □ Silicone rubber matrix—medroxyprogesterone acetate release □ Matrix boundary diffusion layer model—equations derived □ Partition coefficient—silicone, medroxyprogesterone acetate □ Vapor phase chromatography—determination

The use of a rubber material as a delivery system for various chemicals has been a subject of considerable interest. The B. F. Goodrich Co. (1) has recently incorporated toxic substances into a rubber matrix and observed effective antifouling activity for prolonged periods. Some therapeutic implications of silicone rubber as a drug delivery system have been described previously (2).

The advantage of silicone rubber as a dosage form for medroxyprogesterone acetate has been discussed by Mishell *et al.* (3). It was shown that medroxyprogesterone acetate was readily absorbed from a vaginal device in sufficient quantity to inhibit ovulation. This drug delivery system promises to be a unique approach in the field of contraception.

Although other investigators (4, 5) have studied the diffusion of drugs across silicone membranes, an *in vitro* study on the release of a drug embedded in a silicone matrix has not been presented. Therefore, the present study was designed to investigate the physicochemical factors involved in the release of medroxyprogesterone acetate from a silicone matrix system. The interdependence of various parameters can be described by mathematical relationships based upon a physical model which is an extension of concepts set forth by Higuchi (6).

EXPERIMENTAL

Medroxyprogesterone acetate[1]–silicone[2] cylinders, 4 cm. by 0.5 cm., were prepared by levigating the required amount of drug into the elastomer and polymerizing with catalyst. The mixture was then forced into prewashed vinyl tubing and allowed to cure. After the cylinders were removed from the tubing and weighed, 24 were mounted between two circular disks and secured in a 3-l. jacketed beaker. Figure 1 is a schematic diagram of the *in vitro* dissolution apparatus. Distilled water from eight 5-gal. carboys was pumped at a rate of about 60 l./day through a 37° water bath, which preheated the water, into the beaker. The effluent was discarded into a drain. This constant flow of water approximates a "perfect sink" condition, *i.e.*, there is no significant concentration build-up in the dissolution media. The same water bath provided 37° water which was continuously circulated through the walls of the beaker,

[1] The Upjohn Co.'s trademark for medroxyprogesterone acetate is Provera.
[2] Silastic Elastomer, Dow Corning Corp., Midland, Mich.

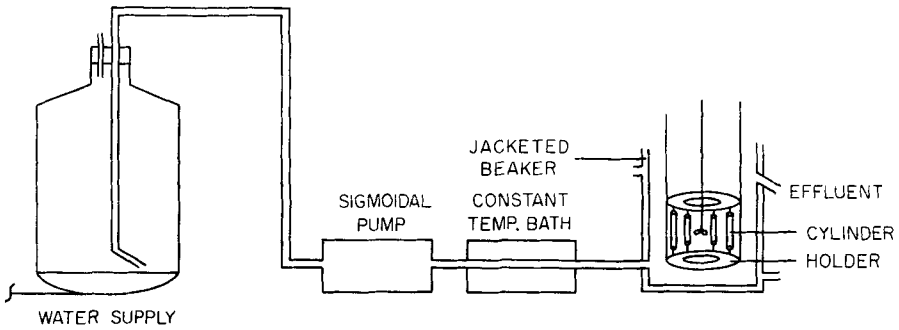


Figure 1—*Dissolution apparatus for silicone cylinders.*

thereby maintaining a constant temperature during dissolution. The solution was agitated by an impeller which was driven by a Servodyne drive system.[3] The impeller was situated in the center of the beaker and extended to the bottom of the cylinders. Its location was fixed for all experiments.

In order to quantitate the initial release rates, the effluent was collected, extracted, and assayed for its medroxyprogesterone acetate content by a modified USP procedure (7). The long time release data (1 week or greater) was obtained by withdrawing the cylinders and determining the residual medroxyprogesterone acetate content by vapor phase chromatography. The difference between the initial and final values gave the amount lost at any given time.

The zones of depletion were measured microscopically with a calibrated reticule. Cross-sectional slices of the cylinder were used for the measurement.

The partition coefficient of medroxyprogesterone acetate was determined by equilibrating flat sheets (about 7 × 2 × 0.05 cm.) of the silicone material in a solution of tritium-labeled medroxyprogesterone acetate at 37°. Sheets were removed at 1-day and 4-day intervals to ensure that equilibrium had resulted. They were then extracted with methylene chloride and the solvent evaporated to dryness. After the addition of 15 ml. of Diotol counting solvent, the samples were counted in a liquid scintillation spectrometer.[4] The partition coefficient was calculated by dividing the counts per volume in the equilibrated solution by counts per volume in the silicone sheet.

THEORETICAL CONSIDERATIONS

The mechanisms of drug release from various matrix systems have previously been discussed (6). It was assumed that the rate-limiting step was the diffusion of drug from the matrix (matrix controlled). Under certain conditions, it is conceivable that the rate of diffusion from the surface of the matrix to the surrounding bulk solution will make a significant contribution to the total diffusional process. Therefore, the mathematics for this system (matrix-boundary diffusion layer model) is presented for two geometric cases. The assumptions in the derivations are: (*a*) A pseudo-steady state exists; (*b*) $A >> C_s$, the concentration of drug in the matrix is much greater than its solubility in the matrix; (*c*) the diffusion coefficients are constant; (*d*) diffusion is the rate-controlling step, rather than dissolution; (*e*) the diffusional process occurs through the matrix phase rather than through pores or channels within the matrix.

Planar Case—Figure 2 is a hypothetical diagram of the matrix-boundary diffusion layer model. The rate of diffusion across a plane of unit area is given by Fick's law,

$$G = -D\frac{dC}{dx} \qquad \text{(Eq. 1)}$$

where G is the rate of diffusion across the plane, dC/dx is the concentration gradient, and D is the diffusion coefficient. The amount depleted from the matrix per unit time per unit area becomes

$$\frac{dQ}{dt} = -G = \frac{D_e}{l}(C_s - C_s') \qquad \text{(Eq. 2)}$$

where C_s and D_e are the solubility and effective diffusion coefficient, respectively, in the matrix phase, C_s' is the concentration in the matrix at $x = 0$, and l is the diffusional distance (zone of depletion). The effective diffusion coefficient is given by

$$D_e = \frac{D_s\epsilon}{\tau} \qquad \text{(Eq. 3)}$$

where ϵ is the volume fraction of the matrix, τ is the tortuosity of the matrix, and D_s is the diffusion coefficient in the matrix phase. It follows that the rate of diffusion across the diffusion boundary layer (h_a) is given by

$$\frac{dQ}{dt} = \frac{D_a}{h_a}[C_a' - C_B(t)] \qquad \text{(Eq. 4)}$$

where D_a is the diffusion coefficient in the aqueous phase, C_a' the concentration in water at $x = 0$, and C_B the concentration at $x = h_a$. Equating Eqs. 2 and 4, under steady-state conditions, utilizing the relationship

$$K = \frac{C_a}{C_s} = \frac{C_a'}{C_s'} \qquad \text{(Eq. 5)}$$

where C_a is the solubility in the aqueous phase, and K is the partition coefficient, and solving for C_s', the rate becomes

$$\frac{dQ}{dt} = \frac{D_e}{l}\left[C_s - \frac{C_sD_eh_a + D_alC_B(t)}{KD_al + D_eh_a}\right] \qquad \text{(Eq. 6)}$$

For the condition $A >> C_s$, where A is the concentration of drug in

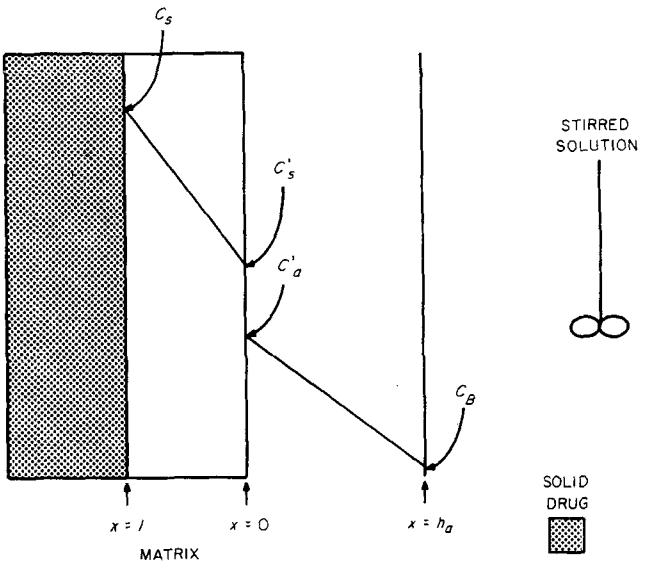


Figure 2—*Hypothetical diagram for the matrix-boundary diffusion layer model.*

[3] Cole Parmer, Chicago, Ill.
[4] Packard Tri-Carb, Packard Instrument Co.

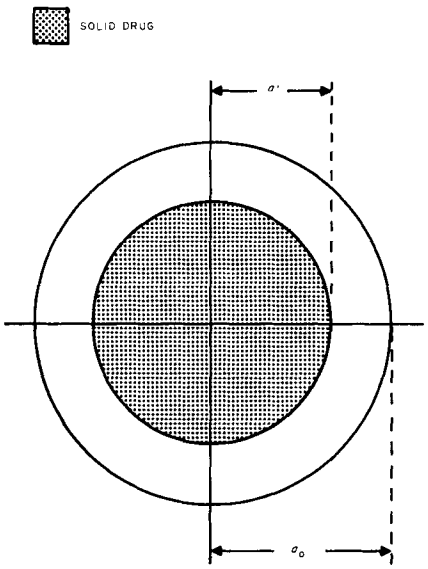


Figure 3—*Schematic diagram of a cross-sectional view of a cylinder where* $l = a_0 - a'$.

the matrix, the rate of release per unit area is

$$\frac{dQ}{dt} = A\frac{dl}{dt} \qquad \text{(Eq. 7)}$$

Equating Eqs. 5 and 6 and simplifying give

$$\left(Kl + \frac{D_e}{D_a}h_a\right)\int_0^l Adl = D_eC_sK\int_0^t dt + D_e\int_0^t C_B(t)dt \qquad \text{(Eq. 8)}$$

Integrating and setting $C_B = 0$ ("perfect sink" condition) the above reduces to

$$l^2 + \frac{2D_eh_al}{KD_a} = \frac{2D_eC_st}{A} \qquad \text{(Eq. 9)}$$

Since

$$Q = Al \qquad \text{(Eq. 10)}$$

Eqs. 9 and 10 define the Q *versus* t plots. When $l^2 >> 2D_eh_al/KD_a$, Eq. 9 reduces to the matrix-controlled process,

$$l^2 = \frac{2D_eC_st}{A} \qquad \text{(Eq. 11)}$$

It follows that

$$Q = (2AD_eC_st)^{1/2} \qquad \text{(Eq. 12)}$$

Equations 11 and 12 have been derived previously (6).

Cylindrical Case—The equations describing the release of a drug from a cylindrical matrix can be derived using the basic relationships that exist in the planar system. The amount (Q') depleted per unit time is

$$\frac{dQ'}{dt} = -2\pi hD_e a\frac{dC}{da} \qquad \text{(Eq. 13)}$$

where h is the height of the cylinder and a is the radius of the area under consideration. All other symbols have been defined previously. Since, according to Fig. 3, $C = C_s$ at $a = a'$ and $C = C_s'$ at $a = a_0$, integration of Eq. 13 yields

$$\frac{dQ'}{dt} = \frac{-2\pi hD_e(C_s' - C_s)}{\ln\dfrac{a'}{a_0}} \qquad \text{(Eq. 14)}$$

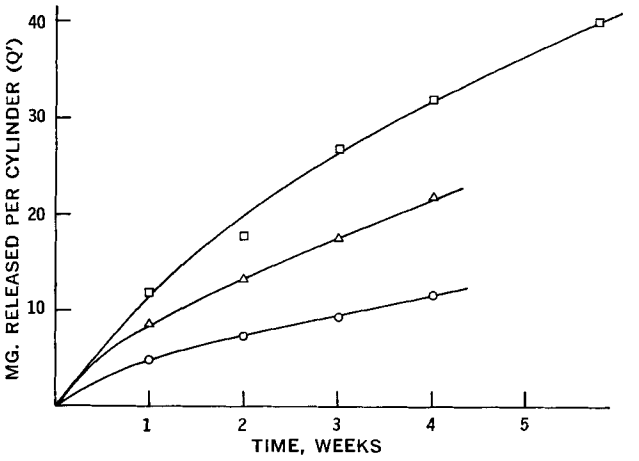


Figure 4—*Total amount of medroxyprogesterone acetate released from silicone cylinders as a function of time for three concentrations. Key:* ○ = 3.0%; △ = 12.0%; □ = 24.0%.

Assuming $C_B = 0$, the rate of diffusion from the surface is given by

$$\frac{dQ'}{dt} = \frac{2\pi ha_0D_a}{h_a}C_a' \qquad \text{(Eq. 15)}$$

After equating Eqs. 14 and 15, substituting Eq. 5, solving for C_s', and rearranging, the rate becomes

$$\frac{dQ'}{dt} = \frac{2\pi ha_0KD_a}{h_a}\left(\frac{D_eC_sh_a}{-KD_aa_0\ln\dfrac{a'}{a_0} + D_eh_a}\right) \qquad \text{(Eq. 16)}$$

For $A >> C_s$

$$\frac{dQ'}{dt} = -2\pi hAa'\frac{da'}{dt} \qquad \text{(Eq. 17)}$$

Equating Eqs. 16 and 17:

$$\int_{a_0}^{a'}\left(KD_aa_0\ln\frac{a'}{a_0} - D_eh_a\right)a'da' = \frac{KD_eC_sD_aa_0}{A}\int_0^t dt \qquad \text{(Eq. 18)}$$

Integration and substitution yield

$$\frac{a'^2}{2}\ln\frac{a'}{a_0} + \frac{1}{4}(a_0^2 - a'^2) + \frac{D_eh_a}{2KD_aa_0}(a_0^2 - a'^2) = \frac{C_sD_et}{A} \qquad \text{(Eq. 19)}$$

For the matrix-controlled system, this reduces to

$$\frac{a'^2}{2}\ln\frac{a'}{a_0} + \frac{1}{4}(a_0^2 - a'^2) = \frac{C_sD_et}{A} \qquad \text{(Eq. 20)}$$

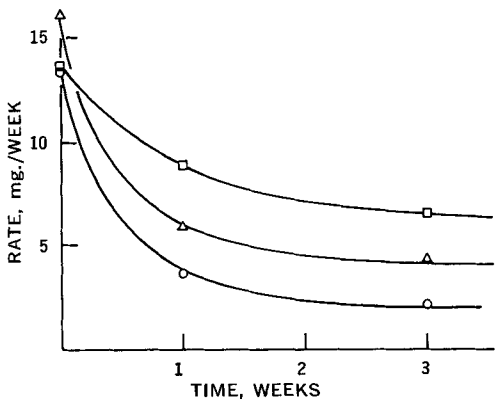


Figure 5—*Rate as a function of time for three medroxyprogesterone acetate concentrations in silicone cylinders. Key:* ○ = 3.0%; △ = 12.0%; □ = 24.0%.

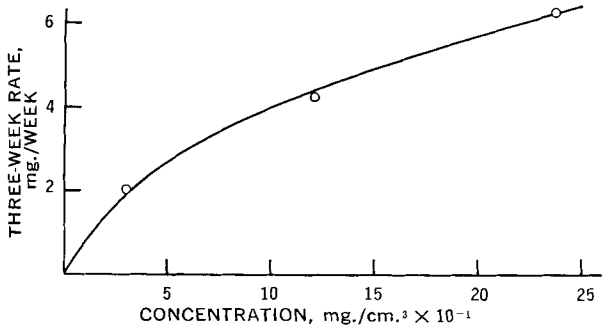


Figure 6—*Three-week release rate from silicone cylinders* versus *medroxyprogesterone acetate concentration.*

Since

$$Q' = \pi h A (a_0^2 - a'^2) \qquad \text{(Eq. 21)}$$

Eqs. 19 or 20 and 21 define the Q' *versus* t plots.

RESULTS AND DISCUSSION

Figures 4 and 5 show the amount of medroxyprogesterone acetate released (Q') and the rate of release respectively as a function of time for the silicone cylinders at three medroxyprogesterone acetate concentrations. It is interesting to note that the initial rates (Fig. 5) are relatively constant while at later times there is a nonlinear dependence of rate upon concentration. Figure 6 shows this dependence at 3 weeks. As the medroxyprogesterone acetate diffuses from the matrix, rather well-defined zones of depletion develop. An example of the zones at various times is illustrated in Fig. 7 for a transparent silicone material. Initially, the transparent material is rendered opaque by the presence of medroxyprogesterone acetate. As the medroxyprogesterone acetate diffuses from the matrix, clear zones result. The longer release times show the larger zones. The unique advantage of this system is that the zones can be measured directly. These then can be compared to theoretical values. The value of Q' can be evaluated from Eq. 21 as long as $A >> C_s$.

Although Eq. 20 was not reported by Higuchi (6), its derivation follows from the other geometric cases which were presented for the release from a homogeneous matrix. Equation 19 represents an extension of Eq. 20 which includes the aqueous boundary diffusion layer. To compare the theories, the following values were assigned:

C_a — The solubility of medroxyprogesterone acetate in water at 37° is 3.25×10^{-3} mg./ml.

D_a — The diffusion coefficient in water was determined from the Sutherland-Einstein equation (8). The calculated value was 6.54×10^{-6} cm.²/sec.

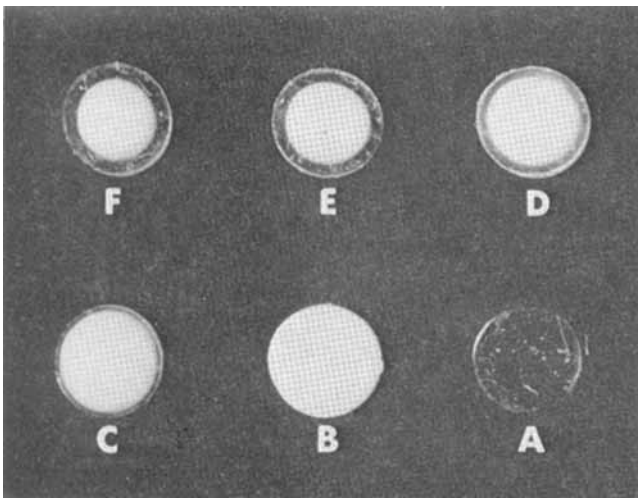


Figure 7—*Cross-sectional views of silicone (transparent) cylinders. Key:* A = *placebo;* B = *drug-filled initial;* C = *1 week;* D = *2 weeks;* E = *3 weeks;* F = *4 weeks.*

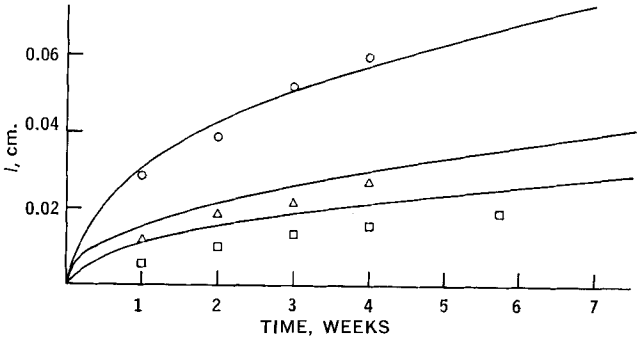


Figure 8—*Thickness of depletion zone as a function of time for medroxyprogesterone acetate-silicone cylinders. Curves drawn are based upon theoretical calculations for the matrix-controlled model. The symbols represent experimental data. Key:* ○ = *3.0%;* △ = *12.0%;* □ = *24.0%.*

h_a — The thickness of the diffusion layer, calculated from the expression $h_a = D_a C_a / G_i$ where G_i is the initial rate, was 66.8×10^{-4} cm.

K — The value of K was determined to be 0.033 ± 0.003.

The values of ϵ, volume fraction of the polymer, and τ, tortuosity, have not been given since these are incorporated into an effective diffusion coefficient. Due to the presence of vacuoles, drug, and filler, the value of ϵ will be less than one. The tortuosity factor accounts for indirect pathways that may result from presence of filler within the matrix.

Since $l = a_0 - a'$, Eqs. 19 and 20 were compared considering l as the variable. The continuous lines in Fig. 8 show l as a function of time as calculated from Eq. 20, for the matrix-controlled case, at three different medroxyprogesterone acetate concentrations. The value of D_e was chosen to be 2.0×10^{-7} cm.²/sec. to fit the data for $A = 30$ mg./cm.³ The symbols represent the experimental points. A similar plot is given in Fig. 9, the l values being calculated from Eq. 19, for the matrix-boundary diffusion layer model. In this case D_e was chosen to be 2.6×10^{-7} cm.²/sec. to fit the data for $A = 30$ mg./cm.³ Comparison of Figs. 8 and 9 indicates that the matrix-boundary diffusion layer model gives a better fit of theory with data. If Eq. 19 represents the correct model, then an increase in h_a should increase the time to reach a given l distance. Since h_a is dependent on hydrodynamic flow, a decrease in stirring rate should result in a higher h_a value. The effect of stirring for $A = 30$ mg./cm.³ is illustrated in Fig. 10. The corresponding h_a value, calculated from initial rate data for the 70 r.p.m. experiment, is 146×10^{-4} cm. Inserting this value back into Eq. 19 and recalculating l as a function of time resulted in good agreement of theory with experimentally measured l values. Further support of the matrix-boundary diffusion

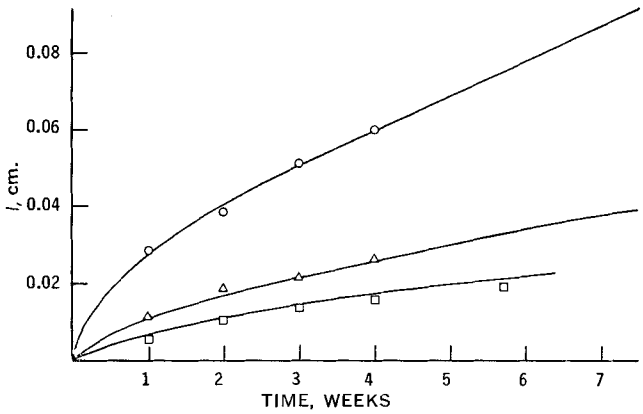


Figure 9—*Thickness of depletion zone as a function of time for medroxyprogesterone acetate-silicone cylinders. Curves drawn are based upon theoretical calculations for the matrix-boundary diffusion model. The symbols represent experimental data. Key:* ○ = *3.0%;* △ = *12.0%;* □ = *24.0%.*

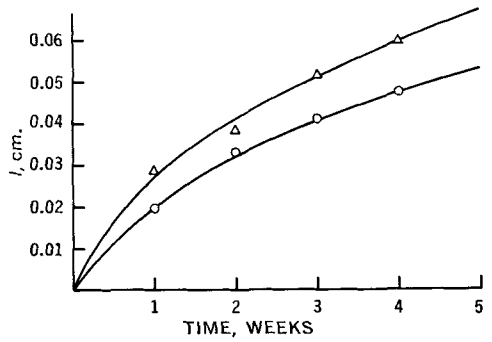


Figure 10—*Thickness of depletion zone as a function of time for 3.0% medroxyprogesterone acetate-silicone cylinders. Key: △ = 700 r.p.m.; ○ = 70 r.p.m.*

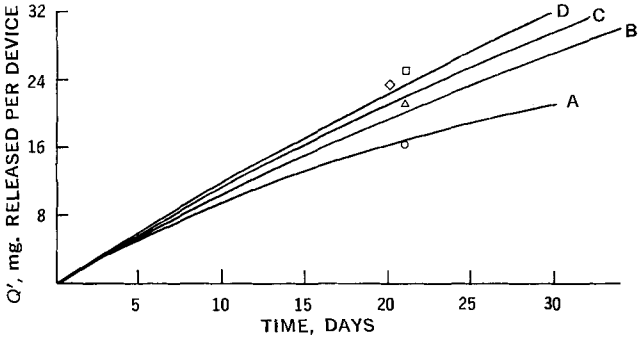


Figure 11—*Release profiles for medroxyprogesterone acetate-silicone devices. Curves are theoretical while symbols are* in vivo *data. Key: ○ = 10 mg./cm.³–A; △ = 20 mg./cm.³–B; □ = 40 mg./cm.³–C; ◇ = 80 mg./cm.³–D.*

layer model stems from an examination of initial rate data. For a purely matrix-controlled system, initial steady-state rates would be dependent upon $(A)^{1/2}$.[5] Figure 5 shows that this is clearly not the case. The initial rates are relatively constant.

Although it appears that Eq. 19 is operating, it would be of interest to determine the limits of its applicability. Setting $\alpha = D_e C_s h_a / D_a C_a$ for the planar case Eq. 9 (recalling that $K = C_a/C_s$), it can be shown that when $\alpha \ll l$, variation in stirring rates would not affect the release rates, since the release of the drug would be matrix controlled. For drugs with high values for α, *i.e.*, $\alpha \cong l$, the effects of agitation would be significant, and therefore the matrix-boundary diffusion layer model would apply. It can be seen that the values of C_s, D_e, C_a, and D_a determine which model is operating. An analogous situation exists in the area of the kinetics of dyeing (9). It would be expected that compounds of similar molecular size and weight would exhibit relatively constant D_e/D_a values. Since the C_s/C_a (K) ratios are more sensitive to molecular structure, they can vary quite markedly (10). This would suggest that the partition coefficient can substantially influence the drug release mechanism. It follows then that compounds which have relatively small K values would follow the matrix-boundary diffusion layer model, while those with large values would be solely matrix controlled. Future studies will be designed to explore the relationship of K to the release mechanism.

Analyses of *in vivo* data from medroxyprogesterone acetate-silicone vaginal devices suggest that the amount released per time per unit area is considerably less than the *in vitro* system. If it is assumed that the contributing factor[6] to these results is a larger aqueous diffusion layer, then the *in vivo* data can be compared to theory by utilizing Eq. 19. Based upon the data, h_a can be estimated at 580×10^{-4} cm. This value can now be inserted back into Eq. 19 and utilizing Eq. 21, a theoretical plot of Q' *versus* t can be obtained, Fig. 11. All other parameters have been previously defined. The plot is made for four concentrations: 10 mg./cm.³, 20 mg./cm.³, 40 mg./cm.³, and 80 mg./cm.³. The symbols in Fig. 11 represent the *in vivo* data, determined by residual analyses of the device for its drug content. Considering the assumptions which were made, the agreement of theory with data is acceptable. It is interesting to note that the curves are initially similar while at 21 days the lower concentration gives a substantially lower dose of drug. It is apparent that the effect of concentration is relatively minor until the zones of depletion are sufficiently large.

CONCLUSIONS

A model system was presented for the release of a water-insoluble steroid, medroxyprogesterone acetate, embedded in a solid silicone rubber. The experimental findings were not totally consistent with concepts already set forth for matrix systems. Therefore, a model was developed which considered the boundary diffusion layer. In this instance, equations were consistent with the experimental results. The amount of drug present within the matrix (A), the diffusion coefficients in the solid and aqueous phases, and the partition coefficient (K), were included. The dependence of the amount released (Q') upon A was in agreement with the values calculated from theory. It was suggested that the partition coefficient strongly influenced the release mechanism.

The applicability of the model to an *in vivo* system was also evaluated. Based on the assumption that the slower release observed *in vivo* was due to a larger boundary diffusion layer, plots were made for the theoretical *in vivo* release at four drug levels within the matrix. Except for the thickness of the diffusion layer, all parameters were the same as the *in vitro* system. The amount of medroxyprogesterone acetate lost after 3 weeks from vaginal devices was qualitatively in agreement with the theoretical values.

REFERENCES

(1) Technical background on B. F. Goodrich "No-Foul" Antifouling Rubber, B. F. Goodrich Aerospace and Defense Products, Oct. 5, 1967.

(2) J. Folkman and V. H. Mark, "Transactions New York Academy of Sciences," Division of Instrumentation, 1968, p. 1187.

(3) D. R. Mishell, Jr., A. F. Parlow, and D. L. Moyer, oral presentation, American Association of Planned Parenthood Physicians, San Francisco, Calif., Apr. 7–9, 1969.

(4) K. Sundaram and F. A. Kincl, *Steroids*, **12**, 517(1968).

(5) E. R. Garrett and P. B. Chemburkar, *J. Pharm. Sci.*, **57**, 1401(1968).

(6) T. Higuchi, *ibid.*, **52**, 1145(1963).

(7) "United States Pharmacopeia" 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 360.

(8) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 525.

(9) J. Crank and G. S. Park, "Diffusion in Polymers," Academic, New York, N. Y., 1968, Chapter 9.

(10) T. Higuchi, *J. Soc. Cosmetic Chemists*, **11**, 85(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 8, 1969, from the *Research Laboratories, The Upjohn Co., Kalamazoo, MI 49001*

Accepted for publication October 9, 1969.

The authors gratefully acknowledge the laboratory assistance of Mr. John Dykehouse and Mr. Glenn Bumgardner. Thanks are also given to Dr. B. B. Pharriss for his assistance in the radioactive study.

* The Upjohn Co., Kalamazoo, MI 49001

† College of Pharmacy, The University of Michigan, Ann Arbor, MI 48104

[5] This can be derived from Eq. 12. It can also be shown that up to 50% release the plane surface is a good approximation for the cylindrical surface.

[6] Other factors such as the formation of an interfacial layer or lack of "perfect sink" conditions could also be important.

Pharmacokinetic Profile of Trimethoprim in Dog and Man

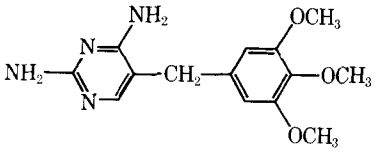
S. A. KAPLAN, R. E. WEINFELD, S. COTLER, C. W. ABRUZZO, and K. ALEXANDER

Abstract □ A pharmacokinetic profile of trimethoprim was determined in man and in the dog. The dog was shown to absorb the drug completely, and distribute the drug into the tissues to a great extent. The low recovery of intact trimethoprim in the urine is indicative of biotransformation and/or alternate routes of excretion. The drug is eliminated in man with a half-life of 15–17 hr. In addition, steady-state blood levels were maintained on daily oral dosing over a 13-week period. The dog was shown to eliminate trimethoprim 4 to 5 times faster than man.

Keyphrases □ Trimethoprim—pharmacokinetic profile □ Pharmacokinetics, trimethoprim—man, dog □ Absorption, distribution—trimethoprim □ Fluorometry—analysis

Trimethoprim is an inhibitor of dihydrofolate reductase which potentiates the activity of sulfonamides against a wide variety of bacterial species (1,2).

Chemically, trimethoprim is 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine with the following structural formula (Structure I):



Structure I

It is a weak difunctional base with both basic groups titrating almost simultaneously with a pKa of 7.2 (3).

This study was designed to elucidate the physiological disposition of trimethoprim in the dog following i.v. and oral administration and in man following single and chronic oral administration.

EXPERIMENTAL

***In Vivo* Dog Study**—A 5.7-mg./kg. dose of trimethoprim was administered intravenously and orally 2 weeks apart to two different male dogs. Five-milliliter oxalated blood specimens were collected at the time intervals indicated in Table I. The total volumes of urine voided were collected at 24-hr. intervals from 0 to 48 hr. following i.v. administration and from 0 to 72 hr. following oral administration. All specimens were frozen for subsequent analyses.

Human Study—Clinical studies were conducted in two human female subjects.[1] Each subject was given a single oral 200-mg. dose of trimethoprim on Day 1. Repeated oral doses of 50 mg. q.i.d. at 7:00 a.m., 12 noon, 5:00 p.m., and 10:00 p.m. commenced on Day 8 and continued through the 7:00 a.m. dose of Day 22 of the study for Subject R. R. Subject M. H. began the repeated oral dosing of 50 mg. q.i.d. on Day 5 and continued only through the first dose of Day 8.

The dosing, blood, and urine sampling schedules are presented in Table II. Five-milliliter oxalated blood specimens were obtained, the total volume of urine voided was measured, and 50-ml. aliquots were frozen for subsequent analyses.

Table I—Blood and Urinary Excretion Trimethoprim Levels in Two Dogs following the i.v. and Oral Administration of 5.7 mg./kg.

Blood Levels					
i.v., mcg./ml.			Oral, mcg./ml.		
Time	Dog 1	Dog 2	Time	Dog 1	Dog 2
1 min.	5.0	6.6	20 min.	Nil	Nil
2.5	4.7	5.4	40	1.8	0.10
5	3.7	4.6	1 hr.	2.8	0.13
10	3.6	4.5	1.5	2.7	0.18
20	3.0	3.7	2	2.7	1.3
40	2.8	3.4	3	2.1	2.3
1 hr.	2.4	2.8	4	1.8	1.9
1.5	2.2	2.4	6	1.4	1.3
2	1.7	1.9	7.5	1.0	—
3	1.6	1.5	8.0	—	0.8
4	1.3	1.1	11.5	0.3	—
6	1.0	0.7	13	—	0.2
7.5	0.8	0.4	23	Nil	Nil
11	—	0.2	48	Nil	Nil
12	0.3	—	72	Nil	Nil
24	Nil[a]	Nil			
48	Nil	Nil			
55	Nil	—			

Urinary Excretion of Intact Trimethoprim

i.v.			Oral		
Time Interval, hr.	Cum. % of Dose Dog 1	Dog 2	Time Interval, hr.	Cum. % of Dose Dog 1	Dog 2
0–24	16.5	28.4	0–24	11.7	29.9
24–48	18.8	29.0	24–48	14.2	31.9
			48–72	14.5	31.9

[a] Nil is less than 0.1 mcg./ml.

In a separate study, a group of 13 healthy adult male volunteers[2] each received a dose of 50 mg. trimethoprim q.i.d. for 13 weeks. Blood specimens were obtained before and once weekly during drug administration, always at the same time of day with regard to drug administration, just prior to the first dose in the morning, and were frozen for subsequent analysis.

Analytical Methods—Trimethoprim was determined in the blood and urine by a specific spectrofluorometric procedure (4) with a sensitivity of 0.1 mcg./ml.

One-milliliter blood or urine (1 to 10 dilution) specimens were placed in 50-ml. glass-stoppered centrifuge tubes. The specimens were diluted with 7 ml. distilled water and 1 ml. of 1 *N* aqueous sodium carbonate solution. Ten milliliters of chloroform (reagent grade) was added, the tubes stoppered, and extracted gently for 10 min. on a reciprocating shaker. The stoppers were removed and the specimens were then centrifuged at 2000 r.p.m. for 10 min. The aqueous phase was aspirated and the chloroform washed with 5 ml. distilled water. The water was aspirated and an 8-ml. aliquot of the chloroform phase was transferred into a 15-ml. glass-stoppered centrifuge tube. Four milliliters of 0.01 *N* sulfuric acid was then added and the sample extracted by shaking and the phases separated by centrifuging for 10 min. each. A 3-ml. aliquot of the acid phase was placed in a fresh 15-ml. glass-stoppered centrifuge tube to which 1 ml. of a 0.1 *M* potassium permanganate solution in 0.1 *N* sodium hydroxide was added. The solution was mixed, and the test tube was stoppered and heated in a 60° water bath for 20 min. The tubes were cooled to room temperature and 0.1 ml. of a 37% formaldehyde solution (reagent grade) was added. The solution was mixed

[1] The Hoffmann-La Roche Special Treatment Units: one subject at the Newark Beth Israel Hospital under the supervision of Dr. A. Leon and the other subject at Martland Hospital under the direction of Dr. H. Solomon.

[2] The Experimental Therapeutics Unit of Oklahoma State Penitentiary, under the supervision of Dr. J. P. Colmore.

Table II—Trimethoprim Dosing Schedule and Blood Levels in Two Human Subjects Following Single and Chronic Dosing Administration

Day of Study	Hour	Blood Level, mcg./ml. R. R. Dose	R. R. Blood Level	M. H. Dose	M. H. Blood Level
1	0	200 mg.	—[a]	200 mg.	—
	2		2.0		1.4
	4		2.1		1.8
	6		2.0		1.3
	8		1.8		1.2
	12		1.3		1.0
	15		0.9		1.0
2	24		0.7		0.7
	30		0.6		0.7
	36		0.4		0.3
3	48		0.3		0.3
4	72		Nil[b]		Nil
5	96		—	50 mg.	Nil
	98		—	q.i.d.	0.4
	100		—		0.4
	102		—		0.5
	104		—		0.8
	108		—		1.2
6	120		—		1.5
	126		—		1.8
	132		—		1.6
7	144		—		1.5
	156		—		1.8
8	168	50 mg[c]	—		1.6
	170	q.i.d.	0.6		1.9
	172		0.6		1.3
	174		1.2		1.0
	176		1.1		0.9
	178		—		0.9
	180		1.1		0.7
	183		1.4		—
9	192		1.5		—
	198		1.8		—
	204		2.5		—
10	216		2.2		—
11	240		2.3		—
12	264		2.2		—
13	288		2.2		—
14	312		2.2		—
16	360		1.6		—
18	408		2.2		—
21	480		1.8		—
	492		—		—
	495		2.1		—
22	504		—		—
	506		2.5		—
	508		2.8		—
	510		2.3		—
	512		2.1		—
	514	50 mg.	1.8		—
	516		2.3		—
	519	50 mg.	2.2		—
23	528		2.2		—
	534		1.6		—
	540		1.2		—
24	552		0.7		—
25	576		0.3		—
	580		—		—
26	600		Nil		—

[a] —indicates no specimen taken. [b] Nil is below 0.1 mcg./ml. [c] q.i.d. at 7:00 a.m., 12 noon, 5:00 p.m., and 10:00 p.m.

and allowed to stand for 5 min. The solution was acidified by the addition of 1 ml. of 1 *N* sulfuric acid and extracted with 3 ml. of chloroform by shaking, and the phases were separated by centrifuging for 10 min. each. The aqueous phase was aspirated and the chloroform phase transferred to a small test tube.

The fluorescence of the chloroform extract was measured in a 1-cm. path. cell at 345 mμ in a Farrand spectrofluorometer (MK-1) with activation at 295 mμ. Duplicate control specimens of blood or urine and control specimens to which 5 and 10 mcg. of trimethoprim were added as internal standards were carried through the entire procedure. The concentrations in the unknown specimens were calculated on the basis of the internal standard after correction for the control values.

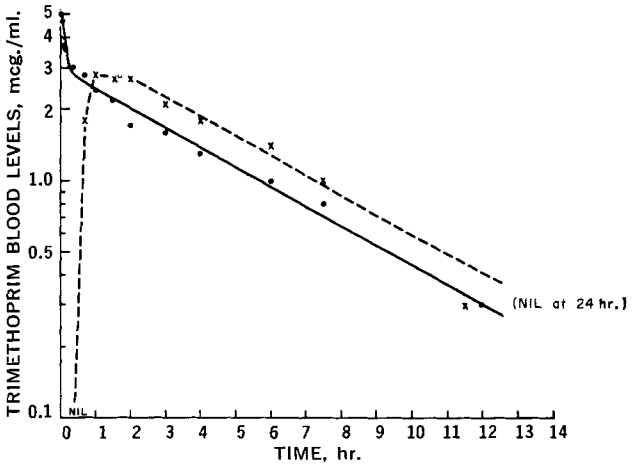


Figure 1—*Trimethoprim blood levels in Dog 1 following i.v. and oral administration. Key: 5.7 mg./kg. trimethoprim to Dog 1:* ●—●, *i.v.; and* ×—×, *oral.*

RESULTS AND DISCUSSION

The dog blood level and urinary excretion data following i.v. and oral administration of trimethoprim are presented in Table I and Figs. 1 and 2. The pharmacokinetic profile of trimethoprim in the dog is summarized in Table III.

The human blood level data of the two subjects receiving both single and chronic administrations of the drug are presented in Table II and Figs. 3 and 4. The corresponding urinary excretion data are presented in Tables IV and V and Fig. 5. The pharmacokinetic profile following the single and chronic oral dosing of trimethoprim is summarized in Table VI. The blood level data of the subjects receiving trimethoprim daily over a 13-week period are presented in Table VII.

Theoretical Considerations—Following the intravenous injection of trimethoprim to two dogs, the resulting blood level curves could be adequately described by the biexponential equations as shown

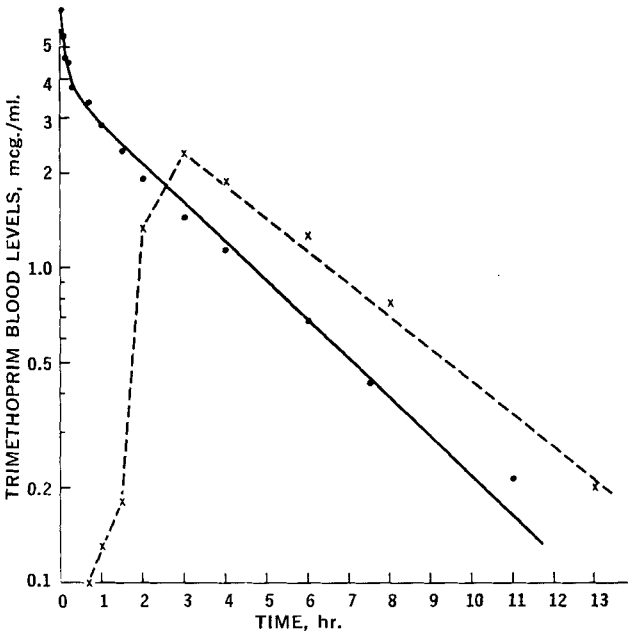


Figure 2—*Trimethoprim blood levels in Dog 2 following i.v. and oral administration. Key: 5.7 mg./kg. trimethoprim to Dog 2:* ●—●, *i.v.; and* ×—×, *oral.*

Table III—Pharmacokinetic Parameters Describing the Physiological Disposition of a 5.7-mg./kg. Dose of Trimethoprim in Two Dogs in Terms of a Two-Compartment Open-System Model

General Equation: $C_p = Ae^{-\alpha t} + Be^{-\beta t}$

Following i.v. Administration	Dog 1	Dog 2
A, mcg./ml.	2.3	3.6
B, mcg./ml.	2.9	3.6
α, hr.$^{-1}$	6.6	13.6
0.693[a]/α, hr.	0.105	0.51
β, hr.$^{-1}$	0.187	0.281
0.693/β, hr.	3.71	2.46
$C_p^0 = A + B$, mcg./ml.	5.2	7.2
Rate Constants		
k_{13}, hr.$^{-1}$	0.327	0.555
0.693/k_{13}, hr.	2.1	1.2
k_{12}, hr.$^{-1}$	2.69	6.46
0.693/k_{12}, hr.	0.26	0.11
k_{21}, hr.$^{-1}$	3.78	6.91
0.693/k_{21}, hr.	0.18	0.10
Volume of Distribution		
V_p, volume of central compartment, l.	8.5	10.9
$(V_D)_{ss}$, total volume of distribution, l.	14.5	21.0
$(V_D)_\beta$, total volume of distribution, l.	14.8	21.5
Total volume as percent of body weight, %	184	154
Following Oral Administration		
β, apparent elimination rate, hr.$^{-1}$	0.224	0.245
0.693/β, hr.	3.1	2.8
Ratio of areas under oral/i.v. blood level curves	1.12	0.94
Estimated percent of dose absorbed[b]	113	112
Absorption Characteristics (Percent of dose absorbed with time)	60% in 40 min. 85% in 1 hr. 100% in 2 hr.	Initial lag period Then 50% in 2 hr. 100% in 3 hr.

Urinary Excretion

Time, hr.	Cumulative Percent of Dose Excreted as Intact Trimethoprim			
	i.v.	Oral	i.v.	Oral
0–24	16.5	11.7	28.4	29.9
24–28	18.8	14.2	29.0	31.8
48–72	—	14.5	—	31.9

[a] 0.693/constant = half-life. [b] Calculated from absorption rate equation (6).

in Figs. 1 and 2. The first portion of the biexponential curve will be referred to as the fast disposition rate, with a rate constant α. The second portion of the curve will be referred to as the slow disposition rate, with a rate constant β. It should be noted that α and β are both hybrid rate constants, each influenced by all the individual processes of the disposition of the drug. The rate constant α reflects the distribution phase of the compound from the central to the peripheral (tissue) compartment. The rate constant β reflects the elimination rate of the drug from the body. The physiological

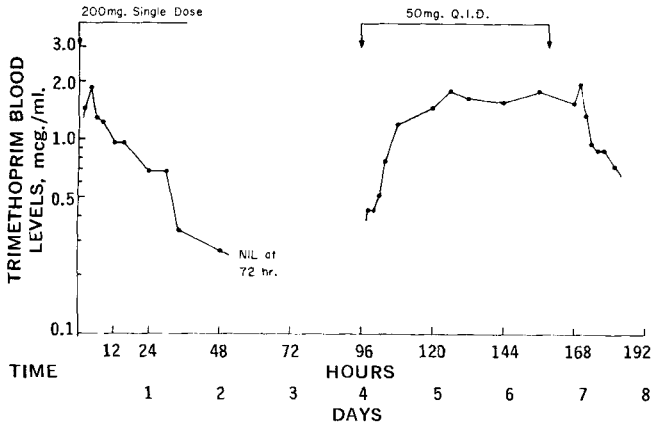


Figure 3—*Trimethoprim blood levels in Subject M. H. following single and chronic oral administration.*

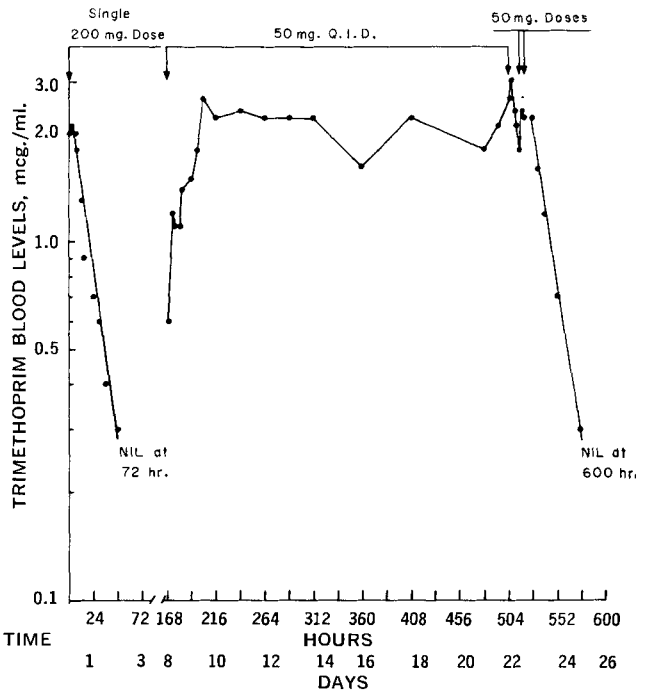
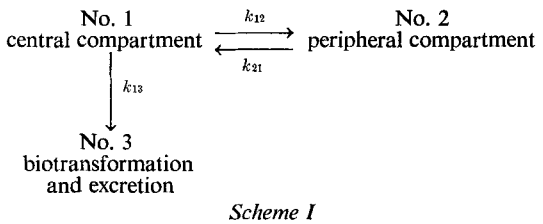


Figure 4—*Trimethoprim blood levels in subject R. R. following single and chronic oral administration.*

Table IV—Urinary Excretion Levels of Intact Trimethoprim in Subject R. R.

Day	Dosing Schedule	Hour Interval	Total mg. Excreted	Percent of Dose Excreted as Intact Drug
1	200-mg. single dose	0–2	0.1	
		2–4	3.5	
		4–6	4.2	
		6–8	8.4	
		8–12	5.1	
		12–15	8.5	
		15–24	12.7	21.3 in 24 hr.
2		24–30	4.1	
		30–36	5.0	
		36–48	12.8	
3		48–72	8.5	
4		72–96	2.2	
5		96–120	0.9	
6		120–144	Nil	
7		144–168	Nil	38.0 of single 200-mg. dose
8	50 mg. q.i.d.	168–170	0.6	
		170–172	1.9	
		172–174	—	
		174–176	4.3	
		176–180	5.9	
		180–183	8.1	
		183–192	14.7	17.8 in 24 hr.
9		192–198	10.7	
		198–204	9.7	
		204–216	31.0	20.4 in 24 hr.
10		216–240	67.0	33.5 in 24 yr.
11		240–264	78.0	39.0 in 24 hr.
12		264–288	63.3	31.7 in 24 hr.
13		288–312	57.3	28.7 in 24 hr.
14		312–336	90.1	45.1 in 24 hr.
15		336–360	69.1	34.6 in 24 hr.
17		384–408	63.0	31.5 in 24 hr.
20		456–480	55.4	27.7 in 24 hr.
22		504–506	4.1	
		506–508	6.0	
		508–510	5.3	
		510–514	7.3	
	50 mg.	514–516	4.3	
		516–519	7.3	
	50 mg.	519–528	22.8	
23		528–534	11.1	
		534–540	10.7	
		540–552	36.7	
24		552–576	23.5	
25		576–600	10.4	
26		600–624	3.1	

disposition of trimethoprim may therefore be defined in terms of the two-compartment open-system model shown in Scheme I (5):



Scheme I

Solution of the differential equations resulting from such a model yields the following integrated equation describing the blood level-time curve after a single intravenous injection as seen in Figs. 1 and 2:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_p is the concentration of drug in the plasma, and A and B are the ordinate axis intercepts.

The α and β are both hybrid rate constants reflecting all the individual rate processes; however, k_{12}, k_{21}, and k_{13} are individual rate constants calculable from this equation (5). The k_{12} and k_{21} are first-order rate constants of distribution and k_{13} is the sum of the simultaneous processes of biotransformation and excretion, all assumed to be first-order processes. This is the rate constant of the elimination reaction *per se*.

The absorption rate is calculated from the following relationship (6):

$$\left(\frac{A}{V_p}\right)_{tn} = C_{p_{tn}} + k_{13} \int_{t_0}^{tn} C_p\,dt + C_{T_{tn}}$$

which indicates that the amount absorbed per unit volume of distribution (A/V_p) at time tn equals the plasma level $(C_p)_{tn}$ plus the tissue level $(C_T)_{tn}$ plus the amount eliminated $(k_{13} \int_{t_0}^{tn} C_p\,dt)$ at time tn.

In a two-compartment open-system model the total volume of distribution of the entire system $(V_D)_{ss}$, when calculated with reference to the concentration of drug in the central compartment, is the sum of the volume of the central compartment, V_p, and of the peripheral (tissue) compartment, V_T (7), where V_p = administered dose/$A + B$ and $(V_D)_{ss} = V_p\,(k_{12} + k_{21})/(k_{21})$.

The volume of distribution can also be calculated using the equation as presented by Gibaldi *et al.* (8) and is referred to as $(V_D)_\beta$.

In this case, $(V_D)_\beta = V_p/f$ where: $f = C_2/(C_2 + C_2^1)$, $C_2 = B/(A + B)$, and $C_2^1 = k_{12}/(\alpha - \beta)$.

Physiological Disposition of Trimethoprim in the Dog—The pharmacokinetic parameters summarized in Table III indicate that trimethoprim has fast disposition rate constants, α, of 6.6 and 13.6 hr.$^{-1}$, respectively, in Dogs 1 and 2. The slow disposition rate constants, β, were 0.187 and 0.281 hr.$^{-1}$ corresponding to half-

Table V—Urinary Excretion Levels of Intact Trimethoprim in Subject M. H.

Day	Dosing Schedule	Hour Interval	Total mg. Excreted	Percent of Dose Excreted as Intact Drug
1	200-mg. single dose	0–2	1.0	
		2–4	2.9	
		4–6	3.3	
		6–8	1.7	
		8–12	6.2	
		12–15	2.2	
		15–24	10.7	14.0 in 24 hr.
2		24–30	4.9	
		30–36	2.7	
		36–48	8.3	
3		48–72	7.3	
4		72–96	Nil[a]	25.6 of single 200-mg. dose
5	50 mg. q.i.d.	96–98	0.09	
		98–100	0.09	
		100–102	0.3	
		102–104	2.9	
		104–108	2.7	
		108–120	12.0	9.0 in 24 hr.
6		120–126	4.0	
		126–132	6.4	
		132–144	20.7	15.6 in 24 hr.
7		144–168	48.1	24.1 in 24 hr.
8		168–170	4.0	
		170–172	1.1	
		172–174	3.1	
		174–176	4.2	
		176–180	7.4	
		180–183	2.6	
		183–192	8.2	

[a] Nil below 0.1 mcg./ml.

lives of 3.7 and 2.5 hrs, and reflect the apparent elimination rates of the drug from the body.

The volumes of the central compartment, V_p, were calculated to be 8.5 and 10.9 l., respectively. The total volumes of distribution, $(V_D)_{ss}$, of trimethoprim were calculated to be 184 and 154% of body weight in the two dogs, suggesting significant tissue uptake of trimethoprim. $(V_D)_\beta$ was calculated and found to be almost identical with the $(V_D)_{ss}$ calculation (Table III).

Only 18.8% of the intravenously administered dose was recovered as intact drug in the 0 to 48-hr. urine of Dog 1 and 29% in Dog 2, indicating biotransformation and/or alternate routes of excretion. Almost all the recovered trimethoprim was excreted in the urine during the 0 to 24-hr. interval.

The data indicate fairly rapid and complete absorption of the orally administered drug in the dog (Table III) with Dog 2 exhibiting a lag in the start of absorption. Absorption was essentially complete in 2 hr. in Dog 1 and in 3 hr. in Dog 2.

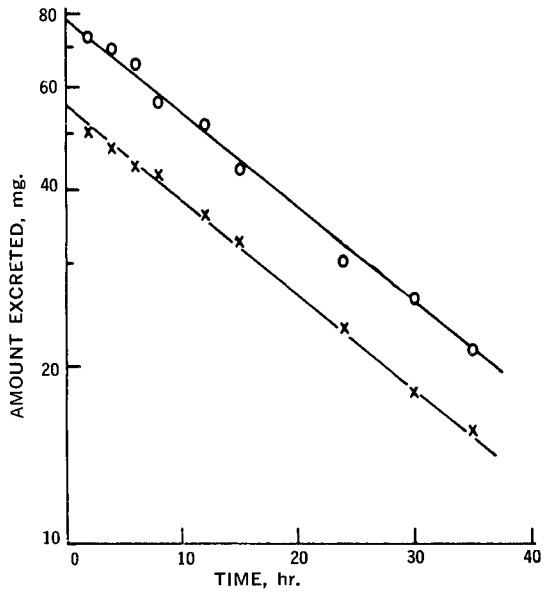


Figure 5—*Urinary excretion of intact trimethoprim in two human subjects. Key:* O—O, *R. R.; and* ×—×, *M. H.*

Physiological Disposition of Trimethoprim in Man—The pharmacokinetic profile following the administration of a single 200-mg. oral dose of trimethoprim to two subjects is presented in Table VI.

In relation to body weight the single doses represented 2.3 mg./kg. for M. H. and 3.4 mg./kg. for R. R.

In each case the blood level curves (Figs. 3 and 4) peaked at 4 hr. postadministration. Subjects R. R. and M. H. exhibited blood level peaks of 1.8 and 2.1 mcg./ml. and elimination rates of 4.0 and 4.6%/hr. corresponding to half-lives of 17.3 and 15 hr., respectively.[4]

Estimates of the volume of distribution following single oral

Table VI—Pharmacokinetic Evaluation of Trimethoprim in Two Human Subjects

	Subject	
	M. H.	R. R.
Single oral dose		
Dose, mg.	200	200
Weight, kg.	87.5	58.9
Dose, mg./kg.	2.29	3.40
Blood level peak		
mcg./ml.	1.84	2.1
Time, hr.	4	4
Elimination rate constant from bloodstream, $hr.^{-1}$	0.040	0.046
Corresponding half-life, hr.	17.3	15.0
Estimated % volume of distribution	158	177
Percent of dose recovered as intact drug in urine	25.6	36.4
Elimination rate constant from urinary excretion of intact trimethoprim, $hr.^{-1}$	0.036	0.037
Corresponding half-life	19.5	18.7
Chronic oral dose of 50 mg. q.i.d.		
Minimum steady-state blood level, mcg./ml.	1.5	2.2
Elimination rate constant following last dose, $hr.^{-1}$	—	0.047
Corresponding half-life, hr.		14.6

Table VII—Mean Trimethoprim Blood Levels[a] following the Daily Administration of 50 mg. q.i.d. to 13 Human Subjects for 13 Weeks

Subject No.	Wt., kg.	[a]Mean ± *SE*
1	86.4	0.867 ± 0.054
2	65.0	1.522 ± 0.047
3	75.9	1.839 ± 0.088
4	100.0	0.325 ± 0.033
5	63.6	1.348 ± 0.077
6	84.5	1.166 ± 0.094
7	99.1	0.558 ± 0.055
8	76.4	1.319 ± 0.099
9	76.8	0.972 ± 0.047
10	75.0	1.138 ± 0.064
11	70.9	0.721 ± 0.066
12	72.7	1.320 ± 0.099
13	70.5	1.420 ± 0.036

[a] Mean of 13 weekly determinations.

doses were made by means of the area equation (9) and are presented in Table VI. It should be noted that $(V_D)_{area}$ has been shown to be mathematically identical to the $(V_D)_\beta$ term previously defined (8):

$$(V_D)_{area} = \frac{\text{amount of drug absorbed}}{\beta(\text{area})}$$

where complete absorption of the dose is assumed, β is the apparent elimination rate constant, and the (area) is the area under a blood level curve from time = 0 to time = ∞. The percent volume of distribution for Subjects M. H. and R. R. were calculated to be 158 and 177% of body weight, indicative of tissue uptake and possible localization of the drug.

The percent of dose excreted as intact drug following oral administration of single 200-mg. doses as seen in Tables IV and V were 25.6 and 36.4% for Subjects M. H. and R. R., with urinary elimination rates of 3.6 and 3.7%/hr. corresponding to half-lives of 19.5 and 18.7 hr. (Fig. 5). The low recovery of the intact drug in the urine is an indication of biotransformation and/or alternate routes of excretion.

Twenty-four-hour urine specimens were collected during the chronic dosing period for the above two subjects and analyzed for intact trimethoprim. The results are presented in Tables IV and V. The daily intact trimethoprim urine levels ranged from 18 to 45% of the administered dose in Subject R. R. and from 9 to 20% in Subject M. H.

Following the chronic administration of 50 mg. trimethoprim q.i.d. (every 5 hr.) to Subjects M. H. and R. R., they exhibited minimum steady-state blood levels of approximately 1.5 and 2.2 mcg./ml., respectively, as indicated in Figs. 3 and 4.

It is interesting to note that the steady-state blood levels are achieved within 2–3 days of chronic administration and that the steady-state levels achieved on dosing 50 mg. q.i.d. (every 5 hr.) are approximately the same as the single dose, 200 mg., peak blood level. Estimated steady-state blood levels were calculated from the elimination rate data utilizing a q.i.d. dosing regimen of every 6 hr. based on the equations of Boxer *et al.* (10). The calculated steady-state minimum–maximum blood levels are 1.7–2.1 mcg./ml. for Subject M. H. and 2.0–2.6 mcg./ml. for Subject R. R. These simulated values are in excellent agreement with the experimental finding indicated above.

The trimethoprim blood levels reported in Table VII following the daily administration of 50 mg. q.i.d. for 13 weeks indicate that steady-state levels were evident by the end of the 1st week of treatment and maintained throughout the experimental period. This finding is substantiated by the small standard error about the mean blood level in each subject which is consistent with steady-state conditions. This would, therefore, preclude drug cumulation or enzyme induction by trimethoprim over a 13-week period.

The mean minimum steady-state trimethoprim blood level of the 13 subjects was 1.1 mcg./ml. with a range from 0.33 to 1.84 mcg./ml. It should be noted that the subjects with the lower blood levels represented those with the higher body weights. The minimum steady-state trimethoprim blood levels seen in the 13-subject study group corresponds with the steady-state blood levels seen in the two-subject study group. In view of these findings one may predict blood levels resulting from multiple dosing, based on pharmacokinetic data obtained from a single dose.

In attempting to compare the disposition profiles of trimethoprim in the dog and in man, it appears that both species absorb the drug well and distribute the drug extensively into the tissues. However, differences are discernible in the rates of elimination of trimethoprim in that the dog eliminated the drug 4–5 times faster than the human Subjects M. H. and R. R.

SUMMARY

1. Trimethoprim appears to be absorbed well and is highly distributed in both the dog and man.

2. In two human subjects the intact drug was eliminated from the bloodstream with half-lives of 15 and 17.3 hr.

3. In man, 50 mg. administered q.i.d. every 5 hr. gives rise to a steady-state blood level approximately the same as the maximum blood level achieved with a single 200-mg. dose.

4. Steady-state blood levels were maintained on daily dosing over a 13-week interval.

5. The low recovery of the intact drug in the urine is indicative of a high degree of biotransformation and/or alternate routes of excretion.

6. The dog eliminated the drug 4 to 5 times faster than the two human subjects studied.

REFERENCES

(1) E. Grunberg and W. F. DeLorenzo, "Antimicrobial Agents and Chemotherapy," 1966, pp. 430–433.

(2) S. R. M. Bushby and G. H. Hitchings, *Brit. J. Pharmacol. Chemother.*, **33**, 72(1968).

(3) V. Toome, unpublished data on file, Hoffmann-La Roche Inc., Nutley, N. J., 1968.

(4) B. A. Koechlin and R. Weinfeld, unpublished data on file, Hoffmann-La Roche Inc., Nutley, N. J., 1966.

(5) S. Riegelman, J. C. K. Loo, and M. Rowland, *J. Pharm. Sci.*, **57**, 117(1968).

(6) J. C. K. Loo and S. Riegelman, *ibid.*, **57**, 917(1968).

(7) S. Riegelman, J. C. K. Loo, and M. Rowland, *ibid.*, **57**, 128 (1968).

(8) M. Gibaldi, R. Nagashima, and G. Levy, *ibid.*, **58**, 193(1969).

(9) J. G. Wagner and E. Nelson, *ibid.*, **53**, 1392(1964).

(10) G. E. Boxer, V. C. Jelinek, R. Tompsett, R. Du Bois, and A. O. Edison, *J. Pharmacol. Exp. Ther.*, **92**, 226(1948).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 7, 1969, from the *Department of Clinical Pharmacology, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication October 22, 1969.

Two-Compartment Model for a Drug and Its Metabolite: Application to Acetylsalicylic Acid Pharmacokinetics

MALCOLM ROWLAND, LESLIE Z. BENET, and SIDNEY RIEGELMAN

Abstract □ A general solution is presented for solving the pharmacokinetic parameters which describe a drug and its metabolite in a two-compartment open model. The method is specifically applied to the treatment of plasma data for acetylsalicylic acid and its metabolite, salicylic acid, following intravenous administration of acetylsalicylic acid. The acetylsalicylic acid data were found to be adequately described by a model in which elimination occurs solely from the central compartment.

Keyphrases □ Model, two-compartment, open—pharmacokinetic parameters □ Kinetic equations—two-compartment open model □ Acetylsalicylic acid—pharmacokinetics □ Concentration–time curve areas, ratio—drug-metabolite

In a previous publication a comparison of two models was made in an attempt to describe the pharmacokinetics of acetylsalicylic acid (ASA) following an intravenous dose to man (1). An essential feature in both of these models was the distribution of ASA between a central and peripheral compartment while the comparison revolved around deciding whether metabolism to salicylic acid (SA) occurred exclusively in the central compartment (Model A) or in both compartments (Model B, Fig. 1). The third possibility, *i.e.*, metabolism solely in the peripheral compartment, was excluded as hydrolysis is known to occur in plasma, which is the reference system for the central compartment. In addition, it was tacitly assumed that Model A also described SA kinetics for an intravenous dose. Both models were simulated on an analog computer, and from the results it was suggested that only Model A adequately fitted the observed ASA and resultant SA plasma data. Subsequent reexamination of this problem shows that by using both the ASA and SA data, an analytical solution is available for determining all the rate constants for ASA in Model B.

DISCUSSION

Before proceeding with the specific problem, a number of pertinent points can be made relating to the above two simple models, A and B. In either case the same biexponential concentration–time curve results from an intravenous dose of the drug into Compartment 1 and this is characterized by the exponents α' and β' together with the coefficients A′ and B′ as shown in Table I. (The double prime terms used in Model B are numerically equivalent to those in Model A, and serve only to distinguish between the models.) However, whereas these exponents and coefficients allow a complete solution of all the rate constants in Model A, *i.e.*, k_{21}, k_{12}, and k_{13}, only $(k_{21} + k_{24})$, $(k_{12} + k_{13})$, together with the product of k_{12} and k_{21}, can be calculated in Model B. Failure to recognize these boundary conditions in Model B resulted in an incorrect analysis of the analog computer calculations which appeared in Fig. 6 of *Reference 1*. Consequently, by just measuring the unchanged drug, it is impossible to obtain a solution for the specific rate constants in Model B. This may only be achieved by measuring the metabolite derived by biotransformation of the parent drug and by injection of this metabolite on a separate occasion. Such an approach, namely, following a drug and one of its metabolites was used in determining the model necessary to describe the pharmacokinetics of ASA. As expected the intravenous ASA data can be fitted by either Model A or B.

Enlarging this discussion leads to consideration of the more general situation which is represented in Model C (Fig. 2). Here elimination can proceed in both the tissue and peripheral compartments for drug and any metabolite that one measures. Despite this complexity, drug plasma levels still decline biexponentially, but with more rate constants involved in any one term in the equation describing these results (Table II). In this case, as in Model B, if drug alone is measured, it is only possible to calculate the sum of various rate constants, *i.e.*, $(k_{12} + k_{13} + k_{17})$ and $(k_{21} + k_{24} + k_{28})$ together with the product of k_{12} and k_{21}. Likewise, an injection of the metabolite only allows the solution of $(k_{43} + k_{46})$, $(k_{34} + k_{25})$ together with the product of k_{34} and k_{43}. An additional number of rate constants in Model C can, however, be solved by determining the fraction of a dose in Compartment 3 with time following the injection of the parent drug. The general form of this function can be readily derived by solving the differential equations which describe Model C and which are as follows:

$$\dot{F}_1 = -(k_{12} + k_{13} + k_{17})\cdot F_1 + k_{21}\cdot F_2 \quad \text{(Eq. 1)}$$

$$\dot{F}_2 = k_{12}\cdot F_1 - (k_{21} + k_{24} + k_{28})\cdot F_2 \quad \text{(Eq. 2)}$$

$$\dot{F}_3 = k_{13}\cdot F_1 + k_{43}\cdot F_4 - (k_{34} + k_{35})\cdot F_3 \quad \text{(Eq. 3)}$$

$$\dot{F}_4 = k_{24}\cdot F_2 + k_{34}\cdot F_3 - (k_{43} + k_{46})\cdot F_4 \quad \text{(Eq. 4)}$$

$$\dot{F}_5 = k_{35}\cdot F_3 \quad \text{(Eq. 5)}$$

$$\dot{F}_6 = k_{46}\cdot F_4 \quad \text{(Eq. 6)}$$

Table I—Various Parameters Which Define Models A and B in Fig. 1

	Model A	Model B
Plasma (blood) concentration time curve	$C_1 = A'e^{-\alpha' t} + B'e^{-\beta' t}$	$C_1 = A''e^{-\alpha'' t} + B''e^{-\beta'' t}$
Fraction of dose in central compartment	$F_1 = \frac{m_1}{\text{dose}} = \frac{C_1}{A' + B'}$ $= X'_\alpha e^{-\alpha' t} + X'_\beta e^{-\beta' t}$	$F_1 = \frac{m_1}{\text{dose}} = \frac{C_1}{A'' + B''}$ $= X''_\alpha e^{-\alpha'' t} + X''_\beta e^{-\beta'' t}$
Definition of terms with respect to the appropriate model	$X'_\alpha = \frac{k_{21} - \alpha'}{\beta' - \alpha'}$ $\alpha' + \beta' = k_{12} + k_{13} + k_{21}$ $\alpha'\beta' = k_{13}\cdot k_{21}$	$X''_\alpha = \frac{(k_{21} + k_{24}) - \alpha''}{\beta' + \alpha'}$ $\alpha'' + \beta'' = (k_{12} + k_{13}) + (k_{21} + k_{24})$ $\alpha''\beta'' = (k_{12} + k_{13})(k_{21} + k_{24}) - k_{12}\cdot k_{21}$

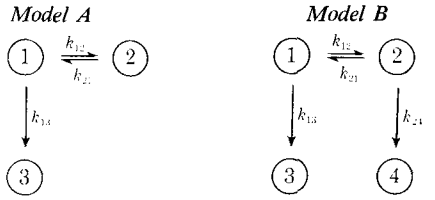


Figure 1—*Two-compartment open system in which elimination of drug, introduced into Compartment 1, occurs either from that compartment (Model A) or from both compartments (Model B).*

$$\dot{F}_7 = k_{17} \cdot F_1 \qquad \text{(Eq. 7)}$$

$$\dot{F}_8 = k_{28} \cdot F_2 \qquad \text{(Eq. 8)}$$

$$\sum_{n=1}^{8} F_n = 1 \qquad \text{(Eq. 9)}$$

$$F = \frac{m_3}{\text{dose}} = X_\alpha e^{-\alpha t} + X_\beta e^{-\beta t} + X_\gamma e^{-\gamma t} + X_\delta e^{-\delta t} \qquad \text{(Eq. 10)}$$

where

$$X_i = \frac{k_{13}(k_{21} + k_{24} + k_{28} - i)(k_{43} + k_{46} - i) + k_{12} \cdot k_{24} \cdot k_{43}}{(j - i)(k - i)(l - i)}$$

and i equals either α, β, γ, or δ, while j, k, and l are the superscripted values not equal to i; *i.e.*, $i = \alpha$:

$$X_\alpha = \frac{k_{13}(k_{21} + k_{24} + k_{28} - \alpha)(k_{43} + k_{46} - \alpha) + k_{12} \cdot k_{24} \cdot k_{43}}{(\beta - \alpha)(\gamma - \alpha)(\delta - \alpha)}$$

Letting $E_2 = k_{21} + k_{24} + k_{28}$; $E_4 = k_{43} + k_{46}$; $A_{13} = k_{12} \cdot k_{24} \cdot k_{43}$, $\therefore$

$$F = k_{13} \sum_{i=\alpha}^{\delta} \frac{(E_2 - i)(E_4 - i)}{(j - i)(k - i)(l - i)} e^{-it} + A_{13} \sum_{i=\alpha}^{\delta} \frac{1}{(j - i)(k - i)(l - i)} e^{-it} \qquad \text{(Eq. 11)}$$

Rearranging

$$\frac{F_3}{\sum_{i=\alpha}^{\delta} \frac{1}{(j - i)(k - i)(l - i)} e^{-it}} = k_{13} \frac{\sum_{i=\alpha}^{\delta} [(E_2 - i)(E_4 - i)]e^{-it}/[(j - i)(k - i)(l - i)]}{\sum_{i=\alpha}^{\delta} e^{-it}/[(j - i)(k - i)(l - i)]} + A_{13} \qquad \text{(Eq. 12)}$$

Since α, β, γ, and δ are known, and the terms $(k_{21} + k_{24} + k_{28})$ and $(k_{43} + k_{46})$ can be determined from the values of the coefficients described in the intravenous data, it is possible to calculate at any given time all the summation terms in Eq. 11, and in the rearranged Eq. 12, an equation for a straight line. Also, since the corresponding values of F_3 may be derived directly from the experimental metabolite plasma data following injection of the parent drug, it is possible to calculate both of the time variable functions on either side of Eq. 12. A plot of these functions will then give a slope of k_{13} and an intercept equal to the product $k_{12}k_{24}k_{43}$. Therefore, even in this relatively complex model, the rate constant k_{13}, describing metabolism within the central compartment to the measured metabolite, can be determined.

Amount of Drug Loss from Compartments 1 and 2—In looking at the general model, it is equally important to know the fraction of the drug which is lost from Compartment 1 and Compartment 2, respectively. These values are simply given as follows:

fraction drug lost from Compartment 1 =

$$(k_{13} + k_{17}) \int_0^\infty F_1 \, dt \qquad \text{(Eq. 13)}$$

Table II—Various Parameters Which are Characteristic of Model C in Fig. 2

Concentration–time curve of drug in Compartment 1.	$C_1 = Ae^{-\alpha t} + Be^{-\beta t}$
Fraction of dose in Compartment 1.	$F_1 = X_\alpha e^{-\alpha t} + X_\beta e^{-\beta t}$
Definition of coefficient and exponents for drug introduced as a bolus *via* Compartment 1.	$X_\alpha = \frac{(k_{21} + k_{24} + k_{28}) - \alpha}{\beta - \alpha}$ $\alpha\beta = (k_{12} + k_{13} + k_{17}) \times (k_{21} + k_{24} + k_{28}) - k_{12} \cdot k_{21}$
Concentration–time curve of metabolite introduced as a bolus *via* Compartment 3.	$C_3 = Ce^{-\gamma t} + De^{-\delta t}$
Corresponding fraction of metabolite in Compartment 3.	$F_3 = X_\gamma e^{-\gamma t} + X_\delta e^{-\delta t}$
Corresponding definition of coefficient and exponents for metabolite.	$X_\gamma = \frac{(k_{43} + k_{46}) - \gamma}{\delta - \gamma}$ $\gamma + \delta = (k_{43} + k_{46}) + (k_{34} + k_{35})$ $\gamma\delta = (k_{43} + k_{46})(k_{34} + k_{35}) - k_{34} \cdot k_{43}$

fraction drug lost from Compartment 2 =

$$(k_{24} + k_{28}) \int_0^\infty F_2 \, dt \qquad \text{(Eq. 14)}$$

Since the values of F_1 and F_2 are given by:

$$F_1 = \frac{E_2 - \alpha}{\beta - \alpha} e^{-\alpha t} + \frac{E_2 - \beta}{\alpha - \beta} e^{-\beta t} \qquad \text{(Eq. 15)}$$

$$F_2 = \frac{k_{12}}{\beta - \alpha} e^{-\alpha t} + \frac{k_{12}}{\alpha - \beta} e^{-\beta t} \qquad \text{(Eq. 16)}$$

it follows that:

$$\text{fraction drug lost from Compartment 1} = \frac{(k_{13} + k_{17})E_2}{\alpha\beta} \qquad \text{(Eq. 17)}$$

$$\text{fraction drug lost from Compartment 2} = \frac{k_{12}(k_{24} + k_{28})}{\alpha\beta} \qquad \text{(Eq. 18)}$$

Also, as expected, addition of Eqs. 17 and 18 and appropriate substitution shows that the sum of the fractions of drug lost from Compartments 1 and 2 is unity.

Clearance of Drug from Compartments 1 and 2—The total body clearance (TBC) of drug, defined by Eq. 19, is model independent for an i.v. dose no matter where drug loss occurs.

$$\text{TBC} = \frac{\text{dose}}{\int_0^\infty C_1 \, dt} \qquad \text{(Eq. 19)}$$

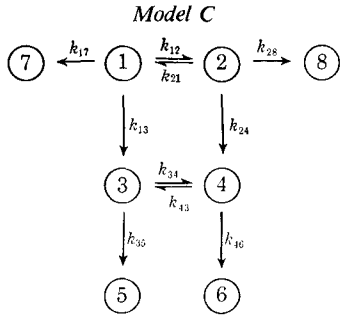


Figure 2—*Compartmental model describing the distribution and elimination of a drug injected into Compartment 1, elimination proceeding by various routes from Compartments 1 and 2, with a metabolite, 3, undergoing analogous disposition.*

Table III—Ratio of the Total Areas under the Metabolite Concentration–Time Curve following an Equimolar i.v. Dose of Drug and Metabolite

Case	Model: $k_{46}>0$	Model: $k_{46}=0$	Ratio of Areas[a]: $k_{46}>0$	Ratio of Areas[a]: $k_{46}=0$
General	I	VI	I $\frac{k_{13}(k_{21}+k_{24}+k_{28})(k_{43}+k_{46})+k_{12}k_{24}k_{43}}{\alpha\beta(k_{43}+k_{46})}$	VI $\frac{k_{13}(k_{21}+k_{24}+k_{28})+k_{12}k_{24}}{\alpha\beta}$
$k_{28}=0$	II	VII	II $\frac{k_{13}(k_{21}+k_{24})(k_{43}+k_{46})+k_{12}k_{24}k_{43}}{\alpha\beta(k_{43}+k_{46})}$	VII $\frac{k_{13}(k_{21}+k_{24})+k_{12}k_{24}}{\alpha\beta}$
$k_{17}=k_{28}=0$	III	VIII	III $\frac{k_{13}(k_{21}+k_{24})(k_{43}+k_{46})+k_{12}k_{24}k_{43}}{\alpha\beta(k_{43}+k_{46})}$	VIII 1
$k_{24}=k_{28}=0$	IV	IX	IV $\frac{k_{13}}{k_{13}+k_{17}}$	IX $\frac{k_{13}}{k_{13}+k_{17}}$
$k_{17}=k_{24}=k_{28}=0$	V	X	V 1	X 1

[a] In the formulas, $\alpha\beta = E_1E_2 - k_{12}\cdot k_{21}$

Multiplying both sides of Eq. 13 by dose divided by V_1, the volume constant for Compartment 1 [*i.e.*, V_1 = dose/$(A + B)$], gives:

$$\frac{\text{(amount of drug lost from Compartment 1)}}{V_1} = (k_{13} + k_{17})\int_0^\infty C_1 dt \qquad \text{(Eq. 20)}$$

Rearranging Eq. 20 results in:

clearance of drug from Compartment 1 = $(k_{13} + k_{17})\,V_1$ (Eq. 21)

Subtracting Eq. 21 from Eq. 19 yields the clearance of drug from Compartment 2, which may also be numerically given by:

clearance of drug from Compartment 2 = $(k_{24} + k_{28})\,V_2$ (Eq. 22)

where V_2, the volume constant for distribution in Compartment 2 with reference to the concentration in Compartment 1, is defined as

$$V_2 = \frac{k_{12}}{E_2}V_1 \qquad \text{(Eq. 23)}$$

Although the volume terms V_1 and V_2 may not be equal to a real physiological space, the clearances from Compartments 1 and 2 (as defined in Eqs. 21 and 22) are measures of an actual clearance for organs and/or tissues within the body.

Area Analysis—Assuming that the rate constants defining the kinetics of the metabolite are independent of whether it is given directly or formed by the biotransformation of the parent compound, one can calculate the ratio of the total areas under the metabolite concentration–time curve in Compartment 3 following an equivalent dose of parent drug and metabolite on separate occasions. This is given by

$$\text{ratio of metabolite areas} = \frac{\left[\int_0^\infty F_3\,dt\right]_{\text{i.v. drug}}}{\left[\int_0^\infty F_3\,dt\right]_{\text{i.v. metabolite}}} = \frac{k_{13}E_2E_4 + A_{13}}{\alpha\beta E_4} \qquad \text{(Eq. 24)}$$

Utilizing such an equation, it is possible to investigate the influence of the route and magnitude of elimination and distribution on the area under the metabolite curve. Several such cases are illustrated in Table III. Cases IX and X represent the well-known models in which elimination of the drug (and metabolite) occurs solely from

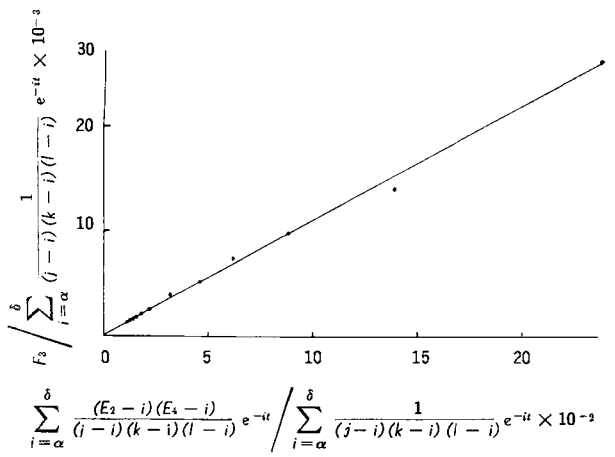


Figure 3—*Plot of the time variable functions in Eq. 13, Subject A. Slope equal to* k_{13}.

Table IV—Values Calculated to Test the Model Appropriate for ASA Pharmacokinetics

Subject	k_{13} (min.$^{-1}$) Model A[a]	k_{13} (min.$^{-1}$) Model C[b]	$k_{12}k_{24}k_{43}$[b]	k_{24}[c]	Fraction ASA Metabolized in Compartment 1[d]	Fraction ASA Metabolized in Compartment 2[e]
A	0.117	0.113	1×10^{-4}	0.005	0.95	0.05
B	0.101[f]	0.095	3×10^{-4}	0.009	0.94	0.06
C	0.096[f]	0.110	-4×10^{-4}	−0.025	1.14	−0.14
D	0.105	0.095	2×10^{-4}	0.014	0.91	0.09

[a] Taken from Reference 1. [b] Calculated by graphical methods utilizing Eq. 12. [c] Calculated using equations defined for Model B in Table I and graphical solution for k_{13}. [d] Calculated using Eq. 17 when $k_{17} = 0$. [e] Calculated using Eq. 18 when $k_{28} = 0$. [f] These values differ slightly from those reported previously (1) due to calculation errors.

the central compartment. Case IX is normally used to calculate the rate constant, k_{13}, when the sum $(k_{13} + k_{17})$ has been determined from previous i.v. data. When the ratio of areas is unity, it is impossible to distinguish between Cases V, VIII, and X. The remaining cases illustrate those conditions in which elimination of an unchanged drug also occurs from Compartment 2 and metabolite may or may not be eliminated from Compartment 4. Table III clearly shows that the corresponding area analysis cannot be used to calculate exclusively the rate constant k_{13}. Rather this ratio of areas under the metabolite concentration–time curve is a complex function which results from the interplay of all the constants in the appropriate model.

Examination of ASA Kinetics—Returning to the problem associated with the ASA data, the total area under the SA plasma concentration–time curve was the same following either injection of ASA or an equivalent dose of SA. Also, it has been reported that only approximately 1% of ASA is eliminated unchanged in the urine (2). These data, in themselves, suggest that all the models in Table III can be excluded with the exception of Cases V, VIII, and X. To distinguish Case VIII from the other two, it is necessary to determine whether the rate constant k_{24} exists. This was ascertained for each of the four subjects in the study (1) by graphically solving Eq. 12. The values for the fraction of the dose in Compartment 3 were obtained by dividing the plasma concentration values for SA by the sum of the coefficients C and D, *i.e.*, $F_3 = C_3/C + D$, while each of the time variable summation terms was calculated at each time point. Figure 3 illustrates such a plot for Subject A of *Reference 1*. As expected, a straight line was obtained for this and the other subjects and resultant values for k_{13} are shown in Table IV together with the intercept $k_{12}k_{43}k_{24}$. In addition, the values for k_{13}, assuming Model A for ASA, are presented. As can be seen the intercept was essentially zero for all subjects, indicating that the term k_{24} is small since the other rate constants, k_{43} and k_{12}, must be positive and significant values as they are related to the distribution of ASA and SA where both of these drugs exhibit biexponential curves. Knowing k_{13}, it is also possible to obtain an exact solution of k_{24} for the ASA data by appropriate substitution into the defined terms for Model B in Table I. These values are presented in Table IV.

From the data in Table IV, it is obvious that the pathway designated by k_{24} may be considered of only minor importance in ASA pharmacokinetics. It is likely that the values reported for k_{24} and the "Fraction ASA Metabolized in Compartment 2" differ from zero only as a result of the errors inherent in the biological experiment. This is reinforced by the negative values calculated for Subject C. Consequently, we are led to the conclusion that Model A rather than Model B describes the ASA data, and this is confirmed by the close similarity between the values of the rate constant, k_{13}, determined by the graphical solution of Eq. 12 and those given by simple calculation based on Model A. Accordingly, as previously suggested (1) the ASA i.v. data can be adequately described by the model in which elimination occurs solely in the central compartment. However, because the rate constant k_{24} appears to be zero, one cannot distinguish between Cases V and X where SA is eliminated exclusively from the central compartment or from both compartments. Obviously were the value of k_{24} a significant number, then k_{43} and hence k_{46} could have been calculated allowing the entire ASA model to be characterized.

TERMINOLOGY

n	refers to Compartment n.
C_n, M_n, F_n	are the corresponding concentration, mass, and fraction of administered dose of a species in compartment n.
k_{nm}	first-order rate constant for the transfer of species from Compartment n to m.
α, β, A, B	are the exponents and coefficients of the equation describing the changes with time for the species in Compartment 1 following an injection of drug into that compartment.
γ, δ, C, D	are the exponents and coefficients of the equation describing the changes with time for the species in Compartment 3 following an injection of metabolite into that compartment.
$\alpha, \alpha', \alpha'', etc.$	are numerically equivalent and the prime terms are solely used to distinguish between various models describing the observed data.
E_n	is the sum of all the rate constants out of compartment n.
A_{nm}	is the product of the rate constants involved in the alternate route between compartments n and m, *i.e.*, $A_{13} = k_{12} \cdot k_{24} \cdot k_{43}$.

REFERENCES

(1) M. Rowland and S. Riegelman, *J. Pharm. Sci.*, **57**, 1313 (1968).

(2) A. J. Cummings and M. L. King, *Nature*, **209**, 620(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 4, 1969, from the *Department of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122*

Accepted for publication October 20, 1969.

Supported by Grant NIGMS 16496 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Comparison of Whole-Body Liquid Scintillometry, Radiography, and Clinical Chemical Tests in the Evaluation of the Effect of Chronic Corticoid Dosing on Calcium in Beagles

M. C. BHATTI*, S. M. SHAW, R. E. LEWIS, and J. E. CHRISTIAN

Abstract □ Chronic doses of 6α-methylprednisolone were administered to beagles for 106 days to bring about changes in calcium balance. During the initial 64 days of corticoid treatment, no outstanding changes were observed in the blood levels of calcium, phosphorus, or alkaline phosphatase. Radiographic changes in the skeleton were not seen after 64 days of treatment. Significant changes in ^{47}Ca retention were detected by whole-body liquid scintillometry after only 13 and 28 days of corticoid administration. On day 64 of the study, the dosage of 6α-methylprednisolone was increased. Decreased levels of calcium in blood plasma and increased levels of plasma alkaline phosphatase were observed after the dose of corticoid was increased. A great reduction in whole-body ^{47}Ca retention was also observed, and thinning of the cortices of the long bones of the legs was apparent. The results of the investigation indicate that whole-body liquid scintillation counting of ^{47}Ca is a sensitive indicator of alterations of calcium metabolism in relation to other diagnostic methodology.

Keyphrases □ Corticoid administration, chronic—Ca effect evaluation □ ^{47}Ca retention—chronic corticoid administration □ Scintillometry—analysis □ Atomic absorpion spectroscopy—analysis

The diagnosis of pathologic conditions affecting the skeleton is difficult. Clinical chemical methods and radiography can rarely detect skeletal disease until a critical stage has developed. Routine radiographic methods are sensitive to rarefying osteopathies only after a 30–50% loss of skeletal mineral (1, 2). There is a definite need for superior diagnostic methodology.

Experimentation in this laboratory (3) and that conducted by others (4–10) have demonstrated that ^{47}Ca and whole-body counting techniques show great promise for the evaluation of skeletal calcification. The purpose of the present investigation was to compare the abilities of whole-body liquid scintillation counting of ^{47}Ca, routine clinical chemical methods, radiography, and ^{45}Ca specific activity determinations of bone sample for the detection of changes in calcium balance brought about in beagle dogs by chronic dosing with the corticoid, 6α-methylprednisolone. Chronic administration of corticoid was chosen because one of the most serious complications of sustained glucocorticoid therapy is the development of osteoporosis (11). The development of osteoporosis from glucocorticoid administration is an insidious process, taking many months to become severe enough to be capable of clear radiological recognition (12, 13). Skeletal alterations resulting from corticoid treatment have been well documented in animals (14–16).

EXPERIMENTAL

Five pure-bred male beagles[1] were used for the investigation. The dogs were housed together in a large indoor kennel which permitted them to exercise freely. The dogs were maintained on a balanced commercial diet.[2] Tap water was allowed *ad libitum*. At 10 weeks of age, each dog received 100 μc. of ^{45}Ca in two subcutaneous doses 2 days apart. Approximately 50 days were allowed for equilibration of the isotope with the stable skeletal calcium pool. At various intervals throughout the study, bone samples were obtained from each dog by amputation of the third digit of a foot. The first phalanx of each digit was analyzed for ^{45}Ca and total calcium.

The bone was air dried, weighed, and ashed in a muffle furnace. The ash was dissolved in 1 *N* HCl. Calcium determinations were carried out in triplicate by atomic absorption spectroscopy[3] according to the methodology developed for the clinical use of the spectrophotometer (17, 18). Determinations of ^{45}Ca were made in triplicate by placing aliquots of the dissolved bone ash in a toluene modified XDC scintillator solution (19) and counting the samples with a Packard Tri-Carb scintillation spectrometer.[4] An internal standardization procedure was utilized to correct the counting data for quenching (20).

Following the ^{45}Ca equilibration period, corticoid administration was initiated. Four of the dogs received a daily oral dose of 2 mg./kg. of body weight of 6α-methylprednisolone for 63 days. On the 64th day, the corticoid dose for these four dogs was raised to 10 mg./kg. of body weight daily and maintained until day 106. The fifth dog served as a control and received doses of lactose in place of corticoid during the entire study.

Before and during corticoid administration, the dogs were radiographed at approximately 2-week intervals. The areas radiographed were the lumbar spine, foreleg, and mandible. All radiographs were made using regular-speed film and ultradetail intensifying screens.

Clinical chemical determinations were performed prior to and at various times during corticoid dosing. After 24 hr. of fasting, a 10-ml. sample of whole blood was obtained from the external jugular vein of each dog in heparinized syringes. Plasma alkaline phosphatase determinations were made using Phosphatabs-Alkaline Quantitative.[5] This simplified test for alkaline phosphate makes use of reagent tablets containing phenolphthalein phosphate substrate (21). The reagent tablets have been reported to produce results which are in excellent agreement with more sophisticated techniques for alkaline phosphatase determination (22–24). An analysis using a standardized enzyme solution[6] was run with each series of alkaline phosphatase determinations as a check against errors in methodology. Serum inorganic phosphorus was determined with Hycel phosphorus reagent.[7] A standardized serum[8] was analyzed with each series of phosphorus determinations to assure against technical errors. Serum calcium was determined by adding 0.2 ml. of plasma to 5 ml. of a solution of 1% lanthanum as $LaCl_3 \cdot 7H_2O$ and 5% trichloroacetic acid in double distilled water. The mixture was centrifuged to precipitate the plasma proteins. The calcium contained in the supernatant was determined by atomic absorption spectroscopy. All clinical chemical determinations were made in triplicate. Each time clinical chemical analyses were conducted, the average value obtained for the corticoid-treated dogs was compared to the values for the control dog.

[1] American Animal Industries, Indianapolis, Ind.

[2] Wayne Dog Krumettes, Allied Mills, Inc., Chicago, Ill.

[3] Perkin-Elmer Atomic Absorption Spectrophotometer, model 303, Perkin-Elmer, Norwalk, Conn.

[4] Model 574, Packard Instrument Company, Inc., Downers Grove, Ill.

[5] General Diagnostic Division, Warner-Chilcott Laboratories, Morris Plains, N. J.

[6] Versatol-E-N, General Diagnostic Division, Warner-Chilcott Laboratories, Morris Plains, N. J.

[7] Hycel Incorporated, Houston, Tex.

[8] Versatol-A, General Diagnostic Division, Warner-Chilcott Laboratories, Morris Plains, N. J.

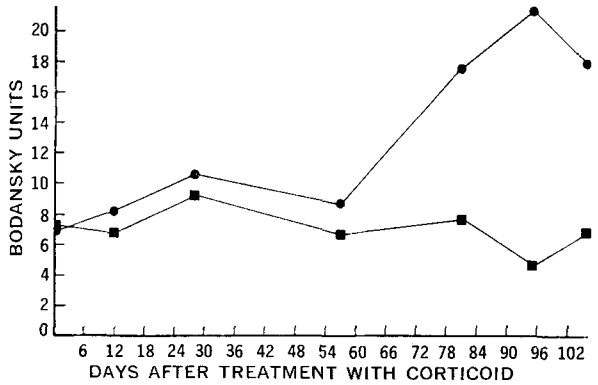


Figure 1—*Plasma alkaline phosphatase levels. Key: ●, 6α-methylprednisolone-treated dogs; and ■, control dog.*

A 4-pi whole-body liquid scintillation counter (3, 25) was used to determine whole-body retention of ^{47}Ca. Retention of ^{47}Ca was determined before corticoid administration and at four intervals during corticoid dosing. For each whole-body counting study, each dog was administered 0.2 μc. of ^{47}Ca intravenously into the external jugular vein. Fifteen minutes after isotope administration, ^{47}Ca retention was determined in each dog. This was considered to be time 0 and the count rate to be 100% retention of ^{47}Ca. Each dog was counted at given time intervals for 144 hr. after time 0. The ^{47}Ca retention values for the four corticoid-treated dogs were averaged at each time interval. Comparisons were made between the average retention values after various periods of corticoid dosing and the average retention values before corticoid dosing. Whole-body counting data were examined statistically using an analysis of variance and a Newman–Keuls Sequential Range test. At the termination of the study, the dogs were euthanitized with pentobarbital, and necropsies were performed.

RESULTS AND DISCUSSION

During the period of corticoid dosing at a level of 2 mg./kg. of body weight, no outstanding changes were observed in the plasma alkaline phosphatase (Fig. 1), serum calcium (Fig. 2), or serum inorganic phosphorus (Fig. 3) levels of the treated dogs as compared to the control dog. No apparent radiographic changes in the treated dogs in relation to the control dog were observed at this lower dosage level of corticoid. However, by whole-body liquid scintillometry, it was possible to detect significant ($p = 0.01$) changes in ^{47}Ca whole-body retention in the dogs after 13 and 28 days of corticoid administration as compared to ^{47}Ca retention in the same dogs previous to the initiation of drug treatment (Fig. 4). At each time period investigated, statistical differences between whole-body ^{47}Ca retention curves occurred 60 hr. and thereon after ^{47}Ca administration. The results of the investigation during the period of corticoid dosing at a level of 2 mg./kg. illustrate the ability of whole-body liquid scintillation counting of ^{47}Ca to detect alterations of calcium

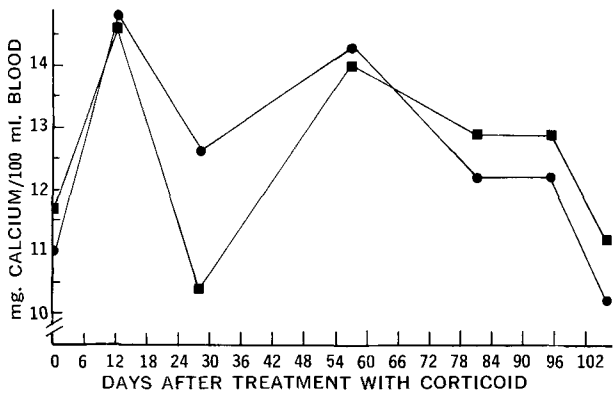


Figure 2—*Serum calcium levels. Key: ●, 6α-methylprednisolone-treated dogs; and ■, control dog.*

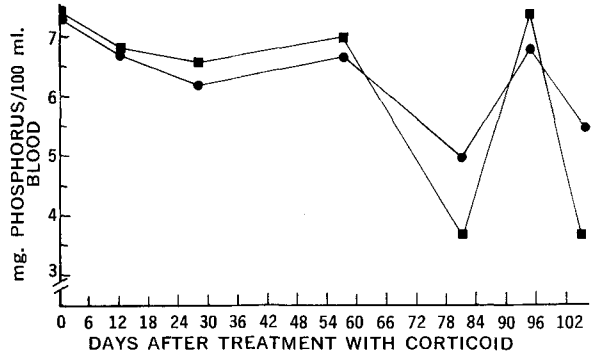


Figure 3—*Serum inorganic phosphorus levels. Key: ●, 6α-methylprednisolone-treated dogs; and ■, control dog.*

metabolism in the absence of radiographic and clinical chemical changes.

As may be seen in Fig. 4, after 54 days of corticoid administration at the lower dosage level the whole-body retention of ^{47}Ca began to approach the values observed before drug treatment. It appeared that the treated dogs were adapting to the pharmacologic effect of the drug. When the dosage level of 6α-methylprednisolone was raised to 10 mg./kg. of body weight, the whole-body retention of ^{47}Ca was found to be greatly decreased in the treated dogs in relation to ^{47}Ca retention in the dogs before corticoid treatment. Following the increase in dosage level the serum calcium in the treated dogs was decreased in relation to the control dog. Also, plasma alkaline phosphatase was elevated in the treated dogs as compared to the control dog. No conspicuous changes were observed in serum inorganic phosphorus during the entire study.

Radiographs taken at termination of the study (after 42 days of corticoid at a dose of 10 mg./kg. of body weight) showed thinning of the cortices of the long bones in the forelegs of the corticoid-treated dogs. Figure 5 illustrates the difference between the radiographic appearance of the long bones of the forelegs of the corticoid-treated dogs as compared to the control dog. It was also noted that the dogs treated with 6α-methylprednisolone developed curvature of the forelegs with the convex side anterior. Periosteal thickening of both the radius and the ulna on the concave side (posterior) occurred. In the middle third of the ulna there was an increased transverse trabecular pattern.

The ^{45}Ca specific activity of bone samples was expressed as c.p.m. ^{45}Ca/mg. Ca/g. bone. For each dog, the bone specific activity at the initiation of corticoid or control treatment was considered

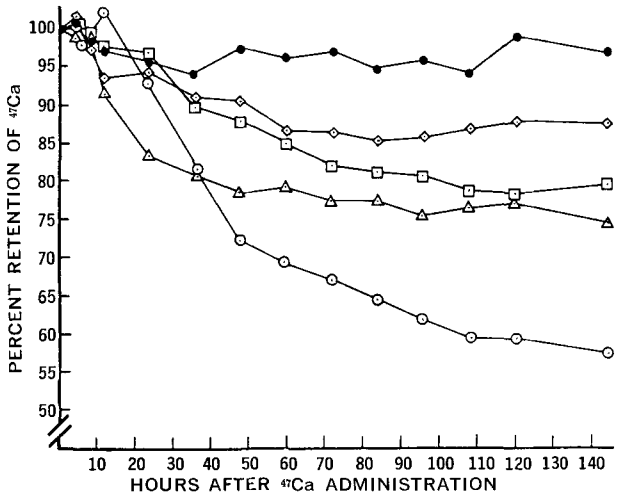


Figure 4—*Whole-body liquid scintillation determination of ^{47}Ca retention by dogs receiving chronic doses of 6α-methylprednisolone. Key: ●, dogs before corticoid treatment; ◇, dogs after 13 days of corticoid; □, dogs after 54 days of corticoid; △, dogs after 28 days of corticoid; and ○, dogs after 98 days of corticoid.*

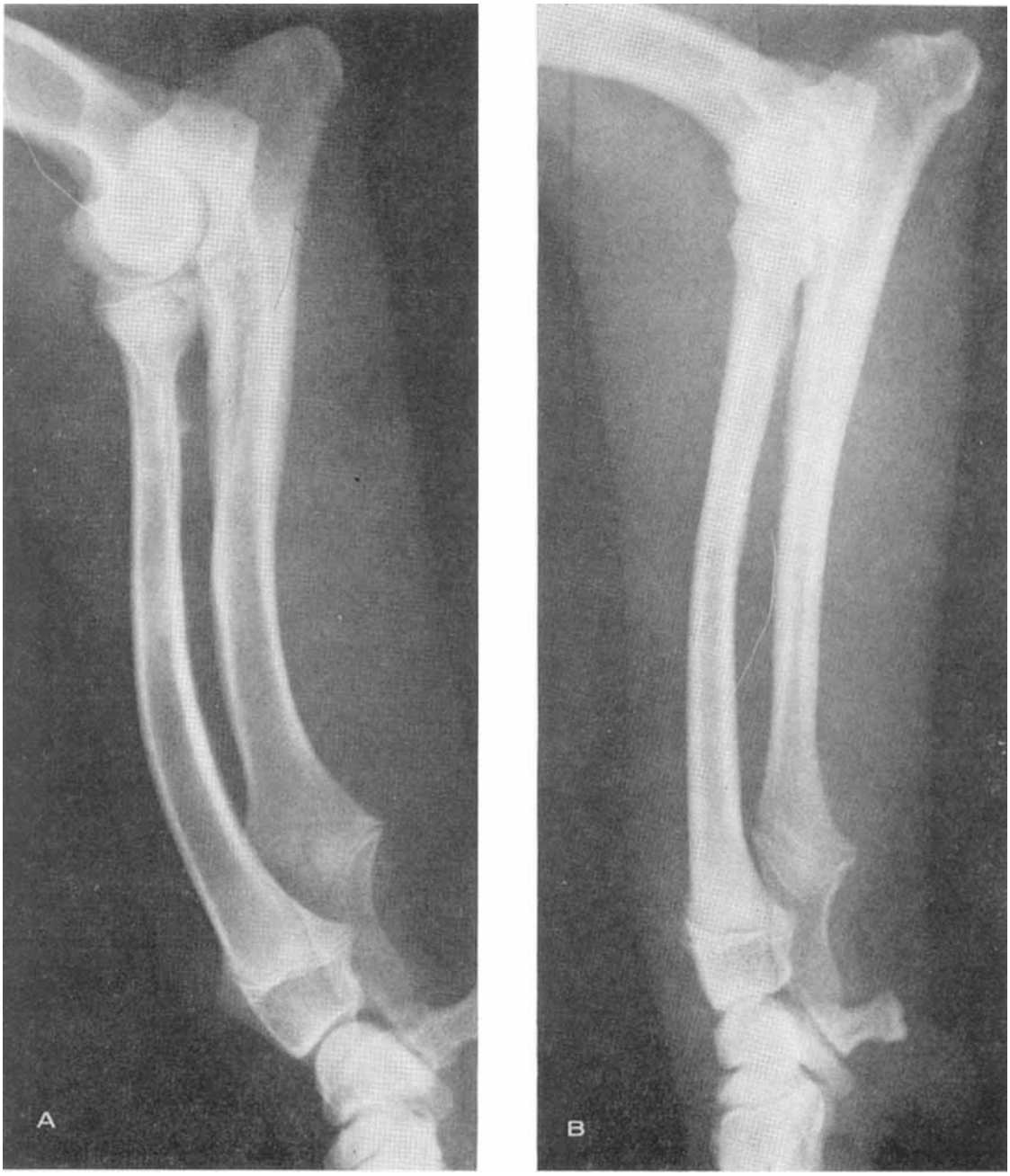


Figure 5—*Radiograph of the foreleg. Key:* A, *dog after chronic doses of 6α-methylprednisolone for 106 days; and* B, *control dog.*

as 100% and subsequent samples were expressed as the percent of the initial specific activity remaining. After 59 days of corticoid dosing at a level of 2 mg./kg. of body weight, the specific activity of the bone samples from the treated dogs was 64% of the initial specific activity while the specific activity of the control dog was 59%. After a total of 106 days of corticoid dosing the specific activity of the bone samples from the treated dogs was 63% of the specific activity found previous to the initiation of corticoid administration while the specific activity of the control dog was 49%. Necropsy examinations at the termination of the study revealed no gross pathology in any of the dogs. However, gonadal atrophy was evident in the corticoid-treated dogs.

Whole-body liquid scintillation detection of ^{47}Ca retention in this investigation indicated that corticoid administration results in a decreased calcium retention. Collins *et al.* (26, 27) and Garrett *et al.* (28, 29) observed that chronic corticoid administration to beagle dogs resulted in decreased ^{47}Ca uptake by bone and an increased fecal excretion of ^{47}Ca. These workers concluded that decreased bone formation rather than increased destruction was the major effect of administered corticoids.

It would appear from the whole-body counting studies that either osteoblastic activity was depressed so that osteoclastic activity, normally held in balance, could show a greater net effect of resorption on bone, or that osteoclastic activity was enhanced resulting in increased bone resorption. Since osteoclasts have been shown to be associated with phosphatase enzymes (30), increased osteoclastic activity may account for the increased alkaline phosphatase levels observed in the corticoid-treated dogs. Conversely, the increased alkaline phosphatase levels may have been due to an increased osteoblastic activity attempting to heal bone damaged caused by the 6α-methylprednisolone (31). However, Frost and Vilaneuva (32) examined the bones of a group of 21 human patients who had received cortisone or ACTH for more than 2 weeks. Only two cases showed normal osteoblastic activity. The remaining 19 cases showed depression or cessation of osteoblastic activity. Frost (32, 33) has concluded that human patients receiving phar-

macodynamic doses of corticoids go into negative calcium balance as a result of an unequal inhibition of both bone formation and bone resorption. The inhibition of formation is greater than the inhibition of resorption with the result that a negative skeletal balance ensues, leading to osteoporosis. In the present investigation, it is possible that an inhibition of bone formation by the corticoid may account for the decreased whole-body retention of ^{47}Ca and that inhibition of resorption may account for the increased retention of ^{45}Ca by the bone samples analyzed. The foreleg curvature developed by the dogs treated with 6α-methylprednisolone presents further evidence of inhibited bone formation. It has been shown in these laboratories that retardation of the distal ulnar epiphysis in dogs will result in curvature of the bones of the foreleg accompanied by periosteal thickening of these bones on the concave area.

SUMMARY

From these studies it can be seen that whole-body liquid scintillation counting of ^{47}Ca is a sensitive indicator of alterations of calcium metabolism in relation to other diagnostic methodology. During the first 64 days of corticoid treatment, no outstanding changes were observed in the blood levels of calcium, phosphorus, or alkaline phosphatase. Radiographic changes in the skeleton were not apparent after 64 days of treatment. However, it was possible to detect significant changes in ^{47}Ca retention by whole-body liquid scintillometry after only 13 days of corticoid administration. Significant radiographic and clinical chemical changes occurred only after elevated doses of corticoid were administered.

REFERENCES

(1) M. Urist, "Bone as a Tissue," McGraw-Hill, New York, N. Y., 1960, pp. 18–45.

(2) G. Bauer, *Proc. Intern. Conf. Sci. Res. (U.M.E.S.C.O.), Ist*, **4,** 232(1958).

(3) M. C. Bhatti, S. M. Shaw, J. E. Christian, and T. S. Miya, *J. Pharm. Sci.*, **58,** 502(1969).

(4) G. Bauer, "Bone as a Tissue," McGraw-Hill, New York, N. Y., 1960, pp. 98–127.

(5) M. Rinsler, G. Dyche, and N. Trott, "Radioaktive Isotope in Klinik and Forschung IV," Urban and Schwarzenberg, Munich-Berlin, W. Germany, 1960, pp. 19–28.

(6) A. Budy, *J. Bone Joint Surg.*, **45A,** 1073(1963).

(7) E. Belcher and R. Dudley, "Medical Uses of ^{47}Ca," International Atomic Energy Agency, Vienna, Austria, 1964, pp. 9–12.

(8) T. Sargent, J. Linfoot, and E. Isaac, "Clinical Uses of Whole Body Counting," International Atomic Energy Agency, Vienna, Austria, 1966, pp. 187–211.

(9) L. Lutwak and J. R. Shapiro, *Science*, **144,** 1155(1964).

(10) F. K. Curtis, H. Fellows, and C. Rich, *J. Lab. Clin. Med.*, **69,** 1036(1967).

(11) P. Fourman, "Calcium Metabolism and the Bone," Charles C Thomas, Springfield, Ill., 1960, pp. 97–106.

(12) E. Rosenberg, *Acta Med. Scand.*, **162,** 211(1958).

(13) J. Lievre, *Rhumatologie*, **12,** 208(1960).

(14) E. Storey, *Endocrinology*, **68,** 533(1961).

(15) E. Storey, *Australian N. Z. J. Surg.*, **27,** 19(1957).

(16) E. Storey, *Brit. J. Exp. Pathol.*, **41,** 207(1960).

(17) J. Willis, *Spectrochim. Acta*, **16,** 259(1960).

(18) A. Zettner, *Advan. Clin. Chem.*, **7,** 41(1964).

(19) G. Bruno and J. Christian, *Anal. Chem.*, **33,** 1215(1961).

(20) C. H. Wang and D. L. Willis, "Radiotracer Methodology in Biological Science," Prentice-Hall, Engelwood Cliffs, N. J., 1965, p. 131.

(21) M. Benjamin, "Outline of Veterinary Clinical Pathology," 2nd ed., Iowa State University Press, Ames, Iowa, 1961, pp. 129–134.

(22) M. Eibler and C. Nelson, *Amer. J. Med. Technol.*, **26,** 137 (1960).

(23) N. Sidoti, *ibid.*, **25,** 339(1959).

(24) P. Feldman, *ibid.*, **25,** 143(1959).

(25) W. V. Kessler, P. L. Ziemer, J. E. Christian, and S. M. Shaw, *Intern. J. Appl. Radiation Isotopes*, **19,** 287(1968).

(26) E. Collins, E. R. Garrett, and R. Johnston, *Metabolism*, **11,** 716(1962).

(27) E. Collins and O. Carpenter, *Acta Endocrinol.*, **42,** 348 (1963).

(28) E. R. Garrett, R. Johnston, and E. Collins, *J. Pharm. Sci.*, **51,** 1050(1962).

(29) E. R. Garrett, R. Johnston, and E. Collins, *ibid.*, **52,** 668 (1963).

(30) A. Morse and R. Greep, *Anat. Record*, **111,** 193(1951).

(31) G. Bourne, "Biochemistry and Physiology of Bone," Academic, New York, N. Y., 1956, pp. 251–283.

(32) H. Frost and A. Villaneuva, *Henry Ford Hosp. Med. Bull.*, **9,** 97(1962).

(33) H. Frost, *ibid.*, **10,** 119(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 30, 1969, from the *Department of Bionucleonics, School of Pharmacy and Pharmacal Sciences, and the Department of Radiology, School of Veterinary Science and Medicine, Purdue University, Lafayette, IN 47907*

Accepted for publication October 13, 1969.

This investigation was supported in part by the National Center for Radiological Health, U. S. Public Health Service, and was conducted under the auspices of the Institute for Environmental Health.

The authors are indebted to The Upjohn Company for the supply of 6α-methylprednisolone which was used for these investigations. The authors also wish to thank Dr. Virgil Anderson and Mrs. Shirley Wolfe for their help with the statistical evaluation of the data and Dr. Duane Brobst for his assistance with the clinical chemical determinations.

* Present address: Nuclear Medicine Section, Department of Radiology, University General Hospital, Iowa City, IA 52240

Heterogeneous Systems II: Influence of Partitioning and Molecular Interactions on *In Vitro* Biologic Activity of Preservatives in Emulsions

NAGIN K. PATEL* and JOYCE M. ROMANOWSKI

Abstract □ The preservative activity of *p*-hydroxybenzoic acid esters (parabens) in the presence of a polysorbate 80–sorbitan monooleate water system was found to be related to the concentration of the free or unbound paraben. Prediction of required preservative concentration for an emulsified system from a knowledge of oil–water partition coefficients and preservative-macromolecules binding data is illustrated. It was demonstrated by an *in vitro* microbiological procedure and using a glass dialysis cell that the fungistatic activity against *Aspergillus niger* of methyl- and propylparaben in emulsified systems was primarily a function of the free paraben concentration in the aqueous phase.

Keyphrases □ *p*-Hydroxybenzoic acid esters activity—partitioning, molecular interaction, effects □ Biological activity—emulsion preservatives *in vitro* □ Emulsions, effect—preservatives □ Inhibition determination—dialysis cell

Biological activity of antimicrobial agents in a heterogeneous system such as an emulsion is much more complex than in a simple aqueous medium. These agents, under the classification of preservatives, are incorporated in emulsified systems—medicinal, cosmetic, pharmaceutical, and nonpharmaceutical emulsions; ointments, creams, *etc.*—to protect against deterioration from bacterial and fungal attack. During the past 2 decades, pharmaceutical and cosmetic industries have recognized an increasing number of failures of the preservative to protect against microbial spoilage. This period coincides with the time during which nonionic emulsifiers, those containing polyoxyethylene groups and others, have become increasingly popular. A multiplicity of factors governs the efficacy of a preservative in the final formulation, and some notable reviews on the subject have appeared in the literature (1–5).

Emulsion and cream formulations contain a variety of ingredients which collectively form excellent substrates for microbial growth (6, 7). Under some conditions, fixed oils, fats (6, 7), hydrocarbon oils (8), the emulsifier (9), or the preservative (10) may be metabolized by certain microorganisms.

Previous investigations (11–16) have shown that many of the more commonly used preservatives in pharmaceutical and cosmetic emulsions are adsorbed to, bound to, or solubilized by nonionic emulsifiers in aqueous solution. The bound or solubilized (12) preservative in such a system has been shown to be devoid of antimicrobial activity (13, 15, 17, 18). In emulsified systems an additional factor, partitioning of the preservative between the oil and water phases, must be considered for the effective preservation of the entire formulation.

It is generally recognized that emulsified preparations containing different oil phases require higher concentration of preservatives (19) than normally used. The activity of some phenols in a two-phase system, liquid petrolatum–water, has been studied by Bean *et al.* (20). Recently, Anderson and Chow reported on the distribution and activity of benzoic acid in oil–water systems, emulsified with 0.1% polyoxyethylene lauryl ether[1] (21); the antifungal activity of benzoic acid was related to its concentration in aqueous phase.

As pointed out by Atkins (22), the problem of deterioration of cosmetic emulsion systems during storage, due to either mold formation or bacterial growth, is confined mainly to oil-in-water emulsions; it is rarely a problem with water-in-oil emulsions, the reason being that most troublesome organisms require an aqueous medium for favorable growth. In a water-in-oil emulsion, the water is surrounded by an inhospitable oil phase and this is not conducive to growth. The less common organisms which can infect the oil phase apparently do not cause product decomposition or spoilage, so that, even if they exist, their presence is not suspected (22). The most important factors affecting the activity of preservatives in oil-in-water emulsions containing nonionic emulsifying agents are those governing the availability of the preservatives in the aqueous phase. In such a system the preservative would partition into the oil phase and would also interact with the nonionic emulsifiers, thus reducing the activity of the preservative. The principal aim of this investigation was to show how the partition coefficient and binding data can readily and satisfactorily be used to determine the effective concentration in aqueous phase of methyl- and propylparaben in typical oil-in-water emulsions containing polysorbate 80[2] and sorbitan monooleate[3] (or polysorbate 20[4] and sorbitan monolaurate[5]) as emulsifying agents. It will also be demonstrated by an *in vitro* microbiological procedure that the activity of the preservatives is related to their concentrations in the aqueous phase. Selection of the oils was based on their varying oil–water partition coefficients (23).

THEORY

The theory behind the partitioning of a preservative in an oil–water system has been described in Part I (23). The equilibria con-

[1] Brij 35, marketed by Atlas Chemical Industries, Inc., Wilmington, Del.

[2] Polyoxyethylene (20) sorbitan monooleate, marketed as Tween 80 by Atlas Chemical Industries, Canada, Ltd., Brantford, Ontario.

[3] Span 80, marketed by Atlas Chemical Industries, Canada, Ltd., Brantford, Ontario.

[4] Polyoxyethylene sorbitan monolaurate, marketed as Tween 20 by Atlas Chemical Industries, Canada, Ltd., Brantford, Ontario.

[5] Span 20, marketed by Atlas Chemical Industries, Canada, Ltd., Brantford, Ontario.

trolling the partitioning of a preservative between two phases and its binding to surfactant have also been discussed (2,12,13,15).

When a preservative distributes between oil and aqueous phases, the transfer is governed by its distribution coefficient or partition coefficient, D (23), as follows:

$$C_{oil} = DC_{H_2O} \quad \text{(Eq. 1)}$$

where C_{oil} and C_{H_2O} are the preservative concentrations in oil and water phases at equilibrium. Assuming that the amount in the aqueous phase is active and if some transfers to the oil phase, sufficient additional preservative should be provided to maintain the required concentration in the water. Thus the total preservative, C_{ts}, to be added to a two-phase system can be calculated by knowing the usual concentration required in the aqueous phase (C_{H_2O}), and knowing the volume of each phase (V_{oil} and V_{H_2O}) and the distribution coefficient:

$$\text{total preservative} = C_{H_2O}V_{H_2O} + C_{oil}V_{oil} \quad \text{(Eq. 2)}$$

Substituting the value of C_{oil} from Eq. 1:

$$C_{ts} = C_{H_2O}V_{H_2O} + DC_{H_2O}V_{oil} \quad \text{(Eq. 3)}$$

If the preservative binds to the emulsifier in aqueous solution, the total preservative in water can be given by the following equation as described elsewhere (13, 15):

$$C_{t_{H_2O}} = RC_{H_2O} \quad \text{(Eq. 4)}$$

where C_{tH_2O} and C_{H_2O} are the concentrations of total (free + bound or solubilized) and free preservative in water, and R is the binding or solubilization constant as explained previously (13,15). The total preservative in this study was taken as the free plus the amount bound to or solubilized by the polysorbate–sorbitan ester dispersion.

If the failure of a preservative in a system consisting of oil and water phases emulsified with nonionic surfactants can be attributed to the partitioning as well as the binding of the preservative, the total quantity of the preservative which is theoretically needed for preservation, C_{ts}, can be computed by combining Eqs. 3 and 4 as represented by the following equation:

$$C_{ts} = C_{H_2O}(RV_{H_2O} + DV_{oil}) \quad \text{(Eq. 5)}$$

In the present work it was assumed that the minimum inhibitory concentration of paraben in the absence of surfactants and oil corresponds to the concentration of free paraben.

MATERIALS

The oil phases and preservatives were the same as employed in earlier papers (15, 23). Polysorbate 80, polysorbate 20, sorbitan monooleate, and sorbitan monolaurate were commercial samples.

EXPERIMENTAL PROCEDURES

Determination of Interaction between Preservative and Nonionic Surfactants—Equilibrium dialysis method using a cellophane membrane[6] was employed to determine this interaction, and the general approach and experimental procedure for this part have been detailed in previous communications (12, 24). Preliminary experimentation showed that this membrane (23) was impermeable to sorbitan monooleate. It was found previously to be impermeable to polysorbate 80 (16) and to the oils (23) used in this study. The general procedure was the same as that described earlier (24) with the following exception. A 10-ml. portion of aqueous polysorbate–sorbitan ester dispersion was pipeted into one of the compartments of the dialysis cell (24) and an equal volume of paraben solution was placed into the opposite compartment. In order to increase the total paraben content of the system, it was necessary in some cases to dissolve additional paraben in the dispersion. Overnight agitation of the assembled cells was sufficient for attainment of equilibrium. Any sorption of the parabens by the membrane and dialysis cells was found to be insignificant (25) under experimental conditions.

[6] Fisher Scientific Co.

Table I—R Values Based on the Interaction Between Parabens and Macromolecules at 30°

Paraben	Macromolecules: Proportion	%	HLB	R[a]
Polysorbate 20–Sorbitan Monolaurate				
Methyl	92:8	5	16.0	3.84
Propyl	92:8	5	16.0	16.6
Polysorbate 80–Sorbitan Monooleate				
Methyl	70:30	5	11.8	3.44
Methyl	70:30	4	11.8	3.03
Methyl	58:42	5	10.5	3.15
Propyl	70:30	5	11.8	14.5
Propyl	58:42	5	10.5	15.5

[a] Each R value represents an average of five to eight determinations.

After equilibration, an aliquot was removed from the nonmacromolecule side of the membrane and the concentration of the preservative was ascertained spectrophotometrically (16). Since the quantity of paraben added to the system was known, the amount on the macromolecule side was readily calculated by simple subtraction and the difference was taken as the amount bound or solubilized (12) by the macromolecules.

Preparation of Emulsion—The emulsions were prepared according to the formula 25% oil, 5% w/v emulsifier (4% in the case of castor oil), and sufficient distilled water or culture medium (as described in the following section) to make 100 ml. Because of high partitioning of propylparaben into almond oil and due to its high binding with the emulsifiers, 10% almond oil was used for microbiological work involving propylparaben–almond oil emulsion system. The emulsifier concentrations were selected to give the most stable emulsion with a minimum concentration of the emulsifying agents. Appropriate amounts of polysorbate 80 and sorbitan monooleate were blended to give the HLB (hydrophile–lipophile balance) values of: 10.5 for mineral oil, 16 for oleic acid, and 11.8 for almond oil and castor oil. Preliminary experiments using the procedure described in the literature (26) gave the above HLB values yielding the most stable emulsions. Polysorbate 80 was dissolved in warm aqueous phase, and sorbitan monooleate was dissolved in warm oil phase; both the phases were blended and passed 2–3 times through a hand homogenizer.

Microbiological Procedures—*Aspergillus niger*[7] was selected as the principal microbe, and the synthetic culture medium used in this study was the same as described elsewhere (15).

Procedure for Dispersions—The minimum inhibitory concentrations (MIC) for methyl- and propylparaben were determined in the presence of dispersions of polysorbate 80 and sorbitan monooleate (or polysorbate 20 and sorbitan monolaurate) in culture medium. The emulsifiers were blended and aqueous dispersion was passed twice through a hand homogenizer. The binding data in terms of R values (total preservative/free preservative) as presented in Table I were used to predict the inhibitory concentration for the surfactant dispersions. The method of prediction and the general procedure have been described earlier (15). The growth of *A. niger* was observed for a 2-week period in the form of mycelial hyphae which were readily visible on the surface in the test tube.

Procedure for Emulsions—A convenient method for approximating the free preservative or the preservative in the aqueous phase in an emulsified system is an equilibrium dialysis technique which utilizes a membrane that permits the free passage of the preservative but is impermeable to the emulsifier and the oil. At equilibrium the activity of the preservative would be identical on both sides of the membrane; for reasonably dilute solutions, it may be assumed that the concentration of the free preservative on both sides of the membrane will be essentially equal. Thus, by placing an emulsion on one side of the membrane and determining the concentration of the free preservative on the opposite side, it is possible to approximate the concentration of free preservative in equilibrium with the emulsifier and the oil phase. Assumption is made that the

[7] UAMH No. 1456, Provincial Laboratory of Public Health, Edmonton. Stock culture was grown on slants of potato dextrose agar.

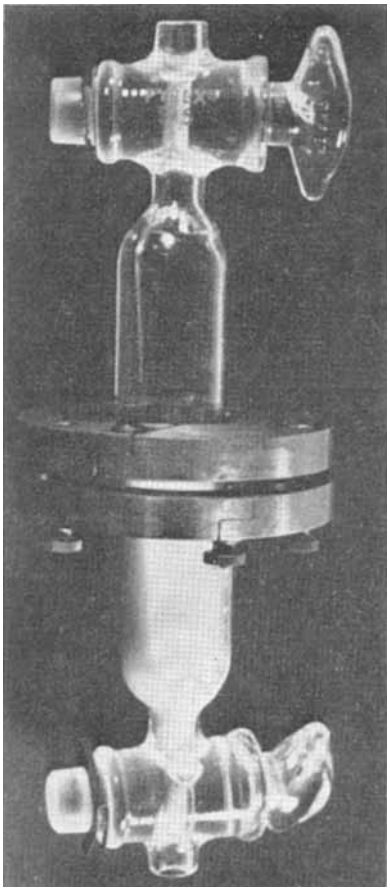


Figure 1—*Assembled dialysis cell containing emulsion in one compartment and culture medium in the other, separated by a cellophane membrane.*

biologic activity of the preservative would parallel the concentration of free preservative.

A glass dialysis cell was constructed for growth studies; it consisted of two halves, each with a capacity of about 30 ml. Each half was prepared by fusing a 6-mm. ground-glass stopcock with the cylindrical end of a flat flange.[8] To assemble the unit, a cellophane membrane was placed between the two halves and secured with the aid of a brass bracket. The assembled dialysis cell is portrayed in Fig. 1. Twenty milliliters of an appropriate concentration (0.065% methylparaben or 0.020% propylparaben) of the paraben in the culture medium was pipeted into one of the compartments. It should be noted that these concentrations correspond to the MIC of these agents for *A. niger* in the culture medium. The entire unit was sterilized by autoclaving at 15 lb. pressure for 15 min. (15). During sterilization, it was necessary to place the dialysis cell in a slant position with both ends kept open to avoid the rupture of the membrane due to pressure buildup at autoclaving temperature. The cell was allowed to attain room temperature with aseptic precautions and the compartment containing preservative solution was closed. Four such dialysis cells were prepared. A predicted inhibitory concentration of the preservative in the emulsion was computed using Eq. 5 and from the knowledge of binding and partition coefficient (23) data. A required amount of the preservative was dissolved in the emulsion. Three additional concentrations of the agent were prepared, one of which was lower and two higher than the above value (15). A 20-ml. sample of the emulsion was pipeted into the empty compartment of the dialysis cell. The unit was closed and equilibrated by overnight agitation at 30°.

The nonemulsion side of the dialysis cell was inoculated with two loopfuls of a spore suspension (15) of *A. niger*. The cells were incubated at 30°, and the growth in the form of mycelial hyphae was observed visually each day for a total period of 2 weeks. In some

[8] Quickfit FG 25.

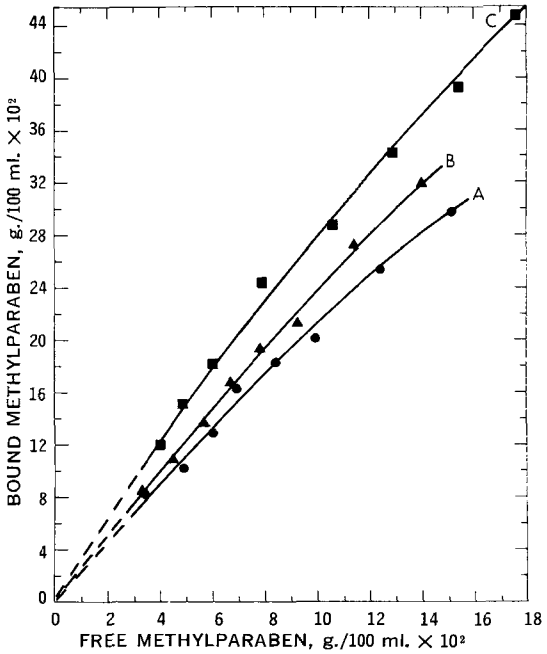


Figure 2—*Adsorption isotherms based on the interaction of methylparaben with 5% aqueous dispersion of surfactants. Key:* A, *polysorbate 80–sorbitan monooleate (58:42), HLB, 10.5;* B, *polysorbate 80–sorbitan monooleate (70:30), HLB, 11.8; and* C, *polysorbate 20–sorbitan monolaurate (92:8), HLB, 16.0.*

cases there was a marked separation of the emulsion during incubation. This separation was prevented by agitation of the cells in a water bath at 30° during the 2-week period. Preliminary tests showed that the membrane was impermeable to *A. niger*, to any other organism which might be present in the emulsion, and to the emulsion itself. At the end of this experiment, a 1-ml. aliquot was moved from the nonemulsion side of the cell and the paraben content analyzed spectrophotometrically. The exact paraben concentration in the emulsion was then calculated.

RESULTS AND DISCUSSION

Interaction of Parabens with Surfactants—The interactions of methyl- and propylparaben with a dispersion of polysorbate 80 and

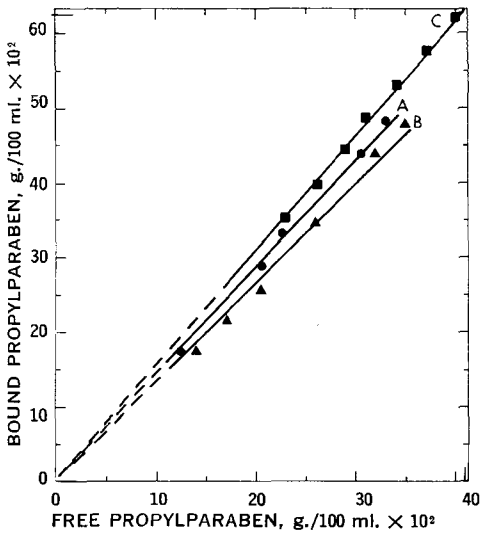


Figure 3—*Adsorption isotherms based on the interaction of propylparaben with 5% dispersion of surfactants. Key:* A, B, *and* C *as defined in Fig. 2.*

sorbitan monooleate (or polysorbate 20 and sorbitan monolaurate) are shown in Figs. 2 and 3. These plots represent typical adsorption isotherms and similar isotherms for a methylparaben–polysorbate system have been reported (12). More useful data for the formulation work are presented in Table I. The ratio, R, of total/free paraben over the concentration range studied as shown in Figs. 2 and 3 is fairly constant. The results also demonstrate that varying proportions of the emulsifiers show a difference in the extent of binding. The R values can easily be obtained from these figures and the average of these values in each case was computed. The average R values are listed in Table I and have been found to be useful in computing the free preservative for a definite concentration of a macromolecule (15).

Inactivation by Surfactants Related to Binding—It has been established that parabens are inactivated in the presence of polysorbate 80 in aqueous solution due to binding or solubilization (12–15). A correlation between the predicted and experimental inhibitory concentrations of methyl- and propylparaben in the presence of 5% dispersion containing various proportions of polysorbate 80 and sorbitan monooleate (or polysorbate 20 and sorbitan monolaurate) is illustrated in Fig. 4. The proportion and the concentrations of the surfactants correspond to the HLB values and the concentrations used in the emulsions for microbiological work. The figure also shows the MIC of the parabens in the absence of the surfactants for the purpose of comparison.

As is evident, there is a significant inactivation of the parabens due to solubilization or binding by the surfactant dispersion. It can be noticed that the magnitude of inactivation increases from methyl- to propylparaben. This is reasonable since propylparaben is bound by the surfactants to a greater extent than methylparaben as shown in Figs. 3 and 4 and Table I (R values).

Correlation of the Binding and Partitioning Data with Inhibitory Concentrations—Figure 5 depicts a comparison of the experimental inhibitory concentrations of methyl- and propylparaben for emulsified systems and the concentrations predicted (using Eq. 5) from a knowledge of MIC in culture medium, the partitioning data, and the binding data. The results show that there is a reasonably good agreement between the predicted and experimental values. The difference in these values can be attributed to small variations in the partition coefficients and R values.

There are two factors responsible for the biologic availability of the antimicrobial agent in the emulsified system, namely the binding to the emulsifier and the partitioning into the oil. If one compares the magnitude of inactivation in Fig. 4 with that in Fig. 5, it is observed that for mineral oil emulsion the inactivation is due solely to the surfactants. This is to be expected since both methylparaben and propylparaben have mineral oil–water partition coefficient values lower than 1 (23). This might also explain the observed lower MIC values for both methyl- and propylparaben in

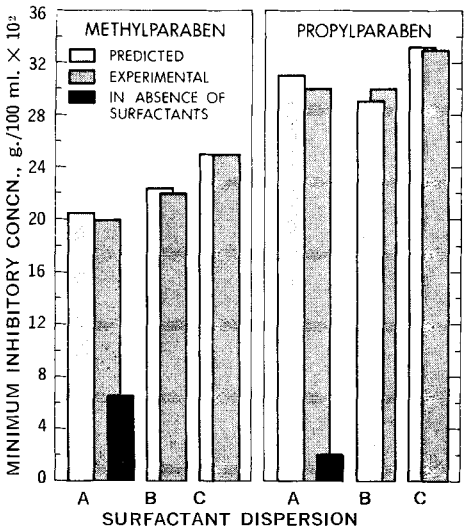


Figure 4—*Comparison of predicted and experimental minimum inhibitory concentrations of parabens in the presence of 5% aqueous dispersion of surfactants. For comparison, the figure also shows the minimum inhibitory concentrations of the parabens in the absence of the surfactants. Key: A, B, and C as defined in Fig. 2.*

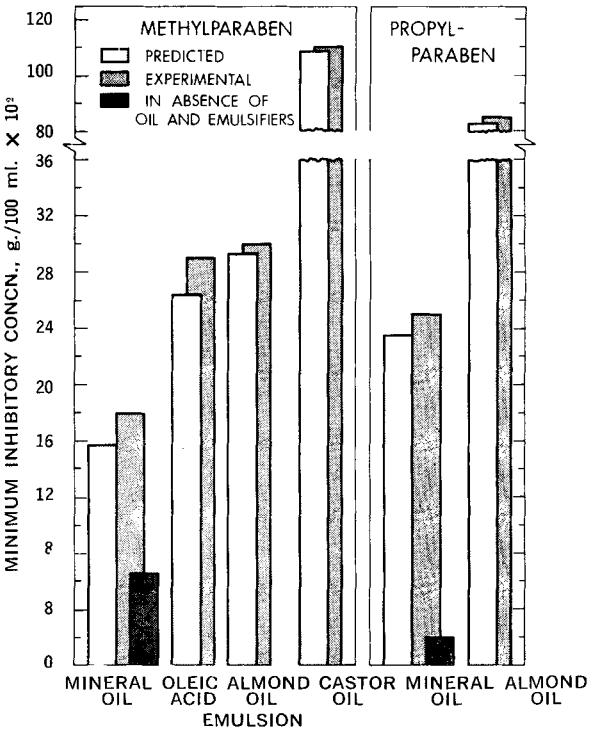


Figure 5—*Comparison of predicted and experimental minimum inhibitory concentrations of parabens in emulsified systems. Concentrations of oils and emulsifiers were 25% and 5% with the following exception: castor oil emulsion contained 4% total emulsifiers, and almond oil emulsion in the case of propylparaben consisted of 10% of the oil.*

the case of mineral oil emulsions (Fig. 5) than those in the aqueous dispersion of the surfactants (Fig. 4).

The magnitude of inactivation of the parabens increased from oleic acid to almond oil to castor oil emulsion, primarily due to the increase in the partition coefficients of these oils. In Part I, the authors reported the oil–water partition coefficients for these oils as 4.4, 7.6, and 58, respectively. From the partitioning data, one would expect a greater difference in the MIC values of oleic acid and almond oil emulsions. However, this difference was reduced due to the higher binding capacity of the emulsifiers (Table I) in the case of oleic acid than those in almond oil emulsion. The greatest inactivation was observed in the case of castor oil emulsion because of the very high partitioning of methylparaben in this oil.

The degrees of binding and partitioning for propylparaben (23) are greater than those of methylparaben. Thus propylparaben was inactivated to a greater extent than methylparaben.

Although emulsions containing nonionic agents can be effectively preserved, there may be some practical limitations. The amount of preservative in emulsified systems with oil of high partition coefficient, and especially when the emulsifier has a high binding tendency, may affect the stability of the product or may be in the range to cause contact dermatitis (27) or may be prohibitive economically. Preliminary work indicated that the stability of emulsions containing nonionic surfactants which have a complexing tendency for parabens was seriously affected with increase in the concentration of paraben (28). Nevertheless, the knowledge of partitioning and binding data should permit evaluation in a product development laboratory so that the most efficient preservative is selected for a particular system. For example, it was found that the almond oil–water partition coefficients of methylparaben and sorbic acid were 7.5 and 3.3. Of the two preservatives, the nonionic emusifier has less affinity for sorbic acid (15). Sorbic acid would be preferred as a preservative for vegetable oil emulsions, although pH of the system must be considered. Furthermore, as suggested by Riegelman (29), rather than using 5–10% of nonionic surfactants as the emulsifier, it would be advisable to attempt to formulate the emulsion with a much lower concentration of emulsifying agent and to attempt to stabilize the system with another agent with less affinity for the preservative.

REFERENCES

(1) L. F. Tice and M. Barr, *J. Soc. Cosmetic Chemists*, **9**, 171 (1958).

(2) H. B. Kostenbauder, in "Developments in Industrial Microbiology," vol. 3, Plenum Press, New York, N. Y., 1962, p. 286.

(3) E. O. Bennett, *ibid.*, p. 273.

(4) M. G. deNavarre, "The Chemistry and Manufacture of Cosmetics," vol. 1, D. Van Nostrand, Princeton, N. J., 1962, p. 257.

(5) D. L. Wedderburn, in "Advances in Pharmaceutical Sciences", vol. 1, H. S. Bean, A. H. Beckett, and J. E. Carless, Eds., Academic, New York, N. Y., 1964, p. 195.

(6) W. B. Hugo and E. G. Beveridge, *J. Appl. Bacteriol.*, **25**, 72(1962).

(7) G. E. Myers and G. A. Leslie, *Can. J. Pharm. Sci.*, **4**, 64(1969).

(8) J. B. Davis, "Petroleum Microbiology," Elsevier, New York, N. Y., 1967, p. 519.

(9) M. Barr and L. F. Tice, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 442(1957).

(10) W. T. Sokolski, C. G. Chidester, and G. E. Honeywell, in "Developments in Industrial Microbiology," vol. 3, Plenum Press, New York, N. Y., 1962, p. 179.

(11) M. Aoki, A. Kamata, I. Yoshioka, and T. Matsuzaki, *J. Pharm. Soc. Japan*, **76**, 939(1956).

(12) N. K. Patel and H. B. Kostenbauder, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 289(1958).

(13) F. D. Pisano and H. B. Kostenbauder, *ibid.*, **48**, 310(1959).

(14) S. M. Blaug and S. S. Ahsan, *J. Pharm. Sci.*, **50**, 441(1961).

(15) N. K. Patel, *Can. J. Pharm. Sci.*, **2**, 77(1967).

(16) *Ibid.*, **2**, 95(1967).

(17) M. G. deNavarre and H. E. Bailey, *J. Soc. Cosmetic Chemists*, **7**, 427(1956).

(18) M. G. deNavarre, *ibid.*, **8**, 68(1957).

(19) L. M. Spalton, "Pharmaceutical Emulsions and Emulsifying Agents," 2nd ed., The Chemist and Druggist, London, England, 1956, p. 100.

(20) H. S. Bean, S. M. Heman-Ackah, and J. Thomas, *J. Soc. Cosmetic Chemists*, **16**, 15(1965).

(21) R. A. Anderson and C. E. Chow, *ibid.*, **18**, 207(1967).

(22) F. Atkins, *Mfg. Chemist*, **21**, 51(1950).

(23) N. K. Patel and J. M. Romanowski, *Can. J. Pharm. Sci.*, **4**, 66(1969).

(24) N. K. Patel and N. E. Foss, *J. Pharm. Sci.*, **54**, 1495(1965).

(25) N. K. Patel, P. C. Sheen, and K. E. Taylor, *ibid.*, **57**, 1370 (1968).

(26) Atlas Chemical Industries, Inc., "The Atlas HLB System," Wilmington, Del., 1962, p. 7.

(27) W. P. Schorr and A. H. Mahajerin, *Arch. Dermatol.*, **93**, 721(1966).

(28) N. K. Patel, unpublished data.

(29) S. Riegelman, "The Pharmaceutical Sciences," Second Annual Visiting Lecturer Series, University of Texas, Austin, Tex., 1959, p. 135; through *Ref. 2*.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton 7, Alberta, Canada.*

Accepted for publication October 8, 1969.

Presented in part at the XVth Canadian Conference on Pharmaceutical Research, Regina, Canada, August 1968.

This work was supported by research grant GR-766 from the University of Alberta General Research Fund.

The authors wish to thank Miss Marilynne S. Bergos for technical assistance during the initial phase of the work and Dr. Gordon E. Myers, Professor (Pharmaceutical Microbiology), for helpful suggestions.

* Present address: Frank W. Horner Limited, Research Laboratories, Montreal 307, P.Q., Canada.

Kinetics of Hydrous Aluminum Oxide Conversion in Mixtures of Amorphous Alumina Gels of Various Acid Reactivities

STANLEY L. HEM*, EMANUEL J. RUSSO, RICHARD J. HARWOOD†, BEHRAM H. TEJANI, SURENDRA M. BAHAL, and RALPH S. LEVI

Abstract □ Mixtures of two amorphous alumina gels of different acid reactivity change physically and chemically upon aging until a constant state is reached. The gels, when individually aged, retain their initial properties. The end-point appearance, viscosity, X-ray diffraction pattern, DTA thermogram, acid-insoluble fraction, and acid reactivity of the mixture are identical to those of the gel having the lower acid reactivity. The change in properties appears to be due to the conversion of the gel having the higher reactivity into the less reactive form. The rate of conversion is first-order, temperature-dependent, and directly dependent on the initial concentration of the less reactive gel.

Keyphrases □ Hydrous aluminum oxide conversion—kinetics □ Alumina gels, amorphous, effect—Al_2O_3 conversion □ X-ray diffraction—identity □ Calorimetry—anlysis, gels

The physical and chemical properties of hydrous aluminum oxide depend on the nature of the reactants used in its formation (1, 2), the pH and temperature of its precipitation (3–5), and the conditions under which it is aged (6, 7). The effects of mixing alumina gels of different physical and chemical properties have not been reported. Preliminary experiments in the authors' laboratories have indicated that when amorphous alumina gels of various acid reactivities are mixed, there are changes in the physical and chemical properties of the mixtures during aging. The purpose of the present study was to examine these changes in greater depth.

EXPERIMENTAL

Source of Gels—One alumina gel was prepared by the reaction of aluminum chloride with sodium carbonate and sodium bicarbonate at 25° and pH 5.8, according to the procedure of Papée *et al.* (8), and washed with deionized water until the concentration of chloride ion in the filtrate, as determined by the Volhard method (9), was less than 0.1%. This gel (I) possessed the full theoretical acid reactivity in terms of its Al_2O_3 content. Gels with reactivity less

REFERENCES

(1) L. F. Tice and M. Barr, *J. Soc. Cosmetic Chemists*, **9**, 171 (1958).

(2) H. B. Kostenbauder, in "Developments in Industrial Microbiology," vol. 3, Plenum Press, New York, N. Y., 1962, p. 286.

(3) E. O. Bennett, *ibid.*, p. 273.

(4) M. G. deNavarre, "The Chemistry and Manufacture of Cosmetics," vol. 1, D. Van Nostrand, Princeton, N. J., 1962, p. 257.

(5) D. L. Wedderburn, in "Advances in Pharmaceutical Sciences", vol. 1, H. S. Bean, A. H. Beckett, and J. E. Carless, Eds., Academic, New York, N. Y., 1964, p. 195.

(6) W. B. Hugo and E. G. Beveridge, *J. Appl. Bacteriol.*, **25**, 72(1962).

(7) G. E. Myers and G. A. Leslie, *Can. J. Pharm. Sci.*, **4**, 64(1969).

(8) J. B. Davis, "Petroleum Microbiology," Elsevier, New York, N. Y., 1967, p. 519.

(9) M. Barr and L. F. Tice, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 442(1957).

(10) W. T. Sokolski, C. G. Chidester, and G. E. Honeywell, in "Developments in Industrial Microbiology," vol. 3, Plenum Press, New York, N. Y., 1962, p. 179.

(11) M. Aoki, A. Kamata, I. Yoshioka, and T. Matsuzaki, *J. Pharm. Soc. Japan*, **76**, 939(1956).

(12) N. K. Patel and H. B. Kostenbauder, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 289(1958).

(13) F. D. Pisano and H. B. Kostenbauder, *ibid.*, **48**, 310(1959).

(14) S. M. Blaug and S. S. Ahsan, *J. Pharm. Sci.*, **50**, 441(1961).

(15) N. K. Patel, *Can. J. Pharm. Sci.*, **2**, 77(1967).

(16) *Ibid.*, **2**, 95(1967).

(17) M. G. deNavarre and H. E. Bailey, *J. Soc. Cosmetic Chemists*, **7**, 427(1956).

(18) M. G. deNavarre, *ibid.*, **8**, 68(1957).

(19) L. M. Spalton, "Pharmaceutical Emulsions and Emulsifying Agents," 2nd ed., The Chemist and Druggist, London, England, 1956, p. 100.

(20) H. S. Bean, S. M. Heman-Ackah, and J. Thomas, *J. Soc. Cosmetic Chemists*, **16**, 15(1965).

(21) R. A. Anderson and C. E. Chow, *ibid.*, **18**, 207(1967).

(22) F. Atkins, *Mfg. Chemist*, **21**, 51(1950).

(23) N. K. Patel and J. M. Romanowski, *Can. J. Pharm. Sci.*, **4**, 66(1969).

(24) N. K. Patel and N. E. Foss, *J. Pharm. Sci.*, **54**, 1495(1965).

(25) N. K. Patel, P. C. Sheen, and K. E. Taylor, *ibid.*, **57**, 1370 (1968).

(26) Atlas Chemical Industries, Inc., "The Atlas HLB System," Wilmington, Del., 1962, p. 7.

(27) W. P. Schorr and A. H. Mahajerin, *Arch. Dermatol.*, **93**, 721(1966).

(28) N. K. Patel, unpublished data.

(29) S. Riegelman, "The Pharmaceutical Sciences," Second Annual Visiting Lecturer Series, University of Texas, Austin, Tex., 1959, p. 135; through *Ref. 2*.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton 7, Alberta, Canada.*

Accepted for publication October 8, 1969.

Presented in part at the XVth Canadian Conference on Pharmaceutical Research, Regina, Canada, August 1968.

This work was supported by research grant GR-766 from the University of Alberta General Research Fund.

The authors wish to thank Miss Marilynne S. Bergos for technical assistance during the initial phase of the work and Dr. Gordon E. Myers, Professor (Pharmaceutical Microbiology), for helpful suggestions.

* Present address: Frank W. Horner Limited, Research Laboratories, Montreal 307, P.Q., Canada.

Kinetics of Hydrous Aluminum Oxide Conversion in Mixtures of Amorphous Alumina Gels of Various Acid Reactivities

STANLEY L. HEM*, EMANUEL J. RUSSO, RICHARD J. HARWOOD†, BEHRAM H. TEJANI, SURENDRA M. BAHAL, and RALPH S. LEVI

Abstract □ Mixtures of two amorphous alumina gels of different acid reactivity change physically and chemically upon aging until a constant state is reached. The gels, when individually aged, retain their initial properties. The end-point appearance, viscosity, X-ray diffraction pattern, DTA thermogram, acid-insoluble fraction, and acid reactivity of the mixture are identical to those of the gel having the lower acid reactivity. The change in properties appears to be due to the conversion of the gel having the higher reactivity into the less reactive form. The rate of conversion is first-order, temperature-dependent, and directly dependent on the initial concentration of the less reactive gel.

Keyphrases □ Hydrous aluminum oxide conversion—kinetics □ Alumina gels, amorphous, effect—Al_2O_3 conversion □ X-ray diffraction—identity □ Calorimetry—anlysis, gels

The physical and chemical properties of hydrous aluminum oxide depend on the nature of the reactants used in its formation (1, 2), the pH and temperature of its precipitation (3–5), and the conditions under which it is aged (6, 7). The effects of mixing alumina gels of different physical and chemical properties have not been reported. Preliminary experiments in the authors' laboratories have indicated that when amorphous alumina gels of various acid reactivities are mixed, there are changes in the physical and chemical properties of the mixtures during aging. The purpose of the present study was to examine these changes in greater depth.

EXPERIMENTAL

Source of Gels—One alumina gel was prepared by the reaction of aluminum chloride with sodium carbonate and sodium bicarbonate at 25° and pH 5.8, according to the procedure of Papée *et al.* (8), and washed with deionized water until the concentration of chloride ion in the filtrate, as determined by the Volhard method (9), was less than 0.1%. This gel (I) possessed the full theoretical acid reactivity in terms of its Al_2O_3 content. Gels with reactivity less

Table I—Initial Properties of Alumina Gels I and II and Their 1:1 Mixture

Property	Alumina Gel I	Alumina Gel II	1:1 Mixture of Alumina Gels I and II
Physical appearance of 4% suspension	White, dense suspension	Opaque semisolid	White, dense suspension
Form	Amorphous	Amorphous	Amorphous
Percentage of theoretical acid-consuming capacity	100	25	62.5
Appearance after acid-consuming capacity test	Clear solution	Insoluble residue	Slight insoluble residue
Viscosity at 25° (c.p.s.)	140	3700	800
Endotherms by DTA[a]	140°	140°, 320°	140°, 320°

[a] Differential thermal analysis.

Table II—Acid Reactivity, Both Initially and after Storage at Elevated Temperatures, of Mixtures Containing Various Initial Ratios of Alumina Gels I and II

Initial Concentration of II in Mixture %	Initial Acid Reactivity[a], %	End-Point Acid Reactivity[a] when Aged at 35°	45°	60°
10	92.5	25%	25%	25%
20	85	25%	25%	25%
40	70	25%	25%	25%
50	62.5	25%	25%	25%

[a] Expressed as percent of theoretical acid-consuming capacity.

than theory were prepared by the addition of strong ammonia solution NF to aluminum chloride solutions at 25° and controlled pH. Gels reacting at 25% of theory (II) and 70% of theory (III) were obtained by precipitating at pH 7.7 and 4.9, respectively; washing as in (I); and aging until the end-point reactivity was reached (10). Aluminum hydroxide compressed gel (F-5000)[1] (IV) was obtained through normal commercial channels. This gel possesses 10% of theoretical acid reactivity. Aluminum hydroxide compressed gel (F-500)[1] (V) and aluminum hydroxide gel[2] (VI) were likewise obtained from commercial sources and were found to react stoichiometrically with 0.1 *N* HCl.

Gel Mixtures—Binary mixtures containing 4% w/w Al_2O_3 were prepared from the gels so as to contain various concentrations of the less acid-reactive components. Appropriate weights of I, II, and III were used, depending on the Al_2O_3 content. Gels IV, V, and VI were diluted with distilled water to form 4% w/w suspensions prior to the preparation of the mixtures.

The mixtures were aged in sealed, screw-capped glass bottles at 60, 45, and 35°. The sample bottles were tared and the weight checked at every assay. No weight losses were noted.

Tests—The acid reactivity of each gel was described by its percentage of the theoretical acid-consuming capacity, based on the stoichiometric reaction of the Al_2O_3 with 0.1 *N* HCl. The precision of the USP acid-consuming capacity test (11) was improved by titrating to pH 3.5 rather than by using bromophenol blue T.S.

The X-ray diffraction pattern of each gel was obtained in the colloidal state by irradiating a sample in dialyzer tubing.

The insoluble gel fraction which remained after the acid-consuming capacity test was collected by filtration on a tared 0.45-μ Millipore filter.[3] Two filters were used so that one could serve as a control. The control filter and the filter with the residue were dried to constant weight at 90° in a vacuum desiccator. The results were reported as the residue obtained from 1 g. of alumina gel.

The viscosity of the alumina gels was determined at 25° using the Haake Rotovisco,[4] system mv. II, head 500, speed 162.

Thermograms of each alumina gel were obtained using a DuPont model 900 differential thermal analyzer.[5] The gels were dried at 25° in a vacuum desiccator. A 2- to 3-mg. sample was introduced into a microcell and heated at a rate of 20° per min. to obtain the DTA thermograms.

RESULTS AND DISCUSSION

Mixtures of I and II—*Initial Properties*—Gels I and II differed physically and chemically in several significant aspects, as seen in Table I. X-ray diffractometer scans indicate that the solid material in the gels is noncrystalline. The broad diffuse band is typical of

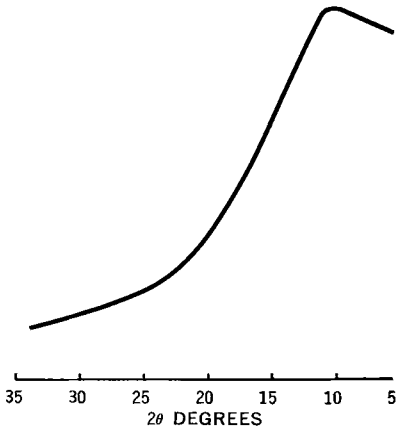


Figure 1—*Initial X-ray diffraction pattern of Alumina Gel I.*

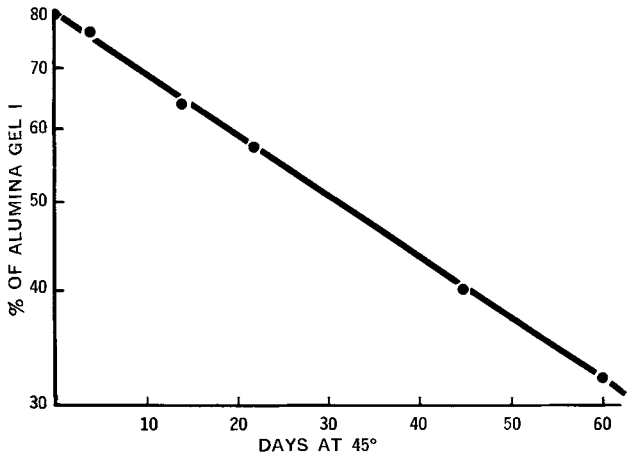


Figure 2—*Change in concentration of Alumina Gel I during aging of a mixture of 80% I and 20% II at 45°.*

[1] Reheis Chemical Co., Division of Armour Pharmaceutical Co., Chicago, Ill.
[2] Merlum No. 8031, Merck Chemical Division, Merck & Co., Inc., Rahway, N. J.
[3] Millipore Filter Corp., Bedford, Mass.
[4] Brinkmann Instruments, Inc., Great Neck, N. Y.
[5] DuPont Instrument Products Division, Wilmington, Del.

Table III—Comparison of the Properties of an Aged 1:1 Mixture of Alumina Gels I and II with Those of II

Property	1:1 Mixture		Alumina Gel II
	Initial	Aged at 45° to End-Point Properties	
Physical appearance of 4% suspension	White, dense suspension	Opaque semisolid	Opaque semisolid
Form	Amorphous	Amorphous	Amorphous
Percentage of theoretical acid-consuming capacity	62.5	25	25
Appearance after acid-consuming capacity test	Slight insoluble residue	Insoluble residue	Insoluble residue
Viscosity at 25° (c.p.s.)	800	3700	3700
Endotherms by DTA	140°, 320°	140°, 320°	140°, 320°

scattering by amorphous material (Fig. 1). Figure 1 is typical of the scans obtained for both individual gels and mixtures throughout this study. These properties of the individual gels did not change upon aging, even at elevated temperatures. The 1:1 mixture of I and II had properties equivalent to the sum of those of the individual gels. Subsequent examinations of this mixture were conducted to determine the changes which occurred during aging.

Changes in Acid Reactivity upon Aging—At 45°, all mixtures lost acid reactivity until a minimum (end-point) level was reached. For the 1:1 mixture of Gels I and II, this end-point level was 25% of theory and was observed after aging for 12 days at 45°. For mixtures of Gels I and II containing 10, 20, 40, or 50% of Gel II, prepared and aged at 35, 45, and 60° (Table II), the end-point reactivities were also 25% of theory. Since the end-point reactivity was independent of the temperature or the ratio of I to II, but was always identical to the acid reactivity of II, it is assumed that I converts to II during the aging of the mixtures.

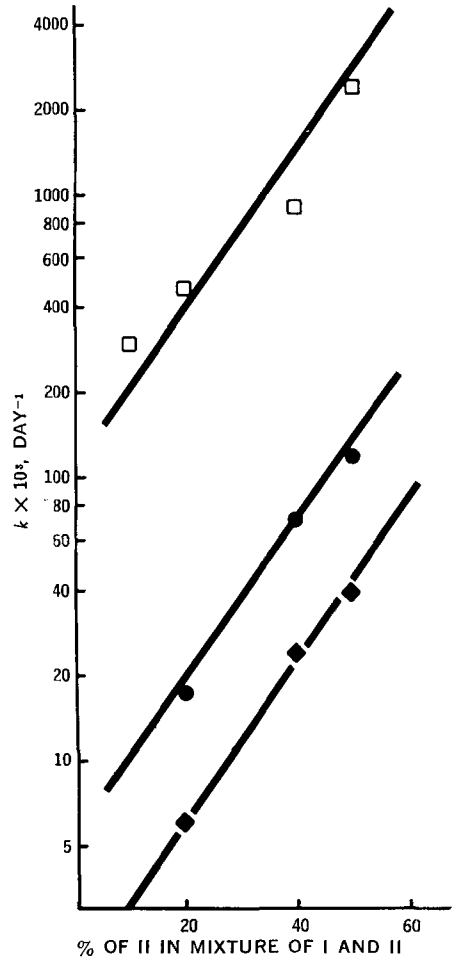


Figure 3—*Effect of percentage of II on the rate of change of I in a mixture of Alumina Gels I and II. Key:* □, *60°;* ●, *45°; and* ■, *35°.*

Under this assumption, the reactivity of mixtures of Gels I and II would be related to the amount of each present at time t by the following equation:

$$R_t = \frac{100I_t + 25\,[II_{init.} + (I_{init.} - I_t)]}{100}$$

where R_t = percent of theoretical acid-consuming capacity of the mixture at time t; I_t = percent of I unchanged at time t; $II_{init.}$ = percent of II in the initial mixture; $I_{init.}$ = percent of I in the initial mixture.

By rearranging the above equation, the amount of I present at any time in a mixture of I and II can be calculated and plotted. Such a plot shows that in a mixture initially containing 80% of I and 20% of II, I converts to II according to apparent first-order kinetics (Fig. 2).

Rate constants for the conversion of I to II in mixtures with various initial concentrations of II were calculated for mixtures aged at 35, 45, and 60°. The rate of conversion of I to II increased at all temperatures and was directly related to the initial concentration of II (Fig. 3).

The heat of activation for the conversion of I to II in a 1:1 mixture was calculated from Fig. 4 to be 26.2 kcal. mole^{-1}.

The effect of a change in temperature at which the acid reactivity test was conducted for the end-point mixture of I and II and for Gel II further indicates a conversion of I to II during aging. For example, the reactivity of II, which is 25% of theory at a test temperature of 37°, increased to 29.3% of theory when tested at 60°. The reactivity

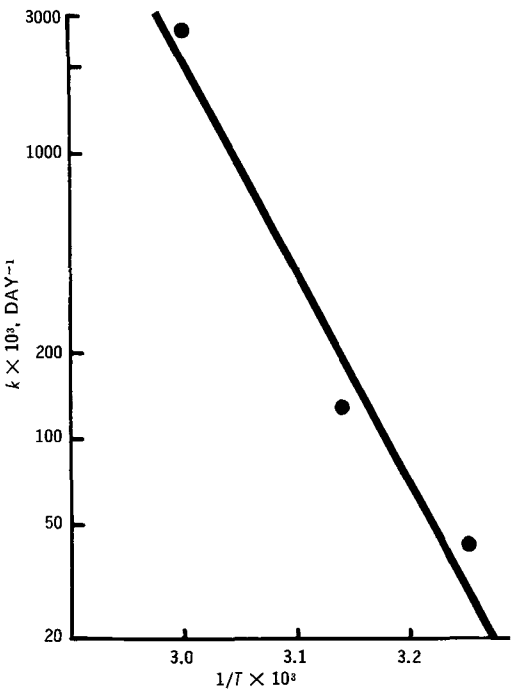


Figure 4—*Effect of temperature on the rate of change of I in a 1:1 mixture of Alumina Gels I and II.*

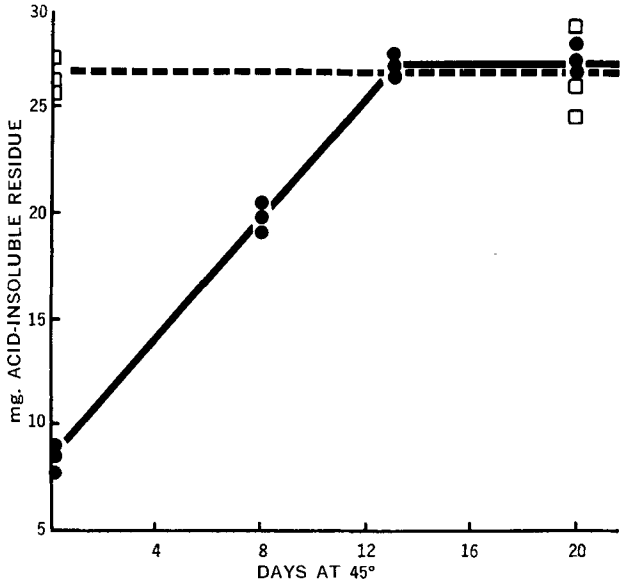


Figure 5—*Effect of aging of alumina gels on the weight of the acid-insoluble residues. Key:* ●, *2:1 mixture of Alumina Gels I and II; and* □, *II.*

of the end-point mixture of I and II also increased from 25 to 29.3% of theory when the test temperature was increased from 37 to 60°.

Effect of Aging on Other Physical and Chemical Properties—Table III shows that the physical and chemical properties of the 1:1 mixture of Gels I and II changed during aging at 45° until they reached the indicated end-point values. The end-point physical appearance, acid reactivity, residue remaining after the acid-consuming capacity test, and viscosity of the mixture were identical to those of Gel II. The same changes occurred in the 1:1 mixture of Gels I and II aged at 35 and 60° and in various other mixtures of I and II aged at various temperatures. The only effect of temperature or ratio of gels was to change the rate of conversion of I to II. In each instance, identical end-point properties were observed.

Acid-Insoluble Fraction after Aging—All gels having less than the theoretical acid-consuming capacity left an acid-insoluble residue at the completion of the acid-consuming capacity test. This residue was believed to consist of completely acid-refractory aluminum oxide. Its weight was determined during the aging at 45° of a 2:1 mixture of Gels I and II and of Gel II. The values obtained from triplicate assays, and the lines determined by least-squares analyses which best fit these points, are given in Fig. 5.

As shown in the figure, 8.6 mg. of acid-insoluble residue was recovered from 1 g. of a freshly prepared 2:1 mixture of Gels I and II containing 4% w/w Al_2O_3. This agrees well with the expected result of 10 mg., which assumes that I leaves no residue and that II is 75% acid-insoluble. The weight of acid-insoluble residue increased as the mixture aged at 45° until a constant value of 27 mg. was reached, beginning at 13 days, which is approximately the same time that the acid reactivity of the mixture reached its end-point reactivity of 25% of theory. From this point on, the quantity of acid-insoluble residue recovered from the mixture was virtually identical to the weight of residue recovered from a 4% w/w suspension of II. For II, the acid-insoluble residue remained constant during aging. These data again indicate a conversion of I to II in the 1:1 mixture of Gels I and II.

Properties of Mixtures of Other Alumina Gels—To test the general hypothesis that in mixtures of amorphous alumina gels of various acid reactivities, the more reactive gel is converted into the less reactive, a series of mixtures were prepared and aged at 60° (Table IV). In all cases when two alumina gels of different acid reactivities were mixed, the mixture lost reactivity until it reached a constant end-point reactivity equal to that of the less reactive gel.

Clearly, in an aged mixture, the more acid-reactive gel is converted into the less reactive form. Since these gels are amorphous, the conversion does not involve a change in form but instead may be analogous to the polymorphic transformations which occur as a system converts to its more stable form upon aging. It can be assumed that a completely acid-refractory form is thermodynamically the most stable, and that acid reactivity and related properties are inversely proportional to the thermodynamic stability. Thus the observed conversion of the system reflects its attainment of the most stable state.

Table IV—End-Point Acid Reactivity of Aged 1:1 Alumina Gel Mixtures[a]

Alumina Gels in Mixture, together with % of Theoretical Acid-Consuming Capacity of Each Gel (in Parentheses)	% Theoretical Acid-Consuming Capacity after Mixture Aged at 60° for 2 Weeks	3 Weeks
I(100):II(25)	25	25
I(100):III(70)	68	69
III(70):II(25)	30	25
I(100):IV(10)	14	12
V(100):IV(10)	8	8
VI(100):IV(10)	8	8

[a] All gels and the aged mixtures in this table were amorphous.

CONCLUSIONS

Mixtures composed of two individually stable amorphous alumina gels of different acid reactivities change physically and chemically upon aging until they reach a constant state. At this point, their properties are identical to those of the less acid-reactive gel. The same constant state is obtained independent of temperature, but the rate of change depends on temperature and on the initial concentration of the less reactive gel. It is hypothesized that the more acid-reactive gel converts to the less reactive gel according to apparent first-order kinetics. Since no changes in form are observed, the change may be thought of as analogous to the polymorphic transformations which occur as a system converts to its most stable state.

REFERENCES

(1) P. H. Hsu and T. F. Bates, *Mineral. Mag.*, **33,** 749(1964).

(2) L. Erdey and F. Paulik, *Acta Chim. Acad. Sci. Hung.*, **7,** 45(1955).

(3) H. Ginsberg, W. Hüttig, and H. Stiehl, *Z. Anorg. Allgem. Chem.*, **318,** 238(1962).

(4) K. Pohl, D. Meissner, and W. Steinert, *ibid.*, **343,** 39(1966).

(5) G. C. Bye and J. G. Robinson, *Kolloid-Z.*, **198,** 53(1964).

(6) R. C. MacKenzie, R. Meldau, and J. A. Gard, *Mineral. Mag.*, **33,** 145(1962)

(7) P. Souza Santos, A. Vallejo-Freire, and H. L. Souza Santos, *Kolloid-Z.*, **133,** 101(1953).

(8) D. Papée, R. Tertian, and R. Biais, *Bull. Soc. Chim. France*, **1958,** 1301.

(9) H. A. Laitinen, "Chemical Analysis," McGraw-Hill, New York, N. Y., 1960, pp. 214–216.

(10) S. L. Hem, E. J. Russo, S. M. Bahal, and R. S. Levi, to be published.

(11) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 26.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Research and Development Department, Wyeth Laboratories Inc., Philadelphia, PA 19101*

Accepted for publication October 13, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors wish to thank Mr. J. A. Lash, Wyeth Laboratories, Inc., for chemical analyses and Mr. N. J. DeAngelis, Wyeth Laboratories, Inc., for thermal analyses. They also acknowledge the participation of Dr. T. H. Doyne, Department of Chemistry, Villanova University, Villanova, PA 19085, in analyzing the X-ray diffraction patterns.

* Present address: School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907

† Present address: Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104

Determination of Radioactive-Labeled Codeine, Morphine, Dihydromorphine, and Their Metabolites in Biological Materials

S. Y. YEH and L. A. WOODS

Abstract □ Recoveries of ^{14}C-labeled morphine from aqueous solutions containing 1 to 500 ng. of drug (containing no ammonium ions) by extraction with three volumes of ethylene chloride containing 10, 20, or 30% of *n*-amyl alcohol were 82.9, 91, and 96%, respectively. Dihydromorphine was 79.1% extracted by ethylene chloride containing 30% *n*-amyl alcohol, and codeine, 97%, with all the above mentioned solvent mixtures. Complete hydrolysis of conjugated metabolites of morphine, codeine, or dihydromorphine was achieved by autoclaving samples in a portable autoclave at 18–20 lb. pressure for 2 hr. in a solution of 1.1 *N* HCl, or for 1 hr. in 2.2 *N* HCl, or 0.5 hr. in 3.3 *N* HCl. The conjugated metabolites were also completely hydrolyzed by treatment in a steam-jacketed autoclave at 20 lb. pressure for 0.5 hr. in 2.2 *N* HCl or 1 hr. in 1.1 *N* HCl. A procedure is described for the estimation of codeine and its metabolites, including morphine, in the same samples.

Keyphrases □ Codeine, metabolites—determination, biological samples □ Morphine, metabolites—determination, biological samples □ Dihydromorphine, metabolites—determination, biological samples □ Radioctive labeling—codeine, morphine, dihydromorphine, metabolites □ Scintillometry, liquid—analysis

Methods for determining codeine metabolites in biological samples have been described (1, 2). Johannesson and Woods (3) modified the procedure for estimating radioactive morphine (4, 5) to quantitate codeine metabolites in biological samples. Low recoveries of codeine metabolites, especially conjugated codeine and conjugated morphine, were obtained when using the hydrolysis procedure of Chernov and Woods (5) for biological samples. Incomplete hydrolysis of conjugated morphine in the early studies has been noted (6). The present paper describes conditions for complete hydrolysis of conjugated morphine, conjugated codeine, and conjugated dihydromorphine, and maximum extraction of codeine, morphine, and dihydromorphine in biological materials.

EXPERIMENTAL

Reagents—All reagents used were analytical grade. Toluene counting solution: 100 mg. of 1,4-bis-2(5-phenyloxazolyl)benzene (POPOP), and 3 g. of 2,5-diphenyloxazole (PPO) per liter of toluene; phenethylamine–methanol–toluene counting solution: 100 mg. of 1,4-bis-2(5-phenyloxazolyl)benzene (POPOP), 3 g. of 2,5-diphenyloxazole (PPO), 270 ml. of redistilled colorless phenethylamine (stored in an amber bottle less than 1 month after redistillation), 270 ml. of absolute methanol, and toluene to make 1 l.; naphthalene–dioxane counting solution (7): 60 g. of naphthalene, 4 g. of PPO, 200 mg. of POPOP, 20 ml. of ethylene glycol, 100 ml. of methanol, and dioxane to make 1 l.; NaOH solutions: 1.0–10.0 *N;* morphine carrier, 1 mg./ml. of morphine sulfate in distilled H_2O; codeine carrier, 1 mg./ml. of codeine phosphate in distilled H_2O; dihydromorphine carrier, 1 mg./ml. of dihydromorphine HCl in distilled H_2O; *N*-$^{14}CH_3$-morphine (5) solution in 0.01 *N* HCl; *N*-$^{14}CH_3$-codeine (8) in 0.01 *N* HCl; *N*-$^{14}CH_3$-dihydromorphine (9) in 0.01 *N* HCl; 40 and 4% anhydrous K_2HPO_4 solutions.

Determination of Free and Conjugated Morphine—*Estimation of Free Morphine*—Duplicates of 1-ml. aliquots of biological samples were placed in 40-ml. centrifuge tubes containing 1 ml. of H_2O and 0.5 ml. of nonlabeled morphine carrier. The samples were buffered with 2 ml. of 40% K_2HPO_4 solution, shaken with 15 ml. of ethylene chloride containing 30% *n*-amyl alcohol in an International shaker at 280–300 oscillations per minute for 30 min., and centrifuged at 1700 r.p.m. for 15 min. The aqueous phase was removed by aspiration.[1] The organic layer was shaken by hand for 30 sec. with 5 ml. of 4% K_2HPO_4 solution. After the mixture was centrifuged for 15 min., the aqueous phase was removed by aspiration.

Ten-milliliter aliquots of the organic phase from each sample were transferred to 20-ml. scintillation-counting vials and evaporated to dryness on a Fisher slide-warmer at 55°. The residue was dissolved in 0.8 ml. *n*-amyl alcohol, 10 ml. of toluene counting solution was added, and the radioactivity of each sample was determined in a Nuclear-Chicago model 720 series liquid scintillator spectrometer for 3 × 10 min. Controls for background and known amounts of labeled morphine were run concurrently to serve as a check on the technique and the performance of the counter.

Known concentrations of 1–500 ng. of *N*-$^{14}CH_3$-morphine were carried through the described extraction procedure and a linear relationship was obtained with the curve passing through the origin when net counts per minute (c.p.m.) or disintegrations per minute (d.p.m.) were plotted against the concentration. From the graph a factor converting c.p.m. or d.p.m. values to concentration was obtained for use in calculation of the drug present in biological materials.

Estimation of Total (Free plus Conjugated) Morphine—Duplicates of 1-ml. aliquots of biological samples, obtained after administration of morphine, were placed in 40-ml. centrifuge tubes containing 0.5 ml. of nonlabeled morphine. The mixture was autoclaved 1 hr. in 2.2 *N* HCl (final normality) at 18–20 lb. pressure in a portable autoclave (All-American portable pressure sterilizer, No. 25X, Wisconsin Aluminum Foundry Co.) and remained in the autoclave until the pressure dropped to 1–2 lb. The hydrolyzed samples were first carefully adjusted to pH 9 by saturating with $NaHCO_3$ powder and then buffered with 2 ml. of 40% K_2HPO_4 solution. The procedure for the determination of free morphine was then followed.

Conjugated morphine was calculated from the difference between the autoclaved and nonautoclaved biological samples.

Determination of Free Dihydromorphine and Conjugated Dihydromorphine—Free and conjugated dihydromorphine in biological specimens were determined according to the above procedure except using 0.5 ml. of nonlabeled dihydromorphine carrier instead of morphine carrier.

Determination of Codeine and Its Metabolites—*Estimation of Free Codeine plus Free Morphine*—Free codeine and the free morphine, metabolite of codeine in the biological samples, were estimated according to the procedure for determination of free morphine except that 0.5 ml. of nonlabeled codeine carrier instead of morphine carrier was used.

Estimation of Free Codeine—Free codeine in the biological samples was determined by modifying slightly the method for determination of free morphine and free codeine described above. Two milliliters of 0.5 *N* NaOH solution, instead of 2 ml. of 40% K_2HPO_4 solution, was added to the sample. The apparent morphine remaining in the aqueous phase as sodium morphinate was removed by aspiration. The organic phase was transferred to another tube and shaken with 4 ml. of 0.1 *N* NaOH solution instead of 4 ml. of 4% K_2HPO_4 solution. The counting procedure for determination of morphine was then followed.

Estimation of Total (Free plus Conjugated) Codeine and Total Morphine—Duplicates of 1-ml. aliquots of the biological samples were placed in 40-ml. centrifuge tubes containing 0.5 ml. nonlabeled

[1] If radioactivity remaining in the aqueous was to be estimated, this phase was completely separated and 0.5-ml. aliquots were counted with 10 ml. naphthalene–dioxane or phenethylamine–methanol–toluene solution.

Table I—Recovery of N-$^{14}CH_3$-morphine, N-$^{14}CH_3$-dihydromorphine, and N-$^{14}CH_3$-codeine in Aqueous Solution with and without NH_4OH by Extraction with Ethylene Chloride Containing Varying Amounts of *n*-Amyl Alcohol[a]

Solvents Containing *n*-Amyl Alcohol	Percentage of Added Radioactivity, Mean ± *SD* Extracted into the Organic Phase	Remained in the Aqueous Phase
I. Samples contained no NH_4OH		
A. Morphine		
10%	82.9 ± 3.3	11.4 ± 0.5
20%	91.0 ± 3.3	5.2 ± 0.3
30%	96.0 ± 1.8	4.8 ± 0.3
B. Dihydromorphine		
30%	79.1 ± 0.8	9.0 ± 0.2
C. Codeine		
30%[b]	99.8–103.2	2.9–3.1
II. Samples contained 1 ml. of 1.8 *N* NH_4OH		
A. Morphine		
10%	67.9 ± 1.8	17.8 ± 0.2
20%	88.2 ± 2.9	9.3 ± 0.5
30%	93.8 ± 1.0	8.1 ± 0.5
B. Dihydromorphine		
30%	78.3 ± 0.6	9.0 ± 0.2
III. Samples contained 1 ml. of 3.6 *N* NH_4OH		
A. Morphine		
10%[c]	33.5 ± 4.6	45.1 ± 1.6[d]
20%	56.5 ± 0.9	24.4 ± 0.3
30%	66.4 ± 0.7	17.0 ± 0.1
B. Dihydromorphine		
30%	74.9 ± 1.6	11.3 ± 0.5
C. Codeine		
10%	96.9 ± 1.9	3.7 ± 0.4
20%	96.3 ± 0.7	3.0 ± 0.5
30%	97.5 ± 2.2	3.8 ± 0.1

[a] Samples contained 1 ml. of N-$^{14}CH_3$-morphine (5080 ± 107 d.p.m.) or N-$^{14}CH_3$-dihydromorphine (8191 ± 156 d.p.m.), or N-$^{14}CH_3$-codeine (4778 ± 79 d.p.m.) and 0.5 ml. nonlabeled drug carrier (1 mg./ml.), 2 ml. of 40% K_2HPO_4 solution and 1 ml. of distilled water or ammonium hydroxide solution as indicated. Quadruplicate samples were used for each experiment. [b] Duplicate samples were analyzed. [c] Four experiments (16 samples) were carried out. [d] Radioactivity in the aqueous phase of 4 out of 16 samples was measured in duplicate.

codeine carrier. The mixture was autoclaved in 2.6 *N* HCl at 18–20 lb. pressure in a portable autoclave for 1 hr. The hydrolyzed samples were carefully adjusted to pH 9.0 by saturating with $NaHCO_3$ powder and buffered with 2 ml. of 40% K_2HPO_4 solution. The procedure for estimation of free codeine and free morphine was then followed.

Estimation of Total Codeine—Total codeine in samples, which had been autoclaved and the pH adjusted to above 7.0, was estimated as free codeine.

Calculation of Codeine Metabolites—Free codeine was calculated from a standard curve prepared from known concentrations of N-$^{14}CH_3$-codeine. Conjugated codeine was calculated from the difference between the samples determined for total codeine and for free codeine. Free morphine was estimated from the difference between samples extracted for free morphine plus free codeine and that for free codeine only. Conjugated morphine was obtained from the difference between samples analyzed for total morphine plus total codeine and for total codeine only, followed by the subtraction of the calculated amount of free morphine.

Estimation of Total Radioactivity—Total radioactivity in the sample was determined by counting 0.5-ml. aliquots with 10 ml. of naphthalene–dioxane counting solution or 10 ml. of phenethylamine–methanol–toluene solution. Toluene-^{14}C was used as the internal standard. Disintegrations per minute (d.p.m.) of each sample were determined with the FORTRAN IV computer (10).

Statistical Evaluation—Duncan's new multiple-range test (11) was used for comparison of all the means.

RESULTS

Recoveries of Morphine, Dihydromorphine, and Codeine—The recoveries of morphine, dihydromorphine, and codeine from aqueous solution containing 0.5 mg. alkaloids and 1–500 ng. of radioactive-labeled drug by the described procedures were 96.0 ± 1.8, 79.1 ± 0.8, and 99.8–103.2%, respectively (Table I). The mean recoveries of morphine from aqueous solution containing 2.5 to 10.0 mg. of morphine were 94.0 ± 1.5%. The results indicate that the solvent mixture is very efficient for extraction of these alkaloids.

The recovery of morphine from aqueous solution improved as the proportion of *n*-amyl alcohol was increased in the solvent mixture. Eighty-three percent of morphine was recovered with ethylene chloride containing 10% of *n*-amyl alcohol (Table I). One hundred-percent recovery of morphine with the same solvent mixture has also been reported (4). The higher recovery presumably was due to the salting-out effect of Na_2HPO_4 (5). The effect of saturation of NaCl or $(NH_4)_2SO_4$ on extraction of alkaloids from acidic solution has also been described (12). The presence of ammonium ions was found to decrease the extraction of morphine (Table I).

Separation of Codeine from Morphine—Codeine was extracted into the organic phase from an alkaline aqueous solution containing both codeine and morphine. The effect of alkalinity of the aqueous phase on the removal of morphine by the organic solvent mixture was such that it was necessary to add 2 ml. of 0.5 *N* NaOH solution and to transfer the organic phase to another tube to be washed with 4 ml. of 0.1 *N* NaOH solution.

Recoveries of morphine and codeine, or codeine alone, from samples containing known amounts of both codeine and morphine indicated that both codeine and morphine were quantitatively extracted into the organic phase when the pH of samples was adjusted to 9.0, and only codeine was extracted when the pH was adjusted to 12–13.

Hydrolysis of Conjugated Morphine, Dihydromorphine, or Codeine—The plasma, bile, and urine of dogs collected after s.c. injection of N-$^{14}CH_3$-morphine and urine of rats after s.c. injection of N-$^{14}CH_3$-codeine and N-$^{14}CH_3$-dihydromorphine were used for hydrolysis studies. Autoclaving samples in 1.1 *N* HCl at 18–20 lb. pressure in a portable sterilizer for 0.5 hr. gave 60–70% hydrolysis of conjugated metabolites in biological samples. By increasing either the acid strength or the autoclaving time, hydrolysis of conjugated metabolites of each drug was increased (Tables II and III).

Complete hydrolysis of the morphine conjugate was achieved by autoclaving samples (*a*) in a portable autoclave at 18–20 lb. pressure in 3.3 *N* HCl for 0.5 hr., in 2.2 or 3.3 *N* HCl for 1.0 hr., or with 1.1, 2.2, or 3.3 *N* HCl for 2.0 hr. (Table II), or (*b*) in a steam-jacketed autoclave (American Sterilizer Co.) at 20 lb. pressure with 2.2 or 3.3 *N* HCl for 0.5 and 1.0 hr. or with 1.1 *N* HCl for 1.5 hr. (Table

Table II—Comparison of the Effect of Acidic Strength and the Autoclaving Time on Hydrolysis of Morphine, Dihydromorphine, and Codeine Conjugated Metabolites[a] in Biological Samples Using a Portable Autoclave[b]

	Maximal Mean, d.p.m.[c]	Percentage of Hydrolyzed Conjugates in the Biological Specimens, Mean ± *SD*[d]								
Time of autoclaving, hr.		0.5			1.0			2.0		
Normality of HCl solution		1.1	2.2	3.3	1.1	2.2	3.3	1.1	2.2	3.3
Plasma of dog after morphine	927	64.1 ± 3.1*	96.1 ± 6.0	101.0 ± 1.4	76.2 ± 1.9*	97.2 ± 1.4	100.5 ± 2.4	100.8 ± 4.9	100.5 ± 4.1	101.6 ± 1.9
Bile of dog after morphine	17972	69.7 ± 7.5*	84.4 ± 16.1	104.3 ± 1.9	89.7 ± 5.7*	100.9 ± 5.0	105.0 ± 10.0	91.0 ± 14.2	92.4 ± 8.9	100.5 ± 6.4
Urine of dog after morphine	1627	63.6 ± 1.5*	86.4 ± 6.6	99.6 ± 0.9	91.6 ± 4.8*	98.7 ± 0.4	96.7 ± 3.6	101.5 ± 1.8	97.8 ± 4.0	100.6 ± 3.5
Urine of rat after codeine	2023	60.3 ± 5.7*	89.4 ± 7.3*	96.3 ± 5.5	91.4 ± 10.3*	98.4 ± 3.6	97.6 ± 2.6	98.8 ± 4.8	104.0 ± 3.6	99.2 ± 2.6
Urine of rat after dihydromorphine	3867	73.4 ± 8.4*[e]	91.8 ± 3.1*[e]	104.1 ± 1.4[e]	89.9 ± 5.4*	104.9 ± 11.4	101.7 ± 3.4	94.4 ± 3.4	95.7 ± 3.6	99.1 ± 3.8

[a] Quadruplicate samples were analyzed in each experiment. [b] All-American portable pressure sterilizer, No. 25X, Wisconsin Aluminum Foundry Co. Inc., Manitowoc, Wis. [c] The maximal mean was calculated from the means of the conjugate obtained upon autoclaving the sample in a portable autocalve at 17–20 lb. pressure with 3.3 *N* HCl for 0.5, 1.0, and 2.0 hr.; 2.2 and 3.3 *N* HCl for 1.0 and 2.0 hr., 1.1, 2.2 and 3.3 *N* HCl for 2 hr., and in a steam-jacketed autoclave at 20 lb. pressure with 2.2 and 3.3 *N* HCl for 0.5 and 1.0 hr., and 1.1 *N* HCl for 1.5 hr. [d] The * indicates a significant difference from the maximal mean at the 0.05 level. [e] Samples after autoclaving were left overnight in the sterilizer.

Table III—Comparison of the Effect of Acidic Strength and the Autoclaving Time on Hydrolysis of Morphine, Dihydromorphine, and Codeine Conjugated Metabolites[a] in Biological Samples Using a Steam-Jacketed Autoclave[b]

	Maximal Mean, d.p.m.[c]	Percentage of Hydrolyzed Conjugates in the Biological Specimens, Mean ± *SD*[d]								
Time of autoclaving, hr.		0.5			1.0			2.0		
Normality of HCl solution		1.1	2.2	3.3	1.1	2.2	3.3	1.1	2.2	3.3
Plasma of dog after morphine	927	93.5 ± 3.6	101.4 ± 1.9	100.8 ± 0.9	101.8 ± 2.3	99.2 ± 2.3	96.5 ± 5.8	98.3 ± 2.8	96.2 ± 1.2	88.1 ± 8.2
Bile of dog after morphine	17972	101.3 ± 2	100.3 ± 4.8	104.6 ± 4.8	103.0 ± 2.1	102.0 ± 2.1	99.0 ± 0.8	100.2 ± 3.3	101.1 ± 1.8	94.4 ± 3.2
Urine of dog after morphine	1627	97.5 ± 2.5	100.5 ± 1.8	99.5 ± 2.0	102.9 ± 1.5	100.1 ± 1.2	102.1 ± 0.8	99.7 ± 1.0	100.4 ± 1.5	97.2 ± 3.1
Urine of rat after codeine	2023	84.9 ± 5.7*	101.1 ± 5.1	102.6 ± 3.7	89.6 ± 4.8	103.9 ± 4.2	98.4 ± 4.7	91.7 ± 1.7	91.7 ± 1.7	79.2 ± 2.0

[a] Quadruplicate samples were analyzed in each experiment. [b] American Sterilizer Company, Erie, Pa., [c] The maximal mean was calculated from the means of the conjugate obtained upon autoclaving the sample in a portable autoclave at 17–20 lb. pressure with 3.3 *N* HCl for 0.5, 1.0, and 2.0 hr.; 2.2 and 3.3 *N* HCl for 1.0 and 2.0 hr., 1.1, 2.2, and 3.3 *N* HCl for 2 hr., and in a steam-jacketed autoclave at 20 lb. pressure with 2.2 and 3.3 *N* HCl for 0.5 and 1.0 hr., and 1.1 *N* HCl for 1.5 hr. [d] The * indicates a significant difference from the maximal mean at the 0.05 level.

Table IV—Hydrolysis of Pure Drug Conjugates: Codeine-6-glucuronide, Morphine-3-glucuronide, and Morphine-3-ethereal Sulfate[a]

Sample	HCl, *N*	Autoclaving Time, hr.	Liberated Drug, mcg., Mean ± *SE*	Conjugate Hydrolyzed, %
Codeine-6-glucuronide dihydrate, 105 mcg.	1.1	0.5[b]	33.5 ± 0.2	57.6
		2.0	31.7	54.5
	2.6	0.5	36.6	62.9
		1.0[b]	55.1 ± 0.8	94.7
		2.0	54.5	93.7
	5.3	0.5	52.5	90.2
		1.0	53.1	91.3
Morphine-3-glucuronide dihydrate, 70 mcg.	2.2	1.0	41.4	98.3
Morphine-3-ethereal sulfate, 210 mcg.	2.2	1.0	150.5	92.3

[a] Quadruplicate samples were autoclaved with HCl in a portable autoclave at 18–20 lb. pressure. The liberated drug was determined with the modified methyl orange dye method (16). [b] Three experiments with four samples each were done.

III). The recovery of morphine was lower than maximum when the samples were autoclaved with 2.2 or 3.3 *N* HCl in a steam-jacketed autoclave at 20 lb. pressure for 1.5 hr. and allowed to remain in the autoclave for 2 hr. (Table III). After extraction, the radioactivity remaining in the aqueous phase was approximately 10% of the total in the biological samples. This radioactivity may represent morphine altered chemically by the conditions of autoclaving.

Complete hydrolysis of isolated pure morphine-3-glucuronide, the major metabolite of codeine in rats or of morphine in rats, dogs, and man, and that of isolated pure morphine-3-ethereal sulfate, the major metabolite of morphine in cats, is accomplished under similar conditions as described above (Table IV).

Codeine-6-glucuronide, the major metabolite of the drug isolated from the urine of dogs (13), is hydrolyzed completely in 2.6 *N* HCl at 18–20 lb. pressure in a portable autoclave for 1 hr., or in 5.3 *N* HCl for 0.5 hr. (Table IV).

Determination of Radioactivity in Aqueous Samples—Total radioactivity was determined successfully by mixing 0.5-ml. aliquots of aqueous solutions, plasma, bile, urine, or fecal homogenate with either 10 ml. of dioxane–naphthalene solution or 10 ml. phenethylamine–methanol–toluene solution.

The results with the described procedure for analysis of *N*-$^{14}CH_3$-codeine metabolites in the bile, urine, and fecal homogenate of rats have previously been reported (8).

DISCUSSION

The recovery of total morphine, dihydromorphine, or codeine by extraction with ethylene chloride containing *n*-amyl alcohol is dependent on complete hydrolysis of conjugated drug and the proper adjustment of pH and salt concentration in the hydrolyzed samples. Using NaOH solutions to adjust the hydrolyzed samples to pH 9–10 was somewhat tedious and laborious, involving some loss of samples due to testing the solution with pH paper. Ammonium hydroxide, instead of NaOH solution, had been used in these experiments to bring the hydrolyzed samples to pH 9–10. However, lower and inconsistent recovery of total morphine was observed, presumably due to the formation of ammonium morphinate salt which remained in the aqueous phase (Table I). The adjustment of autoclaved biological samples to pH 9 by saturating with $NaHCO_3$ was found to give most satisfactory results. The recovery of codeine was not affected by either the presence of NH_4OH ions in the system or by the amount of *n*-amyl alcohol.

Conditions used in previous studies for hydrolysis of conjugated morphine and its analogs varied considerably. The original work on hydrolysis of conjugated narcotics reported by Gross and Thompson (14) stated that 35–92% of the injected morphine could be recovered in the urine and feces of the dog after the biological samples had been autoclaved in 1.1 *N* HCl at 15 lb. pressure for 0.5 hr. Longer hydrolysis or use of stronger acid (up to 5.5 *N*) did not change the yield. The autoclave used by Gross and Thompson was presumed to be a steam-jacketed one. Identical conditions were followed by most subsequent investigators (1, 15). Data have also been reported on hydrolysis of morphine conjugate in 1.1 *N* HCl in a steam-jacketed autoclave at 18 lb. pressure for 0.5 hr. (2, 16) or 25 min. (4); in 0.8 *N* HCl at 15–18 lb. pressure in a portable autoclave for 20 min. (5, 17); and in 4.1 *N* HCl on a boiling water bath for 0.5 hr. (18). The different results are probably due to the variablility of hydrolysis conditions. The elevated temperature is prolonged in a steam-jacketed autoclave which has been switched off, while the temperature in a portable autoclave drops quickly to room temperature. Furthermore, the kinetics of degradation of morphine in aqueous solution have been shown to be dependent on the morphine concentration, on the concentration of oxygen, and on temperature (19).

The strength of the alkaline solution used by Johannesson and Woods (3) for extraction of codeine from aqueous solution containing codeine and morphine was found inadequate to remove morphine completely. In addition, with their procedure, 0.5 to 1.0 ml. of the alkaline solution residue remained on the top of the organic phase. Ten to twenty-five percent of the apparent morphine was extracted back to the organic phase when it was shaken with 4 ml. of 4% K_2HPO_4 solution. Conjugated morphine [45% of codeine injected in the rat (8)], a major metabolite of codeine, therefore would be mistakenly estimated as conjugated codeine.

REFERENCES

(1) T. K. Adler, J. M. Fujimoto, E. L. Way, and E. Baker, *J. Pharmacol. Exp. Ther.*, **114,** 251(1955).

(2) L. A. Woods, H. E. Muehlenbeck, and L. B. Mellett, *ibid.*, **117,** 117(1956).

(3) T. Johannesson and L. A. Woods, *Acta Pharmacol. Toxicol.*, **21,** 381(1964).

(4) S. J. Mule and L. A. Woods, *J. Pharmacol. Exp. Ther.*, **136,** 232(1962).

(5) H. I. Chernov and L. A. Woods, *ibid.*, **149,** 146(1965).

(6) E. L. Way and T. K. Adler, personal communications of H. Rapoport, *Bull. WHO*, **25,** 227(1961).

(7) G. A. Bray, *Anal. Biochem.*, **1,** 279(1960).

(8) S. Y. Yeh and L. A. Woods, *J. Pharmacol. Exp. Ther.*, **166,** 86(1969).

(9) *Ibid.*, **169,** 168(1969).

(10) J. L. Spratt, *Int. J. Appl. Radiat. Isotop.*, **16,** 439(1965).

(11) R. G. D. Steel and J. H. Torrie, "Principles and Procedures of Statistics," McGraw-Hill, New York, N. Y., 1960.

(12) V. F. Framarenko, *Chem. Abstr.*, **62,** 11625h(1965).

(13) S. Y. Yeh and L. A. Woods, *Federation Proc.*, **26,** 742(1967); *J. Pharmacol. Exp. Ther.*, to be published.

(14) E. G. Gross and V. Thompson, *J. Pharmacol. Exp. Ther.*, **68,** 413(1940).

(15) J. M. Fujimoto, E. L. Way, and C. H. Hine, *J. Lab. Clin. Med.*, **44,** 627(1954).

(16) L. A. Woods, *J. Pharmacol. Exp. Ther.*, **112**, 158(1954).

(17) J. H. Sanner and L. A. Woods, *ibid.*, **148**, 176(1965).

(18) H. Yoshimura, K. Oguri, and H. Tsukamoto, *Biochem. Pharmacol.*, **18**, 279(1969).

(19) S. Y. Yeh and J. L. Lach, *J. Pharm. Sci.*, **50**, 35(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 13, 1969, from the *Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, IA 52240*

Accepted for publication October 24, 1969.

This study was supported by U. S. Public Health Service Grant NB 02928.

Complexes of Ergot Alkaloids and Derivatives III: Interaction of Dihydroergocristine with Xanthine Analogs in Aqueous Media

H. V. MAULDING and M. A. ZOGLIO

Abstract □ The capacity of the poorly water-soluble ergot derivative, dihydroergocristine methanesulfonate, to form intermolecular complexes with caffeine, theophylline, and 7-β-hydroxypropyltheophylline was studied. Upon inclusion of xanthine, dihydroergocristine exhibited elevated solubility at pH 6.65, a larger dissolution rate constant in 0.1 *N* HCl, and a change in partitioning-rate constants for transfer of the alkaloid from aqueous to organic phases. These alterations of physicochemical properties appear to be a consequence of mutual interaction between the two components in solution. The effect of dihydroergocristine was enhanced on enteral administration with each of the three complexing agents as measured on α-adrenergic blockade in cats. When tritiated dihydroergocristine was given orally to humans along with 7-β-hydroxypropyl-theophylline, blood levels went higher and stayed higher than when the alkaloid was administered alone. The same situation was true of total urinary excretion of tritium.

Keyphrases □ Ergot alkaloids—physicochemical analyses □ Dihydroergocristine, interacting—xanthine analogs □ Xanthine-dihydroergocristine complex formation—solubility effect □ Biological activity, dihydroergocristine—xanthines, effect □ Partitioning rates—dihydroergocristine-xanthine complex □ Colorimetric analysis—spectrophotometer

Previous studies in this area (1, 2) have attempted to correlate *in vitro* data with physiological responses observed on addition of caffeine and other xanthines to several ergot alkaloids and their congeners (3–5).

The present report is concerned with interactions occurring in solution between three complexing agents—caffeine, theophylline, and the soluble derivative 7-β-hydroxypropyl-theophylline—and dihydroergocristine methanesulfonate. Dihydroergocristine is one of the hydrogenated alkaloids of the ergotoxine group (6).

This work points to a good accord between physicochemical data derived from solubility, dissolution rate, and partitioning-rate studies and pharmacological results from human and animal investigations. The evidence appears to indicate complex formation leading to increased absorption rate, as well as the amount of absorption of many ergot derivatives in the presence of xanthines.

EXPERIMENTAL

Materials—Dihydroergocristine methanesulfonate[1] (mol. wt. 707.8) showed only traces of contaminants when subjected to thin-layer chromatography.

The various xanthines utilized were: 7-β-hydroxypropyl-theophylline,[2] m.p. 135–138°; theophylline,[3] m.p. 272–274°; and caffeine anhydrous powder USP,[4] m.p. 238°.

Melting points are uncorrected. Reagent grade chloroform (Mallinckrodt Chemical Works) was employed in the partitioning studies. A pH 6.65 buffer was made by dissolving 13.6 g. KH_2PO_4 in 500 ml. water, adjusting the pH with concentrated KOH, and diluting to 1 l. (ionic strength, 0.2).

The pH measurements were taken on a Metrohm pH meter and spectrophotometric data were obtained from a Cary model 14 spectrophotometer.

Partitioning Studies—A solution was prepared by placing dihydroergocristine methanesulfonate (150 mg.) in 950 ml. of pH 6.65 phosphate buffer, stirring magnetically for 30 min. to 1 hr, followed by filtration (Whatman No. 1 filter paper) into a flask immersed in a water bath maintained at 30°, and finally addition of pH 6.65 buffer to make 1 l. of solution.

This solution was immediately analyzed for dihydroergocristine (7) and read at 585 mμ (absorbance of 0.902 equivalent to 0.1 mg./ml.). The usual concentrations of alkaloid obtained in this manner were in the range of 0.06 to 0.09 mg./ml.; 500 ml. (half) of this solution of known concentration was kept and 500 ml. had xanthine added to it.

Fifteen milliliters of the freshly prepared aqueous phase—either with or without xanthine—was added carefully to 15 ml. chloroform in screw-capped vials (50 ml.). The vials were sealed and rotated at 6 r.p.m. in a 30° (±0.1°) water bath. Five-milliliter samples were taken at 3, 5, 7, 9, 11, 13, and 15 min. from the aqueous phase and analyzed for dihydroergocristine by the Van Urk method (7).

Solubility Studies—Dihydroergocristine (50 mg.) was placed in watertight, amber, screw-capped vials (50 ml.) containing exactly 10 ml. of pH 6.65 phosphate buffer and varying quantities of the three xanthines being considered. The vials were clamped onto the edge of metal disks (15-cm. diameter) mounted on a motor-driven shaft and rotated vertically at 6 r.p.m. in a constant-temperature bath, 30 ± 0.1°. After 24 hr., samples were taken using pipets with filters attached and analyzed for dihydroergocristine by the Van Urk method (7).

[1] Sandoz, A.-G., Basel, Switzerland.
[2] Ganes Chemical Works, Inc., New York, N. Y.
[3] Matheson, Coleman and Bell.
[4] Chas. Pfizer Co.

(16) L. A. Woods, *J. Pharmacol. Exp. Ther.*, **112**, 158(1954).

(17) J. H. Sanner and L. A. Woods, *ibid.*, **148**, 176(1965).

(18) H. Yoshimura, K. Oguri, and H. Tsukamoto, *Biochem. Pharmacol.*, **18**, 279(1969).

(19) S. Y. Yeh and J. L. Lach, *J. Pharm. Sci.*, **50**, 35(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 13, 1969, from the *Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, IA 52240*

Accepted for publication October 24, 1969.

This study was supported by U. S. Public Health Service Grant NB 02928.

Complexes of Ergot Alkaloids and Derivatives III: Interaction of Dihydroergocristine with Xanthine Analogs in Aqueous Media

H. V. MAULDING and M. A. ZOGLIO

Abstract □ The capacity of the poorly water-soluble ergot derivative, dihydroergocristine methanesulfonate, to form intermolecular complexes with caffeine, theophylline, and 7-β-hydroxypropyltheophylline was studied. Upon inclusion of xanthine, dihydroergocristine exhibited elevated solubility at pH 6.65, a larger dissolution rate constant in 0.1 *N* HCl, and a change in partitioning-rate constants for transfer of the alkaloid from aqueous to organic phases. These alterations of physicochemical properties appear to be a consequence of mutual interaction between the two components in solution. The effect of dihydroergocristine was enhanced on enteral administration with each of the three complexing agents as measured on α-adrenergic blockade in cats. When tritiated dihydroergocristine was given orally to humans along with 7-β-hydroxypropyl-theophylline, blood levels went higher and stayed higher than when the alkaloid was administered alone. The same situation was true of total urinary excretion of tritium.

Keyphrases □ Ergot alkaloids—physicochemical analyses □ Dihydroergocristine, interacting—xanthine analogs □ Xanthine-dihydroergocristine complex formation—solubility effect □ Biological activity, dihydroergocristine—xanthines, effect □ Partitioning rates—dihydroergocristine-xanthine complex □ Colorimetric analysis—spectrophotometer

Previous studies in this area (1, 2) have attempted to correlate *in vitro* data with physiological responses observed on addition of caffeine and other xanthines to several ergot alkaloids and their congeners (3–5).

The present report is concerned with interactions occurring in solution between three complexing agents—caffeine, theophylline, and the soluble derivative 7-β-hydroxypropyl-theophylline—and dihydroergocristine methanesulfonate. Dihydroergocristine is one of the hydrogenated alkaloids of the ergotoxine group (6).

This work points to a good accord between physicochemical data derived from solubility, dissolution rate, and partitioning-rate studies and pharmacological results from human and animal investigations. The evidence appears to indicate complex formation leading to increased absorption rate, as well as the amount of absorption of many ergot derivatives in the presence of xanthines.

EXPERIMENTAL

Materials—Dihydroergocristine methanesulfonate[1] (mol. wt. 707.8) showed only traces of contaminants when subjected to thin-layer chromatography.

The various xanthines utilized were: 7-β-hydroxypropyl-theophylline,[2] m.p. 135–138°; theophylline,[3] m.p. 272–274°; and caffeine anhydrous powder USP,[4] m.p. 238°.

Melting points are uncorrected. Reagent grade chloroform (Mallinckrodt Chemical Works) was employed in the partitioning studies. A pH 6.65 buffer was made by dissolving 13.6 g. KH_2PO_4 in 500 ml. water, adjusting the pH with concentrated KOH, and diluting to 1 l. (ionic strength, 0.2).

The pH measurements were taken on a Metrohm pH meter and spectrophotometric data were obtained from a Cary model 14 spectrophotometer.

Partitioning Studies—A solution was prepared by placing dihydroergocristine methanesulfonate (150 mg.) in 950 ml. of pH 6.65 phosphate buffer, stirring magnetically for 30 min. to 1 hr, followed by filtration (Whatman No. 1 filter paper) into a flask immersed in a water bath maintained at 30°, and finally addition of pH 6.65 buffer to make 1 l. of solution.

This solution was immediately analyzed for dihydroergocristine (7) and read at 585 mμ (absorbance of 0.902 equivalent to 0.1 mg./ml.). The usual concentrations of alkaloid obtained in this manner were in the range of 0.06 to 0.09 mg./ml.; 500 ml. (half) of this solution of known concentration was kept and 500 ml. had xanthine added to it.

Fifteen milliliters of the freshly prepared aqueous phase—either with or without xanthine—was added carefully to 15 ml. chloroform in screw-capped vials (50 ml.). The vials were sealed and rotated at 6 r.p.m. in a 30° (±0.1°) water bath. Five-milliliter samples were taken at 3, 5, 7, 9, 11, 13, and 15 min. from the aqueous phase and analyzed for dihydroergocristine by the Van Urk method (7).

Solubility Studies—Dihydroergocristine (50 mg.) was placed in watertight, amber, screw-capped vials (50 ml.) containing exactly 10 ml. of pH 6.65 phosphate buffer and varying quantities of the three xanthines being considered. The vials were clamped onto the edge of metal disks (15-cm. diameter) mounted on a motor-driven shaft and rotated vertically at 6 r.p.m. in a constant-temperature bath, 30 ± 0.1°. After 24 hr., samples were taken using pipets with filters attached and analyzed for dihydroergocristine by the Van Urk method (7).

[1] Sandoz, A.-G., Basel, Switzerland.
[2] Ganes Chemical Works, Inc., New York, N. Y.
[3] Matheson, Coleman and Bell.
[4] Chas. Pfizer Co.

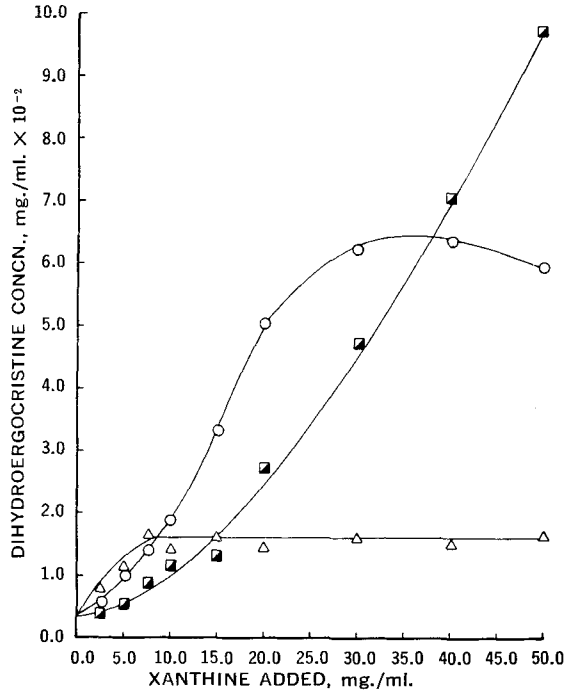


Figure 1—*Solubilizing action of xanthine on dihydroergocristine in phosphate buffer (pH 6.65) at 30° for 24 hr. Key: ◪, 7-β-hydroxypropyl-theophylline; ○, caffeine; and △, theophylline.*

Dissolution Rates—A 25-r.p.m. stirrer motor fitted with a 2.54-cm. propeller blade placed 4 cm. from the bottom of an 800-ml. beaker containing 500 ml. 0.1 *N* HCl solution was employed for these determinations. Dihydroergocristine (50 mg.) or the alkaloid in combination with xanthine, prepared by mixing 50 mg. alkaloid with 5 g. xanthine in a mortar, was placed into the stirred solution from a height of about 1.5 cm. The temperature was maintained at 37 ± 0.1° by immersing the beaker in a constant-temperature bath. Samples were withdrawn periodically for dihydroergocristine analysis in the usual manner (7).

RESULTS AND DISCUSSION

The solubility of dihydroergocristine methanesulfonate is elevated to some degree by all three xanthines examined at pH 6.65 as may be seen from Fig. 1. It is apparent (Fig. 1) that 7-β-hydroxypropyl-theophylline ultimately allows a greater solubilization of the alkaloid than caffeine or theophylline; however, this was not shown in concentrations greater than 50 mg./ml. xanthine because extremely large amounts are of little practical consequence. Caffeine leads to greater solubilization of the alkaloid than theophylline as it elevates the amount of dihydroergocristine in solution at this pH and temperature (30°) by almost 12 times, while theophylline increases the amount about 3 times. This was previously shown to be the situation with dihydroergotoxine (2). Curves as seen in Fig. 1 do not readily allow analysis by elegant treatments as those of Connors *et al.* (8, 9), thus leaving the exact nature of the complex in doubt.

It is recognized that dissolution rates of some solids may be the rate-limiting step in their absorption (10, 11). When ergot derivatives were subjected to dissolution-rate studies with and without the presence of xanthines, the rate constant was found to be increased nearly threefold in the first instance (1, 2, 5). The authors have previously found HCl (500 ml., 0.1 *N*) allows approximation of a first-order process with these substances. Figure 2 gives an example where a 100:1 ratio of 7-β-hydroxypropyl-theophylline to dihydroergocristine was utilized in the dissolution rate at a stirrer speed of 25 r.p.m. and 37°. The rate constants vary by a factor of 2.5:1 (0.053 to 0.138 $min.^{-1}$), which is in agreement with those previously reported (1, 2). Caffeine and theophylline gave almost the same ratios and results as those reported in Fig. 2 relative to the alkaloid, although they are not reported here. Therefore, for the conditions employed, there is evidence of a significant change in dissolution-rate constant on addition of xanthine to dihydroergocristine.

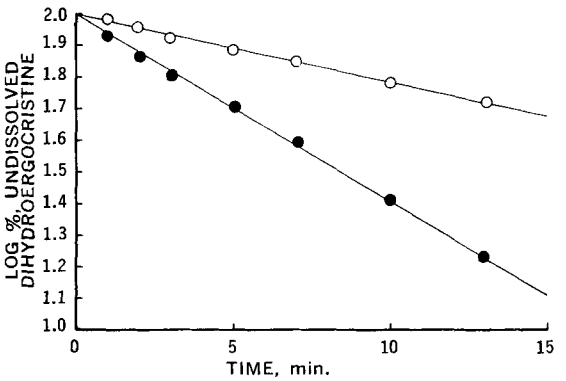


Figure 2—*Effect of 7-β-hydroxypropyl-theophylline on the dissolution rate of dihydroergocristine in 0.1 N HCl (500 ml.) at 37° and 25 r.p.m. Key: ○, the dissolution of dihydroergocristine, 50 mg.; and ●, the dissolution of dihydroergocristine, 50 mg., and 7-β-hydroxypropyl-theophylline, 5 g.*

It was previously observed that xanthines had the effect of increasing the partitioning rate of ergot derivatives from an aqueous to an organic phase under certain conditions, although the equilibrium distribution was not disturbed (1, 2). Figure 3 gives an example of the action of 7-β-hydroxypropyl-theophylline in altering the partitioning rate of dihydroergotoxine methanesulfonate of which dihydroergocristine methanesulfonate is one of the three components (2). This phenomenon was noted with dihydroergocristine methanesulfonate itself in the presence of 7-β-hydroxypropyl-theophylline in a weight–weight ratio of 100:1 (xanthine–alkaloid) and the results may be seen in Fig. 4. The results in Fig. 4 show slopes of 2.5×10^{-2} $min.^{-1}$ and 5.1×10^{-2} $min.^{-1}$ for the alkaloid alone and with xanthine, respectively. Similar results were obtained with caffeine and theophylline and are not reported here. In 0.1 *N* HCl the rate was reversed as was found to be the case with ergotamine tartrate (1).

An investigation was made of the effect of increasing 7-β-hydroxypropyl-theophylline concentration on the partitioning-rate constants of dihydroergocristine methanesulfonate as the amount of xanthine goes from 0 to 500 times the weight of the ergot derivative

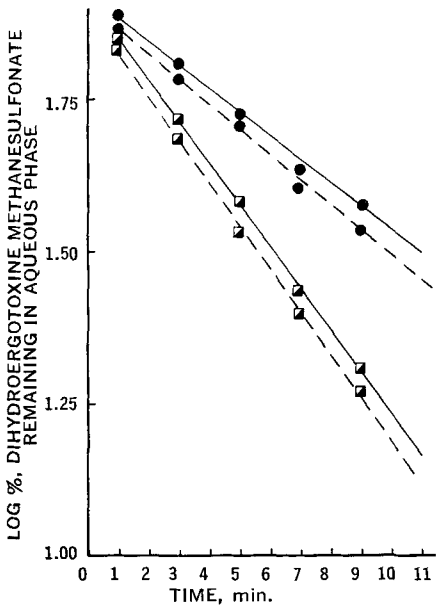


Figure 3—*Effect of 7-β-hydroxypropyl-theophylline on the partitioning rate of dihydroergotoxine methanesulfonate from an aqueous (phosphate buffer, pH 6.65) to an organic phase (chloroform). Key: ●, dihydroergotoxine, 0.1 mg./ml.; ◪, dihydroergotoxine, 0.1 mg./ml., and 7-β-hydroxypropyl-theophylline, 10.0 mg./ml.; - - -, experimental run No. 1; and ——, experimental run No. 2.*

Table I—First-Order Partitioning Rate Constants Showing Partitioning of Dihydroergocristine Methanesulfonate from pH 6.65 Phosphate Buffer into Chloroform at 30°[a]

Dihydroergocristine methanesulfonate[b]	1	1	1	1	1	1
7-β-Hydroxypropyl-theophylline[c]	0	10	50	100	200	500
Rate constant × 10^{-2} $min.^{-1}$[d]	2.5	3.0	4.4	5.1	5.8	6.6

[a] All experiments carried out as mentioned in *Experimental* section with xanthine added after assay to determine alkaloid present. Concentrations of dihydroergocristine varied from 0.06 mg./ml. as mentioned. [b,c] Quantities given are measured on a weight to weight basis, *i.e.*, 1:50 is 1 mg. dihydroergocristine and 50 mg. 7-β-hydroxypropyl-theophylline. [d] Rate constants and slopes thereof obtained by linear regression analysis from four experiments utilizing each ratio and run consecutively. Values were measured between 3 and 15 min.

in solution. The results are listed in Table I and show a general trend upward for the rate constants as the amount of xanthine is increased. A dose–effect relationship of this same type was encountered on enteral administration of dihydroergocristine to cats with a general increase in effect up to a 50:1 ratio (w/w) of xanthine to alkaloid followed by leveling up to a ratio of 200:1 (5).

Biological—The α-adrenergic blockade of dihydroergocristine methanesulfonate in cats was studied with and without xanthine present and this activity was enhanced on enteral administration of the alkaloid together with caffeine, theophylline, and 7-β-hydroxypropyl-theophylline. Significant increases in blocking action were noted at 7-β-hydroxypropyl-theophylline to dihydroergocristine ratios of 12.5:1 (w/w), the maximum effect being at 50:1 with no further increases on addition of excess complexing agent. This phenomenon was characterized by: (*a*) faster onset, (*b*) higher maximum, and (*c*) longer duration of action when the alkaloid and xanthine were administered concurrently. These differences were not encountered on i.v. dosing and are a result of increased dihydroergocristine absorption from the gut (5).

Tritiated dihydroergocristine was administered orally to five patients along with 7-β-hydroxypropyl-theophylline (1 mg. alkaloid and 100 mg. xanthine) and plasma levels were found to go higher and stay higher longer than when dihydroergocristine (1 mg.) was given alone. Total urinary tritium excretion was appreciably higher in the combination than in the case of the alkaloid itself; however, not much appears to be unchanged dihydroergocristine (5).

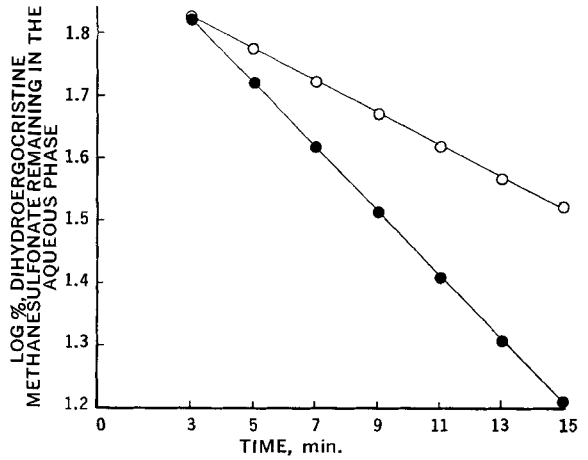


Figure 4—*Effect of 7-β-hydroxypropyl-theophylline on the partitioning rate of dihydroergocristine from an aqueous (phosphate buffer, pH 6.65, 30°, 6 r.p.m.) to an organic phase (chloroform). Key:* ○, *dihydroergocristine, 0.072 mg./ml.; and* ●, *dihydroergocristine, 0.072 mg./ml., and 7-β-hydroxypropyl-theophylline, 7.2 mg./ml.*

REFERENCES

(1) M. A. Zoglio, H. V. Maulding, and J. J. Windheuser, *J. Pharm. Sci.*, **58**, 222(1969).

(2) M. A. Zoglio and H. V. Maulding, to be published.

(3) N. A. Bercel, *Calif. Med.*, **72**, 234(1950).

(4) A. P. Friedman, *New Engl. J. Med.*, **250**, 600(1954).

(5) B. Berde, A. Cerletti, M. J. Dengler, and M. A. Zoglio, presented at Third Migraine Trust Symposium, London, April 24–25, 1969, vol. III, "Background to Migraine," to be published.

(6) C. O. Wilson and O. Gisvold, "Textbook of Organic Medicinal and Pharmaceutical Chemistry," J. B. Lippincott, Philadelphia, Pa., 1962, p. 476.

(7) N. J. Allport and T. T. Cocking, *Quart. J. Pharm. Pharmacol.*, **5**, 341(1932).

(8) K. A. Connors and J. A. Mollica, *J. Pharm. Sci.*, **55**, 772 (1966).

(9) K. A. Connors, M. H. Infeld, and B. J. Kline, *J. Amer. Chem. Soc.*, **91**, 3597(1969).

(10) J. G. Wagner, *J. Pharm. Sci.*, **50**, 359(1961).

(11) D. E. Wurster and P. W. Taylor, *ibid.*, **54**, 169(1965)

ACKNOWLEDGMENTS AND ADDRESSES

Received August 14, 1969, from *Pharmacy Research and Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication October 9, 1969.

The valuable technical assistance of D. C. Murdock is gratefully acknowledged as is aid from M. Wagner and Jane Pearson in carrying out the experimental portion of this work.

Effect of pH on the Micellar Properties of a Nonionic Surfactant

J. R. BLOOR*, J. C. MORRISON, and C. T. RHODES†

Abstract □ The effect of change in pH on the critical micelle concentration of a nonionic surfactant (Tween 40) has been investigated by surface tension and light-scattering methods. It is shown that a linear relationship exists between the free energy of micellization and pH. Significance of changes in the enthalpy and entropy of micellization are discussed. A computer program, written in Algol 803, is presented to convert raw experimental light-scattering data to micellar molecular weights. It is shown that both micellar molecular weight and the hydration per unit mass of surfactant decrease with increase in pH value. Interpretation of these results is discussed.

Keyphrases □ Nonionic surfactant—micellar properties, pH effect □ Micellar properties, nonionic surfactants—pH effect □ Surface tension method—pH effect, micelle concentration □ Turbidity method—pH effect, micelle concentration □ Refractive index—determination □ NMR—identification □ Computer program—conversion of light-scattering data to micellar molecular weights

Surfactants are widely used in pharmaceutical technology as wetting agents, solubilizers, and emulsifiers. Thus, studies of the properties of surfactants are of assistance in placing pharmaceutical formulation on a rational rather than an empirical basis (1). Also, naturally occurring surfactants, as found for example in bile and gastric juice, can have a significant effect upon drug absorption rates and other aspects of drug action (2). Kakemi *et al.* have attributed the retarded absorption of sulfonamides from aqueous solutions of a nonionic surfactant to micellar entrapment of drug (3). It has also been suggested that surface-active bile salts transport lipids by micellar solubilization (4). Surfactants can therefore play an important part in the biotransport of drugs. In the present paper the effects of pH upon a number of micellar properties of a nonionic surfactant are reported. This work forms part of a study of drug diffusion in micellar solution, further results of which will be published shortly (5). In addition to the value which the results reported in this paper have for diffusion studies, the data are of use in further elucidating the micellar structure of nonionic surfactants and in identifying the forces involved in micellar aggregation.

EXPERIMENTAL

Materials—A polyoxyethylene sorbitan monopalmitate[1] was characterized by NMR spectroscopy. The solvent used was D_2O, and the sodium salt of tetramethylsilane was used as the internal reference. Mean molecular surfactant formula was estimated by the method previously described by Rhodes (6).

The water used in this investigation was double distilled from an all-glass still.

Both the sodium chloride and benzene used in this work were of AnalaR grade.

[1] Tween 40, supplied by Honeywill-Atlas Ltd., Carshalton, England.

Table I—Characterization of the Surfactant by NMR Spectroscopy

Formula Claimed by Manufacturers	Formula Calculated Using Methyl Protons as Reference	Formula Calculated Using Sorbitan Ring Protons as Reference
Alkyl protons 31	30 (.4)	31 (.1)
Polyoxyethylene protons 20	20 (.0)	19 (.5)

Surface Tension Measurements—The critical micelle concentrations (CMC) values of the surfactant at various temperatures and pH values were determined by use of a Du Nouy tensiometer (7). Experimental technique was checked by measuring the surface tension of water which was found to be 72.0 dynes cm.$^{-1}$ at 25°. This was in excellent agreement with the literature value (8). The temperature was controlled at all times to ±0.1° of that required.

Light Scattering—Turbidities were measured using a Brice-Phoenix Universal light-scattering photometer[2] using unpolarized incident light, wavelength 436 mμ, and a standard 30 × 30-mm. turbidity cell. Temperature during determination of turbidities was maintained at ±0.1° of that required. The photometer was calibrated against an opal glass diffuser as a primary reference standard. For benzene the observed Rayleigh ratio was found to be 48.8 × 10^{-6} cm.$^{-1}$ which was in good agreement with the previously reported value (9). All solutions were clarified by filtration through Millipore cellulose membrane filters (0.1 μm pore size). Dissymmetries Z (I 45°/I 135° where I is the intensity of scattered light) were determined for each solution using a standard 40 × 40-mm. semioctagonal cell.

Refractive Index Measurements—The refractive indices of surfactant solutions were determined using a Hilger and Watts interference refractometer[3] with ±0.1° temperature control. Calibration was effected using sodium chloride as standard (10).

RESULTS AND DISCUSSION

The results of the NMR characterization of the surfactant (Table I) show that the mean molecular formula determined experimentally is in good agreement with that stated by the manufacturers. Absence of signals, other than those assigned to the surfactant (5.8 p.p.m. sorbitan ring protons, 6.3 p.p.m. polyoxyethylene protons, 8.7 p.p.m. alkyl protons, and 9.1 p.p.m. terminal methyl protons), is supporting evidence for the manufacturers' formula.

Plots of surface tension and turbidity against surfactant concentration showed abrupt changes of slope at the CMC; typical results are shown in Figs. 1 and 2. Results obtained by the two methods are in good agreement with one another and literature values (11).

Table II records the CMC values for the surfactant at various pH values and temperatures. Methods for calculating the thermodynamic parameters of micellization from CMC values and solubilization data have been discussed in some detail by Molyneaux *et al.* (12) and Humphreys and Rhodes (13). Subject to activity corrections, which for a nonionic surfactant are probably small, the free energy of micellization, ΔG_m, may be calculated by use of Eq. 1:

$$\Delta G_m = -RT \ln K \qquad \text{(Eq. 1)}$$

[2] Supplied by Techmation Ltd., London, England.
[3] Supplied by Hilger and Watts Ltd., London, England.

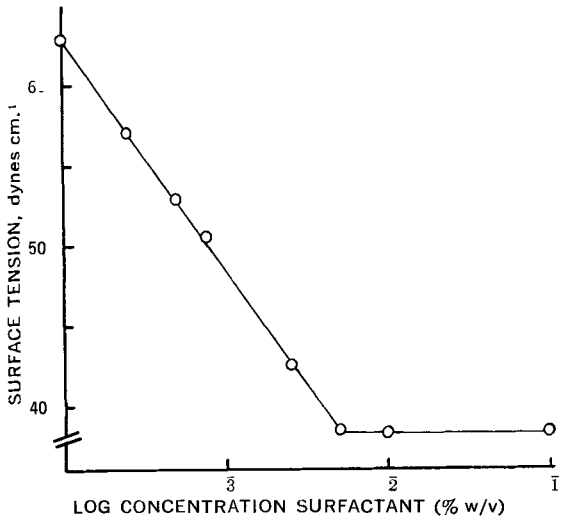


Figure 1—*Surface tension as a function of log surfactant concentration at 25°.*

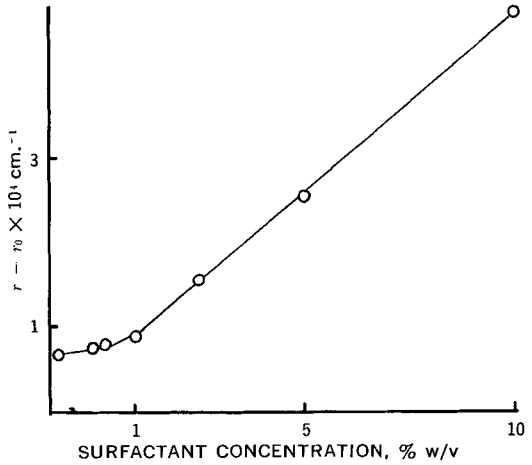


Figure 2—*Turbidity as a function of surfactant concentration at 25° and pH 2.1.*

where R is the gas constant, T the absolute temperature, and K the CMC expressed in terms of mole fraction. Figure 3 shows the ΔG_m values for the surfactant as a function of pH. A linear relationship between these two quantities is apparent. When data are available on the variation of K with temperature, it is possible to resolve ΔG_m into the enthalpic and entropic, ΔH_m and ΔS_m, factors by means of Eq. 2:

$$\Delta G_m = \Delta H_m - T\Delta S_m \qquad \text{(Eq. 2)}$$

Estimates of these quantities have been made (Table III). Because of the small temperature difference, 15°, used in this work, high-precision estimates of ΔH_m and ΔS_m are not possible. However, the values are of use for qualitative interpretation of micellar structure.

The average micellar molecular weight, $\bar{M}_w$, was determined from turbidities measured at 90° by extrapolating the Debye function to zero micellar concentration (14, 15):

$$H(C - C_0)/(\tau - \tau_0) = 1/\bar{M}_w \lim (C - C_0) \to 0 \qquad \text{(Eq. 3)}$$

where H is a constant for any given surfactant system under specified instrumental and environmental conditions, C is the surfactant concentration, C_0 is the CMC, τ is the turbidity at 90°, and τ_0 is the turbidity at 90° at the CMC. Plots of the Debye function against $(C - C_0)$ are governed by Eq. 4:

$$H(C - C_0)/(\tau - \tau_0) = 1/\bar{M}_w + 2B(C - C_0) \qquad \text{(Eq. 4)}$$

The term B, the second virial coefficient, represents the deviation from ideal behavior. The value of H is evaluated from Eq. 5:

$$H = \{32\pi^3 n^2[(n - n_0)/C]^2\}/(3\lambda^4 N) \qquad \text{(Eq. 5)}$$

where C is the solution concentration in g./ml.; n is the refractive index of the solution, n_0 is the refractive index of the solvent, λ is the wavelength of the incident light in cm., and N is Avogadro's number.

A computer program has been written in Algol 803 to substitute raw imput data, surfactant concentration, turbidities, refractive index measurements, and relevant constants into the above equations. Using least-squares procedures the program causes the determination of the best line for the refractive index against concentration plot and the plots of Debye function against concentration. Samples of imput and output data together with the program are given in the Appendix. Dissymmetry ratios were always close to unity, less than 1.03. These values confirmed the clarity of the solutions and substantiated the validity of the experimental procedure. Debye plots are shown in Fig. 4.

Figure 5 shows that a substantial change in micellar molecular weight occurs with change in pH. The values of $\bar{M}_w$ reported do not include water of hydration because the refractive index of the hydrated water is very close to that of the solvent. However, as shown in Fig. 6, the second virial coefficient shows a significant decrease in value with increase in pH. This shows that the solvent–solute interaction is greater at low pH values, indicating more micellar hydration than in alkaline solution. Thus, both the aggregation number and amount of water bound per unit mass of surfactant are greater in acid solution.

In interpreting these results the effect of pH upon water structure is probably a critical factor. There is evidence that sodium and hydroxyl ions are structure-formers which thus increase the extent of water structure around the monomeric surfactant (16).

Table II—Critical Micelle Concentrations of the Surfactant

Temperature	pH	Critical Micelle Concentration (molar) × 10^5
25	2.1	6.5
25	5.0	4.1
25	7.4	3.7
25	9.2	2.6
25	12.1	2.2
40	2.1	4.9
40	12.1	1.4

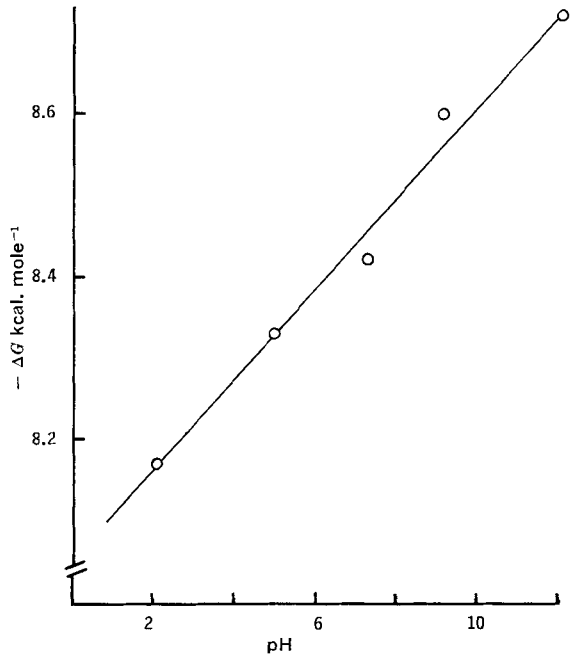


Figure 3—*Free energy of micellization at 25° as a function of pH.*

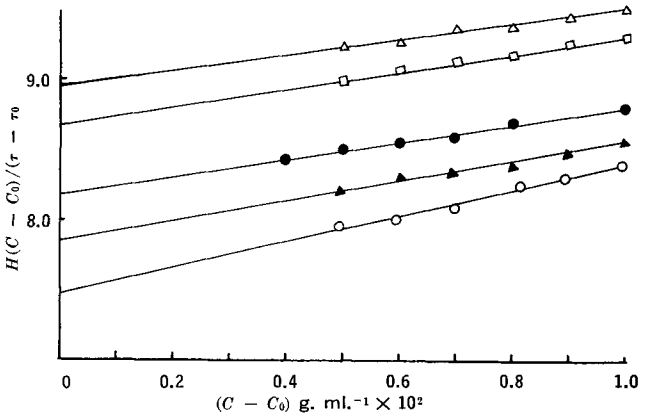


Figure 4—*Debye plots. Key: pH 2.1,* ○; *pH 5.2,* ▲; *pH 7.3,* ●; *pH 9.8,* □; *and pH 11.9,* △.

Chloride ions, however, are considered to be structure-breakers. The observed values for entropy of micellization will, of course, be greatly dependent upon the extent of water structure around the monomeric surfactant, as well as the structure and order of the micelle. Since the micelle is hydrated to a greater extent in acid solution, it is most likely that the palisade layer has a more open and flexible, and thus less ordered, structure than at high pH values. It is thus unlikely that the micelle in acid solution has a lower configurational entropy than that in alkaline solution since the structure of the hydrocarbon core is very probably independent of pH (12). This discussion is supported by the experimentally observed values of ΔS_m, 5 e.u. at pH 2.1 and 18 e.u. at pH 12.1, the larger entropy change being provisionally assigned as due to the greater degree of water structure at higher pH.

The enthalpy of micellization may be further resolved as shown in Eq. 6:

$$\Delta H_m = \Delta H_m^1 + \Delta H_m^2 \qquad \text{(Eq. 6)}$$

where ΔH_m^1 is the enthalpy change associated with the hydrophobic part of the surfactant and ΔH_m^2 is the enthalpy change associated with the head group. The difference in the estimated ΔH_m at pH values 2.1 and 12.1 which are −6.7 and −3.4 kcal. mole^{-1}, respectively, may therefore be expected to be due to several factors. Wurzshmitt (17) has suggested that nonionic surfactants are in fact weakly cationic in nature. The ionic nature of the head group and the extent of palisade layer hydration may thus be expected to be pH dependent. Since a greater amount of water structure is believed to exist around the surfactant molecules at high pH, the enthalpy change associated with the destruction of this "iceberg sheath" (18) would be expected to be greater than at low pH values. However, the enthalpy values shown in Table III are greater at low pH. Thus the other factors involved must more than compensate for this effect.

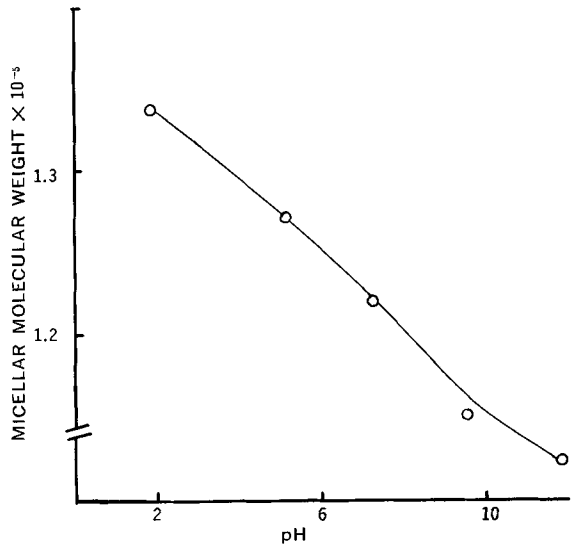


Figure 5—*Change in micellar molecular weight of surfactant with pH at 25°.*

Table III—Thermodynamic Parameters Controlling the Micellization

pH	ΔG_m kcal. mole^{-1}	ΔH_m kcal. mole^{-1}	ΔS_m cal. mole^{-1} $^0K^{-1}$
2.1	8.2	6.7	5
12.1	8.7	3.4	18

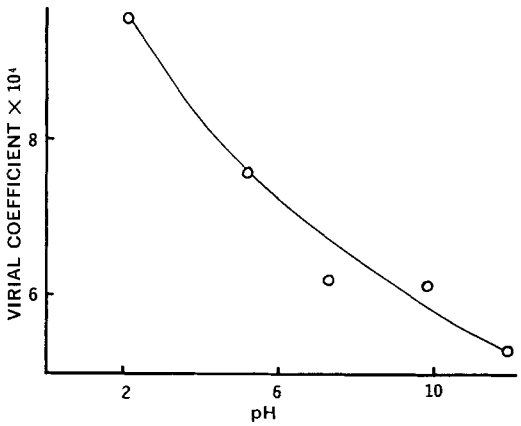


Figure 6—*Variation of second virial coefficient with pH.*

The reasons for the higher aggregation number at low pH cannot be precisely delineated at the present time. It may be that geometric limitations imposed by the differences in hydration with pH play a significant role. For a spherical micelle, changes in the hydration and thus in the size of the palisade layer must be accompanied by alteration in the aggregation number if the density of the hydrocarbon core is to remain constant.

There is no reason to suppose that the nonionic surfactant investigated in this study is unique of its type. It is therefore probable that other nonionic surfactants show similar significant changes in micellar properties with pH. Further work is necessary to see if the hypotheses outlined in this paper are of general applicability. In particular, information about the pH dependence of the interaction with drugs and the effect upon drug diffusion and transport is of considerable pharmaceutical relevance.

APPENDIX

The following computer program converts experimental light-scattering data into micellar molecular weights. The comment statement in the programs defines the symbols and explains the form of the imput data.

Imput Data

```
7
436
0.0327
1.333000
1.023
0.490   0.221   0.109   0.0336
5  4.85  4.73  4.70  4.72  4.78  0.0
5  2.55  2.53  2.57  2.52  2.55  0.0
1  0  0  0  0  0  0
1  0  0  0  0  0  0
0.000063
1.333657  0.005
1.333788  0.006
1.333920  0.007
1.334051  0.008
1.33418   0.009
1.334314  0.010
3  5.27  5.25  5.24  0  0  0
```

```
3  4.88  4.87  4.88  0 0 0
1  0  0  0  0  0  0
1  0  0  0  0  0  0
1  1  1  0.0336

3  5.13  5.12  5.12  0  0  0
3  4.00  4.00  3.99  0  0  0
1  0  0  0  0  0  0
1  0  0  0  0  0  0
1  1  1  0.0336

3  5.25  5.23  5.22  0  0  0
3  3.54  3.53  3.54  0  0  0
1  0  0  0  0  0  0
1  0  0  0  0  0  0
1  1  1  0.0336

2  5.20  5.20  0  0  0  0
2  5.00  5.00  0  0  0  0
1  0  0  0  0  0  0
1  0  0  0  0  0  0
0.490  1  0.109  1

3  5.17  5.16  5.16  0  0  0
3  4.43  4.43  4.42  0  0  0
1  0  0  0  0  0  0
1  0  0  0  0  0  0
0.490  1  0.109  1

3  5.21  5.20  5.20  0  0  0
3  4.08  4.07  4.07  0  0  0
1  0  0  0  0  0  0
1  0  0  0  0  0  0
0.490  1  0.109  1
```

Program

```
FMT. LIGHT SCATTERING ANALYSIS'
BEGIN
REAL R,Q,K,KI,FS,FF,Z,T,P,H,C1,C2,F1,F2,F3,F4,FS1,FS2,
  FS3,FS4,
A,B,AA,AI,BI,CCMC,TS'
INTEGER W,M,I,L,NN,J'
SWITCH S1:=LI'
READ M'
BEGIN
    REAL ARRAY S(1:4),SUM(1:4),D,C,CM,N(1:M),
      G(1:4,1:6),GS(1:4,1:6)'
    REAL PROCEDURE INNERPRODUCT (A,B,M,I)'
    REAL A,B' INTEGER M,I'
    BEGIN
        REAL SUM' SUM:=O'
        FOR I:=1 STEP 1 UNTIL M DO SUM:=SUM+
          (A*B)'
        INNERPRODUCT:=SUM'
    END OF INNERPRODUCT PROCEDURE'
    PROCEDURE F(X,Y,M,F,A,B)'
    REAL ARRAY X,Y' INTEGER M' REAL F,A,B'
    BEGIN
        REAL XBAR,YBAR' INTEGER I'
        XBAR:=YBAR:=O' FOR I:=1 STEP 1 UNTIL
          M DO
        BEGIN
            XBAR:=XBAR+X(I)'
            YBAR:=YBAR+Y(I)'
        END'
        XBAR:=XBAR/M' YBAR:=YBAR/M'
        F:=(INNERPRODUCT(X(I)-XBAR,Y(I)-YBAR,
          M,I))/
        (SQRT(INNERPRODUCT(X(I)-XBAR-X(I)
          -XBAR,M,I)*
        INNERPRODUCT(Y(I)-YBAR,Y(I)-YBAR,M,1)))'
        A:=INNERPRODUCT(X(I) -XBAR,Y(I)-YBAR,M,
        I)/INNERPRODUCT(X(I)-XBAR,X(I)-XBAR,M,I)'
        B:=(INNERPRODUCT(X(I),1,M,I)*
          INNERPRODUCT(X(I),Y(I),M,I)-
        INNERPRODUCT(Y(I),1M,I)*INNERPRODUCT
          (X(I),X(I),M,I))/
        (INNERPRODUCT(X(I),1,M,I)**2-M*
          INNERPRODUCT(X(I),X(I),M,I))'
    END OF F PROCEDURE'
COMMENT DATA IS GIVEN IN FORM
M- NUMBER OF SOLUTIONS INCLUDING SOLVENT
W- WAVELENGTH
AA- CONSTANT VARIABLE A
N(M) -REFRACTIVE INDEX OF SOLVENT
R- RW/RC, CORRECTION FACTOR
F1,F2,F3,F4- FILTERS USED FOR SOLVENT—IF NO
  FILTER USED
THEN PUT VALUE EQUAL TO 1
NN- NUMBER OF GALVO READINGS FOLLOWED BY
  GALVO READINGS FOR EACH OF
GS,GW,G45,G135,FOR SOLVENT—IF NUMBER OF
  READINGS LESS THAN 6 THEN ADD APPROPRIATE
  NUMBER OF ZEROS
CCMC-CONCENTRATION AT CMC
N- REFRACTIVE INDEX OF SOLUTION FOLLOWED BY
  CONCENTRATION C OF
SOLUTION FOR EACH OF THE M SOLUTIONS
NN - NUMBER OF GALVO READINGS FOLLOWED BY
  GALVO READINGS
FOR EACH OF GS GW G45 G135 FOR SOLUTION
  FOLLOWED BY FILTERS
USED (AS ABOVE) FOR EACH OF THE M SOLUTIONS'
READ W,AA,N(M),R,F1,F2,F3,F4' C(M):=O'
IF W=436 THEN Q:=1.25 ELSE IF W=546 THEN Q:=1.41'
K:=Q*R*AA'
KI:=(32*3.1428571**3)/(3*W**4*6.023*10**(-5))'
FOR J:=1 STEP 1 UNTIL 4 DO
BEGIN
    READ NN' S(J):=O'
    FOR I:=1 STEP 1 UNTIL 6 DO
    BEGIN
        READ G(J,I)'
        S(J):=S(J)+G(J,I)'
    END'
    S(J):=S(J)/NN'
END'
FF:=F1*F2*F3*F4' TS:=K*N(M)*N(M)*FF*S(1)/S(2)'
  READ CCMC'
FOR I:=1 STEP 1 UNTIL M-1 DO
    BEGIN
        READ N(I), C(I)'
        CM(I):=C(I)-CCMC'
    END'
F(C,N,M,C1,A,B)'
IF C1 LESSEQ 0.9 THEN
BEGIN
    PRINT £NO CORRELATION?'
    GOTO LI'
END ELSE PRINT FREEPOINT(5), £
CORRELATION RI VERSUS C= ?,SAMELINE,C1,£
AI= ?,SAMELINE,A,£ BI= ?,SAMELINE,B'
FOR L:=1 STEP 1 UNTIL M-1 DO
BEGIN SWITCH S2:=L2'
    FOR I:=1 STEP 1 UNTIL 4 DO
    BEGIN
        SUM(I):=O' READ NN'
        FOR J:=1 STEP 1 UNTIL 6 DO
        BEGIN
            READ GS(I,J)'
            SUM(I):=SUM(I)+GS(I,J)'
        END'
        SUM(I):=SUM(I)/NN'
    END'
    READ FS1,FS2,FS3,FS4'
    FS:=FS1*FS2*FS3*FS4'
    IF G(3,1)=0 AND G(4,1)=0 AND GS(3,1)=0 AND
    GS(4,1)=0 THEN GOTO L2 ELSE
    Z:=(FS*SUM(3)/SUM(2)-FF*S(3)/S(2))/
    (FS*SUM(4)/SUM(2)-FF*S(4)/S(2))'
    IF Z GREQ 0.7 AND Z LESSEQ 1.3 THEN GOTO LI'
    L2:P:=A*C(L)+B'
    T:=K*P*FS*SUM(1)/SUM(2)' T:=T-TS'
    H:=(KI*P*P*(P-N(M))**2)/(C(L)*C(L))'
    D(L):=(H*CM(L))/T'
END'
F(CM,D,M-1,C2,AI,BI)'
PRINT FREEPOINT (5),£
CORRELATION HCM/T VERSUS CM= ?, SAMELINE,C2,£
A2= ?,SAMELINE,AI,£ B2= ?,SAMELINE,BI' PRINT £
MOLECULAR WEIGHT= ?,SAMELINE,1/BI'
END'
LI:END OF PROGRAM'
```

Data Output

```
CORRELATION RI VERSUS C=1.0000
AI= .13142 BI = 1.3330
CORRELATION HCM/T VERSUS CM= .98405
A2= .00010 B2= .00001
MOLECULAR WEIGHT = 121761.14
END OF PROGRAM
```

REFERENCES

(1) B. A. Mulley, "Advances in Pharmaceutical Sciences," vol. I, Academic, New York, N. Y., 1964.

(2) P. H. Ellworthy, A. T. Florence, and C. B. Macfarlane, "Solubilization by Surface Active Agents," Chapman & Hall Ltd. London, England, 1968.

(3) K. Kakemi, T. Arita, and J. Muramishi, *Chem. Pharm. Bull.*, **13,** 976(1965).

(4) A. F. Hofman and B. Borgstrom, *Federation Proc.*, **21,** 43(1962).

(5) J. R. Bloor and J. C. Morrison, to be published.

(6) C. T. Rhodes, *Can. J. Pharm. Soc.*, **2,** 16(1967).

(7) J. Harkins and K. Jordan, *J. Amer. Chem. Soc.*, **52,** 1751 (1932).

(8) "Handbook of Chemistry and Physics," 46th ed., Chemical Rubber Co., 1962.

(9) B. A. Brice, M. Halwer, and R. Speiser, *J. Opt. Soc. Amer.*, **40,** 768(1950).

(10) N. A. Lange, "Handbook of Chemistry," 10th ed., McGraw-Hill, New York (1961).

(11) "Non Ionic Surfactants," M. J. Schick, Ed., E. Arnold Ltd., London, England, 1967.

(12) P. Molyneaux, C. T. Rhodes, and J. Swarbrick, *Trans. Faraday Soc.*, **61,** 1043(1965).

(13) K. J. Humphreys and C. T. Rhodes, *J. Pharm. Sci.*, **57,** 79(1968).

(14) P. Debye, *J. Phys. Chem.*, **53,** 1(1949).

(15) K. A. Stacey, "Light Scattering in Physical Chemistry," Butterworths, London, England, 1956.

(16) H. S. Frank and Y. W. Wen, *Discuss. Faraday Soc.*, **24,** 133(1957).

(17) B. A. Wurzshmitt, *Anal. Chem.*, **30,** 118(1950).

(18) H. S. Frank and P. Evans, *J. Chem. Phys.*, **13,** 507(1945).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *School of Pharmacy, Portsmouth College of Technology, Hampshire, England.*

Accepted for publication October 31, 1969.

Presented to the Pharmaceutical Chemistry Section of the International Pharmaceutical Conference, London, September 1969.

The authors thank the Science Research Council for a grant enabling the purchase of the light-scattering photometer. Miss F. Tringham is thanked for her valuable assistance in devising the computer program.

* Present address: Sterling-Winthrop Research and Development, Newcastle-Upon-Tyne, England.

† Present address: Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada.

DRUG STANDARDS

Trifluoperazine Tablets: Alternative Methods of Analysis

J. R. WATSON, FUMI MATSUI, and W. N. FRENCH

Abstract □ Procedures utilizing a direct spectrophotometric measurement (such as described in the British Pharmacopoeia) for the analysis of trifluoperazine tablets suffer from several disadvantages. The possibility of excipient interference is not precluded and this factor, coupled with use of a fixed reference absorptivity value, can sometimes lead to erroneous results. Therefore, alternative assay procedures are required to assess accurately the drug content. Two such methods are described. The first is the acid-dye type and involves the partitioning of a trifluoperazine-bromocresol purple complex between an aqueous buffer pH 6 and benzene containing 1% isoamyl alcohol and subsequent measurement of the yellow-colored organic phase at 410 mμ. Acidic and neutral compounds as well as the common excipients do not interfere. The second method employs an alkaline siliceous earth column through which the drug is eluted into a chloroform–methanol–HCl system and the absorbance measured at 259 mμ. The precision and accuracy of the alternative methods, as well as the pharmacopeial procedure are compared using commercial dosage forms and simulated drug–excipient mixtures.

Keyphrases □ Trifluoperazine tablets—analyses □ Acid-dye method—trifluoperazine analysis □ Column chromatography—analysis □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer

The current BP method for the analysis of trifluoperazine hydrochloride tablets (1) involves dissolution of an aliquot sample of 20 powdered tablets and direct spectrophotometric measurement of the filtrate at 256 mμ using a fixed reference absorptivity value. Although the method is satisfactory in most cases, it suffers from the disadvantage of possible interference from excipients and also from both inter- and intrainstrumental variations. The latter variations may be considerable (2, 3) and introduce unsuspected error into the assay. Therefore, alternative assay procedures which are relatively free from interference and instrumental variation are required on occasion for products with assay results that are suspect by the pharmacopeial method.

While several methods have been reported in the literature as general procedures suitable for the analysis of piperazinyl phenothiazine drugs in pharmaceutical dosage forms and in biological media, there are virtually no data on their direct application to the analysis of trifluoperazine hydrochloride tablets. Blazek and Mares (4) determined drugs of the piperazinyl phenothiazine type gravimetrically by precipitation with silicotungstic acid or by electrochemical titration against the same reagent. Other electrochemical methods include controlled-potential coulometric analysis (5) and polarography (6), the latter being reported as having an accuracy of ±5% when applied to trifluoperazine hydro-

REFERENCES

(1) B. A. Mulley, "Advances in Pharmaceutical Sciences," vol. I, Academic, New York, N. Y., 1964.

(2) P. H. Ellworthy, A. T. Florence, and C. B. Macfarlane, "Solubilization by Surface Active Agents," Chapman & Hall Ltd. London, England, 1968.

(3) K. Kakemi, T. Arita, and J. Muramishi, *Chem. Pharm. Bull.*, **13,** 976(1965).

(4) A. F. Hofman and B. Borgstrom, *Federation Proc.*, **21,** 43(1962).

(5) J. R. Bloor and J. C. Morrison, to be published.

(6) C. T. Rhodes, *Can. J. Pharm. Soc.*, **2,** 16(1967).

(7) J. Harkins and K. Jordan, *J. Amer. Chem. Soc.*, **52,** 1751 (1932).

(8) "Handbook of Chemistry and Physics," 46th ed., Chemical Rubber Co., 1962.

(9) B. A. Brice, M. Halwer, and R. Speiser, *J. Opt. Soc. Amer.*, **40,** 768(1950).

(10) N. A. Lange, "Handbook of Chemistry," 10th ed., McGraw-Hill, New York (1961).

(11) "Non Ionic Surfactants," M. J. Schick, Ed., E. Arnold Ltd., London, England, 1967.

(12) P. Molyneaux, C. T. Rhodes, and J. Swarbrick, *Trans. Faraday Soc.*, **61,** 1043(1965).

(13) K. J. Humphreys and C. T. Rhodes, *J. Pharm. Sci.*, **57,** 79(1968).

(14) P. Debye, *J. Phys. Chem.*, **53,** 1(1949).

(15) K. A. Stacey, "Light Scattering in Physical Chemistry," Butterworths, London, England, 1956.

(16) H. S. Frank and Y. W. Wen, *Discuss. Faraday Soc.*, **24,** 133(1957).

(17) B. A. Wurzshmitt, *Anal. Chem.*, **30,** 118(1950).

(18) H. S. Frank and P. Evans, *J. Chem. Phys.*, **13,** 507(1945).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *School of Pharmacy, Portsmouth College of Technology, Hampshire, England.*

Accepted for publication October 31, 1969.

Presented to the Pharmaceutical Chemistry Section of the International Pharmaceutical Conference, London, September 1969.

The authors thank the Science Research Council for a grant enabling the purchase of the light-scattering photometer. Miss F. Tringham is thanked for her valuable assistance in devising the computer program.

* Present address: Sterling-Winthrop Research and Development, Newcastle-Upon-Tyne, England.

† Present address: Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada.

DRUG STANDARDS

Trifluoperazine Tablets: Alternative Methods of Analysis

J. R. WATSON, FUMI MATSUI, and W. N. FRENCH

Abstract □ Procedures utilizing a direct spectrophotometric measurement (such as described in the British Pharmacopoeia) for the analysis of trifluoperazine tablets suffer from several disadvantages. The possibility of excipient interference is not precluded and this factor, coupled with use of a fixed reference absorptivity value, can sometimes lead to erroneous results. Therefore, alternative assay procedures are required to assess accurately the drug content. Two such methods are described. The first is the acid-dye type and involves the partitioning of a trifluoperazine-bromocresol purple complex between an aqueous buffer pH 6 and benzene containing 1% isoamyl alcohol and subsequent measurement of the yellow-colored organic phase at 410 mμ. Acidic and neutral compounds as well as the common excipients do not interfere. The second method employs an alkaline siliceous earth column through which the drug is eluted into a chloroform–methanol–HCl system and the absorbance measured at 259 mμ. The precision and accuracy of the alternative methods, as well as the pharmacopeial procedure are compared using commercial dosage forms and simulated drug–excipient mixtures.

Keyphrases □ Trifluoperazine tablets—analyses □ Acid-dye method—trifluoperazine analysis □ Column chromatography—analysis □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer

The current BP method for the analysis of trifluoperazine hydrochloride tablets (1) involves dissolution of an aliquot sample of 20 powdered tablets and direct spectrophotometric measurement of the filtrate at 256 mμ using a fixed reference absorptivity value. Although the method is satisfactory in most cases, it suffers from the disadvantage of possible interference from excipients and also from both inter- and intrainstrumental variations. The latter variations may be considerable (2, 3) and introduce unsuspected error into the assay. Therefore, alternative assay procedures which are relatively free from interference and instrumental variation are required on occasion for products with assay results that are suspect by the pharmacopeial method.

While several methods have been reported in the literature as general procedures suitable for the analysis of piperazinyl phenothiazine drugs in pharmaceutical dosage forms and in biological media, there are virtually no data on their direct application to the analysis of trifluoperazine hydrochloride tablets. Blazek and Mares (4) determined drugs of the piperazinyl phenothiazine type gravimetrically by precipitation with silicotungstic acid or by electrochemical titration against the same reagent. Other electrochemical methods include controlled-potential coulometric analysis (5) and polarography (6), the latter being reported as having an accuracy of ±5% when applied to trifluoperazine hydro-

Table I—Analysis of Simulated Drug–Excipient Mixtures

Simulated Mixture No.	% Recovery[a] Direct UV[b]	Acid Dye[c]	Column[d]
1	102.9	101.0	101.6
2	103.8	100.4	101.8
3	104.3	100.8	100.1
4	106.2	100.7	100.8
5	107.2	100.1	100.5
6	109.1	100.3	101.2

[a] Each quoted value in a given row represents the average results of duplicate runs using the same stock solution. Maximum range for duplicates on each sample were: [b] 0.5%, [c] 0.5%, and [d] 0.6%.

chloride formulations. Quantitative paper chromatography involving colorimetric measurement at 610 mμ of the complex formed between the drug and potassium iodoplatinate after elution from the chromatogram was applied to several of the phenothiazine-type drugs by Nadeau and Sobolewski (7). Piperazinyl phenothiazine compounds could be detected in submicrogram quantities by gas chromatography (8) and spectrofluorometry (9), but these techniques have yet to be developed into precise analytical methods.

While the above procedures perhaps could be employed in some instances for the analysis of trifluoperazine hydrochloride tablets, in general they are not sufficiently rapid and accurate or else require the use of equipment which may not be readily available to the analyst.

In the present study, two alternative assay procedures were considered. The first assay procedure is of the acid-dye type and involves the partitioning of a trifluoperazine-bromocresol purple complex between an aqueous buffer of pH 6 and benzene containing 1% isoamyl alcohol, and subsequent measurement of the organic phase at 410 mμ. The second method employs an alkaline-siliceous earth chromatographic column from which the drug is eluted with chloroform (10) and the absorbance measured at 259 mμ. In addition, the use of ultrasonic energy was investigated as a rapid and convenient means to break up the solid dosage form and prepare the sample solution.

In order to measure the accuracy and precision of each technique and to compare against the direct UV procedure (BP method), stock sample solutions were prepared using the ultrasonic disintegration apparatus and aliquots of the filtered solution treated simultaneously by each of the assay procedures.

EXPERIMENTAL

A. Preparation of Sample Solutions—For each product examined, 10 tablets were selected at random and the active ingredient dissolved in a sufficient volume of 1% (v/v) HCl to give a concentration (based on label claim[1]) of approximately 23.6 mg. of salt or the equivalent of 20 mg. of base per 100 ml. (*i.e.*, 10 × 1-mg. tablets in 50 ml, 10 × 2 mg. in 100 ml., 10 × 5 mg. in 250 ml., 10 × 10 mg. in 500 ml., and 10 × 20 mg. in 1000 ml.).

Disintegration of the tablets and dissolution of the drug were accomplished with an ultrasonic probe of 1.27-cm. (0.5-in.) diameter (clamped in a vertical position, tip downward) powered by a 200-w. generator.[2] A basket assembly constructed of stainless steel wire mesh (30 mesh), 5.08 cm. (2 in.) in length and internal diameter slightly larger than the probe, was used to hold each tablet in the area of maximum sonic energy emission. A small rod attached to the basket assembly served as an arm to hold the basket in place during insonation.

In practice, each of the 10 tablets was insonated individually. The first tablet was placed in the wire basket assembly and the basket brought up over the probe so that the tablet came in contact with the tip. A beaker containing the appropriate amount of solvent was raised so that the basket was about half immersed. Ultrasonic energy was applied for 30 sec. at a power setting of 30% maximum with manual tuning for optimum output. This insonation process was repeated until all 10 tablets had been disintegrated. The resulting suspension was stirred briefly and then filtered through a 25-mm. membrane filter of 0.45-μ porosity (with a 1-mm. thick prefilter) using a 5-ml. syringe fitted with a continuous pipeting device. Aliquots of this solution after filtration were subsequently taken for analysis by the chromatographic column procedure, by direct UV measurement, and by the acid-dye technique.

Simulated drug–excipient mixtures were prepared by adding 10 ml. of a solution of trifluoperazine hydrochloride (accurately weighed and made up to a concentration of about 11.8 mg./10 ml. 1% HCl) to 25 ml. of filtered solution prepared as above from insonation of placebos, and adjusting the volume to 50 ml. with 1% HCl (final concentration approximately 23.6 mg. salt/100 ml.). For each successive mixture (Table I), the number of placebos was increased to give about 9% overestimation with the direct UV procedure.

B. Assay by Direct UV Measurement—An aliquot of the filtered sample solution prepared in Part A was diluted twentyfold with 1% HCl to give a concentration (based on label claim) of about 1.18 mg. of salt/100 ml. Dilution was facilitated by the use of an automatic dilutor[3] adjusted for a diluent to sample solution ratio of 19:1. The absorbance of the final solution was measured at 256 mμ against a blank of 1% HCl. The average drug content per tablet was determined by comparison against the average absorptivity value obtained from three separate solutions containing trifluoperazine hydrochloride reference material. For each solution, about 50 mg. of standard (accurately weighed) was made to a volume of 250 ml. in 1% HCl. A twentyfold dilution was made as described with the automatic dilutor.

C. Assay by the Acid-Dye Technique—*Reagents*—(*a*) Buffer solution, pH 6.0 (McIlvaine): 7.37 ml. of 0.1 *M* citric acid plus 12.63 ml. of 0.2 *M* Na_2HPO_4. (*b*) Bromocresol purple (BCP) in buffer solution: bromocresol purple dissolved in buffer solution of pH 6.0 to give a concentration of 37.5 mg./100 ml. This solution was extracted with benzene containing 1% isoamyl alcohol until the extract was virtually colorless. (*c*) Photometric solvent: benzene (A.R.) containing 1% isoamyl alcohol (A.R.) by volume.

Procedure—An aliquot of the filtered sample solution prepared in Part A was diluted tenfold with 1% HCl to give a concentration (based on label claim) of about 118 mcg. of salt per 5 ml. A 5.0-ml. aliquot of this final solution was pipeted into a 42-ml. centrifuge tube along with 5.0 ml. of buffer pH 6, 5.0 ml. of BCP solution, and 10 ml. of photometric solvent (the latter dispensed from an automatic dispenser for convenience). The tube was stoppered and tumbled end-over-end for 5 min. in a device constructed to rotate 15 tubes simultaneously at 100 r.p.m. (alternatively, the stoppered tubes may be shaken vigorously by hand for 1 min.). After mixing, the tube was centrifuged for 2 min.; then the clear supernatant was decanted carefully into a clean dry cell for measurement of absorbance at 410 mμ against a blank prepared in the same manner using 5 ml. of 1% HCl. At the same time, 5.0 ml. of standard reference solution containing approximately 120 mcg. of trifluoperazine hydrochloride (accurately determined from the initial weighing) was carried through the assay procedure. The average drug content per tablet was determined by comparison of the absorbance of the sample solution against that of the standard reference solution.

D. Assay by the Alkaline-Siliceous Earth Column Procedure—*Pretreatment of Diatomaceous Earth*[4]—Acid-washed diatomaceous earth (100 g.) was placed in a 64 × 150-mm. medium-porosity

[1] Tablets were labeled to contain trifluoperazine hydrochloride with the dosage level expressed as the equivalent amount of base.

[2] Blackstone Ultrasonics, model BP-2.
[3] Labindustries, Automatic Dilutor.
[4] Celite 545, Johns-Manville, New York, N. Y.

sintered-glass funnel and 1000-ml. portions of 1:1 hot (boiling) aqueous HCl passed through with the aid of gentle suction. Five hundred milliliters of hot water was then passed through, followed by 1000 ml. of hot 1 *N* NaOH, 500 ml. of chloroform, and 1000 ml. of water. The treated diatomaceous earth was dried for 15 hr. at 110°.

Preparation of Partition Columns—Six grams of the purified diatomaceous earth was mixed well with 2 ml. of 20% NaOH in a small beaker and the damp mass transferred to a 25 × 220-mm. glass column containing a pledget of glass wool at the base to support the diatomaceous earth. The mixture was firmly tamped to produce a column free of air pockets and irregularities.

Procedure—A 4.0-ml. aliquot of the filtered sample solution prepared in Part A was pipeted directly on the surface of the column and allowed to soak in. Water-washed chloroform (90 ml.) was then passed through the column and collected in a 100-ml. volumetric flask containing 8 ml. of methanol and 4 drops of concentrated HCl. The contents of the volumetric flask were made to volume with water-washed chloroform and the absorbance measured at 259 mμ against a blank prepared in the same manner. The average drug content per tablet was determined by comparison against the absorptivity of a reference standard solution prepared in the same manner.

RESULTS AND DISCUSSION

Sample Preparation—Spectrophotometric analysis of drugs in a solid dosage form involves two main steps: the first is to effect quantitative dissolution of the active ingredient from the tablet matrix and the second is to prepare the solution in a suitable form (*i.e.*, clarity and concentration) for final measurement. The techniques used to achieve these goals ultimately govern the overall accuracy and precision of the method.

The current pharmacopeial directive for extracting trifluoperazine hydrochloride from excipient material entails finely grinding 20 weighed tablets and shaking an aliquot of the powder equivalent to about 5 mg. of the drug for 15 min. with 5% HCl. The mixture is then made up to a specified volume with the same solvent, mixed, filtered, and the absorbance measured at 256 mμ. Several factors inherent to this type of sampling sequence tend to diminish the accuracy and precision of the analysis. First are the errors in weighing and those associated with mechanical loss and nonhomogeneous grind. In some cases, there is the added problem of incomplete dissolution of the active drug because of insufficient particle breakup. The use of ultrasonic disintegration as a sampling tool not only precludes the above difficulties but is more rapid and less tedious than the conventional technique. The fact that 1% HCl invariably gave the same assay results as 5% HCl, and because the former acidity was more compatible with the requirements of the acid-dye extraction method and the alkaline-siliceous earth column procedure, militated against the use of the stronger pharmacopeial solvent. Before adopting the ultrasonic technique for routine analytical use, it was necessary to establish its feasibility for the phenothiazine drugs in general and trifluoperazine in particular because there are several well-documented cases in the literature where the ultrasonic field induced chemical changes in the sonified material. For example, the rate of hydrolysis of aspirin was increased when the drug was subjected to ultrasound (11, 12). Skauen (13) has reviewed other instances where ultrasonic waves under certain conditions affected the chemical integrity of medicinal compounds. Because of the lability of most compounds of the phenothiazine family, it was deemed essential to monitor the absence or presence of any destructive action on the trifluoperazine molecule associated with the high-energy emission from the probe. This was done by: (*a*) comparing the thin-layered chromatograms (14, 15) of powdered and sonified tablets; developing systems used were benzene–dioxane–aq. NH_3(60:35:5) giving R_f = 0.69, and *n*-BuOH saturated with 1 *N* NH_3 giving R_f = 0.51; in no instance were any spots found in the sonified material which were not present in the powdered sample; and (*b*) comparing the assay results using the shakeout procedure against those using the ultrasonic-disintegration technique; within experimental error and content uniformity variations, these were in good agreement (usually within 2%). The data, therefore, gave no evidence to suggest any decomposition of any brand of trifluoperazine tablets resulting from exposure to ultrasonic energy under the conditions employed.

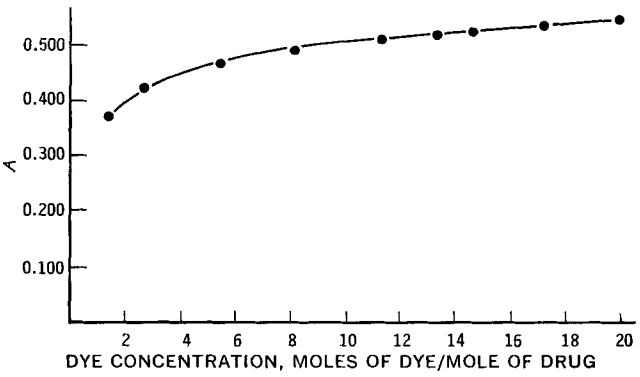


Figure 1—*Effect of dye concentration on absorbance (varying quantity of dye but fixed concentration of drug).*

The official compendium does not specify the type of filtration in the monograph for trifluoperazine tablets. With certain formulations where gravity filtration through Whatman No. 2 paper was employed, the filtrate appeared as a fine opalescent suspension, a situation which could conceivably lead to erroneously high results despite subsequent dilution. While the use of finer porosity paper usually eliminated this difficulty, the filtration step became excessively time consuming and, as a result, the possibility of drug decomposition became an important consideration. Suction filtration even through a thin pad of acid-washed diatomaceous earth was sometimes subject to clogging and was therefore not always satisfactory. Maximum clarity was readily achieved with the use of a membrane filter of micron-range porosity.

Methods—Under the experimental conditions of sample preparation, the effective dye concentration expressed as a molar ratio of dye to drug with the acid-dye technique was approximately 12:1. A plot of this ratio against absorbance is given in Fig. 1. With a fixed concentration of drug, in the case of trifluoperazine hydrochloride, the optimum dye concentration expressed in moles of dye per mole of drug is restricted to a relatively narrow band between 10:1 and 16:1. At lower ratios the color intensity was significantly decreased and was more sensitive to small variations in dye concentration, while higher ratios (*i.e.*, more concentrated dye solution) resulted in the formation of a precipitate. Beer's law was obeyed for drug concentrations in the range of 30 to 150 mcg./5 ml. of sample solution. All solutions which were analyzed by the acid-dye technique in this study fell within this concentration range.

The extent of partition of the drug–dye complex from an aqueous phase of given pH into the organic phase appears to be related to the molecular structure of the particular drug and dye. In the ideal situation, the pH values at which partition of the colored ion pair into the organic phase is a maximum and blank readings a minimum are coincident. This is rarely the case in practice but the pH should usually be kept as high as possible to prevent extraction of the sulfonic acid form of the dye into the organic layer. An optimum

Table II—Analysis of Commercial Dosage Forms

Sample No.	Manufacturer	Dosage Level, mg. Base/Tablet	% of Label Claim[a]		
			Direct UV[b]	Acid Dye[c]	Column[d]
1	A	1	93.9	92.6	90.3
2	B	2	99.7	99.0	100.2
3	B	2	98.6	95.3	97.5
4	C	1	92.6	90.6	91.0
5	C	2	100.7	98.7	100.0
6	D	1	96.9	98.0	95.7
7	D	1	95.1	95.1	93.4
8	E	1	105.7	99.5	100.4
9	E	5	108.8	109.2	106.9

[a] Each quoted value in a given row represents the average results of duplicate runs using the same stock solution. Maximum range for duplicates on each sample were: [b] 0.6%, [c] 0.8%, and [d] 0.9%.

buffer pH of 6.0 was indicated for the trifluoperazine-bromocresol purple system.

In the chromatographic method, the solvent system, methanol (8 ml.) containing concentrated hydrochloric acid (4 drops) and diluted to 100 ml. with water-washed chloroform, is completely transparent down to the cutoff point of 242 mμ. In this solvent system, the absorption maximum for trifluoperazine is at 259 mμ. Nine determinations of the absorptivity of trifluoperazine hydrochloride following partition through the alkaline-siliceous earth column gave an average value of 59.80 with a coefficient of variation of 1%. The commercial diatomaceous earth (see *Footnote 4*), which is often suitable as such for analysis by column chromatography, in this instance gave blank readings which were high and variable (0.072–0.114) and, as a consequence, was not satisfactory for use without further treatment. Washing with hydrochloric acid, then consecutively with aqueous alkali and chloroform to simulate the analytical conditions, led to almost total removal of UV-absorbing impurities and lowered the reference column absorbance values to 0.005 or less. This value, established by several replicate measurements on each treated batch of diatomaceous earth, was quite reproducible and was subtracted as a fixed quantity from the absorbance reading of each solution of the drug eluted from the column.

Results—Studies on the extent of the quantitative interference by excipient substances encountered with the direct spectrophotometric procedure for four brands of trifluoperazine tablets showed that the absorbance contribution of the water-soluble coating materials is generally negligible with the higher dosage forms but is significant at the 1-mg. level where the dilution factor is less. With some of these tablets, the drug content can be overestimated by 2.5%. With placebos of Brand V, for example, an absorbance measurement equivalent to approximately 5% interference was observed. The soluble (in 1% HCl) coating materials of Brands I–IV, and the soluble excipient substances of the placebos of Brand V, however, gave zero absorbance on spectrophotometric analysis by either of the two alternative methods.

To substantiate the validity of these results and to ascertain that complete drug recovery could be effected by the acid dye and the alkaline column procedures, several simulated drug–excipient mixtures were prepared from a stock solution of trifluoperazine hydrochloride and filtered excipient solutions made from sonification of placebos. The data in Table I clearly demonstrate that, as expected with the pharmacopeial method, the presence of excipient material in the simulated solutions resulted in varying levels of interference corresponding to the number of placebos employed, but that no detectable interference and essentially 100% recovery were achieved with all six solutions by the two alternative procedures.

Inspection of the results in Table II for nine commercial formulations shows that, where the extent of interference is about 2.5% or less, the direct spectrophotometric method is of comparable accuracy to that of the two proposed procedures. For most of the lot numbers examined, the assay values are comparable for all three assay procedures, except in the case of sample No. 8 where the degree of overestimation was about 6% by the direct UV method.

The above data underline the fact that with some trifluoperazine tablet formulations, the pharmacopeial method can give erroneously high results, and in these instances where suspicious assay results are encountered, the two alternative procedures described herein should prove useful to assess accurately the drug content.

REFERENCES

(1) "British Pharmacopoeia 1968," General Medical Council, The Pharmaceutical Press, London, England, 1968, p. 1039.

(2) G. Wernimont, *Anal. Chem.*, **39** 554(1967).

(3) M. Winstead, *Amer. J. Med. Technol.*, **32** (1966).

(4) J. Blazek and V. Mares, *Cesk. Farm.*, **15,** 349(1966).

(5) F. H. Merkle and C. A. Discher, *Anal. Chem.*, **36,** 1639 (1964).

(6) N. Chuen and B. E. Riedel, *Can. Pharm. J., Sci. Sect.*, **94,** 51(1961).

(7) G. Nadeau and G. Sobolewski, *J. Chromatogr.*, **2,** 544(1959).

(8) C. McMartin and H. V. Street, *ibid.*, **22,** 274(1966).

(9) J. B. Ragland and V. J. Kinross Wright, *Anal. Chem.*, **36,** 1356(1964).

(10) J. Levine, *J. Pharm. Sci.*, **54,** 485(1965).

(11) E. Mario and R. J. Gerraughty, *ibid.*, **54,** 321(1965).

(12) T. E. Needham, Jr., and R. J. Gerraughty, *ibid.*, **58,** 62(1969).

(13) D. M. Skauen, *ibid.*, **56,** 1373(1967).

(14) J. Cochin and J. W. Daly, *J. Pharmacol. Exp. Ther.*, **139,** 160(1963).

(15) T. J. Mellinger and C. E. Keeler, *J. Pharm. Sci.*, **51,** 1169 (1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 14, 1969, from the *Research Laboratories, Food and Drug Directorate, Ottawa, Canada.*

Accepted for publication October 9, 1969.

Presented in part to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutial Sciences, Montreal meeting, May 1969.

Optimal Ferric Hydroxamate Method for Determination of Intact Pilocarpine

IRWIN S. GIBBS* and MURRAY M. TUCKERMAN

Abstract □ The ferric hydroxamate colorimetric method for lactones has been optimized for the determination of pilocarpine in the presence of its degradation product by careful control of pH, reagent concentration, and time to yield a method which has the following advantages of similar methods previously proposed: (*a*) direct sampling of the usual 1 or 2% solutions without dilution; (*b*) a stable blank with relatively low absorbance; and (*c*) production of a color with a minimal rate of fading, suitable for quantitative determination. Analysis of 20 independent sets of calibrations by linear regression analysis yields a regression coefficient of 0.98, an absorbance (*y*) intercept of −0.007, and a slope of 0.51.

Keyphrases □ Pilocarpine solutions—analysis □ Ferric hydroxamic acid method—color formation □ Colorimetry analysis—spectrophotometer

Various analytical methods for the determination of pilocarpine hydrochloride have appeared in the literature including titrimetric (1–4), ion-exchange (5), colorimetric (6–12), and measurement of the UV absorption of the tetraphenylboride (13). For the routine quality control of pilocarpine hydrochloride ophthalmic solutions, the method of Brochmann-Hanssen *et al.* (12) appears most useful. The method consists of making the sample alkaline in the presence of hydroxylamine hydrochloride, allowing the reaction to proceed for a period of time, and adding ferric chloride to the acidified mixture to yield a colored ferric hydroxamate. After allowing the color to develop, the absorbance is read at 500 mμ in a suitable instrument. The advantages of this method are specificity for the intact lactone ring of the pilocarpine molecule and more than sufficient sensitivity.

In using the technique, this sensitivity has been found to be a burden because samples cannot be taken directly from the solutions of pilocarpine hydrochloride commonly appearing in commerce, thus entailing several dilutions during analysis of samples and preparation of calibration curves. In addition, variations in absorbance with time for treated samples, gas bubble formation on the walls of the cells, and the small volume of the final sample, making adequate rinsing of cells difficult, all serve to make this method less than ideal.

The proposed method reduces the disadvantages, allowing for direct sampling from aqueous solutions of pilocarpine hydrochloride in concentrations of 1 and 2% and yielding absorbance values which conform to the usual conditions for photometric measurement.

EXPERIMENTAL

Apparatus—The Cary model 15 recording spectrophotometer and the Gilford apparatus with the Beckman DUR monochromator were used for the absorbance measurements.

Samples—The samples consisted of aqueous solutions of pilocarpine hydrochloride USP XVII in a concentration of 0.7%.

Table I—Change in Absorbance with Time against Water

Time	Sample	Reagent Blank
	Brochmann-Hanssen Method[a]	
	Trial A	
Initial	0.732	0.205
+ 10 min.	0.844	0.320
+ 20 min.	0.894	0.392
	Trial B	
Initial	0.779	0.133
+ 10 min.	0.777	0.304
+ 20 min.	0.819	0.361
	Trial C	
Initial	0.872	0.159
+ 10 min.	0.991	0.308
+ 20 min.	1.020	0.316
	Trial D	
Initial	0.667	0.161
+ 10 min.	0.692	0.060
+ 20 min.	0.672	0.100
	Proposed Method[b]	
	Trial A	
Initial	0.504	0.005
+ 10 min.	0.489	0.007
+ 20 min.	0.472	0.007
	Trial B	
Initial	0.457	0.007
+ 10 min.	0.442	0.006
+ 24 min.	0.418	0.006

[a] $\%\Delta A$ for sample/min. for 1st 10 min. based on initial net sample value = −0.06, Trial A; −2.7, Trial B; −0.42, Trial C; and +2.49 Trial D. [b] $\%\Delta A$ for sample/min. for 1st 10 min. based on initial net sample value = −0.34, Trial A; and −0.31, Trial B.

Solutions used in the performance of the method suggested by Brochmann-Hanssen *et al.* (12) were 0.04%. The pilocarpine hydrochloride employed was assayed by the USP method (14), yielding a recovery of 99.85 ± 0.11%.

Reagents—Hydroxylamine hydrochloride, 1 *M* in water; alkaline phosphate consisting of 10 volumes of 7% trisodium phosphate and 2 volumes of 3.5 *M* sodium hydroxide; and ferric chloride solution, 0.3 *M* in 0.1 *N* hydrochloric acid.

Proposed Method—Transfer an accurately measured volume of pilocarpine hydrochloride solution containing between 10 and 20 mg. of pilocarpine hydrochloride to a 25.0-ml. volumetric flask and add water to make approximately 10 ml. Add 1.0 ml. of 1 *M* hydroxylamine hydrochloride and 3.0 ml. of alkaline phosphate solution, mixing well after each addition, and allow the solution to stand for 10 min. Add 1.0 ml. of 5.25 *M* hydrochloric acid followed by 1.0 ml. of ferric chloride solution. Dilute with water after 10 min. and read the absorbance in a suitable spectrophotometer at 480 mμ. A reagent blank is treated in the same manner. A calibration curve may be conveniently prepared by taking 1.00, 2.00, and 3.00 ml. of a 0.7% pilocarpine hydrochloride solution (which yields absorbance values equivalent to 0.35, 0.70, and 1.05% solutions, respectively) and treating in the described manner.

RESULTS

Twenty calibration curves were prepared as indicated on separate days. Each curve was prepared using fresh reagents and a fresh 0.7% pilocarpine hydrochloride standard solution. The following

Table II—Absorbance *versus* Time Obtained with Treated Sample (3.00 ml. of 0.7% Pilocarpine Hydrochloride) and Reagent Blank

Time (Minutes after Addition of Ferric Chloride Solution)	Sample Absorbance	Reagent Blank Absorbance
2	0.563	0.011
3	0.557	0.011
4	0.553	0.011
5	0.551	0.010
6	0.550	0.011
7	0.548	0.010
8	0.547	0.009
9	0.546	0.010
10	0.545	0.009
11	0.544	0.012
12	0.545	0.012
13	0.544	0.012
14	0.543	0.012
15	0.541	0.011
16	0.541	0.011
17	0.541	0.009
18	0.538	0.010
19	0.536	0.011
20	0.535	0.010
21	0.531	0.011
22	0.532	0.011

significant values were obtained upon calculation using regression analysis: regression coefficient, 0.98; absorbance (y) intercept, −0.007; and slope, 0.51.

Fading Rate—As has been pointed out (12, 15), ferric hydroxamic acid procedures, in general, yield unstable colors. Comparison of the change in absorbance with time between the proposed method and the Brochmann-Hanssen method for pilocarpine hydrochloride was rendered difficult by formation of gas bubbles on the walls of the cell immediately upon introduction of the sample when using the Brochmann-Hanssen technique. These bubbles grew larger with time. The colored solutions arising from the proposed method, however, are homogeneous and no difficulty was experienced.

Absorbance spectra of the solutions obtained with the Brochmann-Hanssen method showed a maximum at 500 mμ as reported (12), while those of solutions obtained with the proposed method showed a slightly broader maximum in the region of 480 mμ. Recorded absorbance spectra obtained with the solutions treated in accordance with the Brochmann-Hanssen technique 10 and 20 min. after the initial spectrum invariably showed a total increase in absorbance at the wavelength of maximum absorption, although the +20-min. spectrum sometimes showed a decrease in absorbance from that at +10 min. Recorded absorbance spectra of solutions obtained with the proposed method showed only a slight decrease in the absorbance maximum 10 and 20 min. after the initial spectrum.

Due to the paradoxical behavior of the solutions obtained in the Brochmann-Hanssen method and also to the fact that both the sample and blank are highly colored, the changes in absorbance with time of both samples and blanks were determined independently against water at 500 mμ. The same was done for solutions obtained with the proposed method at 480 mμ. The results are shown in Table I.

As can be seen, the method proposed by Brochmann-Hanssen *et al.* (12) yields rather large changes in absorbance with time for both the sample and blank. In addition, the overall net change in absorbance of the sample is not predictable with respect to direction or magnitude. On the other hand, the absorbance of the reagent blank used in the proposed method is constant within the limits of accuracy of the digital read-out meter of the Gilford apparatus employed. The absorbance of the sample obtained by the proposed method shows only a slight decrease over the time period studied.

In order to determine the optimal time period between combination of the sample with the reagents and determination of the absorbance, an absorbance *versus* time study for the sample and reagent blank was performed. Water was used to establish a baseline absorbance of 0. The results are shown in Table II.

As can be seen by perusal of Table II, the absorbance of the reagent blank again remains constant within the limits of accuracy of the instrument throughout the time period employed. The sample shows an initial accelerated fading, which is somewhat stabilized at approximately 10 min. following the addition of the ferric chloride solution. Thus, by following the directions given for the proposed method, the operator has sufficient time to make several solutions to volume, rinse the cells, and take absorbance readings without fear of significant fading during the time required.

CONCLUSIONS

A method has been proposed for the colorimetric analysis of aqueous solutions of pilocarpine hydrochloride which exhibits: (*a*) ease of sampling; (*b*) no change in absorbance of the reagent blank and only slight fading of the sample over the time suggested after addition of the last reagent required in sample treatment and reading of the sample absorbance; and (*c*) no gas bubble formation in the treated sample as was obtained with a previously proposed method (12).

While the proposed method has not been applied to samples of commercially available solutions, no marked deviations are expected in the results, provided that interfering substances are removed prior to sample treatment by methods similar to that prescribed by Brochmann-Hanssen *et al.* (12).

REFERENCES

(1) T. Higuchi and J. Concha, *J. Amer. Pharm. Ass., Sci. Ed.*, **40,** 173(1951).

(2) C. A. Johnson and R. E. King, *J. Pharm. Pharmacol.*, **15,** 584(1963).

(3) J. Tolgyessy and M. Sarsunova, *Z. Anal. Chem.*, **196,** 192(1963).

(4) P. J. Cooper and P. W. Hammond, *Analyst*, **92,** 180(1967).

(5) W. Manikowski and L. Niezgodski, *Poznan. Towarz. Przyjaciol Nauk, Wydzial Lekar., Prace Komisji Farm.*, **4,** 165 (1966); through *Chem. Abstr.*, **65,** 15159h(1966).

(6) I. S. Shupe, *J. Ass. Offic. Agr. Chemists*, **24,** 757(1941).

(7) J. W. Webb, R. S. Kelley, and A. J. McBay, *J. Amer. Pharm. Ass., Sci. Ed.*, **41,** 278(1952).

(8) J. Levine and E. Horrocks, *J. Ass. Offic. Agr. Chemists*, **43,** 233(1960).

(9) R. Fagerstrom, *J. Pharm. Pharmacol.*, **15,** 479(1963).

(10) J. Siman, *Gyogyszereszet*, **9,** 221(1965); through *Chem. Abstr.*, **63,** 11252h(1965).

(11) I. Calafeteanu, V. Stoicesco, P. Grintesco, H. Beral, E. Dumitresco, and C. Ioan, *Acta Pharm. Jugoslav.*, **15,** 83(1965).

(12) E. Brochmann-Hanssen, P. Schmid, and J. D. Benmaman, *J. Pharm. Sci.*, **54,** 783(1965).

(13) J.-A. Gautier, J. Renault, and J. Rabiant, *Ann. Pharm. Franc.*, **17,** 491(1959).

(14) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 471.

(15) S. Siggia, "Quantitative Organic Analysis Via Functional Groups," 3rd ed., Wiley, New York, N. Y., 1963, p. 140.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 4, 1969, from *Temple University, School of Pharmacy, Health Sciences Center, Philadelphia, PA 19140*

Accepted for publication September 29, 1969.

* American Foundation for Pharmaceutical Education fellow, 1966–1969.

Fluorometric Determination of Salicylic Acid in Aspirin Products Including Noninterfering, Interfering, and Buffered Substances

NORMAN SHANE and DENNIS MIELE

Abstract □ A rapid, fluorometric method for the determination of salicylic acid in aspirin products in combination with noninterfering fluorescent substances, such as phenyltoloxamine citrate, glyceryl guaiacolate, and caffeine, interfering fluorescent materials, such as hydrogenated castor oil (Castorwax) and salicylamide, and buffering agents, such as aluminum glycinate and magnesium carbonate, gave accurate and precise results. The procedure is based on a simple dissolution of the crushed tablet in a pH 4.0 buffer solution and reading of the filtered solution at activation and fluorescent wavelengths of 310 and 410 mμ, respectively. An additional operation of a chloroform and buffer solution extraction is necessary when interfering substances are present. In the case of salicylamide, a constant background reading has to be subtracted; however, an accuracy of 95.5% was established. For nonaspirin salicylates in buffered-aspirin products, the procedure gave results similar to those reported by Guttman and Salomon.

Keyphrases □ Aspirin dosage forms—salicylic acid determination □ Salicylic acid determination—aspirin dosage form, buffered, unbuffered □ Interfering substances—salicylic acid determination □ Fluorometry—analysis

There are many methods in literature for the determination of small amounts of salicylic acid in aspirin and aspirin products. These involve colorimetry (1–3), chromatography (4–7), spectrophotometry (5–10), and titrimetry (11). Fluorometric determinations (12–14) have been described for the determination of salicylate in biological tissues by measurement of the characteristic fluorescence of the salicylate ion on exposure to UV light.

The present paper describes a simple, fast, fluorometric determination of salicylic acid in aspirin products. With no interference of other fluorescent compounds, separations are not necessary. The ingredients can be dispersed in a pH 4 buffer solution, filtered, and the salicylic acid content determined fluorometrically. With interfering substances that have solubility differences in organic systems, a simple extraction can be performed.

For buffered-aspirin tablets, a variety of methods (15–18) is described for the determination of total nonaspirin salicylates. By treating the sample with HCl–citric acid solution and extracting with chloroform and then with pH 4 buffer solution, the salicylate content can be determined fluorometrically. Results similar to the Guttman and Salomon method (18) were obtainable.

EXPERIMENTAL

Apparatus and Reagents—*Fluorometer*—A G. K. Turner Associates model 2 fluorometer with a 7-60 primary filter (band pass 310 mμ) and a No. 2A secondary filter (band pass 410 mμ) was used to obtain the fluorescent readings. A Beckman research pH meter was used to measure the pH of the buffer solutions.

A Beckman DK2 recording spectrophotometer was used to obtain spectra of the chloroform solution. The results for the buffered-aspirin tablets were obtained on the Aminco-Bowman spectrofluorometer (4-8100). The activation and emission wavelengths were set at 310 and 410 mμ, respectively.

Materials—Spectral grade, analytical reagent chloroform (Merck & Co.) was used. All analgesic compounds, *i.e.*, acetylsalicylic acid, USP grade; salicylamide, NF grade; acetaminophen, NF grade; phenyltoloxamine citrate, USP grade; glyceryl guaiacolate, USP grade; salicylic acid, USP grade, were checked by IR spectrophotometry. Castor oil[1] was also used. The filter units employed in the experiment were Whatman folded filter paper No. 2V and the Millipore Swinnex-13 using celotate (cellulose acetate) No. ECPO-1300 filter.

Solutions—(*a*) pH 4 buffer; prepared by combining a ratio of 7.71 ml. of 0.2 *M* disodium hydrogen phosphate and 12.29 ml. of 0.1 *M* citric acid. (*b*) Seventeen milliliters of concentrated hydrochloric acid and 1000 ml. of 0.1 *M* citric acid.

Tablets—Powdered mixtures of the following composition were analyzed: (*a*) 325 mg. of aspirin; (*b*) 325 mg. of aspirin and 146 mg. of castor oil; (*c*) 195 mg. of aspirin, 130 mg. of salicylamide, 98 mg. of acetaminophen, and 65 mg. of caffeine; and (*d*) 325 mg. of aspirin, 97 mg. of magnesium carbonate, and 49 mg. of aluminum glycinate.

PROCEDURE

Absence of Interfering Substances—*Calibration*—To five 50-ml. volumetric flasks, 0.1, 0.2, 0.3, 0.5, and 1.0 mg. of salicylic acid were added. Buffer solution, pH 4, was added to each flask, which was then shaken for 30 sec. and filtered. Five milliliters of the filtered solution was added to a cell and placed in the fluorometer which was initially set to zero with the buffer solution. When a steady state was achieved (approximately 1 min.), readings were taken.

Method—A tablet was crushed and transferred to a 50-ml. volumetric flask. The contents were brought to volume with pH 4 buffer solution, shaken vigorously for 30 sec., and then filtered. Proceed as described in the previous calibration beginning: "Five milliliters of the filtered solution was added. . . ."

Presence of Interfering Substances—*Calibration for Tablets Coated with Castor Oil*—To five separators, 0.1, 0.2, 0.3, 0.5, and 1.0 mg. of salicylic acid were added. To each separator, 25 ml. of chloroform was added and shaken for 30 sec. The chloroform solution was extracted with three 15-ml. portions of buffer solution; the buffer extracts were transferred to a 50-ml. volumetric flask and adjusted to volume. Five milliliters of the solution was added to the cell and read in the fluorometer that had been previously standardized with buffer solution. Readings were taken at equilibrium.

A tablet was crushed and transferred to a 125-ml. separator. Proceed as described in the previous calibration beginning: "To each separator, 25 ml. of chloroform was added and shaken for 30 sec. . . ."

For tablets containing aspirin, acetaminophen, salicylamide, and caffeine, a tablet was crushed and put into a 50-ml. volumetric flask which was brought to volume with chloroform. The solution was shaken vigorously for 1 min. and transferred with as much residue material as possible to the separator, allowing a few seconds for the complete drainage of the flask. Fifty milliliters of buffer solution was then added, and the separator was shaken vigorously for 30 sec. The lower chloroform layer was completely drained, including as much filmy interface as possible. (The clarity of the division of the two layers is enhanced by gently swirling the flasks as the layers separate. This enables the trapped air bubbles in the chloroform layer to escape to the surface. If these bubbles do not disappear,

[1] Castorwax, The Baker Castor Oil Co., Bayonne, N. J.

Table I—Recovery Efficiency of Salicylic Acid from Recrystallized Aspirin[a]

Recrystd. Aspirin, mg.	Salicylic Acid Added, mg.	Total Wt. Salicylic Acid Found, mg.	Corrected Wt. Salicylic Acid Found, mg.	Recovery, %
305.8	None	0.097 (0.032%)	—	—
315.0	None	0.097 (0.031%)	—	—
325.1	None	0.109 (0.034%)	—	—
335.5	None	0.113 (0.034%)	—	—
345.3	None	0.117 (0.034%)	—	—
356.1	None	0.120 (0.034%)	—	—
300.2	0.127	0.225	0.126	99.2
325.3	0.127	0.238	0.131	103.2
345.5	0.254	0.371	0.257	101.2
301.1	0.254	0.351	0.252	99.2
324.4	0.508	0.617	0.510	100.4
345.0	0.508	0.625	0.511	100.6
355.9	1.016	1.135	1.018	100.2
325.1	1.016	1.126	1.019	100.3

[a] The average of salicylic acid found in recrystallized aspirin was 0.033%; this amount was used to calculate the weight found in the composition composed of both aspirin and added salicylic acid in Tables I and II.

they should be drained with the chloroform.) The buffer solution was transferred to a 100-ml. volumetric flask through the top of the separator. The separator was rinsed with an additional 10 ml. of buffer, which was then transferred to the previous flask that was brought to volume with additional buffer solution. The solution was filtered using the Millipore filter. Five milliliters of the filtered solution is read on the fluorometer that has been standardized with buffer solution. Two samples should be read, and if the readings differ by more than one unit, a third sample is required. Readings should be taken after allowing the instrument to equilibrate with the sample inside for 1 min.

For buffered-aspirin tablets containing aspirin, magnesium carbonate, and aluminum glycinate, a tablet was crushed and placed in a 125-ml. separator. HCl–citric acid solution, 25 ml., was added. After the effervescence ceased, two 25-ml. chloroform extractions of the mixture were made and saved. The combined chloroform extracts were then extracted with two 25-ml. portions of buffer. The buffer extracts were placed in a 100-ml. volumetric flask and brought to volume. Some of this aqueous solution was filtered, and a 5-ml. aliquot was added to the cell and read on the Aminco spectrofluorometer. Readings were taken after the instrument was equilibrated with the sample inside for 1 min.

RESULTS AND DISCUSSION

Studies of the fluorescent properties of salicylic acid as a function of pH show that the fluorescent spectra arise from different ionic forms (19). The stable spectrum of the acid arises from the singly ionized form. Figure 1 shows the plots of pH *versus* fluorescence

Table II—Recovery of Salicylic Acid from Recrystallized Aspirin and Castor Oil

Recrystd. Aspirin, mg.	Castor Oil, mg.	Salicylic Acid Added, mg.	Total Wt. Salicylic Acid Found, mg.	Corrected Wt. Salicylic Acid Found, mg.	Recovery, %
300.5	126.5	0.101	0.200	0.101	100.0
316.0	136.1	0.101	0.203	0.099	98.0
324.5	176.3	0.202	0.312	0.205	101.5
335.4	155.8	0.202	0.315	0.204	101.0
345.7	166.1	0.404	0.523	0.409	101.2
356.1	177.0	0.404	0.522	0.405	100.3
301.1	177.1	0.808	0.903	0.804	99.5
325.2	125.4	0.808	0.927	0.820	101.5

Table III—Recovery of Salicylic Acid in Powdered Mixtures of Aspirin, Salicylamide, Acetaminophen, and Caffeine

Addition Salicylic Acid to Powdered Mixtures, mg.	Fluorometric Reading Adjusted for Background	Reading/mg. Salicylic Acid
1.98	21.5	10.86
2.58	25.4	9.84
3.45	34.5	10.00
4.00	39.1	9.75
4.16	40.2	9.66
4.84	48.3	9.98
5.07	49.2	9.68
5.58	54.3	9.73

(19). At pH 4, the monosalicylate ion levels off in fluorescent intensity. The detection limit of the salicylic acid at pH 4 is around 0.1 p.p.m.

Aspirin shows a rate constant of about 0.1 day^{-1} at 17° at pH 4 (8). At room temperature during the time period of the above experiments, aspirin showed a negligible decomposition.

The determination of salicylic acid in aspirin products in these experiments is based on the fact that the aspirin compound does not fluoresce while salicylic acid does at pH 4. At this pH, aspirin decomposition is negligible. For the experiment on noninterfering substances, aspirin, having starch as the excipient, gave results as shown in Table I.

In the analysis of aspirin tablets coated with castor oil, the tablets were dissolved in chloroform and extracted with pH 4 buffer solution. The salicylic acid forms the monosodium salts with the buffer solution and remains in the aqueous layer. Results are shown in Table II.

For the determination of salicylic acid in tablets containing aspirin, salicylamide, acetaminophen, and caffeine, a fluorometric procedure was developed in which the ingredients were dissolved in chloroform, and aspirin and salicylic acid were extracted into a buffer solution. The interference of the major ingredient is limited by the chloroform extraction and the use of the buffer. Salicylic

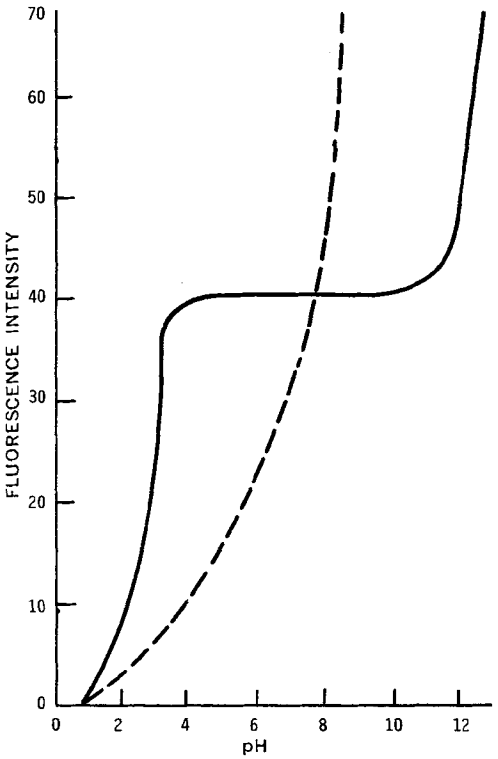


Figure 1—*Plot of pH* versus *fluorescence for salicylic acid and salicylamide. Key:—, salicylic acid; and - - -, salicylamide.*

acid can be determined with an accuracy of ±0.15 mg. at levels of 1 mg. The values may be simply calculated from a suitable calibration curve.

The fluorometer is especially useful in the determination of very small amounts of salicylic acid in the presence of large amounts of the ingredients of the tablet because its fluorescence per unit weight is much larger than that of the active ingredients. If the amount of salicylic acid in the tablet is as small as 2 mg., it would represent only 1.6% of the respective standard amount of salicylamide, the only ingredient which exhibits fluorescence at this pH. Figure 1 shows the effect of pH *versus* fluorescence of a solution containing 1 mg. of salicylic acid and 10 mg. of salicylamide. The optimum condition for determining salicylic acid in the presence of salicylamide was at pH 4. The actual amount of the fluorescence of salicylamide is limited by chloroform extraction and buffer operation. The reading of the salicylamide is depressed because 90% of it has been removed. The ratio of anionic to undissociated species is 10:1 in both aspirin and salicylic acid and only $1:10^4$ in salicylamide, and it is the anionic species which fluoresces to the largest extent.

The background reading of the tablet has been carefully determined by using a large number of runs. Tablet mixtures were simulated by weighing the standard amounts of the four components. The quantity of salicylic acid in each of the two components (aspirin and salicylamide, which gave readings) were carefully determined. Neither acetaminophen nor caffeine gave fluorescence under the conditions of this analysis. The salicylic acid content of aspirin was determined by using an iron (III) complexing technique (20)—0.128 mg./195 mg. of aspirin. Using a titration in nonaqueous medium with methanolic potassium hydroxide, it was determined that there was a negligible amount of salicylic acid in salicylamide, and the reading of salicylamide was a result of salicylamide fluorescence. The observed background of the composed tablet is 32.5. The salicylic acid content of the composed tablet gave a reading of 1.2; therefore, the adjusted background is 31.3 (four components with no salicylic acid).

The observed readings of fresh commercial tablets varied from 32.5 to 35.8. Individual batches were consistent among themselves, alternating between 0.13 and 0.45 mg. of salicylic acid in the tablets.

The data, summarized in Table III, were acquired through the addition of salicylic acid to a weighed portion, equivalent to one tablet, of a powdered mixture made from 10 tablets. The tablet background was obtained from the measurement of a given amount of the mixture and subtracted from the observed reading to determine the increase resulting from the addition of salicylic acid. The data are plotted in Fig. 2.

The best straight line through the data points is obtained by using a least-squares method (21). The calculated straight line is where $y = 9.63x + 0.85$, y = adjusted fluorescence reading, and x = milligrams of salicylic acid added.

Adjusting the background and initial salicylic acid content, the equation can be solved to find the total amount of salicylic acid per tablet to give

$$x = y_1 - \frac{32.1}{9.63}$$

where y_1 is the observed fluorometer reading.

The average deviation of calculated values from the actual values is 0.06 mg., representing an average relative error of 2.00% with a standard deviation of 0.074; one can be 95.5% confident of being accurate to within 0.15 mg. and 99.7% confident of being accurate within 0.22 mg.

However, the deviations in the observed data are not the only errors involved. An error of ±0.05 mg. is introduced by uncertainties in the fluorometer readings (readings are good only to ±0.5 division). The major error is a result of the tolerance limits of the ingredients. Limits of ±10% significantly alter the readings of the background. This introduces an error of ±0.3 mg. in the final determination of salicylic acid. However, as there are extremes, salicylamide and aspirin must both be either high or low by 10% to obtain such a large error; this large deviation is unlikely. This large error can only be eliminated by: (*a*) determining the salicylamide and aspirin contents of the tablets, and (*b*) adjusting the background accordingly.

By employing a high source intensity xenon lamp and a grating instrument rather than the one incorporating band filters and a mercury lamp source (as used in these experiments), a more monochromatic system for maintaining an exact activation and fluorescent spectrum would permit a lesser background effect and a higher intensified signal at the appropriate selected wavelengths.

Table IV—Results of Salicylic Acid Determination by the Method of Guttman and Salomon and by Fluorescence[a]

Batch	Salicylic Acid Found	$\bar{x}$[b]	SD[c]
	Guttman and Salomon Method, mg.		
A	0.6, 0.6, 0.7, 0.6, 0.95	0.690	0.1516
B	1.9, 1.6, 2.1, 1.8, 1.7	1.820	0.1924
C	4.2, 3.7, 4.3, 3.7, 3.8	3.940	0.2881
	Fluorescence, mg.		
A	0.7, 0.6, 0.8, 0.7, 0.6	0.680	0.0837
B	2.0, 1.8, 1.7, 1.9, 1.8	0.680	0.0837
C	4.3, 4.0, 3.7, 4.0, 3.7	3.940	0.0630

[a] Results obtained on the analysis of salicylic acid content on buffered aspirin composed of 325 mg. of aspirin, 97 mg. of magnesium carbonate, and 49 mg. of aluminum glycinate. The different batches contained buffered aspirin at various environmental conditions. [b] $\bar{x}$ = means of results. [c] SD = standard deviation.

Table IV shows the determination of salicylic acid in buffered-aspirin tablets by fluorescence and the Guttman and Salomon

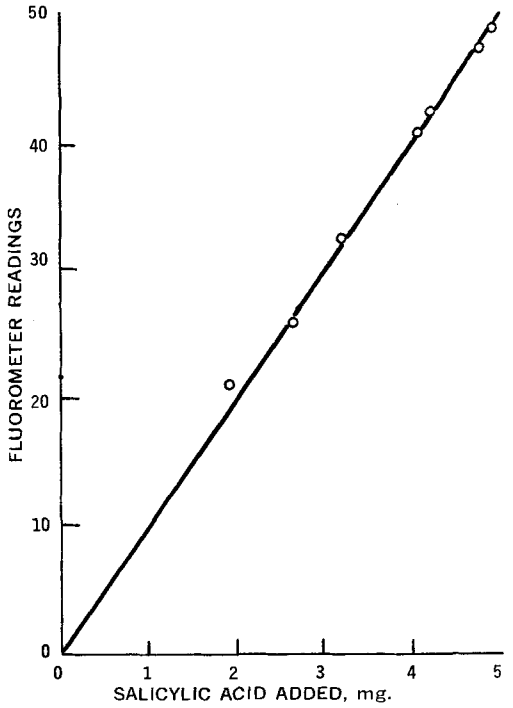


Figure 2—*Calibration curve for salicylic acid and powdered mixture containing aspirin, acetaminophen, salicylamide, and caffeine. Key:* O—O, *least-squares straight line.*

method. As shown in the table, there is no statistical difference between the methods with respect to the means and variances.

After the HCl–citric acid solution is added to the crushed tablet, a pH of 0.9–1 is observed. Slight effervescence occurs but subsides in less than 1–2 min. The extraction of the undissociated salicylic acid and aspirin by chloroform is then accomplished. The aspirin and salicylic acid are extracted from the chloroform solution by the buffer solution. The whole experiment takes about 10 min.

To ensure complete extraction of salicylic acid and aspirin into the chloroform solution, some of the aqueous phase was treated with 1 *N* NaOH and tested for the absence of salicylate fluorescence. Also, to be assured of complete extraction of salicylic acid from the chloroformic solution, some of the organic phase was run on the spectrophotometer to observe the absence of salicylic acid.

REFERENCES

(1) M. L. Dow, *J. Ass. Offic. Agr. Chemists*, **43**, 239(1960).

(2) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 51.

(3) L. J. Leeson and A. M. Mattocks, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 329(1958).

(4) T. Higuchi and K. P. Patel, *ibid.*, **41**, 171(1952).

(5) J. Levine, *ibid.*, **46**, 687(1957).

(6) R. F. Heuermann and J. Levine, *ibid.*, **47**, 276(1958).

(7) J. Levine, *J. Pharm. Sci.*, **50**, 506(1961).

(8) L. J. Edwards, *Trans. Faraday Soc.*, **46**, 723(1950).

(9) R. B. Tinker and A. J. McBay, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 315(1954).

(10) R. C. Reed and W. W. Dams, *J. Pharm. Sci.*, **54**, 1533 (1965).

(11) S. L. Lin, *ibid.*, **56**, 1130(1967).

(12) A. Saltzman, *J. Biol. Chem.*, **174**, 399(1948).

(13) M. A. Chirigos and S. Udenfriend, *J. Lab. Clin. Med.*, **54**, 769(1959).

(14) M. E. Lange and S. A. Bell, *J. Pharm. Sci.*, **55**, 386(1966).

(15) J. Levine and J. D. Weber, *ibid.*, **57**, 631(1968).

(16) J. D. Weber and J. Levine, *ibid.*, **55**, 78(1966).

(17) D. E. Guttman, *ibid.*, **57**, 1685(1968).

(18) D. E. Guttman and G. W. Salomon, *ibid.*, **58**, 120(1969).

(19) G. A. Thommes and E. Leininger, *Anal. Chem.*, **30**, 1361 (1958).

(20) R. E. Pankratz and F. J. Bandelin, *J. Amer. Pharm. Ass., Sci. Ed.*, **41**, 267(1952).

(21) L. G. Parratt, "Probability and Experimental Errors in Science," Wiley, New York, N. Y., 1961.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 16, 1969, from *Analytical Research, Bristol-Myers Products, Hillside, NJ 07207*

Accepted for publication September 19, 1969.

The authors gratefully acknowledge the technical assistance of Richard Bates and A. L. Seiman for statistical analysis of the results; also the assistance of Dr. A. Marcus in writing this paper.

Gas-Liquid Chromatography of *d*-Biotin

V. VISWANATHAN, F. P. MAHN, V. S. VENTURELLA, and B. Z. SENKOWSKI

Abstract □ A rapid and specific gas chromatographic method has been developed for the detection and determination of *d*-biotin. This technique has been found applicable to agricultural premixes and pharmaceutical injectable preparations. After suitable preliminary extraction, the biotin silyl ester is prepared using bis-(trimethylsilyl)acetamide (BSA) reagent. The silyl ester of *d*-biotin exhibited good peak symmetry and a linear response when utilizing a hydrogen flame ionization detector with a 2% OV-17 column operated at a temperature of 190°. The conditions for the assay of *d*-biotin in several preparations are described employing *n*-octacosane as the internal standard. The standard deviation of the developed procedure under the conditions studied was ±2.7%.

Keyphrases □ *d*-Biotin—determination □ Parenterals, agricultural premixes—*d*-biotin analysis □ *n*-Octacosane—internal standard □ GLC—analysis

d-Biotin is widely distributed in animals and plants and was first isolated from egg yolk (1). It is required in comparatively small amounts for the growth of bacteria, plants, and animals and appears to be related to the process of cell development. In 1940, György *et al.* (2) published their work on the identity of biotin. In the following 2 years, du Vigneaud *et al.* (3, 4) established the empirical and structural formulas. In 1943, Harris *et al.* (5) synthesized *d*-biotin (the naturally occurring form) and in 1949, Goldberg and Sternbach (6) patented their findings of a more economical synthesis.

While several microbiological techniques are currently employed for the determination of *d*-biotin, a comprehensive survey of the literature indicated that GLC had heretofore not been employed for quantitative analysis. A considerable number of satisfactory GLC procedures have been reported for various water-soluble vitamins. These procedures involve the preparation of the appropriate trimethylsilyl derivatives (ester or ether) prior to GLC analysis.

Carboxylic organic compounds, such as biotin, are not sufficiently volatile for direct analysis employing GLC. However, the chemical structure of *d*-biotin indicated the possibility of substituting the active hydrogen of the carboxyl group with a silyl group, thereby making the gas chromatographic analysis possible. Horning *et al.* (7) mentioned the qualitative response of *d*-biotin silyl ester in their study of urinary acids and related compounds.

This paper describes the quantitative GLC determination of *d*-biotin after the conversion to its silyl ester with bis-(trimethylsilyl)acetamide (BSA). The derivative is easily formed and serves very well for quantitative analysis. The linearity of detector response with concentration injected, reproducibility, recovery data, and procedures for handling several types of dosage forms are described.

method. As shown in the table, there is no statistical difference between the methods with respect to the means and variances.

After the HCl–citric acid solution is added to the crushed tablet, a pH of 0.9–1 is observed. Slight effervescence occurs but subsides in less than 1–2 min. The extraction of the undissociated salicylic acid and aspirin by chloroform is then accomplished. The aspirin and salicylic acid are extracted from the chloroform solution by the buffer solution. The whole experiment takes about 10 min.

To ensure complete extraction of salicylic acid and aspirin into the chloroform solution, some of the aqueous phase was treated with 1 *N* NaOH and tested for the absence of salicylate fluorescence. Also, to be assured of complete extraction of salicylic acid from the chloroformic solution, some of the organic phase was run on the spectrophotometer to observe the absence of salicylic acid.

REFERENCES

(1) M. L. Dow, *J. Ass. Offic. Agr. Chemists*, **43**, 239(1960).

(2) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 51.

(3) L. J. Leeson and A. M. Mattocks, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 329(1958).

(4) T. Higuchi and K. P. Patel, *ibid.*, **41**, 171(1952).

(5) J. Levine, *ibid.*, **46**, 687(1957).

(6) R. F. Heuermann and J. Levine, *ibid.*, **47**, 276(1958).

(7) J. Levine, *J. Pharm. Sci.*, **50**, 506(1961).

(8) L. J. Edwards, *Trans. Faraday Soc.*, **46**, 723(1950).

(9) R. B. Tinker and A. J. McBay, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 315(1954).

(10) R. C. Reed and W. W. Dams, *J. Pharm. Sci.*, **54**, 1533 (1965).

(11) S. L. Lin, *ibid.*, **56**, 1130(1967).

(12) A. Saltzman, *J. Biol. Chem.*, **174**, 399(1948).

(13) M. A. Chirigos and S. Udenfriend, *J. Lab. Clin. Med.*, **54**, 769(1959).

(14) M. E. Lange and S. A. Bell, *J. Pharm. Sci.*, **55**, 386(1966).

(15) J. Levine and J. D. Weber, *ibid.*, **57**, 631(1968).

(16) J. D. Weber and J. Levine, *ibid.*, **55**, 78(1966).

(17) D. E. Guttman, *ibid.*, **57**, 1685(1968).

(18) D. E. Guttman and G. W. Salomon, *ibid.*, **58**, 120(1969).

(19) G. A. Thommes and E. Leininger, *Anal. Chem.*, **30**, 1361 (1958).

(20) R. E. Pankratz and F. J. Bandelin, *J. Amer. Pharm. Ass., Sci. Ed.*, **41**, 267(1952).

(21) L. G. Parratt, "Probability and Experimental Errors in Science," Wiley, New York, N. Y., 1961.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 16, 1969, from *Analytical Research, Bristol-Myers Products, Hillside, NJ 07207*

Accepted for publication September 19, 1969.

The authors gratefully acknowledge the technical assistance of Richard Bates and A. L. Seiman for statistical analysis of the results; also the assistance of Dr. A. Marcus in writing this paper.

Gas-Liquid Chromatography of *d*-Biotin

V. VISWANATHAN, F. P. MAHN, V. S. VENTURELLA, and B. Z. SENKOWSKI

Abstract □ A rapid and specific gas chromatographic method has been developed for the detection and determination of *d*-biotin. This technique has been found applicable to agricultural premixes and pharmaceutical injectable preparations. After suitable preliminary extraction, the biotin silyl ester is prepared using bis-(trimethylsilyl)acetamide (BSA) reagent. The silyl ester of *d*-biotin exhibited good peak symmetry and a linear response when utilizing a hydrogen flame ionization detector with a 2% OV-17 column operated at a temperature of 190°. The conditions for the assay of *d*-biotin in several preparations are described employing *n*-octacosane as the internal standard. The standard deviation of the developed procedure under the conditions studied was ±2.7%.

Keyphrases □ *d*-Biotin—determination □ Parenterals, agricultural premixes—*d*-biotin analysis □ *n*-Octacosane—internal standard □ GLC—analysis

d-Biotin is widely distributed in animals and plants and was first isolated from egg yolk (1). It is required in comparatively small amounts for the growth of bacteria, plants, and animals and appears to be related to the process of cell development. In 1940, György *et al.* (2) published their work on the identity of biotin. In the following 2 years, du Vigneaud *et al.* (3, 4) established the empirical and structural formulas. In 1943, Harris *et al.* (5) synthesized *d*-biotin (the naturally occurring form) and in 1949, Goldberg and Sternbach (6) patented their findings of a more economical synthesis.

While several microbiological techniques are currently employed for the determination of *d*-biotin, a comprehensive survey of the literature indicated that GLC had heretofore not been employed for quantitative analysis. A considerable number of satisfactory GLC procedures have been reported for various water-soluble vitamins. These procedures involve the preparation of the appropriate trimethylsilyl derivatives (ester or ether) prior to GLC analysis.

Carboxylic organic compounds, such as biotin, are not sufficiently volatile for direct analysis employing GLC. However, the chemical structure of *d*-biotin indicated the possibility of substituting the active hydrogen of the carboxyl group with a silyl group, thereby making the gas chromatographic analysis possible. Horning *et al.* (7) mentioned the qualitative response of *d*-biotin silyl ester in their study of urinary acids and related compounds.

This paper describes the quantitative GLC determination of *d*-biotin after the conversion to its silyl ester with bis-(trimethylsilyl)acetamide (BSA). The derivative is easily formed and serves very well for quantitative analysis. The linearity of detector response with concentration injected, reproducibility, recovery data, and procedures for handling several types of dosage forms are described.

EXPERIMENTAL

Operational Parameters—The instrument used for this work was a Varian Aerograph model 204 B, equipped with a hydrogen-flame ionization detector. The column used was a stainless steel coil, 121.9 cm. (4 ft.) long and 3 mm. o.d., packed with 2% OV-17 on diatomite (Chromosorb G), AW/HMDS treated, 70/80 mesh. Prior to use, the column was flow conditioned at 225° for 16–20 hr. with a stream of nitrogen. The temperatures were: column, 190° (or adjusted accordingly to obtain a retention time of approximately 19 min. for *n*-octacosane); injector port, 275° with a Pyrex insert; detector, 275°. The flow rates were: carrier gas—nitrogen, 60 ml./min.; detector gas—hydrogen, 30 ml./min.; and air, 300 ml./min. All injections were made using a 10-μl. Hamilton syringe with an injection volume of approximately 5 μl. The instrument was operated at a range of 10 and 32× attenuation (or sufficient to obtain a 50% peak response on the recorder scale). The recorder used was 0–1 mv. (Texas Instrument) with a pen response of 0.4 sec. and a chart speed of 12 in./hr. All peak areas were measured using a disk integrator. Under the conditions stated the relative retention time of *d*-biotin silyl ester is 0.68 with respect to the internal standard, *n*-octacosane, which has a specific retention time of approximately 19 min. A sample chromatogram is shown in Fig. 1.

Reagents and Chemicals—Liquid phase phenylmethylsilicone fluid, OV-17, *n*-octacosane, and bis-(trimethylsilyl)acetamide (BSA) were commercial grade (Applied Science Laboratories, Inc.). The solid support, diatomite (Chromosorb G), AW/HMDS treated, 70/80 mesh, was used as purchased (F & M Scientific Corp.). All solvents were analytical reagent grade, requiring no further purification.

Standard Preparation—*Internal Standard Solution*—Five hundred milligrams of *n*-octacosane, accurately weighed into a 50-ml. volumetric flask, was dissolved and diluted to volume with *n*-hexane.

Reference Standard Solution—An accurately weighed 50-mg. portion of standard *d*-biotin was placed in a 50-ml. volumetric flask and treated with 5.00 ml. of bis-(trimethylsilyl)acetamide reagent. The flask was stoppered and heated on a steam bath with frequent agitation until the reaction was complete and the solution clear (approximately 20–30 min.). The mixture was cooled, 5.00 ml. of internal standard solution added, and the solution diluted to volume with *n*-hexane.

Sample Preparation—*A.* d-*Biotin Premix: 1%*—An accurately weighed portion of premix (equivalent to about 10 mg. of *d*-biotin) was placed in a 50-ml. centrifuge tube. Fifteen milliliters of absolute ethanol was added and the mixture warmed on a steam bath for 10 min., with occasional agitation. The mixture was centrifuged and the clear supernatant liquid transferred to a 125-ml. conical flask without disturbing the residue. The extraction was repeated four more times and the extracts combined. The combined extracts were evaporated to dryness on a steam bath under a stream of nitrogen. Exactly 2.0 ml. of bis-(trimethylsilyl)acetamide reagent was added. The vessel was stoppered and heated on a steam bath for 20–30 min. with frequent agitation. The sample preparation was cooled and 1.00 ml. of internal standard solution (containing 10 mg. of *n*-octacosane) was added. Quantitative transfer of the entire mixture into a 10-ml. volumetric flask was attained with the aid of *n*-hexane and the solution was diluted to volume with *n*-hexane (working sample solution).

B. d-*Biotin Parenteral: 0.05%*—A 20.0-ml. portion of sample preparation was transferred to a 125-ml. conical flask. Concentrated hydrochloric acid, 0.5 ml., and 30 ml. of anhydrous methanol were added. The solution was evaporated to dryness on a steam bath under a stream of nitrogen. Exactly 2.0 ml. of bis-(trimethylsilyl)-acetamide reagent was added; the flask was stoppered and heated on a steam bath for 20–30 min. with frequent agitation. The solution was cooled and 1.00 ml. of internal standard solution (containing 10 mg. of *n*-octacosane) was added. The entire mixture was quantitatively transferred into a 10-ml. volumetric flask with the aid of *n*-hexane and diluted to volume with *n*-hexane.

Standard Calibration and Sample Analysis—Chromatographic preruns of several 5-μl. injections of working standard solution was carried out in order to condition the column to determine the instrument sensitivity and the peak retention times.

Five-microliter volumes of the working standard and sample solutions, equivalent to approximately 5 mcg. of *d*-biotin, were alternately injected into the instrument using the aforementioned conditions. After the elution of the *n*-octacosane peak, the instrument was ready for another injection. Duplicate samples and standards were chromatographed and the respective areas determined from the integrator trace.

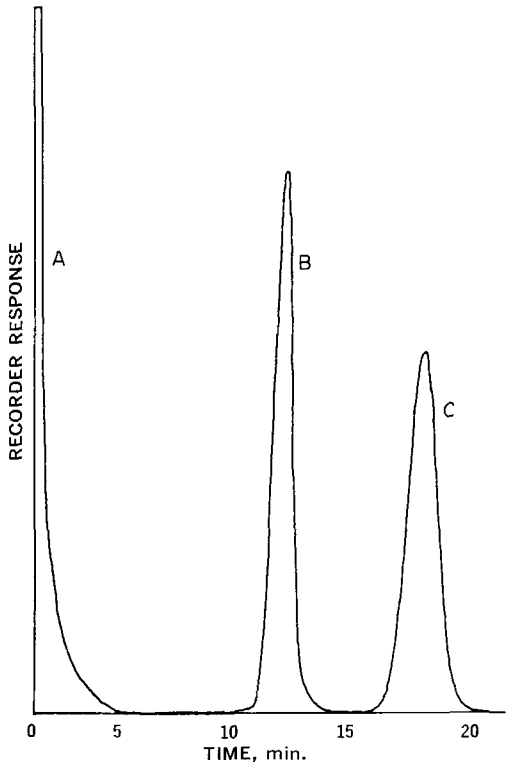


Figure 1—*Standard chromatogram showing relative retention times of biotin TMS derivative and internal standard. Key:* A, *solvent;* B, *biotin TMS; and* C, n-*octacosane.*

Calculations—*Response Factor for Biotin* (RF_B)—

$$RF_B = \frac{A_B\,(\text{std}) \times C_O}{A_O\,(\text{std}) \times C_B}$$

where A_B = peak area of biotin in the working standard, A_O = peak area of *n*-octacosane in the working standard, C_O = mg. of *n*-octacosane per ml. of working standard, and C_B = mg. of biotin per ml. of working standard.

Concentration of Biotin—

$$\frac{A_B\,(\text{spl}) \times C_O \times 10}{A_O\,(\text{spl}) \times RF_B \times \text{spl taken (g. or ml.)}} = \text{mg. biotin/g. (ml.)}$$

where *A* is area, *C* is concentration, and RF_B is the response factor for biotin; 10 = dilution factor.

RESULTS AND DISCUSSION

Hexamethyldisilazane and trimethylchlorosilane in the ratio of 2:1 was found unsuitable for the silylation of biotin, while bis-(trimethylsilyl)acetamide reagent was found satisfactory for complete and rapid silylation. This behavior was also reported by Horning (7). The selection of diatomaceous earth (Gas Chrom G), AW/HMDS treated, was arbitrary and any support properly

Table I—*d*-Biotin Premix Recovery Data

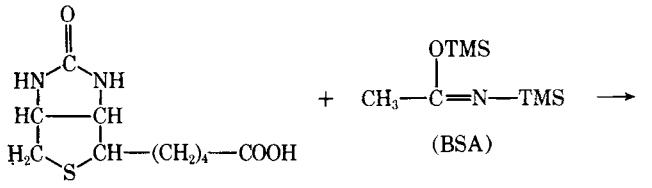
Sample No.	Amount Present, mg./g.	Experimentally Found, mg./g.	Recovery, %
1	10.00	10.05	100.5
2	11.05	10.85	98.2
3	12.05	12.02	99.8
4	13.05	13.25	101.5

Table II—Replicate Recoveries of *d*-Biotin

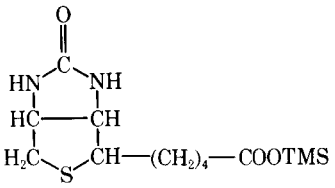
Run No.	Premix, mg./g. Found[a]	Injectable, mg./ml. Found[b]
1	10.37	0.532
2	10.27	0.543
3	9.87	0.547
4	10.23	0.567
5	10.58	0.556
6	10.64	0.573
Mean	10.33	0.553
σ	±0.28	±0.015
ts:95%	±0.71	±0.040

[a] Label claim: 10 mg./g. [b] Label claim: 0.500 mg./ml.

silanized may be suitable for good peak symmetry and quantitative recovery. Results obtained using either copper tubing or stainless steel tubing were equivalent. *d*-Biotin forms a TMS derivative when the carboxyl group exists in the acidic form. The sodium salt of *d*-biotin did not yield a TMS derivative. Therefore, it is assumed that reaction takes place at the carboxyl group to form the biotin silyl ester. The reaction apparent in the derivatization can then take the following form:



biotin



biotin silyl ester

Since the derivatization was shown to occur at the carboxyl center, concentrated hydrochloric acid was added to the parenteral sample prior to derivatization because the *d*-biotin present in the parenteral preparation is in a water-soluble salt form.

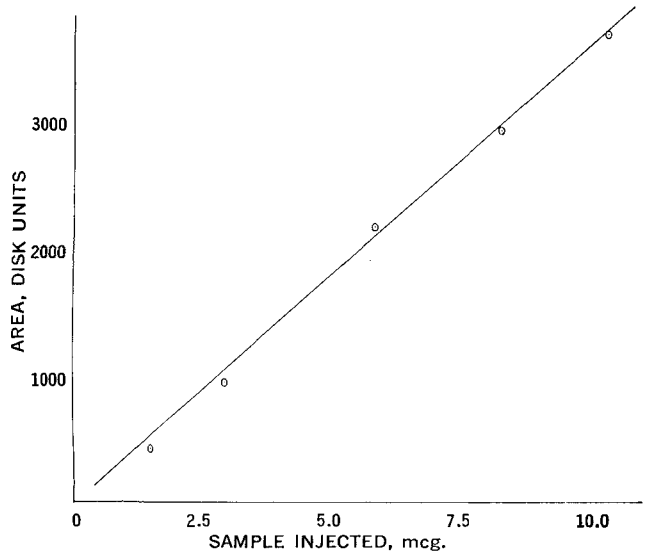


Figure 2—*Linearity of response with concentration. Column: 2% OV-17 on Chromosorb G, length 121.9 cm. (4 ft.); temperature 190°; 5 μl. injected; attenuation 32×. Micrograms of* d-*biotin injected as the silyl ester with the internal standard as a constant.*

Table III—Comparative Analyses of *d*-Biotin in Premix Samples[a,b]

Sample No.	Gas-Liquid Chromatography	Microbiological	Colorimetric (8)
1	9.5	11.7	9.2
2	11.1	11.6	10.8
3	10.9	11.9	10.4
4	10.9	10.4	10.2
5	10.1	10.9	10.2
6	10.2	10.0	9.2
7	10.8	10.4	10.8
8	10.4	10.3	10.5
9	10.7	11.2	10.7

[a] Label claim: 10 mg./g. [b] Results in mg./g.

Linearity data for *d*-biotin–TMS ester *versus* *n*-octacosane as the internal standard are shown in Fig. 2. The minimum detectable level for the *d*-biotin–TMS ester was approximately 0.3 mcg. at range 10, 1X. The recovery of known amounts of *d*-biotin added to a sample premix is given in Table I.

Initially, 5α-cholestane was chosen as the internal standard. However, it had a specific retention time of approximately 28 min. In order to reduce the chromatographic time, *n*-octacosane (C_{28} hydrocarbon), which had a specific retention time of approximately 19 min. under the prescribed conditions, was preferable. In addition, the latter proved more economical.

Six replicates of a premix containing 10 mg. of biotin per gram showed a mean of 10.33 with a relative standard deviation from the mean of ±0.28. The precision (*ts*:95%) was ±0.71 (Table II).

Six replicates of a sample of injectable containing 0.500 mg. of *d*-biotin per milliliter showed a mean of 0.553 mg. with a relative standard deviation from the mean of ±0.015. The precision (*ts*: 95%) was ±0.040 (Table II).

Table III shows the results of analyses of nine samples of *d*-biotin premix by the described GLC method compared to the microbiological assay and to the colorimetric procedure previously reported (8).

Investigation is still being carried out on the application of this procedure towards the determination of *d*-biotin, present in microgram levels, in multivitamin preparations. In these preparations, as well as certain types of premixes, additional suitable isolation techniques may be required in order to remove interfering components.

SUMMARY

A rapid and specific GLC technique has been developed for the detection and determination of *d*-biotin. The method involves the preparation of the TMS ester, addition of *n*-octacosane as an internal standard, and analysis employing a hydrogen-flame ionization detector. Conditions are outlined for the determination of *d*-biotin in premixes and in parenteral preparations.

REFERENCES

(1) W. G. Bateman, *J. Biol. Chem.*, **26,** 263(1916).

(2) P. György, *Science*, **92,** 609(1940).

(3) V. du Vigneaud, K. Hofmann, D. Melville, and P. György, *J. Biol. Chem.*, **140,** 643(1941).

(4) V. du Vigneaud, *Science*, **96,** 455(1942).

(5) S. A. Harris, D. E. Wolf, R. Mozingo, and K. Folkers, *ibid.*, **97,** 447(1943).

(6) M. W. Goldberg and L. H. Sternbach, U. S. pat. 2,489,232, Nov. 1949.

(7) M. G. Horning, E. A. Boucher, and A. M. Moss, *J. Gas Chromatogr.*, **5,** 297(1967).

(8) C. Plinton, F. P. Mahn, M. Hawrylyshyn, V. S. Venturella, and B. Z. Senkowski, *J. Pharm. Sci.*, **58,** 875(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 13, 1969, from the *Analytical Research Laboratory, Quality Control Department, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication September 22, 1969.

Formulation and Computation of Compartment Models

CARL F. EVERT and MICHAEL J. RANDALL

Abstract □ In the formulation of compartment models for describing biological phenomena, two separate approaches have usually been employed for isotope dilution systems and systems for which no tracers are introduced. In the former, steady state is assumed to exist and small perturbations are introduced for solution of the system. In the latter, sets of simultaneous differential equations are solved for the complete time course of the drug kinetic system. For linear systems which can be described by first-order kinetics, it is shown that the isotope dilution problem can be cast into the more general approach of simultaneous linear differential equations and the restriction of steady state removed. Solutions to these equations are shown to be easily obtainable using the state-space approach. For systems in which linearity cannot be assumed, digital computer techniques are presented which greatly facilitate numerical solutions. These concepts are demonstrated with two examples. A third example shows how these concepts and others can be employed with isotope dilution to find the initial pool sizes and rate constants in a six-compartment system.

Keyphrases □ Models, compartment—formulation, computation □ Theoretical considerations—data fitting, models □ Kinetic equations—first-order model system □ Computer program—data fitting, models

The use of mathematical descriptions called models that seek to capture the essence of biological phenomena has been emerging over the last 20 years. These concepts have been widely reported in the literature from a general and theoretical point of view, as well as in relation to specific applications. Most frequently, some form of dynamic behavior has been of concern so that the areas of pharmacokinetics and radioactive tracers have been the major contributors of applications. From these sources two points of view have arisen. One is concerned with the dynamics of a system described by a set of differential equations and seeks to evaluate the time course of the substance as a steady state is approached. The other presumes that steady state exists and that small perturbations about this value are introduced in order to evaluate flow rates, dilution, *etc.* These two points of view have placed emphasis on different variables, and the formulation of the descriptions of the system behavior in terms of these different variables has given the appearance of different computational requirements. It will be shown for first-order systems that these differences are superficial and that new advances in computer algorithms and computing languages now provide conveniences not generally recognized or utilized in biological modeling.

COMPUTATION FOR COMPARTMENT MODELS

The observable behavior of the distribution, absorption, or elimination of drugs has led to the concept that a biological system can be treated as if there were boundaries called "compartments" which separate the system into parts and that the drug or other material is transferred from one compartment to another in conformance with first-order kinetics. This assumes that the rate of change of material is proportional to the amount of material that is present in the compartment. The application of Fick's law in the case of diffusion can lead to similar sets of linear first-order differential equations. Although the assumption of first-order kinetics, linearity, and rate coefficients that are constants is clearly a gross approximation of a complex biological phenomenon, the approach has proved beneficial in numerous instances when it is utilized with discretion and understanding. The assumptions are so widely accepted that one textbook (1) states: "Most drugs disappear from the body in this fashion."

The use of mathematical models as an aid to the better understanding of biological phenomena requires that some form of computation be executed. While there have been advances from time to time in the conceptual features of modeling, little attention seems to have been paid to keeping the computational procedures up-to-date and to making the techniques more attractive to the biological researchers who could take advantage of modeling as a tool. Basic to a dynamic model is the solution of a set of differential equations. This job has been variously relegated to the analog computer or the digital computer. The work that has been assigned to the digital computer has been that of solving the equations by the techniques of numerical integration, the computation of statistical parameters, or perhaps some curve fitting. Most laboratories seem to have developed their own libraries of programs associated with modeling and, except for utilizing some common language such as FORTRAN, they appear to have overlooked the availability of general-purpose programs such as CSMP (Continuous System Modeling Program) (2). It is hoped that this paper will suggest some new approaches to meeting the computational requirements of modeling and thus reduce the effort required in the utilization of models for biological systems.

The recent emergence of the ideas of organizing the description of systems characterized by sets of first-order differential equations under the heading of "state space" has fostered the development of algorithms (3) which provide analytical solutions. The user is not required to manipulate matrices or to use the Laplace transform. The algorithms may be programmed for digital computers and thus provide the proper analytical form and the associated coefficients in a more convenient fashion than most other methods. This is also the form most familiar to those applying compartment theory, and the approach should therefore have appeal to anyone interested in biological dynamics. The use of the state-space formulation of the equations has the added advantages that it identifies the basic assumptions of linearity and clarifies the use of superposition. The state-space method yields solutions in functional form which gives the opportunity of locating maxima by differentiation and permits comparisons in terms of time constants. When comparing the functional solutions with experimental data points, one need only evaluate the function at the specific points in question. All other points need not be computed. An analog computer or numerical integration technique would continuously evaluate the complete range of the solution. There is the disadvantage, however, that when the system parameters are altered, an entirely new state-space solution must be executed, but corresponding disadvantages are present in obtaining the solutions of numerical integration procedures. Thus there are trade-offs in the computational aspects of modeling, and it is desirable to have alternative techniques available so that one may select the tool that is most appropriate for the requirements. An analog computer run generates a continuous solution in the desired range.

The analog computer is convenient for use in matching models to data when the number of parameters to be changed is small, say five or less. However, to evaluate a single point, the analog computer must compute a continuous solution at least to the desired point. Digital programs such as CSMP to be illustrated here have been written to simulate the operation of the analog computer. They are in fact conveniently packaged numerical integration routines with the ability to be interspersed with FORTRAN statements. With this program, one may run successive cases and make decisions automatically at the end of each case for purposes of optimization or curve fitting. Good initial estimates of system parameters will reduce the number of successive runs required. These analog simulators usually incorporate nonlinear components and thus provide a convenient way of solving nonlinear differential equations without special programming. Here again the solutions are in the form of tables of values or digitally plotted curves rather than explicit functions of the independent parameter. Evaluation of specific data points requires computation of all previous points. As a means of illustrating the use of CSMP and later the state-space approach, a problem which has been published (4) in a nonlinear formulation requiring extensive special programming will be reviewed. However, the ramifications of the method go far beyond this particular application.

The usual problem in drug dynamics seeks to evaluate the time course of the amount of drug in the various compartments of interest. The initial conditions are usually assumed to be zero except in the compartment into which the drug is introduced. The resulting formulation is a dynamic system with the drug being distributed among the compartments and perhaps being eliminated from the system at several points. The conditions of steady state may not be of particular interest because steady state could be the condition in which all the drug has been cleared from the system. The length of time for the system to reach steady state may be prohibitively long for clinical verification. The point of interest may well be the early rates of the dynamic behavior, and there may be little concern with the final conditions of steady state.

In the case of the mathematical description of phenomena in which radioactive tracers are of interest, the emphasis has been on the small variations from an existing steady state. In fact an effort is made not to disturb the steady state appreciably. This concept of steady state in a biological system does not mean that there is no exchange of material among the compartments, but rather that the amount transferred into a compartment in a given unit of time is exactly the same as the amount which leaves the compartment in the given unit of time. Thus there can be a flow of material in the system, and the point of interest is in establishing the flow rates or pool sizes through dilution measurements. Radioactive tracers are suited to this task because they are not distinguishable by the body from the parent substance. Tracers may be injected in small enough quantities so as not to upset the equilibrium conditions and yet in sufficient amount so as to be measurable. The usual measurement is that of specific activity. Therefore, it was a natural approach to formulate the description in these well-known terms. The equations have been published (4) before and are of the general form:

$$S_i \frac{da_i}{dt} = \sum_{\substack{j=1 \\ j \neq i}}^{n} \rho_{ij}(a_j - a_i)$$

where a_i = specific activity, ρ_{ij} = flow constant, and S_i = compartment size (amount of parent substance).

This formulation is most convenient when the conditions of steady state are met. However, when one wishes to investigate the behavior in nonsteady state, it requires that the equations be viewed as having time-varying coefficients. A solution which leads to a form of the generalized Riccati-type equation can be found in the reference given. The solution was re-presented by Rescigno and Segre (5). The system solved in these references was a three-compartment closed system. The flow constants were assumed fixed with the exception of one which was specified as a function of time.

The general form of these equations can be derived from the equations that describe the same system with first-order kinetics (see *Appendix 1*). The specific activity parameter is introduced by defining specific activity as $a = R/S$, differentiating and substituting for the derivatives of R and S in the equations. Here R is the amount of radioactivity and S is the amount of parent substance. If S is assumed to be constant, it can be associated with the first-order rate constant to give a new coefficient ρ. However, if S changes with time and the new coefficients ρ are specified as constants, as in the example referred to, one is forced to allow the normally fixed-rate constant to vary. It is not clear what physical reality was intended to be represented in the example problem. Nevertheless, assume that a problem of this form is to be solved and its biological justification has been established. For the three-compartment closed system the equations for nonsteady state, when written in terms of specific activity, take the form:

$$S_1 \frac{da_1}{dt} = \rho_{12}(a_2 - a_1) + \rho_{13}(a_3 - a_1)$$

$$S_2 \frac{da_2}{dt} = \rho_{21}(a_1 - a_2) + \rho_{23}(a_3 - a_1)$$

$$S_3 \frac{da_3}{dt} = \rho_{31}(a_1 - a_3) + \rho_{32}(a_2 - a_3)$$

$$\frac{dS_1}{dt} = \rho_{12} - \rho_{21} + \rho_{13} - \rho_{31}$$

$$\frac{dS_2}{dt} = \rho_{21} - \rho_{12} + \rho_{23} - \rho_{32}$$

$$\frac{dS_3}{dt} = \rho_{31} - \rho_{13} + \rho_{32} - \rho_{23}$$

where S_i = amount of parent substance, a_i = specific activity, and ρ_{ij} = flow coefficients.

If some of the ρ's are functions of time, it is necessary to carry out the simultaneous solution of these six differential equations. Any suitable numerical integration algorithm may be used. However, the Continuous System Modeling Program is typical of the family of similar programs available for such solutions. The program shown in *Appendix 2* is all that is required to solve this problem. Problems of much greater complexity can be solved in the same fashion and require a minimum of effort or programming skill.

The formulation of the three-compartment closed system, when applied to a system with first-order kinetics, really falls into the more usual category of the nonsteady-state linear systems. Since in such systems the theorem of superposition is applicable, one may solve the equations for the behavior of the parent substance S, followed by the solution for the activity R. The division of the curve for R by that for S gives a curve of specific activity as a function of time. No assumption of steady state need be made in the formulation. The origins of the two curves which are to be divided may be offset relative to each other. That is to say, the system may be initially loaded with the unlabeled species so that all compartments have initial conditions or finite pool sizes at the time the radioactive tracer is introduced. In fact there may be little interest in how the system got to the condition which prevailed at the time of the introduction of the tracer. The subsequent values of specific activity are the important considerations. A complete example of a two-compartment closed model is carried out in the state-space form in *Appendix 3*. Note that the solution is in the form of a function of exponential terms. If the rate constants are known, they can be combined into numerical coefficients. Computer solutions of the state-space matrix $\bar{A}$, of course, require numerical values.

It commonly occurs that the rate constants of the system are not known and that an experiment must be conducted to evaluate these constants. Many papers have treated the problem of curve fitting of assumed functions or the iterative searching for unknown parameters. One of the basic uses of the tracer method is that of establishing pool size through the technique of introducing tracers and then computing the pool size from the dilution indicated by the specific activity. This is easily done in a single compartment or more complicated system, if the rate constants are known or where extensive measurements can be made. Unfortunately, in many systems some rate constants may be unknown or certain internal compartments are not accessible for measurement. The accompanying model considered in *Appendix 4* is one in which it was desired to establish the pool size existing at the time of the introduction of the tracer and none of the rate constants was known. The approach was one that could be of benefit in most drug metabolism studies. A model was established based on the best judgment of what might be appropriate and an analytical solution was derived. The state-space numerical solution would have yielded the final exponential form if rate constant values were given. Nevertheless, here it was desirable to identify

the terms of each coefficient comprising the final solution form, and a complete derivation was made. The derivation developed showed that it would be possible to determine the desired quantities from clinical test data. Then to demonstrate that the procedure was feasible, a simulation of the model using assumed parameters was run and data were taken on the model which corresponded to the clinical data expected from the actual experiment. Then from these data the pool size and rate constants were evaluated and shown to agree with the assumed values. One, of course, is not assured that the model is a unique representation of the biological phenomena, but if the experimental data indicates that the model is acceptable, then the parameters can be evaluated. Thus, in this fashion one can be assured that an experiment is feasible and that it will yield the desired parameters. Additional knowledge was gained regarding the requirements for data collection in the clinical experiment, the sensitivity of the parameters in the model was determined, and the general level of confidence regarding the experiment was raised.

While there is no completely general approach to this kind of modeling, this example utilizes relationships that might be overlooked in some situations. Rate constants were separated, based on the final value of the compartment measurements, and the use of a derivative of the analytical form of the solution yielded a transcendental equation that gave the sum of two rate constants. The fortuitous circumstances inherent in this model were that there was an output compartment directly linked to the input compartment and the fact that the parent drug could be assayed as well as the specific activity measured.

CONCLUSIONS

These considerations have shown that it is not necessary to formulate the tracer kinetics for systems governed by first-order linear differential equations in the form of nonlinear equations in order to evaluate the nonsteady-state behavior in terms of specific activity. The solutions may be obtained routinely through the use of the state-space formulation for the separate evaluations of the parent substance and the radioactive tracer, followed by a division of the curves or data points if specific activity is desired. Furthermore, if the case for nonlinear formulation of nonsteady-state conditions is justified on some biological grounds, the solution can be conveniently obtained through the use of a continuous system modeling program such as CSMP which provides a numerical integration algorithm and suitable selection of nonlinear elements. The model in *Appendix 4*, chosen to exhibit the procedures mentioned above, also exhibited other important features. Through the use of final value and curve fitting or solution of transcendental equations, the initial pool size was evaluated in a model of three internal compartments. The usual model in which dilution techniques are used through the measurement of specific activity to evaluate pool size consists of a single internal compartment linked to an external excretory compartment. An interesting problem exists in the extension of this approach to more complex systems.

It is to be noted that the formalism of postulating a model, assuming parameters, and executing trial runs to produce simulated clinical data, followed by the use of the data to check the previously assumed parameters, provides an assurance that the entire process is well defined. In this fashion the requirements for data collection and processing may be set forth well in advance of the clinical phase of the experimentation.

APPENDIX 1

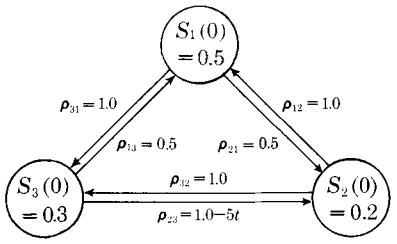
For an n-compartment, closed, first-order system, one can write

$$\frac{dS_i}{dt} = \sum_{\substack{j=1 \\ j\neq i}}^{n} K_{ij}S_j - \sum_{\substack{j=1 \\ j\neq i}}^{n} K_{ji}S_i \quad i = 1,2,\ldots,n$$

or

$$\frac{dS_i}{dt} = \sum_{\substack{j=1 \\ j\neq i}}^{n} K_{ij}S_j - K_{ji}S_i \quad i = 1,2,\ldots,n$$

where K_{ij} is the rate constant with units hours^{-1} for the transfer of substances S from compartment j to compartment i. S_i is the *total* amount of substance in compartment i with units of moles.



Scheme I—Three-compartment system.

One can write similar equations for the activity R_i of compartment i since the body will process labeled and unlabeled material in like manner. R has units of counts per minute.

$$\frac{dR_i}{dt} = \sum_{\substack{j=1 \\ j\neq i}}^{n} K_{ij}R_j - K_{ji}R_i \quad i = 1,2,\ldots,n$$

Specific activity a_i of compartment i is defined as

$$a_i = \frac{R_i}{S_i} \quad i = 1,2,\ldots,n$$

Differentiating,

$$\frac{da_i}{dt} = \frac{S_i(dR_i/dt) - R_i(dS_i/dt)}{S_i^2}$$

$$\frac{da_i}{dt} = \frac{1}{S_i}\frac{dR_i}{dt} - \frac{R_i}{S_i^2}\frac{dS_i}{dt}$$

Substituting for the derivatives of R_i and S_i results in

$$\frac{da_i}{dt} = \frac{1}{S_i}\sum_{\substack{j=1 \\ j\neq i}}^{n} K_{ij}R_j - K_{ji}R_i - \frac{R_i}{S_i^2}\sum_{\substack{j=1 \\ j\neq i}}^{n} K_{ij}S_j - K_{ji}S_i$$

$$\frac{da_i}{dt} = S_j\sum_{\substack{j=1 \\ j\neq i}}^{n} K_{ij}\frac{1}{S_i}\frac{R_j}{S_j} - K_{ji}\frac{1}{S_j}\frac{R_i}{S_i} - S_j\sum_{\substack{j=1 \\ j\neq i}}^{n} K_{ij}\frac{1}{S_i}\frac{R_i}{S_i} - K_{ji}\frac{1}{S_j}\frac{R_i}{S_i}$$

$$\frac{da_i}{dt} = S_j\sum_{\substack{j=1 \\ j\neq i}}^{n} \frac{K_{ij}}{S_i}a_j - \frac{K_{ji}}{S_j}a_i - \frac{K_{ij}}{S_i}a_i + \frac{K_{ji}}{S_j}a_i$$

$$S_i\frac{da_i}{dt} = \sum_{\substack{j=1 \\ j\neq i}}^{n} K_{ij}S_j(a_j - a_i)$$

If one defines $\rho_{ij} = K_{ij}S_j$ as a flow variable with variability due to S_j and not the rate constant K_{ij},

$$S_i\frac{da_i}{dt} = \sum_{\substack{j=1 \\ j\neq i}}^{n} \rho_{ij}(a_j - a_i)$$

If steady-state conditions exist, all S's will be constant and ρ becomes a constant.

APPENDIX 2

For the system in Scheme I with the substances S_i at steady state, it is desired to find the specific activities a_i of each compartment after injecting one unit of labeled material into Compartment 1 at $t = 0$. One cannot resort to known general solutions of linear differential equations (see *Appendix 3*) because the flow "constant" $\rho_{23} = 1 - 5t$ makes the differential equations which describe the system have time-varying coefficients. For the system in Scheme I, one can write for the specific activities:

$$\frac{da_1}{dt} = \frac{1}{S_1}[\rho_{12}(a_2 - a_1) + \rho_{13}(a_3 - a_1)]$$

$$\frac{da_2}{dt} = \frac{1}{S_2}[\rho_{21}(a_1 - a_2) + \rho_{23}(a_3 - a_2)]$$

$$\frac{da_3}{dt} = \frac{1}{S_3}[\rho_{31}(a_1 - a_3) + \rho_{32}(a_2 - a_3)]$$

```
****CONTINUOUS SYSTEM MODELING PROGRAM****
      ***PROBLEM INPUT STATEMENTS***
LABEL    TRACER KINETICS EXAMPLE PAGE 148 SHEPPARD
PARAM  R12=1.0,R13=.5,R21=.5,R32=1.0,R31=1.0
       R23=1.-5.*TIME
       DA1=(R12*(A2-A1)+R13*(A3-A1))/S1
       DA2=(R21*(A1-A2)+R23*(A3-A2))/S2
       DA3=(R31*(A1-A3)+R32*(A2-A3))/S3
       DS1=0.
       DS2=-.5-5.*TIME
       DS3=.5+5.*TIME
       A1=INTGRL(1.,DA1)
       A2=INTGRL(0.,DA2)
       A3=INTGRL(0.,DA3)
       S1=INTGRL(.5,DS1)
       S2=INTGRL(.2,DS2)
       S3=INTGRL(.3,DS3)
TIMER OUTDEL=.01,FINTIM=.2,DELT=.0001
PRTPLT A1(0.,1.,S1)
PRTPLT A2(0.,1.,S2)
PRTPLT A3(0.,1.,S3)
END
STOP
```

Figure 1—*CSMP program.*

where ρ_{23} is time varying.

For the substance S_i in each compartment the equations are:

$$\frac{dS_1}{dt} = \rho_{12} + \rho_{13} - \rho_{21} - \rho_{31} = 0$$

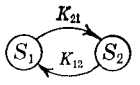
$$\frac{dS_2}{dt} = \rho_{21} + \rho_{23} - \rho_{12} - \rho_{32} = -0.5 - 5t$$

$$\frac{dS_3}{dt} = \rho_{31} + \rho_{32} - \rho_{13} - \rho_{23} = 0.5 + 5t$$

The six equations shown can be solved simultaneously in a straightforward manner with numerical techniques by using a digital computer language such as CSMP which simulates an analog computer. The simple CSMP program required to solve the above equations is shown in Fig. 1. Integration is performed by using the CSMP function "INTGRL(IC,X)", where IC is the initial condition and X is the function to be integrated. The functions to be integrated such as da_1/dt, which is shown in DA1 in Fig. 1, are defined by FORTRAN IV statements and/or with other CSMP functions. Plots of functions may be called for as well as function values at discrete points. Results of the above example are shown in Figs. 2–4. It is important to note that the above approach makes solutions easily obtainable for a large class of problems. The assumption of steady state, for instance, could be removed and add little complexity to the CSMP program.

APPENDIX 3

Consider a two-compartment, closed, first-order system with initial conditions $S_1(0)$ and $S_2(0)$:



As in *Appendix I*, one can write:

$$\frac{dS_1}{dt} = K_{12}S_2 - K_{21}S_1$$

$$\frac{dS_2}{dt} = K_{21}S_1 - K_{12}S_2 \qquad \text{(Eq. 1)}$$

or in matrix form

$$\begin{bmatrix}\dot{S}_1\\ \dot{S}_2\end{bmatrix} = \begin{bmatrix}-K_{21} & K_{12}\\ K_{21} & -K_{12}\end{bmatrix}\cdot\begin{bmatrix}S_1\\ S_2\end{bmatrix} \qquad \text{(Eq. 2)}$$

which may be written as

$$\dot{\bar{S}} = \bar{A}\bar{S}$$

These equations are in the form of the canonical state equations for which solutions are well known (3). The solution is

$$\bar{S} = e^{\bar{A}t}\bar{S}_0$$

where $\bar{S}_0$ is a constant column vector

$$\begin{bmatrix}S_1(0)\\ S_2(0)\end{bmatrix}$$

of initial conditions. The matrix $e^{\bar{A}t}$ is defined as

$$e^{\bar{A}t} = \bar{I} + \bar{A}t + \cdots + \frac{\bar{A}^K t^K}{K!} + \cdots$$

where $\bar{I}$ is an $n \times n$ identity matrix and $\bar{A}$ is an $n \times n$ constant matrix, which, for this example, are rate constants as in Eq. 2.

All that is necessary for a complete analytical solution to Eq. 1 is evaluating $e^{\bar{A}t}$. Several methods suited to computer implementation exist in the literature (3), but the method of Laplace transforms will be used here for convenience. It can be shown (3) that $e^{\bar{A}t}$ is the inverse Laplace transform of the matrix

$$[s\bar{I} - \bar{A}]^{-1}$$

where s is the Laplace variable and $\bar{I}$ is the identity matrix. One can write

$$[s\bar{I} - \bar{A}] = s\begin{bmatrix}1 & 0\\ 0 & 1\end{bmatrix} - \begin{bmatrix}-K_{21} & K_{12}\\ K_{21} & -K_{12}\end{bmatrix} = \begin{bmatrix}s + K_{21} & -K_{12}\\ -K_{21} & s + K_{12}\end{bmatrix}$$

Now,

$$[s\bar{I} - \bar{A}]^{-1} = \begin{bmatrix}\dfrac{(s + K_{12})}{s(s + K_{12} + K_{21})} & \dfrac{K_{12}}{s(s + K_{12} + K_{21})}\\ \dfrac{K_{21}}{s(s + K_{12} + K_{21})} & \dfrac{(s + K_{21})}{s(s + K_{12} + K_{21})}\end{bmatrix}$$

by normal methods. Taking the inverse Laplace transform yields[1]

$$L^{-1}([s\bar{I} - \bar{A}]^{-1}) = e^{\bar{A}t}$$

$$= \begin{bmatrix}\dfrac{K_{12}}{K_{12} + K_{21}} + \dfrac{K_{21}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}; & \dfrac{K_{12}}{K_{12} + K_{21}} - \dfrac{K_{12}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}\\ \dfrac{K_{21}}{K_{12} + K_{21}} - \dfrac{K_{21}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}; & \dfrac{K_{21}}{K_{12} + K_{21}} + \dfrac{K_{12}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}\end{bmatrix}$$

Thus the solutions for Eq. 2 can be written as

$$\begin{bmatrix}S_1\\ S_2\end{bmatrix} = \begin{bmatrix}\dfrac{K_{12}}{K_{12} + K_{21}} + \dfrac{K_{21}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}; & \dfrac{K_{12}}{K_{12} + K_{21}} - \dfrac{K_{12}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}\\ \dfrac{K_{21}}{K_{12} + K_{21}} - \dfrac{K_{21}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}; & \dfrac{K_{21}}{K_{12} + K_{21}} + \dfrac{K_{12}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}\end{bmatrix}\begin{bmatrix}S_1(0)\\ S_2(0)\end{bmatrix}$$

or

$$S_1 = S_1(0)\left[\frac{K_{12}}{K_{12} + K_{21}} + \frac{K_{21}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}\right] + S_2(0)\left[\frac{K_{12}}{K_{12} + K_{21}} - \frac{K_{12}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}\right]$$

$$S_2 = S_1(0)\left[\frac{K_{21}}{K_{12} + K_{21}} - \frac{K_{21}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}\right] + S_2(0)\left[\frac{K_{21}}{K_{12} + K_{21}} + \frac{K_{12}}{K_{12} + K_{21}}e^{-(K_{12}+K_{21})t}\right]$$

[1] A matrix of the type

$$\begin{bmatrix}1 & 0\\ 0 & 1\end{bmatrix}$$

is written as

$$\begin{bmatrix}1; & 0\\ 0; & 1\end{bmatrix}$$

because of format restrictions.

MINIMUM A1 VERSUS TIME MAXIMUM

TIME	A1	0.0 I	1.0000E 00 I	S1
0.0	1.0000E 00			5.0000E-01
1.0000E-02	9.7085E-01			5.0000E-01
2.0000E-02	9.4335E-01			5.0000E-01
3.0000E-02	9.1741E-01			5.0000E-01
4.0000E-02	8.9296E-01			5.0000E-01
5.0000E-02	8.6993E-01			5.0000E-01
6.0000E-02	8.4824E-01			5.0000E-01
7.0000E-02	8.2783E-01			5.0000E-01
8.0000E-02	8.0865E-01			5.0000E-01
9.0000E-02	7.9063E-01			5.0000E-01
1.0000E-01	7.7373E-01			5.0000E-01
1.1000E-01	7.5789E-01			5.0000E-01
1.2000E-01	7.4309E-01			5.0000E-01
1.3000E-01	7.2928E-01			5.0000E-01
1.4000E-01	7.1644E-01			5.0000E-01
1.5000E-01	7.0454E-01			5.0000E-01
1.6000E-01	6.9359E-01			5.0000E-01
1.7000E-01	6.8359E-01			5.0000E-01
1.8000E-01	6.7460E-01			5.0000E-01
1.9000E-01	6.6675E-01			5.0000E-01
2.0000E-01	6.6062E-01			5.0000E-01

Figure 2—$a_1(t)$ *from CSMP program.*

MINIMUM A2 VERSUS TIME MAXIMUM

TIME	A2	0.0 I	1.0000E 00 I	S2
0.0	0.0			2.0000E-01
1.0000E-02	2.4822E-02			1.9475E-01
2.0000E-02	4.9252E-02			1.8899E-01
3.0000E-02	7.3252E-02			1.8274E-01
4.0000E-02	9.6807E-02			1.7599E-01
5.0000E-02	1.1992E-01			1.6874E-01
6.0000E-02	1.4262E-01			1.6098E-01
7.0000E-02	1.6493E-01			1.5273E-01
8.0000E-02	1.8692E-01			1.4398E-01
9.0000E-02	2.0865E-01			1.3473E-01
1.0000E-01	2.3022E-01			1.2497E-01
1.1000E-01	2.5176E-01			1.1472E-01
1.2000E-01	2.7341E-01			1.0397E-01
1.3000E-01	2.9540E-01			9.2716E-02
1.4000E-01	3.1803E-01			8.0964E-02
1.5000E-01	3.4173E-01			6.8711E-02
1.6000E-01	3.6721E-01			5.5960E-02
1.7000E-01	3.9570E-01			4.2710E-02
1.8000E-01	4.2975E-01			2.8960E-02
1.9000E-01	4.7662E-01			1.4709E-02
2.0000E-01	6.4537E-01			−4.0663E-05

Figure 3—$a_2(t)$ *from CSMP program.*

MINIMUM A3 VERSUS TIME MAXIMUM

TIME	A3	0.0 I	1.0000E 00 I	S3
0.0	0.0			3.0000E-01
1.0000E-02	3.1908E-02			3.0525E-01
2.0000E-02	6.1143E-02			3.1099E-01
3.0000E-02	8.7954E-02			3.1724E-01
4.0000E-02	1.1258E-01			3.2399E-01
5.0000E-02	1.3522E-01			3.3123E-01
6.0000E-02	1.5607E-01			3.3898E-01
7.0000E-02	1.7531E-01			3.4723E-01
8.0000E-02	1.9310E-01			3.5598E-01
9.0000E-02	2.0959E-01			3.6522E-01
1.0000E-01	2.2491E-01			3.7497E-01
1.1000E-01	2.3918E-01			3.8522E-01
1.2000E-01	2.5252E-01			3.9596E-01
1.3000E-01	2.6504E-01			4.0721E-01
1.4000E-01	2.7684E-01			4.1896E-01
1.5000E-01	2.8802E-01			4.3120E-01
1.6000E-01	2.9868E-01			4.4395E-01
1.7000E-01	3.0893E-01			4.5720E-01
1.8000E-01	3.1891E-01			4.7095E-01
1.9000E-01	3.2883E-01			4.8519E-01
2.0000E-01	3.3933E-01			4.9994E-01

Figure 4—$a_3(t)$ *from CSMP program.*

If one does not know $S_1(0)$ and $S_2(0)$ but knows them at some time, t_1, earlier [e.g., 100 mg. of substance was injected into Compartment 1 with no initial substance in the system, then $S_1(t_1) = 100$ and $S_2(t_1) = 0$], one can use the above solutions with different initial conditions and evaluate at the later time which has been defined as zero to obtain $S_1(0)$ and $S_2(0)$.

If labeled substance is now injected into the system at $t = 0$, it is possible to write for the activities, as in *Appendix 1*,

$$\frac{dR_1}{dt} = K_{12}R_2 - K_{21}R_1$$

$$\frac{dR_2}{dt} = K_{21}R_1 - K_{12}R_2$$

The solutions to the above follow in the same manner as for the unlabeled substance S_1, *i.e.*,

$$R_1 = R_1(0)\left(\frac{K_{12}}{K_{12}+K_{21}} + \frac{K_{21}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right) + R_2(0)\left(\frac{K_{12}}{K_{12}+K_{21}} - \frac{K_{12}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right)$$

$$R_2 = R_1(0)\left(\frac{K_{21}}{K_{12}+K_{21}} - \frac{K_{12}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right) + R_2(0)\left(\frac{K_{21}}{K_{12}+K_{21}} + \frac{K_{12}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right)$$

The above solutions could be obtained by other methods, but the state-space method has the advantage of being easily relegated to a computer. One can now obtain a complete analytical solution for the specific activities simply by dividing:

$$a_1 = \frac{R_1}{S_1}$$

$$a_1 = \frac{R_1(0)\left(\frac{K_{12}}{K_{12}+K_{21}} + \frac{K_{21}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right) + R_2(0)\left(\frac{K_{12}}{K_{12}+K_{21}} - \frac{K_{12}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right)}{S_1(0)\left(\frac{K_{12}}{K_{12}+K_{21}} + \frac{K_{21}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right) + S_2(0)\left(\frac{K_{12}}{K_{12}+K_{21}} - \frac{K_{12}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right)}$$

and

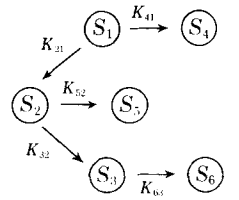
$$a_2 = \frac{R_2}{S_2}$$

$$a_2 = \frac{R_1(0)\left(\frac{K_{21}}{K_{12}+K_{21}} - \frac{K_{12}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right) + R_2(0)\left(\frac{K_{21}}{K_{12}+K_{21}} + \frac{K_{12}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right)}{S_1(0)\left(\frac{K_{21}}{K_{12}+K_{21}} - \frac{K_{21}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right) + S_2(0)\left(\frac{K_{21}}{K_{12}+K_{21}} + \frac{K_{12}}{K_{12}+K_{21}}e^{-(K_{12}+K_{21})t}\right)}$$

Note that the amount of radioactive drug injected must be added to $S_1(0)$ and $S_2(0)$ since S is defined as the total amount of drug present. Also note that the second term in the numerator of the a_i is zero if the labeled material is injected into Compartment 1 only, as is the usual case.

APPENDIX 4

In the following first-order compartment model each compartment contains an initial condition at time $t = 0$. Measurements are possible for Compartments 4, 5, and 6 only, and hence the initial conditions of Compartments 1, 2, and 3 are unknown. Also, all rate constants are unknown. It will be shown that all of the unknowns may be uniquely determined by using the method of isotope dilution.



One can write

$$\frac{dS_1}{dt} = -(K_{21} + K_{41})S_1$$

$$\frac{dS_2}{dt} = K_{21}S_1 - (K_{32} + K_{52})S_2$$

$$\frac{dS_3}{dt} = K_{32}S_2 - K_{63}S_3$$

The solutions to the above three equations are

$$S_1 = S_1(0)e^{-(K_{21}+K_{41})t}$$

$$S_2 = \frac{K_{21}S_1(0)}{(K_{52}+K_{32}) - (K_{21}+K_{41})}(e^{-(K_{21}+K_{41})t} - e^{(K_{32}+K_{52})t}) + S_2(0)e^{-(K_{32}+K_{52})t}$$

$$S_3 = S_3(0)e^{-K_{63}t} + \frac{K_{32}S_2(0)}{(K_{52}+K_{32}) - K_{63}}(e^{-K_{63}t} - e^{-(K_{52}+K_{32})t}) + K_{21}K_{32}S_1(0)\left(\frac{e^{-K_{63}t}}{(K_{52}+K_{32}-K_{63})(K_{41}+K_{21}-K_{63})} + \frac{e^{-(K_{32}+K_{52})t}}{(K_{63}-K_{32}-K_{52})(K_{41}+K_{21}-K_{32}-K_{52})} + \frac{e^{-(K_{41}+K_{21})t}}{(K_{63}-K_{41}-K_{21})(K_{52}+K_{32}-K_{41}-K_{21})}\right)$$

The above three equations describe the time dependency of mass.

Suppose an amount of labeled material is injected into Compartment 1 at $t = 0$. One can write rate equations for the amount of activity in each compartment as:

$$\frac{dR_1}{dt} = -(K_{41} + K_{21})R_1$$

$$\frac{dR_2}{dt} = K_{21}R_1 - (K_{52} + K_{32})R_2$$

$$\frac{dR_3}{dt} = K_{32}R_2 - K_{63}R_3$$

The solutions to the activity equations above are of the same form as the mass equation solutions except that no initial conditions of activity exist on any compartments except 1. Thus the solutions are

$$R_1 = R_1(0)e^{-(K_{21}+K_{41})t}$$

$$R_2 = \frac{K_{21}R_1(0)}{(K_{21}+K_{41}) - (K_{52}+K_{32})}(e^{-(K_{32}+K_{52})t} - e^{-(K_{21}+K_{41})t})$$

$$R_3 = K_{21}K_{32}R_1(0)\left(\frac{e^{-K_{63}t}}{(K_{52}+K_{32}-K_{63})(K_{21}+K_{41}-K_{63})} + \frac{e^{-(K_{32}+K_{52})t}}{(K_{63}-K_{32}-K_{52})(K_{21}+K_{41}-K_{32}-K_{52})} + \frac{e^{-(K_{21}+K_{41})t}}{(K_{63}-K_{41}-K_{21})(K_{32}+K_{52}-K_{41}-K_{21})}\right)$$

Now if specific activity is defined as

$$a = \frac{R}{S}$$

one has for Compartment 1

$$a_1 = \frac{R_1}{S_1} = \frac{R_1(0)\ e^{-(K_{41}+K_{21})t}}{S_1(0)\ e^{-(K_{41}+K_{21})t}}$$

which is a constant. $K_{41}S_1$ and $K_{41}R_1$ can be measured so that

$$a_1 = \frac{K_{41}R_1}{K_{41}S_1}$$

can be determined. Since $R_1(0)$ and a_1 are known,

$$S_1(0) = \frac{R_1(0)}{a_1}$$

which is one of the unknowns to be determined. Note that one must subtract that amount of labeled substance introduced from the above value.

In addition it is possible now also to evaluate K_{41} from

$$K_{41} = \frac{K_{41}S_1(0)}{S_1(0)}$$

K_{21} may be determined from a curve fitting of the $K_{41}S_1$ data with K_{41} as the only unknown. K_{21} could also be determined from the final values of Compartments 4, 5, and 6, *i.e.*,

$$\frac{K_{41}}{K_{21}} = \frac{S_4(\infty)}{S_5(\infty) + S_6(\infty)}$$

The sum $(K_{32} + K_{52})$ can be found from a curve fit of the $K_{52}R_2$ data with the sum as the only unknown or by solving a transcendental equation derived as

$$K_{52}R_2 = \frac{K_{52}K_{32}R_1(0)}{(K_{21} + K_{41}) - (K_{52} + K_{32})}\left(e^{-(K_{41}+K_{21})t} - e^{-(K_{32}+K_{52})t}\right)$$

$$\frac{dK_{52}R_2}{dt} = \frac{K_{52}K_{32}R_1(0)}{(K_{21} + K_{41}) - (K_{52} + K_{32})}\left(-(K_{41} + K_{21})\,e^{-(K_{41}+K_{21})t} + (K_{32} + K_{52})\,e^{-(K_{32}+K_{52})t}\right)$$

at the time T_m, $K_{52}R_2$ reaches its maximum and $(dK_{52}R_2/dt) = 0$. Thus

$$(K_{41} + K_{21})\,e^{-(K_{41}+K_{21})T_m} = (K_{32} + K_{52})\,e^{-(K_{32}+K_{52})T_m}$$

which is a transcendental equation which must be solved iteratively. All terms are known except the sum $(K_{32} + K_{52})$.

The sum $(K_{32} + K_{52})$ may now be split into its parts by making use of the relationship

$$\frac{K_{52}}{K_{32}} = \frac{S_5(\infty)}{S_6(\infty)}$$

and thus K_{52} and K_{32} are determined explicitly. One can now determine the unknown initial conditions, $S_2(0)$. It is possible to measure

$$K_{52}S_2 = K_{52}S_2(0)e^{-(K_{32}+K_{52})t} + \frac{K_{52}K_{21}S_1(0)}{(K_{32} + K_{52}) - (K_{41} + K_{21})}\left(e^{-(K_{41}+K_{21})t} - e^{-(K_{32}+K_{52})t}\right)$$

and thus at $t = 0$ one can be sure he is measuring $K_{52}S_2(0)$ alone. Since K_{52} is known,

$$S_2(0) = \frac{K_{52}S_2(0)}{K_{52}}$$

Only two unknowns remain for the system, $S_3(0)$ and K_{63}. K_{63} can be found with a curve fit of the measured $K_{63}R_3$ data with K_{63} as the only unknown, or one can solve another transcendental equation derived in a manner similar to the first.

From the $K_{63}S_3$ data at $t = 0$, one can find $K_{63}S_3(0)$ in the same manner as $K_{52}S_2(0)$ and hence

$$S_3(0) = \frac{K_{63}S_3(0)}{K_{63}}$$

Thus all unknowns have been uniquely determined. Two methods of evaluating several of the unknowns are possible and can be used as a check.

REFERENCES

(1) D. S. Riggs, "The Mathematical Approach to Physiological Problems," Williams & Wilkins, Baltimore, Md., 1963, p. 131.

(2) I.B.M., *System 360 Continuous System Modeling Program H20-0367-2.*

(3) L. A. Zadeh and C. A. Desoer, "Linear System Theory," McGraw-Hill, New York, N. Y., 1963, p. 300.

(4) C. W. Sheppard, "Basic Principles of the Tracer Method," Wiley, New York, N. Y., 1962, p. 148.

(5) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Waltham, Mass., 1966, p. 52.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 31, 1969, from *The William S. Merrell Company, Division of Richardson-Merrell, Inc., Lockland Station, Cincinnati, OH 45215*

Accepted for publication October 21, 1969.

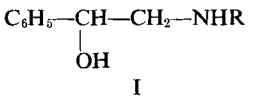
Some Derivatives of Phenethanolamine as MAO Inhibitors

J. N. WELLS and J. K. SHIELDS*

Abstract □ A series of *N*-methyl and *o*-alkyl phenethanolamine derivatives has been prepared and tested against monoamine oxidase *in vitro*. A substantial increase in MAO inhibition over that of the parent phenethanolamine derivatives was noted in the *o*-benzyl analogs.

Keyphrases □ Monoamine oxidase inhibition *in vitro*—phenethanolamine derivatives □ *N*-Methyl, *o*-alkyl derivatives—monoamine oxidase inhibition *in vitro* □ Inhibition analysis—radioactive content method

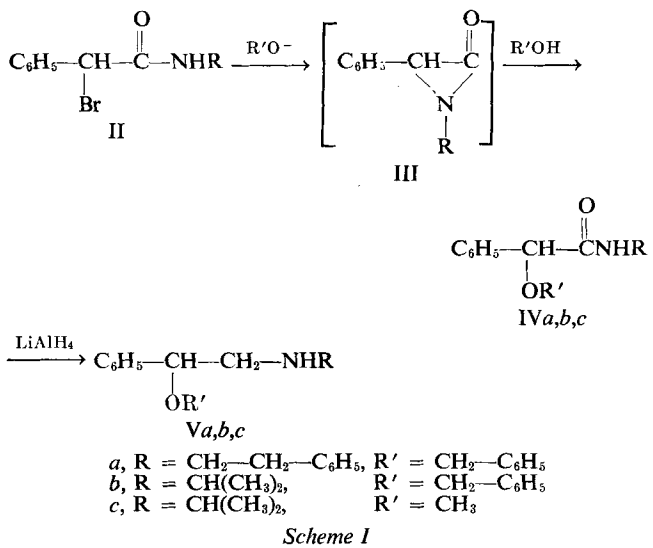
A previous report (1) from these laboratories indicated that 2-phenethylamino-1-phenylethanol (I*a*) and 2-isopropylamino-1-phenylethanol (I*b*) were good monoamine oxidase (MAO) inhibitors *in vitro*. A more recent investigation has shown that the *in vivo* duration of action of I*a* is very short and characterized by potent CNS stimulant properties (2).



I

a, R = CH_2—CH_2—C_6H_5
b, R = $CH(CH_3)_2$

The purpose of the studies reported here was to improve the inherent MAO-inhibiting properties of I*a* and I*b* by structural modification. Based on the report (3) that etherification of the hydroxyl group in a hydrazinoalkanol series increased the *in vivo* potency, the authors have prepared a few ether and *N*-alkyl analogs of I*a* and I*b* and tested their effectiveness against MAO *in vitro*.



Scheme I

CHEMISTRY

Preparation of *N*-methyl derivatives of I*a* and I*b* was accomplished by a standard reductive amination procedure (4).

Synthesis of the desired amino-ethers (V*a,b,c*) was accomplished *via* the corresponding α-bromophenylacetamide (II) (Scheme I). α-Bromoamides have been shown to form α-lactams when treated with an alkoxide (5). It was assumed that the α-lactam was formed as an intermediate in this sequence since product ratios and yields under various conditions correspond to those predicted from studies of α-lactam reactivity (6). For example, α-lactam formation could have occurred when the appropriate α-bromoamide (II) was reacted with an equimolar amount of either sodium methoxide or benzoxide suspended in the respective alcohol or benzene. However, yields of the ether-amides decreased when benzene was the solvent. This would be expected from proposed ring opening mechanisms attributing formation of the amino-ester (VI) to alkoxide attack on the carbonyl carbon of α-lactams and ether-amide (VII) formation to solvolysis of the α-carbon by an alcohol molecule (Scheme II) (5–8).

One experiment was conducted in an attempt to isolate both the products expected if an α-lactam was involved. When *N*-phenethyl α-bromophenylacetamide (II*a*) was reacted with an equimolar amount of sodium benzoxide in ether at room temperature, both the amino-ester (VI, R′ = CH_2—C_6H_5, R = CH_2—CH_2—C_6H_5) and ether-amide (IV*a*) were isolated. In no other case was an attempt made to recover the amino-ester or to quantify the yield. It is interesting to note that in the reaction of sodium methoxide with II*a*, only the amino-ester (methyl α-phenethylaminophenylacetate) was isolated.

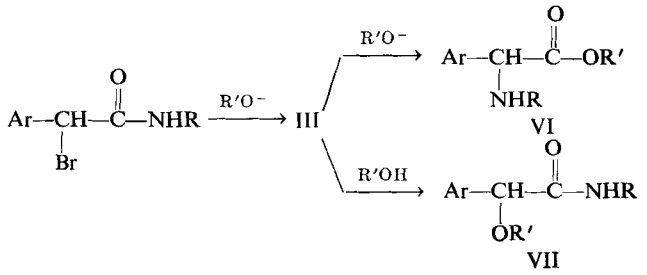
The desired amino-ether compounds (V*a,b,c*) were readily obtained by lithium aluminum hydride reduction of the corresponding ether-amides (IV*a,b,c*).

***In Vitro* Monoamine Oxidase Inhibition**—Inhibition of monoamine oxidase *in vitro* was assessed by the method of Wurtman and Axelrod (9). The potential MAO inhibitor and the substrate (2-^{14}C-tryptamine) were incubated with rat liver homogenate. After a suitable length of time the deaminated product (^{14}C-indolacetic acid) was extracted and counted for its radioactive content. The compounds were screened at 5×10^{-4} *M* concentration as the hydrochloride salt by using: 1 ml. of a 10^{-3} *M* solution of the potential inhibitor, 0.625 ml. of phosphate buffer, 0.10 ml. (0.02 μc.) of ^{14}C-tryptamine substrate, and 0.25 ml. of rat liver homogenate to give a final volume of 2 ml. The degree of inhibition was compared to 5×10^{-4} *M* iproniazid standard. The results are given in Table I.

EXPERIMENTAL

Melting points were determined on a Buchi apparatus with open capillary tubes and are uncorrected. NMR spectra were obtained with a Varian Associates A-60-A spectrophotometer in $CDCl_3$ with tetramethylsilane as an internal standard. Combustion analyses were conducted by Galbraith Laboratories, Inc., Knoxville, Tenn. IR spectra were determined on a Perkin-Elmer model 21 spectrophotometer and were consistent with structures given.

***N*-Methyl-2-phenethylamino-1-phenylethanol Hydrochloride**—Formic acid (25.6 g. of 90%, 0.50 mole), formaldehyde (9.0 g. of 40%, 0.30 mole), and 2-phenethylamino-1-phenylethanol (24.1 g., 0.10 mole) were reacted by standard procedures (4). The product was isolated as the hydrochloride salt and recrystallized from hexane–acetone to give a white solid (8.6 g., 30%), m.p. 153–154°; NMR



Scheme II

(D_2O) δ7.25 (m, 10, Ar), 5.25 (m, 1, C<u>H</u>), 3.22 (b m, 6, CH_2), 2.95 (s, 3, CH_3).

Anal.—Calcd. for $C_{17}H_{22}ClNO$: C, 69.97; H, 7.60. Found: C, 69.93; H, 7.76.

***N*-Methyl-2-isopropylamino-1-phenylethanol Hydrochloride**—Formic acid (51.2 g. of 90%, 1.0 mole), formaldehyde (18.0 g. of 40%, 0.60 mole), and 2-isopropylamino-1-phenylethanol (35.8 g., 0.20 mole) were reacted as above. Recrystallization of the hydrochloride salt from hexane–acetone gave 16.5 g. (36%) of white solid, m.p. 134–135°; NMR (D_2O) δ7.4 (s, 5, Ar), 5.15 (t, 1, C<u>H</u>), 3.7 (m, 1, CH), 3.25 (d, 2, CH_2), 1.27 (m, 6, CH_3).

Anal.—Calcd. for $C_{12}H_{20}ClNO$: C, 62.73; H, 8.77. Found: C, 62.86; H, 8.97.

***N*-Phenethyl-α-benzoxyphenylacetamide (IV*a*)**—*Method A*—*N*-Phenethyl α-bromophenylacetamide (11) (20.0 g., 0.06 mole) in 150 ml. of benzyl alcohol was added over 1 hr. to a heated solution of sodium benzoxide (7.8 g., 0.06 mole) in 50 ml. of benzyl alcohol. The mixture was heated under reflux for 10 hr. and then filtered. The filtrate was added to water and extracted with ether. The dried organic extract was evaporated *in vacuo* and the residue distilled under reduced pressure to remove benzyl alcohol. The thick residue solidified after 2 days. The solid was recrystallized from hexane to give 5.0 g. (23%) of white crystalline solid, m.p. 86–88°; NMR ($CDCl_3$) δ7.25 (m, 9, Ar), 6.8 (b m, 1, NH), 4.75 (s, 1, CH), 4.40 (d, 2, C<u>H</u>$_2$), 3.5 (q, 2, NHC<u>H</u>$_2$), 2.75 (t, 2, CH_2).

Anal.—Calcd. for $C_{23}H_{23}NO_2$: C, 79.97; H, 6.71. Found: C, 79.75; H, 6.33.

Method B—*N*-Phenethyl-α-bromophenylacetamide (25.0 g., 0.08 mole) in 100 ml. of benzene was added dropwise to a suspension of 10.4 g. (0.08 mole) of sodium benzoxide in 100 ml. of benzene. After 24 hr. at room temperature the precipitate which had formed was removed and the filtrate was evaporated *in vacuo*. The solid residue was recrystallized from hexane to give 2.5 g. (9%) of white solid identical to that obtained by *Method A*.

***N*-Isopropyl-α-methoxyphenylacetamide (IV*c*)**—Utilizing *Method A* above, *N*-isopropyl-α-bromophenylacetamide (40.0 g., 0.16 mole) was reacted to give 5 g. (15%) of recrystallized material, m.p. 51–56°; NMR ($CDCl_3$); δ7.3 (m, 5, Ar), 6.43 (m, 1, NH), 4.51 (s, 1, CH), 4.0 (t, 1, CH), 3.3 (s, 3, OCH_3), 1.15 (m, 6, CH_3). This material was used without further purification.

***N*-Phenethyl-α-benzoxyphenylacetamide (IV*a*) and Benzyl-α-phenethylaminophenylacetate**—To a solution of sodium benzoxide (13.0 g., 0.10 mole) in ether was added in one portion an ether solution of *N*-phenethyl-α-bromophenylacetamide (32.0 g., 0.1 mole). After 1 hr. the solid was collected and discarded. Ethereal HCl was added dropwise to the filtrate. The precipitate was recrystallized from acetone–ethanol (4:1) to give 2.3 g. (6.2%) of VI·HCl, m.p. 224–225°.

The ether filtrate was evaporated *in vacuo*. The resulting residue was treated with water and extracted with ether. The ether extracts were dried and evaporated to give an oil which could not be purified. The aqueous phase was chilled and a precipitate formed. The solid was recrystallized from hexane to give 1.0 g. (2.1%) of IV*a*.

Attempted Synthesis of *N*-Phenethyl-β-methoxyphenethylamine Hydrochloride—Following *Method B*,*N*-phenethyl-α-bromophenylacetamide (32.0 g., 0.10 mole) was reacted with sodium methoxide (5.4 g., 0.10 mole) to give a crude solid, m.p. 222–225°. Without further purification (7.5 g., 0.028 mole) was reacted with lithium aluminum hydride according to the general reduction procedure. The product obtained was found to be 2-phenyl-2-phenethylaminoethanol hydrochloride, 1.7 g. (19%), m.p. 131–132°; NMR ($CDCl_3$), δ6.40 (m, 10, Ar), 5.6 (m, 1, CH), 4.35 (m, 3, C<u>H</u>$_2$—OH), 3.15 (m, 4, C<u>H</u>$_2$—Ar, C<u>H</u>$_2$—N).

Table I—Results of *In Vitro* Screening for MAO Inhibition Using Rat Homogenate

Compound	% Inhibition
Iproniazid	100
I*a*	36 ± 7[a,b]
I*b*	36[c]
V*a*	93 ± 2[a]
V*b*	70 ± 5[a]
V*c*	15 ± 4[a]
N-Methyl I*a*	51 ± 4[a]
N-Methyl I*b*	24 ± 6[d]

[a] Triplicate determinations reported as the mean and average deviation from the mean value. [b] Found to be less active than previously reported (1). Dioxane was used in the earlier testing and if not properly purified would contain ethanol which has been reported to inhibit MAO *in vitro* (10). [c] Single determination. [d] Duplicate determinations reported as the mean and average deviation of an individual result from this mean value.

Anal.—Calcd. for $C_{16}H_{20}ClNO$: C, 69.18; H, 7.27. Found: C, 69.47; H, 7.24.

***N*-Phenethyl-β-benzoxyphenethylamine Hydrochloride (V*a*)**—*General Reduction Procedure*—*N*-Phenethyl-α-benzoxyphenylacetamide (8.0 g., 0.023 mole) suspended in 200 ml. of dry ether was added in 10-ml. portions to a suspension of lithium aluminum hydride (1.74 g., 0.046 mole) in 100 ml. of ether. The mixture was heated under reflux for 12 hr. and then 9.6 ml. of water was added. The resulting precipitate was removed by filtration and the ether filtrate dried. The ether was placed in a dry-ice acetone bath and dry ethereal HCl was added dropwise. The solid that precipitated was recrystallized from acetone–methanol (4:1) by cooling the solution to 0° to give 1.6 g. (19%) of pure V*a*·HCl, m.p. 136–137.5°; NMR ($CDCl_3$) δ7.37 (m, 15, Ar), 5.15 (q, 1, CH), 4.5 (dd, 2, O—C<u>H</u>$_2$—Ar), 3.3 (m, 6, C<u>H</u>$_2$—N—C<u>H</u>$_2$—C<u>H</u>$_2$—Ar).

Anal.—Calcd. for $C_{23}H_{26}ClNO$: C, 75.08; H, 7.12. Found: C, 75.26; H, 7.21.

***N*-Isopropyl-β-benzoxyphenethylamine Hydrochloride (V*b*)**—*N*-Isopropyl-α-benzoxyphenylacetamide (6.0 g., 0.021 mole) was reacted according to the general reduction procedure to give 1.3 g. (22%) of V*b*, m.p. 188–189°; NMR ($CDCl_3$) δ7.4 (m, 10, Ar), 5.17 (q, 1, CH), 4.5 (dd, 2, O—CH_2), 3.33 (m, 3, CH, N—CH_2), 1.5 (d, 6, CH_3).

Anal.—Calcd. for $C_{18}H_{24}ClNO$: C, 70.69; H, 7.91. Found: C, 70.39; H, 7.90.

***N*-Isopropyl-β-methoxyphenethylamine Hydrochloride (V*b*)**—*N*-Isopropyl-α-methoxyphenylacetamide (5.0 g, 0.024 mole) was reacted according to the general reduction procedure to give 1.1 g. (20%) of V*c*·HCl, m.p. 225–226°; NMR (D_2O) δ7.3 (m, 5, Ar), 4.65 (m, 1, CH), 3.3 (m, 6, CH, N—C<u>H</u>$_2$, OCH_3), 1.50 (d, 6, CH_3).

Anal.—Calcd. for $C_{12}H_{20}ClNO$: C, 62.73; H, 8.77. Found: C, 62.61, H. 8.69.

DISCUSSION

From Table I it can be observed that *in vitro* inhibition of MAO by the benzyl ether compounds V*a* and V*b* was substantially increased over that of the parent compounds (I*a* and I*b*). It is interesting to note that the methyl ether of 2-isopropylamino-1-phenylethanol causes a decrease in *in vitro* MAO inhibition from the parent compound which corresponds to observations in the hydrazinoalkanol series of Schuler and Wyss (3). Methylation of the nitrogen of the parent compounds increased MAO inhibition in one case (II*a*) over the parent but decreased inhibition in relation to the parent with compound II*b*. *In vivo* testing of these compounds will be carried out at a later date.

REFERENCES

(1) J. N. Wells, A. V. Shirdokar, and A. M. Knevel, *J. Med. Chem.*, **9**, 195(1966).

(2) J. N. Wells and W. G. Andrus, *J. Pharm. Sci.*, **58**, 1553(1969).

(3) W. Schuler and E. Wyss, *Arch. Intern. Pharmacodyn.*, **128**, 431(1960).

(4) R. N. Icke, B. B. Wisegarver, and G. A. Alles, *Org. Syn.*, **25**, 89(1945).
(5) H. E. Baumgarten, *J. Amer. Chem. Soc.*, **84**, 4975(1962).
(6) J. C. Sheehan and I. Lengyel, *ibid.*, **86**, 1356(1964).
(7) J. C. Sheehan and J. J. Beeson, *ibid.*, **89**, 362(1967).
(8) H. E. Baumgarten, *ibid.*, **85**, 3303(1963).
(9) R. J. Wurtman and J. Axelrod, *J. Biochem. Pharmacol.*, **12**, 1439(1963).
(10) J. C. Towne, *Nature*, **201**, 709(1964).
(11) P. Truitt, E. E. Richardson, L. N. Long, and W. J. Middleton, *J. Amer. Chem. Soc.*, **71**, 3479(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 8, 1969, from the *Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication October 10, 1969.

Abstracted in part from a thesis submitted by J. K. Shields in partial fulfillment of Master of Science degree requirements.

* NDEA Title IV Fellow.

Some Cyclic Ketals and Acetals of Digitoxin, Digoxin, and Ouabain

KHALID S. ISHAQ and OLE GISVOLD

Abstract □ A new and milder method has been utilized to reinvestigate the preparation, properties, and biological activities of the mono-*o*-isopropylidene (acetonides) derivatives of digitoxin, digoxin, and ouabain. The preparation, properties, and biological activities of the mono-*o*-ethylidene derivatives of digitoxin and digoxin also are described. The latter derivatives show greater activity than the former.

Keyphrases □ Digitoxin, digoxin cyclic ketals, acetals—synthesis □ Ouabain cyclic ketals, acetals—synthesis □ Bioanalysis—digitoxin, digoxin, ouabain derivatives □ TLC, paper chromatography—separation, identification □ UV spectrophotometry—identity

In a previous publication the preparation and activities of the mono-*o*-isopropylidene derivatives (glycoside acetonides) of digitoxin, digoxin, and ouabain have been reported (1). Their biological activities were much less than had been expected on the basis of their physical properties and, in the cases of digitoxin and of digoxin, the position of the isopropylidene group. Because these activities were so unexpectedly low, a reinvestigation of the preparation of these derivatives by much milder methods was sought and one such was found. Acetonation can be effected in a very short time at room temperature *via* the use of 2,2-dimethoxypropane, acetone, a trace of acid, and room temperature conditions. The yields are almost quantitative and derivatives readily can be crystallized. Bioassay of these derivatives prepared by this new, very mild method gave comparable results in the case of digoxin but not in the case of digitoxin or ouabain. The ease with which the isopropylidene derivatives (dioxolanes) of digitoxin and digoxin could be prepared *via* the use of ketal-interchange techniques suggested the preparation of other cyclic ketals and cyclic acetals (dioxolanes) to test further the contribution to activity such derivatives might exert. Because the isopropylidene group, even though it was the simplest type of a cyclic ketal and on the terminal digitoxose residue, reduced activity so markedly that the preparation of a cyclic acetal as a second type of a dioxolane derivative was investigated. The ethylidene derivative readily could be prepared *via* 1,1-dimethoxyethane, acetaldehyde, a trace of acid, and room temperature conditions. Bioassays of these ethylidene derivatives *via* LD_{50} intravenous assays in cats showed that mono-*o*-ethylidene digoxin had the same activity as that of acetyldigoxin whereas acetyldigitoxin was 2.35 times as active as mono-*o*-ethylidene digitoxin. Thus the activity (toxicity) of digitoxin was reduced to a greater extent by both types of derivatives than was the case with digoxin. Also it is of interest to note that the mono-*o*-ethylidene digoxin activity was the same as that of acetyldigoxin.

In the case of digoxin, its usefulness is improved in its acetyl derivatives.[1] Acetyldigoxin is considered by some to be the drug of choice because it is well absorbed orally, is less toxic to the CNS, and has a shorter duration of action than digitoxin or acetyldigitoxin. Acetyldigoxin was isolated directly from *Digitalis lanata* by Hopponen and Gisvold (2) in 1952, and its good oral absorption properties in cats were first reported by White and Gisvold (3).

It now appears that the physical properties of the monoethylidene derivative of digoxin, *i.e.*, R_f value measuring liposolubility, should enhance its degree of absorption upon oral administration. Its toxicity and duration of action would depend in part upon its distribution and metabolism *in vivo*. This derivative is sensitive to hydrolysis at condition below pH 7 but is quite stable above pH 7. Dioxolanes are usually stable *in vivo* and thus such derivatives of the cardiac glycosides might exert some interesting effects upon their metabolism *in vivo*. Increased stability without a significant increase in stereochemical bulk would be effected by the substitution of F for H in the ethylidene residue. Such investigations are now in progress.

EXPERIMENTAL

The details of some of the paper chromatographic techniques used in these studies have been previously described (4). Thin-layer

[1] Marketed in Europe as Novadigal and Lanatilin.

(4) R. N. Icke, B. B. Wisegarver, and G. A. Alles, *Org. Syn.*, **25**, 89(1945).
(5) H. E. Baumgarten, *J. Amer. Chem. Soc.*, **84**, 4975(1962).
(6) J. C. Sheehan and I. Lengyel, *ibid.*, **86**, 1356(1964).
(7) J. C. Sheehan and J. J. Beeson, *ibid.*, **89**, 362(1967).
(8) H. E. Baumgarten, *ibid.*, **85**, 3303(1963).
(9) R. J. Wurtman and J. Axelrod, *J. Biochem. Pharmacol.*, **12**, 1439(1963).
(10) J. C. Towne, *Nature*, **201**, 709(1964).
(11) P. Truitt, E. E. Richardson, L. N. Long, and W. J. Middleton, *J. Amer. Chem. Soc.*, **71**, 3479(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 8, 1969, from the *Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication October 10, 1969.

Abstracted in part from a thesis submitted by J. K. Shields in partial fulfillment of Master of Science degree requirements.

* NDEA Title IV Fellow.

Some Cyclic Ketals and Acetals of Digitoxin, Digoxin, and Ouabain

KHALID S. ISHAQ and OLE GISVOLD

Abstract □ A new and milder method has been utilized to reinvestigate the preparation, properties, and biological activities of the mono-*o*-isopropylidene (acetonides) derivatives of digitoxin, digoxin, and ouabain. The preparation, properties, and biological activities of the mono-*o*-ethylidene derivatives of digitoxin and digoxin also are described. The latter derivatives show greater activity than the former.

Keyphrases □ Digitoxin, digoxin cyclic ketals, acetals—synthesis □ Ouabain cyclic ketals, acetals—synthesis □ Bioanalysis—digitoxin, digoxin, ouabain derivatives □ TLC, paper chromatography—separation, identification □ UV spectrophotometry—identity

In a previous publication the preparation and activities of the mono-*o*-isopropylidene derivatives (glycoside acetonides) of digitoxin, digoxin, and ouabain have been reported (1). Their biological activities were much less than had been expected on the basis of their physical properties and, in the cases of digitoxin and of digoxin, the position of the isopropylidene group. Because these activities were so unexpectedly low, a reinvestigation of the preparation of these derivatives by much milder methods was sought and one such was found. Acetonation can be effected in a very short time at room temperature *via* the use of 2,2-dimethoxypropane, acetone, a trace of acid, and room temperature conditions. The yields are almost quantitative and derivatives readily can be crystallized. Bioassay of these derivatives prepared by this new, very mild method gave comparable results in the case of digoxin but not in the case of digitoxin or ouabain. The ease with which the isopropylidene derivatives (dioxolanes) of digitoxin and digoxin could be prepared *via* the use of ketal-interchange techniques suggested the preparation of other cyclic ketals and cyclic acetals (dioxolanes) to test further the contribution to activity such derivatives might exert. Because the isopropylidene group, even though it was the simplest type of a cyclic ketal and on the terminal digitoxose residue, reduced activity so markedly that the preparation of a cyclic acetal as a second type of a dioxolane derivative was investigated. The ethylidene derivative readily could be prepared *via* 1,1-dimethoxyethane, acetaldehyde, a trace of acid, and room temperature conditions. Bioassays of these ethylidene derivatives *via* LD_{50} intravenous assays in cats showed that mono-*o*-ethylidene digoxin had the same activity as that of acetyldigoxin whereas acetyldigitoxin was 2.35 times as active as mono-*o*-ethylidene digitoxin. Thus the activity (toxicity) of digitoxin was reduced to a greater extent by both types of derivatives than was the case with digoxin. Also it is of interest to note that the mono-*o*-ethylidene digoxin activity was the same as that of acetyldigoxin.

In the case of digoxin, its usefulness is improved in its acetyl derivatives.[1] Acetyldigoxin is considered by some to be the drug of choice because it is well absorbed orally, is less toxic to the CNS, and has a shorter duration of action than digitoxin or acetyldigitoxin. Acetyldigoxin was isolated directly from *Digitalis lanata* by Hopponen and Gisvold (2) in 1952, and its good oral absorption properties in cats were first reported by White and Gisvold (3).

It now appears that the physical properties of the monoethylidene derivative of digoxin, *i.e.*, R_f value measuring liposolubility, should enhance its degree of absorption upon oral administration. Its toxicity and duration of action would depend in part upon its distribution and metabolism *in vivo*. This derivative is sensitive to hydrolysis at condition below pH 7 but is quite stable above pH 7. Dioxolanes are usually stable *in vivo* and thus such derivatives of the cardiac glycosides might exert some interesting effects upon their metabolism *in vivo*. Increased stability without a significant increase in stereochemical bulk would be effected by the substitution of F for H in the ethylidene residue. Such investigations are now in progress.

EXPERIMENTAL

The details of some of the paper chromatographic techniques used in these studies have been previously described (4). Thin-layer

[1] Marketed in Europe as Novadigal and Lanatilin.

chromatography (5) using silica gel and development with benzene–95% ethyl alcohol was used to good advantage. The Raymond reagent was used to detect the glycosides and their reaction products on both paper and silica gel.

Preparation of Isopropylidene Derivatives—The dry glycosides, digoxin, digitoxin, or ouabain (65 mg. each), were dissolved in 130, 65, and 60 ml., respectively, of dried acetone followed by the addition of 15 ml. of 2,2-dimethoxypropane. Dry hydrogen chloride, 12–14 bubbles, was added *via* a glass tube that had a 4-mm. internal diameter. The course of the reaction could be followed by the chromatographic examination of a few milliliters of the reaction mixture. At room temperature, 95 min., 90 min., and 27 hr., respectively, gave the optimum conversion to the desired derivative which was at least 90% in the case of digoxin and digitoxin and 80% plus in the case of ouabain. The remainder was the glycoside and a faster moving substance on TLC silica gel. Hydrogen chloride was chosen as the condensing agent because it readily could be removed under a vacuum on the Buchi during the removal of the acetone and excess 2,2-dimethoxypropane. This is a critical factor since nonvolatile acids such as *p*-toluene sulfonic acid, although effective as a catalyst, are troublesome to remove and may lead to some cleavage of the acid-sensitive dioxolane derivatives.

Although conversion to the desired isopropylidene derivative was obtained in very high yield, preparation of a highly purified analytically pure sample *via* direct crystallization was not readily possible because of the formation of isomorphous crystals. Chromatographic purification on 1-mm. silica gel plates using benzene–95% ethyl alcohol (7:3) yielded analytically pure samples. Such samples gave a positive Raymond test, negative periodate-benzidine test (6), and showed a UV $\lambda_{max.}$ at 218 mμ. Thus the lactone ring was intact and the isopropylidene residue was on the terminal digitoxose residue. The R_f value of these analytically pure samples was the same as that obtained from the initial acetonated glycoside product.

Mono-*o*-isopropylidene digoxin after recrystallization from benzene and naphtha (Skellysolve B) melted at 241–244.5° with decomposition.

Anal.—Calcd. for mono-*o*-isopropylidene digoxin, $C_{44}H_{68}O_{14}$: C, 64.36; H, 8.34. Found: C, 64.13; H, 8.54.

Mono-*o*-isopropylidene digitoxin after recrystallization from benzene and naphtha melted at 243.5–246.5° with decomposition.

Anal.—Calcd. for mono-*o*-isopropylidene digitoxin, $C_{44}H_{68}O_{13}$: C, 65.65; H, 8.50. Found: C, 65.43; H, 8.36.

Mono-*o*-isopropylidene ouabain after recrystallization from methanol, benzene, and naphtha melted at 221–223° with decomposition.

Anal.—Calcd. for mono-*o*-isopropylidene ouabain, $C_{32}H_{48}O_{12}$: C, 61.55; H, 7.74. Found: C, 61.50; H, 7.96.

Preparation of Ethylidene Derivatives—Dry digoxin, 80 mg., was dissolved with the aid of heat in 170 ml. of dried methylene chloride in a 250-ml. three-necked flask fitted with a condenser equipped with a drying tube. The flask was cooled to 5° in an ice bath and 5 ml. of 1,1-dimethoxyethane added. Dried and cooled acetaldehyde, 10 ml., was added *via* a syringe and finally dry hydrogen chloride, 33–55 bubbles, was introduced *via* a glass tube having an internal diameter of 4 mm. After 17 hr. the solvents were removed under vacuum on the Buchi. Analysis *via* TLC on silica gel using benzene–95% ethyl alcohol (7:3) showed the presence of some digoxin R_f value 0.50 and two very closely moving substances having an average R_f value of 0.67. The latter might well be two conformers or isomers at the 2-position of the dioxolane ring. Purification *via* thick-layer silica gel plates as described above gave a crystalline product after crystallization from methanol, benzene, and water that melted at 128–148°; $[\alpha]_D^{22}$ = +9.6 (C = 0.5 in methanol); $\lambda_{max.}$ at 218 mμ.

Anal.—Calcd. for mono-*o*-ethylidene digoxin, $C_{44}H_{66}O_{14}$: C, 63.99; H, 8.24. Found: C, 63.44; H, 8.53.

Because digitoxin was more soluble in methylene chloride, 80 mg. was dissolved in 40 ml. of methylene chloride and a 200-ml. three-necked flask was used. The remainder of the experimental conditions were the same as those used for the preparation of mono-*o*-ethylidene digoxin. The purified mono-*o*-ethylidene digitoxin was crystallized from methanol, benzene, and water. It melted at 113–127°, $[\alpha]_D^{22}$ = + 8.5 (C = 0.5 in methanol); $\lambda_{max.}$ at 218 mμ. Its R_f value on silica gel using benzene–95% ethyl alcohol (7:3) was 0.72. As in the case of the digoxin derivative, two very closely moving spots were obtained. Thus here also two conformers or isomers at the 2-position of the dioxolane ring are possible to account for the two very closely moving spots on TLC since the R_f value of digitoxin was 0.57.

Anal.—Calcd. for mono-*o*-ethylidene digitoxin, $C_{43}H_{66}O_{13}$: C, 65.29; H, 8.41. Found: C, 64.73; H, 8.62.

Bioassay[2] of the Glycoside Acetonides—The following mean LD_{50} ± *SE* mg./kg. was obtained: digoxin acetonide, 0.582 ± 0.04 (4 cats); digitoxin acetonide, 1.38 ± 0.03 (4 cats); ouabain acetonide, no minimum lethal dose could be reached (2 cats). Thus the digoxin acetonide was slightly more active than that previously reported whereas digitoxin acetonide was about 1.8 times as active as that previously reported. The inactivity of the ouabain acetonide differed from that previously reported which was 0.8837 ± 0.0714 (10 cats).

Bioassay[2] of the Glycoside Mono-*o*-ethylidene Derivatives—The following mean LD_{50} ± *SE* mg./kg. was obtained: mono-*o*-ethylidene digoxin, 0.375 ± 0.02 (4 cats), and mono-*o*-ethylidene digitoxin, 1.05 ± 0.06.

REFERENCES

(1) O. Gisvold, *J. Pharm. Sci.*, **55,** 1350(1966).

(2) R. Hopponen and O. Gisvold, *J. Amer. Pharm. Ass., Sci. Ed.*, **41,** 146(1952).

(3) W. F. White and O. Gisvold, *ibid.*, **41,** 42(1952).

(4) O. Gisvold, *ibid.*, **47,** 594(1958); **47,** 600(1958); O. Gisvold and S. E. Wright, *ibid.*, **46,** 535(1957).

(5) E. J. Johnston, *J. Pharm. Sci.*, **55,** 531(1966).

(6) J. M. Clark, Jr., "Experimental Biochemistry," Freeman, San Francisco, Calif., 1964, p. 213.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 21, 1969, from the *College of Pharmacy, Department of Medical Chemistry, University of Minnesota, Minneapolis, MN 55455*

Accepted for publication September 25, 1969.

This investigation was supported in part by research grant HE-06569 from the National Heart Institute, U. S. Public Health Service, Bethesda, Md.

[2] The authors wish to acknowledge the assistance of Dr. W. F. White and Dr. S. A. El-Refrawy in performing the bioassays of these derivatives.

Estimation of Steroid Solubility: Use of Fractional Molar Attraction Constants

J A. OSTRENGA and C. STEINMETZ

Abstract □ The fractional molar attraction constant (f_p), an empirical constant related to the solubility parameter, was defined and chosen to be related to the solubilizing capacity of solvents for a given steroid because it was thought to assess the relative polarity of solvent molecules. Experimental solubilities for two steroids are reported which indicate that f_p may be a useful parameter for solubility estimations.

Keyphrases □ Steroid solubility—estimation □ Fractional molar attraction constants—steroid solubility □ X-ray diffraction—identification □ Scintillometry—analysis

The solubility of a drug in one or more components of a pharmaceutical dosage form is valuable information which can be used to characterize its behavior in such a physical system. The solubility is usually not difficult to determine and often it is one of a few physical parameters readily available to the formulator which can aid him in the design of suitable dosage forms. This is particularly true in the case of topical dosage forms and where the solubility of the active ingredient can be related to phenomena such as thermodynamic activity, vehicle release rate, dissolution rate, and apparent penetration rate.

THEORETICAL

Methods or techniques for estimating or predicting the solubilities of drugs in various pharmaceutical solvents have obvious practical implications. One of the most useful concepts has been that of the solubility parameter (δ) as defined by Hildebrand and Scott (1) in Eq. 1 for nonelectrolytes:

$$\delta = (E/V)^{1/2} = (EV)^{1/2}/V = F/V \qquad \text{(Eq. 1)}$$

where E is the molar cohesive energy, V the molar volume, and F the molar attraction constant (2). It was originally derived for regular solutions ($\Delta H > 0$) where a good solvent for a given solute has a δ value close to that of the solute according to

$$\Delta G_m = V_m \phi_1 \phi_2 (\delta_1 - \delta_2)^2 - T\Delta S_m \qquad \text{(Eq. 2)}$$

where ΔG_m is the free energy of mixing, T the absolute temperature, S_m the entropy for mixing, V_m the volume of the mixture, and ϕ the volume fraction of component 1 or 2. This requirement corresponds to fulfilling the thermodynamic condition that ΔG_m be large and negative for good mixing. Burrell (3) has explored its potential for application in the paint and polymer coatings industry. The main limitation, however, has been that the solubility parameter concept is strictly applicable only to nonpolar systems where dispersion or London forces are predominant and other forces such as those related to hydrogen bonding and polarity are absent. In attempting to increase its applicability, these other forces and their effects have been considered by Burrell (4) and Gardon (5) and have proven to be worthwhile efforts.

In the case of steroids one encounters a somewhat specific solubility problem regarding prediction or estimation. The solubility parameter is not very useful for estimating the solubilizing capability of solvents for steroids because the circumstances are such that certain assumptions associated with δ are no longer valid; namely, that the solutes are crystalline, and specific interactions become highly significant in defining the solubility characteristics (both of these conditions tend to make $\Delta H < 0$). For steroids one finds that a proper hydrophilic-hydrophobic balance on the part of the solvent is associated with good solvent properties. That is, steroids are relatively insoluble in nonpolar solvents such as hexane and very polar solvents such as water but have appreciable solubility in solvents with partial polarity. This realization suggested that what is required is a parameter which assesses the fraction of the solvent molecule which can participate in the solubilization of a steroid and thereby hopefully place solvency on a common scale. This notion led to the defining of an empirical quantity called the fractional molar attraction constant (f_p). That is, since the presence of certain functional groups (*e.g.*, esters, ketones, ethers, alcohols) play a critical role in governing the solubility capacity of solvents for steroids and since apparently the relative abundancies of these groups are also important, the separation of δ into two components was considered according to Eqs. 3 and 4:

$$\delta = \delta_p + \delta_n = (F_p + F_n)/V \qquad \text{(Eq. 3)}$$

$$\delta_p = F_p/V;\ \delta_n = F_n/V \qquad \text{(Eq. 4)}$$

where δ_p and δ_n are the contributions made to δ by participating and nonparticipating functional groups, respectively. F_p and F_n are similarly defined for F; that is, F_p is the sum of F for all participating groups in the solvent molecule and F_n is the corresponding sum for all nonparticipating groups ($F = F_p + F_n$). The fractional molar attraction constant then was defined as in Eq. 5.

$$f_p = \delta_p/\delta = F_p/(F_p + F_n) = F_p/F \qquad \text{(Eq. 5)}$$

In order to demonstrate the possible usefulness of f_p, the solubilities of two steroids in various solvents were determined at 25°. The steroids employed in this report were fluocinolone acetonide[1] and fluocinolide.[2]

EXPERIMENTAL

Materials—Cellosolve acetate,[3] carbitol acetate,[3] *n*-butyl carbitol,[3] glyceryl triacetate,[3] methyl cellosolve acetate,[3] ethylene glycol diacetate,[3] isopropyl myristate,[4] cellosolve solvent,[3] methyl cellosolve,[3] diethylene glycol,[3] propylene glycol,[3] Ucar solvent LM,[3] 1,4-butanediol,[4] polyethylene glycol 400,[3] carbitol,[3] propylene carbonate,[5] toluene (scintillation grade),[6] PPO,[7] POPOP,[7] dioxane (scintillation grade),[6] naphthalene,[8] fluocinolone acetonide, and fluocinolide were used as received. All other materials were of analytical reagent grade. ^{14}C-labeled (acetonide label) steroids were used in all determinations and were provided by the Institute of Organic Chemistry, Syntex Research, Palo Alto, Calif. The radioactive purity was checked by developing a radioactive sample on a TLC plate with chloroform–methanol (95:5 for the acetate and 100:5 for the alcohol) and then scanning on a Vanguard model 880-D glass plate scanner. These radiochromatograms indicated that the purity was $\geq 98\%$.

Solubility Determinations—Solubilities were determined in the various solvents in duplicate by one of two methods. In the first method, an excess quantity of radioactive steroid with known spe-

[1] 6α, 9α-Difluoro-11β, 16α, 17α, 21-tetrahydroxy-1,4-diene-3,20-dione 16, 17-acetonide.

[2] 6α, 9α-Difluoro-11β, 16α, 17α, 21-tetrahydroxy-1, 4-diene-3,20-dione 16, 17-acetonide 21-acetate.

[3] Union Carbide Chemicals Co., New York, N. Y.

[4] General Aniline and Film Corp., New York, N. Y.

[5] Jefferson Chemical Co., Inc., Houston, Tex.

[6] Matheson, Coleman & Bell, Div. Matheson Co., Inc., Norwood, Ohio.

[7] Arapahoe Chemicals, Div. Syntex Corp., Boulder, Colo.

[8] Baker Analyzed Reagent Grade from J. T. Baker Chemical Co., Phillipsburg, N. J.

Table I—Solubilities of Phase I Fluocinolone Acetonide (Compd. *A*) and Fluocinolide (Compd. *B*) in Various Solvents at 25°

Solvent	f_p	Solubility (mg./ml.) at 25°: Compd. *A* Exp.	Compd. *A* Calcd.	Compd. *B* Exp.	Compd. *B* Calcd.
Isopropyl myristate	0.109	0.80[a]	0.71	0.14	0.16
n-Amyl acetate	0.244	8.65[a]	11.71	3.35	2.82
n-Butyl carbitol	0.289	51.84	21.09	5.95	5.14
Carbitol acetate	0.292	17.66	21.86	5.75	5.33
Cellosolve acetate	0.315	16.39	28.44	9.01	6.98
Polyethylene glycol 400	0.330	—	—	8.0[b]	8.23
Methyl cellosolve acetate	0.352	43.03	41.83	9.75	10.36
Ethyl acetate	0.356	20.77	43.51	7.80	10.78
Carbitol	0.364	—	—	14.0[b]	11.67
Cellosolve solvent	0.392	82.26	60.80	11.44	15.18
Ucar solvent LM	0.416	118.5	107.5	13.55	32.62
Methyl cellosolve	0.451	66.87	48.82	18.99	12.16
Propylene carbonate	0.460	—	—	14.0	9.55
Ethylene glycol diacetate	0.472	16.97	31.29	5.28	6.97
Glyceryl triacetate	0.498	24.42[a]	18.53	2.96	3.62
1,4-Butanediol	0.508	14.55[a]	15.26	—	—
Diethylene glycol	0.538	8.20[a]	8.71	1.95	1.41
Propylene glycol	0.595	—	—	0.69	0.41
Water	1.000	0.021	0.020	0.00053	0.00072

[a] The most stable phase is the clathrate; values for the solubility of Phase I were obtained by extrapolation (see text). [b] Values obtained by extrapolation to 100% of solvent in a plot of log solubility *versus* percent solvent in water.

cific activity was added to approximately 10 ml. of each solvent in a culture tube. The preparations were capped, sealed with rubber tape, and agitated on a Vibro-Mixer[9] at 25° in a thermostated bath until equilibrium was attained (2–5 days). All preparations were protected from the light. The only difference in the second method was that prior to agitation the preparations were heated to 60° to aid solubilization. Equivalent results were obtained by the two methods. Samples were withdrawn at appropriate times and passed through a suitable Gelman Metricel filter[10] (13 mm., 0.2μ). One milliliter of each filtrate was then analyzed on a liquid scintillation counter. Upon termination of the solubility experiments, the excess steroid was collected from each preparation by filtration and dried. X-ray powder diffraction patterns using copper K_α radiations on a Stoe Weissenberg goniometer system with a 57.3-mm. diameter camera were obtained on each dried sample as a means for identifying the polymorphic phase at equilibrium.

Radiochemical Assays—The specific activities of the radiochemicals were 20.7 mc./mmole for both steroids. Samples of the filtrates for each solvent were assayed for steroid utilizing a Nuclear Chicago Unilux II liquid scintillation counter. One-milliliter samples were mixed directly with either 15 ml. of scintillation fluid consisting of 13 g. of PPO (2,5-diphenyloxazol), 0.26 g. of dimethyl POPOP (1,4-bis-2-[4-methyl-5-phenyloxazolyl] benzene), 208 g. of naphthalene, 0.6 l. of methanol, 1 l. of dioxane, and 1 l. of toluene or 10 ml. of scintillation fluid consisting of 4 g. of POP, 0.1 g. of dimethyl POPOP, and 1 l. of toluene. The extent of quenching was determined from a channels ratio analysis utilizing a standard quench correction curve which included the correction factor for counter efficiency. The steroid content for each sample was calculated from the known specific activities and the disintegrations per unit time as obtained from the standard correction curve.

RESULTS

The solubilities of Phase I fluocinolone acetonide (*A*) and Phase I fluocinolide (*B*) at 25° in the various solvents are given in Table I and correspond to the mean value of two determinations. Solvents were chosen so as to give a wide range of f_p values. In the case of *B*, X-ray diffraction powder patterns of the steroid before and after equilibration indicated that no polymorphic conversion had occurred in any of the solvents. In the case of *A*, however, a polymorphic conversion to the clathrate was detected in five solvents (Table I). In these instances the solubilities for Phase I were obtained by extrapolation of a plot of solubility *versus* time (*t*) to $t = 0$ where such plots correspond to experiments employing pure Phase I as the starting material. Consequently, the reported values in these instances are approximations.

The f_p values for the various solvents in Table I were calculated according to the definition ($f_p = F_p/F = F_p/\delta V$). F_p values were calculated from structure alone by summing the *F* values corresponding to each participating group present in the solvent molecule (esters, ethers, alcohols). Such group *F* values for many functional groups have been reported by Small (6). It should be pointed out that for diols such as ethylene glycol, propylene glycol, *etc.*, the value of *F* for a single OH group is 275 as calculated from

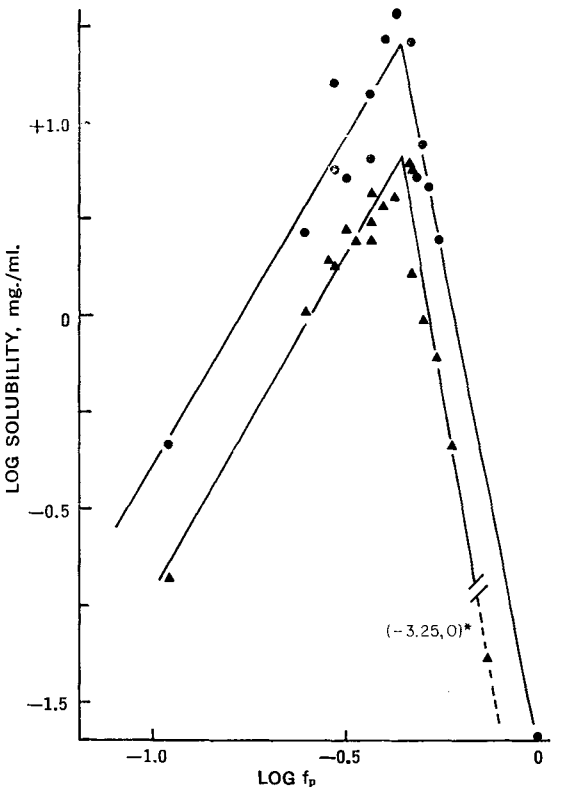


Figure 1—*Apparent linear relationship between log solubility and log* f_p *for fluocinolone acetonide* (●) *and fluocinolide* (▲) *in various solvents at 25°. * Coordinates for data point (water) which did not fit on axes.*

[9] Chemapec Corp., Hoboken, N. J.
[10] Gelman Instruments Inc., Ann Arbor, Mich.

$F = \delta V$ for the whole molecule and subtracting the reported values for the other groups, while for monofunctional alcohols such as carbitol, cellosolve, *etc.*, the corresponding value is 325 (7). To obtain $F = \delta V$, δ values taken from the technical literature of the supplier or from *Reference 2* were used and molar volumes ($V = \rho$/mol.wt.) were obtained utilizing densities (ρ) at 20° from the technical literature of the supplier or *Reference 8*. In a few instances where δ values were not available, F was calculated in the same manner as F_p. Fractional molar attraction constants so calculated are included in Table I for each solvent.

The solubility data for both steroids were found to be related to the calculated f_p values of the solvents and an optimum value of f_p was evident. For a log solubility–log f_p correlation the relationship appeared to exhibit linearity separately on each side of a maximum for both steroids. These empirical relationships are shown in Fig. 1 along with the calculated regression lines. Linear equations as determined by the method of least squares which best fit the plots in Fig. 1 are for *A*

	r	n	
$\log S = 3.474 \log f_p + 3.197$	0.939	9	(Eq. 6)
$\log S = -9.773 \log f_p - 1.691$	0.994	7	(Eq. 7)

and for *B*

$\log S = 3.554 \log f_p + 2.627$	0.989	10	(Eq. 8)
$\log S = -12.215 \log f_p - 3.139$	0.990	8	(Eq. 9)

where n is the number of data points. The correlation coefficients (r) indicated a high degree of linearity for the apparent relationships cited. Solubilities have been calculated employing Eqs. 6–9 and are included in Table I for comparison with the experimental values. A linear correlation between calculated and experimental values gave $r = 0.916$ at $p = 0.01$ for *A* and $r = 0.732$ at $p = 0.01$ for *B*. This result indicated that the relationship is significant but that much of the deviation is masked by the log–log relationship. A relationship between solubility for f_p is apparent, however, from linear plots. Perhaps a more meaningful and practical use of this relationship can be obtained by graphical estimation from linear plots and an empirical log–log relationship need not be forced or assumed.

Fractional molar attraction constants were defined with the intention of placing the solubilizing capability of solvents on a common scale in order hopefully to assess the importance of specific interactions and their relative abundance in various solvents. For strictly nonpolar solvents such as heptane, $F_p = 0$ and $f_p = 0$, while for a strictly polar solvent such as water, $F_p = F$ and $f_p = 1$. It can be shown that F values are additive on a molar basis (6, 9). They are at best only approximately additive on a constitutive and/or atomic basis but nonetheless the limited additivity of F can be useful in calculating the value of F for solvent molecules if certain limitations and rules are recognized (7). Equations 6–9 indicate the maximum solubility occurs at approximately the same solvent f_p value for *A* and *B* (0.428 and 0.431, respectively). Thus, the solvents studied are effective in solubilizing both steroids, the difference being the magnitude of their effect. An optimum in a plot of solubility *versus* f_p is in agreement with the notion that a proper hydrophobic–hydrophilic balance is required for good solvent properties.

The described techniques and relationships may be useful in estimating the solubilities of test compounds in additional solvents, once limited solubility data have been obtained. It should be pointed out that solubilities obtained utilizing these relationships can give predicted values which are substantially in error. Nonetheless, the relationships can be used to aid in the choice of solvents in appropriate situations and should find application in formulation work. In addition, it is anticipated that these relationships will be applicable to semisolid systems such as creams and ointments where solubility determinations are experimentally impractical or very difficult. Since the solubility of a topical drug in pharmaceutical solvents is necessarily required for the intelligent design of efficacious creams and ointments, additional methods for estimating solubility are an asset to the formulator.

REFERENCES

(1) J. H. Hildebrand and R. L. Scott, "The Solubility of Non-Electrolytes," 3rd ed., Dover Publications, New York, N. Y., 1964, p. 129.

(2) J. Brandrap and E. H. Immergut, "Polymer Handbook," Interscience, New York, N. Y., 1966, p. IV-341.

(3) H. Burrell, *Offic. Dig. Fed. Soc. Paint Technol.*, **27** (369), 726(1955).

(4) H. Burrell, *E.A T.I.P.E.C.*, **21** (1962).

(5) J. L. Gardon, *J. Paint Technol.*, **38,** 43(1966).

(6) P. A. Small, *J. Appl. Chem.*, **3,** 71(1953).

(7) J. A. Ostrenga, *J. Pharm. Sci.*, **58,** 1281(1969).

(8) C. D. Hodgman *et al.*, Eds., "Handbook of Chemistry and Physics," 44th ed., The Chemical Rubber Publishing Co., Cleveland, Ohio, 1961.

(9) J. A. Ostrenga, *J. Med. Chem.*, **12,** 349(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 13, 1969, from *Syntex Research, Institute of Pharmaceutical Sciences, Stanford Industrial Park, Palo Alto, CA 94304*

Accepted for publication October 10, 1969.

Stabilization of Homatropine Methylbromide–Iodobismuthate Complex in Solution for Quantitative Analysis of Homatropine Methylbromide

S. AHUJA, D. SPIEGEL, and F. R. BROFAZI

Abstract □ The colorimetric reaction between homatropine methylbromide (HMB) and the Dragendorff reagent was investigated as a means of quantitative analysis for HMB. The reaction was found to be unstable under normal laboratory lighting conditions. Stabilization of the colorimetric reaction was achieved by the addition of methanol to an acetone solution of the HMB–iodobismuthate complex in the ratio of 1:1. The stabilization was accompanied by a hypsochromic shift from 474 to 382 mμ. The stabilized colorimetric reaction follows the Beer–Lambert law in the range of concentrations of HMB studied for this investigation. The sensitivity of the method was found to be 0.1 mg. of HMB under the experimental conditions described. However, with slight modifications, this method should be useful for much smaller concentrations of HMB.

Keyphrases □ Homatropine methylbromide analysis—reaction mechanism □ Iodobismuthate complex stabilization—homatropine methylbromide analysis □ Colorimetric reaction—stabilization

For many years, the Dragendorff reagent has been used as a qualitative test reagent for alkaloids. However, it is only recently that the applications of this reagent for quantitative analysis have been reported (1–4). Pfordte (1) used the Dragendorff reagent, as modified by Trabert (5), in the paper chromatographic analysis of atropine, hyoscyamine, and scopolamine. The orange-colored alkaloid spot was eluted from the sprayed paper chromatographic strips with acetone, and the absorbance of this solution was measured at 400 mμ, because the maximum at 475 mμ faded quickly. Paris and Saint-Firmin (2, 3) employed a thin-layer chromatographic method instead of the paper chromatographic method for quantitative analysis of hyoscyamine and scopolamine bromide with the Dragendorff reagent, as modified by Trabert (5). They used 365 mμ as the analytical wavelength and noted that the colorimetric solutions were stable in the dark for 30 min. (2) to several days (3).

The present investigation was initiated with the objective of studying the colorimetric reaction of homatropine methylbromide and the Dragendorff reagent as a means of quantitative analysis for homatropine methylbromide (HMB). In the earlier stages of investigation, it was discovered that the colorimetric reaction was unstable, so efforts were made to stabilize the colorimetric reaction in order to achieve a more reliable method of analysis.

EXPERIMENTAL

Reagents—Acetone and methanol, spectroquality, Baker; homatropine methylbromide NF; 5% sulfuric acid; Dragendorff reagent, as modified by Munier and Macheboeuf (6). Ten milliliters of glacial acetic acid and 40 ml. of water are added to 850 mg. of bismuth subnitrate. To 40 ml. of this slurry, 40 ml. of 40% aqueous potassium iodide solution, 125 ml. of glacial acetic acid, and 250 ml. of water are added.

Table I—Stability of HMB–Iodobismuthate Complex in Solution

Solvent Acetone: Methanol	$\lambda_{max.}$ (mμ)	Absorbance at Indicated Time, min. 0	15	30	60
100:0	474	0.438	0.453	0.406	0.260
90:10	464	0.448	0.420	0.276	0.199
80:20	459	0.420	0.400	0.350	0.295
70:30	449	0.351	0.329	0.301	0.285
60:40	380	0.497	0.500	0.502	0.634
50:50	382	0.505	0.503	0.502	0.501
40:60	383	0.492	0.488	0.487	0.487
30:70	387	0.460	0.427	0.449	0.756

Method—An accurately weighed amount of homatropine methylbromide was dissolved in distilled water to yield a 0.25-mg./ml. solution. Five milliliters of this solution (equivalent to 1.25 mg. of homatropine methylbromide) was transferred to a 100-ml. beaker, and 2 drops of 5% sulfuric acid and 1 ml. of freshly prepared modified Dragendorff reagent were added. The resulting precipitate was allowed to stand for 1 hr. to facilitate filtration. (The precipitation is quantitative and cannot be readily extracted into any of the common immiscible organic solvents; therefore, filtration is used.) The precipitate was filtered through a fine-porosity sintered-glass funnel with the aid of vacuum. The precipitate was dried by suction and dissolved in acetone directly in the sintered-glass funnel and the solution was collected in a 50-ml. volumetric flask containing a known volume of methanol. The volume was made up with acetone. The resulting solutions were scanned with a recording spectrophotometer[1] in 1-cm. cells.

RESULTS AND DISCUSSION

The Dragendorff reagent, as modified by Munier and Macheboeuf (6), was used for this investigation. This reagent is commonly used in these laboratories as a spray reagent for TLC plates (7) and in that respect, it is similar to that used by previous workers (1–3).

The reaction product of an alkaloid and the Dragendorff reagent has been called alkaloid–iodobismuthate complex (1, 8, 9) and will be so referred to in this paper. The HMB–iodobismuthate complex is obtained as an orange precipitate when HMB is reacted with the Dragendorff reagent, as modified by Munier and Macheboeuf (6). This precipitate, when solubilized in acetone, yields an orange-colored solution with a maximum at 474 mμ. Due to some change in the iodobismuthate complex, the absorbance at 474 mμ decreases rapidly with a resulting shift of the maximum to 362.5 mμ. From investigations in these laboratories, the stability of the maximum at 362.5 mμ under regular laboratory lighting conditions, appears to be questionable. [The complex obtained with Dragendorff reagent, as modified by Trabert (5), was also found to be unstable.] Therefore, stabilization of the iodobismuthate complex was considered desirable so that quantitation could be achieved by the use of the complex itself rather than an unknown by-product.

Studies were conducted with several mixtures of solvents to determine a suitable solvent system which would offer greatest stability characteristics for the complex.

Stabilization of the HMB–iodobismuthate complex could be achieved by the addition of methanol to the acetone solution of the complex in the ratio of 1:1. The resulting 50% acetone–methanol

[1] Cary 15 recording spectrophotometer, Cary Instruments, Monrovia, Calif.

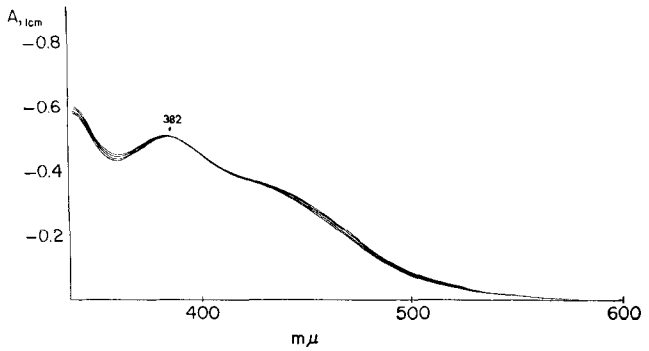


Figure 1—*Stability of homatropine methylbromide–iodobismuthate complex in 50% acetone–methanol solution (recorded at 15-min. intervals over a period of 1 hr.).*

solution was stable for at least 1 hr. in normal laboratory lighting conditions. This time period was considered adequate even if a large number of samples have to be analyzed at the same time; therefore, longer time periods were not checked.

The stabilization was accompanied by a hypsochromic shift from 474 to 382 mμ. Stabilization of the complex could be demonstrated by allowing the acetone–methanol solution to stand for half an hour, evaporating the solvents, dissolving the residue in acetone, and observing the return of the maximum at 474 mμ. This was not the case with solution of the complex in acetone alone, thereby suggesting a definite change in the complex. Table I shows the hypsochromic shifts caused by the addition of various amounts of methanol to the acetone solution of HMB–iodobismuthate complex. The stability of these solutions under normal laboratory lighting conditions, as indicated by change in absorbance readings at $\lambda_{max.}$, is also shown in Table I. (The reagent blank readings were found to be negligible.)

From these results, it is apparent that stabilization can be achieved when 50 to 60% methanol is present in the acetone–methanol solution with greatest stability being indicated for 50% methanol content. The stability of 50% acetone–methanol solution of the HMB–iodobismuthate complex over a 1-hr. period can be seen in Fig. 1. These data also show that greater sensitivity is obtained with 50% acetone–methanol solution. This was confirmed by studying acetone–methanol solutions of the HMB–iodobismuthate complex containing 55.0, 52.5, 47.5, and 45.0% of methanol. Therefore, it is logical to conclude that of the various acetone–methanol solutions investigated, 50% acetone–methanol solution offers the greatest stability and sensitivity for the quantitation of iodobismuthate complex.

Table II shows that the colorimetric reaction follows the Beer–Lambert law from 0.1 to 1.8-mg. concentrations of HMB. Higher concentrations than 1.8 mg. were not investigated. Lower workable limits were found to be 0.1 mg. under the experimental conditions described. However, with slight modifications such as final dilution volume and the size of the spectrophotometric cell, it should be possible to work with much smaller concentrations of HMB.

These investigations have shown that Dragendorff reagent, as modified by Munier and Macheboeuf (6), can prove to be a useful reagent for quantitative analysis of homatropine methylbromide. The colorimetric reaction is stable and sensitive to small concentrations of HMB. Furthermore, the colorimetric reaction should prove useful for quantitative analysis of other alkaloids and compounds reactive with the Dragendorff reagent.

Table II—Relationship between Concentration of HMB and Absorbance

Concentration of HMB, mg.	Absorbance at 382 mμ
0.1	0.035
0.2	0.070
0.4	0.152
0.6	0.245
1.0	0.390
1.2	0.480
1.4	0.540
1.6	0.600
1.8	0.680

CONCLUSIONS

The Dragendorff reagent, as modified by Munier and Macheboeuf (6), has been found to be a useful reagent for quantitative analysis of homatropine methylbromide. The resulting homatropine methylbromide–iodobismuthate complex in acetone solution has been stabilized by the addition of methanol in the ratio of 1:1. The colorimetric solution obtained has a maximum at 382 mμ and is stable for at least 1 hr. under normal laboratory lighting conditions. The colorimetric reaction follows the Beer–Lambert law down to a 0.1-mg. concentration of HMB, under the working conditions described in this publication. With small modifications, this reaction could become sensitive for much smaller concentrations of HMB. Furthermore, it may be logically concluded that this method should prove useful for chemical compounds reactive with the Dragendorff reagent.

REFERENCES

(1) K. Pfordte, *J. Chromatogr.*, **21,** 495(1966).

(2) R. R. Paris and A. R. Saint-Firmin, *Compt. Rend.*, **264,** 825(1967).

(3) A. R. Saint-Firmin and R. R. Paris, *J. Chromatogr.*, **31,** 252(1967).

(4) K. Blaim and R. Cizewska, *Chem. Anal. (Warsaw)*, **12,** 761(1967).

(5) V. H. Trabert, *Pharm. Zentralhalle*, **93,** 462(1954).

(6) R. Munier and M. Macheboeuf, *Bull. Soc. Chim. Biol.*, **33,** 846(1951).

(7) L. G. Chatten, "Pharmaceutical Chemistry," vol. 1, Marcel Dekker, New York, N. Y., 1966, p. 387.

(8) M. Francois and L. G. Blanc, *Compt. Rend.*, **175,** 273(1922).

(9) M. Francois and L. G. Blanc, *Bull. Soc. Chim.*, **33,** 640(1923).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 30, 1969, from *Geigy Chemical Corporation, Ardsley, NY 10502*

Accepted for publication October 8, 1969.

Effect of Heavy Water on Three High Producing Strains of *Penicillium chrysogenum*

KASHEED MOHAMMED, DANIEL A. NONA, and MARTIN I. BLAKE

Abstract □ Three high antibiotic-producing strains of *Penicillium chrysogenum* (Wisconsin Q176, 51-20F3, and 49-133) were examined for their ability to grow and produce penicillin in nutrient solutions containing pure heavy water. Penicillin titer values and pH changes were observed over the culture study period. Wisconsin Q176 responded most favorably. Continuous feeding of dextrose and potassium phenylacetate gave better antibiotic titers than a single addition of the two components. With either feeding regimen, potassium phenylacetate was more effective as a precursor than *N*-(β-hydroxyethyl)phenylacetamide or α-phenylacetamide. L-Leucine added together with potassium phenylacetate further enhanced penicillin production. The production of the yellow pigment associated with Wisconsin Q176 in protio nutrient cultures was suppressed in deuterium oxide nutrient cultures.

Keyphrases □ Deuterium oxide effect—*Penicillium chrysogenum* penicillin production □ Penicillin production, *P. chrysogenum*—deuterium oxide effect □ Media additives, effect—penicillin production □ Additives, single, multiple feedings—penicillin production, *P. chrysogenum*

Extensive studies have been reported in the literature pertaining to the effects of deuterium oxide on various organisms. In each case, deuterium oxide was substituted for water either partially or *in toto* in the nutrient medium. These investigations have been summarized by Flaumenhaft *et al.* (1) and by Katz (2), who thoroughly surveyed deuterium oxide effects on organisms.

Nona *et al.* (3) studied the effect of deuterium oxide on the culturing of *Penicillium janczewskii* and the antifungal activity of fully deuteriated griseofulvin. In an earlier report, Mohammed *et al.* (4) investigated the effect on the growth and antibiotic production of a number of low producing strains of *Penicillium*. Mycelial dry weights, pH variations, and penicillin potency over a 90-day study period were included in that study.

In the present study three high producing strains of *Penicillium*, the parent strain and two mutants, were cultured in water and deuterium oxide by a continuous feeding technique and by a single addition of dextrose and a precursor.

EXPERIMENTAL

Strains of *Penicillium chrysogenum*.—Three high producing strains of *Penicillium* were used in the present study: Wisconsin Q176[1] and two of its mutants, Wisconsin 51-20F3[2] and Wisconsin 49-133.[2] Two methods for preserving the stock cultures were utilized: on agar slants (4) at 5° and by lyophilization of healthy spores. The spores were suspended in 1.0 ml. of calf serum and the suspension was freeze-dried. The lyophil cultures were stored at 5°. Propagation of the spores from the lyophil tube was effected by first suspending the freeze-dried material in Sabouraud liquid medium. Agar slants were then prepared from this enriched medium. Fungal spores obtained from these slants were used for inoculation of the seed inoculum medium.

Preparation of the Seed Inoculum.—A standard salt mixture was used throughout this study. The composition of the mixture was (g./100 ml. of solvent): $NaNO_3$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.025; KH_2PO_4, 0.05; $ZnSO_4 \cdot 7H_2O$, 0.005; $CuSO_4 \cdot 5H_2O$, 0.0003; $FeSO_4 \cdot 7H_2O$, 0.005. To prepare the seed inoculum 7.0 g. of dextrose was added to the basic salt mixture. The pH of the solution was adjusted to 6.5 with 25% potassium hydroxide solution. Finally calcium carbonate, 1.2 g., was added. Ninety milliliters of this medium was placed in a 1-l. conical flask; the flask was stoppered with nonabsorbent cotton, capped with aluminum foil, and then sterilized. One loopful of spores obtained from an agar slant prepared as described under *Strains of Penicillium chrysogenum* was used to inoculate the flask. A thick vegetative growth was obtained within 48–54 hr. which was then used for inoculating the fermentation nutrient medium.

Protio and Deuterio Nutrient Media.—The protio and the deuterio fermentation media both had the same nutrient composition: the basic salt mixture as indicated under *Preparation of Seed Inoculum*, together with dextrose and a precursor, or a combination of precursors, as listed under *Procedures*. The protio nutrient medium and a deuterio nutrient medium contained distilled water and pure deuterium oxide (99.6%) as the solvents, respectively. However, in preparing the deuterio nutrient medium, the exchangeable hydrogen of the components was replaced with deuterium by dissolving the nutrient mixture in deuterium oxide and lyophilizing. This procedure was repeated to ensure complete exchange. Finally the pH was adjusted to 6.5 with 25% (w/v) potassium hydroxide in deuterium oxide, and 1.0 g. of calcium carbonate/100 ml. of solution was added. After sterilization, 10 ml. of inoculum was added to each flask/100 ml. of nutrient medium. In the case of the deuterio inoculation, the seed inoculum was filtered through a Seitz pressure filter, and the vegetative mycelial growth was washed with deuterium oxide under aseptic conditions prior to inoculation of the sterilized deuterio fermentation nutrient medium.

Procedures.—The sterilization techniques, the measurement of pH, and penicillin bioassay determinations have been described in an earlier paper (4). Preparation of the seed inoculum and all fermentations were conducted on an Eberbach rotary shaker at 210 r.p.m. Incubation temperature was kept at 26–28°, and the relative humidity was between 20 and 30%. In all cases, the pH of the fermentation medium was adjusted to 6.5 with 25% potassium hydroxide solution, and 1.0 g. of calcium carbonate was added/100 ml. of medium. One hundred milliliters of nutrient medium was added to a 3-l. conical flask; the flask was plugged with nonabsorbent cotton, capped with aluminum foil, and sterilized.

One series of experiments involved a single addition of dextrose and a precursor or precursors to the basic salt mixture prior to sterilization. The concentration of dextrose was 3.0%. The following precursors were studied (g./100 ml. of nutrient solution): potassium phenylacetate, 0.3; α-phenylacetamide, 0.3; *N*-(β-hydroxyethyl)phenylacetamide, 0.3; and L-leucine, 0.06.

In a second study, dextrose, 2.0 g., and potassium phenylacetate, 0.3 g., or *N*-(β-hydroxyethyl)phenylacetamide, 0.3 g., dissolved in 8.0 ml. of solvent were fed continuously to the basic salt mixture which contained 1.0% dextrose.

In a third experiment, a solution of dextrose containing 2.0 g. in 8.0 ml. of solvent was fed continuously to the basic salt mixture which contained, in addition to 1.0% dextrose, 0.3% α-phenylacetamide.

Following sterilization, the nutrient medium in each flask was inoculated with 10 ml. of seed inoculum. After fermentation had

[1] Obtained from Northern Utilization Research and Development Div., Peoria, IL 61604

[2] Supplied by Dr. J. F. Stauffer and Dr. M. P. Backus, Professors of Botany, University of Wisconsin, Madison, WI 53706

Table I—Penicillin Production, pH Plateau, and Time for Maximum Penicillin Production by *Penicillium chrysogenum* Strain, Wisconsin Q176

Additive	Peak Penicillin Production, u./ml.	Time for Maximum Production, days	pH Plateau
Potassium phenylacetate (H_2O)[a]	849	6.5	7.81–7.84
Potassium phenylacetate (D_2O)[a]	601	6.5	7.00–7.10
Potassium phenylacetate (H_2O)[b]	755	6.5	7.81–7.90
Potassium phenylacetate (D_2O)[b]	511	6.5	6.91–6.92
L-Leucine + Potassium phenylacetate (H_2O)[b]	896	7.5	7.82–7.91
L-Leucine + Potassium phenylacetate (D_2O)[b]	742	6.5	7.10–7.11
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[a]	812	7.5	7.79–7.84
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[a]	348	8.5	7.02–7.08
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[b]	751	7.5	7.89–7.95
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[b]	375	8.5	7.01–7.05
α-Phenylacetamide (H_2O)[a]	785	6.5	7.81–7.91
α-Phenylacetamide (D_2O)[a]	306	7.5	7.12–7.22
α-Phenylacetamide (H_2O)[b]	780	6.5	7.78–7.93
α-Phenylacetamide (D_2O)[b]	284	5.5	7.12–7.21

[a] Continuous feeding. [b] Single addition, prior to sterilization.

proceeded for 20–30 hr., the flasks containing 1.0% dextrose in the basic salt formula were fed continuously as indicated in the second and third experiments above. The pH of the continuous feed mixture was adjusted to 6.5 with 25% potassium hydroxide in water or deuterium oxide and sterilized separately in a 250-ml. conical flask. A 240-mm. length of rubber tubing was connected to the tip of a 10-ml. microburet equipped with a Teflon stopcock, and the top was plugged with nonabsorbent cotton. The apparatus was wrapped in aluminum foil and sterilized in a dry heat oven at 180° for 4–6 hr. A sterile transfer chamber was used for transferring the feed mixture to the buret. The rubber tubing from the buret was wrapped with the flask's sterile cotton plug and inserted into the flask in such a way as to have at least 100–120 mm. of the tubing extending inside the flask below the cotton plug. The culture nutrient flask was placed on the shaker with the buret fitted securely with a vinylized three-finger jaws clamp attached to a heavy-based buret stand. The solution was introduced at the rate of 0.08 ml. per hour and required 100 ± 4 hr. for the entire solution to be added. Triplicate flasks were prepared for each single addition and continuous feeding experiment.

RESULTS AND DISCUSSION

Growth Observation—The three strains of *Penicillium* studied grew well in the protio seed nutrient medium. Profuse growth was evident within 24 hr. The culture appeared very thick and gelatinous after 48 hr. Fermentation of the protio or deuterio culture medium which contained all the additives introduced at one time produced greater mycelial growth than the corresponding continuous slow feeding culture. Numerous globular pellets were produced in the protio fermentation cultures that had the nutrients added in a single addition. These pellets were tan in color and varied in size from 1 to 5 mm. in diameter. When deuterium oxide was used as the solvent, the organisms manifested morphological changes. Small spherical pellets appeared after 60–70 hr. of fermentation. These pellets were white to gray in color and varied in size from 1 to 3 mm. in diameter.

After 24 hr. fermentation, both the protio and deuterio cultures which contained an initial 1.0% dextrose in the basic salt mixture showed sparse pellet formation. These pellets varied in size from 1 to 4 mm. in diameter in the protio culture and 1 to 2 mm. in diameter in the deuterio culture. Continuous slow addition of the feed solution to either the protio or deuterio fermentation medium resulted in an enlargement rather than an increase in the number of pellets. After 150–160 hr., the increase in size of the pellets ranged from 4 to 8 mm. in diameter in the protio culture and 2 to 6 mm. in diameter in the deuterio culture.

A yellow pigment was secreted in all protio cultures fermented by Wisconsin Q176. This phenomenon characteristic of the strain was not observed in any of the deuterio fermentation cultures.

Penicillin Production—The three strains of *Penicillium* under study responded differently in protio and deuterio fermentation culture. Overall, penicillin production and the pH plateau were higher for the protio fermentation culture than for the corresponding deuterio fermentation culture.

Tables I, II, and III show penicillin production, pH plateau, and time for maximum penicillin production for strains Q176, 51-20F3, and 49–133. Continuous feeding of dextrose and potassium phenylacetate gave a higher penicillin assay than single addition. Soltero and Johnson (5) obtained increased penicillin titers by the continuous feeding of glucose or sucrose to the protio fermentation media. These workers used a single addition of lactose in the fermentation media as the control. In the present study, a repressive effect in penicillin yield was noted in all deuterio fermentation cultures. Similar observations were reported in an earlier paper (4). However, at that time, low antibiotic-producing strains of *Penicillium* were examined, and the additives were introduced in a single addi-

Table II—Penicillin Production, pH Plateau, and Time for Maximum Penicillin Production by *Penicillium chrysogenum* Strain, Wisconsin 51-20F3

Additive	Peak Penicillin Production, u./ml.	Time for Maximum Production, days	pH Plateau
Potassium phenylacetate (H_2O)[a]	1952	7.5	7.76–7.80
Potassium phenylacetate (D_2O)[a]	79	6.5	7.11–7.13
Potassium phenylacetate (H_2O)[b]	1352	6.5	7.63–7.65
Potassium phenylacetate (D_2O)[b]	21	6.5	7.11–7.15
L-Leucine + Potassium phenylacetate (H_2O)[b]	2173	7.5	7.87–7.91
L-Leucine + Potassium phenylacetate (D_2O)[b]	40	6.5	6.96–6.98
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[a]	1701	7.5	7.86–7.90
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[a]	79	6.5	6.94–6.97
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[b]	1607	6.5	7.89–7.91
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[b]	68	6.5	6.99–7.00
α-Phenylacetamide (H_2O)[a]	944	7.5	7.68–7.71
α-Phenylacetamide (D_2O)[a]	69	6.5	6.96–6.98
α-Phenylacetamide (H_2O)[b]	898	7.5	7.80–7.81
α-Phenylacetamide (D_2O)[b]	56	6.5	6.80–7.01

[a] Continuous feeding. [b] Single addition, prior to sterilization.

Table III—Penicillin Production, pH Plateau, and Time for Maximum Penicillin Production by *Penicillium chrysogenum* Strain, Wisconsin 49-133

Additive	Peak Penicillin Production, u./ml.	Time for Maximum Production, days	pH Plateau
Potassium phenylacetate (H_2O)[a]	1993	6.5	7.40–7.80
Potassium phenylacetate (D_2O)[a]	120	6.5	6.97–6.99
Potassium phenylacetate (H_2O)[b]	1372	6.5	7.19–7.32
Potassium phenylacetate (D_2O)[b]	20	6.5	7.00–7.02
L-Leucine + Potassium phenylacetate (H_2O)[b]	2397	6.5	7.86–7.91
L-Leucine + Potassium phenylacetate (D_2O)[b]	50	7.5	7.02–7.04
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[a]	1731	7.5	7.28–7.80
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[a]	80	7.5	6.98–7.00
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[b]	1545	7.5	7.76–7.80
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[b]	71	6.5	6.98–6.99
α-Phenylacetamide (H_2O)[a]	1164	7.5	7.69–7.74
α-Phenylacetamide (D_2O)[a]	112	7.5	7.07–7.10
α-Phenylacetamide (H_2O)[b]	1101	7.5	7.68–7.73
α-Phenylacetamide (D_2O)[b]	39	6.5	6.93–7.48

[a] Continuous feeding. [b] Single addition, prior to sterilization.

tion. Table I shows that continuous feeding of dextrose and potassium phenylacetate to the deuterio fermentation medium gave higher penicillin production than a single addition, 601 u./ml. *versus* 511 u./ml. In the corresponding deuterio experiments with mutant strains 51-20F3 and 49–133, the penicillin titers were drastically reduced. The two mutants gave higher penicillin titers than the parent strain (Q176) in protio fermentation culture. In deuterio cultures, the parent strain yielded more penicillin than the mutants. Christensen (6) indicated that the virulence of mutants placed in a different environment may equal, exceed, or be less than the parent strain. Changes in the original characteristics of the mutants usually result in altered production of enzymes and organic acids.

L-Leucine significantly stimulated penicillin production in protio fermentation culture (Tables I, II, and III). In deuterio fermentation culture enriched with the amino acid, the stimulating effect in penicillin production did not occur with strains 51-20F3 and 49–133. Strain Q176 produced 742 u./ml. with L-leucine and 511 u./ml. without L-leucine. This represents an increase of 45.2%. Foster (7) explained that leucine served as a precursor for the azolactone moiety of the penicillin molecule.

Continuous feeding or a single addition of dextrose and *N*-(β-hydroxyethyl)phenylacetamide in protio culture showed relatively high penicillin titers when fermented with either of the three strains of *Penicillium* (Tables I, II, and III). Penicillin production in the related deuterio fermentation culture with strains 51-20F3 and 49–133 (Tables II and III) showed about a twentyfold reduction in antibiotic production and about a twofold reduction with strain Q176 (Table I).

Dextrose administered by either feeding regime to α-phenylacetamide-enriched protio culture did not greatly affect antibiotic production. In deuterio fermentation culture, Wisconsin Q176 (Table I) gave higher penicillin titers than the other two strains.

Tables II and III show that strains 51–20F3 and 49–133 were limited in their capacity to produce antibiotic in a D_2O environment. This severe reduction in antibiotic production in each case is apparently related to a low pH plateau. With these two mutants, it appears higher pH plateaus are necessary to activate the enzyme system required for penicillin biosynthesis. The parent strain Q176 consistently maintained a higher pH plateau and higher penicillin titers in each deuterio fermentation culture studied.

Table I indicates that in deuterio fermentation culture, addition of the precursor potassium phenylacetate gave the highest penicillin titers among the precursors added singly. A combination of L-leucine and potassium phenylacetate further enhanced penicillin production. In deuterio fermentation culture (Tables II and III), severe repression in antibiotic production was evident with each precursor investigated. However, no significant differences were observed in the quantities of penicillin produced. Apparently the amount of penicillin produced in deuterio fermentation culture is not as dependent on the type of precursor used as on the strain of *Penicillium*.

REFERENCES

(1) E. Flaumenhaft, S. Bose, H. L. Crespi, and J. J. Katz, *Intern. Rev. Cytol.*, **18**, 313(1965).

(2) J. J. Katz, "Chemical and Biological Studies with Deuterium," 39th Annual Priestley Lecture Series, Pennsylvania State University, State College, Pa.

(3) D. A. Nona, M. I. Blake, H. L. Crespi, and J. J. Katz, *J. Pharm. Sci.*, **57**, 1993(1968).

(4) K. Mohammed, D. A. Nona, and M. I. Blake, *ibid.*, **57**, 2049(1968).

(5) F. V. Soltero and M. J. Johnson, *Appl. Microbiol.*, **2**, 41 (1954).

(6) J. J. Christensen, *Publ. Amer. Ass. Advan. Sci.*, **12**, 77(1940).

(7) J. W. Foster, "Chemical Activities of Fungi," Academic, New York, N. Y., 1949, p. 589.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Department of Pharmacy, University of Illinois, Medical Center, Chicago, IL 60612*

Accepted for publication September 30, 1969.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported by grant AI 06825-03 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Certain Disubstituted *o*-Aminoacetoxy- and Propoxybenzoic and Cinnamic Acids and their *tert*-Butyl Esters

ALEX GRINGAUZ

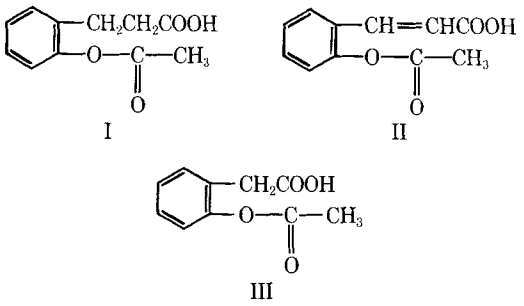
Abstract □ Several *o*-aminoacetoxy- and propoxy derivatives of benzoic and cinnamic acids as well as certain of their *tert*-butyl esters have been prepared as potential analgesics and local anesthetics. The syntheses and biological evaluation of representative compounds are presented.

Keyphrases □ Analgesic properties—derivatives of benzoic, cinnamic acids, esters □ Anesthetic properties—derivatives of benzoic, cinnamic acids, esters □ Benzoic acid, esters (derivatives)—synthesis, biological evaluation □ Cinnamic acid, esters (derivatives)—synthesis, biological evaluation □ IR—analysis □ Bradykinin-induced writhing test—biological analgetic screening

The objective of this work was to synthesize several types of compounds structurally related to acetylsalicylic acid in the hope of obtaining a drug which would overcome some of the disadvantages of aspirin. Ideally the desired agent should have analgesic-antipyretic activity equal to or exceeding that of aspirin. It should have a longer duration of action, possibly as a result of greater stability toward hydrolytic degradation both *in vivo* and *in vitro;* this would make liquid dosage forms possible. It should also exhibit good aqueous solubility. It is also desirable that such a compound produce no, or at least greatly reduced, gastric irritation, ulceration, or bleeding.

DISCUSSION

o-Acetoxyphenylpropionic acid (I) was reported (1) to have analgesic-antipyretic properties similar to aspirin. A recent report (2), however, could not verify this potency. Molecular models indicate that the *trans* isomer of II should be incapable of intramolecularly catalyzed hydrolysis and therefore be more stable. Of the two vinylogs of aspirin (II), only the *cis* isomer showed activity (2). For similar reasons the preparation of an acetyl derivative of *o*-hydroxyphenylpropiolic acid was attempted. Synthesis of this compound has thus far been unsuccessful. *o*-Acetoxyphenylacetic acid (III), the first homolog of aspirin, was recently prepared (2) and found to lack significant activity.

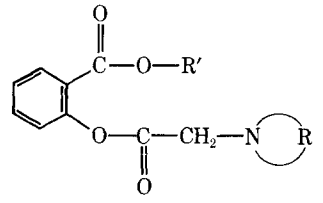


I

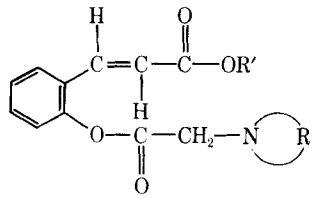
II

III

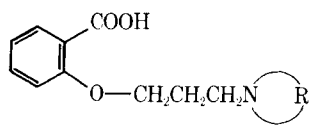
Compounds of Type IV (R′ = H) were synthesized (3) but failed to show pharmacological activity of any significance. Their hydrolytic decomposition rate was considerably greater than aspirin (5–42 times). Since acid functions such as carboxyl groups tend to sharply lower biological potency (4), several esters of IV (R′ = *tert*-butyl) were prepared. Two such esters showed relatively good resistance to *in vitro* hydrolysis (3). This factor alone could alter the absorption and distribution picture in the animal body—and thus possibly its pharmacology. The vinology principle (5) was the rationale for the preparation of several cinnamates V (R′ = H and *tert*-butyl).



IV



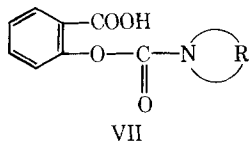
V



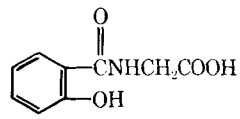
VI

p-Methoxyacetylsalicylic acid has good antipyretic activity (6). *o*-Methoxybenzoic acid also exhibits some activity (7). More recently, it was found (8) that 2-(3-carboxy-4-methoxyphenyl)alanine, a precursor to "phenylalanine aspirin," possessed good analgesic activity. It was therefore deemed of interest to prepare compounds of the *o*-aminopropoxybenzoic acid type (VI) to determine the presence of biological activity. The one member of this series tested for analgesia (XXIV) was, however, devoid of this property.

The synthesis of salicylic acid carbamates of Type VII was unsuccessful (see *Chemistry*). Salicyluric acid (VIII) being the main metabolite of both aspirin and salicylic acid appeared to offer interesting possibilities as a structural moiety. Analgesic-antipyretic activity for the 4-nitro derivative has been claimed (9). Attempts to prepare *o*-aminoacetoxy derivatives were undertaken but were unsuccessful.



VII

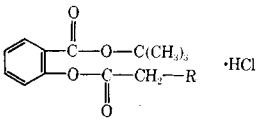


VIII

CHEMISTRY

tert-Butyl aminoacetoxysalicylates (IV) were synthesized by acylation of *tert*-butyl salicylate with bromoacetyl chloride followed by treatment with the appropriate secondary amine. *o*-Coumaric acid was converted to *o*-chloroacetoxycinnamic acid (XVIII) by reaction with chloroacetic anhydride and sodium chloroacetate. Conversion of XVIII to its *tert*-butyl ester (XIX) was accomplished in poor yields by either treatment with isobutylene and H_2SO_4 (9%) or by conversion to the acid chloride with $SOCl_2$ followed by treatment with *tert*-butyl alcohol and pyridine (13%). Reaction of XIX with NaI in acetone followed by the desired amine afforded

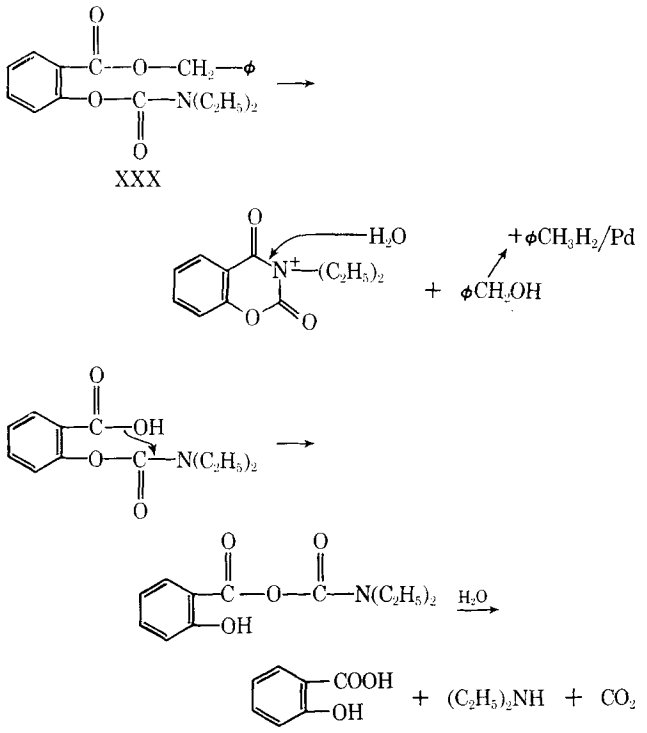
Table I—Disubstituted *tert*-Butyl Aminoacetoxysalicylate Hydrochlorides



Compd.	R	Recrystn.[a] Solvent	M.p. °C.	Anal., % Calcd.	Found
XI	Thiamorpholino	A	177–178[b]	C, 54.60 H, 6.47 N, 3.75	C, 54.49 H, 6.37 N, 3.77[d]
XII	3-Pyrrolino	A	158–159[b]	C, 60.11 H, 6.53 N, 4.12	C, 62.46 H, 6.49 N, 4.28
XIII	Diallylamino	B	141–143[b]	C, 62.03 H, 7.12 N, 3.81	C, 61.42 H, 7.07 N, 3.86
XIV	*cis*-2,6-Dimethylpiperidino	C	186–187[b]	C, 62.55 H, 7.88 Cl, 9.24	C, 62.46 H, 7.73 Cl, 8.92
XV	*cis*-2,5-Dimethylpyrrolidino	C	170–172[b]	C, 61.69 H, 7.63 Cl, 9.59	C, 61.70 H, 7.70 Cl, 9.20
XVI	3-Azabicyclo-[3.2.2]-nonano	D	174–175[b,c]	C, 63.70 H, 7.64 N, 3.54	C, 63.81 H, 7.85 N, 3.43

[a] A = ethyl acetate–ethanol; B = ethanol (95%); C = ethyl acetate–methanol; D = ethyl acetate. [b] Melted with decomposition. [c] Sealed capillary. [d] Sulfur analysis: Calcd. 8.57. Found: 8.55.

V (R′ = *tert*-butyl) as the free base. Conversion to the hydrochloride salt resulted in cleavage of the *tert*-butyl group in at least one instance (see *Experimental*) yielding the aminoacid hydrochloride V (R′ = H). Methyl *o*-(3-bromopropoxy)benzoate (XXIII) was prepared by treatment of methyl salicylate with $NaOCH_3$ and refluxing the product with 1,3-dibromopropane. Reaction of XXIII with the appropriate amine followed by acid hydrolysis of the methyl ester gave VI as the hydrochloride salt. Alternately, VI could be prepared by reaction of methyl salicylate (as the Na salt) with dialkylaminopropyl chloride followed by hydrolysis of the methyl ester.



Scheme I

The carbamate VII (R = diethyl) was attempted but did not lead to the desired product. Benzyl diethylcarbamoylsalicylate (XXX) was synthesized by treating benzyl salicylate with phosgene and reacting the resulting chloroformate with diethylamine. Alternately XXX was also prepared by direct reaction with diethylcarbamoyl chloride. Reductive debenzylation of XXX, however, did not afford VII; salicylic acid was the isolable product. A possible mechanism for this unexpected result is proposed (Scheme I).

EXPERIMENTAL

All melting and boiling points are uncorrected; melting points were determined in capillary tubes. Infrared spectra were obtained in KBr pellets on a Perkin-Elmer 337 instrument. Elemental analyses were done by Smith, Kline & French Laboratories and Alfred Bernhardt, Max-Planck Institute, West Germany. Animal testing was carried out by the Pharmacology Department, Menley & James Laboratories.

***tert*-Butyl Dialkyl or Cycloalkylaminoacetoxysalicylate Hydrochlorides**—tert-*Butyl Salicylate* (*IX*)—This was prepared in 40–50% yield by either treatment of salicyloyl chloride with *tert*-butyl alcohol as described by Cwalina and Gringauz (3) or by H_2SO_4 catalyzed reaction of salicylic acid with excess isobutylene according to McCloskey and Fonken (10).

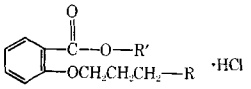
tert-*Butyl Bromoacetoxysalicylate* (*X*)—This was prepared in 68% yield from IX and bromoacetyl chloride according to Cwalina and Gringauz (3).

tert-*Butyl Thiamorpholinoacetoxysalicylate Hydrochloride* (*XI*)—To a solution of 8.10 g. (0.0257 mole) of X in 50 ml. absolute ether there was added dropwise and with stirring 5.15 g. (0.0514 mole) of thiamorpholine[1] in 10 ml. of dry ether. The stirred mixture was refluxed for 6 hr. and filtered. Addition of ethereal HCl precipitated 6.0 g. (62.5%) of the desired salt. Repeated recrystallizations from ethyl acetate–ethanol (70:30) gave m.p. 177–178° dec. The free base could also be obtained in 53% yield, m.p. 83–90° (crude). The five additional analogs similarly prepared are listed in Table I.

***o*-Disubstituted Alkylaminoacetoxycinnamic Acid Hydrochloride**—o-*Coumaric Acid* (*XVII*)—This was prepared in 75–80% yield from coumarin by treatment with aqueous NaOH and yellow HgO according to Seshardi and Rao (11).

[1] A sample of thiamorpholine was supplied by Dr. W. Horrom of Abbott Laboratories, N. Chicago, Ill.

Table II—Disubstituted *o*-3-Aminopropoxybenzoates and Benzoic Acid Hydrochlorides



Compd.	R′	R	Method[a]	M.p. °C.[b]	Anal., % Calcd.	Found
XXIV	CH_3	Diethylamino	A and B	86–88	C, 59.69 H, 8.02	C, 59.79 H, 8.08
XXV	H	Diethylamino		135–137	C, 58.43 H, 7.71	C, 58.19 H, 7.68
XXVI	CH_3	Pyrrolidino	A	139.5–140	C, 60.10 H, 7.40	C, 60.16 H, 7.45
XXVII	H	Pyrrolidino		139–141	C, 58.84 H, 7.05	C, 59.08 H, 7.04
XXVIII	CH_3	Dimethylamino	B	[c]	C, 57.04 H, 7.36	C, — H, —
XXIX	H	Dimethylamino		[d]	C, 55.49 H, 6.99	C, 55.62 H, 7.52

[a] Methods A and B as described in *Experimental* section. [b] Solvent of recrystallization was ethyl acetate–2-propanol, 80:20 for the methyl esters, 50:50 for the acids. [c] Could not be crystallized. [d] Extremely hygroscopic; no valid m.p. was obtainable.

o-*Chloroacetoxycinnamic Acid* (*XVIII*)—A mixture of 50.0 g. (0.302 mole) XVII, 102.5 g. (0.60 mole) of freshly distilled chloroacetic anhydride and 35.6 g. (0.302 mole) of sodium chloroacetate was stirred 15 hr. at 60°. The resultant melt was poured into ice water and stirred until solidification. The crude product obtained was air dried and extracted from unreacted XVII with CH_2Cl_2. Treatment with charcoal and evaporation of solvent afforded 41.0 g. (56.5%) of product which, after recrystallization from benzene–ether melted at 135.0–137.5°.

Anal.—Calcd. for $C_{11}H_9ClO_4$: C, 54.90; H, 3.77; Cl, 14.74. Found: C, 55.16; H, 3.77; Cl, 14.68.

tert-*Butyl* o-*Chloroacetoxycinnamate* (*XIX*)—Direct reaction of XVIII with isobutylene and H_2SO_4 according to McCloskey and Fonken (10) gave a crude yield of 9% with accompanying recovery of XVII and XVIII. A somewhat better yield was obtained by the dropwise addition, at room temperature, of the acid chloride prepared from 34.5 g. (0.143 mole) of XVIII with $SOCl_2$ in dry benzene to a stirred solution of 10.6 g. (0.143 mole) *tert*-butyl alcohol and 11.3 g. (0.143 mole) pyridine. After 6 hr. the mixture was poured into ice water and extracted with ether. Evaporation of the combined, dried (Na_2SO_4) ether extracts afforded a dark oil. Repeated extractions with boiling petroleum ether (30–60°) yielded, on cooling, 5.6 g. (13.3%) of product. Repeated recrystallization from the above solvent gave a m.p. 55–57°.

Anal.—Calcd. for $C_{15}H_{17}ClO_4$: C, 60.71; H, 5.78; Cl, 11.95. Found: C, 60.68; H, 5.75; Cl, 12.06.

tert-*Butyl* o-*Morpholinoacetoxycinnamate Hydrochloride* (*XX*)—To a solution of 4.4 g. (0.00149 mole) XIX in 50 ml. of N_2-purged acetone there was added 2.22 g. NaI similarly dissolved and the mixture refluxed several hours under N_2. The resulting product was treated with 2.58 g. (0.0298 mole) of morpholine in 100 ml. absolute ether and refluxed an additional 3 hr. After filtering the precipitate the residue was treated with HCl gas to yield, after crystallization from 2-propanol, 3.71 g. (65%) of product. Four recrystallizations gave a m.p. 165–167°.

Anal.—Calcd. for $C_{19}H_{26}ClNO_5$: C, 59.47; H, 6.83; Cl, 9.24; N, 3.65. Found: C, 59.00; H, 6.87; Cl, 9.12; N, 3.57.

tert-*Butyl* o-*3-Azabicyclo*-[*3.2.2*]-*nonan-3-ylacetoxycinnamate Hydrochloride* (*XXI*)—This was similarly prepared and recrystallized from ethyl acetate–2-propanol, m.p. 146–147°.

Anal.—Calcd. for $C_{23}H_{32}ClNO_4 \cdot H_2O$: C, 62.85; H, 7.78; N, 3.18. Found: C, 62.70; H, 7.75; N, 3.18.

o-*Diethylaminoacetoxycinnamic Acid Hydrochloride* (*XXII*)—This compound was obtained in 67% yield from XIX and diethylamine. Attempted conversion of the *tert*-butyl ester to its hydrochloride apparently cleaved the ester[2] directly to the free carboxyl group. Repeated recrystallization from 2-propanol gave a m.p. 183–185° dec.

Anal.—Calcd. for $C_{15}H_{20}ClNO_4$: C, 57.41; H, 6.43; N, 4.47 Found: C, 57.59; H, 6.52; N, 4.49.

o-Disubstituted Alkylaminopropoxybenzoic Acid Hydrochlorides—*Methyl* o-*3-Bromopropoxybenzoate* (*XXIII*)—To 152 g. (1 mole) methyl salicylate there was added a freshly prepared solution of 23.0 g. (1 mole) Na in 400 ml. absolute methanol. After the addition of 487 g. (2.4 moles) of 1,3-dibromopropane, 300 ml. xylene, and 100 ml. dimethylformamide, the methanol was distilled off and the remaining mixture refluxed 14 hr. Following workup with water, the mixture was washed with 5% NaOH, saturated NaCl, and dried (Na_2SO_4). Evaporation of the solvent *in vacuo* gave 118 g. (43%) of an oil, b.p. 135–136° (1.2 mm.), n_D^{25} 1.5381, d_4^{25} 1.363.

Methyl o-*3-Diethylaminopropoxybenzoate* (*XXIV*)—*Method A*—To 13.65 g. (0.05 mole) XXIII dissolved in absolute ether there was added 7.31 g. (0.10 mole) diethylamine and the mixture refluxed 18 hr. Filtration of the precipitate, followed by removal of solvent gave 8.3 g. (62.7%) of an oil, b.p. 120–124° (2 mm.), n_D^{30} 1.5094. Treatment with ethereal HCl gave the hydrochloride, m.p. 85–86° (ethyl acetate–2-propanol).

Method B—To a suspension of 0.25 mole of sodium methyl salicylate (prepared as above) in 250 ml. of toluene there was added 37.2 g. (0.25 mole) of 3-diethylaminopropyl chloride and the mixture refluxed 20 hr. Workup as above afforded 14.2 g. (22%) XXIV, b.p. 140–145° (2.5 mm.), n_D^{30} 1.5038. The hydrochloride melted at 86–88°. A mixed melting point with the product obtained by Method A gave no depression. The IR spectra of both salts were identical.

Anal.—Calcd. for $C_{15}H_{24}ClNO_3$: C, 59.69; H, 8.02. Found: C, 59.79, H, 8.08.

o-*3-Diethylaminopropoxybenzoic Acid Hydrochloride* (*XXV*)—Refluxing 10.9 g. (0.0361 mole) XXIV with 5 ml. concentrated HCl and 40 ml. H_2O for 4 hr. gave, after evaporation of the solvent *in vacuo*, an oil. Cooling and repeated scratching with absolute ether afforded 9.1 g. (88%) of product. Three recrystallizations from ethyl acetate–2-propanol (50:50) gave a m.p. 135–137°.

Anal.—Calcd. for $C_{14}H_{22}ClNO_3$: C, 58.43; H, 7.71. Found: C, 58.19; H, 7.68.

Additional analogs of XXIV and XXV were similarly prepared and are listed in Table II.

PHARMACOLOGY

Analgetic screening was performed using the modified bradykinin induced writhing test in mice (12). The compounds were administered at doses equivalent to the ED_{50}[3] of aspirin, on a molecular weight basis, in a volume of 10 ml./kg. Ten mice per group were used for each compound and three groups of controls received the appropriate vehicle. Compounds XI, XV, XVI, XX, XXI, XXII,

[2] Cwalina and Gringauz (*Reference 3*) have shown the ease with which similar *tert*-butyl esters are cleaved under analogous conditions.

[3] The dose which produces analgesia in 50% of the animals tested.

and XXV were tested. Only XI exhibited any activity. When compared to aspirin this activity was insignificant.

Local anesthetic activity was assayed as the ability of the test agent to inhibit the blink reflex in the rabbit when the cornea was lightly stimulated. Butacaine sulfate (Butyn sulfate) was used as a control local anesthetic. Compounds XI, XV, XVI, XX, and XXI were tested and found to have no local anesthetic activity.

REFERENCES

(1) C. W. Bauer and E. F. Lasala, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 48(1960).

(2) A. Gringauz, *J. Med. Chem.*, **11**, 611(1968).

(3) G. E. Cwalina and A. Gringauz, *J. Org. Chem.*, **26**, 3344 (1961).

(4) "Medicinal Chemistry," 2nd ed., A. Burger, Ed., Interscience, New York, N. Y., 1960, p. 43.

(5) R. C. Fuson, *Chem. Rev.*, **16**, 1(1934).

(6) F. Schönohöfer, *Chem. Ber.*, **84**, 13(1951).

(7) "Mays Chemistry of Synthetic Drugs," 5th ed., Longmans, London, England, 1959, p. 41.

(8) F. Leonard, *J. Med. Chem.*, **8**, 813(1965).

(9) J. Parrod and J. Cottet, French pat. 1,068,636(1954).

(10) A. L. McCloskey and G. S. Fonken, "Organic Syntheses," vol. IV, Wiley, New York, N. Y., 1963, p. 261.

(11) T. S. Seshardi and P. S. Rao, *Proc. Indian Acad. Sci.*, **3A**, 293(1936).

(12) R. B. P. Burns, N. Aliota, and K. E. Hurley, *Arch. Int. Pharmacodyn. Ther.*, **175**, 41(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 22, 1969, from the *Brooklyn College of Pharmacy, Long Island University, Brooklyn, NY 11126*

Accepted for publication November 6, 1969.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

The author wishes to express his gratitude to Menley & James Laboratories for their financial support and especially to Dr. Kenneth R. Heimlich of Smith, Kline & French Laboratories for his personal help and interest in this project. Grateful acknowledgement is also hereby made to Dr. Robert B. P. Burns for the pharmacologic data and pertinent suggestions.

COMMUNICATIONS

Evaluation of an Improved Heidelberg Telemetry Capsule for the Study of Antacids

Keyphrases ☐ Heidelberg FM-transmitting capsule—evaluation ☐ Gastric function assessment—Heidelberg telemetry capsule, evaluation

Sir:

Development of a telemetry system employing the Heidelberg FM-transmitting capsule has provided a convenient methodological approach to the clinical assessment of normal gastric function, as well as disease- and drug-induced alterations in gastric activity. The Heidelberg capsule, while elegant in concept, has been found lacking in dependability, a defect related possibly to transmitter construction. One factor involved in the lack of reliability may have been the relatively large hydrogen-ion sensor that permitted accumulation of particulate matter (*e.g.*, antacids) and mucous debris, thus interfering with normal operation. Furthermore, a relatively high percentage of the capsules did not exhibit a linear response throughout the functional range of pH 1 to 7 during *in vitro* standardization (1).

A modified Heidelberg probe,[1] having a smaller hydrogen-ion sensitive area, has recently been made available. These redesigned telemetry capsules have demonstrated a high degree of *in vitro* reliability with regard to linearity of response in the workable pH range of 1 to 7. The purpose of this study was to evaluate the *in vivo* performance of this modified device.

A FM-signal receiving unit,[1] in belt form, was positioned externally over the stomach area of healthy adult human subjects and connected to a recorder for continuous monitoring of transmitted pH values. Throughout the experimental period the subjects sat erect in an arm chair. Each telemetry capsule was activated by saturation of the hydrogen-ion sensitive end-plate with 0.9% sodium chloride solution, and calibrated in Beckman buffer solutions of pH 2 and 7 at 37°. The capsule was swallowed by the fasted subject, and gastric pH was monitored during the subsequent 50-min. period. After recording baseline pH values (3 to 10 min.), a specified dose of one of four commercial antacid preparations (designated A, B, C, and D) was administered with 30 ml. of water at room temperature: antacid A, 22 ml., suspension; B, 22 ml., suspension; C, 5 ml., suspension; D, two tablets. Each antacid preparation contained aluminum and magnesium hydroxides; formulation C also contained magnesium carbonate and methyl polysiloxane.

The apparent onset and duration of gastric acid buffering activity following administration of the three liquid and one solid antacid formulations are reported in Table I.

Although all of the "improved" Heidelberg capsules were apparently operative, as determined by *in vitro* calibration in buffer solutions and recording of pH signal immediately after swallowing the device, in approximately 14% of the trials (5 of 35 experiments) the anticipated elevation of gastric pH after administration of antacid was not perceived. In 86% of the trials the onset of buffering activity (*i.e.*, elevation of gastric pH above 3.0) following administration of antacid was

[1] Medintron Corp. of America, New York, N. Y.

and XXV were tested. Only XI exhibited any activity. When compared to aspirin this activity was insignificant.

Local anesthetic activity was assayed as the ability of the test agent to inhibit the blink reflex in the rabbit when the cornea was lightly stimulated. Butacaine sulfate (Butyn sulfate) was used as a control local anesthetic. Compounds XI, XV, XVI, XX, and XXI were tested and found to have no local anesthetic activity.

REFERENCES

(1) C. W. Bauer and E. F. Lasala, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 48(1960).

(2) A. Gringauz, *J. Med. Chem.*, **11**, 611(1968).

(3) G. E. Cwalina and A. Gringauz, *J. Org. Chem.*, **26**, 3344 (1961).

(4) "Medicinal Chemistry," 2nd ed., A. Burger, Ed., Interscience, New York, N. Y., 1960, p. 43.

(5) R. C. Fuson, *Chem. Rev.*, **16**, 1(1934).

(6) F. Schönohöfer, *Chem. Ber.*, **84**, 13(1951).

(7) "Mays Chemistry of Synthetic Drugs," 5th ed., Longmans, London, England, 1959, p. 41.

(8) F. Leonard, *J. Med. Chem.*, **8**, 813(1965).

(9) J. Parrod and J. Cottet, French pat. 1,068,636(1954).

(10) A. L. McCloskey and G. S. Fonken, "Organic Syntheses," vol. IV, Wiley, New York, N. Y., 1963, p. 261.

(11) T. S. Seshardi and P. S. Rao, *Proc. Indian Acad. Sci.*, **3A**, 293(1936).

(12) R. B. P. Burns, N. Aliota, and K. E. Hurley, *Arch. Int. Pharmacodyn. Ther.*, **175**, 41(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 22, 1969, from the *Brooklyn College of Pharmacy, Long Island University, Brooklyn, NY 11126*

Accepted for publication November 6, 1969.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

The author wishes to express his gratitude to Menley & James Laboratories for their financial support and especially to Dr. Kenneth R. Heimlich of Smith, Kline & French Laboratories for his personal help and interest in this project. Grateful acknowledgement is also hereby made to Dr. Robert B. P. Burns for the pharmacologic data and pertinent suggestions.

COMMUNICATIONS

Evaluation of an Improved Heidelberg Telemetry Capsule for the Study of Antacids

Keyphrases ☐ Heidelberg FM-transmitting capsule—evaluation ☐ Gastric function assessment—Heidelberg telemetry capsule, evaluation

Sir:

Development of a telemetry system employing the Heidelberg FM-transmitting capsule has provided a convenient methodological approach to the clinical assessment of normal gastric function, as well as disease- and drug-induced alterations in gastric activity. The Heidelberg capsule, while elegant in concept, has been found lacking in dependability, a defect related possibly to transmitter construction. One factor involved in the lack of reliability may have been the relatively large hydrogen-ion sensor that permitted accumulation of particulate matter (*e.g.*, antacids) and mucous debris, thus interfering with normal operation. Furthermore, a relatively high percentage of the capsules did not exhibit a linear response throughout the functional range of pH 1 to 7 during *in vitro* standardization (1).

A modified Heidelberg probe,[1] having a smaller hydrogen-ion sensitive area, has recently been made available. These redesigned telemetry capsules have demonstrated a high degree of *in vitro* reliability with regard to linearity of response in the workable pH range of 1 to 7. The purpose of this study was to evaluate the *in vivo* performance of this modified device.

A FM-signal receiving unit,[1] in belt form, was positioned externally over the stomach area of healthy adult human subjects and connected to a recorder for continuous monitoring of transmitted pH values. Throughout the experimental period the subjects sat erect in an arm chair. Each telemetry capsule was activated by saturation of the hydrogen-ion sensitive end-plate with 0.9% sodium chloride solution, and calibrated in Beckman buffer solutions of pH 2 and 7 at 37°. The capsule was swallowed by the fasted subject, and gastric pH was monitored during the subsequent 50-min. period. After recording baseline pH values (3 to 10 min.), a specified dose of one of four commercial antacid preparations (designated A, B, C, and D) was administered with 30 ml. of water at room temperature: antacid A, 22 ml., suspension; B, 22 ml., suspension; C, 5 ml., suspension; D, two tablets. Each antacid preparation contained aluminum and magnesium hydroxides; formulation C also contained magnesium carbonate and methyl polysiloxane.

The apparent onset and duration of gastric acid buffering activity following administration of the three liquid and one solid antacid formulations are reported in Table I.

Although all of the "improved" Heidelberg capsules were apparently operative, as determined by *in vitro* calibration in buffer solutions and recording of pH signal immediately after swallowing the device, in approximately 14% of the trials (5 of 35 experiments) the anticipated elevation of gastric pH after administration of antacid was not perceived. In 86% of the trials the onset of buffering activity (*i.e.*, elevation of gastric pH above 3.0) following administration of antacid was

[1] Medintron Corp. of America, New York, N. Y.

Table I—Onset and Duration (Time in Minutes) of Buffering Action following Administration of Gastric Antacid Preparations in Human Subjects as Recorded by Heidelberg Capsule Telemetry

Antacid A—Suspension			Antacid B—Suspension			Antacid C—Suspension			Antacid D—Tablet		
Subject	Onset[a]	Duration	Subject	Onset	Duration	Subject	Onset	Duration	Subject	Onset	Duration
1	1	2[d]	1	3	40	1	31	>16	1	2	>46
2	1	13[d]	3	5	24	5	1	12	12[b]	0	0
3[b]	0	0	5	9	8[d]	10[b]	0	0	13	1	6[d]
5	6	12[d]	6	1	11	11	37	>10	15	3	1[d]
6	7	>40	7	2	2[d]	12	2	>40	18	7	2[d]
7[c]	43	> 4	10	3	2[d]	14	7	28			
9	1	10[d]	11	7	8[d]	15[b]	0	0			
10	5	4[d]	12	1	22[d]	16	1	34			
11[b]	0	0				17	27	>20			
12	2	>46				18	25	>22			
13	4	4[d]				19	29	4			
Average onset	3.4(n=8)			3.9(n=8)			17.8(n=9)			3.3(n=4)	

[a] Gastric pH >3.0. [b] No response, subject not included in calculation of average value. [c] Atypical response, subject not included in calculation of average value. [d] Gastric pH nonrecordable after indicated time.

readily detectable. The apparent *in vivo* "failure rate" (14%) encountered in this study was, however, less than the approximately 50% "failure rate" found by other investigators (2) who examined capsules of an earlier design under essentially similar conditions (*i.e.*, unrestrained device in subjects sitting erect).

Considering the amount and composition of the antacid formulations used in this study, an initial rise in gastric pH following ingestion of any of the four preparations may be considered a predictable phenomenon. This applies also to those individuals who may have been subject to gastric hyperacidity at the time of antacid administration. That a pH rise was not detectable in some cases is consistent with the assumption of *in vivo* capsule failure. Transmitter failure in certain cases may have been attributable to location in the stomach, *i.e.*, the device may have lodged in an area of the mucosa whereby contact of the sensor with the gastric contents was prevented or impaired.

Evaluation of the data obtained in those cases where transmission of gastric pH values was confirmed suggests that the duration of the buffering action of antacids cannot be measured reliably by this technique. Approximately 40% of the capsules used in this investigation ceased normal transmission shortly after signaling onset of antacid-induced elevation of gastric pH. In view of the observed rapid increase in the gastric pH of fasted subjects to values which exceeded the capsule range (pH above 7), it is presumed that those capsules were emptied from the stomach together with the gastric contents. Emptying of the stomach is often accelerated by significant elevations in gastric pH (3). The capsule may be retained in the stomach for an arbitrary period by securing one end of a measured length of string to the capsule and the other end to the teeth after swallowing the transmitter. Since attachment of a restraining device would create an artificial situation in which the location of the capsule would be unrelated to retention of antacid in the stomach, it was not employed in this investigation.

The results of this investigation suggest that the *in vivo* performance of the modified Heidelberg telemetric capsule is not completely dependable. It may be considered a convenient and useful device for monitoring changes in gastric pH in the period immediately following administration of antacids whose buffering activity is qualitatively predictable. Duration of antacid-induced buffering effect could not, under the conditions of this study, be accurately ascertained, due possibly to acceleration of gastric emptying and consequent ejection of transmitter from the stomach.

(1) E. W. Packman, unpublished data.
(2) R. Brogle, personal communication, May 1969.
(3) J. M. Marcussen, *Acta Med. Scand.*, **172**, 451(1962).

FREDERICK J. GOLDSTEIN
ELIAS W. PACKMAN
Department of Biological Sciences
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104

Received May 29, 1969.
Accepted for publication November 6, 1969.
Presented to the Pharmacology and Biochemistry Section, APhA, Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.
This study was supported in part by a grant from the Warner-Lambert Research Institute.

Formulation of a Morphine Implantation Pellet Suitable for Tolerance–Physical Dependence Studies in Mice

Keyphrases ☐ Morphine implantation pellet, formulation—tolerance, physical dependence studies ☐ Dependence studies—morphine pellet formulation ☐ Cellulose, microcrystalline—implantation pellets

Sir:

There is considerable current interest in the study of morphine addiction, tolerance, and physical dependence. When using laboratory animals (*e.g.*, mice) for such studies, an essential part of the procedure is

Table I—Onset and Duration (Time in Minutes) of Buffering Action following Administration of Gastric Antacid Preparations in Human Subjects as Recorded by Heidelberg Capsule Telemetry

Antacid A—Suspension			Antacid B—Suspension			Antacid C—Suspension			Antacid D—Tablet		
Subject	Onset[a]	Duration	Subject	Onset	Duration	Subject	Onset	Duration	Subject	Onset	Duration
1	1	2[d]	1	3	40	1	31	>16	1	2	>46
2	1	13[d]	3	5	24	5	1	12	12[b]	0	0
3[b]	0	0	5	9	8[d]	10[b]	0	0	13	1	6[d]
5	6	12[d]	6	1	11	11	37	>10	15	3	1[d]
6	7	>40	7	2	2[d]	12	2	>40	18	7	2[d]
7[c]	43	> 4	10	3	2[d]	14	7	28			
9	1	10[d]	11	7	8[d]	15[b]	0	0			
10	5	4[d]	12	1	22[d]	16	1	34			
11[b]	0	0				17	27	>20			
12	2	>46				18	25	>22			
13	4	4[d]				19	29	4			
Average onset	3.4(n=8)			3.9(n=8)			17.8(n=9)			3.3(n=4)	

[a] Gastric pH >3.0. [b] No response, subject not included in calculation of average value. [c] Atypical response, subject not included in calculation of average value. [d] Gastric pH nonrecordable after indicated time.

readily detectable. The apparent *in vivo* "failure rate" (14%) encountered in this study was, however, less than the approximately 50% "failure rate" found by other investigators (2) who examined capsules of an earlier design under essentially similar conditions (*i.e.*, unrestrained device in subjects sitting erect).

Considering the amount and composition of the antacid formulations used in this study, an initial rise in gastric pH following ingestion of any of the four preparations may be considered a predictable phenomenon. This applies also to those individuals who may have been subject to gastric hyperacidity at the time of antacid administration. That a pH rise was not detectable in some cases is consistent with the assumption of *in vivo* capsule failure. Transmitter failure in certain cases may have been attributable to location in the stomach, *i.e.*, the device may have lodged in an area of the mucosa whereby contact of the sensor with the gastric contents was prevented or impaired.

Evaluation of the data obtained in those cases where transmission of gastric pH values was confirmed suggests that the duration of the buffering action of antacids cannot be measured reliably by this technique. Approximately 40% of the capsules used in this investigation ceased normal transmission shortly after signaling onset of antacid-induced elevation of gastric pH. In view of the observed rapid increase in the gastric pH of fasted subjects to values which exceeded the capsule range (pH above 7), it is presumed that those capsules were emptied from the stomach together with the gastric contents. Emptying of the stomach is often accelerated by significant elevations in gastric pH (3). The capsule may be retained in the stomach for an arbitrary period by securing one end of a measured length of string to the capsule and the other end to the teeth after swallowing the transmitter. Since attachment of a restraining device would create an artificial situation in which the location of the capsule would be unrelated to retention of antacid in the stomach, it was not employed in this investigation.

The results of this investigation suggest that the *in vivo* performance of the modified Heidelberg telemetric capsule is not completely dependable. It may be considered a convenient and useful device for monitoring changes in gastric pH in the period immediately following administration of antacids whose buffering activity is qualitatively predictable. Duration of antacid-induced buffering effect could not, under the conditions of this study, be accurately ascertained, due possibly to acceleration of gastric emptying and consequent ejection of transmitter from the stomach.

(1) E. W. Packman, unpublished data.
(2) R. Brogle, personal communication, May 1969.
(3) J. M. Marcussen, *Acta Med. Scand.*, **172**, 451(1962).

FREDERICK J. GOLDSTEIN
ELIAS W. PACKMAN
Department of Biological Sciences
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104

Received May 29, 1969.
Accepted for publication November 6, 1969.
Presented to the Pharmacology and Biochemistry Section, APhA, Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.
This study was supported in part by a grant from the Warner-Lambert Research Institute.

Formulation of a Morphine Implantation Pellet Suitable for Tolerance–Physical Dependence Studies in Mice

Keyphrases ☐ Morphine implantation pellet, formulation—tolerance, physical dependence studies ☐ Dependence studies—morphine pellet formulation ☐ Cellulose, microcrystalline—implantation pellets

Sir:

There is considerable current interest in the study of morphine addiction, tolerance, and physical dependence. When using laboratory animals (*e.g.*, mice) for such studies, an essential part of the procedure is

administering the morphine in such a way that the animals receive adequate doses of the drug at frequent enough intervals to reach and maintain the desired levels of tolerance and physical dependence. This can be done by repeated daily injections, but this consumes too much time, both in administering the doses and in the time (weeks) it takes for tolerance and dependence to develop. A more convenient alternate procedure is to implant a pellet of morphine subcutaneously, thus allowing a continual gradual release of the drug. Maggiolo and Huidobro (1) have reported such a technique utilizing a pellet of pure morphine base compressed at high pressure. While this pellet proved useful for their studies, it released drug very slowly (25% of the dose in 8 days and 50% in 16 days).

Way *et al.* (2, 3) produced morphine tolerance and dependence in mice by subcutaneously injecting increasing doses of morphine three times a day for 3 consecutive weeks; but from a time-convenience standpoint, they were interested in using the pellet implantation method. However, with the Maggiolo-Huidobro pellet, the mice developed the desired level of tolerance and dependence too slowly. By using a modified formulation developed by these laboratories, Way *et al.* could induce a high degree of tolerance and physical dependence in 2 or 3 days (the modified pellet released 25–50% of the drug in the first 2 days after implantation). Since the publication of the Way articles, we have received a number of requests for manufacturing details. Therefore, the purpose of this Communication is to provide these formulation details pertaining to the experimental products utilized in the previously published paper emanating from these laboratories. For each tablet:

Morphine, purified powder	0.075 g.
Microcrystalline cellulose (Avicel)	0.075 g.
Fumed silicon dioxide (Cab-O-Sil)	0.00075 g.
Calcium stearate	0.0015 g.

Directions: Screen the morphine, microcrystalline cellulose, fumed silicon dioxide, and calcium stearate through 60-mesh screen. Slug using 1.91-cm. (0.75-in.) FFBE punch and die, obtaining thin, firm wafers. Screen, No. 16 mesh, *via* Stokes oscillating granulator. Mix well in a twin shell blender. Compress *via* Colton tablet press model 330:

Tablet weight	0.152 g.
Tablet hardness	15 Strong-Cobb Units
Tablet thickness	3 mm.

Because of the possibility of particle segregation in the hopper of the tablet press, the slugging step was introduced. This is especially important in view of the small batch sizes produced.

The determining factor in selecting microcrystalline cellulose as the diluent was the desire to control pellet density or hardness. Because morphine is soluble in tissue fluids and cellulose is not, it is reasonable to expect that, after some initial rapid release of the drug, the absorption rate would become constant as soon as the material at the surface of the pellet has dissolved. While it is quite possible that other materials could also give the desired effect, the microcrystalline cellulose was chosen because: (*a*) it is easily compressed to varying degrees of density while providing a physically stable pellet; (*b*) it proved a satisfactory vehicle for providing a reasonably constant absorption rate for the drug; and (*c*) its physical characteristics are such that after 5 days' implantation the pellet remained as a semisolid palpable mass, facilitating easy removal from the animal.

This work suggests that microcrystalline cellulose is a possible vehicle for long-term implantation pellets for human use, provided, of course, it proves to be safe from a toxicity standpoint. Preliminary information suggests that microcrystalline cellulose may be relatively innocuous since implantation of pellets in mice containing no morphine elicited no overt toxic effects. Whether or not the body can adequately eliminate the cellulose from the tissues remains to be seen.

(1) C. Maggiolo and F. Huidobro, *Acta Physiol. Lat. Amer.*, **11**, 70(1961).
(2) E. L. Way, H. H. Loh, and F. Shen, *Science*, **162**, 1290(1968).
(3) E. L. Way, H. H. Loh, and F. Shen, *J. Pharmacol. Exp. Ther.*, **167**, 1(1969).

ROBERT D. GIBSON
JAMES E. TINGSTAD
Pharmaceutical Technology Laboratory
School of Pharmacy
University of California
San Francisco, CA 94122

Received August 8, 1969.
Accepted for publication October 29, 1969.

Rearrangement of Chloramphenicol-3-monosuccinate

Keyphrases ☐ Chloramphenicol-3-monosuccinate—rearrangement ☐ Chromatography, liquid–liquid, thin-layer—separation, analysis ☐ NMR spectroscopy—structure ☐ Optical rotatory dispersion—identity ☐ IR spectrophotometry—structure

Sir:

Investigation of the behavior of aqueous solutions of chloramphenicol-3-monosuccinate (I) indicated that at pH's near neutrality the compound is incapable of independent existence but rather exists as an equilibrium mixture of itself and a different molecular form. This report concerns the isolation and characterization of the rearranged compound. Details of the chemistry of this process will be reported in a subsequent communication.

Components of the equilibrium mixture were separated by reverse phase liquid–liquid chromatography using a fatty acid *N,N'*-dimethylamide[1] on silanized diatomaceous earth[2] as stationary phase and 0.05 *N*

[1] Hallcomid M-18, The C. P. Hall Co., Akron Ohio.
[2] Celite 545, Johns-Manville, New York, N. Y.

administering the morphine in such a way that the animals receive adequate doses of the drug at frequent enough intervals to reach and maintain the desired levels of tolerance and physical dependence. This can be done by repeated daily injections, but this consumes too much time, both in administering the doses and in the time (weeks) it takes for tolerance and dependence to develop. A more convenient alternate procedure is to implant a pellet of morphine subcutaneously, thus allowing a continual gradual release of the drug. Maggiolo and Huidobro (1) have reported such a technique utilizing a pellet of pure morphine base compressed at high pressure. While this pellet proved useful for their studies, it released drug very slowly (25% of the dose in 8 days and 50% in 16 days).

Way *et al.* (2, 3) produced morphine tolerance and dependence in mice by subcutaneously injecting increasing doses of morphine three times a day for 3 consecutive weeks; but from a time-convenience standpoint, they were interested in using the pellet implantation method. However, with the Maggiolo-Huidobro pellet, the mice developed the desired level of tolerance and dependence too slowly. By using a modified formulation developed by these laboratories, Way *et al.* could induce a high degree of tolerance and physical dependence in 2 or 3 days (the modified pellet released 25–50% of the drug in the first 2 days after implantation). Since the publication of the Way articles, we have received a number of requests for manufacturing details. Therefore, the purpose of this Communication is to provide these formulation details pertaining to the experimental products utilized in the previously published paper emanating from these laboratories. For each tablet:

Morphine, purified powder	0.075 g.
Microcrystalline cellulose (Avicel)	0.075 g.
Fumed silicon dioxide (Cab-O-Sil)	0.00075 g.
Calcium stearate	0.0015 g.

Directions: Screen the morphine, microcrystalline cellulose, fumed silicon dioxide, and calcium stearate through 60-mesh screen. Slug using 1.91-cm. (0.75-in.) FFBE punch and die, obtaining thin, firm wafers. Screen, No. 16 mesh, *via* Stokes oscillating granulator. Mix well in a twin shell blender. Compress *via* Colton tablet press model 330:

Tablet weight	0.152 g.
Tablet hardness	15 Strong-Cobb Units
Tablet thickness	3 mm.

Because of the possibility of particle segregation in the hopper of the tablet press, the slugging step was introduced. This is especially important in view of the small batch sizes produced.

The determining factor in selecting microcrystalline cellulose as the diluent was the desire to control pellet density or hardness. Because morphine is soluble in tissue fluids and cellulose is not, it is reasonable to expect that, after some initial rapid release of the drug, the absorption rate would become constant as soon as the material at the surface of the pellet has dissolved. While it is quite possible that other materials could also give the desired effect, the microcrystalline cellulose was chosen because: (*a*) it is easily compressed to varying degrees of density while providing a physically stable pellet; (*b*) it proved a satisfactory vehicle for providing a reasonably constant absorption rate for the drug; and (*c*) its physical characteristics are such that after 5 days' implantation the pellet remained as a semisolid palpable mass, facilitating easy removal from the animal.

This work suggests that microcrystalline cellulose is a possible vehicle for long-term implantation pellets for human use, provided, of course, it proves to be safe from a toxicity standpoint. Preliminary information suggests that microcrystalline cellulose may be relatively innocuous since implantation of pellets in mice containing no morphine elicited no overt toxic effects. Whether or not the body can adequately eliminate the cellulose from the tissues remains to be seen.

(1) C. Maggiolo and F. Huidobro, *Acta Physiol. Lat. Amer.*, **11**, 70(1961).
(2) E. L. Way, H. H. Loh, and F. Shen, *Science*, **162**, 1290(1968).
(3) E. L. Way, H. H. Loh, and F. Shen, *J. Pharmacol. Exp. Ther.*, **167**, 1(1969).

ROBERT D. GIBSON
JAMES E. TINGSTAD
Pharmaceutical Technology Laboratory
School of Pharmacy
University of California
San Francisco, CA 94122

Received August 8, 1969.
Accepted for publication October 29, 1969.

Rearrangement of Chloramphenicol-3-monosuccinate

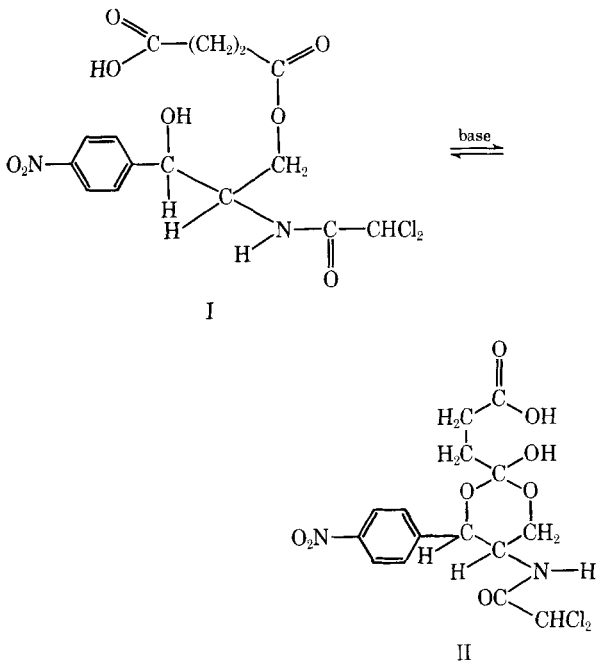
Keyphrases ☐ Chloramphenicol-3-monosuccinate—rearrangement ☐ Chromatography, liquid–liquid, thin-layer—separation, analysis ☐ NMR spectroscopy—structure ☐ Optical rotatory dispersion—identity ☐ IR spectrophotometry—structure

Sir:

Investigation of the behavior of aqueous solutions of chloramphenicol-3-monosuccinate (I) indicated that at pH's near neutrality the compound is incapable of independent existence but rather exists as an equilibrium mixture of itself and a different molecular form. This report concerns the isolation and characterization of the rearranged compound. Details of the chemistry of this process will be reported in a subsequent communication.

Components of the equilibrium mixture were separated by reverse phase liquid–liquid chromatography using a fatty acid *N,N'*-dimethylamide[1] on silanized diatomaceous earth[2] as stationary phase and 0.05 *N*

[1] Hallcomid M-18, The C. P. Hall Co., Akron Ohio.
[2] Celite 545, Johns-Manville, New York, N. Y.



Scheme I

HCl as eluent. TLC using silica plates with an acidic developer indicated that the isolated material was a single entity which could be converted to I by treatment with base (see Scheme I).

Spectral and kinetic evidence suggest that the rearranged material is a cyclic hemi-*ortho* ester (II) of the type postulated as an unstable intermediate in 1–3 acyl migrations (1, 2).

Structural determination was primarily achieved by using NMR. A comparison of the NMR spectra of I with II revealed certain significant differences in the chemical shifts and splitting patterns of several protons (Table I).

The peak positions for the amide nitrogen, the aromatic ring protons, and the $CHCl_2$ hydrogen were identical in both linear and cyclic form. The peak position of the C(1)—H proton in the cyclic compound is 57 c.p.s. downfield from that of the linear form. The deshielding effect produced on esterifying a hydroxyl group on the proton or the protons on the carbon holding the hydroxyl group is well documented (3). Also for the cyclic ester, the splitting of the C(2)—H hydrogen by the C(1)—H and the two C(3)—H protons gives a quartet centered at 4.44 p.p.m. The two hydrogens on C(3) have shifted upfield to 3.7 p.p.m. as a pair of overlapping doublets, where in the linear form these three protons form a complex multiplet.

Table I—Chemical Shift Values (p.p.m.) Using D_6Acetone for Solvent, TMS as Internal Standard

Proton	Chemical Shift (p.p.m.) Chloramphenicol-3-monosuccinate	Chemical Shift (p.p.m.) Cyclic Succinate
C_1—H	5.26	6.23
C_2—H	4.4	4.44
C_3—H_2	4.4	3.7
C_3—OH	6	—
—$(CH_2)_2$—	2.63 (singlet)	2.7 (A_2B_2)
ring—H_4	7.95 (A_2B_2)	7.95 (A_2B_2)
N—H	7.46	7.46
CCl_2—H	6.35	6.35

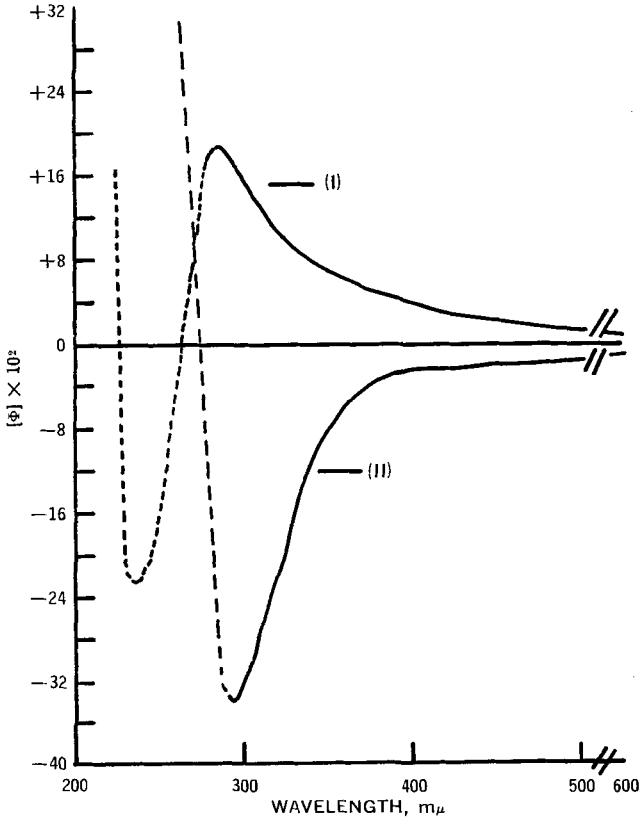


Figure 1—*The ORD curves of chloramphenicol-3-monosuccinate (I) and the cyclic hemi-*ortho *ester (II). Anhydrous ethanol is the solvent. Dashed portion of lines indicates areas of high absorbance.*

The succinate methylene protons in the cyclic *ortho* ester do not give rise to a singlet as is the case in the linear molecule. The signal for these hydrogens in the cyclic compound is an A_2B_2 system which arises from a pair of overlapping triplets. It is thought that different electronic effects associated with sp^2 and sp^3 hybridized carbon atoms adjacent to the succinate methylenes cause splitting of signal.

Additional evidence for the proposed cyclic isomer was obtained from IR and optical rotatory dispersion (ORD) data. Whereas the IR spectrum for I showed three distinct carbonyl absorptions at 1690, 1718, and 1745 $cm.^{-1}$, the spectrum for II showed only two carbonyl peaks at 1690 and 1745 $cm.^{-1}$. This apparent loss of ester carbonyl absorption and a change in absorbance in the C—O—C stretch region in the IR spectrum of II could be explained on the basis of the cyclic isomer (II).

The ORD curve for the cyclic and linear ester is shown in Fig. 1. Due to high absorption of both compounds below a wavelength of 280 mμ, this region is an approximation based on repeated determinations and several dilutions. Linear chloramphenicol-3-monosuccinate shows a positive Cotton effect, whereas the cyclic ester shows a negative Cotton effect. The ORD curves of o^1-acetylated chloramphenicol-3-monosuccinate and o^1-dichloroacetylchloramphenicol-3-mono-

succinate also shows a negative Cotton effect. Esterification of the secondary hydroxyl group is associated with a change in optical rotation resulting in a negative ORD curve for the o^1-ester compound whose ORD curve prior to esterification was positive. Also characteristic of esterification of the o^1-hydroxyl group is a paramagnetic shift of the C(1)—H. Both of these phenomena were observed in the rearrangement of chloramphenicol-3-monosuccinate.

Complete migration of the succinyl function was ruled out on the basis of NMR and chemical data. Complete migration would have given a terminal hydroxyl. No peak corresponding to that for the C(3)—OH proton of chloramphenicol was observed. Chemical evidence indicated that the 0 → 0 migration product was incapable of existence in equilibrium with chloramphenicol-3-monosuccinate under experimental conditions.

Attempts to acetylate or methylate the isomeric compound resulted in complex reaction mixtures, presumably due to the breakdown of the cyclic structure by the reacting anion. All spectral and chemical evidence support the proposed structure.

(1) H. Hibbert and M. E. Grieg, *Can. J. Res.*, **4**, 252(1931).
(2) P. E. Verkade and O. E. Van Lobinsen, *Kon. Vlaam. Meded. Acad. Wetensch. Proc., Ser. B*, **56**, 324(1953).
(3) C. R. Narayanan and M. R. Sarma, *Tetrahedron Lett.*, **1968**, 1553.

BEVERLY SANDMANN*
DALE SZULCZEWSKI
JOHN WINDHEUSER
T. HIGUCHI†
School of Pharmacy
University of Wisconsin
Madison, Wis.;
Parke, Davis and Company
Detroit, Mich.; and
Department of Pharmaceutical Chemistry
School of Pharmacy
University of Kansas
Lawrence, Kan.

Received September 11, 1969
Accepted for publication October 20, 1969.
* Present address: University of Missouri, School of Pharmacy, Kansas City, Mo.
† All correspondence relative to the paper should be addressed to Professor T. Higuchi at University of Kansas, Lawrence, KS 66044

Covalent Addition of *N*-Chlorosaccharin to Cyclohexene

Keyphrases □ *N*-Chlorosaccharin—cyclohexene addition □ Cyclohexene—*N*-chlorosaccharin addition □ *N*-(2-Chlorocyclohexyl)-saccharin—synthesis, structure, formation rate □ IR—identification, structure □ NMR—identification, structure □ UV—rate of formation

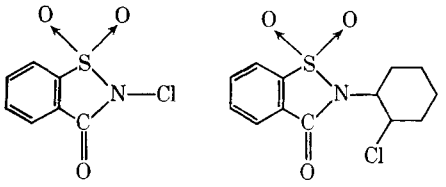
Sir:

It has recently been reported (1) that *N*-chloro-*N*-methylethanesulfonamide will add covalently across the >C=C< double bond of 1-hexene under photoirradiation. In similar, but apparently more facile, reactions, *N,N*-dichlorobenzenesulfonamide (2) and *N*-aryl-*N*-halosulfonamides (3) will add covalently to cyclohexene. Because *N*-chloro compounds are used as chlorinating and oxidizing agents for a wide variety of compounds, additional reactions of the above type must be expected to occur if the molecules to be chlorinated contain unsaturated groups.

Table I—Rate Constants for the Covalent Addition of *N*-Chlorosaccharin to Cyclohexene in Carbon Tetrachloride at 25°

[Cyclohexene]$_{added}$ × 10 *M*	[*N*-Chlorosaccharin]$_{added}$ × 10^4 *M*	$10^2 k_{obs.}$ sec^{-1}	$10^2 k_1$ M^{-1} $sec.^{-1}$
4.96	6.30	1.69	3.41
3.22	10.00	1.16	3.60

We have recently discussed (4) the possible usefulness of *N*-chlorosaccharin (I) as an organochlorinating agent on the basis of its low chlorine potential in water and its solubility and stability in a variety of organic solvents. However, we now present evidence that I will also covalently add to cyclohexene in a facile reaction at room temperature to yield *N*-(2-chlorocyclohexyl)-saccharin (II).



I II

When I (400 mg.) was added to cyclohexene (15 ml.) at 25°, it gradually dissolved and simultaneously a white powder crystallized out of solution. After recrystallization from acetone–water, this powder had m.p. 171–172.5° and the same elemental analysis as II. (Found: C, 52.07; H, 4.86; Cl, 11.94; N, 4.78; S, 10.97. II, $C_{13}H_{14}NClSO_3$, requires C, 52.0; H, 4.67; Cl, 11.85; N, 4.67; S, 10.70.) Its structure was confirmed by NMR and IR spectroscopy. Its NMR spectrum showed the presence of 4 benzene protons and 10 cyclohexene protons, but no cyclohexene-ethylene protons were evident. The IR spectrum of the compound was consistent with that of Structure II and contained a strong band at the carbonyl-stretching frequency region. This latter piece of evidence ruled out the possibility that an O—C bond existed between saccharin and cyclohexene. The product did not release iodine from aqueous solutions of potassium iodide, thereby indicating that it was not in equilibrium with *N*-chlorosaccharin and that its chlorine was fixed and no longer "active."

The rate of formation of the adduct was determined by measuring changes in UV absorbance at 270 mμ after carbon tetrachloride solutions of I and cyclohexene (which had been equilibrated at 25.0 ± 0.2°) were mixed in a 1-cm. spectrophotometer cell. The rate of change of absorbance was first order when [cyclohexene]$_{added}$ was much greater than [I]$_{added}$ and pseudo first-order rate constant, $k_{obs.}$, values were calculated. At two different cyclohexene concentrations the value of $k_{obs.}/[\text{cyclohexene}]_{added} = k_1$ was constant and thus

succinate also shows a negative Cotton effect. Esterification of the secondary hydroxyl group is associated with a change in optical rotation resulting in a negative ORD curve for the o^1-ester compound whose ORD curve prior to esterification was positive. Also characteristic of esterification of the o^1-hydroxyl group is a paramagnetic shift of the C(1)—H. Both of these phenomena were observed in the rearrangement of chloramphenicol-3-monosuccinate.

Complete migration of the succinyl function was ruled out on the basis of NMR and chemical data. Complete migration would have given a terminal hydroxyl. No peak corresponding to that for the C(3)—OH proton of chloramphenicol was observed. Chemical evidence indicated that the 0 → 0 migration product was incapable of existence in equilibrium with chloramphenicol-3-monosuccinate under experimental conditions.

Attempts to acetylate or methylate the isomeric compound resulted in complex reaction mixtures, presumably due to the breakdown of the cyclic structure by the reacting anion. All spectral and chemical evidence support the proposed structure.

(1) H. Hibbert and M. E. Grieg, *Can. J. Res.*, **4**, 252(1931).
(2) P. E. Verkade and O. E. Van Lobinsen, *Kon. Vlaam. Meded. Acad. Wetensch. Proc., Ser. B*, **56**, 324(1953).
(3) C. R. Narayanan and M. R. Sarma, *Tetrahedron Lett.*, **1968**, 1553.

BEVERLY SANDMANN*
DALE SZULCZEWSKI
JOHN WINDHEUSER
T. HIGUCHI†
School of Pharmacy
University of Wisconsin
Madison, Wis.;
Parke, Davis and Company
Detroit, Mich.; and
Department of Pharmaceutical Chemistry
School of Pharmacy
University of Kansas
Lawrence, Kan.

Received September 11, 1969
Accepted for publication October 20, 1969.
* Present address: University of Missouri, School of Pharmacy, Kansas City, Mo.
† All correspondence relative to the paper should be addressed to Professor T. Higuchi at University of Kansas, Lawrence, KS 66044

Covalent Addition of *N*-Chlorosaccharin to Cyclohexene

Keyphrases □ *N*-Chlorosaccharin—cyclohexene addition □ Cyclohexene—*N*-chlorosaccharin addition □ *N*-(2-Chlorocyclohexyl)-saccharin—synthesis, structure, formation rate □ IR—identification, structure □ NMR—identification, structure □ UV—rate of formation

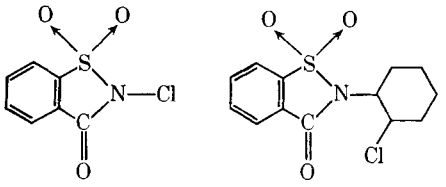
Sir:

It has recently been reported (1) that *N*-chloro-*N*-methylethanesulfonamide will add covalently across the >C=C< double bond of 1-hexene under photoirradiation. In similar, but apparently more facile, reactions, *N,N*-dichlorobenzenesulfonamide (2) and *N*-aryl-*N*-halosulfonamides (3) will add covalently to cyclohexene. Because *N*-chloro compounds are used as chlorinating and oxidizing agents for a wide variety of compounds, additional reactions of the above type must be expected to occur if the molecules to be chlorinated contain unsaturated groups.

Table I—Rate Constants for the Covalent Addition of *N*-Chlorosaccharin to Cyclohexene in Carbon Tetrachloride at 25°

[Cyclohexene]$_{added}$ × 10 *M*	[*N*-Chlorosaccharin]$_{added}$ × 10^4 *M*	$10^2 k_{obs.}$ sec^{-1}	$10^2 k_1$ M^{-1} $sec.^{-1}$
4.96	6.30	1.69	3.41
3.22	10.00	1.16	3.60

We have recently discussed (4) the possible usefulness of *N*-chlorosaccharin (I) as an organochlorinating agent on the basis of its low chlorine potential in water and its solubility and stability in a variety of organic solvents. However, we now present evidence that I will also covalently add to cyclohexene in a facile reaction at room temperature to yield *N*-(2-chlorocyclohexyl)-saccharin (II).



I II

When I (400 mg.) was added to cyclohexene (15 ml.) at 25°, it gradually dissolved and simultaneously a white powder crystallized out of solution. After recrystallization from acetone–water, this powder had m.p. 171–172.5° and the same elemental analysis as II. (Found: C, 52.07; H, 4.86; Cl, 11.94; N, 4.78; S, 10.97. II, $C_{13}H_{14}NClSO_3$, requires C, 52.0; H, 4.67; Cl, 11.85; N, 4.67; S, 10.70.) Its structure was confirmed by NMR and IR spectroscopy. Its NMR spectrum showed the presence of 4 benzene protons and 10 cyclohexene protons, but no cyclohexene-ethylene protons were evident. The IR spectrum of the compound was consistent with that of Structure II and contained a strong band at the carbonyl-stretching frequency region. This latter piece of evidence ruled out the possibility that an O—C bond existed between saccharin and cyclohexene. The product did not release iodine from aqueous solutions of potassium iodide, thereby indicating that it was not in equilibrium with *N*-chlorosaccharin and that its chlorine was fixed and no longer "active."

The rate of formation of the adduct was determined by measuring changes in UV absorbance at 270 mμ after carbon tetrachloride solutions of I and cyclohexene (which had been equilibrated at 25.0 ± 0.2°) were mixed in a 1-cm. spectrophotometer cell. The rate of change of absorbance was first order when [cyclohexene]$_{added}$ was much greater than [I]$_{added}$ and pseudo first-order rate constant, $k_{obs.}$, values were calculated. At two different cyclohexene concentrations the value of $k_{obs.}/[\text{cyclohexene}]_{added} = k_1$ was constant and thus

the reaction appeared to be first order in cyclohexene and I. Results of two experiments are presented in Table I.

(1) T. Ohashi, M. Sugie, M. Okahara, and S. Kowori, *Tetrahedron Lett.*, **1968,** 4195.

(2) W. Theilacker and H. Wessel, *Justus Liebigs Ann. Chem.*, **703,** 34(1967).

(3) M. S. Kharasch and H. M. Priestley, *J. Amer. Chem. Soc.*, **61,** 3425(1939).

(4) H. Dawn, T. Higuchi, and I. H. Pitman, to be published.

HAN-SON DAWN*
IAN H. PITMAN
TAKERU HIGUCHI

Department of Analytical Pharmaceutical Chemistry and Pharmaceutics
School of Pharmacy
The University of Kansas
Lawrence, KS 66044

Received September 19, 1969.
Accepted for publication November 4, 1969.
This work was included in a presentation made to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.
Supported in part by the Department of the Army, Contract DAAA-15-67-C-0638.

* Present address: Wyeth Laboratories, Research and Development Department, Box 8299, Philadelphia, PA 19101

Books

REVIEWS

The Molecular Orbital Theory of Organic Chemistry. By MICHAEL J. S. DEWAR. McGraw-Hill Book Company, New York, NY 10036, 1969. vii + 484 pp. 15.5 × 23.5 cm. Price $16.50.

This book is representative of a general class of books written over the past decade in which quantum chemistry is applied to organic chemistry, with major emphasis on molecular orbital theory. The book is divided into ten chapters, the first four of which develop the concepts of molecular orbital theory useful to the organic chemist. In subsequent chapters, molecules of increasing complexity are treated with considerable emphasis on the conceptualization of the theory. The continual relationship to more classical concepts such as reaction types based upon valence bond mechanisms and the Hammett relationships makes the entrance into molecular orbital theory particularly useful.

There is much emphasis on self-consistent field theory, with very little attention paid to the less exact but simpler Hückel schemes in common use. This is particularly true of Hückel all-valence electron methods which are completely ignored. In places, the level of sophistication of treatment exceeds the needs of the medicinal chemist. There is much written about hydrocarbons but perhaps too little about heteroatom-containing molecules, which, of course, are the tools of the medicinal chemist's trade.

In summary, this book is a useful reference for the organic or medicinal chemist who already has some background in molecular orbital theory.

Reviewed by Lemont B. Kier
Battelle Memorial Institute
Columbus Laboratories
505 King Avenue
Columbus, OH 43201 ■

Principles of Physical, Organic, and Biological Chemistry. By JOHN R. HOLUM. John Wiley and Sons, Inc., New York, NY 10016, 1969. x +728 pp. 17.2 × 23.5 cm. Price $10.95.

This book consists essentially of two parts. The first deals with basic physical and chemical principles, including the concepts of energy, atomic structure, thermodynamics, chemical bonds, spontaneity, equilibria, and organic chemistry. The second comprises the application of these principles in developing an understanding of and an appreciation for the molecular basis of life.

The material, which contains appropriate illustrations and examples, is well organized and easily understood. Particularly noteworthy is the author's lucid treatment of the rather difficult subject of thermodynamics. His qualitative discussions of such concepts as internal energy, entropy, enthalpy, and spontaneity are commendable. Indeed, the student of physical chemistry would do well to read his account first and then proceed to the advanced texts.

The latter chapters deal with such topics as, "Biochemical Regulation and Defense," "Important Fluids of the Body," "Energy for Living," "Metabolism of Lipids, Carbohydrates and Proteins," and the "Chemistry of Heredity." These chapters apply the fundamental principles presented in earlier chapters to explain the compositions and functions of living matter. The author's approach in explaining life on a molecular basis should prove to be not only informative to the student but stimulating and fascinating as well.

As stated in the Preface, this book "...is intended for use in a two- or three-term terminal college course in chemistry for students in the humanities, the social sciences, and the paramedical sciences, including nursing, home economics, physical therapy, many areas of biology, and many programs in the agricultural sciences." It is well suited for that purpose.

Reviewed by Edward O. Magarian
Department of Pharmaceutical Chemistry
College of Pharmacy
University of Kentucky
Lexington, KY 40506 ■

the reaction appeared to be first order in cyclohexene and I. Results of two experiments are presented in Table I.

(1) T. Ohashi, M. Sugie, M. Okahara, and S. Kowori, *Tetrahedron Lett.*, **1968,** 4195.

(2) W. Theilacker and H. Wessel, *Justus Liebigs Ann. Chem.*, **703,** 34(1967).

(3) M. S. Kharasch and H. M. Priestley, *J. Amer. Chem. Soc.*, **61,** 3425(1939).

(4) H. Dawn, T. Higuchi, and I. H. Pitman, to be published.

HAN-SON DAWN*
IAN H. PITMAN
TAKERU HIGUCHI

Department of Analytical Pharmaceutical Chemistry and Pharmaceutics
School of Pharmacy
The University of Kansas
Lawrence, KS 66044

Received September 19, 1969.
Accepted for publication November 4, 1969.
This work was included in a presentation made to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.
Supported in part by the Department of the Army, Contract DAAA-15-67-C-0638.

* Present address: Wyeth Laboratories, Research and Development Department, Box 8299, Philadelphia, PA 19101

Books

REVIEWS

The Molecular Orbital Theory of Organic Chemistry. By MICHAEL J. S. DEWAR. McGraw-Hill Book Company, New York, NY 10036, 1969. vii + 484 pp. 15.5 × 23.5 cm. Price $16.50.

This book is representative of a general class of books written over the past decade in which quantum chemistry is applied to organic chemistry, with major emphasis on molecular orbital theory. The book is divided into ten chapters, the first four of which develop the concepts of molecular orbital theory useful to the organic chemist. In subsequent chapters, molecules of increasing complexity are treated with considerable emphasis on the conceptualization of the theory. The continual relationship to more classical concepts such as reaction types based upon valence bond mechanisms and the Hammett relationships makes the entrance into molecular orbital theory particularly useful.

There is much emphasis on self-consistent field theory, with very little attention paid to the less exact but simpler Hückel schemes in common use. This is particularly true of Hückel all-valence electron methods which are completely ignored. In places, the level of sophistication of treatment exceeds the needs of the medicinal chemist. There is much written about hydrocarbons but perhaps too little about heteroatom-containing molecules, which, of course, are the tools of the medicinal chemist's trade.

In summary, this book is a useful reference for the organic or medicinal chemist who already has some background in molecular orbital theory.

Reviewed by Lemont B. Kier
Battelle Memorial Institute
Columbus Laboratories
505 King Avenue
Columbus, OH 43201 ■

Principles of Physical, Organic, and Biological Chemistry. By JOHN R. HOLUM. John Wiley and Sons, Inc., New York, NY 10016, 1969. x +728 pp. 17.2 × 23.5 cm. Price $10.95.

This book consists essentially of two parts. The first deals with basic physical and chemical principles, including the concepts of energy, atomic structure, thermodynamics, chemical bonds, spontaneity, equilibria, and organic chemistry. The second comprises the application of these principles in developing an understanding of and an appreciation for the molecular basis of life.

The material, which contains appropriate illustrations and examples, is well organized and easily understood. Particularly noteworthy is the author's lucid treatment of the rather difficult subject of thermodynamics. His qualitative discussions of such concepts as internal energy, entropy, enthalpy, and spontaneity are commendable. Indeed, the student of physical chemistry would do well to read his account first and then proceed to the advanced texts.

The latter chapters deal with such topics as, "Biochemical Regulation and Defense," "Important Fluids of the Body," "Energy for Living," "Metabolism of Lipids, Carbohydrates and Proteins," and the "Chemistry of Heredity." These chapters apply the fundamental principles presented in earlier chapters to explain the compositions and functions of living matter. The author's approach in explaining life on a molecular basis should prove to be not only informative to the student but stimulating and fascinating as well.

As stated in the Preface, this book "...is intended for use in a two- or three-term terminal college course in chemistry for students in the humanities, the social sciences, and the paramedical sciences, including nursing, home economics, physical therapy, many areas of biology, and many programs in the agricultural sciences." It is well suited for that purpose.

Reviewed by Edward O. Magarian
Department of Pharmaceutical Chemistry
College of Pharmacy
University of Kentucky
Lexington, KY 40506 ■

the reaction appeared to be first order in cyclohexene and I. Results of two experiments are presented in Table I.

(1) T. Ohashi, M. Sugie, M. Okahara, and S. Kowori, *Tetrahedron Lett.*, **1968,** 4195.

(2) W. Theilacker and H. Wessel, *Justus Liebigs Ann. Chem.*, **703,** 34(1967).

(3) M. S. Kharasch and H. M. Priestley, *J. Amer. Chem. Soc.*, **61,** 3425(1939).

(4) H. Dawn, T. Higuchi, and I. H. Pitman, to be published.

HAN-SON DAWN*
IAN H. PITMAN
TAKERU HIGUCHI

Department of Analytical Pharmaceutical Chemistry and Pharmaceutics
School of Pharmacy
The University of Kansas
Lawrence, KS 66044

Received September 19, 1969.
Accepted for publication November 4, 1969.
This work was included in a presentation made to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.
Supported in part by the Department of the Army, Contract DAAA-15-67-C-0638.

* Present address: Wyeth Laboratories, Research and Development Department, Box 8299, Philadelphia, PA 19101

Books

REVIEWS

The Molecular Orbital Theory of Organic Chemistry. By MICHAEL J. S. DEWAR. McGraw-Hill Book Company, New York, NY 10036, 1969. vii + 484 pp. 15.5 × 23.5 cm. Price $16.50.

This book is representative of a general class of books written over the past decade in which quantum chemistry is applied to organic chemistry, with major emphasis on molecular orbital theory. The book is divided into ten chapters, the first four of which develop the concepts of molecular orbital theory useful to the organic chemist. In subsequent chapters, molecules of increasing complexity are treated with considerable emphasis on the conceptualization of the theory. The continual relationship to more classical concepts such as reaction types based upon valence bond mechanisms and the Hammett relationships makes the entrance into molecular orbital theory particularly useful.

There is much emphasis on self-consistent field theory, with very little attention paid to the less exact but simpler Hückel schemes in common use. This is particularly true of Hückel all-valence electron methods which are completely ignored. In places, the level of sophistication of treatment exceeds the needs of the medicinal chemist. There is much written about hydrocarbons but perhaps too little about heteroatom-containing molecules, which, of course, are the tools of the medicinal chemist's trade.

In summary, this book is a useful reference for the organic or medicinal chemist who already has some background in molecular orbital theory.

Reviewed by Lemont B. Kier
Battelle Memorial Institute
Columbus Laboratories
505 King Avenue
Columbus, OH 43201 ■

Principles of Physical, Organic, and Biological Chemistry. By JOHN R. HOLUM. John Wiley and Sons, Inc., New York, NY 10016, 1969. x +728 pp. 17.2 × 23.5 cm. Price $10.95.

This book consists essentially of two parts. The first deals with basic physical and chemical principles, including the concepts of energy, atomic structure, thermodynamics, chemical bonds, spontaneity, equilibria, and organic chemistry. The second comprises the application of these principles in developing an understanding of and an appreciation for the molecular basis of life.

The material, which contains appropriate illustrations and examples, is well organized and easily understood. Particularly noteworthy is the author's lucid treatment of the rather difficult subject of thermodynamics. His qualitative discussions of such concepts as internal energy, entropy, enthalpy, and spontaneity are commendable. Indeed, the student of physical chemistry would do well to read his account first and then proceed to the advanced texts.

The latter chapters deal with such topics as, "Biochemical Regulation and Defense," "Important Fluids of the Body," "Energy for Living," "Metabolism of Lipids, Carbohydrates and Proteins," and the "Chemistry of Heredity." These chapters apply the fundamental principles presented in earlier chapters to explain the compositions and functions of living matter. The author's approach in explaining life on a molecular basis should prove to be not only informative to the student but stimulating and fascinating as well.

As stated in the Preface, this book "...is intended for use in a two- or three-term terminal college course in chemistry for students in the humanities, the social sciences, and the paramedical sciences, including nursing, home economics, physical therapy, many areas of biology, and many programs in the agricultural sciences." It is well suited for that purpose.

Reviewed by Edward O. Magarian
Department of Pharmaceutical Chemistry
College of Pharmacy
University of Kentucky
Lexington, KY 40506 ■

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

APRIL 1970
VOLUME 59 NUMBER 4

REVIEW ARTICLE

Small Molecule–Macromolecule Interactions as Studied by Optical Rotatory Dispersion–Circular Dichroism

J. H. PERRIN and P. A. HART

Keyphrases ☐ Small molecule–macromolecule interactions—review ☐ Cotton effect—molecular binding ☐ Metachromasia—molecular binding ☐ Dye effects—optical rotatory dispersion ☐ Optical rotatory dispersion–circular dichroism—molecular binding determination

This review is intended to summarize the study of specific binding of low molecular weight substances to macromolecular systems by the application of optical rotatory dispersion–circular dichroism (ORD–CD). The authors have specifically not included inorganic ions. In addition, solvent and cosolute effects have not been covered because these phenomena are not necessarily related specifically to binding.

The review includes brief discussions of the phenomena of ORD–CD as well as applications to the proteins, nucleic acids, and polysaccharides. Metachromasia, the color-change phenomenon associated with binding, is discussed from the historical viewpoint and in terms of the more recent spectroscopic studies.

Extrinsic (induced) Cotton effects are discussed for dyes, coenzymes, and related ligands when bound to proteins, nucleic acids, polysaccharides, and membranes. The specific structural information (if any) that can be obtained from these studies is presented.

ORD, or the study of the change of optical rotation with wavelength, goes back to the early work of Biot (1) and Fresnel (2), who showed that the rotation of an optically active medium increases with decreasing wavelength of light. Thirty years later came Pasteur's classical work (3) on the resolution of racemic tartrates. The discovery of the Bunsen burner is frequently considered the major reason that ORD studies did not advance significantly until the 1930's, because measurements were too easily made at the sodium D-line, a wavelength of little significance for most colorless compounds; however, much use was made of these measurements as analytical and characterization tools. In the 1930's, much pioneer work in the field of ORD was done by Lowry (4) and Kuhn (5, 6); they, as well as Mitchell (7), were the first to apply CD to organic chemical problems. Instrumentation still prevented the widespread use of the techniques until the 1950's when commercial ORD instruments became available and, more recently, when CD-measuring devices were introduced. Measurements by both techniques are frequently made down to wavelengths of 185 mμ. For detailed discussions on the theory, instrumentation, and applications of ORD–CD, the reader is referred to books by Crabbé (8), Djerassi (9), and Velluz *et al.* (10), and to reviews by Heller and Fitts (11), Klyne and Parker (12), Moscowitz (13), Tinoco (14), Schellman (15), and Eyring *et al.* (16).

The rotation at any constant wavelength is usually expressed as a specific rotation defined as

$$[\alpha]_\lambda^t = \frac{100\alpha\lambda}{lc} \qquad \text{(Eq. 1)}$$

where $[a]_\lambda^t$ is the specific rotation at a temperature t and a wavelength λ, $\alpha\lambda$ is the observed rotation, l is the pathlength in decimeters, and c is the concentration in grams per 100 ml. of solution; or as a molecular rota-

tion $[M]_\lambda$ defined as

$$[M]_\lambda = \frac{[\alpha]M}{100} \qquad \text{(Eq. 2)}$$

where M is the molecular weight. The optical rotation of polymeric substances is usually considered a function of individual residues rather than the total molecular weight, and the mean residue rotation is defined as

$$[M]_\lambda = \frac{MRW}{100}[\alpha]_\lambda \qquad \text{(Eq. 3)}$$

where MRW is the mean residue weight which is around 115 for proteins. Optical rotation is dependent on the refractive index of the solvent; the observed rotation can be approximated to vacuum conditions by the Lorentz correction factor, enabling a reduced mean residue rotation:

$$[M']_\lambda = \frac{3}{n^2 + 2}\frac{MRW}{100}[\alpha]_\lambda \qquad \text{(Eq. 4)}$$

to be obtained. For more precise work, the dispersion in the refractive index of the solvent must be considered.

More information concerning the primary or secondary structure of a compound is gained by measuring rotations at a series of wavelengths, and the resultant plot of rotation against wavelength is called the rotatory dispersion curve for the solute.

ORIGIN OF OPTICAL ACTIVITY

A beam of plane polarized light can be considered to be made up of a right circularly polarized wave E_R and a left circularly polarized wave E_L. No optical rotation is observed if these two components are transmitted with equal velocity through the medium. However, if the two vectors pass through the medium with unequal velocity, then the plane of their resultant will have rotated through an angle α. If the right circularly polarized component travels faster, then the medium is dextrorotatory; conversely, if the left travels faster, then it is levorotatory. The speed of a lightwave is a function of the refractive index of the medium, and the medium is optically active if it has different indexes of refraction (n_L and n_R) for the left and right circularly polarized light. Fresnel (2) has shown that the rotation in radians per unit length is given by

$$\alpha = \frac{\pi}{\lambda}(n_L - n_R) \qquad \text{(Eq. 5)}$$

and, using the more familiar specific rotation in degrees per decimeter,

$$[\alpha] = \frac{1800}{\lambda}\left(\frac{n_L - n_R}{c}\right) \qquad \text{(Eq. 6)}$$

The refractive indexes n_L and n_R vary differently with the wavelength and are approximated by

$$n = 1 + \frac{a\lambda^2}{\lambda^2 - \lambda_v^2} \qquad \text{(Eq. 7)}$$

where a is a constant function of the strength of the oscillator of characteristic wavelength λ_v. λ_v is a constant and is the same as that in London's equation for intermolecular attraction (17). This change in refractive index with wavelength means that the specific rotations also are a function of wavelength; in the regions far from optically active absorption bands, they give rise to plain curves, the equations for which have been given by Drude (18):

$$[\alpha] = \sum_i \frac{K_i}{\lambda^2 - \lambda_i^2} \qquad \text{(Eq. 8)}$$

where λ_i's are the wavelengths of the optically active electronic transition and K_i's are constants proportional to the rotatory strength of the ith transition. In the simplest case this modifies to the single-term Drude, giving

$$[\alpha] = \frac{K}{\lambda^2 - \lambda_0^2} \qquad \text{(Eq. 9)}$$

where λ_0 is the wavelength of the closest absorption maximum. The equation clearly indicates an increase in optical rotation as the wavelength is decreased.

Suppose, however, the medium shows unequal absorption of the incident right and left circularly polarized light; then the emerging light is also elliptically polarized and the phenomenon of unequal absorption is known as circular dichroism. To a good approximation (8), it can be shown that the angle of ellipticity ψ is given by

$$\psi = \frac{\pi}{\lambda}(K_L - K_R) \qquad \text{(Eq. 10)}$$

where K_L and K_R are the absorption coefficients of the left and right circularly polarized light. A specific ellipticity $[\psi]$ is analogous to the specific rotation, and a molar ellipticity $[\theta]$ is given by (19)

$$[\theta] = \frac{[\psi]M}{100} = 2.303\frac{4500}{\pi}(\epsilon_L - \epsilon_R) = 3300(\epsilon_L - \epsilon_R) = 3300\Delta\epsilon \qquad \text{(Eq. 11)}$$

where ϵ_L and ϵ_R are the molar extinction coefficients for the left and right circularly polarized light. The extinction coefficients also vary with wavelength, and the ellipticity of the medium varies with wavelength, enabling a circular dichroism curve to be obtained. A CD curve is only a function of the unequal absorption of the incident light, but the ORD curve is a function of the unequal transmission as well as the unequal absorption of light, the shape of the ORD curve depending on the proximity of any optically active chromophores (8, 9).

The combination of unequal absorption and unequal transmission of the left and right circularly polarized light in the region in which optically active absorption bands occur is known as the "Cotton effect," after the man who first observed these anomalous rotations (7, 20, 21). Whether single or multiple Cotton effects are noticed depends on the number of optically active absorption bands involved; obviously, the wavelength regions of the Cotton effect are of most interest in ORD studies. The molecular amplitude, a, of a Cotton effect is defined as the difference between the molecular rotation at a peak or trough and the molecular rotation of the next extremum of shorter wavelength divided by 100.

This molar amplitude has been shown as a corollary of the Kronig-Kramer theorem (8, 22, 23) to be ap-

proximated to the dichroic absorption ($\Delta\epsilon$) of a CD curve by

$$a = 40.28\Delta\epsilon \qquad \text{(Eq. 12)}$$

or, using molar ellipticity $[\theta]$,

$$a = 0.0122[\theta] \qquad \text{(Eq. 13)}$$

This relationship allows at least semiquantitation between ORD and CD, but it is emphasized that the relationships were obtained for the n-π^* transition of a saturated carbonyl group and should be used with caution for other chromophores (8).

To transform CD spectra into ORD spectra, one must use the more general form of the Kronig-Kramer equation (9, 24):

$$[M]_\lambda = \frac{2}{\pi}\int_0^\infty [\theta]\left[\frac{\lambda'}{\lambda^2 - \lambda'^2}\right]d\lambda' \qquad \text{(Eq. 14)}$$

This integral can be approximated by summation, but the interval must be small, preferably 1 mμ or less.

ORD OF PROTEINS

For a fuller treatment and references, the reader is referred to reviews by Urnes and Doty (25), Yang (26), and Gratzer and Cowburn (27).

Visible and Near UV Region—The $[\alpha]_D$ as an oversimplification can be considered to be made up of two contributions, first a levorotatory one from the amino acid residues and second a dextrorotatory one arising from a secondary and tertiary structure. Typically, a protein shows levorotation $[\alpha_D]$, which is lowered to the range -80 to $-120°$ upon denaturation (28). It was always realized that because the sodium D-line was so far from the absorbing groups and the rotations measured were small and difficult to quantitate, ORD would give better quantitative data.

Early studies were performed on the synthetic poly-γ-benzyl-L-glutamate (29) and poly-L-glutamic acid (30, 31) at wavelengths through the visible region down to 300 mμ. These investigations showed that in helix-promoting solvents the polypeptides had anomalous dispersion which was dextrorotatory at long wavelengths, passing through a maximum and then dropping steeply at shorter wavelengths, whereas in the coil-promoting solvents the ORD curve was simple and levorotatory in the visible region. These observations suggested that ORD would be a powerful tool in elucidating the secondary structure of proteins. The β-forms of polypeptides were also shown to contribute to the optical rotation (29). The oligomers of γ-benzyl-L-glutamate were known to form β-aggregates in a poor solvent such as chloroform, and the ORD of the concentrated solution was dextrorotatory throughout the visible region, in strong contrast to the anomalous dispersion of the α-helix and the levorotation of the coiled forms. The β-aggregates dissociated on dilution, and ORD curves similar to the coiled forms were obtained.

UV Regions—The laboratories of Blout (32, 33) and Doty (34, 35) in the early 1960's reported ORD and CD measurements on polypeptides in the UV region; Doty's group also measured the CD of polypeptides

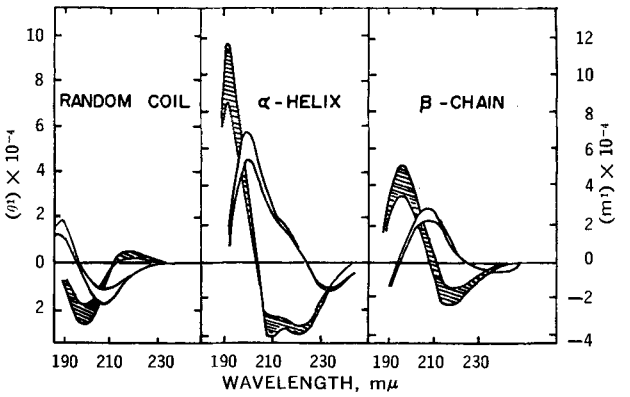


Figure 1—*CD (shaded) and ORD of homopolypeptides in the random coil, α-helical, and β-conformations. Taken from* Reference 27 *with permission of MacMillans (Journals) Ltd., London, England.*

down to 190 mμ, and they calculated the ORD curve using the Kronig-Kramer transform and obtained reasonable agreement with the experimental curve. The use of ORD in protein chemistry is mainly to determine conformations; the currently accepted curves for the various forms, taken from *Reference 27*, are shown in Fig. 1. Cotton effects may also arise due to aromatic groups such as tyrosine, tryptophane, and phenylalanine, as well as disulfide bonds (26, 36, 37).

Polypeptides containing less than 50% α-helix obey the simple Drude equation, and the constant wavelength term is usually denoted by λ_c; however, λ_c lacks the physical meaning of the wavelength of the nearest optically active absorption band, and it is the result of a mathematical approximation. For denatured or nonglobular proteins, λ_c is near 220 mμ and it is from 230 to 270 mμ for native proteins. λ_c, like $[\alpha_D]$, can be used to estimate the helix content if it is assumed that only the helix and a coil are responsible for the changes. These methods are of only historical interest, and the interested reader is referred to the excellent paper of Yang and Doty (29). In 1956, Moffitt (38) used a quantum mechanical approach to the rotatory dispersion of helices; he, together with Yang (39), published a phenomenological equation to explain the ORD of helical macromolecules to enable an estimate of the percentage of the α-helix to be made:

$$[M']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \qquad \text{(Eq. 15)}$$

The constants, λ_0 and b_0, are primarily functions of the helical content alone and therefore were regarded as independent of environmental factors such as solvent or side chains; a_0, however, represents the intrinsic residue rotations and varies with the environment. Using the suggested value of 212 mμ for λ_0 (40, 41), λ_0 has been set at 212 mμ and gives a value for b_0 for the 100% helical conformation of -630; assuming that b_0 for the coiled form is close to zero, the fraction in the helical form is given by

$$\frac{-b_0}{630}$$

Numerous modifications of the Moffitt equation have been proposed (see *References 25* and *26* for discussion

and further references), but the method gives reliable results if only random coil and α-helical conformations are present. In days when Cotton effects can be directly observed and of readily available CD measurements, the Moffitt equation is really obsolescent. Also, obsolescent are the numerous attempts to use various modification of the multiterm Drude equations to explain the ORD of polypeptides and calculate the percentage α-helix (25, 26). For a critical evaluation of Moffitt's equation, the two-term Drude, and a modified two-term Drude, see the work of Blout's group (40–42).

Wada *et al.* (43) have modified the Moffitt equation to include terms for the β-conformation which had previously been ignored; however, such interpretation is complex, but it appears that the b_0 value is only slightly affected by aggregation. It should be pointed out that Cotton effects due to aromatic residues which appear in many proteins probably invalidate the use of Moffitt's and the various Drude equations for quantitative interpretation.

The advent of equipment capable of measuring ORD spectra down to 185 mμ enables the Cotton effects to be observed directly, and attempts to calculate the percentage helix can be made by obtaining the curve of a model compound in the fully α-helical form or in the fully random coiled form and finding the conformation of the protein under investigation by simple proportion. Using poly-L-glutamic acid as a model compound, measurements at 233 and 198 mμ have been recommended (44); again the presence of β-aggregates complicates the picture, particularly as the β-form contribution is variable (45).

CIRCULAR DICHROISM OF PROTEINS

For a general discussion, see the excellent articles by Beychok (37, 46). CD curves for homopolypeptides in the random coil α-helical and β-conformations are shown in Fig. 1. The percentage of any form in a protein can be estimated from the values of the ellipticities at specific wavelengths of reference synthetic materials known to be in the various configurations. This technique is simplified if the β-form is again ignored as is often the case (35, 37, 47). The CD of β-forms has been investigated in detail by Fasman and Potter (45) and Quadrifoglio and Urry (48). Aromatic side chains and disulfides in polypeptides give rise to CD spectra in the 250-mμ → 350-mμ region (37, 49). It should also be noted that the amino acids, tyrosine, tryptophane, phenylalanine, and cystine, show intense side-chain optical activity near 220 mμ at all pH's and whether free or as amides and esters (37). This means that estimating the α-helix content and sense for proteins containing a high proportion of these residues is very risky because this side-chain absorption closely overlaps the most important peptide bands. CD has recently been shown to provide a quick criterion of nonidentity between two proteins having similar electrophoretic mobilities and elution profiles (50).

OPTICAL ACTIVITY OF NUCLEIC ACIDS

The origin of optical activity in the monomeric components of the nucleic acids, the purine and pyrimidine

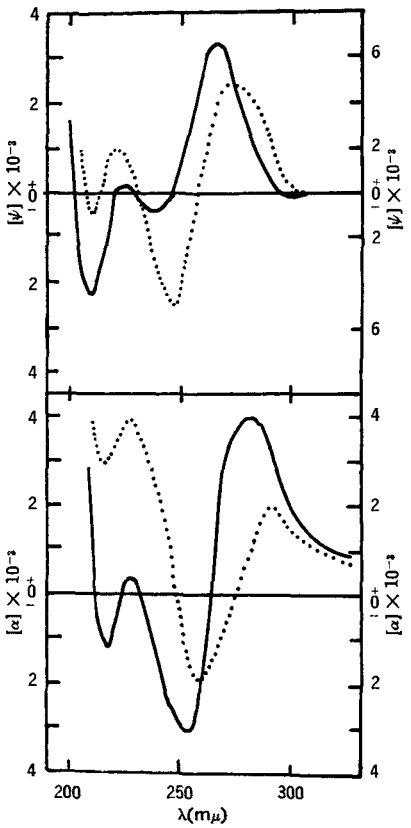


Figure 2—*CD (upper) and ORD (lower) of salmon DNA (. . .) and* E. coli *ribosomal RNA* (——). *Taken from* Reference 54 *with permission of Dr. J. T. Yang and Academic Press, New York, N. Y.*

ribosides and 2′-deoxyribosides, at the characteristic UV absorption bands of the aglycones is in the asymmetric perturbation of the aglycone chromophore by the glycosidic portion. The rotation varies as a function of substituent changes in the sugar moiety and as a function of changes in the torsion angle (the angle describing the conformation about the glycosidic bond). It has been generally assumed that the torsion angle for most monomeric nucleosides and nucleotides is in the range corresponding to the *anti*-conformation, but recent work (51, 52) demonstrates that for a number of nucleosides (both purine and pyrimidine ribosides) a significant proportion of the conformer equilibrium is composed of the *syn*-form. It is unknown, at present, whether this new information will significantly alter the current status of the nucleic acid conformation problem to be discussed.

When the monomers are joined by the familiar 3′,5′-phosphodiester linkage to form nucleic acids, one might expect the polymer rotation to be similar to the monomer rotation, differing perhaps by the small perturbation occasioned by the functional group change at C-3′, C-5′. Indeed, that is the observation for unordered polymers (53), but the helical order that can be assumed under appropriate conditions by DNA and some RNA's is characterized by an enhanced optical activity. The complex subject of optical activity of the nucleic acids has been reviewed recently (27, 54) and only the highlights will be discussed here.

Nucleic acid structure can be considered on three different levels, the single-stranded "random" coil, the

single-stranded stacked-base form, and the double-helical form of which there is an A and a B form. The A form of DNA and the double-helical forms of RNA are thought to have similar structural features differing from the B form of DNA in a 15–20° tilt of the base pairs relative to the helix axis compared with virtually no tilt in the B form.

The ORD and CD differences of DNA and RNA may in some cases be rationalized on the basis of the various fundamentally different forms available, but in some cases the observed differences have not been rationalized. Extensive ORD studies of DNA and RNA (55–62) have demonstrated that DNA and RNA from different sources show fairly typical behavior, being characterized by two peaks and a trough in the vicinity of the 260-mμ absorption band (Fig. 2). The relative magnitudes of these characteristic maxima and minima are the distinguishing characteristics that usually allow differentiation between DNA and RNA. The characteristics are best seen in the respective circular dichroism spectra that show a positive ellipticity followed by an intense and a weak negative curve for salmon sperm DNA and a positive effect followed by weak and strong negative effects for double-stranded RNA (63). Extensive prior circular dichroism studies (64–68) recorded similar results.

The first and second ORD peaks of DNA are temperature-dependent as is the first peak of helical RNA. When the variation between rotation at 290 mμ and temperature is studied for DNA, a sharp drop in rotation is seen at the helix–coil transition temperature determined by other means. The same study on helical RNA shows the typical broad melting behavior of that system.

The gross differences in the ORD–CD spectra of DNA and RNA do not arise from double helix–single helix random coil differences, although these forms have typical rotational characteristics. The differences have not been fully rationalized, but some correlations between structure and rotation phenomena do exist. The reader is referred to the review by Yang and Samejima (54) as well as the recent work of Johnson and Tinoco (69 and references therein).

OPTICAL ACTIVITY OF POLYSACCHARIDES

The study of polysaccharide structure *via* the optical rotation method has historically been carried out at the sodium D-line. The current status of the problem has been reviewed (70). Even when ORD and CD were applied, the non-UV absorbance of most sugars and many polysaccharides made interpretation difficult. It is, therefore, not surprising that less is known about the solution conformation of polysaccharides than about proteins and nucleic acids. Recent reports (71–73) show that the mucopolysaccharides, chondroitin sulfate and heparin, have Cotton effects in the region below 220 mμ arising from n-π^* and π-π^* amide transitions.

The mucopolysaccharide ORD spectra are of two different types depending on structure. The 3-1-linked glycosamine sugars show a negative Cotton effect (trough at 217–220 mμ), whereas the 4-1-linked glycosamine sugars (α- or β-glycosidic link) show a dominance of the positive Cotton effect in the π-π^* region (~198 mμ) (see Fig. 3). It is thought that the differences may be derived from a preferential association of the 'C-2' nitrogen with the prior C-2 hydroxyl in the first case and of the C-2 nitrogen with the succeeding 2', 3', or 4' hydroxyl in the latter case.

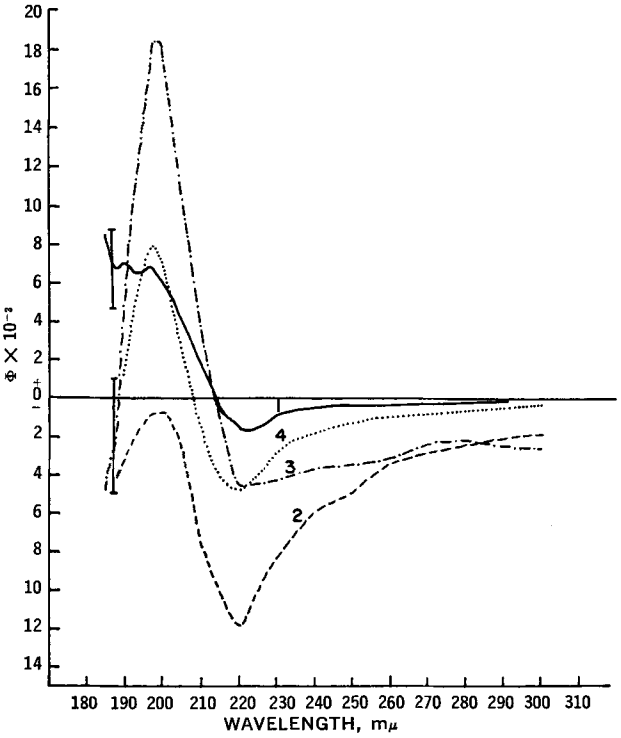


Figure 3—*ORD of mucopolysaccharides containing* N-*acetyl glucosamine. Curve 1,* N-*acetyl glucosamine; 2, heparin A; 3, chondroitin sulfate-I; 4,* S-*chondroitin sulfate. Taken from* Reference 70 *with permission of Marcel Dekker, Inc., New York, N. Y.*

That the mucopolysaccharides appear to have optical properties that depend largely on the way the sugars are linked in the polymer regardless of the configuration of the glycosidic bond indicates that rotatory properties derived from the monomer (74) are probably insufficient indexes of the polymer structure. It is concluded that there is a preferred twisting of the polymer strand imposing unique perturbations on the amide chromophores. The observation of these new amide transitions will no doubt allow more informative studies of polysaccharide secondary structure in solution to be made in the future.

SURVEY OF OTHER METHODS

The reader is referred to the classical review on protein binding by Goldstein (75) for details and references on other experimental methods and to the review by Meyer and Guttman (76) for references concerning specific drugs. These methods, summarized below, can be used for the study of binding of small molecules to macromolecules in general.

Dialysis—The equilibrium method is by far the most widely applied and is capable of giving good quantitative data, provided reasonable experimental precautions, such as a careful pretreatment of the dialysis tub-

ing, are followed. Faster kinetic dialysis techniques have been reported by several workers (77–79).

Ultrafiltration—This is also a widely practiced equilibrium technique which is quicker than equilibrium dialysis but is quantitatively inferior because of the continually changing macromolecule concentration and the accumulation of the macromolecule on the membrane surface.

EMF and Conductivity—Here the incorporation of buffers in the system is an obvious problem and the methods are only useful for studying the binding of ions.

Electrophoresis—This method is qualitatively useful in that it can be possible to find to which macromolecule fraction the small molecule is bound. Precise quantitation is difficult.

Spectroscopy—The method can be useful if the spectra of either the small molecule or the macromolecule is modified in the presence of the other, but in many the interactions cause little or no spectral changes.

NMR and ESR—The use of NMR to study biological interactions has been pioneered by Burgen (80) and Jardetzky (81). The differential changes of the relaxation rates in the high-resolution NMR spectra of both small and large molecules can be used to identify the area of the molecule directly involved in the formation of complexes. Other references can be found in the reviews (82, 83). Electron spin resonance spectroscopy has been applied by studying the ESR spectra of bound free radicals (84). The nature of the ESR spectra is sensitive to the environment, and rapid molecular motion can be detected.

Other methods which have been used include polarography, gel filtration, fluorescence quenching, adsorption, diffusion, the measurement of partition coefficients, and Kerr constant dispersion.

METACHROMASIA

A recurrent phenomenon that arises in the study of binding processes by ORD–CD is metachromasia (metachromasy). Metachromatic complexes are characterized by a shift to lower wavelength of the absorption maximum of the bound species as well as a decrease in its absorbance (hypochromism). The substance that can induce metachromasia in a dye is called a chromotrope. The usual chromotropic substances of biological origin are high-molecular weight polyanions. Heparin, chondroitin sulfate, hyaluronate, nucleic acids, agar, and alginate are such substances. Some inorganic and synthetic chromotropes are silicates, polyphosphates, chitin sulfate, carboxymethylcellulose, alginate sulfate, pectin sulfate, and polyacrylates. Some relatively low-molecular weight substances are chromotropic such as soaps and anionic detergents, *e.g.*, myristate, surface-active agents (Duponol), and phospholipids (85, 86).

Dyes that stain metachromatically are ordinarily metachromatic by themselves and do not obey Beer's law; it is concluded that metachromasia, whether in the presence or absence of macromolecules, is a function of intermolecular dye–dye association. When chromotropes are present, the initial binding of either the dye aggregate or the dye monomer followed by aggregation at the appropriate concentration is thought to be the sequence of events (87, 88). In fact, when a normally metachromatic dye (methylene blue, toluidine blue) is mixed at low concentration with a substance that normally stains orthochromatically (fibrin, gelatin, and most tissue and cellular structures), the orthochromatic color is seen. Orthochromicity refers to the lack of absorbance maximum shift on binding. Only if the dye concentration is increased does one eventually see metachromasia (89). In addition, ligands that do not ordinarily aggregate in the absence of binding agents (acranil, atebrin, neomonacrin, proflavin, 9-aminoacridine, and some other aminoacridines) show a shift to higher wavelength when bound to nucleic acids, and this bathochromic shift is thought to involve dye–polymer interactions (85, 90, 91). The same observation has recently been made in a study of the binding of proflavin to poly(styrene sulfonic acid) (92).

Elegant studies of the binding of DBTC (4,5,4′,5′-dibenzo-3,3′-diethyl-9-methylthiacarbocyanine) to a large variety of polyanions (93–95) revealed that the dye molecule reacts to form at least five distinct complexes depending on the macromolecule, the polymer–dye ratio, and the pH. Four of the complexes are characterized by a shift of the dye absorbance to higher wavelengths and only one is metachromatic. The complexes are thought to involve dye–polymer interactions, resulting in conformational distortion of the dye, dye–counter ion interactions on the polymer, and perhaps in some cases dye–dye interactions, although these are thought to be of minor importance. Dye–counter ion interactions have been invoked as an alternative rationalization for metachromasia by other workers (96).

PROTEIN INTERACTIONS

Detergents—Putnam and Neurath (97–99) pioneered the investigations into the denaturation of proteins by detergents using techniques such as viscosity, electrophoresis, and precipitation. In these investigations of the binding of dodecyl sulfate to horse serum albumin, they concluded that two distinct complexes were formed, the first occurring without detectable changes in the shape of the albumin but the second only occurring with considerable change in asymmetry of the protein. The complexes were said to be due to the binding of detergent anions to cationic protein groups, this erroneous conclusion coming from the similarity between the number of detergent ions bound and the number of binding sites available. Equilibrium dialysis (100–102) was later used to determine accurately the number of detergent anions bound to serum albumin.

Foster (103), among others, suggested that correlation between the number of anions bound and the number of cationic sites was fortuitous, suggesting that the hydrophobic nature of the anion plays an important role in the binding to the proteins. The application of optical rotatory dispersion to the detergent-binding problem has been made mainly by Bruno Jirgensons at the University of Texas. Using a sodium lamp in his earlier work (104, 105), he noticed that detergents produced a negative shift in the specific rotation and this shift was accompanied by an increase in viscosity. Using the same technique (106) to investigate the $[\alpha]_D$ of the plant pro-

teins, tuburin and pea legumin, denatured with sodium dodecyl benzene sulfonate, he again observed an increased negative rotation, and the effect of the detergent became more marked as the pH increased. In this paper he states that optical rotatory power seems to be one of the surest methods of measuring denaturation, a strong statement when one considers he was measuring at a single wavelength far from the absorbing groups in the proteins; one is not surprised at his immediate interest in obtaining dispersion curves when instrumentation became available.

Using the Rudolf instrument (107), he found that all the proteins he investigated obeyed the single-term Drude equation and that on denaturing a metal-binding β-globulin with AOT and heating to 50°, the dispersion constant of the denatured material was shifted from 243 to 220–230 mμ, whereas the constants for the Bence-Jones protein and γ-globulin increased under the same conditions from 200–215 to 220 mμ. Realizing the work of Foster (103), Kauzmann (108), and Tanford (109), emphasizing the importance of hydrophobic bonds in the folding of proteins, he proceeded to investigate the possibility that the extreme efficiency of the unfolding of globular proteins by surface-active agents (greater than urea, guanidine salts, and alcohol on a molar basis) might be due to the hydrophobic nature of the tail of the detergent molecule (110). Using the Yang and Doty (29) method of obtaining λ_c from the single-term Drude, and suggesting that an increase in λ_c above 220 mμ reflects an α-helix formation, he found that detergent-treated γ-globulins, pepsin, and soybean trypsin inhibitor showed a limited formation of α-helices. b_0 values from the equation of Moffitt and Yang (39) rose to values close to -80, indicating α-helix formation.

In the treatment of α- and β-caseins with AOT, the λ_c of α-casein was enhanced considerably while the value for the β-form was not affected. It was also observed that the detergents were more active the longer their hydrophobic chain; for example, the 10-carbon chain of sodium caprate caused λ_c of γ-equine to shift to 234 mμ from the native value of 210, whereas the same concentration of the 6-carbon chain of sodium caproate caused little or no change in λ_c. He concluded that the hydrocarbon chain of the detergent penetrates into the interior of the proteins, partially unfolding them, these conformational changes being greater the longer the hydrophobic tail of the detergent. Heating the systems to 50° always caused an increased denaturation, presumably due to the fact that at the elevated temperature the micelle-monomer equilibrium of the detergent is shifted toward the free molecules, facilitating the intervention between the protein and the detergent. In an earlier inconclusive paper (111), Jirgensons had found that a bovine albumin, a human albumin, and mercapto albumin had not been denaturated by several long-chain sulfosuccinates at 50 or 70°, using no change in λ_c as the criterion. Low reduced viscosities seemed to confirm these observations. Similar observations had been made regarding the effect of detergents on taka-amylase (112), another helical protein. Using improved instrumentation capable of observing the trough of the Cotton effect at 225–240 mμ due to the α-helical conformation, Jirgensons (113) again turned his attention to effects of detergents on the nonhelical proteins. He found that the proteins, reduced and carboxymethylated serum albumin, normal serum γ-globulin, myeloma globulin, pepsin, trypsin, trypsinogen, α-chymotrypsin, chymotrypsinogen, and soybean trypsin inhibitor did not show the Cotton effect, but that the Cotton effect was observed with troughs at 225 to 240 mμ if the hydrophobic chain of the added detergent was long enough. Sodium dodecyl, sodium decyl, and sodium octyl sulfates, as well as sodium dioctyl sulfosuccinate, were used in the investigations. These observations supported the view that the chains of the detergent disturbed the hydrophobic forces essential for the conformation of the nonhelical proteins.

A paper, again involving the nonhelical proteins and anionic detergents, also published in 1962 (114), contained similar observations. Instrumentation allowing the α-helical trough around 230 mμ to be investigated led Jirgensons (115) to look now at the α-helical proteins such as serum albumin, insulin, growth hormone, lysozyme, bacterial α-amylase, lactic dehydrogenase, and glutamic dehydrogenase. All these proteins showed a definite Cotton effect at 225–235 mμ, and dodecyl sulfate diminished the levorotation of these helical proteins to a greater or lesser extent. The effects were greater with dodecyl than with decyl sulfate. These effects are opposite to those observed earlier for the nonhelical proteins and suggest that the detergent can promote disorganization of helical proteins. The disulfide-linked macromolecules seemed more resistant to this disorganization than did the cystine-free bacterial amylase. This supported the hypothesis that the conformation of the helical proteins also depends upon hydrophobic bonds. In this paper Jirgensons mentions the existence of the β-conformation (25) for the first time. Again turning to the nonhelical proteins, human serum γ-globulin, and myeloma protein (116), treating them with sodium decyl sulfate in alkaline conditions of pH 10.2–11.0 at an elevated temperature of 50°, he found that the b_0 values were initially close to zero but became negative on denaturation and fragmentation by the detergent. The levorotation was also increased; although b_0 returned almost to zero on removing the detergent by dialysis, the levorotation stayed enhanced. He concluded structural changes in the following steps: native ordered → strongly disorganized and fragmented → partially organized → strong disorganized and aggregated fragments. The γ-globulin is split into fragments of molecular weight 56,000 to 75,000 by detergent; however, the observations are complicated by the fact that disulfide and ester bonds are sensitive to alkali.

In another paper (117), again looking at detergents and nonhelical proteins, including the plant proteins, α-conarachin and edestin, he found b_0 to be near zero for the native proteins and negative on treatment with detergent. The specific rotation also became more negative, but he agreed with the statement by Tanford *et al.* (109) that no reliable conclusions concerning conformation can be drawn from the changes of $[\alpha]_D$, especially in the instances when the properties of the solvent are drastically changed. In the same paper it was found that the nonionic surfactant (Brij 35) was less effective than the anionic detergent with the same chain length,

and that lysozyme and pituitary growth hormone are only slightly affected by detergents even on heating. He concluded that the native globular proteins having a b_0 value of near zero are not α-helical yet possess some order in the folding of the polypeptide chains. So at this time there was a possibility that the structure of these proteins in aqueous solution included the β-form. Wada *et al.* (43) had expanded the Moffitt equation in 1961 to take into account the β-configuration; Troitski (118), a Russian worker using a manual spectropolarimeter and a lowest wavelength of 365 mμ, used it to interpret his investigations into the effect of sodium decyl sulfate on γ-globulin, egg albumin, and serum albumin. He concluded that γ-globulin loses its β-structure and retains its α-structure on treatment with detergent. On the other hand, egg albumin lost 35% of its β-form, but the α-helix content did not change and there was no change at all in the conformation of serum albumin. The author concluded that the detergent degrades the β-structure but not the α-helix, and he suggested that detergents may be specific reagents for the β-configuration in the correct conditions. These observations regarding the α-helix were contrary to those of Jirgensons, and this, together with the claims for the β-structure in aqueous solutions of specially treated poly-α-L-lysine (119, 120) and silk (121), stimulated Jirgensons (122, 123) to further investigations. The proteins having high α-helix content are slightly disorganized, as shown by changes in the amplitudes of the Cotton effect and b_0 values. The disordered proteins such as histones are partially disorganized by the detergents, whereas those with considerable β-conformation like desoxyribonuclease and β-lactoglobulin seem to lose their β-form and acquire some α-helical form. Several other proteins having some β-structure as well as the α-helical form were also investigated, but no clear interpretation is presented; however, the possibility of a somewhat different β-structure is mentioned. In other publications concerning the histone F1 (124, 125), he again reported that decyl and dodecyl sulfate convert the disordered histone partially into the α-helical form. Decker and Foster (126), investigating the interaction of bovine plasma albumin with alkyl benzenesulfonates at pH 6.5, found an initial binding involving 11 $\pm$ 1 sites and two other complexes involving 38 and 76 detergent molecules. This is in agreement with the complexes of Putnam and Neurath (98) who used dialysis and moving-boundary electrophoresis experiments. The formation of the higher complexes was found by ORD experiments to be accompanied by some loss of α-helix content of the protein.

Again looking at the nonhelical proteins in 1967 (127), Jirgensons found that tetradecyl sulfate was more effective than dodecyl sulfate in promoting helical formation, again confirming that the hydrocarbon chain plays a decisive role in protein conformation. In their classical paper on the β-form of poly-L-lysine in aqueous solution, Sarkar and Doty (119) found that disordered poly-L-lysine in alkaline solution went to the β-configuration on heating; whereas treatment with sodium dodecyl sulfate in neutral solution seemed to produce a β-form, forming a peak at 205 mμ and a trough at 230 mμ. However, the magnitude of the trough was greatly reduced. Supporting evidence for the β-form was obtained from IR, UV, and CD. These observations stimulated further work on synthetic polypeptides, and Grouke and Gibbs (128) found that sodium dodecyl sulfate shifted the random-coiled form of poly-L-ornithine in neutral solution of the α-helical form using ORD and CD methods. This α-helix formation is accompanied by the shift of the ORD minimum to 235 mμ. The α-helical form of poly-L-ornithine was unchanged on heating; a similar treatment on poly-L-lysine had caused the formation of a β-structure (119).

Velluz and Legrand (129), using entirely CD techniques, found that treatment of human serum albumin (HSA) with dodecyl sulfate decreases the helical content to 40%. Treatment of HSA with detergent and 2-mercaptoethanol (reducing the S—S bonds) causes disappearance of the dichroism in the 250–300-mμ region (due to loss of S—S bonds) whereas the dichroism in 200–250 mμ remains constant, suggesting no change in α-helix content. The S—S bonds act as protectors of the rigid structures before denaturation, probably by maintaining a conformation which isolates the sensitive groups from the medium; the detergent seems to prevent the collapse of the rigid structures when the S—S bonds are broken. This is not so on denaturation with urea. The effect of delipidization of lipoproteins has been investigated by ORD and CD (130), but the results are confusing. CD, ORD, and IR show that β-lipoprotein is disordered and probably has some helical structure; the β-apoprotein retains its β-structure, but on treatment with dodecyl sulfate the proteins become more disordered and some α-helix formation may be induced. The conformational changes which had been reported earlier on delipidization (131, 132) are probably caused by the presence of dodecyl sulfate. Dodecyl sulfate has no effect on the configuration of β-lactoglobulin polymorphs A, B, and C as measured by CD (133).

The Cotton effect at near 280 mμ due to tryptophane residues in egg white lysozyme disappears on exposure to sodium dodecyl sulfate (134) at concentrations which do not affect the α-helix content but do completely inhibit the enzyme activity. This suggests that tryptophane is involved in the formation of the enzyme substrate complex. Similarly, dimethyl benzylmyristylammonium chloride did not affect the secondary structure of lysozyme (135) or acetyl lysozyme (136) but did reduce the enzyme activity. The binding of various sulfates and sulfonates to bovine serum albumin has been investigated (137), and it was found that $[\alpha]_{233}$ changes in a linear manner up until 10 moles of detergent are bound per protein molecule; the rotation remains constant as more detergent is added until a large configuration change takes place when between 45 and 60 moles are bound. This may again be due to the alteration of conformation of the tryptophane residues.

As can be seen from this, the interpretation of the ORD and CD spectra of detergent–protein complexes is difficult and is complicated by the fact that detergents may increase α-helical content (30), convert helical to β-structure (27), abolish β but not helical structure (26), or act as a denaturating agent (19) with various proteins.

Dyes—In 1958, Markus and Karush (138) studied the effect of anionic dyes on the rotatory dispersion of HSA at pH 7.4 from 725 mμ to 550 mμ and found that strongly anomalous dispersion curves were obtained. Using a manual polarimeter, it was found that the $[\alpha]_D$ depended on the substituent in the *p*-position of the *p*-benzene-azo-benzoylamino acetic acid dyes. On removing the dyes, all changes were completely reversible. Correlation with viscosity and dialysis experiments suggested to the authors that the changes in the observed $[\alpha]_D$ were due either to structural changes in the serum albumin involving interaction with the end groups of the dyes or the stabilization of resonance forms of the dyes. However, in a later paper, having obtained improved instrumentation, Winkler and Markus (139) showed that the earlier reported anomalous rotation was due to an artifact (stray light) and that the sudden changes in rotation between 500 and 600 mμ were no longer observed. The curves obtained still showed very strong deviations from the dispersion of HSA in the absence of dye. The earlier conclusions obtained from the sodium D-line measurements were, of course, not affected by these observations.

Blout and Stryer (140) were the first to report a Cotton effect from a polypeptide–dye complex at wavelengths near those of the absorption bands of the dyes. This indicated that the chromophoric group of the dye had acquired asymmetry. L-Polyglutamic acid (141, 142) was used with acriflavine and neutral rhodamine 6G, and it was found that complexing, and the resulting Cotton effects at wavelengths greater than 400 mμ, only resulted when the peptide was in the helical form (below pH 6.0); above pH 6.0 the peptide is in a random configuration, and no anomalous dispersion is observed. The magnitude of the Cotton effect of acriflavine-L-PGA complex as a function of pH showed good agreement with the helix content as determined from $[\alpha]_{546}$. In further investigations with a wider range of dyes, Stryer and Blout (143) again found complexing only in the pH region of high helical content and found molar rotations up to a million for the induced Cotton effects. The signs of the Cotton effects were opposite for the L- and D-polyglutamic acids, leading to the conclusion that helices of opposite screw sense show Cotton effects of opposite sign. Three models were suggested (Fig. 4) for the interaction between the peptide and dye; the first involved an unaggregated dye interacting with the asymmetric α-carbon atom of the peptide residue; the second involved dye end-to-end aggregation giving rise to a super helix around the peptide helix. In the third, the asymmetric polypeptide acts as a sterically determined "seeding center" to favor one of the screw senses of a dye helix which forms tangential to the polypeptide α-helix. None of the models satisfied all the observations (143, 144), although the latter two seem more feasible.

Blout (144) reported that covalent binding of a single dye molecule to a helical macromolecule results in an extrinsic Cotton effect; this does not prove, however, that all dye–polypeptide Cotton effects are the result of a single dye molecule bound to an asymmetric site in the macromolecule. Using the streaming dichroism technique (145), Ballard *et al.* (146) investigated the binding of acridine orange with the α-helical form of poly-α-L-

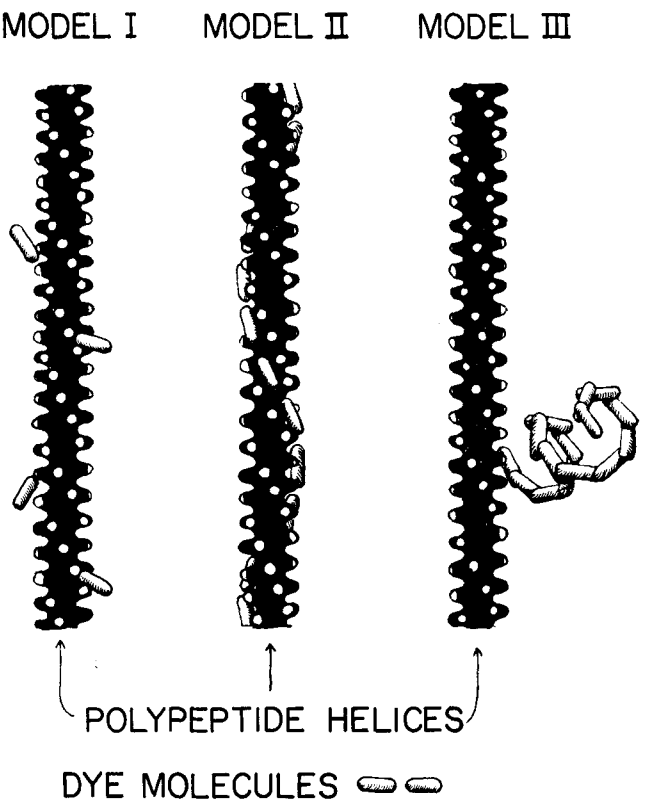


Figure 4—*Three suggested modes of interaction between dye and polypeptide. Model I, unaggregated dye interacts near the asymmetric α-carbon atom of the peptide residue; model II, dye end-to-end aggregate giving rise to dye superhelix; model III, tangential dye helix. Taken from* Reference 144 *with permission of the editors and Interscience Publishers, New York, N. Y.*

glutamic acid. They found three circular dichroism bands associated with the long wavelength absorption band of AO at 495 mμ and further CD bands associated with the 270-mμ absorption band of the dye but none associated with the 295-mμ absorption band. The induced activity was relatively insensitive to the glutamate residue-to-dye ratio but was dependent on the ionic strength of the solution. It was concluded that the dye aggregate in the L-PGA complex has the form of a left-handed super helix bound to the core of a right-handed α-helix of L-PGA. AO binds strongly to PGA over the pH region of the transition from a random coil to α-helix, and Myhr and Foss (147) decided to reinvestigate the random coil region of binding. They stress that the method of preparing the complex influences the ORD properties; complexes prepared by changing the PGA conformation in the presence of AO are not the same as those prepared by first changing the PGA conformation and then adding the dye. Their observations suggest that the dye prevents conformational changes in the random coil pH region, and they state that this probably prevented Stryer and Blout from observing optical activity of the random coil complex. Preparing complexes by titrating the L-PGA to the desired pH before adding the dye, they found large Cotton effects in the region of the AO absorption bands for both the helical and random coil pH regions. The curve for the helical form was very similar to that of Stryer and Blout. Yamaoka and Resnik (148) reinvestigated the interaction between helical α-PGA and

acridine orange at several dye-to-polymer ratios (D/P) using ORD. Even at a D/P of 0.0001, a broad positive peak was located at 525 mμ, a steep negative trough with a shoulder around 480 mμ, and a second peak at 450 mμ was also found. The curves were in good agreement with those of Stryer and Blout; however, the complexing at very low D/P ratios agreed with the observations of Mason's group (146), which reported binding of approximately two AO molecules per three polymer molecules. This led to support for Blout's first model, *i.e.*, binding of unaggregated dye near the asymmetric α-carbon atom of the peptide residue. The resultant CD curves from the Kronig-Kramer transform, when compared with the measurements of Mason's group (146), show good agreement with the positions and signs of all three extrema, but there is not good agreement on the magnitude of the rotations. Yamaoka and Resnik (149) also investigated the interaction of helical L-PGA and proflavine and found extrinsic optical activity throughout the absorption bands of the dye, but no new peaks or shoulders were found. The induced Cotton effects were shown to be multiple by the Kronig-Kramer transform. This analysis also shows a weak background rotation which may suggest a slight alteration in the polypeptide conformation when the dye is bound.

Eyring *et al.* (150), reinvestigating the PGA–acridine orange system, found significant differences in the ORD curves depending on whether the pH was adjusted before or after mixing. Heating had little or no effect on the curves except in the case of the helical form above 55°, where a decrease in rotation was observed; this was reversible on cooling. Their complexes with helical PGA are very similar to those of Stryer and Blout (143), and they correctly point out that Stryer and Blout overlooked the Cotton effects of the coiled PGA–AO complex by approximating their data with a smooth curve. Yang's group reported Cotton effects with the coiled PGA, although they were much smaller in magnitude than those found with the helical form. There are considerable differences between their data and that of Myhr and Foss, possibly due to differences in experimental conditions.

Others—The binding of coenzymes, substrates, inhibitors, *etc.*, has been investigated by ORD and CD, and particular interest has been shown in extrinsic Cotton effects generated by a chromophoric molecule interacting with an asymmetric site of the protein as in the case of the dyes. Considerable interest has been shown in the interaction of liver alcohol dehydrogenase and nucleotides where a Cotton effect is seen close to the absorption maximum of the complex (151–153). The stoichiometry of this binding has been shown to be two moles of nucleotide per mole of enzyme.

Heme has a plane of symmetry, but the iron atom constitutes a center of asymmetry for a prosthetic group since different ligands may occupy the fifth and sixth coordination sites of the metal atom and because the protein may be linked both to iron and the porphyrin side chains; thus considerable interest has been shown in the optical dispersion of heme proteins (154–157), particularly concerning the Cotton effect in the Soret band. The extrinsic effects of these and other protein-substrate bindings were reviewed by Ulmer and Vallee in 1965 (158) and only the more recent developments, particularly the obvious usage of CD, will be discussed here. Myoglobin and hemoglobin exhibit a positive Cotton effect in the Soret band of the visible spectrum, regardless of the state of iron oxidation or nature of the ligand in the sixth coordination position of the Fe atom (156, 159). As heme is directly bound to the histidine residue which is part of a right-hand helix segment (160), Beychok (37) investigated the poly-L-histidine–hemin complex but found little correlation with the myoglobin spectra, the situation being complicated by the fact that heme aggregates and binding occurs with monomers as well as aggregates. Urry (161), in a theoretical paper concerning the heme–protein interactions, predicts that the rotational strengths of the proteins change on the interaction and that the changes in the peak and trough of the ORD of apomyoglobin upon binding of heme (162, 163) should not be interpreted as changes in helical content. He also proposed that a substantial contribution to the rotational strength of the Soret transition in these heme proteins arises from coupling with the protein transitions. Concentrating on the troublesome region below 300 mμ, Beychok's group (164) concluded that the major contribution to the 260-mμ CD band of hemoglobin was from heme rather than aromatic residues. The difference in ellipticity in the 260-mμ region between the α- and β-chains reflected a considerable difference in the heme environment in the two cases, or a difference in tightness of the attachment, or both. Side-chain chromophores, rather than heme, were found to generate the longer wavelength bands near 280 to 290 mμ. In a later paper, Javaherian and Beychok (165) found that binding of one hemin per apohemoglobulin dimer derived from horse hemoglobin caused restoration of the helix content, not only of the chain having hemin bound but almost all of the helix content in the other chain as well, even without occupation of the heme binding site of that chain. In the same paper they investigated the CD of hemoglobin Gun-Hill (166) and again found that heme is bound only to α-chains. Others (167), investigating the same UV region of human hemoglobin and its subunits, have shown that binding of oxygen and carbon monoxide causes large changes in optical rotation. The changes at 233 mμ were taken to prove a conformational change in the protein but whether both chains are involved was not clear. Similar observations were not made with myoglobulin. Darnall *et al.* (168) have recently investigated the CD spectra of methemerythrin with the iron coordinated to a variety of ligands. They concluded that contributions in the 290-mμ region were from tyrosine residues and from the iron atoms. In the peptide n-π^* and π-π^* region, the CD was similar to that of an α-helical conformation, and this did not change with the nature of the iron bound ligand or upon dissociation of the protein into monomers. The extrinsic spectra above 300 mμ due to the heme binding have been well investigated by CD. Myer (169) found that the different oxidation states of the iron in heme of horse heart cytochrome-C caused differences in the Soret region, as well as in the aromatic dichroic bands, and there was an increase of all transitions in the δ absorption region seen in the lower oxida-

tion states. He said that the change in the valence state induces significant alterations in the disymmetrical environments of the prosthetic heme group and the aromatic side chains, possibly due to the increased ligand-binding affinity of the ferrous state. Strickland (170) investigated the CD spectra of horseradish peroxidase complexed with its enzyme substrate compounds and found wavelength shifts in the Soret region similar to those observed by UV absorption spectrophotometry. In the 280-mμ region the CD of the complex differed from the native enzyme, indicating that heme may contribute to the ultraviolet CD and that the orientation of an aromatic side chain may change. In the 207–222-mμ region the CD spectrum of the protein is unchanged on complexing and suggests the α-helical conformation.

Recently, Japanese workers (171, 172) found that oxidized cytochrome cc′ shows three types of reversible CD spectra in the 300–450-mμ region depending on pH: type I (neutral), type II (intermediate), type III (alkaline). In the reduced state it has an abnormal spectrum in the pH regions of types I and II, but it has normal spectrum at more alkaline pH's. Alcohols, phenols, and ketones convert type I and type II spectra to type III at fixed pH values, and the efficiency is parallel to the hydrophobicity of the ligand. At pH 7.4, 25% of 2-propanol converts the oxidized form from the neutral to the alkaline type but causes no change in the far UV region, whereas urea at pH 7.0 causes destruction of the α-helix but only a slight shift to the alkaline type in the extrinsic regions. These observations show that a shift from neutral to the alkaline type is not necessarily accompanied by a change in the gross helical conformation which might protect the special environment of the heme. Similar observations were made by Tsong and Sturtevant (173) using an apoenzyme from cytochrome b_2 free of flavin mononucleotide (FMN). They found a CD spectrum strongly dependent on the oxidation states of heme, and the CD spectrum below 250 mμ was that of an α-helix and independent of the heme. When the enzyme was treated with FMN and *l*-lactate, 80% of the original enzyme activity was recovered and the CD spectrum of the original enzyme was obtained. Denaturing by aging changed the CD spectra below 250 mμ but not above. The enzyme could be partially reactivated to give the original CD spectra. These results suggest direct heme–FMN interaction is responsible for the catalytic and optical properties of cytochrome b_2. Van Holde (174) found that the oxygenated hemocyanin gave two dichroic bands in the visible region; on comparison of this spectrum with some peptide–copper (II) complexes, he saw a similarity between the hemocyanins and histidine-containing peptides, suggesting that perhaps the histidine residues are ligands in hemocyanin. Li and Johnson (175) have recently measured the ORD properties of several fetal and abnormal hemoglobins.

The ORD of rhodopsin, before and after irradiation, has been measured (176) and the α-helical content appeared to be diminished after bleaching, and there was a possibility that unbleached rhodopsin also showed a weak Cotton effect in the visible region. Later CD investigations (177) confirmed the extrinsic Cotton effects for rhodopsin and isorhodopsin near the absorption wavelengths of the retinal chromophore. Crescitelli

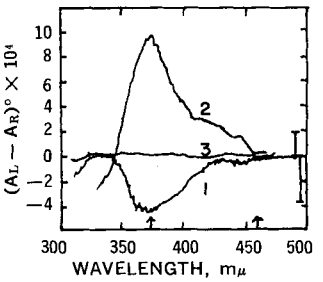


Figure 5—*CD spectra of free FAD 1 and the bound FAD 2. Curve 3, base line. Vertical bars indicate scales corresponding to* $\epsilon_L - \epsilon_R = 1$ *for Curves 1 and 2, respectively. Taken from* Reference 180 *with permission of Academic Press, New York, N. Y.*

et al. (178) also observed the extrinsic CD bands with rhodopsin and porphyrodopsin, although they differ in behavior in the near UV region; the extrinsic CD bands disappear upon bleaching. The extrinsic CD spectra of squid and cattle rhodopsin (179) have been found to differ, probably due to the difference in protein conformation around the site of attachment of the retinal chromophore. Upon illumination the helical structure of the proteins was converted to a disordered one, and the retinal chromophore lost its optical activity. This change in protein conformation upon illumination is considered to be an important role in initiating the visual process.

One of the more interesting investigations concerning enzymes and their substrates is the binding of flavin adenine dinucleotide to D-amino acid oxidase (180). There is a conformational change of the dinucleotide as a result of the binding to the apoenzyme; this is shown by the inversion of the dinucleotide CD spectrum as seen in Fig. 5 without any appreciable wavelength shift. Apparently the binding between the two rings is preserved, but the mutual steric orientation is markedly changed when the nucleotide is bound to the apoenzyme. Other polypeptides whose interactions have been investigated by ORD or CD include glutamine synthetase (181), ribonuclease (182), bakers yeast *l*-lactate dehydrogenase (183), horse liver alcohol dehydrogenase (184), aspartate transcarbamylase (185), lypolydehydrogenase (186), glutamate dehydrogenase (187–189), pig heart lactate dehydrogenase (190), avidin (191), immunoglobulin (192), muscle phosphorylase B (193), glutamate-aspartate transaminase (194), and lipoproteins (195, 196).

Sonenberg (197) has found that human growth hormone induces changes in the ellipticity of human erythrocyte membranes at 222 mμ, observing a decrease in the negative band of about 30% in a phosphate buffer but not in water. The necessary presence of phosphate suggests that some organization of the membrane is necessary. Similar effects were not observed with bovine growth hormone, bovine serum albumin, bovine insulin, or cortisol. Lenard and Singer (198) had previously investigated the ORD and CD of red blood cells and found approximately one-third in the helical conformation and the remainder in the random coil; however, Urry and Ji (199) have shown that particulate systems can show CD patterns characteristic of an α-helix and caution must be exercised in interpreting data from particulate systems.

Hansch *et al.* (200) have correlated protein binding of a variety of organic compounds of miscellaneous structure with their octanol–water partition coefficients, so demonstrating the nonspecific nature of the process. In the same paper this partition coefficient is related to the

protein binding and the rotation at the Na_D line; however, whether the change in rotation is due to a change in configuration and/or to any induced optical activity of the organic molecule is not apparent.

Chignell (201–203) has studied the interaction of phenylbutazone and some of its analogs as well as a number of other anti-inflammatory substances with BSA and HSA and has found that many of these substances exhibit extrinsic Cotton effects. When phenylbutazone was mixed with human serum albumin, a positive ellipticity band was generated at 287 mμ. Similar observations were made for oxyphenbutazone and sulfapyrazone, although the rotational strengths of the latter substances were smaller. More polar derivatives gave greatly reduced rotational strengths, and the reduction was positively correlated with a poorer distribution of the polar derivatives into nonpolar media.

Fifteen *N*-arylanthranilates, including flufenamic and meclofenamic acids as complexes with human serum albumin, were studied (203), and extrinsic Cotton effects were observed for all of them. Metachromasia was not observed. The binding of flufenamic acid to human, porcine, equine, bovine, canine, ovine, and rabbit serum albumins induced optical activity in all cases, but there were significant ellipticity differences observed and, in particular, a reversal of the sign of the long wavelength band was seen for the ovine and rabbit proteins.

NUCLEIC ACIDS

Acridines—Acridine binding to nucleic acids has been actively studied almost from the time that selective staining of nuclear material by dyes was observed. Interest stems from the function of some of these dyes as antibacterial agents (204) and mutagens (205, 206). Studies of the nucleic acid–dye complexes have involved most of the methods mentioned previously, and a portion of those studies has been reviewed, including some of the optical rotatory dispersion–circular dichroism work to be described (54, 207). A large proportion of the more recent work has been devoted to a determination of the stoichiometry of binding as well as to the structure determination of the complexes

The stoichiometry of acridine dye binding to nucleic acids has been studied extensively and can be expressed in terms of the quantity (r) of dye (ligand) bound per mole of nucleic acid phosphorus against the concentration of free aminoacridine. The definition of r is

$$r = \sum_{J=1}^{J=P} \frac{n_J k_J c}{1 + k_J c} \qquad \text{(Eq. 16)}$$

where n_J is the number of P classes of binding sites (of intrinsic binding constant k_J) for the ligand on the nucleic acid (207). If there are two classes of binding sites, Eq. 16 simplifies to

$$r = \frac{n_I k_I c}{1 + k_I c} + \frac{n_{II} k_{II} c}{1 + k_{II} c} \qquad \text{(Eq. 17)}$$

and in the case of a single type of binding site, Eq. 17 becomes

$$r = \frac{nkc}{1 + kc} \qquad \text{(Eq. 18)}$$

When two or three binding sites are present, it is sometimes possible to select certain linear portions of the *r/c versus r* plots and attribute the linear portions of them to specific binding sites, allowing calculation of individual n_J's and k_J's. However, if the assumption of Langmuir-type binding (implicit in Eq. 18) does not hold because of cooperative phenomena in which a bound ligand promotes the binding of further ligands, it may not be possible to recognize the contribution of each binding site in the *r/c* plot.

The binding of acridine orange to nucleic acids apparently involves two processes as shown by the curved *r/c versus r* plots found by Peacocke (90), a strong binding process (I) involving about 0.2 molecule of dye per nucleic acid monomer and a weaker process (II) with a 1:1 dye–monomer ratio. It has recently been shown (208) that the binding is adenyl or guanyl specific and that the weak complex (II) can be distinguished from the strong (I) by hot dialysis or by fluorescence spectroscopy (209). The weak complex dissociates at room temperature and fluoresces at 620 mμ, whereas the strong complex is stable as high as the denaturation temperature of DNA and fluoresces at 515 mμ.

Efforts to define the structure of the various nucleic acid–dye complexes were abetted when an extrinsic Cotton effect between 450 and 540 mμ (the wavelength region in which DNA-bound acridine orange absorbs) was observed (210) (see Fig. 6). The observed finite rotation must have been due to asymmetric perturbation of the dye chromophore by the DNA, because, by itself, acridine orange is optically inactive. At the same time (210) the observation was made that heat-denatured DNA induced no Cotton effect in bound acridine orange, but later work (211, 212) using denatured calf thymus DNA disclosed an induced Cotton effect in the bound dye. In addition, it has been shown that acridine orange binds to poly A, poly U, and poly C (213).

It was further shown that proflavin displayed an extrinsic Cotton effect when bound to both DNA and RNA (214), followed by similar observations on 1,2,3,9-tetraaminoacridine (215).

Given the observation of induced Cotton effects, it was natural to exploit the observations further as they held a high potential for allowing one to learn more about the nucleic acid–dye complexes. What follows is an historical account of the attempts to establish the structure of the previously mentioned nucleic acid–dye complexes by considerations of the induced optical activity at the absorption maxima of the dye and the dependence of that rotation on temperature, polymer/dye ratios, pH, and ionic strength.

The originally observed induced optical activity in native DNA-bound acridine orange was quite weak at very high polymer-to-dye ratios of around 56. As the P/D ratio decreased from 13 to 1 (maximum dye binding), a strong anomalous dispersion curve developed at 504 mμ, then gave way to a weaker Cotton effect at 465 mμ. The observation corresponds to the metachromatic and hypochromic phenomena that had previously been observed spectroscopically. The observed optical activity was thought, at that time, to arise from neighboring, weakly coupled dyes in the case of the higher P/D ratios and to arise from bound dye-

aggregates (strongly coupled) in the case of the metachromatic and hypochromic spectrum at low P/D ratios.

At about the same time, Lerman (216) studied the binding of several dyes (acridine orange among them) in terms of viscosities and sedimentation coefficients. The studies were done in the concentration range that would correspond to the high polymer/dye ratio (strong binding). Because of increased viscosity, a decreased sedimentation coefficient (reduced mass per unit polymer length) and a loss of helicity, the hypothesis was advanced that the complex involved intrusion (intercalation) of the dye between two adjacent base pairs causing an extension of the sugar–phosphate backbone. The intercalation hypothesis was presumably supported by later polarized fluorescence work (217) showing that the dye was oriented perpendicularly to the helix axis and by chemical studies (218) that showed an inhibition of diazotization of the primary amino groups of some acridine dyes (proflavin but not acridine orange). The latter observation was a unique consequence of binding to DNA because other polyanions had no effect on diazotization rate.

Circular dichroism of the DNA–acridine orange complex (one dye molecule per base pair) under static and streaming conditions (145, 219) showed a strong positive band at higher wavelengths (505 mμ) and a weaker negative band at shorter wavelengths (467 mμ). These bands increased and decreased, respectively, on streaming, indicating that the 505-mμ band is polarized perpendicularly to the helix axis and the 467-mμ band is parallel polarized.

Since the 500-mμ band of the dye is a π-π^* transition requiring a component along the long axis of the dye, the conclusion was reached that the molecular planes of the acridine orange molecules are tipped relative to the helix axis. It was further concluded that the sign of the ellipticity required a left-handed helical arrangement of the dyes and the best such arrangement involves binding of the acridine ring nitrogen (conjugate acid) to the sugar–phosphate backbone.

The concentration range at which these streaming circular dichroism studies were run was probably in the intermediate range between monomer binding (strong) and aggregate binding (weak), however, and Blake and Peacocke (220) have suggested that the streaming measurements were made on a mixed complex, making the results appropriately ambiguous.

More extensive work by Gardner and Mason (221) dealt specifically with the ambiguities. By measuring circular dichroism over a range of ionic strength, pH, and P/D ratios, they were able to detect three ellipticity bands. Two negative bands at 465 and 488 mμ and one positive band at 505 mμ were observed, and they had optimum intensities at P/D ratios of 3, 9, and 4, respectively. Only the 505- and 465-mμ bands were observed at P/D ratios of less than 3 at low ionic strength and low pH, and those bands are diminished at large P/D ratios. The 488-mμ band was optimum at high ionic strength, neutral pH, and a larger P/D ratio. The 505- and 465-mμ bands were assigned to bound dimers, bound in the form of a skewed (dissymmetric) sandwich, and the 488-mμ band was thought to arise from the bound monomer. It was

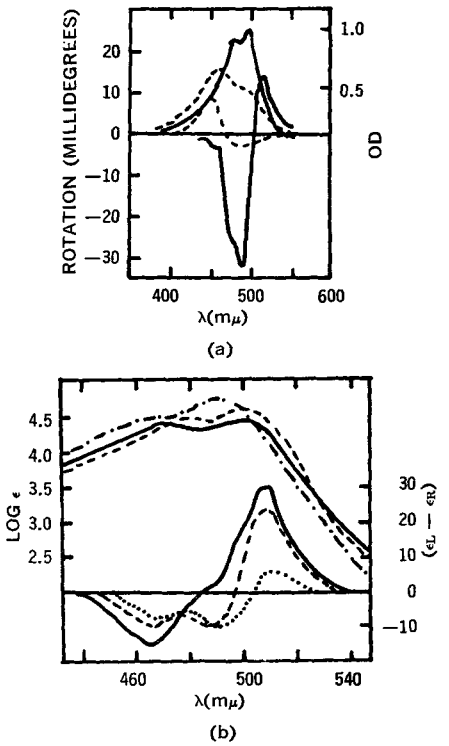


Figure 6—(a) *ORD and absorption spectra of acridine orange bound to native DNA at low (- - -), 28.48 to 15.68 μM, and high (——), 85.44 to 15.68 μM, polymer/dye ratios. The upper curve in each pair is the absorption spectrum.* (b) *CD and absorption spectra of acridine orange bound to native DNA at polymer/dye ratios: (——), 3 and (- - -), 9 and (. . .), 15. The upper curves are absorption spectra; (·—·) absorption spectrum of acridine orange in ethanol. Taken from* Reference 54 *with permission of Dr. J. T. Yang and Academic Press, New York, N. Y.*

proposed that the monomer was bound only partly between adjacent base pairs rather than completely occluded (modified intercalation hypothesis) on the basis of the present studies and the previous streaming circular dichroism studies (145).

The binding of proflavin to both DNA and RNA has been studied beginning with observations by Blake and Peacocke (222, 223). These authors studied the optical rotatory dispersion of proflavin bound to DNA and RNA in the 400–500-mμ spectral range. As in the case of acridine orange, no optical activity was observed for the free dye, but a large unsymmetrical Cotton effect (more intense trough than peak) was observed in the presence of DNA and RNA.

The proflavin–DNA complex was studied under conditions of changing ionic strength, DNA denaturation, and P/D ratio. Decreasing the ionic strength increased the extent of binding by a factor of 1.4 but increased the magnitude of the Cotton effect by a factor of 2. At all ionic strengths the magnitude of the Cotton effect increased linearly as the P/D ratio increased (change from type II to type I binding) to a value of about 4, then decreased to noise level with a further increase in the P/D ratio. A statistical treatment of the data indicated that only a small number of proximate dye molecules are required for the observation of optical activity, not an extended helical array as some workers have proposed in polysaccharide studies (224, 225). Denatur-

ation of the DNA did not destroy the optical activity, ruling out the need for long segments of double helix in order to observe optical activity, another requirement that was thought to prevail (140).

Because of the observation of optical activity in the denatured DNA complex, and because RNA is similar in structure to denatured DNA, optical rotation studies were conducted (222) on the RNA–proflavin complex and virtually identical observations were made. However, whereas in the DNA system a reduction of pH to 2.7 obliterated optical activity, which activity returned to higher values when the original pH was restored, the same pH titration in the RNA case was smoothly reversible from pH 7 to pH 2.7 and back to pH 7.

The differences between acridine orange and proflavin probably arise because of the much greater tendency for acridine orange to aggregate (226), and it was concluded that the "monomer" binding of acridine orange is identical with the strong binding of proflavin (type I, high P/D). Because strong binding was observed in denatured systems where double-helical structures are precluded, it was felt that the double-helical structure was not essential for strong binding and that instead a "close contact" between the dye molecules and the nucleic acid bases was the only requirement, leading only incidentally to intercalation in the double-helical structures. A study of the binding of proflavin to poly A and poly U at neutral pH, to poly A at acid pH, and to poly (A + U) led (227) to the conclusion that a relatively rigid conformation of the binding macromolecule is required for the induction of Cotton effects similar to the ones seen in denatured DNA systems. The observations that led to the conclusion were that the homopolymers at neutral pH induced no optical activity in the dye (even though it was bound as shown by other spectroscopic criteria) whereas at lower pH, a Cotton effect was induced. It was further found by a statistical treatment that interaction between as few as two ligands on denatured DNA and RNA was sufficient to generate optical activity.

Further work on the DNA–proflavin complex (149) showed that the unsymmetrical long-wavelength Cotton effect reported by Blake and Peacocke (222) was a composite curve. The latter workers applied the Kronig-Kramers transform to ORD data obtained under conditions similar to those used by Blake and Peacocke, and the calculated CD curves showed the presence of two transitions. The presence of two transitions in the high wavelength region was confirmed by a CD curve of the proflavin–DNA complex determined by Blake and Peacocke (228) which showed an unsymmetrical double-ellipticity curve characteristic of exciton interactions (229–236), confirming the optical activity was a function, in part, of interaction between relatively small numbers of bound chromophores.

Some work has been done (228) on the extent of acridine structure variation tolerated for the induction of Cotton effects by DNA and it has been found that 1,2,3-aminoacridines are optically active at most of the intrinsic ultraviolet absorption bands, but that 9-aminoacridine has optical activity induced only at lower wavelengths and that optical activity is not induced in the 350–500-mμ region.

Additional conclusions outside the scope of this review have been reached regarding the structure of the DNA–dye complexes (237–239).

Other Antibiotics—General studies of the binding of actinomycin D to DNA (240–243) and some related monomeric species have revealed the following characteristics of the complex: (*a*) binding is guanine specific; (*b*) binding occurs adjacent to a guanyl–cytidyl pair; (*c*) binding causes helix distortion; (*d*) several complexes exist in equilibrium; (*e*) the peptide side chains conform to the sugar–phosphate backbone so that there is a high energy barrier to conformational change within the complex; and (*f*) the complexes dissociate slowly. It was concluded that complexation was by intercalation. It has been further shown that actinomycin D is dimeric down to concentrations of 10^{-5} *M* and that it binds to nucleoside species in the following order of preference: deoxyguanosine, guanosine, adenosine, deoxyadenosine, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, inosine, and xanthosine.

The circular dichroism of free actinomycin D (211, 244, 245) has been determined in both water (dimer) and ethanol (monomer). The water spectrum shows one strong negative band at 380 mμ and two weaker negative ellipticity bands between 450 and 440 mμ. The ethanol spectrum is composed largely of one negative band. In the presence of DNA (211, 245, 246) the once weak 440-mμ band becomes strongly optically active and is shifted to around 460 mμ. There is a concomitant hypochromic effect that appears in the UV spectrum of the complex, indicating that the changes observed in the inherently optically active ligand are induced as a result of the binding process. The question of the structure of the complex has not been settled although Homer (245) proposes that either the actinomycin D dimer is bound or that the monomer is bound and the metachromatic-hypochromic effect is the result of association between the antibiotic and the purine moieties of the nucleic acid.

The cytotoxic antibiotic kanchanomycin, of unknown structure and possessing inherent optical activity, has been studied with respect to its binding to DNA, RNA, and synthetic polynucleotides by spectrophotometric methods (247) and by optical rotatory dispersion–circular dichroism (248). The binding process requires magnesium ion and, apparently, two different complexes are formed, an initial complex (I) that eventually rearranges to a second complex (II). The initial complex dissociates readily when magnesium ion is removed with EDTA and complex II dissociates more slowly. Circular dichroism spectra of the two forms represent significant departures from the spectrum of the free antibiotic. The latter, in the presence of magnesium ion, is composed of a small positive peak at around 405 mμ and two shallow negative extrema at 365 and 336 mμ. In the presence of magnesium and DNA, two extrema are apparent, a positive one at around 400 mμ and a negative one at 350 mμ. After 20 hr., a complex, more intense negative band at around 375 mμ appears, the spectrum of complex II. Binding of kanchanomycin to poly A gives similar results.

Assorted Ligands—The interaction of DNA with malouetine [5α-pregnan-3β,20α-ylenebis (trimethylammonium iodide)] and irehdiamine (pregn-5-ene-

3β,20α-diamine) has been thoroughly studied (249) by following changes in the rotatory properties of the nucleic acid. At low ionic strength, two different complexes are formed. The first, requiring a steroid/DNA-P ratio of about 0.2, is characterized by enhanced thermal stability, increased hyperchromicity at 260 mμ, and a shift of the long-wavelength circular dichroic band toward longer wavelengths with an increase in the rotational strength. It is thought that the disordering shown by the hyperchromicity is ligand-induced and may involve a transition of the double helical structure away from the β-form (see under *Optical Activity of Nucleic Acids*). The second complex requires a higher steroid/polymer ratio (greater than 0.2) and the nucleic acid rotatory values of this complex resemble disoriented DNA. The two forms are thought to resemble those assumed by DNA in the presence of planar aromatic rings and copper (II) ions, respectively.

The binding of purines as well as purine and pyrimidine nucleosides has been examined by a number of techniques, including rotation at a single wavelength (250, 251); although the rotation studies do not conform exactly with the subject of this review, the work is important and deserves mention. The interaction of poly U with many of the previously mentioned compounds has been studied. A large positive increase of the rotation at 350 mμ was observed when binding occurred and it was found that 1:1 complexes that were temperature sensitive formed between poly U and adenosine, deoxyadenosine, L-adenosine, 3-isoadenosine, 9-(γ-hydroxypropyl) adenine, and 9-(hydroxypentyl) adenine. No interaction was found for *N*-6-methyladenosine, *N*-1-methyladenosine, and the nucleoside antibiotic tubercidin.

A large number of aryl-substituted diammonium compounds have been observed to display extrinsic Cotton effects when bound to native and synthetic nucleic acids and polynucleotides (252–254) and have been adopted as reporter molecules utilizing CD as the spectroscopic probe.

For nitroanilines of the type $Ar(CH_2)_MN^+(CH_3)_2$-$(CH_2)_3N^+(CH_3)_3 \cdot 2Br^-$, a red shift and hypochromism are observed for the complexed (DNA and RNA) state. Of further interest is the induction of a positive and a negative Cotton effect in the aromatic chromophore by RNA and DNA, respectively. The evidence seems to indicate a binding of the diammonium group to the anionic phosphate backbone and that the aryl portion lies in the minor groove of the helix. The origin of the oppositely signed Cotton effects is thought to arise from differential conformational preference of the 2,4-dinitroaniline function, depending on the nucleic acid used.

Polysaccharides—The original work of Stone *et al.* (71, 224, 225) on extrinsic Cotton effects in acidic polysaccharide-bound dyes has been reviewed (70). Extrinsic Cotton effects have been observed in dyes bound to λ-carrageenate, heparin, and the chondroitin sulfates (150). The polysaccharide-dye ORD spectra are complex in the region of the dye metachromatic absorption region. Acridine orange, methylene blue, and neutral red all show induced Cotton effects when bound to heparin and the effect disappears at high P/D ratios. At the time, the conclusion was reached that the effect arose from a helical array of dyes along the polysaccharide chain, but subsequent work in the nucleic acid series showed that a long helical array was not necessary, allowing other rationalizations in the polysaccharide area.

More recent work (150) has shown that chondroitin sulfate and acridine orange form two different complexes (neutral pH) depending on the order of mixing. The induced Cotton effect was more intense when the polysaccharide was added to the dye, the apparent reason being related to dye self-association. This order-of-mixing dependence disappeared at low pH.

The binding of a number of azodyes to α-cyclodextrin has been studied (255) by the ORD technique and only congo red displayed reasonably strong extrinsic Cotton effects that appeared outside the rather strong positive background rotation of α-cyclodextrin.

CONCLUSION

It is apparent from the foregoing that the application of optical rotatory dispersion–circular dichroism to the study of small-molecule binding by macromolecules is able to provide certain specific information and is potentially able to provide much more. It is possible to decide when binding occurs by observing the induction of optical activity in symmetrical chromophores by asymmetrical macromolecules. Of greater interest is the ability to decide whether the chromophore is intimately involved in the binding process and how it is involved. Further, a determination of the effects of binding on the macromolecule is important. Both the latter kinds of information can potentially be obtained from ORD–CD measurements, but greater knowledge of the correlation between macromolecular structure and rotational data is needed as is a more thorough knowledge of the origin of the induced Cotton effect.

A noteworthy recent development that will prove useful in the future, particularly in the study of ligand effects on macromolecular structure, is the difference spectropolarimetric method adopted by Adkins and Yang (256), a method useful in the study of small conformational changes. The method was used to estimate the equilibrium constant for the lysozyme–*N*-acetyl-D-glucosamine interaction.

The interpretation of measurements made on protein systems, if in terms of conformational changes, must take into account recent precautions regarding Cotton effects derived from intrinsic aromatic functional groups as well as recent measurements (257) that reveal how environment alone can change the rotatory properties of cyclic mono- and dipeptides, compounds that are thought to be electronic analogs of polypeptides. The study of membrane-bound small molecules requires that particular attention be paid to the problem of artifacts arising from absorption, flattening, and scattering treated by Urry and Ji (199).

Future studies should concentrate on the careful accumulation of a broader variety of good data and a more rigorous study of the question of optical activity induced by intermolecular interaction. Further, as better correlations between optical activity and conformation in macromolecular systems become available, ligand

effects on that conformation should be studied, perhaps by application of the difference technique mentioned previously.

REFERENCES

(1) J. B. Biot, *Mem. Acad. Sci.*, **2,** 41(1817).

(2) A. Fresnel, *Ann. Chim. Phys.*, **28,** 147(1825).

(3) L. Pasteur, *ibid.*, **24,** 442(1848).

(4) T. M. Lowry, "Optical Rotatory Power," Longmans, Green and Co., Ltd., London, England, 1935.

(5) W. Kuhn, in "Stereochemie," K. Freundenberg, Ed., Deulicke, Leipzig, Germany, 1933.

(6) W. Kuhn, *Ann. Rev. Phys. Chem.*, **9,** 417(1958).

(7) S. Mitchell, "The Cotton Effect," G. Bell, London, England, 1933.

(8) P. Crabbé, "ORD and CD in Organic Chemistry," Holden-Day, San Francisco, Calif., 1965.

(9) C. Djerassi, "Optical Rotatory Dispersion Applications to Organic Chemistry," McGraw-Hill, New York, N. Y., 1960.

(10) L. Velluz, M. Legrand, and M. Grosjean, "Optical Circular Dichroism," Academic, New York, N. Y., 1965.

(11) W. Heller and D. D. Fitts, "Physical Methods of Organic Chemistry," 3rd ed., vol. 1, part III, Interscience, New York, N. Y., 1960, p. 2147.

(12) W. Klyne and A. C. Parker, "Physical Methods of Organic Chemistry," 3rd ed., vol. 1, part III, Interscience, New York, N. Y., 1960, p. 2335.

(13) A. Moscowitz, *Advan. Chem. Phys.*, **4,** 67(1962).

(14) I. Tinoco, *ibid.*, **4,** 113(1962).

(15) J. A. Schellman, *Accounts Chem. Res.*, **1,** 144(1968).

(16) H. Eyring, H.-C. Liu, and D. Caldwell, *Chem. Rev.*, **68,** 525(1968).

(17) S. Glasstone, "Textbook of Physical Chemistry," 2nd ed., D. Van Nostrand, New York, N. Y., 1946.

(18) P. Drude, "Lehrbuch der Optik," S. Hirzel, Leipzig, Germany, 1906.

(19) E. Bunnenberg, C. Djerassi, K. Mislow, and A. Moscowitz, *J. Amer. Chem. Soc.*, **84,** 2823(1962).

(20) A. Cotton, *Compt. Rend.*, **120,** 989(1895).

(21) A. Cotton, *Ann. Chim. Phys.*, **8,** 347(1896).

(22) R. de L. Kronig, *J. Opt. Soc. Amer.*, **12,** 547(1926).

(23) H. A. Kramer, *Atti Congr. Int. Fisici.*, **2,** 545(1927).

(24) W. Moffitt and A. Moscowitz, *J. Chem. Phys.*, **30,** 648 (1959).

(25) P. Urnes and P. Doty, *Advan. Protein Chem.*, **16,** 401 (1961).

(26) J. T. Yang, in "Poly α Amino Acids," G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1967.

(27) W. B. Gratzer and D. A. Cowburn, *Nature*, **222,** 426 (1969).

(28) C. Cohen, *ibid.*, **175,** 129(1955).

(29) J. T. Yang and P. Doty, *J. Amer. Chem. Soc.*, **79,** 761(1957).

(30) P. Doty, A. Wada, J. T. Yang, and E. R. Blout, *J. Polym. Sci.*, **23,** 851(1957).

(31) E. Tizuka and J. T. Yang, *Biochemistry*, **4,** 1249(1965).

(32) N. S. Simmons and E. R. Blout, *Biophys. J.*, **1,** 55(1960).

(33) E. R. Blout, I. Schmier, and N. S. Simmons, *J. Amer. Chem. Soc.*, **84,** 3193(1962).

(34) G. M. Holzwarth, W. B. Gratzer, and P. Doty, *ibid.*, **84,** 3194(1962).

(35) G. M. Holzwarth and P. Doty, *ibid.*, **87,** 218(1965).

(36) D. V. Myers and J. T. Edsall, *Proc. Nat. Acad. Sci., U. S.*, **53,** 169(1965).

(37) S. Beychok, in "Poly α Amino Acids," G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1967.

(38) W. Moffitt, *J. Chem. Phys.*, **25,** 467(1956).

(39) W. Moffitt and J. T. Yang, *Proc. Nat. Acad. Sci., U. S.*, **42,** 596(1956).

(40) E. Shechter and E. R. Blout, *ibid.*, **51,** 695(1964).

(41) M. Sogami, W. J. Leonard, Jr., and J. F. Foster, *Arch. Biochem. Biophys.*, **100,** 260(1963).

(42) J. P. Carver, E. Shechter, and E. R. Blout, *J. Amer. Chem. Soc.*, **88,** 2562(1966).

(43) A. Wada, M. Tsubio, and E. Konishi, *J. Phys. Chem.*, **65,** 1119(1961).

(44) J. T. Yang and W. J. McCabe, *Biopolymers*, **3,** 209(1965).

(45) G. D. Fasman and J. Potter, *Biochem. Biophys. Res. Commun.*, **27,** 209(1967).

(46) S. Beychok, *Science*, **154,** 1288(1966).

(47) M. Grosjean and M. Tari, *Compt. Rend.*, **258,** 2034(1964).

(48) F. Quadrifoglio and D. W. Urry, *J. Amer. Chem. Soc.*, **90,** 2760(1968).

(49) S. Beychok and G. D. Fasman, *Biochemistry*, **3,** 1675(1964).

(50) S. E. Kornguth and J. H. Perrin, to be published.

(51) P. A. Hart and J. P. Davis, *J. Amer. Chem. Soc.*, **91,** 512(1969).

(52) P. A. Hart and J. P. Davis, *Biochem. Biophys. Res. Commun.*, **34,** 733(1969).

(53) J. T. Yang, T. Samejima, and P. K. Sarkar, *Biopolymers*, **4,** 623(1966).

(54) J. T. Yang and T. Samejima, in "Progress in Nucleic Acid Research and Molecular Biology," J. N. Davidson and W. E. Cohn, Eds., Academic, New York, N. Y., 1969.

(55) T. Samejima and J. T. Yang, *Biochemistry*, **3,** 613(1964).

(56) M. R. Lambarg, P. C. Zamecnik, T. K. Li, J. Kagi, and B. L. Vallee, *ibid.*, **4,** 63(1965).

(57) M. M. Warshaw, C. A. Bush, and I. Tinoco, Jr., *Biochem. Biophys. Res. Commun.*, **18,** 633(1965).

(58) T. Samejima and J. T. Yang, *J. Biol. Chem.*, **240,** 2094 (1965).

(59) G. D. Fasman, C. Lindblow, and E. Seaman, *J. Mol. Biol.*, **12,** 630(1965).

(60) M. R. Lambarg and P. C. Zamecnik, *Biochem. Biophys. Res. Commun.*, **20,** 328(1965).

(61) C. M. Kay and K. Oikawa, *Biochemistry*, **5,** 213(1966).

(62) J. N. Vournakis and H. A. Sheraga, *ibid.*, **5,** 2997(1966).

(63) P. K. Sarkar, B. Wells, and J. T. Yang, *J. Mol. Biol.*, **25,** 563(1967).

(64) J. Brahms, *J. Amer. Chem. Soc.*, **85,** 3298(1963).

(65) J. Brahms and W. F. H. M. Mommaerts, *J. Mol. Biol.*, **10,** 73(1964).

(66) J. Brahms, *Nature*, **202,** 797(1964).

(67) J. Brahms, *J. Mol. Biol.*, **11,** 785(1965).

(68) J. Brahms, *Proc. Roy. Soc., Ser. A*, **297,** 150(1967).

(69) W. C. Johnson and I. Tinoco, *Biopolymers*, **7,** 727(1969).

(70) A. L. Stone, in "Structure and Stability of Biological Macromolecules," S. N. Timasheff and G. D. Fasman, Eds., Marcel Dekker, New York, N. Y., 1969, p. 353.

(71) A. L. Stone, *Biopolymers*, **3,** 617(1965).

(72) *Ibid.*, **7,** 173(1969).

(73) A. L. Stone, *Nature*, **216,** 551(1967).

(74) S. Beychok and E. A. Kabat, *Biochemistry*, **4,** 2625(1965).

(75) A. Goldstein, *Pharmacol. Rev.*, **1,** 102(1949).

(76) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57,** 895 (1968).

(77) H. H. Stein, *Anal. Biochem.*, **13,** 305(1965).

(78) A. Agren and R. Elofsson, *Acta Pharm. Suecica*, **4,** 281 (1967).

(79) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57,** 1627 (1968).

(80) J. C. Metcalfe, H. Marlow, and A. S. V. Burgen, *Nature*, **209,** 1142(1966).

(81) O. Jardetzky, N. G. Wade, and J. J. Fischer, *ibid.*, **197,** 183(1963).

(82) J. C. Metcalfe, A. S. V. Burgen, and O. Jardetzky, in "Molecular Associations in Biology," B. Pullman, Ed., Academic, New York, N. Y., 1968, p. 487.

(83) H. G. Mautner, *Ann. Rep. Med. Chem.*, **1968,** 240.

(84) O. H. Griffith and A. S. Waggoner, *Accounts Chem. Res.*, **2,** 17(1968), and references therein.

(85) L. Michaelis, *Cold Spring Harbor Symp. Quant. Biol.*, **12,** 131(1947).

(86) M. Schubert and D. Hamerman, *J. Histochem. Cytochem.*, **4,** 159(1956).

(87) B. Sylvén, *Quart. J. Microsc. Sci.*, **95,** 327(1954).

(88) D. F. Bradley and M. K. Wolf, *Proc. Nat. Acad. Sci., U. S.*, **45,** 944(1959).

(89) J. A. Bergeron and M. Singer, *J. Biophys. Biochem. Cytol.*, **4,** 433(1958).

(90) A. R. Peacocke and J. N. H. Skerrett, *Trans. Faraday Soc.*, **52,** 261(1956).

(91) D. S. Drummond, V. F. W. Simpson-Gildemeister, and A. R. Peacocke, *Biopolymers*, **3,** 135(1965).
(92) D. O. Jordan, T. Kurucsev, and M. L. Martin, *Trans. Faraday Soc.*, **65,** 612(1969).
(93) R. E. Kay, E. R. Walwick, and C. K. Gifford, *J. Phys. Chem.*, **68,** 1896(1964).
(94) *Ibid.*, **68,** 1907(1964).
(95) R. C. Bean, W. C. Shepard, R. E. Kay, and E. R. Walwick, *ibid.*, **69,** 4368(1965).
(96) P. J. Hillson and R. B. McKay, *Nature*, **210,** 297(1966).
(97) F. W. Putnam and H. Neurath, *J. Amer. Chem. Soc.*, **66,** 692(1944).
(98) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **159,** 195 (1945).
(99) *Ibid.*, **160,** 397(1945).
(100) F. Karush and M. Sonenberg, *J. Amer. Chem. Soc.*, **71,** 1369(1949).
(101) J. T. Yang and J. F. Foster, *ibid.*, **75,** 5560(1953).
(102) M. J. Pallansch and D. R. Briggs, *ibid.*, **76,** 1396(1954).
(103) J. F. Foster, in "The Plasma Proteins," F. W. Putnam, Ed., Academic, New York, N. Y., 1960.
(104) B. Jirgensons, *Arch. Biochem. Biophys.*, **39,** 261(1952).
(105) *Ibid.*, **48,** 154(1954).
(106) B. Jirgensons, *J. Polym. Sci.*, **5,** 179(1950).
(107) B. Jirgensons, *Arch. Biochem. Biophys.*, **78,** 235(1958).
(108) W. Kauzmann, *Advan. Protein Chem.*, **14,** 1(1959).
(109) C. Tanford, P. K. De, and V. G. Taggart, *J. Amer. Chem. Soc.*, **82,** 6028(1960).
(110) B. Jirgensons, *Arch. Biochem. Biophys.*, **94,** 59(1961).
(111) B. Jirgensons, *Tex. Rep. Biol. Med.*, **17,** 106(1959).
(112) T. Isemura and T. Takagi, *J. Biochem. Tokyo*, **46,** 1637 (1959).
(113) B. Jirgensons, *Arch. Biochem. Biophys.*, **96,** 321(1962).
(114) B. Jirgensons, *Makromol. Chem.*, **51,** 137(1962).
(115) *Ibid.*, **56,** 138(1962).
(116) B. Jirgensons, D. Vomezawa, and G. Orguraki, *Makromol. Chem.*, **60,** 25(1963).
(117) B. Jirgensons, *J. Biol. Chem.*, **238,** 2716(1963).
(118) G. V. Troitski, *Biofizika*, **10,** 895(1965).
(119) P. K. Sarkar and P. Doty, *Proc. Nat. Acad. Sci., U. S.*, **55,** 981(1966).
(120) B. Davidson, N. Tooney, and G. D. Fasman, *Biochem. Biophys. Res. Commun.*, **23,** 156(1966).
(121) E. Tizuka and J. T. Yang, *Proc. Nat. Acad. Sci., U. S.*, **55,** 1175(1966).
(122) B. Jirgensons, *J. Biol. Chem.*, **241,** 147(1966).
(123) *Ibid.*, **241,** 4855(1966).
(124) B. Jirgensons and L. S. Hnilica, *J. Amer. Chem. Soc.*, **88,** 2342(1966).
(125) B. Jirgensons and L. S. Hnilica, *Biochim. Biophys. Acta*, **109,** 241(1965).
(126) R. V. Decker and J. F. Foster, *Biochemistry*, **5,** 1242 (1966).
(127) B. Jirgensons, *J. Biol. Chem.*, **242,** 912(1967).
(128) M. J. Grouke and J. H. Gibbs, *Biopolymers*, **5,** 586(1967).
(129) L. Velluz and M. Legrand, *Angew. Chem. Int. Ed., Engl.*, **4,** 838(1965).
(130) A. M. Gotto, R. I. Levy, and D. S. Fredrickson, *Proc. Nat. Acad. Sci., U. S.*, **60,** 1436(1968).
(131) A. Scanu and J. L. Granda, *Progr. Biochem. Pharmacol.*, **4,** 153(1968).
(132) A. Scanu and R. Hirz, *Nature*, **218,** 200(1968).
(133) T. Seibles, *Biochemistry*, **8,** 2949(1969).
(134) A. N. Glazer and N. S. Simmons, *J. Amer. Chem. Soc.*, **87,** 2288(1965).
(135) K. Hayashi, M. Kugimiya, T. Imoto, M. Funatsu, and C. C. Bigelow, *Biochemistry*, **7,** 1461(1968).
(136) *Ibid.*, **7,** 1467(1968).
(137) J. A. Reynolds, S. Herbert, H. Polet, and J. Steinhardt, *Biochemistry*, **6,** 937(1967).
(138) G. Markus and F. Karush, *J. Amer. Chem. Soc.*, **80,** 89(1958).
(139) M. Winkler and G. Markus, *ibid.*, **81,** 1873(1959).
(140) E. R. Blout and L. Stryer, *Proc. Nat. Acad. Sci., U. S.*, **45,** 1591(1959).
(141) E. R. Blout and M. Idelson, *J. Amer. Chem. Soc.*, **78,** 497(1956).
(142) M. Idelson and E. R. Blout, *ibid.*, **80,** 4631(1958).
(143) L. Stryer and E. R. Blout, *ibid.*, **83,** 1411(1961).
(144) E. R. Blout, *Biopolym. Symp.*, No. 1, **1964,** 397.
(145) S. F. Mason and A. J. McCaffery, *Nature*, **204,** 468(1964).
(146) R. E. Ballard, A. J. McCaffery, and S. F. Mason, *Biopolymers*, **4,** 97(1966).
(147) B. C. Myhr and J. G. Foss, *ibid.*, **4,** 949(1966).
(148) K. Yamaoka and R. A. Resnik, *J. Phys. Chem.*, **70,** 4051 (1966).
(149) K. Yamaoka and R. A. Resnik, *Nature*, **213,** 1031(1967).
(150) E. J. Eyring, H. Kraus, and J. T. Yang, *Biopolymers*, **6,** 703(1968).
(151) D. D. Ulmer, T. K. Li, and B. L. Vallee, *Proc. Nat. Acad. Sci., U. S.*, **47,** 1155(1961).
(152) H. Theorell and R. Bonnichsen, *Acta Chem. Scand.*, **5,** 1105(1951).
(153) T. K. Li, D. D. Ulmer, and B. L. Vallee, *Biochemistry*, **1,** 114(1962).
(154) A. J. Osbahor and G. L. Eichhorn, *J. Biol. Chem.*, **237,** 1820(1962).
(155) G. Eichhorn, *Tetrahedron*, **13,** 208(1961).
(156) S. Beychok and E. R. Blout, *J. Mol. Biol.*, **3,** 659(1961).
(157) T. Samejima and J. T. Yang, *ibid.*, **8,** 863(1964).
(158) D. D. Ulmer and B. L. Vallee, *Advan. Enzymol.*, **27,** 37 (1965).
(159) S. Beychok, *Biopolymers*, **2,** 575(1964).
(160) J. C. Kendrew, R. E. Dickerson, B. E. Strandberg, R. G. Hart, D. R. Davies, D. C. Phillips, and V. C. Store, *Nature*, **185,** 422(1960).
(161) D. W. Urry, *Proc. Nat. Acad. Sci., U. S.*, **54,** 640(1965).
(162) S. C. Harrison and E. R. Blout, *J. Biol. Chem.*, **240,** 299 (1965).
(163) E. Breslow, S. Beychok, K. D. Mardman, and F. R. N. Gurd, *ibid.*, **240,** 304(1965).
(164) S. Beychok, I. Tyuma, R. E. Benesch, and R. Benesch, *ibid.*, **240,** 2460(1967).
(165) K. Javaherian and S. Beychok, *J. Mol. Biol.*, **37,** 1(1968).
(166) T. B. Bradley, R. C. Wohl, and R. F. Reider, *Science*, **157,** 1581(1967).
(167) M. Brunori, J. Engel, and T. M. Schuster, *J. Biol. Chem.*, **242,** 773(1967).
(168) D. W. Darnall, K. Garbett, and I. Klotz, *Arch. Biochem. Biophys.*, **133,** 103(1969).
(169) Y. Myer, *J. Biol. Chem.*, **243,** 2115(1968).
(170) E. H. Strickland, *Biochim. Biophys. Acta*, **151,** 70(1968).
(171) Y. Imai, K. Imai, R. Sato, and T. Horio, *J. Biochem. Tokyo*, **65,** 225(1969).
(172) Y. Imai, K. Imai, K. Ikeda, K. Hamuguchi, and T. Horio, *ibid.*, **65,** 629(1969).
(173) T. Tsong and J. Sturtevant, *J. Biol. Chem.*, **244,** 2397 (1969).
(174) K. E. Van Holde, *Biochemistry*, **6,** 93(1967).
(175) T. K. Li and B. P. Johnson, *ibid.*, **8,** 2083(1969).
(176) Y. Kito and M. Takezaki, *Nature*, **211,** 197(1966).
(177) *Ibid.*, **215,** 1197(1967).
(178) F. Crescitelli, W. F. H. M. Mommaerts, and T. I. Shaw, *Proc. Nat. Acad. Sci., U. S.*, **56,** 1729(1966).
(179) Y. Kito, M. Azuma, and Y. Maeda, *Biochim. Biophys. Acta*, **154,** 352(1968).
(180) T. Takagi, K. Aki, T. Isemura, and T. Yamano, *Biochem. Biophys. Res. Commun.*, **24,** 501(1966).
(181) V. P. Wellner and A. Meister, *Biochemistry*, **5,** 872(1966).
(182) R. E. Cathou, G. C. Hammes, and P. R. Schimmel, *ibid.*, **4,** 2687(1965).
(183) M. Iwatsubo and J. L. Risler, *Eur. J. Biochem.*, **9,** 280 (1969).
(184) A. Rosenberg, H. Theorell, and T. Yonetani, *Arch. Biochem. Biophys.*, **110,** 413(1965).
(185) E. A. Dratz and M. Calvin, *Nature*, **211,** 497(1966).
(186) A. Brady and S. Beychok, *Biochem. Biophys. Res. Commun.*, **32,** 186(1968).
(187) P. M. Bayley and G. K. Radda, *Biochem. J.*, **94,** 31(1965).
(188) *Ibid.*, **98,** 105(1966).
(189) M. E. Magar, *Biochim. Biophys. Acta*, **96,** 345(1965).
(190) H. d'A. Heck, *J. Biol. Chem.*, **244,** 4375(1969).
(191) A. B. Pritchard, D. B. McCormick, and L. D. Wright, *Biochem. Biophys. Res. Commun.*, **25,** 524(1966).

(192) R. E. Cathou, A. Kulczycki, Jr., and E. Haber, *Biochemistry*, **7,** 3958(1968).
(193) Y. M. Torchinskii, N. B. Levanova, and V. Y. Pikkelgas, *Mol. Biol. U.S.S.R.*, **1,** 23(1969).
(194) V. I. Ivanov, Yu. An Breusov, M. Ya. Karpeiskii, and O. L. Polyanovskii, *ibid.*, **1,** 588(1967).
(195) A. Scanu and R. Hiz, *Proc. Nat. Acad. Sci., U. S.*, **59,** 890(1968).
(196) A. M. Gotto, R. I. Levy, and D. S. Fredrickson, *ibid.*, **60,** 1437(1968).
(197) M. Sonenberg, *Biochem. Biophys. Res. Commun.*, **36,** 450(1969).
(198) J. Lenard and S. J. Singer, *Proc. Nat. Acad. Sci., U. S.*, **56,** 1828(1966).
(199) D. W. Urry and T. H. Ji, *Arch. Biochem. Biophys.*, **128,** 802(1968).
(200) F. Helmer, K. Kiehs, and C. Hansch, *Biochemistry*, **7,** 2858(1969).
(201) C. F. Chignell, *Life Sciences*, **7,** 1181(1968).
(202) C. F. Chignell, *Mol. Pharmacol.*, **5,** 244(1969).
(203) *Ibid.*, **5,** 455(1969).
(204) A. Albert, "The Acridines," Arnold, London, England, 1966.
(205) S. Brenner, L. Barnett, F. H. C. Crick, and A. Orgel, *J. Mol. Biol.*, **3,** 121(1961).
(206) F. H. C. Crick, L. Barnett, S. Brenner, and R. J. Watts-Tobin, *Nature*, **192,** 1227(1961).
(207) A. Blake and A. R. Peacocke, *Biopolymers*, **6,** 1225(1968).
(208) S. Yamabe, *Mol. Pharmacol.*, **3,** 556(1967).
(209) S. C. Riva, L. G. Silvestri, and L. R. Hill, *ibid.*, **3,** 327 (1967).
(210) D. M. Neville and D. F. Bradley, *Biochem. Biophys. Acta*, **50,** 397(1961).
(211) V. I. Permogorov, Y. S. Lazurkin, and S. Z. Shmurak, *Dokl. Biophys.* (Engl. Trans.), **155,** 71(1964).
(212) V. G. Alekseyev, M. A. Mokulskii, and V. I. Permogorov, *Biofizika*, **10,** 382(1965).
(213) R. F. Biers, Jr., and G. Armilei, *Nature*, **208,** 466(1965).
(214) A. Blake and A. R. Peacocke, *ibid.*, **206,** 1009(1965).
(215) A. Blake and A. R. Peacocke, *Biopolymers*, **5,** 871(1967).
(216) L. S. Lerman, *J. Mol. Biol.*, **3,** 18(1961).
(217) L. S. Lerman, *Proc. Nat. Acad. Sci., U. S.*, **49,** 94(1963).
(218) L. S. Lerman, *J. Mol. Biol.*, **10,** 367(1964).
(219) S. F. Mason, *Collog. Spectrosc. Int.*, **12,** 129(1965).
(220) A. Blake and A. R. Peacocke, *Biopolymers*, **4,** 1091(1966).
(221) B. J. Gardner and S. F. Mason, *ibid.*, **5,** 79(1967).
(222) A. Blake and A. R. Peacocke, *Nature*, **206,** 1009(1965).
(223) A. Blake and A. R. Peacocke, *Biopolymers*, **4,** 1091(1966).
(224) A. L. Stone, L. G. Childers, and D. F. Bradley, *ibid.*, **1,** 111(1963).
(225) A. L. Stone, *ibid.*, **2,** 315(1964).
(226) V. Zanker, *Z. Physik. Chem. (Leipzig)*, **199,** 225(1952).
(227) A. Blake and A. R. Peacocke, *Biopolymers*, **5,** 383(1967).
(228) *Ibid.*, **5,** 871(1967).
(229) W. Moffitt, *Proc. Nat. Acad. Sci., U. S.*, **42,** 736(1956).
(230) W. Moffitt, D. D. Fitts, and J. G. Kirkwood, *ibid.*, **43,** 723(1957).
(231) I. Tinoco, Jr., *J. Chem. Phys.*, **33,** 1332(1960).
(232) I. Tinoco, Jr., *Radiation Res.*, **20,** 133(1963).
(233) I. Tinoco, Jr., *J. Amer. Chem. Soc.*, **86,** 297(1964).
(234) I. Tinoco, Jr., R. W. Woody, and D. F. Bradley, *J. Chem. Phys.*, **38,** 1317(1963).
(235) D. F. Bradley, I. Tinoco, Jr., and R. W. Woody, *Biopolymers*, **1,** 239(1963).
(236) D. F. Bradley, S. Lifson, and B. Honig, in "Electronic Aspects of Biochemistry," Academic, New York, N. Y., 1964, p. 77.
(237) D. O. Jordan, in "Molecular Associations in Biology," B. Pullman, Ed., Academic, New York, N. Y., 1968, p. 221.
(238) M. Gilbert and P. Claverie, *ibid.*, p. 245.
(239) D. F. Bradley and S. Lifson, *ibid.*, p. 261.
(240) I. H. Goldberg, M. Rabinowitz, and E. Reich, *Proc. Nat. Acad. Sci., U. S.*, **48,** 2094(1961).
(241) W. Kersten, *Biochem. Biophys. Acta*, **47,** 610(1961).
(242) E. Reich, in "Biochemical Studies of Antimicrobial Drugs," B. A. Newton and P. E. Reynolds, Eds., Cambridge University Press, 1966.
(243) W. Müller and D. M. Crothers, *J. Mol. Biol.*, **35,** 251 (1968).
(244) H. Ziffer, K. Yamaoka, and A. B. Mauger, *Biochemistry*, **7,** 996(1968).
(245) R. B. Homer, *Arch. Biochem. Biophys.*, **129,** 405(1969).
(246) K. Yamaoka and H. Ziffer, *Biochemistry*, **7,** 1001(1968).
(247) P. A. Friedman, P. B. Joel, and I. H. Goldberg, *ibid.*, **8,** 1535(1969).
(248) P. A. Friedman, T. K. Li, and I. H. Goldberg, *ibid.*, **8,** 1545(1969).
(249) H. R. Mahler, G. Green, R. Goutarel, and O. Khuong-Huu, *ibid.*, **7,** 1568(1968).
(250) W. M. Huang and P. O. P. Ts'o, *J. Mol. Biol.*, **16,** 523 (1966).
(251) P. M. Pitha, W. M. Huang, and P. O. P. Ts'o, *Proc. Nat. Acad. Sci., U. S.*, **61,** 332(1968).
(252) E. J. Gabbay, *J. Amer. Chem. Soc.*, **90,** 6574(1968).
(253) E. J. Gabbay and J. Mitschele, *Biochem. Biophys. Res. Commun.*, **34,** 53(1969).
(254) E. J. Gabbay, *J. Amer. Chem. Soc.*, **91,** 5136(1969).
(255) K. Sensse and F. Cramer, *Chem. Ber.*, **102,** 509(1969).
(256) B. J. Adkins and J. T. Yang, *Biochemistry*, **7,** 266(1968).
(257) N. J. Greenfield and G. D. Fasman, *Biopolymers*, **7,** 595 (1969), and references therein.

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*

19-Norsteroids of Unnatural Configuration from Ergosterol

AHMED H. ElMASRY and OLE GISVOLD

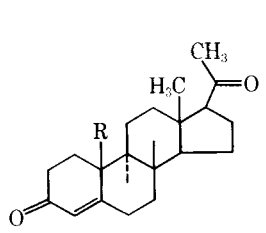
Abstract □ A new method for the preparation of a 19-norsteroid of unnatural configuration from ergosterol has been described. This method led to the preparation of 5α,8α,9α,10α-19-norpregnan-3,20-dione which possesses unnatural configuration at positions 8 and 10. The following reaction sequence was utilized to prepare this steroid. Ergosterol was photo-oxidized to give bisergostatrienol that was pyrolyzed to yield neoergosterol whose side chain was cleaved by ozonolysis to yield 3β-hydroxy-19-norpregna-5,7,9(10)-20α-aldehyde. This aldehyde, for the first time obtained in a crystalline state, was degraded *via* ozonolysis of the 3β-hydroxy-20-morpholino-methylene-19-norpregna-5,7,9(10)-triene derivative to give 3β-hydroxy-19-norpregna-5,7,9(10)-triene-20-one. The latter was reduced to the corresponding diol with Ru/carbon, and its Ring B in turn successfully hydrogenated in cyclohexane with Rh/alumina as the catalyst. The diol was oxidized to the diketone with the Jones reagent. Although some of the above transformations previously have been recorded, this report describes marked improvements in the techniques and yields of most of the intermediates.

Keyphrases □ 19-Norsteroids, unnatural configuration—synthesis □ Ergosterol—19-norsteroid synthesis □ IR spectrophotometry—structure □ NMR spectroscopy—identity □ Polarimetry—identity □ Optical rotatory dispersion—identity

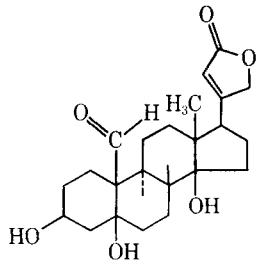
It is well established that progesterone (I), the naturally occurring progestin, possesses a wide range of biological activities together with other metabolic effects when administered parenterally (1). *Via* the oral route, however, it is essentially inactive. Ehrenstein's multistep transformation of strophanthidin (II) (2) to yield 14β,17α-19-norprogesterone (III) gave an opportunity to study the biological activity of a 19-norsteroid (3). It also gave an opportunity to study structure–activity relationships in the progestins that had certain unnatural configurations. In the case of progesterone (I), it has been shown that inversion of configuration at C-17 (from β to α), or at C-14 (from α to β), or at both centers destroys the biological activity, while inverting the configuration at C-8 (from β to α) only reduces it (4). However, activity is retained by inversion of the configuration at C-10 to 10α-progesterone;[1] also, double inversion at both C-9 and C-10 gives retroprogesterone (9β,10α-progesterone) whose progestational activities are more potent than progesterone (5).

In the case of 19-norprogesterone (IV), which is orally active, inversion at either C-17, C-14, or C-8 provides progestational agents of high order of activity (2,4,6).

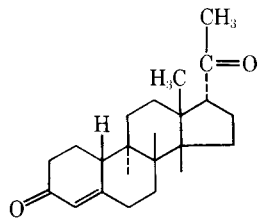
It is evident that progestational activity has been retained in progestins that have an unnatural configuration at C-8 or C-10. Also, loss of the C-19 methyl group exerts a favorable biological effect which is great enough to overcome the unfavorable changes in 14β,17α-progesterone (inactive). Thus, 14β,17α-19-norprogesterone (III) is 8 times as active as progesterone (I) (7). Therefore, the preparation and testing of progestins and 19-norprogestins that have other multiple unnatural configurational centers are of considerable interest. One of these is 8α,10α-19-norprogesterone (V).



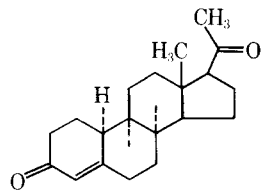
I, R = CH_3
IV, R = H



II



III



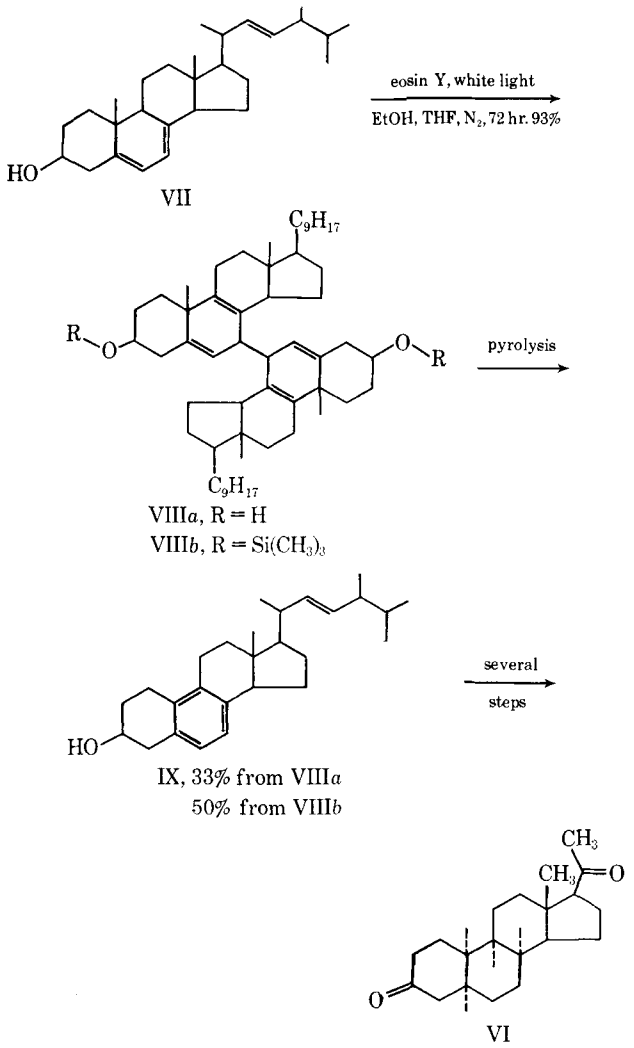
V

A key intermediate for the preparation of this isomer of 19-norprogesterone is the 5α,8α,9α,10α-19-norpregnan-3,20-dione (VI). This article describes the transformation of ergosterol (VII), *via* neoergosterol (IX)—a Ring B aromatic sterol (8), to the key intermediate (VI) possessing the unnatural configuration at C-8 and C-10[2] (Scheme I).

Ergosterol (VII) was chosen as the most suitable starting sterol because the presence of a double bond in the side chain would permit its degradation by suitable techniques to the C-17 methyl ketone found in the pregnane series. Also the presence of the diene system in Ring B enables one to prepare 19-nor Ring B aromatic steroids *via* a previously known method (8). This route of partial synthesis was preferred over a totally synthetic one because, generally, the former would lead to a pure *d*-enantiomer which is twice as active biologically as the racemic (*dl*-) form expected from the latter (9).

[1] Cited as footnote 5 in *J. Amer. Chem. Soc.*, **88**, 4538(1966) by M. Uskokovic *et al.* that in Belgian Patent 634,693 (1964), Ciba, 10α-progesterone was reported to be a progestational agent.

[2] The transformation of 5α,8α,9α,10α-19-norpregnan-3,20-dione (VI) to 8α,10α-19-norprogesterone (V), with the biological results, will be the subject of a forthcoming communication.



Scheme I

DISCUSSION

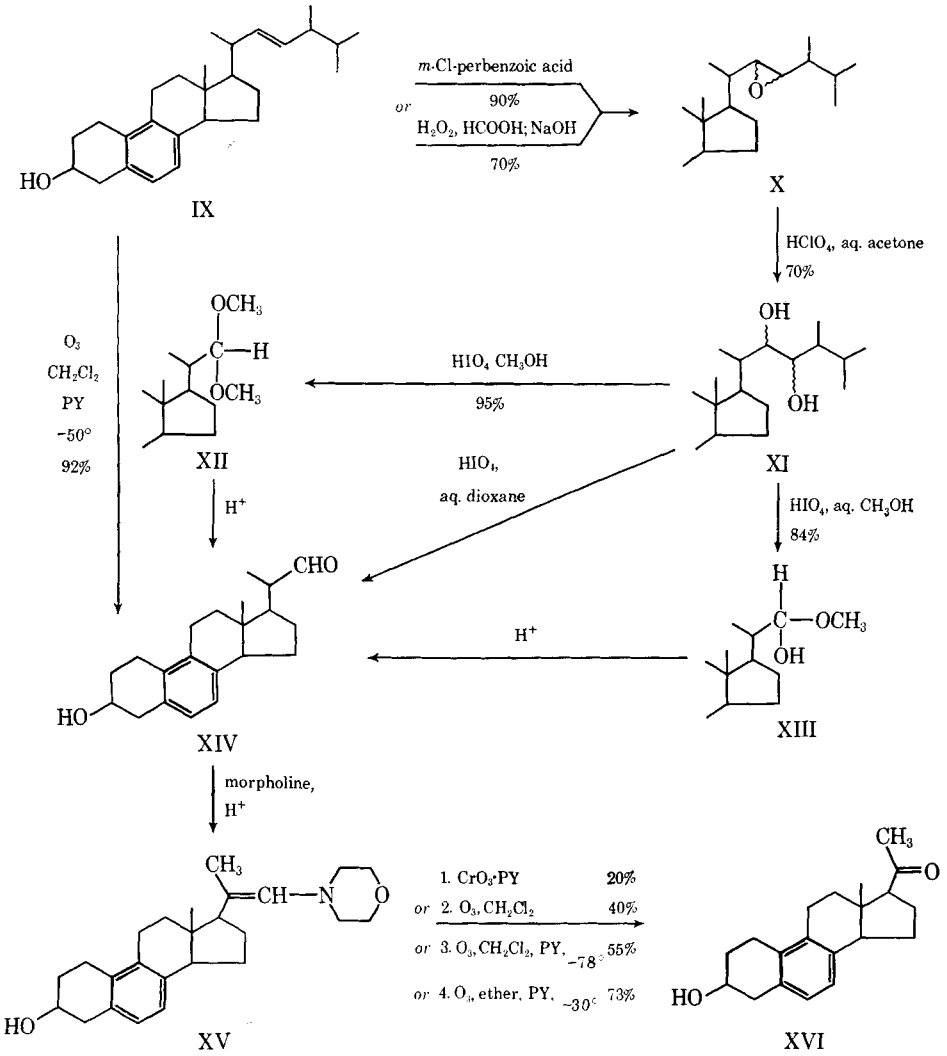
Preparation of Neoergosterol—Ergosterol (VII) (Scheme I) was photo-oxidized with white light in the presence of eosin Y as a sensitizer by a modification of the method of Mosettig and Scheer (8). The time of exposure to the light was increased from 48 to 72 hr., and also a more diluted solution was used to effect better exposure of ergosterol and eosin to the light. This was necessary because as bisergostatrienol (VIII*a*) was formed, it produced a dense white flocculent precipitate. In this way, bisergostatrienol (VIII*a*) was obtained in an average yield of 90–96%. Decomposing bisergostatrienol by refluxing in diethylene glycol monoethyl ether (Carbitol) afforded neoergosterol (IX) in 33% yield. Realizing that such a low yield for the second step in the synthesis might mitigate its practical aspects, it was decided to investigate possible variations in this step to increase the yield of neoergosterol. It was reported that pyrolysis of the acetates of these biscompounds affords the Ring B aromatic sterol acetates in 50% yield (8). However, the preparation of the acetate of bisergostatrienol requires large volumes of pyridine (1/180) and a reaction period of 1 week (10), which gave 80% yield. Because protection of the alcohol group had a certain advantage in the pyrolysis step, another method to protect this group, such as the trimethylsilylation method, was sought. Bisergostatrienol (VIII*a*) was silylated with bis-(trimethylsilyl)-acetamide (11) by refluxing in benzene under anhydrous conditions. The corresponding bis-(trimethylsilyl)-ether derivative (VIII*b*) was obtained in 95% yield. Decomposition of this silyl ether derivative by refluxing in diethylene glycol diethyl ether (Diethyl Carbitol) and by treating the reaction mixture with water and a small amount of *p*-toluenesulfonic acid, to cleave the silyl ether, afforded neoergosterol (IX) in 50% yield.

Degradation of the Side Chain of Neoergosterol—Prior to these studies, neoergosterol (IX) was subjected to ozonolysis conditions in carbon tetrachloride, or to the action of osmium tetroxide followed by periodate cleavage in methanol and acid hydrolysis of the resulting acetal (12). The aldehyde (XIV) obtained in both cases was an oil characterized as the 2,4-dinitrophenylhydrazone. Other investigators (13) ozonized neoergosterol in a mixture of chloroform and acetic acid and purified the crude product by separation of the aldehyde (XIV) with sodium bisulfite. However, no physical data for this aldehyde were reported. Because both ozone and osmium tetroxide cleavage methods did not lead to the separation of a characterizable aldehyde, a different method was sought. Neoergosterol (IX) (Scheme II) was treated with *m*-chloroperbenzoic acid in a mixture of chloroform and benzene at room temperature. The epoxides (X) (mixture of two possible isomers) were obtained by direct crystallization in 90% yield. Opening of the epoxide was accomplished by perchloric acid at the reflux temperature of aqueous acetone. The triols (XI) (two erythro isomers) were obtained in 70% yield. Fieser (14) reacted cholesterol with formic acid and hydrogen peroxide where 3β,5α,6β-cholestatriol was obtained in 91% yield. This method was applied on neoergosterol hoping to obtain the triol (XI) in an overall yield higher than in the route *via* epoxide formation and perchloric acid cleavage. However, the epoxide (X) was obtained instead, in about 70% yield. Furthermore, the epoxide (X) proved very resistant to cleavage with hot formic acid. Cleavage of the triol (XI) with periodic acid (15) in methanol afforded the dimethyl acetal (XII) in 95% yield, identical with that previously prepared (12). This indicated that the erythro diol structure underwent the periodate cleavage as readily as the *threo* isomer. Cleavage with periodic acid in aqueous methanol gave the hemiacetal (XIII) as a crystalline compound in high yields. A similar hemiacetal of a C-22 steroidal aldehyde has been reported to be formed under comparable conditions (16). Acetic acid hydrolysis of either the acetal (XII) or the hemiacetal (XIII) in aqueous dioxane gave the aldehyde (XIV) but as a semisolid material. All attempts to induce it to crystallize were unsuccessful; however, it exhibited one spot on TLC using different solvent systems. It showed the characteristic bands for aldehydes in the infrared and gave the reported (12) 2,4-dinitrophenylhydrazone. Cleavage of the triol (XI) with periodic acid in aqueous dioxane gave the aldehyde (XIV), again as an oil possessing the same properties as the one prepared by hydrolysis of the acetals.

These unsuccessful trials in obtaining the aldehyde (XIV) in a pure form prompted a reinvestigation of the ozonolysis of neoergosterol (IX). Slomp (17) has found that methylene chloride is a superior solvent for ozonolysis studies, and that the presence of pyridine, on a mole for mole basis, favors the selective ozonolysis of isolated double bonds over the conjugated ones. It also was reported (18) that a 2% KI solution could be used to determine accurately the cessation of the consumption of ozone by the double bond of isosafrol. A distinct yellow color appears in the KI solution after the reaction of ozone with the double bond has occurred and before ozone attacks the benzene ring. These conditions were applied to the ozonolysis of neoergosterol (IX) which was conducted in methylene chloride at −50° using one equivalent of pyridine. The pure crystalline aldehyde (XIV) was isolated in 92% yield. It was found that both the rate of passing ozone through the reaction and the rate of stirring affect the detection of a correct endpoint in the KI solution. Details are included in the experimental part.

Treatment of the aldehyde (XIV) with morpholine in the presence of a catalytic amount of *p*-toluenesulfonic acid at the reflux temperature of benzene afforded the enamine (XV). Molecular sieve also was used to absorb the water formed during the reaction.

Oxidation of the enamine (XV) with chromic acid in pyridine by the method of Elks (13) afforded, after chromatographic purification and Girard's separation, the C-20 keto compound (XVI) in 20% yield. Such a low yield, together with the successful result of the ozonolysis of neoergosterol (IX), prompted an investigation of the ozonolysis of this enamine. Ozonolysis of the enamine (XV) in methylene chloride at −78° afforded the ketone (XVI), after Girard's separation, in about 40% yield. When pyridine was present, on a mole for mole basis, the ketone (XVI) was obtained in 55% yield. The uptake of ozone was only half of that when pyridine was absent. Greenwood and Rubinstein (19) have found that ether had



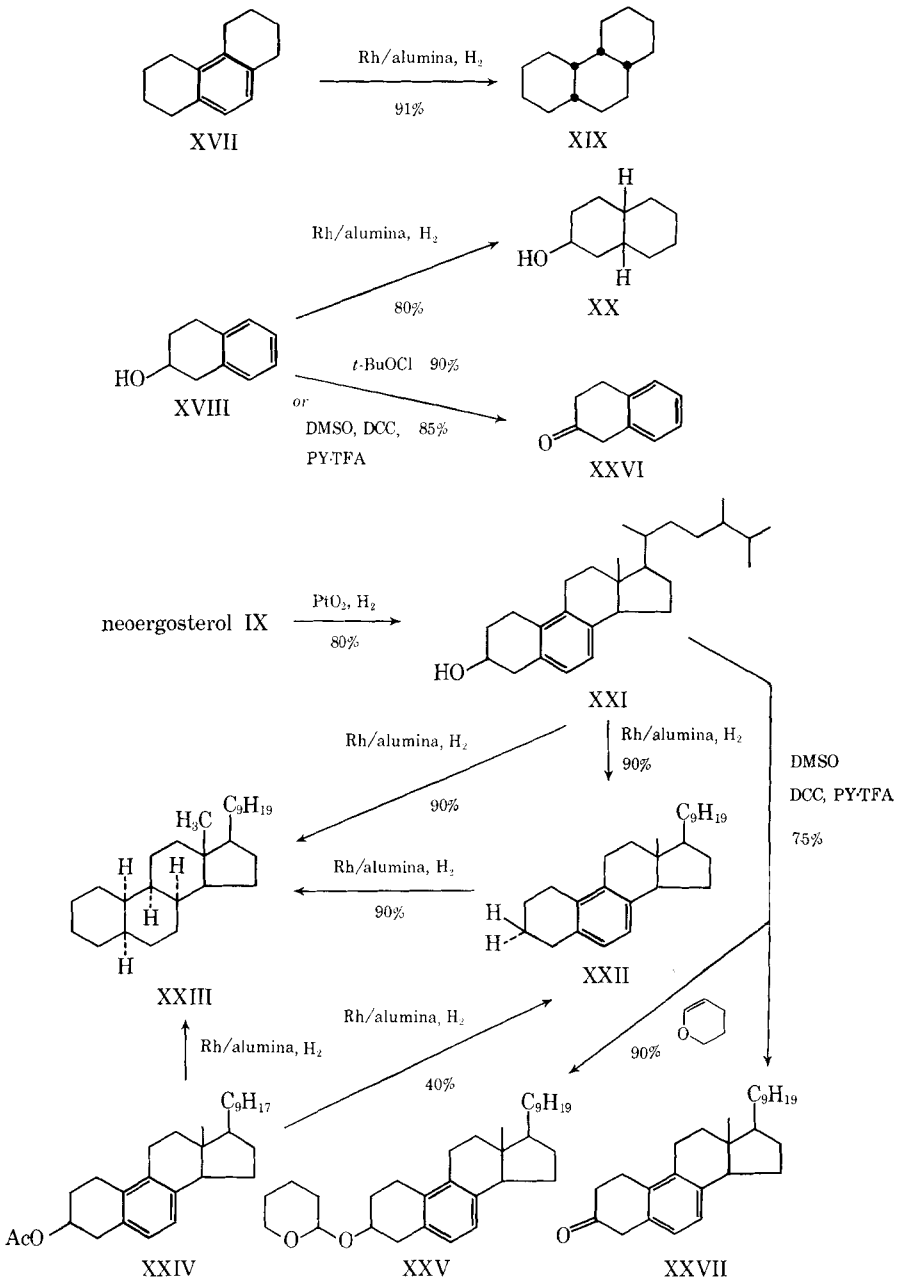
Scheme II

some advantages over methylene chloride in increasing the yields of aldehydes and ozonides, while minimizing the formation of other side reactions, during ozonolysis of certain alkenes. Also, raising the temperature of the reaction had a similar effect. Therefore, the enamine (XV) was ozonized in ether at $-30°$ in the presence of 1 mole of pyridine. The ketone (XVI) was obtained in 73% yield after the usual Girard's separation process.

Hydrogenation Studies—For the conversion of the ketone (XVI) to the key intermediate 5α,8α,9α,10α-19-norpregnan-3,20-dione (VI), the aromatic Ring B must be reduced by suitable methods. The assignment of the α-configuration at positions 5, 8, 9, and 10 as a result of catalytic reduction methods arises from the fact that the β-side of the molecule of the Ring B aromatic steroids is considerably hindered by the C-18 angular methyl group. This will require that the catalyst approaches the molecule from the less hindered side, the α-side. This is further supported by the report of Farkas and Rapala (20) who found that hydrogenation of equilenin and neoergosterol over ruthenium afforded 40% yield of 5α,8α,9α,10α-estrane-3β,17β-diol and 19-nor-24-methyl-5α,8α,9α,10α-cholestan-3β-ol in 10% yield, respectively. This denotes that such hydrogenations are from the α-side.

Two model compounds (Scheme III), 1,2,3,4,5,6,7,8-octahydrophenanthrene (XVII) and 1,2,3,4-tetrahydro-β-naphthol (XVIII), were selected for catalytic hydrogenation studies. Both are related in structure to the ketone (XVI) in that the former possesses the carbon skeleton of Rings A, B, and C and the latter contains Rings A and B. It is reasonable to assume that conditions needed to hydrogenate these two compounds should be of value in designing experiments to reduce dihydroneoergosterol (XXI) and then the ketone (XVI) with a minimum of undesirable side reactions. Hydrogenation of the octahydrophenanthrene (XVII) in ethanol containing 1% acetic acid over Rh/alumina at room temperature and 60 p.s.i. proceeded smoothly to provide the *cis*-syn-*cis*-perhydrophenanthrene (XIX) in excellent yields. The reduction of this compound was previously reported to occur only at 240° and 200 atmospheric pressures over Raney Ni (21). Reduction of 1,2,3,4-tetrahydro-β-naphthol (XVIII) in the same solvent system and under the same conditions afforded *cis,cis*-2-decalol in about 80% yield. Dihydroneoergosterol (XXI) was obtained in 80% yield by hydrogenating neoergosterol (IX) over Adam's catalyst. When dihydroneoergosterol (XXI) was subjected to the hydrogenation conditions used for the octahydrophenanthrene (XVII), the hydrocarbon (XXII) was obtained in about 90% yield, indicating complete hydrogenolysis of the hydroxyl group. Even when a hydrogen pressure of 250 p.s.i. was applied, reduction of this hydrocarbon went very slowly. However, when the amount of catalyst was increased to 10 times that needed to reduce an equimolar amount of octahydrophenanthrene (XVII), reduction went at the usual rate at room temperature and 60 p.s.i. The hydrocarbon (XXIII) was obtained in about 90% yield. The successful hydrogenation of the aromatic B Ring without hydrogenolysis of the hydroxyl group at C-3 is of prime importance to prepare ultimately the key intermediate (VI). Different forms of the oxygen function were prepared in order to prevent hydrogenolysis during the reduction reaction. Hydrogenation of neoergosteryl acetate (XXIV) afforded, as a final product, the hydrocarbon (XXIII), with the aromatic compound (XXII) as the intermediate product of hydrogenation.

Dihydroneoergosterol (XXI) was treated with dihydropyran according to the method of Petersen and Gisvold (22) where the tetrahydropyranyl ether (XXV) was obtained. Hydrogenation of

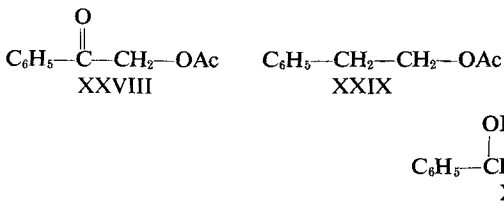


Scheme III

this derivative did not proceed under the previously employed conditions. Even when the amount of catalyst was increased to twice that needed to reduce neoergosteryl acetate and the reaction mixture shaken for 2 days, the tetrahydropyranyl ether derivative (XXV) was recovered unchanged. Because the effectiveness of rhodium in hydrogenating ketones is not conclusive (23), it was thought that hydrogenating dihydroneoergosterone (XXVII) might proceed by reducing the aromatic ring with the preservation of the oxygen function at C-3. To prepare dihydroneoergosterone (XXVII) with a minimum of by-products, preliminary studies were performed on 1,2,3,4-tetrahydro-β-naphthol (XVIII). Oxidation with *t*-butyl hypochlorite (24) afforded the ketone (XXVI) in 90% yield. Oxidation with dimethyl sulfoxide by the method of Pfitzner and Moffatt (25) afforded the ketone (XXVI) in 85% yield. The DMSO method proved more fruitful than *tert*-butyl hypochlorite in oxidizing dihydroneoergosterol, and the ketone (XXVII) was obtained, after purification through Girard's separation, in 75% yield. Hydrogenation of this ketone at room temperature and under ordinary pressures over Rh/alumina proved very difficult, and practically no noticeable absorption of hydrogen occurred during a period of 32 hr. The ketone was recovered from the reaction, *via* the use of Girard's reagent, in 50% yield. No other substances could be separated from the nonketonic fraction.

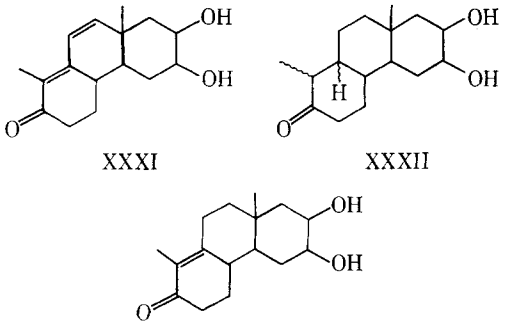
In a study of the factors affecting hydrogenation and those favoring hydrogenation over hydrogenolysis, Nishimura (26) reported the usefulness of the catalyst rhodium oxide–platinum oxide (7:3) in hydrogenations where hydrogenolysis is to be avoided. The catalyst consists of rhodium oxide and platinum oxide in a 7:3 ratio by weights of the metals. When this catalyst was employed in the hydrogenation of 1,2,3,4-tetrahydro-β-naphthol (XVIII), dihydroneoergosterol (XXI), neoergosteryl acetate (XXIV), the tetrahydropyranyl ether of dihydroneoergosterol (XXV), and the ketone (XXVII), results similar to those with Rh/alumina reductions were obtained. Nishimura also reported that the hydrogenolysis reaction is acid-catalyzed. Therefore, the amount of acetic acid (1%) used in the hydrogenation of dihydroneoergosterol (XXI) was decreased gradually in an attempt to favor hydrogenation over hydrogenolysis. At a concentration of 0.1%, neither hydrogenation nor hydrogenolysis occurred. With a gradual increase in the amount of acid, very slow absorption of hydrogen occurred which was due chiefly to hydrogenolysis. This was found to be the case with either Rh/alumina or Rh–Pt oxides (7:3) as the catalyst.

These unsuccessful results prompted the investigation of other factors, such as the solvent effect, influencing the extents of hydrogenation and hydrogenolysis reactions. Kindler and Blaas (27) hydrogenated benzoylcarbinyl acetate (XXVIII) over palladium in different solvents. They found that by using an oxygenated solvent such as methanol, dioxane, ethyl acetate, or acetic acid, 2 moles of hydrogen were absorbed and XXIX was obtained in 70–80% yield. However, when a nonoxygenated solvent such as cyclohexane, benzene, or toluene was used, reduction ceased after absorption of 1 mole of hydrogen and XXX was the main product formed. A similar effect was also found in the hydrogena-



XXVIII XXIX XXX

tion of 4-chloro- or 4-bromo-2-allylphenol (28). Reduction of the double bond in oxygenated solvents results in appreciable loss of the halogen; while if benzene or cyclohexane is used, elimination of the halogen does not occur. Woodward *et al.*, in their total synthesis of the steroids (29), obtained the glycol (XXXI) as one of the intermediates. Hydrogenation of this glycol, or its acetate or the acetonide, over supported Pd in alcohol or ethyl acetate did not cease with the absorption of 1 mole of hydrogen. But, if the reduction was arbitrarily stopped at that point, mixtures of the starting material product of reduction of the disubstituted double bond (XXXIII), and products of reduction of both double bonds (XXXII) were

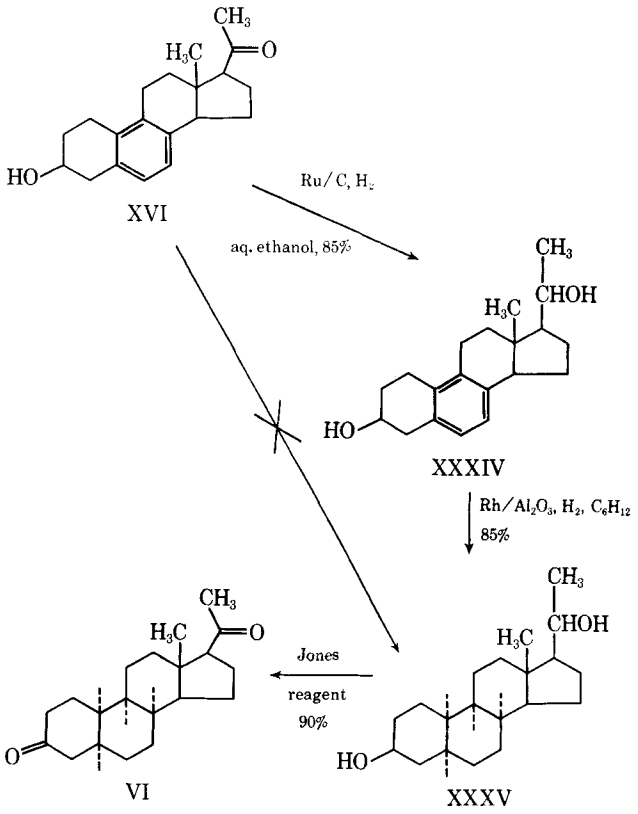


XXXI XXXII

XXXIII

obtained. When the hydrogenation of XXXI was allowed to proceed to completion, 2 moles of hydrogen were absorbed, giving a complicated mixture of stereoisomeric saturated keto compounds (XXXII). They found that by changing the solvent to benzene, hydrogenation of XXXI, or its diacetate or the acetonide, stopped completely after absorption of 1 mole of hydrogen to furnish the corresponding monounsaturated compound (XXXIII) in excellent yields. Therefore, cyclohexane was chosen as the solvent for the reduction of Ring B of the aromatic ketone (XVI).

Hydrogenation of the ketone (XVI) over Rh/alumina in cyclohexane did not proceed even at 1400 p.s.i. hydrogen pressure and the ketone was recovered unchanged (Scheme IV). This ketone was hydrogenated in 65% ethanol at room temperature and 60 p.s.i. over Ru/carbon where 1 mole of hydrogen was absorbed in 2 days. The diols (XXXIV) (mixture of 20α- and 20β-) were obtained in 85% yield. When the reduction was conducted in the same way except under 1200 p.s.i., 10 hr. was enough to afford the diols in the same yield. Hydrogenation of this aromatic diol over Rh/alumina in cyclohexane at room temperature and 1400 p.s.i. for 12 hr. afforded the aliphatic diol (XXXV) in 85% yield. The crude reaction product gave a negative tetranitromethane test, indicating the absence of a double bond (30). The IR spectrum of the diol (XXXV) showed the presence of strong OH and C—O stretching vibrations, absence of the phenyl C=C stretching, and the out of plane aromatic C—H bending. NMR spectrum of the crude reaction product showed no aromatic or olefin protons and also the absence of allylic protons at 1.85–2.20 δ. By integration it showed the presence of a multiplet of two protons centering at 3.67 δ corresponding to



XVI XXXIV VI XXXV

Scheme IV

the two protons at C-3 and C-20. By deuterium exchange there was a decrease of two protons in the total value of the integration. This indicates that the hydrogenation of the benzene ring of the aromatic diol (XXXIV) in cyclohexane did not cause hydrogenolysis of the C-3 hydroxyl group.

Oxidation of the diol (XXXV) with Jones reagent (31) in acetone afforded the diketone 5α,8α,9α,10α-19-norpregnan-3,20-dione (VI) in 90% yield.

Since this compound contains two carbonyl groups not interacting with each other, its rotatory dispersion curve should be the summation of the RD curves of the two separate carbonyl groups (32). However, the specific rotation at the peak (313 mμ) is +2566° (a value of about +3300° was expected). This order of rotation could be accounted for through the contribution of the 17β-methyl ketone alone, which would indicate that no contribution, or a weak positive Cotton effect, is provided by the 3-keto group. A careful examination of a conformational model of VI in its octant projection would indicate that a positive contribution can be attributed to C-6. Carbon atoms 7 and 8 lying in the horizontal B plane of the octant rule (32) will make no substantial contribution. Carbon atoms 13, 14, 15, 16, 17, and 18 will appear in the lower-left octant and hence will make a negative contribution. These carbon atoms, being more distant from the keto group than C-6 is, will have a smaller influence. Therefore, a resultant weak positive Cotton effect might be obtained for the C-3 keto group, which in turn might explain the RD curve of the diketone (VI).

EXPERIMENTAL

All melting points were determined in capillary tubes in a Thomas-Hoover melting point apparatus and are uncorrected. Optical rotations were obtained using a Perkin-Elmer 114 polarimeter, with a 1-dm. cell at 22°. Microanalyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. IR spectra (KBr pellets) were determined on a Perkin-Elmer 237B grating IR spectrophotometer. NMR spectra[3] were recorded on a

[3] The authors are grateful to Daniel A. Koechel and Thomas N. Riley for preparing the NMR spectra and the ORD curves, respectively.

Varian Associates A-60D spectrometer using concentrations of about 10% and tetramethylsilane as the internal reference standard. ORD curves were determined with a Cary recording spectropolarimeter model 60, using a 1-cm. cell at 25°. Hydrogenations at ordinary pressures were done in a Parr low-pressure hydrogenation apparatus (Parr Instrument Co., Moline, Ill.). Hydrogenations at high pressures were carried out in an autoclave (Autoclave Engineers Inc., Erie, Pa.), and the hydrogenation mixture was stirred magnetically at 500–600 r.p.m. Supported rhodium or ruthenium catalysts (5%) were purchased from Engelhard Industries, Inc., Newark, N. J.

Bisergostatrienol (VIII*a*)—The photo-oxidation apparatus consisted of four horizontally fixed, 32-w. white circular fluorescent lamps, with a minimum space between lamps, and enclosed with a reflecting shield of highly polished white interior. The reaction vessel, usually a suitably sized round-bottom flask fitted with a reflux condenser and an efficient stirrer, was placed in the center of the circle of lamps. Fifty grams of Eosin Y[4] was dissolved in 1500 ml. of ethanol. To this solution, 3.84 ml. of concentrated sulfuric acid (sp. gr. 1.84) was added slowly and with stirring to liberate the free acid. The solution was filtered from the formed sodium sulfate. After bubbling nitrogen through the solution for 30 min., 750 ml. of tetrahydrofuran (THF) (previously passed through activated alumina column to decompose the peroxides) and 50 g. of ergosterol[5] were added in the dark. The mixture was stirred and irradiated with the white light for 72 hr. under a nitrogen atmosphere. During the reaction time, bisergostatrienol precipitated as dense fine white needles. The mixture was diluted with 4.3 l. of ethanol and the THF was allowed to evaporate spontaneously under the hood overnight. The fine needles were filtered, washed with ethanol and with ether, and dried in a vacuum desiccator. A yield of 45.5 g. (93%) of bisergostatrienol (VIII*a*), m.p. 201–203° with decomposition [reported (33) 202–203°], was obtained. The yields of several such reactions averaged 90–96%.

Neoergosterol (IX)—A mixture of bisergostatrienol (VIII*a*) (5.0 g.) and diethylene glycol monoethyl ether (Carbitol) (250 ml.) was refluxed for 5 min. in a nitrogen atmosphere. The solution was rapidly cooled to approximately 100° and then added to a mixture of water and crushed ice (250 g.) while stirring. The mixture was kept at −15° for 1 day and filtered while cold; the residue was washed with cold water, dissolved in hot methanol (60 ml.), and allowed to cool slowly. The fine needles of neoergosterol were collected and recrystallized from methanol (60 ml.). The yield was 1.65 g. (33%), m.p. 151–153° [reported (33) 151–152°]; $[\alpha]_D$ −10° (c 1.0). The IR spectrum showed characteristic peaks at 3330 cm.$^{-1}$ (OH); 3055, 3030 cm.$^{-1}$ (aromatic C—H); 1485, 810 cm.$^{-1}$ (aromatic ring); and 970 cm.$^{-1}$ (*trans* C=C).

Acetylation of Bisergostatrienol—A solution of bisergostatrienol (VIII*a*) (1 g.) in pyridine (180 ml.) was mixed with acetic anhydride (15 ml.). After a week the solution was poured into cold water (400 ml.); the separated crystals were collected and washed with dilute acetic acid and with water. After two recrystallizations from benzene–alcohol and drying over P_2O_5 *in vacuo*, colorless fine needles, 830 mg., 80%, m.p. 203–205°, were obtained [reported (10) m.p. 205.5–206°].

Pyrolysis of the Diacetate of the Biscompound; Preparation of Neoergosteryl Acetate (XXIV)—The diacetate of the biscompound (500 mg.) was pyrolyzed by the method described for the biscompound using 40 ml. of diethylene glycol monoethyl ether as the solvent. The yield of neoergosteryl acetate (XXIV) after two recrystallizations from methanol was 250 mg. (50%), m.p. 121–123° [reported (10) 123.5–124°]. The IR spectrum (KBr pellet) showed the absence of the OH group, presence of the ester carbony group band (1735 cm.$^{-1}$), presence of acetate ester characteristic band (1240 cm.$^{-1}$), and also the bands of the aromatic ring and *trans* double bond were present.

Bis(trimethylsilyl)ether of Bisergostatrienol (VIII*b*)—Bisergostatrienol (VIII*a*) (2 g.; 0.0025 mole) was suspended in anhydrous benzene (100 ml.), and bis(trimethylsilyl)-acetamide (Aldrich Chem. Co., Milwaukee, Wis.) (11) (1.12 g.; 0.0055 mole) was added. The stirred mixture was refluxed gently for 3 hr. under atmosphere of dry nitrogen. The solution was allowed to cool to room temperature. The solvent was removed *in vacuo*, and to the residue dry acetone (60 ml.) was added. After cooling at 5° for 1 hr., the material that precipitated was collected and dried *in vacuo* for 24 hr. at room temperature. The yield was 2.2 g. (95%), m.p. 190–192°. The IR spectrum showed the absence of OH absorbance and the presence of the following characteristic bands (34): 1250, 840, 750 cm.$^{-1}$ [$Si(CH_3)_3$]; 1090–1075 cm.$^{-1}$ (Si—O—C).

Anal.—Calcd. for $C_{89}H_{102}O_2Si_2$: C, 79.58; H, 10.99; Si, 6.00. Found: C, 79.13; H, 10.84; Si, 6.26.

Pyrolysis of the Bis(trimethylsilyl)ether of the Biscompound (VIII*b*)—A mixture of bisergostatrienol bis(trimethylsilyl)ether (VIII*b*) (1 g.) and diethylene glycol diethyl ether (Diethyl Carbitol) (50 ml.) was refluxed for 5 min. in a nitrogen atmosphere. The solution was cooled rapidly to approximately 100° and added to a stirred mixture of water and crushed ice (50 g.) containing a few crystals of *p*-toluenesulfonic acid. The mixture was worked up in the same way as mentioned under pyrolysis in diethylene glycol monoethyl ether. The yield of neoergosterol (IX) was 490 mg. (50%), m.p. 151–153°.

Neoergosterol-22,23-epoxide (X)—*A. With* m-*Chloroperbenzoic Acid*—Neoergosterol (IX) (2 g.; 0.0052 mole) was dissolved in dry olefin-free benzene (20 ml.) and chloroform (10 ml.). To this solution was added slowly a cold solution of *m*-chloroperbenzoic acid (FMC Corp., New York, N. Y.) (1 g.; 0.0058 mole) in benzene (15 ml.) and chloroform (10 ml.) and the solution was kept in the dark for 9 hr. at 25°. The solution was diluted with ether (200 ml.), washed with a 10% sodium sulfite solution, sodium bicarbonate solution, and with water until the washings were neutral. The organic solvents were dried over anhydrous sodium sulfate and distilled under reduced pressure. To the solid glassy residue was added 88% methanol (20 ml.) after which a white dense flocculent precipitate was obtained upon trituration. The precipitate was collected, dried *in vacuo* for 48 hr. at 30°. The yield was 1.8 g. (90%), m.p. 105–109° (the wide range is possibly due to the presence of a mixture of the two stereoisomeric epoxides); $[\alpha]_D$ −13.5° (c 1.0). The IR spectrum showed the absence of the 970 cm.$^{-1}$ band (*trans* C=C) and the presence of a new band at 909 cm.$^{-1}$ (so-called "11-μ band") characteristic of epoxide rings (35).

Anal.—Calcd. for $C_{27}H_{40}O_2$: C, 81.76; H, 10.17. Found: C, 81.48; H, 10.30.

B. With Formic Acid and Hydrogen Peroxide—A suspension of neoergosterol (1 g.) in 88% formic acid (20 ml.) was heated at 80° with stirring for 20 min. during which a clear solution was obtained. When the solution came to room temperature, it was treated with 30% hydrogen peroxide solution (3 ml.) and 88% formic acid (10 ml.). The mixture was stirred in the dark at room temperature for 24 hr. The mixture was treated with boiling water (100 ml.), stirred, and kept at 80° for 1 hr., then cooled to room temperature. The oil that separated was collected, dissolved in methanol (50 ml.), and treated with 1 ml. of 25% sodium hydroxide solution and warmed on a steam bath for 15 min. The solution was cooled to room temperature, acidified with dilute HCl, diluted with water (200 ml.), and extracted with ether. The ether extract was washed with water until the washings were neutral, dried, and the ether removed *in vacuo*. The glassy residue was crystallized from 88% methanol. The yield was 710 mg. (70%), m.p. 112–115° (possibly a different ratio of the two isomeric epoxides was obtained). Mixed melting point with the epoxide prepared by the *m*-chloroperbenzoic acid method gave a m.p. of 110–113°. The IR spectrum was identical to that prepared by *m*-chloroperbenzoic acid.

Anal.—Calcd. for $C_{27}H_{40}O_2$: C, 81.76; H, 10.17. Found: C, 81.95; H, 10.44.

Erythro-22,23-dihydroxy-22,23-dihydroneoergosterol (XI)—To a solution of neoergosterol epoxide (1 g.) in acetone (40 ml.) was added a solution of 2.5 ml. of 70% $HClO_4$ in 10 ml. water. The solution was refluxed in a nitrogen atmosphere for 3 hr. The solution was diluted with water and extracted with ethyl acetate. The ethyl acetate extract was washed with water until the washings were neutral, dried, and evaporated *in vacuo*. The residue was crystallized from ether when 700 mg. (70%) of colorless crystals were obtained, m.p. 173–175°, that gave a positive periodate–benzidine test (36), indicative of the presence of vicinal diol structure. One of the two erythro isomers was obtained after two recrystallizations from ether–petroleum ether. It was dried *in vacuo* at 70° for 5 hr.; m.p. 206–208°, $[\alpha]_D$ +29° (c 1.0). The IR spectrum showed the absence of the epoxide band and the presence of strong hydroxyl absorbance at 3375 cm.$^{-1}$

[4] Color index 45380, National Aniline Division, Allied Chemical & Dye Corp., New York, N. Y.
[5] Melting point 161–163°; Aldrich Chem. Co., Milwaukee, Wis.

Anal.—Calcd. for $C_{27}H_{42}O_3$: C, 78.21; H, 10.21. Found: C, 77.99; H, 9.93.

3β-Hydroxy-19-norpregna-5,7,9(10)-triene-20α-aldehyde Dimethyl Acetal (XII)—A solution of the triol (XI) (1 g.) in methanol (40 ml.) was treated with a solution of periodic acid (0.6 g.) in methanol (7 ml.). The solution was stirred in the dark for 18 hr., neutralized with 1*N* NaOH solution, and filtered. The residue on the filter was washed with methanol. The filtrate and washings were concentrated *in vacuo* to a volume of 20 ml., cooled, and 850 mg. (95%) of white crystals of the dimethyl acetal (XII) were obtained; m.p. 177–179°, lit. (12) 178.6–180.9°.

3β-Hydroxy-19-norpregna-5,7,9(10)-triene-20α-aldehyde Methyl Hemiacetal (XIII)—A solution of the triol (XI) (1 g.) in methanol (50 ml.) was treated with a solution of periodic acid (0.6 g.) in water (9 ml.). The solution was stirred in the dark for 18 hr. after which time the precipitate that formed was collected and dried *in vacuo*. The yield was 750 mg. (84%); m.p. 142–143°. The IR spectrum showed the absence of a carbonyl absorbance, presence of strong hydroxyl absorbance at 3380 cm.$^{-1}$ and at 3430 cm.$^{-1}$. It also showed several sharp peaks at 2820, 1145, 1100, and 1075 cm.$^{-1}$, characteristic of ethers (35).

Anal.—Calcd. for $C_{22}H_{32}O_3$: C, 76.70; H, 9.36. Found: C, 76.47; H, 9.48.

3β-Hydroxy-19-norpregna-5,7,9(10)-triene-20α-aldehyde (XIV)—*A. From the Triol (XI)*—A solution of the triol (XI) (1 g.) in peroxide-free dioxane (45 ml.) was treated with a solution of periodic acid (0.6 g.) in water (10 ml.). The solution was stirred in the dark for 24 hr. after which time it was diluted with ethyl acetate (200 ml.) and ether (100 ml.) The solution was washed with water till neutral, dried over anhydrous sodium sulfate, and the organic solvents evaporated *in vacuo* at a temperature less than 40°. The glassy pale yellowish mass that remained was dried *in vacuo* over P_2O_5 for 24 hr. when 620 mg. (82%) was obtained. It was very soluble in the organic solvents and failed to crystallize from most of them. With ether–petroleum ether as solvent for crystallization, an oil was always obtained, $[\alpha]_D$ −17° (c 1.0). The IR spectrum showed the characteristic bands of the aldehyde group at 2700 cm.$^{-1}$ (H of CHO) and at 1725 cm.$^{-1}$ (C=O).

B. By Hydrolysis of the Acetal (XII) or the Hemiacetal (XIII)—A solution of the acetal (XII) or the hemiacetal (XIII) (500 mg.) in dioxane (20 ml.) was refluxed in a nitrogen atmosphere with a solution of acetic acid (2 ml.) in water (10 ml.) for 5 min. The solution was allowed to cool to room temperature, mixed with ether and ethyl acetate (100 ml. each), and the organic layer washed with water until it was free of acid. The solution was dried over anhydrous sodium sulfate and concentrated to dryness *in vacuo*. The glassy mass that remained was dried *in vacuo* over P_2O_5 for 24 hr. when 420 mg. was obtained. This material was identical to that prepared in Method A in every respect. The 2,4-dinitrophenylhydrazone of this aldehyde melted at 230–231° after one recrystallization from ethyl acetate–methanol, reported (12) 230–232°.

C. By Ozonolysis of Neoergosterol (IX)—A solution of neoergosterol (IX) (5 g.; 0.013 mole) in methylene chloride (400 ml.) and pyridine (1.1 ml.; 0.014 mole) in a tubular reactor equipped with a magnetic stirrer, a gas inlet, and an outlet leading to a $CaSO_4$ drying tube which in turn led to a 2% KI solution, was cooled to −50° in a dry ice–acetone bath. A stream of ozone-rich dry oxygen (5.40% w/w; 7.77 mg. O_3/100 ml.) (generated by a Welsbach model T-23 ozonator) was passed at a rate of 0.0026 cu. m./min. (0.03 cu. ft./min.) into the stirred solution, and the outcoming gases from the reaction were bubbled through the KI solution. After 15 min. a yellow color was obtained in the KI solution, indicating that about 95% of attack on the double bond had occurred. After an additional 45 sec., the flow of ozone was stopped (1.65 mole equivalents of ozone) and zinc dust (6 g.) and glacial acetic acid (27 ml.) were added. The mixture was allowed to stir at 0–10° for 1 hr. and finally for 5 min. at 35°. The bright-yellow mixture was filtered and washed with two 150 ml. portions of water. It then was cooled by the addition of crushed ice (100 g.) and washed with two 20-ml. portions of cold 10% sodium carbonate, 15 ml. of cold 10% sodium hydroxide, and five 150-ml. portions of cold water, all aqueous washings being back-washed with 20 ml. of methylene chloride. The combined methylene chloride solutions were dried over anhydrous sodium sulfate and the solvent removed under reduced pressure at a temperature less than 40° when a white crystalline residue was obtained. This solid material was dried *in vacuo* over P_2O_5 for 24 hr. when 3.8 g. (92%), m.p. 132–134°, was obtained; $[\alpha]_D$ −20° (c 1.0.). The IR spectrum showed the following characteristic bands: 3515, 3400 cm.$^{-1}$ (OH); 3040, 3010 cm.$^{-1}$ (aromatic C—H); 2700, 2680 (H of CHO); 1725 cm.$^{-1}$ (C=O); 1480, 810 cm.$^{-1}$ (aromatic ring), beside the other aliphatic C—H and C—O stretching vibrations. The NMR spectrum showed the following characteristic peaks: singlet at 0.63 δ (C-18 CH_3); doublet centering at 1.20 δ J = 7 c.p.s. (C-21 CH_3); singlet at 6.83 δ (2 aromatic protons); and a doublet centering at 9.60 δ J = 2.9 c.p.s. (H of CHO). A portion of this material was washed on a funnel with ethyl acetate, anhydrous ether, dried *in vacuo* for 24 hr., m.p. 136–138°; $[\alpha]_D$ −21° (c 1.0).

Anal.—Calcd. for $C_{21}H_{28}O_2$: C, 80.73; H, 9.03. Found: C, 80.54; H, 9.13. The 2,4-dinitrophenylhydrazone of this aldehyde did not depress the melting point when present in a mixture with that prepared from the aldehyde of the periodate reactions. The following observations are significant:

1. During the ozonolysis, if the rate of passing O_3 was increased [0.0035 cu. m./min. (0.04 cu. ft./min.)], an earlier end-point might show in the KI solution and neoergosterol will be separated along with the aldehyde.

2. Also if the rate of stirring is too fast, O_3 will be forced to saturate the solution, and further attack the benzene ring and an incorrect end-point will be obtained. An average rate of stirring of 200–250 r.p.m. with either a magnetic or a mechanical stirrer should be maintained during the ozonolysis.

3. If temperatures colder than −50° are used, the intermediate ozonide will precipitate along with some neoergosterol, causing an incomplete reaction.

3β-Hydroxy-20-morpholino-methylene-19-norpregna-5,7,9(10)-triene (XV)—A solution of the aldehyde (XIV) (3 g.) in anhydrous benzene (80 ml.) was distilled under nitrogen atmosphere until 20 ml. of distillate was collected (to remove the traces of moisture). To the benzene solution was added 2 ml. of dry morpholine and 12 mg. of *p*-toluenesulfonic acid. This solution was refluxed, while stirred magnetically, under dry nitrogen atmosphere for 4 hr. in the presence of 7 g. of conditioned (by heating at 320° for 5 hr.) Linde 4A molecular sieve 0.160-cm. (0.063-in.) pellets in a continuous-extraction apparatus placed between the condenser and the reaction flask. This assembly permits the condensed vapors of water and benzene to pass through the molecular sieve, so that water will be adsorbed, before it returns to the reaction flask. The solution was cooled, benzene and excess morpholine distilled *in vacuo*, and the pale brown glassy residue dried *in vacuo* over P_2O_5 for 24 hr. The yield was 3.64 g. (99%). The IR spectrum of this enamine showed the absence of carbonyl absorbance and the presence of the enamine double bond absorption frequency at 1645 cm.$^{-1}$ (37).

3β-Hydroxy-19-norpregna-5,7,9(10)-triene-20-one (XVI)—*A. By Oxidizing the Enamine (XV) with CrO_3·PY*—Finely powdered chromic acid (1.65 g.) was added, over a period of 15 min., to stirred anhydrous pyridine (25 ml.) at 0° under nitrogen. A solution of the enamine (XV) (2 g.) in pyridine (25 ml.) was added over 5 min. The mixture was stirred for 4 hr. at 0° and then was allowed to stand overnight at room temperature. Benzene (200 ml.) was added and the mixture was filtered through a column of alumina (25 g.) and the column was eluted with benzene. Evaporation of the eluate *in vacuo* yielded 1 g. of a semisolid material. The ketone (XVI) was separated from this material through the formation of Girard "T" derivative as follows: this semisolid material was dissolved in absolute ethanol (10 ml.), glacial acetic acid (1 ml.), and 1 g. of Girard T reagent was added. The mixture was refluxed under dry nitrogen atmosphere for 1 hr. The solution then was added to sodium bicarbonate (1.26 g.) in water (40 ml.) and the resulting colloidal solution was extracted twice with ether. The clear aqueous layer was acidified to pH 1 with hydrochloric acid and extracted with ether. The ether extract was washed with water until it was free of acid, dried over anhydrous sodium sulfate, and the solvent removed *in vacuo*. The residue was crystallized twice from ether when 0.31 g. (20%) of white rosettes was obtained: m.p. 150–151°, $[\alpha]_D$ +57° (c 1.0 in $CHCl_3$); lit. (13) m.p. 151–152°, $[\alpha]_D$ +58° (c 0.9 in $CHCl_3$). The IR spectrum showed the following characteristic bands: 3340 cm.$^{-1}$ (OH); 3040, 3015 cm.$^{-1}$ (aromatic C—H); 1700 cm.$^{-1}$ (C=O); 1420, 1355 cm.$^{-1}$ (CH_3—C(=O)—); 1480, 810 cm.$^{-1}$ (aromatic ring), beside the other aliphatic C—H and C—O stretching vibrations. The NMR spectrum (in CCl_4) showed the following peaks: singlet at 0.51 δ (C-18 CH_3); singlet at 2.11 δ (C-21 CH_3);

multiplet centering at 3.95 δ (H of C-3); and a singlet at 6.73 δ (2 aromatic protons).

B. By Ozonolysis of the Enamine (XV)—Using methylene chloride, a solution of the enamine (XV) (2 g.; 0.0052 mole) in dry methylene chloride (200 ml.) was put in the same tubular reactor with the same outfit used for ozonizing neoergosterol. A stream of ozone-rich dry oxygen (5.40% w/w) was passed at a rate of 0.0026 cu. m./min. (0.03 cu. ft./min.) into the stirred solution at −78°. After 17 min. a yellow color was obtained in the KI solution, indicating about 95% attack on the double bond had occurred. Ozone was stopped after 30 sec. more (4.6 mole equivalents of ozone were absorbed); zinc dust (3 g.) and glacial acetic acid (14 ml.) were added. The solution was worked up and processed for the neutral fraction as previously described under the ozonolysis of neoergosterol. The semisolid material (1.4 g.), that was obtained after distilling the methylene chloride, was purified through Girard T separation procedure as previously described. The residue that remained, after distilling the ether extract of the ketonic fraction, was crystallized twice from ether when 0.62 g. (40%) of white rosettes was obtained; m.p. 151–152°, mixed melting point with the ketone (XVI) prepared by $CrO_3 \cdot PY$ method showed no depression.

Using methylene chloride with pyridine, the above mentioned experiment was conducted in the same way except that pyridine (0.44 ml., 0.0056 mole) was added to the enamine solution before the ozonolysis. A yellow color appeared in the KI solution after passing ozone through the solution for 10 min. The flow of ozone was stopped after 30 sec. more (2.7 mole equivalents of ozone were absorbed). The yield of the ketone (XVI), after Girard T separation and two crystallizations from ether, was 0.85 g. (55%), m.p. 150–151°. The IR spectrum was identical to that prepared by $CrO_3 \cdot PY$ oxidation method and to that prepared by the previously mentioned ozonolysis method.

Using ether, the previous experiment was conducted in the same way except that anhydrous ether (300 ml.) was used as the solvent instead of methylene chloride, and the temperature of the reaction during ozonolysis was adjusted at −30°. The yellow color appeared in the KI solution after 10.8 min. and the flow of ozone was stopped after 30 sec. more (2.9 mole equivalents of ozone were absorbed). The yield of the ketone (XVI), after evaporating the ether extract from Girard T separation under reduced pressure and drying the residue *in vacuo* over P_2O_5 for 24 hr., was 1.28 g., m.p. 142–144°. One recrystallization from ether afforded 1.13 g. (73%), m.p. 151–152°. The IR spectrum was identical to that prepared by the previous methods.

Model Procedure for Hydrogenation—The described amount of catalyst was soaked with a few milliliters of the solvent.[6] The compound to be hydrogenated was dissolved in the rest of the solvent, mixed with the presoaked catalyst, and the mixture was hydrogenated under the conditions described. After the time used to effect reduction had elapsed, the solution was filtered from the catalyst and diluted with ether (four times the amount of solvent used), washed with 5% sodium bicarbonate solution, water, and then dried over anhydrous sodium sulfate. The organic solvents were distilled under reduced pressure and the products in each case separated as described below.

Perhydrophenanthrene (XIX)—A mixture of 1,2,3,4,5,6,7,8-octahydrophenanthrene (Aldrich Chem. Co., Milwaukee, Wis.) (XVII) (930 mg.; 0.005 mole), ethanol (50 ml.) containing 1% glacial acetic acid, and 5% Rh/alumina (400 mg.) was hydrogenated at room temperature and 60 p.s.i. initial pressure for 9 hr. The hydrogen uptake was 0.016 mole. The crude product was distilled at 87–89°/2 mm. when 870 mg. (91%) of colorless oil, n_D^{20} 1.5010 was obtained; lit. (38) b.p. 109–111°/4 mm. The IR spectrum (liquid film) showed the absence of the aromatic bands (3050, 3025, 3000 cm.$^{-1}$, and 1480, 800 cm.$^{-1}$) and the presence of the aliphatic C—H stretching frequencies at 2890, 2835 cm.$^{-1}$, and C—H bending frequency at 1440 cm.$^{-1}$.

***cis,cis*-2-Decalol (XX)**—A mixture of 1,2,3,4-tetrahydro-β-naphthol (XVIII) (K & K Laboratories, Inc., Plainview, N. Y.) (740 mg.; 0.005 mole), ethanol (50 ml.) containing 1% glacial acetic acid, and 5% Rh/alumina (200 mg.) was hydrogenated at room temperature and 60 p.s.i. initial pressure for 5.5 hr. The hydrogen uptake was 0.015 mole. The crude product was crystallized twice from petroleum ether when 615 mg. (80%), m.p. 102–104°, lit. (39) m.p. 105°, of XX was obtained. The IR spectrum showed the absence of the aromatic bands (3040, 3000 cm.$^{-1}$ and 1485, 735 cm.$^{-1}$) that were present in XVIII and the presence of the following characteristic bands: 3300 cm.$^{-1}$ (OH); 1050, 1026 cm.$^{-1}$ (C—O), and the other aliphatic C—H bands.

3β-Hydroxy-19-norergosta-5,7,9(10)-triene; Dihydroneoergosterol (XXI)—A solution of neoergosterol (IX) (5 g.) in ethyl acetate (135 ml.) containing perchloric acid [0.05 ml. of a solution prepared from 70% perchloric acid (10 ml.) and ethyl acetate (90 ml.)] was hydrogenated at room temperature and atmospheric pressure over PtO_2 (100 mg.) for 0.5 hr. The solution was filtered from the catalyst, washed with water till neutral, dried over anhydrous sodium sulfate, and the solvent removed *in vacuo*. The residue was crystallized from methanol when 4.01 g. (80%) of XXI was obtained; m.p. 145–147°, lit. (40) m.p. 146–148°. The IR spectrum showed the absence of the *trans* double bond band at 970 cm.$^{-1}$.

19-Norergosta-5,7,9(10)-triene (XXII)—A mixture of dihydroneoergosterol (XXI) (1.14 g.; 0.003 mole), ethanol (50 ml.) containing 1% glacial acetic acid, and 5% Rh/alumina (500 mg.) was hydrogenated at room temperature and 60 p.s.i. for 7 hr. The crude product was crystallized from methanol when 0.99 g. (90%), m.p. 66–67°, lit. (40) 67–68°, of XXII was obtained. The IR spectrum showed the absence of OH and C—O absorbance and the presence of the aromatic characteristic bands at 3040, 3010, 1485, and 810 cm.$^{-1}$. When the pressure in the above experiment was raised to 250 p.s.i., absorption of H_2 was very slow, and when the reaction was worked up as usual the product (XXII) was isolated in 75% yield (820 mg.).

5α,8α,9α,10α-19-Norergostane (XXIII)—A mixture of 19-norergosta-5,7,9(10)-triene (XXII) (1.1 g.; 0.003 mole) of dihydroneoergosterol (XXI) (1.14 g.; 0.003 mole), ethanol (50 ml.) containing 1% glacial acetic acid, and 5% Rh/alumina (2.4 g.) was hydrogenated at room temperature and 60 p.s.i. initial pressure for 12 hr. The hydrogen uptake was 0.009 mole, 0.012 mole in case of dihydroneoergosterol. The crude product, 0.99 g. (90%), was a heavy colorless oil that showed no OH bands or aromatic bands in the IR, and it only showed the usual aliphatic C—H absorbances at 2930, 2900, 2840, 1465, 1445, 1380, 1375, and 1365 cm.$^{-1}$ This compound was not investigated further.

Hydrogenation of Neoergosteryl Acetate (XXIV)—A mixture of neoergosteryl acetate (XXIV) (420 mg.; 0.001 mole), ethanol (25 ml.) containing 1% glacial acetic acid, and 5% Rh/alumina (800 mg.) was hydrogenated at room temperature and 60 p.s.i. for 12 hr. The hydrogen uptake was 0.005 mole. The crude product, 330 mg. (90%), was a colorless oil that had an IR spectrum similar to that of XXIII prepared from dihydroneoergosterol (XXI). When the experiment was conducted in the same way except for only 3 hr., 19-norergosta-5,7,9(10)-triene (XXII) was isolated from the crude product by crystallization from methanol in 40% yield (150 mg.), m.p. 65–67°. The IR spectrum was similar to that of the product previously prepared.

3β-2′-Tetrahydropyranyloxy-19-norergosta-5,7,9(10)-triene (XXV)—Dihydroneoergosterol (XXI) (500 mg.) was dissolved in chloroform (3 ml.) and dihydropyran (200 mg.) (previously dried over KOH, distilled from KOH, and then distilled from sodium). As one drop of a solution containing one drop of phosphorus oxychloride in 5 ml. of ethyl acetate was added, the solution became warm. This solution was warmed to 50° for 20 min., cooled to room temperature, and diluted with ether (100 ml.). The solution was washed with sodium bicarbonate solution, water, dried over sodium sulfate, and solvents removed *in vacuo*. The residue was dried *in vacuo* over P_2O_5 for 24 hr. and then crystallized from methanol. The yield was 540 mg. (90%) of colorless needles, m.p. 125–127°, $[\alpha]_D$ +13.5° (c 1.0). The IR spectrum showed the absence of OH absorbance and the presence of the following characteristic sharp bands: 1195, 1130, 1115, 1070, 1050, and 1025 cm.$^{-1}$ (C—O—C—O—C and C—O vibrations) (35). Additional aromatic and aliphatic C—H bands also were present.

Anal.—Calcd. for $C_{32}H_{50}O_2$: C, 82.35; H, 10.80. Found: C, 82.35; H, 10.94.

Attempted Hydrogenation of the Tetrahydropyranyl Derivative of Dihydroneoergosterol (XXV)—A mixture of the tetrahydropyranyl ether derivative (XXV) (200 mg.), absolute ethanol (40 ml.) containing 1% glacial acetic acid, and 5% Rh/alumina (400 mg.; and in another similar experiment 800 mg.) was shaken in a Parr hydrogenator at room temperature and under 60 p.s.i. H_2 pressure

[6] All the solvents used were boiled for 5 min. to drive off all the dissolved O_2 and then were allowed to cool in an atmosphere of N_2.

for 2 days. After the usual workup of the reaction, the crude product was crystallized from methanol when 180 mg. of white needles, m.p. 124–126°, that had an IR spectrum similar to that of XXV, were obtained.

3,4-Dihydro-2(1-*H*)-naphthalenone (XXVI)—*A. With tert-Butyl Hypochlorite*—A solution of 1,2,3,4-tetrahydro-β-naphthol (XVIII) (595 mg.; 0.004 mole) in carbon tetrachloride (2 ml.) and pyridine (0.32 ml.; 0.004 mole) was cooled to −5°. From a capillary, *tert*-butyl hypochlorite (0.47 ml.; 0.004 mole) was added and the temperature was allowed to stay at 0° for 15 min. The PY·HCl that precipitated was filtered and washed with cold CCl_4 (2 ml.). The filtrate and washings were evaporated at reduced pressure under N_2. A pale yellow liquid remained, 525 mg. (90%), which yielded a semicarbazone (crystallized from ethanol), m.p. 192–194°, lit. (41) m.p. 193–194°. The IR spectrum (liquid film) showed the absence of OH bands, the presence of strong and sharp carbonyl band at 1720 $cm.^{-1}$ together with the other aliphatic and aromatic C—H bands. The NMR spectrum showed the following characteristic peaks: multiplet centering at 2.60 δ (2 protons at C-3), multiplet centering at 3.05 δ (2 protons at C-4), a singlet at 3.61 δ (2 protons at C-1), and a multiplet centering at 7.24 δ (4 aromatic protons).

B. With DMSO—1,2,3,4-Tetrahydro-β-naphthol (XVIII) (595 mg.; 0.004 mole) was dissolved in anhydrous dimethyl sulfoxide (6 ml.) and benzene (6 ml.) containing pyridine (0.32 ml.; 0.004 mole) and trifluoroacetic acid (0.16 ml.; 0.002 mole). After the addition of dicyclohexylcarbodiimide (2.48 g.; 0.012 mole) the reaction mixture was stirred magnetically, under anhydrous conditions, at room temperature for 18 hr. when a fine white precipitate of dicyclohexylurea was being formed throughout the reaction. The mixture was diluted with ether (100 ml.) followed by the dropwise addition of a solution of oxalic acid (1.08 g.; 0.012 mole) in methanol (10 ml.) and the solution was stirred till gas evolution had ceased (0.5 hr.). The solution was cooled, filtered from the urea derivative (m.p. 234°), washed with 5% sodium bicarbonate and twice with water, dried over anhydrous sodium sulfate, and the solvents removed under reduced pressure. The yellow oil that remained was distilled at 117–118°/2 mm. under N_2. The yield was 495 mg. (85%), its semicarbazone had a m.p. 193–194° which did not depress the melting point of the semicarbazone of the material prepared by *tert*-butyl hypochlorite. The IR and NMR spectra were also identical with the material prepared by *tert*-butyl hypochloride.

19-Norergosta-5,7,9(10)-triene-3-one (XXVII)—Dihydroneoergosterol (XXI) (1.52 g.; 0.004 mole) was treated as under DMSO oxidation of 1,2,3,4-tetrahydro-β-naphthol (XVIII). The duration, workup of the reaction, was exactly the same as that previously described. The crude residue left after evaporating the solvents under reduced pressure was subjected to Girard's separation procedure as mentioned before under the ketone (XVI) except that a nitrogen atmosphere always was used. The residue that remained after distilling the ether extract of the ketonic fraction was crystallized from methanol. The yield was 1.13 g. (75%) of white needles, m.p. 90–91°, reported (40) m.p. 91–92°. The IR spectrum showed the absence of OH absorbance, presence of carbonyl band at 1720 $cm.^{-1}$ and the other aliphatic and aromatic C—H bands.

Attempted Reduction of the Ketone (XXVII)—A mixture of the ketone (XXVII) (380 mg.; 0.001 mole), ethanol (40 ml.) containing 1% glacial acetic acid, and 5% Rh/alumina (750 mg.) was hydrogenated at room temperature and 60 p.s.i. initial pressure for 32 hr. when no noticeable absorption of H_2 occurred. After the usual workup of the reaction mixture, the residue (340 mg.) was subjected to Girard's separation where the ketonic fraction gave 190 mg. (50%) of the ketone (XXVII) identified by its melting point and IR spectrum. Distillation of the ether extract of the nonketonic fraction of Girard's separation did not afford any residue.

Rhodium–Platinum (7:3) Oxides—A mixture of rhodium trichloride trihydrate (769 mg.), chloroplatinic acid (345 mg.), and sodium nitrate (20 g.) was heated slowly until a temperature of 300° was reached in 30 min. The mixture started to melt and the red fumes of the oxides of nitrogen evolved. After the evolution of the gases had subsided (temperature about 400°), the temperature was raised and kept at 460–480° for about 10 min. After cooling, the solid mass was rinsed with distilled water. The solid was collected, washed with 100 ml. of 0.5% aqueous sodium nitrate, and then dried over calcium chloride for 48 hr. The yield was 525 mg. (80%) of fine brown powder.

General Procedure for Reduction over Rh–Pt (7:3) Oxides—The solvent, to be used in the hydrogenation, was boiled for 5 min. to drive off all the dissolved O_2, then allowed to cool in an atmosphere of N_2. The amount of glacial acetic acid was added to make a 1% solution. The catalyst was suspended in few milliliters of this solvent and reduced to the metals by hydrogenation at room temperature and 20 p.s.i. for 0.5 hr. The substance to be reduced was dissolved in the rest of the solvent and then was added to the reduced catalyst and the hydrogenation conducted under the prescribed conditions. The mixture then was filtered, diluted with ether (*ca.* 3 times as the amount of solvent used), washed with sodium bicarbonate solution, water, and dried over anhydrous sodium sulfate. The solvents were removed *in vacuo* and the product was worked up as the conditions warrented.

***cis,cis*-2-Decalol (XX)**—A mixture of 1,2,3,4-tetrahydro-β-naphthol (XVIII) (370 mg.; 0.0025 mole), ethanol (25 ml.), and the catalyst (25 mg.) was hydrogenated at room temperature and 60 p.s.i. The hydrogen uptake was 0.0075 mole in 70 min. The crude product crystallized after the removal of the ether, m.p. 85–89°, one recrystallization from petroleum ether afforded 345 mg. (90%), m.p. 103–104°. The IR spectrum was identical with that of the product prepared from the reduction with Rh/alumina.

Hydrogenation of Dihydroneoergosterol (XXI)—A mixture of dihydroneoergosterol (XXI) (380 mg.; 0.001 mole), ethanol (30 ml.), and the catalyst (50 mg.) was hydrogenated at room temperature and 60 p.s.i. for 24 hr. The crude product was an oil whose IR was similar to that of XXIII. When the above experiment was conducted in the same way and the hydrogenation was stopped after 6 hr., 19-norergosta-5,7,9(10)-triene (XXII) was isolated from the crude product by crystallization from methanol. The yield was 160 mg. (50%), m.p. 65–67°.

Hydrogenation of Neoergosteryl Acetate (XXIV)—The previous two experiments were repeated in the same way except using neoergosteryl acetate (XXIV) (420 mg., 0.001 mole) instead of dihydroneoergosterol. From the first experiment the same oily material was separated, and from the second experiment 19-norergosta-5,7,9(10)-triene (XXII) was obtained in a similar manner.

Attempted Hydrogenation of the Tetrahydropyranyl Derivative of Dihydroneoergosterol (XXV)—A mixture of the tetrahydropyranyl ether derivative (XXV) (200 mg.), absolute ethanol (40 ml.) containing 1% glacial acetic acid (the boiling step was omitted), and the catalyst (40 mg.) was shaken under 60 p.s.i. H_2 pressure and at room temperature for 1 day. Standard manipulation afforded 185 mg. of the ether (XXV) identified by its melting point and IR spectrum.

Attempted Hydrogenation of the Ketone (XXVII)—A mixture of the ketone (XXVII) (250 mg.), ethanol (30 ml.), and the catalyst (40 mg.) was shaken under 60 p.s.i. H_2 and at room temperature for 1 day. Standard manipulation afforded 200 mg. which upon Girard's separation process gave 110 mg. (45%) of the ketone (XXVII), m.p. 89–91°. Nothing could be obtained from the nonketonic fraction after the use of the Girard reagent.

Effect of Acid—A series of experiments was conducted using in each a mixture of dihydroneoergosterol (XXI) (200 mg.), ethanol (30 ml.), and the catalyst (30 mg.) or Rh/alumina (400 mg.). Glacial acetic acid in 0.1, 0.2, 0.3, 0.4, or 0.5% concentration was used. The mixtures were hydrogenated at room temperature and 60 p.s.i. for 8 hr. After the usual workup, the experiments with 0.1% and 0.2% acetic acid concentrations yielded dihydroneoergosterol (XXI). The experiments with higher acid concentrations (they had a slow absorption of H_2) afforded 19-norergosta-5,7,9(10)-triene (XXII) in 40–60% yields, identified by its melting point and IR spectrum.

3β,20β-Dihydroxy-19-norpregna-5,7,9(10)-triene (XXXIV)—A mixture of 3β-hydroxy-19-norpregna-5,7,9(10)-triene-20-one (XVI) (894 mg.; 0.003 mole), ethanol 65% (80 ml.) (without acetic acid), and 5% Ru/carbon (1.0 g.) was hydrogenated at room temperature at 60 p.s.i. for 2 days. The hydrogen uptake was 0.003 mole. The crude solid (870 mg.) that was obtained after evaporating the organic solvents was crystallized from ether when 760 mg. (85%) of colorless rosettes of the 20α- and 20β-diols were obtained, m.p. 169–171°. One hundred milligrams of this mixture was recrystallized twice from methylene chloride to yield 20 mg. (of the 20β-isomer), m.p. 209–210°, $[\alpha]_D$ −23° (c 1.0). The IR spectrum showed the absence of carbonyl band, presence of the following characteristic bands: 3330, 3295 $cm.^{-1}$ (OH); 3040, 3000 $cm.^{-1}$ (aromatic C—H); 1485, 810 $cm.^{-1}$ (aromatic ring); and 1140, 1115, 1080, 1040, and 1020 $cm.^{-1}$ (C—O), beside the aliphatic C—H vibrations.

Anal.—Calcd. for $C_{20}H_{28}O_2$: C, 79.96; H, 9.39. Found: C, 79.66; H, 9.45. When this experiment was conducted in the same way, except at 1200 p.s.i. for 10 hr., the same product was separated and the same yield of the diols (XXXIV) was obtained.

3β-20β-Dihydroxy-5α,8α,9α,10α-19-norpregnanediol (XXXV)—3β-20β-(& 20α-)Dihydroxy-19-norpregna-5,7,9(10)-triene (XXXIV), m.p. 169–171° (600 mg.; 0.002 mole), finely powdered, was dissolved in cyclohexane (600 ml.) (previously boiled then cooled under N_2) with the aid of few drops of ethyl acetate. This solution was hydrogenated at room temperature and 1400 p.s.i. over 5% Rh/alumina (1.2 g.) for 12 hr. The catalyst was filtered from the solution which was distilled under reduced pressure and the solid residue (600 mg.) was recrystallized from ether. The yield was 515 mg. (85%), m.p. 185–188°. One hundred milligrams of this mixture was recrystallized twice from methylene chloride when 25 mg. (of the 20β-isomer) dried for 24 hr. *in vacuo* at 60°, m.p. 230–232°, was obtained, $[\alpha]_D$ −6.5° (c 0.5). The IR spectrum showed the absence of the aromatic bands and the presence of the following characteristic bands: 3300, 3200 cm.$^{-1}$ (OH); 1140, 1095, 1065–1055, 1040, and 1010 cm.$^{-1}$ (C—O); and the other aliphatic C—H bands. The NMR spectrum (of the crude reaction product) did not show any peaks in the aromatic, olefinic, or the allylic (1.85–2.20 δ) regions. Also the crude reaction product gave a negative tetranitromethane test (30). Mass spectral molecular ion at *m/e* 306.

Anal.—Calcd. for $C_{20}H_{34}O_2$: C, 78.38; H, 11.18. Found: C, 78.38; H, 10.92.

5α,8α,9α,10α-19-Norpregnan-3,20-dione (VI)—3β-20β-(& 20α-)-Dihydroxy-5α,8α,9α,10α-19-norpregnanediol (XXXV), m.p. 185–188° (306 mg.; 0.001 mole), was dissolved in acetone (40 ml.) previously distilled from potassium permanganate. The solution was cooled to 10° and 0.28 ml. of Jones reagent (31) was added rapidly while stirring the solution under N_2. After 4 min. the solution was diluted with ether (150 ml.) and washed with water, dried over anhydrous sodium sulfate, and the ether evaporated *in vacuo*. The semisolid residue that remained (298 mg.) was dried *in vacuo* over P_2O_5 for 2 days when colorless fine needles were formed. This residue was recrystallized from ether (10 ml.) when 270 mg. (90%), m.p. 159–161°, was obtained. Another recrystallization afforded 220 mg., dried *in vacuo* at room temperature for 48 hr., m.p. 163–164°, $[\alpha]_D$ + 32° (c 0.5). The IR spectrum showed the absence of OH bands, and the presence of the following characteristic bands: 1710–1700 cm.$^{-1}$ (C=O); 1427, 1420 cm.$^{-1}$ (CH_2 at C-2 and C-4; and 1355 cm.$^{-1}$ (CH_3—C(=O)—). The NMR spectrum showed the following peaks: 0.82 δ (C-18 CH_3), 2.15 δ (C-21 CH_3), and a multiplet centering at 2.25 δ (methylene protons at C-2, C-4, and C-17). The ORD (c 0.03 g./100 ml. $CHCl_3$), t = 27°, $[\alpha]_{600}$ +33°, $[\alpha]_{589}$ +33°, $[\alpha]_{400}$ +226.6°, $[\alpha]_{350}$ +566.6°, $[\alpha]_{320}$ +2162°, $[\alpha]_{315}$ +2450°, $[\alpha]_{313}$ +2566° (peak), $[\alpha]_{310}$ +2430°, $[\alpha]_{294}$ 0000°, $[\alpha]_{270}$ −3333°, $[\alpha]_{260}$ −3433° (trough), and $[\alpha]_{250}$ −3433°. The dispersion curve of this diketone is a "single, positive Cotton-effect curve."

Anal.—Calcd. for $C_{20}H_{30}O_2$: C, 79.42; H, 10.00. Found: C, 79.30; H, 9.79.

REFERENCES

(1) O. Gisvold, in "Textbook of Organic Medicinal and Pharmaceutical Chemistry," C. O. Wilson, O. Gisvold, and R. F. Doerge, Eds., J. B. Lippincott, Philadelphia, Pa., 1966, p. 701.

(2) G. W. Barber and M. Ehrenstein, *Ann. Chem.*, **603,** 89(1957).

(3) C. Djerassi, *Science*, **151,** 1055(1966).

(4) T. Miyake and W. H. Rooks, II, in "Methods in Hormone Research," R. I. Dorfman, Ed., vol. V, 59(1966).

(5) E. H. Reerink, H. F. L. Scholer, P. Westerhof, A. Querido, A. A. H. Kassenaar, E. Diczfalusy, and K. C. Tillinger, *Nature (London)*, **186,** 168(1960).

(6) L. Velluz, J. Mathieu, and G. Nominé, *Tetrahedron Suppl.*, **8,** part II, 495(1966).

(7) M. Ehrenstein, G. W. Barber, and H. Hertz, *Endocrinology*, **60,** 681(1957).

(8) E. Mosettig and I. Scheer, *J. Org. Chem.*, **17,** 764(1952).

(9) R. A. Edgren, R. C. Jones, and D. L. Peterson, *Fert. Steril.*, **18,** 238(1967).

(10) T. Ando, *Bull. Chem. Soc. Jap.*, **13,** 371(1938).

(11) J. F. Klebe, H. Finkbeiner, and D. M. White, *J. Amer. Chem. Soc.*, **88,** 3390(1966).

(12) W. E. Bachmann, M. P. Cava, and A. S. Dreiding, *Helv. Chim. Acta*, **42,** 1790(1959).

(13) J. Elks, J. F. Oughton, and L. Stephenson, Brit. pat. 864,231, 1961.

(14) L. F. Fieser and S. Rajagopalan, *J. Amer. Chem. Soc.*, **71,** 3938(1949).

(15) J. M. Bobbitt, in "Advances in Carbohydrate Chemistry," vol. 11, Academic, New York, N. Y., 1956, p. 1.

(16) G. Slomp, Jr., Y. F. Shealy, J. L. Johnson, R. A. Donia, B. A. Johnson, R. P. Holysz, R. L. Pederson, A. O. Jensen, and A. C. Ott, *J. Amer. Chem. Soc.*, **77,** 1216(1955).

(17) G. Slomp, Jr., and J. L. Johnson, *ibid.*, **80,** 915(1958).

(18) A. Maggiolo and A. Tumolo, *J. Amer. Oil Chemists Soc.*, **38,** 279(1961).

(19) F. L. Greenwood and H. Rubinstein, *J. Org. Chem.*, **32,** 3369(1967).

(20) E. Farkas and R. T. Rapala, U. S. pat. 3,015,666, 1958.

(21) J. R. Durland and H. Adkins, *J. Amer. Chem. Soc.*, **59,** 135(1937); **60,** 1501(1938).

(22) R. V. Petersen and O. Gisvold, *J. Amer. Pharm. Ass., Sci. Ed.*, **45,** 572(1956).

(23) P. N. Rylander and D. R. Steele, *Engelhard Ind. Tech. Bull.*, **3,** 125(1963).

(24) L. F. Fieser and M. Fieser, in "Reagents for Organic Synthesis," Wiley, New York, N. Y., 1967, p. 90.

(25) K. E. Pfitzner and J. G. Moffatt, *J. Amer. Chem. Soc.*, **85,** 3027(1963); **87,** 5661(1965); **87,** 5670(1965).

(26) S. Nishimura and H. Taguchi, *Bull. Chem. Soc. Jap.*, **36,** 353(1963).

(27) K. Kindler and L. Blaas, *Chem. Ber.*, **77B,** 585(1944).

(28) K. Kindler, H. Oelschlager, and P. Henrich, *ibid.*, **86,** 167(1953).

(29) R. B. Woodward, F. Sondheimer, D. Taub, K. Heusler, and W. M. McLamore, *J. Amer. Chem. Soc.*, **74,** 4223(1952).

(30) "Elucidation of Structures by Physical and Chemical Methods," K. W. Bentley, Ed., Interscience, New York, N. Y., 1963, 34, 161–163.

(31) C. Djerassi, R. R. Engle, and A. Bowers, *J. Org. Chem.*, **21,** 1547(1956).

(32) C. Djerassi, in "Optical Rotatory Dispersion," McGraw-Hill, New York, N. Y., 1960, pp. 51, 71–72, 83, 178, 189.

(33) A. Windaus and P. Borgeaud, *Ann.*, **460,** 235(1928).

(34) L. J. Bellamy, in "The Infra-red Spectra of Complex Molecules," Wiley, New York, N. Y., 1954, p. 274.

(35) K. Nakanishi in "Infrared Absorption Spectroscopy," Holden-Day, San Francisco, Calif., 1964, pp. 21, 36.

(36) J. A. Cifonelli and F. Smith, *Anal. Chem.*, **26,** 1132(1954).

(37) J. Szmuszkovicz, in "Advances in Organic Chemistry," vol. 4, Interscience, New York, N. Y., 1963, p. 1.

(38) R. P. Linstead, R. R. Whetstone, and P. Levine, *J. Amer. Chem. Soc.*, **64,** 2014(1942).

(39) W. Huckel, R. Mentzel, E. Brinkmann, and E. Kamenz, *Ann.*, **451,** 109(1927).

(40) E. L. McGinnis, G. D. Meakins, and D. J. Morris, *J. Chem. Soc.*, **1967,** 1238.

(41) G. Stork and E. L. Foreman, *J. Amer. Chem. Soc.*, **68,** 2172 (1946).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 7, 1969, from the *Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455*

Accepted for publication October 31, 1969.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Abstracted in part from a dissertation submitted to the Graduate School of the University of Minnesota by Ahmed H. El Masry, in partial fulfillment of the Doctor of Philosophy degree requirements.

This investigation was supported in part by Research Grant HE-06569 from the National Heart Institute, U. S. Public Health Service, Bethesda, Md.

Hydrolysis of Procaine and Its Quaternary Derivatives within Lyotropic Smectic Mesophases

K. S. MURTHY* and E. G. RIPPIE

Abstract □ The effect of solvent anisotropicity on ester hydrolysis was studied in lyotropic liquid crystalline phases. Procaine hydrochloride and two of its quaternary derivatives, procaine methyl chloride and procaine ethyl chloride, were employed as substrates, and an aqueous gel consisting of 55% polyoxyethylene tridecyl ether, identified as a neat smectic system, was chosen as the solvent medium. Pseudo-first-order rate constants for the hydrolysis were obtained in the apparent pH range 8.80–11.40, at a temperature of from 30 to 50°, over a surfactant span of 50–65% in the mesophase. Rate measurements were also carried out in aqueous media and in mixed aqueous systems containing polyethylene glycol 300 and 400. The esters were located within the aqueous polyoxyethylene layers as indicated by their UV spectral characteristics. The reaction rates were found to be considerably slower (300- to 1100-fold) in the liquid crystalline phases than in aqueous media. The reactions, as they occur within the smectic phase, are characterized by relatively low apparent activation energies and by large negative entropies of activation.

Keyphrases □ Procaine, quaternary derivatives, hydrolysis—lyotropic smectic mesophases □ Activation energies, entropies—procaine, derivatives, in lyotropic mesophases □ Hydrolysis rate constants—procaine, derivatives, in lyotropic mesophases □ UV spectrophotometry—solubilizate location, homogeneous gels

Mesophases, also called mesomorphs or liquid crystals, are states of matter intermediate between crystalline solids and isotropic liquids (1). Molecules in this anisotropic state are orientated with their long axes parallel to one another. The molecules are typically elongated and are generally characterized by the presence of strong dipoles near the centers and weak dipoles near the ends. The parallel orientation of the molecules results both from intermolecular attractions and from the shapes of the molecules. Due to differences in the strengths of the molecular linkages in different directions, for these systems, the transition from the solid to the liquid state takes place in stages, with rising temperature. At lower temperatures the weaker bonds break first, resulting in the formation of meosphases. This is followed at higher temperatures by the loosening of the residual molecular associations when a true liquid state is attained (2).

There are three different types of liquid crystalline phases (3). These are: smectic in which the molecules are arranged in the form of layers with their long axes parallel to each other in the layers and nearly normal to the plane of the layers; nematic where the molecules maintain parallel arrangement along their long axes but are not stratified; and cholesteric where the molecular axes are parallel to the plane of the layers, but the direction of the long axes of the molecules changes continuously in going from one layer to another, resulting in a helical structure. Cholesteric mesomorphism is exhibited by compounds derived from cholesterol, although cholesterol itself is not mesomorphic.

Liquid crystalline structures may be prepared either by heating the crystalline solid (thermotropic mesomorphism) or by the addition of controlled amounts of polar solvents, generally water, to certain organic compounds (lyotropic mesomorphism). Many amphiphilic compounds, such as soaps and surfactants, having a tendency to form lyotropic mesophases on the addition of water, show the following general sequence of phases (4):

crystalline solid → neat phase → viscous isotropic phase → middle phase → micellar solution → molecular dispersion in water

The term "neat phase" corresponds to the smectic mesophase where the molecules are arranged in the form of lamellar micelles consisting of parallel equidistant sheets of amphiphilic molecules separated by layers of water. In such systems, contact between the polar–polar and nonpolar–nonpolar portions of the molecules is maximized, resulting in a stable configuration. The "middle phase" is composed of long cylindrical micelles arranged in a two-dimensional hexagonal array with the polar groups forming the surface of the cylinders (5).

Several books and reviews dealing with liquid crystals have appeared in recent years indicating the importance of these structures to scientists in different disciplines (6–8). It is generally agreed that lipids exist in biological membranes as mesophases. The structural model for the plasma membrane proposed by Davson and Danielli (9) and Robertson (10) rests on the foundations of a bimolecular lipid leaflet with protein absorbed on both faces. Artificially constituted bimolecular lipid membranes composed of neat smectic structures are currently employed as models for the study of properties of natural membranes (11). In spite of the recognition of the role of mesophases in biological systems, reports of systematic studies of reaction kinetics utilizing these structures as solvent media have been scarce. This is probably related to the fact that nonmesomorphic solutes in relatively low concentrations often tend to destroy or disrupt the parallel orientation of the molecules composing the mesophases.

The only reported kinetic studies employing thermotropic mesophases as solvents were conducted by Svedberg (12, 13) nearly 50 years ago. He studied the unimolecular thermal decomposition of picric acid, pyrogallol, and trinitroresorcinol, at a temperature of 140°, using *p*-azoxyphenetole as the solvent system. Swarbrick and Carless (14) studied the rate of oxidation of benzaldehyde in lyotropic mesophases encountered in ternary systems consisting of betaine–benzaldehyde–water. The reaction rates were found to be significantly lower in the mesophases when compared with the corresponding rates in isotropic systems. Barry and Shotton (15) studied the acid-catalyzed hydrolysis of sodium dodecyl sulfate in the presence of 1-hexadecanol in a solvent medium containing perchloric acid at 60°. They

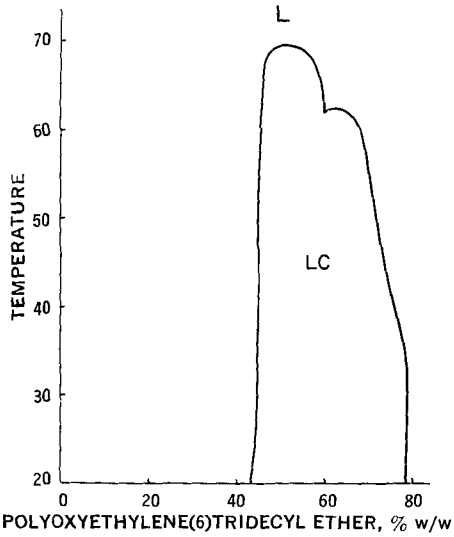


Figure 1—*Equilibrium phase diagram for the system polyoxyethylene (6) tridecyl ether + water showing regions of isotropic liquid (L) and liquid crystalline (LC) phases.*

found an increase in the rate of hydrolysis of the substrate in the liquid crystalline phase and attributed this to a decrease in the dielectric constant at the surface of the lamellar micelles constituting the phase.

Because of the ordered arrangement of molecules within mesophases, reaction rates in these systems would be expected to exhibit significant solvent effects in many instances. The aim of this study was to investigate the effect of the anisotropicity of the solvent on the kinetics of ester hydrolysis, utilizing lyotropic liquid crystalline phases as the solvent media. Procaine hydrochloride (PHC) was chosen as the ester substrate, and an aqueous gel consisting of 55% polyoxyethylene (6) tridecyl ether was selected as the solvent phase. In order to determine the importance of steric and inductive effects in mesophases, hydrolysis rates of two quaternary derivatives of procaine—procaine methyl chloride (PMC) and procaine ethyl chloride (PEC)—were also studied. For purposes of comparison, hydrolysis rates were also determined in aqueous and polyethylene glycol 300–water solutions.

EXPERIMENTAL

Materials—Polyoxyethylene (6) tridecyl ether.[1] As obtained commercially, this surfactant contained impurities exhibiting absorbance in the UV region. Attempts to eliminate these UV-absorbing materials by treatment with ion-exchange resins and washing with strong alkalies and acids proved to be unsuccessful. Polyethylene glycol 300[2] (PEG 300) and polyethylene glycol 400[2] (PEG 400) USP, procaine hydrochloride[3] USP. The two quaternary derivatives of procaine, PMC (m.p. 171–173°) and PEC (m.p. 202°), were synthesized according to the procedure of Einhorn and Uhlfelder (16). The compounds were recrystallized from a mixture of ethyl alcohol and ethyl acetate and dried under vacuum at 80° for 12 hr.

Anal.—Calcd. for $C_{14}H_{23}ClN_2O_2$: C, 58.63; H, 8.08; Cl, 12.36, N, 9.77. Found: C, 58.75; H, 8.35; Cl, 11.80; N, 9.38.

Anal.—Calcd. for $C_{15}H_{25}ClN_2O_2$: C, 59.89; H, 8.38; Cl, 11.79. Found: C, 60.01; H, 8.75; Cl, 11.50.

[1] Marketed as Renex 36, lot no. 8404 B, Atlas Chemical Industries Wilmington, Del.
[2] Ruger Chemical Co., Irvington, N. Y.
[3] Matheson, Coleman & Bell, East Rutherford, N. J.

Table I—Ultraviolet Absorption Maxima of the Substrates in Various Solvent Media

Substrate	Solvent Medium	$\lambda_{max.}$
PHC	Distilled water[a]	286.0
	PEG 300–water (42%)[a]	295.0
	Mesophase (55% surfactant)[a]	295.0
	n-Hexane	270.5
PMC	Distilled water	290.5
	PEG 300–water (42%)	298.0
	Mesophase (55% surfactant)	298.0
PEC	Distilled water	290.5
	PEG 300–water (42%)	298.5
	Mesophase (55% surfactant)	298.0

[a] Conditions: solvents buffered to pH 11.40.

The buffers employed were as follows: for studies at pH 8.80 and 9.70, borate; for pH 10.60 and 11.40, carbonate. *n*-Hexane[4] spectroquality reagent. All other chemicals were reagent grade.

Equipment—Polarizing microscope[5] equipped with Koeffler hot stage, centrifuge,[6] spectrophotometer,[7] pH meter.[8] For kinetic measurements in aqueous buffer solutions at pH 11.40 where the reactions are relatively rapid (half-lives of less than 20 min.), the reactions were conducted directly in silica cells housed within the thermostated compartment of the recording spectrophotometer.[9]

Procedure—*Phase Diagram*—Mixtures containing different concentrations of surfactant were prepared by accurately weighing the surfactant and distilled water in small weighing bottles, melting in a water bath to obtain a homogeneous mass, stirring, and cooling to room temperature. The samples were allowed to equilibrate at room temperature for 48 hr. before being examined for liquid crystalline characteristics.

The identification of the sample as isotropic or liquid crystalline was made by visual and microscopic observations. In this system the mesophase was found to be a viscous, transluscent gel. The isotropic liquid was found to be fluid and transparent. Microscopic examination was conducted on a small quantity of the sample placed on a clean glass slide with a coverslip placed lightly on top. The existence of birefringence was verified by observation under crossed polars employing a magnification of 100×. The temperature of phase transition was determined by heating the sample on the hot stage. The rate of heating was at first rapid; but as temperatures came close to the transition temperatures, the rate was maintained at 1° for every 5 min. The temperature at which there was complete disappearance of birefringence, when viewed in the microscope under polarized light, was taken as the temperature of transition. At each surfactant concentration, three such determinations were made and the average value was used. The phase diagram was also determined with the same concentrations of buffer salts as were later used in the kinetic experiments. No significant differences in visual appearance or microscopic features of the mixtures were observed due to the presence of the buffer salts. In all cases there was a slight lowering of transition temperatures of the order of 2° or less.

Assay—The concentrations of the substrates, PHC, PMC, and PEC, in the various solvent systems were followed by measuring the absorbance at 306 mμ. Para-aminobenzoic acid, the degradation product of the substrates, does not absorb appreciably at this wavelength and the observed absorbance change was, therefore, taken as exclusively due to the presence of the substrates. Due to the insolubility of the surfactant in dilute aqueous solutions, the absorbance measurements were made for systems containing the surfactant in 20% ethanol–water solutions. All the kinetic studies were performed at an ionic strength of 0.1 adjusted with potassium chloride.

Systems containing polyoxyethylene chains are generally found to be susceptible to auto-oxidative degradation (17, 18). Such a breakdown of the polyoxyethylene in the systems under study would result

[4] Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Bausch & Lomb, C. Reichert Optische Werke A. G.
[6] Servall Superspeed Centrifuge, type ss, Ivan Servall, Inc., Norwalk, Conn.
[7] Beckman model DU, Beckman Instruments, Inc., Fullerton, Calif.
[8] Beckman model GS, Beckman Instuments, Inc., Fullerton, Calif.
[9] Cary model 14, Applied Physics Corp., Monrovia, Calif.

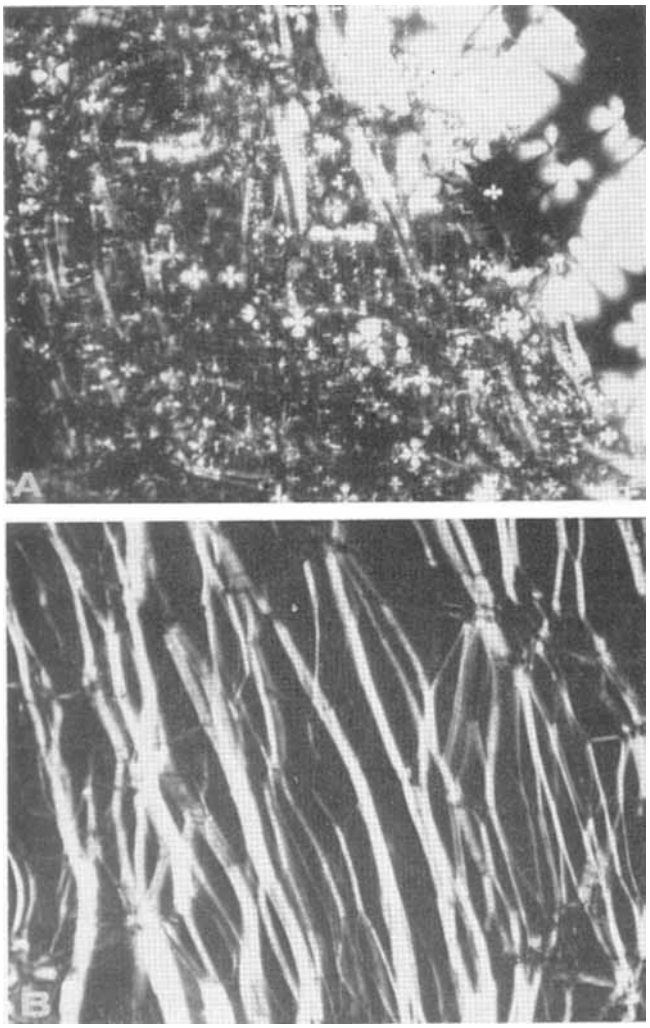


Figure 2—*Typical photomicrographs of the mesophase containing 55% surfactant, polyoxyethylene (6) tridecyl ether, at room temperature under crossed polars and magnification 100×;* (A) *typical anisotropic spherulites;* (B) *network of anisotropic threads and oily streaks.*

in a significant drop in pH. After some preliminary experimentation, a combination of sodium sulfite and hypophosphorous acid in concentrations of 0.1% each was found to be effective in stabilizing against oxidation. In the course of preliminary experiments, it was also found that reaction rates were extremely low below an apparent pH of 8.00. Studies were, therefore, conducted at higher pH's.

In a typical experiment the following procedure was employed. The theoretically calculated quantities of the buffer salts and the substrates were dissolved in distilled water. A measured volume of this buffer solution was pipeted into a conical flask containing a

Table II—Effect of the Solvent Medium on the Apparent First-Order Rate Constants for the Hydrolysis of the Substrates

Substrate	Mesophase Apparent First-Order Rate Constant,[a] $k \times 10^3$ hr.$^{-1}$	Buffered Aqueous Solution Apparent First-Order Rate Constant,[b] k, hr.$^{-1}$
PHC	5.53[c]	1.69[f]
PMC	3.70[d]	4.09[g]
PEC	7.39[e]	4.34[h]

[a] Conditions: pH 11.40, temperature 50°, surfactant concentration 55%. [b] Conditions: pH 11.40, temperature 50°. [c] $SD = \pm 3.0\%$. [d] $SD = \pm 1.9\%$. [e] $SD = \pm 3.9\%$. [f] $SD = \pm 1.8\%$. [g] $SD = \pm 2.3\%$. [h] $SD = \pm 2.1\%$.

Table III—pH Dependency of the Apparent First-Order Rate Constants for the Hydrolysis of PHC in the Mesophase[a]

Apparent pH of the Mesophase	Apparent First-Order Rate Constant, $k \times 10^3$ hr.$^{-1}$
8.80	1.19[b]
9.70	1.29[c]
10.60	3.35[d]
11.40	5.52[e]

[a] Conditions: temperature 50°, surfactant concentration in the mesophase 55%. [b] $SD = \pm 4.1\%$. [c] $SD = \pm 4.5\%$. [d] $SD = \pm 2.5\%$. [e] $SD = \pm 3.0\%$.

previously weighed quantity of the surfactant such that the resultant gel contained the required concentration of the surfactant and was of the desired apparent pH. The ionic strength of the gel was maintained at 0.1 by the addition of potassium chloride to the aqueous solution whenever necessary. In order to obtain a homogeneous gel, the mixture of the surfactant and the aqueous salt solution was melted in a water bath and cooled. The necessary adjustments in the apparent pH of the mesophase were made by the addition of concentrated solutions of sodium hydroxide or hydrochloric acid to the aqueous buffer solution. The gel was then subdivided accurately into 5-g. portions in flasks of 10-ml. capacity. A similar set of flasks containing reference gel was also prepared at the same time in a manner identical to that of the sample gel but without substrate. To suppress any possible oxidation of the surfactants, the gels were degassed with a stream of nitrogen and the flasks were tightly stoppered, sealed, and protected from light. At appropriate intervals, duplicate samples were withdrawn, diluted with 40% ethanol–water solution, and analyzed spectrophotometrically with an appropriately treated blank in the reference compartment of the spectrophotometer.

The apparent pH of the mesophase was determined at a room temperature of about 25° at the start of each kinetic experiment. At subsequent intervals, the pH of the solutions used for the assay were measured to monitor the pH of the gel.

UV Absorption Spectra—The UV absorption spectra of solubilizates have been utilized for determining their location within the different regions of the micelle (19, 20). To prepare the mesophase for UV examination, the homogeneous gel was introduced into the cells with the aid of a syringe. The cells were held in a water bath at 75° until the air bubbles entrapped in the gel were driven off. Because of this procedure, the theoretically added amounts of the substrates could not be retained in the samples. Comparisons

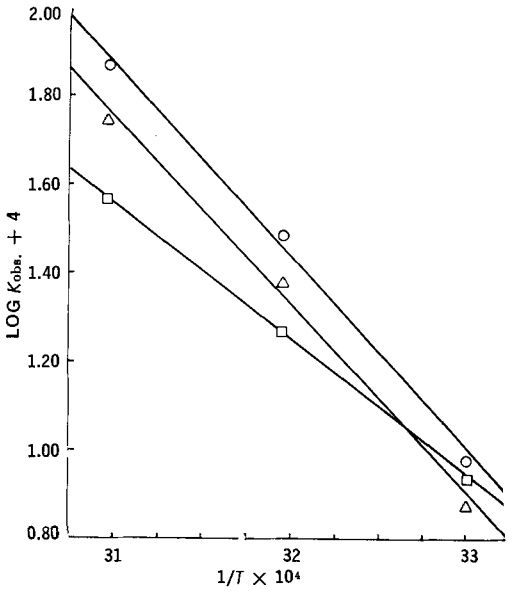


Figure 3—*Arrhenius plots of the hydrolysis reaction in the mesophase containing 55% surfactant at pH 11.40. Key:* △, *PHC;* □, *PMC; and* ○, *PEC.*

Table IV—Activation Parameters for the Hydrolysis Reactions in the Mesophase and in the Aqueous Solution

Substrate	Energy of Activation, kcal./mole	Entropy, e.u.	Frequency Factor, sec.$^{-1}$
PHC[a]	7.60	−26.9	3.3×10^6
PMC[a]	2.21	−43.8	1.9×10^3
PEC[a]	8.08	−24.8	2.5×10^6
PHC[b]	13.80	−23.8	4.0×10^7

[a] Conditions: solvent, mesophase, pH 11.40, surfactant concentration 55%. [b] Conditions: solvent, aqueous solution, parameters obtained from *Reference 25*.

Table V—Apparent First-Order Rate Constants for the Hydrolysis Reaction of the Substrates in PEG 300–Water (42%) Medium[a]

Substrate	Apparent First-Order Rate Constant, $k \times 10^2$ hr.$^{-1}$
PHC	3.06[b]
PMC	2.09[c]
PEC	1.99[d]

[a] Conditions: pH 11.40, temperature 50°. [b] $SD = \pm 3.8\%$. [c] $SD = \pm 3.1\%$. [d] $SD = \pm 1.8\%$.

were, therefore, made on the basis of wavelength of maximum absorption.

RESULTS AND DISCUSSION

The regions of temperature and composition for the occurrence of mesophase and isotropic liquid in the surfactant–water mixtures are presented in Fig. 1. The general features of the diagram are similar to that reported in the literature for systems of this type (21). At room temperature, liquid crystalline phases were found to occur in the surfactant concentration range 44–76% w/w. It has not been possible to determine the region where both the phases, isotropic liquid and mesophase, coexist. Typical photomicrographs of the mesophase observed under crossed polars are presented in Fig. 2. The anisotropic spherulites, both positive (extinction arms narrowest at the center of the cross) and negative (extinction arms broadest at the center of the cross), encountered in this system represent one form of focal conic

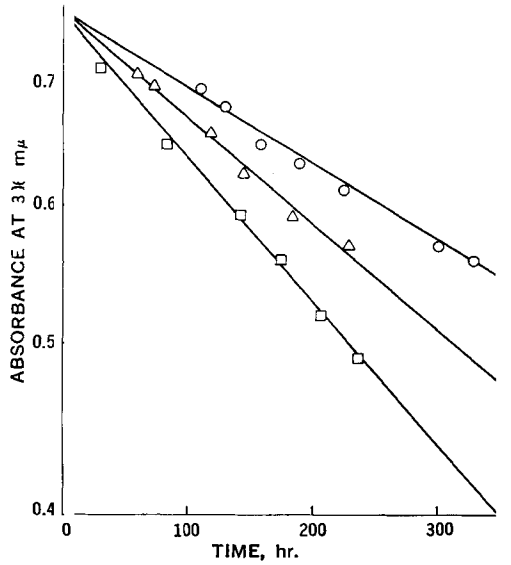


Figure 4—*Plot showing the effect of surfactant concentration in the mesophase on the rate of hydrolysis of PHC at 50° and at pH 9.70. Key: ○, 65%; △, 55%; and □, 50%.*

Table VI—Effect of PEG 400 on the Apparent First-Order Rate Constants for the Hydrolysis of PHC[a]

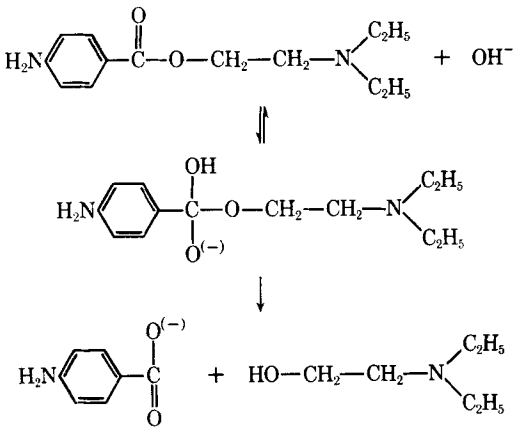
Surfactant Concn., % w/w	Apparent First-Order Rate Constant, $k \times 10^3$ hr.$^{-1}$
50	2.04[b]
55	1.29[c]
65	0.97[d]

[a] Conditions: apparent pH of the mesophase 9.70, temperature 50°. [b] $SD = \pm 4.9\%$. [c] $SD = \pm 4.5\%$. [d] $SD = \pm 5.1\%$.

texture characteristic of smectic structures. Current theories do not consider the middle phase to be smectic (22) since it does not assume any focal conic arrangement. Mixtures containing 50, 55, and 65% surfactant did not show any phase separation when equilibrated at 50° or when centrifuged at room temperature for 7 hr. at 7000 r.p.m. The temperature of transition from the mesophase to the isotropic liquid at 50, 55, and 65% surfactant concentrations are 69, 69, and 64°, respectively. For nonionic surfactant systems, the two-phase region consisting of isotropic liquid and liquid crystalline phases is narrow, and it is reasonable to assume that at 50°, the highest temperature at which the rate studies were carried out, the system is composed of neat smetic phase only.

From the data presented in Table I, it is apparent that the wavelength of maximum absorption for all three substrates in the mesophase are very close to those in the PEG 300–water mixtures but significantly different from those in *n*-hexane and in distilled water. This indicates that the esters are located within the polyoxyethylene layers of the lamellar micelle.

Kinetic Studies—Pseudo-first-order rate constants for the hydrolysis of the substrates were obtained from least-squares calculations of the slopes of the plots of log absorbance *versus* time. The apparent first-order rate constants for the hydrolysis of the substrates in the mesophase containing 55% surfactant and the corresponding rate constants in simple aqueous buffered solutions at pH 11.40 are presented in Table II. The pH dependence of the pseudo-first-order rate constants in the mesophase is shown in Table III. A typical Arrhenius plot for the hydrolysis reaction in the mesophase is shown in Fig. 3. The value of the heat of ionization of water taken from the literature (23) (12.27 kcal./mole) was subtracted from the observed values to obtain the apparent energies of activation as given in Table IV. The entropy of activation and the frequency factors were calculated from the well-known Eyring equation on absolute reaction rates (24). All data are consistent with the mechanism of hydrolysis (see Scheme I) proposed by Higuchi *et al.* (25) for procaine in strongly alkaline solutions (Fig. 4).



Scheme I

Since the UV absorption data of the esters in the various solvent phases indicated that these substrates were located within the aqueous polyoxyethylene layers of the micelle, the reaction rates were also determined in a medium simulating the environment of the substrates in the mesophase. If the substrates are protected from hydrolytic reaction by the polyoxyethylene chains, then a medium which corresponds in molecular composition to the polyoxyethylene

Table VII—Effect of PEG 400 on the Apparent First-Order Rate Constants for the Hydrolysis of PHC[a]

PEG 400 Concn., % w/v	Apparent First-Order Rate Constant, $k \times 10^2$ hr.$^{-1}$
35	4.46[b]
40	2.87[c]
45	2.32[d]

[a] Conditions: pH 11.40, temperature 50°. [b] $SD = \pm 2.2\%$. [c] $SD = \pm 1.8\%$. [d] $SD = 1.7\%$.

portion of the surfactant in the mesophase should result in a significant lowering of the observed rates. The results of the rate constants determined in such a medium are given in Table V.

Also, under these conditions, increase in the concentration of ethylene oxide in the solvent system should lower the observed rates. Such an increase in the ethylene oxide concentration can be brought about either by an increase in the PEG concentration or by an increase in the surfactant concentration. Rate constants obtained in both cases are presented in Tables VI and VII. An examination of this data shows that, although polyoxyethylene exerts a shielding action, reducing the rates of decomposition, the observed rates in PEG 300–water systems are higher than in the mesophase. While no definite arguments can be offered to explain the observed differences in the two media, it is probable that these results may reflect differences in the precise orientation of the esters in the two media and their accessibility to hydrolytic attack. This is especially important in view of the results indicating that the reactions are strongly inhibited with an increase in surfactant concentration in the medium.

The degree of protection conferred by polyoxyethylene chains appears to be dependent more on the concentration than on the chain length. The small differences observed in the rates of hydrolysis between the two quaternary compounds, PMC and PEC, both in aqueous and in PEG 300–water mixtures, are within the range of experimental error, and no significance can be attached to them.

SUMMARY

Equilibrium conditions of temperature and composition at which lyotropic mesophases occur were established for the system polyoxyethylene (6) tridecyl ether–water. Employing substrate concentrations of the order of 1×10^{-4} *M*, hydrolytic decomposition rates of procaine and two of its quaternary derivatives, PMC and PEC, were determined spectrophotometrically in a medium consisting of 55% surfactant and identified to be of neat smectic type. Apparent energies and entropies of activation were calculated for the reactions in the mesophase from rate measurements in the temperature range 30–50°. The reactions, as they occur in the mesophase, appear to be characterized by low Arrhenius energies of activation and large negative entropies. On the basis of UV spectral characteristics, it was inferred that the substrates are located in the polyoxyethylene region of the lamellar micelles. Kinetic measurements in a 42% PEG 300–water system, which corresponds in composition to the polyoxyethylene portion of the micelle, indicate that the reaction rates are significantly retarded by the polyoxyethylene chains. The degree of retardation appears to be dependent upon the concentration of PEG in the system. The available data do not appear to indicate any change in the reaction mechanism for the hydrolysis of procaine in the mesophase from that in simple aqueous solutions.

REFERENCES

(1) O. Lehmann, *Z. Phys. Chem.* (Leipzig), **4,** 462(1889).
(2) G. H. Brown and W. G. Shaw, *Chem. Rev.*, **57,** 1049(1957).
(3) G. Friedel, *Ann. Phys. (Paris)*, **18,** 273(1922).
(4) R. R. Balambra, J. S. Clunie, and J. F. Goodman, *Proc. Roy. Soc. London*, **A285,** 535(1965).
(5) P. A. Winsor, *Chem. Rev.*, **68,** 1(1968).
(6) G. W. Gray, "Molecular Structure and the Properties of Liquid Crystals," Academic, New York, N. Y., 1962.
(7) "Ordered Fluids and Liquid Crystals," Advan. Chem. Ser. 63, Amer. Chem. Soc., Washington, D. C., 1967.
(8) A. Saupe, *Angew. Chem. Int. Ed. Engl.*, **7,** 97(1968).
(9) J. F. Danielli and H. Davson, *J. Cell. Comp. Physiol.*, **5,** 495(1935).
(10) J. D. Robertson, in "Cellular Membranes in Development," M. Locke, Ed., Academic, New York, N. Y., 1964, p. 1.
(11) H. T. Tien and A. L. Diana, *Chem. Phys. Lipids*, **2,** 55 (1968).
(12) T. Svedberg, *Kolloid Z.*, **18,** 54, 101(1916).
(13) *Ibid.*, **21,** 19(1917).
(14) J. Swarbrick and J. E. Carless, *J. Pharm. Pharmacol.*, **16,** 596(1964).
(15) B. W. Barry and E. Shotton, *ibid.*, **19,** 785(1968).
(16) A. Einhorn and E. Uhlfelder, *Ann. Chem.*, **372,** 138(1910).
(17) F. E. Bailey, Jr., and J. V. Koleske, in "Nonionic Surfactants," vol. 1, M. J. Schick, Ed., Marcel Dekker, New York, N. Y., 1967, p. 807.
(18) C. W. McGary, *J. Polym. Sci.*, **46,** 51(1960).
(19) S. Riegelman, N. A. Allawala, M. K. Hrenoff, and L. A. Strait, *J. Colloid. Sci.*, **13,** 208(1958).
(20) M. Donbrow and C. T. Rhodes, *J. Pharm. Pharmacol.*, **18,** 424(1966).
(21) B. A. Mulley and A. D. Metcalf, *J. Colloid. Sci.*, **19,** 501 (1964).
(22) F. B. Rosevear, *J. Soc. Cosmet. Chem.*, **19,** 581(1968).
(23) H. S. Harned and B. B. Owens, "The Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold, New York, N. Y., 1967, p. 754.
(24) S. Glasstone, "Text Book of Physical Chemistry," 2nd ed., D. Van Nostrand, Princeton, N. J., 1946, p. 1103.
(25) T. Higuchi, A. Havinga, and L. W. Busse, *J. Amer. Pharm. Ass., Sci. Ed.*, **39,** 405(1950).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 29, 1969, from the *Department of Pharmaceutics, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455*

Accepted for publication November 21, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Abstracted in part from a thesis submitted by K. S. Murthy to the University of Minnesota in partial fulfillment of Doctor of Philosophy degree requirements.

* Present address: Product Development Department, Parke Davis & Co., Detroit, MI 48232

Statistical Techniques in Predicting Thermal Stability

DONALD L. BENTLEY

Abstract □ In developing new pharmaceutical products it is often necessary to predict degradation rates at marketing temperatures from data collected on accelerated degradation taken at elevated temperatures. A technique for predicting degradation rate based on the Arrhenius equation was presented by Garrett in 1956. While his method is characterized by ease of computation involved (necessary due to scarcity of computer facilities at that time), it violates a number of assumptions upon which least-squares analysis is based, and hence inferences made from the results can be misleading. This report presents a method based on weighted least-squares analysis which can easily be adapted for computer analysis. Comparisons are made with the method suggested by Garrett to illustrate differences in technique and the effect the basic assumptions have upon the results obtained by the two methods. A statistical test is presented for determining the applicability of the Arrhenius relation to the data at hand. Finally, the technique is illustrated by application to chloramphenicol.

Keyphrases □ Thermal stability—statistical techniques for prediction, equations derived □ Arrhenius least-squares equations, thermal stability—comparison, evaluation □ Chloramphenicol, thermal stability—statistical determination □ Degradation rates—chloramphenicol

In 1956 Garrett (1) published a paper recognizing the economic benefits of predicting thermal stability of a drug from data collected at elevated temperatures. The method outlined by Garrett was a simple approximation to a method of fitting the Arrhenius relation by weighted least-squares analyses suggested by McBride and Villars (2) in 1954. The approximation suggested by Garrett gained a great deal in ease of computation, yet sacrificed in the extent to which valid inferences could be derived from the results. Until 1960 ease of computation was of primary concern due to scarcity and cost of computer time, but today even the smallest company can easily rent computer time at a reasonable cost. However, the approximate solution suggested by Garrett is the standard method used today (3, 4).

The purposes of this paper are to outline a method for predicting stability as based on the weighted least-squares technique, to illustrate why weighted least squares should be used in lieu of the unweighted approximation, and to present a statistical test for the validity of the Arrhenius assumption which can easily be computed from the results of the weighted method.

Appendix 1 contains the mathematical formulas involved in the weighted least-squares analysis.

THEORETICAL

Arrhenius Relationship—The functional relationship between time and concentration of a drug stored under constant conditions is dependent upon order of reaction and a rate constant which determines speed of reaction. A thorough discussion of methods for picking proper order is outside the scope of this paper and the assumption will be made that correct order can be determined. An example of a typical situation is the first-order reaction given by Eq. 1 where the logarithm of concentration at time t, denoted C_t, is linearly related to time by

$$\ln C_t = \ln C_0 - k_\tau t \qquad \text{(Eq. 1)}$$

where τ is the temperature at which storage took place and k_τ is the rate constant.

The Arrhenius relationship:

$$\ln k_\tau = \gamma + \delta/\tau \qquad \text{(Eq. 2)}$$

states that speed of reaction is dependent upon temperature; that is the logarithm of the reaction rate is a linear function of the reciprocal of absolute temperature. It is this relationship which allows data taken at elevated temperatures to be used to predict the degradation rate at room temperature and hence estimate shelf life of the drug. The rate constants obtained at the elevated temperatures can be used to estimate the parameters in the Arrhenius equation, which in turn can be used to estimate reaction rate at room temperature (or any other desired temperature).

The methods of estimation used in the previous procedure can vary from simple (and quick) eyeball techniques to a thorough statistical analysis based on weighted least squares. The remaining discussion points out the advantages of the latter in comparison to less rigorous techniques.

Simple Linear Regression—Assume a situation in which a variable Y is linearly dependent upon a second variable X. Examples of such a situation include both Eqs. 1 and 2. A general expression for such a relationship is $Y = \alpha + \beta X$. If Y could be measured without error for any value of X, α and β could be determined from two sample points and any further observations would fall on the determined line.

In practice, experimental error enters due to measurement error, biological variation, etc. Hence, an observed Y_i is related to its corresponding X_i by Eq. 3,

$$Y_i = \alpha + \beta X_i + \epsilon_i \qquad \text{(Eq. 3)}$$

where $\alpha + \beta X_i$ is the underlying relationship and ϵ_i is the error term. This situation is illustrated in Fig. 1 in which the dotted line represents the true but unknown relationship and the error is the vertical distance from the point to the dotted line. The statistician's problem is to estimate α and β. A number of methods are available such as: arbitrarily saying $\alpha = 47$ and $\beta = -10$, fitting the data by eye, and least-squares analysis. The first method is obviously of no value, the second might prove useful if rough guesses are desired; but if a thorough analysis including extrapolation, confidence statements, or tests of hypotheses is desired, a proper least-squares analysis must be performed to make better use of the data.

The conditions of a simple least-squares analysis are as follows: assume a sequence of n observation pairs $(Y_1, X_1), (Y_2, X_2), \ldots (Y_n, X_n)$ represented by the points in Fig. 1. If Eq. 3 holds for all $i = 1, \ldots, n$, and if further (*a*) the additive errors ϵ are independent of one another; (*b*) the distribution of the error term has mean zero; and (*c*) the variance of each ϵ_i is σ^2, a constant not depending upon i or X_i; then the "best" estimates (5) for α and β are those which will minimize the right-hand side of Eq. 4.

$$\sum_{i=1}^{n} \epsilon_i^2 = \sum_{i=1}^{n} (Y_i - \alpha - \beta X_i)^2 \qquad \text{(Eq. 4)}$$

In Fig. 1 the least-squares line is represented by the solid line and is the one which minimizes the sum of squared distances in the vertical direction from sample points to fitted line.

Note again assumptions (*a*) that the error ϵ_i is additive, and (*c*) that the ϵ_i's have a common variance; that is, dispersion of error is not proportional to X and hence to the expected value of Y.

The results of a least-squares analysis are not restricted to α and $\hat{\beta}$, the estimates of α and β, respectively. Equation 5 gives the variance

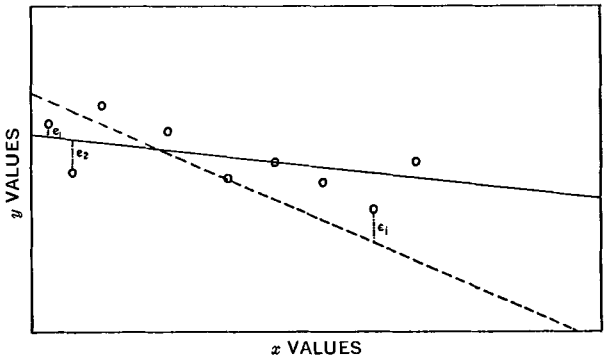


Figure 1—*Example of regression situation. Broken line represents true unknown relationship; solid line represents least-squares fit to data.*

of the estimate of β in which σ^2 is the variance of the ϵ_i's.

$$\sigma_{\hat{\beta}}^2 = \sigma^2 / \sum_{i=1}^{n} (x_i - \sum_{i=1}^{n} x_i/n)^2 \qquad \text{(Eq. 5)}$$

The smaller the variance of the estimate the greater is the confidence which can be placed in it being close to the true unknown parameter.

One further estimate which can be obtained is given by Eq. 6.

$$S^2 = \sum_{i=1}^{n} (Y_i - \hat{\alpha} - \hat{\beta} X_i)^2/(n - 2) \qquad \text{(Eq. 6)}$$

This is an estimate of σ^2, the variance of the error term. This estimate is just an average of the squared deviations of the sample points from the fitted line.

Application of Weighted Least-Squares to Stability Studies—Assume an experiment in which a drug has been stored at various temperatures. Assays are made throughout the period of the experiment to estimate concentration as a function of time. According to the Arrhenius relation a faster degradation is expected to occur at higher temperatures; hence, assays for the higher temperature data might be made more frequently but for a shorter period of time.

A simple least-squares analysis is made by fitting Eq. 1 to the data collected at each temperature τ to determine $\hat{k}_\tau$, the estimate of degradation rate for that temperature. The assumptions mentioned previously as required for a valid least-squares analysis will be sufficiently satisfied except for those nonzero-order reactions in which the range over which concentration varies is extremely large.

Along with each estimate of a k_τ is an estimate of the experimental error variance σ^2 as given by Eq. 6 and the coefficient by which σ^2 must be multiplied in order to obtain the variance of $\hat{k}_\tau$, the estimate of k_τ. This coefficient is given by

$$1/\sum_{i=1}^{n_\tau} (t_i - \bar{t})^2 \qquad \text{(Eq. 7)}$$

where t_i represents the times at which the assays are made, n_τ represents the number of assays made at temperature τ, and $\bar{t} = \sum_{i=1}^{n\tau} (t_i/n_\tau)$.

Figure 2 shows the least-square curves for data collected on chloramphenicol at temperatures 32, 34, 42, 58, and 71°. As each analysis yields an estimate of the same experimental error variance σ^2, these estimates can be combined to form one single estimate of error variance. This estimate will be used later in the analysis and will be referred to as the combined variance estimate denoted S_c^2.

Figures 3 and 4 illustrate the reason for using weighted least squares for fitting the Arrhenius relationship. Figure 3 is a plot of the various k_τ estimates against reciprocal absolute temperature using a linear scale on both axes. Each vertical line represents a 95% confidence interval for the corresponding k_τ. The horizontal mark indicates the point estimate. The width of each confidence interval depends upon the coefficient given by Eq. 7, that is the times and number of observations taken at each temperature.

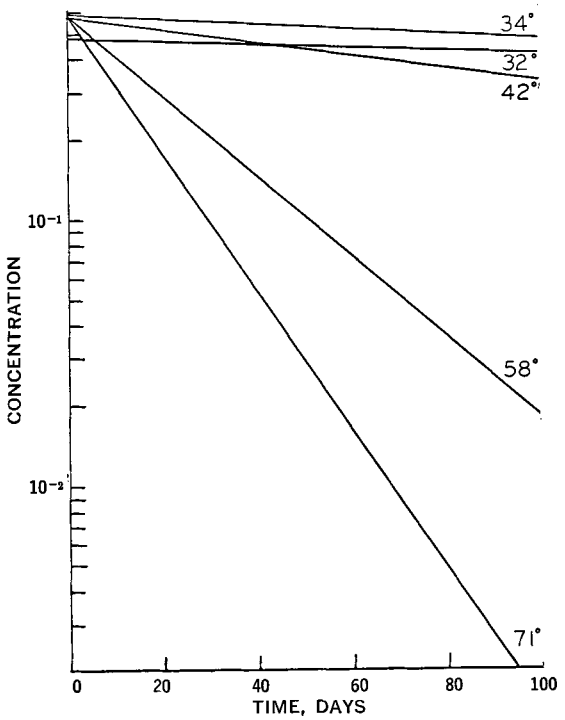


Figure 2—*Least-squares degradation of chloramphenicol data.*

Figure 4 is a plot of the same estimates and same confidence intervals, but drawn on semilogarithm paper. Hence the scale on the vertical axis is $\log_{10} k_\tau$. Note that the length of the confidence intervals for the k_τ at lower temperatures have been lengthened relative to the higher temperatures.

A simple least-squares fit, represented by the broken lines in Fig. 4, was applied to the model:

$$\hat{k}_\tau = e^{\gamma + \delta/\tau + \epsilon} \qquad \text{(Eq. 8)}$$

that is, $\ln \hat{k}_\tau = \gamma + \delta/\tau + \epsilon$ where $\hat{k}_\tau$ is the estimate of k_τ obtained in the previous analyses, and ϵ is an additive error to $\ln k_\tau$ with com-

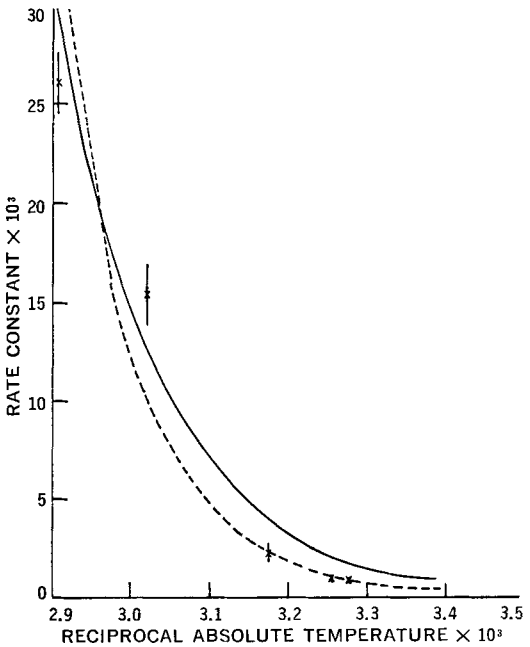


Figure 3—*Arrhenius fit to degradation constants with 71° data included. Vertical lines represent 95% confidence intervals. Solid line is the weighted least-squares line; broken line is the unweighted least-squares line.*

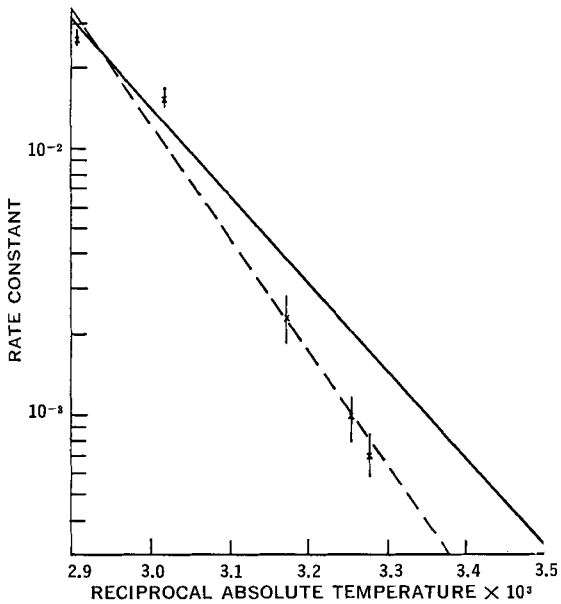


Figure 4—*Arrhenius fit to degradation constants with 71° data included. Vertical lines represent 95% confidence intervals. Solid line is the weighted least-squares line; broken line is the unweighted least-squares line.*

mon variance for all τ. But the true error in $\hat{k}_\tau$ is additive to k_τ, not ln k_τ, because of the method of derivation of $\hat{k}_\tau$; further, the variances of the errors in the $\hat{k}_\tau$ differ according to the coefficient given in Eq. 7. This is illustrated by the extreme difference in the lengths of the lines representing the confidence intervals in Fig. 4. The simple least-squares analysis assumes these lengths to be the same. The effect of the simple least-squares analysis is to force the Arrhenius equation through the low temperature data and essentially ignore the high temperature data. Hence, much more faith is placed in the point estimates of the low temperature $\hat{k}_\tau$ than is warranted. Finally, the usual confidence statements on extrapolated

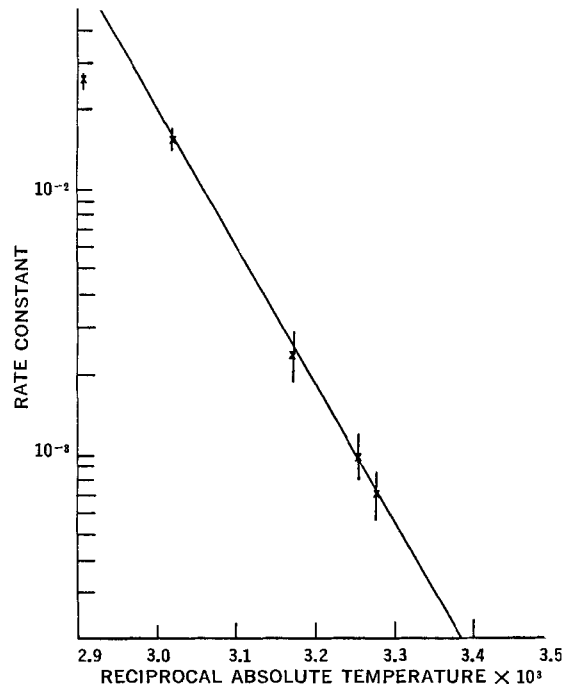


Figure 5—*Arrhenius fit to degradation constants with 71° data excluded. Vertical lines represent 95% confidence intervals; solid line is the weighted least-squares line.*

degradation rates (such as at room temperature) cannot validly be made.

Appendix 2 outlines justification for applying the method of weighted least squares to the model:

$$\hat{k}_\tau = e^{\gamma + \delta/\tau} + \epsilon_\tau \qquad \text{(Eq. 9)}$$

where ϵ_τ is now an additive error to k_τ in the estimate $\hat{k}_\tau$ and has variance σ_τ^2. The method of weighted least squares weights each ln $\hat{k}_\tau$ in inverse proportion to the square of the width of its confidence interval. The weighted least squares fit is represented by the solid lines in Figs. 3 and 4.

Statistical Test of Arrhenius Assumption—The benefits of the weighted least-squares analysis are many. First, the estimates of the parameters of the Arrhenius equation meet the statistician's requirements as the "best" which can be obtained. Second, a confidence interval can easily be constructed around the rate constant for any desired temperature, such as room temperature. Finally, a second estimate of σ^2, the variance of the original experimental error, can be obtained from the fit of the $\hat{k}_\tau$ to the Arrhenius equation. This estimate of variance will be independent of the combined variance estimate S_c^2 based on the original individual temperature analyses, and it will be referred to as the Arrhenius variance estimate and will be denoted S_a^2. Its formula is included in Appendix 1.

The advantage of the two estimates of error variance is as follows. S_a^2 is dependent upon the validity of the Arrhenius assumption. If the Arrhenius relationship does not hold, S_a^2 will tend to be large relative to the true experimental error variance, and therefore large relative to S_c^2 which does not depend upon the Arrhenius assumption. By dividing the Arrhenius variance estimate S_a^2 by the combined variance estimate S_c^2, one obtains an F statistic. By comparing the computed F with a tabled F, which can be found in any elementary statistics book (6), the experimenter can determine the validity of the Arrhenius assumption.

A significantly large F ratio would indicate the Arrhenius relationship does not hold. If the Arrhenius assumption is shown to be significantly invalid (that is the dispersion around the Arrhenius line is greater than could be attributed to chance variation in the $\hat{k}_\tau$), then the least-squares line is not valid and should not be used for predictive purposes. Further, no method of fitting a straight line would be valid for predictive purposes.

On the other hand, if the Arrhenius fit does not yield a significant F value, the high as well as low temperature rate constants fit the Arrhenius line within the measurement error involved in the $\hat{k}_\tau$, and hence all rate constants should be weighted according to their error variances.

Example—The above analysis was performed on a chloramphenicol solution. The results of the analysis are given in Table I. The initial analysis was performed at five temperatures: 32, 34, 42, 58, and 71°. Both the weighted and unweighted least-squares analyses were performed in order to make a comparison between the two methods. The two Arrhenius curves are presented in Figs. 3 and 4. Note that the estimate of degradation from the unweighted method for 23° would have been 2.91×10^{-4} and the analysis terminated at this point. This compares with an estimate of 8.01×10^{-4} computed by the weighted method on the same data.

At this point in the weighted analysis the F test was applied. The computed value was 36.95 with 3 degrees of freedom in the numerator and 167 in the denominator. This value is extremely significant ($F = 5.70$ is significant for $\alpha = 0.001$), indicating the Arrhenius assumption invalid. Upon investigation a precipitate was found to have formed in the 71° data soon after termination of collecting data at that temperature. Hence, these data were excluded from the analysis and another weighted least-squares analysis was performed. The F ratio testing the fit of the four remaining temperatures to the Arrhenius relationship yielded a value $F = 0.1110$ with 2 degrees of freedom for the numerator and 136 for the denominator. Such a small value indicates an extremely good fit to the Arrhenius relationship. The weighted least-squares line is plotted in Fig. 5. No analysis was performed by the unweighted method omitting the 71° temperature to emphasize the fact that the unweighted method would not have led to the detection of lack of fit to the Arrhenius relationship.

The new estimate of degradation rate at 23° was 2.12×10^{-4}. Of primary concern is how well this estimate, based on accelerated data collected over an 89-day period, compares with the value observed on production batches. The actual values observed on two separate

Table I—Results of Analysis on Chloramphenicol Study

Temperature, °C	Reciprocal Absolute Temperature	Computed from Raw Data: $\hat{\alpha}$	Computed from Raw Data: $\hat{K}_\tau \pm$ 95% Confidence Limits	K_τ Predicted from Arrhenius Fit: Unweighted with 71° Data	K_τ Predicted from Arrhenius Fit: Weighted with 71° Data	K_τ Predicted from Arrhenius Fit: Weighted without 71° Data
32	3.28×10^{-3}	−0.313	$7.03 \times 10^{-4} \pm 1.32 \times 10^{-4}$	7.96×10^{-4}	1.72×10^{-3}	7.02×10^{-4}
34	3.26×10^{-3}	−0.227	$9.72 \times 10^{-4} \pm 1.94 \times 10^{-4}$	9.87×10^{-4}	2.03×10^{-3}	9.07×10^{-4}
42	3.18×10^{-3}	−0.232	$2.36 \times 10^{-3} \pm 4.80 \times 10^{-4}$	2.28×10^{-3}	3.82×10^{-3}	2.45×10^{-3}
58	3.02×10^{-3}	−0.215	$1.55 \times 10^{-2} \pm 1.49 \times 10^{-3}$	1.07×10^{-2}	1.24×10^{-2}	1.54×10^{-2}
71	2.91×10^{-3}	−0.234	$2.61 \times 10^{-2} \pm 1.67 \times 10^{-3}$	3.39×10^{-2}	2.98×10^{-2}	6.12×10^{-2}
23	3.38×10^{-3}			2.91×10^{-4}	8.01×10^{-4}	2.12×10^{-4}

production batches, with more than 2 years of data on each, are 2.07×10^{-4} and 2.10×10^{-4}.

APPENDIX 1

Suppose $Y_i = X_{i1}\beta_1 + X_{i2}\beta_2 + \cdots + X_{im}\beta_m + \epsilon_i$ for $i = 1, 2, \ldots n$. Define Y to be the $n \times 1$ matrix $[Y_i]$, X the $n \times m$ matrix $[X_{ij}]$, β the $m \times 1$ matrix $[\beta_i]$, and ϵ the $n \times 1$ matrix $[\epsilon_i]$. Then the n observations satisfy $Y = X\beta + \epsilon$. Further, suppose the covariance matrix for ϵ is $\sigma^2 V$ for a known $n \times n$ matrix V and unknown scalar σ^2. That is the expectation of $[\epsilon\epsilon'] = \sigma^2 V$ where ϵ' is the transpose of ϵ.

The estimate of β is

$$\hat{\beta} = (X'V^{-1}X)^{-1}X'V^{-1}Y \qquad \text{(Eq. 10)}$$

The estimate of σ^2 is

$$S^2 = (Y'V^{-1}Y - Y'V^{-1}X\hat{\beta})/(n - m) \qquad \text{(Eq. 11)}$$

The covariance matrix for the $\hat{\beta}$ vector is

$$(X'V^{-1}X)^{-1}\sigma^2 \qquad \text{(Eq. 12)}$$

which can be estimated by replacing σ^2 by S^2. To estimate a value on the true line $Y_0 = X_{01}\beta_1 + X_{02}\beta_2 + \cdots + X_{0m}\beta_m = X_0\beta$, use the estimate:

$$\hat{Y}_0 = X_0\hat{\beta} \qquad \text{(Eq. 13)}$$

The variance of the estimate $X_0\hat{\beta}$ is

$$\sigma_{Y_0}^2 = X_0(X'V^{-1}X)^{-1}X_0'\sigma^2 \qquad \text{(Eq. 14)}$$

which can be estimated by replacing σ^2 by S^2. A $(1 - \alpha)$ 100% confidence interval on Y_0 can be constructed as

$$X_0\hat{\beta} \pm t_{n-m}^{(1-\alpha/2)}[X_0(X'V^{-1}X)^{-1}X_0'S^2]^{1/2} \qquad \text{(Eq. 15)}$$

where $t_{n-m}^{(1-\alpha/2)}$ is the point on Student's t distribution with $n - m$ degrees of freedom which is exceeded with probability $\alpha/2$.

To apply the weighted analysis to the degradation problem, first perform an individual analysis on the data at each temperature τ setting $V = I$, the $n \times n$ identity matrix. The matrix X is $n \times 2$ with each element of the first column one (1) and the second column the respective times t_i at which assays were made. Y is the vector of the appropriate function of concentration (for a first-order reaction, Y is the vector of the logarithms of concentration). The second element of the $\hat{\beta}$ vector in Eq. 10 is the estimate $\hat{k}_\tau$ of the rate constant.

Call ω_τ the second row, second column element of $(X'V^{-1}X)^{-1}$, $v_\tau = (\hat{k}_\tau)^2/\omega_\tau$ and S_τ^2 the computed value for Eq. 11 at temperature τ based on $n = n_\tau$ and $m = 2$.

The combined variance estimate for the weighted least-squares analysis is

$$S_c^2 = \sum_{\text{all } \tau}(n_\tau - 2)S_\tau^2 / \sum_{\text{all } \tau}(n_\tau - 2) \qquad \text{(Eq. 16)}$$

The method of fitting the Arrhenius relationship to the $\hat{k}_\tau$ is as follows. Set the Y vector to be the logarithms of the $\hat{k}_\tau$'s. The X matrix has each element of the first column one (1) and the second column the reciprocal absolute temperatures, *i.e.*, $1/\tau$. The matrix V, which is $n \times n$ where n is now the number of temperatures at which data were collected, has the corresponding v_τ on the main diagonal and zeros everywhere else.

The Arrhenius variance estimate S_a^2 is computed at this point by Eq. 11 where n is the number of temperatures and $m = 2$. The F statistic is

$$F = S_a^2/S_c^2 \qquad \text{(Eq. 17)}$$

having $n - 2$ degrees of freedom in the numerator and $\sum_{\text{all } \tau}(n_\tau - 2)$ degrees of freedom in the denominator. Reject the applicability of the Arrhenius relationship for large F.

Estimates of logarithm of rate constant at absolute temperature τ_0 can be made from the results of the Arrhenius fit by setting $X_0 = (1/\tau_0)$ in Eq. 13. A confidence interval for logarithm rate constant at τ_0 can be established by Eq. 15. Once these have been done the estimate of time for a given drug to degrade from an initial concentration C_0 to a minimal concentration C_m can be estimated and a confidence interval for length of time for such a degradation can be constructed.

APPENDIX 2

Consider a set of n observations $(y_1, x_1), (y_2, x_2), \ldots, (y_R, x_R)$ where x_i and y_i are related by

$$y_i = e^{\alpha+\beta x_i} + \epsilon_i \qquad \text{(Eq. 18)}$$

Denote by $\tilde{y}_i$ the true value of y associated with x_i; that is $\tilde{y}_i = e^{\alpha+\beta x_i}$. Then $\tilde{y}_i = y_i - \epsilon_i$. Expanding $\ln\tilde{y}_i = \ln(y_i - \epsilon_i)$ in a Taylors series about y_i gives

$$\ln\tilde{y}_i = \alpha + \beta x_i = \ln y_i - \epsilon_i\frac{1}{\tilde{y}_i} + \text{remainder} \qquad \text{(Eq. 19)}$$

where the remainder is a term of order $[\epsilon_i/\tilde{y}_i]^2$. Provided $\epsilon_i/\tilde{y}_i$ is much less than 1, the remainder can be ignored. This gives

$$\ln y_i \simeq \alpha + \beta x_i + \epsilon_i/\tilde{y}_i \qquad \text{(Eq. 20)}$$

where the new error term, call it ϵ_i^*, is now $\epsilon_i/\tilde{y}_i$. It follows immediately that the mean of ϵ_i^* is zero and the variance of ϵ_i^* is $\sigma_{\epsilon_i}^2/\tilde{y}_i^2$. Approximating $\tilde{y}_i$ by y_i gives the weighting used in the Arrhenius analysis described in Appendix 1.

REFERENCES

(1) E. R. Garrett, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 171(1956).

(2) W. R. McBride and D. S. Villars, *Anal. Chem.*, **26**, 901(1954).

(3) J. E. Tingstad, L. H. Mac Donald, and P. D. Meister, *J. Pharm. Sci.*, **52**, 343(1963).

(4) E. L. Parrott, *J. Amer. Pharm. Ass.*, **NS 6**, 73(1966).

(5) F. A. Graybill, "An Introduction to Linear Statistical Models," vol. I, McGraw-Hill, New York, N. Y., 1961, p. 115.

(6) G. W. Snedecor, "Statistical Methods," The Iowa State University Press, Ames, Iowa, 1961.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 28, 1968, from the *Department of Mathematics, Pomona College, Claremont, CA 91711*

Accepted for publication October 31, 1969.

This investigation was supported by Public Health Service Research Grant No. GM16010 01 from the National Institutes of Health.

The author is indebted to Malcolm P. Boghosian of Allergan Pharmaceuticals for suggesting this work and for furnishing the chloramphenicol data.

Whole Body Measurements of ^{131}I-Tetracycline as an Index of Skeletal Growth

R. G. WOLFANGEL*, S. M. SHAW, and J. E. CHRISTIAN

Abstract □ A derivative of tetracycline was tagged with ^{131}I and administered to rats. Whole body retention of the tetracycline was determined by sequential measurements of whole body radioactivity. Statistically significant differences of whole body burdens were found for two age groups of rats (100 g. *versus* 200 g.); the younger animals retaining a greater portion of the administered tetracycline. Subsequent distribution analysis indicated that whole body radioactivity measurements did not precisely assess skeletal burdens of ^{131}I-labeled tetracycline because variable amounts of tetracycline persisted in soft tissue for prolonged intervals after injection, but did provide estimates of skeletal burdens which could be used to recognize differences in skeletal growth rate between groups of young and mature rats. The whole body counting technique may be applicable for the study of metabolic skeletal disorders.

Keyphrases □ ^{131}I-Tetracycline—skeletal growth index, whole body measurements □ Growth, skeletal—index, radioiodinated tetracycline □ Paper chromatography—analysis, identity □ UV spectrophotometry—analysis, identity □ Scintillation counting, whole body—analysis

Since the initial observations of tetracycline-induced fluorescence of bones by Rall *et al.*, investigators have been examining tetracycline fixation in mineralized tissue (1). The following has been reported concerning the deposition of tetracycline in bones and teeth; deposition occurs after introduction by any route, but is greatest following parenteral administration (2); tetracyclines are actively deposited at all sites of newly mineralizing bone and are relatively permanently fixed in the bone until resorption occurs (3, 4). The quantity of tetracycline deposited in bone is proportional to animal age and the dose administered (5, 6), and the presence of tetracycline in bone or teeth can be readily detected by the appearance of a bright yellow fluorescence under UV irradiation (7, 8).

Tetracycline bone labeling, followed by microscopic measurements of the width, area, or volume of yellow fluorescent zones found in bone sections, is used as an index of skeletal metabolic activity (9–11) such as appositional growth rate, radial rate of osteon closure, and osteon maturation rate. Direct determinations of the total quantity of tetracycline bound to the skeleton might also provide an index of skeletal metabolic activity; if so, the necessity of skeletal biopsy, sectioning, and tedious fluorescence microscopy currently employed for tetracycline skeletal observations would be alleviated. Thus, ^{131}I-labeled tetracycline was prepared and used to conduct animal studies. Whole body measurements of tetracycline retention, following administration of labeled tetracycline, were investigated for possible value to assess skeletal metabolic activity in young growing rats as compared to older mature rats. The accuracy of whole body counting for the determination of the total quantity of tetracycline bound to the skeleton was established by the direct measurement of labeled tetracycline bound to the entire skeleton of the two age groups of rats, as well as the residual amount of tetracycline remaining in the soft tissue of the animals.

METHODS

Synthesis and Purity—Hlavka *et al.* (12) reported the preparation of 7-iodo-6-demethyl-6-deoxytetracycline (766 tet) by dissolving 6-demethyl-6-deoxytetracycline (66 tet) and *N*-iodosuccinimide (NIS) in concentrated sulfuric acid at 0°. By substitution of ^{131}I for stable iodine, 7-radioiodo-6-demethyl-6-deoxytetracycline (*766 tet) was prepared in this laboratory according to Hlavka's directions. The ^{131}I-label was introduced by the preparation of *N*-^{131}iodosuccinimide (N*IS) by modification of the method of Benson *et al.* (13). Aqueous solutions of Na^{131}I[1] (25–75 mc.) were transferred to a test tube containing 2 ml. of carbon tetrachloride, and 1 ml. of NaI carrier (5 mg./ml.) was added. The test tube was fitted with a rubber stopper through which a dropping pipet, filled with concentrated nitric acid, had been inserted. Nitric acid was then added to the water–carbon tetrachloride mixture. The closed tube was left for 18–24 hr., during which time free iodine was formed and dissolved in the organic liquid. The aqueous overlayer was removed with a micropipet allowing the $^{131}I_2$ in the carbon tetrachloride to remain in the test tube. Stable elemental iodine (1 g.) was placed in an amber 5-dr. vial, the cap lined with Teflon, and 5 ml. of sodium-dried, distilled dioxane added. The solution of carbon tetrachloride, containing $^{131}I_2$, was transferred to the vial. The test tube was rinsed with 1 ml. additional carbon tetrachloride, and the 1 ml.

[1] Purchased from Nuclear Science and Engineering Corp., Pittsburgh, Pa.

(6) G. W. Snedecor, "Statistical Methods," The Iowa State University Press, Ames, Iowa, 1961.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 28, 1968, from the *Department of Mathematics, Pomona College, Claremont, CA 91711*

Accepted for publication October 31, 1969.

This investigation was supported by Public Health Service Research Grant No. GM16010 01 from the National Institutes of Health.

The author is indebted to Malcolm P. Boghosian of Allergan Pharmaceuticals for suggesting this work and for furnishing the chloramphenicol data.

Whole Body Measurements of ^{131}I-Tetracycline as an Index of Skeletal Growth

R. G. WOLFANGEL*, S. M. SHAW, and J. E. CHRISTIAN

Abstract □ A derivative of tetracycline was tagged with ^{131}I and administered to rats. Whole body retention of the tetracycline was determined by sequential measurements of whole body radioactivity. Statistically significant differences of whole body burdens were found for two age groups of rats (100 g. *versus* 200 g.); the younger animals retaining a greater portion of the administered tetracycline. Subsequent distribution analysis indicated that whole body radioactivity measurements did not precisely assess skeletal burdens of ^{131}I-labeled tetracycline because variable amounts of tetracycline persisted in soft tissue for prolonged intervals after injection, but did provide estimates of skeletal burdens which could be used to recognize differences in skeletal growth rate between groups of young and mature rats. The whole body counting technique may be applicable for the study of metabolic skeletal disorders.

Keyphrases □ ^{131}I-Tetracycline—skeletal growth index, whole body measurements □ Growth, skeletal—index, radioiodinated tetracycline □ Paper chromatography—analysis, identity □ UV spectrophotometry—analysis, identity □ Scintillation counting, whole body—analysis

Since the initial observations of tetracycline-induced fluorescence of bones by Rall *et al.*, investigators have been examining tetracycline fixation in mineralized tissue (1). The following has been reported concerning the deposition of tetracycline in bones and teeth; deposition occurs after introduction by any route, but is greatest following parenteral administration (2); tetracyclines are actively deposited at all sites of newly mineralizing bone and are relatively permanently fixed in the bone until resorption occurs (3, 4). The quantity of tetracycline deposited in bone is proportional to animal age and the dose administered (5, 6), and the presence of tetracycline in bone or teeth can be readily detected by the appearance of a bright yellow fluorescence under UV irradiation (7, 8).

Tetracycline bone labeling, followed by microscopic measurements of the width, area, or volume of yellow fluorescent zones found in bone sections, is used as an index of skeletal metabolic activity (9–11) such as appositional growth rate, radial rate of osteon closure, and osteon maturation rate. Direct determinations of the total quantity of tetracycline bound to the skeleton might also provide an index of skeletal metabolic activity; if so, the necessity of skeletal biopsy, sectioning, and tedious fluorescence microscopy currently employed for tetracycline skeletal observations would be alleviated. Thus, ^{131}I-labeled tetracycline was prepared and used to conduct animal studies. Whole body measurements of tetracycline retention, following administration of labeled tetracycline, were investigated for possible value to assess skeletal metabolic activity in young growing rats as compared to older mature rats. The accuracy of whole body counting for the determination of the total quantity of tetracycline bound to the skeleton was established by the direct measurement of labeled tetracycline bound to the entire skeleton of the two age groups of rats, as well as the residual amount of tetracycline remaining in the soft tissue of the animals.

METHODS

Synthesis and Purity—Hlavka *et al.* (12) reported the preparation of 7-iodo-6-demethyl-6-deoxytetracycline (766 tet) by dissolving 6-demethyl-6-deoxytetracycline (66 tet) and *N*-iodosuccinimide (NIS) in concentrated sulfuric acid at 0°. By substitution of ^{131}I for stable iodine, 7-radioiodo-6-demethyl-6-deoxytetracycline (*766 tet) was prepared in this laboratory according to Hlavka's directions. The ^{131}I-label was introduced by the preparation of *N*-^{131}iodosuccinimide (N*IS) by modification of the method of Benson *et al.* (13). Aqueous solutions of Na^{131}I[1] (25–75 mc.) were transferred to a test tube containing 2 ml. of carbon tetrachloride, and 1 ml. of NaI carrier (5 mg./ml.) was added. The test tube was fitted with a rubber stopper through which a dropping pipet, filled with concentrated nitric acid, had been inserted. Nitric acid was then added to the water–carbon tetrachloride mixture. The closed tube was left for 18–24 hr., during which time free iodine was formed and dissolved in the organic liquid. The aqueous overlayer was removed with a micropipet allowing the $^{131}I_2$ in the carbon tetrachloride to remain in the test tube. Stable elemental iodine (1 g.) was placed in an amber 5-dr. vial, the cap lined with Teflon, and 5 ml. of sodium-dried, distilled dioxane added. The solution of carbon tetrachloride, containing $^{131}I_2$, was transferred to the vial. The test tube was rinsed with 1 ml. additional carbon tetrachloride, and the 1 ml.

[1] Purchased from Nuclear Science and Engineering Corp., Pittsburgh, Pa.

rinse added to the reaction vial. Then 1.2 g. of *N*-silver succinimide was added in one portion, the lined cap replaced, and the closed vial agitated continuously for 90 min. The closed vial was immersed in a water bath (50°) for 5 min., removed, and the contents filtered through a sintered-glass filter. The reaction vial and silver iodide precipitate were washed with 2-, 2-, and 1-ml. portions of hot dioxane. The dioxane wash was filtered and added to the filtrate. Carbon tetrachloride (25 ml.) was added to the filtrate, the flask stoppered, covered with aluminum foil, and placed in a freezer for 12–18 hr. The contents of the flask were filtered to obtain $N^{131}IS$ crystals which were washed with 30-, 20-, and 10-ml. portions of carbon tetrachloride. The $N^{131}IS$ crystals were stored in a vacuum desiccator, in the dark, for at least 24 hr. prior to utilization for the preparation of *766. The *766 was prepared with the 66 tet and the $N^{131}IS$ according to the procedures of Hlavka (12).

Analyses of the *766 tet for chemical purity were performed as described by Hlavka *et al.* (12). These analyses included the preparation of spectral absorbance curves and paper chromatographic procedures. Solutions of known concentration (2–15 mcg./ml.) were prepared by dissolving the labeled tetracycline in 0.1 *N* HCl. A Bausch and Lomb Spectronic 505 was used to record a continuous spectral absorbance curve, between the wavelengths of 220 and 370 mμ, for each solution of *766 tet. The absorbance peaks were located and log epsilon values calculated. For the chromatographic procedures, Whatman No. 1 chromatography paper was immersed in a 0.2 *M* phosphate buffer (pH 2) solution and allowed to dry. Labeled tetracycline samples were applied to the buffer-treated chromatography paper as 1–2% solutions (aqueous and/or methanolic). Volumes of 2–25 μl. of the tetracycline solutions were applied to the paper. The tetracycline-spotted chromatography paper was placed in a standard chromatography jar and developed by the descending movement of *n*-butanol saturated with 0.2 *M* phosphate buffer (pH 2). Developing the paper for 19–20 hr. afforded a solvent movement of approximately 45 cm. The atmosphere of the chromatography jar was saturated with *n*-butanol and water vapor at least 24 hr. before using the jar to develop a chromatogram. Location of the tetracycline on the developed chromatograms was performed by UV light irradiation.

Hlavka *et al.* (12) reported maximum absorbance at 230 and 345 mμ with log epsilon values of 4.48 and 4.12, respectively, for 766 tet dissolved in 0.1 *N* HCl. UV spectral absorbance curves of the *766 tet prepared in this laboratory showed peaks at 239 and 345 mμ. The log epsilon values calculated for the absorbance peaks of *766 tet were 4.35 and 4.14, respectively. The UV absorbance and log epsilon values were not in exact agreement with the literature. This disagreement is attributed to chemical contamination of the *766 tet with minute quantities of 66 tet. The small amount of chemical contaminant appeared on the developed chromatograms and was identified as 66 tet using R_f values. The degree of chemical contamination was considered insignificant.

To determine the radiochemical purity of the *766, paper chromatographic procedures utilized for the determination of chemical purity were employed. Autoradiography was used to detect the labeled tetracycline. The developed chromatograms were overlayed with No Screen Kodak Medical X-Ray film.[2] After sufficient exposure time (10^8 d./cm.^{2}), the film was developed in accordance with the recommendations supplied by the Kodak X-ray developer.[2] The area of the chromatogram bearing the *766 tet (as observed from the autoradiogram) was sectioned. The remaining portion of the chromatogram was considered to contain radiochemical impurities. The sections were counted individually in a 7.6-cm. (3-in.), sodium iodide, thallium-activated well crystal scintillation counting system. Ninety-nine percent of the radioactivity appeared at the R_f of 766 tet.

Whole Body Retention—Two groups of female Sprague-Dawley[3] strain albino rats were utilized in this phase of the investigation. The first group of six animals was comprised of young, rapidly growing animals (100 ± 5 g.) and the second group of six rats contained adult rats (200 ± 10 g.). Animals were housed in individual metabolism cages for 24 hr. prior to *766 tet administration and during the remainder of the study. Food and water were allowed *ad libitum*. A dose of 20 mg. of *766 tet (0.25 μc./mg.) per kilogram of body weight was given to each rat intraperitoneally. Distilled water served as the solvent for the labeled tetracycline.

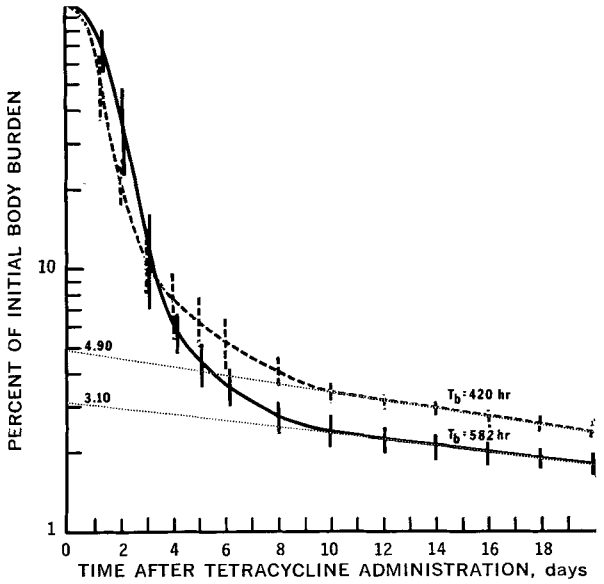


Figure 1—*Whole body retention of labeled tetracycline in rats. Key: - - -, 100 g.; and ——, 200 g.*

Whole body radioactivity counts of each rat were taken using an Armac liquid scintillation counting system.[4] Just prior to placing the rats in the counter, they were imprisoned in a cylindrical cage which inhibited virtually all motion and allowed for reproducible positioning of the rats within the detector. The initial measurement of whole body radioactivity was made 45–60 min. after the dose of radioiodinated tetracycline was given and was considered as the initial or 100% body burden. Whole body counts were repeated at various time intervals for 480 hr. after drug administration. Subsequent counts were corrected for variation in counter efficiency, background, and radioactive decay to the time of the initial determination. Corrected counts were expressed as a percent of the initial body burden.

During the course of the whole body counting study, urine and feces were collected separately from each rat at 24-hr. intervals. Each intact 24-hr. specimen was counted in the Armac liquid scintillation counting system. The fraction of the administered *766 tet excreted per time interval was calculated in a manner similar to the whole body counts and the data expressed as a percent of the initial body burden.

Distribution—The distribution and retention of *766 tet were determined in young and adult rats at various intervals after intraperitoneal administration of the labeled tetracycline. Sixteen 40-day-old female Sprague-Dawley rats (100 ± 10 g.) were administered a dose of 20 mg. of *766 tet (0.40 μc./mg.) per kilogram of body weight. Similarly, eighteen 90-day-old female rats (205 ± 10 g.) were each injected with the labeled tetracycline solution. The rats were housed, watered, and fed as in the previous study. Three[5] animals from each age group were sacrificed 24 hr. after tetracycline injection, and at 72-hr. intervals thereafter, with the final group sacrificed 384 hr. after tetracycline administration. The rats were ether anesthetized and a 1–3-ml. blood sample withdrawn by intracardiac puncture. The rats were then sacrificed by an overdose of ether. Each rat was weighed to the nearest 0.5 g. The following samples were excised for analysis: heart, liver, lung, kidney, spleen, thyroid, humerus, femur, and a 1–3-g. sample of muscle. Each sample, except blood, was rinsed in distilled water and weighed. In most cases, the entire organ was removed and weighed. Each tissue sample was analyzed for tetracycline content by counting in the 7.6-cm. (3-in.), well crystal scintillation counting system. The counting data were expressed as the micrograms of tetracycline in the tissue specimen. Subsequent calculations, using the weight of the tissue specimen and the milligrams of tetracycline initially administered to each rat, were performed to express the data as the percent of

[2] Eastman Kodak Co., Rochester, N. Y.
[3] Sprague-Dawley, Inc., Madison, Wis.
[4] Packard Model 440 Armac Scintillation detector in conjunction with a Packard model 410A Auto-Gamma spectrometer, Packard Instrument Co., La Grange, Ill.
[5] Only two rats were sacrificed at 24 and 96 hr. for the younger group.

Table I—Percent of the Dose of Iodinated Tetracycline (20 mg./kg.) Retained in Various Tissues[a] of Young Female Rats (100 g.) following Drug Administration

Organ	Time after Administration, hr.[b]					
	24	96	168	240	312	384
Heart	0.173 ± 0.010	0.007 ± 0.003	—	—	—	—
Liver	3.223 ± 0.616	1.556 ± 1.052	0.304 ± 0.404	0.437 ± 0.406	0.218 ± 0.170	0.129 ± 0.183
Lung	0.207 ± 0.010	0.012 ± 0.003	0.006 ± 0.002	—	—	—
Kidney	1.593 ± 0.172	0.036 ± 0.003	0.019 ± 0.006	0.009 ± 0.002	0.007 ± 0.002	0.006 ± 0.002
Spleen	0.133 ± 0.000	0.170 ± 0.052	0.038 ± 0.019	—	—	—
Thyroid	0.128 ± 0.054	0.204 ± 0.018	0.126 ± 0.040	0.088 ± 0.016	0.055 ± 0.017	0.048 ± 0.026
Skeletal[c]	3.748 ± 0.145	3.030 ± 0.266	2.794 ± 0.228	2.759 ± 0.601	2.333 ± 0.130	1.856 ± 0.032

[a] Data expressed as the percent of dose per entire organ or tissue. [b] Intervals of 24 and 96 hr. represent the means of two rats; all others are the means of three rats. [c] Skeletal is average percent per gram of right humerus and femur multiplied by the number of grams of skeletal tissue estimated from body weight.

Table II—Percent of the Dose of Iodinated Tetracycline (20 mg./kg.) Retained in Various Tissues[a] of Mature Female Rats (200 g.) following Drug Administration

Organ	Time after Administration, hr.[b]					
	24	96	168	240	312	384
Heart	0.212 ± 0.019	0.007 ± 0.004	—	—	—	—
Liver	2.974 ± 0.850	1.092 ± 0.692	0.322 ± 0.350	0.235 ± 0.172	0.198 ± 0.155	0.254 ± 0.262
Lung	0.364 ± 0.135	0.013 ± 0.003	0.005 ± 0.112	—	—	—
Kidney	1.504 ± 0.162	0.051 ± 0.019	0.017 ± 0.003	0.010 ± 0.002	0.007 ± 0.000	0.006 ± 0.001
Spleen	0.178 ± 0.089	0.009 ± 0.004	0.004 ± 0.002	—	—	—
Thyroid	0.138 ± 0.052	0.167 ± 0.033	0.115 ± 0.006	0.104 ± 0.019	0.066 ± 0.020	0.084 ± 0.038
Skeletal[c]	1.821 ± 0.205	1.754 ± 0.194	1.614 ± 0.232	1.447 ± 0.198	1.578 ± 0.163	1.216 ± 0.178

[a] Data expressed as the percent of dose per entire organ or tissue. [b] Data represent the means of three rats. [c] Skeletal is average percent per gram of right humerus and femur multiplied by the number of grams of skeletal tissue estimated from body weight.

the administered dose of tetracycline retained per gram of tissue or organ, as well as per the entire tissue or organ.

Skeletal Retention—Following the distribution study, six 40-day-old female Sprague-Dawley rats (100 ± 5 g.) and six 90-day-old rats (200 ± 10 g.) received *766 tet in the same manner as described for the distribution study. Three of the rats from each age group were sacrificed at 312 hr. (13 days) and 384 hr. (16 days) after injection of the labeled tetracycline. The entire skeleton was excised from each rat and the total mass of nonskeletal tissue assembled. The skeleton and composited soft tissue from each rat were separately assayed for tetracycline by counting in the Armac liquid scintillation counting system. The quantity of the administered dose of labeled tetracycline retained by the skeletal mass and by the nonskeletal tissue was expressed as a percent of the initial body burden.

Excretion—Examination of excreta obtained from rats dosed with *766 tet was undertaken in order to determine the metabolic fate of the tetracycline. Three 40-day-old female Sprague-Dawley rats (100 ± 10 g.) were injected intraperitoneally with a dose of 20 mg. of *766 tet (2.4 μc./mg.) per kilogram of body weight. Three 90-day-old female rats (200 ± 10 g.) were also given a dose of 20 mg. of *766 tet (2.4 μc./mg.) per kilogram of body weight. A higher specific activity tetracycline was necessary for this phase of the investigation in order to ensure the detection of metabolic products, if any, in the excreta. Following labeled-tetracycline administration, the rats were housed in individual metabolism cages and the excreta collected 48 hr. after injection of the tetracycline. Radioactive products appearing in the 48 hr. excreta were qualitatively analyzed using chromatographic techniques. Thick-layer chromatograms were prepared with an aqueous slurry of MN-Cellulose powder-300.[6] The cellulose-coated plates were air dried and impregnated with a mixture of 0.2 *M* phosphate buffer (pH 2.0) and glycerol (19:1). The plates were allowed to air dry before spotting. Untreated urine was applied to the thick-layer chromatograms in amounts ranging from 2.5 to 30 μl. per application. Following thorough homogenation of feces, portions of the fecal homogenates were applied to the thick-layer chromatograms. Each plate was also spotted with a sample of the *766 tet used to dose the rats. After solvent development,[7] the chromatograms were autoradiographed for 4 weeks.

RESULTS AND DISCUSSION

Whole body retention of tetracycline for the two different age groups of rats is presented in Fig. 1. Whole body retention of tetracycline was similar during the first 4 days of the experiment, but then the body burden of tetracycline in the younger (100 g.) rats became 1% greater than the mature (200 g.) animals. A Student's *t* difference of means test showed the body burden of tetracycline to be statistically greater ($p = 0.005$) in the younger rats from Day 8 until termination of the study at Day 20. The curves shown in Fig. 1 were fitted by a standard exponential analysis in the sense of least squares. The intercepts and slopes are reported for the long-lived exponentials. The young rats intercept is 1.8% greater and has a half-time 162 hr. faster than the mature rats. The elevated whole body tetracycline retention shown by the young rats was considered to be the result of increased tetracycline fixation to the faster growing skeletal tissue of the younger rats, but this remained to be demonstrated from the distribution and skeletal studies.

The results of the distribution study, expressed in terms of the percent of the administered dose of tetracycline retained by the entire organ or tissue, are presented in Tables I and II and in Fig. 2. In Fig. 2 the curves were fitted by a standard exponential analysis in the sense of least squares. The percent of the administered dose of tetracycline present in the entire skeleton was calculated according to the method of Myers and Jaffe (6). They averaged the micrograms of tetracycline retained per gram of humerus bone and femur bone, then multiplied the value by the total skeletal weight. The values of total skeletal weight were obtained from the body weight of the rats by using data from a quantitative study of skeletal growth in the albino rat performed by Donaldson (14). As may be observed from Tables I and II, the amount of tetracycline decreased to undetectable levels by Day 7 or 10 for the spleen, lung, and heart. The skeleton, liver, thyroid, and kidney persistently contained tetra-

[6] Brinkmann Instruments, Inc., Westbury, N. Y.

[7] The organic phase of an *n*-butanol–0.2 *M* phosphate buffer (pH 2.0) mixture.

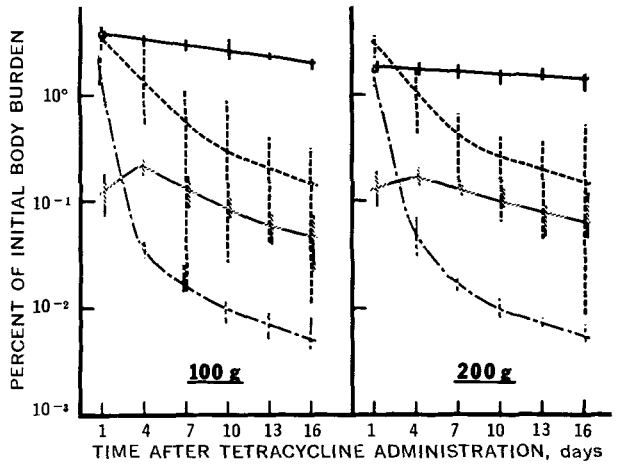


Figure 2—*Retention of labeled tetracycline in rat organs. Key: ——, skeleton; - - -, liver; —|||—, thyroid; and - — - —, kidney.*

cyline until the termination of the study. For both age groups, bone contained greater amounts of the tetracycline than any of the other tissues studied. After Day 10, the quantity of tetracycline in the skeleton of either age group exceeded the level of all other organs by a factor of 10 or greater.

The intercepts and biological half-times of the whole body retention curves (Fig. 1) and organ distribution curves (Fig. 2) have been summarized and compared in Table III. Both the intercepts, as percent of the dose, and half-times, in hours, are listed for the long-lived exponentials. Differences have been computed by subtracting the value of the mature rats from that of the young rats. Whole body retention curves show an intercept difference of 1.80%. Intercepts of the liver, kidney, and thyroid are very similar for young and adult rats; however, the skeleton shows an intercept difference of 1.92% which is very comparable to the intercept difference seen in whole body retention. Examination of the biological half-time data shows that the long-term whole body retention half-time of the younger rats was 162 hr. shorter than that observed for the older animals. Age differences in the biological half-times of the liver and kidney were not great. Thyroids show a difference in biological half-times between the age groups, but the skeleton, by far, displayed the greatest difference (the younger group 319 hr. faster). Although the intercepts and half-times obtained for whole body retention would not seem to be precise measurements of the tetracycline bone compartment, there is a high correlation between whole body retention intercepts and the skeletal intercepts obtained from the distribution study. This correlation becomes less evident when biological half-times are examined in the same manner.

In addition to the distribution analyses previously described, the levels of tetracycline in entire skeletons were investigated in that time domain where the whole body burdens were statistically greater in the young rats as compared to the mature rats. At Day 13 and Day 16 postinjection, complete skeletons of three females per age group were separated from their respective soft tissue. The skeletons and soft tissues were counted separately for tetracycline content. In Table IV, skeletal and soft tissue retention as a

Table III—Summary of Age-Related Differences of Long-Term ^{131}I-Tetracycline Retention Intercepts and Half-Times[a]

	Intercepts, % of Initial Body Burden			Biological Half-Times, hr.		
	100 g.	200 g.	Δ[b]	100 g.	200 g.	Δ
Whole body retention	4.90	3.10	1.80	420	582	−162
Liver	1.01	0.80	0.21	125	180	− 55
Kidney	0.03	0.03	—	135	150	− 15
Thyroid	0.20	0.16	0.04	180	327	−147
Skeleton	3.82	1.90	1.92	405	724	−319

[a] Obtained from the long-lived exponentials of the whole body retention and organ distribution curves. [b] Value from mature rat substracted from that of the young rat.

Table IV—Percent of the Initial Body Burden of Tetracycline Retained by the Skeleton and Nonskeletal Tissue

Time, hr.	Skeletal Retention,[a] % Young, 100 g.	Mature, 200 g.	$t_{obs.}$[b]
312	1.600 ± 0.021	1.098 ± 0.088	9.61
384	1.520 ± 0.058	0.998 ± 0.171	5.012
	Retention by Nonskeletal Tissue,[a] %		
312	1.780 ± 1.144	1.110 ± 0.626	1.182
384	0.901 ± 0.351	0.634 ± 0.153	1.207

[a] Mean and standard deviation of three rats per age group. [b] Statistic calculated from the experimental data according to a Student's t difference of means test. Any $t_{obs.}$ exceeding 4.60 provides statistical proof (4 degrees of freedom, 99.5 percentile point) of greater retention in the younger animals.

percent of the initial body burden is shown for the young and mature rats both at 312 hr. (Day 13) and 384 hr. (Day 16). The younger rats retained more tetracycline in both hard and soft tissue compartments at each time interval. A difference of means test indicated that the skeleton of young rats contained significantly greater amounts of tetracycline than the skeleton of older rats. Skeletal values were also noted to be highly consistent as evidenced by the low standard deviations. Regarding soft tissue retention of tetracycline, considerable variations were present among the members of each group resulting in standard deviations much larger than those obtained for skeletal retention. A difference of means test did not indicate that the observed differences in soft tissue retention between age groups were statistically significant.

The results of this phase of the study indicate that the statistically greater whole body retention of tetracycline previously shown by the young group of rats resulted from localization of increased amounts of tetracycline in the skeletons of the young. This supports the hypothesis that increased skeletal mineralization in young animals can be determined using whole body measurements of radioiodinated tetracycline, but points out that the validity of the test remains dependent upon obtaining either a complete or uniform clearance of tetracycline from the soft tissue compartment prior to making whole body measurements.

Figure 3 graphically represents the percent of the administered tetracycline accumulated in the urine and the feces of rats which were used in the whole body retention study. No significant difference in excretion of the tetracycline was noted between age groups. The data show clearly the importance of fecal excretion as the route of tetracycline elimination.

As described in the methods section, examination of excreta obtained from both age groups was undertaken in order to deter-

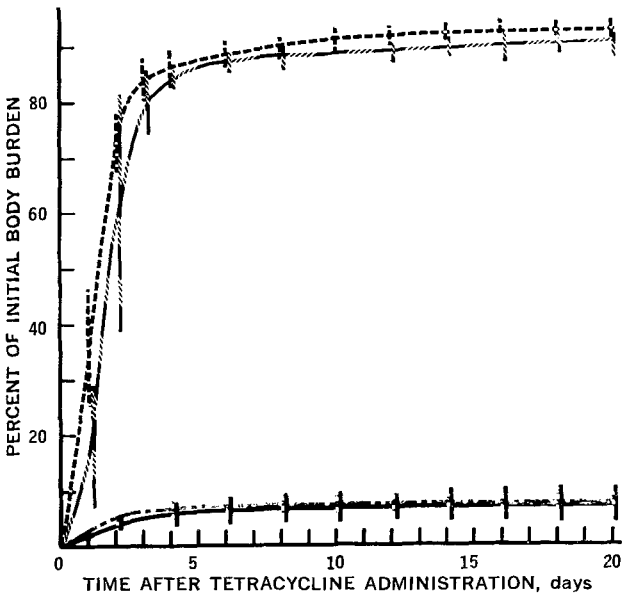


Figure 3—*Accumulated excretion of labeled tetracycline in the rat. Key: - - -, feces, 100 g.; —|||—, feces, 200 g.; - — - —, urine, 100 g.; and ——, urine, 200 g.*

Table V—Comparison of *In Vivo* Properties of ^{131}I-Labeled Tetracycline to Commerical Tetracyclines

Criteria of Comparison, %	Chlortetracycline	Demethyl-chlortetracycline	Tetracycline	^{131}I-Labeled Tetracycline
Whole body retention	3–23% at 72 hr.[a] (18)	10% at 168 hr.[a] (16)	9.5% at 72 hr.[a] 6.7% at 168 hr.[a] (15)	10% at 72 hr. 3–4% at 168 hr.
Dose eliminated in feces	50% at 168 hr. (18)	15% at 168 hr. (16)	23–54% at 168 hr. (15)	90% at 168 hr.
Dose in skeleton	—	—	3–5% in 60-g. male rats at 4 days; 1–2% in 340-g. males (6)	3% in 100-g. female rats at 4 days; 1.8% in 200-g. females
Dose per gram of bone after intraperitoneal injection of 20 mg./kg.	—	—	0.16% per gram of bone at 24 hr. in adult males (19)	0.15% per gram of bone at 24 hr. in adult females
Dose recovered unmetabolized in excreta	Not metabolized to any significant extent (18)	93% (16)	95% (15)	Not metabolized to any significant extent[b]

[a] Values listed are based upon quantitative recovery of excreta products. Numbers in parentheses are references. [b] Analysis performed on excreta collected within 48 hr. after administration of the drug.

mine the metabolic fate of the labeled tetracycline. For both age groups the autoradiograms prepared from chromatograms of urine disclosed the presence of a single labeled compound with an R_f value almost identical with that of the *766 tet utilized in the various studies. In feces, the autoradiograms indicated the presence of minute amounts of ^{131}I-activity other than that identical to the *766 tet standard. Even though the film had been exposed to the chromatogram for 4 weeks the image produced on the film by radioactivity other than that associated with the *766 tet was barely detectable. The *766 tet utilized in these studies was recoverable in the excreta of the animals in essentially unaltered form. This strongly signifies that ^{131}I-activity remaining in the animal tissue is associated with tetracycline, but does not provide unequivocal proof of this assumption. It would appear that the *766 tet utilized in the various studies was essentially unmetabolized.

Aware that iodination of tetracycline might result in a compound that was not biologically representative of the tetracycline family, attention was devoted to assembling *in vivo* parameters of retention, excretion, and distribution of the iodinated tetracycline. In Table V, biological observations for the iodinated tetracycline are compared to results obtained by other investigators using several commercially available tetracycline antibiotics. There is good correlation between the radioiodinated tetracycline prepared in this laboratory and the other tetracyclines for whole body retention, skeleton and bone uptake, and metabolism. Examination of the percent of the dose eliminated in the feces shows that the radioiodinated tetracycline is excreted in the feces in far greater quantities than the other tetracyclines. Since in most cases the radioiodinated tetracycline compared favorably to the other tetracyclines, the radioiodinated tetracycline is considered to be representative of the tetracycline family of compounds.

SUMMARY AND CONCLUSIONS

Investigators have reported that tetracyclines are rapidly excreted within 7 days of administration and that the majority of tetracycline retained after 7 days remains bound to the skeleton (15, 16). Also, the quantity of tetracycline fixed and retained by the skeleton of animals is proportional to the age of the animal and/or skeletal turnover rate (2, 6, 17). Based upon these reports, an attempt was made to assess skeletal burdens of tetracycline in rats by measurements of whole body radioactivity following administration of radioiodinated tetracycline.

When the whole body retention of labeled tetracycline was studied in young and mature rats, statistically greater body burdens were observed in the younger group. Distribution experiments showed that the higher body burdens of the younger animals were related to their increased skeletal uptake of tetracycline. In subsequent experiments, skeletons of several test rats from each age group were surgically cleansed of soft tissue. Tetracycline skeletal burdens were determined and found to be very uniform within each group, and statistically higher levels of skeletal tetracycline were found in the younger animals when compared to the older. Tetracycline levels within soft tissue were obtained for the same animals. While skeletal levels were very consistent within each experimental group, the soft tissue values showed relatively large variations among members of each group. The younger rats retained larger amounts of tetracycline in soft tissues, but differences in soft tissue values between age groups could not be statistically verified. The data from the various experiments indicate that age differences in whole body retention of labeled tetracycline did reflect age differences of skeletal tetracycline fixation; however, persistent localization of tetracycline in soft tissue compartments, and the animal-to-animal variation of soft tissue tetracycline residue, reduced the accuracy of predicting skeletal levels by whole body measurements. The proposed technique may be of extreme value in the study of skeletal metabolic disorders provided a rapid, complete, or uniform clearance of tetracycline from soft tissue compartments could be obtained.

In conclusion, the results of this investigation indicate:

1. The labeled tetracycline synthesized in this laboratory was representative of the tetracycline antibiotics.
2. Whole body retention of iodinated tetracycline in young growing rats was greater than in mature rats, and resulted from enhanced skeletal deposition of tetracycline in the young rats as contrasted to the older animals.
3. Residual and variable amounts of tetracycline persisted in soft tissues for prolonged intervals after injection, but the skeleton showed the greatest uptake and consistent binding of the drug.
4. Skeletal fixation of tetracycline can be estimated by whole body measurements of radioiodinated tetracycline, but the accuracy of the estimates obtained is dependent upon a uniform soft tissue clearance and this has been shown to be variable. The technique may be of value for assessment of skeletal metabolic activity, especially when comparing normal to pathological states, providing a uniform clearance of tetracycline from soft tissues can be achieved.

REFERENCES

(1) D. P. Rall, T. L. Lee, M. Lane, and M. G. Kelly, *J. Nat. Cancer Inst.*, **19**, 79(1957).

(2) R. A. Milch, D. P. Rall, and J. E. Tobie, *ibid.*, **19**, 87(1957).

(3) W. H. Harris, R. H. Jackson, and J. Jowsey, *J. Bone Joint Surg.*, **44A**, 1308(1962).

(4) H. M. Frost, *Can. J. Biochem. Physiol.*, **40**, 485(1962).

(5) W. H. Harris, *Nature*, **188**, 1038(1960).

(6) H. M. Myers and S. M. Jaffe, *J. Dent. Res.*, **44**, 502(1965).

(7) R. A. Milch, D. P. Rall, and J. E. Tobie, *J. Bone Joint Surg.*, **40A**, 897(1958).

(8) L. Blomquist and A. Hanngren, *Biochem. Pharmacol.*, **15**, 215(1966).

(9) H. M. Frost, *Can. J. Biochem. Physiol.*, **41**, 31(1963).

(10) W. R. Lee, *J. Anat.*, **98**, 665(1964).

(11) O. Landeros and H. M. Frost, *Henry Ford Hosp. Med. Bull.*, **12**, 499(1964).

(12) J. J. Hlavka, A. Schneller, H. Kranzinski, and J. H. Boothe, *J. Amer. Chem. Soc.*, **84**, 1426(1962).

(13) W. R. Benson, E. T. McBee, and L. Rand, *Org. Syn.*, **42**, 73(1962).

(14) H. H. Donaldson, *Amer. J. Anat.*, **26**, 237(1919–1920).
(15) R. G. Kelly and D. A. Buyake, *J. Pharmacol. Exp. Ther.*, **130**, 144(1960).
(16) R. G. Kelly, L. A. Kanegis, and D. A. Buyake, *ibid.*, **134**, 320(1961).
(17) W. R. Lee, J. H. Marshall, and H. A. Sissions, *J. Bone Joint Surg.*, **47B**, 157(1965).
(18) H. J. Eisner and R. J. Wulf, *J. Pharmacol. Exp. Ther.*, **142**, 122(1963).
(19) D. A. Buyake, H. J. Eisner, and R. G. Kelly, *ibid.*, **130**, 150(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 13, 1968, from the *Department of Bionucleonics, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication November 12, 1969.

This investigation was supported in part by the National Center for Radiological Health, U. S. Public Health Service.

* Present address: Hazelton Labs, Falls Church, VA 22046

Effect of Probenecid on Renal Clearance of Riboflavin in Man

WILLIAM J. JUSKO, GERHARD LEVY*, SUMNER J. YAFFE, and RAFAEL GORODISCHER

Abstract □ The renal clearance of riboflavin was determined in three human subjects at various serum concentrations of the vitamin with and without prior administration of probenecid. Renal clearances of riboflavin exceeded (up to three times) the endogenous creatinine clearances, which indicates that riboflavin excretion involves renal tubular secretion. The clearance of riboflavin was less at low than at high serum concentrations of the vitamin, characteristic of a saturable tubular reabsorption process. Probenecid decreased the renal clearance of riboflavin, and this effect was directly related to the serum concentration of the inhibitor. The serum protein binding of the vitamin was essentially constant (60%) over the concentration range encountered and was unaffected by the presence of probenecid.

Keyphrases □ Riboflavin, renal clearance, man—probenecid effect □ Probenecid, effects—riboflavin, renal clearance, plasma protein binding □ Spectrophotometry—analysis

A pharmacokinetic analysis of literature data by Levy and Jusko (1) yielded renal clearance values for riboflavin in a human subject which were appreciably higher than the normal glomerular filtration rate. Subsequently, it was found that probenecid, an inhibitor of certain specialized renal transport processes (2), decreased the initial rates of urinary excretion of oral and parenteral doses of the vitamin in human subjects (3). These studies suggested that the renal excretion of riboflavin in man involves tubular secretion. The existence of such a mechanism for riboflavin has already been demonstrated in the chicken by Rennick (4) who also noted an inhibitory effect of probenecid on this process.

The purposes of the study to be described were to determine directly the renal clearance of riboflavin in man and to assess quantitatively the effect of probenecid on this process as well as on the plasma protein binding of the vitamin.

EXPERIMENTAL

The studies were carried out in three healthy human subjects: an adult male (Subject J), age 26 years, and two female children (Subjects A and C), ages 8 and 11 years, respectively. The adult subject received a single intravenous dose of riboflavin-5′-phosphate (FMN)[1] equivalent to about 30 mg. of riboflavin (FR) with and without 1 g. probenecid[2] given orally in suspension 1 hr. prior to FMN injection. Urine was collected at appropriate intervals for a total of 48 hr. Blood samples were drawn from the antecubital vein at −0.5, 0.25, 0.75, 1.25, 2.25, 3.25, 4.5, 6.5, 8.5, 13.0, and 25.0 hr. relative to the time of FMN injection. These times were midpoints of urine collection periods.

The two younger subjects received an initial intramuscular dose of FMN[3] equivalent to 16 mg. FR followed by three hourly oral doses of 6 mg. FR as FMN in solution. Urine was collected at hourly intervals and blood was drawn from the antecubital vein at the midpoints of the three urine-collection periods. A 0.5-g. dose of probenecid in tablet form was administered in crossover fashion to the two children 1 hr. prior to the initial dose of FMN. There was an interval of 7 days or more between the control and probenecid studies.

Protein-Binding Determinations—The ultrafiltration technique described in an earlier publication (5) was used to determine the extent of protein binding of the flavins and probenecid in the serum samples.

FMN Stability Study—A series of samples, each containing 1 ml. of FMN in pH 7.4 isotonic Sorensen's buffer and 1 ml. of freshly drawn whole blood from Subject J, were incubated at 37° in the dark. The concentrations of FMN and FR in the samples were determined as a function of time. Control solutions, without blood, were similarly analyzed.

Analytical Method—Riboflavin and FMN were determined fluorometrically by methods previously described (5, 6). Endogenous creatinine levels in urine and serum were determined colorimetrically by the alkaline picrate method (7). Probenecid in serum was assayed by the spectrophotometric method of Dayton *et al.* (8). Initial serum samples were assayed for albumin content as described previously (5).

Data for flavins and probenecid were corrected for blank readings of urine and serum samples obtained prior to administration of the compounds to the test subjects. There was no interference in the assay of any of the compounds due to the presence of the others.

RESULTS

Renal Clearances of Riboflavin and Effect of Probenecid—Riboflavin clearances in Subjects A and C (Table I) were determined

[1] Sodium riboflavin-5′-phosphate, Hoffmann-LaRoche, Nutley, N. J.
[2] Benemid tablets, Merck Sharpe and Dohme, West Point, Pa.
[3] Hyrye injection, S. F. Durst and Co., Inc., Philadelphia, Pa.

(14) H. H. Donaldson, *Amer. J. Anat.*, **26**, 237(1919–1920).
(15) R. G. Kelly and D. A. Buyake, *J. Pharmacol. Exp. Ther.*, **130**, 144(1960).
(16) R. G. Kelly, L. A. Kanegis, and D. A. Buyake, *ibid.*, **134**, 320(1961).
(17) W. R. Lee, J. H. Marshall, and H. A. Sissions, *J. Bone Joint Surg.*, **47B**, 157(1965).
(18) H. J. Eisner and R. J. Wulf, *J. Pharmacol. Exp. Ther.*, **142**, 122(1963).
(19) D. A. Buyake, H. J. Eisner, and R. G. Kelly, *ibid.*, **130**, 150(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 13, 1968, from the *Department of Bionucleonics, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication November 12, 1969.

This investigation was supported in part by the National Center for Radiological Health, U. S. Public Health Service.

* Present address: Hazelton Labs, Falls Church, VA 22046

Effect of Probenecid on Renal Clearance of Riboflavin in Man

WILLIAM J. JUSKO, GERHARD LEVY*, SUMNER J. YAFFE, and RAFAEL GORODISCHER

Abstract □ The renal clearance of riboflavin was determined in three human subjects at various serum concentrations of the vitamin with and without prior administration of probenecid. Renal clearances of riboflavin exceeded (up to three times) the endogenous creatinine clearances, which indicates that riboflavin excretion involves renal tubular secretion. The clearance of riboflavin was less at low than at high serum concentrations of the vitamin, characteristic of a saturable tubular reabsorption process. Probenecid decreased the renal clearance of riboflavin, and this effect was directly related to the serum concentration of the inhibitor. The serum protein binding of the vitamin was essentially constant (60%) over the concentration range encountered and was unaffected by the presence of probenecid.

Keyphrases □ Riboflavin, renal clearance, man—probenecid effect □ Probenecid, effects—riboflavin, renal clearance, plasma protein binding □ Spectrophotometry—analysis

A pharmacokinetic analysis of literature data by Levy and Jusko (1) yielded renal clearance values for riboflavin in a human subject which were appreciably higher than the normal glomerular filtration rate. Subsequently, it was found that probenecid, an inhibitor of certain specialized renal transport processes (2), decreased the initial rates of urinary excretion of oral and parenteral doses of the vitamin in human subjects (3). These studies suggested that the renal excretion of riboflavin in man involves tubular secretion. The existence of such a mechanism for riboflavin has already been demonstrated in the chicken by Rennick (4) who also noted an inhibitory effect of probenecid on this process.

The purposes of the study to be described were to determine directly the renal clearance of riboflavin in man and to assess quantitatively the effect of probenecid on this process as well as on the plasma protein binding of the vitamin.

EXPERIMENTAL

The studies were carried out in three healthy human subjects: an adult male (Subject J), age 26 years, and two female children (Subjects A and C), ages 8 and 11 years, respectively. The adult subject received a single intravenous dose of riboflavin-5′-phosphate (FMN)[1] equivalent to about 30 mg. of riboflavin (FR) with and without 1 g. probenecid[2] given orally in suspension 1 hr. prior to FMN injection. Urine was collected at appropriate intervals for a total of 48 hr. Blood samples were drawn from the antecubital vein at −0.5, 0.25, 0.75, 1.25, 2.25, 3.25, 4.5, 6.5, 8.5, 13.0, and 25.0 hr. relative to the time of FMN injection. These times were midpoints of urine collection periods.

The two younger subjects received an initial intramuscular dose of FMN[3] equivalent to 16 mg. FR followed by three hourly oral doses of 6 mg. FR as FMN in solution. Urine was collected at hourly intervals and blood was drawn from the antecubital vein at the midpoints of the three urine-collection periods. A 0.5-g. dose of probenecid in tablet form was administered in crossover fashion to the two children 1 hr. prior to the initial dose of FMN. There was an interval of 7 days or more between the control and probenecid studies.

Protein-Binding Determinations—The ultrafiltration technique described in an earlier publication (5) was used to determine the extent of protein binding of the flavins and probenecid in the serum samples.

FMN Stability Study—A series of samples, each containing 1 ml. of FMN in pH 7.4 isotonic Sorensen's buffer and 1 ml. of freshly drawn whole blood from Subject J, were incubated at 37° in the dark. The concentrations of FMN and FR in the samples were determined as a function of time. Control solutions, without blood, were similarly analyzed.

Analytical Method—Riboflavin and FMN were determined fluorometrically by methods previously described (5, 6). Endogenous creatinine levels in urine and serum were determined colorimetrically by the alkaline picrate method (7). Probenecid in serum was assayed by the spectrophotometric method of Dayton *et al.* (8). Initial serum samples were assayed for albumin content as described previously (5).

Data for flavins and probenecid were corrected for blank readings of urine and serum samples obtained prior to administration of the compounds to the test subjects. There was no interference in the assay of any of the compounds due to the presence of the others.

RESULTS

Renal Clearances of Riboflavin and Effect of Probenecid—Riboflavin clearances in Subjects A and C (Table I) were determined

[1] Sodium riboflavin-5′-phosphate, Hoffmann-LaRoche, Nutley, N. J.
[2] Benemid tablets, Merck Sharpe and Dohme, West Point, Pa.
[3] Hyrye injection, S. F. Durst and Co., Inc., Philadelphia, Pa.

Table I—Effect of Probenecid on Riboflavin and Creatinine Clearances in Two Normal Children

Subject	Serum Concentrations[a] Probenecid, mg. %	Total Flavin, mcg./ml.	Clearances, ml./min./1.73 m.² Total Flavin	Creatinine	Clearance Ratio
A (F, 8 yr.)	0.0	0.413	401	142	2.82
	0.0	0.364	337	130	2.59
	0.0	0.298	313	100	3.13
	2.22	0.554	255	98	2.60
	2.34	0.485	136	62	2.19
	3.82	0.422	255	123	2.07
C (F, 11 yr.)	0.0	0.440	291	114	2.55
	0.0	0.318	301	101	2.98
	0.0	0.277	293	99	2.96
	4.13	0.535	153	86	1.78
	5.39	0.369	128	78	1.64
	5.05	0.354	132	114	1.16

[a] Serum samples were obtained at 1.5, 2.5, and 3.5 hr. after initial riboflavin administration and data from each study are listed in that order.

after parenteral administration of the vitamin, with sustaining oral doses given to prevent the usual rapid fall in serum flavin levels. The renal clearances of riboflavin and endogenous creatinine were calculated in the usual way by dividing the urinary excretion rate by the midtime serum concentration. The endogenous creatinine clearance was determined as a measure of glomerular filtration rate (9). For purposes of intersubject comparison, the renal clearance values for the two children were corrected to standard body size of 1.73 m.² by the method of Dubois and Dubois (10).

The riboflavin-creatinine clearance ratios for Subjects A and C ranged from 2.6 to 3.1 in the control periods (Table I). This shows that the vitamin is secreted in the renal tubules. Probenecid caused a decrease in riboflavin clearance and clearance ratios but had no significant effect on the clearance of creatinine. The 0.5-g. dose of probenecid was apparently better absorbed in Subject C than in Subject A as seen by the time course of probenecid serum concentrations in the two children. The relationship between the serum probenecid concentration and riboflavin clearance ratio in the two subjects is shown in Fig. 1.

The experiments in Subject J were designed to determine the renal clearance of riboflavin and the effect of probenecid over a wide range of rapidly changing serum concentrations of the vitamin. The serum concentrations of total riboflavin (FR and FMN) and of FMN alone as a function of time after rapid intravenous injection of FMN with and without prior administration of probenecid are shown in Fig. 2. The corresponding urinary excretion rates as a function of time are shown in Fig. 3. Total riboflavin concentrations in the serum were appreciably higher (Fig. 2) after administration of probenecid while the urinary excretion rates were similar (Fig. 3). Seventy-six percent of the dose was recovered as riboflavin and 14% as FMN in the control experiment. During probenecid administration, 71% was recovered as riboflavin and there was no measurable excretion of FMN.

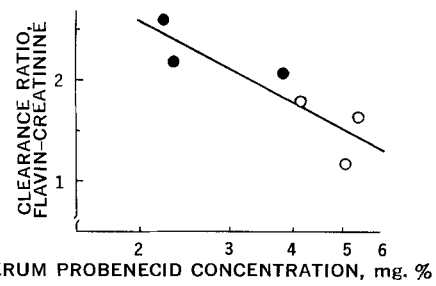


Figure 1—*Effect of probenecid on renal clearances of riboflavin (total flavin) in Subjects A (●) and C (○). The clearance ratio, flavin-creatinine, is plotted as a function of the log serum probenecid concentration, and a least-squares regression line was fitted to the data.*

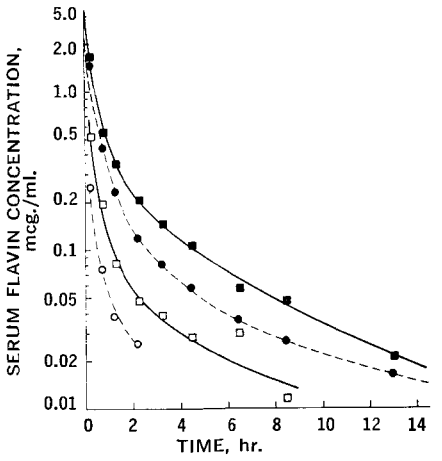


Figure 2—*Serum concentrations of flavins as a function of time after rapid i.v. injection of about 30 mg. FR as FMN to Subject J. Total flavin (solid symbols) and FMN (open symbols) concentrations were determined in a control experiment (circles) and following oral administration of 1 g. probenecid (squares). Total flavin curves were obtained by a computer nonlinear least-squares fit to the data.*

The curves for total riboflavin concentrations in the serum (Fig. 2) and for urinary excretion rates (Fig. 3) were obtained by nonlinear least-squares computer fit to the experimental data. Preliminary graphical analysis by the method of residuals (11) indicated that these data declined in a triexponential manner. Because the serum and excretion values ranged over more than two orders of magnitude, they were converted to their respective logarithmic values to reduce bias. The "NLIN" digital computer program of Marquardt (12) was then used to fit a triexponential curve of the type:

$$F = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + C \cdot e^{-\gamma t} \qquad \text{(Eq. 1)}$$

to the experimental data where F represents either total riboflavin serum concentrations or urinary excretion rates, t is time, and the remaining symbols are constants. The values of the six constants for each of the four sets of data are listed in Table II. The apparent volume of the central compartment, V_c, was calculated from the serum data using the equation:

$$V_c = \text{dose}/(A + B + C) \qquad \text{(Eq. 2)}$$

where the denominator represents the computer derived zero-time serum concentration of the flavins ($C_p°$). This apparent volume

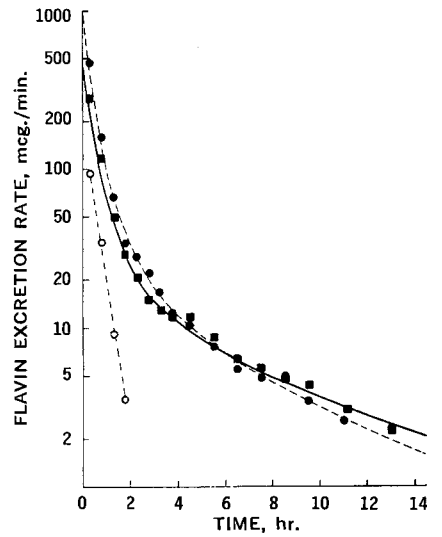


Figure 3—*Urinary excretion rate of flavins as a function of time after rapid i.v. injection of about 30 mg. FR as FMN to Subject J. Lines and symbols are defined as in Fig. 2. No FMN was found in the urine after administration of probenecid.*

Table II—*Pharmacokinetic Coefficients Determined*[a] *from Serum Concentrations and Urinary Excretion Rates of Total Riboflavin after I.V. Administration of Riboflavin-5′-phosphate to Subject J*

Parameter[b]	Serum Levels[c] Control Experiment		Serum Levels[c] During Probenecid	Urinary Excretion Rates[c] Control Experiment		Urinary Excretion Rates[c] During Probenecid
A	1.83	*	3.09	803	*	423
α, hr.$^{-1}$	3.24		3.99	3.02		2.28
B	0.316		0.435	94.7		39.6
β, hr.$^{-1}$	0.717		0.616	0.824		0.956
C	0.072	*	0.140	14.6		18.0
γ, hr.$^{-1}$	0.120	*	0.144	0.148		0.159
Dose, mg.	31.0		30.4			
Apparent volume of central compartment,[d] liter	14.0	*	8.27			

[a] By a least-squares computer fit of Eq. 1. [b] A, B, and C have units of mcg./ml. for serum data and mcg./min. for urinary excretion rates. [c] Asterisk designates those coefficients which do not overlap in the range of their calculated value ± the standard error. [d] Calculated using Eq. 2.

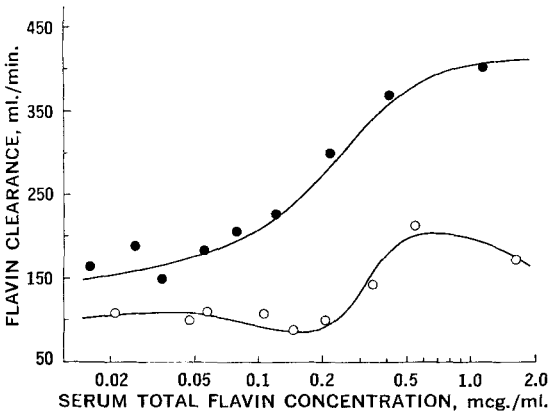


Figure 4—*Renal clearance of total riboflavin plotted as a function of the serum riboflavin concentration in a control experiment (solid symbols) and after oral administration of 1 g. probenecid (open symbols). Curves represent clearances calculated from the least-squares fit of the serum and urinary excretion data. Simultaneously determined endogenous creatinine clearances averaged 124 ± 12 ml./min. and were independent of the serum riboflavin and probenecid concentrations.*

was 14.0 l. in the control experiment and only 8.3 l. in the presence of probenecid.

FMN concentrations in the serum and urine could be determined only during the early times after injection of the vitamin. Available assay methods are inadequate (5) to detect FMN at low concentrations of the vitamin and in the presence of a large excess of FR. It was thus necessary to determine renal clearance in terms of total riboflavin rather than for FMN and FR separately. However, the clearances of FR and FMN determined separately during the early times in the control experiment were quite similar.

The renal clearances of total riboflavin in the presence and absence of probenecid are plotted in Fig. 4 as a function of the total riboflavin concentration in the serum. The points are experimental values and the curves are the results of clearance calculations based on urinary excretion rate and serum concentration values calculated from the parameters of Eq. 1 listed in Table II. The glomerular filtration rate of Subject J averaged 130 ± 10 ml./min. in the presence and 120 ± 11 ml./min. in the absence of probenecid as measured by the endogenous creatinine clearances. As in Subjects A and C, the riboflavin and creatinine clearance values for Subject J indicate that riboflavin excretion involves tubular secretion. Probenecid has a pronounced inhibitory effect on riboflavin excretion as shown by the appreciable decrease in renal clearance when probenecid was given.

The continuous decline in renal clearance of riboflavin during the course of decreasing serum levels of the vitamin is characteristic of a compound which undergoes saturable tubular reabsorption (9). This mechanism occurs with at least three other water-soluble vitamins: thiamine (13), pantothenic acid (14), and ascorbic acid (9). A multiple-compartment pharmacokinetic model embodying saturable renal tubular reabsorption has been developed and applied to riboflavin clearance data from man and dog (15). The relationship which describes the observed (net) renal clearance (Cl_T) of compounds which undergo apparent first-order excretion (clearance) from serum to urine (Cl_f) and saturable tubular reabsorption is

$$Cl_T = Cl_f - \frac{T_m C_u}{C_p(K_m + C_u)} \qquad \text{(Eq. 3)}$$

where C_p and C_u are the serum and urine concentrations of the drug, T_m is the tubular reabsorption transport maximum, and K_m is the Michaelis-Menten constant for the saturable tubular reabsorption process (15). Graphical and computer analysis of the experimental renal clearance data obtained in the absence of probenecid has yielded values of 420 ml./min. for Cl_f, 33.3 mcg./min. for T_m, and 16.3 mcg./ml. for K_m (15). It was not possible to determine these parameters when probenecid was administered because the inhibitory effect of this drug was not constant during the experiment.

Probenecid serum concentrations were determined for Subject J and are shown as a function of time in Fig. 5. These data were used in conjunction with the riboflavin clearance values to construct a drug concentration–response plot which is depicted in Fig. 6. Because of the concentration dependence of riboflavin clearance and the wide concentration range of the data, it was necessary to express the clearances during probenecid administration as a percent of the riboflavin clearance at similar serum concentrations of the vitamin in the control experiment. It should be noted that the pharmacologic effect–serum concentration relationship presented in Fig. 6 (where both riboflavin and probenecid serum concentrations varied markedly) is of a type different from the effect–concentration relationship depicted in Fig. 1 (where the riboflavin concentration was relatively constant). It is of interest, however, that the maximum inhibition of riboflavin clearance[4] in Subject J occurred at a probenecid serum concentration of about 8 mg. % and that the same maximum inhibitory concentration is obtained upon extrapolating the data from Subjects A and C in Fig. 1 to a clearance ratio of unity. Renal clearance studies in dogs (16) also showed that a similar serum concentration of probenecid (7 mg. %) produced maximum inhibition of renal clearance of riboflavin and PAH.

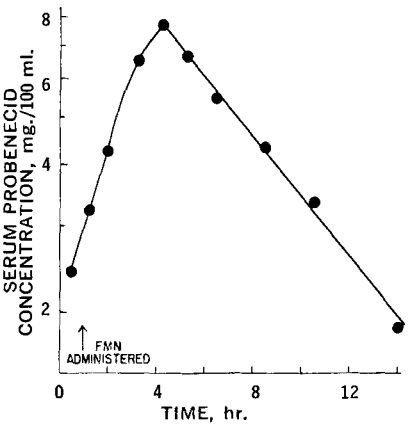


Figure 5—*Concentrations of probenecid in the serum as a function of time after oral administration of 1 g. in aqueous suspension to Subject J. Decline in serum concentration of probenecid represents a half-life of 5 hr.*

[4] This refers to maximum inhibition in terms of actual clearance values (ml./min.) rather than in terms of relative values (percent of control clearance).

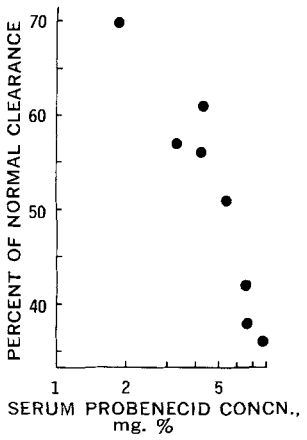


Figure 6—*Effect of probenecid on renal clearance of flavin in Subject J. The percent of normal clearance was calculated (see text) from the data of Fig. 4 and is plotted as a function of the log serum probenecid concentration.*

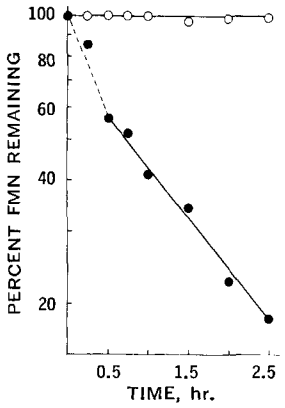


Figure 7—*Rate of dephosphorylation of riboflavin-5'-phosphate during incubation of 72 mcg. % FMN in fresh whole blood (●) and in pH 7.4 Sorensen's buffer (○). Total riboflavin concentration (FR + FMN) remained constant for the duration of the experiment.*

Protein-Binding Results—The serum samples which were obtained in each of the experiments in Subjects A and C were pooled to obtain adequate volume for a determination of the extent of protein binding of the flavins. In Subject A, the flavins were 64 and 57% bound in the absence and presence of probenecid, respectively. The corresponding values for Subject C were 52 and 54%. These data indicate that probenecid had little or no displacing effect on the protein binding of the flavins. The data show also that if the renal clearance values of riboflavin were corrected for protein binding, the net secretion of the flavins would appear to be even more pronounced than indicated by the data in Table I.

In Subject J, individual serum samples were used to determine the extent of protein binding over an appreciable range of serum concentrations of the vitamin. The results of these determinations are summarized in Table III. Over a 10- to 20-fold range in serum riboflavin concentrations, the fraction of total riboflavin bound remained essentially constant and was similar in the control and probenecid experiments. The albumin concentration of the serum was 4.6 g./100 ml., and it was possible to calculate the theoretical degree of binding of total riboflavin using known values (5) for the albumin-FR and albumin-FMN association constants (1.3×10^3 and 3.2×10^4 liter/mole, respectively, at 30°) and the assayed serum concentrations of FR and FMN (Table 3). The basis for such calculations is given in an earlier report on the plasma protein binding of riboflavin and FMN (5). These calculations show that no change in the degree of protein binding of total riboflavin is to be expected over the concentration range of riboflavin encountered in these experiments. Despite the existence of the two forms of the vitamin in the serum, the extent of binding of total riboflavin remained constant due to the relatively constant concentration ratio of FR and FMN in the serum.

The serum protein binding of probenecid was also determined in the serum samples obtained from Subject J. The extent of protein binding of probenecid remained essentially constant during the course of the experiment and the change in serum concentrations of riboflavin did not appear to affect the protein binding of probenecid. Over the serum concentration range of 2 to 8 mg./100 ml., probenecid was 90 ± 4% protein bound, which is in excellent agreement with the data reported by Dayton *et al.* (8).

Table III—Protein Binding of Riboflavin in Human Serum[a] in the Presence and Absence of Probenecid

Serum Flavin Concn., mcg./ml.			Serum Probenecid Concn.,	Fraction of Total Flavin Bound	
FR	FMN[b]	Total[b]	mg. %	Experimental	Theoretical[c]
0.859	0.282	1.141	0.0	0.58	0.58
0.330	0.085	0.415	0.0	0.60	0.56
0.175	0.043	0.218	0.0	0.61	0.56
0.091	0.029	0.120	0.0	0.63	0.58
1.139	0.511	1.650	3.23	0.60	0.59
0.356	0.194	0.551	4.25	0.58	0.62
0.264	0.081	0.346	6.53	0.53	0.55
0.158	0.048	0.206	7.71	0.59	0.55
0.107	0.039	0.146	6.68	0.60	0.57
0.078	0.028	0.106	5.47	0.57	0.57

[a] Albumin content: 4.63 g./100 ml. [b] Riboflavin equivalent. [c] See text.

FMN Dephosphorylation—The data shown in Figs. 2 and 3 indicate that intravenously administered FMN is rapidly converted to riboflavin until a FR–FMN concentration ratio of about 3:1 is attained. This appears to reflect an equilibrium between the rate of dephosphorylation and the rate of phosphorylation of the vitamin. A similar phenomenon has been observed in the rat by Christensen (17). Phosphatase (18) and flavokinase (19) enzymes in the liver may account for these processes, but enzymes associated with erythrocytes also contribute to the very rapid loss of serum FMN and appearance of FR. Incubation of FMN with fresh whole blood from Subject J and with buffer showed that FMN was rapidly converted to FR in whole blood (Fig. 7), but that there was no dephosphorylation of FMN in the control buffer solution. The concentration of total riboflavin in the blood samples remained constant for the duration of the experiment. Previous experiments have shown (5) that incubation of FMN with human plasma produced no change in the relative protein binding of the vitamin which is indirect evidence that FMN is not dephosphorylated in blood plasma.

DISCUSSION

The results of the present study confirm earlier indications (1, 3) that the renal excretion of riboflavin in man involves tubular secretion. Renal clearances of the vitamin were found to exceed markedly the glomerular filtration rate in three human subjects and it was shown that probenecid has a pronounced inhibitory effect on riboflavin excretion. In Subjects A and C, where the riboflavin serum levels were maintained relatively constant, the effect of probenecid could be characterized by a typical dose–response relationship (Fig. 1). In Subject J, the dose–response relationship (Fig. 6) is complicated by the serum concentration dependence of riboflavin clearances resulting from the saturable tubular reabsorption process. Therefore, the clearances during probenecid administration had to be expressed as a percent of the clearance value obtained in the control experiment at the same riboflavin concentration in the serum.

The riboflavin clearances in Fig. 4 have a constant value of about 100 ml./min. during the time when probenecid serum levels declined from 7.7 to 1.8 mg. % and riboflavin serum concentration ranged from 0.2 to 0.02 mcg./ml. This suggests that probenecid inhibits both the tubular secretion and the specialized reabsorption of the vitamin. With inhibition of both active processes, the clearance of flavin would be expected to remain constant at less than the glomerular filtration rate (124 ml./min.) because the vitamin is bound to some extent to serum proteins. In view of the difference in the probenecid serum concentration–effect relationship during increasing and decreasing probenecid serum levels, respectively, it appears that the time course of probenecid concentration in the serum does not adequately reflect the time course of probenecid concentration at its site of action, particularly in the elimination phase of probenecid. The fact that maximum inhibition of riboflavin clearance was observed while probenecid concentration in the

serum declined from 7.7 to 1.8 mg. % suggests that probenecid is retained at its site of action considerably longer than in the serum.

Probenecid had no serum protein-displacing effect on riboflavin and FMN under the experimental conditions. The protein-binding determinations were also done to determine whether the renal clearance of flavin could change as a result of saturation of protein-binding sites at high serum concentrations of riboflavin. This might result in a greater fraction of unbound riboflavin with concomitant increase in renal clearances at high serum riboflavin levels. The actual measurements and theoretically calculated protein-binding data (Table III) showed that the extent of protein binding of total riboflavin is quite constant over the concentration range used in these studies. The protein-binding data do, however, indicate that correction of the renal clearance values for the extent of riboflavin binding would result in even higher clearance values than shown in Table I and Fig. 4 for the three subjects.

The data in Fig. 2 and Table I show that serum concentrations of riboflavin were substantially higher during probenecid administration than during control experiments. Pharmacokinetic analysis of these data indicates that the apparent volume of the central compartment for the vitamin decreased from 14.0 to 8.3 l. when probenecid was administered. This may be a real effect of probenecid on the distribution of riboflavin or it may reflect the limitations of presently available pharmacokinetic techniques in that an inhibition of renal excretion may modify the apparent distribution constants of the pharmacokinetic model. Studies are now in progress to determine the effect, if any, of probenecid on the distribution of riboflavin in anephric subjects (20). It should be possible in this way to determine if probenecid does in fact modify pharmacokinetic parameters other than those reflecting the renal excretion of riboflavin.

In order to examine the mechanism and kinetics of the renal excretion of riboflavin and the nature of the effect of probenecid on these processes in more detail than is feasible in man, additional studies have been carried out in the dog. The results of these studies, which will be reported in other communications (15, 16), demonstrate that riboflavin excretion in the dog also involves both tubular secretion and saturable tubular reabsorption. After attainment of constant serum levels of riboflavin and PAH, a typical log-linear concentration–response relationship was found for the inhibition of renal clearance of these compounds by probenecid. Interestingly, the maximum inhibitory effect of probenecid on both riboflavin and PAH renal clearance in these dogs was reached at about 7 mg. % serum probenecid concentration, similar to the maximum effective concentration found in the subjects of the present study.

Probenecid inhibits the renal excretion of riboflavin in species other than man and dog. Rennick (4) has demonstrated inhibition of tubular secretion of riboflavin by probenecid in the chicken. Markkanen *et al.* (21) have shown that probenecid decreases the basal excretion of riboflavin in man and rabbits whose riboflavin intake was limited to that derived from the normal diet.

There is evidence (16, 22) that the renal transport mechanism for riboflavin may involve a phosphorylation–dephosphorylation process. Probenecid, besides decreasing the renal clearance of various compounds by competitive inhibition, also inhibits directly those enzymes which require a source of high-energy phosphate bond energy (23). This may be the mechanism of the effect of probenecid on the renal clearance of riboflavin, since no FMN was found in the urine when probenecid was administered in the present study. It is of interest that a similar phosphorylation–dephosphorylation mechanism may be responsible for the specialized intestinal absorption of riboflavin (24) which also appears to be inhibited by probenecid (3).

REFERENCES

(1) G. Levy and W. J. Jusko, *J. Pharm. Sci.*, **55,** 1322(1966).

(2) I. M. Weiner, J. A. Washington II, and G. H. Mudge, *Bull. Johns Hopkins Hosp.*, **106,** 333(1960).

(3) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **56,** 1145(1967).

(4) B. R. Rennick, *Proc. Soc. Exp. Biol. Med.*, **103,** 241(1960).

(5) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **58,** 58(1969).

(6) H. B. Burch, O. A. Bessey, and O. H. Lowry, *J. Biol. Chem.*, **175,** 457(1948).

(7) "Laboratory Manual of Pediatric Micro- and Ultramicro-Biochemical Techniques," 3rd ed., D. O'Brien and F. A. Ibbott, Eds., Harper and Row, New York, N. Y., 1961, p. 100.

(8) P. G. Dayton, T. F. Yu, W. Chen, L. Berger, L. A. West, and A. B. Gutman, *J. Pharmacol. Exp. Ther.*, **140,** 278(1963).

(9) R. F. Pitts, "Physiology of the Kidney and Body Fluids," Yearbook Medical Publishers, Chicago, Ill., 1963, p. 66.

(10) "Documenta Geigy: Scientific Tables," 6th ed., K. Diem, Ed., Geigy Pharmaceuticals, Ardsley, N. Y., supplementary nomograms.

(11) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Toronto, Ontario, Canada, 1966, p. 20.

(12) D. W. Marquardt, "DPE-NLIN," Share General Library Program No. 7-1354.

(13) H. N. Haugen, *Scand. J. Clin. Lab. Invest.*, **13,** 61(1961).

(14) K. Roholt and V. Schmidt, *ibid.*, **3,** 108(1951).

(15) W. J. Jusko and G. Levy, to be published.

(16) W. J. Jusko, B. R. Rennick, and G. Levy, to be published.

(17) S. Christensen, *Acta Pharmacol. Toxicol.*, **27,** 41(1969).

(18) D. B. McCormick and M. Russell, *Comp. Biochem. Physiol.*, **5,** 113(1962).

(19) D. B. McCormick, *Proc. Soc. Exp. Biol. Med.*, **107,** 784 (1961).

(20) W. J. Jusko, J. A. Leonards, and G. Levy, to be published.

(21) T. Markkanen, P. Toivanen, A. Toivanen, and E. Sotaniemi, *Scand. J. Clin. Lab. Invest.*, **15,** 511(1963).

(22) I. Magyar, *Hung. Acta Med.*, **1,** 37(1948).

(23) K. H. Beyer, H. F. Russo, E. K. Tillson, A. K. Miller, W. F. Verwey, and S. R. Gass, *Amer. J. Physiol.*, **166,** 625(1951).

(24) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **56,** 58(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 17, 1969, from the *Department of Pharmaceutics, School of Pharmacy* and the *Department of Pediatrics, School of Medicine, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication November 3, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported in part by Public Health Service Fellowship 5-F1-GM-33,073 for W. J. J. and by Grant No. FR-77 from the National Institute of General Medical Sciences, Bethesda, Md.

* To whom requests for reprints should be directed.

Influence of Dimethyl Sulfoxide on the Hemolytic Activity of Antimicrobial Preservatives I

HOWARD C. ANSEL and GARY E. CABRE*

Abstract □ The influence of dimethyl sulfoxide (DMSO) on the hemolytic activity of various chemical types of antimicrobial preservatives has been investigated *in vitro*, using rabbit erythrocytes. Irrespective of the chemical type of preservative, DMSO causes a depression in the hemolytic response of the erythrocytes to the preservative agents. This depression, which is greatest when the DMSO concentration is between 10 and 20%, is dependent upon preservative concentration and time. The degree of the hemolytic depression caused by the presence of DMSO is decreased with an increase in the concentration of the preservative agent or with an increase in the time of exposure. It was concluded that DMSO affects the rate of preservative-induced hemolysis, probably due to a cellular mechanism rather than to an extracellular preservative–DMSO chemical interaction.

Keyphrases □ Antimicrobial preservatives—hemolytic activity □ Dimethyl sulfoxide effect—hemolytic activity, antimicrobial preservatives □ Erythrocyte protection, hemolysis—dimethyl sulfoxide □ UV spectrophotometry—analysis

In a previous communication (1), it was reported that dimethyl sulfoxide (DMSO) in concentrations up to about 20% increasingly depressed the hemolytic response of rabbit erythrocytes *in vitro* to phenol. At greater DMSO concentrations, hemolysis was increased from its depressed level with an increase in the concentration of DMSO, presumably due to the commencement of the hemolytic effects of the DMSO itself.

The present study is an expansion of the previous work, undertaken to ascertain if the curious hemolytic profile obtained for combinations of DMSO and phenol is peculiar to that pair of agents or whether a similar alteration in the hemolytic response of erythrocytes to other chemical types of antimicrobial preservatives occurs in the presence of DMSO. This information would indicate the specificity or lack of specificity of the action of DMSO toward phenol-induced hemolysis and would help set the direction of subsequent studies aimed at the mechanism of the action, since the data obtained would suggest either a cellular-based, general action of DMSO or a specific extracellular drug–drug interaction.

The group of commonly employed antimicrobial preservatives selected for this study includes members representing the various chemical classes of preservatives and for which the individual hemolytic activities could be readily characterized and quantitated (2).

EXPERIMENTAL

Materials—The dimethyl sulfoxide and the antimicrobial preservatives employed in this investigation were reagent grade and were obtained commercially.

Blood Samples—Rabbit blood, obtained by cardiac puncture and defibrinated in the manner previously described (3), was used throughout this investigation. Each blood sample was collected immediately prior to its use and was verified for osmotic normalcy during the course of each experiment (2).

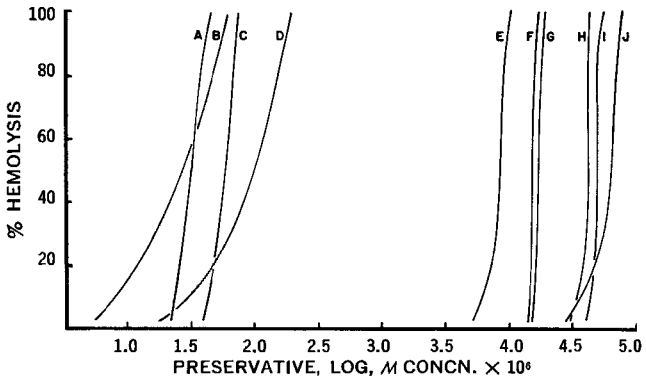


Figure 1—*Hemolytic activities of various preservative agents in 0.6% NaCl. Key:* A, *benzalkonium chloride;* B, *phenylmercuric nitrate;* C, *thimerosal;* D, *chlorhexidine diacetate;* E, p-*chlorophenol;* F, *chlorobutanol;* G, m-*cresol;* H, *phenylethyl alcohol;* I, *phenol; and* J, *benzyl alcohol.*

Quantitative Determination of Percent Hemolysis—The colorimetric method employed for the determination of the degree of hemolysis occurring in each test solution has recently been described (2). In brief, it involved the addition of 0.05 ml. of defibrinated blood to duplicate pairs of colorimeter tubes, each containing 5 ml. of test solution. The test mixtures were incubated in a water bath for 45 min. (except where noted) at 37° after which the unhemolyzed cells were settled by centrifugation and the absorbance readings of the hemolysate determined with a Klett-Summerson photoelectric colorimeter. Each absorbance reading was compared with a total hemolysis reading obtained by laking red cells in distilled water. The degree of hemolysis occurring in each test solution was calculated as a percent of total hemolysis. The data reported represent the average of a minimum of two, but usually four, like experiments. During and after the incubation period, each test mixture was macroscopically observed for color changes, precipitation, and other signs of denaturation.

Experimental Solutions—Aqueous solutions were employed throughout the investigation. In determining the hemolytic activity of each of the test solutions, 0.6% sodium chloride was added as an extracellular agent to protect the erythrocytes from simple osmotic hemolysis (1). Thus, any hemolysis occurring in the test solutions could be attributed to the activity of the antimicrobial preservative and/or DMSO. Appropriate control solutions were processed concurrently with the experimental solutions throughout the investigation (1, 2).

In order to assess the influence of DMSO on the hemolytic activity of the various antimicrobial preservatives, it was initially desirable to use concentration levels of the preservatives in the test solutions which would normally exert an intermediate (approximately 50%) degree of hemolysis in the absence of DMSO. Once the upward or downward influence of DMSO on hemolysis was established, the level of antimicrobial agent in the test solution was adjusted accordingly to permit the collection of the widest range of data.

Kinetic Studies—The general experimental design of the research series is such that the degree of hemolysis is determined after a constant 45-min. incubation period. In the current project the hemolytic activities of certain representative test solutions were also determined at incubation intervals of from 5 to 20 min., during periods up to 120 min.

Spectral Studies—Three preservatives which are diverse in chemical structure, benzalkonium chloride, chlorhexidine diacetate, and *m*-cresol, were subjected to spectral examination

Table I—Molar Concentrations of Preservatives Causing 10%, 50%, and 100% Hemolysis of Rabbit Erythrocytes *In Vitro* in the Presence of 0.6% Sodium Chloride

Preservative	Molar Concn. of Preservative Causing Hemolysis[a] 10%	50%	100%
Phenol	4.3×10^{-2}	4.6×10^{-2}	5.0×10^{-2}
Benzyl alcohol	3.8×10^{-2}	6.3×10^{-2}	7.0×10^{-2}
Phenylethyl alcohol	3.5×10^{-2}	4.6×10^{-2}	4.9×10^{-2}
m-Cresol	1.7×10^{-2}	1.8×10^{-2}	1.9×10^{-2}
Chlorobutanol	1.6×10^{-2}	1.7×10^{-2}	1.8×10^{-2}
p-Chlorophenol	6.2×10^{-3}	8.0×10^{-3}	9.2×10^{-3}
Thimerosal	4.8×10^{-5}	6.1×10^{-5}	6.8×10^{-5}
Chlorhexidine $(Ac)_2$	3.2×10^{-5}	1.1×10^{-4}	1.9×10^{-4}
Benzalkonium Cl	2.2×10^{-5}	3.3×10^{-5}	4.2×10^{-5}
Phenylmercuric NO_3	7.9×10^{-6}	3.1×10^{-5}	4.7×10^{-5}

[a] Data are the average of a minimum of two, but usually four, separate determinations conducted at 37° for 45 min.

alone and in combination with DMSO in order to detect evidence of chemical interaction. Such interaction could account for the decrease in hemolytic activity by the preservatives upon the addition of DMSO.

A Perkin-Elmer 212 and a Beckman DU spectrophotometer were employed in this study, and the peak absorbance for each preservative was determined at 282, 256, and 272 mμ, respectively, for benzalkonium chloride, chlorhexidine diacetate, and *m*-cresol. Dimethyl sulfoxide exhibited no absorbance above 250 mμ and thus in combination with the preservatives was noninterfering with the determinations of preservative concentrations. Reference cells were appropriately prepared for each experiment.

RESULTS AND DISCUSSION

Hemolytic Activity of the Antimicrobial Preservatives—The hemolytic activities of the antimicrobial preservatives employed in this study are depicted in Fig. 1 and summarized in Table I. The data were collected for solutions of preservatives in 0.6% sodium chloride and compare well with the hemolytic data obtained previously for certain of the preservatives in 0.9% sodium chloride (2). By reducing the level of sodium chloride in the present study, dissolution of the various preservative agents was enhanced and yet the tonicity of the solution was maintained at a level sufficient to protect the erythrocytes from simple osmotic hemolysis.

The individual hemolysis curves for the preservatives not only indicate the relative hemolytic potency of these agents, but they also provided the means by which the appropriate (intermediate or near-totally hemolytic) concentrations of the preservatives were selected for study in combination with DMSO to determine the influence of the latter agent. Thus, for each preservative, the strength which caused an intermediate degree of hemolysis (approximately 50%) was combined in initial experiments with various amounts of DMSO to determine whether the latter agent promoted a decrease or an

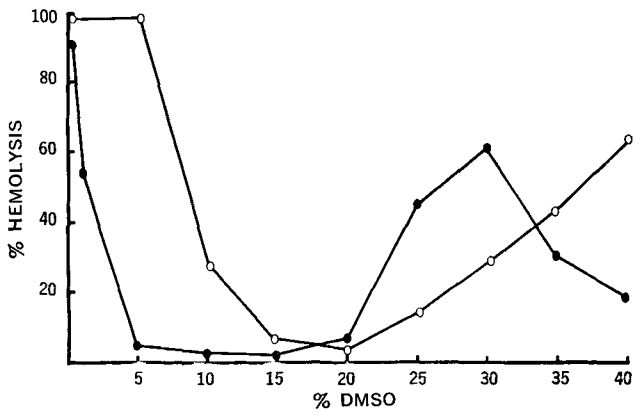


Figure 2—*Influence of DMSO on the hemolytic activity of* m-*cresol and* p-*chlorophenol in the presence of 0.6% NaCl. Key:* ●, *1.8* × 10^{-2} M m-*cresol; and* ○, *8.0* × 10^{-3} M p-*chlorophenol.*

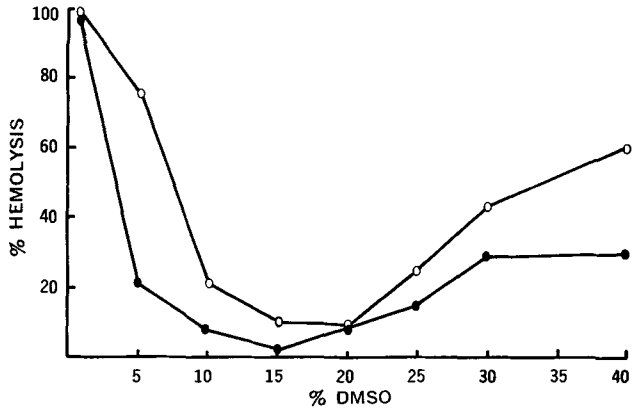


Figure 3—*Influence of DMSO on the hemolytic activity of phenylethyl alcohol and benzyl alcohol in the presence of 0.6% NaCl. Key:* ●, *5.0* × 10^{-2} M *phenylethyl alcohol; and* ○, *7.0* × 10^{-2} M *benzyl alcohol.*

increase in the degree of hemolysis compared to the preservative-saline control solution. It was found, in each instance, that the presence of increasing amounts of DMSO to approximately 10–20% caused a progressive decrease in the degree of hemolysis for each preservative agent within the 45-min. period of each experiment. Thus, the studies were continued using an amount of preservative inducing a greater degree of hemolysis (80–100%) so that the influence of DMSO in reducing hemolysis could be followed over a more extensive course.

Influence of DMSO on Preservative-Induced Hemolysis—Figures 2 through 5 depict the influence of DMSO on the hemolytic activities of various preservative agents. It is interesting to note that in each instance hemolysis is dramatically decreased, and indeed almost prevented, as the DMSO level is increased to between 10 and 20%, irrespective of the chemical type of preservative and its effective hemolytic concentration.

Figures 2 through 5 show that as the level of DMSO is increased beyond that amount which induces optimum retardation of hemolysis, hemolysis is increased and may be quantitated until denaturation of the blood occurs at DMSO concentrations of about 30%. It has been previously shown (1) that DMSO in the presence of 0.6% sodium chloride induces a hemolytic response in erythrocytes at DMSO levels of 25% and causes increased hemolysis with increasing concentrations of DMSO until denaturation occurs at approximately 50% DMSO levels. Thus, it would seem as though the portions of the curves in Figs. 2 through 5 beyond the areas of optimum depression reflect the combined cytotoxic activities of DMSO and the preservatives on the red blood cells, with the high concentrations of DMSO being too destructive to lend further any protective influence to the erythrocytes against preservative-induced hemolysis.

The deleterious effects of high levels of DMSO on blood are observed as amber-brown discoloration of the extracellular liquid,

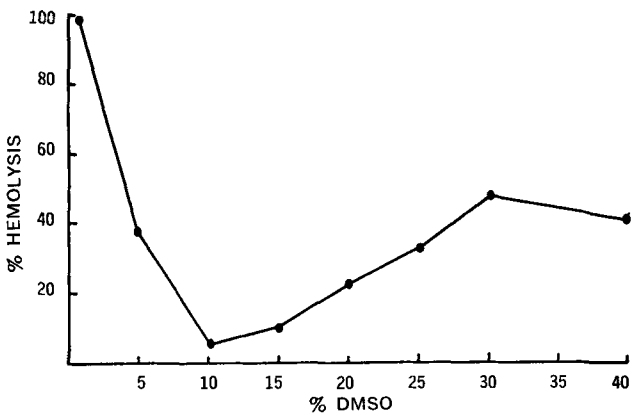


Figure 4—*Influence of DMSO on the hemolytic activity of 1.8* × 10^{-2} M *chlorobutanol in the presence of 0.6% NaCl.*

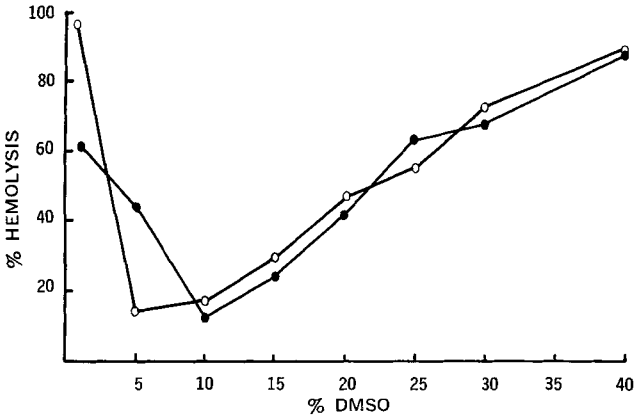


Figure 5—*Influence of DMSO on the hemolytic activity of phenylmercuric nitrate and thimerosal in the presence of 0.6% NaCl. Key:* ●, *3.1* × 10^{-5} M *phenylmercuric nitrate; and* ○, *6.1* × 10^{-5} M *thimerosal.*

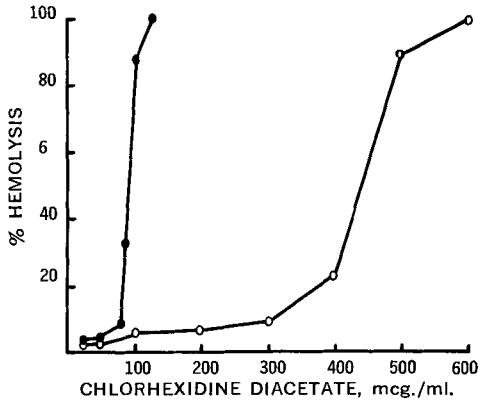


Figure 7—*Influence of DMSO on the hemolytic activity of chlorhexidine diacetate. Key:* ●, *chlorhexidine diacetate in 0.6% NaCl; and* ○, *chlorhexidine diacetate in 0.6% NaCl and 15% DMSO.*

the flocculation of the blood's components, and the precipitation of the hemoglobin from laked erythrocytes. Huggins (4) reported on his observations of the interaction between DMSO and human plasma proteins, noting that true denaturation of plasma protein seems to occur at DMSO concentrations approximating 50%. He stated that precipitation of protein by DMSO occurred with individual plasma fractions—albumin, fibrinogen, gamma globulin—and appears to be a general effect rather than denaturation of a specific plasma component.

At the time it was reported (1) that DMSO interfered with the hemolytic activity of phenol, it was suggested that such an effect might be due to either an extracellular interaction between the two chemical agents, or due to some cellular action of DMSO which retards the ability of phenol to exert its cytotoxic activity.

Since the present work demonstrates that greatly different molar concentrations of the various chemical types of preservative agents are hindered in their effort to destroy the integrity of the erythrocyte by a comparatively large and consistant level of DMSO, it would seem to suggest that the mode of this activity is cellular-based rather than by virtue of extracellular chemical interactions between DMSO and the preservative agents. This hypothesis was supported by the spectral analysis of combinations of DMSO with benzalkonium chloride, chlorhexidine diacetate, and *m*-cresol, which failed to indicate any sign of chemical association between DMSO and the preservatives.

Influence of Preservative Concentration—Figure 6 depicts the pattern generally followed as DMSO is added in increasing amounts to various hemolytic concentrations of a preservative agent. It can be seen that the effectiveness of DMSO in causing a reduction in hemolysis is decreased as the concentration of preservative is increased. This pattern was found for the other preservatives studied, although in instances in which the range of concentration of preservative used was narrow (because of the slight difference in the quantity required to cause trace, intermediate, and total hemolysis) some of the data collected resulted in overlapping and crossing curves when plotted.

Figure 7 shows the influence of 15% DMSO on the hemolytic activity of various concentrations of chlorhexidine diacetate. In the absence of DMSO, chlorhexidine diacetate induces trace hemolysis at 3.2 × 10^{-5} *M* (20 mcg./ml.) concentration and total hemolysis at levels of 1.9 × 10^{-4} *M* (120 mcg./ml.). Dimethyl sulfoxide is effective in keeping the level of hemolysis depressed at chlorhexidine diacetate concentrations below about 6.4 × 10^{-4} *M* (400 mcg./ml.), but is ineffective at higher preservative levels.

The data of Figs. 6 and 7 convincingly show that although DMSO is effective in depressing the level of hemolysis induced by low hemolytic levels of preservative agents, it is ineffective against preservative concentrations of great hemolytic potency. The difference in the concentration of a preservative required to cause total hemolysis compared to that amount needed to cause trace hemolysis in the presence of DMSO does not seem to contribute to the molar strength sufficiently to suggest that the comparatively massive proportion of DMSO present would be any less able to associate in complexation with the greater amount than with the lower preservative level. Thus, the data would seem to suggest that a cellular-based mechanism for the action of DMSO in preventing preservative-induced hemolysis is more likely than an extracellular drug–drug interaction mechanism.

Kinetic Studies—In order to determine if the effect of DMSO in lowering the level of preservative-induced hemolysis was time-related, experiments were conducted using a constant preservative concentration and several levels of DMSO, with hemolytic activity

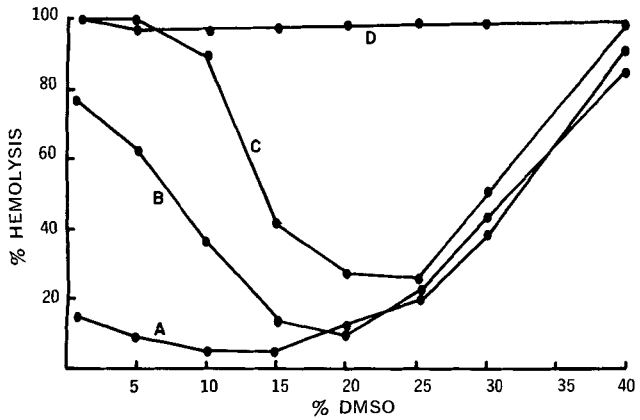


Figure 6—*Influence of DMSO on the hemolytic activity of benzalkonium chloride in the presence of 0.6% NaCl. Key:* A, *2.2* × 10^{-6} M *benzalkonium chloride;* B, *3.3* × 10^{-5} M *benzalkonium chloride;* C, *4.2* × 10^{-5} M *benzalkonium chloride; and* D, *6.5* × 10^{-5} M *benzalkonium chloride.*

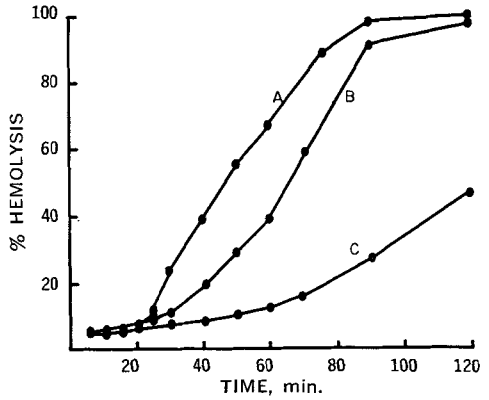


Figure 8—*Influence of time and DMSO on the hemolytic activity of benzalkonium chloride. Key:* A, *3.3* × 10^{-5} M *benzalkonium chloride in 0.6% NaCl;* B, *3.3* × 10^{-5} M *benzalkonium chloride in 0.6% NaCl and 5% DMSO; and* C, *3.3* × 10^{-5} M *benzalkoninm chloride in 0.6% NaCl and 15% DMSO.*

determined at intervals of from 5 to 20 min. during periods up to 120 min.

As can be seen in Fig. 8, the addition of DMSO to solutions of 3.3×10^{-5} *M* benzalkonium chloride in saline slows the rate of hemolysis, with 15% concentrations of DMSO being more effective than 5% concentrations in this effort. Thus, whereas earlier data seemed to indicate that 15% DMSO actually had the ability to reduce or even prevent preservative-induced hemolysis, the present results show that it is a time-related phenomenon. The same pattern of data was obtained in experiments using DMSO–phenol and DMSO–chlorhexidine diacetate systems. This would seem to reinforce the premise that DMSO exerts a cellular effect on the erythrocyte which results in a reduction in the rate of preservative-induced hemolysis.

Work soon to be reported from this laboratory shows that erythrocytes take up DMSO from solution and resist attempts to remove it by washing. Gerhards and Gibian (5) found that in human blood about 30% of injected DMSO is bound to plasma protein and 25% to the formed elements of the blood, the remainder remaining free. Thus, it is not unlikely that the attachment of DMSO to the erythrocyte in some manner affects the vulnerability of the cell toward the preservative agents or alters the permeability barriers of the cell affecting the permeation of the preservative agents into the cell or the exit of hemoglobin from the cell.

Spectral Studies—Preliminary studies of the UV absorption spectra of three preservatives, benzalkonium chloride, chlorhexidine diacetate, and *m*-cresol, alone and in combination with DMSO, were performed to indicate whether or not there is a likelihood of a chemical interaction between DMSO and the preservative agents. The preservatives and DMSO were studied at concentration combinations found to be most inhibitory on preservative-induced hemolysis during the present work. Preliminary data suggest that complexes were not formed. However, more complete studies to rule out complex formation need yet to be made.

In conclusion, the evidence collected more strongly suggests that DMSO interferes with the hemolytic activity of antimicrobial preservatives through a direct action on the erythrocyte or by alteration of its permeability barriers rather than by chemical interaction with the various preservative agents. Further, the degree of this interference is dependent upon the concentrations of both the preservative and DMSO and also upon the length of exposure to these agents.

REFERENCES

(1) H. C. Ansel and W. F. Leake, *J. Pharm. Sci.*, **55**, 685(1966).

(2) H. C. Ansel and D. E. Cadwallader, *ibid.*, **53**, 169(1964).

(3) T. S. Grosicki and W. J. Husa, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 632(1954).

(4) C. E. Huggins, *Transfusion*, **3**, 483(1963).

(5) E. Gerhards and H. Gibian, *Ann. N. Y. Acad. Sci.*, **141**, 65 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 10, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication November 21, 1969.

This paper is based in part on the manuscript submitted by Gary E. Cabre which received first prize in the Southern Section in the 1967 Undergraduate Lunsford Richardson Pharmacy Awards competition.

* Participant, 1967, Undergraduate Research Program, School of Pharmacy, University of Georgia, Athens, GA 30601

Interaction of Amine Drugs with a Polycarboxylic Acid Ion-Exchange Resin

SAUL BORODKIN and MARTIN H. YUNKER

Abstract □ The interaction of polacrilin potassium, the salt of a polycarboxylic acid ion-exchange resin, with 11 amine drugs was studied. All drugs showed maximum interaction at pH 4.5–5.5. Tertiary amines exhibited a much greater affinity for the resin than primary, secondary, and quaternary amines. Selectivity coefficients were used to express the degree of interaction, with experiments showing that these values remain constant over wide variations in resin, drug, and alkali metal concentrations. Rate studies demonstrated that both adsorption of drug onto the resin and elution from the resin are very rapid. The rapid elution rates, along with decreasing resin affinity for amine drugs above pH 6.0, indicate that the presence of polacrilin potassium in a dosage form should not affect total drug availability in the gastrointestinal tract.

Keyphrases □ Polacrilin potassium—interaction, amine drugs □ Amine drugs—interaction, polycarboxylic acid ion-exchange resin □ Ion-exchange chromatography—separation □ UV spectrophotometry—analysis

The use of synthetic polycarboxylic ion-exchange resins in pharmacy and medicine has been quite extensive. Their use in congestive heart failure and edema (1), isolation and purification of streptomycin and other drugs (2), and analysis of drugs (3) is well documented. Adsorbates of amine drugs with carboxylic ion-exchange resins for sustained release (4–7) and taste coverage (8) have been prepared, although to a lesser extent than complexes with sulfonic acid resins.

Because of their unusually large swelling capacities, polymethacrylic carboxylic acid ion-exchange resins have found usage in pharmacy as tablet disintegrants. Van Abbe and Rees (9) reported on the effectiveness of polacrilin potassium[1] as a disintegrating agent, while a later patent (10) describes the use of a similar resin in the acidic form for the same purpose. Being cation exchangers, however, these insoluble polymers have the capability of adsorbing amine drugs, thus possibly interfering with drug availability and assay. This potential incompatibility may have limited the use of these resins as tablet disintegrants to some extent.

The present investigation was undertaken to study the interaction between amine drugs and polacrilin potassium. Eleven commonly used drugs were selected to include primary, secondary, tertiary, and quaternary

[1] Amberlite IRP-88, Rohm and Haas Co., Philadelphia, Pa. Previously sold as Amberlite XE-88.

determined at intervals of from 5 to 20 min. during periods up to 120 min.

As can be seen in Fig. 8, the addition of DMSO to solutions of 3.3×10^{-5} *M* benzalkonium chloride in saline slows the rate of hemolysis, with 15% concentrations of DMSO being more effective than 5% concentrations in this effort. Thus, whereas earlier data seemed to indicate that 15% DMSO actually had the ability to reduce or even prevent preservative-induced hemolysis, the present results show that it is a time-related phenomenon. The same pattern of data was obtained in experiments using DMSO–phenol and DMSO–chlorhexidine diacetate systems. This would seem to reinforce the premise that DMSO exerts a cellular effect on the erythrocyte which results in a reduction in the rate of preservative-induced hemolysis.

Work soon to be reported from this laboratory shows that erythrocytes take up DMSO from solution and resist attempts to remove it by washing. Gerhards and Gibian (5) found that in human blood about 30% of injected DMSO is bound to plasma protein and 25% to the formed elements of the blood, the remainder remaining free. Thus, it is not unlikely that the attachment of DMSO to the erythrocyte in some manner affects the vulnerability of the cell toward the preservative agents or alters the permeability barriers of the cell affecting the permeation of the preservative agents into the cell or the exit of hemoglobin from the cell.

Spectral Studies—Preliminary studies of the UV absorption spectra of three preservatives, benzalkonium chloride, chlorhexidine diacetate, and *m*-cresol, alone and in combination with DMSO, were performed to indicate whether or not there is a likelihood of a chemical interaction between DMSO and the preservative agents. The preservatives and DMSO were studied at concentration combinations found to be most inhibitory on preservative-induced hemolysis during the present work. Preliminary data suggest that complexes were not formed. However, more complete studies to rule out complex formation need yet to be made.

In conclusion, the evidence collected more strongly suggests that DMSO interferes with the hemolytic activity of antimicrobial preservatives through a direct action on the erythrocyte or by alteration of its permeability barriers rather than by chemical interaction with the various preservative agents. Further, the degree of this interference is dependent upon the concentrations of both the preservative and DMSO and also upon the length of exposure to these agents.

REFERENCES

(1) H. C. Ansel and W. F. Leake, *J. Pharm. Sci.*, **55**, 685(1966).

(2) H. C. Ansel and D. E. Cadwallader, *ibid.*, **53**, 169(1964).

(3) T. S. Grosicki and W. J. Husa, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 632(1954).

(4) C. E. Huggins, *Transfusion*, **3**, 483(1963).

(5) E. Gerhards and H. Gibian, *Ann. N. Y. Acad. Sci.*, **141**, 65 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 10, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication November 21, 1969.

This paper is based in part on the manuscript submitted by Gary E. Cabre which received first prize in the Southern Section in the 1967 Undergraduate Lunsford Richardson Pharmacy Awards competition.

* Participant, 1967, Undergraduate Research Program, School of Pharmacy, University of Georgia, Athens, GA 30601

Interaction of Amine Drugs with a Polycarboxylic Acid Ion-Exchange Resin

SAUL BORODKIN and MARTIN H. YUNKER

Abstract □ The interaction of polacrilin potassium, the salt of a polycarboxylic acid ion-exchange resin, with 11 amine drugs was studied. All drugs showed maximum interaction at pH 4.5–5.5. Tertiary amines exhibited a much greater affinity for the resin than primary, secondary, and quaternary amines. Selectivity coefficients were used to express the degree of interaction, with experiments showing that these values remain constant over wide variations in resin, drug, and alkali metal concentrations. Rate studies demonstrated that both adsorption of drug onto the resin and elution from the resin are very rapid. The rapid elution rates, along with decreasing resin affinity for amine drugs above pH 6.0, indicate that the presence of polacrilin potassium in a dosage form should not affect total drug availability in the gastrointestinal tract.

Keyphrases □ Polacrilin potassium—interaction, amine drugs □ Amine drugs—interaction, polycarboxylic acid ion-exchange resin □ Ion-exchange chromatography—separation □ UV spectrophotometry—analysis

The use of synthetic polycarboxylic ion-exchange resins in pharmacy and medicine has been quite extensive. Their use in congestive heart failure and edema (1), isolation and purification of streptomycin and other drugs (2), and analysis of drugs (3) is well documented. Adsorbates of amine drugs with carboxylic ion-exchange resins for sustained release (4–7) and taste coverage (8) have been prepared, although to a lesser extent than complexes with sulfonic acid resins.

Because of their unusually large swelling capacities, polymethacrylic carboxylic acid ion-exchange resins have found usage in pharmacy as tablet disintegrants. Van Abbe and Rees (9) reported on the effectiveness of polacrilin potassium[1] as a disintegrating agent, while a later patent (10) describes the use of a similar resin in the acidic form for the same purpose. Being cation exchangers, however, these insoluble polymers have the capability of adsorbing amine drugs, thus possibly interfering with drug availability and assay. This potential incompatibility may have limited the use of these resins as tablet disintegrants to some extent.

The present investigation was undertaken to study the interaction between amine drugs and polacrilin potassium. Eleven commonly used drugs were selected to include primary, secondary, tertiary, and quaternary

[1] Amberlite IRP-88, Rohm and Haas Co., Philadelphia, Pa. Previously sold as Amberlite XE-88.

Table I—Drugs Used in Study

Drug	pKa[a]	$\lambda_{max.}$ (0.08 *N* HCl), mμ
Phenylpropanolamine hydrochloride	9.4	257
Ephedrine base	9.5	257
Pseudoephedrine hydrochloride	9.7	257
Desoxyephedrine hydrochloride	9.5	257
Carbinoxamine maleate	8.1	262
Quinidine sulfate	8.8	280
Methapyrilene hydrochloride	8.8	312
Chromonar hydrochloride	8.3	320
Dextromethorphan hydrobromide	8.3	278
Thiamine mononitrate	—[b]	245
Neostigmine bromide	—[b]	260

[a] The pKa's for carbinoximine and chromonar were determined in this laboratory by potentiometric titration while the others were obtained from the literature. Only the pKa for the most basic amine group in the molecule is listed. [b] Thiamine and neostigmine are quaternary amines.

amines. Although the primary goal of this study was to assess the extent of the potential incompatability in regard to the use of carboxylic acid resins as disintegrating agents, it was believed that the approach taken and information generated might also be useful for other ion-exchange resin applications.

EXPERIMENTAL

Resin—Polacrilin potassium is the potassium salt of a weakly acidic cation-exchange resin with a polymethacrylic acid–divinylbenzene matrix having a particle size range of 100–500 mesh. A single lot was used in all experiments. Assays showed a potassium content of 5.38 meq./g., a total exchange capacity of 6.97 meq./g., and a water content of 18.0% (Karl Fischer).

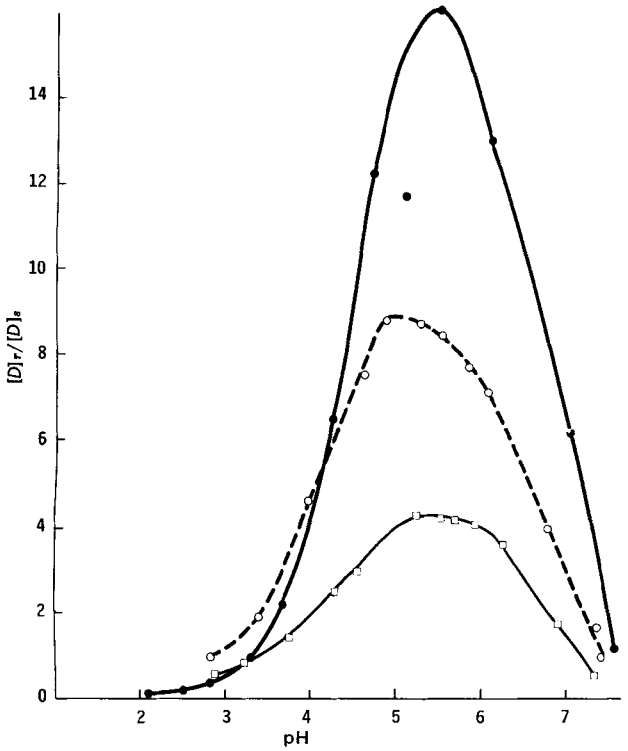


Figure 1—*Effect of pH on drug distribution ratio. Key: ●, quinidine sulfate (1 mg./ml.) + polacrilin K (10 mg./ml.); ○, dextromethorphan HBr (1 mg./ml.) + polacrilin K (10 mg./ml.); and □, dextromethorphan HBr (1 mg./ml.) + polacrilin K (5 mg./ml.).*

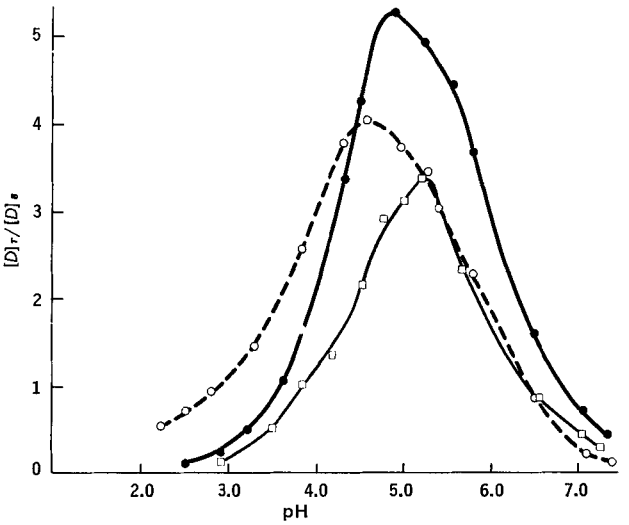


Figure 2—*Effect of pH on drug distribution ratio. Key: ●, methapyrilene HCl (1 mg./ml.) + polacrilin K (10 mg./ml.); ○, chromonar HCl (1 mg./ml.) + polacrilin K (10 mg./ml.); and □, carbinoxamine maleate (0.25 mg./ml.) + polacrilin K (2.5 mg./ml.).*

Drugs—Table I lists the drugs used in this study. Other salt forms would be expected to behave similarly. The UV absorbance maxima used in their analysis and the pKa's are included.

Equilibrium Studies—To establish the relationship between interaction of a drug and pH, a series of 12–14 solutions was prepared to contain 200 mg. drug (except for carbinoxamine maleate where 50 mg. was used), 1.8 g. of sodium chloride (isotonic saline concentration), and various predetermined amounts of hydrochloric acid in 200 ml. of solution. Each solution was added to a 300-ml. bottle along with constant amount (0.5–5.0 g. depending on the drug) of polacrilin potassium, and the bottles were rolled at 24–25°. To improve accuracy, the resin–drug ratios in these experiments were much higher than would normally be used in tablets. After 24 hr. the pH was determined, the resin was separated by filtration, and the drug concentration in the filtrate was determined by UV absorbance using a Beckman DU spectrophotometer after the required dilution with 0.08 *N* HCl. In the case of chromonar, the samples were drawn after 4 hr. as a precaution because of the drug's reported instability. Rate studies with other drugs indicated that adsorption should be close to equilibrium after that time period.

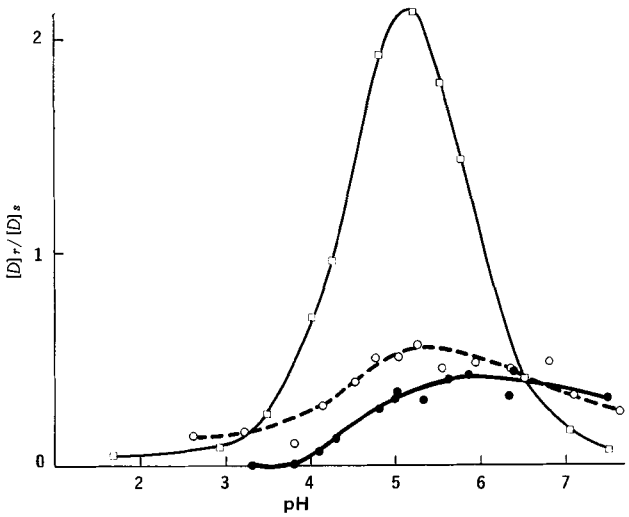


Figure 3—*Effect of pH on drug distribution ratio. Key: ●, phenylpropanolamine HCl (1 mg./ml.) + polacrilin K (25 mg./ml.); ○, ephedrine (1 mg./ml.) + polacrilin K (25 mg./ml.); and □, thiamine mononitrate (1 mg./ml.) + polacrilin K (10 mg./ml.).*

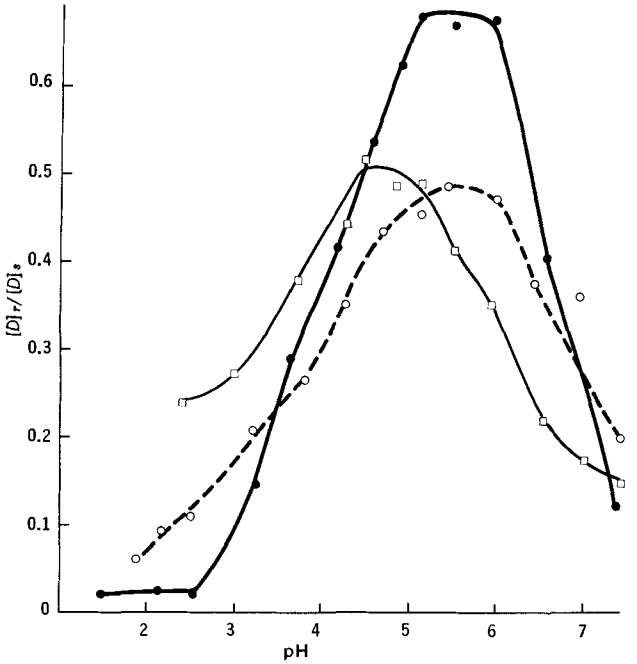


Figure 4—*Effect of pH on drug distribution ratio. Key:* ●, *desoxyephedrine HCl (1 mg./ml.) + polacrilin K (25 mg./ml.);* ○, *pseudoephedrine HCl (1 mg./ml.) + polacrilin K (25 mg./ml.); and* □, *neostigmine bromide (1 mg./ml.) + polacrilin K (10 mg./ml.).*

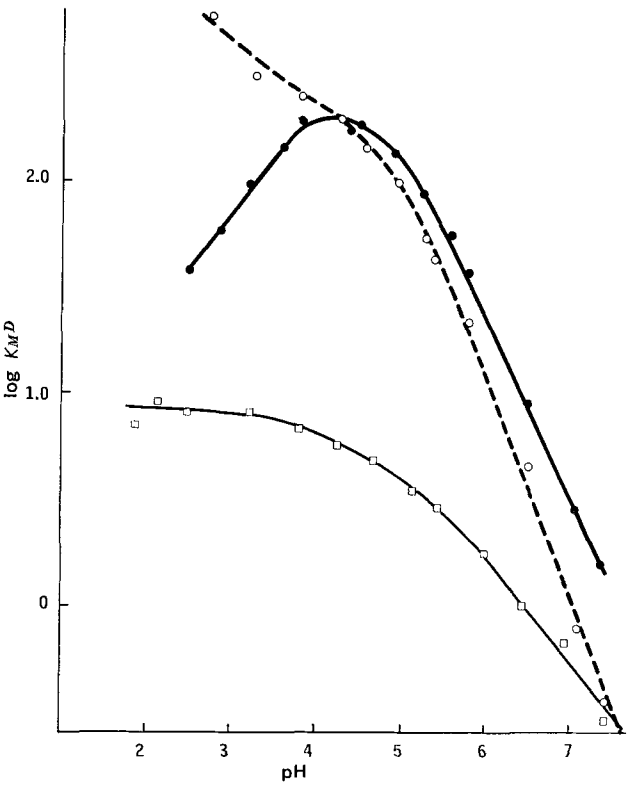


Figure 6—*Effect of pH on the selectivity coefficient. Key:* ●, *methapyrilene;* ○, *chromonar; and* □, *pseudoephedrine.*

The procedures used to determine the effects of alkali metal, resin, and drug concentrations were identical to the above except for varying concentrations of the component being studied. To ensure the constant pH required in each series, fine adjustments were made with HCl after 4 and 6 hr.

In the temperature study experiments, the equilibrations were run in flasks equipped with mechanical stirrers and submerged in constant-temperature baths.

Relationship between Degree of Resin Dissociation, α, and pH (Fig. 5)—A series of 13 solutions was prepared to contain 1.8 g. of sodium chloride and various predetermined amounts of HCl (for an equilibrium pH range of 2.5–7.5) in 200 ml. of solution. Each solution was added to a 300-ml. bottle along with 2.0 g. of polacrilin potassium and the bottles were rolled at 24–25° as in the equilibrium studies. After 24 hr., the pH of each mixture was determined and the resin was separated from the solution phase by filtration. The resin was then reslurried in 200 ml. of 1 *N* HCl for 2 hr. to elute the cations that had remained in the resin phase. Aliquot samples (20 ml.) were pipeted from both the equilibrium and acid elution solutions and evaporated to constant weight under an IR lamp. Since the solids were a mixture of sodium and potassium chlorides in the molar ratio 154 to 54, the weight concentrations were converted to equivalents per liter by dividing by the weighted average molecular weight (62.63). The concentrations in the equilibrium and elution solutions were equal to $[M]_s$ and $[M]_r$, respectively. Their sum varied from 205–209 meq./l. (theoretical 208) at the 13 different pH's. The fraction of resin in the dissociated form, α, was obtained by dividing $[M]_r$ by the total exchange capacity of the polacrilin (69.7 meq./l.).

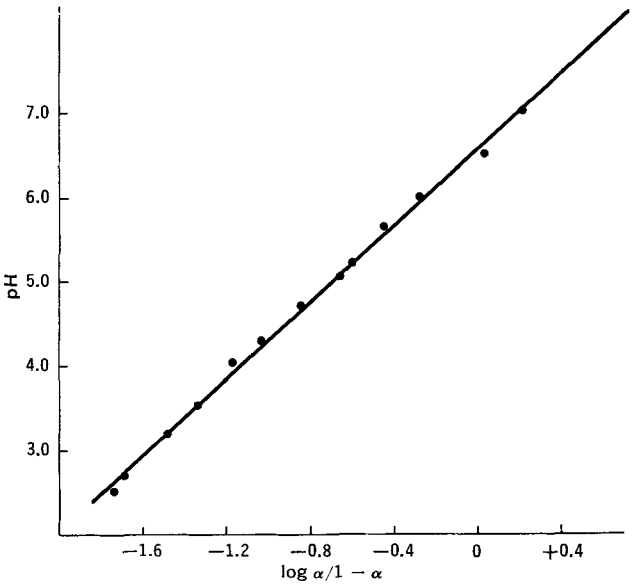


Figure 5—*Relationship between pH and polacrilin K dissociation in 0.154* M *NaCl* (n = *2.28, pKa = 6.60*).

Rate Studies—In the adsorption rate studies, 500 ml. of buffer containing 0.154 eq. of sodium was prepared by dissolving 1.94 g. of dibasic sodium phosphate and 19.2 g. of monobasic sodium phosphate in water. Ten grams of polacrilin potassium was added and the pH of the slurry was adjusted to 5.50–5.60 with HCl over a 4-hr. period. One gram of drug was dissolved in 500 ml. of water and the solution was added to initiate adsorption. The slurry was

Table II—Selectivity Coefficients (K_M^D) at Various pH's

Drug	pH 4.5	pH 5.0	pH 5.5	pH 6.0
Phenylpropanolamine	2.48	2.67	2.13	1.45
Desoxyephedrine	7.44	5.81	3.80	2.38
Ephedrine	5.37	4.49	2.77	1.72
Pseudoephedrine	5.55	3.87	2.61	1.64
Carbinoxamine	267	236	131	49.2
Chromonar	151	77.7	17.1	15.4
Quinidine	353	308	208	116
Methapyrilene	179	122	61.9	24.4
Dextromethorphan	276	196	112	68.6
Thiamine	52.5	44.3	23.7	9.03
Neostigmine	6.42	4.22	2.22	1.14

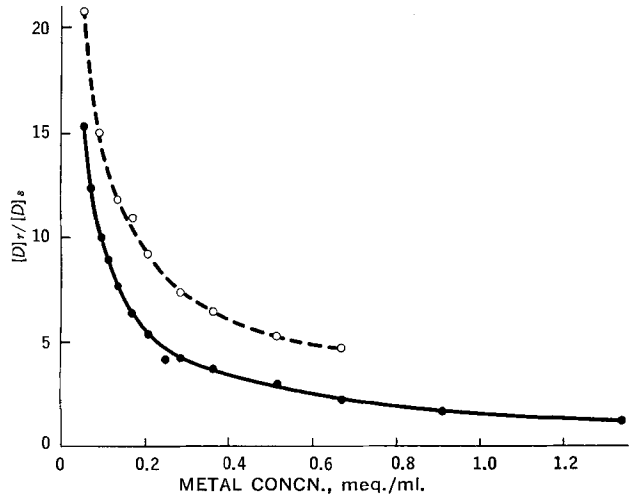


Figure 7—*Effect of alkali metal concentration on drug distribution ratio. Key:* ●, *methapyrilene HCl (1 mg./ml.), polacrilin K (10 mg./ml.), NaCl (0–1.283 meq./ml.), pH 5.3; and* ○, *dextromethorphan HBr (1 mg./ml.), polacrilin K (10 mg./ml.), NaCl (0–0.616 meq./ml.), pH 5.1.*

stirred at 25° for 4 days with methapyrilene and dextromethorphan and for 2 weeks in the case of carbinoxamine and quinidine. Ten-milliliter samples were removed at 2, 5, 10, and 30 min.; at 1, 4, and 24 hr.; and at the end of the run. The solution phase was filtered from the resin and assayed for drug by UV spectroscopy.

In the elution rate studies, 1 g. of methapyrilene hydrochloride, 1.94 g. of dibasic sodium phosphate, and 19.2 g. of monobasic sodium phosphate were dissolved in enough water to make 1 l. Ten grams of polacrilin potassium was added, the pH was adjusted to 5.5 with HCl, and the slurry was stirred for 24 hr. at 25°. The resin, containing approximately 82.0% of the drug, was filtered from the solution and added to 1 l. of fresh buffer solution containing 0.154 meq./ml. of sodium. The pH 6.8 elution-rate run used a buffer containing 18.8 g. of dibasic and 1.86 g. of monobasic sodium phosphate. The buffer in the pH 5.4 study contained 1.94 g. of dibasic and 19.2 g. of monobasic sodium phosphate. Stirring was continued at 25° for 24 hr. with samples being removed after 2, 5, 10, and 30 min. and after 1, 3, and 24 hr. The solution phase was filtered from the resin and assayed for drug by UV spectroscopy.

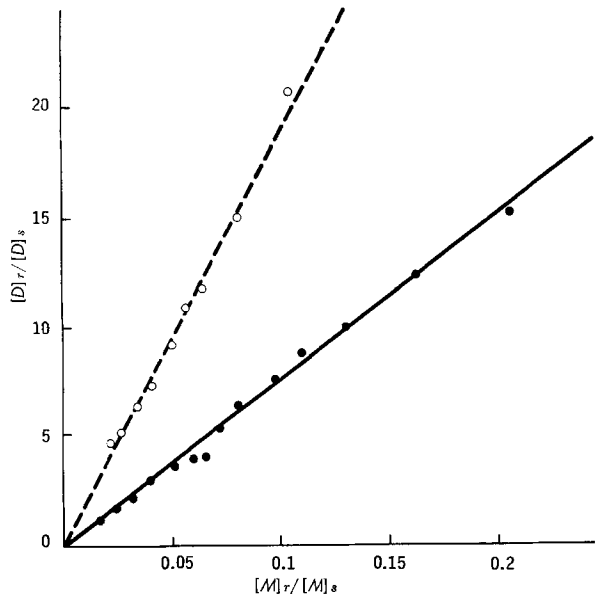


Figure 8—*Relationship of alkali metal distribution ratio to drug distribution ratio. Key:* ●, *methapyrilene at pH 5.3* (K_M^D = *77.2); and* ○, *dextromethorphan at pH 5.1* (K_M^D = *195).*

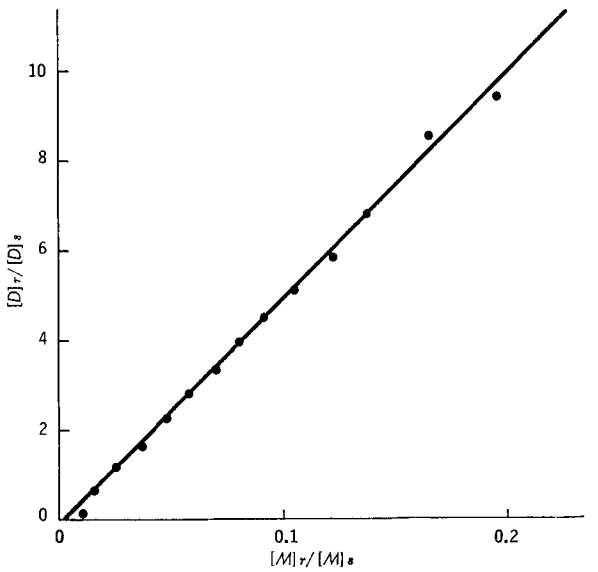


Figure 9—*Effect of polacrilin potassium concentration on drug distribution ratio, methapyrilene HCl (1 mg./ml.), KCl (0.154 meq./ml.), polacrilin K (1.25–30.0 mg./ml.), pH 5.4,* K_M^D = *50.4.*

RESULTS AND DISCUSSION

Effect of pH—Figures 1–4 show the effect of pH on the interaction at equilibrium between polacrilin potassium and the eleven drugs tested. Drug interaction is expressed as a distribution ratio, drug in resin phase, $[D]_r$, divided by drug in solution phase, $[D]_s$, both expressed as meq./ml. of solution. Although the extent of interaction varied considerably with the drug, in all cases the ratio showed a maximum at pH 4.5–5.5. The sharp decrease in complexation below pH 4.5 is apparently caused by the diminishing number of anionic sites on the resin due to its low dissociation constant. The decreasing interaction above pH 5.5, despite the greater ionization of the resin, can be attributed to a decreasing affinity of the polacrilin for amine drugs relative to alkali metal cations. The increasing portion of drug in the unionized form may contribute to this.

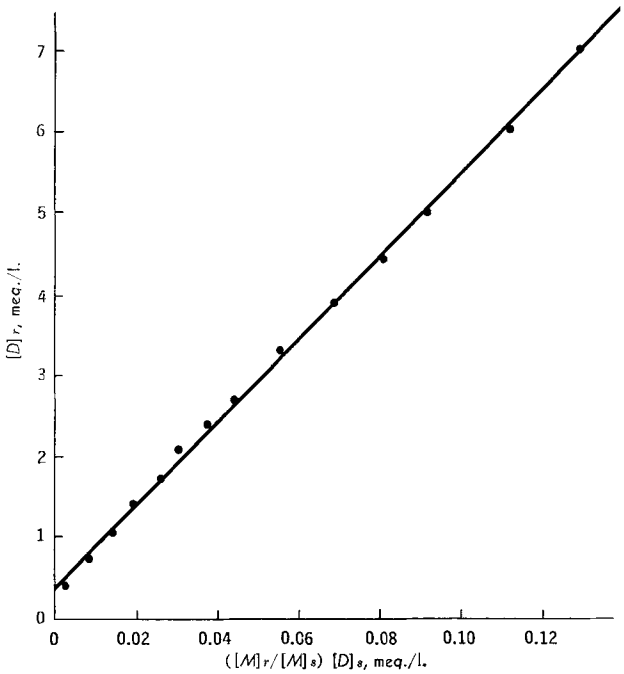


Figure 10—*Effect of drug concentration on interaction, methapyrilene HCl (0.42–10.08 meq./l.), polacrilin K (10 mg./ml.), NaCl (0.154 meq./ml.), pH = 5.4,* K_M^D = *50.9.*

Table III—Rates of Equilibration at 25°

Drug	Process	pH	% Drug in Resin Phase after— 2 min.	1 hr.[a]	4 hr.	24 hr.	Final
Methapyrilene	Adsorption	5.5	71.7	79.8	80.7	82.0	82.3[b]
Dextromethorphan	Adsorption	5.6	64.5	75.9	77.1	79.2	79.2[b]
Carbinoxamine	Adsorption	5.5	86.9	93.2	95.1	95.1	94.8[c]
Quinidine	Adsorption	5.5	80.3	86.0	87.9	89.7	89.6[c]
Methapyriline	Elution	6.8	68.7	64.9	62.1[d]	63.4	—
Methapyrilene	Elution	5.4	90.5	89.5	89.1[d]	89.0	—

[a] Results of samples at 5, 10, and 30 min. were all intermediate between the 2-min. and 1-hr. percentages. [b] Sampled after 4 days. [c] Sampled after 3 weeks. [d] Sampled at 3 hr.

Although the drug distribution ratio is a convenient term for expressing drug interaction, it will vary with drug, resin, and sodium ion concentrations, as well as with pH. This is demonstrated in Fig. 1 where two different resin concentrations were used with dextromethorphan. Samuelson (3) suggests the use of a selectivity coefficient to express the relative affinities of resins for different cations. In this study the selectivity coefficient, $K_M{}^D$, would be defined by the following equation:

$$K_M{}^D = \frac{[D]_r\,[M]_s}{[D]_s\,[M]_r} \qquad \text{(Eq. 1)}$$

where $[M]_s$ and $[M]_r$ represent the concentrations, in meq./ml., of alkali metal in the solution and resin phases, respectively. The alkali metal concentration includes both the sodium from added sodium chloride and potassium contributed by the polacrilin potassium, with the sodium ion predominating. The selectivity coefficients would be expected to remain constant with variations in drug, resin, and metal concentrations, although it does change with pH.

Calculations of selectivity coefficients from the distribution ratios found in Figs. 1–4 require accurate estimations of $[M]_r$ ($[M]_s$ can be obtained by difference once $[M]_r$ is known). Since the meq. of alkali metal in the resin phase is approximately equal to the meq. of ionized sites on the resin, less those occupied by drug cations, the following equation may be written:

$$[M]_r = \alpha[Re] - [D]_r \qquad \text{(Eq. 2)}$$

where α is the fraction of total resin equivalents in the dissociated form while $[Re]$ is the resin concentration expressed in meq. total exchange capacity per ml. of solution. Gustafson (11) has demonstrated that polymethacrylic carboxylic ion-exchange resins follow the empirical relationship:

$$\text{pH} = \text{pKa} + n \log \frac{\alpha}{1-\alpha} \qquad \text{(Eq. 3)}$$

where pKa is an apparent dissociation constant which changes with ionic strength, while n is a second constant specific for the resin and independent of ionic strength. The linear relationship obtained between pH and log $\alpha/1-\alpha$ (Fig. 5) shows that polacrilin potassium follows Eq. 3. The values from this experiment allow calculation of selectivity coefficients from the drug distribution ratios shown in Figs. 1–4 using Eqs. 1–3.

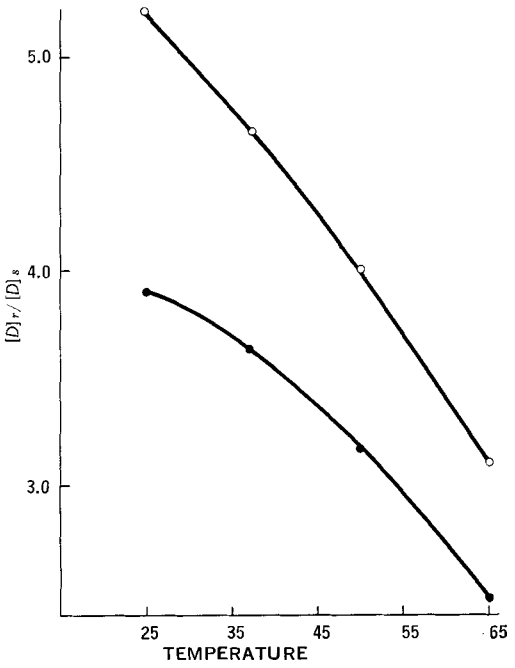


Figure 11—*Effect of temperature on the drug distribution ratio, methapyrilene HCl (1 mg./ml.), polacrilin K (10 mg./ml.), NaCl (0.154 meq./ml.). Key: ● pH 4.6, and ○, pH 5.3.*

The relationship between the selectivity coefficients and hydrogen ion concentration was obtained by plotting log $K_M{}^D$ *versus* pH for each drug. Figure 6 shows some typical results. In all cases the relationship appeared linear between pH 5.5 and 7.5 with slopes of 0.5–1.0. This linearity indicates that the selectivity coefficient is proportional to a power (equal to the slope) of the hydrogen ion concentration. Results below pH 5.5 were inconsistent in that maxima, levelings, and nonlinear increases were observed in the graphs.

Comparison of Drugs—Table II shows the selectivity coefficients obtained for each drug at pH 4.5, 5.0, 5.5, and 6.0. Since all drugs should be essentially in the cationic form at the pH's used, the considerable variations in selectivity coefficients between drugs cannot be attributed to their dissociation constants. The differences are more likely related to steric and resonance effects within the drug cation. Nevertheless the magnitude of the selectivity coefficients can be related to the degree of nitrogen substitution. The five tertiary amine drugs (dextromethorphan, methapyrilene, quinidine, carbinoxamine, and chromonar) showed substantially greater interaction than the other six drugs. At pH 5.0, the average selectivity coefficient for the tertiary amines was approximately 40 times greater than the average for the three secondary amine drugs (ephedrine, pseudoephedrine, and desoxyephedrine). Phenylpropanolamine, the only primary amine studied, showed the smallest interaction. The two quaternary amine drugs varied somewhat in their behavior. Neostigmine gave a selectivity coefficient similar to the secondary amine drugs while the value for thiamine was intermediate between secondary and tertiary amines.

Effects of Alkali Metal, Resin, and Drug Concentrations—The relationship between the drug distribution ratio and the sodium ion concentration is shown in Fig. 7 for methapyrilene and dextromethorphan. Similar curves were obtained with the other drugs tested. To show that these results are consistent with the selectivity coefficient relationship (Eq. 1), the drug distribution ratios were plotted against the calculated alkali metal ratios (Fig. 8). Since Eq. 1 may be rearranged to

$$\frac{[D]_r}{[D]_s} = K_M{}^D \frac{[M]_r}{[M]_s} \qquad \text{(Eq. 4)}$$

the linearity obtained demonstrates that the selectivity coefficients remain constant over a wide alkali metal concentration range. $[M]_r$ and $[M]_s$ were calculated from Eqs. 2 and 3, with the variability of pKa with ionic strength being estimated from Gustafson's relationship (11).

Experiments in which the potassium ion concentration was varied gave similar results to those obtained with sodium ion variation. Potassium gave approximately the same selectivity coefficients. The similarity indicates that small changes in the potassium–sodium

ratio within a system should have negligible effect on drug distribution ratios.

Figure 9 shows the effect of polacrilin potassium concentration on the methapyrilene distribution ratio. The distribution ratio was plotted against the calculated $[M]_r/[M]_s$ to attain the linearity predicted by Eq. 4. The results show that the selectivity coefficient also remains constant over a wide range of resin concentration.

In evaluating the effect of drug concentration, a plot of the methapyrilene distribution ratio *versus* $[M]_r/[M]_s$ showed deviation from linearity at low drug concentration. However a straight line was obtained when the concentration of resin-adsorbed drug, $[D]_r$, was plotted against $[D]_s[M]_r/[M]_s$ (Fig. 10). Although the linearity is consistent with Eq. 1, the intercept is not. Apparently the total drug adsorbed is the sum of that adsorbed according to the selectivity coefficient relationship plus a constant:

$$[D]_r = K_M{}^D \frac{[M]_r}{[M]_s}[D]_s + \text{constant} \qquad \text{(Eq. 5)}$$

This constant, which becomes significant only at very low drug–resin ratios, can be attributed to a small quantity of drug bound to the resin by forces other than ionic bonding.

Effect of Temperature—The effect of temperature on the methapyrilene distribution ratio at two different pH's is shown in Fig. 11. The decrease in drug–resin interaction with rising temperature is in agreement with Samuelson's generalization (3) that an increase in temperature results in a decrease in ion selectivity.

Rate of Equilibration—Although the large size of the drug cations might be expected to affect exchange kinetics, the rate of equilibration was found to be quite rapid, both for adsorption and elution. Table III summarizes the results of rate studies at 25°. The lack of significant change after 24 hr. in the adsorption runs emphasizes the fact that equilibrium is attained in that time.

The rapid exchange rates are consistent with the results reported by Kunin (12) for carboxylic ion-exchange resins. He noted that, although the exchange rate in going from acid to metal cycle is slow and may require weeks for completion, attainment of equilibrium in going from one metal cycle to another occurs in minutes or hours. Since the latter process most closely resembles both adsorption onto polacrilin potassium (potassium to drug cycle) and elution from the resin (drug to sodium cycle) the rapid rates are not surprising. Further, the fine particle size of polacrilin potassium might contribute significantly to acceleration of the exchange rates.

The rapid exchange rates along with the selectivity coefficient results indicate that the presence of polacrilin potassium in a dosage form should have insignificant effect on the total drug availability. Although appreciable resin adsorption of drug may occur in the gastrointestinal tract during pH 4.5–6.0 exposure, rapid desorption should result as the adsorbate passes into the higher pH of the small intestine. The decreased selectivity coefficients at higher pH along with the rapid elution rates ensure drug availability. Even in the pH 4.5–6.0 range, continuous absorption of drug into the blood would result in elution from the resin. The higher temperatures that would be encountered in the body compared with the 24–25° data obtained in this study would promote greater drug availability, both through lower selectivity coefficients and faster exchange rates.

SUMMARY AND CONCLUSIONS

1. The interaction between amine drugs and polacrilin potassium follows the selectivity coefficient relationship. It would be expected that other carboxylic acid ion-exchange resins would behave similarly.

2. Tertiary amines show a greater affinity for polacrilin potassium than other amine drugs. In all cases, maximum interaction occurs at pH 4.5–5.5.

3. The rapid elution rates, along with decreasing interaction above pH 6.0, indicate that the presence of polacrilin potassium in a dosage form should not affect total *in vivo* availability. It is questionable whether any significant delay in absorption would occur.

4. The high selectivity coefficients obtained with some drugs indicate that precautions must be taken in assaying for amine drugs in the presence of polacrilin potassium. Buffers above pH 7.0 or below pH 3.0 or solutions with high cation concentrations may be used to effect complete drug elution.

REFERENCES

(1) G. J. Martin, "Ion Exchange and Adsorption Agents in Medicine," Little, Brown, Boston, Mass., 1955, p. 124.

(2) C. Calmon and T. R. E. Kressman, "Ion Exchangers in Organic and Biochemistry," Interscience, New York, N. Y., 1957, p. 502.

(3) O. Samuelson, "Ion Exchange Separations in Analytical Chemistry," Wiley, New York, N. Y., 1963, pp. 58, 85.

(4) W. C. Fiedler and G. J. Sperandio, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 45(1957).

(5) J. B. Ward and G. J. Sperandio, *Amer. Perfum. Cosmet.*, **81**, 23(1966).

(6) D. A. Schlichting, *J. Pharm. Sci.*, **51**, 134(1962).

(7) H. A. Smith, R. V. Evanson, and G. J. Sperandio, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 94(1960).

(8) French pat. 2155M (1963).

(9) N. J. Van Abbe and J. T. Rees, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 487(1958).

(10) V. Coletta and R. B. Wurfield, U.S. pat. 3,091,574 (1963).

(11) R. L. Gustafson, *J. Phys. Chem.*, **68**, 1563(1964).

(12) R. Kunin, "Ion Exchange Resins," Wiley, New York, N. Y., 158, p. 53.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Pharmaceutical Products Division, Abbott Laboratories, North Chicago, IL 60064*

Accepted for publication October 28, 1969.

Effect of Age on Intestinal Absorption of Riboflavin in Humans

WILLIAM J. JUSKO, GERHARD LEVY,* and SUMNER J. YAFFE

Abstract □ The development of gastrointestinal absorption function in humans was studied using riboflavin, a vitamin which is absorbed by a site-specific (proximal small intestine) and saturable transport process. Oral doses of 150 mg./m.² body surface area of riboflavin-5′-phosphate were administered in solution to subjects ranging in age from 0.25 to 40 years. The urinary recovery of the vitamin increased significantly (from 6 to 12% of the dose) over this age range. The ratio of maximum excretion rate to dose and the time of occurrence of the maximum excretion rate were independent of age. The kinetics of riboflavin elimination also did not show any appreciable change with age. These observations suggest that, in the age range studied, younger subjects retain the vitamin at intestinal absorption sites for a shorter period of time than do older subjects. This appears to be due to decreased intestinal transit rate with increasing age. Prompt release of drugs from pharmaceutical dosage forms seems therefore even more important in children than in adults in order to assure adequate absorption.

Keyphrases □ Riboflavin absorption—intestinal □ Age effect—riboflavin intestinal absorption □ Kinetic equations—urinary excretion parameters relationship, gastrointestinal absorption □ Elimination half-life, riboflavin—age effect

There is little biopharmaceutical information available to aid in the design of oral dosage forms for infants and children. Of the various physiologic characteristics which might change with age, those related to gastrointestinal absorption are among the least studied. Several recent reviews have pointed to the need for quantitative studies of intestinal absorption function at various ages (1–3).

In the course of investigations of factors affecting the gastrointestinal absorption of riboflavin (4) and riboflavin-5′-phosphate (5) in man, it was found that this vitamin is absorbed in the proximal small intestine by a saturable transport process. The absorption of riboflavin can therefore serve as an index of gastrointestinal transit rate since absorption only occurs while the vitamin is in the proximal small intestine. Since the gastrointestinal absorption of drugs administered in solid dosage forms can be impaired if the solid drug is not dissolved before or during passage through the small intestine (6), the results of absorption studies with riboflavin are applicable in general terms to other drugs inasmuch as their absorption is affected by the rate of gastrointestinal transit. The use of riboflavin is particularly advantageous in this study involving infants and children since the absorption of this vitamin can be assessed from urinary excretion data (4, 5).

THEORETICAL

In order to understand more clearly the relationship of urinary excretion parameters (time course of excretion rates and urinary recovery) to gastrointestinal factors involved in absorption by a specialized transport process (maximum absorptive capacity and intestinal transit rate), it is useful to evolve mathematical relationships for a simple pharmacokinetic model which is consistent with the proposed mechanism of intestinal absorption.

If a substance is absorbed by a saturable specialized process in the small intestine and the dose administered (X_D) is large enough to saturate the absorption process, then the rate of absorption can be considered to be zero order ($V_{max.}$). Assuming the body to be a single compartment and the rate of elimination to be first order (k_E), the appropriate pharmacokinetic model is:

$$X_A \xrightarrow{V_{max.}} X_B \begin{array}{l} \xrightarrow{k_e} X_U \\ \xrightarrow{k_m} X_M \end{array}$$

Scheme I

where X represents amounts of drug, subscripts refer to amount absorbed (A), amount in the body (B), and amount eliminated *via* urine (U), and by metabolism and/or extrarenal routes (M), and $X_D \gg X_A$. First-order rate constants for the elimination processes are represented by k_e and k_m and:

$$k_E = k_e + k_m \qquad \text{(Eq. 1)}$$

The differential equations which are applicable are:

$$-dX_A/dt = V_{max.} \qquad \text{(Eq. 2)}$$

$$dX_B/dt = V_{max.} - k_E \cdot X_B \qquad \text{(Eq. 3)}$$

$$dX_U/dt = k_e \cdot X_B \qquad \text{(Eq. 4)}$$

While absorption is occurring, solution of these equations for X_B yields:

$$X_B = (V_{max.}/k_E) \cdot [1 - \exp(-k_E \cdot t)] \qquad \text{(Eq. 5)}$$

and from Eqs. 4 and 5, it can be seen that:

$$dX_U/dt = (V_{max.} \cdot k_e/k_E) \cdot [1 - \exp(-k_E \cdot t)] \qquad \text{(Eq. 6)}$$

The maximum amount of drug in the body, $(X_B)_{max.}$, occurs when Eq. 3 equals zero or:

$$(X_B)_{max.} = V_{max.}/k_E \qquad \text{(Eq. 7)}$$

and the corresponding maximum urinary excretion rate, $(dX_U/dt)_{max.}$, from Eqs. 4 and 7 is therefore:

$$(dX_U/dt)_{max.} = V_{max.} \cdot k_e/k_E \qquad \text{(Eq. 8)}$$

The actual peak excretion rate (ER_p) will be proportional to $V_{max.}$ (Eq. 8) only after absorption at $V_{max.}$ has proceeded over a period of at least 4 elimination half-lives, *i.e.*, when:

$$\exp(-k_E \cdot t) \to 0$$

so that Eq. 5 reduces to Eq. 8. If the time of occurrence (t_p) of ER_p is $<4 \cdot t_{1/2}$, then $V_{max.}$ can be determined by rearranging Eq. 6 to yield:

$$V_{max.} = (ER_p \cdot k_E/k_e)/[1 - \exp(-k_E \cdot t_p)] \qquad \text{(Eq. 9)}$$

The amount of drug absorbed (X_A), from Eq. 2, is:

$$X_A = V_{max.} \cdot t_q \qquad \text{(Eq. 10)}$$

where t_q is the time during which drug absorption proceeds. This equation shows that two intestinal factors can modify the amount of drug absorbed under the conditions of the model: (*a*) the maxi-

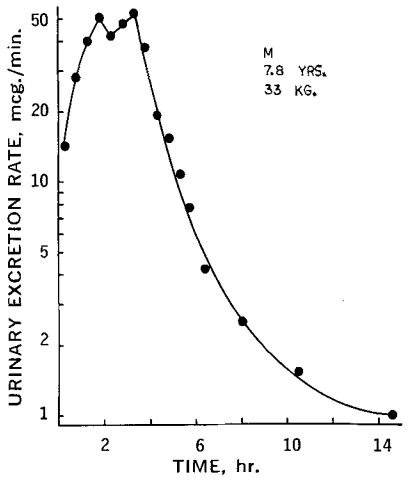


Figure 1—*Urinary excretion rate as a function of time after oral administration of 150 mg./m.² riboflavin to a human subject.*

mum absorption rate ($V_{max.}$), and (*b*) the residence time of drug at the absorption site (t_g). Since the urinary recovery of drug is proportional to the amount absorbed:

$$X_U^\infty = X_A \cdot k_e/k_E \qquad \text{(Eq. 11)}$$

it will also be dependent on the contribution of $V_{max.}$ and t_g to intestinal absorption of the drug.

Although the kinetics of absorption and excretion of riboflavin are more complex than the theoretical model, the mathematical relationships developed here will facilitate interpretation of the experimental results.

EXPERIMENTAL

Thirteen male and 10 female human subjects in apparent good health served as test subjects. The age of the subjects ranged from 3 months to 40 years. After an overnight fast, each subject received an oral dose of 150-mg. riboflavin, administered as riboflavin-5′-phosphate[1] (FMN), per square meter of body surface area. The surface area was calculated from the weight and height of each subject by the method of Dubois and Dubois (7). The vitamin was given in 15 to 40 ml. of aqueous solution immediately following a standard breakfast consisting of 30 g. cereal and 250 ml. milk or formula per square meter surface area. An equal volume of orange juice was usually mixed with the FMN solution to mask the taste. Urine was usually collected in older children and adults at 0.5-hr. intervals for 4 hr., 1-hr. intervals for the next 4 hr., and then at convenient intervals for a total of 36 hr. The subjects were moderately hydrated with oral fluids during the course of experiments to maintain adequate urine output. Studies on infants and children were carried out at the Children's Hospital of Buffalo under medical supervision. A plastic urine collection device was taped to the genital region of the infants and permitted quantitative collection of samples. The subjects were required not to take any drugs or vitamin preparations for at least 3 days prior to or during the experiments.

Total riboflavin in urine was determined fluorometrically by methods previously described (4, 5). An 18- to 24-hr. blank urine collection was obtained in each subject, and all data were corrected for blank values.

RESULTS

The typical time course of urinary excretion rate of riboflavin after oral administration of FMN is shown in Fig. 1. From such excretion data, the following parameters were determined for each subject: half-lives of elimination, urinary recovery, peak excretion rate, and time of occurrence of the peak excretion rate.

[1] Sodium riboflavin-5′-phosphate, Lot No. 414085, Hoffmann-La Roche, Nutley, N. J.

The results shown in Fig. 1 indicate that the decline in urinary excretion rate was biexponential but the initial "rapid" phase with a half-life of about 1.4 hr. accounts for elimination of most of the vitamin. A smaller fraction of the excreted vitamin was recovered in the urine during the terminal "slow" phase. To estimate the rapid and slow half-lives of elimination, semilogarithmic plots of amount of riboflavin remaining unexcreted as a function of time were constructed. Typical results obtained in four of the younger subjects are shown in Fig. 2. After the absorptive phase, which affects the first four hours of elimination, the decline in excretion was resolved into the rapid and slow phases by the method of residuals (8). The data are shown for the younger subjects because these were generally more variable than results obtained in older children and adults. This is partially due to the difficulty in ensuring complete urine voiding at the desired collection times for such subjects.

The relationship of the rapid and slow half-life of elimination to age of the subjects is shown in Fig. 3. In this and subsequent plots and statistical treatments, the logarithm of age was used. The rapid half-life of elimination exhibits a limited range of values, averaging 1.4 hr., and neither the least-squares regression nor the correlation coefficient shows any significant change of this parameter with age. The mean values, least-squares regression slopes and intercepts, correlation coefficients, and statistical calculations for this parameter, as well as others to be considered, are summarized in Table I. The slow half-life of elimination was quite variable but shows a barely statistically significant ($p = 0.05$) tendency to increase with age. Tests were repeated in five of the subjects as shown in Fig. 3. In four of these, where the age of the subject was similar in both tests, an average of the pairs of parameters was used with the mean of the logarithm of age in the statistical calculations.

There was a statistically significant ($p < 0.01$) increase in urinary recovery of riboflavin with age (Fig. 4), from a mean of about 6% at 0.25 years to about 12% at 40 years of age. Since the dose of riboflavin administered (150 mg./m.²) was greatly in excess of that

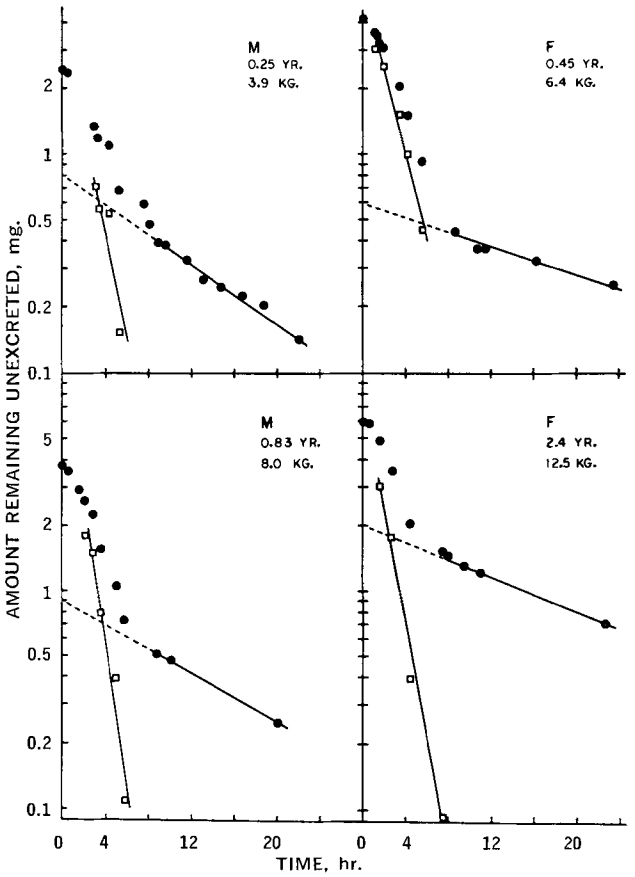


Figure 2—*Amount of riboflavin remaining unexcreted (●) as a function of time in four children. The rapid half-life of elimination was determined (□) from the postabsorptive data using the method of residuals by extrapolating (dashed line) the linear slow phase of elimination to the ordinate. Sex, age, and weight of the subjects are shown.*

Table I—Statistics of Various Riboflavin Absorption and Elimination Parameters as a Function of the Logarithm of Age

Parameter	Mean, SD	Least-Squares Linear Regression: Intercept[a]	Slope	95% Confidence Limits of Slope	Correlation Coefficient: r	t	Significance Level
Rapid half-life, hr.	1.35(0.60)	1.42	−0.197	−0.582 to 0.187	−0.161	0.82	50
Slow half-life, hr.	[b]	14.1	4.18	0.20 to 8.17	0.395	2.16	95
Percent urinary recovery	[b]	8.34	2.22	1.00 to 3.44	0.570	3.33	99
Peak excretion rate/dose	0.254(0.073)	0.245	0.032	−0.010 to 0.075	0.280	1.33	80
Time of peak excretion rate, hr.	2.18(0.89)	2.33	−0.458	−1.04 to 0.128	−0.280	1.27	75

[a] Intercept on parameter axis at age = 1 yr. [b] Significantly age dependent.

which can be absorbed (4, 5), the percent urinary recovery of the vitamin was correspondingly low.

The relationship of the peak excretion rate of riboflavin to the age of the subjects is shown in Fig. 5. The actual peak excretion rate was divided by the dose of riboflavin administered to permit comparison of the data over the entire age range. This parameter shows a slight and statistically insignificant (p = 0.2) tendency to increase with age. The value chosen for the peak excretion rate for the individual subjects is the average of the three highest excretion rates found. This method was chosen to reduce bias caused by occasional high excretion rate values resulting from incomplete voiding of an earlier sample by the younger subjects. The use of only the single highest values yielded a slope of essentially zero and indicated therefore no change with age.

The time of occurrence of the peak excretion rate is plotted as a function of age in Fig. 6 and shows a slight, but insignificant (p = 0.25), tendency to decrease with age.[2]

DISCUSSION

The intestinal absorption of riboflavin was studied as a function of age in normal subjects under conditions where maximum absorption of the vitamin will occur (*i.e.*, with food). The dose is at least five times greater than that needed to saturate the absorption process (4, 5) which accounts for the relatively low urinary recovery of riboflavin (Fig. 4). Because of the large dose employed, an assumption that riboflavin absorption occurs at a maximum rate ($V_{max.}$) during the early times after administration appears justified.

The urinary recovery of riboflavin shows a definite, though small, increase with age of the subjects (Fig 4). As shown in the *Theoretical* section, the increase in urinary recovery is a direct measure of the effect of age on intestinal absorption providing that the elimination kinetics of the vitamin are not also altered in the age range studied (Eq. 11). The kinetics of elimination of riboflavin are complex, but the data suggest that the renal excretion of the vitamin does not significantly change with age. A biexponential decline in elimination of riboflavin was found (Fig. 2), but it can also be seen (Fig. 1) that most of the vitamin is excreted during the phase characterized by a rapid half-life. This parameter remained essentially constant over the age range studied (Fig. 3). Although the slow half-life of elimination showed a tendency to increase with age, this phase contributed less than 30% to the overall urinary recovery of riboflavin. Furthermore, since the urinary excretion of the vitamin was only followed for 36 hr., it is probable that the age dependency of the slow half-life would cause the urinary recoveries of riboflavin to be even greater in the older subjects if urine was collected over a longer time period. Also, the length of the slow half-life relative to the duration of urine collections limits considerably the accuracy of this parameter.

Two factors should primarily affect the intestinal absorption of a substance such as riboflavin under the conditions of this study: the maximum absorptive capacity and the intestinal transit rate (Eq. 10). Since the peak excretion rate to dose ratio (Fig. 5), the time of its occurrence (Fig. 6), and the rapid half-life of elimination (Fig. 3) all seem unaffected by age, it may be concluded that the maximum absorptive capacity of riboflavin, when corrected for body surface area, is probably quite constant in the age range studied (Eq. 9). It therefore appears that an age-dependent increase in retention of the vitamin at intestinal absorption sites is responsible for the increased urinary recovery of riboflavin with age. This may be primarily due to decreased intestinal transit rate in older subjects. The possibility that the relative length of intestine capable of absorbing riboflavin increases with age cannot be ruled out although the age independence of $V_{max.}$ and t_p do not support this possibility.

Although the vitamin was administered as FMN in the present study, there is extensive evidence showing that FMN is rapidly and almost completely dephosphorylated to free riboflavin in the small intestine (5). The specialized transport process appears to involve subsequent rephosphorylation of riboflavin to FMN in the

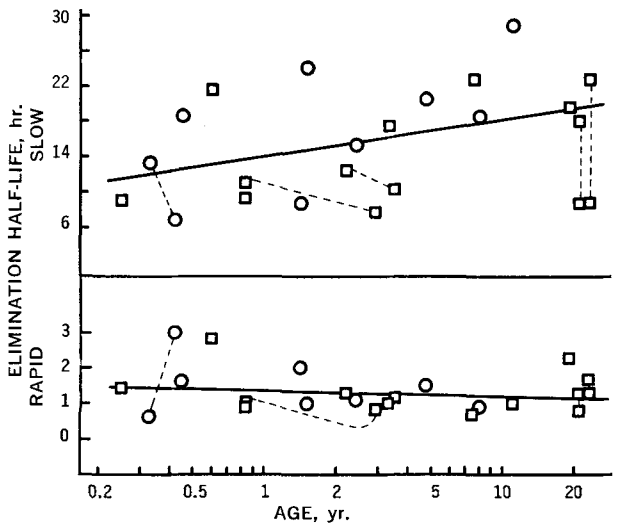


Figure 3—*Relationship between the rapid and slow half-life of elimination and the age (logarithmic scale) of the subjects. Squares and circles represent male and female subjects, respectively. Dashed lines connect repeated tests in the same subject and the solid line shows the least-squares regression fit of the data (Table I).*

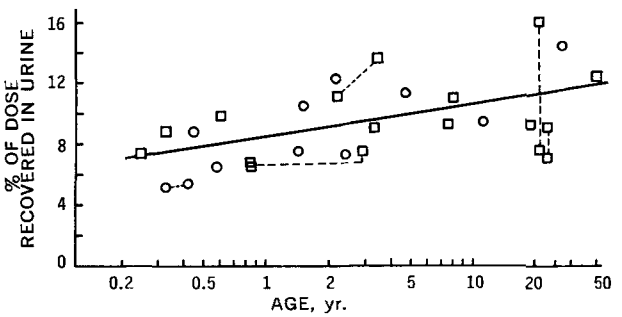


Figure 4—*Relationship between the percent urinary recovery of riboflavin and age of the subjects. Symbols are defined as in Fig. 3.*

[2] The difference in the number of data points shown in Figs. 3–6 is due to the lack of availability of some of the values, usually because of not frequent enough urine collections, particularly in the younger subjects.

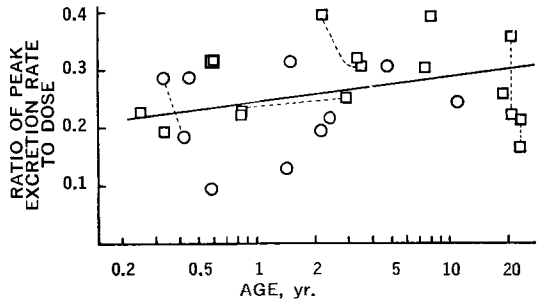


Figure 5—*Relationship between the ratio of peak excretion rate to dose and age of the subjects. Symbols are defined as in Fig. 3.*

intestinal wall (5, 9). The possibility that conversion of the oral dose of FMN to riboflavin could be rate-limiting in the absorption process appears to be ruled out by identical urinary excretion data obtained after oral administration of either form of the vitamin (4, 5). The lack of change in the peak excretion rate to dose ratio with age and the observation (9) that the activity of intestinal phosphatases decreases rather than increases with age (at least in the rat) further rule out the FMN dephosphorylation rate as a factor causing increased absorption of riboflavin with age.

The dependence of absorption on age and intestinal transit rate is not unique for riboflavin. D-Xylose, used commonly in clinical tests of intestinal absorption (10), exhibits absorption characteristics very similar to riboflavin. This sugar appears to be absorbed by a specialized process chiefly in the duodenum and proximal jejunum (10, 11). Lanzkowsky *et al.*, using both urinary excretion (12) and blood level (13) measurements, have shown a significant (twofold) increase in D-xylose absorption over an age range similar to that used in this study. Barreiro *et al.* (14) have further demonstrated a correlation of D-xylose absorption with intestinal transit time using direct measurements of intestinal motility in human subjects.

Many physiologic factors can influence drug absorption from the gastrointestinal tract (15), but little information is available on their role at various ages. A number of intestinal enzymes exhibit age-dependence in activity (1). Bender (16) has described a pronounced decrease in splanchnic blood flow in the elderly. Gastric emptying rate, though dependent on numerous factors and usually quite variable, is considered to be relatively constant throughout the childhood growth period (17). Intestinal secretions, the segmental activity of the bowel, the type of intestinal motility, the degree of vascularity, and the relative length of the intestine could also vary with age (1, 2, 9) and affect drug absorption. The increase in riboflavin absorption with age may, in part, be related to one or more of these factors which may also alter the apparent retention of the vitamin at intestinal absorption sites.

The results of this study should not be extrapolated to ages outside of the age range investigated. Bender (18) has cited possible factors which may account for decreased specialized intestinal absorption of several compounds in the elderly and it is known that many physiologic processes undergo rapid quantitative changes in the neonate (17). The results of the present study do suggest, however, that prompt release of drugs from pharmaceutical dosage forms to assure absorption is even more important in young children than in adults, in view of the apparently shorter residence time of such drugs at intestinal absorption sites.

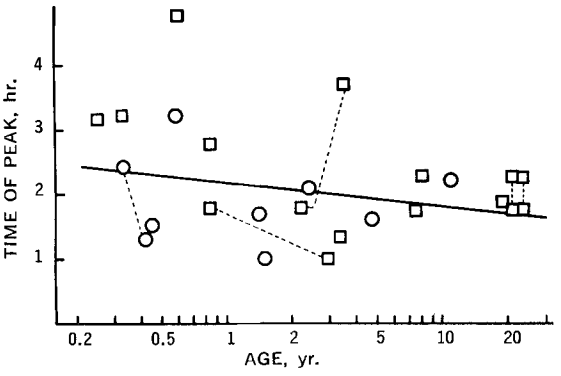


Figure 6—*Relationship between the time of occurrence of the peak excretion and age of the subjects. Symbols are defined as in Fig. 3.*

REFERENCES

(1) R. P. Spencer, *Yale J. Biol. Med.*, **37**, 105(1964).
(2) C. Morin and M. Davidson, *Gastroenterology*, **52**, 565(1967).
(3) S. J. Yaffe and N. Back, *Pedia. Clin. N. Amer.*, **13**, 527 (1966).
(4) G. Levy and W. J. Jusko, *J. Pharm. Sci.*, **55**, 285(1966).
(5) W. J. Jusko and G. Levy, *ibid.*, **56**, 58(1967).
(6) G. Levy and W. J. Jusko, *Clin. Pharmacol. Ther.*, **8**, 887 (1967).
(7) "Documenta Geigy: Scientific Tables," K. Dien, Ed., 6th ed., Geigy Pharmaceutical, Ardsley, N. Y., supplementary nomograms.
(8) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Toronto, Ontario, Canada, 1966, p. 20.
(9) S. Englard, *Fed. Proc.*, **11**, 208(1952).
(10) R. P. Spencer, "The Intestinal Tract," Charles C Thomas, Springfield, Ill., 1960, p. 154.
(11) T. H. Wilson, "Intestinal Absorption," W. B. Saunders, Philadelphia, Pa., 1962, p. 94.
(12) P. Lanzkowsky, M. Madenlioglu, J. F. Wilson, and M. E. Lahey, *New Engl. J. Med.*, **268**, 1441(1963).
(13) P. Lanzkowsky, E. A. Lloyd, and M. E. Lahey, *J. Amer. Med. Ass.*, **186**, 163(1963).
(14) M. A. Barreiro, R. D. McKenna, and I. T. Beck, *Amer. J. Dig. Dis.*, **13**, 234(1968).
(15) J. G. Wagner, *Drug. Intel.*, **2**, 30(1968).
(16) A. D. Bender, *J. Amer. Geriat. Soc.*, **13**, 192(1965).
(17) M. Davidson, in "The Biologic Basis of Pediatric Practice," R. E. Cooke, Ed., McGraw-Hill, New York, N. Y., 1968, p. 814.
(18) A. D. Bender, *J. Amer. Geriat. Soc.*, **16**, 1331(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 11, 1969, from the *Department of Pharmaceutics, School of Pharmacy*, and the *Department of Pediatrics, School of Medicine, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication October 31, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported in part by a Public Health Service Fellowship 5-F1-GM-33,073 for W.J.J. and Research Grant No. FR-77 from the National Institute of General Medical Sciences, Bethesda, Md.

The authors wish to express their appreciation for the technical assistance of Miss Mary Yeager, R.N., and the nurses of the Clinical Research Center of Children's Hospital of Buffalo.

* To whom requests for reprints should be directed.

Synthesis of Potential Antimalarials: Primaquine Analogs

S. M. TALATI, M. R. LATHAM, E. G. MOORE, G. W. HARGREAVES, and C. DeWITT BLANTON, Jr.*

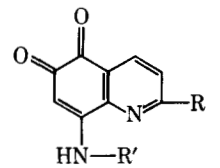
Abstract □ A series of 8-aminoquinolines, analogous to primaquine and carrying a substituent in position two, have been synthesized by standard methods and tested for activity against *Plasmodium berghei* in mice. The results of preliminary biological tests are reported.

Keyphrases □ 8-Aminoquinolines—synthesis □ Antimalarial activity—8-aminoquinolines □ TLC—separation □ IR spectrophotometry—identity

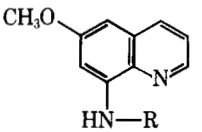
Quinoline quinones may offer a lead in the search for better antimalarials, since the mechanism of action for the important 6-methoxy-8-aminoquinolines appears to involve their *in vivo* conversion to the quinoline-*o*-quinone (1, 2). Pamaquine-5,6-quinone (I) is apparently identical with a product isolated from the feces of chickens which have been fed the drug (3, 4), and this metabolite has been reported to be 16 times as active as the parent *in vitro* (5, 6). It is likely, therefore, that the antiplasmodial activity (*Plasmodium gallinaceum*) was associated with the metabolic intermediate. Thus, the high *in vitro* antimalarial activities of 6-hydroxypentaquine, pentaquine-5,6-quinone, and pamaquine-5,6-quinone, in contrast to the *in vitro* inactivity of pentaquine (II) and pamaquine (III), lend support to Schonhofer's theory (7–9) that the *in vivo* action of the 8-aminoquinolines upon the erythrocytic or tissue stages of malaria plasmodia is due to the quinonoid products to which these drugs are converted by the host.

Much of the early work directed toward the synthesis of various 5,6-quinones was apparently abandoned because of compound instability. However, a clue to a technique for stabilizing the quinoline-*o*-quinone may be found in a paper by Holmes (10), involving introduction of a 2-hydroxyl function into the quinoline nucleus. Furthermore, the introduction of such a group suggests a modification which might be made in the 8-aminoquinolines in order to lower toxicity. Holmes (10) speculated that the oxidative detoxication of quinine by rabbit liver, far from indicating, as usually assumed, the advantage of blocking the 2-position of the quinoline antimalarials, might equally well be construed as an argument in favor of deliberately introducing an oxygen at this position. Specifically, it appears possible that substances which are structurally related to the 8-aminoquinoline drugs, and which are both quinoline quinones and carbostyrils, might exhibit a desirable combination of low toxicity and high antimalarial activity.

This report describes experiments directed toward the synthesis of substituted 8-aminocarbostyril-5,6-quinones (IV) with particular attention given to the synthesis of analogs of primaquine (V), as this agent is among the more active 8-aminoquinoline antimalarials. The target compounds (IV) were not obtained, but a series of 2-benzyloxy- or 2-hydroxy-6-alkoxy-8-(aminoalkylamino)quinolines were successfully prepared. These represent a new group of 8-aminoquinolines, which are the immediate precursor of the carbostyril-*o*-quinones.



I; R = H, R′ = $-CH(CH_3)(CH_2)_3N(C_2H_5)_2$
IV; R = OH, R′ = $-CH(R'')(CH_2)_nNH_2$
R″ = H, CH_3 $n = 1–4$



II; R = $-(CH_2)_5NHCH(CH_3)_2$
III; R = $-CH(CH_3)(CH_2)_3N(C_2H_5)_2$
V; R = $-CH(CH_3)(CH_2)_3NH_2$

To achieve the synthesis of the desired carbostyrils, it was necessary to prepare 2-benzyloxy-6-alkoxy-8-aminoquinoline (VI) (11), followed by incorporation of an appropriate side chain at position-8 (VII, VIII) (11, 12) and cleavage of the ether (IX) (11) or ethers (X) (11, 13) (see Scheme I). Theoretically, oxidation (1, 10) of this latter product (IX or X) should give the carbostyril-*o*-quinone (XI). Present evidence (IR, elemental analysis, molecular weight, *etc.*) suggests that the quinoline nucleus was destroyed in attempts to prepare the carbostyril-5,6-quinones.

In addition to the 6-methoxy derivatives, the authors have prepared several 6-ethoxy derivatives, since reports (13) in the literature indicate that ethyl ethers are more readily cleaved to the hydroxyl compound. The nature of the substituent on the 8-amino function was varied by incorporating different homologs of the alkylamino moiety (VIII), as well as some arylalkyl amino moieties (XII) (see Scheme II).

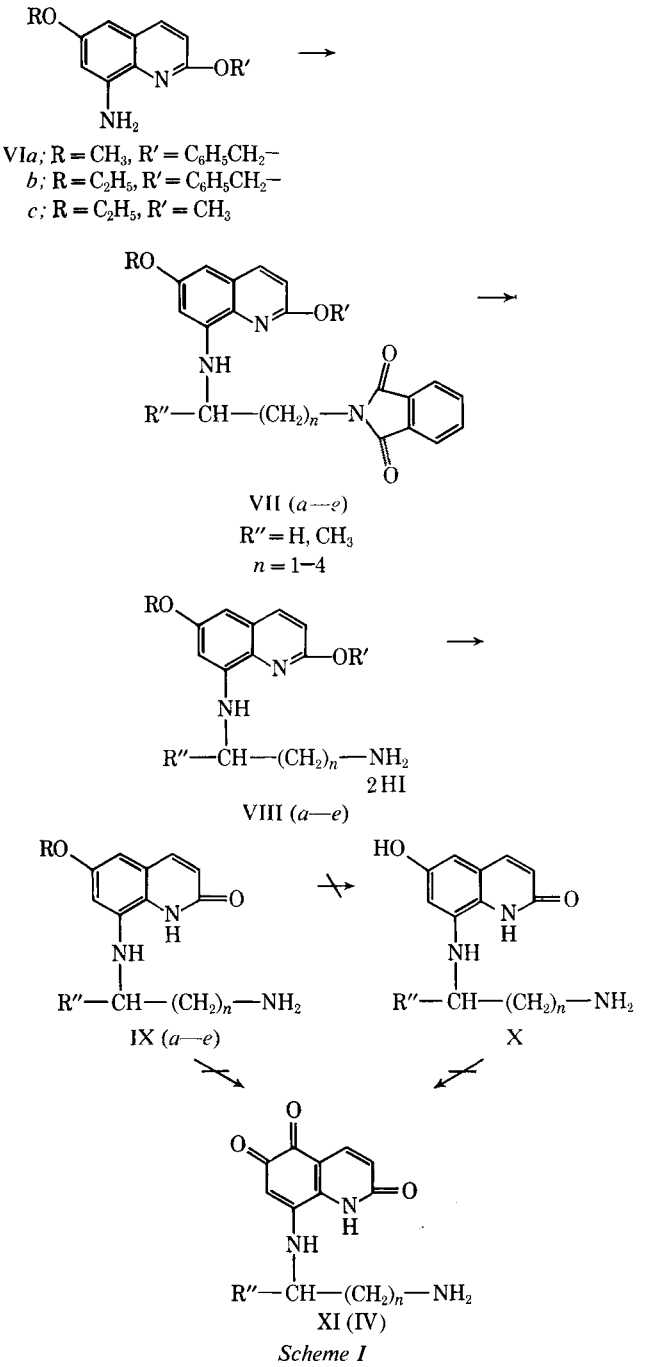
As cleavage of the 2-benzyloxy analogs produced erratic results during attempted hydrogenolysis to the 2-hydroxy analogs (IX*a–e*) (11),[1] a 2-methoxy analog (VIII*d*) was prepared in hope of achieving simultaneous acid cleavage of the 2,6-dialkoxy moieties (X). Preliminary experiments for this latter reaction have not been successful.

PHARMACOLOGICAL RESULTS

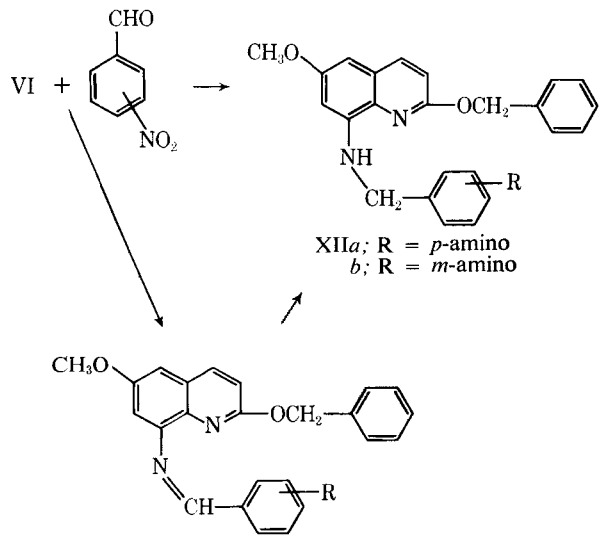
Test Methods—The compounds were tested against *P. berghei* in mice and *P. gallinaceum* in chicks and *P. gallinaceum* in mosquitos.[2]

[1] Only one of the 2-hydroxy-6-alkoxy-8-aminoquinoline derivatives was obtained by this procedure—*viz.*, IX*d*.

[2] These test data were supplied by the Walter Reed Army Institute of Research, Washington, D. C.



Scheme I



Scheme II

Mice were infected by two routes: (*a*) intraperitoneal injection of parasitized blood, and (*b*) intraperitoneal injection of a sporozoite suspension. The first test was conducted by Dr. L. Rane, University of Miami (14–16) and the other by Dr. Maurice King, Illinois Institute of Technology.[3]

The testing in chicks was also conducted by Dr. Rane[4] and the mosquito test by Dr. E. J. Gerberg, Insect Control and Research, Inc., Baltimore, Md. (17).

Testing Results—Table I summarizes the results of the Compounds VIII*a–e*. Chloroquine, quinine, and primaquine are included for comparison. The other compounds discussed in this paper are not included because they were found inactive in these test systems.

Compound VIII*a* was also tested as a prophylactic agent by Dr. King. It was found inactive at 480 mg./kg. while primaquine demonstrated activity at 90 mg./kg.

None of the compounds was active in the mosquito test.

EXPERIMENTAL

All melting points were taken on a Mel-Temp capillary melting point apparatus and are uncorrected. The microanalyses and molecular weight determinations were by Galbraith Laboratories, Inc., Knoxville, Tennessee. IR spectra were determined with a Perkin-Elmer model 137 Infracord. TLC was performed on plates coated with silica gel G and Eastman chromatogram sheets, type 6060.

6-Methoxy-8-nitroquinoline and 6-Ethoxy-8-nitroquinoline (XIII)—These quinolines were prepared from 4-methoxy-2-nitroaniline and 4-ethoxy-2-nitroaniline by the procedure of Mosher *et al.* (18) or by the procedure of Yale *et al.* (19). (6-Methoxy-8-nitroquinoline was also purchased from Winthrop Laboratories, N. Y.)

Table I—Increase in Mean Survival Time (Days)

Compound	Dosage, mg./kg. *P. berghei* in Mice[a] 40	160	640	*P. gallinaceum* in Chicks[b] 120
VIII*a*	1.7	3.5	15.7[c]	6.4
	1.7	3.3	10.2[d]	
VIII*b*	—		Inactive	6.8
VIII*c*	—		Inactive	6.4[e]
VIII*d*	0.5	3.9 (2/5)[f]	(5/5)[f]	9.5 (3/5)[f]
VIII*e*	0.5	4.1	8.6[d]	7.6
Chloroquine	4.6	10.0	(5/5)[f]	
Quinine	1.2	3.4	6.5	
Primaquine phosphate	4.5	8.8 (2/5)[f]	(5/5)[f]	

[a] A compound is considered to be active if the mean survival time of the treated group is more than double the mean survival time of the control group (5 animals were used in a group). Controls normally live 6–7 days. [b] Controls normally live 3–3.5 days. [c] Curative for one animal. [d] Curative for two animals. [e] At 140 mg./kg. [f] Drug toxicity is considered the cause of death when treated animals die before controls; fractions in parentheses represent toxicity death over total animals in treated group.

[3] The detailed test method will be published elsewhere. Mice were treated with drug on Days -1, 0, and 1 days of infection. Blood smears for parasite determination are made on Days 6, 10, 14, and 21 after infection. Drug effectiveness was evaluated by comparing the mean parasitemia of the drug-treated group to that of the nondrug-treated controls at slide Day 14. A value of less than 0.25 of the control value was considered for activity. Primaquine was active under these conditions.

[4] Chicks (9–12 days old) were infected with a uniform disease fatal to 100% of untreated controls within 3–4 days. Compounds under test were dissolved or suspended in peanut oil and administered subcutaneously or *per os* immediately after infection of the chicks. An increase of 100% in survival time was considered to be the minimum effective response to the antimalarial activity of a drug. Chicks that survived for 30 days were recorded as cured.

Table II—Substituted 8-(Phthalimidoalkylamino)quinolines (VII)

Structure (VII)	R	R′	R″	*n*	M.p.	Yield, %	Anal., % Calcd.	Found
a $C_{30}H_{29}N_3O_4$	CH_3	$C_6H_5CH_2$-	CH_3	3	121–123	25–37	C, 72.73 H, 5.86 N, 8.47	C, 72.48 H, 5.97 N, 8.58
b $C_{29}H_{27}N_3O_4$	C_2H_5	$C_6H_5CH_2$-	H	2	117	46.5	C, 72.04 H, 6.00 N, 8.69[a]	C, 71.95 H, 5.82 N, 8.54
c $C_{29}H_{27}N_3O_4$	CH_3	$C_6H_5CH_2$-	H	3	139–140	42	C, 72.30 H, 5.60 N, 8.75[b]	C, 72.41 H, 5.86 N, 5.75
d $C_{23}H_{23}N_3O_4$	C_2H_5	CH_3-	H	2	101–102	50[c]		
e $C_{30}H_{29}N_3O_4$	CH_3	$C_6H_5CH_2$-	H	4	65–68	38[c]		

[a] Molecular weight calculated for 483; found 947. (Dimer calculated mol./wt. = 966.) [b] Molecular weight calculated for 481; found 493. [c] This intermediate not analyzed at this step.

6-Methoxy-8-nitroquinoline and 6-Ethoxy-8-nitroquinoline Methiodides (XIV)—The quaternization procedure of Mislow and Koepfli (11) was utilized. The yield of the 6-methoxy derivative (XIV*a*) was 90–98% of theory, melting at 143–144° (lit. m.p. 149°). 6-Ethoxy-8-nitroquinoline methiodide (XIV*b*) was prepared in a crude yield of 98% and, after recrystallization from butanol, melted at 143–145°.

6-Methoxy-1-methyl-8-nitro-2-quinolone and 6-Ethoxy-1-methyl-8-nitro-2-quinolone (XV)—The crude methiodides (XIV) were oxidized by the procedure of Mislow and Koepfli (11). 6-Methoxy-1-methyl-8-nitro-2-quinolone (XV*a*), prepared in a 61.5% yield, melted at 190–191° (lit. m.p. 186–187°). 6-Ethoxy-1-methyl-8-nitro-2-quinolone (XV*b*) was recrystallized from methanol to give a 50% yield, m.p. 154–155°. (If pure methiodide was used, the yield could be raised to 87%.)

2-Chloro-6-methoxy-8-nitroquinoline and 2-Chloro-6-ethoxy-8-nitroquinoline (XVI)—Chlorination of the quinolone (XV) was carried out by the procedure of Mislow and Koepfli (11). The 2-chloro-6-methoxy derivative (XVI*a*) was obtained in a 71% yield, m.p. 224–226° (lit. m.p. 225–226°). 2-Chloro-6-ethoxy-8-nitroquinoline (XVI*b*) was prepared in a 77% yield, m.p. 162–162.5°.

Anal.—Calcd. for $C_{11}H_9ClN_2O_3$: C, 52.28; H, 3.56; N, 11.09. Found: C, 52.30; H, 3.60; N, 10.92.

6-Methoxy-8-nitrocarbostyril and 6-Ethoxy-8-nitrocarbostyril (XVII)—Hydrolysis of the chloro compounds (XVI) was accomplished using the procedure of Mislow and Koepfli (11). The 6-methoxy derivative (XVII*a*) melted at 209–211° (lit. m.p. 210–211°). The ethoxy derivative (XVII*b*) was obtained in a yield of 76% and melted at 162–164°.

Anal.—Calcd. for $C_{11}H_{10}N_2O_4$: C, 56.39; H, 4.30; N, 11.87. Found: C, 56.18; H, 4.33; N, 11.90.

2-Benzyloxy-6-methoxy-8-nitroquinoline and 2-Benzyloxy-6-ethoxy-8-nitroquinoline (XVIII)—The carbostyrils (XVII) were benzylated by standard procedures (11). The 6-methoxy derivatives (XVIII*a*; 65–76% yield) melted at 137–139° (lit. m.p. 139–140°). 2-Benzyloxy-6-ethoxy-8-nitroquinoline (XVIII*b*), obtained in a 52% yield, melted at 125–126°.

Anal.—Calcd. for $C_{18}H_{16}N_2O_4$: C, 66.66; H, 4.93; N, 8.64. Found: C, 66.82; H, 4.83; N, 8.62.

2-Benzyloxy-6-methoxy-8-aminoquinoline and 2-Benzyloxy-6-ethoxy-8-aminoquinoline (VI)—Reduction of the nitro compound (XVIII) was accomplished by the procedure of Mislow and Koepfli (11). Yields of 40–60% were obtained for the 6-methoxy derivative (VI*a*), which melted at 84–85° (lit. m.p. 86–87°). 2-Benzyloxy-6-ethoxy-8-aminoquinoline, obtained as a gray powder (74%), melted at 120–122°.

Anal.—Calcd. for $C_{18}H_{18}N_2O_2$: C, 72.98; H, 6.08; N, 9.46. Found: C, 73.54; H, 6.37; N, 9.27.

Sample revealed only one spot on TLC (silica gel G plate eluted with chloroform–ethanol, 15:1).

Preparation of 2-Methoxy-6-ethoxy-8-nitroquinoline (XIX)—To a solution of 23.85 g. of 6-ethoxy-8-nitrocarbostyril (XVII*b*) in 500 ml. of hot 2.5 *N* sodium hydroxide was added 200 ml. of dimethylsulfate in 25-ml. portions, the solution being basified after each addition. On cooling, light-yellow needles crystallized out. Recrystallization from ethanol gave a product which had three spots on TLC. The material was placed on an alumina column and eluted with benzene–chloroform, chloroform–ethanol, and ethanol. Fraction I (benzene–chloroform) gave 12.8 g. of shining yellow plates, m.p. 128–129°. Fraction II (chloroform–ethanol) gave 4.4 g. of yellow needles, m.p. 155–156°. The melting point and IR spectrum revealed that the product from Fraction II was identical to 1-methyl-6-ethoxy-8-nitro-2-quinolone (XV*b*). The last fraction gave a small amount of starting material, m.p. 164–165°. The product from Fraction I was recrystallized as the 2-methoxy-6-ethoxy-8-nitroquinoline. This product was homogeneous on TLC (benzene–chloroform, 15:3).

Anal.—Calcd. for $C_{12}H_{12}N_2O_4$: C, 58.06; H, 4.83; N, 11.29. Found: C, 57.95; H, 4.92; N, 11.15.

Table III—Substituted 8-(Aminoalkylamino)quinolines (VIII)

Structure (VIII)	R	R′	R″	*n*	M.p.	Yield	Anal., % Calcd.	Found
a $C_{22}H_{29}I_2N_3O_2$	CH_3	$C_6H_5CH_2$-	CH_3	3	125 dec.	74–92	C, 42.35 H, 4.67 N, 6.76	C, 42.51 H, 4.71 N, 6.66
b $C_{21}H_{27}I_2N_3O_2$	C_2H_5	$C_6H_5CH_2$-	H	2	119–120 dec.	85	C, 41.52 H, 4.45 N, 6.92	C, 41.17 H, 4.41 N, 6.71[a]
c $C_{21}H_{27}I_2N_3O_2$	CH_3	$C_6H_5CH_2$-	H	3	130 dec.	91	C, 41.51 H, 4.44 N, 6.91	C, 41.53 H, 4.65 N, 7.07[b]
d $C_{15}H_{23}I_2N_3O_2$	C_2H_5	CH_3-	H	2	127–128 dec.	64.4	C, 33.89 H, 4.33 N, 7.91	C, 33.63 H, 4.56 N, 7.80
e $C_{22}H_{29}I_2N_3O_2$	CH_3	$C_6H_5CH_2$-	H	4	92–95 dec.	79.1	C, 42.55 H, 4.71 N, 6.86	C, 42.27 H, 4.98 N, 6.56

[a] This compound (VIII*b*) was also prepared as the monohydrated diphosphate salt. Calcd. for $C_{21}H_{31}N_3O_{11}P_2$: C, 44.62; H, 5.84; N, 7.43. Found: C, 44.59; H, 6.29; N, 7.43. [b] Percent iodine: calcd., 41.68; found, 41.68.

If care was taken to ensure complete solution of the 6-ethoxy-8-nitrocarbostyril before addition of the dimethylsulfate, it was possible to raise the yield of desired product. In one case, tetrahydrofuran was employed to facilitate solution in the hot alkali solution (65–70°). With this modification, the procedure gave 86.6% 2-methoxy-6-ethoxy-8-nitroquinoline, m.p. 125–126°.

2-Methoxy-6-ethoxy-8-aminoquinoline (VI*c*)—A suspension of 2-methoxy-6-ethoxy-8-nitroquinoline was reduced on the Parr apparatus with platinum oxide. The product, which melted at 140–143° (72%), was used in the next step without elemental analysis (preparation of VIII*d*).

Preparation of *N*-(Bromoalkyl)phthalimides (XX)—The procedure of Elderfield *et al.* (20) was employed. [*N*-(3-Bromopropyl)-phthalimide was obtained commercially.]

Preparation of 2-Benzyloxy(or methoxy)-6-methoxy(or ethoxy)-8-(phthalimidoalkylamino)quinolines (VII*a–e*, Table II)—The procedure of Elderfield (20) was slightly modified for this condensation. A solution of 0.08 mole of 2-benzyloxy(or methoxy)-6-methoxy(or ethoxy)-8-aminoquinoline, 0.4 mole of sodium acetate, and 0.08 mole of the appropriate *N*-(bromoalkyl)phthalimide in 300 ml. of 66% ethanol was refluxed for 3 days. An additional quantity of the phthalimide (0.08 mole) was added on the second day and 0.4 mole of sodium acetate was added each day. The pH was maintained near 7–8. A dark-brown oil separated from the reaction. The reaction mixture was cooled, diluted with 600 ml. of water, saturated with potassium carbonate, and extracted with ether. (Any solid, which appeared, was collected with the ether fraction.) The ether extract was concentrated to one-half its original volume and chilled to give a yellow precipitate. [In some cases, it was necessary to dry the ether extract ($MgSO_4$), evaporate to dryness, and add anhydrous ether to achieve a precipitate.] The product which dissolved in the ether was found to be a mixture of starting materials. This procedure generally yielded a product of sufficient purity for the next step—TLC showed the product to be homogeneous. Analytical samples were prepared by recrystallization from 95% ethanol or aqueous DMF (see Table II for physical data).

Preparation of 2-Benzyloxy(or methoxy)-6-methoxy(or ethoxy)-8-(aminoalkylamino)quinoline Dihydriodides (VIII*a–e*, Table III)—The procedure of Mosher (12) was utilized. In some cases, the phthalimido product was not readily soluble in ethanol, and solution was achieved by the addition of chloroform. TLC showed the product to be homogeneous (methanol–diethylamine, 19:1) (see Table III for physical data).

Preparation of 6-Ethoxy-8-(3-aminopropylamino)carbostyril Monoacetate (IX*b*)—A solution of 2-benzyloxy-6-ethoxy-8-(3-aminopropylamino)quinoline was reduced on the Parr apparatus with palladium oxide. The product was isolated as the monoacetate and recrystallized from absolute ethanol to give light-yellow needles (47.7%), m.p. 183–184° (IR 3450, 1670, 1610, and 1550 $cm.^{-1}$).

Anal.—Calcd. for $C_{16}H_{23}N_3O_4$: C, 58.22; H, 7.16; N, 13.08. Found: C, 58.00; H, 7.33; N, 12.87.

2-Benzyloxy-6-methoxy-8-(4-amino-1-methylbutylamino)quinoline (VIII*a*) and 2-benzyloxy-6-methoxy-8-(4-aminobutylamino)quinoline (VIII*c*) were subjected to similar reaction conditions, but the analytical data were inconclusive and structural assignments have not been made at this time.

(VIII*a* to IX*a*) *Anal.*—Calcd. for $C_{15}H_{21}N_3O_2 \cdot CH_3COOH$ (IX*a*): C, 61.07; H, 7.18; N, 12.57; mol. wt. 334. Found: C, 57.29; H, 6.89; N, 13.35; mol. wt. 210 (CH_3OH).

(VIII*c* to IX*c*) *Anal.*—Calcd. for $C_{14}H_{19}N_3O_2 \cdot CH_3COOH$ (IX*c*): C, 60.00; H, 6.87; N, 13.12. Found: C, 55.06; H, 6.70; N, 13.49.

In one experiment, attempts were made to isolate the carbostyril as the hydriodide salt. It was not possible to obtain a product that was homogeneous on TLC.

Anal.—Calcd. for $C_{15}H_{21}N_3O_2 \cdot 2HI$: C, 33.96; H, 4.15; I, 48.21; N, 7.92. Found: C, 36.44; H, 4.54; I, 46.22; N, 7.10.

Attempted Preparation of 2,6-Dihydroxy-8-(3-aminopropylamino)quinoline (X)—A solution of 8 g. (0.022 mole) of 2-benzyloxy-6-ethoxy-8-(3-aminopropylamino)quinoline in 50 ml. of methyl cellosolve was heated and stirred with 80 ml. of 48% hydrobromic acid at 120–130° for 4 hr. The solvent was evaporated, *in vacuo*, to give the hydrobromide which was suspended in water and neutralized with sodium bicarbonate to give a product of unknown structure. This substance was recrystallized from pyridine–water to give yellow platelets, m.p. 202–203°. IR spectrum indicated ether cleavage, showing a band at 1680 $cm.^{-1}$, characteristic for carbonyl absorption. However, molecular weight and elemental analysis did not correlate with calculated values. TLC showed the product to be homogeneous (methanol–diethylamine, 15:2).

Anal.—Calcd. for $C_{12}H_{15}N_3O_2$: C, 61.80; H, 6.43; N, 18.02; mol. wt., 233. Found: C, 70.90; H, 7.33; N, 11.83; mol. wt. (DMF), 480.

2-Benzyloxy-6-methoxy-8-(4-aminobenzylamino)quinoline Dihydrochloride (XII)—This procedure was adapted after that of Tipson and Clapp (21). Two grams (0.007 mole) of 2-benzyloxy-6-methoxy-8-aminoquinoline was mixed with 1.5 g. of *p*-nitrobenzaldehyde in 25 ml. of DMF and 5 drops of piperidine. This mixture was allowed to stand for 1 hr. with stirring after heating just briefly on a water bath. The solution was then shaken on the Parr apparatus, under hydrogen, with platinum oxide until 3 p.s.i. of pressure had been lost. Filtration of the catalyst and dilution of the filtrate with water gave a greenish-yellow product. This substance was dissolved in ether, dried, and treated with charcoal before removal of the drying agent. Hydrogen chloride was passed through the ethereal solution to give a product melting at 225–227° (78% yield). TLC revealed only one spot (methanol–diethylamine, 19:1) (IR 3400, 2850, 1610, and 1580 $cm.^{-1}$). Analytical sample recrystallized from ethanol–ether.

Anal.—Calcd. for $C_{24}H_{21}N_3O_2 \cdot 2HCl$: C, 62.88; H, 5.46; N, 9.17. Found: C, 62.76; H, 5.61; N, 9.20.

It was possible to isolate and characterize the intermediate, 2-benzyloxy-6-methoxy-8-(*p*-nitrobenzylideneamino)quinoline, m.p. 118–122° (XII*c*).

Anal.—Calcd. for $C_{24}H_{19}N_3O_4$: C, 69.73; H, 4.60; N, 10.16. Found: C, 69.65; H, 4.73; N, 9.92.

2-Benzyloxy-6-methoxy-8-(3-aminobenzylamino)quinoline dihydrochloride (XII*b*), m.p. 149–152°, was prepared by a similar procedure.

Anal.—Calcd. for $C_{24}H_{21}N_3O_2 \cdot 2HCl$ (XII*b*): C, 62.88; H, 5.46; N, 9.17. Found: C, 62.68; H, 5.32; N, 9.09.

REFERENCES

(1) N. L. Drake and Y. T. Pratt, *J. Amer. Chem. Soc.*, **73,** 544(1951).

(2) R. C. Elderfield, "Heterocyclic Compounds," vol. 4, Wiley, New York, N. Y., 1952, p. 198.

(3) E. S. Josephson, J. Greenberg, D. J. Taylor, and H. L. Bami, *J. Pharmacol. Exp. Ther.*, **103,** 7(1951).

(4) J. Greenberg, D. J. Taylor, and E. S. Josephson, *J. Infec. Dis.*, **88,** 163(1951).

(5) W. Schulemann, *Proc. Roy. Soc. Med.*, **25,** 897(1932).

(6) P. B. Russell, in "Antimalarials," Medicinal Chemistry, 2nd ed., A. Burger, Ed., Interscience, New York, N. Y., 1960, pp. 814, 830.

(7) F. Schonhofer, *Z. Physiol. Chem.*, **274,** 1(1942).

(8) F. Schonhofer, *Med. Chem.*, **3,** 64(1936).

(9) K. C. Blanchard, in "A Survey of Antimalarial Drugs, 1941–1945," vol. 1, F. Y. Wiselogle, Ed., J. W. Edwards, Ann Arbor, Mich., 1946, pp. 129–138.

(10) R. R. Holmes, J. Conrady, J. Guthrie, and R. McKay, *J. Amer. Chem. Soc.*, **76,** 2400(1954).

(11) K. Mislow and J. B. Koepfli, *J. Amer. Chem. Soc.*, **68,** 1553(1946).

(12) H. S. Mosher, *J. Amer. Chem. Soc.*, **68,** 1565(1946).

(13) O. Y. Magidson and I. T. Strukov, *Arch. Pharm.*, **271,** 364(1933).

(14) T. S. Osdene, P. B. Russell, and L. Rane, *J. Med. Chem.*, **10,** 431(1967).

(15) W. T. Colwell, J. H. Lange, and D. W. Henry, *ibid.*, **11,** 282(1968).

(16) W. G. Duncan and D. W. Henry, *ibid.*, **12,** 25(1969).

(17) E. J. Gerberg, L. T. Richard, and J. B. Poole, *Mosquito News*, **26,** 359(1966).

(18) H. S. Mosher, W. H. Yanko, and F. Whitmore, "Organic Syntheses, Collective Volume III," Wiley, New York, N. Y., 1955, p. 580.

(19) H. L. Yale and J. Berustein, *J. Amer. Chem. Soc.*, **70,** 254 (1948).

(20) R. C. Elderfield, H. E. Mertel, R. T. Mitch, I. M. Wempen, and E. Werble, *ibid.*, **77,** 4816(1955).

(21) R. S. Tipson and M. A. Clapp, *J. Org. Chem.*, **11,** 292(1946).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from *Auburn University, School of Pharmacy, Auburn, AL 36830*

Accepted for publication November 17, 1969.

This work was supported by Contract Number DA-49-193-MD-2985 from the U. S. Army Medical Research and Development Command, and in part by the office of General Research, The University of Georgia, Athens, Georgia.

This is contribution Number 710 from the Army Research Program on Malaria.

* To whom inquiries should be addressed. Present address: School of Pharmacy, University of Georgia, Athens, GA 30601

Odor Threshold and Gas-Chromatographic Assays of Vaginal Odors: Changes with Nitrofurazone Treatment

A. DRAVNIEKS, B. K. KROTOSZYNSKI, L. KEITH*, and I. M. BUSH*

Abstract □ Although vaginal disorders are frequently accompanied by malodors, the efficiency of drugs to reduce the malodors by reducing the infection has been difficult to estimate. Recently, techniques have become available for combining sensory and gas-chromatographic approaches to compare odor intensities of complex vapor mixtures. These techniques were applied to vaginal odors. An apparatus was devised to collect vaginal vapors in a form suitable for odor-revelant gas-chromatographic analyses, as well as for odor-threshold determinations. Odor changes in vaginal vapors upon treatment with nitrofurazone vaginal suppositories were studied on five patients with several types of disorders (hematuria, endometritis, and stress incontinence). The distribution of odorous components in the gas chromatograms reflected, through disappearance of many malodorous peaks, a significant reduction in the content of the malodorous volatile compounds in the vaginal vapors. Odor-threshold measurements were conducted in an apparatus where nonodorous methane tracer and a hydrogen-flame ionization detector were used to measure the degree of vapor dilution needed to reach the threshold, using the ASTM odor-threshold test design combined with ED_{50} statistics. These measurements similarly indicated that odors were reduced by the drug.

Keyphrases □ Vaginal odor threshold, intensity—analysis □ Odors, vaginal—threshold, intensity determination □ Nitrofurazone effect—vaginal odors □ GLC—analysis

It is usually noted that a reduction in bacterial population in suitable media is accompanied by a reduction in the odors. Odor reduction is often subjectively observed when the antibacterial agent nitrofurazone[1] is used in the treatment of vaginal disorders accompanied by malodor. In the absence of a satisfactory method for quantitative or precise qualitative assay of odors, such observations have remained in the form of testimonials.

Recently, techniques have become available for more detailed studies of the odors in complex mixtures. These techniques are based on combinations of psychophysical (sensory) and gas-chromatographic methods, the latter providing means for collecting and separating the components of odorous vapors. In the present work these techniques were adapted to assay the vaginal odors and their changes upon treatment with an antibacterial preparation. Odor thresholds of vaginal vapors were also determined.

[1] Furacin Vaginal Suppositories, Eaton Laboratories, Division of the Norwich Pharmacal Co.

PRINCIPLES OF ODOR MEASUREMENT

The odor results from interaction of vapors with the observer's chemoreceptors (primarily olfactory and to some extent trigeminal). Substances differ in odor thresholds, *i.e.*, in the lowest concentrations in air at which the odors of their vapors can be detected. Observers also differ in sensitivity (olfactory acuity), so that odor thresholds are represented not by sharp concentration levels but rather by concentration zones, within which the thresholds for different observers can vary severalfold. To a lesser extent, thresholds as estimated by the same observer may vary from time to time.

Above threshold levels, odor intensity increases in proportion to a fractional power (0.2–0.7) of the odorant concentration in air. These relations are expressed in Stevens' law (1, 2):

$$I = k\,(C - C_{\text{thr.}})^x \qquad \text{(Eq. 1)}$$

where I is psychophysical odor intensity, k is a coefficient (small for weak odors, large for strong odors), C is concentration of the odorant in air, $C_{\text{thr.}}$ is threshold concentration of the odorant, and x is an exponent. The values of k, $C_{\text{thr.}}$, and x are not necessarily related. Corollaries of this expression are: (*a*) odor intensity experienced by an observer does less than double when the odorant's concentration is doubled; *e.g.*, for $x = 0.5$, the concentration must increase by a factor of 4 to increase the intensity by a factor of 2; (*b*) if an odorant A has smaller values of k and x than another odorant B, an undiluted A can smell weaker than undiluted B, while diluted A can smell stronger than similarly diluted B; (*c*) since the highest possible concentration of an odorant is at its saturation pressure, odorants with low x, or high threshold and an average x, may never reach very high odor intensity.

Odor-threshold values, therefore, do not necessarily indicate the undiluted odor intensity; rather, the odor of a lower threshold odorant will be noticed farther from the source than will that of a higher threshold odorant. In addition, above the threshold, the character of the odor can be objectionable, as with the malodors, or acceptable, as with fragrances.

The composition of odorous vapors of biochemical origin is complex, with many odorants participating. Odorants present at subthreshold concentrations can summate (sometimes even synergistically) to reach threshold and can modify the character of odors of other substances which are present at suprathreshold levels. In odor assay, therefore, all those substances that are present at levels exceeding reasonable fractions, *e.g.*, one-tenth of their threshold concentrations, must be considered. Gas-chromatographic techniques permit the delivery of separated components for sensory

ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from *Auburn University, School of Pharmacy, Auburn, AL 36830*

Accepted for publication November 17, 1969.

This work was supported by Contract Number DA-49-193-MD-2985 from the U. S. Army Medical Research and Development Command, and in part by the office of General Research, The University of Georgia, Athens, Georgia.

This is contribution Number 710 from the Army Research Program on Malaria.

* To whom inquiries should be addressed. Present address: School of Pharmacy, University of Georgia, Athens, GA 30601

Odor Threshold and Gas-Chromatographic Assays of Vaginal Odors: Changes with Nitrofurazone Treatment

A. DRAVNIEKS, B. K. KROTOSZYNSKI, L. KEITH*, and I. M. BUSH*

Abstract □ Although vaginal disorders are frequently accompanied by malodors, the efficiency of drugs to reduce the malodors by reducing the infection has been difficult to estimate. Recently, techniques have become available for combining sensory and gas-chromatographic approaches to compare odor intensities of complex vapor mixtures. These techniques were applied to vaginal odors. An apparatus was devised to collect vaginal vapors in a form suitable for odor-revelant gas-chromatographic analyses, as well as for odor-threshold determinations. Odor changes in vaginal vapors upon treatment with nitrofurazone vaginal suppositories were studied on five patients with several types of disorders (hematuria, endometritis, and stress incontinence). The distribution of odorous components in the gas chromatograms reflected, through disappearance of many malodorous peaks, a significant reduction in the content of the malodorous volatile compounds in the vaginal vapors. Odor-threshold measurements were conducted in an apparatus where nonodorous methane tracer and a hydrogen-flame ionization detector were used to measure the degree of vapor dilution needed to reach the threshold, using the ASTM odor-threshold test design combined with ED_{50} statistics. These measurements similarly indicated that odors were reduced by the drug.

Keyphrases □ Vaginal odor threshold, intensity—analysis □ Odors, vaginal—threshold, intensity determination □ Nitrofurazone effect—vaginal odors □ GLC—analysis

It is usually noted that a reduction in bacterial population in suitable media is accompanied by a reduction in the odors. Odor reduction is often subjectively observed when the antibacterial agent nitrofurazone[1] is used in the treatment of vaginal disorders accompanied by malodor. In the absence of a satisfactory method for quantitative or precise qualitative assay of odors, such observations have remained in the form of testimonials.

Recently, techniques have become available for more detailed studies of the odors in complex mixtures. These techniques are based on combinations of psychophysical (sensory) and gas-chromatographic methods, the latter providing means for collecting and separating the components of odorous vapors. In the present work these techniques were adapted to assay the vaginal odors and their changes upon treatment with an antibacterial preparation. Odor thresholds of vaginal vapors were also determined.

[1] Furacin Vaginal Suppositories, Eaton Laboratories, Division of the Norwich Pharmacal Co.

PRINCIPLES OF ODOR MEASUREMENT

The odor results from interaction of vapors with the observer's chemoreceptors (primarily olfactory and to some extent trigeminal). Substances differ in odor thresholds, *i.e.*, in the lowest concentrations in air at which the odors of their vapors can be detected. Observers also differ in sensitivity (olfactory acuity), so that odor thresholds are represented not by sharp concentration levels but rather by concentration zones, within which the thresholds for different observers can vary severalfold. To a lesser extent, thresholds as estimated by the same observer may vary from time to time.

Above threshold levels, odor intensity increases in proportion to a fractional power (0.2–0.7) of the odorant concentration in air. These relations are expressed in Stevens' law (1, 2):

$$I = k\,(C - C_{\text{thr.}})^x \qquad \text{(Eq. 1)}$$

where I is psychophysical odor intensity, k is a coefficient (small for weak odors, large for strong odors), C is concentration of the odorant in air, $C_{\text{thr.}}$ is threshold concentration of the odorant, and x is an exponent. The values of k, $C_{\text{thr.}}$, and x are not necessarily related. Corollaries of this expression are: (*a*) odor intensity experienced by an observer does less than double when the odorant's concentration is doubled; *e.g.*, for $x = 0.5$, the concentration must increase by a factor of 4 to increase the intensity by a factor of 2; (*b*) if an odorant A has smaller values of k and x than another odorant B, an undiluted A can smell weaker than undiluted B, while diluted A can smell stronger than similarly diluted B; (*c*) since the highest possible concentration of an odorant is at its saturation pressure, odorants with low x, or high threshold and an average x, may never reach very high odor intensity.

Odor-threshold values, therefore, do not necessarily indicate the undiluted odor intensity; rather, the odor of a lower threshold odorant will be noticed farther from the source than will that of a higher threshold odorant. In addition, above the threshold, the character of the odor can be objectionable, as with the malodors, or acceptable, as with fragrances.

The composition of odorous vapors of biochemical origin is complex, with many odorants participating. Odorants present at subthreshold concentrations can summate (sometimes even synergistically) to reach threshold and can modify the character of odors of other substances which are present at suprathreshold levels. In odor assay, therefore, all those substances that are present at levels exceeding reasonable fractions, *e.g.*, one-tenth of their threshold concentrations, must be considered. Gas-chromatographic techniques permit the delivery of separated components for sensory

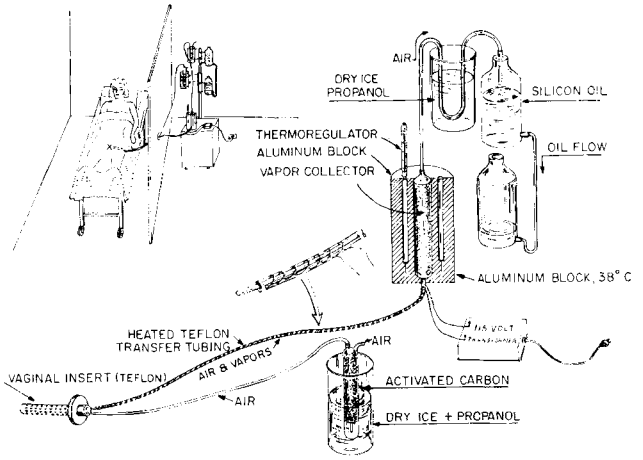


Figure 1—*Apparatus for collection of vaginal vapors.*

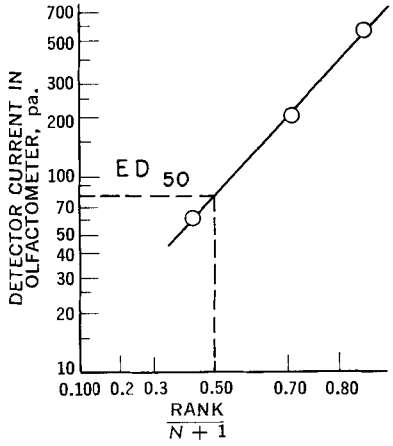


Figure 2—*Example of ED_{50} odor-threshold determination of vaginal vapors.*

assay at concentrations 10–20 times higher than those in the original odorous air mixtures. In this way, mixtures can be studied for predominance and intensities of certain odor notes (*e.g.*, obviously objectionable, fragrant, *etc.*).

METHODS

Vaginal Vapor Collection—Figure 1 shows the arrangement for vaginal vapor collection. A perforated Teflon tube 1.91-cm. (0.75-in.) i.d. and 9.52 cm. (3.75 in.) long (with the disklike collar) was provided with two openings at the outer end. One of these was connected to a vapor collector by Teflon tubing heated along its entire length by low-voltage Teflon-insulated resistance wire. The collector was made of stainless-steel tubing 0.97-cm. (0.38-in.) i.d. and 25.4 cm. (10 in.) long, packed with Teflon powder coated with 10% hydrocarbon grease, mol. wt. about 1000 (Apiezon L). Prior to use, the organic vapors were removed from the collector by heating it in a stream of highly purified helium until a satisfactory base line gas chromatogram was obtained. During the sample collection from the vagina, the collector was kept in an aluminum block at 38° to prevent the condensation of water from vapors. Vaginal vapors were made to flow into the collector by allowing silicone oil to flow from the upper to the lower of the two flasks shown. A dry ice–propanol trap between the collector and the oil prevented atmospheric or oil vapors from reaching the collector. Organic vapors from the vagina dissolved in the hydrocarbon grease coating of the collector, while water vapor passed through.

The other Teflon tube leading to the vaginal insert allowed air to replace that which was removed into the collector. To block room vapors from entering the collector *via* this Teflon tube, room air was first passed through an activated carbon adsorber cooled to −80° by a dry ice–propanol mixture. Usually 2 l. of air was sampled during a collection period of 20 min. In addition to the collected vapors, the Teflon insert with its vaginal debris was used for odor-threshold studies. Immediately after removal from the patient, the insert was placed into an odorless glass vessel provided with inlet and outlet tubing. If odor study on the insert was postponed, the vessel and contained insert were stored in a freezer and brought back to room temperature shortly before study.

Table I—Example of Data Treatment of Vaginal Vapors by a Small Panel [Quantal Response Method (4)][a]

Stimulus Level as Methane Tracer Detector Current, pa.[b]	No. of Panelists Beginning to Detect Odor	Tolerance Level,[c] pa.	Frequency of Observation Rank	Rank $\frac{}{N+1}$
9	0			
30	0	16		
125	4	60	2.5[d]	0.355
360	1	210	5.0	0.72
900	1	570	6.0	0.86

[a] Six panelists, $N = 6$. [b] pa. = picoampere (10^{-12} amp.). [c] Calculated as logarithmic average of the adjoining levels. [d] Ranks 1, 2, 3, and 4; average rank 2.5.

Odor-Threshold Measurements—The statistical design followed a combination of triangle test (3) and quantal response (4) methods. The vapor from the insert with vaginal debris, in known dilutions with nitrogen, was led in a special olfactometer to one of three sniffing ports. In this olfactometer, nonodorous methane carries the vapor into a nitrogen stream. The amount of subsequent dilution by cryogenically deodorized nitrogen is monitored continuously by a hydrogen-flame ionization detector. The detector indicated the methane content by a proportional increase in the ionization current. The measurements permitted calculations of the degree of dilution of odorous vapors in the sniffing port.

The panel of observers sniffed at each of the three sniffing ports by using the statistical design of the ASTM method (5). A negative response was recorded if no odor was reported at any of the ports, or if the observer reported odor at a wrong port. A positive response was recorded if an odor was reported at a correct port (ports were changed randomly). Concentrations of odorous vapors were increased threefold for the next observations, and so on. At each of the concentrations, several observers reported their judgments independently.

Data were tabulated, ranked, and plotted as shown in Table I and Fig. 2. This approach is the same as that used in the study of responses to poisons or other drugs, except that here the response is a positive observation of odor. The concentration level at probability = 0.50 is the odor ED_{50}, the lowest level at which half of the observers would sense an odor. The detector units of the olfactometer were converted to p.p.m. of headspace, using the methane calibration factor.

Gas-Chromatographic Sensory Assay—The vapors in the collector (Fig. 1) were transferred by a special injection needle to a gas chromatograph, using procedures described elsewhere (6). The transfer was effected in a device in which high-purity helium was passed through the collector and injection needle, connected in series with a short length of Teflon tubing. The needle was cooled with liquid nitrogen while a heated copper block traveled along the collector at the velocity of 2.54 cm. (1 in.)/min. in the direction of He flow. The motion was provided by means of a motor-actuated string device (7). Vapors from the collector were eluted in the direction opposite to that which was followed during collection. This method minimized the contamination of the sample by impurities generated by decomposition of the collector's hydrocarbon-grease phase, since each portion of the collector was heated for only a short time.

In the gas chromatograph the carrier gas was He at a flow rate of 10 ml./min. An open tubular column 15.24 m. (50 ft.) long, coated with polyethylene glycol (Carbowax 20 M), was used, and temperature was programmed from 60 to 180° at 4°/min. A hydrogen-flame ionization detector was used, with a sensitivity of approximately 4000

Table II—List of Diagnoses and Samples

Description	Patient Number	Test Number
Normal	1	1
Hematuria	2	2-1 Before Rx[a]
		2-2 4 hr. after Rx
Stress incontinence	3	3-1 Before Rx
		3-2 4 hr. after Rx
Endometritis	4	4-1 8 hr. before Rx
		4-2 Immediately before Rx
		4-3 15 hr. after Rx
		4-4 24 hr. after Rx
Endometritis	5	5-1 7 hr. before Rx
		5-2 Immediately before Rx
		5-3 16 hr. after Rx
		5-4 24 hr. after Rx
Endometritis	6	6-1 7 hr. before Rx
		6-2 Immediately before Rx
		6-3 17 hr. after Rx
		6-4 24 hr. after Rx
Bacterial vaginitis	7	7-1 7 hr. before Rx
		7-2 Immediately before Rx
		7-3 20 hr. after Rx

[a] See *Schedule of Tests* section for definition.

Table III—Odor Thresholds, EG_{50}, of Vaginal Vapors

Case No.	Odor Threshold[a] Vapor in Nitrogen, p.p.m. Before	After	Av. Ratio[b]
2	360	3,400	9.5
3	3,400	80,000	23.0
4	290	480	1.6
5	940		
	15,000	17,000	4.0
		13,000	
6	25,000		
	34,000	42,000	1.1
		25,000	
7	1,700	2,400	2.7
	480		

[a] When two collections of vapors were made, the value for the second collection is placed below that for the first. [b] Odor thresholds averaged geometrically—considered correct procedure for dealing with thresholds covering several orders of magnitude.

pg./cm.[2] of the gas-chromatographic peak area[2] at 0.1 × 1 electrometer setting (the highest sensitivity setting available). A series of *n*-alkanes was run as standard for conversion of retention times to approximate Kovats Indices (8) using a fifth-order polynomial curve and a computer to interpolate values.

At the exit from the gas-chromatographic column 110 ml./min. of He was added to the column effluent and the combined effluent of 120 ml./min. was split 1:1 so that 60 ml./min. was delivered, respectively, to the detector and to a sniffing port. A chemist experienced in this technique observed and recorded the odor of the gas chromatographically separated components (9, 10).

By this technique the components of the vapors are delivered at their respective concentrations at the ports in a sequence which depends on vapor pressures and polarities (in a gas chromatographic sense) of the components. The following concentration relations apply. For example,[3] let the original concentration of a component in the air = *n*-g./ml. At perfect efficiency the collection and transfer from 2000 ml. will deliver 2000 *n*-g. into the gas chromatograph. Of this, 1000 *n*-g. goes to the sniffing port, diluted in He carrier and subsequently, on leaving the port, to some extent also diluted by room air. A typical peak was carried out in 20–40 ml. He, and if the substance was evenly distributed (in mixing with air before reaching the nose), the actual concentration reaching the nose was 10 *n*–20 *n*-g./ml. This was higher, by an order of magnitude than that in the original vaginal air sample. Due to losses in collection and transfer, however, the actual concentration factor was somewhat less.

Potent odorants with low-odor thresholds can exhibit odor at concentrations in the range 0.1–10 pg./ml. of air. Calculations indicate that by collecting organic vapors from 2 l. of air and using a detector with sensitivity on the order of 1000 pg./cm.[2] of chart area, most odor-relevant compounds will be adequately represented in the gas chromatograms. A few, however, still can be odorous without exhibiting visible peaks.

The odor characters of the various components are tabulated in broad categories. The most convenient categorization is a hedonic one; it is based on a scale of "obviously fragrant" and "pleasant" at one end to "objectionable" and "highly objectionable" (repulsive or nauseating) at the other. Between these extremes there is a category of odors of slight, mild, or moderate character. These do not impress observers as being significantly fragrant or objectionable. This intermediate category consists of odors, some of which do not immediately resemble a familiar odor and others which do have an obviously distinct recognizable character ("musty," "sour," "mushroomlike," "burnt," "medicinal," *etc.*).

Because of differences in the character and intensity of odors of the components, the number of distinguishable peaks and their prominence are not the best guides for the comparison of odors of different samples. In addition, some peaks may represent more than one component, each with different sensory characteristics. Most organic materials are odorous at sufficient concentrations. However, some substances, such as hydrocarbons, have high thresholds and can produce large peaks in chromatograms but are not odorous on elution from the sniffing port, and, therefore, contribute little to the initial (mixed) odor. Also, a rich pattern with many larger peaks can indicate strength of an odor but not its objectionability.

Although overall richness can be a guide to the comparison of samples which are similar in composition, much more odor-relevant information is obtained by tabulating peaks by their odor categories and comparing the number of peaks in different categories. As unpleasant odors come under control during treatment, the number of objectionably odorous peaks decreases. Such effects occur even in those chart locations where no pleasantly odorous peaks emerge.[4] In locations where both objectionable and nonobjectionable and/or

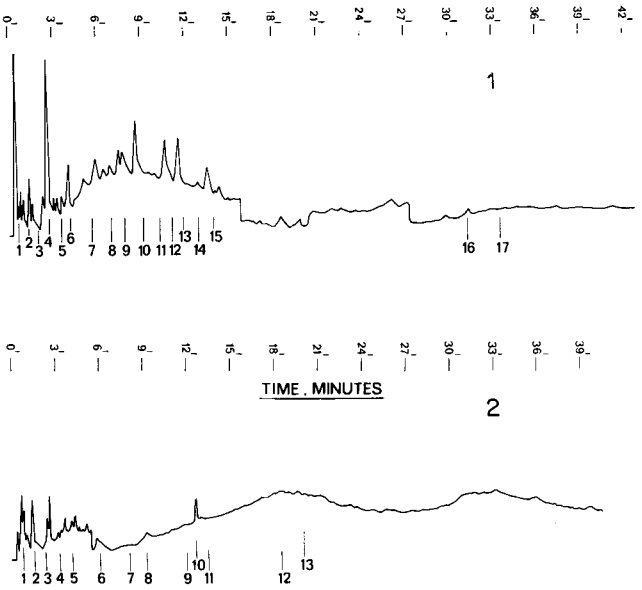


Figure 3—*Typical gas chromatograms of vaginal vapors. 1, Before treatment—odors: 1, pungent unpleasant; 2, very strong, unpleasant; 3, very unpleasant, cut liverlike; 4, very strong; 5, very unpleasant, nauseating; 6, no odor; 7, no odor; 8, very unpleasant; 9, sweet, unpleasant, nauseating; 10, very unpleasant; 11, very bad, nauseating; 12, very unpleasant, nauseating, repulsive; 13, very bad, repulsive, nauseating; 14, very unpleasant; 15, bad; 16, sharp; 17, slight, not unpleasant. 2, After treatment—odors: 1, moderate; 2, very pleasant, caramellike; 3, slight; 4, slight; 5, moderate; 6, slight; 7, no odor; 8, moderate; 9, slight; 10, not unpleasant, 11, no odor; 12, no odor; 13, moderate, not unpleasant. Some "no odor" peaks not listed.*

[2] Picrogram (pg.) = 10^{-12} g.
[3] Where "*n*" is a particular value in the system being analyzed.
[4] *cf.* Table V, Subject 6, Kovats Index range 500–600.

Table IV—Odorograms of Vaginal Vapors (Subjects 1–4)[a]

Kovats Index Ranges	Subject 1	Subject 2		Subject 3		Subject 4			
	1	1	2	1	2	1	2	3	4
<400	V	—		V				0	
400– 500					—	V		0	0
500– 600	X	V							
600– 650					—	VV	X		
650– 700				XL				0,0	0,0
700– 750	X,X	V	—	XL	—	V	X,X		0
750– 800				X		V	X	&,&	0
800– 850			—	X	—	V	X	&	0
850– 900	X,X				&	XXXL	X,X		&
900– 920				X		XXXL			
920– 940			&			XXX	X		&
940– 960	0		&			XXX			
960– 980	0			X		XXX	XXXX	&	0
980–1000	0			X			XXXX	&,&	0,0
1000–1020								&	
1020–1040				X		XXXX	XXXL		0
1040–1060	&	X		V			XXXL	0,0	0
1060–1080	&,&			XXX	&&	XXXX		0,0	0
1080–1100						XXXX		0,0	
1100–1120	XL			XXX		—		0	0,0
1120–1140	0,0	XXX				—	XXXL	&	0,0
1140–1160	X			XXX	&&		XXXL,XXX	&	0,0
1160–1180	X,X		&			—	XXXX	&	
1180–1200	XXX			X		XXX	XXX	&	
1200–1220	XXX,XXX					XXX	XXX	&	
1220–1240					—	XXXX	XXXX	0	
1240–1260	XXXX	XXX							—
1260–1280					0			0	
1280–1300	XXX					XXX	X	—	—
1300–1320	XXX			XXX		XXXX,XXXX	X	—,0	
1320–1340							XXXX	—	
1340–1360	XXXX					XXXX		0	
1360–1380						XXXX			
1380–1400					0	XXXX	X	—	0
1400–1420	0			XXXX		XXX,XXX	—	—	0
1420–1440	XXX							—,0	—
1440–1460				XXXX		XXX			
1460–1480	X							—	
1480–1500					0			—	
1500–1520				W		—			
1520–1540						—,—		0	
1540–1560	X	XXX			0				
1560–1580	XX			X					
1580–1600						—			
1600–1620		XXXX			—	—		—	
1620–1640	0					—	—	—	—
1640–1660		V							
1660–1680	—				—	—,—		(S)	0
1680–1700				XX					
1700–1720	W	XXXX			&	—	X		
1720–1740		X							
1740–1760	0,0					—,—	X	—	
1760–1780	0								
1780–1800						—			
1800–1820	0			V					
1820–1840									
1840–1860									
1860–1880									
1880–1900					&&				
1900–1920									
Seconds:									
1400–1550	XXXb	B		V	&&				
1550–1600				B		—	X		
1600–1650	—			B		—			
1650–1700	—			B	—		X		
1700–1750							X		
1750–1800	—			W		—,V			
1800–1850	0			—	B				
1850–1900	X			W		0			
1900–1950	—			W				B	
1950–2000					B	0	B		
2000–2050	—								
2050–2100	—								
2100–2150					—				
2150–2200									
2200	M			—					

[a] Weak, mild, not unpleasant, 0; unpleasant, X; bloodlike, XL; very unpleasant, XX; strong, sweatlike, XXX; nauseating, repulsively sweet, XXXX; pungent, sharp, V; waxy, W; medicinal, M; earthy, mushroomlike, E; sour, S; burnt, B; peak but no odor, —; in parentheses, odor, but no peak ( ); pleasant, & ; very pleasant, fragrant, & &. Comma separation used if two or more peaks were observed in the range.

Table V—Odorograms of Vaginal Vapors (Subjects 5–6)[a]

Kovats Index Ranges	Subject 5				Subject 6			
	1	2	3	4	1	2	3	4
<400								
400– 500			—			0	—,—	
500– 600	XXX	XXX,V		0	X	XXX	—	—
600– 650	XXX				X,XX		—	—
650– 700			&			X	—	
700– 750	V	XXX,XXX	&	&,&	XXX	0	&	—,—
750– 800	X				XXX	0,0,XXX		—
800– 850		XXXX	0	&		XX		&
850– 900	XX,X		0	&	XXXX	XXXX	&&	&
900– 920				0	—	XX		
920– 940			0					0
940– 960	XX			X		XXXX	0	
960– 980	XXX	M.M	0,M,&			XXX	0	
980–1000	XXX			0,&	XXX	XXXX	&	—
1000–1020					XXX	0	&	
1020–1040	M	M,X,XXXX	—	XXX	XX		&	X
1040–1060	M,M		—	X	XXXX	V	&	0
1060–1080	XXXX	XXXX,XXXX	—	X,X	XXXX	0	—	0
1080–1100	XXXX	XXXX,XXXX,XXX			XXXX	0,0	—,—	0,&
1100–1120	XXXX	XXX,Xb			XXX	0,0	—,—	&
1120–1140		Xb,Xb,Xb	—	X	0,0	E	—	0
1140–1160	X	XXXX,XXXX			0	E,B	—,—,—	
1160–1180	—,—				0	XXX	—,0	
1180–1200		0	0,0,W		0		X,—	—
1200–1220	XXXX	0		0	0			
1220–1240	XXXX			0,0			—	
1240–1260	0	XXXL				S		
1260–1280								X
1280–1300	0,0			—	XXX		—	
1300–1320	—,X	0		0	Xb			
1320–1340								
1340–1360	Xb			0	XXX			
1360–1380		X	0				—	
1380–1400	V			M				
1400–1420					X			
1420–1440			—					
1440–1460							—	
1460–1480	X							
1480–1500		0					—	
1500–1520	XXX		0			XXXX		
1520–1540				0			—	
1540–1560	E						—	
1560–1580			&		XXXX			
1580–1600								
1600–1620	XXXX	&	0	—				
1620–1640					0	XXXX	0	0
1640–1660		V						
1660–1680								
1680–1700		XXXX		—				
1700–1720	0		0		X			
1720–1740						0	—,(0)	—
1740–1760		XXXX			XXX			
1760–1780			0					
1780–1800		B	0		XXXX	X		0
1800–1820	V							
1820–1840								
1840–1860								
1860–1880								
1880–1900								
1900–1920	X				S	XXX	0	—
Seconds:								
1400–1550	V				X	XXXX	0,0	
1550–1600	B	B			—			0
1600–1650	V		B		XXX			
1650–1700	B			0		S		
1700–1750								
1750–1800				—				—
1800–1850	V				B	XXXX	B	—
1850–1900			—	—	V,B		—	0
1900–1950			—					0
1950–2000	B			B				
2000–2050			—		V	B		B
1050–2100	V,—				B	B		B
2100–2150							B	
2150–2200	B			B	0,0	E		
2200	V,B		—,—,—,—	B				

[a] Weak, mild, not unpleasant, 0; unpleasant, X; bloodlike, XL; very unpleasant, XX; strong, sweatlike, XXX; nauseating, repulsively sweet, XXXX; pungent, sharp, V; waxy, W; medicinal, M; earthy, mushroomlike, E; sour, S; burnt, B; peak but no odor, —; in parentheses, odor, but no peak ( ); pleasant, &; very pleasant, fragrant, & &. Comma separation used if two or more peaks were observed in the range.

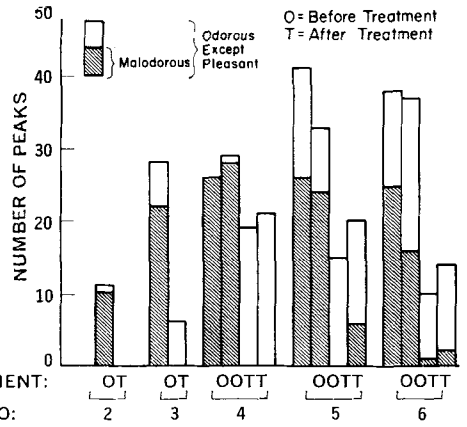


Figure 4—*Changes in number of odorous peaks in vaginal-vapor chromatograms upon nitrofurazone treatment.*

pleasant peaks coexist, and may be only partially resolved, malodor control leads to a decrease of malodorous components. As a result overall odor at that zone of the chromatogram changes from malodorous toward the neutral or fragrant. Also, odorous peaks can become smaller and nondetectable at the sniffing port whenever the odorant there falls below its odor threshold. In essence the process serves as a "spectrograph" for the odorants, with magnification and focussing to aid the odor observations.

Figure 3 illustrates gas chromatograms obtained in a case in which overall odor intensity of vaginal vapors decreased sharply upon treatment with nitrofurazone. The number of components observed in the gas chromatogram also decreased. In this case, the most significant change occurred in the odor character of the peaks:

Chromatogram No.	Number of Peaks			
	Objectionable	No Odor	Neutral	Fragrant
1	14	13	2	0
2	—	4	9	1

Schedule of Tests—Table II shows the diagnoses and the arrangement of test samplings. All subjects were selected from hospitalized patients.

The suppositories (Rx) contained nitrofurazone, 0.3%, in a base of glyceryl monolaurate and polyoxyethylene (4) sorbitan monostearate. They melt at body temperature and are self-emulsifying in vaginal fluids. It is assumed that the 2-g. suppository was spread fairly evenly over the vaginal mucosa. The suppository did not exhibit fragrant or objectionable odor.

RESULTS AND DISCUSSION

Odor Thresholds—Measurements of odor thresholds are summarized in Table III. In all cases the threshold increased upon treatment with nitrofurazone. To make the odor unnoticeable, less nitrogen was needed to dilute the vapors after treatment than before. For the six subjects this difference reaches the 95% confidence level (error probability of 0.05 or less by the Wilcoxon test).

There was a tendency for this effect to diminish as thesholds increased, with a rank-correlation coefficient of +0.74, somewhat short of the +0.83 value required for the 95% confidence level.

Since during odor-threshold studies the insert was at room rather than at body temperature, the absolute threshold values are lower; differences between them, however, are valid for comparisons. Also, the character (quality) of an odor is not considered in measurement of threshold.

Reciprocals of odor thresholds cannot, *per se*, uniquely describe odor intensities. Even in the absence of fragrant components, vapors with lower thresholds do not necessarily have higher odor intensities when not so diluted. The odor threshold data were instructive and indicated that odor was controlled by treatment. However, a better insight can be gained into what occurs with the odor complex during treatment from inspection of the odorograms.

Gas-Chromatographic Assays—Data from odor assays combined with gas-chromatographic separation of vapor components are compiled in Tables IV and V. The Kovats Index coordinates' numerical increase indicates that the vapor pressures of the corresponding odorants were decreasing, or their solubilities in the polar stationary phase (polyethylene glycols)[5] were increasing. No calibration was available toward the end of the scale, where values are listed simply as seconds of retention time and therefore are valid only relatively. Coding of odor characters is indicated in the legends to Tables IV and V.

These odorograms indicate clearly changes in vapor composition associated with use of nitrofurazone suppositories.

Case 1 was that of a normal subject. Vapor-component odors were distributed widely over the range from objectionable (only two peaks were in the most objectionable category) through many neutral to some pleasant components.

In the other five cases investigated, pretreatment odorograms were dominated by components of varying objectionability. After nitrofurazone treatment few, if any, objectionable odorants were observed. Some fragrant notes occurred after treatment, and many components were represented by small peaks unassociated with odor; these components were present at levels far below their odor thresholds. Regions where malodors were present before treatment were not populated by odorous peaks after treatment, or showed peaks with lower odor intensities. These may correspond either to the same substances as were present before treatment, now at lower concentrations, or to new (*e.g.*, fragrant) substances, or to a mixture of the two with objectionable components in part reduced.

As is evident from inspection of Tables IV and V, the effect of treatment cannot be explained by mere masking of unpleasant peaks by fragrant components. In several gas-chromatographic positions where highly objectionable peaks were observed before treatment, no fragrant or other odorous peaks appeared after treatment.

Figure 4 shows the frequency of occurrence of various categories of odors in the vaginal vapors studied, regardless of positions of the gas-chromatographic peaks of these odorants.

The chemical identification of the malodorous components would be significant and should represent a natural extension of this work. However, because of the very low concentrations of the odorants, this is a formidable task and was not undertaken at the present time. For an orientation (see Tables IV and V), the components with Kovats Index of 1000–1100 correspond to the substances boiling in the range of 100–180° and having molecular weight of 80–150. Substances with larger indexes boil higher and have larger molecular weights.

The corresponding relative areas are not useful for comparison of odor relevance since substances vary so greatly in thresholds and intensities of odors. A small peak may be associated with a strong odor and a large peak may be odorless. Even comparison of what appears to be the same peak in two gas chromatograms can be misleading. For example, a peak may correspond to an unresolved mixture of two substances, one contributing most of the area but no odor, the other being responsible for the odor but for only a small fraction of the peak's area. If no odor is observed in the presence of a peak from the sniffing port, this can indicate the presence of components which might still be odorous at a higher concentration.

In odor assays by the technique outlined previously, the method should be considered as a help in separating various odor notes so that they can be surveyed more accurately. The gas chromatograms indicate approximate chromatographic locations of component odorants, convenient for systematization and display.

SUMMARY AND CONCLUSIONS

A technique for gas-chromatographic separation and assay of the components of complex vaginal odors, assumed to be due in large part to bacterial action, was applied to the study of vaginal odors before and after treatment with nitrofurazone antibacterial vaginal suppositories. Odor thresholds changed under treatment in a direction which indicated reductions in the noticeability of odors at a distance. The assays indicated that major changes occurred in the composition of the complex odors, with significant reduction in the occurrences of objectionably odorous components at odor-threshold

[5] *n*-Alkanes are designated by their number of carbon atoms times 100; thus the number for *n*-decane is 1000. Polar compounds' numbers are higher, relative to the number of C atoms (*e.g.*, *n*-butanol's number of 1113).

and suprathreshold concentrations. Under treatment of the vagina with the suppository, the respective odorograms became populated by nonobjectionably odorous components and some fragrant ones which may have been derived from the suppository preparation (although its composition and odor qualities provide no support for this statement). In some gas-chromatographic positions which were populated by malodors before treatment, no odorants were found after treatment; the effect of treatment therefore is not a simple masking but rather the result of simultaneous reduction in malodorous components and increased dominance of milder odors and fragrances.

REFERENCES

(1) S. S. Stevens, in "Psychophysics of Sensory Function in Sensory Communication," Rosenblith, Ed., M.I.T. Press, Cambridge, Mass., 1961, p. 1.

(2) S. S. Stevens, *Psychol. Rev.*, **64,** 153(1957).

(3) ASTM Manual on Sensory Testing Methods, ASTM, No. 434, 1968, p. 24.

(4) B. H. Ellis, *Food Sci. Technol.*, **3,** 307(1967).

(5) ASTM, Tentative Test for Odor in Industrial Water and Waste Water, ASTM Spec. Publ. 148-F, ASTM D-1292-617 (1962).

(6) A. Dravnieks and B. K. Krotoszynski, *J. Gas Chromatogr.*, **6,** 144(1968).

(7) A. Dravnieks and B. K. Krotoszynski, to be published.

(8) E. sz Kovats, *Advan. Gas Chromatogr.*, **1,** 229(1965).

(9) A. Dravnieks, B. K. Krotoszynski, W. E. Lieb, and E. Jungermann, *J. Soc. Cosmet. Chem.*, **19,** 611(1968).

(10) A. Dravnieks, B. K. Krotoszynski, R. E. Caseley, and D. Taber, *Proc. Sci. Sect. Toilet Goods Ass.*, **1,** 14(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 27, 1969, from *IIT Research Institute, Chicago, IL 60616*

Accepted for publication December 8, 1969.

* Present address: Department of Urology, Cook County Hospital, and the Chicago Medical School, Chicago, Ill.

Spectral Studies of Trimethylsilyl Ether of Chloramphenicol

MICHEL MARGOSIS

Abstract □ The trimethylsilyl ether of chloramphenicol used in GLC analysis has been characterized by UV, near IR, IR, NMR, and mass spectroscopy, confirming the structure of the *O,O*-ditrimethyl siloxy derivative. Pyridine and acetonitrile, used as solvents in the silylation reaction, promote formation of diastereomers. Addition of hydroxylic solvents promotes the inversion of configuration and causes partial solvolysis. To verify this, two fractions were collected from a GLC column and analyzed by IR and mass spectroscopy. The experimental evidence presented establishes the two fractions as diastereomers.

Keyphrases □ *O,O*-Ditrimethyl siloxy chloramphenicol diastereomers—structure confirmation, effect of pyridine, acetonitrile □ Trimethylsilyl ether—chloramphenicol derivative, effect of pyridine, acetonitrile □ IR—structure, identification □ UV—structure, identification □ NMR—structure, identification □ Mass spectrometry—structure, identification □ GLC—analysis, separation

Chloramphenicol, D(−)-*threo*-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol, is a certifiable antibiotic for which neither of the current official assay methods (microbiological and UV procedures) (1) is specific. Gas-liquid chromatographic (GLC) procedures were investigated for chloramphenicol assay; however, when chloramphenicol was chromatographed on a QF-1 column above 200°, poorly resolved peaks were obtained, which were probably due to thermal breakdown products (Fig. 1). When it was chromatographed on a DC-200 or SE-30 column, no peaks of any kind were obtained.

Several workers (2–4) have successfully applied the silylation method of Bentley *et al.* (5) to this determination. However, several problems arose when the method was tried in this laboratory. The principal difficulties included contamination of the anode of the flame-ionization detector and solvent tailing, similar to that previously observed in the GLC of lincomycin (6). To overcome these difficulties, the technique was modified as follows: a previously developed extraction procedure (6) was used; methanol or other hydroxylic solvent was added to the sample immediately before injection into the gas chromatograph; different solvent systems were used; and the reaction mixture was evaporated to dryness and reconstituted in an inert solvent.

The TMS derivative prepared by the procedure of Bentley *et al.* (5) exhibited a single symmetrical chromatographic peak at 5.7 min. (Fig. 2) on each of the three columns (7), and recovery was quantitative. Freshly prepared solutions of the derivative gave no other significant peaks when injected directly. After standing (at least overnight), these solutions began to exhibit secondary peaks at 9.2 min., which reached limiting values of 2–3.5% of the area in the case of ethyl acetate or methylene chloride solutions but increased to about 50% in pyridine or acetonitrile. The addition of small amounts of methanol or other hydroxylic solvents tended to accelerate this reaction. With increasing amounts of methanol, additional peaks at 4.3 min. were noted on the QF-1 column (Fig. 3) but not on the DC-200 or SE-30 columns. When the methanol solution of the derivative was partitioned between water and carbon tetrachloride, the chromatogram of the nonpolar fraction showed two peaks (at 5.7 and 9.2 min.; Fig. 4) attributed to the TMS ether of chloramphenicol and its *erythro* isomer, while the chromatogram of the polar fraction was similar to that of the

and suprathreshold concentrations. Under treatment of the vagina with the suppository, the respective odorograms became populated by nonobjectionably odorous components and some fragrant ones which may have been derived from the suppository preparation (although its composition and odor qualities provide no support for this statement). In some gas-chromatographic positions which were populated by malodors before treatment, no odorants were found after treatment; the effect of treatment therefore is not a simple masking but rather the result of simultaneous reduction in malodorous components and increased dominance of milder odors and fragrances.

REFERENCES

(1) S. S. Stevens, in "Psychophysics of Sensory Function in Sensory Communication," Rosenblith, Ed., M.I.T. Press, Cambridge, Mass., 1961, p. 1.

(2) S. S. Stevens, *Psychol. Rev.*, **64,** 153(1957).

(3) ASTM Manual on Sensory Testing Methods, ASTM, No. 434, 1968, p. 24.

(4) B. H. Ellis, *Food Sci. Technol.*, **3,** 307(1967).

(5) ASTM, Tentative Test for Odor in Industrial Water and Waste Water, ASTM Spec. Publ. 148-F, ASTM D-1292-617 (1962).

(6) A. Dravnieks and B. K. Krotoszynski, *J. Gas Chromatogr.*, **6,** 144(1968).

(7) A. Dravnieks and B. K. Krotoszynski, to be published.

(8) E. sz Kovats, *Advan. Gas Chromatogr.*, **1,** 229(1965).

(9) A. Dravnieks, B. K. Krotoszynski, W. E. Lieb, and E. Jungermann, *J. Soc. Cosmet. Chem.*, **19,** 611(1968).

(10) A. Dravnieks, B. K. Krotoszynski, R. E. Caseley, and D. Taber, *Proc. Sci. Sect. Toilet Goods Ass.*, **1,** 14(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 27, 1969, from *IIT Research Institute, Chicago, IL 60616*

Accepted for publication December 8, 1969.

* Present address: Department of Urology, Cook County Hospital, and the Chicago Medical School, Chicago, Ill.

Spectral Studies of Trimethylsilyl Ether of Chloramphenicol

MICHEL MARGOSIS

Abstract □ The trimethylsilyl ether of chloramphenicol used in GLC analysis has been characterized by UV, near IR, IR, NMR, and mass spectroscopy, confirming the structure of the *O,O*-ditrimethyl siloxy derivative. Pyridine and acetonitrile, used as solvents in the silylation reaction, promote formation of diastereomers. Addition of hydroxylic solvents promotes the inversion of configuration and causes partial solvolysis. To verify this, two fractions were collected from a GLC column and analyzed by IR and mass spectroscopy. The experimental evidence presented establishes the two fractions as diastereomers.

Keyphrases □ *O,O*-Ditrimethyl siloxy chloramphenicol diastereomers—structure confirmation, effect of pyridine, acetonitrile □ Trimethylsilyl ether—chloramphenicol derivative, effect of pyridine, acetonitrile □ IR—structure, identification □ UV—structure, identification □ NMR—structure, identification □ Mass spectrometry—structure, identification □ GLC—analysis, separation

Chloramphenicol, D(−)-*threo*-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol, is a certifiable antibiotic for which neither of the current official assay methods (microbiological and UV procedures) (1) is specific. Gas-liquid chromatographic (GLC) procedures were investigated for chloramphenicol assay; however, when chloramphenicol was chromatographed on a QF-1 column above 200°, poorly resolved peaks were obtained, which were probably due to thermal breakdown products (Fig. 1). When it was chromatographed on a DC-200 or SE-30 column, no peaks of any kind were obtained.

Several workers (2–4) have successfully applied the silylation method of Bentley *et al.* (5) to this determination. However, several problems arose when the method was tried in this laboratory. The principal difficulties included contamination of the anode of the flame-ionization detector and solvent tailing, similar to that previously observed in the GLC of lincomycin (6). To overcome these difficulties, the technique was modified as follows: a previously developed extraction procedure (6) was used; methanol or other hydroxylic solvent was added to the sample immediately before injection into the gas chromatograph; different solvent systems were used; and the reaction mixture was evaporated to dryness and reconstituted in an inert solvent.

The TMS derivative prepared by the procedure of Bentley *et al.* (5) exhibited a single symmetrical chromatographic peak at 5.7 min. (Fig. 2) on each of the three columns (7), and recovery was quantitative. Freshly prepared solutions of the derivative gave no other significant peaks when injected directly. After standing (at least overnight), these solutions began to exhibit secondary peaks at 9.2 min., which reached limiting values of 2–3.5% of the area in the case of ethyl acetate or methylene chloride solutions but increased to about 50% in pyridine or acetonitrile. The addition of small amounts of methanol or other hydroxylic solvents tended to accelerate this reaction. With increasing amounts of methanol, additional peaks at 4.3 min. were noted on the QF-1 column (Fig. 3) but not on the DC-200 or SE-30 columns. When the methanol solution of the derivative was partitioned between water and carbon tetrachloride, the chromatogram of the nonpolar fraction showed two peaks (at 5.7 and 9.2 min.; Fig. 4) attributed to the TMS ether of chloramphenicol and its *erythro* isomer, while the chromatogram of the polar fraction was similar to that of the

Figure 1—*Chloramphenicol on QF-1 column at 238°.*

parent compound, with its major fragment peak at 4.3 min., reverted by solvolysis.

By chromatographing chloramphenicol in the three different columns, it has also been determined that the same TMS derivative is obtained using either the procedure of Bentley *et al.* (5) (see *Experimental*) or *N,O*-bis(trimethylsilyl) acetamide.

In the first phase of this study, the TMS derivative, as originally prepared for GLC analysis, was characterized and identified by UV, IR, NMR, and mass spectroscopy. In the second phase, the two nonpolar chromatographic effluents were trapped with a simple collection system and identified by IR and mass spectroscopy.

EXPERIMENTAL

Instrumental Analysis—*Gas Chromatography*—A Barber-Colman model 5000 was used with a flame-ionization detector and a 1.83-m. (6-ft.) × 3-mm. U-shaped glass column packed with 5% QF-1 on 80–100-mesh Gas Chrom Q. The carrier gas was nitrogen at 23 p.s.i., 58 ml./min., and the column temperature was 238°. For fraction collection, a 23:1 splitter was connected to the exit of the column, with the column temperature at 250° and the collector tube temperature at 240°.

Gas Chromatography–Mass Spectroscopy—An LKB model 9000 combination gas chromatograph–mass spectrometer equipped with a 1.83-m. (6-ft.) × 0.635-cm. (0.25-in.) stainless steel column packed with 3% OV-17 on Gas Chrom Q was used. The temperatures were: flash heater, 270°; column, programmed from 200°; and the separator and source, 300°. The carrier gas was helium and the ionization potential was set at 70 ev.

Mass Spectrometry—The mass spectrometer was an Atlas CH4 equipped with a probe inlet system permitting the sample temperature to be maintained independently from the ion source. The conditions were as follows: ion source regulated at 250°; the ionization potential, 70 ev.; magnetic scanning; accelerating voltage, 3 kv.; and sample temperature, 60–80°.

Nuclear Magnetic Resonance—Varian A-60 spectrometers were used.

Spectrophotometry—UV and near IR spectra were obtained with a Cary 14 spectrophotometer with 1-cm. and 10-cm. cells, respectively. The IR spectra were obtained with Beckman IR-12 and Perkin-Elmer 457 spectrophotometers.

Sample Preparation—*Sample 1 (S1)*—Deuterated chloramphenicol was obtained by treating chloramphenicol with D_2O and freeze-drying.

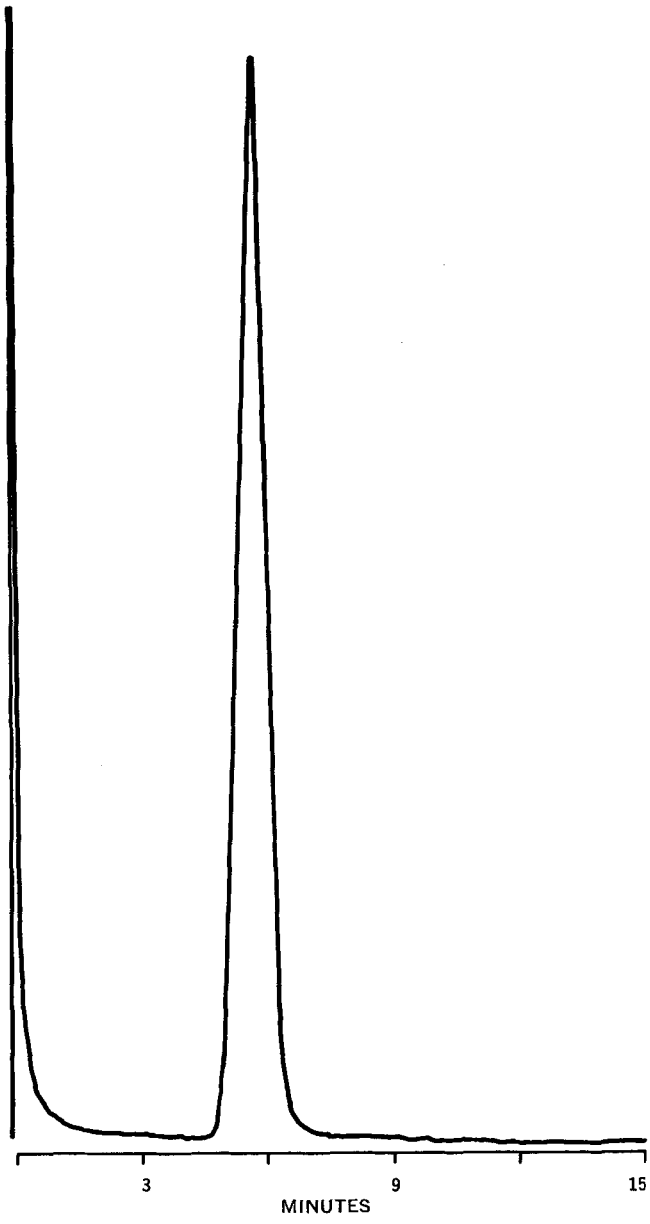


Figure 2—*TMS ether of chloramphenicol (S2) on QF-1 column at 238°.*

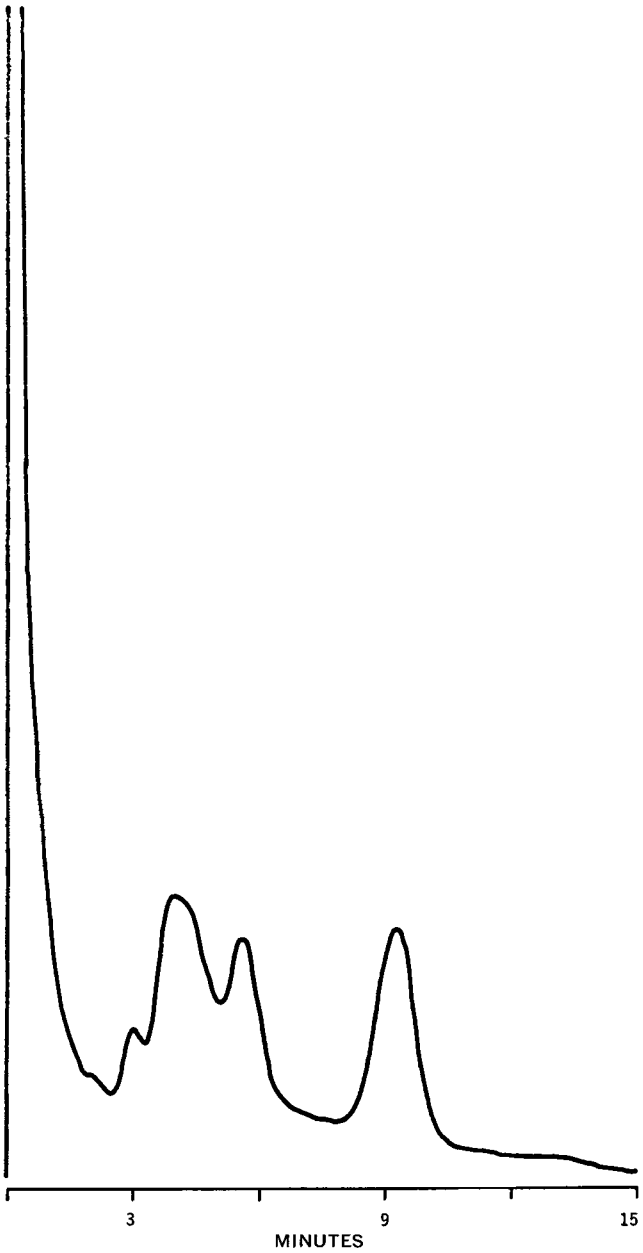


Figure 3—*TMS ether of chloramphenicol with methanol added (S3 before extraction) on QF-1 column at 238°.*

Sample 2 (S2)—The derivative "standard" used for this qualitative analysis was prepared by the Bentley *et al.* (2) procedure which consists of dissolving an appropriate amount of chloramphenicol in pyridine, adding an excess mixture of hexamethyldisilazane and trimethylchlorosilane (9:1), and allowing the mixture to stand 30–60 min. About 75% of the mixture was evaporated with heat and air to dryness; the residue was dissolved in carbon tetrachloride, filtered, and reevaporated to dryness. Solutions were made from this stock sample in appropriate solvents for GLC and spectral analyses.

Sample 3 (S3)—The sample for effluent analysis was prepared as described for S2, but after standing for 30 min., methanol was added to about 5% of original volume, and the solution was allowed to stand overnight. An equal amount of CCl_4 was added and the mixture was shaken. Water was then added and the CCl_4 was extracted. The extract was washed twice with water and passed through a CCl_4-saturated wad of glass wool. About 50 μl. of this CCl_4 solution was injected into the chromatograph. Fraction collection was started as soon as the first peak began to appear by connecting an open-end glass capillary tube to the split end of the column exit with a sleeve of Teflon "spaghetti" tubing and leaving it connected until near the end of the peak. Another glass capillary was similarly connected to collect the second fraction. The separations and collections were repeated until enough material was collected for the various analyses.

The effluent samples were prepared for mass spectrometry by rinsing each capillary tube with 10 μl. of CCl_4 into a gold microcup which was placed in the instrument inlet probe after air evaporation. Samples for IR analyses were prepared by rinsing each capillary tube with 10 μl. of CCl_4 onto a small amount of KBr. The KBr was then ground in a boron carbide mortar until dry, pressed into a 1-mm. disk, and scanned on an IR spectrophotometer equipped with a beam condenser.

RESULTS AND DISCUSSION

Characterization of the TMS Derivative—There are four possible isomers of *p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol: two enantiomers each of the *threo* and of the *erythro* configuration. However, only the D (—)-*threo* conformer (chloramphenicol) possesses therapeutic antibiotic activity.

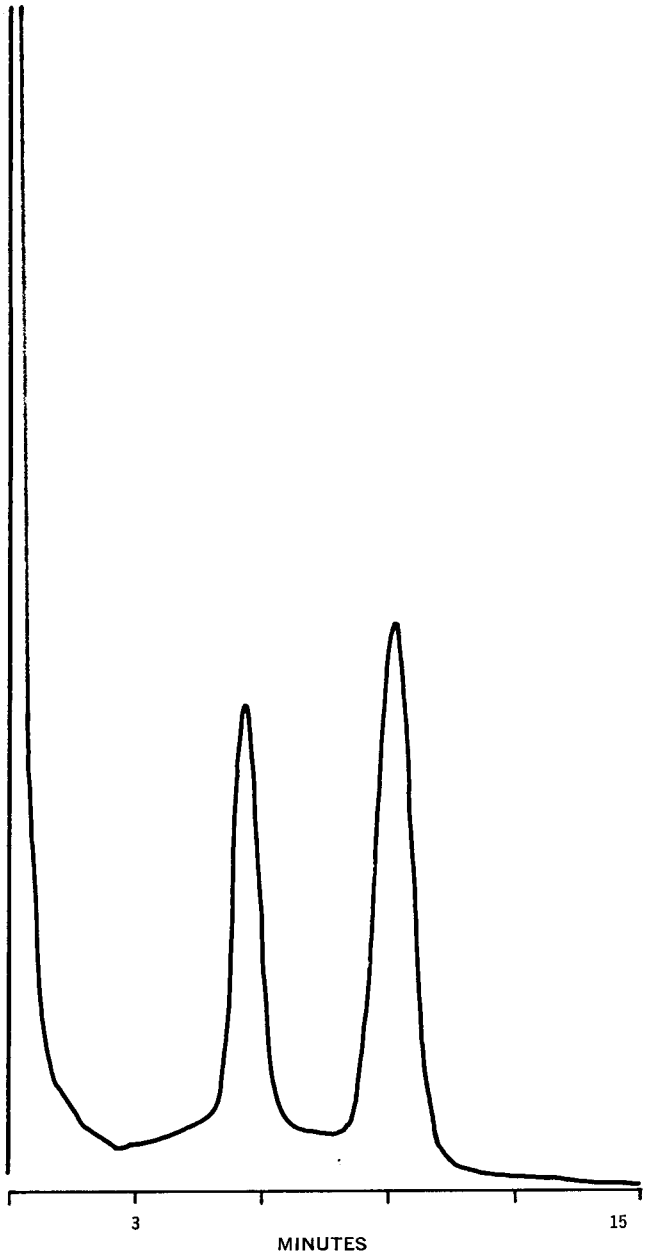


Figure 4—*Carbon tetrachloride extract of mixture in Fig. 3 (S3) on QF-1 column at 238°.*

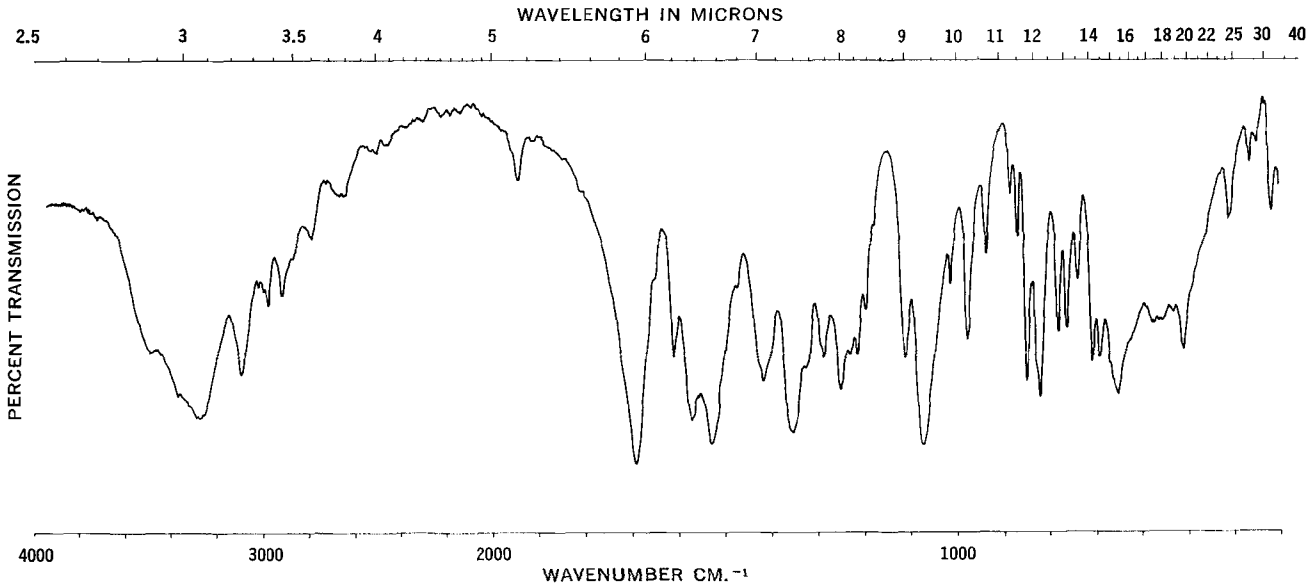
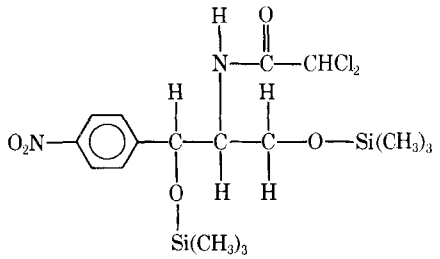


Figure 5—*IR spectrum of chloramphenicol KBr disk.*

The silylation reaction that produces a derivative sufficiently volatile and stable for use in GLC analysis yields the *O,O*-ditrimethylsilyl ether as represented in Structure I. Substantiation of this structure forms the main part of this study.



I

Morphology—The trimethylsilyl ether of chloramphenicol (S2) is a colorless, amorphous solid that crystallizes slowly over a period of weeks and melts below 55°. In contrast, chloramphenicol is a white crystalline solid melting at around 152°.

Ultraviolet Analyses—The UV spectrum of S2 in cyclohexane exhibited a single absorption band with a maximum at 264 mμ and an absorptivity of 10,900 l./mole cm. A methanolic solution showed a maximum at 273 mμ whereas chloramphenicol itself showed a maximum at 278 mμ in water with absorptivity of 9630 l./mole cm.

The UV spectrophotometric method has been demonstrated to be rather nonspecific (8), since the single absorption band near 280 mμ ascribed to the n-π transition of the nitrophenyl nucleus is also exhibited by many structurally related compounds (9, 10). Yamamoto *et al.* (4) reported that chloramphenicol, *p*-nitrophenylserinol (1-phenyl-2-amino-1,3-propanediol), and *N*-acetyl-*p*-nitrophenylserinol were separated on a SE-52 column above 200°. These workers collected the effluents and decided that they were not degradation products on the basis of UV spectrophotometry. This seems to contradict the fact that chloramphenicol was previously shown by thermogravimetry to decompose rapidly at about 160° (11) as well as the author's findings of thermolysis of chloramphenicol on the QF-1 column.

Infrared Analyses—A near IR spectrum of S2 in CCl_4 showed the presence of an amide proton at 6702 and 4926 cm.$^{-1}$, aro-

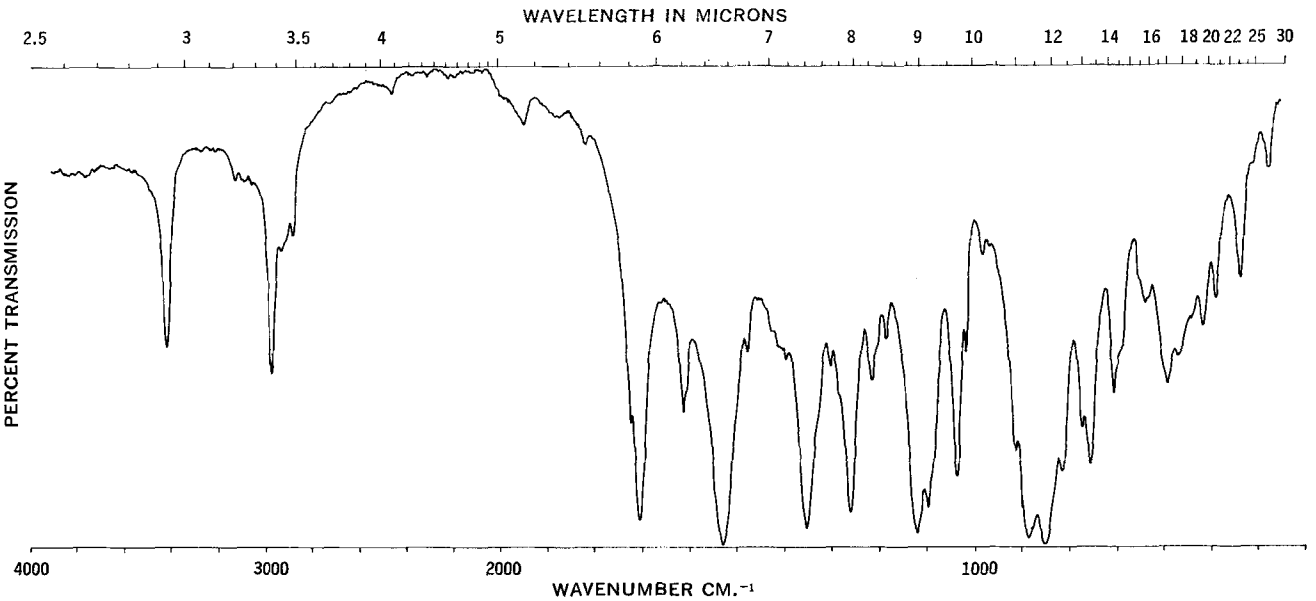


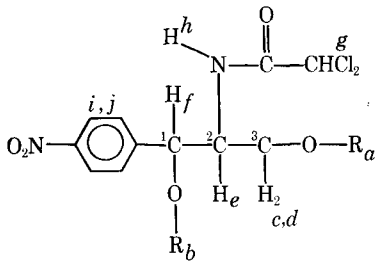
Figure 6—*IR spectrum of TMS ether of chloramphenicol (S2).*

matic protons at 6050, 5907, and 4615 cm.$^{-1}$, and methyl protons at 4355 cm.$^{-1}$

IR spectra of chloramphenicol and S2 were obtained as KBr disks (Figs. 5 and 6). Significant absorption bands common to both spectra were those of the amide proton stretching mode at about 3400 cm.$^{-1}$, the amide carbonyl fundamental at about 1695 cm.$^{-1}$, the aromatic nucleus at 1610 cm.$^{-1}$, the nitro group at 1527 and 1351 cm.$^{-1}$, and the aromatic substitution pattern at 2000–1667 and 758 cm.$^{-1}$

The spectrum of S2 showed additional new intense bands at 2967 and 2882 cm.$^{-1}$ attributed to the symmetric and asymmetric stretching mode of the six methyl groups; at 1259 cm.$^{-1}$ due to the rocking deformation mode of the methyl groups; at 1135 and 1120 cm.$^{-1}$ due to the Si—O—C stretching; at 850 and 755 cm.$^{-1}$ due to the Si—C bond. The position, shape, and intensity of the absorption bands at 1259, 850, and 755 cm.$^{-1}$ are particularly characteristic of Si—$(CH_3)_3$ groups. The absorption bands due to the hydroxyl functions near 3330 cm.$^{-1}$ have disappeared.

Nuclear Magnetic Resonance—The NMR spectra of S2 in CCl_4 and in deuterated acetone were obtained and compared to that of deuterated chloramphenicol (S1) in deuterated acetone. Replacing the three readily exchangeable protons with deuterium clarified the spectrum to some extent by eliminating three peaks and reducing the splitting pattern and peak overlap. A typical spectrum obtained from a CCl_4 solution is shown in Fig. 7 and assignments of peaks are given in Table I.



A comparison of spectra obtained from deuterated acetone solutions shows that the spectrum of S2 was, as expected, similar to that of S1 except for the NH proton at 7.45 p.p.m., and the two trimethylsilyl groups of 0.10 and 0.15 p.p.m.

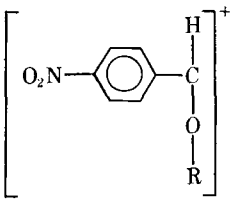
The C_3 proton peaks were found at 3.63 and 3.75 p.p.m. compared with 3.75 and 3.85 p.p.m. in the deuterated compound. Similarly, the C_2 proton peak appeared at 4.07 *versus* 4.25 p.p.m., the C_1 proton peak at 5.35 *versus* 5.33 p.p.m., the $\underline{C}HCl_2$ proton peak at 6.25 *versus* 6.35 p.p.m., and the aromatic proton peaks at 7.95 p.p.m. for both compounds. Thus, the silylation of chloramphenicol results in a variable upfield shift of the peaks, believed to be most likely due to the shielding effects of the silyl function.

The NH proton coupling with the C_2 proton (J = 9 c.p.s.) shows up as a doublet. The aromatic proton splitting is typical of *p*-nitro substitution. The $\underline{C}HCl_2$ and the methyl protons of the siloxy groups at C_1 and C_3 gave rise to singlets. The C_1 proton doublet peak is indicative of a coupling with the C_2 proton. The latter, in turn, exhibited a more complex multiplet because it couples with the amide proton as well as with one at C_1 and two at C_3. The two C_3 protons may also be expected to be nonequivalent because of their adjacent position to an asymmetric carbon.

The two singlet peaks observed for the trimethylsilyl groups are clear evidence of the nonequivalence of the two groups. This nonequivalence may be due partly to the diamagnetic anisotropy of the benzene nucleus. Similarly, the two doublets centered at 4.16 p.p.m. indicate that the two C_3 protons are nonequivalent. The relative values of the integrated peaks are also consistent with the proposed structure.

Mass Spectroscopy—In the electron-impact mass spectrum of chloramphenicol (Fig. 8) and S2 (Fig. 9), some fragmentation may be common to both compounds or may be associated solely with silyl ethers (12–15).

One mode of scission common to both compounds occurs between C_1 and C_2, resulting in two major fragments (I and II), where R is H in chloramphenicol and $Si(CH_3)_3$ in S2.



I

(*a*) R = H, *m/e* 153

(*b*) R = $Si(C_3H)_3$, *m/e* 225

$ROCH_2CH=\overset{+}{N}HCOCHCl_2$

II

(*a*) R = H, *m/e* 170

(*b*) R = $Si(CH_3)_3$, *m/e* 242

In chloramphenicol, the two fragments appear as the base peak at *m/e* 153 (RI = 100%; Σ_{35}, the calculated percentage of total ionization over the range *m/e* 35 to M^+, = 6.16%) and at *m/e* 170 (RI = 56%; Σ_{35} = 3.42%). High resolution has shown the *m/e* 153 peak to be a doublet due to the loss of HO from Fragment II as the main fraction, whereas the other is ascribed to protonated Fragment I*a* (16). S2 is ruptured at the same site with protonated Fragment I*b* at *m/e* 225 (RI = 58%; Σ_{35} = 14.94%), and Fragment II*b* at *m/e* 242 (RI = 4.9%; Σ_{35} = 1.27%).

Fragmentations generally ascribed to trimethylsilyl ethers are expectedly found in the spectrum of S2. The molecular ion is barely perceptible at *m/e* 466 (RI = 0.012%; Σ_{35} = 0.003%) at

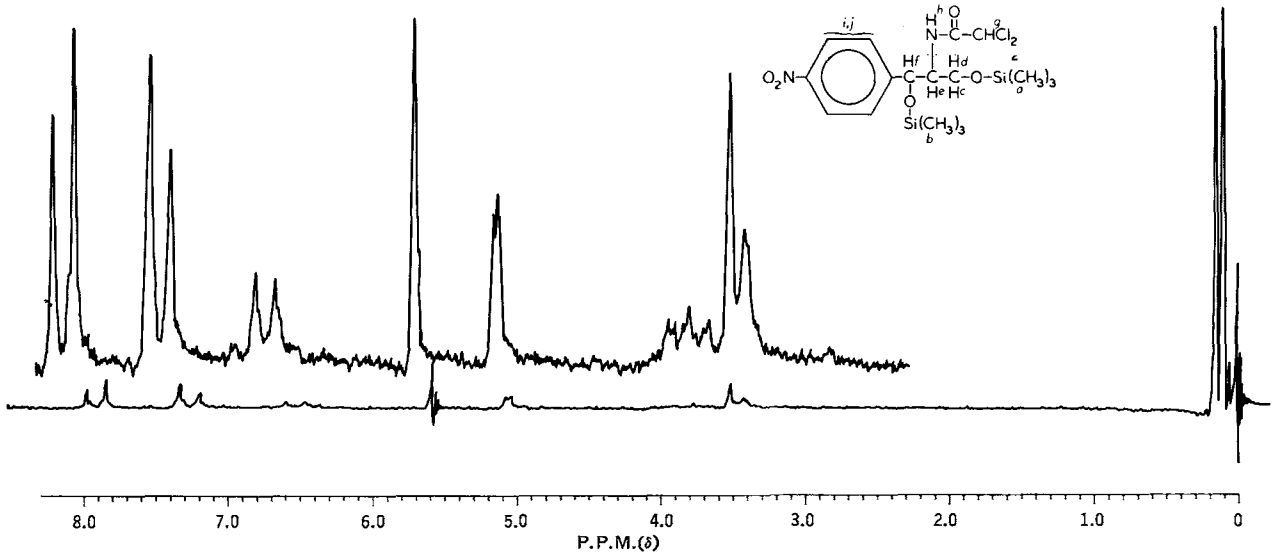


Figure 7—*NMR spectrum of TMS ether of chloramphenicol (S2) in CCl_4.*

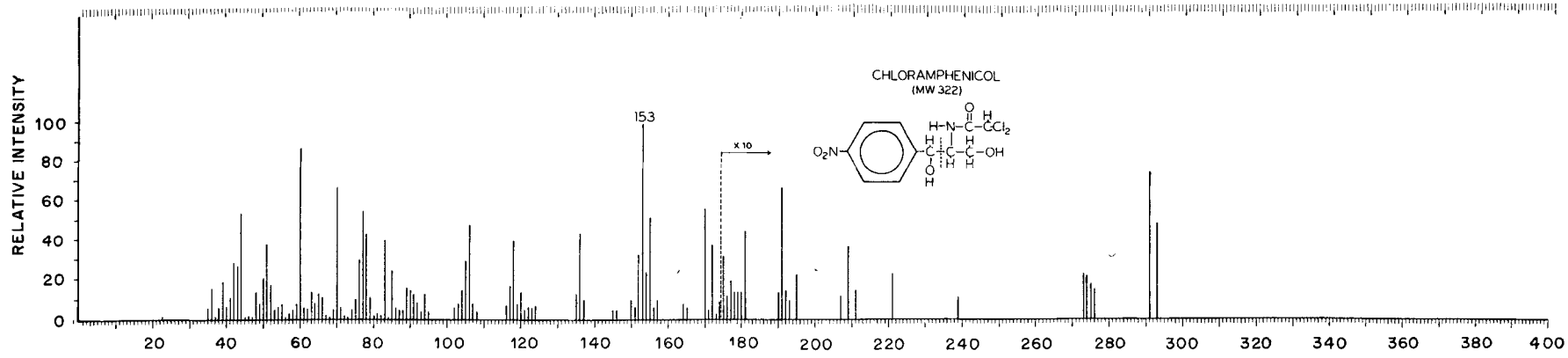


Figure 8—*Mass spectrum of chloramphenicol.*

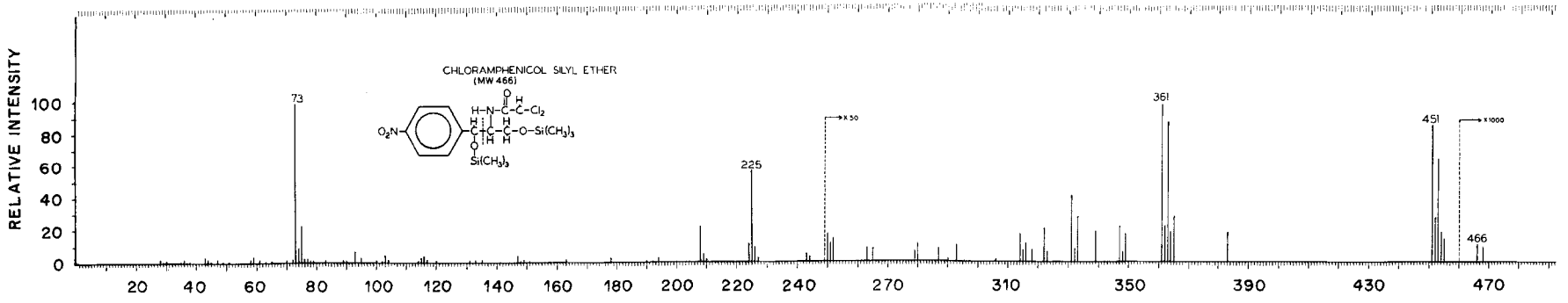


Figure 9—*Mass spectrum of TMS ether of chloramphenicol (S2).*

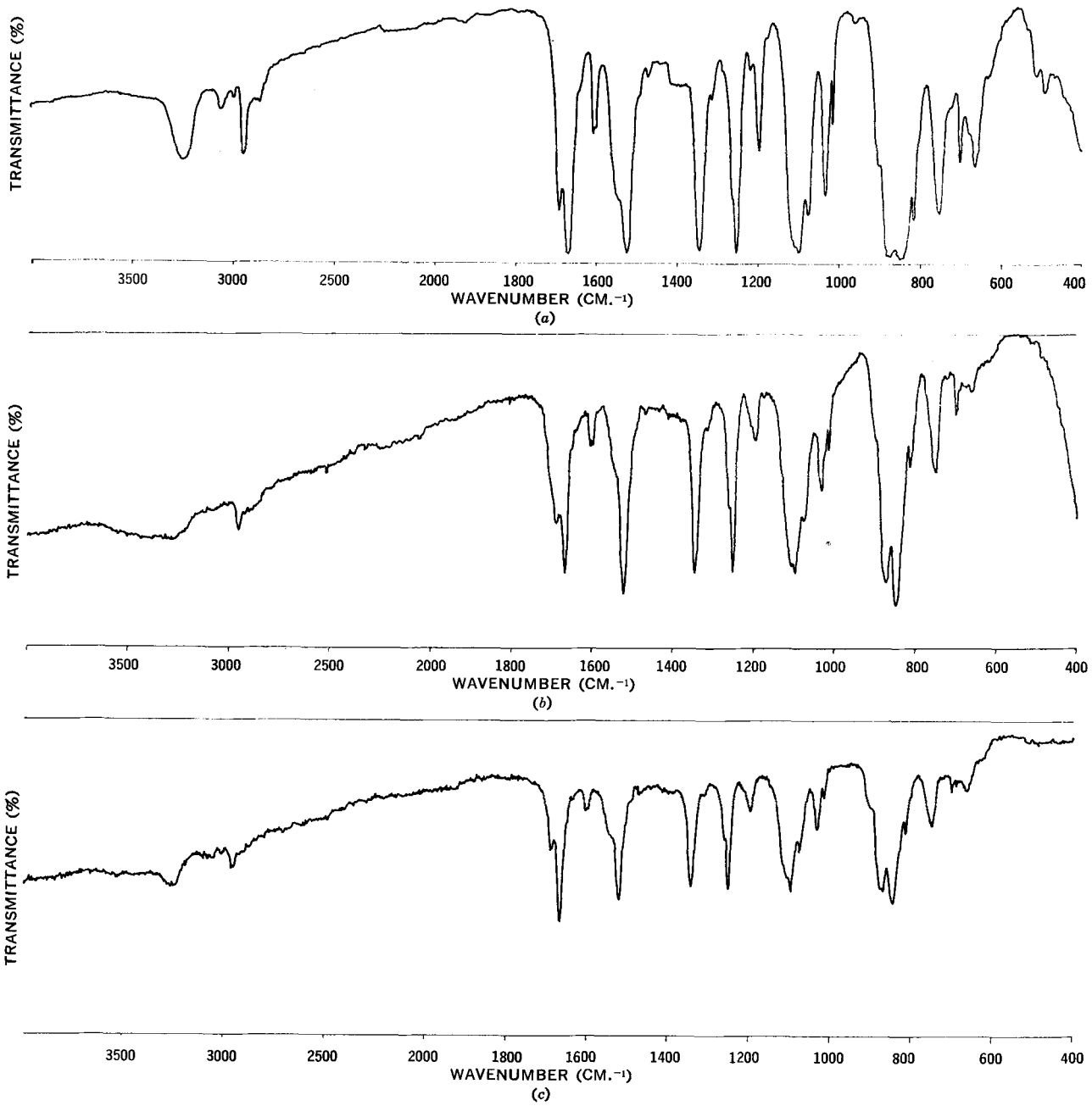


Figure 10—*IR spectra of microsamples:* (a) *Standard TMS ether of chloramphenicol (S2).* (b) *Chromatographic Fraction I from S3.* (c) *Chromatographic Fraction II from S3.*

a high instrument sensitivity. The loss of one methyl group to yield the $(M\text{-}15)^+$ peak at *m/e* 451 (RI = 1.8%; Σ_{35} = 0.45%) and the subsequent elimination of trimethylsilanol to yield a $(M\text{-}15\text{-}90)^+$ peak at *m/e* 361 (RI = 2.0%; Σ_{35} = 0.52%) are particularly useful in establishing the proper location of the molecular ion. A broad metastable peak at *m/e* 290 (calculated $361^2/451 = 288.96$ and $363^2/453 = 290.88$ for the +2 peak), probably due to the coalescence of two metastable peaks from each of the two chlorine isotopes, was observed corresponding to this sequence.

Fragment I*b* (*m/e* 225) seems to undergo losses typical of *O*-silyl ethers, M-15, -73, or -90, in addition to the loss of atomic oxygen from the nitro group to show peaks at *m/e* 194 (RI = 3.3%; Σ_{35} = 0.84%), *m/e* 136 (RI = 2.2%; Σ_{35} = 0.58%), and *m/e* 119 (RI = 0.86%; Σ_{35} = 0.22%), respectively.

Fragment II*b* (*m/e* 242) shows a similar pattern of an *O*-silyl ether with the loss of 15, 30, or 73, simultaneous with the elimination of $COCHCl_2$, to give peaks at *m/e* 116 (RI = 4.3%; Σ_{35} = 1.11%), *m/e* 101 (RI = 0.93%; Σ_{35} = 0.24%), and *m/e* 58 (RI = 2.4; Σ_{35} = 0.61%).

Characterization of Gas Chromatographic Effluents—A preliminary melting point of an evaporated nonpolar mixture of S3 taken before chromatography was about 71–72°, in contrast to about 55° for the standard S2.

An IR spectrum of chloramphenicol obtained with a Beckman IR-12 spectrophotometer matched that reported by Sensi (17) and by Suzuki (18) but differed somewhat from that reported by Suzuki and Shindo (19). However, the latter authors did indicate spectral differences between the *threo* and *erythro* conformers in the crystalline state in the 5.93–5.97, 6.40–6.54, and 10.00–11.10-μ regions. Although these differences were expected to appear in the silyl ethers, none was detected in the KBr spectra of the collected fractions (Figs. 10*a*, *b*, *c*). In fact, both fractions from S3 appeared to be identical to the standard S2.

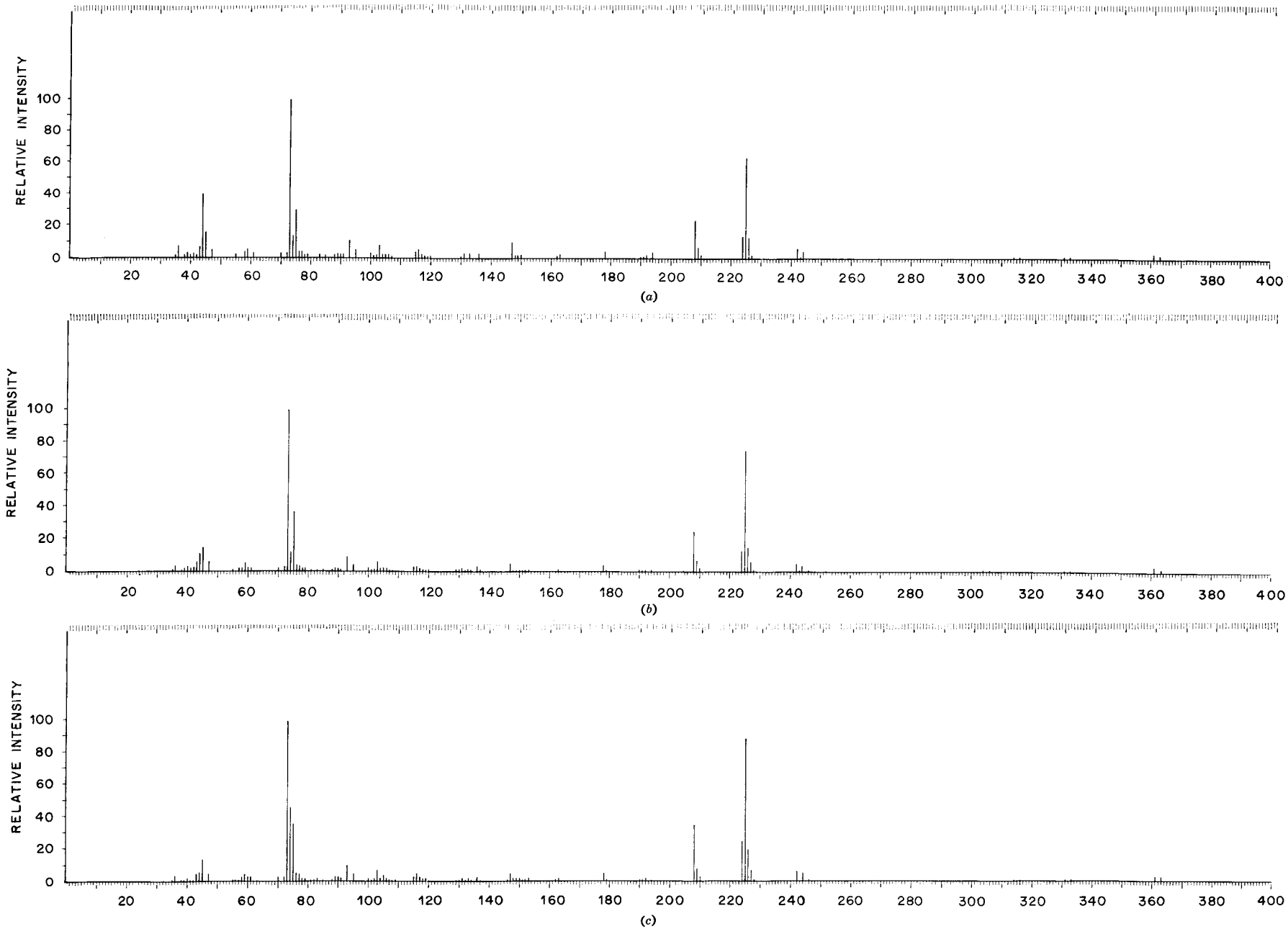


Figure 11—*Mass spectra of microsamples:* (a) *Standard TMS ether of chloramphenicol (S1).* (b) *Chromatographic Fraction I from S3.* (c) *Chromatographic Fraction II from S3.*

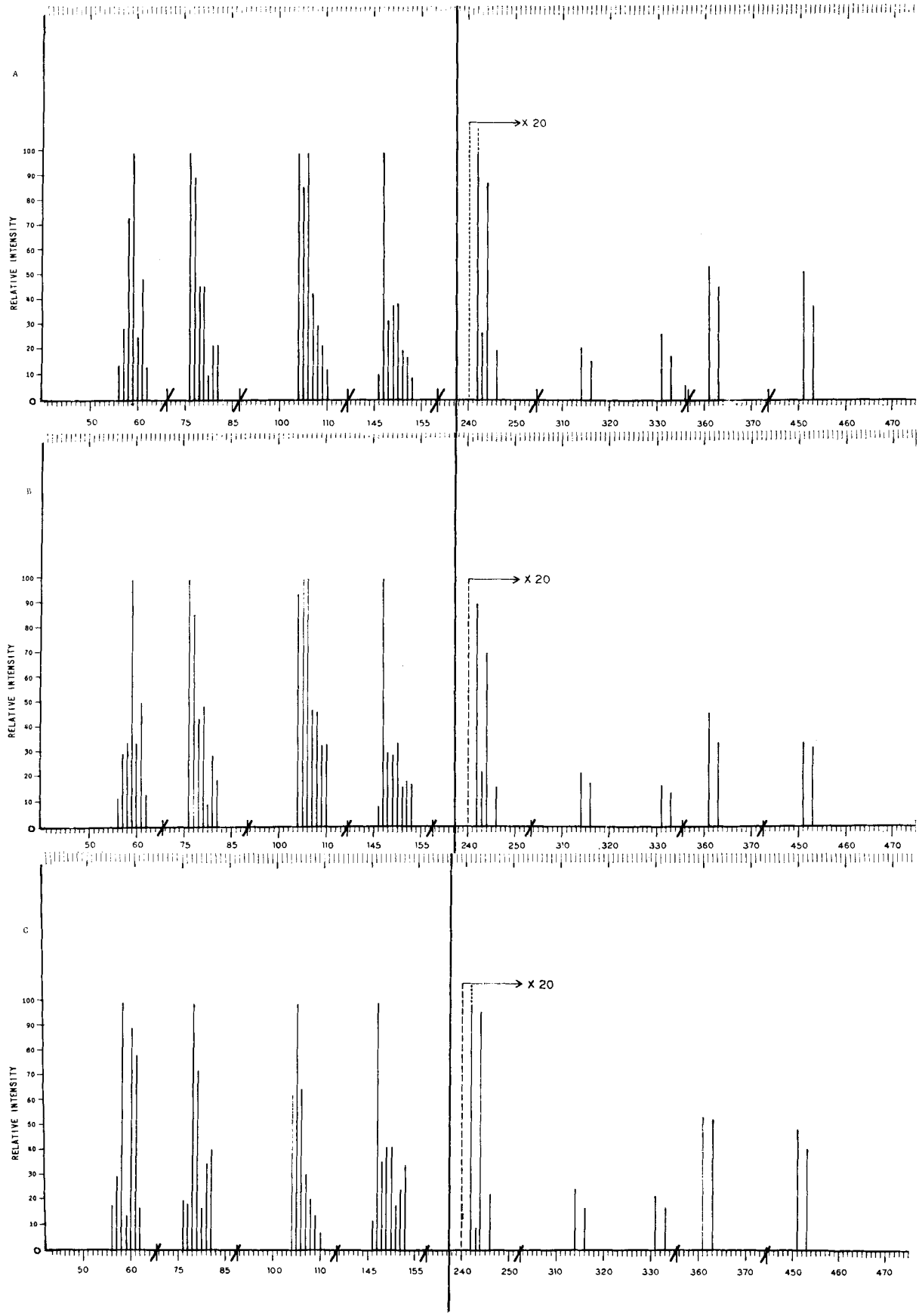


Figure 12—*Partial mass spectra of microsamples:* (a) *TMS ether of standard, S2.* (b) *Chromatographic Fraction I from S3.* (c) *Chromatographic Fraction II from S3.*

Table I—Observed Shift and Splitting of S2 in CCl_4

Assignment	ν^a	δ^a	No. Protons	Splitting
a	6	0.10	18	Singlet
b	9	0.15		Singlet
c	205	3.42	1	Doublet
d	211	3.52	1	Singlet
e	228	3.80	1	Multiplet
f	304	5.06	1	Doublet
g	335	5.58	1	Singlet
h	392	6.53	1	Doublet
i,j	455	7.58	4	Quartet

[a] Chemical shift (ν) measured in c.p.s. from tetramethylsilane used as internal standard in CCl_4 and in p.p.m. (δ).

Standard S2, a fresh derivative solution, and S3 were injected into the LKB 9000 and the Barber-Colman 5000 gas chromatographs. The resultant chromatograms showed identical retention times for standard S2, the fresh derivative solution, and the first fraction from S3. The mass spectra of S2 and of the fresh derivative solution obtained after injection into the LKB 9000 were identical to S2 placed directly into the inlet probe, indicating that the three samples were identical.

The mass spectrum of each collected fraction was compared to that of the S2 and the same fragmentation pattern was obtained peak for peak (Fig. 11), confirming the identity of each as the *O,O*-ditrimethylsilyl ether of *p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol. However, S2, although essentially identical to Fraction I, differed from Fraction II in the relative intensity of several fragment peaks and clusters (Fig. 12).

The conclusion that Fraction II is the diastereomer of chloramphenicol silyl ether is substantiated by the available data as follows: the methanol-treated silyl solution (S3) yielded two GLC peaks and enantiomers are not known to be separable under these conditions; IR spectra showed that both effluent fractions were identical to S2; and mass spectra showed that Fraction I was identical with the S2 but differed from Fraction II only in the relative abundance of some peaks.

Petersson *et al.* (12) and Capella and Zorzut (14) have shown that mass spectra of diastereomers have the same fragmentation pattern but differ in the relative intensities of some fragment peaks. They report that the *m/e* 147 disiloxonium ion exhibits a very high abundance, particularly when the sample material contains more than one trimethylsilyl group. The *m/e* 147 peak was also reported to be more intense in the *erythro* than in the *threo* conformer in the case of oleic and elaidic acid derivatives. With regard to chloramphenicol silyl ether, the *m/e* 147 peak was found to be relatively weak but significant at about RI = 5% and Σ_{35} = 1% for both fractions of S3 as well as for S2. Walden inversions of structures related to chloramphenicol have been reported to occur with phosphorus halides (20), in esterification reactions (21), with heat (22), with hydrolysis (23), and with other nucleophilic attack by solvolysis (24).

REFERENCES

(1) Code of Federal Regulations, Title 21, Part 141d, 301 (1968).

(2) P. D. Shaw, *Anal. Chem.*, **35**, 1580(1963).

(3) G. L. Resnick, D. Corbin, and D. H. Sandberg, *ibid.*, **38**, 582(1966).

(4) M. Yamamoto, S. Iguchi, and T. Aoyama, *Chem. Pharm. Bull.*, **15**, 123(1967).

(5) R. Bentley, C. C. Sweeley, M. Makita, and W. W. Wells, *Biochem. Biophys. Res. Commun.*, **11**, 14(1963).

(6) M. Margosis, *J. Chromatogr.*, **37**, 46(1968).

(7) M. Margosis, FDA, NCAIA, unpublished data.

(8) E. F. Schwarm, C. Dabner, J. W. Wilson, Jr., and M. P. Boghosian, *J. Pharm. Sci.*, **55**, 744(1966).

(9) D. Masterson, Jr., *ibid.*, **57**, 305(1968).

(10) L. Doub and J. M. Vandenbelt, *J. Amer. Chem. Soc.*, **69**, 2714(1947).

(11) W. W. Wendlandt and M. Zief, *Naturwissenschaften*, **45**, 467(1958).

(12) G. Petersson, O. Samuelson, K. Anjou, and E. von Sydow, *Acta Chem. Scand.*, **21**, 1251(1967).

(13) J. Diekman, J. B. Thomson, and C. Djerassi, *J. Org. Chem.*, **32**, 3904(1967).

(14) P. Capella and C. M. Zorzut, *Anal. Chem.*, **40**, 1458(1968).

(15) J. A. McCloskey, R. N. Stillwell, and A. M. Lawson, *ibid.*, **40**, 233(1968).

(16) C. Brunnee, G. Kappus, and K.-H. Maurer, *Fresenius' Z. Anal. Chem.*, **232**, 17(1967).

(17) P. Sensi, *Boll. Soc. Ital. Biol. Sper.*, **29**, 592(1953).

(18) M. Suzuki, *J. Antibiotics (Tokyo), Ser. B*, **14**, 323(1961).

(19) M. Suzuki and H. Shindo, *J. Pharm. Soc. Japan*, **76**, 927 (1956).

(20) A. Iliceto, *Ric. Sci.*, **23**, 1621(1953); through *Chem. Abstr.*, **48**, 12702*c*(1954).

(21) W. Logemann, F. Lauria, and E. Pella, *Gazz. Chim. Ital.*, **83**, 407(1953); through *Chem. Abstr.*, **49**, 222*h*(1955).

(22) T. Taguchi, M. Tomoeda, and I. Aratani, *J. Amer. Chem. Soc.*, **78**, 1468(1956).

(23) M. P. Kerop'yan and A. S. Elina, *Zh. Obshch. Khim.*, **31**, 3298(1961); through *Chem. Abstr.*, **57**, 2114*f*(1962).

(24) L. H. Sommer and F. A. Carey, *J. Org. Chem.*, **32**, 2473 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 29, 1969, from the *National Center for Antibiotics and Insulin Analysis, Division of Pharmaceutical Sciences, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, DC 20204*

Accepted for publication November 6, 1969.

The author wishes to express his appreciation to Dr. J. Y. T. Chen, Mr. J. N. Damico, and Mrs. B. S. Fisher of the Food and Drug Administration, for obtaining some of the spectra and for most helpful discussions, and to Mr. R. Pittman of the National Institutes of Health, for the determinations with the LKB 9000.

This study was initiated at the National Center for Antibiotics and Insulin Analysis and pursued at the FDA Institute for Advanced Analytical Chemistry at Georgetown University under the supervision of Dr. Charles F. Hammer, Director.

Release Study of Sulfaethylthiadiazole (SETD) from a Tablet Dosage Form Prepared from Spray-Congealed Formulations of SETD and Wax

IMTIAZ S. HAMID and CHARLES H. BECKER*

Abstract □ *In vitro* dissolution patterns of some spray-congealed products of sulfaethylthiadiazole–wax made into compressed tablets are reported. The tablets were made with a Carver laboratory press, employing a single force of compression and being approximately the same weight. The rate of release of sulfaethylthiadiazole (SETD) from the tablets was evaluated in acid pepsin medium and alkaline pancreatin medium using a rotating-bottle method. There was a gradual decrease in the amount of SETD released from the tablets as the concentration of sorbitan monooleate increased in the formulation, using acid pepsin medium. In alkaline pancreatin medium, an increase in the percentage of SETD released was noted as the concentration of surfactant increased. The mechanism of release of SETD appeared to be due to erosion, solubilization, and leaching of the drug from the tablet. The Higuchi model for drug release from inert matrices could describe the release pattern only for the initial few hours when apparently the variables other than the amount of drug released and time were essentially constant.

Keyphrases □ Sulfaethylthiadiazole–wax tablets—drug release □ Spray-congealed formulations—SETD–wax compressed tablets □ Dissolution, *in vitro*—SETD–wax compressed tablets □ Surfactant effect—SETD release, tablets □ Wax matrix effect—SETD release, tablets

The purpose of this investigation was to study *in vitro* the dissolution patterns of some spray-congealed sulfaethylthiadiazole–wax products made into compressed tablets. The spray-congealed products, in powder form, were prepared in this laboratory by John and Becker (1), and the dissolution patterns of the various powdered products were reported. Other workers (2–4) studied release patterns of tablet formulations which were simply made by mixing the active drug with inert matrices and compressing the materials into tablets.

Inasmuch as the release patterns of sulfaethylthiadiazole (SETD) from the spray-congealed drug–wax products, in powder form, were known in this study, with and without surfactant, it would be of value to know the release pattern of the drug from the same products when compressed into tablet form. Such factors as force of compression, possible fusion of the material on compression, and degree of disintegration or erosion (or both) of the tablets during dissolution should have decided effects on the release pattern of the drug. Other investigators (2–4) have reported dissolution patterns of drug from inert matrices in tablet form, but they did not report the release patterns of the drug imbedded in the matrices in the powder form.

EXPERIMENTAL

Materials—The spray-congealed SETD–wax products, in powder form, used to make the tablets in this study were prepared and reported by John and Becker (1). All other materials employed conformed to USP or NF specifications or were of analytical reagent quality.

White wax, a synthetic waxlike ester,[1] and a combination of equal parts of these waxes were employed in the formulations of John and Becker (1) as matrices, and these are designated as W_1, W_2, and W_3, respectively. The surfactant, sorbitan monooleate,[2] was used in some of the formulations in concentrations of 0, 1, 4, and 10%, and these are designated as S_0, S_1, S_4, and S_{10}, respectively. The tablets were made from three finenesses of spray-congealed powders, since these were atomized with three different nozzle sizes, namely, 0.05, 0.10, and 0.15. cm., and these are designated as N_2, N_4, and N_6, respectively.

Manufacture of Tablets—There were no additives incorporated in the tablets which were made with a Carver laboratory press. Accurately weighed, 0.5000 g. of the spray-congealed powder was put into the die. The die and the punches, 1.27 cm. in diameter, were previously lubricated with a small amount of talc to avoid sticking of the tablet to the punches. The 4000-p.s.i. gauge pressure was maintained for 1 min. All tablets were accurately weighed after compression, and they did not vary by more than 10 mg. It could be assumed that the surface area was essentially the same for a particular wax product made into tablets.

Assay Method—The SETD released was assayed by the Bratton-Marshall (5) procedure using a Klett-Summerson photoelectric colorimeter with a No. 54 filter.

***In Vitro* Dissolution Studies**—The rotating-bottle apparatus (6) was used to determine the dissolution behavior of the SETD from the tablets. Tablets weighing approximately 0.5000 g., accurately weighed, were put in 90-ml. screw-capped bottles, and to each bottle 60 ml. of acid pepsin medium (simulated gastric fluid USP, pH 1.1) was added. Duplicate samples were allowed to rotate end-over-end at a speed of 40 to 45 r.p.m. in a water bath maintained at 37 ± 1°. Samples were removed at specific time intervals, *i.e.*, at 0.25, 0.5, 1, 1.5, 2, 4, and 8 hr. Dissolution studies were also conducted in an alkaline pancreatin medium, test fluid B, pH 8.3, of Robinson and Swintosky (7), in a similar manner. Samples were removed for assay at 0.5, 1, 1.5, 2, 4, 8, and 12 hr.

Samples were also run for extended periods of time, *i.e.*, 24 and 48 hr., although equilibrium was not attained in acid pepsin medium or alkaline pancreatin medium.

RESULTS AND DISCUSSION

Spray-congealed products with white wax as the matrix were very tacky powders. The tackiness increased as the concentration of sorbitan monooleate in the formulations was increased, causing difficulty in transferring these powders into the die. The punches and the die had to be polished with a lubricant, talc, in order to obtain smooth and uniform tablets.

Spray-congealed products containing a combination of white wax and the synthetic waxlike ester in a 1 to 1 ratio were free flowing powders, but the ones with a higher concentration of the surfactant, sorbitan monooleate, were not free flowing. However, the tablets were easier to make from these products than those containing white wax alone as the matrix. In all of these instances, the punches and die had to be polished with talc to obtain smooth tablets.

All of the spray-congealed products containing the synthetic waxlike ester alone as the matrix, including the product having as high as 10% sorbitan monooleate, were free-flowing powders. However, the ones without surfactant could not be compressed into tablets at 4000-p.s.i. gauge pressure. This appeared to be due

[1] Marketed as Glycowax S-932 by Glyco Chemicals, Inc., New York, N. Y.

[2] Marketed as Span 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

Table I—*In Vitro* Dissolution Data Expressed as Percent SETD Released for SETD–White Wax Tablets and Powder Containing Sorbitan Monooleate and Atomized through a 0.15-cm. Pneumatic Nozzle

Time, hr.	Sample Symbol $W_1N_6S_0$ Tablet	Powder	Sample Symbol $W_1N_6S_4$ Tablet	Powder	Sample Symbol $W_1N_6S_{10}$ Tablet	Powder
			Dissolution in Acid Pepsin Medium			
0.25	0.28	8.00	0.32	10.05	0.25	3.66
0.50	0.35	9.76	0.33	16.91	0. 2	7.77
1.00	0.44	11.43	0.39	28.79	0.37	11.43
1.50	0.49	—	0.49	33.36	0.43	14.63
2.00	0.56	15.08	0.51	37.11	0.48	16.00
4.00	0.67	—	0.64	—	0.78	—
8.00	0.73	—	0.60	—	0.78	—
24.00	—	42.50	1.53	50.16	1.73	47.53
48.00	1.34	—	—	—	—	—
			Dissolution in Alkaline Pancreatin Medium			
0.50	1.60	38.16	3.41	74.04	3.74	65.81
1.00	2.15	53.47	4.37	80.44	6.48	74.73
1.50	4.03	—	9.42	—	11.70	—
2.00	7.00	74.79	11.59	83.18	14.08	87.75
4.00	12.60	90.27	21.31	85.50	25.61	89.81
6.00	22.50	90.72	33.25	87.29	39.50	—
8.00	31.40	—	34.99	—	43.78	95.52
12.00	50.80	—	56.54	—	64.02	—
24.00	—	94.15	—	—	—	—
48.00	86.50	—	91.00	92.32	97.52	96.89

to the friability of the finished tablet, as well as adherence or sticking of the tablet to the punch surface. The products with surfactant, on the other hand, could be compressed into tablets easily.

Tables I, II, and III give the dissolution data in acid pepsin medium and alkaline pancreatin medium from tablets with matrices of white wax (W_1), synthetic waxlike ester (W_2), and a combination of equal parts of these waxes (W_3) and varying concentrations of sorbitan monooleate. The tables also include dissolution data from the powder forms of the same formulations, reported earlier (1), for comparison.

Effect of Sorbitan Monooleate—There was an increase in the amount of SETD released with time from all tablets, with and without surfactant, in acid pepsin medium. However, there was a decrease in the rate of release of SETD from the tablets in acid pepsin medium as the concentration of surfactant in the tablets increased, particularly over the early dissolution testing periods, that is, from 0.25 to 4 hr. In most cases of powder forms of the same products (1) and for the same time period, there was an increase in the rate of release of SETD as the concentration level of sorbitan monooleate increased from 0 to 4%, but the rate of release decreased with the level of surfactant concentration at 10%. The magnitude of release of SETD was anywhere from 25 to 100 times less in tablet dosage form than in powder form. The decrease in the percent release of SETD from the tablets, as the concentration of sorbitan monooleate was increased, could be due to more cohesiveness of the products which increased as the concentration of surfactant was increased in the formulations. This can cause agglomeration of particles, which in turn can result in less surface area exposed to the dissolution medium. In addition, there could have been some fusion of particles under the compressional force used in tableting which could result in less porosity and more compactness of particles, thereby decreasing the release of SETD. Although sorbitan monooleate lowers surface tension and should theoretically increase the release of drug from a tablet, the surfactant apparently is not effective in an acid medium of low pH such as acid pepsin medium, nor is it as effective with tablets which are excessively compact.

An increase in the percentage of SETD released from tablets with time was noted in alkaline pancreatin medium, as the concentration of surfactant was increased in all the formulations of different waxes. This was also true for the same products in the powder form, except the percentage of release was of a larger magnitude (1). The release of SETD from white wax was anywhere from 35 to 60 times greater in powder form than that in tablet dosage form. The

Table II—*In Vitro* Dissolution Data Expressed as Percent SETD Released for SETD–Synthetic Waxlike Ester Tablets and Powder Containing Sorbitan Monooleate and Atomized through a 0.15-cm. Pneumatic Nozzle

Time, hr.	Sample Symbol $W_2N_6S_0$ Tablet	Powder	Sample Symbol $W_2N_6S_4$ Tablet	Powder	Sample Symbol $W_2N_6S_{10}$ Tablet	Powder
			Dissolution in Acid Pepsin Medium			
0.25	0.75	11.88	0.70	22.62	0.41	9.37
0.50	1.01	24.68	1.17	32.22	0.55	13.71
1.00	1.60	34.96	1.46	40.68	1.00	24.45
1.50	2.63	39.76	2.09	43.42	1.30	30.62
2.00	2.98	45.48	2.78	45.93	1.58	34.73
4.00	4.45	—	3.34	—	3.17	—
8.00	4.05	—	4.35	—	3.65	—
24.00	—	59.87	—	—	—	61.82
48.00	6.68	—	6.91	66.39	8.14	63.07
			Dissolution in Alkaline Pancreatin Medium			
0.50	2.70	30.00	2.42	69.70	2.85	56.44
1.00	3.20	45.25	2.59	80.40	4.45	65.36
1.50	3.80	—	4.00	—	6.21	—
2.00	4.40	48.67	4.88	85.01	7.64	76.78
4.00	6.00	53.70	6.70	90.95	9.76	92.32
6.00	7.80	65.36	7.09	95.06	12.46	94.61
8.00	9.50	—	9.72	—	19.09	—
12.00	9.57	—	12.89	—	22.14	—
48.00	18.22	97.81	20.79	97.35	46.67	96.89

Table III—*In Vitro* Dissolution Data Expressed as Percent SETD Released for SETD–Synthetic Waxlike Ester–White Wax (1:1) Tablets and Powder Containing Sorbitan Monooleate and Atomized through a 0.15-cm. Pneumatic Nozzle

Time, hr.	Sample Symbol $W_3N_6S_0$ Tablet	Powder	Sample Symbol $W_3N_6S_4$ Tablet	Powder	Sample Symbol $W_3N_6S_{10}$ Tablet	Powder
			Dissolution in Acid Pepsin Medium			
0.25	0.40	18.74	0.34	8.23	0.27	5.71
0.50	0.48	24.68	0.45	14.63	0.31	9.37
1.00	0.60	—	0.50	19.65	0.45	12.57
1.50	0.64	33.36	0.61	20.57	0.50	16.00
2.00	0.67	33.59	0.64	21.94	0.57	18.97
4.00	0.88	—	0.87	—	0.79	—
8.00	1.15	—	1.25	—	1.55	—
24.00	—	—	—	34.39	—	36.57
48.00	2.51	54.04	4.07	—	6.83	—
			Dissolution in Alkaline Pancreatin Medium			
0.50	2.15	38.19	1.99	31.54	3.50	55.30
1.00	2.19	58.27	3.26	46.39	5.16	68.78
1.50	3.01	—	5.61	—	8.30	—
2.00	4.00	58.73	8.10	56.67	9.89	78.38
4.00	8.60	82.27	13.52	69.24	15.50	86.61
6.00	14.88	82.50	17.88	74.73	20.00	90.27
8.00	17.99	—	23.68	—	29.00	—
12.00	33.99	—	37.63	—	30.31	—
48.00	76.50	96.89	62.32	95.98	70.99	95.06

increase in percent of SETD release in both tablet and powder forms may be due, partially, to surface tension lowering effect of sorbitan monooleate on the dissolution medium. Sorbitan monooleate probably enhances the action of alkaline pancreatin medium to accelerate softening and erosion of the wax-matrix tablets which did occur. It should be pointed out, also, that alkaline pancreatin medium contains ox bile extract which is a surfactant due to the presence of bile salts; this, combined with sorbitan monooleate, could have been more effective than either surfactant used alone.

Effect of Wax Matrix—Those tablets which could be made from formulations of spray-congealed SETD–synthetic waxlike ester powders gave the highest drug release of the three matrices investigated in an 8-hr. dissolution study in acid pepsin medium. Tablets with white wax alone as the matrix gave the lowest drug release. A combination of synthetic waxlike ester and white wax in a 1 to 1 proportion as the matrix ranged between the two above matrices in regard to release of SETD. This was true with and without surfactant. This effect may be due to the chemical composition of the two waxes. The synthetic waxlike ester contains a high percentage of glyceryl tristearate but no free fatty acids. The ester might have been hydrolyzed sufficiently in the acid pepsin medium to account for the highest percentage of SETD released of the three matrices studied On the other hand, white wax, not a glyceryl ester and not as easily hydrolyzed by acids, contains free fatty acids which are practically insoluble in the acid pepsin medium, hence showing the lowest drug release from this matrix. The products in powder form were also observed to possess similar characteristics (1).

As compared to the acid pepsin medium dissolution study, the percent of SETD released from tablets was found to be the highest from the white wax matrix in alkaline pancreatin medium over a 12-hr. period. Tablets with the synthetic waxlike ester alone as the matrix gave the lowest drug release. A combination of the synthetic waxlike ester and white wax in a 1 to 1 proportion was intermediate in release of SETD. Similar results were obtained when these products were studied in powder form by John and Becker (1). The high release of SETD from tablets containing the white wax matrix alone can most likely be attributed to the presence of free fatty acids in the wax which react with the alkalinity of the dissolution medium to form soaps that act as surfactants. The synthetic waxlike ester is apparently not as easily eroded and dispersed in alkaline pancreatin medium as white wax due to the lack of free fatty acids.

The percent of drug released from tablets of all the waxes, after any comparable time interval in alkaline pancreatin medium, was greater than in acid pepsin medium. One explanation is that the drug SETD is more soluble in an alkaline medium than in an acid medium. Secondly, the constituents of the waxes, both free fatty acids and the esters, are apparently more soluble and dispersible in an alkaline medium.

Effect of Nozzle Size—No definite conclusions could be drawn as to effect of nozzle size used in the pneumatic atomization of the various spray-congealed SETD–wax products and the influence of their particle sizes when made into tablets on the release of the drug in acid pepsin medium as well as in alkaline pancreatin medium. As reported earlier (1), the average particle size of SETD–white wax powders spray congealed with different nozzle sizes, namely, 0.05, 0.10, and 0.15 cm., was 9.2, 13.4, and 14.8 μ, respectively; for the SETD–synthetic waxlike ester powders, the average particle size was 7.8, 12.2, and 14.6 μ; and for the SETD–synthetic waxlike ester–white wax powders in a 1 to 1 proportion, the average particle size was 8.9, 13.1, and 18.8 μ, respectively. Apparently the difference in ranges of particle sizes was not sufficient to show any significant pattern in release of SETD from the respective tablets. It appears likely that the force of compression employed in making the tablets, resulting in some fusion of the waxy materials, removed any possible effects of particle size if such exist in this instance.

***In Vitro* Dissolution Pattern of SETD from Tablets Made from Inert Wax Matrices**—Higuchi (8) derived the following equation for drug release from granular inert matrices:

$$Q = A\left[\frac{DK}{r}(2 - KC_s)C_s t\right]^{1/2} \qquad \text{(Eq. 1)}$$

where Q is the amount of drug released after time t per unit exposed area, D is the diffusitivity of drug in the homogeneous matrix media, A is the total amount of drug present in the matrix per unit volume, C_s is the solubility of the drug in the matrix system, r is the tortuosity factor of the capillary system, and K equals the specific

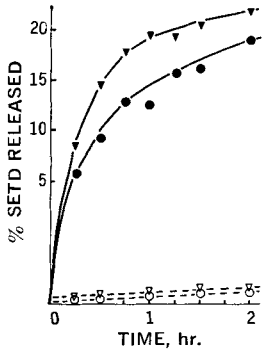


Figure 1—*Plot of percent SETD released as a function of time for dissolution from SETD–synthetic waxlike ester–white wax (1:1) tablets and powder in acid pepsin medium. Key:* ▼ ▽, $W_3N_6S_{10}$; ● ○, $W_3N_6S_4$. *Solid line: powder; dashed line: tablets.*

volume of the drug. Under the conditions where shape, size, weight, degree of compression, and surface area remain essentially unchanged, the above equation reduces to:

$$Q = kt^{1/2} \qquad \text{(Eq. 2)}$$

where k is a constant taking account of all the factors held constant in Eq. 1.

The percent of SETD released from the tablets after dissolution in the acid pepsin medium from all three matrices evaluated in this investigation was plotted as a function of square root of time. The linear relationship according to Eq. 2 was observed to be valid for a few hours where the release was less than 2%. After that the dissolution data did not follow this pattern, apparently due to considerable change in the effective surface area of the tablets. Similarly, the percent of SETD released from the tablets after dissolution in alkaline pancreatin medium from all three matrices was plotted as a function of square root of time. Likewise, in this instance, a deviation from linearity was observed after a few hours due to change in effective surface area of the tablets as erosion and some dispersion of the tablets occurred. Tablets compressed from spray-congealed products containing white wax alone as the matrix were observed to be more soluble than the other wax matrices employed and, hence, showed more deviation. After 6 hr. of exposure in the alkaline pancreatin medium, the SETD–white wax tablets were about half of the original size.

Dissolution of SETD from Spray-Congealed SETD–Wax Powders *versus* Dissolution of SETD from Tablets—It would be of interest to compare the dissolution of SETD from spray-congealed SETD–wax powders to the dissolution of SETD from tablets prepared from the same products. Figure 1 shows the percent of SETD-released against time from SETD-synthetic waxlike ester–white wax, in a 1 to 1 proportion, powder and tablets. It is apparent that the magnitude of release of SETD from the powdered forms is much greater than that in the tablet dosage form. The compressional force required in tableting resulted in some fusion, less porosity, and more compactness of the particles which seems to be responsible for the small amount of SETD release from the tablets.

Further work on tablets employing spray-congealed products of drug in wax matrices with modifier, which are free flowing, suitable for direct compression, and which release the active ingredient more completely, is presently under investigation. The results of this study will be reported separately in a later paper.

REFERENCES

(1) P. M. John and C. H. Becker, *J. Pharm. Sci.*, **57**, 584(1968).

(2) S. J. Desai, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **54**, 1459(1965).

(3) P. Singh, S. J. Desai, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **56**, 1542(1967).

(4) J. B. Schwartz, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **57**, 274(1968).

(5) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).

(6) J. C. Souder and W. C. Ellenbogen, *Drug Std.*, **26**, 77(1958).

(7) M. J. Robinson and J. V. Swintosky, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 473(1959).

(8) T. Higuchi, *J. Pharm. Sci.*, **52**, 1145(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 26, 1968, from the *College of Pharmacy, University of Florida, Gainesville, FL 32601*

Accepted for publication December 9, 1968.

Abstracted in part from a thesis presented by Imtiaz S. Hamid to the Graduate School of the University of Florida, Gainesville, in partial fulfillment of Master of Science in Pharmacy degree requirements.

* To whom inquires concerning this manuscript should be addressed.

Fitting a Double-Exponential Curve to Observed Salicylate Concentrations in Blood

F. W. MUELLER and S. V. LIEBERMAN

Abstract □ For interpretation of the results of blood concentration data to be meaningful, a very careful evaluation of the basic aspects of data collection, data description, and analysis is essential. The importance of these considerations is illustrated by the magnitude of observed differences in rate constants obtained under a variety of possible data-handling methods. The method of curve fitting presented, which minimizes squared logarithmic deviations, offers a different approach by utilizing relative error rather than absolute error. If truly equal weights are desired for data points, it is felt that this is the more appropriate definition of best fit. In any case, no mathematical technique for fitting a model to the data can compensate for an inadequate description of drug activity.

Keyphrases □ Blood concentration data—evaluation, basic aspects □ Rate constants—double-exponential curve fitting □ Salicylate concentration levels—rate constants, curve fitting, example

A desire to determine rate constants for drug absorption and elimination has resulted in the development of numerous analytical and mathematical techniques for pharmacokinetic analysis. In recent years, the applications of pharmacokinetic analysis have progressed rapidly from graphical solutions of concentration *versus* time plots to computer programs applied to increasingly sophisticated mathematical models. The latter yield apparent first-order rate constants for various processes of distribution and elimination [*e.g.*, Levy *et al.* (1) and Wagner (2)]. In many publications, the estimated values obtained for the parameters of the models have been presented without any indication that other values are possible. Where a number of apparent first-order rate constants are derived by a series of arithmetic manipulations from these estimated parameters, any inaccuracy in these estimates will be magnified in the subsequent computations. For a given set of observations, there are several important statistical considerations which merit careful attention before beginning the process of fitting a specific model to observed data.

volume of the drug. Under the conditions where shape, size, weight, degree of compression, and surface area remain essentially unchanged, the above equation reduces to:

$$Q = kt^{1/2} \qquad \text{(Eq. 2)}$$

where k is a constant taking account of all the factors held constant in Eq. 1.

The percent of SETD released from the tablets after dissolution in the acid pepsin medium from all three matrices evaluated in this investigation was plotted as a function of square root of time. The linear relationship according to Eq. 2 was observed to be valid for a few hours where the release was less than 2%. After that the dissolution data did not follow this pattern, apparently due to considerable change in the effective surface area of the tablets. Similarly, the percent of SETD released from the tablets after dissolution in alkaline pancreatin medium from all three matrices was plotted as a function of square root of time. Likewise, in this instance, a deviation from linearity was observed after a few hours due to change in effective surface area of the tablets as erosion and some dispersion of the tablets occurred. Tablets compressed from spray-congealed products containing white wax alone as the matrix were observed to be more soluble than the other wax matrices employed and, hence, showed more deviation. After 6 hr. of exposure in the alkaline pancreatin medium, the SETD–white wax tablets were about half of the original size.

Dissolution of SETD from Spray-Congealed SETD–Wax Powders *versus* Dissolution of SETD from Tablets—It would be of interest to compare the dissolution of SETD from spray-congealed SETD–wax powders to the dissolution of SETD from tablets prepared from the same products. Figure 1 shows the percent of SETD-released against time from SETD-synthetic waxlike ester–white wax, in a 1 to 1 proportion, powder and tablets. It is apparent that the magnitude of release of SETD from the powdered forms is much greater than that in the tablet dosage form. The compressional force required in tableting resulted in some fusion, less porosity, and more compactness of the particles which seems to be responsible for the small amount of SETD release from the tablets.

Further work on tablets employing spray-congealed products of drug in wax matrices with modifier, which are free flowing, suitable for direct compression, and which release the active ingredient more completely, is presently under investigation. The results of this study will be reported separately in a later paper.

REFERENCES

(1) P. M. John and C. H. Becker, *J. Pharm. Sci.*, **57**, 584(1968).

(2) S. J. Desai, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **54**, 1459(1965).

(3) P. Singh, S. J. Desai, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **56**, 1542(1967).

(4) J. B. Schwartz, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **57**, 274(1968).

(5) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).

(6) J. C. Souder and W. C. Ellenbogen, *Drug Std.*, **26**, 77(1958).

(7) M. J. Robinson and J. V. Swintosky, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 473(1959).

(8) T. Higuchi, *J. Pharm. Sci.*, **52**, 1145(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 26, 1968, from the *College of Pharmacy, University of Florida, Gainesville, FL 32601*

Accepted for publication December 9, 1968.

Abstracted in part from a thesis presented by Imtiaz S. Hamid to the Graduate School of the University of Florida, Gainesville, in partial fulfillment of Master of Science in Pharmacy degree requirements.

* To whom inquires concerning this manuscript should be addressed.

Fitting a Double-Exponential Curve to Observed Salicylate Concentrations in Blood

F. W. MUELLER and S. V. LIEBERMAN

Abstract □ For interpretation of the results of blood concentration data to be meaningful, a very careful evaluation of the basic aspects of data collection, data description, and analysis is essential. The importance of these considerations is illustrated by the magnitude of observed differences in rate constants obtained under a variety of possible data-handling methods. The method of curve fitting presented, which minimizes squared logarithmic deviations, offers a different approach by utilizing relative error rather than absolute error. If truly equal weights are desired for data points, it is felt that this is the more appropriate definition of best fit. In any case, no mathematical technique for fitting a model to the data can compensate for an inadequate description of drug activity.

Keyphrases □ Blood concentration data—evaluation, basic aspects □ Rate constants—double-exponential curve fitting □ Salicylate concentration levels—rate constants, curve fitting, example

A desire to determine rate constants for drug absorption and elimination has resulted in the development of numerous analytical and mathematical techniques for pharmacokinetic analysis. In recent years, the applications of pharmacokinetic analysis have progressed rapidly from graphical solutions of concentration *versus* time plots to computer programs applied to increasingly sophisticated mathematical models. The latter yield apparent first-order rate constants for various processes of distribution and elimination [*e.g.*, Levy *et al.* (1) and Wagner (2)]. In many publications, the estimated values obtained for the parameters of the models have been presented without any indication that other values are possible. Where a number of apparent first-order rate constants are derived by a series of arithmetic manipulations from these estimated parameters, any inaccuracy in these estimates will be magnified in the subsequent computations. For a given set of observations, there are several important statistical considerations which merit careful attention before beginning the process of fitting a specific model to observed data.

STATISTICAL CONSIDERATIONS

An equation, describing the biological system, is used as a model, and an iterative curve-fitting procedure is necessary to obtain the estimated rate constants. This requires: (*a*) a selection of a descriptive statistic for the data, (*b*) a definition of best fit, and (*c*) a choice of the method of deriving the best fit. All three affect the estimated equation and, as a result, the conclusions drawn from an evaluation of the results. The average is the statistic most frequently used to describe concentration levels for a group of individuals. The best fit is commonly defined as the curve which minimizes the sum of the squared deviations between observed and computed concentrations. Under certain conditions, however, other alternatives are equally rational.

The selection of a statistic should obviously be based upon its descriptive quality. Physiological characteristics of individuals are such that, many times, salicylate concentration levels at a given time are not normally distributed. Consequently, the median of a group may be a better choice than the mean. If drug concentrations at observed time periods follow the normal or any other symmetric distribution, results with mean or median will be very similar. As for best fit, either the sum of the squared differences between observed and computed concentrations or the sum of the squared differences between the logarithms of these concentrations can be minimized. Comparisons of these latter differences are equivalent to relative percent deviations. Usually, accuracy of analytical methods is in terms of percentage error rather than absolute error, and a definition of best fit using differences in logarithms may be more appropriate. As basic as all these considerations seem, very little attention has been given to how they affect results.

In order to estimate the kinetic rate constants for orally administered drugs in tablet form, a biexponential curve-fitting procedure was developed for Teorell's equation 25 (3) for two consecutive monomolecular reactions giving the relationship of drug concentration in blood with time. Both averages and medians were used in fitting, and results based on each of the previously mentioned definitions of best fit were compared. The purposes of this paper are: to present this fitting procedure; to discuss briefly other available methods; and to illustrate the effect of data point selection, the definition of best fit, and the descriptive statistic chosen on the estimated rate constants.

METHODS AND DISCUSSION

The Model—Teorell's equation 25, describing the kinetics of drug appearance in the blood and disappearance from the blood when administered other than intravenously, was used:

$$C = \frac{a_0 Ka}{Vd(Ka - Kd)}\left(e^{-Kdt} - e^{-Kat}\right) = \frac{\gamma Ka}{\delta}\left(e^{-Kdt} - e^{-Kat}\right) \qquad \text{(Eq. 1)}$$

where: C = blood drug concentration in mcg./ml.; a_0 = dose in mg./kg.; Vd = the specific apparent volume of distribution (l./kg.); Ka = apparent drug appearance rate constant in hr.$^{-1}$; Kd = apparent drug disappearance rate constant in hr.$^{-1}$; t = time after ingestion in hours; γ = proportionality constant (a_0/Vd); and $\delta = Ka - Kd$.

In the manner of Lowenthal and Vitsky (4), rearranging Eq. 1 gives Eq. 2,

$$C = \frac{\gamma Ka}{\delta} e^{-Kat}\left(e^{\delta t} - 1\right) \qquad \text{(Eq. 2)}$$

and taking natural logs of Eq. 2 gives the linear Eq. 3:

$$\ln \frac{C}{e^{\delta t} - 1} = \ln \frac{\gamma Ka}{\delta} - Kat \qquad \text{(Eq. 3)}$$

The Delta Search—A least-squares best fit for Eq. 3 is derived by a search of delta (δ) values from an initially estimated δ in steps of 0.1. For each delta, a residual sum of squares about the fitted line is obtained. Once the direction of delta, from the initial estimate is determined, the 0.1 increment or decrement is repetitively applied for as long as a decrease in variability is observed. When an increase occurs, the search step is reduced to 0.005 and the value of the delta which gives the best fit is obtained. The absolute value of the slope of the best fit line is the estimated Ka. Subtracting δ from Ka yields Kd, and by substituting the Ka and Kd values the intercept ($\ln \gamma\, Ka/\delta$) can be solved for gamma (γ).

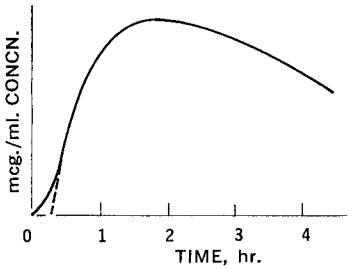


Figure 1—*Typical salicylate concentration curve for aspirin, 650 mg. (10 gr.).*

Time Axis Intercept—When the dose is administered as tablets, a form which does not provide immediate availability of the complete dose, early moments are not characterized by the first-order biexponential model. Instead, a time gap exists between the time of administration (time zero) and an extrapolated "kinetic time zero," the time at which the best fitting curve crosses the time axis. Termination of the fitting process at time zero assumes that the model is immediately applicable upon administration of the drug, and that the extrapolated curve should pass through the origin. As illustrated by Fig. 1, this is not true. The best fit to the observed data is based on a curve passing through the time axis at a point greater than zero.

The next phase of the search is directed toward finding the time axis intercept which gives the overall best fit. This is accomplished by subtracting a time increment, in this case 1 min., from each of the observed time periods, and once again searching for a delta to obtain the best fit to the data. The squared deviations for this fit and the previous fit are compared. If the current fit is superior, an additional time increment is subtracted and the delta search is repeated. This is continued until the sum of squared deviations for the current fit shows an increase. At this point, 0.5-min. time increments are applied, and the estimate of the time axis intercept is determined to the nearest half minute (Fig. 2). The search is now complete and an optimum Ka, Kd, γ, and time axis intercept (Δt) are available.[1]

Best Fit—The procedure just described was designed to obtain a best fit by minimizing the sum of squared logarithmic deviations between observed and computed concentrations. Three papers, Lowenthal and Vitsky (4), Wagner (5), and Wiegand and Sanders (6), have presented other mathematical techniques for fitting the double-exponential model. All of these published methods are designed to obtain a set of constants minimizing the sum of squares for deviations between observed and computed concentrations.

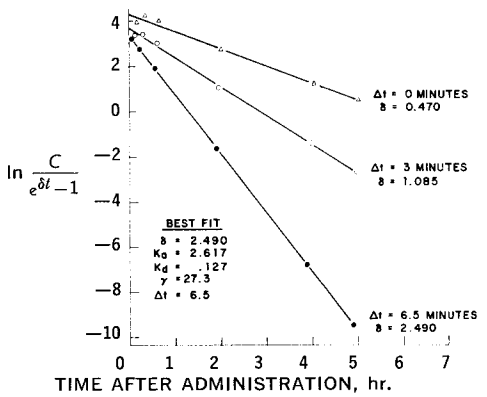


Figure 2—*Illustration of delta search for time axis intercept* (Δt). *Data points are from average salicylate concentration following 650-mg. (10-gr.) dose of aspirin. Best fit results are* $\delta = 2.490$, Ka = *2.617*, Kd = *0.127*, $\gamma = 27.3$, *and* $\Delta t = 6.5$.

[1] This computer program will be supplied upon request.

Table I—Computed Concentrations (mcg./ml.) from Best Fit Curve with and without 10-min. Data Point

Time, min.	Buffered Aspirin, 650 mg. (10 gr.)—Averages—With 10-min. Data Point	Observed Average Concn.	Excluding 10-min. Data Point
10	5.87	5.88	7.95
20	19.21	19.20	19.19
40	25.79	25.63	25.64
120	23.52	23.45	23.67
240	18.95	19.58	18.96
300	17.01	16.64	16.99
	Ka = 5.61		Ka = 4.91
	Kd = 0.108		Kd = 0.111
	γ = 28.3		γ = 28.6
	Δt = 7.5		Δt = 6.0

Selections of a definition of best fit, of observation times for data points, and of a descriptive statistics are, to a degree, a matter of choice and certainly subject to more than one opinion. The evaluation of which method provides the best fit is much more objective. For a given definition of best fit, the method can be considered correct only if it generates a set of constants meeting the least-squares criterion, *i.e.*, minimal residual sum of squares.

A valid comparison of these methods would require the application of each program to a common set of data. Although this has not been done, a general review of these procedures suggests that the results would differ. The Lowenthal and Vitsky method uses both definitions of best fit during the search for the constants. A two-dimensional grid search for *Ka* and *Kd* is performed. For each *Ka* and *Kd*, a gamma to complete the set is computed from the intercept of the best fit to the linearized Eq. 3 previously shown. The best set of constants is selected on the basis of minimal sum of squares of differences in observed and computed concentrations. Obtaining gamma by one definition of best fit and the best set by the other definition gives a different result than one from a three-dimensional search.

Wagner submits too little detail to evaluate the iterative routine for estimating the parameters. Essentially, his procedure yields a set of constants, *Ka*, *Kd*, *A*, and *B* which are used to determine γ and Δt by subsequent computations. The third procedure, proposed by Wiegand and Sanders, a modification of an iterative least squares by Deming (7), also simultaneously searches for a set of constants and should lead to a best fit. Although the time axis intercept can be computed by the Wiegand and Sanders procedures, their published data are for drug administered in solution with immediate application of first-order processes assumed. If, instead of this zero-time assumption, an intercept is computed by the delta search program, an 8-min. value is obtained with a substantially larger *Ka* providing the best fit. The suitability of assuming a zero-time intercept and essentially forcing a curve through the origin leads to other areas of importance: selection of data time points, limitations of curve extrapolation, and interpretation of computed rate constants.

Data Point Selection—Curve fitting is undertaken to obtain estimated rate constants which offer an adequate description of drug activity. It is, therefore, vital that the period of activity be sufficiently defined if the resulting constants are to be meaningful.

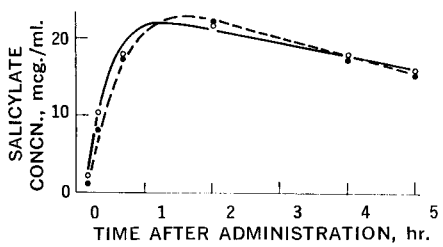


Figure 3—*Computed biexponential curves with minimized* $\Sigma(ln\ C' - ln\ C)^2$ *for 650-mg. (10-gr.) dose of aspirin. Solid line for fit to averages:* Ka = *2.62*, Kd = *0.107*, γ = *25.3*, *and* Δt = *8.0. Broken line for fit to medians:* Ka = *1.68*, Kd = *0.160*, γ = *29.4*, *and* Δt = *8.5.*

Selection of time periods, obviously, depends upon the characteristic curve for the drug. At least two or three time periods on the ascending and descending portion of the curve and one point near the peak should be selected. To establish the ascending portion accurately, the first observation should be taken as early as possible. Likewise, the descending portion should be clearly determined by later time points. Early observations are extremely critical for rapidly absorbed drugs characterized by a sharp early rise in concentration. Failure to obtain an early observation often leads to an inaccurate computed value for *Ka*. Under these circumstances, *Ka* will not be representative, and extrapolation of the curve at early moments will be misleading. Because of the mutual dependence of the constants, inadequate description of any portion of the curve can impair the accuracy of all constants.

Table I provides an example of the effect of inadequately defining the early portion of the curve. A best fit was determined for average salicylate levels from 650 mg. (10 gr.) of a buffered aspirin measured at 10, 20, 40, 120, 240, and 300 min. A best fit for the same data without the 10-min. observed average reduced the computed *Ka* from 5.61 to 4.91; in this case *Kd* and γ were not appreciably changed. The observed 10-min. concentration was 5.88 mcg./ml., and the computed 10-min. level for the fit to all points was 5.87 mcg./ml. Removing the 10-min. reading and extrapolating the computed curve result in a 10-min. estimated value of 7.95 mcg./ml., a 35% deviation from the observed value.

The descriptive quality of computed constants cannot be separated from the careful selection of data points. Drug description by pharmacokinetic rate constants is applicable only over the period observed. Therefore, the activity period of interest must be adequately covered by the observed data.

Medians and Means—To evaluate differences between fitting the model to medians and to means, a set of constants was determined for each. Salicylate levels for 18 subjects on a 650-mg. (10-gr.) dose of buffered aspirin and two separate trials of a 650-mg. (10-gr.) dose of commercial aspirin were used.

The choice of averages or medians is highly dependent upon the data. Averages are more stable and, when symmetrical distributions of concentrations are observed, deserve preference. Frequently, however, physiological characteristics of individuals are such that salicylate concentrations at early time periods fall in nonsymmetrical distributions. A few rapid drug absorbers produce averages considerably higher than the medians. Figure 3 illustrates this situation using salicylate concentrations for a 650-mg. (10-gr.) dose of aspirin. The best fit was obtained using the delta search. The fit to medians produced a lower *Ka* and a higher *Kd* than the fit to averages. Because of the skewness of the concentration distributions at early time periods, choice of the medians in this case might be desirable, since it, by definition, represents the middle value.

Table II gives the calculated constants and residual sum of squares for the best fit for the three sets of data. A comparison of sums of squares indicates that two of these three fits to averages were better than the corresponding fits to medians. This obviously does *not* imply that averages should be chosen inasmuch as the most representative statistic will not necessarily provide the lowest residual sum of squares.

Weighting of Data Points—Weighting of data points is often suggested in pharmacokinetic publications, but weighting factors are rarely proposed. Actually, the two definitions of best fit mentioned earlier represent forms of weighting. The best fit definition minimizing the sum of squared deviations appears to give equal weight to all points, but, to a degree, it is really giving more weight to the larger data values. It assumes that the reliability of all points, on the basis of absolute error, is the same. Since from an analytical point of view it is probably more accurate to assume that the reliability of all points is the same with respect to percentage error, minimizing the total squared deviations would give more weight to the larger observed values (*i.e.*, the middle portion of the curve). In contrast, minimizing the sum of squared differences in logarithms, under the assumption of equal absolute error, would give more weight to the lower observed values (*i.e.*, the ends of the curve). The choice of definition of best fit depends upon which viewpoint is more appropriate. Fitting to minimize sum of squares of differences gives a curve with greater percentage deviations of observed and computed concentrations for the low values. Minimizing squared differences in logarithms, on the other hand, presents a curve with a more uniform allocation of percent differ-

Table II—Best Fit Kinetic Constants and Residual Sum of Squares [$\Sigma(\ln C' - \ln C)^2$]

	Fit to Averages					Fit to Medians				
	Ka	*Kd*	γ	Δt	*SS*	*Ka*	*Kd*	γ	Δt	*SS*
Buffered aspirin, 650 mg. (10 gr.)	5.61	0.108	28.3	7.5	0.001596	5.45	0.110	30.0	8.5	0.005022
Aspirin, 650 mg. (10 gr.)	2.62	0.127	27.3	6.5	0.004740	1.48	0.167	30.7	6.0	0.051946
Aspirin, 650 mg. (10 gr.)	2.62	0.107	25.3	8.0	0.005381	1.68	0.160	29.4	8.5	0.001439

Table III—Comparison of Rate Constants for Best Fit

	Minimizing $\Sigma(C' - C)^2$				Minimizing $\Sigma(\ln C' - \ln C)^2$			
	Ka	*Kd*	γ	Δt, min.	*Ka*	*Kd*	γ	Δt, min.
Wagner data (tetracycline)	0.72	0.149	2.65	25.3	0.87	0.132	1.92	27.5
Lowenthal and Vitsky data (aspirin formulation D)	2.07	0.246	63.41	0	1.30	0.452	47.18	0
					4.36	0.130	53.90	6.0 (Best fit)
Wiegand and Sanders data (HT 1479)	2.30	0.254	7.70	0	2.40	0.245	7.54	0
					3.26	0.237	7.30	8.0 (Best fit)

ences of observed and computed values. Table III shows the results of fitting for each definition of best fit using data from each of the previously mentioned publications.

When choosing a definition of best fit or weighting factors, variation in the physiological characteristics among individuals should not be confused with the reliability of analytical determinations. Greater variation at early time periods of the concentration curve does not necessarily mean that the analytical accuracy is poorer at these time periods. The inaccuracy of *Ka* estimates may be partially a result of fitting for minimal sum of squared deviations or the lack of an early observation, since giving less weight to the lower observations or the lack of an early point could create greater error in estimating this portion of the curve. Minimizing sum of squared differences in logarithms should give more validity to the fit at the early and late time periods.

Other Alternatives—Using individuals or the geometric mean can present a problem for the delta search method if any observations at the early time period give a zero concentration level. If no zero levels are observed, fitting to the geometric means is equivalent to fitting data groups for individuals when minimizing the sum of squares of differences in natural logs. Obtaining a set of constants for each subject also presents fitting problems when all individual curves do not adequately fit the model. Due to the mutual dependency of a set of constants, the appropriateness of separately obtaining an average or median *Ka*, *Kd*, γ, and Δt from the group of individual sets must also be considered.

REFERENCES

(1) G. Levy, M. Gibaldi, and W. J. Jusko, *J. Pharm. Sci.*, **58,** 422(1969).

(2) J. G. Wagner, *J. Theor. Biol.*, **20,** 173(1968).

(3) T. Teorell, *Arch. Int. Pharmacodyn. Ther.*, **57,** 205(1937).

(4) W. Lowenthal and B. L. Vitsky, *J. Pharm. Sci.*, **56,** 169(1967).

(5) J. G. Wagner, *Clin. Pharmacol. Ther.*, **8,** 201(1967).

(6) R. G. Wiegand and P. G. Sanders, *J. Pharmacol. Exp. Ther.*, **146,** 271(1964).

(7) W. E. Deming, "Statistical Adjustment of Data," Dover, N. Y., 1964 (Wiley, New York, N. Y., 1943).

(8) R. G. Wiegand and J. D. Taylor, *Biochem. Pharmacol.*, **3,** 256(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 11, 1969, from *Statistical Services Department, Technical Services, Bristol-Myers Products, Hillside, NJ 07207*

Accepted for publication November 5, 1969.

The blood concentration data appearing in Tables I and II were obtained from the Biochemistry Department, Research and Development, Bristol-Myers Products.

Physical Stability of Sulfaguanidine Suspensions

R. D. C. JONES*, B. A. MATTHEWS†, and C. T. RHODES‡

Abstract □ The effects of several variables—electrolyte, type, and concentration of surfactant and nature of vehicle—upon the physical properties of sulfaguanidine suspensions have been investigated. It is shown that the concept of controlled flocculation may usefully be applied to the formulation of pharmaceutically acceptable suspensions of this drug.

Keyphrases □ Sulfaguanidine suspension—physical stability □ Stability, sulfaguanidine suspension—electrolyte, surfactant, vehicle effects □ Sedimentation volume—sulfaguanidine suspension □ Redispersibility—sulfaguanidine suspension

Sulfaguanidine is an extremely useful antibacterial drug which is virtually nonabsorbed from the gastrointestinal tract. It is therefore particularly effective in the treatment of localized gastrointestinal infections. Because the drug does not enter the blood stream, it may be given in quite large doses, even to very young children. In the treatment of dysentery, doses of 3 g. three times daily are common and doses of 5 g. are sometimes used for severe cases.

The choice of a suspension rather than a tablet or capsule is often controlled by patient acceptability. While solid dosage forms are often supplied to adults, the treatment of children, particularly the very young, is easier with a suitably flavored suspension. There are a number of proprietary suspensions containing sulfaguanidine available for both pediatric and adult use.

In order to ensure uniformity of dosage, it is the aim of the pharmaceutical formulator to produce a preparation for which the minimum of shaking will cause homogeneous distribution of drug. It has been shown that the concept of controlled flocculation may be usefully applied to pharmaceutical suspensions (1–4). In the present paper the authors report studies which show how controlled flocculation may be applied to the formulation of sulfaguanidine suspensions.

EXPERIMENTAL

Materials—Sulfaguanidine BPC,[1] cetyltrimethylammonium bromide,[2] polysorbate 80,[2] aluminum chloride reagent grade, glycerol BP, glass-distilled water.

Particle Size—The particle size of the sulfaguanidine used in this investigation was determined using a model B Coulter counter in the manner previously described by Matthews and Rhodes (5) and Short *et al.* (6). Raw Coulter data were converted to percentage weight and percentage number oversize by a computer program. The results shown in Fig. 1 represent the mean of three determinations.

Sedimentation Volume—The sedimentation volumes of test suspensions were determined at intervals during storage by the method previously described (3).

Sedimentation volume is defined as the ratio of the ultimate settled height, H_u, to the original height, H_o.

[1] Supplied by British Drug Houses, Poole, England.
[2] Tween 80, supplied by Honeywell Atlas Ltd., London, England.

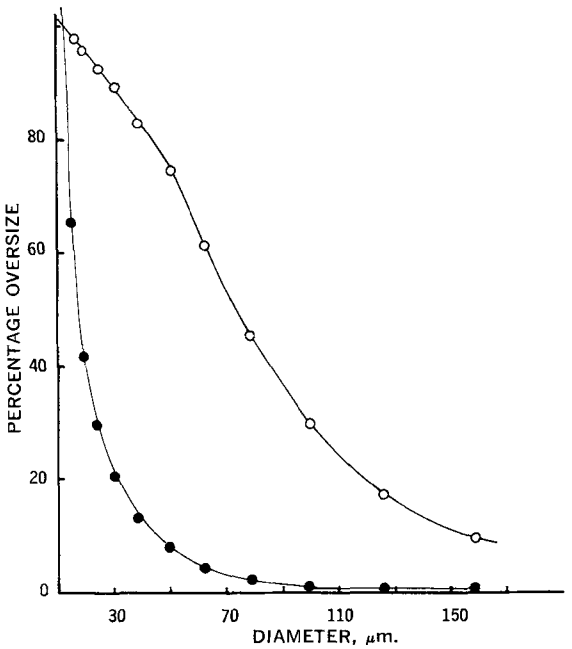


Figure 1—*Particle size spectrum of sulfaguanidine BPC. Key:* ●, *number; and* ○, *weight.*

Redispersibility—To standardize evaluation of redispersibility, the machine described by Matthews and Rhodes (3) was used. This rotates the measuring cylinders in which the suspensions were stored through 360° at a constant speed of 20 r.p.m.

Preparation of Suspensions—Preliminary tests were performed to determine the type and concentration of surfactant to wet the drug. Of the surfactants screened, cetyltrimethylammonium bromide (CETAB) and polysorbate 80 were shown to have satisfactory wetting properties at 1×10^{-2} *M* and were compatible with the aluminum chloride used as the flocculating agent.

Suspensions were prepared by dispersing 5 g. of drug in 50 ml. of double strength surfactant solution (2×10^{-2} *M*) by means of a Silverson mixer (Silverson Ltd., London, England); 40 ml. of water and 10 ml. of electrolyte solution (10 times the required concentration) were then added. The measuring cylinders containing the suspensions were then stoppered and shaken well before storage at laboratory temperature (about 18°). Suspensions containing glycerol were prepared as before, being made to volume with a suitable glycerol–water mixture.

RESULTS AND DISCUSSION

The particle size spectrum of the sulfaguanidine BPC used in this project is shown in Fig. 1. Although official, this material is of a comparatively large particle size, larger than many formulators would choose to use in suspensions. This further demonstrates the need for more official standards on particle size (5–7). However, the studies of controlled flocculation reported in this paper are substantially independent of particle size. Of course, with a different concentration of drug or with a drug of different particle size, minor modification in electrolyte or surfactant concentration might be required.

Results of the study of suspensions of sulfaguanidine wetted by 10^{-2} *M* polysorbate 80 in the presence of different concentrations of aluminum chloride as flocculating agent are shown in Fig. 2.

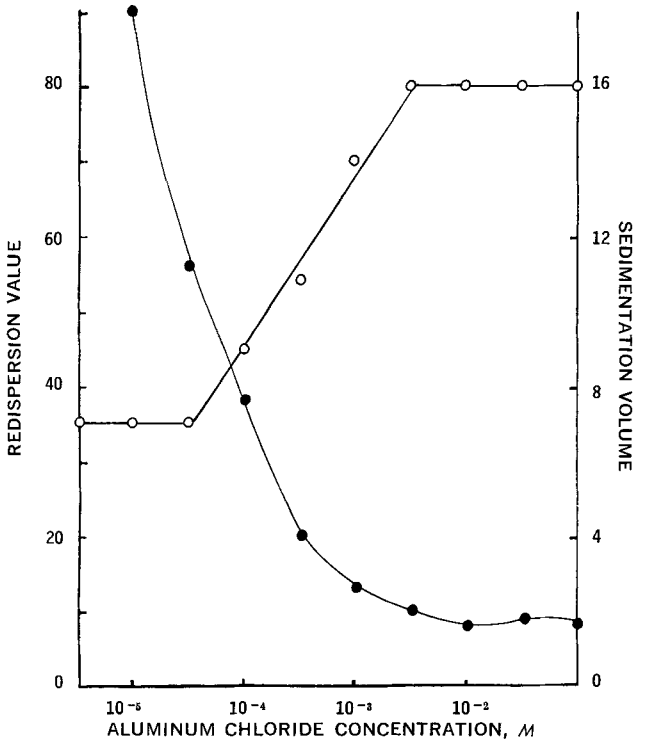


Figure 2—*Flocculation of sulfaguanidine, wetted by polysorbate 80, by aluminum chloride. Key: ○, sedimentation volume; and ●, redispersion value.*

It can be seen that there was a steady increase in sedimentation volume from about 5×10^{-5} *M* aluminum chloride until a maximum value of about 16% at 5×10^{-3} *M*. Increase in concentration of aluminum chloride above this concentration produced no corresponding increase in sedimentation volume. The progressive rise in sedimentation volume was accompanied by an increase in the ease of redispersibility, as is evidenced by the number of revolutions of the redispersing apparatus required to return the system to a homogeneous state. These results may readily be interpreted in terms of

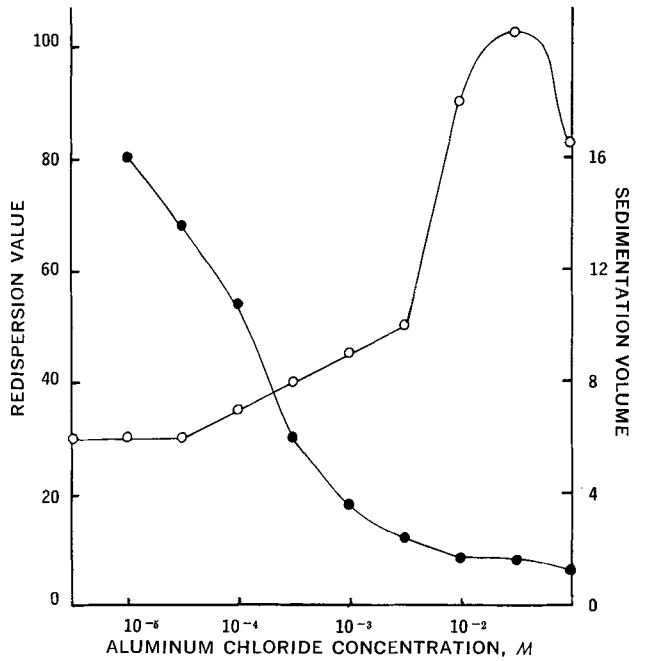


Figure 3—*Flocculation of sulfaguanidine, wetted by CETAB, by aluminum chloride. Key: ○, sedimentation volume; and ●, redispersion value.*

Table I—Effect of Glycerol upon Suspensions of Sulfaguanidine Wetted by CETAB and Partially Flocculated by Aluminum Chloride, 10^{-2} *M*

Glycerol Concentration, % w/v	Sedimentation Volume	Redispersion Value 1 week	Redispersion Value 6 weeks
0	18.0	8	8
10	17.6	8	8
15	17.6	8	9
20	17.8	8	9
25	18.4	10	11
30	18.6	12	13
35	19.0	14	14
40	19.3	16	16
45	19.5	16	16
50	Diffuse, inelegant suspension	—	—

the changes in energies of interaction between the suspended particles caused by adsorption of ions from solution. It has recently been shown that calculated energies of interaction for griseofulvin are in qualitative agreement with experimental stability results (8, 9). The general theory relating such values as zeta potential sedimentation volume and redispersibility has been previously discussed in some detail (1, 3). The sedimentation volumes of the flocculated systems are high because the floccules form rapidly and insufficient time is available for the formation of a tight, impacted cake. Thus, the ease of redispersibility increases with electrolyte concentration since relatively small forces can accomplish destruction of the nonimpacted floc, whereas the reverse situation obtains in the deflocculated system. The results shown in Fig. 3 for suspensions wetted by CETAB are similar. However, it is noteworthy that with this surfactant an increase in the concentration of flocculating agent above 5×10^{-2} *M* produced a reduction in sedimentation volume.

Having determined the optimum aluminum chloride concentration for the two surfactants, some studies were made of the effect of glycerol upon the sulfaguanidine suspensions. Four series of suspensions were prepared for this study, two sets for both surfactants, with and without aluminum chloride. The results are shown in Tables I–IV.

In the series of suspensions wetted by CETAB, in the presence of aluminum chloride, there was a small but significant increase of sedimentation volume as the glycerol concentration is increased until at 50% glycerol the products are quite inelegant. There was, however, a substantial increase in the redispersion value. A possible factor to be taken into account in considering this finding is that with the increase in viscosity of the medium the speed at which the redispersing machine operated was not great enough to redisperse the floccules. It is suggested that when redispersibility tests are used on suspensions in which the vehicle is other than aqueous, considerable care should be taken in the theoretical interpretation of such data since the effective shear at the particle–particle junctions in the floccules will vary with the viscosity and density of the continuous phase. In detailed studies of such systems, it might be useful to vary the redispersing force by modifications in apparatus design or speed of operation.

Table II—Effect of Glycerol upon Suspensions of Sulfaguanidine Wetted by CETAB, with No Added Electrolyte

Glycerol Concentration, % w/v	Sedimentation Volume	Redispersion Value 1 week	Redispersion Value 6 weeks
0	6.0	100+	100+
10	7.4	83	100+
15	9.7	76	100+
20	10.3	64	100+
25	11.1	58	100+
30	11.6	41	100+
35	12.0	36	100+
40	13.0	24	100
45	13.0	18	94
50	Diffuse, inelegant suspension	—	86

Table III—Effect of Glycerol upon Suspensions of Sulfaguanidine Wetted by Polysorbate 80 and Partially Flocculated by Aluminum Chloride, 5×10^{-3} *M*

Glycerol Concentration, % w/v	Sedimentation Volume	Redispersion Value 1 week	Redispersion Value 6 weeks
0	16.1	9	9
10	16.1	9	9
15	16.2	9	10
20	16.5	11	11
25	16.8	13	12
30	17.0	14	14
35	17.2	15	16
40	17.2	16	17

Table IV—Effect of Glycerol upon Suspensions of Sulfaguanidine Wetted by Polysorbate 80, with No Added Electrolyte

Glycerol Concentration, % w/v	Sedimentation Volume	Redispersion Value 1 week	Redispersion Value 6 weeks
0	6.0	100+	100+
10	7.1	100+	100+
15	7.7	89	100+
20	8.6	74	100+
25	10.1	58	100+
30	10.6	42	100+
35	11.2	34	100+
40	11.6	26	98

The change in sedimentation volume recorded in Table I is considerably less than had been expected. It is possible that this finding could be due to interaction between the aluminum ion and glycerol which would affect the availability of flocculant electrolyte, reducing the degree of flocculation of these systems. However, the authors have not been able to find literature substantiation of this hypothesis. This possible explanation is supported by the difference in effect which the glycerol had upon the redispersion values of the flocculated and unflocculated systems. Values increased for the systems with electrolyte but decreased for the deflocculated systems.

From the results shown in Table II, it can be seen that in the absence of flocculating agent, there was a marked increase in the sedimentation volume with increase in glycerol concentration. There was also a substantial increase in the ease of redispersibility as the glycerol concentration was increased. In the absence of electrolyte the sedimentation volume is controlled by such factors as viscosity and density of vehicle and the degree of heterogeneity of the dispersed phase. For any given system there is a critical particle size at which sedimentation will occur. Comparison of the redispersion values shown in Tables I and II shows that while the electrolyte-flocculated systems show no change in redispersion value over a 5-week period, the other systems show a marked decrease in the ease of redispersibility. This finding further points up the advantage of controlled flocculation.

Tables III and IV show the results obtained when polysorbate 80 was used as the wetting agent. The results are broadly similar to those obtained with the CETAB systems. Again, the change in sedimentation value with increasing glycerol concentration is very much greater in the absence of electrolyte, indicating the possibility of interaction between the flocculant and glycerol. Also, the effect of glycerol upon the redispersion value differs for the two systems in the same way as in the CETAB study.

The results reported in this paper further demonstrate the utility of the controlled flocculation technique in suspension formulation. Sulfaguanidine, wetted by a suitable surfactant, may be flocculated by aluminum chloride to give a pharmaceutically elegant preparation. Such suspensions may be readily redispersed, and tests have shown that even after prolonged storage such products retain satisfactory physical properties; whereas in the absence of electrolyte, impaction becomes increasingly evident. The addition of glycerol to the flocculated systems did not produce the added effect which was expected. Reasons for this behavior are the subject of further investigation.

REFERENCES

(1) B. A. Haines, Jr., and A. N. Martin, *J. Pharm. Sci.*, **50,** 228 (1961).

(2) B. A. Matthews and C. T. Rhodes, *ibid.*, **57,** 557(1968).

(3) *Ibid.*, **57,** 569(1968).

(4) B. A. Matthews and C. T. Rhodes, *J. Pharm. Pharmacol.*, **20,** 2045(1968).

(5) B. A. Matthews and C. T. Rhodes, *J. Pharm. Sci.*, **56,** 838 (1967).

(6) P. M. Short, S. V. Lincoln, and C. T. Rhodes, *Die Pharm.*, **6,** 319(1969).

(7) P. M. Short, E. T. Abbs, and C. T. Rhodes, *Can. J. Pharm. Sci.*, **4,** 8(1969).

(8) B. A. Matthews and C. T. Rhodes, *Pharm. Acta Helv.*, **45,** 52 (1969).

(9) B. A. Matthews and C. T. Rhodes, paper presented to the International Pharmaceutical Conference, London, England, 1969.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 8, 1969, from the *School of Pharmacy, Portsmouth College of Technology, Portsmouth, Hampshire, England.*

Accepted for publication October 31, 1969.

The authors thank P. M. Short for assistance in particle size analysis.

* Present address: Hafod, Heol Isaf, Penparcau, Aberystwyth, Wales.

† Present address: J. R. Geigy A. G., CH-4000 Basel 21, Switzerland.

‡ Present address: Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver 8, BC, Canada.

Use of the Derjaguin, Landau, Verwey, and Overbeek Theory to Interpret Pharmaceutical Suspension Stability

B. A. MATTHEWS* and C. T. RHODES†

Abstract □ Earlier work on the coagulation of drug suspensions is extended to other types of drugs and electrolytes. The coagulation mechanism is interpreted using the Derjaguin, Landau, Verwey, and Overbeek theory to predict energy of interaction curves. It is suggested that coagulation occurs at the primary minimum but that the depth of this is restricted due to steric stabilization by the surfactant film. A quantitative estimation of this stabilization is made using a recently published technique. The practical implications of these findings for the pharmaceutical formulator are discussed.

Keyphrases □ Suspensions, pharmaceutical—stability □ Coagulation—suspensions □ Interaction energies—computer prediction □ Stability, suspension—theory, experimental findings

The formulation of a solid-in-liquid drug suspension, which will remain free from impaction on storage, presents a challenging problem to the development pharmacist. One method which has been suggested for approaching this problem has been to use the principle of controlled flocculation (1). Although some workers (2) have questioned whether this procedure can be applied to macroscopic particles, the present authors have shown (3) that both flocculation, probably involving chemical bridging, and coagulation, resulting from zeta potential reduction, can be used to prevent impaction. It has also been shown (4) that comparisons may be made between coagulation in model monodisperse systems and in heterodisperse drug suspensions.

It has been demonstrated previously (3) that Brownian motion and differential sedimentation rates can cause particle collisions and, in the present paper, the coagulation mechanism is investigated further in terms of energy of interaction curves. Earlier work on flocculation and coagulation in suspensions of griseofulvin by aluminum chloride is extended to a sulfonamide (sulfamerazine) and a corticosteroid (hydrocortisone). Coagulation by mono- and divalent electrolytes is also examined to determine the extent of applicability of the Schultze-Hardy rule to supracolloidal suspensions.

THEORY

The theory which describes quantitatively the stability of lyophobic colloids and which enjoys almost universal acceptance is that propounded by Derjaguin and Landau (5) and, independently, by Verwey and Overbeek (6). This theory, known as the D.L.V.O. theory, involves a comparison of the forces of electrostatic repulsion and of van der Waals attraction. The theory ignores the effect on stability of materials such as polymers but, within this restricted framework, it provides an extremely useful basis for understanding stability phenomena.

Potential Energy of Electrostatic Repulsion—The potential energy of repulsion V_R may be expressed in D.L.V.O. theory by the following equation:

$$V_R = \epsilon a \psi_\delta^2 \exp(-Kh)/[(h/a) + 2] \qquad \text{(Eq. 1)}$$

where ψ_δ = the potential at the Stern layer which is usually identified with the experimentally determined zeta potential; a = the particle radius; K = the Debye-Hückel thickness; h = the interparticular distance; and ϵ = dielectric constant. Equation 1 holds for small Ka values and small potentials. For large Ka values and $\psi_\delta < 50$ mv., a condition which may be expected in many pharmaceutical suspensions, Kruyt (7) has given the following approximate expression:

$$V_R = \frac{\epsilon a \psi_\delta^2}{2} \ln[1 + \exp(-Kh)] \qquad \text{(Eq. 2)}$$

van der Waals Attraction—It was suggested by Kallman and Willstatter (8) that the attractive forces between colloidal particles could be quantitized by integrating the van der Waals attraction between their constituent atoms or molecules. Although the attractive potential energy between two atoms is low and decays with the inverse sixth power of the distance, it was shown by de Boer (9) and Hamaker (10) that the total interaction energy between two colloidal particles was sufficient to compete with double-layer repulsion.

The expression derived for the attraction energy, V_A, is

$$V_A = -\frac{Aa}{12h} \qquad \text{(Eq. 3)}$$

where A is the Hamaker constant describing the attraction between two similar particles in a given medium and h is the distance between the particles. This simple equation is not valid when the London forces operate over distances comparable with or larger than 0.1 λ where λ is the wavelength of intrinsic oscillations of the atoms. This is due to the "retardation effect" which is caused by the finite time necessary for electromagnetic waves to travel from one atom to the other atom in which it is inducing a dipole.

Schenkel and Kitchener (11) have analyzed the retardation effect and have derived empirical equations which enable attractive energies to be calculated allowing for this effect. These may be applied to coarse suspensions and aerosols. The equations are:

$$V_A \text{ (fully retarded)} \simeq -(2.45Aa)/(120\pi h^2) \qquad \text{(Eq. 4)}$$

$$V_A \text{ (partially retarded)} = -\frac{Aa}{\pi}\frac{2.45\lambda}{120h^2} - \frac{\lambda^2}{1045h^2} + \frac{\lambda^3}{5.62 \times 10^4 h^4} \qquad \text{(Eq. 5)}$$

Equation 4 may be usefully applied for particle separations greater than 150 Å and Eq. 5 for shorter distances.

Total Energy of Interaction—Since the repulsive and attractive energies are in the same units, they may be summated to produce total energy of interaction curves:

$$V_{\text{total}} = V_A + V_R \qquad \text{(Eq. 6)}$$

These may be of three general types and are illustrated in Fig. 1.

Curve A pertains when $V_R \gg V_A$, *i.e.*, where there is a large repulsive potential at the double layer. In such cases the dispersion will be indefinitely stable provided that the particles are not sufficiently large to sediment under gravity. Curve B shows a high potential barrier which must be surmounted if particles are to approach sufficiently closely to enter the deep primary energy minimum P. If the height of this barrier V_M greatly exceeds the mean thermal energy of the particles, they will not be able to enter P. The value of V_M necessary to prevent this is considered to be approximately 10–20 kT [Napper (12)], corresponding to a zeta potential of approximately 50 mv. The curve reaches a minimum at P, a very small interparticular distance, because of the Born repulsion between adjoining electron clouds.

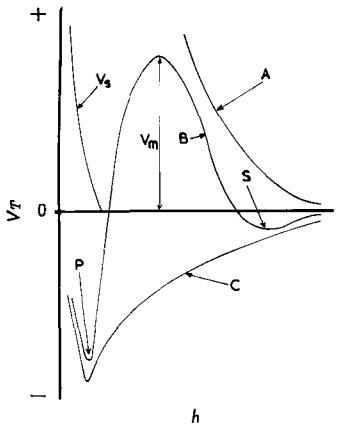


Figure 1—*Total energy of interaction between two particles,* V_T *as a function of interparticulate distance* h.

Curve *B* also has a secondary minimum, *S*, at greater distances; if this is deep enough, approximately 5 kT or greater, loose coagulation is possible. Aggregates formed by particles in the secondary minimum would be readily broken up by dilution or shaking. Schenkel and Kitchener (11) showed that 10-μ polystyrene particles were able to undergo secondary minimum coagulation.

When Curve *C* pertains, the London attraction completely overwhelms electrostatic repulsion and rapid coagulation occurs.

Other Stabilizing Mechanisms—It has been known for many years that substances such as nonionic surfactants may, if adsorbed at the particle surface, stabilize a dispersion in the absence of a significant zeta potential. The structure of various types of adsorbed layers has been considered by Ottewill (13). Early attempts to quantize the steric stabilization due to an adsorbed film have been summarized by Lyklema (14). A more recent approach is that of Ottewill and Walker (15) whose method has been used in the present paper.

The shape of the potential energy of steric stabilization curve V_S is shown also on Fig. 7 (15). It possesses a sharp cutoff at an interparticular distance of $2d$, where d is the thickness of the adsorbed layers and is approximately equal to the fully extended surfactant chain length at nearly monolayer coverage. These authors have studied the effect of a film of the nonionic surfactant $C_{12}H_{25}(OC_2H_4)_6OH$ on the stability of polystyrene latex. It was realized that there are distinct similarities between this surfactant and the anionic alkyl ether sulfates used by the present authors. It is reasonable to suppose that if the repulsion due to the anionic grouping is neutralized by a polyvalent cation, the surfactant will behave similarly to a nonionic. Ottewill (20) has suggested that the same treatment could be applied quantitatively to the system used above with modifications for the shorter chain length of $C_{12}H_{23}(OC_2H_4)_2SO_4$. The repulsive force V_S due to the adsorbed film is expressed in the equation:

$$V_S = \frac{4\pi C^2}{3V_1\rho_2^2}\left(\psi_1 - \chi_1\right)\left(d - \frac{h}{2}\right)^2\left(3a + 2d + \frac{h}{2}\right) \quad \text{(Eq. 7)}$$

where C is the concentration of surface-active agent in the adsorbed layer, V_1 is the molecular volume of the solvent molecules,[1] ρ_2 is the density of the adsorbed film, ψ_1 is an entropy parameter, χ_1 is a dimensionless quantity characterizing the interaction energy of the surface-active agent, d is the length of the surfactant molecule, h is the distance between the particles, and a is the particle radius.

EXPERIMENTAL

Materials—Griseofulvin, fine and coarse particles, ammonium dioxyethylated dodecyl sulfate (ADDS), sodium dioxyethylated dodecyl sulfate (SDDS), and aluminum chloride were those described earlier (3, 4, 16). Sulfamerazine BPC 1954 (Ferryman & Co. Ltd.), m.p. 236° (BPC 1954, 235–239°), specific gravity 1.393, specific surface area 0.60 m.²/g., determined on the Fisher subsieve sizer (16). Hydrocortisone BP (micromilled, Merck Sharp and Dohme Ltd.), m.p. 213.5° (BP 1968, 214°), specific gravity 1.277 ("Merck Index" gives 1.289 for the acetate), specific surface area 5.53 m.²/g. (16). Sodium chloride, calcium chloride (British Drug Houses, Ltd.) Analar. Distilled water was freshly redistilled from an all-glass still immediately prior to use.

Methods—Suspensions were prepared as described previously (4). They were stored in 100-ml. measuring cylinders at laboratory temperatures. Sedimentation volumes were measured as before (4); they are expressed as percentages. Sedimentation volume is defined as the ratio of the ultimate settled height, H_u, to the original height, H_o. Zeta potentials were measured also as before (4).

RESULTS AND DISCUSSION

Effect of Electrolyte Valency on Coagulation—Moderately acidic pH values were necessary (3, 4) for aluminum chloride to induce coagulation because of precipitation of aluminum hydroxide above pH 4.0. This indicated that the coagulation principle might not be applicable to pharmaceutical suspensions, even if problems of taste and toxicity could be overcome. It was decided, therefore, to see whether a divalent or monovalent electrolyte could produce the same effect. Since the earlier experiments had indicated that the mechanism involved was probably similar to that in colloids, it was realized that greater concentration of lower valency electrolytes would be necessary.

To determine whether pH would significantly affect coagulation with these electrolytes, the experiments were performed at pH 3.0 and at natural pH (5–6). Sodium chloride and calcium chloride were chosen as electrolytes and the technique used was as described before (4). The results are shown in Fig. 2, together with the results on aluminum chloride at pH 3.0 for comparison. The results indicate that pH has little effect on the coagulation produced by these salts. The minimum concentration of electrolyte required to produce maximum coagulation was molar for the monovalent salt, 5×10^{-2} *M* for the divalent, and 5×10^{-4} *M* for the trivalent. These are in the ratio of 100:5:0.05 which is in reasonable agreement with the Schultze-Hardy rule values of 100:1.6:0.13. It was noticed that the results obtained with calcium chloride were similar to those obtained with the other electrolytes, *i.e.*, that concentrations of electrolyte greater than the critical value produced little further increase in sedimentation volume. However, with the maximum concentration of calcium chloride employed, 2 *M*, a sudden further increase in sedimentation volume occurred. Since no compatibility tests had been performed with ADDS and mono- and divalent electrolytes, the suspensions experiment was repeated leaving out the griseofulvin.

It was noticed that this large concentration of calcium chloride, 2 *M*, produced a distinct opalescence whereas all of the other solutions remained clear. This precipitation of the surfactant was evidently the cause of the increase in sedimentation height, and this point on the curve can be considered as the point of transition between a coagulation and a flocculation reaction (3).

Reversibility of Coagulation in Suspensions—If coagulation in the system investigated above is dependent primarily on the effect of the cations on the repulsive potential caused by the surfactant, and

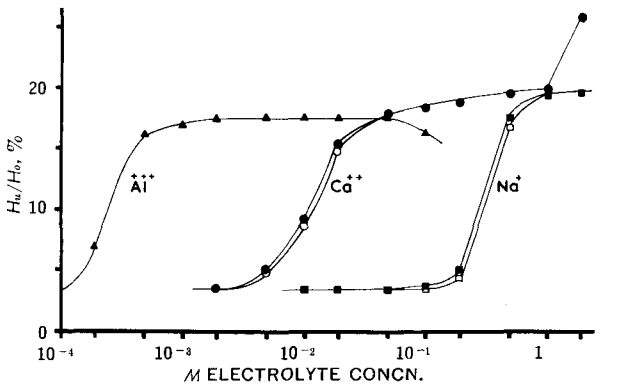


Figure 2—*Coagulation of fine-particle griseofulvin in* 10^{-3} M *ammonium dioxyethylated dodecyl sulfate by aluminum, calcium, and sodium chloride. Key: solid symbols, pH 3.0; open symbols, natural pH.*

[1] This is given as v_1 in the paper which would be the volume fraction of the solvent, but this is a misprint (20).

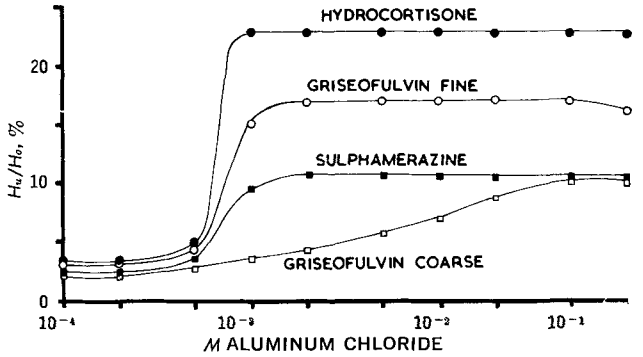


Figure 3—*Coagulation of several drugs in 10^{-3} M ammonium dioxyethylated dodecyl sulfate by aluminum chloride at pH 2.0.*

if no precipitation reaction occurs, the phenomenon should be reversible. This was investigated by preparing a 2.5% suspension of fine-particle griseofulvin in 10^{-3} *M* SDDS and adding 10^{-3} *M* aluminum chloride. The suspension became coagulated and sedimented to leave a clear supernatant. After storage overnight the suspension had a sedimentation volume of 18%. The supernatant (80 ml.) was decanted and replaced by 80 ml. of 10^{-3} *M* SDDS solution at the same pH (4.3). The suspension was redispersed and allowed to sediment again. The suspension was observed to be a typical uncoagulated dispersion and sedimented slowly leaving an opalescent supernatant. After 2 weeks the sedimentation volume was 5.0% and the griseofulvin had impacted on the base of the cylinder. This was considered good evidence that coagulation is reversible and that no irreversible reaction occurs between the surfactant and the electrolyte.

Coagulation in Suspensions of Other Drugs—Since Haines and Martin (1, 17) and Wilson and Ecanow (2) have studied sulfamerazine suspensions, it was decided to extend these investigations to this drug also. Hydrocortisone was also selected as a representative of another important class of drug, which is formulated as suspensions. It was found that ADDS wetted both of these materials at the previously used concentration, 10^{-3} *M*, so suspensions were prepared as before at pH 2.0. The results are shown in Fig. 3, together with results on coarse and fine griseofulvin taken from *Reference 4* for comparison.

The shape of the sedimentation volume–electrolyte concentration graph is identical in each case, the only real difference being the heights of the plateaus. These were, in order of increasing height, griseofulvin coarse particle, sulfamerazine, griseofulvin fine particle, and hydrocortisone. It was considered that this difference between drugs was probably a reflection of their particle sizes and, since the specific surface area was considered to be the most appropriate parameter for comparison, this was determined for each on the Fisher sub-sieve sizer. The results are shown in Table I.

The results confirm that the height of the sedimentation curve plateau is a function of the particle size, although other properties such as the contact angle between the drug and the surfactant may have some influence. This same phenomenon has also been reported in suspensions of inorganic salts by Wolf and Kurtz (18). These results suggest that coagulation can be applied to a wide range of insoluble drugs as a formulation technique.

Interpretation of Coagulation in Pharmaceutical Suspensions by Means of the D.L.V.O. Theory—A number of attempts have been made in the literature to relate the stability of monodisperse latex suspensions to the D.L.V.O. theory, *e.g.*, Schenkel and Kitchener

Table I—Specific Surface Area Results on Drugs Used in Suspension Studies

	Griseofulvin Coarse[a]	Sulfamerazine	Griseofulvin Fine[a]	Hydrocortisone
Specific Surface Area, $m.^2/g.$	0.38	0.60	1.32	5.53

[a] Taken from *Reference 4*.

Table II—Data Used in Determining the Potential Energy of Repulsion

Suspension	Concentration of $AlCl_3$, *M*	Condition	Sedimentation Volumes, %	Debye-Hückel *K*, cm.	Zeta Potential, mv.
1	10^{-5}	Uncoagulated	3.5	1.287×10^6	−46.4
2	10^{-4}	Uncoagulated	4.0	1.835×10^6	−32.7
3	10^{-3}	Coagulated	17.0	3.565×10^6	−17.0
4	10^{-2}	Coagulated	17.5	9.046×10^6	−4.5

(11) and Ho and Higuchi (19). As far as is known, however, no published attempts have been made to compare coagulation in pharmaceutical suspensions with this theory.

Equation 6 can be combined with Eq. 7 to give an expression for the total energy of interaction:

$$V_{total} = V_A + V_R + V_S \qquad \text{(Eq. 8)}$$

A computer program was written to evaluate V_{total} from these equations using the following data.

V_A—The Hamaker constant *A*—Two values of this have been taken, 10^{-13} and 5×10^{-13}, which are stated to be reasonable values for an organic substance in water (11, 19). The Hamaker constant for the surfactant film has been assumed to be the same as for the solvent.

The characteristic wavelength λ of intrinsic oscillations of the atoms was assumed to be 10^{-5} cm. (19).

The particle radius *a*—Three values were studied, 0.5, 1.0, and 2.5 μ. The results are expresssed for particle diameters.

The interparticulate distance *h*—Values from 10 to 800 Å were studied.

V_R—The dielectric constant was taken as 78.54. Since ψ_δ is difficult to determine experimentally, it was approximated to the zeta potential. Watillon and Joseph-Petit (21) have listed papers where this approximation is made.

Four suspensions were prepared containing 2.5% w/v fine particle griseofulvin and 10^{-3} *M* SDDS. The concentrations of aluminum chloride were 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} *M*, and suspensions were left at their natural pH values. Zeta potentials and sedimentation volumes were measured as before and the results are shown in Table II.

V_S—The chain length *d* of the surfactant was calculated from molecular models. It was found to be about 25 Å. All other data were taken from Ottewill and Walker (15): $C = 0.26$ g./ml., $\rho_2 = 1.0$ g./ml., $\psi_1 = 0.5$, and $\chi_1 = 0.25$.

The curves obtained for 1-μ particles are shown in Figs. 4 and 5, and the complete results showing the position and depth of the various minima are given in Table III.

The results indicate that, regardless of which value of the Hamaker constant is used, Suspensions 1 and 2 should be highly uncoagulated. This is found in practice. If the higher value of *A* is taken, a secondary minimum should occur between particles in Suspension 2. Verwey and Overbeek (6) give an example of a suspension of 1-μ

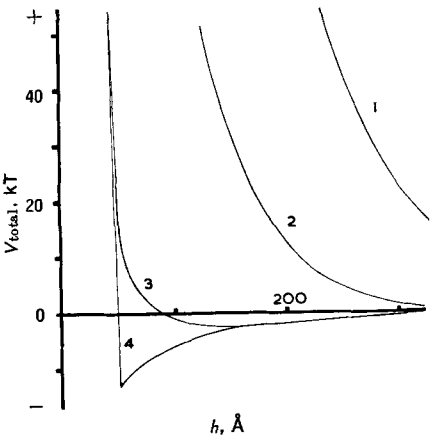


Figure 4—*Total energy of interaction curves for particles of 1-μ diameter in Suspensions 1–4. Hamaker constant:* $A = 10^{-13}$.

Table III—Depth and Position of Primary and Secondary Minima in the Energy of Interaction Curves for Griseofulvin Suspensions

Suspension and Zeta Potential, mv.	Hamaker Constant, $A = 10^{-13}$					
	$d = 1\ \mu$		$d = 2\ \mu$		$d = 5\ \mu$	
	Primary Minimum	Secondary Minimum	Primary Minimum	Secondary Minimum	Primary Minimum	Secondary Minimum
46.4[1]	—	—	—	—	—	—
3.27[2]	—	—	—	—	—	—
17.0[3]	—	−1.82 kT 150 Å	—	−3.61 kT 150 Å	—	−9.0 kT 150 Å
4.5[4]	−12.9 kT 50 Å	—	−25.8 kT 50 Å	—	−64.5 kT 50 Å	
46.4[1]	—	—	—	—	—	−3.1 kT 700 Å
32.7[2]	—	−1.91 kT 360 Å	—	−3.82 kT 360 Å	—	−9.6 kT 360 Å
17.0[3]	−41 kT 50 Å	—	−83 kT 50 Å	—	−206 kT 50 Å	
4.5[4]	−65 kT 50 Å	—	−130 kT 50 Å	—	−325 kT 50 Å	

particles in a solution of 10^{-3} M 1:1 electrolyte where there was a secondary minimum of 6 kT and where loose coagulation could occur. The secondary minimum found above reached a depth of 9 kT for 5-μ radius particles, and this might be an explanation of the slight increase in sedimentation volume that occurred in this suspension (see Table II).

Suspensions 3 and 4 were found in practice to be coagulated to practically the same extent; *i.e.*, they had similar sedimentation volumes and similar redispersibility characteristics. The energy of interaction curves should therefore be very similar in shape. This is only possible if the higher value of the Hamaker constant is taken. If the lower value is taken, Suspension 3 still has a very high maximum and a secondary minimum of similar depth to Suspension 2 described above.

With the value of A of 5×10^{-13}, Suspensions 3 and 4 both show coagulation in the primary minimum but the depth of this is restricted due to the contribution of V_S, which is zero at 50 Å but rises rapidly at lower interparticulate distances. The primary minimum would otherwise plunge to many hundreds of kT at interparticulate distances of about 10 Å. Coagulation in the primary minimum in colloidal suspensions is usually accepted to be irreversible. It was pointed out by Hamaker (22) that the frictional and inertial forces exerted by shaking increase much more rapidly with particle size than forces of adhesion. He gave an example of a suspension of 1-μ particles where the depth of the minimum was about 25 kT or 10^{-12} ergs. Hamaker calculated that a force of 10^{-6} dyne or 10^{-9} g. would be necessary to supply this energy over a distance of 10^{-6} cm. This, he pointed out, was equivalent to 300 times the particle weight. He stated: "Whether it is actually possible to supply forces of this order of magnitude by shaking is, in itself, open to question, though it is not excluded."

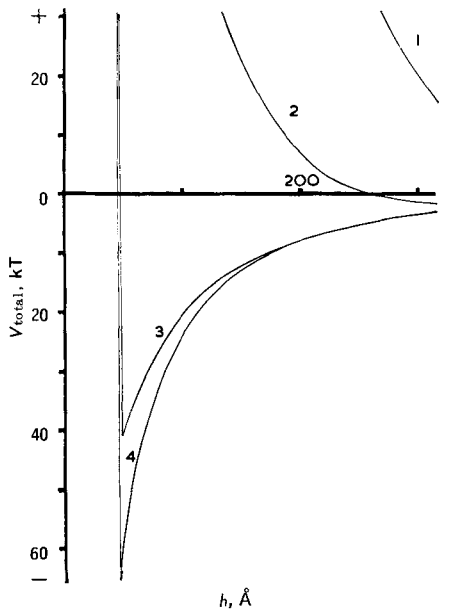


Figure 5—*Total energy of interaction curves for particles of 1-μ diameter in Suspensions 1–4. Hamaker constant:* A = 5×10^{-13}.

The situation would appear to be very similar in the suspensions described above. It should be stressed, however, that because of the irregular shape of the particles and the very open structure of the coagulated cake, the number of points of contact will be very small. In addition to this, surface irregularities, impurities, or sorption of the surfactant in the micellar form may prevent particles from approaching even to a distance of 50 Å and, if this situation pertains, the depth of the minimum will be restricted even further.

These results may also explain why the sedimentation volume–electrolyte concentration graph exhibited a rapid change with fine-particle griseofulvin and a more progressive change with the coarse-particle material. Examination of the two drugs under a microscope showed that the particles in the fine sample were very smooth, whereas in the coarse drug they were much rougher. This might enable the particles in the fine material to approach each other to within the surfactant-restricted primary minimum at a lower concentration of electrolyte than in the case of the coarse particles.

Rheological Examination of the Coagulated Suspensions—It was considered that examination of these suspensions in a viscometer, with a means of applying a variable shear rate, would provide evidence of the interparticular forces occurring in them. Although a Haake Rotovisco apparatus was available, there was no suitable attachment for handling these suspensions.[2] Samples were therefore sent to Baird and Tatlock Ltd. Suspensions 1–4 were prepared as before but the concentration of fine-particle griseofulvin was increased to 25% w/v to give a suitable reading on the instrument. Four readings were taken at different shear rates and commencing with the largest shear rate. The results are shown in Table IV.

Although these results are not conclusive, they do agree with the earlier data. Suspensions 1 and 2 are both basically Newtonian and show the same viscosity at all shear settings. Suspensions 3 and 4 both exhibit pseudoplasticity or plasticity, and there is very little difference between the readings obtained from each. The results on Suspensions 3 and 4 tend to confirm that there are attractive forces holding the particles together but that these forces can be overcome by the application of shear and the suspension can become more fluid. The absence of a complete set of results for increasing and decreasing rates or shear means that it is impossible to tell if the systems are thixotropic. There is clearly scope for further work on the rheology of coagulated suspensions.

[2] The authors thank J. K. Watkins of Baird and Tatlock Ltd., London, for the rheological measurements on the Haake Rotovisco apparatus.

Table IV—Rheological Examination of Griseofulvin Suspensions

Suspension	Shear Rate, sec.$^{-1}$	Viscosity, c.p.s.
1	2620	2.74
	1310	2.74
	873	2.75
	436	2.58
2	2620	2.67
	1310	2.86
	873	2.83
	436	2.83
3	2620	5.08
	1310	5.99
	873	6.94
	436	9.55
4	2620	5.31
	1310	6.20
	873	7.16
	436	9.78

CONCLUSIONS

It is considered that these results provide a good semiquantitative explanation of the coagulation phenomena found, if allowance is made for all the nonideal factors which occur in these suspensions and for the likely errors in ascribing values to some of the parameters.

The following guidelines may be set out for the formulation of a suspension by the coagulation technique.

1. Select a nontoxic anionic surfactant which will wet the drug or drugs and determine the minimum concentration needed. It is desirable to choose a surfactant of an appropriate chain length to ensure that the depth of the energy of interaction curve minimum is of the right order in comparison with the size of the particles. Test the electrolyte for compatibility with the surfactant.
2. Select a suspending agent, if necessary, which will not interact with the surfactant or coagulating electrolyte.
3. Add just sufficient of the electrolyte to produce coagulation in the surfactant-restricted primary minimum.
4. Ensure that the balance between the attractive and repulsive forces is not altered by any additional excipients such as color or preservatives.
5. The state of the coagulated suspension after a comparatively short interval may be considered as a good guide to the long-term storage results of such a suspension. The type of quality control standards applicable to such a suspension are:

(*a*) Control of the particle size of the suspended material by a Coulter counter, Fisher sub-sieve sizer, or other techniques.

(*b*) Measurement of the zeta potential of the final suspension. The standard should probably be a maximum value since the work described above has suggested that further lowering of the parameter has little effect on the nature of the system.

(*c*) Determination of the ratio between the H_u/H_o values of a coagulated and uncoagulated suspension.

(*d*) Evaluation of the rheological properties of the suspension.

(*e*) Measurement of redispersibility after storage.

It is believed that the work in this paper has shown that an adequate comprehension of the interfacial properties of suspensions enables their formulation to be approached from truly rational principles rather than by empirical techniques. As Elworthy (23) has remarked: "The principal point is to understand the interparticulate forces in order to be able to control them." It is the hope of the authors that this paper has contributed, in some measure, to an increase in the understanding of these forces as they affect the interfacial properties of pharmaceutical suspensions.

REFERENCES

(1) B. A. Haines and A. N. Martin, *J. Pharm. Sci.*, **50,** 753 (1961).

(2) R. G. Wilson and B. Ecanow, *ibid.*, **52,** 757(1963).

(3) B. A. Matthews and C. T. Rhodes, *J. Pharm. Pharmacol.*, **205,** 2045(1968).

(4) B. A. Matthews and C. T. Rhodes, *J. Pharm. Sci.*, **57,** 569 (1968).

(5) B. Derjaguin and L. Landau, *Acta Physiochim.*, **14,** 633 (1941).

(6) E. J. W. Verwey and J. T. G. Overbeek, "Theory of Stability of Lyophobic Colloids," Elsevier, New York, N. Y., 1948.

(7) H. R. Kruyt, "Colloid Science," Elsevier, New York, N. Y., 1952.

(8) H. Kallman and M. Willstatter, *Naturwissenschaften*, **20,** 952(1932).

(9) J. H. de Boer, *Trans. Faraday Soc.*, **32,** 10(1936).

(10) H. C. Hamaker, *Physica*, **4,** 1058(1937).

(11) J. H. Schenkel and J. A. Kitchener, *Trans. Faraday Soc.*, **56,** 161(1960).

(12) D. H. Napper, *Sci. Progr.*, **55,** 91(1967).

(13) R. H. Ottewill, *Proc. Congr. Int. Fed. Soc. Cosmet. Chemists*, London, England, **1962,** 209.

(14) J. Lyklema, *Advan. Colloid Interf. Sci.*, **2,** 65(1968).

(15) R. H. Ottewill and T. Walker, *Kolloid Z.*, **227,** 108(1968).

(16) B. A. Matthews and C. T. Rhodes, *J. Pharm. Sci.*, **56,** 836 (1967).

(17) B. A Haines and A. N. Martin, *ibid.*, **50,** 228(1961).

(18) L. K. Wolf and R. Kurtz, *Angew. Chem.*, **66,** 739(1954).

(19) N. F. H. Ho and W. I. Higuchi, *J. Pharm. Sci.*, **57,** 436 (1968).

(20) R. H. Ottewill, private communication (1968).

(21) A. Watillon and A. M. Joseph-Petit, *Discuss. Faraday Soc.*, **42,** 143(1966).

(22) H. C. Hamaker, *Trans. Faraday Soc.*, **36,** 186(1940).

(23) P. H. Elworthy, *Pharm. J.* (England), **199,** 107(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *School of Pharmacy, Portsmouth College of Technology, Portsmouth, Hampshire, England.*

Accepted for publication October 31, 1969.

This work forms part of a thesis submitted by B.A.M. for the Ph.D. degree of the University of London; it was presented to the 29th International Congress of Pharmaceutical Sciences, London, England, September 1969.

The authors thank Dr. R. H. Ottewill of the School of Chemistry, University of Bristol, England, for helpful discussion on the interpretation of the data reported in this paper.

* Present address: J. R. Geigy A. G., CH-4000, Basel 21, Switzerland.

† Present address: Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver 8, BC, Canada.

Substituted Tetralins II: Preparation of 2-Amino-4-carbamyl-6-methoxy-4-methyltetralin

DONALD E. GREEN*, ARNOLD R. MARTIN, and ALLEN I. WHITE†

Abstract □ The synthesis of 2-amino-4-carbamyl-6-methoxy-4-methyltetralin was accomplished by isonitrosation of the correspondingly substituted precursor 1-tetralone and subsequent catalytic reduction. This structural analog of meperidine exhibited only very slight analgesic activity.

Keyphrases □ 2-Amino-4-carbamyl-6-methoxy-4-methyltetralin—synthesis □ Analgesic activity—2-amino-4-carbamyl-6-methoxy-4-methyltetralin □ IR spectrophotometry—structure

Recently the preparation and analgesic potency of some 2-aminotetralin derivatives of general structure, I*a*, were reported (1). As part of a continuing study of structure activity relationships in the series, it was decided to prepare compounds having a carbonyl function (R_2 = C—R) in the 4-position. These derivatives are of
‖
O
interest because of their relationship to the meperidine and ketobemidone series of analgesics (III*a* and III*b*, respectively). The title compound (II) represents the first 4-carbonyl-containing derivative prepared in the authors' laboratory. It is anticipated that II can be converted to either I*b* or I*c* (X = H; Y = OCH_3) by conventional procedures and that separation of stereoisomers can be effected (see structures).

Attention had been focused since the early stages of the work in the authors' laboratory on the utility of 1-tetralones as useful intermediates for the introduction of 2-amino substituents into the tetralin moiety. A five-step sequence, beginning with a glyoxylation and ending with a Curtius reaction (26% overall yield), has been described previously (1). A more direct route, the isonitrosation of 1-tetralones followed by a catalytic reduction using the methods of Kindler and Peschke (2) and Rosenmund and Karg (3), appeared to be more suitable for the preparation of 4-carbonyl-2-aminotetralins.

PROCEDURE

A number of routes may be employed for the synthesis of 1-tetralones. The most common procedures are the cyclodehydration of γ-arylcarboxylic acids and the oxidation of the corresponding tetralins. Since a 6-methoxy substituent on the tetralin nucleus was also desired for the authors' purposes,[1] any direct cyclization to a 1-tetralone must, of necessity, proceed from less readily available *meta*-substituted phenyl intermediates. Moreover, the additional structural requirement imposed by the desire for a quaternary carbon atom to become C_4 in the tetralin system required the inclusion of an alkyl group on this carbon as well as the carbonyl moiety. A common general method for the preparation of a γ-quaternary carboxyl compound is a Michael condensation using acrylonitrile or methylacrylate on a tertiary carbon bearing a labile hydrogen atom. Thus, the starting material chosen for the sequence was *m*-methoxyhydratroponitrile (IV), which was prepared by the procedure of Kugita and Oine (4).

The synthesis of II is outlined in Scheme I. Compound V was prepared directly by carbomethoxyethylation of IV. The best results were obtained when IV was treated with methylacrylate and *N*-benzyltrimethylammonium hydroxide (5) without a solvent and by heating the mixture to reflux briefly after allowing it to stand overnight. In this manner, yields of up to 63% of V were obtained. The saponification of the ester (V), employing the procedure of Fuson and Miller (6), gave nearly quantitative yields of the cyano acid (VI) without affecting the nitrile group.

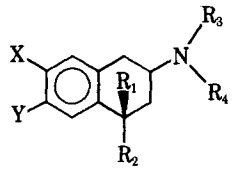
Cyclization of VI to a 1-tetralone was accomplished using several conventional cyclizing agents (stannic chloride, anhydrous hydrogen fluoride, and polyphosphoric acid). Aluminum chloride was not investigated because of its propensity to cause cleavage of ethers. The initial reagent employed was polyphosphoric acid, which has found extensive use in similar cyclizations (7–9). Although nearly a quantitative yield of crude ketonic material was obtained, the removal of a low-melting contaminant afforded only about a 75% yield of a pure tetralone, which was subsequently shown to be the amidotetralone (VIII) rather than the cyanotetralone (VII). Cyclodehydration by hydrogen fluoride (9–11) likewise gave about 75% yields of the same amidotetralone. This unexpected result had been obtained also by Price and Kaplan (11) in the cyclization of β-cyano-γ-(*p*-methoxyphenyl)-butyric acid. No doubt, entry of moisture into both the polyphosphoric acid and hydrogen fluoride-catalyzed reactions caused hydrolysis of the nitrile group. By far the most commonly employed reagent for cyclizations to tetralones is stannic chloride, either on the free acid (10, 11) or on the derived acid chloride (7, 8, 11–13). Moreover, from γ-cyano-γ-arylbutyric acids, Horning and Schock (8, 13) obtained good yields of 1-cyano-4-oxotetralins (hereafter designated 4-cyano-1-tetralones for simplicity and uniformity of nomenclature). By utilizing their procedure, yields well in excess of 90% of a quite pure product were obtained routinely.[2]

Although one of the ultimate goals of this investigation is to introduce an ester group at C_4 of a 2-aminotetralin system, no attempt was made in this synthetic scheme to convert either the amidotetralone (VIII) or the cyanotetralone (VII) to the corresponding ester because of the possibility of intramolecular aminolysis of the ester at C_4 by the primary amino group at C_2. Because the amide group does not react with primary amines and is resistant to catalytic reduction, it was decided to convert the cyanotetralone (VII) to the amidotetralone (VIII). The hindered nature of the nitrile group of VII became apparent when it was subjected to vigorous acid-catalyzed hydrolysis conditions without alteration. The nitrile (VII) was converted to the amide (VIII) in yields of 85–90% using alkaline hydrogen peroxide in a Radziszewski reaction (7, 15).

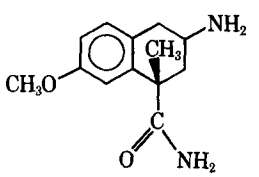
Nitrosations of aliphatic ketones have been reported using nitrous acid, nitrosyl chloride, nitrosyl sulfuric acid, nitrous fumes, and esters of nitrous acid (16). Acid or base is usually required as a catalyst with the last two reagents named. For the nitrosation of a carbon atom attached to a single activating group, such as a carbonyl, base catalysis is ordinarily not effective and acid must be used. However, cyclic ketones and phenones can be nitrosated in good yields using basic catalysts. 1-Tetralones and 1-indanones, being both cyclic and aromatic, are especially reactive toward nitrosation. Tetralones, however, seem to be quite susceptible to side reactions. Basic conditions promote oxidation of the products (dihydronaphthoquinone monoximes) to the quinoneimine tau-

[1] Substitution of an oxygen function in the 6-position of the tetralin system corresponds to a 3-oxygen function in the morphine series.

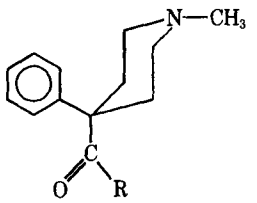
[2] The cyclization of γ-*m*-methoxyphenylbutyric acids or their chlorides can give rise to both 6- and 8-methoxy-1-tetralones. Although there have been occasional reports (12,14) of the formation of small amounts of the 8-methoxy analogs by cyclization into the *ortho*-position with reference to the methoxyl group, it has been shown (9,12) that excellent yields of the 6-methoxy analogs formed by cyclization into the *para*-position only are the usual result.



Ia, $R_1, R_2 = CH_3$;
R_3, R_4 = H or alkyl
b, $R_1, R_4 = CH_3$;
$R_2 = CO_2Et$
c, $R_1, R_3, R_4 = CH_3$; R_2 = COEt
X and Y = H, OH, OCH_3



II



IIIa, R = OEt
b, R = Et

tomers of 2-nitroso-1-naphthols. Acid reagents often cause rearrangements of substituents on the alicyclic ring.

The tetralones synthesized in this laboratory are resistant to aromatization because of the presence of a quaternary carbon at C_4. Therefore, the early attempts at the nitrosation of these compounds were conducted using concentrated hydrochloric acid and amyl nitrite in methanol according to the procedure of Perkin and Robinson (17). However, these initial experiments were uniformly unsuccessful as had been those of Horning and Schock (8) on a similar tetralone. Reports of successful nitrosations of variously substituted tetralones by the use of base (7, 18) prompted a trial of this catalyst. Using the procedure of Kornfeld *et al.* (7) a yield of about 30% of the desired isonitrosoamidotetralone (IX) was obtained in the initial trial run. Subsequent nitrosation experiments employing both acid- and base-catalyzed reactions on a number of tetralones revealed several beneficial modifications in both procedures[3] and led to good yields of IX by both methods.

The last step in the reaction sequence, leading to the substituted 2-aminotetralin (II), consists of the reduction of the enol-nitroso (or keto-isonitroso) moiety. Nitrosation of aryl ketones followed by reduction of the resulting product has long been used as a standard method for the preparation of various derivatives of β-phenethylamine (19). However, catalytic reductions often lead to aryl α-aminoketones, aryl α-aminoalkylcarbinols, or diarylpiperazines (20). Lithium aluminum hydride reductions give aryl α-aminoalkylcarbinols (21).

The synthesis of nonoxygenated amines by hydrogenations using palladium catalysts was studied extensively by Rosenmund and Karg (3). It was found that strong Lewis acids such as sulfuric acid, boron trifluoride, zinc chloride, hydrochloric acid, and (most especially) perchloric acid promoted the hydrogenolysis to the benzyl group. The use of nonaqueous fatty acid solutions and elevated temperatures also was found to be beneficial. However, the fact that these conditions were not always necessary is demonstrated by the reduction of 3-carboxamido-7-methoxy-1-tetralone to 2-carboxamido-6-methoxytetralin by the use of palladium on carbon in absolute alcohol without the use of either an acid catalyst or solvent (11).

The reduction of IX to the corresponding 2-aminotetralin (II) was accomplished using essentially the original procedure of Rosenmund and Karg (3). The use of 10% palladium on carbon and anhydrous perchloric acid (22) caused the reduction to proceed with much greater ease. The first 2 moles of hydrogen (reduction of the oxime) were taken up in 2–3 min. and the uptake of the third mole (reduction of the carbonyl) required only another 15 min. at room temperature. Gentle heating was required to cause the hydrogenolysis to occur, the stoichiometric quantity being taken up in about 2 hr. at 45° but requiring less time if the temperature is raised slightly more. Rosenmund and Karg required a temperature of 80–90°; but a report (19) of reduction of the aromatic ring at about 100°, using essentially the same other reaction conditions, prompted a search for means to accomplish the desired reduction at a lower temperature. The aminotetralin (II) was isolated by the same procedure as used by Rosenmund and Karg. However, it was discovered that a large quantity of organic material (as the perchlorate salt?) remained adsorbed to the catalyst. A methanol extraction of the catalyst, using a continuous extraction apparatus, recovered 0.48 g. of material from reduction in which 1.05 g. of nitrosated tetralone had been used. The IR spectrum of the hydrochloride salt of reduction product was consistent with that of a primary amine salt. No evidence of OH absorption was observed.

When examined by the method of Eddy and Leimbach (23), Compound II (as the hydrochloride) exhibited only very slight analgesic activity at a dose of 0.34 mmole/kg. This order of activity is roughly equivalent to that previously reported (1) for primary amines of the tetralin series.

EXPERIMENTAL[4]

Methyl-γ-cyano-γ-(*m*-methoxyphenyl)valerate (V)—When the dropwise addition, with vigorous stirring, of 35.8 g. (0.417 mole) of freshly distilled methyl acrylate into 56.4 g. (0.350 mole) of *m*-methoxyhydratroponitrile (IV) and 1 ml. of a 35% *N*-benzyltrimethylammonium hydroxide in methanol solution was started, the temperature immediately rose to about 60°. After cooling the mixture to 45°, further additions of methyl acrylate did not produce a rise in temperature, but the addition of another 0.5 ml. of *N*-benzyltrimethylammonium hydroxide solution again caused the temperature to rise sharply. Subsequently, 1 drop of *N*-benzyltrimethylammonium hydroxide solution was added occasionally while the remaining methyl acrylate was being added, the temperature being held at 45–50° by external cooling. After the addition was completed, the mixture was stirred at 55° for 1 hr. and then allowed to stand overnight at room temperature. The solution was then refluxed (110°) for 15 min., cooled, diluted with ether, washed with water, and dried with sodium sulfate. Flash distillation of the ether followed by vacuum distillation of the residual oil gave 15.8 g. of a colorless oil, b.p. 95–97° (1.0 mm.); 54.4 g. (62.8%) of cyanoester (V), b.p. 133–136° (0.3 mm.) and 122° (0.15 mm.); and a high-boiling residue which solidified upon cooling.

The combined product from several runs was fractionally distilled through a 0.91-m. (3-ft.) Podbielniak column to obtain the analytical sample, b.p. 158.1° (corrected) (1.5 mm.); n_D^{20}, 1.5129; d^{20}, 1.117; $\lambda_{max.}^{CCl_4}$, 2232 cm.$^{-1}$ (C≡N) and 1733 cm.$^{-1}$ (ester C=O).

Anal.—Calcd. for $C_{14}H_{17}NO_3$: C, 68.00; H, 6.93; N, 5.66; saponification equiv., 247. Found : C, 67.65; H, 7.08; N, 5.66; saponification equiv., 250.

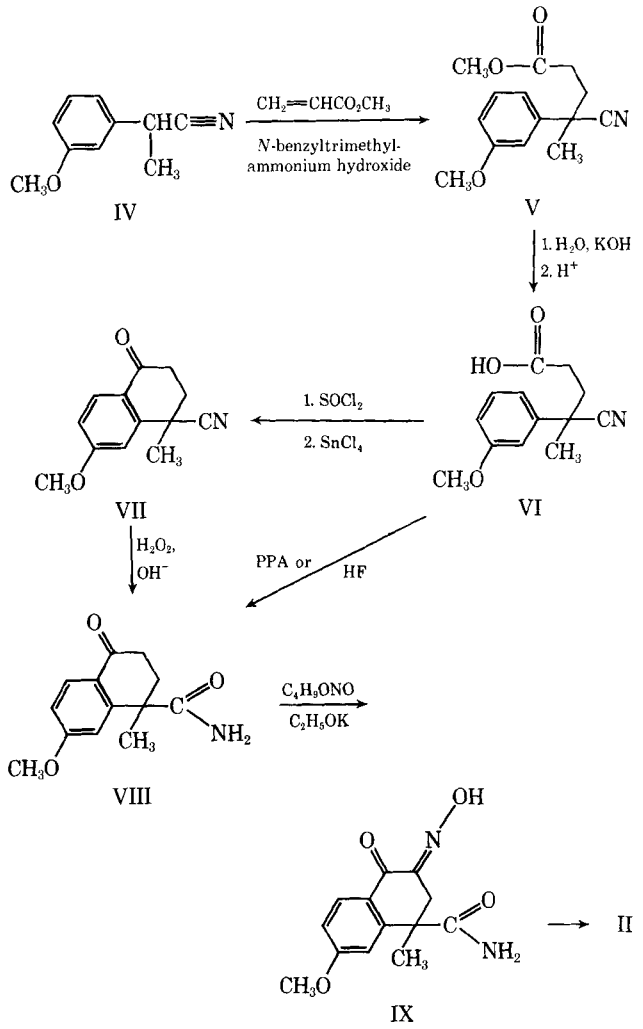
γ-Cyano-γ-(*m*-methoxyphenyl)valeric Acid (VI)—A mixture of 30.9 g. (0.125 mole) of V, 12 g. of potassium hydroxide, 12 ml. of water, and 60 ml. of methanol was warmed and then allowed to stand overnight at room temperature. After removing most of the methanol, the solution was diluted with water and washed with ether. The aqueous phase was acidified with 6 *N* hydrochloric acid, the oil which separated was removed, and the aqueous layer was extracted three times with ether. The organic phases were combined and washed with a saturated solution of sodium chloride. Concentration of the ethereal solution gave 28.9 g. (99.3%) of the cyanoacid as white prisms, m.p. 101.5–102.0° (ethanol–water), continuous submaxima between $\lambda_{max.}^{CHCl_3}$ 2440 and 3330 cm.$^{-1}$ (COOH), 2232 cm.$^{-1}$ (C≡N), and 1716 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_{13}H_{15}NO_3$: C, 66.94; H, 6.48; N, 6.00; neut. equiv., 233. Found: C, 66.70; H, 6.43; N, 5.91; neut. equiv., 230.

1,2,3,4-Tetrahydro-7-methoxy-1-methyl-4-oxonaphthonitrile (VII)—To a solution of 34.9 g. (0.150 mole) of cyanoacid (VI), in

[3] A more complete discussion of the acid- and base-catalyzed isonitrosation of 1-tetralones, plus an attempt to interpret the structure of the oxamino ketones formed on the basis of their spectral properties, will be the subject of a future publication from the authors' laboratory.

[4] Melting points were determined on a calibrated Fisher-Johns melting point block and are corrected unless otherwise specified. The IR spectra were obtained with a Beckman IR-5 spectrophotometer; all spectra of solutions were determined in a cell with a 0.1-mm. pathlength. The analyses were performed by the Weiler and Strauss Microanalytical Laboratory, Oxford, England, or by the Galbraith Laboratories, Inc., Oxford, Tenn.



Scheme I

150 ml. of anhydrous ether and 10 drops of pyridine, was added 19.5 g. (0.165 mole) of thionyl chloride. The mixture was allowed to stand overnight and then the ether and excess thionyl chloride were removed *in vacuo* (aspirator) from a 40° water bath. Forty milliliters of anhydrous benzene was added and removed *in vacuo* as above; then another 40 ml. of benzene was added and removed similarly, removing the last traces *in vacuo*. The residual yellow oil was dissolved in 20 ml. of anhydrous benzene and chilled, with stirring, in an ice–brine bath until about half the benzene had frozen to the wall of the flask. A solution of 35 ml. of stannic chloride in 35 ml. of anhydrous benzene was added rapidly, producing a sticky red complex which was not stirrable. After standing for 30 min., the complex was decomposed with 100 ml. of concentrated hydrochloric acid, 100 g. of ice, and 100 ml. of ether. The aqueous layer was separated and extracted with ether and then with benzene. The combined organic phases were washed with 5% hydrochloric acid, with 5% sodium hydroxide solution, and finally with a saturated solution of sodium chloride. Evaporation of the volatile solvents deposited 31.1 g. (96.2%) of VII as long white needles, tinged with yellow, m.p. 95°. (The yellow color could be washed off with cold ethanol without significant loss of cyanotetralone.) Two recrystallizations from methanol afforded the analytical sample, m.p. 101.5–102.0°, $\lambda_{max.}^{CHCl_3}$ 2235 cm.$^{-1}$ (C≡N) and 1689 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_{13}H_{13}NO_2$: C, 72.54; H, 6.09; N, 6.51. Found: C, 72.95; H, 6.52; N, 6.44.

1,2,3,4-Tetrahydro-7-methoxy-1-methyl-4-oxo-1-naphthamide (VIII)—*By Cyclization of VI with Polyphosphoric Acid*—A mixture of 3.0 g. (13 mmoles) of VI with 25 ml. of polyphosphoric acid was stirred at 90–100° for 1 hr. When the orange-brown reaction mixture was poured into 250 ml. of water and 250 ml. of chloroform, stirred by a magnetic stirrer, the viscous mass quickly dissolved. The phases were separated and the greenish-yellow fluorescent aqueous layer was extracted twice with chloroform; then the combined extracts were washed twice with 5% sodium hydroxide and twice with water. After drying with sodium sulfate, evaporation of the chloroform extracts afforded 2.9 g. (97%) of yellowish amidotetralone (VIII). Two recrystallizations from water with the use of decolorizing carbon afforded white crystals, m.p. 179.0–179.5°; $\lambda_{max.}^{CHCl_3}$ 3484 cm.$^{-1}$ (free NH), 3378 cm.$^{-1}$ (bonded NH), and 1675 cm.$^{-1}$ (amide C=O).

Anal.—Calcd. for $C_{13}H_{15}NO_3$: C, 66.94; H, 6.48; N, 6.00. Found: C, 67.16; H, 6.39; N, 5.98.

By Cyclization of VI with Hydrogen Fluoride—About 75 ml. of anhydrous hydrogen fluoride was added to 5.0 g (21 mmoles) of cyanoacid (VI) in a polyethylene bottle. When all of the hydrogen fluoride had evaporated, the residue was poured onto ice, the solution was made alkaline, and the solid was removed by filtration and washed with ice water. The dried solid, 4 g. (75%), m.p. 178–180°, did not depress the melting point of the amidotetralone obtained from the polyphosphoric acid cyclization, and the IR spectra of the two samples were identical.

By the Radziszewski Reaction on VII—A suspension of 21.5 g. (0.100 mole) of cyanotetralone (VII) in 41 ml. of 30% hydrogen peroxide and 200 ml. of 95% ethanol was warmed to 60° with stirring. The addition of 2 ml. of 7.5 *N* sodium hydroxide caused the solution to reflux, accompanied by a rapid evolution of oxygen. The clear, colorless solution was refluxed a total of 45 min. with the addition of more sodium hydroxide solution, as was necessary, to maintain the pH at about 9.5. After neutralizing the mixture, the ethanol was removed *in vacuo*, water was added, and the mixture was heated to boiling and filtered before chilling to crystallize the product. The white crystals of crude amidotetralone amounted to 20.4 g. (88%). Its identity was established by comparison of its IR spectrum with an authentic sample prepared as previously described and by mixed melting point (no depression).

1,2,3,4-Tetrahydro-3-isonitroso-7-methoxy-1-methyl-4-oxo-1-naphthamide (IX)—*By Acid Catalysis*—A chilled mixture of 1.8 ml. (22 mmoles) of precooled concentrated hydrochloric acid and 2.8 ml. (2.6 g., 25 mmoles) of cold, dry *n*-butyl nitrite (24) was added rapidly, with vigorous stirring, to a suspension of 4.7 g. (20 mmoles) of amidotetralone (VIII) in 30 ml. of reagent methanol chilled in ice. The ice bath was removed and the white suspension was allowed to warm spontaneously. When the temperature reached 15° the mixture took on a green tint and a mild exothermic reaction set in. At 25°, evolution of a colorless gas began and the exothermic reaction became more vigorous; the temperature rose to 42° and then fell to 35° while most of the solid dissolved. Warming the yellowish-green suspension at 40° for 2 hr. produced a copious white precipitate which was removed by filtration and washed with ice-cold methanol. After drying, the white microcrystalline oxime (IX) weighed 3.1 g. (59%), m.p. 210–218° (dec.).

The filtrate from the reaction mixture and the methanol washings of the crystals were returned to the flask and warmed at 40–45° for another hour. Adding 0.5 ml. of *n*-butyl nitrite to the dark-green solution and raising the bath temperature slightly caused the color of the solution to change to yellow and caused more solid to separate. After another 45 min. at 50°, 0.1 g. (2%) of IX, m.p. 217–222° (dec.), was filtered off. The product was soluble in aqueous alkali and sodium carbonate, giving a deep-yellow solution, but was almost insoluble in water and all common organic solvents except methanol, acetic acid, pyridine, dimethylformamide, and methyl cellosolve. Recrystallization from methanol–methylene chloride (1:1) gave stubby, very pale needles which darkened at about 205°, sintered at about 215°, and melted (in an evacuated capillary) at 220.1–224.1° (corrected) with evolution of a gas. The oximinotetralone (IX) gave an immediate strong orange color with ferric chloride in ethanol which faded upon warming or acidification. It dissolved in concentrated sulfuric acid to give a deep-yellow color rather than the intense red color which is typical of oximes of α-diketones (17). A yellow solution of its sodium or potassium salt gave the characteristic intense blue color with ferrous sulfate (25).

Anal.—Calcd. for $C_{13}H_{14}N_2O_4$: C, 59.53; H, 5.38; N, 10.68 Found: C, 60.06; H, 5.68; N, 10.49.

The solubility and melting behavior of the crude product suggested the presence of a less-polar, lower-melting isomer. Attempts to separate the suspected mixture on anionotropic alumina (Woelm) were unsuccessful.

By Alkaline Catalysis—A solution prepared by dissolving 0.86 g.

(0.022 g. atoms) of potassium in 4 ml. of absolute ethanol and 8 ml. of anhydrous toluene was added to a suspension of 4.7 g. (20 mmoles) of VIII in 75 ml. of anhydrous toluene with vigorous stirring in a nitrogen atmosphere. The clear, dark solution was chilled to 5° in an ice bath, and 2.8 ml. (2.6 g., 25 mmoles) of cold *n*-butyl nitrite was added slowly. When the exothermic reaction ceased, the ice bath was removed and the suspension was allowed to warm spontaneously to room temperature. The brown mixture was stirred at 45° for 1.5 hr., cooled, diluted with an equal volume of anhydrous ether, allowed to stand overnight, and dried in a vacuum oven.

The brown salt (5.9 g., 97% yield) was dissolved in 20 ml. of water containing 1 ml. of 10% sodium hydroxide solution and filtered to remove a few milligrams of dark-green solid. (An ether extraction of the filtrate removed more of the green compound.) Chilling the aqueous phase and acidifying to pH 4 with glacial acetic acid caused 3.9 g. (74%) of light-brown, gummy solid, m.p. 161–179°, to separate. Triturating the solid with ice-cold methanol in a chilled mortar afforded 1.8 g. of buff-colored powder which softened at 200° and melted from 208–219°. Recrystallization from methyl cellosolve–water (1:1) gave yellow needles, m.p. 216–220°, which did not depress the melting point of IX prepared by acid catalysis and which had an identical IR spectrum. Its sodium salt gave the characteristic deep-blue color with Fe^{++} (24).

Evaporation of the methanol filtrate from the trituration of the gummy solid left a dark-brown gum which was dissolved in chloroform and from which a buff-colored solid was precipitated by petroleum ether. Recrystallization of the material from dilute ethanol gave a yellow solid, m.p. 156–165°; $\lambda_{max.}^{mineral\ oil}$ 3185 and 1692 cm.$^{-1}$; $\lambda_{max.}^{CHCl_3}$ 3663, 3344, 3205, and 1692 cm.$^{-1}$. This material has not yet been identified.

3-Amino-1,2,3,4-tetrahydro-7-methoxy-1-naphthamide Hydrochloride (II)—A mixture of 1.050 g. (4.00 mmoles) of IX, 1.05 g. of 10% palladium on carbon, 4.4 ml. of a 2 *N* solution of anhydrous perchloric acid in glacial acetic acid (8.8 mmoles), and 45 ml. of glacial acetic acid were placed in a Parr hydrogenator at an initial pressure of 3.5 atm. When shaking was started, the first 2 moles of hydrogen were taken up in 3–4 min., the next 0.5 mole in about 10 min., and the next 0.5 mole in about 30 min., by which time the uptake of hydrogen had practically ceased. Warming the mixture to 40–45° caused the uptake of hydrogen to recommence. After 2 more hours the slow, steady uptake ceased with a total consumption of 108% of the theoretical amount of hydrogen. (Increasing the temperature causes the reduction to become complete in a shorter time.) The catalyst was removed by centrifugation and the solution was concentrated to dryness at the water pump. The residue, which was dissolved in water, was rendered distinctly alkaline and extracted first with ether and then with chloroform. The organic extracts were dried separately with potassium carbonate followed by potassium hydroxide pellets and then treated with anhydrous hydrogen chloride. Only 7 mg. of amine hydrochloride was obtained from the ether extract, and only 27 mg. was obtained from the chloroform extract. Therefore, the alkaline aqueous phase was placed in a liquid–liquid continuous extractor and extracted overnight with ether. After drying the ether phase as above, 315 mg. of the amine hydrochloride was precipitated with hydrogen chloride. The total recovery was 349 mg. (32.2%). (From the methanol extraction of the catalyst in a continuous extraction apparatus, 0.48 g. of crystalline material was obtained. Assuming that the material was the perchlorate salt of II, that weight represents another 37% of product.)

The amine hydrochloride was dissolved in 12–15 ml. of absolute alcohol, a small amount of insoluble material was filtered off, and the solution was diluted with about two to three volumes of anhydrous ether. The product separated as shiny white microcrystalline plates. A second recrystallization afforded the analytical sample, which darkened at 255°, softened at 263°, and melted (in an evacuated capillary) at 267.5–270.0°; $\lambda_{max.}^{mineral\ oil}$ 3390 and 3279 cm.$^{-1}$ (NH), 2012 cm.$^{-1}$ (NH_3^+), and 1647 cm.$^{-1}$ (amide C=O).

Anal.—Calcd. for $C_{13}H_{18}N_2O_2 \cdot HCl$: C, 57.67; H, 7.07; N, 10.35; Cl, 13.10. Found: C, 57.23; H, 7.12; N, 10.21; Cl, 12.94.

REFERENCES

(1) A. R. Martin, A. P. Parulkar, D. J. Gusseck, L. J. Anderson, G. L. Grunewald, and A. I. White, *J. Pharm. Sci.*, **58,** 340(1969).

(2) K. Kindler and W. Peschke, *Ann.*, **519,** 291(1935).

(3) K. W. Rosenmund and E. Karg, *Ber.*, **75,** 1850(1943).

(4) H. Kugita and T. Oine, *Chem. Pharm. Bull.*, **11,** 253(1963).

(5) E. C. Horning and A. F. Finelli, *Org. Syn.*, **30,** 80(1963).

(6) R. C. Fuson and T. G. Miller, *J. Org. Chem.*, **17,** 886(1952).

(7) E. C. Kornfeld, E. J. Fornefeld, G. B. Kline, M. J. Mann, D. E. Morrison, R. G. Jones, and R. B. Woodward, *J. Amer. Chem. Soc.*, **78,** 3087(1956).

(8) E. C. Horning and R. U. Schock, Jr., *ibid.*, **70,** 2945(1948).

(9) W. S. Johnson, *Org. React.*, **2,** 114(1944).

(10) D. H. Hey and K. A. Nagdy, *J. Chem. Soc.*, **1953,** 1894.

(11) C. C. Price and W. Kaplan, *J. Amer. Chem. Soc.*, **66,** 477 (1944).

(12) W. E. Bachmann and W. J. Horton, *ibid.*, **69,** 58(1947).

(13) E. C. Horning and R. U. Schock, Jr., *ibid.*, **71,** 1359(1949).

(14) D. K. Ingold and H. A. Piggott, *J. Chem. Soc.*, **1923,** 1469.

(15) G. B. Payne and P. H. Williams, *J. Org. Chem.*, **26,** 651 (1961).

(16) O. Touster, *Org. React.*, **7,** 327(1953).

(17) W. H. Perkin and R. Robinson, *J. Chem. Soc.*, **91,** 1073 (1907).

(18) D. Ginsburg and R. Pappo, *ibid.*, **1953,** 1524.

(19) W. H. Hartung and R. Simonoff, *Org. React.*, **7,** 263(1953).

(20) R. Adkins and R. L. Shriner, in "Organic Chemistry," vol. 1, 2nd ed., H. Gilman, Ed., 1943, p. 807.

(21) M. E. Wolff and J. F. Oneto, *J. Amer. Chem. Soc.*, **78,** 2615(1956).

(22) R. Mozingo, "Organic Synthesis, Collective Volume III," Wiley, New York, N. Y., 1955, p. 685.

(23) N. B. Eddy and D. J. Leimbach, *J. Pharmacol. Exp. Ther.*, **107,** 385(1953).

(24) C. F. Koelsch, *J. Org. Chem.*, **26,** 1291(1961).

(25) M. A. Whiteley, *J. Chem. Soc.*, **83,** 24(1903).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 8, 1969, from the *College of Pharmacy, Washington State University, Pullman, WA 99163*

Accepted for publication November 25, 1969.

This investigation was supported in part by Grant B-2054 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and by funds provided for medical and biological research by the State of Washington Initiative Measure 171.

* Present address: Analytical Instrument Division, Varian Associates, Palo Alto, CA 94303.

† To whom all correspondence should be addressed.

Testing for Uniformity: Sampling Plans in Pharmacopeias for Weight, Volume, and Content Uniformity

VALERIA PIETRA and IVO SETNIKAR

Abstract □ A procedure for analyzing the unit-to-unit uniformity specifications given by several pharmacopeias and for translating them into coefficients of variation is presented. Since the pharmacopeias fail to give the probability level of the compliance with the specifications, two largely adopted probability levels were considered: the 95% level which is important to the producer and the 10% level which is important to the consumer. The coefficients of variation implied by official uniformity specifications for the weights of tablets, capsules, miscellaneous oral forms, and sterile solids; for injection volume; and for content were calculated. The examined pharmacopeias show remarkable differences, both with regard to the sampling strategy and to the allowed variability of the considered dosage forms.

Keyphrases □ Pharmacopeias, dosage form uniformity testing—comparison □ Uniformity specifications—coefficient of variation calculations □ Coefficient of variation, dosage forms—equations □ Variability determination—dosage form units

The USP and NF introduced their first uniformity test, for unit-to-unit weight variability of tablets, in the 1950 editions. Since then uniformity specifications have been extended to the weight of other dosage forms (capsules and sterile solids) and, in other pharmacopeias, to the volume of injectable solutions (1–3). Finally, a uniformity specification for the content of the active ingredient of some tablets was introduced by USP XVII and NF XII (4).

As a general rule the official uniformity tests state the sample size and limit the number of specimens which may be outside certain limits. The allowed variability is difficult to evaluate since it depends on two factors: on the sampling plan and on the limits which discriminate the "inside" from the "outside" specimens. The pharmacopeias do not inform either on the maximum variability that a product complying with the specification may have (important information for the consumer) or on the maximum variability compatible with the compliance of the product (important information for the producer).

The uniformity specifications given by several pharmacopeias were therefore analyzed, translated into coefficients of variation, compared, and their efficacy and weaknesses commented upon.

THEORY

Although concerned with measurements of continuous variables, uniformity specifications of pharmacopeias involve sampling plans worked out for attributes, *i.e.*, for the restriction of "defectives." However, since in the context defectives are the specimens which have a weight, volume, content, *etc.*, outside some established limits, there is nothing intrinsically wrong with these specimens, provided that their incidence in the product does not exceed a certain percentage. Defectives in the context is therefore a misnomer and is properly substituted by the word "outsiders."

When the acceptance of a lot is based on a sampling plan defined by the sample size n and the acceptance number c, the probability of acceptance P_a depends on the percentage of outsiders in the submitted lot and is shown by the operating characteristic curve (*OC* curve) which may be calculated by the binomial expansion. The *OC* curve does not, of itself, give information about the percentage of outsiders which is considered critical for accepting a lot, but rather gives a general picture of the performance of the sampling plan on which it is based. In order to define, through the *OC* curve, the maximum percentage of outsiders allowed, the P_a must be agreed on and specified. Conventionally, two P_a values are considered as particularly important in sampling-inspection procedures (5): the P_a of 95%, representative of the "producer's risk" (R_P), and the P_a of 10%, used for defining the "consumer's risk" (R_C). A possible alternative value for defining the R_C may be the 5% acceptance probability.

In Table I the percentages of outsiders in the population corresponding to these three levels are given for the most common sample sizes asked by official specifications or used in pharmaceutical inspections.

The values of Table I were calculated using the central F distribution (6) for sample sizes $n \leqslant 30$, owing to the fact that in the expansion of the binomial:

$$\tfrac{1}{2}(n_1 + n_2 - 2)$$

$$[p + (1 - p)]$$

P_a is the sum of the first $n_1/2$ terms and $(1 - P_a)$ is the sum of the remaining $n_2/2$ terms when:

$$\frac{p}{1-p} = \frac{n_1}{n_2} \cdot F_{P_a};\ n_1,\ n_2 \qquad \text{(Eq. 1)}$$

For sample sizes n = 50, 60, and 100, the percentages of outsiders were calculated with the aid of the tables of Cameron (7), *i.e.*, using Poisson's approximation (6).

Table I shows the capability of a plan to detect and to limit the outsiders in a lot. But this is only one step toward the assessment of variability, which depends also on the limits used for discriminating the insiders from the outsiders. These limits are symmetrically set about the mean and are expressed as fractions of the mean. The official uniformity specifications can be converted into an appropriate measure of variability (coefficient of variation, *CV*) by means of the factors given in Table II, the entries of which are the reciprocals of the abscissas of the normal curve, corresponding to the fractions of outsiders, multiplied by 100. A numerical example of this conversion is shown in the section *Specifications for Uniformity of Tablet Weights*.

In fact, for the evaluation of the variability through the percentage of outsiders and the amplitude of limits, the distribution pattern of the variable must be recognized. Unfortunately the sample sizes prescribed by official codexes are inadequate for the identification of the distribution type and larger samples may not be available in field inspections, in inspections performed by the

Table I—Percentage of Outsiders which Will Be Accepted with the Stated Probabilities[a] for Samples of Stated Sample Size and Rejection Number[b]

$O_{tol.}$	Accept. Prob.	Sample Size 5	6	7	8	9	10	11	12	13	14	15	18	20	25	30	50	60	100
0	0.95	1	0.9	0.8	0.7	0.6	0.5	0.5	0.4	0.4	0.4	0.3	0.27	0.25	0.20	0.17	0.10	0.09	0.05
	0.10	37	32	28	25	23	21	19	17	16	15	14	12	11	9	7.5	4.5	3.8	2.5
	0.05	45	39	35	31	28	26	24	22	21	19	18	15	14	11	9.5	6	5	3
1	0.95	8	6	5	4.5	4	3.5	3.3	3	2.8	2.5	2.5	2.2	2	1.5	1	0.7	0.6	0.35
	0.10	58	51	45	41	37	34	31	29	27	25	24	20	18	15	12.5	8	6.5	4
	0.05	66	58	52	47	43	39	36	34	32	30	28	24	22	18	15	9.5	8	4.5
2	0.95	19	15	13	11	10	9	8	7	7	6	5.5	5	4.5	3.5	2.5	1.5	1.4	0.8
	0.10	75	67	60	54	49	45	42	39	36	34	32	27	24.5	20	17	10.5	9	5.5
	0.05	81	73	66	60	55	51	47	44	41	39	36	31	28	23	20	12.5	10.5	6.5
3	0.95	34	27	23	19	17	15	14	12	11	10	9.5	7.5	7	5.5	4.5	2.5	2.3	1.5
	0.10	89	80	72	66	60	55	51	48	45	42	39	33	30.5	25	21	13.5	11.1	6.5
	0.05	92	85	77	71	66	61	56	53	49	47	44	38	34	28	24	15.5	13	8
4	0.95		42	34	29	25	22	20	18	17	15	14	12	10.5	8.5	7	4	3.3	2
	0.10			83	76	70	65	60	55	52	49	46	40	36	30	25	16	13.3	8
	0.05			87	81	75	70	65	61	57	54	51	44	40	33	28	18.5	15.3	9
5	0.95				40	34	30	27	25	22	21	19	16	14	11	9	5	4.4	2.5
	0.10					79	73	68	63	59	56	53	46	41	34	29	18.5	15.5	9.5
	0.05					83	78	73	68	64	61	58	50	46	38	32	21	17.5	10.5
6	0.95							35	32	29	26	24	20	18	14	12	6.5	5.5	3.5
	0.10										63	60	51	46	38	33	21	17.6	10.5
	0.05										67	64	55	51	42	36	23.5	19.7	12
7	0.95										33	30	24	22	17	14	8	6.6	4
	0.10												56	51	43	36	23.5	19.6	12
	0.05												61	56	46	39	26.5	22	13
8	0.95												29	26	21	17	9.5	7.8	4.5
	0.10													56	47	40	26	21.7	13
	0.05													61	50	43	29	24.1	14.5
9	0.95													30	24	19	11	9	5.5
	0.10														50	43	28.5	23.7	14
	0.05														54	47	31.5	26.2	15.5
10	0.95														27	22	12.5	10.3	6
	0.10														55	47	31	25.7	15.5
	0.05														58	50	34	28.3	17
11	0.95														31	25	14	11.5	7.0
	0.10															50	33	27.7	16.5
	0.05															53	36.5	30.3	18.0
12	0.95															28	15.5	12.8	7.5
	0.10																35.5	29.6	18
	0.05																39	32.4	19.5

[a] Accept. prob. [b] $O_{tol.}$

average consumer, or in outgoing quality inspection of the producer. This obstacle may be overcome assuming a normal distribution. But the variables involved do not always conform to the normal distribution, since sometimes they are truncated normal, skew, lepto- or platy-curtic, or bimodal, *etc.* (8–10). Nevertheless, a normal distribution is very frequent, and therefore it is still meaningful to assume it for analyzing uniformity specifications and for comparing those of different official codexes. Obviously this assumption must be kept in mind in critical situations and in borderline conditions. In these cases the distribution type ought to be checked with large samples before drawing final conclusions.

Table II—Factors (u) for Calculating CV[a] in Relation to the Percentage of Outsiders

Outsiders, %[b]	0	1	2	3	4	5	6	7	8	9
0	[c]	39	43	46	49	51	53	55	57	59
10	61	63	64	66	68	69	71	73	75	76
20	78	80	81	83	85	87	89	91	93	95
30	97	99	101	103	105	107	109	112	114	116
40	119	121	124	127	130	132	135	138	142	145

[a] $CV = u \cdot L$; L is the limit about the mean and expressed as fraction of the mean. [b] *E.g.*, for 22% outsiders, $u = 81$. [c] Factors for percentages between 0.05 and 0.90:

Percent	0.05	0.10	0.20	0.25	0.30	0.40
Outsiders, %	29	30	32	33	34	35
Percent	0.50	0.60	0.70	0.80	0.90	
Outsiders, %	36	36	37	38	38	

ANALYSIS OF THE UNIFORMITY SPECIFICATIONS OF PHARMACOPEIAS

With the exception of the "Pharmacopée Française" (11), the most important recent pharmacopeias demand the compliance with uniformity tests for several dosage forms. In the present study the pharmacopeias listed in Table III were examined, their uniformity tests analyzed, and the percentages of outsiders calculated with the aid of Table I at the acceptance probability levels of $R_P = 95\%$ and of $R_C = 10\%$ and then converted into CV values using Table II.

Table III—Pharmacopeias Examined and Abbreviations Used

Pharmacopeias	Year of Issue	Abbreviation
British Pharmacopoeia	1968	BP
Deutsches Arzneibuch 7 (of the DDR)	1964	DA-E
Deutsches Arzneibuch 7 (of the BR)	1968	DA-W
Farmacopea Ufficiale della Repubblica Italiana 7	1965	FU
Österreichisches Arzneibuch 9	1960	OA
Pharmacopée Belge 5	1962	PB
Pharmacopoeia of Japan 7	1961	PJ
Pharmacopoea Nordica	1964	PN
State Pharmacopoeia of the USSR 9	1961	PUSSR
Spécifications pour le Contrôle de la Qualité des Préparations Pharmaceutiques (WHO)	1967	WHO
United States Pharmacopeia XVII	1965	USP
National Formulary XII	1965	NF

Table IV—Official Specifications for Tablet Weight Uniformity

Codex	Sample Size	Acceptance Conditions[a]								CV[b]			
		I Condition				II Condition				Producer		Consumer	
		Plan	Limits	O_P	O_C	Plan	Limits	O_P	O_C	I	II	I	II
BP	20	2/20	$m \pm L$	4.5	24.5	0/20	$m \pm L'$	0.25	11.0	2.5–5.0	3.3–5.0	4.3– 8.6	6.3– 9.5
DA-E[c]	10	1/10	$m \pm L$	3.5	34.0	0/10	$m \pm 2L$	0.50	21.0	2.4–7.2	3.6–10.8	5.2–15.7	8.0–24.0
DA-W	20	2/20	$m \pm L$	4.5	24.5	0/20	$m \pm 2L$	0.25	11.0	2.5–7.5	3.3–9.9	4.3–12.9	6.3–18.9
FU	20	2/20	$m \pm L$	4.5	24.5	0/20	$m \pm 2L$	0.25	11.0	2.5–5.0	3.3–6.6	4.3– 8.6	6.3–12.6
OA	20	2/20	$m \pm L$	4.5	24.5	0/20	$m \pm 1.5L$	0.25	11.0	2.5–5.0	2.5–4.9	4.3– 8.6	4.7– 9.5
PB	20	2/20	$m \pm L$	4.5	24.5	0/20	$m \pm 2L$	0.25	11.0	2.5–7.5	3.3–9.9	4.3–12.9	6.3–18.9
PJ	20	2/20	$m \pm L$	4.5	24.5	0/20	$m \pm 2L$	0.25	11.0	2.5–5.0	3.3–6.6	4.3– 8.6	6.3–12.6
PN	100, 30	3/30	$M^d \pm L$	4.5	21.0	0/30	$M^d \pm 2L$	0.17	7.5	2.9–5.0	3.8–6.4	4.6– 8.0	6.5–11.2
PUSSR	10	0/10	$m \pm L$	0.5	21.0					1.8–3.6		4.0– 8.0	
WHO	20	2/20	$m \pm L$	4.5	24.5	0/20	$m \pm 2L$	0.25	11.0	2.5–7.5	3.3–9.9	4.3–12.9	6.3–18.9
USP–NF	20	2/20	$m \pm L$	4.5	24.5	0/20	$m \pm 2L$	0.25	11.0	2.5–5.0	3.3–6.6	4.3– 8.6	6.3–12.6

[a] The actual values of L are given in Table V. [b] The ranges shown reflect the ranges of L values for different tablet weights. [c] *Cf. Footnote*[a] of Table V. [d] *Cf. Footnote*[b] of Table V.

Table V—Limits for Tablets

Codex	L					
	0.05 m	0.075 m	0.08 m	0.10 m	0.125 m	0.15 m
BP	>250	81–249		⩽80		
BP (L′)				⩾250	81–249	⩽80
DA-E[a]	>300	151–300		51–150		⩽50
DA-W	>300	151–300		26–150		⩽25
FU	>300	151–300		⩽150		
OA	>500		251–500	⩽251		
PB	⩾300	150–299		25–149		<25
PJ	⩾300	120–299		<120		
PN[b]	Sliding[b]					
PUSSR	⩾120			<120		
WHO	>324	131–324		14–130		⩽13
USP–NF	>324	131–324		⩽130		

[a] Same specifications for granules, dragee-cores, and pastilles. [b] Weigh 100 tablets and calculate the average weight M. $L = 0.10\ M$ for tablets weighing less than 80 mg.; $L = 4$ mg. $+ 0.05\ M$ for tablets weighing 80 mg. or more; values of L from 0.058 to 0.10 M were considered for calculating CV.

Some specifications set their limits about the sample mean m and some about the true mean μ. In the first case the same specimen may be sometimes an outsider and sometimes an insider, according to the value of the mean of the sample which includes it. As a consequence the probability of acceptance depends also on the random difference between the sample mean and the true mean, as shown in a previous paper (12). No correction was made, however, for this situation or for the influence of double-sampling plans on the OC curve, since these corrections require propositions not given in the examined pharmacopeias.

Specifications for Uniformity of Tablet Weights—Table IV summarizes the uniformity specifications and the acceptance conditions given for tablets.

Most pharmacopeias set two interlinked conditions shown in Table IV under the headings I and II. These conditions may be analyzed even by the trinomial expansion (13).

Table VII—Notes for Capsules

(*a*) Type A specification for contents of hard capsules. Limits: $L = 0.10\ m$ for contents of 120 mg. or less; $L = 0.075\ m$ for contents of more than 120 mg. Type B specification for contents of soft capsules (chlortrianisene, ethchlorvynol, ethosuximide, halibut liver oil, paramethadion, phytonanadione, tetrachloroethylene).

(*b*) For hard and for soft capsules.

Content, mg.	>300	151–300	51–150	⩽50
L	0.05 m	0.075 m	0.10 m	0.15 m

If the weight of content is labeled, m is the label weight. If the weight of content is not labeled, m is the sample average weight.

(*c*) $L = 0.05\ m + 10$ mg. Values from 100 to 1000 mg. were considered for calculating CV.

(*d*) Type A specification for the weights of whole capsules. In the event of noncompliance, type B specification is applied to contents. The definition of specification B is obscure.

(*e*) Type A specification for the weights of whole capsules. In the event of noncompliance, type B specification is applied to the contents. If the contents fail to comply with test B1, test B2 is allowed, provided that not more than 6/20 contents fall outside the limits $m \pm 0.10\ m$. These conditions correspond to a producer's CV ⩽7.5 and a consumer's CV ⩽13.5.

The implied maximum percentages of outsiders are given in the O_P column (for a $P_a = 95\%$, *i.e.*, the producer's risk) and in the O_C column (for a $P_a = 10\%$, *i.e.*, the consumer's risk). The limits which discriminate the outsiders are set by most pharmacopeias symmetrically about the sample mean. Usually different limits are given according to the average tablet weight. The "Pharmacopoea Nordica" makes an exception since it relates the weight limits to the mean obtained on a larger sample than that used for measuring variability and considers a sliding variation instead of a step-by-step one. From Tables IV and V it may be noted that both the limits and the tablet weights which define them are quite different in the examined codexes.

The actually allowed unit-to-unit variabilities are given as CV values in Table IV, the ranges of which reflect the ranges of the

Table VI—Official Specifications for Capsule Weight Uniformity

Codex	Step or Type	Sample Size	Acceptance Conditions[a]								CV[b]			
			I Condition				II Condition				Producer		Consumer	
			Plan	Limits	O_P	O_C	Plan	Limits	O_P	O_C	I	II	I	II
BP[c]	A	20	2/20	$m \pm L$	4.5	24.5	0/20	$m \pm 2L$	0.25	11.0	3.8–5.0	4.9– 6.6	6.4– 8.6	9.5–12.6
	B	10	1/10	$m \pm 0.075\ m$	3.5	34.0	0/10	$m \pm 0.15\ m$	0.5	21.0	3.6	5.4	7.9	12.0
DA-E		10	1/10	$m \pm L^d$	3.5	34.0	0/10	$m \pm 2L^d$	0.5	21.0	2.4–7.2	3.6–10.8	5.3–15.7	8.0–24.0
DA-W		20	2/20	$m \pm 0.10\ m$	4.5	24.5	0/20	$m \pm 0.15\ m$	0.25	11.0	5.0	5.1	8.6	9.5
PB		20	2/20	$m \pm L^e$	4.5	24.5	0/20	$m \pm 2L^e$	0.25	11.0	3.0–7.5	4.0– 9.9	5.2–12.9	7.6–18.9
PN		20	2/20	$m \pm 0.10\ m$	4.5	24.5	0/20	$m \pm 0.20\ m$	0.25	11.0	5.0	6.6	8.6	12.6
WHO[f]	A	20	0/20	$m \pm 0.10\ m$	0.25	14.0					3.3		6.8	
	B	20	2/20	$m \pm 0.10\ m$	4.5	24.5	0/20	$m \pm 0.20\ m$	0.25	11.0	5.0	6.6	8.6	12.6
USP–NF[g]	A	20	0/20	$m \pm 0.10\ m$	0.25	14.0					3.3		6.8	
	B1	20	2/20	$m \pm 0.10\ m$	4.5	24.5	0/20	$m \pm 0.25\ m$	0.25	11.0	5.0	8.2	8.6	15.8
	B2	60	6/60	$m \pm 0.10\ m$	5.5	17.6	0/60	$m \pm 0.25\ m$	0.09	3.8	5.2	7.5	7.4	12.0

[a] The actual values of L are given in Table VII. [b] The ranges shown reflect the ranges of L values for different capsule weights. [c] *Cf. Note* (*a*) in Table VII. [d] *Cf. Note* (*b*) in Table VII. [e] *Cf. Note* (*c*) in Table VII. [f] *Cf. Note* (*d*) in Table VII. [g] *Cf. Note* (*e*) in Table VII.

Table VIII—Official Specification for Weight Uniformity of Miscellaneous Oral Dosage Forms

Dosage Form	Acceptance Conditions: I Condition Plan	I Condition Limits	O_P	O_C	II Condition Plan	II Condition Limits	O_P	O_C	CV: Producer I	Producer II	Consumer I	Consumer II
DA-E												
Dragees	1/10	$m \pm 0.15\,m$	3.5	34.0					7.2		15.8	
Pills[a]	2/20	$m \pm 0.10\,m$	4.5	24.5	0/20	$m \pm 0.15\,m$	0.25	11.0	5.0	5.0	8.6	9.5
DA-W												
Pills	3/30	$m \pm 0.10\,m$	4.5	21.0	0/30	$m \pm 0.30\,m$	0.17	7.5	5.0	9.6	8.0	16.8
OA												
Pills	3/30	$m \pm 0.10\,m$	4.5	21.0	0/30	$m \pm 0.30\,m$	0.17	7.5	5.0	9.6	8.0	16.8
Divided powders	0/10	$m \pm L$[b]	0.5	21.0					2.9–5.4		6.4–12.0	
PB												
Cachet-gelules	2/20	$m \pm L$[c]	4.5	24.5	0 20	$m \pm 2\,L$[c]	0.25	11.0	2.8–5.0	3.7–6.6	4.8– 8.6	7.1–12.6
PN												
Pills	3/30	M[d] $\pm 0.15\,M$	4.5	21.0					7.5		15.0	
Dragees and boles	2/20	$m \pm 0.10\,m$	4.5	24.5	0 20	$m \pm 0.20\,m$	0.25	11.0	5.0	6.6	8.6	12.6

[a] Comprehensive of all formed oral dosage forms.

[b] Content, mg. >1000 | 501–1000 | 201–500 | ≤200
L 0.08 m | 0.10 m | 0.12 m | 0.15 m

m must be within ±10% the labeled weight. [c] $L = 0.05\,m + 5$ mg. Values from 100 to 2000 mg. were considered for calculating CV. [d] The grand mean M is calculated on 100 pills.

L values given in Table V for weights of different tablets. Remarkable differences may be noted between the uniformity levels required by the different codexes.

The following example, based on the BP uniformity specifications for tablets greater than 250 mg., shows how the CV values were obtained. The sampling plan is defined by $n = 20$ and $c = 2$ (Table IV). According to Table I there is a 95% probability of accepting the submitted lot if 4.5% (O_P) of the items of the lot are outside the limits, which are $m \pm 0.05\,m$ (Table V). From the table of the normal distribution, it is found that 4.5% outsiders (O_P), namely, 2.25% at each end, lie beyond the limits $m \pm 2\,SD$. Therefore, $2\,SD = 0.05\,m$, so that $CV = 100\,SD/m = 2.5$.

The CV values may be obtained using the factors of Table II:

$$CV = u \cdot L = 50 \cdot 0.05 = 2.5 \qquad \text{(Eq. 2)}$$

Specification for Uniformity of Capsule Weights, for Weights of Miscellaneous Oral Forms, and for Suppository Weights—Tables VI and VII show the weight uniformity specification for capsule weights, Table VIII shows those for miscellaneous oral dosage forms, and Table IX shows those for suppositories.

These tables were prepared following the same criteria adopted for tablets and, as for tablets, there is little similarity between the different sampling plan strategies and procedures.

Specifications for Uniformity of Injectables—Tables X and XI summarize the specifications for weight uniformity of sterile solids. There are large differences with regard to the allowed CV values and still more with regard to the weight classes for which the sizes of limits change (Table XI).

The specifications for uniformity of volume of injectables are more regular (Table XII). These specifications are given in four of the examined pharmacopeias.

Content Uniformity—USP–NF are the first pharmacopeias to introduce a specification on content uniformity. The prescribed two-step procedure is summarized in Table XII and the operating characteristic curve is given in Fig. 1.

Table IX—Official Specifications for Suppository Weight Uniformity

Codex	Sample Size	Acceptance Conditions: I Condition Plan	I Condition Limits	O_P	O_C	II Condition Plan	II Condition Limits	O_P	O_C	CV: Producer I	Producer II	Consumer I	Consumer II
DA-E	5	0/5	$m \pm 0.05\,m$	1.0	37.0					2.0		5.6	
DA-W	10	1/10	$m \pm 0.05\,m$	3.5	34.0	0/10	$m \pm 0.10\,m$	0.5	21.0	2.4	3.6	5.3	8.0
PB	20	2/20	$m \pm 0.05\,m$	4.5	24.5	0/20	$m \pm 0.10\,m$	0.25	11.0	2.5	3.3	4.3	6.3
PN	20	2/20	$m \pm 0.10\,m$	4.5	24.5	0/20	$m \pm 0.20\,m$	0.25	11.0	5.0	6.6	8.6	12.6
PUSSR	10	0/10	$m \pm 0.05\,m$	0.5	21.0					1.8		4.0	

Table X—Official Specifications for Weight Uniformity of Sterile Solids

Codex	Step	Sample Size	Acceptance Conditions: I Condition Plan	I Condition Limits	O_P	O_S	II Condition Plan	II Condition Limits	O_P	O_C	CV: Producer I	Producer II	Consumer I	Consumer II
BP		10	1/10	μ[a] $\pm L$	3.5	34.0	0/10	$m \pm 2\,L$	0.5	21.0	2.4– 4.8	3.6– 7.2	5.3–10.6	8.0–16.0
DA-E[b]		10	0/10	$m \pm L$	0.5	21.0					2.2– 5.4		4.8–12.0	
DA-E[c]		5	1/5	$m \pm L$	8.0	58.0	0/5	$m \pm 2\,L$	1.0	37.0	2.8– 8.6	3.9–11.7	9.1–27.1	11.2–33.6
PB		10	0/10	$m \pm L$	0.5	21.0					1.1– 3.6		2.4– 8.0	
PJ		10	0/10	μ[d] $\pm L$	0.5	21.0					5.0–10.8		11.2–24.0	
USP–NF[e]	1	20	2/20	$m \pm 0.10\,m$	4.5	24.5	0/20	$m \pm 0.15\,m$	0.25	11.0	5.0	5.0	8.6	9.5
	2	60	6/60	$m \pm 0.10\,m$	5.5	17.6	1/60	$m \pm 0.15\,m$	0.6	6.5	5.2	5.4	7.4	8.1

For the values of L *cf.* Table XI.

[a] μ is the labeled weight. [b] The pooled mass of the 10 units must be within ±10% of the labeled mass, if this is smaller than 2000 mg., and within ±15% of the labeled mass if this is larger than 2000 mg. [c] The specification concerns the implants. [d] The sample average must be within $\mu \pm 0.5\,L$ (*cf.* Table XI). μ is the labeled weight. [e] Step 2 is allowed when the sample does not comply with Step 1 and when less than 7/20 weights are outside $m \pm 0.10\,m$. The producer's CV may therefore reach a value of 7.5 and the consumer's CV a value of 13.5. The sample average m must be between $\mu \pm 0.07\,\mu$, where μ is the labeled weight. The USP–NF do not state if the last rule applies to the sample of 20, or of 60, or to both.

Table XI—*L* Values for Sterile Solids

Codex	BP	DA-E	DA-E	PB	PJ
L =					
0.03 *m*				>300	
0.05 *m*	⩾300		>300	151–300	
0.06 *m*		>1000		51–150	
0.075 *m*	121–299		151–300	26–50	
0.08 *m*		501–1000			
0.10 *m*	⩽120	201–500	51–150	<26	
0.12 *m*		101–200			
0.14 *m*					⩾300
0.15 *m*		⩽100	⩽50		120–299
0.20 *m*					15–119
0.30 *m*					<15

DISCUSSION

The specifications for uniformity tests prescribed in several important pharmacopeias are remarkably different, even in the same codex for different dosage forms, and lack essential information for establishing the variability actually allowed. If the specifications are analyzed with constant criteria, their comparison becomes possible and reveals discrepancies as *CV* at the consumer's risk level from 8.0 to 15.7 for tablets, from 6.8 to 15.7 for capsules, from 8.0 to 12.0 for miscellaneous oral forms, from 4.3 to 8.6 for suppositories, from 8.6 to 27.1 for sterile solids, and from 8.0 to 15.8 for injection volume.

For some dosage forms, different variabilities are allowed, depending on the average weight. The criteria adopted for establishing these weight classes differ markedly in the examined pharmacopeias as shown by Table V for tablets, Table VII for capsules, and Table XI for sterile solids. The differences are difficult to understand since they are not related to technical reasons because most dosage forms are produced with a narrower weight variability than that necessary for the compliance with the official specifications. The differences are even not justified by some needs of the consumer, who may be prepared to accept differences in uniformity according to the safety margin or to the therapeutic efficacy of a drug, but not according to the dosage form or to the size of the dosage form by which the drug is administered.

Uniformity specifications with two interlinked compliance conditions (I Condition and II Condition in the tables) imply a larger *CV* for the second condition than for the first, both for the producer and for the consumer. Probably the second condition aims to establish absolute limits to variability or to protect from abnormal variability. Compliance with absolute limits, however, cannot be assured by sample inspections, and abnormal variability can be investigated only with much larger sample sizes than those considered by the official specifications.

Most specifications consist of a one-step sampling plan. There are some exceptions, *e.g.*, USP–NF describe a two-step sampling plan for capsules, for sterile solids, and for content of active ingredient. These double plans are difficult to comment on since the second step in the three plans has different effects, both on the variability allowed to the producer and on the protection for the consumer.

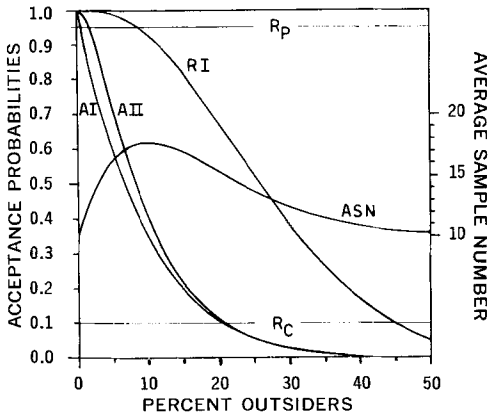


Figure 1—*Characteristics of two-step sampling plan for content uniformity of USP–NF. Key:* AI, *operating characteristic* (OC) *curve for acceptance after the first sampling* {*10,0*}; RI, OC *curve for rejection after the first sampling* {*10,2*}; AII, OC *curve for acceptance after the second sampling* {*10,1;20,0*}; ASN, *average sample number related to percent outsiders;* R_p, *producer's risk level* (P_a = *0.95*); R_c, *consumer's risk level* (P_a = *0.10*).

Perhaps the most critical uniformity specification is the two-step sampling plan for content, required by USP–NF for some drugs dosed in tablets. The operating characteristic (*OC*) curves, given in Fig. 1, show that the second step adds a very small amount of tolerated outsiders at the level of the R_P and that there is practically no difference of tolerated outsiders at the level of the R_C. The advantage of the second step, therefore, is not clear.

The specification for content uniformity apparently restricts variability to the same order of magnitude as that allowed for tablet weights. The *CV* allowed for content, however, is comprehensive both for variability of actual content and for the apparent variability, *i.e.*, that linked to the analytical error, which may reach values of 3% and more and is different for each analytical method. Therefore, the allowances for content variability are actually different for each drug, depending on the precision of the analytical method.

In conclusion, the official uniformity specifications may be analyzed in order to obtain useful information on the variabilities actually allowed. The relevant *CV* values may be calculated and used for production-control charts and for acceptance inspections performed with other sample sizes. They inform also the consumer about the variability which may inhere in accepted products.

The approach presented in this paper complements that of Roberts (14) who studied the relationship between *CV* of the lot and the probability that a sample fails the USP–NF uniformity tests for weight of tablets, capsules, or sterile solids.

SYMBOLS AND DEFINITIONS

P_a = acceptance probability
n = sample size
c = acceptance number
p = fraction of defectives (or outsiders) in the population

Table XII—Official Specifications for Uniformity of Injection Volume and for Content Uniformity

Codex	Sample Size	Acceptance Conditions				*CV*	
		Plan	Limits	O_P	O_C	Producer	Consumer
BP	10	0/10	$\mu \pm L^a$	0.5	21.0	1.8–3.6	4.0–8.0
PB	10	1/10	$\mu^b \pm 0.15\,\mu$	3.5	34.0	7.2	15.8
PJ	10	1/10	$\mu^b \pm 0.15\,\mu$	3.5	34.0	7.2	15.8
WHO	10	1/10	$\mu^b \pm 0.15\,\mu$	3.5	34.0	7.2	15.8
USP–NF	10	0/10	$\mu^c \pm 0.15\,\mu$	0.5	21	5.4	12
	30	1/30	$\mu^c \pm 0.15\,\mu$	1.5	21.5	6.2	12

[a] For labeled volumes ⩽2.0 ml., L = 0.10 μ (μ = prescribed volume) and the sample average volume within $\mu \pm 0.05\,\mu$. For labeled volumes >2.0 ml., L must be within the labeled volume and $+0.05\,\mu$. The prescribed volume μ is given in a table. [b] μ is the prescribed volume given in a table of the codex. Directions are given for the limits of the average sample volume. [c] Specification for content uniformity. The second step, with additional 20 contents, is allowed when not more than one value in Step 1 exceeds $\mu \pm 0.15\,\mu$. This implies a producer's *CV* of 7.2 and a consumer's *CV* of 15.7. μ is the labeled content. The specification applies to tablets of hydrocortisone, prednisolone, prednisone, chlorpromazine, prochlorperazine, digitoxin, ergonovine, and phenobarbital.

OC = operating characteristic curve
R_C = consumer's risk (set at a 10% level)
R_P = producer's risk (set at a 95% level)
CV = coefficient of variation [$CV = (s/m) \cdot 100$]
s = sample standard deviation
m = sample mean
μ = population or true mean
T = prescribed value
L = limit symmetrically set about the mean
k = fraction of the mean by which limits are expressed ($L = km$)
u = factor for calculating CV from k when a normal distribution is assumed ($CV = uk$)

Outsiders = specimens outside mean $\pm L$
Insiders = specimens inside mean $\pm L$

REFERENCES

(1) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1968.

(2) "Pharmacopée Belge," **5**, 1962.

(3) "Pharmacopoeia of Japan," 7th ed., Hirokawa Publishing, Tokyo, Japan, 1961.

(4) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965; "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.

(5) E. L. Grant, "Statistical Quality Control," McGraw-Hill, New York, N. Y., 1964, p. 344.

(6) A. Hald, "Statistical Theory with Engineering Applications," Wiley, New York, N. Y., 1965, pp. 674, 688.

(7) J. M. Cameron, *Ind. Quality Control*, **9**, 37(1952).

(8) P. M. A. section meetings, 1963, Quality Control Section, pp. 458, 471.

(9) J. D. Haynes, M. Schnall, and A. S. Doniger, "Types of Unit-to-Unit Variation and Their Implications," Third Annual Eastern Regional Meeting, Industrial Pharmacy Section, APhA, New York, N. Y., 1963.

(10) C. W. Dunnett and R. Crisafio, *J. Pharm. Pharmacol.*, **7**, 314(1955).

(11) "Pharmacopée Francaise," 8th ed., Impr. Maisonneuve, St. Ruffine, France, 1965.

(12) I. Setnikar and V. Pietra, *J. Pharm. Sci.*, **58**, 112(1969).

(13) J. D. Haynes, M. Schnall, and R. A. Lamm, "Variability Tests in Acceptance Sampling from Non-normal Populations," presented at the American Statistical Association annual meeting, Cleveland, Ohio, 1963.

(14) C. Roberts, *Technometrics*, **11**, 161(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 26, 1969, from the *Research Division, Recordati s.a.s., Milano, Italy.*

Accepted for publication September 12, 1969.

Quantitative Gas-Liquid Chromatographic Determination of Estrone in Dermatological Products

PRAMOD P. KARKHANIS and JON R. ANFINSEN

Abstract □ A gas-liquid chromatographic procedure employing an internal-external standard ratioing technique is described for the analyses of estrone in dermatological preparations. The analysis of a cream or lotion is performed by the addition of an internal standard, extraction of sample with 10% sodium hydroxide, filtration, adjustment of the filtrate pH to 9–9.5, and chromatography on a 3% OV-1 column.

Keyphrases □ Estrone dermatological products—analysis □ Extraction procedure, estrone—internal-external standard ratioing technique □ GLC—analysis □ Equilenin solution—internal standard

Estrone has been incorporated in creams primarily for the treatment of senile vaginitis, pruritus vulvae, leukoplakia vulvae, and in emollients for the relief of local antikeratotic and trophic therapy in skin of the climacteric. In addition to the base, these preparations frequently contain vitamin A, hydrocortisone, and pyrilamine maleate for local antihistaminic and analgesic effect.

Several chemical methods for the estrone are found in the literature (1–5). However, due to the small amount of the steroid and the interference from the other ingredients in these pharmaceutical preparations, the results obtained with some of these methods were unreliable. The biological assay of estrone (6), based on the cellular change in the vagina of the spayed mouse or rat, gave erratic results.

Kroman *et al.* have quantitatively determined the concentration of estrone in the human plasma using a combination of chemical extraction and gas chromatography (7) and Wotiz and Chattoraj have described a method to determine estrone in low- and high-titer urine employing TLC and gas chromatography (8).

A GLC procedure has been described for ethinyl estradiol in both sesame oil solutions and solid dosage forms, using estrone as an internal standard, by Talmage *et al.* (9); Boughton *et al.* have determined ethinyl estradiol in tablets and granulations by gas chromatography using estrone as an internal standard (10). The proposed method, with a simple clean-up procedure, allows the separation and determination of estrone by gas chromatography while eliminating interferences from excipients commonly present in the creams and lotions.

EXPERIMENTAL

Instrument—Hewlett-Packard 5754A research chromatograph equipped with Hewlett-Packard 3370A electronic integrator and Honeywell Electronic 16 recorder.

Column—A 1.22-m. (4-ft.) helical glass column, 4 mm. i.d.

Liquid Phase—Three percent OV-1 on diatomite aggregate[1] (HP), 80–100 mesh (Supelco, Inc., Bellefonte, Pa.). The column is conditioned overnight at 300° with a helium flow rate of 45 ml./min.

[1] Chromosorb G, Johns-Manville Products Corp., New York, N. Y.

OC = operating characteristic curve
R_C = consumer's risk (set at a 10% level)
R_P = producer's risk (set at a 95% level)
CV = coefficient of variation [$CV = (s/m) \cdot 100$]
s = sample standard deviation
m = sample mean
μ = population or true mean
T = prescribed value
L = limit symmetrically set about the mean
k = fraction of the mean by which limits are expressed ($L = km$)
u = factor for calculating CV from k when a normal distribution is assumed ($CV = uk$)

Outsiders = specimens outside mean $\pm L$
Insiders = specimens inside mean $\pm L$

REFERENCES

(1) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1968.

(2) "Pharmacopée Belge," **5**, 1962.

(3) "Pharmacopoeia of Japan," 7th ed., Hirokawa Publishing, Tokyo, Japan, 1961.

(4) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965; "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.

(5) E. L. Grant, "Statistical Quality Control," McGraw-Hill, New York, N. Y., 1964, p. 344.

(6) A. Hald, "Statistical Theory with Engineering Applications," Wiley, New York, N. Y., 1965, pp. 674, 688.

(7) J. M. Cameron, *Ind. Quality Control*, **9**, 37(1952).

(8) P. M. A. section meetings, 1963, Quality Control Section, pp. 458, 471.

(9) J. D. Haynes, M. Schnall, and A. S. Doniger, "Types of Unit-to-Unit Variation and Their Implications," Third Annual Eastern Regional Meeting, Industrial Pharmacy Section, APhA, New York, N. Y., 1963.

(10) C. W. Dunnett and R. Crisafio, *J. Pharm. Pharmacol.*, **7**, 314(1955).

(11) "Pharmacopée Francaise," 8th ed., Impr. Maisonneuve, St. Ruffine, France, 1965.

(12) I. Setnikar and V. Pietra, *J. Pharm. Sci.*, **58**, 112(1969).

(13) J. D. Haynes, M. Schnall, and R. A. Lamm, "Variability Tests in Acceptance Sampling from Non-normal Populations," presented at the American Statistical Association annual meeting, Cleveland, Ohio, 1963.

(14) C. Roberts, *Technometrics*, **11**, 161(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 26, 1969, from the *Research Division, Recordati s.a.s., Milano, Italy.*

Accepted for publication September 12, 1969.

Quantitative Gas-Liquid Chromatographic Determination of Estrone in Dermatological Products

PRAMOD P. KARKHANIS and JON R. ANFINSEN

Abstract □ A gas-liquid chromatographic procedure employing an internal-external standard ratioing technique is described for the analyses of estrone in dermatological preparations. The analysis of a cream or lotion is performed by the addition of an internal standard, extraction of sample with 10% sodium hydroxide, filtration, adjustment of the filtrate pH to 9–9.5, and chromatography on a 3% OV-1 column.

Keyphrases □ Estrone dermatological products—analysis □ Extraction procedure, estrone—internal-external standard ratioing technique □ GLC—analysis □ Equilenin solution—internal standard

Estrone has been incorporated in creams primarily for the treatment of senile vaginitis, pruritus vulvae, leukoplakia vulvae, and in emollients for the relief of local antikeratotic and trophic therapy in skin of the climacteric. In addition to the base, these preparations frequently contain vitamin A, hydrocortisone, and pyrilamine maleate for local antihistaminic and analgesic effect.

Several chemical methods for the estrone are found in the literature (1–5). However, due to the small amount of the steroid and the interference from the other ingredients in these pharmaceutical preparations, the results obtained with some of these methods were unreliable. The biological assay of estrone (6), based on the cellular change in the vagina of the spayed mouse or rat, gave erratic results.

Kroman *et al.* have quantitatively determined the concentration of estrone in the human plasma using a combination of chemical extraction and gas chromatography (7) and Wotiz and Chattoraj have described a method to determine estrone in low- and high-titer urine employing TLC and gas chromatography (8).

A GLC procedure has been described for ethinyl estradiol in both sesame oil solutions and solid dosage forms, using estrone as an internal standard, by Talmage *et al.* (9); Boughton *et al.* have determined ethinyl estradiol in tablets and granulations by gas chromatography using estrone as an internal standard (10). The proposed method, with a simple clean-up procedure, allows the separation and determination of estrone by gas chromatography while eliminating interferences from excipients commonly present in the creams and lotions.

EXPERIMENTAL

Instrument—Hewlett-Packard 5754A research chromatograph equipped with Hewlett-Packard 3370A electronic integrator and Honeywell Electronic 16 recorder.

Column—A 1.22-m. (4-ft.) helical glass column, 4 mm. i.d.

Liquid Phase—Three percent OV-1 on diatomite aggregate[1] (HP), 80–100 mesh (Supelco, Inc., Bellefonte, Pa.). The column is conditioned overnight at 300° with a helium flow rate of 45 ml./min.

[1] Chromosorb G, Johns-Manville Products Corp., New York, N. Y.

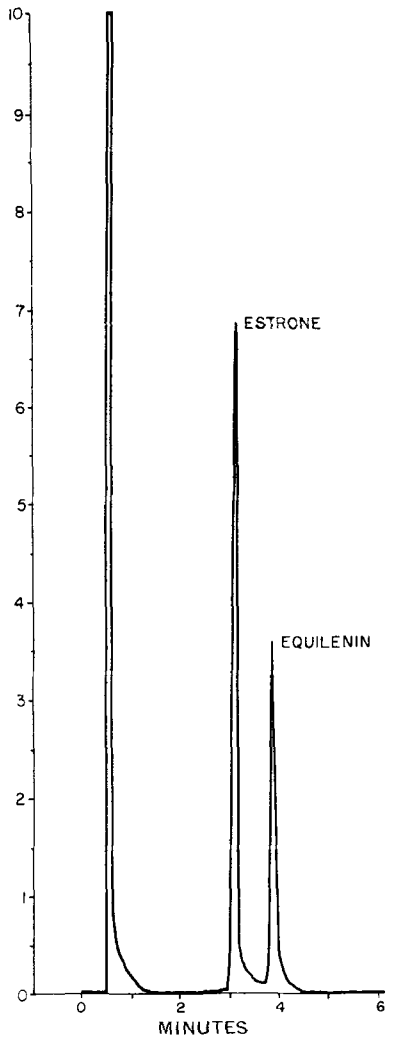


Figure 1—*A typical chromatograph.*

Operating Conditions—Hydrogen pressure 14 p.s.i.; air pressure, 30 p.s.i.; helium flow rate, 70 ml./min.; column oven temperature, 245°; detector temperature, 290°; injection port temperature, 290°; detector, flame ionization.

Reagents and Solutions—Estrone NF[2]; equilenin (K and K Laboratories, Inc.); chloroform NF; sodium hydroxide 10% aqueous solution; 6 *N* sulfuric acid.

Standard Estrone Solution—Weigh accurately 50.0 mg. of estrone NF into a 25-ml. volumetric flask. Dissolve in and make to volume with methanol–methylene chloride (1:1).

Internal Standard Equilenin Solution—Weigh accurately 50.0 mg. of equilenin into a 50-ml. volumetric flask. Dissolve in and make to volume with methanol.

Working Standard Solution—Pipet 1 ml. each of standard and internal standard solutions into a small stoppered flask. Evaporate the solvent to dryness on a steam bath. To the residue add 0.5 ml. of chloroform. Mix well.

Preparation of Sample Solution—Into a glass or polypropylene stoppered tube, weigh accurately an amount of sample containing approximately 1.0 mg. of estrone. With a pipet add 1 ml. of internal standard solution followed by 20 ml. of 10% sodium hydroxide solution. Heat on a steam bath for about 5 min. and then shake the tube on a mechanical shaker for about 10–15 min. or until the sample is well dispersed. Cool to room temperature. Filter the solution under vacuum through a 0.64-cm. (0.25-in.) layer of diatomaceous earth[3] spread over glass-fiber filter paper (2.1 cm.) in a Gooch crucible. Rinse the tube with 10 ml. of water and filter the rinsing.

Transfer the filtrate to a 100-ml. beaker; rinse the vacuum flask with 10 ml. of water and transfer it to the beaker. Adjust the pH of the solution to 9–9.5 with 6 *N* sulfuric acid while mechanically stirring. Transfer this solution to a 125-ml. separator and extract three times with 25 ml. chloroform. Collect the chloroform layer and discard the aqueous layer after the third extraction. Filter the chloroform extract through about 5–6 g. of anhydrous sodium sulfate over a glass-fiber filter paper into a 125-ml. flask. Wash with 10 ml. chloroform. Evaporate the solvent to about 10 ml. on a Rinco evaporator. Transfer the solution to a small tube, rinse the flask with 5 ml. of chloroform, and transfer it to the tube. Then evaporate the solvent to about 1 ml. by heating in a stream of nitrogen. This is the working sample solution.

Chromatography—Balance the instrument after it has reached the operating temperatures and then set the range at 10^2 and attenuator to 8. Inject aproximately 3–4 μl. of working standard solution and working sample solution, using a 10-μl. Hamilton syringe. The estrone peak has a retention time of about 3.0 min., whereas the equilenin peak has a retention time of about 4.0 min. The area under each peak is determined with an electronic integrator or by employing a manual technique (11).

Calculations—Es = estrone peak area, Eq = equilenin peak area, A = micrograms of estrone in the working standard solution, and W = sample weight in grams:

$$\frac{A}{W} \times \frac{Es/Eq \text{ for sample}}{Es/Eq \text{ for std.}} = \text{mcg. estrone/g.} \qquad \text{(Eq. 1)}$$

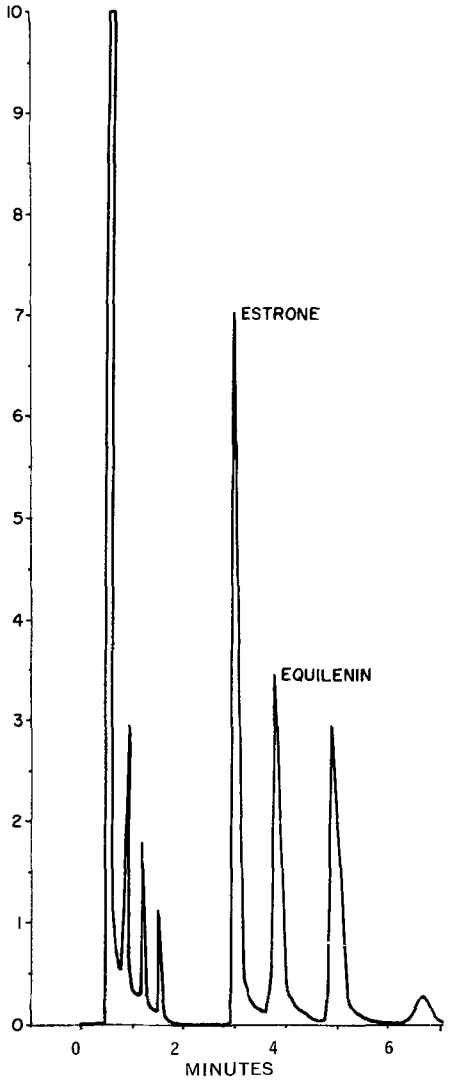


Figure 2—*A chromatograph obtained from the extract of dermatological preparation.*

[2] Supplied by the Dome Laboratories, Division of Miles Laboratories, Inc., West Haven, Conn.
[3] Celite 545, Johns-Manville Products Corp.

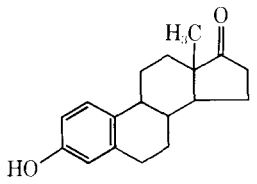
Table I—Determination of Estrone in Commercial Preparations

Product	Label Claim, mcg. Estrone	Estrone Found, mcg./g.
Cream I	200.0	205.4
Cream II	200.0	202.9
Emollient	200.0	204.8

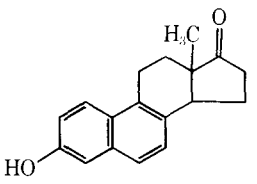
RESULTS AND DISCUSSION

The calibration curve for concentration of estrone *versus* integrated area of estrone/integrated area for equilenin gave a straight line. A typical chromatograph is shown in Fig. 1, and a chromatograph obtained from the extract of the dermatological preparation is shown in Fig. 2. Liquid phases such as SE-30, OV-1, and XE-60 were investigated. OV-1 [methyl silicone (Supelco, Inc.)] was found to be most satisfactory because of its stability at high temperatures.

The accuracy of a gas chromatographic procedure is dependent upon the ability to compensate for errors resulting from extraction, filtration, and injection. The internal–external ratioing technique makes it possible to minimize these errors when an internal standard with chemical properties similar to the compound being quantitated can be identified. The most important chemical similarity to achieve is that of partition coefficient since large errors are introduced by extraction losses. Equilenin was selected as an internal standard due to its very close structural relationship with estrone (Structures I and II) so that the losses incurred during the entire operation were compensated. The value of R, the response factor as described by Celeste and Turczan (12), was found constant; however, it should be noted that when employing the internal–external ratioing procedure, the determination of gas chromatographic response factor is not necessary.



estrone
I



equilenin
II

The sample cream base formulated was composed of cetyl alcohol, stearyl alcohol, spermaceti, beeswax, white petrolatum, mineral oil, glycerin sodium lauryl sulfate, ascorbyl palmitate, BHT, BHA, and methyl- and propylparabens. Vitamin A palmitate and hydrocortisone alcohol were also present. To this cream base a fixed amount of internal standard of equilenin and known amounts of estrone at different levels were added. For 18 replicate samples, average recovery and coefficients of variation were found to be 98.9% and 1.1%, respectively. Three commercial preparations[4] were assayed for their estrone content. The results obtained are shown in Table I.

REFERENCES

(1) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 159.

(2) E. J. Roy, *J. Endocrinol.*, **25,** 361(1962).

(3) J. Carol, F. M. Kunze, D. Banes, and J. H. Graham, *J. Pharm. Sci.*, **50,** 550(1961).

(4) J. H. Graham, *ibid.*, **54,** 1665(1965).

(5) T. Urbanyi and C. R. Rohm, *ibid.*, **55,** 501(1966).

(6) Private communications with Dome Laboratories, West Haven, Conn.

(7) H. S. Kroman, S. R. Bender, and R. L. Capizzi, *Clin. Chim. Acta*, **9,** 73(1957).

(8) H. H. Wotiz and S. C. Chattoraj, *Anal. Chem.*, **36,** 1466 (1964).

(9) J. M. Talmage, M. H. Penner, and M. Geller, *J. Pharm. Sci.*, **54,** 1194(1965).

(10) O. D. Boughton, R. Bryant, W. J. Ludwig, and D. L. Timma, *ibid.*, **55,** 951(1966).

(11) E. M. Emery, *J. Gas Chromatogr.*, **5,** 596(1967).

(12) A. Celeste and J. Turczan, *J. Ass. Offic. Agr. Chem.*, **46,** 1055(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 16, 1969, from *Methods Development Laboratories, Miles Laboratories, Inc., Elkhart, IN 46514*

Accepted for publication November 19, 1969.

[4] Supplied by Dome Laboratories.

Simultaneous Determination of Nonderivatized Phenylpropanolamine, Glyceryl Guaiacolate, Chlorpheniramine, and Dextromethorphan by Gas Chromatography

ERNEST MARIO and LAWRENCE G. MEEHAN

Abstract □ A gas chromatographic procedure is described for the rapid and quantitative determination of phenylpropanolamine, glyceryl guaiacolate, chlorpheniramine, and dextromethorphan in a commercially available cough-cold preparation. Pramoxine is used as the internal standard. The active ingredients are extracted in chloroform and injected without further treatment. A single chromatogram, obtained isothermally, quantitatively resolves all four ingredients.

Keyphrases □ Phenylpropanolamine, glyceryl guaiacolate, chlorpheniramine, dextromethorphan—simultaneous determination □ GLC—analysis □ Pramoxine—GLC internal standard

In recent years, the introduction of cough-cold preparations containing several active ingredients has increased sharply. The combining of antitussive, antipyretic, and antihistaminic drugs has distinct commercial advantages in that several cough-cold symptoms can be treated simultaneously with a single preparation. However, these combination-dose forms present serious difficulties to the quality control analyst who is concerned with identification and independent quantitation of each active ingredient. Classical wet methods are time consuming, expensive, and frequently of little use when the physical-chemical properties of two or more ingredients are relatively similar. The applicability of gas chromatography to pharmaceutical analysis is well documented in the literature (1–10), and the resolution of many types of mixtures has been published. Serious difficulties are often encountered when relatively polar compounds of high molecular weight are chromatographed (11). These materials often exhibit excessive tailing, and the selection of the liquid phase becomes very critical if resolution is to be obtained. The problem is compounded when active ingredients of widely divergent polarities are present in a given mixture. In a recent article, Hishta and Laubach (12) analyzed a mixture of phenylpropanolamine, phenylephrine, phenyltoloxamine, and chlorpheniramine by gas chromatography after forming the bistrimethylsilylacetamide (BSA) derivative.

This investigation was undertaken to study the advisability of using a low liquid load of nonpolar silicon rubber SE-30 in a modified all-glass system for the resolution of a multicomponent drug mixture containing ingredients of widely divergent polarities without the use of derivatives. In initial studies utilizing metal columns, it was found that the results were often inconsistent, showing incomplete recovery and excessive peak tailing. Apparently the active ingredients, when present in the free base form, interact in some way with metal surfaces. On modification to an all-glass, on-column injection system, these problems were eliminated.

EXPERIMENTAL

Equipment—A Varian Aerograph model 1200-1 with flame ionization detector was used for the experimental work. The column used was a 2.44-m. (8-ft.) coiled Pyrex glass, 0.32-cm. (0.125-in.) o.d., packed with 2% SE-30 on 80/100-mesh diatomite [Chromosorb W (HP), acid washed and silanized]. Gas flow rates of 30 ml./min. for hydrogen, 30 ml./min. for helium, and 300 ml./min. for air were used. The instrument was modified by replacing the 0.64-cm. (0.25-in.) injection port containing 0.32-cm. (0.125 in.) metal inserts with an 0.32-cm. (0.125-in.) injection port, and the column was extended through the port to the septum. Injection was on-column, and therefore the sample did not come in contact with any metal surface except for the brief instant it passed through the syringe needle during injection. The detector oven and injection port were maintained at 270°. The column oven temperature was held isothermal at 180° through the analysis.

Reagents—The pramoxine internal standard was prepared by weighing approximately 1 g. of pramoxine hydrochloride of known purity into a 250-ml. separator containing 25 ml. of deionized water. After solution was effected, 25 ml. of 50% sodium hydroxide solution was added cautiously to the aqueous phase in the separator while cooling under cold tap water. This solution was then extracted with four 20-ml. portions of chloroform. The chloroform extracts were filtered through cotton and pooled in a 100-ml. volumetric flask. The total volume was adjusted to 100 ml. with chloroform. The solution, when refrigerated, was stable for approximately 6 weeks.

Sample Preparation—A commercially available cough-cold preparation was analyzed in this study.[1] The sample was prepared by pipeting 25.0 ml. of the "cough syrup" into a 250-ml. separator. Five milliliters of concentrated hydrochloric acid was added and the sample extracted with three 30-ml. portions of carbon tetrachloride. The carbon tetrachloride extracts were pooled and backwashed once with 30 ml. of deionized water. The aqueous extracts were pooled in a 250-ml. separator and the carbon tetrachloride phase discarded. Twenty-five milliliters of 50% sodium hydroxide was then added cautiously to the aqueous phase and cooled periodically under cold tap water. The sample was then extracted with seven 30-ml. portions of chloroform, adding 5 g. of sodium chloride to the combined phases during each of the first four extracts. The chloroform extracts were filtered through cotton and pooled in a 250-ml. wide-mouth beaker. The extracts were evaporated continuously (*but never to dryness*) on a steam bath with a stream of forced air. Evaporation was continued until approximately 25 ml. of the combined chloroform extracts remained. This volume was transferred quantitatively to a 50-ml. volumetric flask to which 5.0 ml. of the pramoxine internal standard reagent had been added. The total volume was adjusted to 50 ml. with chloroform.

Standard Preparation—A standard was prepared by weighing to the nearest tenth of a milligram approximately 55 mg. of phenylpropanolamine hydrochloride, 75 mg. of glyceryl guaiacolate, 5 mg. of chlorpheniramine maleate (prepared by weighing and dissolving 100 mg. of chlorpheniramine maleate into 100.0 ml. of deionized water and pipeting 5 ml. of this stock solution), and 37.5 mg. of dextro-

[1] Coldene Cough and Cold, trade name of Pharmacraft Division, Pennwalt Corp., Rochester, NY 14623

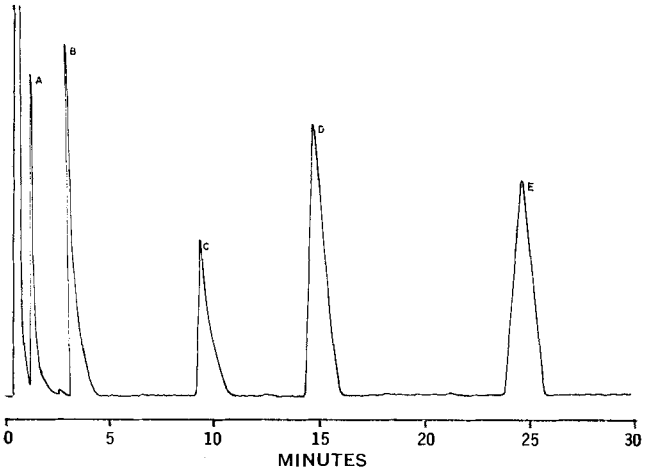


Figure 1—*Typical chromatogram. Key: A, phenylpropanolamine; B, glyceryl guaiacolate; C, chlorpheniramine; D, dextromethorphan; and E, pramoxine.*

methorphan hydrobromide into a 250-ml. separator containing approximately 25 ml. of deionized water. After solution was effected, this standard solution was treated exactly as described for the sample solution, including the addition of 5.0 ml. of pramoxine.

Procedure and Analysis—A 10-μl. Hamilton syringe equipped with a Chaney adapter was used to inject 2.0 μl. of the sample and standard solutions alternately. Values were calculated by peak height measurement. Since a completely synthetic standard was prepared in a manner identical to the procedure used to obtain the sample, and the detector response was linear within concentrations of ±25% of theory (see *Results and Discussion*), the ratio of the peak heights in the internal standard to each unknown could be stated in constant proportion to the ratio of their amounts, that is

$$\left[\frac{H_x}{H_s}\right]_\alpha = \left[\frac{C_x}{C_s}\right]_\alpha \qquad \text{(Eq. 1)}$$

and

$$\left[\frac{H_x}{H_s}\right]_\beta = \left[\frac{C_x}{C_s}\right]_\beta \qquad \text{(Eq. 2)}$$

where H_x is the peak height of the individual component to be analyzed, H_s is the peak height of the pramoxine internal standard, C_x is the concentration of the component in mg./ml., C_s is the concentration of the pramoxine internal standard in mg./ml., α is the ratio within the standard solution, and β is the ratio within the sample solution. Combining Eqs. 1 and 2,

$$\left[\frac{H_x}{H_s}\right]_\alpha \left[\frac{C_x}{C_s}\right]_\beta = \left[\frac{H_x}{H_s}\right]_\beta \left[\frac{C_x}{C_s}\right]_\alpha \qquad \text{(Eq. 3)}$$

Rearranging Eq. 3,

$$\left[\frac{C_x}{C_s}\right]_\beta = \frac{\left[\frac{H_x}{H_s}\right]_\beta \left[\frac{C_x}{C_s}\right]_\alpha}{\left[\frac{H_x}{H_s}\right]_\alpha} \qquad \text{(Eq. 4)}$$

But the concentration of the internal standard is held constant in both the sample (β) and the standard (α) solutions. Therefore, Eq. 4 simplifies to

$$[C_x]_\beta = \frac{\left[\frac{H_x}{H_s}\right]_\beta [C_x]_\alpha}{\left[\frac{H_x}{H_s}\right]_\alpha} \qquad \text{(Eq. 5)}$$

Since the term $[C_x]_\alpha$ is known, and $[H_x/H_s]_\alpha$ and $[H_x/H_s]_\beta$ are measured experimentally, Eq. 5 can be solved for $[C_x]_\beta$ (the mg./ml. of unknown in sample). The values obtained from Eq. 5 were compared to the theoretical amounts of each component and the percent recovered calculated.

Table I—Evaluation of the Linearity of Detector Response in Samples Containing 75 and 125% of Theoretical Drug Quantities

Ingredient	Theoretical, mg./5 ml.	Found, mg./5 ml.	% Theory Recovered
Phenylpropanolamine	8.25	8.44	102.30
	13.75	13.94	101.38
Glyceryl guaiacolate	11.25	11.06	98.31
	18.75	18.93	100.96
Chlorpheniramine	0.75	0.74	98.66
	1.25	1.25	100.00
Dextromethorphan	5.63	5.72	101.60
	9.38	9.33	99.47

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1. All the peaks are symmetrical and well resolved. Table I contains data of the detector response in a concentration range of ±25% of the theoretical drug quantities contained in the sample preparation. Table II contains the recovery data for the four active ingredients in duplicate samples of 10 different lots of the cough preparation calculated from peak height values. Precision and accuracy data are also presented and indicate excellent reproducibility.

The initial preextraction of the cough preparation with carbon tetrachloride was necessary to remove the organic soluble flavor and coloring additives which interfere with the chromatogram.

A simplified single-injection, gas chromatographic method for determining phenylpropanolamine, glyceryl guaiacolate, chlorpheniramine, and dextromethorphan in a commercially available cough preparation has been presented. The use of an isothermal method results in excellent resolution of all four components not withstanding their widely divergent polarities and boiling points. The modification of the chromatograph to an all-glass, on-column injection system yields quantitative, reproducible results. The pro-

Table II—Experimental *versus* Theoretical Quantities of Drug Actives Calculated from Peak Height Data

	mg./5 ml.			
	Phenyl-propanol-amine	Glyceryl Guaiaco-late	Chlor-phen-iramine	Dextro-methor-phan
Theoretical quantity	11.0	15.0	1.00	7.5
Lot A (1)	11.0	14.6	0.98	7.4
(2)	11.2	15.0	1.02	7.6
Lot B (1)	11.1	15.2	1.00	8.0
(2)	10.6	14.6	1.01	7.8
Lot C (1)	11.0	15.6	1.04	7.2
(2)	11.0	15.0	1.01	7.4
Lot D (1)	10.7	15.9	1.02	7.8
(2)	10.7	15.5	1.04	7.6
Lot E (1)	11.1	15.3	1.05	7.4
(2)	11.0	15.5	1.03	7.2
Lot F (1)	11.2	15.6	1.01	7.5
(2)	10.5	14.6	0.96	7.3
Lot G (1)	11.0	14.7	0.97	7.2
(2)	11.0	15.3	0.97	7.4
Lot H (1)	10.8	15.4	1.02	7.4
(2)	10.8	15.2	0.99	7.6
Lot I (1)	11.1	15.7	1.04	7.4
(2)	11.0	15.6	1.03	7.7
Lot J (1)	10.0	15.0	0.99	7.4
(2)	10.6	14.9	0.98	7.4
Average	10.9	15.2	1.01	7.5
Relative *SD* of preparation, %	1.99	2.55	2.51	2.74
Relative *SD* of preparation from label claim, %	2.28	2.98	2.79	2.74

cedure described in this paper is being applied to a series of complex pharmaceutical mixtures which will be the subjects of future articles.

REFERENCES

(1) L. D. Quinn, *J. Org. Chem.*, **24,** 911(1959).

(2) H. M. Fales and J. J. Pisano, *Anal. Biochem.*, **3,** 337(1962).

(3) E. Brochmann-Hanssen and A. B. Svendsen, *J. Pharm. Sci.*, **51,** 318(1962).

(4) *Ibid.*, **51,** 1095(1962).

(5) K. D. Parker, C. R. Fontan, and P. L. Kirk, *Anal. Chem.*, **34,** 1345(1962).

(6) C. R. Fontan, W. C. Smith, and P. L. Kirk, *ibid.*, **35,** 591 (1963).

(7) E. W. Cieplinski, *ibid.*, **35,** 256(1963).

(8) A. MacDonald and R. T. Pflaum, *J. Pharm. Sci.*, **53,** 887 (1964).

(9) G. B. Lawless, J. J. Sciarra, and A. Monte-Bovi, *ibid.*, **54,** 273(1965).

(10) M. Elefant, L. Chafetz, and J. M. Talmage, *ibid.*, **56,** 1181 (1967).

(11) E. Brochmann-Hanssen and C. R. Fontan, *J. Chromatogr.*, **19,** 296(1965).

(12) C. Hishta and R. G. Laubach, *J. Pharm. Sci.*, **58,** 745(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *Quality Control Department, Strasenburgh Laboratories, Rochester, NY 14623*

Accepted for publication October 13, 1969.

TECHNICAL ARTICLES

Utilization of the Solids Processor for Preparation of Tablet Granulations

FRANK W. GOODHART, J. RONALD DRAPER, and FRED C. NINGER

Abstract □ Various factors relating to the use of vacuum tumble dryers for the preparation of tablet granulations were studied. A dryer was employed (the 1-cu. ft. solids processor) which has a working capacity of 15–18 kg. for most pharmaceutical granulations. A typical process involves the wetting of the substrate with a drug solution and then drying at a predetermined temperature and vacuum. It was found that uniform drug distribution for low-dose tablet formulations could be obtained. Drying rates were determined for spray-dried lactose and dicalcium phosphate dihydrate at two temperatures and three tumbling speeds. Drying times varied from 35 to 60 min. A series of modified direct compression formulations was studied in which microcrystalline cellulose was used at two levels; two lubricants, calcium stearate and stearic acid, were employed; and lubricant addition was carried out by two methods, internal and external. The effects of these factors on tableting characteristics were monitored by an instrumented tablet machine (Stokes BB-2), and the final physical properties of the tablets were determined. A series of antacid granulations was prepared in which the influence of mixing time and the amount of granulating fluid was varied. The resulting granulations and tablets were characterized as described above.

Keyphrases □ Solids processor—preparation, tablet granulations □ Tablet granulations, preparation—solids processor □ Vacuum tumble dryers—data, mixing, drying, formulation, processing factors

New technology for the formulation of solid dosage forms has been developed over the past 5–10 years. The availability of new materials *per se*, new forms of old materials, and the invention and utilization of new machinery have allowed the formulation and manufacture of many products by simplified methods. Thus, the use of direct compression of medicinals, especially those in the low- and medium-dose range, has overtaken older traditional methods of wet granulation and slugging. Emphasis on faster dissolution rate and providing the drug in a readily available form are other reasons for updating tablet formulation and technology.

Some methods for simplified processing of tablet granulations have been described. A spray-drying process was reported by Raff *et al.* (1). A placebo granulation was prepared by spray drying and the drug, colorant, and tablet lubricant were added to the granulation and blended. A one-step spray-drying process could conceivably be feasible if the high inlet temperature would not physically or chemically affect the drug. Later, Kornblum described a spray-drying process for the preparation of a granulation for the formulation of sustained-action tablets (2).

The Littleford-Lodge mixer has been shown to be of value in mixing small quantities of active ingredients with inert diluents (3). In experiments using 250 mcg. of micronized salicylamide per 100 mg. of terra alba, coefficients of variation for drug content were from about 1 to 4% over a 0.5–10-min. interval of mixing. Mixing unmicronized salicylamide gave a significantly higher coefficient of variation.

An air-suspension technique for the preparation of tablet granulations was described by Wurster (4). These granulations were 16–20% active by weight, and deviation of content from theory was about −1 to +6%. The amount of solids lost was 1–8%.

Some of these methods might be satisfactory for active products in the low-dose range, arbitrarily in the

cedure described in this paper is being applied to a series of complex pharmaceutical mixtures which will be the subjects of future articles.

REFERENCES

(1) L. D. Quinn, *J. Org. Chem.*, **24,** 911(1959).

(2) H. M. Fales and J. J. Pisano, *Anal. Biochem.*, **3,** 337(1962).

(3) E. Brochmann-Hanssen and A. B. Svendsen, *J. Pharm. Sci.*, **51,** 318(1962).

(4) *Ibid.*, **51,** 1095(1962).

(5) K. D. Parker, C. R. Fontan, and P. L. Kirk, *Anal. Chem.*, **34,** 1345(1962).

(6) C. R. Fontan, W. C. Smith, and P. L. Kirk, *ibid.*, **35,** 591 (1963).

(7) E. W. Cieplinski, *ibid.*, **35,** 256(1963).

(8) A. MacDonald and R. T. Pflaum, *J. Pharm. Sci.*, **53,** 887 (1964).

(9) G. B. Lawless, J. J. Sciarra, and A. Monte-Bovi, *ibid.*, **54,** 273(1965).

(10) M. Elefant, L. Chafetz, and J. M. Talmage, *ibid.*, **56,** 1181 (1967).

(11) E. Brochmann-Hanssen and C. R. Fontan, *J. Chromatogr.*, **19,** 296(1965).

(12) C. Hishta and R. G. Laubach, *J. Pharm. Sci.*, **58,** 745(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *Quality Control Department, Strasenburgh Laboratories, Rochester, NY 14623*

Accepted for publication October 13, 1969.

TECHNICAL ARTICLES

Utilization of the Solids Processor for Preparation of Tablet Granulations

FRANK W. GOODHART, J. RONALD DRAPER, and FRED C. NINGER

Abstract □ Various factors relating to the use of vacuum tumble dryers for the preparation of tablet granulations were studied. A dryer was employed (the 1-cu. ft. solids processor) which has a working capacity of 15–18 kg. for most pharmaceutical granulations. A typical process involves the wetting of the substrate with a drug solution and then drying at a predetermined temperature and vacuum. It was found that uniform drug distribution for low-dose tablet formulations could be obtained. Drying rates were determined for spray-dried lactose and dicalcium phosphate dihydrate at two temperatures and three tumbling speeds. Drying times varied from 35 to 60 min. A series of modified direct compression formulations was studied in which microcrystalline cellulose was used at two levels; two lubricants, calcium stearate and stearic acid, were employed; and lubricant addition was carried out by two methods, internal and external. The effects of these factors on tableting characteristics were monitored by an instrumented tablet machine (Stokes BB-2), and the final physical properties of the tablets were determined. A series of antacid granulations was prepared in which the influence of mixing time and the amount of granulating fluid was varied. The resulting granulations and tablets were characterized as described above.

Keyphrases □ Solids processor—preparation, tablet granulations □ Tablet granulations, preparation—solids processor □ Vacuum tumble dryers—data, mixing, drying, formulation, processing factors

New technology for the formulation of solid dosage forms has been developed over the past 5–10 years. The availability of new materials *per se*, new forms of old materials, and the invention and utilization of new machinery have allowed the formulation and manufacture of many products by simplified methods. Thus, the use of direct compression of medicinals, especially those in the low- and medium-dose range, has overtaken older traditional methods of wet granulation and slugging. Emphasis on faster dissolution rate and providing the drug in a readily available form are other reasons for updating tablet formulation and technology.

Some methods for simplified processing of tablet granulations have been described. A spray-drying process was reported by Raff *et al.* (1). A placebo granulation was prepared by spray drying and the drug, colorant, and tablet lubricant were added to the granulation and blended. A one-step spray-drying process could conceivably be feasible if the high inlet temperature would not physically or chemically affect the drug. Later, Kornblum described a spray-drying process for the preparation of a granulation for the formulation of sustained-action tablets (2).

The Littleford-Lodge mixer has been shown to be of value in mixing small quantities of active ingredients with inert diluents (3). In experiments using 250 mcg. of micronized salicylamide per 100 mg. of terra alba, coefficients of variation for drug content were from about 1 to 4% over a 0.5–10-min. interval of mixing. Mixing unmicronized salicylamide gave a significantly higher coefficient of variation.

An air-suspension technique for the preparation of tablet granulations was described by Wurster (4). These granulations were 16–20% active by weight, and deviation of content from theory was about −1 to +6%. The amount of solids lost was 1–8%.

Some of these methods might be satisfactory for active products in the low-dose range, arbitrarily in the

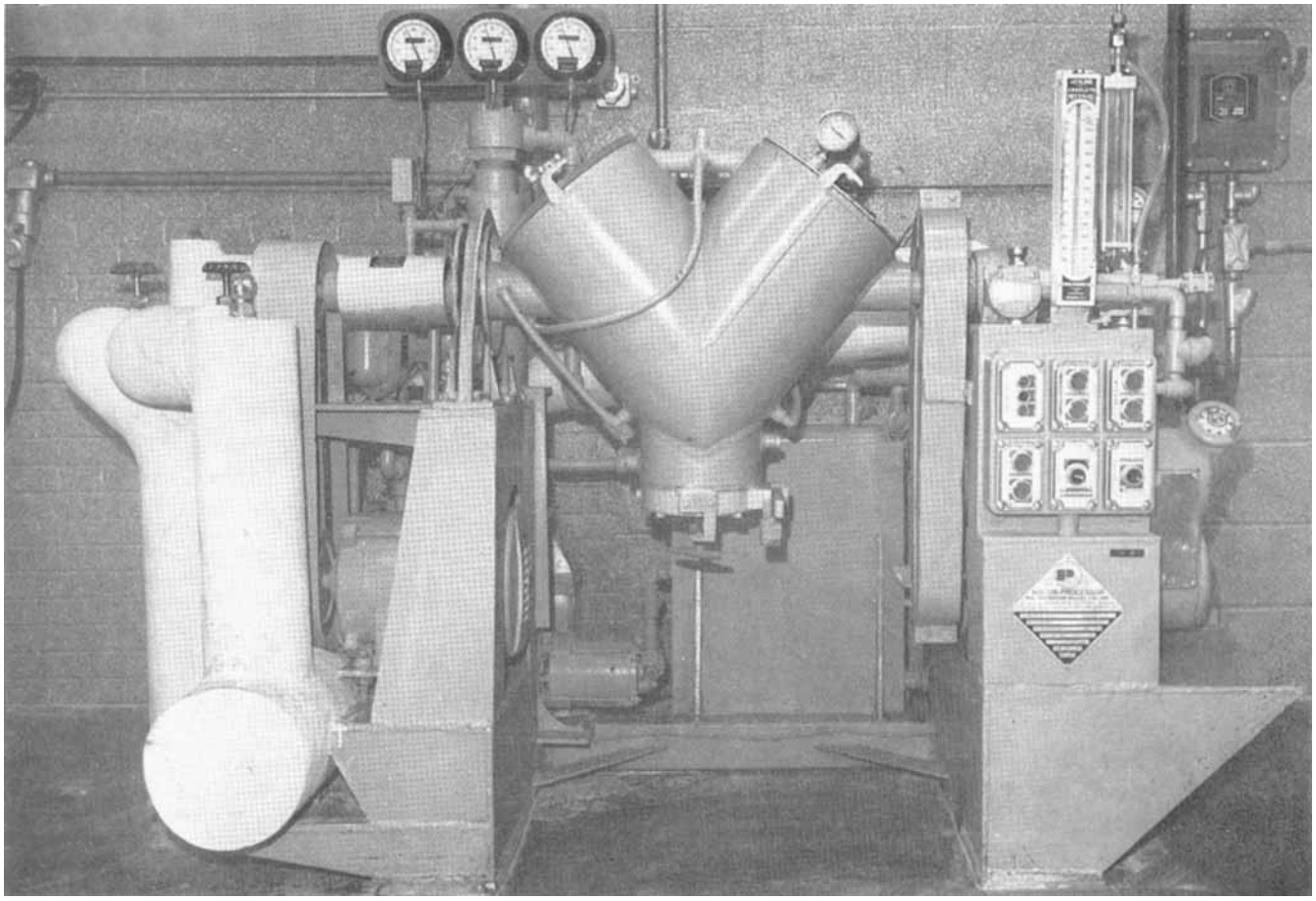


Figure 1—*Photograph of the 1-cu. ft. vacuum tumble dryer used throughout the study.*

area of 0.5–5 mg. per tablet. Ordinarily, tablet weights for these drug potencies would be 100–200 mg. When low microgram (10–100) quantities of drug are to be incorporated into a tablet, it is the generally accepted procedure to dissolve the drug in an appropriate solvent and apply the solution to the tablet substrate. The reasoning for this procedure is that drug particles often vary substantially in physical characteristics from the tablet substrate. Adequate dry blending of low-dose drugs would be dependent on a number of factors. Among these are the relative size distribution of the substrate compared to the drug, the ratio of drug to the substrate, the degree of affinity of the substrate for the drug, and the degree of static charge. The type of mixer used and mixing time are additional practical considerations. In one report, the mixing technology for 0.1-mg. reserpine tablets weighing 100 mg. (5) was studied. It was found that 100 mg. of the ultimate dry mix obtained had a coefficient of variation of 3.5%. However, on wetting this mix and granulating with water, the resulting granules had a coefficient of variation of 4.3%. The explanation for the slight segregation in the mix was the fact that the various components had different affinities for the water.

From these considerations, it was believed that vacuum tumble dryers (VTD) would provide advantages to the total processing of pharmaceutical granulations. Formerly, two reports on the use of twin-cone vacuum dryers for pharmaceutical material had appeared (6, 7), but no data on the total processing of tablet granulations were reported. VTD provides a method for addition of drug from solution, thus eliminating the various problems encountered in dry mixing procedures; among these are colorant blending and static charge. Since the material is agitated during drying, the tendency for the drugs to migrate, a factor which might produce nonuniformity within the powder bed, is minimized. Other advantages which are readily apparent are: (*a*) drying in vacuum can be carried out at lower temperatures, thus heat-labile drugs may be processed more favorably; (*b*) installations are compact; (*c*) solvent can be recovered; (*d*) complete enclosure of the mixing chamber virtually eliminates the possibility of contaminating adjacent areas with potent drug; (*e*) tumble dryers are easy to clean; (*f*) discharging of material from the VTD is easy and this results in high yields of finished product. One-step processing of some formulations might be feasible and this alone greatly simplifies manufacturing procedures. It was also believed that overall processing time might be shorter than could be achieved with other equipment. In opposition to these advantages are the relatively high cost of vacuum dryers and the fact that this equipment is not available in many pharmaceutical processing plants.

A general study of the technology for using VTD was undertaken in order to better understand its overall applicability to the processing of pharmaceutical

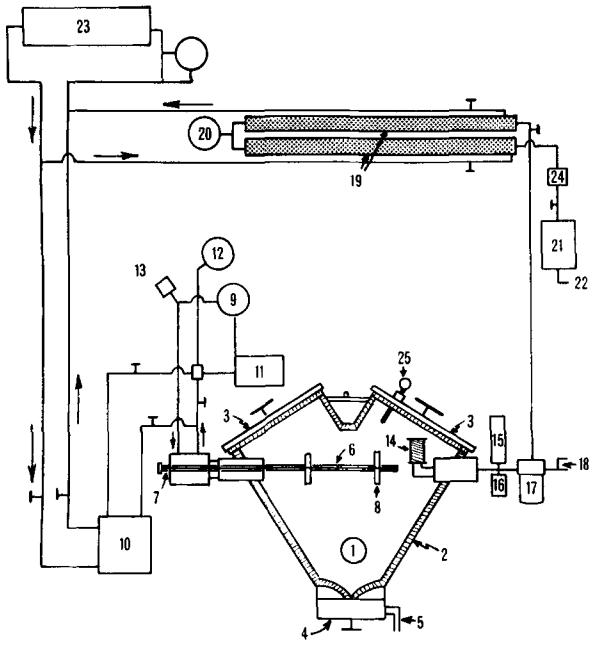


Figure 2—*Diagrammatic view of the 1-cu. ft. vacuum tumble dryer. Key: 1, shell; 2, jacket; 3, access covers; 4, discharge cover; 5, butterfly valve; 6, intensifier bar; 7, liquid feed tube; 8, dispersing disks; 9, chromalox immersion heater; 10, heat exchanger; 11, pump; 12, expansion tank; 13, thermometer; 14, felt ring filter; 15, mercury manometer; 16, vapor temperature thermometer; 17, solids separator; 18, ball valve; 19, condensers; 20, receiver; 21, vacuum pump; 22, vent; 23, chilling unit; 24, expansion tank; 25, solenoid valve; and 26, vessel thermometer.*

granulations. Experiments on mixing, drying, formulation factors, and processing factors were carried out and are reported here.

EXPERIMENTAL

General Description of Equipment—The equipment employed was a 1-cu. ft. vacuum tumble dryer[1] which is shown in Fig. 1. A schematic representation is illustrated in Fig. 2. The shell, 1, is fabricated of 304 stainless steel, and all pipes and fittings are 304 stainless steel. It has a working capacity of about 15–18 kg. for most pharmaceutical materials and is driven by a $^3/_4$-hp. Reeves Vari-Speed Motodrive which can turn the shell at speeds to 47 r.p.m. The shell is jacketed, thus providing a method of heating or cooling the material being processed. Access covers are provided on both sides and a butterfly valve and additional cover are used for discharging the material. The intensifier bar is operated by a separate 3-hp. motor. The bar functions as a disperser for added liquid material. A liquid feed tube is used in conjunction with the intensifier bar for the addition of liquids. Liquids are dispersed between two sets of disks on the intensifier bar. Spacings between the disks may be varied from 0.013 to 0.051 cm. (0.005 to 0.020 in.). The prongs on the dispersing disks are used for more vigorous mixing or for the reduction of agglomerates.

The jacket is heated by means of chromalox immersion heater and a liquid circulating pump. Heated liquid may be chilled by shunting the circulation through a heat exchanger which is connected to the same chiller that is used for the condensing system.

The vacuum and condensing system consists of a felt ring-type filter in the vessel, a vapor temperature thermometer, an absolute mercury manometer, a solids separator, two slanted condensers leading to a receiver, a Kinney KC-8 vacuum pump, and a Carrier model 30EA005 liquid chilling package. An ethylene glycol–water mixture was used in the chilling unit and the temperature of the circulating liquid was generally −2 to −5°. A solenoid valve between the vacuum pump and the condensers closed when the vacuum pump was turned off, thus preventing the condensers from being fouled with oil. The vacuum pump was vented to the atmosphere for removal of vapors which collect in the oil.

[1] Solids Processor, Patterson-Kelley Co., Inc., East Stroudsburg, Pa.

Table I—Composition of Tablet Granulation for the Determination of Uniformity of Mixing

	One Tablet	110,000 Tablets
Methylparaben USP	0.20 mg.	0.0220 kg.
Isopropyl alcohol NF		1.500 l.
Microcrystalline cellulose NF	52.50 mg.	5.775 kg.
Starch USP	15.00 mg.	1.650 kg.
Spray-dried lactose USP	80.80 mg.	8.888 kg.
Calcium stearate NF	1.50 mg.	0.165 kg.
	150.00 mg.	16.500 kg.

General Operation—Material is placed in the shell and the access covers are fitted and sealed against the recessed O-rings. Care must be taken to obtain a good seal because small amounts of material between the lid and O-ring may cause a leak which interferes with the operation of the processor. The material may be mixed by tumbling, and if lumps are present the intensifier bar may be turned on. Dispersion blades on the intensifier bar effectively break up agglomerates and give additional agitation for better mixing. Liquids are then added *via* the liquid feed tube. For the preparation of low-dose tablets, typically 1000–1500 ml. of drug solution is added to 15–18 kg. of substrate. Usually the time period of addition is 5 min., but shorter intervals are also satisfactory. Part of the solvent containing no drug is retained for flushing the liquid feed tube and dispersing disks. Heat and vacuum are then applied to the shell. The degree of vacuum that may be used is determined by the inspection of the vapor pressure–temperature curve for the solvent being used and the temperature of the condensate. For isopropanol the condensed liquid had a temperature of about 23°; thus, a vacuum of 40 mm. of Hg could be applied without causing the isopropanol to boil. Upon reaching a vacuum of 40 mm. of Hg, the vacuum pump is shut off. Evaporation and condensation of the solvent continue at about this level of vacuum until most of the solvent is collected. The receiver is emptied at this point and full vacuum is applied to the system to remove the last traces of solvent. Generally, this process using isopropanol takes about 30–50 min. and solvent recoveries are 90–95%. Ordinary materials used for producing low-dose tablet formulations are easily emptied from the shell, giving very high yields of finished product. No problems with the sticking of materials to the shell were encountered in this work.

Materials—Spray-dried lactose USP XVII, dibasic calcium phosphate dihydrate USP XVII, isopropyl alcohol NF XII, stearic acid USP XVII, calcium stearate NF XII, starch USP XVII, microcrystalline cellulose NF XII (Avicel PH 101), methyl *p*-hydroxybenzoate, an estrogenic steroid, and an antacid powder containing magnesium trisilicate, aluminum hydroxide, and magnesium hydroxide.

Equipment—Stokes BB-2 27 station tablet machine instrumented to detect and measure compression force (CF), ejection force (EF), and lower punch pulldown force (LPPF); an air-actuated pressure-regulated hardness tester; a friabilator (8); and a USP XVII tablet disintegration tester.

Evaluation of VTD for the Distribution of Low-Dose Ingredients—Preliminary experimentation on the preparation of drug concentrates, which were to be diluted for the production of tablets by the direct compression method, indicated that homogeneous blends could be obtained. The drug concentrates varied in composition from about 0.20 to 0.75% w/w. An experiment was designed for checking typical drug distribution in a 150-mg. tablet containing 200 mcg. of active ingredient. A model drug, methylparaben, was chosen for this work in order to minimize any assay difficulties which might occur. Tablets were prepared according to the formula shown in Table I.

Microcrystalline cellulose, starch, and spray-dried lactose were placed in the VTD and mixed well by tumbling. Methylparaben was dissolved in 1 l. of isopropyl alcohol and added to the powder mix.

Table II—Percentage Composition of Granulations Used in Preparing a Low-Dose Tablet and Method of Lubricant Addition

	Experiment Number							
	1	2	3	4	5	6	7	8
Starch USP	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Microcrystalline cellulose NF	20.00	20.00	20.00	20.00	35.00	35.00	35.00	35.00
Spray-dried lactose USP	68.87	68.87	68.87	68.87	53.87	53.87	53.87	53.87
D & C red No. 30 lake	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Stearic acid USP	1.00	1.00	—	—	1.00	—	1.00	—
Calcium stearate NF	—	—	1.00	1.00	—	1.00	—	1.00
Isopropyl alcohol NF								
Lubricant addition[a]	I	E	I	E	I	I	E	E

[a] I—internal; E—external.

An additional 500 ml. of isopropyl alcohol was used for rinsing the liquid feed tube and intensifier bar. The general procedure previously given was used to dry the granulation. Jacket temperature was set at 66° and the entire process was completed in 40 min. At the end of the run, three 100-g. samples of granulation were withdrawn from the two sides and bottom of the processor. Samples of granulation weighing approximately 150 mg. each were assayed in duplicate in order to determine uniformity of mixing. A spectrophotometric procedure was utilized for assay of methylparaben. This involved treating the sample with 0.05 *N* sodium hydroxide, filtering, and reading at 295 mμ. A blank was similarly prepared from the active granulation, but the solution was neutralized with 0.05 *N* HCl to quench the absorbance of methylparaben at 295 mμ. The precision of the method was estimated by assaying six samples of the bulk 100-g. sample from the bottom position.

Factors Affecting the Drying Rate of Spray-Dried Lactose and Dicalcium Phosphate Dihydrate—Preliminary work on drying 16.5–18-kg. charges of these materials indicated that two machine factors might affect drying rate. Temperature, of course, was one factor and speed of tumbling the second factor. Two temperatures, 42 and 66°, and three tumbling speeds, 7, 17, and 31 r.p.m., were studied.

Isopropyl alcohol (1500 ml.) was added to 16.5 kg. of spray-dried lactose or 18.0 kg. of dicalcium phosphate dihydrate over a 5-min. period. The choice of the material to be run, the temperature, and tumbling speeds were randomly selected. After liquid addition *via* the liquid feed tube, a vacuum of 60 mm. Hg was applied and the jacket temperature was turned on. At the end of an additional 5 min., the vacuum was reduced to about 40 mm. Hg and the processing continued until 1400–1450 ml. of isopropanol had been collected. Measurements of the condensate collected were made every 5 min. by means of measuring the height of the liquid in the receiver which had been previously calibrated. The condensate was drained from the receiver and full vacuum was applied. Usually an additional 10–40 ml. of isopropanol was collected.

Preparation of Low-Dose Tablet Granulations—The problem of mixing very small quantities of drugs with diluent is usually overcome by adding a solution of the drug to the diluent. VTD seemed ideal for such granulations since the drug could be added through the liquid feed tube and the mixture could then be conveniently dried in a closed system. Furthermore, it also seemed reasonable to add colorants and tablet lubricant by means of solution or suspension, thus allowing a one-step processing method.

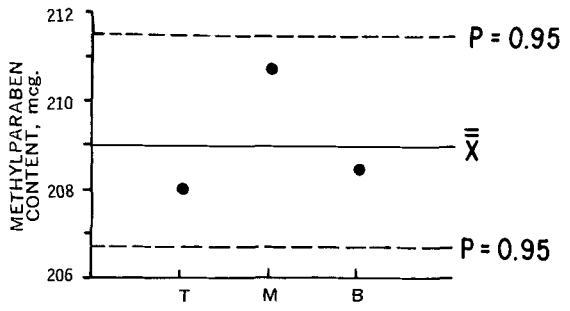


Figure 3—*Evaluation of mixing for methylparaben, 200 mcg./150 mg., prepared in the vacuum tumble dryer. Key: T, top; M, middle; and B, bottom.*

A factorial experiment was designed in order to test a number of formulation variables. These were the level of microcrystalline cellulose, 20 and 35%; kind of lubricant, stearic acid and calcium stearate; and method of lubricant addition, internal or external. Other excipients common to all formulas were spray-dried lactose and starch; the overall formulas are given in Table II with the method of lubricant addition.

A steroid drug was used at 10- and 25-mcg. levels during initial investigation. It was decided to carry out formulation screening without drug since excessive quantities were required and the formulations not containing drug had the same physical and compression characteristics as active granulations.

The general procedure for preparing these granulations was to blend the lactose, starch, and microcrystalline cellulose in the VTD. In the case of internally lubricated formulations, a suspension of the lubricant and the colorant was made in isopropanol and added to the powder blend by means of the liquid feed tube and rotating intensifier bar. In formulations where the lubricant was added externally, only the colorants were added from suspension in isopropanol. The same procedure was used for drying as described previously under drying rate experimentation, but a temperature of 66° and a speed of 7 r.p.m. were used for all runs. Lubricants, when added externally, were passed through a No. 60 screen onto a portion of the dried granulation, preblended, and then added to the bulk of the granulation and drum rolled for 20 min.

Finished granulations were characterized by determining flow rate through a standard Stokes BB-2 hopper, by compression on an instrumented Stokes BB-2 tablet machine, and by measuring finished tablet characteristics. Tablets were prepared using 0.71-cm. ($^{9}/_{32}$-in.) flat-faced beveled-edge punches at a weight of 150 mg. and a speed of 1400 tablets per minute.

Preparation of an Antacid Granulation by the Standard Wet Granulation Technique—The components of this granulation were

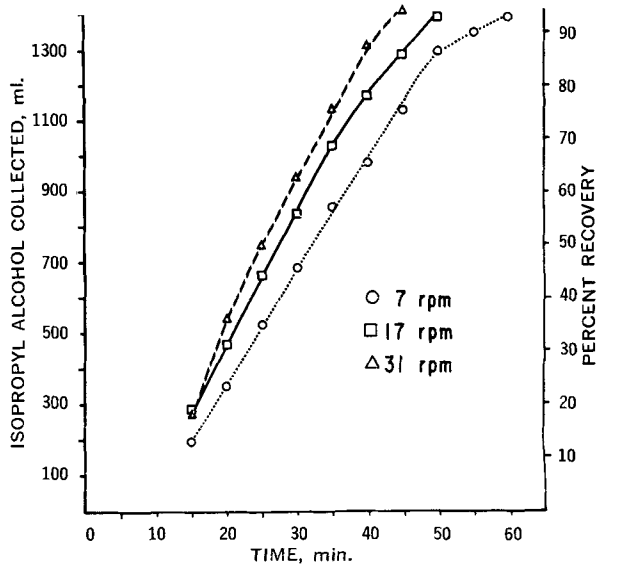


Figure 4—*Isopropyl alcohol recovery in the vacuum tumble dryer for dicalcium phosphate dihydrate at three speeds. Drying temperature, 42°.*

Table III—Typical Process Record for Vacuum Tumble Dryer[a]

Total Time, min.	Temperature: Jacket	Batch	Vapor	Vacuum, mm. Hg	Total Condensate, ml.	Remarks
0	—	—	—	—	—	Set jacket temperature to 66°
5	—	—	—	—	—	Set vacuum to 60 mm.
10	46	27	27	78	—	Set vacuum to 40 mm.
15	57	32	27	42	370	
20	66	37	28	44	860	
25	66	44	29	39	1310	
30	68	51	30	36	1430	
35	66	57	30	39	1470	Empty receiver, apply full vacuum
40	66	58	29	8	1470	

[a] Material: spray-dried lactose, 16.5 kg. Date: June 4, 1968. Liquid: isopropanol, 1500 ml. Tumbling speed: 31 r.p.m. Jacket temperature: 66°.

mixed in the VTD by means of the impeller bar while tumbling. Batch size was 15 kg. Varying amounts of water from 2100 to 2800 ml. were added, and agitation time with the intensifier bar was varied to determine whether these were important factors in the actual processing of the granulation. The water was added over a 5-min. period *via* the liquid feed tube and rotating intensifier bar. Drying temperature was at a jacket setting of 60° and tumbling speed was 25 r.p.m. Mesh sizes, bulk density, and flow rates were measured on all samples. Tablets were compressed on the Stokes BB-2 instrumented tablet machine using 1.58-cm. (0.63-in.) flat-faced beveled-edge punches at a theoretical weight of 1.33 g.

RESULTS AND DISCUSSION

Evaluation of Mixing—Replicate assays of a well-mixed granulation from the bottom position resulted in a mean content of 202.2 mcg. and a standard deviation of 1.40. Results from duplicate positional assays are plotted in Fig. 3 along with 95% confidence limits derived from the standard deviation obtained from the replicate measurements. There is a lack of any significant difference in the content of the three positional samples. In view of the turbulent condition of the powder bed during liquid addition, uniform mixing was expected. The VTD was fitted with lucite lids rather than the standard steel access covers. This allowed a viewing of the condition of the contents under the various processing conditions. Liquid addition at the rate of 200–300 ml. per minute was observed, and it was noted that the high degree of turbulence produced by the intensifier bar in conjunction with tumbling of the VTD effectively wetted only the powder. It seemed unlikely that localized wetted areas could be produced under these processing conditions. On drying, the mixture was free flowing, and localized sticking was not experienced.

Drying Rates—Little information has been published on drying pharmaceutical materials and particularly complete tablet granulations in VTD. Swartz and Suydam (7) described the drying of calcium sulfate dihydrate, lactose, and mannitol in the Rovac twin-shell processor after addition of 1000 ml. of water to 10 kg. of substrate. Under the conditions of heat and vacuum employed in the Rovac drying, a rising rate, constant rate, and falling rate drying cycle was observed. Heat transfer coefficients for the system were calculated and reported.

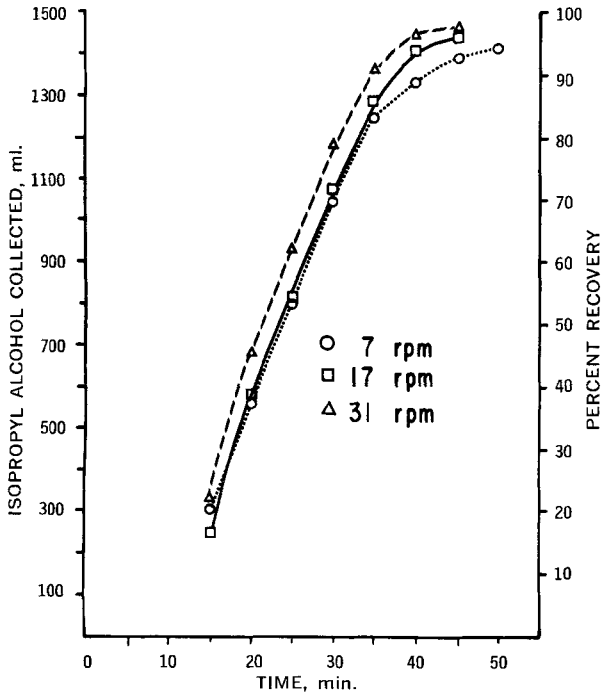


Figure 5—*Isopropyl alcohol recovery in the vacuum tumble dryer for spray-dried lactose at three speeds. Drying temperature, 66°.*

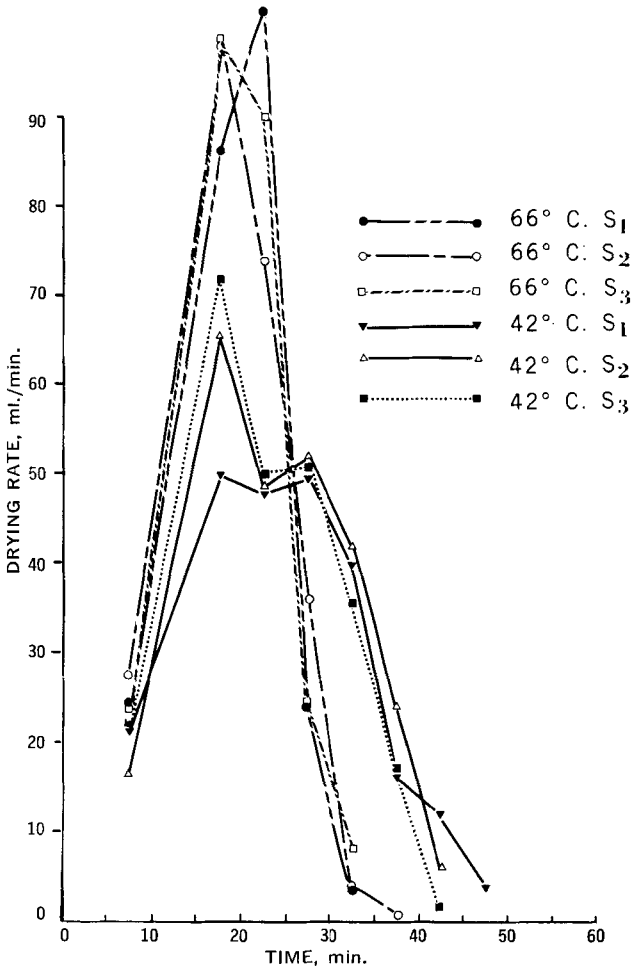


Figure 6—*Drying rates for spray-dried lactose using 1500 ml. of isopropyl alcohol and a 16.5-kg. load. Key: S_1, 7 r.p.m.; S_2, 17 r.p.m.; and S_3, 31 r.p.m.*

Table IV—Drying Time Factors for Vacuum Tumble Dryers

Size, cu. ft.	Drying Time Factor
1	1
10	2.10
20	2.70
30	3.01
40	3.37
50	3.70

Isopropyl alcohol is often the solvent of choice for addition of drugs to a tablet substrate; therefore, this solvent was chosen for experimentation. A typical processing sheet for these experiments is shown in Table III. Liquid was added over the first 5-min. period and the jacket temperature was turned on. After about 15 min. the jacket circulating fluid reached the setting of 66°. Batch temperature was 46° after 10 min. of operation and eventually reached 66° by the end of the run. Condensate begins to flow well after 10 min. and is rapidly collected over the next 25 min. of processing. The increased vapor temperature through the run indicated that isopropyl alcohol vapors were being collected in a somewhat superheated condition. During some processes it is advisable to backflush the felt filter by means of quickly opening the ball valve (item 18 in Fig. 1). Inrushing air forces dry powdered material off the filter, thus allowing better vapor flow from the vessel.

Comparative condensate volumes collected as a function of time are shown in Fig. 4 for dicalcium phosphate dihydrate at 42°. These data clearly point out the advantage of drying at a high tumbling speed over slow tumbling. The process is completed in 45 min. at a tumbling speed of 31 r.p.m., but 60 min. is required for drying at 7 r.p.m. Similar data were obtained for drying spray-dried lactose, Fig. 5. When drying was carried out at 66° the effect of tumbling speed was much diminished and the drying time becomes almost entirely temperature dependent.

Drying rates were calculated and are shown in Figs. 6 and 7 for spray-dried lactose and dicalcium phosphate dihydrate. At 66° the drying rate reached a maximum of about 100 ml./min. for lactose and between 67 and 87 ml./min. for dicalcium phosphate. At this higher temperature setting, a sharply rising drying rate occurs followed by a very sharp drop in rate. Drying at 42° gives a somewhat lower drying rate and a subsequent increase in overall processing time of about 10–15 min. A nearly constant drying rate was noted for dicalcium phosphate at the two lower speeds and for lactose only at the lowest speed. The effect of tumbling speed on drying rates is probably due to the greater surface area exposed at the higher speeds. At these higher speeds the effect of centrifugal force on holding the material to the walls might be considered a factor which would retard drying rate. However, the materials employed in this study were in motion even at high speed, and this factor would then contribute to the somewhat increased drying rates.

Larger VTD requires longer drying periods, but drying time does not increase proportionately with size. The manufacturer was asked for information on drying time in larger processors, and the factors for various sizes are listed in Table IV (9).

Predicted drying time for a process in a 30-cu. ft. VTD would be three times the time in a 1-cu. ft. VTD. Thus, a process requiring 35 min. in the 1-cu. ft. VTD would require 105 min. in a 30-cu. ft. VTD or 135 min. for a process requiring 45 min. in the smaller unit. These drying times are quite reasonable in view of drying times and extra handling needed by alternate methods.

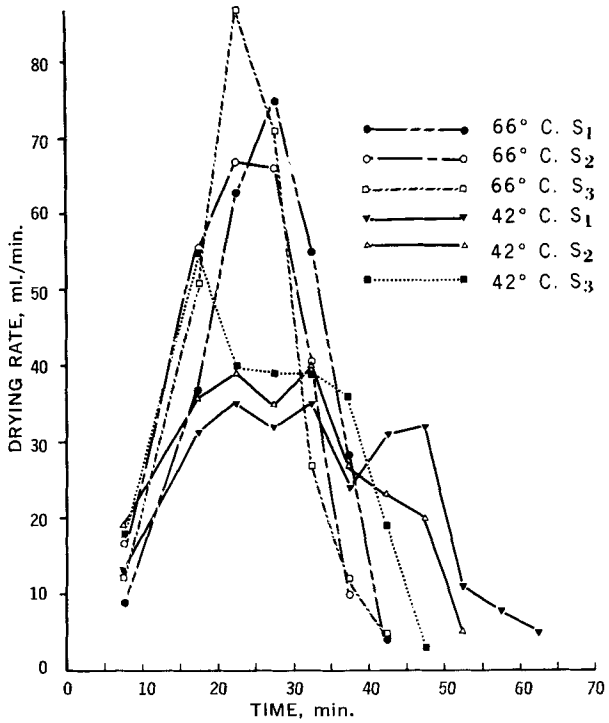


Figure 7—*Drying rates for dicalcium phosphate dihydrate using 1500 ml. of isopropyl alcohol and a 18.0-kg. load. Key: S_1, 7 r.p.m.; S_2, 17 r.p.m.; and S_3, 31 r.p.m.*

Low-Dose Tablet Granulations—Granulations were processed in a routine manner similar to those carried out for spray-dried lactose in the drying rate experiments. Overall processing time was 45 min. and about 93–97% of the added isopropanol was recovered. All materials were free flowing when dried and no sticking to the lids or vessel itself occurred. Yields were very high.

Flow rates were determined by loading 4.0 kg. of granulation into the standard hopper used for the BB-2 machine and noting the weight delivery onto a balance at 15- or 30-sec. intervals. Distance from the bottom of the hopper to the balance platform was 15.24 cm. (6 in.). This type of experiment gives an indication of the glidant activity of the granulation and also whether or not flow is sluggish. Sluggish or nonlinear flow rates have been shown to increase variability of tablet weights under some conditions of tableting (10). All eight of the granulations prepared exhibited linear flow rates as calculated by regression analysis. Flow rates for the various granulations are given in Table V in g./sec.

The highest flow rates are for granulations containing stearic acid added from alcoholic solution. Lubricant added internally generally gives higher flow rates except for calcium stearate at the high level of microcrystalline cellulose. These data might be indicative of a lubricant coating around the substrate particles.

An attempt was made to compress tablets at hardnesses of about 6 and to measure the resulting compression properties and physical

Table V—Compression Data and Physical Data for Granulations Prepared in the Vacuum Tumble Dryer

	Experiment Number							
	1	2	3	4	5	6	7	8
Flow rate of granulation, g./sec.	28	27	16	10	32	19	15	21
Compressive force, lb.	1732	1549	2488	1854	1128	1506	634	952
% Coefficient of variation, compressive force	3.44	7.23	5.54	5.34	5.51	3.28	4.60	5.11
Hardness, kg./sq. in.	6.1	6.8	4.6	3.2	6.4	6.8	5.9	5.6
Friability, %	0.11	0.07	10.98	9.82	0.00	0.00	0.00	0.00
Disintegration time, sec.	45–60	30–45	60–75	60–90	60–120	150–210	15–30	15–30
Average ejection force, μSt.	77	82	83	66	71	74	49	69
Coefficient of wt. variation	1.74	1.01	0.60	0.56	0.90	0.74	0.98	0.92

Table VI—Summary of Physical and Compression Data for Antacid Tablets

Formula	Water, ml.	Mixing Time, min.	Flow Rate, g./sec.	Bulk Density,[a] g./ml.	Average Compressive Force	Coefficient of Compressive Force Variation, %	Hardness, kg./sq. in.	Coefficient of Weight Variation, %
1	2100	5	37.4	0.813	3291	1.65	10.2	0.538
2	2100	7	32.8	0.793	3301	1.52	11.2	0.538
3	2300	5	21.6	0.819	3762	8.91	9.8	0.538
4	2300	7	35.7	0.813	3801	2.95	11.1	0.361
5	2500	5	40.6	0.806	3586	2.89	11.3	0.313
6	2500	7	42.9	0.813	3536	2.75	10.7	0.277
7	2700	5	40.7	0.819	3402	2.47	11.6	0.387
8	2700	7	68.8	0.819	3644	2.65	9.5	0.470

[a] Determined using the Numinco bulk density tester.

properties. These data are summarized in Table V. Tablets were compressed from all eight granulations, but two of these, 3 and 4, demonstrated very high friability of 10–11%. A considerable amount of capping occurred during the friability test. While a relatively high compressive force was used to tablet these formulations, average hardnesses of only 4.6 and 3.2 were attained. Each of these formulations contained calcium stearate and a lower level of microcrystalline cellulose.

Formulation factors were quite significant in the amount of compressive force needed to make a tablet and to a lesser extent on the magnitude of ejection force. In Fig. 8, average compressive force is shown as a function of the formulation factors. All three factors were very significant statistically. The most significant factor was the level of microcrystalline cellulose. Low level (20%) formulations required compression at an average of 1906 lb. but the higher level (35%) formulations were compressed at an average of 1055 lb. Formulations with the higher microcrystalline cellulose levels had hardnesses of 5.6–6.8 and were virtually nonfriable. Granulations containing internally added lubricant (I) required higher compressive force than externally added lubricant (E), Fig. 8. The higher force for the internally lubricated substrate is related to crystal fracture and realignment in order to obtain a particle bonding. The calcium stearate formulations were compressed on the average at higher compressive force levels than stearic acid formulations.

The effect of formulation factors on the resultant ejection force is shown in Fig. 9. The type of lubricant is seen to have no significant effect on ejection force, but both method of lubricant addition and microcrystalline cellulose level were highly significant. The degree of difference between externally and internally lubricated granulations is small from the practical viewpoint. It appears that internally added lubricant may be satisfactory over a long run and that the final mix step, so common for all tablet granulations, could be omitted. Since microcrystalline cellulose itself does not adhere to die walls when directly compressed, it seems reasonable that increasing its level in tablet formulations reduces ejection force. It is conceivable that savings in both tool and machine wear are possible by working at lower compressive forces. Whether or not this factor is worth considering over the use of more expensive raw materials has not been determined.

Disintegration times on all formulations were good. Formulas 5 and 6 indicate a tendency toward longer disintegration times on these internally lubricated granulations. Drug dissolution studies would be required to determine further the extent of drug availability as a function of lubricant addition method.

The coefficient of weight variation for all formulas seemed to be reasonable but Formulation 1 was somewhat higher than the others. During the flow rate studies, it was noted that this formula tended to plug the hopper on several occasions. On other runs, uniform flow was observed. The somewhat sluggish nature of this granulation probably accounts for the higher weight variability.

Preparation of Antacid Tablets—Granulations were produced using various levels of water and two mixing times as noted in Table VI. Increments of water were increased by 200 ml. in successive experiments. Preliminary experiments indicated that at least 2000 ml. of water was required to produce a granulation that could be tableted and that 2800 ml. produced an overwetted granulation which balled up in the processor. Tablets were compressed at hardnesses of about 10 kg./sq. in. and the resultant compressive force was measured.

Statistical comparison of flow rates of the granulations showed a significant difference in slopes between experiments. A trend towards higher flow was noted as the amount of water used to granulate increased. The mesh sizes of all granulations were about the same with the exception of Formula 8 which was coarser. In Formula 8 the highest level of water and the longest mixing time were used. This coarser mesh pattern could account for higher flow rate. Formula 3 exhibited the lowest flow rate and also the highest

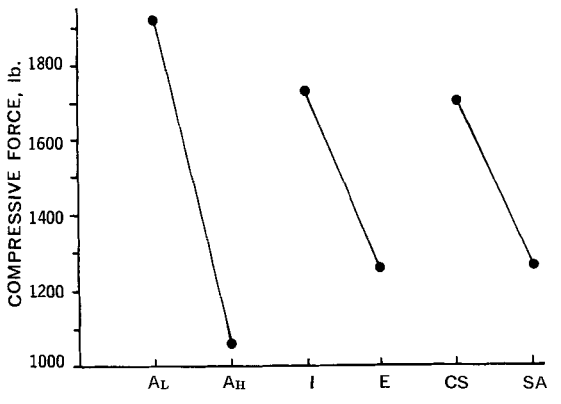


Figure 8—*Average compressive force as a function of formulation factors for direct compression granulations. Key: A_L, microcrystalline cellulose 20%; A_H, microcrystalline cellulose 35%; I, internal lubrication; E, external lubrication; CS, calcium stearate; and SA, stearic acid.*

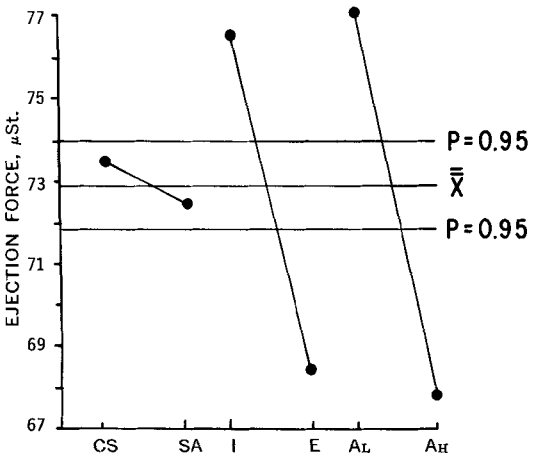


Figure 9—*Effect of formulation factors on ejection force. Key: CS, calcium stearate; SA, stearic acid; I, internal lubrication; E, external lubrication; A_L, microcrystalline cellulose 20%; and A_H, microcrystalline cellulose 35%.*

coefficient of variation of compressive force. However, weight variation of Formula 3 was comparable to other experiments.

Comparing experiments, hardness was not directly proportional to compressive force, indicating that these formulations require a somewhat different compression force for preparing tablets of an average hardness of 10. It is believed the variability in required compression force between different granulations of the same formula might be large enough to preclude using compressive force as a possible specification in this type of product. The reasons for shifts in required compression force have not been reported at this time nor their importance described.

All formulations were satisfactory with respect to chew and mouthfeel characteristics, and no substantial difference could be found between formulations when tasted by a small taste panel.

Moisture recovery on drying was 98–99%, and processing times varied from 105 min. for the lowest amount of added water to 120 min. for the highest amount of added water. Whether these relatively long processing times, compared with an isopropanol process, are acceptable depends to a large degree on the kind of product being made and the required rate of production.

SUMMARY AND CONCLUSIONS

Various data on the operation of a 1-cu. ft. VTD as related to general tableting technology have been reported. The results indicate that vacuum tumble drying is a satisfactory process for some particularly common types of tablet formulations. It was found that adequate mixing could be obtained and that the short processing time coupled with good yields were other advantages. Obviously the use of such equipment on a larger scale requires the usual considerations of loading, unloading, cleaning, *etc.* While the authors' experience in working with the VTD on a pilot scale was highly satisfactory, other alternate methods are available. However, the convenience factor as well as other advantages listed earlier seems to indicate the VTD may often be the process of choice.

REFERENCES

(1) A. M. Raff, M. J. Robinson, and E. V. Svedres, *J. Pharm. Sci.*, **50**, 76(1961).

(2) S. S. Kornblum, *ibid.*, **58**, 125(1969).

(3) A. M. Mattocks and K. A. Patel, "Evaluation of the Littleford-Lodge Mixer in Pharmaceuticals," 1962.

(4) D. E. Wurster, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 82(1960).

(5) R. Tawaski and P. Spieser, *Pharm. Acta Helv.*, **39**, 734 (1964).

(6) J. Cooper, C. J. Swartz, and W. L. Suydam, Jr., *J. Pharm. Sci.*, **50**, 67(1961).

(7) C. J. Swartz and W. L. Suydam, Jr., *ibid.*, **54**, 1050(1965).

(8) E. G. E. Shafer, E. G. Wollish, and C. E. Engel, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 114(1956).

(9) Personal communication, The Patterson-Kelley Co., East Stroudsburg, Pa.

(10) G. Gold, R. N. Duvall, B. T. Palermo, and J. G. Slater, *J. Pharm. Sci.*, **57**, 2153(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950*

Accepted for publication November 3, 1969.

Encapsulation of Clomacran Phosphate {2-Chloro-9-[3-(dimethylamino)propyl]acridan Phosphate} I: Effect of Flowability of Powder Blends, Lot-to-Lot Variability, and Concentration of Active Ingredient on Weight Variation of Capsules Filled on an Automatic Capsule-Filling Machine

GEORGE M. IRWIN*, GERALD J. DODSON, and LOUIS J. RAVIN

Abstract □ A correlation has been shown to exist between the flow properties of clomacran phosphate powder blends and capsule fill weight variation when an automatic capsule-filling machine is used. A correlation of 0.96 was obtained for the capsule mixes tested. Weight variation of the finished capsules is also affected by lot-to-lot variability and concentration of clomacran phosphate. Granulation of the active ingredient overcomes most of the problems normally encountered and provides finished capsules of uniform weight.

Keyphrases □ Clomacran PO_4—encapsulation □ Weight variation—clomacran PO_4 □ Powder blends flowability, lot variability effects—capsule weight variation □ Concentration effect, clomacran PO_4—capsule weight variation

Automatic capsule-filling machines are a recent addition to the equipment available to the industrial pharmacist for the encapsulation of powders. A detailed description of the operation of one of these machines (the Zanasi capsule-filling machine) was reported by Stoyle (1). He pointed out that, with a well-formulated product, capsules can be filled with a high degree of filling accuracy. Recently Reier *et al.* (2) evaluated the factors affecting the encapsulation of powders using a semiautomatic filling machine, but the literature has little information on the development of formulations to be used with an automatic capsule-filling machine. The purpose of this paper is to report data related to some of the problems encountered during the development of a capsule mix containing clomacran phosphate for use with the Zanasi capsule-filling machine.

In these studies, an attempt has been made to correlate the flowability of powder mixes, as measured with the flowometer described by Gold *et al.* (3), with the weight variation of capsules observed during encapsulation with the Zanasi. In addition, the way in which lot-to-lot variability, the concentration of clomacran

coefficient of variation of compressive force. However, weight variation of Formula 3 was comparable to other experiments.

Comparing experiments, hardness was not directly proportional to compressive force, indicating that these formulations require a somewhat different compression force for preparing tablets of an average hardness of 10. It is believed the variability in required compression force between different granulations of the same formula might be large enough to preclude using compressive force as a possible specification in this type of product. The reasons for shifts in required compression force have not been reported at this time nor their importance described.

All formulations were satisfactory with respect to chew and mouthfeel characteristics, and no substantial difference could be found between formulations when tasted by a small taste panel.

Moisture recovery on drying was 98–99%, and processing times varied from 105 min. for the lowest amount of added water to 120 min. for the highest amount of added water. Whether these relatively long processing times, compared with an isopropanol process, are acceptable depends to a large degree on the kind of product being made and the required rate of production.

SUMMARY AND CONCLUSIONS

Various data on the operation of a 1-cu. ft. VTD as related to general tableting technology have been reported. The results indicate that vacuum tumble drying is a satisfactory process for some particularly common types of tablet formulations. It was found that adequate mixing could be obtained and that the short processing time coupled with good yields were other advantages. Obviously the use of such equipment on a larger scale requires the usual considerations of loading, unloading, cleaning, *etc.* While the authors' experience in working with the VTD on a pilot scale was highly satisfactory, other alternate methods are available. However, the convenience factor as well as other advantages listed earlier seems to indicate the VTD may often be the process of choice.

REFERENCES

(1) A. M. Raff, M. J. Robinson, and E. V. Svedres, *J. Pharm. Sci.*, **50**, 76(1961).

(2) S. S. Kornblum, *ibid.*, **58**, 125(1969).

(3) A. M. Mattocks and K. A. Patel, "Evaluation of the Littleford-Lodge Mixer in Pharmaceuticals," 1962.

(4) D. E. Wurster, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 82(1960).

(5) R. Tawaski and P. Spieser, *Pharm. Acta Helv.*, **39**, 734 (1964).

(6) J. Cooper, C. J. Swartz, and W. L. Suydam, Jr., *J. Pharm. Sci.*, **50**, 67(1961).

(7) C. J. Swartz and W. L. Suydam, Jr., *ibid.*, **54**, 1050(1965).

(8) E. G. E. Shafer, E. G. Wollish, and C. E. Engel, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 114(1956).

(9) Personal communication, The Patterson-Kelley Co., East Stroudsburg, Pa.

(10) G. Gold, R. N. Duvall, B. T. Palermo, and J. G. Slater, *J. Pharm. Sci.*, **57**, 2153(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950*

Accepted for publication November 3, 1969.

Encapsulation of Clomacran Phosphate {2-Chloro-9-[3-(dimethylamino)propyl]acridan Phosphate} I: Effect of Flowability of Powder Blends, Lot-to-Lot Variability, and Concentration of Active Ingredient on Weight Variation of Capsules Filled on an Automatic Capsule-Filling Machine

GEORGE M. IRWIN*, GERALD J. DODSON, and LOUIS J. RAVIN

Abstract □ A correlation has been shown to exist between the flow properties of clomacran phosphate powder blends and capsule fill weight variation when an automatic capsule-filling machine is used. A correlation of 0.96 was obtained for the capsule mixes tested. Weight variation of the finished capsules is also affected by lot-to-lot variability and concentration of clomacran phosphate. Granulation of the active ingredient overcomes most of the problems normally encountered and provides finished capsules of uniform weight.

Keyphrases □ Clomacran PO_4—encapsulation □ Weight variation—clomacran PO_4 □ Powder blends flowability, lot variability effects—capsule weight variation □ Concentration effect, clomacran PO_4—capsule weight variation

Automatic capsule-filling machines are a recent addition to the equipment available to the industrial pharmacist for the encapsulation of powders. A detailed description of the operation of one of these machines (the Zanasi capsule-filling machine) was reported by Stoyle (1). He pointed out that, with a well-formulated product, capsules can be filled with a high degree of filling accuracy. Recently Reier *et al.* (2) evaluated the factors affecting the encapsulation of powders using a semiautomatic filling machine, but the literature has little information on the development of formulations to be used with an automatic capsule-filling machine. The purpose of this paper is to report data related to some of the problems encountered during the development of a capsule mix containing clomacran phosphate for use with the Zanasi capsule-filling machine.

In these studies, an attempt has been made to correlate the flowability of powder mixes, as measured with the flowometer described by Gold *et al.* (3), with the weight variation of capsules observed during encapsulation with the Zanasi. In addition, the way in which lot-to-lot variability, the concentration of clomacran

Table I—Percent Composition of Powder Blends Used in Flowability Studies

Ingredients	1	2	3	4	5	6	7
Clomacran phosphate, SK&F	—	22.0	22.0	22.0	22.0	22.0	22.0
Lactose USP	—	—	—	—	—	—	72.0
Dicalcium phosphate	—	—	72.0	—	—	—	—
Lactose, spray-dried	96.0	72.0	—	36.0	—	—	—
Free-flowing starch	—	—	—	36.0	73.0	72.0	—
Magnesium stearate USP	3.0	4.0	4.0	4.0	4.0	4.0	4.0
Fumed silica	—	1.0	1.0	1.0	—	1.0	1.0
Sodium lauryl sulfate USP	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Table II—Percent Composition of Powder Blends Used in Effect of Lot-to-Lot Variability Studies

Ingredients	8, 9, 10
Clomacran phosphate, SK&F[a]	42.0
Lactose, spray-dried	55.0
Magnesium stearate USP	3.0

[a] Chemical Lots A, B, and C were used in Formulas 8, 9, and 10, respectively.

Table III—Percent Composition of Powder Blends Used in Effect of Concentration Studies

Ingredients	11	12	13
Clomacran phosphate, SK&F	17.5	35.0	70.0
Lactose, spray-dried	79.5	62.0	27.0
Magnesium stearate USP	3.0	3.0	3.0

phosphate in the powder mixes, and different strength granulations affected the weight variation of the capsules were also evaluated.

EXPERIMENTAL

Materials—Clomacran phosphate, SK&F; lactose USP; magnesium stearate USP; sodium lauryl sulfate USP; free-flowing starch[1]; lactose, spray-dried[2]; terra alba, English[3]; calcium phosphate, dibasic[4]; polyvinylpyrrolidone[5]; fumed silica.[6]

Apparatus—Zanasi automatic capsule-filling machine was used.[7] The recording powder flowometer described by Gold *et al.* (3) was used throughout these studies for the flowability measurements. Since the apparatus was designed to measure flow rates of granulated materials, it was necessary to use a Vibrolator[8] to facilitate the flow of the powder blends from the hopper.

Preparation of Powder Blends—Six powder blends containing a constant concentration of drug, lubricant, and excipient were prepared; different excipients were used in each formula. A formulation without active ingredient was also prepared. The formulas for the various powder blends are listed in Table I.

Each powder blend was prepared in the same manner prior to flow-rate measurements. Several additional powder blends were prepared in order to determine the effect of lot-to-lot variability and concentration of the active ingredient on the weight variation of finished capsules. The formulas for these blends are listed in Tables II and III. Flow-rate measurements were not obtained for powder blends listed in Tables II and III.

[1] Dry-Flo Starch, National Starch and Chemical Corp., New York, N. Y.
[2] Foremost Foods Company, Industrial Div., San Francisco, Calif.
[3] Whittaker, Clark and Daniels, Inc., New York, N. Y.
[4] Kind & Knox Gelatin Co., Camden, N. J.
[5] Plasdone C, GAF Corp., Industrial Products Div., New York, N. Y.
[6] Cab-O-Sil, Cabot Corp., Boston, Mass.
[7] Model LZ 164, United Shoe Machinery Corp., Boston, Mass.
[8] Model UCV-6, Martin Engineering Co., Neponset, Ill.

Clomacran phosphate granulations were prepared with different lots of chemical at several concentrations of active ingredient to determine what effect granulating would have on the weight variation of finished capsules. The formulas for the granulations are listed in Table IV.

Flow-Rate Measurements—Flow rates expressed in g./sec. were determined with the flowometer for the seven powder blends. Each value represents an average of four determinations. Samples were run on 2 different days to minimize variations due to ambient conditions.

Preparation of Capsules—The powder blends evaluated for their flow properties were filled into No. 4 capsules. Any necessary adjustments were made for the first formulation and kept constant throughout the study.

Twenty groups of 10 capsules each were collected from each of the seven formulations encapsulated. These capsules were in turn divided into groups of five according to the dosator from which they had been obtained. All capsules were weighed on a torsion balance to establish the control weight during the filling operation, and one of the five capsules from each group was randomly selected and weighed on an analytical balance. Accurate weights were obtained for 20 capsules from each dosator for the seven powder blends.

Similar procedures were employed during the encapsulation of the formulations prepared to evaluate the effect of lot-to-lot variability and concentration of active ingredient. The granulation was treated in a similar manner. Nos. 1, 3, and 4 capsules were used to encapsulate the 100-, 50-, and 25-mg. strengths, respectively.

RESULTS AND DISCUSSION

Rate of Flow Measurements—Stoyle (1) has outlined the essential characteristics for powder blends which are to be encapsulated with the Zanasi capsule-filling machine. During the authors' preliminary investigation it was observed that whenever capsules prepared from a particular powder blend had a high coefficient of weight variation, a cavity was left in the powder bed in the hopper which was not completely refilled after the dosator removed the charge. This observation prompted these flow-rate measurement studies. The flow-rate measurements served as a screen to select a combination of

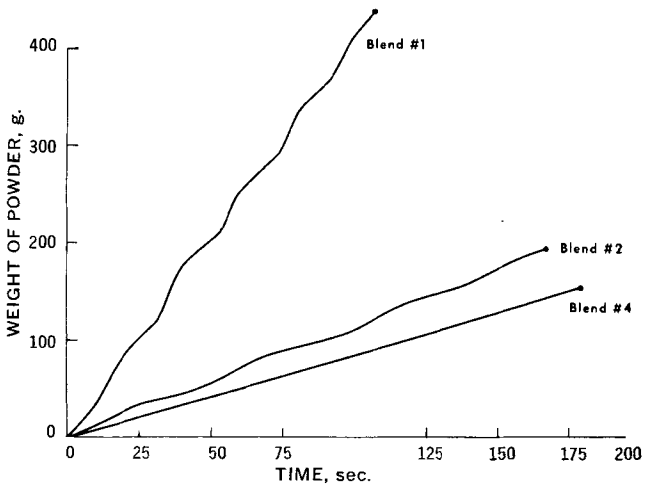


Figure 1—*Flowometer recording for several powder blends.*

Table IV—Percent Composition of Clomacran Phosphate Granulations

Ingredients	14	15	16	17	18	19
Clomacran phosphate, SK&F (Lot C)	22.0	37.0	36.0	—	—	—
Clomacran phosphate, SK&F (Lot D)	—	—	—	19.0	32.0	36.0
Terra alba, English	54.0	39.0	40.0	53.0	45.0	40.0
Sucrose USP	11.0	11.0	11.0	16.0	12.0	11.0
Starch USP	8.0	8.0	8.0	7.0	7.0	8.0
Polyvinylpyrrolidone	4.0	4.0	4.0	4.0	3.0	3.0
Magnesium stearate USP	1.0	1.0	1.0	1.0	1.0	2.0

Table V—Rate of Flow Measurements for Various Powder Blends

Blend	Rate of Flow, g./sec.
1	4.08
2	1.01
3	0.95
4	0.75
5	0.58
6	0.52
7	Did not flow

Table VI—Capsule Weight Variation Data for Seven Clomacran Phosphate Blends

Blends	$\bar{X}$, mg.	σ, mg.	CV
1	175	1.8	1.0
2	168	2.8	1.7
3	164	3.9	2.4
4	136	5.0	3.7
5	114	4.5	3.9
6	120	5.4	4.3
7	169	8.3	4.9

ingredients having the best flow properties. Flow-rate data obtained for seven capsule blends are shown in Table V.

It is evident from these data that the flow-rate varies with the different blends. Figure 1 illustrates the relative order of flow rate for three of the powder blends evaluated. The actual rates were determined by dividing the total weight flowing through the hopper by the total elapsed time.

Capsule Weight Variation—The weight variation for the seven capsule blends was calculated as a percent of capsule fill, since the density of the mixes differed, depending upon the excipients used in each case. For the lot of empty capsules used to encapsulate the powder blends, the mean and standard deviation were determined to be 37.1 and 1.3 mg., respectively. Utilizing these values and the standard deviation for the finished capsules, it was possible to calculate the standard deviation for the capsule fill alone. The values for the mean and standard deviation and the coefficient of variation for the seven powder blends are listed in Table VI.

Figure 2 illustrates a plot of rate of flow *versus* coefficient of variation for five blends.[9] A linear response was obtained and a correlation coefficient of 0.96 was calculated which indicates a good fit of the data. It is evident from this correlation that the flowability of the powder blends is related to the weight variation of the clomacran phosphate capsules filled on the Zanasi capsule-filling machine.

On the basis of flowability and weight variability data, powder blend No. 2 appeared to have the essential characteristics for encapsulation using the Zanasi. Subsequent powder blends containing varying concentrations of ingredients similar to those of powder blend No. 2 were evaluated on the basis of coefficient of weight variation of finished capsules. Since a correlation of 0.96 had been obtained between flowability and coefficient of variation for several capsule blends, it was felt that weight variation of finished capsules could be used to evaluate future powder blends. A coefficient of weight variation of 3% was arbitrarily set as a maximum. Any formulation that exceeded this limit was considered unsatisfactory.

Effect of Different Lots of Clomacran Phosphate Chemical—Table VII illustrates what effect various lots of clomacran phosphate chemical had on the capsule weight variation of finished capsules. A variation was noted when different lots of chemical were used, and further examination of the chemical indicated a difference in particle size. Figure 3 shows photomicrographs of several lots of clomacran phosphate chemical. The data suggest that a difference in particle size is responsible in part for the observed weight variation. The chemical of larger particle size shows less variation. Formula No. 10 prepared with chemical Lot C had a coefficient of weight variation outside the 3% limit.

Effect of Concentration of Clomacran Phosphate—The data in Table VIII indicate that the concentration of clomacran phosphate

[9] Data for powder blends Nos. 1 and 7 were not included in this figure. Blend No. 1 had no active ingredient and Blend No. 7 did not flow.

Table VII—Capsule Weight Variation for Powder Blends Containing Different Lots of Clomacran Phosphate Chemical

Formula	Lot of Chemical	$\bar{X}$, mg.	σ, mg.	CV
8	A	281	1.6	0.6
9	B	229	4.9	2.1
10	C	219	7.3	3.3

Table VIII—Capsule Weight Variation for Powder Blends Containing Different Concentrations of Clomacran Phosphate

Formula	Dosage Strength, mg.	$\bar{X}$, mg.	σ, mg.	CV
11	25	258	3.9	1.5
12	50	235	10.8	4.6
13	100	153	12.4	8.1

in the powder blend has an effect on the weight variation of finished capsules. The greater the concentration of active ingredient in the blend, the greater the weight variation. The differences in fill weight are due to the change in density of the capsule blends as the concentration of active ingredient is increased. The formulas for these blends are listed in Table III. A coefficient of variation greater than 3% was observed for Formulas No. 12 and No. 13; therefore they were considered unsatisfactory for encapsulation.

Effect of Granulating Clomacran Phosphate—When granulations of different strengths of clomacran phosphate were prepared using different lots of chemical as shown in Table IV and the resulting

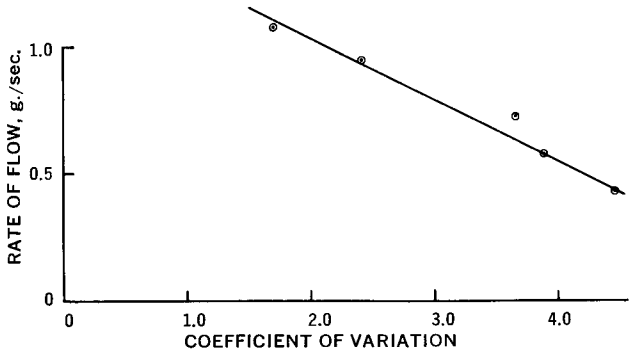


Figure 2—*A plot of rate of flow* versus *coefficient of variation for five powder blends.*

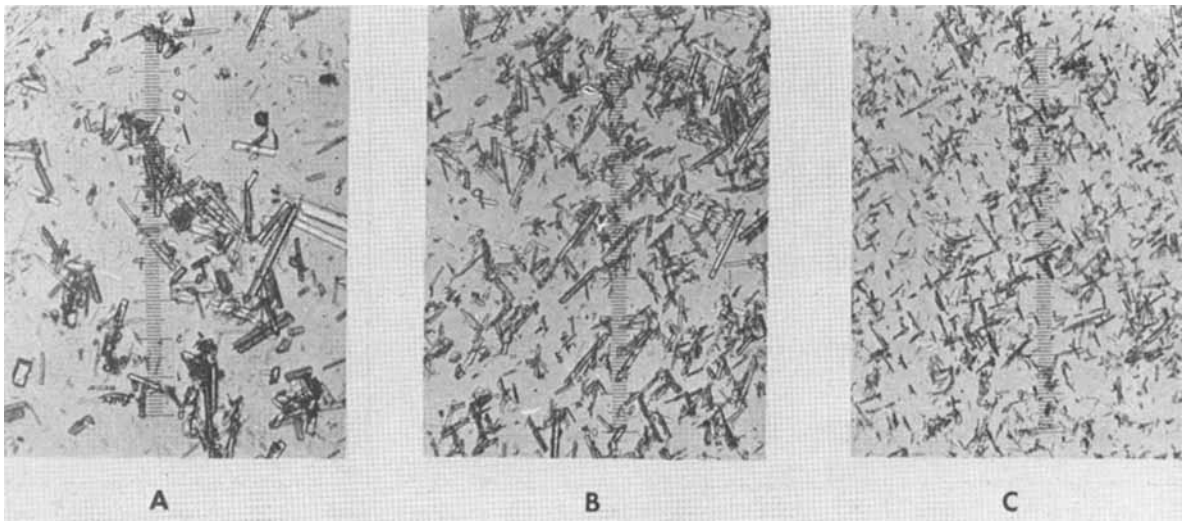


Figure 3—*Photomicrographs for several lots of clomacran phosphate chemical. Each scale division is equivalent to 6 μ.*

Table IX—Capsule Weight Variation for Clomacran Phosphate Granulations

Formula	Dosage Strength, mg.	$\bar{X}$, mg.	σ, mg.	CV
14	25	187	2.4	1.3
15	50	228	2.0	0.9
16	100	443	3.8	0.9
17	25	213	2.0	1.0
18	50	263	2.8	1.1
19	100	452	4.0	0.9

powder was filled into capsules, there was no appreciable difference in the weight variation of the finished capsules. These data are shown in Table IX.

The granulation process essentially eliminated the effect of lot-to-lot variability and concentration of the active ingredient. In every instance the granulated formulas showed a coefficient of variation of less than 1.5%, regardless of the lot of chemical or the concentration of active ingredient in the powder blend.

SUMMARY

1. The flowometer is a useful device for evaluating the flow properties of powder blends intended for encapsulation with an automatic capsule-filling machine.

2. A correlation of 0.96 was obtained between the rate of flow for five powder blends prepared for encapsulation and the coefficient of variation of the finished capsule fill weight.

3. Capsule weight variation was affected when different lots of clomacran phosphate were used. The particle size of the chemical contributed in part to the weight variation of the finished capsule.

4. The concentration of active ingredient in the capsule mix affected the weight variation. When formulating capsule blends of different strengths for encapsulation with an automatic capsule-filling machine, it may be necessary to reformulate to maintain satisfactory weight control.

5. The use of a granulated powder overcomes most of the problems normally encountered and provides finished capsules of uniform weight.

REFERENCES

(1) L. E. Stoyle, "Evaluation of the Zanasi Automatic Capsule Filler," Preprint, Industrial Pharmacy Section, APhA 113th Annual Meeting, Dallas, Tex., 1966.

(2) G. Reier, R. Cohn, S. Rock, and F. Wagenblast, *J. Pharm. Sci.*, **57**, 660(1968).

(3) G. Gold, R. N. Duvall, and B. T. Palermo, *ibid.*, **55**, 1133 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101*

Accepted for publication December 11, 1969.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors wish to acknowledge the assistance and cooperation provided by Dr. G. Gold, Dr. R. N. Duvall, and Mr. J. G. Slater of Miles Laboratories, Elkhart, Ind.

* Present address: Wyeth Laboratories, Radnor, Pa.

Some Coumarin Constituents of *Prunus mahaleb* L. Fruit Kernels V

M. EL-DAKHAKHNY

Abstract □ It was possible to isolate 2-glucosyloxy-4-methoxyethyl *trans*-cinnamate from the alcoholic extract of the defatted *Prunus mahaleb* L. fruit kernels. The structure of this compound was confirmed by IR, NMR, and mass spectra. Further evidence was sought through its de-ethylation into 2-glucosyloxy-4-methoxy *trans*-cinnamic acid which was identical in every respect with authentic material prepared synthetically. Through acetylation it afforded a tetraacetate, the structure of which was also confirmed by NMR and mass spectra. The identity of the sugar as glucose was ascertained by enzymatic and acid hydrolysis and subsequent paper chromatography in three different solvent systems.

Keyphrases □ Coumarins—*Prunus mahaleb* fruit kernels □ Paper chromatography—separation, identification □ Optical rotation—identity □ IR spectrophotometry—structure □ NMR spectroscopy—structure □ Mass spectroscopy—structure

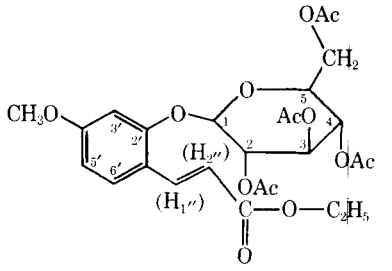
In a previous communication (1), it was reported that *Prunus mahaleb* L. kernels contain herniarin (7-methoxycoumarin) both in a free form and as a glucoside. The present author, during examination of the alkaloidal fraction of the kernels, was able to isolate a crystalline compound which appears not to have been reported before. Although this compound was isolated from the alkaloidal fraction, it was found to be free of nitrogen and gave negative color reactions for alkaloids. The aim of this work was to study the structure of this compound. Pharmacological studies on this compound have been carried out and will appear shortly.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. IR spectra were determined in KBr using a Leitz Unicam S.P. 200 G. NMR spectra were determined in DMSO using a 60-Mc. instrument. Mass spectra were determined with CH_4, instrument at 70°, using an electron energy of 70 eV. Optical rotations were determined with a Perkin-Elmer 141 polarimeter.

Isolation of 2-Glucosyloxy-4-methoxyethyl *trans*-Cinnamate (I)—Five hundred grams dried and defatted mahaleb kernels was extracted with 95% ethanol to exhaustion. After concentration of the alcoholic extract, ether was added to precipitate choline and other glycosides. After decantation of ether, the precipitate was dissolved once more in alcohol and ether was added once again. The combined ethereal extracts were concentrated and extracted with diluted HCl to remove the alkaloids. The aqueous acidic solution was then made alkaline with ammonia and extracted with chloroform. The chloroform was dried over anhydrous sodium sulfate and then concentrated. After evaporation of chloroform, a yellow semisolid residue with a strong characteristic odor was left. Repeated crystallization from chloroform–methanol gave colorless needles, m.p. 180–182°, $[\alpha]_D^{22} = -58.8°$ (in pyridine, yield 90 mg. about 0.2% of alcoholic extract, C = 56.22%; H = 6.30%; $C_{18}H_{24}O_9$ requires C = 56.24% and H = 6.29%). It gave a single spot on silica gel G developed with chloroform–methanol (85:15; R_f, 0.267) which could be seen as a dark blue fluorescent spot in UV light. It gave a positive reaction for Molisch test and negative reactions for alkaloids and flavonoids.

The UV spectrum in ethanol showed maxima at λ 239, λ 296, and λ 322 mμ ($\epsilon = 8.83 \times 10^3$, 1.23×10^4, and 1.55×10^4, respectively). The IR spectrum showed bands characteristic for hydroxy groups (3380 cm.$^{-1}$), carbonyl in conjugation with a double bond (1680 and 1615 cm.$^{-1}$), 1,2,4-trisubstituted aromatic structure (1570, 772, 766, and 760 cm.$^{-1}$) (2). This compound was identified as 2-glucosyloxy-4-methoxyethyl *trans*-cinnamate (Structure I).



2-glucosyloxy-4-methoxyethyl *trans*-cinnamate(acetate)

I

The mass spectrum of this compound (Fig. 1) showed a molecular ion peak at *m/e* 384 (46.7%) from which the expulsion of methanol gave a peak at *m/e* 352 (8.5%). The important fragmentation behavior of the molecule includes the following features (Scheme I); expulsion of 162 mass units by loss of $C_6H_{10}O_5$ for the glucose moiety leads to an ion at *m/e* 222 (22.3%) formulated as "A." Subsequent expulsion of ethanol by the possible mechanism indicated by the arrows leads to the highly conjugated ion "B" which appears as the strongest ion in the spectrum (base peak). The latter ion loses carbon monoxide presumably from the cyclohexadienone system with the formation of ion "C" which appears at *m/e* 148 (81.5%). Further degradation of the latter ion happens by expulsion of methyl radical giving the stabilized ozonium ion "D" at *m/e* 133 (58.7%). Subsequent expulsion of carbon monoxide gives a $C_7H_5O^+$ ion at *m/e* 105 (11.7%) which may be represented by the constituent "E."

Enzymatic Hydrolysis of the Glucoside—Twenty milligrams of Compound I was dissolved in about 20 ml. of water, about 20 mg. of B-glucosidase in 20 ml. water was added, one drop of toluene for preservation, and the mixture was kept for about 10 hr. at 37° with occasional shaking. The mixture was then extracted with ether. The ethereal layer was dried over anhydrous sodium sulfate and then examined chromatographically on paper (Whatman No. 1) using the upper phase of *n*-butanol–acetic acid–water (4:1:5) for developing. Spots were located under UV light. The liberated aglycone gave one spot (R_f, 0.863) and was different from the original compound (R_f, 0.79) and from herniarin (R_f, 0.905).

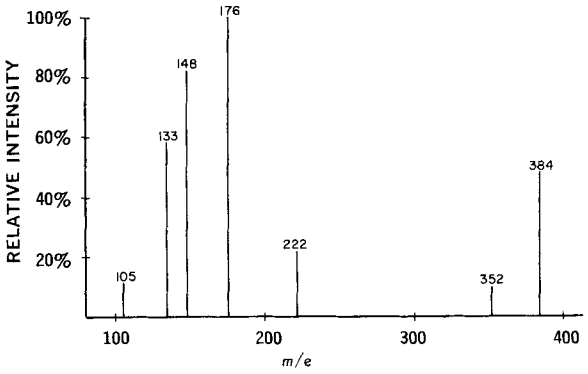
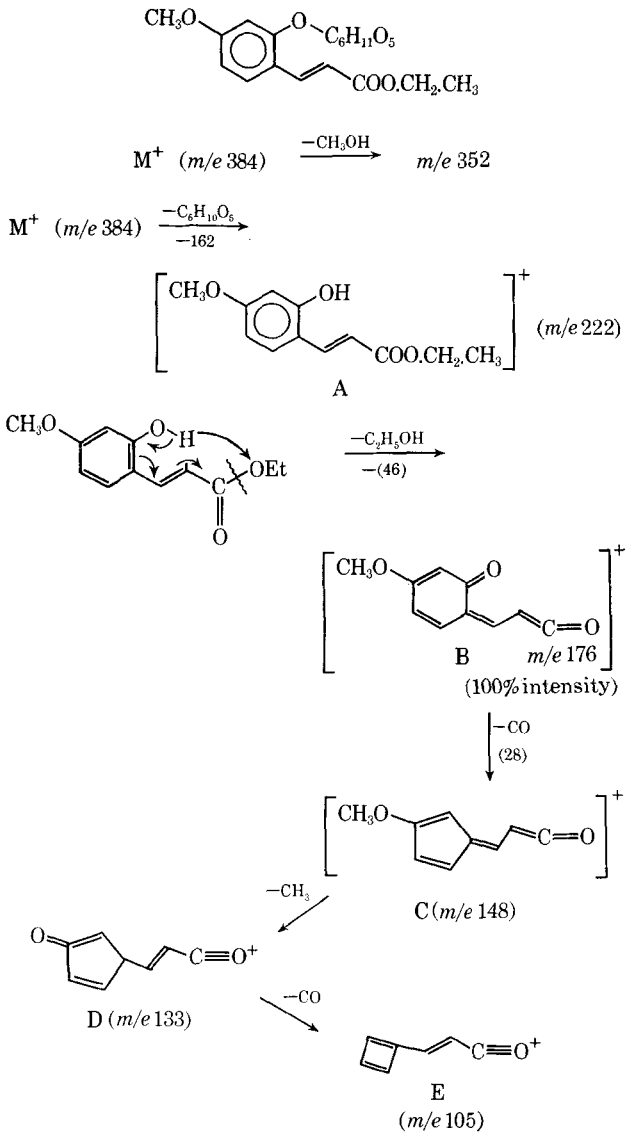


Figure 1—*Mass spectrum of glucoside.*



Scheme I—Fragmentation pattern of glucoside.

The aqueous layer after filtration and concentration under reduced pressure was tested chromatographically on paper (Whatman No. 1) against reference sugars using the following solvent systems: (*a*) *n*-butanol–acetic acid–water, 4:1:5 (3); (*b*) ethyl acetate–pyridine–water, 5:2:5 (4); and (*c*) collidine saturated with water (5).

The chromatograms were developed for about 24 hr. (descending). After drying at room temperature, they were sprayed with *p*-anisidine–phosphoric acid reagent (6). In the three solvent systems, the aqueous phase gave a brown spot which was identical to that of glucose run as a reference material.

Acid Hydrolysis of the Glucoside—To the alcoholic solution (20 ml.) of about 20 mg. of the glucoside, about 20 ml. 2 *N* sulfuric acid was added and the mixture was refluxed for about 1 hr. The reaction mixture was then diluted with water and extracted with ether. The ethereal layer was tested chromatographically on paper (Whatman No. 1, solvent: upper phase of *n*-butanol–acetic acid–water, 4:1:5) and on silica gel G (solvent: benzene–3% methanol). It gave one spot identical to herniarin.

The aqueous layer, after being neutralized with barium carbonate, was passed through a column of ion-exchange resin (Dowex 50WX, 50–100 mesh, hydrogen form) to remove any barium ions and then concentrated under reduced pressure and tested chromatographically on paper using the same solvents mentioned previously. The only brown spot was identical to reference glucose.

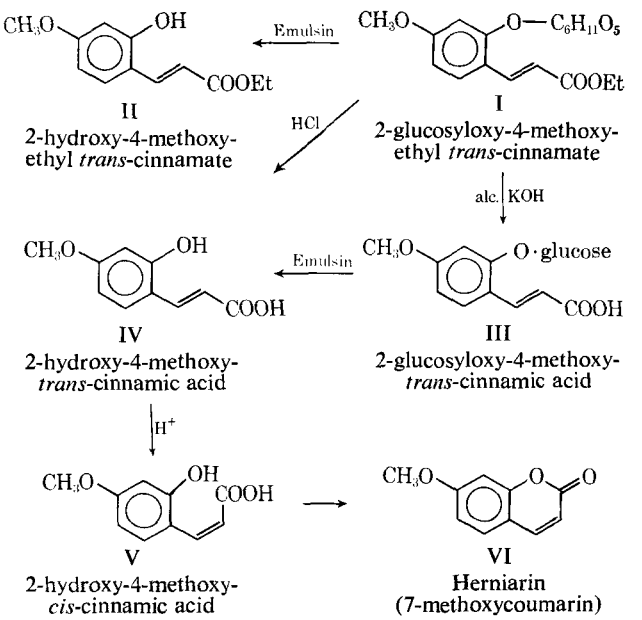
Acetylation of the Glucoside—One hundred milligrams of the crystalline compound was dissolved in 1 ml. of anhydrous pyridine, then 1 ml. of acetic anhydride was added and the mixture was heated at 100° for about 2 hr. The pyridine and acetic anhydride were evaporated under reduced pressure leaving a colorless crystalline mass. Recrystallization from aqueous methanol gave colorless needles, m.p. 160–162°, $[\alpha]_D^{22} = -56.5°$ (in chloroform). Tested chromatographically on silica gel G, it gave one fluorescent spot (solvent: chloroform–methanol, 85:15; R_f, 0.35). The IR spectrum in KBr showed the disappearance of the hydroxyl band. The NMR spectrum showed three acetyl groups at $\delta = 2.17$ and another one at $\delta = 2.14$ accounting for the acetyl group attached to C_6 of the sugar moiety (Structure I), one methoxy group at $\delta = 4$. There were bands indicating the presence of an ethyl group at $\delta = 1.42$ (triplet, $J = 7$ c.p.s.) and $\delta = 4.3$. It showed also three aromatic protons: H(3′) at $\delta = 6.98$ (singlet), H(5′) at $\delta = 6.9$ (doublet, $J = 9$ c.p.s.), and H(6′) at $\delta = 7.9$ (doublet, $J = 9$ c.p.s.). Two *trans*-ethylenic protons appeared: H(1″) at $\delta = 7.91$ (doublet, $J = 16$ c.p.s.) and H(2″) at $\delta = 6.55$ (doublet, $J = 16$ c.p.s.). The sugar moiety appeared as a complex multiplet ($\delta = 5$–6).

The mass spectrum showed the presence of a molecular ion at *m/e* 552. Further fragmentation showed the loss of four molecules of acetic acid successively and then behaved like the mother compound.

Deethylation of the Glucoside—Twenty-five milligrams of the crystalline glucoside was dissolved in about 25 ml. aqueous alcohol (20%), then 25 ml. of 1% KOH in aqueous alcohol was added and the mixture was left overnight at room temperature. The next day the solution was passed through a column of ion-exchange resin (Dowex 50WX, 50–100 mesh, hydrogen form) to neutralize the potassium hydroxide. The effluent neutral solution was evaporated under reduced pressure. The colorless mass left, when crystallized from water, gave plates m.p. 196–198°, $[\alpha]_D^{22} = -50.7°$ (in pyridine) (12 mg. yield); reported for 2-glucosyloxy-4-methoxy *trans*-cinnamic acid (III), 194–196° (7). When tested chromatographically on paper (Whatman No. 1, solvent: *n*-butanol–acetic acid–water, 4:1:5), it gave one spot (R_f, 0.635) which was identical with authentic 2-glucosyl-4-methoxy *trans*-cinnamic acid prepared synthetically (7). IR spectra of both products were also identical.

DISCUSSION

2-Glucosyl-4-methoxyethyl *trans*-cinnamate (I, Scheme II) was isolated from the alkaloidal fraction of the alcoholic extract of *Prunus mahaleb* L. kernels. NMR spectra of the compound and its tetraacetate showed signals for an ethyl group. These signals were found to be persistent even after thorough drying or using solvents other than ethanol or methanol for crystallization. The presence of the ethyl group as a part of the molecule and not as impurity was proved by mass spectra of both the compound and its acetate. That the compound occurs in the *trans*-form was shown by the IR spectrum [985 cm.$^{-1}$ (2)] and by the NMR spectra showing the presence of two *trans*-ethylenic protons, H(1″) at $\delta = 7.91$ (doublet, $J = 16$ c.p.s.) and H(2″) at $\delta = 6.55$ (doublet, $J = 16$ c.p.s.).



Scheme II—Reactions of the glucoside.

Nevertheless, on acid hydrolysis the compound gave herniarin. The formation of herniarin may be accounted for by the transformation of the liberated 2-hydroxy-4-methoxy-*trans*-cinnamic acid (IV, Scheme II) to the *cis*-form in presence of hydrogen ions. However, enzymatic hydrolysis did not give herniarin but gave 2-hydroxy-4-methoxyethyl *trans*-cinnamate (IV) which was found to be less mobile on paper than herniarin. The structure of the glucoside (I) was also ascertained through its de-ethylation to the free-acid glucoside (III) which was identical in every respect with authentic material prepared synthetically (7).

SUMMARY

2-Glucosyloxy-4-methoxyethyl *trans*-cinnamate was isolated in a crystalline form from the alcoholic extract of *Prunus mahaleb* L. fruit kernels. The structure of this compound was proved by IR, NMR, and mass spectra. It was further ascertained by de-ethylation of the compound to the free-acid glucoside which was identical with authentic material. On acetylation, it afforded a tetraacetate, the spectral studies of which (IR, NMR, and mass spectra) assured the suggested structure. On acid and enzymatic hydrolysis, it afforded glucose which was identified by chromatographic techniques.

REFERENCES

(1) M. El-Dakhakhny, *Planta Med.*, **12**, 181(1964).

(2) L. J. Bellamy, "The Infra-red Spectra of Complex Molecules," 2nd ed., Methuen & Co. Ltd., London, England, 1964, pp. 8, 34, 96, 132.

(3) S. M. Partridge, *Biochem. J.*, **24**, 238(1948).

(4) M. A. Jermyn and F. A. Isherwood, *ibid.*, **44**, 402(1949).

(5) H. F. Linskens, "Papierchromatographie in der Botanik," 2nd ed., Springer-Verlag, Berlin, West Germany, 1959.

(6) S. M. Mukherjee and H. C. Stivastana, *Nature*, **169**, 330 (1952).

(7) S. A. Brown, *Phytochemistry*, **2**, 137(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from *Trent University, Biochemistry Department, Peterborough, Ontario, Canada.*

Accepted for publication October 10, 1969.

The author thanks S. A. Brown, Professor of Chemistry, Trent University, for his interest and for permitting the completion of this work in his department. Thanks are extended to Prof. R. Hänsel and Dr. H. Rimpler of the Institute Pharmacognosy, Free University of Berlin, and Prof. M. B. E. Fayez of the National Research Centre, Cairo, UAR, for their interest; also to Dr. Schulz of Schering, Berlin, for help with NMR and mass spectra interpretations.

Present address: Department of Pharmacology, Medical Faculty, Alexandria University, Alexandria, UAR.

Tablet-to-Tablet Variation of Drug Content of Sugar-Coated Tablets Containing Drug in the Sugar Coat

J. THUR(|) CARSTENSEN*, ARNOLD KOFF, J. B. JOHNSON, and S. H. RUBIN

Abstract □ In dosage forms where active drug is added to a sugar coat, it may be shown that the distribution of assays should be normal and the standard deviation should be proportional to the square root of the number of coats. Although, in a series of batches, the standard deviation was found linearly related to the square root of the number of coats, the plot failed to intersect at the origin and did not have the required slope, presumably due to secondary contributions to the variation such as pan build-up.

Keyphrases □ Drug content variation—sugar coating, tablets □ Tablet coatings—drug content variation □ Core size, tablets—coating drug content variation □ Coatings, tablet—drug content relationship

In recent years, several publications have dealt with tablet-to-tablet variation of uncoated tablets or compression coated tablets. Garrett (1) and Garrett and Olson (2) have studied the problem from the point of view of content of drug; Brochmann-Hanssen and Medina (3), Smith *et al.* (4), Lazarus and Lachman (5), and Airth *et al.* (6) analyzed the situation from the point of view of weight variation. Some publications, *e.g.*, those of Lachman *et al.* (7) and Kaplan (8), conclude that statistical variation may be used as a means of evaluating processes; whereas others, *e.g.*, those by Grundman and Ecanow (9) and French *et al.* (10), have been concerned with the problem of statistical sampling.

The inherent variations in sugar-coated tablets have been touched upon by Bhatia (11), but this paper aims not at the statistical variation to be expected but rather on the effect of incompatibilities. Anderson and Sakr (12), in a comprehensive treatment of the statistics of sugar coating, studied the mean line average as a parameter, since smoothness of the tablet was their main point of discussion. Since some coated tablets contain the active component in the sugar coat, it would appear important to know whether expected statistical variations might apply in such a situation. Mattocks (13) has pointed out the problem associated with uniformity of coating, and Butensky (14) has found that, weightwise, the coefficient of variation rises to a maximum of 7% at a stage prior to the final subcoating. The work by Anderson and Sakr (12) also implies that the coefficient of variation of the mean line average appears to level off at a certain stage of the coating operation,

Table I—Tablet-to-Tablet Variation of Sugar-Coated Tablets, Showing Standard Deviation as a Function of Number of Coats, *n*

Number of Coats (*n*)	$\sqrt{n}$	$10^2 \times SD$ (Σ), mg.	Average Drug Content per Tablet ($n\mu$), mg.	$\sqrt{n\mu}$
1	1.00	1.6	0.120	0.347
2	1.41	3.0	0.244	0.494
6	2.45	12.5	0.693	0.835
11	3.32	19.3	1.214	1.102
13	3.61	23.6	1.456	1.208
16	4.00	22.5	1.790	1.340

Nevertheless, on acid hydrolysis the compound gave herniarin. The formation of herniarin may be accounted for by the transformation of the liberated 2-hydroxy-4-methoxy-*trans*-cinnamic acid (IV, Scheme II) to the *cis*-form in presence of hydrogen ions. However, enzymatic hydrolysis did not give herniarin but gave 2-hydroxy-4-methoxyethyl *trans*-cinnamate (IV) which was found to be less mobile on paper than herniarin. The structure of the glucoside (I) was also ascertained through its de-ethylation to the free-acid glucoside (III) which was identical in every respect with authentic material prepared synthetically (7).

SUMMARY

2-Glucosyloxy-4-methoxyethyl *trans*-cinnamate was isolated in a crystalline form from the alcoholic extract of *Prunus mahaleb* L. fruit kernels. The structure of this compound was proved by IR, NMR, and mass spectra. It was further ascertained by de-ethylation of the compound to the free-acid glucoside which was identical with authentic material. On acetylation, it afforded a tetraacetate, the spectral studies of which (IR, NMR, and mass spectra) assured the suggested structure. On acid and enzymatic hydrolysis, it afforded glucose which was identified by chromatographic techniques.

REFERENCES

(1) M. El-Dakhakhny, *Planta Med.*, **12**, 181(1964).

(2) L. J. Bellamy, "The Infra-red Spectra of Complex Molecules," 2nd ed., Methuen & Co. Ltd., London, England, 1964, pp. 8, 34, 96, 132.

(3) S. M. Partridge, *Biochem. J.*, **24**, 238(1948).

(4) M. A. Jermyn and F. A. Isherwood, *ibid.*, **44**, 402(1949).

(5) H. F. Linskens, "Papierchromatographie in der Botanik," 2nd ed., Springer-Verlag, Berlin, West Germany, 1959.

(6) S. M. Mukherjee and H. C. Stivastana, *Nature*, **169**, 330 (1952).

(7) S. A. Brown, *Phytochemistry*, **2**, 137(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from *Trent University, Biochemistry Department, Peterborough, Ontario, Canada.*

Accepted for publication October 10, 1969.

The author thanks S. A. Brown, Professor of Chemistry, Trent University, for his interest and for permitting the completion of this work in his department. Thanks are extended to Prof. R. Hänsel and Dr. H. Rimpler of the Institute Pharmacognosy, Free University of Berlin, and Prof. M. B. E. Fayez of the National Research Centre, Cairo, UAR, for their interest; also to Dr. Schulz of Schering, Berlin, for help with NMR and mass spectra interpretations.

Present address: Department of Pharmacology, Medical Faculty, Alexandria University, Alexandria, UAR.

Tablet-to-Tablet Variation of Drug Content of Sugar-Coated Tablets Containing Drug in the Sugar Coat

J. THUR(|) CARSTENSEN*, ARNOLD KOFF, J. B. JOHNSON, and S. H. RUBIN

Abstract □ In dosage forms where active drug is added to a sugar coat, it may be shown that the distribution of assays should be normal and the standard deviation should be proportional to the square root of the number of coats. Although, in a series of batches, the standard deviation was found linearly related to the square root of the number of coats, the plot failed to intersect at the origin and did not have the required slope, presumably due to secondary contributions to the variation such as pan build-up.

Keyphrases □ Drug content variation—sugar coating, tablets □ Tablet coatings—drug content variation □ Core size, tablets—coating drug content variation □ Coatings, tablet—drug content relationship

In recent years, several publications have dealt with tablet-to-tablet variation of uncoated tablets or compression coated tablets. Garrett (1) and Garrett and Olson (2) have studied the problem from the point of view of content of drug; Brochmann-Hanssen and Medina (3), Smith *et al.* (4), Lazarus and Lachman (5), and Airth *et al.* (6) analyzed the situation from the point of view of weight variation. Some publications, *e.g.*, those of Lachman *et al.* (7) and Kaplan (8), conclude that statistical variation may be used as a means of evaluating processes; whereas others, *e.g.*, those by Grundman and Ecanow (9) and French *et al.* (10), have been concerned with the problem of statistical sampling.

The inherent variations in sugar-coated tablets have been touched upon by Bhatia (11), but this paper aims not at the statistical variation to be expected but rather on the effect of incompatibilities. Anderson and Sakr (12), in a comprehensive treatment of the statistics of sugar coating, studied the mean line average as a parameter, since smoothness of the tablet was their main point of discussion. Since some coated tablets contain the active component in the sugar coat, it would appear important to know whether expected statistical variations might apply in such a situation. Mattocks (13) has pointed out the problem associated with uniformity of coating, and Butensky (14) has found that, weightwise, the coefficient of variation rises to a maximum of 7% at a stage prior to the final subcoating. The work by Anderson and Sakr (12) also implies that the coefficient of variation of the mean line average appears to level off at a certain stage of the coating operation,

Table I—Tablet-to-Tablet Variation of Sugar-Coated Tablets, Showing Standard Deviation as a Function of Number of Coats, *n*

Number of Coats (*n*)	$\sqrt{n}$	$10^2 \times SD$ (Σ), mg.	Average Drug Content per Tablet ($n\mu$), mg.	$\sqrt{n\mu}$
1	1.00	1.6	0.120	0.347
2	1.41	3.0	0.244	0.494
6	2.45	12.5	0.693	0.835
11	3.32	19.3	1.214	1.102
13	3.61	23.6	1.456	1.208
16	4.00	22.5	1.790	1.340

Table II—Results of Applying an Active Drug in Sugar Coating, Showing Distribution of Assays after the First Coats

Range, mg.	Actual Assays, mg.[a]	Number of Occurrences	Fraction of Total	Fraction Expected if Normally Distributed
<0.087		0	0	0.001
0.087–0.103	0.099, 0.100, 0.096, 0.101, 0.102	5	0.25	0.34
0.104–0.119	0.118, 0.107, 0.113 0.117, 0.116, 0.109	6	0.55	0.50
0.119–0.135	0.123, 0.132, 0.123 0.128, 0.125	5	0.80	0.84
0.135–0.154	0.149, 0.135, 0.143 0.152	4	1.00	0.999
>0.154		0	1.00	1.00

[a] Average assay 0.119 mg., *SD* 0.016 mg.

and, thereafter, the coefficient of variation remains fairly constant with increasing weight.

In studying the drug content uniformity in the coating of tablets, it has been the experience of the authors that the size of the tablet affects the final tablet-to-tablet variation. The aim of the investigation reported here is an analysis of this point and of the statistical assumptions which can and cannot be made.

EXPERIMENTAL

Deep concave placebo tablet cores were enteric coated, and subcoats were added in conventional fashion in an 81.31-cm. (32-in.) tablet-coating pan. The drug, which had an exceedingly low water solubility, was micronized and then added in form of a suspension in a gelatin–acacia syrup. Sixteen applications of equal weight were used to accomplish a total addition of 1.79 mg. of drug per tablet.

Twenty tablets were removed and assayed individually after the first and 16th applications, and 10 tablets were removed and assayed after the 2nd, 6th, 11th, and 13th applications. The average drug content and the standard deviations were calculated for each sample. The standard deviations obtained after the various coats are listed in Table I. The results are shown graphically in Fig. 1. The individual results from the sample taken after the first coat are listed in Table II.

Four additional batches were made, using the same coating procedure and tablet core (120 mg.), but assays were only performed on the final tablet (20 individual tablets). One batch was made with a 100-mg. core, two batches were made with a 160-mg. core, and three batches were made with a 175-mg. core, again employing the same manufacturing procedure but only assaying 20 individual tablets of the final product. Seven batches of tablets of four other active compounds were produced and assayed in a similar fashion, using different core sizes (120, 175, 300, and 390 mg.). The tablet-to-tablet variation of the final tablets appeared normally distributed; the results from all 18 batches are shown in Figs. 2 and 3.

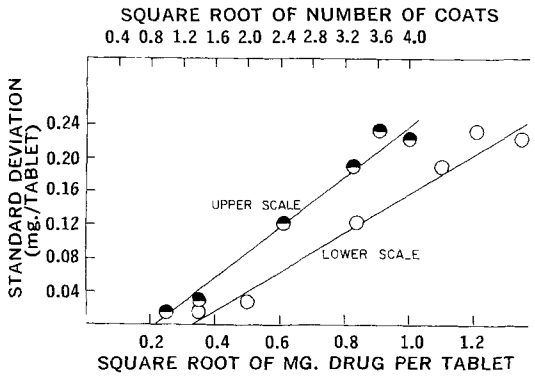


Figure 1—*The standard deviation of assayed drug content of tablets as a function of the square root of the number of coats (upper scale) and the square root of the amount of drug found per tablet (lower scale).*

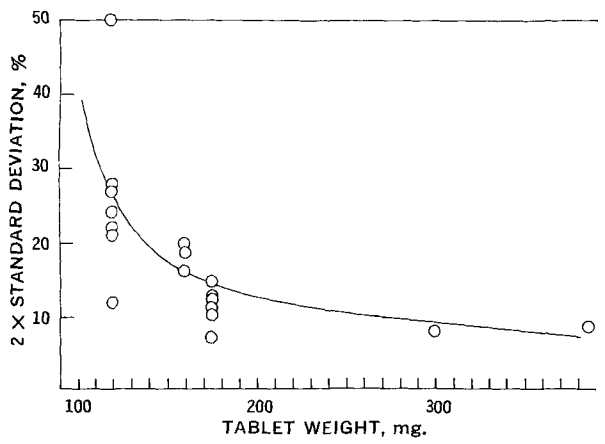


Figure 2—*Sugar-coated tablets with drug in sugar coat: 2 × standard deviation as a function of tablet size.*

RESULTS AND DISCUSSION

The data in Table II and Fig. 4 imply a normal distribution of the assays of the first coat; the variance associated with this will be denoted as σ^2 in the following. The test for normality is not rigorous, but suffices to justify the assumption of normality as a starting point. As shall be shown, this assumption implies a normal distribution of the final tablet assays as well. Other conceivable distributions would be binomial (fraction p coated, $[1-p]$ not coated). This for a large number of applications would lead to a Poisson distribution and eventually, for a very large number of coatings, would approximate a normal distribution. In this light, 16 applications is not a large number. If one assumes that the distribution of the first coat can be described by the normal deviate x_1, the second coat by x_2, and the n^{th} coat by x_n, each with an average μ, and if one assumes $x_1, x_2, \ldots x_{16}$ to be independent variables, then (as shown in the *Appendix*) it is straightforward to show that the variance after n coats (Σ^2) should be given by $n\sigma^2$, where σ is assumed to be identical for each coat, *i.e.*, equal to that of the first coat. A plot of the standard deviation as a function of the square root of the number of coats should, therefore, produce a straight line through the origin with a slope identical to the standard deviation of the first coat: $\Sigma = \sigma \cdot \sqrt{n}$. The coefficient of variation will decrease with increasing number of coats since

$$\frac{\sqrt{n\sigma^2}}{\mu n} = \frac{\sigma}{\mu\sqrt{n}} \qquad \text{(Eq. 1)}$$

It is seen from Fig. 2 that the standard deviation of individual tablet assays from all 18 batches (of varying tablet size) exhibited great scatter. The trend, however, is unmistakably that the standard deviation decreases with increasing tablet size. Although it is difficult to draw conclusions from such data, some speculation may be offered. Figure 3 represents the data of Fig. 2 in logarithmic form, and a line of slope 1 is more compatible with the data than a line of slope $\frac{2}{3}$, so that the standard deviation might be related to the volume (or weight) of the tablet rather than to the surface. If this

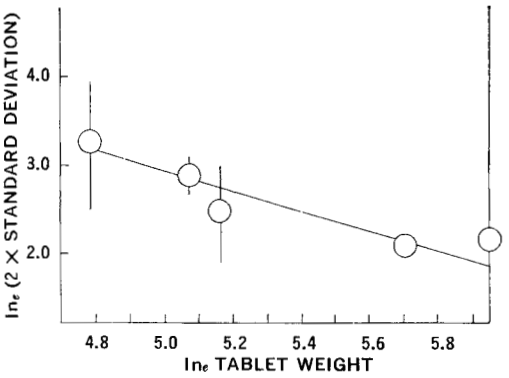


Figure 3—*Variation of drug content as a function of tablet size on a natural logarithmic scale. The standard deviation is on a percentage basis, the tablet weight is in milligrams.*

indeed is the case, then the assumption that σ is the same for all 16 applications may not be true.

The volume increase during the 16 coats is small; however, it would appear from Fig. 1 that the size increase during the application of the active coat does not play an important role, since linearity seems to prevail. On the other hand, it is obvious that the line neither passes through the origin nor has a slope equal to the standard deviation of the first application. It is possible, since there is always build-up on the pan, that in the earlier stages some drug is deposited on the build-up on the pan and that in later coats some drug is applied back to the tablets by attrition, and thus a second contribution to the standard deviation occurs.

The findings have significance in the sense that the tablet-to-tablet variation will be smaller the larger the number of coats, *i.e.*, the application of a given amount of drug is best accomplished with a larger number of coatings of more dilute coating mixture than with a smaller number of coatings with a more concentrated mixture. The data also imply that a large tablet core yields a better drug distribution in the active coat than a small core. It is obvious that other factors (*e.g.*, viscosity and surface activity of the syrup-suspension containing the active ingredient, as well as core shape) are of significance. These have not been a subject of this study and have been kept constant in order to evaluate the statistical, rather than the physicochemical, aspect of sugar coating.

APPENDIX

Suppose n coats of active material are applied to a panload of tablets. Let the distribution of the first coat be designated by the normal deviate x_1, the second x_2, and the n^{th} x_n, and, assuming $x_1, x_2, \ldots x_n$ to be independent variables with identical variances, then the moment-generating function for the distribution of $x_1 + x_2 + \cdots + x_n$ is (15)

$$M_{x_1+x_2+\cdots+x_n}(\theta) = M_{(x_1)}M_{(x_2)}\ldots M_{(x_n)} =$$

$$\prod_{i=1}^{n} \frac{1}{\sigma\sqrt{2\pi}} \int_{-\infty}^{\infty} \exp[x_i\theta] \cdot \exp\left[-\frac{[x_i-\mu]^2}{2\sigma^2}\right] dx_i = e^{\theta n\mu} \cdot e^{n\sigma^2\theta^2/2} \quad \text{(Eq. 2)}$$

where μ is the amount of drug applied. The first moment about $\theta = 0$ is the mean $n\mu$ and is given by:

$$\left[\frac{\partial M}{\partial \theta}\right]_{\theta=0} = n \cdot \mu \quad \text{(Eq. 3)}$$

The second moment is

$$\left[\frac{\partial^2 M}{\partial \theta^2}\right]_{\theta=0} = [n\mu]^2 + n \cdot \sigma^2 \quad \text{(Eq. 4)}$$

The variance is the second moment minus the square of the first moment, both at $\theta = 0$, *i.e.*:

$$\Sigma^2 = [n\mu]^2 + n\sigma^2 - [n\mu]^2 = n\sigma^2 \quad \text{(Eq. 5)}$$

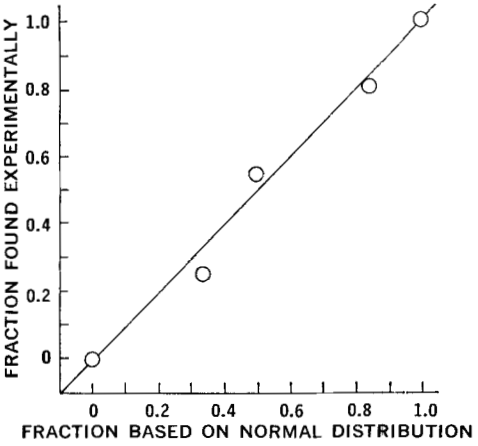


Figure 4—*Results from the last two columns of Table II. The ordinate is the cumulative fraction larger than a certain figure, and the abscissa is the fraction expected if the distribution were normal with average content 0.119 mg. and* SD *0.016 mg.*

If one takes the square root:

$$\Sigma = \sigma \cdot \sqrt{n} \quad \text{(Eq. 6)}$$

i.e., the standard deviation after n coats (Σ) is proportional to the square root of the number of coats with σ, the standard deviation of the first coat, as the proportionality factor. Furthermore the distribution after n coats will be normal if it was normal originally. For large n, this may be expected for any type distribution (central limit theorem).

REFERENCES

(1) E. R. Garrett, *J. Pharm. Sci.*, **51,** 672(1962).
(2) E. R. Garrett and E. C. Olson, *ibid.*, **51,** 764(1962).
(3) E. Brochmann-Hanssen and J. C. Medina, *ibid.*, **52,** 630 (1963).
(4) C. D. Smith, T. P. Michaels, M. M. Chertkoff, and L. P. Sinotte, *ibid.*, **52,** 1183(1963).
(5) J. Lazarus and L. Lachman, *ibid.*, **55,** 1121(1966).
(6) J. M. Airth, D. F. Bray, and C. Radecka, *ibid.*, **56,** 233(1967).
(7) L. Lachman, H. D. Sylwestrowicz, and P. P. Speiser, *ibid.*, **55,** 959(1966).
(8) L. L. Kaplan, *ibid.*, **56,** 1323(1967).
(9) R. A. Grundman and B. Ecanow, *ibid.*, **53,** 1527(1964).
(10) W. N. French, F. Matsui, D. Cook, and L. Levi, *ibid.*, **56,** 1622(1967).
(11) D. G. Bhatia, T. D. Sokoloski, and V. N. Bhatia, *ibid.*, **55,** 1116(1966).
(12) W. Anderson and A. M. Sakr, *J. Pharm. Pharmacol.*, **18,** 783(1966).
(13) A. M. Mattocks, *Proc. Production Conf. Amer. Pharm. Mfr. Ass.*, **1958,** 196–209.
(14) I. Butensky, "Automatic Coating of Tablets," Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1961.
(15) C. Bennett and N. Franklin, "Statistical Analysis in Chemistry and the Chemical Industry," Wiley, New York, N. Y., 1954, p. 85.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 5, 1969, from *Hoffmann-La Roche, Inc., Nutley, NJ 07110 and the School of Pharmacy, University of Wisconsin, Madison, WI 53706*

Accepted for publication October 17, 1969.

* To whom request for reprints should be directed. Present address: School of Pharmacy and Extension Services in Pharmacy, University of Wisconsin, Madison, WI 53706

Action of Antibiotics on Respiratory Tract I: Ampicillin

G. BENZI, E. BERMUDEZ, and E. ARRIGONI

Abstract □ *In vitro* ampicillin antagonizes the barium chloride-, histamine-, and acetylcholine-induced spasm on the dog bronchial chains. Ampicillin is more effective in preventing a histamine than an acetylcholine bronchospasm, while isoprenaline and adrenaline are similarly potent against the effects of the two agonists. *In situ* the antibiotic induces at first a prompt but short increase in ventilation; subsequently, a lesser but persistent activity remains for 2 or 3 hr.

Keyphrases □ Ampicillin effect—respiratory tract □ Histamine-, barium chloride-, acetylcholine-induced bronchospasm—ampicillin effect □ Respiratory ventilation—ampicillin effect

In previous research, the action of some antibiotics *in vitro* and *in situ* upon the motility and tone of the extrahepatic biliary tract (rifamycin SV, erythromycin, tetracyclines, ampicillin, aminosidin, spiramycin, and dicloxacillin) and the ureter (ampicillin and dicloxacillin) was studied. It was particularly observed that ampicillin relaxed the above-mentioned musculatures, normal or hypertonized by barium chloride, histamine, serotonin, carbachol, or cholecystokinin (1, 2). This behavior points out the problem of the action of the antibiotics on various tissues, including the respiratory tract.

The purpose of the present paper is to report the ampicillin action on the airways *in vitro* and *in situ*. In this field, the *in vitro* pharmacological preparation consisting of a chain of tracheal or bronchial rings has been used (3, 4). Human, guinea pig, and dog preparations are equally sensitive to contracting agents, such as histamine and acetylcholine (5), and the activity of the drugs on isolated human bronchial chains agrees closely with that found by other workers using preparations from animals, including the dog (6). *In situ* the changes in airway smooth muscle tone may be measured: (*a*) by recording the dynamic pressure–volume relationship of the lungs (7), the resistance to airflow in the trachea isolated *in situ* or in the respiratory tract (8, 9); and (*b*) by the radiography of the airways (10).

The direct record of the ventilation by a rubber balloon inserted into a primary or secondary bronchus was used. For the experiments *in vitro* and *in situ*, the dog was chosen because of its size and availability.

METHOD

The experiments were performed in the dog both *in vitro* and *in situ*. Ampicillin sodium salt, of which amounts are expressed in terms of D(−)-6-(α-amino-α-phenylacetamido)-penicillanic acid, was used.

Experiments *In Vitro*—Bronchi of 32 mongrel dogs of either sex (weighing 7.4 to 10.9 kg.) were removed immediately after death. All tissues were dissected and cut in rings which were tied together in chains with loops of cotton. The mucosa was removed to allow greater freedom of movement of the muscle. Four to six rings were suspended in a 50-ml. organ bath containing Tyrode solution gassed with 95% oxygen and 5% carbon dioxide; the temperature was 36.5–37.5°. The tonus level of preparations was continuously recorded by a strain-gauge lever, giving a magnification of ×15 to 20, tension 200 mg., writing on a kimograph drum. The preparations were left 2 hr. before any drugs were given. Three submaximal doses of acetylcholine were given until regular responses were obtained, before any doses of acetylcholine itself or other agonists were tested.

The action of ampicillin (62.5–2000 mcg./ml.) was evaluated against the stimulation by barium chloride (100–400 mcg./ml.); for the construction of the dose–response curve, ampicillin activity was taken as the percent inhibition of the recorded response area by barium chloride stimulation during a 20-min. period of contact.

For comparative assay with other well-known bronchodilators, the activity of ampicillin (2048–16,384 mcg./ml.), *l*-adrenaline (0.5–4 mcg./ml.), and *dl*-isoprenaline (0.125–2.0 mcg./ml.) was evaluated against the stimulation by acetylcholine chloride (0.2–2 mcg./ml.) and histamine acid phosphate (1.0–10 mcg./ml.). Two-dose levels of agonist and antagonist were usually used; the agonists were left in contact with the preparation for 0.5–1 min. Ampicillin was introduced into the bath 10 min., and adrenaline or isoprenaline 30 sec. before the acetylcholine or histamine doses. The ED_{50}, the slope of the curve, the potency ratio, and their 95% confidence limits were calculated (11).

Experiments *In Situ*—The experiments were carried out on 28 beagle dogs of either sex (weighing 10.7–16.8 kg.) preanesthetized with urethan (0.4 g./kg. i.p.). Anesthesia was induced and maintained by chloralose (80 mg./kg. i.v.); the arterial blood pressure was measured from a cannula inserted into a femoral artery; the intestinal movement and tone were recorded by a rubber balloon inserted into the jejunum.

During the succinylcholine chloride (1 mg./kg. i.v.) action, an intratracheal Warne tube was set in place. Through this tube a little rubber balloon was pushed into a primary or, if possible, secondary bronchus under X-ray examination.

Arterial blood pressure and intestinal and bronchial activities were recorded by a polygraph Physioscript EE12 Schwarzer.

Ampicillin (1.25–80 mg./kg.) was administered intravenously by a polystan tube inserted into the femoral vein. Two submaximal doses of *dl*-isoprenaline or *l*-adrenaline were given i.v. until regular responses were obtained before any doses of ampicillin sodium salt were tested. To analyze the mechanism of action, ampicillin was tested after: (*a*) cutting the vagi, and (*b*) treatment with the following drugs: morphine hydrochloride (4–8 mg./kg. i.v.), atropine sulfate (2–3 mg./kg. s.c.), dibenamine hydrochloride (2–5 mg./kg. i.v.), D(−)INPEA (4–8 mg./kg. i.v.), chlorpheniramine maleate (2–3 mg./kg. s.c.), cyproheptadine hydrochloride (300–600 mcg./kg. i.v.), hexamethonium bromide (300–600 mcg./kg. i.v.).

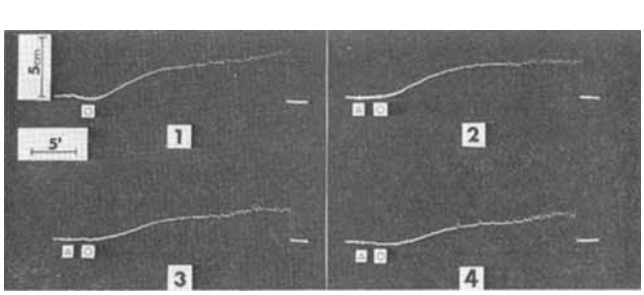


Figure 1—*Action of ampicillin on dog bronchial chain stimulated by barium chloride. The barium chloride (250 mcg./ml.) was added to the bath at the marks* (○). *The antibiotic* (△) *was present in the bath at the following concentrations: 1 = 0 mcg./ml.; 2 = 250 mcg./ml.; 3 = 500 mcg./ml.; 4 = 1000 mcg./ml. At* △ *the recording apparatus was stopped for the first 8 min. of contact with ampicillin.*

Table I—Dog Bronchial Chain *In Vitro:* ED_{50}, Slope Function of the Line (*S*), and Estimated Relative Potency (ERP), with 95% Confidence Limits, of *dl*-Isoprenaline, *l*-Adrenaline, and Ampicillin against the Stimulation by Acetylcholine Chloride (0.2–2 mcg./ml.) and Histamine Acid Phosphate (1.0–10 mcg./ml.)

Antagonist	Agonist: Acetylcholine ED_{50}[a]	Acetylcholine S[b]	Acetylcholine ERP[c]	Histamine ED_{50}[a]	Histamine S[b]	Histamine ERP[c]
Isoprenaline	0.40 (0.20–0.80)	2.30 (0.92–5.75)	28,000 (9650–81,200) D	0.49 (0.25–0.95)	2.57 (1.03–6.42)	9490 (3490–25,810) D
Adrenaline	1.30 (0.62–2.73)	2.29 (1.04–5.04)	8615 (2920–25,415) D	1.05 (0.51–2.15)	2.25 (1.00–5.06)	4430 (1575–12,450) D
Ampicillin	11,200 (5090–24,640)	2.39 (0.68–8.36)	1	4650 (2214–9765)	2.80 (0.62–12.60)	1

[a] ED_{50} = dose (mcg./ml.) of bronchodilator necessary to reduce by 50% the contraction produced by acetylcholine or histamine. [b] S = fold change in dose required to produce a unit standard deviation change in response along the line; thus S = antilog s = antilog $1/b$, where b and s are, respectively, the slope constant and standard deviation of a line relating log dose of antagonist, and probit percent reduction of agonist activity. [c] D = ampicillin and compared drug differ significantly in potency ($p < 0.05$).

Table II—Dog Ventilation *In Situ:* Range of the Percent Changes both in Amplitude of the Recorded Tracing and in Frequency of the Respiratory Rate Induced by the i.v. Injection of Ampicillin

Ampicillin Dose Levels Injected i.v., mg./kg.	Number of Tested Dogs	Period of Observation after Injection of Ampicillin: From 0 to 20 min. Increase in Amplitude, %	From 0 to 20 min. Decrease in Frequency, %	From 20 to 60 min. Increase in Amplitude, %	From 20 to 60 min. Decrease in Frequency, %	From 1 to 2 hr. Increase in Amplitude, %	From 1 to 2 hr. Decrease in Frequency, %
1.25–5	6	24–115	8–28	10–62	8–27	0–29	0–24
10 –20	4	62–150	18–35	22–75	12–40	0–40	4–42
40 –80	4	48–174	13–38	14–94	16–43	13–48	12–37

RESULTS AND DISCUSSION

On the dog bronchial chains *in vitro*, ampicillin (62.5–2000 mcg./ml.) reduces or inhibits the stimulation by barium chloride (Fig. 1); the log dose–response curve is indicated in Fig. 2.

The comparative assay of ampicillin, isoprenaline, and adrenaline action against the stimulation by acetylcholine or histamine is summarized in Table I and shows that isoprenaline is more active than adrenaline and ampicillin in reducing the agonist-induced spasm. It is possible to observe that against acetylcholine-induced contraction, ampicillin is about 1/8600 as active as *l*-adrenaline and about 1/28,000 as active as *dl*-isoprenaline. According to Mc Dougal and West (5), on dog tracheal rings aminophilline possesses about 1/10,000 the activity of adrenaline and about 1/40,000 the activity of isoprenaline. On the other hand, ampicillin, as is aminophilline (5), is more effective in preventing a histamine than an acetylcholine bronchospasm, while adrenaline and isoprenaline are similarly potent against the effects of the two agonists.

Obviously the *in vitro* technique employed provides information concerning potency ratio or mechanisms operant in the airway wall; nevertheless it is unphysiological, the studies being limited to denervated portions of the airways. However, it is impossible to correlate directly the present results with clinical conditions. In fact it is well known that many bronchoconstrictive stimuli act, at least in part, *via* reflex mechanisms, and these effects are absent in the *in vitro* preparation. Nevertheless, the results *in vitro* related to isoprenaline and adrenaline agree with the findings of previous workers (12) using *in vivo* the test of the antagonism of the effects of a histamine aerosol in guinea pigs.

The observations *in situ* indicate that, after intravenous injection of 1.25–80 mg./kg., ampicillin induces a biphasic action. At first the antibiotic induces a prompt, high, but short (5–20 min.) increase in ventilation, and subsequently a lesser but persistent activity remains for 2 or 3 hr., as summarized in Table II and exemplified in Fig. 3. No change in systemic blood pressure occurs during the ampicillin action on the airways.

The increase in ventilation induced by ampicillin during its first phase of action is similar to those caused by the i.v. injection of isoprenaline (0.125–2 mcg./kg.) and adrenaline (0.5–5 mcg./kg.). Nevertheless, related to the very different biotransformation, the total time of activity of the sympathomimetic amines is much shorter (2–8 min.) than that of ampicillin, and furthermore the well-known changes in systemic blood pressure occur.

The *in situ* preparation utilized by the authors does not provide any information on drug effects on peripheral airways which are vitally concerned with the gas-exchange process. The pressures recorded by the bronchial balloon must have been changes in the depth of respiration, and it is completely possible that the results

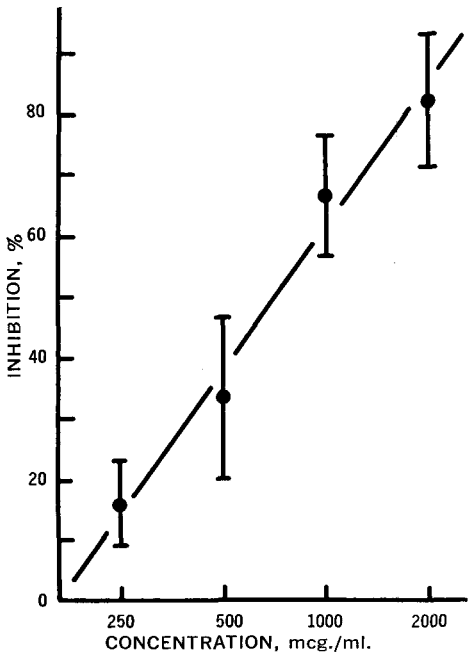


Figure 2—*Log dose–response curve of ampicillin action against the stimulation by barium chloride. The ordinate shows the inhibition percent of the contracting action by barium. The concentrations of the antibiotic (mcg./ml.) are plotted on the abscissa in logarithmic scale. The vertical lines indicate standard errors of the means. Eight preparations at each dose level.*

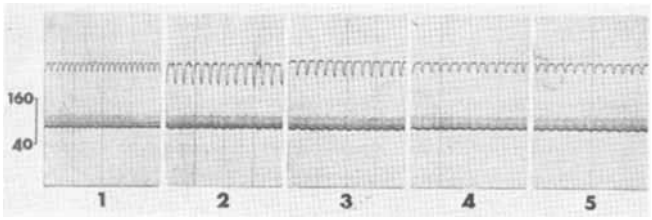


Figure 3—*Action of ampicillin i.v. on the ventilation of a dog* in situ. *From top to bottom: tracing recorded by a balloon inserted into the bronchus; blood pressure (mm. Hg); 1 = control condition; 2 = 2 min. after injection i.v. of 10 mg./kg. of ampicillin; 3 = 30 min. later; 4 = 1 hr. later; 5 = 2 hr. later.*

are only in part associated with changes in bronchial tone. In fact, it should be noted that: (*a*) the baseline of bronchial balloon pressure did not change appreciably in response to ampicillin, indicating that the resting tone of bronchial muscle was slightly or not affected by the drug; (*b*) the ampicillin action was antagonized by the high respiratory depression by morphine; and (*c*) the antibiotic action was unaffected after treatment with atropine, dibenamine, INPEA, chlorpheniramine, cyproheptadine, and hexamethonium.

In conclusion, only *in vitro* is it possible to postulate the ability of ampicillin to relax directly the bronchial musculature. *In vivo* the pressure fluctuations noted with each breath were probably not caused by rapid breath-to-breath changes in bronchial tone. It is more likely that ampicillin increased the activity of the respiratory center, causing an increase in ventilation and a subsequent increase in anatomic deadspace.

REFERENCES

(1) G. Benzi, A. Crema, F. Berté, G. M. Frigo, and E. Arrigoni, *Arch. Int. Pharmacodyn.*, **174,** 74(1968).

(2) G. Benzi, E. Bermudez, E. Arrigoni, and F. Berté, to be published.

(3) J. C. Castillo and E. J. De Beer, *J. Pharmacol Exp. Ther.*, **90,** 104(1947).

(4) D. F. Hawkins, *Brit. J. Pharmacol.*, **10,** 230(1955).

(5) M. Mc Dougal and G. B. West, *ibid.*, **8,** 26(1953).

(6) D. F. Hawkins and H. O. Schild, *ibid.*, **6,** 682(1951).

(7) H. Konzett and R. Rössler, *Arch. Exp. Pathol. Pharmakol.*, **195,** 71(1940).

(8) J. Mead, *Physiol. Rev.*, **41,** 281(1961).

(9) J. A. Nadel and J. G. Widdicombe, *J. Physiol.*, **163,** 13 (1962).

(10) K. H. Kilburn, *J. Appl. Physiol.*, **15,** 229(1960).

(11) J. T. Litchfield, Jr., and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96,** 99(1949).

(12) G. Chen, R. Portman, D. Russel, and C. R. Ensor, *J. Amer. Pharm. Ass., Sci. Ed.*, **40,** 273(1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 14, 1969, from the *Instituto di Farmacologia, Universita di Pavia, Piazza Botta, 10, 27100-Pavia, Italy.*

Accepted for publication December 8, 1969.

Determination of Epitetracycline and Chlortetracycline in Tetracycline by Quantitative Thin-Layer Chromatography

IDO C. DIJKHUIS and MARTHA R. BROMMET

Abstract □ Small amounts of epitetracycline and chlortetracycline in tetracycline were determined by quantitative thin-layer chromatography on kieselguhr layers, impregnated with a citrate–phosphate solution, pH 5.5, containing 10% glycerin. The method involves chromatography under sharply defined conditions (relative humidity, temperature, and rapidity of spotting) in order to obtain a good separation of the different zones and to prevent rapid epimerization during development. After elution with 0.1 *N* HCl, epitetracycline was measured at 356 mμ, while for chlortetracycline the fluorimetric method of Chicarelli was used. The possible identities of three other impurities—with R_f values between epitetracycline and tetracycline—are discussed; their percentages were calculated as epitetracycline.

Keyphrases □ Epitetracycline—analysis, separation in tetracycline □ Chlortetracycline—analysis, separation in tetracycline □ TLC—analysis □ Spectrophotometry—analysis

In 1963, Remmers *et al.* (1) described a spectrophotometric determination of 4-epitetracycline (epi-TC) in tetracycline (TC), based on the absorbance-ratio difference at 254 and 267 mμ. However, this method is not suitable for measuring small amounts of epi-TC in commercial samples; accurate measurements at those wavelengths are disadvantageously affected by the presence of anhydrotetracyclines (ATC, epi-ATC). The paper chromatographic method, reported by Addison and Clark (2), gives better information about the percentage of epi-TC.

When small amounts of chlortetracycline (CTC) are to be determined, the spectrophotometric procedure, given by Woolford *et al.* (3), is not preferable. It is also not possible to apply the fluorimetric procedure [Chicarelli (4)] because the fluorescence of the CTC is quenched by the TC solution.

In 1964, Sonanini and Anker (5) described the identification of three tetracyclines on kieselguhr layers, impregnated with a solution (pH 3.7) containing 5% glycerin. In 1968, Ascione *et al.* (6) reported the separation of some tetracyclines on the same support, impregnated with a EDTA–PEG 400–glycerin solution, pH 7.0.

The present authors used the idea of Sonanini and Anker as a starting point for a quantitative determination of epi-TC and CTC present in commercial samples of TC.

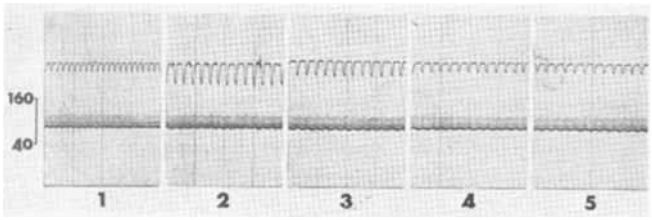


Figure 3—*Action of ampicillin i.v. on the ventilation of a dog* in situ. *From top to bottom: tracing recorded by a balloon inserted into the bronchus; blood pressure (mm. Hg); 1 = control condition; 2 = 2 min. after injection i.v. of 10 mg./kg. of ampicillin; 3 = 30 min. later; 4 = 1 hr. later; 5 = 2 hr. later.*

are only in part associated with changes in bronchial tone. In fact, it should be noted that: (*a*) the baseline of bronchial balloon pressure did not change appreciably in response to ampicillin, indicating that the resting tone of bronchial muscle was slightly or not affected by the drug; (*b*) the ampicillin action was antagonized by the high respiratory depression by morphine; and (*c*) the antibiotic action was unaffected after treatment with atropine, dibenamine, INPEA, chlorpheniramine, cyproheptadine, and hexamethonium.

In conclusion, only *in vitro* is it possible to postulate the ability of ampicillin to relax directly the bronchial musculature. *In vivo* the pressure fluctuations noted with each breath were probably not caused by rapid breath-to-breath changes in bronchial tone. It is more likely that ampicillin increased the activity of the respiratory center, causing an increase in ventilation and a subsequent increase in anatomic deadspace.

REFERENCES

(1) G. Benzi, A. Crema, F. Berté, G. M. Frigo, and E. Arrigoni, *Arch. Int. Pharmacodyn.*, **174,** 74(1968).

(2) G. Benzi, E. Bermudez, E. Arrigoni, and F. Berté, to be published.

(3) J. C. Castillo and E. J. De Beer, *J. Pharmacol Exp. Ther.*, **90,** 104(1947).

(4) D. F. Hawkins, *Brit. J. Pharmacol.*, **10,** 230(1955).

(5) M. Mc Dougal and G. B. West, *ibid.*, **8,** 26(1953).

(6) D. F. Hawkins and H. O. Schild, *ibid.*, **6,** 682(1951).

(7) H. Konzett and R. Rössler, *Arch. Exp. Pathol. Pharmakol.*, **195,** 71(1940).

(8) J. Mead, *Physiol. Rev.*, **41,** 281(1961).

(9) J. A. Nadel and J. G. Widdicombe, *J. Physiol.*, **163,** 13 (1962).

(10) K. H. Kilburn, *J. Appl. Physiol.*, **15,** 229(1960).

(11) J. T. Litchfield, Jr., and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96,** 99(1949).

(12) G. Chen, R. Portman, D. Russel, and C. R. Ensor, *J. Amer. Pharm. Ass., Sci. Ed.*, **40,** 273(1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 14, 1969, from the *Instituto di Farmacologia, Universita di Pavia, Piazza Botta, 10, 27100-Pavia, Italy.*

Accepted for publication December 8, 1969.

Determination of Epitetracycline and Chlortetracycline in Tetracycline by Quantitative Thin-Layer Chromatography

IDO C. DIJKHUIS and MARTHA R. BROMMET

Abstract □ Small amounts of epitetracycline and chlortetracycline in tetracycline were determined by quantitative thin-layer chromatography on kieselguhr layers, impregnated with a citrate–phosphate solution, pH 5.5, containing 10% glycerin. The method involves chromatography under sharply defined conditions (relative humidity, temperature, and rapidity of spotting) in order to obtain a good separation of the different zones and to prevent rapid epimerization during development. After elution with 0.1 *N* HCl, epitetracycline was measured at 356 mμ, while for chlortetracycline the fluorimetric method of Chicarelli was used. The possible identities of three other impurities—with R_f values between epitetracycline and tetracycline—are discussed; their percentages were calculated as epitetracycline.

Keyphrases □ Epitetracycline—analysis, separation in tetracycline □ Chlortetracycline—analysis, separation in tetracycline □ TLC—analysis □ Spectrophotometry—analysis

In 1963, Remmers *et al.* (1) described a spectrophotometric determination of 4-epitetracycline (epi-TC) in tetracycline (TC), based on the absorbance-ratio difference at 254 and 267 mμ. However, this method is not suitable for measuring small amounts of epi-TC in commercial samples; accurate measurements at those wavelengths are disadvantageously affected by the presence of anhydrotetracyclines (ATC, epi-ATC). The paper chromatographic method, reported by Addison and Clark (2), gives better information about the percentage of epi-TC.

When small amounts of chlortetracycline (CTC) are to be determined, the spectrophotometric procedure, given by Woolford *et al.* (3), is not preferable. It is also not possible to apply the fluorimetric procedure [Chicarelli (4)] because the fluorescence of the CTC is quenched by the TC solution.

In 1964, Sonanini and Anker (5) described the identification of three tetracyclines on kieselguhr layers, impregnated with a solution (pH 3.7) containing 5% glycerin. In 1968, Ascione *et al.* (6) reported the separation of some tetracyclines on the same support, impregnated with a EDTA–PEG 400–glycerin solution, pH 7.0.

The present authors used the idea of Sonanini and Anker as a starting point for a quantitative determination of epi-TC and CTC present in commercial samples of TC.

Table I—Percentages of epi-TC and CTC and Compounds A, B, and C in Three Commercial Samples of TC

Sample. No.	epi-TC, %	CTC, %	A, %	B, %	C, %
I	2.4	0	0.3	0.6	0.6
	2.4	0	0.3	0.7	0.7
	2.5	0	0.2	0.9	0.7
II	1.1	3.4	0.3	0.5	0.5
	1.2	3.4	0.2	0.5	0.5
	1.4	3.5	0.3	0.6	0.5
III	5.8	0.1	0.4	0.5	0.4
	5.6	0.1	0.4	0.4	0.5
	5.5	0.1	0.4	0.5	0.6

EXPERIMENTAL

Materials—*Kieselguhr, Purified*—One-hundred grams of Kieselguhr-G (Merck) is suspended in 500 ml. of 4 *N* HCl, boiled, and filtered; this procedure is done three times to remove the binder ($CaSO_4$) and most of the iron. After washing with distilled water, the product is suspended in 500 ml. of a 1% aqueous EDTA solution, pH 3, and boiled again to eliminate traces of Fe^{+++} and Ca^{++} ions. The purified kieselguhr is finally washed with distilled water and methanol and dried at 100°. As a control for purity, 500 mg. is suspended in 5 ml. of 0.1 *N* HCl and centrifuged; the absorbance of the clear solution at 356 mμ is <0.005.

Solution pH 5.5—Forty-three milliliters of citric acid (0.1 *M*), 57.0 ml. of $Na_2HPO_4 \cdot 2H_2O$ (0.2 *M*), and 10 ml. of glycerin (sp. gr. 1.23) were used.

Tetracycline Hydrochloride (USP Reference Standard, Packaged July 1968)—Dried over P_2O_5 at 60° *in vacuo*. Absorbance of a 1% solution in 0.1 *N* HCl in a 1-cm. cell at 356 mμ is 308. Impurities: ATC, 0.1% [spectrophotometric (7)]; CTC, 0.3%; epi-TC, 0.5%; Compound A, 0.1%; Compound B, 0.2%; Compound C, 0.2%.

Epitetracycline Ammonium Salt[1]—Absorbance of a 1% solution in 0.1 *N* HCl in a 1-cm. cell at 356 mμ is 316. Impurities: epi-ATC, 0.3% [spectrophotometric (7)]; TC, 0.5%. Because of its instability, epi-TC was only used in calculating the recoveries of epi-TC and TC after spotting and elution.

Chlortetracycline Hydrochloride—From capsules (Aureomycin), dried over P_2O_5 at 60° *in vacuo*. Absorbance of a 1% solution in 0.1 *N* HCl in a 1-cm. cell at 269 mμ is 194; this value has been corrected for the absorbance of TC present in CTC (0.6%, determined by quantitative TLC).

Oxytetracycline (OTC), 2-acetyl-2-decarboxamido-tetracycline (ADTC),[1] methacycline (MTC), anhydrotetracycline (ATC),[1] and epi-anhydrotetracycline (epi-ATC)[1] were used for the thin-layer chromatographic identification of other impurities in tetracycline.

Kieselguhr Layers—Layers (0.25 mm.) were prepared with the Desaga apparatus by application of a slurry of 4 g. purified kieselguhr in 8 ml. of the solution, pH 5.5, per 20 × 20-cm. glass plate. The plates were stored at 15° and a relative humidity of 65–75% during 2 days before use.

All chemicals used were of analytical grade.

Microliter pipets, 5-μl., 55-mm. length (microcaps, Drummond Scientific), were used.

Apparatus—Beckman DU spectrophotometer. Filter-fluorimeter, primary filter 364 mμ, secondary filter 414 mμ. TLC-tank, 20 × 20 × 8 cm., saturated with dichloromethane–ethanol 95% (9:1) at 15°.

PROCEDURES

Chromatography—The sample of TC is dissolved in methanol at 0° in a concentration of 20 mg. per ml. Approximately 1 mg. of the sample (= *p* mg. dried substance) is spotted rapidly over 15 cm. and the plate is placed immediately in the developing solvent [dichloromethane–ethanol 95% (9:1) at 15°]. The time between spotting and developing must not exceed 3 min. The fluorescing zones are marked under longwave UV light.

[1] The authors are grateful to Dr. J. Keiner (Jena, D.D.R.) for his gift of ADTC and to Mr. J. Ribbers (Organon, Holland) for the preparation of highly purified epi-TC, ATC, and epi-ATC.

R_f values are: epi-TC, 0.1; Compound A, 0.17; Compound B, 0.30; Compound C, 0.38; TC, 0.55–0.65; CTC, 0.80; epi-ATC, 0.85; ATC, 1.

If necessary, the fluorescence of CTC can be activated by spraying a few milliliters of 0.01 *N* NaOH on the plate. Activation of the fluorescence of epi-TC, by spraying with NaOH or by holding the plate above ammonia vapor, is not recommended because of rapid decomposition of this compound.

Assay of Epitetracycline (Hydrochloride)—The epi-TC zone is scratched off, transferred into a stoppered centrifuge tube, and extracted with 5.00 ml. of 0.1 *N* HCl. After centrifuging, the absorbance (*A*) of the clear solution is determined at 356 mμ. To eliminate inaccuracies in spotting and elution, 50 mcg. of the TC standard (concentration 1 mg. per ml.) is spotted over 15 cm.; after elution with 5.00 ml. of 0.1 *N* HCl, the absorbance at 356 mμ is determined (= $A_{stand.}$).

$$\% \text{ epi-TC} = \frac{A \times 5}{A_{stand.} \times p} \qquad \text{(Eq. 1)}$$

In this laboratory the $A_{stand.}$ for TC was 0.277 ± 0.003 (recovery 90%), while for epi-TC with the same procedure a recovery of 91% was obtained. The absorbance of the blank—250 mg. of developed kieselguhr per 5.00 ml. of 0.1 *N* HCl—was negligible; also the background absorbance between epi-TC and Compound A was very small (about 0.005), and therefore no correction had to be made. This background absorbance, due to epimerization during development, increased at higher R_f values; the values of the percentages of Compounds A, B, and C in Table I were corrected with 0.1, 0.2, and 0.2, respectively.

Assay of Chlortetracycline (Hydrochloride)—The CTC zone is scratched off, transferred into a stoppered centrifuge tube, and extracted with 5.00 ml. of 0.2 *N* NaOH. After centrifuging, the fluorescence of the clear solution is determined about 15 min. after NaOH addition, adjusting the reading of the galvanometer at a suitable value. The percentage of CTC (hydrochloride) is calculated from the standard curve.

CTC Standard Curve—To eliminate inaccuracies in spotting and elution, 5, 10, 15, and 20 mcg. of the CTC standard (concentration 0.5 mg. per ml.) are spotted over 15 cm. After elution with 5.00 ml. of 0.2 *N* NaOH the fluorescence is measured. In this laboratory the recovery of the CTC standard was 97 ± 3%, while Beer's law was obeyed. The fluorescence of the blank was nil.

DISCUSSION

Before good results could be obtained in the determination of epi-TC and CTC in TC, many problems had to be solved. While the qualitative examination of these substances was easy—because only 1–5 mcg. of a sample of TC can be spotted on the plate—it was rather difficult to separate them when 1–1.5 mg. had to be examined. (These great quantities were necessary for spectrophotometric measurements.) Moreover, epi-TC and CTC also had to be separated from five other impurities present in most commercial samples of TC: ATC, epi-ATC, and the Compounds A, B, and C. Finally, chromatography on one plate and with one developing sol-

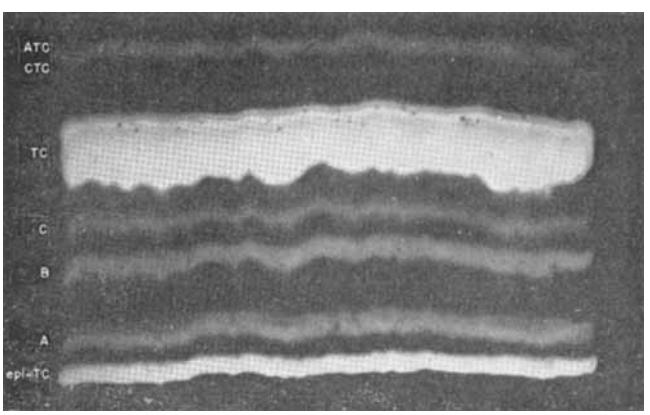


Figure 1—*The quantitative TLC separation of the impurities in commercial tetracycline hydrochloride.*

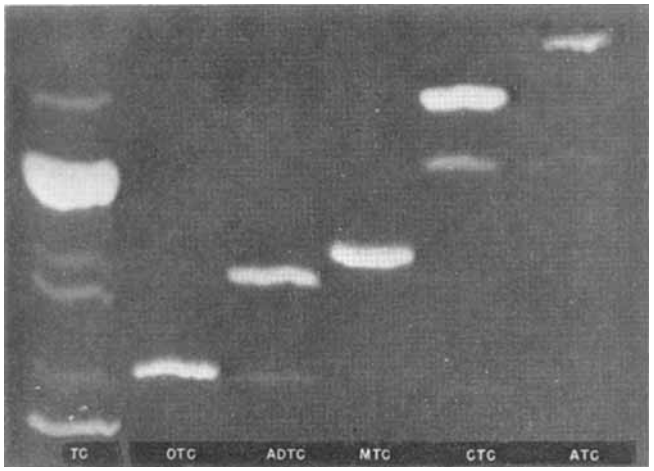


Figure 2—*Chromatogram of five possible impurities in commercial tetracycline (OTC, ADTC, MTC, CTC, and ATC are standard substances).*

vent was tried. After rejecting ethyl acetate and chloroform–acetone (1:1) and kieselguhr layers pH 3.7 (5), dichloromethane–ethanol 95% (9:1) was selected, in combination with kieselguhr impregnated with a buffer solution pH 5.5, containing 10% glycerin. In order to obtain reproducible values in the determination of epi-TC, the influence of the chemicals used and the influence of small variations in the technique were studied. It was found that the separation of the zones depended greatly on the amount of purification of the kieselguhr and on the condition of the layer before and during development (relation of relative humidity to temperature). Further, it was observed that the partial conversion of TC into epi-TC during the determination was strongly influenced by the amount of time between spotting and development and by the temperature of the methanolic TC solution, Working under the conditions described under *Procedures*, the epimerization is negligible and the chromatogram shows sharply defined zones of about 0.5 cm. width. Because the recoveries of epi-TC and TC are practically equal, TC is preferred as a standard substance for epi-TC determinations, being more stable than epi-TC.

As to CTC, the fluorimetric procedure is preferable because of its high sensitivity. Only very high percentages of epi-ATC (R_f 0.85) might quench the fluorescence.

In nearly all samples of TC, three yellow fluorescing zones between epi-TC and TC were observed (Fig. 1) After elution with 0.1 *N* HCl, maximum absorbances were found at about 355 mμ. Two-dimensional TLC using methyl ethyl ketone saturated with the buffer solution, pH 5.5, as second phase suggests that their identities might be oxytetracycline (R_f 0.17), 2-acetyl-2-decarboxamido-tetracycline (R_f 0.30), and methacycline (R_f 0.38) (Fig. 2). Keiner *et al.* (8) used this developing solvent, pH 4.7, to separate ADTC from epi-TC and TC. However, the authors did not succeed in a definite identification by IR, mainly due to the large quantity of eluted glycerin and to the decomposition of these three compounds during the isolation. They were determined quantitatively—calculated as epi-TC—to give an idea of the possible errors in epi-TC determination if no sharply defined zones can be obtained.

The methods for the determination of epi-TC and CTC are not limited to tetracycline substances but can also be performed in some TC formulations. For aqueous solutions and suspensions of TC, it is recommended in most cases to freeze-dry the preparation and to spot after dissolving in methanol.

Finally, this quantitative TLC method can also be applied to determine impurities in other tetracyclines like chlortetracycline, demethylchlortetracycline, and methacycline. In oxytetracycline studies, however, the solvent system used in development should be replaced by another one.

REFERENCES

(1) E. G. Remmers, G. M. Sieger, and A. P. Doerschuk, *J. Pharm. Sci.*, **52,** 752(1963).

(2) E. Addison and R. G. Clark, *J. Pharm. Pharmacol.*, **15,** 268 (1963).

(3) M. H. Woolford and F. S. Chicarelli, *J. Amer. Pharm. Ass., Sci. Ed.*, **45,** 400(1956).

(4) F. S. Chicarelli, P. van Gieson, and M. H. Woolford, *ibid.*, **45,** 418(1956).

(5) D. Sonanini and L. Anker, *Pharm. Acta Helv.*, **39,** 518(1964).

(6) P. P. Ascione, J. B. Zagar, and G. P. Chrekian, *J. Pharm. Sci.*, **56,** 1393(1967).

(7) I. C. Dijkhuis, *Pharm. Weekbl.*, **102,** 1308(1967).

(8) J. Keiner, R. Hüttenrauch, and W. Poethke, *Arch. Pharm.*, **300,** 840(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 31, 1968, from *The Municipal Pharmacy for the Hospitals of the Hague, The Hague, Netherlands.*

Accepted for publication October 30, 1969.

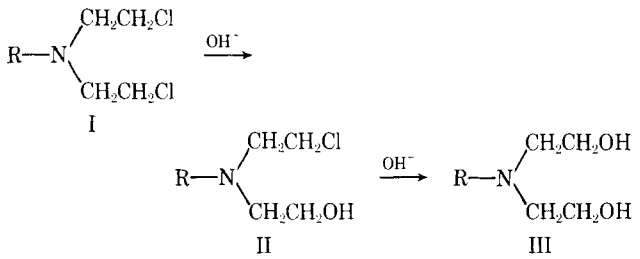
Gas-Liquid Chromatographic Determination of Melphalan

J. T. GORAS*, J. B. KNIGHT†, R. H. IWAMOTO†, and P. LIM†

Abstract □ The gas-liquid chromatography of melphalan and its decomposition products, determined as trimethylsilyl derivatives, is reported. The application of this procedure to the quantification of intact melphalan in a pharmaceutical preparation is described.

Keyphrases Melphalan and dosage forms—analysis □ GLC—analysis □ Chrysene—internal standard □ Mass spectroscopy—identification

The compound *p*-di(2-chloroethyl)amino-L-phenylalanine,[1] NSC 8806 (melphalan, L-sarcolysin, L-phenylalanine mustard), is a chemotherapeutic agent used in the treatment of various tumors (1–7). Because the drug is susceptible to hydrolytic degradation, an assay procedure that quantitatively measures intact melphalan in the presence of its hydrolysis products was desired. The hydrolytic degradation of melphalan (I) (8, 9) and related nitrogen mustards (10–12) has been reported as a replacement of one or both chlorine atoms by hydroxyl groups to give Forms II and III (see Scheme I).



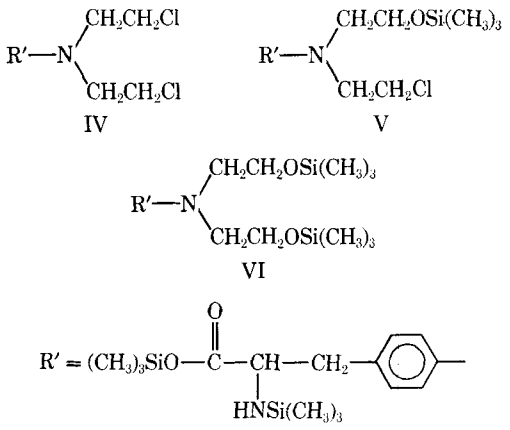
R = L-phenylalanine for melphalan

Scheme I

Quantitative procedures that have been applied to melphalan include direct UV spectrophotometry, ionic and total chlorine determination, spectrophotofluorimetry (8), and a colorimetric assay utilizing γ-(4-nitrobenzyl)pyridine (NBP) (12, 13). The first three procedures are inherently nonspecific in this application. The NBP assay has been employed by Friedman *et al.* (12) to study the hydrolysis of two simpler nitrogen mustards. However, it has been recently demonstrated that the NBP reagent is reactive with both Forms I and II of a mustard similar to melphalan and cannot be considered specific for the intact molecule (14).

Klebe *et al.* (15) have shown that, in the absence of moisture, many amino acids form stabile and volatile *N*- and *O*-bonded trimethylsilyl derivatives with bis(trimethylsilyl) acetamide (BSA) and may be assayed by GLC. Other silylating reagents have been used for quantitative preparations of trimethylsilyl derivatives of amino acids (16), but BSA was preferred for these experiments because of its higher reactivity as a silyl donor.

Quantitative conversion of amino acids to trimethylsilyl derivatives is accomplished simply by dissolving either the free amino acids or their hydrochlorides in an excess of BSA reagent. Degradation products II and III are capable of further reaction with BSA to form Compounds V and VI, respectively (see Scheme II).



Scheme II

Melphalan bulk, which served as a reference standard, was silylated with BSA and chromatographed as the trimethylsilyl derivative of the intact compound. A mixture of hydrolytic decomposition products was prepared, silylated, and chromatographed under the same conditions. Peaks having retention times of 1.1 and 1.3 relative to the intact melphalan derivative (IV) were recorded and identified as trimethylsilyl derivatives V and VI, respectively. The separation permitted the quantitative determination of intact melphalan in a freeze-dried dosage formulation containing mannitol. With the use of an internal standard, the assay is accurate as well as rapid and specific.

EXPERIMENTAL

Reagents and Materials—Melphalan bulk used in this study was provided by the Drug Distribution Unit of Cancer Chemotherapy National Service Center, National Institutes of Health. A sterile, freeze-dried intravenous dosage formulation of melphalan dihydrochloride was prepared from bulk material by this laboratory. Each vial contained 50 mg. of melphalan and 100 mg. of mannitol. A purified grade of BSA[2] in septum-stoppered vials was obtained for the experiment. A solution of chrysene[3] in chloroform (2.5 mg./ml.) was prepared fresh daily for use as an internal standard. The column packing employed for GLC was a laboratory preparation consisting of 2.5% (w/w) of a silicone polymer[4] on acid-washed,

[1] Manufactured as Alkeran by Burroughs Wellcome and Co.

[2] Pierce Chemical Co., Rockford, Ill.

[3] Aldrich Chemical Co., Milwaukee, Wis.

[4] SE-54, General Electric Co., Waterford, N. Y.

Table I—Mass Spectral Analysis of Fractions

Compound	Retention Time, min.	—Molecular Weight— Theory	Found
IV	12.6	448	447
V	15.2	502	502
VI	17.5	556	556

Table II—Retention Times

Compound	Relative Retention Time
IV	1.0
V	1.1
VI	1.3
Mannitol-TMS	0.13
Chrysene	0.66

silanized, 80/100 mesh diatomaceous earth.[5] All solvents were reagent grade quality and used as supplied.

Instrumentation—A gas chromatograph[6] equipped with a flame-ionization detector and an all-glass column (1.8 m. long, 3 mm. i.d.) was employed for quantitative work. Prepurified nitrogen was used as the carrier gas at a flow rate of 30 ml./min. The column oven was operated isothermally at 210°, the injector at 250°, and the detector at 215°. A similar gas chromatography system[7] was used to obtain fractions for mass spectrometry analysis. The mass spectral data were obtained from a modified mass spectrometer[8] with an upper limit of *m/e* 500 and with resolution capabilities of 1/1000.

Procedures—Derivatives were prepared for qualitative GLC analysis by reacting approximately 10 mg. of dry sample with 0.5 ml. of BSA. The reactions were carried out in 1-ml. septum-stoppered vials, and BSA was introduced with a hypodermic syringe to exclude atmospheric moisture. The reactions were complete in 15 min. at room temperature, and 3-μl. portions of the solutions were injected onto the column with a microliter syringe.[9] This procedure was followed using samples of melphalan bulk, melphalan freeze-dried dosage formulation, and hydrolytically degraded melphalan. The latter material was obtained by heating an aqueous solution of bulk melphalan at 40° and carefully monitoring the extent of the hydrolysis by measuring the amount of ionic chlorine liberated. When the concentration of ionic chlorine represented one-half of the total chlorine, the solution was cooled and lyophilized.

The quantification of intact melphalan in the freeze-dried dosage formulation was accomplished with the aid of an internal standard. Exactly 10.0 ml. of internal standard solution was added to the contents of each vial. The vials were fitted with septum-stoppered caps and 2.5 ml. of BSA was introduced with a hypodermic syringe. The vials were shaken and allowed to stand at room temperature for 1 hr. A syringe was used to withdraw 4-μl. portions of the solutions for injection onto the column. A standard solution was prepared for each series of samples by following the same procedure with an accurately weighed quantity of melphalan bulk approximately equivalent to that in the samples. The initial injection was used only to condition the column and the associated chromatogram was disregarded. Samples and standard solution were alternately chromatographed.

Calculations—Peak heights were measured from adjusted baselines and the peak height ratios of intact melphalan to chrysene were determined. The quantity of intact melphalan in the samples was determined by direct comparison of peak height ratios between samples and standard solution.

Identification of Derivatives—Samples of the trimethylsilyl derivatives were collected at the exit port of the gas chromatograph for identification. The collected fractions were protected from atmospheric moisture and immediately analyzed by mass spectrometry. The mass spectral data are summarized in Table I. In the case of the fraction representing the derivative of melphalan reference material, sufficient sample was isolated to allow recovery of the parent compound by hydrolysis of the trimethylsilyl groups. The resulting product, melphalan, was identified by IR spectroscopy and paper chromatography.

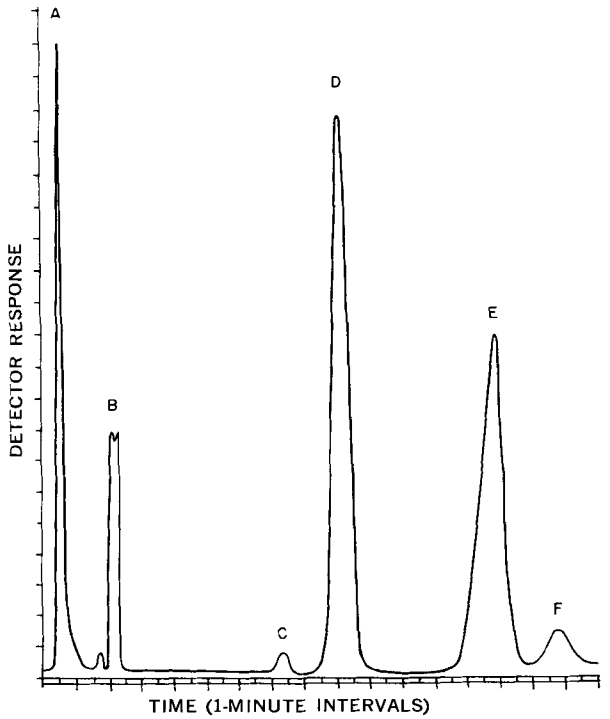


Figure 1—*Chromatogram of an assay preparation from freeze-dried dosage formulation: A = solvent, B = mannitol-TMS, C = impurity in chrysene, D = chrysene, E = melphalan-TMS (IV), F = decomposition product-TMS (V).*

RESULTS AND DISCUSSION

Qualitative Determinations—The silylated melphalan reference sample chromatographed as a single component. Three components, the first of which corresponded to the reference sample, were obtained when partially hydrolyzed material was silylated and chromatographed. Fragments common to *N,O*-bis(trimethylsilyl) derivatives of amino acids (17) and aryl amino acids (18) were prominent in the mass spectrum of each fraction. Molecular weights determined from the mass spectral data were in agreement with those calculated for IV, V, and VI. In addition, paper chromatographic and IR spectroscopic analyses established that the parent compound of the reference sample derivative was intact melphalan.

GLC analysis of silylated dosage formulation revealed the presence of V. Mannitol in the formulation eluted early as a silylated product and posed no problem. Relative retention times are given in Table II.

The same elution order was found when the derivatives were chromatographed using a relatively less polar liquid phase;[10] however, the order was reversed when a relatively more polar phase[11] was employed.

Quantification of Intact Melphalan in Dosage Formulation—A typical chromatogram for the assay of a freeze-dried dosage formulation preparation is shown in Fig. 1 (in this particular assay, Compound VI is not present). Chrysene was selected for the internal standard because it is nonreactive with BSA and no decomposition products elute at or near its retention time (0.66 relative to intact melphalan).

[5] Chromosorb W, Johns-Manville Corp., New York, N. Y.
[6] Perkin-Elmer model 801.
[7] Beckman model GC-5.
[8] Consolidated Electrodynamics Corp. model CEC-105.
[9] Hamilton Co., Whittier, Calif.
[10] SE-30, General Electric Co.
[11] OV-17, Applied Science Labs., Inc., State College, Pa.

The average of five replicate determinations for percent intact melphalan in a single vial of dosage formulation was 88.7% with a standard deviation of 1 (range = 2.5%).

Variations of less than 3% were found in the assay of several vials from the same production lot. The samples and reference solutions may be used for quantitative work up to 24 hr. after preparation.

REFERENCES

(1) F. Bergel and J. A. Stock, *J. Chem. Soc.*, **1954,** 2409.

(2) F. Bergel, *J. Pharm. Pharmacol.*, **7,** 297(1955).

(3) L. F. Larinov, A. S. Khoklov, E. N. Shodinskaja, O. S. Vasina, V. I. Troosheikina, and M. A. Navikova, *Lancet*, **269,** 169 (1955).

(4) L. I. Chebotareva, *Vop. Onkol.*, **2,** 323(1956).

(5) J. M. Luck, *Science*, **123,** 984(1956).

(6) H. Seliger, *Krebsarzt Z.*, **11,** 342(1956).

(7) J. F. Holland and W. Regelson, *Ann. N. Y. Acad. Sci.*, **68,** 1122(1958).

(8) M. A. Chirigos and J. A. Mead, *Anal. Biochem.*, **7,** 259 (1965).

(9) K. A. Stacey, M. Cobb, S. F. Cousens, and B. Alexander, *Ann. N. Y. Acad. Sci.*, **68,** 682(1958).

(10) P. D. Bartlett, S. D. Ross, and C. G. Swain, *J. Amer. Chem. Soc.*, **68,** 682(1958).

(11) C. Golumbic, J. S. Fruton, and M. Bergman, *J. Org. Chem.*, **11,** 518(1946).

(12) O. R. Friedman and E. Boger, *Anal. Chem.*, **33,** 906(1961).

(13) J. Epstein, R. W. Rosenthal, and R. J. Ess, *ibid.*, **27,** 1435 (1955).

(14) M. K. Balazs, C. A. Anderson, R. H. Iwamoto, and P. Lim, to be published.

(15) J. F. Klebe, H. Finkbeiner, and D. M. White, *J. Amer. Chem. Soc.*, **88,** 3390(1966).

(16) E. D. Smith and H. Sheppard, *Nature*, **208,** 878(1965).

(17) R. M. Teeter, presented at the Tenth Annual Conference on Mass Spectrometry and Allied Topics, New Orleans, La., June 1962.

(18) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," vol. II, Holden-Day, San Francisco, Calif., 1964, p. 187.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1969, from * *The John L. Smith Memorial for Cancer Research, Chas. Pfizer and Co., Inc., Maywood, NJ 07607,* and the † *Life Sciences Division, Stanford Research Institute, Palo Alto, CA 94025*

Accepted for publication September 5, 1969.

This investigation was supported by contracts PH 43-67-687 and PH 43-64-500 with Chemotherapy, National Cancer Institute, National Institutes of Health, Public Health Service. The opinions expressed in this paper are those of the authors and are not necessarily those of Chemotherapy.

The first author wishes to thank Mr. Frederick J. Carleton, Chas. Pfizer and Co., for allotting time for this project and providing administrative support.

Synthesis of 4-{*p*-[(2-Chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric Acid and Its Behavior in the 4-(4-Nitrobenzyl)pyridine Assay Procedure

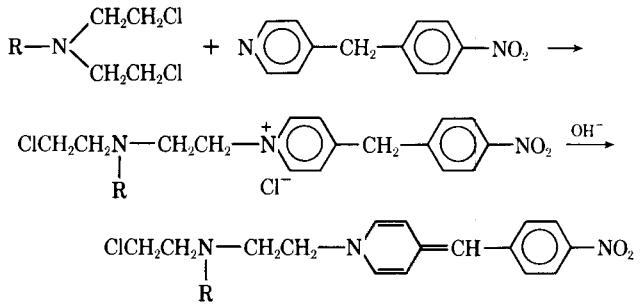
M. K. BALAZS*, C. A. ANDERSON, R. H. IWAMOTO, and P. LIM†

Abstract □ The synthesis of 4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)-amino]phenyl}butyric acid is reported. Investigation of the behavior of this compound in the NBP assay procedure showed that it alkylates NBP in a manner similar to chlorambucil. Therefore the results obtained from the assay of chlorambucil and similar aryl nitrogen mustards by the NBP method must be interpreted with due caution.

Keyphrases □ 4-{*p*-[(2-Chloroethyl)-(2-hydroxyethyl)amino]-phenyl}butyric acid—synthesis □ 4-(4-Nitrobenzyl)pyridine alkylation—4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}-butyric acid □ Chlorambucil analysis—4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric acid interference □ Colorimetric analysis—spectrophotometer □ TLC—separation, identification □ IR spectrophotometry—structure □ NMR spectroscopy—structure

The compound 4-(4-nitrobenzyl)pyridine (NBP) has been used as an analytical reagent for alkylating agents, among which are the nitrogen mustards such as melphalan,[1] chlorambucil,[2] and uracil mustard.[3] The general method involves the alkylation of NBP; subsequent basification results in the formation of a chromophore whose intensity can be measured photometrically.

The following reaction sequence has been suggested by Petering and Van Giessen (1) (Scheme I).



Scheme I

Holtzman (2) reportedly was able to isolate the product obtained from the reaction of mustard gas with NBP; he identified it as the mono-NBP product.

The NBP procedure has been considered in the authors' laboratories for the assay of chlorambucil. One

[1] 4-{*p*-[Bis(2-chloroethyl)amino]}phenyl-L-alanine.

[2] 4-{*p*-[Bis(2-chloroethyl)amino]}phenyl butyric acid was furnished by Cancer Chemotherapy National Service Center.

[3] 5-[Bis(2-chloroethyl)amino]uracil.

The average of five replicate determinations for percent intact melphalan in a single vial of dosage formulation was 88.7% with a standard deviation of 1 (range = 2.5%).

Variations of less than 3% were found in the assay of several vials from the same production lot. The samples and reference solutions may be used for quantitative work up to 24 hr. after preparation.

REFERENCES

(1) F. Bergel and J. A. Stock, *J. Chem. Soc.*, **1954,** 2409.

(2) F. Bergel, *J. Pharm. Pharmacol.*, **7,** 297(1955).

(3) L. F. Larinov, A. S. Khoklov, E. N. Shodinskaja, O. S. Vasina, V. I. Troosheikina, and M. A. Navikova, *Lancet*, **269,** 169 (1955).

(4) L. I. Chebotareva, *Vop. Onkol.*, **2,** 323(1956).

(5) J. M. Luck, *Science*, **123,** 984(1956).

(6) H. Seliger, *Krebsarzt Z.*, **11,** 342(1956).

(7) J. F. Holland and W. Regelson, *Ann. N. Y. Acad. Sci.*, **68,** 1122(1958).

(8) M. A. Chirigos and J. A. Mead, *Anal. Biochem.*, **7,** 259 (1965).

(9) K. A. Stacey, M. Cobb, S. F. Cousens, and B. Alexander, *Ann. N. Y. Acad. Sci.*, **68,** 682(1958).

(10) P. D. Bartlett, S. D. Ross, and C. G. Swain, *J. Amer. Chem. Soc.*, **68,** 682(1958).

(11) C. Golumbic, J. S. Fruton, and M. Bergman, *J. Org. Chem.*, **11,** 518(1946).

(12) O. R. Friedman and E. Boger, *Anal. Chem.*, **33,** 906(1961).

(13) J. Epstein, R. W. Rosenthal, and R. J. Ess, *ibid.*, **27,** 1435 (1955).

(14) M. K. Balazs, C. A. Anderson, R. H. Iwamoto, and P. Lim, to be published.

(15) J. F. Klebe, H. Finkbeiner, and D. M. White, *J. Amer. Chem. Soc.*, **88,** 3390(1966).

(16) E. D. Smith and H. Sheppard, *Nature*, **208,** 878(1965).

(17) R. M. Teeter, presented at the Tenth Annual Conference on Mass Spectrometry and Allied Topics, New Orleans, La., June 1962.

(18) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," vol. II, Holden-Day, San Francisco, Calif., 1964, p. 187.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1969, from * *The John L. Smith Memorial for Cancer Research, Chas. Pfizer and Co., Inc., Maywood, NJ 07607,* and the † *Life Sciences Division, Stanford Research Institute, Palo Alto, CA 94025*

Accepted for publication September 5, 1969.

This investigation was supported by contracts PH 43-67-687 and PH 43-64-500 with Chemotherapy, National Cancer Institute, National Institutes of Health, Public Health Service. The opinions expressed in this paper are those of the authors and are not necessarily those of Chemotherapy.

The first author wishes to thank Mr. Frederick J. Carleton, Chas. Pfizer and Co., for allotting time for this project and providing administrative support.

Synthesis of 4-{*p*-[(2-Chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric Acid and Its Behavior in the 4-(4-Nitrobenzyl)pyridine Assay Procedure

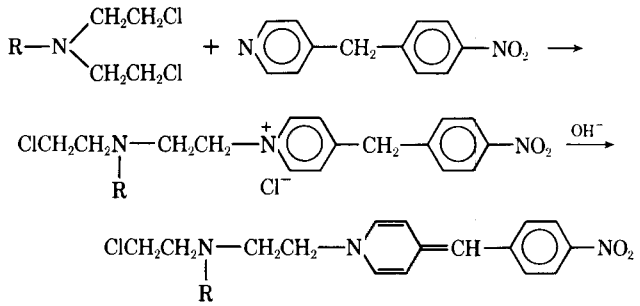
M. K. BALAZS*, C. A. ANDERSON, R. H. IWAMOTO, and P. LIM†

Abstract □ The synthesis of 4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)-amino]phenyl}butyric acid is reported. Investigation of the behavior of this compound in the NBP assay procedure showed that it alkylates NBP in a manner similar to chlorambucil. Therefore the results obtained from the assay of chlorambucil and similar aryl nitrogen mustards by the NBP method must be interpreted with due caution.

Keyphrases □ 4-{*p*-[(2-Chloroethyl)-(2-hydroxyethyl)amino]-phenyl}butyric acid—synthesis □ 4-(4-Nitrobenzyl)pyridine alkylation—4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}-butyric acid □ Chlorambucil analysis—4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric acid interference □ Colorimetric analysis—spectrophotometer □ TLC—separation, identification □ IR spectrophotometry—structure □ NMR spectroscopy—structure

The compound 4-(4-nitrobenzyl)pyridine (NBP) has been used as an analytical reagent for alkylating agents, among which are the nitrogen mustards such as melphalan,[1] chlorambucil,[2] and uracil mustard.[3] The general method involves the alkylation of NBP; subsequent basification results in the formation of a chromophore whose intensity can be measured photometrically.

The following reaction sequence has been suggested by Petering and Van Giessen (1) (Scheme I).



Scheme I

Holtzman (2) reportedly was able to isolate the product obtained from the reaction of mustard gas with NBP; he identified it as the mono-NBP product.

The NBP procedure has been considered in the authors' laboratories for the assay of chlorambucil. One

[1] 4-{*p*-[Bis(2-chloroethyl)amino]}phenyl-L-alanine.

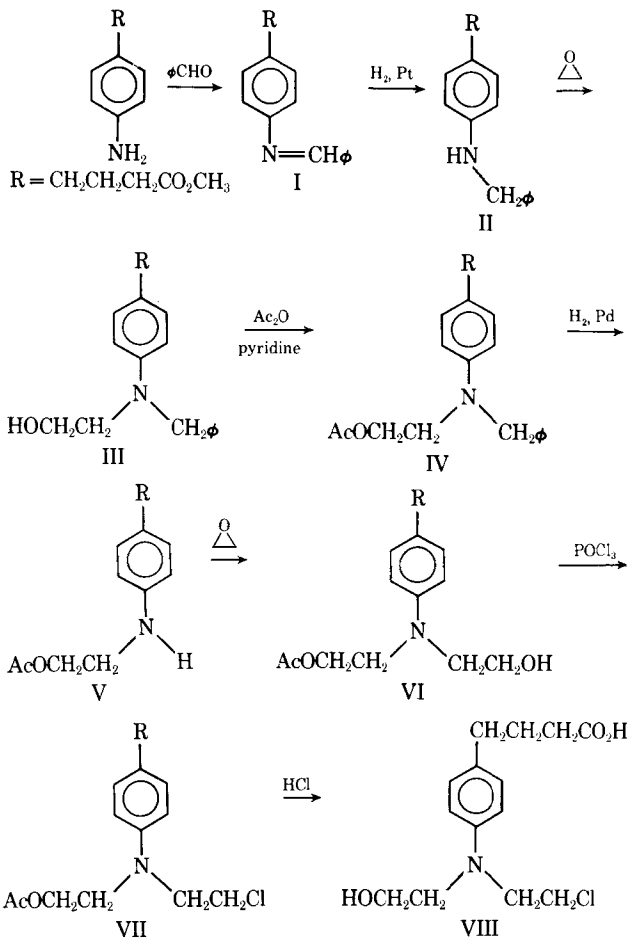
[2] 4-{*p*-[Bis(2-chloroethyl)amino]}phenyl butyric acid was furnished by Cancer Chemotherapy National Service Center.

[3] 5-[Bis(2-chloroethyl)amino]uracil.

possible contaminant is 4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric acid, the chlorambucil hemimustard, VIII. This can result from hydrolysis of chlorambucil or from incomplete chlorination during the preparation of the mustard. Since VIII is still an alkylating agent, it may react with NBP to yield a chromophore characteristically similar to that resulting from the reaction between NBP and chlorambucil. If this happens, the usefulness of the NBP assay of aryl bis-mustards may be questionable. To clarify this situation, the authors have prepared the chlorambucil hemimustard and studied its behavior in the NBP procedure.

RESULTS AND DISCUSSION

The synthesis of VIII was carried out as described in Scheme II; the details are given in the *Experimental* section.



Scheme II

Chlorambucil and its hemimustard were treated with NBP under identical, optimal conditions, which were experimentally determined by varying the substrate–NBP ratio and the period of heating. The results, expressed in molar absorptivities, are given in Tables I and II.

The data show that the hemimustard, VIII, does react with NBP to yield a product whose visible absorption characteristics are similar to those of the chlorambucil–NBP chromophore. Therefore, it is felt that the data obtained from the NBP assay procedure of aryl nitrogen mustards must be interpreted with due caution.

A comparison of the respective molar absorptivities indicates that the intensity of the hemimustard NBP product is only 0.8 that of the chlorambucil–NBP value. The hemimustard appears to reach its maximum color faster than does chlorambucil. At this time, no attempt has been made to seek an explanation for either of these observations because such information is beyond the scope of this communication.

Table I—Color Intensity Developed when the Ratio of Alkylating Compound to NBP was Varied

Moles Compd./ Moles NBP	$\epsilon \times 10^{-4}$, 563 mμ	
	Chlorambucil	Hemimustard, VIII
1:1.2	2.38	1.76
1:2.3	3.14	2.70
1:4.6	3.20	2.84
1:9.2	3.00	2.80

EXPERIMENTAL[4]

NBP Assay Procedure—Chlorambucil (1.2 mg.) or VIII (1.1 mg.) was dissolved in 25.0 ml. of 95% EtOH. A 1.0-ml. aliquot of this solution was placed in a 5.0-ml. volumetric flask together with 1.0 ml. of an NBP solution (3.55 g./50 ml. of acetone) and 1.0 ml. of buffer solution (1.0 g. of potassium hydrogen phthalate/100 ml. of H_2O).

The flask was kept at 85° in a water bath for 30 min. The solution was cooled immediately for 2–3 min. in an ice bath, and 0.1 ml. of a KOH solution (1.4 g./25 ml. of 95% EtOH) was added.[5] The solution was diluted to 5.0 ml. with 95% EtOH and shaken. After 2 min. the absorbance from 560–570 mμ was recorded. This procedure was used by varying the heating time to obtain the data tabulated in Table II. The molar ratios were varied by changing the concentration of NBP solutions so that the data in Table I could be obtained.

Materials—4-Nitrobenzylpyridine (NBP) (Aldrich Chemical Co.) was used without further purification.

The chlorambucil used was chromatographically homogeneous on Whatman No. 1 paper, solvent system *n*-BuOH–HOAc–H_2O, 5:2:3, and by GLC as its silyl derivative on a 1.52-m. (5-ft.) 5% SE-54-diatomaceous earth (Gas Chrom W, Applied Science Laboratories, Inc.) column at 210°. Elemental analysis for the chlorambucil sample was satisfactory.

Anal.—Calcd. for $C_{14}H_{19}Cl_2NO_2$: C, 55.27; H, 6.30; Cl, 23.31; N, 4.60. Found: C, 55.57; H, 6.25; Cl, 23.05; N, 4.60. The IR spectrum of this material was identical to that of an authentic sample. The NMR spectrum is consistent with that expected for the compound; 1.90 δ (m, 2H, $\underline{CH_2}$—CO_2H), 2.40 δ (m, 4H, φ—CH_2—CH_2—), 3.65 δ [s (broad), 8H, $ClCH_2\underline{CH_2}$—], 6.80 δ (q, 4H, aromatic), and 11.0 δ [s (broad), 1H, $CO_2\underline{H}$].

Methyl 4-[(*p*-Benzylidineamino)phenyl]butyrate (I)—A mixture of 6.15 g. (0.03 mole) of methyl 4-(*p*-aminophenyl)butyrate, 0.50 g. of fused potassium acetate, and 3.71 g. of benzaldehyde was refluxed in 50 ml. of absolute EtOH for 4 hr. The cooled solution was diluted with 75 ml. of H_2O and extracted with ether. The ether extracts were combined, dried ($MgSO_4$), filtered, and evaporated at reduced pressure (first at 20 mm. and then at 0.6 mm.) for 7 hr. The product, 8.45 g. (94%), was characterized by spectral and chromatographic evidence. The IR spectrum showed the expected absorption at 6.24 μ, indicative of a benzal imino system. The NMR spectrum, showing expected increase in aryl protons, was consistent with the proposed structure. TLC on silica gel in Solvent System 1 showed only a minor contaminant in addition to a main spot.

Methyl 4-[*p*-(*N*-Benzylamino)phenyl]butyrate (II)—A solution of 8.45 g. (0.03 mole) of crude benzylidene compound in 90 ml. of 95% EtOH was stirred in an atmosphere of hydrogen gas in the presence of 1.0 g. of 5% Pd-C catalyst for 85 min., during which time the theoretical amount of hydrogen was consumed. The catalyst was removed by filtration; the reaction mixture was concentrated to a tan oil, 7.56 g. (89%), which was of sufficient purity to use in the

[4] The IR spectra were recorded as neat liquid films, as mineral oil mulls, or as $CHCl_3$ solutions. The NMR spectra were taken as $CDCl_3$ solutions with internal TMS (δ = 0.0) using a Varian A60-A spectrometer. Where multiplets are involved the chemical shift is measured from TMS to the center of the multiplet. In TLC, Solvent System 1 is benzene–ether, 1:1, and System 2 is $CHCl_3$–MeOH, 2:1.

[5] The quantity of added base has been found to be quite critical, since the addition of insufficient or excess base in this experiment causes a decrease of the absorbance at 560–570 mμ.

Table II—Color Intensity Developed when the Time of the Alkylation Reaction was Varied[a]

Time, min.	$\epsilon \times 10^{-4}$, 563 mμ	
	Chlorambucil	Hemimustard, VIII
10	2.54	2.76
20	3.06	2.75
30	3.32	2.75
40	3.36	2.76
50	3.36	2.78

[a] The ratio of alkylating agent to NBP was 1:2.3.

next step. The IR spectrum showed an absorption at 2.90 μ attributed to N—H. The NMR spectrum exhibited to a two-proton singlet at 4.3 δ and a five-proton singlet at 7.3 δ that were assigned to the benzyl group.

SiO_2 TLC (Solvent System 1) of the reaction mixture shows two major spots. One of these is the desired *N*-benzyl compound, II, while the slower moving spot travels identically as methyl 4-(*p*-aminophenyl)butyrate. In smaller scale preparation, column chromatography on SiO_2 (Solvent System 1) was employed to remove the primary amine. In the subsequent hydroxyethylation reaction, it was found that the bis-hydroxyethylated compound, methyl 4-{*p*-[bis-hydroxyethyl)amino]phenyl}butyrate could be easily separated from the desired compound, III, by chromatography so that extensive purification at this reduction step is not necessary.

Methyl 4-{[*p*-(*N*-Benzyl)-(2-hydroxyethyl)amino]phenyl}butyrate (III)—A 100-ml. round-bottom flask containing 7.6 g. (0.028 mole) of the *N*-benzyl compound, II, 40 ml. of 50% HOAc, and 7.7 ml. of ethylene oxide at 0° was stoppered and kept at room temperature for 16 hr. The reaction mixture was then poured into 75 ml. of H_2O and neutralized with solid $NaHCO_3$. This solution was extracted with several portions of EtOAc. The combined EtOAc extracts were washed thoroughly with H_2O, dried ($MgSO_4$), filtered, and evaporated to a brown oil (7.89 g.).

The crude hydroxyethyl compound was purified by chromatography on a 40 × 2.2-cm. SiO_2 column. The column was developed first with φH-Et_2O, 9:1, followed by φH-Et_2O, 4:1, which eluted 5.22 g. (66%) of Compound III as an oil. The IR spectrum exhibited the expected —OH bands at 2.9 and 9.55 μ. The NMR spectrum exhibited, in addition to those resonances observed for Compound II, a four-proton multiplet centered at 3.65 δ which was assigned to the methylene groups of the newly added 2-hydroxyethyl moiety.

Methyl 4-{*p*-[(*N*-Benzyl)-(2-acetoxyethyl)amino]phenyl}butyrate (IV)—A solution of 5.22 g. (0.016 mole) of the hydroxyethyl Compound III, 15 ml. of dry pyridine, and 15 ml. of acetic anhydride was kept overnight in a 100-ml. round-bottom flask. The reaction mixture was poured into 100 ml. of H_2O, neutralized with $NaHCO_3$, and extracted with EtOAc. The combined EtOAc extracts were washed with *N* HCl (until the aqueous wash remained at pH 1), H_2O, saturated $NaHCO_3$, and again with H_2O. The EtOAc solution was dried ($MgSO_4$), filtered, and evaporated to an oil, 4.97 g. (84%). The material was homogeneous on SiO_2 TLC (Solvent System 1) and its IR showed the typical acetoxy band at 8.10 μ. The NMR spectrum exhibited a three-proton singlet at 1.99 δ which was assigned to the acyl methyl group. In addition, there was a two-proton triplet at 4.25 δ (J = 6 c.p.s.) that was assigned to the AcO—$\underline{CH_2}$— protons.

Methyl 4-{[*p*-(2-Acetoxyethyl)amino]phenyl}butyrate (V)—A solution of 4.95 g. (0.013 mole) of the *N*-benzyl-acetoxyethylamino Compound IV in 35 ml. of 95% EtOH was stirred with 0.56 g. of 5% Pd–C catalyst in the presence of hydrogen gas at atmospheric pressure until sligh ly more than the theoretical amount of gas was taken up (16 hr.). The catalyst was removed by filtration and the ethanol was evaporated under reduced pressure to yield 3.49 g. (93%) of an oil which was homogeneous on SiO_2 TLC (Solvent System 1). The NMR spectrum showed that the benzyl group was removed since the two singlets attributed to the protons of the benzyl group were absent in the spectrum. The IR spectrum showed the expected N—H band at 2.90 μ.

Methyl 4-{*p*-[(2-Hydroxyethyl)-(2-acetoxyethyl)amino]phenyl}butyrate (VI)—Hydroxyethylation of V with ethylene oxide was carried out in a manner similar to that used for the preparation of III. A 2.69-g. (0.009-mole) portion of V gave 2.18 g. (70%) of an oil whose NMR spectrum was consistent with that expected for the desired compound. The additional methylene groups of the hydroxyethyl group were observed as a nine-proton multiplet at 3.6 δ (the integration includes the three protons of the O—CH_3 singlet). At 4.2 δ a two-proton triplet was assigned to the methylene group adjacent to the acetoxy moiety. The IR spectrum showed absorptions at 5.70 and 5.80 μ, the former being assigned to the acetate, and at 2.90 and 9.55 μ, both of which were assigned to the OH.

On SiO_2 TLC (Solvent System 1) the compound was nearly homogeneous and showed three minor contaminants in addition to a large major spot.

Methyl 4-{*p*-[(2-Chloroethyl)-(2-acetoxyethyl)amino]phenyl}butyrate (VII)—Freshly distilled $POCl_3$ (20 ml.) and 2.61 g. (8.0 mmoles) of the hydroxyethyl compound, (VI), were heated at reflux for 1 hr. and then poured into 200 ml. of crushed ice. The mixture was stirred for 20 min., with ice being added periodically, and then extracted with several portions of chloroform. The combined $CHCl_3$ extracts were dried ($MgSO_4$), filtered, and evaporated to give 2.45 g. (88%) of a yellow oil. The oil was essentially homogeneous on silica gel TLC in Solvent System 1. The NMR spectrum exhibited the following resonances: 2.00 δ (s, 3H, $C\underline{CH_3}$); 3.62 δ (m, 6H, —$\underline{CH_2}$—$\underline{CH_2}$— and —$\underline{CH_2}$—CH_2 of *N* substituents); 3.67 δ (s, 3H, O—$\underline{CH_3}$); 4.21 δ (t, J = 6 c.p.s., assigned to $\underline{CH_2}$ adjacent to Cl or OAc); and 6.85 δ (q, 4H, J = 8.5 c.p.s., aromatic). The IR spectrum (neat) showed no absorption at 2.90 μ and an absorption at 13.3 μ which was assigned to C—Cl. The crude material was purified by preparative silica gel plates (Solvent System 1) to yield 1.29 g. (68%) of an analytically pure oil.

Anal.—Calcd. for $C_{17}H_{24}ClNO_4$: C, 59.73; H, 7.08; N, 4.10. Found: C, 59.76; H, 7.23; N, 4.15.

A smaller scale reaction, carried out prior to the above experiment, gave material that had identical IR, NMR, and TLC to the material prepared above. A satisfactory chlorine analysis was obtained.

Anal.—Calcd.: 10.37. Found: 10.3 for this material.

4-{*p*-[(2-Chloroethyl)-(hydroxyethyl)amino]phenyl}butyric Acid "Hemimustard" (VIII)—The hydrolysis of the blocking groups was performed in refluxing HCl. A solution of 1.405 g. (4.1 mmoles) of Compound VII and 3.0 ml. of concentrated HCl was heated at reflux for 4 hr. The reaction mixture was cooled, diluted with H_2O, buffered at pH 5 by the addition of NaOAc, and extracted with several portions of CH_2Cl_2. The CH_2Cl_2 extracts were combined, dried ($MgSO_4$), filtered, and concentrated to a colorless oil, 1.20 g. (84%).

A portion of the crude material was dissolved in Et_2O–petroleum ether (b. p. 30–60°). After 3 days in the cold, there was deposited crystalline VIII; recrystallization from the same solvent yielded an analytically pure product, m.p. 51–53° (uncorrected). The material was homogeneous on silica gel TLC (Solvent System 2).

Anal.—Calcd. for $C_{14}H_{20}ClNO_3$: C, 58.84; H, 7.05; Cl, 12.40; Cl^-, 0.0; N, 4.90. Found: C, 58.72; H, 6.93; Cl, 12.54; Cl^-, 0.0, N, 4.86.

REFERENCES

(1) H. G. Petering and G. J. Van Giessen, *J. Pharm. Sci.*, **52**, 1159(1963).

(2) G. Holtzman, Office of Scientific Research and Development, OSTD 4288, Oct. 27, 1944, unpublished data.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1969, from *Stanford Research Institute, Menlo Park, CA 94025*

Accepted for publication December 2, 1969.

This work was carried out under the auspices of Chemotherapy, National Cancer Institute, National Institutes of Health, Public Health Service, Contract No. PH-43-64-500. The opinions expressed in this paper are those of the authors and not necessarily those of the Cancer ChemotherapyNational Service Center.

The authors are indebted to the Cancer Chemotherapy National Service Center for furnishing chlorambucil and to Dr. L. Goodman for his encouragement and stimulating discussions.

* Present address: Applied Materials Technology, Inc., 2999 San Ysidro Way, Santa Clara, Calif.

† To whom inquiries should be directed.

Riboflavin Distribution and Elimination in Two Functionally Anephric Human Patients

WILLIAM J. JUSKO*, JACK R. LEONARDS†, and GERHARD LEVY*‡

Abstract □ Pharmacokinetic studies in normal human subjects have suggested that probenecid modifies appreciably the distribution in the body of riboflavin and certain drugs. To exclude the effects of probenecid on renal excretion, the distribution and elimination of riboflavin with and without probenecid were studied in two functionally anephric patients during hemodialysis. A pharmacokinetic analysis of the time course of plasma levels of the vitamin, based on a two-compartment open model, indicates no appreciable effect of probenecid on the distribution and transfer rate constants of riboflavin. Riboflavin was eliminated more slowly by hemodialysis of the patients than by renal excretion in normal subjects. The rate of removal of riboflavin by hemodialysis appears to be primarily a function of the rate of blood flow through the dialyzer.

Keyphrases □ Riboflavin distribution, elimination—anephric patients □ Anephric condition—riboflavin distribution, elimination □ Probenecid effect—riboflavin distribution, elimination □ Hemodialysis—riboflavin elimination

The possible effect of probenecid on the distribution of various compounds in the body has been of recent interest. Gibaldi *et al.* (1, 2) have shown in studies with penicillins that, in addition to the well-known inhibitory effect on the renal excretion of this antibiotic, probenecid also seems to increase significantly the fraction of drug in the apparent body compartment from which elimination occurs. The results of recent studies in this laboratory suggest that probenecid affects also the distribution of riboflavin in the body (3). Since riboflavin is eliminated primarily by renal excretion, it was of interest to pursue further studies in functionally anephric patients in order to exclude the primary renal effect of probenecid. These studies were carried out during the course of hemodialysis of the anephric patients and may also be of nutritional interest since it has been suggested that vitamin deficiencies could develop due to the removal of water-soluble vitamins by dialysis (4).

EXPERIMENTAL

Two functionally anephric[1] male human patients, who were undergoing hemodialysis, served as test subjects. Subject S, age 30, with chronic membranous glomerulonephritis, and Subject K, age 34, with collagen disease, were maintained on a Kiil and a Skeggs-Leonards dialyzer, respectively. Both systems involved dialysate flow of approximately 580 ml./min. and blood flow of about 270 ml./min. Hematocrit values of each subject ranged from 0.22 to 0.24, and the plasma albumin concentration was about 3.2 g. % in each subject.

An intravenous dose of 38.9 mg. riboflavin as riboflavin-5′-phosphate[2] was administered 3 to 5 hr. after the start of hemodialysis. One gram of probenecid[3] in aqueous suspension was given orally, in crossover fashion, 1.5 to 2.0 hr. prior to riboflavin. Blood samples were collected proximal to the arterial shunt 0.5 hr. before and at 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 hr. after injection of riboflavin. Studies in each patient were separated by a 7-day interval.

[1] Less than 3% of normal renal function.
[2] Hyrye Injection, S.F. Durst Co., Philadelphia, Pa.
[3] Benemid, Merck Sharp and Dohme, West Point, Pa.

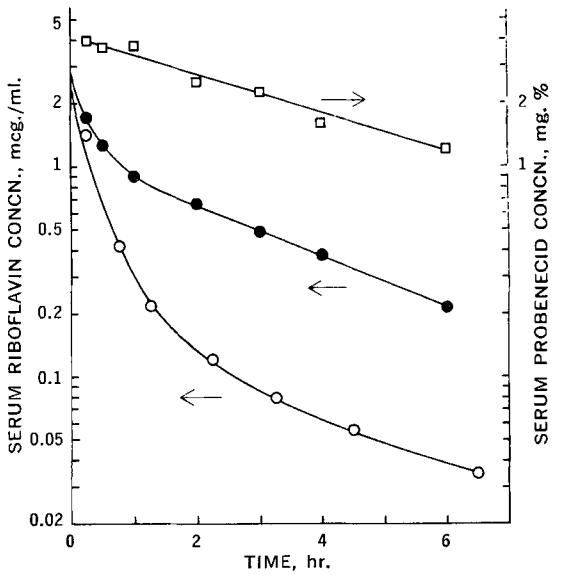


Figure 1—*Time course of total riboflavin (●) plasma concentrations in an anephric subject (S) after intravenous injection of 38.9 mg. riboflavin as riboflavin-5′-phosphate (FMN). Also shown for comparative purposes are riboflavin serum levels in a normal subject (○), 85 kg., who received an i.v. dose of 31 mg. riboflavin as FMN; and (□), probenecid plasma concentrations in the anephric patient after oral administration of 1 g. probenecid at −2 hr.*

Total riboflavin (5) and probenecid (6) concentrations in plasma were determined by methods described previously. No mutual interference in the analyses of the two compounds was found (3). Plasma concentrations were corrected for blank values of samples obtained prior to the administration of either drug.

RESULTS

The time course of total riboflavin and probenecid plasma concentrations in Subject S are shown in Fig. 1. Also depicted for purposes of comparison are riboflavin plasma concentrations obtained in a normal subject. The plasma concentrations of riboflavin (C_p) as a function of time (t) in the anephric subjects were fitted to a biexponential equation:

$$C_p = A \cdot \exp(-\alpha t) + B \cdot \exp(-\beta t) \qquad \text{(Eq. 1)}$$

using the "NLIN" digital computer program of Marquardt (7). The distribution (k_{12}, k_{21}) and elimination (k_{el}) rate constants of the typical two-compartment open model (elimination occurring from the central compartment) were obtained from the parameters A, α, B, and β as described by Rescigno and Segre (8). The apparent volume of the central compartment (V_c) was calculated from:

$$V_c = \text{dose}/(A + B) \qquad \text{(Eq. 2)}$$

and an additional "volume" parameter, $(V_d)_\beta$, was determined from:

$$(V_d)_\beta = V_c \cdot k_{el}/\beta \qquad \text{(Eq. 3)}$$

as described by Gibaldi *et al.* (9). The body clearance (Cl_b) of the vitamin was calculated from:

$$Cl_b = k_{el} \cdot V_c \qquad \text{(Eq. 4)}$$

All of the parameters and rate constants described, which were obtained from riboflavin plasma levels in the presence and

Table I—Pharmacokinetic Parameters[a] of Riboflavin Distribution and Elimination in Anephric Subjects in the Presence and Absence of Probenecid

	Subject[b]			
	S		K	
	Probenecid,[c] g.			
	0	1	0	1
Parameter				
A, mcg./ml.	1.52	1.88	1.38	1.23
B, mcg./ml.	1.13	0.971	0.740	0.757
α, hr.$^{-1}$	3.39	4.81	2.92	2.34
β, hr.$^{-1}$	0.275	0.211	0.0534	0.142
CD[d]	0.9999	0.9997	0.9988	0.9990
k_{12}, hr.$^{-1}$	1.48	2.67	1.77	1.16
k_{21}, hr.$^{-1}$	1.60	1.78	1.05	0.978
k_{el}, hr.$^{-1}$	0.581	0.571	0.148	0.339
V_c, l.	14.7	13.6	18.3	19.5
V_c, percent of body weight	26.1	24.2	25.1	26.7
V_d-β, l.	31.1	36.7	50.8	46.5
V_d-β, percent of body weight	55.5	65.5	69.6	63.7
Cl_b, ml./min.	142	129	45	110

[a] Symbols are defined in the text. [b] Body weights: S, 56 kg.; K, 73 kg. [c] Plasma concentration range of probenecid: Subject S: 2.47 to 0.95 mg. %; Subject K: 3.96 to 1.22 mg. %. [d] Coefficient of determination: $(\Sigma obs.^2 - \Sigma dev.^2)/\Sigma obs.^2$

absence of probenecid, are listed in Table I. The coefficient of determination (CD) for each of the four sets of data is close to unity, indicative of the excellent fit of the experimental data to the curve calculated from Eq. 1.

It was also of interest to determine if blood flow through the dialyzer or diffusion of riboflavin across the dialysis membrane may be rate limiting in the elimination of the vitamin by hemodialysis. The effective plasma flow rate (Q_E) through the dialyzer can be calculated from the relationship:

$$Q_E = Q \cdot (1 - \text{HCT}) \cdot F_f \qquad \text{(Eq. 5)}$$

where Q is the actual blood flow rate (about 270 ml./min.), HCT is the hematocrit (0.23), and F_f is the fraction of nonprotein-bound vitamin in the plasma. The value of the latter was calculated to be about 0.5 based on the actual albumin concentration in the plasma (3.2 g. %) and the previously determined (10) association constants of the vitamin with human albumin. The effective plasma flow rate thus estimated from Eq. 5 is about 110 ml./min., which is quite similar to the body clearance values observed in three of the four studies in the anephric patients (Table I). It was not possible to determine dialysance directly due to the very rapid rate of flow of the dialysis fluid (580 ml./min.) which resulted in such extensive dilution of riboflavin, making its determination in the dialysis fluid impossible.

DISCUSSION

The distribution parameters V_c and $(V_d)_\beta$ are very similar in all four tests when expressed as a percent of body weight (Table I). Probenecid, therefore, had no apparent effect on the distribution of riboflavin in the body under the experimental conditions. The rate constants k_{12} and k_{21} were somewhat more variable but also showed no consistent differences as a function of probenecid administration. It may be concluded, therefore, that the apparent effect of probenecid on riboflavin distribution in a normal subject (3) is probably an indirect result of its effect on the renal excretion of the vitamin.

The distribution parameter V_c ranged from 24.2 to 26.7% of body weight in the present study which is appreciably larger than the 16% value reported previously for a normal subject (3). This difference is consistent with the low hematocrit and low plasma albumin concentration in the anephric patients.

The similarity of three of the four body clearance (Cl_b) values to the calculated effective plasma flow rate through the dialysis system suggests that blood flow through the dialyzer is either the rate-limiting step or at least one of the rate-determining processes in the elimination of riboflavin by hemodialysis. This assumes that metabolism and extrarenal excretion (other than hemodialysis) contribute little to the elimination of the vitamin in anephric patients as is the case in normal subjects (1, 3).

Renal clearance of riboflavin in normal subjects involves glomerular filtration, renal tubular secretion, and renal tubular reabsorption by a specialized saturable process (11). Clearance is, therefore, concentration dependent, ranging from as high as 400 ml./min. at high plasma concentrations of riboflavin to 150 ml./min. or less at low plasma concentrations in normal adults (11). On the other hand, body clearance of riboflavin is not concentration dependent in functionally anephric patients due to the absence of a renal reabsorption process. A direct comparison of renal clearance in normal subjects with body clearance in hemodialyzed patients is therefore impossible.

The pharmacokinetics of riboflavin elimination in anephric patients and normal subjects differ also in that the time course of riboflavin concentration decline in the plasma of the patients is describable by a biexponential expression while a triexponential expression is required for normal subjects (11). This appears to be due to the absence of the renal reabsorption capability in the patients. A similar effect has been found in newborn infants whose renal mechanisms are immature (12).

It is of interest that probenecid, which is eliminated in normal subjects almost exclusively by biotransformation and which because of its high affinity to plasma proteins (6) is not likely to be removed effectively by hemodialysis, was as rapidly eliminated in the two patients (half-life of 2 to 4 hr.) as in normal subjects at a comparable dosage level (3, 6). On the other hand, drugs that are eliminated solely or primarily by renal excretion have a much longer half-life in anephric patients than in normal subjects (13, 14).

REFERENCES

(1) M. Gibaldi and M. A. Schwartz, *Clin. Pharmacol. Ther.*, **9**, 345(1968).

(2) M. Gibaldi, D. Davidson, M. E. Plaut, and M. A. Schwartz, to be published.

(3) W. J. Jusko, G. Levy, S. J. Yaffe, and R. Gorodischer, to be published.

(4) N. Lasker, A. Harvey, and H. Baker, *Trans. Amer. Soc. Artif. Intern. Organs*, **9**, 51(1963).

(5) H. B. Burch, O. A. Bessey, and O. H. Lowry, *J. Biol. Chem.*, **175**, 457(1948).

(6) P. G. Dayton, T. F. Yu, W. Chen, L. Berger, L. A. West, and A. B. Gutman, *J. Pharmacol. Exp. Ther.*, **140**, 278(1963).

(7) D. W. Marquardt, DPE-NLIN, Share General Library Program No. 7-1354 (1964).

(8) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Toronto, Ontario, Canada, 1966, p. 20.

(9) M. Gibaldi, R. Nagashima, and G. Levy, *J. Pharm. Sci.*, **58**, 193(1969).

(10) W. J. Jusko and G. Levy, *ibid.*, **58**, 58(1969).

(11) W. J. Jusko and G. Levy, to be published.

(12) W. J. Jusko, N. Khanna, G. Levy, L. Stern, and S. J. Yaffe, to be published.

(13) A. L. Linton, D. H. Lawson, I. MacVarish, and J. S. Eakin, *Proc. Eur. Dialysis Ren. Transplant. Ass.*, **5**, 153(1968).

(14) B. T. Williams, P. Dawson-Edwards, D. D. Hilton, K. M. Simpson, and H. J. Black, *ibid.*, **5**, 158(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 29, 1969, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214 and †Department of Biochemistry, Case-Western Reserve University, Cleveland, Ohio.

Accepted for publication October 21, 1969.

The authors are indebted to Dr. William Cumming for medical supervision, to Mr. Vernon Rudder for technical assistance, and to the patient-volunteers for their willing cooperation.

‡ To whom requests for reprints should be directed.

Improved Procedure for Determination of Microamounts of Sulfamethazine and Procaine Penicillin Admixed with Chlortetracycline and *p*-Arsanilic Acid in Medicated Feeds

MURUGAN MALAIYANDI and JEAN P. BARRETTE

Abstract □ An improved technique is presented for the separation and determination of both sulfamethazine and procaine penicillin in medicated feeds. This method utilizes diazotization of the free amino compounds with nitrite and coupling with the Bratton-Marshall reagent to produce a soluble red dye. The absorbance of this dye at 545 mμ is measured. Results on six laboratory-mixed medicated feeds are reported.

Keyphrases □ Sulfamethazine—analysis, medicated feeds □ Procaine penicillin—analysis, medicated feeds □ TLC—separation □ Bratton-Marshall reaction—colorimetric analysis □ UV spectrophotometry—analysis

Sulfamethazine and procaine penicillin in combination with other drugs, such as chlortetracycline, tylosine, and *p*-arsanilic acid, are admixed in swine and cattle feeds for the prevention of infection associated with hemorrhagic septicemia, swine dysentery, bacterial scours in calves (1) and coccidiosis in chickens (2). Gerriets (3) observed that chickens fed with sulfamethazine-incorporated, riboflavin-deficient fodder developed typical clinical riboflavin avitaminosis, and he concluded that in sulfamethazine therapy both riboflavin and vitamin K_3 should be supplemented in the feed. It has been shown that the excretion of sulfamethazine is not complete and the concentration of the sulfa drug in bovine milk (4, 5) and plasma proteins (6) can be relatively high. Borsnk (7) noted that injection of sulfamethazine in hen eggs affected embryonic development, resulting in poor hatchability, several growth disturbances, and prenatal malformations. Because of these and other harmful side effects recorded in the literature, it has become necessary for the regulatory agencies to develop reliable analytical methods for estimating these drugs in feeds.

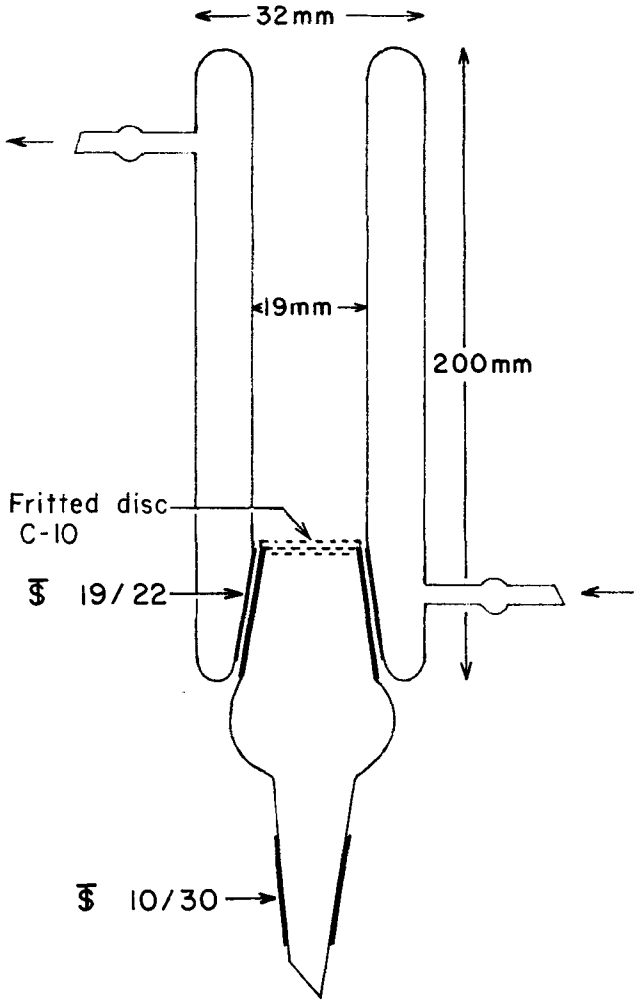


Figure 1—*Extraction apparatus.*

Several reports have appeared in the literature concerning the separation and determination of sulfamethazine and procaine penicillin in combination with other drugs, and these methods have been summarized in a previous communication (8). The present paper describes an improvement over the procedure outlined in the earlier method (8) for the estimation of sulfamethazine and procaine penicillin, and their extraction from the adsorbent alumina is accomplished with considerable saving of time. By this procedure, six laboratory-prepared samples containing varying amounts of *p*-arsanilic acid, chlortetracycline, sulfamethazine, and procaine penicillin have been extracted and the components separated cleanly from one another on thin-layer alumina plates and estimated spectrophotometrically.

EXPERIMENTAL

Apparatus—The extraction apparatus for eluting the drugs from thin-layer alumina is illustrated in Fig. 1. The alumina chromatoplates were prepared as previously described (9).

Reagents—An analytical sample of sulfamethazine (m.p. 198–198.5°) was obtained by crystallizing a technical sample[1] twice from a mixture of methanol–petroleum ether (b.p. 66–68°) followed by a final recrystallization from benzene–petroleum ether (b.p. 66–68°) mixture. The sample was shown to be identical to an authentic sample by determination of mixed melting point, elemental analysis, R_f values, and IR and NMR spectra. From the UV absorption spectrum, the sample was found to be 99.8% pure.

Procaine Penicillin[2]—Analytical standard was used without further purification. All organic solvents were glass-distilled. The alumina[3] for thin-layer chromatography was processed according to the method of Malaiyandi *et al.* (9) with plates activated at 120°

[1] American Cyanamid Co.
[2] Chas. Pfizer Co.
[3] Camag alumina supplied by Mondray Co. Ltd., Montreal, Quebec, Canada.

instead of 145°. Aqueous hydrochloric acid, 6 *N*; aqueous sodium nitrite solution, 1.0%; aqueous ammonium sulfamate solution, 5.0%; Bratton-Marshall reagent (aqueous *N*-(1-naphthyl)ethylenediamine dihydrochloride), 0.1%. The last three reagents were all freshly prepared each week and stored in a refrigerator (5°).

PROCEDURE

Standard and Recovery Curves for Procaine Penicillin and Sulfamethazine—*Standard Curve*—Ten aliquots (in duplicate) containing known amounts of different concentrations of procaine penicillin and sulfamethazine in ethanol were transferred to separate 50-ml. volumetric flasks. To these flasks was added 3.0 ml. of 6 *N* HCl followed by 15 ml. of distilled water. The flasks were heated on a water bath at 70–80° for 15 min. and the ethanol was evaporated off with a gentle stream of nitrogen (about 45 min.). The solutions were diluted with 10 ml. water and estimated by the procedure described for the preparation of standard curve for *p*-arsanilic acid (9).

Recovery Curve—A concentrated ethanolic solution containing known amounts of a mixture of procaine penicillin and sulfamethazine was prepared. Eight aliquots (in duplicate) containing amounts varying from 10 to 200 mcg. of drugs were applied to thin-layer alumina plates (0.25–0.30 mm. thick) along with a clearly visible reference spot at one side of the plate. The plates were developed as described in the earlier method (8).[4]

The areas[5] containing the drugs were separately scraped off the plate, and the adhering alumina was swept with a camel hair brush onto glazed paper. The alumina was transferred to the hot water-circulated extraction apparatus over a bed of prewashed methanol-moistened diatomaceous earth (Celite 545, about 1.5–2.0 cm. high). The drug was eluted with 175 ml. of hot 2.0% methanolic hydrochloric acid. The eluate was evaporated using a rotary vacuum evaporator (bath temperature below 37°) to a volume of about 15 ml. The concentrate was quantitatively transferred to a 50-ml. volumetric flask with three rinsings of about 5-ml. portions of aqueous methanol. The flask was heated on a water bath at 70–80° and the methanol evaporated with a gentle stream of nitrogen. When the evaporation of methanol was complete, the contents of the flask were cooled, diazotized, and coupled with the Bratton-Marshall reagent as described previously. After 30 min. the solutions were diluted to volume, shaken thoroughly, and centrifuged. The absorbance was then measured and plotted to obtain the recovery curve.

Preparation of Laboratory Premixes—Six laboratory-blended samples of medicated feed containing *p*-arsanilic acid, chlortetracycline, procaine penicillin, and sulfamethazine at levels ranging from 120 to 1250 mcg./g. of feed were prepared by grinding 50-g. portions of unmedicated feed with varying amounts of the four drugs in a Sorvall-Omni mixer. After mixing for about 5 min., the mixture was tumbled on a Fisher-Kendall mixer for about 15 min. These operations were repeated twice and the sample was carefully transferred to a container which was then tumbled for 1 hr. to achieve thorough mixing.

Extraction of Drugs and Their Estimation—Two 1.0-g. portions (a_1 and a_1') and one 5.0-g. portion (a_5) of the medicated feed were extracted with 50–55 ml. of a solvent mixture containing ammonium hydroxide–dimethylformamide–chloroform–ethanol (5:5:20:20). (In the case of a_5, 200 ml. of the same solvent mixture was used.) After refluxing for about 8 hr. the warm extracts were filtered through a bed of diatomaceous earth and concentrated under reduced pressure to about 5 ml. (20 ml. in the case of a_5). The concentrates were then quantitatively transferred to 10-ml. (50 ml. in the case of a_5) volumetric flasks with several rinsings with absolute ethanol and made to volume. Aliquots (4 × 250 μl.) of this extract were applied to a thin-layer alumina plate and developed, and the drugs were extracted and estimated as described in the previous section.

[4] If the humidity of the laboratory is very high, use of a 60:30:8:2 solvent mixture is recommended. When fresh dimethylamine solution is available, a 60:30:5:5 solvent composition is used.

[5] Along with the solvent front, a fluorescent lipid band moved. This lipid band should be excluded to avoid intractable turbidity during centrifugation.

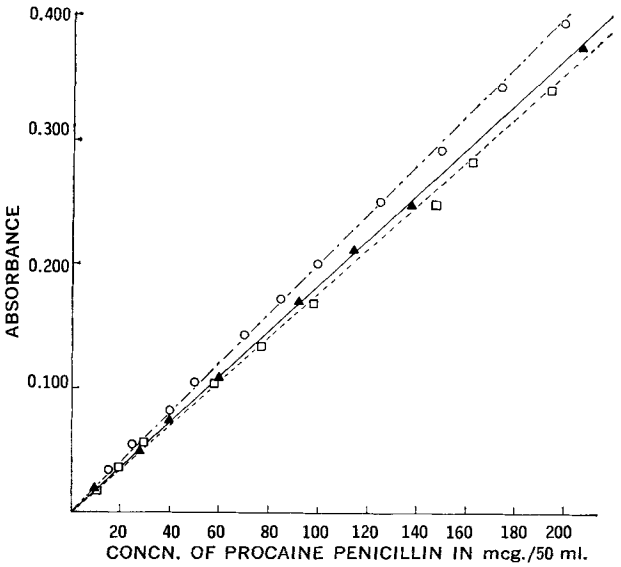


Figure 2—*Standard and recovery curves for procaine penicillin. Key:* ○, *standard curve;* □, *recovery curve by previous method; and* ▲, *recovery curve by present method.*

RESULTS AND DISCUSSION

The need for processed alumina and the choice of range of pH (7.2–7.4) of the adsorbent and the solvent systems to effect a clean separation of procaine penicillin and sulfamethazine from other drugs have been discussed in the earlier report (8). To avoid the laborious procedure of extraction of the drugs from the adsorbent alumina, a convenient technique was developed using a hot water-jacketed filtering funnel. By this expedient the alumina powder containing the drug was directly transferred over the bed of diatomaceous earth in the filtering funnel. The drug was eluted with 2% hot methanolic hydrochloric acid which, by circulating hot water in the jacket, was maintained above 50° during filtration.

It had been previously observed that neither procaine penicillin nor sulfamethazine was completely recovered (88–91%) (8) from adsorbent alumina. Being highly polar compounds, it is not surprising that some of the drugs were firmly adsorbed on the substrate. To evaluate the extent of elution by the current method and compare it with the method described earlier (8), eight aliquots (in duplicate) of a standard mixture containing varying amounts ranging from 10 to 200 mcg. of each drug were applied to thin-

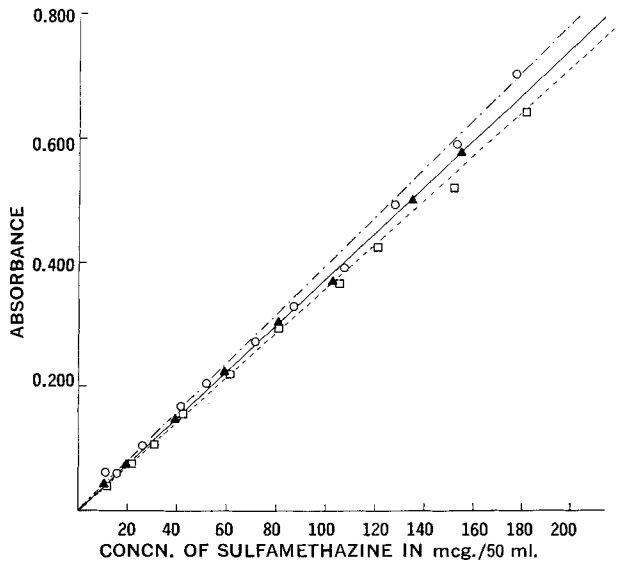


Figure 3—*Standard and recovery curves for sulfamethazine. Key:* ○, *standard curve;* □, *recovery curve by previous method; and* ▲, *recovery curve by present method.*

Table I—Analysis of Procaine Penicillin and Sulfamethazine in Medicated Feeds

Sample	Procaine Penicillin, mcg./g. Added	Found	± SD	Sulfamethazine, mcg./g. Added	Found	±SD
a_1	394	380	19.7	299	290	3.0
a_1'		373	5.0		286	3.5
a_5		386	13.6		285	5.2
b_1	375	402	3.6	437	433	3.6
b_1'		395	5.0		438	3.6
b_5		390	10.0		435	5.0
c_1	161	180	11.6	288	295	1.0
c_1'		174	4.0		306	7.0
c_5		186	8.3		292	5.7
d_1	241	261	7.8	226	237	6.0
d_1'		275	3.6		237	5.0
d_5		258	7.8		231	2.2
e_1	529	541	9.0	1031	999	4.0
e_1'		552	8.0		1009	2.5
e_5		549	17.1		1017	5.7
f_1	1240	1181	12.3	746	744	7.6
f_1'		1207	16.4		749	16.4
f_5		1145	11.2		733	8.3

layer plates, developed, extracted, and estimated. Also, 10 aliquots (in duplicate) of separate standard solutions of procaine penicillin and sulfamethazine were directly determined to obtain the standard curves.

It can be seen from Fig. 2 that by the previous technique, extraction of procaine penicillin at low levels was almost in agreement with the amounts obtained with the standards; whereas at levels above 40 mcg., the recoveries were considerably poorer. This seems to indicate that with the former procedure the alumina was firmly retaining some of the drug. By the current technique, however, it is apparent that the recovery of the drug is very consistent, and better than 93% recoveries are obtained in the range 10–200 mcg.

Examination of Figs. 2 and 3 reveals that the recovery of sulfamethazine by the previous procedure (91%) was better than that of procaine penicillin. It is evident from Fig. 3 that more than 95% recovery of the sulfa drug was obtained by the current technique. By employing the present procedure, two 1-g. and one 5-g. portions of Sample *a* were analyzed and found to be homogeneous.

To test the validity of the method, six laboratory-blended samples were analyzed and the averages of the results of two duplicate analyses of each sample are given in Table I. A close examination of the data reveals that the estimated contents of procaine penicillin are slightly higher than the amount actually added. The standard deviation[6] varies from ±3.0 to 20.0 for the six samples, indicating that although the variation is large it is still acceptable. On the other hand, in the case of sulfamethazine the results are highly reproducible with exceedingly small standard deviations (within ±10.0) and are in close agreement with the amounts actually added.

A comparison of results of the two 1-g. and the 5-g. portions of each sample shows that there is complete agreement between these analyses. For routine work, a 5-g. sample extraction is recommended for the convenience in extraction and makeup of solutions. This extraction procedure can be profitably extended to many other estimations involving thin-layer chromatography.

REFERENCES

(1) *Federal Register*, USDA, **28,** 3694(April 1, 1963).

(2) J. L. Gardiner, *Poultry Sci.*, **37,** 549(1958).

(3) E. Gerriets, *Berlin Muench. Tieraerztl. Wischr.*, **75,** 346 (1962); through *Chem. Abstr.*, **58,** 4857*e*(1963).

(4) F. Rasmussen, *Acta Pharmacol. Toxicol.*, **15,** 139(1958).

(5) C. S. Sisodia and C. M. Stowe, *Indian Vet. J.*, **41,** 711(1964).

(6) B. Toth, B. Kelentei, and K. C. Zinder, *Arzneimittel-Forsch.*, **14,** 188(1964).

(7) I. Borsnk, *Folia Morphol.*, **15,** 297(1964); through *Chem. Abstr.*, **62,** 16800*h*(1964).

(8) M. Malaiyandi, S. A. MacDonald, and J. P. Barrette, *J. Chromatogr.*, **38,** 160(1968).

(9) M. Malaiyandi, S. A. MacDonald, and J. P. Barrette, *J. Agr. Food Chem.*, **17,** 51(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 26, 1969, from *Canada Department of Agriculture, Scientific Services Laboratory, Ottawa, Canada.*

Accepted for publication October 28, 1969.

The authors thank Miss S. A. MacDonald for technical assistance, and Drs. D. A. Shearer and K. J. Jenkins and Mr. E. J. Doyle for reviewing the manuscript.

[6] The standard deviation was calculated using the formula $SD = \sqrt{\Sigma(x - \bar{x})^2/n}$ where x is the actual value, $\bar{x}$ the absolute arithme average, and n the number of estimations.

Factors Affecting a Fluorometric Assay of Folic Acid

JOHN R. HAZLETT and DANE O. KILDSIG

Abstract □ A fluorometric method of assaying folic acid, based on the oxidation of folic acid by potassium permanganate, has been investigated. Various factors were found to affect the relative fluorescence obtained in the assay. These include the concentration of potassium permanganate, the length of oxidation time, and the pH and temperature of the solution.

Keyphrases □ Folic acid—analysis □ Potassium permanganate effect—folic acid fluorescence □ pH effect—oxidized folic acid fluorescence □ Temperature effect—oxidized folic acid fluorescence □ Fluorometry—analysis

The oxidative degradation of folic acid by potassium permanganate yields a fluorescent pterine (1). As a result, this oxidation has been used in analytical procedures for the quantitative determination of folic acid (2, 3). The formation of the free pterine derivative provides an approximately 20-fold increase in fluorescent intensity over that of native folic acid fluorescence.

Table I—Final Fluorescent Intensity

Concentration of $KMnO_4$ (moles/l. × 10^{-5})	Fluorescent Intensity (F_∞)
4.43	80.5
6.33	78.0
9.49	75.0
31.6	80.5

In a comprehensive study, Allfrey *et al.* (2) measured directly the fluorescence of the oxidized mixture as well as that of the chromatographically isolated oxidation

Table I—Analysis of Procaine Penicillin and Sulfamethazine in Medicated Feeds

Sample	Procaine Penicillin, mcg./g. Added	Found	± SD	Sulfamethazine, mcg./g. Added	Found	±SD
a_1	394	380	19.7	299	290	3.0
a_1'		373	5.0		286	3.5
a_5		386	13.6		285	5.2
b_1	375	402	3.6	437	433	3.6
b_1'		395	5.0		438	3.6
b_5		390	10.0		435	5.0
c_1	161	180	11.6	288	295	1.0
c_1'		174	4.0		306	7.0
c_5		186	8.3		292	5.7
d_1	241	261	7.8	226	237	6.0
d_1'		275	3.6		237	5.0
d_5		258	7.8		231	2.2
e_1	529	541	9.0	1031	999	4.0
e_1'		552	8.0		1009	2.5
e_5		549	17.1		1017	5.7
f_1	1240	1181	12.3	746	744	7.6
f_1'		1207	16.4		749	16.4
f_5		1145	11.2		733	8.3

layer plates, developed, extracted, and estimated. Also, 10 aliquots (in duplicate) of separate standard solutions of procaine penicillin and sulfamethazine were directly determined to obtain the standard curves.

It can be seen from Fig. 2 that by the previous technique, extraction of procaine penicillin at low levels was almost in agreement with the amounts obtained with the standards; whereas at levels above 40 mcg., the recoveries were considerably poorer. This seems to indicate that with the former procedure the alumina was firmly retaining some of the drug. By the current technique, however, it is apparent that the recovery of the drug is very consistent, and better than 93% recoveries are obtained in the range 10–200 mcg.

Examination of Figs. 2 and 3 reveals that the recovery of sulfamethazine by the previous procedure (91%) was better than that of procaine penicillin. It is evident from Fig. 3 that more than 95% recovery of the sulfa drug was obtained by the current technique. By employing the present procedure, two 1-g. and one 5-g. portions of Sample *a* were analyzed and found to be homogeneous.

To test the validity of the method, six laboratory-blended samples were analyzed and the averages of the results of two duplicate analyses of each sample are given in Table I. A close examination of the data reveals that the estimated contents of procaine penicillin are slightly higher than the amount actually added. The standard deviation[6] varies from ±3.0 to 20.0 for the six samples, indicating that although the variation is large it is still acceptable. On the other hand, in the case of sulfamethazine the results are highly reproducible with exceedingly small standard deviations (within ±10.0) and are in close agreement with the amounts actually added.

A comparison of results of the two 1-g. and the 5-g. portions of each sample shows that there is complete agreement between these analyses. For routine work, a 5-g. sample extraction is recommended for the convenience in extraction and makeup of solutions. This extraction procedure can be profitably extended to many other estimations involving thin-layer chromatography.

REFERENCES

(1) *Federal Register*, USDA, **28,** 3694(April 1, 1963).

(2) J. L. Gardiner, *Poultry Sci.*, **37,** 549(1958).

(3) E. Gerriets, *Berlin Muench. Tieraerztl. Wischr.*, **75,** 346 (1962); through *Chem. Abstr.*, **58,** 4857*e*(1963).

(4) F. Rasmussen, *Acta Pharmacol. Toxicol.*, **15,** 139(1958).

(5) C. S. Sisodia and C. M. Stowe, *Indian Vet. J.*, **41,** 711(1964).

(6) B. Toth, B. Kelentei, and K. C. Zinder, *Arzneimittel-Forsch.*, **14,** 188(1964).

(7) I. Borsnk, *Folia Morphol.*, **15,** 297(1964); through *Chem. Abstr.*, **62,** 16800*h*(1964).

(8) M. Malaiyandi, S. A. MacDonald, and J. P. Barrette, *J. Chromatogr.*, **38,** 160(1968).

(9) M. Malaiyandi, S. A. MacDonald, and J. P. Barrette, *J. Agr. Food Chem.*, **17,** 51(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 26, 1969, from *Canada Department of Agriculture, Scientific Services Laboratory, Ottawa, Canada.*

Accepted for publication October 28, 1969.

The authors thank Miss S. A. MacDonald for technical assistance, and Drs. D. A. Shearer and K. J. Jenkins and Mr. E. J. Doyle for reviewing the manuscript.

[6] The standard deviation was calculated using the formula $SD = \sqrt{\Sigma(x - \bar{x})^2/n}$ where x is the actual value, $\bar{x}$ the absolute arithme average, and n the number of estimations.

Factors Affecting a Fluorometric Assay of Folic Acid

JOHN R. HAZLETT and DANE O. KILDSIG

Abstract □ A fluorometric method of assaying folic acid, based on the oxidation of folic acid by potassium permanganate, has been investigated. Various factors were found to affect the relative fluorescence obtained in the assay. These include the concentration of potassium permanganate, the length of oxidation time, and the pH and temperature of the solution.

Keyphrases □ Folic acid—analysis □ Potassium permanganate effect—folic acid fluorescence □ pH effect—oxidized folic acid fluorescence □ Temperature effect—oxidized folic acid fluorescence □ Fluorometry—analysis

The oxidative degradation of folic acid by potassium permanganate yields a fluorescent pterine (1). As a result, this oxidation has been used in analytical procedures for the quantitative determination of folic acid (2, 3). The formation of the free pterine derivative provides an approximately 20-fold increase in fluorescent intensity over that of native folic acid fluorescence.

Table I—Final Fluorescent Intensity

Concentration of $KMnO_4$ (moles/l. × 10^{-5})	Fluorescent Intensity (F_∞)
4.43	80.5
6.33	78.0
9.49	75.0
31.6	80.5

In a comprehensive study, Allfrey *et al.* (2) measured directly the fluorescence of the oxidized mixture as well as that of the chromatographically isolated oxidation

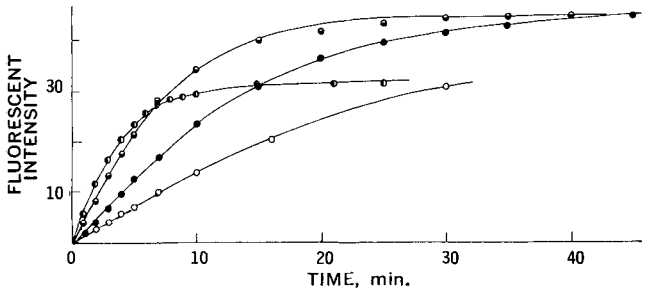


Figure 1—*Effect of $KMnO_4$ concentration on the fluorescent intensity of folic acid solutions. Key: ○, 2.54 × 10^{-5} moles/l.; ●, 4.43 × 10^{-5} moles/l.; ◒, 9.49 × 10^{-5} moles/l.; and ◐, 3.16 × 10^{-4} moles/l.*

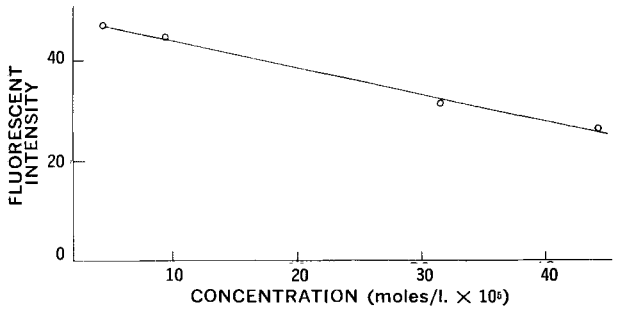


Figure 2—*Decrease in maximum fluorescence with increasing $KMnO_4$ concentration.*

product. Their simplest direct procedure was valid only over the narrow pH range of 3.9 to 4.4, and there was no evidence of fluorescence dependence on potassium permanganate concentration. In the present investigation, this direct method is more thoroughly investigated with respect to the influence of the potassium permanganate concentration on fluorescent intensity.

EXPERIMENTAL

The fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer. Excitation maxima were found at 275 and 375 mμ with maximum fluorescence observed at 445 mμ.

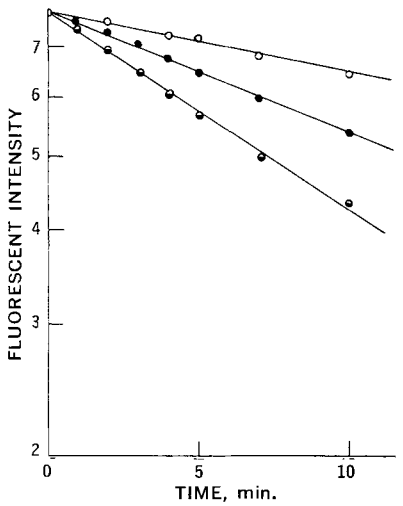


Figure 3—*First-order plots of the oxidation of folic acid with varying concentration of $KMnO_4$. Key: ○, 2.54 × 10^{-5} moles/l.; ●, 6.33 × 10^{-5} moles/l.; and ◒, 9.49 × 10^{-5} moles/l.*

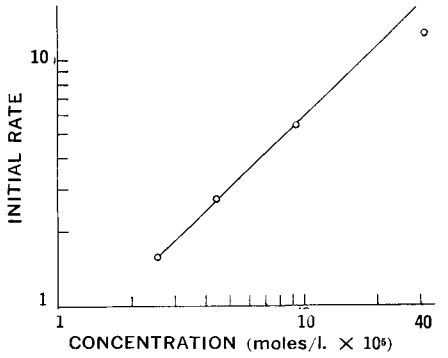


Figure 4—*Log-log plot of the initial rate of the oxidation reaction as affected by the concentration of $KMnO_4$.*

To determine the effect of $KMnO_4$ concentration on the fluorescent intensity of folic acid solutions, the concentration of $KMnO_4$ was varied from 2.54 × 10^{-5} to 4.43 × 10^{-4} moles/l. All solutions contained 4.53 × 10^{-6} moles/l., of folic acid and were buffered at pH 8.12 with a borate buffer. The spectrophotofluorometer was standardized and the fluorescence measured at 25°. The standard solution was obtained by filtering the oxidized folic acid solution to remove the residual manganese dioxide, resulting in a clear colorless solution. If necessary, 1.0 ml. of 3% hydrogen peroxide was added to reduce the remaining $KMnO_4$.

RESULTS AND DISCUSSION

Oxidation of Folic Acid—The oxidation of folic acid may be followed by determining the increase in fluorescence of the solution due to the formation of a pterine.[1] The increase in fluorescent intensity as related to the concentration of $KMnO_4$ is shown in Fig. 1. The rate of fluorophor formation is seen to increase with increasing concentration of $KMnO_4$. However, as the concentration is increased above 4.43 × 10^{-5} moles/l., the total magnitude of the fluorescence decreases. The decrease in maximum fluorescence with increasing $KMnO_4$ concentration is shown in Fig. 2. This decrease in

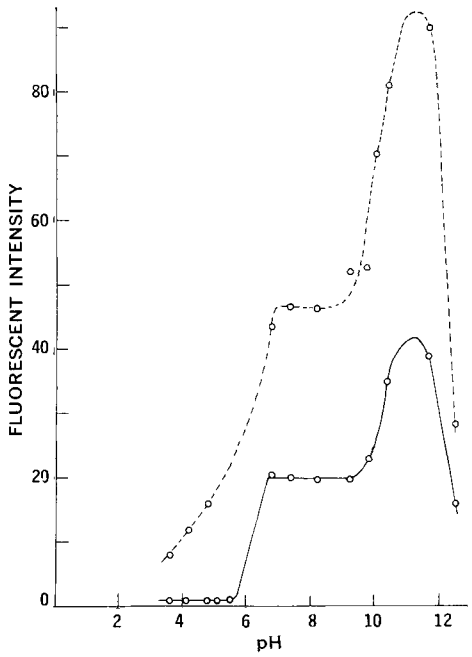


Figure 5—*Effect of pH on the fluorescent intensity of the folic acid oxidation product. Key: - - -, excitation 375 mμ; and ——, excitation 275 mμ.*

[1] 2-Amino-4-hydroxy-6-carboxypteridine (*Reference 1*).

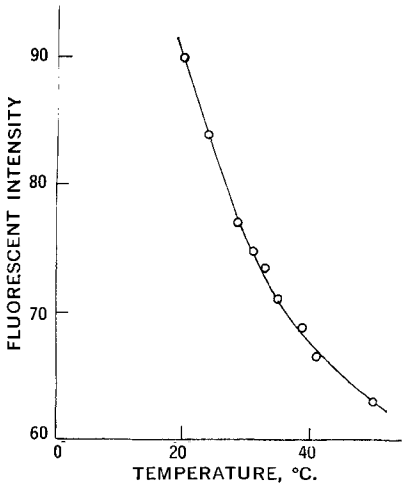


Figure 6—*Effect of temperature on the fluorescent intensity of the folic acid oxidation product.*

fluorescence is probably due to quenching by the MnO_2 formed during the reaction. This effect of $KMnO_4$ concentration probably was not observed by Allfrey *et al.* (2) because of the magnitude of the $KMnO_4$ concentration used (1.23×10^{-3} moles/l.).

A plot of the logarithm of the difference $F_\infty - F_t$, where F_∞ is the final fluorescence and F_t is the fluorescence at time t, is shown in Fig. 3. The linearity of the plot indicates the reaction is first order with respect to folic acid. The value of F_∞ used in this plot was obtained by allowing the solutions to equilibrate for 48 hr. and filtering the solutions to remove the MnO_2. In some cases, 1.0 ml. of 3% hydrogen peroxide was added to reduce any remaining $KMnO_4$. The resulting divalent manganese ions do not appear to quench the fluorescence of the oxidation product. The values obtained for F_∞ are shown in Table I. The close agreement of the final values, essentially independent of $KMnO_4$ concentration, justifies the use of the expression $F_\infty - F_t$ in the logarithmic plot.

The order of the reaction with respect to $KMnO_4$ can be determined from the slope of a plot of log initial rate of reaction *versus* log $KMnO_4$ concentration as shown in Fig. 4. The slope of this line, 0.986, indicates a first-order reaction with respect to $KMnO_4$. The effect of quenching is seen at the highest concentration. The mechanism of this oxidation reaction, which is apparently second order, is being investigated.

Fluorescence *versus* pH—The effect of pH on the fluorescence of the oxidized folic acid is shown in Fig. 5. The existence of a plateau between pH 7 and 9 differs from the narrow plateau between pH 3.9 and 4.4 previously reported (2). This again could be due to the greater concentration of $KMnO_4$ used in the earlier investigation, 1.23×10^{-3} moles/l., which as seen in Fig. 2 would have resulted in a considerable decrease in the fluorescent intensity. The increase in fluorescence near pH 11 may be due to the ionization of the lactim hydrogen of the pterine.

Fluorescence *versus* Temperature—As discussed by Udenfriend (4), fluorescence can be very sensitive to temperature. The effect of temperature on the fluorescence of the oxidation product is shown in Fig. 6. The greatest temperature dependence occurs between 20 and 28° and represents an error of approximately 2% per degree.

CONCLUSIONS

The oxidation of folic acid with $KMnO_4$ has been found to produce a fluorescence whose magnitude is dependent on the concentration of $KMnO_4$. An optimum concentration of $KMnO_4$ was found, above which a decrease in fluorescence occurred due to quenching. The time required for maximum fluorescence also varied with the concentration of $KMnO_4$. As expected, both pH and temperature markedly influenced the fluorescence obtained, the optimum pH being between 6 and 9.

REFERENCES

(1) E. Wittle, B. O'Dell, J. Vandenbelt, and J. Pfiffner, *J. Amer. Chem. Soc.*, **69**, 1786(1947).

(2) V. Allfrey, L. Teply, C. Geffen, and C. King, *J. Biol. Chem.*, **178**, 465(1949).

(3) K. Okamoto, *Bitamin (Kyoto)*, **28**, 49(1963).

(4) S. Udenfriend, "Fluorescence Assay in Biology and Medicine," Academic, New York, N. Y., 1962, p. 106.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 22, 1969, from the *Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication October 20, 1969.

Hydroxyindole-*o*-methyltransferase IV: Inhibitory Activities of Some *N*-Acrylyltryptamines and *N*-Crotonyltryptamines

BENG T. HO, MICHAEL B. NOEL, and WILLIAM M. McISAAC

Abstract □ Several *N*-acrylyltryptamines and *N*-crotonyltryptamines were synthesized to explore the mode of binding of the double bond in *N*-(1-cyclohexenecarbonyl)tryptamine to hydroxyindole-*o*-methyltransferase. Chlorine substitution at the β-position of the acrylyl or crotonyl group increased inhibition of the enzyme.

Keyphrases □ *N*-Acrylyltryptamines and *N*-crotonyltryptamines—synthesis □ *N*-(1-Cyclohexenecarbonyl)tryptamine double-bond binding—hydroxyindole-*o*-methyltransferase □ Chlorine substitution, *N*-acrylyltryptamines and *N*-crotonyltryptamines—hydroxyindole-*o*-methyltransferase inhibition □ IR spectrophotometry—structure □ NMR spectroscopy—structure

In previous works from this laboratory (1, 2), *N*-(1-cyclohexenecarbonyl)tryptamine (I) and *N*-benzoyltryptamine (II) were found to be, respectively, 3.4 and 2.3 times better inhibitors of hydroxyindole-*o*-methyltransferase (HIOMT) than *N*-cyclohexanecarbonyltryptamine (III). To explore the mode of binding of the carbon-carbon double bond in I, *N*-acrylyltryptamine (IV) and *N*-crotonyltryptamine (V) were synthesized. Since chlorine substitution on the *ortho* position of the phenyl group of II resulted in a sixfold increase in inhibitory activity (2), this study also includes Compounds VII–IX for investigating the possible enhancement of binding of the acrylyl (CH_2=CH—C(=O)—) and crotonyl (CH_3—CH=CH—C(=O)—) groups by a chlorine atom at the beta position.

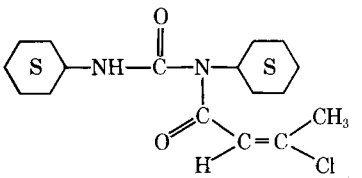
Results showed that IV and V were inhibitors of equal activity (Table I). The terminal CH_3 of the crotonyl moiety of V did not appear to contribute to the binding of this inhibitor to the enzyme. Substitution of the "methylene" hydrogen of the acrylyl group of IV by a chlorine atom slightly increased the activity (see VII). A greater increase in inhibition resulted when the chlorine atom was introduced to the "methine" carbon of the crotonyl moiety of V; VIII and IX were about twice as active as V.

The relative weakness in activities of those acrylyl and crotonyl derivatives compared to I could be attributed to the absence of several saturated methylene carbons which are capable of binding to the enzyme *via* hydrophobic interaction. The finding that *ortho*-chlorophenyl derivative (VI) is a much better inhibitor than the phenyl derivative (II), whereas the chlorocrotonyl derivatives (VII and VIII) are only slightly more active than the crotonyl derivative (V), can be visualized as a difference in the effect of a chlorine atom on the two series. A chlorine atom would be expected to exert stronger effect on the binding of an aromatic phenyl ring, as in the case of VI, than on an isolated double bond, as in VII or VIII. The observation that I was a better inhibitor than III would indicate that the C=C of the cyclohexenyl moiety of I provided additional binding to the enzyme. This type of unsaturated linkage is capable of complexing with the enzyme *via* a mixture of donor-acceptor, hydrogen-bonding, and even hydrophobic interactions (3).

PROCEDURES

N-Acyltryptamines IV, V, VII, VIII, and IX were prepared by the treatment of tryptamine with the appropriate acid chlorides obtained commercially or prepared from the corresponding acids. Attempts to prepare VIII from tryptamine and *trans*-3-chlorocrotonic acid in the presence of dicyclohexylcarbodiimide (DCC) were unsuccessful. Tryptamine and chlorocrotonic acid formed a salt which was insoluble in most organic solvents except aqueous tetrahydrofuran and aqueous acetonitrile. In the latter two cases, the only compound isolated from the reaction was the acylurea XI (m.p. 163–164°, MeOH $\nu_{max.}$ (KBr): 1700, 1645 (C=O); 1615 cm.$^{-1}$ (C=C).

Anal.—Calcd. for $C_{17}H_{27}ClN_2O_2$: C, 62.5; H, 8.33. Found: C, 62.4; H, 8.35.



XI

In the preparation of V, a second, higher melting crystalline compound was isolated. Dissimilarities in the IR spectra of the two products led to the initial assumption that one of them was disubstituted amide, *N*,*N*-dicrotonyltryptamine. However, elemental analyses indicated that they were isomeric, whereupon the melting points of both analytical samples were retaken. The compound, which originally had a m.p. of 83–84°, now melted over a range of 96–110°. When subjected to IR analysis, the spectrum of this substance changed to that of the higher melting compound in 2 hr. Further investigation was hampered by the unsuccessful attempts to isolate the lower melting product in later preparations.

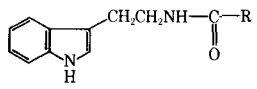
EXPERIMENTAL

Melting points are corrected and were taken on a melting-point apparatus.[1] IR spectra were obtained with a spectrophotometer.[2]

***N*-Acrylyltryptamine (IV)**—To a solution of 1.46 g. (10 mmoles) of tryptamine and 1.5 ml. (10.8 mmoles) of triethylamine in 75 ml. of chloroform was added, at 0°, 0.73 ml. (10.2 mmoles) of acrylyl chloride. The mixture was stirred at ambient temperature for 4 hr.; then 400 mg. (28%) of tryptamine hydrochloride was filtered off. The filtrate was extracted successively with 25-ml. portions of water, 2 *N* HCl, 2 *N* NaOH, and again water. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo* to a yellow oil. Water was added to a solution of the oil in MeOH until turbidity occurred. The oily layer, which deposited upon standing, was separated by decantation and dissolved in 100 ml. of boiling water. After treating with char-

[1] Mel-Temp.
[2] Perkin-Elmer, model 237B.

Table I—Inhibition of HIOMT by



Compound	R	I_{50}[a] mM
I	1-Cyclohexenyl	0.25[b]
III	C_6H_{11}—	0.85[c]
II	C_6H_5—	0.37[c]
VI	*o*—ClC_6H_4—	0.055[b]
IV	$CH_2=CH$—	0.90
V	CH_3—$CH=CH$—	0.95
VII	Cl—$CH=CH$—(*trans*)	0.75
VIII	Cl, CH_3—$C=CH$—(*trans*)[d]	0.50
IX	Cl, CH_3—$C=CH$—(*cis*)	0.45

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Data from *Reference 2*. [c] Data from *Reference 1*. [d] *Trans* refers to the configuration in which the methyl and carbonyl groups are opposite one another.

coal (Norit) and filtering through diatomaceous earth (Celite), the solution was allowed to evaporate on bench top, yielding 650 mg. (31% after correction for recovery of tryptamine) of white, lustrous plates, m.p. 77–79°. Several recrystallizations from water gave an analytical sample, m.p. 79–80°; $\nu_{max.}$ (KBr): 1620 (C=O); 745 cm.$^{-1}$ (indole CH).

Anal.—Calcd. for $C_{13}H_{14}N_2O$: C, 72.9; H, 6.59; N, 13.1. Found: C, 72.6; H, 6.45; N, 13.0.

***N*-Crotonyltryptamine (V)**—In a similar manner as described in the preparation of IV, 10 mmoles of tryptamine was treated with crontoyl chloride in chloroform in the presence of triethylamine. After stirring at ambient temperature for 17 hr., the mixture was washed successively with water, 2 *N* HCl, 2 *N* NaOH, and water. The organic layer, after drying (Na_2SO_4), was evaporated *in vacuo*, leaving a yellow oil which did not crystallize. Distillation of this oil under reduced pressure yielded a pale-yellow, viscous oil which became semisolid upon setting, m.p. 76–78°. Extraction of the oily distillate with boiling water and subsequent evaporation of the extracts led to the formation of 660 mg. (30%) of small, lustrous plates, m.p. 78–80°. Several recrystallizations from water gave an analytical sample, m.p. 83–84°; $\nu_{max.}$ (KBr): 1620 (C=O); 745 cm.$^{-1}$ (indole CH).

Anal.—Calcd. for $C_{14}H_{16}N_2O$: C, 73.6; H, 7.06; N, 12.3. Found: C, 73.7; H, 7.10; N, 12.2.

Continuous extraction of this residual oil with boiling water yielded additional crops of crystals. The combined crops were recrystallized from water to give a higher melting V, m.p. 116–120°. An analytical sample was obtained upon further recrystallization of this product from water to a constant m.p. 129–130°; $\nu_{max.}$ (KBr): 1630 (C=O); 745 cm.$^{-1}$ (indole CH).

Anal.—Calcd. for $C_{14}H_{16}N_2O$: C, 73.6; H, 7.06; N, 12.3. Found: C, 73.9; H, 7.16; N, 12.3.

***N*-*trans*-(3-Chloroacrylyl)tryptamine (VII)**—A solution of 2.13 g. (20 mmoles) of *trans*-3-chloroacrylic acid and 3.0 ml. (23.5 mmoles) of benzenesulfonyl chloride in 15 ml. of xylene was heated at 90° for 30 min. and then distilled at 115–128°. This fraction of distillate, containing the acid chloride and xylene, was diluted with 15 ml. of chloroform and added at 0° to a solution of 3.2 g. (20 mmoles) of tryptamine and 4 ml. (29 mmoles) of triethylamine in 40 ml. of chloroform. The mixture was stirred at ambient temperature for 17 hr.; filtered to remove the tryptamine hydrochloride; and treated with water, 2 *N* HCl, 2 *N* NaOH, and water as described in the preparation of IV. The chloroform layer was dried (Na_2SO_4), treated with charcoal, filtered, and then evaporated *in vacuo*, leaving a pale-yellow semisolid. This product was dissolved in ether and treated with petroleum ether (b.p. 30–60°) to cloudiness. Upon chilling, 1.25 g. (25% after correction for recovery of tryptamine) of pale-yellow needles, m.p. 108–111°, was yielded. Subsequent recrystallization from ether–petroleum ether (b.p. 30–60°) gave an analytical sample as clumps of white needles, m.p. 109–110.5°; $\nu_{max.}$ (KBr): 1640 (C=O); 740 (indole CH).

Anal.—Calcd. for $C_{13}H_{13}ClN_2O$: C, 62.8; H, 5.27; Cl, 14.3; N, 11.3. Found: C, 62.7; H, 5.13; Cl, 14.0; N, 11.1.

***N*-*trans*-(3-Chlorocrotonyl)tryptamine (VIII)**—A mixture of 1.0 g. (8.3 mmoles) of *trans*-3-chlorocrotonic acid, 0.75 ml. (10.4 mmoles) of $SOCl_2$, and 6 ml. of $CHCl_3$ was refluxed for 45 min. After cooling, the solution was added to a chilled solution of 2.4 g. (15 mmoles) of tryptamine and 2.0 ml. (14.4 mmoles) of triethylamine in 25 ml. of $CHCl_3$. The mixture was stirred at ambient temperature for 4 hr., filtered to remove tryptamine hydrochloride, and concentrated to about 5 ml. Addition of 20 ml. acetone to this $CHCl_3$ solution precipitated a mixture of triethylamine hydrochloride and tryptamine hydrochloride which was removed by filtration. The acetone filtrate was evaporated *in vacuo* leaving an oil. A solution of this oil in chloroform was treated with water, 1 *N* HCl, 1 *N* NaOH, and water as described in the preparation of IV; dried (Na_2SO_4); treated with charcoal; and again evaporated *in vacuo*. The residual pale-yellow oil was extracted repeatedly with hot ether. When the combined ethereal extract was concentrated to about 20 ml., it turned cloudy and thick, shiny needles deposited upon standing on the bench top. Addition of petroleum ether (b.p. 30–60°) to this ethereal mixture caused the precipitation of more product; total first crop was 800 mg. (38%), m.p. 73–75.5°. Subsequent extractions of the original oil with ether afforded an additional crop of 390 mg. (18%). Thus, the total yield was 56%. Recrystallization of the first crop several times from ether–petroleum ether gave an analytical sample as shiny platelets, m.p. 77–78.5°; $\nu_{max.}$ (KBr): 1620 (C=O); 745 cm.$^{-1}$ (indole CH). NMR ($CDCl_3$): δ 2.18 (doublet, CH_3); 5.91 (doublet, olefinic H). Assignment of *trans* configuration was based on 3-chlorocrotonic acid (4).

Anal.—Calcd. for $C_{14}H_{15}ClN_2O$: C, 64.0; H, 5.75; N, 10.7. Found: C, 64.1; H, 5.86; N, 10.8.

***N*-*cis*-(3-Chlorocrotonyl)tryptamine (IX)**—The procedure was similar to that used for VII, except that the *cis*-3-chlorocrotonyl chloride was prepared by refluxing the corresponding acid with PCl_3 in xylene and distilled at 135–140°. The yellow oily product from the reaction was dried thoroughly *in vacuo* and then dissolved in 15 ml. of anhydrous ether. Concentration of the ethereal solution to about 5 ml. caused the deposition of a 44% yield (after correction for recovery of tryptamine hydrochloride) of yellow crystals, m.p. 98–101°. Addition of petroleum ether (b.p. 30–60°) to the filtrate of the first crop gave another 44% yield of less pure material, m.p. 89–93°. Recrystallization of the first crop several times from ether–petroleum ether afforded an analytical sample as transparent prisms, m.p. 102.5–103.5°; $\nu_{max.}$ (KBr): 1630 (C=O); 740 cm.$^{-1}$ (indole CH). NMR ($CDCl_3$): δ2.85 (doublet, CH_3); 5.83 (doublet, olefinic H). Assignment of *cis* configuration was based on 3-chloroisocrotonic acid (4).

Anal.—Calcd. for $C_{14}H_{15}ClN_2O$: C, 64.0; H, 5.75; N, 10.7. Found: C, 63.9; H, 5.67; N, 10.6.

Assay—Hydroxyindole-*o*-methyltransferase was isolated from beef pineal gland and purified according to the method of Axelrod and Weissbach (5).

The stock solutions of all the inhibitors were prepared in 50% aqueous propylene glycol. Incubation was carried out with *N*-acetylserotonin and *S*-adenosyl-L-methionine-methyl-^{14}C according to the previously described procedure (6).

REFERENCES

(1) B. T. Ho, W. M. McIsaac, L. W. Tansey, and P. M. Kralik, *J. Pharm. Sci.*, **57**, 1998(1968).

(2) B. T. Ho, W. M. McIsaac, and L. W. Tansey, *ibid.*, **58**, 563 (1969).

(3) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967, p. 44.

(4) "High Resolution NMR Spectra Catalog," vol. 1, Varian, Palo Alto, Calif., 1962, Spectra Nos. 53 and 54.

(5) J. Axelrod and H. Weissbach, *J. Biol. Chem.*, **236**, 211(1961).

(6) B. T. Ho, W. M. McIsaac, and L. W. Tansey, *J. Pharm. Sci.*, **58**, 130(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 9, 1969, from the *Texas Research Institute of Mental Sciences, Houston, TX 77025*

Accepted for publication December 11, 1969.

This work was supported by Grants MH-11168 and MH-12959, U. S. Public Health Service, and the Britton Fund.

The authors thank Miss Patricia M. Kralik and Mr. Edward Fritchie for the enzyme assays and Miss Geraldine Hagger for technical assistance.

Enhancement of Steroid Absorption by Dialkylamides

Keyphrases ☐ Steroids—absorption ☐ Absorption, steroids—*N*,*N*-dialkylalkylamide effect ☐ Prednisone absorption enhancement—*N*,*N*-di-*n*-propylpropionamide

Sir:

Amides having two alkyl substituents on the nitrogen atom can interact in nonaqueous solvents with a number of organic nonelectrolytes to form molecular complexes (1). This suggests that the lipoid phase of biologic membranes might provide an environment conducive to complex formation between nitrogen-substituted amides and certain drugs. Pronounced drug–amide complex formation in the lipoid phase of biologic membranes might be expected to influence the rate of drug transfer across such membranes. The direction and magnitude of this effect would presumably depend on the partition coefficient and diffusion characteristics of the drug relative to those of the drug–amide complex in the membrane and on the extent of the drug–amide interaction.

The influence of nitrogen-substituted amides on drug absorption has not been investigated extensively. There is evidence, however, that *N*,*N*-dialkylalkylamides can influence the percutaneous absorption rate of certain drugs. Munro and Stoughton, for example, reported *N*,*N*-dimethylacetamide (DMA) and *N*,*N*-dimethylformamide to be superior to ethanol, benzene, and a cream base in promoting the penetration of hydrocortisone and griseofulvin into human skin (2). Reid and Brookes found that the percutaneous absorption of three corticosteroids was more rapid from an ointment base containing 25% DMA than from three other ointment bases (3). These observations are explainable on the basis of drug–amide complex formation, although a physical alteration of the cutaneous barrier may have occurred since amide concentrations of 25–100% were used.

We have studied the effect of relatively low *N*,*N*-dialkylalkylamide concentrations on steroid absorption from the rat small intestine. This preliminary communication describes one representative system: prednisone and *N*,*N*-di-*n*-propylpropionamide (DPP). Male Sprague-Dawley rats, starved 14–22 hr., were prepared as described by Doluisio *et al.* for studying drug absorption from solution by the *in situ* rat small intestine (4). Seven milliliters of drug solution was placed in the intestine, and 0.2-ml. samples were removed at 10-min. intervals for 1 hr. The volume of the luminal prednisone solution was maintained constant by adding solvent (isotonic saline) immediately prior to sample removal. About 4 ml. additional solvent was required for this purpose. The concentration of prednisone in the sample was determined by the colorimetric procedure of Porter and Silber (5). The initial prednisone and DPP concentrations in the luminal solution were 5×10^{-4} *M* and 2.8×10^{-2} *M* (0.5% v/v), respectively. Prednisone concentrations determined in the serial samples were corrected for previously removed drug.

Table I—Effect of 0.028 *M* *N*,*N*-di-*n*-Propylpropionamide (DPP) on the Absorption of Prednisone from the Rat Intestine

Percent Absorbed[a]					
10 min.		20 min.		60 min.	
Control	With DPP	Control	With DPP	Control	With DPP
26.0	56.1	37.5	72.3	61.6	90.4
26.4	46.6	42.3	64.0	70.3	86.5
23.6	47.1	36.2	63.3	58.5	88.1
23.2	49.0	37.8	65.4	62.0	87.4
		Mean			
24.8	49.7	38.4	66.2	63.1	88.1

[a] The effect of DPP was statistically significant ($p < 0.001$) at all times.

The results of the absorption experiments are summarized in Table I. The presence of amide enhanced significantly ($p < 0.001$) the absorption of the steroid. A detailed kinetic analysis of the entire data will be presented in a subsequent report. The nature of the prednisone–DPP interaction was studied in both water and isopropyl myristate (IPM) by equilibrium solubility and equilibrium partition methods. The results indicate that a molecular complex of stoichiometry other than 1:1 formed between prednisone and DPP. The interaction was more extensive in a nonpolar than in a polar medium. For example, 2% v/v DPP increased the solubility of prednisone in IPM by 137%, compared to a 27% increase obtained under similar conditions in water. In addition, the IPM–water partition coefficient was increased 110% when the IPM phase contained 2% v/v DPP. The IPM–water partition coefficient of DPP is about 4.

The integrity of the intestinal membranes did not appear to be affected by DPP in the concentration used in this study. The gross appearance of the gut did not change; the absorption rate of caffeine, a drug that does not appear to interact with DPP in a nonaqueous solvent (IPM), was the same in the presence and absence of the amide. (Caffeine and DPP may interact slightly in water; the aqueous solubility of caffeine is increased 15% by 2% v/v DPP.)

Additional data, to be presented in a future report, indicate a relationship between the alkyl chain length of the nitrogen substituent and the absorption-enhancing effect of the dialkylpropionamides. These findings are significant because they may lead to a better understanding of intestinal absorption processes, particularly with respect to enhancing the absorption rate of certain poorly absorbed drugs.

(1) T. Higuchi and S. Chulkaratana, through T. Higuchi and K. A. Connors, in "Advances in Analytical Chemistry and Instrumentation," 4th ed., C. N. Reilley, Ed., Interscience, New York, N. Y., 1965, p. 117.
(2) D. P. Munro and R. B. Stoughton, *Arch. Dermatal.*, **92**, 585(1965).
(3) J. Reid and D. B. Brookes, *Brit. J. Dermatol.*, **80**, 328(1968).
(4) J. T. Doluisio, N. F. Billups, L. W. Dittert, E. T. Sugita, and J. V. Swintosky, *J. Pharm. Sci.*, **58**, 1196(1969).
(5) C. C. Porter and R. H. Silber, *J. Biol. Chem.*, **185**, 201(1950).

WILLIAM L. HAYTON
DAVID E. GUTTMAN*
GERHARD LEVY
Department of Pharmaceutics
School of Pharmacy
State University of New York at Buffalo
Buffalo, NY 14214

Received November 14, 1969.
Accepted for publication January 13, 1970.
Supported in part by Fellowship No. 1 FO1 GM-43160-01 for W.L.H. from the U.S. Public Health Service.

* Present address: Smith Kline & French Labs., Philadelphia, PA 19101

Synthesis and Antibacterial Activity of 1-Styryl-3,4-dihydroisoquinolines

Keyphrases □ 1-Styryl-3,4-dihydroisoquinolines—synthesis □ Antibacterial activity—1-styryl-3,4-dihydroisoquinolines

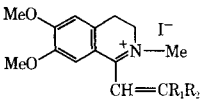
Sir:

So far, the synthesis of 1-styrylisoquinolines has been achieved either by the cyclization of the Schiff bases derived from cinnamaldehyde (1, 2) or by the condensation of 1-methylisoquinoline with aromatic aldehydes (3). In this communication, we wish to report a new procedure for the synthesis of 1-styryl-3,4-dihydroisoquinolines which were isolated as methiodide salts. The procedure involves the cyclodehydration of substituted β-phenethylamides to 3,4-dihydroisoquinolines through the Bischler-Napieralski reaction (4).

Details about the synthesis and characterization of 1-styryl-3,4-dihydroisoquinoline methiodides (Compounds 1–7) will be published (5).

The methiodide salts (Compounds 1–7) were subjected to *in vitro* screening for antimetabolites by a new method (6). In this method, the detection system utilizes the gram-positive *Bacillus subtilis* and gram-negative *Escherichia coli*. Both organisms were grown in two types of agar: nutrient agar and a completely synthetic medium with glucose as the only source of carbon.

Table I—Inhibition of *B. subtilis* Grown in Two Different Media[a]



Compound	R_1	R_2	Nutrient Agar	Synthetic Agar
1	Ph	Ph	24	35
2	Ph	*p*-Methylphenyl	25	36
3	*p*-Methylphenyl	*p*-Methylphenyl	29	35
4	*p*-Ethylphenyl	*p*-Ethylphenyl	36	39
5	*p*-Chlorophenyl	*p*-Chlorophenyl	28	35
6	Me	Ph	16	22
7	Me	Me	0	0

[a] The numbers in the body of the table are zones of growth inhibition in mm. around a 13-mm. paper disk.

These seven compounds were tested at concentrations of 1 mg./ml., and the results are presented in Table I.

The inhibition of test organism by Compounds 1–6 was stronger on synthetic agar than on nutrient agar. However, the difference was not large enough to suggest an antimetabolitelike mode of action (5). Compound 7 was essentially inactive against *B. subtilis*. None of the compounds inhibited the growth of *E. coli*. These results indicate that 1-styryl-3,4-dihydroisoquinoline methiodides possess some antibacterial activity.

A more extensive testing will be required before any structure–activity correlation can be drawn.

(1) E. C. Weinback and W. H. Hartung, *J. Org. Chem.*, **15**, 676(1950); W. M. Whaley and T. R. Govindachari, "Organic Reactions," vol. 6, Wiley, New York, N. Y., 1962, p. 151.
(2) W. H. Mills and J. L. B. Smith, *J. Chem. Soc.*, **121**, 2724(1922).
(3) T. Kametani, T. Terui, T. Ogino, and K. Fukumoto, *J. Chem. Soc. (C)*, **1969**, 874.
(4) F. Bergmann, M. Weizmann, E. Dimant, J. Patai, and J. Szmuskowica, *J. Amer. Chem. Soc.*, **70**, 1612(1948).
(5) R. E. Harmon, B. L. Jensen, S. K. Gupta, and J. Nelson, to be published.
(6) L. J. Hanka, "Abstracts," Fifth International Congress of Chemotherapy, Vienna, Austria, July 1967, B g/2, 351.

R. E. HARMON
B. L. JENSEN
S. K. GUPTA
Department of Chemistry
Western Michigan University
Kalamazoo, MI 49001
and
L. J. HANKA
Research Laboratories
The Upjohn Co.
Kalamazoo, MI 49001

Received November 24, 1969.
Accepted for publication February 3, 1970.
The work at Western Michigan University was supported by Grant CA-06140 from the National Cancer Institute. The work at The Upjohn Co. was in part supported by Contract PH 43-68-1023 with Chemotherapy, National Cancer Institute, National Institutes of Health, Bethesda, Md.

(1) T. Higuchi and S. Chulkaratana, through T. Higuchi and K. A. Connors, in "Advances in Analytical Chemistry and Instrumentation," 4th ed., C. N. Reilley, Ed., Interscience, New York, N. Y., 1965, p. 117.
(2) D. P. Munro and R. B. Stoughton, *Arch. Dermatal.*, **92**, 585(1965).
(3) J. Reid and D. B. Brookes, *Brit. J. Dermatol.*, **80**, 328(1968).
(4) J. T. Doluisio, N. F. Billups, L. W. Dittert, E. T. Sugita, and J. V. Swintosky, *J. Pharm. Sci.*, **58**, 1196(1969).
(5) C. C. Porter and R. H. Silber, *J. Biol. Chem.*, **185**, 201(1950).

WILLIAM L. HAYTON
DAVID E. GUTTMAN*
GERHARD LEVY
Department of Pharmaceutics
School of Pharmacy
State University of New York at Buffalo
Buffalo, NY 14214

Received November 14, 1969.
Accepted for publication January 13, 1970.
Supported in part by Fellowship No. 1 FO1 GM-43160-01 for W.L.H. from the U.S. Public Health Service.

* Present address: Smith Kline & French Labs., Philadelphia, PA 19101

Synthesis and Antibacterial Activity of 1-Styryl-3,4-dihydroisoquinolines

Keyphrases □ 1-Styryl-3,4-dihydroisoquinolines—synthesis □ Antibacterial activity—1-styryl-3,4-dihydroisoquinolines

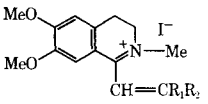
Sir:

So far, the synthesis of 1-styrylisoquinolines has been achieved either by the cyclization of the Schiff bases derived from cinnamaldehyde (1, 2) or by the condensation of 1-methylisoquinoline with aromatic aldehydes (3). In this communication, we wish to report a new procedure for the synthesis of 1-styryl-3,4-dihydroisoquinolines which were isolated as methiodide salts. The procedure involves the cyclodehydration of substituted β-phenethylamides to 3,4-dihydroisoquinolines through the Bischler-Napieralski reaction (4).

Details about the synthesis and characterization of 1-styryl-3,4-dihydroisoquinoline methiodides (Compounds 1–7) will be published (5).

The methiodide salts (Compounds 1–7) were subjected to *in vitro* screening for antimetabolites by a new method (6). In this method, the detection system utilizes the gram-positive *Bacillus subtilis* and gram-negative *Escherichia coli*. Both organisms were grown in two types of agar: nutrient agar and a completely synthetic medium with glucose as the only source of carbon.

Table I—Inhibition of *B. subtilis* Grown in Two Different Media[a]



Compound	R_1	R_2	Nutrient Agar	Synthetic Agar
1	Ph	Ph	24	35
2	Ph	*p*-Methylphenyl	25	36
3	*p*-Methylphenyl	*p*-Methylphenyl	29	35
4	*p*-Ethylphenyl	*p*-Ethylphenyl	36	39
5	*p*-Chlorophenyl	*p*-Chlorophenyl	28	35
6	Me	Ph	16	22
7	Me	Me	0	0

[a] The numbers in the body of the table are zones of growth inhibition in mm. around a 13-mm. paper disk.

These seven compounds were tested at concentrations of 1 mg./ml., and the results are presented in Table I.

The inhibition of test organism by Compounds 1–6 was stronger on synthetic agar than on nutrient agar. However, the difference was not large enough to suggest an antimetabolitelike mode of action (5). Compound 7 was essentially inactive against *B. subtilis*. None of the compounds inhibited the growth of *E. coli*. These results indicate that 1-styryl-3,4-dihydroisoquinoline methiodides possess some antibacterial activity.

A more extensive testing will be required before any structure–activity correlation can be drawn.

(1) E. C. Weinback and W. H. Hartung, *J. Org. Chem.*, **15**, 676(1950); W. M. Whaley and T. R. Govindachari, "Organic Reactions," vol. 6, Wiley, New York, N. Y., 1962, p. 151.
(2) W. H. Mills and J. L. B. Smith, *J. Chem. Soc.*, **121**, 2724(1922).
(3) T. Kametani, T. Terui, T. Ogino, and K. Fukumoto, *J. Chem. Soc. (C)*, **1969**, 874.
(4) F. Bergmann, M. Weizmann, E. Dimant, J. Patai, and J. Szmuskowica, *J. Amer. Chem. Soc.*, **70**, 1612(1948).
(5) R. E. Harmon, B. L. Jensen, S. K. Gupta, and J. Nelson, to be published.
(6) L. J. Hanka, "Abstracts," Fifth International Congress of Chemotherapy, Vienna, Austria, July 1967, B g/2, 351.

R. E. HARMON
B. L. JENSEN
S. K. GUPTA
Department of Chemistry
Western Michigan University
Kalamazoo, MI 49001
and
L. J. HANKA
Research Laboratories
The Upjohn Co.
Kalamazoo, MI 49001

Received November 24, 1969.
Accepted for publication February 3, 1970.
The work at Western Michigan University was supported by Grant CA-06140 from the National Cancer Institute. The work at The Upjohn Co. was in part supported by Contract PH 43-68-1023 with Chemotherapy, National Cancer Institute, National Institutes of Health, Bethesda, Md.

Books

REVIEWS

Methodenlehre der therapeutisch-klinischen Forschung. By P. MARTINI, G. OBERHOFFER, and E. WELTE. 4th ed. Springer-Verlag, New York, N. Y., 1968. viii + 495 pp. Price $37.00. (In German)

The 4th, new, and completely revised edition of the original book of the late P. Martini is the legacy of this pioneer of exact clinical-therapeutic research. The three basic requirements for meaningful research are reliable clinical standards, the exclusion of secondary complicating factors, and the use of mathematical comparisons for the objective evaluation of clinical results. The discussion of these aspects is the thematic center of the book of Martini, Oberhoffer, and Welte. The large amount of scientific information is very well organized and clearly written.

After an interesting historical review the fundamentals of clinical therapeutic research in the experimental phase and the unity of clinical and experimental research are discussed in Chapters 2 and 3. In particular the causality and expediency of medical research and especially the common necessary suppositions in experiments are treated. In a special, very instructive chapter, important co-factors ("Mitursachen") in therapeutic problems are described. In particular the exclusion of these disturbing factors and the role of side effects in clinical research are characterized. A large part of the book deals with the problems of statistics, data processing, and documentation. It is up-to-date and presented in such a way that it is easily understandable even for nonspecialized readers. The fundamental principles of statistics, the influence of variables on the mean value and deviation, data processing, and programming as applied in clinical research are presented. The largest part of the book is concerned with the special methodology of clinical-therapeutic research in selected diseases such as scarlet fever, diphtheria, malaria, hepatic and lung diseases, high blood pressure, heart insufficiency, kidney and gastric diseases, and many others. These discussions are particularly oriented toward the selection of reliable clinical standards and the application of mathematical comparisons. The influence of individual, collective, and psychological factors in some special organ diseases are also discussed.

The appendix contains instructive statistical tables, computer programs, and a detailed literature index and also a separate author register. This arrangement appears to be effective and useful. The copiously equipped book is recommended to everyone working in the field of medical research and data processing.

Reviewed by: Joachim K. Seydel
Department of Pharmaceutical Chemistry
Forschungsinstitut Borstel
Institut für Experimentelle Biologie und Medizin
Hamburg, West Germany ■

Aromatic Nucleophilic Substitution. By JOSEPH MILLER, in **Reaction Mechanisms in Organic Chemistry,** monograph 8. Edited by C. EABORN and N. B. CHAPMAN, Elsevier Publishing Co., 335 Jarvan Galenstraat, P.O. Box 211, Amsterdam, The Netherlands, 1968. xi + 408 pp. 14.5 × 22 cm. Price $23.50.

This monograph represents a comprehensive review and interpretation, both quantitative and qualitative, of a multiplicity of aromatic nucleophilic substitution reactions including S_N1, activated S_N2 and benzyne mechanisms, charge-transfer and covalent complexes. These mechanisms as well as a brief summary of side reactions, kinetic evidence, and energetics of substitution reactions are introduced in Chapter 1 (26 pp.). Chapter 2 (11 pp.) considers the S_N1 mechanism in greater detail; Chapter 3 (18 pp.) similarly discusses the benzyne or elimination-addition mechanism. The last Chapter 9 (23 pp.) considers less common aromatic S_N reactions while Chapter 7 (73 pp.) summarizes and interprets results obtained when studying nucleophilic substitution in nonbenzenoid aromatic systems. Comparison is made between homo- and heterocyclic organic systems; heterocyclic systems are desirably categorized as π-excessive and π-deficient. Considerations of inorganic and other nonbenzenoid aromatic systems (tropylium ions, quinones, *etc.*) conclude Chapter 7.

While the entire monograph is well written, this reviewer found Chapter 4 (72 pp.) concerning substituent effects in aromatic S_N reactions to be presented in a particularly interesting and informative manner. Pan-activating substituents, which is a term introduced by the author to "denote a substituent which is able to activate electrophilic, nucleophilic and radical substitution in an aromatic ring to which it is attached," are discussed along with a consideration of the effects of most functional groups on S_N *versus* S_E reactions. Hammett and related treatments, Dewar's proposal concerning the mode by which a substituent may affect a distant reaction center, the effect of *o*, *p*, *m* and two or more substituents on aromatic S_N reactions are topics discussed in a complete and concise manner. Substituents are classified into eight groups dependent upon their chemical nature; the influence of each group of functions is comparatively discussed when they are bonded *p*, *o*, and *m* to the leaving group.

Chapter 5 (40 pp.) dealing with the leaving group, Chapter 6 (30 pp.) concerning the nucleophile, and Chapter 8 (47 pp.), which considers the medium (solvent), catalytic and steric effects, are very informative and are of interest to synthetic as well as theoretical chemists. Subjects discussed in these chapters are treated quantitatively and qualitatively in terms of aromatic S_N1 and S_N2 reactions. Chapter 8 also considers photo and metal ion catalysis. Experimental results, along with their interpretation, are presented in all chapters. The monograph is well referenced into 1967 and is not only of value to the practicing chemist, but to the student who is presently studying theoretical organic chemistry on a graduate level.

Reviewed by Donald T. Witiak
Division of Medicinal Chemistry
College of Pharmacy
The Ohio State University
Columbus, OH 43210 ■

The Chemistry and Physics of High Energy Reactions. By ERNEST J. HENLEY and EVERETT R. JOHNSON. University Press, 6411 Chillum Place, N.W., Washington, D.C., 1969. vi + 475 pp. 15 × 23 cm. Price $18.50.

This volume is intended to provide a coherent textbook as an easy entree for the novice in the field of radiation chemistry. The material is presented in thirteen chapters in a high level, mathematical manner. Material is presented on units, radiation, radioactivity, and general nomenclature; interaction of radiation with matter; radiation sources; and radiation dosimetry. Other topics include radiation chemistry of gases, solids, water, and aqueous systems, as well as the effects of radiation on liquid hydrocarbons, polymers, and radiation-induced polymerizations.

Throughout the book the authors insert numerous example problems which are very useful for the comprehension of concepts. Also included are a multitude of tables and figures containing significant data. The text is broad in scope, rather cursory on certain topics, but provides excellent in-depth information on many areas of radiation chemistry. Although it is the stated intent of the authors to limit references, additional citations in areas discussed briefly would be helpful to the reader.

In summary, this book is a complete, well-prepared text which is most valuable as a reference or textbook for the radiation chemist and as a reference for the radiobiologist.

Reviewed by Stanley M. Shaw
School of Pharmacy
and Pharmacal Sciences
Purdue University
Lafayette, IN 47907 ■

Pharmaceutics—Galenical Pharmacy. By ERIK SANDELL, translated from Swedish by R. PAMELA ZACHARIAS. 364 pp. 16 × 23 cm. May be obtained by payment through international money order addressed to E. Sandell, postal giro account number 15 84 49, Postgirokontaret, Stockholm 1, Sweden.

Professor Sandell intends to present a "basic course in Pharmaceutics and Galenical Pharmacy and . . . to direct more specialized

Books

REVIEWS

Methodenlehre der therapeutisch-klinischen Forschung. By P. MARTINI, G. OBERHOFFER, and E. WELTE. 4th ed. Springer-Verlag, New York, N. Y., 1968. viii + 495 pp. Price $37.00. (In German)

The 4th, new, and completely revised edition of the original book of the late P. Martini is the legacy of this pioneer of exact clinical-therapeutic research. The three basic requirements for meaningful research are reliable clinical standards, the exclusion of secondary complicating factors, and the use of mathematical comparisons for the objective evaluation of clinical results. The discussion of these aspects is the thematic center of the book of Martini, Oberhoffer, and Welte. The large amount of scientific information is very well organized and clearly written.

After an interesting historical review the fundamentals of clinical therapeutic research in the experimental phase and the unity of clinical and experimental research are discussed in Chapters 2 and 3. In particular the causality and expediency of medical research and especially the common necessary suppositions in experiments are treated. In a special, very instructive chapter, important co-factors ("Mitursachen") in therapeutic problems are described. In particular the exclusion of these disturbing factors and the role of side effects in clinical research are characterized. A large part of the book deals with the problems of statistics, data processing, and documentation. It is up-to-date and presented in such a way that it is easily understandable even for nonspecialized readers. The fundamental principles of statistics, the influence of variables on the mean value and deviation, data processing, and programming as applied in clinical research are presented. The largest part of the book is concerned with the special methodology of clinical-therapeutic research in selected diseases such as scarlet fever, diphtheria, malaria, hepatic and lung diseases, high blood pressure, heart insufficiency, kidney and gastric diseases, and many others. These discussions are particularly oriented toward the selection of reliable clinical standards and the application of mathematical comparisons. The influence of individual, collective, and psychological factors in some special organ diseases are also discussed.

The appendix contains instructive statistical tables, computer programs, and a detailed literature index and also a separate author register. This arrangement appears to be effective and useful. The copiously equipped book is recommended to everyone working in the field of medical research and data processing.

Reviewed by: Joachim K. Seydel
Department of Pharmaceutical Chemistry
Forschungsinstitut Borstel
Institut für Experimentelle Biologie und Medizin
Hamburg, West Germany ■

Aromatic Nucleophilic Substitution. By JOSEPH MILLER, in **Reaction Mechanisms in Organic Chemistry,** monograph 8. Edited by C. EABORN and N. B. CHAPMAN, Elsevier Publishing Co., 335 Jarvan Galenstraat, P.O. Box 211, Amsterdam, The Netherlands, 1968. xi + 408 pp. 14.5 × 22 cm. Price $23.50.

This monograph represents a comprehensive review and interpretation, both quantitative and qualitative, of a multiplicity of aromatic nucleophilic substitution reactions including S_N1, activated S_N2 and benzyne mechanisms, charge-transfer and covalent complexes. These mechanisms as well as a brief summary of side reactions, kinetic evidence, and energetics of substitution reactions are introduced in Chapter 1 (26 pp.). Chapter 2 (11 pp.) considers the S_N1 mechanism in greater detail; Chapter 3 (18 pp.) similarly discusses the benzyne or elimination-addition mechanism. The last Chapter 9 (23 pp.) considers less common aromatic S_N reactions while Chapter 7 (73 pp.) summarizes and interprets results obtained when studying nucleophilic substitution in nonbenzenoid aromatic systems. Comparison is made between homo- and heterocyclic organic systems; heterocyclic systems are desirably categorized as π-excessive and π-deficient. Considerations of inorganic and other nonbenzenoid aromatic systems (tropylium ions, quinones, *etc.*) conclude Chapter 7.

While the entire monograph is well written, this reviewer found Chapter 4 (72 pp.) concerning substituent effects in aromatic S_N reactions to be presented in a particularly interesting and informative manner. Pan-activating substituents, which is a term introduced by the author to "denote a substituent which is able to activate electrophilic, nucleophilic and radical substitution in an aromatic ring to which it is attached," are discussed along with a consideration of the effects of most functional groups on S_N *versus* S_E reactions. Hammett and related treatments, Dewar's proposal concerning the mode by which a substituent may affect a distant reaction center, the effect of *o*, *p*, *m* and two or more substituents on aromatic S_N reactions are topics discussed in a complete and concise manner. Substituents are classified into eight groups dependent upon their chemical nature; the influence of each group of functions is comparatively discussed when they are bonded *p*, *o*, and *m* to the leaving group.

Chapter 5 (40 pp.) dealing with the leaving group, Chapter 6 (30 pp.) concerning the nucleophile, and Chapter 8 (47 pp.), which considers the medium (solvent), catalytic and steric effects, are very informative and are of interest to synthetic as well as theoretical chemists. Subjects discussed in these chapters are treated quantitatively and qualitatively in terms of aromatic S_N1 and S_N2 reactions. Chapter 8 also considers photo and metal ion catalysis. Experimental results, along with their interpretation, are presented in all chapters. The monograph is well referenced into 1967 and is not only of value to the practicing chemist, but to the student who is presently studying theoretical organic chemistry on a graduate level.

Reviewed by Donald T. Witiak
Division of Medicinal Chemistry
College of Pharmacy
The Ohio State University
Columbus, OH 43210 ■

The Chemistry and Physics of High Energy Reactions. By ERNEST J. HENLEY and EVERETT R. JOHNSON. University Press, 6411 Chillum Place, N.W., Washington, D.C., 1969. vi + 475 pp. 15 × 23 cm. Price $18.50.

This volume is intended to provide a coherent textbook as an easy entree for the novice in the field of radiation chemistry. The material is presented in thirteen chapters in a high level, mathematical manner. Material is presented on units, radiation, radioactivity, and general nomenclature; interaction of radiation with matter; radiation sources; and radiation dosimetry. Other topics include radiation chemistry of gases, solids, water, and aqueous systems, as well as the effects of radiation on liquid hydrocarbons, polymers, and radiation-induced polymerizations.

Throughout the book the authors insert numerous example problems which are very useful for the comprehension of concepts. Also included are a multitude of tables and figures containing significant data. The text is broad in scope, rather cursory on certain topics, but provides excellent in-depth information on many areas of radiation chemistry. Although it is the stated intent of the authors to limit references, additional citations in areas discussed briefly would be helpful to the reader.

In summary, this book is a complete, well-prepared text which is most valuable as a reference or textbook for the radiation chemist and as a reference for the radiobiologist.

Reviewed by Stanley M. Shaw
School of Pharmacy
and Pharmacal Sciences
Purdue University
Lafayette, IN 47907 ■

Pharmaceutics—Galenical Pharmacy. By ERIK SANDELL, translated from Swedish by R. PAMELA ZACHARIAS. 364 pp. 16 × 23 cm. May be obtained by payment through international money order addressed to E. Sandell, postal giro account number 15 84 49, Postgirokontaret, Stockholm 1, Sweden.

Professor Sandell intends to present a "basic course in Pharmaceutics and Galenical Pharmacy and . . . to direct more specialized

Books

REVIEWS

Methodenlehre der therapeutisch-klinischen Forschung. By P. MARTINI, G. OBERHOFFER, and E. WELTE. 4th ed. Springer-Verlag, New York, N. Y., 1968. viii + 495 pp. Price $37.00. (In German)

The 4th, new, and completely revised edition of the original book of the late P. Martini is the legacy of this pioneer of exact clinical-therapeutic research. The three basic requirements for meaningful research are reliable clinical standards, the exclusion of secondary complicating factors, and the use of mathematical comparisons for the objective evaluation of clinical results. The discussion of these aspects is the thematic center of the book of Martini, Oberhoffer, and Welte. The large amount of scientific information is very well organized and clearly written.

After an interesting historical review the fundamentals of clinical therapeutic research in the experimental phase and the unity of clinical and experimental research are discussed in Chapters 2 and 3. In particular the causality and expediency of medical research and especially the common necessary suppositions in experiments are treated. In a special, very instructive chapter, important co-factors ("Mitursachen") in therapeutic problems are described. In particular the exclusion of these disturbing factors and the role of side effects in clinical research are characterized. A large part of the book deals with the problems of statistics, data processing, and documentation. It is up-to-date and presented in such a way that it is easily understandable even for nonspecialized readers. The fundamental principles of statistics, the influence of variables on the mean value and deviation, data processing, and programming as applied in clinical research are presented. The largest part of the book is concerned with the special methodology of clinical-therapeutic research in selected diseases such as scarlet fever, diphtheria, malaria, hepatic and lung diseases, high blood pressure, heart insufficiency, kidney and gastric diseases, and many others. These discussions are particularly oriented toward the selection of reliable clinical standards and the application of mathematical comparisons. The influence of individual, collective, and psychological factors in some special organ diseases are also discussed.

The appendix contains instructive statistical tables, computer programs, and a detailed literature index and also a separate author register. This arrangement appears to be effective and useful. The copiously equipped book is recommended to everyone working in the field of medical research and data processing.

Reviewed by: Joachim K. Seydel
Department of Pharmaceutical Chemistry
Forschungsinstitut Borstel
Institut für Experimentelle Biologie und Medizin
Hamburg, West Germany ■

Aromatic Nucleophilic Substitution. By JOSEPH MILLER, in **Reaction Mechanisms in Organic Chemistry,** monograph 8. Edited by C. EABORN and N. B. CHAPMAN, Elsevier Publishing Co., 335 Jarvan Galenstraat, P.O. Box 211, Amsterdam, The Netherlands, 1968. xi + 408 pp. 14.5 × 22 cm. Price $23.50.

This monograph represents a comprehensive review and interpretation, both quantitative and qualitative, of a multiplicity of aromatic nucleophilic substitution reactions including S_N1, activated S_N2 and benzyne mechanisms, charge-transfer and covalent complexes. These mechanisms as well as a brief summary of side reactions, kinetic evidence, and energetics of substitution reactions are introduced in Chapter 1 (26 pp.). Chapter 2 (11 pp.) considers the S_N1 mechanism in greater detail; Chapter 3 (18 pp.) similarly discusses the benzyne or elimination-addition mechanism. The last Chapter 9 (23 pp.) considers less common aromatic S_N reactions while Chapter 7 (73 pp.) summarizes and interprets results obtained when studying nucleophilic substitution in nonbenzenoid aromatic systems. Comparison is made between homo- and heterocyclic organic systems; heterocyclic systems are desirably categorized as π-excessive and π-deficient. Considerations of inorganic and other nonbenzenoid aromatic systems (tropylium ions, quinones, *etc.*) conclude Chapter 7.

While the entire monograph is well written, this reviewer found Chapter 4 (72 pp.) concerning substituent effects in aromatic S_N reactions to be presented in a particularly interesting and informative manner. Pan-activating substituents, which is a term introduced by the author to "denote a substituent which is able to activate electrophilic, nucleophilic and radical substitution in an aromatic ring to which it is attached," are discussed along with a consideration of the effects of most functional groups on S_N *versus* S_E reactions. Hammett and related treatments, Dewar's proposal concerning the mode by which a substituent may affect a distant reaction center, the effect of *o*, *p*, *m* and two or more substituents on aromatic S_N reactions are topics discussed in a complete and concise manner. Substituents are classified into eight groups dependent upon their chemical nature; the influence of each group of functions is comparatively discussed when they are bonded *p*, *o*, and *m* to the leaving group.

Chapter 5 (40 pp.) dealing with the leaving group, Chapter 6 (30 pp.) concerning the nucleophile, and Chapter 8 (47 pp.), which considers the medium (solvent), catalytic and steric effects, are very informative and are of interest to synthetic as well as theoretical chemists. Subjects discussed in these chapters are treated quantitatively and qualitatively in terms of aromatic S_N1 and S_N2 reactions. Chapter 8 also considers photo and metal ion catalysis. Experimental results, along with their interpretation, are presented in all chapters. The monograph is well referenced into 1967 and is not only of value to the practicing chemist, but to the student who is presently studying theoretical organic chemistry on a graduate level.

Reviewed by Donald T. Witiak
Division of Medicinal Chemistry
College of Pharmacy
The Ohio State University
Columbus, OH 43210 ■

The Chemistry and Physics of High Energy Reactions. By ERNEST J. HENLEY and EVERETT R. JOHNSON. University Press, 6411 Chillum Place, N.W., Washington, D.C., 1969. vi + 475 pp. 15 × 23 cm. Price $18.50.

This volume is intended to provide a coherent textbook as an easy entree for the novice in the field of radiation chemistry. The material is presented in thirteen chapters in a high level, mathematical manner. Material is presented on units, radiation, radioactivity, and general nomenclature; interaction of radiation with matter; radiation sources; and radiation dosimetry. Other topics include radiation chemistry of gases, solids, water, and aqueous systems, as well as the effects of radiation on liquid hydrocarbons, polymers, and radiation-induced polymerizations.

Throughout the book the authors insert numerous example problems which are very useful for the comprehension of concepts. Also included are a multitude of tables and figures containing significant data. The text is broad in scope, rather cursory on certain topics, but provides excellent in-depth information on many areas of radiation chemistry. Although it is the stated intent of the authors to limit references, additional citations in areas discussed briefly would be helpful to the reader.

In summary, this book is a complete, well-prepared text which is most valuable as a reference or textbook for the radiation chemist and as a reference for the radiobiologist.

Reviewed by Stanley M. Shaw
School of Pharmacy
and Pharmacal Sciences
Purdue University
Lafayette, IN 47907 ■

Pharmaceutics—Galenical Pharmacy. By ERIK SANDELL, translated from Swedish by R. PAMELA ZACHARIAS. 364 pp. 16 × 23 cm. May be obtained by payment through international money order addressed to E. Sandell, postal giro account number 15 84 49, Postgirokontaret, Stockholm 1, Sweden.

Professor Sandell intends to present a "basic course in Pharmaceutics and Galenical Pharmacy and . . . to direct more specialized

Books

REVIEWS

Methodenlehre der therapeutisch-klinischen Forschung. By P. MARTINI, G. OBERHOFFER, and E. WELTE. 4th ed. Springer-Verlag, New York, N. Y., 1968. viii + 495 pp. Price $37.00. (In German)

The 4th, new, and completely revised edition of the original book of the late P. Martini is the legacy of this pioneer of exact clinical-therapeutic research. The three basic requirements for meaningful research are reliable clinical standards, the exclusion of secondary complicating factors, and the use of mathematical comparisons for the objective evaluation of clinical results. The discussion of these aspects is the thematic center of the book of Martini, Oberhoffer, and Welte. The large amount of scientific information is very well organized and clearly written.

After an interesting historical review the fundamentals of clinical therapeutic research in the experimental phase and the unity of clinical and experimental research are discussed in Chapters 2 and 3. In particular the causality and expediency of medical research and especially the common necessary suppositions in experiments are treated. In a special, very instructive chapter, important co-factors ("Mitursachen") in therapeutic problems are described. In particular the exclusion of these disturbing factors and the role of side effects in clinical research are characterized. A large part of the book deals with the problems of statistics, data processing, and documentation. It is up-to-date and presented in such a way that it is easily understandable even for nonspecialized readers. The fundamental principles of statistics, the influence of variables on the mean value and deviation, data processing, and programming as applied in clinical research are presented. The largest part of the book is concerned with the special methodology of clinical-therapeutic research in selected diseases such as scarlet fever, diphtheria, malaria, hepatic and lung diseases, high blood pressure, heart insufficiency, kidney and gastric diseases, and many others. These discussions are particularly oriented toward the selection of reliable clinical standards and the application of mathematical comparisons. The influence of individual, collective, and psychological factors in some special organ diseases are also discussed.

The appendix contains instructive statistical tables, computer programs, and a detailed literature index and also a separate author register. This arrangement appears to be effective and useful. The copiously equipped book is recommended to everyone working in the field of medical research and data processing.

Reviewed by: Joachim K. Seydel
Department of Pharmaceutical Chemistry
Forschungsinstitut Borstel
Institut für Experimentelle Biologie und Medizin
Hamburg, West Germany ■

Aromatic Nucleophilic Substitution. By JOSEPH MILLER, in **Reaction Mechanisms in Organic Chemistry,** monograph 8. Edited by C. EABORN and N. B. CHAPMAN, Elsevier Publishing Co., 335 Jarvan Galenstraat, P.O. Box 211, Amsterdam, The Netherlands, 1968. xi + 408 pp. 14.5 × 22 cm. Price $23.50.

This monograph represents a comprehensive review and interpretation, both quantitative and qualitative, of a multiplicity of aromatic nucleophilic substitution reactions including S_N1, activated S_N2 and benzyne mechanisms, charge-transfer and covalent complexes. These mechanisms as well as a brief summary of side reactions, kinetic evidence, and energetics of substitution reactions are introduced in Chapter 1 (26 pp.). Chapter 2 (11 pp.) considers the S_N1 mechanism in greater detail; Chapter 3 (18 pp.) similarly discusses the benzyne or elimination-addition mechanism. The last Chapter 9 (23 pp.) considers less common aromatic S_N reactions while Chapter 7 (73 pp.) summarizes and interprets results obtained when studying nucleophilic substitution in nonbenzenoid aromatic systems. Comparison is made between homo- and heterocyclic organic systems; heterocyclic systems are desirably categorized as π-excessive and π-deficient. Considerations of inorganic and other nonbenzenoid aromatic systems (tropylium ions, quinones, *etc.*) conclude Chapter 7.

While the entire monograph is well written, this reviewer found Chapter 4 (72 pp.) concerning substituent effects in aromatic S_N reactions to be presented in a particularly interesting and informative manner. Pan-activating substituents, which is a term introduced by the author to "denote a substituent which is able to activate electrophilic, nucleophilic and radical substitution in an aromatic ring to which it is attached," are discussed along with a consideration of the effects of most functional groups on S_N *versus* S_E reactions. Hammett and related treatments, Dewar's proposal concerning the mode by which a substituent may affect a distant reaction center, the effect of *o*, *p*, *m* and two or more substituents on aromatic S_N reactions are topics discussed in a complete and concise manner. Substituents are classified into eight groups dependent upon their chemical nature; the influence of each group of functions is comparatively discussed when they are bonded *p*, *o*, and *m* to the leaving group.

Chapter 5 (40 pp.) dealing with the leaving group, Chapter 6 (30 pp.) concerning the nucleophile, and Chapter 8 (47 pp.), which considers the medium (solvent), catalytic and steric effects, are very informative and are of interest to synthetic as well as theoretical chemists. Subjects discussed in these chapters are treated quantitatively and qualitatively in terms of aromatic S_N1 and S_N2 reactions. Chapter 8 also considers photo and metal ion catalysis. Experimental results, along with their interpretation, are presented in all chapters. The monograph is well referenced into 1967 and is not only of value to the practicing chemist, but to the student who is presently studying theoretical organic chemistry on a graduate level.

Reviewed by Donald T. Witiak
Division of Medicinal Chemistry
College of Pharmacy
The Ohio State University
Columbus, OH 43210 ■

The Chemistry and Physics of High Energy Reactions. By ERNEST J. HENLEY and EVERETT R. JOHNSON. University Press, 6411 Chillum Place, N.W., Washington, D.C., 1969. vi + 475 pp. 15 × 23 cm. Price $18.50.

This volume is intended to provide a coherent textbook as an easy entree for the novice in the field of radiation chemistry. The material is presented in thirteen chapters in a high level, mathematical manner. Material is presented on units, radiation, radioactivity, and general nomenclature; interaction of radiation with matter; radiation sources; and radiation dosimetry. Other topics include radiation chemistry of gases, solids, water, and aqueous systems, as well as the effects of radiation on liquid hydrocarbons, polymers, and radiation-induced polymerizations.

Throughout the book the authors insert numerous example problems which are very useful for the comprehension of concepts. Also included are a multitude of tables and figures containing significant data. The text is broad in scope, rather cursory on certain topics, but provides excellent in-depth information on many areas of radiation chemistry. Although it is the stated intent of the authors to limit references, additional citations in areas discussed briefly would be helpful to the reader.

In summary, this book is a complete, well-prepared text which is most valuable as a reference or textbook for the radiation chemist and as a reference for the radiobiologist.

Reviewed by Stanley M. Shaw
School of Pharmacy
and Pharmacal Sciences
Purdue University
Lafayette, IN 47907 ■

Pharmaceutics—Galenical Pharmacy. By ERIK SANDELL, translated from Swedish by R. PAMELA ZACHARIAS. 364 pp. 16 × 23 cm. May be obtained by payment through international money order addressed to E. Sandell, postal giro account number 15 84 49, Postgirokontaret, Stockholm 1, Sweden.

Professor Sandell intends to present a "basic course in Pharmaceutics and Galenical Pharmacy and . . . to direct more specialized

studies through reference to monographs, other texts and journal publications."

His text touches on many areas of pharmaceutics: small scale manufacturing, preparation of medicinals official in the Scandinavian countries and, in certain instances, rationale of dosage form.

Professor Sandell has had various levels of success in covering a wide range of topics; some, in a manner not usual, but in a way which would be welcomed, in the typical "American" text. However, the treatment of theory and certain topics is somewhat abbreviated and the approach to some of the topics is at an intermediate rather than at a basic level. It seems that the author, in his attempt to cover a wide range of topics, has included, for example, areas such as *Honeys* and *Lamillae* and has given only a cursory treatment of *Isotonicity* and *Hydrogen Ion Concentration.* On the other hand there is a short, but welcomed, discussion of *Jet Injection.*

Almost every section has some references which may as intended, direct the reader to a more specialized study of the topic being considered. However, students in the United States would find it difficult to abstract the many foreign language publications that are cited.

Generally speaking, the choice of topics and their sequence of presentation is logical. What is disturbing in a basic text is to find different levels of treatment of the material. It may be, as Professor Sandell states that, "this text-book is . . . typically Scandinavian . . .," *i.e.*, geared to their educational systems.

Although this reviewer cannot recommend *Pharmaceutics—Galenical Pharmacy* as a text for undergraduates in this country, there is merit in the author's approach to several topics.

Reviewed by L. A. Luzzi
School of Pharmacy
University of Georgia
Athens, GA 30601 ■

An Introduction to Chromatography on Impregnated Glass Fiber. By FREDERICK C. HAER. Ann Arbor Science Publishers, Inc., Box 1425, Ann Arbor, MI 48106, 1969. xiv + 173 pp. 15 × 23 cm. Price $2.50.

The author describes the nature and application of a variation of thin-layer chromatography known commercially as "instant TLC." This rapid method has attracted minor usage, but is growing due to the present availability of several forms of impregnated adsorbents.

The information is set out in eleven short chapters. Theory, general techniques, equipment, and history are described in the first four chapters. Individual chapters are devoted to major chemical groupings of lipids, amino acids, steroids, sugars, and nucleic acids. A separate chapter treats toxicological problems. Each chapter includes complete procedures for sample handling, isolation, and purification prior to chromatography. General and theoretical aspects are handled clearly and are supported by illustrations that are well chosen and executed. Drugs are not treated extensively, and pharmaceuticals not at all. The reference value of this book is limited largely to biochemistry, although other applications have been published.

This book is a handy laboratory manual and should be available in any laboratory using instant TLC. It supplements the well-known texts on TLC and paper chromatography. It is written clearly and illustrated well, and it has adequate indexing and referencing.

Reviewed by Lee T. Grady
Drug Standards Laboratory
American Pharmaceutical Association Foundation
2215 Constitution Ave., N.W.
Washington, DC 20037 ■

Proceedings of the Institute on Drug Literature Evaluation. Conducted by the American Society of Hospital Pharmacists. American Society of Hospital Pharmacists, 4630 Montgomery Ave., Washington, DC 20014, 1969. x + 192 pp. 21.3 × 27.7 cm. Price $7.00.

The hospital pharmacist, as a drug information specialist, confronts the problem of locating sources of good clinical information on drugs and then interpreting and evaluating this information.

As stated in the Preface, the American Society of Hospital Pharmacists conducted the Institute on Drug Literature Evaluation in March 1968, to offer presentations and a forum for discussion of topics designed to assist interested hospital pharmacists in becoming more proficient in the evaluation, understanding, and use of the clinical drug literature.

Seventeen presentations of the Institute are given in this book, covering various topics of clinical pharmacy and literature evaluation.

Staff Review ■

NOTICES

Subsidia Pharmaceutica 1968. Edited by Laboratory of the Swiss Pharmaceutical Society. Swiss Pharmaceutical Society, Marktgasse 52, 3011 Bern, Switzerland, 1968. 253 pp. 17.4 × 25 cm. Price Sw. fr. 75.—plus postage.

WHO Expert Committee on Specifications for Pharmaceutical Preparations: Twenty-second Report. World Health Organization Technical Report Series, No. 418. World Health Organization, 1211 Geneva 27, Switzerland, 1969. 29 pp. 16 × 24 cm. Price 6/-. $1.00, Sw. fr. 3.—.

A Comprehensive Guide to the English-Language Literature on Cannabis. By JAMES R. GAMAGE and EDMUND L. ZERKIN. Stash Press, The Student Association for the Study of Hallucinogens, Inc., 638 Pleasant St., Beloit, WI 53511, 1969. xi + 265 pp. 22 × 30 cm. Price $5.95.

Addendum 1969 to the British Pharmacopoeia 1968. By General Medical Council. The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England, 1969. xx + 100 pp. 14 × 22 cm. Price £3 5s.

Deutsches Arzneibuch 7. Ausg. 1968 Kommentar. By H. BOHME and K. HARTKE. Wissenschaftliche Verlagsgesellschaft MBH Stuttgart 1, Postfach 40, Birkenwaldstrabe 44, 1969. xvi + 1619 pp. 17.2 × 24.4 cm. Price DM 196,-.

The Biochemistry of Folic Acid and Related Pteridines. By R. L. BLAKLEY. North Holland Import, Wiley, 605 Third Ave., New York, NY 10016, 1969. xxi + 569 pp. 17.3 × 24.7 cm. Price $29.00.

Welfare Medical Care: An Experiment. By CHARLES H. GOODRICH, M. C. OLENDZKI, and G. G. READER. Harvard University Press, Cambridge, Mass., 1970. viii + 343 pp. 16.5 × 24 cm. Price $7.00.

Genetics of Antibiotic-producing Microorganisms. By G. SERMONTI. Wiley, 605 Third Ave., New York, NY 10016, 1969. xiii + 389 pp. 15.5 × 23.5 cm. Price $18.00.

Practical Optical Crystallography, 2nd Edition. By N. H. HARTSHORNE and A. STUART. American Elsevier Publishing Co., Inc., 52 Vanderbilt Ave., New York, NY 10017, 1970. ix + 326 pp. 14 × 22 cm. Price $11.50 [First edition reviewed in *J. Pharm. Sci.*, **54,** 166(1965)].

Medicaments Organiques De Synthese II. By LEON VELLUZ. Masson et Cie, Editeurs, 120 Boulevard Saint-Germain, Paris, France, 1970. iii + 236 pp. 15 × 23 cm. Price 80 fr.

Encyclopedia of Industrial Chemical Analysis. Vol 8. By FOSTER DEE SNELL and LESLIE S. ETTRE. Wiley, 605 Third Ave., New York, NY 10016, 1969. xvi + 737 pp. 18.5 × 26 cm. Price $35.00, Subscription; $45.00, Single Copy.

Pharmaceutical Abstracts. Vol. IX, No. 4, Vol. X, No. 1 & 2, and Vol. X, No. 3. Edited by HENRY M. BURLAGE. College of Pharmacy, The University of Texas, Austin, TX 78712, 1968. 21.7 × 27.5 cm. Price $6.00, $5.80, and $4.50, respectively. Paperbound.

Verstandliche Wissenschaft, Band 99. By H. WAGNER and RAUSCHGIFT-DROGEN. Springer-Verlag, 1 Berlin 33, 1969. vii + 141 pp. 11.5 × 18.5 cm. Price $2.20.

Clinical Pharmacology (Dilling). By STANLEY ALSTEAD, J. GORDON MACARTHUR, and THOMAS J. THOMSON. Bailliere Tindall & Cassell, 7–8 Henrietta St., London, W.C. 2, England, 1969. xii + 760 pp. 12.5 × 19 cm. Price $7.25.

Bacterial Episomes and Plasmids. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Co., 34 Beacon St., Boston, MA 02106, 1969. xi + 268 pp. 15.5 × 23.5 cm. Price $12.50.

studies through reference to monographs, other texts and journal publications."

His text touches on many areas of pharmaceutics: small scale manufacturing, preparation of medicinals official in the Scandinavian countries and, in certain instances, rationale of dosage form.

Professor Sandell has had various levels of success in covering a wide range of topics; some, in a manner not usual, but in a way which would be welcomed, in the typical "American" text. However, the treatment of theory and certain topics is somewhat abbreviated and the approach to some of the topics is at an intermediate rather than at a basic level. It seems that the author, in his attempt to cover a wide range of topics, has included, for example, areas such as *Honeys* and *Lamillae* and has given only a cursory treatment of *Isotonicity* and *Hydrogen Ion Concentration.* On the other hand there is a short, but welcomed, discussion of *Jet Injection.*

Almost every section has some references which may as intended, direct the reader to a more specialized study of the topic being considered. However, students in the United States would find it difficult to abstract the many foreign language publications that are cited.

Generally speaking, the choice of topics and their sequence of presentation is logical. What is disturbing in a basic text is to find different levels of treatment of the material. It may be, as Professor Sandell states that, "this text-book is . . . typically Scandinavian . . .," *i.e.*, geared to their educational systems.

Although this reviewer cannot recommend *Pharmaceutics—Galenical Pharmacy* as a text for undergraduates in this country, there is merit in the author's approach to several topics.

Reviewed by L. A. Luzzi
School of Pharmacy
University of Georgia
Athens, GA 30601 ■

An Introduction to Chromatography on Impregnated Glass Fiber. By FREDERICK C. HAER. Ann Arbor Science Publishers, Inc., Box 1425, Ann Arbor, MI 48106, 1969. xiv + 173 pp. 15 × 23 cm. Price $2.50.

The author describes the nature and application of a variation of thin-layer chromatography known commercially as "instant TLC." This rapid method has attracted minor usage, but is growing due to the present availability of several forms of impregnated adsorbents.

The information is set out in eleven short chapters. Theory, general techniques, equipment, and history are described in the first four chapters. Individual chapters are devoted to major chemical groupings of lipids, amino acids, steroids, sugars, and nucleic acids. A separate chapter treats toxicological problems. Each chapter includes complete procedures for sample handling, isolation, and purification prior to chromatography. General and theoretical aspects are handled clearly and are supported by illustrations that are well chosen and executed. Drugs are not treated extensively, and pharmaceuticals not at all. The reference value of this book is limited largely to biochemistry, although other applications have been published.

This book is a handy laboratory manual and should be available in any laboratory using instant TLC. It supplements the well-known texts on TLC and paper chromatography. It is written clearly and illustrated well, and it has adequate indexing and referencing.

Reviewed by Lee T. Grady
Drug Standards Laboratory
American Pharmaceutical Association Foundation
2215 Constitution Ave., N.W.
Washington, DC 20037 ■

Proceedings of the Institute on Drug Literature Evaluation. Conducted by the American Society of Hospital Pharmacists. American Society of Hospital Pharmacists, 4630 Montgomery Ave., Washington, DC 20014, 1969. x + 192 pp. 21.3 × 27.7 cm. Price $7.00.

The hospital pharmacist, as a drug information specialist, confronts the problem of locating sources of good clinical information on drugs and then interpreting and evaluating this information.

As stated in the Preface, the American Society of Hospital Pharmacists conducted the Institute on Drug Literature Evaluation in March 1968, to offer presentations and a forum for discussion of topics designed to assist interested hospital pharmacists in becoming more proficient in the evaluation, understanding, and use of the clinical drug literature.

Seventeen presentations of the Institute are given in this book, covering various topics of clinical pharmacy and literature evaluation.

Staff Review ■

NOTICES

Subsidia Pharmaceutica 1968. Edited by Laboratory of the Swiss Pharmaceutical Society. Swiss Pharmaceutical Society, Marktgasse 52, 3011 Bern, Switzerland, 1968. 253 pp. 17.4 × 25 cm. Price Sw. fr. 75.—plus postage.

WHO Expert Committee on Specifications for Pharmaceutical Preparations: Twenty-second Report. World Health Organization Technical Report Series, No. 418. World Health Organization, 1211 Geneva 27, Switzerland, 1969. 29 pp. 16 × 24 cm. Price 6/-. $1.00, Sw. fr. 3.—.

A Comprehensive Guide to the English-Language Literature on Cannabis. By JAMES R. GAMAGE and EDMUND L. ZERKIN. Stash Press, The Student Association for the Study of Hallucinogens, Inc., 638 Pleasant St., Beloit, WI 53511, 1969. xi + 265 pp. 22 × 30 cm. Price $5.95.

Addendum 1969 to the British Pharmacopoeia 1968. By General Medical Council. The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England, 1969. xx + 100 pp. 14 × 22 cm. Price £3 5s.

Deutsches Arzneibuch 7. Ausg. 1968 Kommentar. By H. BOHME and K. HARTKE. Wissenschaftliche Verlagsgesellschaft MBH Stuttgart 1, Postfach 40, Birkenwaldstrabe 44, 1969. xvi + 1619 pp. 17.2 × 24.4 cm. Price DM 196,-.

The Biochemistry of Folic Acid and Related Pteridines. By R. L. BLAKLEY. North Holland Import, Wiley, 605 Third Ave., New York, NY 10016, 1969. xxi + 569 pp. 17.3 × 24.7 cm. Price $29.00.

Welfare Medical Care: An Experiment. By CHARLES H. GOODRICH, M. C. OLENDZKI, and G. G. READER. Harvard University Press, Cambridge, Mass., 1970. viii + 343 pp. 16.5 × 24 cm. Price $7.00.

Genetics of Antibiotic-producing Microorganisms. By G. SERMONTI. Wiley, 605 Third Ave., New York, NY 10016, 1969. xiii + 389 pp. 15.5 × 23.5 cm. Price $18.00.

Practical Optical Crystallography, 2nd Edition. By N. H. HARTSHORNE and A. STUART. American Elsevier Publishing Co., Inc., 52 Vanderbilt Ave., New York, NY 10017, 1970. ix + 326 pp. 14 × 22 cm. Price $11.50 [First edition reviewed in *J. Pharm. Sci.*, **54,** 166(1965)].

Medicaments Organiques De Synthese II. By LEON VELLUZ. Masson et Cie, Editeurs, 120 Boulevard Saint-Germain, Paris, France, 1970. iii + 236 pp. 15 × 23 cm. Price 80 fr.

Encyclopedia of Industrial Chemical Analysis. Vol 8. By FOSTER DEE SNELL and LESLIE S. ETTRE. Wiley, 605 Third Ave., New York, NY 10016, 1969. xvi + 737 pp. 18.5 × 26 cm. Price $35.00, Subscription; $45.00, Single Copy.

Pharmaceutical Abstracts. Vol. IX, No. 4, Vol. X, No. 1 & 2, and Vol. X, No. 3. Edited by HENRY M. BURLAGE. College of Pharmacy, The University of Texas, Austin, TX 78712, 1968. 21.7 × 27.5 cm. Price $6.00, $5.80, and $4.50, respectively. Paperbound.

Verstandliche Wissenschaft, Band 99. By H. WAGNER and RAUSCHGIFT-DROGEN. Springer-Verlag, 1 Berlin 33, 1969. vii + 141 pp. 11.5 × 18.5 cm. Price $2.20.

Clinical Pharmacology (Dilling). By STANLEY ALSTEAD, J. GORDON MACARTHUR, and THOMAS J. THOMSON. Bailliere Tindall & Cassell, 7–8 Henrietta St., London, W.C. 2, England, 1969. xii + 760 pp. 12.5 × 19 cm. Price $7.25.

Bacterial Episomes and Plasmids. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Co., 34 Beacon St., Boston, MA 02106, 1969. xi + 268 pp. 15.5 × 23.5 cm. Price $12.50.

studies through reference to monographs, other texts and journal publications."

His text touches on many areas of pharmaceutics: small scale manufacturing, preparation of medicinals official in the Scandinavian countries and, in certain instances, rationale of dosage form.

Professor Sandell has had various levels of success in covering a wide range of topics; some, in a manner not usual, but in a way which would be welcomed, in the typical "American" text. However, the treatment of theory and certain topics is somewhat abbreviated and the approach to some of the topics is at an intermediate rather than at a basic level. It seems that the author, in his attempt to cover a wide range of topics, has included, for example, areas such as *Honeys* and *Lamillae* and has given only a cursory treatment of *Isotonicity* and *Hydrogen Ion Concentration.* On the other hand there is a short, but welcomed, discussion of *Jet Injection.*

Almost every section has some references which may as intended, direct the reader to a more specialized study of the topic being considered. However, students in the United States would find it difficult to abstract the many foreign language publications that are cited.

Generally speaking, the choice of topics and their sequence of presentation is logical. What is disturbing in a basic text is to find different levels of treatment of the material. It may be, as Professor Sandell states that, "this text-book is . . . typically Scandinavian . . .," *i.e.*, geared to their educational systems.

Although this reviewer cannot recommend *Pharmaceutics—Galenical Pharmacy* as a text for undergraduates in this country, there is merit in the author's approach to several topics.

Reviewed by L. A. Luzzi
School of Pharmacy
University of Georgia
Athens, GA 30601 ■

An Introduction to Chromatography on Impregnated Glass Fiber. By FREDERICK C. HAER. Ann Arbor Science Publishers, Inc., Box 1425, Ann Arbor, MI 48106, 1969. xiv + 173 pp. 15 × 23 cm. Price $2.50.

The author describes the nature and application of a variation of thin-layer chromatography known commercially as "instant TLC." This rapid method has attracted minor usage, but is growing due to the present availability of several forms of impregnated adsorbents.

The information is set out in eleven short chapters. Theory, general techniques, equipment, and history are described in the first four chapters. Individual chapters are devoted to major chemical groupings of lipids, amino acids, steroids, sugars, and nucleic acids. A separate chapter treats toxicological problems. Each chapter includes complete procedures for sample handling, isolation, and purification prior to chromatography. General and theoretical aspects are handled clearly and are supported by illustrations that are well chosen and executed. Drugs are not treated extensively, and pharmaceuticals not at all. The reference value of this book is limited largely to biochemistry, although other applications have been published.

This book is a handy laboratory manual and should be available in any laboratory using instant TLC. It supplements the well-known texts on TLC and paper chromatography. It is written clearly and illustrated well, and it has adequate indexing and referencing.

Reviewed by Lee T. Grady
Drug Standards Laboratory
American Pharmaceutical Association Foundation
2215 Constitution Ave., N.W.
Washington, DC 20037 ■

Proceedings of the Institute on Drug Literature Evaluation. Conducted by the American Society of Hospital Pharmacists. American Society of Hospital Pharmacists, 4630 Montgomery Ave., Washington, DC 20014, 1969. x + 192 pp. 21.3 × 27.7 cm. Price $7.00.

The hospital pharmacist, as a drug information specialist, confronts the problem of locating sources of good clinical information on drugs and then interpreting and evaluating this information.

As stated in the Preface, the American Society of Hospital Pharmacists conducted the Institute on Drug Literature Evaluation in March 1968, to offer presentations and a forum for discussion of topics designed to assist interested hospital pharmacists in becoming more proficient in the evaluation, understanding, and use of the clinical drug literature.

Seventeen presentations of the Institute are given in this book, covering various topics of clinical pharmacy and literature evaluation.

Staff Review ■

NOTICES

Subsidia Pharmaceutica 1968. Edited by Laboratory of the Swiss Pharmaceutical Society. Swiss Pharmaceutical Society, Marktgasse 52, 3011 Bern, Switzerland, 1968. 253 pp. 17.4 × 25 cm. Price Sw. fr. 75.—plus postage.

WHO Expert Committee on Specifications for Pharmaceutical Preparations: Twenty-second Report. World Health Organization Technical Report Series, No. 418. World Health Organization, 1211 Geneva 27, Switzerland, 1969. 29 pp. 16 × 24 cm. Price 6/-. $1.00, Sw. fr. 3.—.

A Comprehensive Guide to the English-Language Literature on Cannabis. By JAMES R. GAMAGE and EDMUND L. ZERKIN. Stash Press, The Student Association for the Study of Hallucinogens, Inc., 638 Pleasant St., Beloit, WI 53511, 1969. xi + 265 pp. 22 × 30 cm. Price $5.95.

Addendum 1969 to the British Pharmacopoeia 1968. By General Medical Council. The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England, 1969. xx + 100 pp. 14 × 22 cm. Price £3 5s.

Deutsches Arzneibuch 7. Ausg. 1968 Kommentar. By H. BOHME and K. HARTKE. Wissenschaftliche Verlagsgesellschaft MBH Stuttgart 1, Postfach 40, Birkenwaldstrabe 44, 1969. xvi + 1619 pp. 17.2 × 24.4 cm. Price DM 196,-.

The Biochemistry of Folic Acid and Related Pteridines. By R. L. BLAKLEY. North Holland Import, Wiley, 605 Third Ave., New York, NY 10016, 1969. xxi + 569 pp. 17.3 × 24.7 cm. Price $29.00.

Welfare Medical Care: An Experiment. By CHARLES H. GOODRICH, M. C. OLENDZKI, and G. G. READER. Harvard University Press, Cambridge, Mass., 1970. viii + 343 pp. 16.5 × 24 cm. Price $7.00.

Genetics of Antibiotic-producing Microorganisms. By G. SERMONTI. Wiley, 605 Third Ave., New York, NY 10016, 1969. xiii + 389 pp. 15.5 × 23.5 cm. Price $18.00.

Practical Optical Crystallography, 2nd Edition. By N. H. HARTSHORNE and A. STUART. American Elsevier Publishing Co., Inc., 52 Vanderbilt Ave., New York, NY 10017, 1970. ix + 326 pp. 14 × 22 cm. Price $11.50 [First edition reviewed in *J. Pharm. Sci.*, **54,** 166(1965)].

Medicaments Organiques De Synthese II. By LEON VELLUZ. Masson et Cie, Editeurs, 120 Boulevard Saint-Germain, Paris, France, 1970. iii + 236 pp. 15 × 23 cm. Price 80 fr.

Encyclopedia of Industrial Chemical Analysis. Vol 8. By FOSTER DEE SNELL and LESLIE S. ETTRE. Wiley, 605 Third Ave., New York, NY 10016, 1969. xvi + 737 pp. 18.5 × 26 cm. Price $35.00, Subscription; $45.00, Single Copy.

Pharmaceutical Abstracts. Vol. IX, No. 4, Vol. X, No. 1 & 2, and Vol. X, No. 3. Edited by HENRY M. BURLAGE. College of Pharmacy, The University of Texas, Austin, TX 78712, 1968. 21.7 × 27.5 cm. Price $6.00, $5.80, and $4.50, respectively. Paperbound.

Verstandliche Wissenschaft, Band 99. By H. WAGNER and RAUSCHGIFT-DROGEN. Springer-Verlag, 1 Berlin 33, 1969. vii + 141 pp. 11.5 × 18.5 cm. Price $2.20.

Clinical Pharmacology (Dilling). By STANLEY ALSTEAD, J. GORDON MACARTHUR, and THOMAS J. THOMSON. Bailliere Tindall & Cassell, 7–8 Henrietta St., London, W.C. 2, England, 1969. xii + 760 pp. 12.5 × 19 cm. Price $7.25.

Bacterial Episomes and Plasmids. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Co., 34 Beacon St., Boston, MA 02106, 1969. xi + 268 pp. 15.5 × 23.5 cm. Price $12.50.

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

MAY 1970
VOLUME 59 NUMBER 5

REVIEW ARTICLE

Mechanisms of Surfactant Effects on Drug Absorption

MILO GIBALDI and STUART FELDMAN

Keyphrases ☐ Absorption, drug—surfactant effect ☐ Surfactant effect, drug absorption—mechanism ☐ Solubility, drug—surfactant effect ☐ Dissolution rate—surfactant effect ☐ Gastric emptying—surfactant effect ☐ Membrane permeation—surfactant effect ☐ Physiologic surfactants—gastrointestinal drug absorption

Surface-active agents are one of the most important groups of adjuvants in pharmaceutical preparations. One or another of these agents has been used, for diverse reasons, in almost every type of dosage form. The widespread use of surfactants as well as their unique physical-chemical properties has prompted considerable interest in the possibile influence of these agents on drug absorption. Attention has been focused to inadvertent effects on drug absorption which may result from the inclusion of a surface-active agent in a given formulation as well as to the use of surfactants in a deliberate attempt to modify drug absorption.

The drug literature contains a number of reports which clearly demonstrate that surface-active agents can influence the rate and extent of absorption of certain drugs. However, enhancement as well as inhibition of the absorption and pharmacologic activity of drugs has been observed in the presence of surfactants. Many of these reports have been reviewed by Blanpin (1). A more recent compilation has been provided by Swisher (2) in a review primarily concerned with the environmental exposure levels and toxicity of surfactants. As noted by Levy (3), much of the difficulty in interpreting some of these studies has been due to the different types of effects which surface-active agents can exert. These effects include interaction with biologic membranes and modification of membrane permeability, interaction with the drug, interaction with the dosage form, and interaction with the organism itself resulting in a pharmacologic effect which may in turn influence drug absorption. These effects may be operative at the same time, some tending to enhance drug absorption, others tending to retard it, and the net effect dependent on the relative magnitude of each.

This article is intended as a selective, rather than exhaustive, review of the drug literature which illustrates the major mechanisms of surfactant activity affecting drug absorption. Several critical determinants of the rate and extent of drug absorption—*viz.*, drug solubility and dissolution rate, gastric emptying, and membrane permeation, are considered with respect to the influence of surface-active agents. Finally, attention is given to the role of physiologic surfactants in the gastrointestinal absorption of drugs.

ROLE OF DRUG SOLUBILITY AND DISSOLUTION RATE IN GASTROINTESTINAL ABSORPTION

When a drug is administered orally in solid form (tablet, capsule, or suspension) or intramuscularly as a pellet or suspension, one frequently finds that the rate of absorption is controlled by the slowest step in the following sequence:

$$\text{solid drug} \xrightarrow{\text{dissolution}} \text{drug in solution} \xrightarrow{\text{absorption}} \text{absorbed drug}$$

In many instances the slowest or rate-limiting step is found to be dissolution of drug in the fluids at the ab-

sorption site. When dissolution is the controlling step in the overall process, absorption is said to be dissolution rate limited. Since the rate-limiting step in the absorption process is the dissolution step, any factor influencing the rate of solution must influence also the rate of absorption. According to dissolution theory (4–6), two important parameters determining the dissolution rate of a solid in a given solvent are the solubility of the drug in the dissolution medium and the surface area of the drug exposed to the medium.

The molecules of many surface-active agents tend to aggregate in aqueous solutions when some bulk concentration, termed the critical micelle concentration (CMC), is exceeded. These aggregates, known as micelles, frequently demonstrate a marked tendency to associate with and solubilize organic and inorganic solutes. The phenomenon of micellar solubilization has been reviewed by Swarbrick (7) and is the subject of a recent text (8). In view of the relationship between solubility and dissolution rate as formulated in the Noyes-Whitney equation (4), the enhanced solubility of a drug in a micellar solution of surfactant should result in a proportional increase in the dissolution rate. While this proportionality is never realized in practice (because of the failure of the Noyes-Whitney relationship to account for changes in the effective diffusion coefficient of the drug), the increase in apparent solubility will usually result in an increase in dissolution rate.

The dissolution rate of a drug, regardless of dissolution mechanism, is always directly proportional to the effective surface area of the drug, *i.e.*, the surface area of drug available to the dissolution fluids. The relationship between surface area, dissolution rates, and gastrointestinal absorption rates has been reviewed by Levy (9) and, more recently, by Fincher (10).

The effective surface area of a drug is usually much smaller than the specific surface area which is an idealized *in vitro* measurement. Many drugs whose dissolution characteristics could be improved by particle size reduction are extremely hydrophobic and may resist wetting by gastrointestinal fluids. Therefore, the gastrointestinal fluids may come in intimate contact with only a fraction of the potentially available surface area. The effective surface area of hydrophobic drug particles can often be increased by the addition of a surface-active agent to the formulation, which functions to reduce the contact angle between the solid and the gastrointestinal fluids. Reduction in contact angle permits more intimate contact of drug and fluids, thereby increasing effective surface area and dissolution rate.

In 1948, Kellner *et al.* (11) studied the effect of polysorbate 80 (polyoxyethylene sorbitan monooleate) on the *in vivo* absorption of cholesterol. These workers reported that rabbits fed polysorbate 80 and cholesterol developed blood cholesterol levels that were two to three times as high as those obtained by feeding cholesterol alone. The reason suggested for this marked increase in cholesterol absorption in the presence of the surfactant was an improved emulsification of cholesterol in the intestinal tract and more efficient absorption. However, solubilization of cholesterol by polysorbate 80 and increased dissolution rate of the water-insoluble compound could also account for the observed effects.

Another example of a possible surfactant effect on drug absorption involving solubilization and increased dissolution rate is found in the work of Fuchs and Ingelfinger (12). These workers observed that sodium lauryl sulfate "hastened the appearance and increased the levels of vitamin A in the blood of human subjects." Krause (13) reported that incorporation of sodium lauryl sulfate in G-strophanthin pills resulted in an increase in absorption of the drug in dogs, guinea pigs, rabbits, and cats. The author postulated that an increase in solubility and a higher rate of dissolution of the drug in the presence of the surfactant were responsible for the increase in pharmacologic effect noted with the formulation.

In a study concerned with the oral absorption of spironolactone, Gantt *et al.* (14) found that when polysorbate 80 was administered with the steroid, gastrointestinal absorption was markedly improved. One explanation for the observed effects is an increase in the dissolution rate of the drug due to solubilization and/or wetting effects by the surfactant. However, changes in the formulation and manufacture of the dosage form upon incorporation of the surfactant may have also played a role in the enhanced absorption (15).

Kakemi *et al.* (16) studied the rectal absorption of sulfonamides in the presence of several nonionic surface-active agents. In experiments where sulfisoxazole was administered in the form of suspensions containing varying concentrations of polysorbate 80, it was found that the blood level after 1 hr. increased with increasing concentrations of surface-active agent up to a maximum polysorbate 80 concentration of 20%, the concentration of surfactant which completely solubilized the excess drug. Comparison of relative drug solubility in surfactant solutions and relative blood levels indicates that an 18-fold increase in sulfisoxazole solubility in the presence of 20% polysorbate 80 results in a threefold increase in initial blood level compared to the level following the administration of the control suspension.

A number of studies have attempted to quantitate the relationship between drug solubility in micellar solutions and dissolution rate. Bates *et al.* (17) reported substantial increases in the dissolution rates of griseofulvin and hexestrol in micellar solutions of bile salts. Bates *et al.* (18) have also shown that physiologic concentrations of lysolecithin, a phospholipid, produce marked increases in the solubility and dissolution rate of hexestrol, dienestrol, and griseofulvin. The results of these studies are in general agreement with the Noyes-Whitney relationship (4). However, in each case, because of the extremely low solubilities of the drugs, dissolution was followed over a concentration range which was above the saturation solubility of the drug in water.

Dissolution studies conducted under "sink conditions" (*i.e.*, drug concentration in solution does not exceed 10 to 20% of saturation solubility in buffer) in micellar solutions clearly show that dissolution rate is not proportional to the apparent solubility of the drug. This was first demonstrated by Higuchi (5), who found that the ratio of dissolution rate of benzocaine in polysorbate 80 solution to that without the surfactant was substantially lower than the ratio predicted by the Noyes-Whitney theory. These results have been sup-

ported by the findings of Parrott and Sharma (19), Gibaldi *et al.* (20), and Elworthy and Lipscomb (21).

In 1964, Higuchi (22) presented a theoretical analysis pertinent to the influence of interacting colloids, such as micelles, on mass transport. Higuchi concluded that dissolution rate (d.r.) in micellar solution under sink conditions could be described by

$$\text{d.r.} = \frac{DC_s}{h} + \frac{D_mC_m}{h} \qquad \text{(Eq. 1)}$$

where D and D_m are the diffusion coefficients for the free drug and micelle-solubilized drug species, respectively, C_s is the solubility of the drug, C_m is the solubility increase due to solubilization, and h is the effective diffusion layer thickness. The influence of solubilization is determined by calculating the ratio (R) of dissolution rate in surfactant solution to that in pure solvent and by assuming that certain constants, such as h, have essentially the same value under each experimental condition. It follows that

$$R = \frac{DC_s + D_mC_m}{DC_s} \qquad \text{(Eq. 2)}$$

Rewriting Eq. 2 according to Singh *et al.* (23):

$$R = \frac{D_{\text{eff.}}C_t}{DC_s} \qquad \text{(Eq. 3)}$$

where C_t is the total solubility of the drug in the surfactant solution $D_{\text{eff.}} = (DC_s + D_mC_m)/C_t$. According to Eq. 3, at any degree of micellar solubilization the larger the molecular size of the micelle-drug species formed upon interaction, the smaller will be the influence of solubilization on the dissolution rate. Hence the potential influence of enhanced solubility on dissolution rate in a micellar solution is offset by the expected small diffusion coefficient of the micelle-drug species. In fact, it is plausible to consider that in some cases $D_{\text{eff.}} \cong DC_s/C_t$ and that the surfactant would have virtually no effect on dissolution rate. Higuchi (22) further predicted that the magnitude of effects of interacting colloids on dissolution rate will approach that on solubility only when the drug concentration in solution approaches or exceeds the solubility in pure solvent. The larger effects on dissolution rate predicted above saturation solubility are evident in the reports of Bates *et al.* (17, 18) and Wurster and Polli (24).

Gibaldi *et al.* (20) studied the effect of micellar solutions of a nonionic surfactant on the dissolution rate of benzoic acid using the rotating-disk and static-disk methods. Their results suggested that Eq. 3 was not applicable to all dissolution systems since the influence of the surfactant on dissolution rate was substantially greater in the static-disk method studies than in the rotating-disk method studies. The role of hydrodynamics in assessing the influence of colloidal solubilizers on dissolution rate was subsequently quantified by Singh *et al.* (23). These workers found that the influence of surfactants on dissolution rate using the stirred and static-disk methods could be described by Eqs. 4 and 5, respectively:

$$R = \frac{(D_{\text{eff.}})^{2/3}C_t}{D^{2/3}C_s} \qquad \text{(Eq. 4)}$$

$$R = \frac{(D_{\text{eff.}})^{1/2}C_t}{D^{1/2}C_s} \qquad \text{(Eq. 5)}$$

Equation 3 described the situation reasonably well only when dissolution occurred under conditions of apparent laminar flow provided by a propeller-driven stirrer apparatus.

These findings were confirmed by Gibaldi *et al.* (25) in their studies on the dissolution of benzoic and salicylic acids in micellar solutions of polyoxyethylene lauryl ether. Their results also suggest that the influence of micellar solubilization on the dissolution rate of drugs from conventional dosage forms will be significantly greater than that predicted by diffusion layer theory (Eq. 3).

While the influence of micellar solubilization on dissolution rate has been studied rather extensively, the effect of low concentrations (below the CMC) of surface-active agent on the dissolution of drugs from powders and other solid dosage forms has been given limited attention. Finholt and Solvang (26) studied the dissolution of phenacetin powder dusted on the surface of 0.1 *N* HCl containing low concentrations of polysorbate 80. An increase in the polysorbate 80 concentration from 0 to 0.01% causes a significant increase in the dissolution rate. A linear relationship was observed when the time required to dissolve 10% of the powder was plotted as a function of the surface tension of the test solution. The effect of polysorbate 80 on the dissolution rate of phenacetin is caused mainly by its ability to reduce the contact angle between the powder and the dissolution medium.

More recently, Weintraub and Gibaldi (27) studied the influence of premicellar concentrations of a nonionic surfactant and certain physiologic surfactants on the dissolution rate of drugs from powders and from commercial dosage forms. Polyoxyethylene lauryl ether and lysolecithin enhanced the dissolution rate of powdered salicylic acid in 0.1 *N* HCl, and sodium glycocholate increased the dissolution rate of powdered salicylamide in pH 6.0 buffer. In each case the effect principally involved a "wetting" phenomenon rather than solubilization. Both the nonionic ether surfactant and lysolecithin enhanced the dissolution rate of aspirin from a tablet dosage form but were without effect on the dissolution rate of the drug from a capsule dosage form. Good correlation was found between surface tension lowering and the dissolution rate of aspirin from the tablet.

ROLE OF GASTRIC EMPTYING AND INTESTINAL TRANSIT IN DRUG ABSORPTION

The rate at which a drug leaves the stomach may have a profound influence on the overall rate and extent of drug absorption. For example, a weak base such as quinine will be absorbed primarily from the small intestine rather than from the stomach. Slow gastric emptying can also affect the biological availability of drugs that are unstable in gastric fluids.

An example of the effect of gastric emptying on drug absorption can be found in the work of Levy and Jusko (28). These workers studied the absorption of riboflavin in human subjects and found that administration of the vitamin after a test meal increased the urinary recovery

Table I—Influence of Bile Salts on Gastric Emptying of Phenol Red in the Rat

Test Solution	No. of Animals	Gastric Emptying,[a] % ± 1 *SD*
Control	5	76 ± 3
Sodium taurodeoxycholate		
26 m*M*	5	80 ± 5
50 m*M*	5	67 ± 10
100 m*M*	5	49 ± 8
Sodium deoxycholate		
5 m*M*	3	69 ± 4
10 m*M*	1	56
26 m*M*	4	17 ± 5

[a] Results expressed as percent of dose emptied 0.5 hr. after gastric intubation.

of the vitamin. Riboflavin is apparently absorbed by a specialized process high in the jejunum (28). Since the absorption process for riboflavin is capacity limited, the rate at which the vitamin passes the absorption site may have an influence on the overall extent of absorption. The authors postulate that a meal reduces the rate of gastric emptying and in turn the rate at which riboflavin reaches the site of specialized transport. This in turn results in an increase in the gastrointestinal absorption of the vitamin.

Varga (29) studied the effect of route of administration on the LD_{50} of chloroquine in rats. He found that there was a marked difference between the LD_{50} of chloroquine after gastric intubation (1080 mg./kg.) and intestinal intubation (210 mg./kg.). The difference in LD_{50} was attributed to the delayed gastric emptying resulting from a pharmacologic effect of chloroquine since little absorption of the drug occurs from the stomach.

Surface-active agents, particularly polyoxyethylene derivatives, may influence gastric emptying rate and intestinal transit by physically altering the viscosity of the gastrointestinal fluids. Levy and Jusko (30) have shown that an increase in the viscosity of the gastrointestinal fluids can decrease the absorption rate of certain drugs by retarding the diffusion of drug molecules to the absorbing membranes and by reducing gastrointestinal transit. Okuda *et al.* (31) studied the effects of nonionic surfactants on the intestinal absorption of vitamin B_{12}. These workers found that three of the surfactants studied enhanced the gastrointestinal absorption of the vitamin when the surfactant was administered undiluted in high doses. The enhancing effect of polysorbate 80, polysorbate 85 (polyoxyethylene sorbitan trioleate), and G-1096 (polyoxyethylene sorbitan hexaoleate) was postulated to be due to the formation of a highly viscous mass in the gastric and intestinal lumen which resulted in a delay in gastric emptying and an enhancement in the gastrointestinal absorption of vitamin B_{12}.

A surfactant may also exert a specific pharmacologic effect on the gastrointestinal tract which may influence drug absorption. For example, Lish (32) reported that dioctyl sodium sulfosuccinate, an anionic agent, inhibits the rate of propulsion of a test meal through the gastrointestinal tract of the rat. The effect was ascribed to a slowing of gastric emptying by the surfactant. The mechanism of action was thought to be mediated by a substance (or substances) formed after contact of the intestinal mucosa with the surface-active agent. In a recent study, Necheles and Sporn (33) found that there was an inhibition of gastric motility in the dog following introduction of certain detergents into the gastric pouch.

The influence of bile salts on gastrointestinal motility and transit has been the subject of several reports. Pannett and Wilson (34) first reported in 1921 that the addition of a small quantity of sodium taurocholate to a test meal is followed by an abnormally rapid evacuation of the stomach contents. They also found an increase in the secretion of acid in the presence of the bile salt. In a detailed study, Sasaki (35, 36) reported the effects of orally administered bile salts on the motility of the rabbit gastrointestinal tract. Sasaki (35) found that the effects of bile salts on gastric motility were extremely variable, with a slight increase in motility noted at low doses of bile salts and a small decrease in motility at higher dose levels. The effect of bile salts on intestinal motility (36) also was of a small order of magnitude. The bile salts usually produced a small increase in motility.

Recently, Feldman and Gibaldi (37) reported on the effect of orally administered bile salts on gastric emptying in the rat. Using phenol red as a marker substance, these workers found that both sodium deoxycholate and its taurine conjugate, sodium taurodeoxycholate, significantly decreased gastric emptying of the phenol red test solution (Table I). A significant difference was observed, however, in the relative influence of the bile salts on gastric emptying. The unconjugated bile salt produced results comparable to the conjugated derivative at about 0.1 to 0.2 the dose of the latter. Further studies (38) as to the influence of sodium deoxycholate on gastric emptying as a function of time after administration indicate that the bile salt markedly alters the pattern of gastric emptying. Gastric emptying of intubated phenol red in control rats proceeds in an apparent first-order manner with a half-life of about 13 min. In rats given sodium deoxycholate, an initially rapid gastric emptying phase, followed by a transition region where little emptying occurs, is noted over the 1st hour. The 2nd hour after intubation is characterized by an exponential decline in phenol red in the stomach, with a half-life of 36 min.

Intubation of sodium deoxycholate and sodium taurodeoxycholate also results in a large net secretion of fluids into the gastric pouch for at least 1 hr. after administration. The increase in gastric secretion and volume in the presence of bile salts offers an explanation for the observed decrease in gastric emptying. Hunt and MacDonald (39) have shown that as the volume of a test meal increases, the percentage of the gastric contents leaving the stomach per unit time becomes smaller. In addition, these workers noted that distension of the intestine reduces the coordinated propulsive activity of the gastric antrum as well as the duodenum. Thus, it appears likely that the reduced gastric emptying and transit rate through the proximal intestine induced by the administration of bile salts are related to the increase in the volume of fluid secreted into the gastric pouch.

The possibility exists that oral administration of bile salts may influence the absorption of drugs admin-

istered concurrently by modifying the pattern of gastric emptying. For example, the data of Mayersohn *et al.* (40) suggest that the increased absorption of riboflavin in man observed upon coadministration of sodium deoxycholate may be due, in part, to a decrease in gastric emptying. Administration of riboflavin with sodium deoxycholate results in both a pronounced increase in the peak urinary excretion rate of flavins as well as a marked delay in the occurrence of the peak rate compared to control studies (Fig. 1). A reduced rate of gastric emptying would result in prolonged and more complete absorption of the vitamin since absorption is limited to the proximal intestine and occurs *via* a "saturable" process (28, 41).

FACTORS INFLUENCING DRUG PERMEATION OF BIOLOGIC MEMBRANES

Two possible ways come to mind by which the rate of drug transfer from solution across biologic membranes may be altered. First, a change in the physical-chemical properties of the drug due to the presence of an additive could reduce or enhance the rate of transport and, second, an alteration in the permeability of the membrane could also influence transfer rate. Each of these possibilities is developed further in the subsequent paragraphs.

1. Drug–Adjuvant Interactions—A significant change in the ability of a drug to permeate a biologic membrane may result from an interaction with another molecule. A molecular complex consists of constituents held together by weak forces such as hydrogen bonds. This type of interaction is usually reversible, provided that the complex is sufficiently soluble in the biologic fluids. The properties of drug complexes, including solubility, molecular size, diffusiveness, and lipid–water partition coefficient, can differ significantly from the properties of the respective free drugs. These differences are responsible for the fact that many drug complexes cannot penetrate biologic membranes and, therefore, have no biologic activity (42). In such cases, the fraction of drug in the complex, which is in equilibrium with the noncomplexed drug, will be in an essentially nonabsorbable form and the effective concentration of drug will be less than the total concentration.

Surfactants may also interact with drug molecules and affect the gastrointestinal absorption of these compounds. Riegelman and Crowell (43–45) studied the effects of surfactants on the rectal absorption of iodoform, triiodophenol, and iodide in rats. Polysorbate 80 and sodium lauryl sulfate were found to decrease the rectal absorption rate of iodoform and triiodophenol, but to increase the absorption rate of iodide. The decrease in rectal absorption rate of iodoform and triiodophenol was attributed to micellar complexation of the drugs, while the increase in iodide absorption rate was postulated to be due to a cleansing action of the surfactant on the intestinal mucosa surface. Since iodide ion is lipid insoluble, it would not be expected to be incorporated into the surfactant micelles. Retardation of iodoform and triiodophenol absorption in the presence of micellar concentrations of the surfactants is in accord with the following model: (*a*) a micellar solution consists of two phases; (*b*) the partition ratio of drug between the micellar phase and the aqueous phase is constant, independent of drug concentration; and (*c*) absorption of the drug incorporated in the micelle is negligible. Since the drug in the micellar phase is unavailable for absorption, the effective concentration of the drug is less than the apparent concentration, and a decreased absorption rate is observed.

Levy and Reuning (46) studied the effect of micellar solutions of polysorbate 60 (polyoxyethylene sorbitan monostearate) on the absorption of ethanol and salicylic acid from the rat gastric pouch. These workers found that in the presence of 2% polysorbate 60 the absorption of salicylic acid was decreased from 50% in 1 hr. to 33% in 1 hr., while ethanol absorption remained unchanged. The observed effect was due to a decrease in activity of salicylic acid as a result of micellar complexation. The absorption of ethanol (which would not be incorporated into the surfactant micelles) was unaffected by the presence of the surfactant.

Kakemi *et al.* (16) studied the effect of various nonionic surface-active agents on the rectal absorption of sulfonamides from solution in the rat. At concentrations of the surfactant above the CMC, a reduction in the absorption rate of the sulfonamides was observed due to "entrapment" of drug in micelles. The experimental results were in agreement with the limiting case of the following theoretical equation, assuming that the solubilized drug was not absorbed to any appreciable extent.

$$A_T = \frac{A_f}{1 + K_m S} + \frac{A_m K_m S}{1 + K_m S} \qquad \text{(Eq. 6)}$$

where A_T, A_f, and A_m represent the absorption rates of both free and micelle-solubilized drug, free drug, and solubilized drug, respectively; S is the surfactant concentration (g./100 ml.); and K_m is a distribution constant equal to [drug-micelle]/[free drug]·[S].

Yamada and Yamamoto (47) found similar effects of micellar solutions of polysorbate 80 on the intestinal absorption of salicylamide in the perfused rat small intestine. Also, they observed no apparent effect of polysorbate 80 on the mucosal membrane, as determined by permeability experiments with salicylamide before and after a prolonged perfusion of the intestine with a

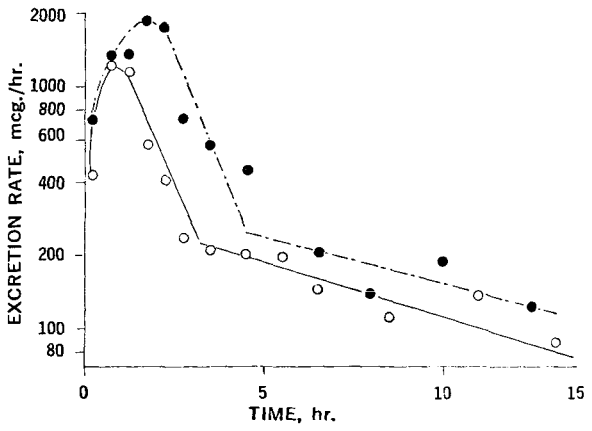


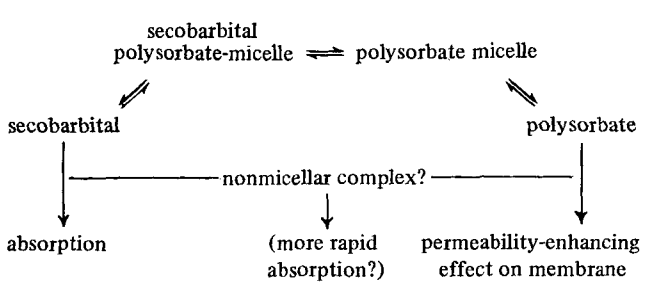
Figure 1—*Urinary excretion rate of apparent riboflavin after oral administration of 30 mg. riboflavin (Subject W.J.). Key: ○, control, and ●, 600 mg. sodium deoxycholate.*

polysorbate 80 solution. This technique, however, can only detect irreversible effects on membrane permeability. Matsumoto (48) offered essentially the same mechanism of micellar solubilization and a corresponding decrease in free drug concentration to explain the effect of polysorbate 80 on the intestinal absorption of sulfisoxazole in the rat. Saski (49) studied the effect of micellar solutions of tyloxapol, a nonionic surface-active polymer, on the transfer of hydrocortisone across the everted rat intestine. Drug transfer rate was inversely proportional to the surfactant concentration and the viscosity of the solution tested. The data suggest that the membrane is impermeable to the drug-micelle species.

2. Membrane Permeability—A number of substances have been found to "interact" with biologic membranes and thereby alter permeability or transport characteristics. For example, Schanker and Johnson (50) and Windsor and Cronheim (51) have shown that the chelating agent, ethylenediaminetetraacetic acid, can increase the *in vivo* intestinal absorption of a number of lipid-insoluble compounds in the rat. The depletion of calcium from the intestinal membranes by the chelating agent was suggested as the reason for these results.

Surfactants may also be capable of modifying the properties of biologic membranes. Alexander and Trim (52) reported that the penetration of hexylresorcinol into *Ascaris lumbricoides* can be affected by ionic surfactants in two different ways. Surfactant concentrations below the CMC increased the penetration of hexylresorcinol into the worm. These workers suggested that a complex between hexylresorcinol and surfactant monomer was responsible for the increase in concentration of the drug at the membrane surface. They did not consider the possibility that the site of action of the surfactant could be the membrane itself and that the increased penetration of the drug in the presence of the surfactant could be due to alteration of membrane permeability. At concentrations of surfactant above the CMC, the absorption of hexylresorcinol was reduced due to the formation of an unabsorbable micelle-drug complex.

Levy *et al.* (53) studied the effect of a nonionic surfactant, polysorbate 80, on the absorption of a number of barbiturates across the goldfish membranes. The absorption rate of the barbiturates was found to increase significantly in the presence of low concentrations (below the CMC) of surfactant and to decrease at higher concentrations of the surfactant. Scheme I was proposed to explain the results. Further studies by Levy and Anello (54) showed that the increase in absorption rate of secobarbital at concentrations of



Scheme I

polysorbate 80 below the CMC was due to an increase in the permeability of the biologic membranes rather than to formation of a more rapidly absorbed nonmicellar polysorbate–secobarbital complex. Anello and Levy (55) have also shown that premicellar concentrations of polysorbate 80 enhance the absorption and exsorption of 4-aminoantipyrine across the goldfish membranes. Gibaldi and Nightingale (56) studied the influence of sodium taurodeoxycholate on pharmacologic effect (time required to produce overturn) of pentobarbital and ethanol in goldfish. These workers found that the bile salt significantly potentiated the pharmacologic effect, presumably by modifying membrane permeability. Further studies (57) indicated that the bile salt exerts an all-or-none effect on the uptake of 4-aminoantipyrine in goldfish; an alteration in membrane permeability is observed above a certain bulk concentration but below the CMC of the surfactant. Whitworth and Yantis (58) found an increase in the absorption of salicylic acid across the external membranes of the frog in the presence of 0.1% polysorbate 80.

In a study of the percutaneous absorption of ionic surfactants, Scala *et al.* (59) found that the salt of a long-chain fatty acid, an alkylbenzene sulfonate, and dodecyltrimethyl ammonium chloride alter skin permeability as they diffuse into and through the skin. When nicotinate and thiourea were placed in the surfactant solution, the rate of diffusion of these compounds was found to increase with time, similar to the diffusion characteristics of the surfactants themselves. In the absence of surfactant, nicotinate and thiourea showed linear diffusion which remained constant over the time period studied. In a study of the effects of polysorbate 80 on the *in vitro* metabolism of Ehrlich-Lettre ascites carcinoma cells, Kay (60) found that the permeability of the cells was increased greatly in the presence of the surfactant as shown by the uptake of a dye, Lissamine green.

Appel *et al.* (61) found that simultaneous feeding of sodium lauryl sulfonate and inulin to rats results in up to a 10-fold increase in urinary inulin excretion over control values. The enhanced urinary excretion of inulin may be the result of an increase in intestinal permeability to inulin in the presence of the surface-active agent.

Mori *et al.* (62) reported that rats and hamsters fed polysorbate 20 (polyoxyethylene sorbitan monolaurate) showed increased gastrointestinal absorption of iron. However, Brise (63) in a later study reported that there was no effect of polysorbate 20 on iron absorption in man. He further postulated that the increase in absorption of iron in hamsters in the presence of polysorbate 20 observed by Mori *et al.* may have been due to some "toxic" action of the surfactant.

Suzuki *et al.* (64) found increased capillary permeability, as measured by a circulating dye, at the site of an intracutaneous injection of various nonionic surfactants. The authors concluded that this increase was mainly due to the wetting and solubilizing effects of the surfactants on the lipid structure of the capillary wall. Penzotti and Mattocks (65) found an increase in the rate of peritoneal dialysis of urea and creatinine in rabbits in the presence of surface-active agents. It was found that the order of magnitude of effects decreased in the following manner: cationic > anionic ≫ nonionic.

The possible mechanisms of action were not discussed, but it appears likely that the mechanism may involve a permeability alteration of the peritoneal membrane.

Nissim (66) studied the effect of feeding cationic, anionic, and nonionic surface-active agents on the histology of the mouse gastrointestinal tract. He found marked pathological changes when the ionic surfactants (cationic and anionic) were fed to mice but no effects when nonionic surfactants were tested. Taylor (67) studied the effects of cetyltrimethylammonium bromide on transport and metabolism in the small intestine of the rat. The surfactant was found to produce no histological damage to everted rat intestine sacs at concentrations of 10^{-4} *M*, but caused considerable injury to the mucosa at concentrations of 5×10^{-4} *M* and 10^{-3} *M*. Concomitant with the mucosal damage by the higher concentrations of the surfactant was an inhibition of the everted intestinal transport of glucose, methionine, and water.

Lish and Weikel (68) studied the effects of surface-active agents on the absorption of an anionic dye, phenol red, from the colon of the anesthetized rat. Anionic surfactants were found to enhance greatly absorption of the dye while a nonionic surfactant had no effect. None of the surfactants studied influenced the absorption of a cationic dye, methyl violet.

Matsuzawa *et al.* (69) found that the addition of nonionic surfactants (polysorbate 80 and a series of polyoxyethylene derivatives of hydrogenated castor oil) to solutions of the antibiotic, enduracidin hydrochloride, resulted in an increase in absorption of the antibiotic from the femoral muscles of the rat. The mechanism of action was not elucidated, but there is a strong possibility that the surfactant may have altered the permeability of the muscle to the antibiotic.

Engel and Riggi (70, 71) studied the effect of surfactants on the intestinal absorption of heparin in the rat. They found that intraduodenal administration of heparin with either sodium lauryl sulfate, dioctyl sodium sulfosuccinate, or G-3300 (an alkyl aryl sulfonate) resulted in an increase in heparin absorption over that observed when heparin was administered alone. These workers also reported enhanced heparin absorption in the presence of 0.4% sodium taurocholate. The authors postulate that the increase in heparin absorption is due to an effect of the surfactant on the intestinal mucosa.

Davenport (72) has reported that bile salts are capable of increasing the permeability of the gastric mucosa as judged by hydrogen-ion flux. The influence of an unconjugated bile salt, sodium deoxycholate, on the absorption of phenol red in the rat was recently studied by Feldman *et al.* (73) using three different techniques to assess absorption—*viz.*, urinary excretion after oral administration to intact animals, loss of drug from *in situ* intestinal loops, and transfer of drug across the isolated everted intestine. Each method showed that the bile salt markedly enhances the absorption or transfer rate of phenol red across the gastrointestinal membranes. The studies in intact animals indicated that the effect of sodium deoxycholate on gastrointestinal permeability was reversible.

Few studies of surfactant effects on drug absorption in man have been reported. Mayersohn *et al.* (40) found that when 600 mg. sodium deoxycholate is administered 30 min. prior to a 30-mg. dose of riboflavin, there is a 50 to 80% increase in total urinary recovery of apparent riboflavin. A similar but less marked enhancement was observed when the same dose of flavin mononucleotide was given with the bile salt. It is likely that change in the permeability of the gastrointestinal membranes is the principal mechanism of these effects, although in the case of riboflavin a reduction in gastric emptying may also play a role.

PHYSIOLOGIC SURFACTANTS AND DRUG ABSORPTION

Interest in the possible existence of a surfactant(s) in gastric fluid has been stimulated by the recent work of Finholt and Solvang (26). Samples of gastric juice obtained from patients under examination for diseases of the stomach showed low surface-tension values (38 to 47 dynes/cm.) and marked wetting activity as judged by powder dissolution studies. The rates of dissolution of powdered phenacetin in diluted gastric juice and in diluted HCl at the same pH and adjusted to the same surface tension with polysorbate 80 were similar but markedly faster than the rates observed in diluted HCl alone. Hence, the dissolution of hydrophobic drugs in the stomach may be facilitated by the presence of physiologic wetting agents.

The source of this surface activity in gastric fluids is not known, nor is it known whether or not gastric secretions themselves manifest surface activity. The presence of surface-active compounds in the gastric juice of some *Crustacea* was established by Vonk (74). Van den Oord *et al.* (75) reported that no bile salts could be detected in extracts of crab gastric juice. However, material with emulsifying properties was isolated; later, these compounds were shown to be fatty acylsarcosyltaurines (76). Further work by Van den Oord (77) suggested that the emulsifiers occurring in the gastric juice of the crab are of endogenous origin. Unfortunately, similar studies with human gastric juice are not available.

A possible source of the surface activity observed in human gastric fluid may be contamination from the duodenum. Reflux of duodenal contents would result in the presence of conjugated bile salts and lysolecithin, both highly surface active, in the gastric fluid. This possibility is strengthened by a recent report of Rhodes *et al.* (78) on the concentration of bile acids in the human stomach upon fasting and after a test meal.

Gastric fluid from normal fasting subjects contained a mean bile acid concentration of 0.08 ± 0.03 (*SE*) m*M*. The levels observed after a test meal were essentially the same. On the other hand, gastric fluids from fasting subjects with gastric ulcers contained a mean bile acid concentration of 0.65 ± 0.34 (*SE*) m*M*. For gastric ulcer patients, in contrast to normal subjects, the concentration of bile acids after food was always greater than the immediate preprandial value. These findings indicate that a small amount of reflux of intestinal contents into the stomach occurs normally and that the reflux of bile is increased considerably in patients with gastric ulcers. Since Finholt and Solvang (26) obtained gastric juice from patients under examination for diseases

Table II—Influence of Bile on Sulfadiazine Absorption from Intestinal Loops in the Rat

Experimental Condition	No. of Animals	% Absorbed in 3 hr. ± 1 *SD*
Controls	6	43 ± 8
Sham bile duct ligation	6	44 ± 7
Bile duct ligation	10	26 ± 17
Choleresis	6	63 ± 8

of the stomach, the extrapolation of their results to normal gastric fluid requires further investigation.

One of the most important groups of surfactants present in man is the bile salts. Bile is chiefly composed of conjugated bile salts, cholesterol, calcium, and lecithin, a phospholipid (79). There are six bile salts present in man—*viz.*, the taurine and glycine conjugates of deoxycholic acid, chenodeoxycholic acid, and cholic acid, present as the sodium salts. Endogenous bile salts represent the end products of cholesterol metabolism in the liver and are stored in the gall bladder after conjugation in the liver until required for digestion. The conjugation of bile salts by the liver with glycine and taurine is an essential physiologic process, since unconjugated bile salts are insoluble at the relatively low pH (6.3–6.6) present in the upper portion of the small intestine. Although the bile salts secreted into the intestinal lumen are, for the most part, conjugated, free acids may be present in the feces due to cleavage of the conjugated bile salts by microorganisms present in the large intestine. The concentrations of conjugated bile salts in the upper jejunum of the fasting human are above the critical micelle concentration and range between 5 and 10 m*M* (80). During fat digestion, concentrations of conjugated bile salts in the order of 40 m*M* are commonly found in the jejunum (81).

Bile salts have long been implicated in the absorption of fat from the small intestine. They are known to have an important role in the emulsification of water-insoluble, long-chain triglycerides and in stimulating the hydrolytic action of pancreatic lipase, resulting in a mixture consisting of fatty acids, monoglycerides, diglycerides, and triglycerides (82). A powerful emulsifying agent is also formed by removal of a fatty acid moiety from a molecule of lecithin by a pancreatic phospholipase to give lysolecithin (83).

The ability of bile salts to solubilize lipid material has also been known for many years. The two lipids that are appreciably solubilized by the bile salt micelles are the lipolysis products—*viz.*, monoglycerides and fatty acids (82). Hofmann and Borgstrom (84) have shown that the presence of monoolein in a bile salt micelle greatly increases its ability to solubilize saturated fatty acids. Feldman and Borgstrom (85) have recently shown that small amounts of diglyceride and triglyceride can also enter such micelles. Fatty acids and monoglycerides contained in the micelles are delivered to the brush border of the mucosal cells of the proximal small intestine, where they are absorbed by a process which is not understood at present. The bile salts, however, are not appreciably absorbed from the proximal intestine and become available for further solubilization of fat digestion products. Intestinal motility eventually carries the bile salts to the ileum where they are absorbed by an active transport process (86).

Bile salts have also been shown to be involved in the absorption of materials other than fats. Bernhard *et al.* (87) showed that in the biliary fistula rat less than 1% of a dose of vitamin A was absorbed. However, if a solution of sodium cholate or taurocholate was introduced into the duodenum, the absorption of vitamin A was increased significantly. Greaves (88) has also reported that bile salts are essential for the intestinal absorption of vitamin K in the rat. Adequate absorption of vitamin D by the rat was shown by Greaves and Schmidt (89) to require bile. When the bile duct was implanted into the colon, the animals absorbed little or none of the vitamin. Oral administration of deoxycholic acid greatly improved absorption of the vitamin in these rats. Taylor (90) confirmed the need for bile for adequate vitamin D absorption in dogs. Heymann (91) also found that dogs did not absorb crystalline vitamin D_2 when bile was not present in the small intestine.

Several reports have appeared in the literature (92–94) as to the importance of bile salts in the intestinal absorption of cholesterol. These studies also showed that the addition of exogenous bile salts enhances the absorption of this sterol.

Lengemann and Dobbins (95) found that intraperitoneal injections and large oral doses of sodium taurocholate enhanced the absorption of calcium by the rat, provided that bile was allowed to flow freely into the small intestine. Seyfried and Lutz (96) reported that the intestinal absorption of tetraiodophenolphthalein is greatly diminished in the absence of bile. Simultaneous administration of bile or bile salts increases the absorption of this substance. Pekanmaki and Salmi (97) found that the absence of bile from the intestine of cats reduced the absorption of free phenolphthalein but had no influence on the amount of absorbed phenolphthalein glucuronide, a water-soluble conjugate. The effect of the endogenous bile on free phenolphthalein absorption was attributed to the solubilizing effect of bile salts on the free drug.

Recently, Meli *et al.* (98) reported that endogenous bile influences the rate of intestinal absorption of ethynylestradiol-6,7-^{3}H-3-cyclopentyl ether in rats. The rate of absorption of the estrogen was considerably lower in biliary-cannulated rats than in control animals. Since the steroid is relatively water insoluble, it is reasonable to consider that the presence of bile salts increased the solubility of the drug in the intestinal lumen and thereby enhanced the dissolution and absorption rate.

Nightingale *et al.* (99) studied the absorption of sulfadiazine in aqueous suspension from proximal intestinal loops in the rat under four experimental conditions —*viz.*, in control animals with intact bile flow, in bile duct-ligated animals, in sham-ligated animals, and in animals where bile flow was stimulated by intraperitoneal administration of sodium dehydrocholate. The results are shown in Table II and clearly indicate that bile flow is an important factor in sulfadiazine absorption from intestinal loops. The mechanism by which bile enhanced sulfadiazine absorption is probably due to micellar solubilization, resulting in an increase in the

dissolution rate of the drug. This possibility is supported by *in vitro* solubility studies.

As noted, conjugation of free bile acids with glycine and taurine results in a considerable lowering of the pKa. Consequently, the conjugated bile acids exist in the intestinal lumen almost entirely as negatively charged ions. In the presence of amphipathic cations, bile salts behave as typical amphipathic anions and an insoluble molecular complex is the frequent result.

Neomycin, a tetrasaccharide with two positively charged amine groups, precipitates glycine and taurine conjugates of dihydroxy and trihydroxy bile salts from aqueous solution (100). Addition of kanamycin, a cationic disaccharide antibiotic, to human bile also results in precipitation (101). Formation of an insoluble complex between a drug and a component of the luminal contents will probably result in decreased absorption of the drug. The poor oral availability of certain drugs such as streptomycin may well be due to bile salt interaction.

Although the detergent properties of bile salts are well recognized, their potential for altering or regulating membrane permeability in the intestine has been given little attention. It is perhaps significant that the ileum, which is the site of active transport of conjugated bile salts, is the region of the small intestine in which the mucosal cells have the shortest life span (102).

Levels of conjugated bile salts in the order of 10^{-2} *M*, comparable to the levels normally found in the proximal intestine, alter the permeability of the everted rat intestine to salicylate (103), salicylamide (104), riboflavin (40), and several other drugs. The salicylate data are shown in Table III. There is a greater than twofold increase in the steady-state transfer rate of salicylate when micellar concentrations (>5 m*M*) of taurodeoxycholate are initially present in the mucosal solution. There are no significant differences between the transfer rates observed at different sodium taurodeoxycholate concentrations above the CMC. Mucosal solutions containing 1 and 5 m*M* taurodeoxycholate produced considerably smaller changes in membrane permeability, an increase of 20–40% over control steady-state transfer rates. Incubation of the intestinal segments in drug-free mucosal solution containing 10 m*M* bile salt followed by the determination of salicylate transfer from bile salt–free drug solution yielded transfer rates essentially equivalent to those observed when 10 m*M* taurodeoxycholate was initially present with the drug.

The data in Table III suggest that the bile salt effect is mediated *via* two different mechanisms. One mechanism appears to be operative at concentrations of sodium taurodeoxycholate below or about the CMC and results in small increases in salicylate transfer rates. A second mechanism, which results in large increases in the permeability of the intestinal membrane, appears to be operative at sodium taurodeoxycholate concentrations above the CMC.

The possibility that monomeric and micellar species of bile salt affect the biologic membrane in a significantly different manner is supported by recent studies on bile salt transfer in isolated jejunal loops of the rat. Dietschy (105) reports that at concentrations below the CMC the rate of passive transfer of sodium taurocholate increased

Table III—Effect of Sodium Taurodeoxycholate (STDC) on Mucosal-to-Serosal Steady-State Transfer of Salicylate[a] Across the Everted Rat Small Intestine at pH 6.0

	No. of Intestinal Segments	Transfer Rate ± *SD*, mcg./min.
Control	19	40 ± 5
STDC		
100 m*M*	2	90 (87, 93)
50 m*M*	4	89 ± 10
10 m*M*	11	87 ± 10
5 m*M*	6	57 ± 9
1 m*M*	7	48 ± 7
Incubation		
Control	4	40 ± 4
STDC, 10 m*M*	4	85 ± 7

[a] Mucosal salicylate concentration maintained essentially constant, 2 mg./ml. Serosal salicylate concentration never exceeded 0.25 mg./ml.

linearly with increasing mucosal concentrations of bile salt. According to Dietschy (105), "since the concentration of monomer becomes almost constant as the total concentration of bile acid is raised to and beyond the CMC, the rate of passive diffusion similarly should reach a limiting value at this same point if bile acid monomer were the only species diffusing passively across the bowel wall." In fact, the permeability coefficient of the membrane actually increased at taurocholate concentrations above the CMC. "Micellar" taurocholate appears to move across the intestinal membrane twice as fast as the monomeric form. Based on the authors' findings, one need not conclude that micellar transport of taurocholate proceeds at a faster rate than monomer transport or indeed that it occurs at all. It is possible that micellar concentrations of bile salt in contact with the mucosa significantly enhance the permeability of the membrane toward the monomeric species.

The latter possibility is further supported by Nogami *et al.* (106) who studied the sorption of surface-active agents by isolated rat intestine. The sorption of sodium lauryl sulfate and cetyltrimethylammonium bromide, with time, followed first-order kinetics at bulk concentrations below the CMC and zero-order kinetics at concentrations exceeding the CMC. This change in order is consistent with a mechanism involving penetration of

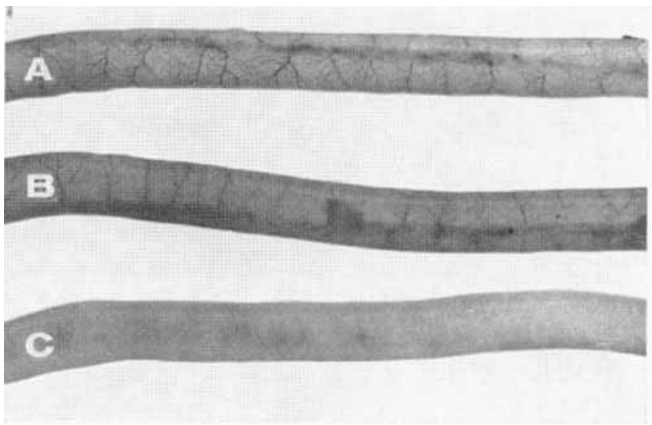


Figure 2—*Segments of everted rat small intestine after 1-hr. incubation. Key: A, physiologic buffer; B, buffer with 10 m*M *sodium taurodeoxycholate and 5 m*M *egg lecithin; and C, buffer with 10 m*M *sodium taurodeoxycholate.*

Table IV—Influence of Egg Lecithin, Oleic Acid, and Glyceryl Monooleate (GMO) in Modifying the Effect of 10 m*M* Sodium Taurodeoxycholate (STDC) on the Transfer Rate of Salicylate Across the Everted Rat Small Intestine at pH 6.0

Incubation Media	No. of Intestinal Segments	Mean Transfer Rate ± *SD*, mcg./min.
Control	6	30 ± 3
STDC	15	78 ± 8
STDC + 5 m*M* lecithin	4	55 ± 9[a]
STDC + 10 m*M* lecithin	4	51 ± 3[a]
STDC + 3 m*M* oleic acid + 1 m*M* GMO	4	72 ± 5[a]
STDC + 6 m*M* oleic acid + 4 m*M* GMO	2	64 (63, 66)

[a] Results significantly different from STDC alone ($p < 0.05$, Student's *t* test, method of paired comparisons).

the monomer but not the micellar species. However, in each case the observed zero-order rate constant was larger than the zero-order rate constant calculated from the results of experiments using premicellar concentrations. Hence, the micellar species appear to alter the permeability of the tissues for the monomeric species.

When the intestinal tissue is incubated in solutions exceeding 5 m*M* bile salt concentration, the changes in permeability are invariably accompanied by pronounced changes in the gross appearance of the mucosal surface (Fig. 2). These observations suggest the possibility that micellar concentrations of sodium taurodeoxycholate produce pronounced changes in membrane structure, conceivably by solubilizing lipid components of the membrane such as phospholipids. These observations also raise an intriguing question as to why such toxic effects are not observed in the intact animal where the intestinal concentration of conjugated bile salts is comparable to that used in the *in vitro* studies.

The micellar species in the proximal intestine normally contains a significant amount of lecithin in addition to the conjugated bile salts. During digestion the micelle will also contain considerable amounts of fat digestion products. Feldman and Gibaldi (107) compared the effect of micellar solutions of pure bile salt and bile salt with lecithin or fat digestion products on the permeability of the everted rat intestine to salicylate. Their results are shown in Table IV. Addition of either phospholipid (egg lecithin) or fat digestion products (oleic acid and glyceryl monooleate) to 10 m*M* solutions of sodium taurodeoxycholate resulted in a protective effect on the everted intestinal membrane. There was a significant decrease in the transfer rate of salicylate after exposure of the everted intestine segment to 10 m*M* sodium taurodeoxycholate containing either phospholipid or fat digestion products when compared to the transfer rates in 10 m*M* sodium taurodeoxycholate alone. As shown in Fig. 2, egg lecithin also protected the intestinal mucosa from the gross histological effects of the bile salt. Similar protective effects were observed with the fat digestion products. The results of this study may explain why conjugated bile salts are highly "toxic" to intestinal tissue *in vitro* but are apparently innocuous *in vivo*.

REFERENCES

(1) O. Blanpin, *Prod. Pharm.*, **13,** 425(1968).
(2) R. D. Swisher, *Arch. Environ. Health*, **17,** 232(1968).
(3) G. Levy, in "Prescription Pharmacy," J. B. Sprowls, Ed., Lippincott, Philadelphia, Pa., 1963, pp. 66–69.
(4) A. A. Noyes and W. R. Whitney, *J. Amer. Chem. Soc.*, **19,** 930(1897).
(5) W. I. Higuchi, *J. Pharm. Sci.*, **56,** 315(1967).
(6) D. E. Wurster and P. W. Taylor, *ibid.*, **54,** 169(1965).
(7) J. Swarbrick, *ibid.*, **54,** 1229(1965).
(8) P. H. Elworthy, A. T. Florence, and C. B. Macfarlane, "Solubilization by Surface Active Agents," Chapman and Hall Ltd., London, England, 1968.
(9) G. Levy, *Amer. J. Pharm.*, **135,** 78(1963).
(10) J. H. Fincher, *J. Pharm. Sci.*, **57,** 1825(1968).
(11) A. Kellner, J. W. Correll, and A. T. Ladd, *Proc. Soc. Exp. Biol. Med.*, **67,** 25(1948).
(12) B. Fuchs and F. J. Ingelfinger, *Gastroenterology*, **27,** 802 (1954).
(13) D. Krause, *Arzneimittel-Forsch.*, **5,** 428(1955).
(14) C. L. Gantt, N. Gochman, and J. M. Dyniewicz, *Lancet*, **1,** 486(1960).
(15) *Ibid.*, **1,** 1130(1962).
(16) K. Kakemi, T. Arita, and S. Muranishi, *Chem. Pharm. Bull.*, **13,** 976(1965).
(17) T. R. Bates, M. Gibaldi, and J. L. Kanig, *Nature*, **210,** 1331(1966).
(18) T. R. Bates, S. L. Lin, and M. Gibaldi, *J. Pharm. Sci.*, **56,** 1492(1967).
(19) E. L. Parrott and V. K. Sharma, *ibid.*, **56,** 1341(1967).
(20) M. Gibaldi, S. Feldman, R. Wynn, and N. D. Weiner, *ibid.*, **57,** 787(1968).
(21) P. H. Elworthy and F. J. Lipscomb, *J. Pharm. Pharmacol.*, **20,** 923(1968).
(22) W. I. Higuchi, *J. Pharm. Sci.*, **53,** 532(1964).
(23) P. Singh, S. J. Desai, D. R. Flanagan, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **57,** 959(1968).
(24) D. E. Wurster and G. P. Polli, *ibid.*, **50,** 403(1961).
(25) M. Gibaldi, S. Feldman, and N. D. Weiner, to be published.
(26) P. Finholt and S. Solvang, *J. Pharm. Sci.*, **57,** 1322(1968).
(27) H. Weintraub and M. Gibaldi, *ibid.*, **58,** 1368(1969).
(28) G. Levy and W. J. Jusko, *ibid.*, **55,** 285(1966).
(29) F. Varga, *Arch. Int. Pharmacodyn.*, **163,** 38(1966).
(30) G. Levy and W. J. Jusko, *J. Pharm. Sci.*, **54,** 219(1965).
(31) K. Okuda, E. V. Duran, and B. F. Chow, *Proc. Soc. Exp. Biol. Med.*, **103,** 588(1960).
(32) P. M. Lish, *Gastroenterology*, **41,** 580(1961).
(33) H. Necheles and J. Sporn, *Amer. J. Gastroenterol.*, **46,** 481(1966).
(34) C. A. Pannett and C. M. Wilson, *Brit. J. Exp. Pathol.*, **2,** 70(1920).
(35) K. Sasaki, *Hiroshima J. Med. Sci.*, **3,** 187(1954).
(36) *Ibid.*, **3,** 195(1954).
(37) S. Feldman and M. Gibaldi, *Gastroenterology*, **54,** 918 (1968).
(38) S. Feldman, R. J. Wynn, and M. Gibaldi, *J. Pharm. Sci.*, **57,** 1493(1968).
(39) J. N. Hunt and I. MacDonald, *J. Physiol.*, **126,** 459(1954).
(40) M. Mayersohn, S. Feldman, and M. Gibaldi, *J. Nutr.*, **98,** 288(1969).
(41) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **56,** 58(1967).
(42) G. Levy, in "Prescription Pharmacy," J. B. Sprowls, Ed., Lippincott, Philadelphia, Pa., 1963, pp. 64–66.
(43) S. Riegelman and W. J. Crowell, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 115(1958).
(44) *Ibid.*, **47,** 123(1958).
(45) *Ibid.*, **47,** 127(1958).
(46) G. Levy and R. H. Reuning, *J. Pharm. Sci.*, **53,** 1471(1964).
(47) H. Yamada and R. Yamamoto, *Chem. Pharm. Bull.*, **13,** 1279(1965).
(48) H. Matsumoto, *Yakugaku Zasshi*, **86,** 590(1966).
(49) W. Saski, *J. Pharm. Sci.*, **57,** 836(1968).
(50) L. S. Schanker and J. M. Johnson, *Biochem. Pharmacol.*, **8,** 421(1961).
(51) E. Windsor and G. E. Cronheim, *Nature*, **190,** 263(1961).

(52) A. E. Alexander and A. R. Trim, *Proc. Royal Soc., Ser. B*, **533,** 220(1946).
(53) G. Levy, K. E. Miller, and R. H. Reuning, *J. Pharm. Sci.*, **5,** 394(1 96 6).
(54) G. Levy and J. A. Anello, *ibid.*, **57,** 101(1968).
(55) J. A. Anello and G. Levy, *ibid.*, **58,** 721(1969).
(56) M. Gibaldi and C. H. Nightingale, *ibid.*, **57,** 1354(1968).
(57) C. H. Nightingale, R. J. Wynn, and M. Gibaldi, *ibid.*, **58,** 1005(1969).
(58) C. W. Whitworth and L. D. Yantis, *ibid.*, **56,** 1661(1967).
(59) J. Scala, D. E. McOsher, and H. H. Reller, *J. Invest. Dermatol.*, **50,** 371(1968).
(60) E. R. M. Kay, *Cancer Res.*, **25,** 764(1965).
(61) W. Appel, H. Schievelbein, and E. Werle, *Arzneimittel-Forsch.*, **7,** 742(1956).
(62) H. D. Mori, P. A. Barker, D. S. Juras, and R. W. Wissler, *Lab. Invest.*, **6,** 421(1957).
(63) H. Brise, *Acta Med. Scand.*, **376,** 47(1962).
(64) M. Suzuki, K. Motoyoshi, H. Arai, and H. Horikawa, *Jap. J. Pharmacol.*, **17,** 525(1967).
(65) S. C. Penzotti and A. M. Mattocks, *J. Pharm. Sci.*, **57,** 1192(1968).
(66) J. A. Nissim, *Nature*, **187,** 305(1960).
(67) C. B. Taylor, *J. Physiol.*, **165,** 199(1963).
(68) P. M. Lish and J. H. Weikel, Jr., *Toxicol. Appl. Pharmacol.*, **1,** 501(1959).
(69) T. Matsuzawa, H. Fujisawa, K. Aoki, and H. Mima, *Chem. Pharm. Bull.*, **17,** 999(1969).
(70) R. H. Engel and S. J. Riggi, *Proc. Soc. Exp. Biol. Med.*, **130,** 879(1969).
(71) R. H. Engel and S. J. Riggi, *J. Pharm. Sci.*, **58,** 706(1969).
(72) H. W. Davenport, *Proc. Soc. Exp. Biol. Med.*, **125,** 670(1967).
(73) S. Feldman, M. Salvino, and M. Gibaldi, to be published.
(74) H. J. Vonk, *Z. Vergleich. Physiol.*, **21,** 717(1935).
(75) A. van den Oord, H. Danielsson, and R. Ryhage, *Nature*, **203,** 301(1964).
(76) A. van den Oord, H. Danielsson, and R. Ryhage, *J. Biol. Chem.*, **240,** 2242(1965).
(77) A. van den Oord, *Comp. Biochem. Physiol.*, **17,** 715(1966).
(78) J. Rhodes, D. E. Barnardo, S. F. Phillips, R. A. Rovelstad, and A. F. Hofmann, *Gastroenterology*, **57,** 241(1969).
(79) A. F. Hofmann and D. M. Small, *Ann. Rev. Med.*, **18,** 333(1967).
(80) S. Tebaqchali, J. Hatzioannou, and C. C. Booth, *Lancet*, **2,** 12(1968).
(81) J. Sjovall, *Acta Physiol. Scand.*, **46,** 339(1959).
(82) A. M. Dawson, *Brit. Med. Bull.*, **23,** 247(1967).
(83) G. A. D. Haslewood, "Bile Salts," Methuen and Co., Ltd., London, England, 1967, p. 3.
(84) A. F. Hofmann and B. Borgstrom, *Fed. Proc.*, **21,** 43 (1962).
(85) E. B. Feldman and B. Borgstrom, *Lipids*, **1,** 430(1966).
(86) L. Lack and I. M. Weiner, *Amer. J. Physiol.*, **200,** 313 (1961).
(87) K. Bernhard, G. Ritzel, and E. Scheitlin, *Helv. Physiol. Pharmacol. Acta*, **10,** C47(1952).
(88) J. D. Greaves, *Amer. J. Physiol.*, **125,** 423(1939).
(89) J. D. Greaves and C. L. A. Schmidt, *J. Biol. Chem.*, **102,** 101(1933).
(90) N. B. Taylor, C. B. Weld, and J. F. Sykes, *Brit. J. Exp. Pathol.*, **16,** 302(1935).
(91) W. Heymann, *J. Biol. Chem.*, **122,** 249(1937–38).
(92) M. D. Siperstein, I. L. Chaikoff, and W. O. Reinhardt, *ibid.*, **198,** 111(1952).
(93) A. C. Ivy, R. Suzuki, and C. R. Prasad, *Amer. J. Physiol.*, **193,** 521(1958).
(94) G. V. Vahouny, C. H. Woo, and C. R. Treadwell, *ibid.*, **193,** 41(1958).
(95) F. W. Lengemann and J. W. Dobbins, *J. Nutr.*, **66,** 45 (1958).
(96) H. Seyfried and W. Lutz, *Wien. Klin. Wochenschr.*, **52,** 226 (1939).
(97) K. Pekanmaki and H. A. Salmi, *Acta Pharmacol. Toxicol.*, **18,** 133(1961).
(98) A. Meli, D. I. Cargill, T. Giannina, and B. G. Steinetz, *Proc. Soc. Exp. Biol. Med.*, **129,** 937(1968).
(99) C. H. Nightingale, J. Axelson, and M. Gibaldi, to be published.
(100) P. De Somer, H. Vanderhaege, and H. Eyssen, *Nature*, **204,** 1306(1964).
(101) W. W. Faloon, I. C. Paes, D. Woolfolk, H. Nankin, K. Wallace, and E. N. Haro, *Ann. N. Y. Acad. Sci.*, **132,** 879(1966).
(102) R. J. M. Fry and E. Staffeldt, *Nature*, **203,** 1396(1964).
(103) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **58,** 425(1969).
(104) *Ibid.*, **58,** 967(1969).
(105) J. M. Dietschy, *J. Lipid Res.*, **9,** 297(1968).
(106) H. Nogami, J. Hasegawa, M. Hanano, and T. Fuwa, *Chem. Pharm. Bull.*, **16,** 2101(1968).
(107) S. Feldman and M. Gibaldi, *Proc. Soc. Exp. Biol. Med.*, **132,** 1031(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214* and the *Division of Pharmaceutics, School of Pharmacy, Temple University, Philadelphia, PA 19140*

Supported by Grant No. AM-11498 from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, Bethesda, Md.

Apparent Directional Permeability Coefficients for Drug Ions: *In Vitro* Intestinal Perfusion Studies

ROBERT H. TURNER*†+, CHANDRAKANT S. MEHTA*‡, and LESLIE Z. BENET+

Abstract □ The *in vitro* absorption kinetics for nine drugs were followed using a perfusion apparatus. Identical perfusion runs were made on everted and noneverted segments of the same rat intestine so that the ratio of directional permeability constants (reported as everted rate to noneverted rate) could be calculated. Both negatively and positively charged drug ions (including the quaternary ammonium compound pralidoxime chloride) exhibited permeability coefficient ratios around 1.3, while completely unionized drugs showed the expected ratio of 1.0. In light of the similarity in the ratio of permeability constants for drug ions and sodium ions, salicylate was tested in a sodium-free buffer resulting in a ratio of 1.08. It appears that the difference in directional permeability constants observed with ionized drugs in the intestine may be explained in relation to sodium transport. It is pointed out that *in vitro* intestinal transport studies could lead to erroneous conclusions concerning the degree of absorption of ionizable drugs *in vivo*.

Keyphrases □ Absorption kinetics—*in vitro* intestinal perfusion □ Perfused intestine—drug transport □ Everted, noneverted intestine—directional permeability constants □ Permeability constants, directional—ratios □ Ionization effect—drug permeability coefficient ratios

Considerable work is being and has been carried out on the transport of various drugs across intestinal membranes. This work has been adequately reviewed by Wilson (1) and more recently by Benson and Rampone (2). The forces causing movement across the intestinal membrane can be divided into five classes: cellular metabolic energy, activity gradients, electrical gradients, hydrogen-ion gradients, and solvent drag. One of the first areas studied extensively was that of hydrogen-ion gradients from which a theory evolved to explain the absorption of ionized and unionized drugs. This theory, called the "pH partition hypothesis," was developed by Brodie *et al.* (3–7). These authors concluded from their investigations that most drugs are absorbed from the gastrointestinal tract by a process of passive diffusion of the unionized drug species across a lipoidal membrane. Thus, in line with this hypothesis, Hogben *et al.* (4) suggested that the unequal distribution of weak organic acids or bases across the gut wall is due to: (*a*) much greater permeability of the gut wall to the unionized form of the compound than to its ionized form, and (*b*) a difference in pH on the two sides of the intestinal wall. They further postulated that the distribution of weak acid or base is dependent on the "virtual" pH of the mucosal solution (the pH of a narrow microclimate adjacent to the mucosal intestinal surface) rather than the pH of the bulk mucosal solution. They pointed out that such a microclimate of fluid with a low pH calculated to be 5.3 would lead to a relatively high concentration of unionized acid next to the mucosa (as compared to the concentration of unionized acid in the bulk mucosal solution at a higher pH, normally 6.6) and consequently lead to increased mucosal to serosal movement of the acid by means of nonionic diffusion. By this mechanism, values greater than one for the steady-state concentration ratio, C_{plasma}/C_{gut}, of a weak acid could be explained without postulating a specific active-transport mechanism.

This hypothesis seems to be consistent with the *in vivo* data of Schanker *et al.* (5) who found that the lowest pKa of an acidic drug showing rapid absorption was about 3, while the corresponding highest pKa for a basic drug was 8. However, there seems to be a great discrepancy between the results obtained *in vitro* and those obtained *in vivo*. It would appear that ionized drug species are much more easily absorbed when membrane permeability is measured using one of the three commonly employed *in vitro* techniques: the everted sac method of Wilson and Wiseman (8), the Crane and Wilson method (9), and use of a perfusion appartus (10, 11). In 1966, Benson and Rampone (2) stated: "This is the era of the everted sac, developed by Wilson and Wiseman, and much of the investigation reported in this review was based upon this *in vitro* technique." Most intestinal absorption studies in the pharmaceutical sciences (see *Reference 12* for review) are still run using the *in vitro* techniques already mentioned. Therefore, this paper is directed toward identifying the discrepancy between *in vivo* and *in vitro* results and to point out the possible unapplicability of *in vitro* intestinal studies, especially when the transport of ionized drugs is observed.

There are a number of studies in the literature which show large differences between *in vitro* results and those expected on the basis of the *in vivo* hypotheses. For example, Hogben *et al.* (4) calculated the ratio of permeability coefficients for unionized and ionized (P_u/P_i) salicylic acid to be 4500; but Nogami and Matsuzawa (13), using absorption kinetics with *in vitro* perfused segments, found this ratio to be 6. Likewise for the drug aminopyrine, Hogben *et al.* (4) calculated a permeability coefficient ratio approaching infinity, but Nogami and Matsuzawa (14) found P_u/P_i equal to 11 in their *in vitro* studies. Nogami *et al.* (15) also found that the *in vitro* permeability coefficients for sulfathia-

zole in ionized forms exceeded the permeability coefficient for the unionized drug.

More recently, Kakemi *et al.* (16) studied the absorption of barbituric acid derivatives through the *in vitro* rat small intestine. Here they found that the pH partition hypothesis was only partially operative, in that absorption rate constants increased in the pH range 6.5 to 7.5 over those rate constants determined at pH 5.5 where barbituric acid derivatives are almost completely unionized. These authors also found that plots of percent barbiturate bound to intestinal mucosa against initial pH of the perfusate, all had maxima in the pH 6.5 to 7.5 region. They speculated that the complexation of barbituric acid derivatives to proteins of the mucosal surface raised the concentration of drug at the membrane surface, which in turn favored absorption in the serosal direction. This proposal is similar to that suggested by Singh *et al.* (17) and Levy and Matsuzawa (18) where drugs complexed to inert ingredients still show measurable absorption rates.

It became apparent to the present investigators that in these *in vitro* studies, the assumption was made that drugs were transported through the intestine by an identical process in each direction. Permeability constants for mucosal (gut) to serosal (plasma) transport were assumed to be the same as for serosal to mucosal transport. However, there were some interesting aspects of the reported data which might lead to the assumption that there was a difference in directional permeability constants. For instance, Nogami and Matsuzawa (13) found that the permeability coefficients for ionized salicylate and unionized salicylic acid increased with the lapse of time. They attributed this increase to the fact that salicylic acid is a toxic substance for living cells and might cause a physiological change in the intestinal tissue. However, this increase in calculated permeability coefficients could also be explained by the fact that the permeability coefficients for mucosal to serosal transport are greater than those for serosal to mucosal transport. The Nogami and Matsuzawa *in vitro* data for aminopyrine (14) would also be consistent with a difference in directional permeability coefficients; and the hypothesis of Kakemi *et al.* (16) implies that in a certain pH range, mucosal to serosal transport of barbituric acid derivatives is favored. Therefore, in this study, the authors have undertaken to determine experimentally whether there is a basis for assuming a difference in directional permeability coefficients and, if such a difference exists, to identify its mode of action.

EXPERIMENTAL

Materials—The following reagent, USP, or NF grade drugs and chemicals were tested: salicylic acid, salicylamide, aminopyrine, aniline, antipyrine, acetanilide, quinine sulfate, pralidoxime chloride (2-PAM chloride), and 5-nitrosalicylic acid. Monobasic sodium phosphate with one water of hydration, dibasic sodium phosphate anhydrous, monobasic potassium phosphate anhydrous, and dibasic potassium phosphate anhydrous were used in preparing the isotonic buffers.

Buffer Solutions—Three considerations entered into the selection of the buffer solutions to be used in these experiments. First, the authors wished to prepare an isotonic buffer solution having the greatest possible buffer capacity, since it is known that the jejunal epithelium of the rat maintains a slightly acidic pH within its lumen by hydrogen-ion secretion (4) and since there is the possibility of an acidic zone adjacent to the mucosal surface as was proposed by Hogben *et al.* (4). A buffer with a very high buffering capacity should eliminate the possibility of this acidic layer influencing drug absorption since Hogben *et al.* (4) have shown *in vivo* that strong buffers decrease the effect of the virtual pH. Second, the authors wanted to use only buffers having pH values which corresponded to the physiological values found either within the intestine or in its intact blood supply. Therefore, the buffers utilized were only those at pH 6.6 and pH 7.4. In light of the unusual results described in the Kakemi *et al.* (16) study of barbituric acid derivatives, it was felt that working at pH's other than 6.6 and 7.4 could possibly introduce effects not normally seen in the intestine. Third, since the authors wish to identify a possible cause for the difference in directional permeability coefficients, they wanted to use buffer solutions containing a minimum number of compounds. Therefore, they chose to make the isotonic buffers using dibasic and monobasic sodium phosphate exclusively.

Table I—Isotonic Buffer Formulas per Liter

	$NaH_2PO_4 \cdot H_2O$, g.	Na_2HPO_4, g.	KH_2PO_4, g.	K_2HPO_4, g.
Sodium phosphate buffer, pH 6.6	11.44	8.73		
Sodium phosphate buffer, pH 7.4	3.06	14.70		
Potassium phosphate buffer, pH 6.6			11.03	10.46
Potassium phosphate buffer, pH 7.4			3.07	18.36

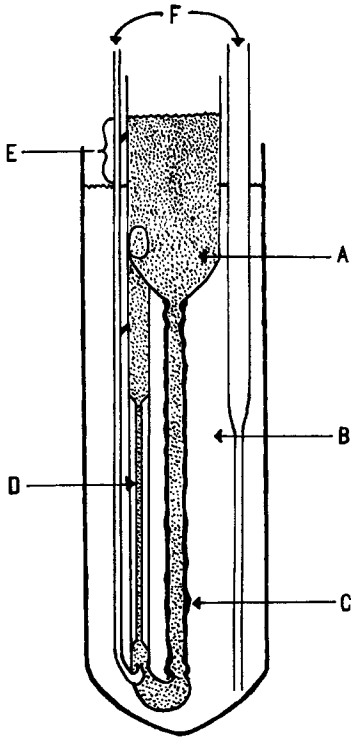


Figure 1—*Diagrammatic representation of the* in vitro *perfusion apparatus. Key: A, inside compartment, 15 ml.; B, outside compartment, approximately 140 ml.; C, intestinal segment, 10-cm. length available for absorption; D, capillary bubble pump; E, difference between heights of inside and outside compartments, 2 cm.; and F, 100% oxygen.*

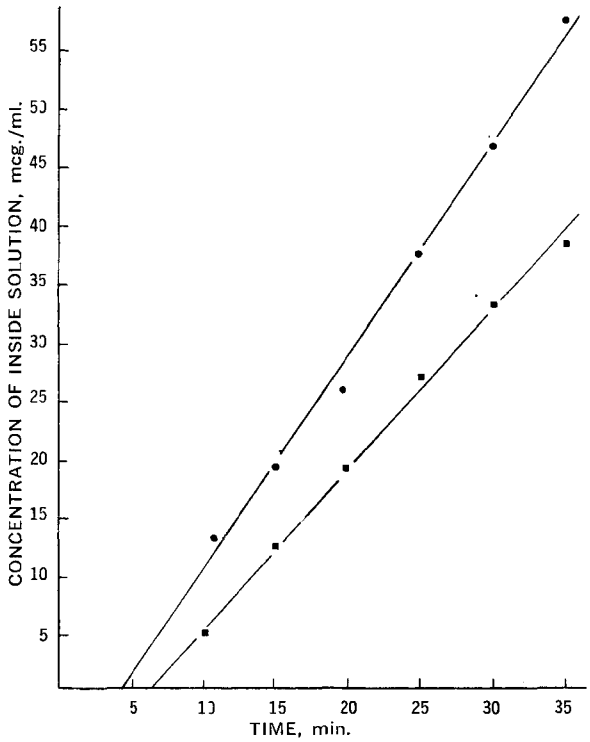


Figure 2—*Sample data plot for the absorption of salicylate through the rat intestine. Outside solution, 1000 mcg./ml. drug in isotonic pH 7.4 sodium phosphate buffer. Inside solution, isotonic pH 7.4 sodium phosphate buffer. Initial absorption rate through: everted segment* (●) = *1.76 mcg./ml./min.; noneverted segment* (■) = *1.39 mcg./ml./min. Permeability coefficient ratio* P_E/P_{NE} = *1.27.*

Using the proper sodium chloride equivalents, a reasonable activity coefficient value for hydrogen ions in an isotonic solution, and the Henderson-Hasselbalch equation, it is possible to calculate (19) the required amount of sodium phosphates in each buffer, as presented in Table I. Using the Van Slyke equation, the pH 6.6 sodium buffer was found to have a buffer capacity of 0.078 and the pH 7.4 buffer had a capacity of 0.048. The buffer capacities of these solutions are approximately twice those for the isotonic buffer solutions at similar pH's listed by Martin (19). During the course of a 40–60-min. perfusion run, the buffering solutions were found to vary by no more than 0.03 pH units, a value well within the accuracy expected in making pH measurements (20). In addition, some perfusion studies were run in a sodium-free buffer, where monobasic potassium phosphate and dibasic potassium phosphate were utilized in preparing the buffers. The formulas for these isotonic buffers, pH 7.4 and 6.6, are presented in Table I.

Table II—Analytical Procedures

Drug	pH of Perfusion Study	Diluent	Dilution, ml.[a]	Apparatus and Wavelengths[b]
Acetanilide	6.6	pH 6.6 buffer	1.0	UV at 237
Aminopyrine	7.4	pH 7.4 buffer	1.0	UV at 260
Aniline	7.4	pH 7.4 buffer	3.0	SPF: *Ex* 285, *Em* 354
Antipyrine	6.6	pH 6.6 buffer	1.0	UV at 255
5-Nitrosalicylic acid	7.4	0.1 *N* HCl	2.0	UV at 308
Quinine sulfate	6.6	0.1 *N* H_2SO_4	3.0	SPF: *Ex* 352, *Em* 454
2-PAM chloride	7.4	0.1 *N* NaOH	1.0	UV at 335
Salicylic acid	7.4	0.1*N* NaOH	5.0	SPF: *Ex* 300, *Em* 408
Salicylamide	6.6	0.1 *N* NaOH	4.0	SPF: *Ex* 332 *Em* 421

[a] Added to the 0.1-ml. samples. [b] UV, ultraviolet measurement at wavelength in mμ; SPF, spectrophotofluorometric measurement; *Ex*, excitation wavelength in mμ; *Em*, emission wavelength in mμ.

Perfusion Studies—Male Sprague-Dawley rats weighing about 250 g. were starved 24 hr. prior to the experiment but allowed free access to water. The rats were sacrificed with a sharp blow; the intestines (jejunum and ileum) were immediately removed and flushed with 40 ml. of the buffer solution to be used in the absorption run. The upper portion of the intestine was cut into two segments of 12 cm. each, and one of the two segments was everted. (In a series of experiments, the first segment of the intestine was everted alternatively.) The *in vitro* absorption kinetics for various drugs were followed using a perfusion apparatus (see Fig. 1) modified slightly from that described by Dietschy *et al.* (21). Each of the two intestinal segments (one everted and one noneverted) was tied securely onto a perfusion apparatus, being certain to maintain the same relative tautness in the stretched segments. The perfusion apparatuses were so designed that only a 10-cm. segment of the intestine was available for absorption. The inside compartment of each apparatus was then filled with 15 ml. of the appropriate isotonic phosphate buffer solution. The inside compartments were placed in large test tube shaped vessels that were suspended in a controlled-temperature water bath, which was maintained at 37.0 ± 0.5°. The outside compartment was filled initially with 130 ml. of a solution containing 1000 mcg./ml. of the drug dissolved in the same isotonic buffer as was used in the inside compartment. Additional drug solution was then added to the outside compartment until a 2-cm. difference in height resulted between the solution in the inside compartment and that in the outside compartment (see Fig. 1). Oxygen was bubbled through the drug solution by means of a capillary tube, and through the solution in the inside compartment by means of a built-in capillary bubble pump which circulated the buffer through the intestinal segment. The rates of bubbling in the two separate apparatuses were adjusted to approximately the same rate, one bubble/second. At regular intervals, 0.1-ml. samples were removed from the inside solution of each apparatus. For each drug, the time intervals were adjusted so that the sixth and final sample was removed from the inside compartment before the concentration in that compartment reached 100 mcg./ml. (*i.e.*, less than 10% of the outside concentration).

Assay Procedures—The 0.1-ml. samples taken from the inside compartments were diluted and assayed by either a UV measurement on the Cary 15 spectrophotometer or by a spectrofluorometric procedure on the Aminco-Bowman spectrofluorometer. Table II lists the various drugs studied, the pH of the buffer in which they were run, the diluent used in the analytical procedure, the amount of diluent added to the 0.1-ml. sample, and the apparatus and wavelengths at which the drug was measured. Standard plots for each drug were made using five known concentrations of the drug in the appropriate diluent.

RESULTS

After assaying each of the drug samples, plots were made of concentration of the inside solution *versus* time for both absorption through the everted and through the noneverted intestinal segments. Figure 2 contains a sample plot of data for the absorption of salicylate ions through two adjacent segments of rat intestine.

Riggs (22) has shown that the transfer of substances by simple diffusion across thin membranes may be described by a useful simplification of Fick's law as presented in Eq. 1:

$$\frac{dQ_i}{dt} = D_m A_m R_{m/s} (C_o - C_i)/\Delta x_m \qquad \text{(Eq. 1)}$$

When this equation is specifically applied to this experiment:

Q_i = the amount of drug in the inside compartment at any time, t
D_m = the effective diffusivity of the drug in the intestinal membrane
A_m = the area of the membrane available for free diffusion
$R_{m/s}$ = partition coefficient between membrane and solvent

Table III—Absorption Rates and Permeability Coefficient Ratios for Nine Drugs Using Buffers Containing Sodium Ions

Drug, pKa	pH of Perfusion Study	Number of Paired Runs	Average Rates ± *SD* in mcg./ml./min. Everted	Average Rates ± *SD* in mcg./ml./min. Noneverted	Permeability Coefficient Ratio ± *SD* P_E/P_{NE}	Level of Significance Ratio *vs.* 1.00
Acetanilide (0.6)	6.6	5	6.60 ± 0.82	6.59 ± 0.93	1.00 ± 0.04	N.S.[a]
Aminopyrine (5.0)	7.4	8	4.23 ± 0.90	3.21 ± 0.61	1.31 ± 0.12	$p < 0.001$
Aniline (4.6)	7.4	8	11.67 ± 1.95	10.67 ± 2.10	1.12 ± 0.22	N.S.
Antipyrine (1.4)	6.6	9	3.10 ± 0.39	3.19 ± 0.52	0.98 ± 0.11	N.S.
5-Nitrosalicylic acid (2.3)	7.4	3	1.94 ± 0.62	1.50 ± 0.41	1.29 ± 0.10	$p < 0.05$
Quinine sulfate (4.1, 8.4)	6.6	5	1.34 ± 0.31	1.11 ± 0.17	1.21 ± 0.13	$p < 0.02$
2-PAM chloride (quaternary ammonium compound)	7.4	8	1.36 ± 0.12	1.15 ± 0.15	1.27 ± 0.11	$p < 0.0001$
Salicylic acid (3.0)	7.4	7	1.78 ± 0.22	1.33 ± 0.21	1.35 ± 0.17	$p < 0.002$
Salicylamide (8.4)	6.6	6	5.82 ± 0.61	5.19 ± 0.66	1.13 ± 0.09	$p < 0.02$
Salicylic acid	7.4 Potassium phosphate buffer	10	1.35 ± 0.31	1.27 ± 0.31	1.08 ± 0.16	N.S.

[a] Not significant.

Δx_m = the thickness of the membrane
C_o = concentration of drug in the outside compartment at any time, t
C_i = concentration of drug in the inside compartment at any time, t

For the experiments run in this work, the last sample of the inside compartment of the perfusion apparatus was always taken at a time when the concentration of that compartment could be considered negligible compared to the concentration of the outside compartment. For example, in Fig. 2 the highest concentration reached in the inside compartment was 57.6 mcg./ml. as compared to an outside concentration of 1000 mcg./ml. Therefore, it may safely be assumed that the backward flow of drug from the inside compartment to the outside compartment is negligible during the time of the experiment and, therefore, C_i can be eliminated from the equation. In addition, since the outside solutions were of such a high concentration and of such a large volume (approximately 140 ml.), it is safe to assume that C_o is a constant throughout the absorption run. Therefore, for these experiments, Eq. 1 may be written as Eq. 2 when absorption is studied through the everted gut and as Eq. 3 when absorption is studied through the noneverted gut:

$$V_i \frac{dC_{iE}}{dt} = P_E C_o \qquad \text{(Eq. 2)}$$

$$V_i \frac{dC_{iNE}}{dt} = P_{NE} C_o \qquad \text{(Eq. 3)}$$

where P_E is defined as the permeability coefficient for absorption through the everted gut—this permeability coefficient contains the diffusion constant, D, the area of the membrane, A, the partition coefficient, $R_{m/s}$, and the thickness of the membrane Δx. The left-hand sides of Eqs. 2 and 3 have been converted into concentration units to correspond with the method of assay where C_{iE} refers to the concentration of the inside compartment when absorption is studied through the everted gut, and NE refers to measurements taken when the gut is not everted. Thus, it may be seen from Eq. 2 that as per the design of the experiment, the absorption rates through the everted and noneverted gut should appear to follow zero-order kinetics as is shown by the straight-line relationships in Fig. 2. Since the concentrations of drug in the outside solutions were always the same (1000 mcg./ml.), and since the volumes of the inside compartments (V_i) were always the same, dividing Eq. 2 by Eq. 3 shows that the ratio of the absorption rates will equal the ratio of apparent permeability coefficients. Table III contains the absorption rates and permeability coefficient ratios for the nine drugs followed in this study. The table contains the pKa of each drug, the pH of the buffer solutions used in the tests, the number of paired trials (each trial refers to one everted and one noneverted absorption run through intestinal segments of the same rat), the average everted and noneverted absorption rates plus or minus the standard deviations for these average rates, and the average of the ratios of permeability coefficients for individual paired runs, plus or minus the standard deviation. As will be noted from the data in Table III, the standard deviations for the average rates of absorption are relatively much higher than the standard deviations for the averages of the permeability coefficient ratios. This would be expected since the absorption rates are a function of the biological variability of the absorption of a drug through the intestine of a number of different rats. However, each of the ratios of permeability coefficients was determined from absorption studies through intestinal segments from the same rat. Therefore, the biological variability of differences in absorption rates due to the individual physiological characteristics of the intestine should be cancelled out to a large extent in the permeability coefficient ratios. Table III also compares the level of significance between the average ratio of permeability coefficients for each drug and the value 1.00 (the value expected if drug passage through the intestinal membrane was identical in each direction). Student's t test was used and 0.05 was taken as the minimum level of significance.

Table IV presents the data for the individual paired runs for salicylic acid in pH 7.4 sodium phosphate buffer. The regression coefficient for each set of data points is also presented in parentheses after the rate determined from the slope of the line drawn through these points. Figure 2 is the plot for the data listed as Run 6 in Table IV.

At the end of the perfusion runs, the difference in heights between the solution in the inside compartment and that in the outside tube was measured and found to have decreased by 0.5 cm. Measurements show that this decrease corresponds to a loss of approximately 1.4 ml. from the inside compartment. Since six 0.1-ml. samples were taken out of the inside compartment during the course of the experiment, there was an overall diffusional loss of 0.8 ml. from the inside compartment to the outside during the course of an absorption run. Since solvent movement was always against the drug flux, it may safely be assumed that solvent drag was not a significant factor in the permeability coefficient ratio.

DISCUSSION

The resulting permeability ratios presented in Table III may be classified into three areas: those very close to a ratio of 1.0, those close to a ratio of 1.3, and an indeterminate group with a ratio midway between 1 and 1.3. The two compounds with ratios very close to 1.0, acetanilide and antipyrine, can be considered to be completely unionized at the pH of the perfusion study. However, a compound such as 2-PAM chloride, which must be completely ionized because it is a quaternary ammonium compound, exhibits a permeability ratio that significantly differs from 1.0. The two other compounds in the study, which may be assumed to be completely ionized at the pH of the perfusion run, salicylic acid and 5-nitrosalicylic acid, also show permeability coefficient ratios very close to 1.3. Quinine sulfate, which is also ionized to a large extent (at least 97%, the majority of which will be the singly positively charged species) exhibits a ratio closer to those of the other ionized drugs than to the ratios around 1.0 for the unionized drugs. Aniline and salicylamide, two compounds which would be expected

Table IV—Individual Paired Data for Salicylic Acid in pH 7.4 Sodium Phosphate Buffer

Run	Everted Rate, mcg./ml./min.	(Regression Coefficient)	Noneverted Rate, mcg./ml./min.	(Regression Coefficient)	Ratio
1	1.790	(0.9935)	1.311	(0.9924)	1.365
2	1.467	(0.9990)	1.143	(0.9945)	1.283
3	2.054	(0.9992)	1.594	(0.9971)	1.289
4	1.835	(0.9908)	1.055	(0.9873)	1.739
5	2.010	(0.9996)	1.598	(0.9931)	1.258
6	1.762	(0.9932)	1.391	(0.9977)	1.267
7	1.542	(0.9956)	1.222	(0.9987)	1.262
Av.	1.780		1.331		1.352

to be at least 97% unionized, give permeability ratios intermediate between those values expected for unionized drugs and those ratios expected for ionized drugs. The aminopyrine (pKa 5.0) run at pH 7.4 appeared to be an exception, since although the drug would be only slightly ionized at this pH, a ratio of 1.31 was found. Excluding the data for aminopyrine and realizing that, as yet, not enough drugs have been run to substantiate a general theory, the data would suggest the following: drugs that are known to be incapable of ionization show the typical uniform path of distribution of drug molecules through the intestine. That is, the mucosal to serosal transfer was equal to the serosal to mucosal transfer, giving a permeability coefficient ratio of 1. If, however, the drug was capable of ionizing to some degree (salicylamide and aniline), the permeability coefficient ratios increased slightly. However, if the drug was completely ionized, whether positively or negatively charged, the permeability coefficient ratio was found to be very close to 1.3. From the data in Table III, it may be concluded that the passage of ionized drugs through the *in vitro* intestine is not a completely passive process.

In looking for an explanation for the 1.3 value for the permeability ratio of ionized drugs, the authors were struck by the similarity of this ratio to the permeability ratio observed by Curran and Solomon for sodium flux in the rat intestine (23). Recently, there have been a number of studies describing sodium ion dependent transport in the intestine as reviewed by Crane (24) and Curran (25). Therefore, an attempt was made to run a similar absorption rate study for salicylic acid in potassium phosphate buffer where sodium ions were completely excluded. This study (as presented at the bottom of Table III) gave a ratio of 1.08, a value very close to the ratios determined for unionized drugs. Although this result must be considered preliminary, there seems to be good evidence that sodium ion transport may be involved in the difference in apparent directional permeability coefficients which have been found in the *in vitro* rat intestine. Further work in this area is now being carried out.

Another interesting result which is obvious from Table III is that the absorption rates for ionized drugs through the intestine are very similar for all of the compounds studied, whether they are positively or negatively charged drug ions. The rate of 2-PAM chloride is especially significant since this compound obviously exists as an ionized species while it is being transported through the *in vitro* intestine. Previously, on the basis of the virtual pH theory of Hogben *et al.* (4), it might have been assumed that ionized drugs apparently pass through the intestine because a sufficient number of the ionized species become unionized at the virtual pH of the acidic microlayer adjacent to the mucosal surface. However, the similarity in rates between 2-PAM chloride, salicylate ions, and quinine ions seems to indicate that ionized drugs do pass through the *in vitro* intestine.

Another interesting similarity may be seen between the absorption rate of salicylate ion and 5-nitrosalicylate ion. Schanker *et al.* (5) found that there was a wide difference between the absorption of these two acids from the rat intestine *in vivo*. Over a given time interval, 60% of the salicylic acid was absorbed, while only 9% of the 5-nitrosalicylic acid was absorbed. The difference in the absorption of these two compounds was used in the explanation of the virtual pH hypothesis. Hogben *et al.* (4) state: "An absorbing surface with a pH of 5.3 would explain why the lowest pKa of an acidic drug compatible with rapid absorption is about 3, while the highest pKa for bases is about 7.8. In each case, the ratio of unionized to ionized drug is 1:200. This appears to be the minimal proportion of unionized drug necessary to insure rapid absorption under the conditions of our experiments."

Although the validity of these data cannot be questioned with respect to *in vivo* absorption, it would appear that this 1:200 ratio is not significant when considering passage through the *in vitro* rat intestine. Furthermore, it leads the authors to believe that the virtual pH hypothesis should not be applied to passage through the *in vitro* intestine.

It is obvious from the data in Table III that drugs which are assumed to be absorbed as unionized species show absorption rates which are significantly higher than those for ionized drugs. This is especially pertinent when considering the permeability ratios for aniline and salicylamide. It is believed that it is safe to assume that these drugs are being absorbed as unionized species and, therefore, the indeterminate ratios (1.12 and 1.13) are assigned as being related to the experimental error inherent in the methods utilized when the absorption rates become large. It is interesting to note that aniline, the compound absorbed at the highest rate in this study, was only found to have an absorption rate about 9 times higher than that for ionized drugs. This difference in rate between unionized and ionized drugs in the *in vitro* intestine is very similar to that reported by Nogami and Matsuzawa (10, 13), but it is quite different from the relative rates of absorption predicted by Hogben *et al.* in the *in vivo* intestine.

Because of the unusual nature of the permeability coefficient ratio for aminopyrine, a drug which would be expected to be unionized and therefore to have a ratio close to 1, preliminary paired runs of aminopyrine in a potassium phosphate buffer have been run. These runs give an average ratio of 1.10. Although work is continuing on this compound, it would appear that aminopyrine is being transported by a process which is different than the other compounds in this study. The authors believe that this result might be significant with respect to *in vivo* transport as well as to *in vitro* transport, since aminopyrine was one of the two drugs studied by Hogben *et al.* (4) which yielded results consistent with a calculated virtual pH of 5.3 at the intestinal wall.

Since the permeability coefficient ratios for the completely unionized drugs, acetanilide and antipyrine, are so close to 1.0, it would appear that the difference in surface area between the mucosal and serosal membranes is not important. This leads the authors to believe that the rate-limiting step in intestinal transport is passage through the membrane rather than passage into or out of the membrane. Under these conditions the surface area of the membrane exposed to the drug solution does not affect the rate of passage. This, in fact, is an implicit assumption in Rigg's simplification of Fick's law as was presented in Eq. 1.

CONCLUSIONS AND SUMMARY

The initial absorption rates for nine drugs were determined *in vitro* through both the everted and noneverted rat intestine using a perfusion apparatus. Two of these drugs, acetanilide and antipyrine, may be considered to be completely in the unionized form at the pH of the buffers used in this work, 6.6 and 7.4. Two other compounds, an acid, salicylamide, and a base, aniline, should be at least 97% unionized in the buffers in which they were studied. The other five drugs studied, including a quaternary ammonium compound—pralidoxime chloride—may be considered to be completely ionized in these studies. On the basis of the nine drugs observed in this work, the authors feel that the following conclusions can be made (with full realization that more extensive studies in different buffer solutions must be undertaken to confirm these results):

1. Drug ions do pass through the *in vitro* rat intestine.
2. These ions pass through the *in vitro* rat intestine at a rate which is much faster than that predicted on the basis of *in vivo* results.
3. Drug ions show a difference in directional permeability coefficients in the *in vitro* rat intestine, with mucosal to serosal transfer occurring about 1.3 times faster than serosal to mucosal transfer. This difference is not observed for the completely unionized drugs.
4. It appears that this difference in directional permeability coefficients might be related to a sodium ion coupled transport.

since when sodium was completely replaced by potassium in the bathing buffer solutions, only a 1.08 value was obtained for the ratio of permeability coefficients for salicylate ion.

5. It would appear that the virtual pH hypothesis of Hogben *et al.* (4) is inoperative when considering *in vitro* intestinal transport.

6. If the presently accepted hypotheses concerning the *in vivo* intestinal absorption of ionizable drugs are valid, it would appear that *in vitro* intestinal transport studies could lead to erroneous conclusions concerning the degree of absorption of ionizable drugs *in vivo*.

REFERENCES

(1) T. H. Wilson, "Intestinal Absorption," W. B. Saunders, Philadelphia, Pa., 1962.

(2) J. A. Benson, Jr., and A. J. Rampone, *Ann. Rev. Physiol.*, **28,** 201(1966).

(3) P. A. Shore, B. B. Brodie, and C. A. M. Hogben, *J. Pharmacol. Exp. Ther.*, **119,** 361(1957).

(4) C. A. M. Hogben, D. J. Tocco, B. B. Brodie, and L. S. Schanker, *ibid.*, **125,** 275(1959).

(5) L. S. Schanker, D. J. Tocco, B. B. Brodie, and C. A. M. Hogben, *ibid.*, **123,** 81(1958).

(6) L. S. Schanker, *J. Med. Chem.*, **2,** 343(1960).

(7) L. S. Schanker, *Ann. Rev. Pharmacol.*, **1,** 29(1961).

(8) T. H. Wilson and G. Wiseman, *J. Physiol., London*, **123,** 116(1954).

(9) R. K. Crane and T. H. Wilson, *J. Appl. Physiol.*, **12,** 145 (1958).

(10) H. Nogami and T. Matsuzawa, *Chem. Pharm. Bull.*, **9,** 532(1961).

(11) J. M. Dietschy, H. S. Salomon, and M. D. Siperstein, *J. Clin. Invest.*, **45,** 832(1966).

(12) L. J. Ravin and P. D. Bernardo, *J. Pharm. Sci.*, **57,** 1075 (1968).

(13) H. Nogami and T. Matsuzawa, *Chem. Pharm. Bull.*, **9,** 532 (1961).

(14) *Ibid.*, **10,** 1055(1962).

(15) H. Nogami, M. Hanano, and J. Watanabe, *ibid.*, **10,** 1161 (1962).

(16) K. Kakemi, T. Arita, R. Hori, and R. Konishi, *ibid.*, **15,** 1883(1967).

(17) P. Singh, J. K. Guillory, T. D. Sokoloski, L. Z. Benet, and V. N. Bhatia, *J. Pharm. Sci.*, **55,** 63(1966).

(18) G. Levy and T. Matsuzawa, *ibid.*, **54,** 1003(1965).

(19) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, Chap. 11.

(20) L. Z. Benet and J. E. Goyan, *J. Pharm. Sci.*, **56,** 665(1967).

(21) J. M. Dietschy, H. S. Salomon, and M. D. Siperstein, *J. Clin. Invest.*, **45,** 832(1966).

(22) D. S. Riggs, "The Mathematical Approach to Physiological Problems," Williams and Wilkins, Baltimore, Md., 1963, Chap. 7.

(23) P. F. Curran and A. K. Solomon, *J. Gen. Physiol.*, **41,** 143 (1957).

(24) R. K. Crane, *Fed. Proc.*, **24,** 1000(1965).

(25) P. F. Curran, *ibid.*, **24,** 993(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 8, 1969, from the *College of Pharmacy, Washington State University, Pullman, WA 99163*

Accepted for publication January 14, 1970.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Abstracted from the National Award of Merit manuscript submitted by R.H.T. and C.S.M. to the 1969 Lunsford Richardson Pharmacy Awards for Undergraduate Research.

This investigation was supported by PHS Research Grants GM 15093 and 17243 from the National Institute of General Medical Sciences.

* Recipient of the Lunsford Richardson Pharmacy Award—National Award of Merit.

† Undergraduate Research Participant supported by funds provided for biological and medical research by State of Washington Initiative Measure No. 171.

‡ National Science Foundation Undergraduate Research Fellow.

+ Present address: School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

Parameters Affecting Absorption of Griseofulvin in a Human Subject Using Urinary Metabolite Excretion Data

PETER KABASAKALIAN, MICAELA KATZ, BERNARD ROSENKRANTZ, and EDWARD TOWNLEY

Abstract □ Using the urinary excretion of 6-demethylgriseofulvin as the index of absorption, the effects of three previously studied parameters (high-fat breakfast, particle size, and dissolution rate) on the absorption of griseofulvin in a single subject are compared with published blood level data derived from many subjects. The results of the effects of three new parameters (time of dose administration, low-dose level, and gastrointestinal transit time) are also reported.

Keyphrases □ Physiological availability—griseofulvin, man □ Griseofulvin absorption parameters—urinary excretion data, man □ 6-Demethylgriseofulvin metabolite—griseofulvin absorption parameters, man □ Trimethylsilyl ether derivative—6-demethylgriseofulvin, griseofulvin metabolite □ GLC—analysis

The factors (1–12) affecting the absorption of orally administered griseofulvin in man have been studied by measuring (using a fluorometric chemical analytical procedure) the level of griseofulvin in blood at various times after drug administration. The calculation of the degree of absorption is based on the assumption that the physiological availability of the drug is proportional to the area (usually determined by the trapezoidal rule) under the blood level–time curve (3, 5, 13–15).

The factors affecting absorption were found to be a function of the diet (high-fat meal) (1, 2), the dosage formulation [particle size (3–7), dissolution rate (8, 9), solubility (7, 10), and presence of surfactants (4, 7)], and the dosage regimen [dosage level (3, 11, 12) and repetitive dosage schedule (6, 7, 9, 11)].

The nonlinear (logarithmic) relationship (3, 11, 12) between the dose ingested and the area under the blood level–time curve has raised questions concerning the validity of the model on which this method of calculating absorption is based. Atkinson *et al.* (11) suggested

since when sodium was completely replaced by potassium in the bathing buffer solutions, only a 1.08 value was obtained for the ratio of permeability coefficients for salicylate ion.

5. It would appear that the virtual pH hypothesis of Hogben *et al.* (4) is inoperative when considering *in vitro* intestinal transport.

6. If the presently accepted hypotheses concerning the *in vivo* intestinal absorption of ionizable drugs are valid, it would appear that *in vitro* intestinal transport studies could lead to erroneous conclusions concerning the degree of absorption of ionizable drugs *in vivo*.

REFERENCES

(1) T. H. Wilson, "Intestinal Absorption," W. B. Saunders, Philadelphia, Pa., 1962.

(2) J. A. Benson, Jr., and A. J. Rampone, *Ann. Rev. Physiol.*, **28,** 201(1966).

(3) P. A. Shore, B. B. Brodie, and C. A. M. Hogben, *J. Pharmacol. Exp. Ther.*, **119,** 361(1957).

(4) C. A. M. Hogben, D. J. Tocco, B. B. Brodie, and L. S. Schanker, *ibid.*, **125,** 275(1959).

(5) L. S. Schanker, D. J. Tocco, B. B. Brodie, and C. A. M. Hogben, *ibid.*, **123,** 81(1958).

(6) L. S. Schanker, *J. Med. Chem.*, **2,** 343(1960).

(7) L. S. Schanker, *Ann. Rev. Pharmacol.*, **1,** 29(1961).

(8) T. H. Wilson and G. Wiseman, *J. Physiol., London*, **123,** 116(1954).

(9) R. K. Crane and T. H. Wilson, *J. Appl. Physiol.*, **12,** 145 (1958).

(10) H. Nogami and T. Matsuzawa, *Chem. Pharm. Bull.*, **9,** 532(1961).

(11) J. M. Dietschy, H. S. Salomon, and M. D. Siperstein, *J. Clin. Invest.*, **45,** 832(1966).

(12) L. J. Ravin and P. D. Bernardo, *J. Pharm. Sci.*, **57,** 1075 (1968).

(13) H. Nogami and T. Matsuzawa, *Chem. Pharm. Bull.*, **9,** 532 (1961).

(14) *Ibid.*, **10,** 1055(1962).

(15) H. Nogami, M. Hanano, and J. Watanabe, *ibid.*, **10,** 1161 (1962).

(16) K. Kakemi, T. Arita, R. Hori, and R. Konishi, *ibid.*, **15,** 1883(1967).

(17) P. Singh, J. K. Guillory, T. D. Sokoloski, L. Z. Benet, and V. N. Bhatia, *J. Pharm. Sci.*, **55,** 63(1966).

(18) G. Levy and T. Matsuzawa, *ibid.*, **54,** 1003(1965).

(19) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, Chap. 11.

(20) L. Z. Benet and J. E. Goyan, *J. Pharm. Sci.*, **56,** 665(1967).

(21) J. M. Dietschy, H. S. Salomon, and M. D. Siperstein, *J. Clin. Invest.*, **45,** 832(1966).

(22) D. S. Riggs, "The Mathematical Approach to Physiological Problems," Williams and Wilkins, Baltimore, Md., 1963, Chap. 7.

(23) P. F. Curran and A. K. Solomon, *J. Gen. Physiol.*, **41,** 143 (1957).

(24) R. K. Crane, *Fed. Proc.*, **24,** 1000(1965).

(25) P. F. Curran, *ibid.*, **24,** 993(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 8, 1969, from the *College of Pharmacy, Washington State University, Pullman, WA 99163*

Accepted for publication January 14, 1970.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Abstracted from the National Award of Merit manuscript submitted by R.H.T. and C.S.M. to the 1969 Lunsford Richardson Pharmacy Awards for Undergraduate Research.

This investigation was supported by PHS Research Grants GM 15093 and 17243 from the National Institute of General Medical Sciences.

* Recipient of the Lunsford Richardson Pharmacy Award—National Award of Merit.

† Undergraduate Research Participant supported by funds provided for biological and medical research by State of Washington Initiative Measure No. 171.

‡ National Science Foundation Undergraduate Research Fellow.

+ Present address: School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

Parameters Affecting Absorption of Griseofulvin in a Human Subject Using Urinary Metabolite Excretion Data

PETER KABASAKALIAN, MICAELA KATZ, BERNARD ROSENKRANTZ, and EDWARD TOWNLEY

Abstract □ Using the urinary excretion of 6-demethylgriseofulvin as the index of absorption, the effects of three previously studied parameters (high-fat breakfast, particle size, and dissolution rate) on the absorption of griseofulvin in a single subject are compared with published blood level data derived from many subjects. The results of the effects of three new parameters (time of dose administration, low-dose level, and gastrointestinal transit time) are also reported.

Keyphrases □ Physiological availability—griseofulvin, man □ Griseofulvin absorption parameters—urinary excretion data, man □ 6-Demethylgriseofulvin metabolite—griseofulvin absorption parameters, man □ Trimethylsilyl ether derivative—6-demethylgriseofulvin, griseofulvin metabolite □ GLC—analysis

The factors (1–12) affecting the absorption of orally administered griseofulvin in man have been studied by measuring (using a fluorometric chemical analytical procedure) the level of griseofulvin in blood at various times after drug administration. The calculation of the degree of absorption is based on the assumption that the physiological availability of the drug is proportional to the area (usually determined by the trapezoidal rule) under the blood level–time curve (3, 5, 13–15).

The factors affecting absorption were found to be a function of the diet (high-fat meal) (1, 2), the dosage formulation [particle size (3–7), dissolution rate (8, 9), solubility (7, 10), and presence of surfactants (4, 7)], and the dosage regimen [dosage level (3, 11, 12) and repetitive dosage schedule (6, 7, 9, 11)].

The nonlinear (logarithmic) relationship (3, 11, 12) between the dose ingested and the area under the blood level–time curve has raised questions concerning the validity of the model on which this method of calculating absorption is based. Atkinson *et al.* (11) suggested

the existence of a linear relationship between the half-life and logarithm of the ingested dose. This dependence of half-life on the amount of drug in the blood is unusual if elimination was following a simple model containing first-order rate constants. Riegelman has shown that one basic assumption of the model, the constancy of the apparent volume of distribution, is invalid for griseofulvin in the rabbit (16). He has also questioned the single-compartment basis of the model in man (17). However, Riegelman has recently shown that, although a multicompartment model is necessary for calculating absorption, the plasma level–time curve for man (18) was proportional to the dose administered.

A further complication relating to the use of blood data has been the fact that most investigations have used a single-dosage regimen. Marvel *et al.* (7) have shown that in the case of griseofulvin, a factor (surfactant in formulation) which enhanced absorption of griseofulvin under a single-dosage regimen did not do so under a multiple-dosage regimen for which this drug is intended.

Besides the use of blood level data, physiological availability can be estimated from urinary excretion data (19, 20). A significant amount of drug must be excreted unchanged in the urine if questions of valid use of this method are not to be raised (21). Unfortunately, the amount of free griseofulvin excreted in the urine is only about 0.1% of the ingested dose (22). Fortunately, the urinary excretion of the reported metabolite (23) (6-demethylgriseofulvin) of griseofulvin can be used for this purpose. Absorbed griseofulvin is reported to be completely excreted as 6-demethylgriseofulvin (10).

This investigation was undertaken to determine whether urinary metabolite excretion data as first used by Riegelman (10) can be used to study the parameters that affect the absorption of griseofulvin in man, but which may not conveniently be studied by the blood level method.

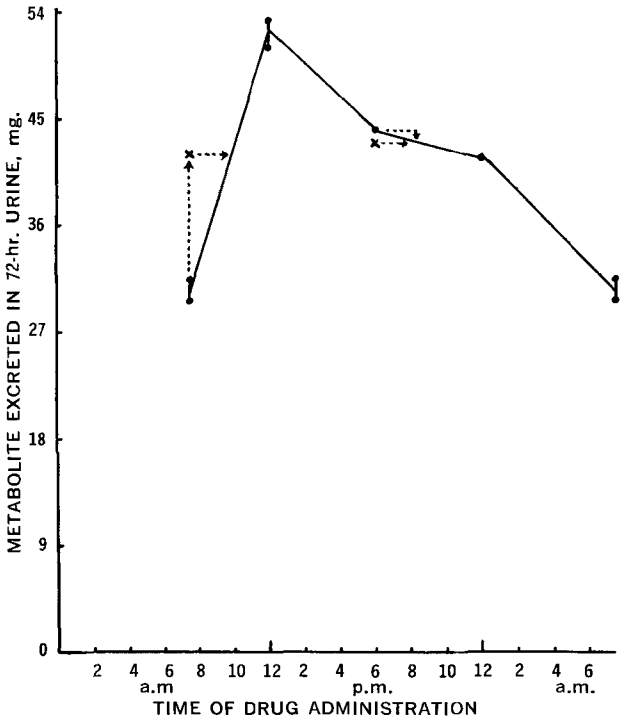


Figure 1—*Plot of metabolite yield after the subject has taken a single dose of 250-mg. microsize griseofulvin experimental Product A at various times during the day: ●, with a nonfat meal; and ×, with a fat meal.*

Table I—Urinary Excretion of Metabolite[a]

Time of Administration	Diet, Meal	Time after Drug Administration, days	Metabolite: In 24-hr. Urine, mg.	Metabolite: In 72-hr. Urine, mg.
7:30 a.m.	Nonfat	1.0	23.1	—
		2.0	5.7	—
		3.0	0.9	29.7
7:30 a.m. repeat[b]	Nonfat	1.0	20.9	—
		2.0	8.7	—
		3.0	1.9	31.5
7:30 a.m.	Fat	1.0	28.1	—
		2.0	10.4	—
		3.0	3.5	42.0
12:00 noon	Nonfat	1.0	37.6	—
		2.0	13.0	—
		3.0	2.8	53.4
12:00 noon repeat[b]	Nonfat	1.0	36.6	—
		2.0	11.5	—
		3.0	3.0	51.1
6:00 p.m.	Nonfat	1.0	29.2	—
		2.0	11.7	—
		3.0	3.3	44.2
6:00 p.m.	Fat	1.0	25.0	—
		2.0	13.9	—
		3.0	4.1	43.0
12:00 midnight	Nonfat	1.0	31.3	—
		2.0	9.4	—
		3.0	1.1	41.8

[a] By subject receiving a single dose of 250 mg. of microsize griseofulvin experimental Product A at various times of drug administration and with or without a fat meal.
[b] Repeat run 1 month later.

EXPERIMENTAL[1]

Material—Commercially available 250-mg. regular size and microsize griseofulvin formulations of three manufacturers, together with one 500-mg. regular size and one 250-mg. and seven 125-mg. microsize experimental griseofulvin formulations, were used in this study.

***In Vitro* Dissolution Test**—Katchen's (8, 24) modified oscillatory tube method was used with simulated intestinal fluid.

Subject—A single healthy ambulatory subject was used in this study. During the experimental period, there were no dietary restrictions except for fat intake during the time of dosage administration and high-fat meal experiments. The normal diet was bland. During the gastrointestinal transit time study, the diet was altered as noted in Table V.

Dosage Regimen—A single-dosage regimen was used. The dose was administered at 7:30 a.m., 12:00 noon, 6:00 p.m., or 12:00 midnight as noted in the time of dose administration study immediately prior to the three regular daily meals; no meals were skipped. After this study, 12:00 noon was chosen as a fixed parameter for the rest of the studies.

Urine Collection—The urine was collected normally for 3 days subsequent to drug administration and combined into 24-hr. samples. The urine volume and pH were noted. The urine was analyzed right after collection. No change in metabolite concentration was observed over several months on repeated assays.

Analytical Method—The urine was analyzed for 6-demethylgriseofulvin[2] by gas-liquid chromatography. Twenty-five milliliters of urine was acidified to pH 1 and extracted with 25 ml. of chloroform.

[1] All gas chromatograms were obtained on a F and M model 400 gas chromatograph with a flame-ionization detector and a Minneapolis Honeywell recorder.
[2] β-Glucuronidase and sulfatase hydrolysis did not indicate the presence of any conjugated metabolite.

Table II—Urinary Excretion of Metabolite[a]

Dose, mg.	Time after Drug Administered, days	Urine ml.	Urine pH	Metabolite In 24-hr. Urine, mg.	Metabolite In 72-hr. Urine, mg.	Metabolite/Dose Ratio in 72-hr. Urine
500	1.0	1860	6.4	35.2	—	—
	2.0	1220	5.6	6.7	—	—
	3.0	1080	5.7	2.0	43.9	0.088
250	1.0	1750	6.3	15.2	—	—
	2.0	960	5.7	6.1	—	—
	3.0	1630	6.4	2.9	24.2	0.097
125	1.0	1650	6.5	9.3	—	—
	2.0	1150	6.6	3.3	—	—
	3.0	1100	6.2	0.7	13.3	0.106
45	1.0	790	6.6	3.3	—	—
	2.0	700	6.0	1.3	—	—
	3.0	1120	6.2	0.2	4.8	0.107
15	1.0	860	5.7	1.02	—	—
	2.0	1620	6.8	0.28	—	—
	3.0	810	6.3	0.11	1.41	0.094
5	1.0	1180	6.2	0.46	—	—
	2.0	1820	6.3	0.00	—	—
	3.0	1700	6.6	0.00	0.46	0.092

[a] By subject receiving at 12:00 noon a single dose or fractions of 500 mg. of regular size griseofulvin Product B.

A 10-ml. aliquot of the chloroform extract was evaporated to a residue on a steam bath under a nitrogen atmosphere. The residue was dried in a draft oven at 60° for 10 min. The trimethylsilyl ether derivative was formed according to the procedure of Horning *et al.* (25), using 100 μl. of B.S.A. reagent,[3] mixing, and heating for 10 min. at 60°. After the addition of 100 μl. of pyridine, a 5-μl. sample was injected into a diatomaceous earth[4] column containing 1% OV-17[4] (a phenylmethyl siloxane polymer) as liquid phase. A 1.22-m. (4.0-ft.) column exhibited at 225° a retention time of 12.4 min. and 1300 theoretical plates for 6-demethylgriseofulvin.

RESULTS AND DISCUSSION

Time of Dosage Administration—The amount of metabolite excreted in the urine in a 3-day period after the administration of an experimental 250-mg. microsize griseofulvin product, A, is recorded in Table I. While the subject's diet was kept low in fats, the time of dosage administration was varied. The results of these experiments are also displayed in the form of a graph in Fig. 1.

The absorption as indicated by the amount of metabolite yield is a function of the time of drug administration. The absorption is lowest early in the morning after a night of fasting. In all the previous (1–12) studies of absorption of griseofulvin in man, the drug was administered early in the morning, *i.e.*, at breakfast time. The absorption reached a maximum when the drug was administered at noon. Griseofulvin administered at supper time or at midnight yielded intermediate absorption values.

The effect of time of griseofulvin administration has not previously been reported. It is not evident why there should be a maximum and minimum absorption when the drug is administered at noon and breakfast time, respectively. That this is not a spurious response but very real was revealed by the fact that the amount of metabolite excreted when the drug was again administered at noon and breakfast time after a lapse of a month was nearly identical to the initial values as shown in Table I.

Perhaps a circadian rhythm (26) involved in the drug absorption [such as intestinal motility or the 24-hr. bile cycle (27)] is responsible for this phenomenon.

High-Fat Meal—Using the experimental Product A, the effect of a high-fat meal at breakfast and supper time just prior to drug administration yielded the results shown in Table I. It should be noted that the high-fat meal followed by drug administration at breakfast increased drug absorption. There was no similar effect when the high-fat meal before drug administration was at supper time.

The previous (1, 2) reported effects of a high-fat meal were determined at breakfast time.

Gastric-emptying time (28) is affected by the fat content of the meal. Since griseofulvin is reported to be absorbed (29) mainly in the intestines, the effect of the high-fat meal would be equivalent to taking the drug at a later time.

Using the graph in Fig. 1, the metabolite yield when a high-fat breakfast was consumed by the subject, and assuming a linear relationship to be in existence between the maximum and minimum absorption points, one can calculate a delay of about 2.5 hr. A similar equivalent delay at supper time would cause no increase in absorption (Fig. 1).

Dose Level—A single dose or fractions of the regular size 500-mg. griseofulvin Product B was administered at noon. The metabolite yields are listed in Table II. The results of these experiments are also displayed in the form of a graph in Fig. 2 using the metabolite-to-griseofulvin dose ratio as an indicator. The urine pH and volumes are also presented and show no effect.

As the dose level of the griseofulvin increased 100-fold, the amount of metabolite excreted increased proportionately, as indicated by the constancy of the metabolite-to-griseofulvin ratio. It is apparent that the percentage of the drug absorbed is independent of the dose level. The constancy of this metabolite yield-to-dose ratio served as an internal calibration of absorption, even though the metabolite yield was low in this individual. In previous (3, 11, 12) studies in man, the logarithm of the dose and blood level have been linearly correlated. Atkinson *et al.* (3, 11) have used dose levels of 250 mg. to 1.0 g., while Grin and Denic (12) have used dose levels of approximately 250 mg. to 8.0 g. A plot of logarithm of dose administered and amount of metabolite excreted is shown in Fig. 3. There appears to be no linear relationship as with the blood level data.

These results cannot be compared with blood level data since no experimental data with low griseofulvin levels have been previously reported.

The absorption of drugs from the gastrointestinal tract are generally assumed to undergo two steps as shown in Eq. 1:

$$A_{\text{solid}} \rightarrow A_{\text{solution}} \rightarrow A_{\text{body}} \qquad \text{(Eq. 1)}$$

The first is the dissolution of the solid dosage and the second is the transport of the drug in solution in the gastrointestinal lumen through a lipoidal permeable membrane into the blood.

Although the extremely low water solubility of griseofulvin probably makes the dissolution step the rate-limiting step in the absorption process, one might have expected that the water solubility would not be self-limiting any longer at the lower doses and that at least the regular size griseofulvin would approach the microsize griseofulvin in its yield of metabolite (a metabolite-to-griseofulvin dose ratio of 0.10 *vs.* 0.21 from the time of dose administration study). Instead, the regular size griseofulvin acted as if it obeyed the cube root law of dissolution of Hixson and Crowell (30).

Hixson and Crowell derived an equation for the dissolution of a single particle in which the surface area was allowed to change with time while its shape factor (ratio of dimensions) remained constant. Niebergall and Goyan (31) extended the equation for use

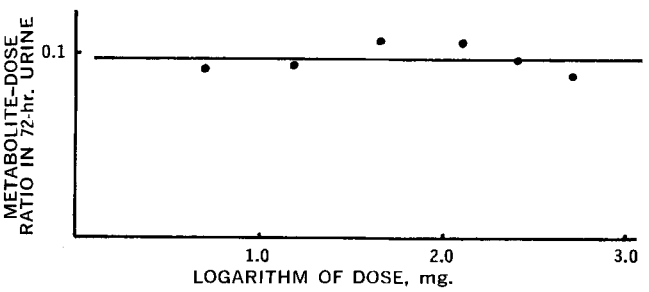


Figure 2—*Plot of metabolite-dose ratio* vs. *logarithm of dose for subject receiving at 12:00 noon a single dose or fraction of 500-mg. regular size griseofulvin Product B.*

[3] B.S.A. reagent (bistrimethylsilyl acetamide) is available from Mann Research, New York, N. Y.

[4] Gas-Chrom Q, Applied Science Laboratories, Inc., State College, PA 16801

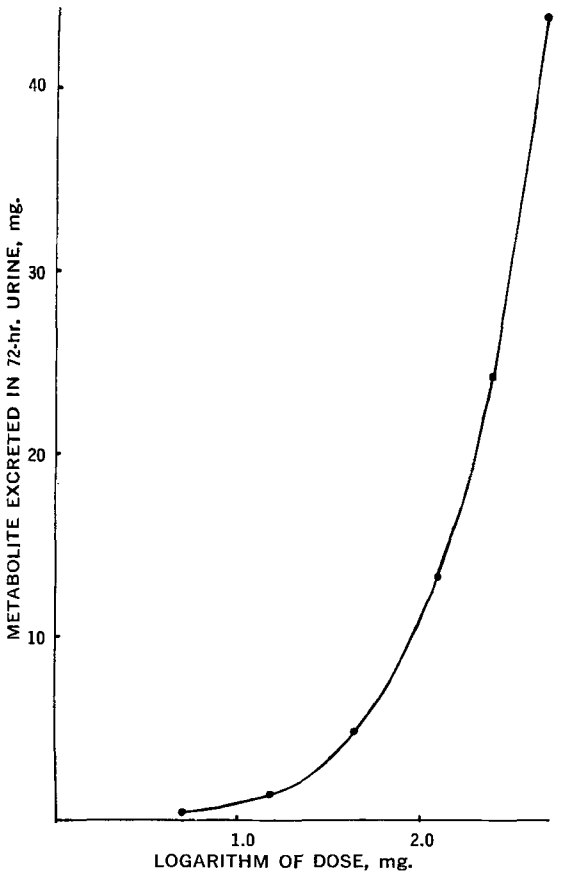


Figure 3—*Plot of metabolite yield* vs. *logarithm of dose for subject receiving at 12:00 noon a single dose or fraction of 500-mg. regular size griseofulvin Product B.*

in multiparticulate systems by assuming N equal-size particles. When the amount of solute needed to saturate a given volume of solvent is much greater than the concentration of material in solution, the integrated form becomes

$$N^{1/3}(w_0^{1/3} - w^{1/3}) = N^{1/3}(Kt) \qquad \text{(Eq. 2)}$$

where N is the number of particles, w_0 is the initial weight of each particle, w is the weight of the particle at time t, and K is the product of the intrinsic dissolution rate constant, solubility, density, and shape factor for the solute.

Therefore, no matter what the total number of particles is, the cube-root law indicates that all particles of identical size obey the fundamental relationship. A graphic presentation of this law using the fraction dissolved, $(w_0 - w)/w_0$, is shown in Fig. 4. A consequence of this relationship is that if the gastrointestinal transit time is constant, the percentage absorption of the drug will be independent of the amount of drug administered.

Particle Size—Table III contains the results of six commercially available 250-mg. griseofulvin products. Three products, C, D, and E, were manufactured with microsize griseofulvin; three products, F, G, and H, were produced with regular size griseofulvin. These drugs were administered at noon time.

The microsize griseofulvin products yielded about twice as much metabolite as the regular size griseofulvin products.

Although the results within each group appeared to differ from each other, these differences are not considered significant since they include formulation content and analytical variations. The mean amount of metabolite obtained from the use of the microsize griseofulvin products was found to be 50.7 mg., while the corresponding value from the regular size griseofulvin products was found to be 26.6 mg. This indicates that the regular size griseofulvin is equivalent to about one-half the microsize griseofulvin.

This equivalency was difficult to determine when blood level data were used because of the nonlinear response of blood level to dose administered. However, blood level data (3–7) did yield this information when dosages of microsize and regular size griseofulvin were compared at identical blood levels.

Table III—Urinary Excretion of Metabolite[a]

Product	Griseofulvin	Time after Drug Administered, days	Metabolite: In 24-hr. Urine, mg.	Metabolite: In 72-hr. Urine, mg.
C	Microsize	1.0	37.1	—
		2.0	12.3	—
		3.0	2.9	52.3
D	Microsize	1.0	30.4	—
		2.0	16.9	—
		3.0	4.1	51.4
E	Microsize	1.0	37.9	—
		2.0	9.4	—
		3.0	1.1	48.4
F	Regular	1.0	17.1	—
		2.0	7.4	—
		3.0	1.5	26.0
G	Regular	1.0	15.2	—
		2.0	8.3	—
		3.0	2.4	25.9
H	Regular	1.0	19.8	—
		2.0	6.7	—
		3.0	1.5	28.0

[a] By subject receiving a single dose of commercially available 250-mg. griseofulvin products administered at 12:00 noon.

Although the *Federal Register* (32) classifies griseofulvin into large particles (regular size) and microsize material, there isn't as large a difference between the material as their names imply. The specification of the specific surface area for the large particle griseofulvin is that it should not be less than 0.35 and not more than 0.65 m.²/g., while that for the microsize griseofulvin is that it should not be less than 1.3 and not more than 1.7 m.²/g. The ratio of the average of these limits is 1:3 with respect to surface area.

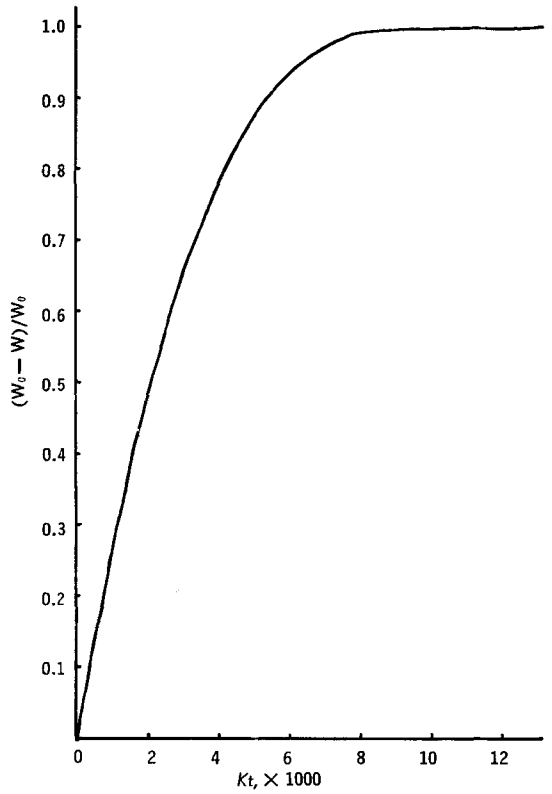


Figure 4—*Plot of the cube-root law of dissolution of Hixson and Crowell in terms of the fraction of solute,* $(W_0 - W)/W_0$, *dissolved* vs. Kt.

Table IV—Urinary Excretion of Metabolite[a]

Product	Dissolution Rates Time, min.	% Dissolved	Time after Drug Administered, days	Metabolite In 24-hr. Urine, mg.	Metabolite In 72-hr. Urine, mg.
I	30	88	1.0	18.2	—
	60	93	2.0	6.1	—
	120	95	3.0	2.0	26.3
J	30	60	1.0	13.8	—
	60	80	2.0	7.1	—
	120	91	3.0	1.9	23.1
K	30	34	1.0	15.9	—
	60	54	2.0	4.6	—
	120	76	3.0	1.4	21.9
L	30	19	1.0	18.9	—
	60	39	2.0	6.1	—
	120	59	3.0	1.7	26.7
M	30	8	1.0	18.4	—
	60	16	2.0	5.9	—
	120	32	3.0	2.1	26.4
N	30	2	1.0	8.8	—
	60	5	2.0	2.5	—
	120	9	3.0	0.8	12.1

[a] By subject receiving at 12:00 noon a single dose of 125 mg. of microsize griseofulvin products having different dissolution rates.

Matthews and Rhodes (33) reported surface area of 0.38 m.²/g. of regular size griseofulvin and 1.32 m.²/g. of microsize griseofulvin. The ratio of surface area of regular size to microsize griseofulvin in this case is 1:3.5.

An inspection of Fig. 4 indicates that the rate of dissolution is essentially constant up to the time when about 40% of the solute has dissolved. Therefore, the ratio of percentage absorptions of microsize and regular size griseofulvin can be calculated by the use of a form of the Noyes-Whitney (34) equation, Eq. 3 (where S is the surface area, V the volume of solvent, K_1 the rate constant, and w_s the weight of solute needed to saturate the solution), when the total absorption is below 40% and the amount of solute in solution is very much less than that required to saturate the solution.

$$V(dw/dt) = -K_1S(w_s) \qquad \text{(Eq. 3)}$$

$$\frac{[N_1(dw_1/dt)]}{[N_2(dw_2/dt)]} = \frac{N_1S_1}{N_2S_2} \qquad \text{(Eq. 4)}$$

Substituting the reported (33) surface areas of 1.32 and 0.38 m.²/g. of microsize and regular size griseofulvin, respectively, yields the following ratio:

$$(N_1S_1)/(N_2S_2) = 1.32/0.38 = 3.5 \qquad \text{(Eq. 5)}$$

The ratio of metabolite yield actually found was 2. This may be due to the fact that the griseofulvin has a hydrophobic surface which has not been completely converted to a hydrophilic surface by granulation (35, 36), and/or that there is significant absorption of air on its surface (37), and/or the particles do not regain their surface area on disintegration of the dosage formulation due to particle aggregation.

Of course, the model used may be incorrect because of the nonuniformity of particle size and/or departure from the concept of retention of constant shape factor during the course of the dissolution process.

Dissolution Rate—Comparison of the amount of metabolite excreted after the ingestion of six experimental microsize griseofulvin formulations, having different dissolution rates (Table IV), did not reveal any relationship between the logarithm of the dissolution rate (Fig. 5) and absorption (except for the slowest dissoluting Product N). These observations appear to differ from those of Katchen and Symchowicz (8, 9). They reported a positive correlation between the logarithm of dissolution rate (amount dissolved in 30 min.) in simulated intestinal fluid and the 24-hr. mean griseofulvin plasma level. However, a closer scrutiny of Katchen and Symchowicz's papers indicates that under the single-dosage regimen there is a large intersubject variation in the effect of dissolution rates. In fact, two (Subjects 4 and 8) out of their 18 subjects show

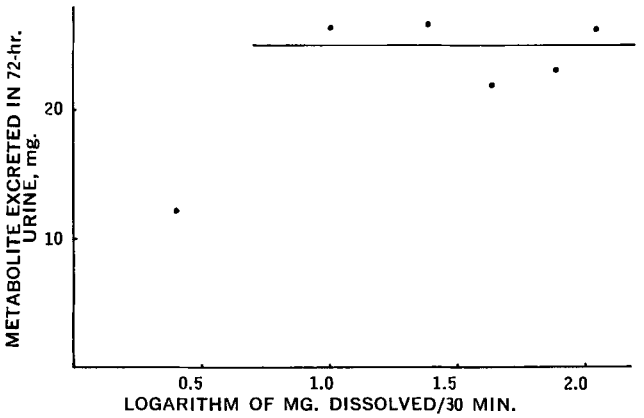


Figure 5—*Plot of metabolite yield* vs. *logarithm of dissolution rate (mg. dissolved/30 min.) for subject receiving at 12:00 noon a single dose of 125 mg. of microsize griseofulvin experimental products having different dissolution rates.*

no correlation between dissolution rate and drug absorption. The subject used in this investigation evidently falls into this minority group.

Recently there has been a flurry of investigations to increase the dissolution rate of griseofulvin and thus supposedly of increasing gastrointestinal absorption. Goldberg *et al.* (38) have reported a eutectic mixture with considerable solid solubility of griseofulvin in succinic acid having dissolution rates about seven times faster than the pure material. Mayersohn and Gibaldi (39) have published a new method of solid-state dispersion of griseofulvin in polyvinylpyrrolidone. The resultant product is reported as having greatly increased dissolution rates over micronized griseofulvin. A recent patent (40) reports that higher griseofulvin blood levels in the rat can be obtained by the use of an intimate mixture of griseofulvin and isogriseofulvin. The success of all these approaches has yet to be proven in man.

There are basically two types of drugs (41), those in which the sequence of drug concentration in the body and pharmacologic activity coincides and those in which there is a considerable delay. In the former case, rapid onset of pharmacological activity is desired; therefore, a dosage form which permits the drug to be

Table V—Urinary Excretion of Metabolite[a]

Irritating Diet Component	Time after Drug Administered, days	Metabolite In 24-hr. Urine, mg.	Metabolite In 72-hr. Urine, mg.
None	1.0	16.6	—
	2.0	6.6	—
	3.0	2.0	25.2
Red pepper	1.0	20.2	—
	2.0	5.9	—
	3.0	1.8	27.9
Milk	1.0	21.8	—
	2.0	3.8	—
	3.0	1.2	26.8
Pepperoni	1.0	20.6	—
	2.0	6.1	—
	3.0	2.0	28.7
Greasy fried food	1.0	24.2	—
	2.0	5.8	—
	3.0	1.5	31.5
Alcohol	1.0	20.2	—
	2.0	2.8	—
	3.0	4.7	27.7
Nuts	1.0	24.4	—
	2.0	6.7	—
	3.0	1.6	32.7
None	1.0	18.5	—
	2.0	5.0	—
	3.0	1.4	24.9

[a] By subject receiving at 12:00 noon a single dose of 125 mg. of microsize griseofulvin Product O followed by alterations in diet.

absorbed rapidly is required. These drugs are usually taken in a *single-dosage regimen* and may be considered to belong to the fast-action "aspirin" type drugs.

In the second case, where there is considerable delay in pharmacologic response, there is little advantage in rapid drug release from the dosage form as long as release is not delayed to the point of reducing drug absorption. The drugs in this category are usually taken in a *multiple-dosage regimen*. Griseofulvin fits into this category. The usually recommended treatment with griseofulvin is a multiple-dosage regimen lasting from 4 to 6 weeks.

Gastrointestinal Transit Time—The results of the attempt to vary the gastrointestinal transit time by the use of alterations in diet (red peppers, excess milk, pepperoncini, greasy fried foods, nuts, and alcohol) for the noon meal immediately after ingestion of 125-mg. microsize griseofulvin experimental Product O are shown in Table V. These variant components of the diet were used independently. Red peppers, excess milk, and pepperoni had a diarrheic effect; excess nuts had a constipating effect; the greasy fried food had a nauseating and a sense-of-fullness effect; and the alcohol had a relaxing effect on the subject.

Product O had a dissolution rate which was slightly faster than that for Product N whose absorption appeared to be impaired by its low-dissolution rate. The percentages dissolved in 30, 60, and 120 min. were 3, 6, and 13%, respectively.

Although these diets may have varied the transit time through the whole gastrointestinal tract, there is little evidence that the transit time through the absorption site was affected much (except in the cases of greasy fried food and nuts), as evidenced by the relatively constant metabolite yield which was comparable to that obtained when the subject had his normal meal. The absorption increased significantly when the irritating diet components were greasy fried foods and nuts.

SUMMARY AND CONCLUSIONS

The effects of six parameters on the physiological availability of orally administered griseofulvin formulations were studied by the use of urinary metabolite excretion data from a single subject. The first parameter, time of dosage administration, had an effect on griseofulvin absorption in the manner of a circadian rhythm, the absorption being least in the morning and the most at noon time. The second parameter, high-fat meal, appeared to be equivalent to a delay in time of dosage administration and subsequently had its effect dependent on the previous factor. The third parameter, dose level, indicated that the percentage absorption of drug was independent of the amount of drug administered. The fourth parameter, particle size, showed that microsize griseofulvin was absorbed about twice as much as regular size griseofulvin. The fifth parameter, dissolution rate of the griseofulvin formulation, had little effect on absorption of griseofulvin as long as the dissolution rate was above that for Product N. The sixth parameter, gastrointestinal transit time, could not be varied enough by dietary means (except in the cases of greasy fried foods and nuts) to affect significantly the residence time of the griseofulvin at the absorption sites.

Obviously, these effects cannot be considered representative of the general population. The urinary metabolite excretion data were in agreement with published blood level data estimation of physiological availability of griseofulvin in formulations for the effect of particle size, high-fat breakfast, and dissolution rate (minority subjects). No published blood level data exist for the time of dosage administration, gastrointestinal transit time, and low-level dose factors. It is inconvenient or impracticable to study these parameters by the blood level method.

REFERENCES

(1) R. G. Crouse, *J. Invest. Dermatol.*, **37,** 529(1961).

(2) R. G. Crouse, *Arch. Dermatol.*, **87,** 176(1963).

(3) R. M. Atkinson, C. Bedford, K. J. Child, and E. G. Tomich, *Antibiot. Chemother.*, **13,** 232(1962).

(4) W. A. M. Duncan, G. MacDonald, and M. J. Thornton, *J. Pharm. Pharmacol.*, **14,** 217(1962).

(5) M. Kraml, J. Dubuc, and R. Gaudry, *Antibiot. Chemother.*, **12,** 239(1962).

(6) M. Kraml, J. Dubuc, and D. Dvornik, *Arch. Dermatol.*, **87,** 179(1963).

(7) J. R. Marvel, D. A. Schlichting, C. Denton, E. J. Levy, and M. M. Cahn, *J. Invest. Dermatol.*, **42,** 197(1964).

(8) B. Katchen and S. Symchowicz, *J. Pharm. Sci.*, **56,** 1108 (1967).

(9) S. Symchowicz and B. Katchen, *ibid.*, **57,** 1383(1968).

(10) S. Riegelman, W. L. Epstein, and R. J. Dayan, presented to the American Pharmaceutical Association, Las Vegas meeting, March 1962.

(11) R. M. Atkinson, C. Bedford, K. J. Child, and E. G. Tomich, *Antibiot. Chemother.*, **12,** 225(1962).

(12) E. I. Grin and M. Denic, *Acta Med. Jugoslav.*, **19,** 62(1965).

(13) J. G. Wagner and E. Nelson, *J. Pharm. Sci.*, **52,** 610(1963).

(14) J. G. Wagner, O. S. Carpenter, and E. J. Collins, *J. Pharmacol. Exp. Ther.*, **129,** 101(1960).

(15) D. G. Chapman, L. G. Chatten, and J. A. Campbell, *Can. Med. Ass. J.*, **76,** 102(1957).

(16) L. J. Fischer and S. Riegelman, *J. Pharm. Sci.*, **54,** 1571 (1965).

(17) S. Riegelman, J. C. K. Loo, and M. Rowland, *ibid.*, **57,** 117, 128(1968).

(18) M. Rowland, S. Riegelman, and W. L. Epstein, *ibid.*, **57,** 984(1968).

(19) A. B. Morrison and J. A. Campbell, *ibid.*, **54,** 1(1965).

(20) E. Nelson and I. Schaldemose, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 489(1959).

(21) E. Nelson, *ibid.*, **49,** 437(1960).

(22) J. M. Robinson, Jr., R. C. V. Robinson, E. S. Bereston, L. L. Manchey, and F. K. Bell, *Arch. Dermatol.*, **81,** 66(1960).

(23) M. J. Barnes and B. Boothroyd, *Biochem. J.*, **78,** 41(1961).

(24) B. Calesnick, B. Katchen, and J. Black, *J. Pharm. Sci.*, **54,** 1277(1965).

(25) A. Zlatkis, "Advances in Gas Chromatography 1967," Proceedings of the Fourth International Symposium held in New York, N. Y., April 3–6, 1967. Preston Technical Abstracts Co., Evanston, Ill., 1967, p. 129.

(26) J. A. Martinez-O'Ferrall, *J. Occup. Med.*, **10,** 305(1968).

(27) B. Josephson and H. Larsson, *Skand. Arch. Physiol.*, **69,** 227(1934).

(28) W. H. Bachrach, *Physiology and Pathologic Physiology of the Stomach*, Ciba Found. Symp., **11,** 3(1959).

(29) B. Davis, K. J. Child, and E. G. Tomich, *J. Pharm. Pharmacol.*, **13,** 166(1961).

(30) A. W. Hixson and J. H. Crowell, *Ind. Eng. Chem.*, **23,** 923 (1931).

(31) P. J. Niebergall and J. E. Goyan, *J. Pharm. Sci.*, **52,** 29 (1963).

(32) *Fed. Regist.*, **31** (12), Jan. 19, 1966 (21 CFR 148 g.).

(33) B. A. Matthews and C. T. Rhodes, *J. Pharm. Sci.*, **56,** 838 (1967).

(34) A. A. Noyes and W. R. Whitney, *J. Amer. Chem. Soc.*, **19,** 930(1897).

(35) P. Finholt, H. Kristiansen, O. C. Schmidt, and K. Wold, *Medd. Nor. Farm. Selsk.*, **28,** 31(1966).

(36) P. Finholt, R. H. Pedersen, S. Solvang, and K. Wold, *ibid.*, **28,** 238(1966).

(37) D. E. Wurster and J. A. Seitz, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 335(1960).

(38) A. H. Goldberg, M. Gibaldi, and J. L. Kanig, *J. Pharm. Sci.*, **55,** 487(1966).

(39) M. Mayersohn and M. Gibaldi, *ibid.*, **55,** 1323(1966).

(40) J. W. Poole, U. S. pat. 3,325,362, June 13, 1967.

(41) G. Levy, *Amer. J. Pharm.*, **135,** 78(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 3, 1969, from the *Research and Development Division, Schering Corp., Bloomfield, NJ 07003*

Accepted for publication November 12, 1969.

The authors thank Mr. S. Strausser for his technical assistance.

Microelectrophoresis and Adsorption Studies of Cholesterol Particles in Bile Salt and Alkyl Surfactant Systems

DANIEL MUFSON* and WILLIAM I. HIGUCHI

Abstract □ As part of a continuing program to understand better the behavior of cholesterol particles in physiological situations, an investigation of the interactions of the cholesterol surface with bile salts and alkyl surfactants was undertaken. Microelectrophoretic techniques and adsorption experiments were employed to characterize the adsorption behavior of these agents. In contrast to the alkyl surfactants, the adsorption of bile salts on the cholesterol particle surfaces was much less than expected in the concentration ranges investigated. These results were initially surprising in light of earlier studies where bile salts were found to inhibit greatly the growth of cholesterol crystals. However, the present data are consistent with the idea that the relatively rigid bile salt molecules can adsorb only onto specific sites on the cholesterol surfaces while the more flexible alkyl surfactants can more readily interact.

Keyphrases □ Cholesterol surface, interaction—bile salts, alkyl surfactants □ Electrophoretic mobility—cholesterol particles □ pH-mobility profiles—cholesterol dispersions □ Microelectrophoresis—cholesterol–bile salts, surfactant interactions □ Adsorption studies—cholesterol–bile salts, surfactant interactions

Cholesterol[1] has been implicated as the cause of many diseases, from atherosclerosis to xanthomatoses. While intensive research has been directed toward the understanding of the role cholesterol plays in these disease states, most of it is of a biological or clinical nature. There is surprisingly little reported work on the quantitative physical chemistry of these systems. For example, even the water solubility of cholesterol was an unknown quantity until recently (1). It, therefore, seems that *in vitro* physical-chemical investigations could be used to advantage to solve some of the very pressing problems of cholesterol deposition, namely, gallstone formation and atherosclerosis.

Cholesterol levels in the body are the results of absorption and synthesis on the one hand and excretion on the other. Removal occurs almost exclusively in the feces *via* the gallbladder. The cholesterol being virtually insoluble in water must be rendered soluble for removal. This function is normally performed by the bile. However, the pathological condition in which cholesterol deposits in the gallbladder, cholelithiasis, is widespread; surgical removal of gallstones is one of the most common operations performed today (2). Gallstone operations today outnumber appendectomies (3). A recent study showed that 59.6% of the women and 41.5% of the men (aged 60 to 100) exhibited gallstones at autopsy (4). "As more people live to develop biliary disease at an older age, surgeons can expect to see and treat an increasing number of patients with difficult biliary problems, complicated by all the other medical problems common to older patients." (5)

The mechanisms leading to the formation of gallstones are imperfectly understood. This is due in part to the many conflicting reports which are to be found in the literature. Bile is a supersaturated solution of cholesterol. Factors responsible for increasing cholesterol's concentration in the gallbladder bile may lead to its precipitation as stones (6). Stones will show almost infinite variation in their composition, appearance, and physical properties, dependent upon the factors involved in their formation (7). Stasis of the bile, pH changes, irritation, coagulation, infection, and the bile salt–phospholipid–cholesterol ratio are some of the factors intimated in the cholesterol deposition process. These factors often interact to produce stone formation. For example, infection can produce a change in pH and irritation of the gallbladder wall. This may allow the absorption of a significant amount of bile salt through the gallbladder wall, seriously affecting the bile salt–cholesterol ratio. The high energy of the supersaturated system is then relieved by the precipitation of cholesterol.

Stones have been found during surgery (8) which suggested that they were formed by the aggregation of smaller spherical particles of cholesterol. Frey *et al.* (9) have observed clusters of cholesterol crystals in the gallbladders of mice fed a lithogenic diet. Thus it appears that in the later stages of stone formation, aggregation of cholesterol particles plays an important role.

There have been physical-chemical studies concerned with the interactions of bile salts and cholesterol in micellar solutions (10–12), at interfaces (13), and at crystalline surfaces (14, 15).

Saad and Higuchi approached the problem from the point of view of cholesterol crystal growth. The influence of cholate, taurocholate, and glycocholate on the precipitation behavior of cholesterol in aqueous media was investigated as a function of pH (14). The Coulter counter was utilized to follow the particle-size distribution changes with time in the supersaturated and undersaturated cholesterol suspension systems. With increasing pH, the rates of growth and dissolution became progressively retarded, with complete inhibition of these processes at pH 8 and higher. The bile salts seemed to exhibit specificity of action which suggested strong binding to specific sites on the cholesterol crystal surface. Conventional long-chain surfactants, sodium dodecyl sulfate and myristyl-γ-picolinium chloride, at 1% concentration had no effect on the crystal growth rate.

Although surgical observations have suggested aggregation of cholesterol crystals as a mechanism for gallstone growth, this aspect has not been pursued from a colloidal-chemical point of view. It would seem that

[1] Systematic nomenclature for compounds given trivial names in the text includes: cholest-5-en-3β-ol, cholesterol; 3α,7α,12α-trihydroxy-5β-cholanoyl glycine, glycocholic acid; 3α,12α-dihydroxy-5β-cholanoyl glycine, glycodeoxycholic acid; 3α,7α,12α-trihydroxy-5β-cholanoyl taurine, taurocholic acid; 3α,7α,12α-trihydroxy-5β-cholanoic acid, cholic acid.

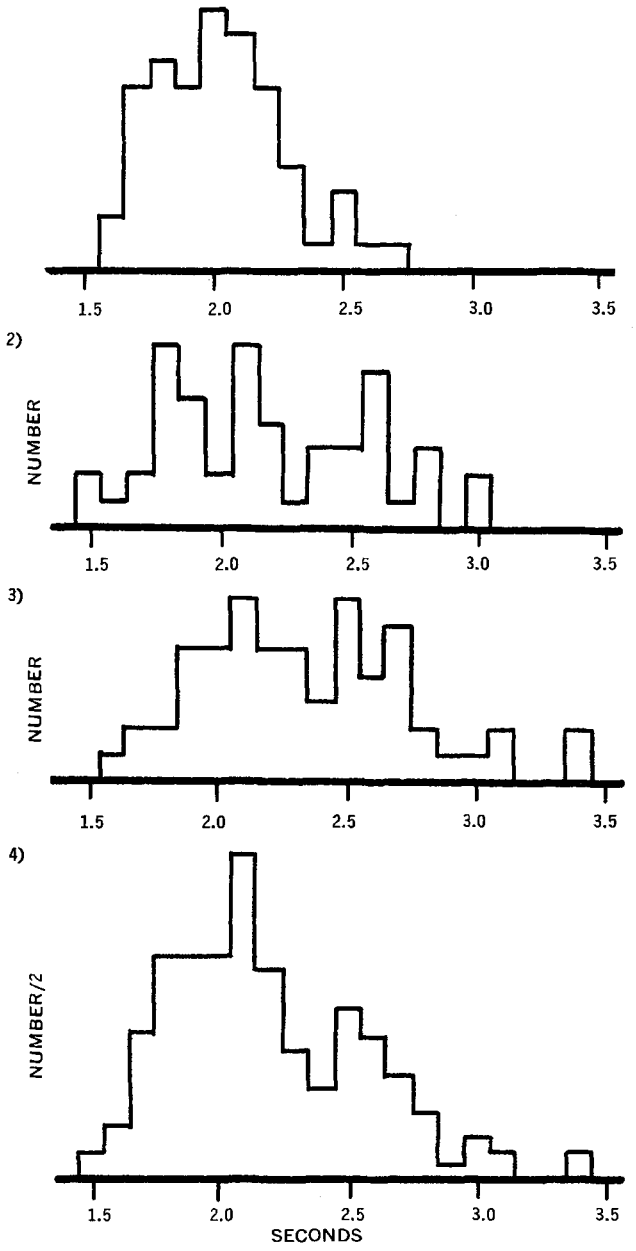


Figure 1—*Histograms of the velocity distribution of cholesterol particles in 10^{-2} M NaCl (time required to travel 0.5 division of Zeta-Meter eyepiece): (1), Experimenter I; (2), Experimenter II; (3), Experimenter II (4-day-old sample); and (4), summation of A, B, and C.*

such a study of the interfacial chemistry of the crystalline cholesterol–bile salt system should be helpful. An investigation of this nature might yield information concerning the etiology and prophylaxis of cholelithiasis.

Microelectrophoresis can be used as a valuable tool to measure interactions at the liquid–solid interface (16–18). Investigations of cholesterol dispersions by microelectrophoretic techniques have been performed. In 1930, Remezov published a voluminous work on the physical-chemical properties of the colloidal state of cholesterol, cholesterol esters, and lecithin (19–23). It is difficult to interpret and correlate his procedures and data. Moyer (24) found Remezov's directions for sol preparation to be too general to reproduce. Douglas and Shaw (25) studied the effect of pH on the electrophoretic mobility of cholesterol, finding a substantial increase in mobility as the pH was raised. Seaman investigated the effects of pH and salts on the mobility of cholesterol particles (26).

In the present studies, the effects of both bile salts and alkyl surfactants on the electrophoretic mobility of cholesterol particles have been determined. Adsorption studies on the same systems were used to complement this data and to allow explanation of some of the phenomena observed.

EXPERIMENTAL

General Considerations—Because the goal of this study was to monitor and interpret the surface properties of cholesterol particles in aqueous media, the presence of minute impurities and the manner of preparation of the dispersant were expected to be important. Cholesterol is known to be unstable towards light, heat, and other radiation in the presence of air. Until the advent of thin-layer chromatography, the widespread nature of cholesterol's autoxidation was not realized (27). Many products are produced during autoxidation, almost all of which are more polar than cholesterol. Oxidation of cholesterol in the presence of UV light has been reported to produce acids of unknown structure (28, 29).

The purity of experimental materials is always of importance in studies of surface properties (30–32). The cholesterol dispersions of some earlier investigations had shown a pH–mobility dependency. This is surprising in light of the nonionogenic nature of cholesterol. The effect of pH on the mobility of cholesterol dispersions of varying purity was therefore initiated prior to the studies of the cholesterol–surfactant systems.

Materials—Purified cholesterol was prepared by subjecting commercial cholesterol (Fisher Scientific Co. and Eastman Organic Chemicals) to the dibromination purification procedure of Fieser (33). Upon the termination of this method, the sample was further recrystallized from ether–methanol, ether–ethanol, and ethanol–water. The purified crystalline sample (m.p. 150°) was stored in a refrigerator under nitrogen. It was found by TLC to be homogeneous. The carbon–hydrogen analysis of this material agreed well with theory: theoretical C, 83.37; H, 12.03; observed C, 84.04; H, 11.99.

The IR spectra[2] of this sample, run in $CHCl_3$, agreed with those in the literature (34–36).

Distilled water of <2 μmho specific conductance was employed in all studies.

All inorganic salts were of analytical reagent quality. Most of the organic additives were used as received unless otherwise noted; *n*-octanol (Fisher Scientific Co.), sodium taurocholate (Mann Research Laboratories, Inc., New York, N. Y., and Calbiochem, Los Angeles, Calif.), sodium cholate (Mann special enzyme grade), cholic acid (Eastman), sodium glycocholate [Calbiochem (A grade)], sodium glycodeoxycholate [Calbiochem (A grade)], sodium dodecyl sulfate-^{35}S (Nuclear-Chicago Corp., Des Plaines, Ill.), sodium dodecyl sulfate (supplied by Dr. K. J. Mysels), cholic-carboxyl-^{14}C acid (Tracerlab, Waltham, Mass.), naphthalene A.R. (J. T. Baker Chemical Co., Phillipsburg, N. J.), 2,5-diphenyloxazole (PPO) (Packard Instrument Co., Inc., Downers Grove, Ill.), octanol-^{14}C (ICN, City of Industry, Calif.).

Buffers used were prepared by titration of the appropriate components in a beaker. The pH was monitored with a Corning model 7 pH meter.[3] Buffers were stored under refrigeration to retard mold growth. Sodium acetate, 1.0 *N*, was added to 10^{-3} *M* acetic acid to produce pH 5 buffer. A pH 6.8 stock buffer was made by dissolving 0.340 g. of potassium phosphate (KH_2PO_4) and 0.254 g. of sodium phosphate dibasic anhydrous (Na_2HPO_4) in enough water to make 100 ml. One milliliter of this stock buffer was used for each 100 ml. of dispersion. Sodium hydroxide (0.1 *N*) was added to 10^{-2} *M* boric acid to produce the pH 9 buffer and to 10^{-4} *M* sodium borate to produce the pH 11 buffer.

Preparation of Dispersions for Microelectrophoresis—Dispersions of cholesterol for microelectrophoretic evaluation were prepared

[2] Perkin-Elmer model 337, Norwalk, Conn.
[3] Corning Glass Works, Corning, N. Y.

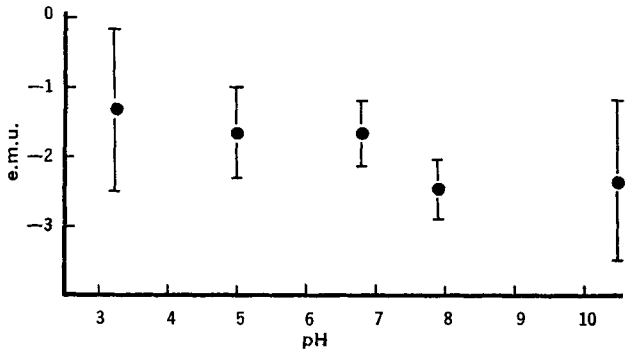


Figure 2—*The pH–mobility profile of cholesterol (bars represent ± 1.96 σ of the population).*

by dilution of concentrated stock dispersions or by precipitation *in situ*.

Stock Dispersion—To prepare 100 ml. of 0.5% w/v dispersion, 500 mg. of the purified cholesterol was first dissolved in 10 ml. of hot ethanol. This solution was subjected to ultrasonic irradiation as 10 ml. of water was quickly added. Irradiation was continued for 30 sec. The resultant dispersion was then added to 80 ml. of water, ultrasonified for 30 sec., treated with nitrogen, and stored in the dark. Twenty-four hours was allowed to elapse before this material was used. Two milliliters of this stock was used to prepare 100 ml. of dispersion containing 0.01% cholesterol. The system was ultrasonified prior to use to disperse any aggregates which may have formed.

***In Situ* Precipitation**—Enough purified cholesterol was dissolved in ethanol so that 1 ml. of this solution yielded a 0.01% dispersion when properly reacted; 1 ml. of water was added to an equal quantity of the alcoholic solution. Ultrasonic energy was supplied during mixing. To this dispersion were added the other components of the study, *i.e.*, sodium chloride, buffer, and test additive.

Electrophoretic Mobility Measurements—The electrophoretic mobility of individual particles was measured by the use of a commercial microelectrophoresis apparatus, the Zeta-Meter.[4] Descriptions of the apparatus appeared recently (37, 38) and will not be repeated here.

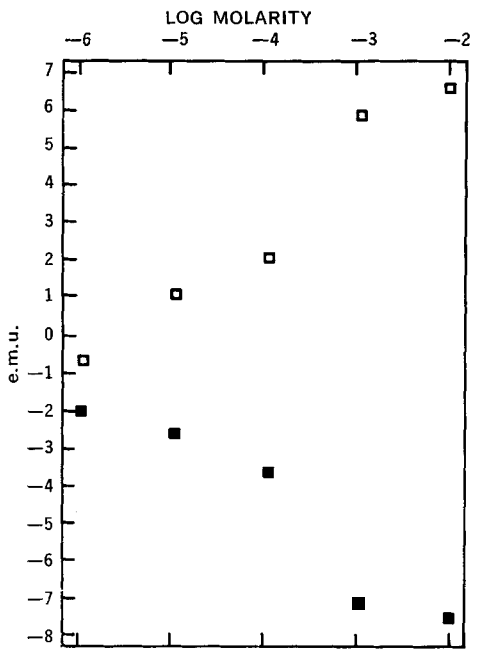


Figure 3—*Concentration–mobility profile of the dodecyl pyridinium chloride–cholesterol (□) and the sodium dodecyl sulfate–cholesterol (■) systems.*

[4] The units of electrophoretic mobility are micron/sec. per volt/cm. Since it is awkward to constantly restate these units, the use of the electrophoretic mobility unit, e.m.u., is proposed; 1 e.m.u. = 1 (micron-cm.)/(volt-sec).

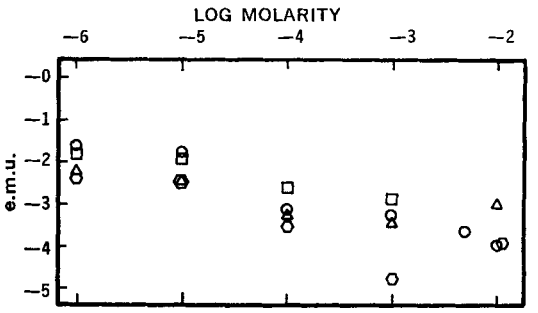


Figure 4—*Concentration–mobility profile of cholesterol–bile salt systems: cholate (○), taurocholate (□), glycocholate (△), glycodeoxycholate (○), at pH 6.8.*

The mobility is determined by measuring the time required for a particle to move over a given distance under a known potential gradient. Twenty particles were observed per experiment. Riddick (39) has stated that: "In tracking colloids to determine *average* EM or zeta potential, one should select *only* particles which appear to be migrating at very close to average velocity—scrupulously avoiding those which approach maxima or minima. However, some systems will show such extreme variation that selection of *average* velocity is difficult to impossible."

During the course of preliminary work, it was found that the mobility was dependent on location within the cell. As current is passed through the cell, oxidation and reduction take place at the appropriate electrodes. It is then possible for these products to migrate in the cell. The concentration of these materials will be highest in the region of the cell closest to the ends. It is for this reason that all electrophoretic mobility readings were made in the center of the cell. Furthermore, that portion of a sample under investigation that had been used for a mobility measurement was discarded after use. The cell was then thoroughly cleaned and wiped.

All mobility measurements were made at 25 ± 2° unless otherwise stated. Mobility readings were conducted within 72 hr. of sample preparation. Twenty-four hours was allowed for equilibration between cholesterol and the test additives. Electrophoresis systems containing organic liquids, *i.e.*, *n*-octanol, were examined in the glass electrophoresis cell. Earlier tests had shown the glass and plastic cells to yield similar data.

Adsorption Studies—The protocol for adsorption studies was as follows: buffer, sodium chloride, and adsorbate were added to 1.0 g. of cholesterol. The complete adsorption system was shaken on a Burrell shaker at 25 ± 2°. One-milliliter aliquots were withdrawn after the suspensions had been filtered through silver membrane filters[5] (1.2 μ). The 1.0-ml. aliquot was placed into glass scintillation vials containing a dioxane "cocktail" (naphthalene and PPO). The number of counts per minute (c.p.m.) was determined in a Beckman liquid scintillation system.[6]

The cholesterol used for all adsorption studies was prepared by the recrystallization of commercial cholesterol, twice from ethanol and once from ethanol–water. The suspensions used for the adsorption studies were prepared by the following method. The recrystallized cholesterol which had been dried under vacuum was ground in a porcelain mortar. One-gram samples were placed in flasks. The other components of the adsorption system were then added. Grinding was shown not to affect the adsorption properties of the cholesterol.

Throughout all experiments, electrophoretic and adsorption, unless specifically noted otherwise, the ionic strength was maintained at a constant level with 10^{-2} *M* NaCl.

RESULTS AND DISCUSSION

Baseline pH–Mobility Profiles—The distributions of particle velocities in a typical cholesterol dispersion are given in Fig. 1.

[5] Selas Flotronics, Spring House, Pa.
[6] Beckman Instruments, Inc., Fullerton, Calif.

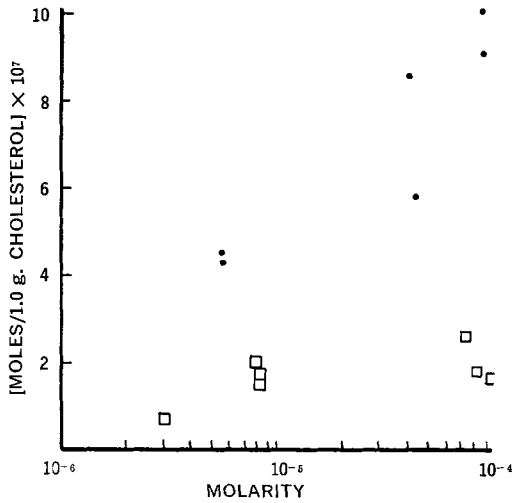


Figure 5—*The adsorption isotherm of sodium dodecyl sulfate* (●) *and sodium cholate* (□) *on cholesterol.*

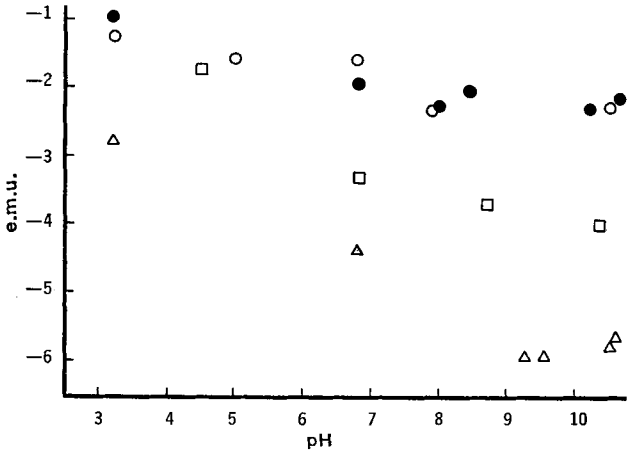


Figure 7—*The effect of octanol* (●), *10⁻³ M sodium cholate* (□), *and octanol–sodium cholate* (△) *on the pH–mobility profile of cholesterol* (○).

Note that the average velocities are quite similar, yet the distributions are broad, and in agreement with the observations of Seaman (26) who found the mobilities of alcohol-precipitated cholesterol particles in a given batch to exhibit considerable scatter. Electrophoretic data reported in the literature usually signify the average mobility of 20 particles in a single experiment. Due to the broad distributions encountered in the cholesterol system, it was decided to repeat each experiment several times and record the mean of the mean mobilities. (The standard deviation was generally ≤ 0.5.)

Figure 2 shows the influence of pH on the electrophoretic mobility. As can be seen, there appeared to be little or no influence of buffer concentration upon the mobility at constant pH. It is of interest to compare these results with those of Seaman (26), Moyer (24), and Douglas and Shaw (25). At high pH's, Douglas and Shaw found e.m.u. values as high as −5. These are contradictory to the authors' results and to those of Seaman. The large mobility observed by Douglas and Shaw may have been related to the presence of oxidation products of cholesterol. Aging and heating of cholesterol were found to increase the pH–mobility dependence of cholesterol (40).

Effects of Bile Salts and of Alkyl Surfactants on the Electrophoretic Mobility of Cholesterol Particles—Figure 3 shows the concentration–mobility profiles for cholesterol particles in the sodium dodecyl sulfate–cholesterol and the dodecyl pyridinium chloride[7]–cholesterol systems for which substantial mobility dependencies upon concentration were noted. Similar experiments with hexadecyl pyridinium chloride and dodecyltrimethylammonium bromide showed that the large concentration dependencies are typical of the alkyl surfactants.

Figure 4 gives the concentration mobility profiles of cholesterol particles in bile salt systems. It is of interest to note that although these salts have different side chains and varying numbers of hydroxyl groups on the four-ring nucleus, they all have similar concentration effects on electrophoretic mobility. This similarity might be related to the fact that although the hydrophilic portions are different, the hydrophobic "backs" of all these bile salts are nearly the same (42).

It was, however, surprising that the electrophoretic mobility produced by all the bile salts remained rather small even at the highest bile salt concentrations. These results initially appeared contradictory to the earlier findings based upon the studies of cholesterol crystal growth inhibition by cholates which suggested strong interaction of bile salt anions with the cholesterol surface. However, as will be seen, these data are consistent with the idea that cholates may exhibit limited adsorption on cholesterol surfaces while conventional long-chain alkyl surfactants are able to adsorb more generally.

Adsorption Studies—Figure 5 represents the data on the adsorption of sodium dodecyl sulfate and of sodium cholate from aqueous solutions onto cholesterol. Experiments at 1×10^{-3} *M* cholate were not meaningful because of the large uncertainties in the calculated amounts adsorbed at high concentration.

These results show that cholate reaches an adsorption plateau at around 10^{-5} *M*. This plateau is reached at the same concentration levels used previously in the Coulter counter studies of crystal growth inhibition. These results are also consistent with the micro-

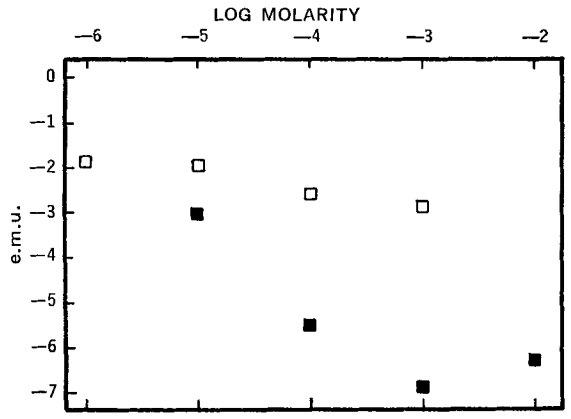


Figure 6—*Concentration–mobility profile of cholesterol–sodium taurocholate systems at pH 6.8 showing the effect of purity of commercial samples: Sample* A (□) *and Sample* B (■).

[7] Purified by extraction and recrystallization (41).

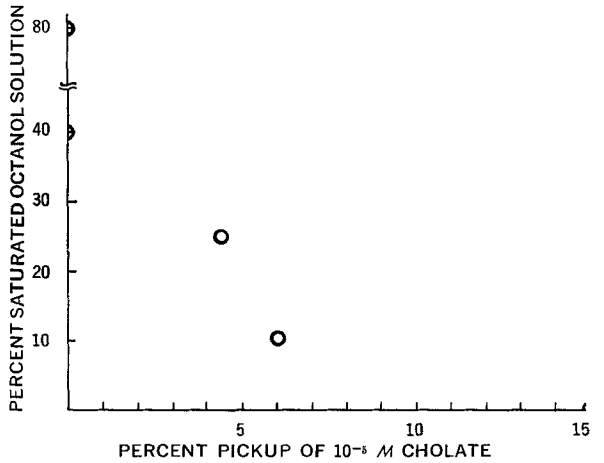


Figure 8—*The effect of octanol concentration on the adsorption of 10^{-5} M sodium cholate on cholesterol (80% saturated octanol solution signifies that 80 ml. of saturated solution has been added to an electrophoretic or adsorption system which contains a total of 100 ml.).*

Table I—Effect of Octanol on the Mobility of the Sodium Taurocholate–Cholesterol and Sodium Dodecyl Sulfate–Cholesterol Systems at pH 6.8

	− e.m.u.
Taurocholate (10^{-3} *M*)	1.92
Taurocholate (10^{-3} *M*) + octanol	4.44
Sodium dodecyl sulfate (10^{-3} *M*)	6.86
Sodium dodecyl sulfate (10^{-3} *M*) + octanol	7.22
Sodium dodecyl sulfate (10^{-4} *M*)	2.35
Sodium dodecyl sulfate (10^{-4} *M*) + octanol	2.65

electrophoretic data in which the bile salts showed very little change in effect on mobility over the range of concentrations studied.

For sodium dodecyl sulfate, adsorption increased sharply between 10^{-5} and 10^{-4} *M*, and reference to Fig. 3 indicates that adsorption should continue to increase up to about 10^{-3} *M*. Thus it appears that the adsorption plateau of sodium cholate occurs at a relatively low surface coverage and is consistent with the microelectrophoretic data present in Fig. 4.

Influence of Octanol and Other Additives on the Adsorption Behavior of Cholates—The low electrophoretic mobility of cholesterol particles in cholate solutions (Fig. 4) and the low extent of adsorption of cholates onto cholesterol (Fig. 5), in contrast to the behavior of the alkyl surfactants, suggested that cholates, probably because of their rigid nuclear structures, cannot be accommodated conveniently by all sites on cholesterol particle surfaces. This idea suggested that an investigation of the influence of additives upon the adsorption behavior of the cholates might be worthwhile, particularly in light of the observation that an unpurified sample of sodium taurocholate gave much higher limiting electrophoretic values than the purified sample (Fig. 6).

Thus the influence of a large number of compounds upon the electrophoretic mobility of cholesterol particles in cholate solutions was investigated (40). It was found that only octanol substantially altered the electrophoretic behavior at 10^{-3} *M* cholate. As can be seen in Fig. 7, octanol did not increase the electrophoretic mobility of the cholesterol particles. However, in combination with millimolar cholate, electrophoretic mobility values as large as those obtained with the alkyl surfactants were observed. It is noteworthy that the influence of octanol upon the electrophoretic mobility was negligible at 10^{-4} *M* cholate.

Experiments were also carried out to determine the influence of octanol on the adsorption of cholate. However, due to large uncertainties, meaningful data were obtained only for cholate concentrations up to 10^{-4} *M* cholate. Figure 8 shows that at 10^{-5} *M* cholate, octanol actually reduced the adsorption of cholate upon the surface of cholesterol. Similar results were obtained at 10^{-4} *M* cholate.

These findings suggest strongly that octanol is an inhibitor for cholate adsorption at low concentrations, but at high cholate concentrations it is able to assist in the adsorption of cholate. At low cholate concentrations, octanol may compete with cholate for those sites that would normally accommodate cholate; at high cholate concentrations ($> 10^{-3}$ *M*), octanol may act as a mediator.

The extent of the mediation which occurs at the higher cholate concentration might be expected to be greatest in a system where both the adsorbent and the adsorbate are rigid molecules as in the case here. Indeed it was found (Table I) that octanol had relatively little effect upon the electrophoretic mobility of cholesterol particles in sodium dodecyl sulfate solutions. It is worthwhile to recognize that many biological situations can involve such "three body" interplay, *e.g.*, cholesterol–protein interactions might be enhanced by the presence of flexible lipid molecules.

REFERENCES

(1) H. Y. Saad and W. I. Higuchi, *J. Pharm. Sci.*, **54,** 1205(1965).

(2) A. J. H. Rains, *Proc. Roy. Soc. Med.*, **62,** 129(1969).

(3) H. F. Newman, J. D. Northup, M. Rosenblum, and H. Abrams, *Amer. J. Gastroenterol.*, **50,** 476(1968).

(4) E. Hove and T. Geill, *Geriatrics*, **23,** 114(1968).

(5) R. E. Hermann and J. C. Martin, *ibid.*, **24,** 139(1969).

(6) H. M. Rothman, in "Gastroenterology," vol. 3, 2nd ed., H. L. Bockus, Ed., W. B. Saunders, Philadelphia, Pa., 1965.

(7) H. B. Weiser and G. R. Gray, *J. Phys. Chem.*, **36,** 286(1932).

(8) A. J. H. Rains, "Gallstones," Charles C Thomas, Springfield, Ill., 1964.

(9) C. Frey, C. Thorpe, and G. Abrams, *Amer. J. Surg.*, **115,** 75(1968).

(10) D. M. Small, *J. Amer. Oil Chem. Soc.*, **45,** 108(1967).

(11) M. Bourges, D. M. Small, and D. G. Dervichian, *Biochem. Biophys. Acta*, **144,** 189(1967).

(12) W. H. Admirand and D. M. Small, *J. Clin. Invest.*, **47,** 1043(1968).

(13) K. D. Dreher, J. H. Schulman, and A. F. Hofmann, *J. Colloid Interface Sci.*, **25,** 71(1967).

(14) H. Y. Saad, thesis, University of Michigan, 1965.

(15) H. Y. Saad and W. I. Higuchi, *J. Pharm. Sci.*, **54,** 1303 (1965).

(16) H. C. Li and P. L. De Bruyn, *Surface Sci.*, **5,** 203(1966).

(17) P. Somasundaran and D. W. Fuerstenau, *J. Phys. Chem.*, **70,** 90(1966).

(18) P. Somasundaran, T. W. Healy, and D. W. Fuerstenau, *J. Colloid Interface Sci.*, **22,** 599(1966).

(19) I. Remezov, *Biochem. Z.*, **218,** 86(1930).

(20) *Ibid.*, **218,** 134(1930).

(21) I. Remezov and N. Travaststyerna, *ibid.*, **218,** 147(1930).

(22) I. Remezov, *ibid.*, **218,** 157(1930).

(23) *Ibid.*, **218,** 173(1930).

(24) L. S. Moyer, *ibid.*, **273,** 122(1934).

(25) H. W. Douglas and D. J. Shaw, *Trans. Faraday Soc.*, **53,** 512 (1957).

(26) G. V. F. Seaman, thesis, Cambridge, 1958.

(27) L. L. Smith, W. S. Matthews, J. C. Price, R. C. Bachmann, and B. Reynolds, *J. Chromatogr.*, **27,** 187(1967).

(28) H. E. Stavely and W. Bergmann, *Amer. J. Cancer*, **30,** 749 (1937).

(29) W. Bergmann, H. E. Stavely, L. C. Strong, and G. M. Smith, *ibid.*, **38,** 81(1940).

(30) H. W. Douglas, *Trans. Faraday Soc.*, **39,** 305(1943).

(31) S. Hollingshead, G. A. Johnson, and B. A. Pethica, *ibid.*, **61,** 577(1965).

(32) J. N. Mebrishi and G. V. F. Seaman, *ibid.*, **64,** 3152(1968).

(33) L. F. Fieser, "Organic Synthesis," coll. vol. 4, Wiley, New York, N. Y., 1963.

(34) D. R. Johnson, R. Idler, V. W. Meloche, and C. A. Baumann, *J. Amer. Chem. Soc.*, **75,** 52(1953).

(35) T. D. Fontaine, J. S. Ard, and R. M. Ma, *ibid.*, **73,** 879 (1951).

(36) E. P. Guth and Z. Mansour, *J. Pharm. Sci.*, **56,** 376(1967).

(37) R. M. Rock and N. C. Burbank, Jr., *J. Amer. Water Works Ass.*, **58,** 676(1966).

(38) R. M. Nash and B. E. Haeger, *J. Pharm. Sci.*, **55,** 829(1966).

(39) T. M. Riddick, "Control of Colloidal Stability Through Zeta Potential," vol. I, Livingston, Wynnewood, Pa., 1968.

(40) D. Mufson, thesis, University of Michigan, 1968.

(41) T. J. Roseman, thesis, University of Michigan, 1968.

(42) A. F. Hofmann, thesis, University of Lund, Sweden, 1964.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 31, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication December 29, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Abstracted from a thesis submitted by Daniel Mufson to the Horace H. Rackham Graduate School, University of Michigan, Ann Arbor, in partial fulfillment of the Doctor of Philosophy degree requirements.

This investigation was supported in part by Training Grant 1T1-GM-1367-03 from the U. S. Public Health Service, and in part by Grant HE-07690 from the National Heart Institute, U. S. Public Health Service, Bethesda, Md.

* Present address: Division of Scientific and Medical Affairs, Parke, Davis and Co., Detroit, MI 48232

Dissolution of Poorly Water-Soluble Drugs I: Some Physical Parameters Related to Method of Micronization and Tablet Manufacture of a Quinazolinone Compound

SAUL S. KORNBLUM and JEFFREY O. HIRSCHORN

Abstract □ An investigation was conducted to analyze the dissolution behavior of a poorly water-soluble drug. Two specific methods of micronization were employed which provided drug forms of different physical characteristics, specific surface area, and particle-size range. The drug, a quinazolinone compound, was micronized by employing spray drying and air attrition. The micron-sized drugs, pelletized pure drugs, and tablets prepared from same by direct compaction were subjected to dissolution-rate studies at pH 1.2 and 3. *In vitro* dissolution data obtained have demonstrated a significant variation for the pure spray-dried and air-attritioned drugs at pH 1.2, although physical specifications had suggested the contrary of that reported. The pure drug forms, when pelletized, resulted in elimination of the difference observed for dissolution rates with the pure drug powders. Dilution of the pure drugs with tablet excipients and subsequent direct compaction into tablets resulted in an improvement of dissolution behavior compared to that obtained with the pure drug forms.

Keyphrases □ Dissolution behavior—poorly water-soluble drugs □ 1 - Isopropyl - 7 - methyl - 4 - phenylquinazolin - 2(1*H*) - one—dissolution □ Micronization effect—poorly water-soluble drug □ Spray-drying effect—poorly water-soluble drug □ Pellets, poorly water-soluble drug—dissolution

During the past decade, numerous publications have related biological availability data with the particle size of various drugs which are orally administered. The importance of the technological understanding and improvement of the dissolution rate for poorly water-soluble drugs has been well documented (1). *In vitro* dissolution-rate information has readily permitted distinction between different dosage forms containing the same drug with relationship to their gastrointestinal absorption characteristics. Solubility rate of a substance has been quantified directly to surface area for nearly a century (2). Reduction to a smaller workable particle-size range for poorly water-soluble drugs has been the general practice during the past decade. The research pharmacist, when formulating a solid or liquid dosage form of an insoluble drug, has relied upon a variety of processes, *i.e.*, grinding, air attrition, ball milling, and controlled precipitation, to obtain the ultimate in particle-size reduction. Air attrition has gained the most widespread acceptance, which has led to the availability of a variety of fluid energy mills (3).

A review article (1) was recently published which dealt in depth with particle size of drugs and its relationship to absorption and activity. Sulfadiazine, griseofulvin, and sulfaethidole (4–6) are a few of those compounds studied in an effort to establish the clinical effect of reduced particle size.

The twofold purpose of the present research was to study the dissolution behavior for a pure water-insoluble drug, which was micronized by spray-drying and air-attrition processes, with a compressed dosage form of the same, and to investigate the acceptability of spray drying as an alternative method for the preparation of micron-sized particles. Dissolution behavior has been compared for both physical forms with relationship to two different physical parameters: particle-size range and specific surface area.

EXPERIMENTAL

Spray-drying and air-attrition processes were selected for the manufacture of micron-sized particles of a poorly water-soluble drug. Spray drying appeared interesting to study in this instance, since it was thought that the process would impart certain desirable physical characteristics not typical of air-attritioned material such as unrestricted particle flow and ease of uniform dispersion of hydrophobic particles in an aqueous system as well as the possible presence of small holes in the spherical particles which would provide additional surface area.

Materials—1-Isopropyl-7-methyl-4-phenylquinazolin-2(1*H*)-one was synthesized in accordance with quality control standards for a pure drug.[1] The drug chosen for this study is indicative of a heterocyclic nitrogen base which does not readily form salts except at extremely low pH. The saturation concentration of the spray-dried and air-attritioned drugs in distilled water with a pH 6.0 and after 6-hr. exposure was found to be 2.7×10^{-4} moles/l. at 37° employing a rotational dissolution apparatus. Initially, the drug was twice passed through a laboratory hammer mill[2] which utilized a plate with 1-mm. perforations. The drug was then micronized by air attrition, employing a conventional fluid energy mill.[3] The micronized drug exhibited large particle aggregates which resulted from the high degree of surface energy developed by particle friction during processing. Additional drug from the same batch was used for preparing material by a spray-drying process which is described in the next section.

Spray-Drying Process—A 50-g. quantity of the quinazolinone compound was dissolved in 50 ml. chloroform, reagent grade, employing mechanical stirring. Special denatured alcohol (90% ethyl alcohol–10% methyl alcohol) was added to bring the solution to 125 ml.

A portable laboratory spray dryer[4] equipped with a centrifugal atomizing wheel, which functioned from air-turbine drive with a velocity of about 40,000 r.p.m., was employed in this study. The heated chamber of the spray dryer was maintained at 100–120° and the outlet temperature at 75–85°. The drug solution was introduced to the atomizing wheel by employing a Sigmamotor pump[5] which was adjusted to a flow rate of 15 ml./min. A cyclone collector separated the spray-dried drug from the solvent system. The solvent was not recovered; however, if scale-up was considered, a solvent-recovery method could be designed. The micron-sized drugs were both stored in tightly closed glass containers.

Physical Form and Particle-Size Range of the Pure Drug Forms—The particle-size range for the spray-dried and air-attritioned quinazolinone compounds was determined by a photomicroscopic method utilizing a grid graduated in microns. Observation of the photographs of the spray-dried drug depicted sphere-shaped amorphous particles which ranged in diameter from 1 to 15 μ. The irregular crystalline fragments of the air-attritioned drug measured from 1 to 5 μ.

[1] Sandoz Pharmaceuticals, Chemical Development Dept., Hanover, N. J.
[2] Raymond Mill, Combustion Engineering Co., Chicago, Ill.
[3] Reductionizer, Reduction Engineering Corp., Newark, N. J.
[4] Nerco-Niro, distributed by Nichols Engineering and Research Corp., New York, N. Y.
[5] Sigmamotor, Inc., Middleport, N. Y.

Specific Surface Area of the Pure Drug Forms—The spray-dried and air-attritioned quinazolinone compounds were subjected to the classical low-temperature adsorption Brunaner, Emmett, and Teller (B.E.T.) technique[6] for specific surface area measurement. Krypton gas was used as the adsorbate. The method permitted sample outgassing to less than 1 μ of pressure. One-gram samples of the spray-dried and air-attritioned materials were heated to 50° and outgassed for 8 hr. to evacuate gasses and vapors acquired from exposure to the atmosphere. The surface area analysis involved incremental gas volume adsorption measurements which were made by accurately determining the equilibrium pressure following the introduction of gas quantities into the system. The specific surface area was found to be 1.37 $m.^2/g.$ for the spray-dried drug and 2.78 $m.^2/g.$ for the air-attritioned drug.

Pellets of the Pure Drug Forms—A comparative study was designed to determine the effect on dissolution when the two pure drug forms were compressed into pellets. Fifty milligrams of the drug was compressed using a single station tablet press,[7] employing 6-mm. shallow-concavity punches. Each pellet was formed with a thickness that was maintained at 1.80 mm., which assured equivalent surface area for both materials tested. Although the hardness was measured as 3 kg. with an air-operated Strong-Cobb tester, the pellets did not fracture during the dissolution runs but remained intact with slow dissolution.

Tablet Manufacture Employing Spray-Dried and Air-Attritioned Quinazolinone Compounds by Direct Compaction—The composition for tablets manufactured by direct compaction (D.C.) utilizing spray-dried and air-attritioned drugs are given in Table I.

Direct Compaction Manufacturing Procedure—The ingredients listed in Table I were accurately weighed and passed through a No. 20-mesh screen. The materials were transferred to a glass mortar and gently blended so that the drug's particle structure would not be altered. Then 160 mg. of the blended powders was placed into the die cavity of the single station tablet press and compressed, using 8-mm. flat, beveled-edge punches. The tablet hardness was maintained at 6.5 ± 0.5 kg. which was monitored by using a compressed air-operated Strong-Cobb tester. The tablet thickness was measured using a micrometer; each tablet employed in this study had to adhere to a measurement of 2.75 ± 0.01 mm. This physical measurement assured compliance of each tablet to the hardness range established for this study. The direct compacted tablets were subjected to the USP disintegration test and were found to disintegrate within 25 ± 5 sec. These tablets were formulated to give fast disintegration in an attempt to observe uninhibited drug dissolution. Tablet disintegration occurred with the rapid formation of discrete particles of drug-diluent which resulted from rupture of the tablet matrix.

Dissolution Rate Analysis of the Pure Drugs, Pellets, and Tablets—The dissolution apparatus that was initially employed in this study was the beaker-stirrer method. The poor wetting property exhibited by the pure drug forms required selection of another method that would permit a more uniform contact of solvent and powders. The rotating-bottle method was found most suitable for attaining reasonably precise data for consecutive experiments, whereas with the beaker-stirrer method it was not possible. Preliminary experiments revealed that 20 r.p.m. was a suitable speed for demonstrating definite differences in dissolution behavior for the pure materials investigated. Rotational rates less than 20 r.p.m. did cause a substantial decrease in the dissolution rate from that reported in this paper. Each sample bottle was immersed in a water bath maintained at 37 ± 0.5°. The powder, pellet, or tablet was placed in a 75-ml. capacity, amber glass bottle containing 50 ml. of aqueous solvent which had been previously adjusted to a pH of 1.2 or 3 with hydrochloric acid. The solvent had been preheated to 37° prior to the addition of the sample. At the moment of sample addition, the time was recorded as $t = 0$. The vessel was tightly closed with a bakelite cap lined with a polyethylene sealer. Dissolution of the drug and tablets was studied over a 30-min. interval, and each sample was carried through a 60-min. period to establish whether total availability of the drug in solution was reached. Preliminary studies indicated that a 60-min. test period was sufficient, since two half-lives were encompassed which permitted valid comparison of the $t_{25\%}$, $t_{50\%}$, and $t_{75\%}$.

[6] Model 2100 surface-area analyzer, Micromeritics Instrument Corp., Norcross, Ga.

[7] Stokes E Machine, Pennsalt Chemicals Corp., Warminster, Pa.

Table I—Tablet Composition for D.C. Tablets Prepared from Spray-Dried and Air-Attritioned Quinazolinone Compounds

Materials	Quantity per 160-mg. Tablet
Isopropylmethylphenylquinazolinone, spray-dried or air-attritioned	50.0 mg.
Microcrystalline cellulose[a]	25.0 mg.
Colloidal silica[b]	0.5 mg.
Corn starch USP	12.0 mg.
Lactose, spray-dried USP	69.5 mg.
Stearic acid powder USP	3.0 mg.

[a] Avicel, FMC Corp., Marcus Hook, Pa. [b] Cab-o-Sil, Cabot Corp., Boston, Mass.

The dissolution studies for the pelletized substances were carried out for 6 hr. at pH 1.2 in the same manner employed for the tablets. A quantity of 50 mg. of the quinazolinone compound was chosen since it represented a concentration below the aqueous saturation point of 60.5 mg./50 ml. at pH 1.2. The closer the quantity of drug in solution is to the saturation equilibrium the slower the approach to total solubility. The present investigation included the following test samples: 50 mg. spray-dried and air-attritioned drugs. In addition, pellets and tablets were studied which contained 50 mg. of spray-dried and air-attritioned drugs. These materials were all subjected to the outlined dissolution rate analysis at pH 1.2 and, in most cases, pH 3.

Nonsink conditions were employed in these experiments to observe the dependence upon limited hydrogen-ion concentration as related to dissolution. An unusual situation was observed: the pH's of the systems at the termination of the dissolution runs did not vary more than ±0.1 unit from the starting value for both pH 1.2 and 3. This was explained by the particular pKa of the drug, which was determined by spectrophotometric and solubility–pH data to be 2.51.

The analytical details of the dissolution experiments involved the withdrawal of a 1-ml. sample at the specified time interval using a pipet with filter-tip. The aliquot was diluted with the specified acid solution, utilized in the dissolution study, to 100 ml. The adsorption of the diluted solution was measured in a Cary recording spectrophotometer over the UV spectra, and the maximum absorbance at 232 mμ was used to calculate the drug concentration per 50 ml. of test solution. A correction factor was calculated for each sequential sampling as a result of the volume change that occurred because the test solution withdrawn for the pellet and tablet runs was not replaced after each sampling.

Individual sample containers were employed for the pure drug forms for each time segment; otherwise, some of the drug would have adhered to the filter-tip of the pipet with each withdrawal, which would have introduced a recurring error.

RESULTS AND DISCUSSION

The quinazolinone compound utilized in this study may be classified as a heterocyclic nitrogen base which exhibited poor aqueous solubility that was shown to be a function of hydrogen-ion concentration and method of micronization. The drug undergoes solubilization by interaction with hydronium ion to form a salt.

The concentration of the compound (mol. wt. 278) at 50 mg./50 ml. was calculated to be 3.6×10^{-3} moles/l. The unprotonated form of the compound formed a saturated solution in distilled water with a concentration of 2.7×10^{-4} moles/l. The hydrochloric acid solution at pH 1.2 contained about 6.3×10^{-2} moles/l. hydrogen ion, which provided about an 18 times greater hydrogen ion than the concentration of the compound. Therefore, complete solution of the 50 mg. at this pH was obtained. At pH 3 the hydrogen-ion concentration was about 1×10^{-3} moles/l., which limited the solubility of the compound since there was a fourfold excess of drug over hydrogen ion. Henceforth, one would expect only about 13 mg. of the compound to go into solution in the protonated form at pH 3. This approximates the values recorded in Table II, which were obtained experimentally.

The dissolution data obtained for the spray-dried and air-attritioned drugs at pH 1.2 and 3 can be compared by observing Table II. The spray-dried and air-attritioned drugs possessed dissimilar particle-size distributions in the low micron range. These

Table II—Dissolution Data at pH 1.2 and 3 of Spray-Dried and Air-Attritioned Quinazolinone Compounds

Sample Intervals, min.	Amount Dissolved, mg./50 ml.[a] Spray-Dried pH 1.2	Spray-Dried pH 3	Air-Attritioned pH 1.2	Air-Attritioned pH 3
1	9.1		6.1	
3	19.2		10.5	
5	27.8	5.6	13.6	4.1
10	34.1	12.6	20.6	9.0
15	37.9		24.1	
20	39.3	12.2	29.5	11.5
30	41.5	12.4	34.1	12.4
60	45.3	12.6	43.3	12.9

[a] Represents the average of four experiments with standard deviation of not more than 1.4 for all data recorded.

data indicate that at pH 1.2 an increase in dissolution rate was observed for the drug when micronized by spray drying as compared with the air-attritioned form. The results were unexpected since the specific surface area of the air-attritioned drug was determined to be twofold that found for the spray-dried drug. The apparent surface area of a poorly water-soluble material in contact with the aqueous medium appears to be unrelated to its specific surface area. Based on this factor, correlation of specific surface area with dissolution behavior without experimental verification could be quite deceptive. It was thought possible that the smaller air-attritioned particles were not subjected to the same intensity of agitation as were the large spray-dried particles; however, by physical observation it was quite apparent this was not the case. In fact, the air-attritioned drug had a definite tendency to form large particle aggregates which diminished the surface area, thereby resulting in a dissolution rate that was less than expected. Furthermore, it was also possible that surface hydration and solubility characteristics varied for both physical forms as a result of their different physical characteristics.

Pellets prepared from the spray-dried and air-attritioned materials, possessing equal surface area, were evaluated with regard to their respective dissolution rates, and Fig. 1 indicates the results obtained. The intended incorporation of the drugs, at a later stage, into a tablet matrix required an interpretation of the interparticle bonding forces that existed for each of the physical drug forms. If the specific particle structure of the drug resulted in dissimilar interparticle adhesion, subsequent inclusion into a tablet could possibly influence the dissolution behavior. The interparticle adhesion that occurs in a solid system may result from either partial fusion or partial dissolution at the surface of the particles induced by energy added during the compression (7).

From Fig. 1 it appears evident that pellets from the two physical drug forms have no significant difference in their dissolution rates.

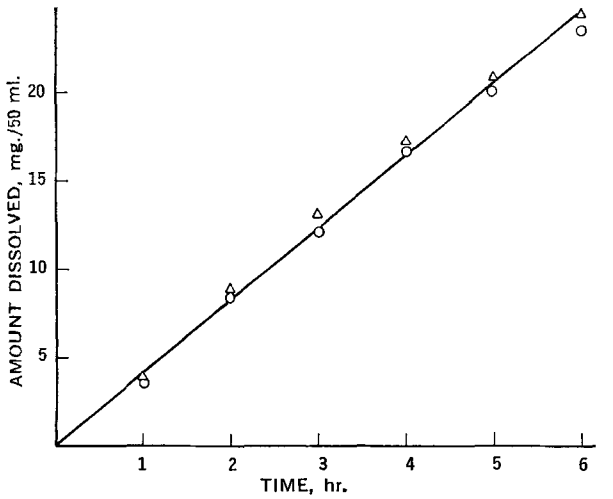


Figure 1—*Apparent zero-order dissolution rates of pelletized spray-dried and air-attritioned drugs were performed in 50 ml. aqueous solution at pH 1.2. Each plot is corrected to origin and the average of three experiments with standard deviation of not more than 0.7 for all data recorded. Key: △, spray-dried drug; and ○, air-attritioned drug.*

Table III—Dissolution Data at pH 1.2 and 3 of Spray-Dried and Air-Attritioned Quinazolinone Compounds Contained in Tablets Manufactured by Direct Compaction

Solution Time, min.	Amount Dissolved,[a] mg./50 ml. Direct Compression Spray-Dried pH 1.2	Spray-Dried pH 3	Air-Attritioned pH 1.2	Air-Attritioned pH 3
1	11.2	1.1	13.3	1.3
3	22.7	3.4	28.3	2.5
5	28.6	3.1	34.6	3.8
10	36.9	4.8	42.2	3.5
15	40.3		46.7	
20	42.9	4.4	50.4	4.8
60	46.4	13.0	50.4	12.9

[a] Represents the average of three experiments with standard deviation of not more than 1.1 for all data recorded.

An apparent zero-order equation for the dissolution data for both pellets was adhered to, which is in accord with the experimental conditions of constant dissolution surface area. By pelletizing the drug forms, the variation of surface wetting observed with the pure drug powders was eliminated. This phenomenon resulted in a need for clarification of the effect on dissolution when the pure drug form was diluted with common tablet excipients and then compressed into tablets.

The experiments designed to study the effect on dissolution with the drug forms in directly compressed tablets revealed certain definite departures from the results obtained with the pure drugs. The direct compaction method was utilized to prepare the tablets, since it permitted dilution of the drug without significant alteration of their physical forms. For this reason, the wet granulation method data were not included in this paper since it was shown to manifest additional complexities in following dissolution of the drug. A retarding effect on dissolution was experienced as a result of materials utilized in wet granulation, *i.e.*, granulating liquid and water-insoluble excipients, which tend to form a barrier layer on the surface of the drug particles during the granulating process.

The physical parameters, *i.e.*, tablet disintegration and thickness, were strictly controlled for the tablets prepared by direct compac-

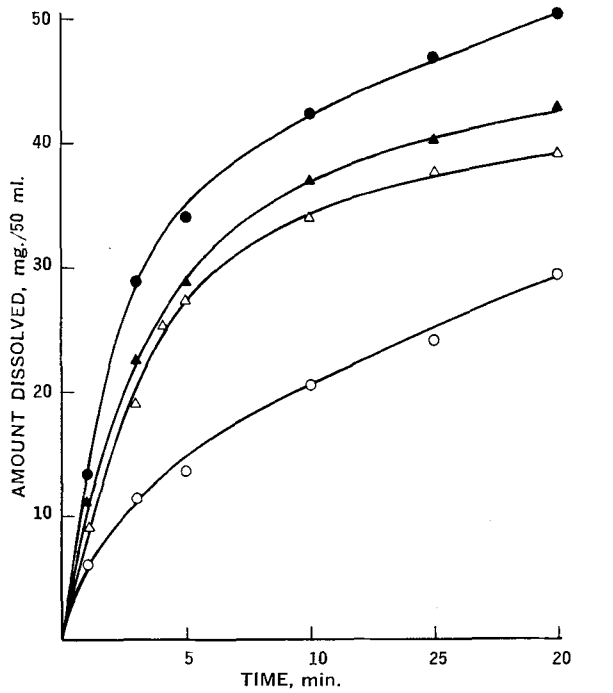


Figure 2—*Dissolution of spray-dried and air-attritioned powders and compressed tablets in 50 ml. aqueous solution at pH 1.2. Key: △, spray-dried powder; ○, air-attritioned powder; ▲, spray-dried tablet; and ●, air-attritioned tablet.*

Table IV—Amount of Spray-Dried and Air-Attritioned Drugs Dissolved (mg.) within a Specific Time Interval[a] in 50 ml. Aqueous Solution at pH 1.2, 37 ± 0.5°, and Rotated at 20 r.p.m.

	$t_{25\%}$, min.	$t_{50\%}$, min.	$t_{75\%}$, min.
Pure Drug			
Spray-dried	2.0	4.1	16
Air-attritioned	5.7	15.5	40
Compressed Tablets			
Spray-dried	1.6	3.5	10.5
Air-attritioned	1.3	2.6	6.5

[a] Each time interval was determined from the plotted data in Fig. 2.

tion. The tablets were formulated to give rapid disintegration, 25 ± 5 sec., in an attempt to observe uninhibited drug dissolution.

Dissolution data obtained for both sets of direct compacted tablets manufactured from the two drug forms have been tabulated in Table III for pH 1.2 and 3.

The dissolution rate obtained for the tablets prepared from air-attritioned and spray-dried drug proved to be quite similar in dissolution behavior (Fig. 2). These results indicate a substantial change from the comparative dissolution rates obtained with the pure air-attritioned drug under the same experimental conditions. The incorporation of the air-attritioned drug into a tablet matrix resulted in omission of the particle aggregates experienced with the pure drug. In addition, the improved dissolution rates for the tablets were probably a result of the hydrophilic property of the solid system and the increased surface area of the drug due to the force of compression.

Dissolution data obtained with the spray-dried drug and tablets of the same at pH 1.2 appear to have parallel dissolution patterns. The dissolution behavior experienced with both tablets compares more closely with the particle size and specific surface area data obtained for the pure drugs.

Table IV provides a tabulation of the time required for the dissolution of 25, 50, and 75% of the drug from both the powders and tablets, which is a convenient method for comparing dissolution data. The solid systems studied in this research were too complex for the application of reaction-order equations and, if utilized, would have possibly led to their misuse.

SUMMARY AND CONCLUSIONS

The results of dissolution studies at low pH provided information that rationalized the practical application of spray drying for the micronization of poorly water-soluble drugs. The air-attritioned drug had a tendency to form large particle aggregates which hindered its apparent dissolution rate in the pure form. In addition, the air-attritioned particles differed from the spray-dried inasmuch as they possessed a high degree of surface energy which gave rise to their sticking to each other and the sides of the storage container. Difficulty existed in handling and formulating the drug into the various forms.

The significantly greater dissolution rate that was observed for the pure spray-dried drug at pH 1.2 despite the smaller particle-size range of the air-attritioned drug would lead one to criticize the selection of a particular physical form of a poorly water-soluble drug by analyzing only physical dimension data. Particle-size range and specific surface area measurements for different physical forms of the drug studied were misleading and quite unrelated to their dissolution behavior. More pertinent is the apparent surface area of the drug available for solvent wetting for a poorly water-soluble material. The apparent surface area was not directly measured in these studies; however, dissolution is an indirect approach to the matter. The large particle aggregates, such as those observed with the air-attritioned drug, interfered with the apparent dissolution rate and gave data that would not normally be expected.

With this hydrophobic drug, it was found that the rotating-bottle method permitted acceptable reproducibility of the dissolution results. On the other hand, the beaker-stirrer method failed in this aspect as a result of discontinuous wetting of the powder by the acidic medium. With the appearance of a variety of dissolution systems in the literature (8), the authors would like to emphasize that the ability to repeat an experiment and obtain valid reproducibility should be the prime objective. A base line for dissolution behavior should always be established with the pure drug powder by employing an *in vitro* dissolution technique, and this should be done prior to fabrication of the dosage form. In this manner, the necessary properties of the tablet or capsule matrix can be more accurately determined.

The quinazolinone compound studied was insoluble in distilled water; however, by reducing the pH, limited solution did occur. The heterocyclic nitrogen in the quinazolinone structure was protonated in solution and the lower the pH of the aqueous system, the greater was its total solubility under nonsink conditions.

The dissolution data obtained for the two pure drug forms could have resulted in selection of the spray-dried drug for use in a solid dosage form; however, with additional experimentation employing a compressed tablet formulation, greater insight was obtained. Incorporation of the drug powders into a compressed tablet was helpful in clarifying possible false conclusions that could have been drawn from the dissolution data for the pure drugs. The dilution factor which resulted when the air-attritioned drug was combined with tablet excipients decreased the interparticle aggregation effect experienced with the pure form. When the tablet was placed in the aqueous medium, it released discrete particles, composed of drug and diluent, upon disintegration. This phenomenon greatly improved the dissolution behavior for the air-attritioned drug in the tablet form, since the discrete particles eliminated the particle aggregation observed with the pure drug powder. The dissolution rates for the pure spray-dried drug and tablets (Fig. 2) were not greatly different, since particle aggregation was not operative during the powder's dissolution experiments. The tablet diluents employed for the compressed tablets were selected where possible for their water-soluble or hydrophilic properties. This particular consideration can assist the diffusion-dissolution-diffusion processes required for dissolution of a drug from the discrete particles resulting from tablet disintegration. Water-insoluble components in the tablet matrix would tend to retard the overall dissolution process of a poorly water-soluble drug. This aspect has been utilized in the design of sustained-release dosage forms by employing a water-insoluble material to reduce dissolution rate. When a tablet disintegrates, the discrete particles formed should be as hydrophilic in property as possible in order to assist the dissolution process.

The variation of dissolution behavior shown in this paper for the pure drug, and tablets of same, underline the fallacy of performing dissolution rate analysis with various physical forms of the pure drug without inclusion of a proposed solid dosage form in such studies. The early initiation of preformulation kinetic studies for new drugs quite often incorrectly forces one to select the supposed best physical form of a drug prior to the consideration of dosage form research. The physical form and its dissolution behavior must be considered by the research pharmacist in organizing the development protocol for new drugs which fall into the category of poorly water soluble.

REFERENCES

(1) J. H. Fincher, *J. Pharm. Sci.*, **57**, 1825(1968).

(2) A. A. Noyes and W. R. Whitney, *J. Amer. Chem. Soc.*, **19**, 930(1897).

(3) J. L. Kanig, in "Remington's Pharmaceutical Sciences," 13th ed., Mack Publishing Co., Easton, Pa., 1965, chap. 13.

(4) J. G. Reinhold, *Amer. J. Med. Sci.*, **210**, 141(1945).

(5) R. M. Atkinson, *Nature*, **193**, 588(1962).

(6) K. Kakemi, T. Arita, and S. Ohashi, *Yakugaku Zasshi*, **82**, 261(1962).

(7) A. S. Rankell and T. Higuchi, *J. Pharm. Sci.*, **57**, 574(1968).

(8) M. Pernarowski, W. Woo, and R. O. Searl, *ibid.*, **57**, 1419 (1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 11, 1969, from the *Pharmacy Research and Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication December 16, 1969.

The technical assistance of C. Kenworthy, B. Dunn, and M. Tikal is gratefully acknowledged.

Controlled Drug Release through Polymeric Films

A. L. FITES, G. S. BANKER, and V. F. SMOLEN

Abstract □ The drug-permeability properties of several water-insoluble films were studied with respect to their potential application for the control of drug release from solid pharmaceutical dosage forms. Films composed of poly(methylvinylether)-maleic anhydride copolymer, crosslinked with polysorbate 20 (Tween 20), appeared as the most promising of the systems studied for the film-controlled drug-release applications. The permeability of the films can be controlled by appropriate adjustment of their polysorbate 20 content, molecular weight of the polymer, and humidity pretreatment. The permeability of the films was, however, found to be affected also by the pH of the diffusion medium.

Keyphrases □ Drug release, controlled—through polymeric films □ Polysorbate 20 effect—polymeric film formation □ Polymeric films—drug permeability □ Permeability coefficients—polymeric films □ Diagram—permeation cell □ UV spectrophotometry—analysis

The importance and pharmaceutical applicability of polymer films are well established in providing protective coatings and controlling drug release from oral dosage forms. In general, two basic factors may be involved in the film-controlled release of medicaments; *i.e.*, the dissolution of the film in the gastrointestinal fluids and its permeability (1, 2). With insoluble films, which maintain their integrity in traversing the gastrointestinal tract, only the latter need be considered. Therefore, the application of an insoluble film coat of desired permeability characteristics to an ordinary compressed tablet could conceivably be utilized to control drug release. This method would be advantageous in its economy and simplicity, provided predictable and controllable release rates are attainable.

In the present study, several synthetic polymeric films were investigated with respect to their drug-permeability properties for the purpose of determining their feasibility for controlled drug-release applications. One film which appeared promising was further investigated with respect to the factors influencing its permeability.

MATERIALS AND METHODS

Polymers, Drugs, and Diffusion Media—Table I describes the polymer systems used in this study. These systems were selected primarily on the basis of the insolubility of the resulting films in gastric and intestinal fluids. The cellulose acid phthalate (CAP) film system contains the maximum amount of plasticizer, PEG 400, consistent with insolubility of the film and absence of tackiness. The proportion of ethylcellulose (EC) to the methylcellulose (MC) was chosen, on the basis of preliminary studies, to produce a maximum permeability and insolubility. The observation in this laboratory that the addition of polysorbate 20 (Tween 20) renders the otherwise soluble poly(methylvinylether)-maleic anhydride (PVM-MA) films insoluble was the basis for its inclusion in this study.

Caffeine was chosen for these studies due to its relatively nonionogenic nature, stability, and ease of assay. Nicotinic acid was also used as a model drug in some of the preliminary experiments.

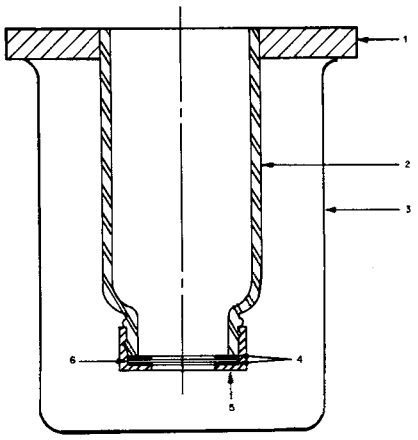


Figure 1—*Permeation apparatus employed in the study of drug permeation through free films. Key: 1, adjustable cell clamp; 2, 120-ml. amber bottle; 3, 600-ml. beaker; 4, polyethylene gaskets; 5, screw cap; and 6, film sample.*

The media in which the drugs were allowed to diffuse consisted of distilled water and buffer solutions of the following composition:

pH	Composition (mmole/l.)	
1.30	HCl, 1.4	NaCl, 34.2
5.83	KH_2PO_4, 25	NaOH, 1.8
7.20	KH_2PO_4, 25	NaOH, 17.4

The pH values of the solutions approximate that of gastric and intestinal fluids.

Permeation Cells—Figures 1 and 2 illustrate the permeation cells and the experimental arrangement. The cells consisted of approximately 120-ml. glass screw-capped chambers. The polymer films were placed between two polyethylene gaskets and were held in place by the plastic screw caps which were drilled to contain a 1.91-cm. (0.75-in.) diameter opening. These cells were inverted into their respective 600-ml. beakers, which contained the receiving medium for the diffusing drugs, where they were held by lucite clamps which allowed adjustment of their depth in the beakers. The medium in each beaker was maintained at 37° and was agitated with a magnetic stirrer employing the setup described in Fig. 2. The magnetic stirrers were connected to a central control; each was synchronized to 325 r.p.m. with a strobe timing light and was rechecked periodically. Fluctuation was minimized during operation by use of a voltage stabilizer and by allowing a warm-up period before proceeding with an experiment.

Preparation and Treatment of the Film—The films of composition listed in Table I were prepared by a casting technique employing a glass substrate. A fixed area on the smooth glass surface was marked off with masking tape. In each case the polymer was evenly spread into the area with a brass film-casting knife as described by Munden (3). The resulting film possessed a uniform thickness, which was controlled by adjustment of the blade level in the film-casting knife and by the number of tape layers. The average thickness of completed films was determined with a micrometer.[1]

Prior to testing, all films were stored over anhydrous calcium sulfate[2] under vacuum at 25° for a minimum of 48 hr. In addition, the PVM-MA films were stored for precisely 48 hr. at a humidity of 52.4%, maintained by a saturated solution of sodium bromide at 41°.

[1] Lufkin Rule Corp., Saginaw, Mich.
[2] Drierite, W. A. Hammond Drierite Co., Xenia, Ohio.

Table I—Film Systems Selected for Permeation Studies

Polymer	Polymer Concn., % w/v of Solution	Plasticizer Concn., % w/v of Solution	Composition of Solvent Systems, ml.
Cellulose acetate hydrogen phthalate (CAP)[a]	7	Polyethylene glycol 400 (PEG 400),[b] 0 & 3	Methylene chloride—50 Acetone *q.s.*—100
Ethyl cellulose (EC)[c]	6	Propylene glycol, 2.8	Methyl chloride—50 Methanol *q.s.*—100
Methyl hydroxypropyl cellulose (MC)[d]	3.5		
Poly(methylvinylether) maleic anhydride 139 & 169 (PVM–MA)[e]	8	Glyceryl triacetate,[f] 5	
		Polyoxyethylene sorbitan monolaurate,[g] 2, 4, and 8	Acetone *q.s.*—100

[a] Cellulose acetate phthalate; Eastman Organic Chemicals, Rochester, N. Y. [b] Carbowax 400; Union Carbide Chemicals, New York, N. Y. [c] Ethocel; Dow Chemical Company, Midland, Mich. [d] Methocel; Dow Chemical Company, Midland, Mich. [e] Gantrez A N; G.A.F. Corp., New York, N. Y. [f] Triacetin; Eastman Organic Chemicals, Rochester, N. Y. [g] Tween 20; Atlas Chemical Company, Chicago, Ill.

Procedures—The pretreated polymer film was secured in the cell which was immersed into the beaker containing 400–500 ml. of the receiving medium, either distilled water or buffer solution, previously equilibrated to 37°. The upper chamber of the cell was filled with drug solution, usually 25 ml., and covered; the level of the fluids was adjusted to the same height to prevent a hydrostatic pressure difference between the two compartments. Four units of this type were set up in the same thermostated water bath and agitated as described previously.

At selected time intervals over an 8- to 10-hr. period, samples of the receiving media were removed and assayed spectrophotometrically for transferred drug. The samples were read on a Beckman DU spectrophotometer at 273 and 262 mμ for caffeine and nicotinic acid, respectively. The blank consisted of fluid taken from a cell containing only the diffusion medium. The volume of sample removed from the receiving chamber was, in each case, replaced by an equal volume of diffusion medium. Six to eight replications were performed on each run. When linear relationships were expected, the data were treated by the method of least squares and the lines of best fit were reported.

RESULTS AND DISCUSSION

Preliminary Screening of Film Systems—The selection of films for further study of their permeability properties was primarily based upon a preliminary study of selected film formulations. The results are presented in Table II and Fig. 3.

The permeability of the cellulose acetate phthalate films studied was severely limited, as is apparent from the relatively small quantities of the drug that traversed the films over the 8-hr. period. Although plasticization of the CAP films by the inclusion of polyethylene glycol 400 (43% w/w polymer) produced approximately a tenfold difference in drug permeability relative to the unplasticized film, total drug permeability in the plasticized film was still less than 3%. The increase in permeability was likely a consequence of the unbound water-soluble plasticizer being leached from the membrane. The practical upper limit of polyethylene glycol content in the films was 43%. Higher concentrations imparted tackiness to the film.

Ethycellulose produced films which were quite brittle and showed negligible drug permeability. The addition of methyl(hydroxypropyl)cellulose to the ethylcellulose film enhanced film clarity, decreased brittleness, and increased drug permeability.

The addition of methyl(hydroxypropyl)cellulose improved the overall properties of the ethylcellulose films, particularly with regard to brittleness. However, the films were observed to lose their integrity at methyl(hydroxypropyl)cellulose contents greater than that contained in the tested film (58% w/w polymer). This was quite apparent from the rapid passage of the drug across the disintegrating films.

Prior to the 2-hr. period, a relatively small quantity of drug was found to permeate the untreated PVM–MA film (Table II and Fig. 3). The rate of drug passage became greatly accelerated between

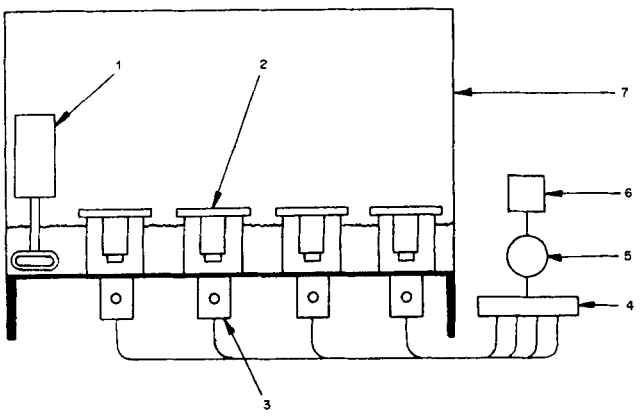


Figure 2—*Diagram of experimental arrangement for permeability studies. Key: 1, constant-temperature bath (stirrer constant-temperature regulator); 2, cell apparatus (Fig. 1); 3, magnetic stirrers; 4, control box; 5, variac; 6, line voltage regulator; and 7, water bath.*

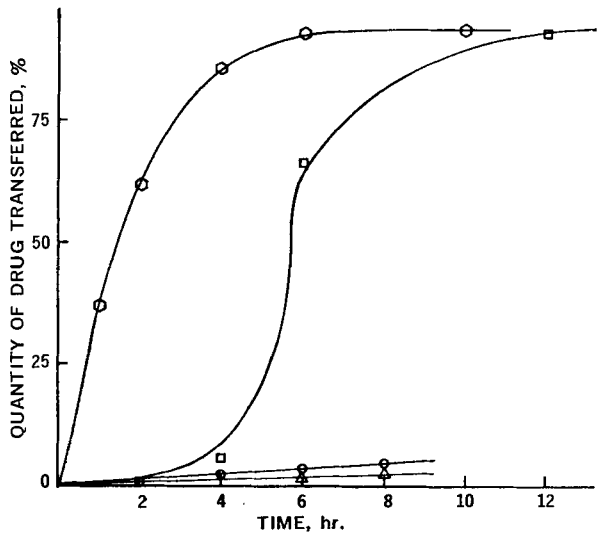


Figure 3—*Drug permeability properties of polymeric film membranes included in preliminary studies. The films studied included PVM–MA (4:1) pretreated, —○—; PVM–MA (4:1) untreated, —□—; EC–MC, —○—; and CAP + propylene glycol, —▽—.*

Table II—Results of Preliminary Investigations of Permeability Properties of Polymeric Films in Distilled Water

Film System[a]	Thickness, mm.	Time, hr.	Cumulative Percent of Drug Transferred across Membrane at Given Time
CAP[b]	0.030	8	0.2
CAP + PEG 400[b]	0.040	2	0.8
		4	1.6
		6	2.2
		8	2.7
EC–MC[c]	0.040	2	0.8
		4	1.8
		6	3.0
		8	4.1
PVM–MA 169[d]	0.045	2	0.9
(4:1)[e]	0.045	2	0.9
		4	5.3
		6	64.9
		12	92.5

[a] Film systems as designated in Table I. [b] Initial concentration of drug 0.25% nicotinic acid. [c] Initial concentration of drug 2% caffeine. [d] Initial concentration of drug 1.5% caffeine. [e] Refers to four parts polymer and one part polysorbate 20.

2 and 4 hr. The increase in permeability is attributed to the observed swelling of the film resulting from the hydration of acid anhydride groups on the polymer. In addition to a Donnan-type swelling effect (4), a polymer chain expansion likely arises from the mutual electrostatic repulsion of the resulting ionized carboxyl groups (5). The effect serves to diminish further the consolidation of the membrane and thereby increases its permeability. The apparent acidic pK's of the carboxyl groups have been reported (5, 6) as having values of 4.85 and 8.95. The swelling of the films may therefore be expected to become increasingly pronounced with an increase in pH of the diffusion media.

It was found that the initial delay in the enhanced permeability of PVM–MA films could be essentially abolished through pretreatment of the films by storage in a humidity chamber, for 48 hr., at 25° and 52.4% relative humidity, to allow film hydration prior to testing. Figure 3 presents a comparison of results obtained with caffeine using treated and untreated films.

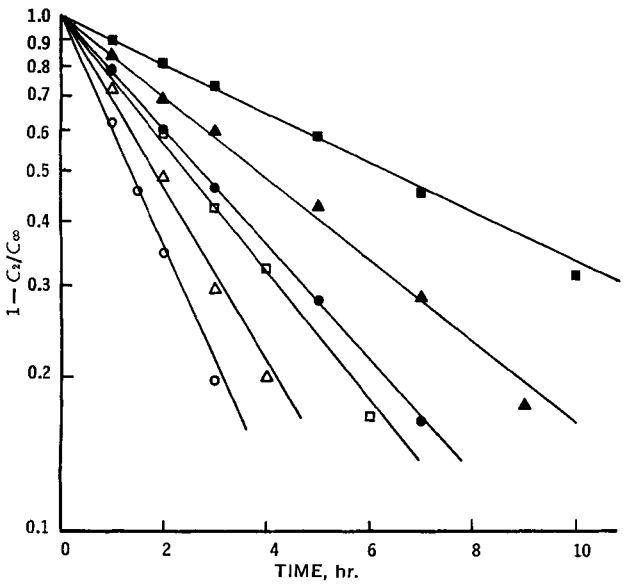


Figure 4—*Permeation of caffeine through PVM–MA 169 polymer films in distilled water and pH 1.30 buffer solution as influenced by the polysorbate 20 content and thickness of the films. At pH 1.30 the films studied included polymer: polysorbate 20 weight of 9:1 (—●—), 2:1 (—▲—), and 1:1 (—■—), with corresponding thicknesses of 0.069, 0.071, and 0.066 mm., respectively. In distilled water the films included were 4:1 (—○—), 2:1 (—△—), and 1:1 (—□—) with corresponding thicknesses of 0.068, 0.068, and 0.066 mm., respectively.*

Table III—Permeation (P) and Correlation (r) Coefficients for PVM–MA Films of Varying Weight Ratio of Polymer to Polysorbate 20 (R)

	PVM–MA 169 Films					
	1.30		5.83		7.20	
R/pH	P[a]	r	P	r	P	r
4:1	0.0024	−1.00	0.0066	−0.99	—	—
2:1	0.0017	−1.00	0.0085	−1.00	0.022	−0.83[b]
1:1	0.0012	−1.00	0.0059	−0.98	—	—

	PVM–MA Films in Distilled Water		PVM–MA Films in pH 1.30 Buffer	
R	P	r	P	r
4:1	0.0060	−1.00	—	—
2:1	0.0037	−0.99	0.0018	−1.00
1:1	0.0030	−1.00	0.0089	−1.00

[a] P is defined by Eq. 3 and has units of hr.$^{-1}$. [b] This relatively low value of the correlation coefficient is attributable to the considerable swelling of the films observed at this pH, with accompanying changes in thickness and area, which vitiates the vigorous applicability of Eq. 1.

Among the films included in the preliminary study, the PVM–MA films appeared most promising with respect to their potential applicability for controlled drug release. Films formed from the PVM–MA and polysorbate 20 were therefore selected for further study with respect to the influence of various factors anticipated to affect their permeability properties. Only caffeine at an initial concentration of 2% w/v and humidity-chamber pretreated films were employed in this further work.

Effect of Polysorbate 20 Content on the Permeability of PVM–MA Films—The PVM–MA polymer itself is water dispersible to a considerable extent. The presence of polysorbate 20 in films formed from the polymer renders them insoluble in water. However, they are still subject to appreciable swelling. Powell (7) has shown that the insolubilization of PVM–MA films by polysorbate 20 results from crosslinking of polymer chains by the polysorbate 20. The crosslinking occurs through esterification of acid anhydride groups on the polymer and hydroxyl groups of the sorbitan ester. Figure 4 illustrates the influence of polysorbate 20 content on the permeability of the PVM–MA films. The results correspond to films prepared from weight ratios of polymer to polysorbate 20 of 9:1, 2:1, and 1:1, having thicknesses from 0.066 to 0.071 mm. Films studied in distilled water (pH 6.70) were found more permeable to caffeine as compared to results obtained with a solution buffered at pH 1.30 with hydrochloric acid. As expected, less swelling of the films was noted for the films immersed in the diffusion medium of lower pH. Table III summarizes these results. Assuming that the permeability constants of the films are directly related to the reciprocal of the film thicknesses for the small range of differences involved allows their normalization to a constant standard thickness. The values for the permeability constants listed in Table III have been corrected to a constant film thickness of 0.071 mm. to allow the relative magnitude of the effects studied to be more readily discerned.

The permeability coefficients, P, listed in Table III are defined by the following equations where V_1, V_2, and C_1, C_2 refer to the volumes and drug concentration within the compartments of the permeation cell at any time, t. The subscript 2 refers to the receiving cell that was devoid of drug at zero time. The final concentration ultimately approached in the receiving compartment, designated with the subscript 2, is symbolized by C_∞ and is defined by Eq. 2 where C_0 refers to the initial concentration of drug in Compartment 1.

$$\log\left(1-\frac{C_2}{C_\infty}\right) = -\frac{V_2+V_1}{V_1}\,2.303\,Pt \qquad \text{(Eq. 1)}$$

$$C_\infty = \frac{C_0V_1}{V_2+V_1} \qquad \text{(Eq. 2)}$$

$$P = -\frac{0.071V_1\,(\text{slope})}{h\,(V_2+V_1)\,2.303} \qquad \text{(Eq. 3)}$$

Values of P obtained from Eq. 3 are normalized to a thickness of 0.071 mm.; the symbol h in the equation denotes the actual mea-

sured thickness of a film. The slope referred to in Eq. 3 is a least-squares regression value obtained from plots of C_2 as a function of time in accordance with Eq. 1. Such plots were always observed to be reasonably linear as indicated by the values of the corresponding correlation coefficients listed in Table II. This indicates that sufficient agitation was maintained in the diffusion cell to allow its description in terms of two compartments.

Greater extents of crosslinking in the films are likely responsible for the observed reduction of swelling and permeability of the films observed to accompany an increased polysorbate 20 content.

Effect of PVM–MA Polymer Molecular Weight—Films were prepared with a ratio of polymer to polysorbate 20 of 2:1 and 1:1 using higher (169) and lower (139) molecular weight grades of the PVM–MA polymer. Using pH 1.30 diffusion medium, the film composed of the lower molecular grade polymer was more permeable to the drug. The permeability coefficients, corrected to constant film thickness, are listed in Table III. The greater permeability of the lower weight grade polymer film is not surprising, considering their greater solubility and potential for being leached from the film. The effectiveness of polysorbate 20 crosslinking in maintaining the integrity of the film may be expected to be diminished as a consequence of the relative reduction in the size of the interacted components.

Effect of pH on PVM–MA Film Properties—As could be predicted from the theoretical considerations previously mentioned, the swelling of PVM–MA 169 (2:1 and 4:1) films was considerably retarded in the pH 1.30 buffered diffusion medium as compared to its immersion in buffers of pH 5.83 and 7.20. The pH of the latter medium exceeds the first acidic pK of the polymeric fixed carboxyl groups and allows the establishment of a fixed anionic charge density within the film. The fixed charge is undoubtedly responsible for the observed swelling and the marked increase in the permeability of the films at higher pH values. The permeability constants are listed in Table III. Although it is not readily apparent from these values, the effect of pH might be expected to be retarded by a further increase in polysorbate 20 crosslinking within the films.

SUMMARY AND CONCLUSIONS

The application of insoluble films to the coating of tablets or for the manufacture or coating of capsules must be accompanied by an ability to predict and to control the permeability properties of the films. The present study is a first exploratory step toward this goal. The study revealed that the PVM–MA films possess promise with regard to their application to the control of drug release. The permeability properties of the PVM–MA films can be controlled through adjustment of the polysorbate 20 content, molecular weight of the polymer, and the initial extent of hydration of the film. However, the influence of pH on the permeability of the films precludes a film-controlled uniform drug-release rate in all regions of the gastrointestinal tract. In cases where it may be desirable to delay drug release until the dosage form reaches the intestinal tract, the increased permeability of the film observed at higher pH could prove to be advantageous. It is conceivable, however, that when a uniform drug-release rate is desired, the expected changes in permeability of the film in response to variation of pH in the gastrointestinal tract may in part be circumvented by the inclusion of buffering agents in the drug formulation contained within the film envelope. The continual presence of a constant pH in at least one side of the film could function to reduce the change in film swelling and allow the permeability of the film to approximate more closely the relatively uniform rates obtained in either acidic or alkaline media. In this manner, the effects of the variations in the external environment could be ameliorated. The PVM–MA films do possess sufficient potential for eventual practical application and thereby warrant further studies into the influence of environment (particularly *in vivo*), film formulation, and the effect of the drug and other components of the dosage form on the controlled drug release imposed by the insoluble PVM–MA films. However, in any practical application, the elastic properties of the films and their ability to resist rupture due to mechanical abuse and osmotic pressure gradients are considerations of fundamental importance to the safety of any proposed product. These factors must be considered in future work.

REFERENCES

(1) E. Stempel, *Drug Cosmet. Ind.*, **98,** 44(1966).

(2) *Ibid.*, **98,** 36(1966).

(3) B. J. Munden, H. G. DeKay, and G. S. Banker, *J. Pharm. Sci.*, **53,** 395(1964).

(4) F. Helferich, "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, pp. 100–123.

(5) H. Eisenberg and R. M. Fuoss, in "Modern Aspects of Electrochemistry," J. M. Bockris, Ed., Plenum Press, New York, N. Y., 1968, p. 1.

(6) L. C. Lappas and W. McKeehan, *J. Pharm. Sci.*, **54,** 176 (1965).

(7) D. R. Powell, "Polyalkylvinyl Ether Copolymers as Protective and Controlled Release Pharmaceutical Film Coatings," Ph.D. thesis, Purdue University, 1967.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 20, 1969, from the *Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication December 9, 1969.

Abstracted in part from a thesis presented to the Graduate School, Purdue University, by Alan L. Fites in partial fulfillment of the Master of Science degree requirements.

Bead Polymerization Technique for Sustained-Release Dosage Form

S. C. KHANNA*, T. JECKLIN†, and P. SPEISER

Abstract □ The bead polymerization technique has been studied for the preparation of a sustained-release dosage form. This technique has been employed in aqueous outer phase for many water-insoluble monomers with and without drug. Different concentrations of water-soluble acidic monomers (α- and β-methacrylic acids) have been incorporated with these water-insoluble monomers in an attempt to improve the solubility and swelling property of the beads and hence the release of drug embedded in them in artificial gastrointestinal buffer juices. To prove the applicability of this method for the preparation of a pharmaceutical dosage form, the beads of various monomers and their mixtures in the presence of different drugs have been prepared. The influence of drug on the property of the beads has also been studied.

Keyphrases □ Sustained-release dosage form—bead polymerization □ Polymerization process—sustained-release dosage form □ Diagram—bead polymerization apparatus □ Particle-size determination—bead polymers □ Drug effect—bead polymer formation

The sustained-release dosage form constitutes an important part of modern pharmaceutical technology. Such an oral dosage form is often desirable to impart sustained-release or long-acting therapeutic effects and is commercially useful. A variety of methods and techniques has been employed for preparing such a dosage form, and approximately 1000 pharmaceutical patents have been issued in the last 15 years. The natural and synthetic polymers, waxes, and many other materials, either alone or combined, have been frequently employed for coating powder particles or granules, using conventional (1), spray drying (2), and other methods of coating in an attempt to prepare sustained-release dosage forms. Beads of different waxes (3) and epoxy resins (4) have also been reported for obtaining such products. The methods so far have mostly employed polymers, but polymerization techniques in which the drug can be embedded during polymerization of a single monomer or the combination of a few monomers have received little or no attention.

The monomers and catalyst used for polymerization are relatively toxic substances. However, the pure polymers obtained from these monomers are nontoxic and harmless to human beings. Most monomers employed in this study are those whose polymers in one form or another have already been in use for pharmaceutical purposes, *e.g.*, acrylates (5) and vinyl acetate (6).

In the present study, the possibility of using one of the methods of polymerization, known as bead polymerization or pearl polymerization, for the preparation of a sustained-release dosage form has been explored (7). Various monomers alone or in combination with others have been prepared in bead form with and without drugs. The effect of drugs on the properties of the beads obtained has also been investigated.

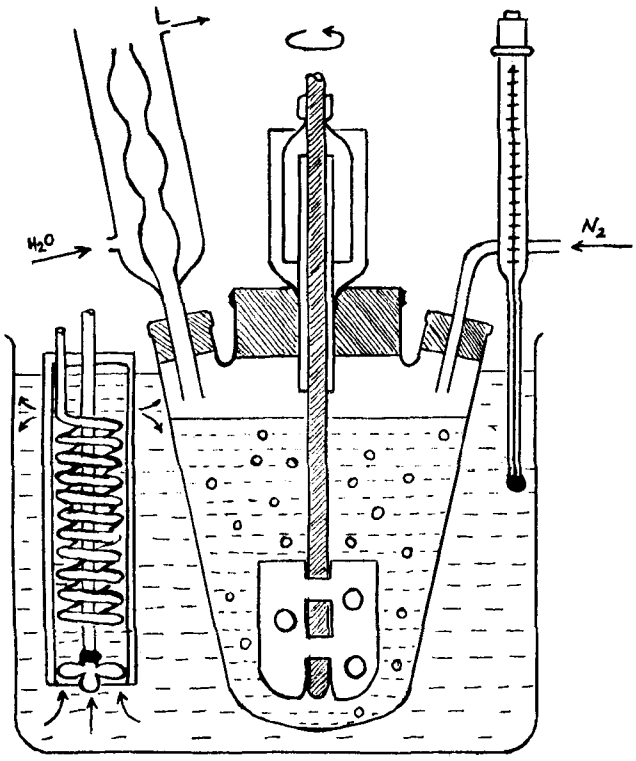


Figure 1—*Apparatus for bead polymerization.*

METHODS OF POLYMERIZATION

Polymerization can be carried out by the following four methods: (*a*) bulk or block polymerization; (*b*) bead or pearl polymerization; (*c*) emulsion polymerization; and (*d*) solution polymerization. The latter two polymerization methods can be employed for pharmaceutical purposes but are less common because of the high content of the monomers left in the polymers after the polymerization is over. The bulk polymerization method may become inconvenient for thermolabile drugs because heat of polymerization is evolved during the process. This can be quite high for many monomers and may cause decomposition of the drug.

Bead polymerization was first carried out in 1931 (8). However, this method was successfully applied only after effective suspension stabilizers were found. The bead polymerization method is similar to the bulk polymerization with respect to reaction kinetics, but the monomer or the mixture of monomers is dispersed by strong mechanical agitation into droplets in a second liquid phase in which both the monomer and the polymer formed are essentially insoluble. The monomer droplets, which are larger than those of a true emulsion, are then polymerized by heating with the catalyst. The dispersion is maintained during this time by continuous agitation. The suspending liquid, mostly water, acts as a cooler when the high amount of the heat of polymerization is evolved during the process. Suitable agents are added to the suspended liquid to hinder the coalescence of the droplets during polymerization. The polymerization takes place in the individual monomer droplets; these polymerized droplets are called beads or pearls. These beads can easily be separated from the aqueous phase when stirring is discontinued. Thus the bead polymerization method overcomes the

Table I—Beads of Methyl Methacrylate and α-Methacrylic Acid with Chloramphenicol

Materials	Formula 1	1.1	2	2.1	3	3.1	4	4.1
Inner phase								
Methyl methacrylate, g.	100	80	66.6	53.0	33.3	27.0	—	—
α-Methacrylic acid, g.	—	—	33.3	27.0	66.6	53.0	100	80
Chloramphenicol, g.	—	20	—	20.0	—	20.0	—	20
Outer aqueous phase								
Carboxyvinyl polymer, g.	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Sodium sulfate, anhyd., g.	—	—	15.0	15.0	30.0	30.0	60.0	60.0
Sulfuric acid, concn., ml.	—	—	6.0	6.0	6.0	6.0	—	—
Water, ml.	300	300	300.0	300.0	300.0	300.0	300.0	300
Catalyst (benzoyl peroxide), g.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Speed of stirrer, r.p.m.	120	120	120	120	120	120	120	120
Temp. of polymerizn.	70–75	70–75	74–80	74–80	74–80	74–80	80–95	80–95
Time of polymerizn., hr.	24	24	7	7	7	7	6	6
Drying temperature	60	60	60	60	60	60	60	60
Washing medium	Water	Water	Water	Water	Water	Water	HCl, 20% v/v	HCl, 20% v/v
Yield	94	80	93	87	95	75	96	80
Amount of drug in beads, %	—	7.0	—	19.5	—	15.0	—	9.5

Table II—Beads[a] of Vinyl Acetate and Crotonic Acid with Chlorothiazide

Materials	Formula 5	5.1	6	6.1	7	7.1	8	8.1	9	9.1
Inner phase										
Vinyl acetate, g.	98.0	78.4	95.0	76.0	92.0	73.6	89.0	71.2	86.0	68.8
Crotonic acid, g.	2.0	1.6	5.0	4.0	8.0	6.4	11.0	8.8	14.0	11.2
Chlorothiazide, g.	—	20.0	—	20.0	—	20.0	—	20.0	—	20.0
Outer aqueous phase[b]	300 ml.									
Yield, g.	85	71	95	78	88	80	95	82	86	76
Amount of drug in beads, %[c]										

[a] General conditions for polymerization: catalyst (α,α′-axo-isobutyronitrile), 1.0 g.; speed of stirrer, 100 r.p.m.; temperature of polymerization, 60–65°; time of polymerization, 36–38 hr.; washing medium, ice cold water; drying temperature, in vacuum at 30°. [b] Composition of the outer aqueous phase: polyvinylpyrrolidone 0.2 g., sodium hypophosphite 2.1 g.; water 300 ml. [c] In all cases, negligible (perhaps just on the surface of the beads).

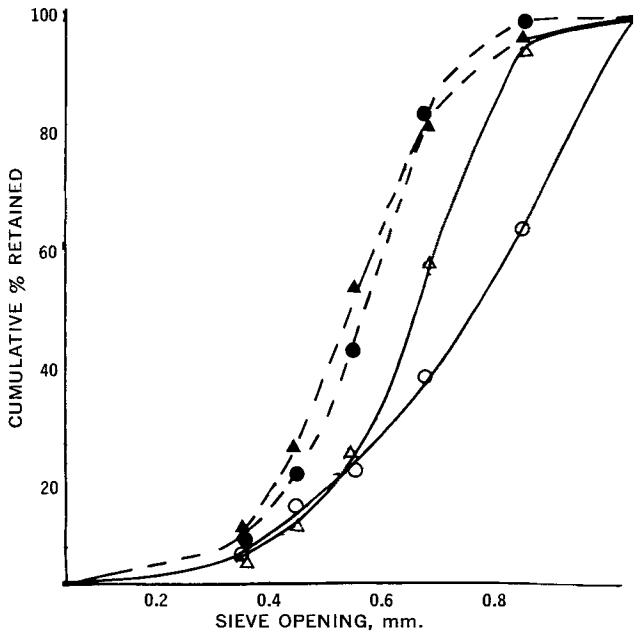


Figure 2—*Percent cumulative undersize of bead formulations 1, 1.1, 2, and 2.1. Key: ○, 1; ●, 1.1; △, 2; and ▲, 2.1.*

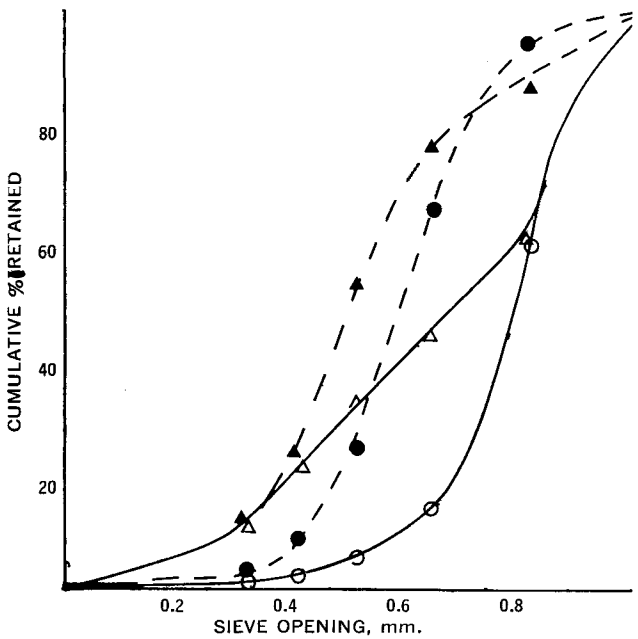


Figure 3—*Percent cumulative undersize of bead formulations 6, 6.1, 8, and 8.1. Key: ○, 6; ●, 6.1; △, 8; and ▲, 8.1.*

Table III—Beads of Composed Copolymers Containing N_1-6-Methoxy-2-(methoxymeth yl)-4-pyrimidinyl Sulfanilamide

Materials[a]	Formula 10	10.1	11.1	12.1	13.0	13.1	14	14.1	15	15.1	16	16.1	17	17.1
Inner phase														
Methyl methacrylate, g.	30.0	22.5	21.0	15.0	—	—	—	—	—	—	—	—	—	—
α-Methacrylic acid, g.	40.0	30.0	30.0	30.0	50	40	50	40	50	40	50	40	50	40
Polyvinylacetate-crotonic acid (92:8), g.	30	22.5	22.5	22.5	30	24	25	20	20	16	15	12	10	8
Divinylbenz. (50%)	—	—	1.5	2.5	20	16	25	20	30	24	35	28	40	32
Sulfa-drug	—	27.0	27.0	27.0	—	20	—	20	—	20	—	20	—	20
Outer aqueous phase														
Carboxyvinyl polymer, g.	0.80	0.96	0.96	0.96	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Sodium sulfate, anhydrous, g.	40.0	48.0	48.0	48.0	60.0	60	60	60	60	60	60	60	60	60
Sodium hydroxide, g.	0.5	0.8	0.8	0.8	—	—	—	—	—	—	—	—	—	—
Water, ml.	200	240	240	240	300	300	300	300	300	300	300	300	300	300
Catalyst (α,α′-axo-isobutyronitrile), g.	—	0.1	0.1	0.1	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Speed of stirrer, r.p.m.	300	300	300	300	300	300	300	300	300	300	300	300	300	300
Temp. of polymerizn.	60	60–70	60–70	60–70	70	70	70	70	70	70	70	70	70	70
Time of polymerizn., hr.	2	2	2	2	5–6	5–6	5–6	5–6	5–6	5–6	5–6	5–6	5–6	5–6
Drying temperature	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Yield	—	88.0	81	86	96	92	95	92	95	89	98	92	95	85
Amount of drug in beads, %	—	26.5	25.8	26.0	—	18.6	—	18.8	—	15.8	—	18.8	—	15.9

[a] Washing medium—water in all cases.

disadvantages of the other polymerization methods and has the further advantage that the drug is homogeneously distributed in the uniform tiny spheres.

EXPERIMENTAL

Materials Used—*Monomers*—α-Methacrylic acid,[1] methyl methacrylate,[1] vinyl acetate,[1] crotonic acid[1] (β-methacrylic acid), and divinylbenzene in ethylvinylbenzene as 45–55% solution.[1]

Polymers—Polyvinylacetate with 8% crotonic acid.[2]

Catalyst—α,α′-Axo-diisobutyronitrile[1] and benzoyl peroxide.[1]

Suspension Stabilizers—Carboxyvinyl polymers[3] and polyvinylpyrrolidone.[4]

Drugs—Chloramphenicol USP, N_1-6-methoxy-2-(methoxymethyl)-4-pyrimidinyl sulfanilamide,[5] chlorothiazide, pentobarbital and its sodium salt, papaverine base, hydrocortisone, and 1-ethyl-1-phenylglutarimide.[6]

Method of Preparation—*Apparatus*—A three-necked flask connected to a reflux condenser and an inert gas (nitrogen or carbon dioxide) supply was used. Through the remaining central wide opening, a stirrer was inserted. The flask was maintained at a constant temperature with a thermostatic water bath to control the reaction of the polymerization process (Fig. 1).

Procedure—The continuous outer phase consisting of water, protective colloid, and other additives (described under individual preparations) was heated in the three-necked flask to the desired temperature. The monomer, with or without drug, was added to the continuous phase and stirred at the optimum speed to form the desired size of monomer droplets. The catalyst was then added to effect the polymerization reaction under the inert gas flow. When the droplets were polymerized completely into solid beads, they were removed and washed with excess water or acidic aqueous solution to remove the impurities on the surface. These were then dried at an appropriate temperature with or without a vacuum. The different formulas prepared are given in Tables I–IV.

Determinations—*Yield of Beads*—The total amount of beads obtained after washing and drying was weighed, and this weight was taken as the yield for that preparation (Tables I–IV).

Amount of Drug in Beads—The polymer beads obtained were powdered and passed through a fine sieve. Approximately 0.5 g. accurately weighed powder was suspended in different 1-l. buffer solutions for 2 days. The amount of the drug dissolved in these buffer solutions from these powders was determined spectrophotometrically (Tables I–IV).

Particle-Size Distribution—A known amount of beads was used for sieve analyses to determine the particle-size distribution. The results for a few preparations of Tables I and II are presented as percent cumulative undersize of beads (Figs. 2 and 3).

RESULTS AND DISCUSSION

Choice of Monomers and Polymers for Polymerization—The choice of the monomers and the polymer to be added to them for polymerization in bead form depends on the following considerations:

1. The composition employed should be easily capable of polymerization at a relatively low temperature in the presence of the drug.
2. The polymer obtained should be mechanically hard, chemically stable, and free from monomers.
3. The beads should show specific properties of dissolution and/or diffusion of the embedded drug in gastrointestinal fluids. The presence of acidic groups in the beads increases the solubility in the neutral and alkaline buffer solutions (artificial duodenum and intestinal juices, *i.e.*, sustained dose) (9). The presence of network-

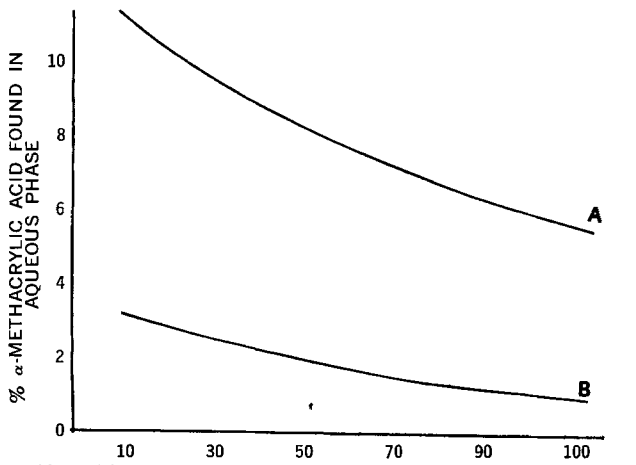


Figure 4—*Solubility of α-methacrylic acid in water and sodium sulfate solution. Key: A, in water; B, in 20% sodium sulfate solution.*

[1] Purchased from Fluka AG, Buchs, Switzerland. Pure monomers are stabilized with 0.01% hydroquinone. These were distilled under vacuum at appropriate temperature before use in presence of copper powder to remove hydroquinone.
[2] Supplied by Ciba AG, Basel, Switzerland.
[3] Carbopol 934 and 941, B. F. Goodrich Chemical Co., Ohio.
[4] Kollidone 25, BASF, Ludwigshafen, West Germany.
[5] Ba 35 092, Ciba AG, Basel, Switzerland.
[6] Doriden, Ciba AG, Basel, Switzerland.

Table IV—Beads[a] of Composed Copolymer in Presence of Different Drugs in Various Concentrations

Materials	Formula 18.1	18.2	18.3	19.1	20.1	20.2	20.3	20.4	21.1	22.1
	Pentobarbital			Sodium Pentobarbital	Papaverine				Hydrocortisone	1-Ethyl-1-phenyl Glutarimide
Inner phase										
Methyl methacrylate, g.	27	24	21	21	27	24	21	18	27	15
α-Methacrylic acid, g.	36	32	28	28	36	32	28	24	36	20
Polyvinylacetate-crotonic acid, g. (92:8)	27	24	21	21	27	24	21	18	27	15
Drug, g.	10	20	30	30	10	20	30	40	10	50
Outer aqueous phase										
Carboxyvinyl polymer, g.	1.0	1.0	1.0	0.8	1.2	1.2	1.6	1.6	0.8	0.8
Sodium sulfate, anhydrous, g.	48.0	48.0	48.0	40.0	60.0	60.0	75.0	75.0	40.0	40.0
Sodium hydroxide, g.	0.5	0.5	0.5	—	0.35	0.4	0.5	0.7	0.35	0.6
Water, ml.	240	240	240	200	300	300	375	375	200	200
Yield, g.	96	89	73	91	79	94	95	66	97	94
Amount of drug in beads, %	9.3	19.2	29	32.7	9.8	19.3	29.1	39.5	10.2	47

[a] General conditions for polymerization: catalyst (benzoyl peroxide), 0.6 g.; speed of stirrer, 250–300 r.p.m.; temperature of polymerization, 60°; time of polymerization, 2–2.5 hr.; drying temperature, 40°; and washing medium, water.

forming monomers in copolymers such as divinylbenzene decreases the swelling and solubility of copolymers in buffer solution.

Choice of Additives in the Outer Phase—The right type and amount of protective colloids in the aqueous outer phase prevent the monomer droplets from deforming and sticking to each other during polymerization. The choice in the outer phase depends mainly on the properties of the monomers used, the protective property at the selected pH, and the presence of electrolytes. The concentration of the protective colloid in the outer phase affects the bead size and may even lead to the formation of emulsions in the high concentrations. Hence, for each preparation, the optimum amount of protective colloid must be determined. The presence of the electrolyte, sodium sulfate, in the aqueous phase is necessary to decrease the solubility of the water-soluble monomer such as α-methacrylic acid in water (salting-out effect). Figure 4 shows the solubility of α-methacrylic acid from the mixtures of methyl methacrylate and α-methycrylic acid in water and 20% w/v sodium sulfate solution. However, the presence of such an electrolyte in water increases the interfacial tension between the monomeric phase and the outer aqueous phase. This latter effect may be strong enough to disturb the bead polymerization system, so it is always desirable to balance these two effects carefully. In principle, many protective colloids can be used, provided the electrolyte causes no salting-out effect on them in the aqueous phase. Carboxyvinyl polymers are stable in the presence of electrolytes between pH 4 and 11 and are employed in this study when electrolytes are present in the aqueous outer phase; otherwise, polyvinylpyrrolidone is the protective colloid used.

When the right type and amount of the protective colloid are used in the outer aqueous phase, the polymers prepared show mostly spherical form. However, the formation of a certain percentage of small agglomerates, twins, and deformed beads cannot be completely avoided. The appearance of the beads, whether transparent or opaque, depends on the solubility of the drugs in the inner monomeric phase. The preparation takes mostly the color of the drug used.

The yield of the beads ranges from 75 to 95% for different preparations. This loss may be due either to the formation of micelles or tiny spherical particles which are lost during filtration and washing of the beads or to the evaporation of a small amount of monomer at the polymerization temperature. Hence the polymerization should be carried out below the boiling temperature of the monomer to avoid the loss due to the latter reason. Further, it may be observed from Tables I–IV that the yield in the presence of drug is always less than when the drug is absent. This loss can be due to the preferential solubility of the drug in, or the affinity of the drug for, the aqueous outer phase rather than those in or for the monomeric phase. This type of loss is further proved by determining the amount of drug present in the beads after polymerization is over (Tables I–IV). Drugs having no affinity for the monomeric phase could not even be embedded in the beads during polymerization as in the examples of chlorothiazide with vinyl acetate and crotonic acid (Table II).

The beads show a large range of particle size distribution varying between 0.3- and 1.0-mm. diameter. In the presence of drugs—either soluble in both the phases as chloramphenicol or insoluble in both the phases as chlorothiazide—the beads obtained are smaller in size than in the absence of drugs (Figs. 2 and 3). These two examples are chosen to investigate the influence of incorporation of soluble and insoluble drugs in the monomeric phase on the size distribution of beads. Chloramphenicol is soluble in the monomeric phase as well as in the outer aqueous phase while chlorothiazide is practically insoluble in both phases. The drug soluble in the outer phase may be affecting the interfacial tension between the two phases while the insoluble drug may be acting as a fine-mesh barrier between droplets and hence decreasing the particle size of the beads (10).

SUMMARY

The method of bead polymerization in aqueous phase has been studied for embedding drugs in polymers. Various polymers and copolymers, either in the presence or in the absence of the drugs, have been studied to prove the widespread applicability of the bead polymerization method. The presence of the drug, either soluble or insoluble, in the outer aqueous phase decreased the bead size and yield of the products. The amount of the drug which could be embedded in the polymer beads depended on the affinity and solubility of the drug for the monomers.

REFERENCES

(1) F. Rosenthal, U. S. pat. 2,895,880 (1959).
(2) A. F. Asker and C. H. Becker, *J. Pharm. Sci.*, **55**, 90(1966).
(3) E. B. Draper and C. H. Becker, *ibid.*, **55**, 376(1966).
(4) S. C. Khanna and P. Speiser, *ibid.*, **58**, 1114(1969).
(5) K. Lehmann, *Pharm. Ind.*, **29**, 396(1967).
(6) W. A. Ritschel, "Die Tablette," Editio Cantor KG, Aulendorg i. Württ, p. 104.
(7) U. S. pat. 3,390,050 (1968).
(8) W. Bauer and H. Lauth, DRP 656 134, 1931.

(9) H. Logemann, "Methoden der Organischen Chemie (Houben-Weyl)," vol. 14/1, Makromolekulare Chemie, Themie/ Stuttgart, p. 414.

(10) E. Trommsdroff and C. Schildknecht, "High Polymers," vol. X, Interscience, New York, N. Y., 1956, pp. 69–109.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from the *School of Pharmacy, Federal Institute of Technology, Zurich, Switzerland.*

Accepted for publication December 2, 1969.

Abstracted from the dissertations submitted by S. C. Khanna and T. Jecklin to the Swiss Federal Institute of Technology in fulfillment of Doctor of Natural Sciences degree requirements.

* Present address: J. R. Geigy S. A., Galenic Research Section, Basel, Switzerland.

† Present address: Sihlfeld Apotheke, Zurich, Switzerland.

Mechanism of Dimethyl Sulfoxide-Induced Hemolysis

WILLIAM P. NORRED, HOWARD C. ANSEL, IVAN L. ROTH*, and JAMES J. PEIFER†

Abstract □ A study has been made of the possible mechanism by which dimethyl sulfoxide induces hemolysis of rabbit erythrocytes *in vitro*. Erythrocytes were shown by spectral analysis to remove dimethyl sulfoxide from aqueous solution and to resist the agent's release upon washing. Electron microscopy revealed the increased formation of lesions in the erythrocyte membrane with increasing concentrations of dimethyl sulfoxide. Dimethyl sulfoxide was also shown to be increasingly capable of removing fatty acids from the erythrocyte membrane with an increase in concentration. Results of the study indicate that dimethyl sulfoxide is capable of causing the hemolysis of erythrocytes by virtue of its affinity for the erythrocyte membrane and the disruption of its integrity, in part due to its lipid solvency action.

Keyphrases □ Erythrocyte hemolysis—dimethyl sulfoxide induced □ Mechanism—dimethyl sulfoxide-induced hemolysis □ Dimethyl sulfoxide retention—erythrocytes □ Fatty acid removal, erythrocytes—dimethyl sulfoxide □ Electron microscopy—erythrocyte lesion determination □ UV spectrophotometry—analysis

Although dimethyl sulfoxide (DMSO) was first synthesized in 1867 (1), it has only recently become the object of intensive scientific investigation. Used for years by the chemical industry as a solvent, the compound became of special interest to biologists after it was found to have a wide range of solvent action for chemicals employed in various laboratory procedures (2–7). DMSO was subsequently utilized investigationally as the solvent for poorly soluble drugs to be employed parenterally in the clinical treatment of cancer and leprosy (8, 9).

Much of the recent flourish of investigation activity centered about DMSO has been prompted by reports of the agent's apparent great ability to traverse biologic membranes and exert its pharmacologic activity (10–14), as well as to increase the degree and rate of penetration of other drugs across biologic membranes (15–17).

DMSO has also received considerable attention concerning its ability to serve effectively as a cryophylactic agent in the preservation of various body organs, tissues, and cells (7, 17–35), including red blood cells (31–35).

One difficulty concerning the use of DMSO as a cryophylactic agent in the preservation of blood for transfusion has been its nature to permeate the erythrocyte along with the subsequent difficulty experienced in its removal from the blood preparative to transfusion (32, 34, 35). Cellular destruction, resulting in hemolysis, commonly accompanies attempts to rid the cells of DMSO by washing (25, 36).

The hemolytic activity of DMSO, *in vitro* and *in vivo*, has been noted in reports from this (36) and from other laboratories (25, 37, 38). It was the purpose of the present work to investigate the mechanism by which DMSO exerts its hemolytic effects against the erythrocyte.

EXPERIMENTAL

Materials—The DMSO employed in this investigation was reagent grade and was obtained commercially.

Blood Samples—In previously reported studies the hemolytic activity of DMSO against human and rabbit erythrocytes was quite comparable (36, 37). This was verified in preliminary experiments to the present work using blood obtained from the forearm veins of several Caucasian donors, 20–25 years of age, and from rabbits by cardiac puncture. For convenience, rabbit blood was employed throughout this study. Fresh blood was obtained immediately prior to each experiment and defibrinated by gentle swirling with glass beads for approximately 5 min. The defibrinated blood was then transferred by decantation to a clean container and employed in the following procedures.

Absorption of DMSO by Red Blood Cells—To assess the capabilities of DMSO to enter and/or bind with the erythrocyte, red blood cells were incubated with solutions of DMSO, and any alteration in the absorbance of the original DMSO test solution was determined spectrophotometrically.

Erythrocytes were separated from the defibrinated blood sample by centrifugation and decantation. Then the cells were washed five times with normal saline solution and reconstituted to the original blood volume. This cell suspension was then employed to prepare test samples containing volumes of erythrocytes ranging in concentration from 0.06 to 0.48%, with 0.004% DMSO in normal saline. The suspensions were allowed to incubate for 30 min. at 37°, after which time the cells were settled by centrifugation at 13,500×g for 15 min. The supernatant was then examined spectrophotometrically with a Beckman DU for its DMSO content, using a wavelength of 208 mμ. The amounts found were compared to the amounts originally present in the test solutions, and the amounts absorbed determined by difference.

Preparation of Erythrocyte Ghosts for Electron Microscopy—One percent suspensions of defibrinated blood were prepared in 0.9% sodium chloride and varying proportions of DMSO, and incubated at 37° for 45 min. Control suspensions of blood in 0.9% sodium chloride were prepared and concurrently processed with the DMSO-containing suspensions.

(9) H. Logemann, "Methoden der Organischen Chemie (Houben-Weyl)," vol. 14/1, Makromolekulare Chemie, Themie/ Stuttgart, p. 414.

(10) E. Trommsdroff and C. Schildknecht, "High Polymers," vol. X, Interscience, New York, N. Y., 1956, pp. 69–109.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from the *School of Pharmacy, Federal Institute of Technology, Zurich, Switzerland.*

Accepted for publication December 2, 1969.

Abstracted from the dissertations submitted by S. C. Khanna and T. Jecklin to the Swiss Federal Institute of Technology in fulfillment of Doctor of Natural Sciences degree requirements.

* Present address: J. R. Geigy S. A., Galenic Research Section, Basel, Switzerland.

† Present address: Sihlfeld Apotheke, Zurich, Switzerland.

Mechanism of Dimethyl Sulfoxide-Induced Hemolysis

WILLIAM P. NORRED, HOWARD C. ANSEL, IVAN L. ROTH*, and JAMES J. PEIFER†

Abstract □ A study has been made of the possible mechanism by which dimethyl sulfoxide induces hemolysis of rabbit erythrocytes *in vitro*. Erythrocytes were shown by spectral analysis to remove dimethyl sulfoxide from aqueous solution and to resist the agent's release upon washing. Electron microscopy revealed the increased formation of lesions in the erythrocyte membrane with increasing concentrations of dimethyl sulfoxide. Dimethyl sulfoxide was also shown to be increasingly capable of removing fatty acids from the erythrocyte membrane with an increase in concentration. Results of the study indicate that dimethyl sulfoxide is capable of causing the hemolysis of erythrocytes by virtue of its affinity for the erythrocyte membrane and the disruption of its integrity, in part due to its lipid solvency action.

Keyphrases □ Erythrocyte hemolysis—dimethyl sulfoxide induced □ Mechanism—dimethyl sulfoxide-induced hemolysis □ Dimethyl sulfoxide retention—erythrocytes □ Fatty acid removal, erythrocytes—dimethyl sulfoxide □ Electron microscopy—erythrocyte lesion determination □ UV spectrophotometry—analysis

Although dimethyl sulfoxide (DMSO) was first synthesized in 1867 (1), it has only recently become the object of intensive scientific investigation. Used for years by the chemical industry as a solvent, the compound became of special interest to biologists after it was found to have a wide range of solvent action for chemicals employed in various laboratory procedures (2–7). DMSO was subsequently utilized investigationally as the solvent for poorly soluble drugs to be employed parenterally in the clinical treatment of cancer and leprosy (8, 9).

Much of the recent flourish of investigation activity centered about DMSO has been prompted by reports of the agent's apparent great ability to traverse biologic membranes and exert its pharmacologic activity (10–14), as well as to increase the degree and rate of penetration of other drugs across biologic membranes (15–17).

DMSO has also received considerable attention concerning its ability to serve effectively as a cryophylactic agent in the preservation of various body organs, tissues, and cells (7, 17–35), including red blood cells (31–35).

One difficulty concerning the use of DMSO as a cryophylactic agent in the preservation of blood for transfusion has been its nature to permeate the erythrocyte along with the subsequent difficulty experienced in its removal from the blood preparative to transfusion (32, 34, 35). Cellular destruction, resulting in hemolysis, commonly accompanies attempts to rid the cells of DMSO by washing (25, 36).

The hemolytic activity of DMSO, *in vitro* and *in vivo*, has been noted in reports from this (36) and from other laboratories (25, 37, 38). It was the purpose of the present work to investigate the mechanism by which DMSO exerts its hemolytic effects against the erythrocyte.

EXPERIMENTAL

Materials—The DMSO employed in this investigation was reagent grade and was obtained commercially.

Blood Samples—In previously reported studies the hemolytic activity of DMSO against human and rabbit erythrocytes was quite comparable (36, 37). This was verified in preliminary experiments to the present work using blood obtained from the forearm veins of several Caucasian donors, 20–25 years of age, and from rabbits by cardiac puncture. For convenience, rabbit blood was employed throughout this study. Fresh blood was obtained immediately prior to each experiment and defibrinated by gentle swirling with glass beads for approximately 5 min. The defibrinated blood was then transferred by decantation to a clean container and employed in the following procedures.

Absorption of DMSO by Red Blood Cells—To assess the capabilities of DMSO to enter and/or bind with the erythrocyte, red blood cells were incubated with solutions of DMSO, and any alteration in the absorbance of the original DMSO test solution was determined spectrophotometrically.

Erythrocytes were separated from the defibrinated blood sample by centrifugation and decantation. Then the cells were washed five times with normal saline solution and reconstituted to the original blood volume. This cell suspension was then employed to prepare test samples containing volumes of erythrocytes ranging in concentration from 0.06 to 0.48%, with 0.004% DMSO in normal saline. The suspensions were allowed to incubate for 30 min. at 37°, after which time the cells were settled by centrifugation at 13,500×g for 15 min. The supernatant was then examined spectrophotometrically with a Beckman DU for its DMSO content, using a wavelength of 208 mμ. The amounts found were compared to the amounts originally present in the test solutions, and the amounts absorbed determined by difference.

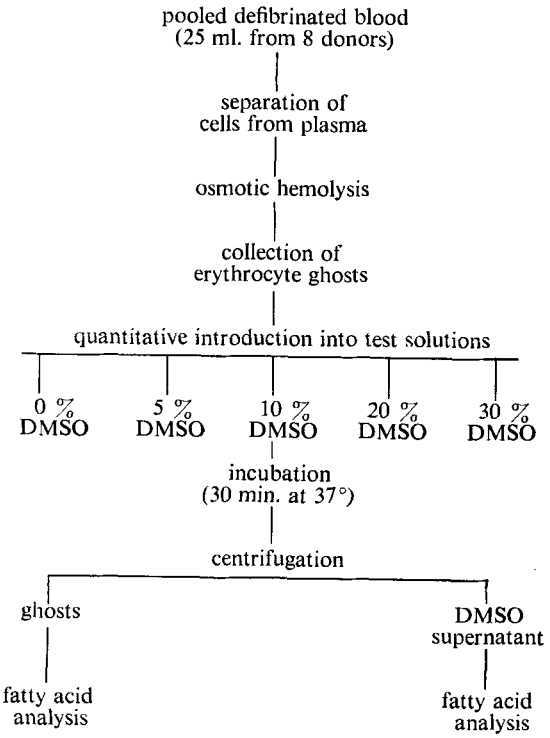
Preparation of Erythrocyte Ghosts for Electron Microscopy—One percent suspensions of defibrinated blood were prepared in 0.9% sodium chloride and varying proportions of DMSO, and incubated at 37° for 45 min. Control suspensions of blood in 0.9% sodium chloride were prepared and concurrently processed with the DMSO-containing suspensions.

After the incubation period, erythrocyte ghosts were prepared essentially by the procedure used by Hillier and Hoffman (39) in their study of the ultrastructure of the human and animal erythrocyte membrane. By this method, hemoglobin-free ghosts are prepared by subjecting the cells to a series of osmotic shocks by washing successively in sodium chloride solutions of decreasing tonicity. The present method differed from that of Hillier and Hoffman only in that the final wash was conducted with distilled water in the present instance rather than with saline of extremely low tonicity. Following each saline washing the cells were resuspended in a solution of lower tonicity, centrifuged at 3500×g for 5 min., until finally they were triple-washed with distilled water and resuspended to one-fourth of the volume of the original suspension of defibrinated blood.

In mounting the ghosts for examination by electron microscopy, one drop of the aqueous suspension was placed on each of several collodion-coated copper grids of 200 mesh and allowed to remain undisturbed for approximately 5 min. Excess liquid was then drawn off the grid using absorbant paper, and the grid was allowed to dry overnight.

It was found unnecessary to employ staining or shadowing procedures. The grids were examined in an RCA EMU 2 electron microscope operating at a voltage of 50 kw. Micrographs were photographically enlarged as noted.

Fatty Acid Analysis—To determine the solvent characteristics of the various DMSO solutions on the erythrocyte membrane, an analysis was performed to establish the degree of fatty acid removal from the membrane. Ghosts were prepared from 25 ml. of pooled defibrinated blood (Scheme I) by successive exposure to solutions of decreasing tonicity and resuspended in 25 ml. of distilled water. One milliliter of the ghost suspension was introduced into 24 ml. of each of the various DMSO test solutions. The samples were incubated 30 min. at 37°, then centrifuged at 20,000×g for 20 min. to settle the ghosts. The supernatant was decanted and the ghosts were resuspended in 10 ml. of 50% ethanol to inhibit enzyme activity Ethanol was also added to the supernatant to a concentration of 50%. The samples were saponified with 2 *M* KOH and the total fatty acids isolated with chloroform. The fatty acids were converted to methyl esters using BF_3-reagent and procedures described by Metcalfe and Schmitz (40). Quantitation of total fatty acids was based on addition of behenic acid (22:0) as an internal standard and gas-liquid chromatography analysis of the methyl esters using a Packard model 845 instrument equipped with dual hydrogen flame detectors and techniques previously described (41).



Scheme I—Preparation of erythrocytes for fatty acid analysis

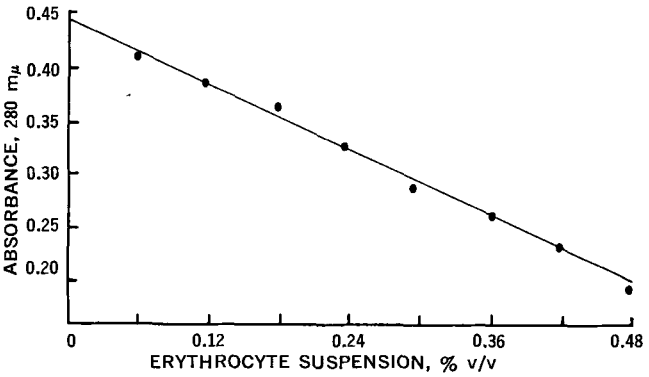


Figure 1—*Spectral absorbance of DMSO (0.004%) in the presence of washed rabbit erythrocytes in 0.9% NaCl. Data are the averages of three samples.*

RESULTS AND DISCUSSION

Absorption of DMSO by Red Blood Cells—Ansel and Leake (36), reporting on the *in vitro* hemolysis of erythrocytes in the presence of DMSO, noted that without the presence of added saline, DMSO is incapable of maintaining the integrity of the erythrocytes, and hemolysis is total at all DMSO concentrations. To prevent osmotic hemolysis, the presence of an extracellular material to which the red blood cell membrane is impermeable is required. In the presence of 0 6% sodium chloride the red blood cells are protected from hemolysis until the DMSO concentration is raised to above 25%. At DMSO concentrations above 30% in the absence of saline and above 45% in the presence of saline, the blood was denatured as observed by brown discoloration of blood cells and released hemoglobin and the flocculation of the cell components.

The ability of erythrocytes to hold DMSO was examined in the present study by incubating previously washed erythrocytes in 0.9% saline solutions containing a fixed quantity of DMSO, and comparing the spectrophotometric absorbance for DMSO in the control sample (without cells) and in the supernatant of the various cell samples. In each of the experiments conducted, the spectral absorbance of extracellular DMSO decreased with increased concentration of cells present. Figure 1 represents one experiment which typifies the data obtained in this series. The data indicate that DMSO is removed from the extracellular medium and held by the erythrocytes. This would tend to support the experiences of others (25, 36) who have found the removal of DMSO from blood cells prior to transfusion so difficult.

The hygroscopic nature of DMSO undoubtedly plays a role in its ability to traverse biologic membranes and perhaps to affect the configuration of membrane proteins. DMSO has a strong affinity for water molecules, with a maximum hydration of DMSO in aqueous solution being three water molecules per DMSO molecule (42). It has been suggested that the hydrogen bonds which exist between water and DMSO are stronger than the hydrogen bonds which exist between water molecules (43). Because of its comparatively small size, the DMSO molecule is thought to be capable of penetrating regions on certain protein subunit interfaces more readily than other bulkier solvents (44).

Rammler and Zaffaroni (44) suggest that the ability of DMSO in high concentrations to cross rapidly the dermal protein barrier, whose conformational integrity is dependent upon bound water, is the result of reversible configuration changes of these proteins due to water substitution by DMSO.

Since the principal component of the living cell is water, and the native form of biopolymers such as proteins, polysaccharides, and nucleic acids is surrounded by arrays of water molecules (the hydration sheath), these seem to suggest the manner in which DMSO gains entrance to the cell and is bound within (44). Substitution or removal of the hydration sheath would be expected to alter the configuration of the biopolymers and consequently the cell or tissue. Since in many instances it has been found that the cells apparently so affected are not permanently altered, the reversible process could be envisaged as occurring either as diffusion or active transport of the major amount of DMSO from the tissue and the gradual removal of the protein-bound DMSO by competitive bonding with cellular water (44).

Figure 2—*Erythrocyte ghosts (× 6900) hemolyzed by osmotic pressure.*

In studying the metabolism and distribution of DMSO in man and in animals, Gerhards and Gibian (45) found that in human blood about 30% of DMSO is bound to plasma protein and 25% to the formed elements, the remainder being free. Denko *et al.* (46), using ^{35}S-labeled DMSO and rat blood, found that DMSO radioactivity is associated predominantly with the serum albumin. They reported that the biologic half-life of DMSO-^{35}S is prolonged by 25% in hard tissues and thus may indicate some tissue binding.

Huggins (31) reported on his observations of the interaction between DMSO and human plasma proteins, noting that true denaturation of plasma protein seems to occur at DMSO concentrations approximating 50%. He stated that precipitation of protein by DMSO occurred with individual plasma fractions—albumin, fibrinogen, and gamma globulin—and appears to be a general effect rather than denaturation of a specific plasma component. DMSO has also been reported to be a good solvent for a number of proteins (47).

DiStefano and Klahn (38) concluded that DMSO probably exerts its hemolytic effect by direct action on the blood cells, although they hesitated to suggest a mechanism for this action.

It is likely that part of the means by which DMSO is capable of acting as a hemolytic agent is its initial ability to gain entrance into the red blood cell (due to its hygroscopicity and small size) and its affinity for proteins, resulting in their disfiguration, denaturation, or dissolution, and thus a loss of cellular integrity.

Electron Microscopy—An ultrastructural examination of the erythrocyte membrane was made following exposure to various DMSO concentrations. It is important to note that the particular cells photographed in each instance are representative of the general appearance of the field of cells examined.

Figure 2 shows the control erythrocytes which were osmotically hemolyzed in the presence of hypotonic saline and then mounted on a collodion-coated copper grid. These cells are intact, with a fine granular appearance. This type of granularity has been noted by other investigators (39, 48, 49). Hillier and Hoffman (39) observed erythrocyte membranes at much higher magnification (× 100,000 to × 200,000) and noted that the granularity is due to closely packed "cylinders" approximately 30 Å thick with a diameter of 100 to 500 Å. They termed these structures "plaques."

Figure 3 shows the appearance of erythrocytes exposed to 5% DMSO and then osmotically hemolyzed. The membranes are intact and have the same general appearance as the osmotically hemolyzed control cells. Some of the cells show the presence of

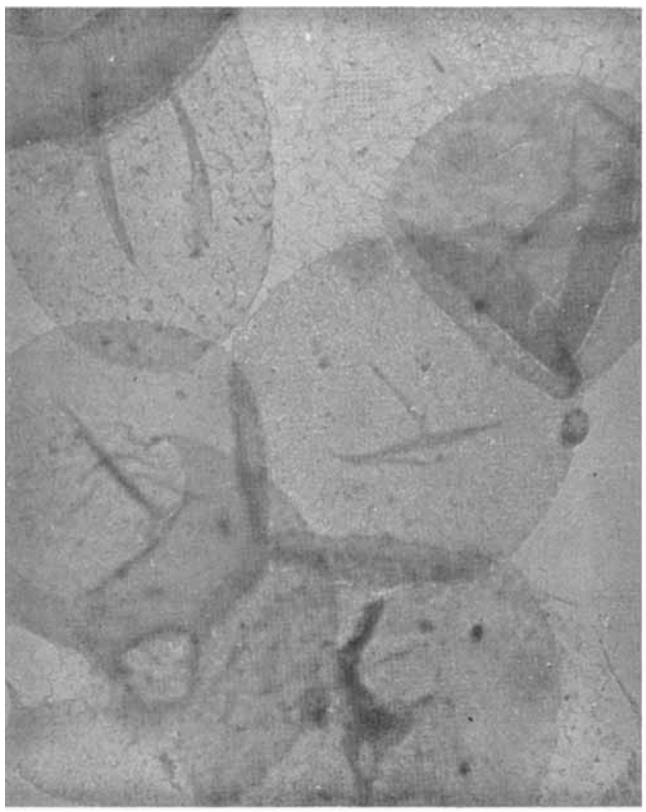


Figure 3—*Erythrocyte ghosts (× 7350) exposed to 5% DMSO in 0.9% NaCl for 45 min. at 37°.*

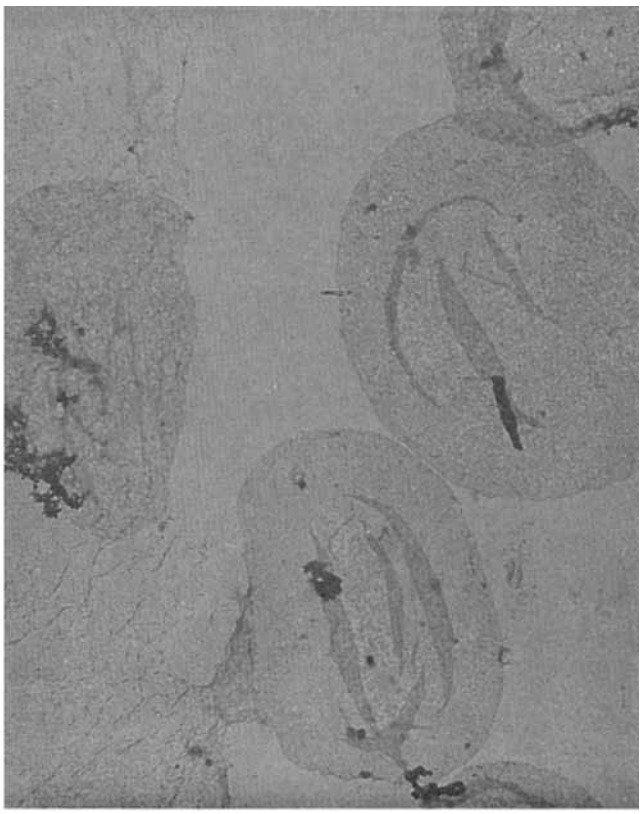


Figure 4—*Erythrocyte ghosts (× 6750) exposed to 20% DMSO in 0.9% NaCl for 45 min. at 37°.*

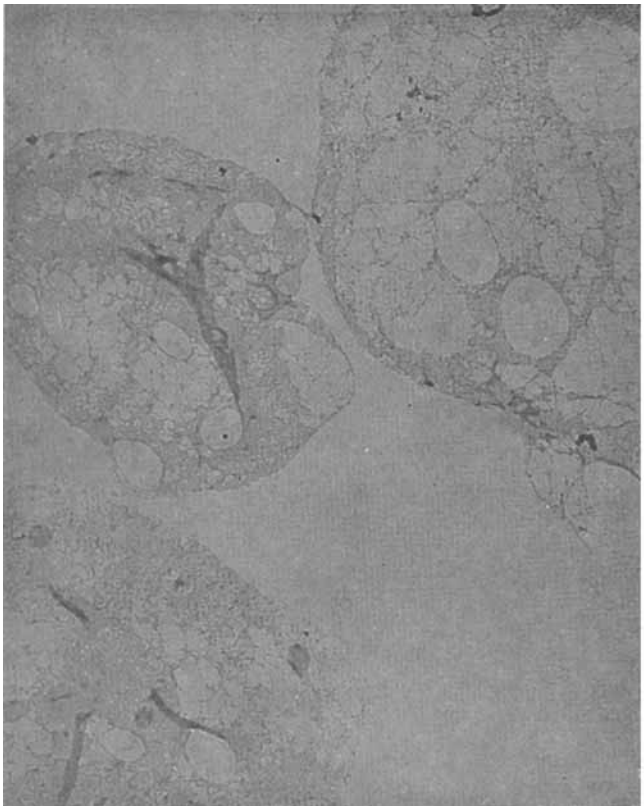


Figure 5—*Erythrocyte ghosts (× 6400) exposed to 30% DMSO in 0.9% NaCl for 45 min. at 37°.*

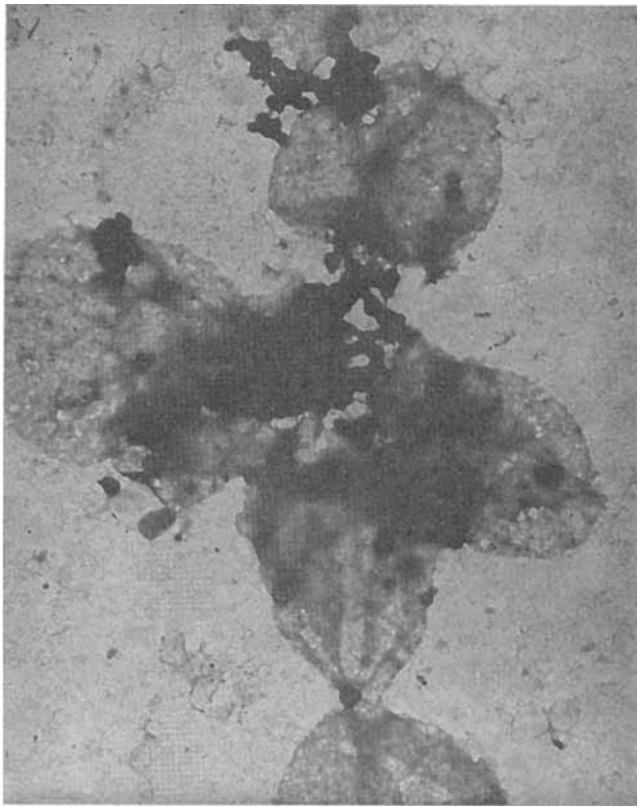


Figure 6—*Erythrocyte ghosts (× 7200) exposed to 40% DMSO in 0.9% NaCl for 45 min. at 37°.*

folds, which probably occurred as the saclike membane collapsed upon itself in attaching to the surface of the collodion membrane.

Figure 4 shows cells after treatment with 20% DMSO, followed by osmotic hemolysis. Definite lesions in the membrane are visible, and more cell fragments are present than in the previous samples. The particles seen on the collodion membrane surrounding the ghosts are interesting. A possible explanation is that these particles are cellular plaques which have been removed from the membrane surface by the action of the DMSO. Hillier and Hoffman (39) noted a similar phenomenon when treating cells with a variety of lipid solvents such as alcohol–ether–chloroform. They suggested that the plaques themselves may be insoluble in the solvent, but are attached to an underlying fibrous membrane by some type of lipid. A lipid solvent may then remove the lipid component, releasing the plaques and thereby cause lesions in the membrane surface. Since the total lipid of the red cell lies mainly within the membrane (50), the solvency of DMSO toward lipoidal and nonlipoidal materials (51) and the concepts of Hillier and Hoffman would seem to support the suggestion that the loss of the erythrocyte's integrity in the presence of DMSO is due to the dissolution of one or more of the membrane components. Another possible mechanism for the disruptive influence of DMSO on the cell membrane has been hypothesized by Puig Muset and Martin-Esteve (52). They propose that DMSO might produce some change in the isomeric conformation of cellular fatty acids, as oleic acid and linoleic acid, with a resulting alteration in the permeability barriers of the cell.

Table I—Fatty Acid Content of Erythrocyte Ghosts Following Incubation with DMSO[a]

% DMSO	Fatty Acid Content	
	mcg.	% of Total (Control)
0 (Control)	646	100 (Standard)
5	562	87.0
10	478	74.0
30	382	59.2

[a] Each sample was incubated for 30 min. with the stated concentration of DMSO.

Figure 5 shows erythrocytes after 30% DMSO treatment, followed by hemolysis. Gross disruption of the membrane has resulted. As could be expected, these cells were partially hemolyzed after incubation with the DMSO, even though the surrounding medium was isotonic with sodium chloride (36).

When cells were treated with 40% and higher concentrations of DMSO, a brown flocculation was observed in the test tubes. The appearance of the cells is shown in Fig. 6. Hemoglobin could not be removed by introduction of the cells into solutions of decreasing tonicity, and in the micrograph appears to be outside the cells but coagulated. The diameter of the ghosts is smaller than the osmotic controls, and the general appearance is that of coagulation of membrane components, possibly due to an alteration in protein configuration.

Fatty Acid Analysis—The lipid studies in this work were limited to the determination of total fatty acid removal from erythrocyte ghosts following incubation for 30 min. in various concentrations of DMSO. As can be seen in Table I, increasing amounts of fatty acids were removed from erythrocyte ghosts with increasing concentrations of DMSO. Work now in progress will attempt to identify the specific fatty acids removed from the erythrocyte by DMSO and to quantitate the rate and extent of such removal.

In conclusion, it appears as though the affinity of DMSO for the erythrocyte membrane and its lipid solvency contribute to the disruption of the integrity of the erythrocyte, resulting in hemolysis, and at high concentrations, in denaturation.

REFERENCES

(1) A. Saytzeff, *Ann. Chem. Pharm.*, **144,** 148(1867).

(2) E. D. Rees and S. J. Singer, *Arch. Biochem.*, **63,** 144(1956).

(3) S. M. Vratsanos, *ibid.*, **90,** 132(1960).

(4) J. B. Caulfield, *J. Biophys. Biochem. Cytol.*, **3,** 827(1957).

(5) R. J. Herschler and S. W. Jacob, *Tech. Ass. Pap.*, **48,** 43A (1965).

(6) M. C. Braude and R. R. Monroe, *Curr. Ther. Res. Clin. Exp.*, **7,** 502(1965).

(7) J. E. Lovelock and M. W. H. Bishop, *Nature*, **183,** 1394 (1959).

(8) R. O. Yeats, *Ann. N. Y. Acad. Sci.*, **141,** 668(1967).
(9) H. Rosenkrantz, Z. Hadidian, H. Seay, and M. M. Mason, *Cancer Chemother. Rep.*, **31,** 7(1963).
(10) L. S. Blumenthal and M. Fuchs, *Ann. N. Y. Acad. Sci.*, **141,** 572(1967).
(11) L. M. Lockie and B. M. Norcross, *ibid.*, **141,** 599(1967).
(12) H. D. Ogden, *ibid.*, **141,** 646(1967).
(13) J. Goldman, *ibid.*, **141,** 653(1967).
(14) E. Ramirez and S. Luza, *ibid.*, **141,** 655(1967).
(15) D. C. Pope and W. T. Oliver, *Can. J. Comp. Med. Vet. Sci.*, **30,** 3(1966).
(16) G. E. Schumacher, *Drug Intel.*, **1,** 188(1967).
(17) E. E. Rosenbaum, R. J. Herschler, and S. W. Jacob, *J. Amer. Med. Ass.*, **192,** 309(1965).
(18) C. E. Huggins, *Surg. Forum*, **13,** 51(1962).
(19) J. Farrant, *J. Pharm. Pharmacol.*, **16,** 472(1964).
(20) J. Farrant, *Nature*, **205,** 1284(1966).
(21) J. A. Armstrong, J. Farrant, and C. A. Walter, *Cryobiology*, **2,** 301(1966).
(22) A. M. Karow, O. Carrier, and W. C. Holland, *ibid.*, **3,** 464(1967).
(23) J. V. Levy, V. Richards, and M. Persidsky, *Proc. Soc. Exp. Biol. Med.*, **110,** 789(1962).
(24) M. J. Ashwood-Smith, *Nature*, **190,** 1204(1961).
(25) H. M. Pyle and H. Boyer, *Vox Sang.*, **8,** 100(1963).
(26) H. M. Pyle and H. Boyer, *Blood*, **21,** 770(1963).
(27) D. E. Pegg, *J. Appl. Physiol.*, **19,** 123(1964).
(28) J. S. Porterfield and M. J. Ashwood-Smith, *Nature*, **193,** 548(1962).
(29) R. Dougherty, *ibid.*, **193,** 550(1962).
(30) B. A. Bouroncle, *Cryobiology*, **3,** 445(1967).
(31) C. E. Huggins, *Transfusion*, **3,** 483(1963).
(32) A. P. Rinfret, C. W. Cowley, G. F. Doebbler, and H. R. Schreiner, *Proc. 9th Congr. Int. Soc. Blood Transfus.*, Mexico (1962), **1964,** 80.
(33) C. E. Huggins, *ibid.*, **1964,** 69.
(34) C. E. Huggins, *Science*, **139,** 504(1963).
(35) C. E. Huggins, *Surgery*, **54,** 191(1963).
(36) H. C. Ansel and W. F. Leake, *J. Pharm. Sci.*, **55,** 685(1966).
(37) D. E. Cadwallader and J. P. Drinkard, *ibid.*, **56,** 583(1967).
(38) V. DiStefano and J. J. Klahn, *Toxicol. Appl. Pharmacol.*, **7,** 660(1965).
(39) J. Hillier and J. F. Hoffman, *J. Cell. Comp. Physiol.*, **42,** 203(1953).
(40) L. D. Metcalfe and A. H. Schmitz, *Anal. Chem.*, **33,** 363 (1961).
(41) J. J. Peifer, *J. Lipid Res.*, **9,** 193(1968).
(42) H. Schott, *J. Pharm. Sci.*, **58,** 946(1969).
(43) J. M. G. Cowie and P. M. Toporowski, *Can. J. Chem.*, **39,** 2240(1961).
(44) D. H. Rammler and A. Zaffaroni, *Ann. N. Y. Acad. Sci.*, **141,** 13(1967).
(45) E. Gerhards and H. Gibian, *ibid.*, **141,** 65(1967).
(46) C. W. Denko, R. M. Goodman, R. Miller, and T. Donovan, *ibid.*, **141,** 77(1967).
(47) S. J. Singer, *Arch. Biochem. Biophys.*, **63,** 144(1956).
(48) D. N. Misra, J. Chakraborty, N. H. Sarkar, and J. B. Chatterjea, *J. Royal Microsc. Soc.*, **85,** 485(1966).
(49) H. Latta, *Blood*, **7,** 508(1952).
(50) B. N. Erickson, H. H. Williams, S. S. Bernstein, I. Avrin, R. L. Jones, and I. G. Macy, *J. Biol. Chem.*, **122,** 575(1938).
(51) M. M. Wolfred, *Quart. Rep. Sulfur Chem.*, **1,** 93(1966).
(52) P. Puig Muset and J. Martin-Esteve, *Experientia*, **21,** 649(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Georgia, Athens, GA 30601*
Accepted for publication December 8, 1969.
* Department of Microbiology, University of Georgia.
† Department of Biochemistry and Nutrition, University of Georgia.

Constituents from *Gymnema sylvestre* Leaves V: Isolation and Preliminary Characterization of the Gymnemic Acids

JOSEPH E. SINSHEIMER, G. SUBBA RAO*, and HUGH M. McILHENNY†

Abstract □ The objectives of this investigation were to isolate and characterize the constituents of gymnemic acid, the antisweet principle of *Gymnema sylvestre* leaves, and to make them available for further biological testing. Gymnemic acid was found to be a complex mixture of at least nine closely related acidic glycosides. Solvent extraction and chromatography of gymnemic acid resulted in the isolation of gymnemic acids A–D (the major constituents) and V in crystalline form. Acids A–D are glycosides which yield glucuronic acid on hydrolysis while acids C and D also yield glucose. The gymnemic acids isolated in this study are compared to those described in the literature.

Keyphrases □ *Gymnema sylvestre* leaves—constituents □ Gymnemic acids—isolation characterization □ Column chromatography—separation □ TLC—separation □ Reverse phase chromatography—separation □ IR spectrophotometry—glycosidic structure □ UV spectrophotometry—glycosidic structure □ NMR spectroscopy—glycosidic structure

While the unique property of the leaves of *Gymnema sylvestre* R. Br. (*Asclepiadaceae*) to inhibit temporarily the ability to taste sweet substances has been known in India since antiquity (1, 2), the first such report to be published in the Western literature appears to be that of Falconer (3) in 1847. Chemical investigations were initiated by Hooper (4, 5) who isolated the antisweet principle as an amorphous monobasic acid, $C_{32}H_{55}O_{12}$, which he named gymnemic acid. Further, Hooper described gymnemic acid as a glycoside since it reduced Fehling's solution after treatment with dilute hydrochloric acid. In 1892, Shore (6) reported a modified procedure to isolate gymnemic acid in white crystalline form and suggested the acid to be a derivative of anthracene. Several years later, Power and Tutin (7) isolated racemic glucose as its osazone from the leaves but were unable to detect any sugar after acidic hydrolysis of gymnemic acid. Upon potassium hydroxide fusion of gymnemic acid, Power and Tutin obtained acetic acid and a mixture of protocatechuic and *p*-hydroxybenzoic acids, while alkaline potassium permanganate oxidation afforded formic acid.

(8) R. O. Yeats, *Ann. N. Y. Acad. Sci.*, **141,** 668(1967).
(9) H. Rosenkrantz, Z. Hadidian, H. Seay, and M. M. Mason, *Cancer Chemother. Rep.*, **31,** 7(1963).
(10) L. S. Blumenthal and M. Fuchs, *Ann. N. Y. Acad. Sci.*, **141,** 572(1967).
(11) L. M. Lockie and B. M. Norcross, *ibid.*, **141,** 599(1967).
(12) H. D. Ogden, *ibid.*, **141,** 646(1967).
(13) J. Goldman, *ibid.*, **141,** 653(1967).
(14) E. Ramirez and S. Luza, *ibid.*, **141,** 655(1967).
(15) D. C. Pope and W. T. Oliver, *Can. J. Comp. Med. Vet. Sci.*, **30,** 3(1966).
(16) G. E. Schumacher, *Drug Intel.*, **1,** 188(1967).
(17) E. E. Rosenbaum, R. J. Herschler, and S. W. Jacob, *J. Amer. Med. Ass.*, **192,** 309(1965).
(18) C. E. Huggins, *Surg. Forum*, **13,** 51(1962).
(19) J. Farrant, *J. Pharm. Pharmacol.*, **16,** 472(1964).
(20) J. Farrant, *Nature*, **205,** 1284(1966).
(21) J. A. Armstrong, J. Farrant, and C. A. Walter, *Cryobiology*, **2,** 301(1966).
(22) A. M. Karow, O. Carrier, and W. C. Holland, *ibid.*, **3,** 464(1967).
(23) J. V. Levy, V. Richards, and M. Persidsky, *Proc. Soc. Exp. Biol. Med.*, **110,** 789(1962).
(24) M. J. Ashwood-Smith, *Nature*, **190,** 1204(1961).
(25) H. M. Pyle and H. Boyer, *Vox Sang.*, **8,** 100(1963).
(26) H. M. Pyle and H. Boyer, *Blood*, **21,** 770(1963).
(27) D. E. Pegg, *J. Appl. Physiol.*, **19,** 123(1964).
(28) J. S. Porterfield and M. J. Ashwood-Smith, *Nature*, **193,** 548(1962).
(29) R. Dougherty, *ibid.*, **193,** 550(1962).
(30) B. A. Bouroncle, *Cryobiology*, **3,** 445(1967).
(31) C. E. Huggins, *Transfusion*, **3,** 483(1963).
(32) A. P. Rinfret, C. W. Cowley, G. F. Doebbler, and H. R. Schreiner, *Proc. 9th Congr. Int. Soc. Blood Transfus.*, Mexico (1962), **1964,** 80.
(33) C. E. Huggins, *ibid.*, **1964,** 69.
(34) C. E. Huggins, *Science*, **139,** 504(1963).
(35) C. E. Huggins, *Surgery*, **54,** 191(1963).
(36) H. C. Ansel and W. F. Leake, *J. Pharm. Sci.*, **55,** 685(1966).
(37) D. E. Cadwallader and J. P. Drinkard, *ibid.*, **56,** 583(1967).
(38) V. DiStefano and J. J. Klahn, *Toxicol. Appl. Pharmacol.*, **7,** 660(1965).
(39) J. Hillier and J. F. Hoffman, *J. Cell. Comp. Physiol.*, **42,** 203(1953).
(40) L. D. Metcalfe and A. H. Schmitz, *Anal. Chem.*, **33,** 363 (1961).
(41) J. J. Peifer, *J. Lipid Res.*, **9,** 193(1968).
(42) H. Schott, *J. Pharm. Sci.*, **58,** 946(1969).
(43) J. M. G. Cowie and P. M. Toporowski, *Can. J. Chem.*, **39,** 2240(1961).
(44) D. H. Rammler and A. Zaffaroni, *Ann. N. Y. Acad. Sci.*, **141,** 13(1967).
(45) E. Gerhards and H. Gibian, *ibid.*, **141,** 65(1967).
(46) C. W. Denko, R. M. Goodman, R. Miller, and T. Donovan, *ibid.*, **141,** 77(1967).
(47) S. J. Singer, *Arch. Biochem. Biophys.*, **63,** 144(1956).
(48) D. N. Misra, J. Chakraborty, N. H. Sarkar, and J. B. Chatterjea, *J. Royal Microsc. Soc.*, **85,** 485(1966).
(49) H. Latta, *Blood*, **7,** 508(1952).
(50) B. N. Erickson, H. H. Williams, S. S. Bernstein, I. Avrin, R. L. Jones, and I. G. Macy, *J. Biol. Chem.*, **122,** 575(1938).
(51) M. M. Wolfred, *Quart. Rep. Sulfur Chem.*, **1,** 93(1966).
(52) P. Puig Muset and J. Martin-Esteve, *Experientia*, **21,** 649(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Georgia, Athens, GA 30601*
Accepted for publication December 8, 1969.
* Department of Microbiology, University of Georgia.
† Department of Biochemistry and Nutrition, University of Georgia.

Constituents from *Gymnema sylvestre* Leaves V: Isolation and Preliminary Characterization of the Gymnemic Acids

JOSEPH E. SINSHEIMER, G. SUBBA RAO*, and HUGH M. McILHENNY†

Abstract □ The objectives of this investigation were to isolate and characterize the constituents of gymnemic acid, the antisweet principle of *Gymnema sylvestre* leaves, and to make them available for further biological testing. Gymnemic acid was found to be a complex mixture of at least nine closely related acidic glycosides. Solvent extraction and chromatography of gymnemic acid resulted in the isolation of gymnemic acids A–D (the major constituents) and V in crystalline form. Acids A–D are glycosides which yield glucuronic acid on hydrolysis while acids C and D also yield glucose. The gymnemic acids isolated in this study are compared to those described in the literature.

Keyphrases □ *Gymnema sylvestre* leaves—constituents □ Gymnemic acids—isolation characterization □ Column chromatography—separation □ TLC—separation □ Reverse phase chromatography—separation □ IR spectrophotometry—glycosidic structure □ UV spectrophotometry—glycosidic structure □ NMR spectroscopy—glycosidic structure

While the unique property of the leaves of *Gymnema sylvestre* R. Br. (*Asclepiadaceae*) to inhibit temporarily the ability to taste sweet substances has been known in India since antiquity (1, 2), the first such report to be published in the Western literature appears to be that of Falconer (3) in 1847. Chemical investigations were initiated by Hooper (4, 5) who isolated the antisweet principle as an amorphous monobasic acid, $C_{32}H_{55}O_{12}$, which he named gymnemic acid. Further, Hooper described gymnemic acid as a glycoside since it reduced Fehling's solution after treatment with dilute hydrochloric acid. In 1892, Shore (6) reported a modified procedure to isolate gymnemic acid in white crystalline form and suggested the acid to be a derivative of anthracene. Several years later, Power and Tutin (7) isolated racemic glucose as its osazone from the leaves but were unable to detect any sugar after acidic hydrolysis of gymnemic acid. Upon potassium hydroxide fusion of gymnemic acid, Power and Tutin obtained acetic acid and a mixture of protocatechuic and *p*-hydroxybenzoic acids, while alkaline potassium permanganate oxidation afforded formic acid.

In 1958, Khastgir *et al.* (8) reinvestigated Hooper's gymnemic acid but failed to obtain a crystalline product even after silicic-acid chromatography. However, the following year Warren and Pfaffmann (9) isolated the antisweet principle in crystalline form, m.p. 199° (dec.) and, subsequently, Pfaffmann (10) reported the detection of glucose, arabinose, and glucuronolactone upon acidic hydrolysis of gymnemic acid. Recently Yackzan (11, 12), while studying the biological effects of *G. sylvestre* fractions, carried out limited chemical work on gymnemic acid and indicated the presence of hydroxyl and carboxyl groups and one or more glycosidic linkages. Yackzan also assigned a molecular weight of about 600 for gymnemic acid based upon an ultracentrifuge technique.

Thus, at the beginning of the present investigation, knowledge regarding the chemical nature of gymnemic acid was scanty, and its antisweet activity was the only established biological property. However, during the course of this investigation, this situation has undergone considerable change. Cochran and Maassab (13) have observed significant antiviral activity for gymnemic acids made available from these laboratories. Also, Stöcklin (14, 15) has greatly advanced the chemistry of gymnemic acid.

It is the purpose of this paper to describe the isolation and preliminary characterization of the gymnemic acids. This is in support of potential interest in the antiviral field, continuing interest as antisweet compounds, and as a comparison to the acids and methods reported by Stöcklin *et al.* (14).

EXPERIMENTAL[1]

Plant Material—Dry leaves of *G. sylvestre* were purchased from Prachi Gobeson Co., Calcutta, India, and the Himalaya Drug Co., Bombay, India. Identification of the plant material was established through an examination of a flowering top specimen as reported in the previous investigation from these laboratories (16) and through its property of suppressing selectively the ability to taste sugar.

Reagents—All common reagents and solvents utilized in this investigation were of analytical reagent grade. The following reagents were from the sources indicated: silica gel G (Warner-Chilcott Labs.); deuterium oxide, tetramethyl silane, silicic acid (100 mesh), ion-exchange resins (Rohm & Haas), IR 120 (H^+) and IRA 401 (Cl^-) (Mallinckrodt); Woelm neutral alumina, activity grade 1 (Alupharm Chemicals); Teflon-6 (70/80 mesh) (Analabs); Glucostat reagent (Worthington Biochemical Corp.); and D-glucuronolactone, D-glucuronic acid, D-glucose, and D-arabinose (Calbiochem).

Thin-Layer Chromatography—Silica gel G TLC plates were prepared by coating five 20 × 20-cm. glass plates at a thickness of 250 μ with a slurry composed of 18 g. of adsorbent and 45 ml. of water. After air drying for 30 min. at room temperature (27°), plates were activated in an oven at 105° for 1 hr. prior to use.

Activated TLC plates were scored into 1-cm. channels before sample application. Sample size was usually 10–50 mcg. for purified compounds and 100–250 mcg. for crude fractions. Chromatograms were developed to a distance of 13–14 cm. by ascending technique. Developing tanks were lined with filter paper and were saturated with appropriate solvent mixture for at least 30 min. Solvent systems employed for the gymnemic acids are listed in Table I. Spray reagents used for visualizing components on chromatograms were: benzoyl chloride–sulfuric acid reagent (17), ceric sulfate–sulfuric acid reagent (15, 18), and modified Liebermann-Burchard reagent (19).

Table I—Thin-Layer Chromatography of the Gymnemic Acids

Gymnemic Acid[a]	R_f Value in Solvent System[b]						
	I	II	III	IV	V	VI	VII
V	0.28	0.62	0.68	0.59	0.75	0.72	0.38
W	0.25	0.49	0.63	0.28	0.55	0.36	0.32
X	0.22	0.43	0.62	0.24	0.54	0.31	0.29
A	0.20	0.35	0.60	0.18	0.51	0.28	0.27
B	0.17	0.31	0.49	0.15	0.47	0.24	0.21
Y	0.16	0.29	0.38	0.13	0.44	0.21	0.19
Z	0.13	0.17	0.36	0.12	0.43	0.19	0.18
C	0.12	0.14	0.30	0.10	0.38	0.17	0.16
D	0.09	0.10	0.25	0.09	0.35	0.14	0.12

[a] Listed in the decreasing order of R_f values. [b] Solvent Systems: I, chloroform–formic acid–methanol (4:1:1) (aged for 3 hr. at 27°); II, chloroform–acetic acid–methanol (5:1:1); III, chloroform–formic acid–methanol–*t*-butanol (4:1:1:1) (aged for 3 hr. at 27°); IV, isopropanol–ammonium hydroxide–chloroform–*t*-butanol (5:2:1:1); V, isopropanol–ammonium hydroxide–isoamyl alcohol (3:2:1); VI, isopropanol–ammonium hydroxide–diethyl carbonate–isoamyl alcohol–*t*-butanol (3:2:2:1:1); and VII (14), butyl formate–methyl ethyl ketone–formic acid–water (5:3:1:1).

Isolation of Crude Gymnemic Acid—Dry, powdered leaves of *G. sylvestre* (260–280 g.) were wrapped in cheese cloth in bundles of about 40 g. each and were loosely packed in the side chamber of a continuous extractor. Initial defatting of leaves was carried out with 4 l. of petroleum ether (b.p. 30–60°) for 18 hr. This was followed by extraction with either water or 95% ethanol, 4 l., for a period of 18 hr. The extracts were filtered and adjusted to pH 2 with 10% HCl to precipitate Hooper's gymnemic acid (4) in a yield of 3–4% of the dry leaves. These fractions were found to consist of at least nine components upon TLC in Solvent Systems I–VII.

Warren and Pfaffmann's gymnemic acid (9), recrystallized three times from diethyl carbonate, was obtained as white microcrystals, m.p. 199–201° (dec.) [lit. (9) m.p. 199° (dec.)]. This gymnemic acid sample gave two spots during TLC in Solvent System VII.

Preliminary Separation of the Gymnemic Acids—*Acetone Extraction: Silicic-Acid Chromatography*—With the aid of a mechanical shaker, 51 g. of crude gymnemic acid mixture was extracted with 175 ml. of acetone in a glass-stoppered 250-ml. conical flask for 48 hr. The acetone extract was filtered and evaporated to dryness to yield 22.17 g. of acetone-soluble acids. No further investigation of the acetone-insoluble fraction was undertaken.

The acetone-soluble fraction was chromatographed over 450 g. of silicic acid in a glass column (34 × 1200 mm.) prepared with the aid of chloroform. The acetone-soluble fraction, plated on 50 g. of silicic acid with the help of 200 ml. of methanol, was applied to the silicic acid column by the use of chloroform for transfers. Elution was commenced with chloroform at a flow rate of 16–17 ml./hr., while collecting one fraction of 400 ml./day. Polarity of the eluting solvent was gradually increased (as shown in Table II) and all of the fractions collected were analyzed by TLC in Solvent System III.

Ethyl Acetate: Continuous Extraction Separation—In a glass mortar, 20 g. of crude gymnemic acid mixture was finely ground, dissolved in 200 ml. of methanol, and then plated on 100 g. of silicic acid. Two 50-g. portions of silicic acid plated material were first extracted with chloroform (1 l.) and then with ethyl acetate (1 l.), each for 18 hr. in two separate continuous extractors.

The combined chloroform extract, upon evaporation to dryness, yielded 4.15 g. of a green residue which was found to contain only trace amounts of gymnemic acids upon TLC in Solvent System III. However, removal of solvent from the pooled ethyl acetate extract gave 4.86 g. of a light-yellow residue which was observed to be rich in gymnemic acids A–D by TLC. This fraction was named "ethyl acetate acids."

A 4.2-g. quantity of ethyl acetate acids was chromatographed on 100 g. of silicic acid by means of a glass column (23 × 500 mm.).

[1] Melting points were taken on a Kofler hot stage and are uncorrected. Spectra were recorded on Perkin-Elmer models 137B and 337 infrared, Beckman DK-2A UV, and Varian A-60A NMR spectrometers. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. Extraction of the plant material was carried out in a Pyrex glass (Corning 3885) side-chamber continuous extraction apparatus. Adjustments of pH were followed with a Beckman Zeromatic pH meter. Centrifugation was carried out with an International Centrifuge model UV at about 2500 r.p.m. All concentrations and evaporations in this investigation were performed under reduced pressure at temperatures not exceeding 50° in either a Buchler Rotary Evapo-Mix (test tube model) or a Rinco flash evaporator.

Table II—Silicic-Acid Chromatography of Acetone-Soluble Acids

Solvent	Fractions (400 ml. each)	Weight, g.	Gymnemic Acids Detected[a]	Purification Procedure[b]	Purified[c] Gymnemic Acid Isolated	Weight, g.
Chloroform	1–6	0.54	None			
Ethyl acetate	1–2	0.09	None[d]			
	3–4	0.76	A,V,W,X	(iii)	A	0.15
					V	0.01
					W[e]	0.02
					X[e]	0.03
	5–12	3.05	A,B	(i)	A	2.40
	13–18	0.89	A,B,Y	(i)	A	0.32
					B	0.04
	19–23	0.61	B,Y,Z	(iii)	B	0.04
					Y[e]	0.03
					Z[e]	0.02
	24–36	1.10	B,C,Y,Z			
	37–49	0.47	C,D,Y,Z	(i)	C	0.02
Acetone–ethyl acetate (1:100)	1–3	0.38	C,D,Y,Z	(iii)	C	0.03
					D	0.02
					Y[e]	0.01
					Z[e]	0.01
Acetone–ethyl acetate (5:100)	1–25	1.10	C,D,Y,Z	(ii)	D	0.04

[a] Detection by TLC in Solvent System III. [b] Purification procedures: (i), adsorption chromatography on deactivated silica gel; (ii), reverse phase partition chromatography on Teflon-6; (iii), preparative TLC. [c] Homogeneous by TLC in Solvent Systems I–VII. [d] Stearic acid isolated from these fractions. [e] Isolated as amorphous solid.

The ethyl acetate fraction in 50 ml. of methanol was plated on 10 g. of silicic acid prior to its application to the column. Development of the column was initiated by chloroform followed by solvents with increasing polarity (as shown in Table III). Solvent flow rate was maintained at 10 ml./hr. and fractions (1/hr.) were collected by means of a fraction collector. All fractions collected were individually analyzed by TLC in Solvent System III.

Ethanol Extraction Method—Ethanol (95%) extraction of petroleum ether defatted leaves (105 g.) was performed as described under *Isolation of Crude Gymnemic Acid.* The filtered ethanol extract was concentrated to about 1 l., and petroleum ether was added until no more precipitation occurred (2 l.). The precipitate was collected by centrifugation which, after drying, gave 11.25 g. of a dark-green residue (17.1% of dry leaves).

The residue in 100 ml. of ethanol was plated on 50 g. of silicic acid and the dry plated material was extracted in a continuous extractor, first with chloroform (2 l.) and then with ethyl acetate (2 l.), each for 18 hr. Evaporation of the ethyl acetate extract to dryness afforded 1.93 g. of a light-green residue (1.8% of dry leaves). The presence of gymnemic acids A–D in this ethyl acetate residue was confirmed by TLC in Solvent Systems I–III, while similar TLC analysis of the original ethanol extract of leaves revealed it to contain gymnemic acids A–D and V–Z.

PURIFICATION OF THE GYMNEMIC ACIDS

Adsorption Chromatography on Deactivated Silica Gel—Samples of gymnemic acid mixtures (0.8 g. each) from the preliminary separation procedures rich in acids A, B, or C, as indicated in Tables II and III, were individually chromatographed on 125 g. of deactivated silica gel (125 g. of silicic acid blended with 25 ml. of water) in glass columns[2] (23 × 600 mm.). These columns were prepared with ethyl acetate, and gymnemic acid mixtures were applied after plating them on 1-g. quantities of deactivated silica gel with the help of 25-ml. portions of methanol. Columns were eluted with ethyl acetate at a flow rate of 10 ml./hr., and 250 fractions (5 ml. each) were collected in each case. All fractions were analyzed by TLC in Solvent System III.

Gymnemic Acid A—A total of 4.16 g. of gymnemic acid A was obtained by eight separate deactivated silica gel adsorption chromatographic separations. Acid A was found to be homogeneous by TLC in Solvent Systems I–VII. An analytical sample was prepared by four recrystallizations from ethyl acetate and was obtained as white microcrystals, m.p. 279–283° (dec.) [lit. (14) m.p. 285° (dec.)]; $\lambda_{max.}^{MeOH}$ 201 mμ (*a* 15.92); $\nu_{max.}^{KBr}$ 3450 (intermolecular H-bonded, polymeric O—H), 2990 (C—H), 1710 (C=O), 1640, 855, 810 (trisubstituted C=C), 1384 (methyl of —O—$COCH_3$), 1440 (methylene of —CH_2—O—COR), 1260 (H-bonded O—H), 1070, 1045, (C—O of C—OH), and 920 $cm.^{-1}$ (β-pyranose ring vibration) (20).

Anal.—Found: C, 63.78; H, 8.31.

Gymnemic acid A was found to be identical to gymnemic acid A_1 isolated by Reichstein's group (14) by TLC in Solvent Systems I–VII and by IR spectroscopy.

Gymnemic Acid B—Gymnemic acid B was isolated in a combined yield of 0.09 g. by deactivated silica gel chromatography of two separate fractions indicated in Tables II and III. Acid B was observed to be homogeneous by TLC in Solvent Systems I–VII. When recrystallized three times from ethyl acetate, gymnemic acid B was obtained as white microcrystals, m.p. 220–225° (dec.); $\lambda_{max.}^{MeOH}$ 201 mμ (*a* 15.42); $\nu_{max.}^{KBr}$ 3450 (O—H), 2990 (C—H), 1710 (C=O), 1625 (trisubstituted C=C), 1450 (side-chain methylene), 1375 (side-chain methyl), 1260 (H-bonded O—H), 1080 and 1040 $cm.^{-1}$ (C—O of C—OH).

Anal.—Found: C, 63.94; H, 8.53.

Gymnemic Acid C—Two separate deactivated silica gel chromatographic separations of fractions described in Tables II and III gave a total of 0.05 g. of gymnemic acid C. Acid C was found to be homogeneous by TLC in Solvent Systems I–VII. After three recrystallizations from ethyl acetate containing a few drops of methanol, acid C was obtained as white microcrystals, m.p. 215–220° (dec.); $\lambda_{max.}^{MeOH}$ 201 mμ (*a* 8.18); $\gamma_{max.}^{KBr}$ 3500 (O—H), 2980 (C—H), 1710 (C=O), 1625 (trisubstituted C=C), 1440 (side-chain methylene), 1370 (side-chain methyl), 1250 (H-bonded O—H), 1070 and 1040 $cm.^{-1}$ (C—O of C—OH).

Anal.—Found: C, 57.22; H, 7.70.

Reverse Phase Partition Chromatography on Teflon-6—Teflon-6 (100 g.), which had been left at 0° overnight, was added to 100 ml. of the organic phase of a *n*-butanol–water–methanol (10:10:1) mixture cooled to 0°. The uniform slurry formed by vigorous stirring was immediately transferred to a jacketed glass column[2] maintained at 17 ± 0.5°. The column was allowed to equilibrate by a flow of 100 ml. of the aqueous phase of the above solvent mixture at a rate of 20 ml./hr.

The sample to be chromatographed (0.5–0.8 g.) was dissolved in a minimum amount of methanol and diluted to four times its volume with the aqueous phase of the solvent mixture. This sample

[2] Columns with minimum hold-up volumes below packing and to the tip of the column were required to avoid remixing of separated components and to obtain desired resolution.

Table III—Silicic-Acid Chromatography of Ethyl Acetate Acids

Solvent	Fractions (10 ml. each)	Weight, g.	Gymnemic Acids Detected[a]	Purification Procedure[b]	Purified[c] Gymnemic Acid Isolated	Weight, g.
Ethyl acetate	1–5	0.08	None			
	6–45	1.46	A,B	(i)	A	1.15
	46–58	0.85	A,B,Y	(i)	A	0.29
					B	0.05
	59–76	0.32	B,C,Y,Z			
	77–91	0.79	C,D,Y,Z	(i)	C	0.02
Acetone–ethyl acetate (1:100)	1–10	0.41	C,D,Y,Z	(ii)	D	0.04
Acetone	1–10	0.14	C,D,Y,Z			

[a] Detection by TLC in Solvent System III. [b] Purification procedures: (i), adsorption chromatography on deactivated silica gel; (ii), reverse phase partition chromatography on Teflon-6. [c] Homogeneous by TLC in Solvent Systems I–VII.

solution was then applied to the Teflon-6 column and developed with the aqueous phase of the solvent mixture. A flow rate of 10 ml./hr. was maintained and 5-ml. fractions were collected with the aid of a fraction collector. Fractions were combined on the basis of TLC results in Solvent System III.

Gymnemic Acid D—Column RPC of the fractions rich in acid D from preliminary separation methods (see Tables II and III) yielded a total of 0.08 g. of gymnemic acid D. Acid D was found to be homogeneous by TLC in Solvent Systems I–VII. Three recrystallizations from ethyl acetate containing a few drops of methanol yielded acid D as white microcrystals, m.p. 210–220° (dec.); $\lambda_{max.}^{MeOH}$ 201 mμ (*a* 9.69); $\nu_{max.}^{KBr}$ 3440 (O—H), 2940 (C—H), 1715 (C=O), 1620 (trisubstituted C=C), 1450 (side-chain methylene), 1380 (side-chain methyl), 1265 (H-bonded O—H), 1070 and 1040 cm.$^{-1}$ (C—O of C—OH).

Anal.—Found: C, 59.90; H, 8.00.

Preparative Thin-Layer Chromatography—Preparative TLC plates[3] were activated at 100° for 15 min. prior to use. With the help of a microcapillary, 130 mg. of gymnemic acid mixture in about 0.5 ml. of methanol was applied as a streak at a distance of 2 cm. from the bottom of the plate. Chromatograms were developed in Solvent System II to a distance of 15 cm. Resolved components were located as dark bands against a fluorescent background. Separated bands were individually removed from the chromatograms by means of a razor blade and collected in separate 125-ml. conical flasks. To each flask, 50 ml. of ethyl acetate–methanol mixture (50:1) was added and allowed to stand at room temperature for 12 hr. with occasional shaking. Ethyl acetate–methanol extracts were then filtered through a fine sintered-glass funnel and solvents removed *in vacuo*.

Gymnemic acids A–D, isolated by preparative TLC (14 chromatograms) from the various fractions shown in Table II, were found to be identical to those obtained by adsorption and partition chromatographic procedures. Yields of acids A–D secured by the preparative TLC method are shown in Table II.

Gymnemic Acid V—Gymnemic acid V was isolated in a combined yield of 0.01 g. from the fractions indicated in Table II by chromatography on six preparative TLC plates. Acid V was found to be homogeneous by TLC in Solvent Systems I–VII. Recrystallization from ethyl acetate gave acid V as white microcrystals, m.p. 310–312°; $\lambda_{max.}^{EtOH}$ 201 mμ (*a* 3.38); $\nu_{max.}^{KBr}$ 3445 (O—H), 2945 (C—H), 1720 (C=O), 1640 (trisubstituted C=C), 1460 (side-chain methylene), 1385 (side-chain methyl), 1265 (H-bonded O—H), 1075 and 1045 cm.$^{-1}$ (C—O of C—OH).

Anal.—Found: C, 68.94; H, 9.68.

Gymnemic Acids W–Z—Isolation of gymnemic acids W–Z was accomplished by preparative TLC of the various fractions described in Table II; 14 plates were utilized. Acids W–Z were obtained as light-yellow, amorphous solids, and several recrystallizations from ethyl acetate–methanol failed to yield better products. However, acids W–Z were observed to be homogeneous by TLC in Solvent Systems I–VII. Table II summarizes yields of gymnemic acids W–Z. No further investigation of these acids was carried out.

[3] E. Merck AG precoated silica gel GF254 plates (thickness 2 mm.).

Isolation and Characterization of Stearic Acid—Evaporation of solvent from ethyl acetate fractions 1 and 2 obtained during silicic-acid chromatography of acetone-soluble gymnemic acids (Table II) gave a yellow, oily residue (84.6 mg.) which upon four recrystallizations from methanol deposited 14.3 mg. of white crystals, m.p. 69–70°; $\nu_{max.}^{KBr}$ 1700, 1300, 940 (carboxylic acid dimer), eight evenly spaced bands between 1320 and 1190 (16 methylene groups in long-chain *n*-alkyl fatty acid) (21), 728 and 720 cm.$^{-1}$ (methylene chain rocking).

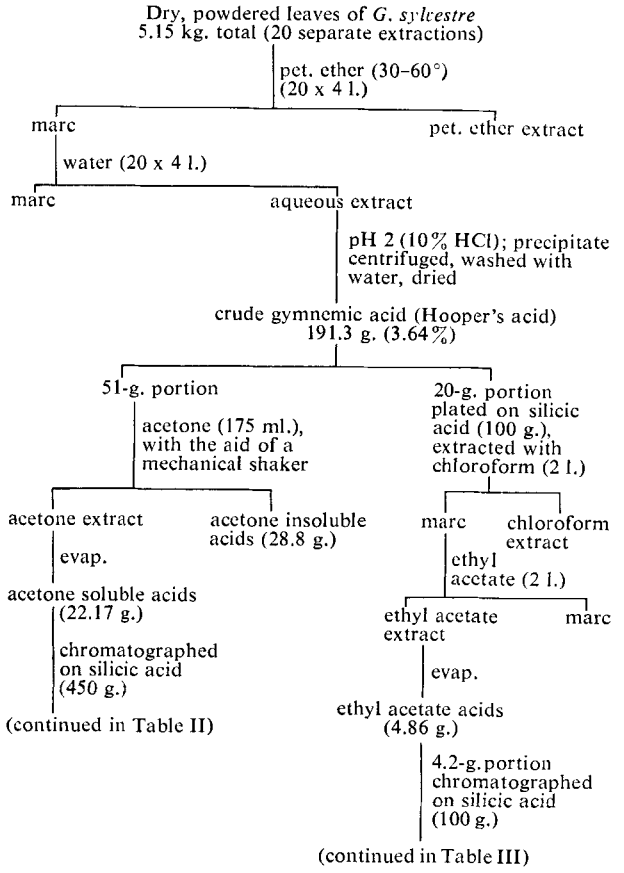
Anal.—Calcd. for $C_{18}H_{36}O_2$: C, 75.99; H, 12.76. Found: C, 75.52; H, 12.96.

An 8-mg. sample of the isolated acid was treated with methanolic boron trifluoride (22), and the reaction product was recrystallized twice from ethanol to give white crystals of methyl ester, m.p. 38–38.5°; $\nu_{max.}^{KBr}$ 1730 cm.$^{-1}$ (ester carbonyl).

The isolated acid and its methyl ester were identified as stearic acid and methyl stearate, respectively, upon comparision with reference compounds, undepressed mixed melting point, and superimposable IR spectra being the criteria for identity.

Identification of Gymnemic Acid Sugars—Thirty-milligram quantities of the gymnemic acids were refluxed with 4 ml. of 3 *N* ethanolic hydrochloric acid for 72 hr. The warm hydrolysates were treated with 5 mg. of activated charcoal,[4] filtered, and concentrated to 3-ml. volumes *in vacuo*. The concentrates were then diluted to 30 ml. with water to separate insoluble aglycone substances which were removed by centrifugation after 1 hr. of refrigeration. The supernatants, adjusted to pH 5–5.5 with 5 *N* sodium hydroxide, were evaporated to dryness, the residues extracted with 2.5 ml. of pyridine–water (4:1), and filtered from sodium chloride solids. The filtrates were evaporated to dryness and residues shaken with 10 ml. of water. The yellow aqueous extracts were filtered onto columns containing 5 ml. of an anion-exchange resin.[5] These columns were preconditioned with 10 volumes of 3 *N* hydrochloric acid and washed with water to neutrality prior to use. Column effluents and 25 ml. of water washings were collected and applied to a second column containing 5 ml. of a strongly basic ion-exchange resin.[6] Neutral sugar residues were recovered from the column effluents and 25 ml. of additional water washings by evaporation to dryness. Elution of the basic ion-exchange resin columns with 25 ml. of 1 *N* hydrochloric acid and evaporation of the effluents to dryness gave the acidic sugar fractions. Neutral and acidic sugar fractions were prepared as 2% solutions in 10% isopropanol in water for PC examination in System A (23), ethyl acetate–pyridine–water (12:5:4), and in System B (24), *n*-butanol–benzene–formic acid–water (100:19:10:25) (aged for 3 days at 27°). Detection of sugars was carried out with silver nitrate, aniline diphenylamine, aniline phthalate (23), and modified aniline hydrogen phthalate (24) reagents. Sugars in hydrolysate fractions were identified by direct comparison to reference sugar samples and to reference sugar

[4] Norit, American Norit Co.
[5] Amberlite IR 120(H^+), Rohm & Haas Co.
[6] Amberlite IRA 401(CO_3^-), prepared by converting 5 ml. of Amberlite IRA 401(Cl^-) (Rohm & Haas Co.) to the carbonate form with 50 ml. of 5% sodium carbonate.



Scheme I—Flow sheet for the isolation of gymnemic acids

samples which had been subjected to the ethanolysis procedure.

Five-milligram quantities of the gymnemic acids were also hydrolyzed by the following procedure.

The sample was suspended in 1 ml. of Kiliani mixture (acetic acid–water–hydrochloric acid, 35:55:10) (25, 26) and refluxed for 5 hr. The residue obtained after evaporation of the reaction mixture was dissolved in 1 ml. of water and extracted with chloroform (3 × 2.5 ml.). The aqueous layer was evaporated to dryness to give the sugar residue which was then chromatographed as described previously.

Characterization of Glucuronic Acid—The ethyl acetate acids fraction (3 g.) (Scheme I) was hydrolyzed and an acidic sugar fraction was isolated in a manner similar to that described for the individual gymnemic acids (ethanolysis procedure). Methyl-β-D-glucofuranosidurono-γ-lactone was prepared from both acidic sugar fraction and reference D-glucuronolactone by the following procedure.

To 108 mg. of acidic sugar fraction in 4 ml. of methanol was added 100 mg. of a cation-exchange resin.[7] The suspension was refluxed with stirring for 6 hr., clarified with 10 mg. of activated charcoal,[4] and filtered. The yellow filtrate was evaporated to dryness *in vacuo* and dried over anhydrous calcium sulfate to yield a yellow syrup (95 mg.). This syrup was plated on 0.75 g. of activated silica gel[8] and chromatographed on a column (13 × 230 mm.) of activated silica gel (10 g.). Elution was carried out with 6% methanol in benzene.

Fractions were monitored by silica gel G TLC with development by 20% methanol in benzene and detection with silver nitrate reagent (23).

Fractions containing material of R_f 0.36 were combined to give 18 mg. of a colorless syrup which was dried over anhydrous calcium sulfate under vacuum. The IR spectrum (neat) of this product exhibited an absorption band at 1790 cm.$^{-1}$ (γ-lactone) and the spectrum was superimposable with that of reference methyl D-glucuronolactone. When 10.8 mg. of the product was seeded with a trace of reference crystalline methyl D-glucuronolactone, gradual crystallization occurred. Recrystallization of the product from ethanol yielded large prisms (3 mg.), m.p. 139°. The melting point of the product was not depressed on admixture with reference methyl-β-D-glucofuranosidurono-γ-lactone and the NMR (D_2O) spectra of the two samples were identical.

RESULTS AND DISCUSSION

Isolation of the Gymnemic Acids—Early in this investigation it became apparent that gymnemic acid described in the literature was a mixture of acidic glycosides. TLC of Hooper's (4) gymnemic acid[9] indicated the presence of at least nine components which were named[10] gymnemic acids A–D and V–Z. The R_f values of these nine gymnemic acids in seven TLC systems are listed in Table I. Gymnemic acid as described by Warren and Pfaffmann (9) with its established antisweet property was found by TLC analysis to consist of acids A and B, the former being the major constituent. Indications of antiviral activity were noted for the gymnemic acid fractions rich in acid A (13).

Plant Extraction and Preliminary Separation—Based upon this information, attempts were made to secure gymnemic acid fractions rich in acid A. By following Hooper's (4) procedure, a crude gymnemic acid mixture was obtained from the aqueous extracts of petroleum ether–defatted *G. sylvestre* leaves by precipitation with 10% hydrochloric acid. Preliminary separation of the gymnemic acids was achieved by (*a*) acetone extraction: silicic-acid chromatography and (*b*) ethyl acetate: continuous extraction as summarized in Scheme I. A rapid and milder procedure involving direct ethanol extraction of defatted leaves and precipitation of the acidic glycosides with petroleum ether was also employed to confirm that previously isolated gymnemic acids represented products present in the dried leaves and not artifacts produced during the process of isolation.

Purification of the Gymnemic Acids—The gymnemic acid fractions, obtained during the preliminary separation step *via* silicic acid chromatography, were repeatedly decolorized with activated charcoal[4] in methanolic solutions until cream-white products were secured. Further purification of the gymnemic acids was carried out by following one of the three procedures: (*a*) adsorption chromatography on deactivated silica gel; (*b*) reverse phase partition chromatography (RPC) on Teflon-6; or (*c*) preparative TLC as summarized in Tables II and III.

In general, preparative TLC procedure was found to be useful for purifying all the gymnemic acids. Gymnemic acids A–D and V were obtained in crystalline form by this procedure. However, gymnemic acids W–Z could be obtained only as amorphous solids despite several recrystallizations. All nine gymnemic acids isolated by preparative TLC were shown to be homogeneous by TLC in seven solvent systems, I–VII. Table II summarizes the results obtained with preparative TLC. Adsorption chromatography on deactivated silica gel proved to be a convenient method for the rapid purification of gymnemic acid A, and to a lesser extent acids B and C, while RPC on Teflon-6 was valuable for obtaining pure acid D. The Teflon-6 RPC was modified from the procedure of Fritz and Hendrick (27) and found to be superior to the silanized diatomaceous earth[11] RPC for retaining the stationary phase with prolonged use.

Final purification of the isolated gymnemic acids was carried out by several recrystallizations from either ethyl acetate alone or ethyl acetate containing a few drops of methanol.

Preliminary Characterization of the Gymnemic Acids—This report is limited to preliminary characterization of the major gymnemic acids A–D. Initial characterization work was performed

[7] Dowex 50(H^+), Dow Chemical Co.

[8] Silicic acid, 100 mesh (Mallinckrodt), was activated at 105° for 24 hr.

[9] Equivalent results were obtained with gymnemic acid commercially available from K & K Laboratories, Inc.

[10] This nomenclature is based upon the order of elution of the components during silicic-acid column chromatography and their abundance in the crude acid mixture. Thus, the four major constituents were named gymnemic acids A–D, acid A being the first major component to elute from the column. Similarly, the minor constituents were named gymnemic acids V–Z, the sequence in which they eluted during chromatographic separation.

[11] Celite 545, Johns-Manville.

Table IV—Sugar Content of the Gymnemic Acids

Sample	Treatment[a]	Glucostat Test	Sugars Detected[b] by Paper Chromatography
Ethyl acetate fraction	Ethanolysis	Positive	Arabinose Glucose Glucuronic acid Glucuronolactone
Gymnemic acid A	Ethanolysis, hydrolysis	Negative	Glucuronic acid Glucuronolactone
Gymnemic acid B	Ethanolysis, hydrolysis	Negative	Glucuronic acid Glucuronolactone
Gymnemic acid C	Ethanolysis, hydrolysis	Positive	Glucose Glucuronic acid Glucuronolactone
Gymnemic acid D	Ethanolysis, hydrolysis	Positive	Glucose Glucuronic acid Glucuronolactone

[a] Ethanolysis was carried out by refluxing with 3 *N* ethanolic HCl for 72 hr. and hydrolysis by refluxing with Kiliani mixture (25) for 5 hr. [b] Colors produced by sugars after treating with modified aniline hydrogen phthalate reagent (24) and heating for 3 min. at 105° were visible (UV): arabinose, red (reddish brown); glucose, green (brown); glucuronic acid and glucuronolactone, brown (brown).

with acid A and with the ethyl acetate acids fraction which represented an enrichment of acids A–D. That the remaining gymnemic acids were closely related to acid A was evident by their various physical properties. Of particular significance was their IR spectra which were remarkably similar except for minor variations in the intensity of absorption peaks.

Glycosidic Properties of the Gymnemic Acids—With the aid of anthrone (28) and chromotropic acid (29) tests, reducing sugars were detected in hydrochloric acid hydrolysates of the gymnemic acids. Neutralized and desalted sugar fractions were then separated into neutral and acidic components. Neutral sugars were collected as effluents from a strongly basic ion-exchange resin in the carbonate form (30). Elution of the anionic resin with hydrochloric acid released sugar acids.

The conditions employed for acidic hydrolysis of the gymnemic acids were dependent upon the subsequent use of the hydrolysates. Thus, while hydrolysis in ethanolic hydrochloric acid was found to be faster and less destructive, it led to formation of ethyl glycosides of the sugars. This limited the detection of free reducing sugars and also gave rise to artifacts during chromatographic analysis. Hydrolyses with the Kiliani mixture (25, 26) were performed as a supplement to ethanolysis studies which assured that the sugars released were in the free reducing state. This procedure had value as a direct route for the identification of sugars. However, the harsh conditions of the Kiliani method led to degradation of both sugar and aglycone moieties, limiting its use in sugar isolations.

All sugar fractions were examined by PC and compared to suspected reference sugars which had been subjected to the similar hydrolysis procedures. Chromogens used were: silver nitrate as a nonspecific but sensitive reagent, aniline diphenylamine for its characteristic color variations with sugars, aniline phthalate for its specificity for reducing sugars, and modified aniline hydrogen phthalate for its specificity as well as distinct color production with reducing sugars. The presence of glucose in the neutral sugar fractions was confirmed by the Glucostat test,[12] a specific enzymatic procedure for β-D-glucose.

The procedures applied for the detection of sugars in various gymnemic acid fractions and their results are summarized in Table IV. Glucuronic acid was found in the hydrolysates of gymnemic acids A–D. Identification of glucuronic acid by PC was aided by the presence of an additional highly mobile spot due to glucuronolactone, which arises during hydrolysis as an equilibrium product of glucuronic acid (31). Glucose was observed to be the only neutral sugar constituent in the hydrolysates of gymnemic acids C and D.

Acidic hydrolysis of the ethyl acetate parent fraction gave rise to arabinose in addition to glucose, glucuronic acid, and glucuronolactone. In order to substantiate that arabinose did originate from the ethyl acetate fraction, a mixture of reference glucose and glucuronic acid was subjected to identical acid hydrolysis, and neutral and acidic sugar fractions were isolated as in the case of gymnemic acid sugars. Analysis of the reference neutral sugar fraction by PC failed to detect any trace of arabinose. Since gymnemic acids A–D do not contain arabinose, its origin is assigned to at least one of the minor gymnemic acids. Pfaffmann (10) reported the detection of arabinose along with glucose and glucuronolactone from the hydrolysate of his sample of gymnemic acid which has subsequently been shown to be a mixture (14).

The ethyl acetate parent fraction was also utilized in the present study as a source for the isolation of glucuronic acid to confirm the PC results. Isolation was in the form of methyl-β-D-glucofurano-sidurono-γ-lactone by the method of Osman *et al.* (32). The isolated compound was identical with the methyl glycoside of reference glucuronolactone by undepressed mixed melting point, chromatography, IR, and NMR analyses.

Positive color reactions with Liebermann-Burchard (19) and benzoyl chloride–sulfuric acid (17) reagents suggested gymnemic acids A–D to be glycosides of either steroid or triterpenoid alcohols. Further, the strong hydroxyl absorption around 3500 $cm.^{-1}$ and the characteristic bands in the region 700–1200 $cm.^{-1}$ (33) in their IR spectra, and a high percentage of oxygen content (about 30%) as indicated by elemental analyses, were consistent with the glycosidic character of the gymnemic acids.

Comparison of Gymnemic Acids Isolated by Various Investigators—While the current investigation was in progress, Stöcklin *et al.* (14) reported their isolation of the antisweet principle of *G. sylvestre* leaves, which they named gymnemic acid A_1. This isolation in a yield of about 0.004% was achieved by a complex procedure involving several treatments with both acid and base.

Reichstein's group called their parent fraction, from which gymnemic acid A_1 was secured, gymnemic acid A. Fraction A showed a single spot during TLC in four solvent systems.[13] However, in Solvent System VII, their gymnemic acid A was found to be a mixture composed of acids A_1, A_2, A_3, and A_4. Gymnemic acids A_1 and A_2 were shown to be identical to the two spots produced by Warren and Pfaffmanns' gymnemic acid in the same TLC system. Acid A_1 is the major constituent in both the fractions isolated by the Swiss group and by Warren and Pfaffmann.

Direct TLC comparison of gymnemic acids A–D, isolated in the present investigation, with acids A_1–A_4 obtained by Reichstein's group established that in TLC Systems I–VII acids A and B were identical to acids A_1 and A_2. However, acids C and D did not correspond to acids A_3 and A_4.

Stöcklin *et al.* (14) felt that gymnemic acid A_1 was the native antisweet principle of *G. sylvestre* leaves. Because of the harsh conditions employed in the isolation procedure, there existed the possibility of their acids A_2 and A_3 being artifacts originating from acid A_1. In this study, the ethanol extraction and petroleum ether precipitation method is presented as evidence for the natural occurrence of gym-

[12] Glucostat test was performed according to Method 1-A described in the literature accompanying the reagent kit (Worthington Biochemical Corp.).

[13] Adsorbent, silica gel G; solvent systems: ethanol–water–ammonium hydroxide (18:2:1), isopropanol–water–ammonium hydroxide (9:1:1), chloroform–methanol–ammonium hydroxide (5:1:1), and *n*-butanol–water–acetic acid (10:1:1).

nemic acids A–D. This fact, coupled with the nonidentity of gymnemic acids A_3 and A_4 with acids C and D from these laboratories or any of the minor acids, suggests that acids A_3 and A_4 are degradation products.

Further, gymnemic acid A (A_1–A_4) was shown by the Swiss group to contain glucuronic acid as the sole sugar residue. This is in agreement with the authors' gymnemic acids A and B which also contain only glucuronic acid. In contrast, presence of both glucuronic acid and glucose in gymnemic acids C and D from this study has been established.

Structural elucidation of gymnemic acids A–D will be the subject of forthcoming papers from the authors.

REFERENCES

(1) R. M. Chopra, "Indigenous Drugs of India," 2nd ed., Art Press, Calcutta, India, 1958, p. 336.

(2) K. S. Mhaskar and J. F. Caius, *Indian Med. Res. Mem.*, **16,** 1(1930).

(3) Falconer, *Pharm. J. Trans.*, **VII,** 351(1847).

(4) D. Hooper, *Pharm. J.*, **17,** 867(1886–1887); through *Chem. Zentrlbl.*, **1887,** 800.

(5) D. Hooper, *Chem. News*, **59,** 159(1889).

(6) L. E. Shore, *J. Physiol.*, **13,** 191(1892).

(7) F. B. Power and F. Tutin, *Pharm. J.*, **19,** 234(1904).

(8) H. N. Khastgir, S. K. Sengupta, and P. Sengupta, *J. Indian Chem. Soc.*, **35,** 650(1958).

(9) R. M. Warren and C. Pfaffmann, *J. Appl. Physiol.*, **14,** 40 (1959).

(10) C. Pfaffmann, "Handbook of Physiology. Sec. 1: Neurophysiology," vol. 1, American Physiological Society, Washington, D. C., 1959, p. 507.

(11) K. S. Yackzan, Ph.D. thesis, University of Alabama, 1964; through *Diss. Abstr.*, **25,** 7354(1965).

(12) K. S. Yackzan, *Alabama J. Med. Sci.*, **3,** 1(1966).

(13) J. E. Sinsheimer, G. S. Rao, H. M. McIlhenny, R. V. Smith, H. F. Maassab, and K. W. Cochran, *Experientia*, **24,** 302(1968).

(14) W. Stöcklin, E. Weiss, and T. Reichstein, *Helv. Chim. Acta*, **50,** 474(1967).

(15) W. Stöcklin, *ibid.*, **50,** 491(1967)

(16) P. E. Manni and J. E. Sinsheimer, *J. Pharm. Sci.*, **54,** 1541 (1965).

(17) T. Simano and K. Taki, *Gifu Yakka Daigaku Kiyo*, **8,** 24(1958); through *Chem. Abstr.*, **53,** 9572*e*(1959).

(18) D. Waldi, in "Thin-Layer Chromatography," E. Stahl, Ed., Springer-Verlag, Berlin, Germany, 1965, p. 487.

(19) I. Belic, *Nature*, **178,** 538(1956).

(20) K. Nakanishi, "Infrared Absorption Spectroscopy—Practical," Holden-Day, San Francisco, Calif., 1962, p. 34.

(21) *Ibid.*, p. 178.

(22) L. D. Metcalfe and A. A. Schmitz, *Anal. Chem.*, **33,** 363 (1961).

(23) I. Smith, "Chromatographic and Electrophoretic Techniques," vol. 1, Interscience, New York, N. Y., 1960, p. 246.

(24) T. Fuleki and F. J. Francis, *J. Chromatogr.*, **26,** 404(1967).

(25) H. Kiliani, *Ber.*, **63,** 2866(1930).

(26) E. Abisch and T. Reichstein, *Helv. Chim. Acta*, **43,** 1844 (1960).

(27) J. S. Fritz and C. E. Hendrick, *Anal. Chem.*, **37,** 1015 (1965).

(28) N. D. Cheronis and J. B. Etrikin, "Identification of Organic Compounds," Interscience, New York, N. Y., 1963, p. 134.

(29) B. Klein and M. Weissman, *Anal. Chem.*, **25,** 771(1953).

(30) J. D. Phillips and A. Pollard, *Nature*, **171,** 41(1953).

(31) R. J. Morris and E. W. Gussey, *J. Org. Chem.*, **30,** 166 (1965).

(32) E. M. Osman, K. C. Hobbs, and W. E. Walston, *J. Amer. Chem. Soc.*, **73,** 2726(1951).

(33) K. Nakanishi, "Infrared Absorption Spectroscopy—Practical," Holden-Day, San Francisco, Calif., 1962, p. 150.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 2, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication November 17, 1969.

Abstracted from dissertations submitted by Hugh M. McIlhenny and G. Subba Rao to the Horace H. Rackham School of Graduate Studies, University of Michigan, in partial fulfillment of Doctor of Philosophy degree requirements.

This work was supported in part by Grant AM 06224 from the National Institute of Arthritis and Metabolic Diseases, and Training Grant GM 1367, U. S. Public Health Service, Bethesda, Md.

The authors thank Professor T. Reichstein and Dr. Walter Stöcklin, University of Basel, Basel, Switzerland, for reference samples of their gymnemic acid and gratefully acknowledge the assistance of Dr. Robert V. Smith who carried out preliminary studies.

* Lilly Foundation Fellow. Present address: National Heart Institute, Bethesda, Md.

† American Foundation for Pharmaceutical Education Fellow. Present address: Chas. Pfizer & Co., Inc., Groton, Conn.

Constituents from *Gymnema sylvestre* Leaves VI: Acylated Genins of the Gymnemic Acids—Isolation and Preliminary Characterization

JOSEPH E. SINSHEIMER and G. SUBBA RAO*

Abstract □ With the aid of a selective enzyme system, genins G, K, N, and gymnestrogenin were isolated and shown to be the aglycones of gymnemic acids A–D, respectively. Genin G was found to be an acylated derivative of gymnemagenin containing formic, acetic, isovaleric, and tiglic acids, while genin K differed from G by the absence of the acetic acid residue. Genin N was observed to be gymnestrogenin tiglate. Genin J, probably an artifact originating from genin G, was also isolated and indicated to be gymnemagenin esterified with acetic, isovaleric, and tiglic acids. The sugar moieties of acids A and B are not acylated, while those of acids C and D are indicated to be esterified with ferulic acid.

Keyphrases □ *Gymnema sylvestre* leaves—acylated genins, isolation □ Genins, acylated—*G. sylvestre* gymnemic acids □ Paper chromatography—identity □ TLC—identity □ IR spectrophotometry—structure □ GLC—identity

In the preceding paper (1), the authors described the isolation and preliminary characterization of the major constituents, acids A–D, of gymnemic acid, the anti-sweet principle of *Gymnema sylvestre* R. Br. (*Asclepiadaceae*) leaves. Gymnemic acids A–D were shown to be acidic glycosides containing glucuronic acid as the sugar component, while acids C and D were also observed to possess glucose. This paper deals with further developments in the structural elucidation of these gymnemic acids.

Acidic or enzymatic hydrolysis of individual gymnemic acids yielded genin mixtures consistent with the presence of acylated genins in the parent glycosides. Characterization of neutral components, obtained after acidic and basic hydrolyses of individual acids A–D, was carried out to establish their genin structure after complete deacylation. Identification of carboxylic acids in the basic hydrolysates of gymnemic acids A–D was then performed to characterize further each gymnemic acid. A selective enzyme procedure, recently developed by Kapadia (2), was utilized to secure the intact acylated genins of the gymnemic acids which were then available for structural investigation. A comparative study of the acylated genins obtained in this investigation with those isolated by Stöcklin *et al.* (3) was also conducted.

EXPERIMENTAL[1]

Reagents—Firebrick (Gas-Chrom RA, 80/100 mesh) and neopentylglycolsuccinate, regular (HI-EFF) (both from Applied Science Labs.); β-glucuronidase aryl sulfatase from *Helix pomatia*, B grade, 160,000 Fishman units/ml. (Calbiochem); and a commercial insecticide ("Real-Kill," Real-Kill Products, Division of Cook Chemical Co., Kansas City, Mo.).

Paper Chromatography—Hydroxamic derivatives (4) (from 10 mcg. of reference carboxylic acids and 100–500 mcg. of sample fractions) were spotted on Whatman No. 1 paper (57 × 19.5 cm.) and saturated for 16 hr. with the aqueous phase of solvent systems employed for development. Chromatograms were developed by descending technique to a distance of 48 cm. For analysis of hydroxamic derivatives of carboxylic acids, Solvent System A, *n*-butanol–dimethyl formamide–water (9:1:1), and Solvent System B, *n*-butanol–acetic acid–water (4:1:5) (4), were employed. Ferric chloride spray reagent was used to visualize hydroxamic acids on chromatograms (4).

Thin-Layer Chromatography—Silica gel G TLC of the genins was performed as in the case of the gymnemic acids (1). Solvent Systems I–IV are described in Table I. Ceric sulfate–sulfuric acid reagent (6) was used as the chromogen.

Identification of ferulic acid by silica gel G TLC was carried out in the following solvent systems: V, ethanol–water–ammonium hydroxide (90:25:4); VI, benzene–dioxane–acetic acid (90:25:4); and VII, *n*-butyl ether (water-saturated)–acetic acid (10:1) (7). Detection of ferulic acid on chromatograms was by spraying with diazotized sulfanilic acid reagent (8).

Gas-Liquid Chromatography—Preparation of a stationary phase of firebrick (Gas-Chrom RA, 80/100 mesh) treated with 10% neopentylglycolsuccinate and 2% phosphoric acid, and packing of columns [1.52 m. × 0.32 cm. (5 ft. × 0.125 in.) copper] were carried out as described in the literature (9–11). Columns were conditioned for 12 hr. as follows: oven temperature, 195°; detector temperature, 200°; injection port temperature, 200°, and carrier gas, helium, at 40 p.s.i. Carboxylic acids (sample size: reference acids, 15 mcg.; sample fraction, ~200 mcg.) were chromatographed under the following conditions: oven temperature, 125°; detector temperature, 210°; injection port temperature, 200°; carrier gas, helium, 60 ml./min. (80 p.s.i.); hydrogen, 50 ml./min. (16 p.s.i.); air, 380 ml./min. (16 p.s.i.); attenuation, 1; and range, 50.

Acidic Hydrolysis—Thirty-milligram quantities of the gymnemic acids were refluxed with 4 ml. of 3 *N* ethanolic hydrochloric acid for 72 hr. The warm hydrolysates were treated with 5 mg. of activated charcoal[2] and concentrated to 3 ml. The concentrates were diluted with water to precipitate genins which were collected by centrifugation after 1 hr. of refrigeration. After washing with water twice, genin precipitates were dried to yield white residues weighing 12–15 mg. TLC Systems I–III (Table I) were employed to analyze these genin residues.

Basic Hydrolysis and Identification of Carboxylic Acids—Basic hydrolysis of gymnemic acids was performed by refluxing 20-mg. samples in 5 ml. of 5% methanolic potassium hydroxide solution for 2 hr. Cooled reaction mixtures were poured into 10-ml. portions of water and precipitated genins were filtered off. After removal of methanol from alkaline filtrates *in vacuo*, they were acidified with 2 *N* sulfuric acid and extracted with ether (6 × 10 ml.). Ether extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness *in vacuo*. Yellow oily residues weighing 4–7 mg. were obtained in each case. These residues were dissolved in minimal amount (~0.1 ml.) of either ether or methanol, and 3–6 μl. of these fractions were analyzed for the presence of free carboxylic acids by GLC. Table II summarizes GLC results. Identification of ferulic acid was carried out by TLC in Solvent Systems V–VII.

Methyl esters of carboxylic acids present in these described fractions were prepared by treatment with diazomethane (12) and

[1] Melting points were recorded on a Kofler hot stage and are uncorrected. IR spectra were taken on Perkin-Elmer spectrophotometer models 137B and 337. F&M model 700 gas chromatograph was employed for GLC of carboxylic acids. Enzymatic hydrolyses were carried out in either a Precision constant-temperature water bath or a Precision incubator maintained at the desired temperature. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

[2] Norit, American Norit Co.

Table I—Thin-Layer Chromatography of the Genins

Genin	R_f Value in Solvent System[a] I	II	III	IV
G	0.64	0.63	0.70	0.73
J	0.55	0.49	0.62	0.67
K	0.47	0.38	0.54	0.63
N	0.39	0.35	0.50	0.61
Gymnestrogenin	0.32	0.16	0.44	0.56
Gymnemagenin	0.23	0.10	0.27	0.40

[a] Solvent Systems: I, benzene–methanol–acetic acid (45:8:4) (5); II, chloroform–methanol (9:1) (3); III, benzene–chloroform–methanol (5:8:3); and IV, benzene–methanol (7:3).

Table II—Gas-Liquid Chromatography of the Carboxylic Acids from the Gymnemic Acids

Acid	R_t, min.[a]
Formic, acetic[b]	1.57
Propionic	2.51
Isobutyric	3.06
n-Butyric	4.15
Isovaleric	4.93
Tiglic	10.81
Unidentified	20.05
Unidentified	21.54

[a] Relative to solvent front. Ferulic acid could not be chromatographed under the conditions employed. [b] Identification of these two acids was accomplished by paper chromatography of their hydroxamic acid derivatives (3, 4).

then converted to hydroxamic acids by reacting with hydroxylamine according to the procedure of Bayer and Reuther (4). These hydroxamic acids were analyzed by PC in Solvent Systems A and B for the presence of formic and acetic acid derivatives.

Results of these chromatographic identifications of carboxylic acids in the hydrolysates of various gymnemic acids are given in Table III.

Isolation of Genins—*Gymnemagenin*—Gymnemic acid A (30 mg.) was refluxed with 5 ml. of 8% sulfuric acid in 50% aqueous ethanol for 6 hr. The reaction mixture was then concentrated to about 1 ml. *in vacuo* and poured into 20 ml. of ice water. The precipitated material was collected by centrifugation, dried, and refluxed with 5 ml. of 5% methanolic potassium hydroxide for 6 hr. Concentration of the reaction mixture to about 1 ml. and pouring into 20 ml. of ice water yielded 18.1 mg. of a white residue. Recrystallization of this residue from chloroform–methanol gave gymnemagenin as white needles, m.p. 329.5–331° [lit. (3) m.p. 328–335°]; $\nu_{max.}^{KBr}$ 3450 (O—H), 2900 (C—H), 1635 (trisubstituted C=C), 1250 (H-bonded O—H), 1070 and 1040 cm.$^{-1}$ (C—O or C—OH).

Anal.—Calcd. for $C_{30}H_{50}O_6$: C, 71.11; H, 9.95. Found: C, 70.95; H, 9.91.

Identity of gymnemagenin with that isolated by Reichstein's group (3) was established by undepressed mixed melting point, superimposable IR spectra, and TLC in Solvent Systems I–IV.

Gymnestrogenin—To a suspension of 10 mg. of gymnemic acid D in 10 ml. of water, 0.1 ml. of β-glucuronidase (*Helix pomatia*) preparation and 2 drops of insecticide (Real-Kill) were added and kept at 40 ± 1° for 5 days. The enzyme digest was then boiled for 1 min., cooled, diluted with 50 ml. of ethanol, and filtered through a bed of diatomaceous earth.[3] The filtrate was evaporated *in vacuo* and the residue obtained was extracted with chloroform (4 × 10 ml.). Pooled chloroform extracts, upon removal of solvent, gave 2.9 mg. of a white residue which was recrystallized from chloroform–methanol to yield fine needles of gymnestrogenin, m.p. 286–288° [lit. (13) m.p. 288–289°]; $\nu_{max.}^{KBr}$ 3470 (O—H), 2945 (C—H), 1635 (trisubstituted C=C), 1070 and 1040 cm.$^{-1}$ (C—OH).

Gymnestrogenin was observed to be homogeneous by TLC in Solvent Systems I–IV and identical to a reference sample (13) by TLC, undepressed mixed melting point, and superimposable IR spectra.

Genin G—To a suspension of 100 mg. of gymnemic acid A in 50 ml. of water, 0.2 ml. of β-glucuronidase (*Helix pomatia*) and 5 drops of insecticide were added and kept at 40 ± 1° for 5 days. Isolation of 34.6 mg. (34.6%) of hydrolysis product was carried out as in the case of gymnestrogenin. This product was observed to be homogeneous by TLC in Solvent Systems I–IV. Recrystallization of the hydrolysis product from petroleum ether–chloroform gave genin G as shiny white crystals, m.p. 164–166°; $\nu_{max.}^{KBr}$ 3450 (O—H), 2960 (C—H), 1740 (C=O), 1460 and 1385 (side-chain methylene and methyl of $-CH_2-O-COCH_3$, respectively), 1260 (H-bonded O—H), 1080 and 1040 cm.$^{-1}$ (C—OH).

Anal.—Calcd. for $C_{43}H_{66}O_{11}$: C, 69.51; H, 8.95. Found: C, 69.52; H, 9.11.

Genin J—A 450-mg. sample of the parent gymnemic acid mixture isolated by the ethyl acetate procedure (1) was dissolved in 150 ml. of 0.01 *N* potassium bicarbonate, 1 ml. of β-glucuronidase (*Helix pomatia*) preparation added, and kept at 37 ± 0.5° for 5 days (3). The enzyme digest was then diluted with 750 ml. of ethanol, filtered through a bed of diatomaceous earth and the filtrate evaporated *in vacuo* to yield 398.7 mg. of a pale-yellow residue. This residue was found by TLC (Table I) to be a mixture composed of genins G, J, K, gymnestrogenin, and gymnemagenin.

A 2.6-mg. quantity of genin J was isolated from the above genin mixture by preparative TLC[4] using Solvent System II for development. Recrystallization from petroleum ether–chloroform yielded genin J as white crystals, m.p. 195–196° [lit. (3) m.p. 193–196°]; $\nu_{max.}^{KBr}$ 3450 (O—H), 2960 (C—H), 1740 (C=O), 1460 (side-chain methylene), 1390 (side-chain methyl), 1260 (H-bonded O—H), 1080 and 1040 cm.$^{-1}$ (C—OH). Genin J was found to be homogeneous by TLC in Solvent Systems I–IV.

Genin K—To a suspension of 20 mg. of gymnemic acid B in 10 ml. of water, 0.1 ml. of β-glucuronidase (*Helix pomatia*) and 2 drops of insecticide were added and kept at 40 ± 1° for 5 days. Isolation of genin K from the hydrolysate was carried out as described for gymnestrogenin. A yield of 6.1 mg. of genin K was obtained. Recrystallization from petroleum ether–chloroform gave genin K as white crystals, m.p. 146–148°; $\nu_{max.}^{KBr}$ 3450 (O—H), 2950 (C—H), 1710 (C=O), 1455 and 1390 (side-chain methylene and methyl, respectively), 1265 (H-bonded O—H), 1080 and 1045 cm.$^{-1}$ (C—OH). Genin K was observed to be homogeneous by TLC in Solvent Systems I–IV.

Genin N—A 10-mg. quantity of gymnemic acid C was subjected to enzymatic hydrolysis with the selective β-glucuronidase (*Helix pomatia*) preparation as described in the isolation of genin K. The hydrolysis product (3.6 mg.) was found to consist of genin N with trace amounts of genins G and K, based upon TLC in Solvent Systems I–IV.

RESULTS AND DISCUSSION

Detection of the Acylated Genins—Stöcklin *et al.* (3) observed that hydrolysis of their gymnemic acid A mixture[5] by the snail (*Helix pomatia*) β-glucuronidase yielded a genin mixture composed of genins G, J, K, and gymnemagenin. A direct TLC comparison of this genin mixture with that obtained during similar enzymatic hydrolysis of gymnemic acid A from these studies established the two genin mixtures to consist of identical components. Also, TLC analysis of nonsugar fractions secured by acidic hydrolysis of gymnemic acids A–D from these studies revealed them to be mixtures of closely related genins.

The production of genin mixtures during acidic hydrolysis of the gymnemic acids is consistent with the reported (3) presence of acyl groups which would undergo partial hydrolysis in acid medium (14). Indeed, when the genin mixture resulting from gymnemic acid A was refluxed with 5% methanolic potassium hydroxide solution, it yielded a single compound, gymnemagenin,[6] in a completion of

[3] Celite 535, Johns-Manville Co.

[4] E. Merck silica gel GF 254 preparative plates (thickness 2 mm.).

[5] The Reichstein group described this mixture to be composed of acids A_1–A_4, of which acids A_1 and A_2 were found to be identical to the authors' acids A and B (1).

[6] Gymnemagenin was found to be identical to a reference sample isolated by Reichstein's group (3) by TLC, undepressed mixed melting point, and superimposable IR spectra. Structure I has been recently assigned for gymnemagenin (15, 16).

Table III—Constituents of Gymnemic Acids A–D

Gymnemic Acid	Sugar	Acylated Aglycone	Parent Genin	Sugar Residue	Carboxylic Acids[a] in Aglycone: Formic	Acetic	Isovaleric	Tiglic
A	Glucuronic acid	G	Gymnemagenin	None	+	+	+	+
		J	Gymnemagenin	—		+	+	+
B	Glucuronic acid	K	Gymnemagenin	None	+		+	+
C	Glucuronic acid Glucose	N	Gymnestrogenin	Ferulic[b]				+[b]
D	Glucuronic acid Glucose	—	Gymnestrogenin	Ferulic				

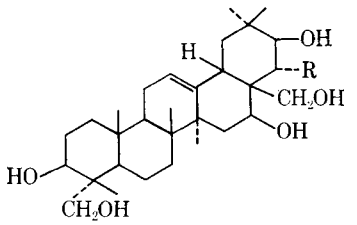
[a] Results obtained from alkaline hydrolysis of genins G, J, and K have been supported by preliminary NMR and mass spectrometric studies (26). [b] This distribution of ferulic and tiglic acid is deduced by comparison to gymnemic acid C and not by direct identification of the acids in the hydrolysate of genin N.

the hydrolysis. Alkaline hydrolysis of the genin mixture obtained by the Swiss investigators also gave them gymnemagenin. Similarly, acidic and basic hydrolyses of gymnemic acid B afforded gymnemagenin, while acids C and D upon identical treatment produced gymnestrogenin.[7] Chromatographic examination of alkaline hydrolysates of the gymnemic acids confirmed the presence of carboxylic acids, thus establishing these glycosides to be acylated derivatives.

Identification of the Carboxylic Acids—From the hydrolysate obtained after enzymatic and alkaline hydrolyses of their gymnemic acid A_1–A_4 mixture, Reichstein's group (3) identified formic, acetic, *n*-butyric, isovaleric, and tiglic acids by GLC and PC. Gymnemic acid A (the present authors' designation), when subjected to a similar detection procedure, revealed the presence of formic, acetic, isovaleric, and tiglic acids in the basic hydrolysate, while gymnemic acid B yielded all these acids except acetic acid. Ferulic and tiglic acids were found in the alkaline hydrolysate of gymnemic acid C while acid D yielded only ferulic acid. The basic hydrolysate of the ethyl acetate parent mixture (1) of gymnemic acids A–D was also examined by GLC and PC. In addition to those compounds given above as arising from acids A–D, trace amounts of *n*-butyric (as noted by the Swiss investigators), isobutyric, propionic, and two unidentified acids were detected (Table II)

Isolation of the Acylated Genins—During enzymatic hydrolysis of their gymnemic acid A mixture, Stöcklin *et al.* (3) found that cleavage of acyl groups present in these glycosides had occurred along with liberation of glucuronic acid. These investigators indicated that this cleavage of acyl groups could be due to either saponification under the alkaline condition (0.01 *N* potassium bicarbonate) employed or to the action of esterase enzymes known to be present in snail β-glucuronidase preparations (3, 17–19). In the present investigation, hydrolysis of individual gymnemic acids by similar enzyme preparation[8] in neutral solutions also cleaved acyl groups along with glycosidic linkages. This suggested esterase activity of the β-glucuronidase preparation to be responsible for the hydrolysis of acyl groups.

During a structural elucidation of the cardenolide glycoside, acospectoside A, Kapadia (2) recently developed a procedure for a selective enzymatic hydrolysis of the glycosidic linkage in the presence of an acyl group. This was accomplished by inhibiting esterase activity of the enzyme preparation through addition of a few drops of the insecticide which contains an esterase inhibitor, *O,O*-dimethyl-*O*-(2,2-dichlorovinyl) phosphate (DDVP) (25). In the present investigation, a similar selective enzyme system made from the commercial β-glucuronidase (*Helix pomatia*) preparation was also found to cleave selectively glycosidic linkages in the gymnemic acids.



I, R = OH
II, R = H

By use of this selective enzyme preparation, gymnemic acids A, B, and D were observed to produce single genins G, K, and gymnestrogenin, respectively, in neutral solutions at 40 ± 1°. However, if hydrolysis was allowed to proceed beyond 6 days, degradation products of the initially formed genins also appeared. Accordingly, crystalline genins G, K, and gymnestrogenin were isolated from the corresponding enzyme digests by allowing hydrolysis to proceed for 5 days. Similar hydrolysis of gymnemic acid C yielded a new genin, N,[9] as a major product with trace amounts of genins G and K. The latter two genins can be assigned to traces of acids A and B originally present in the gymnemic acid C sample employed. These enzymatic hydrolyses were followed by TLC in Solvent System II.

Stöcklin *et al.* (3) attempted to isolate the acylated genins from the snail enzyme hydrolysate of their gymnemic acid A mixture by silicic-acid chromatography. This resulted in the isolation of genin J in crystalline form, m.p. 193–196°, while genins G and K were obtained as amorphous solids. However, the latter two genins yielded crystalline acetate derivatives.

Due to its apparent structural similarity and potential utility in the structure proof of the acylated genins, genin J was also isolated in this investigation. This was accomplished by preparative TLC from the genin mixture obtained by nonselective enzymatic hydrolysis of the parent mixture of acids obtained by the ethyl acetate procedure (1).

Characterization of Acyl Functions—Reichstein's group (3), based upon elemental analysis and the identification of carboxylic acids in their gymnemic acid mixture, postulated gymnemic acid A_1

[7] Gymnestrogenin was observed to be identical to a reference sample isolated by Stöcklin (13) directly from the leaves of *G. sylvestre* and he has also designated Structure II to this compound.

[8] The β-glucuronidase preparation from the snail, *Helix pomatia*, has been demonstrated to effect the cleavage of β-linked D-glucose (20) as well as D-glucuronic acid (21) present in several steroid and triterpenoid glycosides. In addition, this snail enzyme preparation has been found to cleave D-glucose residues from various glycosides that were resistant to similar hydrolysis by other enzymes known to cleave β-linked D-glucose, *e.g.*, strophanthobiase (20, 22, 23). It may be mentioned here that gymnemic acids A–D could not be hydrolyzed by either β-glucuronidase (bovine liver) or β-glucosidase (almond emulsion) preparations (24).

[9] Naming of the new aglycone, genin N, is a continuation of Stöcklin's nomenclature (13).

(the present authors' acid A), $C_{49}H_{74}O_{16}$, to be the glucuronide of gymnemagenin containing 1 mole of formic, acetic, isovaleric, and tiglic acids through ester linkages. Similarly, genin J, $C_{40}H_{64}O_8$, was hypothesized to be mono-*o*-isovaleryl-mono-*o*-tiglyl-gymnemagenin. Except for the IR spectra, no further work on genins G, J, K, and their acetates has been reported by Reichstein's group.

The present authors' experiments utilizing the selective enzyme preparation clearly established genins G, K, N, and gymnestrogenin to be the aglycones of gymnemic acids A, B, C, and D, respectively. Basic hydrolysis of genins G, J, and K showed them to be acylated derivatives of gymnemagenin, while similar hydrolysis of genin N proved it to be an acylated derivative of gymnestrogenin. These relationships are summarized in Table III. It may be noted that genin J differs from genin G only by absence of a formyl group. It is suggested that genin J arises primarily as an artifact of the further hydrolysis of genin G rather than from a minor gymnemic acid. This is based upon the isolation of genin G in significant yields after nonspecific enzymatic hydrolysis of the ethyl acetate-extracted acids, a fraction relatively free of minor gymnemic acids.

A distribution of carboxylic acids between sugar and aglycone portions of gymnemic acids A–D is also indicated in Table III. Basic hydrolysis of both gymnemic acid A and genin G yielded identical mixtures of formic, acetic, isovaleric, and tiglic acids. Similarly, alkaline hydrolysis of gymnemic acid B and genin K gave identical carboxylic acid mixtures (formic, isovaleric, and tiglic acids). This deleted the possibility of hydroxyl groups in the sugar moieties of gymnemic acids A and B being esterified. However, since gymnemic acid D yields gymnestrogenin upon selective enzyme treatment and liberates ferulic acid only after its basic hydrolysis, it is indicated that ferulic acid is present in the sugar part of this glycoside. Based upon the similarity between gymnemic acids C and D, both in their genin and sugar portions, it is postulated that the ferulic acid found in the basic hydrolysate of acid C is also present in the sugar residue of this acid. However, an identification of carboxylic acids in a basic hydrolysate of genin N, the aglycone of gymnemic acid C, has not been carried out due to a lack of pure sample.

Investigations directed toward the position assignment of acyl and sugar residues in the basic genin structure of gymnemic acids A–D are currently in progress.

REFERENCES

(1) J. E. Sinsheimer, G. S. Rao, and H. M. McIlhenny, *J. Pharm. Sci.*, **59,** 622(1970).

(2) G. J. Kapadia and T. B. Zalucky, *Lloydia*, **31,** 424(1968).

(3) W. Stöcklin, E. Weiss, and T. Reichstein, *Helv. Chim. Acta*, **50,** 474(1967).

(4) E. Bayer and K. H. Reuther, *Angew. Chem.*, **68,** 689(1956).

(5) J. M. Bobbitt, "Thin-Layer Chromatography," Reinhold, New York, N. Y., 1963, p. 138.

(6) "Thin-Layer Chromatography," E. Stahl, Ed., Springer-Verlag, Berlin, Germany, 1965, p. 487.

(7) *Ibid.*, p. 385.

(8) *Ibid.*, p. 488.

(9) L. D. Metcalf, *Nature*, **188,** 142(1960).

(10) B. R. Baumgardt, Dept. Bull. No. 1, Dept. of Dairy Sci., University of Wisconsin, Madison, Wis., 1964.

(11) M. J. deFaubert Maunder, H. Egan, and J. Roburn, *Analyst*, **89,** 157(1964).

(12) F. Arndt, in "Organic Syntheses," A. H. Blatt, Ed., coll. vol. II, Wiley, New York, N. Y., 1943, p. 165.

(13) W. Stöcklin, *Helv. Chim. Acta*, **51,** 1235(1968).

(14) L. F. Fieser and M. Fieser, "Advanced Organic Chemistry," Reinhold, New York, N. Y., 1962, p. 372.

(15) G. S. Rao and J. E. Sinsheimer, *Chem. Commun.*, **1968,** 1681.

(16) W. Stöcklin, *Helv. Chim. Acta*, **52,** 365(1969).

(17) P. Jarrige and R. Henry, *Bull. Soc. Chim. Biol.*, **34,** 872 (1952).

(18) R. Henry and M. Thevenet, *ibid.*, **34,** 897(1952).

(19) F. L. Myers and D. H. Northcote, *J. Exp. Biol.*, **35,** 639 (1958).

(20) H. Huber, F. Blindenbacher, K. Mohr, P. Speiser, and T. Reichstein, *Helv. Chim. Acta*, **34,** 46(1951).

(21) M. Wakabayashi and W. H. Fishman, *J. Biol. Chem.*, **236,** 996(1961).

(22) J. Schmutz and T. Reichstein, *Pharm. Acta Helv.*, **22,** 359 (1947).

(23) P. Hauschild-Rogat, J. v. Euw, O. Schindler, E. Weiss, and T. Reichstein, *Helv. Chim. Acta*, **45,** 2116(1962).

(24) H. M. McIlhenny, Ph.D. thesis, University of Michigan, 1966; through *Diss. Abstr.*, **B28,** 125(1967).

(25) "The Merck Index," 8th ed., Merck & Co., Rahway, N. J., 1968, p. 322.

(26) G. S. Rao, Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1969.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 2, 1969, from the *College of Pharmacy, The University of Michigan, Ann Arbor, MI 48104*

Accepted for publication December 29, 1969.

Abstracted from a dissertation submitted by G. Subba Rao to the Horace H. Rackham School of Graduate Studies, The University of Michigan, in partial fulfillment of Doctor of Philosophy degree requirements.

The authors thank Professor T. Reichstein and Dr. Walter Stöcklin, The University of Basel, Basel, Switzerland, for reference samples of genins. They also thank Dr. Govind J. Kapadia, Howard University, for providing materials and procedure to carry out selective enzymatic hydrolysis of the gymnemic acids.

* Lilly Foundation fellow. Present address: Section on Chemistry, Molecular Disease Branch, National Heart Institute, Bethesda, Md.

Inhibition of Sulfathiazole Crystal Growth by Polyvinylpyrrolidone

A. P. SIMONELLI, S. C. MEHTA, and W. I. HIGUCHI

Abstract □ The results of polyvinylpyrrolidone inhibition of sulfathiazole single crystal growth are presented. It was found that the minimum concentration of polyvinylpyrrolidone required to inhibit completely crystal growth was a linear function of the supersaturation ratio with an intercept of ~1.15 exhibited on the latter axis. It was also found that the minimum concentration of polyvinylpyrrolidone required for complete inhibition of crystal growth was a function of the molecular weight of polyvinylpyrrolidone used for the inhibition study. The results of inhibition studies using molecular weights of polyvinylpyrrolidone of 10,000, 40,000, and 360,000 are presented. The data suggest that the inhibition point depends on the relative rates of transport of polyvinylpyrrolidone and sulfathiazole to the crystal surface from the bulk of the solution. A model is presented that appears to be consistent with the data.

Keyphrases □ Sulfathiazole crystal growth—polyvinylpyrrolidone effect □ Crystal growth, sulfathiazole—mechanism, polyvinylpyrrolidone inhibition □ Polyvinylpyrrolidone molecular weight effect—sulfathiazole crystal growth □ Model, kinetic—polyvinylpyrrolidone effect, sulfathiazole crystal growth

The growth of crystals may create undesirable changes in many pharmaceutical preparations such as suspensions for intramuscular or subcutaneous injection, oral suspensions, suppositories, and ointments containing undissolved drug particles. Crystal growth may cause these preparations to change drastically their particle-size distribution. Associated crystal growth formulation problems include adverse changes in syringeability, dispersibility, and irritability to the patient upon administration (1–3).

Crystal growth may also play an important role in polymorphic reversions. When polymorphism is present, the various crystal modifications of solid drugs can exhibit drastically different physical characteristics. Most important of these characteristics from a drug-availability standpoint are differing solubilities and dissolution rates as they can significantly alter important pharmacokinetic factors such as rates of absorption, drug availability, and resulting physiological concentrations. Since the higher energy crystal form of the drug exhibits higher activity, maintaining the physical stability of the high energy polymorphic form of drugs in suspension despite their instability becomes very important and has posed many problems to formulators. The importance of this problem is compounded by the fact that the reversion of the unstable form to the more stable form produces a less soluble form of the drug and in doing so increases the rate of crystal growth due to the resulting higher supersaturations. Such changes are realized if the unstable modification is exposed to the conditions of temperature and solvent (4–7) which permit rapid crystal growth of the more stable phase. Unless there is inhibition, such changes may lead to marked variances in a formulation's performance as a physical or drug delivery system.

Crystal growth and the associated polymorphic reversions have been extensively studied, and it has been found that a number of additives such as polymers and surfactants prevent these undesirable changes in pharmaceutical preparations (8–10). A survey of the literature, however, shows that very little is known regarding the role played by these agents in these situations except for their classifications as dispersion or suspending agents. Recognizing the need for studies designed to shed some light on the mechanism of this phenomenon, this study was initiated as a logical extension of the previous investigation regarding the growth of single crystals. While this report deals only with the sulfathiazole–polyvinylpyrrolidone (PVP) system, the underlying principles are sufficiently general to be extrapolated to other drug–polymer systems. Sulfathiazole was chosen because it has exhibited polymorphic behavior and is widely used as a suspension.

APPARATUS AND PROCEDURE

The apparatus and procedure used to study crystal growth rates have been described in another communication (11). The effect of additives upon the rate of crystal growth was determined by adding polymer in increments as a function of time through a sampling hole in the container lid. A stopwatch was used to obtain the time of each measurement of the crystal dimension and the time of inhibitor addition.

RESULTS AND DISCUSSION

Since the growth of the long axis of a single crystal of sulfathiazole in the absence of inhibitor must serve as a base line and integral part of this study, Fig. 1, which illustrates the growth rate of sulfathiazole as a function of the supersaturation ratio (11), is included. The supersaturation ratio, S, is the supersaturated solution concentration of sulfathiazole divided by the solubility of sulfathiazole. Figure 1 shows that the crystal growth rate of sulfathiazole is a linear function of the supersaturation ratio at higher supersaturations and that extrapolation of the linear portion of the curve yields an intercept on the supersaturation ratio axis at about a value of 1.15. Below a supersaturation ratio of 1.15, crystal growth may occur and may be surface controlled (11). Figure 1 also clearly shows that the stirring rate exerts a strong influence on the observed crystal growth rate above this supersaturation ratio and strongly indicates that in this region the growth rate must be diffusion controlled.

The effect of addition of PVP to the supersaturated solution was next investigated. It was found that the resulting effect on the sulfathiazole crystal growth rate was dependent upon the concentration of PVP in solution. To investigate this aspect further the polymer was added in lambda increments as a function of time. Between additions of polymer, the length of the crystal was also measured as a function of time and in this way the effect of PVP on the growth rate was established. Figure 2 shows typical results of such an experiment. The crystal length is plotted *versus* time and each arrow indicates the time of an addition of a solution of PVP by means of a lambda pipet. Interestingly the direction as well as the magnitude of the PVP effect was apparently dependent on the PVP concentration.

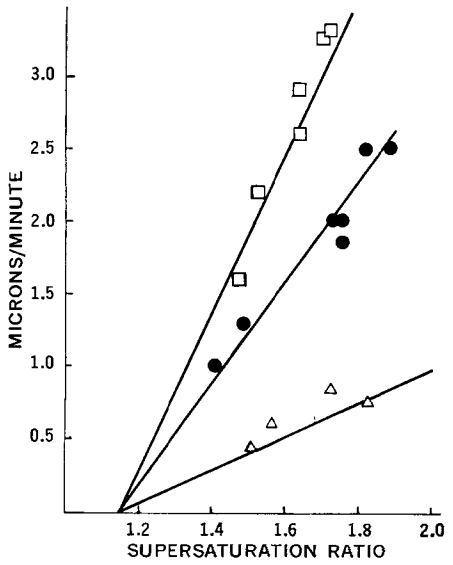


Figure 1—*Crystal growth of sulfathiazole in water along the long axis as a function of supersaturation at different stirring speeds. Key: △, 10 r.p.m.; ●, 150 r.p.m.; and □, 400 r.p.m.*

The initial additions of PVP showed no apparent effect; then further additions of PVP apparently decreased the growth rate; the rate of growth then apparently increased with other additions of PVP; and finally, still further additions of PVP caused complete crystal growth inhibition. This experiment was repeated a large number of times, and the results showed that this behavior was qualitatively reproducible, *i.e.*, the acceleration of growth before complete inhibition. The rate of acceleration, however, was not quantitatively reproducible. The reason for this becomes apparent if the growth pattern of the crystal is noted during the experiment, because the addition of PVP not only caused changes in the rate of crystal growth but also caused changes in the growth pattern. This is illustrated in Fig. 3 which shows the appearance of the crystals at various stages of the experiment. Initially the crystal face outline has smooth linear lines which converge to sharp points. This outline is maintained during its

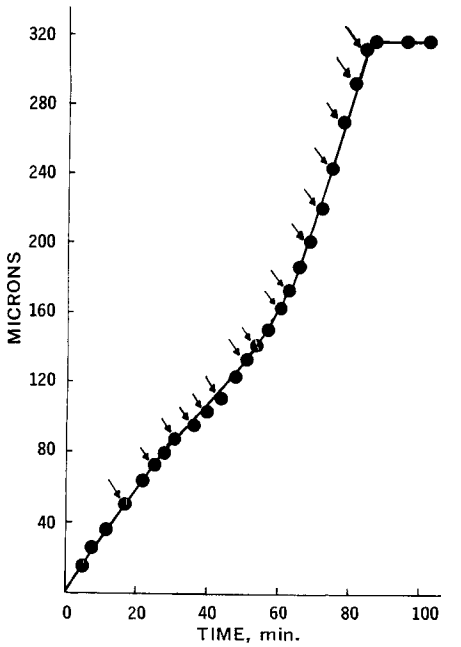


Figure 2—*A typical plot of crystal growth of sulfathiazole as a function of time, with incremental additions of PVP to the solution made also as a function of time. Each arrow indicates the time of addition of PVP solution in lambda increments.*

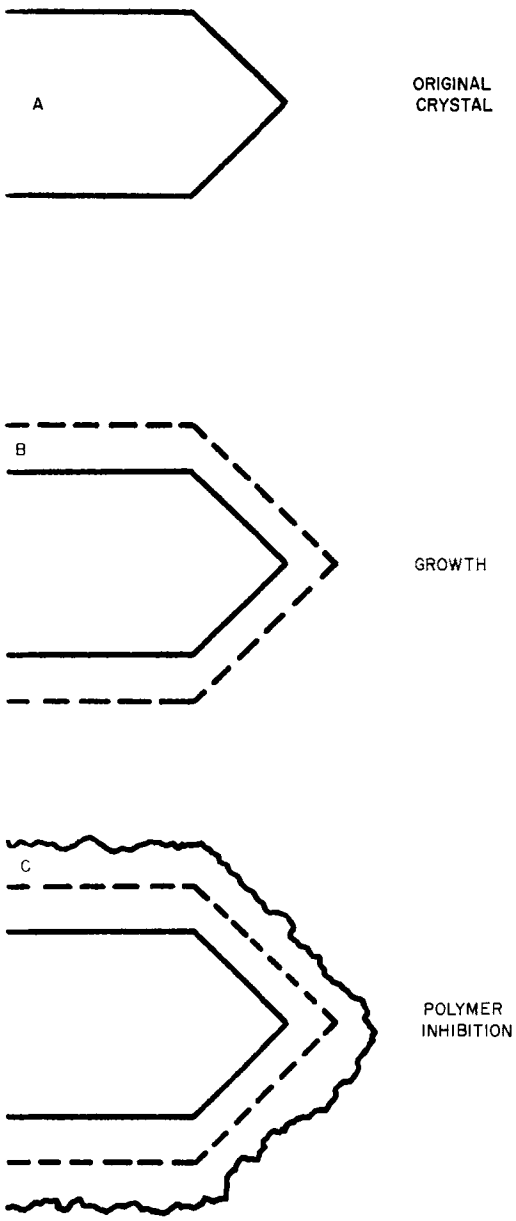


Figure 3—*Appearance of crystal surface outline at various stages of the experiment: A, original crystal at t = 0; B, after normal growth; and C, after the addition of PVP, showing polymer inhibition.*

enlargement as crystal growth proceeds in the absence of PVP. After the addition of PVP, however, the smooth linear lines during the accelerated growth period are replaced by randomly jagged lines which converge to indefinite intersection points. These intersection points are rounded as opposed to the sharp points previously observed.

Although the accelerated growth rate was not quantitative, the point of complete inhibition was reproducible and indicated that it would provide meaningful data regarding the influence of PVP. For this reason the minimum concentration of PVP for complete inhibition was noted as a function of the supersaturation ratio (Fig. 4). The relationship shown by Fig. 4 appears to be linear with an x-axis intercept and, as expected, it shows that higher PVP concentrations are needed as the supersaturation increases. It should be emphasized that each point plotted in Fig. 4 was the result of a large number of PVP additions to the solution with subsequent observation of the crystal length between additions to determine its rate of crystal growth. When the crystal ceased to grow, the sum of the PVP additions was calculated and the PVP concentration in the solution determined. This value was checked by mounting a new crystal and exposing it to the above inhibitory concentration of PVP to elimi-

Table I—Comparison of PVP Equivalent and Molar Weights Needed for Crystal Growth Inhibition

PVP Molecular Weight	Ratio of Slopes[a] Equiv. Weight Basis (ψ)[b]	Molar Weight Basis (α)[c]
10,000	1.00	1.00
40,000	1.49	0.37
360,000	4.30	0.12

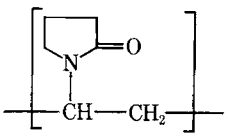
[a] Relative to the 10,000 molecular weight slope. [b] ψ, also equal to the number of equivalents needed for inhibition relative to the 10,000 molecular weight PVP. [c] α, also equal to the number of moles needed for inhibition relative to the 10,000 molecular weight PVP.

nate the possible error of dilution introduced by the frequent addition of PVP in the initial experiment.

As a result the values of the minimum concentration of PVP for complete inhibition plotted in Fig. 4 should be considered only as upper limits as the process of adding discrete amounts almost assures some degree of overshooting the minimum value. To eliminate or to decrease significantly the magnitude of this overshoot would require a countless number of additional experiments for each point. However, this was not done as the data at hand are sufficiently precise to provide firm establishment of the model which will be subsequently proposed.

This inhibition was further investigated to include the effect of PVP molecular weight. Figure 4 shows the resulting plots from different experiments using PVP molecular weights of 10,000, 40,000, and 360,000. As can be seen, the necessary grams of PVP per 100 ml. of solution to cause inhibition of crystal growth at any supersaturation ratio increases with increasing molecular weight of PVP. In addition the rate of increase of the grams of PVP per 100 ml. of solution needed as a function of increasing supersaturation ratio is much faster for PVP polymers of high molecular weight than those of lower molecular weight.

At this point of discussion, it was felt that the polymers should be compared on the basis of their relative concentrations of the recurring vinyl pyrrolidone segment,



rather than relative concentrations of molecules because it was desired to compare their effectiveness on an equivalent weight basis. For this reason, concentrations were expressed in terms of gram percent rather than moles/liter. It should be noted, however, that if the

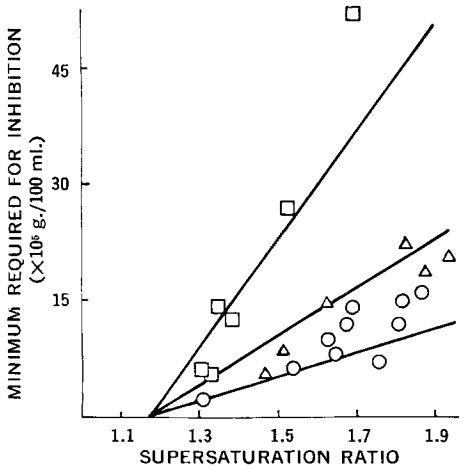


Figure 4—*Minimum concentration of different molecular weights of PVP required to cause inhibition of crystal growth of sulfathiazole as a function of supersaturation. Key: O, 10,000 molecular weight; Δ, 40,000 molecular weight; and □, 360,000 molecular weight.*

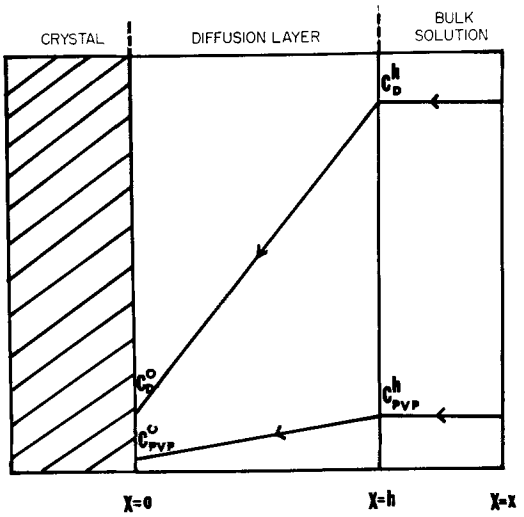


Figure 5—*Kinetic model describing the concentrations of sulfathiazole and PVP as a function of distance from the crystal surface.*

necessary concentrations of PVP were expressed in terms of molarity, the effects of polymer molecular weight would be very different from those noted using gram percent. As a matter of fact, the range of their relative molecular weights, 1:4:36, is sufficiently large to cause the relationship to be reversed, *i.e.*, the higher molecular weight PVP requires less moles for inhibition. Table I shows the results of a comparison of the slopes of best fit linear curves drawn through the corresponding data points.

These relationships imply that this is not an equilibrium process but must involve dominating kinetic factors. If an equilibrium process was in control, it would not be expected that more equivalents of the higher molecular weight PVP would be needed due to the better adsorption tendencies of the higher molecular weight polymers. This would be true if all segments have equal covering ability. This can be rationalized on the basis that a higher molecular weight PVP molecule contains more units per molecule than its lower molecular weight counterpart and therefore can make more attachments to a crystal surface with which to anchor itself.

It can be inferred that diffusional processes are controlling the effective concentration of PVP required for inhibition by considering the following:

1. The rate of crystal growth appears to be controlled by the rate of diffusion of sulfathiazole to the surface as shown by the stirring rate dependence of the crystal growth studies.

2. The PVP molecular weight dependence of the PVP inhibition suggests that the rate of PVP diffusion to the surface may be rate determining; otherwise, it whould be expected that the minimum equivalent weight of PVP needed to inhibit sulfathiazole crystal growth should be independent of the molecular weight as the PVP polymer consists of identical recurring units.

3. Finally the marked inhibition concentration dependency of PVP on the supersaturation ratio also supports the hypothesis that the rate of diffusion of PVP to the surface is rate determining. For a nondiffusional model, it would be expected that the PVP concentration needed should be invariant and not a function of the supersaturation ratio.

This rationalization prompted the proposal of the kinetic model which is illustrated in Fig. 5. It is easily seen that in this model the relative concentrations of drug and PVP at the surface of the crystal will depend on the relative concentration in the bulk of the solution and the relative rates of diffusion through the diffusion layer. If this is true the relative rates of deposition of both species on the surface of the crystal must also depend on the relative rates of diffusion to the surface, as it would be expected that the relative rates of deposition must be dependent on the relative concentrations of both species at the surface. Since the data show that a minimum concentration of PVP is needed for inhibition to occur, it can be postulated that if the rate of deposition of PVP is relatively slow as compared to that of the sulfathiazole, it is buried by the avalanche of precipitating sulfathiazole molecules. If, on the other hand, its rate is relatively rapid, it in turn can bury the precipitating sulfathiazole

molecules and sufficiently cover the crystal surface to cause inhibition of crystal growth. This model can be used to explain adequately all the results reported in this study. For example this model would predict that a higher PVP concentration would be needed to cause crystal growth inhibition at higher supersaturation ratios of sulfathiazole as the sulfathiazole molecules would increase their diffusional rate to the surface, necessitating that the PVP do so also.

Since this model readily lends itself to a mathematical treatment, quantitative relationships were derived to test the validity of the model. Assuming a steady state, the rate of diffusion of drug, G_D, and PVP, G_p, are given by the following equations:

$$G_D = K_D D_D (C_D^h - C_D^0) \quad \text{(Eq. 1)}$$

$$G_p = K_p D_p (C_p^h - C_p^0) \quad \text{(Eq. 2)}$$

where C_D and C_p denote the concentration of drug and PVP, respectively; the superscripts h and 0 denote the concentrations in the bulk of the solution and at the crystal surface, respectively; and K_D and K_p are the corresponding proportionality constants.

Since the model assumes that inhibition occurs when the relative diffusional rate of PVP to the surface is sufficiently rapid, it can be stated that for complete inhibition to occur the following condition must prevail:

$$G_p \geq k' G_D \quad \text{(Eq. 3)}$$

where k' is the proportionality constant relating the minimum PVP rate of diffusion for inhibition to the drug rate of diffusion. Substitution of Eqs. 1 and 2 into Eq. 3 and rearrangement of the resulting equation lead to the following equation for the minimum bulk concentration of PVP, $(C_p^h)_M$, needed for crystal growth inhibition of sulfathiazole:

$$(C_p^h)_M = \frac{kD_D}{D_p}[C_D^h - C_D^0] + C_p^0 \quad \text{(Eq. 4)}$$

where[1] $k = (K_D/K_p)k'$.

The supersaturation ratio, S, is defined as

$$S = \frac{C_D^h}{C_D^s} \quad \text{(Eq. 5)}$$

where C_D^s is the solubility of the drug.

Equations 4 and 5 yield

$$(C_p^h)_M = \frac{kD_D C_D^s (S)}{D_p} + C_p^0 - \frac{kD_D C_D^0}{D_p} \quad \text{(Eq. 6)}$$

Equation 6 predicts that a plot of $(C_p^h)_M$ *versus* S is a straight line with a slope of $kK_D C_D^s / D_p$ which intercepts the supersaturation ratio axis at:

$$S = \frac{C_D^0}{C_D^s} - \frac{C_p^0 D_p}{kD_D C_D^s} \quad \text{(Eq. 7)}$$

The above predicts that in the absence of PVP the intercept is equal to C_D^0/C_D^s when $(C_p^h)_M = 0$, and experimentally this appears to be equal to 1.15. In the presence of PVP, however, experimental results indicate that the intercept essentially remains unchanged and this appears to differ with the results predicted by Eq. 7. If one examines the relative magnitudes of the first and second terms of Eq. 7, however, it can easily be shown that the second term is negligible. The first term is equal to $\simeq$1.15, assuming this is independent of the presence of PVP. The second term can be set equal to C_p^0/slope. Since C_p^0 is $<<< 10^{-5}$ g./100 ml. and the slope is 1.5 to 5.5 $\times$ 10^{-6}, Eq. 7 predicts that the intercept is essentially unchanged with the addition of PVP.

Equation 6 was derived for the sulfathiazole–PVP system but will be applicable to any drug-inhibitor system that is described by this model. If the drug is changed, Eq. 6 predicts that the minimum concentration of inhibitor needed for complete crystal growth inhibition, $(C_p^h)_M$, would be directly proportional to the solubility and diffusion coefficient of the drug. Perhaps of more significance is that Eq. 6 predicts that $(C_p^h)_M$ is directly proportional to the supersaturation ratio and inversely proportional to the diffusion coefficient of the polymer.

[1] In an idealized situation, $K_D = K_p$ and therefore $k = k'$. The more general treatment presented in this discussion—*viz.*, $k \neq k'$, is preferred as it might allow for nonideal factors such as nonlinear concentration gradients and different sites of deposition.

Table II—Comparison of Experimental PVP Molecular Effectiveness to the Expected Effectiveness[a]

Relative Comparison[b]	PVP Molecular Weight Polymer 10,000	40,000	360,000
Molar slopes[c]	1	0.37	0.12
Apparent molecular effectiveness	1	2.70	8.33
Diffusion coefficient factor	1	2.11	4.37
Diffusion coefficient adjusted experimental molecular effectiveness	1	5.70	36.40
Expected molecular effectiveness	1	4.00	36.00

[a] Assuming equivalency of molecular segments. [b] Relative to 10,000 molecular weight polymer. [c] See Table I.

These predictions can be used as a test for the applicability of the model to a given system. The data previously presented in this study clearly show that $(C_p^h)_M$ is directly proportional to the supersaturation ratio. If $(C_p^h)_M$ is also inversely proportional to the respective PVP diffusion coefficients, then the slopes of the three straight lines obtained for the three different molecular weight PVP compounds should be in the same ratio as their reciprocal diffusion coefficients assuming that each segment has equal covering capability regardless of the molecular size.

For this purpose, the diffusion coefficients of the three species of PVP used in this study, 10,000, 40,000, and 360,000 molecular weight were independently determined in a diffusion cell using a membrane filter by a method previously reported (12). The diffusion coefficients were found to be 1.55 $\times$ 10^{-6}, 7.33 $\times$ 10^{-7}, and 3.55 $\times$ 10^{-7} cm.^{2}/sec. for the 10,000, 40,000, and 360,000 molecular weight polymers. A set of straight lines were simultaneously fitted to the data points of all three PVP compounds using a common intercept of 1.15 and ratio of slopes that were proportional to their respective reciprocal diffusion coefficients. A common intercept was used as predicted by Eq. 6. Figure 4 shows that the agreement is well within experimental error and lends strong support for the proposed model.

Although the diffusional rate of PVP to the surface has been established as the controlling process, it would be of interest to determine the relative effectiveness of the different molecular weight PVP molecules once they arrive at the surface. It would be expected that the 360,000 molecular weight PVP would be 9 times more effective than the 40,000 molecular weight PVP and 36 times more effective than the 10,000 molecular weight PVP. Table II shows the results of calculations which strongly support the equivalency of molecular segments in covering the surface regardless of molecular weight and that the apparent relative molecular effectiveness is due to the relative PVP rates of transport to the crystal surface.

Yet to be explained is the small amount of PVP needed for complete inhibition of crystal growth of sulfathiazole. This result suggests that the PVP must be strongly bound to the sulfathiazole crystal surface. This should not be unexpected as the PVP consists of a recurring group which allows it to form many attachments to the surface. This possibility was tested out by running an inhibited crystal from a PVP solution of a given sulfathiazole supersaturation ratio in a solution of comparable supersaturation ratio but without PVP. The crystal did not grow, indicating that the PVP was not removed from the crystal surface and therefore it was indeed strongly bound.

To investigate this further, calculations were made using Eqs. 1 and 2 to estimate the relative rates of transport of sulfathiazole and PVP from the bulk of the solution to the crystal surface. The results of these calculations confirmed the above, as they clearly showed that the relative rates of deposition of PVP as compared to sulfathiazole was not sufficiently large to provide for a tightly packed film over the sulfathiazole crystal surface.

Although no further evidence was obtained, sufficient data are at hand to permit a mechanistic speculation. It is believed that these results can be explained on the basis that the polymer forms a noncondensed netlike film over the crystal surface of sulfathiazole. This

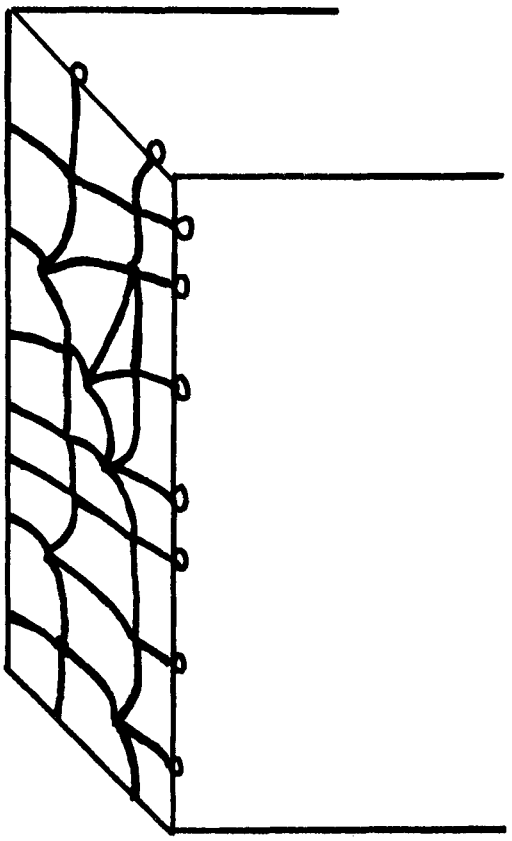


Figure 6—*Net inhibition model illustrating the inhibition by PVP on one surface of the crystal.*

is illustrated by Fig. 6. This model, of course, will permit the sulfathiazole to grow out through the openings of the net. In order to do so, however, the crystal will need to grow in fingerlike protrusions (Fig. 7). In addition the radii of these protrusions will be governed by the effective pore size of the polymer net. If this is true the curvature of these protrusions will require higher supersaturation ratios to grow, as can be shown using the Kelvin equation (12):

$$\ln \frac{P}{P_0} = \frac{2\gamma M}{RTdr} \qquad \text{(Eq. 8)}$$

where P = vapor pressure of a small drop or particle of radius r (~micron), P_0 = vapor pressure of a planar surface ($r \sim$ large), γ = interfacial tension or free energy of the crystal, M = molecular weight, d = density, R = gas constant, and T = absolute temperature.

In the case of a particle suspended in a saturated solution, it can be assumed that the ratio P/P_0 can be approximated by the ratio of the respective activities in the solution, a/a_0, where a is the activity of a small spherical particle and a_0 is the activity of a large particle. If activity coefficients of both particles are the same, the activities can be replaced by their respective solubilities. This ratio, however, defines the supersaturation ratio that a large crystal will be exposed to if placed in a solution which is saturated with respect to the small particle. This, of course, is the supersaturation ratio which has been utilized in all previous graphs, as the single crystal used in the previous experiments can be considered the equivalent of a large particle. As a result, Eq. 8 can be expressed by the following equation:

$$\ln S = \frac{2\gamma M}{RTdr_p} \qquad \text{(Eq. 9)}$$

where S is the supersaturation ratio (relative to the solubility of a large particle) of a solution in which a protrusion with a curvature that is equivalent to a particle of radius r_p will have equal probabilities of growth or dissolution.

Equation 9 shows that as the effective radius of a protrusion decreases, the protrusion will require a correspondingly larger supersaturation ratio before it will grow. This indicates that a netlike coverage of PVP that controls the effective radius of protrusions from the crystal surface can cause the crystal to require a higher supersaturation ratio than if it were absent in order for the crystal to grow. Furthermore, the tighter the net the smaller the effective radii of the resulting protrusions and the higher the minimum supersaturation ratio required for crystal growth to occur.

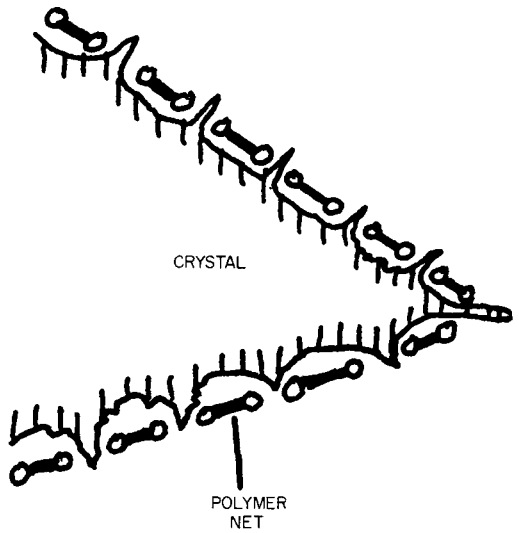


Figure 7—*Net inhibition model illustrating a cross section of the protrusions growing through the openings of a polymer net.*

This speculation can be combined with the kinetic diffusion model to describe a possible mechanism of inhibition by PVP of a sulfathiazole crystal exposed to its supersaturated solution. The experimental data previously presented in this study showed that the crystal growth inhibition of sulfathiazole by PVP is related to the relative transport rates to the crystal surface. This implies that the point of inhibition is related to the relative rates of deposition. The PVP reaching the surface forms a netlike structure (since complete coverage does not occur at the point of inhibition) which allows the sulfathiazole to grow out in fingerlike protrusions (suggested by fingerlike growth before the point of inhibition). Due to the higher curvature of these protrusions, however, the minimum supersaturation ratio for growth increases as shown by Eq. 9. This curvature, on the other hand, is determined by the effective pore size of the polymer net. The effective pore size of the net is determined by the relative PVP to sulfathiazole transport rate. Since the relative PVP to sulfathiazole transport rate governs the relative ratio of PVP and sulfathiazole deposited on the crystal surface, it follows that the higher the relative PVP rate, the smaller the effective pore size of the polymer net and the higher the supersaturation required for growth to be maintained. This model is consistent with all the data reported in this study.

REFERENCES

(1) T. J. Macek, U. S. pat. 2,671,750, Mar. 9, 1954.

(2) B. J. Magerlein and J. K. Dale, U. S. pat. 2,828,319, Mar. 25, 1958.

(3) *Ibid.*, U. S. pat. 2,841,599, July 1, 1958.

(4) H. Nogami and T. Nagai, *Yakkyoku*, **9**, 29(1958).

(5) J. Hasegawa and T. Nagai, *Chem. Pharm. Bull. (Tokyo)*, **6**, 129(1958).

(6) W. I. Higuchi and H. Y. Saad, *J. Pharm. Sci.*, **54**, 74(1965).

(7) H. Y. Saad and W. I. Higuchi, *ibid.*, **54**, 1205(1965).

(8) J. D. Mullins and T. J. Macek, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 245(1960).

(9) J. K. Dale and S. L. Ross, U. S. pat. 2,861,920, Nov. 25, 1958.

(10) J. K. Dale and A. R. Hanze, U. S. pat. 3,062,712, Nov. 6, 1962.

(11) S. C. Mehta, P. D. Bernardo, W. I. Higuchi, and A. P. Simonelli, *J. Pharm. Sci.*, **59**, 638(1970).

(12) A. H. Goldberg and W. I. Higuchi, *ibid.*, **57**, 1583(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication September 10, 1969.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami meeting, May 1968.

Supported by Contract PH 43-68-1284 with Chemotherapy, National Cancer Institute, National Institutes of Health.

The authors thank the reviewer for suggesting Table II.

Rate of Crystal Growth of Sulfathiazole and Methylprednisolone

S. C. MEHTA, P. D. BERNARDO*, W. I. HIGUCHI, and A. P. SIMONELLI

Abstract □ The results of crystal growth rate studies using single crystals of sulfathiazole and methylprednisolone are presented. The growth rate of sulfathiazole crystals growing in a supersaturated aqueous solution showed stirring rate dependence from 10 to 400 r.p.m. The same stirring rate dependence was found for all three faces studied. In alcohol solutions, however, the stirring rate dependence appeared to disappear above 150 r.p.m., suggesting that at the higher stirring rates the rate of crystal growth of sulfathiazole is surface controlled. A plot of the growth rate *versus* the supersaturation ratio appeared to be linear for all studies with intercepts exhibited on the supersaturation ratio axis. The intercept appeared to be a function of its solvent varying from 1.07 to 1.43 and to be a function of the polarity of the alcohol. The crystal growth rate of methylprednisolone, on the other hand, showed no stirring rate dependence in the range from 20 to 400 r.p.m. and appears to be surface controlled even at low stirring rates.

Keyphrases □ Crystal growth rates—single crystal method □ Sulfathiazole crystals—growth rate □ Methylprednisolone crystals—growth rate □ Stirring rate effect—crystal growth □ Refractive index—crystal axes determination

There are a number of situations where an understanding of the growth behavior of crystals may provide a basis for improving pharmaceutical formulations. Crystal growth in a suspension formulation, as a result of temperature fluctuations or Ostwald ripening, may lead to undesirable changes in its particle-size distribution. Such changes whether they affect the ease of administration, efficacy, or the esthetic appearance of suspensions constitute "physical instability." Investigations (1–5) of crystal growth behavior involving systems of pharmaceutical interest are relatively few. Furthermore, most of these studies have not been mechanistically oriented, *i.e.*, aimed at establishing the molecular mechanisms. Generally such studies have only answered the question of whether or not crystal growth occurs under the particular prevailing condition.

The present report describes the results of crystal growth studies involving the two drugs, sulfathiazole and methylprednisolone. A simple but convenient method to study crystal growth is described. The crystal growth rate data were obtained as a function of supersaturation, crystal orientation, and stirring rate and provided much insight as to the probable rate-determining processes governing crystal growth. Furthermore, this method is well suited for the screening and evaluation of potential crystal growth inhibitors.

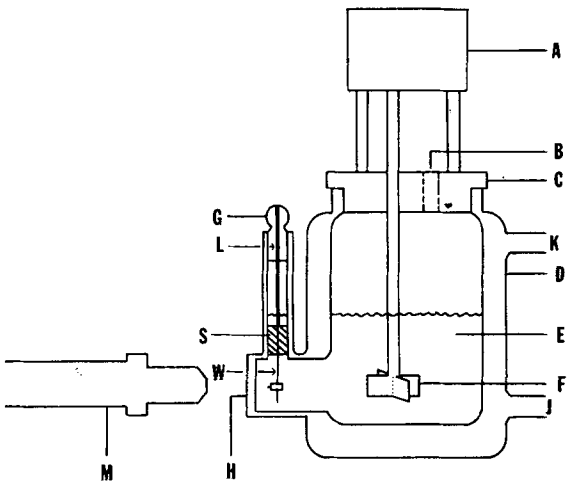


Figure 1—*Crystal growth apparatus. Key: A, synchronous motor; B, sampling port; C, Teflon lid; D, jacketed beaker; E, supersaturated solution; F, stirrer; G, glass stopper with a fused stainless steel rod (L); H, optical glass window; J, inlet for water (30°); K, outlet for water (30°); M, microscope; S, stainless steel coupling to hold removable crystal holder shown in Fig. 2; and W, tungsten wire holding crystal holder described in Fig. 2.*

GENERAL CONSIDERATIONS

Previous experiences have indicated the need to develop a technique utilizing a relatively convenient experimental system yet capable of providing much insight into the factors governing the rates of growth of crystals. Since the prime purposes of this study were the quantitation of crystal growth rates and the determination of the effect of various factors upon this growth, it was decided that a single crystal technique which measures linear growth rather than techniques which utilize gross volume changes should be employed. The single crystal technique is more reproducible and therefore more quantitative because of the following reasons:

1. Surface area is not important, as linear growth is a function of the chemical potential of the solution relative to the solid surface which can be expressed in terms of unit area. Furthermore, the

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication September 10, 1969.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami meeting, May 1968.

Supported by Contract PH 43-68-1284 with Chemotherapy, National Cancer Institute, National Institutes of Health.

The authors thank the reviewer for suggesting Table II.

Rate of Crystal Growth of Sulfathiazole and Methylprednisolone

S. C. MEHTA, P. D. BERNARDO*, W. I. HIGUCHI, and A. P. SIMONELLI

Abstract □ The results of crystal growth rate studies using single crystals of sulfathiazole and methylprednisolone are presented. The growth rate of sulfathiazole crystals growing in a supersaturated aqueous solution showed stirring rate dependence from 10 to 400 r.p.m. The same stirring rate dependence was found for all three faces studied. In alcohol solutions, however, the stirring rate dependence appeared to disappear above 150 r.p.m., suggesting that at the higher stirring rates the rate of crystal growth of sulfathiazole is surface controlled. A plot of the growth rate *versus* the supersaturation ratio appeared to be linear for all studies with intercepts exhibited on the supersaturation ratio axis. The intercept appeared to be a function of its solvent varying from 1.07 to 1.43 and to be a function of the polarity of the alcohol. The crystal growth rate of methylprednisolone, on the other hand, showed no stirring rate dependence in the range from 20 to 400 r.p.m. and appears to be surface controlled even at low stirring rates.

Keyphrases □ Crystal growth rates—single crystal method □ Sulfathiazole crystals—growth rate □ Methylprednisolone crystals—growth rate □ Stirring rate effect—crystal growth □ Refractive index—crystal axes determination

There are a number of situations where an understanding of the growth behavior of crystals may provide a basis for improving pharmaceutical formulations. Crystal growth in a suspension formulation, as a result of temperature fluctuations or Ostwald ripening, may lead to undesirable changes in its particle-size distribution. Such changes whether they affect the ease of administration, efficacy, or the esthetic appearance of suspensions constitute "physical instability." Investigations (1–5) of crystal growth behavior involving systems of pharmaceutical interest are relatively few. Furthermore, most of these studies have not been mechanistically oriented, *i.e.*, aimed at establishing the molecular mechanisms. Generally such studies have only answered the question of whether or not crystal growth occurs under the particular prevailing condition.

The present report describes the results of crystal growth studies involving the two drugs, sulfathiazole and methylprednisolone. A simple but convenient method to study crystal growth is described. The crystal growth rate data were obtained as a function of supersaturation, crystal orientation, and stirring rate and provided much insight as to the probable rate-determining processes governing crystal growth. Furthermore, this method is well suited for the screening and evaluation of potential crystal growth inhibitors.

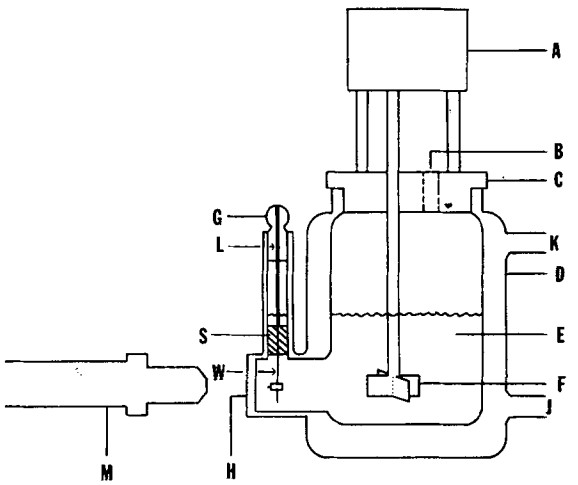


Figure 1—*Crystal growth apparatus. Key: A, synchronous motor; B, sampling port; C, Teflon lid; D, jacketed beaker; E, supersaturated solution; F, stirrer; G, glass stopper with a fused stainless steel rod (L); H, optical glass window; J, inlet for water (30°); K, outlet for water (30°); M, microscope; S, stainless steel coupling to hold removable crystal holder shown in Fig. 2; and W, tungsten wire holding crystal holder described in Fig. 2.*

GENERAL CONSIDERATIONS

Previous experiences have indicated the need to develop a technique utilizing a relatively convenient experimental system yet capable of providing much insight into the factors governing the rates of growth of crystals. Since the prime purposes of this study were the quantitation of crystal growth rates and the determination of the effect of various factors upon this growth, it was decided that a single crystal technique which measures linear growth rather than techniques which utilize gross volume changes should be employed. The single crystal technique is more reproducible and therefore more quantitative because of the following reasons:

1. Surface area is not important, as linear growth is a function of the chemical potential of the solution relative to the solid surface which can be expressed in terms of unit area. Furthermore, the

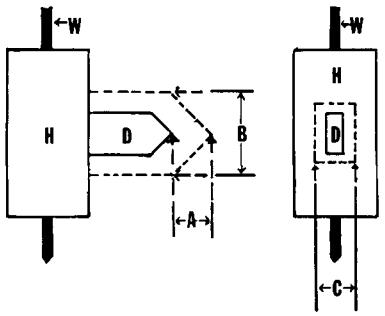


Figure 2—*Measurement of crystal growth. Key: H, inert rubber of crystal holder to keep crystal in position; D, original crystal; A, increase in half the length; B, increase in width; C, increase in thickness; and W, tungsten wire attached to stainless steel coupling (S) shown in Fig 1.*

same crystal can be exposed to a variety of conditions, all of which are affecting the same surface.

2. Nucleation does not present a problem as it is readily detectable. This is not true for the gross volume technique, and its presence must be determined by separate means such as a Coulter counter technique. If the proper growth rate is to be determined in a gross volume technique, the rate of nucleation must also be determined and its contribution subtracted.

3. The random heterogeneity of the surface free energy at the solid–liquid interface of all particles is not a problem in single crystal methods.

4. The need for a significant quantity of material to be transferred from the solution phase to the solid phase which causes the supersaturation ratio to be significantly decreased during experiments using the gross volume technique is also not present in single crystal work.

In addition, single crystal techniques, unlike gross volume techniques, not only permit the linear growth rate of each face of a crystal to be studied independently but also under varying conditions. This is essential in mechanistic studies. Finally the single crystal technique will also permit the crystal growth rate experiments to serve as a baseline for subsequent crystal growth inhibition studies. Furthermore, in order to separate diffusion-controlled cases from surface-controlled rates, it was decided to employ variable stirring conditions as well as varying supersaturation conditions.

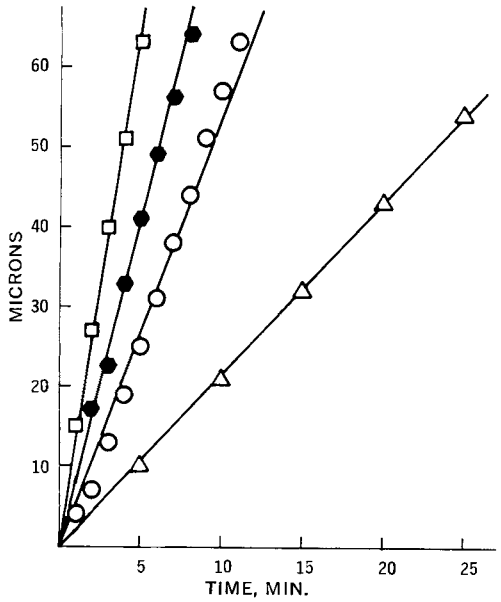


Figure 3—*Crystal growth of sulfathiazole in 95% v/v ethanol along the long axis at different supersaturation ratios. Key: △, S = 1.26; ○, S = 1.37; ●, S = 1.52; and □, S = 1.73.*

Table I—Solubility of Sulfathiazole and Methylprednisolone in Different Solvent Systems at 30°

Solvent	Solubility, g./100 g.	$\lambda_{max.}$, mμ
Sulfathiazole		
Water	0.065	282
95% v/v Ethanol in water	1.06	288
50% v/v Ethanol in water	1.30	
60% v/v Ethanol in *sec*-butanol	0.555	
Methylprednisolone		
Water	0.0072	249
1% v/v Ethanol in water	0.0077	
2% v/v Ethanol in water	0.0085	

MATERIALS

All solvents utilized in the experiments were distilled. The sulfathiazole crystals used in the crystal growth experiments were prepared by double recrystallization either from 95% ethanol or from water. The methylprednisolone crystals used in these studies

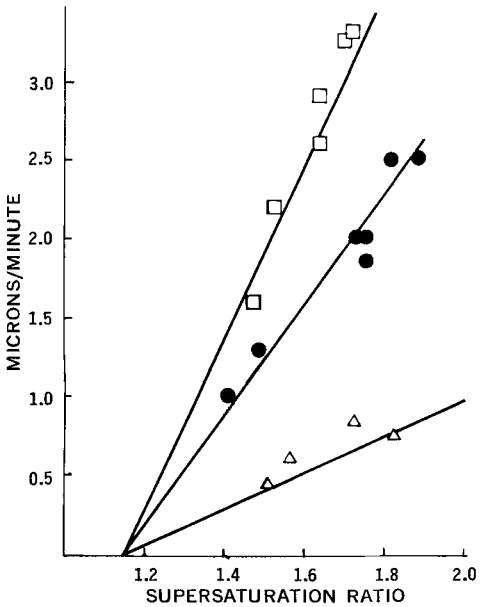


Figure 4—*Crystal growth rate of sulfathiazole in water along the long axis of the crystal as a function of supersaturation at different stirring speeds. Key: △, 10 r.p.m.; ●, 150 r.p.m.; and □, 400 r.p.m.*

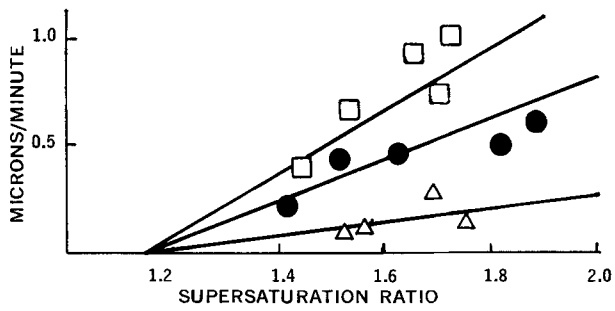


Figure 5—*Crystal growth rate of sulfathiazole in water along the dimension designated as width as a function of supersaturation at different stirring speeds. Key: △, 10 r.p.m.; ●, 150 r.p.m.; and □, 400 r.p.m.*

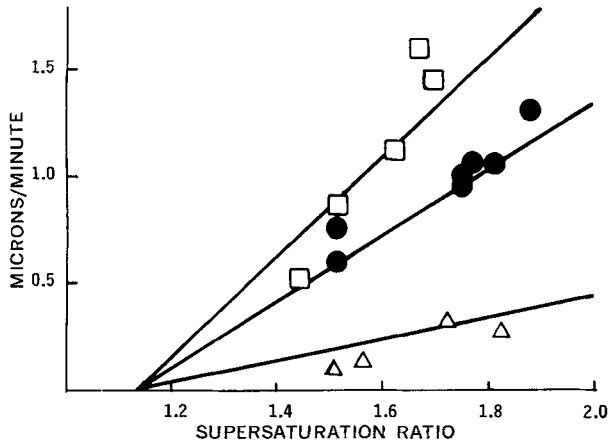


Figure 6—*Crystal growth rate of sulfathiazole in water along the dimension designated as thickness as a function of supersaturation at different stirring speeds. Key: △, 10 r.p.m.; ●, 150 r.p.m.; and □, 400 r.p.m.*

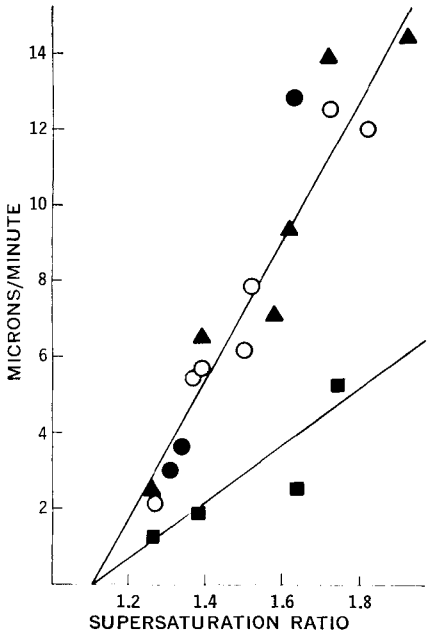


Figure 7—*Crystal growth rate of sulfathiazole in 95% v/v ethanol along the dimension designated as the long axis as a function of supersaturation at different stirring speeds. Key: ■, 10 r.p.m.; ○, 150 r.p.m.; ●, 240 r.p.m.; and ▲, 400 r.p.m.*

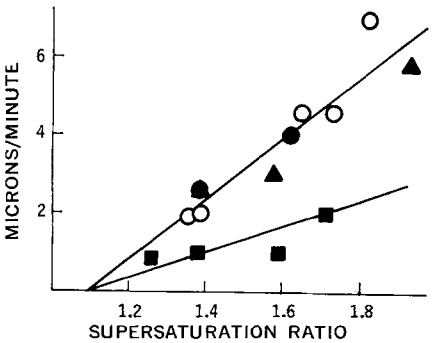


Figure 8—*Crystal growth rate of sulfathiazole in 95% v/v ethanol along the dimension designated as width as a function of supersaturation at different stirring speeds. Key: ■, 10 r.p.m.; ○, 150 r.p.m.; ●, 240 r.p.m.; and ▲, 400 r.p.m.*

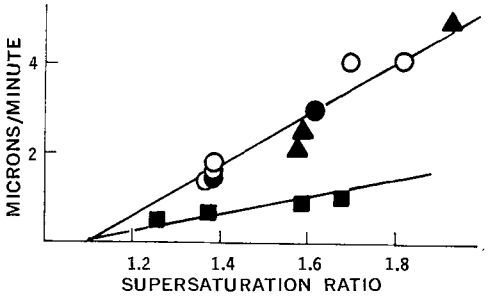


Figure 9—*Crystal growth rate of sulfathiazole in 95% v/v ethanol along the dimension designated as thickness as a function o supersaturation at different stirring speeds. Key: ■, 10 r.p.m.; ○, 150 r.p.m.; ●, 240 r.p.m.; and ▲, 400 r.p.m.*

were prepared by recrystallization either from a 50% v/v alcohol–water mixture or from acetone.

The crystalline modifications of both compounds were the same as those designated as the most stable forms (Form I) in previous studies (6, 7) and were confirmed by melting point, refractive index, X-ray diffraction, and IR spectra.

In addition, refractive index determinations were used to characterize the long axes of the individual crystals of both compounds. The refractive index of the long axis of the hexagonally shaped face of sulfathiazole crystal was found to be 1.674, in agreement with the reported value (8). For methylprednisolone the refractive index along the long axis of the rod-shaped crystal was found to be 1.602 (9).

The supersaturated solutions used in these studies were prepared by either of two methods, saturating at higher temperatures using steam heat or by solvent mixing. Because of chemical instability, supersaturated methylprednisolone solutions were always prepared by solvent mixing.

APPARATUS AND PROCEDURE

Figure 1 shows the apparatus used to study the growth rate of sulfathiazole. The temperature of the system was controlled at 30°

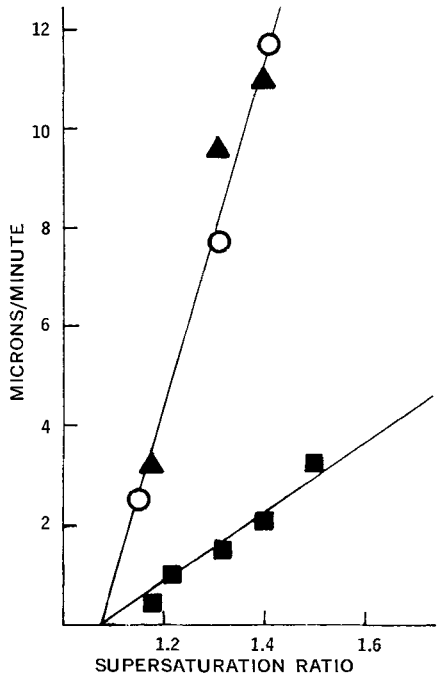


Figure 10—*Crystal growth rate of sulfathiazole in 50% v/v ethanol–water along the long axis as a function of supersaturation at different stirring speeds. Key: ■, 10 r.p.m.; ○, 150 r.p.m.; and ▲, 400 r.p.m.*

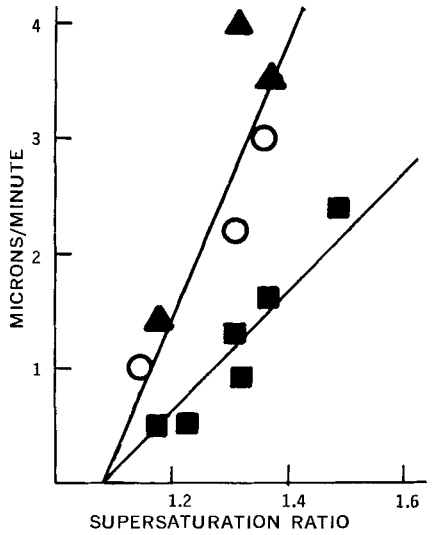


Figure 11—*Crystal growth rate of sulfathiazole in 50% v/v ethanol–water along the dimension designated as width as a function of supersaturation at different stirring speeds. Key: ■, 10 r.p.m.; ○, 150 r.p.m.; and ▲, 400 r.p.m.*

by means of a water jacket through which 30° water was circulated. For crystal growth studies, a single crystal of sulfathiazole is mounted in a slit made in a piece of rubber for this purpose (10). See Fig. 2. This piece of rubber is held by a tungsten wire attached to a stainless steel coupling. This coupling permits the crystal holder to be removed to facilitate the delicate and tedious process of mounting the crystal. In addition the coupling prevents the crystal from lateral movement during the experiment after the crystal holder is mounted in place. After a mounted crystal is immersed in the supersaturated solution, a microscope calibrated in microns is used to study crystal growth through an optical-glass window. Figure 2 shows the manner in which the length, width, and thickness of a crystal are followed as a functon of time. The supersaturated solution was stirred at a constant rate during an experiment by means of a synchronous motor mounted on the container lid. The effect of the stirring rate on crystal growth rate, on the other hand, was studied by interchanging motors of different speeds. A stopwatch was used to obtain the time of each crystal measurement. During the experiment, samples were periodically withdrawn from the solution and assayed spectrophotometrically in order to determine the concentration of the solution.

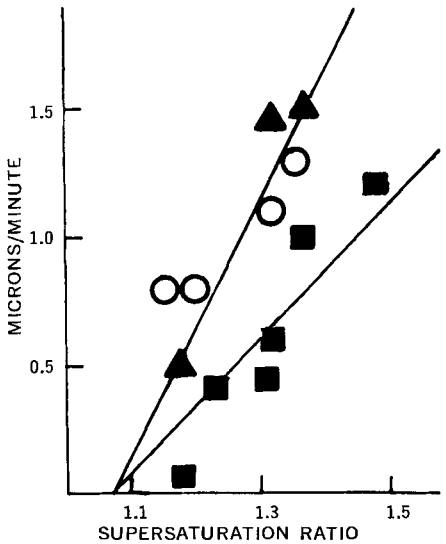


Figure 12—*Crystal growth rate of sulfathiazole in 50% v/v ethanol–water along the dimension designated as thickness as a function of supersaturation at different stirring speeds. Key: ■, 10 r.p.m.; ○, 150 r.p.m.; and ▲, 400 r.p.m.*

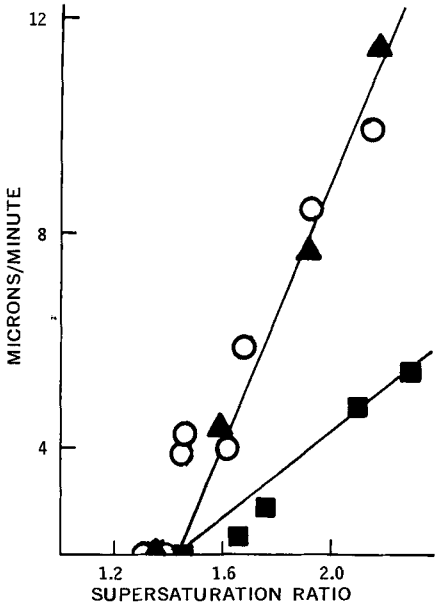


Figure 13—*Crystal growth rate of sulfathiazole in 40% v/v sec-butanol–60% v/v ethanol along the long axis as a function of supersaturation at different stirring speeds. Key: ■, 10 r.p.m.; ○, 150 r.p.m.; and ▲, 400 r.p.m.*

RESULTS AND DISCUSSION

The solubilities of sulfathiazole and methylprednisolone were determined in the following manner. Excesses of the amounts of the recrystallized compounds needed to produce saturated solutions

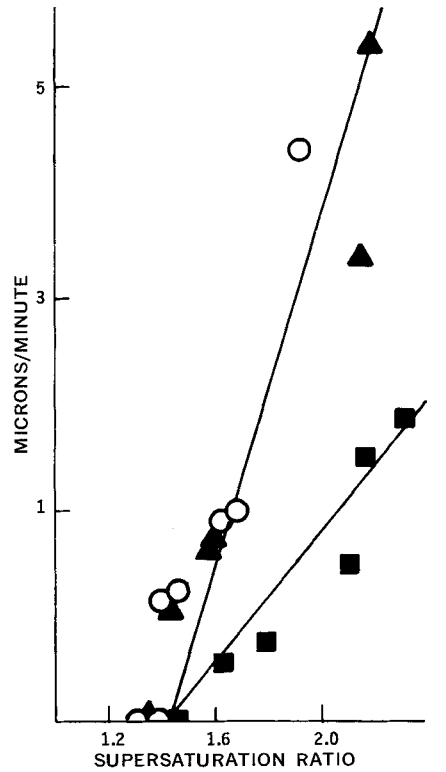


Figure 14—*Crystal growth rate of sulfathiazole in 40% v/v sec-butanol–60% v/v ethanol along the axis designated as width as a function of supersaturation at different stirring speeds. Key: ■, 10 r.p.m.; ○, 150 r.p.m.; and ▲, 400 r.p.m.*

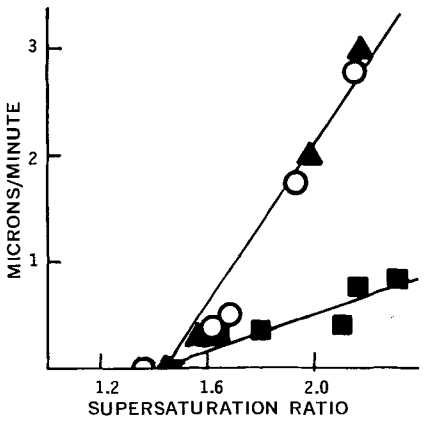


Figure 15—*Crystal growth rate of sulfathiazole in 40% v/v sec-butanol–60% v/v ethanol along the axis designated as thickness as a function of supersaturation at different stirring speeds. Key:* ■, *10 r.p.m.;* ○, *150 r.p.m.; and* ▲, *400 r.p.m.*

were placed in volumetric flasks with the solvents and agitated in a water bath at 30°. Duplicate samples were withdrawn at 12–24-hr. intervals, filtered through a 0.45-μ Millipore filter, and analyzed spectrophotometrically. The solubilities of sulfathiazole and of methylprednisolone in the various solvents used are listed in Table I.

Sulfathiazole Studies—Figure 3 represents the results of a typical sulfathiazole crystal growth experiment in 95% v/v ethanol where the growth in microns along the long axis was measured and plotted as a function of time. The growth–time plots were always found to be linear with little scatter, except in cases where the apex of the crystals was initially not sharp due to prior washing in a solvent. In the latter situations the slopes of the plots became linear after a short initial period during which time sharpening of the crystal edges occurred. Size variations of the crystals employed in these experiments were found to have little or no effect upon the rate of growth over a crystal length range of 450 to 2000 μ. Also the method of preparation of the seed crystals usually had no effect upon the growth rates.

From limiting linear portions of the data such as those presented in Fig. 3, the rates were obtained by determining the slopes for each experiment and are plotted in Figs. 4–15 for each of the three dimensions of the sulfathiazole crystal in a number of solvents under different stirring rates. These rates have been presented as a function of the supersaturation ratio, S, which is the supersaturated solution concentration divided by the solubility. The data for sulfathiazole crystal growth studies in water (Figs. 4–6) appeared to be linearly dependent upon the supersaturation ratio, S, for all stirring speeds and for all three dimensions. Least-squares parameters calculated for these studies are tabulated in Table II and showed that a common intercept of $S = 1.17$ was consistent with all of the water experiments.

The stirring rate dependence found in these experiments strongly supports a diffusion-controlled process in region $S > 1.17$.

Table II—Linear Least-Squares Parameters[a] for Sulfathiazole Using Water as a Solvent

Axis	Stirring, r.p.m.	Intercept[b]	Av. Intercept	Slope[c]
Length	10	0.99	1.10	1.26
	150	1.09		3.41
	400	1.22		5.56
Width	10	1.33	1.24	0.04
	150	1.08		1.67
	400	1.31		2.54
Thickness	10	1.27	1.16	0.29
	150	1.01		0.84
	400	1.20		1.59
Av. for all data		1.17		

[a] See *Appendix*. [b] Both slope and intercept were allowed to vary. [c] Curve was forced through average intercept for all three axes, $S = 1.17$.

Table III—Linear Least-Squares Parameters[a] for Sulfathiazole Using 95% Hydroalcoholic Solution as a Solvent

Axis	Stirring, r.p.m.	Intercept[b]	Av. Intercept	Slope[c]
Length	10	1.15	1.14	6.89
	150	1.10		18.00
	240	1.22		21.63
	400	1.09		18.46
	150, 240, 400	1.12		18.62
Width	10	0.94	1.05	2.95
	150	1.17		8.40
	400	1.04		7.20
	150, 400	1.11		7.91
Thickness	10	0.80	1.08	1.90
	150	1.16		5.90
	400	1.29		5.50
	150, 400	1.17		5.70
Av. for all data		1.10		

[a] See *Appendix*. [b] Both slope and intercept were allowed to vary. [c] Curve was forced through average intercept for all three axes, $S = 1.10$.

The growth rates of all of the dimensions appear to be determined by the transport of solute through the solvent at even the highest stirring speeds. For comparison purposes, the growth rate curves as a function of the stirring rate shown by Figs. 4, 5, and 6 were arbitrarily fitted by linear curves passing through the 1.17 intercept, with arbitrary slopes chosen so that a constant slope ratio as a function of the stirring rate was maintained in all three figures; *i.e.*, the ratios of the slope at 400 r.p.m. to that at 150 r.p.m. to that at 10 r.p.m. was constant. The agreement of these arbitrary curves with the experimental data points strongly suggests that the stirring rate dependence is the same for all three dimensions.

The intercept value of about $S = 1.17$ might be related to a "critical" supersaturation for two-dimensional nucleation. Therefore, at very low supersaturation ratios, crystal growth may occur extremely slowly or may be dependent upon the existence of screw dislocations.

Sulfathiazole crystal growth experiments with the other solvent systems studied (Figs. 7–15) generally appeared to show stirring rate dependence at the lower speeds but interestingly the rates were much less stirring dependent at the higher speeds in these solvents than in the water experiments. The growth rates observed in 95% v/v ethanol, 50% ethanol–water, and 40% butanol–60% ethanol were found to be essentially independent of stirring rates above 150 r.p.m. The growth rates at 10 r.p.m. were generally much slower in these studies See Tables III–V.

These experiments in the alcohol-containing solvents appear to be consistent with the interpretation that at lower agitation conditions (~10 r.p.m.) the crystal growth rates are solvent diffusion controlled at the higher supersaturations. However, at higher

Table IV—Linear Least-Squares Parameters[a] for Sulfathiazole Using 50% Hydroalcoholic Solution as a Solvent

Axis	Stirring, r.p.m.	Intercept[b]	Av. Intercept	Slope[c]
Length	10	1.13	1.10	6.97
	150	1.08		33.55
	400	1.08		34.78
	150, 400	1.08		34.17
Width	10	1.14	1.08	5.08
	150	1.04		9.98
	400	1.05		13.34
	150, 400	1.05		11.77
Thickness	10	1.15	1.02	2.70
	150	0.84		4.87
	400	1.08		5.27
	150, 400	0.98		5.07
Av. for all data		1.07		

[a] See *Appendix*. [b] Both slope and intercept were allowed to vary. [c] Curve was forced through average intercept for all three axes, $S = 1.07$.

Table V—Linear Least-Squares Parameters[a] for Sulfathiazole Using 40% *sec*-Butanol–60% Ethanol as a Solvent

Axis	Stirring, r.p.m.	Intercept[b]	Av. Intercept	Slope[c]
Length	10	1.53	1.39	6.27
	150	1.33		21.04
	400	1.30		15.39
	150, 400	1.29		13.13
Width	10	1.52	1.40	2.01
	150	1.27		5.91
	400	1.42		6.49
	150, 400	1.31		5.17
Thickness	10	1.41	1.50	0.90
	150	1.56		3.57
	400	1.52		3.86
	150, 400	1.54		4.67
Av. for all data		1.43		

[a] See *Appendix*. [b] Both slope and intercept were allowed to vary. [c] Curve was forced through average intercept for all three axes, $S = 1.43$.

stirring rates, the crystal growth rates are surface controlled rather than diffusion controlled.

In all the plots shown in Figs. 7–15, there appear to be significant intercepts on the supersaturation axes as were found to be the case in the water experiments Figs. 4–6. In the cases of ethanol and ethanol–water experiments, the intercepts are the order of $S \simeq 1.1$. For the 40% *sec*-butanol–60% ethanol the intercept is much larger, the order of $S \simeq 1.43$. These intercepts might again be related to the "critical" supersaturation for two-dimensional nucleation in the respective solvents. It is noteworthy to mention at this point that in *n*-propanol, crystal growth of sulfathiazole did not occur even at supersaturation of 2.2, and that in *sec*-butyl alcohol a supersaturation ratio of 5.5 showed no growth. The observations in conjunction with the *S*-intercepts found in the present studies suggest a direct relationship between the "critical *S*" for two-dimensional nucleation and alcohol solvent polarity—*viz.*, the higher the alcohol solvent polarity the smaller the *S*-intercept.

METHYLPREDNISOLONE CRYSTAL GROWTH IN WATER

The growth rate along the rod axis (refractive index = 1.602) of the methylprednisolone crystal in water at various supersaturations and stirring speeds was also studied. The results are shown in Fig. 16. The data at all stirring speeds appear to be linear with supersaturation and show a small intercept on the *S*-axis. The least-squares linear curves were calculated and the slopes and intercepts tabulated in Table VI. As opposed to sulfathiazole crystals, Table VI clearly shows that the rates for methylprednisolone appeared to be virtually independent of stirring for all stirring rates over the range of 20 to 400 r.p.m. This supports a mechanism in which the crystal growth rate of methylprednisolone in water is surface controlled in the range of agitation rates employed in these studies.

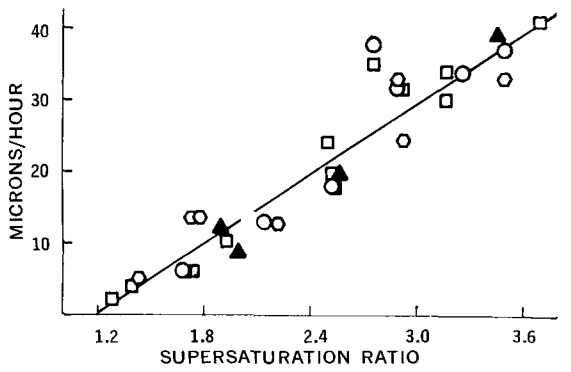


Figure 16—*Crystal growth rate of methylprednisolone in water as a function of supersaturation at different stirring speeds. Key:* ▲, *20 r.p.m.;* □, *150 r.p.m.;* ○, *240 r.p.m.; and* ⬡, *400 r.p.m.*

Table VI—Linear Least-Squares Parameters for Methylprednisolone Using Water as the Solvent

Stirring, r.p.m.	Intercept[a]	Slope[a]
20	1.43	18.732
150	1.27	17.498
240	1.37	19.163
400	0.90	13.329
All data points	1.23	16.896

[a] Both slope and intercept were allowed to vary.

For this reason a least-squares linear curve was calculated using all the methylprednisolone growth rates regardless of stirring rate, and this curve was drawn in Fig. 16.

SUMMARY

These results show the advantage of using a single crystal technique to obtain quantitative data regarding the growth rate of crystalline drugs. In particular, it permits meaningful quantitation of the stirring rate dependence and the effect of the composition of the solution on the crystal growth rate. This permits an insight as to the controlling mechanism operative under the conditions of the experiment.

APPENDIX: LEAST-SQUARES PARAMETERS

It is generally accepted that the results of a least-squares optimization of the data will depend on the function chosen, *i.e.*, straight line, parabola, exponential, *etc.* It is not always recognized, however, that one must be careful to use the appropriate least-squares treatment if one is to fit the best linear curve to the experimental data. For example, one can assume that the experimental error resides solely in the y data, in the x data, or in both. Obviously the best fit curve obtained will depend on which approach was used.

It also should be noted that the least-squares approach (a mathematical interpretation as opposed to a physical interpretation) should not contradict any known facts and/or limitations set by the experimental system. For example, if it is known that the data plot passes through the origin, then a least-squares set of equations which ignores this requirement may not only result in an artificial intercept but also an incorrect slope.

By definition, a crystal must be exposed to a supersaturation ratio greater than 1 in order for it to grow. Therefore, any optimization of the authors' data that yields a curve which displays a positive y-intercept must be incorrect. This means the least-squares approach to be used must yield an x-intercept of the supersaturation ratio axis if the most correct slope is to be calculated.

In addition, it is reasonable to assume that all curves of a given system should be represented by the same intercept for the following reason. At the x-intercept, the crystal growth is obviously independent of the stirring rate and most likely is regulated by a surface-controlled process. If this is true, all curves must intercept at the same point as the only difference between curves is the stirring rate.

With the above in mind, the following approach was arbitrarily used to determine the intercepts and slopes for the systems studied. First the least-squares parameters were determined allowing both the intercept and slope to vary, *i.e.*, $y = mx + b$ where m is the slope and the x-intercept is b/m. The average x-intercept for all curves in a given system was calculated and assumed to be the best estimate of the supersaturation ratio axis intercept. The best estimate for the slope of each curve was then determined using the least-squares equations for $y = m(x - \bar{I})$, where $\bar{I}$ is the average intercept for the system.

REFERENCES

(1) J. K. Dale and S. L. Ross, U. S. pat. 2,861,920, Nov. 25, 1958.

(2) J. K. Dale and R. A. Hanze, U. S. pat. 3,062,712, Nov. 6, 1962.

(3) J. Polderman, *Bull. Chim. Pharm.*, **101**, 105(1962).

(4) J. E. Carless and A. A. Foster, *J. Pharm. Pharmacol.*, **18**, 697(1966).
(5) J. Glasby and K. Ridgway, *ibid.*, **20**, 94S(1968).
(6) H. Nogami and T. Nagai, *Yakkyoku*, **9**, 29(1958).
(7) J. Hasegawa and T. Nagai, *Chem. Pharm. Bull. (Tokyo)*, **6**, 129(1958).
(8) D. C. Grove and G. L. Keenan, *J. Amer. Chem. Soc.*, **63**, 97(1941).
(9) J. A. Biles, *J. Pharm. Sci.*, **50**, 464(1961).
(10) D. J. Allen, G. Milosovich, and A. M. Mattocks, *ibid.*, **54**, 383(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication November 20, 1969.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

* Present address: Smith Kline & French Laboratories, Philadelphia, Pa.

Theoretical Model Studies of Drug Absorption and Transport in the Gastrointestinal Tract I

AKIRA SUZUKI, W. I. HIGUCHI, and N. F. H. HO

Abstract □ The simultaneous chemical equilibria and mass transfer of basic and acidic drugs through a two-phase compartment model were theoretically investigated. The model consisted of a well-stirred bulk aqueous phase, an aqueous diffusion layer, and a lipid barrier for perfect and imperfect sink cases. The nonsteady and quasi-steady-state changes in the concentration–distance distributions in the lipid phase were studied. The rate of change of the total drug concentration in the bulk aqueous phase was described in the general form of a first-order equation useful for the evaluation of experiments. A limiting steady-state relationship involving the transport rate with the partition coefficient, pH at the aqueous–lipid interface, dissociation constant of the drug, aqueous and lipid diffusion coefficients, and thickness of the diffusion layer was derived. Increasing the agitation rate in the aqueous phase markedly affects the pH profiles for the rate of transport. The pH-partition theory is shown to be a limiting case of the more general approach presented.

Keyphrases □ Drug absorption, transport—theoretical model □ Model, two-phase compartment—theoretical investigation □ Chemical equilibria, mass transfer—two-phase compartment model □ Agitation rate effect—rate transport pH profiles

The increasing interest in the mechanistic understanding rather than in only a mathematical representation of drug transport and absorption phenomena should dictate systematic physical model analyses of various *in vitro* situations. Thus, detailed theoretical considerations of diffusion and equilibria involving multibarrier systems and the carrying out of appropriate model experiments are necessary for the isolation of the important *in vivo* factors.

Recent investigations (1–4) in these laboratories have been devoted to the physical model approach to a number of situations in this regard. The present paper is concerned with the problem of treating the time dependency and the pH-buffer dependency for the transport of basic and acidic solutes into and across lipoidal barriers. It is, to some extent, an extension of the works of Howard *et al.* (3) and Stehle (4) and should be useful in the understanding of gastrointestinal and buccal absorption problems. In the accompanying paper (5), the techniques developed here are applied to some of the data on *in situ* drug absorption published by Koizumi *et al.* (6, 7).

THEORY

General Description of the Model—The simultaneous mass transfer and chemical equilibrium reactions in a system consisting of two homogeneous phases will follow the one-dimensional model in Fig. 1. The bulk aqueous phase is well stirred and consists of a basic drug and buffer. At $x \leq -h$,

$$(TR)_{-h} = (R)_{-h} + (RH^+)_{-h} \quad \text{(Eq. 1)}$$

$$(TB)_{-h} = (B^-)_{-h} + (HB)_{-h} \quad \text{(Eq. 2)}$$

where (TR) is the total drug concentration of R and RH^+ species and (TB) is the total buffer concentration of B^- and HB species. It is assumed that electrical neutrality holds everywhere in the aqueous phase. Consequently, at $x \leq 0$,

$$(H^+) + (RH^+) + (Na^+) - (OH^-) - (B^-) = 0 \quad \text{(Eq. 3)}$$

where (Na^+) is the cation concentration derived from the buffer. It is further assumed that the following equilibrium reactions are instantaneous,

$$\frac{(R)(H^+)}{(RH^+)} = K_{a,R} \quad \text{(Eq. 4a)}$$

$$\frac{(B^-)(H^+)}{(HB)} = K_{a,HB} \quad \text{(Eq. 4b)}$$

$$(H^+)(OH^-) = K_w \quad \text{(Eq. 4c)}$$

where $K_{a,R}$, $K_{a,HB}$, and K_w are the dissociation constants of drug, buffer, and water, respectively.

Under the assumption of quasi-steady-state conditions existing within the aqueous diffusion layer, the total flux of the drug to the water–lipid interface is expressed by the equation

$$G = -D_{RH}\frac{d(RH^+)}{dx} - D_R\frac{d(R)}{dx} \quad \text{(Eq. 5)}$$

$$(-h \leq x \leq -0)$$

where G is the total flux of the drug and D_{RH} and D_R are the diffusion coefficients; upon integration, the solution is

$$Gh = D_{RH}(RH^+)_{-h} + D_R(R)_{-h} - D_{RH}(RH^+)_{-0} - D_R(R)_{-0} \quad \text{(Eq. 6)}$$

In an analogous procedure for the buffer species,

$$D_{HB}(HB)_{-h} + D_B(B^-)_{-h} - D_{HB}(HB)_{-0} - D_B(B^-)_{-0} = 0 \quad \text{(Eq. 7)}$$

(4) J. E. Carless and A. A. Foster, *J. Pharm. Pharmacol.*, **18**, 697(1966).
(5) J. Glasby and K. Ridgway, *ibid.*, **20**, 94S(1968).
(6) H. Nogami and T. Nagai, *Yakkyoku*, **9**, 29(1958).
(7) J. Hasegawa and T. Nagai, *Chem. Pharm. Bull. (Tokyo)*, **6**, 129(1958).
(8) D. C. Grove and G. L. Keenan, *J. Amer. Chem. Soc.*, **63**, 97(1941).
(9) J. A. Biles, *J. Pharm. Sci.*, **50**, 464(1961).
(10) D. J. Allen, G. Milosovich, and A. M. Mattocks, *ibid.*, **54**, 383(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication November 20, 1969.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

* Present address: Smith Kline & French Laboratories, Philadelphia, Pa.

Theoretical Model Studies of Drug Absorption and Transport in the Gastrointestinal Tract I

AKIRA SUZUKI, W. I. HIGUCHI, and N. F. H. HO

Abstract □ The simultaneous chemical equilibria and mass transfer of basic and acidic drugs through a two-phase compartment model were theoretically investigated. The model consisted of a well-stirred bulk aqueous phase, an aqueous diffusion layer, and a lipid barrier for perfect and imperfect sink cases. The nonsteady and quasi-steady-state changes in the concentration–distance distributions in the lipid phase were studied. The rate of change of the total drug concentration in the bulk aqueous phase was described in the general form of a first-order equation useful for the evaluation of experiments. A limiting steady-state relationship involving the transport rate with the partition coefficient, pH at the aqueous–lipid interface, dissociation constant of the drug, aqueous and lipid diffusion coefficients, and thickness of the diffusion layer was derived. Increasing the agitation rate in the aqueous phase markedly affects the pH profiles for the rate of transport. The pH-partition theory is shown to be a limiting case of the more general approach presented.

Keyphrases □ Drug absorption, transport—theoretical model □ Model, two-phase compartment—theoretical investigation □ Chemical equilibria, mass transfer—two-phase compartment model □ Agitation rate effect—rate transport pH profiles

The increasing interest in the mechanistic understanding rather than in only a mathematical representation of drug transport and absorption phenomena should dictate systematic physical model analyses of various *in vitro* situations. Thus, detailed theoretical considerations of diffusion and equilibria involving multibarrier systems and the carrying out of appropriate model experiments are necessary for the isolation of the important *in vivo* factors.

Recent investigations (1–4) in these laboratories have been devoted to the physical model approach to a number of situations in this regard. The present paper is concerned with the problem of treating the time dependency and the pH-buffer dependency for the transport of basic and acidic solutes into and across lipoidal barriers. It is, to some extent, an extension of the works of Howard *et al.* (3) and Stehle (4) and should be useful in the understanding of gastrointestinal and buccal absorption problems. In the accompanying paper (5), the techniques developed here are applied to some of the data on *in situ* drug absorption published by Koizumi *et al.* (6, 7).

THEORY

General Description of the Model—The simultaneous mass transfer and chemical equilibrium reactions in a system consisting of two homogeneous phases will follow the one-dimensional model in Fig. 1. The bulk aqueous phase is well stirred and consists of a basic drug and buffer. At $x \leq -h$,

$$(TR)_{-h} = (R)_{-h} + (RH^+)_{-h} \quad \text{(Eq. 1)}$$

$$(TB)_{-h} = (B^-)_{-h} + (HB)_{-h} \quad \text{(Eq. 2)}$$

where (TR) is the total drug concentration of R and RH^+ species and (TB) is the total buffer concentration of B^- and HB species. It is assumed that electrical neutrality holds everywhere in the aqueous phase. Consequently, at $x \leq 0$,

$$(H^+) + (RH^+) + (Na^+) - (OH^-) - (B^-) = 0 \quad \text{(Eq. 3)}$$

where (Na^+) is the cation concentration derived from the buffer. It is further assumed that the following equilibrium reactions are instantaneous,

$$\frac{(R)(H^+)}{(RH^+)} = K_{a,R} \quad \text{(Eq. 4a)}$$

$$\frac{(B^-)(H^+)}{(HB)} = K_{a,HB} \quad \text{(Eq. 4b)}$$

$$(H^+)(OH^-) = K_w \quad \text{(Eq. 4c)}$$

where $K_{a,R}$, $K_{a,HB}$, and K_w are the dissociation constants of drug, buffer, and water, respectively.

Under the assumption of quasi-steady-state conditions existing within the aqueous diffusion layer, the total flux of the drug to the water–lipid interface is expressed by the equation

$$G = -D_{RH}\frac{d(RH^+)}{dx} - D_R\frac{d(R)}{dx} \quad \text{(Eq. 5)}$$

$$(-h \leq x \leq -0)$$

where G is the total flux of the drug and D_{RH} and D_R are the diffusion coefficients; upon integration, the solution is

$$Gh = D_{RH}(RH^+)_{-h} + D_R(R)_{-h} - D_{RH}(RH^+)_{-0} - D_R(R)_{-0} \quad \text{(Eq. 6)}$$

In an analogous procedure for the buffer species,

$$D_{HB}(HB)_{-h} + D_B(B^-)_{-h} - D_{HB}(HB)_{-0} - D_B(B^-)_{-0} = 0 \quad \text{(Eq. 7)}$$

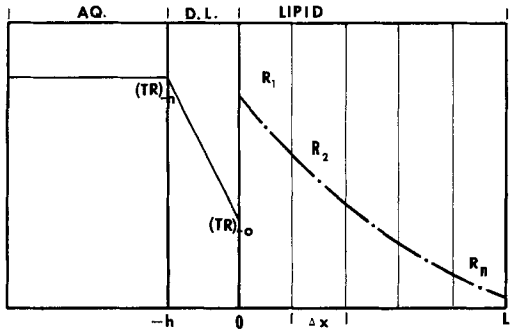


Figure 1—*Two-phase diffusion model consisting of a bulk aqueous phase, aqueous diffusion layer, and a lipid phase. Concentration distribution of total drug species is governed by steady-state rate into the diffusion layer, and concentration of the diffusing nonionized drug in the lipid phase is governed by nonsteady-state rate determined by numerical finite-difference methods.*

in which case the total flux of the buffer is zero.

If only the nonprotonated drug molecule is capable of diffusing into the lipid phase, the equilibrium assumed to be established instantaneously at the aqueous–lipid interface is expressed by the partition coefficient,

$$P = (R)_{+0}/(R)_{-0} \quad (x = 0) \qquad \text{(Eq. 8)}$$

where P is the partition coefficient; (R) is the concentration of the nonionized drug; and the subscripts, -0 and $+0$, refer to the aqueous and lipid side of the interface, respectively. The continuity of flow through the interface is given by

$$G = -D_{R,oil}\left(\frac{\partial(R)}{\partial x}\right)_{x=0} \qquad \text{(Eq. 9)}$$

where $D_{R,oil}$ is the diffusion coefficient of R in lipid. In this model the flux of the total drug species in the aqueous diffusion layer is taken to be the same as the flux of the nonionized drug from the interface to the lipid phase. Within the lipid, Fick's second law applies:

$$\frac{\partial(R)}{\partial t} = D_{R,oil}\frac{\partial^2(R)}{\partial x^2} \quad (x > 0) \qquad \text{(Eq. 10)}$$

and at $x = L$ two extreme boundary conditions can exist, that is, (*a*) for the impermeable boundary,

$$\left(\frac{\partial(R)}{\partial x}\right)_{x=L} = 0 \qquad \text{(Eq. 11}a\text{)}$$

(*b*) for the perfect sink,

$$(R)_{x=L} = 0 \qquad \text{(Eq. 11}b\text{)}$$

Changes in the Concentration–Distance Distribution in the Aqueous and Lipid Phases with Time—The simultaneous time change of the total drug concentration–distance distribution in the aqueous phase in which steady-state conditions are assumed and that for the diffusing basic drug molecule in the lipid phase in which nonsteady-state conditions exist cannot be solved analytically. Numerical methods and the utilization of a high-speed computer are necessary.

In using the finite-difference method (8), the lipid compartment is divided into a number of cells of equal intervals as shown in Fig. 1. Accordingly, the concentration of drug in the bulk aqueous phase and each cell in the lipid phase and at the interface can be calculated by solving the following set of differential equations:

$$\frac{d(TR)_{-h}}{dt} = -\frac{A}{V}G \qquad \text{(Eq. 12)}$$

$$\frac{d(R_1)}{dt} = \frac{G}{\Delta x} - \frac{D_{R,oil}[(R_1) - (R_2)]}{(\Delta x)^2} \qquad \text{(Eq. 13)}$$

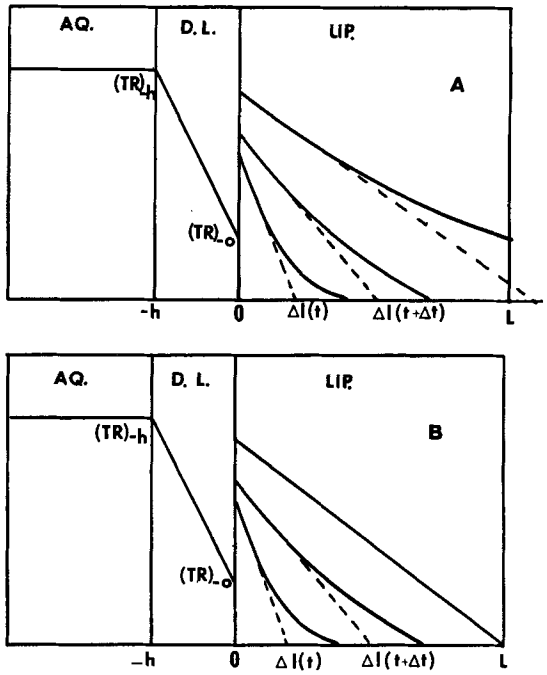


Figure 2—*Model used to estimate* F(t) *by linear approximation of the concentration–distance curves in the lipid phase. Key: A, impermeable boundary case; and B, perfect-sink case.*

$$\frac{d(R_i)}{dt} = \frac{D_{R,oil}}{(\Delta x)^2}[(R_{i-1}) - 2(R_i) + (R_{i+1})] \quad (i = 2, 3, 4 \ldots n-1) \qquad \text{(Eq. 14)}$$

and at $x = L$, for the impermeable boundary or no-sink case,

$$\frac{d(R_n)}{dt} = \frac{D_{R,oil}}{(\Delta x)^2}[(R_{n-1}) - (R_n)] \qquad \text{(Eq. 15}a\text{)}$$

and for the perfect-sink case,

$$\frac{d(R_n)}{dt} = \frac{D_{R,oil}}{(\Delta x)^2}[(R_{n-1}) - 3(R_n)] \qquad \text{(Eq. 15}b\text{)}$$

where A is the surface area, V is the volume of the bulk aqueous phase, (R_i) is the concentration of neutral drug in the ith cell, Δx is the length of each cell, n is the total number of cells, and the other terms are defined as before.

Since the concentration of drug species at the various aqueous boundaries is dependent upon pH, the hydrogen-ion concentration must be known. From Eqs. 1 through 4 the hydrogen-ion concentration in the bulk phase is given by

$$(H^+)^4_{-h} + \{K_{a,R} + K_{a,HB} + (TR)_{-h} + (Na^+)_{-h}\}(H^+)^3_{-h} + \{[(TR)_{-h} + K_{a,R} - (TB)_{-h}]K_{a,HB} - (K_{a,R} + K_{a,HB})(Na^+)_{-h}K_w\}(H^+)^2_{-h} - \{K_{a,HB}(TB)_{-h} + (K_{a,R} + K_{a,HB})K_w + K_{a,R}K_{a,HB}(Na^+)_{-h}\}(H^+)_{-h} - K_{a,R}K_{a,HB}K_w = 0 \qquad \text{(Eq. 16)}$$

Table I—Numerical Dimensions of Constants and Initial Drug and Buffer Concentrations Used for Computation

$V = 10$ cm.^{3}	$A = 10$ cm.^{2}	$h = 10^{-2}$ cm.
$K_{a,R} = 10^{-8}$	$D = 10^{-5}$ cm.^{2} sec.$^{-1}$ for all diffusion coefficients	
Initial concentrations: $(TB)_{-h} = 10^{-2}$ M		
$(TR)_{-h} = 10^{-4}$ M		

	No-Sink Case	Perfect-Sink Case
L	10^{-1} cm.	5×10^{-2} cm.
$(Na^+)_{-h}$	0	$^1/_2(TB)_{-h}$
P	1, 100	100
$pK_{a,HB}$	4, 6, 8, 10, 12	4, 6, 8, 10

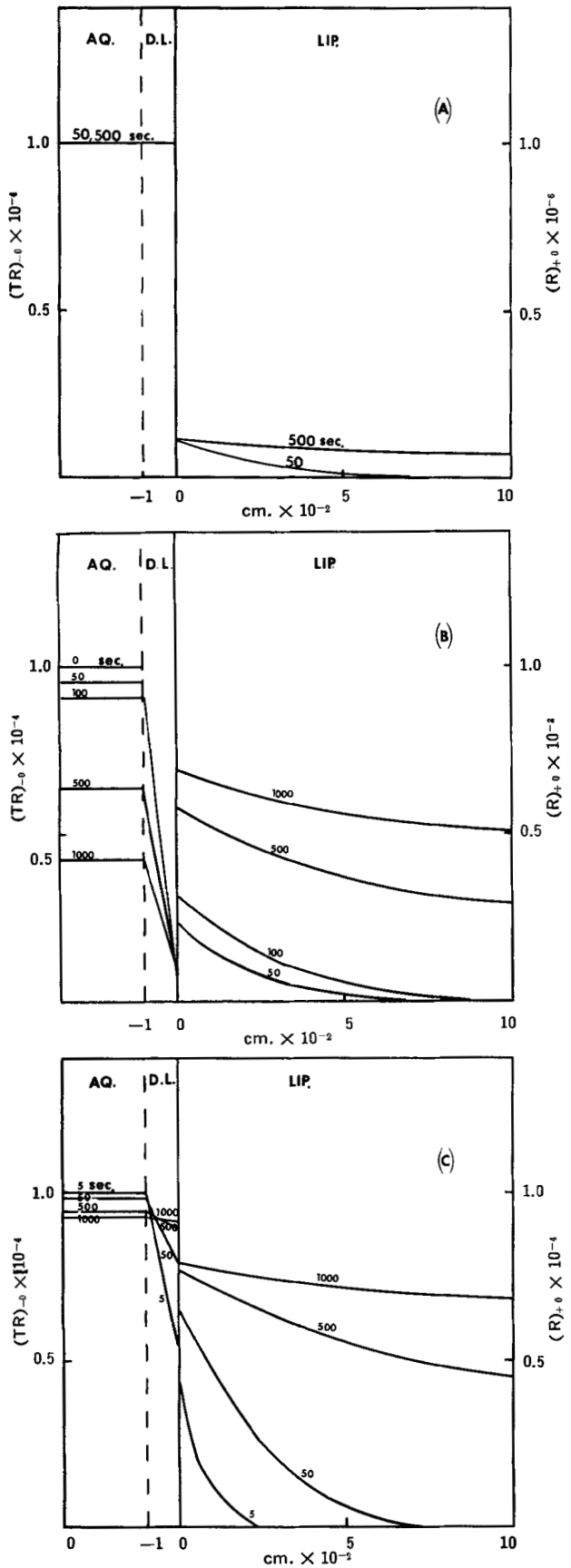


Figure 3—*Time-dependent concentration distribution curves of total drug species (TR) in the aqueous phase and the nonprotonated drug (R) in the oil phase for the no-sink case: (A) initial bulk aqueous pH = 3.05 and partition coefficient* P = *100; (B) pH = 8.82,* P = *100; (C) pH = 8.82,* P = *1.0.*

Table II—Change in the Bulk and Surface pH with Time for the No-Sink Case in a Low Buffer Capacity System with $P = 100$

$K_{a,HB}$	Initial pH$_{-h}$	Initial pH$_{-0}$	After 500 sec pH$_{-h}$	After 500 sec pH$_{-0}$
10^{-6}	3.05	3.05	3.05	3.05
10^{-8}	6.01	5.71	5.98	5.95
10^{-10}	7.79	6.84	7.67	7.33
10^{-12}	8.82	7.70	8.71	8.22

The equation for the hydrogen-ion concentration at the interface is derived from Eqs. 3, 4, and 6–9; thus,

$$(H^+)^4_{-0} + [\beta + \gamma\eta + \alpha + \gamma(Na^+)_{-0}](H^+)^3_{-0} + [(\alpha + \beta)\eta - \gamma K_w - \gamma\delta + (\gamma\eta + \beta)(Na^+)_{-0}](H^+)^2_{-0} - [(\beta + \gamma\eta)K_w - \beta\delta + \beta\eta(Na^+)_{-0}](H^+)_{-0} - \beta\eta K_w = 0 \quad \text{(Eq. 17)}$$

where

$$\alpha = \frac{1}{K_{a,R}}\left\{[D_R(R)_{-h} + D_{RH}(RH^+)_{-h}] + \frac{2hD_{R,oil}(R_I)}{\Delta x}\right\}$$

$$\beta = D_R + \frac{2hPD_{R,oil}}{\Delta x}$$

$$\gamma = D_{RH}/K_{a,R}$$

$$\delta = \frac{K_{a,HB}}{D_B}[D_B(B^-)_{-h} + D_{HB}(HB)_{-h}]$$

$$\eta = \frac{K_{a,HB}D_B}{D_{HB}}$$

Here, it is assumed that $(Na^+)_{-h} = (Na^+)_{-0}$. Finally, the concentration of the diffusing specie at the aqueous side of the interface, *i.e.* $(R)_{-0}$, is expressed by

$$(R)_{-0} = \frac{\alpha K_{a,R}}{\beta + \gamma(H^+)_{-0}} \quad \text{(Eq. 18)}$$

Rate of Change of the Total Concentration of Drug in the Bulk Aqueous Phase—To relate the theory of the diffusion model to the usual treatment of experimental data, *i.e.*, the rate of change of the total drug concentration in the bulk aqueous phase, it is useful to rewrite Eq. 12 in the following manner,

$$\frac{d(TR)_{-h}}{dt} = -\frac{AD_R}{Vh}F(t)(TR)_{-h} \quad \text{(Eq. 19)}$$

where $F(t)$ is defined as

$$F(t) = G/G_{max.} \quad \text{(Eq. 20)}$$

and

$$G_{max.} = \frac{D_R(TR)_{-h}}{h} \quad \text{(Eq. 21)}$$

Equation 20 is the ratio of the actual flux to the maximum flux which takes place when all of the drug species in the bulk aqueous phase is nonprotonated and the concentration of R at the interface is zero. By performing the integration, Eq. 19 becomes

$$\ln\frac{(TR)_{-h}}{(TR)_{-h,t=0}} = -\frac{AD_R}{Vh}\int_0^t F(t)dt \quad \text{(Eq. 22)}$$

$$(0 < F(t) \leq 1)$$

and by utilizing the mean-value theorem to evaluate the integral,

$$\ln\frac{(TR)_{-h}}{(TR)_{-h,t=0}} = -\frac{AD_R}{Vh}F(\Phi)t \quad \text{(Eq. 23)}$$

$$(0 \leq \Phi \leq t)$$

If $F(\Phi)$ is relatively invariant with Φ, an apparent first-order decrease in the drug concentration with time will be observed. In general, the slope of ln $(TR)_{-h}$ *versus t* plots is

$$K_u = -\frac{AD_R}{Vh}F(\Phi) \quad \text{(Eq. 24)}$$

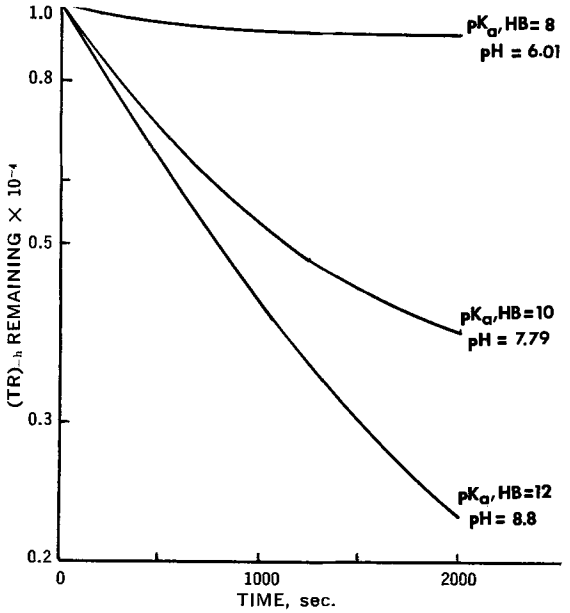


Figure 4—*First-order plot of the change in the total drug concentration in the bulk aqueous phase with time. No-sink case for different initial bulk aqueous pH.*

Approximation of the Function $F(t)$—Since the function $F(t)$ influences the apparent first-order rate constant, the elucidation of the nature of the function in terms of the partition coefficient, pH at the interface, diffusion coefficients, and thickness of the diffusion layer would lead to meaningful physical interpretation. For this purpose, an approximation of $F(t)$ is derived for two cases, perfect-sink and no-sink situations.

No-Sink Case—The analysis is based on the model for the concentration–distance distribution changes with time in Fig. 2A. It assumes a linear approximation of the nonsteady-state concentration profile of R at time t in the lipid phase. From Eqs. 8 and 9,

$$G = \frac{D_{R,oil}\, P(R)_{-0}}{\Delta 1} \qquad \text{(Eq. 25)}$$

where $\Delta 1$ is the distance from the interface to the point where $(R)_{+0}$ is zero and changes with time such that $0 < \Delta 1 > L$. Furthermore, assuming that the flux G has not changed appreciably during the period needed to build up the concentration distribution in the lipid phase, the total amount of drug transported through the interface is approximated by

$$\int_0^t G\, dt = \int_0^1 (R)_{oil}\, dx \qquad \text{(Eq. 26}a\text{)}$$

Thus,

$$Gt = \tfrac{1}{2}(R)_{-0} P \Delta 1 \qquad \text{(Eq. 26}b\text{)}$$

and it follows from Eqs. 25 and 26b that

$$\Delta 1 = (2D_{R,oil}t)^{1/2} \qquad \text{(Eq. 27)}$$

Solving for $(R)_{-0}$ with the aid of Eqs. 6 and 25 and $D_{RH} = D_R$,

$$(R)_{-0} = \frac{(TR)_{-h}}{1 + [(H^+)_{-0}]/(K_{a,R}) + (hD_{R,oil}P)/(\Delta 1\ D_R)} \qquad \text{(Eq. 28)}$$

and with Eq. 27,

$$(R)_{-0} = \frac{(TR)_{-h}}{1 + [(H^+)_{-0}]/(K_{a,R}) + (hD_{R,oil}P)/(D_R\sqrt{2D_{R,oil}t})} \qquad \text{(Eq. 29)}$$

It is convenient to define a new function $f(t)$ as the approximation of $F(t)$. By means of Eqs. 1, 4, 6, 20, and 21, the function can be simply expressed by

$$f(t) \sim F(t) = G/G_{max.} =$$

$$1 - \frac{\{1 + [(H^+)_{-0}]/(K_{a,R})\}(R)_{-0}}{(TR)_{-h}} \qquad \text{(Eq. 30)}$$

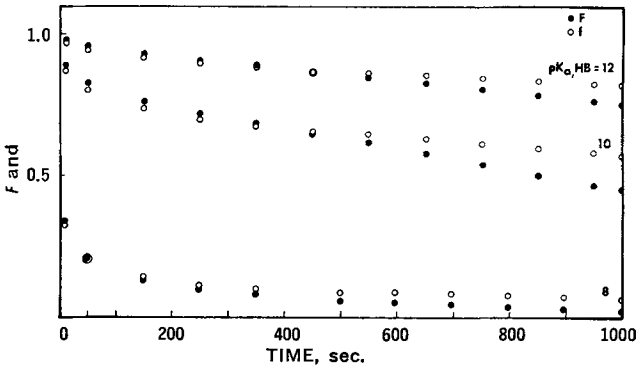


Figure 5—*Change in the diffusion efficiency coefficient with time for the no-sink case. The numerically calculated coefficient* F(t) *by Eq. 20 is compared with the approximation* f(t) *by Eq. 31.*

and the substitution of Eq. 29 leads to

$$f(t) = \frac{1}{\{1 + [(H^+)_{-0}]/(K_{a,R})\}\ T + 1} \qquad \text{(Eq. 31)}$$

where

$$T = \frac{D_R}{hP}\left(\frac{2t}{D_{R,oil}}\right)^{1/2} \qquad \text{(Eq. 32)}$$

Therefore, in this case of an impermeable boundary in the lipid phase, the time-dependent nature of the function $f(t)$ makes it evident that first-order diffusion kinetics are not applicable.

Perfect-Sink Case—In this situation the diffusion of the drug can be divided into two stages (Fig. 2B). The first stage is the period of nonsteady-state rate in the lipid phase leading to the second stage of quasi-steady-state conditions. In other words, $F(t)$ will eventually be constant (time independent) after an initial lag period.

In the nonsteady-state period the approximate function $f(t)$ given by Eqs. 31 and 32 can be applied. From Eq. 27, with $\Delta 1 = L$ always, the time lag is

$$\tau = \frac{L^2}{2D_{R,oil}} \qquad \text{(Eq. 33)}$$

where τ is the lag time, and L is the thickness of the lipid phase.

In the steady-state period, Eq. 25 can be rewritten as

$$G = \frac{D_{R,oil}P(R)_{-0}}{L} \qquad \text{(Eq. 34)}$$

After the substitution of Eqs. 21, 28, and 34 into 20, the function[1] $f(T)$ takes the same form as Eq. 31; that is,[2]

$$f(T) = \frac{1}{\{1 + [(H^+)_{-0}]/(K_{a,R})\}\ T + 1} \qquad \text{(Eq. 35)}$$

where, in this case,

$$T = \frac{D_R L}{PhD_{R,oil}} \qquad \text{(Eq. 36)}$$

Therefore, in the perfect-sink case, a log $(TR)_{-h}$ *versus* t plot should be linear after a lag period.

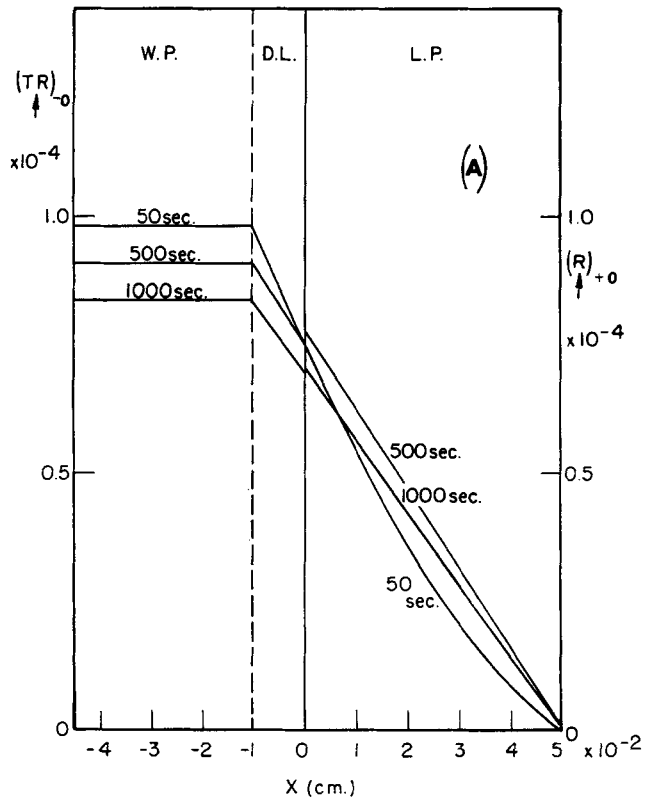
---

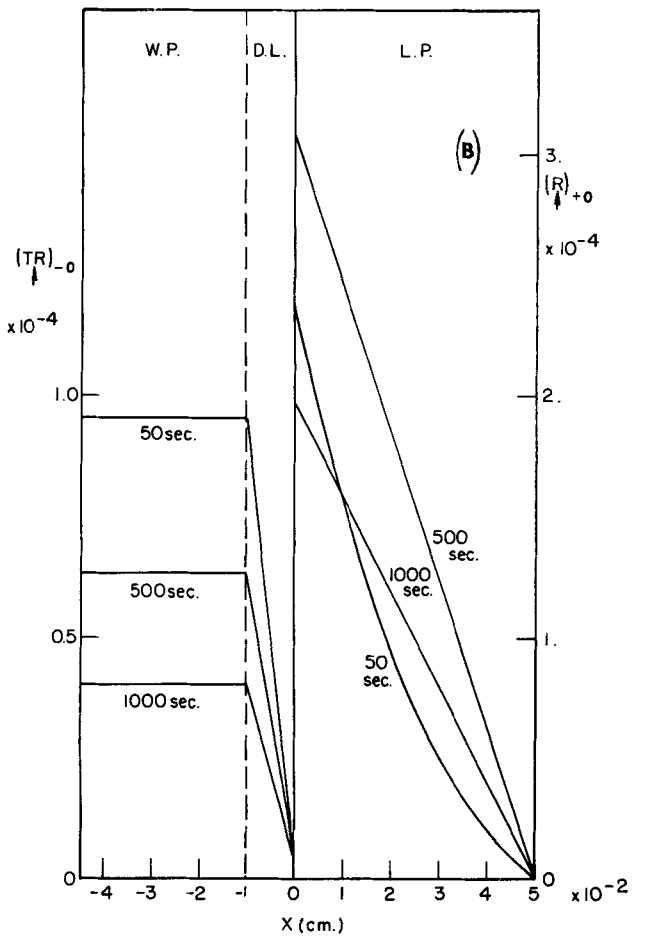
[1] Note that $f(t)$ is replaced by $f(T)$ since the function is time-independent in the steady-state period for the perfect-sink case.

[2] While this theoretical treatment is based on an amine drug, the following equation can also be derived in an analogous manner for an acidic drug, like a barbiturate,

$$f(t) = \frac{1}{\{1 + (K_{a,R})/[(H^+)_{-0}]\}\ T + 1} \qquad \text{(Eq. 35}a\text{)}$$

where $K_{a,R}$ is the dissociation constant of the acidic drug and T is either Eq. 32 or 36, depending upon the perfect- or no-sink case.





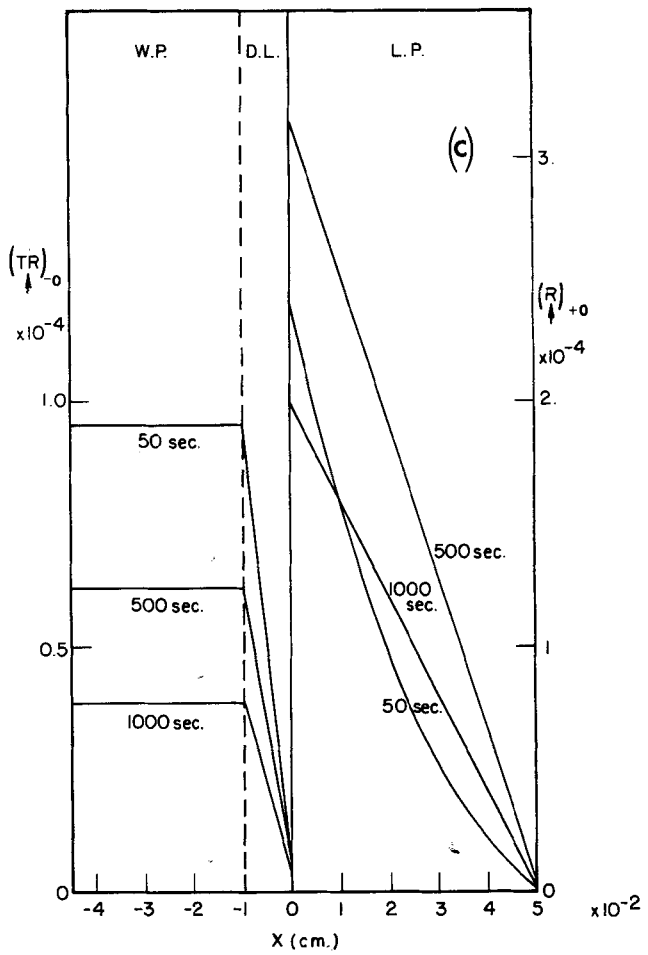


Figure 6—*Time-dependent concentration distribution profiles in aqueous and lipid phases for the perfect-sink case. Partition coefficient* P = *100. (A) Dissociation constant of buffer,* $K_{a,HB} = 10^{-6}$. *(B)* $K_{a,HB} = 10^{-8}$. *(C)* $K_{a,HB} = 10^{-10}$. *Sodium-ion concentration was one-half of the total buffer concentration always.*

CALCULATIONS

Computations were carried out for a range of parameters with the aid of the IBM 360/67 digital computer. Table I gives the dimensions of the constants and initial drug and buffer concentrations. The dissociation constant of the buffer and partition coefficient were varied. The detailed method of computation is given in the *Appendix*.

RESULTS AND DISCUSSION

In this section the interphase diffusional transport of an amine drug is analyzed for a wide range of pH and partition coefficients, other parameters being constant. It is discussed in relation to two extreme situations, the no-sink (impermeable lipid boundary at x = L) and the perfect-sink cases. In contrast to the later, the former case simulates the situation of retarded drug absorption in a simple way when the rate-determining step is due to one kind of interfacial barrier, like an impermeable membrane. There are also intermediate situations, *i.e.*,

$$\left(\frac{\partial(R)}{\partial x}\right)_{x=L} = C \qquad \text{(Eq. 37)}$$

where C is some nonzero value.

No-Sink Case—Typical changes of drug concentration–distance distribution curves in the aqueous and lipid phases with time are shown in Fig. 3. Steady-state and nonsteady-state conditions prevail

Table III—The Initial Slope K_u and $F(t)$ Values for the No-Sink Case with $P = 100$

$K_{a,HB}$	pH_{-h} at $t = 0$	K_u	$F(\Phi)$	$F(t)$	$f(t)$
10^{-4}	3.05	~0	~0	0.121×10^{-3}	0.157×10^{-3}
10^{-8}	6.01	0.123×10^{-3}	0.123	0.0973	0.1096
10^{-10}	7.79	0.736×10^{-3}	0.736	0.7163	0.6969
10^{-12}	8.82	0.915×10^{-3}	0.915	0.9053	0.8946

Table IV—Comparison of the Function $f(T)$ during the Steady-State Period and the Lag Time τ[a] with Theory for the Perfect-Sink Case in a Strong Buffer System

Initial			Steady-State		Lag Time, sec.	
$K_{a,HB}$	pH_{-h}	pH_{-0}	$F(T)$	$f(T)$	τ	$\tau_{obs.}$[b]
10^{-4}	4.03	4.03	2.16×10^{-3}	2.2×10^{-3}	125	100
10^{-6}	6.02	6.01	0.167	0.17	125	100
10^{-8}	8.01	8.00	0.902	0.91	125	100–200
10^{-10}	9.98	9.98	0.948	0.95	125	100–200

[a] τ calculated by Eq. 33. [b] $\tau_{obs.}$ estimated from Fig. 9.

in the respective diffusion layer and lipid phase. Because of the impermeable barrier, at sufficiently long times there will be a concentration buildup in the lipid that approaches some equilibrium concentration as determined by the partition coefficient.

Upon comparing Figs. 3A and B, the pH effect on the distribution profiles is evident. When $pK_{a,HB} = 4$ [and the bulk pH ~ 3 with $(Na^+) = 0$], the ratio of the initial bulk aqueous concentration of nonprotonated drug to the total concentration is only 1.11×10^{-5}. This results in a relatively small concentration gradient in the diffusion layer and, consequently, in a small amount of nonprotonated drug being transported into the lipid phase, even though the partition coefficient is favorable. On the other hand, when $K_{a,HB} = 10^{-12}$ (and the bulk pH ~ 8.8), the initial $(R)_{-h}/(TR)_{-h}$ is 0.887. Here the flux in the diffusion layer and the amount transported into the lipid are large. In Figs. 3A and C the results indicate that the rate of diffusion is influenced more by the amount of the free amine drug available for transport at the interface rather than the partition coefficient. In this regard, the pH profile of the diffusion model should be considered. Based on Eqs. 16 and 17, Table II shows the pH of the bulk aqueous phase and the interface for various $K_{a,HB}$ values and at different times with $P = 100$. Since $(Na^+) = 0$, the buffer capacity is very low. It is found that $pH_{-0} < pH_{-h}$ initially and can be explained by the fact that RH^+ and R species, as well as buffer, have some flux according to the concentration gradient within the diffusion layer and some RH^+ arriving at the interface dissociates into H^+ and R. The pH_{-h} will decrease in time and eventually will be equal to pH_{-0} when the diffusion rate is zero.

The semilogarithmic plots of $(TR)_{-h}$ *versus* t (Fig. 4) do not show a true linear relationship, as expected from Eq. 22, since the slope K_u is time dependent. When pH_{-0} of the system is approximately equal to or greater than the pKa of the drug, the initial rate is very rapid; however, due to the backup drug concentration in the lipid later on, the rate approaches zero. In Table III the initial apparent first-order rate constant is given and the function F, determined in various ways for the first 250-sec. period, shows good agreement. $F(\Phi)$ was calculated by using Eq. 23 and the initial slope from Fig. 4, $F(t)$ by the computer-simulated transport program from the general Eqs. 20 and 21, and $f(t)$ by Eqs. 31 and 32. However, in Fig. 5 the functions F and, therefore, the rate constants are always changing with time. That the rate constant in early period (~ 500 sec.) from the log $(TR)_{-h}$ *versus* t plot is apparently constant with time can be explained by the fact that it is less sensitive to time than the differentially calculated K_u. Also, after 500 sec., the $F(t)$ and $f(t)$ tend to diverge. Since the derivation of $f(t)$ is based on a linear approximation of the flux in the lipid (see Fig. 2A), the function $f(t)$ becomes a poorer approximation of $F(t)$ when backup occurs in the lipid compartment at the impermeable boundary.

Perfect-Sink Case—In Fig. 6 the concentration distribution curves are shown for various values of $K_{a,HB}$ with P = 100. Nonsteady-state diffusion in the lipid occurs in the initial period. Later, when the concentration–distance profile is linear, the system is at steady state and the diffusional rate is first order with respect to the total drug concentration in the bulk aqueous phase (Fig. 7). From Fig. 8, which shows the time change in $(TR)_{-h}$ and Q, the amount of nonprotonated drug in the sink, the lag time can be obtained by extrapolation and also predicted by Eq. 33. The results of this perfect-sink case are summarized in Table IV. Because of the strong buffer capacity, $(Na^+)_{-h} = (Na^+)_{-0} = 1/2(TB)$, the pH of the bulk aqueous phase and at the interface is nearly the same. There is good agreement between the lag time values obtained from the theory and the computer-simulated experiments and between the steady-state rate constants obtained in part from rigorous calculation by Eq. 19 and the approximation by Eqs. 35 and 36. Referring to Fig. 9, one can readily follow the course of the interphase transport by an analysis of $f(T)$. The curve is characterized by a rapid change with time, followed by an asymptotic relationship during the steady-state period. As the pH is more alkaline, the function $f(T)$ approaches 1 and the apparent first-order rate constant increases in magnitude.

Significance of the Function $f(T)$ for the Perfect-Sink Case—Thus far, the rate-determining factors have been discussed from the general viewpoint of the partition coefficient and the pH at the interface influencing the amount of nonionized drug available

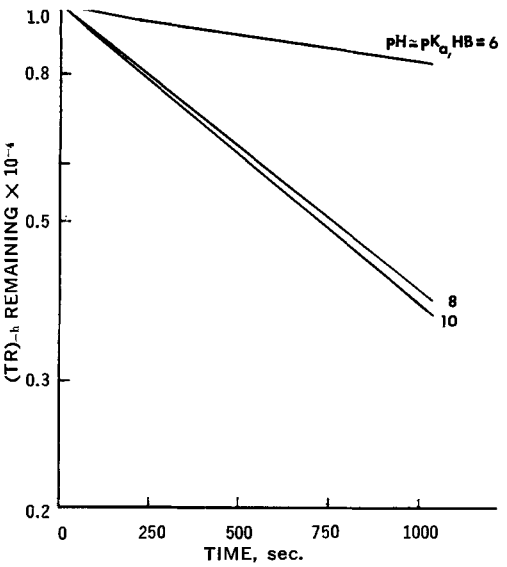


Figure 7—*First-order change in the total drug concentration in the bulk aqueous phase with time. Perfect-sink case for different initial bulk aqueous pH.*

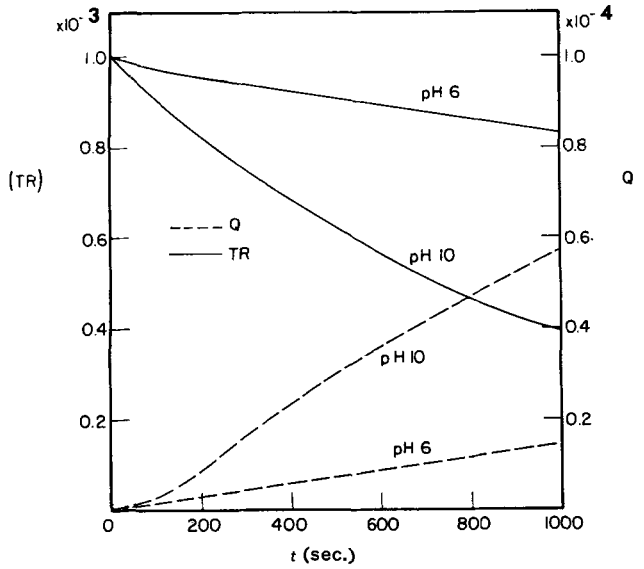


Figure 8—*Time change in the total amount of drug in the bulk aqueous phase and in the sink.*

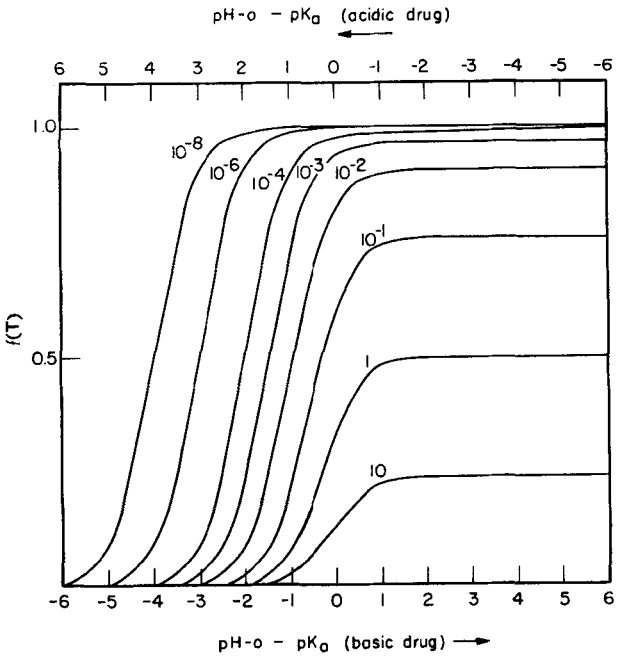


Figure 10—*Relationship of the pH at the aqueous–lipid interface and the pK of the drug with the diffusion efficiency coefficient for various T, which includes all transport parameters such as diffusion and partition coefficients, thickness of diffusion layer, and lipid phases.*

for diffusion across the lipid phase. It is useful to examine the nature of the time-independent function $f(T)$ by Eqs. 35 and 36 and the steady-state rate constant K_u (Eq. 24) relative to pH_{-0}.

As shown in Fig. 10, when the (pH_{-0} − pK_a) for a basic drug becomes increasingly positive and T is sufficiently small, say 10^{-6} to 10^{-8}, $f(T)$ is unity in the limit. Consequently, a few selected and interesting cases can be pointed out. If

$$\lim f(T) = 1, \quad \text{then } K_u = -\frac{AD_R}{Vh}$$

$$P \to \infty$$

$$(H^+)_{-0} \lesssim K_a$$

h = constant

On the other hand, if

$$\lim f(T) = 1, \quad \text{then } K_u \sim 0$$

$$h \to \infty$$

$$P \to \infty$$

$$(H^+)_{-0} \lesssim K_a$$

In both of these cases the rate-determining factor is the flux across the aqueous diffusion layer. These examples emphasize the importance of the aqueous diffusion layer, which is affected by the degree of stirring or agitation. More significantly, it shows that the pH-partition theory (9) is only a special case of the theory presented here. A very small value of $D_{R,oil}$ can also slow down the rate. In accordance with the pH-partition theory, the $f(T)$ and, consequently, $K_u \sim 0$ when the (pH_{-0} − pKa) becomes more negative.

Another interesting point in Fig. 10 is the shifting of the $f(T)$ *versus* (pH_{-0} − pKa) profiles with various T values. For T = 10, the profile approaches the dissociation curve characteristic of the basic drug; for other T values the profile deviates to the left of the dissociation curve.

From Eq. 36 it can be seen that increasing the partition coefficient, increasing h, increasing $D_{R,oil}$, decreasing D_R, or decreasing L, all have the effect of shifting the profile leftward away from the dissociation curve. The effect of agitation mentioned previously is particularly noteworthy in this regard. Thus it is important for investigators to recognize that the degree of agitation may not only influence the drug-absorption rate but that it can significantly influence the rate *versus* pH profiles. Another interpretation of the curves in Fig. 10 can be given; that is, at a constant diffusion layer thickness and a given rate of diffusion, the effect of a low concentration of non-

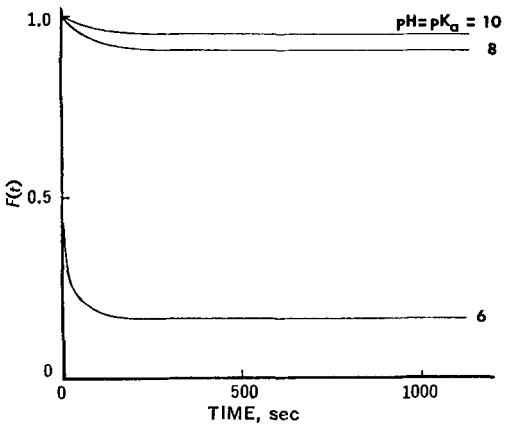
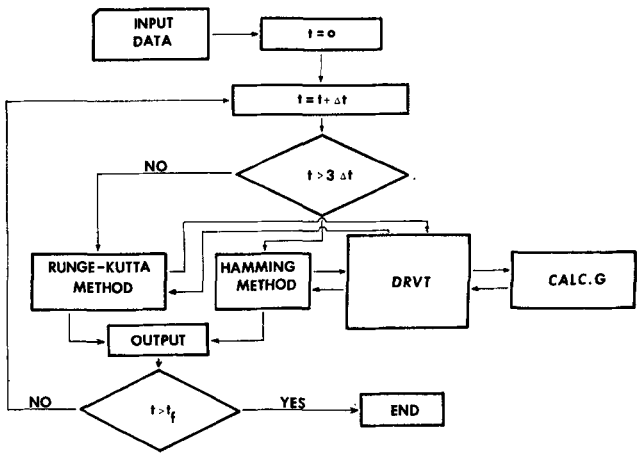


Figure 9—*Change in the diffusion efficiency coefficient with time for the perfect-sink case. After lag time,* F(t) *calculated is the same as the approximation* f(t).



Scheme I—Flow diagram for the computation of concentration distributions

ionized species at the interface is balanced by a high partition coefficient and vice versa. All of these conclusions also apply to the case of acidic drugs in an analogous way.

In the study of rat intestinal and gastric absorption of sulfonamides, Koizumi *et al.* (6, 7) derived a first-order rate constant,

$$K_u \sqrt{M} = \frac{abP}{1 + aP} \qquad \text{(Eq. 38)}$$

where M is the molecular weight of the sulfonamide, K_u is the absorption rate of the nonionized moiety, a and b are constants, and P is the partition coefficient.

Equation 38 was found to be in good agreement with a large number of *in situ* experiments. It is noteworthy that the substitution of Eq. 35 or 35*a* into 24 gives

$$K_u = -\frac{AD_R}{Vh} \cdot \frac{BP}{1 + BP} \qquad \text{(Eq. 39)}$$

Both equations have the same form, although the methods of derivation are different. In the next paper, the results of Koizumi *et al.* and others will be discussed and compared with a similar model as presented in this study but modified to simulate the gastric and intestinal membrane.

APPENDIX

Numerical Calculating Procedure—To calculate the change of $(TR)_{-h}$, the concentration profile of R in the lipid phase with time and other parameters, the procedure shown in Scheme I is used. The input data are given in Table I. After $t = 0$, a series of calculation procedures undergo integration for each time increment, $t + \Delta t$. The $(TR)_{-h}$ and (R_i) at time t are determined by the stepwise integration of Eqs. 12–15*a* or 15*b*, depending upon the choice of the perfect-sink or no-sink case, by the Runge-Kutta technique for the initial period, $t \leq 3\Delta t$, and thereafter by the predictor-corrector method of Hamming (10). The calculation of the derivatives in Eqs. 12–15 is performed in the subroutine DRVT after evaluating G in the subroutine CALCG.

The procedure of subroutine CALCG is as follows. The first step involves the calculation of $(H^+)_{-h}$ from the fourth-power polynominal Eq. 16 by the Newton-Raphson method. Then $(R)_{-h}$, $(RH^+)_{-h}$, $(B^-)_{-h}$, and $(HB)_{-h}$ are obtained from Eqs. 1, 2, and 4, respectively. The next step is the evaluation of $(H^+)_{-0}$ from Eq. 17. In turn, $(B^-)_{-0}$, $(HB)_{-0}$, $(RH^+)_{-0}$ and $(R)_{-0}$ are found, using Eqs. 4, 7, and 18 and finally G by Eq. 6.

REFERENCES

(1) A. H. Goldberg, W. I. Higuchi, N. F. H. Ho, and G. Zografi, *J. Pharm. Sci.*, **56,** 1432(1967).

(2) A. Ghanem, W. I. Higuchi, and A. P. Simonelli, *ibid.*, **58,** 165(1969).

(3) S. A. Howard, A. Suzuki, M. A. Farvar, and W. I. Higuchi, to be published.

(4) R. G. Stehle and W. I. Higuchi, *J. Pharm. Sci.*, **56,** 1367(1967).

(5) A. Suzuki, W. I. Higuchi, and N. F. H. Ho, *ibid.*, **59,** 651(1970).

(6) T. Koizumi, T. Arita, and K. Kakemi, *Chem. Pharm. Bull.*, **12,** 413(1964).

(7) *Ibid.*, **12,** 421(1964).

(8) J. Crank, "Mathematics of Diffusion," Oxford, New York, N. Y., 1956.

(9) P. Shore, B. Brodie, and C. Hogben, *J. Pharmacol. Exp. Ther.*, **119,** 361(1957).

(10) A. Ralston and H. Wilf, "Mathematical Methods for Digital Computers," Wiley, New York, N. Y., 1960.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication December 16, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Theoretical Model Studies of Drug Absorption and Transport in the Gastrointestinal Tract II

AKIRA SUZUKI*, W. I. HIGUCHI, and N. F. H. HO

Abstract □ Multicompartment diffusional models for the absorption of neutral, acidic, basic, and amphoteric drugs were investigated. The general model consisted of a bulk aqueous phase, an aqueous diffusion layer, n-compartments of homogeneous and heterogeneous phases, and a perfect sink. With the mathematical techniques reported previously, equations were derived in general terms for the nonsteady- and steady-state periods. Utilizing the steady-state diffusion efficiency function of the barrier systems, the first-order rate constants for various examples of two- and three-compartment models were obtained from the general model and some computations were given. Various sets of *in situ* experimental rat data have been analyzed by means of the different models. These include the intestinal, gastric, and rectal absorption of sulfonamides and barbituric acid derivatives. Self-consistent dimensional constants and diffusion coefficients were arrived at and the correlations obtained with the models have been found to be generally satisfactory.

Keyphrases □ Theoretical models—drug absorption, transport, gastrointestinal tract □ Drug absorption, transport, gastrointestinal tract—theoretical models, equations derived □ Kinetics—drug absorption, transport □ Sulfonamides—absorption, diffusion data, rats □ Barbituric acid derivatives—absorption, diffusion data, rats

In a previous paper the diffusion of basic and acidic drugs across an aqueous diffusion layer and a lipid compartment in a homogeneous two-phase model was presented (1). It provided a mathematical technique whereby more complicated models can be handled. A function was also derived which was found useful in analyzing the diffusion rate with respect to the partition coefficient, surface and bulk pH, dissociation constant, diffusion coefficients, and diffusion layer thickness.

ionized species at the interface is balanced by a high partition coefficient and vice versa. All of these conclusions also apply to the case of acidic drugs in an analogous way.

In the study of rat intestinal and gastric absorption of sulfonamides, Koizumi *et al.* (6, 7) derived a first-order rate constant,

$$K_u \sqrt{M} = \frac{abP}{1 + aP} \qquad \text{(Eq. 38)}$$

where M is the molecular weight of the sulfonamide, K_u is the absorption rate of the nonionized moiety, a and b are constants, and P is the partition coefficient.

Equation 38 was found to be in good agreement with a large number of *in situ* experiments. It is noteworthy that the substitution of Eq. 35 or 35*a* into 24 gives

$$K_u = -\frac{AD_R}{Vh} \cdot \frac{BP}{1 + BP} \qquad \text{(Eq. 39)}$$

Both equations have the same form, although the methods of derivation are different. In the next paper, the results of Koizumi *et al.* and others will be discussed and compared with a similar model as presented in this study but modified to simulate the gastric and intestinal membrane.

APPENDIX

Numerical Calculating Procedure—To calculate the change of $(TR)_{-h}$, the concentration profile of R in the lipid phase with time and other parameters, the procedure shown in Scheme I is used. The input data are given in Table I. After $t = 0$, a series of calculation procedures undergo integration for each time increment, $t + \Delta t$. The $(TR)_{-h}$ and (R_i) at time t are determined by the stepwise integration of Eqs. 12–15*a* or 15*b*, depending upon the choice of the perfect-sink or no-sink case, by the Runge-Kutta technique for the initial period, $t \leq 3\Delta t$, and thereafter by the predictor-corrector method of Hamming (10). The calculation of the derivatives in Eqs. 12–15 is performed in the subroutine DRVT after evaluating G in the subroutine CALCG.

The procedure of subroutine CALCG is as follows. The first step involves the calculation of $(H^+)_{-h}$ from the fourth-power polynominal Eq. 16 by the Newton-Raphson method. Then $(R)_{-h}$, $(RH^+)_{-h}$, $(B^-)_{-h}$, and $(HB)_{-h}$ are obtained from Eqs. 1, 2, and 4, respectively. The next step is the evaluation of $(H^+)_{-0}$ from Eq. 17. In turn, $(B^-)_{-0}$, $(HB)_{-0}$, $(RH^+)_{-0}$ and $(R)_{-0}$ are found, using Eqs. 4, 7, and 18 and finally G by Eq. 6.

REFERENCES

(1) A. H. Goldberg, W. I. Higuchi, N. F. H. Ho, and G. Zografi, *J. Pharm. Sci.*, **56,** 1432(1967).

(2) A. Ghanem, W. I. Higuchi, and A. P. Simonelli, *ibid.*, **58,** 165(1969).

(3) S. A. Howard, A. Suzuki, M. A. Farvar, and W. I. Higuchi, to be published.

(4) R. G. Stehle and W. I. Higuchi, *J. Pharm. Sci.*, **56,** 1367(1967).

(5) A. Suzuki, W. I. Higuchi, and N. F. H. Ho, *ibid.*, **59,** 651(1970).

(6) T. Koizumi, T. Arita, and K. Kakemi, *Chem. Pharm. Bull.*, **12,** 413(1964).

(7) *Ibid.*, **12,** 421(1964).

(8) J. Crank, "Mathematics of Diffusion," Oxford, New York, N. Y., 1956.

(9) P. Shore, B. Brodie, and C. Hogben, *J. Pharmacol. Exp. Ther.*, **119,** 361(1957).

(10) A. Ralston and H. Wilf, "Mathematical Methods for Digital Computers," Wiley, New York, N. Y., 1960.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication December 16, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Theoretical Model Studies of Drug Absorption and Transport in the Gastrointestinal Tract II

AKIRA SUZUKI*, W. I. HIGUCHI, and N. F. H. HO

Abstract □ Multicompartment diffusional models for the absorption of neutral, acidic, basic, and amphoteric drugs were investigated. The general model consisted of a bulk aqueous phase, an aqueous diffusion layer, n-compartments of homogeneous and heterogeneous phases, and a perfect sink. With the mathematical techniques reported previously, equations were derived in general terms for the nonsteady- and steady-state periods. Utilizing the steady-state diffusion efficiency function of the barrier systems, the first-order rate constants for various examples of two- and three-compartment models were obtained from the general model and some computations were given. Various sets of *in situ* experimental rat data have been analyzed by means of the different models. These include the intestinal, gastric, and rectal absorption of sulfonamides and barbituric acid derivatives. Self-consistent dimensional constants and diffusion coefficients were arrived at and the correlations obtained with the models have been found to be generally satisfactory.

Keyphrases □ Theoretical models—drug absorption, transport, gastrointestinal tract □ Drug absorption, transport, gastrointestinal tract—theoretical models, equations derived □ Kinetics—drug absorption, transport □ Sulfonamides—absorption, diffusion data, rats □ Barbituric acid derivatives—absorption, diffusion data, rats

In a previous paper the diffusion of basic and acidic drugs across an aqueous diffusion layer and a lipid compartment in a homogeneous two-phase model was presented (1). It provided a mathematical technique whereby more complicated models can be handled. A function was also derived which was found useful in analyzing the diffusion rate with respect to the partition coefficient, surface and bulk pH, dissociation constant, diffusion coefficients, and diffusion layer thickness.

In contrast to the two-phase model, this study describes a more general model in an attempt to simulate the diffusion of drug across the biological membrane and is easily adaptable to various conditions as the case may be. It utilizes the same mathematical techniques and computer method of numerical calculation heretofore mentioned. The appropriate theoretical relationships are also applied to some *in situ* experiments on absorption of sulfonamides and barbiturates in the rat intestine, stomach, and rectum.

THEORY

General Description of the Multicompartment Model and Nonsteady-State Diffusion—The general theory involves the simple diffusion in one dimension through multicompartments, each compartment being homogeneous or heterogeneous in phase according to the situation and representing different structural regions of the system (Fig. 1). The first compartment consists of a bulk aqueous phase and a diffusion layer of thickness L_1. The ith compartment ($i = 2, 3 \ldots n$) represents a portion of the membrane having distinct but uniform diffusional characteristics. The present general treatment will be assumed to be heterogeneous, *i.e.*, consisting of aqueous and lipid phases. Its thickness is L_i and the volume fraction of lipid is α_i. After the nth compartment, there is a perfect sink.

Let us now consider the drug also in general terms. The ionic equilibria of an amphoteric drug in water are

$$R_w^+ \overset{K_1}{\rightleftharpoons} R_w^0 + H^+ \overset{K_2}{\rightleftharpoons} R_w^- + 2H^+ \qquad \text{(Eq. 1)}$$

and

$$K_1 = \frac{(R_w^0)(H^+)}{(R_w^+)} \qquad \text{(Eq. 2)}$$

$$K_2 = \frac{(R_w^-)(H^+)}{(R_w^0)} \qquad \text{(Eq. 3)}$$

where (R_w^+), (R_w^-), and (R_w^0) are the concentrations of the cationic, anionic, and unionized drug species, respectively, in water (subscript w); K_1 and K_2 are the dissociation constants; and (H^+) is the hydrogen-ion concentration. For a basic drug, K_1 is its dissociation constant with $K_2 = 0$; for an acidic drug, K_2 is its dissociation constant with $K_1 = \infty$; for a neutral drug, $K_1 = \infty$ and $K_2 = 0$.

In the initial period, one assumes steady-state fluxes in the diffusion layer and nonsteady-state fluxes in the outer compartments. Therefore, applying Fick's first law,

$$G = -D_w^+ \frac{d(R_w^+)}{dx} - D_w^- \frac{d(R_w^-)}{dx} - D_w^0 \frac{d(R_w^0)}{dx} \qquad \text{(Eq. 4)}$$

where G is the total flux of the drug species in the diffusion layer and D is the diffusion coefficient.

The description of the concentration–distance change with time is complex. Each compartment, except the first, is divided into unit cells of equal intervals, Δx_i. The distribution of drug between the lipid and aqueous phases in each cell is assumed to be instantaneously established and follows the Nernst relationship:

$$(R_0^+)_{ij} = P_i^+(R_w^+)_{ij} \qquad \text{(Eq. 5a)}$$

$$(R_0^0)_{ij} = P_i^0(R_w^0)_{ij} \qquad \text{(Eq. 5b)}$$

$$(R_0^-)_{ij} = P_i^-(R_w^-)_{ij} \qquad \text{(Eq. 5c)}$$

where P_i^+, P_i^0, and P_i^- are the partition coefficients of the cationic, nonionic, and anionic species, respectively; and the subscripts i, j, o, and w denote the ith compartment, jth cell, lipid, and aqueous phases, respectively. It is also assumed that the hydrogen-ion concentration in each compartment is constant; *i.e.*, the buffer capacity is large. The total drug concentration Y_{ij} of the jth cell in the ith compartment is

$$Y_{ij} = \alpha_i[(R_0^+)_{ij} + (R_0^0)_{ij} + (R_0^-)_{ij}] + (1 - \alpha_i)[(R_w^+)_{ij} + (R_w^0)_{ij} + (R_w^-)_{ij}] \qquad \text{(Eq. 6)}$$

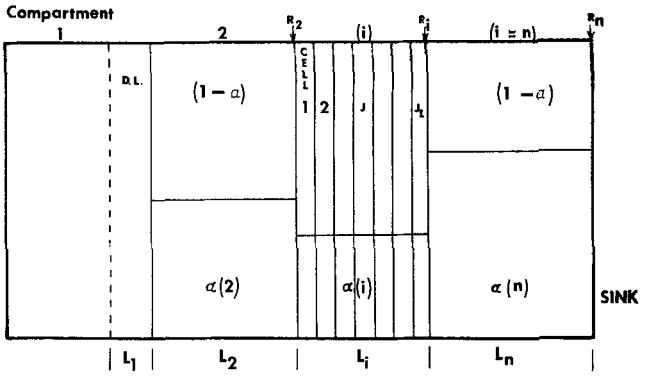


Figure 1—*General diffusion model consisting of an aqueous compartment with a diffusion layer followed by* n *compartments and a perfect sink. Each heterogeneous compartment contains lipid of volume fraction* α *and an aqueous phase* $(1 - \alpha)$. *Each compartment of thickness* L *is subdivided into* j *number of cells.* R *is the ratio of the true interfacial area to the geometrical surface area between compartments.*

From Eqs. 2, 3, 5, and 6, the following are obtained

$$(R_0^+)_{ij} = C_{0,i}^+ \cdot Y_{ij} \qquad \text{(Eq. 7a)}$$

$$(R_0^0)_{ij} = C_{0,i}^0 \cdot Y_{ij} \qquad \text{(Eq. 7b)}$$

$$(R_0^-)_{ij} = C_{0,i}^- \cdot Y_{ij} \qquad \text{(Eq. 7c)}$$

$$(R_w^+)_{ij} = C_{w,i}^+ \cdot Y_{ij} \qquad \text{(Eq. 7d)}$$

$$(R_w^0)_{ij} = C_{w,i}^0 \cdot Y_{ij} \qquad \text{(Eq. 7e)}$$

$$(R_w^-)_{ij} = C_{w,i}^- \cdot Y_{ij} \qquad \text{(Eq. 7f)}$$

where

$$C_{0,i}^+ = P_i^+(H^+)_i^2/\beta_i \qquad \text{(Eq. 8a)}$$

$$C_{0,i}^0 = P_i^0(H^+)_i K_1/\beta_i \qquad \text{(Eq. 8b)}$$

$$C_{0,i}^- = P_i^- K_1 K_2/\beta_i \qquad \text{(Eq. 8c)}$$

$$C_{w,i}^+ = (H^+)_i^2/\beta_i \qquad \text{(Eq. 8d)}$$

$$C_{w,i}^0 = (H^+)_i K_1/\beta_i \qquad \text{(Eq. 8e)}$$

$$C_{w,i}^- = K_1 K_2/\beta_i \qquad \text{(Eq. 8f)}$$

$$\beta_i = \alpha_i[P_i^+(H^+)_i^2 + P_i^0 K_1(H^+)_i + P_i^- K_1 K_2] + (1 - \alpha_i)[(H^+)_i^2 + K_1(H^+)_i + K_1 K_2] \qquad \text{(Eq. 9)}$$

where $C_{w,i}^-$, $C_{w,i}^0$, and $C_{w,i}^+$ are the fractions in the respective anionic, nonionic, and cationic forms of the total concentration of the particular species in the aqueous phase of the ith compartment; and $C_{0,i}^-$, *etc.*, are defined in the same manner. Equations 7–9 indicate that the concentration of drug species in a particular phase of a unit cell, for example, $(R_0^0)_{ij}$, is interdependent with other species in the various phases through Y_{ij} and its magnitude is determined by the coefficient $C_{0,i}^0$, which in turn is directly influenced by the $(H^+)_i$ and the partition coefficient of the species.

The flux from the j to the $j + 1$th cell in the ith compartment is expressed by

$$G_{i(j \to j+1)} = \{\alpha_i[D_0^+(R_{0,ij}^+ - R_{0,ij+1}^+) + D_0^0(R_{0,ij}^0 - R_{0,ij+1}^0) + D_0^-(R_{0,ij}^- - R_{0,ij+1}^-)] + (1 - \alpha_i)[D_w^+(R_{w,ij}^+ - R_{w,ij+1}^+) + D_w^0(R_{w,ij}^0 - R_{w,ij+1}^0) + D_w^-(R_{w,ij}^- - R_{w,ij+1}^-)]\}/\Delta x_i \qquad \text{(Eq. 10)}$$

In terms of the total concentration in the cell, Eq. 10 is rewritten as follows:

$$G_{i(j \to j+1)} = \frac{D_{eff(i)}(Y_{ij} - Y_{i,j+1})}{\Delta x_i} \qquad \text{(Eq. 11)}$$

where the effective diffusion coefficient in the compartment, $D_{eff(i)}$, is

$$D_{eff(i)} = \alpha_i[D_0^+ C_{0i}^+ + D_0^0 C_{0i}^0 + D_0^- C_{0i}^-] + (1 - \alpha_i)[D_w^+ C_{wi}^+ + D_w^0 C_{wi}^0 + D_w^- C_{wi}^-] \qquad \text{(Eq. 12)}$$

Using the finite-difference method and Eq. 11, the nonsteady-state concentration change in the jth cell of the ith compartment with time may be expressed by the following equation,

$$\frac{dY_{ij}}{dt} = \frac{G_{i(j-1\rightarrow j)} - G_{i(j\rightarrow j+1)}}{\Delta x_i} = \frac{D_{\mathrm{eff}(i)}}{(\Delta x_i)^2}(Y_{i,j-1} - 2Y_{ij} + Y_{i,j+1}) \quad (j = 2, 3, 4 \ldots j1 - 1) \qquad \text{(Eq. 13)}$$

Now that the diffusional movement from cell to cell in a compartment has been described, one must further account for the continuity of the flow between compartments by applying the boundary condition that the total flux to the interface is equal to the total flux from the interface. Therefore,

$$G_{T(i\rightarrow i+1)} = \frac{AR_iD_{\mathrm{eff}(i)}}{0.5\Delta x_i}(Y_{i,j1} - Y_{\mathrm{int}(i,i+1)}) = \frac{AR_{i+1}D_{\mathrm{eff}(i+1)}}{0.5\Delta x_{i+1}}(P_{\mathrm{eff}(i,i+1)}Y_{\mathrm{int}(i,i+1)} - Y_{i+1,1}) \qquad \text{(Eq. 14)}$$

where $G_{T(i\rightarrow i+1)}$ is the total flux immediately to or from the boundary, the subscript $j1$ is the last cell of the ith compartment, R_i and R_{i+1} are the ratio of the actual surface area of the ith and $i+1$th compartment, respectively, to the geometrical surface area A and, accordingly, R_i and $R_{i+1} \geq 1$. The $Y_{\mathrm{int}(i,i+1)}$ is the total concentration at the interface on the ith-compartment side and $P_{\mathrm{eff}(i,i+1)}Y_{\mathrm{int}(i,i+1)}$ is the total concentration at the interface on the $i+1$th compartment side where $P_{\mathrm{eff}(i,i+1)}$ is the effective partition coefficient between the respective compartments. Under the assumption that the activity of the unionized drug species on both sides of the boundary is the same, it follows that

$$C_{w,i}{}^0Y_{\mathrm{int}(i,i+1)} = C_{w,i+1}{}^0P_{\mathrm{eff}(i,i+1)}Y_{\mathrm{int}(i,i+1)} \qquad \text{(Eq. 15}a\text{)}$$

and, consequently,

$$P_{\mathrm{eff}(i,i+1)} = \frac{C^0_{w,i}}{C^0_{w,i+1}} \qquad \text{(Eq. 15}b\text{)}$$

The average rate of change in concentration in the last cell of the ith compartment is

$$\frac{dY_{i,j1}}{dt} = \frac{1}{\Delta x_i}\left[G_{i(j1-1\rightarrow j1)} - \frac{G_{T(i\rightarrow i+1)}}{AR_i}\right] \qquad \text{(Eq. 16}a\text{)}$$

and in the first cell of the $i+1$th compartment is

$$\frac{dY_{i+1,1}}{dt} = \frac{1}{\Delta x_{i+1}}\left[\frac{G_{T(i\rightarrow i+1)}}{AR_{i+1}} - G_{i+1,(1\rightarrow 2)}\right] \qquad \text{(Eq. 16}b\text{)}$$

By substitution of Eqs. 11 and 14, the corresponding equations for 16a and b are

$$\frac{dY_{i,j1}}{dt} = \frac{D_{\mathrm{eff}(i)}}{\Delta x_i}\left[\frac{Y_{i,j1-1} - Y_{i,j1}}{\Delta x_i} - \frac{Y_{i,j1} - Y_{\mathrm{int}(i,i+1)}}{0.5\Delta x_i}\right] \qquad \text{(Eq. 17}a\text{)}$$

$$\frac{dY_{i+1,1}}{dt} = \frac{D_{\mathrm{eff}(i+1)}}{\Delta x_{i+1}}\left[\frac{P_{\mathrm{eff}(i,i+1)}Y_{\mathrm{int}(i,i+1)} - Y_{i+1,1}}{0.5\Delta x_{i+1}} - \frac{Y_{i+1,1} - Y_{i+1,2}}{\Delta x_{i+1}}\right] \qquad \text{(Eq. 17}b\text{)}$$

Thus, Eqs. 13, 17a, and 17b describe the nonsteady-state diffusion across the barrier system in a general manner. The method of numerical calculation of the rate and concentration–distance distribution was described previously (1).

Steady-State Diffusion—Previously, the mathematical technique of obtaining the steady-state rate, lag time, and rate constant for the diffusion of drug across a two-phase homogeneous barrier was described under the assumption of an initial quasi-steady-state flux in the aqueous diffusion and a perfect sink. Similarly, one derives the steady-state rate equation,

$$\frac{d(TR)_{w,1}}{dt} = -K_u(TR)_{w,1} \qquad \text{(Eq. 18)}$$

where $(TR)_{w,1}$ is the total concentration of drug in the water phase (Compartment 1) and the rate constant K_u is

$$K_u = \frac{AD_{\mathrm{eff}(1)}}{VL_1} \qquad \text{(Eq. 19)}$$

where V is the volume of the bulk aqueous phase, L_1 is the thickness of the aqueous diffusion layer, and the function f is the diffusion efficiency coefficient of the barriers in the system. In the nonstationary-state period, the function f is time dependent; however, after the lag time, τ, *i.e.*,

$$\tau = 0.5\left[\frac{L_2{}^2}{D_{\mathrm{eff}(2)}} + \frac{L_3{}^2}{D_{\mathrm{eff}(3)}} + 2\frac{(R_2/R_3)L_2L_3}{P_{\mathrm{eff}(2\rightarrow 3)}D_{\mathrm{eff}(3)}}\right] \qquad \text{(Eq. 20)}$$

for a three-compartment model,[1] the f is time independent and is given by the general relationship,

$$f = \frac{L_1/D_{\mathrm{eff}(1)}}{L_1/D_{\mathrm{eff}(1)} + L_2/[R_2P_{\mathrm{eff}(1\rightarrow 2)}D_{\mathrm{eff}(2)}] + L_3/[R_3P_{\mathrm{eff}(1\rightarrow 2)}P_{\mathrm{eff}(2\rightarrow 3)}D_{\mathrm{eff}(3)}] + L_4/[R_4P_{\mathrm{eff}(1\rightarrow 2)}P_{\mathrm{eff}(2\rightarrow 3)}P_{\mathrm{eff}(3\rightarrow 4)}D_{\mathrm{eff}(4)}] + \cdots} \qquad \text{(Eq. 21)}$$

APPLICATION OF THE STEADY-STATE THEORY TO SOME SPECIFIC MODELS

The foregoing general steady-state theory can be applied to some specific multicompartment models. To reiterate some of the assumptions made, (*a*) after a lag period the concentration gradient of the diffusing species in each compartment is linear and, subsequently, (*b*) the drug diffuses across the barriers at a steady-state rate into a perfect sink. The various models are shown in Figs. 2–3 and the lag time can be estimated from Eq. 20 for each model. In addition to the previous assumptions, it is specified that only unionized molecules can partition into the lipid phase, *i.e.*, $P_i{}^+ = P_i{}^- = 0$, and that the aqueous diffusion coefficients in a compartment ($D_{w,i}$) of all ionized and unionized species are equal. Also, let the partition coefficient $P_i{}^0 = P$ and the fraction of undissociated molecules in the aqueous phase in a compartment $X_i = C_{w,i}{}^0$ (see Eq. 8). The effective diffusion coefficient in a compartment ($D_{\mathrm{eff}(i)}$) consisting of a homogeneous phase is simply the diffusion coefficient of the drug in that phase; otherwise, in a heterogeneous compartment it is given by Eq. 12.

With the appropriate substitution of Eqs. 2, 3, 8, 9, 12, 15b, and 21 according to the model into Eq. 19, the absorption rate constant for basic, acidic, or amphoteric drugs can be derived in such a form that is convenient for analysis with experimental data.

Model I: Aqueous–Lipid Compartments—This model consists of a bulk aqueous phase with a diffusion layer and a lipid phase ($\alpha_2 = 1$). With the effective partition coefficient between compartment $P_{\mathrm{eff}(1\rightarrow 2)} = PX_1$ and $D_{\mathrm{eff}(2)} = D_o$, the rate constant is given by

$$K_u = B_1 \cdot \frac{1}{1 + B_2/PX_1} \qquad \text{(Eq. 22)}$$

where

$$B_1 = \frac{AD_{w,1}}{VL_1} \qquad \text{(Eq. 22}a\text{)}$$

$$B_2 = \frac{L_2D_{w,1}}{L_1R_2D_0} \qquad \text{(Eq. 22}b\text{)}$$

Model II: (*a*) Aqueous–Lipid–Aqueous and (*b*) Aqueous–Aqueous–Lipid Compartments—In Model IIa, there are three homogeneous compartments in which the two aqueous phases are separated by a lipid one. The aqueous phases may be of different composition and pH. The relationships, $P_{\mathrm{eff}(1\rightarrow 2)} = PX_1$ and $P_{\mathrm{eff}(2\rightarrow 3)} = 1/PX_3$

[1] For more than three compartments, τ will be of the same form except that the cross terms will be more complicated.

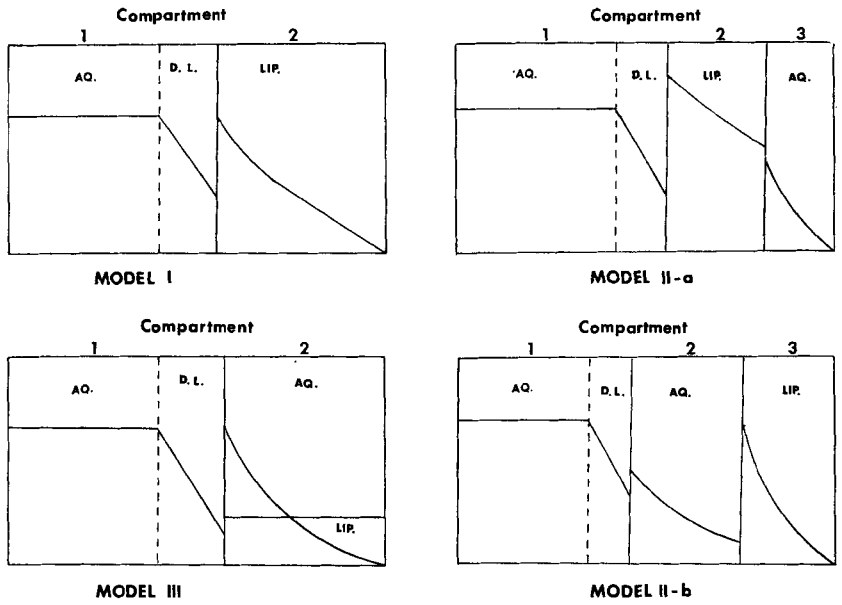


Figure 2—*Some specific models used for the application of the steady-state transport theory.*

are readily derived and, when introduced into Eq. 19,

$$K_u = B_1 \cdot \frac{1}{1 + (B_2/PX_1) + (B_3X_3/X_1)} \qquad \text{(Eq. 23)}$$

where B_1 and B_2 are defined by Eqs. 22*a*, 22*b*, and

$$B_3 = \frac{L_3D_{w,1}}{L_1R_3D_{w,3}} \qquad \text{(Eq. 23}a\text{)}$$

It is noted that the first and second compartments of Model II*a* are the same as in Model I. Comparing Eqs. 22 and 23, the third compartment in the series is accounted for by the addition of a third term in the denominator of the rate-constant equation. The rate-determining factors may be easily deduced by Eq. 23. When the lipid compartment is the rate-determining barrier, the determining factors may be low lipid diffusivity and/or the concentration of unionized drug at the aqueous–lipid interface through X_1 and the partition coefficient. In turn, X_1 depends upon the pH and the nature of the drug, *i.e.*, whether it is amphoteric, acidic, basic, or neutral. Also, if the partition coefficient is high, the transport across the first and the third compartments will be rate determining and the lipid compartment behaves essentially as a control reservoir.

In Model II*b* the sequence of the compartments is different from the previous model. The second aqueous compartment may be thought to be analogous to the presence of a mucoid or thick proteinaceous layer intervening between the bulk aqueous and lipid phases. This model is similar to the membrane described by Robertson (2) in which he assumed that it consisted of phospholipids with a protein envelope. Although identical in form to Eq. 23, the rate-constant equation is

$$K_u = B_1 \cdot \frac{1}{1 + (B_2X_2/X_1) + (B_3/PX_1)} \qquad \text{(Eq. 24)}$$

and the coefficients are

$$B_2 = \frac{L_2D_{w,1}}{L_1R_2D_{w,2}} \qquad \text{(Eq. 24}a\text{)}$$

$$B_3 = \frac{L_3D_{w,1}}{L_1R_3D_0} \qquad \text{(Eq. 24}b\text{)}$$

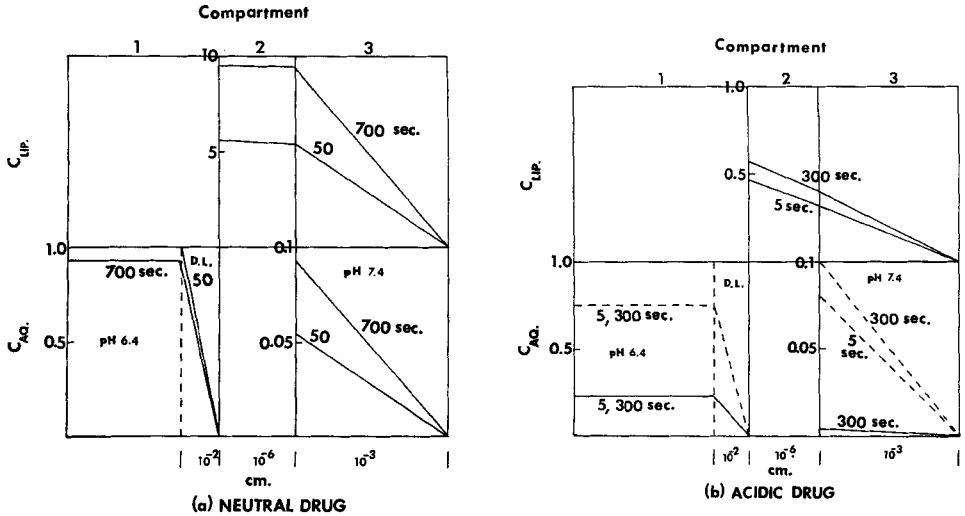


Figure 3—*Time-dependent concentration distribution profiles in the aqueous and lipid phases of Model IV. The model consists of an aqueous compartment followed by lipid and heterogeneous compartments.* V = *10 cm.*^{3}, A = *10 cm.*^{2}, R_2 = *100*, R_3 = *1*, α_1 = *0*, α_2 = *1*, α_3 = *0.1*, D_w = 10^{-6} *cm.*^{2} *sec.*$^{-1}$, D_0 = 10^{-12} *cm.*^{2} *sec.*$^{-1}$, P = *100. In 3a, only a neutral drug is used. In 3b an acidic drug (pKa = 6) is used. Key:* ——, *unionized form; and - - -, ionized form.*

Table I—Comparison Between the Numerically Calculated and Estimated Values of the Lag Time τ and the Steady-State Diffusion Efficiency Coefficient F in Model IV

Drug	$\tau_{calc.}$, sec.	$F_{calc.}$	$\tau_{est.}$,[a] sec.	f
Acidic	10	0.976	8	0.98
Neutral	200	0.90	140	0.90

[a] $\tau_{est.}$ calculated from Eq. 20 and f from Eq. 21.

Table II—Maximum Rate Constant of Gastric Absorption in Rats and Some Physical Constants of Sulfonamides[a]

	Sulfonamide	K_u, hr.$^{-1}$	P[b]	pKa_1	pKa_2
1	Sulfanilamide	0.075	0.36	3.36	10.43
2	Sulfanilacetamide	0.068	0.87	1.78	5.38
3	Sulfaguanidine	0.010	0.03	2.75	12.05
4	Sulfapyridine	0.087	2.24	2.58	8.43
5	Sulfadiazine	0.090	1.54	2.00	6.48
6	Sulfamethoxazole	0.200	22.00	1.76	5.80
7	Sulfathiazole	0.061	0.52	2.36	7.12
8	Sulfamerazine	0.070	2.10	2.26	7.06
9	Sulfisoxazole	0.210	22.40	1.55	5.10
10	Sulfamethizole	0.094	2.20	2.00	5.45
11	Sulfisomidine	0.027	0.40	2.36	7.5
12	Sulfamethazine	0.140	3.61	2.36	7.38
13	Sulfamethoxy-pyridazine	0.079	1.31	2.06	7.00
14	Sulfamono-methoxine	0.200	14.70	2.00	5.90
15	Sulfaethidole	0.180	8.00	1.93	5.60
16	Sulfadimethoxine	0.190	77.80	2.02	6.70
17	Sulfaphenazole	0.200	87.90	1.9	6.50

[a] Data taken from *Reference 4*. [b] P is the partition coefficient of unionized drug between isoamyl alcohol and water at 37°.

Model III: Aqueous-Lipid/Aqueous Compartments—Unlike the previous cases, this model provides for a heterogeneous phase system to simulate a membrane consisting of lipoidal cells in an aqueous intercellular fluid environment. In this way, all molecular species existing in the aqueous diffusion layer are able to permeate through the heterogeneous compartment; however, the rate is determined by the effective permeability coefficient,

$$K_{eff(1\to2)} = P_{eff(1\to2)}D_{eff(2)} = \alpha_2 D_0 P X_1 + (1-\alpha_2)D_{w,2}X_1\left[1 + \frac{(H^+)_2}{K_1} + \frac{K_2}{(H^+)_2}\right] \quad \text{(Eq. 25)}$$

and, with $X_2 = (R_w^0)_2/[(R_w^0)_2 + (R_w^+)_2 + (R_w^-)_2]$ and Eqs. 2–3, it follows that

$$K_{eff(1\to2)} = \alpha_2 D_0 P X_1 + (1-\alpha_2)D_{w,2}\frac{X_1}{X_2} \quad \text{(Eq. 26)}$$

Therefore,

$$K_u = B_1 \cdot \frac{1}{1 + B_2/[PX_1 + B_3(X_1/X_2)]} \quad \text{(Eq. 27)}$$

where B_1 is defined as before,

$$B_2 = \frac{L_2 D_{w,1}}{L_1 R_2 D_0 \alpha_2} \quad \text{(Eq. 27}a\text{)}$$

$$B_3 = \frac{(1-\alpha_2)\,D_{w,2}}{\alpha_2 D_0} \quad \text{(Eq. 27}b\text{)}$$

As the volume fraction of lipid approaches unity ($\alpha_2 \to 1$), the model becomes more like Model I. Higuchi and Higuchi (3) also made a theoretical analysis of the diffusional movement of drugs through heterogeneous barriers; however, their results are somewhat different from the authors'. They also considered the effect of the shape and size of the internal phase and drug interactions with the internal phase.

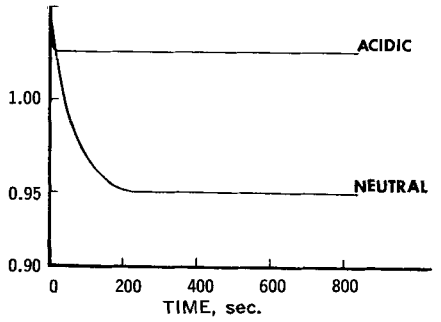


Figure 4—*Change in the function* F *with time during the nonsteady- and steady-state transport of neutral and acidic drugs in Model IV.*

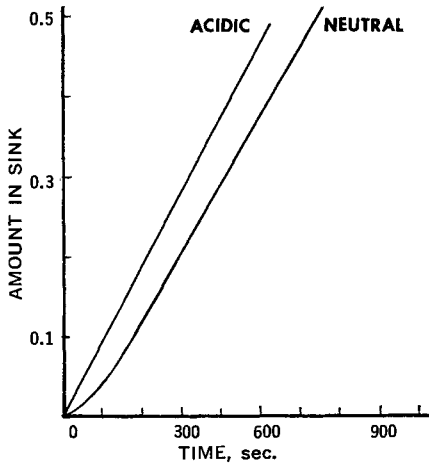


Figure 5—*Total amount of drug accumulated in the perfect sink with time according to Model IV.*

Model IV: Aqueous-Lipid-Lipid/Aqueous Compartments—For this model, consisting of barrier compartments of homogeneous aqueous and lipid phases in series followed by a heterogeneous compartment, or parallel barrier, numerical calculations were performed. Figures 3*a* and *b* show the concentration–distance distribution changes with time for a neutral drug, such as dibutylphthalate or cholesterol, and an acidic drug, pKa = 6. A pH_1 of 6.4 in the bulk aqueous phase and pH_3 of 7.4 in the heterogeneous phase were chosen for their close association with physiological

Table III—Maximum Rate Constants of Gastric Absorption in Rats and Some Physical Constants of Barbituric Acids[a]

	Barbiturate	K_u, hr.$^{-1}$	P[b]	pKa_1
1	Barbital	0.053	3.82	7.91
2	Probarbital	0.082	8.81	8.01
3	Allobarbital	0.092	16.80	7.79
4	Phenobarbital	0.135	34.40	7.41
5	Cyclobarbital	0.142	4.14	7.50
6	Pentobarbital	0.194	106.00	8.11
7	Amobarbital	0.195	113.00	7.94
8	Metharbital	0.178	20.60	8.17
9	Hexobarbital	0.276	73.20	8.34
10	Mephobarbital	0.354	55.80	7.70
11	Thiopental	0.475	991.00	7.45
12	Thiamylal	0.417	1700.00	7.48
13	5-Cyclohexen-1-yl-5-ethyl-1-methylbarbituric acid	0.276	187.00	8.14
14	5,5-Diallyl-1-methylbarbituric acid	0.290	85.50	8.06
15	5-Ethyl-5-iso-pentyl-1-methyl-barbituric acid	0.421	402.00	8.31

[a] Data taken from *Reference 7*. [b] P is the partition coefficient of unionized drug between isoamyl alcohol and water at 37°.

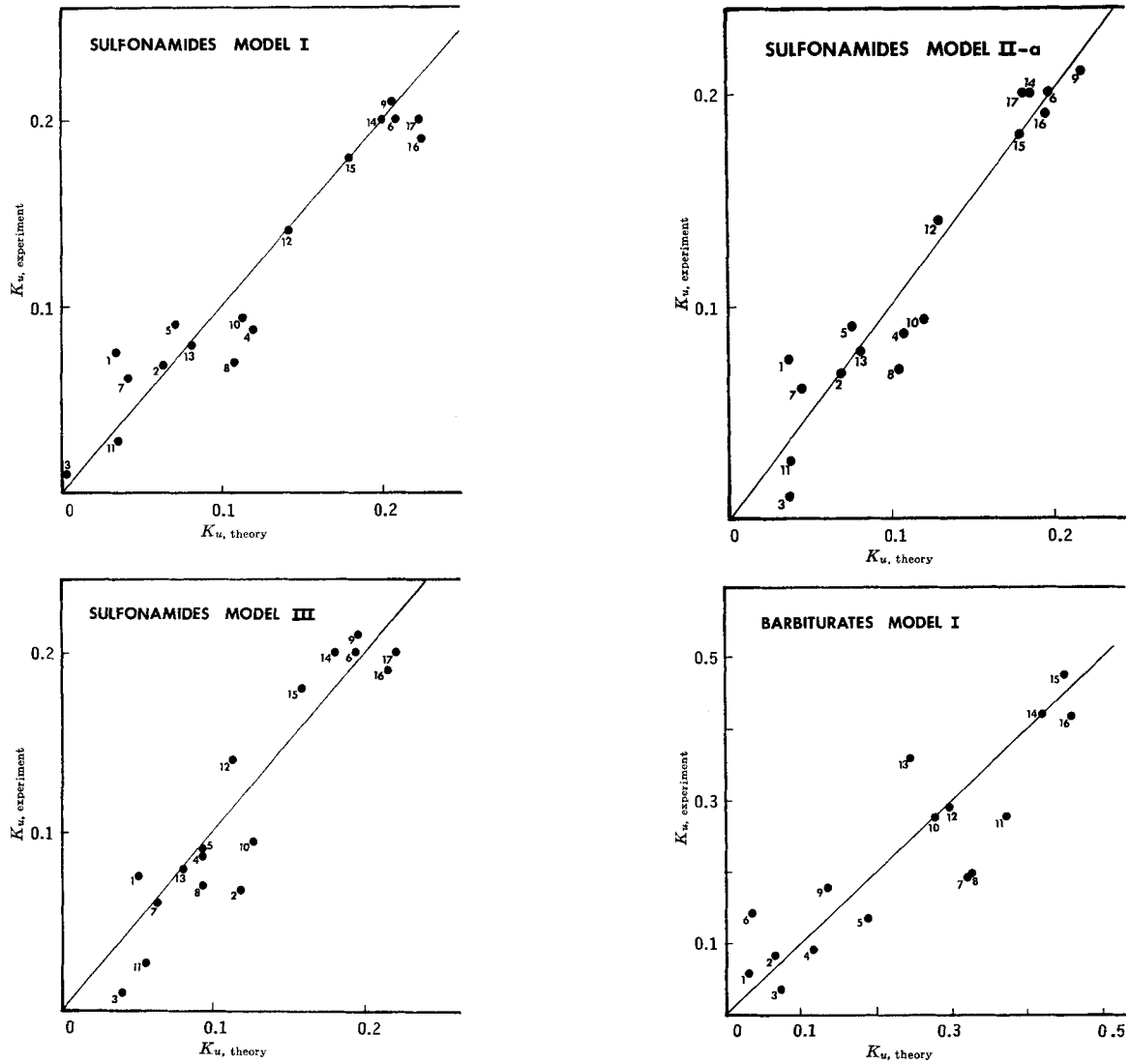


Figure 6—*Comparison of experimental rate constants for the gastric absorption of sulfonamides and barbiturates with the theoretical rate constants calculated for various models using self-consistent dimensional and diffusion constants. Numbers refer to those drugs in Tables II and III.*

conditions. Other dimensional values are given in Fig. 3*a*.

The steady-state rate constant is

$$K_u = B_1 \cdot \frac{1}{1 + (B_2/PX_1) + B_3/[PX_1 + (B_4X_1/X_3)]} \quad \text{(Eq. 28)}$$

where

$$B_3 = \frac{L_3 D_{w,1}}{L_1 R_3 D_{0,3} \alpha_3} \quad \text{(Eq. 28}a\text{)}$$

$$B_4 = \frac{(1 - \alpha_3) D_{w,3}}{\alpha_3 D_{0,3}} \quad \text{(Eq. 28}b\text{)}$$

and B_1 and B_2 are the same as in Eqs. 22*a* and *b*. The magnitude of X_1 and X_3 depends upon the environment of the first and third compartments, respectively, and the nature of the drug.

Between the cases of the acidic and neutral drugs, the total rate of diffusion is slightly faster with the acidic drug. Despite the low concentration of the unionized form of the acidic drug in the bulk aqueous phase, the instantaneous equilibrium conversion of unionized to anionic species at the boundary of the lipid and heterogeneous compartments ($pH_3 - pKa > 1$) and the rapid effective diffusivity of both anionic and nonionic species in the last compartment led to a higher concentration gradient in the lipid than that for the neutral drug case. Thus, the "push-pull" effect is shown. In the case of the neutral drug, the relatively low partitioning from the lipid to the heterogeneous compartment, which is essentially aqueous in character ($\alpha_3 = 0.1$), resulted in a back-up effect or a small lipid concentration gradient.

Figure 4 gives the change in the total rate in the form of $F = (VL_1)/(AD_{w,1}) \cdot K_u$ with time. Previously (1), it was shown that the function F comes from the rigorous numerical calculation of the

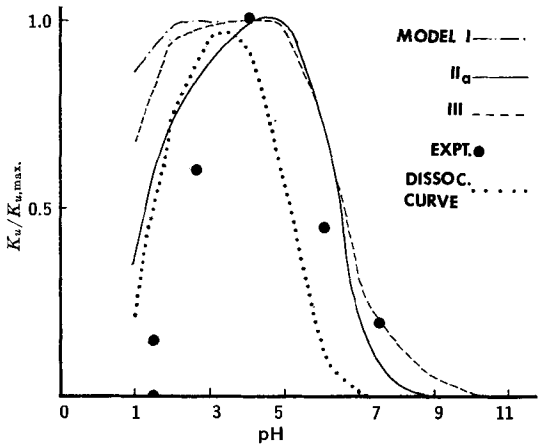


Figure 7—*Comparison of* in situ *and theoretical absorption rates of sulfisoxazole through the intestinal tract from solutions of various pH.*

Table IV—Coefficients of Eq. 22 of Model I Calculated by Regression Analysis of *In Situ* Experimental Absorption Data

Drug	Tract[a]	B_1	B_2
Sulfonamides	*I*	1.87 (1.8)	1.13 (1.0)
Sulfonamides	*G*	0.21 (0.23)	1.91 (2.0)
Barbiturates	*G*	0.43 (0.46)	51.1 (50.0)
Sulfonamides	*R*	1.03 (1.00)	45.1 (41.6)

[a] *I*, *G*, and *R* denote the intestine, stomach, and rectum, respectively. Numerical values of the coefficients in parentheses were calculated using the self-consistent dimensional constants and diffusion coefficients in Table VII.

Table V—Coefficients of Eq. 23 of Model II*a* Calculated by Regression Analysis of *In Situ* Experimental Absorption Data

Drug	Tract	B_1	B_2	B_3
Sulfonamides	*I*	2.25 (2.37)[a]	1.13 (1.0)	0.50 (0.5)
Sulfonamides	*G*	0.247 (0.24)	2.14 (2.0)	0.34 (0.5)
Barbiturates	*G*	0.66 (0.52)	50.0 (50.0)	0.67 (0.5)
Sulfonamides	*R*	1.03 (1.0)	45.1 (42.0)	0.48 (0.5)

[a] Numerical values of the coefficients in parentheses were calculated using the self-consistent dimensional constants and diffusion coefficients in Table VIII.

dynamic flux of drug through the system and the function f is the useful approximation of F in analytic form. At steady state, both F and f should give the same results. The numerical calculations of the lag time τ and the function F at quasisteady state in Table I are in good agreement with the estimated τ (Eq. 20) and f (Eq. 21). Figure 5 shows the total amount of neutral and acidic drug in the sink as a function of time.

APPLICATION OF THEORETICAL MODELS TO ABSORPTION OF SULFONAMIDES AND BARBITURATES IN RATS

In this section an attempt is made to analyze the movement of drugs across cell membranes in the studies of Kakemi *et al.* of the kinetics of absorption of sulfonamides and barbiturates in the intestinal, gastric, and rectal tracts (4–7). Utilizing theoretical models and introducing physical dimensions, some of which are associated with various membranes and others determined by *in vitro* experiments, the authors attempt to calculate for physical constants self-consistent with experimental data and to discuss factors controlling the rate of movement.

Regression Analysis Results and Dimensional Constants—Tables II and III give the experimental results of Kakemi *et al.* only in the gastric absorption of sulfonamides and barbiturates, while the results of intestinal and rectal absorption are found elsewhere

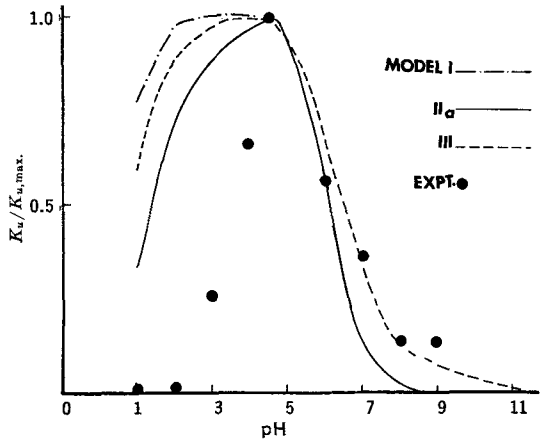


Figure 8—*Comparison of* in situ *and theoretical absorption rates of sulfisoxazole through the stomach from solutions of various pH.*

Table VI—Coefficients of Eq. 27 of Model III Calculated by Regression Analysis of *In Situ* Experimental Absorption Data

Drug	Tract	B_1	B_2	B_3
Sulfonamides	*I*	2.03 (2.0)[a]	3.53 (3.3)	1 03 (1.0)
Sulfonamides	*G*	0.23 (0.25)	4.80 (5.0)	0.95 (1.0)
Barbiturates	*G*	0.46 (0.50)	60.65 (60.7)	1.10 (1.0)
Sulfonamides	*R*	4.23 (4.0)	230.75 (230.0)	1.07 (1.0)

[a] Numerical values of the coefficients in parentheses were calculated using the self-consistent dimensional constants and diffusion coefficients in Table IX.

(5, 6). Their steady-state first-order rate constant,[2] K_u, for an aqueous–lipoidal barrier model has the same general form as Eq. 19, although derived differently. Before applying Models I, II*a*, and III to their results, it is necessary to specify the pH of the compartments following the first bulk aqueous compartment. To account for the possibility of the simultaneous diffusion of buffer species and the subsequent effect on the pH of the aqueous phase in the membrane compartments, it is arbitrarily assumed that $pH_i = (pH_1 + 7.4)/2$, where $i = 2$ or 3 for the respective Model II*a* or III. Also, the volume fraction of lipid in the heterogeneous compartment of Model III was taken to be 0.5. Through the use of (*a*) the values of the maximum experimental K_u, pKa_1, and pKa_2, (*b*) the partition coefficient relative to isoamyl acetate–water, such as those found in Tables II and III, and (*c*)

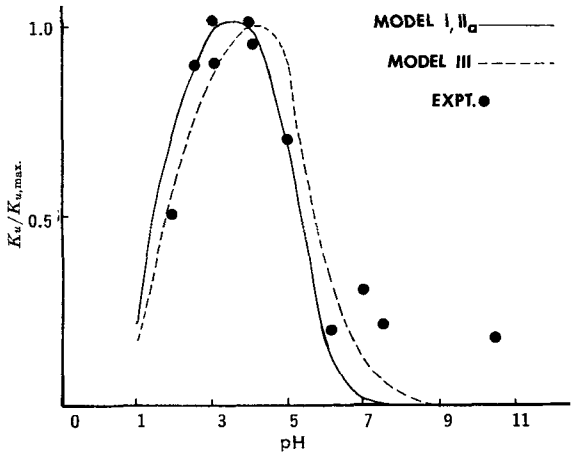


Figure 9—*Comparison of* in situ *and theoretical absorption rates of sulfisoxazole through the rectum from solutions of various pH.*

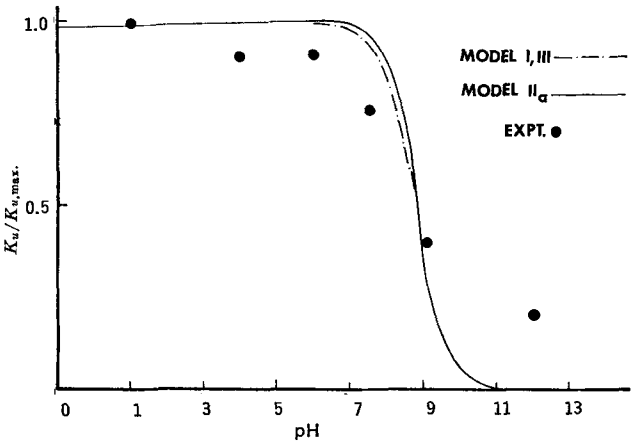


Figure 10—*Comparison of* in situ *and theoretical absorption rates of hexobarbital through the stomach from solutions of various pH.*

[2] According to Kakemi *et al.*, $(M)^{1/2} K_u = (abP)/(1 + aP)$, where a and b are constants, P is the partition coefficient, and M is the molecular weight.

Table VII—Dimensional Constants and Diffusion Coefficients for Model I

Drug	Tract	A/V	L_1	L_2	D_w	D_0	R_2
Sulfonamides	*I*	1	10^{-2}	10^{-6}	5×10^{-6}	10^{-12}	500
Sulfonamides	*G*	0.13	10^{-2}	10^{-6}	5×10^{-6}	10^{-12}	250
Barbituric acid derivatives	*G*	0.13	10^{-2}	10^{-6}	10^{-5}	8×10^{-14}	250
Sulfonamides	*R*	0.56	10^{-2}	10^{-6}	5×10^{-6}	10^{-12}	12

Table VIII—Dimensional Constants and Diffusion Coefficients for Model II*a*

Drug	Tract	A/V	L_1	L_2	L_3	R_2	D_w	D_0
Sulfonamides	*I*	1.3	10^{-2}	10^{-6}	5×10^{-3}	500	5×10^{-6}	10^{-12}
Sulfonamides	*G*	0.15	10^{-2}	10^{-6}	5×10^{-3}	250	5×10^{-6}	10^{-12}
Barbituric acid derivatives	*G*	0.15	10^{-2}	10^{-6}	5×10^{-3}	250	10^{-5}	8×10^{-14}
Sulfonamides	*R*	0.56	10^{-2}	10^{-6}	5×10^{-3}	12	5×10^{-6}	10^{-12}

Table IX—Dimensional Constants and Diffusion Coefficients for Model III

Drug	Tract	A/V	L_1	L_2	R_2	D_0	D_w	$D_{w'}$	α
Sulfonamides	*I*	1.1	10^{-2}	10^{-6}	300	10^{-12}	5×10^{-6}	10^{-12}	0.5
Sulfonamides	*G*	0.14	10^{-2}	10^{-6}	200	10^{-12}	5×10^{-6}	10^{-12}	0.5
Barbituric acid derivatives	*G*	0.14	10^{-2}	10^{-6}	200	1.7×10^{-13}	10^{-5}	1.7×10^{-13}	0.5
Sulfonamides	*R*	2.2	10^{-2}	10^{-6}	4.4	10^{-12}	5×10^{-6}	10^{-12}	0.5

the application of Hartley's method of nonlinear regression analysis (8), the coefficients B_1, B_2, and B_3 of the rate-constant Eqs. 22 (Model I), 23 (Model II*a*), and 27 (Model III) were determined and these are found in Tables IV–VI. From these coefficients, self-consistent combinations of dimensional constants and diffusion coefficients for each model were calculated. To derive the results in Tables VII–IX, the following procedure was taken: (*a*) assume $L_1 = 10^{-2}$ cm., $L_2 = 10^{-6}$ cm., $\alpha = 0.5$ in the case of Model III, and $R_2 = 500$ for the intestinal tract in the case of Model I (9); (*b*) assume $D_{w,1} = 1 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ for the barbituric acid derivatives[3] and by comparing B_1 of the sulfonamides and barbiturates in the gastric tract, take $D_{w,1} = 5 \times 10^{-6}$ cm.^{2} sec.$^{-1}$ for the sulfonamides; (*c*) calculate D_0 for the sulfonamides from B_2 of the intestinal tract, R_2 of the gastric and rectal tract, and the rest of the parameters. Between the barbiturates and the sulfonamides, the $D_{w,1}$ of the latter drug does not seem unreasonable on the basis of the higher average molecular weight. The $D_0 = 1 \times 10^{-12}$ cm.^{2} sec.$^{-1}$ found for the sulfonamides compares well with the diffusion coefficients (10^{-10}–10^{-12} cm.^{2} sec.$^{-1}$) of some organic solutes through lipid bilayer membranes (10) and of various sulfonamides through red blood cells (11). It is observed that the D_0 for the barbiturates is smaller than that for the sulfonamides by a factor of 10. This cannot be easily explained by the usual Stokes-Einstein diffusion equation.

The theoretical K_u of the sulfonamides in the intestinal, gastric, and rectal tracts and of the barbiturates in the gastric tract for the various models was calculated, using the dimensional constants and the diffusion coefficients in Tables VII–IX, and plotted against the K_u of the *in situ* experiments. Figure 6 shows the $K_{u,\text{expt.}}$ *versus* $K_{u,\text{theory}}$ plot only for the gastric absorption of sulfonamides according to Models I, II*a*, and III and of barbiturates for Model I. The strong correlation observed here in these figures was also consistent throughout the other cases. However, at this point one cannot determine which physical model is more applicable, except to say that the constants determined previously are self-consistent.

Comparison of the pH Profile of the Absorption Rate with Experiments and Physical Models—The relative absorption rates *versus* bulk aqueous pH for sulfisoxazole in the intestinal, gastric, and rectal tracts are shown in Figs. 7–9 and for hexobarbital in the gastric tract in Fig. 10. The calculated results of the physical models are compared with experimental data.

In the cases of gastric and intestinal absorption of sulfisoxazole, the physical models show a large deviation in the acidic region and a relatively good fit on the alkaline side of the profile, particularly with Model III. The profile of Model I is symmetrical about the dissociation curve. This is not surprising in view of the lengthy discussion given earlier (1). On the other hand, asymmetry of the profiles of Models II*a* and III about the dissociation curve is found. This is explainable by the influence of the effective pH in the aqueouslike compartments of the membrane, which was assumed to be the arithmetic average of the pH of the bulk drug solution and the physiologic pH of 7.4 on the distribution and transport of drug species in the membrane.

Because of the restriction that the (H^+) in any compartment (particularly, the first compartment) is constant, the present models cannot adequately account for the shift of the maximum experimental rates in Figs. 7 and 8 to higher pH values of the bulk solution relative to the pH of the dissociation curve at which the fraction of the unionized drug form is maximum. There is experimental evidence[4] in the case of gastric absorption of sulfisoxazole that increasing the buffer capacity of the drug solution tends to shift the maximum rate closer to the pH maximum of the dissociation curve. Thus, an accurate accounting of the pH at the surface and within the aqueous phases of the membrane and also protein binding in the physical models may explain the shift of the experimental maximum rate to the more alkaline side. Although there can be a difference between the surface and bulk solution pH, it seems doubtful that they are related through the surface potential (12) in *in situ* experiments; that is, $pH_s = pH_{soln.} + e\psi_0/kT$. Instead it is suggested that surface pH might be related to acid production in the cells and diffusion into the bulk solution.[5]

In the absorption rate *versus* bulk pH profile for the rectal absorption of sulfisoxazole (Fig. 9) and gastric absorption of hexobarbital (Fig. 10), there was relatively good agreement between the *in situ* experimental results and physical models. Maximum absorption of sulfisoxazole was observed at the isoionic point and the absorption rate of hexobarbital corresponded with the pKa.

In conclusion it is difficult to select the physical model that best describes the *in situ* absorption experiments in the intestinal, gastric,

[3] Kakemi *et al.* found $D_{w,1} = 0.815 - 1.27 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ at 37° for the barbiturates.

[4] Refer to Fig. 5 in *Reference 4*.

[5] Theoretical models involving the effect of the surface potential on the one hand and acid production with simultaneous reaction and diffusion on the other hand are being investigated.

or rectal tracts since all of the models studied showed fairly good agreement. More knowledge of the structure and microenvironment of the membrane as well as drug interactions with cellular substances is necessary before modification of the physical models can be made. Furthermore, the models generally have more quantitative parameters in detail which the usual *in situ* experiments alone cannot provide. However, the physical model approach to drug transport studies is being further investigated.

REFERENCES

(1) A. Suzuki, W. I. Higuchi, and N. F. H. Ho, *J. Pharm. Sci.*, **59,** 644(1970).

(2) J. Kavanau, "Structure and Function of Biological Membranes," vol. I, Holden-Day, San Francisco, Calif., 1965, p. 145.

(3) W. I. Higuchi and T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 568(1960).

(4) T. Koizumi, T. Arita, and K. Kakemi, *Chem. Pharm. Bull.*, **12,** 413(1964).

(5) *Ibid.*, **12,** 421(1964).

(6) K. Kakemi, T. Arita, and S. Muranishi, *ibid.*, **13,** 861(1965).

(7) K. Kakemi, T. Arita, R. Hori, and R. Konishi, *ibid.*, **15,** 1534(1967).

(8) N. R. Draper and H. Smith, "Applied Regression Analysis," Wiley, New York, N. Y., 1966, p. 269.

(9) T. H. Wilson, "Intestinal Absorption," W. B. Saunders, Philadelphia, Pa., 1962.

(10) R. C. Bean, W. C. Shepperd, and H. Chan, *J. Gen. Physiol.*, **52,** 495(1968).

(11) L. B. Holder and S. L. Hayes, *Mol. Pharmacol.*, **1,** 266 (1965).

(12) A. Albert, *Pharmacol. Rev.*, **4,** 136(1952).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 23, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication October 30, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969

* Present address: Tanabe Seiyaku, Osaka, Japan.

Interfacial Barriers in Interphase Transport III: Transport of Cholesterol and Other Organic Solutes into Hexadecane–Gelatin–Water Matrices

ABDEL-HALIM GHANEM, W. I. HIGUCHI, and A. P. SIMONELLI

Abstract □ The purpose of this study was to quantitate the transport behavior of several organic solutes in matrix systems composed of micron-size hexadecane droplets dispersed in an aqueous gelatin gel where the oil–water interfacial barrier to transport was expected to play an important role. Two interrelated experiments were conducted. The first was the one-dimensional aqueous uptake of the solute by the matrix which was a continuous layer placed at the bottom of a beaker. The other experiment was solute uptake and release from aggregates of oil droplets suspended in an aqueous medium. Solutes investigated were ^{14}C-labeled cholesterol, diethylphthalate, ^{14}C-labeled octanol, and ^{14}C-labeled progesterone. The data have been analyzed by various physical models. It was found that cholesterol transport essentially was controlled by the oil–water interfacial barrier in both kinds of experiments—even when the matrix thickness was as large as 3.7 mm. For the other solutes, the oil–water interfacial barriers were found to be controlling in the experiments with aggregates (10–1000 μ). However, in the experiments with the continuous matrix layers, bulk matrix diffusion factors as well as the oil–water interphase transport were found to be important for these solutes. The techniques developed in this investigation should be useful: (*a*) in the quantitation of interfacial barriers in oil–water interphase transport of solutes, and (*b*) in the separation of various bulk diffusional resistances from interfacial resistances in complex multiphase matrices.

Keyphrases □ Interphase transport—interfacial barriers □ Hexadecane–gelatin–water matrix—organic solute transport □ Matrix layer—solute uptake □ Aggregated gelatin encapsulated hexadecane droplets—solute transport □ Electrolyte, polysorbate 80, concentration effects—interphase transport

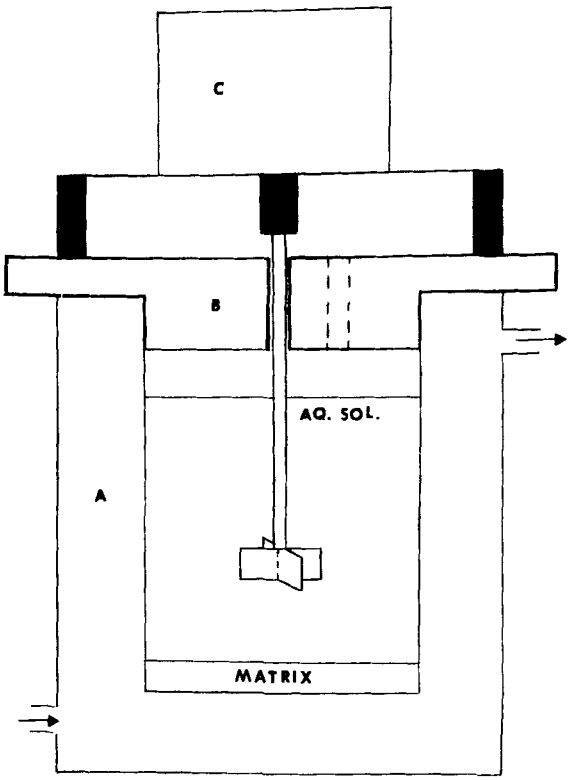


Figure 1—*Schematic diagram showing the apparatus used for the continuous matrix layer (CML) uptake experiments.*

Recent studies in these laboratories have been aimed at the mechanistic understanding of various factors influencing the interphase transport of drugs and other biologically interesting substances. These investigations (1–4) have considered, for example, the simultaneous multiphase interactions involving pH and the buffer parameters, the oil–water partition coefficients, and the diffusion coefficients. More recently (5–8), utilizing a novel technique, the existence and the importance of the

or rectal tracts since all of the models studied showed fairly good agreement. More knowledge of the structure and microenvironment of the membrane as well as drug interactions with cellular substances is necessary before modification of the physical models can be made. Furthermore, the models generally have more quantitative parameters in detail which the usual *in situ* experiments alone cannot provide. However, the physical model approach to drug transport studies is being further investigated.

REFERENCES

(1) A. Suzuki, W. I. Higuchi, and N. F. H. Ho, *J. Pharm. Sci.*, **59,** 644(1970).

(2) J. Kavanau, "Structure and Function of Biological Membranes," vol. I, Holden-Day, San Francisco, Calif., 1965, p. 145.

(3) W. I. Higuchi and T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 568(1960).

(4) T. Koizumi, T. Arita, and K. Kakemi, *Chem. Pharm. Bull.*, **12,** 413(1964).

(5) *Ibid.*, **12,** 421(1964).

(6) K. Kakemi, T. Arita, and S. Muranishi, *ibid.*, **13,** 861(1965).

(7) K. Kakemi, T. Arita, R. Hori, and R. Konishi, *ibid.*, **15,** 1534(1967).

(8) N. R. Draper and H. Smith, "Applied Regression Analysis," Wiley, New York, N. Y., 1966, p. 269.

(9) T. H. Wilson, "Intestinal Absorption," W. B. Saunders, Philadelphia, Pa., 1962.

(10) R. C. Bean, W. C. Shepperd, and H. Chan, *J. Gen. Physiol.*, **52,** 495(1968).

(11) L. B. Holder and S. L. Hayes, *Mol. Pharmacol.*, **1,** 266 (1965).

(12) A. Albert, *Pharmacol. Rev.*, **4,** 136(1952).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 23, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication October 30, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969

* Present address: Tanabe Seiyaku, Osaka, Japan.

Interfacial Barriers in Interphase Transport III: Transport of Cholesterol and Other Organic Solutes into Hexadecane–Gelatin–Water Matrices

ABDEL-HALIM GHANEM, W. I. HIGUCHI, and A. P. SIMONELLI

Abstract □ The purpose of this study was to quantitate the transport behavior of several organic solutes in matrix systems composed of micron-size hexadecane droplets dispersed in an aqueous gelatin gel where the oil–water interfacial barrier to transport was expected to play an important role. Two interrelated experiments were conducted. The first was the one-dimensional aqueous uptake of the solute by the matrix which was a continuous layer placed at the bottom of a beaker. The other experiment was solute uptake and release from aggregates of oil droplets suspended in an aqueous medium. Solutes investigated were ^{14}C-labeled cholesterol, diethylphthalate, ^{14}C-labeled octanol, and ^{14}C-labeled progesterone. The data have been analyzed by various physical models. It was found that cholesterol transport essentially was controlled by the oil–water interfacial barrier in both kinds of experiments—even when the matrix thickness was as large as 3.7 mm. For the other solutes, the oil–water interfacial barriers were found to be controlling in the experiments with aggregates (10–1000 μ). However, in the experiments with the continuous matrix layers, bulk matrix diffusion factors as well as the oil–water interphase transport were found to be important for these solutes. The techniques developed in this investigation should be useful: (*a*) in the quantitation of interfacial barriers in oil–water interphase transport of solutes, and (*b*) in the separation of various bulk diffusional resistances from interfacial resistances in complex multiphase matrices.

Keyphrases □ Interphase transport—interfacial barriers □ Hexadecane–gelatin–water matrix—organic solute transport □ Matrix layer—solute uptake □ Aggregated gelatin encapsulated hexadecane droplets—solute transport □ Electrolyte, polysorbate 80, concentration effects—interphase transport

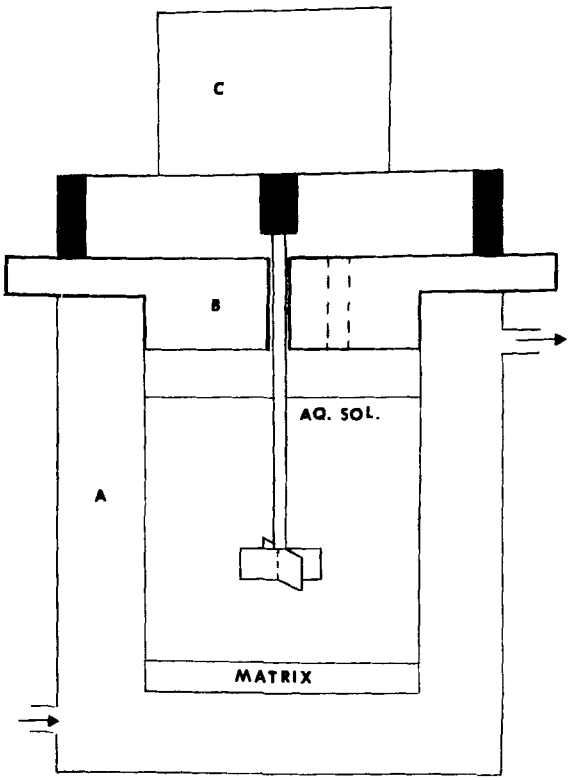


Figure 1—*Schematic diagram showing the apparatus used for the continuous matrix layer (CML) uptake experiments.*

Recent studies in these laboratories have been aimed at the mechanistic understanding of various factors influencing the interphase transport of drugs and other biologically interesting substances. These investigations (1–4) have considered, for example, the simultaneous multiphase interactions involving pH and the buffer parameters, the oil–water partition coefficients, and the diffusion coefficients. More recently (5–8), utilizing a novel technique, the existence and the importance of the

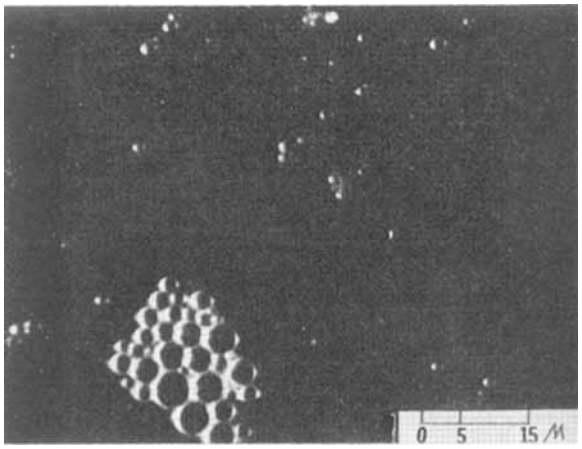


Figure 2—*Dark-field photomicrograph of aggregates before treatment with formaldehyde solution.*

oil–water interfacial barriers have been quantitated for several systems.

The present communication describes the results of experiments on the transport of solutes into and out of gelatin matrices in which micron-size droplets of hexadecane were dispersed. The findings of this study have been correlated with the previous research in these laboratories and demonstrate when an oil–water interfacial barrier is rate determining (5–7) and when bulk matrix diffusional factors are dominant (1–3).

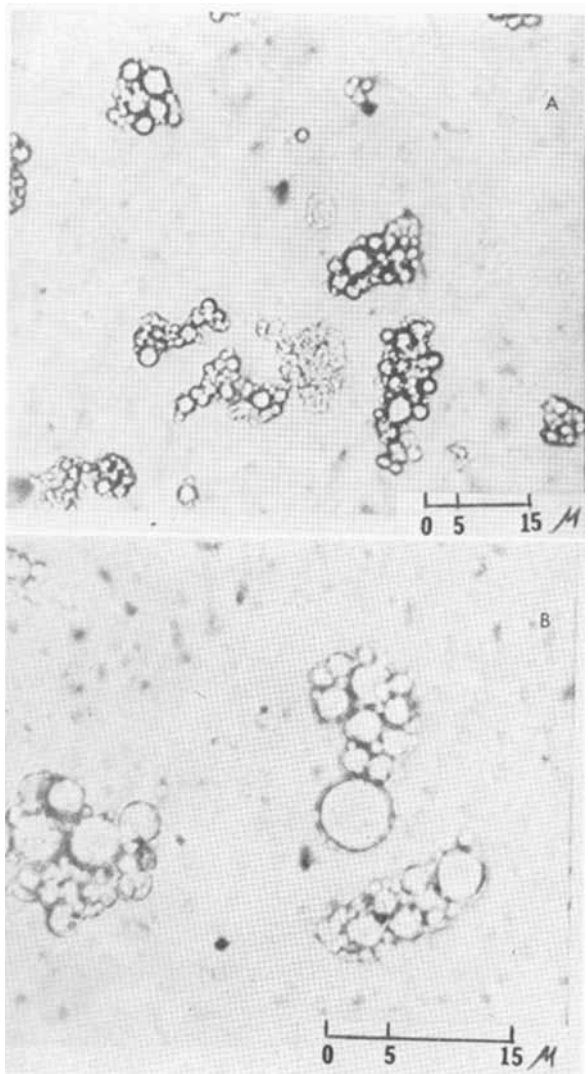


Figure 3—*Photomicrograph of the aggregates after treatment with an 18.5% formaldehyde solution.*

EXPERIMENTAL

General Considerations—Essentially two kinds of interrelated experiments were conceived. The first of these was the one-dimensional aqueous uptake of the solute by a dispersion of oil droplets in a continuous gelatin matrix layer polymerized with a formaldehyde solution. The second type of experiment was the determination of the uptake and release of solute from an aqueous suspension of aggregates of the oil droplets prepared by first treating the gelatin stabilized oil-in-water emulsion with concentrated sodium sulfate (5) and then with a formaldehyde solution. As will be seen, this combined approach provides the means to determine the relative importances of oil–water interfacial barriers and bulk diffusion in complex heterogeneous matrices of this type.

Materials—The gelatin, hexadecane, sodium sulfate, and diethylphthalate employed in these studies were previously described

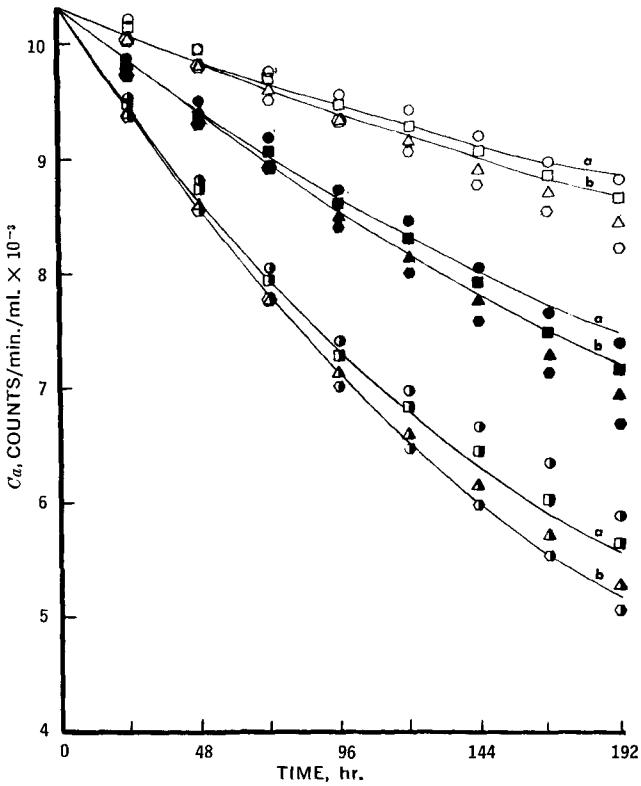


Figure 4—*Comparison of experimental ^{14}C-labeled cholesterol uptakes (CML and AGE techniques) with theoretically computed values based upon the interfacial barrier model as a function of oil volumes and polysorbate 80.* C_a *is counts/min./ml. of solute in the aqueous polysorbate 80 remaining* versus *time in hours. Initial* (t = 0) ^{14}C*-4-cholesterol concentration was* 3.4×10^{-8} *g./ml. and initial aqueous volumes were 50 ml. in all experiments. Key:*

Oil Volume, ml.	*0.1% Polysorbate 80 Solution (a)*		*0.05% Polysorbate 80 Solution (b)*	
	CML	*AGE*	*CML*	*AGE*
0.2	○	□	△	⬡
0.4	●	■	▲	⬢
0.8	◑	◨	◭	◑

Curves are theoretically computed values using K = *140 for Curve (a),* K = *250 for Curve (b), and* P = 5×10^{-9} *cm./sec.*

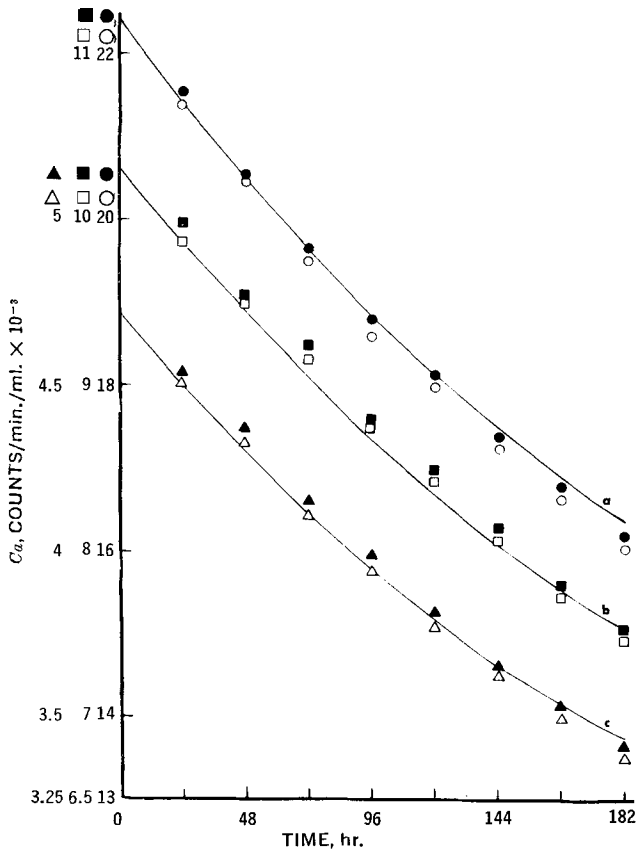


Figure 5—*Comparison of experimental ^{14}C-labeled cholesterol uptake (CML technique) with theoretically computed values (interfacial barrier model) at two stirring speeds and three ^{14}C-4-cholesterol concentrations in 0.1% polysorbate 80. C_a is counts/min./ml. of solute in the aqueous polysorbate 80 remaining* versus *time in hours. Oil volume was 0.4 ml. Initial aqueous volumes were 50 ml. Key:*

Stirring Speed, r.p.m.	*8.5 × 10^{-8} g./ml. (a)*	*3.4 × 10^{-8} g./ml. (b)*	*1.36 × 10^{-8} g./ml. (c)*
50	●	■	▲
150	○	□	△

Curves are theoretically computed values with K = *140 and* P = *5 × 10^{-9} cm./sec.*

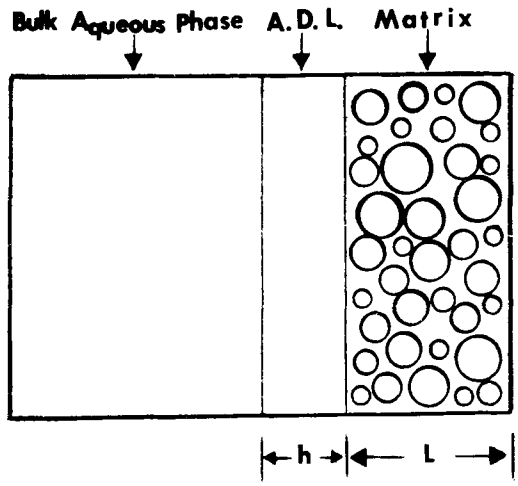
(5, 6). Formaldehyde AR grade[1] was used. Solutes other than diethylphthalate investigated in these studies were ^{14}C-4-cholesterol,[2] ^{14}C-4-progesterone,[3] and ^{14}C-1-octanol.[4]

Procedure for the Continuous Matrix Layer (CML) Uptake—Figure 1 shows a schematic diagram of the experimental apparatus which consists of a water-jacketed beaker (A) with a plastic cover (B) that prevents evaporation and supports a synchronous motor (C).[5] Two holes have been drilled in the cover, one for sampling and the other for a four-blade glass stirring rod.

A specified amount of the hexadecane-in-water emulsion stabilized with gelatin was prepared as previously reported (6). The emulsion at 40° was transferred to the beaker, and the temperature was immediately dropped to 10° by placing the beaker in an ice water bath for 1 hr. Then 25 ml. of a 3.7% formaldehyde solution (pH 9.0) at 10° was added to the beaker and allowed to interact with the gel for 1 hour at the same temperature. The formaldehyde was then leached out by washing with water several times over a period of 12–18 hr. In this manner, matrices of different thicknesses (volumes corresponding to 1, 2, 4, and 8 ml.) were prepared and were found to be stable in water or in polysorbate 80 solution (0.05–0.1%) over a period of 10 days.

The uptake experiments were carried out in the following manner. The aqueous solution of the organic solute was added to the beaker containing the prepared matrix system. Stirring was immediately initiated using either a 50 or a 150 r.p.m. synchronous motor. The temperature was kept at 23 ± 2°. Samples of 1.0 ml. were removed at predetermined time intervals for quantitative analysis.

Procedure for the Transport Studies with Aggregated "Gelatin-Encapsulated" Hexadecane Droplets (AGE)—Fifty grams of an 18% solution of sodium sulfate was added to 70 g. of the previously described (5) hexadecane–gelatin emulsion. The mixture was stirred for 6–8 hr. at 50° (5). The mixture was then transferred to an ice water jacketed Waring blender operating at a speed of 1500–2000 r.p.m. and cooled to 10°. Then 100 ml. of an 18.5% formaldehyde solution (pH 9.0, temperature 10°) was added to the mixture and allowed to react for 1 hr. with continuous stirring. The material was then washed with water and stored in water until use in the uptake experiment.



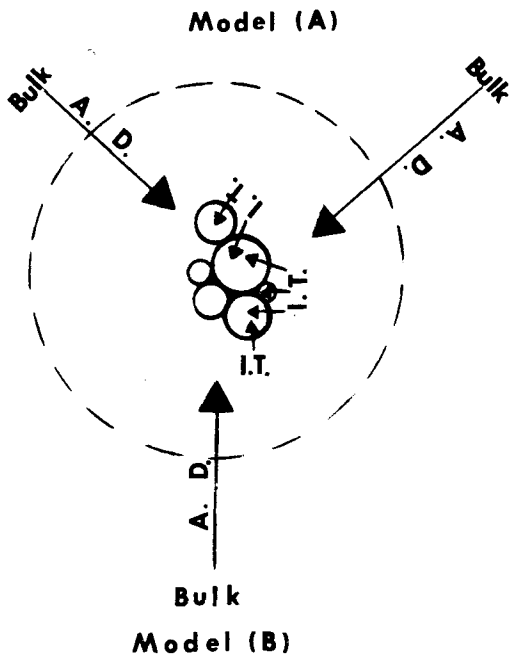


Figure 6—*The physical models that describe the uptakes of the solute from the aqueous phase to the CML, Model (A), and the AGE, Model (B). A.D.L. = aqueous diffusion layer. A.D. = aqueous diffusion. I.T. = interfacial transport.*

[1] Mallinckrodt Chemical Works, New York, N. Y.
[2] New England Nuclear Corp., Boston, Mass.
[3] Amersham/Searle Corp., Chicago, Ill.
[4] International Chemical and Nuclear Corp., City of Industry, Calif.
[5] Hurst Manufacturing Corp., Princeton, Ind.

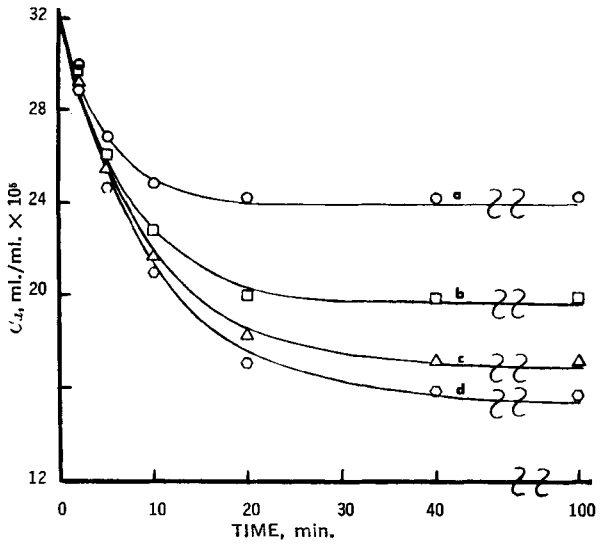


Figure 7—*Comparison of experimental diethylphthalate uptakes (AGE technique) with theoretically computed values (interfacial barrier model) and the influence of different sodium sulfate concentrations.* C_a *is ml./ml. of solute in the aqueous medium remaining* versus *time in minutes. To 1.5-g. aggregates was added 100 ml. of diethylphthalate in:* ○, *water (a);* □, *2.5% sodium sulfate (b);* △, *5% sodium sulfate (c); and* ⬡, *7% sodium sulfate (d). Curves are theoretically computed values with* K = *50, 95, 132, and 160 for (a), (b), (c), and (d), respectively; and* P = 1.0×10^{-5} *cm./sec.*

"Fine" aggregates of supramicron sizes were produced by this procedure and these were found to be stable in water for a month. These aggregates were also found to be stable in polysorbate 80 solution (0.05–0.1%) for at least 7 days. Figure 2 shows a dark-field photomicrograph of the aggregates before treatment with formaldehyde. Figure 3 shows a photomicrograph of the aggregates after treatment with formaldehyde solution. Visual examination of these aggregates showed that about 10% were singlets, about 20% were aggregates of 2–10, about 40% were from 10 to 20, 20% were from 20 to 100, and 10% from 100 to 200.

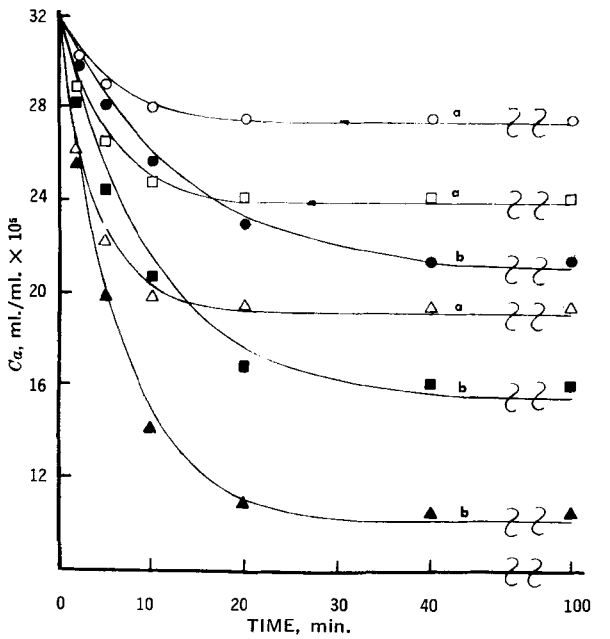


Figure 8—*Comparison of experimental diethylphthalate uptakes (AGE technique) with theoretically computed values (interfacial barrier model), and the effect of different aggregate weights in water and 7% sodium sulfate.* C_a *is ml./ml. of solute in the aqueous medium remaining* versus *time in minutes. Key:*

Medium, 100 ml.	*0.75 g.*	*1.5 g.*	*3.0 g.*
Water (a)	○	□	△
7% Sodium sulfate (b)	●	■	▲

Curves are theoretically computed values with K = *50 for Curve (a),* K = *160 for Curve (b), and* P = 1.0×10^{-5} *cm./sec.*

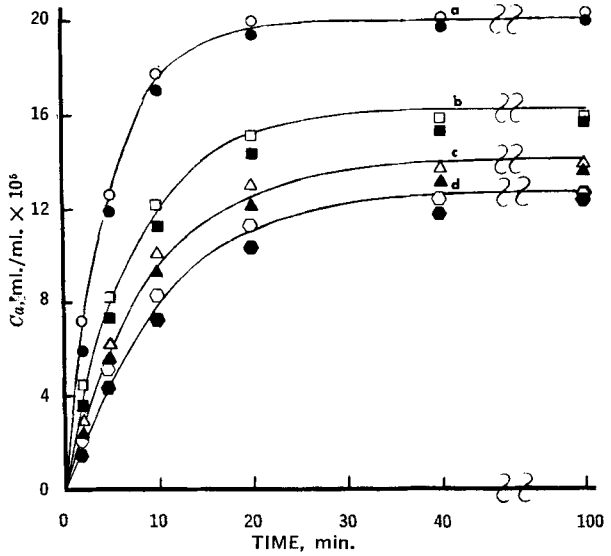


Figure 9—*Comparison of experimental diethylphthalate releases (AGE technique) with theoretically computed values (interfacial barrier model) and the influence of different sodium sulfate concentrations.* C_a *is ml./ml. of solute in the aqueous medium released* versus *time in minutes. Volume of the aqueous medium was 100 ml. Key:*

1.5-g. Aggregates	*Water (a)*	*2.5% Sodium Sulfate (b)*	*5% Sodium Sulfate (c)*	*7% Sodium Sulfate (d)*
Fine	○	□	△	⬡
Coarse	●	■	▲	●

Curves are theoretically computed values with the same parameters as previously mentioned under Fig. 7.

"Coarse" aggregates were also prepared in a similar manner but by using a 22% sodium sulfate solution instead of 18% as was used in the case of the "fine" aggregate preparation. Visual examination of this preparation indicated that about 10% were singlets, 20% were from 2 to 10, 20% from 10 to 50, 30% from 50 to 200, and 30% from 200 to 500.

The solute uptake experiments were carried out as follows. The stock aggregates in water were stirred for a few minutes, a portion was filtered, and then allowed to remain for 2 hr. on the filter. A predetermined amount (0.75, 1.5, or 3.0 g.) was transferred to a 150-ml. plastic beaker[6] which was found to resist sticking of the aggregates to the beaker wall—a problem encountered with glassware. Exactly 100 ml. of the aqueous medium containing the solute was then added to the beaker. Stirring was initiated immediately by means of a synchronous motor (150 or 300 r.p.m.) with a magnetic head coupling to a magnetic stirring bar. In order to achieve good dispersion of the aggregates, the magnetic head was placed slightly off-center of the beaker. The temperature was kept at 23 ± 2°. At different time intervals, a sample of 5 ml. was withdrawn by a 10-ml. syringe, and approximately half of it was filtered through a silver filter membrane[7] using a 10-mm. diameter stainless steel filter holder.[8] Part of this filtrate was used for analysis.

The diethylphthalate release studies with these aggregates were carried out in the same way except that the prepared aggregates were treated in the following manner. A suspension of about 60 g. of the aggregates in about 200 ml. of water containing 1.2 ml. of

[6] Nalgene, Nalge Co., Rochester, N. Y.
[7] Selas Flotronics, Spring House, Pa.
[8] Millipore Filter Corp., Bedford, Mass.

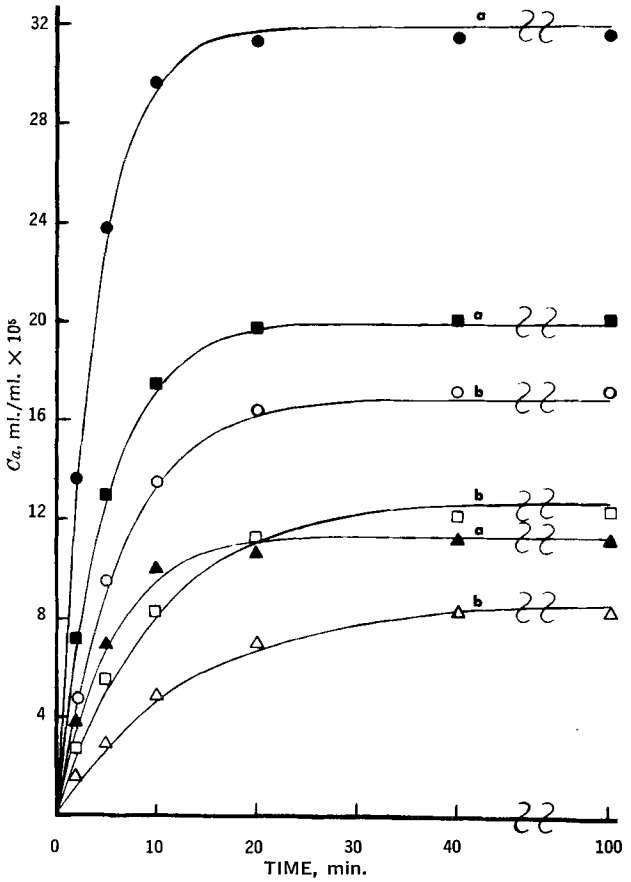


Figure 10—*Comparison of experimental diethylphthalate releases (AGE technique) with theoretically computed values (interfacial barrier model) and the effect of different aggregate weights in water and 7% sodium sulfate.* C_a *is ml./ml. of solute in the aqueous medium released* versus *time in minutes. Key:*

Medium, 100 ml.	*0.75 g.*	*1.5 g.*	*3.0 g.*
Water (a)	▲	■	●
7% Sodium sulfate (b)	△	□	○

Curves are theoretically computed values with the same parameters as previously mentioned under Fig. 8.

diethylphthalate was slowly stirred for 48 hr. with a magnetic stirrer. The mixture was then filtered, washed twice with 50 ml. of water, and stored in 200 ml. of a 0.03% diethylphthalate solution. The concentration of diethylphthalate in the encapsulated oil was determined by total extraction of diethylphthalate (5).

Assay Procedure—Where radioactive-labeled materials were employed, the Beckman LS 200B liquid scintillation system[9] was used. In the diethylphthalate experiments, a spectrophotometric analysis[10] (λ = 273.5 mμ) was employed.

EXPERIMENTAL RESULTS AND THEORETICAL TREATMENT

^{14}C-Labeled Cholesterol Transport from Aqueous Polysorbate 80 Solution into the Hexadecane–Gelatin Matrix Layer (CML) and into Dispersed Aggregates (AGE)—The results of the ^{14}C-4-cholesterol uptake experiments using both techniques (CML and AGE) for three oil volumes and two polysorbate 80 concentrations[11] (0.05 and 0.1%) are presented in Figs. 4 and 5. The uptake rates were found to be generally slow and, for the CML as well as the AGE experiments, the rates were dependent strongly on the oil volumes in the system. Figure 5 presents the influence of the stirring rates and the aqueous ^{14}C-4-cholesterol concentration upon the CML uptake experiments. As can be seen, stirring had a negligible effect upon the rates. Also the uptake rates were found to be directly proportional (first order) to the cholesterol concentration.

The CML and the AGE uptake rates were found to be essentially identical when the mass of the oil phases were the same, as was the case for the experiments shown in Fig. 4. As the primary oil particle-size distributions were the same for the CML and AGE studies, this finding is particularly significant.

In order to explain these data, let us consider the various possible rate-determining mechanisms by referring to the models given in Fig. 6. In general, for the CML case there are three possible processes, any of which can be the rate-determining one. These are: (*a*) diffusion through the aqueous diffusion layer of thickness *h*, (*b*) diffusion through the matrix itself, or (*c*) interfacial barrier transport. For the AGE experiment these three processes may still apply, but the magnitudes of aqueous diffusion and the matrix diffusion resistances would be expected to be much smaller than for those in the CML situation. The interfacial barrier, however, would be expected to be about the same for both the CML and the AGE situations, even though the preparation procedures differed somewhat (see *Experimental*).

All of these experimental results for cholesterol uptake in both systems support the mechanism in which an interfacial barrier is rate determining. First, the absolute rates were essentially the same for the CML and the AGE experiments in every situation. Secondly, the rate of stirring had no influence on the CML uptake rates. Thirdly, the uptake rates showed a strong oil volume (or a layer thickness) dependence with the CML experiments. This would not have been expected if either transport in the aqueous diffusion layer or transport through the matrix itself was rate determining. Thus all of the data support the overwhelming importance of the interfacial process in both situations.

These conclusions justify the quantitative treatment based upon an interfacial barrier control developed previously (5). Thus employing the primary oil droplet size distribution data obtained earlier (6) and the independently determined partition coefficients, the theoretical uptake rates were calculated using different interfacial barrier permeability coefficients. These theoretical calcula-

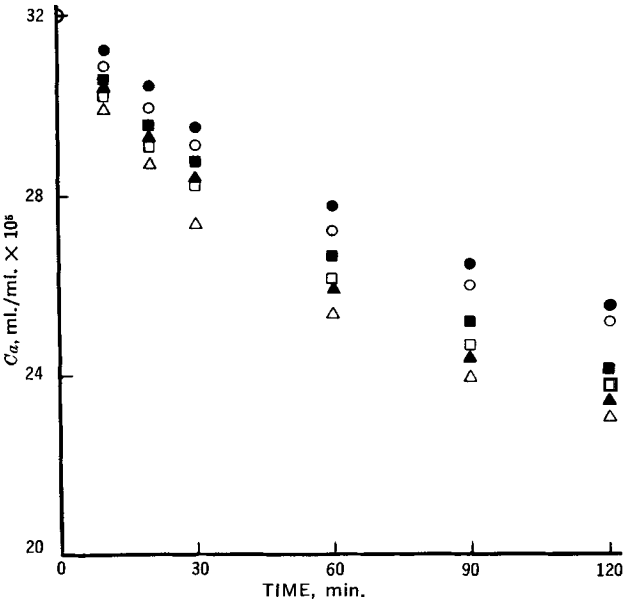


Figure 11—*Experimental diethylphthalate uptakes using the CML method as a function of matrix volume and stirring.* C_a *is ml./ml. of solute in the aqueous medium remaining* versus *time in minutes. Volume of the aqueous medium was 50 ml. of water. Key:*

Stirring Speed, r.p.m.	*2.0 ml.*	*4.0 ml.*	*8.0 ml.*
50	●	■	▲
150	○	□	△

[9] Beckman Instruments, Inc., Fullerton, Calif.
[10] Hitachi, Ltd., Tokyo, Japan.
[11] Preliminary experiments showed that in the absence of polysorbate 80, appreciable adsorption of cholesterol occurred on the beaker walls.

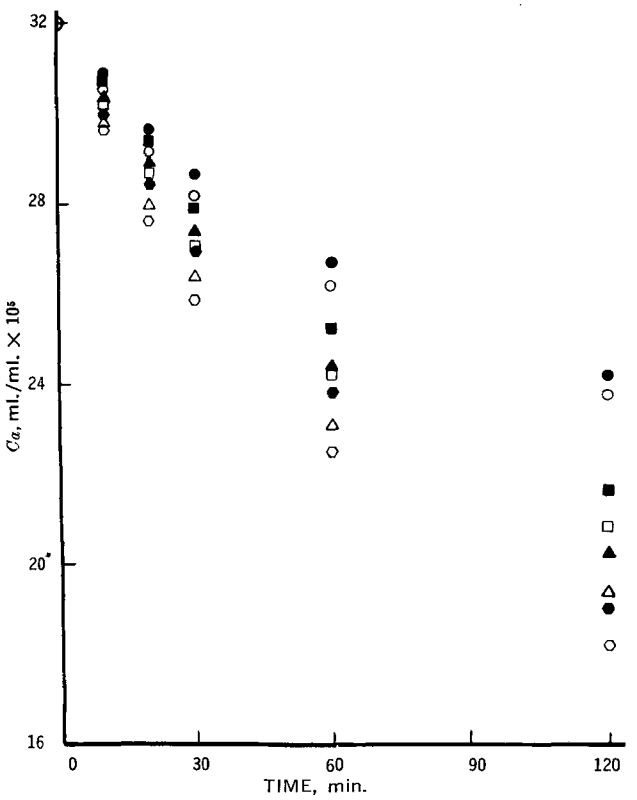


Figure 12—*Experimental diethylphthalate uptakes using CML method as a function of sodium sulfate concentrations and stirring.* C_a *is ml./ml. of solute in the aqueous medium remaining* versus *time in minutes. Matrix volume was 4.0 ml. and aqueous volume was 50 ml. Key:*

Stirring Speed, r.p.m.	*Water*	*2.5% Sodium Sulfate*	*5% Sodium Sulfate*	*7% Sodium Sulfate*
50	●	■	▲	●
150	○	□	△	○

tions have revealed that a single permeability coefficient value of $P = 5 \times 10^{-3}$ cm./sec. quantitatively describes all of the data (Figs. 4 and 5). The smooth curves presented in these plots correspond to the theoretical uptake calculations utilizing this P value. The agreement, as can be seen, is quite satisfactory.

Transport of Diethylphthalate into the Hexadecane–Gelatin Matrix Layer (CML) and into and out of Dispersed Aggregates (AGE)—The results of the diethylphthalate uptake experiments employing the AGE method are presented in Figs. 7 and 8. In Fig. 7, the influence of the electrolyte concentrations on the uptake rate is demonstrated. As can be seen, the significant effects are large at times, *i.e.*, near equilibrium. The initial rate appears to be relatively independent of the electrolyte concentration. In Fig. 8, comparisons of the uptake behavior are shown for three different concentrations of the aggregates in water and in 7% sodium sulfate. The strong dependence upon the oil volume is apparent.

The curves given in Figs. 7 and 8 are the theoretically computed values based upon the known primary particle distribution (6), the independently determined partition coefficients, and a permeability coefficient, $P = 1.0 \times 10^{-5}$ cm./sec. The good agreement of the experimental data with theoretical computations in regard to the time dependence and the oil volume dependence demonstrates that the interfacial barrier model (5, 6) is operative. Furthermore, this good correlation of the experiment with the theoretical calculations states that the electrolyte influence upon the uptake rate is a partition coefficient effect and not an effect upon the permeability coefficient, as was also found with the nonaggregated, nonencapsulated, hexadecane–gelatin–water systems (6).

Results of the diethylphthalate release rate experiments with the dispersed aggregates are given in Figs. 9 and 10. The effects of different electrolyte concentrations upon the release rate are shown in Fig. 9 for both the "fine" and "coarse" aggregate systems. Figure 10 presents the data on the oil volume effect for the release of diethylphthalate into water and 7% sodium sulfate.

The curves in Figs. 9 and 10 give the theoretically computed values for the release of diethylphthalate using the primary droplet size distribution data (6), the independently determined partition coefficient for each situation, and a P value of 1.0×10^{-5} cm./sec. The good agreement obtained for the uptake (Figs. 7 and 8) using the same P value clearly shows that the same interfacial barrier model is governing the transport of the solute in both situations. It is noteworthy that the aggregate size differences between the "fine" and "coarse" aggregates do not appear to be significant (Fig. 9). It was also found that experiments at 150 and 300 r.p.m. showed no differences in these experiments.

Results of some of the diethylphthalate uptake experiments employing the matrix layer method (CML) are presented in Figs. 11 and 12. Figure 11 shows the matrix volume and the stirring rate effects upon the solute uptake rates. Figure 12 presents the influence of electrolyte concentration on the diethylphthalate CML uptake rates.

In contrast to the cholesterol CML and AGE experiments (Figs. 4 and 5), in which the rates in both kinds of experiments were comparable and in good agreement with the interfacial barrier model, the diethylphthalate CML and AGE experiments show large differences in several respects. First, the diethylphthalate CML rates are generally much slower than the AGE rates. Secondly, there is an appreciable initial stirring rate effect in the diethylphthalate CML experiments. Finally, the CML matrix volume effects (Fig. 11) are relatively much smaller than those observed with the diethylphthalate AGE experiments and the cholesterol CML and AGE experiments. All of these findings dictate, that in the diethylphthalate CML experiments, the interfacial barrier is not, or at least not by itself, rate determining.

The CML data in Figs. 11 and 12 have been further theoretically treated employing a matrix-bulk diffusion model developed in these laboratories (3) using the mixture relationships (1, 2) that assume rapid equilibrium between the oil droplets and the gel matrix. The comparison of the experimental data with this theory has so far not been very satisfactory. Consequently, a model in-

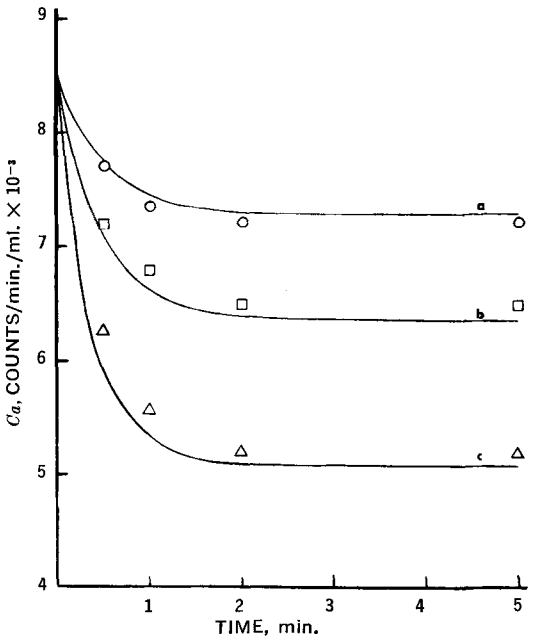


Figure 13—*Comparison of experimental* ^{14}C*-labeled octanol uptakes (AGE technique) with theoretically computed values (interfacial barrier model) as a function of aggregate weights.* C_a *is counts/min./ml. of the solute in water remaining* versus *time in minutes. One hundred milliliters of* ^{14}C*-1-octanol solution in water was added to:* ○, *0.75 g. (a);* □, *1.5 g. (b); and* △, *3.0 g. (c) of the aggregates. Curves are theoretically computed values with* K = *50 and* P = *1.0* × 10^{-4} *cm./sec.*

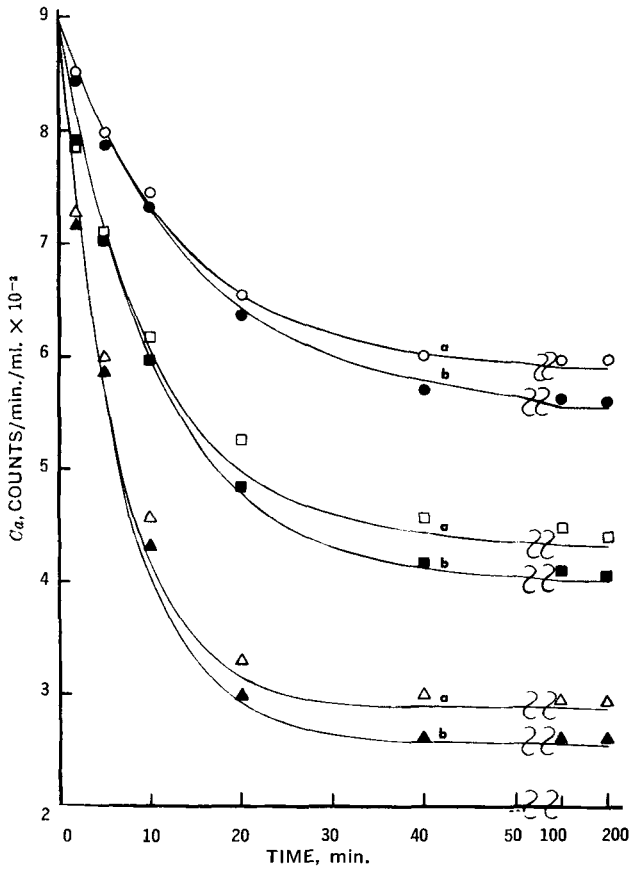


Figure 14—*Comparison of experimental* ^{14}C*-labeled progesterone uptakes (AGE technique) with theoretically computed values (interfacial barrier model) as a function of the aggregate weights and polysorbate 80 solutions.* C_a *is counts/min./ml. of the solute in the polysorbate 80 remaining* versus *time in minutes. Key:*

Medium, 100 ml.	*0.75 g.*	*1.5 g.*	*3.0 g.*
0.1% Polysorbate 80 (a)	○	□	△
0.05% Polysorbate 80 (b)	●	■	▲

Curves are theoretically computed values with K = *160 for Curve (a),* K = *185 for Curve (b), and* P = *1.0* × 10^{-5} *cm./sec.*

volving the simultaneous consideration of (*a*) the aqueous diffusion layer, (*b*) bulk gelatin-matrix diffusional parameters, and (*c*) the interfacial transport barrier of the oil droplets is being developed (9).

Transport Studies with Other Solutes Employing the AGE Technique—Uptake studies with ^{14}C-1-octanol and ^{14}C-4-progesterone are shown in Figs. 13 and 14, respectively. These experiments with octanol (Fig. 13) compare very well with the theoretically computed values using the primary droplet size distribution (6), independently determined partition coefficient, and a *P* value of 1 × 10^{-4} cm./sec. Figure 14 compares the experimental ^{14}C-4-progesterone uptake rates from polysorbate 80 solutions[12] by the aggregates with theoretically computed values employing the same primary droplet size distribution (6), the partition coefficients, and a *P* value of 1.0 × 10^{-5} cm./sec. The ^{14}C-labeled progesterone results which show good agreement with the theoretical calculations indicate that the polysorbate 80 concentration effect is primarily a partition coefficient effect rather than a permeability coefficient effect. More work is underway to distinguish whether the free solute, the micelle-solubilized solute, or both are involved in the interfacial event.

DISCUSSION

These techniques and their general applicability to various solute transport situations should be uniquely helpful in many biological and pharmaceutical problems. One aspect of the studies underway in these laboratories is aimed at the molecular mechanistic understanding of transport factors at biological membranes and tissues.

REFERENCES

(1) T. Koizumi and W. I. Higuchi, *J. Pharm. Sci.*, **57**, 87(1968).

(2) *Ibid.*, **57**, 93(1968).

(3) S. A. Howard, "A Two Phase Technique for the Investigation of Interphase Transport," Ph.D. dissertation, University of Michigan, Ann Arbor, Mich., 1968.

(4) R. Stehle and W. I. Higuchi, to be published.

(5) A. H. Ghanem, W. I. Higuchi, and A. P. Simonelli, *J. Pharm. Sci.*, **58**, 165(1969).

(6) *Ibid.*, **59**, 232(1970).

(7) A. Bikhazi and W. I. Higuchi, to be published.

(8) A. H. Goldberg, W. I. Higuchi, N. F. H. Ho, and G. Zografi, *J. Pharm. Sci.*, **56**, 1432(1967).

(9) A. H. Ghanem, W. I. Higuchi, and A. P. Simonelli, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 19, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication December 29, 1969.

This investigation was supported by Grant GM-13368 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.

Presented at the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington meeting, April 1970.

[12] In the absence of polysorbate 80, appreciable adsorption of progesterone to the beaker walls was noticed.

Sedimentation Kinetics of Flocculated Suspensions I: Initial Sedimentation Region

J. THURØ CARSTENSEN* and KENNETH S. E. SU

Abstract □ Depending on the concentration of a suspension, it will exhibit one of three sedimentation patterns pertaining to low, intermediate, and high concentrations of solids, respectively. Of these, only the dilute region has been thoroughly investigated in the past from a theoretical and experimental standpoint. Pharmaceutical suspensions are mostly of the intermediate type. It is shown here that if the models by Kynch, Michaels, and Bolger pertain, and if the sedimented cake experiences an exponential compaction from the onset, experimental data are consistent with theory and lead to the relation: $x = x_0 \exp[-kt] + C \cdot [1 - \exp(-kt)] \cdot \exp[-\omega t]$, where k is a sedimentation rate constant pertaining to a constant density plug, and ω is a sedimentation rate constant pertaining to the cake. It has also been found that, empirically, the sedimentation heights in the initial stage may be presented by the relation: $[x_0^2 - x^2] = \beta t$, where x denotes height, and t time.

Keyphrases □ Sedimentation kinetics—flocculated suspensions □ Kinetic equations—suspension sedimentation □ Suspensions, flocculated—sedimentation cakes □ Viscosity effect—suspension cake concentrations

Stokes's law has been known for over a century (1) but, nevertheless, as pointed out by Kynch (2), a satisfactory theory of sedimentation of multiparticle, coarse suspensions has never been put forth. Although it might appear that sedimentation should follow Stokes's law, Higuchi (3) and Hiestand (4) have shown that both in peptized and flocculated systems such a simple point of view is in agreement with neither theory nor fact. In most of the investigations reported in the past, attempts have been made to modify Stokes's law in some way (5–9) to account for sedimentation behaviors. The more dilute a suspension is, the more closely should the fall of each particle or floc adhere to Stokes's law. Pharmaceutical suspensions are, however, rarely very dilute, and their behavior would not be expected to fall in this category.

Michaels and Bolger (10) have reported a linear pattern for sedimentation rates for dilute suspensions (<1%). These authors, as well as Haines and Martin (7), point to the fact that very concentrated suspensions follow yet another pattern, a fact that will not be a point of discussion here but will be reserved for a subsequent study (11). Suspensions of "intermediate" concentration, however, have a downward curvature, *i.e.*, the sedimentation boundary moves downward with greater and greater velocity until a certain critical height, H_0, is reached, at which time (t_0) the rate decreases abruptly.

Examples of this pattern may be found described occasionally in pharmaceutical literature, the work by Benedict *et al.* (12) being an example. Pharmaceutical suspensions are mostly of the intermediate type. They, also, are of the floc-aggregate type described by Michaels and Bolger (10), and the considerations in the following apply to this type system.

Robinson (8) considered the sediment to be of uniformly increasing concentration during the descent of the boundary; Ward and Kammermeyer (9) showed that the ensuing equations apply only to special systems. Michaels and Bolger (10) and Gaudin and Fuerstenau (13, 14) have, furthermore, demonstrated that the density of the sediment remains constant at and for some (time-dependent) distance below the boundary, with a cake of higher density building up at the bottom, and that, therefore, the system is uniform at time zero only. The terms cake and sediment will be used for these two phases in the following. These views correlate with the simplest of the models proposed by Kynch (2).

From a pharmaceutical point of view, it is important to determine the pattern (and predict ultimate heights) of suspension sedimentation. It is one of the objectives of this communication to establish graphical means of describing the descent of the sedimentation boundary in the initial stage, *i.e.*, prior to the critical height, H_0. Another objective is to attempt to correlate the initial sedimentation pattern with existing views on the sedimentation process and arrive at a general equation for the initial sedimentation of flocculated suspensions in the intermediate concentration range.

EXPERIMENTAL

The system used here is the same as that employed by Michaels and Bolger (10) except that concentrations are higher. At higher concentrations, air entrapment might be a source of variation, and an apparatus such as shown in Fig. 1 was used. Previous investigators of dilute suspensions (8) have pointed out that initial turbulence was a source of variation, without spelling out the extent of this variation. The method of providing a uniform starting suspension used here was the same as that employed by Michaels and Bolger (10) (turning the tube end-over-end 10 times). This, of course, causes initial turbulence; the extent to which this affected results was gauged by performing each experiment at least three times.

The size of aggregates is a dynamic property and forming the aggregate under high shear (Waring blender) will not necessarily give a representative floc-aggregate (10) in the sense that the size will subsequently remain constant during settling. Some experiments were, therefore, conducted by allowing a mildly agitated suspension to equilibrate. The sedimentation experiment was repeated until the curves were reproducible. This appeared to require about 24 hr. Reproducible curves all show the convexity noted in the top curve of Fig. 2. Where suspensions were made in a blender, they were also checked on succesive days until the sedimentation rates had changed to a constant value.

The general procedure used, then, was as follows: 25 g. of colloidal kaolin NF[1] was rinsed with 500 ml. of water which had previously been distilled over potassium permanganate. The volume was then adjusted to 500 ml. and the suspension transferred to a tube of the type shown in Fig. 1. The suspension was thoroughly deaerated by applying aspirator vacuum to one of the outlets of the two-way stopcock. The tube was occasionally turned end-over-end, and finally after no more visible escape of air, the tube was turned end-over-end 10 times, then opened to the atmosphere and placed vertically; the movement of the interface was followed by use of a high precision cathetometer[2] and an electric timer. After 24 hr., the

[1] Merck and Co., Log No. 781603-62325, Rahway, N. J.
[2] Gaertner Scientific Corp., Chicago, Ill.

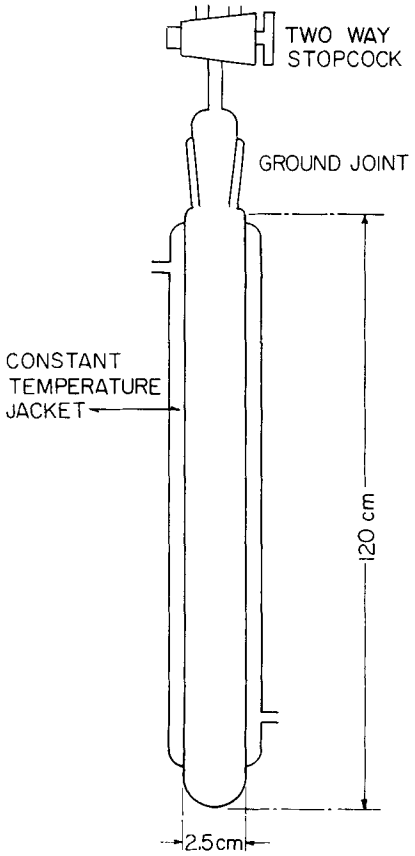


Figure 1—*Apparatus set-up for sedimentation studies.*

tube was again evacuated, turned end-over-end 10 times, opened to the atmosphere, and the procedure carried out again. This was then repeated until three successive, reproducible curves were obtained.

Part of the supernatant was then replaced by an equal volume of glycerin and the procedure repeated. Data were obtained at four glycerin concentrations; at each point where supernatant was replaced by glycerin, the viscosity of the supernatant was checked by means of an Ostwald-Fenske viscometer. All experiments were carried out in a constant-temperature room (25 ± 0.3°). In one set of experiments, lower (constant) temperatures were obtained by circulating constant temperature water through the jacket of the tube. It should be noted that the apparatus cannot be insulated and that good temperature control throughout the length of the tube is only possible at temperatures less than 5° above or below the temperature of the surrounding area.

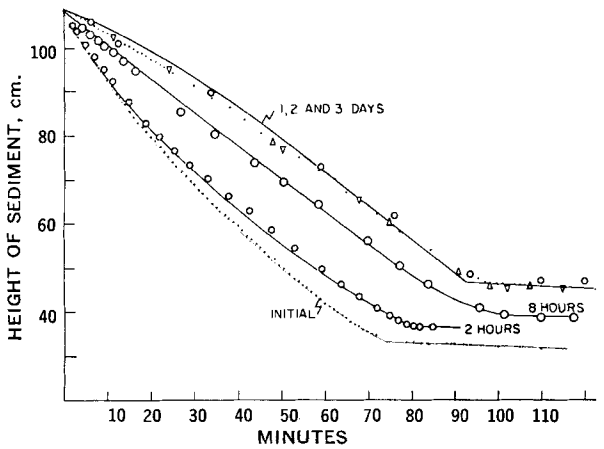


Figure 2—*Sedimentation curves in water as a function of time of a suspension made at low shear. Triangles and small circles by the upper curve are points from different runs.*

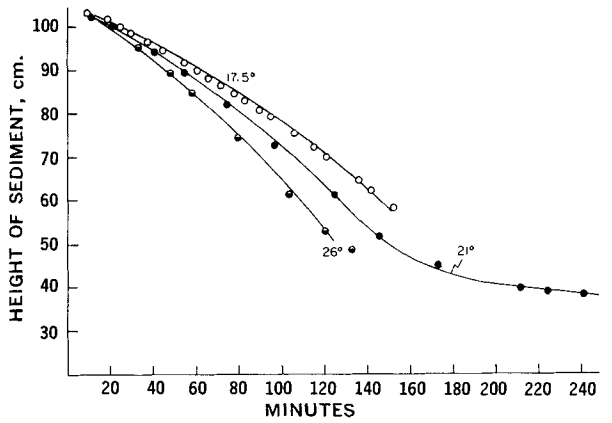


Figure 3—*Sedimentation curves in 10% v/v glycerin in water at various temperatures. The temperature was varied from experiment to experiment as a means of varying the viscosity.*

A note on some visual manifestations may be in order here. The interface is well defined and horizontal during the free-fall period. When the point of transition into the second, slower phase of the sedimentation is approached, a fairly heavy concentration of fine particles appears above the interface for a short while. At the critical time t_0, which is reproducible to within 5%, the surface suddenly becomes ragged, and at that particular time the second phase starts. The patterns associated with the second phase are the subject of a separately reported study (11). A set of runs was performed at 7, 9, and 11% by weight of kaolin in water as well.

RESULTS AND DISCUSSION

All of the tested systems showed convex curvature in the initial phase, such as shown in Figs. 2 and 3. This evidently differs from the linearity exhibited by more dilute systems.

If the sedimentation patterns found here for flocculated suspensions in the intermediate concentration range are analogous to those suggested for dilute systems by Kynch (2), Michaels and Bolger (10), and Gaudin and Fuerstenau (13–15), then, at time t, there will be a-centimeters of suspension containing the initial volume fraction ϕ_0 of solids, and b-centimeters of suspension containing the volume fraction in the cake (ϕ). The height of the sediment will be $x = a + b$. As time progresses, a will diminish at the expense of b. The a-values deduced from the data by Michaels and Bolger (Fig. 6, *Reference 10*) are reproduced in Fig. 4, and it is seen that an equation of the form $a = x_0 \exp[-kt]$ is a good fit. The decrease in a may, therefore, be considered to be of the form:

$$a = x_0 \exp[-kt] \qquad \text{(Eq. 1)}$$

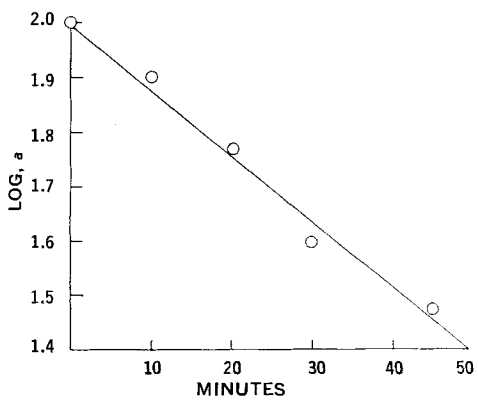


Figure 4—*The graph shows the result of logarithmic treatment of data reported by Michaels and Bolger (10). If the logarithm of the length of the constant-density plug is plotted as a function of time, then a straight line results, as shown in the plotting used here. The data refer to a 1.9% calcium oxide suspension.*

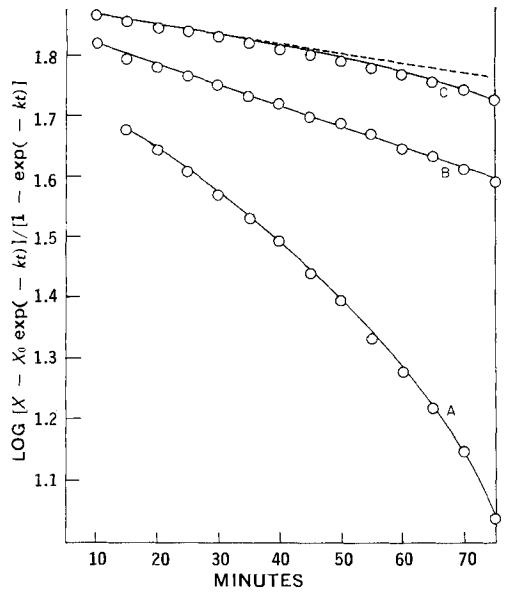


Figure 5—*Sedimentation data from a 7% by weight kaolin suspension plotted according to Eq. 7, using three values of* k. *Key: Curve A, $k = 0.005$ min.$^{-1}$; Curve B,* k = *0.01 min.$^{-1}$; Curve C,* k = *0.025 min.$^{-1}$. Temperature: 28°.*

To describe the time dependence of x, it is necessary to know how b changes with time. It has been shown (11, 16) that the cake (in the final phase) experiences an exponential decay, so $db/dt = -\omega \cdot b$. In the initial phase, it also experiences a build-up from the temporal contribution of the sediment. If the cross-sectional area is denoted Q, then the amount of solids in the cake at time t is $b \cdot Q \cdot \phi$; the amount of solids in the suspension above the cake is $Q \cdot a \cdot \phi_0$ (since it contains the initial volume fraction of solids). The original amount of solids was $x_0 \cdot Q \cdot \phi_0$, so material balance dictates that $Q \cdot a \cdot \phi_0 + b \cdot Q \cdot \phi = x_0 \cdot \phi_0 \cdot Q$, or $a \cdot \phi_0 + b \cdot \phi = x_0 \cdot \phi_0$. This may be rewritten:

$$\phi = \frac{[x_0 - a] \cdot \phi_0}{b} \qquad \text{(Eq. 2)}$$

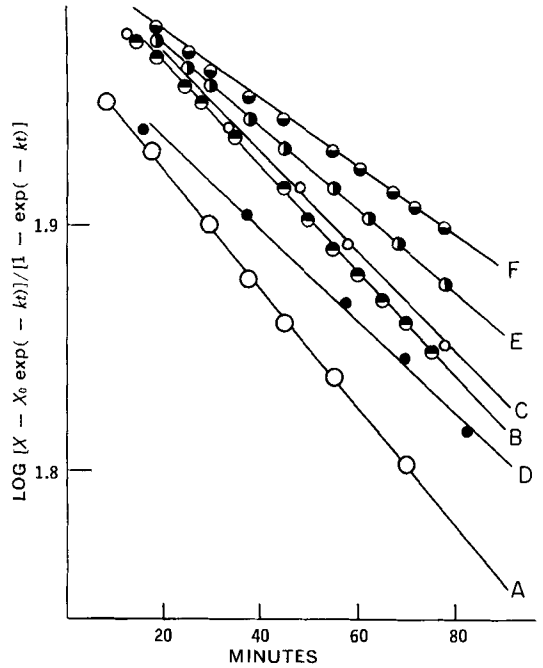


Figure 6—*Sedimentation data from 5% kaolin suspensions in aqueous glycerin vehicles, plotted according to Eq. 7. Curve notations are shown in Table I, indicating the employed* k-*values and the viscosity corresponding to each line.*

Table I—k- and ω-Values at Various Viscosities

Curve in Fig. 6	Viscosity, cps.	Rate Constant k, min.$^{-1}$, $\pm 10^{-3}$	Compaction Constant ω, min.$^{-1}$, $\pm 10^{-4}$
A	0.87	0.035	0.0055
B	0.96	0.032	0.0049
C	1.12	0.028	0.0046
D	1.19	0.024	0.0046
E	1.35	0.023	0.0039
F	1.40	0.022	0.0032
	2.40	0.013	0.0023

The total change of b with time, then, is:

$$\frac{db}{dt} = \frac{k \cdot \phi_0 \cdot a}{\phi} - \omega \cdot b \qquad \text{(Eq. 3)}$$

Inserting Eqs. 1 and 2 into Eq. 3 yields:

$$\frac{db}{dt} = k \cdot \frac{\exp[-kt]}{1 - \exp[-kt]} \cdot b - \omega \cdot b \qquad \text{(Eq. 4)}$$

which has the solution:

$$b = C \cdot \{1 - \exp[-kt]\} \cdot \exp[-\omega t] \qquad \text{(Eq. 5)}$$

The expression for $x = a + b$, then is:

$$x = x_0 \exp[-kt] + C \cdot \{1 - \exp[-kt]\} \cdot \exp[-\omega t] \qquad \text{(Eq. 6)}$$

For the purpose of plotting, this is rearranged:

$$\log\left(\frac{x - x_0 \exp[-kt]}{1 - \exp[-kt]}\right) = -\frac{\omega}{2.3} \cdot t + \log C \qquad \text{(Eq. 7)}$$

The data may now be plotted using estimates of k,[3] and by applying successive values of k, a best k-value (*i.e.*, the k-value that imparts linearity[4] to the data when treated according to Eq. 7) can be arrived at. An example of this is shown in Fig. 5. If done manually, two or three k-values are arrived at (*e.g.*, 0.034, 0.035, and 0.036 min.$^{-1}$) which produce lines without apparent curvature. The value giving the best statistical fit (17, 18) is then chosen. With the aid of a computer, the iteration procedure can be accomplished rapidly. Figure 6 and Table I show data at various viscosities plotted according to Eq. 7. With the proper k-value, linearity prevails to within 15 cm. of the critical height.

The dependency of k on the viscosity is of interest, and Fig. 7 shows that the logarithm of the rate constant is linearly related to the logarithm of the viscosity. The slope is reasonably close to minus unity, so that k is inversely proportional to viscosity. This type behavior might suggest that Stokes's law, with some modification, applies, since the stokes velocity is also inversely proportional to viscosity. In a suspension, the forces involved are both of van der Waals and electrical nature (15, 19–23). In causing flocculation they might be playing a part directly in the sedimentation process, *i.e.*, the rate constant might be associated with a flocculation rate. By such a visualization, the initial perturbance causes an equilibrium floc, A_j (containing on the average j single particles) to be broken up into smaller flocs, A_i. This, of course, is a highly simplified picture, because $A_j \rightarrow A_i$ could not be a single-step process. Furthermore, both i and j would present averages of a population of numbers.

It would also have to be assumed that A_j, once formed, would appear immediately in the cake; in spite of these shortcomings, the viewpoint explains the inverse relationship between rate constant and viscosity, and can not be eliminated as a possibility. W. Higuchi *et al.* (24, 25) have shown that k_{ij} approaches some factor times k_{11}, the rate constant for combination of single particles, and have also shown that $k_{11} = 8k_0T/3\eta$. It is not possible to test this hypothesis on more concentrated systems with the data presented here;

[3] First estimates of k (and ω) can be obtained from Eq. 6 by feathering technique if k and ω are not of the same order of magnitude.

[4] The value giving the best statistical fit (17, 18), *i.e.*, the one producing the least residual sum of squares.

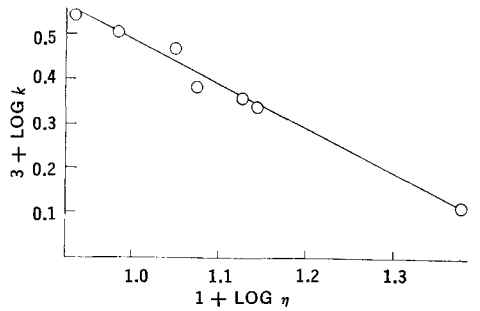


Figure 7—*A plot of the logarithm of the rate constant* k versus *the logarithm of the viscosity. The drawn line has a slope of minus unity:* k *is in units min.*$^{-1}$ *and viscosity is in cps.*

to such an end it would be necessary to perform experiments where the initial perturbance (deflocculation) could be varied in a quantitative way. Experimental procedures in previous publications as well as this work have simply been designed to achieve a reproducible perturbance, so that data are comparable; it may be, for the reasons quoted, that data may not be comparable from author to author.

The values of ω are obtained from the slopes in Fig. 6 and are listed in Table I. The values are plotted as a function of viscosity in Fig. 8. The general range of the ω-values is in good agreement with data on cake contraction reported by Carstensen and Su (11) who found ω_1-values in the range of 0.2–0.4 hr.$^{-1}$ (*i.e.*, 0.003–0.007 min.$^{-1}$). It would appear from Fig. 8 that ω is inversely proportional to the viscosity.

At higher concentrations the critical height, H_0, increases and the curves show less distinct breaks. Evaluation of data by Eq. 7 becomes less exacting, and k values are at best $\pm 20\%$. The k-values seem to taper off at a value of 0.01 hr.$^{-1}$ as shown in Fig. 9, but ω-values decrease with increasing concentration.

As a last comment on the treatment of the preceding, attention should be called to the fact that an obvious approximation is made in assuming the cake to be of uniform concentration along the entire length b. However, attempts to present ϕ as a function of x lead to forms of Eq. 3 that cannot be solved analytically. That the approximation is not unrealistic is apparent both from the X-ray data by Gaudin and Fuerstenau (13, 14) and by the linearity which can be achieved by inserting proper k-values in Eq. 7. On the other hand, deviation from linearity close to the critical height undoubtedly reflects the effect of the approximation.

It is often convenient, in the laboratory, to have a rapid method available for plotting; the treatment just outlined, clearly, is not rapid. For routine plotting, the initial convex portion of the curve may be approximated by a parabola, *i.e.*, $x_0^2 - x^2 = \beta t$, where x_0 is the initial height. Data are plotted in this fashion in Figs. 10 and 11, and, with exception of the initial turbulent period (8), the data fit such a relationship well. It should be noted that this means of presentation is purely empirical, and that the comments to follow may not have general applicability but may merely apply to the kaolin–glycerin–water system. The value of this type of practical approach

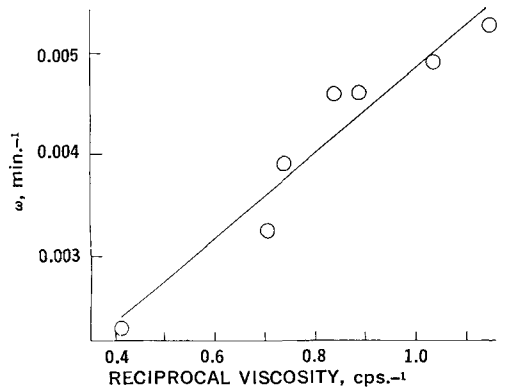


Figure 8—*A plot of the compaction-rate constant,* ω, versus *reciprocal viscosity.*

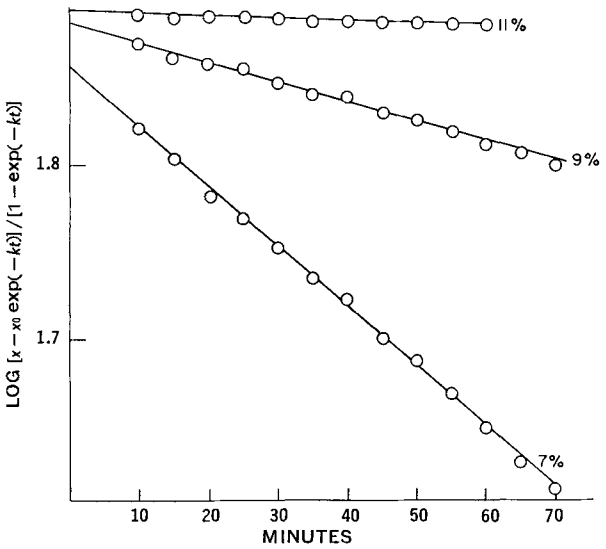


Figure 9—*Sedimentation data from 7, 9, and 11% kaolin suspensions in water, plotted according to Eq. 7, all using a* k*-estimate of 0.01 min.*$^{-1}$.

is that it allows rapid extrapolation and implies at what time the critical height is being approached.

Analogies have been made in the past (26) between the fall of the boundary to the flow of liquid through a porous plug in an infinitely long medium. By applying the Poiseiuille equation[5] such a view would require that $V/t = \pi Pr^4/8L\eta$, where V/t is volume flow per unit time, P is pressure head, r is a capillary radius parameter, and L is the length of the plug. Since each milliliter flowing through the plug is associated with a height decrease of $1/\pi R^2$, the velocity of the interface would be: $V/(t\pi R^2) = (Pr^4)/(8L\eta R^2) = \beta/(2.L) = (dx)/(dt)$, where R is the radius of the tube and the sedimentation constant $\beta = Pr^4/4\eta R^2$. If L approximately equals x, a formal integration would yield $x^2 - x_0^2 = -\beta t$, assuming the pressure head to be constant. This is not theoretically justifiable. Although, as required by the treatment, the sedimentation constant appears to be inversely proportional to the viscosity, it should also be inversely proportional to R^2; data reported elsewhere (11) show this not to be the case. The squared-heights fitting is, therefore, only empirical.

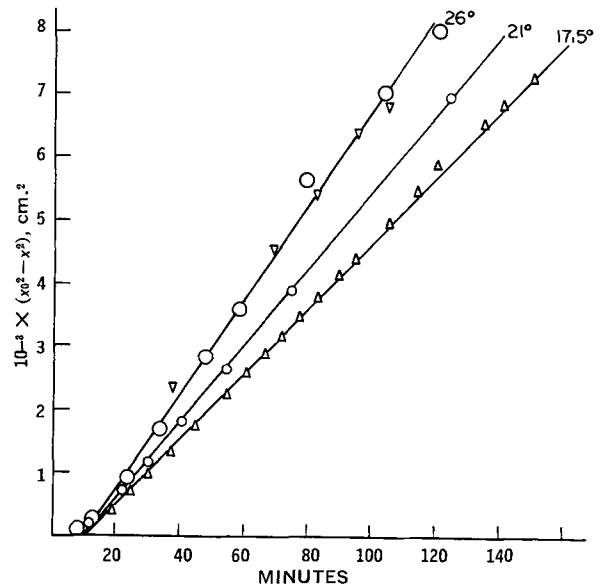


Figure 10—*Squared-heights curves for 5% kaolin suspensions in 10% v/v glycerin in water at three temperatures.*

[5] Use of the Kozeny-Carmen equation (27, 28) does not change the consequences of the arguments outlined.

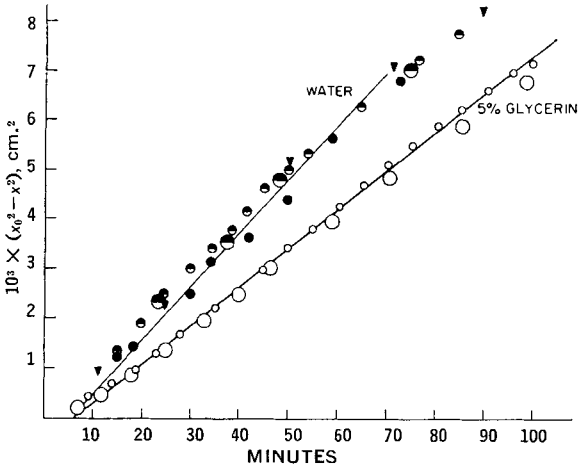


Figure 11—*Squared-heights curves for 5% kaolin suspensions in water and 5% v/v glycerin in water.*

SUMMARY

By assuming the sedimentation process to consist of the descent of a constant density plug and simultaneous build-up of a higher density cake, it has been shown that sedimentation data are consistent with theory, if it is assumed that the exponential contraction of the cake is initiated at zero time. The point in time, t_0, the critical time, where the boundary of the sediment and the cake coincide, denotes the end of the initial stage of sedimentation.

Empirically, sedimentation data in the initial stage are amenable to plotting by graphing the square of the height as a function of time. The slope of these lines appears to be proportional to the ratio of density difference to viscosity.

NOMENCLATURE

a = length of sediment (cm.) (constant-density plug).
b = height of cake (cm.).
C = preexponential factor for cake contraction (cm.).
H_0 = critical height (cm.) at which point the first phase of sedimentation ends and the second phase starts.
k = rate constant for sediment (min.$^{-1}$).
L = length of constant-density plug falling through infinitely long column of liquid (cm.).
P = pressure head (dynes/cm.^{2}).
R = radius of tube (cm.).
t_0 = time at which H_0 occurs; critical time (min.).
V = volume (cm.^{3}).
x = height of sediment interface above bottom of tube (cm.).
x_0 = initial height (cm.).
β = sedimentation constant (cm.^{2}/min.).
η = viscosity, centipoise or poise, as indicated.
ϕ = volume fraction of suspended matter in cake.
ϕ_0 = volume fraction of suspended matter in sediment.
ω = compaction rate constant (min.$^{-1}$), *i.e.*, exponential decay constant for cake height.

REFERENCES

(1) R. Stokes, *Proc. Cambridge Phil. Soc.*, **9,** 5(1856).
(2) G. Kynch, *Trans. Faraday Soc.*, **48,** 166(1952).
(3) T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 657(1958).
(4) E. N. Hiestand, *J. Pharm. Sci.*, **53,** 1(1964).
(5) H. Steinour, *Ind. Eng. Chem.*, **36,** 840(1944).
(6) *Ibid.*, **35,** 901(1944).
(7) B. A. Haines, Jr., and A. N. Martin, *J. Pharm. Sci.*, **50,** 228(1961).
(8) C. S. Robinson, *Ind. Eng. Chem.*, **18,** 869(1926).
(9) H. Ward and K. Kammermeyer, *ibid.*, **32,** 622(1940).
(10) A. S. Michaels and J. C. Bolger, *Ind. Eng. Chem. Fundam.*, **1,** 24(1962).
(11) J. T. Carstensen and K. Su, to be published.
(12) L. K. Benedict, G. S. Banker, and W. V. Kessler, *J. Pharm. Sci.*, **54,** 1659(1965).
(13) A. M. Gaudin and M. C. Fuerstenau, *Eng. Mining J.*, **159,** 110(1958).
(14) A. M. Gaudin and M. C. Fuerstenau, *Int. Mining Proc. Congr.*, London, England, Apr. 1960.
(15) A. M. Gaudin, M. C. Fuerstenau, and S. R. Mitchell, *Mining Eng. (London)*, **11,** 613(1959).
(16) C. B. Egolf and W. L. McCabe, *Trans. Amer. Inst. Chem. Eng.*, **33,** 620(1937).
(17) C. R. Wylie, Jr., "Advanced Engineering Mathematics," McGraw-Hill, New York, N. Y., 1960.
(18) J. T. Carstensen, J. B. Johnson, D. C. Spera, and M. J. Frank, *J. Pharm. Sci.*, **57,** 23(1968).
(19) J. deBoer, *Advan. Colloid Interface Sci.*, **3,** 21(1950).
(20) F. London, *Z. Phys.*, **63,** 245(1930).
(21) B. Deryagin, A. Titijevskaia, I. Abriscova, and E. Lifshitz, *Discuss. Faraday Soc.*, **18,** 24(1954).
(22) B. Deryagin, I. Abriscova, and E. Lifshitz, *Quart. Rev. (London)*, **10,** 295(1956).
(23) E. Verway and J. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier, Amsterdam, The Netherlands, 1948.
(24) W. Higuchi, R. Okada, G. Stelter, and A. Lemberger, *J. Pharm. Sci.*, **52,** 49(1963).
(25) W. Higuchi, R. Okada, and A. Lemberger, *ibid.*, **51,** 683 (1962).
(26) P. G. W. Hawksley, "Some Aspects of Fluid Flow," Arnold, London, England, 1951, p. 114.
(27) J. Kozeny, *S. B. Akad. Wiss. Wien. Abh. Math. Naturwiss. Kl. IIa*, **136,** 271(1927).
(28) P. C. Carman, *Trans. Inst. Chem. Eng.*, **15,** 150(1937).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 12, 1969, from the *School of Pharmacy and Extension Services in Pharmacy, University Extension, University of Wisconsin, Madison, WI 53706*

Accepted for publication December 11, 1969.

This work was supported in part by grants from Hoffmann-La Roche, Nutley, N. J., and R. T. Vanderbilt Co., Inc., New York, NY 10017

* To whom requests for reprints should be directed.

Sedimentation Kinetics of Flocculated Suspensions II: Sedimentation below the Critical Height

J. THURÖ CARSTENSEN* and KENNETH S. E. SU

Abstract □ Aside from the phenomenological equation by Egolf and McCabe and by Robinson, no attempts have appeared in literature in the past to quantitate terminal sedimentation characteristics of flocculated suspensions. The physical factors involved are suggested here, taking into account electrical effects and geometric factors contributing to friction. The equation of settling emerges as a linear combination of exponential decays—*viz.*, $[x - H_u] = A_1e^{-\omega_1\tau} + A_2e^{-\omega_2\tau}$, where x is the height of the sediment at time τ, H_u is the final height, and A and ω are constants. From the data it would appear that the friction, B, exerted is both viscosity (η) and dimension (R) dependent and of the form $B(\eta,R) = \Gamma \cdot e^{\xi\eta}e^{\mu R}$, where μ, ξ, and Γ are system-dependent constants.

Keyphrases □ Sedimentation kinetics—flocculated suspensions, equations □ Kinetics, sedimentation, flocculated suspensions—critical height, equations derived □ Suspensions, flocculated—sedimentation kinetics, equations

Sedimentation of flocculated suspensions has been the subject of several publications in the past (1–12); such systems have been shown (1, 7, 9–13) to sediment first at a rapid rate, primarily governed by gravitational and frictional forces. At a particular, well-reproducible point, the rate changes abruptly, and further sedimentation appears to be governed by forces over and above those just mentioned. With a few exceptions (1, 3, 13), all of the cited references attempt to modify Stokes's law to explain experimental data. In no case [except indirectly by Robinson (7)] has cognizance been taken of interparticle forces in relation to sedimentation rates.[1]

The treatment to follow deals with the second phase of sedimentation of flocculated suspensions of intermediate concentration, and attempts to account for the effect of such forces.

As shall be shown the final sedimentation pattern follows a linear combination of exponential decays. No previous treatment has led to such a pattern for the second stage of sedimentation or to the type plot encountered in concentrated suspensions, although the data in several examples in literature (2, 6, 7, 9) imply such a relationship.

THEORETICAL

London-van der Waals forces (14, 15) are responsible for the stability of the flocculated state, and in general, at small separations, the potential energy between two particles will be negative. At intermediate distances (16–19), however, the potential energy becomes positive because of the repulsion of double layers. Since the true charge of the particle surface cannot be determined by experiment, quantitation of such forces is difficult, and the upper limit for distances over which these forces will be effective cannot be stated *a priori*.

[1] A sizable amount of information regarding interparticle forces has been obtained by rheological approaches, notably the studies by Higuchi and Stehle (25), Gillespie (26, 27), Goodeve (28), and Vand (19).

Since the authors are dealing with the phase of sedimentation which occurs at a critical height, H_0, it may be advantageous, at the onset, to use a coordinate system slightly different from the one customarily used. Usually the position of the boundary is measured from the bottom of the tube and denoted x; time, t, is measured from the time sedimentation starts. Here, if the height H_0 occurs at time t_0, the position and time axes are chosen with the points t_0 and H_0 as the origin. Distance is now denoted y ($y = H_0 - x$), *i.e.*, positive in downward direction and positive for the times τ ($\tau = t - t_0$) in question. It shall be assumed that the center of gravity of the sediment is at one-half the height of the boundary of the sediment, *i.e.*, its position is $y = H_0/2$ originally, and at time τ it is $H_0 - x + x/2 = H_0 - x/2 = y$.

The forces exerted on the sediment are gravitational forces, frictional and reactional forces, and forces that are electrical in nature. The gravitational force is in the y-direction and of magnitude $M[1 - (\rho_0/\rho)]g$, where ρ and ρ_0 are the densities of solid and liquid, M is the mass of the sediment, and g is the gravitational acceleration. The frictional and reactional forces are in a direction opposite to y and must be related to viscosity and to wall and bottom effects, *i.e.*, to the geometry of the vessel. The functional relationship is not equated to $6\pi\eta r$, nor is an attempt made at this particular point in the development to formulate the diameter dependency, but the force is described as a velocity-dependent $[-B(\eta,R)dy/d\tau]$ and a velocity-independent $[-\psi(R)]$ component. The electrical force is assumed to be a repulsion (*i.e.*, in a direction opposite to the direction of the y-axis) and with regard to magnitude it is assumed to increase the closer the floc-aggregates are to one another; they are related to distance between flocs which in turn is related to the liquid volume of the cake, *i.e.*, $\pi R^2(x) - 4\pi r^3 n/3$, where R is the radius of the tube, and where n is the number and r the "radius" of the floc-aggregates. These forces are assumed to be of the form θy. The sum of all the forces then equals the mass of the sediment times its acceleration, *i.e.*,

$$M[1 - \rho_0/\rho]g - B(\eta,R)\frac{dy}{d\tau} - \psi(R) - \theta y = M\frac{d^2y}{d\tau^2} \quad \text{(Eq. 1)}$$

This may be rewritten

$$\frac{d^2y}{d\tau^2} + \frac{B(\eta,R)}{M}\frac{dy}{d\tau} + \frac{\theta}{M}y = [1 - \rho_0/\rho]g - \frac{\psi(R)}{M} \quad \text{(Eq. 2)}$$

It is apparent from inspection of Eqs. 1 and 2 that

$$y^* = \frac{M[1 - \rho_0/\rho]g - \psi(R)}{\theta}$$

is a particular solution to the differential equation. The remaining solutions are obtained by inserting an expected solution of the form $y = -A \cdot e^{-\omega\tau}$ into the homogeneous equation corresponding to Eq. 2. This yields:

$$-A \cdot e^{-\omega\tau}\left[\omega^2 - \frac{B(\eta,R)}{M}\omega + \frac{\theta}{M}\right] = 0 \quad \text{(Eq. 3)}$$

When the expression in brackets is equated to zero, Eq. 3 is satisfied; the characteristic roots are:

$$\omega = \frac{B(\eta,R)}{2M} \pm \sqrt{\left[\frac{B(\eta,R)}{2M}\right]^2 - \frac{\theta}{M}} \quad \text{(Eq. 4)}$$

The roots shown in Eq. 4 may be simplified if $B(\eta,R)/M \gg \theta/B(\eta,R)$ in which case they are:

$$\omega_1 = \frac{B(\eta,R)}{M} \quad \text{(Eq. 5)}$$

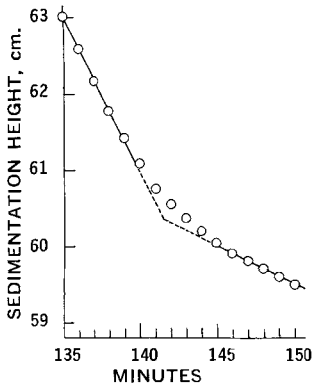


Figure 1—*Graph showing the method for determining the critical height* (H_0) *in a 5.54-cm. i.d. tube, 10% v/v glycerin and 11% w/v kaolin, and the critical time* (t_0).

and

$$\omega_2 = \frac{\theta}{B(\eta,R)} \qquad \text{(Eq. 6)}$$

The complete solution, in terms of y, is:

$$y = \frac{M[1 - \rho_0/\rho]g - \psi(R)}{\theta} - A_1' \cdot e^{-\omega_1 \tau} - A_2' \cdot e^{-\omega_2 \tau} \qquad \text{(Eq. 7)}$$

where the exponents in Eq. 7 have the meaning denoted in Eqs. 5 and 6. It is noted that $y^* = M[1 - \rho_0/\rho]g - \psi(R)/\theta$ corresponds to y_∞ by virtue of Eq. 7, and, therefore, is related to H_u, the ultimate height, by the relation $y_\infty = y^* = H_0 - H_u/2$. Inserting this into Eq. 7 and rearranging, yields:

$$x - H_u = A_1 e^{-\omega_1 \tau} + A_2 e^{-\omega_2 \tau} \qquad \text{(Eq. 8)}$$

where $A_1 = 2A_1'$ and $A_2 = 2A_2'$.

It is apparent from Eq. 8 that the terminal sedimentation pattern should be characterized by a linear combination of two exponential decays. By the assumption leading to Eqs. 5 and 6, $\omega_1 \gg \omega_2$ and the first term on the right hand side of Eq. 8 should be predominant at small values of τ and the last term should predominate at high values of τ.

EXPERIMENTAL

A previously described setup and methodology (1) were used: tubes 1.25 m. high, and of inside diameter 2.46, 2.72, 4.60, or 5.54 cm. were used as sedimentation vessels. The exact inside dimension was determined for each tube by introducing known amounts of water and checking heights with a precision cathetometer. The system studied was an 11% weight per volume kaolin[2] suspension

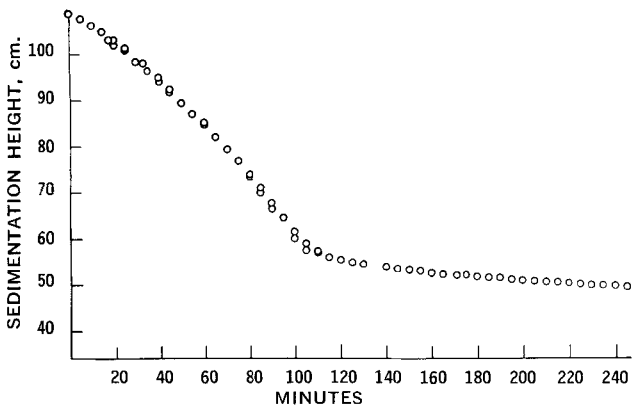


Figure 2—*Sedimentation curve of 11% w/v of kaolin in water in a tube of 5.54-cm. i.d. Duplicate points are shown.*

[2] Colloidal kaolin NF, lot No. 781603, Merck and Co., Rahway, N. J.

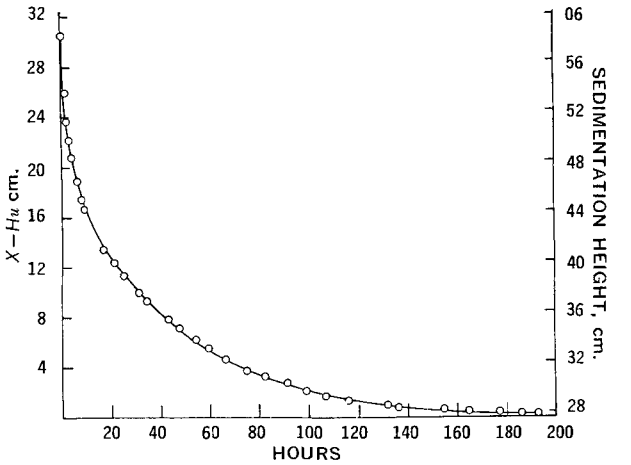


Figure 3—*Sedimentation pattern in a tube of 4.60-cm. i.d. of an 11% w/v suspension of kaolin in 5% v/v glycerin solution at heights below the critical height,* H_0. *The left ordinate is adjusted for final height,* H_u; *the right ordinate axis shows the actual heights* (x). *The curve shown in full is the theoretical curve;* $10 \cdot e^{-0.31\tau} + 20 \cdot e^{-0.0233\tau}$. *The points shown are experimental points.*

in water, 5% v/v glycerin, or 10% v/v glycerin. The suspensions were made in a Waring blender and transferred to the sedimentation tubes. The tops of the tubes were connected by means of a 24/40 ground joint to a stopcock, and after transfer, the stopcock was attached to the tube, and the suspension thoroughly deaerated by means of aspirator vacuum. Once deaerated, the tubes were turned end-over-end 10 times, the stopcock opened, the tube placed vertically and securely on a stand, and the position of the sedimentation boundary noted every 5 min.; timing intervals were made shorter when the critical height, H_0, was approached. This height, as well as the critical time, t_0, was then determined by graphical interpolation as shown in Fig. 1. Following this the position of the boundary was noted every 5 min. for 0.5 hr., and then hourly for 50 hr. (except for necessary schedule interruptions). Subsequently, the sedimentation height was recorded twice daily until a point in time had been reached where less than 0.2 mm. change occurred in a 24-hr. period; this usually required 7–13 days. Each set was obtained in duplicate. All sedimentation heights were determined by means of a cathetometer, and readings were accurate to 0.1 mm. The tubes used were not jacketed, but the experiments were carried out in a constant-temperature room at 25 ± 0.6°. The viscosity of the super-

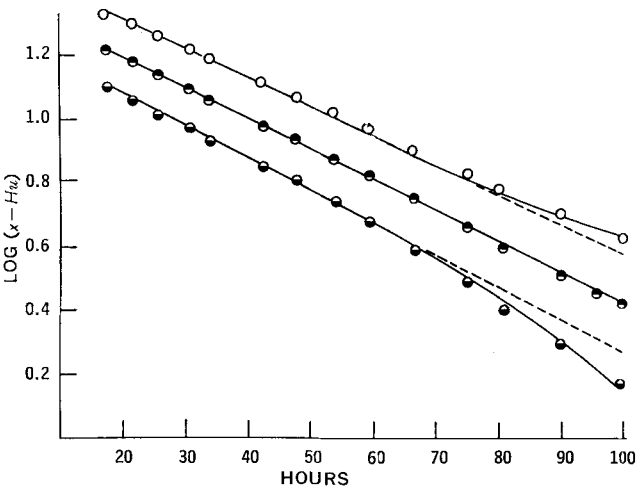


Figure 4—*Decay pattern of 11% w/v kaolin suspension in 5% v/v glycerin solution at high time values where* ω_2 *and* A_2 *predominate. Test was performed in a tube of 4.60-cm. i.d. The curves are separated by 0.1 and 0.2 logarithmic units as indicated. The plots demonstrate estimation of final sedimentation height prior to the time when it is reached. Key:* ○, H_u = *27.3 cm. (scale minus 0.2);* ◓, H_u = *27.8 cm. (scale minus 0.1), and* ◒, H_u = *28.4 cm.*

Table I—Theoretical Sedimentation Curve for 11% w/v Kaolin in 5% v/v Aqueous Glycerin in 4.6-cm. i.d. Tube[a]

Hr.	-0.137τ	$e^{-0.31\tau}$	$10e^{-0.31\tau}$	-0.097τ	$e^{-0.0223\tau}$	$20e^{-0.0223\tau}$	$10e^{-0.31\tau} + 20e^{-0.0223\tau}$
1	0.8630–1	0.730	7.30	0.9903–1	0.9780	19.56	26.86
2	0.7260–1	0.532	5.32	0.9806–1	0.9563	19.13	24.45
3	0.5890–1	0.388	3.88	0.9709–1	0.9352	18.70	22.58
4	0.4520–1	0.283	2.83	0.9612–1	0.9146	18.29	21.12
5	0.3150–1	0.207	2.07	0.9515–1	0.8944	17.89	19.96
6	0.1780–1	0.151	1.51	0.9418–1	0.8746	17.49	19.00
8	0.9280–2	0.085	0.85	0.9224–1	0.8361	16.72	17.57
10	0.6300–2	0.043	0.42	0.9030–1	0.8000	16.00	16.43
12	0.3560–2	0.023	0.23	0.8836–1	0.7649	15.30	15.53
14	0.2600–2	0.018	0.18	0.8642–1	0.7315	14.63	14.81
15	0.9450–3	0.009	0.09	0.8545–1	0.7164	14.33	14.42
20	0.260 –3	0.002	0.02	0.8060–1	0.6398	12.80	12.82
40				0.6120–1	0.4093	8.19	8.19
80				0.224 –1	0.1675	3.35	3.35
160				0.448 –2	0.0281	0.56	0.56

[a] $A_1 = 10$ cm., $\omega_1 = 0.31$ hr.$^{-1}$, $A_2 = 20$ cm., $\omega_2 = 0.0223$ hr.$^{-1}$.

natant was checked after each experiment by means of an Ostwald-Fenske viscometer.

RESULTS

A typical sedimentation curve is shown in Fig. 2; the shape is of the same nature as the one described elsewhere (1); the scale is selected so as to show both the initial and the beginning of the second phases of sedimentation, and the characteristic point separating the two curves. Even with the scale used it is apparent that some slight curvature exists in the second phase. In proper scale, all of the sedimentation curves have the shape shown in Fig. 3 when x is plotted as a function of τ.

Once the ultimate height, H_u, is attained, a first estimate of the parameters ω_2 and A_2 can be made, by using values of τ higher than 15 hr. In this case (as seen in Table I) $A_2e^{-\omega_2\tau} \gg A_1e^{-\omega_1\tau}$ and Eq. 8 becomes $[x - H_u] = A_2e^{-\omega_2\tau}$. The logarithmic form of this is

$$\log [x - H_u] = -\frac{\omega_2}{2.3}\tau + \log A_2 \ (\tau > 15 \text{ hr.}) \quad \text{(Eq. 9)}$$

An example of this is shown in Table II and Fig. 4. It should be noted that the final height may be estimated well in advance of its actual occurrence from linearity as opposed to curvature of this type plot. Estimated values were always within 0.5 mm. of the actual, experimentally determined, final height.

By means of the estimates of A_2 and ω_2 the value of $[x - H_u] - A_2e^{-\omega_2\tau}$ can be determined at values of τ less than 15 hr., so the two other parameters may be estimated by rearrangement of Eq. 8:

$$\log \{[x - H_u] - A_2e^{-\omega_2\tau}\} = -\frac{\omega_1}{2.3}\tau + \log A_1 \quad \text{(Eq. 10)}$$

An example of this is shown in Table III and Fig. 5. By this plotting a better estimate of A_2 is obtained as shown in Fig. 5. The difference between this estimate and the original estimate was always within error of the graphical estimate in Fig. 4. With the estimates of A_1, ω_1, A_2, and ω_2, statistically best values may be obtained by computer, using iteration techniques (20–22). It should be noted, however, that graphical and manually generated estimates are quite good. Construction of the theoretical curve from the parameter estimates arrived at in Tables II and III and Figs. 4 and 5 is shown in Table I, and the curve drawn in full in Fig. 3 is taken from this table. It is quite obvious from the figure that the data fit the curve well. Averages of duplicate determinations of A_1, ω_1, A_2, and ω_2 from all the sedimentation tests are listed in Table IV.

DISCUSSION

In not too concentrated suspensions the sedimentation pattern is characterized first by a settling pattern which is linear (9) or parabolic with time (1) caused, respectively, by free fall or constant-density plug descent (1, 9). Attempts to describe mathematically the phase that follows the initial settling have been made by Robinson (7) and by Egolf and McCabe (13). Robinson found the dependency of the initial settling rates on concentration and then

Table II—Sedimentation Data for High Values of τ when ω_2 and A_2 Are the Predominant Terms

Hr.	Height, x, cm.	$x - 28.4$, cm.	log $[x - 28.4]$	$x - 27.8$, cm.	log $[x - 27.8]$	$x - 27.3$, cm.	log $[x - 27.3]$
17.67	41.06	12.76	1.1059	13.36	1.1258	13.86	1.1318
21.50	40.02	11.62	1.0652	12.22	1.0871	12.72	1.1045
25.65	38.96	10.56	1.0237	11.16	1.0477	11.66	1.0630
30.42	37.88	9.48	0.9768	10.08	1.0035	10.58	1.0245
34.20	37.12	8.72	0.9405	9.32	0.9694	9.82	0.9921
42.70	35.56	7.16	0.8549	7.76	0.8899	8.26	0.9170
47.22	34.87	6.47	0.8109	7.07	0.8494	7.57	0.8791
53.98	33.90	5.50	0.7404	6.10	0.7853	6.60	0.8195
59.30	33.23	4.83	0.6840	5.43	0.7348	5.93	0.7731
66.70	32.32	3.92	0.5933	4.52	0.6551	5.02	0.7007
75.23	31.51	3.11	0.4928	3.71	0.5694	4.21	0.6243
82.30	30.93	2.53	0.4031	3.13	0.4955	3.63	0.5599
90.18	30.39	1.99	0.2989	2.59	0.4133	3.09	0.4900
98.90	39.90	1.50	0.1761	2.10	0.3222	2.60	0.4150
107.12				1.70			
115.55				1.34			
132.23				0.92			
136.70				0.70			
155.12				0.52			
165.17				0.41			
176.93				0.32			
186.00				0.20			
193.50				0.19			

Table III—Sedimentation Data at Low Values of τ[a]

Hr.	Height, x, cm.	$[x - 27.8]$, cm.	0.0097τ	$Q = e^{-0.0223\tau}$	$[x-27.8-19.2Q]$, cm.	$[x-27.8-20Q]$, cm.	$[x-27.8-20.5Q]$, cm.
0	58.3	30.5	0	1	11.3	10.5	10.0
0.27	56.55	28.75	0.9974–1	0.9940	9.67	8.87	8.37
0.52	55.33	27.53	0.9950–1	0.9885	8.55	7.76	7.27
1.30	52.87	25.07	0.9874–1	0.9714	6.42	5.64	5.16
1.97	51.41	23.61	0.9809–1	0.9570	5.24	4.47	3.99
2.47	50.56	22.76	0.9760–1	0.9463	4.59	3.83	3.36
2.92	49.85	22.05	0.9717–1	0.9369	4.06	3.31	2.84
3.48	49.15	21.35	0.9662–1	0.9252	3.59	2.85	2.38
4.05	48.49	20.69	0.9607–1	0.9135	3.15	2.42	1.96
4.52	48.01	20.21	0.9562–1	0.9040	2.85	2.13	1.68
5.02	47.54	19.74	0.9513–1	0.8939	2.58	1.86	1.42
5.52	47.12	19.32	0.9465–1	0.8840	2.35	1.64	1.20
6.03	46.68	18.88	0.9415–1	0.8740	2.10	1.40	0.96
6.50	46.33	18.53	0.9370–1	0.8648	1.93	1.23	0.80
7.02	45.96	18.16	0.9319–1	0.8549	1.75	1.06	0.63
7.45	45.66	17.88	0.9277–1	0.8466	1.63	0.95	0.52
8.03	45.32	17.52	0.9221–1	0.8358	1.47	0.80	0.39
8.62	44.97	17.17	0.9164–1	0.8249	1.33	0.67	0.20
9.45	44.50	16.70	0.9083–1	0.9097	1.15	0.51	0.10

[a] Eleven percent w/v of kaolin in 5% v/v glycerin in a tube of 4.60-cm. i.d.

applied these findings to experimental data; since in that context, the sedimentation volume represents a suspension of time-dependent concentration (disregarding the supernatant) the settling rates should constantly change in a predictable fashion.

Egolf and McCabe suggested a phenomenological log–log relationship between absolute height and time for the phase following the initial settling. Ward and Kammermeyer (8) tested both models in different type systems (carbonates, silica, and magnesium oxide), and found fair agreement with regard to the shape of the curves, but large (20–40%) quantitative deviations in the second phase of sedimentation. These authors concluded that the application of the approaches of Robinson (7) and Egolf and McCabe (13) is limited to prediction of settling of only such systems that are closely related to the ones used to develop the appropriate equations and factors. Reflecting on the later findings that the initial settling is in the form of a constant-density plug (1, 9–11), it would appear that it may be erroneous to assume that the sediment may be viewed as a uniform suspension of constantly changing concentration; this may be the source of the cited quantitative deviations.

The approach taken here is to account for the forces involved (even though all of them cannot be expressed explicitly *a priori*) and then to try to gain insight into them, quantitatively and qualitatively, by way of experimental results. Experimental variation limits the number of conclusions that may be drawn from the A_1- and ω_1-values. It will be noted from Table IV that the coefficient of variation for these two parameters is 5%; this prohibits any conclusions that may be drawn regarding the viscosity dependency of ω_1 (and, therefore, also of $B[\eta,R]$). The data would imply that either ω_1 does not vary with changing R, or, possibly, it decreases with increasing R.

On the other hand, ω_2 is quite reproducible (±1%); it appears from the results in Table IV that $\omega_2\{\omega_2 = \theta \cdot [B(\eta,R)]^{-1}\}$ decreases with both increasing R- and η-values, and suggest the radial dependence of $B(\eta,R)$ to be of the form $e^{\mu R}$; in this case Eq. 6 takes the form:

$$\log \omega_2 = \log \theta - \log C^* - \frac{\mu}{2.3} R \qquad \text{(Eq. 11)}$$

where C^* is a constant. Figure 6 shows the data plotted in this fashion, and the plots are fairly linear; the slopes (calculated by least-squares fit of the data in Table IV) are shown in Table V, and are, as seen, fairly close to one another.

The data in Table IV would also suggest the dependence of ω_2 on viscosity to be an exponential decay; if this is the case, then $B(\eta,R) = \Gamma \cdot e^{\mu R} e^{\zeta \eta}$; inserting this in Eq. 6 yields:

$$\log \omega_2 = \log \frac{\theta}{\Gamma} - \frac{\mu}{2.3} R - \frac{\zeta}{2.3}\eta \qquad \text{(Eq. 12)}$$

If $\log \omega_2$ is plotted as a function of viscosity, four straight lines should result, one for each tube. The data plotted in this fashion are shown in Fig. 7. It is seen that fairly good linearity is exhibited, and the values of the slopes as shown in Table V are close to each other. Least-squares values of the intercepts from Figs. 6 and 7 are also shown in this table. The intercepts in Fig. 6 should be $[2 + \log \theta/\Gamma - (\mu \cdot R/2.3)]$ and the intercepts in Fig. 7 should be $[2 + \log \theta/\Gamma - (\zeta \cdot \eta/2.3)]$. It is, therefore, possible to get a crude estimate of $\log [\theta/\Gamma]$ by means of the intercept values. Results of this type calculation are shown in Table V and values derived from the

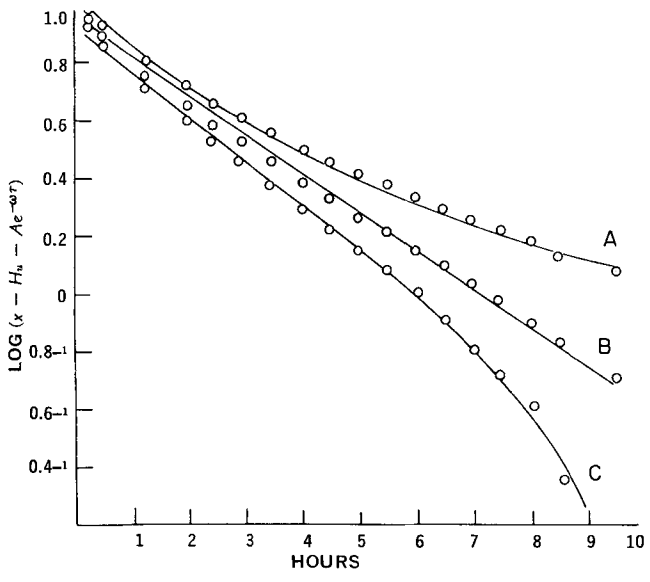


Figure 5—*Decay pattern of 11% w/v kaolin suspension in 5% v/v glycerin solution at low time values where ω_1 and A_1 predominate. Data pertain to 4.60-cm. i.d. tube. The plots demonstrate refinement of the estimate of A_2. Key: A, A_2 = 19.2 cm.; B, A_2 = 20.0 cm.; and C, A_2 = 20.5 cm.*

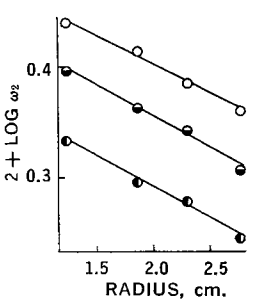


Figure 6—*Plot of the logarithm of the smaller characteristic root, ω_2, as a function of the tube diameter. ○, 0.89 cps.; ◒, 1.05 cps.; ◐, 1.20 cps.*

Table IV—Value of A_1, A_2, ω_1, and ω_2 as a Function of Viscosity and Tube Diameter

Glycerin, % v/v	Viscosity, poises	Radius, cm.	A_1, cm. ±10%	ω_1, hr.$^{-1}$ ±10%	A_2, cm. ±6%	ω_2, hr.$^{-1}$ ±1%
0	0.0089	1.23	4.9	0.42	24.1	0.0276
0	—	1.86	10.3	0.38	16.0	0.0260
0	—	2.30	10.2	0.29	18.7	0.0242
0	—	2.77	8.2	0.13	20.0	0.0229
5	0.0105	1.23	6.0	0.41	31.2	0.0248
5	—	1.86	9.3	0.38	31.3	0.0230
5	—	2.30	10.0	0.31	20.5	0.0220
5	—	2.77	9.8	0.29	18.6	0.0202
10	0.0120	1.23	7.1	0.38	41.0	0.0216
10	—	1.86	9.6	0.30	19.3	0.0197
10	—	2.30	8.6	0.33	17.4	0.0190
10	—	2.77	8.8	0.15	20.0	0.0175

Table V—Slopes and Intercepts from Figs. 6 and 7

Line	Fig.	Slope (ζ/2.3), cps.$^{-1}$	Slope (μ/2.3), cm.$^{-1}$	Intercept	log [θ/Γ]
R = 1.23 cm.	7	−0.36		0.77	0.84–2[a]
R = 1.86 cm.	7	−0.40		0.78	0.88–2[a]
R = 2.30 cm.	7	−0.35		0.70	0.82–2[a]
T = 2.77 cm.	7	−0.39		0.71	0.86–2[a]
η = 0.89 cps.	6		−0.054	0.51	0.88–2[b]
η = 1.05 cps.	6		−0.057	0.46	0.85–2[b]
η = 1.20 cps.	6		−0.057	0.40	0.84–2[b]

[a] Based on an average value of μ/2.3 = 0.055. [b] Based on an average value of ζ/2.3 = 0.37.

two figures show acceptably good agreement, especially considering that they have been obtained from extrapolated figures (intercepts).

This fact in combination with the linearity of the plots in Figs. 6 and 7, the closeness of the values of the slopes, and the adherence of all the data to an equation of the type of Eq. 8 lends credence to the views presented here.

It should be mentioned, qualitatively, that increasing concentrations tend to decrease (or mask) the values of A_1, and that this parameter eventually is of such small magnitude that it can no longer be detected within experimental error. In the same vein, H_0 becomes larger with increasing solids content and, eventually, at a critical concentration the initial constant-density plug phase will disappear, and at a second critical concentration, the A_1-term will disappear. The entire sedimentation pattern will then be a simple logarithmic decay curve. The critical concentration for kaolin in 10% v/v glycerin in a tube of 2.5-cm. i.d. is 8–10 w/v percent. Data of this nature have been reported occasionally in literature in the past, the data by Haines and Martin (6) being a notable example.

The final height, H_u, as reported here, may still be subject to some decrease by consolidation processes such as described by Ratcliff (23, 24). This phase has not been a subject of this investigation.

SUMMARY

The sedimentation of flocculated suspensions takes place in successive stages; the sedimentation phase following the initial phase has been studied and found, based on consideration of the forces involved, to be confined to follow the following equation: $[x-H_u] = A_1e^{-\omega_1\tau} + A_2e^{-\omega_2\tau}$. Experimental data have supported the presented concepts and suggest that the frictional term in the equation of motion is of the form $B(\eta,R) = \Gamma \cdot e^{\mu R}e^{\zeta\eta}$.

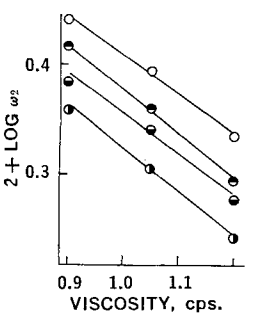


Figure 7—*Plot of the logarithm of the smaller characteristic root, ω_2, as a function of viscosity. Key: ○, 2.46-cm. i.d. tube; ◓, 3.72-cm. i.d. tube; ◒, 4.60-cm i.d. tube; and ◑, 5.54-cm. i.d. tube.*

NOMENCLATURE

A, A_1, A_2, A_1', A_2'	= preexponential factors in descent of sedimentation boundary
$B(\eta,R)$	= viscosity dependent component of frictional force
C^*	= preexponential factor for radial dependence of ω_2
g	= gravitational acceleration
H_0	= critical height
M	= mass of sediment
r	= radius of floc
R	= radius of tube
t	= time measured from start of sedimentation
t_0	= critical time
x	= height of sedimentation boundary above bottom of tube
y	= distance of sedimentation boundary from critical height ($= H_0 - x/2$)
y_∞, y^*	= y-value at infinite time ($= H_0 - H_u/2$)
$\psi(R)$	= viscosity independent component of frictional force
ρ_0	= density of fluid
ρ	= density of suspended solid
τ	= time measured from critical time ($= t - t_0$)
μ	= exponential factor to radial dependence of ω_2
Γ	= preexponential factor to radial dependence of ω_2
θ	= dimensional factor in electrical repulsion term
ζ	= exponential factor to viscosity dependence of ω_2
ω, ω_1, ω_2	= exponential decay constants in descent of sedimentation boundary

REFERENCES

(1) J. T. Carstensen and K. Su, *J. Pharm. Sci.*, **59**, 666(1970).
(2) R. Stokes, *Proc. Cambridge Phil. Soc.*, **9**, 5(1856).
(3) G. Kynch, *Trans. Faraday Soc.*, **48**, 166(1952).
(4) H. Steinour, *Ind. Eng. Chem.*, **36**, 840(1944).
(5) *Ibid.*, **35**, 901(1944).
(6) B. A. Haines, Jr., and A. N. Martin, *J. Pharm. Sci.*, **50**, 228 (1961).
(7) C. S. Robinson, *Ind. Eng. Chem.*, **18**, 869(1926).

(9) A. S. Michaels and J. C. Bolger, *Ind. Eng. Chem. Fundam.*, **1,** 24(1962).
(10) A. M. Gaudin and M. C. Fuerstenau, *Eng. Mining J.*, **159,** 110(1958).
(11) A. M. Gaudin and M. C. Fuerstenau, *Int. Mining Proc. Congr.*, London, England, Apr. 1960.
(12) A. M. Gaudin, M. C. Fuerstenau, and S. R. Mitchell, *Mining Eng. (London)*, **11,** 613(1959).
(13) C. B. Egolf and W. L. McCabe, *Trans. Amer. Inst. Chem. Eng.*, **33,** 620(1937).
(14) J. deBoer, *Advan. Colloid Interface Sci.*, **3,** 21(1950).
(15) F. London, *Z. Phys.*, **63,** 245(1930).
(16) B. Deryagin, A. Titijevskaia, I. Abriscova, and E. Lifshitz, *Discuss. Faraday Soc.*, **18,** 24(1954).
(17) B. Deryagin, I. Abriscova, and E. Lifshitz, *Quart. Rev. (London)*, **10,** 295(1956).
(18) E. Verway and J. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier, Amsterdam, The Netherlands, 1948.
(19) V. Vand, *J. Phys. Colloid Chem.*, **52,** 277(1948).
(20) C. R. Wylie, Jr., "Advanced Engineering Mathematics," McGraw-Hill, New York, N. Y., 1960, p. 177.
(21) C. M. Metzler, APhA Academy of Pharmaceutical Sciences, 5th National Meeting, Washington, D. C., November 20, 1968.
(22) J. T. Carstensen, J. B. Johnson, D. C. Spera, and M. J. Frank, *J. Pharm. Sci.*, **57,** 23(1968).
(23) G. A. Ratcliff, D. A. Blackader, and D. N. Sutherland, *Chem. Eng. Sci.*, **22,** 201(1967).
(24) G. A. Ratcliff, *J. Colloid Interface Sci.*, **25,** 586(1967).
(25) W. Higuchi and R. G. Stehle, *J. Pharm. Sci.*, **54,** 265(1965).
(26) T. Gillespie, *J. Phys. Chem.*, **66,** 1077(1962).
(27) T. Gillespie, *J. Colloid Sci.*, **15,** 219(1960).
(28) C. F. Goodeve, *Trans. Faraday Soc.*, **35,** 342(1939).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 21, 1969, from the *School of Pharmacy and Extension Services in Pharmacy, University Extension, University of Wisconsin, Madison, WI 53706*

Accepted for publication November 14, 1969.

This investigation was supported by grants from R. T. Vanderbilt Co., New York, NY 10017 and Hoffmann-La Roche, Inc., Nutley, NJ 07110

* To whom requests for reprints should be addressed.

Cholesterol Biosynthesis and Lipid Biochemistry in the Scolex of *Echinococcus granulosus*

GEORGE A. DIGENIS*, RALPH E. THORSON†, and ARPINE KONYALIAN

Abstract □ When live scolices of *Echinococcus granulosus* were incubated with labeled mevalonate *in vitro*, no radioactivity was incorporated into the parasites' cholesterol fraction. However, labeled cholesterol was recovered after a scolices' homogenate was incubated with 4-^{14}C-cholesteryl acetate. It is suggested that the scolex of *E. granulosus* is unable to biosynthesize cholesterol and that it obtains cholesterol in an esterified form from the host, which is subsequently hydrolyzed within the scolex to free cholesterol. The fatty acid content of *E. granulosus* was examined by gas-liquid chromatography.

Keyphrases □ *Echinococcus granulosus* scolices—cholesterol biosynthesis □ Cholesterol biosynthesis—*E. granulosus* scolices □ Lipid biochemistry—*E. granulosus* scolices □ Hydrolytic activity, *E. granulosus* scolices—4-^{14}C-cholesteryl acetate □ TLC—separation □ GLC—identity □ Scintillometry—analysis

It is a well-known fact that, in vertebrates, cholesterol is biosynthesized from acetate or mevalonate. In many insects, however, radioactive acetate or mevalonate is not incorporated into cholesterol; some insects do not even synthesize squalene, the noncyclic precursor of sterols (1, 2).

In 1954, Butenandt and Karlson (3) first isolated 25 mg. of ecdysone, the molting hormone, from 500 kg. of silkworm pupae. The compound [shown later to be a steroid (4)] was effective in accelerating molting, even in quantities below 7.5×10^{-3} mcg., when injected into ligated fly abdomens. In 1963, Karlson and Hoffmeister (5) demonstrated the incorporation of radioactivity from tritium-labeled cholesterol into ecdysone in *Calliphora* larvae, indicating that cholesterol is a precursor to ecdysone. Clark and Bloch (6) showed that 95% of the dietary cholesterol in the beetle *Dermestes vulpinus* could be replaced by β-sitosterol for structural utilization, but 5% of it remained essential for metabolic function; Clayton (7), working with the cockroach *Blattella germanica*, obtained similar results. According to Karlson (8), only a small part of the cholesterol injected into insects was converted to ecdysone; the majority still fulfilled cellular functions. Smissman *et al.* (9) isolated dehydroepiandrosterone, pregnenolone, and progesterone as the products of normal insect metabolism of dietary sterols in the confused flour beetle, *Tribolium confusum*. Horn *et al.* (4) isolated crustecdysone from crayfish, a compound which proved to be both chemically and biologically similar to ecdysone. Carlisle (10) demonstrated the biological activity of extracts from crabs, copepods, and locusts on the immature shore crab *Carcinus maenas*, indicating the presence of a common set of interactive ecdysones in crustaceans and insects.

The hormone that has an effect opposite to that of ecdysone on the molting stages of insects was first isolated from the abdomens of *Cecropia* males by Williams (11). Karlson and Schmialek (12) injected extracts of the excretions of the beetle *Tenebrio molitor* into mature larvae of the same species and noted the retardation of pupation in 88% of the treated larvae. In

(9) A. S. Michaels and J. C. Bolger, *Ind. Eng. Chem. Fundam.*, **1,** 24(1962).
(10) A. M. Gaudin and M. C. Fuerstenau, *Eng. Mining J.*, **159,** 110(1958).
(11) A. M. Gaudin and M. C. Fuerstenau, *Int. Mining Proc. Congr.*, London, England, Apr. 1960.
(12) A. M. Gaudin, M. C. Fuerstenau, and S. R. Mitchell, *Mining Eng. (London)*, **11,** 613(1959).
(13) C. B. Egolf and W. L. McCabe, *Trans. Amer. Inst. Chem. Eng.*, **33,** 620(1937).
(14) J. deBoer, *Advan. Colloid Interface Sci.*, **3,** 21(1950).
(15) F. London, *Z. Phys.*, **63,** 245(1930).
(16) B. Deryagin, A. Titijevskaia, I. Abriscova, and E. Lifshitz, *Discuss. Faraday Soc.*, **18,** 24(1954).
(17) B. Deryagin, I. Abriscova, and E. Lifshitz, *Quart. Rev. (London)*, **10,** 295(1956).
(18) E. Verway and J. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier, Amsterdam, The Netherlands, 1948.
(19) V. Vand, *J. Phys. Colloid Chem.*, **52,** 277(1948).
(20) C. R. Wylie, Jr., "Advanced Engineering Mathematics," McGraw-Hill, New York, N. Y., 1960, p. 177.
(21) C. M. Metzler, APhA Academy of Pharmaceutical Sciences, 5th National Meeting, Washington, D. C., November 20, 1968.
(22) J. T. Carstensen, J. B. Johnson, D. C. Spera, and M. J. Frank, *J. Pharm. Sci.*, **57,** 23(1968).
(23) G. A. Ratcliff, D. A. Blackader, and D. N. Sutherland, *Chem. Eng. Sci.*, **22,** 201(1967).
(24) G. A. Ratcliff, *J. Colloid Interface Sci.*, **25,** 586(1967).
(25) W. Higuchi and R. G. Stehle, *J. Pharm. Sci.*, **54,** 265(1965).
(26) T. Gillespie, *J. Phys. Chem.*, **66,** 1077(1962).
(27) T. Gillespie, *J. Colloid Sci.*, **15,** 219(1960).
(28) C. F. Goodeve, *Trans. Faraday Soc.*, **35,** 342(1939).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 21, 1969, from the *School of Pharmacy and Extension Services in Pharmacy, University Extension, University of Wisconsin, Madison, WI 53706*

Accepted for publication November 14, 1969.

This investigation was supported by grants from R. T. Vanderbilt Co., New York, NY 10017 and Hoffmann-La Roche, Inc., Nutley, NJ 07110

* To whom requests for reprints should be addressed.

Cholesterol Biosynthesis and Lipid Biochemistry in the Scolex of *Echinococcus granulosus*

GEORGE A. DIGENIS*, RALPH E. THORSON†, and ARPINE KONYALIAN

Abstract □ When live scolices of *Echinococcus granulosus* were incubated with labeled mevalonate *in vitro*, no radioactivity was incorporated into the parasites' cholesterol fraction. However, labeled cholesterol was recovered after a scolices' homogenate was incubated with 4-^{14}C-cholesteryl acetate. It is suggested that the scolex of *E. granulosus* is unable to biosynthesize cholesterol and that it obtains cholesterol in an esterified form from the host, which is subsequently hydrolyzed within the scolex to free cholesterol. The fatty acid content of *E. granulosus* was examined by gas-liquid chromatography.

Keyphrases □ *Echinococcus granulosus* scolices—cholesterol biosynthesis □ Cholesterol biosynthesis—*E. granulosus* scolices □ Lipid biochemistry—*E. granulosus* scolices □ Hydrolytic activity, *E. granulosus* scolices—4-^{14}C-cholesteryl acetate □ TLC—separation □ GLC—identity □ Scintillometry—analysis

It is a well-known fact that, in vertebrates, cholesterol is biosynthesized from acetate or mevalonate. In many insects, however, radioactive acetate or mevalonate is not incorporated into cholesterol; some insects do not even synthesize squalene, the noncyclic precursor of sterols (1, 2).

In 1954, Butenandt and Karlson (3) first isolated 25 mg. of ecdysone, the molting hormone, from 500 kg. of silkworm pupae. The compound [shown later to be a steroid (4)] was effective in accelerating molting, even in quantities below 7.5×10^{-3} mcg., when injected into ligated fly abdomens. In 1963, Karlson and Hoffmeister (5) demonstrated the incorporation of radioactivity from tritium-labeled cholesterol into ecdysone in *Calliphora* larvae, indicating that cholesterol is a precursor to ecdysone. Clark and Bloch (6) showed that 95% of the dietary cholesterol in the beetle *Dermestes vulpinus* could be replaced by β-sitosterol for structural utilization, but 5% of it remained essential for metabolic function; Clayton (7), working with the cockroach *Blattella germanica*, obtained similar results. According to Karlson (8), only a small part of the cholesterol injected into insects was converted to ecdysone; the majority still fulfilled cellular functions. Smissman *et al.* (9) isolated dehydroepiandrosterone, pregnenolone, and progesterone as the products of normal insect metabolism of dietary sterols in the confused flour beetle, *Tribolium confusum*. Horn *et al.* (4) isolated crustecdysone from crayfish, a compound which proved to be both chemically and biologically similar to ecdysone. Carlisle (10) demonstrated the biological activity of extracts from crabs, copepods, and locusts on the immature shore crab *Carcinus maenas*, indicating the presence of a common set of interactive ecdysones in crustaceans and insects.

The hormone that has an effect opposite to that of ecdysone on the molting stages of insects was first isolated from the abdomens of *Cecropia* males by Williams (11). Karlson and Schmialek (12) injected extracts of the excretions of the beetle *Tenebrio molitor* into mature larvae of the same species and noted the retardation of pupation in 88% of the treated larvae. In

1961, Schmialek (13) isolated 60 mg. of an oil from 80 kg. of *Tenebrio* feces, which was identified as farnesol and its oxidation product farnesal. Later it was shown that insects can biosynthesize labeled farnesol and farnesal from tagged mevalonic acid (14). Using the farnesol isolated by Schmialek (13), as well as the commercial compound, Wigglesworth (15) showed that farnesol reproduces all the effects of a juvenile hormone when applied to the surface of the cuticle of the insect *Rhodnius prolixus*. The main effects were retention of larval characters and partial reversal of metamorphosis in the molting adult. Other active compounds also have been isolated from the microsporidian *Tribolium castaneum* (16), various other microorganisms (17), and even from the balsam fir tree (18). It appears from these studies that in insects the pathway of mevalonate is directed toward the synthesis of specific hormones and not cholesterol, whereas in vertebrates part of the mevalonate is incorporated into cholesterol.[1]

The work on cholesterol biosynthesis in cestodes is still in its infancy. Frayha (20) observed that 1-^{14}C-acetate did not incorporate into the cholesterol fraction of *E. granulosus* scolices; but when 26-^{14}C-cholesterol was fed to mice infected with the parasite, the label did appear in the cholesterol isolated from the scolices. Indeed, Meyer *et al.* (21) reported the *Spirometra mansonoides* lacks the mechanisms required for the synthesis *de novo* of its sterols.[2]

The authors of this article (23) have recently shown that lipid extracts of *Echinococcus granulosus*, a cestode, contained substances with both stimulatory and depressive effects on the growth of excysted cysticercoids of *Hymenolepis diminuta* in *in vitro* cultures. Since mevalonate has been shown to be a precursor of farnesol and farnesal in insects (14), an attempt was made to correlate the biological activity of the different lipid fractions of *E. granulosus* scolices with their biosynthesis from mevalonate.

MATERIALS AND METHODS

Collection and Preparation of Scolices—The livers and lungs of the sheep and cattle infected with cysts of *E. granulosus* were obtained within 2 hr. of slaughter. The hydatid fluid and scolices[3] were collected aseptically from the cysts according to the previously published methods (23) to be used in both the authors' biosynthetic studies and hydrolytic studies.

Biosynthetic Studies—For the biosynthetic studies, the live[3] scolices were washed four times with an aqueous buffer solution (pH 7.4) of disodium monohydrogen phosphate and potassium dihydrogen phosphate (25).

The washed scolices were then incubated in a water bath shaker for 5 hr. at 37° with 4 ml. of phosphate buffer (pH 7.0), 1 ml. of antibiotic solution,[4] and 1 ml. of 2-^{14}C-DL-mevalolactone[5] (specific activity 3.11×10^5 c.p.m.[6]/0.04 μM/ml.). At the end of the incubation period, 2 ml. of 3 N H_2SO_4 was added to the flask.[7] The scolices were separated by low-speed centrifugation, washed several times with distilled water, and then transferred to a 125-ml. conical flask. A quantitative sample taken from a combination of the incubation supernatant fluid and the scolices washings was analyzed for radioactivity. From this figure the amount of the label actually taken up by the scolices was determined to be 1.33×10^5 c.p.m.

Isolation and Fractionation of the Lipids—The total lipids were extracted from the scolices and fractionated by thin-layer chromatography (TLC); the cholesterol was isolated according to the previously published methods (23).

Saponification of Neutral Fats—The lipids of Band III were saponified by refluxing them in 5 ml. of 5% ethanolic KOH for 24 hr. under nitrogen. At the end of the time, 5 ml. of distilled water was added to the mixture, and the ethanol was evaporated by bubbling nitrogen into the gently warmed (ambient 45°) flask. The solution was then extracted with three separate 5-ml. portions of ether. The ether extracts were dried over sodium sulfate, and the solvent was evaporated under nitrogen. The residue was taken up in a minimal amount of chloroform which was transferred into a liquid scintillation vial, evaporated, and assayed for ^{14}C.

The aqueous layer, containing the saponifiable fraction of the lipids, was acidified to pH 2.0 with 2 N H_2SO_4 and then extracted four times with ether. The ether fractions were dried overnight over anhydrous Na_2SO_4 and, subsequently, concentrated to a volume of about 5 ml. An aliquot from the solution was assayed for ^{14}C.

In another experiment the ether solution of the saponifiable fraction of lipids from scolices (not treated with radioactive mevalonate) was prepared for GLC analysis by prior treatment with diazomethane according to the method of Beames (26).

Gas-Liquid Chromatography—Gas-liquid chromatography (GLC) of the fatty acids was performed by a Pye argon chromatography apparatus equipped with a 1.21-m. (4-ft.) glass column (4 mm. in diameter) packed with 10% ethylene glycol adipate on diatomaceous earth (Celite 545, 80/100 mesh). Ten-microliter samples were injected. The gas-flow rate was 30 ml./min. and the column temperature was 180°. The calibration of the GLC instrument, as well as the quantitation and identification of chromatograms, was performed according to previously published procedures (27).

Enzyme Activity of a Scolex Homogenate—The hydrolytic action of homogenates from *E. granulosus* scolices on cholesteryl acetate was investigated as follows: 1 g. of live scolices[3] was homogenized in a Potter-Elvehjem homogenizer for 3 min. The homogenate was incubated with 1 ml. of antibiotic solution,[4] 4 ml. of phosphate buffer pH 7.0, and 6.0 mg. of radioactive 4-^{14}C-cholesteryl acetate[8] in a water bath at 37° for 24 hr. with vigorous and continuous shaking. At the end of the incubation period, the lipids were extracted from the mixture with chloroform. The residue, obtained by evaporation of the chloroform extracts, was subjected to TLC, and its cholesterol fraction was isolated as described before (23) and analyzed for its radioactivity content.

Scintillation Counting—All radioactivity measurements of the cholesterol samples were performed in a Packard Tricarb scintillation spectrometer model 3003 with an efficiency of 70% and a background count of 8 c.p.m. The samples were counted in liquid scintillation glass vials (20-ml. capacity) with 18 ml. of liquid scintillation cocktail containing 4 g. of 2,5-diphenyloxazol (PPO) as primary scintillator and 50 mg. of dimethyl-1,4-bis[2(5-phenyloxazol)benzene] (POPOP) as secondary scintillator per liter of toluene. Samples that were insoluble in the cocktail were first solubilized in 3

[1] Durr (19) recently provided evidence for the incorporation of mevalonic acid into the nonsaponifiable lipids of omental and subcutaneous adipose tissue of man, epididymal fat pad of rat, and the fat tail of the Syrian sheep. His stoichiometric studies showed that about one-half of the incorporated radioactivity in the nonsaponifiable lipids was in squalene, 20% in lanosterol and cholesterol, and the remainder in unidentified substances.

[2] However, Ginger and Fairbairn (22) working with *Hymenolepis diminuta* demonstrated the incorporation of 1-^{14}C-acetate into the cestode's cholesterol fraction.

[3] The viability of the collected scolices was determined by their motility and staining properties (1:1000 eosin solution) according to the procedure described by Meymerian *et al.* (24).

[4] The antibiotic solution was prepared by diluting with distilled water, 200,000 units of penicillin G and 200 mg. of streptomycin to a final volume of 400 ml.

[5] Purchased from the Radiochemical Centre, Amersham, Bucks, England.

[6] This abbreviation will be used throughout this paper to denote the number of counts per minute.

[7] Microscopic examination (24) of a sample of the scolices showed them to be totally killed by the sulfuric acid treatment.

[8] Radioactive cholesteryl acetate with a specific activity of 1.55×10^4 c.p.m./mg. was synthesized by treating 4-^{14}C-cholesterol with acetic anhydride in the presence of anhydrous pyridine (28). The ester was shown to be pure by mixed melting point (115–116°) with authentic cholesteryl acetate and by TLC in two solvents (29). Subsequent radioautography of the chromatograms exhibited only one radioactive spot.

Table I—Incorporation of 2-^{14}C-DL-Mevalolactone into Various Lipid Fractions of *E. granulosus* Scolices

Band	c.p.m.	Incorporation, %
I	1096	0.82
I*a*	5	Negligible
I*b*	115	0.08
I*c*	148	0.20
I*d*	502	0.37
II	171	0.13
II*a*	Negligible	Negligible
II*b* (ecdysone level)	8	0.01
II*c*	34	0.02
II*d*	93	0.07
II*e*	9	0.01
III	826	0.62
Unsaponifiable fraction of Band III	Negligible	Negligible
Saponifiable fraction of Band III	827	0.62

ml. of dioxane and then added to 15 ml. of the cocktail. In the case of samples that were too small to be eluted from the TLC plates, the silica gel was scraped off, mixed with the cocktail, and counted according to the method of Snyder and Stephens (30). All samples were counted twice for at least 10 min.

RESULTS

In a duplicate determination, 3.0 g. of live scolices of *E. granulosus* was incubated at 37° with 1 ml. of 2-^{14}C-mevalolactone (specific activity 3.11×10^5 c.p.m./0.04 μM/ml.). At the end of the incubation period (5 hr.), the lipids[9] were extracted from the scolices and fractionated by TLC using 0.25-mm. thick layers of silica gel G with methylene chloride–acetone (92:8, v/v) as a developer. The three zones obtained[10] were designated Bands I, II, and III (23), Bands II and III being the origin and front, respectively.

It was observed that the eluate from Band I inhibited growth markedly (length 0.5 to 1.0 mm.) as compared with the growth of worms under the influence of Band III or in a control (9.5 to 10 mm.) (23). Band I was subjected to further chromatography on a 20 × 20-cm. silica gel[11] plate with chloroform–acetone (90:10, v/v) as eluant. Four different zones were observed which were designated Band I*a* (origin), I*b* (R_f = 0.36), I*c* (R_f = 0.74), and I*d* (R_f = 0.93). The extent of the incorporation of 2-^{14}C-mevalonate into the various lipid fractions of *E. granulosus* scolices is presented in Table I.

When Band I*c*, containing the free cholesterol,[12,13] was eluted from the TLC chromatogram, it yielded 4 mg. of residue which was dissolved in 10.0 ml. of benzene. When 2.5 ml. from this solution was mixed with scintillation cocktail and its radioactivity counted, it was found to have a specific activity of 37 c.p.m./mg. To show whether or not the label was incorporated into cholesterol, 7.5 ml. from the cholesterol solution was mixed with 10 ml. of benzene containing 0.047 g. of pure, unlabeled cholesterol. The benzene was evaporated under a stream of nitrogen and the residue recrystallized once from 95% ethanol. No radioactivity was detected in the recrystallized cholesterol. In contrast, however, the supernatant solution was found to contain all the radioactivity (110 c.p.m.), indicating that I*c* contained compound(s) other than cholesterol[14] to which the label was incorporated.

Band III, which was shown to contain the neutral fat fraction of the lipids (31), was eluted and saponified. Aliquots from the saponifiable and nonsaponifiable fractions were counted for radioactivity. The results in Table I demonstrate that the entire radioactivity of Band III from the 2-^{14}C-mevalonate was incorporated into the saponifiable fraction.

With the significance of the saponifiable fraction in mind, the authors proceeded to determine by GLC the type and quantity of the fatty acids in this fraction. The results are shown in Table II.

The presence of hydrolytic enzymes in the scolices was indicated when a homogenate from 1 g. of live scolices was incubated with 6.0 mg. of radioactive cholesteryl acetate[8] at 37°. After incubation, free cholesterol was isolated from the mixture, and it was found to be radioactive (1056 c.p.m.). No radioactive cholesterol was detected in a control mixture[15] without the scolex homogenate.

DISCUSSION

With the recent findings on insect lipid biochemistry in mind, the authors have attempted to elucidate the mevalonate–cholesterol biosynthetic pathway of *E. granulosus*. First, however, a very brief description of the parasite's life cycle is necessary. The adult worm discharges eggs in the feces of the primary host, often a dog. When the eggs are ingested by an intermediate host, such as cow, sheep, or man, the eggs hatch in the duodenum. The released oncospheres then travel *via* the lymphatics and bloodstream to various parts of the body, usually the liver or lungs, where they become hydatid cysts. In man these cysts may grow to 15–20 cm. in diameter after several years. Within the cysts are found scolices which, if ingested by a suitable host, become adult worms within 7 weeks.

The only effective[16] treatment of the hydatid disease (echinococcosis), surgical removal of the cyst, suffers from both (*a*) a relatively high risk if secondary cyst infection by the viable hydatid scolices within the cyst and (*b*) the possibility of a violent and sometimes fatal anaphylactic reaction by the patient to the cyst fluid.

It seems to the authors that a more knowledgeable approach to the treatment and (or) control of echinococcosis could be made if more was known about the biochemistry of the parasite. Also, the possibility of finding an endocrine system in parasites similar to the one producing ecdysis in insects presents itself.

Cholesterol appears to be the major sterol in helminths. Fairbairn and Jones (34) showed that 75% of the unsaturated sterols of *Ascaris lumbricoides* was cholesterol. Thompson *et al.* (35) reported that 98 and 85% of the unsaponifiable matter of adult *Taenia taeniaeformis* and *Moniezia* sp. (respectively) were cholesterol. The authors' studies recently showed that in *E. granulosus* scolices, free cholesterol per dry weight constituted a high 3.03% (23, 32) as compared to 1.4% for *Taenia taeniaeformis* reported by von Brand *et al.* (36).

The authors' results showing the lack of incorporation of 2-^{14}C-mevalonate into cholesterol by the scolices suggest that the terpenic pathway is directed towards compounds other than cholesterol. The scolices' apparent "inability to synthesize" their own cholesterol is substantiated by the results of Frayha (20) who demonstrated that 1-^{14}C-acetate did not incorporate into the cholesterol fraction of *E. granulosus* scolices. He also demonstrated the passage of 26-^{14}C-

[9] The classic saponification of the lipids prior to fractionation by TLC was avoided because a correlation between the biological activity of the various lipid fractions and their biosynthesis from mevalonate was sought.

[10] The visualization and the elution of the substances from the zones obtained by TLC fractionation were performed as described previously (23).

[11] Chromagram, Eastman Kodak, Co., Inc., Rochester, N. Y.

[12] It was previously shown (23, 32) that when the lipid fraction of 0.495 g. of lyophilized scolices[13] was subjected to a similar TLC fractionation, it yielded 0.022 g. of residue from Band I. Further purification of the residue showed that 0.015 g. of it was cholesterol in its free form. Only trace amounts of cholesterol were shown to exist in their esterified form in Band III.

[13] One gram of lyophilized scolices was found equivalent to 14.5 g. of wet live scolices (32).

[14] Preliminary examination of I*c* by combined GLC–mass spectroscopy revealed that this fraction consisted of cholesterol and other compounds, the identities of which are under investigation.

[15] The control mixture consisted of 6.0 mg. of radioactive cholesteryl acetate, 1 ml. of antibiotic solution, and 4 ml. of phosphate buffer (pH 7.0).

[16] Chemotherapy appears to be difficult since the permeability of the membrane (particularly the internal germinal membrane) surrounding the hydatid cyst is known to be highly selective (33).

Table II—Major Fatty Acid Composition of the Scolex of *E. granulosus*

Fatty Acid	Shortened Designation	Methyl Esters as Percentage of Total Esters
n-Dodecanoic	12:0[a]	0.35
Tetradecanoic	14:0	1.67
	14:1	1.34
Hexadecanoic	16:0	8.17
	16:1	2.95
Heptadecanoic	17:0	0.63
	17:1	1.78
Octadecanoic	18:0	23.5
	18:1	27.7
	18:2	7.44
	18 un[b]	1.86
Eicosanoic	20:0	6.03
	20:1	2.4
	20:2	0.67
	20:3	0.74
	20:4	5.21
	20 un[b]	2.60

[a] The numerals following the colon refer to the number of double bonds in the molecule, whereas the numerals before the colon refer to the chain length (number of carbon atoms) of the fatty acid. [b] This abbreviation denotes unknown number of unsaturations.

cholesterol from the host (infected mice) to the scolices of *E. granulosus* (20). Furthermore, Meyer *et al.* (21) reported that the cestode *Spirometra mansonoides* lacked mechanisms required for the *de novo* synthesis of sterols.

The fact that free radioactive cholesterol was isolated from the scolices upon incubation with radioactive cholesteryl acetate indicates the presence of an enzyme system within the cyst responsible for the hydrolysis of the cholesteryl ester. This finding was consistent with the results of Lee *et al.* (37) who reported the presence of nonspecific esterases in various tapeworms.

Based on these recent findings, it seems reasonable to assume that cholesterol passes, probably in an esterified[17] form, from the host through the cyst membrane into the scolices where the cholesteryl ester is hydrolyzed to free cholesterol by an esterase system within the scolices.

REFERENCES

(1) A. J. Clark and K. Bloch, *J. Biol. Chem.*, **234,** 2578(1959).

(2) W. E. Robbins, J. N. Kaplanis, S. J. Louloudes, and R. E. Monroe, *Ann. Entomol. Soc. Amer.*, **53,** 128(1960).

(3) A. von Butenandt and P. Karlson, *Z. Naturforsch.*, **9b,** 389 (1954).

(4) D. H. S. Horn, E. J. Middleton, J. A. Wunderlich, and F. Hampshire, *Chem. Commun.*, **11,** 339(1966).

(5) P. Karlson and H. Hoffmeister, *Hoppe-Seylers Z. Physiol. Chem.*, **331,** 298(1963).

(6) A. J. Clark and K. Bloch, *J. Biol. Chem.*, **234,** 2583(1959).

(7) R. B. Clayton, *ibid.*, **235,** 3421(1960).

(8) P. Karlson, *Angew. Chem., Intern. Ed.*, **2,** 175(1963).

(9) E. E. Smissman, N. A. Jenny, and S. D. Beck, *J. Pharm. Sci.*, **53,** 1515(1964).

(10) D. B. Carlisle, *Gen. Comp. Endocrinol.*, **5,** 366(1965).

(11) C. M. Williams, *Nature*, **178,** 212(1956).

(12) P. Karlson and H. Schmialek, *Z. Naturforsch.*, **14b,** 821 (1959).

(13) H. Schmialek, *ibid.*, **16b,** 461(1961).

(14) *Ibid.*, **18b,** 462(1963).

(15) V. B. Wigglesworth, *J. Insect Physiol.*, **7,** 73(1961).

(16) F. M. Fisher and R. C. Sanborn, *Nature*, **194,** 1193(1962).

(17) H. A. Schneiderman, L. I. Gilbert, and M. Weinstein, *ibid.*, **188,** 1041(1960).

(18) W. S. Bowers, H. M. Fales, M. J. Thompson, and E. C. Uebel, *Science*, **154,** 1020(1966).

(19) I. F. Durr, *Biochem. J.*, **98,** 317(1966).

(20) G. J. Frayha, *Comp. Biochem. Physiol.*, **27,** 875(1968).

(21) F. Meyer, S. Kimura, and J. F. Mueller, *J. Biol. Chem.*, **241,** 4224(1966).

(22) C. D. Ginger and D. Fairbairn, *J. Parasitol.*, **52,** 1097(1966).

(23) R. E. Thorson, G. A. Digenis, A. Berntzen, and A. Konyalian, *ibid.*, **54,** 970(1968).

(24) E. Meymerian, G. W. Luttermoser, G. J. Frayha, C. W. Schwabe, and B. Prescott, *Ann. Surg.*, **158,** 211(1963).

(25) W. M. Clark, "The Determination of Hydrogen Ions," Williams and Wilkins, Baltimore, Md., 1920, p. 144.

(26) C. G. Beames, Jr., *Exp. Parasitol.*, **16,** 291(1965).

(27) J. W. Farguhar, W. Insull, Jr., P. Rosen, W. Stoffel, and E. H. Ahrens, *Nutr. Rev.*, **17,** Part II, 1, Supplement (Aug. 1959).

(28) R. L. Shriner, R. C. Fuson, and D. Y. Curtin, "The Systematic Identification of Organic Compounds," 4th ed., Wiley, New York, N. Y., 1959, p. 212.

(29) D. Waldi, in "Thin-Layer Chromatography," E. Stahl, Ed., Academic, New York, N. Y., 1965, p. 252.

(30) F. Snyder and N. Stephens, *Anal. Biochem.*, **4,** 128(1962).

(31) G. A. Digenis and B. A. Faraj, unpublished data.

(32) A. K. Konyalian, M.S. dissertation, American University of Beirut, Beirut, Lebanon, 1967.

(33) C. W. Schwabe, *Amer. J. Trop. Med. Hyg.*, **8,** 20(1959).

(34) D. Fairbairn and R. N. Jones, *Can. J. Chem.*, **34,** 182(1956).

(35) M. J. Thompson, E. Mosettig, and T. von Brand, *Exp. Parasitol.*, **9,** 127(1960).

(36) T. von Brand, Y. Sato, and R. Overton, *J. Parasitol.*, **51,** 483 (1965).

(37) D. L. Lee, A. H. Rothman, and J. B. Senturia, *Exp. Parasitol.*, **14,** 285(1963).

(38) D. Waldi, in "Thin-Layer Chromatography," E. Stahl, Ed., Academic, New York, N. Y., 1965, p. 254.

[17] 70–75% of human serum cholesterol is found in an esterified form (38).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 13, 1969, from the *Departments of Pharmaceutical Chemistry and Tropical Health, Schools of Pharmacy and Public Health, American University of Beirut, Beirut, Lebanon.*

Accepted for publication December 29, 1969.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Miami meeting, May 1968.

These studies were supported in part by research grants from the Medical Research Committee, American University of Beirut, and from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service (AI-02205-08 and AI-07218-01).

Thanks are extended to Dr. George J. Frayha, Department of Tropical Health, American University of Beirut, for useful discussions. The assistance of Miss Diana Walstad in the preparation of this manuscript is gratefully acknowledged.

* Present address: School of Pharmacy, University of Kentucky, Lexington, Ky. To whom requests for reprints should be directed.

† Present address: Department of Biology, University of Notre Dame, Notre Dame, Ind.

Metabolic Fate of *N*-γ-Phenylpropyl-*N*-benzyloxy Acetamide (W-1372) in Rats, Dogs, and Monkeys

JEROME EDELSON, J. F. DOUGLAS, and B. J. LUDWIG

Abstract □ The absorption, distribution, and metabolic fate of *N*-γ-phenylpropyl-*N*-benzyloxy acetamide (W-1372) was studied in the rat, dog, and monkey. W-1372-^{14}C was readily absorbed by all three species. Following an oral dose, peak blood levels of radioactivity were attained within 1 hr. in the monkey and in 2 hr. in the dog. The blood half-life of radioactivity following oral administration was 11.5 hr. in the dog. In the rat, maximum blood concentration of ^{14}C was reached 1 hr. following intraperitoneal administration, and the half-life was about 2 hr. Distribution studies of labeled drug in the rat indicate that the administered radioactivity is largely excreted within 24 hr. The major metabolites of W-1372 are hippuric acid, benzoic acid, *N*-γ-phenylpropyl-*N*-benzyloxyamine, and carbon dioxide.

Keyphrases □ *N*-γ-Phenylpropyl-*N*-benzyloxy acetamide (W-1372)—metabolism □ Absorption, distribution, metabolic fate—W-1372-^{14}C □ Inverse isotope dilution—analysis □ Hippuric acid, benzoic acid, *N*-γ-phenylpropyl-*N*-benzyloxyamine, carbon dioxide—W-1372-^{14}C metabolites □ TLC—identification □ IR spectrophotometry—identification

N-γ-Phenylpropyl-*N*-benzyloxy acetamide (W-1372) is a new hypolipidemic agent that has been shown by Berger *et al.* (1, 2) to reduce the extent of atherosclerotic lesions and to lower serum cholesterol levels in animals maintained on a high-cholesterol diet. This manuscript describes studies from this laboratory on the absorption, distribution, and metabolic fate of W-1372 in the rat, dog, and squirrel monkey.

METHODS

Preparation of *N*-γ-Phenylpropyl-*N*-benzyloxy-benzyl-7-^{14}C Acetamide (W-1372-Benzyl-^{14}C)—*N*-γ-Phenylpropyl-*N*-hydroxy acetamide, 965 mg., was added to a solution of 115 mg. of sodium in 10 ml. of anhydrous ethanol. After 2 mc., 633 mg., of benzyl chloride-7-^{14}C was added, the reaction mixture was heated to reflux for 4 hr. and left at room temperature overnight. The solvent was removed *in vacuo* and the residue was stirred with 40 ml. of water and 40 ml. of ethyl ether. The organic phase was separated and the aqueous phase was again extracted with an equal volume of ether. The organic phases were combined and dried over anhydrous sodium sulfate. After removal of the solvent, the residue was molecularly distilled (90°/0.004 mm.) to yield 681 mg. of W-1372-benzyl-^{14}C, specific activity 1.69×10^6 d.p.m./mg.

The product gave only one radioactive spot in TLC in methanol–acetone–acetic acid (95:95:10), and its IR spectrum was identical to that of authentic W-1372.

Preparation of *N*-γ-Phenylpropyl-*N*-benzyloxy Acetamide-acetyl-1-^{14}C (W-1372-Acetyl-^{14}C)—Acetyl chloride-1-^{14}C, 1.0 mc., 791 mg., was added to a solution of 4.84 g. of *N*-γ-phenylpropyl-*N*-benzyloxyamine in 20 ml. of ethyl ether. The reaction mixture was heated to reflux for 30 min., and the solid which formed, *N*-γ-phenylpropyl-*N*-benzyloxyamine hydrochloride, was removed by filtration. The filtrate was washed consecutively with 10 ml. of 0.1 *N* hydrochloric acid, 10 ml. of 0.1 *N* sodium hydroxide, and 10 ml. of water. The remaining organic phase was dried and molecularly distilled, as previously described, to yield 2.73 g. of W-1372-acetyl-^{14}C, specific activity 8.24×10^5 d.p.m./mg. Its purity was verified by TLC as previously described.

Absorption and Blood Concentration—Three male Sprague-Dawley rats, weighing about 200 g. each, received intraperitoneally a solution containing 40 mg. of W-1372-benzyl-^{14}C in 0.5 ml. of polyethylene glycol 400. Blood samples were taken from the tail vein at appropriate intervals and assayed for radioactivity by liquid scintillation counting.[1]

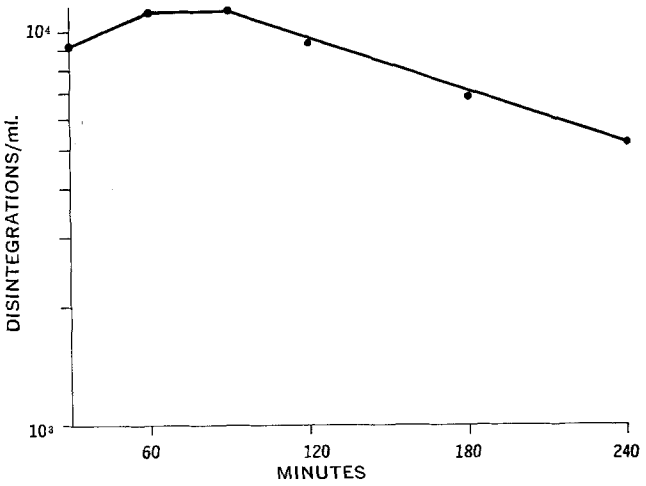


Figure 1—*Blood radioactivity after intraperitoneal administration of W-1372-benzyl-^{14}C to the rat.*

A male beagle hound, weighing 11 kg., was given a capsule containing 1.258 g. of W-1372-benzyl-^{14}C. Blood samples were taken from the vein to the toe nail at intervals and assayed for radioactivity.

Two 800-g., male squirrel monkeys received, by stomach tube, 50.8 mg. of W-1372-benzyl-^{14}C dissolved in 2.0 ml. of polyethylene glycol 400. At appropriate intervals, blood was removed from the jugular vein and assayed for radioactivity.

Tissue Distribution in the Rat—A male Sprague-Dawley rat, weighing 150 g., received intraperitoneally 42.2 mg. of W-1372-benzyl-^{14}C dissolved in 1.0 ml. of polyethylene glycol 400. Urine and feces were collected for 24 hr. The animal was then sacrificed and the organs were excised. The tissues and the remainder of the carcass were dissolved in 6 volumes of 1 *M* hydroxide of hyamine (Packard Instrument Co., Downers Grove, Ill.) by digesting for 18 hr. at 50°. An aliquot of each solution was taken and assayed for radioactivity in a liquid scintillation spectrometer.

A second rat, weighing 180 g., was given intraperitoneally a solution containing 51.2 mg. of acetyl-labeled W-1372 in 1.0 ml. of propylene glycol. The animal was placed in a metabolism chamber for 24 hr. and then sacrificed. The tissues were processed and analyzed for radioactivity. The carbon dioxide of respired air was trapped in 2.5 *M* sodium hydroxide, and the radioactivity of an aliquot of this solution was measured.

Inverse Isotope Dilutions—Typical inverse isotope dilution experiments were carried out as follows: a male Sprague-Dawley rat, weighing about 200 g., received intraperitoneally 12.7 mg. of W-1372-benzyl-^{14}C dissolved in 1.0 ml. of polyethylene glycol 400. Urine was collected for 24 hr. and its radioactivity measured. Isotope dilutions were carried out on aliquots of urine as follows.

1. Nonradioactive hippuric acid, 1000 mg., was added to the first 1.0-ml. aliquot, followed by the addition of 20 ml. of water and

[1] The scintillating fluid consisted of a solution of 7 g. of 2,5-diphenyloxazole, 250 mg. of 1,4-bis-2-(4-methyl-5-phenyloxazoyl)benzene, and 125 g. naphthalene in 1 l. dioxane.

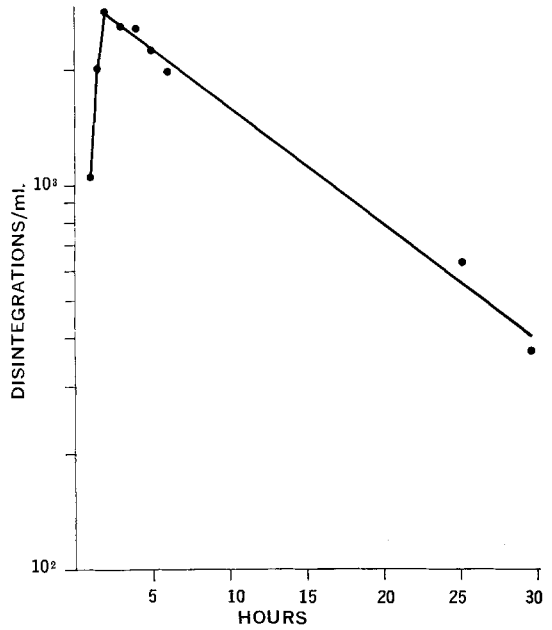


Figure 2—*Blood radioactivity after oral administration of W-1372-benzyl-^{14}C to the dog.*

sufficient 50% sodium hydroxide to yield a clear solution. Acidification of the mixture with concentrated hydrochloric acid precipitated the hippuric acid. The solid was removed and recrystallized from water to constant specific activity; m.p. 189–190°.

2. Nonradioactive benzoic acid, 1000 mg., was added to a second 1.0-ml. aliquot of urine and the mixture was diluted with water and heated until homogeneous. Upon cooling, benzoic acid crystallized and was removed by filtration. It was recrystallized from water to constant specific activity; m.p. 121–122°.

3. Nonradioactive *N*-γ-phenylpropyl-*N*-benzyloxyamine hydrochloride, 1000 mg., was added to a 2.0-ml. aliquot of urine. A few drops of concentrated hydrochloric acid were added, followed by sufficient hot ethanol to dissolve the solid. Water, 100 ml., was

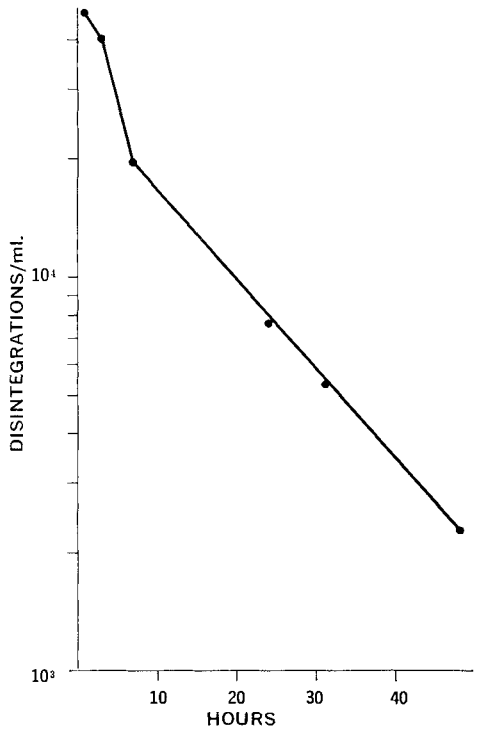


Figure 3—*Blood radioactivity after oral administration of W-1372-benzyl-^{14}C to the squirrel monkey.*

Table I—Tissue Distribution of ^{14}C in a Rat 24 hr. after Intraperitoneal Administration of 42.2 mg. W-1372-Benzyl-^{14}C

Organ	Radioactivity Recovered— d.p.m. × 10^3	Radioactivity Recovered— %
Urine	39,100	54.7
Carcass	23,250	32.7
Stomach and intestines	7,980	11.1
Liver	1,135	1.6
Kidney	661	0.9
Lung	71.4	0.1
Heart	27.9	0.04
Spleen	17.4	0.02
Total		101.3

added, and the mixture was made alkaline with 25 ml. of 10% sodium hydroxide and extracted with two 150-ml. portions of ether. The ether phase was dried over anhydrous sodium sulfate, and hydrogen chloride gas was bubbled into the ether solution to precipitate the hydrochloride of *N*-γ-phenylpropyl-*N*-benzyloxyamine. The solid was separated and recrystallized from ethanol to constant specific activity; m.p. 134–135°.

4. Nonradioactive benzaldehyde, 1000 mg., was added to a 2.0-ml. aliquot of urine; ethanol was added until the mixture was homogeneous. Sodium acetate, 1.5 g., and 1.0 g. semicarbazide hydrochloride were added, and the mixture was vigorously shaken and heated in a boiling water bath for 10 min. On cooling, the semicarbazone of benzaldehyde crystallized. The derivative was repeatedly recrystallized from 50% aqueous ethanol to constant specific activity; m.p. 221–223°.

To quantitate the various metabolites occurring in the urine in a combined form, an aliquot of urine was hydrolyzed by the addition of an equal volume of concentrated hydrochloric acid, and the mixture was heated on a steam bath for 10 min. Nonradioactive carrier was then added and the inverse isotope dilution carried out for total metabolites as described for the free metabolites.

RESULTS AND DISCUSSION

Absorption and Blood Concentration in the Rat, Dog, and Squirrel Monkey—After intraperitoneal administration to rats, benzyl-labeled W-1372 was rapidly absorbed, and a maximal blood concentration of radioactivity was reached after 1 hr. This level of blood radioactivity was maintained for 30 min. and was followed by a first-order decline. The half-life of ^{14}C in the blood was approximately 2 hr. (Fig. 1).

In the dog the drug was readily absorbed after an oral dose of W-1372-benzyl-^{14}C, and a peak blood level of ^{14}C was obtained within 2 hr. Under these conditions the disappearance of radioactivity from the blood followed first-order kinetics and the ^{14}C half-life was estimated to be 11.5 hr. (Fig. 2).

W-1372 was also rapidly absorbed by the squirrel monkey since the maximum blood concentration of radioactivity was reached during the 1st hour after oral administration of benzyl-labeled drug (Fig. 3). In this study the disappearance of radioactivity from blood did not follow the kinetics of a single-compartment model but occurred at two distinct rates. It is difficult to estimate the half-life during the initial depletion pattern. However, after 7 hr., the elim-

Table II—Tissue Distribution of ^{14}C in a Rat 24 hr. after Intraperitoneal Administration of 51.2 mg. W-1372-Acetyl-^{14}C

Organ	Radioactivity Recovered— d.p.m. × 10^3	Radioactivity Recovered— %
Respired CO_2	26,900	62.1
Carcass	5,320	12.3
Stomach and intestines	4,950	11.4
Urine	2,436	5.6
Liver	583	1.4
Kidney	135	0.3
Lung	52	0.1
Heart	22	0.05
Spleen	20	0.05
Brain	6	0.01
Total		93.4

Table III—Urinary Excretion of Hippuric and Benzoic Acids by Animals Given W-1372-Benzyl-^{14}C

Species	No. of Animals	Dose (mg.), Route	% of Urinary Radioactivity as Hippuric and Benzoic Acids
Rat, Sprague-Dawley	5	12–71, i.p.	85.6
Dog, beagle hound	5	25–520, i.p. and p.o.	65.8
Monkey, squirrel	3	16–32, i.p.	68.7

ination of blood radioactivity follows first-order kinetics with a half-life of approximately 13.5 hr.

Tissue Distribution in the Rat—Radioactivity from the intraperitoneal administration of W-1372-benzyl-^{14}C was principally excreted in the urine within 24 hr. Significant amounts of ^{14}C were also found in the carcass and gastrointestinal tract at this time (Table I).

Radioactivity from W-1372-acetyl-1-^{14}C was largely eliminated as respiratory carbon dioxide. Over 60% of the radioactivity of the dose administered was exhaled within 24 hr. while less than 6% was voided in the urine during this period. The distribution of the residual radioactivity is shown in Table II.

Identification and Quantitation of Urinary End Products—The major urinary end products of W-1372 in the three species studied, rat, dog, and monkey, were benzoic acid and hippuric acid. Quantitatively these compounds accounted for at least 65% of the urinary radioactivity. Table III shows the average values obtained with a number of animals of each species. In a single experiment on dog urine, the authors found an additional 1.9% of the urinary radioactivity present as benzoate conjugated with glucuronic acid. This value was determined by the increase in the amount of free benzoic acid present after hydrolysis with β-glucuronidase.

These conversions of W-1372 in the animal body would be anticipated from the metabolic fate of the related compound, benzyl *N*-benzyl carbethoxyhydroxamate (W-398), which is converted into benzoic acid and hippuric acid in both the rat and man (3, 4).

The deacetylated derivative of W-1372, *N*-γ-phenylpropyl-*N*-benzyloxyamine, was also identified and quantitated in the urine of animals receiving W-1372-benzyl-^{14}C. The *N*-γ-phenylpropyl-*N*-benzyloxyamine was present in both the free and bound form since acid hydrolysis of the urine liberated additional free amine. This increase was particularly significant for the dog. The nature of the conjugate has not been elucidated. Trace amounts of benzaldehyde were also identified in acid-hydrolyzed urine, but this may have arisen from the degradation of some metabolite. The quantities of the various metabolites occurring in the urines of various animal species are summarized in Table IV.

Table IV—Excretion of W-1372-Benzyl-^{14}C Urinary Metabolites in the Rat, Dog, and Monkey

Urinary End Product	% of Urinary Radioactivity[a] Rat No. 185	Dog No. 192	Monkey No. 195
Hippuric acid	87.1	54.8	61.0
Benzoic acid	0.32	0.84	1.54
N-γ-Phenylpropyl-*N*-benzyloxyamine (uncombined)	2.04	0.55	6.22
N-γ-Phenylpropyl-*N*-benzyloxyamine (total)[b]	2.23	19.22	7.69
Benzaldehyde (uncombined)	0.18	0.042	1.54
Benzaldehyde (total)[b]	—	0.28	—
Total radioactivity recovered	89.83	75.14	71.77

[a] Values obtained by inverse isotope dilution. [b] Total metabolite after acid hydrolysis of urine.

REFERENCES

(1) F. M. Berger, J. F. Douglas, B. J. Ludwig, and S. Margolin, *J. Pharmacol. Exp. Ther.*, **170,** 371(1969).

(2) F. M. Berger, J. F. Douglas, G. Lu, and B. J. Ludwig, *Proc. Soc. Exp. Biol. Med.*, **132,** 293(1969).

(3) J. F. Douglas, *J. Pharmacol. Exp. Ther.*, **150,** 105(1965).

(4) J. Edelson, A. Schlosser, and J. F. Douglas, *Biochem. Pharmacol.*, **17,** 779(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 10, 1969, from *Wallace Laboratories, Division of Carter-Wallace, Inc., Cranbury, NJ 08512*

Accepted for publication December 16, 1969.

A preliminary report of this paper was presented at the 1969 Spring Meeting of the American Society for Pharmacology and Experimental Therapeutics.

The authors are indebted to Mr. A. Schlosser and Mr. A. Maggio for their technical assistance, to the Organic Chemistry Department of Wallace Laboratories for providing the intermediates needed for the radiochemical syntheses, and to Mr. M. Meyer for the IR spectral data.

Analysis of *p*-Aminosalicylic Acid, Its Salts and Dosage Forms, by Nonaqueous Titration

JAMES HUNT and MARTIN I. BLAKE

Abstract □ *p*-Aminosalicylic acid, its salts, and its dosage forms are determined by visual or potentiometric titration with sodium methoxide in benzene–methanol using dimethylformamide as the titration solvent. *p*-Aminosalicylic acid and its decomposition product *m*-aminophenol may be differentiated with this titration system. Salts of *p*-aminosalicylic acid are converted to the acid form by ion-exchange chromatography prior to titration. The procedure is applied to several dosage forms.

Keyphrases □ *p*-Aminosalicylic acid and salts—analysis □ Dosage forms, p-aminosalicylic acid and salts—analysis □ Column chromatography—separation □ Potentiometric titration—analysis

The USP XVII method (1) for the determination of *p*-aminosalicylic acid (PAS), its salts and dosage forms, involves the diazotization reaction and is based on procedures developed by Tarnoky and Brews (2) and Pesez (3, 4). The use of an external indicator and the nonselectivity of the reaction have been noted by Chatten (5) as shortcomings of the procedure. *m*-Aminophenol (MAP), the major breakdown product of PAS, is also diazotized in the titration process; in the official monographs the MAP content is determined separately by colorimetric analysis.

A variety of analytical methods has been proposed for the determination of PAS and its salts. These have been extensively reviewed by Lach and Cohen (6).

A number of nonaqueous titrimetric procedures have appeared in the literature. They have been reviewed by Kucharsky and Safarik (7) and are briefly noted here. Chatten (8) reported the visual titration of PAS with potassium hydroxide in methanol as titrant, acetone as the titration solvent, and thymol blue as indicator. For sodium *p*-aminosalicylate, methanol served as the titration medium and perchloric acid in dioxane as titrant. These methods were reported to be specific even in the presence of MAP. Butler and Ramsey (9) titrated PAS and its sodium salt potentiometrically with perchloric acid in glacial acetic acid. An acetic acid–carbon tetrachloride solvent mixture served as the titration medium. Stockton and Zuckerman (10) determined sodium *p*-aminosalicylate and its solutions by potentiometric titration with perchloric acid in propylene glycol and isopropyl alcohol (1:1), using the same solvent mixture as the titration medium. The decomposition products MAP and sodium bicarbonate did not interfere. Das and Palit (11) employed the same titrant and solvent system.

In the present study, PAS is titrated with sodium methoxide in benzene–methanol (10:1). The titration medium is dimethylformamide. The difference in the acidic properties of PAS and MAP permitted a differentiating titration of the two components. The salts of PAS are converted to the acid by ion-exchange chromatography prior to titration. The techniques are applied to several dosage forms.

Table I—Comparative Study of Proposed and Official Assays for *p*-Aminosalicylate Content in Dosage Forms

Dosage Form	Labeled Amount, g./Unit Dose	Label Claim Found, %: Proposed Method	Label Claim Found, %: Official Assay
		***p*-Aminosalicylic Acid**	
Powder	—	99.86 ± 0.24[a]	100.04 ± 0.37
		Sodium *p*-Aminosalicylate	
Powder	—	99.62 ± 0.29	100.52 ± 0.06
Capsule	0.50	100.71 ± 0.54	100.43 ± 0.26
Tablet	0.50	100.77 ± 0.86	99.82 ± 0.29
Tablet	0.69	98.70 ± 0.57	99.98 ± 0.26
Tablet	1.00	97.40 ± 0.32	100.50 ± 0.38
		Calcium *p*-Aminosalicylate	
Powder	—	99.45 ± 0.17	99.40 ± 0.52
Tablet (chocolate coated)	0.50	100.43 ± 0.72	100.21 ± 0.18
Capsule	0.50	96.99 ± 0.42	100.58 ± 0.71
Solution	1.00 (5 ml.)	96.87 ± 0.89	102.18 ± 0.40

[a] Average deviation based on at least five determinations.

EXPERIMENTAL

Apparatus—Titrations were performed visually or potentiometrically with a Fisher titrimeter, model 35, equipped with a sleeve-type calomel and platinum electrode system. A 50-ml. buret (1 cm. i.d.) was employed as a chromatographic column. It was plugged at the base with glass wool to support the resin column.

Reagents—(*a*) *p*-Aminosalicylic acid, *m*-aminophenol, and sodium and calcium *p*-aminosalicylate were the best quality available from commercial sources. The *m*-aminophenol was further purified by recrystallizing from hot water. (*b*) Tenth normal sodium methoxide in benzene–methanol (10:1) was prepared and standardized as described earlier (12). (*c*) Other chemicals and all solvents used in this study were reagent grade and were used without further purification. (*d*) Powder, capsule, and tablet dosage forms were supplied by Dorsey Laboratories, Lincoln, Neb. A solution of sodium *p*-aminosalicylate was obtained from Hines Veterans Administration Hospital, Hines, Ill., prepared as directed in their hospital pharmacy formulary.

Preparation of Column—A weak cation-exchange resin (Amberlite IRC-50) and a strong cation-exchange resin (Dowex 50W-X8) were used in this study. The resin columns were prepared as described earlier (13, 14).

General Assay Procedure—One-half to one milliequivalent of sodium or calcium *p*-aminosalicylate, accurately weighed, was dissolved in the smallest volume of dimethylformamide and the solution passed through the column containing the weak resin. Effluent was collected in a 100-ml. graduated cylinder. Additional dimethylformamide was added to the column until 75 ml. of ef-

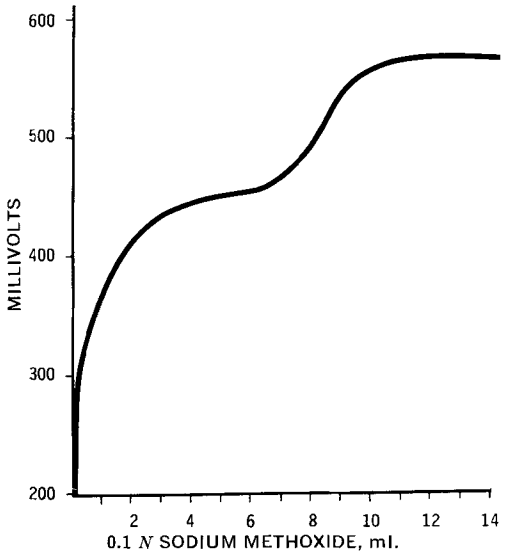


Figure 1—*Typical titration curve for MAP.*

fluent was collected. The effluent was titrated visually to the first permanent blue color using thymol blue as indicator (0.3% in methanol) or potentiometrically with the Fisher titrimeter. By the latter method, the end-point was determined from the inflection of the curve obtained by plotting volume of titrant *versus* millivolt readings.

Analysis of Dosage Forms—*Capsules and Tablets*—Twenty tablets were weighed and powdered, or 20 capsules were emptied as completely as possible and the contents weighed. A sample of the powder mass equivalent to between 0.5 and 1.0 meq. of *p*-aminosalicylate salt was dissolved in 25 ml. of dimethylformamide with the aid of magnetic stirring. The solution was treated as described under *General Assay Procedure*.

Chocolate-coated tablets of calcium *p*-aminosalicylate were soaked in distilled water until the coating dissolved. This required no longer than 5 min. The tablets were dried rapidly between filter paper and the dried tablets were assayed as described previously.

Solution—One milliliter of a solution, labeled to contain 1 g. of sodium *p*-aminosalicylate per 5 ml., was transferred by pipet to the column containing the weak resin. The column was eluted with dimethylformamide until a total of 75 ml. of effluent was collected. The effluent was titrated as described previously.

Official Assay Procedure—*p*-Aminosalicylic acid, the sodium and calcium salts, and their dosage forms were assayed as described in USP XVII (1).

Differentiating Titration of *p*-Aminosalicylic Acid and *m*-Aminophenol—Samples containing 1 meq. of MAP and a series of mixtures containing 1 meq. of PAS and varying amounts of MAP were titrated visually and potentiometrically in dimethylformamide with 0.1 *N* sodium methoxide as titrant.

Table II—Analysis of PAS and MAP Mixtures

Meq. Ratio of Components, PAS–MAP	Recovery, % PAS	Recovery, % MAP
	Visual Titration	
1.00:1.00	101.41 ± 0.98[a]	
1.00:0.50	99.66 ± 0.08	
1.00:0.33	97.55 ± 0.65	
1.00:0.25	99.97 ± 0.48	
1.00:0.20	99.47 ± 0.10	
	Potentiometric Titration	
1.00:1.00	96.57 ± 0.42	99.21 ± 0.45
1.00:0.50	98.26 ± 0.37	99.74 ± 0.85
1.00:0.33	97.93 ± 0.22	101.11 ± 0.87
1.00:0.25	98.85 ± 0.54	103.84 ± 0.23
1.00:0.20	97.86 ± 0.34	102.35 ± 0.08

[a] Average deviation based on at least five determinations.

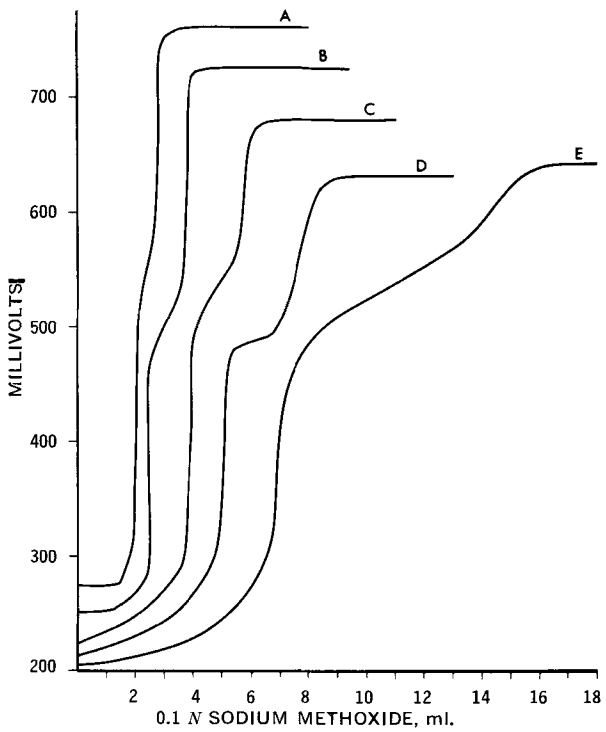


Figure 2—*Differentiating titration of mixtures of PAS and MAP in varying meq. ratios: A, 1:0.20; B, 1:0.25; C, 1:0.33; D, 1:0.50; and E, 1:1.0.*

Chromatographic Separation of *p*-Aminosalicylic Acid and *m*-Aminophenol—A series of mixtures corresponding to those described in the previous section was passed through a chromatographic column containing a strong cation-exchange resin. Aliquots of the mixture were treated as described in the *General Assay Procedure*, except that a strong resin (Dowex 50W-X8) was used in place of the weak resin.

RESULTS AND DISCUSSION

A major drawback to the official assay procedure for PAS, its salts and dosage forms, is that the breakdown product MAP also responds to the assay. As a result, apparently quantitative recoveries may be obtained even though extensive decomposition may have taken place. Although the USP includes a limits test for MAP based on colorimetric analysis, it would be desirable to have a simple titrimetric procedure which would be specific for PAS. Most nonaqueous titration procedures proposed in the literature involve the titration of the amino group with perchloric acid in a suitable solvent system. Although Chatten (5, 8) titrated the carboxyl group in PAS with potassium hydroxide in methanol, the sodium salt was determined with perchloric acid.

In the present study, PAS was titrated visually with sodium methoxide using thymol blue as the indicator. The end-point was readily detectable with one drop of titrant. The sodium and calcium salts and their dosage forms were titrated similarly after treatment with the weak cation-exchange resin (Amberlite IRC-50), which converted the salt to the free PAS. The results of the analyses are recorded in Table I. Comparative assays were performed by the official procedure. In almost every case the percent recovery by the official assay was essentially the same or higher than by the proposed method.

MAP was not titratable visually with sodium methoxide. Samples dissolved in dimethylformamide produced an indicator color change with the first drop of titrant. However, suitable inflections were obtained by potentiometric titration. A typical titration curve is shown in Fig. 1. The average percent recovery based on a series of 10 titrations was 99.22 ± 1.09%.

The effect of the presence of added MAP on the titration of PAS was tested. A series of mixtures of PAS and MAP, listed in Table II, was titrated visually with sodium methoxide. The presence

of MAP did not interfere with the visual end-point determination in the titration of PAS. This is probably attributable to the large difference in pKa values for these compounds: 3.25 for PAS (15) and 9.71 for MAP (16). When the mixtures were titrated potentiometrically, two inflections in the titration curve were obtained, indicating the feasibility of a differentiating titration procedure. The titration curves for the series of mixtures are shown in Fig. 2, and the analysis data are listed in Table II. In the titration curves the first inflection is attributable to PAS, while the second end-point is due to the MAP. Although the differentiating titration may not be useful for determining the MAP content when present in low concentrations (*e.g.*, 10% or less), both the visual and the potentiometric titration procedures are specific for the PAS content.

The weak cation-exchange resin (Amberlite IRC-50) was found to be effective for converting the salts of PAS to the free acid. The PAS was eluted from the column with dimethylformamide and the eluate titrated with sodium methoxide. When mixtures of PAS (or its salt form) and MAP listed in Table II were passed through the resin column, both PAS and MAP appeared in the eluate. A differentiating titration yielded quantitative recoveries for both components. Apparently the PAS and MAP are too weakly basic to be retained by a carboxylic acid-type resin. When the strong cation-exchange resin (Dowex 50W-X8) was used in place of the weak resin, the MAP was retained by the column while the PAS passed through and was recovered quantitatively in the eluate. The percent recovery based on the potentiometric titration of seven samples was 100.22 ± 0.56%. A sulfonic acid-type resin (Dowex 50W-X8) is a sufficiently strong acid to extract the MAP from the mixture.

In preliminary studies, ethylenediamine, acetone, isopropyl alcohol, methyl isobutyl ketone, acetonitrile, and dimethylformamide were examined as solvents for the differentiating titration of PAS and MAP. Dimethylformamide was found to produce the most reproducible and clearly defined end-points. The sleeve-type calomel and platinum electrode system produced good differentiating titration curves where the conventional sleeve-type and glass electrode system was unsuccessful in differentiating PAS and MAP.

SUMMARY

The proposed assay procedure has advantage over the official assay in that the PAS content is determined by direct visual titration. MAP, if present, does not interfere with the end-point detection. Quantitative separation of MAP from PAS may be achieved by passing the mixture through a column of strong cation-exchange resin (Dowex 50W-X8). The MAP is retained by the column while the PAS appears in the eluate. Mixtures of MAP and PAS may be differentiated by potentiometric titration. While this procedure is not suitable for determining low concentrations of MAP, as is required for the official dosage forms (1% or less), it is useful for studying the kinetics of the decomposition of PAS in dosage forms, particularly solutions.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 36, 37, 586, 587, 882.

(2) A. Tarnoky and B. A. Brews, *Biochem. J.*, **45**, 508(1949).

(3) M. Pesez, *Bull. Soc. Chim. Fr.*, **30**, 918(1949).

(4) M. Pesez, *Bull. Soc. Chim. Biol.*, **31**, 1369(1949).

(5) L. G. Chatten, in "Titrimetric Methods," D. S. Jackson, Ed., Plenum Press, New York, N. Y., 1969, pp. 146–154.

(6) J. L. Lach and J. Cohen, *Drug Stand.*, **28**, 65(1960).

(7) J. Kucharsky and L. Safarik, "Titrations in Nonaqueous Solvents," Elsevier, New York, N. Y., 1965, p. 182.

(8) L. G. Chatten, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 556 (1956).

(9) A. Q. Butler and J. C. Ramsey, *ibid.*, **42**, 338(1953).

(10) J. R. Stockton and R. Zuckerman, *ibid.*, **43**, 273(1954).

(11) M. N. Das and S. R. Palit, *J. Ind. Chem. Soc.*, **31**, 34(1954).

(12) M. I. Blake, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 287(1957).

(13) M. C. Vincent and M. I. Blake, *Drug Stand.*, **26**, 206(1958).

(14) M. I. Blake and D. A. Nona, *J. Pharm. Sci.*, **52**, 945(1963).

(15) "The Merck Index," 8th ed., Merck and Co., Rahway, N. J., 1968, p. 62.

(16) S. Diembeck, E. Grauer, and H. Jager, *Archiv Pharm.*, **301**, 628(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 17, 1969, from the *Department of Pharmacy, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612*

Accepted for publication December 29, 1969.

The authors thank Dr. Walter F. Charnicki, Dorsey Laboratories, and Mr. James Tio, Hines Veterans Administration Hospital, for supplying samples for analysis.

Improved Differential Spectrophotometric Determination of Rifamycins

CARMINE R. PASQUALUCCI*, ARISTIDE VIGEVANI, PIETRO RADAELLI, and GIAN G. GALLO

Abstract □ A differential spectrophotometric method for the determination of rifamycin B, rifamide, rifamycin SV, and rifampin, based on the oxidation by $NaNO_2$ of the hydroquinone moiety of the compounds, is described. The application of the method to the determination of rifamycin B in fermentation broths and of rifampin in capsules and in syrup is reported. Precision and accuracy data are given.

Keyphrases □ Rifamycins, dosage forms—differential determination □ Fermentation broths—rifamycin determination □ Hydrolytic oxidation—rifamycin determination □ Colorimetric analysis—spectrophotometry

Rifamycin B (I) (1) is a metabolite produced by *S. mediterranei*, while rifamide (II) (2), rifamycin SV (III) (3), and rifampin[1] (IV) (4) are the derived semisynthetic antibiotics presently in therapeutic use.[2] All these rifamycins[3] possess a characteristic chromophoric group which permits their spectrophotometric determination (2, 5–7). Furthermore, based on the quinone–hydroquinone nature of the chromophore, a differential spectrophotometric method has been described for rifamycin B and SV (8). The subjects of the present

[1] The international nonproprietary name for this compound is rifampicin.

[2] Rifamide as Rifocin M; rifamycin SV as Rifocin; rifampin as Rifadin.

[3] Rifamycins LXIII (rifamycin LXII: G. C. Lancini, G. G. Gallo, G. Sartori, and P. Sensi, *J. Antibiot.* (Tokyo), **22**, 369(1969).

of MAP did not interfere with the visual end-point determination in the titration of PAS. This is probably attributable to the large difference in pKa values for these compounds: 3.25 for PAS (15) and 9.71 for MAP (16). When the mixtures were titrated potentiometrically, two inflections in the titration curve were obtained, indicating the feasibility of a differentiating titration procedure. The titration curves for the series of mixtures are shown in Fig. 2, and the analysis data are listed in Table II. In the titration curves the first inflection is attributable to PAS, while the second end-point is due to the MAP. Although the differentiating titration may not be useful for determining the MAP content when present in low concentrations (*e.g.*, 10% or less), both the visual and the potentiometric titration procedures are specific for the PAS content.

The weak cation-exchange resin (Amberlite IRC-50) was found to be effective for converting the salts of PAS to the free acid. The PAS was eluted from the column with dimethylformamide and the eluate titrated with sodium methoxide. When mixtures of PAS (or its salt form) and MAP listed in Table II were passed through the resin column, both PAS and MAP appeared in the eluate. A differentiating titration yielded quantitative recoveries for both components. Apparently the PAS and MAP are too weakly basic to be retained by a carboxylic acid-type resin. When the strong cation-exchange resin (Dowex 50W-X8) was used in place of the weak resin, the MAP was retained by the column while the PAS passed through and was recovered quantitatively in the eluate. The percent recovery based on the potentiometric titration of seven samples was 100.22 ± 0.56%. A sulfonic acid-type resin (Dowex 50W-X8) is a sufficiently strong acid to extract the MAP from the mixture.

In preliminary studies, ethylenediamine, acetone, isopropyl alcohol, methyl isobutyl ketone, acetonitrile, and dimethylformamide were examined as solvents for the differentiating titration of PAS and MAP. Dimethylformamide was found to produce the most reproducible and clearly defined end-points. The sleeve-type calomel and platinum electrode system produced good differentiating titration curves where the conventional sleeve-type and glass electrode system was unsuccessful in differentiating PAS and MAP.

SUMMARY

The proposed assay procedure has advantage over the official assay in that the PAS content is determined by direct visual titration. MAP, if present, does not interfere with the end-point detection. Quantitative separation of MAP from PAS may be achieved by passing the mixture through a column of strong cation-exchange resin (Dowex 50W-X8). The MAP is retained by the column while the PAS appears in the eluate. Mixtures of MAP and PAS may be differentiated by potentiometric titration. While this procedure is not suitable for determining low concentrations of MAP, as is required for the official dosage forms (1% or less), it is useful for studying the kinetics of the decomposition of PAS in dosage forms, particularly solutions.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 36, 37, 586, 587, 882.

(2) A. Tarnoky and B. A. Brews, *Biochem. J.*, **45**, 508(1949).

(3) M. Pesez, *Bull. Soc. Chim. Fr.*, **30**, 918(1949).

(4) M. Pesez, *Bull. Soc. Chim. Biol.*, **31**, 1369(1949).

(5) L. G. Chatten, in "Titrimetric Methods," D. S. Jackson, Ed., Plenum Press, New York, N. Y., 1969, pp. 146–154.

(6) J. L. Lach and J. Cohen, *Drug Stand.*, **28**, 65(1960).

(7) J. Kucharsky and L. Safarik, "Titrations in Nonaqueous Solvents," Elsevier, New York, N. Y., 1965, p. 182.

(8) L. G. Chatten, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 556 (1956).

(9) A. Q. Butler and J. C. Ramsey, *ibid.*, **42**, 338(1953).

(10) J. R. Stockton and R. Zuckerman, *ibid.*, **43**, 273(1954).

(11) M. N. Das and S. R. Palit, *J. Ind. Chem. Soc.*, **31**, 34(1954).

(12) M. I. Blake, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 287(1957).

(13) M. C. Vincent and M. I. Blake, *Drug Stand.*, **26**, 206(1958).

(14) M. I. Blake and D. A. Nona, *J. Pharm. Sci.*, **52**, 945(1963).

(15) "The Merck Index," 8th ed., Merck and Co., Rahway, N. J., 1968, p. 62.

(16) S. Diembeck, E. Grauer, and H. Jager, *Archiv Pharm.*, **301**, 628(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 17, 1969, from the *Department of Pharmacy, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612*

Accepted for publication December 29, 1969.

The authors thank Dr. Walter F. Charnicki, Dorsey Laboratories, and Mr. James Tio, Hines Veterans Administration Hospital, for supplying samples for analysis.

Improved Differential Spectrophotometric Determination of Rifamycins

CARMINE R. PASQUALUCCI*, ARISTIDE VIGEVANI, PIETRO RADAELLI, and GIAN G. GALLO

Abstract □ A differential spectrophotometric method for the determination of rifamycin B, rifamide, rifamycin SV, and rifampin, based on the oxidation by $NaNO_2$ of the hydroquinone moiety of the compounds, is described. The application of the method to the determination of rifamycin B in fermentation broths and of rifampin in capsules and in syrup is reported. Precision and accuracy data are given.

Keyphrases □ Rifamycins, dosage forms—differential determination □ Fermentation broths—rifamycin determination □ Hydrolytic oxidation—rifamycin determination □ Colorimetric analysis—spectrophotometry

Rifamycin B (I) (1) is a metabolite produced by *S. mediterranei*, while rifamide (II) (2), rifamycin SV (III) (3), and rifampin[1] (IV) (4) are the derived semisynthetic antibiotics presently in therapeutic use.[2] All these rifamycins[3] possess a characteristic chromophoric group which permits their spectrophotometric determination (2, 5–7). Furthermore, based on the quinone–hydroquinone nature of the chromophore, a differential spectrophotometric method has been described for rifamycin B and SV (8). The subjects of the present

[1] The international nonproprietary name for this compound is rifampicin.

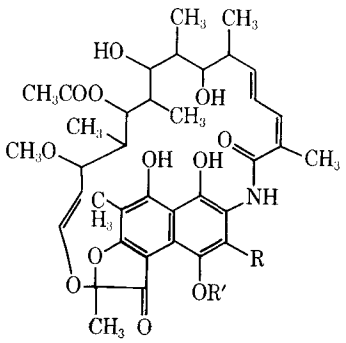
[2] Rifamide as Rifocin M; rifamycin SV as Rifocin; rifampin as Rifadin.

[3] Rifamycins LXIII (rifamycin LXII: G. C. Lancini, G. G. Gallo, G. Sartori, and P. Sensi, *J. Antibiot.* (Tokyo), **22**, 369(1969).

Table I—Differential Absorptivities of the Rifamycins as a Function of Reaction Time and of $NaNO_3$ Concentration, at the Given Wavelengths

Time, min.	$NaNO_2$, % 0.01	0.02	0.1	0.01	0.05	0.1	0.01	0.05	0.1	0.01	0.05	0.1
	Rifamycin B, 425 mμ			Rifamide, 424 mμ			Rifamycin SV, 447 mμ			Rifampin, 473 mμ		
2	15.81	17.90	21.00	1.18	12.90	14.00	2.31	15.19	15.60	0.95	7.76	14.29
5	18.12	21.30	21.51	—	14.00	13.90	—	15.60	15.58	5.72	14.63	14.70
10	20.35	21.48	21.47	10.30	13.97	14.06	14.28	15.67	15.65	—	14.71	14.69
15	21.02	21.50	21.50	12.43	14.02	13.97	15.52	15.55	15.57	11.84	14.67	14.71
20	21.47	—	21.50	13.53	13.98	14.00	15.58	—	15.60	13.34	14.70	14.70
30	—	21.52	20.90	13.92	14.00	13.94	15.60	15.58	15.55	14.16	14.73	—
40	21.51	21.39	20.40	14.03	—	13.98	15.62	15.60	15.60	14.43	14.65	14.68

paper are the modification of the differential spectrophotometric method, its extension to rifamide and rifampin, and its application to pharmaceutical preparations.



	R	R′
I,	H	CH_2COOH
II,	H	$CH_2CON(C_2H_5)_2$
III,	H	H
IV,	$CH{=}N{-}N$ ⟨piperazine⟩ $N{-}CH_3$	H

THEORY AND PROCEDURE

The differential spectrophotometric methods are suitable for the determination of substances in the presence of high concentrations of foreign-absorbing components. In the case of rifamycins the quinone–hydroquinone system, together with the position of the absorption maximum in the visible region, makes the method specific for this family of antibiotics. Thus, rifamycins can be determined in fermentation broths, in pharmaceutical preparations, and in biological fluids, all cases in which the presence of foreign components must be overcome. The authors have studied the differential method in the case of rifamycin B, rifamide, rifamycin SV, and rifampin, and they describe its application to the determination of rifamycin B in the fermentation broth and of rifampin in some pharmaceutical forms.

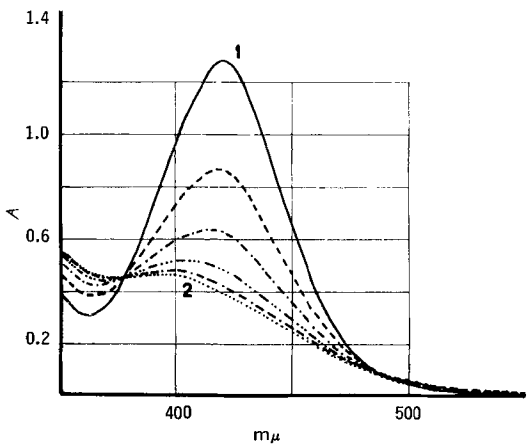


Figure 1—*Visible spectra of the hydrolytic oxidation of rifamide into rifamycin S (4.00 mcg./ml. of rifamide in pH 4.63 acetate buffer containing 0.01% w/v $NaNO_2$). 1: initial curve (rifamide), 2: final curve (rifamycin S); the curves were recorded at 5-min. intervals.*

Table II—Differential Absorptivities (*a*) of the Rifamycins

	$\lambda_{max.}$	*a*
Rifamycin B	425 mμ	21.5
Rifamide	424 mμ	14.0
Rifamycin SV	447 mμ	15.6
Rifampin	473 mμ	14.7

The previous differential spectrophotometric method (8) is not applicable for the determination of rifamycin B in the fermentation broth. In fact, the use of $HAuCl_4$ as the oxidizing agent and of alcoholic benzoate buffer causes the formation of precipitates, which hinder the photometric reading. The authors adopted as the oxidizing agent a solution of $NaNO_2$ in aqueous acetate buffer, pH 4.63. The same oxidizing agent was also chosen for the determination of rifamycin SV, rifampin, and rifamide. With rifamide it must be pointed out that a hydrolytic oxidation to rifamycin S occurs, with the splitting of the glycolic amide moiety. This was demonstrated by the visible spectrum of the reaction solution (Fig. 1) and by isolation and identification (TLC, IR, NMR, and UV) of the final product.

The effect of the concentration of the oxidizing agent on the reaction time was studied for all the rifamycins and the results are given in Table I. On the basis of these data, a reaction time of 5 min. and a $NaNO_2$ concentration of 0.1% were chosen. In fact, under these conditions the rifamycins are completely transformed to the oxidized forms. In Table II the wavelengths and the differential absorptivities of the examined rifamycins, determined on the pure compounds in the conditions of the method, are reported.

The general procedure of the differential determination is as follows: the rifamycin to be determined (as powder, suspension, or solution) is dissolved in a water-miscible solvent at a concentration of about 1 mg./ml. Two equal portions of this solution are separately diluted to a concentration of 20–40 mcg./ml., one with the pH 4.63

Table III—Precision Data of the Differential Method[a]

	Rifamycin B in Fermentation Broths, mcg./ml.	Rifampin Capsules, % w/w	Rifampin Syrup, % w/w
Values obtained	1251; 1251; 1260; 1256; 1256; 1237; 1242; 1256; 1257; 1237	78.9; 78.3; 78.2; 78.5; 78.6; 79.0; 78.5; 79.2; 77.6; 78.2	1.88; 1.89; 1.85; 1.90; 1.88; 1.87; 1.89; 1.85; 1.88; 1.88
Mean	1249.7	78.50	1.877
SD	8.25	0.464	0.0164
RSD	0.66	0.59	0.87

[a] Data for rifamide and rifamycin SV are not reported, but they are of the same order of magnitude as rifamycin B and rifampin.

Table IV—Accuracy Data of Differential Method for Rifamycin B in Fermentation Broth

Sample	Rifamycin B Broth, mcg./ml. Present[a]	Added	Total Content	Found	Error, mcg./ml.	$\frac{\text{Found}}{\text{Total Content}} \cdot 100$
A	777.3	804.3	1581.6	1545.5	−36.1	97.7
B	813.6	967.9	1781.5	1772.7	− 8.8	99.5
C	613.6	967.9	1581.5	1581.8	+ 0.3	100.0
Mean error, −14.87						
Relative error, − 0.90						

[a] Determined by this method.

Table V—Accuracy Data of Differential Method for Rifampin in Capsules and Syrup

Weighed, mcg./ml.	Found, mcg./ml.	Error, mcg./ml.	$\frac{\text{Found}}{\text{Weighed}} \cdot 100$
Rifampin in Capsules			
35.60	35.10	−0.5	98.6
32.86	32.53	−0.33	99.0
32.40	32.32	−0.08	99.8
35.74	35.59	−0.15	99.6
34.20	33.50	−0.7	98.0
33.56	33.02	−0.54	98.4
32.08	31.63	−0.45	98.6
33.20	32.67	−0.53	98.4
31.96	31.84	−0.12	99.6
31.90	31.28	−0.62	98.1
Mean error, −0.402			
Relative error, −1.21			
Rifampin in Syrup			
17.35	17.38	+0.03	100.2
18.65	18.84	+0.19	101.0
17.55	17.93	+0.38	102.2
19.00	19.05	+0.05	100.3
20.80	20.71	−0.09	99.6
27.75	27.32	−0.43	98.5
19.70	19.53	−0.17	99.1
24.00	23.49	−0.51	97.9
18.15	18.35	+0.20	101.1
18.40	18.49	+0.09	100.5
Mean error, −0.026			
Relative error, −0.13			

acetate buffer solution (Solution A) and the other one with the oxidizing pH 4.63 acetate buffer solution containing 0.1% w/v $NaNO_2$ (Solution B). The absorbance of the solution diluted with Solution A is determined against the solution diluted with Solution B with a spectrophotometer at the appropriate wavelength. The amount of rifamycin is calculated from the measured absorbance.

EXPERIMENTAL[4]

Reagents and Solutions—*Solution A*—Acetate buffer at pH 4.63, prepared according to *Reference 9*.

Solution B—Solution A containing 0.1% w/v of $NaNO_2$.

All the reagents and solvents were pure grade (obtained from C. Erba).

Procedures—*Determination of Rifamycin B in the Fermentation Broth*—Two 1-ml. portions of the broth in two 50-ml. volumetric flasks were made to volume, one with Solution A and the other with Solution B. The absorbance of the solution diluted with Solution A was determined at 425 mμ in a 1-cm. cell against the solution diluted with Solution B. The rifamycin B concentration in the fermentation broth was obtained by the formula:

$$\text{rifamycin B (mcg./ml.)} = \frac{A_{425} \cdot 50{,}000}{21.5} \qquad \text{(Eq. 1)}$$

where A_{425} is the absorbance at 425 mμ and 21.5 is the absorptivity of rifamycin B determined by this method. If the concentration of rifamycin B is higher than 1500 mcg./ml., a higher dilution of the broth is necessary. The precision and accuracy data are reported in Tables III and IV, respectively.

Determination of Rifampin in Capsules—The capsules were opened; about 30 mg. of the powder, accurately weighed, was placed in a 20-ml. volumetric flask and made to volume with methanol. Two 1-ml. portions of this solution were treated and examined at 473 mμ, as described for rifamycin B. The content of rifampin was obtained by the formula:

$$\text{rifampin \% (w/w)} = \frac{A_{473} \cdot 100}{14.7 \cdot p} \qquad \text{(Eq. 2)}$$

where A_{473} is the absorbance at 473 mμ, 14.7 is the absorptivity of rifampin determined by this method, and p is the weight of the powder expressed in grams.

Determination of Rifampin in Syrup—About 1.5 g. of syrup, exactly weighed, was placed in a 20-ml. volumetric flask and made to volume with methanol. Two 1-ml. portions of this solution were treated and examined at 473 mμ, as described for rifamycin B. The titer of rifampin was obtained by the formula:

$$\text{rifampin \% (w/w)} = \frac{A_{473} \cdot 100}{14.7 \cdot p} \qquad \text{(Eq. 3)}$$

where the symbols have the same meaning reported in the preceding method.

The precision and accuracy data of the rifampin determination are reported in Tables III and V, respectively.

[4] A Beckman DU spectrophotometer was used in the determinations.

REFERENCES

(1) P. Sensi, A. M. Greco, and R. Ballotta, "Antibiotics Annual 1959–1960," Medical Encyclopedia, Inc., New York, N. Y., 1960, p. 262.

(2) N. Maggi, G. G. Gallo, and P. Sensi, *Farmaco, Ed. Prat.*, **20,** 147(1965).

(3) P. Sensi, R. Ballotta, A. M. Greco, and G. G. Gallo, *Farmaco, Ed. Sci.*, **16,** 165(1961).

(4) N. Maggi, C. R. Pasqualucci, R. Ballotta, and P. Sensi, *Chemotherapia*, **11,** 285(1966).

(5) G. G. Gallo, P. Sensi, and P. Radaelli, *Farmaco, Ed. Prat.*, **15,** 283(1960).

(6) G. G. Gallo, C. R. Pasqualucci, and P. Radaelli, *ibid.*, **18,** 78(1963).

(7) C. R. Pasqualucci, A. Vigevani, P. Radaelli, and N. Maggi, *ibid.*, **24,** 46(1969).

(8) G. Sferruzza and R. Rangone, *ibid.*, **19,** 491(1964).

(9) H. T. S. Britton, "Hydrogen Ions," vol. I, Chapman and Hall Ltd., London, England, 1955, p. 357.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 8, 1969, from *Laboratori Ricerche Lepetit S.p.A., 20158 Milano, Italy.*

Accepted for publication January 6, 1970.

The authors are indebted to Dr. N. Maggi for helpful discussions.

* Present address: Archifar S.p.A., 20146 Milano, Italy.

Determination of Tablet Strength by the Diametral-Compression Test

J. T. FELL* and J. M. NEWTON†

Abstract □ The strength of lactose tablets has been measured by application of the diametral-compression test. The relative value of tensile, compressive, and shear stresses within the tablet varies, depending on the characteristics of the tablets and the surface providing the applied compression. It has been shown that to obtain reproducible results for the strength of tablets prepared at a given compression force, the tablet must break in such a manner that the tensile stress is the major stress. For a given tablet, this may require the placing of suitable padding material between the tablet and the compressing surfaces. Assessment of the type of failure can be made visually and under the correct conditions, the results expressed as a tensile strength. There are, however, a range of conditions which ensure tensile failure resulting in different values for the tensile strength. These values are characteristic of the tablet and test conditions and are not absolute values of tensile strength.

Keyphrases □ Tablet strength determination—diametral-compression test □ Tensile strength, tablets—measurements □ Compression effect—tablet fracture □ Fracture mode, tablets—testing

The authors have previously described the use of the diametral-compression test to assess the tensile strength of lactose tablets (1). This test is carried out by a procedure similar to that often used to assess the crushing strength of tablets, *i.e.*, diametral compression between two flat platens. The determination of a tensile strength, as opposed to a crushing strength, from this procedure depends upon the correct state of stress developing within a specimen of known shape and dimensions. Consider the stress distribution within a tablet, which is in the form of a cylinder, placed between the platens of a loading system. Under conditions of ideal line loading, the values of tensile σ_1, compressive σ_2, and shear stress τ can be calculated by elastic theory (2) and are illustrated in Fig. 1. The value for the maximum tensile stress σ_0 is constant over the whole of the load diameter and has a magnitude:

$$\sigma_0 = \frac{2P}{\pi D t} \qquad \text{(Eq. 1)}$$

where

P = applied load
D = tablet diameter
t = tablet thickness

The values for the compressive and shear stresses are a minimum at the center of the load diameter and infinitely high immediately under the load points (Fig. 1). These high values for shear and compressive stresses will prevent the initiation of failure in tension. In practice, ideal line loading will never occur, the load will be distributed over an actual contact area. The stress distribution across the load diameter, when the contact

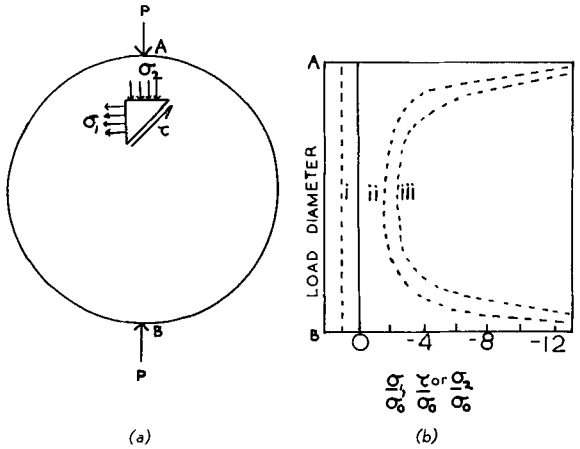


Figure 1—*Stress distribution across loaded diameter for a cylinder between two-line loads—ideal line loading (3). Key:* (a) *Loading system:* A + B = *points of loading;* σ_1 = *tensile stress;* σ_2 = *compressive stress;* τ = *shear stress.* (b) *Relative magnitude of tensile* σ_1, *compressive* σ_2, *and shear* τ *stresses compared with maximum tensile stress* σ_0. *Curve i* = σ_1/σ_0; *Curve ii* = τ/σ_0; *Curve iii* = σ_2/σ_0. P = *applied load.*

width is $^1/_{10}$ of the specimen diameter and uniform contact pressure is applied, calculated from elastic theory (2), is shown in Fig. 2. The tensile stress is constant over most of the load diameter, except for the regions near the loading area, but the shear and compressive stresses have been considerably reduced in this area. Thus, it is possible to have failure of the specimen initiated in tension, and the tensile strength can be calculated from Eq. 1. In practical terms, therefore, to obtain tensile failure of constant magnitude, the conditions of the test must ensure that a maximum length of the load diameter is under constant tensile stress, associated with minimum values for shear and compressive stresses, below the loading area.

The mechanical properties of the specimen and load platens determine the stress distribution within the specimen (3). In the case of pharmaceutical tablets compressed between metal platens, tablets are often soft compared with the platens. Hence, there will be a spreading of the load at the contact points due to flattening of the tablet, preventing line loading and reducing shear and compressive stresses. Under these conditions, failure will occur in tension and the results should be reproducible, providing that distribution of the load is not so great that the stresses within the central portion of the tablet are affected. When, however, tablets have a high elastic modulus, the conditions of ideal line loading are approached, and failure may be initiated by shear or

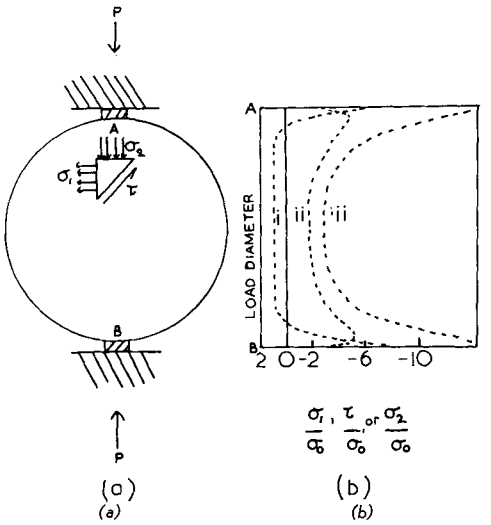


Figure 2—*Stress distribution across loaded diameter for a cylinder compressed between plates to give a constant width of $^1/_{10}$ of the diameter, with uniform contact pressure (3). Key:* (a) *Loading system:* A + B = *loading area;* σ_1 = *tensile stress;* σ_2 = *compressive stress;* τ = *shear stress.* (b) *Relative magnitude of tensile* σ_1, *compressive* σ_2, *and shear* τ *stresses compared with the maximum tensile stress* σ_0. *Curve i* = σ_1/σ_0; *Curve ii* = τ/σ_0; *Curve iii* = σ_2/σ_0. *P = applied load.*

compression. To obtain the correct conditions for tensile failure of such specimens, a narrow pad of a soft material is placed between platens and the specimen (3–6). The pad should be soft enough to allow distribution of the load over a reasonable area, minimizing shear and compressive stresses but not so soft that the distribution of load is excessive. Various suggestions have been made for the type and dimension of padding (4–6). Rudnick *et al.* (3) consider that the choice is best made from experimental observation, the type of failure being recognizable by examination of the specimen after testing. The three types of failure listed by these workers (3) are: compression and/or shear failure—here the specimen fractures in an irregular manner resulting in several irregular fragments; normal tensile failure—here the specimen splits into two halves along the loaded diameter; and triple cleft failure—the specimen splits symmetrically about the loaded diameter into four pieces. The tongue and groove shape of the outer surface and a clean central fracture are characteristics of the last method of failure. Rudnick *et al.* (3) consider this type to be a variation of the normal tensile fracture due to experimental conditions, and data from such systems can be used to calculate tensile strength. These three types of failure are illustrated by the photographs of tablets subjected to diametral compression (Fig. 3).

The applications of these principles to the determination of the strength of tablets prepared from lactose are reported in this paper.

EXPERIMENTAL

Materials—The samples of lactose used were: (*a*) a < 32-μ size fraction from a single batch of crystalline lactose (British Drug House laboratory reagent grade), (*b*) spray-dried lactose A, a < 32-μ size fraction from a single batch of spray-dried lactose (McKesson Robbins), and (*c*) spray-dried lactose, Samples B, C, and D, which were produced under known conditions using an experimental spray drier.

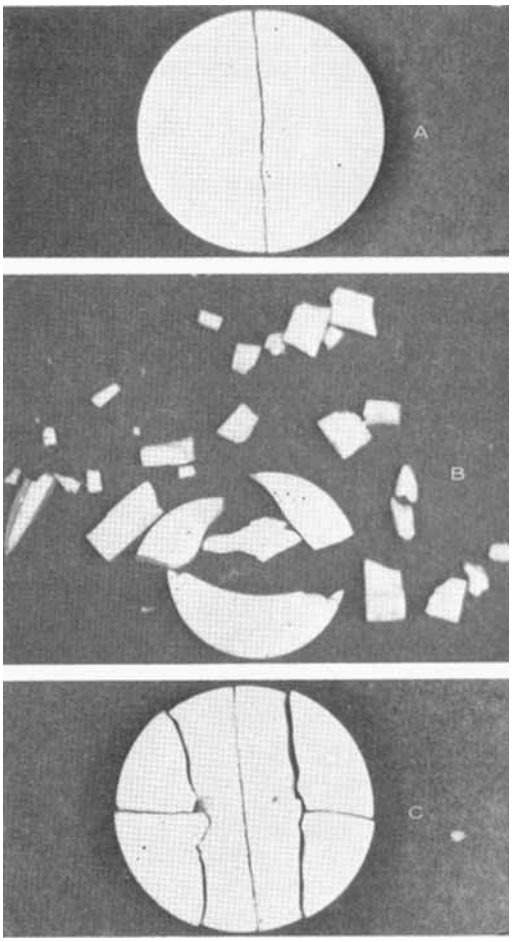


Figure 3—*Fractured tablets after diametral compression. (A) Normal tensile failure—obtained with crystalline lactose and spray-dried lactose A. (B) Shear and compressive failure—obtained with spray-dried lactose samples B, C, and D. (C) Tensile failure of spray-dried lactose samples B, C, and D using padding material.*

Methods—*Compression of Tablets*—Tablets of the different samples of lactose were prepared on an Instron physical testing instrument, floor model, modified to take a conventional tablet punch and die system [1.27-cm. (0.5-in.) diameter flat-faced punches] (Fig. 4). The powder samples (0.5 g.) were dried at 90° for 15 hr.

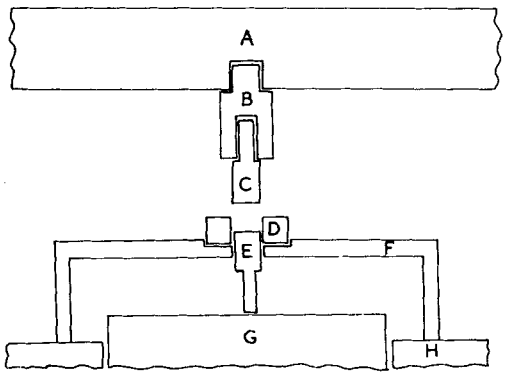


Figure 4—*Schematic illustration of modification to enable tablets to be prepared on Instron physical testing instrument. Key: A = crosshead of Instron physical testing instrument; B = upper punch holder; C = upper punch [1.27-cm. (0.5-in.) diameter flat face]; D = die; E = lower punch [1.27-cm. (0.5-in.) diameter flat face]; F = die support table; G = load cell; H = lower table of Instron physical testing instrument.*

Table I—Breaking Loads of Lactose Tablets Prepared at Different Applied Loads

Type of Lactose	Load Applied to Form Tablet, kg.	Without Padding: Mean Breaking Load of Tablet, kg.	Without Padding: Variance, V_1	Without Padding: No. of Tablets Breaking in Tension	With Padding: Mean Breaking Load of Tablet, kg.	With Padding: Variance, V_2	With Padding: No. of Tablets Breaking in Tension	Variance[a] Ratio
Crystalline	500	1.1	0.003	5	1.9	0.010	5	3.33
	1000	2.9	0.012	5	5.0	0.060	5	5.00
	2000	6.0	0.081	5	12.0	0.440	5	5.45
	3000	9.7	0.370	5	18.9	1.770	5	4.78
	4000	13.1	0.530	5	27.6	0.790	5	1.49
Spray-dried lactose A	500	2.0	0.005	5	2.7	0.011	5	2.20
	1000	4.3	0.030	5	6.7	0.050	5	1.66
	2000	8.5	0.040	5	14.8	0.970	5	24.25
	3000	13.1	0.510	5	22.3	1.900	5	3.73
	4000	17.3	0.400	5	33.1	0.640	5	1.60
Spray-dried lactose B	500	6.0	0.175	5	2.9	0.150	5	1.17
	1000	16.3	4.200	5	12.6	1.000	5	4.20
	2000	28.5	45.500	0	35.9	0.450	5	101.11
	3000	31.4	94.200	1	41.7	3.680	5	25.60
	4000	23.7	59.000	2	46.1	7.600	5	7.76
Spray-dried lactose C	500	5.3	0.150	5	6.5	0.125	5	1.20
	1000	17.0	5.700	5	22.6	1.000	5	5.70
	2000	28.8	20.050	1	41.7	1.150	5	17.43
	3000	33.7	47.100	0	53.3	4.170	5	11.29
	4000	31.1	81.600	0	62.8	3.440	5	23.72
Spray-dried lactose D	500	5.1	0.197	5	4.9	0.125	5	1.58
	1000	15.9	1.653	4	17.3	0.740	5	2.23
	2000	25.1	43.800	1	25.7	0.750	5	58.40
	3000	37.6	1.270	3	35.2	6.400	5	5.04
	4000	37.3	21.240	0	49.1	1.570	5	13.53

[a] The variance ratios are expressed as the ratio of the largest to smallest variance according to normal statistical convention ("Statistical Analysis in Chemistry and Chemical Industry," by C. A. Bennett and N. C. Franklin, John Wiley, New York, N. Y., 1954, p. 109). A significant difference, at 0.05% level, in the variance ratio is indicated by underlining the value.

and then stored in a desiccator over silica gel until required. The samples were quickly transferred to the die, previously painted with a 1% suspension of magnesium stearate in carbon tetrachloride, and compressed at a crosshead speed of 0.1 cm./min. When the required load level had been reached, the crosshead was reversed at the same speed. The tablets were carefully removed from the die, weighed, their diameter and thickness measured using a micrometer, and stored in a desiccator over silica gel until required. Tablets at a range of compression loads were produced.

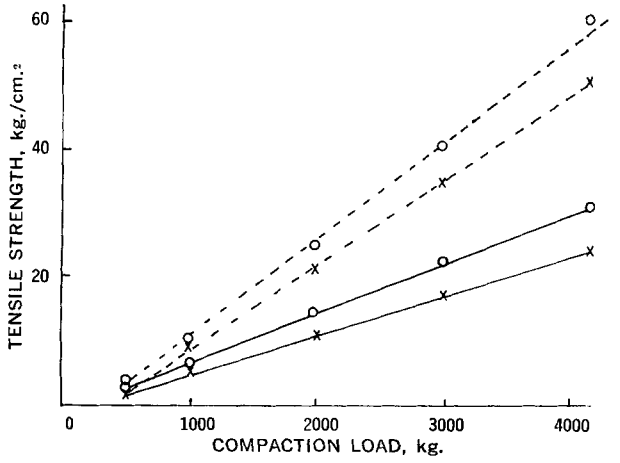


Figure 5—*The tensile strength of tablets of crystalline and spray-dried lactose A, prepared at known compression forces, when tested in the absence and presence of padding material. Key: ×, crystalline lactose; ○, spray-dried lactose A; - - -, in the presence of padding material; and —, in the absence of padding material.*

Strength of Tablets—The tensile strength of the tablets was measured by the application of the diametral-compression test described previously (1). This test consisted of compressing tablets diametrically between the platens of an Instron physical testing instrument at the rate of 0.1 cm./min. Tablets prepared from crystalline lactose and spray-dried lactose A fractured in tension between the steel platens of the Instron physical testing instrument (Fig. 3A). Tablets prepared at loads above 500 kg. from spray-dried lactose B, C, and D, however, fractured as described by Rudnick *et al.* (3) as compression or shear failure (Fig. 3B). To ensure that these tablets fractured in tension, various padding materials, *e.g.*, various thickness of paper, cardboard, and blotting paper, were placed between the tablet and the steel platens and the mode of fracture observed. Of the materials tested, it was found that three sheets of blotting paper, each 0.03 cm. thick, produced the conditions which resulted in tensile failure of the specimens. The breaking load of five tablets of each sample of lactose was determined with and without the addition of padding material.

RESULTS AND DISCUSSION

The assessment of the strength of tablets is an important aspect of the control of pharmaceutical tablets and investigations into the process of compaction. Various techniques have been used including fracture resistance (7), bending strength (8), tensile strength (9),

and crushing strength (10). Of these tests, the crushing strength is extensively used and most commercial instruments are designed to be used for this particular assessment. Brook and Marshall (11) have recently investigated the variability of such instruments, which record in different ways the load at which the tablet breaks when compressed by some means between two metal surfaces. The distribution of forces within the tablet and the related mode of fracture when tablets are tested by this procedure have not, however, been taken into account. In many cases, fracture can occur by tensile failure, resulting from correct stress conditions in the tablet. Here, variation in strength values will be due to tablet and testing instrument variation. In other cases, however, conditions of tensile failure will not apply, resulting in an additional variable of uncertain magnitude. The importance of considering the mode of fracture can be seen from the introductory remarks on the distribution of forces within the test specimen. When tablets do not break in tension in a diametral-compression test, variation in the breaking load can occur, caused by variations in the relative shear, compression, and tension forces involved. This effect is illustrated in Table I where the mean values of the breaking load of compacts when subjected to the diametral-compression test are recorded. Also given are the variance and variance ratio of the results when the tablets are tested with and without the presence of padding between the platens and the tablet. The important feature of the results is that when failure of the tablets occurs in tension, irrespective of the test conditions, there is always a low variance of the value of the load at which the tablet breaks (Table I). There is usually no significant difference, at the 5% level, between the variance of the breaking load with and without padding. When tablets do not break in tension (spray-dried lactose B, C, and D), there is a high variance of the breaking load and a significant difference in the variance of the breaking with and without padding (Table I). Only when the tablets break in tension is it possible to apply Eq. 1 and express the results for tablet strength as tensile strength.

Introduction of too soft a padding can, in fact, have the opposite effect to that just noted. Rudnick *et al.* (3) predicted from statistical theory that the amount of material subjected to a maximum tensile stress is greater when a hard rather than a soft contact surface is used. This would result in a lower strength and variance when a hard contact surface is used. This prediction was confirmed experimentally by Addinall and Hackett (12) in a detailed study of the effect of different packing pieces on the tensile strength of autoclaved plaster. The results in Fig. 5, which show the tensile strength calculated from Eq. 1, confirm these findings. The tensile strength of compacts prepared from crystalline lactose and spray-dried lactose A increased when padding was introduced. There is also a slightly greater variance of the breaking load when padding is present. In the presence or absence of padding, all tablets of these two materials fractured in tension. This raises the question as to which of the values represents the tensile strength of the tablet. Rudnick *et al.* (3) consider that there is no such value as a true tensile strength, but values obtained under any conditions are true values for those conditions. If it is required to compare the tensile strength of tablets, the conditions of the test must be the same, and tensile failure must be assured in all cases. Thus, to compare tablets prepared from spray-dried lactose B, C, and D with crystalline and spray-dried lactose A, the results in which a padding was used must be considered. For comparisons between tablets of crystalline and spray-dried lactose A, the values without padding are preferable.

A further consideration in the choice of test conditions is the actual strength of the tablets. Thus, tablets produced at low-compression forces, producing weaker tablets, often failed in tension without the need for padding. Therefore, if the tablets have a low tensile strength, it is unlikely that padding will be required.

CONCLUSIONS

The authors consider that the results indicate that it should be possible to choose test conditions, ensuring their validity by simple observation of the mode of fracture, which will improve the reproducibility of the assessment of the strength of tablets and enable meaningful comparisons to be made between the strength of tablets of different materials. For routine evaluation the results need not be expressed in terms of tensile strength, the breaking load under conditions of tensile failure being satisfactory.

REFERENCES

(1) J. T. Fell and J. M. Newton, *J. Pharm. Pharmacol.*, **20,** 657(1968).

(2) R. Peltier, RILEM Bulletin No. 19, 1954.

(3) W. C. Rudnick, A. R. Hunter, and F. C. Holden, *Mater. Res. Stand.*, **1,** 283(1963).

(4) N. B. Mitchell, *ibid.*, **1,** 780(1961).

(5) P. J. F. Wright, *Mag. Concr. Res.*, **7,** 87(1955).

(6) L. L. Simon, *Constructional Rev.*, **29,** 23(1956).

(7) C. J. Endicott, W. Lowenthal, and H. M. Gross, *J. Pharm. Sci.*, **50,** 343(1961).

(8) K. Munzel and W. Kagi, *Pharm. Acta Helv.*, **32,** 305(1957).

(9) E. Nelson, *Drug Std.*, **24,** 1(1956).

(10) E. Shotton and D. Ganderton, *J. Pharm. Pharmacol.*, **12,** 87T(1960).

(11) D. P. Brook and K. Marshall, *J. Pharm. Sci.*, **57,** 481(1968).

(12) E. Addinall and P. Hackett, *Civil Eng. Pub. Works Rev.*, **59,** 699(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 31, 1969, from the * *Pharmacy Department, The University, Manchester 13, England,* and † *The Lilly Research Centre Ltd., Erl Wood Manor, Windlesham, Surrey, England.*

Accepted for publication November 19, 1969.

The authors wish to express their thanks to Dr. D. C. Smith and Dr. E. C. Combe, Turner Dental School, Manchester, for the use of the Instron physical testing instrument, and to the Science Research Council for the award of a research grant to J.T.F., and the award of a grant to Dr. D. C. Smith for the purchase of the Instron physical testing instrument.

Dissolution Rate Studies I: Design and Evaluation of a Continuous Flow Apparatus

JAMES E. TINGSTAD and SIDNEY RIEGELMAN

Abstract □ An apparatus for the study of dissolution rate under conditions of continuous flow is described. Typical data are presented; the effect of sample size, surfactant, and flow rate on the dissolution behavior of prednisone powder and prednisone tablets is shown. The requirements of an ideal standard method for studying dissolution rates are discussed, and the major advantages of this method over present methods are pointed out. The method: (*a*) is more flexible, (*b*) produces data in a differential form, (*c*) utilizes a small-volume system which assures greater homogeneity, (*d*) prevents excessive accumulation of solute in the system, and (*e*) provides agitation and solvent flow in a controlled, measurable, and physically meaningful manner.

Keyphrases □ Dissolution apparatus—design, evaluation □ Diagram—dissolution apparatus □ Prednisone—dissolution rate profile □ Surfactant concentration effect—prednisone dissolution □ Sample size effect—prednisone dissolution □ Flow rate effect—prednisone dissolution

There is little doubt that the determination of dissolution rates is an important tool in the design, evaluation, and control of solid dosage forms. Parrott *et al.* (1) has stated: "The release of a drug from the primary particle and its subsequent availability to the body is governed by the dissolution rate of the particle." Nelson (2–4) also pointed to the significance of dissolution rate in determining drug availability in his series of publications on the dissolution rates of weak acids and bases and their salts.

For obvious reasons, it would be ideal if one relatively simple and inexpensive apparatus and method could be used to determine the dissolution rates of most drugs and drug products. However, standing in the way of the one-method concept is the fact that a great variety of factors influence the results obtained from dissolution rate tests. These include factors intrinsic to the products, such as physical-chemical properties of the drug and the variations in the composition of the formulation, and factors extrinsic to the products, such as the type and volume of solvent, the degree, type, and uniformity of agitation, the geometry of the container, the state of homogeneity existing in the system, and adsorption or adherence of the drug to solid surfaces of the apparatus. In light of these factors, a single method is feasible only if it has adequate flexibility to allow sufficient controlled variation of experimental conditions to ensure, regardless of what drug is studied, that the results will reflect primarily the intrinsic variables rather than the extrinsic.

Flexibility is a necessary requirement for a standard dissolution rate method for another important reason. There is presently an acknowledged scarcity of data showing correlation between *in vitro* dissolution rates and *in vivo* absorption, but the great current interest and activity in this area indicate that more and more data will be forthcoming. As the knowledge in this area accumulates, problems and deficiencies in the *in vitro* methods will come to light, necessitating changes in equipment and procedures. The more flexible the standard method is, the more easily such changes can be made to accommodate the new findings.

In addition to flexibility, there are two other basic requirements that a standard dissolution rate method should meet. It should yield accurate, meaningful, and reproducible data that can be quantitatively related to theoretical dissolution rate equations, and it should be sensitive enough to detect small differences among various drugs and among different formulations of the same drug. Unfortunately, present dissolution rate methods lack the necessary flexibility, their basic design introduces far too many uncontrolled variables into the dissolution process, their method of agitation cannot be quantitatively related to dissolution rate equations except by including it in the constant, and they produce data in such a way that only gross differences in formulations can be detected.

The serious deficiencies in these methods can best be shown by first looking at the Noyes-Whitney equation (5) which, in theory, describes the dissolution process: $da/dt = KS(C_s - C)$, where da/dt is the dissolution rate expressed as amount dissolved per unit time, S is the surface area of undissolved solute presented to the solvent, C_s is the concentration of solute in a saturated solution, C is the solute concentration in the bulk of the medium at any time, and K is a constant dependent on a variety of factors including the temperature, viscosity, pH characteristics, degree of agitation of the medium, and the diffusion coefficient of the solute molecules. While numerous attempts have been made to expand this equation to include various additional factors (6–9), none of these has led to an expression which adequately takes into account all the variables involved.

The dissolution process is particularly complicated when considering solid dosage forms. In the case of pure powdered drug, assuming immediate wetting, the surface area will continually decline as more and more particles dissolve. With capsules and tablets, however, the situation becomes more involved because, as they disintegrate or disperse in the medium, the amount of exposed surface area initially increases, going through a maximum and then decreasing in the conventional pattern.[1] How quickly a tablet disintegrates and the granules and drug particles disperse depends, among other factors, upon the composition of the formulation, the amount of pressure used in compression, the

[1] Wagner (10) has suggested an interesting statistical approach of the empirical linearization of the dissolution data using product or log product paper, which apparently takes into account the variation of dissolution surface area.

amount of entrapped air (which affects both the wetting and the bulk density of the particles), the volume of the liquid, the geometry of the container, and the degree and type of agitation.

Since these systems are so variable and complex, a dissolution rate method which seeks to meet the requirements listed for a universal method must help solve, not add to, the inherent problems involved in studying the dissolution process. Unfortunately, present methods are almost exclusively based on a static system involving solvent present in "bulk" form; the system is stirred by a motor-driven propeller or wire mesh basket or by a rocking device, and discrete samples are withdrawn and analyzed at various times (11). The basic disadvantages of these methods, inherent in the basic design, are: (*a*) a lack of flexibility, (*b*) a lack of homogeneity (caused by both the large volumes and agitation methods employed), (*c*) a variable concentration gradient, (*d*) a method of agitation that is semiquantitative at best and relates poorly to theoretical dissolution rate equations, and (*e*) the data produced tend to obscure the details of the dissolution process.

The inherent lack of homogeneity in these methods results from both the agitation methods and large volumes employed. Agitating the liquid by stirring with a wire mesh basket[2] or a propeller, or by a rocking motion, causes a variable shear rate of transfer over the surface of the particles, which results in excessive variations in their individual rates of dissolution. The movement of solvent over any particle will depend on the position of the particle in the vessel and the character of the stirring process at each position within the container.

The latter varies markedly with the geometry of the vessel, the volume of the liquid, and the speed and form of motion created by the agitator. While it is possible to standardize many of the geometric and mechanical factors, nevertheless the agitation varies at different positions in the container. In addition, various sized granules and particles disperse differently throughout the system. While a tablet is disintegrating, the granules tend to collect at the bottom of the container, the remainder of the tablet is in the basket in the center of the system, some of the granules are stuck in the screen, and the fine particles are dispersed throughout the medium. As a result, the apparatus introduces an inherent variability into the dissolution process, a variability that is extrinsic to the product under study, and one which cannot be eliminated merely by standardizing the procedure.

The lack of homogeneity caused by the agitation methods could be minimized by keeping the volume of the system small (*e.g.*, 50 ml. or less), but this is not feasible with present methods. As mentioned earlier, the basic equations describing the dissolution process include a term for concentration gradient ($C_s - C$), and since C_s remains constant, it is important that C (the solute concentration in the bulk of the system) be kept as low as possible. With the present static methods, and considering the low solubility of many drugs, using small volumes would result in a significant increase in C as the experiment progressed.

There is another reason why it is important to keep C as low as possible. The ultimate objective in dissolution rate studies is to discern dosage form effects that may later influence the absorption of the drug within the gastrointestinal tract. In the *in vivo* situation, the dissolution process most likely takes place from particles adhering to or very near the mucosal surface. Therefore, the diffusional pathway to the absorption site is very short, and the drug molecules are almost instantly absorbed into what is, for all practical purposes, a perfect sink—the body fluids. Consequently, the chances of achieving good *in vitro–in vivo* correlations in this area are better if the *in vitro* system more closely approaches these perfect sink conditions.

The need for such a perfect sink (*i.e.*, a relatively large volume of solvent) necessitates using a relatively high rate of agitation. But studies of Levy *et al.* (13) have shown that quite often the rate of agitation must be kept low in order to establish meaningful *in vitro–in vivo* correlations. In addition, it is often necessary to use low agitation in order to detect subtle differences between formulations. But low agitation of a relatively high-volume system results in poor homogeneity, and the sample withdrawn for analysis might not be representative of the whole system. Thus, an inherent disharmony exists in these systems between the requirements for homogeneity, large volumes, and low stirring rates.

A further disadvantage of present methods has to do with the empirical nature of stirring or rocking as a device for ensuring homogeneity and moving solvent/solute molecules. Since these agitation methods are empirical, they cannot be related to fundamental dissolution rate equations except by including them in a catchall constant, which is a very superficial solution to the problem. This makes it critical that the various test systems be standardized as much as possible—one stirring rate, one type of container, one volume of solvent, *etc.* But this greatly reduces investigative flexibility, which is a prime requisite for a good standard method, as pointed out previously. Furthermore, the results obtained with such rigid standard procedures will be less and less quantitative and meaningful as drugs of lower and lower solubility are studied, because at fixed volumes the concentration gradient will vary more significantly. And yet it is precisely these drugs that are often most important to study from a drug availability standpoint.

There is one other disadvantage of present methods which should be mentioned. Because they are based on a concept of a static fixed volume, they produce data expressed as an integral function. That is, since the dissolved molecules are accumulating in the solution, the resultant data represent an integral function of the dissolution process rather than a differential function. Thus, these methods produce average dissolution rates at best, and this makes it difficult to detect subtle but possibly important differences in formulations. In other words, two formulations may differ significantly in their dissolution rate behavior; but with present methods these differences would be, in effect, hidden under the

[2] This refers to the proposed USP–NF Dissolution Test, Method I (12).

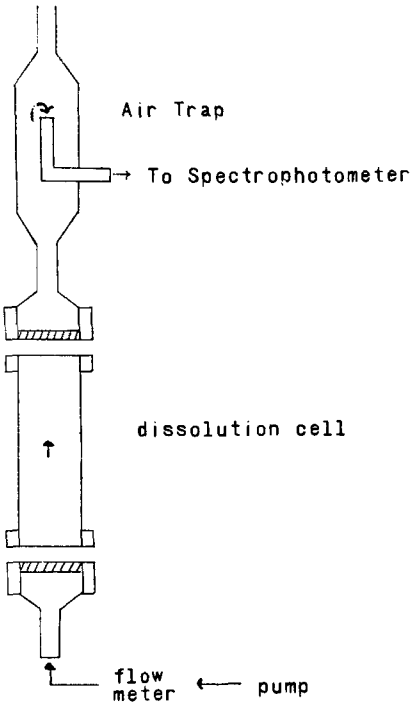


Figure 1—*Sketch of the dissolution rate apparatus. Arrows indicate direction of solvent flow.*

integral curve representing the accumulated drug dissolved up to that time. This will become more apparent in viewing the following experimental results.

In light of the serious deficiences in present methods, it is apparent that a new "standard" dissolution rate method is needed, one that: (*a*) has a much higher degree of flexibility, (*b*) yields data in a differential form which can then be converted to the integral form if desired, (*c*) utilizes a relatively homogeneous low-volume system, (*d*) prevents excessive accumulations of solute in the system, and (*e*) provides solvent flow in a controlled, precise, measurable manner which can be mathematically related to fundamental dissolution rate equations. This communication describes just such an apparatus and method, the basic idea for which was first suggested by Olson (14) and subsequently investigated by Hamlin and Rowe (15) and Langenbucher (16). The apparatus described herein is very similar to that used by these previous workers, with certain modifications.

EXPERIMENTAL

Description of Apparatus—The apparatus is described in Fig. 1. The dissolution cell is a glass cylinder 6.1 cm. long, 1.9 cm. in diameter, and has a volume of 17 ml. This cell was constructed from two small-volume glass filter funnels (Millipore catalog number XXLO-025.00), cut down in height and attached back to back. This allows use of filter membranes of sufficient retentive characteristics to limit the dissolution process to the dissolution cell and prevent solid particles from reaching the spectrophotometer. The dissolution chamber can be dismantled, cleaned, and dried for subsequent determinations with minimal effort. The pump is a centrifugal constant-capacity pump (Cole-Parmer) of low cost. The solvent flow to the system is controlled by external valves, with the excess capacity of the pump being recirculated to the reservoir from which the pump draws the solvent. The flowmeter is a Gilmont size 3 and the filters (shaded area) are standard coarse-porosity sintered glass. The spectrophotometer (Beckman model DB) has an attached recorder. The air trap prevents air bubbles from distorting the spectrophotometric reading.

General Procedure—The dissolution cell and the filters are thoroughly cleaned of residue from the previous run and the apparatus is assembled. The pump is then turned on and the entire system flushed with solvent until the spectrophotometric reading recedes to zero (a few minutes). Then the dissolution cell and the filters are disassembled and dried, and the sample (in these experiments, a tablet or weighed quantity of powder) is placed on the lower filter. The dissolution cell is then clamped into place and the upper filterpiece/air trap attached with a similar clamp. The apparatus is then submerged in a constant-temperature bath, including the flowmeter, lower filter, dissolution cell, and the lower part of the upper filterpiece/air trap. The solvent reservoir and as much connecting tubing as possible are also in the bath. As soon as the cell has reached the bath temperature, the pump is turned on and the flow regulated to the desired flow rate, which is then held constant. At lower flow rates, the flow remained constant; at higher flow rates, occasional adjustments in the valve system were necessary to maintain a constant flow rate. The air outlet, open when the pump is turned on, is closed as soon as the liquid level in the air trap is above the outlet tube to the spectrophotometer. The absorbance is continuously recorded on the strip chart, and the "waste" solution is either discarded or saved in an appropriate receptacle.

For these experiments, prednisone USP[3] (screened 80 mesh) and prednisone tablets USP, 5 mg.,[4] were studied using as solvents either distilled water or various aqueous solutions of sodium lauryl sulfate USP. The bath temperature was 25.0 ± 0.1°. The absorbance was read at 239 mμ using a 1-cm. cell and a molar absorptivity of 15,500. In the concentration ranges studied, the Beer-Lambert law was found to be valid. All determinations were in duplicate. The following experiments were performed primarily to test the usefulness of this apparatus and method. More complete and detailed studies, where the emphasis is on investigating the dissolution process rather than evaluating the method, are in progress.

Effect of Surfactant Concentration—The dissolution behavior of a 5-mg. sample of prednisone powder was studied using various concentrations of sodium lauryl sulfate in distilled water as the solvent. The flow rate was 20.2 ml./min.

Effect of Sample Size—The dissolution behavior of various sized samples of prednisone powder (5, 10, 15, and 20 mg.) was studied using 0.02% w/v sodium lauryl sulfate as the solvent. The flow rate was 20.2 ml./min.

Effect of Flow Rate—The dissolution behavior of 5-mg. prednisone tablets was studied using distilled water as the solvent and varying the flow rate between 10 and 54 ml./min.

RESULTS AND DISCUSSION

Typical dissolution curves for prednisone powder (upper plot) and prednisone tablets (lower plot) as recorded on the strip chart are reproduced in Fig. 2. The tracings are presented for illustrative purposes. No quantitative comparison of the two should be made, since different experimental conditions were used (tablet: 5 mg. prednisone and water as the solvent; powder: 10 mg. prednisone and 0.02% sodium lauryl sulfate as the solvent; in both cases, the flow rate was 20.2 ml./min.). The curves record absorbance, but this is easily converted to dissolution rate (mg./min.) by converting absorbance to concentration (mg./ml.) and multiplying by flow rate (ml./min.).

The roughness of the tablet curve, contrasted with the smooth curve for the powder, is not surprising, since the granules in the tablet do not release the drug at an even rate. Figure 2 shows that a very meaningful and revealing quantitative "dissolution rate profile" (a continuous tracing of the differential function) can be directly obtained with this method. Such a profile has obvious advantages in the development, evaluation, and control of solid dosage forms. The profile gives the formulator and the control analyst a good closeup view of how the tablet or capsule is performing in the dissolution system.

[3] Supplied by The Upjohn Co.
[4] Supplied by the Pharmaceutical Technology Laboratory, School of Pharmacy, University of California, San Francisco Medical Center.

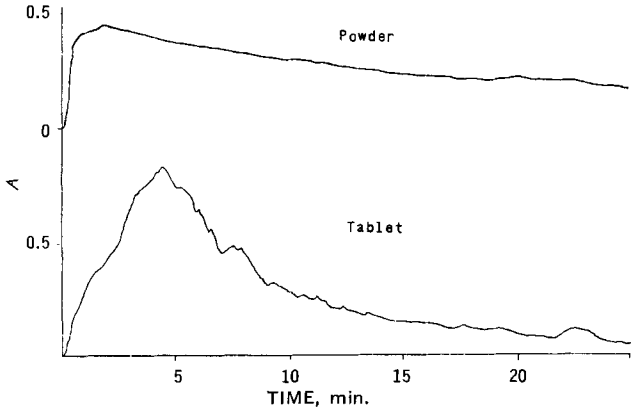


Figure 2—*Typical dissolution rate curves for prednisone powder (upper plot) and prednisone tablets (lower plot) as recorded on the spectrophotometer strip chart.*

The tablet curve in Fig. 2 shows that a definite maximum dissolution rate is reached. In these experiments the peak dissolution rate correlates reasonably well with the total amount of drug dissolved at the end of 5, 10, and 15 min. The total amount of drug dissolved (the integral of da/dt) was obtained by cutting out and weighing the area under each curve. Figure 3 shows the correlation between peak dissolution rate and amount dissolved in the first 15 min. in the tablet experiments. Since (as expected) the values for the integral were more reproducible than the peak dissolution rate, the area under the curve for various time periods was used in subsequent plots.

Effect of Surfactant Concentration—When first working with prednisone powder using water as the solvent, it was observed that the powder agglomerated into one mass in which a considerable amount of air was entrapped. The problem was greatly reduced by including a surfactant in the solvent, but concentrations had to be kept low because of excessive foaming. Wetting was not complete at the highest (0.05%) concentration employed. The influence of surfactant (Fig. 4) emphasizes that the dissolution processes (as commonly considered) is really a combination of two distinct processes: wetting of the solid and subsequent dissolution. As long as the powder is not completely wet, the effective surface area can be considerably less than the total surface area, because a significant amount of the solid is involved in solid–air and solid–solid interfaces rather than in a solid–liquid interface. In such cases, attempts should be made to wet the sample first before trying to perform highly definitive quantitative dissolution rate studies. The desirability of considering the wetting process in dissolution rate studies has been emphasized by Finholt (17).

Since the amount of sodium lauryl sulfate used in the system is well below the critical micelle concentration (~0.2%), it is likely that the surfactant used in these experiments had negligible effect on the saturation solubility of the prednisone. While surfactant solutions were used in studying the dissolution behavior of prednisone powder, water proved to be satisfactory for the tablets.

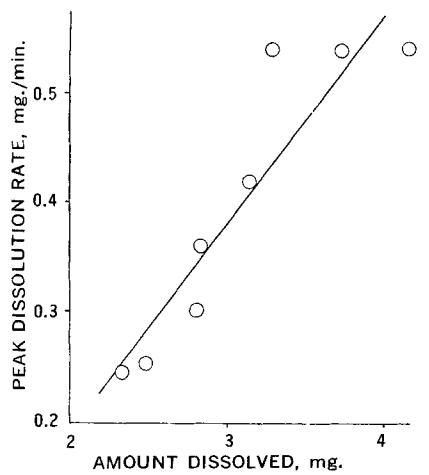


Figure 3—*Plot illustrating the correlation between peak dissolution rate and amount dissolved in the first 15 min. for prednisone tablets.*

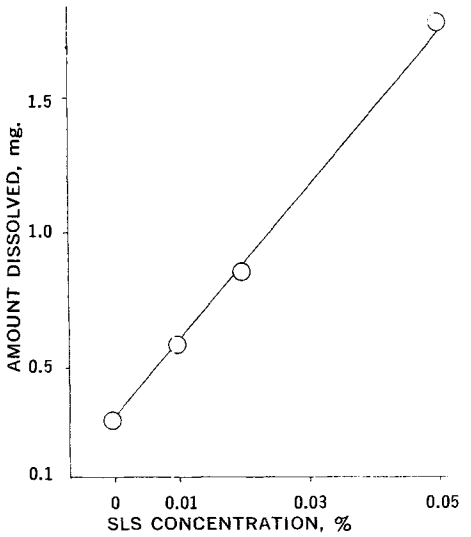


Figure 4—*Plot showing the effect of the sodium lauryl sulfate (SLS) concentration on the dissolution behavior of prednisone powder. The amount dissolved represents the area under the curve for the first 5 min.*

Effect of Sample Size—As mentioned earlier, it is important to keep the concentration of dissolved drug well below saturation. This will depend upon the dissolution rate, the solubility of the drug, and the flow rate. To get some indication of the degree of saturation in these systems, the sample size was varied in the prednisone powder studies and its effect on dissolution rate observed. If the system was close to saturation, increasing the sample size would have only a small effect on the dissolution rate because the concentration gradient ($C_s - C$) would rapidly disappear.[5] Figure 5 shows this effect on the total amount dissolved in 5 min., using sample weight to the two-thirds power as the abscissa. While this coordinate is not entirely accurate (it assumes that the solid is composed of uniform spheres), it does suffice to show that added surface area does significantly increase the dissolution rate, a clear indica-

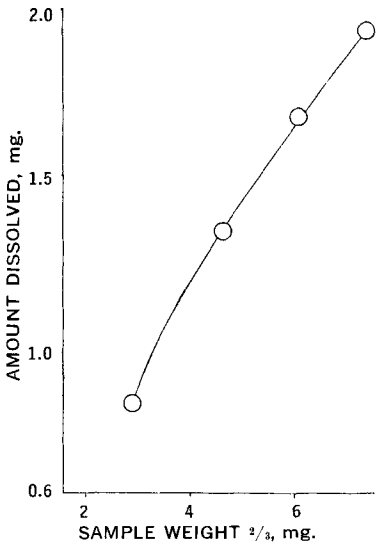


Figure 5—*Plot illustrating the effect of sample size on the dissolution behavior of prednisone powder. The amount dissolved represents the area under the curve for the first 5 min.*

[5] With this flow method, ($C_s - C$) would remain essentially constant using a constant surface pellet. With tablets, powders, *etc.*, the concentration gradient does not remain constant, but the continuous flow of fresh solvent into the system will help keep the concentration gradient at a high level.

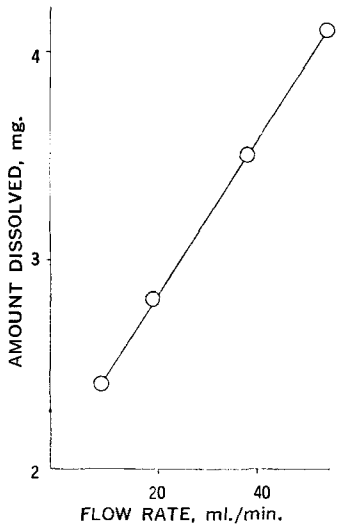


Figure 6—*Plot showing the effect of flow rate on the dissolution behavior of prednisone tablets. The amount dissolved represents the area under the curve for the first 15 min.*

tion that the system is not close to saturation. This was confirmed by determining, in a separate experiment, that these systems were about one-tenth saturated. It should be pointed out that the flow rate in these studies was 20.2 ml./min. It is desirable to repeat this study at lower flow rates for various systems, since the lower the flow rate, the more critical the problem with saturation. However, these experiments have been deferred until more modifications have been made in the equipment.

This emphasizes the flexibility inherent in this method as compared to present methods. If two drugs had widely different solubilities, present methods would dictate changing the volume of the system (*i.e.*, the "dissolution chamber") and the stirring rate (to retain some measure of homogeneity), which would very likely result in a major change in many undefinable variables. With the authors' flow method, drugs of different solubilities can easily be accommodated simply by changing the flow rate (a clearly defined and easily measured parameter) and the amount of solvent used; the size of the dissolution cell would remain the same. While it is true that such changes would produce some undefinable changes in the flow system, it is also true that such changes are very likely to be much less than with present systems.

Effect of Flow Rate—As mentioned, one of the major advantages of this method is that solvent flow around the solid can be carefully and quantitatively controlled. Solvent flow affects the dissolution process by: (*a*) physical abrasion of the solid, (*b*) affecting the solution concentration in the effluent, and (*c*) probably reducing the diffusion layer thickness around each particle. Thus, flow rate, a physically relatable parameter, affects dissolution rate in a quantitative, definable way. Precisely what that effect is must await further studies, but Fig. 6 illustrates that flow rate does, in fact, have the expected significant effect on dissolution rate.

While the development of this apparatus is in its initial stages, it is apparent that the basic design offers many advantages over present methods, many of which have already been discussed. The vertical alignment of the dissolution cell helps ensure that the liquid flow through the cell will be reasonably homogeneous, even when flow rates are very low or comparatively high. Of course, some inherent variation still exists in the flow method because: (*a*) at times it may be necessary to increase flow rate to the point where the flow becomes turbulent, and (*b*) the flow of solvent will not be exactly the same past each and every particle. However, it is obvious that such problems of deviations from ideal will be much less with a smaller, more homogeneous system such as the authors' flow system. The small (17 ml.) volume of the system also ensures homogeneity. If a change of solvent pH is desired in the same experiment (simulating the *in vivo* change from stomach to duodenum), this can quickly and easily be done by switching from one reservoir to another. In contrast, this would be a major problem with present methods.

The number of definitive studies on the apparatus has been minimal, but even with the limited data gathered to date, several postulates arise:

1. The ascending curve for a tablet represents the disintegration and dispersal processes, although they still continue after the peak dissolution rate is reached.
2. At a fixed flow rate, the maximum dissolution rate and/or the time needed to achieve maximum dissolution rate may be useful parameters for comparison of tablet additives relative to the pure (wetted) powder.
3. The descending curve appears to be exponential in shape and, therefore, it may be possible to express the slope in terms of a rate constant or a half-life.

It should be emphasized that the described apparatus is a prototype, and numerous improvements are both possible and probable as more experience is gained. For example, as Langenbucher (16) points out, the cross-sectional area of solvent flow is a variable that must be taken into account. It is also probable that the volume of the dissolution cell should be reduced to a minimum to optimize homogeneity of the system. Fortunately, with this method, reducing system volume is no problem; cell size is limited only by the physical size of the sample and the need to keep the filters of sufficient cross-sectional area to avoid excessive clogging. In addition, the size and shape of the air trap should probably be altered toward a minimum size and optimum shape, because any mixing and pooling of solution beyond the dissolution cell tend to distort the spectrophotometric tracing. An inexpensive integrater can easily be attached to the spectrophotometer to obtain the integral curve as well as the differential tracing.[6] It would be better if the solvent flow was electronically controlled to keep flow rate constant automatically. Finally, the system should lend itself easily to automation.

But despite the preliminary nature of this work, there is little doubt that, based on theoretical considerations, and the results of these experiments, this method is far superior to present methods both for fundamental and practical studies. It is by far the best candidate for a "standard" method among the presently available procedures. Not only is it superior to present methods, but it possibly has the inherent flexibility that may allow it to meet, with appropriate modifications, most or all of the requirements listed for the ideal dissolution rate method.

REFERENCES

(1) E. L. Parrott, D. E. Wurster, and T. Higuchi, *J. Amer. Pharm Ass., Sci. Ed.*, **44**, 269(1955).
(2) E. Nelson, *ibid.*, **46**, 607(1957).
(3) *Ibid.*, **47**, 297(1958).
(4) *Ibid.*, **47**, 300(1958).
(5) A. Noyes and W. Whitney, *Z. Phys. Chem.*, **23**, 689(1897).
(6) L. Bruner and S. Tolloczko, *ibid.*, **35**, 283(1900).
(7) W. Nernst, *ibid.*, **47**, 52(1904).
(8) E. Brunner, *ibid.*, **47**, 56(1904).
(9) P. J. Niebergall and J. E. Goyan, *J. Pharm. Sci.*, **52**, 29 (1963).
(10) J. Wagner, private communication, 1969.
(11) J. A. Hersey, *Mfg. Chem. Aerosol News*, **40**, 32(1969).
(12) E. G. Feldmann, "Memorandum to USP–NF Joint Panel on Physiological Availability," Dec. 23, 1968.
(13) G. Levy, J. R. Leonards, and J. A. Procknal, *J. Pharm. Sci.*, **56**, 1365(1967).
(14) C. Olson, personal communication, The Upjohn Co. (1965).
(15) W. E. Hamlin and E. L. Rowe, personal communication, The Upjohn Co. (1965).
(16) F. Langenbucher, to be published.
(17) P. Finholt and S. Solvang, *J. Pharm. Sci.*, **57**, 1322(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *University of California School of Pharmacy, San Francisco Medical Center, San Francisco, CA 94122*
Accepted for publication October 31, 1969.

[6] With present dissolution rate methods, the integral curves obtained can be differentiated by appropriate means, but these would be only average figures unless measurements were made on a continuous basis—and this requires a flow system. But for continuous measurement, the homogeneity of the system becomes even more critical for assay purposes, and it is likely that stirring rates would have to be increased.

Automated Fluorometric Procedure for Unit Dose Analysis of Digitoxin and Digoxin in Tablet Formulations

L. F. CULLEN, D. L. PACKMAN, and G. J. PAPARIELLO

Abstract □ A sensitive and specific automated procedure is described for the unit dose analysis of digitoxin and digoxin in tablet formulations. The technique is based on the fluorometric measurement of the dehydration products of the cardiotonic steroids, resulting from their reaction with hydrogen peroxide in concentrated hydrochloric acid. The automated system is capable of analyzing 12 tablets per hour with a relative standard deviation of ±1.4% at the 0.05-mg. digitoxin level. Accuracy of the procedure was determined by collecting comparative data on digitoxin tablets by both the automated method and the manual USP procedure. Specificity of the method, with respect to the analysis of intact digitoxin in the presence of the products of photochemical, alkaline hydrolytic, and thermal degradation, was demonstrated by comparison to quantitative TLC values. This method has also been shown to be applicable to the unit dose analysis of digoxin in the 0.02 to 1.0 mg./tablet range.

Keyphrases □ Digitoxin—automated fluorometric method, tablets □ Digoxin—automated fluorometric method, tablets □ Automated methods—digitoxin, digoxin, unit dose analysis in tablets, diagram □ Automatic analyzer—diagram, digitoxin, digoxin determination □ TLC—analysis

Determining the precise amount of digitoxin in tablets is of the utmost interest to the pharmaceutical industry. These tablets are of low dosage, *i.e.*, 0.05–0.20 mg. per tablet, and, consequently, require unit dose analysis as a control on the homogeneity of the product. An attempt was made to automate these unit dose assays to gain the advantages of reproducibility, rapidity, and sensitivity offered by an automatic analyzer system.

Khoury (1) described an automated sulfuric acid-induced fluorescence method for the single-tablet analysis of cardiac glycosides. However, it was found that this procedure was not applicable to the analysis of digitoxin tablets which contain sugars and dyes. Since these interfering inactive components are common formulating materials, the present investigation was undertaken to develop a technique which would overcome these shortcomings.

The majority of the analytical methods reported for the quantitative determination of digitoxin are either colorimetric (2–9) or fluorometric (10–12). The colorimetric procedures did not exhibit the necessary sensitivity or specificity for single-tablet analysis at the normal digitoxin dosage levels. Thus, fluorometric procedures were investigated.

Wells *et al.* (12) presented a sensitive and specific fluorometric method for the analysis of digitoxin and digoxin in biological fluids and tissues. The technique is based on the fluorometric measurement of the dehydration products of the cardiotonic steroids resulting from the reaction of the glycosides with hydrogen peroxide in concentrated hydrochloric acid. Detailed discussions of the influence of time, temperature, and reagent concentrations on the excitation and emission properties of the glycosides and the mechanism of fluorescence formation were described by Wells *et al.* (12) and also Jelliffe (13). This procedure has been adapted and modified for continuous analysis by use of an automatic analyzer system.[1] Applicability of this method to the unit dose analysis of digoxin in the 0.02–1.0 mg. per tablet range requires only a simple change in the fluorometer filter system.

EXPERIMENTAL

Apparatus—A standard Technicon automatic analyzer system consisting of the following modules: (*a*) solid-prep sampler, programmed at 12 samples/hr.; (*b*) proportioning pumps[2] (two required); (*c*) continuous filter, speed 2; (*d*) fluorometer,[3] equipped with fluorescent lamp (Turner 110-853), quartz flowcell,[4] and filters: digitoxin analysis, primary—Corning[5] No. 7-59 and secondary—Wratten[6] No. 8; digoxin analysis, primary—Corning No. 7-60 and secondary—Wratten No. 3; and (*e*) linear recorder.

Reagents and Solutions—(*a*) Seventy percent SDA No. 30 alcohol (ethanol–methanol, 10:1) in water (v/v); (*b*) concentrated hydrochloric acid; (*c*) 0.1% ascorbic acid in methanol; (*d*) 0.065 *M* hydrogen peroxide in methanol (prepared by adding 1.0 ml. of reagent grade 30% hydrogen peroxide to 200 ml. of absolute methanol)—this reagent should be prepared fresh daily and maintained in an ice bath on the automated manifold; and (*e*) standard digitoxin and digoxin solutions—prepare a 0.5 mg./ml. solution of analytical reference standard[7] digitoxin or digoxin in 70% denatured alcohol were used. The standard calibration curve is derived from a series of diluted solutions ranging in concentration from 0.05–0.25 mg./ml.

Automated Methodology—A diagram of the flow system indicating the automated equipment arrangement for the analytical procedure is shown in Fig. 1. In performing the analyses, three standards of the appropriate levels of the cardiotonic steroid are placed on the sample plate, followed by samples of the intact or powdered tablets. At the end of a series of 12 samples, two standards are inserted to minimize the effects of reagent changes and instrumental variations. Samples are introduced into the solid-prep unit, programmed at 12 samples/hr. with its diluent volume precalibrated to deliver 150 ml., and homogenized in the 70% denatured alcohol. During the period of homogenization, the sample is diluted and dissolution of the drug occurs. A portion of the mixture is aspirated into the flow system and automatically filtered to remove insolubles. To prevent mechanical obstruction from the insoluble excipient materials at the tubing connections, a decantation trap is placed between the solid-prep unit and the proportioning pump to remove the majority of the solid matter, thus extending the operational time of the automated system before a breakdown period is required. The residual insolubles are removed by the continuous filter module. After passage through the filter module, the filtrate is segmented with air and then combined with streams of the ascorbic acid reagent, concentrated hydrochloric acid, and the hydrogen peroxide reagent. The sample stream is passed through time-delay coils to permit complete fluorometric development. Since

[1] AutoAnalyzer, Technicon Corp., Tarrytown, N. Y.
[2] Technicon model I.
[3] Technicon model I.
[4] Technicon catalog No. 126-0125.
[5] Corning Glass Works, Corning, N. Y.
[6] Eastman Kodak Co., Rochester, N. Y.
[7] USP Reference Standards, Bethesda, Md.

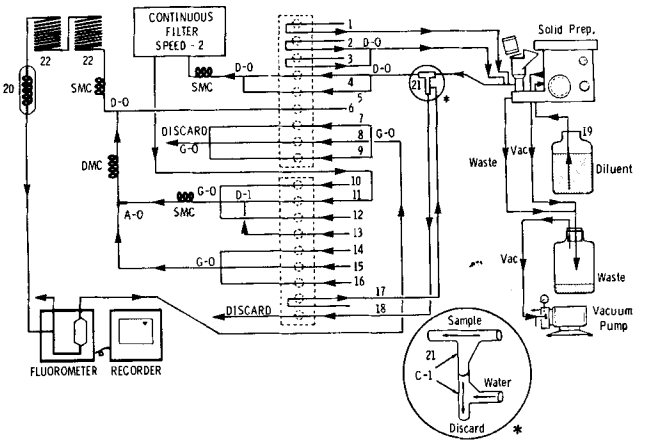


Figure 1—*Automated flow diagram. Key: 1, 0.8 ml./min. air (Tygon); 2,3, 2.76 ml./min. 70% SDA No. 30 alcohol (Acidflex); 4,5, 2.03 ml./min. sample (Acidflex); 6, 0.56 ml./min. H_2O_2 reagent (Solvaflex); 7,8,9, 2.03 ml./min. flowcell (Acidflex); 10, 2.42 ml./min. ascorbic acid reagent (Solvaflex); 11, 2.76 ml./min. sample (Acidflex); 12, 2.42 ml./min. ascorbic acid reagent (Solvaflex); 13, 1.60 ml./min. air (Tygon); 14,15,16, 2.76 ml./min. HCl (Acidflex); 17, 0.42 ml./min. water (Tygon); 18, 0.60 ml./min. (Tygon); 19, reservoir-70% SDA No. 30 alcohol; 20, water-cooled mixing coil (No. 114-209-1); 21, decantation trap; and 22, 12.19-m. (40-ft.) time-delay coils, 2.0 mm. i.d. (No. 105-1173-01).*

a slight fluctuation in the temperature of the final solution at the time of sample measurement in the flowcell has a significant effect on the noise level in the sample response, it is necessary to introduce a water-cooled mixing coil into the system prior to the entrance of the sample stream into the flowcell. The constant and controlled temperature of the final solution results in a significant increase in the precision of the procedure by eliminating the noise in the sample response. Calculations are made using corresponding fluorescent intensities of standards and solid dosage formulation samples.

Under the exact analytical conditions described, digitoxin exhibits excitation and emission wavelength maxima at 395 and 570 mμ, respectively. Excitation and emission wavelength maxima for digoxin are 350 and 490 mμ, respectively. These wavelength maxima are centered well within the optimum spectral transmission characteristics of the selected filters. Spectral measurements were made on a Farrand spectrofluorometer,[8] following preparation of the samples with the automatic analyzer flow system. The spectral data are in exact agreement with those reported by Wells *et al.* (12).

TLC System—Silica gel GF precoated glass plates (10 × 20 cm. with a 250-μ adsorbent layer)[9] were activated by heating at 105° for 20 min. prior to use. The chromatogram was developed in a saturated

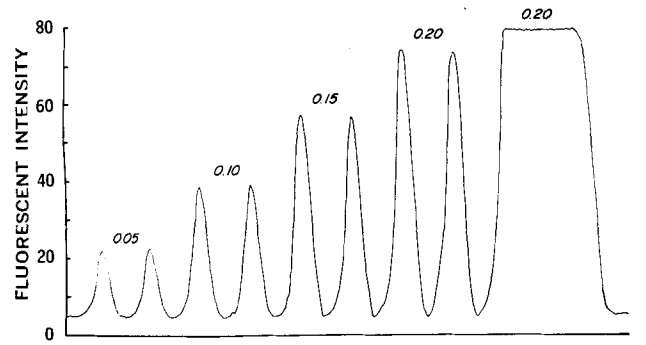


Figure 2—*Reproduction of recording curve on digitoxin standards (mg.) at the rate of 12 samples/hr.*

[8] Farrand catalog No. 104242, Farrand Optical Co., Inc., Mount Vernon, N. Y.
[9] Analtech, Inc., Wilmington, Del.

Table I—Comparison of Fluorometric and Quantitative TLC Analyses of Intentionally Degraded Digitoxin Samples

Sample Treatment	% Initial—Fluorometric Method	% Initial—TLC Method
Digitoxin powder stored at 100° for 1 month	85	87
Digitoxin powder stored at 185° for 16 hr.	76	77
Digitoxin powder exposed to shortwave UV light for 1 month at room temperature	65	63
Suspension of the digitoxin heated in 1 *N* NaOH for 1.5 hr. at 75°	46	43
Digitoxin tablet formulation stored at 100° for 1 month	87	88
Digitoxin tablet formulation exposed to shortwave UV light for 1 month at room temperature	89	91

chamber with a benzene–95% ethanol (7:3, v/v) solvent system. When the solvent front ascended approximately 15 cm. from the origin, the plate was removed and air-dried. Visualization was effected by spraying the plate with chloramine-trichloroacetic acid reagent (14) and heating at approximately 105° for 10 min. Following this heating period, the plates were inspected under shortwave (253.7 mμ) and longwave (366.0 mμ) UV light.

RESULTS AND DISCUSSION

Suitability as Stability Method—Specificity of the method for analysis of intact digitoxin in the presence of its photochemical, alkaline hydrolytic, and thermal degradation products was demonstrated by comparing analytical values of intentionally degraded samples to those obtained by quantitative TLC. In the TLC procedure, the digitoxin was separated from its degradation products on the chromatographic plate. A zone of the silica gel encompassing the intact digitoxin spot was scraped from the plate. The digitoxin was eluted from the adsorbent with 70% denatured alcohol and then assayed by the automated procedure to obtain a

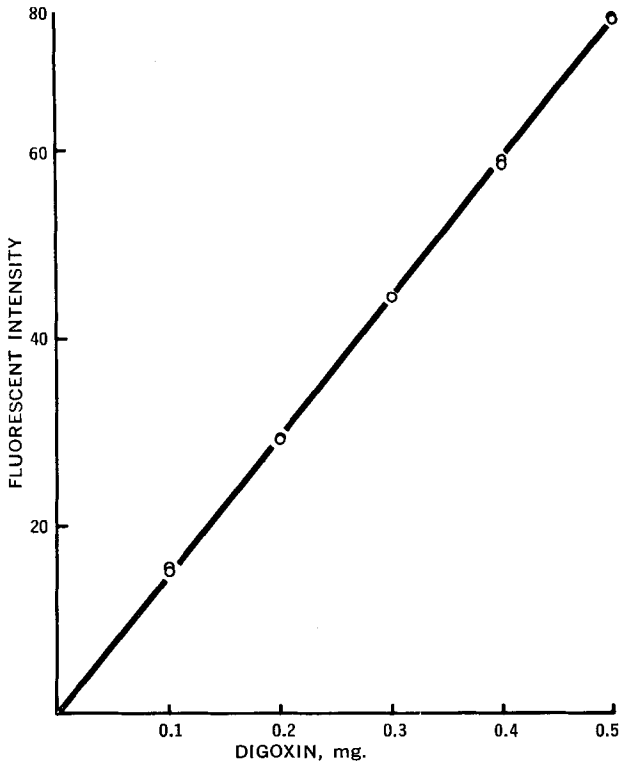


Figure 3—*Relationship of fluorescent intensity to digoxin level.*

Table II—Comparative Data Obtained on Digitoxin Tablets by Automated Procedure and Manual USP XVII Procedure

Product[a]	Labeled Amount, mg./tablet	% of Claim[b] Automated	% of Claim[b] USP
A	0.10	102	101
	0.20	100	100
B	0.10	97	98
C	0.15	100	100
	0.20	103	102
D	0.10	103	102
	0.20	101	101
E	0.10	102	102
	0.20	103	102
F	0.10	103	103
G	0.05	101	100
	0.10	100	99
	0.15	100	100
	0.20	99	100

[a] Tablets analyzed were prepared by the following manufacturers: Wyeth Labs., Inc.; The Upjohn Co.; E. R. Squibb & Sons; Parke, Davis & Co.; Eli Lilly & Co.; Davies, Rose-Hoyt; and Abbott Labs. Companies are not necessarily listed in order as given above. [b] Data from a single assay on a 10-tablet composite sample.

quantitative value. The data obtained are summarized in Table I. Since there is good agreement between the values by the two techniques, it is concluded that the fluorometric procedure is stability indicating.

Linearity and Sensitivity—Figure 2 is a reproduction of an actual recording curve obtained by analyzing standards of digitoxin in the 0.05–0.20-mg. range. The uniformity of replicate analyses at the various concentrations and the return to the base line between samples, indicating no sample overlap, can be noted. When the values for the standards of Fig. 2 are plotted, a linear relationship exists between fluorescent intensity and digitoxin concentration in the range studied. This curve also contains a recording of the steady state, during which time a 0.20-mg. standard was continuously sampled, which further defines the excellent flow characteristics of this automated system.

In the application of the method to the analysis of digoxin samples, it was also observed that a linear relationship exists between fluorescent intensity and digoxin concentration (Fig. 3).

The flow diagram of Fig. 1 is applicable to the analysis of digitoxin and digoxin samples which initially contain 0.02–1.0 mg. of the cardiac glycosides. At the 0.02-mg. level, the final concentration of the drug in the flowcell is approximately 0.07 mcg./ml.

Precision—Repeatability of the automated technique was demonstrated by performing 30 replicate assays on aliquots of both digitoxin and digoxin standard solutions. Relative standard deviations of ±1.4 and ±1.0% were determined at the 0.05- and 0.20-mg. digitoxin levels, respectively, and ±1.2% at the 0.25-mg. digoxin level. In addition, a series of 20 replicate assays run on a composite sample of a tablet formulation at the 0.10-mg. digitoxin per tablet level produced a relative standard deviation of ±1.0%.

Accuracy—The accuracy of the proposed procedure was evaluated by comparing the results obtained by the automated system with the manual USP XVII colorimetric procedure (9) on several different marketed digitoxin tablet formulations. Comparative results are shown in Table II. The agreement between the two techniques demonstrates that the automated method provides an accurate means of evaluating digitoxin content.

The effect of common inert tablet components on this fluorometric procedure for digoxin and digitoxin was investigated to uncover any possible interfering material. The inactive components evaluated were lactose, sucrose, calcium sulfate, magnesium stearate, stearic acid, alginic acid, microcrystalline cellulose (Avicel), ion-exchange resin (Amberlite IRP-88), methylcellulose (Methocel), and starch. It was found that none of these materials interfered.

REFERENCES

(1) A. J. Khoury, "Automation in Analytical Chemistry, Technicon Symposia, 1966," vol. I, Mediad, Inc., New York, N. Y., 1967.

(2) A. E. James, F. O. Laquer, and J. D. McIntyre, *J. Amer. Pharm. Ass., Sci. Ed.*, **36**, 1(1947).

(3) F. K. Bell and J. C. Krantz, *ibid.*, **37**, 297(1948).

(4) E. Soos, *Sci. Pharm.*, **16**, 29(1948).

(5) E. E. Kennedy, *J. Amer. Pharm. Ass., Sci. Ed.*, **39**, 25(1950).

(6) J. M. Rowson, *J. Pharm. Pharmacol.*, **4**, 814(1952).

(7) D. H. Tattje, *Ann. Pharm. Fr.*, **12**, 267(1954).

(8) D. H. Tattje, *J. Pharm. Pharmacol.*, **9**, 29(1957).

(9) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

(10) L. J. Sciarini and W. T. Salter, *J. Pharmacol. Exp. Ther.*, **101**, 167(1951).

(11) I. M. Jakovljevic, *Anal. Chem.*, **35**, 1513(1963).

(12) D. Wells, B. Katzung, and F. H. Meyers, *J. Pharm. Pharmacol.*, **13**, 389(1961).

(13) R. W. Jelliffe, *J. Chromatogr.*, **27**, 172(1967).

(14) "Thin-Layer Chromatography," E. Stahl, ed., Academic, New York, N. Y., 1965.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 16, 1969, from *Wyeth Laboratories, Pharmacy Research and Development Division, Radnor, PA 19101*

Accepted for publication October 31, 1969.

Presented to the Technicon International Congress on Automated Analysis, Chicago meeting, June 1969.

Physical Chemistry of Ergot Alkaloids and Derivatives I: Ionization Constants of Several Medicinally Active Bases

H. V. MAULDING and M. A. ZOGLIO

Abstract □ A novel procedure is presented for determination of the ionization constants of several pharmacologically active naturally occurring ergot alkaloids and their congeners. For this purpose use was made of the complexation and consequent solubilization of these poorly soluble molecules by certain xanthines with special emphasis on 7-β-hydroxypropyltheophylline. This method allows an approach to investigation of the approximate pKa values of the proteinaceous alkaloids such as ergotamine which resisted the usual means available for measurement of dissociation constants. It is also illustrative of the weak attractive forces present in complexes of this nature.

Keyphrases □ Ergot alkaloids, congeners—ionization constants, determination □ Dissociation-constant determination—methodology □ Titration, aqueous—dissociation-constant determination

Difficulties were experienced on attempted evaluation of the ionization constants for various ergot alkaloids and their analogs. The insolubility of the bases in aqueous media has been discussed previously by the authors (1–3). This limitation is likewise encountered in ethanol–water solutions sometimes employed with apparent success in pKa measurements (4). The alkaloidal base, ergotamine, exhibits little solubility in polar solvents such as ethanol and propylene glycol, and this is the situation with many related compounds. Ergotamine and other ergot alkaloids possess an UV spectrum but only small differences are discernible between the ionized species at pH 2 and the neutral species at pH 11, thus precluding this route for analysis of the ionization constants. Somewhat erratic solubility properties have been noted in this laboratory for ergot alkaloids and derivatives when the cyclic tripeptide moiety is intact, leading to a lack of precision and confidence in a pKa obtained by this technique.

Preliminary investigations and previous communications (1–3) indicated an elevation in solubility of the neutral molecule as well as prevention of its precipitation upon inclusion of certain xanthines. A study was undertaken to detect if this phenomenon would have applicability in measurement of ionization constants of these relatively complex molecules.

EXPERIMENTAL

Dissociation constants were determined by titration of the hydrochlorides or methanesulfonates of the bases in aqueous solution (0.005 mole of the methanesulfonate salt or 0.005 mole of the base solubilized by 5 ml. 0.1 *N* HCl in 92.5 ml. freshly boiled water) along with 10–20 g. 7-β-hydroxypropyltheophylline (see Table I). This solution was titrated with 10 equal increments (0.5 ml.) of carbonate-free 0.1 *N* KOH. The pH was taken initially, following each portion of KOH titrant, and nine values for the ionization constant calculated in each case (5).

The same procedure was carried out with the two relatively soluble bases, methysergide and methylergonovine, except the xanthine was omitted.

Table I—Ionization Constants of Ergot Alkaloids and Derivatives[a]

Compound	% Xanthine[b]	pKa
1. Methysergide	0	6.62 (± 0.02)[c]
2. Methylergonovine	0	6.65 (± 0.03)[c]
3. Dihydroergocristine	10	6.74 (± 0.02)[d, e]
4. Dihydroergocriptine	10	6.74 (± 0.02)[d, e]
5. Dihydroergocornine	10	6.76 (± 0.02)[d, e]
6. Dihydroergotamine	10	6.75 (± 0.03)[d, e]
7. Ergostine[f]	15–20	6.30 (± 0.04)[d, e]
8. Ergotamine[f]	15–20	6.25 (± 0.04)[d, e]
9. Ergotaminine[f]	15–20	6.72 (± 0.04)[d, e]

[a] All measurements at 24°. [b] 7-β-Hydroxypropyltheophylline. [c] Thermodynamic ionization constant. [d] Measured pKa values in presence of corresponding xanthine percentage (Column 2). [e] Corrected pKa values for Compounds 3–9 are 6.89 (±0.07), 6.89 (±0.07), 6.91 (±0.07), 6.90 (±0.08), 6.45 (±0.09), 6.40 (±0.09), and 6.87 (±0.09), respectively, and are calculated by addition of 0.15 (±0.05) to the measured values (Column 3) and should closely approach the thermodynamic pKa. See under *Results and Discussion*. [f] Excess HCl added for dissolution of base followed by neutralization with KOH.

MATERIALS

Ergotamine base was twice recrystallized from acetone–water (90 ml. acetone: 10 ml. water); methysergide base was recrystallized four times from methanol–water (10 ml. methanol: 40 ml. water), followed by drying at 50° (1 mm.) for 24 hr.

Other alkaloidal bases and methanesulfonate salts were chromatographically pure (traces of contaminants) and were dried overnight at 50° (1 mm.).

7-β-Hydroxypropyltheophylline, m.p. 135–138° (Ganes Chemical Works, Inc., New York) was used.

Tris(hydroxymethyl)aminomethane (THAM) (primary standard), pKa = 8.18 at 20° (Fisher Sci. Co., Fairlawn, N. J.) was used.

The pH values were measured on a Metrohm pH meter using electrodes[1] standardized on 0.05 *M* potassium hydrogen phthalate (pH 4.0, 24°) and 0.05 *M* sodium borate (pH 9.20, 24°). All measurements were made at 24°.

RESULTS AND DISCUSSION

Utilization of mixed solvent systems in evaluation of dissociation constants for substances showing poor aqueous solubility has been made rather frequently since the procedure was first reported (6). Although this expediency should probably be avoided whenever

[1] Corning triple-purpose glass electrode No. 476020 and calomel electrode No. 476002, Corning Glass Works, Medfield, Mass.

possible (5), there are circumstances where it must be resorted to for obtaining anything resembling satisfactory pKa values.

It has been seen in this laboratory that ergotamine and other alkaloids of ergot having a cyclic tripeptide attached to the lysergic or isolysergic portions of the molecule generally exhibit low solubility not only in water but in many water-miscible solvents. This fact tends to complicate titrimetric analysis. Fortunately the ionization constants of protonated amines, such as dihydroergocristine methanesulfonate, are frequently little different in mixed solvents than in water alone.

Neutral xanthines possess the ability to solubilize the free alkaloids to some extent (1), and it was hoped by this means titration could be carried out. The xanthine, 7-β-hydroxypropyltheophylline, was chosen for extensive work because of its high water solubility relative to caffeine, theophylline, and others. The results of the study are listed in Table I.

The compounds in Table I have three or more nitrogen atoms present in their structures; however, only the ring nitrogen located in the tetrahydropyridine or hexahydropyridine moieties of the lysergic and isolysergic acid portions (7–9) need be considered in this work. The indole nitrogen (amine) in the lysergic or isolysergic acid fragment is weakly basic and of no consequence in normal pH ranges (7). The amide nitrogen present in methysergide and methylergonovine (Table I, Compounds 1 and 2) displays insignificant pH effect over the interval investigated, and this holds for the additional peptide nitrogens of the more complex molecules (Table I, Compounds 3–9).

The pKa values were calculated using the expression

$$pKa = pH + \log \frac{BH^+}{B} \qquad \text{(Eq. 1)}$$

where BH^+ = alkaloidal salt concentration (HCl or methanesulfonate) and B = alkaloidal base concentration. No correction was necessary for OH^- or H^+ ions in the range of pH's studied. KOH titrant was added in 10 equal increments and the pKa value obtained for each of the nine points (5). Results with a precision of less than ±0.04 were rejected.

A slight perturbation of the ionization constant was evidenced with the more soluble compounds in Table I, methysergide and methylergonovine, on inclusion of xanthine. Since these materials have the capacity to complex as well as ergotamine and the other ergot derivatives studied (1–3), this parameter is present in all compounds under consideration. Measured pKa values for these two substances declined upon introduction of 7-β-hydroxypropyltheophylline in quantities of 2 to 30 g./100 ml. solvent. There was little change in the measured pKa (Table I, Column 3) following this initial drop of 0.1–0.2 units, 0.15 (±0.05),[2] on addition of xanthine. Extrapolations of the data as a plot of pKa *versus* percent xanthine would certainly produce erroneous values. This may be seen in Fig. 1 in the case of methysergide, where extrapolation would lead to a pKa somewhat lower than it actually is. It may be noted in Fig. 1 that there appears this small initial alteration of the ionization constant on addition of the xanthine followed by a degree of leveling.

In some of the proteinaceous alkaloids, such as dihydroergocristine and dihydroergocriptine, the end values could be obtained for the previously stated equation before precipitation of the free base took place, where BH^+/B = 39/1 to 9/1. In these instances the dissociation constants derived from these calculations were found to be greater by the previously stated increment, 0.15 (±0.05)[2] than the observed pKa when sufficient xanthine was present to solubilize the alkaloid. The quantities of 7-β-hydroxypropyltheophylline used (Table I, Column 2) were those experimentally found to hold the base in solution. This lowering of the ionization constant occurred with THAM and remains fairly constant over a wide proportion of xanthine levels. Caffeine, theophylline, and other analogs give satisfactory results for many of these ergot derivatives when employed for pKa determination.

[2] The value, 0.15 (±0.05), does not refer to the precision of the experimental results but to a lowering of the pKa value by 0.1 to 0.2 on addition of xanthine. The precision of all values obtained with xanthine are listed in Column 2, Table I.

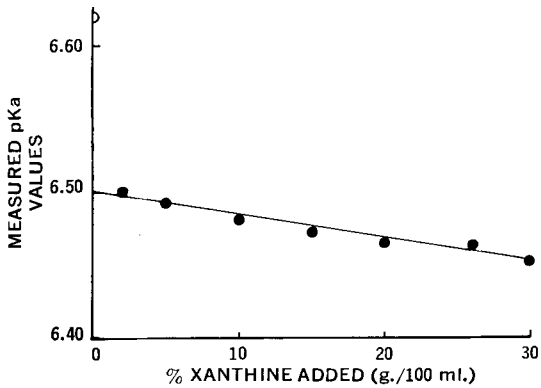


Figure 1—*Plot of −log Ka (pKa) measured for methysergide (0.01 mole of HCl salt in 100 ml. water; precision of experimental results not indicated but greater than ±0.03 in every case) against the concentration of 7-β-hydroxypropyltheophylline (g./100 ml.). Key: ○, pKa at zero xanthine concentration—thermodynamic pKa; and ●, measured pKa in presence of amount of xanthine indicated on abscissa.*

The numbers in Table I (superscript ^{e}) are the corrected pKa values for Compounds 3–9 and are the best obtainable from available data. Each of these values is derived by addition of this factor[2] to the measured pKa (Column 3), excepting Compounds 1 and 2 where the thermodynamic ionization constant could be found by titration. Although no claim is made regarding their exactness, these values should closely approach the thermodynamic ionization constant.

This method offers a serviceable approach to determination of the ionization constants of the ergot alkaloids and derivatives of proteinaceous nature. It is extremely reproducible and easy to apply. The small effects demonstrated by rather large quantities of complexing agent on the pKa give more circumstantial evidence for the weak attractive forces present between the two types of molecules.

REFERENCES

(1) M. A. Zoglio, H. V. Maulding, and J. J. Windheuser, *J. Pharm. Sci.*, **58**, 222(1969).

(2) M. A. Zoglio and H. V. Maulding, to be published.

(3) H. V. Maulding and M. A. Zoglio, to be published.

(4) S. P. Agarwal and M. I. Blake, *J. Pharm. Sci.*, **57**, 1434(1968).

(5) A. Albert and E. P. Sergeant, "Ionization Constants of Acids and Bases," Wiley, New York, N. Y., 1962, pp. 17–68.

(6) M. Mizutani, *Z. Phys. Chem.*, **116**, 230(1925).

(7) J. Chilton and J. P. Stenlake, *J. Pharm. Pharmacol.*, **14**, 367 (1962).

(8) C. Wilson and O. Gisvold, "Textbook of Organic Pharmaceutical and Medicinal Chemistry," 4th ed., J. B. Lippincott, Philadelphia, Pa., 1962, p. 471.

(9) A. Burger, "Medicinal Chemistry," Interscience, New York, N. Y., 1960, p. 583.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 13, 1969, from the *Pharmacy Research and Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication November 20, 1969.

The authors acknowledge the technical assistance of M. Wagner and V. Nieman.

Physiological Studies on Ergot: Influence of 5-Methyltryptophan on Alkaloid Biosynthesis and the Incorporation of Tryptophan Analogs into Protein

J. E. ROBBERS and H. G. FLOSS*

Abstract □ Time-course studies were performed which compared the influence of 5-methyltryptophan to tryptophan on alkaloid formation in ergot strain SD-58. Evidence was obtained that confirms the role of tryptophan as an inducer of alkaloid synthesis. Additional studies were conducted to determine the incorporation into protein of the tryptophan analogs: 5-methyltryptophan, 7-methyltryptophan, and 1-*N*-methyltryptophan, when they are supplied exogenously to the fungus. Incorporation was observed; however, it was insignificant when compared to the amount of tryptophan incorporated.

Keyphrases □ Ergot alkaloid production—shake cultures □ Tryptophan, 5-methyltryptophan effect, alkaloid production—comparison □ Protein incorporation—tryptophan and analogs

The important role of tryptophan in ergot alkaloid biosynthesis has been clearly established. Floss and Mothes (1) have obtained data which suggest that tryptophan is also involved in an induction phenomenon besides serving as a precursor in the formation of the ergoline ring system. These workers found that tryptophan stimulates alkaloid formation when added at the beginning of the culture period but not when added after alkaloid production had started, that a variety of tryptophan analogs stimulate alkaloid production even though they are not incorporated into the alkaloids, and that mycelia grown in the presence of tryptophan or its analogs retain the ability to produce more alkaloid than the controls even after replacement into fresh culture medium.

One of the disadvantages of this earlier work was that the percentage increase in alkaloid production due to tryptophan analogs was small because these experiments were carried out with cultures that produced large amounts of alkaloids. By using a fully synthetic culture medium, it was thought that the effect of tryptophan and its analogs could be demonstrated more dramatically. Eliminating yeast extract from the medium reduces the growth of the organism to about one-fourth that of the complete medium; however, the amount of alkaloid produced per gram of mycelium is unchanged.

The purposes of this investigation were to study the induction phenomenon in a more precise manner over the time period in which alkaloid production is initiated in the fungus and to compare the effect of 5-methyltryptophan with that of tryptophan.

In the series of experiments previously mentioned, Floss and Mothes (1) also found uptake into the mycelia of radioactivity from the methyltryptophan analogs. However, the metabolic fate of the analogs was not determined except for nonincorporation into alkaloids. Recent studies by Lark (2) have shown that *Escherichia coli* can incorporate 5-methyltryptophan into protein. Consequently, the present authors have attempted to determine if methyl analogs of tryptophan are incorporated into protein by the ergot fungus.

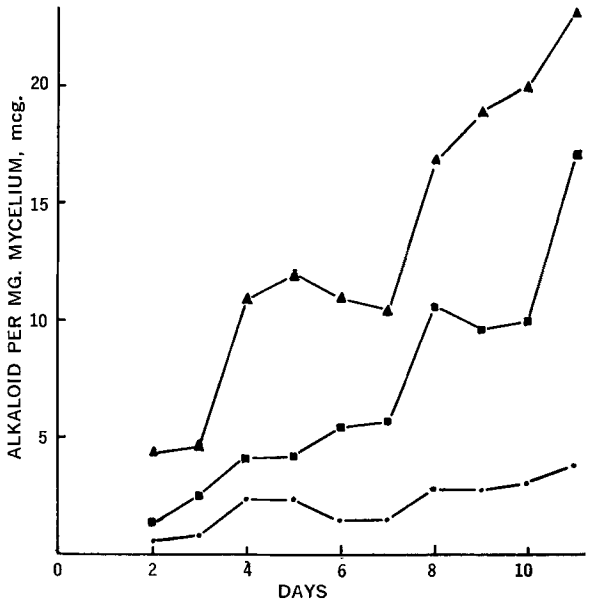


Figure 1—*Time-course study comparing the influence of tryptophan and 5-methyltryptophan on alkaloid production in* Claviceps *strain SD-58. Key:* ●, *control;* ▲, *tryptophan; and* ■, *5-methyltryptophan.*

EXPERIMENTAL

Induction of Alkaloid Production—*Claviceps* strain SD-58 was grown for 5 days in shake culture in a medium containing the following ingredients: 50 g. mannitol, 50 g. sucrose, 5.4 g. succinic acid, 3.0 g. Difco yeast extract, 0.1 g. KH_2PO_4, 0.3 g. $MgSO_4 \cdot 7H_2O$, 0.0044 g. $ZnSO_4 \cdot 7H_2O$, and boiled tap water, 1 l. The mycelia from one culture were placed into 0.01 *M* phosphate buffer and resuspended. Using aliquots of this suspension as inoculum, three groups of flasks containing the inoculum medium without yeast extract were prepared. One group of cultures also contained 4×10^{-3} *M* DL-tryptophan and a second group contained 4×10^{-3} *M* DL-5-methyltryptophan. A flask from each of the three groups was harvested every day starting at the 2nd day of growth. Quantitation of the alkaloids in the culture filtrate was effected by extracting the alkaline culture filtrate with $CHCl_3$ and redissolving the dried $CHCl_3$ extract in 2% succinic acid solution. This procedure prevented tryptophan and 5-methyltryptophan from interfering in the quantitation. A colorimetric determination was performed using van Urk's reagent (3, 4).

Analog Incorporation into Protein—Ergot strain SD-58 was grown in shake culture in the mannitol–sucrose–succinic acid–yeast extract medium previously described. Tritiated tryptophan, 5-methyltryptophan, 7-methyltryptophan, and 1-*N*-methyltryptophan were added, one to a culture, at the start of the growth period. The tritiated compounds were from the same source as in a previous study (1). On the 3rd and 6th days of growth the mycelia were removed by filtration and dried. The protein was extracted from the mycelia using trichloroacetic acid precipitation methods (5) and quantitated with the biuret reaction. A second purification was effected by redissolving the protein in 1 *N* NaOH and repeating the

Table I—Incorporation of ^{3}H-Labeled Tryptophan and Methyltryptophans into Elymoclavine and Protein

	Tryptophan	5-Methyl-tryptophan	7-Methyl-tryptophan	1-*N*-Methyl-tryptophan
Specific activity of added compound	7.0 mc./mmole	1.27 mc./mmole	0.39 mc./mmole	1.1 mc./mmole
Total activity fed	2.8 μc.	1.91 μc.	1.93 μc.	1.82 μc.
Absolute incorporation into alkaloids	35.3%	0.9%	0.2%	1.8%
Absolute incorporation into protein, 3rd day	39.6%	0.3%	0.7%	0.5%
Absolute incorporation into protein, 6th day	5.0%	0.8%	0.8%	0.5%

precipitation and quantitation methods. Using a measured amount of the repurified protein, which was dissolved in a 1 *N* NaOH solution, the incorporation of radioactivity into the protein was determined.

Approximately 50 mg. of protein powder obtained from each culture was hydrolyzed for 24 hr. at 125° with a saturated solution of barium hydroxide in an evacuated sealed glass tube. The barium was removed from the hydrolysate through the addition of 2 *N* sulfuric acid to pH 6 and subsequent centrifugation to remove the barium sulfate.

To investigate the presence of tritiated tryptophan and tryptophan analogs in the protein hydrolysates, ascending paper chromatography in *n*-butanol–acetic acid–water (4:1:1) was employed. By cutting the chromatogram into 1-cm. horizontal strips starting at the point of origin, placing these strips in toluene scintillation counting solution, and counting the radioactivity, the zones of activity on the chromatograms were determined, and the R_f values were compared with reference compounds.

Elymoclavine was isolated from 6-day-old culture filtrates by partitioning and recrystallizing from methanol to a constant specific radioactivity.

RESULTS AND DISCUSSION

The time–course study showing that tryptophan and 5-methyltryptophan stimulate alkaloid production early in the growth phase of the fungus (Fig. 1) confirms and extends earlier observations (1). Under the modified conditions, the effect of 5-methyltryptophan can be seen more clearly than in the earlier experiments. At Day 11, tryptophan induces a 495% increase and 5-methyltryptophan a 341% increase in alkaloid production over the control culture, whereas the previous work (1) shows only a 61% increase in alkaloids for each compound after 22 days. The time–course experiment was repeated four times and the results were found to be reproducible. Figure 1 represents the results from a single experiment. Stimulation of alkaloid production in *Claviceps* has also been reported by Baxter and Zahid (6). These data give supportive evidence to the postulation (1) that tryptophan is an inducer of alkaloid biosynthesis.

An explanation of this phenomenon becomes more difficult when one considers the work recently reported by Lingens *et al.* (7, 8) on the regulation of aromatic amino acid biosynthesis in *Claviceps*. Using alkaloid-producing mycelia of a strain of *Claviceps paspali*, they found (7) that L-tryptophan inhibited one of the isoenzymes of 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP) synthetase, reversed the inhibition of chorismate mutase by phenylalanine and tyrosine, and also activated this enzyme; but unlike the situation in other microorganisms, it did not inhibit anthranilate synthetase. 5-Methyltryptophan elicited the same effect as tryptophan on DAHP synthetase and apparently (8) also on chorismate mutase. In similar studies with *Claviceps* strain SD-58, however, it was found that anthranilate synthetase was inhibited by tryptophan (8). A weak point of the latter result is that it was not known whether the mycelium produced alkaloids under the culture conditions used.

The results clearly show that in strain SD-58 the addition of 5-methyltryptophan leads to increased alkaloid production. On the other hand, the incorporation study shows that 5-methyltryptophan cannot replace tryptophan as alkaloid precursor (Table I). Likewise, the organism can grow in the presence of 5-methyltryptophan without added tryptophan, yet 5-methyltryptophan is not incorporated into protein to any significant extent (Table I). This leaves unexplained the source of the endogenous tryptophan. One simple explanation would be that in ergot strain SD-58, 5-methyltryptophan cannot replace tryptophan as feedback regulator of aromatic amino acid biosynthesis. As another possibility, an anthranilate synthetase isoenzyme could exist in the fungus which is not inhibited by tryptophan or 5-methyltryptophan.

There is a low level of incorporation of radioactivity into protein when tritiated methyl analogs of tryptophan are fed to ergot strain SD-58 (Table I). However, their absolute incorporation appears insignificant when compared to the incorporation of tryptophan (approximately 80 times greater in the 3-day-old cultures). The decreased incorporation into protein of labeled tryptophan between Day 3 and Day 6 can be attributed to a dilution effect caused by the synthesis of nonlabeled endogenous tryptophan and has been observed before (5). Because of this low level of incorporation, it was not possible to determine conclusively if the activity was due to the direct incorporation of the analogs or if the analogs were metabolized to intermediates that could be utilized in amino acid biosynthesis and thus be incorporated into protein in this manner. However, the results from the chromatographic investigation of the protein hydrolysate tend to support the former viewpoint.

REFERENCES

(1) H. G. Floss and U. Mothes, *Arch. Mikrobiol.*, **48**, 213 (1964).

(2) K. G. Lark, *J. Bacteriol.*, **97**, 980(1969).

(3) H. W. van Urk, *Pharm. Weekbl.*, **66**, 473(1929).

(4) M. L. Smith, *Pub. Health Rep.*, **45**, 1466(1930).

(5) H. Kaplan, U. Hornemann, K. M. Kelley, and H. G. Floss, to be published.

(6) R. M. Baxter and N. D. Zahid, "Biosynthesis of Ergot Alkaloids: Regulatory Factors, Significance of the Effect of Certain Analogues of Tryptophan," presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Miami meeting, May 1968.

(7) F. Lingens, W. Goebel, and H. Ulesseler, *Eur. J. Biochem.*, **2**, 442(1967).

(8) F. Lingens, "4. Internationales Symposium Biochemie und Physiologie der Alkaloide," Akademie-Verlag, Berlin, East Germany, 1969, p. 55.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 2, 1969, from the *Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication November 17, 1969.

Presented at the 10th annual meeting of the American Society of Pharmacognosy, Corvallis, Ore., August 1969.

This work was supported by National Institutes of Health Research Grant AM 11662 and by Eli Lilly & Company. The authors are indebted to Mrs. Arisine Basmadjian for her laboratory assistance.

* Recipient of Research Career Development Award from the National Institutes of Health.

Quinine—Effect on *Tetrahymena pyriformis* II: Comparative Activity of the Stereoisomers, Quinidine and Quinine

K. A. CONKLIN*, P. HEU, and S. C. CHOU

Abstract □ Treatment of heat-synchronized *Tetrahymena* with quinine or quinidine resulted in inhibition of synchronized cell division. Syntheses of DNA, RNA, protein, and lipids were also effectively blocked by both drugs. The effects of quinine and quinidine were quantitatively the same in all instances, indicating that the intracellular action of the two drugs in *Tetrahymena* is similar.

Keyphrases □ Quinine, quinidine effect—*Tetrahymena pyriformis* □ Quinidine, quinine effect—RNA, DNA, protein, lipid synthesis □ Thymidine-, uracil-, amino acids-^{14}C—*Tetrahymena pyriformis* utilization

Quinine and quinidine are, respectively, the levo- and dextrorotatory stereoisomers of 6-methoxy-α-(5-vinyl-2-quinuclidinyl)-4-quinolinemethanol and exhibit similar physical and chemical properties. Biologically the drugs are similar with respect to absorption, distribution, excretion, and antimalarial activity (1), but quinidine is more effective than quinine as an antifibrillatory agent (2). Whether this difference in biological activity is due to different actions at the subcellular level has yet to be determined.

The authors investigated the action of the two drugs on the free-living protozoan *Tetrahymena pyriformis*. *Tetrahymena* is ideal for the study of drug action because: (*a*) this organism is easily grown in culture medium; (*b*) the cells can be synchronized which allows for the study of the effects of drugs on a population of cells that are all in the same growth phase; and (*c*) the direct action of drugs on DNA, RNA, protein, and lipid synthesis can be studied while the cells are in their normal growth environment.

EXPERIMENTAL

Cultures of *T. pyriformis*, strain GL, were grown, synchronized by heat treatment (3), harvested, and washed in Ringer's buffer as described previously (4). The following procedures were used to determine the effect of quinine and quinidine on synchronized *Tetrahymena* in Ringer's buffer.

Effect of Quinine and Quinidine on Synchronized Cell Division—At the end of the heat treatment (EHT), 5-ml. aliquots of washed cells were pipeted into 25-ml. conical flasks containing either quinine hydrochloride or quinidine sulfate and into a control flask without drug. The drug concentrations were 1.3, 2.5, and 3.8×10^{-4} *M*. The flasks were placed on a rotator at 28° for the duration of the experiment. Cell samples were taken at regular intervals and fixed in 0.7% formalin in Ringer's buffer; the number of cells per milliliter was determined by direct cell counts (4). The effectiveness of the drugs was determined by the observed degree of inhibition of the first (80 min. after EHT) and second (200 min. after EHT) synchronized divisions.

Effect of Quinine and Quinidine on DNA, RNA, Protein, and Lipid Syntheses after EHT—At EHT, 2-ml. aliquots of washed cells were pipeted into 25-ml. flasks containing measured amounts of quinine or quinidine plus 2.5 μc./ml. of ^{3}H-thymidine, 2.0 μc./ml. of ^{14}C-uracil, 0.25 μc./ml. of ^{14}C-amino acids, or 0.25 μc./ml. of ^{14}C-acetate. The drugs were used at 1.3, 2.5, and 3.8×10^{-4} *M*, and flasks containing just the radioactive precursor served as controls. The flasks were incubated on a rotator at 28°, and incorporation of radioactive precursors was followed for 80 min. by the filter paper disk procedures of Byfield and Scherbaum for DNA, RNA, and protein (5) or Byfield *et al.* for lipids (6).

Table I—Inhibition of Synchronized Cell Division by Quinine (Q) and Quinidine (QD)

		Concentration, moles/l. × 10^4 1.3	2.5	3.8
		Mean % Inhibition ± *SE*[a]		
First division	Q	15 ± 2.3	40 ± 0.6	108 ± 2.3[b]
	QD	19 ± 1.5	40 ± 2.2	101 ± 4.1[b]
Second division	Q	50 ± 3.2	95 ± 3.6	102 ± 3.1[b]
	QD	50 ± 3.9	102 ± 1.5[b]	110 ± 4.3[b]

[a] Each value from three determinations. In no instance is p less than 0.2 for any pair of values. [b] Values greater than 100% inhibition are due to cell death and lysis in the treated samples.

RESULTS AND DISCUSSION

Table I shows the effect of quinine and quinidine on the first and second synchronized cell divisions. The actions of the drugs at all levels tested were quantitatively the same, both drugs showing partial inhibition at 1.3 and 2.5×10^{-4} *M* and complete inhibition at 3.8×10^{-4} *M*.

To determine if arrest of cell division was due to inhibition of DNA, RNA, or protein biosyntheses, the effect of the drugs on incorporation of radioactive precursors (thymidine, uracil, and amino acids, respectively) into the macromolecules was followed. The results (Table II) show that both quinine and quinidine were effective inhibitors of all three pathways and that equal concentrations of the two drugs produced approximately the same degree of inhibition. These results indicate that both drugs have similar actions on these biosynthetic pathways and that the inhibition of cell division is not due to inhibition of one specific cellular function.

One possible explanation for inhibition of both nucleic acid and protein syntheses is that the drugs block energy metabolism in *Tetrahymena*. To investigate this possibility, the authors studied the incorporation of acetate into the acid-insoluble lipid fraction. This test has been used by others to determine the state of the energy-generating systems in *Tetrahymena* (7, 8). The results (Table II) show that both drugs were effective inhibitors of lipid synthesis, and the degree of inhibition was the same for equal concentrations of the two drugs. As stated previously for quinine (4), these results indicate that the primary action of the drugs in *Tetrahymena* may be inhibition of energy production with the action on DNA, RNA, and protein syntheses being secondary. In support of this, quinidine is known to inhibit oxygen uptake (9, 10) and the oxidation of free fatty acids (11) and carbohydrates (11, 12) in cardiac muscle, and quinine is known to inhibit pyruvate oxidation through the citric acid cycle (13), isolated respiratory enzymes (14), and oxygen uptake (15) of *Plasmodium*.

Tetrahymena has proved to be a very useful tool for studying the mechanism of action of certain drugs (4, 7, 16, 17). The results presented using this system demonstrate that, for each parameter investigated, there was no significant difference in the action of quinine or quinidine (p always greater than 0.2). These data suggest that the intracellular action of the two drugs in *Tetrahymena* is the

Table II—Inhibition of DNA, RNA, Protein, and Lipid Synthesis by Quinine (Q) and Quinidine (QD)

		Concentration, moles/l. × 10^4 1.3	2.5	3.8
		Mean % Inhibition ± *SE*[a]		
DNA synthesis	Q	34 ± 3.5	69 ± 6.1	79 ± 2.6
	QD	32 ± 5.7	75 ± 2.1	78 ± 3.2
RNA synthesis	Q	33 ± 0.5	55 ± 2.4	62 ± 1.9
	QD	36 ± 2.9	56 ± 2.1	61 ± 1.9
Protein synthesis	Q	51 ± 3.9	60 ± 0.2	73 ± 1.4
	QD	50 ± 3.4	63 ± 3.8	71 ± 1.1
Lipid synthesis	Q	8 ± 1.8	14 ± 3.3	27 ± 4.1
	QD	7 ± 2.3	15 ± 1.8	35 ± 3.7

[a] Each value from four determinations. In no instance is p less than 0.2 for any pair of values.

same. The difference in antifibrillatory action of the drugs may be due in part to differences in chemical structure which, in turn, may reflect cardiac receptor specificity.

REFERENCES

(1) J. V. Taggart, D. P. Earle, Jr., R. W. Berliner, C. G. Zubrod, W. J. Welch, N. B. Wise, E. F. Schroeder, I. M. London, and J. A. Shannon, *J. Clin. Invest.*, **27,** 80(1948).

(2) K. F. Wenckelbach, *J. Amer. Med. Ass.*, **81,** 472(1923).

(3) O. H. Scherbaum and E. Zeuthen, *Exp. Cell Res.*, **6,** 221 (1954).

(4) K. A. Conklin, S. C. Chou, and S. Ramanathan, *Pharmacology*, **2,** 247(1969).

(5) J. E. Byfield and O. H. Scherbaum, *Anal. Biochem.*, **17,** 434(1966).

(6) J. E. Byfield, H. Henze, and O. H. Scherbaum, *Life Sci.*, **6,** 1099(1967).

(7) S. C. Chou and S. Ramanathan, *ibid.*, **7,** 1953(1968).

(8) J. E. Byfield and O. H. Scherbaum, *Science*, **156,** 1504 (1967).

(9) E. Uyeki, E. Geiling, and K. Dubois, *Arch. Int. Pharmacodyn. Ther.*, **97,** 191(1954).

(10) J. Webb, P. Saunders, and K. Nakamura, *J. Pharmacol. Exp. Ther.*, **101,** 287(1951).

(11) L. Crevasse and J. C. Shipp, *Amer. Heart J.*, **68,** 667(1964).

(12) M. E. Hess and N. Hangaard, *Circ. Res.*, **6,** 256(1958).

(13) J. W. Moulder, *J. Infec. Dis.*, **85,** 195(1949).

(14) E. Haas, *J. Biol. Chem.*, **155,** 321(1944).

(15) J. D. Fulton, *Ann. Trop. Med. Parasitol.*, **32,** 77(1938).

(16) K. A. Conklin, S. C. Chou, and S. Ramanathan, *Fed. Proc.*, **28,** 361(1969).

(17) S. C. Chou, S. Ramanathan, and W. C. Cutting, *Pharmacology*, **1,** 60(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 20, 1969, from the *Department of Pharmacology, School of Medicine, University of Hawaii, Honolulu, HI 96816*

Accepted for publication January 6, 1970.

This work submitted by K. A. Conklin in partial fulfillment of Doctor of Philosophy degree requirements.

* National Institutes of Health predoctoral fellow.

Physiologic Surface-Active Agents and Drug Absorption VII: Effect of Sodium Deoxycholate on Phenol Red Absorption in the Rat

STUART FELDMAN*, MICHAEL SALVINO, and MILO GIBALDI

Abstract □ The influence of sodium deoxycholate on the absorption of phenol red in the rat was studied using three techniques to assess absorption—*viz.*, urinary excretion data after oral administration to intact animals, loss of drug from *in situ* intestinal loops, and transfer of drug across the isolated everted intestine. Each of the methods provided evidence that the bile salt markedly enhances the absorption of phenol red by altering the permeability of the intestinal membranes. In the intact rat, these effects appear to be reversible.

Keyphrases □ Phenol red absorption, rat—sodium deoxycholate effect □ Sodium deoxycholate—effect on phenol red absorption, rat □ Colorimetric analysis—phenol red

The role of bile salts in the intestinal solubilization and absorption of fats and fat-soluble vitamins has been studied extensively (1–3). Recently, it has been reported that bile salts may also enhance the intestinal absorption of poorly lipid-soluble substances. Mayersohn *et al.* (4) reported a 1.5- to 1.8-fold increase in the urinary recovery of riboflavin in man when sodium deoxycholate was administered 0.5 hr. prior to the oral ingestion of the vitamin. The results suggest that the bile salt enhancement of riboflavin absorption may be due to changes in the permeability of the gastrointestinal membranes to the transport of the vitamin. Feldman and Gibaldi (5, 6) have shown that relatively low micellar concentrations of sodium taurodeoxycholate markedly increase the permeability of everted rat intestine to salicylate ion.

The purpose of the present investigation was to determine the effects of an unconjugated bile salt, sodium deoxycholate (SDC), on the absorption of a water-soluble, poorly absorbed compound, phenol red, in the rat using several experimental techniques to assess absorption.

EXPERIMENTAL

Absorption Studies in Intact Rats—Male Sprague-Dawley rats weighing between 200 and 300 g. were used in all experiments. The animals were fasted 24 hr. (with water allowed *ad libitum*) prior to gastric intubation of 1.5 ml. of a 1-mg./ml. phenol red solution in distilled water containing 100 m*M* sodium deoxycholate (SDC).

Table II—Inhibition of DNA, RNA, Protein, and Lipid Synthesis by Quinine (Q) and Quinidine (QD)

		Concentration, moles/l. × 10^4 1.3	2.5	3.8
		Mean % Inhibition ± *SE*[a]		
DNA synthesis	Q	34 ± 3.5	69 ± 6.1	79 ± 2.6
	QD	32 ± 5.7	75 ± 2.1	78 ± 3.2
RNA synthesis	Q	33 ± 0.5	55 ± 2.4	62 ± 1.9
	QD	36 ± 2.9	56 ± 2.1	61 ± 1.9
Protein synthesis	Q	51 ± 3.9	60 ± 0.2	73 ± 1.4
	QD	50 ± 3.4	63 ± 3.8	71 ± 1.1
Lipid synthesis	Q	8 ± 1.8	14 ± 3.3	27 ± 4.1
	QD	7 ± 2.3	15 ± 1.8	35 ± 3.7

[a] Each value from four determinations. In no instance is p less than 0.2 for any pair of values.

same. The difference in antifibrillatory action of the drugs may be due in part to differences in chemical structure which, in turn, may reflect cardiac receptor specificity.

REFERENCES

(1) J. V. Taggart, D. P. Earle, Jr., R. W. Berliner, C. G. Zubrod, W. J. Welch, N. B. Wise, E. F. Schroeder, I. M. London, and J. A. Shannon, *J. Clin. Invest.*, **27,** 80(1948).

(2) K. F. Wenckelbach, *J. Amer. Med. Ass.*, **81,** 472(1923).

(3) O. H. Scherbaum and E. Zeuthen, *Exp. Cell Res.*, **6,** 221 (1954).

(4) K. A. Conklin, S. C. Chou, and S. Ramanathan, *Pharmacology*, **2,** 247(1969).

(5) J. E. Byfield and O. H. Scherbaum, *Anal. Biochem.*, **17,** 434(1966).

(6) J. E. Byfield, H. Henze, and O. H. Scherbaum, *Life Sci.*, **6,** 1099(1967).

(7) S. C. Chou and S. Ramanathan, *ibid.*, **7,** 1953(1968).

(8) J. E. Byfield and O. H. Scherbaum, *Science*, **156,** 1504 (1967).

(9) E. Uyeki, E. Geiling, and K. Dubois, *Arch. Int. Pharmacodyn. Ther.*, **97,** 191(1954).

(10) J. Webb, P. Saunders, and K. Nakamura, *J. Pharmacol. Exp. Ther.*, **101,** 287(1951).

(11) L. Crevasse and J. C. Shipp, *Amer. Heart J.*, **68,** 667(1964).

(12) M. E. Hess and N. Hangaard, *Circ. Res.*, **6,** 256(1958).

(13) J. W. Moulder, *J. Infec. Dis.*, **85,** 195(1949).

(14) E. Haas, *J. Biol. Chem.*, **155,** 321(1944).

(15) J. D. Fulton, *Ann. Trop. Med. Parasitol.*, **32,** 77(1938).

(16) K. A. Conklin, S. C. Chou, and S. Ramanathan, *Fed. Proc.*, **28,** 361(1969).

(17) S. C. Chou, S. Ramanathan, and W. C. Cutting, *Pharmacology*, **1,** 60(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 20, 1969, from the *Department of Pharmacology, School of Medicine, University of Hawaii, Honolulu, HI 96816*

Accepted for publication January 6, 1970.

This work submitted by K. A. Conklin in partial fulfillment of Doctor of Philosophy degree requirements.

* National Institutes of Health predoctoral fellow.

Physiologic Surface-Active Agents and Drug Absorption VII: Effect of Sodium Deoxycholate on Phenol Red Absorption in the Rat

STUART FELDMAN*, MICHAEL SALVINO, and MILO GIBALDI

Abstract □ The influence of sodium deoxycholate on the absorption of phenol red in the rat was studied using three techniques to assess absorption—*viz.*, urinary excretion data after oral administration to intact animals, loss of drug from *in situ* intestinal loops, and transfer of drug across the isolated everted intestine. Each of the methods provided evidence that the bile salt markedly enhances the absorption of phenol red by altering the permeability of the intestinal membranes. In the intact rat, these effects appear to be reversible.

Keyphrases □ Phenol red absorption, rat—sodium deoxycholate effect □ Sodium deoxycholate—effect on phenol red absorption, rat □ Colorimetric analysis—phenol red

The role of bile salts in the intestinal solubilization and absorption of fats and fat-soluble vitamins has been studied extensively (1–3). Recently, it has been reported that bile salts may also enhance the intestinal absorption of poorly lipid-soluble substances. Mayersohn *et al.* (4) reported a 1.5- to 1.8-fold increase in the urinary recovery of riboflavin in man when sodium deoxycholate was administered 0.5 hr. prior to the oral ingestion of the vitamin. The results suggest that the bile salt enhancement of riboflavin absorption may be due to changes in the permeability of the gastrointestinal membranes to the transport of the vitamin. Feldman and Gibaldi (5, 6) have shown that relatively low micellar concentrations of sodium taurodeoxycholate markedly increase the permeability of everted rat intestine to salicylate ion.

The purpose of the present investigation was to determine the effects of an unconjugated bile salt, sodium deoxycholate (SDC), on the absorption of a water-soluble, poorly absorbed compound, phenol red, in the rat using several experimental techniques to assess absorption.

EXPERIMENTAL

Absorption Studies in Intact Rats—Male Sprague-Dawley rats weighing between 200 and 300 g. were used in all experiments. The animals were fasted 24 hr. (with water allowed *ad libitum*) prior to gastric intubation of 1.5 ml. of a 1-mg./ml. phenol red solution in distilled water containing 100 m*M* sodium deoxycholate (SDC).

Table I—Total Urinary Excretion of Phenol Red in Individual Rats after Oral Administration of a 1.5-mg. Dose with and without 150 μmole Sodium Deoxycholate (SDC)

Rat	Control, % of Dose	SDC, % of Dose
1	4.9	8.0
2	3.2	9.7
3	2.9	11.7
4	4.6	10.9
5	2.7	6.3
Mean ±*SD*	3.7 ± 1.0	9.3 ± 2.2

A solution of 1 mg./ml. phenol red served as the control. The studies were performed in a crossover fashion with each animal serving as his own control. Following intubation, the animal was placed in a restraining cage. Urine was collected quantitatively every hour for 8 hr. and then at convenient intervals until no measurable levels of phenol red could be detected in the urine (16–24 hr.). Urination was induced by causing the rats to inhale ether vapors for a few seconds. The collected urine was assayed colorimetrically for phenol red using methods described previously (7). The total amount of phenol red excreted in each urine sample was determined by means of standard curves. In a second set of experiments, the test animals were injected intraperitoneally with 1.5 ml. of a 1-mg./ml. phenol red solution. Immediately following the injection, 1.5 ml. of 100 m*M* SDC was administered by gastric intubation.

The same animals were used in control studies in which the phenol red injection was followed by gastric intubation of 1.5 ml. distilled water in place of the bile salt. Urine was collected and assayed as described previously.

Absorption Studies from *In Situ* Intestinal Loops—The method of Levine *et al.* (8) was utilized to study the *in situ* absorption of phenol red from the rat small intestine. Fasted Sprague-Dawley rats weighing between 195 and 240 g. were used for this portion of the study. The animals were anesthetized with ether and maintained under light anesthesia during the surgical procedures. A midline incision was made, the small intestine was located, and two loops, each 5.08 cm. (2 in.) long, were formed. The first loop was approximately 15.24 cm. (6 in.) from the pylorus with 2.54 cm. (1 in.) of intestine separating the two consecutive loops. One-half milliliter of a 1-mg./ml. phenol red solution in the presence or absence of SDC was injected into a loop, through a ligature, by means of a syringe and blunt needle. In experiments utilizing 100 m*M* SDC, the loops in any animal contained either phenol red or phenol red plus the bile salt. The solutions were placed in the intestinal loops in a crossover fashion. The midline incision was then closed and the animals were allowed to recover. The animals were sacrificed after 3 hr. and the loops were excised, homogenized,[1] and assayed for phenol red content according to Levine (9). The percent of phenol red absorbed from each loop was calculated after correcting for assay recovery based on the amount of phenol red recovered from loops after 3 hr. *in vitro* incubation experiments in pH 7.2 buffer at 37°.

Drug Transfer in Isolated Everted Rat Intestine—Two consecutive everted rat small intestine segments, each 10 cm. in length, were prepared by methods previously described (5). Each segment was suspended in 80 ml. of mucosal solution consisting of modified Krebs-Henseleit buffer at pH 7.4 containing 0.2 mg./ml. phenol red and, in bile salt experiments, 100 m*M* SDC. Since both phenol red and SDC are negatively charged at pH 7.4, little or no interaction was expected between the two compounds. All solutions were adjusted to 150 m*M* Na^+ by the addition of sodium chloride.

The mucosal solution was oxygenated continuously by a mixture of oxygen–carbon dioxide (95:5) and maintained at 37 ± 0.1° by means of a water bath. The serosal solution consisted of 2 ml. of modified Krebs-Henseleit buffer, pH 7.4. The experimental procedure for sampling of the serosal solution has been discussed previously (5). Each experiment was run over a 2-hr. period, and the consecutive segments were placed in either the phenol red control buffer or phenol red–SDC–buffer in a crossover fashion.

[1] Eberbach homogenizer, Eberbach Corp.

The serosal samples were diluted appropriately with distilled water, alkalinized with 3 *N* NaOH, and assayed for phenol red spectrophotometrically at 560 mμ. Neither SDC nor "blank" fluid interfered with the assay procedure.

RESULTS

Absorption in Intact Rats—Table I shows the urinary recovery of phenol red, expressed as percent of dose, after oral administration of 1.5 ml. of a 1-mg./ml. phenol red solution in the presence or absence of 150 μmole SDC. The results show that coadministration of the bile salt with phenol red results in an approximately threefold increase in the urinary recovery of phenol red. Differences are significant at the 99.5% level of confidence as determined by Student's *t* test (10). A representative plot of the excretion rate of phenol red *versus* time for both experimental situations is presented in Fig. 1.

Studies in which phenol red was injected intraperitoneally and SDC given orally indicate that the bile salt has no influence on the urinary recovery of phenol red. The mean percent of phenol red recovered in the urine after oral administration of the bile salt was 41.2 ± 11.3% (mean ±*SD*, four rats), essentially identical to the control value of 42.4 ±11.7% (mean ±*SD*, four rats).

This finding rules out an effect of the bile salt on the net distribution and elimination patterns of phenol red and suggests that the increase in urinary recovery of phenol red is due to increased gastrointestinal absorption of the compound in the presence of the bile salt.

Absorption from Intestinal Loops—It was of interest to consider the absorption of phenol red from a well-defined section of the rat small intestine. The intestinal loop preparation was well suited for these experiments. Utilization of this technique made it possible to study the absorption of phenol red in the presence and absence of the bile salt without the complicating problems of enterohepatic circulation and intestinal transit. Also, it was possible by using this technique to control the initial concentration of bile salt present at a specific site within the intestinal lumen.

The results obtained from the intestinal loop experiments are presented in Table II. The percent of phenol red absorbed in control studies was 5.6%. This value is in good agreement with the value of 7% reported by Levine (9). In the presence of 10 m*M* sodium deoxycholate, the mean percent absorbed was 24.3%. A still larger increase in phenol red absorption was observed in the presence of 100 m*M* SDC (69.3%).

Phenol Red Transfer across Everted Rat Intestine—The effect of SDC on the intestinal transfer of phenol red is noted in Table III. The results are consistent with the findings of the *in vivo* and *in*

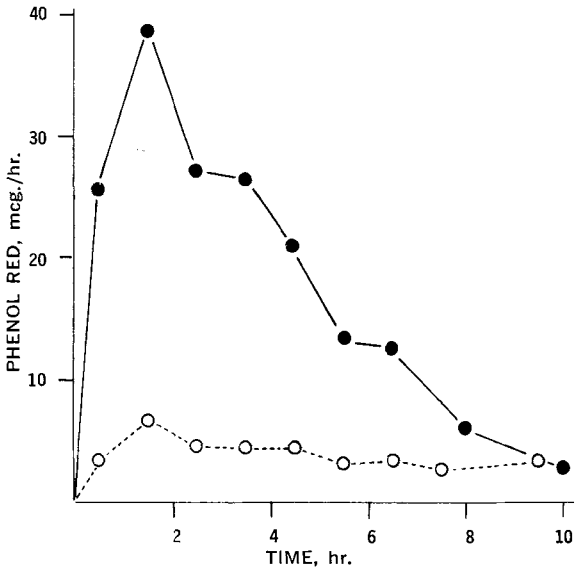


Figure 1—*Urinary excretion rates of phenol red after oral administration with and without sodium deoxycholate (SDC). Key:* ●, *SDC; and* ○, *control.*

Table II—Effect of Sodium Deoxycholate (SDC) on the Absorption of Phenol Red from Rat Intestinal Loops

Solution	No. of Loops	Absorbed ±*SD*, %
Control	3	5.6 ± 1.2
10 m*M* SDC	6	24.3 ± 15.1
100 m*M* SDC	3	69.3 ± 3.5

situ studies. The bile salt produced a 75% increase in the steady-state transfer rate of the drug across the isolated tissue preparation.

DISCUSSION

Each of the three methods used to assess absorption of phenol red in the rat provided evidence that sodium deoxycholate at concentration exceeding the critical micelle concentration markedly alters the permeability of the gastrointestinal membranes to this poorly absorbed compound. It is of particular interest to note that the *in vitro* technique, *i.e.*, the everted intestinal preparation, provided an excellent indication of *in vivo* absorption phenomenon with respect to the bile salt effect. Until recently, the isolated everted intestine technique has been used almost exclusively for studying active accumulation and transport of various nutrients. The present study indicates that this *in vitro* technique may also have considerable value in studying drug absorption which usually involves passive rather than active processes.

Care, of course, must be used in extrapolating the results of any *in vitro* model to predict results in the intact biologic system. Invariably, quantitative differences are noted and, in some instances, qualitative differences may be observed as well. The isolated everted intestine appears to be considerably more permeable to phenol red than the intact intestine. The clearance of phenol red from the mucosal solution, *i.e.*, the steady-state transfer rate divided by the mucosal concentration, is about 0.8 ml./hr. This value is of the same order of magnitude as the clearance of salicylate—*viz.*, 1.2 ml./hr. (5). However, the rate of intestinal absorption of salicylate is several orders of magnitude greater than that of phenol red. Chalfin *et al.* (11), Tidball *et al.* (12), and Baker *et al.* (13) previously found the small intestine to be considerably more permeable when everted than when not everted. Despite this significant qualitative shortcoming of the everted gut technique with respect to phenol red transfer, the effect of the bile salt on membrane permeability is as adequately demonstrated with this preparation as with the other techniques employed.

An important qualitative difference observed between the isolated and intact rat intestine is the ability to reverse the bile salt effect on membrane permeability. The authors have previously demonstrated that the effect of bile salts on the permeability of the everted intestine was not reversible (6). However, the results of *in vivo* absorption studies in rats suggest that this may not be the case in the intact intestine. Oral dosing studies carried out in a crossover fashion gave no indication that the effects of the orally administered bile salt were irreversible. Urinary excretion data obtained from control studies in two rats, 3 days after each had received SDC, were essentially identical to those obtained from control studies in rats who had no previous exposure to SDC.

The intestinal loop experiments summarized in Table II indicate a dose-dependent effect of the bile salt on the intestinal membranes. One-half milliliter of a 10 m*M* SDC solution resulted in a mean absorption rate that was four times greater than control values. The results were extremely variable from loop-to-loop as indicated by the very large coefficient of variation, *i.e.*, about 60%. This finding is in contrast to the twelvefold increase in phenol red absorption (coefficient of variation of 5%) produced by a 100 m*M* solution of SDC. The 5-μmole dose of SDC may be in the range of the minimum effective dose (MED) of deoxycholate needed to alter intestinal permeability while the 50-μmole dose is clearly above the MED.

Table III—Effect of 100 m*M* Sodium Deoxycholate (SDC) on the Steady-State Transfer Rate of Phenol Red across the Everted Rat Small Intestine—Mucosal Drug Concentration Maintained at 0.2 mg./ml.

Rat	Control, mcg./min.	SDC, mcg./min.
1	2.72	4.41
2	2.62	4.90
3	2.38	4.18
Mean ±*SD*	2.57 ± 0.17	4.50 ± 0.37

During the course of these studies, it was observed that there was an increase in the volume of fluid within the intestinal loop after the 3-hr. exposure to 100 m*M* SDC. This is consistent with the earlier observation of Feldman *et al.* (14) who reported an increase in fluid volume within the gastric pouch after exposure to 26 m*M* SDC. Despite the rather marked influx of fluid into the intestinal lumen, enhanced absorption of phenol red occurred in the presence of the bile salt. In control studies, little fluid remained within the intestinal lumen after the 3-hr. absorption period, indicating significant net water absorption in the absence of the bile salt.

The mechanism of action of the bile salt in altering the permeability of the intestinal tract to various drugs is still not certain. It has been suggested that the mechanism may involve an interaction of the bile salt micelle with membrane lipid, particularly phospholipid (15). This aspect of the problem is under further investigation.

REFERENCES

(1) A. M. Dawson, *Brit. Med. Bull.*, **23,** 247(1967).

(2) A. F. Hofmann, *Gastroenterology*, **50,** 56(1966).

(3) A. F. Hofmann, *Biochem. J.*, **89,** 57(1963).

(4) M. Mayersohn, S. Feldman, and M. Gibaldi, *J. Nutr.*, **98,** 288(1969).

(5) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **58,** 425(1969).

(6) *Ibid.*, **58,** 967(1969).

(7) S. Feldman and M. Gibaldi, *Gastroenterology*, **54,** 918 (1968).

(8) R. M. Levine, M. R. Blair, and B. B. Clark, *J. Pharmacol. Exp. Ther.*, **114,** 78(1955).

(9) R. R. Levine, *ibid.*, **131,** 328(1961).

(10) G. W. Snedecor and W. G. Cochran, "Statistical Methods," 6th ed., Iowa State University Press, Ames, Iowa.

(11) D. Chalfin, I. L. Cooperstein, and C. A. M. Hogben, *Proc. Soc. Exp. Biol. Med.*, **99,** 746(1958).

(12) C. S. Tidball, T. R. Liebross, S. Thomas, and M. M. Cassidy, *Physiologist*, **10,** 325(1967).

(13) R. D. Baker, S. Watson, J. L. Long, and M. J. Wall, *Biochem. Biophys. Acta*, **173,** 192(1969).

(14) S. Feldman, R. J. Wynn, and M. Gibaldi, *J. Pharm. Sci.*, **57,** 1493(1968).

(15) S. Feldman and M. Gibaldi, *Proc. Soc. Exp. Biol. Med.*, **132,** 1031(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 7, 1969, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication November 6, 1969.

This investigation was supported in part by Grant AM-11498 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

The authors thank Mr. Ralph J. Wynn for his technical assistance in certain phases of this study.

* Present address: Temple University, School of Pharmacy, Philadelphia, PA 19140

Determination of Dialkyltriazenoimidazoles by Nonaqueous Titration

PAUL D. STERNGLANZ

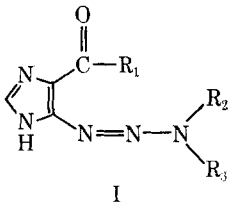
Abstract □ An analytical method for dialkyltriazenoimidazoles has been developed. It serves as a reliable purity check on these anticancer drugs whose effectiveness is a function of their purity. The method consists of titration in nonaqueous media with perchloric acid. The titrant, perchloric acid, is dissolved in a nitroethane–chlorobenzene mixture. Samples are dissolved in glacial acetic acid or in acetonitrile. Poor solubility of some of the compounds is overcome by the selection of an appropriate solvent system, chosen to give also optimum titration end-points. The electrode system consists of conventional glass and calomel electrodes. The method is directly applicable to the unstable 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide, without interference by its transformation product, 1-(2-chloroethyl)-3-[5(or 4)-carbamoylimidazol-4(or 5)-yl]-*v*-triazolinium chloride, usually present in small amounts.

Keyphrases □ Dialkyltriazenoimidazoles—analysis □ Anticancer drugs—purity determination □ Potentiometric nonaqueous titration—analysis

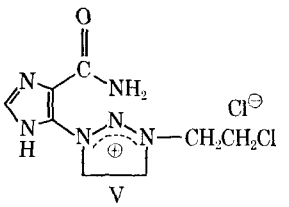
Dialkyltriazenoimidazoles (1, 2) (I) exemplified by II–IV have shown much promise as useful chemotherapeutic drugs (3–7). 5(or 4)-(3,3-Dimethyl-1-triazeno)imidazole-4(or 5)-carboxamide (II) is receiving clinical trial as an anticancer agent (8, 9). 5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (IV) is highly active against experimental leukemia (4, 7), and methyl 5(or 4)-(3,3-dimethyl-1-triazeno)-imidazole-4(or 5)-carboxylate (III) has shown activity as both an antileukemic and an antimicrobial agent (5). For these reasons, a simple assay method that could be applied equally well to all three triazenoimidazole derivatives, as well as to other derivatives of the general structure of I, would be potentially very useful. In addition, Compound IV is unstable at room temperature, but it can be preserved for months at −15°. Its transformation product (V) has been assigned (10) the structure of a *v*-triazolinium salt formed by internal alkylation. Since the transformation product V has no demonstrated biological activity, it is important that its presence does not influence the assay of IV.

A colorimetric method for dialkyltriazenoimidazoles in plasma and urine has been reported recently (11). However, this method may not distinguish between IV and V and is less suitable as an assay method of solid samples. In contrast, the proposed method is applicable equally well to the three imidazole derivatives II, III, or IV; neither the transformation product V nor lactose, a formulating agent for oral administration, interferes. The method is based on a modification (12) of the nonaqueous titration of weak bases with perchloric acid.

Solutions of Compounds III and IV are prepared in acetonitrile and of Compound II in glacial acetic acid, respectively, the choice being dictated by solubility. The titrant is perchloric acid dissolved in acetic acid or in a nitroethane–chlorobenzene mixture. Either titrant can be used for the titration of Compounds II and III. However, perchloric acid must be dissolved in a nitroethane–chlorobenzene mixture for the titration of Compound IV. This titrant, recently recommended by Huber (12), has not been applied to any extent in nonaqueous titrimetry. The special titrant was chosen because the presence of glacial acetic acid causes poor end-points in titrations of Compound IV and mixtures of IV and V.



I



V

II, $R_1 = NH_2$, $R_2 = R_3 = CH_3$
III, $R_1 = OCH_3$, $R_2 = R_3 = CH_3$
IV, $R_1 = NH_2$, $R_2 = R_3 = CH_2CH_2Cl$

EXPERIMENTAL

Apparatus—A Corning model 12 research pH meter (with expanded scale) was used with a Sargent/Jena combination electrode (E. H. Sargent, item S-30072-15 or miniature S-30070-10). The aqueous KCl in the reference electrode compartment was replaced by 0.1 *M* $LiClO_4$ in isopropanol.

Preparation of Titrant (12)—Place about 500 ml. of nitroethane in a 1000-ml. volumetric flask; cool in an ice bath; and add 2.1 ml. perchloric acid (72% w/w) and then, dropwise, 5 ml. acetic anhydride. Fill to the mark with chlorobenzene. Let stand overnight. Standardize against either potassium acid phthalate (National Bureau of Standards Sample No. 84) or against 1,3-diphenylguanidine (Standard Grade, G. F. Smith Chemical Co., Columbus, Ohio).

Procedure—Use 0.05 to 0.07-mmole sample; dissolve III or IV in 15 to 20 ml. acetonitrile and II in 15 to 20 ml. glacial acetic acid. The compounds are only partially soluble but will dissolve completely during titration under magnetic stirring. Titrate with standard 0.025 *N* perchloric acid to the potentiometric end-point.

The titrant is introduced from a 5-ml. buret with 0.01-ml. subdivisions.

If mixtures of Compound IV and lactose are analyzed, the sample size should be adjusted so that the lactose will not exceed 350 mg. Although the lactose is insoluble in acetonitrile and is not expected to interfere, amounts exceeding 350 mg. have been found to affect the electrode response adversely.

RESULTS AND DISCUSSION

Preliminary experiments had shown that the solubility of the imidazole derivatives in solvents suitable for titration was poor; for example, less than 0.1% of Compound IV dissolves in acetonitrile. This seemed to be an obstacle for a titration method but, fortunately, the undissolved particles of the imidazole derivatives go into solution while the stirred suspension is being titrated.

Poorly soluble compounds are generally not assumed to be analyzable with the same precision and accuracy as those of good solubility because titration end-points are less distinct in dilute solu-

Table I—Assay of Triazenoimidazole Derivatives by Nonaqueous Titration

	% Purity Found	Standard Deviation	Number of Determinations
Compound II[a]	99.1	0.79	5
Compound III[a]	99.7	0.20	5
Compound IV[b]	96.8	0.55	5

[a] Absence of impurities in Compounds II and III was confirmed by elemental analysis and TLC. [b] Contamination of IV by V was found by direct determination of V; the amount of V ranged from 2.5 to 4.2% (13).

tion. However, Table I proves that the precision and accuracy of the proposed method are good.

In Table II the analyses of synthetic mixtures of IV with lactose, and of IV with V and lactose, are shown. Comparison of the amount of Compound IV actually present (indicated in Column 4) and found (indicated in Column 5) shows that neither lactose nor Compound V interferes in the analysis of IV. The weighed amount of IV (in Column 1) differs from the actual amount present (in Column 4) because IV contains an average amount of 3% transformation product V.

Compounds II, III, and IV are weaker bases than imidazole (pKa′ = 7.20 for imidazolium ion in water) (14). The base-weakening effect is attributed to the electronegativity of the triazeno and carboxamide or carboxylate groups. The bis(2-chloroethyl)triazeno group of Compound IV is much more base weakening than the dimethyltriazeno group of Compounds II and III.

Glacial acetic acid cannot be used in the titration of IV for two reasons: Compound IV is too weak a base and, therefore, poor titration breaks would be obtained and, furthermore, the transformation product V, present as an impurity in IV, interferes. Bases too weak to be titratable in acetic acid often become titratable in acetic anhydride. This is the case with Compound IV, and good titration breaks are obtained; but acetic anhydride cannot be used in this particular analysis either, because the transformation product V would be simultaneously titrated with IV.

In acetonitrile, the situation is different; Compound IV is sufficiently basic to be titratable (if acetic acid is absent) while the transformation product V remains neutral. Perchloric acid in acetonitrile would be a good titrant but it is unstable. Perchloric acid in acetic acid is very stable but causes poor end-points in the titration of IV. Perchloric acid in nitroethane–chlorobenzene gives the best results and is therefore used.

Table II—Titration of Mixtures of IV and V and Lactose[a]

% IV Weighed	% V Weighed	% V Actual[b]	% IV Actual[b]	% IV Found
100	0			97.3
100	0			97.0
85.6	14.4	17.0	83.0	82.5
69.4	30.6	32.7	67.3	66.8
49.4	50.6	52.1	47.9	48.0
21.0	79.0	79.6	20.4	20.4
14.4	85.6	86.0	14.0	14.4

[a] Lactose (330 mg.) was added to all six samples. [b] The values for "% V Actual" and "% IV Actual" are based on an average assay of 97% purity (or presence of 3% V in the starting material IV, see Table I).

REFERENCES

(1) Y. F. Shealy, C. A. Krauth, and J. A. Montgomery, *J. Org. Chem.*, **27**, 2150(1962).

(2) Y. F. Shealy, C. A. Krauth, S. J. Clayton, A. T. Shortnacy, and W. R. Laster, Jr., *J. Pharm. Sci.*, **57**, 1562(1968).

(3) Y. F. Shealy, J. A. Montgomery, and W. R. Laster, Jr., *Biochem. Pharmacol.*, **11**, 674(1962).

(4) Y. F. Shealy and C. A. Krauth, *Nature*, **210**, 208(1966).

(5) Y. F. Shealy, C. A. Krauth, R. F. Pittillo, and D. E. Hunt, *J. Pharm. Sci.*, **56**, 147(1967).

(6) I. Wodinsky, J. Swiniarski, and C. J. Kensler, *Cancer Chemother. Rep.*, **52**, 393(1968).

(7) G. Hoffman, I. Kline, M. Gang, D. D. Tyrer, J. M. Venditti, and A. Goldin, *ibid.*, **52** (Part 1), 715(1968).

(8) C. McDonald, N. Wollner, F. Ghavimi, and J. Zweig, *Proc. Amer. Ass. Cancer Res.*, **8**, 43(1967).

(9) T. L. Loo, E. A. Stasswender, J. A. Jardine, and E. Frei, III, *ibid.*, **8**, 42(1967).

(10) D. J. Abraham, J. S. Rutherford, and R. D. Rosenstein, *J. Med. Chem.*, **12**, 189(1969).

(11) T. L. Loo and E. A. Stasswender, *J. Pharm. Sci.*, **56**, 1016 (1967).

(12) W. Huber, *Z. Anal. Chem.*, **216**, 260(1966).

(13) R. H. James, P. D. Sternglanz, and Y. F. Shealy, *J. Pharm. Sci.*, **58**, 1193(1969).

(14) C. J. Hawkins and D. D. Perrin, *J. Chem. Soc.*, **1962**, 1351.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *Southern Research Institute, Birmingham, AL 35205*

Accepted for publication December 29, 1969.

This work was supported by Contract PH43-64-51 with Chemotherapy, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The author thanks Dr. Y. F. Shealy for the imidazole derivatives and for helpful criticism.

After the procedures had been devised, some of the titrations were performed by Greenwood Laboratories, Chadds Ford, Pa.

Structure–Activity Relation in Organophosphorus-Inhibited Acetylcholinesterase Reactivators III: Methiodides of Hydroxyimino Derivatives of Pyridylethanes

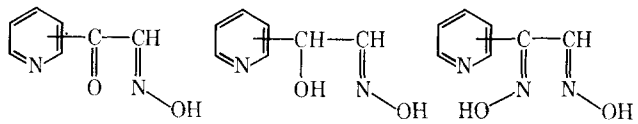
PALMARISA FRANCHETTI*, MARIO GRIFANTINI, and MARIA L. STEIN

Abstract □ The methiodides derived from 2-hydroxyimino-1-pyridyl-ethan-1-ones, 2-hydroxyimino-1-pyridyl-1-hydroxyethanes, and 1,2-di(hydroxyimino)-1-pyridylethanes were tested as reactivators of phosphorylated acetylcholinesterase. The most active member, 2-hydroxyimino-1-(2′-pyridyl)-ethan-1-one, displays a rate of reactivation, in the case of tetraethyl pyrophosphate poisoning, slightly higher than that of the well-known reactivator 2-hydroxyiminomethyl-1-methyl-pyridinium iodide.

Keyphrases □ Acetylcholinesterase reactivators—structure–activity relationship □ Pyridylethane derivatives (methiodide salts)—synthesis □ Reactivators—organophosphorus-inhibited acetylcholinesterase □

It has recently been shown (1, 2) that methiodides of pyridine oximes, containing the ketoxime group on a position of the side chain one carbon atom distant from the ring, are able to reactivate organophosphorus-inhibited acetylcholinesterase. The compounds hitherto considered were 2-hydroxyimino derivatives of 1-pyridylethanes further substituted in position 2 by phenyl or amide groups; because these groups can shield the hydroxyimino group in relation to the active site of the enzyme, it was of interest to replace them with hydrogen.

Therefore, the methiodides derived from the three isomers of each of the following types of bases were synthesized: *anti*-2-hydroxyimino-1-pyridyl-ethan-1-ones (I), 2-hydroxyimino-1-pyridyl-1-hydroxyethanes (II), and *anti*-1,2-di(hydroxyimino)-1-pyridylethanes (III).



Ia, b, c
a = 2-pyridyl–

IIa, b, c
b = 3-pyridyl–

IIIa, b, c
c = 4-pyridyl–

Wilson *et al.* (3) have already examined the reactivating property of the methiodide salts of 1-(3′-pyridyl)-2-hydroxyimino-ethan-1-one and 1-(4′-pyridyl)-2-hydroxyimino-ethan-1-one, finding them less active than 2-hydroxyiminomethyl-1-methyl-pyridinium iodide (2-PAM). The picolinic isomer is a new compound, and its synthesis makes it possible to correlate the activities of the three isomeric methiodides (IV*a–c*) to their structure. It should be noted that Wilson *et al.* have proposed for Products I*b* and I*c* the configuration *syn* to the keto group; however, the authors find that I*b* and I*c*, like the new Compound I*a*, form complexes with copper and nickel salts and, by reaction with hydroxylamine, give the *anti*-1,2-di(hydroxyimino)-1-pyridylethanes. The *anti*-configuration should therefore be assigned to all the isomers I (4).

The methiodide salts of Compounds II and III were synthesized to ascertain how the biological activity varies with changing of the carbonyl adjacent to the ring.

EXPERIMENTAL[1]

***anti*-2-Hydroxyimino-1-(2′-pyridyl)-ethan-1-one (I*a*)**—To a stirred solution of 12 g. of isopentyl nitrite, cooled with ice, 2.3 g. of Na dissolved in 50 ml. of anhydrous EtOH was added dropwise. Stirring was continued for 12 hr.; 250 ml. of water was then added and the solution was extracted with ether. The water solution was acidified to pH 5–6 with dilute acetic acid and extracted with ethyl acetate; after evaporation of the solvent, the residual oil was chromatographed on silica gel, eluting with ethyl acetate. By evaporation of the first eluates, a product was obtained which was recrystallized from benzene; m.p. 113–115°.

Anal.—($C_7H_6N_2O_2$) C,H,N. $\lambda_{max.}^{EtOH}$ (log ε): 254 mμ (4.06).

The product gives colored precipitates with copper and nickel salts.

***anti*-1-Hydroxy-1-pyridyl-2-hydroxyiminoethanes (II*a–c*)**—To 0.3 g. of sodium borohydride in 4 ml. of water and 5 ml. of methanol, 1 g. of the corresponding α-hydroxyiminoketone in 20 ml. of methanol was added dropwise with stirring. After 2 hr. the solution was neutralized with dilute acetic acid and reduced under vacuum to one-fourth of its original volume. The solution was extracted with ethyl acetate, and the products were purified by chromatography on silica gel (ethyl acetate–methanol, 80:20) followed by crystallization from ethyl acetate–ethyl ether; m.p. II*a* 111–112°, II*b* 99–101°, and II*c* 97–99°.

Anal.—($C_7H_8N_2O_2$) C,H,N.

***anti*-1,2-Di(hydroxyimino)-1-pyridylethanes (III*a–c*)**—One gram of Compound I was refluxed for 2 hr. with 0.55 g. of hydroxylamine hydrochloride in 30 ml. of EtOH. The solution was then diluted with water, made basic with aqueous $NaHCO_3$, and extracted with ethyl acetate. The residue obtained after evaporation of the solvent was crystallized from EtOH; m.p. III*a* 143–145°, III*b* 173–175°, and III*c* 184–187°.

Anal.—($C_7H_7N_3O_2$) C,H,N.

The *anti*-configuration of the dioximes was established on the basis of the red-orange colored complexes obtained with the nickel salts (5).

***N*-Methyl Pyridinium Iodides (Table I)**—The Products IV*a* and V*a* were obtained by heating the corresponding bases in anhydrous EtOH with methyl iodide at 60° for 48 hr. in sealed vessels; the residue obtained by evaporation of the solvent was washed with ethyl acetate and crystallized. The Products V*b*, VI*a*, VI*b*, and VI*c* were obtained in a similar manner, performing the reaction in acetone at room temperature; V*c* was obtained from II*c* in ether after 10 days of reaction. Products V*a* and V*c* were very hygroscopic.

All the quaternary salts were subjected to biological assay. The *in vitro* reactivating velocity of acetylcholinesterase inhibited by tetraethyl pyrophosphate (TEPP) or diisopropyl phosphorofluoridate (DFP) was measured according to the technique described

[1] Melting points are uncorrected. When no unusual spectral features were observed with the compounds described, no absorption peaks are reported. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values.

Table I—1-Methylpyridinium Iodides

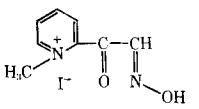
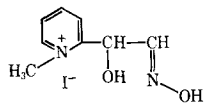
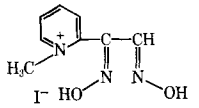
No.	Compd.	Crystn.	M.p.	Formula	Anal.
IV*a*		Me_2CO–MeOH	142–143°	$C_8H_9IN_2O_2$	C, H, N
V*a*		Me_2CO	102–104°	$C_8H_{11}IN_2O_2$	C, H, N
V*b*		MeOH–Et_2O	130–132°	$C_8H_{11}IN_2O_2$	C, H, N
V*c*		Me_2CO	115–117°	$C_8H_{11}IN_2O_2$	C, H, N
VI*a*		MeOH–EtOAc	183–185°	$C_8H_{10}IN_3O_2$	C, H, N
VI*b*		MeOH–EtOAc	156–158°	$C_8H_{10}IN_3O_2$	C, H, N
VI*c*		MeOH–EtOAc	178–180°	$C_8H_{10}IN_3O_2$	C, H, N

Table II—Reactivation of Inhibited Bovine Erythrocyte Acetylcholinesterase by Means of Oximes IV–VI (pH 7.4 and 25°)[a,b]

Oximes (Iodides), 5×10^{-3} *M*	pKa′	pKa″	Inhibiting Group: Diethyl phosphoryl $k_{obs.}$	Diethyl phosphoryl Relative Rate Constant	Diisopropyl phosphoryl $k_{obs.}$	Diisopropyl phosphoryl Relative Rate Constant
2-PAM	7.9		1.1×10^{-2}	1	1.7×10^{-3}	1
IV*a*	6.3		1.6×10^{-2}	1.47	8.3×10^{-4}	0.49
IV*b* (2)	7.2		1.3×10^{-2}	1.21	4.5×10^{-4}	0.26
IV*c* (2)	7.1		4.1×10^{-3}	0.38	8.1×10^{-4}	0.47
V*a*	9.8		None	—	None	—
V*b*	9.9		None	—	None	—
V*c*	9.8		None	—	None	—
VI*a*	7.3	9.8	2.6×10^{-3}	0.23	1.4×10^{-4}	0.08
VI*b*	8.1	9.3	9.2×10^{-4}	0.08	1.3×10^{-4}	0.075
VI*c*	7.5	8.7	1.8×10^{-3}	0.16	1.2×10^{-4}	0.07

[a] $k_{obs.}$ is in $min.^{-1}$. [b] pKa values were obtained by potentiometric titration and, for overlapping values, by application of the calculation method due to Noyes, as given by Albert and Serjeant (7).

by Ashani *et al.* (6). The results are shown in Table II, together with the values of pKa for the same compounds.

RESULTS AND DISCUSSION

The values of the hydrolysis rate in Table II indicate that pyridine derivatives, containing a hydroxyimino group in the β-position of the side chain, show reactivating ability. This is particularly evident in the series of Compounds IV. Among the three positional isomers the most active is the picolinic derivative, which, together with the nicotinic one, has an activity slightly higher than that of 2-PAM in the case of TEPP poisoning. The nearly equivalent activity for the two isomers is remarkable, in contrast to what is observed with the pyridine aldoxime methiodides where the nicotinic isomer is inactive (8). For DFP-poisoned acetylcholinesterase, the three isomers show nearly equivalent activity.

Structural modifications of the carbonyl group lead to a diminution of activity that can be ascribed to the different nucleophilicity of the hydroxyimino groups. This is particularly evident in the series of Compounds V; reduction of the carbonyl function to an alcoholic one results in a strong diminution of acidity, which is probably responsible for the inactivity of these compounds. The introduction of a second hydroxyimino group generally leads to less active compounds in comparison to the ketones.

From these results, compared with those discussed in the preceding notes (1, 2), it is possible to conclude that voluminous groups attached to the terminal carbon atom of the side chain in this type of compound decrease the ability to reactivate inhibited acetylcholinesterase; a shielding effect on the active site of the enzyme by the phenyl and arylamide groups present in the compounds described in preceding notes can then be postulated.

REFERENCES

(1) M. Grifantini, S. Martelli, and M. L. Stein, *J. Pharm. Sci.*, **58**, 460(1969).

(2) P. Franchetti, M. Grifantini, and S. Martelli, *ibid.*, **58**, 857 (1969).

(3) I. B. Wilson, S. Ginsburg, and C. Quan, *Arch. Biochem. Biophys.*, **77**, 286(1958).

(4) Houben-Weyl, "Methoden der organischen Chemie," Band X/4 Stickstoff-Verbindugen I, Teil 4, Georg. Thieme Verlag, Stuttgart, Germany, 1968, p. 294.

(5) H. Diel, "The Application of the Dioximes to Analytical Chemistry," G. F. Smith Chemical Co., Columbus, Ohio, 1940.

(6) Y. Ashani, H. Edery, J. Zahavy, W. Künberg, and S. Cohen, *Isr. J. Chem.*, **3**, 133(1965).

(7) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen and Co. Ltd., London, England (1962).

(8) I. B. Wilson, *Fed. Proc.*, **18**, 752(1959).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 8, 1969, from the *Institute of Pharmaceutical and Organic Chemistry, The University of Camerino, Camerino, Italy.*

Accepted for publication December 29, 1969.

This investigation was supported by the Italian National Research Council.

* Miss Franchetti was granted a fellowship of the Italian National Research Council.

Independence of Antimineralocorticoid and Catatoxic Effects of Various Steroids

B. SOLYMOSS, M. KRAJNY*, and S. VARGA

Abstract □ Several steroids were assayed by means of Kagawa's test to determine whether the catatoxic or detoxifying effect depends upon antimineralocorticoid potency. In adult male rats, ethylestrenol, norbolethone, progesterone, triamcinolone, prednisolone, and hydroxydione were found free of antimineralocorticoid effects at the dose of 1 mg. Similar results were obtained in young female rats pretreated with as much as 10 mg. of these steroids. Thus, this type of nonspecific resistance-increasing effect of certain steroids appears to be unrelated to antimineralocorticoid potency.

Keyphrases □ Steroids—antimineralocorticoid, catatoxic effects, independence □ Antimineralocorticoid activity—steroids □ Catatoxic activity—steroids □ Atomic absorption spectroscopy—analysis

Recently it was observed in this laboratory that pretreatment with antimineralocorticoid or anabolic steroids diminishes the anesthesia induced by pentobarbital, progesterone, or hydroxydione and prevents the toxicity of digitoxin, dimethylbenz(a)anthracene, indomethacin, and a large variety of unrelated compounds (1–3). This protection is at least partly due to the induction of drug-metabolizing enzymes since spironolactone, norbolethone, or ethylestrenol increases the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent aliphatic hydroxylation of pentobarbital and hexobarbital as well as the *N*-dealkylation of aminopyrine in the hepatic microsomes (4, 5).

The pharmacologic classification of steroid hormones and their derivatives is based upon the fact that some of their actions are separable and independent whereas others are merely inseparable subordinate manifestations of one of these basic activities (1, 6). The stimulation of drug-metabolizing enzymes has already been attributed to different hormonal actions. In studying the role of androgens, Quinn *et al.* (7) observed that the enzymatic degradation of hexobarbital is faster in males than in females. Booth and Gillette (8) found that the activation of drug-metabolizing enzymes by testosterone derivatives more closely parallels their anabolic than their androgenic activity. Regarding the role of female sex hormones, it was found that chronic treatment of ovariectomized rats with progesterone increases the hepatic detoxification of chlorpromazine (9). Remmer (10) noted that glucocorticoids also influence the enzymatic degradation of drugs.

It is of interest whether the catatoxic effect of steroids is dependent on one of their specific hormonal properties. Obviously, not all these steroids share androgenic, anabolic, luteoid, or glucocorticoid activities. However, depending on dosage and under special circumstances, anabolic steroids and progesterone can reveal antimineralocorticoid properties (11). To determine whether the antimineralocorticoid activity is a common function of catatoxic steroids, the authors investigated the effect of norbolethone, ethylestrenol, and progesterone as well as of triamcinolone or prednisolone in Kagawa's test (12), and compared the results with the classic antimineralocorticoid action of spironolactone and SC-11927. The hormonally inactive hydroxydione was used as a control. In a repetition of this experiment on young female rats, the steroids were administered in the same manner and at the same dosage as in the previous detoxification studies (1–5).

MATERIALS AND METHODS

First Experiment—One hundred and forty-four male Sprague-Dawley rats of the Holtzman Farms (Madison, Wis.) with a mean initial body weight of 150 g. (range 140–160 g.) were bilaterally adrenalectomized, under ether anesthesia, by the lumbar route and maintained on sucrose and tap water. Approximately 24 hr. postoperatively the rats were divided and treated as indicated in Table I. Sodium chloride[1] (2.5 ml. of 0.85% solution), desoxycorticosterone acetate (DOC-Ac)[2] (6 or 12 mcg. in 0.2 ml. Mazola corn oil), as well as spironolactone,[3] SC-11927,[3] ethylestrenol,[4] norbolethone,[5] progesterone and prednisolone,[6] triamcinolone,[7] or hydroxydione[8] (1 mg. in 0.5 ml. Mazola corn oil) were administered subcutaneously.

The bladder of the animal was emptied by applying slight pressure before placing four or five of them in a metabolic cage. During the period of urine collection, no food or water was provided. Voiding was again induced at the end of a 4-hr. period for complete recovery of urine. Using the Unicam SP 90 atomic absorption spectrophotometer, pooled specimens were analyzed three times for total sodium and potassium excretion (13); these values were used for the calculation of the log (Na × 10)/K ratio. Blocking activity was measured by reversal of the Na/K effect of DOC, as described by Kagawa (12).

Second Experiment—One hundred and seventeen female Sprague-Dawley rats of the Holtzman Farms with a mean initial body weight of 100 g. (range 90–110 g.) were divided and treated as indicated in Table II immediately after adrenalectomy. Spironolactone, SC-11927, ethylestrenol, norbolethone, progesterone, triamcinolone, prednisolone, or hydroxydione was administered twice daily for 3 days (10 mg. in 1 ml. H_2O *per os*). Groups 13–22 were maintained on Purina Laboratory Chow and 0.85% NaCl as drinking fluid during the first 2 days of this pretreatment; on the 3rd day they received sucrose cubes and tap water. Approximately 72 hr. postoperatively, the pretreatment was followed by the administration of NaCl, DOC, and various steroids as well as by urine collection and electrolyte determinations, as described in the first experiment.

The differences between the Na/K excretion values were statistically evaluated by variance analysis.

RESULTS

The end-point for antimineralocorticoid activity is based on the log urinary Na/K ratio, which may reflect various changes in the in-

[1] Fisher Scientific Co.
[2] Ciba Co. Ltd.
[3] G. D. Searle & Co.
[4] Organon Inc.
[5] Wyeth Laboratories Inc.
[6] Schering Corp.
[7] Lederle Laboratories
[8] Pfizer Ltd.

Table I—The Effect of Various Steroids on Desoxycorticosterone Acetate-Induced Urinary Electrolyte Excretion in Kagawa's Test

Group	Number of Animals	Treatment[a] Steroid, 1 mg.	Treatment[a] DOC-Ac, mcg.	Urinary Excretion[b] μequiv. of Na	Urinary Excretion[b] μequiv. of K	log Na × 10/K Ratio in Urine
1	27	—	—	960	230	1.63
2	9	—	6	770[c]	360[c]	1.33[c]
3	27	—	12	590[c]	400[c]	1.17[c]
4[d]	9	Spironolactone	12	790[e]	320	1.39[e]
5	9	SC-11927	12	760[e]	310	1.39[e]
6	18	Ethylestrenol	12	480[e]	360	1.13
7	9	Norbolethone	12	340[e]	260	1.11
8	9	Progesterone	12	370[e]	270	1.14
9	9	Triamcinolone	12	860[e]	1590[e]	0.73[e]
10	9	Prednisolone	12	910[e]	1790[e]	0.71[e]
11	9	Hydroxydione	12	620	400	1.08

[a] In addition all animals were adrenalectomized 24 hr. earlier and were injected with 2.5 ml. 0.85% NaCl s.c. at the beginning of urine collection. [b] Total electrolyte excretion in sample of nine animals. [c] = $p < 0.05$ as compared with Group 1. [d] Groups 4–7 received the most potent catatoxic steroids. [e] = $p < 0.05$ as compared with Group 3.

dividual cations. Accordingly, Tables I and II list both Na and K means in order that distinctive electrolyte effects of the steroids can be surfaced for interpretation.

First Experiment (Table I)—Compared with the adrenalectomized controls (Group 1), 6 mcg. of DOC-Ac (Group 2) lowered the urinary log (Na × 10)/K ratio and 12 mcg. (Group 3) was even more active ($p < 0.05$ as compared with Group 2). As expected, both spironolactone (Group 4) and SC-11927 (Group 5) reversed the effect of 12 mcg. DOC-Ac. No significant antimineralocorticoid activity was revealed by ethylestrenol (Group 6), norbolethone (Group 7), progesterone (Group 8), or hydroxydione (Group 11). Triamcinolone (Group 9) and prednisolone (Group 10) even further diminnished the urinary Na/K ratio, already decreased by 12 mcg. DOC-Ac (Group 3).

Second Experiment (Table II)—In comparison to the adrenalectomized controls (Group 12), 6 mcg. of DOC-Ac (Group 13) also significantly lowered the urinary log (Na × 10)/K ratio in young female rats, and 12 mcg. (Group 14) was still more active ($p < 0.05$ as compared with Group 13). Spironolactone (Group 15) or SC-11927 (Group 16) counteracted the effect of 12 mcg. DOC-Ac (Group 14). None of the other steroids (Groups 17–22) revealed antimineralocorticoid properties.

DISCUSSION

Kagawa (11) showed that the influence of spironolactone and its derivatives on renal electrolyte excretion is due to a specific antagonism of mineralocorticoid effects. Thus, as expected, spironolactone and SC-11927 reversed the action of 12 mcg. DOC-Ac in both experiments.

It was demonstrated that high doses of testosterone and of many of its derivatives have antimineralocorticoid properties; 18,19-dinortestosterone proved to be active even in small amounts (11). Under the experimental conditions reported here, ethylestrenol or norbolethone failed to show antimineralocorticoid activity, even when a high-dose level was maintained over several days as in the second experiment. Progesterone also proved to possess a DOC-Ac blocking potency similar to that of spironolactone; however, it also resembled DOC-Ac in its effect on electrolyte excretion when given alone and in large doses (14). This complex action and the differences in dosage might explain why progesterone failed to exhibit antimineralocorticoid effects in the experiments. In agreement with expectations, triamcinolone, prednisolone, or hydroxydione was completely devoid of any antimineralocorticoid activity in both experiments. After the administration of triamcinolone or prednisolone, there was a marked decrease in urinary log (Na × 10)/K ratio in male (first experiment) but not in female rats (second experiment). This is probably due to the rats receiving large doses of these steroids for 3 days, thus leading to potassium depletion; therefore, in the actual Kagawa test the last dose of triamcinolone or prednisolone did not so markedly influence potassium excretion.

The lack of correlation between the anabolic, androgenic, luteoid, or glucocorticoid activity of some steroids and their resistance-increasing effect against various compounds was obvious without detailed investigations. The present results demonstrate that even under the experimental conditions used in the detoxification experiments (1–5), the highly active microsomal enzyme inducers, ethylestrenol and norbolethone, do not share the antimineralocorticoid activity of spironolactone and its derivatives. It is noteworthy that spironolactone pretreatment prevents the anesthetic effect of progesterone, DOC-Ac, and hydroxydione, even after bilateral nephrectomy (15). Thus, the catatoxic effect of spironolactone is also manifest under conditions in which its classic antimineralocorticoid property could not play any role.

Table II—The Effect on Kagawa's Test of High Doses of Various Steroids Administered to Young Female Rats

Group	Number of Animals	Treatment[a] Steroid, 10 mg.	Treatment[a] DOC-Ac, mcg.	Urinary Excretion[b] μequiv. of Na	Urinary Excretion[b] μequiv. of K	log Na × 10/K Ratio in Urine
12	18	—	—	1250	450	1.44
13	9	—	6	1010[c]	520	1.29[c]
14	18	—	12	800[c]	630[c]	1.11[c]
15[d]	9	Spironolactone	12	1320[e]	480[e]	1.44[e]
16	9	SC-11927	12	1300[e]	510[e]	1.41[e]
17	18	Ethylestrenol	12	940[e]	700	1.13
18	9	Norbolethone	12	590[e]	480	1.09
19	9	Progesterone	12	630[e]	480	1.12
20	9	Triamcinolone	12	1090[e]	850[e]	1.11
21	9	Prednisolone	12	950[e]	830[e]	1.06
22	9	Hydroxydione	12	750	630	1.08

[a] In addition all animals were adrenalectomized 72 hr. earlier and then treated with steroids (10 mg. twice daily) and given 2.5 ml. 0.85% NaCl s.c. at the beginning of urine collection. [b] Total electrolyte excretion in sample of nine animals. [c] = $p < 0.05$ as compared with Group 12. [d] Groups 15–18 received the most potent catatoxic steroids. [e] = $p < 0.05$ as compared with Group 14.

These results support the view that the catatoxic action of different steroids is not subordinate to any presently known specific hormonal action (1).

REFERENCES

(1) H. Selye, *Can. Med. Ass. J.*, **101**, 51(1969).

(2) H. Selye, *Can. J. Physiol. Pharmacol.*, **47**, 981(1969).

(3) K. Kovacs and A. Somogyi, *Proc. Soc. Exp. Biol. Med.*, **131**, 1350(1969).

(4) B. Solymoss, H. G. Classen, and S. Varga, *ibid.*, **132**, 940 (1969).

(5) B. Solymoss, S. Varga, and H. G. Classen, to be published.

(6) H. Selye, "Textbook of Endocrinology," 2nd ed., Acta Inc., Montreal, Canada, 1947.

(7) G. P. Quinn, J. Axelrod, and B. B. Brodie, *Biochem. Pharmacol.*, **1**, 152(1958).

(8) J. Booth and J. R. Gillette, *J. Pharmacol. Exp. Ther.*, **137**, 374(1962).

(9) T. S. Miya, E. H. Adams, and R. Saunders, 113th Annual Meeting, APhA, Dallas, Texas, 1966.

(10) H. Remmer, *Arch. Exp. Pathol. Pharmacol.*, **233**, 184(1958).

(11) C. M. Kagawa, in "Methods of Hormonal Research," R. J. Dorfman, Ed., vol. 3, Academic, New York, N. Y., 1964, p. 351.

(12) C. M. Kagawa, *Endocrinology*, **67**, 125(1960).

(13) Unicam Instruments Ltd., Unicam absorption methods for the Unicam SP 90 atomic absorption spectrophotometer, Series 1, Cambridge, England, 1966.

(14) C. M. Kagawa, *Proc. Soc. Exp. Biol. Med.*, **99**, 705(1958).

(15) H. Selye, *Steroids*, **13**, 803(196).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 27, 1969, from the *Institut de Médecine et de Chirurgie Experimentales, Université de Montreal, Montreal, Canada*

Accepted for publication December 16, 1969.

This work was supported by grants from the Ministére de la Santé, Québec, and from the Medical Research Council of Canada (Block Term Grant MT-1829). The authors wish to thank the companies mentioned in the *Materials and Methods* section for their contributions of the drugs used. The technical assistance received from Mrs. C. Jubert and Miss E. Aclément is also gratefully acknowledged.

* Postdoctoral Fellow of M.R.C. Quebec.

Subcutaneous Absorption Kinetics of Benzyl Alcohol II

BERTON E. BALLARD and EFRAIM MENCZEL*

Abstract □ Under multiple-dosing conditions at a subcutaneous site, equations were derived which permit one to estimate the number of doses, n, required to approach within ± 1% (or any other fixed fraction) of the asymptotic minimum level: $n \geq 3.3219 [(t_{0.5})/\tau] \log_{10} Q$, where $Q > +1$. Here $t_{0.5}$ is the absorption half-life of benzyl alcohol from a subcutaneous absorption cell, τ is the dosing interval, Q (always positive) equals $(\beta - 1)/(\alpha - 1)$, α equals $B^{(n)}_{\text{min.}}/B^{\infty}_{\text{min.}}$ (and is 0.99 or 1.01 in this example), and β equals $B''/B^{\infty}_{\text{min.}}$, where B'' equals $Bi - Bm$. Definitions: $B^{(n)}_{\text{min.}}$ = amount of benzyl alcohol in the cell per unit area of subcutaneous tissue one τ after the nth dose, $B^{\infty}_{\text{min.}}$ = asymptotic minimum amount of benzyl alcohol in the cell per unit area, Bi = initial dose of benzyl alcohol per unit area, and Bm = constant maintenance dose of benzyl alcohol per unit area ($Bi \geq Bm$). The benzyl alcohol disappears from the cell in an apparent monoexponential manner.

Keyphrases □ Benzyl alcohol—subcutaneous absorption □ Kinetics—benzyl alcohol, subcutaneous absorption □ Equations—dosage numbers/subcutaneous area for asymptotic minimum level

In a recent report from this laboratory (1), a multiple-dosing procedure was used in connection with a study of the subcutaneous (s.c.) absorption kinetics of benzyl alcohol (BA) dissolved in normal saline (NS). The BA in NS solution was contained in a glass absorption cell affixed to the moist s.c. tissue of an anesthetized rat by a silicone adhesive. The solution was kept homogeneous by means of a vibrating stirrer. The use of this cell made it possible to control the area of s.c. tissue exposed to the drug solution at all times and to sample the cell's contents periodically.

Although the number of doses per unit area (p.u.a.) of s.c. tissue needed to approach within ±1% of the asymptotic minimum value were shown in Table IV of the previous report (1), details of the mathematical calculations were omitted. The purpose of this note is to derive the equations needed to make this estimation.

THEORETICAL

Under multiple-dosing conditions, where the mean volume of drug solution in the cell was nearly constant throughout the experiment, and the drug was administered at the times zero, τ, 2τ, 3τ, ..., the following equation can be derived (1):

$$Bc = B' \frac{(1 - e^{-Pn\tau})}{(1 - e^{-P\tau})} \qquad \text{(Eq. 1)}$$

where Bc in this case is the amount of drug in the cell p.u.a. just after the administration of the nth dose p.u.a. In Eq. 1 it is assumed that the drug disappears from the cell in an apparent monoexponential manner and the initial dose p.u.a., Bi, equals the constant maintenance dose(s) p.u.a., Bm, in magnitude. Thus

$$B' = Bi = Bm \qquad \text{(Eq. 2)}$$

The term P in Eq. 1 is the mean penetration coefficient having the units of time^{-1}, n is the integer number of initial and maintenance doses administered, and τ is the constant dosing time interval.

When Bi is larger than Bm, then

$$Bi = B'' + Bm \qquad \text{(Eq. 3)}$$

where B'' is the amount of drug p.u.a. in the cell administered along with Bm as a part of the initial dose p.u.a. and $Bi \geq Bm$. The amount

These results support the view that the catatoxic action of different steroids is not subordinate to any presently known specific hormonal action (1).

REFERENCES

(1) H. Selye, *Can. Med. Ass. J.*, **101**, 51(1969).

(2) H. Selye, *Can. J. Physiol. Pharmacol.*, **47**, 981(1969).

(3) K. Kovacs and A. Somogyi, *Proc. Soc. Exp. Biol. Med.*, **131**, 1350(1969).

(4) B. Solymoss, H. G. Classen, and S. Varga, *ibid.*, **132**, 940 (1969).

(5) B. Solymoss, S. Varga, and H. G. Classen, to be published.

(6) H. Selye, "Textbook of Endocrinology," 2nd ed., Acta Inc., Montreal, Canada, 1947.

(7) G. P. Quinn, J. Axelrod, and B. B. Brodie, *Biochem. Pharmacol.*, **1**, 152(1958).

(8) J. Booth and J. R. Gillette, *J. Pharmacol. Exp. Ther.*, **137**, 374(1962).

(9) T. S. Miya, E. H. Adams, and R. Saunders, 113th Annual Meeting, APhA, Dallas, Texas, 1966.

(10) H. Remmer, *Arch. Exp. Pathol. Pharmacol.*, **233**, 184(1958).

(11) C. M. Kagawa, in "Methods of Hormonal Research," R. J. Dorfman, Ed., vol. 3, Academic, New York, N. Y., 1964, p. 351.

(12) C. M. Kagawa, *Endocrinology*, **67**, 125(1960).

(13) Unicam Instruments Ltd., Unicam absorption methods for the Unicam SP 90 atomic absorption spectrophotometer, Series 1, Cambridge, England, 1966.

(14) C. M. Kagawa, *Proc. Soc. Exp. Biol. Med.*, **99**, 705(1958).

(15) H. Selye, *Steroids*, **13**, 803(196).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 27, 1969, from the *Institut de Médecine et de Chirurgie Experimentales, Université de Montreal, Montreal, Canada*

Accepted for publication December 16, 1969.

This work was supported by grants from the Ministére de la Santé, Québec, and from the Medical Research Council of Canada (Block Term Grant MT-1829). The authors wish to thank the companies mentioned in the *Materials and Methods* section for their contributions of the drugs used. The technical assistance received from Mrs. C. Jubert and Miss E. Aclément is also gratefully acknowledged.

* Postdoctoral Fellow of M.R.C. Quebec.

Subcutaneous Absorption Kinetics of Benzyl Alcohol II

BERTON E. BALLARD and EFRAIM MENCZEL*

Abstract □ Under multiple-dosing conditions at a subcutaneous site, equations were derived which permit one to estimate the number of doses, n, required to approach within ± 1% (or any other fixed fraction) of the asymptotic minimum level: $n \geq 3.3219 [(t_{0.5})/\tau] \log_{10} Q$, where $Q > +1$. Here $t_{0.5}$ is the absorption half-life of benzyl alcohol from a subcutaneous absorption cell, τ is the dosing interval, Q (always positive) equals $(\beta - 1)/(\alpha - 1)$, α equals $B^{(n)}_{\text{min.}}/B^{\infty}_{\text{min.}}$ (and is 0.99 or 1.01 in this example), and β equals $B''/B^{\infty}_{\text{min.}}$, where B'' equals $Bi - Bm$. Definitions: $B^{(n)}_{\text{min.}}$ = amount of benzyl alcohol in the cell per unit area of subcutaneous tissue one τ after the nth dose, $B^{\infty}_{\text{min.}}$ = asymptotic minimum amount of benzyl alcohol in the cell per unit area, Bi = initial dose of benzyl alcohol per unit area, and Bm = constant maintenance dose of benzyl alcohol per unit area ($Bi \geq Bm$). The benzyl alcohol disappears from the cell in an apparent monoexponential manner.

Keyphrases □ Benzyl alcohol—subcutaneous absorption □ Kinetics—benzyl alcohol, subcutaneous absorption □ Equations—dosage numbers/subcutaneous area for asymptotic minimum level

In a recent report from this laboratory (1), a multiple-dosing procedure was used in connection with a study of the subcutaneous (s.c.) absorption kinetics of benzyl alcohol (BA) dissolved in normal saline (NS). The BA in NS solution was contained in a glass absorption cell affixed to the moist s.c. tissue of an anesthetized rat by a silicone adhesive. The solution was kept homogeneous by means of a vibrating stirrer. The use of this cell made it possible to control the area of s.c. tissue exposed to the drug solution at all times and to sample the cell's contents periodically.

Although the number of doses per unit area (p.u.a.) of s.c. tissue needed to approach within ±1% of the asymptotic minimum value were shown in Table IV of the previous report (1), details of the mathematical calculations were omitted. The purpose of this note is to derive the equations needed to make this estimation.

THEORETICAL

Under multiple-dosing conditions, where the mean volume of drug solution in the cell was nearly constant throughout the experiment, and the drug was administered at the times zero, τ, 2τ, 3τ, ..., the following equation can be derived (1):

$$Bc = B' \frac{(1 - e^{-Pn\tau})}{(1 - e^{-P\tau})} \qquad \text{(Eq. 1)}$$

where Bc in this case is the amount of drug in the cell p.u.a. just after the administration of the nth dose p.u.a. In Eq. 1 it is assumed that the drug disappears from the cell in an apparent monoexponential manner and the initial dose p.u.a., Bi, equals the constant maintenance dose(s) p.u.a., Bm, in magnitude. Thus

$$B' = Bi = Bm \qquad \text{(Eq. 2)}$$

The term P in Eq. 1 is the mean penetration coefficient having the units of time^{-1}, n is the integer number of initial and maintenance doses administered, and τ is the constant dosing time interval.

When Bi is larger than Bm, then

$$Bi = B'' + Bm \qquad \text{(Eq. 3)}$$

where B'' is the amount of drug p.u.a. in the cell administered along with Bm as a part of the initial dose p.u.a. and $Bi \geq Bm$. The amount

of drug in the cell p.u.a. at any time, Bc, can be calculated from

$$Bc = B''e^{-Pt} + Bm\left[\frac{(1 - e^{-P\tau})}{(1 - e^{-P\tau})}\right]e^{-PT} \qquad \text{(Eq. 4)}$$

where t is clock time starting at zero following the administration of the first dose p.u.a., and T is a new clock time starting just after the administration of the last maintenance dose.

The minimum amount of drug p.u.a. in the cell,[1] $B^{(n)}_{\text{min.}}$, one τ after the administration of the nth dose p.u.a., can be calculated from

$$B^{(n)}_{\text{min.}} = B''e^{-Pn\tau} + Bm\left[\frac{(1 - e^{-Pn\tau})}{(1 - e^{-P\tau})}\right]e^{-P\tau} \qquad \text{(Eq. 5)}$$

After a very large (or infinite) number of maintenance doses p.u.a. has been administered, according to Eq. 5, the asymptotic minimum amount of drug p.u.a.,[1] $B^{\infty}_{\text{min.}}$, can be calculated from

$$B^{\infty}_{\text{min.}} = Bm\left(\frac{e^{-P\tau}}{1 - e^{-P\tau}}\right) \qquad \text{(Eq. 6)}$$

Let

$$\alpha_n = \frac{B^{(n)}_{\text{min.}}}{B^{\infty}_{\text{min.}}} = \frac{B^{(n)}_{\text{min.}}/V}{B^{\infty}_{\text{min.}}/V} \qquad \text{(Eq. 7)}$$

where α_n is a ratio of amounts or concentrations of drug with V being the constant for cell volume (or apparent distribution volume for the body). In this paper the value of $B^{(n)}_{\text{min.}}$ or $(B^{(n)}_{\text{min.}}/V)$ was arbitrarily set equal to $\pm 1\%$ of $B^{\infty}_{\text{min.}}$ or $(B^{\infty}_{\text{min.}}/V)$, so that the value of α_n in Eq. 7 would be either 0.99 or 1.01, depending upon whether $B^{\infty}_{\text{min.}}$ or $(B^{\infty}_{\text{min.}}/V)$ was being approached from below or above.

Substitution of Eq. 7 into Eq. 5 yields

$$\alpha_n B^{\infty}_{\text{min.}} = B''e^{-Pn\tau} + Bm\left[\frac{(1 - e^{-Pn\tau})}{(1 - e^{-P\tau})}\right]e^{-P\tau} \qquad \text{(Eq. 8)}$$

Rearranging Eq. 8 yields

$$\alpha_n = \left(\frac{B''}{B^{\infty}_{\text{min.}}}\right)e^{-Pn\tau} + \left(\frac{Bm}{B^{\infty}_{\text{min.}}}\right)\left(\frac{1 - e^{-Pn\tau}}{1 - e^{-P\tau}}\right)e^{-P\tau} \qquad \text{(Eq. 9)}$$

Let

$$\beta = \frac{B''}{B^{\infty}_{\text{min.}}} = \frac{B''/V}{B^{\infty}_{\text{min.}}/V} \qquad \text{(Eq. 10)}$$

where β is a ratio of amounts or concentrations of drug. Substitution of Eqs. 6 and 10 into Eq. 9 yields

$$\alpha_n = \beta e^{-Pn\tau} + 1 - e^{-Pn\tau} \qquad \text{(Eq. 11)}$$

or

$$(\alpha_n - 1) = e^{-Pn\tau}(\beta - 1) \qquad \text{(Eq. 12)}$$

For α arbitrarily close to 1, say $|\alpha - 1| = 0.01$, it is desired to find the smallest n such that

$$|\alpha_n - 1| = |e^{-Pn\tau}(\beta - 1)| \leqslant |\alpha - 1| \qquad \text{(Eq. 13)}$$

Solving Eq. 13 for n gives

$$n \geqslant \frac{1}{P\tau}\ln\left(\frac{\beta - 1}{\alpha - 1}\right) \qquad \text{(Eq. 14)}$$

Since $P = \ln 2/t_{0.5}$, substitution of P into Eq. 14 gives

$$n \geqslant \left(\frac{t_{0.5}}{\tau}\right)\left(\frac{1}{\ln 2}\right)\ln\left(\frac{\beta - 1}{\alpha - 1}\right) \qquad \text{(Eq. 15)}$$

Let

$$Q = \left(\frac{\beta - 1}{\alpha - 1}\right) \qquad \text{(Eq. 16)}$$

where Q is always positive and greater than 1.[2] Substitution of Eq. 16 into Eq. 15 yields

$$n \geqslant 3.3219\left(\frac{t_{0.5}}{\tau}\right)\log_{10} Q \qquad \text{(Eq. 17)}$$

The time, t, needed for amounts or concentrations to reach within $\pm 1\%$ of the corresponding asymptotic values one τ after the nth dose is

$$t = n\tau \qquad \text{(Eq. 18)}$$

RESULTS AND DISCUSSION

Table I summarizes the data needed to estimate the doses, n, and the time, t, to approach within $\pm 1\%$ of the asymptotic value $B^{\infty}_{\text{min.}}$ or $(B^{\infty}_{\text{min.}}/V)$.

The methods described are exact when it has been demonstrated that monoexponential loss of drug occurs from a compartment such as a subcutaneous absorption cell. However, when the concentration–time course of drug in a compartment is best described by a polyexponential function (2), the methods described for calculating n and t must be used with caution. Also, other phenomena discussed by Wagner *et al.* (3) may invalidate these procedures for multiple-dosing calculations. Two examples using literature data indicate how Eqs. 17 and 18 can be used to solve for the quantities n and t. The first example involves multiple intramuscular injection of drug; the second involves continuous intravenous infusion of drug.

Example 1—Multiple Intramuscular Injection—Boxer *et al.* (4) injected streptomycin hydrochloride solutions intramuscularly into dogs at regular dosing intervals and followed changes in the plasma drug concentration with time. They derived an equation which permitted them to use the serum drug concentrations obtained at a given time after the initial dose was administered, but before the second dose was administered, to predict the serum drug concentration in the body at the same time interval after the last dose given after an infinite number of doses had been administered. The equation is

$$C_H = C_h\left(\frac{1}{1 - e^{-k\tau}}\right) \qquad \text{(Eq. 19)}$$

where C_H is the actual concentration to be expected at the corresponding time interval after a steady state has been established, C_h is an experimentally determined concentration at a time in the interval between the initial and second doses; k is the rate constant for the disposition of drug in the body, assuming it to be a single compartment; and τ is the dosing interval.

In the derivation of Eq. 19, Boxer *et al.* (4) assumed that the absorption rate of streptomycin hydrochloride from the intramuscular site was extremely (or infinitely) rapid, so that the disappearance of drug from the serum could be described by a monoexponential function. There is evidence to indicate that drug absorption rate from this site is not instantaneous (5–8). However, Eq. 19 can be a useful first approximation for the mathematical analysis of some clinical data involving blood or serum levels of drug.

Data in the left half of Table IV of the Boxer *et al.* paper (4) show that the calculated value of C_h 3 hr. after the initial dose, and before the second dose was given, was 26.3 mcg./ml.; τ is 3 hr., the mean value of k is 0.39 (± 0.028) hr.$^{-1}$; and the biological half-life in the dog is 1.78 hr. According to Eq. 19, the calculated value of C_H is 38.1 mcg./ml. The initial dose equals the maintenance dose ($\beta = 0$), and substitution of these values into Eq. 17 gives the following value for n:

[1] The notation in this paper differs somewhat from that used previously (1). The term $B_{\text{min.}}$ has been changed to $B^{(n)}_{\text{min.}}$ because $B_{\text{min.}}$ in these derivations depends in part upon n. The term $Bc_{\text{min.}}$ has been changed to $B^{\infty}_{\text{min.}}$ to indicate more clearly that the asymptotic minimum amount of drug p.u.a. is evaluated at infinite time.

[2] By appropriate substitution into Eq. 17, where $\beta \geqslant 0$, it follows that when $\alpha > \beta$, then $\alpha = 0.99$ and $Q > +1$. When $\alpha < \beta$, then $\alpha = 1.01$ and $Q > +1$. It also follows that $\alpha \neq \beta$, because Q can never equal 1. Should $Q = 1$, then no doses would be required to reach the desired level, which is an obvious impossibility.

Table I—Number of Total Doses Necessary to Approach within ±1% of $B^{\infty}_{min.}$, and the Constants Needed for Its Calculation[a]

Constant	Animal A	Animal B	Animal C	Animal D
$t_{0.5}$ (hr.)[b]	2.58_4	1.79_5	0.95_7	1.33_4
$(t_{0.5})/\tau$[c]	5.16_8	3.59_0	1.91_4	2.66_8
$B^{\infty}_{min.}$ (mg./cm.²)[d]	7.00_3	4.33_5	2.71_1	3.74_6
Bi (mg./cm.²)[e]	7.39	6.74	5.68	5.47
Bm (mg./cm.²)[f]	1.00_5	0.92_3	1.18_3	1.11_1
B'' (mg./cm.²)[g]	6.38_5	5.81_7	4.49_7	4.35_9
$(\alpha - 1)$[h]	−0.01	+0.01	+0.01	+0.01
$(\beta - 1)$[i]	-0.0882_5	$+0.3418_7$	$+0.6588_7$	$+0.1636_4$
Q[j]	8.82	34.2	65.9	16.4
n[k]	>16 (16.28)	>18 (18.33)	>11 (11.57)	>10 (10.75)
t (hr.)[l]	8	9	5.5	5

[a] For some constants in this table, extra digits were obtained by computational means and are indicated by subscripts. Subscripts are listed to minimize rounding-off errors in subsequent calculations, and do not imply that four significant figure experimental accuracy was achieved. [b] BA absorption half-life in cell. [c] The dosing interval, τ, is 0.5 hr. [d] Calculated from Eq. 6. [e] Initial dose p.u.a. [f] Mean maintenance dose p.u.a. [g] Calculated from Eq. 3. [h] The term α is defined in Eq. 7. [i] The term β is defined in Eq. 10. [j] Calculated from Eq. 16. [k] Number of total doses p.u.a. needed for $B^{(n)}_{min.}$ to approach within ±1% of $B^{\infty}_{min.}$ one τ after the nth dose as calculated from Eq. 17. [l] Time needed for amounts in the cell to reach within ±1% of the asymptotic value of $B^{\infty}_{min.}$ calculated from Eq. 18.

$$n \geq 3.3219\,(1.78/3)\log_{10}(-1/-0.01) \geq 3 \quad \text{(Eq. 20)}$$

Thus, about 3 hr. after three doses have been administered, the concentration of drug in the serum should be within 1% of that found 3 hr. after the last dose given after an infinite number of doses had been administered. While the experimental value of 39.4 mcg./ml. found 3 hr. after three doses had been given does not quite fall within ±1% of the theoretical value of 38.1 mcg./ml., it is close, particularly if one takes into account the uncertainty in the value for k.[3] The time needed to reach this level can be calculated from Eq. 18 and is 9 hr.

Example 2—Continuous Intravenous Infusion—Wagner and Alway (9) studied data obtained from the continuous intravenous infusion of lincomycin hydrochloride to humans. Consider the case where the infusion rate, k_0, is 50,000 mcg./hr.; the apparent half-life for the disappearance of the drug from the body by all processes, assuming that it is a single compartment, is 3.91 hr. (k = 0.177 hr.$^{-1}$); and the volume constant, V, or the apparent volume of distribution is 26,000 ml. Using Eq. 21, it is possible to calculate the concentration, C, of lincomycin in the fluids of distribution at any time:

$$C = \frac{k_0}{Vk}(1 - e^{-kt}) \quad \text{(Eq. 21)}$$

The concentration, C^{∞}, in the body at infinite time is

$$C^{\infty} = \frac{k_0}{Vk} \quad \text{(Eq. 22)}$$

Substituting the appropriate values into Eq. 22, the value of C^{∞} is 10.9 mcg./ml.,[4] and a concentration 1% less than this is 10.8 mcg./ml.

How long would the infusion apparatus have to run to reach the value of 10.8 mcg./ml.? As a first approximation, a continuous intravenous infusion of a drug can be thought of as a process where a large number of small doses are administered at very frequent time intervals (*e.g.*, intravenous drip). For the present discussion, let $\tau = 3.91 \times 10^{-4}$ hr. (1.4 sec.), $t_{0.5}/\tau = 10^4$, and $\alpha = 0.99$. The first dose is equal in magnitude to all succeeding doses, so that $\beta = 0$. The amount of drug administered in the first dose equals $k_0\tau$, and is 19.6 mcg. The number of doses needed to reach the 10.8-mcg./ml. level can be calculated from Eq. 17:

$$n \geq 3.3219\,(10^4)\log_{10}(-1/-0.01) \geq 6.64 \times 10^4 \quad \text{(Eq. 23)}$$

and the time needed to reach the serum concentration of 10.8 mcg./ml. calculated from Eq. 18 is 26.0 hr.

If 200,000 mcg. of lincomycin hydrochloride was injected intravenously in an instantaneous manner into the same subject, and the "continuous" infusion was then begun immediately (1.4 sec. later), how long would it take for the serum concentration of the drug to reach 10.8 mcg./ml.? The value of β can be calculated by substitution into Eq. 10:

$$\beta = \frac{B''/V}{B^{\infty}_{min.}/V} = \frac{(200{,}000 \text{ mcg.} - k_0\tau)/26{,}000 \text{ ml.}}{10.9 \text{ mcg./ml.}} = 0.706 \quad \text{(Eq. 24)}$$

To be mathematically exact in Eq. 24, one maintenance dose, $k_0\tau$, must be subtracted from the 200,000-mcg. initial dose. From a practical standpoint in this example, the $k_0\tau$ term can be ignored because it was made negligibly small compared to the 200,000-mcg. first dose. The α-term remains at 0.99. Substitution of α and β and $t_{0.5}/\tau = 10^4$ into Eqs. 16 and 17 gives a value of n of 4.88×10^4. The time needed for the serum concentration to reach 10.8 mcg./ml. as calculated from Eq. 18 is about 19 hr. Thus, the time needed to approach within −1% of C^{∞} was reduced about 7 hr. when the initial dose was set at a value four times greater than the hourly amount delivered by the infusion apparatus.

[3] The value of k in this report is related to the K found by Boxer *et al.* (4) by the following equation: k = (K ± one standard error) (−2.303) = (−0.17 ± 0.012 hr.$^{-1}$) (−2.303) = 0.39 ± 0.028 hr.$^{-1}$.

[4] This value of C^{∞} has been reached or exceeded in some clinical studies with this drug (10–13).

REFERENCES

(1) B. E. Ballard and E. Menczel, *J. Pharm. Sci.*, **56,** 1476(1967).

(2) S. Riegelman, J. C. K. Loo, and M. Rowland, *ibid.*, **57,** 117 (1968).

(3) J. G. Wagner, J. I. Northam, C. D. Alway, and O. S. Carpenter, *Nature*, **207,** 1301(1965).

(4) G. E. Boxer, V. C. Jelinek, R. Tompsett, R. DuBois, and A. O. Edison, *J. Pharmacol. Exp. Ther.*, **92,** 226(1948).

(5) J. D. Adcock and R. A. Hettig, *Arch. Intern. Med.*, **77,** 179(1946).

(6) E. K. Marshall, Jr., *J. Pharmacol. Exp. Ther.*, **92,** 43(1948).

(7) P. P. Koelzer and J. Giesen, *Aerztl. Wochenschr.*, **5,** 969 (1950).

(8) H. R. Black, R. S. Griffith, and A. M. Peabody, *Ann. N. Y. Acad. Sci.*, **135,** 974(1966).

(9) J. G. Wagner and C. D. Alway, *Nature*, **201,** 1101(1964).

(10) J. J. Vavra, W. T. Sokolski, and J. B. Lawson, *Antimicrob. Ag. Chemother.*, **1963,** 176.

(11) P. Ma, M. Lim, and J. H. Nodine, *ibid.*, **1963,** 183.

(12) H. M. Bellamy, Jr., B. B. Bates, and J. A. Reinarz, *ibid.*, **1966,** 36.

(13) J. A. Reinarz and D. A. McIntosh, *ibid.*, **1965,** 232.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 11, 1969, from the *School of Pharmacy, University of California at San Francisco, San Francisco, CA 94122*, and the * *Hebrew University, Faculty of Medicine, School of Pharmacy, Jerusalem, Israel.*

Accepted for publication December 29, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami meeting, May 1968.

This study was supported in part by research funds of the University of California, a general research support grant FR 05453 from the Division of Research Facilities and Resources, National Institutes of Health, U. S. Public Heath Service, Bethesda, Md., and additional research funds allocated by the Eastern Mediterranean Office of WHO, and approved by the Washington WHO Regional Office (Feb. 1967).

Ethylene Oxide Penetration of the Silicone Coating Used as a Lubricant on Disposable Syringe Rubber Plunger Tips and Hypodermic Needles

YU YIN CHEN

Abstract □ The ability of 100% ethylene oxide to penetrate the silicone coating used on disposable hypodermic needles and syringe rubber plunger tips has been determined. It was shown that ethylene oxide has the ability to penetrate through the silicone coating and thus kill the spores of *Bacillus subtilis* var. *niger*, ATCC 9372, which were introduced underneath the silicone coating.

Keyphrases □ Ethylene oxide penetration—silicone lubricant □ Silicone lubricant effect—ethylene oxide bactericidal action □ *Bacillus subtilis*—ethylene oxide penetration determination

With the introduction of medical devices made from comparatively low melting point plastics, the ability to sterilize these instruments by autoclaving was no longer feasible; subsequently, ethylene oxide has been utilized and found effective (without high temperature) as a chemical sterilant.

Recent usage of silicone as a lubricant for disposable hypodermic needles and syringe rubber plunger tips raised the question of whether the ethylene oxide gas can penetrate the silicone coating and sterilize the surface beneath. It was, therefore, the purpose of this study to test the effectiveness of 100% ethylene oxide [4-hr. cycle, 48.8° (120° F.)] as a penetrating sterilant through a silicone coating. A number of preliminary tests were necessary to establish procedures and suitable materials. In the selection of materials it was necessary to obtain a nonbacteriostatic solvent capable of dissolving silicone (to expose the organisms which were covered by it), due to its immiscibility with the water present in the culture medium. Initial tests indicated *n*-hexane (1), purified (Curtin Co.), was an effective solvent. In turn, methanol (Baker analyzed reagent) was added to increase the miscibility of the *n*-hexane. The bacteriostatic properties of *n*-hexane had to be determined to assure that there was no killing factor other than ethylene oxide.

MATERIALS AND METHODS

Bacillus subtilis var. *niger*, ATCC 9372, was selected as the test organism since its spores are known to be highly resistant to ethylene oxide gas [Beeby and Whitehouse (2); Ernst and Shull (3, 4); Kelsey (5); and Phillips (6)]. Sterile trypticase soy broth (TSB) was used as the culture medium (20 ml. per culture tube), and disposable hypodermic syringes (2.5 ml.) and needles [16 gauge × 3.81 cm. (1.5 in.)] were used as the test samples. Commercial silicone fluid M360 (Dow Corning Co.), a colorless, highly water-repelling, nontoxic, nonvolatile, low-surface tension, and chemically thermally inert substance (1) was used as the coating agent.

Spore Strips Treated with *n*-Hexane—To determine whether *n*-hexane had a bacteriostatic effect on *B. subtilis* var. *niger* spores, three different concentrations (10^3, 10^5, and 10^7) of spore strips (American Sterilizer Co.) were immersed in *n*-hexane for 1 hr. These were then divided into two groups. The first group was cultured in TSB; the other was mixed with 1 ml. of 90% methanol (shaken for 20 min. on an automatic shaker) and then cultured in TSB for 48 hr. at 32°.

Vegetative Cells Treated with *n*-Hexane—To determine the bacteriostatic properties of *n*-hexane on vegetative cells of *B. subtilis* var. *niger*, four separate amounts (0.1, 0.2, 0.5, and 1.0 ml.) of *n*-hexane were mixed with 0.5 ml. of 90% methanol and 0.06 ml.

Table I—Effect of *n*-Hexane on Spore Strips of *B. subtilis* var. *niger*

Concentration of Spores	Total No. of Samples	24-hr. Growth	48-hr. Growth
10^3	5 Without methanol	1 Light growth 4 No growth	1 Heavy growth 4 No growth
10^3	5 With methanol	4 Light growth 1 No growth	4 Heavy growth 1 No growth
10^5	5 Without methanol	5 Medium growth	5 Heavy growth
10^5	5 With methanol	5 Heavy growth	5 Heavy growth
10^7	5 Without methanol	5 Heavy growth	5 Heavy growth
10^7	5 With methanol	5 Medium growth	5 Heavy growth

ACKNOWLEDGMENTS AND ADDRESSES

Received July 11, 1969, from the *School of Pharmacy, University of California at San Francisco, San Francisco, CA 94122*, and the * *Hebrew University, Faculty of Medicine, School of Pharmacy, Jerusalem, Israel.*

Accepted for publication December 29, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami meeting, May 1968.

This study was supported in part by research funds of the University of California, a general research support grant FR 05453 from the Division of Research Facilities and Resources, National Institutes of Health, U. S. Public Heath Service, Bethesda, Md., and additional research funds allocated by the Eastern Mediterranean Office of WHO, and approved by the Washington WHO Regional Office (Feb. 1967).

Ethylene Oxide Penetration of the Silicone Coating Used as a Lubricant on Disposable Syringe Rubber Plunger Tips and Hypodermic Needles

YU YIN CHEN

Abstract □ The ability of 100% ethylene oxide to penetrate the silicone coating used on disposable hypodermic needles and syringe rubber plunger tips has been determined. It was shown that ethylene oxide has the ability to penetrate through the silicone coating and thus kill the spores of *Bacillus subtilis* var. *niger*, ATCC 9372, which were introduced underneath the silicone coating.

Keyphrases □ Ethylene oxide penetration—silicone lubricant □ Silicone lubricant effect—ethylene oxide bactericidal action □ *Bacillus subtilis*—ethylene oxide penetration determination

With the introduction of medical devices made from comparatively low melting point plastics, the ability to sterilize these instruments by autoclaving was no longer feasible; subsequently, ethylene oxide has been utilized and found effective (without high temperature) as a chemical sterilant.

Recent usage of silicone as a lubricant for disposable hypodermic needles and syringe rubber plunger tips raised the question of whether the ethylene oxide gas can penetrate the silicone coating and sterilize the surface beneath. It was, therefore, the purpose of this study to test the effectiveness of 100% ethylene oxide [4-hr. cycle, 48.8° (120° F.)] as a penetrating sterilant through a silicone coating. A number of preliminary tests were necessary to establish procedures and suitable materials. In the selection of materials it was necessary to obtain a nonbacteriostatic solvent capable of dissolving silicone (to expose the organisms which were covered by it), due to its immiscibility with the water present in the culture medium. Initial tests indicated *n*-hexane (1), purified (Curtin Co.), was an effective solvent. In turn, methanol (Baker analyzed reagent) was added to increase the miscibility of the *n*-hexane. The bacteriostatic properties of *n*-hexane had to be determined to assure that there was no killing factor other than ethylene oxide.

MATERIALS AND METHODS

Bacillus subtilis var. *niger*, ATCC 9372, was selected as the test organism since its spores are known to be highly resistant to ethylene oxide gas [Beeby and Whitehouse (2); Ernst and Shull (3, 4); Kelsey (5); and Phillips (6)]. Sterile trypticase soy broth (TSB) was used as the culture medium (20 ml. per culture tube), and disposable hypodermic syringes (2.5 ml.) and needles [16 gauge × 3.81 cm. (1.5 in.)] were used as the test samples. Commercial silicone fluid M360 (Dow Corning Co.), a colorless, highly water-repelling, nontoxic, nonvolatile, low-surface tension, and chemically thermally inert substance (1) was used as the coating agent.

Spore Strips Treated with *n*-Hexane—To determine whether *n*-hexane had a bacteriostatic effect on *B. subtilis* var. *niger* spores, three different concentrations (10^3, 10^5, and 10^7) of spore strips (American Sterilizer Co.) were immersed in *n*-hexane for 1 hr. These were then divided into two groups. The first group was cultured in TSB; the other was mixed with 1 ml. of 90% methanol (shaken for 20 min. on an automatic shaker) and then cultured in TSB for 48 hr. at 32°.

Vegetative Cells Treated with *n*-Hexane—To determine the bacteriostatic properties of *n*-hexane on vegetative cells of *B. subtilis* var. *niger*, four separate amounts (0.1, 0.2, 0.5, and 1.0 ml.) of *n*-hexane were mixed with 0.5 ml. of 90% methanol and 0.06 ml.

Table I—Effect of *n*-Hexane on Spore Strips of *B. subtilis* var. *niger*

Concentration of Spores	Total No. of Samples	24-hr. Growth	48-hr. Growth
10^3	5 Without methanol	1 Light growth 4 No growth	1 Heavy growth 4 No growth
10^3	5 With methanol	4 Light growth 1 No growth	4 Heavy growth 1 No growth
10^5	5 Without methanol	5 Medium growth	5 Heavy growth
10^5	5 With methanol	5 Heavy growth	5 Heavy growth
10^7	5 Without methanol	5 Heavy growth	5 Heavy growth
10^7	5 With methanol	5 Medium growth	5 Heavy growth

Table II—Effect of *n*-Hexane on Vegetative Cells of *B. subtilis* var. *niger*

Amount of *n*-Hexane, ml.	Total No. of Samples	Amount of Organisms, ml.	24-hr. Growth
0.1	10	0.06	All heavy growth
0.1	10	0.005	All heavy growth
0.2	10	0.06	All heavy growth
0.2	10	0.005	All heavy growth
0.5	10	0.06	All heavy growth
0.5	10	0.005	All heavy growth
1.0	10	0.06	All heavy growth
1.0	10	0.005	All heavy growth

Table III—Effect of *n*-Hexane on Spores Suspended in Methanol

Amout of *n*-Hexane, ml.	Total No. of Samples	24-hr. Growth	48-hr. Growth
0.1	10	10 Light growth	10 Heavy growth
0.2	10	8 Heavy growth 2 No growth	8 Heavy growth 2 No growth
0.5	10	8 Heavy growth 2 No growth	10 Heavy growth
1.0	10	8 Heavy growth 2 No growth	10 Heavy growth

of a heavy growth culture of *B. subtilis* var. *niger* and were shaken for 1 hr. on an automatic shaker (Group 1). Following the same method, a second group was mixed with a small amount of the heavy growth culture (0.005 ml.). Both groups were then cultured in TSB for 24 hr. at 32°.

Spores in Methanol Treated with *n*-Hexane—To determine a possible bacteriostatic effect of *n*-hexane on spores in the methanol suspension, spores (15×10^6 per ml.) were diluted with 90% methanol to approximately 1000 spores per ml. Spore samples (1 ml. each) were thoroughly mixed for 30 min. (on automatic shaker) with different amounts (0.1, 0.2, 0.5, and 1.0 ml.) of *n*-hexane and then cultured in TSB for 48 hr. at 32°.

Samples Inoculated with Spores and Coated with Silicone—To determine whether silicone fluid inhibits the germination and/or growth of spores, 20 rubber plunger tips were dipped into a spore suspension (15×10^6 per ml.) and then allowed to air dry for 6 hr. at 25°. A measurement (using Sahli-type hemoglobin pipet to pick up the spore solution adhering to the plunger tip) was made which was 20 λ and contained approximately 300,000 spores adhering to the rubber surface.

In addition, 20 needles were immersed into the 15×10^6 spore solution for 4 hr. and then allowed to air dry for 6 hr. at 25°. The pour plate counting method determined that approximately 32,000 spores were under the coating per needle (number of spores under coating = number of spores rinsed off before coating − number of spores rinsed off after coating). Experimental needles and rubber plunger tips were then coated with silicone (the thickness of the coating and technique were as normally used by the manufacturer, which is a maximum of 1 mg./cm.^{2} of exposed surface area of rubber tip and 1 mg. per needle). Controls were not coated with silicone. All the samples were then cultured in TSB for 24 hr. at 32°.

Inoculated Samples Sterilized by Ethylene Oxide—To determine the ability of 100% ethylene oxide gas to penetrate and sterilize through the silicone coating, the rubber tips and needles were inoculated with spores, coated with silicone (same method as mentioned previously), and assembled as a syringe. The syringes were then sterilized with 100% ethylene oxide with a concentration of approximately 2628 mg./l. for 4 hr. at 48.8° (120°F.) and 4 p.s.i. pressure. Then the experimental group had the silicone coating removed with *n*-hexane, and a control group was left coated. Both groups were cultured in TSB at 32° for 10 days.

Table IV—Effect of Silicone Coating on the Inoculated Samples—Not Sterilized

Treatments	Samples	24-hr. Growth
With coating	10 Plungers	All heavy growth
With coating	10 Needles	All heavy growth
Without coating	10 Plungers	All heavy growth
Without coating	10 Needles	All heavy growth

Table V—Effect of Ethylene Oxide Sterilization on Inoculated Samples

Treatment	Samples	10-Day Cultivation
Coating removed	10 Plungers	All no growth
Coating removed	10 Needles	All no growth
Coating not removed	10 Plungers	All no growth
Coating not removed	10 Needles	All no growth

RESULTS

The results are shown in Tables I through V.

DISCUSSION AND CONCLUSION

The results of Table I show that a concentration of 10^3 spores on paper strips, after immersion in *n*-hexane, was not successfully cultured. The *n*-hexane, being nonsoluble in water, formed a layer in the liquid growth medium; however, when mixed with 90% methanol, *n*-hexane solubility was increased, thereby releasing the spores for germination and growth. There is no indication that *n*-hexane acts as a bacteriostatic agent to both the spores and vegetative forms of *B. subtilis* var. *niger;* therefore, *n*-hexane was used as a solvent for the silicone fluid.

The number of microorganisms involved did not prove to be an influencing factor for the bacteriostatic character of *n*-hexane. The results of Table II indicate that a small number of microorganisms was not inhibited by any of the levels of *n*-hexane.

Results of Table IV indicate that the silicone coating (which was coated by the spray method) did not cover a great number of the inoculated spores (10^6); therefore, the silicone coating was not considered a complete masking factor.

Results of this study indicated that 100% ethylene oxide does penetrate the silicone fluid used as a coating agent and does kill the test organisms present on the surface and beneath the coating.

REFERENCES

(1) Medical Materials Data, Dow Corning, Bulletin 14-003.

(2) M. M. Beeby and C. E. Whitehouse, *J. Appl. Bacteriol.*, **28** 349(1965).

(3) R. R. Ernst and J. J. Shull, *Appl. Microbiol.*, **10,** 337(1962).

(4) *Ibid.*, **10,** 342(1962).

(5) J. C. Kelsey, *J. Clin. Pathol.*, **14,** 59(1961).

(6) C. R. Phillips, *Amer. J. Hyg.*, **50,** 280(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 24, 1969, from the *Laboratory Control Center, Sherwood Medical Industries, Inc., DeLand, FL 32720*

Accepted for publication December 29, 1969.

The author thanks R. Wickham, DeLand Plant Engineer, for the helpful coating technique; Keith Hansen, professor at Stetson University, and D. Root, microbiologist, for their help in the preparation of this manuscript; and J. Slade, Director of Laboratory, for his proofreading and suggestions.

Sterol Metabolism X: Epimeric 23-Hydroxycholesterols

JOHAN E. VAN LIER and LELAND L. SMITH

Abstract □ Synthesis of the epimeric 23-hydroxycholesterols has been achieved by sodium borohydride reduction of 3β-hydroxycholest-5-en-23-one. Tentative configurational assignments were made based on specific rotation data. The configurations of the epimeric 24-hydroxycholesterols are discussed, and a reconsideration of their configurational assignment is suggested.

Keyphrases □ 23-Hydroxycholesterols, epimeric—synthesis □ Absolute configuration—23-hydroxycholesterols, epimeric □ IR spectrophotometry—identification □ Adsorption chromatography—identification

Although naturally occurring 20-, 22-, 24-, 25-, and 26-hydroxysterols have been studied extensively, 23-hydroxysterols have received relatively little attention. 23-Hydroxysterols have limited representation in nature in the fungal metabolite 23*S*-hydroxylanosterol (lanosta-8,24-dien-3β,23*S*-diol)(1), the chiograsterols (2), the plant hormone antheridiol (3), and possibly a 23-hydroxycholesterol (cholest-5-ene-3β,23-diol) sulfate in human infant meconium (4). Other 23-hydroxylated steroids include several bile acids (5), the jervine and veratramine classes of steroidal alkaloids (6), and a variety of triterpenoid compounds (7–18). In view of a continuing interest in the autoxidation of cholesterol in the side chain (19, 20) and in the occurrence of side-chain-oxidized cholesterol derivatives in human tissues (21–23), the authors sought to prepare the as yet undescribed epimeric 23-hydroxycholesterols for record purposes.

THEORETICAL

Sodium borohydride reduction of 3β-hydroxycholest-5-en-23-one (24) gave a mixture of epimeric 3β,23-diols separable by chromatography. Twice the yield of the lower melting, chromatographically more mobile, more levorotatory epimer was obtained compared to the higher melting, more polar, more dextrorotatory epimer. Although the epimeric 3β,23-diols were resolved from one another by adsorption chromatography, they were unresolved on gas chromatography; their corresponding 3β,23-diacetates also were not resolved.

The 23-hydroxycholesterol epimers could be distinguished from one another by means of their IR absorption spectra, but except for minor differences in ion intensity the epimers were not distinguishable by their mass spectra, either as the free 3β,23-diols or as their 3β,23-diacetates. Mass spectra of the 3β,23-diols were characterized by a strong molecular ion (*m/e* 402) and by ions at *m/e* 384 $(M-18)^+$ and *m/e* 369 $(M-33)^+$, representing loss of water and of a methyl radical and water, respectively. Mass spectra of the 3β,23-diacetates exhibited no molecular ion; the highest mass observed, also the base peak, was *m/e* 426 $(M-60)^+$, representing loss of the elements of acetic acid. The next highest mass at *m/e* 366 $(M-120)^+$ corresponds to loss of two molecules of acetic acid.

Assignment of absolute configuration to the epimers may be made by analogy to arguments advanced in assignment of absolute configuration to the epimeric 23-lanosterols (25). Since *S*(+)-4-methylpent-3-en-2-ol and *S*(+)-4-methylpentan-2-ol derived therefrom have the same absolute configuration and sign of specific rotation (26, 27), these two secondary alcohols serving as model compounds for assignment of the respective 23-hydroxylanosterol and 23-hydroxycholesterol derivatives imply that the more dextrorotatory epimers in both sterol series have the same absolute configuration. The absolute configuration of 23-hydroxysterols corresponding to that of *S*(+)-4-methylpentan-2-ol and *S*(+)-4-methylpent-3-en-2-ol is the $23\beta_F$-configuration by Plattner's convention (28). The $23\beta_F$-configuration corresponds to the 23*S*-configuration by the Cahn-Ingold-Prelog sequence rule nomenclature for the more dextrorotatory 23-hydroxylanosterol (25). Because of a change in priority of the C_{22}-steroid residue relative to the terminal isobutyl group of 23-hydroxycholesterol, the more dextrorotatory $23\beta_F$-hydroxycholesterol and the lanost-8-ene-3β,$23\beta_F$-diol(25) derived by catalytic reduction of 23*S*-hydroxycholesterol are of the 23R-configuration.

Other than the as yet undescribed cholest-5-ene-3β,21-diol and (25*S*)-cholest-5-ene-3β,26-diol, all possible cholesterol derivatives hydroxylated in the side chain have now been prepared and absolute configurations assigned. However, previously assigned absolute configurations (29, 30) of the epimeric 22-hydroxycholesterols have been recently reversed, the naturally occurring epimer now being termed cholest-5-ene-3β,$22\beta_F$(22R)-diol (31–33). Furthermore, it appears that revision of the previously accepted absolute configurations of the epimeric 24-hydroxycholesterols is in order. The prior assignment of the $24\beta_F$(24*S*)-configuration (29) to the naturally occurring, more levorotatory 24-hydroxycholesterol cerebrosterol (cholest-5-ene-3β,24-diol) (34–36) was made in reference to molecular rotations of model levorotatory *n*-alkylisopropylcarbinols, in the same manner as used by Entwistle and Pratt (25) and by the authors with methylisobutenylcarbinol and methylisobutylcarbinol for assignment of configuration to the 3β,23-diols. The model levorotatory alkylisopropylcarbinols used for this purpose (29) were erroneously given the *S*-configuration[1] which led to a $24\beta_F$-assignment for the levorotatory 24-hydroxycholesterol epimer. However, when proper recognition is made that the levorotatory model compounds used for this assignment are of the R-configuration, a revision of absolute configuration obtains, and the more levorotatory, naturally occurring 24-hydroxycholesterol cerebrosterol becomes cholest-5-ene-3β,$24\alpha_F$-(24R)-diol.[2]

In view of the revised absolute configurational assignments of the 22-hydroxycholesterols (31-33), the presently suggested revised absolute configurational assignments of the 24-hydroxycholesterols, and the absolute configurations of the 23-hydroxycholesterols assigned herein, it is now apparent that the three hydroxycholesterols of β_F-configuration: $22\beta_F$(22R)-hydroxycholesterol, $23\beta_F$(23R)-hydroxycholesterol, and $24\beta_F$(24*S*)-hydroxycholesterol, are the more dextrorotatory of their respective epimeric pairs. This deduction follows from recognition that the dextrorotatory enantiomers of the appropriate model secondary alcohols *S*(+)-6-methylheptan-2-ol (38), *S*(+)-4-methylpentan-2-ol (26, 27), and *S*(+)-3-methylbutan-2-ol (29) have the same *S*-configuration.

[1] Model *n*-alkylisopropylcarbinol Compounds XXX (p. 1987) of Klyne and Stokes (29) used for assignment of absolute configuration to the 24-hydroxycholesterol epimers appear to have the *S*-configuration but are stated to be levorotatory, whereas model Compound XXI [*S*(+)-3-methylbutan-2-ol] is dextrorotatory and its enantiomeric model Compound XXII [R(−)-3-methylbutan-2-ol] is levorotatory (p. 1986). From the printed figures, both Models XXX and XXI have the *S*-configuration, but XXX should have the R-configuration to be levorotatory (37). This point is repeated by Fieser and Fieser (6).

[2] Prior reports from this laboratory (21–23) have used without revision the absolute configuration of cerebrosterol as assigned by Klyne and Stokes (29) but converted to the Cahn-Ingold-Prelog sequence rule nomenclature, thus 24*S*-hydroxycholesterol (cholest-5-ene-3β,24*S*-diol). These prior usages should now be revised. On the same basis the more levorotatory epimer of another 24-hydroxysterol epimeric pair previously assigned $24\beta_F$(24*S*)-configuration (38) should also be revised.

EXPERIMENTAL[3]

3β-Hydroxycholest-5-en-23-one—A solution of 40 mg. of 3β-hydroxynorchol-5-en-23-ic acid, m.p. 188–190°, $\tilde{\nu}_{max.}^{KBr}$ 3300, 2650, 1680, 1270, and 1040 cm.$^{-1}$, in 5 ml. of dry pyridine–acetic anhydride (2:1) was held overnight at room temperature. The mixture then was diluted with 50 ml. of distilled water. The precipitated acetate was filtered, washed with water, dried under vacuum ($\tilde{\nu}_{max.}^{KBr}$ 2650, 1720, 1680, 1250, and 1040 cm.$^{-1}$), and dissolved in 50 ml. of absolute diethyl ether. One drop of dry pyridine and 0.7 ml. of thionyl chloride were added, and after 3 hr. at room temperature the ether was evaporated under vacuum. Crystallization from diethyl ether–hexane give 318 mg. of the acid chloride acetate, m.p. 149–154°, $\tilde{\nu}_{max.}^{KBr}$ 1800, 1720, 1250, 1030, and 760 cm.$^{-1}$ [lit. m.p. 155–156° (24)]. To 240 mg. of magnesium turnings was added under nitrogen 1.45 g. of freshly distilled isobutyl bromide in 30 ml. of absolute diethyl ether. The mixture was stirred vigorously until the reaction started, and gentle stirring was continued until all magnesium dissolved. The Grignard solution was cooled to −5° and 1 g. of cadmium chloride was added. After stirring for 10 min., 30 ml. of dry benzene was added, and after 15 min. the acid chloride acetate (318 mg.) in 10 ml. of dry benzene was added. The stirred mixture was allowed to come to room temperature and was held overnight. The mixture was cooled in an ice bath and the complex destroyed by cautious addition of 10 ml. of 2 *N* sulfuric acid and then 100 ml. of water. The reaction mixture was extracted three times with 100-ml. portions of diethyl ether; the combined ether extracts were washed with sodium bicarbonate solution, with water, and with saturated sodium chloride solution, and were dried over anhydrous sodium sulfate. The dried solution was evaporated under vacuum, redissolved in 50 ml. of methanol containing 50 mg. of sodium methoxide, and refluxed for 45 min. After cooling, 100 ml. of water was added, and the mixture was neutralized with 2 *N* sulfuric acid and extracted three times with 100-ml. portions of diethyl ether. The ether extract was washed with sodium bicarbonate solution, with water, and with saturated sodium chloride solution before drying over anhydrous sodium sulfate and evaporated under vacuum. Gas chromatographic analysis of the product indicated the presence of four components. The reaction products were dissolved in a small amount of methylene chloride and chromatographed on a 2.5 × 60-cm. column packed with synthetic polysaccharide (Sephadex LH-20) prepared with methylene chloride. Elution with neat methylene chloride (13.5-ml. fractions taken automatically) gave several fractions which were analyzed individually by TLC. From Fraction No. 13 there was obtained on evaporation of the solvent 145 mg. of the desired 23-ketone, m.p. 136–140° [lit. m.p. 141–143° (24)]; $\tilde{\nu}_{max.}^{KBr}$ 3400, 1700, and 1050 cm.$^{-1}$; R_C 0.93 in benzene–ethyl acetate (3:2).

An unidentified steroidal ketone was recovered from Fraction No. 14, obtained from the polysaccharide column from which the 23-ketone had just been eluted. Evaporation of the methylene chloride gave 70 mg. of material; upon recrystallization from hexane–diethyl ether, there was obtained 18 mg. of white crystals, m.p. 119–125°; $\tilde{\nu}_{max.}^{KBr}$ 3520, 3450, 1730, 1720, 1060, and 1020 cm.$^{-1}$; $\tilde{\nu}_{max.}^{CCl4}$ 3630, 1740, 1155, 1055 cm.$^{-1}$; R_C 0.89 in benzene–ethyl acetate (3:2); magenta color with 50% sulfuric acid.

Anal.—Calcd. for $C_{24}H_{36}O_3$: C, 77.34; H, 9.74. Found: C, 77.08; H, 9.95.

Cholest-5-ene-3β,23α$_F$(23*S*)-diol—A solution of 150 mg. of 3β-hydroxycholest-5-en-23-one dissolved in 20 ml. of methanol was treated overnight at room temperature with an excess of sodium borohydride. The solvent was evaporated under vacuum and the residue was dissolved in 100 ml. of diethyl ether and washed with 2 *N* sulfuric acid, with water, with sodium bicarbonate solution, with water, and with saturated sodium chloride solution. The ether solution was dried over anhydrous sodium sulfate and evaporated under vacuum. The solid residue was found by TLC to be a mixture of epimeric 23-hydroxycholesterols. The mixed epimers were chromatographed on a 2.5 × 60-cm. column of silica gel prepared and irrigated with hexane–diethyl ether (3:1) with 15-ml. fractions being collected automatically. TLC examination of each fraction established that the more mobile epimer was present in Fractions 36–46, and the more polar epimer in Fractions 48–60. Fractions 46–48 contained both epimers. The mixed fraction was rechromatographed on a 20 × 20-cm. chromatoplate of silica gel HF_{254} 1 mm. thick, irrigated with benzene–ethyl acetate (3:2). Recovery of both resolved 3β,23-diols from the chromatoplate afforded additional material which was added to the appropriate initial fraction. Crystallization from hexane–ethyl acetate of the fractions containing the more mobile epimer gave 91 mg. (67%) of the 3β,23α$_F$(23*S*)-diol. Recrystallization from diisopropyl ether–diethyl ether gave the analytical sample, m.p. 136–137°; [α]$_D$ −30°; $\tilde{\nu}_{max.}^{KBr}$ 3400 and 1050 cm.$^{-1}$; R_C 0.84 in benzene–ethyl acetate (3:2); blue-purple color with 50% sulfuric acid; r_T 2.25 (3% QF-1), 1.66 (3% SE-30); mass spectrum: *m/e* 402 (100%), 384 (32%); 369 (13%), 351 (11%), 300 (14%), 291 (16%), 273 (23%), 255 (17%), *etc.*

Anal.—Calcd. for $C_{27}H_{46}O_2$: C, 80.53; H, 11.52. Found: C, 80.25; H, 11.82.

Cholest-5-ene-3β,23α$_F$(23*S*)-diol 3β,23-Diacetate—A solution of 55 mg. of the more mobile 3β,23α$_F$-diol in 5 ml. of dry pyridine–acetic anhydride (2:1) was held overnight at room temperature and worked up by precipitation with 10 ml. of water. The diacetate was centrifuged down (15 min. at 10,000 × *g*) and the supernatant was discarded. After washing the derivative several times with distilled water, the sample was dried under vacuum and recrystallized from methanol, yielding 50 mg. of 3β,23*S*-diacetate, m.p. 143–146°; [α]$_D$ −35°; $\tilde{\nu}_{max.}^{KBr}$ 1730, 1240, and 1030 cm.$^{-1}$; R_C 1.43 in benzene–ethyl acetate (3:2); blue-purple color with 50% sulfuric acid; r_T 4.62 (3% QF-1), 2.69 (3% SE-30); mass spectrum: *m/e* 426 (100%), 366 (10%), 351 (7.5%), 326 (5.7%), 282 (11%), *etc.*

Anal.—Calcd. for $C_{31}H_{50}O_4$: C, 76.49; H, 10.36. Found: C, 76.26; H, 10.45.

Cholest-5-ene-3β,23β$_F$(23R)-diol—Crystallization from hexane–ethyl acetate of the appropriate fractions containing the more polar epimer afforded 44 mg. (33%) of the 3β,23β$_F$-diol. Recrystallization from diisopropyl ether–diethyl ether gave the analytical sample, m.p. 175–176°; [α]$_D$ −22°; $\tilde{\nu}_{max.}^{KBr}$ 3400 and 1050 cm.$^{-1}$, different from the spectrum of the 23*S*-epimer; R_C 0.74; blue-purple color with 50% sulfuric acid; r_T 2.25 (3% QF-1), 1.66 (3% SE-30), unseparable by gas chromatography from the 23*S*-epimer; mass spectrum: essentially the same as for the 23*S*-epimer.

Anal.—Calcd. for $C_{27}H_{46}O_2$: C, 80.53; H, 11.52. Found: C, 80.35; H, 11.56.

Cholest-5-ene-3β,23β$_F$(23R)-diol 3β,23-Diacetate—Acetylation of 17 mg. of the 3β,23β$_F$-diol in the same manner used for the 3β,23α$_F$-diol gave 11 mg. of the 3β,23β$_F$-diacetate, crystallized from methanol, m.p. 111–114°; [α]$_D$ −9°; $\tilde{\nu}_{max.}^{KBr}$ 1730, 1240, and 1030 cm.$^{-1}$, different from the spectrum of the 23-epimeric diacetate; R_C 1.43; blue-purple color with 50% sulfuric acid; r_T 4.62 (3% QF-1), 2.69 (3% SE-30), inseparable by gas chromatography from the 23*S*-epimer.

Anal.—Calcd. for $C_{31}H_{50}O_4$: C, 76.49; H, 10.36. Found: C, 76.74; H, 10.48.

[3] Melting points were taken on a calibrated Kofler block under microscopic magnification. Optical rotations were obtained in 1.0% solutions in chloroform using a 2-dm. tube. IR absorption spectra were recorded over the range 4000–400 cm.$^{-1}$ with a Perkin-Elmer model 337 spectrophotometer equipped with a beam condenser. Adsorption TLC was conducted using silica gel HF_{254} (E. Merck GmbH., Darmstadt, Germany) and the solvent system benzene–ethyl acetate (3:2). Thin-layer chromatographic mobilities (R_C) are expressed in terms of cholesterol as unit mobility. Gas chromatography was conducted on a Hewlett-Packard F and M model 402 gas chromatograph using 3% QF-1 and 3% SE-30 columns as described previously in detail (22). Retention times (r_T) are given in terms of cholesterol as unit time.

REFERENCES

(1) N. Entwistle and A. D. Pratt, *Tetrahedron*, **24**, 3949(1968).

(2) K. Takeda, H. Minato, and A. Shimaoka, *Chem. Commun.*, **1968**, 104.

(3) G. P. Arsenault, K. Biemann, A. W. Barksdale, and T. C. McMorris, *J. Amer. Chem. Soc.*, **90**, 5635(1968).

(4) P. Eneroth and J.-Å. Gustafsson, *FEBS Lett.*, **3**, 129(1969).

(5) G. A. D. Haslewood, "Bile Salts," Methuen and Co., Ltd., London, England, 1967, pp. 8–9.

(6) L. F. Fieser and M. Fieser, "Steroids," Reinhold, New York, N. Y., 1959, pp. 870–874.

(7) A. J. Birch, D. J. Collins, S. Muhammad, and J. P. Turnbull, *J. Chem. Soc.*, **1963**, 2762.

(8) C. W. L. Bevan, D. E. U. Ekong, T. G. Halsall, and P. Toft, *Chem. Commun.*, **1965**, 636.

(9) D. Lavie, M. K. Jain, and I. Kirson, *Tetrahedron Lett.*, **1966**, 2049.

(10) M. K. Jain, I. Kirson, and D. Lavie, *Isr. J. Chem.*, **4,** 32 (1966).
(11) C. J. Breen, E. Ritchie, W. T. L. Sidwell, and W. C. Taylor, *Aust. J. Chem.*, **19,** 455(1966).
(12) H. K. Adam, T. A. Bryce, I. M. Campbell, N. J. McCorkindale, A. Gaudemer, R. Gmelin, and J. Polonsky, *Tetrahedron Lett.*, **1967,** 1461.
(13) A. Chatterjee and A. B. Kundu, *ibid.*, **1967,** 1471.
(14) J. D. Connolly, K. L. Handa, R. McCrindle, and K. H. Overton, *ibid.*, **1967,** 3449.
(15) D. Lavie, M. K. Jain, and I. Kirson, *J. Chem. Soc.* (*C*), **1967,** 1347.
(16) T. Murata, M. Shinohara, T. Hirata, K. Kamiya, M. Nishikawa, and M. Miyamoto, *Tetrahedron Lett.*, **1968,** 103.
(17) T. Murata, M. Shinohara, T. Hirata, and M. Miyamoto, *ibid.*, **1968,** 849.
(18) D. Lavie and E. C. Levy, *ibid.*, **1968,** 2097.
(19) L. L. Smith, W. S. Matthews, J. C. Price, R. C. Bachmann, and B. Reynolds, *J. Chromatogr.*, **27,** 187(1967).
(20) J. E. van Lier and L. L. Smith, to be published.
(21) J. E. van Lier and L. L. Smith, *Biochemistry*, **6,** 3269(1967).
(22) J. E. van Lier and L. L. Smith, *Anal. Biochem.*, **24,** 419 (1968).
(23) J. E. van Lier and L. L. Smith, *Tex. Rep. Biol. Med.*, **27,** 167(1969).
(24) P. Kurath, F. M. Ganis, and M. Radakovich, *Helv. Chim. Acta*, **40,** 933(1957).
(25) N. Entwistle and A. D. Pratt, *Tetrahedron*, **25,** 1449(1969).
(26) D. I. Duveen and J. Kenyon, *J. Chem. Soc.*, **1936,** 1451.
(27) S. R. Landor, B. J. Miller, and A. R. Tatchell, *J. Chem. Soc.* (*C*), **1966,** 1822, 2280.
(28) P. A. Plattner, *Helv. Chim. Acta*, **45,** 1693(1951); *Chem. Ind.*, **1951,** SNI.
(29) W. Klyne and W. M. Stokes, *J. Chem. Soc.*, **1954,** 1979.
(30) K. Tsuda and R. Hayatsu, *J. Amer. Chem. Soc.*, **81,** 5987 (1959).
(31) H. Mori, K. Shibata, K. Tsuneda, M. Sawai, and K. Tsuda, *Chem. Pharm. Bull. Tokyo*, **16,** 1407(1968).
(32) H. Mori, K. Shibata, K. Tsuneda, and M. Sawai, *ibid.*, **16,** 2416(1968).
(33) E. P. Burrows, G. M. Hornby, and E. Caspi, *J. Org. Chem.*, **34,** 103(1969).
(34) A. Ercoli, S. Di Frisco, and P. de Ruggieri, *Gazz. Chim. Ital.*, **83,** 78(1953).
(35) A. Ercoli and P. de Ruggieri, *ibid.*, **83,** 720(1953).
(36) A. Ercoli and P. de Ruggieri, *J. Amer. Chem. Soc.*, **75,** 3284(1953).
(37) K. Mislow, "Introduction to Stereochemistry," W. A. Benjamin, New York, N. Y., 1966, p. 95.
(38) T. Masui and E. Staple, *Steroids*, **9,** 443(1967).
(39) W. v. E. Doering and R. W. Young, *J. Amer. Chem. Soc.*, **74,** 2997(1952).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 17, 1969, from the *Department of Biochemistry, University of Texas Medical Branch, Galveston, TX 77550*

Accepted for publication December 11, 1969.

The authors acknowledge gifts of 3β-hydroxychol-5-enic acid from Dr. M. J. Thompson, U. S. Department of Agriculture, Beltsville, Md., and Dr. H. L. Herzog, Schering Corp., Bloomfield, N. J. Financial support for these studies was by U. S. Public Health Service Research Grant HE-10160.

Paper IX of the series: L. L. Smith and J. E. van Lier, *J. Atheroscler. Res.*, to be published.

New Compounds: Mannich Bases from 1,2-Diphenylindolizine—*N*-Substituted Cyclohexylaminomethyl Derivatives

WILLIAM B. HARRELL*, SHAO-WEN KUANG, and CROSSLEY O'DELL

Abstract □ Seven new Mannich bases, involving *N*-substituted cyclohexylamines and 1,2-diphenylindolizine, have been synthesized as potential biologically active compounds.

Keyphrases □ Mannich bases—synthesis □ *N*-Substituted cyclohexylamines—synthesis from 1,2-diphenylindolizine □ 1,2-Diphenylindolizine—Mannich base synthesis

In previously reported work, it has been shown that certain Mannich bases derived from indolizines exhibited CNS-depressant activity (1–3). As part of a continuing exploration of indolizines with potential biological activity, a series of Mannich bases involving *N*-substituted cyclohexylamines was synthesized from 1,2-diphenylindolizine (4) (Table I).

EXPERIMENTAL[1]

The appropriate secondary amine (0.045 mole) was combined with 30 ml. of 1,4-dioxane and 2.25 ml. of 40% aqueous formaldehyde (0.030 mole). The mixture was placed in the refrigerator and allowed to stand for 48 hr. To the mixture was then added 4.1 g. of 1,2-diphenylindolizine (0.015 mole); the resulting clear solution was stirred at room temperature for 72 hr., during which

[1] Melting points were taken on a Thomas-Hoover melting-point apparatus and are uncorrected. Elemental analyses were obtained from Strauss Microanalytical Laboratories, Oxford, England.

(10) M. K. Jain, I. Kirson, and D. Lavie, *Isr. J. Chem.*, **4,** 32 (1966).
(11) C. J. Breen, E. Ritchie, W. T. L. Sidwell, and W. C. Taylor, *Aust. J. Chem.*, **19,** 455(1966).
(12) H. K. Adam, T. A. Bryce, I. M. Campbell, N. J. McCorkindale, A. Gaudemer, R. Gmelin, and J. Polonsky, *Tetrahedron Lett.*, **1967,** 1461.
(13) A. Chatterjee and A. B. Kundu, *ibid.*, **1967,** 1471.
(14) J. D. Connolly, K. L. Handa, R. McCrindle, and K. H. Overton, *ibid.*, **1967,** 3449.
(15) D. Lavie, M. K. Jain, and I. Kirson, *J. Chem. Soc.* (*C*), **1967,** 1347.
(16) T. Murata, M. Shinohara, T. Hirata, K. Kamiya, M. Nishikawa, and M. Miyamoto, *Tetrahedron Lett.*, **1968,** 103.
(17) T. Murata, M. Shinohara, T. Hirata, and M. Miyamoto, *ibid.*, **1968,** 849.
(18) D. Lavie and E. C. Levy, *ibid.*, **1968,** 2097.
(19) L. L. Smith, W. S. Matthews, J. C. Price, R. C. Bachmann, and B. Reynolds, *J. Chromatogr.*, **27,** 187(1967).
(20) J. E. van Lier and L. L. Smith, to be published.
(21) J. E. van Lier and L. L. Smith, *Biochemistry*, **6,** 3269(1967).
(22) J. E. van Lier and L. L. Smith, *Anal. Biochem.*, **24,** 419 (1968).
(23) J. E. van Lier and L. L. Smith, *Tex. Rep. Biol. Med.*, **27,** 167(1969).
(24) P. Kurath, F. M. Ganis, and M. Radakovich, *Helv. Chim. Acta*, **40,** 933(1957).
(25) N. Entwistle and A. D. Pratt, *Tetrahedron*, **25,** 1449(1969).
(26) D. I. Duveen and J. Kenyon, *J. Chem. Soc.*, **1936,** 1451.
(27) S. R. Landor, B. J. Miller, and A. R. Tatchell, *J. Chem. Soc.* (*C*), **1966,** 1822, 2280.
(28) P. A. Plattner, *Helv. Chim. Acta*, **45,** 1693(1951); *Chem. Ind.*, **1951,** SNI.
(29) W. Klyne and W. M. Stokes, *J. Chem. Soc.*, **1954,** 1979.
(30) K. Tsuda and R. Hayatsu, *J. Amer. Chem. Soc.*, **81,** 5987 (1959).
(31) H. Mori, K. Shibata, K. Tsuneda, M. Sawai, and K. Tsuda, *Chem. Pharm. Bull. Tokyo*, **16,** 1407(1968).
(32) H. Mori, K. Shibata, K. Tsuneda, and M. Sawai, *ibid.*, **16,** 2416(1968).
(33) E. P. Burrows, G. M. Hornby, and E. Caspi, *J. Org. Chem.*, **34,** 103(1969).
(34) A. Ercoli, S. Di Frisco, and P. de Ruggieri, *Gazz. Chim. Ital.*, **83,** 78(1953).
(35) A. Ercoli and P. de Ruggieri, *ibid.*, **83,** 720(1953).
(36) A. Ercoli and P. de Ruggieri, *J. Amer. Chem. Soc.*, **75,** 3284(1953).
(37) K. Mislow, "Introduction to Stereochemistry," W. A. Benjamin, New York, N. Y., 1966, p. 95.
(38) T. Masui and E. Staple, *Steroids*, **9,** 443(1967).
(39) W. v. E. Doering and R. W. Young, *J. Amer. Chem. Soc.*, **74,** 2997(1952).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 17, 1969, from the *Department of Biochemistry, University of Texas Medical Branch, Galveston, TX 77550*

Accepted for publication December 11, 1969.

The authors acknowledge gifts of 3β-hydroxychol-5-enic acid from Dr. M. J. Thompson, U. S. Department of Agriculture, Beltsville, Md., and Dr. H. L. Herzog, Schering Corp., Bloomfield, N. J. Financial support for these studies was by U. S. Public Health Service Research Grant HE-10160.

Paper IX of the series: L. L. Smith and J. E. van Lier, *J. Atheroscler. Res.*, to be published.

New Compounds: Mannich Bases from 1,2-Diphenylindolizine—*N*-Substituted Cyclohexylaminomethyl Derivatives

WILLIAM B. HARRELL*, SHAO-WEN KUANG, and CROSSLEY O'DELL

Abstract □ Seven new Mannich bases, involving *N*-substituted cyclohexylamines and 1,2-diphenylindolizine, have been synthesized as potential biologically active compounds.

Keyphrases □ Mannich bases—synthesis □ *N*-Substituted cyclohexylamines—synthesis from 1,2-diphenylindolizine □ 1,2-Diphenylindolizine—Mannich base synthesis

In previously reported work, it has been shown that certain Mannich bases derived from indolizines exhibited CNS-depressant activity (1–3). As part of a continuing exploration of indolizines with potential biological activity, a series of Mannich bases involving *N*-substituted cyclohexylamines was synthesized from 1,2-diphenylindolizine (4) (Table I).

EXPERIMENTAL[1]

The appropriate secondary amine (0.045 mole) was combined with 30 ml. of 1,4-dioxane and 2.25 ml. of 40% aqueous formaldehyde (0.030 mole). The mixture was placed in the refrigerator and allowed to stand for 48 hr. To the mixture was then added 4.1 g. of 1,2-diphenylindolizine (0.015 mole); the resulting clear solution was stirred at room temperature for 72 hr., during which

[1] Melting points were taken on a Thomas-Hoover melting-point apparatus and are uncorrected. Elemental analyses were obtained from Strauss Microanalytical Laboratories, Oxford, England.

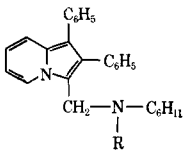
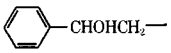


Table I—Mannich Bases

Compound	R	M.p.	Yield, %	Formula	Anal. Calcd.	Anal. Found
I		182–183°	85	$C_{33}H_{38}N_2$	C, 85.67 H, 8.28 N, 6.05	C, 85.72 H, 8.32 N, 6.18
II	CH_3CH_2—	123–124°	90	$C_{29}H_{33}N_2$	C, 85.25 H, 7.89 N, 6.86	C, 85.32 H, 7.92 N, 6.69
III	CH_3—	112–113°	88	$C_{28}H_{30}N_2$	C, 85.24 H, 7.66 N, 7.10	C, 85.36 H, 7.66 N, 7.09
IV	$(CH_3)_2CH$—	127–128°	84	$C_{30}H_{34}N_2$	C, 85.26 H, 8.11 N, 6.63	C, 85.42 H, 8.09 N, 6.42
V	$NCCH_2CH_2$—	152–153°	92	$C_{30}H_{31}N_3$	C, 83.10 H, 7.21 N, 9.69	C, 83.13 H, 7.19 N, 9.48
VI	$HOCH_2CH_2$—	138–139°	74	$C_{29}H_{32}N_2O$	C, 82.04 H, 7.60 N, 6.60	C, 82.11 H, 7.65 N, 6.78
VII		137–138°	84	$C_{35}H_{36}N_2O$	C, 83.96 H, 7.25 N, 5.59	C, 83.92 H, 7.31 N, 5.42

the crystalline product appeared. The product was collected and recrystallized from acetone. Each compound gave a negative color reaction with *p*-dimethylaminobenzaldehyde reagent, indicating that substitution had occurred at the C-3 position (5).

REFERENCES

(1) W. B. Harrell and R. F. Doerge, *J. Pharm. Sci.*, **56**, 225(1967).
(2) W. B. Harrell and R. F. Doerge, *ibid.*, **56**, 1200(1967).
(3) W. B. Harrell and R. F. Doerge, *ibid.*, **57**, 1989(1968).
(4) P. A. Barrett, *J. Chem. Soc.*, **1958**, 325.
(5) D. O. Holland and J. H. C. Nayler, *ibid.*, **1955**, 1657.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 2, 1969, from *The School of Pharmacy, Texas Southern University, Houston, TX 77004*

Accepted for publication November 12, 1969.

Supported by a grant from the Robert A. Welch Foundation.

* To whom correspondence should be addressed.

COMMUNICATIONS

Time Integral of Drug Concentration in the Central (Plasma) Compartment

Keyphrases ☐ Drug concentration time integral—central compartment ☐ Absorption comparison method—different formulations

Sir:

A well-known result in pharmacokinetics is that the time integral of the concentration of a drug in the central (plasma) compartment is equal to the total amount of drug absorbed divided by the product of the volume of distribution for the compartment and the elimination rate constant. The result is of great practical importance in that amounts of drug absorbed from different formulations of a drug can be readily compared by administering the different formulations to the same subject. Standard statistical designs, such as balanced incomplete block designs, can thus be employed. The result has been proved for one-compartment and two-compartment systems under suitable conditions. The usual procedure has been to obtain an expression for the concentration in the central compartment (by solving the appropriate differential equations) and to integrate this expression over time to obtain the stated result. A recent example of this procedure is given in Eqs. 11*a* through 14*a* of Gibaldi *et al.* (1). The purpose of the present note is to show that the result is a direct consequence of two basic assumptions and thus holds under quite general conditions. In fact, the present proof is implicit in the derivation given for nonintravenous routes of administration in Eq. 22*a* of the reference.

The two basic assumptions are:

1. Elimination of the drug takes place only from the central compartment, that is, the compartment over

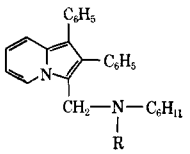
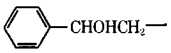


Table I—Mannich Bases

Compound	R	M.p.	Yield, %	Formula	Anal. Calcd.	Anal. Found
I		182–183°	85	$C_{33}H_{38}N_2$	C, 85.67 H, 8.28 N, 6.05	C, 85.72 H, 8.32 N, 6.18
II	CH_3CH_2—	123–124°	90	$C_{29}H_{33}N_2$	C, 85.25 H, 7.89 N, 6.86	C, 85.32 H, 7.92 N, 6.69
III	CH_3—	112–113°	88	$C_{28}H_{30}N_2$	C, 85.24 H, 7.66 N, 7.10	C, 85.36 H, 7.66 N, 7.09
IV	$(CH_3)_2CH$—	127–128°	84	$C_{30}H_{34}N_2$	C, 85.26 H, 8.11 N, 6.63	C, 85.42 H, 8.09 N, 6.42
V	$NCCH_2CH_2$—	152–153°	92	$C_{30}H_{31}N_3$	C, 83.10 H, 7.21 N, 9.69	C, 83.13 H, 7.19 N, 9.48
VI	$HOCH_2CH_2$—	138–139°	74	$C_{29}H_{32}N_2O$	C, 82.04 H, 7.60 N, 6.60	C, 82.11 H, 7.65 N, 6.78
VII		137–138°	84	$C_{35}H_{36}N_2O$	C, 83.96 H, 7.25 N, 5.59	C, 83.92 H, 7.31 N, 5.42

the crystalline product appeared. The product was collected and recrystallized from acetone. Each compound gave a negative color reaction with *p*-dimethylaminobenzaldehyde reagent, indicating that substitution had occurred at the C-3 position (5).

REFERENCES

(1) W. B. Harrell and R. F. Doerge, *J. Pharm. Sci.*, **56**, 225(1967).
(2) W. B. Harrell and R. F. Doerge, *ibid.*, **56**, 1200(1967).
(3) W. B. Harrell and R. F. Doerge, *ibid.*, **57**, 1989(1968).
(4) P. A. Barrett, *J. Chem. Soc.*, **1958**, 325.
(5) D. O. Holland and J. H. C. Nayler, *ibid.*, **1955**, 1657.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 2, 1969, from *The School of Pharmacy, Texas Southern University, Houston, TX 77004*

Accepted for publication November 12, 1969.

Supported by a grant from the Robert A. Welch Foundation.

* To whom correspondence should be addressed.

COMMUNICATIONS

Time Integral of Drug Concentration in the Central (Plasma) Compartment

Keyphrases ☐ Drug concentration time integral—central compartment ☐ Absorption comparison method—different formulations

Sir:

A well-known result in pharmacokinetics is that the time integral of the concentration of a drug in the central (plasma) compartment is equal to the total amount of drug absorbed divided by the product of the volume of distribution for the compartment and the elimination rate constant. The result is of great practical importance in that amounts of drug absorbed from different formulations of a drug can be readily compared by administering the different formulations to the same subject. Standard statistical designs, such as balanced incomplete block designs, can thus be employed. The result has been proved for one-compartment and two-compartment systems under suitable conditions. The usual procedure has been to obtain an expression for the concentration in the central compartment (by solving the appropriate differential equations) and to integrate this expression over time to obtain the stated result. A recent example of this procedure is given in Eqs. 11*a* through 14*a* of Gibaldi *et al.* (1). The purpose of the present note is to show that the result is a direct consequence of two basic assumptions and thus holds under quite general conditions. In fact, the present proof is implicit in the derivation given for nonintravenous routes of administration in Eq. 22*a* of the reference.

The two basic assumptions are:

1. Elimination of the drug takes place only from the central compartment, that is, the compartment over

which the time integral of concentration can be estimated by suitable sampling techniques.

2. Elimination from this compartment is first order.

Suppose that the amount of drug in the central compartment at any time is X, that the volume of distribution for the compartment is V, and that the elimination rate constant is k_e. Then the rate of elimination is k_eX, and the amount of drug eliminated between times t and $(t + dt)$ is $k_eX\,dt$. Consequently the total amount of drug absorbed (D)—which must equal the total amount eliminated—is given by

$$D = \int_0^\infty k_eX\,dt \qquad \text{(Eq. 1)}$$

Dividing both sides of the equation by k_eV and defining the concentration C as X/V give the required result:

$$D/k_eV = \int_0^\infty C\,dt \qquad \text{(Eq. 2)}$$

As is clear from its derivation, the result is independent of the method of administration of the drug and is true for a system comprising any number of compartments with any type of transfer between them, provided only that the two basic assumptions are true. The same argument yields an analogous result for nth-order elimination from the central compartment (*i.e.*, rate of elimination $= k_eX^n$ where $n \neq 0$). In this case, it is seen that

$$D/k_eV^n = \int_0^\infty C^n\,dt \qquad \text{(Eq. 3)}$$

(1) M. Gibaldi, R. Nagashima, and G. Levy, *J. Pharm. Sci.*, **58**, 193(1969).

WILFRED J. WESTLAKE
Biostatistics Department
Smith Kline & French Laboratories
Philadelphia, PA 19101

Received September 26, 1969.
Accepted for publication November 19, 1969.

After this communication was accepted for publication, it was brought to the author's attention that this method of derivation is implicit in some earlier papers; *e.g.*, J. G. Wagner *et al.*, *Nature*, **207**, 1301 (1965).

Acospectoside A III: Selective Conversion into Acovenoside B Using Snail Enzyme with Inhibited Esterase Activity

Keyphrases ☐ Acospectoside A, hydrolysis—snail enzyme ☐ Acovenoside B formation—acospectoside A hydrolysis ☐ TLC—identity ☐ Paper chromatography—identity ☐ IR spectrophotometry—identity ☐

Sir:

The use of snail (*Helix pomatia*) enzyme preparation for the hydrolysis of glucose residues in cardenolide glycosides, first advocated by Reichstein *et al.* (1), is well known. In the author's structural studies (2, 3) on acospectoside A (I) (4), the cleavage of the terminal glucose residue could not be realized using several known glucosidase preparations, *i.e.*, emulsin, strophanthobiase, β-glucosidase, invertase, taka-diastase, and cellulase. However, by using the snail enzyme the splitting of glucose could be accomplished, though to a small extent leading to acovenoside B (II) (5, 6) and the hydrolysate contained acobioside A (III) (7) and acovenoside A (IV) (5, 6) and glucose as major products. It was further shown that II and III result by cleavage of the terminal glucose residue and C-1 ester group, respectively, while IV results by subsequent hydrolysis of II and III with esterase and β-glucosidase, respectively. These and other data enabled the determination of the structure of acospectoside A as 1-*O*-acetyl-acobioside A (I).

The low yield of II may be attributed to the higher order of activity of the esterase component, as compared to the β-glucosidase component, of the snail enzyme preparation. To secure higher yields of II, it was thought that by blocking the esterase component of the mixture the β-glucosidase activity might consequently be favored. This was actually realized by "poisoning" the esterase component by employing a commercial insecticide preparation[1] containing 2,2-dichlorovinyl dimethyl phosphate (a choline esterase inhibitor) as an active ingredient. The result was the formation of acovenoside B (II) as the sole crystalline product in about 27% yield. The identity of the product was established by direct comparison (mixed melting point, TLC, paper chromatography, and IR spectra) with an authentic sample.

In natural product studies, the need is frequently encountered to effect selectively the cleavage of an ester or a glycosidic linkage in the presence of the other. The availability of an enzyme preparation, such as that of snail, which would effect both, and a method to inhibit one and thus promote the other[2] have obvious advantages in structure elucidation work. A recent example of the application of the herein reported technique is the case of gymnemic acid A, a complex triterpenoid ester glucuronoside with reported (9) antisweet and antiviral activities, in which Rao and Sinsheimer (10, 11) induced selective sugar cleavage with the snail enzyme without affecting the ester groups and thus obtained only one product. This stands in contrast to the mixture of at least four components obtained earlier by Stocklin *et al.* (12) by treatment of their gymnemic acids with snail enzyme. The utility of the method was also tested by Rao and Sinsheimer (10, 11) in three other instances, gymnemic acids B, C, and D, which again afforded single genins designated K, N, and M.

[1] Real-Kill, an insecticide marketed by Real-Kill Products, Division of Cook Chemical Co., Kansas City, Mo., was employed as esterase inhibitor. In a typical procedure, a mixture of 75.6 mg. of I, 70 mg. of snail enzyme (Helicase, marketed by Industrie Biologique Francaise, S. A. Gennevilliers, France), and 0.4 ml. of Real-Kill in 12 ml. of water was left with stirring at room temperature for 5 days.

[2] Another method has recently been reported (see *Reference 8*) for the selective inhibition of the β-glucosidase activity in the snail enzyme which resulted in enhanced esterase activity. The results are being separately published.

which the time integral of concentration can be estimated by suitable sampling techniques.

2. Elimination from this compartment is first order.

Suppose that the amount of drug in the central compartment at any time is X, that the volume of distribution for the compartment is V, and that the elimination rate constant is k_e. Then the rate of elimination is k_eX, and the amount of drug eliminated between times t and $(t + dt)$ is $k_eX\,dt$. Consequently the total amount of drug absorbed (D)—which must equal the total amount eliminated—is given by

$$D = \int_0^\infty k_eX\,dt \qquad \text{(Eq. 1)}$$

Dividing both sides of the equation by k_eV and defining the concentration C as X/V give the required result:

$$D/k_eV = \int_0^\infty C\,dt \qquad \text{(Eq. 2)}$$

As is clear from its derivation, the result is independent of the method of administration of the drug and is true for a system comprising any number of compartments with any type of transfer between them, provided only that the two basic assumptions are true. The same argument yields an analogous result for nth-order elimination from the central compartment (*i.e.*, rate of elimination $= k_eX^n$ where $n \neq 0$). In this case, it is seen that

$$D/k_eV^n = \int_0^\infty C^n\,dt \qquad \text{(Eq. 3)}$$

(1) M. Gibaldi, R. Nagashima, and G. Levy, *J. Pharm. Sci.*, **58**, 193(1969).

WILFRED J. WESTLAKE
Biostatistics Department
Smith Kline & French Laboratories
Philadelphia, PA 19101

Received September 26, 1969.
Accepted for publication November 19, 1969.

After this communication was accepted for publication, it was brought to the author's attention that this method of derivation is implicit in some earlier papers; *e.g.*, J. G. Wagner *et al.*, *Nature*, **207**, 1301 (1965).

Acospectoside A III: Selective Conversion into Acovenoside B Using Snail Enzyme with Inhibited Esterase Activity

Keyphrases ☐ Acospectoside A, hydrolysis—snail enzyme ☐ Acovenoside B formation—acospectoside A hydrolysis ☐ TLC—identity ☐ Paper chromatography—identity ☐ IR spectrophotometry—identity ☐

Sir:

The use of snail (*Helix pomatia*) enzyme preparation for the hydrolysis of glucose residues in cardenolide glycosides, first advocated by Reichstein *et al.* (1), is well known. In the author's structural studies (2, 3) on acospectoside A (I) (4), the cleavage of the terminal glucose residue could not be realized using several known glucosidase preparations, *i.e.*, emulsin, strophanthobiase, β-glucosidase, invertase, taka-diastase, and cellulase. However, by using the snail enzyme the splitting of glucose could be accomplished, though to a small extent leading to acovenoside B (II) (5, 6) and the hydrolysate contained acobioside A (III) (7) and acovenoside A (IV) (5, 6) and glucose as major products. It was further shown that II and III result by cleavage of the terminal glucose residue and C-1 ester group, respectively, while IV results by subsequent hydrolysis of II and III with esterase and β-glucosidase, respectively. These and other data enabled the determination of the structure of acospectoside A as 1-*O*-acetyl-acobioside A (I).

The low yield of II may be attributed to the higher order of activity of the esterase component, as compared to the β-glucosidase component, of the snail enzyme preparation. To secure higher yields of II, it was thought that by blocking the esterase component of the mixture the β-glucosidase activity might consequently be favored. This was actually realized by "poisoning" the esterase component by employing a commercial insecticide preparation[1] containing 2,2-dichlorovinyl dimethyl phosphate (a choline esterase inhibitor) as an active ingredient. The result was the formation of acovenoside B (II) as the sole crystalline product in about 27% yield. The identity of the product was established by direct comparison (mixed melting point, TLC, paper chromatography, and IR spectra) with an authentic sample.

In natural product studies, the need is frequently encountered to effect selectively the cleavage of an ester or a glycosidic linkage in the presence of the other. The availability of an enzyme preparation, such as that of snail, which would effect both, and a method to inhibit one and thus promote the other[2] have obvious advantages in structure elucidation work. A recent example of the application of the herein reported technique is the case of gymnemic acid A, a complex triterpenoid ester glucuronoside with reported (9) antisweet and antiviral activities, in which Rao and Sinsheimer (10, 11) induced selective sugar cleavage with the snail enzyme without affecting the ester groups and thus obtained only one product. This stands in contrast to the mixture of at least four components obtained earlier by Stocklin *et al.* (12) by treatment of their gymnemic acids with snail enzyme. The utility of the method was also tested by Rao and Sinsheimer (10, 11) in three other instances, gymnemic acids B, C, and D, which again afforded single genins designated K, N, and M.

[1] Real-Kill, an insecticide marketed by Real-Kill Products, Division of Cook Chemical Co., Kansas City, Mo., was employed as esterase inhibitor. In a typical procedure, a mixture of 75.6 mg. of I, 70 mg. of snail enzyme (Helicase, marketed by Industrie Biologique Francaise, S. A. Gennevilliers, France), and 0.4 ml. of Real-Kill in 12 ml. of water was left with stirring at room temperature for 5 days.

[2] Another method has recently been reported (see *Reference 8*) for the selective inhibition of the β-glucosidase activity in the snail enzyme which resulted in enhanced esterase activity. The results are being separately published.

(1) H. Huber, F. Blindenbacher, K. Mohr, P. Spieser, and T. Reichstein, *Helv. Chim. Acta*, **34**, 46(1951).
(2) G. J. Kapadia, *Lloydia*, **30**, 285(1967).
(3) G. J. Kapadia, *J. Pharm. Sci.*, **58**, 1555(1969).
(4) *Ibid.*, **54**, 1834(1965).
(5) J. v. Euw and T. Reichstein, *Helv. Chim. Acta*, **33**, 485(1950).
(6) P. Hauschild-Rogat, E. Weiss, and T. Reichstein, *ibid.*, **45**, 2612(1962).
(7) P. Hauschild-Rogat, J. v. Euw, O. Schindler, E. Weiss, and T. Reichstein, *ibid.*, **45**, 2117(1962).
(8) G. J. Kapadia and M. L. Sethi, "Abstracts of the Academy of Pharmaceutical Sciences at the 116th Annual Meeting of American Pharmaceutical Association," Montreal, Canada, May 1969, Abstract No. 6, p. 98.
(9) J. E. Sinsheimer, G. S. Rao, H. N. McIlhenny, R. V. Smith, H. F. Maassab, and K. W. Cochran, *Experientia*, **24**, 302(1968).
(10) J. E. Sinsheimer and G. S. Rao, to be published.
(11) G. S. Rao, Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1969.
(12) W. Stocklin, E. Weiss, and T. Reichstein, *Helv. Chim. Acta*, **50**, 474(1967).
(13) G. J. Kapadia and T. B. Zalucky, *Lloydia*, **31**, 424(1968).

GOVIND J. KAPADIA
Department of Pharmacognosy and Natural Products
College of Pharmacy
Howard University
Washington, DC 20001

Received September 4, 1969.
Accepted for publication November 21, 1969.
This communication is a part of the paper presented to the Ninth Annual Meeting of the American Society of Pharmacognosy, Iowa City, Iowa, July 1968 (see *Reference 13* for the published abstract).
This investigation was supported by Grant HE11212 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The author thanks Drs. G. S. Rao and J. E. Sinsheimer, College of Pharmacy, University of Michigan, Ann Arbor, Mich., for communicating the results of their studies on gymnemic acids prior to publication. Thanks are also due to Professor T. Reichstein, University of Basel, for an authentic sample of acovenoside B and the enzyme preparation, cellulase, and to Drs. M. B. E. Fayez and T. B. Zalucky for their kind cooperation in a certain phase of this work.

Cytotoxic Activity of Imidazole Derivatives

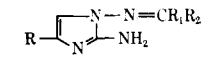
Keyphrases ☐ Imidazole derivatives—synthesis ☐ Cytotoxicity—imidazole derivatives

Sir:

In this communication, we wish to report the synthesis and cytotoxic activity of a number of imidazole derivatives (Compounds 1–8). The synthesis of these imidazole derivatives was accomplished by the reaction of α-haloketones with guanylhydrazones of aromatic aldehydes. The method of preparation of these compounds was very much similar to the one reported by Beyer *et al.* (1). The imidazole derivatives, Compounds 1–8, were characterized by IR and UV spectroscopy.

All these compounds were subjected to L-1210 *in vitro* assay for cytotoxic activity (2). In these screening experiments, the samples were weighed (about 5–10 mg.) into glass homogenizers (32-ml. size) and sterilized with 0.1 ml. of 70% ethanol and about 0.1 ml. of dimethylsulfoxide (DMSO) to help them solubilize. The sample was ground with sterile water to make a suspension containing L-1210 leukemic cells. The tubes were stoppered and incubated at 37° for 3 days; then cell counts were made on each tube by a Coulter counter. The percent inhibition and the ID_{50} and ID_{90} were calculated.

Table I—Screening Data



Compd.	R	R_1	R_2	ID_{50}	ID_{90}
1	Phenyl	H	Phenyl	0.022	0.038
2	Phenyl	Me	Phenyl	0.96	1.5
3	Phenyl	H	3,4-Methylenedioxyphenyl	0.29	0.47
4	3,4-Dihydroxyphenyl	H	3,4-Methylenedioxyphenyl	27	50
5	*m*-Nitrophenyl	Me	Phenyl	40	50
6	*p*-Nitrophenyl	H	*m*-Nitrophenyl	1.25	2.0
7	Methyl	H	*m*-Nitrophenyl	21	50
8	Methyl	H	*o*-Hydroxyphenyl	9.0	21

The assay values for the compounds are shown in Table I. Values of 1 or less for ID_{50} were considered potentially active.

Three compounds showed ID_{50} values less than 1. Based on these encouraging results, attempts are being made in our laboratories to synthesize a wide variety of these imidazole derivatives and to test them for L-1210 *in vitro* assay for possible cytotoxic activity. Obviously, more extensive testing will be required before any structure–activity correlation can be drawn.

(1) H. Beyer, A. Hetzheim, H. Honeck, D. Ling, and T. Pyl, *Chem. Ber.*, **101**, 3151(1968).
(2) H. H. Buskirk, *Proc. Tissue Culture Ass.*, **20**, 23(1969).

ROBERT E. HARMON
S. K. GUPTA
J. L. HANSEN
Department of Chemistry
Western Michigan University
Kalamazoo, MI 49001

Received November 24, 1969.
Accepted for publication February 3, 1970.
This work was supported by Grant CA-6140 from the National Institutes of Health. The assistance of Drs. G. S. Fonken and H. H. Buskirk of The Upjohn Company, Kalamazoo, MI 49001, for testing these compounds is gratefully acknowledged.

Antigenicity of a Polypeptide with a Known Sequence of Amino Acids

Keyphrases ☐ Polypeptides—known amino acid sequence ☐ Antigenicity—polypeptide

Sir:

Random copolymers containing varying amounts of the amino acid residues, alanine, glutamic acid, and

(1) H. Huber, F. Blindenbacher, K. Mohr, P. Spieser, and T. Reichstein, *Helv. Chim. Acta*, **34**, 46(1951).
(2) G. J. Kapadia, *Lloydia*, **30**, 285(1967).
(3) G. J. Kapadia, *J. Pharm. Sci.*, **58**, 1555(1969).
(4) *Ibid.*, **54**, 1834(1965).
(5) J. v. Euw and T. Reichstein, *Helv. Chim. Acta*, **33**, 485(1950).
(6) P. Hauschild-Rogat, E. Weiss, and T. Reichstein, *ibid.*, **45**, 2612(1962).
(7) P. Hauschild-Rogat, J. v. Euw, O. Schindler, E. Weiss, and T. Reichstein, *ibid.*, **45**, 2117(1962).
(8) G. J. Kapadia and M. L. Sethi, "Abstracts of the Academy of Pharmaceutical Sciences at the 116th Annual Meeting of American Pharmaceutical Association," Montreal, Canada, May 1969, Abstract No. 6, p. 98.
(9) J. E. Sinsheimer, G. S. Rao, H. N. McIlhenny, R. V. Smith, H. F. Maassab, and K. W. Cochran, *Experientia*, **24**, 302(1968).
(10) J. E. Sinsheimer and G. S. Rao, to be published.
(11) G. S. Rao, Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1969.
(12) W. Stocklin, E. Weiss, and T. Reichstein, *Helv. Chim. Acta*, **50**, 474(1967).
(13) G. J. Kapadia and T. B. Zalucky, *Lloydia*, **31**, 424(1968).

GOVIND J. KAPADIA
Department of Pharmacognosy and Natural Products
College of Pharmacy
Howard University
Washington, DC 20001

Received September 4, 1969.
Accepted for publication November 21, 1969.
This communication is a part of the paper presented to the Ninth Annual Meeting of the American Society of Pharmacognosy, Iowa City, Iowa, July 1968 (see *Reference 13* for the published abstract).
This investigation was supported by Grant HE11212 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The author thanks Drs. G. S. Rao and J. E. Sinsheimer, College of Pharmacy, University of Michigan, Ann Arbor, Mich., for communicating the results of their studies on gymnemic acids prior to publication. Thanks are also due to Professor T. Reichstein, University of Basel, for an authentic sample of acovenoside B and the enzyme preparation, cellulase, and to Drs. M. B. E. Fayez and T. B. Zalucky for their kind cooperation in a certain phase of this work.

Cytotoxic Activity of Imidazole Derivatives

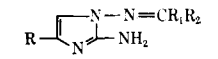
Keyphrases ☐ Imidazole derivatives—synthesis ☐ Cytotoxicity—imidazole derivatives

Sir:

In this communication, we wish to report the synthesis and cytotoxic activity of a number of imidazole derivatives (Compounds 1–8). The synthesis of these imidazole derivatives was accomplished by the reaction of α-haloketones with guanylhydrazones of aromatic aldehydes. The method of preparation of these compounds was very much similar to the one reported by Beyer *et al.* (1). The imidazole derivatives, Compounds 1–8, were characterized by IR and UV spectroscopy.

All these compounds were subjected to L-1210 *in vitro* assay for cytotoxic activity (2). In these screening experiments, the samples were weighed (about 5–10 mg.) into glass homogenizers (32-ml. size) and sterilized with 0.1 ml. of 70% ethanol and about 0.1 ml. of dimethylsulfoxide (DMSO) to help them solubilize. The sample was ground with sterile water to make a suspension containing L-1210 leukemic cells. The tubes were stoppered and incubated at 37° for 3 days; then cell counts were made on each tube by a Coulter counter. The percent inhibition and the ID_{50} and ID_{90} were calculated.

Table I—Screening Data



Compd.	R	R_1	R_2	ID_{50}	ID_{90}
1	Phenyl	H	Phenyl	0.022	0.038
2	Phenyl	Me	Phenyl	0.96	1.5
3	Phenyl	H	3,4-Methylenedioxyphenyl	0.29	0.47
4	3,4-Dihydroxyphenyl	H	3,4-Methylenedioxyphenyl	27	50
5	*m*-Nitrophenyl	Me	Phenyl	40	50
6	*p*-Nitrophenyl	H	*m*-Nitrophenyl	1.25	2.0
7	Methyl	H	*m*-Nitrophenyl	21	50
8	Methyl	H	*o*-Hydroxyphenyl	9.0	21

The assay values for the compounds are shown in Table I. Values of 1 or less for ID_{50} were considered potentially active.

Three compounds showed ID_{50} values less than 1. Based on these encouraging results, attempts are being made in our laboratories to synthesize a wide variety of these imidazole derivatives and to test them for L-1210 *in vitro* assay for possible cytotoxic activity. Obviously, more extensive testing will be required before any structure–activity correlation can be drawn.

(1) H. Beyer, A. Hetzheim, H. Honeck, D. Ling, and T. Pyl, *Chem. Ber.*, **101**, 3151(1968).
(2) H. H. Buskirk, *Proc. Tissue Culture Ass.*, **20**, 23(1969).

ROBERT E. HARMON
S. K. GUPTA
J. L. HANSEN
Department of Chemistry
Western Michigan University
Kalamazoo, MI 49001

Received November 24, 1969.
Accepted for publication February 3, 1970.
This work was supported by Grant CA-6140 from the National Institutes of Health. The assistance of Drs. G. S. Fonken and H. H. Buskirk of The Upjohn Company, Kalamazoo, MI 49001, for testing these compounds is gratefully acknowledged.

Antigenicity of a Polypeptide with a Known Sequence of Amino Acids

Keyphrases ☐ Polypeptides—known amino acid sequence ☐ Antigenicity—polypeptide

Sir:

Random copolymers containing varying amounts of the amino acid residues, alanine, glutamic acid, and

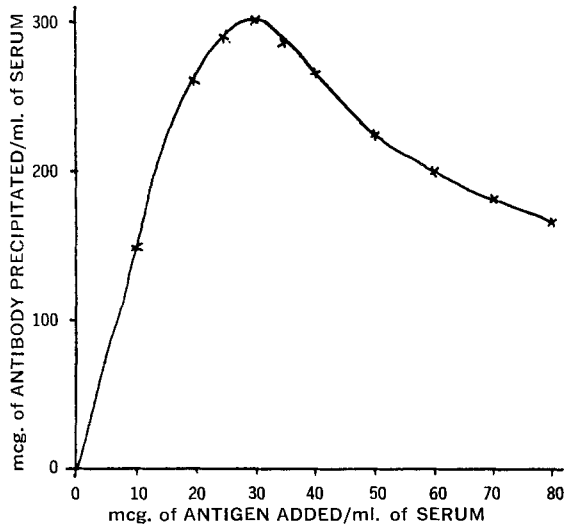


Figure 1—*The precipitin curve.*

tyrosine, have been shown to be antigenic (1–3). However, due to the unknown primary amino acid sequence of these random polymers, it is difficult to describe the locus of the active site of these antigenic polymers. To overcome this difficulty, the use of linear polypeptides with a known repeating sequence of amino acids has been suggested. For this purpose, poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester was recently synthesized (4), and we wish to report the antigenic properties of this polymer.

After obtaining preimmunization sera, four rabbits were treated at weekly intervals with 500 mcg. of poly-(tyr-glu-ala-gly)gly-1-^{14}C ethyl ester. The first 2 weeks they were injected intradermally using complete Freunds adjuvant as suspension medium, and the 3rd week they were injected subcutaneously. The injection on the 4th week was done intravenously using buffered saline. Bleedings were conducted on the following week, and the serum from each animal was found to give a precipitin reaction with the polymer. The preimmunized sera under the same conditions gave a negative precipitin reaction. The quantitative determination of the antibody was obtained by the addition of dilutions of poly-(tyr-glu-ala-gly)gly-1-^{14}C ethyl ester to 1-ml. samples of the pooled rabbit sera. The precipitates were kept at 4° for 48 hr., washed twice with small volumes of buffered saline, and collected by centrifugation. The total amount of protein precipitated was estimated by analysis for nitrogen by a micro-Kjeldahl method (5). The amount of antigen contained in each precipitate was estimated by use of the Folin-Ciocalteu method. From these results the precipitin curve shown in Fig. 1 was obtained.

From these results it can be seen that this polypeptide is antigenic; further studies pertaining to the specificity of its antibodies are presently in progress.

(1) P. H. Maurer, B. F. Gerulat, and P. Pinchuck, *J. Immunol.*, **90**, 388(1963).

(2) P. H. Maurer, B. F. Gerulat, and P. Pinchuck, *J. Biol. Chem.*, **239**, 922(1964).

(3) L. G. Clark and P. H. Maurer, *Int. Arch. Allergy*, **35**, 58 (1969).

(4) B. J. Johnson and E. G. Trask, *J. Chem. Soc.* (*C*), **1969**, 2644.

(5) D. H. Campbell, J. S. Garvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology," W. A. Benjamin, New York, N. Y., 1964, p. 46.

BRIAN J. JOHNSON
ELWOOD G. TRASK

Department of Chemistry
Tufts University
Medford, MA 02155

Received December 8, 1969.

Accepted for publication February 3, 1970.

Presented in part at the 158th American Chemical Society National Meeting, New York, N. Y., 1969.

After submission of this manuscript, a review article by M. Sela, *Science*, **166**, 1365(1969) was published on the molecular aspects of antigenicity.

This work was supported by a grant from the National Science Foundation.

We wish to thank Dr. R. D. Stollar of Tufts Medical School for many helpful suggestions.

Quantitative Correlation of Absorption and *In Vitro* Dissolution Kinetics of Aspirin from Several Dosage Forms

Keyphrases ☐ Aspirin dosage forms—dissolution, absorption ☐ Absorption–dissolution, aspirin—correlation

Sir:

Several types of *in vivo–in vitro* correlation are described in the pharmaceutical literature (1). The most informative of these, but the most difficult to achieve, is the quantitative correlation between *in vitro* dissolution and *in vivo* absorption, particularly one involving several different dosage forms. An example is found in the report of Levy *et al.* (2) who were able to correlate the absorption of aspirin from three different dosage forms with a function of the dissolution rate, using the beaker method (3) at 50 r.p.m. A plot of the percent absorbed at time *T versus* the percent dissolved in (*T*-lag time)/2 gave a straight line with a slope of unity.

The present report introduces a new dissolution method, which has permitted an absolute quantitative correlation between the absorption and *in vitro* dissolution of aspirin from these three dosage forms. These findings extend the work of Levy *et al.* (2) because this type of 1:1 correlation was not attainable with the beaker method.

A schematic diagram of the rotating-flask apparatus used to determine dissolution is shown in Fig. 1. The apparatus consists of a spherical glass flask suspended in a constant-temperature bath. The globe is supported by glass rods, fused to its sides, which form the horizontal axis of the sphere. One support rod is coupled to a constant-speed motor, which provides rotation about the horizontal axis. A sampling port is molded into the sphere to permit introduction of the dosage form and periodic withdrawal of samples. The volume of the dissolution medium (in this case 400 ml.) and the position of the sampling port are such that fluid

(1) H. Huber, F. Blindenbacher, K. Mohr, P. Spieser, and T. Reichstein, *Helv. Chim. Acta*, **34**, 46(1951).
(2) G. J. Kapadia, *Lloydia*, **30**, 285(1967).
(3) G. J. Kapadia, *J. Pharm. Sci.*, **58**, 1555(1969).
(4) *Ibid.*, **54**, 1834(1965).
(5) J. v. Euw and T. Reichstein, *Helv. Chim. Acta*, **33**, 485(1950).
(6) P. Hauschild-Rogat, E. Weiss, and T. Reichstein, *ibid.*, **45**, 2612(1962).
(7) P. Hauschild-Rogat, J. v. Euw, O. Schindler, E. Weiss, and T. Reichstein, *ibid.*, **45**, 2117(1962).
(8) G. J. Kapadia and M. L. Sethi, "Abstracts of the Academy of Pharmaceutical Sciences at the 116th Annual Meeting of American Pharmaceutical Association," Montreal, Canada, May 1969, Abstract No. 6, p. 98.
(9) J. E. Sinsheimer, G. S. Rao, H. N. McIlhenny, R. V. Smith, H. F. Maassab, and K. W. Cochran, *Experientia*, **24**, 302(1968).
(10) J. E. Sinsheimer and G. S. Rao, to be published.
(11) G. S. Rao, Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1969.
(12) W. Stocklin, E. Weiss, and T. Reichstein, *Helv. Chim. Acta*, **50**, 474(1967).
(13) G. J. Kapadia and T. B. Zalucky, *Lloydia*, **31**, 424(1968).

GOVIND J. KAPADIA
Department of Pharmacognosy and Natural Products
College of Pharmacy
Howard University
Washington, DC 20001

Received September 4, 1969.
Accepted for publication November 21, 1969.
This communication is a part of the paper presented to the Ninth Annual Meeting of the American Society of Pharmacognosy, Iowa City, Iowa, July 1968 (see *Reference 13* for the published abstract).
This investigation was supported by Grant HE11212 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The author thanks Drs. G. S. Rao and J. E. Sinsheimer, College of Pharmacy, University of Michigan, Ann Arbor, Mich., for communicating the results of their studies on gymnemic acids prior to publication. Thanks are also due to Professor T. Reichstein, University of Basel, for an authentic sample of acovenoside B and the enzyme preparation, cellulase, and to Drs. M. B. E. Fayez and T. B. Zalucky for their kind cooperation in a certain phase of this work.

Cytotoxic Activity of Imidazole Derivatives

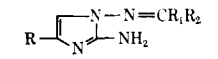
Keyphrases ☐ Imidazole derivatives—synthesis ☐ Cytotoxicity—imidazole derivatives

Sir:

In this communication, we wish to report the synthesis and cytotoxic activity of a number of imidazole derivatives (Compounds 1–8). The synthesis of these imidazole derivatives was accomplished by the reaction of α-haloketones with guanylhydrazones of aromatic aldehydes. The method of preparation of these compounds was very much similar to the one reported by Beyer *et al.* (1). The imidazole derivatives, Compounds 1–8, were characterized by IR and UV spectroscopy.

All these compounds were subjected to L-1210 *in vitro* assay for cytotoxic activity (2). In these screening experiments, the samples were weighed (about 5–10 mg.) into glass homogenizers (32-ml. size) and sterilized with 0.1 ml. of 70% ethanol and about 0.1 ml. of dimethylsulfoxide (DMSO) to help them solubilize. The sample was ground with sterile water to make a suspension containing L-1210 leukemic cells. The tubes were stoppered and incubated at 37° for 3 days; then cell counts were made on each tube by a Coulter counter. The percent inhibition and the ID_{50} and ID_{90} were calculated.

Table I—Screening Data



Compd.	R	R_1	R_2	ID_{50}	ID_{90}
1	Phenyl	H	Phenyl	0.022	0.038
2	Phenyl	Me	Phenyl	0.96	1.5
3	Phenyl	H	3,4-Methylenedioxyphenyl	0.29	0.47
4	3,4-Dihydroxyphenyl	H	3,4-Methylenedioxyphenyl	27	50
5	*m*-Nitrophenyl	Me	Phenyl	40	50
6	*p*-Nitrophenyl	H	*m*-Nitrophenyl	1.25	2.0
7	Methyl	H	*m*-Nitrophenyl	21	50
8	Methyl	H	*o*-Hydroxyphenyl	9.0	21

The assay values for the compounds are shown in Table I. Values of 1 or less for ID_{50} were considered potentially active.

Three compounds showed ID_{50} values less than 1. Based on these encouraging results, attempts are being made in our laboratories to synthesize a wide variety of these imidazole derivatives and to test them for L-1210 *in vitro* assay for possible cytotoxic activity. Obviously, more extensive testing will be required before any structure–activity correlation can be drawn.

(1) H. Beyer, A. Hetzheim, H. Honeck, D. Ling, and T. Pyl, *Chem. Ber.*, **101**, 3151(1968).
(2) H. H. Buskirk, *Proc. Tissue Culture Ass.*, **20**, 23(1969).

ROBERT E. HARMON
S. K. GUPTA
J. L. HANSEN
Department of Chemistry
Western Michigan University
Kalamazoo, MI 49001

Received November 24, 1969.
Accepted for publication February 3, 1970.
This work was supported by Grant CA-6140 from the National Institutes of Health. The assistance of Drs. G. S. Fonken and H. H. Buskirk of The Upjohn Company, Kalamazoo, MI 49001, for testing these compounds is gratefully acknowledged.

Antigenicity of a Polypeptide with a Known Sequence of Amino Acids

Keyphrases ☐ Polypeptides—known amino acid sequence ☐ Antigenicity—polypeptide

Sir:

Random copolymers containing varying amounts of the amino acid residues, alanine, glutamic acid, and

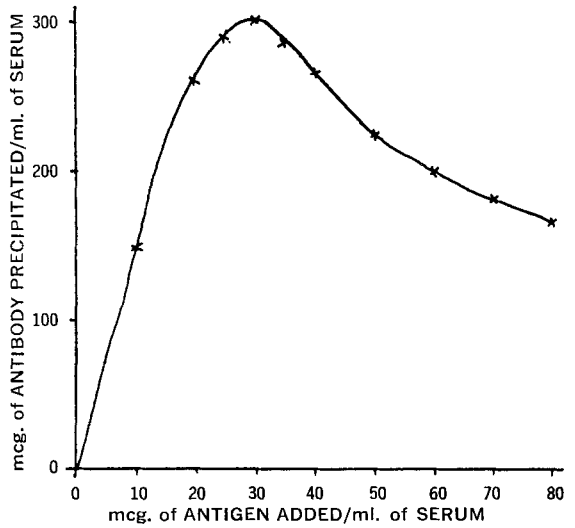


Figure 1—*The precipitin curve.*

tyrosine, have been shown to be antigenic (1–3). However, due to the unknown primary amino acid sequence of these random polymers, it is difficult to describe the locus of the active site of these antigenic polymers. To overcome this difficulty, the use of linear polypeptides with a known repeating sequence of amino acids has been suggested. For this purpose, poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester was recently synthesized (4), and we wish to report the antigenic properties of this polymer.

After obtaining preimmunization sera, four rabbits were treated at weekly intervals with 500 mcg. of poly-(tyr-glu-ala-gly)gly-1-^{14}C ethyl ester. The first 2 weeks they were injected intradermally using complete Freunds adjuvant as suspension medium, and the 3rd week they were injected subcutaneously. The injection on the 4th week was done intravenously using buffered saline. Bleedings were conducted on the following week, and the serum from each animal was found to give a precipitin reaction with the polymer. The preimmunized sera under the same conditions gave a negative precipitin reaction. The quantitative determination of the antibody was obtained by the addition of dilutions of poly-(tyr-glu-ala-gly)gly-1-^{14}C ethyl ester to 1-ml. samples of the pooled rabbit sera. The precipitates were kept at 4° for 48 hr., washed twice with small volumes of buffered saline, and collected by centrifugation. The total amount of protein precipitated was estimated by analysis for nitrogen by a micro-Kjeldahl method (5). The amount of antigen contained in each precipitate was estimated by use of the Folin-Ciocalteu method. From these results the precipitin curve shown in Fig. 1 was obtained.

From these results it can be seen that this polypeptide is antigenic; further studies pertaining to the specificity of its antibodies are presently in progress.

(1) P. H. Maurer, B. F. Gerulat, and P. Pinchuck, *J. Immunol.*, **90**, 388(1963).

(2) P. H. Maurer, B. F. Gerulat, and P. Pinchuck, *J. Biol. Chem.*, **239**, 922(1964).

(3) L. G. Clark and P. H. Maurer, *Int. Arch. Allergy*, **35**, 58 (1969).

(4) B. J. Johnson and E. G. Trask, *J. Chem. Soc.* (*C*), **1969**, 2644.

(5) D. H. Campbell, J. S. Garvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology," W. A. Benjamin, New York, N. Y., 1964, p. 46.

BRIAN J. JOHNSON
ELWOOD G. TRASK

Department of Chemistry
Tufts University
Medford, MA 02155

Received December 8, 1969.

Accepted for publication February 3, 1970.

Presented in part at the 158th American Chemical Society National Meeting, New York, N. Y., 1969.

After submission of this manuscript, a review article by M. Sela, *Science*, **166**, 1365(1969) was published on the molecular aspects of antigenicity.

This work was supported by a grant from the National Science Foundation.

We wish to thank Dr. R. D. Stollar of Tufts Medical School for many helpful suggestions.

Quantitative Correlation of Absorption and *In Vitro* Dissolution Kinetics of Aspirin from Several Dosage Forms

Keyphrases ☐ Aspirin dosage forms—dissolution, absorption ☐ Absorption–dissolution, aspirin—correlation

Sir:

Several types of *in vivo–in vitro* correlation are described in the pharmaceutical literature (1). The most informative of these, but the most difficult to achieve, is the quantitative correlation between *in vitro* dissolution and *in vivo* absorption, particularly one involving several different dosage forms. An example is found in the report of Levy *et al.* (2) who were able to correlate the absorption of aspirin from three different dosage forms with a function of the dissolution rate, using the beaker method (3) at 50 r.p.m. A plot of the percent absorbed at time *T versus* the percent dissolved in (*T*-lag time)/2 gave a straight line with a slope of unity.

The present report introduces a new dissolution method, which has permitted an absolute quantitative correlation between the absorption and *in vitro* dissolution of aspirin from these three dosage forms. These findings extend the work of Levy *et al.* (2) because this type of 1:1 correlation was not attainable with the beaker method.

A schematic diagram of the rotating-flask apparatus used to determine dissolution is shown in Fig. 1. The apparatus consists of a spherical glass flask suspended in a constant-temperature bath. The globe is supported by glass rods, fused to its sides, which form the horizontal axis of the sphere. One support rod is coupled to a constant-speed motor, which provides rotation about the horizontal axis. A sampling port is molded into the sphere to permit introduction of the dosage form and periodic withdrawal of samples. The volume of the dissolution medium (in this case 400 ml.) and the position of the sampling port are such that fluid

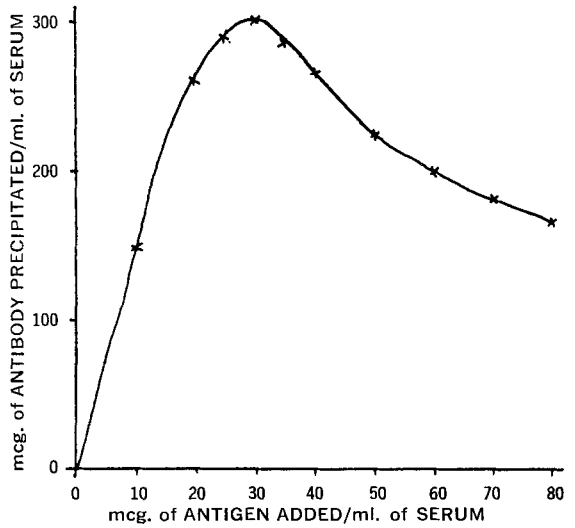


Figure 1—*The precipitin curve.*

tyrosine, have been shown to be antigenic (1–3). However, due to the unknown primary amino acid sequence of these random polymers, it is difficult to describe the locus of the active site of these antigenic polymers. To overcome this difficulty, the use of linear polypeptides with a known repeating sequence of amino acids has been suggested. For this purpose, poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester was recently synthesized (4), and we wish to report the antigenic properties of this polymer.

After obtaining preimmunization sera, four rabbits were treated at weekly intervals with 500 mcg. of poly-(tyr-glu-ala-gly)gly-1-^{14}C ethyl ester. The first 2 weeks they were injected intradermally using complete Freunds adjuvant as suspension medium, and the 3rd week they were injected subcutaneously. The injection on the 4th week was done intravenously using buffered saline. Bleedings were conducted on the following week, and the serum from each animal was found to give a precipitin reaction with the polymer. The preimmunized sera under the same conditions gave a negative precipitin reaction. The quantitative determination of the antibody was obtained by the addition of dilutions of poly-(tyr-glu-ala-gly)gly-1-^{14}C ethyl ester to 1-ml. samples of the pooled rabbit sera. The precipitates were kept at 4° for 48 hr., washed twice with small volumes of buffered saline, and collected by centrifugation. The total amount of protein precipitated was estimated by analysis for nitrogen by a micro-Kjeldahl method (5). The amount of antigen contained in each precipitate was estimated by use of the Folin-Ciocalteu method. From these results the precipitin curve shown in Fig. 1 was obtained.

From these results it can be seen that this polypeptide is antigenic; further studies pertaining to the specificity of its antibodies are presently in progress.

(1) P. H. Maurer, B. F. Gerulat, and P. Pinchuck, *J. Immunol.*, **90**, 388(1963).

(2) P. H. Maurer, B. F. Gerulat, and P. Pinchuck, *J. Biol. Chem.*, **239**, 922(1964).

(3) L. G. Clark and P. H. Maurer, *Int. Arch. Allergy*, **35**, 58 (1969).

(4) B. J. Johnson and E. G. Trask, *J. Chem. Soc.* (*C*), **1969**, 2644.

(5) D. H. Campbell, J. S. Garvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology," W. A. Benjamin, New York, N. Y., 1964, p. 46.

BRIAN J. JOHNSON
ELWOOD G. TRASK

Department of Chemistry
Tufts University
Medford, MA 02155

Received December 8, 1969.

Accepted for publication February 3, 1970.

Presented in part at the 158th American Chemical Society National Meeting, New York, N. Y., 1969.

After submission of this manuscript, a review article by M. Sela, *Science*, **166**, 1365(1969) was published on the molecular aspects of antigenicity.

This work was supported by a grant from the National Science Foundation.

We wish to thank Dr. R. D. Stollar of Tufts Medical School for many helpful suggestions.

Quantitative Correlation of Absorption and *In Vitro* Dissolution Kinetics of Aspirin from Several Dosage Forms

Keyphrases ☐ Aspirin dosage forms—dissolution, absorption ☐ Absorption–dissolution, aspirin—correlation

Sir:

Several types of *in vivo–in vitro* correlation are described in the pharmaceutical literature (1). The most informative of these, but the most difficult to achieve, is the quantitative correlation between *in vitro* dissolution and *in vivo* absorption, particularly one involving several different dosage forms. An example is found in the report of Levy *et al.* (2) who were able to correlate the absorption of aspirin from three different dosage forms with a function of the dissolution rate, using the beaker method (3) at 50 r.p.m. A plot of the percent absorbed at time *T versus* the percent dissolved in (*T*-lag time)/2 gave a straight line with a slope of unity.

The present report introduces a new dissolution method, which has permitted an absolute quantitative correlation between the absorption and *in vitro* dissolution of aspirin from these three dosage forms. These findings extend the work of Levy *et al.* (2) because this type of 1:1 correlation was not attainable with the beaker method.

A schematic diagram of the rotating-flask apparatus used to determine dissolution is shown in Fig. 1. The apparatus consists of a spherical glass flask suspended in a constant-temperature bath. The globe is supported by glass rods, fused to its sides, which form the horizontal axis of the sphere. One support rod is coupled to a constant-speed motor, which provides rotation about the horizontal axis. A sampling port is molded into the sphere to permit introduction of the dosage form and periodic withdrawal of samples. The volume of the dissolution medium (in this case 400 ml.) and the position of the sampling port are such that fluid

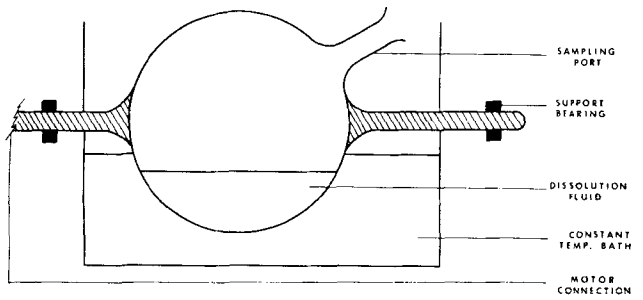


Figure 1—*Schematic diagram of dissolution apparatus. The flask has a diameter of about 16 cm. (6.5 in.)*

does not enter the port, regardless of the position of the flask as it proceeds through a revolution. These measures prevent the accumulation of undissolved solid in the port. The port is stoppered to prevent loss of the dissolution medium to the water bath. A review of the literature on dissolution methodology suggests that this method is unique, although the hydrodynamics of the present system may be similar to that found in the apparatus described by Ferrari and Khoury (4) or in the apparatus utilized by Simoons (5).

The dissolution rate of aspirin from three commercially available dosage forms—conventional tablets, buffered tablets, and timed-release tablets—was determined at 37° and 1.2 r.p.m. in 0.1 *N* HCl. Samples of the dissolution fluid were taken at frequent intervals by means of a filter pipet. The samples were then hydrolyzed and assayed spectrophotometrically at 302.5 mμ for salicylic acid. The dissolution data on each dosage form were compared with percent absorbed–time data on the same dosage forms in man from the literature (2, 6). The correlation is shown in Fig. 2 which is a plot of percent absorbed to time *T versus* percent dissolved *in vitro* at time *T*. Regression lines, calculated by the method of least squares, using all data were as follows:

$$\text{percent absorbed} = 0.79\,(\text{percent dissolved}) + 2.44 \quad \text{(Eq. 1)}$$

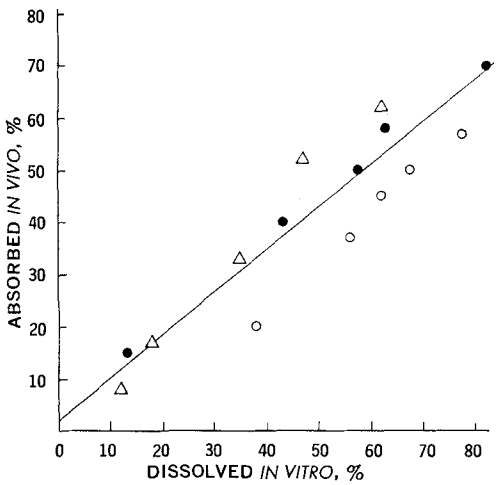


Figure 2—*Plot of percent of dose of aspirin absorbed to time* T *after drug administration* versus *percent dissolved* in vitro *at time* T. *Key:* ○, *conventional tablets;* ●, *buffered tablets, and* △, *timed-release tablets.*

$$\text{percent dissolved} = 1.08\,(\text{percent absorbed}) + 4.48 \quad \text{(Eq. 2)}$$

The solid line in Fig. 2 represents Eq. 1. Using the method described by Mather (7) for interclass correlation where both variables are normally distributed, the correlation coefficient was found to be 0.92 corresponding to $p < 0.001$.

(1) G. Levy, *Proc. Int. Congr. Pharm. Sci. 27th*, **1967**, 58.
(2) G. Levy, J. R. Leonards, and J. A. Procknal, *J. Pharm. Sci.*, **54**, 1719(1965).
(3) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262**, 1053 (1960).
(4) A. Ferrari and A. Khoury, *Ann. N. Y. Acad. Sci.*, **153**, 660 (1968).
(5) J. R. Simoons, Ph.D thesis, University of Amsterdam, The Netherlands, 1962.
(6) G. Levy, *Arch. Int. Pharmacodyn.*, **152**, 59(1964).
(7) K. Mather, "Statistical Analysis in Biology," 4th ed., Methuen and Co., London, England, 164, p. 160.

MILO GIBALDI
HOWARD WEINTRAUB*

Department of Pharmaceutics,
School of Pharmacy,
State University of New York at Buffalo,
Buffalo, NY 14214

Received November 21, 1969.
Accepted for publication January 29, 1970.
Supported in part by Grant AM-11498 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

* Fellow of the American Foundation for Pharmaceutical Education.

Penicillin Stability to Alcohols

Keyphrases □ Penicillin G, potassium, stability—alcoholic solution □ Stability—alcoholic potassium penicillin G solution

Sir:

There is considerable confusion in the literature with regard to the stability of penicillin solutions in the presence of alcohols. Several textbooks and other published works state that penicillin solutions are inactivated by alcohols and glycerol (1–14), and this statement is also found in the most recent volume of the *United States Pharmacopeia* under the monograph for Potassium Penicillin G (15). Perhaps because of this statement, at least one textbook on microbiology cautions that patients with venereal disease being treated with penicillin should avoid an intake of alcoholic beverages (16). Historically, one can trace the probable origin of the belief that penicillin is inactivated by alcohols to a report by Abraham and Chain (17) who reported in 1942 that alkali salts of penicillin rapidly lose their biological activity when dissolved in primary alcohols. Presumably, alcoholysis proceeds with the ultimate formation of the appropriate inactive monoester of penicilloic acid (Scheme I).

However, in 1948, Chain *et al.* (18) conclusively demonstrated that this decomposition of penicillin

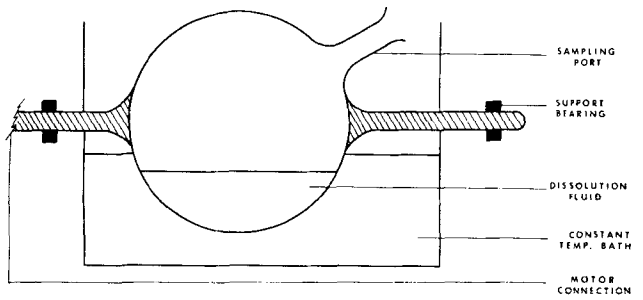


Figure 1—*Schematic diagram of dissolution apparatus. The flask has a diameter of about 16 cm. (6.5 in.)*

does not enter the port, regardless of the position of the flask as it proceeds through a revolution. These measures prevent the accumulation of undissolved solid in the port. The port is stoppered to prevent loss of the dissolution medium to the water bath. A review of the literature on dissolution methodology suggests that this method is unique, although the hydrodynamics of the present system may be similar to that found in the apparatus described by Ferrari and Khoury (4) or in the apparatus utilized by Simoons (5).

The dissolution rate of aspirin from three commercially available dosage forms—conventional tablets, buffered tablets, and timed-release tablets—was determined at 37° and 1.2 r.p.m. in 0.1 *N* HCl. Samples of the dissolution fluid were taken at frequent intervals by means of a filter pipet. The samples were then hydrolyzed and assayed spectrophotometrically at 302.5 mμ for salicylic acid. The dissolution data on each dosage form were compared with percent absorbed–time data on the same dosage forms in man from the literature (2, 6). The correlation is shown in Fig. 2 which is a plot of percent absorbed to time *T versus* percent dissolved *in vitro* at time *T*. Regression lines, calculated by the method of least squares, using all data were as follows:

$$\text{percent absorbed} = 0.79\,(\text{percent dissolved}) + 2.44 \quad \text{(Eq. 1)}$$

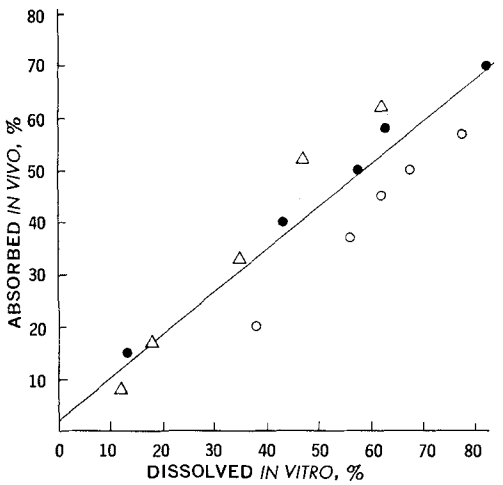


Figure 2—*Plot of percent of dose of aspirin absorbed to time* T *after drug administration* versus *percent dissolved* in vitro *at time* T. *Key:* ○, *conventional tablets;* ●, *buffered tablets, and* △, *timed-release tablets.*

$$\text{percent dissolved} = 1.08\,(\text{percent absorbed}) + 4.48 \quad \text{(Eq. 2)}$$

The solid line in Fig. 2 represents Eq. 1. Using the method described by Mather (7) for interclass correlation where both variables are normally distributed, the correlation coefficient was found to be 0.92 corresponding to $p < 0.001$.

(1) G. Levy, *Proc. Int. Congr. Pharm. Sci. 27th*, **1967**, 58.
(2) G. Levy, J. R. Leonards, and J. A. Procknal, *J. Pharm. Sci.*, **54**, 1719(1965).
(3) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262**, 1053 (1960).
(4) A. Ferrari and A. Khoury, *Ann. N. Y. Acad. Sci.*, **153**, 660 (1968).
(5) J. R. Simoons, Ph.D thesis, University of Amsterdam, The Netherlands, 1962.
(6) G. Levy, *Arch. Int. Pharmacodyn.*, **152**, 59(1964).
(7) K. Mather, "Statistical Analysis in Biology," 4th ed., Methuen and Co., London, England, 164, p. 160.

MILO GIBALDI
HOWARD WEINTRAUB*

Department of Pharmaceutics,
School of Pharmacy,
State University of New York at Buffalo,
Buffalo, NY 14214

Received November 21, 1969.
Accepted for publication January 29, 1970.
Supported in part by Grant AM-11498 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

* Fellow of the American Foundation for Pharmaceutical Education.

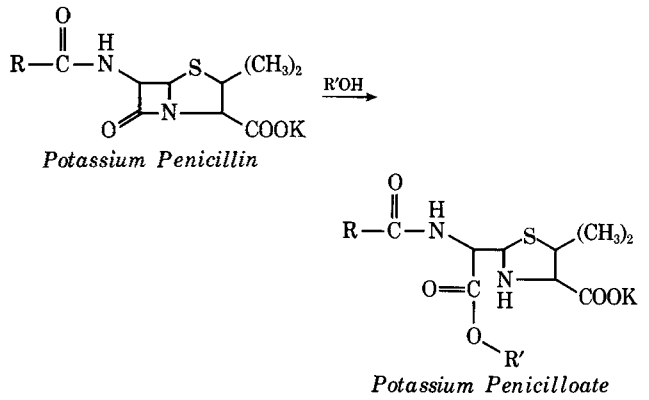
Penicillin Stability to Alcohols

Keyphrases □ Penicillin G, potassium, stability—alcoholic solution □ Stability—alcoholic potassium penicillin G solution

Sir:

There is considerable confusion in the literature with regard to the stability of penicillin solutions in the presence of alcohols. Several textbooks and other published works state that penicillin solutions are inactivated by alcohols and glycerol (1–14), and this statement is also found in the most recent volume of the *United States Pharmacopeia* under the monograph for Potassium Penicillin G (15). Perhaps because of this statement, at least one textbook on microbiology cautions that patients with venereal disease being treated with penicillin should avoid an intake of alcoholic beverages (16). Historically, one can trace the probable origin of the belief that penicillin is inactivated by alcohols to a report by Abraham and Chain (17) who reported in 1942 that alkali salts of penicillin rapidly lose their biological activity when dissolved in primary alcohols. Presumably, alcoholysis proceeds with the ultimate formation of the appropriate inactive monoester of penicilloic acid (Scheme I).

However, in 1948, Chain *et al.* (18) conclusively demonstrated that this decomposition of penicillin



Scheme I—Alcoholysis of potassium penicillin to inactive potassium penicilloate

salts in methanol occurred only in the presence of zinc, copper, or tin, which were contaminants in the alcohol that was used to demonstrate deactivation of penicillin as previously reported (17). They postulated that rather than a direct alcoholytic fission of the penicillin β-lactam ring, alcoholysis probably resulted through an intermediate oxazolone (penicillenic acid), formed by attack of the metal ion on the sulfur of the molecule (18). This inactivation of penicillin by metal-contaminated alcohol was retarded by dimercaptopropanol (18). These findings were later confirmed by Parnaby, who pointed out that alcoholic penicillin solutions were as stable as aqueous penicillin solutions (19).

Brodersen (20), while studying the kinetics of penicillin–penicillinase reactions, found that ethanol could be used to halt the enzymatic reaction without inactivating residual penicillin. At about the same time, other workers were using ethanol to eluate adsorbed penicillin from charcoal chromatographic columns in relatively good yields, which offers additional evidence that alcohols do not adversely affect penicillin stability (21). Indeed, penicillin has even been used in the past as a preservative of beer (22).

Finally, one can point to Fleming's original report on penicillin in which he indicates that penicillin activity could be extracted into ethanol from concentrated fermentation media (23).

Clearly, full consideration of the meaning of these early studies has not been given, and the concept of penicillin–alcohol "incompatibility" remains to this day. Curiously enough, with the voluminous literature available on all aspects of penicillins, a review by Doyle and Nayler (24) is the only report that we have found that clearly points out that penicillins are stable in the presence of alcohols.

Preliminary studies in our laboratory indicated that potassium penicillin G, following incubation with various concentrations of ethanol contained in nutrient broth, human serum, and human plasma, indeed continued to exhibit antibacterial activity against a known penicillin-sensitive strain of *Staphylococcus aureus*. Similar *in vitro* activity of penicillin in the presence of benzyl alcohol was demonstrated as well.

Thus, to derive more quantitative data and to establish that penicillin is stable in the presence of alcohols, we initiated a series of simple confirmatory experiments

Table I—Assay Results for Residual Potassium Penicillin G

Day after Preparation of Solution	Potassium Penicillin G Remaining, %				
	Predicted[a]	Solution A[b]	Solution B[c]	Solution C[d]	Solution D[e]
1	98.3	99.0	97.0	99.0	96.9
2	96.7	96.3	94.0	97.4	97.1
3	95.0	95.5	96.3	93.5	94.4
4	93.4	92.0	94.9	90.0	89.8
5	91.0	92.0	90.0	92.5	88.4
6	90.0	91.8	89.0	89.2	85.0
7	88.0	88.5	87.0	88.0	85.2
8	86.0	84.6	87.0	84.0	83.6
9	84.0	86.4	85.2	84.0	83.2
10	83.0	85.1	84.1	85.0	82.0

[a] Calculated from published tables (20). [b] Potassium penicillin G control solution. [c] 40% ethanolic potassium penicillin G solution. [d] 70% ethanolic potassium penicillin G solution. [e] 0.5% benzyl alcohol potassium penicillin G solution.

on the subject. Ethanol and benzyl alcohol were utilized, the latter because sterile distilled water containing benzyl alcohol as a preservative is used in many hospitals as a diluent for the preparation of solutions of penicillin for parenteral administration.

Experimental—*Materials*—The penicillin used was commercially available potassium penicillin G. All solutions used in this study were prepared with a 1% phosphate buffer pH 6.0 (2.0 g. dibasic potassium phosphate, 8.0 g. monobasic potassium phosphate, and sufficient distilled water to make 1000 ml.) to contain a final concentration of 2000 u./ml. of potassium penicillin G. Subsequent sterilization of these solutions was done by Millipore filtration (0.22-μ pore size), after which they were stored at room temperature in previously sterilized glass flasks fitted with screw caps.

Assay Procedure—The method used to determine residual penicillin was an iodometric titration adapted from Alicino (25) and Mundell *et al.* (26) as described by Grove and Randall (27). At predetermined times (Table I), 2-ml. aliquots from each of the test solutions were aseptically removed and assayed for total penicillin. Each assay was done in duplicate, and the results were averaged. As a precautionary measure to detect accidental contamination by microorganisms, each day a small amount from each penicillin test solution was diluted with sterile saline and the resulting mixture was passed through a Millipore filter apparatus. Three further rinses with saline through the filter served to remove all residual penicillin, after which the membrane filter was removed, incubated on a suitable solid medium according to standard procedures, and observed for evidence of bacterial or fungal growth. No contamination was noted for all solutions throughout the course of this study.

Preparation of Penicillin Test Solutions—Penicillin Control (Solution A)—A sterile and buffered penicillin solution was prepared as described under *Materials*.

Ethanol Penicillin Solutions (Solutions B and C)—Two separate penicillin solutions were prepared as directed for Solution A, except that a portion of the water in each solution was replaced with 95% ethanol to furnish final concentrations of 40% (Solution B) and 70% (Solution C) ethanol, respectively.

Benzyl Alcohol Penicillin Solution (Solution D)—A sterile aqueous solution of benzyl alcohol (0.5%) was used as the vehicle to prepare the buffered penicillin solution.

Results and Discussion—The data from Table I show that the amounts of potassium penicillin G remaining in the control solution (Solution A) at specific times after the start of the experiment coincide rather well with predicted values which were calculated from published tables (20). Furthermore, it is noteworthy that the observed residual penicillin concentrations in the ethanol (Solutions B and C) as well as the benzyl alcohol (Solution D) solutions compared favorably with those of the penicillin control (Solution A).

Thus, these experiments have shown that potassium penicillin G can be mixed with ethanol solutions of concentrations as high as 70% for up to 10 days, with no greater loss than penicillin in aqueous solution. In a similar manner, it has been shown that benzyl alcohol, at a concentration of 0.5%, will not cause a decrease in stability of potassium penicillin G when in contact with the antibiotic for periods up to 10 days.

(1) "Penicillin," Merck and Co., Rahway, N. J., 1944, p. 5.

(2) "A Review of the Present Information Concerning Penicillin," Abbott Laboratories, North Chicago, Ill., 1945, p. 152.

(3) M. C. Rebstock, in "Medicinal Chemistry," 2nd ed., A. Burger, Ed., Interscience, New York, N. Y., 1960, p. 885.

(4) H. Berry, in "Penicillin—Its Practical Application," 2nd ed., A. Fleming, Ed., Butterworth and Co., Ltd., London, England, 1950, p. 61.

(5) W. E. Herrell, "Penicillin and Other Antibiotic Agents," W. B. Saunders, Philadelphia, Pa., 1945, p. 102.

(6) D. E. Wurster, in "Textbook of Pharmaceutical Compounding and Dispensing," 2nd ed., R. A. Lyman and J. B. Sprowls, Eds., J. B. Lippincott, Philadelphia, Pa., 1955, p. 354.

(7) J. K. Dale, in "Husa's Pharmaceutical Dispensing," 6th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1966, pp. 690, 698.

(8) "The United States Dispensatory and Physician's Pharmacology," 26th ed., A. Osol, R. Pratt, and M. D. Altschule, Eds., J. B. Lippincott, Philadelphia, Pa., 1967, p. 830.

(9) L. P. Garrod, in "Experimental Chemotherapy," vol. 3, R. J. Schnitzer and F. Hawking, Eds., Academic, New York, N. Y.' 1964, p. 3.

(10) D. E. Wurster, in "Prescription Pharmacy," J. B. Sprowls, Jr., Ed., J. B. Lippincott, Philadelphia, Pa., 1963, p. 369.

(11) "Martindale Extra Pharmacopoeia," 25th ed., R. G. Todd, Ed., The Pharmaceutical Press, London, England, 1967, p. 930.

(12) L. Neipp, *Schweiz. Z. Allg. Pathol. Bakteriol.*, **20,** 150 (1957).

(13) M. A. Schwartz and F. H. Buckwalter, *J. Pharm. Sci.*, **51,** 1119(1962).

(14) P. P. Koelzer and J. Giesen, *Z. Gesamte Inn. Med. Ihre Grenzgeb.*, **4,** 321(1949).

(15) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 495.

(16) F. R. Gomila and J. E. Linder, in "Antimicrobial Agents Annual 1960," P. Gray, B. Tabenkin, and S. G. Bradley, Eds., Plenum, New York, N. Y., 1961, p. 349.

(17) E. P. Abraham and E. Chain, *Brit. J. Exp. Pathol.*, **23,** 103 (1942).

(18) E. Chain, F. J. Philpot, and D. Callow, *Arch. Biochem.*, **18,** 171(1948).

(19) R. C. Parnaby, *Pharm. J.*, **156,** 55(1946).

(20) R. Brodersen, "Inactivation of Penicillin in Aqueous Solution," Einar Munksgaard, Copenhagen, Denmark, 1949, pp. 115, 122, 148.

(21) Y. Kawano and S. Uno, *J. Ferment. Technol.*, **27,** 166(1949).

(22) G. J. Haas, *Wallerstein Lab. Commun.*, **18,** 253(1955).

(23) A. Fleming, *Brit. J. Exp. Pathol.*, **10,** 226(1929).

(24) F. P. Doyle and J. H. C. Nayler, in "Advances in Drug Research," vol. 1, N. J. Harper and A. B. Simmonds, Eds., Academic, New York, N. Y., 1964, p. 26.

(25) J. F. Alicino, *Ind. Eng. Chem. Anal. Ed.*, **18,** 619(1946).

(26) M. Mundell, H. Fischbach, and T. E. Eble, *J. Amer. Pharm. Ass., Sci. Ed.*, **35,** 373(1946).

(27) D. C. Grove and W. A. Randall, "Assay Methods of Antibiotics, A Laboratory Manual," Medical Encyclopedia, New York, N. Y., 1955, p. 16.

ALVIN B. SEGELMAN
NORMAN R. FARNSWORTH
Department of Pharmacognosy
School of Pharmacy
University of Pittsburgh
Pittsburgh, PA 15213

Received June 6, 1969.
Accepted for publication February 5, 1970.

Books

REVIEWS

Experimental Methods in Pharmacology. By CHARLES O. WARD. St. John's University Press, Jamaica, NY 11432, 1969. 119 pp. 15 x 23 cm.

This small book briefly describes 62 experiments under 18 different chapter headings. Some of the subjects covered are local anesthetics, psychotropic drugs, anticonvulsants, muscle relaxants, topical drugs, antihistamines, and diuretics. Most of the experiments described are time-tested. Unfortunately the book is not of general value even though it is well suited to the author's laboratory. The descriptions of the experiments are very short and in many cases not too informative. Many numbers are missing for dosages and stimulus parameters; no statement of the intent of the blank spaces is given. This reviewer presumes that the student is to furnish the numbers by reference to the literature.

In some cases the experiments are so inadequately described as to be seriously misleading. An example is the experiment on measuring blood flow with a rotameter. The obvious requirement that flow measurements must have simultaneous pressure measurements to have physiological meaning is ignored. In addition the use of the carotid artery is questionable because of the parallel flow (unmeasured) through three other large arteries.

The book is marred by many typographical errors; some of these are quite humorous, such as untravenously and cammula. Dosages are given in variable units; cc. of solution per kgm. or by weight per kgm. Anesthesia seems to have been omitted in some experiments in which it seems indicated. In an experiment in which painful stimuli are applied to a guinea pig, no description of the animal's response is given.

This edition of "Experimental Methods in Pharmacology" cannot be recommended for general use. The ideas behind it are excellent, and a revision based on discussions with other pharmacology teachers would be a welcome addition.

Reviewed by R. P. Ahlquist
Medical College of Georgia
Augusta, GA 30902 ■

Benzyl Alcohol Penicillin Solution (Solution D)—A sterile aqueous solution of benzyl alcohol (0.5%) was used as the vehicle to prepare the buffered penicillin solution.

Results and Discussion—The data from Table I show that the amounts of potassium penicillin G remaining in the control solution (Solution A) at specific times after the start of the experiment coincide rather well with predicted values which were calculated from published tables (20). Furthermore, it is noteworthy that the observed residual penicillin concentrations in the ethanol (Solutions B and C) as well as the benzyl alcohol (Solution D) solutions compared favorably with those of the penicillin control (Solution A).

Thus, these experiments have shown that potassium penicillin G can be mixed with ethanol solutions of concentrations as high as 70% for up to 10 days, with no greater loss than penicillin in aqueous solution. In a similar manner, it has been shown that benzyl alcohol, at a concentration of 0.5%, will not cause a decrease in stability of potassium penicillin G when in contact with the antibiotic for periods up to 10 days.

(1) "Penicillin," Merck and Co., Rahway, N. J., 1944, p. 5.

(2) "A Review of the Present Information Concerning Penicillin," Abbott Laboratories, North Chicago, Ill., 1945, p. 152.

(3) M. C. Rebstock, in "Medicinal Chemistry," 2nd ed., A. Burger, Ed., Interscience, New York, N. Y., 1960, p. 885.

(4) H. Berry, in "Penicillin—Its Practical Application," 2nd ed., A. Fleming, Ed., Butterworth and Co., Ltd., London, England, 1950, p. 61.

(5) W. E. Herrell, "Penicillin and Other Antibiotic Agents," W. B. Saunders, Philadelphia, Pa., 1945, p. 102.

(6) D. E. Wurster, in "Textbook of Pharmaceutical Compounding and Dispensing," 2nd ed., R. A. Lyman and J. B. Sprowls, Eds., J. B. Lippincott, Philadelphia, Pa., 1955, p. 354.

(7) J. K. Dale, in "Husa's Pharmaceutical Dispensing," 6th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1966, pp. 690, 698.

(8) "The United States Dispensatory and Physician's Pharmacology," 26th ed., A. Osol, R. Pratt, and M. D. Altschule, Eds., J. B. Lippincott, Philadelphia, Pa., 1967, p. 830.

(9) L. P. Garrod, in "Experimental Chemotherapy," vol. 3, R. J. Schnitzer and F. Hawking, Eds., Academic, New York, N. Y.' 1964, p. 3.

(10) D. E. Wurster, in "Prescription Pharmacy," J. B. Sprowls, Jr., Ed., J. B. Lippincott, Philadelphia, Pa., 1963, p. 369.

(11) "Martindale Extra Pharmacopoeia," 25th ed., R. G. Todd, Ed., The Pharmaceutical Press, London, England, 1967, p. 930.

(12) L. Neipp, *Schweiz. Z. Allg. Pathol. Bakteriol.*, **20,** 150 (1957).

(13) M. A. Schwartz and F. H. Buckwalter, *J. Pharm. Sci.*, **51,** 1119(1962).

(14) P. P. Koelzer and J. Giesen, *Z. Gesamte Inn. Med. Ihre Grenzgeb.*, **4,** 321(1949).

(15) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 495.

(16) F. R. Gomila and J. E. Linder, in "Antimicrobial Agents Annual 1960," P. Gray, B. Tabenkin, and S. G. Bradley, Eds., Plenum, New York, N. Y., 1961, p. 349.

(17) E. P. Abraham and E. Chain, *Brit. J. Exp. Pathol.*, **23,** 103 (1942).

(18) E. Chain, F. J. Philpot, and D. Callow, *Arch. Biochem.*, **18,** 171(1948).

(19) R. C. Parnaby, *Pharm. J.*, **156,** 55(1946).

(20) R. Brodersen, "Inactivation of Penicillin in Aqueous Solution," Einar Munksgaard, Copenhagen, Denmark, 1949, pp. 115, 122, 148.

(21) Y. Kawano and S. Uno, *J. Ferment. Technol.*, **27,** 166(1949).

(22) G. J. Haas, *Wallerstein Lab. Commun.*, **18,** 253(1955).

(23) A. Fleming, *Brit. J. Exp. Pathol.*, **10,** 226(1929).

(24) F. P. Doyle and J. H. C. Nayler, in "Advances in Drug Research," vol. 1, N. J. Harper and A. B. Simmonds, Eds., Academic, New York, N. Y., 1964, p. 26.

(25) J. F. Alicino, *Ind. Eng. Chem. Anal. Ed.*, **18,** 619(1946).

(26) M. Mundell, H. Fischbach, and T. E. Eble, *J. Amer. Pharm. Ass., Sci. Ed.*, **35,** 373(1946).

(27) D. C. Grove and W. A. Randall, "Assay Methods of Antibiotics, A Laboratory Manual," Medical Encyclopedia, New York, N. Y., 1955, p. 16.

ALVIN B. SEGELMAN
NORMAN R. FARNSWORTH
Department of Pharmacognosy
School of Pharmacy
University of Pittsburgh
Pittsburgh, PA 15213

Received June 6, 1969.
Accepted for publication February 5, 1970.

Books

REVIEWS

Experimental Methods in Pharmacology. By CHARLES O. WARD. St. John's University Press, Jamaica, NY 11432, 1969. 119 pp. 15 x 23 cm.

This small book briefly describes 62 experiments under 18 different chapter headings. Some of the subjects covered are local anesthetics, psychotropic drugs, anticonvulsants, muscle relaxants, topical drugs, antihistamines, and diuretics. Most of the experiments described are time-tested. Unfortunately the book is not of general value even though it is well suited to the author's laboratory. The descriptions of the experiments are very short and in many cases not too informative. Many numbers are missing for dosages and stimulus parameters; no statement of the intent of the blank spaces is given. This reviewer presumes that the student is to furnish the numbers by reference to the literature.

In some cases the experiments are so inadequately described as to be seriously misleading. An example is the experiment on measuring blood flow with a rotameter. The obvious requirement that flow measurements must have simultaneous pressure measurements to have physiological meaning is ignored. In addition the use of the carotid artery is questionable because of the parallel flow (unmeasured) through three other large arteries.

The book is marred by many typographical errors; some of these are quite humorous, such as untravenously and cammula. Dosages are given in variable units; cc. of solution per kgm. or by weight per kgm. Anesthesia seems to have been omitted in some experiments in which it seems indicated. In an experiment in which painful stimuli are applied to a guinea pig, no description of the animal's response is given.

This edition of "Experimental Methods in Pharmacology" cannot be recommended for general use. The ideas behind it are excellent, and a revision based on discussions with other pharmacology teachers would be a welcome addition.

Reviewed by R. P. Ahlquist
Medical College of Georgia
Augusta, GA 30902 ■

Drug Abuse: Social and Psychopharmacological Aspects. Compiled and edited by JONATHAN O. COLE and J. R. WITTENBORN, Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiii + 170 pp. 15.5 × 23.5 cm. Price $9.50.

This book is drawn from reports presented during the Fifth Annual Meeting of the American College of Neuropsychopharmacology. While there is no primary focus to the papers included, they are representative of current research related to the growing social and medical concern of drug abuse.

One paper should be of particular interest because of the controversial nature of the topic and the scarcity of scientific research in this area. This study of the long-term effects of LSD administration in volunteer graduate students suggests that the use of LSD under controlled circumstances with selected individuals may be less hazardous and have less far-reaching consequences than previously thought. However, other studies reported in this book seem to present evidence to the contrary, confirming the need for more extensive research into these problems.

Data from a study of marijuana indicate that marijuana use begins as a group experience engaged in by peers, and not as the pusher engaging the youth.

This book includes eleven studies concerning various aspects of drugs and drug abuse. While the editors admit that no answers are presented, they are hopeful that this volume will serve to stimulate research in these and related areas.

Staff Review ■

Drugs and Youth. Edited by J. R. WITTENBORN, HENRY BRILL, JEAN PAUL SMITH, and SARAH A. WITTENBORN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiv + 485 pp. 18 × 26 cm. Price $22.75.

This book, "Drugs and Youth," is based on the proceedings of the Rutgers Symposium on Drug Abuse held in June, 1968, at the Rutgers Interdisciplinary Research Center. The one hundred fifty participants in the three-day symposium were scientists, law enforcement officials, and members of the judiciary.

The book is divided into six major sections, the first being introductory material of a broad nature. Part II deals with morphine, heroin, and cocaine. Amphetamine-type and barbiturate tranquilizer-type drugs are covered in the third section, followed by marijuana and LSD in Part IV.

The concluding two sections deal with drug abuse and the law, and action to be taken, respectively.

Although the book takes a broad look at the problems of drugs and youth with a sociological emphasis, it should be of interest to all persons working in the area of drug abuse.

Staff Review ■

Techniques of Medication. By ERIC W. MARTIN, STEWART F. ALEXANDER, WILLIAM E. HASSAN, JR., and BURTON L. SHERMAN. J. B. Lippincott, East Washington Square, Philadelphia, PA 19105, 1969. vii + 239 pp. 18.3 × 26.1 cm. Price $18.00.

This book is devoted to discussing those factors that often are not emphasized in the administration of a drug. In addition to the rational selection of a drug, factors such as route of administration and form of medication will often determine the usefulness of a medication. The authors have done well in attempting to discuss this comprehensive area. The book is introduced by discussing the trends in therapeutics and principles of medication. The second chapter is devoted to prescription writing and the factors that must be considered in a prescription. These factors would include dosage, directions to the patient, and legal and professional aspects.

This is followed by sections dealing with various routes of medication (dermatomucosal, parenteral, and gastrointestinal) and outlining the principles of medication that must be considered. These principles include topics such as dosage forms, techniques of administrations, and care of needles and syringes. The next section discusses nicely the handling of medication by nurses. This section is written in clear, easy-to-follow outline form. The book closes with a comprehensive discussion of the legal aspects of medication. These include factors such as prescribing errors, inadequate testing, and sensitivity reactions.

This book incorporates the basic concepts of a book published by Austin Smith in 1948 entitled "Technic of Medication." The authors of this volume are to be complimented on bringing this important topic up to date and providing a practical manual on medication techniques for physicians, medical students, nurses, and pharmacists. All members of the health team must not only know their drugs but, equally important, they must known how to use them.

Reviewed by J. P. Long
Department of Pharmacology
University of Iowa
Iowa City, IA 52240 ■

Industrial Toxicology. By LAWRENCE T. FAIRHALL. Second Edition (Facsimile of 1957 Edition). Hafner Publishing Co., New York, NY 1969. xii + 376 pp. 18.3 × 26.3 cm. Price $12.00.

A continuing demand for this standard reference work in industrial toxicology has justified this reprinting of the 12-year old second edition. It is likely that part of such a demand is based on the desires of some to replace their much-used and worn-out copies. Fairhall has had a well-established and long-standing reputation as a source of information about elements and compounds of toxicologic significance in industry. This book, though hardly expected to be up to date, still contains much data of current value and is a useful guide to some of the outstanding older literature.

With a compendium type of format, this volume is presented in two parts. Part I deals with inorganic substances, mostly elements and their salts, oxides, or compounds with hydrogen. The larger Part II, entitled *Carbon Compounds*, deals with numerous organic chemicals. For each substance or compound, its chemical and physical characteristics, industrial uses, toxicity, and general approach to methods of analysis are described in a concisely documented form. There is little consideration of mechanisms of toxic action and no discussion of the treatment of poisoning by the agents described. An appendix contains tables listing urinary metabolites of 73 compounds, threshold limit values of numerous toxic gases, vapors, fumes and dusts, and the LD_{50}'s by oral administration in rats of 104 organic chemicals.

Obviously, much new knowledge concerning substances of industrial toxicologic importance has developed since this edition was published in 1957, and several more recent books are available that contain such information. Hence, the later works must be relied upon as prime references in the field, with Fairhall serving a valuable supplementary and back-up function. An example of an area in arrears in this book is the timely topic of pesticides. Some of the well-known controversial persistent pesticides such as aldrin, dieldrin, endrin, heptachlor, and heptachlor epoxide are not described, though threshold limit values and LD_{50}'s for aldrin and dieldrin are tabulated in the appendix.

For numerous agents described, however, little or no new information has been produced since 1957, and for these substances Fairhall stands as a definitive reference. This volume is still an important component of any library purporting to cover toxicology comprehensively or industrial toxicology specifically.

Reviewed by Julius Coon
Jefferson Medical College
Philadelphia, PA 19107 ■

The Biochemistry of Alkaloids: Molecular Biology, Biochemistry and Biophysics, Vol. 3. By TREVOR ROBINSON. Springer-Verlag, New York, NY, 1968. 149 pp. 17 × 25 cm. Price $9.75.

I enjoyed the opportunity of reviewing this book which was both readable and interesting. Although the author, in his preface, makes no attempt to state whether the monograph is intended primarily as a text or reference book, it would appear to me that its greatest

Drug Abuse: Social and Psychopharmacological Aspects. Compiled and edited by JONATHAN O. COLE and J. R. WITTENBORN, Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiii + 170 pp. 15.5 × 23.5 cm. Price $9.50.

This book is drawn from reports presented during the Fifth Annual Meeting of the American College of Neuropsychopharmacology. While there is no primary focus to the papers included, they are representative of current research related to the growing social and medical concern of drug abuse.

One paper should be of particular interest because of the controversial nature of the topic and the scarcity of scientific research in this area. This study of the long-term effects of LSD administration in volunteer graduate students suggests that the use of LSD under controlled circumstances with selected individuals may be less hazardous and have less far-reaching consequences than previously thought. However, other studies reported in this book seem to present evidence to the contrary, confirming the need for more extensive research into these problems.

Data from a study of marijuana indicate that marijuana use begins as a group experience engaged in by peers, and not as the pusher engaging the youth.

This book includes eleven studies concerning various aspects of drugs and drug abuse. While the editors admit that no answers are presented, they are hopeful that this volume will serve to stimulate research in these and related areas.

Staff Review ■

Drugs and Youth. Edited by J. R. WITTENBORN, HENRY BRILL, JEAN PAUL SMITH, and SARAH A. WITTENBORN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiv + 485 pp. 18 × 26 cm. Price $22.75.

This book, "Drugs and Youth," is based on the proceedings of the Rutgers Symposium on Drug Abuse held in June, 1968, at the Rutgers Interdisciplinary Research Center. The one hundred fifty participants in the three-day symposium were scientists, law enforcement officials, and members of the judiciary.

The book is divided into six major sections, the first being introductory material of a broad nature. Part II deals with morphine, heroin, and cocaine. Amphetamine-type and barbiturate tranquilizer-type drugs are covered in the third section, followed by marijuana and LSD in Part IV.

The concluding two sections deal with drug abuse and the law, and action to be taken, respectively.

Although the book takes a broad look at the problems of drugs and youth with a sociological emphasis, it should be of interest to all persons working in the area of drug abuse.

Staff Review ■

Techniques of Medication. By ERIC W. MARTIN, STEWART F. ALEXANDER, WILLIAM E. HASSAN, JR., and BURTON L. SHERMAN. J. B. Lippincott, East Washington Square, Philadelphia, PA 19105, 1969. vii + 239 pp. 18.3 × 26.1 cm. Price $18.00.

This book is devoted to discussing those factors that often are not emphasized in the administration of a drug. In addition to the rational selection of a drug, factors such as route of administration and form of medication will often determine the usefulness of a medication. The authors have done well in attempting to discuss this comprehensive area. The book is introduced by discussing the trends in therapeutics and principles of medication. The second chapter is devoted to prescription writing and the factors that must be considered in a prescription. These factors would include dosage, directions to the patient, and legal and professional aspects.

This is followed by sections dealing with various routes of medication (dermatomucosal, parenteral, and gastrointestinal) and outlining the principles of medication that must be considered. These principles include topics such as dosage forms, techniques of administrations, and care of needles and syringes. The next section discusses nicely the handling of medication by nurses. This section is written in clear, easy-to-follow outline form. The book closes with a comprehensive discussion of the legal aspects of medication. These include factors such as prescribing errors, inadequate testing, and sensitivity reactions.

This book incorporates the basic concepts of a book published by Austin Smith in 1948 entitled "Technic of Medication." The authors of this volume are to be complimented on bringing this important topic up to date and providing a practical manual on medication techniques for physicians, medical students, nurses, and pharmacists. All members of the health team must not only know their drugs but, equally important, they must known how to use them.

Reviewed by J. P. Long
Department of Pharmacology
University of Iowa
Iowa City, IA 52240 ■

Industrial Toxicology. By LAWRENCE T. FAIRHALL. Second Edition (Facsimile of 1957 Edition). Hafner Publishing Co., New York, NY 1969. xii + 376 pp. 18.3 × 26.3 cm. Price $12.00.

A continuing demand for this standard reference work in industrial toxicology has justified this reprinting of the 12-year old second edition. It is likely that part of such a demand is based on the desires of some to replace their much-used and worn-out copies. Fairhall has had a well-established and long-standing reputation as a source of information about elements and compounds of toxicologic significance in industry. This book, though hardly expected to be up to date, still contains much data of current value and is a useful guide to some of the outstanding older literature.

With a compendium type of format, this volume is presented in two parts. Part I deals with inorganic substances, mostly elements and their salts, oxides, or compounds with hydrogen. The larger Part II, entitled *Carbon Compounds*, deals with numerous organic chemicals. For each substance or compound, its chemical and physical characteristics, industrial uses, toxicity, and general approach to methods of analysis are described in a concisely documented form. There is little consideration of mechanisms of toxic action and no discussion of the treatment of poisoning by the agents described. An appendix contains tables listing urinary metabolites of 73 compounds, threshold limit values of numerous toxic gases, vapors, fumes and dusts, and the LD_{50}'s by oral administration in rats of 104 organic chemicals.

Obviously, much new knowledge concerning substances of industrial toxicologic importance has developed since this edition was published in 1957, and several more recent books are available that contain such information. Hence, the later works must be relied upon as prime references in the field, with Fairhall serving a valuable supplementary and back-up function. An example of an area in arrears in this book is the timely topic of pesticides. Some of the well-known controversial persistent pesticides such as aldrin, dieldrin, endrin, heptachlor, and heptachlor epoxide are not described, though threshold limit values and LD_{50}'s for aldrin and dieldrin are tabulated in the appendix.

For numerous agents described, however, little or no new information has been produced since 1957, and for these substances Fairhall stands as a definitive reference. This volume is still an important component of any library purporting to cover toxicology comprehensively or industrial toxicology specifically.

Reviewed by Julius Coon
Jefferson Medical College
Philadelphia, PA 19107 ■

The Biochemistry of Alkaloids: Molecular Biology, Biochemistry and Biophysics, Vol. 3. By TREVOR ROBINSON. Springer-Verlag, New York, NY, 1968. 149 pp. 17 × 25 cm. Price $9.75.

I enjoyed the opportunity of reviewing this book which was both readable and interesting. Although the author, in his preface, makes no attempt to state whether the monograph is intended primarily as a text or reference book, it would appear to me that its greatest

Drug Abuse: Social and Psychopharmacological Aspects. Compiled and edited by JONATHAN O. COLE and J. R. WITTENBORN, Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiii + 170 pp. 15.5 × 23.5 cm. Price $9.50.

This book is drawn from reports presented during the Fifth Annual Meeting of the American College of Neuropsychopharmacology. While there is no primary focus to the papers included, they are representative of current research related to the growing social and medical concern of drug abuse.

One paper should be of particular interest because of the controversial nature of the topic and the scarcity of scientific research in this area. This study of the long-term effects of LSD administration in volunteer graduate students suggests that the use of LSD under controlled circumstances with selected individuals may be less hazardous and have less far-reaching consequences than previously thought. However, other studies reported in this book seem to present evidence to the contrary, confirming the need for more extensive research into these problems.

Data from a study of marijuana indicate that marijuana use begins as a group experience engaged in by peers, and not as the pusher engaging the youth.

This book includes eleven studies concerning various aspects of drugs and drug abuse. While the editors admit that no answers are presented, they are hopeful that this volume will serve to stimulate research in these and related areas.

Staff Review ■

Drugs and Youth. Edited by J. R. WITTENBORN, HENRY BRILL, JEAN PAUL SMITH, and SARAH A. WITTENBORN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiv + 485 pp. 18 × 26 cm. Price $22.75.

This book, "Drugs and Youth," is based on the proceedings of the Rutgers Symposium on Drug Abuse held in June, 1968, at the Rutgers Interdisciplinary Research Center. The one hundred fifty participants in the three-day symposium were scientists, law enforcement officials, and members of the judiciary.

The book is divided into six major sections, the first being introductory material of a broad nature. Part II deals with morphine, heroin, and cocaine. Amphetamine-type and barbiturate tranquilizer-type drugs are covered in the third section, followed by marijuana and LSD in Part IV.

The concluding two sections deal with drug abuse and the law, and action to be taken, respectively.

Although the book takes a broad look at the problems of drugs and youth with a sociological emphasis, it should be of interest to all persons working in the area of drug abuse.

Staff Review ■

Techniques of Medication. By ERIC W. MARTIN, STEWART F. ALEXANDER, WILLIAM E. HASSAN, JR., and BURTON L. SHERMAN. J. B. Lippincott, East Washington Square, Philadelphia, PA 19105, 1969. vii + 239 pp. 18.3 × 26.1 cm. Price $18.00.

This book is devoted to discussing those factors that often are not emphasized in the administration of a drug. In addition to the rational selection of a drug, factors such as route of administration and form of medication will often determine the usefulness of a medication. The authors have done well in attempting to discuss this comprehensive area. The book is introduced by discussing the trends in therapeutics and principles of medication. The second chapter is devoted to prescription writing and the factors that must be considered in a prescription. These factors would include dosage, directions to the patient, and legal and professional aspects.

This is followed by sections dealing with various routes of medication (dermatomucosal, parenteral, and gastrointestinal) and outlining the principles of medication that must be considered. These principles include topics such as dosage forms, techniques of administrations, and care of needles and syringes. The next section discusses nicely the handling of medication by nurses. This section is written in clear, easy-to-follow outline form. The book closes with a comprehensive discussion of the legal aspects of medication. These include factors such as prescribing errors, inadequate testing, and sensitivity reactions.

This book incorporates the basic concepts of a book published by Austin Smith in 1948 entitled "Technic of Medication." The authors of this volume are to be complimented on bringing this important topic up to date and providing a practical manual on medication techniques for physicians, medical students, nurses, and pharmacists. All members of the health team must not only know their drugs but, equally important, they must known how to use them.

Reviewed by J. P. Long
Department of Pharmacology
University of Iowa
Iowa City, IA 52240 ■

Industrial Toxicology. By LAWRENCE T. FAIRHALL. Second Edition (Facsimile of 1957 Edition). Hafner Publishing Co., New York, NY 1969. xii + 376 pp. 18.3 × 26.3 cm. Price $12.00.

A continuing demand for this standard reference work in industrial toxicology has justified this reprinting of the 12-year old second edition. It is likely that part of such a demand is based on the desires of some to replace their much-used and worn-out copies. Fairhall has had a well-established and long-standing reputation as a source of information about elements and compounds of toxicologic significance in industry. This book, though hardly expected to be up to date, still contains much data of current value and is a useful guide to some of the outstanding older literature.

With a compendium type of format, this volume is presented in two parts. Part I deals with inorganic substances, mostly elements and their salts, oxides, or compounds with hydrogen. The larger Part II, entitled *Carbon Compounds*, deals with numerous organic chemicals. For each substance or compound, its chemical and physical characteristics, industrial uses, toxicity, and general approach to methods of analysis are described in a concisely documented form. There is little consideration of mechanisms of toxic action and no discussion of the treatment of poisoning by the agents described. An appendix contains tables listing urinary metabolites of 73 compounds, threshold limit values of numerous toxic gases, vapors, fumes and dusts, and the LD_{50}'s by oral administration in rats of 104 organic chemicals.

Obviously, much new knowledge concerning substances of industrial toxicologic importance has developed since this edition was published in 1957, and several more recent books are available that contain such information. Hence, the later works must be relied upon as prime references in the field, with Fairhall serving a valuable supplementary and back-up function. An example of an area in arrears in this book is the timely topic of pesticides. Some of the well-known controversial persistent pesticides such as aldrin, dieldrin, endrin, heptachlor, and heptachlor epoxide are not described, though threshold limit values and LD_{50}'s for aldrin and dieldrin are tabulated in the appendix.

For numerous agents described, however, little or no new information has been produced since 1957, and for these substances Fairhall stands as a definitive reference. This volume is still an important component of any library purporting to cover toxicology comprehensively or industrial toxicology specifically.

Reviewed by Julius Coon
Jefferson Medical College
Philadelphia, PA 19107 ■

The Biochemistry of Alkaloids: Molecular Biology, Biochemistry and Biophysics, Vol. 3. By TREVOR ROBINSON. Springer-Verlag, New York, NY, 1968. 149 pp. 17 × 25 cm. Price $9.75.

I enjoyed the opportunity of reviewing this book which was both readable and interesting. Although the author, in his preface, makes no attempt to state whether the monograph is intended primarily as a text or reference book, it would appear to me that its greatest

Drug Abuse: Social and Psychopharmacological Aspects. Compiled and edited by JONATHAN O. COLE and J. R. WITTENBORN, Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiii + 170 pp. 15.5 × 23.5 cm. Price $9.50.

This book is drawn from reports presented during the Fifth Annual Meeting of the American College of Neuropsychopharmacology. While there is no primary focus to the papers included, they are representative of current research related to the growing social and medical concern of drug abuse.

One paper should be of particular interest because of the controversial nature of the topic and the scarcity of scientific research in this area. This study of the long-term effects of LSD administration in volunteer graduate students suggests that the use of LSD under controlled circumstances with selected individuals may be less hazardous and have less far-reaching consequences than previously thought. However, other studies reported in this book seem to present evidence to the contrary, confirming the need for more extensive research into these problems.

Data from a study of marijuana indicate that marijuana use begins as a group experience engaged in by peers, and not as the pusher engaging the youth.

This book includes eleven studies concerning various aspects of drugs and drug abuse. While the editors admit that no answers are presented, they are hopeful that this volume will serve to stimulate research in these and related areas.

Staff Review ■

Drugs and Youth. Edited by J. R. WITTENBORN, HENRY BRILL, JEAN PAUL SMITH, and SARAH A. WITTENBORN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiv + 485 pp. 18 × 26 cm. Price $22.75.

This book, "Drugs and Youth," is based on the proceedings of the Rutgers Symposium on Drug Abuse held in June, 1968, at the Rutgers Interdisciplinary Research Center. The one hundred fifty participants in the three-day symposium were scientists, law enforcement officials, and members of the judiciary.

The book is divided into six major sections, the first being introductory material of a broad nature. Part II deals with morphine, heroin, and cocaine. Amphetamine-type and barbiturate tranquilizer-type drugs are covered in the third section, followed by marijuana and LSD in Part IV.

The concluding two sections deal with drug abuse and the law, and action to be taken, respectively.

Although the book takes a broad look at the problems of drugs and youth with a sociological emphasis, it should be of interest to all persons working in the area of drug abuse.

Staff Review ■

Techniques of Medication. By ERIC W. MARTIN, STEWART F. ALEXANDER, WILLIAM E. HASSAN, JR., and BURTON L. SHERMAN. J. B. Lippincott, East Washington Square, Philadelphia, PA 19105, 1969. vii + 239 pp. 18.3 × 26.1 cm. Price $18.00.

This book is devoted to discussing those factors that often are not emphasized in the administration of a drug. In addition to the rational selection of a drug, factors such as route of administration and form of medication will often determine the usefulness of a medication. The authors have done well in attempting to discuss this comprehensive area. The book is introduced by discussing the trends in therapeutics and principles of medication. The second chapter is devoted to prescription writing and the factors that must be considered in a prescription. These factors would include dosage, directions to the patient, and legal and professional aspects.

This is followed by sections dealing with various routes of medication (dermatomucosal, parenteral, and gastrointestinal) and outlining the principles of medication that must be considered. These principles include topics such as dosage forms, techniques of administrations, and care of needles and syringes. The next section discusses nicely the handling of medication by nurses. This section is written in clear, easy-to-follow outline form. The book closes with a comprehensive discussion of the legal aspects of medication. These include factors such as prescribing errors, inadequate testing, and sensitivity reactions.

This book incorporates the basic concepts of a book published by Austin Smith in 1948 entitled "Technic of Medication." The authors of this volume are to be complimented on bringing this important topic up to date and providing a practical manual on medication techniques for physicians, medical students, nurses, and pharmacists. All members of the health team must not only know their drugs but, equally important, they must known how to use them.

Reviewed by J. P. Long
Department of Pharmacology
University of Iowa
Iowa City, IA 52240 ■

Industrial Toxicology. By LAWRENCE T. FAIRHALL. Second Edition (Facsimile of 1957 Edition). Hafner Publishing Co., New York, NY 1969. xii + 376 pp. 18.3 × 26.3 cm. Price $12.00.

A continuing demand for this standard reference work in industrial toxicology has justified this reprinting of the 12-year old second edition. It is likely that part of such a demand is based on the desires of some to replace their much-used and worn-out copies. Fairhall has had a well-established and long-standing reputation as a source of information about elements and compounds of toxicologic significance in industry. This book, though hardly expected to be up to date, still contains much data of current value and is a useful guide to some of the outstanding older literature.

With a compendium type of format, this volume is presented in two parts. Part I deals with inorganic substances, mostly elements and their salts, oxides, or compounds with hydrogen. The larger Part II, entitled *Carbon Compounds*, deals with numerous organic chemicals. For each substance or compound, its chemical and physical characteristics, industrial uses, toxicity, and general approach to methods of analysis are described in a concisely documented form. There is little consideration of mechanisms of toxic action and no discussion of the treatment of poisoning by the agents described. An appendix contains tables listing urinary metabolites of 73 compounds, threshold limit values of numerous toxic gases, vapors, fumes and dusts, and the LD_{50}'s by oral administration in rats of 104 organic chemicals.

Obviously, much new knowledge concerning substances of industrial toxicologic importance has developed since this edition was published in 1957, and several more recent books are available that contain such information. Hence, the later works must be relied upon as prime references in the field, with Fairhall serving a valuable supplementary and back-up function. An example of an area in arrears in this book is the timely topic of pesticides. Some of the well-known controversial persistent pesticides such as aldrin, dieldrin, endrin, heptachlor, and heptachlor epoxide are not described, though threshold limit values and LD_{50}'s for aldrin and dieldrin are tabulated in the appendix.

For numerous agents described, however, little or no new information has been produced since 1957, and for these substances Fairhall stands as a definitive reference. This volume is still an important component of any library purporting to cover toxicology comprehensively or industrial toxicology specifically.

Reviewed by Julius Coon
Jefferson Medical College
Philadelphia, PA 19107 ■

The Biochemistry of Alkaloids: Molecular Biology, Biochemistry and Biophysics, Vol. 3. By TREVOR ROBINSON. Springer-Verlag, New York, NY, 1968. 149 pp. 17 × 25 cm. Price $9.75.

I enjoyed the opportunity of reviewing this book which was both readable and interesting. Although the author, in his preface, makes no attempt to state whether the monograph is intended primarily as a text or reference book, it would appear to me that its greatest

Drug Abuse: Social and Psychopharmacological Aspects. Compiled and edited by JONATHAN O. COLE and J. R. WITTENBORN, Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiii + 170 pp. 15.5 × 23.5 cm. Price $9.50.

This book is drawn from reports presented during the Fifth Annual Meeting of the American College of Neuropsychopharmacology. While there is no primary focus to the papers included, they are representative of current research related to the growing social and medical concern of drug abuse.

One paper should be of particular interest because of the controversial nature of the topic and the scarcity of scientific research in this area. This study of the long-term effects of LSD administration in volunteer graduate students suggests that the use of LSD under controlled circumstances with selected individuals may be less hazardous and have less far-reaching consequences than previously thought. However, other studies reported in this book seem to present evidence to the contrary, confirming the need for more extensive research into these problems.

Data from a study of marijuana indicate that marijuana use begins as a group experience engaged in by peers, and not as the pusher engaging the youth.

This book includes eleven studies concerning various aspects of drugs and drug abuse. While the editors admit that no answers are presented, they are hopeful that this volume will serve to stimulate research in these and related areas.

Staff Review ■

Drugs and Youth. Edited by J. R. WITTENBORN, HENRY BRILL, JEAN PAUL SMITH, and SARAH A. WITTENBORN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiv + 485 pp. 18 × 26 cm. Price $22.75.

This book, "Drugs and Youth," is based on the proceedings of the Rutgers Symposium on Drug Abuse held in June, 1968, at the Rutgers Interdisciplinary Research Center. The one hundred fifty participants in the three-day symposium were scientists, law enforcement officials, and members of the judiciary.

The book is divided into six major sections, the first being introductory material of a broad nature. Part II deals with morphine, heroin, and cocaine. Amphetamine-type and barbiturate tranquilizer-type drugs are covered in the third section, followed by marijuana and LSD in Part IV.

The concluding two sections deal with drug abuse and the law, and action to be taken, respectively.

Although the book takes a broad look at the problems of drugs and youth with a sociological emphasis, it should be of interest to all persons working in the area of drug abuse.

Staff Review ■

Techniques of Medication. By ERIC W. MARTIN, STEWART F. ALEXANDER, WILLIAM E. HASSAN, JR., and BURTON L. SHERMAN. J. B. Lippincott, East Washington Square, Philadelphia, PA 19105, 1969. vii + 239 pp. 18.3 × 26.1 cm. Price $18.00.

This book is devoted to discussing those factors that often are not emphasized in the administration of a drug. In addition to the rational selection of a drug, factors such as route of administration and form of medication will often determine the usefulness of a medication. The authors have done well in attempting to discuss this comprehensive area. The book is introduced by discussing the trends in therapeutics and principles of medication. The second chapter is devoted to prescription writing and the factors that must be considered in a prescription. These factors would include dosage, directions to the patient, and legal and professional aspects.

This is followed by sections dealing with various routes of medication (dermatomucosal, parenteral, and gastrointestinal) and outlining the principles of medication that must be considered. These principles include topics such as dosage forms, techniques of administrations, and care of needles and syringes. The next section discusses nicely the handling of medication by nurses. This section is written in clear, easy-to-follow outline form. The book closes with a comprehensive discussion of the legal aspects of medication. These include factors such as prescribing errors, inadequate testing, and sensitivity reactions.

This book incorporates the basic concepts of a book published by Austin Smith in 1948 entitled "Technic of Medication." The authors of this volume are to be complimented on bringing this important topic up to date and providing a practical manual on medication techniques for physicians, medical students, nurses, and pharmacists. All members of the health team must not only know their drugs but, equally important, they must known how to use them.

Reviewed by J. P. Long
Department of Pharmacology
University of Iowa
Iowa City, IA 52240 ■

Industrial Toxicology. By LAWRENCE T. FAIRHALL. Second Edition (Facsimile of 1957 Edition). Hafner Publishing Co., New York, NY 1969. xii + 376 pp. 18.3 × 26.3 cm. Price $12.00.

A continuing demand for this standard reference work in industrial toxicology has justified this reprinting of the 12-year old second edition. It is likely that part of such a demand is based on the desires of some to replace their much-used and worn-out copies. Fairhall has had a well-established and long-standing reputation as a source of information about elements and compounds of toxicologic significance in industry. This book, though hardly expected to be up to date, still contains much data of current value and is a useful guide to some of the outstanding older literature.

With a compendium type of format, this volume is presented in two parts. Part I deals with inorganic substances, mostly elements and their salts, oxides, or compounds with hydrogen. The larger Part II, entitled *Carbon Compounds*, deals with numerous organic chemicals. For each substance or compound, its chemical and physical characteristics, industrial uses, toxicity, and general approach to methods of analysis are described in a concisely documented form. There is little consideration of mechanisms of toxic action and no discussion of the treatment of poisoning by the agents described. An appendix contains tables listing urinary metabolites of 73 compounds, threshold limit values of numerous toxic gases, vapors, fumes and dusts, and the LD_{50}'s by oral administration in rats of 104 organic chemicals.

Obviously, much new knowledge concerning substances of industrial toxicologic importance has developed since this edition was published in 1957, and several more recent books are available that contain such information. Hence, the later works must be relied upon as prime references in the field, with Fairhall serving a valuable supplementary and back-up function. An example of an area in arrears in this book is the timely topic of pesticides. Some of the well-known controversial persistent pesticides such as aldrin, dieldrin, endrin, heptachlor, and heptachlor epoxide are not described, though threshold limit values and LD_{50}'s for aldrin and dieldrin are tabulated in the appendix.

For numerous agents described, however, little or no new information has been produced since 1957, and for these substances Fairhall stands as a definitive reference. This volume is still an important component of any library purporting to cover toxicology comprehensively or industrial toxicology specifically.

Reviewed by Julius Coon
Jefferson Medical College
Philadelphia, PA 19107 ■

The Biochemistry of Alkaloids: Molecular Biology, Biochemistry and Biophysics, Vol. 3. By TREVOR ROBINSON. Springer-Verlag, New York, NY, 1968. 149 pp. 17 × 25 cm. Price $9.75.

I enjoyed the opportunity of reviewing this book which was both readable and interesting. Although the author, in his preface, makes no attempt to state whether the monograph is intended primarily as a text or reference book, it would appear to me that its greatest

utility would be in the former category, especially in a graduate course in alkaloids.

The material covered is thorough and the author's viewpoint regarding the unbalanced progress in the study of alkaloid biochemistry is heartily supported by the reviewer, *i.e.*, quite a bit is known about alkaloid biosynthesis but very little is known about function or degradative pathways. For example, the author cites the work of Fairbairn, who showed that the morphine alkaloids are not end products of metabolism but appear to be actively turned over at a rapid rate. Similar studies of this type are sadly lacking and much more work is needed in this area. It may well be that alkaloids are active metabolites within plants.

In any book there is a certain lag period between the literature survey, writing, and official publication. The lag period works against areas of research which are making active progress. A specific example of material which has been greatly extended since publication of the book is the work on the biosynthesis of indole alkaloids. This problem has now been pretty well cleared up and a monoterpenoid precursor is well established for the C_9 or C_{10} unit involved. This is not a criticism of the author but is cited to point out some problems even with monographs of this type. Indeed, overall I believe the book to be quite up to date.

In summary, the book is clearly written, well organized, and readable. The major alkaloids are covered according to structural types and their biochemistry discussed. I would recommend its use in courses of natural products and especially in courses emphasizing alkaloids. It is a welcome addition to the library of books dealing with natural products.

Reviewed by John Tashiro
College of Pharmacy
University of Rhode Island
Kingston, RI 02881 ■

NOTICES

Chemical and Botanical Guide to Lichen Products. Edited by CHICITA F. CULBERSON. University of North Carolina Press, Chapel Hill, 1969. xi + 628 pp. 16 × 23.5 cm. Price $12.50.

II. Nachtrags-und Erganzungsband Gehes Codex. Edited by FELIX DIEPENBROCK. Wissenschaftliche Verlagsgesellschaft MbH., Stuttgart, Germany, 1969. 538 pp. 16 × 23.5 cm. Price DM 85.-. (*German*)

Drugs in Current Use and New Drugs. Edited by WALTER MODELL. Springer Publishing Co., Inc., 200 Park Ave. S., New York, NY 10003, 1970. v + 201 pp. 14 × 21 cm. Price $3.25.

Handbook of Experimental Pharmacology: Diuretica. Springer-Verlag, 1 Berlin 33, Heidelberger Plaza, Berlin-West, 1969. xviii + 764 pp. 17 × 25.5 cm. Price $68.20. (*German*)

Circulatory and Respiratory Mass Transport. Edited by G. E. W. WOLSTENHOLME and JULIE KNIGHT. Little, Brown and Co., 34 Beacon St., Boston, MA 02106, 1969. x + 310 pp. 15.5 × 23.5 cm. Price $12.50.

The Lynn Index—A Bibliography of Phytochemistry. Edited by NORMAN R. FARNSWORTH, RALPH N. BLOMSTER, MAYNARD W. QUIMBY, and JOHN W. SCHERMERHORN. Norman R. Farnsworth Publisher, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213, 1969. ix + 274 pp. 15 × 23 cm. Price $5.00.

Gas Chromatography in Biology and Medicine. A Ciba Foundation Symposium. Edited by ROTH PORTER. J & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. ix + 213 pp. 15 × 23.5 cm. Price 60s.

Molecular Biology Biochemistry and Biophysics. Edited by A. KLEINZELLER. Springer-Verlag, 175 Fifth Ave., New York, NY 10010, 1969. xi + 166 pp. 16.5 × 25.5 cm. Price $12.70.

Biochemical and Clinical Aspects of Alcohol Metabolism. Edited by VISHWANATH M. SARDESAI. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiii + 319 pp. 18.2 × 26 cm. Price $27.00.

Oral Hypoglycaemic Agents. Edited by G. D. CAMPBELL. Academic Press Inc., London Ltd. Berkeley Square House, Berkeley Square, London, England WIX 6BA, 1969. xviii + 482 pp. 15.7 × 23.7 cm. Price $19.50.

Biochemistry of Mental Disorders: Report of a WHO Scientific Group. World Health Organization Technical Report Series, No. 427, World Health Organization, Geneva, Switzerland, 1969. 40 pp. 16 × 24 cm. Price 6/-, $1.00, Sw. fr. 3.-, Paperbound. (*English*)

Principles for the Testing and Evaluation of Drugs for Carcinogenicity: Report of a WHO Scientific Group. World Health Organization Technical Report Series, No. 426, World Health Organization, Geneva, Switzerland, 1969. 26 pp. 16 × 24 cm. Price $4-, $0.60, Sw. fr. 2.-, Paperbound. (*English*)

Pharmaceutical Advertising, 1968 Revision. Offprint from the International Digest of Health Legislation, Geneva, 1968, Vol. 19, No. 3, World Health Organization, Geneva, Switzerland, 1968. Local distributors: The American Public Health Association, Inc., 1740 Broadway, New York, NY 10019. 100 pp. 16 × 24 cm. Price Sw. fr. 12.-., £1 4s. Od., $4.00, Paperbound. (*English*)

utility would be in the former category, especially in a graduate course in alkaloids.

The material covered is thorough and the author's viewpoint regarding the unbalanced progress in the study of alkaloid biochemistry is heartily supported by the reviewer, *i.e.*, quite a bit is known about alkaloid biosynthesis but very little is known about function or degradative pathways. For example, the author cites the work of Fairbairn, who showed that the morphine alkaloids are not end products of metabolism but appear to be actively turned over at a rapid rate. Similar studies of this type are sadly lacking and much more work is needed in this area. It may well be that alkaloids are active metabolites within plants.

In any book there is a certain lag period between the literature survey, writing, and official publication. The lag period works against areas of research which are making active progress. A specific example of material which has been greatly extended since publication of the book is the work on the biosynthesis of indole alkaloids. This problem has now been pretty well cleared up and a monoterpenoid precursor is well established for the C_9 or C_{10} unit involved. This is not a criticism of the author but is cited to point out some problems even with monographs of this type. Indeed, overall I believe the book to be quite up to date.

In summary, the book is clearly written, well organized, and readable. The major alkaloids are covered according to structural types and their biochemistry discussed. I would recommend its use in courses of natural products and especially in courses emphasizing alkaloids. It is a welcome addition to the library of books dealing with natural products.

Reviewed by John Tashiro
College of Pharmacy
University of Rhode Island
Kingston, RI 02881 ■

NOTICES

Chemical and Botanical Guide to Lichen Products. Edited by CHICITA F. CULBERSON. University of North Carolina Press, Chapel Hill, 1969. xi + 628 pp. 16 × 23.5 cm. Price $12.50.

II. Nachtrags-und Erganzungsband Gehes Codex. Edited by FELIX DIEPENBROCK. Wissenschaftliche Verlagsgesellschaft MbH., Stuttgart, Germany, 1969. 538 pp. 16 × 23.5 cm. Price DM 85.-. (*German*)

Drugs in Current Use and New Drugs. Edited by WALTER MODELL. Springer Publishing Co., Inc., 200 Park Ave. S., New York, NY 10003, 1970. v + 201 pp. 14 × 21 cm. Price $3.25.

Handbook of Experimental Pharmacology: Diuretica. Springer-Verlag, 1 Berlin 33, Heidelberger Plaza, Berlin-West, 1969. xviii + 764 pp. 17 × 25.5 cm. Price $68.20. (*German*)

Circulatory and Respiratory Mass Transport. Edited by G. E. W. WOLSTENHOLME and JULIE KNIGHT. Little, Brown and Co., 34 Beacon St., Boston, MA 02106, 1969. x + 310 pp. 15.5 × 23.5 cm. Price $12.50.

The Lynn Index—A Bibliography of Phytochemistry. Edited by NORMAN R. FARNSWORTH, RALPH N. BLOMSTER, MAYNARD W. QUIMBY, and JOHN W. SCHERMERHORN. Norman R. Farnsworth Publisher, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213, 1969. ix + 274 pp. 15 × 23 cm. Price $5.00.

Gas Chromatography in Biology and Medicine. A Ciba Foundation Symposium. Edited by ROTH PORTER. J & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. ix + 213 pp. 15 × 23.5 cm. Price 60s.

Molecular Biology Biochemistry and Biophysics. Edited by A. KLEINZELLER. Springer-Verlag, 175 Fifth Ave., New York, NY 10010, 1969. xi + 166 pp. 16.5 × 25.5 cm. Price $12.70.

Biochemical and Clinical Aspects of Alcohol Metabolism. Edited by VISHWANATH M. SARDESAI. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, IL 62703, 1969. xiii + 319 pp. 18.2 × 26 cm. Price $27.00.

Oral Hypoglycaemic Agents. Edited by G. D. CAMPBELL. Academic Press Inc., London Ltd. Berkeley Square House, Berkeley Square, London, England WIX 6BA, 1969. xviii + 482 pp. 15.7 × 23.7 cm. Price $19.50.

Biochemistry of Mental Disorders: Report of a WHO Scientific Group. World Health Organization Technical Report Series, No. 427, World Health Organization, Geneva, Switzerland, 1969. 40 pp. 16 × 24 cm. Price 6/-, $1.00, Sw. fr. 3.-, Paperbound. (*English*)

Principles for the Testing and Evaluation of Drugs for Carcinogenicity: Report of a WHO Scientific Group. World Health Organization Technical Report Series, No. 426, World Health Organization, Geneva, Switzerland, 1969. 26 pp. 16 × 24 cm. Price $4-, $0.60, Sw. fr. 2.-, Paperbound. (*English*)

Pharmaceutical Advertising, 1968 Revision. Offprint from the International Digest of Health Legislation, Geneva, 1968, Vol. 19, No. 3, World Health Organization, Geneva, Switzerland, 1968. Local distributors: The American Public Health Association, Inc., 1740 Broadway, New York, NY 10019. 100 pp. 16 × 24 cm. Price Sw. fr. 12.-., £1 4s. Od., $4.00, Paperbound. (*English*)

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

JUNE 1970
VOLUME 59 NUMBER 6

REVIEW ARTICLE

α-Chymotrypsin: A Case Study of Substituent Constants and Regression Analysis in Enzymic Structure–Activity Relationships

CORWIN HANSCH and EUGENE COATS

Keyphrases □ α-Chymotrypsin—structure–activity relationship □ Structure–activity relationship—α-chymotrypsin substituent constants □ Regression analysis—α-chymotrypsin structure–activity relationship □ Inhibitors—α-chymotrypsin □ Substrates—α-chymotrypsin

One of the central problems in drug research is the development of a systematic approach for the elucidation of structure–activity relationships. The problem is so pressing and so complex that it must be approached on many levels simultaneously. In this report the authors are concerned with purified enzyme, one of the simplest systems. The concept of drug-receptor sites, which has developed in recent years (1), is much like that of the active sites on enzymes. In some instances, enzymes are the active sites. Thus it seemed reasonable to make a survey of the structure–activity work on one of the most thoroughly studied enzymes, chymotrypsin.

The hydrolytic enzymes have been the subject of the most extensive studies of structure–activity interrelation. Although an enormous amount of work has been devoted to all aspects of such enzymes, the understanding of their mechanism of action is still quite incomplete (2–4). Of this class of enzymes the most extensively studied is chymotrypsin. Its modes of action in the hydrolysis of esters and amides have been reviewed from various points of view (2–9). The present survey is from a particular point of view, namely that of extrathermodynamic structure–activity relationships.

In surveying the literature, the authors have selected for analysis only those sets of data for which suitable substituent constants are available. Even with this limitation, the data are so voluminous and scattered that they cannot claim to have included all possible examples. Their object has been to try to characterize in a gross fashion some common characteristics of substrates and inhibitors, which have been defined by the constants K_m and K_i. This effort is not intended to be the final definitive study but rather an initial study which hopefully will encourage others to undertake better designed experiments to explore more thoroughly substituent effects for which, at present, ideal data are lacking.

Considerable evidence now supports the view that chymotrypsin operates by what is called a double-displacement mechanism (2, 4, 10, 11); that is, the enzyme is first acylated and then the acyl intermediate is hydrolyzed to products:

$$\mathrm{RC(=O)—OR' + enzyme \rightarrow RC(=O)—enzyme \rightarrow RCOOH + enzyme + R'OH} \quad \text{(Eq. 1)}$$

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} ES' \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P_2 \qquad \text{(Eq. 2)}$$

(ES' is the acyl intermediate; P_1 is the first product released.)

$$ES' + H^+ \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} ES'H \qquad K_i = \frac{k_{-4}}{k_4} \qquad \text{(Eq. 3)}$$

The kinetic implications of this mechanism have been worked out by Gutfreund and Sturtevant (10, 11). In the early stages of the reaction, it is customary to assume that $k_2[ES] \gg k_{-2}[ES'][P_1]$ and that $k_3[ES'] \gg k_{-3}[E][P_2]$. If a large amount of substrate is present, then it may be assumed that $[S] = [S]_0$ where $[S]_0$ is the initial substrate concentration. Application of the steady-state approximation to ES yields:

$$\{(k_2 + k_3')[S]_0 + k_3K_m\}[E] = k_3'K_m[E]_0 + \frac{k_3'K_m[S]_0[E]_0}{K_m + [S]_0} \exp\left[-\left(\frac{(k_2 + k_3')[S]_0 + k_3'K_m}{K_m + [S]_0}\right)t\right] \quad \text{(Eq. 4)}$$

where $[E]_0 = [E] + [ES] + [ES']$ is the total enzyme concentration, t represents time, $K_m = (k_{-1} + k_2)/k_1$, and $k_3' = k_3K_i/(K_i + [H^+])$. The rates of product appearance are:

$$V = \frac{d[P_1]}{dt} = \frac{k_2}{K_m}[E][S]_0 \quad \text{(Eq. 5)}$$

$$\frac{d[P_2]}{dt} = k_3'[E]_0 - k_3'\frac{K_m + [S]_0}{K_m} \quad \text{(Eq. 6)}$$

Measurements which are made at times large enough so that the exponential term in Eq. 3 can be neglected can be treated using Eq. 6:

$$V = \frac{d[P_1]}{dt} = \frac{d[P_2]}{dt} = \frac{k_2k_3'[S]_0[E]_0}{(k_2 + k_3')[S]_0 + k_3'K_m} \quad \text{(Eq. 7)}$$

Lineweaver-Burk plots at different values of $[S]_0$ yield the apparent constant:

$$K_{m(\text{app.})} = \frac{k'_3}{k_2 + k'_3}K_m \quad \text{(Eq. 8)}$$

From the development of Eq. 8, it is clear that the apparent K_m with which the authors are concerned is a complex constant. The problem is even more complicated if one assumes two or more binding sites to be involved which might or might not lead to productive reactions (2). In fact, the picture is so complex that for structure–activity studies, one must proceed with an open mind and examine what empirical evidence is available simply to improve one's ability to design better experiments with which to make still closer approximations. The dangers of relying on $K_{m(\text{app.})}$ as a meaningful constant are apparent from Eq. 8, and they have also been stressed by Bender and Kézdy (2).

While considerable effort (2, 9, 10, 12) has been made to define the kinetic parameters associated with Eqs. 1–3, the present consideration will be limited to the hopefully less complex parameters $K_{m(\text{app.})}$ and K_i. Despite many theoretical points for concern about the complex nature of K_m, Neurath and Hartley (13) have summarized considerable evidence to show that K_m is a close approximation to the simple binding constant K_s. This review further supports this idea and attempts to characterize more sharply the enzymic areas surrounding the catalytic site.

The general approach to the formulation of extrathermodynamic models has been well analyzed for nonenzymic reactions by Leffler and Grunwald (14). For enzymic processes, hydrophobic forces become extremely important, and these must play an important part in any model (15–18). The approach in such studies has been to factor effects of substituents on rate or equilibrium constants into free energy-related terms as follows:

$$-\Delta G = RT \ln K \quad \text{(Eq. 9)}$$

$$\delta_X \Delta G = \delta_X \Delta G_{\text{hydrophobic}} + \delta_X \Delta G_{\text{electronic}} + \delta_X \Delta G_{\text{steric}} \quad \text{(Eq. 10)}$$

That is, the effect of substituent X on the free energy change in a rate or equilibrium process characterized by k is factored in operational terms as shown in Eq. 10. Extrathermodynamic numerical solutions to Eq. 10 can be obtained by the use of suitable substituent constants:

$$\delta_X \log K = a\pi_X + b\sigma_X + cE_{s_X} + d \quad \text{(Eq. 11)}$$

Other free energy-based parameters such as polarizability may also be employed in Eq. 11, so the pertinent substituent parameters determining biological response can be sorted out *via* regression analysis. In Eq. 11, the parameter π is defined (19) as: $\pi_X = \log P_X - \log P_H$, where P_X is the octanol–water partition coefficient of a derivative and P_H is that of a parent molecule. Hydrophobic binding is operationally defined by the octanol–water reference system. Electronic effects of X, represented by Hammett's σ-parameter and its various modifications (14), are related to highly specific electronic effects not contained in π. To represent the steric effects of X, Taft's E_s parameter (20) can be used (21, 22). Here again, as with π and σ, there is overlap between π and E_s. Steric effects are highly specific effects such as those involved in the formulation of E_s. These parameters have been successfully employed in the delineation of the roles of substituents in a variety of systems (18) including a number of interest here, namely, simple proteins (23) and enzymes (16).

While the parameters σ and E_s have been extensively studied and their use justified by many good correlations in relatively simple systems, partition coefficients have been much less studied. That they can be used to correlate quantitatively binding constants of small molecules to proteins is illustrated (17) in Eq. 12:

$$\log 1/C = 0.75(\pm 0.07)\log P + 2.30(\pm 0.15) \quad \begin{matrix} n & r & s \\ 42 & 0.960 & 0.159 \end{matrix} \quad \text{(Eq. 12)}$$

In Eq. 12, C is the molar concentration of organic compound necessary to produce a 1:1 complex with purified bovine serum albumin, and P is the octanol–water partition coefficient. This linear free energy relationship correlates the affinities of 42 miscellaneous (phenols, anilines, naphthalenes, alcohols, *etc.*) organic compounds for a hydrophobic site on serum albumin. This equation and many similar ones (17, 18, 23) appear to justify the octanol–water partition coefficient as a meaningful parameter for estimating hydrophobic character of a molecule. The relative constant π is the corresponding parameter for a substituent (19, 24).

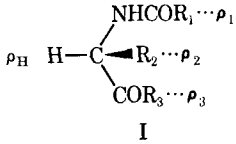
While the present authors have used octanol–water as a reference system, Scholtan has shown in a rather extensive study that the isobutanol–water system appears to yield comparable results (25). Wildnauer and

Canady (26) have recently shown that for certain chymotrypsin inhibitors, aliphatic hydrocarbons such as pentane, hexane, heptane, and water serve as suitable reference systems. They also correlated inhibitor potency with surface area of the inhibitor (26).

Since α-chymotrypsin has been readily available in crystalline form for some time, it has been the subject of extensive investigation. The detailed studies of Niemann, Bender, Baker, and others (see 27–32), dealing with the selectivity and inhibition of the enzyme, along with recent sequence analyses and X-ray crystallographic work (33) provide data for structure–activity analysis. A variety of data is most important; a correlation of one or two sets of data for such a complex process as enzyme substrate interaction leaves one with the feeling that other workers using different molecules might find quite different results. It is only after many sets of quite different data can be treated with a consistent result that one can place much confidence in such extrathermodynamic correlations.

One unfortunate observation of the present survey is that most of the studies of substrates and inhibitors were designed without giving much thought to the present state of the ability to treat substituent effects in quantitative terms; that is, instead of varying one of two parts of substrate or inhibitor molecules with substituents for which reliable physicochemical parameters are known, workers have often studied a small set of congeners in which gross changes have been made which preclude treatment by present methods of analysis. It is hoped that a result of the analyses in this report will be the encouragement of better designed studies for *quantitative* structure–activity analysis.

In attempting to understand substituent effects on the interaction of substrates and inhibitors with α-chymotrypsin, the model of Hein and Niemann (30, 31) is of help in orienting the discussion. Actually, their nomenclature will be employed to designate the space around an asymmetric center held on the active site of the enzyme. As will become apparent, the results of this analysis do not support the necessity of postulating the large number of microscopic binding constants in which each of the less than perfect modes of interaction plays a definite part in holding substrate to enzyme. The view taken here is similar to that of Bender and Kézdy (2) that the 12 possible modes of interaction between, say, an L-substrate and the enzyme violate the rule of "scientific simplicity," and this complexity should not be invoked until absolutely necessary. The general picture of the Hein-Niemann model is shown in Structure I. In this generalized model, $NHCOR_1$ represents the *N*-acyl portion; R_2 is the side chain in the α-position; COR_3 is the ester or amide bond that is hydrolyzed; and ρ_1, ρ_2, ρ_3, and ρ_H are the areas of the active site with which the four substituents on the α-carbon interact. Structure I depicts the most specific



I

Table I—L-$RCHCO_2CH_3$ ($NHCOCH_3$) as Chymotrypsin Substrates

R	E_s[a]	π[b]	σ^*[a]	log $1/K_m$ Obs.[c]	log $1/K_m$ Calcd.[d]
Methyl	0.00	0.50	0.00	−2.87	−2.69
Ethyl	−0.07	1.00	−0.10	−1.72	−1.99
Propyl	−0.36	1.50	−0.12	−1.01	−1.28
Butyl	−0.39	2.00	−0.13	−0.83	−0.57
Pentyl	−0.40	2.50	−0.16	−0.21	0.14
Hexyl[e]	−0.40	3.00	−0.17	−0.47	
Isopropyl	−0.47	1.30	−0.19	−2.05	−1.56
Isobutyl	−0.93	1.80	−0.13	−0.58	−0.85
Benzyl	−0.38	2.03[f]	0.22	−0.10	−0.53
Cyclohexylmethyl	−0.98	2.89[g]	−0.06	0.72	0.69

[a] From *Reference 20.* [b] From *Reference 19.* [c] From *Reference 50; K_m* is in m*M*. [d] Calculated using Eq. 13. [e] Not included in derivation of Eq. 13. [f] Benzyl value of 2.63 minus 0.60 for folding effects (24) observed on measured log *P* for *N*-acetylphenylalanine methyl ester. [g] Calculated using measured log *P* for cyclohexanol minus hydroxyl plus methyl. See text for discussion.

of the 12 possible interactions, with all of the others resulting in a less productive or nonproductive complex termed "wrong-way binding."

Recent experimental evidence now makes it possible to indicate some of the amino acids of the enzyme which are involved in the portions of the active site, as proposed by Hein and Niemann.

ρ_H Area: On the basis of results (34, 35) with α-methylamino acids, it has been concluded that although the hydrolysis rate is lowered, the methyl group has little effect on binding. Hence, the ρ_H area is presumed to be open to solvent.

ρ_1 Area: On the basis of unique dialkylation of the active site by *p*-nitrophenyl-*N*-bromoacetyl-α-aminoisobutyrate, methionine 192 has been identified as being reactive in this area of the active site (36). The ester portion of this reagent was first hydrolyzed by the enzyme, affording *p*-nitrophenol and acylated serine 195. Then the bromoacetyl moiety, now fixed to the enzyme, reacted with methionine 192.

ρ_2 Area: Evidence for a specific role for an amino acid residue interaction with a substrate in this area has not been found. There is some evidence to suggest that a tryptophan residue in this region might participate in a charge transfer reaction (37). X-ray crystallographic studies indicate (33) that tryptophan 215 lies near methionine 192.

ρ_3 Area: By irreversible alkylation studies with diisopropyl fluorophosphate and other reagents, a serine residue has been implicated in this portion of the active site (38). From amino acid sequence studies and X-ray crystallography, it is now fairly certain (33, 39) that this is serine 195. Histidine 57 has also been identi-

Table II—$C_6H_5CH_2CH_2CO_2R$ as Chymotrypsin Substrates

R	π[a]	log $1/K_m$ Obs.[b]	log $1/K_m$ Calcd.[c]
Methyl	0.50	3.27	3.27
Ethyl	1.00	3.36	3.37
Propyl	1.50	3.48	3.48

[a] From *Reference 19.* [b] From *Reference 26; K_m* is in *M*. [c] Calculated using Eq. 14.

Table III—$C_6H_5CONHCH_2CO_2R$ as Chymotrypsin Substrates

R	E_s[a]	π[b]	log $1/K_m$ Obs.[c]	log $1/K_m$ Calcd.[d]
Methyl	0.00	0.50	−0.51	−0.51
Ethyl	−0.07	1.00	−0.36	−0.34
Propyl	−0.36	1.50	−0.28	−0.25
Isopropyl	−0.47	1.30	−0.31	−0.37
Butyl	−0.39	2.00	−0.04	−0.06
Isobutyl	−0.93	1.80	−0.38	−0.36

[a] From *Reference 20.* [b] From *Reference 19.* [c] From *Reference 46; K_m* is in m*M*. [d] Calculated using Eq. 15.

fied (40–42) by alkylation and X-ray crystallography in this portion of the active site, and it lies only 4 Å from the serine 195 residue (33).

METHOD

The general approach is to account for substituent effects on the binding of substrate by enzyme, as represented by log $1/K_m$ or log $1/K_i$, by the linear combination of free energy-based substituent constants (Eq. 11). In Eq. 11, the constants *a*, *b*, *c*, and *d* are found by the method of least squares. (See *Reference 43* for a discussion of this technique and its use in regression analysis.) While E_s has been defined (19) to represent intramolecular steric repulsions, it has been found to be of use in intermolecular interactions as well (21, 22). In testing the value of E_s in chymotrypsin correlations, the authors have also tested Hancock's corrected parameter (44), defined as: $E_s^c = E_s + 0.306(n - 3)$, where n represents the number of α-hydrogens on the substituent alkyl group. In assaying electronic effects of substituents, σ, as well as its variations (14) σ_I, σ^+, and σ^-, has been employed.

For hydrophobic interactions, π or log P has been used. In most of the examples, a large portion of the substrate or inhibitor was held constant, and π can be used to represent the hydrophobic effect of the substituents. For example, in Table I the $-CHCO_2CH_3$ portion
$NHCOCH_3$
of the substrates is constant, and π has been used for each of the R functions attached to the α-carbon. For each CH_3 or CH_2 group, the value of 0.50 for π was used to estimate π for higher alkyl functions. The additive constitutive character of π and log P greatly simplifies such analyses (19, 24).

From the data in Tables I–X, the equations in Table XX have been derived for enzyme substrate binding; from the data in Tables XI–XIX, the equations in Table XXI have been derived for enzyme inhibitor interaction. Only the most significant equations, as determined (43) by an F test ($\alpha \leq 0.10$), are given. In several of the examples, so few data points are available that one cannot be sure that higher order (45) equations are not needed.

Table IV—$C_6H_5CONHCH_2CO_2R$ as Chymotrypsin Substrates

R	π[a]	log $1/K_m$ Obs.[b]	log $1/K_m$ Calcd.[c]
Methyl	0.50	2.57	2.56
Ethyl	1.00	2.63	2.63
Propyl	1.50	2.77	2.75
Isopropyl	1.30	2.66	2.69

[a] From *Reference 19.* [b] From *Reference 26; K_m* is in *M*. [c] Calculated using Eq. 16.

Table V—$XCH(CH_2COOEt)_2$ as Chymotrypsin Substrates

X	π[a]	σ_I	log $1/K_m$ Obs.[b]	log $1/K_m$ Calcd.[c]
—OH	−1.16	0.29	−2.00	−1.91
—$NHCOCH_3$	−1.21[d]	0.24	−1.82	−1.94
—$OCOCH_3$	−0.27	0.33	−1.57	−1.45
—H	0.00	0.00	−1.21	−1.31

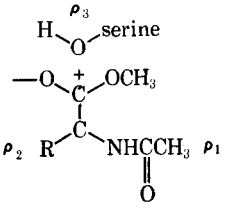
[a] Aliphatic values from *Reference 24.* [b] From *Reference 47; K_m* is in *M*. [c] Calculated using Eq. 17. [d] Aliphatic $CONH_2$ plus 0.50 for methyl group.

The following log P values in the octanol–water reference system have been measured for the first time: *N*-acetyl-DL-phenylalanine methyl ester, 0.92 ± 0.01; ethyl propyl *p*-nitrophenylphosphonate, 2.20 ± 0.01; ethyl furoate, 1.52 ± 0.01; azulene, 3.20 ± 0.02; ethyl isonicotinate, 1.43 ± 0.02; ethyl nicotinate, 1.32 ± 0.01; ethyl picolinate, 0.87 ± 0.01; ethyl anthranilate, 2.57 ± 0.01; and ethyl 2-thiophenecarboxylate, 2.33 ± 0.03. The standard deviations are from four separate determinations using different volume ratios of solvents, except for ethyl picolinate where only three determinations were made. To calculate π-values for the acyl functions of Table VIII, the ethoxy value of −0.23 derived from diethyl ether was subtracted from the measured log P values for the ethyl esters.

The use of group polarizability (P_E) as reflected in atomic molecular refractivities in quantitative correlations was suggested by the recent study of Agin *et al.* (59). They showed that, as a first approximation, binding of small molecules to macromolecules should be logarithmically related to αI, where α is the polarizability and I is the ionization potential. It is found (49) that little is lost in correlation if I is neglected. Niemann and Hein also considered the importance of polarizability (52). Although there is a large amount of cocorrelation between P_E and π since both are dependent on molar volume, the proper selection of derivatives shows enough independence so that they may be used to characterize enzymic binding areas. For example, $r^2 = 0.3$ for the correlation between π and P_E for the substituents in Table IX. For the 21 groups of Table VII, the cocorrelation is $r^2 = 0.4$.

RESULTS

Equation 13 in Table XX correlates the data of Table I. Using the Hein-Niemann model, the substrate fit to the site of action can be pictured as follows:



II

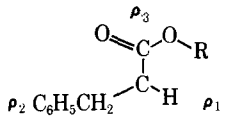
Table VI—$RCOOC_6H_4NO_2$ as Chymotrypsin Substrates

R	π^a	E_s^b	pH 5.92 log k_2/K_m Obs.[c]	pH 5.92 log k_2/K_m Calcd.[d]	pH 7.99 log k_2/K_m Obs.[c]	pH 7.99 log k_2/K_m Calcd.[e]	pH 8.90 log k_2/K_m Obs.[c]	pH 8.90 log k_2/K_m Calcd.[f]
Methyl	0.50	0.00	2.56	2.62	3.13	3.30	3.12	3.14
Ethyl	1.00	−0.07	2.80	2.89	3.53	3.51	—	—
Propyl	1.50	−0.36	2.81	2.78	3.42	3.39	3.25	3.18
Pentyl	2.50	−0.40	3.55	3.49	3.97	3.96	3.80	3.74
Isopropyl	1.30	−0.47	2.49	2.42	3.28	3.09	2.79	2.88
Isobutyl	1.80	−0.93	1.99	2.01	2.60	2.71	2.33	2.45
tert-Butyl	1.98[g]	−1.54	1.38	1.07	2.16	1.90	1.86	1.57
Neopentyl	2.48[h]	−1.74	0.82	1.12	1.69	1.92	1.36	1.56

[a] From *Reference 19*. [b] From *Reference 20*. [c] From *Reference 48*. [d] Calculated using Eq. 19. [e] Calculated using Eq. 20. [f] Calculated using Eq. 21. [g] From measured log *P* for *tert*-butylbenzene. [h] Calculated by adding 0.50 for methyl to the measured *tert*-butyl value.

In Structure II, the α-hydrogen is not shown; it is below the plane of the page. Since adding terms in E_s or σ to Eq. 13 does not result in a significant reduction in the variance of the data, enzyme substrate binding as defined by K_m depends linearly on the hydrophobic character of R as defined by the octanol–water reference system. In deriving Eq. 13, two cases where R = H or hexyl have not been included. Hein and Niemann pointed out that when R = hexyl, a sharp break occurs in both K_m and K_0, indicating that groups as long as hexyl do not fit into the ρ_2 area without some steric hindrance. The length of the chain may be involved since the cyclohexylmethyl group, which is just as lipophilic as hexyl (compare π-values, Table I), is well fit by Eq. 13. However, other explanations are possible (see *Discussion*). This same kind of break comes in data from inhibitor studies (see Eqs. 34 and 35), although at a somewhat longer chain length. The case where R = H is more active than Eq. 13 would predict. Why this is so is not clear. However, in this example, no binding possibility for the ρ_2 area exists.

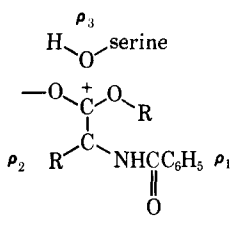
The esters of hydrocinnamic acid of Table II are correlated by Eq. 14. These derivatives can be visualized as fitting the model site as in Structure III:



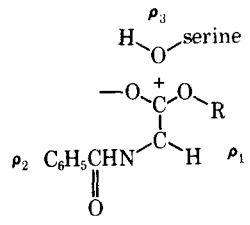
III

Although only three derivatives are in this set, they have been included simply because the hydrophobic character of the area as characterized by the coefficient with π in Eq. 14 can be compared to that in Eqs. 15 and 16. The mean coefficient with π for these three equations is 0.29 ± 0.1. The difference between this dependence of binding on π and that of Eq. 13 indicates the pronounced difference between the ρ_2 and ρ_3 areas.

Two sets of data, in which the alkyl group of the ester function pictured in Structures IV*a* and IV*b* as binding to the ρ_3 area is varied, are correlated by Eqs. 15 and 16.



IV*a*



IV*b*

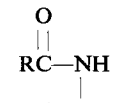


Table VII—R′—CH(L)—$COOCH_3$ as Chymotrypsin Substrates

Acyl-R	Alkyl-R′	π-R′[a]	P_{E-R}[b]	−log $1/K_m$ Obs.[c]	−log $1/K_m$ Calcd.[d]
Benzoyl	Methyl	0.50	25.10	−0.99	−1.12
2-Quinolinyl	Methyl	0.50	41.00	0.66	0.19
2-Furoyl	Methyl	0.50	17.00	−1.69	−1.78
2-Theophenoyl	Methyl	0.50	22.71	−1.18	−1.31
Nicotinyl	Methyl	0.50	23.00	−1.57	−1.29
Isonicotinyl	Methyl	0.50	23.00	−1.46	−1.29
Picolinyl	Methyl	0.50	23.00	−1.25	−1.29
Acetyl	Methyl	0.50	5.72	−2.87	−2.71
o-Aminobenzoyl	Methyl	0.50	29.40	−0.67	−0.76
Chloroacetyl	Propyl	1.50	10.58	−0.70	−0.93
Benzoyl	Propyl	1.50	25.10	0.07	0.27
Acetyl	Isopropyl	1.30	5.72	−2.05	−1.61
Chloroacetyl	Isopropyl	1.30	10.58	−1.64	−1.21
Benzoyl	Isopropyl	1.30	25.10	−0.66	−0.01
Acetyl	Propyl	1.50	5.72	−1.01	−1.33
Acetyl	Benzyl	2.03[e]	5.72	−0.10	−0.60
Acetyl	Isobutyl	1.80	5.72	−0.58	−0.92
Acetyl	Ethyl	1.00	5.72	−1.72	−2.02
Acetyl	Butyl	2.00	5.72	−0.83	−0.64
Acetyl	Pentyl	2.50	5.72	−0.21	0.05
Acetyl	Cyclohexyl-methyl	2.89[f]	5.72	0.72	0.59

[a] Taken from *Reference 19* unless otherwise noted. [b] Calculated from refractive indexes and density or from atomic refractivity; see *Reference 49*. [c] From *References 30* and *50–52*. K_m is in m*M*. [d] Calculated *via* Eq. 22. [e] Benzyl π-value of 2.63 minus 0.60 for folding interactions (24). [f] Calculated by taking the measured log *P* for cyclohexanol, subtracting the π-value for aliphatic hydroxyl, and adding the π-value for methylene.

Table VIII—$CH_3CH(L)COOCH_3$ as Chymotrypsin Substrates

NHAcyl

Acyl	π^a	P_E[b]	−log $1/K_m$ Obs.[c]	−log $1/K_m$ Calcd.[d]
Furoyl	1.75	17.0	−1.69	−1.90
Theophenoyl	2.56	22.7	−1.18	−1.31
Nicotinyl	1.55	23.0	−1.57	−1.28
Isonicotinyl	1.66	23.0	−1.46	−1.28
Picolinyl	1.10	23.0	−1.25	−1.28
Benzoyl	2.75	25.1	−0.99	−1.06
2-Quinolinyl	2.45	41.0	0.66	0.58
2-Aminobenzoyl	2.80	29.4	−0.67	−0.62

[a] See section on *Method*. [b] Calculated using values from *Reference 49*. [c] From *Reference 52*. [d] Calculated using Eq. 23.

Table IX—Acyl-$NHCH_2CO_2CH_3$ as Chymotrypsin Substrates

Acyl	P_E[a]	log $1/K_m$ Obs.[b]	log $1/K_m$ Calcd.[c]
Acetyl	5.72	−1.48	−1.82
Propionyl	10.34	−1.58	−1.63
Isobutyryl	14.96	−1.66	−1.44
Isopentanoyl	19.58	−1.38	−1.25
Chloroacetyl	10.58	−1.82	−1.63
Dichloroacetyl	15.44	−1.34	−1.42
Trifluoroacetyl	6.08	−1.72	−1.81
Phenylacetyl	29.72	−0.90	−0.83
Benzoyl	25.10	−0.88	−1.02
p-Aminobenzoyl	29.40	−0.92	−0.84
Nicotinyl	23.00	−1.49	−1.11
Isonicotinyl	23.00	−1.24	−1.11
2-Furoyl	17.00	−1.29	−1.36
Indole carbonyl	36.47	−0.14	−0.55

[a] Calculated from atomic refractivities or refractive indexes and densities; see *Reference 49.* [b] From *Reference 53.* [c] Calculated using Eq. 24.

In this example the amide moiety might fit into either the ρ_1 or ρ_2 area. In Eq. 15, where a better selection and larger number of derivatives were tested, a dependence is found on both π and E_s. The positive sign of the coefficient with E_s indicates that large functions hinder binding in the ρ_3 area as measured by $1/K_m$. This means that as R becomes larger, interaction of the electron-deficient sp^2 carbon of the ester group with a nucleophile, say serine, becomes unfavorable. Binding is promoted by increasing values of π but to a lesser extent than at ρ_2 (compare slopes in Eqs. 13 and 15). In Eq. 16, the dependence of $1/K_m$ on π is in rough agreement with that of Eqs. 14 and 15. In Eq. 17, good agreement is found between log $1/K_m$ and π. So few points are available that no assessment of the roles of E_s or σ can be made.

In a recent study of *p*-nitrophenyl esters of fatty acids, Milstien and Fife (48) found that a plot of log k_2/K_m *versus* E_s gave a fair correlation. Such a correlation is expressed in numerical form in Eq. 18. It is seen in Eq. 19 that when hydrophobic bonding is also taken into account, a much better correlation results. The coefficient with E_s is larger than that of Eq. 15, as one would expect, since R is closer to the electron-deficient carbonyl carbon in molecules of Table VI than in those of Table III. One would expect more hindrance to binding of the kind depicted in Structure IV. Studies by Milstien and Fife were made at various hydrogen-ion concentrations. Equations 20 and 21, derived from data obtained from runs made under more basic conditions, yield the same quality correlations as at the lower pH. Under each of the three different conditions of pH, the dependence of log k_2/K_m on π and E_s is the same. While there are small differences in the coefficients with these terms from equation to equation, these variations are well within the 95% confidence intervals. There is a significant difference between the intercept of the low pH (Eq. 19) and equations (Eqs. 20 and 21) for work at the higher pH. This is in line with the well-known higher activity of chymotrypsin at higher pH. The work of Milstien and Fife, expressed in units of k_2/K_m, is not directly comparable with the other work using K_m and K_i. It has been included to show that although activity of the enzyme does vary with pH, the relative substituent effects do not; at least this is true for the range of changes and pH range investigated by Milstien and Fife. This would imply that large changes in the geometry of the reaction site, as it is defined by these esters, do not occur with changes in pH. The variations in activity with pH might be attributable to the relative

Table X—Acyl-$NHCH(CH_3)COOCH_3$ as Chymotrypsin Substrates

Acyl	P_E[a]	log $1/K_m$ Obs.[b]	log $1/K_m$ Calcd.[c]
Furoyl	17.00	−1.69	−1.75
Theophenoyl	22.70	−0.87	−1.04
Nicotinyl	23.00	−0.78	−1.00
Isonicotinyl	23.00	−1.43	−1.00
Picolinyl	23.00	−1.23	−1.00
Benzoyl	25.10	−0.52	−0.74
o-Aminobenzoyl	29.40	−0.20	−0.20

[a] See *Reference 49.* [b] From *Reference 52.* [c] Calculated according to Eq. 25.

Table XI—R—$C_6H_4OCH_2COX$ as Chymotrypsin Inhibitors

R(X = CH_3)	π[a]	σ	log $1/I/S$ Obs.[b]	log $1/I/S$ Calcd.[c]
H	0.00	0.00	−1.77	−1.96
4-NO_2	0.24	0.78	−1.30	−1.41
3-NO_2	0.11	0.71	−1.40	−1.55
2-NO_2	−0.23	1.24[d]	−1.48	—[e]
4-CN	−0.32	0.66	−2.22	−1.92
3-CN	−0.30	0.56	−2.05	−1.95
4-OCH_3	−0.04	−0.27	−1.92	−2.12
4-CH_3	0.52	−0.17	−1.96	−1.63
3-CH_3	0.51	−0.07	−1.77	−1.59
4-Cl	0.70	0.23	−1.30	−1.30
3-Cl	0.76	0.37	−0.74	−1.19
2-C_6H_5	2.13	0.00[d]	−0.90	—[e]
3,4-Di-Cl	1.46	0.60	−0.57	−0.52
2,3-Di-Cl	1.35	1.05[d]	−0.41	—[e]
3,4-Benzo	1.34	0.17	−0.95	−0.82
R(X = C_6H_5)				
H	0.00	0.00	−1.40	−1.10
4-OCH_3	−0.04	−0.27	−0.98	−1.25
4-Cl	0.70	0.23	−0.40	−0.43

[a] From *Reference 19.* [b] From *Reference 54.* [c] Calculated using Eq. 26. [d] *Ortho*-values for σ taken from *Reference 55.* [e] *Ortho*-substituents omitted because of unreliable substituent constants.

Table XII—Miscellaneous Chymotrypsin Inhibitors

Compound	log *P*	log $1/K_i$ Obs.[a]	log $1/K_i$ Calcd.[b]
Phenol	1.46	−0.54	−0.52
3-Methoxyphenol	1.58	−0.30	−0.40
4-Methylphenol	1.94	−0.03	−0.05
3-Methylphenol	2.02	0.05	0.03
2-Chlorophenol	2.15	0.07	0.16
2-Bromophenol	2.35	0.21	0.35
4-Chlorophenol	2.39	0.42	0.39
4-Bromophenol	2.59	0.68	0.58
2,4-Dichlorophenol	3.08	1.14	1.06
2,4-Dibromophenol	3.48	1.35	1.45
Acetonitrile	−0.34	−2.86	−2.87
Cyclohexanol	1.23	−1.46	—
Benzene	2.13	−0.49	−0.46
N,N-Dimethylaniline	2.31	−0.22	−0.28
Chlorobenzene	2.84	0.02	0.24
Bromobenzene	2.99	0.29	0.38
Acridine	3.40	0.85	0.78
Naphthalene	3.37	0.96	0.75

[a] From *Reference 56.* [b] Calculated using Eq. 29.

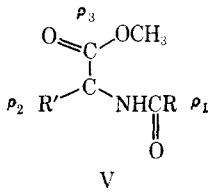
Table XIII—Aromatic Acids as Chymotrypsin Inhibitors

Compound	log P	pK_a	—log $1/K_i$— Obs.[a]	Calcd.[b]
Benzoic acid	1.81	4.17	1.99	2.31
3-Methylbenzoic acid	2.37	4.27	2.42	2.80
4-Methylbenzoic acid	2.27	4.37	2.43	2.71
3-Phenylpropionic acid	1.84	4.66	2.79	2.33
4-Phenylbutyric acid	2.42	4.76	3.22	2.85
2-Naphthoic acid	3.19	4.16	3.86	3.53
4-*tert*-Butylbenzoic acid	3.79[c]	4.41	3.89	4.07

[a] From *Reference 26*; K_m is in *M*. [b] Calculated using Eq. 30. [c] Calculated by adding *tert*-butyl π-value of 1.98 (from *tert*-butylbenzene log *P*) to the measured log *P* for benzoic acid.

protonation of the critical imidazole moiety in the ρ_3 area.

Equation 22 correlates a set of substrates (Table VII) in which changes are being made simultaneously in two positions which would affect binding in the ρ_2 and ρ_1 areas of Structure V:



V

For this set of congeners, poor correlations were obtained using $\Sigma\pi$ for R′ and R. Factoring R into two terms, $\pi_R + \pi_R'$, improved the correlation; however, from many preliminary calculations, it was observed that π does not correlate substituent effects well for the ρ_1 area. This area does not appear to be hydrophobic in character. The best correlations for this area were obtained using the group polarizability (P_E). Thus it would seem that dispersion forces and steric factors are most important for binding in this area.

In Eq. 23, using π instead of P_E yielded a correlation with r of only 0.556! For this set of substrates, where only the acyl portion is being varied, rather large changes in R result in relatively small changes in K_m. In fact, there is only a 10-fold change in binding for the molecules in Table VIII; thus the ρ_1 area does not appear to be apolar in character, nor does it appear to be sterically demanding, since a wide variety of modifications binding in this area are quite active substrates. Equations 24 and 25 are two further examples where better correlations are obtained with P_E than with π. Using π in Eq. 24 instead of P_E gave a correlation with

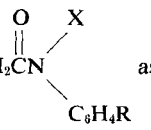
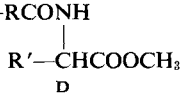


Table XIV—$C_6H_5OCH_2CN$ as Chymotrypsin Inhibitors

X	R	π_R[a]	σ_R	—log $1/I/S$— Obs.[b]	Calcd.[c]
Me	H	0.00	0.00	−1.70	−1.29
H	H	0.00	0.00	−1.11	−1.29
Me	4-NO_2	0.24	0.78	−0.78	−1.00
H	4-Cl	0.70	0.23	−0.18	−0.44
H	4-Br	1.02	0.23	−0.23	−0.05
H	3-Cl	0.76	0.37	−0.43	−0.37

[a] From *Reference 19*. [b] See *b*, Table XI. [c] Calculated using Eq. 31.

Table XV—RCONH as Chymotrypsin Inhibitors

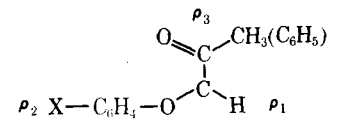


R	R′	P_{E-R}[a]	π_R'[b]	—log $1/K_i$— Obs.[c]	Calcd.[d]
CH_3	$(CH_3)_2CH$	5.72	1.30	−2.30	−2.55
$ClCH_2$	$(CH_3)_2CH$	10.58	1.30	−2.22	−2.11
C_6H_5	$(CH_3)_2CH$	25.10	1.30	−0.72	−0.82
CH_3	C_3H_7	5.72	1.50	−2.05	−1.97
$ClCH_2$	C_3H_7	10.58	1.50	−1.77	−1.54
C_6H_5	C_3H_7	25.10	1.50	−0.25	−0.24
CH_3	$C_6H_5CH_2$	5.72	2.03[e]	−0.36	−0.45

[a] See *Reference 49*. [b] From *Reference 19*. [c] From *Reference 51*. [d] Calculated using Eq. 33. [e] Calculated from benzyl π-value of 2.63 minus folding contribution of 0.60.

$r = 0.627$, while the same procedure with Eq. 25 gave a correlation with $r = 0.709$. The mean coefficient with P_E in the four substrate examples (Eqs. 22–25) is 0.088 ± 0.035.

The equations in Table XXI summarize the structure–activity relationships of the data from Tables XI–XIX on chymotrypsin inhibitors. One of the best designed sets of inhibitors comes from the work of Baker *et al.* (54). The results are summarized in Eq. 26. Most of his derivatives were methyl ketones; however, a few were phenyl ketones. The constant X was given a value of 0 for the methyl ketones and a value of 1 for the phenyl ketones. Both the π- and σ-parameters are necessary to obtain a good correlation. The fit of these inhibitors to the model of the active site can be depicted as Structure VI:



VI

The dependence of the inhibiting power of these derivatives on hydrophobic binding is strong, although somewhat different from Eq. 13. This is probably due to the fact that $1/K_m$ and I_{50} are not strictly comparable ways of comparing binding affinities. The positive coefficient with σ in Eq. 26 means that electron-withdrawing substituents also promote binding. Since π differs slightly from system to system and this difference is related to σ, part of the effect of σ may simply be that

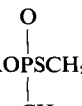


Table XVI—ROPSCH$_2$CH$_2$SEt as Chymotrypsin Inhibitors

R	π[a]	—log K_i— Obs.[b]	Calcd.[c]
Methyl	0.50	−0.47	−0.59
Ethyl	1.00	−0.34	−0.02
Propyl	1.50	0.53	0.55
Butyl	2.00	1.26	1.11
Pentyl	2.50	1.86	1.68
Hexyl	3.00	2.44	2.25
Heptyl	3.50	2.52	2.81
Octyl[d]	4.00	—	—

[a] From *Reference 19*. [b] From *Reference 57*. [c] Calculated using Eq. 34. [d] This point not employed in the regression; see text for discussion.

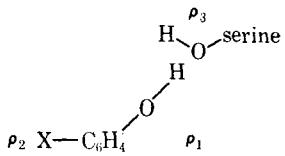
Table XVII—$ROP(=O)(CH_3)-SCH_2CH_2S^+(CH_3)Et\ CH_3SO_4^-$ as Chymotrypsin Inhibitors

R	π[a]	log K_i Obs.[b]	log K_i Calcd.[c]
Methyl	0.50	0.27	0.00
Ethyl	1.00	0.14	0.64
Propyl	1.50	1.24	1.28
Butyl	2.00	2.09	1.92
Pentyl	2.50	2.71	2.57
Hexyl	3.00	3.47	3.21
Heptyl	3.50	3.55	3.85
Octyl[d]	4.00	3.42	—

[a] From *Reference 19.* [b] From *Reference 57.* [c] Calculated using Eq. 35. [d] This point was not used in the regression; see text for discussion.

of correcting π; this could, in fact, explain part of the difference between Eqs. 26 and 13. However, in the present case this difference must be small, since π-constants are from the phenoxyacetic acid system which, of course, is quite closely related to the phenoxyacetones. Whether the phenyl ring is really fitting into the same area (ρ_2) as the R group of Eq. 13 is, of course, open to question. It is possible that the inhibitors bind in another part of the enzyme and bring about their effects allosterically. The dummy parameter X of Eq. 26 is simply a technique (43) for taking into account the stereoelectronic difference between the methyl and phenyl functions. Using P_E in Eq. 26 instead of π yields a poorer correlation (r = 0.779), indicating that this set more closely resembles compounds of Eq. 13 than molecules binding to the ρ_1 area (Eqs. 22–25).

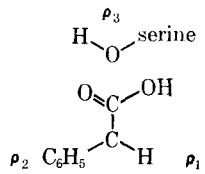
An interesting set of data is that of Berezin *et al.* (56) which gives rise to Eqs. 27–29. Equation 27 correlates the 10 phenols of Table XII, and Eq. 28 correlates the rest of the molecules in Table XII. Cyclohexanol was omitted in the regression analysis because of uncertainty in its hydrogen-bonding ability. The slopes of Eqs. 27 and 28 are quite similar, indicating a common hydrophobic mechanism of action. However, the difference in the intercepts indicates the phenols to be about eight times as effective on an isolipophilic basis. Assuming this to be due to the strong hydrogen-bonding ability of the phenols, the term, HB, has been added for hydrogen bonding, and Eqs. 27 and 28 have been combined into Eq. 29. For phenols, HB is assigned a value of 1; the other molecules were given a value of 0. Cyclohexanol does not fit into the hydrogen-bonding group; that is, giving it a value of 1 for HB results in a poorly calculated log $1/K_i$. However, using a value of 0 for hydrogen bonding results in a good calculated log $1/K_i$. The similar dependence of inhibitory activity of the compounds represented by Eqs. 13 and 29, as indicated by the coefficients associated with the π and log P terms, would suggest a common mode of binding by the inhibitors and substrates. The importance of hydrogen bonding might be rationalized as in Structure VII:



VII

It is possible that the acidic proton of the phenols aids in holding the inhibitors between areas ρ_3 and ρ_2, although there are many other ways in which the phenolic OH could participate in binding inhibitor to enzyme. Structure VII is meant to be suggestive and not to imply that only serine must be involved. Since the slopes of Eqs. 27–29 are the same, one would assume the same mechanism of inhibition as far as the hydrophobic contribution of the inhibitor is concerned. Using P_E in Eq. 29 in place of log P results in a much poorer correlation (r = 0.870). This information also supports binding in the ρ_2 rather than the ρ_1 area.

A smaller set of congeners, acting in a parallel manner to those of Eqs. 26–29, is the group of acids in Table XIII correlated by Eq. 30. This group of acids can be fit to the model as in Structure VIII:



VIII

Confidence limits on the coefficient with the log P term of Eq. 30 are large. While it cannot be said that the dependence on hydrophobic bonding is that of about 1 as was found for substrates and inhibitors reacting with the ρ_2 area, it is reasonably close. The positive coefficient with the pKa term indicates that the more unionized acids are more effective. The poorer correlation with this set (note the confidence intervals and standard deviation) may be due to steric effects involved with the different side-chain lengths between the carboxyl group and the aromatic ring.

Table XVIII—C_6H_5COR as Chymotrypsin Inhibitors

R	π[a]	log $1/K_i$ Obs.[b]	log $1/K_i$ Calcd.[c]
Acetophenone	0.50	3.27	3.27
Propiophenone	1.00	3.36	3.37
Butyrophenone	1.50	3.48	3.48

[a] Values for alkyl portion only. [b] From *Reference 26;* K_m is in *M.* [c] Calculated using Eq. 36.

Table XIX—Hydrocarbons as Chymotrypsin Inhibitors

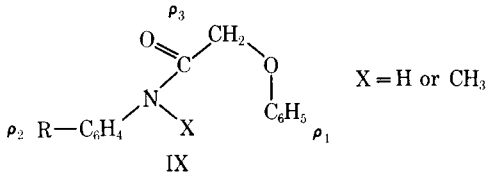
Compound	log P	log $1/K_i$ Obs.[a]	log $1/K_i$ Calcd.[b]
Azulene	3.20	4.22	3.50
Benzene	2.13	2.09	1.93
Toluene	2.69	2.55	2.75
Naphthalene	3.37	3.94	3.75
Chlorobenzene	2.84	3.10	2.97
Indene	3.33	3.67	3.70
Pentane	2.50	2.25	2.47
Cyclohexene	2.22	2.07	2.06
Ethylbenzene	3.15	2.75	3.43
Anthracene	4.45	5.27	5.34

[a] From *References 32* and *58;* K_m is in *M.* [b] Calculated using Eq. 37.

Table XX—Correlation of Structure and Activity of Chymotrypsin Substrates

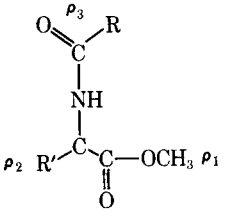
Type Substrate	"Best" Equation	n	r	s	Conditions	Eq. No.
$RCHCO_2CH_3$ $NHCOCH_3$ from Table I	$\log 1/K_m = 1.419(\pm0.40)\pi - 3.409(\pm0.74)$	9	0.955	0.350	In water at 25°, pH 7.90 and 0.10 M in sodium chloride	13
$C_6H_5CH_2CH_2CO_2R$ from Table II	$\log 1/K_m = 0.210(\pm0.22)\pi + 3.160(\pm0.24)$	3	0.997	0.012	0.1 M KCl; 3.3×10^{-3} M Tris buffer; pH 6.9; 25°	14
$C_6H_5CONHCH_2CO_2R$ from Table III	$\log 1/K_m = 0.406(\pm0.18)\pi + 0.400(\pm0.30)E_s - 0.714(\pm0.19)$	6	0.972	0.047	In water at pH 7.0 and 25°	15
$C_6H_5CONHCH_2CO_2R$ from Table IV	$\log 1/K_m = 0.251(\pm0.31)\pi + 3.343(\pm0.36)$	4	0.925	0.055	0.1 M KCl; 3.3×10^{-3} M Tris buffer; pH 6.9; 25°	16
$RCH(CH_2COOEt)_2$ from Table V	$\log 1/K_m = 0.518(\pm0.61)\pi - 1.308(\pm0.52)$	4	0.932	0.152	In water at pH 7.8, 25° and 0.1 M NaCl	17
$RCOC_6H_4NO_2$ from Table VI	$\log k_2/K_m = 1.164(\pm0.65)E_s + 3.101(\pm0.60)$	8	0.872	0.460	25° in 4.68% CH_3CN, pH 5.92	18
	$\log k_2/K_m = 1.762(\pm0.42)E_s + 0.789(\pm0.40)\pi + 2.225(\pm0.52)$	8	0.981	0.201	pH 5.92	19
	$\log k_2/K_m = 1.513(\pm0.42)E_s + 0.632(\pm0.39)\pi + 2.983(\pm0.51)$	8	0.976	0.198	pH 7.99	20
	$\log k_2/K_m = 1.620(\pm0.45)E_s + 0.627(\pm0.42)\pi + 2.823(\pm0.59)$	7	0.982	0.196	pH 8.90	21
RCONH R'—CHCOOCH$_3$ L from Table VII	$\log 1/K_m = 1.382(\pm0.27)\pi R' + 0.082(\pm0.02)P_{E-R} - 3.876(\pm0.58)$	21	0.934	0.331	In water at 25°; pH 7.90 and 0.10–0.05 M NaCl	22
L CH_3CH—$COOCH_3$ $NHCOR$ from Table VIII	$\log 1/K_m = 0.103(\pm0.023)P_E - 3.653(\pm0.62)$	8	0.975	0.179	In water at 25°; pH 7.90 and 0.10 M NaCl	23
RCONH $CH_2CO_2CH_3$ from Table IX	$\log 1/K_m = 0.042(\pm0.015)P_E - 2.068(\pm0.31)$	14	0.873	0.225	In water at 25°; pH 7.90 and 0.50 M NaCl	24
RCONH CH_3—$CHCO_2CH_3$ D from Table X	$\log 1/K_m = 0.125(\pm0.077)P_E - 3.887(\pm1.822)$	7	0.882	0.270	In water at 25°; pH 7.90 and 0.10 M NaCl	25

The amides in Table XIV, described by Eq. 31, can fit the Hein-Niemann model as in Structure IX. Unfortunately, only six derivatives are available to assess complex substituent changes. As Eq. 31 shows, π_R is an important factor, and the slope of this equation is in agreement with that of Eqs. 13 and 29. Using P_E instead of π yields, as expected, a poorer correlation (r = 0.829). So few data points are available that no assessment of the role of X could be made. The differences in activity when X is H or CH_3 are small, so that a reasonable correlation can be achieved with the single-variable Eq. 31.

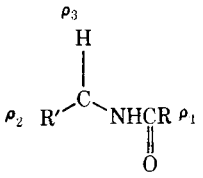


IX

The esters correlated by Eqs. 32 and 33 are comparable to those of Eq. 22, except that D-isomers of Eqs. 32 and 33 are used as inhibitors. Again, using P_E for the acyl function and π for the α-alkyl group gives the best correlation. The higher coefficient with π_R of Eq. 33 indicates binding of this function in the ρ_2 area. Two models of binding (Structures X*a* and X*b*) can be used to rationalize these results:



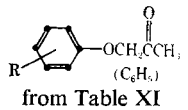
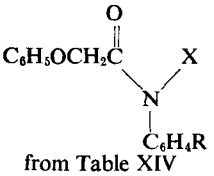
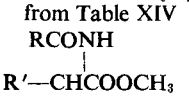
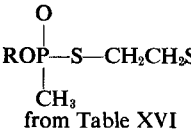
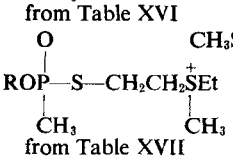
X*a*



X*b*

If binding occurs so that R′ is in the ρ_2 area and the α-H is in the ρ_H area, then the fit of Structure X*a* is obtained. The geometry here is quite different from that of Structure II and may be responsible for the difference in slope for the R term of Eq. 13 and the R′ term of Eq. 33. If this is indeed the way binding occurs, the fact that P_E gives a better correlation than π would imply that ρ_3 is similar to ρ_1 (see *Discussion*). The arrangement in Structure X*b* is based on the premise that R′ must fit into ρ_2 and R into the ρ_1 area. This places the α-H in the ρ_3 area and the ester function in the ρ_H below the plane of the page. It is unfortunate that more data points are not available so that more

Table XXI—Correlation of Structure and Activity of Chymotrypsin Inhibitors

Type Inhibitor	"Best" Equation	n	r	s	Conditions	Eq. No.
 from Table XI	$\log 1/I/S = 0.798(\pm0.28)\pi + 0.459(\pm0.45)\sigma + 0.868(\pm0.40)X - 1.964(\pm0.24)$	15	0.913	0.261	0.05 M Tris buffer; pH 7.4; 10% dimethyl sulfoxide	26
Misc. Compds. from Table XII	$\log 1/K_i = 0.950(\pm0.11)\log P - 1.883(\pm0.26)$	10	0.990	0.089	None given	27
	$\log 1/K_i = 0.996(\pm0.10)\log P - 2.596(\pm0.26)$	8	0.995	0.139		28
	$\log 1/K_i = 0.977(\pm0.06)\log P + 0.592(\pm0.12)HB - 2.537(\pm0.18)$	17	0.994	0.111		29
Aromatic acids	$\log 1/K_i = 0.942(\pm0.58)\log P + 0.960(\pm1.78)pKa - 3.660(\pm8.23)$	7	0.917	0.361	0.1 M KCl; 3.3×10^{-3} M Tris buffer; pH 6.9; 25°	30
 from Table XIV	$\log 1/I/S = 1.216(\pm0.85)\pi_R - 1.289(\pm0.51)$	6	0.893	0.297	0.05 M Tris buffer; pH 7.4; 10% dimethyl sulfoxide	31
 from Table XV	$\log 1/K_i = 0.900(\pm0.85)\pi_{R+R'} - 3.824(\pm2.40)$	7	0.771	0.630	In water at 25°; pH 7.90 and 0.10 M NaCl	32
	$\log 1/K_i = 2.874(\pm0.90)\pi_{R'} + 0.089(\pm0.02)P_{E-R} - 6.793(\pm1.49)$	7	0.984	0.193		33
 from Table XVI	$\log K_i = 1.133(\pm0.24)\pi - 1.151(\pm0.53)$	7	0.984	0.242	25°; pH 7.60	34
 from Table XVII	$\log K_i = 1.284(\pm0.32)\pi - 0.643(\pm0.71)$	7	0.978	0.326	25°; pH 7.60	35
C_6H_5COR from Table XVIII	$\log 1/K_i = 0.31(\pm0.37)\pi + 2.53(\pm0.40)$	3	0.996	0.020	0.1 M KCl; $3.3 \times 10^{-3}M$ Tris buffer; pH 6.9; 25°	36
Hydrocarbons from Table XIX	$\log 1/K_i = 1.473(\pm0.43)\log P - 1.209(\pm1.31)$	10	0.942	0.379	0.1 M KCl; $3.3 \times 10^{-3}M$ Tris buffer; pH 6.9; 25°	37

weight could be placed on this equation and the coefficients could be made sharply defined.

Equations 34 and 35, based on data from Tables XVI and XVII, correlate inhibition by two sets of phosphonates. In each set, the authors have omitted the derivative where R = octyl, since, as with Eq. 13, a break occurs at this point. The slopes of Eqs. 34 and 35 are close to those of the other equations where it is expected that binding in the ρ_2 area is occurring. From a comparison of the intercepts of Eqs. 34 and 35, it is clear that the onium compounds are more effective, despite the fact that the onium compounds are, as a whole, less hydrophobic. It may be that the positively charged sulfur aids in binding by interaction with an electron-rich species of the ρ_1 area and, in this fashion, compensates for the lower hydrophobic character.

The three ketones correlated by Eq. 36 are included to show that dependence of inhibitory power on π when the ρ_3 area is presumably involved is essentially the same as for substrates (Eqs. 14–16).

The correlation obtained with the hydrocarbons in Eq. 37 is not as good as that found by Wildnauer and Canady (26) with the molecular area of the inhibitor. The slope of Eq. 37 is somewhat higher than those for other sets binding in the ρ_2 area. Because of the rather large confidence interval on this slope, one cannot be sure this difference is real.

DISCUSSION

While the sets of data analyzed in this review were by no means ideally designed for assaying the relative importance of hydrophobic, electronic, and steric effects of substituents operating on substrates in the ρ_1, ρ_2, and ρ_3 areas of the enzyme, the overall view obtained with grossly different molecular species from many different laboratories gives a consistent picture.

Hydrophobic binding in the ρ_2 area is defined by the slopes of the hydrophobic terms in Eqs. 13, 22, 26, 29, 31, 34, 35, and 37. The mean and standard deviation for the eight values is 1.21 ± 0.23. There does not appear to be any significant difference for the slopes for the substrates and those for the inhibitors. This finding supports the idea of a common area and mechanism of binding for the two classes of reactants. Inhibition viewed in these terms would appear to be simple occupation of the binding site by the inhibitor.

The data on the ρ_3 area are also gratifyingly uniform. Comparable dependence on hydrophobic binding is seen in the coefficients with the hydrophobic terms in Eqs. 14–16 and 36. The mean and standard deviation for these four sets is 0.29 ± 0.1. Thus the importance of hydrophobic binding in the ρ_3 area is about one-fourth that of the ρ_2 area. Again, it is seen that substrates and inhibitors show the same dependence on π.

Steric inhibition of binding by the sp^2 carbon of the carbonyl group can be quite important, as shown by Eqs. 15 and 19–21. It is of interest to compare the coefficients of E_s in Eqs. 15 and 19–21 with those obtained for the hydrolysis of esters under homogeneous conditions. Taft (20) has defined E_s as:

$$\log k/k_0 = \delta E_s \qquad \text{(Eq. 38)}$$

where k_0 refers to the unsubstituted ester and k refers to the rate of acidic hydrolysis of a corresponding substituted acetate: X—CH_2COOR. Taft (20) found a value of δ of 0.30 for the alkaline hydrolysis of acetate esters

$$\begin{matrix} & & \mathrm{O} & \\ & & \| & \\ \mathrm{RCH_2O} & - & \mathrm{C} & -\mathrm{CH_3} \end{matrix}$$

of the type RCH_2OCCH_3. This compares with the value of 0.40 in Eq. 15. Present evidence indicates that the geometry of the transition state for acid and alkaline hydrolysis of esters is the same, so that one would expect the same dependence on δ. In Eqs. 19–21, an average value of δ of 1.5 is found. This compares with the values of 1.4, 1.7, and 1.9 found for the methanolysis, *n*-propanolysis, and isopropanolysis, respectively, of esters in which the R group of RCO_2—β—$C_{10}H_7$ was varied (20). Methanolysis of *l*-menthyl esters ($RCO_2C_{10}H_{16}$) showed a δ value of 1.7. Thus the serine moiety of chymotrypsin seems to make about the same steric demands on substrates as simple alcohols in homogeneous organic reactions. Of course, in Eqs. 19–21, the effect of E_s on the two constants, K_m and k_2, is being considered; while in Eq. 15, it is the effect on K_m that is correlated. The result with Eq. 15 implies that the formation of *ES* as characterized by K_m must involve the conversion of the sp^2 carbon of the ester into an sp^3 carbon in the *ES* complex; that is, since the $sp^2 \rightarrow sp^3$ change is involved in the homogeneous hydrolysis of esters, and since the δ values are so close for the enzymic and homogeneous processes, one would surmise that tetrahedral transition states are involved in each case. Although the steric effects in Eqs. 19–21 are involved with two processes, the result with Eq. 15 suggests that the effect of E_s may be primarily on K_m rather than k_2.

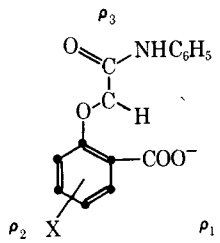
One of the most interesting aspects of this survey is the finding that binding in the ρ_2 area is characterized by π, while that in the ρ_1 area is not well correlated by π but correlates with polarizability as defined by P_E. While such an effect was not anticipated in undertaking the analysis, in retrospect it seems quite logical. This is the area in which a peptide bond of a protein molecule is bound. The binding of such a polarizable group would be facilitated by a polarizing atmosphere.

No doubt, better results would be obtained if, instead of the average polarizability that is embodied in molar refractivity, one could employ directional values. These values for polarization along the three mutually perpendicular directions are at present known for only a relatively few simple species (60).

It is the authors' interpretation of the correlation data that the area must be polar in character and charge interactions aid in holding polarizable groups in this area. One cannot completely rule out desolvation of the acyl moiety with binding in the area since there is some correlation with π; however, this would appear to be of secondary importance. Inhibitors and substrates appear to show the same dependence on P_E (compare Eqs. 22 and 33).

One might expect that the ρ_3 area would also be polar in character. The low coefficients with π in Eqs. 14–16 and 36 suggest that it is not lipophilic. Because only alkyl groups on the congeners are binding in this area, the same quality of correlation is obtained using π or P_E. The results with Eq. 33 in which P_E gives a better correlation than π can be interpreted as in Structure X*a* to mean that the character of the ρ_3 and ρ_1 areas is similar. It would be worthwhile to study a set of congeners binding in this area which has more variance in polarizability.

The partition coefficient of the whole molecule, or even a constant fraction of it, may not be the decisive feature in the binding process. This is illustrated in the recent study by Baker (61) of compounds in Structure XI. With the —COO^- function in the *ortho*-position, an active series of inhibitors is obtained. Only a twofold loss over that of the parent compound occurred. However, placing a —COO^- function in the *para*-position of the phenoxyacetones resulted in a huge loss in activity. That the binding of the phenoxy moiety



XI

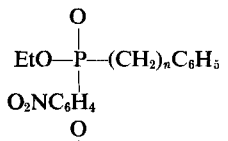
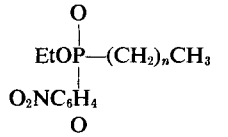
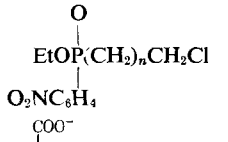
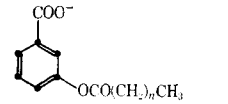
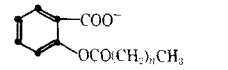
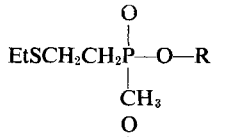
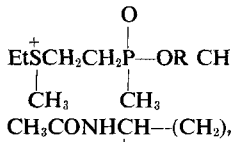
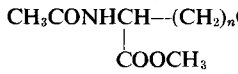
in Structure XI occurs in the ρ_2 area is supported by Eq. 39:

$$\log I_{50} = 0.70(\pm 0.25)\pi + 2.29(\pm 0.18) \quad \overset{n}{4} \quad \overset{r}{0.993} \quad \overset{s}{0.043} \qquad \text{(Eq. 39)}$$

The slope of Eq. 39 is quite close to that of Eq. 26. One finds a constant increase in inhibitory power for each unit of hydrophobicity of X. Apparently, in the *ortho*-position, the —COO^- function can remain free of the area and retain its solvation shell. In fact, it may aid binding by interaction with the polarizing ρ_1 area. This may also be true for the molecules correlated by Eq. 35. However, when in the 4-position, desolvation of the —COO^- appears to be necessary for the phenoxy ring to move into the area.

A point that is not completely clear is the break in activity which occurs when a certain degree of bulkiness or chain length is reached in a set of congeners. This has been mentioned in connection with Eqs. 13, 24, and 35. Other data are available on this point; however, it is not clear whether the break is a function of

Table XXII—Relation between Lipophilic Character of Side Chain and Change in Character of Binding

Compound	π-Value at which Break Occurs	Reaction Constant	Reference
	3.63	I_{50}	62
	3.50	I_{50}	62
	3.39	I_{50}	62
	3.50	Rel. rate hydrolysis	63
	3.50	$V_{max.}$	64
	3.50	K_i	57
	3.50	K_i	57
	2.50	K_m	50

chain length, hydrophobicity, or total molecular volume of the chain. There does seem to be a rather close correlation between the break in activity and the π-value; this is illustrated by the examples in Table XXII. Except for the last example, a close relationship is found between the degree of hydrophobic character and the break in the activity parameter. This break appears to occur at about the same point, regardless of the type functional group involved (*e.g.*, phosphate or acetate). The break also occurs at the same π-value, whether one is considering binding (K_m or K_i) or rate of hydrolysis ($V_{max.}$ or relative rate). This indicates that when a lipophilic moiety of sufficient size is present in the substrate or inhibitor, a decrease in expected activity occurs in a variety of different processes. This could be attributed to a kind of micelle formation[1] on the enzyme of the substrate. While most of the side chains are simple alkyl groups, the two examples where a phenyl and a chlorine moiety are included in the side chain are well in line with the alkyl function, despite the fact that the geometry of the phenyl group, its polarizability, *etc.*, is quite different from the alkane groups. A recheck of Set 8 may be worthwhile since the authors pointed out that a break in activity occurred when the α-alkyl group contained five carbons but, using a cyclohexylmethyl function, gives normal activity (see Table I). This was taken as evidence that chain length was crucial for the area. The other data of Table XXII make this seem unlikely.

Although only a few studies such as the present for chymotrypsin have been made on purified enzymes (16, 18), some data at hand for comparison indicate that other enzymes have hydrophobic pockets which show the same relationship between log P and various activities. Three such examples are:

Inhibition of NADH Oxidase Activity by Barbiturates (45)—

$$\log I_{50} = 1.107 \log P + 1.237 \quad \begin{matrix} n & r & s \\ 6 & 0.921 & 0.261 \end{matrix} \quad \text{(Eq. 40)}$$

Inhibition of Adenosine Deaminase by 9-(1-Hydroxy-2-alkyl)adenines (65)—

$$-\log (I/S)_{0.5} = 0.932\pi - 0.483 \quad \begin{matrix} n & r & s \\ 6 & 0.987 & 0.157 \end{matrix} \quad \text{(Eq. 41)}$$

Relative Rate of Hydrolysis of p-*Nitrophenyl Esters by Serum Esterase (16)*—

$$\log \text{rate} = 0.950 \log P + 3.503 E_s - 0.469 \quad \begin{matrix} n & r & s \\ 6 & 0.976 & 0.497 \end{matrix} \quad \text{(Eq. 42)}$$

In each of the three examples, enzymic activity shows the same dependence on hydrophobic character as defined by the octanol–water reference system. This suggests, but by no means is firm evidence, that the enzymic hydrophobic sites are quite similar to octanol in terms of polarity. This kind of hydrophobic site is quite different from that of serum albumin, hemoglobin, or whole serum when log P is taken as the reference. In many examples of the binding of quite different kinds of organic compounds to serum protein or homogenized tissue, the coefficient with log P or π falls in the range 0.5–0.7 (17). While different ways are used in expressing the binding constants which are not strictly comparable, in 24 such examples (18) a mean and standard deviation of slope of 0.58 ± 0.11 were found.

The importance of having suitable reference standards for determining the relationship between apolar character and mode of interaction of organic compounds with biochemical systems can be further extended. Narcosis under proper conditions is a completely reversible process. Simple binding of organic compounds to nerve tissue causes narcosis, so that the molar concentration of drug producing a standard narcosis can be *roughly* compared to K_m or K_i values from purified enzymes. The following equation illustrates the dependence of such reversible processes on log P.

Tadpole Narcosis by Miscellaneous Organic Compounds—

$$\log 1/C = 0.96 \log P + 0.75 \quad \begin{matrix} n & r & s \\ 44 & 0.967 & 0.303 \end{matrix} \quad \text{(Eq. 43)}$$

In 18 such examples, using various organisms, a mean slope of 1.10 ± 0.14 was found (18). Other processes (17, 18) show the same as well as different dependencies on log P. Too few examples are available at present to draw conclusions of deep significance.

The present study has successfully pulled together a wide variety of data on chymotrypsin substrates and

[1] The authors are indebted to Professor R. Nelson Smith for a number of discussions in which this idea developed.

inhibitors. This extrathermodynamic definition of the characteristics of the areas of interaction in the enzyme is quite consistent, especially when allowance is made for the fact that the information comes from a variety of different laboratories using different experimental techniques with quite a diverse group of organic compounds. It is hoped that this survey of the structure–activity relationship of chymotrypsin will encourage others working in this area to design more suitable substrates and inhibitors which can be used to define the intermolecular interactions more precisely. It seems likely that this approach, tested on chymotrypsin, can be applied to other enzyme substrate or enzyme inhibitor interactions. Also, it seems reasonable to expect that nonenzymic sites of drug action can be mapped using this approach.

REFERENCES

(1) E. J. Ariëns, "Molecular Pharmacology," vol. I, Academic, New York, N. Y., 1964.

(2) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, **34,** 49 (1965).

(3) L. Cunningham, in "Comprehensive Biochemistry," vol. 16, M. Florkin and E. H. Stotz, Eds., Elsevier, Amsterdam, The Netherlands, 1965, p. 85.

(4) T. C. Bruice and S. Benkovic, "Bioorganic Mechanisms," vol. I, W. A. Benjamin, New York, N. Y., 1966, p. 212.

(5) D. E. Koshland, in "The Enzymes," vol. I, P. D. Boyer, H. Lardy, and K. Myrbäck, Eds., Academic, New York, N. Y., 1959, chap. 7.

(6) E. L. Smith, in "The Enzymes," vol. IV, P. D. Boyer, H. Lardy, and K. Myrbäck, Eds., Academic, New York, N. Y., 1959, chap. 1.

(7) P. Desnuelle, in "The Enzymes," vol. IV, P. D. Boyer, H. Lardy, and K. Myrbäck, Eds., Academic, New York, N. Y., 1959, chap. 5.

(8) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967.

(9) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, pp. 218–229.

(10) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63,** 656 (1956).

(11) H. Gutfreund and J. M. Sturtevant, *Proc. Nat. Acad. Sci.*, **42,** 719(1956).

(12) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225,** 1049 (1957).

(13) H. Neurath and B. S. Hartley, *J. Cell. Comp. Physiol.*, **54,** Suppl. 1, 179(1959).

(14) J. E. Leffler and E. Grunwald, "Rates and Equilibria of Organic Reactions," Wiley, New York, N. Y., 1963.

(15) C. Hansch and T. Fujita, *J. Amer. Chem. Soc.*, **86,** 1616 (1964).

(16) C. Hansch, E. W. Deutsch, and R. N. Smith, *ibid.*, **87,** 2738 (1965).

(17) C. Hansch, *Accounts Chem. Res.*, **2,** 232(1969).

(18) C. Hansch, in "Drug Design," vol. I, E. J. Ariëns, Ed., in the series "Medicinal Chemistry," Academic, New York, N. Y., to be published.

(19) T. Fujita, J. Iwasa, and C. Hansch, *J. Amer. Chem. Soc.*, **86,** 5175(1964).

(20) R. W. Taft, in "Steric Effects in Organic Chemistry," M. S. Newman, Ed., Wiley, New York, N. Y., 1956, p. 644.

(21) E. Kutter and C. Hansch, *J. Med. Chem.*, **12,** 647(1969).

(22) E. Kutter and C. Hansch, *Arch. Biochem. Biophys.*, **135,** 126(1970).

(23) F. Helmer, K. Kiehs, and C. Hansch, *Biochemistry*, **7,** 2858(1968).

(24) C. Hansch and S. M. Anderson, *J. Org. Chem.*, **32,** 2583 (1967).

(25) W. Scholtan, *Arzneim.-Forsch.*, **18,** 505(1968).

(26) R. Wildnauer and W. J. Canady, *Biochemistry*, **5,** 2885 (1966).

(27) M. L. Bender and F. J. Kézdy, *J. Amer. Chem. Soc.*, **86,** 3704(1964).

(28) M. L. Bender, F. J. Kézdy, and C. R. Gunter, *ibid.*, **86,** 3714(1964).

(29) B. R. Baker, in "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967, p. 129.

(30) G. E. Hein and C. Niemann, *J. Amer. Chem. Soc.*, **84,** 4487(1962); *ibid.*, **84,** 4495(1962).

(31) C. L. Hamilton, C. Niemann, and G. Hammond, *Proc. Nat. Acad. Sci.*, **55,** 664(1966).

(32) J. L. Miles, D. A. Robinson, and W. J. Canady, *J. Biol. Chem.*, **238,** 2932(1963).

(33) P. B. Sigler, D. M. Blow, B. W. Mathews, and R. Henderson, *J. Mol. Biol.*, **35,** 143(1968).

(34) W. B. Lawson and H. J. Schramm, *Biochemistry*, **4,** 377 (1965).

(35) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967, p. 55.

(36) W. B. Lawson and H. J. Schramm, *J. Amer. Chem. Soc.*, **84,** 2017(1962).

(37) D. S. Sigman and E. R. Blout, *ibid.*, **89,** 1747(1967).

(38) N. K. Schaeffer, S. C. May, and W. H. Summerson, *J. Biol. Chem.*, **202,** 67(1953).

(39) B. S. Hartley and D. L. Kauffman, *Biochem. J.*, **101,** 229 (1966).

(40) Y. Nakagawa and M. L. Bender, *J. Amer. Chem. Soc.*, **91,** 1566 (1969).

(41) G. Schoellman and E. Shaw, *Biochemistry*, **2,** 252(1963).

(42) E. G. Ong, E. Shaw, and G. Schoellman, *J. Biol. Chem.*, **240,** 694(1965).

(43) N. R. Draper and H. Smith, "Applied Regression Analysis," Wiley, New York, N. Y., 1966.

(44) C. K. Hancock, E. A. Meyers, and B. J. Yager, *J. Amer. Chem. Soc.*, **83,** 4211(1961).

(45) C. Hansch and S. M. Anderson, *J. Med. Chem.*, **10,** 745 (1967).

(46) B. Zerner and M. L. Bender, *J. Amer. Chem. Soc.*, **86,** 3669(1964).

(47) S. G. Cohen and J. Crossley, *ibid.*, **86,** 1217(1964).

(48) J. B. Milstien and T. H. Fife, *Biochemistry*, **8,** 623(1969).

(49) A. Leo, C. Hansch, and C. Church, *J. Med. Chem.*, **12,** 766(1969).

(50) J. B. Jones, T. Kunitake, C. Niemann, and G. E. Hein, *J. Amer. Chem. Soc.*, **87,** 1777(1965).

(51) J. B. Jones, C. Niemann, and G. E. Hein, *Biochemistry*, **4,** 1735(1965).

(52) J. R. Rapp, C. Niemann, and G. E. Hein, *ibid.*, **5,** 4100(1966).

(53) J. P. Wolf, III, and C. Niemann, *ibid.*, **2,** 82(1963).

(54) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **10,** 1129 (1967).

(55) G. B. Barlin and D. D. Perrin, *Quart. Rev.*, **20,** 75(1966).

(56) I. V. Berezin, A. V. Levashov, and K. Martinek, "Vth Federated European Biological Meeting, Prague," 1968.

(57) A. A. Aaviksaar, E. V. Rozengart, P. F. Sikk, and R. A. Herbst, *Org. Reactiv.*, **4,** 1059(1968).

(58) A. J. Hymes, D. A. Robinson, and W. J. Canady, *J. Biol. Chem.*, **240,** 134(1965).

(59) D. Agin, L. Hersh, and D. Holtzman, *Proc. Nat. Acad. Sci.*, **53,** 952(1965).

(60) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," 2nd ed., Cornell University Press, Ithaca, N. Y., 1969, p. 152.

(61) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11,** 1054 (1968).

(62) E. L. Becker, T. R. Fukuto, B. Boone, D. C. Canham, and E. Boger, *Biochemistry*, **2,** 72(1963).

(63) H. J. Hofstee, *Biochim. Biophys. Acta*, **24,** 211(1957).

(64) H. J. Hofstee, *ibid.*, **32,** 182(1959).

(65) H. F. Schaeffer, R. N. Johnson, E. Oldin, and C. Hansch, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Chemistry, Pomona College, Claremont, CA 91711*

Interfacial Barrier Limited Interphase Transport of Cholesterol in the Aqueous Polysorbate 80–Hexadecane System

ANWAR B. BIKHAZI and WILLIAM I. HIGUCHI

Abstract □ The kinetics of transfer of cholesterol from an aqueous polysorbate 80 solution into hexadecane and vice versa was studied by means of the multiparticulate dispersion technique. The experimental data were quantitatively analyzed by the physical model which accounts for the effects of bulk diffusion, interfacial resistance, interfacial area, and the lipid–water partition coefficient. For the 0.1% polysorbate 80, a P value around 1.7 to 2.2 × 10^{-7} cm. $sec.^{-1}$ was found that was consistent with all of the data on water-to-oil as well as oil-to-water transfer experiments. These findings suggest such large nonspecific interfacial barriers to be important in many biological and biopharmaceutical situations.

Keyphrases □ Interfacial barrier limited transport—interphase □ Cholesterol interphase transport—aqueous polysorbate 80–hexadecane system □ Emulsions—hexadecane–water–polysorbate 80 □ Particle-size distribution—emulsions □ Partition coefficient, cholesterol—hexadecane–polysorbate 80 system

Recently (1–5), there has been increased interest in the possibility of utilizing physical models to gain an understanding of the transport of drugs and other biologically important substances from body fluids into tissues and across membranes. The main characteristic of the physical model approach is the intimate interaction of realistic physical concepts with well-designed experiments. Thus the previous studies (1–5) have shown that it should be possible to interrelate such factors as the pKa, the partition coefficient and the diffusional characteristics of the drug, the buffer characteristics and the pH of the aqueous phases involved, and the heterogeneous nature of the membrane, and then to test such relationships experimentally.

One of the factors in interphase transport of drugs which has escaped serious consideration until recently (1, 2) is the possible existence of significant interfacial resistances at the oil–water interface. The present studies along with those of Ghanem *et al.* (1) appear to represent for the first time the likelihood of the frequent domination of interfacial barriers in interphase transport.

The purpose of the present communication is to describe the interfacial barrier-controlled transfer of cholesterol across an oil–water interface. As will be shown, very large interfacial resistances were found. Because of the probable nonspecific nature of the interfacial barrier, these findings may be very pertinent to a number of biological and pharmaceutical situations, for example, the deposition of gallstones, the initiation of atherosclerosis, and the transport and the absorption of cholesterol and other steroids (6) from the gastrointestinal tract.

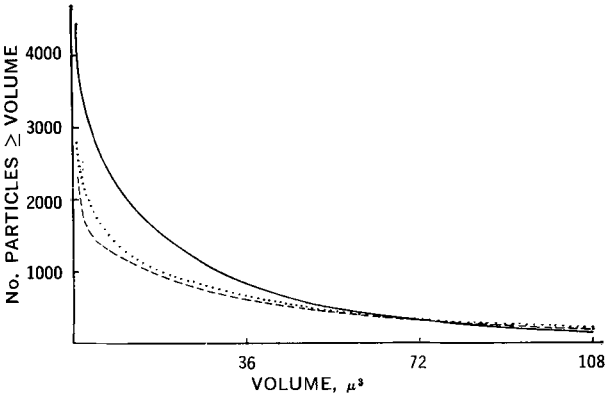


Figure 1—*Cumulative particle-size distribution data from Emulsions I and II obtained using the Coulter counter model A. A plot of number of particles* ≥ *volume* versus *volume gave a 95% mass balance for Emulsion I, a 66% mass balance for Emulsion II as shown in Table I, and a 73% mass balance for Emulsion III. Key: —, Emulsion I; - - -, Emulsion II; and · · ·, Emulsion III.*

EXPERIMENTAL

General Considerations in the Design of Experiments—It was decided to employ the multiparticulate dispersion technique (1, 2) which provides both good reproducibility and sensitivity for interfacial barrier determination. Both water-to-oil ("solute uptake") as well as oil-to-water ("solute release") transport experiments were carried out in order to assure the reliability of the interfacial permeability coefficient. Different oil particle-size distributions were utilized to demonstrate further that an interfacial barrier was rate controlling.

Preparation of Stock Emulsions—Two 8% hexadecane–water emulsion stocks using 0.1% polysorbate 80[1] were prepared in such a manner that the particle-size distributions differed significantly. Emulsion I was prepared by mixing 8 ml. of hexadecane[2] with 1 ml. of 10% aqueous polysorbate 80 solution and then making up to 100 ml. with distilled water. The mixture was then homogenized for 75 sec. in a Waring blender. Emulsion II was prepared by mixing 8 ml. of hexadecane with 0.5 ml. of the 10% polysorbate 80 solution and then making up to 100 ml. with distilled water. This mixture was then homogenized for 45 sec., after which 0.5 ml. of 10% surfactant was added to make the final surfactant concentration of

[1] Obtained from Atlas Chemical Industries, Inc., Wilmington, Del.
[2] Hexadecane Spectroquality Reagent, Matheson Coleman & Bell, Norwood, Ohio.

Table I—Oil Droplet Size Distribution and Data Treatment of Emulsion Systems I and II Given in Fig. 1 with Their Respective Mass Balance

j	Mean Radius (Micron)	Δ(Volume) (Micron)[3]	No. of Particles	Total Volume (Micron)[3]
		System I		
1	0.688	0.90	625.00	850.00
2	0.815	0.91	475.00	1073.50
3	0.912	0.90	300.00	954.00
4	0.990	0.91	200.00	814.00
5	1.060	0.90	162.50	809.25
6	1.120	0.91	137.50	807.13
7	1.175	0.90	125.00	847.50
8	1.225	0.91	100.00	770.00
9	1.275	0.90	100.00	870.00
10	1.335	1.81	162.50	1616.88
11	1.415	1.82	150.00	1777.50
12	1.485	1.81	137.50	1883.75
13	1.545	1.80	112.50	1743.75
14	1.605	1.82	100.00	1690.00
15	1.695	4.53	225.00	4590.00
16	1.815	4.52	187.50	4706.25
17	1.915	4.53	162.50	4761.25
18	2.010	4.53	137.50	4661.25
19	2.095	4.52	112.50	4331.25
20	2.175	4.53	75.00	3240.00
21	2.250	4.53	75.00	3577.50
22	2.325	4.52	50.00	2640.00
23	2.385	4.53	50.00	2870.00
24	2.495	4.55	62.50	4075.00
25	2.505	4.51	62.50	4112.50
26	2.560	4.52	50.00	3520.00
27	2.640	9.06	50.00	3850.00
28	2.740	9.05	37.50	3232.50
29	2.830	9.05	37.50	3562.50
30	2.920	9.06	37.50	3907.50
31	3.085	42.28	137.50	16981.25
			Sum	95126.00
			Mass Balance	95%
		System II		
1	0.688	0.90	525	714.00
2	0.815	0.91	187.50	423.75
3	0.912	0.90	112.50	357.75
4	0.990	0.91	87.50	356.13
5	1.060	0.90	50.00	249.00
6	1.120	0.91	50.00	293.00
7	1.175	0.90	37.00	253.13
8	1.225	0.91	25.00	192.50
9	1.275	0.90	25.00	217.50
10	1.335	1.81	75.00	746.25
11	1.415	1.82	62.50	740.63
12	1.485	1.81	62.50	856.25
13	1.545	1.80	50.00	775.00
14	1.605	1.82	50.00	845.00
15	1.695	4.53	112.50	2295.00
16	1.815	4.52	75.00	1882.50
17	1.915	4.53	75.00	2197.50
18	2.010	4.53	62.50	2118.75
19	2.095	4.52	62.50	2406.25
20	2.175	4.53	62.50	2700.00
21	2.250	4.53	37.50	1788.75
22	2.325	4.52	37.50	1980.00
23	2.385	4.53	25.00	1435.00
24	2.495	4.55	50.00	3260.00
25	2.505	4.51	31.25	2056.25
26	2.560	4.52	37.50	2640.00
27	2.640	9.06	37.50	2887.50
28	2.740	9.05	37.50	3232.50
29	2.830	9.05	37.50	3562.50
30	2.920	9.06	37.50	3907.50
31	3.140	28.97	150.00	19425.00
			Sum	66795.37
			Mass Balance	66%

0.1%. These emulsions were gently shaken for about 15 min. prior to their use in the rate runs.

In addition to these two emulsions, a third emulsion (Emulsion III) was prepared in a similar manner but utilizing a polysorbate 80 ester (Polyol-Free).[1]

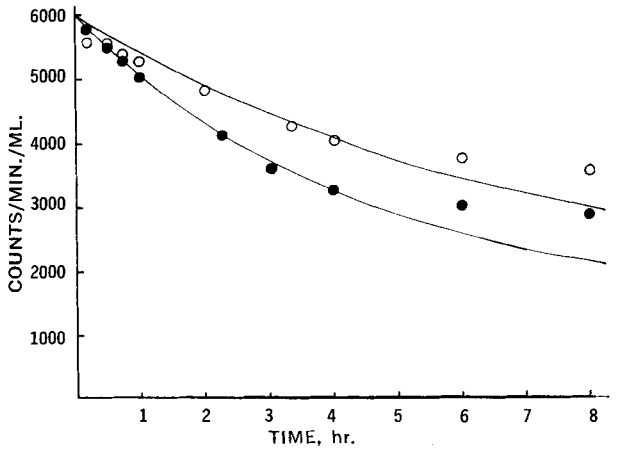


Figure 2—*Comparison of experimental data with theory for the uptake of cholesterol from Emulsion Systems I and II; counts/min./ml. of drug in the aqueous 0.1% polysorbate 80 phase* versus *time in hours. Key: experimental points from 2% oil of System I, ●; and experimental points from 2% oil of System II, ○. Curves are theoretically computed values.* V_W *= 49 ml. of aqueous polysorbate 80 phase;* P *value for both systems is* 1.7×10^{-7} *cm. sec.*$^{-1}$*;* K *value is 200.*

Figure 1 shows the cumulative particle-size distribution data from the three emulsions obtained using the Coulter counter.[3] The data given in Fig. 1 were used to obtain the differential size distribution in Table I. No significant particle-size distribution changes were found with these emulsions up to 8 hr.

Uptake Experiments—Predetermined dilutions of these stocks were made in 0.1% polysorbate 80 solution. Then 1.5×10^{-7} g. of 4-^{14}C-cholesterol contained in 3 ml. of a 0.1% polysorbate 80 solution was added into 47 ml. of the diluted emulsions and the mixture shaken gently at 30° in the Burrell Wrist-Action shaker.[4] Three- or five-milliliter samples were pipeted out at different time intervals and the aqueous phases were analyzed by either filtration (7) of the sample employing 0.20-μ pore size Gelman Metricel filters[5] (GA-8) or by high-speed centrifugation[6] at 21,600×g for 1.5 min. Out of the clear aqueous solution collected, 1 ml. was pipeted into a liquid scintillation vial. To the latter, 10 ml. of a liquid scintillation cocktail was added, and the samples quantitatively analyzed in the Beckman liquid scintillation system.[7] In the filtration procedure it was found that a small amount (≃ 10%) of the cholesterol was lost to the filter during filtration. Therefore, a correction for the adsorption loss was obtained by this technique.

Release Experiments—For the release experiments the same procedure was used for the preparation of the stock emulsions. However, radioactive cholesterol was added to the oil (6.68×10^{-7} g. of 4-^{14}C-cholesterol/ml. of hexadecane) prior to the emulsification step. Aliquots of the stock emulsion containing the 4-^{14}C-cholesterol were then added at zero time to predetermined volumes of 0.1% polysorbate 80 solutions. Sampling and analysis of the aqueous phases were carried out in the same manner as in the uptake experiments.

Partition Coefficient Determinations—The hexadecane–0.1% polysorbate 80 partition coefficient for the 4-^{14}C-cholesterol was determined in emulsion systems containing 0.24 to 8% hexadecane and equilibrating for 48 and 72 hr. A value of 200 ± 10% was found which was used in the analysis of the data.

RESULTS AND ANALYSIS OF THE DATA

In all of the uptake and the release experiments, significant changes in the aqueous cholesterol concentrations were found up to 8 hr. The results of the experiments for uptake and release are

[3] Model A, Coulter Electronics, Hialeah, Fla.
[4] Burrell Corp., Pittsburgh, Pa.
[5] Gelman Instrument Co., Ann Arbor, Mich.
[6] Lourdes Instrument Corp., Brooklyn, N. Y.
[7] Beckman Instruments, Inc., Fullerton, Calif.

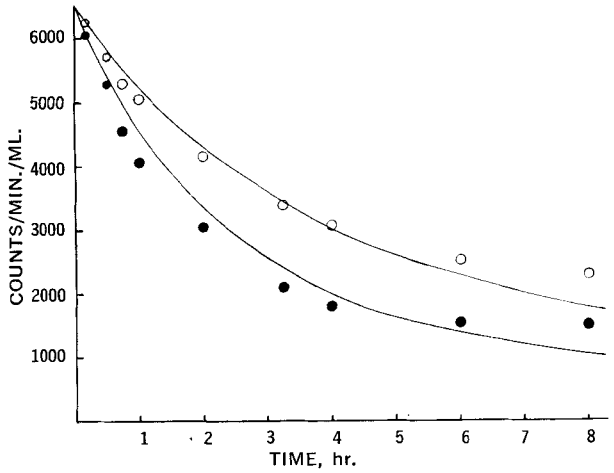


Figure 3—*Comparison of experimental data with theory for the uptake of cholesterol from Emulsion Systems I and II. Counts/min./ml. of drug in the aqueous 0.1% polysorbate 80 phase* versus *time in hours. Key: experimental points from 4% oil of System I, ●; and experimental points from 4% oil of System II, ○. Curves are theoretically computed values.* V_W = *48 ml. aqueous polysorbate 80 phase;* P *value for both systems is* 1.7×10^{-7} *cm. sec.*$^{-1}$; K *value is 200.*

presented in Figs. 2–9. In the uptake plots (Figs. 2–5) the ordinate gives the actual aqueous concentrations of 4-^{14}C-cholesterol as a function of time. In the release plots (Figs. 6–9) the initial (zero time) expected aqueous concentrations were subtracted from all of the determinations of the aqueous cholesterol.

The following procedure was developed for the analysis of the experimental results. The general relationships apply to both uptake and release, the two situations differing only by the difference in sign of the concentration gradient.

It is helpful to refer to the model in Fig. 10. For an oil droplet of radius a_j, the rate of cholesterol transport into (or out of) the droplet is given by Eq. 1:

$$G_j = \frac{4\pi a_j{}^2 PD(C_b - C_{bj}')}{D + a_j P} \qquad \text{(Eq. 1)}$$

where P is the apparent permeability coefficient for the interfacial barrier, D is the relevant diffusion coefficient for cholesterol in the 0.1% polysorbate 80 solution, C_b is the total bulk aqueous cholesterol concentration, and C_{bj}' is defined by Eq. 2:

$$K = \frac{C_{oj}}{C_{bj}'} \qquad \text{(Eq. 2)}$$

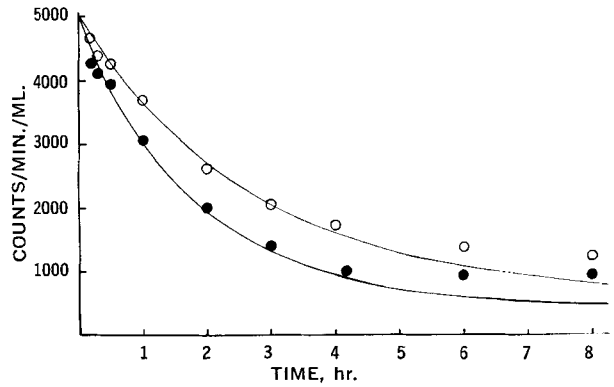


Figure 4—*Comparison of experimental data with theory for the uptake of cholesterol from Emulsion Systems I and II. Counts/min./ml. of drug in the aqueous 0.1% polysorbate 80 phase* versus *time in hours. Key: experimental points from 6% oil of System I, ●; and experimental points from 6% oil of System II, ○. Curves are theoretically computed values.* V_W = *47 ml. aqueous polysorbate 80 phase;* P *value for both systems is* 1.7×10^{-7} *cm. sec.*$^{-1}$; K *value is 200.*

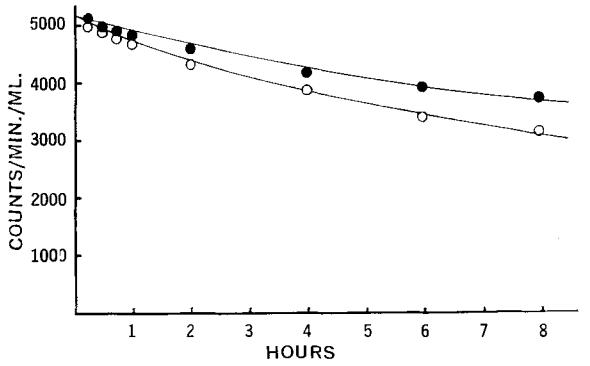


Figure 5—*Comparison of experimental data with theory for the uptake of cholesterol from Emulsion III. Counts/min./ml. of drug in the aqueous 0.1% polysorbate 80 ester phase* versus *time in hours. Key: experimental points from 0.8% oil, ●; and experimental points from 1.2% oil, ○. Curves are theoretically computed values.* P *value for both dilutions is* 2.2×10^{-7} *cm. sec.*$^{-1}$; K *value is 200.*

where K is the effective hexadecane–0.1% polysorbate 80 partition coefficient for cholesterol and C_{oj} is the cholesterol concentration in the oil droplet. When G_j is positive the situation is for uptake; when G_j is negative, one has cholesterol release from the droplet.

It is noteworthy that when $a_jP \ll D$, then a_jP may be neglected in the denominator of Eq. 1. In this case, one may write

$$G_j = 4\pi a_j{}^2 P(C_b - C_{bj}') \qquad \text{(Eq. 3)}$$

which is the appropriate limiting expression for the interfacial barrier-controlled transfer of cholesterol. One may also write:

$$G_j = V_{oj}\frac{dC_{oj}}{dt} \qquad \text{(Eq. 4)}$$

where $V_{oj} = \frac{4}{3}\pi a_j{}^3$ is the volume of the oil droplet and t is the time.

Equations 1, 2, and 4 may be combined to give

$$\frac{dC_{oj}}{dt} = \frac{3DP(C_b - C_{oj}/K)}{a_j(D + a_jP)} \qquad \text{(Eq. 5)}$$

Now, from material balance considerations in the system, one may write

$$\frac{dC_b}{dt} = -\frac{4\pi}{3V_W}\sum_{j=1}^{L} a_j{}^3 \Delta N_j \frac{dC_{oj}}{dt} \qquad \text{(Eq. 6)}$$

where V_W is the volume of the aqueous phase, ΔN_j is the number of droplets of sizes between a_j and a_{j+1}, and L represents the largest oil droplets in the system.

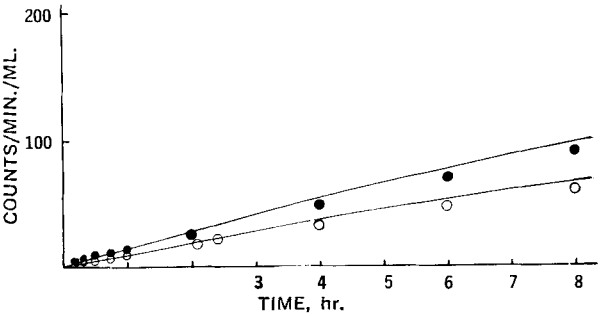


Figure 6—*Comparison of experimental data with theory for the release of cholesterol from Emulsion Systems I and II. Counts/min./ml. of drug in the aqueous 0.1% polysorbate 80 phase* versus *time in hours. Key: experimental points from 0.24% oil of System I, ●; and experimental points from 0.24% oil of System II, ○. Curves are theoretically computed values.* V_W = *49.88 ml. aqueous polysorbate 80 phase;* P *value for both systems is* 1.7×10^{-7} *cm. sec.*$^{-1}$; K *value is 200.*

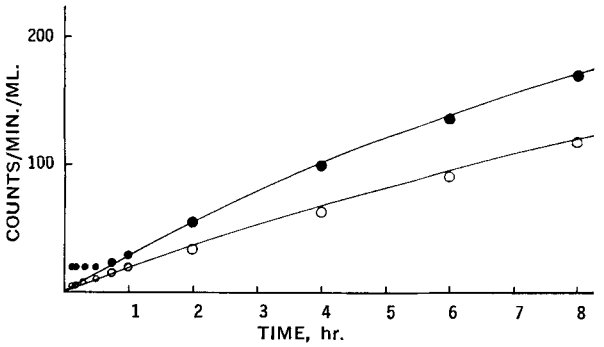


Figure 7—*Comparison of experimental data with theory for the release of cholesterol from Emulsion Systems I and II. Counts/min./ml. of drug in the aqueous 0.1% polysorbate 80 phase* versus *time in hours. Key: experimental points from 0.48% oil of System I, ●; and experimental points from 0.48% oil of System II, ○. Curves are theoretically computed values.* V_W = *49.76 ml. aqueous polysorbate 80 phase;* P *value for both systems is* 1.7×10^{-7} *cm. sec.*$^{-1}$; K *value is 200.*

As is easily seen, the two equations, Eqs. 5 and 6, may be used to solve for C_b as a function of time when V_W, D, P, K, and the particle-size distribution (*e.g.*, Table I) are known. However, this cannot be done analytically and one must resort to numerical methods. The flow diagram based on the FORTRAN IV language (IBM 360 digital computer) for solving Eqs. 5 and 6 is given in Fig. 11.

The computer calculations for the various experimental conditions were carried out for both cholesterol uptake and release. These are presented as the smooth curves in Figs. 2–9. As can be seen, a P value of around 1.7 to 2.2×10^{-7} cm. sec.$^{-1}$ was found to give good agreement of all experiments with the theoretical relations of Eqs. 5 and 6. The experiments with the polysorbate 80 ester sample appeared to correspond to slightly higher rates ($P \simeq 2.2 \times 10^{-7}$ cm. sec.$^{-1}$) than those with the unpurified surfactant ($P \simeq 1.7 \times 10^{-7}$ cm. sec.$^{-1}$).

In all of these calculations, a choice of D from 10^{-6} to 10^{-10} cm.^{2} sec.$^{-1}$ made no significant difference in the results. The lower limit (10^{-10} cm.^{2} sec.$^{-1}$) would be an unexpectedly low value even when the principal species in 0.1% polysorbate 80 is micellar in nature.

An idea of the sensitivity of the fit of the experimental data to the theoretical predictions can be obtained by referring to Fig. 12. It can be seen that the precision in the determination of P is in the neighborhood of ± 10% for the release experiments. For the

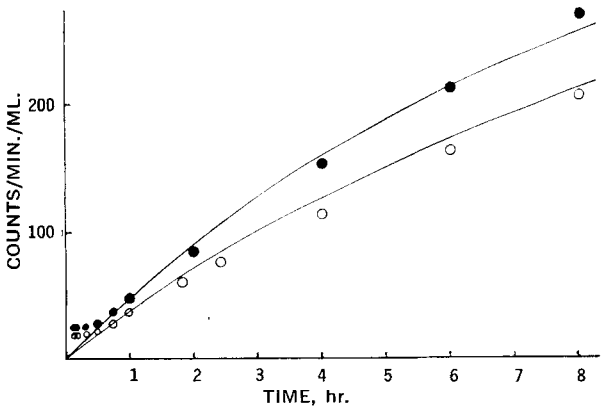


Figure 8—*Comparison of experimental data with theory for the release of cholesterol from Emulsion Systems I and II. Counts/min./ml. of drug in the aqueous 0.1% polysorbate 80 phase* versus *time in hours. Key: experimental points from 0.96% oil of System I, ●; and experimental points from 0.96% oil of System II, ○. Curves are theoretically computed values.* V_W = *49.52 ml. aqueous polysorbate 80 phase;* P *value for both systems is* 1.7×10^{-7} *cm. sec.*$^{-1}$; K *value is 200.*

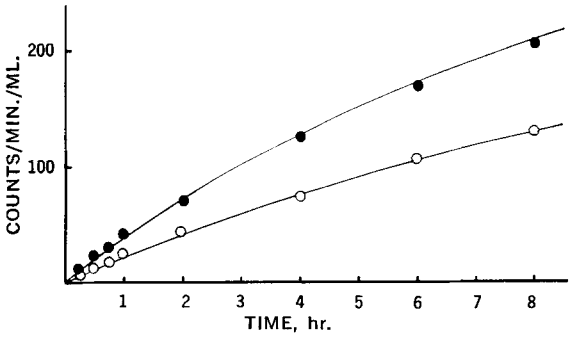


Figure 9—*Comparison of experimental data with theory for the release of cholesterol from Emulsion III. Counts/min./ml. of drug in the aqueous 0.1% polysorbate 80 ester phase* versus *time in hours. Key: experimental points from 0.4% oil, ○; and experimental points from 0.8% oil, ●. Curves are theoretically computed values.* P *value for both dilutions is* 2.2×10^{-7} *cm. sec.*$^{-1}$; K *value is 200.*

uptake experiments, while the general agreement of the experimental data with theory is good, somewhat larger discrepancies were found at larger times.

One of the factors which may limit the accuracy of the treatment of the transport by Eqs. 5 and 6 is the particle-size distribution data obtainable with the Coulter counter. The situation in the case of Emulsion I of this study is quite satisfactory as it has yielded a 95% mass balance. In other experiments, comparably good mass balances have been found (1). However, one should question the accuracy of the analysis procedure when, for example, only 66% (Emulsion II) of the oil phase can be accounted for by the Coulter counter data.

Whenever the mass balance is poor, the cause may be attributed to one of five possibilities: (*a*) volumetric errors in transferring the oil in the stock emulsion to the reaction flask; (*b*) significant number of droplets below the Coulter counter sensitivity; (*c*) significant number of droplets too large to be accurately sized; (*d*) accuracy of the Coulter counter itself; and (*e*) solubilization of the oil in the aqueous media (intrinsic solubility of solubilization by, for example, surfactant).

In the experiments with Emulsion II, it is most likely that the discrepancy is not likely to be due to the following: (*a*) because of good precision of the partition coefficient data obtained with many solutes; (*b*) because this would require an unusual, essentially bimodal, distribution of droplet sizes; (*d*) because many other emulsions have yielded better mass balances than 66%; and (*e*) because no time effects on size distribution were observed. It is therefore proposed that the absence of good mass balance in the case of Emulsion II is the result of (*c*), *i.e.*, the presence of a significant number of large droplets that was not measured by the instrument.

In order to assess the effect of neglecting the large droplets in the calculations with Eqs. 5 and 6, a computation was carried out with a particle-size distribution for Emulsion II (see Table I, Column 9), but for which a 93% mass balance was obtained by including more

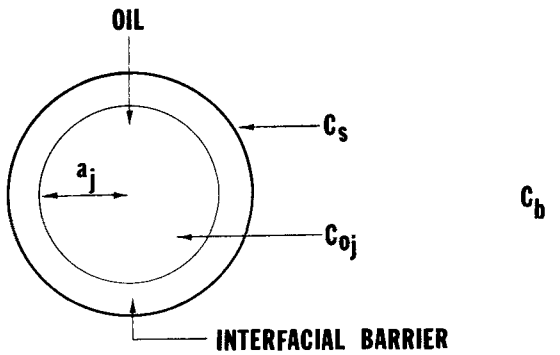


Figure 10—*The physical model that describes the uptake and/or release of the solute (cholesterol) across the oil droplet. Key:* a_j = *droplet radius;* C_{oj} = *solute concentration in the oil phase;* C_s = *aqueous solute concentration just outside the adsorbed film;* C_b = *solute concentration in the aqueous 0.1% polysorbate 80 phase.*

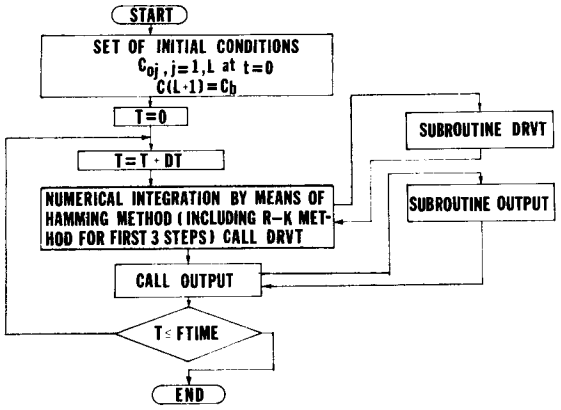


Figure 11—*Computer flow diagram showing the procedure for computation of* C_b *and* C_{oj}.

large droplets in the extrapolation. In Fig. 13, the broken curve shows the computed release-time behavior for this situation. As can be seen, for initial release the difference between the 66% mass balance and the 93% mass balance is relatively small and comparable to the scatter of the experimental data.

DISCUSSION

The results of the analysis of data clearly show that an interfacial barrier was operative in these experiments. The effective permeability coefficient value of 1.7 to 2.2 × 10^{-7} cm. sec.$^{-1}$ corresponds to a rather large interfacial resistance to transport which might appear to be rather surprising for the simple oil–water system in these studies.

It is of interest to compare this value with those reported in the literature for transport of organic compounds across biological and "synthetic" biological membranes. Bean *et al.* (8) reported on the bilayer lipid membrane permeability coefficient of a number of

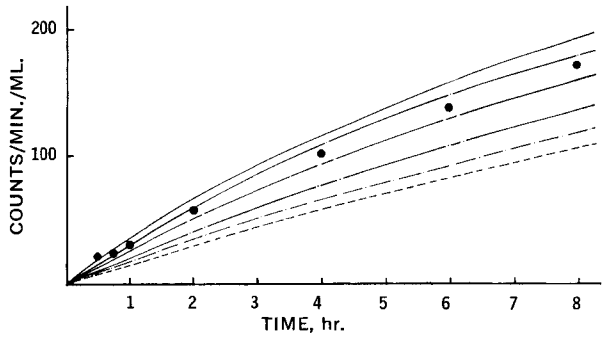


Figure 12—*Sensitivity of the fit of the experimental data to the theoretical predictions. The points are experimental results for 0.48% release experiments whose data gave these theoretical curves with a change in the* P *values. Key:* P *value 9* × 10^{-8} *cm. sec.*$^{-1}$, - - -; P *value 1* × 10^{-7} *cm. sec.*$^{-1}$, —·—; P *value 1.2* × 10^{-7} *cm. sec.*$^{-1}$, ·-·-·; P *value 1.5* × 10^{-7} *cm. sec.*$^{-1}$, — - - —; P *value 1.8* × 10^{-7} *cm. sec.*$^{-1}$, — ·; P *value 2* × 10^{-7} *cm. sec.*$^{-1}$, —; K *value is 200.*

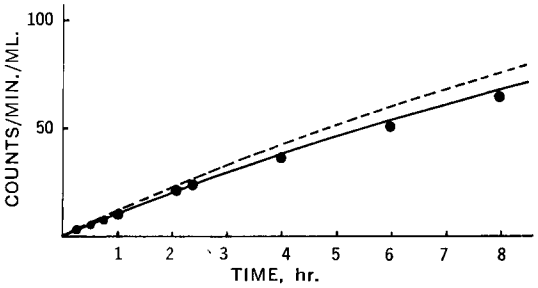


Figure 13—*Effect of neglecting the large droplets in the calculations with Eqs. 5 and 6 as computed for Emulsion II. Key: the broken curve, - - -, shows the computed release-time behavior for a 93% mass balance; and the full curve, —, shows the computed release-time behavior for a 66% mass balance.* P *value for both systems is 1.7* × 10^{-7} *cm. sec.*$^{-1}$; K *value is 200.*

organic compounds. The authors found that the permeability coefficients for most simple organic molecules are in the range of 10^{-4} to 10^{-6} cm. sec.$^{-1}$. Holder and Hayes (9) found red blood cell permeability coefficients of a number of sulfonamides to be in the range of 10^{-4} to 10^{-7} cm. sec.$^{-1}$. Rothblat *et al.* recently (10) reported data on the uptake of cholesterol by $L_{5178}Y$ tissue culture cells. A rough calculation with their data yields a permeability coefficient in the neighborhood of 10^{-6} cm. sec.$^{-1}$. Thus the interfacial barrier for cholesterol at the hexadecane–0.1% polysorbate 80 system is of the same order of magnitude if not somewhat greater than those observed in a number of biological situations.

It is hoped that this investigation and the continuing studies will provide a basis for understanding the detailed molecular factors in the various biological and biopharmaceutical situations.

REFERENCES

(1) A. H. Ghanem, W. I. Higuchi, and A. P. Simonelli, *J. Pharm. Sci.*, **58**, 165(1969).

(2) A. H. Goldberg, W. I. Higuchi, N. F. H. Ho, and G. Zografi, *ibid.*, **56**, 1432(1967).

(3) R. G. Stehle and W. I. Higuchi, *ibid.*, **56**, 1367(1967).

(4) S. A. Howard and W. I. Higuchi, to be published.

(5) A. Suzuki, N. F. H. Ho, and W. I. Higuchi, to be published.

(6) C. Sylvèn and B. Borgström, *J. Lipid Res.*, **9** (1968).

(7) A. H. Goldberg and W. I. Higuchi, *J. Pharm. Sci.*, **57**, 1583 (1968).

(8) R. C. Bean, W. C. Shepherd, and H. Chan, *J. Gen. Physiol.*, **52**, 495(1968).

(9) L. B. Holder and S. L. Hayes, *Mol. Pharmacol.*, **1**, 266 (1965).

(10) G. H. Rothblat, R. W. Hartzell, Jr., H. Mialke, and D. Kritchevsky, *Biochim. Biophys. Acta*, **116**, 133(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 27, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication January 12, 1970.

This investigation was supported by Grants GM-13368 from the National Institute of General Medical Sciences and HE-07690 from the National Heart Institute, U. S. Public Health Service, Bethesda, Md.

Intermolecular Bonding of the Antibiotic Diumycin

JOEL KIRSCHBAUM, WILLIAM A. SLUSARCHYK, and FRANK L. WEISENBORN

Abstract □ The closely related antibiotics diumycin A and B, with monomeric molecular weights of approximately 1800 daltons in ethanol, aggregate in aqueous buffers to form particles with a molecular weight (mol. wt.) of at least 32,000 daltons. The aggregate of diumycin is essentially unaffected by esterification of the acid, acetylation of hydroxyl groups, high ionic strength buffer, or variations in pH from 2.2 to 12.4. These results indicate that salt linkages and hydrogen bonds contribute only slightly to stabilize the aggregate. The aggregate may be disrupted by: (*a*) the addition of such hydrophobic bond-breaking agents as buffered aqueous solutions of guanidinium chloride, urea, or formamide; (*b*) the hydrolytic loss of a lipid side chain (mol. wt. about 400 daltons); or (*c*) the addition of alcohols. The ability of an alcohol to disrupt the aggregate increases with its hydrocarbon content. From these data, it is concluded that lipid–lipid hydrophobic interactions are responsible for the self-association of diumycin. The aggregate is spherical with a mantle of hydrophilic sugars, including glucose and glucosamine, surrounding a tangle of lipid side chains.

Keyphrases □ Diumycin—intermolecular bonding □ Aggregates, diumycin A, B—ethanolic aqueous buffers □ Hydrogen, lipid–lipid hydrophobic bonding—diumycin □ Sedimentation coefficients—diumycin □ Molecular weight—diumycin

The diumycins are a family of closely related antibiotics isolated from *Streptomyces umbrinus*, with formula molecular weights between 1700 and 2100 daltons, as determined by elemental analysis, including phosphorus content (1).

This paper concerns the ability of diumycin to aggregate in aqueous solvents, an ability shared by many natural products (2–5), and the source of intermolecular binding energy for such aggregation.

EXPERIMENTAL

Sources of Diumycin and Diumycin Derivatives—Diumycin A and B were isolated and characterized by Meyers *et al.* (1). Weisenborn *et al.* (6) prepared the derivatives of diumycin. Hydrolysis of purified diumycin, with 1 *M* HCl at 100° for 30 min., yields a 358-dalton lipid (6).

Sedimentation Coefficients—Sedimentation coefficients were determined with the aid of a Spinco model E analytical ultracentrifuge, using a calibrated temperature-control unit. Viscosity was measured with the aid of capillary and Zimm viscometers (7). One-milliliter pycnometers were used for density measurements at 20 ± 0.002°. Viscosity and density corrections were also taken from the data of Kawahara and Tanford (8) for adjusting the sedimentation coefficients to standard conditions (9), which are the viscosity and density of water at 20°.

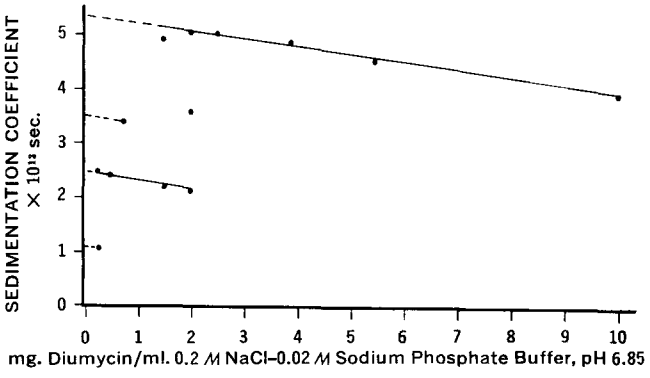


Figure 1—*Dependence of the sedimentation coefficient of diumycin A on its concentration in 0.2* M *NaCl–0.02* M *sodium phosphate buffer, pH 6.85.*

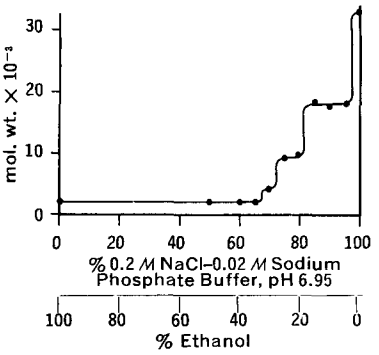


Figure 2—*Molecular weight of diumycin A (10 mg./ml.) in mixtures of ethanol and 0.2* M *NaCl–0.02* M *sodium phosphate buffer, pH 6.85.*

Molecular Weights—Molecular weights were determined by the Archibald approach-to-sedimentation equilibrium method (10), using 5 to 10 mg. solute/ml. solvent. The concentration gradients at the meniscus and cell bottom were measured from schlieren patterns recorded on Kodak metallographic plates and enlarged 10-fold on a Nikon magnifier. Total concentration was measured with the aid of a capillary centerpiece in an interference cell. The integral of the concentration gradient was evaluated by the procedure of Engelberg (11). The partial specific volume of 0.61 cm.³/g., calculated for diumycin from density data using methodology described by Schachman (12), was decreased by 0.01 cm.³/g. for calculations of molecular weight when buffered 8 *M* urea, 9 *M* formamide, or 5 *M* guanidinium chloride was used as the solvent (13).

RESULTS AND DISCUSSION

Although elemental analysis of diumycin, including the determination of phosphorus, indicates a molecular weight of approximately 1900 daltons (1), the apparent molecular weight of diumycin in neutral pH buffer, at a concentration of 3 to 10 mg./ml., is 32,000 daltons (Table I). A minor component has an apparent molecular weight of 65,000 daltons. Dilution disrupts the aggregate (Fig. 1), with several species seen at concentrations below 3 mg./ml. (Table I).

Aggregation in aqueous solvents is observed between pH 2.2 and 12.4, as well as in 1% sodium dodecyl sulfate (Table I). The ineffectiveness of sodium dodecyl sulfate as a disaggregating agent indicates little or no increase in net negative charge on diumycin due to binding of anions (14), with repulsive forces essentially unchanged. The methyl ester and acetate (10 *M* acetate/ 1 *M* diumycin) of diumycin aggregate in aqueous buffer with molecular weights of 32,000 and 38,000 daltons, respectively (Table I). The stability of the 32,000-dalton aggregate under these conditions indicates that ionic forces (noncovalent interactions between polar groups), including salt linkages (14, 15) and hydrogen bonds (16, 17), contribute only slightly to stabilize the 32,000-dalton aggregate. Some involvement of ionic forces is indicated by the molecular weight of 17,000 daltons in 5 *M* NaCl–0.02 *M* phosphate buffer (pH 6.85) and the higher concentration of the 65,000-dalton aggregate than the 32,000-dalton aggregate at pH 2.2 and 4.05 (Table I), a reversal of the relative concentrations of the aggregates that are found in solutions containing 0.2 *M* NaCl.

Stabilization of the aggregate by hydrophobic bonding (18–20) and, in particular, by lipid–lipid hydrophobic bonding (21) is indicated by: (*a*) the ability of the hydrophobic bond-breaking

Table I—Molecular Weights[a] of Diumycin and its Derivatives in Various Solvents

Compound	Concentration, mg./ml.	Solvent	Molecular Weight, daltons Major Component	Minor Component
A, Mixture[b]	10–3	0.2 *M* NaCl–0.02 *M* Na phosphate buffer, pH 6.85 (P[c])	32,000	65,000
A, Mixture	2–0.5	P[c]	32,000	7,000
A, Mixture	0.25	P[c]	1,600	
B	5	90% ethanol–10% P[c]	1,600	
B	5	P[c]	61,000	30,000
Mixture	5	pH 2.2, glycine–HCl buffer, 0.05 *M*	62,000	32,500
Mixture	5	pH 4.05, acetate buffer, 0.1 *M*	65,000	27,000
Mixture	5	pH 10.4, glycine–NaOH buffer, 0.05 *M*	30,200	
Mixture	5	pH 12.4, glycine–NaOH buffer, 0.2 *M*	31,000	
Mixture	5	Isotonic buffer, containing K^+ Na^+, Ca^{2+}, Mg^{2+}, Cl^-, $SO_4^=$, and $PO_4^{\equiv}$	30,800	
Mixture	10	Sodium dodecyl sulfate in P[c]	64,000	
Methyl ester of diumycin A	5	90% ethanol–10% P[c]	1,800	
		P[c]	31,000	
Acetate of diumycin A	5	P[c]	38,000	
Mixture	5	5 *M* NaCl in 0.02 *M* phosphate buffer (pH 6.85)	17,000	
Mixture	5	95% P–5% methanol	34,000	67,000
A, Mixture	5	95% P–5% ethanol	18,000	
Mixture	5	95% P–5% *n*-propanol	9,000	14,000
Mixture	5	95% P–5% *n*-butanol	3,800	
A, Mixture	5	90% ethanol–10% P[c]	1,800	
Mixture	5	5 *M* guanidinium chloride	3,600	1,900
Mixture	5	9 *M* formamide	1,600	
Mixture	5	8 *M* urea	1,900	
Partial hydrolysate of diumycin A (lipidless)	5	90% ethanol–10% P[c]	1,200	400
		P[c]	1,400	

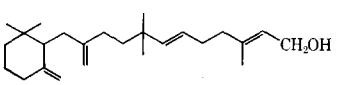
[a] Precision is within 5% accuracy with 15% in low ionic strength solvents. [b] Mixture = diumycin A and diumycin B. [c] P is 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer, pH 6.85.

solvent systems of buffered 8 *M* urea, 9 *M* formamide, or 5 *M* guanidinium chloride to disrupt the aggregate (Table I); (*b*) the inability of a lipidless diumycin to aggregate (Table I); and (*c*) the increasing efficacy of alcohols as disaggregating agents as the length of their hydrocarbon portion increases (Table I). *n*-Butanol is the most effective and methanol is the least effective disaggregating agent, as measured by the apparent molecular weight of diumycin in 95% NaCl–phosphate buffer–5% alcohol (ROH), where R = $-CH_3$, $-CH_2CH_3$, $-CH_2CH_2CH_3$, or $-CH_2CH_2CH_2CH_3$.

The molecular weight of diumycin A (10 mg./ml.) in various mixtures of ethanol and 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer (pH 6.85) is illustrated in Fig. 2. As the concentration of ethanol increases, the molecular weight decreases from 32,000 daltons to 17,000, 10,000, 4000, and finally 2000 daltons. This indicates that: (*a*) disaggregation proceeds by repeated halving of the molecule; (*b*) 16 subunits comprise the 32,000-dalton aggregate; and (*c*) the monomeric concentration term in an equilibrium expression would have an exponent of 16 and 32 for the 32,000-dalton and 65,000-dalton aggregates, respectively, if it is assumed that the law of mass action is valid in these solvent systems.

Table II—Products of Acid Hydrolysis of Diumycin

Glucose
Glucosamine (2 equivalents)
2-Amino-1,3-cyclopentanedione[a]
Acetic acid (2 equivalents)
NH_3 (3 equivalents)
Unknown sugar
H_3PO_4



[3,8,8-trimethyl-11-methylen-12-(2,2-dimethyl-6-methylenecyclohexyl)-2,6-dodecadien-1-ol (diumycinol)]

[a] From diumycin A.

Table II lists the products of the acid hydrolysis of diumycin which includes a 25-carbon lipid (3,8,8-trimethyl-11-methylen-12-(2,2-dimethyl-6-methylenecyclohexyl)-2,6-dodecadien-1-ol) and three hydrophilic sugars. The 32,000-dalton aggregate may be depicted as a core of 16 interacting lipid side chains surrounded by the hydrophilic moieties. This picture is in accord with the X-ray diffraction studies of Luzzati (22), who found that such complex lipids as lysolecithin and phosphatidylethanolamine aggregated in aqueous solution. The aggregate became more spherical as the size of the hydrophilic portion increased.

The 65,000-dalton aggregate may result from the ionic bonding (electrostatic interaction) of two 32,000-dalton aggregates. A less likely interpretation is that a second spherical conformation exists which can accommodate exactly twice the number of lipid side chains found in the 32,000-dalton aggregate.

REFERENCES

(1) E. Meyers, D. S. Slusarchyk, J. L. Bouchard, and F. L. Weisenborn, *J. Antibiot.*, **22**, 490(1969).

(2) L. J. Reed and D. J. Cox, *Ann. Rev. Biochem.*, **35**, 57(1966).

(3) I. M. Klotz and D. W. Darnall, *Science*, **166**, 126(1969).

(4) B. Pullman, "Molecular Association in Biology," Academic, New York, N. Y., 1968.

(5) J. Kirschbaum, *J. Pharm. Sci.*, **57**, 690(1968).

(6) F. L. Weisenborn, W. A. Slusarchyk, and M. Moore, to be published.

(7) B. H. Zimm and D. N. Crothers, *Proc. Natl. Acad. Sci., U. S.*, **48**, 905(1962).

(8) K. Kawahara and C. Tanford, *J. Biol. Chem.*, **241**, 3228 (1966).

(9) H. K. Schachman, "Ultracentrifugation in Biochemistry," Academic, New York, N. Y., 1959, p. 81.

(10) W. J. Archibald, *J. Phys. Colloid Chem.*, **51**, 1204(1947).

(11) J. Engelberg, *Anal. Biochem.*, **6**, 530(1963).

(12) H. K. Schachman, *Methods Enzymol.*, **4**, 32(1957).

(13) C. Tanford, K. Kawahara, and S. Lapanje, *J. Amer. Chem. Soc.*, **89**, 729(1967).
(14) E. Stellwagen and H. K. Schachman, *Biochemistry*, **1**, 1056 (1962).
(15) J. Steinhardt and S. Beychok, in "The Proteins," vol. 2, 2nd ed., H. Neurath, Ed., Academic, New York, N. Y., 1964, p. 287.
(16) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond," Freeman, San Francisco, Calif., 1960.
(17) D. Eisenberg and W. Kauzmann, "The Structure and Properties of Water," Oxford, London, England, 1969, p. 246.
(18) H. A. Scheraga, in "The Proteins," vol. 1, 2nd ed., H. Neurath, Ed., Academic, New York, N. Y., 1963, p. 565.
(19) E. E. Schrier and H. A. Scheraga, *Biochim. Biophys. Acta*, **64**, 406(1962).
(20) W. Kauzmann, *Advan. Protein. Chem.*, **14**, 1(1959).
(21) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p. 421.
(22) V. Luzzati, in "Biological Membranes," Academic, New York, N. Y., 1968, p. 71.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 24, 1969, from *Squibb Institute for Medical Research, New Brunswick, NJ 08903*
Accepted for publication February 3, 1970.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.
The authors thank Dr. K. Florey, Dr. N. Coy, Dr. E. Meyers, and Peter Arnow for helpful suggestions and Dr. David Frost for editorial assistance.

Utilization of a Model Copolymer to Evaluate the Contribution of Hydrophobic Bonding in Drug Binding

JANARDAN B. NAGWEKAR* and HARRY B. KOSTENBAUDER†

Abstract □ A copolymer, having an overall composition of 1 unit of vinylpyridine to 26 units of vinylpyrrolidone, was synthesized. Several alkyl copolymers were made by quaternizing the nitrogen of vinylpyridine units of the copolymer with normal alkyl bromides, ethyl through hexyl. Using an equilibrium dialysis procedure, the interaction of *p*-toluene sulfonic acid sodium with each alkyl copolymer was studied over a temperature range of 15–45°, and the binding constants and thermodynamic parameters were determined. The quaternized vinylpyridine units of the alkyl copolymers constituted the binding sites for the *p*-toluene sulfonate ions. The stability of the complex formed in the reaction increased with an increase in temperature from 15–37° but decreased at 45°. The binding process was found endothermic and associated with positive entropy effects, indicating that the polymeric model system described here demonstrated properties expected for hydrophobic bonding. The effect of urea on the binding of sulfonate ion by one of the alkyl copolymers was studied and found to be in keeping with the claim that urea breaks hydrophobic bond.

Keyphrases □ Vinylpyridine–vinylpyrrolidone copolymers—synthesis □ Equilibrium dialysis—copolymer–*p*-toluene sulfonic acid sodium interaction □ Binding sites—copolymer–*p*-toluene sulfonate ions □ Urea effect—alkyl copolymer binding sulfonate ions □ UV spectrophotometry—identity

During the past 3 decades, several interactions have been reported which involved binding of drugs by plasma proteins; notable examples are penicillins (1), sulfonamides (2–5), methyl orange (6–7), and short- and long-chain fatty acids (8–14). In connection with these binding studies, it has been reported that the interactions primarily take place through ionic forces, but a further contribution to the stability of protein–drug complex is made by the hydrophobic part of a drug molecule. It has also been noticed that the larger the hydrophobic group of a drug molecule, the more stable is the complex. The contributions of hydrophobic groups is attributed to van der Waals forces. However, a close examination of the thermodynamic data has revealed that van der Waals interactions alone cannot account for the stabilizing effect of hydrophobic groups. It is felt that hydrophobic bonding probably plays an important role in drug–protein complexing.

Hydrophobic bonding is a concept introduced by Kauzmann (15), who postulated its thermodynamic properties by extrapolating the behavior of small-size hydrocarbons in an aqueous medium. A hydrophobic bond is defined as the tendency of hydrophobic groups, mainly the hydrocarbons, to adhere to one another in an aqueous solution. The adherence of hydrophobic groups in an aqueous medium is not thought to be merely a manifestation of van der Waals forces; but the structure of water in close proximity to hydrophobic groups is believed to play a significant role, since such adherence processes are accompanied by entropy effects. The concept of hydrophobic bonding was originated to indicate its contribution in stabilizing the folded configuration of globular proteins. On the basis of a physical model, Nemethy and Scheraga (16) have shown that a hydrophobic bond can be formed between two isolated side chains attached to a rigid peptide backbone of protein. Attempts have been made to estimate the thermodynamic contribution of hydrophobic groups to form a drug–protein complex, but the simultaneous contribution of protein molecules due to their "configurational adaptability" obscured such evaluation (17, 18).

It is known that in the event of drug–protein interaction, the thermodynamic activity of a drug in the body is reduced, the biological action of a drug is influenced, and even the metabolism and excretion are hindered. The extent of participation of hydrophobic bonding in exerting such effects is not completely understood. However, one may expect that the concept of hydrophobic bonding can be applied to advan-

(13) C. Tanford, K. Kawahara, and S. Lapanje, *J. Amer. Chem. Soc.*, **89**, 729(1967).
(14) E. Stellwagen and H. K. Schachman, *Biochemistry*, **1**, 1056 (1962).
(15) J. Steinhardt and S. Beychok, in "The Proteins," vol. 2, 2nd ed., H. Neurath, Ed., Academic, New York, N. Y., 1964, p. 287.
(16) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond," Freeman, San Francisco, Calif., 1960.
(17) D. Eisenberg and W. Kauzmann, "The Structure and Properties of Water," Oxford, London, England, 1969, p. 246.
(18) H. A. Scheraga, in "The Proteins," vol. 1, 2nd ed., H. Neurath, Ed., Academic, New York, N. Y., 1963, p. 565.
(19) E. E. Schrier and H. A. Scheraga, *Biochim. Biophys. Acta*, **64**, 406(1962).
(20) W. Kauzmann, *Advan. Protein. Chem.*, **14**, 1(1959).
(21) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p. 421.
(22) V. Luzzati, in "Biological Membranes," Academic, New York, N. Y., 1968, p. 71.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 24, 1969, from *Squibb Institute for Medical Research, New Brunswick, NJ 08903*
Accepted for publication February 3, 1970.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.
The authors thank Dr. K. Florey, Dr. N. Coy, Dr. E. Meyers, and Peter Arnow for helpful suggestions and Dr. David Frost for editorial assistance.

Utilization of a Model Copolymer to Evaluate the Contribution of Hydrophobic Bonding in Drug Binding

JANARDAN B. NAGWEKAR* and HARRY B. KOSTENBAUDER†

Abstract □ A copolymer, having an overall composition of 1 unit of vinylpyridine to 26 units of vinylpyrrolidone, was synthesized. Several alkyl copolymers were made by quaternizing the nitrogen of vinylpyridine units of the copolymer with normal alkyl bromides, ethyl through hexyl. Using an equilibrium dialysis procedure, the interaction of *p*-toluene sulfonic acid sodium with each alkyl copolymer was studied over a temperature range of 15–45°, and the binding constants and thermodynamic parameters were determined. The quaternized vinylpyridine units of the alkyl copolymers constituted the binding sites for the *p*-toluene sulfonate ions. The stability of the complex formed in the reaction increased with an increase in temperature from 15–37° but decreased at 45°. The binding process was found endothermic and associated with positive entropy effects, indicating that the polymeric model system described here demonstrated properties expected for hydrophobic bonding. The effect of urea on the binding of sulfonate ion by one of the alkyl copolymers was studied and found to be in keeping with the claim that urea breaks hydrophobic bond.

Keyphrases □ Vinylpyridine–vinylpyrrolidone copolymers—synthesis □ Equilibrium dialysis—copolymer–*p*-toluene sulfonic acid sodium interaction □ Binding sites—copolymer–*p*-toluene sulfonate ions □ Urea effect—alkyl copolymer binding sulfonate ions □ UV spectrophotometry—identity

During the past 3 decades, several interactions have been reported which involved binding of drugs by plasma proteins; notable examples are penicillins (1), sulfonamides (2–5), methyl orange (6–7), and short- and long-chain fatty acids (8–14). In connection with these binding studies, it has been reported that the interactions primarily take place through ionic forces, but a further contribution to the stability of protein–drug complex is made by the hydrophobic part of a drug molecule. It has also been noticed that the larger the hydrophobic group of a drug molecule, the more stable is the complex. The contributions of hydrophobic groups is attributed to van der Waals forces. However, a close examination of the thermodynamic data has revealed that van der Waals interactions alone cannot account for the stabilizing effect of hydrophobic groups. It is felt that hydrophobic bonding probably plays an important role in drug–protein complexing.

Hydrophobic bonding is a concept introduced by Kauzmann (15), who postulated its thermodynamic properties by extrapolating the behavior of small-size hydrocarbons in an aqueous medium. A hydrophobic bond is defined as the tendency of hydrophobic groups, mainly the hydrocarbons, to adhere to one another in an aqueous solution. The adherence of hydrophobic groups in an aqueous medium is not thought to be merely a manifestation of van der Waals forces; but the structure of water in close proximity to hydrophobic groups is believed to play a significant role, since such adherence processes are accompanied by entropy effects. The concept of hydrophobic bonding was originated to indicate its contribution in stabilizing the folded configuration of globular proteins. On the basis of a physical model, Nemethy and Scheraga (16) have shown that a hydrophobic bond can be formed between two isolated side chains attached to a rigid peptide backbone of protein. Attempts have been made to estimate the thermodynamic contribution of hydrophobic groups to form a drug–protein complex, but the simultaneous contribution of protein molecules due to their "configurational adaptability" obscured such evaluation (17, 18).

It is known that in the event of drug–protein interaction, the thermodynamic activity of a drug in the body is reduced, the biological action of a drug is influenced, and even the metabolism and excretion are hindered. The extent of participation of hydrophobic bonding in exerting such effects is not completely understood. However, one may expect that the concept of hydrophobic bonding can be applied to advan-

tage in drug formulation for purposes of: (*a*) enhancing the stability of certain drugs by complexation with biologically suitable agents capable of forming hydrophobic bonds with drug molecules, and (*b*) manipulating the release rate of drugs incorporated in a sustained-release dosage form. This necessitates extensive understanding of the properties of hydrophobic bonding. Recently, certain model systems have been studied to determine the thermodynamic parameters of hydrophobic bond formation. These model systems consisted of the adsorption of short-chain fatty acids by polystyrene resin at 4° (19), dimerization of small-chain fatty acids at 25° (20), and the interaction of phenol with short-chain fatty acid anions at 25° (21, 22). None of these model systems includes a water-soluble polymeric system containing definite sites for hydrophobic bond formation with suitable small molecules. It was the purpose of this study to devise such a model polymeric system that would make possible the evaluation of the contribution of hydrophobic bonding.

MODEL SYSTEM

The proposed model system consists of *p*-toluene sulfonic acid sodium (PTSAS) salt and a series of relatively simple, water-soluble copolymers containing definite sites for hydrophobic bonding. The interactions of this system are intended to be studied by equilibrium dialysis. Since the model copolymers considered here are not available commercially, they were synthesized in the laboratory. This research project therefore constitutes two distinct phases: (*a*) synthesis and characterization of a model copolymer and its derivatives, and (*b*) binding studies to determine thermodynamic functions of hydrophobic bonding for the model system.

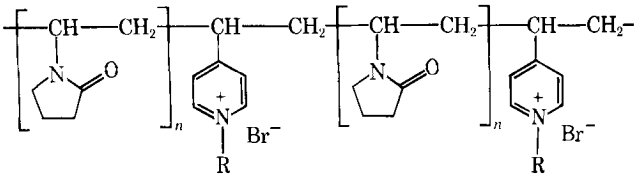
The copolymer is made from two types of monomers, 4-vinylpyridine (hereafter called vinylpyridine) and *N*-vinyl-2-pyrrolidone (hereafter called vinylpyrrolidone); its overall composition is designed to contain about 30 units of vinylpyrrolidone to 1 unit of vinylpyridine. Several alkyl copolymers are prepared by quaternizing the nitrogen of vinylpyridine units of the copolymer with an appropriate *n*-alkyl bromide of a homologous series. The alkyl bromides used are bromoethane through bromohexane. The quaternized units of the copolymer would be expected to constitute the binding sites.

The interactions of PTSAS with each alkyl copolymer were studied at different temperatures using equilibrium dialysis, and the thermodynamic constants were determined. From such data the energy contribution of the methylene groups of the alkyl chain is estimated.

The choice of the model system used was made on the basis of the following considerations. PTSAS is a salt of a strong acid and strong base and will remain completely ionized in aqueous solution. Being an aromatic compound, the complicating feature of micelle formation which occurs with long-chain, aliphatic, water-soluble species (23) can be avoided. Also, PTSAS is amenable to spectrophotometric assay.

Since the phenomenon of hydrophobic bonding has to be studied in aqueous solution, the copolymer employed for this purpose must be water soluble. A copolymer composed mainly of polyvinylpyrrolidone might be expected to be water soluble even when a large fraction of binding sites is occupied. Since both monomers, vinylpyridine and vinylpyrrolidone, are equally reactive, the composition of a copolymer formed should approximate the composition of the feed of reaction (24), which in this case is 1 part of vinylpyridine to 30 parts of vinylpyrrolidone. It may then be reasonably expected that, in this copolymer, any 2 vinylpyridine units would be separated by about 25–35 units of vinylpyrrolidone (Structure I).

It is reasoned that the interaction of negatively charged *p*-toluene sulfonate ions takes place only with positively charged quaternized vinylpyridine units of the copolymer. Thus, the quaternized units represent the binding sites. Electrostatic binding is designed to bring the sulfonate ions sufficiently close to the quaternized units to facilitate hydrophobic bonding taking place between the hydrophobic



I

portion of the sulfonate ion and the alkyl group of a quaternized vinylpyridine unit. [See Structure I for a schematic representation of the structure of a copolymer molecule showing two vinylpyridine units (quaternized with R = ethyl through hexyl bromide) separated by about 26 units (*n*) of vinylpyrrolidone.] The binding energy involved in such interactions is due to two factors, electrostatic binding and hydrophobic bonding. Since all alkyl copolymers are prepared from the same parent copolymer, the charge density of each alkyl copolymer is probably not significantly different, and the energy contribution of electrostatic binding in all cases should remain the same. Therefore, the difference in the binding energy that may be shown in the binding of *p*-toluene sulfonate ion to various alkyl copolymers can be attributed to the difference in the hydrophobicity of alkyl chains attached to the quaternary nitrogen of vinylpyridine units.

The distance between adjacent binding sites on the polymer is such that the binding of a sulfonate ion to one site does not significantly influence the binding of another ion to the next site.

A potential complicating factor in the interpretation of energetics of binding is a possible secondary interaction of the sulfonate ion with the backbone of a copolymer which is primarily composed of polyvinylpyrrolidone. However, the difference in affinity of the anions for the quaternized site and for the copolymer backbone probably would be sufficient to permit isolation of the respective interactions. It is further believed that this tendency of secondary binding will be considerably reduced if the sulfonate ion, like other anions of similar molecular size (23), requires participation of as many as 10 units of vinylpyrrolidone for its binding.

The purpose of studying the system in 5 *M* urea solution was to provide additional means to test the validity of the present model system, since urea is known to weaken hydrophobic bonding (15, 25, 26).

SYNTHESIS AND CHARACTERIZATION OF A MODEL COPOLYMER AND ITS DERIVATIVES

The main characteristics desired of the model copolymer are: (*a*) it should be water soluble; (*b*) its molecular size should be large enough to render it nondiffusible to the cellophane membrane employed during the binding studies; and (*c*) its molecular composition is such as to contain about 30 units of vinylpyrrolidone to 1 unit of vinylpyridine.

Through a series of systematic experiments, the conditions necessary to yield the copolymer of desired properties were determined. Accordingly, 2.7 moles of vinylpyrrolidone and 0.095 mole of vinylpyridine (distilled at 45° under reduced pressure) were dissolved in about 260 g. of alcohol. To this solution was added 7.4 g. of α,α-azobis-isobutyronitrile previously dissolved in 40 g. of acetone. The reaction mixture was contained in a 2.5-l. capacity bottle with a bakelite stopper. Nitrogen gas was bubbled into the reaction mixture for 15 min. and the bottle was stoppered. The copolymerization was carried out with constant stirring at 60° using a heating plate equipped with a magnetic stirrer. When the reaction mixture became quite viscous in about 4 hr., the stirring was discontinued, but the heating was continued at 50° for another 36 hr. The reaction mixture was diluted with about 1600 ml. of acetone and fractionated by adding ether. Three fractions of the copolymer were obtained subsequent to the addition of about 850-, 750-, and 500-ml. quantities of ether, in that order. The second fraction was collected, dissolved in 2.5 l. of distilled water, filled into several cellophane tubes, and placed in a tank containing 8 gal. of distilled water for dialysis. The water in the bath was being constantly stirred and was replaced with freshly distilled water every 24 hr. Dialysis was continued for 4 days until no copolymer was detected in the water outside the tubes. The tubes were removed from the bath, and the contents were concentrated by blowing warm air over the bags. The concentrated copolymer was dissolved in a

mixed solvent containing 1230 ml. of acetone and 150 ml. of ethanol. The copolymer was precipitated with 1500 ml. of ether, which was added to the copolymer solution in 100-ml. portions with constant stirring. The viscous mass thus obtained was separated by decantation of ether. The polymer phase was further precipitated by adding to it 500 ml. of ether with constant stirring. The ether layer was decanted. The copolymer precipitate was then washed twice with ether, using 500 ml. of ether each time. The copolymer was dried at 60° under reduced pressure.

Analysis of Copolymer—Polyvinylpyrrolidone, when present in an acidic medium, does not absorb in the UV region. However, the copolymer shows a characteristic absorption maximum at 254 mμ. Therefore, it was necessary to synthesize polyvinylpyridine, which would serve as a reference compound for the quantitative determination of the vinylpyridine content in a known amount of copolymer by a spectrophotometric method. Polyvinylpyridine was synthesized by polymerizing vinylpyridine (freshly distilled at 45° under reduced pressure) using the conditions similar to those used for the synthesis of the copolymer. A linear relationship observed between the concentration of polyvinylpyridine and absorbance is shown in Fig. 1*a*. This offered an effective analytical tool to quantitate the amount of vinylpyridine present in a given quantity of copolymer. The Beer plot obtained for the copolymer is shown in Fig. 1*b*. With reference to the standard plot (Fig. 1*a*), it was calculated that the copolymer contained 26 units of vinylpyrrolidone to 1 unit of vinylpyridine. For the sake of convenience, the composition of the copolymer may be expressed as 1:26.

Alkyl Copolymers—Twenty grams of copolymer (1:26) was dissolved in 120 g. of nitromethane, and an appropriate quantity of alkyl bromide was added. The quantity of each alkyl bromide, from 1-bromoethane through 1-bromohexane, used was 16, 18, 30, 34, and 40 g., respectively. The reaction was carried out at 70° with constant stirring for 4 days in a closed glass container. The reaction mixture was brought to room temperature and poured in a thin stream within 15 min. into 1 l. of ether to precipitate the alkyl copolymer. The mixture was constantly stirred during the precipitation. The ether was decanted and the precipitate was dissolved in a mixture of 200 ml. of acetone and 20 ml. of ethyl alcohol. The solution was added to 500 ml. of ether to precipitate the alkyl copolymer. The ether was decanted, and the alkyl copolymer thus obtained was washed three times with 200-ml. portions of ether and dried at 50° under reduced pressure.

Assay of Alkyl Copolymer—The bromide content of the quaternized copolymer was quantitatively determined by a potentiometric titration method using 0.01 *N* silver nitrate solution as a titrant (27). From knowledge of the bromide content in a given quantity of alkyl copolymer, the proportion of quaternized units of vinylpyridine was calculated.

The composition of each alkyl copolymer is expressed in terms of units of vinylpyrrolidone present to 1 quaternized unit of vinylpyridine. The composition of various alkyl copolymers is recorded in Table I. In those cases where the alkyl copolymers are shown to contain more than 26 units of vinylpyrrolidone to 1 unit of vinylpyridine, all the vinylpyridine units were not quaternized by the respective alkyl bromide. However, it was experimentally demonstrated that the binding sites are represented solely by the quaternized units of vinylpyridine. Since the difference between the molecular weight of vinylpyridine (mol. wt. 105) and that of vinylpyrrolidone (mol. wt. 111) is small, and since the unquaternized vinylpyridine unit does not constitute a binding site for the sulfonate ion, it is not unreasonable to equate an unquaternized vinylpyridine unit to a vinylpyrrolidone unit while determining the composition of the alkyl copolymers in terms of units of vinylpyrrolidone present to 1 quaternized unit of vinylpyridine, as shown in Table I.

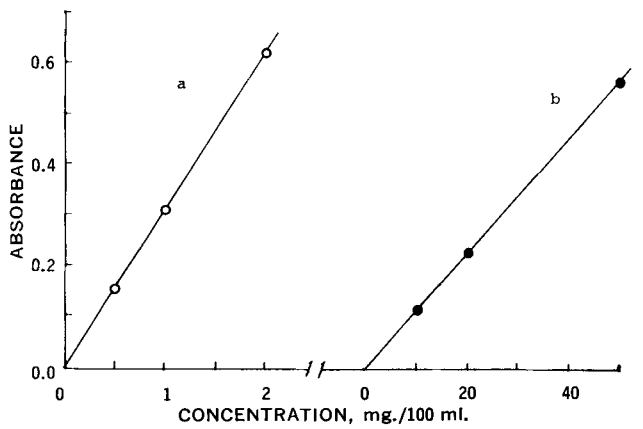


Figure 1—*The Beer plots of absorbance against concentration of synthesized polyvinylpyridine (a) and 1:26 copolymer (b) in diluted hydrochloric acid at 254 mμ.*

Table I—Composition of the Alkyl Copolymers Expressed in Terms of Number of Vinylpyrrolidone Units to One Quaternized Vinylpyridine Unit

Copolymer	Composition
Ethyl	1:26
Propyl	1:29
Butyl	1:29
Pentyl	1:30
Hexyl	1:27

Copolymers of composition 1:20 and 1:34 were also synthesized, and only the hexyl derivatives were prepared.

EXPERIMENTAL

Materials—Ethyl copolymer (1:26), propyl copolymer (1:29), butyl copolymer (1:29), pentyl copolymer (1:30), and hexyl copolymer (1:20, 1:27, and 1:34) were synthesized in the laboratory. *p*-Toluene sulfonic acid sodium salt[1] was recrystallized from acetone–water solution. Sodium chloride (reagent grade); urea USP; and cellophane membrane[2] were used.

Solution of Alkyl Copolymer—A 2% (w/v) solution of each alkyl copolymer was prepared in 0.1 *M* sodium chloride solution (distilled water). A 2% (w/v) solution of pentyl copolymer was also prepared in 5 *M* urea.

Solution of *p*-Toluene Sulfonic Acid Sodium (PTSAS) Salt—The 0.01, 0.015, 0.02, 0.025, and 0.03 *M* solutions of PTSAS were prepared in 0.1 *M* sodium chloride solution. The ionic strength of each solution was adjusted to 0.13 with NaCl. Similar solutions were also prepared with 5 *M* urea.

Dialysis Procedure—Each dialysis cell consisted of two Plexiglas blocks, 6.3 × 6.3 × 2.6 cm., each half with a cavity having a capacity of 20-ml. Threaded Plexiglas plugs provided access to the cell cavities. To assemble the cells, a cellophane membrane previously freed of water-soluble material was clamped between the two symmetrical halves. Solutions were pipeted into each cavity as required; the stoppers, fitted with polyvinyl chloride washers, were screwed in tightly. The cells were then rotated at 9 r.p.m. in a water bath maintained at 15, 22, 30, 37, or 45 ± 0.2°. The system attained equilibrium within 44 hr. The cells were then removed from the bath, and the contents from the nonpolymer side of each cell were immediately transferred to a 20-ml. container provided with a cap. After the solution attained room temperature, each solution was analyzed for PTSAS by a spectrophotometric method.

Assay Method—PTSAS was assayed with a Beckman DU spectrophotometer at 261 mμ. Two milliliters of the sample solution was diluted to 50 ml. with distilled water and its absorbance noted using water as a blank. To correct for any spectral contribution of trace quantities of diffused macromolecule, dialysis cells were set up containing the solution of an appropriate alkyl copolymer on one side of the membrane and 0.13 *M* sodium chloride solution on the other side.

RESULTS

To ascertain that only the quaternized vinylpyridine units of an alkyl copolymer represented the binding sites, the interaction of 0.02 *M* PTSAS with each of three hexyl copolymers of composition 1:20, 1:27, and 1:34 was studied. The concentration of each hexyl copolymer was expressed in terms of its hexyl-4-vinylpyridinium

[1] Eastman Organic Chemicals, Rochester, N. Y.
[2] Visking Cellulose Casing, Viscing Corp., Chicago, Ill.

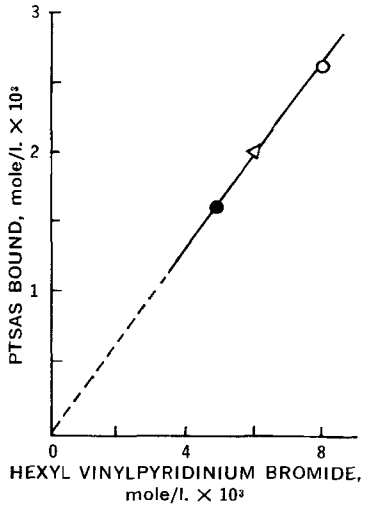


Figure 2—*Binding of 0.02 M PTSAS by hexyl copolymers of three different compositions, 1:34, ●; 1:27, △; and 1:20, ○; at room temperature.*

bromide content. (This practice of expressing concentration was also adopted with other alkyl copolymers.) A plot of concentration of hexyl copolymer *versus* moles of bound PTSAS was made as shown in Fig. 2. The linear plot obtained clearly indicates that the binding of sulfonate ions by the alkyl copolymer is a function of the concentration of quaternized vinylpyridine units of the latter.

The binding data obtained at various temperatures for each alkyl copolymer derivative were treated according to Eq. 1 (6):

$$1/r = 1/nKa + 1/n \qquad \text{(Eq. 1)}$$

where r is the number of moles of PTSAS bound per mole of an alkyl copolymer, n is the number of binding sites per mole of the alkyl copolymer, K is the binding constant (liter/mole), and a is the molar concentration of free PTSAS at equilibrium. From the slope ($1/nK$) and intercept ($1/n$), the binding constant, K, was evaluated. The straight lines of the plots were obtained by the method of least squares. Typical Langmuir plots obtained in the studies are shown in Fig. 3. The binding constants are listed in Table II.

The resulting straight lines indicate that each sulfonate anion binds to an identical group present in the alkyl copolymer and that the electrostatic repulsion between sulfonate ion already bound to one site and that bound to the next binding site is negligible.

It was noticed that in none of the cases was the intercept of Fig. 3 equal to unity, indicating that all the binding sites of an alkyl copolymer are not available for interaction with the sulfonate ions. It is likely that a few binding sites remain buried within the macromolecule, because the positively charged binding sites are so widely spaced that absolute rigidity of the macromolecule cannot be attained to prevent it from coiling completely. It also appears that the extent of availability of the binding sites partly depends on temperature, since out of every 10 binding sites, 7 to 8 sites at 15° and 22°, 8 to 9 binding sites at 30° and 45°, and 6 to 7 binding sites at 37° are available for the interaction. Although the number of available binding sites was not the same at all the temperatures

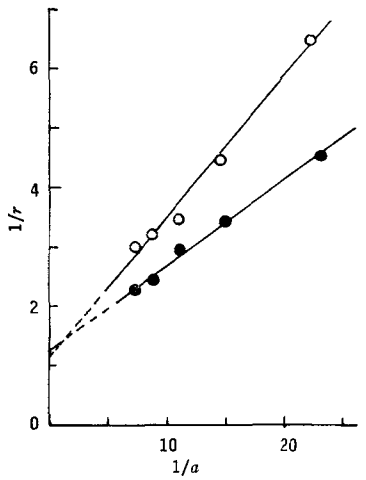


Figure 3 — *Typical Langmuir plots obtained for binding of PTSAS by ethyl copolymer, ○; and hexyl copolymer, ●; at 30°.*

Table II—Binding Constants for the Interaction of PTSAS with Various Alkyl Copolymers over the Temperature Range of 15–45°

Copolymer	Binding Constant, K, l./mole				
	15°	22°	30°	37°	45°
Ethyl	46.00	—	46.66	50.00	32.70
Propyl	61.00	—	85.70	102.14	61.30
Butyl	52.84	54.56	55.00	63.28	42.56
Pentyl	62.80	—	111.00	127.20	58.00
Hexyl	63.36	85.84	94.80	108.08	68.60

studied, the number of binding sites available at any one particular temperature was essentially the same for all the alkyl copolymers.

Thermodynamic Functions—From the data in Table II (excluding the data obtained at 45°) a plot of log K *versus* $1/T$ was made (Fig. 4). The linear curves (least squares) obtained indicated that over the range 15–37° the enthalpy of binding is constant. The data obtained at 45° are discussed later. From the slope ($-\Delta H/2.303$) of the linear curve, the enthalpy of binding, ΔH, was calculated. The free energy of binding, ΔF, was calculated from the following relationship:

$$\Delta F = -RT \ln K \qquad \text{(Eq. 2)}$$

Finally, the entropy of binding, ΔS, was calculated from the following expression:

$$\Delta S = \frac{\Delta H - \Delta F}{T} \qquad \text{(Eq. 3)}$$

The thermodynamic constants obtained for the alkyl copolymers are listed in Table III.

When the corresponding binding constant, K, is plotted against the alkyl copolymer of the ascending homologous series, an ascending zigzag curve is obtained (Fig. 5). The binding constants of the alkyl copolymers with an odd number of carbon atoms in the alkyl chain are higher than those of the alkyl copolymers with the next

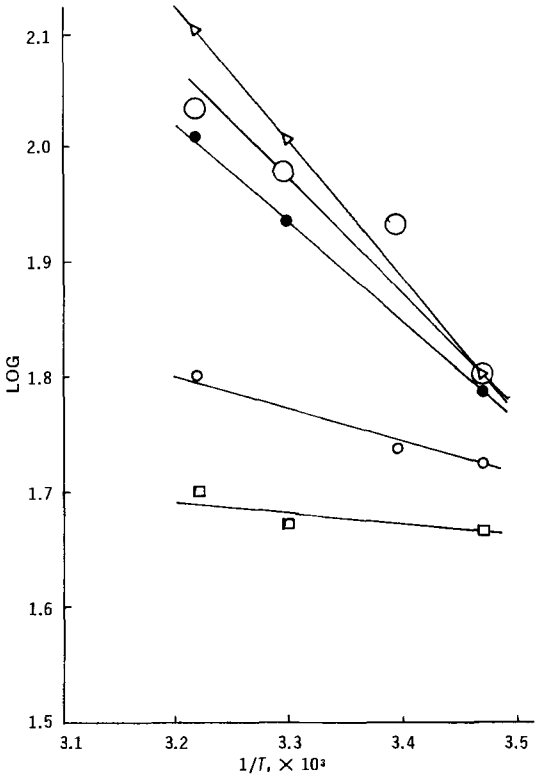


Figure 4—*Plots of logarithm of binding constant* vs. *reciprocal of absolute temperature for the interaction of PTSAS with ethyl copolymer, □; propyl copolymer, ○; butyl copolymer, ●; pentyl copolymer, △; and hexyl copolymer, ○; over the temperature range of 15–37°.*

Table III—Thermodynamic Parameters of Binding for the Interaction of PTSAS with Various Alkyl Copolymers

Copolymer	ΔF, cal./mole 15°	22°	30°	37°	45°	ΔH, cal./mole Temp. Range 15–37°	ΔS, e.u. Temp. Range 15–37°
Ethyl	2193	—	2316	2414	2205	458	9.21
Propyl	2354	—	2683	2853	2602	4068	22.32
Butyl	2272	2345	2430	2558	2372	1373	12.64
Pentyl	2372	—	2837	2987	2567	5611	27.77
Hexyl	2377	2613	2743	2888	2674	4728	24.41

Table IV—Binding Constants and Thermodynamic Parameters of Binding for the Interaction between PTSAS and Pentyl Copolymer in the Absence and in the Presence of 5 *M* Urea over the Temperature Range of 15–37°

5 *M* Urea	K, l./mole 15°	30°	37°	ΔF, cal./mole 15°	30°	37°	ΔH, cal./mole	ΔS, e.u.
Absent	62.8	111.0	127.2	2372	2837	2987	5611	27.77
Present	29.2	40.0	44.0	1932	2221	2331	3433	18.62

even number of carbon atoms. This zigzag pattern is seen to exist at all the temperatures studied. Consequently, the values of thermodynamic functions determined for the series of alkyl copolymers also reflect the same pattern. However, the values of the binding constants, and for that matter those of the thermodynamic functions, increase with increasing alkyl chain length when a series of alkyl copolymers, containing an odd or even number of carbon atoms in the alkyl chain, is considered.

From the comparative data presented in Table IV and Fig. 6, it is clearly seen that the binding of the sulfonate ion by the pentyl copolymer has been influenced very significantly in the presence of 5 *M* urea. It is also noticed that the binding of the sulfonate ion by the pentyl copolymer increased with increase in temperature from 15–37°. The plot of log K against $1/T$ (Fig. 7) gives a straight line (least squares), which again suggests that the enthalpy of binding for the system is constant over this temperature range. In the presence of 5 *M* urea, the free energy of binding for this system became less negative by 400 cal./mole at 15°, 616 cal./mole at 30°, and 556 cal./mole at 37°. Similarly, the enthalpy of binding became less positive by 2200 cal./mole and the positive entropy of binding reduced by about 9 entropy units.

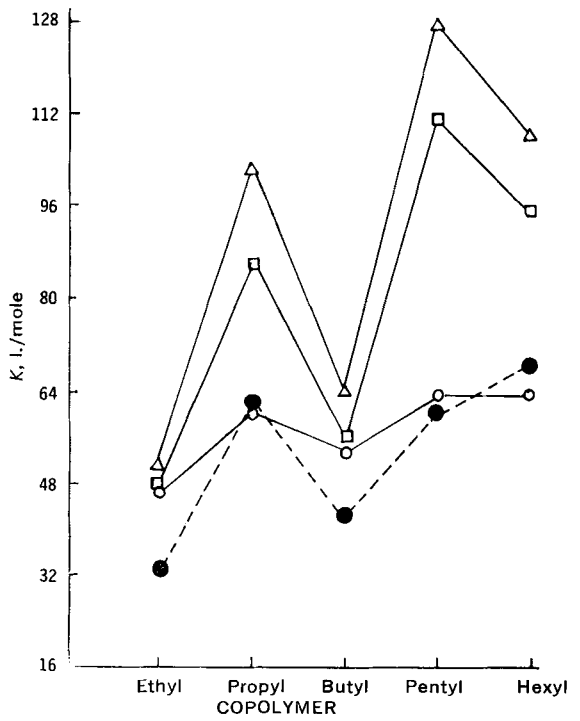


Figure 5—*Plots of binding constant* vs. *alkyl copolymer at 15°, ○; 30°, □; 37°, △; and 45°, ●.*

Another feature that becomes discernible is that the number of binding sites, which remain available on the pentyl copolymer molecule for binding of the sulfonate ions, is practically the same whether or not urea is present in the system. This may be noted by visual inspection of the intercepts of the plots in Fig. 6. This feature may serve to indicate that the intrinsic nature and behavior of the alkyl copolymers are not significantly modified in the presence of 5 *M* urea.

DISCUSSION

An examination of the data shows that the stability of the complex formed between an alkyl copolymer and the sulfonate ion increased with increasing temperature over the range 15–37° and that the binding process was endothermic. More importantly, the binding was associated with an increase in entropy, indicating that the model system demonstrates thermodynamic behavior in common with that described for hydrophobic bonding.

From the data of the composition of alkyl copolymers, it can be seen that the charge density of each alkyl copolymer is not significantly different. Therefore, the energy contribution of electrostatic binding of each alkyl copolymer is considered the same. The difference shown by these alkyl copolymers in the values of their respective thermodynamic constants can be attributed to the contribution of the methylene, $=CH_2$, groups in the alkyl chains.

Despite the fact that the enthalpy of binding, which may be called enthalpy of hydrophobic bonding, was positive and therefore unfavorable, the bond formation was strengthened at relatively higher temperature. The free energy required to strengthen hydrophobic bond was derived from the substantial gain in entropy during the binding process. In aqueous solution, according to the concept of iceberg formation (28), the hydrocarbon groups of alkyl copolymers and *p*-toluene sulfonate ion are considered to be surrounded by one or more layers of water molecules, which are highly ordered with better hydrogen bonding than the molecules in ordinary liquid water. This brings about a lowering of configura-

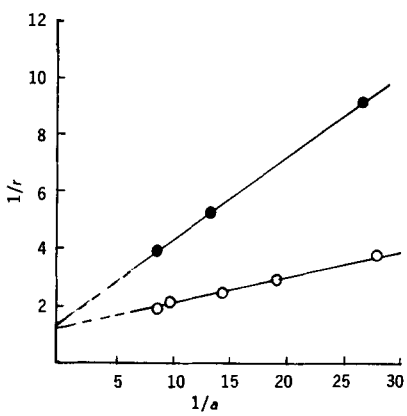


Figure 6—*Comparison of Langmuir plots for binding of PTSAS by pentyl copolymer in the absence of urea, ○, and in the presence of 5 M urea, ●, at 30°.*

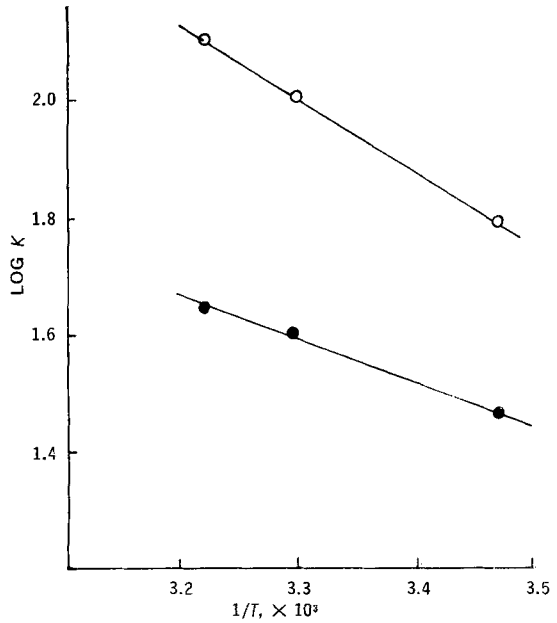


Figure 7—*Comparison of plots of logarithm of binding constant vs. reciprocal of absolute temperature for the binding of PTSAS by pentyl copolymer in the absence of urea, ○, and in the presence of 5 M urea, ●.*

tional entropy of the hydrocarbon groups. There is also a true hydration about the sulfonate group of the anion and the quaternary nitrogen of the vinylpyridinium groups of the alkyl copolymer. When binding occurs between the reactants concerned, the icebergs become either less ordered or contain fewer water molecules. As a result, the water molecules are released from the ordered structure, producing proportional increase in entropy due to the release of the configurational entropy of the hydrocarbon groups of the reactants.

Although the hydrophobic bonding might be expected to become continually stronger with a rise in temperature, there is a limit of maximum temperature for iceberg structure (16). The limit of maximum temperature depends upon the aliphatic or aromatic nature of the hydrophobic group. Since the π-electron orbitals of aromatic hydrocarbon react with the highly hydrogen-bonded water species, the extent of partial "icecage" formed around the aromatic hydrocarbon is smaller than that formed around the aliphatic hydrocarbon (29). Consequently, the lowering of configurational entropy of the aromatic hydrocarbon group is less than that of the aliphatic hydrocarbon groups. Nemethy and Scheraga (16) have theorized that, when the interaction takes place between the aliphatic side chains, the hydrophobic bond becomes stronger up to 58°; but when the interaction takes place between aromatic hydrocarbons, the hydrophobic bond becomes stronger up to 42°. According to this theory, the lowering of configurational entropy of the phenyl group would be negligible at 45°. Therefore, in the present system, when binding takes place at 45°, the contribution to the total entropy gain by the phenyl group of the sulfonate ion due to the release of its configurational entropy would be negligible. In view of this theory, it is conceivable that the decreased stability of the hydrophobic bond in the model system at 45° is due to the possible breakdown of the partial ice cage around the phenyl group of the sulfonate ion (Table III).

As shown in Fig. 5, the zigzag pattern exhibited in the values of the binding constants is intriguing. This pattern points to the possible role the number of contacts or closeness (established between the interacting hydrophobic groups within the Van der Waals radii) plays to determine the strength of hydrophobic bond (29). If the concept that the carbon atoms of the alkyl chain assume an extended zigzag arrangement in the liquid state (30) is extended to explain the zigzag pattern observed in Fig. 5, it seems probable that the terminal methyl group of the propyl and pentyl chain (containing odd numbers of carbon atoms) orient much closer to the phenyl group of the target sulfonate ion than the terminal methyl group of the butyl and hexyl chain (containing even numbers of carbon atoms).

Also, in view of the fact that in a normal hydrocarbon the angle formed by the two consecutive C—C bonds is approximately 110° and the C—C bond length is 1.54 Å (31), it can be estimated that the terminal methyl group of odd carbon alkyl chain, as compared to that of the even carbon alkyl chain, would be closer to the aromatic ring of the sulfonate ion by a geometric distance of about 0.88 Å. It is, therefore, conceivable that more water molecules are released by complexing the propyl group with the sulfonate ion than by complexing the butyl group, thereby resulting in substantial gain in entropy. Similarly, the pentyl group forms a more stable complex with the sulfonate ion than does the hexyl group.

It is seen that the increment in the binding energy with the increase of two methylene groups in an alkyl chain is not uniform and depends on the temperature of the interaction. The increment of the binding energy varied from −18 to −105 cal. at 15°, −97 to −329 cal. at 30°, and −134 to −330 cal. at 37°, depending on what two alkyl chains were considered. Similar nonuniformity in the increment of free energy has been reported by Nemethy and Scheraga (16) even on the basis of a physical model. Such nonuniformity may be attributed to the fact that the stability of hydrophobic bond is governed by several factors, such as the number of contacts and the closeness of contact between the interacting hydrophobic groups, the structure of iceberg, and the extent of partial ice cage around the hydrocarbons involved. A significant variance in the iceberg structure around the alkyl groups of various alkyl copolymers can be expected, since a variance in the iceberg structure around the lower hydrocarbons, methane, ethane, propane, and butane, has been reported by Clausen and Polgase (32) from solubility studies in water. They also observed a nonuniformity in the decrease of entropy values for the solubility of these hydrocarbons of a homologous series. The decrease in entropy with the additional $-CH_2-$ group from methane to butane was 1.4, 6.4, and 1.0 e.u., respectively.

The effect of urea on the binding of sulfonate ion by the pentyl copolymer is in keeping with the claim that urea breaks hydrophobic bond. From Fig. 7 it is noted, however, that the thermodynamic behavior of the present model system in the presence of 5 *M* urea remained qualitatively the same. This suggests that the iceberg structure in the immediate vicinity of the alkyl chains of the alkyl copolymers and phenyl group of the sulfonate ion is not completely disrupted but has been significantly modified by the urea molecules, which are known to undergo hydrogen bonding with water.

REFERENCES

(1) R. Tompsett, S. Shultz, and W. McDermott, *J. Bacteriol.*, **53,** 581(1947).

(2) B. D. Davis, *Science*, **95,** 78(1942).

(3) B. D. Davis, *J. Clin. Invest.*, **22,** 753(1943).

(4) D. R. Gilligan, *J. Pharmacol. Exp. Ther.*, **79,** 320(1943).

(5) I. M. Klotz and F. M. Walker, *J. Amer. Chem. Soc.*, **70,** 943(1948).

(6) I. M. Klotz, F. M. Walker, and R. B. Pivan, *ibid.*, **68,** 1486 (1946).

(7) I. M. Klotz and J. M. Urquhart, *ibid.*, **71,** 847(1949).

(8) G. A. Ballou, P. D. Boyer, J. M. Luck, and F. G. Lum, *J. Biochem.*, **153,** 589(1944).

(9) G. A. Ballou, P. D. Boyer, and J. M. Luck, *ibid.*, **159,** 111 (1945).

(10) P. D. Boyer, F. G. Lum, G. A. Ballou, J. M. Luck, and R. G. Rice, *ibid.*, **162,** 181(1946).

(11) P. D. Boyer, G. A. Ballou, and J. M. Luck, *ibid.* **167,** 407 (1947).

(12) J. D. Teresi and J. M. Luck, *J. Biol. Chem.*, **194,** 823(1952).

(13) D. S. Goodman, *J. Amer. Chem. Soc.*, **80,** 3892(1958).

(14) A. A. Spector, K. John, and J. E. Fletcher, *J. Lipid Res.*, **10,** 56(1969).

(15) W. Kauzmann, *Advan. Protein Chem.*, **14,** 1(1959).

(16) G. Nemethy and H. A. Scheraga, *J. Phys. Chem.*, **66,** 1773 (1962).

(17) F. Karush, *ibid.*, **56,** 70(1952).

(18) R. Loverin, *J. Amer. Chem. Soc.*, **85,** 3677(1963).

(19) H. Schneider, G. C. Kresheck, and H. A. Scheraga, *J. Phys. Chem.*, **69,** 1310(1965).

(20) E. E. Schrier, M. Pottle, and H. A. Scheraga, *J. Amer. Chem. Soc.*, **86,** 3444(1964).

(21) Y. Moon, D. C. Poland, and H. A. Scheraga, *J. Phys. Chem.*, **68**, 2960(1964).
(22) D. K. Kunimitsu, Y. A. Woody, E. R. Stimson, and H. A. Scheraga, *ibid.*, **72**, 856(1968).
(23) P. Molyneux and H. P. Frank, *J. Amer. Chem. Soc.*, **83**, 3169(1961).
(24) H. Mark and A. V. Tabolsky, "Physical Chemistry of Polymeric Systems," 2nd ed., 1950, p. 404.
(25) C. Tanford and K. DeParitosh, *J. Biol. Chem.*, **236**, 1711 (1961).
(26) P. Mukerjee and A. Ray, *J. Phys. Chem.*, **67**, 190(1963).
(27) U. P. Straus and N. L. Gershfield, *ibid.*, **58**, 747(1954).
(28) H. S. Frank and M. W. Evans, *J. Chem. Phys.*, **13**, 507 (1945).
(29) G. Nemethy and H. A. Scheraga, *ibid.*, **36**, 3401(1962).
(30) H. D. Anderson and D. L. Hammick, *J. Chem. Soc.*, **1950**, 1089.
(31) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Holt, Rinehart, and Winston, New York, N. Y., 1960, p. 34.
(32) W. F. Claussen and M. F. Polgase, *J. Amer. Chem. Soc.*, **74**, 4817(1952).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 22, 1969, from the *School of Pharmacy, Temple University, Philadelphia, PA 19140* and the *College of Pharmacy, Wayne State University, Detroit, MI 48202*

Accepted for publication February 9, 1970.

Abstracted in part from the first prize winning manuscript submitted by Janardan B. Nagwekar to the Lunsford Richardson Pharmacy Award Competition, Eastern Region of the United States, 1965.

This study was supported in part by Public Health Service Research Grant AM 04096-03, National Institute of Arthritis and Metabolic Diseases.

* Present address: College of Pharmacy, Wayne State University, Detroit, MI 48202

† Present address: College of Pharmacy, University of Kentucky, Lexington, KY 40506

Determination of Phenoxymethyl Penicilloic Acid and Phenoxyethyl Penicilloic Acid in Urine in the Presence of the Parent Penicillins

JAN BIRNER

Abstract □ Penicillin and the corresponding penicilloic acid are extracted by chloroform from an acidified sample of urine which has been partly saturated by ammonium sulfate. A portion of the extract is dried and redissolved in a small volume of acetone. A measured aliquot is chromatographed on a silica gel thin-layer plate by acetone–acetic acid (19:1). The separated penicilloic acid is detected by starch–iodine spraying. The zones are transferred into glass tubes, nitrated, and neutralized by ammonia. The absorbance of the yellow supernatant, which is proportional to the concentration of penicilloic acid, is measured spectrophotometrically.

Keyphrases □ Penicillin, penicilloic acid in urine—penicilloic acid determination □ Phenoxymethyl penicilloic acid—determination in urine □ Phenoxyethyl penicilloic acid—determination in urine □ TLC—analysis □ Starch–iodine spray—TLC spot identification □ Colorimetric analysis—spectrophotometer

Due to their instability in solution, penicillins taken orally or administered parenterally undergo chemical changes, and the breakdown products are excreted from the body in the urine.

Walkenstein *et al.* (1) found that between 30 and 60% penicillin G in urine remains biologically active, the major degradation product being penicilloic acid. The data were obtained by comparison of radioassays and bioassays of urine. Penicillins can be estimated directly by several methods such as iodometric, hydroxylamine, or biological, but penicilloic acid in the presence of penicillin is usually found by difference. Pan (2) gives a method for the determination of penicilloic acid in penicillin G fermentation broth, which is based on extraction at different pH values and subsequent colorimetric determination. Phenoxymethyl penicillin (penicillin V) and phenoxymethyl penicilloic acid can be separated by paper chromatography; Roehr (3) gives R_f values in four different systems.

McGilveray and Strickland (4) use TLC for identification of about 10 penicillins in four systems.

It was observed that using silica gel G and acetone–acetic acid (19:1) in TLC penicillin V and phenoxyethyl penicillin (phenethicillin) can be separated from the corresponding penicilloic acids. The separated penicilloic acids, containing a phenolic group in the side chain, may be determined subsequently by a colorimetric method (5) by nitration, neutralization by ammonia, and spectrophotometric measurement. Based on this observation, a method has been developed that permits direct determination of penicilloic acid, in the presence of penicillin, in urine.

The sample, partially saturated by ammonium sulfate, is extracted by chloroform at pH 2, using a 1:5 ratio of water to chloroform. The chloroform extract is dried and redissolved in acetone, and an aliquot is taken for chromatography on silica gel using acetone–acetic acid (19:1). The zones of separated penicilloic acid, located by starch–iodine spraying, are transferred into glass tubes and nitrated in the presence of the silica to form quinoid radicals, which are yellow in color in the presence of ammonia. The silica, which does not interfere in this process, is separated by centrifugation, and the supernatant is used for spectrophotometric measurements.

(21) Y. Moon, D. C. Poland, and H. A. Scheraga, *J. Phys. Chem.*, **68**, 2960(1964).
(22) D. K. Kunimitsu, Y. A. Woody, E. R. Stimson, and H. A. Scheraga, *ibid.*, **72**, 856(1968).
(23) P. Molyneux and H. P. Frank, *J. Amer. Chem. Soc.*, **83**, 3169(1961).
(24) H. Mark and A. V. Tabolsky, "Physical Chemistry of Polymeric Systems," 2nd ed., 1950, p. 404.
(25) C. Tanford and K. DeParitosh, *J. Biol. Chem.*, **236**, 1711 (1961).
(26) P. Mukerjee and A. Ray, *J. Phys. Chem.*, **67**, 190(1963).
(27) U. P. Straus and N. L. Gershfield, *ibid.*, **58**, 747(1954).
(28) H. S. Frank and M. W. Evans, *J. Chem. Phys.*, **13**, 507 (1945).
(29) G. Nemethy and H. A. Scheraga, *ibid.*, **36**, 3401(1962).
(30) H. D. Anderson and D. L. Hammick, *J. Chem. Soc.*, **1950**, 1089.
(31) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Holt, Rinehart, and Winston, New York, N. Y., 1960, p. 34.
(32) W. F. Claussen and M. F. Polgase, *J. Amer. Chem. Soc.*, **74**, 4817(1952).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 22, 1969, from the *School of Pharmacy, Temple University, Philadelphia, PA 19140* and the *College of Pharmacy, Wayne State University, Detroit, MI 48202*

Accepted for publication February 9, 1970.

Abstracted in part from the first prize winning manuscript submitted by Janardan B. Nagwekar to the Lunsford Richardson Pharmacy Award Competition, Eastern Region of the United States, 1965.

This study was supported in part by Public Health Service Research Grant AM 04096-03, National Institute of Arthritis and Metabolic Diseases.

* Present address: College of Pharmacy, Wayne State University, Detroit, MI 48202

† Present address: College of Pharmacy, University of Kentucky, Lexington, KY 40506

Determination of Phenoxymethyl Penicilloic Acid and Phenoxyethyl Penicilloic Acid in Urine in the Presence of the Parent Penicillins

JAN BIRNER

Abstract □ Penicillin and the corresponding penicilloic acid are extracted by chloroform from an acidified sample of urine which has been partly saturated by ammonium sulfate. A portion of the extract is dried and redissolved in a small volume of acetone. A measured aliquot is chromatographed on a silica gel thin-layer plate by acetone–acetic acid (19:1). The separated penicilloic acid is detected by starch–iodine spraying. The zones are transferred into glass tubes, nitrated, and neutralized by ammonia. The absorbance of the yellow supernatant, which is proportional to the concentration of penicilloic acid, is measured spectrophotometrically.

Keyphrases □ Penicillin, penicilloic acid in urine—penicilloic acid determination □ Phenoxymethyl penicilloic acid—determination in urine □ Phenoxyethyl penicilloic acid—determination in urine □ TLC—analysis □ Starch–iodine spray—TLC spot identification □ Colorimetric analysis—spectrophotometer

Due to their instability in solution, penicillins taken orally or administered parenterally undergo chemical changes, and the breakdown products are excreted from the body in the urine.

Walkenstein *et al.* (1) found that between 30 and 60% penicillin G in urine remains biologically active, the major degradation product being penicilloic acid. The data were obtained by comparison of radioassays and bioassays of urine. Penicillins can be estimated directly by several methods such as iodometric, hydroxylamine, or biological, but penicilloic acid in the presence of penicillin is usually found by difference. Pan (2) gives a method for the determination of penicilloic acid in penicillin G fermentation broth, which is based on extraction at different pH values and subsequent colorimetric determination. Phenoxymethyl penicillin (penicillin V) and phenoxymethyl penicilloic acid can be separated by paper chromatography; Roehr (3) gives R_f values in four different systems.

McGilveray and Strickland (4) use TLC for identification of about 10 penicillins in four systems.

It was observed that using silica gel G and acetone–acetic acid (19:1) in TLC penicillin V and phenoxyethyl penicillin (phenethicillin) can be separated from the corresponding penicilloic acids. The separated penicilloic acids, containing a phenolic group in the side chain, may be determined subsequently by a colorimetric method (5) by nitration, neutralization by ammonia, and spectrophotometric measurement. Based on this observation, a method has been developed that permits direct determination of penicilloic acid, in the presence of penicillin, in urine.

The sample, partially saturated by ammonium sulfate, is extracted by chloroform at pH 2, using a 1:5 ratio of water to chloroform. The chloroform extract is dried and redissolved in acetone, and an aliquot is taken for chromatography on silica gel using acetone–acetic acid (19:1). The zones of separated penicilloic acid, located by starch–iodine spraying, are transferred into glass tubes and nitrated in the presence of the silica to form quinoid radicals, which are yellow in color in the presence of ammonia. The silica, which does not interfere in this process, is separated by centrifugation, and the supernatant is used for spectrophotometric measurements.

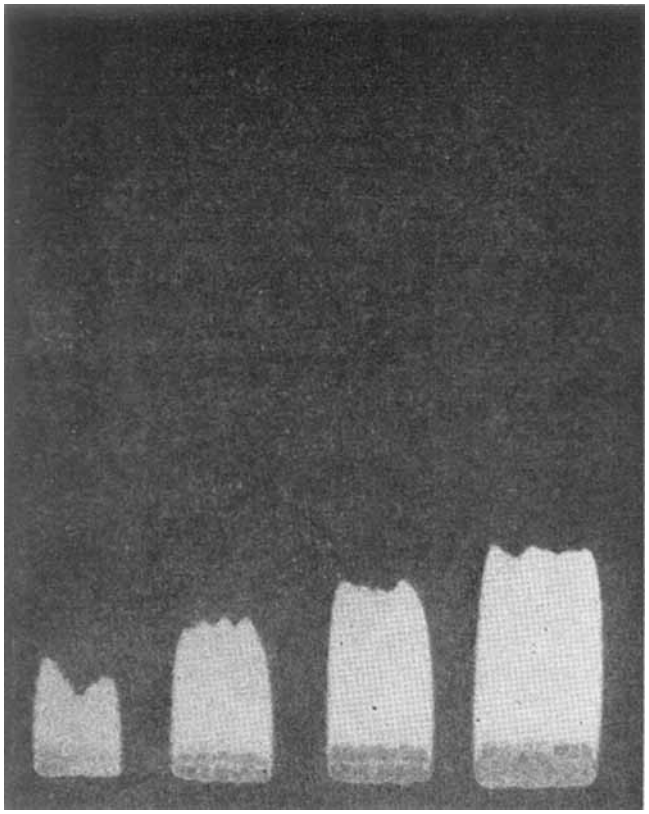


Figure 1—*Starch–iodine-sprayed chromatogram of phenoxymethyl penicilloic acid.*

EXPERIMENTAL

Reagents—Ammonium sulfate A.R.; sodium sulfate anhydrous A.R.; chloroform B.P.; sulfuric acid, 12 *N* A.R.; acetone A.R.; silica gel G according to Stahl for TLC; glacial acetic acid; sodium hydroxide, 2.5 *N;* phosphate buffer, 1%, pH 7; *n*-butanol; 10% potassium nitrate in concentrated sulfuric acid; ammonia 0.880 sp. gr. A.R.–water (1:1); starch, soluble A.R.; 0.1 *N* iodine in 4% potassium iodide A.R.

Procedure—*A. Preparation of Thin-Layer Plates and Starch–Iodine Spray*—Using CAMAG hand-operated apparatus, glass plates, 20 × 10 cm., were coated with silica gel slurry sufficient to produce a thickness of 250–300 μ after drying. The plates were dried in an oven at 80° and finally kept at room temperature exposed to the atmosphere. The starch–iodine solution was prepared by mixing 50 ml. of 1% soluble starch solution, 3 ml. of glacial acetic acid, and 1 ml. of 0.1 *N* iodine.

B. Preparation of Penicilloic Acid—1. Prepare phenoxymethyl penicilloic acid by dissolving 8.5 g. of penicillin V in 80 ml. of water and 9 ml. of 2.5 *N* sodium hydroxide. Keep the solution overnight at 4–8°. After addition of 14 ml. of *n*-butanol, neutralize the solution by addition of dilute sulfuric acid, while mixing and cooling, until a precipitate forms. Filter off the precipitate, wash several times with water, and dry for 4 hr. at 36° and then in a desiccator over P_2O_5; m.p. 121–122° (dec.).

2. Prepare phenoxyethyl penicilloic acid by dissolving 8.5 g. of phenethicillin in 80 ml. of water and 9 ml. of 2.5 *N* sodium hydroxide. Stir the solution for 0.5 hr.; then add 14 ml. of *n*-butanol. Add slowly, while mixing and cooling, 2 *N* sulfuric acid, until the pH reaches approximately 2.8 (approximately 25 ml.). Filter off the precipitate, wash several times with water, and dry at 36° overnight and then in a desiccator over P_2O_5; m.p. 113–114°.

C. Preparation of a Sample—Pipet 2 ml. of urine into a suitable glass-stoppered separator, add 1.5 g. of powdered ammonium sulfate, and mix by swirling to dissolve. Add 10 ml. of chloroform by means of pipet and 0.12 ml. of 12 *N* sulfuric acid which results in a pH of approximately 2. Stopper, and shake immediately for 1 min. After standing for a few minutes to allow the layers to separate, draw off the chloroform layer into a dry test tube, filtering through a small glass funnel containing filter paper on which has been placed about 1 g. of anhydrous sodium sulfate. Transfer 5 ml. of the chloroform extract into a 10–15-ml. test tube fitted with a ground-glass stopper. Remove the chloroform by warming the test tube in a 30–40° water bath while blowing a gentle air current into the tube. Add 0.2 ml. of acetone to the dry residue, stopper immediately with a glass stopper, and gently agitate to dissolve the residue.

D. Separation of Penicilloic Acid from Penicillin and Spectrophotometric Determination of Penicilloic Acid—Stock solutions of the prepared penicilloic acids are made by dissolving in 1% phosphate buffer, pH 7, to furnish a concentration of 10 mg./ml. From these, aliquots are added to normal urine to furnish concentrations ranging from 0.2 to 1.0 mg. acid/ml. Two-milliliter aliquots of these standard solutions are submitted to Procedure C. Using a microsyringe, apply the standards and samples, usually 20-μl. aliquots, each equivalent to 0.1 ml. of original sample or standard, to the plate in duplicate using four application sites for one plate. Develop without delay the plates containing samples and standards by acetone–acetic acid (19:1) in a covered jar lined with filter paper until the solvent front advances 12–14 cm. After drying, spray the plates with starch–iodine solution. The penicilloic acid appears as an elongated spot starting near the point of application, being more elongated at a higher concentration. The penicillin spots, less marked, appear near the liquid front, and the separation is complete. The amount of the sample applied to the plate may be varied depending upon the concentration.

Scrape the white zones containing penicilloic acid onto a square of glossy paper and quantitatively transfer into 11.5 × 1.5-cm. glass centrifuge tubes. Add 0.5 ml. of 10% potassium nitrate in concentrated sulfuric acid to each tube. Immerse the unstoppered tubes in boiling water for 15 min., stirring occasionally by rotation. After cooling, run 1.5 ml. of distilled water into each tube. Mix the solutions by swirling and cool the tubes in ice water. Add 5 ml. of

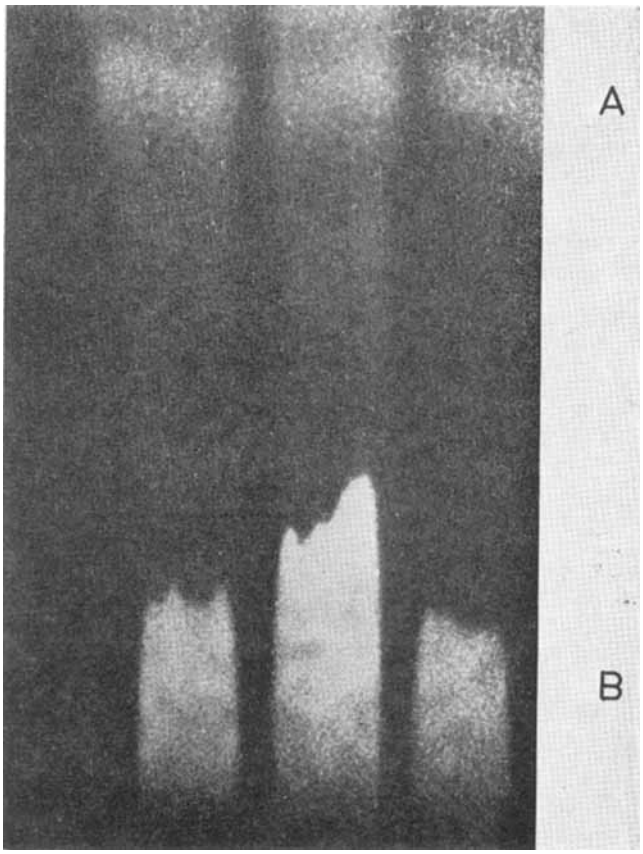


Figure 2—*Chromatogram for chloroform extract from 0.1 ml. urine from Subject 2, phenoxyethyl penicillin-treated. Key: A, phenoxyethyl penicillin; B, phenoxyethyl penicilloic acid.*

Table I—Penicillin and Phenoxymethyl Penicilloic Acid in Urine (mcg./ml.)

Hr.	Donor 1 (79.7 kg.)[a] Pen. V[b]	Pav.[c]	Total	% Pav. in Total	Donor 2 (78.8 kg.) Pen. V	Pav.	Total	% Pav. in Total
1	214	45	259	17.4	308	40	348	11.5
2	214	50	264	18.9	471	160	631	25.4
3	372	290	662	43.8	564	385	949	40.6
4	118	160	278	57.6	322	320	644	49.7
6	48	95	143	66.4	45	60	105	57.1

Hr.	Donor 3 (50.7 kg.) Pen. V	Pav.	Total	% Pav. in Total	Donor 4 (78.8 kg.) Pen. V	Pav.	Total	% Pav. in Total
1	1375	900	2275	39.6	348	250	698	35.8
2	468	850	1318	64.5	855	620	1575	39.4
3	228	540	768	70.3	434	440	874	50.3
4	57	280	337	83.1	224	370	594	62.3
6	22	150	172	87.2	69	320	389	82.3

[a] Numbers in parentheses refer to body weight. [b] Pen. V = phenoxymethyl penicillin. [c] Pav. = phenoxymethyl penicilloic acid.

half-strength ammonia (ammonia 0.880 sp. gr. mixed with water, 1:1) gradually from a buret and again mix the contents of the tubes by swirling and cool. After cooling, occlude the tubes with a thumb protected by rubber and shake several times. Centrifuge the tubes for 10–15 min. at 2000 r.p.m. Remove and measure spectrophotometrically at 420 mμ the yellow supernatant, using 10-mm. cells and setting the instrument on a clear blank obtained by boiling 0.5 ml. of nitrating mixture diluted with 1.5 ml. of water and 5 ml. of diluted ammonia. From the undeveloped part of the glass plate, scrape off, in duplicate, areas of surface equal to the areas extracted for penicilloic acid and put them through the same procedure as the samples to serve as a blank. Any value obtained from this latter is subtracted from the values for samples and standards.

From the values of the standards, plot a curve and obtain data for samples.

To provide typical natural samples for illustration of the method, an experiment was carried out to determine the amount of penicilloic acid present in samples of urine in a group of four volunteers taking penicillin orally. Two penicillins were used in the experiment—phenoxymethyl penicillin (penicillin V) and phenoxyethyl penicillin (phenethicillin). The amount of penicillin taken orally was 0.5 g. of the potassium salt. The urine samples were collected at 0, 1, 2, 3, 4, and 6 hr. and immediately kept in an ice water bath. All volunteers were adult males, coded 1, 2, 3, and 4, weighing 79.7, 78.8, 50.7, and 78.8 kg., respectively. The amount of penicillin in each sample was determined by biological assay. The result for zero-hour samples was nil both for penicilloic acid and penicillin. From the data given by

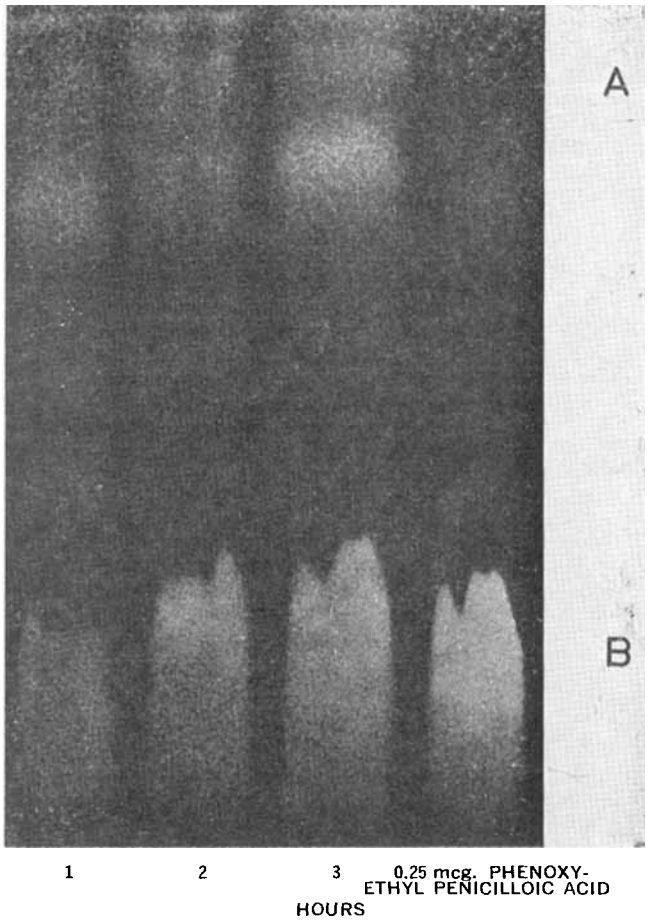


Figure 3—*Chromatogram of chloroform extract from 0.1 ml. urine from Subject 2, phenoxyethyl penicillin-treated. Key: A, phenoxyethyl penicillin; B, phenoxyethyl penicilloic acid.*

assays of penicillin and penicilloic acid, the percentage of the latter was calculated. The data for penicillin V are presented in Table I; the data for phenethicillin are presented in Table II.

A photograph of a developed starch–iodine sprayed plate containing a set of standards of phenoxymethyl penicilloic acid is shown (Fig. 1).

A photograph of a similar chromatogram of samples for phenethicillin from Donor 2 at 0, 1, 2, and 6 hr. is shown (Fig. 2). Samples from this same donor at 1, 2, and 3 hr. and a 25-mcg. standard of penicilloic acid are shown in Fig. 3.

Accuracy of Method—To aliquots of normal urine to which penicillin V had been added to a concentration of 2.5 mg./ml.,

Table II—Phenethicillin and Phenoxyethyl Penicilloic Acid in Urine, mcg./ml.

Hours	Donor 1 (79.7 kg.) Pheneth-icillin	PHA[a]	Total	% of PHA	Donor 2 (78.8 kg.) Pheneth-icillin	PHA	Total	% of PHA
1	254	280	534	52.4	428	170	598	28.4
2	369	380	749	50.7	417	300	717	41.8
3	369	345	714	48.3	494	280	774	36.2
4	204	160	364	44.0	374	220	594	37.0
6	77	40	117	34.2	145	70	215	32.5

Hours	Donor 3 (50.7 kg.) Pheneth-icillin	PHA	Total	% of PHA	Donor 4 (78.8 kg.) Pheneth-icillin	PHA	Total	% of PHA
1	2414	1300	3714	35.0	390	370	760	48.7
2	963	850	1813	46.9	902	550	1452	62.1
3	277	380	657	57.8	471	770	1241	62.0
4	131	200	331	60.4	390	360	750	48.0
6	41	50	91	55.0	101	80	181	44.2

[a] PHA = Phenoxyethyl penicilloic acid.

phenoxymethyl penicilloic acid was added to furnish concentrations of 0, 0.25, and 0.5 mg./ml. These solutions, together with standard phenoxymethyl penicilloic acid preparations as in Procedure D, were submitted to Procedures C and D. Recoveries ranged from 92 to 100%. Similar results were obtained with phenethicillin and phenoxyethyl penicilloic acid.

Extraction Efficiency—Under the described conditions the extraction of phenoxymethyl and phenoxyethyl penicilloic acids from aqueous solutions by chloroform was between 88 and 92% complete. This was confirmed by assaying, by nitration, samples containing 0.5 mg./ml. and 1 mg./ml. directly and after extraction.

DISCUSSION

The yellow quinoid radical produced by nitration of these compounds that originate from the phenolic group could be produced if phenol or its derivatives were present in the sample used for nitration.

During numerous assays of urine samples without penicillin or penicilloic acid, the blank value of the chromatographed chloroform extract was close to or similar to the normal blank value, indicating that phenolic compounds present in urine do not interfere with this assay. The difference in R_f values for penicillins and for the corresponding penicilloic acids probably derives from the fact that the former are monobasic acids, which generally have high values compared with dibasic acids to which class the latter belong. The R_f value of phenoxyacetic acid in the method used is higher than that of penicillin; possible interference by this acid, if present in the extract, is thus eliminated.

The data given in Tables I and II provide an example only of the information obtainable using this method. No attempt has been made to correlate the results with liquid intake, excretion volumes, or other controllable factors.

REFERENCES

(1) S. S. Walkenstein, N. Chumakow, and J. Seifter, *Antibiot. Chemother.*, **4**, 1245(1954).

(2) S. C. Pan, *Anal. Chem.*, **26**, 1438(1954).

(3) M. Roehr, *Microchim. Ichnoanal. Acta*, **1965**, 705.

(4) I. H. McGilveray and R. D. Strickland, *J. Pharm. Sci.*, **56**, 77(1967).

(5) J. Birner, *Anal. Chem.*, **31**, 271(1959).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 28, 1969, from the *Biochemistry Department, Commonwealth Serum Laboratories, Parkville, Victoria, Australia.*

Accepted for publication February 3, 1970.

The author thanks Mr. R. Dickson for technical assistance and Miss M. M. Broderick for biological assays. He is indebted to Mr. E. H. Baxter for assistance in preparing the manuscript.

Kinetics and Mechanisms of Drug Action on Microorganisms X: Action of Spectinomycin on *Escherichia coli* by Microbial Kinetics

JOBST B. MIELCK and EDWARD R. GARRETT*

Abstract □ The steady-state growth of *Escherichia coli* cultures, $N = N_0 e^{k_0 t}$, is slowly inhibited by spectinomycin to a new steady state with a new rate constant, k_{app}. The $k_{app.}$ is linearly dependent on drug concentration, S, above a certain minimum concentration of spectinomycin, S^*; *i.e.*, $k_{app.} = k_0 - k_S(S - S^*)$. This minimum concentration is a function of the concentration of the media and can be assigned to binding or removal of microbiologically effective spectinomycin as protonated material by the components of the media. The logarithm of the inhibitory constant, k_S, linearly increases with the pH of the media to pH 7.6, and this implies that only uncharged material is biologically active. The slow rate of achievement of a drug-equilibrated, steady-state, microbial generation rate can be reconciled with a relatively rapid reequilibrated rate on dilution with fresh media by postulating depletion of a cell-generated vital metabolite linked to the growth rate of the microorganism.

Keyphrases □ Microorganisms—mechanism, kinetics, drug activity □ Kinetics, mechanism—spectinomycin activity □ Spectinomycin action, *E. coli*—kinetics, mechanism □ pH, organism population, nutrient concentration, effects—spectinomycin-affected generation rates □ *E. coli* generation rates—spectinomycin concentration

The aminoglycoside spectinomycin has antibacterial activity against a variety of Gram-positive and Gram-negative microorganisms (1). The inhibitory action is bacteriostatic and is reversed by washing the drug-affected cells (2). The antibiotic forms a stoichiometric 1:1 complex with the 30-S ribosomal subunit extracted from *Escherichia coli*. The formation of this complex blocks some steps in the translocation of the peptidyl-RNA from the acceptor site for aminoacyl *t*-RNA to the peptidyl donor site. Protein synthesis is thus inhibited (2).

The determination of the generation rates of *E. coli* as a function of the concentrations of a variety of antibiotics and chemotherapeutic agents has established a useful procedure to quantify the effects of drugs on the time course of bacterial generation (3–8).

The purposes of this study are to determine kinetically the inhibitory effects of spectinomycin on the generation rate of *E. coli* in defined concentration ranges and to evaluate the effects of variable inoculum size, concentration, and pH of the culture medium on drug activity.

EXPERIMENTAL

Test Organism—Replicate slants of *E. coli* strain B/r were used in all experiments. The slants had been prepared from a single colony and were stored in a refrigerator at 4°.

Culture Media—Antibiotic medium 3[1] was rehydrated according to the specifications of the manufacturer. The media were filtered twice through Millipore 0.45 μ HA filters and autoclaved at 120° for 15 min. The pH of the media was 7.05 ± 0.05. Various amounts of Millipore-filtered 1.7 *N* HCl and 2.0 *N* NaOH, respectively, were added aseptically after the sterilization to obtain pH values within a range of 7.50 to 5.87 for the investigation of the anti-

[1] Bacto Antibiotic Medium 3, Difco Laboratories, Detroit, Mich.

phenoxymethyl penicilloic acid was added to furnish concentrations of 0, 0.25, and 0.5 mg./ml. These solutions, together with standard phenoxymethyl penicilloic acid preparations as in Procedure D, were submitted to Procedures C and D. Recoveries ranged from 92 to 100%. Similar results were obtained with phenethicillin and phenoxyethyl penicilloic acid.

Extraction Efficiency—Under the described conditions the extraction of phenoxymethyl and phenoxyethyl penicilloic acids from aqueous solutions by chloroform was between 88 and 92% complete. This was confirmed by assaying, by nitration, samples containing 0.5 mg./ml. and 1 mg./ml. directly and after extraction.

DISCUSSION

The yellow quinoid radical produced by nitration of these compounds that originate from the phenolic group could be produced if phenol or its derivatives were present in the sample used for nitration.

During numerous assays of urine samples without penicillin or penicilloic acid, the blank value of the chromatographed chloroform extract was close to or similar to the normal blank value, indicating that phenolic compounds present in urine do not interfere with this assay. The difference in R_f values for penicillins and for the corresponding penicilloic acids probably derives from the fact that the former are monobasic acids, which generally have high values compared with dibasic acids to which class the latter belong. The R_f value of phenoxyacetic acid in the method used is higher than that of penicillin; possible interference by this acid, if present in the extract, is thus eliminated.

The data given in Tables I and II provide an example only of the information obtainable using this method. No attempt has been made to correlate the results with liquid intake, excretion volumes, or other controllable factors.

REFERENCES

(1) S. S. Walkenstein, N. Chumakow, and J. Seifter, *Antibiot. Chemother.*, **4**, 1245(1954).

(2) S. C. Pan, *Anal. Chem.*, **26**, 1438(1954).

(3) M. Roehr, *Microchim. Ichnoanal. Acta*, **1965**, 705.

(4) I. H. McGilveray and R. D. Strickland, *J. Pharm. Sci.*, **56**, 77(1967).

(5) J. Birner, *Anal. Chem.*, **31**, 271(1959).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 28, 1969, from the *Biochemistry Department, Commonwealth Serum Laboratories, Parkville, Victoria, Australia.*

Accepted for publication February 3, 1970.

The author thanks Mr. R. Dickson for technical assistance and Miss M. M. Broderick for biological assays. He is indebted to Mr. E. H. Baxter for assistance in preparing the manuscript.

Kinetics and Mechanisms of Drug Action on Microorganisms X: Action of Spectinomycin on *Escherichia coli* by Microbial Kinetics

JOBST B. MIELCK and EDWARD R. GARRETT*

Abstract □ The steady-state growth of *Escherichia coli* cultures, $N = N_0 e^{k_0 t}$, is slowly inhibited by spectinomycin to a new steady state with a new rate constant, k_{app}. The $k_{app.}$ is linearly dependent on drug concentration, S, above a certain minimum concentration of spectinomycin, S^*; *i.e.*, $k_{app.} = k_0 - k_S(S - S^*)$. This minimum concentration is a function of the concentration of the media and can be assigned to binding or removal of microbiologically effective spectinomycin as protonated material by the components of the media. The logarithm of the inhibitory constant, k_S, linearly increases with the pH of the media to pH 7.6, and this implies that only uncharged material is biologically active. The slow rate of achievement of a drug-equilibrated, steady-state, microbial generation rate can be reconciled with a relatively rapid reequilibrated rate on dilution with fresh media by postulating depletion of a cell-generated vital metabolite linked to the growth rate of the microorganism.

Keyphrases □ Microorganisms—mechanism, kinetics, drug activity □ Kinetics, mechanism—spectinomycin activity □ Spectinomycin action, *E. coli*—kinetics, mechanism □ pH, organism population, nutrient concentration, effects—spectinomycin-affected generation rates □ *E. coli* generation rates—spectinomycin concentration

The aminoglycoside spectinomycin has antibacterial activity against a variety of Gram-positive and Gram-negative microorganisms (1). The inhibitory action is bacteriostatic and is reversed by washing the drug-affected cells (2). The antibiotic forms a stoichiometric 1:1 complex with the 30-S ribosomal subunit extracted from *Escherichia coli*. The formation of this complex blocks some steps in the translocation of the peptidyl-RNA from the acceptor site for aminoacyl *t*-RNA to the peptidyl donor site. Protein synthesis is thus inhibited (2).

The determination of the generation rates of *E. coli* as a function of the concentrations of a variety of antibiotics and chemotherapeutic agents has established a useful procedure to quantify the effects of drugs on the time course of bacterial generation (3–8).

The purposes of this study are to determine kinetically the inhibitory effects of spectinomycin on the generation rate of *E. coli* in defined concentration ranges and to evaluate the effects of variable inoculum size, concentration, and pH of the culture medium on drug activity.

EXPERIMENTAL

Test Organism—Replicate slants of *E. coli* strain B/r were used in all experiments. The slants had been prepared from a single colony and were stored in a refrigerator at 4°.

Culture Media—Antibiotic medium 3[1] was rehydrated according to the specifications of the manufacturer. The media were filtered twice through Millipore 0.45 μ HA filters and autoclaved at 120° for 15 min. The pH of the media was 7.05 ± 0.05. Various amounts of Millipore-filtered 1.7 *N* HCl and 2.0 *N* NaOH, respectively, were added aseptically after the sterilization to obtain pH values within a range of 7.50 to 5.87 for the investigation of the anti-

[1] Bacto Antibiotic Medium 3, Difco Laboratories, Detroit, Mich.

bacterial activity of spectinomycin as a function of the pH. Media with half and twice the amount of nutrients, respectively, were prepared and adjusted to a pH of 7.14 ± 0.02 for the study of the binding capacity of the medium for the antibiotic.

Antibiotic—An assayed sample of spectinomycin sulfate[2] was used (640 ± 35 mcg. base/mg.).

Bacterial Generation—A 5-ml. aliquot of culture medium was inoculated from a fresh slant, and the culture was allowed to grow for 12 hr. at 37.5° in an incubator. A 1.0-ml. sample was then diluted with 9.0 ml. broth at 37.5°, which was diluted 100-fold into a fresh medium after 60 min. The growth of this culture was followed up to 10^7 *E. coli*/ml. Samples of this culture were finally appropriately diluted in a single step, or in several steps, into replicate volumes of 49.0 ml. broth in 125-ml. loosely capped conical flasks to achieve the desired organism concentrations. The cultures were maintained at 37.5 ± 0.1° in a 50-gal. constant-temperature water bath equipped with a shaker. All pipets and media used for the dilutions of the cultures were kept at 37.5° to protect the organisms from temperature shocks.

Total Count Method—One-milliliter samples were withdrawn at 20-min. intervals from the cultures. They were diluted to obtain counts within a range of 10,000 to 30,000 counts/50 μl. on the Coulter counter, model B.[3] The diluent used was a Millipore 0.45 μ HA-filtered aqueous solution of 0.85% NaCl and 1% formaldehyde. The instrument was equipped with a 30-μ orifice; the settings were: an aperture current of 5, amplification of 8, gain of 10, lower threshold of 13, and upper threshold at maximum. The total counts were corrected for the background count of the particular batch of medium used, diluted in the same way as the sample. The background counts in general did not exceed 1000 counts/50 μl.

Viable Count Method—Samples of 0.50 ml. were withdrawn from the cultures and appropriately diluted into sterilized 0.85% saline solution so that 50 to 150 colonies per plate would result. From these dilutions, aliquots of 1.00 ml. were pipeted onto each of three replicate agar plates. The plates were incubated for 48 hr. at 37.5° and the colonies counted on a Colony counter model C-110.[4]

Effect of Antibiotic Concentration on Generation Rates—Fresh solutions of spectinomycin in distilled water were aseptically prepared for each experiment. They were sufficiently diluted so that aliquots of 1.00 ml. added to the 49-ml. culture volumes yielded the desired graded drug concentrations between 6.00 and 20.0 mcg./ml. The solutions were added to the cultures growing at 37.5° in the logarithmic growth phase at determined organism concentrations. Samples were withdrawn every 20 min. and counted by the respective method. A culture without drug was studied in each experiment to obtain the generation rate constant in absence of drug as a reference. The generation curves for 21.9, 35.0, and 43.8 mcg./ml. spectinomycin at pH 7.05 were obtained by both the viable and total cell count methods (Fig. 1).

Effect of Organism Population on Drug-Affected Generation Rates—Each flask of four sets of four replicate 49-ml. volumes of culture medium of pH 7.05 was inoculated with 1.00 ml. of appropriately diluted culture growing in the logarithmic growth phase. The organism concentrations in the respective sets were 5×10^3, 5×10^4, 5×10^5, and 5×10^6 *E. coli*/ml. at the time of addition of drug. Drug solutions were added to achieve spectinomycin concentrations of 15.0, 24.0, and 30.0 mcg./ml., respectively. One culture in each set contained no drug. Total cell counts were obtained from samples drawn every 20 min.

Reversibility of Drug Action—A 49-ml. volume of culture growing in the logarithmic growth phase at 37.5° contained at time zero a population of 10^6 *E. coli*/ml. (Curve A in Fig. 2). Drug was added to achieve a concentration of 28 mcg./ml. spectinomycin (Curve B in Fig. 2) at time zero. An aliquot of 2.00 ml. of this culture was diluted into 49 ml. of fresh medium at 50 min. after the addition of drug to give 1.12 mcg./ml. (Curve C in Fig. 2). At the same time, 5.00 ml. of the culture of Curve B was diluted into 45.0 ml. of broth that contained 28.0 mcg./ml. spectinomycin (Curve D in Fig. 2), and 2.00 ml. of the drug-free culture of Curve A was diluted into 49 ml. of drug-free broth (Curve A_1 in Fig. 2). At 240 min., 0.80 ml. of the culture of Curve B was diluted into each of two replicate 49-ml. volumes of fresh medium to give 4.48 mcg./ml. (Curve E in Fig. 2). When these cultures containing 4.48 mcg./ml. drug reached the steady-state growth phase, at 355 min. and 6×10^6 *E. coli*/ml., a 1.00-ml. aliquot of spectinomycin solution was added to one of the replicate cultures to establish a drug concentration of 30.5 mcg./ml. (Curve F in Fig. 2). The total number of organisms in the various cultures was obtained from samples withdrawn every 20 min.

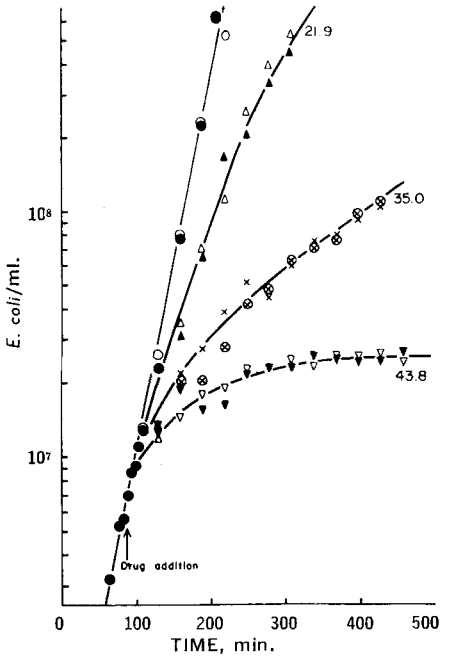


Figure 1—*Generation rate curves of* E. coli *at 37.5° and pH 7.05 obtained by total (closed symbols) and viable (open symbols) cell counts. The curves are labeled with the respective concentrations of spectinomycin (mcg./ml.).*

Effect of pH on Drug-Affected Generation Rates—Sufficient amounts of 1.7 *N* HCl and 2.0 *N* NaOH were added to the culture media to obtain pH values of 5.87, 6.20, 6.53, 6.55, 6.72, 6.87, 7.13,

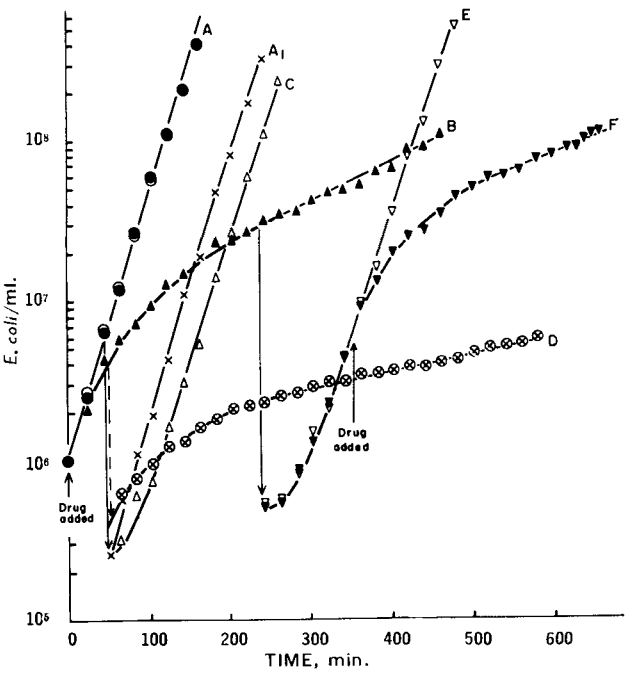


Figure 2—*Generation rate curves of* E. coli *at 37.5° and pH 7.10 on addition and dilution of spectinomycin. The curves, final drug concentrations (mcg./ml.), and apparent steady-state generation rate constants (10^4 $k_{app.}$ in $sec.^{-1}$) were A, 0, 6.20; A_1, 0, 6.24; B, 28.0, 0.96; C, 1.12, 5.95; D, 28.0, 0.42; E, 4.48, 5.90; and F, 30.5, 0.79.*

[2] The Upjohn Co., Kalamazoo, Mich.
[3] Coulter Electronics Co., Hialeah, Fla.
[4] New Brunswick Scientific Co., New Brunswick, N. J.

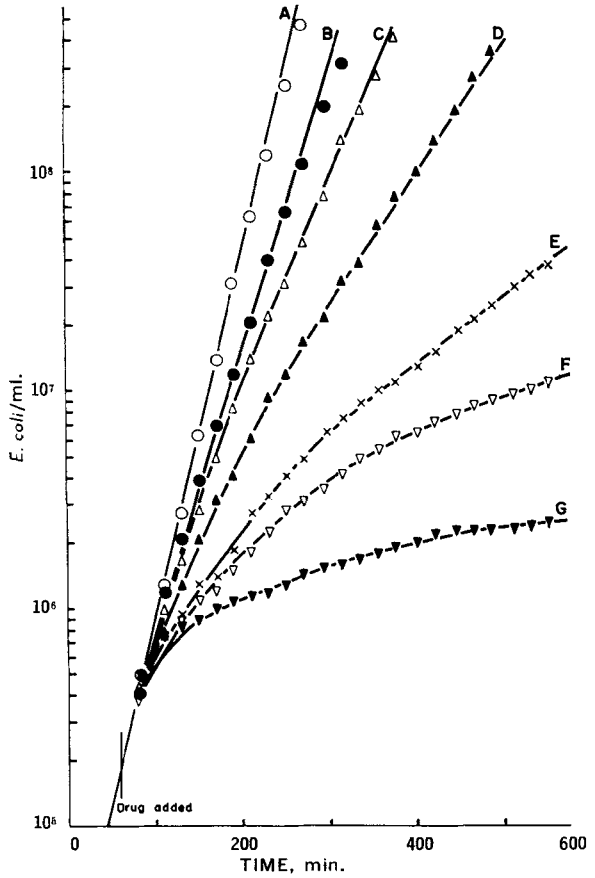


Figure 3—*Typical generation curves for* E. coli *at 37.5° and pH 6.72. The curves, spectinomycin concentrations (mcg./ml.), and generation rate constants (10^4 $k_{app.}$, $sec.^{-1}$) were: A, 0, 6.30; B, 32.0, 4.56; C, 40.0, 3.58; D, 53.3, 2.37; E, 66.7, 1.18; F, 72.7, 0.572; and G, 80, 0.*

7.22, 7.30, and 7.50, respectively. The pH values of these studies were read at 10^7 *E. coli*/ml., using a Schott model U combination glass electrode and a Beckman Zeromatic pH meter with an expanded scale. From a single slant, separate cultures for each pH were prepared as previously described. At organism concentrations in the range from 8×10^4 to 8×10^5 *E. coli*/ml., 1.00-ml. aliquots of spectinomycin solutions were added to achieve the desired drug concentrations. A typical example is given in Fig. 3. In addition to the data obtained for the total number of organisms as a function of time, the pH in some of the cultures was monitored as a function of the organism concentration.

Table I—Bacteriostatic Activity Constants for Spectinomycin against *E. coli* at Various Media pH Values

pH[a]	$10^5(k_S \pm SD)$,[b] ml. mcg.$^{-1}$ sec.$^{-1}$	$10^4\ k_0$,[c] sec.$^{-1}$	$10^4\ k'_0$,[d] sec.$^{-1}$	S^*,[e] mcg. ml.$^{-1}$
5.87	0.142 ± 0.017	5.90	6.16	18.3
6.30	0.294 ± 0.012	6.40	6.26	−4.76
6.30	0.389 ± 0.015	6.30	6.68	9.77
6.53	0.869 ± 0.017	6.55	8.01	16.8
6.55	0.562 ± 0.034	6.29	7.29	17.8
6.72	0.956 ± 0.028	6.30	7.52	12.8
6.87	1.32 ± 0.12	6.09	7.05	7.27
7.13	2.20 ± 0.10	6.23	7.75	6.91
7.22	2.98 ± 0.14	6.50	6.80	1.01
7.30	2.42 ± 0.10	5.91	6.51	2.48
7.50	4.88 ± 0.11	6.19	7.89	3.48

[a] pH of the culture medium at 10^7 *E. coli* $ml.^{-1}$. [b] Slope of a plot of $k_{app.}$ in $sec.^{-1}$ *versus* drug concentration, S, in mcg. $ml.^{-1}$ obtained by a least-squares fit of the data to $k_{app.} = k'_0 - k_S S$, and the standard deviation of that slope. [c] Experimentally observed generation rate constant at $S = 0$. [d] Calculated generation rate constant at $S = 0$. [e] Calculated drug concentration, S, at $k_{app.} = k_0$.

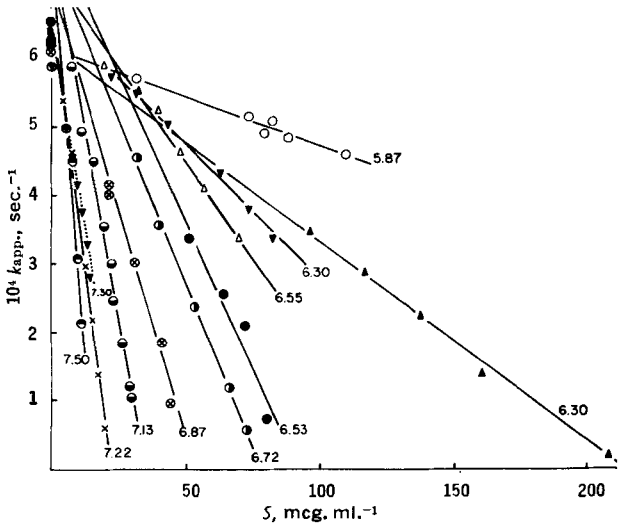


Figure 4—*Apparent first-order generation rate constants,* $k_{app.}$ *in* $sec.^{-1}$, *as functions of the spectinomycin concentration,* S, *in mcg./ml. The curves are labeled with the pH of the media.*

Effects of Nutrient Concentration on Drug-Affected Generation Rates—Culture media of half and twice the normal concentration of nutrients were prepared and adjusted to pH 7.14 ± 0.02 and 6.87 ± 0.02, respectively. Microbial cultures were prepared in the previously described manner using these media separately. The respective relative nutrient concentrations, pH values, and the range of spectinomycin concentrations (mcg./ml.) used were: (*a*) for half the nutrient concentration: at pH 7.10 from 8 to 20 mcg./ml., at pH 7.16 from 6.7 to 12 mcg./ml.; and at pH 6.87 from 23 to 32 mcg./ml.; and (*b*) for twice the nutrient concentration: at pH 7.16 from 7 to 16 mcg./ml. The drug-free and drug-affected generation rate constants were obtained by total cell counts.

RESULTS

Effect of Drug Concentration on Generation Rates—The number of *E. coli* per milliliter obtained by both total and viable cell counts is plotted on a semilogarithmic scale against time in Fig. 1. The numbers from the respective methods are coincident; thus no kill or death of microorganisms is observed between 0 and 44 mcg./ml. over the time period studied. Thus, this study can be based upon total cell counts obtained within this range of drug concentration.

A typical family of generation curves obtained over a wide range of graded drug concentrations and based on total cell counts is given in Fig. 3. Upon addition of drug to the culture growing in the logarithmic phase, the generation rate is decreased slowly until a new spectinomycin-affected steady-state phase is established. Apparent first-order generation rate constants, $k_{app.}$ in $sec.^{-1}$, are obtained from the slopes of this linear portion in accordance with the equation

$$\log N = \log N_0 + \frac{k_{app.}}{2.303}\, t \qquad \text{(Eq. 1)}$$

where N is the number of microorganisms. A plot of the $k_{app.}$ values obtained at various pH values against spectinomycin concentration, S (Fig. 4), demonstrates the linear dependence of $k_{app.}$ on S at lower $k_{app.}$ values in accordance with the equation

$$k_{app.} = k_0' - k_S S;\ k_{app.} < k_0 \qquad \text{(Eq. 2)}$$

where k_0' in $sec.^{-1}$ is the extrapolated value for $k_{app.}$ at $S = 0$, the slope k_S in ml./mcg.-sec. is the inhibitory constant of the antibiotic, and k_0 is the generation rate constant in the absence of the antibiotic. The values for k_S and k_0', calculated from least-squares fits, and the experimentally determined k_0 in $sec.^{-1}$ are listed in Table I.

Effect of pH on Drug-Affected Generation Rate—The apparent first-order generation rate constants, $k_{app.}$, obtained at pH values from 5.87 to 7.50 in the culture media in the absence and presence

of graded spectinomycin concentrations, S, are plotted against S in Fig. 4. The drug-free generation rate constants are independent of pH in this range [Table I and (5)], while the drug-affected generation rate constants decrease with increasing pH at comparable drug concentrations. The calculated inhibitory constant, k_S, and intercepts, k_0', are listed in Table I. A plot of the log k_S against pH is given in Fig. 5. The drug concentrations, $S = S^*$ (Table I), that produce $k_{app.} = k_0$, the drug-free generation rate constant, can be calculated from Eq. 2. The pH values of the media in drug-free and drug-affected growing cultures were constant to within 1% of their initial values up to 10^8 *E. coli*/ml. (5).

Effect of Organism Populations on Drug-Affected Generation Rates—Apparent first-order generation rate constants, $k_{app.}$, for the logarithmic portion of the growth curves obtained in the presence of three different spectinomycin concentrations over a 1000-fold range in inoculum size at the time of drug addition are given in Table II. Although the data show a trend toward smaller rate constants at lower inoculum sizes, the differences appear insignificant compared to the observed values for $k_{app.}$ at $S = 0$. At high organism concentrations ($N > 10^8$ *E. coli*/ml.) or after long periods of drug-affected generation ($t > 600$ min.), the generation rate of the drug-affected cultures increased markedly and approached a new steady state. Apparently, a very small fraction of the bacteria was resistant to spectinomycin. The estimated fraction of the inoculum that was not inhibited was less than 0.01 when the growth curves of the new steady state were extrapolated to the time of inoculation.

On the basis of these observations, it cannot be concluded that the antibiotic is consumed by the organism or that a drug antagonist is excreted as a function of the numbers of organisms (6). It is, therefore, assumed that the drug concentration is in sufficient excess to remain constant over the time period of observation.

Reversibility of Drug Action—Addition of drug to a culture growing in the logarithmic growth phase (Curve A in Fig. 2, $k_0 = 6.20 \times 10^{-4}$ sec.$^{-1}$) to establish 28.0 mcg./ml. spectinomycin (Curve B in Fig. 2) resulted in a gradual decrease in the rate of generation over a period of 200 min. until a new steady state ($k_{app.} = 0.96 \times 10^{-4}$ sec.$^{-1}$) was achieved. Curve A_1 in Fig. 2 ($k_0 = 6.24 \times 10^{-4}$ sec.$^{-1}$) demonstrates that the technique of diluting the cultures does not significantly affect the generation rate constant. Upon dilution of the culture of Curve B in Fig. 2 into fresh medium where the drug concentration of 28.0 mcg./ml. was reduced to 1.12 mcg./ml. (Curve C in Fig. 2), the generation rate gradually increased over a period of 60 min. until a new logarithmic phase with a generation rate constant of 5.95×10^{-4} sec.$^{-1}$ was established.

Table II—Effect of Inoculum Size[a] on Spectinomycin-Affected Growth of *E. coli*

Inoculum Size, *E. coli*/ml.	[Spectinomycin], mcg./ml. — $10^4 k_{app.}$, sec.$^{-1}$				S^*,[b] mcg./ml.
	0	15.0	24.0	30.0	
5×10^3	6.18	3.81	0.96	—	7.7
5×10^4	6.00	3.92	1.37	0.173	5.2
5×10^5	6.00	4.05	1.60	0.238	7.3
5×10^6	6.09	4.04	—	—	—

[a] At time of drug addition. [b] Calculated drug concentration, $S^* = S$, at $k_{app.} = k_0$ from linear relation $k_{app.} = k_0' - k_S S$.

When the culture of Curve B was diluted 10-fold into a fresh medium where the original drug concentration was maintained, the continuous decrease in the generation rate was not interrupted. However, the generation rate constant, 0.42×10^{-4} sec.$^{-1}$ established at 220 min., was lower than that of Curve B. The expected value for this rate constant was that for Curve B in Fig. 2, 0.96×10^{-4} sec.$^{-1}$. The difference in these rate constants could suggest that the activity of spectinomycin is dependent on the concentration of microorganisms. However, the results from the studies of the influence of inoculum size on the generation rate constants over a much wider range of organism concentrations did not show a significant dependence. Dilution of the culture of Curve B at the beginning of its steady-state phase at 240 min. into drug-free medium (Curve E in Fig. 2) yielded a gradual increase in the generation rate over a period of 80 min. as compared to the 60 min. of Curve C in Fig. 2, whereafter a new steady-state phase with a rate constant of 5.90×10^{-4} sec.$^{-1}$ was established.

When drug was added to a replicate culture of that of Curve E in Fig. 2 to yield a drug concentration of 30.5 mcg./ml., a gradual decrease in the generation rate over 160 min. was observed, when a new logarithmic phase with a generation rate constant of 0.79×10^{-4} sec.$^{-1}$ was achieved.

Effect of Nutrient Concentration on Drug-Affected Generation Rates—The concentration of nutrients in the culture media has a significant effect on the drug-affected generation rate constants (Table III and Fig. 6). The calculated values for the spectinomycin concentration, $S = S^*$ when $k_{app.} = k_0$, increase nearly twice when the nutrient concentration is doubled. These S^* values were calculated from least-squares fits of the generation rate constants to the drug concentrations in accordance with Eq. 2.

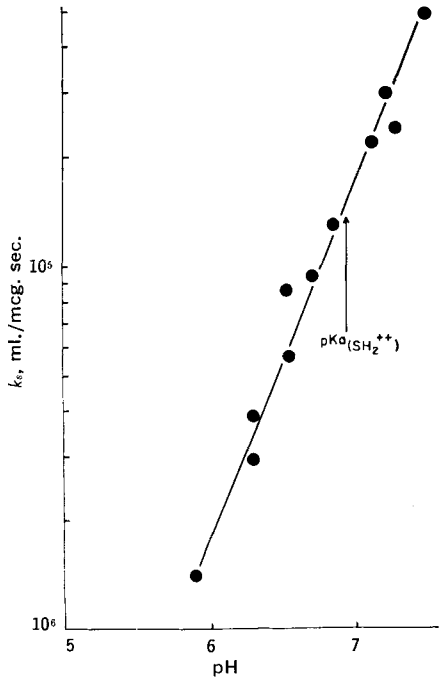


Figure 5—*Dependence of the log of the inhibitory constant,* k_S, *in ml./mcg.-sec. of spectinomycin on the pH of the culture media.*

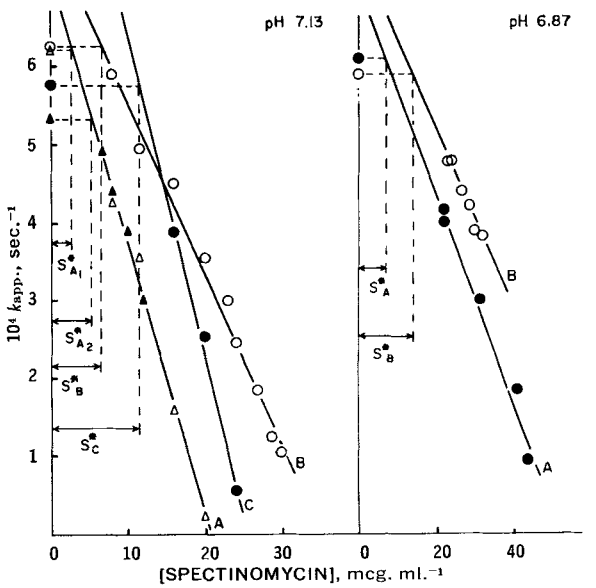


Figure 6—*Effect of various nutrient concentrations on the dependence of the generation rate constants of* E. coli *on the spectinomycin concentration. The curves, relative nutrient concentrations and calculated spectinomycin concentration,* $S = S^*$ *(mcg./ml.) at* $k_{app.} = k_0$, *were at pH 7.13: A, 0.5, 2.96, and 5.49 respectively; B, 1, 6.9; C, 2, 12.0, and at pH 6.87: A, 1, 7.27; and B, 2, 14.1.*

Table III—Effect of Nutrient Concentration on the Biologically Ineffective Spectinomycin Concentration, S^*, against *E. coli*

Nutrients[a]	pH	$10^5\ k_S$[b]	$10^4\ k_0'$[c]	$10^4\ k_0$[d]	S^*[e]
0.5	7.10	0.348	7.23	6.20	2.96
	7.16	0.346	7.23	5.33	5.49
1	7.13	0.260	7.75	6.23	6.91
2	7.16	0.416	10.7	5.73	12.0
1	6.87	0.129	7.05	6.09	7.27
2	6.87	0.118	7.56	5.90	14.1

[a] Relative nutrient concentration with regard to normal antibiotic medium 3. [b] Least-squares slope from the linear relation $k_{app.} = k_0' - k_S S$. [c] Intercept calculated from this relation. [d] Experimentally determined $k_{app.}$ at $S = 0$. [e] Calculated drug concentration, S, at $k_{app.} = k_0$.

DISCUSSION

The drug-affected, apparent first-order generation rate constant, $k_{app.}$, is linearly related to spectinomycin concentration (Fig. 4 and Eq. 2). Similar equations have been successfully employed to define the antibacterial activity of chloramphenicol (9, 10), tetracycline (9, 10), and, in part, lincomycin (5). They are based on the assumption that the extracellular spectinomycin concentration, S, equilibrates with the drug concentration, S', in the receptor site compartment or biophase. Here the antibiotic reversibly binds to receptor sites, R, to form the drug–receptor complex, RS':

$$S \overset{K_1}{\rightleftharpoons} S';\ S' + R \overset{K_2}{\rightleftharpoons} RS' \qquad \text{(Eq. 3)}$$

The equilibria are characterized by the respective equilibrium constants K_1 and K_2 during the drug-affected steady state. The constant K_2 is the affinity constant of spectinomycin for its receptor site, and the product:

$$K_1K_2 = k_b(\text{ml./mcg.}) \qquad \text{(Eq. 4)}$$

is a quantitative measure of the antibacterial activity. It is implied that the fraction of drug-free receptor sites is proportional to the rate of protein synthesis. A certain fraction of this protein synthesis would be proportional to the rate of generation of bacteria, while a large fraction may be necessary for the maintenance of viability (9). The rate of increase in the number of bacteria may be defined in accordance with this concept (9) by

$$\frac{dN}{dt} = \{q\,(k_p - k_p') - qk_pK_1K_2[S]/(1 + K_1K_2[S])\}N \qquad \text{(Eq. 5)}$$

where k_p is the overall rate constant of protein synthesis; k_p' is the minimum rate constant of protein synthesis necessary for bacterial generation; and q is a proportionality constant linking the rate of population increase of N organisms, dN/dt, to the net rate of protein synthesis and to the total number of organisms, N, in a balanced culture. If only a small fraction of the total number of receptor sites has to be reacted with spectinomycin to achieve complete inhibition of bacterial generation,

$$K_1K_2[S] \ll 1 \qquad \text{(Eq. 6)}$$

As a consequence, Eq. 5 reduces to

$$\frac{dN}{dt} = \{q\,(k_p - k_p') - qk_pK_1K_2[S]\}N \qquad \text{(Eq. 7)}$$

Combining the constants in Eq. 7 yields

$$\frac{dN}{dt} = (k_0 - k_S[S])N \qquad \text{(Eq. 8)}$$

where $k_{app.}$ is defined as

$$k_{app.} = (k_0 - k_S[S]) \qquad \text{(Eq. 9)}$$

which is identical with Eq. 2 when k_0' is substituted for k_0. The calculated $k_{app.}$ (Eq. 2) equals k_0 at a concentration S^* (Table I) of spectinomycin. This implies that it takes a definite concentration of antibiotic to be exceeded before antibacterial activity is manifested. It is possible that this amount is bound either by the microorganisms or by the constituents of the culture media. If the bacteria were responsible for this binding, the calculated value for S^* would be a function of the inoculum size. The studies conducted with different inoculum sizes showed no such dependency for S^* (Table II), and permitted the conclusion that the organisms were not responsible for the binding of spectinomycin. If the media were responsible for this binding, S^* would vary with the concentration of nutrients. The experiments carried out with culture media that contained half and twice the normally used concentration of nutrients did show that S^* definitely increased with the nutrient concentration; these experiments permitted the conclusion that the constituents of the media effectively inhibited a definite amount of spectinomycin from exercising biological action (Table III). The relative amounts bound, *i.e.*, the ratio of the estimated S^* values, are of the same order of magnitude as the relative nutrient concentrations for studies conducted at the same pH values (Fig. 6). The S^* values also decreased with increasing pH (Table I and Fig. 4), although the data showed great variability. The nature of the dependency of logarithm S^* on pH (Fig. 7) implied that the charged species were preferably bound to the media.

The inhibitory constant, k_S (Eq. 2 and Table I), varied as a function of pH. The linear plot of log k_S against pH (Fig. 5) has a calculated slope of 1.053 ± 0.056. The pH region studied includes the pKa of the doubly protonated spectinomycin, $pKa_2 = 6.95$ (1). The linearity of this plot is maintained up to significantly higher pH values, *i.e.*, pH 7.5. The pKa of the singly protonated spectinomycin is 8.70 (1). It is, therefore, expected that the pKa_1 influences the antibacterial activity and that the uncharged fraction of the total spectinomycin concentration is the only antibacterially active species. The k_S values cannot be obtained at extracellular pH values higher than 7.5. Beyond this pH, the drug-free generation rate constant changes and the generation rate-determining step may be different from that within a range of pH 5.8 to 7.5 where k_0 is independent of pH. It is expected that the curve in Fig. 5 would approach a pH-independent value for k_S at extracellular pH values that exceed the pKa_1. An intrinsic activity constant, k_S^*, may be defined with regard to the concentration of unprotonated spectinomycin. Thus, it may be calculated (11) from the unprotonated fraction, f_S, of the total antibiotic concentration and the respective $[H^+]$ and the k_S values (Table I):

$$k_S^* = k_S/f_S = k_S([H^+]^2 + K_1[H^+] + K_1K_2)/K_1K_2 \qquad \text{(Eq. 10)}$$

The literature values for the dissociation constants (1) are $K_1 = 1.01 \times 10^{-7}$ and $K_2 = 2.00 \times 10^{-9}$. The mean value obtained for k_S^* was $(2.66 \pm 0.49) \times 10^{-3}$ ml./mcg.-sec.

The rate of achievement of the drug-affected steady-state generation rate with spectinomycin is a slower process than with tetracycline (4), chloramphenicol (7), and lincomycin (5). This is apparent from inspection of Figs. 1–3 where time in excess of 200 min. after drug addition is necessary to obtain a linear semilogarithmic plot of the number of organisms against time. There are several possible explanations for this phenomenon. The assumption of the model of Eq. 3 could imply that the rate of diffusion of drug, S, into the biophase (the compartment where antibiotic concentration, S', is in instantaneous equilibration with receptor sites, R) is a slow process and is rate limiting. However, when the drug-affected culture media are diluted with fresh media (Curves G and E, Fig. 2), the resumption of the new steady-state generation rate occurs in the relatively short time interval of less than 50 min. Although differences in rates of diffusion of spectinomycin in and out of the biophase, *i.e.*,

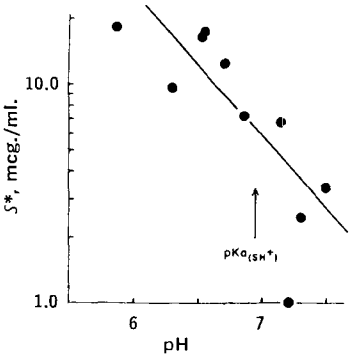


Figure 7—*Correlation of the log of* S*, *the spectinomycin concentration,* S, *at* $k_{app.} = k_0$ *calculated from the linear dependence of* $k_{app.}$ *on* S *with the pH of the culture media.*

a possible "active" process, could be postulated, it appears more reasonable to be critical of the assumption of rate-determining drug diffusion into the biophase.

An alternate explanation may be that drug action, as manifested by the degree of inhibition of generation rate, increases the sensitivity of the organism to further drug action until the steady-state generation rate is achieved. This "feedback" phenomenon is explainable on the premise that the rapidly equilibrated drug concentration in the biophase is competitive for receptor sites with a metabolic intermediate produced by the growing organism.

An analogy can be drawn to the sulfonamide–*p*-aminobenzoic acid competition. The initial fraction of receptor sites that is drug–receptor complex is reflected by an initial decrease in generation rate. This results in a diminution of the production of a vital metabolic intermediate. Subsequent depletion of excess stores of this intermediate in the normal metabolic or generation processes of the organism results in less amounts to compete with drug concentrations in the biophase, greater fractions of drug–receptor complex, and, consequently, further slowing of generation rates. On dilution of the drug with fresh medium, the drug in the organism's biophase rapidly reequilibrates with the consequence of less drug-bound receptor sites. A new steady-state production of metabolic intermediate may occur. This results in the observed reasonably rapid increase in microbial generation to new steady-state conditions (Fig. 2). A kinetically equivalent phenomenon is that the feedback is mediated by decreasing the number of available receptor sites concomitant with decreasing growth rates.

REFERENCES

(1) C. Lewis and H. W. Clapp, *Antibiot. Chemother.*, **11**, 118 (1961).

(2) J. Davies, P. Anderson, and B. D. Davis, *Science*, **149**, 1096(1965).

(3) E. R. Garrett, *Arzneim. Forsch.*, **16**, 1364(1966).

(4) E. R. Garrett and G. H. Miller, *J. Pharm. Sci.*, **54**, 427 (1965).

(5) J. B. Mielck and E. R. Garrett, *Chemotherapy*, **14**, 337 (1969).

(6) E. R. Garrett and O. K. Wright, *J. Pharm. Sci.*, **56**, 1576 (1967).

(7) E. R. Garrett, G. H. Miller, M. R. W. Brown, and K. Smith, *J. Med. Chem.*, **9**, 203(1966).

(8) E. R. Garrett, J. B. Mielck, J. K. Seydel, and H. J. Kessler, *ibid.*, **12**, 740(1969).

(9) E. R. Garrett, G. H. Miller, and M. R. W. Brown, *J. Pharm. Sci.*, **55**, 593(1966).

(10) E. R. Garrett and M. R. W. Brown, *J. Pharm. Pharmacol.*, **15**, 185(1963).

(11) E. R. Garrett, *Arzneim. Forsch.*, **17**, 795(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 3, 1969, from the *College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32601*

Accepted for publication January 2, 1970.

The authors thank Dr. George B. Whitfield, Manager, Infectious Diseases Research, The Upjohn Co., Kalamazoo, Mich., for the supplies of spectinomycin, and Mr. George L. Perry, Sr., for technical assistance.

* To whom communications regarding this paper should be addressed.

Pharmacokinetic Evidence for Saturable Renal Tubular Reabsorption of Riboflavin

WILLIAM J. JUSKO and GERHARD LEVY*

Abstract □ Pharmacokinetic relationships have been developed to characterize a multicompartment drug distribution and elimination model which includes a saturable renal tubular reabsorption process. The derived expressions have been applied to serum concentration and urinary excretion data obtained after rapid intravenous administration of riboflavin to man and dog. The mathematical relationships and experimental data demonstrate the dependence of renal clearance on the serum concentration of the drug and on urine flow rate. The results of this study indicate that the renal excretion of riboflavin, like that of several other water-soluble vitamins, involves saturable tubular reabsorption as well as tubular secretion.

Keyphrases □ Riboflavin—saturable renal tubular reabsorption □ Renal tubular reabsorption, secretion—riboflavin □ Flavin-inulin—clearance ratio □ Pharmacokinetics—riboflavin renal clearance

A number of natural substances are known to be reabsorbed from renal tubules by a saturable process. Among these are the water-soluble vitamins: thiamine (1), pantothenic acid (2), and ascorbic acid (3). The renal excretion of another water-soluble vitamin, riboflavin, has been shown (4) to involve tubular secretion in man, and it has recently been suggested (5) that tubular reabsorption of riboflavin occurs in the chicken. In addition, an analysis of published data (6) indicates that the renal clearance of riboflavin in man decreases at lower serum levels of the vitamin.

A substance that undergoes saturable renal tubular reabsorption will characteristically yield higher renal clearances with increasing serum concentrations (7). The kinetics of this process have not been studied in detail, particularly over a wide concentration range such as is obtained after rapid intravenous injection of the substance. The single-injection technique for the study of renal clearance is often considered unsuitable (7) because of the difficulty of characterizing and accounting for the effect of rapid flux of the drug between plasma and tissue. Thus, this technically simpler method is often rejected in favor of the commonly used constant intravenous infusion method where plasma and tissue levels of the drug are maintained relatively constant. However, the constant infusion method will not reveal a possible concentration dependence of renal clearance unless the study is carried out at several, widely different infusion rates.

The purpose of this report is to present mathematical relationships which may be utilized for pharmacokinetic

a possible "active" process, could be postulated, it appears more reasonable to be critical of the assumption of rate-determining drug diffusion into the biophase.

An alternate explanation may be that drug action, as manifested by the degree of inhibition of generation rate, increases the sensitivity of the organism to further drug action until the steady-state generation rate is achieved. This "feedback" phenomenon is explainable on the premise that the rapidly equilibrated drug concentration in the biophase is competitive for receptor sites with a metabolic intermediate produced by the growing organism.

An analogy can be drawn to the sulfonamide–*p*-aminobenzoic acid competition. The initial fraction of receptor sites that is drug–receptor complex is reflected by an initial decrease in generation rate. This results in a diminution of the production of a vital metabolic intermediate. Subsequent depletion of excess stores of this intermediate in the normal metabolic or generation processes of the organism results in less amounts to compete with drug concentrations in the biophase, greater fractions of drug–receptor complex, and, consequently, further slowing of generation rates. On dilution of the drug with fresh medium, the drug in the organism's biophase rapidly reequilibrates with the consequence of less drug-bound receptor sites. A new steady-state production of metabolic intermediate may occur. This results in the observed reasonably rapid increase in microbial generation to new steady-state conditions (Fig. 2). A kinetically equivalent phenomenon is that the feedback is mediated by decreasing the number of available receptor sites concomitant with decreasing growth rates.

REFERENCES

(1) C. Lewis and H. W. Clapp, *Antibiot. Chemother.*, **11**, 118 (1961).

(2) J. Davies, P. Anderson, and B. D. Davis, *Science*, **149**, 1096(1965).

(3) E. R. Garrett, *Arzneim. Forsch.*, **16**, 1364(1966).

(4) E. R. Garrett and G. H. Miller, *J. Pharm. Sci.*, **54**, 427 (1965).

(5) J. B. Mielck and E. R. Garrett, *Chemotherapy*, **14**, 337 (1969).

(6) E. R. Garrett and O. K. Wright, *J. Pharm. Sci.*, **56**, 1576 (1967).

(7) E. R. Garrett, G. H. Miller, M. R. W. Brown, and K. Smith, *J. Med. Chem.*, **9**, 203(1966).

(8) E. R. Garrett, J. B. Mielck, J. K. Seydel, and H. J. Kessler, *ibid.*, **12**, 740(1969).

(9) E. R. Garrett, G. H. Miller, and M. R. W. Brown, *J. Pharm. Sci.*, **55**, 593(1966).

(10) E. R. Garrett and M. R. W. Brown, *J. Pharm. Pharmacol.*, **15**, 185(1963).

(11) E. R. Garrett, *Arzneim. Forsch.*, **17**, 795(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 3, 1969, from the *College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32601*

Accepted for publication January 2, 1970.

The authors thank Dr. George B. Whitfield, Manager, Infectious Diseases Research, The Upjohn Co., Kalamazoo, Mich., for the supplies of spectinomycin, and Mr. George L. Perry, Sr., for technical assistance.

* To whom communications regarding this paper should be addressed.

Pharmacokinetic Evidence for Saturable Renal Tubular Reabsorption of Riboflavin

WILLIAM J. JUSKO and GERHARD LEVY*

Abstract □ Pharmacokinetic relationships have been developed to characterize a multicompartment drug distribution and elimination model which includes a saturable renal tubular reabsorption process. The derived expressions have been applied to serum concentration and urinary excretion data obtained after rapid intravenous administration of riboflavin to man and dog. The mathematical relationships and experimental data demonstrate the dependence of renal clearance on the serum concentration of the drug and on urine flow rate. The results of this study indicate that the renal excretion of riboflavin, like that of several other water-soluble vitamins, involves saturable tubular reabsorption as well as tubular secretion.

Keyphrases □ Riboflavin—saturable renal tubular reabsorption □ Renal tubular reabsorption, secretion—riboflavin □ Flavin-inulin—clearance ratio □ Pharmacokinetics—riboflavin renal clearance

A number of natural substances are known to be reabsorbed from renal tubules by a saturable process. Among these are the water-soluble vitamins: thiamine (1), pantothenic acid (2), and ascorbic acid (3). The renal excretion of another water-soluble vitamin, riboflavin, has been shown (4) to involve tubular secretion in man, and it has recently been suggested (5) that tubular reabsorption of riboflavin occurs in the chicken. In addition, an analysis of published data (6) indicates that the renal clearance of riboflavin in man decreases at lower serum levels of the vitamin.

A substance that undergoes saturable renal tubular reabsorption will characteristically yield higher renal clearances with increasing serum concentrations (7). The kinetics of this process have not been studied in detail, particularly over a wide concentration range such as is obtained after rapid intravenous injection of the substance. The single-injection technique for the study of renal clearance is often considered unsuitable (7) because of the difficulty of characterizing and accounting for the effect of rapid flux of the drug between plasma and tissue. Thus, this technically simpler method is often rejected in favor of the commonly used constant intravenous infusion method where plasma and tissue levels of the drug are maintained relatively constant. However, the constant infusion method will not reveal a possible concentration dependence of renal clearance unless the study is carried out at several, widely different infusion rates.

The purpose of this report is to present mathematical relationships which may be utilized for pharmacokinetic

characterization, after rapid intravenous injection, of a multiple-compartment model which includes a saturable renal tubular reabsorption process. The derived expressions will be applied to serum concentration and urinary excretion data obtained after rapid intravenous administration of riboflavin to man and dog.

THEORETICAL

Renal Clearance Expressions—A multicompartment model in which drug is administered by single injection into the central compartment (which includes plasma) is depicted in Fig. 1. The distribution of drug between the central compartment (Subscript 1) and the tissue compartment (Subscript 2) is characterized by the apparent first-order transfer rate constants k_{12} and k_{21}. The entry of drug into the renal tubules is assumed to be by a first-order process characterized by the rate constant k_e. This may involve glomerular filtration with or without a tubular secretion component, provided the secretion process is of such high capacity as not to be saturated under the experimental conditions. The tubular reabsorption of the compound (transfer from urine to plasma) is considered to be of relatively low capacity (saturable) so that the Michaelis-Menten parameters T_m (maximum transport capacity) and K_m (Michaelis-Menten constant) are applicable. An extrarenal elimination component, represented by the apparent first-order rate constant k_{13}, is also employed. The renal clearance expressions to be derived are applicable to the described renal excretion mechanisms even if the extrarenal components of the model are more or less complex than those presented here.

The differential equation for the rate of urinary excretion (dX_u/dt) is

$$\frac{dX_u}{dt} = k_e \cdot X_1 - \frac{T_m \cdot C_u}{K_m + C_u} \qquad \text{(Eq. 1)}$$

where C_u is the concentration of drug in the urine. This is assumed to reflect the concentration of drug at the site of tubular reabsorption. Since

$$X_1 = C_p \cdot V_c \qquad \text{(Eq. 2)}$$

and

$$k_e = Cl_f/V_c \qquad \text{(Eq. 3)}$$

therefore,

$$Cl_f \cdot C_p = k_e \cdot X_1 \qquad \text{(Eq. 4)}$$

where C_p is the plasma concentration of drug, V_c is the volume of the central compartment, and Cl_f is the clearance for drug transfer from plasma to urine. Substituting Eq. 4 into Eq. 1 yields

$$\frac{dX_u}{dt} = Cl_f \cdot C_p - \frac{T_m \cdot C_u}{K_m + C_u} \qquad \text{(Eq. 5)}$$

Upon dividing both sides of Eq. 5 by C_p, the net or observed renal clearance (Cl_T) is described by

$$Cl_T = Cl_f - \frac{T_m \cdot C_u}{C_p(K_m + C_u)} \qquad \text{(Eq. 6)}$$

Equation 6 depicts the typical dependence of net clearance on plasma concentration of a drug subject to saturable tubular reabsorption. At relatively high C_p values, the last term of the equation becomes insignificant and $Cl_T \approx Cl_f$, which provides a method of estimating Cl_f.

Equation 6 can be further rearranged to yield

$$C_u = T_m \cdot \frac{C_u}{C_p(Cl_f - Cl_T)} - K_m \qquad \text{(Eq. 7)}$$

which, with a known Cl_f value, permits a linearization of experimental data whereby T_m and K_m can be determined from the slope and intercept, respectively, of a plot of C_u *versus* $C_u/C_p(Cl_f - Cl_T)$.

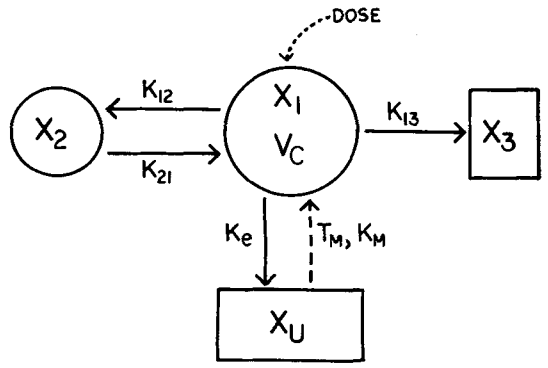


Figure 1—*Multiple-compartment open model with central* (X_1, V_c), *tissue* (X_2), *urine* (X_u), *and extrarenal* (X_3) *compartments. Symbols next to the arrows represent transfer rate constants between compartments. Apparent volume and amounts of drug are designated by* V_c *and* X, *respectively.*

Effect of Urine Flow Rate—At relatively high urine concentrations of drug (where $C_u \gg K_m$), Eq. 6 reduces to

$$Cl_T = Cl_f - \frac{T_m}{C_p} \qquad \text{(Eq. 8)}$$

At relatively low urine concentrations of drug (where $K_m \gg C_u$), Eq. 6 reduces to

$$Cl_T = Cl_f - \frac{C_u \cdot T_m}{C_p \cdot K_m} \qquad \text{(Eq. 9)}$$

Since

$$\frac{C_u}{C_p} = \frac{Cl_T}{R} \qquad \text{(Eq. 10)}$$

where R is the urine flow rate, Eq. 9 may be written as

$$Cl_T = Cl_f - \frac{Cl_T \cdot T_m}{R \cdot K_m} \qquad \text{(Eq. 11)}$$

Equation 8 shows that the net renal clearance (Cl_T) is independent of urine concentration (C_u) at relatively high urine and serum concentrations of drug. Consequently, Cl_T is also independent of urine flow rate under these conditions. On the other hand, Cl_T increases with increasing urine flow rate (R) at low plasma and urine concentrations (Eq. 11). Thus, evaluation of the effect of urine flow on renal clearances will provide further evidence for a saturable renal tubular reabsorption mechanism.

Explicit Solution for Transfer Rate Constants—The remaining differential equations that describe the multicompartment model shown in Fig. 1 are:

$$\frac{dX_1}{dt} = -k_e \cdot X_1 + \frac{T_m \cdot C_u}{K_m + C_u} - k_{12} \cdot X_1 - k_{13} \cdot X_1 + k_{21} \cdot X_2 \qquad \text{(Eq. 12)}$$

$$\frac{dX_2}{dt} = k_{12} \cdot X_1 - k_{21} \cdot X_2 \qquad \text{(Eq. 13)}$$

$$\frac{dX_3}{dt} = k_{13} \cdot X_1 \qquad \text{(Eq. 14)}$$

where the k's are apparent first-order rate constants dictating the rate of change (dX/dt) in the amounts of drug (X) in the respective compartments.

Equation 2 can be substituted into Eq. 14 to yield

$$\frac{dX_3}{dt} = k_{13} \cdot V_c \cdot C_p \qquad \text{(Eq. 15)}$$

The volume of the central compartment, V_c, can be determined after single i.v. injection of a drug from

$$V_c = \text{dose}/C_p^0 \qquad \text{(Eq. 16)}$$

where C_p^0 is the zero-time plasma drug concentration obtained by nonlinear least-squares extrapolation. At time = infinity (∞), X_3^∞ is the difference between the dose of drug administered and the total urinary recovery of the drug. Since

$$X_3^\infty = \int_0^\infty \frac{dX_3}{dt} \cdot dt \qquad \text{(Eq. 17)}$$

then by substituting from Eq. 15:

$$X_3^\infty = k_{13} \cdot V_c \cdot \int_0^\infty C_p \cdot dt \qquad \text{(Eq. 18)}$$

of which the integral portion is the area under the plasma level curve (AUC) which can be readily determined. Thus, k_{13} can be calculated from

$$k_{13} = X_3^\infty / V_c \cdot (\text{AUC}) \qquad \text{(Eq. 19)}$$

Similarly, X_3 as a function of time is

$$X_3^t = k_{13} \cdot V_c \cdot \int_0^t C_p \cdot dt \qquad \text{(Eq. 20)}$$

Substituting Eqs. 1 and 2 into Eq. 12 yields

$$\frac{dC_p}{dt} \cdot V_c = -\frac{dX_u}{dt} - k_{12} \cdot X_1 - k_{13} \cdot C_p \cdot V_c + k_{21} \cdot X_2 \qquad \text{(Eq. 21)}$$

which, upon rearrangement, becomes

$$\frac{dC_p}{dt} \cdot V_c + \frac{dX_u}{dt} + k_{13} \cdot C_p \cdot V_c = -k_{12} \cdot X_1 + k_{21} \cdot X_2 \qquad \text{(Eq. 22)}$$

As evident from Eq. 13, both sides of Eq. 22 are equal to $-dX_2/dt$, or

$$\frac{dX_2}{dt} = -\frac{dC_p}{dt} \cdot V_c - \frac{dX_u}{dt} - k_{13} \cdot C_p \cdot V_c \qquad \text{(Eq. 23)}$$

All parameters on the right-hand side of Eq. 23 can be determined directly from experimental data and from Eqs. 16 and 19. Thus, succcessive values of dX_2/dt as a function of time can be calculated. Since

$$X_2^t = \int_0^t \frac{dX_2}{dt} \cdot dt \qquad \text{(Eq. 24)}$$

values of X_2 as a function of time can be calculated using computer numerical integration[1] of Eqs. 23 and 24. With values of X_2, dX_2/dt, and X_1 thus obtainable at successive times (Eqs. 24, 23, and 2, respectively), rearrangement of Eq. 13 provides a slope–intercept method for determining the transfer rate constants k_{12} and k_{21} since

$$\frac{dX_2/dt}{X_1} = k_{12} - k_{21} \cdot \frac{X_2}{X_1} \qquad \text{(Eq. 25)}$$

Calculation of the amounts of drug in the respective compartments of the model as a function of time can then be achieved by use of Eqs. 2 (for X_1), 24 (for X_2), and 20 (for X_3). X_u as a function of time can be determined directly from the urinary excretion data.

EXPERIMENTAL

The studies were carried out in a healthy male human subject (26 years, 85.4 kg.) and an apparently healthy female mongrel dog (29.5 kg.). The human subject received an intravenous dose of 31 mg. riboflavin (FR) as sodium riboflavin-5′-phosphate (FMN).[2] Urine was collected at appropriate intervals for a total of 48 hr.

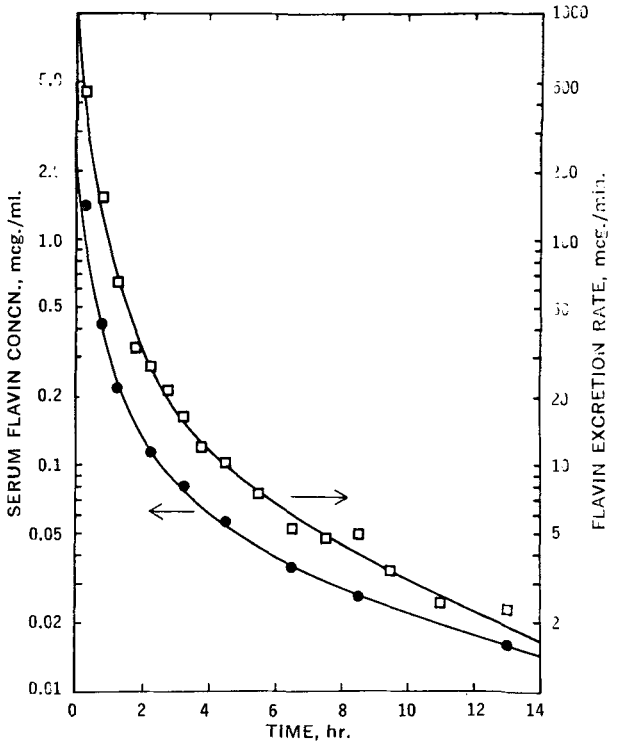


Figure 2—*Serum concentrations* (●) *and urinary excretion rates* (□) *of total riboflavin in a human subject after rapid intravenous injection of 31 mg. riboflavin (FR) as riboflavin-5′-phosphate (FMN). Solid lines represent a least-squares triexponential computer fit of the data.*

Blood samples (16 ml. each) were obtained from the antecubital vein at −0.5, 0.25, 0.75, 1.25, 2.25, 3.25, 4.5, 6.5, 8.5, 13.0, and 25.0 hr. after FMN injection. These were midtimes of urine-collection periods. The serum and urine samples were analyzed for FR, FMN, and endogenous creatinine.

Dog Study[3]—The dog was fasted overnight prior to the experiment but was allowed free access to water. Anesthesia was induced with sodium pentobarbital,[4] 30 mg./kg., and was maintained for the duration of the experiment by administration of 2–4 mg./kg. of the anesthetic approximately every hour as needed.

The animal was prepared for study using standard surgical techniques with care being taken to minimize blood loss. A tracheal cannula was inserted to prevent obstruction of respiration. A venous cannula in the left foreleg was used for infusion of isotonic saline solution to maintain adequate hydration of the dog. A 300-mg. priming dose of inulin[5] was injected intravenously 30 min. prior to rapid intravenous administration of 18.3 mg. of riboflavin.[6] Using a cannula placed in a right foreleg vein, inulin solution (12 mg./ml., 2 ml./min.) was infused for the duration of the experiment. Urine was generally collected every 10 min. for 280 min. from cannulas placed in each ureter. Arterial blood samples (11 ml. each) were collected at the midtimes of the urine-collection periods by means of a cannula placed into a left hindleg artery of the dog. Dilute heparin solution (50 units/ml.) was maintained in the arterial cannula between collection times to prevent clotting of blood in the cannula.

Analytical Methods—Riboflavin and FMN in serum and urine were assayed fluorometrically as described previously (9). Inulin and endogenous creatinine were determined by standard colorimetric techniques (10). There was no interference in the assay of any of the compounds due to the presence of the others.

Data for flavins and inulin were corrected for blank readings of urine and serum obtained prior to administration of the compounds to the test subjects.

[1] Mathematically, this can be done using either the trapezoidal rule or Simpson's rule (8).

[2] Sodium riboflavin-5′-phosphate, Hoffmann-La Roche, Nutley, N. J.

[3] The authors gratefully acknowledge the help of Dr. Barbara R. Rennick in this experiment.

[4] Diabutal, 60 mg./ml., Diamond Laboratories, Des Moines, Iowa.

[5] Inulin, Nutritional Biochemicals Corp., Cleveland, Ohio.

[6] Riboflavin USP, Hoffmann-La Roche, Nutley, N. J.

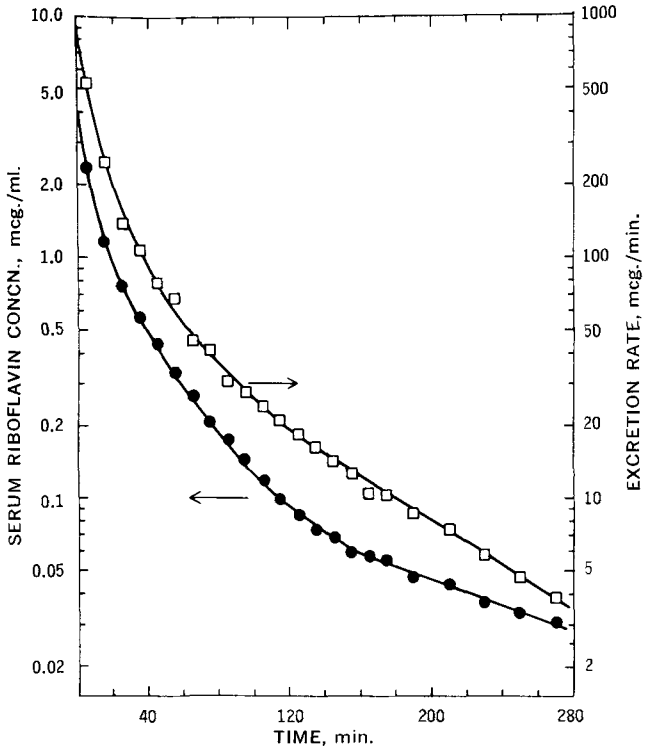


Figure 3—*Serum concentrations and urinary excretion rates of riboflavin in a dog after rapid intravenous injection of 18.3 mg. riboflavin. Symbols and lines are defined as in Fig. 2.*

RESULTS

Renal Clearances—The declines in serum concentrations and urinary excretion rates of flavin after rapid intravenous injection of the vitamin to the man and the dog are shown in Figs. 2 and 3, respectively. The data are plotted semilogarithmically as a function of time. Graphical analysis of the experimental results by the method of residuals (11) indicated that the decline of all data was triexponential. A nonlinear least-squares computer fit[7] of the data was obtained using the "NLIN" digital computer program of Marquardt (12). The general equation which was fit to the data is

$$F = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + C \cdot e^{-\gamma t} \qquad \text{(Eq. 26)}$$

where F represents either flavin plasma levels or urinary excretion rates, t is time, and the remaining symbols are constants. Since the experimental data extended over a range of more than two orders of magnitude, they were converted to their respective logarithm values to reduce bias resulting from the numerical size of the larger values relative to the smaller values. The six parameters which were obtained for each of the four sets of data are shown in Table I; the solid lines of Figs. 2 and 3 represent the computer least-squares fit to the respective data.

All human pharmacokinetic analyses in this study were carried out on the basis of total flavin levels, *i.e.*, riboflavin and riboflavin-5′-phosphate (FMN). This was necessary because of the rapid dephosphorylation of FMN in the blood *in vitro* (13) and because of limited assay sensitivity for FMN in the presence of an excess of riboflavin. However, estimations of renal clearances of FMN during the early sampling periods (when FMN levels were high) showed that the clearance of this substance was similar to that of riboflavin itself.

The renal clearances of flavin in man are shown in Fig. 4 plotted as a function of serum flavin concentration. All renal clearance values were obtained by the usual method of dividing the urinary excretion rate by the midtime serum concentration. The solid line of Fig. 4 represents flavin clearances calculated from the least-squares parameters of Table I. The figure also shows the endogenous creatinine clearances obtained at the same times as a measure of the glomerular filtration rate (GFR). The GFR remained essentially constant for the duration of the experiment, and the average value obtained was 130 ml./min.

The flavin clearances in Fig. 4 show a profile characteristic of saturable tubular reabsorption since the net clearance values progressively diminish as serum levels of the vitamin decline. Tubular secretion of the vitamin also occurs since the net clearance exceeds the GFR. Since the substrate concentration of the tubular secretory process (presumably plasma or cell water concentration of flavin) is appreciably lower than the flavin concentration in the urine, and since the net clearance of flavin increases rather than decreases at

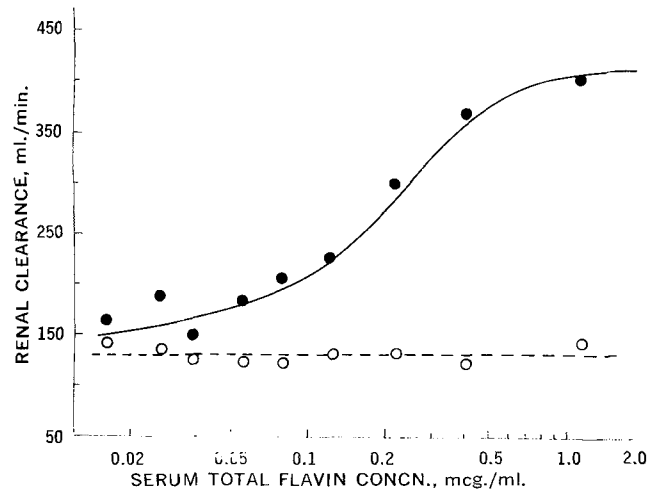


Figure 4—*Renal clearances of total riboflavin (●) in the human subject as a function of serum total riboflavin concentration. Solid line represents a least-squares fit of the data according to the constants of Table I. Open symbols show simultaneously obtained creatinine clearances.*

Table I—Pharmacokinetic Constants[a] Describing the Serum Concentrations and Urinary Excretion Rates of Riboflavin after Its Intravenous Administration to Man and Dog

Constant[b]	Serum Concentrations Man	Serum Concentrations Dog	Urinary Excretion Rates Man	Urinary Excretion Rates Dog
A, mcg./J	1.83	2.40	803	594
α, hr.$^{-1}$	3.24	10.4	3.02	9.69
B, mcg./J	0.316	1.43	94.7	257
β, hr.$^{-1}$	0.717	1.86	0.824	2.46
C, mcg./J	0.0719	0.0975	14.6	58.1
γ, hr.$^{-1}$	0.120	0.258	0.148	0.602

[a] From least-squares computer fits of Eq. 26. [b] For serum: J = ml.; for urinary excretion: J = min.

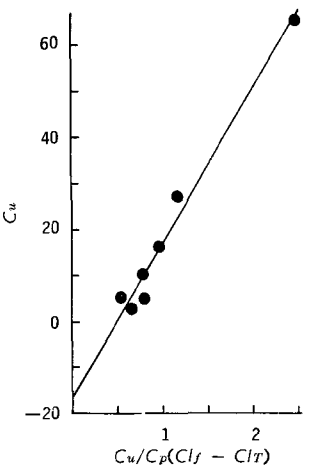


Figure 5—*Linearization method for obtaining the Michaelis-Menten constants of the renal tubular reabsorption process according to Eq. 7. The line fitted to the data has a slope of* T_m *and an intercept of* $-K_m$.

[7] The contribution of C.D.C. 6400 computer time by the State University of New York at Buffalo Computer Center is gratefully acknowledged.

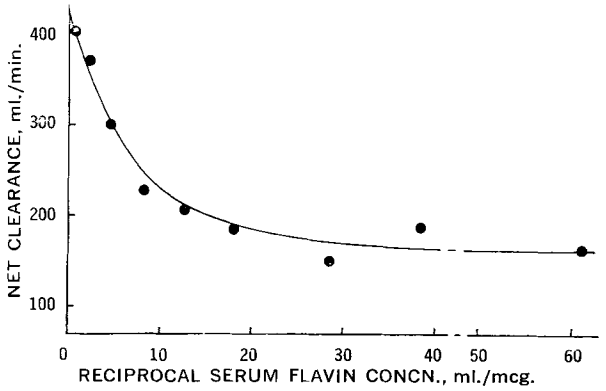


Figure 6—*Net renal clearance* (Cl_T) *of riboflavin in man plotted as a function of reciprocal serum concentration. Symbols are experimental data, and the line represents clearances calculated according to Eq. 6 using the derived values of* Cl_f, T_m, *and* K_m.

high plasma concentrations of the vitamin, it was assumed that the secretory process was not capacity limited under the experimental conditions. Tubular secretion and glomerular filtration of flavin were therefore treated pharmacokinetically as apparent first-order processes. On the assumption that the contribution of the tubular reabsorption process to the net clearance is negligible at relatively high serum concentrations of the vitamin, a theoretical Cl_f was calculated from the C_p^0 and dX_u^0/dt values obtained by computer from plots of these data as a function of time.

This value, 420 ml./min. in man, was taken as an estimate of Cl_f and used in Eq. 7 for linearization of the renal excretion data by a plot of C_u *versus* $C_u/C_p \cdot (Cl_f - Cl_T)$. The graphical results are shown in Fig. 5. A least-squares fit of the data yielded a slope of 33.3 mcg./min. for T_m and an intercept K_m value of 16.3 mcg./ml. The three renal excretion parameters can be more readily obtained using Eq. 6 with a plot such as shown in Fig. 6. A direct computer fit of Eq. 6 to the experimental data using the Marquardt digital computer program (12) yielded nearly identical values for Cl_f, T_m, and K_m (425 ml./min., 34.1 mcg./min., and 17.9 mcg./ml., respectively). In the human subject, the urine flow rates were essentially constant at about 1 ml./min. The curve shown in Fig. 6 represents the calculated net clearance (Cl_T) of flavin, using the graphically derived values of Cl_f, T_m, and K_m in Eq. 6. The excellent fit of the experimental points to the calculated line demonstrates the suitability of the pharmacokinetic expressions for describing the concentration dependence of the renal clearances of riboflavin.

The renal clearances of riboflavin and urine flow rates obtained in the dog are shown in Fig. 7 as a function of time. The solid line depicts renal clearance values calculated from the least-squares parameters for the dog listed in Table I. The inulin clearances which were obtained as a measure of GFR were relatively constant and averaged 98.0 ml./min. with a standard deviation of 7.6. The fact that the flavin–inulin clearance ratio is greater than unity and the

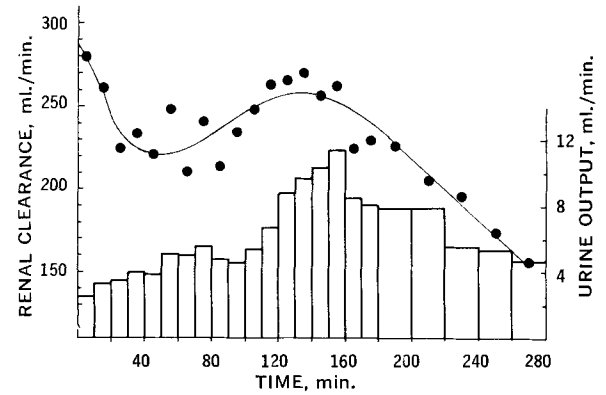


Figure 7—*Renal clearance of riboflavin* (●) *and urine flow rates* (*bars*) *in the dog as a function of time after riboflavin injection. The solid curve is a computer least-squares fit of the renal clearance data according to the constants of Table I.*

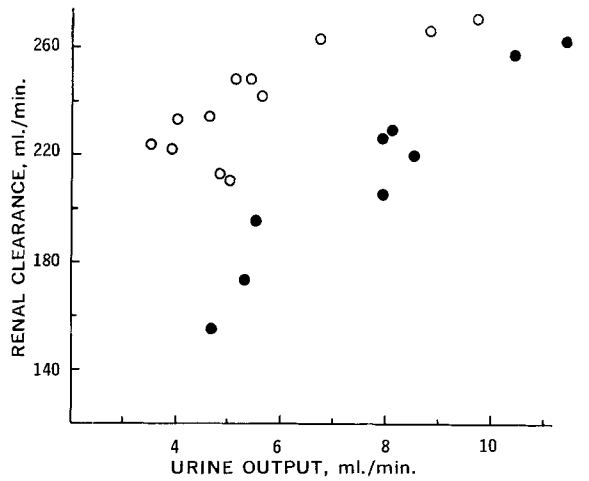


Figure 8—*Effect of urine flow rate on riboflavin clearance in the dog. Open symbols are results obtained during 0–140 min. and solid symbols are results obtained during 140–280 min. after riboflavin administration.*

decrease in the clearance values of riboflavin with decreasing serum concentrations indicate that the renal clearance of riboflavin in the dog, as in man, involves both tubular secretion and saturable tubular reabsorption. These data also demonstrate the dependence of net renal clearance on urine flow rate, but the relationship is better exemplified in Fig. 8. In that figure, the net renal clearance of riboflavin is plotted as a function of urine flow rate with the data obtained in the first 140 min. separated from the data subsequently obtained. As predicted by Eq. 5, there is little effect of urine flow on the early riboflavin clearances ($C_p > 0.06$ mcg./ml.), but net clearance increased markedly with increasing urine flow rate when riboflavin serum and urine levels were relatively low.

For the dog, the earlier described computer program (12) was used to calculate Cl_f, T_m, and K_m and values are listed in Table II.

Distribution and Elimination Parameters of the Model—The last portion of the *Theoretical* section includes methods of calculating the rate constants k_{13}, k_{12}, and k_{21}. The volume of the central compartment for man and dog was obtained using Eq. 16, with C_p^0 determined from the least-squares parameters for the serum level data in Table I where

$$C_p^0 = A + B + C \qquad \text{(Eq. 27)}$$

The areas under the plasma level curves for man and dog were

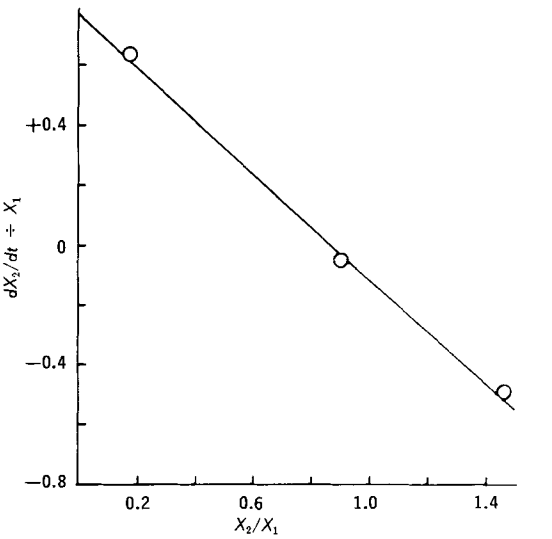


Figure 9—*Plot of pharmacokinetic data from the human according to Eq. 25 to yield an intercept of* k_{12} *on the ordinate and a slope of* $-k_{21}$. *Circles are values calculated directly from the experimental data and the line is the computer regression calculation.*

Table II—Pharmacokinetic Constants of the Multicompartment Model for Riboflavin Distribution and Elimination in Man and Dog

Constant	Man	Dog
Cl_f, ml./min.	420	232
T_m, mcg./min.	33.3	21.9
K_m, mcg./ml.	16.3	9.61
k_e, hr.$^{-1}$	1.80	2.98
k_{13}, hr.$^{-1}$	0.133	0.433
k_{12}, hr.$^{-1}$	0.768	3.01
k_{21}, hr.$^{-1}$	0.872	5.11
V_c, l.	14.0	4.66
V_c, % b.w.	16.4	15.8
Wt., kg.	85.4	29.5
Dose, mg.	31.0	18.3
Urinary excretion, %	89.8	85.0

found to be 1.62 and 1.36 mcg. hr./ml., respectively. The k_{13} values for man and dog, which were calculated by means of Eq. 19, are 0.133 and 0.433 hr.$^{-1}$, respectively. All numerical integrations were carried out using the trapezoidal rule with sequential areas determined at 0.05-hr. increments.

Figure 9 shows the plot of $dX_2/dt/X_1$ *versus* X_2/X_1 for the data obtained in the human subject. There is good agreement between the points calculated directly from the experimental data and the computer-calculated regression line. A similar plot was obtained for the dog; the resultant values of k_{12} and k_{21} which were found in the two experiments are listed in Table II.

Since only one man and one dog were employed in the present study, the rate constants and pharmacokinetic parameters listed in Table II cannot be used as a measure of species differences. However, it is interesting to note several similarities in the data of Table II for man and dog. The volume of the central compartment is about 16% of body weight in both. The percent urinary excretion

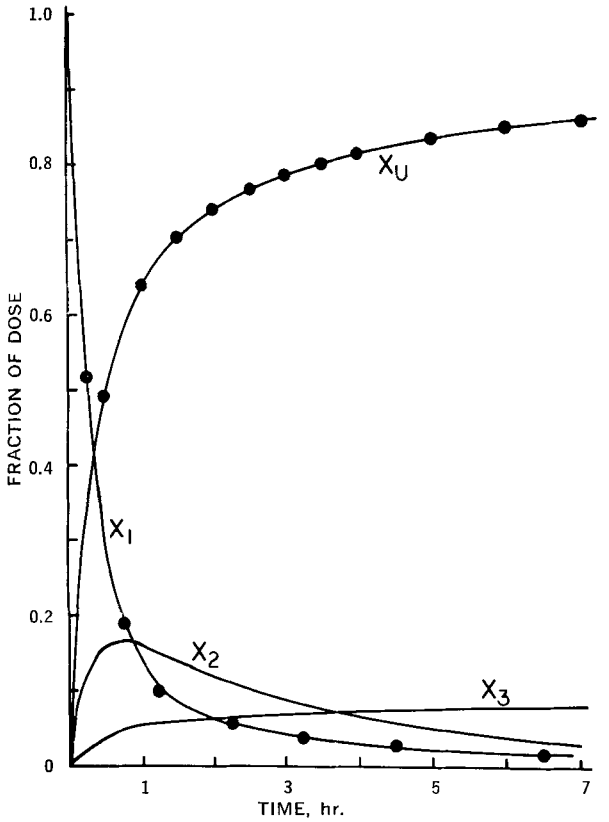


Figure 10—*Amounts of total riboflavin (as fraction of the administered dose) as a function of time in the four compartments of the pharmacokinetic model for man. Data points were obtained experimentally from serum concentrations (for Curve* X_1*) and urinary excretion rates (for Curve* X_u*).*

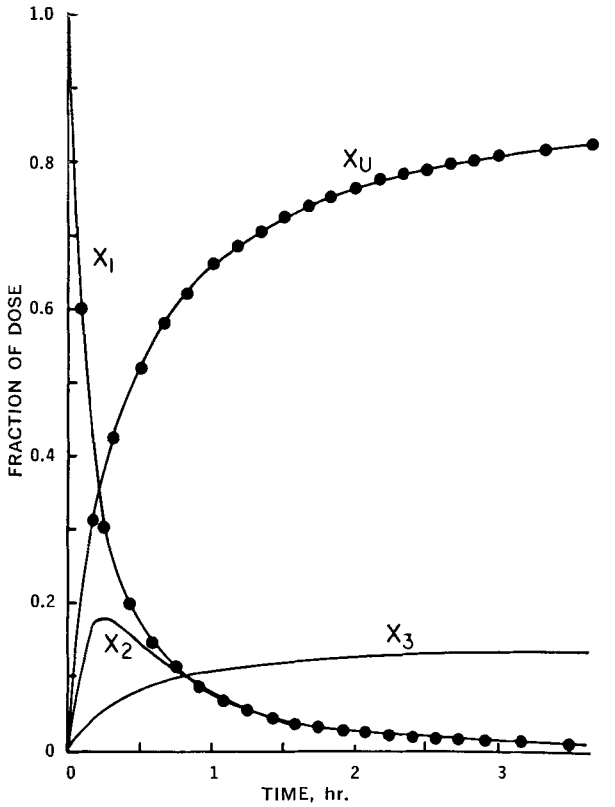


Figure 11—*Fraction of the riboflavin dose as a function of time in the four compartments of the pharmacokinetic model for the dog. Data points and lines were obtained as in Fig. 10.*

and the ratios of $k_e:k_{13}$, $k_{21}:k_{12}$, and $T_m:K_m$ are similar. However, the tubular secretion process (Cl_f) had a relatively much greater capacity in the dog, resulting in more rapid elimination of the vitamin.

The parameters of Table II were used with the appropriate equations of the *Theoretical* section to calculate the fraction of the dose of flavin in the respective compartments of the pharmacokinetic model of Fig. 1. The results of such calculations are shown in Fig. 10 for the man and in Fig. 11 for the dog. The experimental data shown for the central (X_1) and urine (X_u) compartments are in excellent agreement with the calculated curves. As predicted by the several similarities in the parameters listed in Table II, the time courses of fractional drug levels in man and dog are quite similar, except that all processes occur at a more rapid rate in the dog. The parameters and results of Table II and Figs. 10 and 11 were further confirmed using the "MIMED" digital computer analog simulation program (14) with the appropriate differential equations (Eqs. 2 and 12–14).

Calculation of the sum of the fraction of the dose of drug in the various compartments using the derived parameters of the model provides an indication of the overall error involved in the calculations. The discrepancy between the summation of fractional drug levels and the administered dose ranged between 0 and 2%. Since the calculated X_1 and X_u values agreed very well with the experimental data, most of the error can be attributed to calculation of X_3 and particularly X_2. This is to be anticipated because of the three components of Eq. 23 which must be calculated from the experimental data and the subsequent necessity of integrating this equation at discrete time increments to obtain X_2 values.

DISCUSSION

A pharmacokinetic model has been developed to characterize the kinetics of renal clearance by simultaneous first-order renal excretion and saturable renal tubular reabsorption after rapid intravenous administration of a drug. This model and the results obtained experimentally demonstrate the dependence of renal clearance on the serum concentration of a drug and the rate of urine flow.

It was assumed in the calculations that the urine concentration of drug approximates the concentration at the site of renal tubular reabsorption. If tubular reabsorption occurs in the distal renal tubule, this assumption is probably quite reasonable. The simultaneous occurrence of glomerular filtration, tubular secretion, tubular reabsorption, and water reabsorption produces a proximal to distal gradient in tubular concentrations of drug, and the actual drug concentration at the site of reabsorption will lie between the glomerular filtrate concentration ($\approx C_p$) and the urine concentration in the ureter (C_u). If the actual drug concentration (S) at the site of tubular reabsorption is considered to be the average of the filtrate and urine concentrations,

$$S = {}^1/_2(C_p + C_u) \qquad \text{(Eq. 28)}$$

and since $C_u \gg C_p$, then $S \approx {}^1/_2C_u$. Substituting this average concentration into Eq. 6 and rearranging yield

$$Cl_T = Cl_f - \frac{T_m \cdot C_u}{C_p({}^1/_2K_m + C_u)} \qquad \text{(Eq. 29)}$$

The T_m value, but not the value of K_m, is independent of the assumption represented by Eq. 28. Equation 29 indicates that K_m is simply a proportionality constant which is dependent on the assumption of how best to approximate the drug concentration at the site of reabsorption. It, therefore, should not be used for comparison of data for drugs which are reabsorbed at different sites in the renal tubule.

The renal clearance equations which were developed are independent of the complexity of the extrarenal portion of the model. Drug levels are measured in the serum and urine, and the clearance equations are only concerned with the relationship between the two compartments as reflected by these fluids.

The nonlinear relationship which characterizes the tubular reabsorption mechanism does not permit solution of the extrarenal parameters of the model by use of the usual pharmacokinetic treatments such as described by Rescigno and Segre (15). However, because drug levels were simultaneously determined in two compartments (central and urine), it was possible to develop explicit solutions for the rate parameters k_{13}, k_{12}, and k_{21}. The results of such calculations yield a very good fit to the test data, but the values obtained for X_2 as a function of time may be slightly in error. This error arises from experimental variation in the values of dC_p/dt, dX_u/dt, and C_p; the necessity of predetermining V_c and k_{13} for use in Eq. 23; and the probable error due to integrating this equation at discrete time increments. The use of computer calculations and least-squares data treatments, however, tends to minimize such error. It is easier to utilize the mathematical relationships described here than to resort to the analog computer for an empirical resolution of the parameters. The linearity of a plot such as is shown in Fig. 9 also provides a test to determine whether or not a more complex model (*e.g.*, two-tissue compartments) might be more appropriate.

The serum concentration and urinary excretion rate data in man and dog could be fitted effectively to a triexponential equation. For a linear multiple-compartment model, such data would be indicative of a three-compartment open model; parameters such as those shown in Table I could be directly utilized for solution of the microscopic transfer rate constants (15). However, because of the nonlinear tubular reabsorption process, this approach was not feasible in this study, and the least-squares triexponential fit of the data (Table I and Eq. 26) was used only for a suitable description of the data which facilitated computer numerical analyses for solution of the pharmacokinetic equations listed in the *Theoretical* section.

With regard to the test compound used in the present study, the data indicate that the renal excretion of riboflavin in man and dog involves a specialized secretory process of apparent high capacity as well as an easily saturated tubular reabsorption process. The first conclusion is based on the renal clearance data which show that riboflavin clearances are much greater than the glomerular filtration rate, even without correction of the clearance data for the moderate extent of serum protein binding of the flavins. Previous work has shown that riboflavin and FMN, in the serum concentration range encountered in this study, are about 60% bound to plasma proteins in man (9). The dog exhibits 19% binding of riboflavin to serum proteins (16). In both species, the extent of flavin binding is independent of serum concentration over the range used

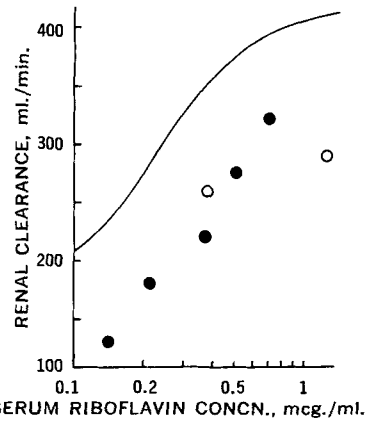


Figure 12—*Relationship between renal clearance and serum concentration of riboflavin in a human subject who received 84 mg. riboflavin by intravenous infusion over 2 hr. The open circles are values obtained during the infusion (when serum concentrations were rising) and the solid circles are values obtained postinfusion (when serum concentrations were falling). Based on data from* Reference 6. *The curve is from Fig. 4 of the present study.*

in the present study. It has been further demonstrated that probenecid, a potent inhibitor of many active transport processes (17), inhibits the renal clearance of riboflavin in man (13) and dog (16). Markkanen *et al.* (18) have also shown that probenecid decreases the basal excretion of riboflavin in man and rabbits whose riboflavin intake was limited to that derived from the normal diet. Rennick (19), whose work first demonstrated the tubular secretion of riboflavin in chickens, also noted that this process was inhibited by probenecid.

The decline in renal clearances of riboflavin with decreasing serum levels of the vitamin indicates that the vitamin undergoes saturable tubular reabsorption. The kinetics of this process are well characterized in man and dog by a model which assumes saturable renal tubular reabsorption. Experimental confirmation of the theoretically predicted effect of urine flow rate on the renal clearance of riboflavin in the dog adds further support to this interpretation. Evidence in the literature also suggests a dependence of riboflavin excretion on urine flow rate. Tucker *et al.* (20) and Johnson (21) reported increases in riboflavin excretion in human subjects after diuresis due to increased water intake. A reexamination of data published by Markkanen (22) shows that diuresis induced by acetazolamide and mercaptomerin also increases the basal excretion of riboflavin in rabbits.

The relationship between renal clearance and serum concentration shown in Fig. 4 was determined under dynamic conditions, *i.e.*, when serum concentrations of riboflavin were changing rapidly. These conditions are quite different from the usual conditions for the determination of clearance values, where relatively constant serum concentrations are maintained by continuous infusion of drug. The question then arises whether the clearance *versus* serum concentration profile obtained in the present study is an artifact due to distribution effects. There is strong evidence that this is not so. First, the magnitude of the clearance changes (almost threefold) is much greater than could be explained by distribution effects, particularly since, according to the pharmacokinetic analysis, no more than 18% of the dose of riboflavin reaches the "tissue" compartment. Second, analysis of riboflavin serum and urinary excretion data from the literature (6) provides independent confirmation of the results of the present study. The renal clearance values calculated from these data, which were obtained by infusing a total of 84 mg. riboflavin over 2 hr., are shown in Fig. 12. The clearance *versus* riboflavin concentration profile is essentially the same as was obtained in the present study, although the numerical values are somewhat lower, probably reflecting differences in body size. The clearance values during infusion of riboflavin (*i.e.*, when the serum concentration of riboflavin was rising) agree well with the postinfusion values (*i.e.*, when the serum concentration of riboflavin was falling). This, and the fact that distribution factors would have much smaller effects when the drug is administered by infusion rather than by rapid intravenous injection, strongly support the conclusions of the present study.

It is now apparent that there exist specialized transport mechanisms for riboflavin, other water-soluble vitamins, and certain amino acids and sugars for renal tubular reabsorption as well as intestinal absorption (3, 7, 23, 24). Both mechanisms are easily saturable. The specialized renal tubular reabsorption process helps to prevent a possible depletion of body levels of these essential nutrients. On the other hand, saturability of transport of nutrients across the small intestine sets an upper limit on the amount of these substances which can be absorbed.

REFERENCES

(1) H. N. Haugen, *Scand. J. Clin. Lab. Invest.*, **13,** 61(1961).

(2) K. Roholt and V. Schmidt, *ibid.*, **3,** 108(1951).

(3) R. F. Pitts, "Physiology of the Kidney and Body Fluids," Yearbook Medical Publishers, Chicago, Ill., 1963, p. 81.

(4) G. Levy and W. J. Jusko, *J. Pharm. Sci.*, **55,** 1322(1966).

(5) J. W. Cowan, R. V. Boucher, and E. G. Buss, *Poultry Sci.*, **45,** 538(1966).

(6) B. Stripp, *Acta Pharmacol. Toxicol.*, **22,** 353(1965).

(7) H. W. Smith, "The Kidney," Oxford University Press, New York, N. Y., 1951, p. 81.

(8) M. H. Protter and C. B. Morrey, "College Calculus with Analytic Geometry," Addison-Wesley, Reading, Mass., 1964, p. 524.

(9) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **58,** 58(1969).

(10) "Laboratory Manual of Pediatric Micro- and Ultramicro-Biochemical Techniques," 3rd ed., D. O'Brien and F. A. Ibbott, Eds., Harper and Row, New York, N. Y., 1961, p. 100.

(11) J. G. Wagner, *Drug Intel.*, **2,** 206(1968).

(12) D. W. Marquardt, DPE-NLIN, Share General Library Program No. 7-1354, 1964.

(13) W. J. Jusko, G. Levy, S. J. Yaffe, and R. Gorodischer, *J. Pharm. Sci.*, **59,** 473(1970).

(14) MIMED, State University of New York at Buffalo Computer Center, adaptation of MIMIC, Control Data Corp., St Paul, Minn., Publ. No. 44610400, 1968.

(15) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Waltham, Mass., 1966, p. 75.

(16) W. J. Jusko, B. R. Rennick, and G. Levy, to be published.

(17) K. H. Beyer, H. F. Russo, E. K. Tillson, A. K. Miller, W. F. Verway, and S. R. Gass, *Amer. J. Physiol.*, **166,** 625(1951).

(18) T. Markkanen, P. Toivanen, A. Toivanen, and E. Sotaniemi, *Scand. J. Clin. Lab. Invest.*, **15,** 511(1963).

(19) B. R. Rennick, *Proc. Soc. Exp. Biol. Med.*, **103,** 241(1960).

(20) R. G. Tucker, O. Mickelson, and A. Keys, *J. Nutr.*, **72,** 251 (1960).

(21) R. E. Johnson, *Fed. Proc.*, **5,** 139(1946).

(22) T. Markkanen, *Z. Vitan. Hormon. Fermentforsch.*, **14,** 72(1965).

(23) T. H. Wilson, "Intestinal Absorption," Saunders, Philadelphia, Pa., 1962, p. 51.

(24) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **56,** 58(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 10, 1969, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication January 12, 1970.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciencies, Montreal meeting, May 1969.

This investigation was supported in part by Public Health Service Fellowship 5-F1-GM-33,073 for W. J. J. from the National Institutes of General Medical Sciences, Bethesda, Md.

* To whom requests for reprints should be directed.

Distribution, Excretion, and Metabolism of ^{14}C-Labeled Quaternary Ammonium Salt of Mepazine and Promethazine in Rats

C. L. HUANG, J. A. YEH, and S. Y. HSU

Abstract □ Studies on the biological fate and antimicrobial activity of mepazine and promethazine methiodide are presented. Synthesis of ^{14}C-methiodide and estimation of the unchanged compound in biological materials are described. After intraperitoneal administration, the majority of these compounds was excreted in feces. The radioactivity in the liver and kidneys prevailed over other organs. Blood levels were low but above the significant level in both compounds. Brain level of promethazine methiodide-^{14}C was detectable but that of mepazine methiodide-^{14}C was insignificant. These compounds displayed a significant antimicrobial activity.

Keyphrases □ Mepazine and promethazine ^{14}C-methiodides—synthesis □ Biological fate—mepazine and promethazine ^{14}C-methiodides □ UV spectrophotometry—identity □ Paper chromatography—separation □ Radiochromatography—purity determination

Mepazine and promethazine are derivatives of phenothiazine but they display quite a different mode of action. Pharmacologically, mepazine is less potent than chlorpromazine (1–5). The drug possesses a neuroleptic effect similar to that of chlorpromazine (6–8); however, mepazine is not a drug of choice for the treatment of psychoses because of the high incidences of side effects such as agranulocytosis, seizures, depression of bone marrow, and jaundice. Promethazine (9, 10) finds its use mainly as a potent antihistaminic drug with a prolonged duration of action and as a drug for motion sickness. The difference in the pharmacological activities between these two drugs appears to be attributable to the effect of the side chains attached to the nitrogen of the phenothiazine ring system. All psychoactive phenothiazine derivatives possess a three-carbon bridge between the terminal and the ring nitrogen, while the presence of a two-carbon bridge between the two nitrogens appears to enhance the antihistaminic effect but diminishes the psychoactive properties of phenothiazine derivatives.

Quaternization of a side-chain nitrogen does not seem to decrease the toxicity of phenothiazine neuroleptics (11, 12). In many cases, toxicity appears to be enhanced.

It is now apparent that there exist specialized transport mechanisms for riboflavin, other water-soluble vitamins, and certain amino acids and sugars for renal tubular reabsorption as well as intestinal absorption (3, 7, 23, 24). Both mechanisms are easily saturable. The specialized renal tubular reabsorption process helps to prevent a possible depletion of body levels of these essential nutrients. On the other hand, saturability of transport of nutrients across the small intestine sets an upper limit on the amount of these substances which can be absorbed.

REFERENCES

(1) H. N. Haugen, *Scand. J. Clin. Lab. Invest.*, **13,** 61(1961).

(2) K. Roholt and V. Schmidt, *ibid.*, **3,** 108(1951).

(3) R. F. Pitts, "Physiology of the Kidney and Body Fluids," Yearbook Medical Publishers, Chicago, Ill., 1963, p. 81.

(4) G. Levy and W. J. Jusko, *J. Pharm. Sci.*, **55,** 1322(1966).

(5) J. W. Cowan, R. V. Boucher, and E. G. Buss, *Poultry Sci.*, **45,** 538(1966).

(6) B. Stripp, *Acta Pharmacol. Toxicol.*, **22,** 353(1965).

(7) H. W. Smith, "The Kidney," Oxford University Press, New York, N. Y., 1951, p. 81.

(8) M. H. Protter and C. B. Morrey, "College Calculus with Analytic Geometry," Addison-Wesley, Reading, Mass., 1964, p. 524.

(9) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **58,** 58(1969).

(10) "Laboratory Manual of Pediatric Micro- and Ultramicro-Biochemical Techniques," 3rd ed., D. O'Brien and F. A. Ibbott, Eds., Harper and Row, New York, N. Y., 1961, p. 100.

(11) J. G. Wagner, *Drug Intel.*, **2,** 206(1968).

(12) D. W. Marquardt, DPE-NLIN, Share General Library Program No. 7-1354, 1964.

(13) W. J. Jusko, G. Levy, S. J. Yaffe, and R. Gorodischer, *J. Pharm. Sci.*, **59,** 473(1970).

(14) MIMED, State University of New York at Buffalo Computer Center, adaptation of MIMIC, Control Data Corp., St Paul, Minn., Publ. No. 44610400, 1968.

(15) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Waltham, Mass., 1966, p. 75.

(16) W. J. Jusko, B. R. Rennick, and G. Levy, to be published.

(17) K. H. Beyer, H. F. Russo, E. K. Tillson, A. K. Miller, W. F. Verway, and S. R. Gass, *Amer. J. Physiol.*, **166,** 625(1951).

(18) T. Markkanen, P. Toivanen, A. Toivanen, and E. Sotaniemi, *Scand. J. Clin. Lab. Invest.*, **15,** 511(1963).

(19) B. R. Rennick, *Proc. Soc. Exp. Biol. Med.*, **103,** 241(1960).

(20) R. G. Tucker, O. Mickelson, and A. Keys, *J. Nutr.*, **72,** 251 (1960).

(21) R. E. Johnson, *Fed. Proc.*, **5,** 139(1946).

(22) T. Markkanen, *Z. Vitan. Hormon. Fermentforsch.*, **14,** 72(1965).

(23) T. H. Wilson, "Intestinal Absorption," Saunders, Philadelphia, Pa., 1962, p. 51.

(24) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **56,** 58(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 10, 1969, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication January 12, 1970.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciencies, Montreal meeting, May 1969.

This investigation was supported in part by Public Health Service Fellowship 5-F1-GM-33,073 for W. J. J. from the National Institutes of General Medical Sciences, Bethesda, Md.

* To whom requests for reprints should be directed.

Distribution, Excretion, and Metabolism of ^{14}C-Labeled Quaternary Ammonium Salt of Mepazine and Promethazine in Rats

C. L. HUANG, J. A. YEH, and S. Y. HSU

Abstract □ Studies on the biological fate and antimicrobial activity of mepazine and promethazine methiodide are presented. Synthesis of ^{14}C-methiodide and estimation of the unchanged compound in biological materials are described. After intraperitoneal administration, the majority of these compounds was excreted in feces. The radioactivity in the liver and kidneys prevailed over other organs. Blood levels were low but above the significant level in both compounds. Brain level of promethazine methiodide-^{14}C was detectable but that of mepazine methiodide-^{14}C was insignificant. These compounds displayed a significant antimicrobial activity.

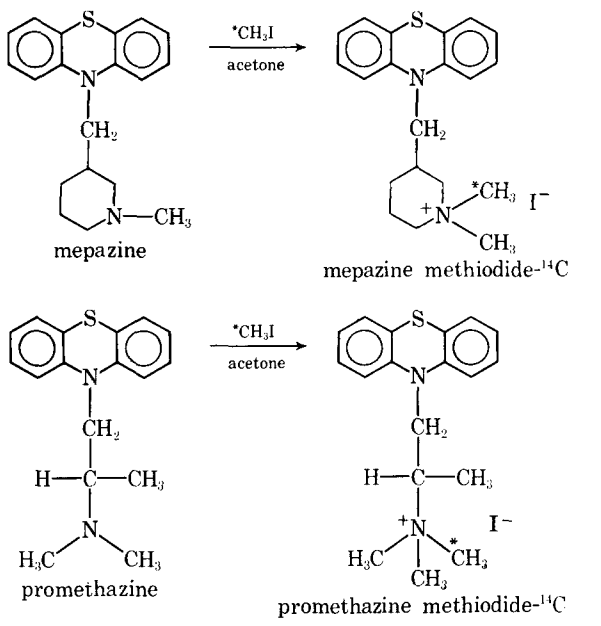
Keyphrases □ Mepazine and promethazine ^{14}C-methiodides—synthesis □ Biological fate—mepazine and promethazine ^{14}C-methiodides □ UV spectrophotometry—identity □ Paper chromatography—separation □ Radiochromatography—purity determination

Mepazine and promethazine are derivatives of phenothiazine but they display quite a different mode of action. Pharmacologically, mepazine is less potent than chlorpromazine (1–5). The drug possesses a neuroleptic effect similar to that of chlorpromazine (6–8); however, mepazine is not a drug of choice for the treatment of psychoses because of the high incidences of side effects such as agranulocytosis, seizures, depression of bone marrow, and jaundice. Promethazine (9, 10) finds its use mainly as a potent antihistaminic drug with a prolonged duration of action and as a drug for motion sickness. The difference in the pharmacological activities between these two drugs appears to be attributable to the effect of the side chains attached to the nitrogen of the phenothiazine ring system. All psychoactive phenothiazine derivatives possess a three-carbon bridge between the terminal and the ring nitrogen, while the presence of a two-carbon bridge between the two nitrogens appears to enhance the antihistaminic effect but diminishes the psychoactive properties of phenothiazine derivatives.

Quaternization of a side-chain nitrogen does not seem to decrease the toxicity of phenothiazine neuroleptics (11, 12). In many cases, toxicity appears to be enhanced.

A comparison was made on the distribution, excretion, and metabolism of ^{35}S-labeled promethazine and a quaternary phenothiazine compound (Aprobit) [1-(10-phenothiazinylmethyl)ethyl - 2 - hydroxyethyldimethylammonium chloride] on mice (13). Fecal excretion was found to be the major route of elimination of the quaternary phenothiazine compound after oral administration, but the drug was excreted mainly unchanged in urine after parenteral administration. Contrary to this report, the majority of the parenterally administered promethazine methiodide was found in the feces.

This study was undertaken to investigate the tissue distribution and excretion pattern in rats and antimicrobial activity of mepazine methiodide-^{14}C and promethazine methiodide-^{14}C. Brain level was low but above the significant level, which indicated a relatively low blood-brain barrier for these compounds. Biliary excretion was revealed to be the major route of elimination of these compounds. These data tend to confirm the belief that the affinity of molecules for the liver cell and consequently the metabolic mechanism of the liver are the decisive factors which determine the biological fate of a compound *in vivo*.



Scheme I—Synthesis of mepazine methiodide-^{14}C and promethazine methiodide-^{14}C

METHOD[1]

Synthesis of Mepazine Methiodide (MPZ-MEI) and Promethazine Methiodide (PMZ-MEI)—Mepazine hydrochloride (0.5 g.) was dissolved in water, and the solution was adjusted to pH 10 by adding sodium hydroxide solution. The oily precipitate which occurred was extracted several times with chloroform; the combined chloroform extracts were evaporated *in vacuo* to yield 0.46 g. of a viscous oil. The free mepazine base thus obtained was dissolved in 50 ml. of acetone, and 0.5 ml. of methyl iodide was added. The mixture was left standing for 15 min. Crystals, which appeared in the container, were collected and recrystallized from methanol to yield 0.56 g. (82%) of the product with m.p. 120–122°; $\lambda_{max.}$ 251 and 255 mμ.

Anal.—Calcd. for $C_{20}H_{25}IN_2S$: C, 53.09; H, 5.53; N, 6.19; I, 28.03; S, 7.08. Found: C, 53.21; H, 5.45; I, 27.92; N, 5.99; S, 7.06.

Similarly, promethazine hydrochloride (0.5 g.) was dissolved in water, adjusted to pH 10, and extracted several times with ether. The combined ether extracts were evaporated *in vacuo* to yield 0.45 g. of an oil. The free base of promethazine was dissolved in 20 ml. of acetone, and 0.3 ml. of methyl iodide in 5 ml. of acetone was added. The mixture was shaken occasionally for 20 min. Then ether was added to the mixture to precipitate the product. The crude product was recrystallized from methanol to yield 0.57 g. (85%) of the quaternary ammonium salt with m.p. 224–225°; $\lambda_{max.}$ 208 and 252 mμ.

Anal.—Calcd. for $C_{18}H_{23}IN_2S$: C, 50.70; H, 5.40; N, 6.57; S, 7.51. Found: C, 50.80; H, 5.51; N, 6.68; S, 7.58.

Synthesis of Mepazine Methiodide-^{14}C (MPZ-MEI-^{14}C) and Promethazine Methiodide-^{14}C (PMZ-MEI-^{14}C)—The synthesis of ^{14}C-labeled quaternary ammonium salt of mepazine and promethazine is shown in Scheme I. The free base of mepazine (0.1 g.) obtained as described was dissolved in 5 ml. of acetone, and ^{14}C-methyl iodide (0.25 mc., 8.5×10^{-3} mmole) in 10 ml. of acetone was added. After 15 min., 0.1 g. of unlabeled methyl iodide was added as a carrier. The mixture was shaken for 15 min. and cooled for 10 min. Crystals which appeared in the mixture were collected and recrystallized from methanol. The product, MPZ-MEI-^{14}C (0.11 g., 82%), had a melting point of 120–122° and specific activity of 0.46 μc./mg. Mixed melting point with an authentic specimen of mepazine methiodide did not show depression (120–122°).

In the same manner, 0.1 g. of the promethazine base obtained as described was dissolved in 5 ml. of acetone, and ^{14}C-methyl iodide (0.25 mc., 8.5×10^{-3} mmole) in 10 ml. of acetone was added. The mixture was shaken for 30 min. and 0.1 g. of unlabeled methyl iodide was added. Then ether was added to the mixture to precipitate the product. The precipitate was collected and recrystallized from ethanol to give 0.12 g. (86%) of the final product, PMZ-MEI-^{14}C, with a melting point of 224–225° and specific activity of 0.12 μc./mg. The physical properties of this compound were found to be identical with those of authentic promethazine methiodide.

The radiochemical purity of both MPZ-MEI-^{14}C and PMZ-MEI-^{14}C was checked by chromatography coupled with a radiochromatogram scanner, Actigraph III. The chromatograms of these compounds showed only one spot with matching R_f values (MPZ-MEI, 0.76; PMZ-MEI, 0.76) and color reactions (MPZ-MEI, pink; PMZ-MEI, purple with 50% sulfuric acid) with the respective parent compound; therefore, both methiodide derivatives were considered to be radiochemically pure.

Tissue-Distribution Studies—For the tissue-distribution studies, each of the preparations (3 mg. of MPZ-MEI-^{14}C and 10 mg. of PMZ-MEI-^{14}C) was suspended in sesame oil (1 ml.) and administered intraperitoneally to three rats weighing 250–300 g. The animals were sacrificed at various time intervals of 0.5, 1, 2, 4, and 8 hr. after injection. The liver, kidneys, spleen, heart, lungs, stomach, intestines, muscle, bone, blood, and brain were isolated, rinsed with normal saline solution and briefly dried, and the weights were recorded. A portion of each organ (1 g.) was weighed out, homogenized (except blood and bone) with 5 ml. of ethanol, and centrifuged. This process was repeated three times and the ethanol extracts were combined. An aliquot (2 ml.) was measured in a planchet and dried, and the activity was recorded. The activity remaining in the residue after the methanol extraction was also recorded. An aliquot of blood specimen was measured and dried directly in a planchet, and a portion of the bone (femur, dried at 250° for 2 hr. and ground to a powder) was placed in a planchet to record the activity. All activity recordings were carried out at a constant geometry and corrections were made for the self-absorption. Recoveries of 0.3–0.9 mcg. of added MPZ-MEI-^{14}C and PMZ-MEI-^{14}C from tissues were 91 ± 4 and 90 ± 5%, respectively.

Excretion Studies—For the excretion studies, a suspension of MPZ-MEI-^{14}C (10 mg./kg.) and PMZ-MEI-^{14}C (25 mg./kg.) in sesame oil was administered intraperitoneally to six rats weighing 280–310 g. The animals were maintained in metabolic cages and were given food and water *ad libitum*. Urine and feces specimens were collected every 12 hr. for the MPZ-MEI-^{14}C group and every 8

[1] Melting points were taken on a Fisher-Johns apparatus and were corrected. UV absorption spectra were recorded in a Perkin-Elmer model 202 spectrophotometer. Paper chromatograms were developed by an ascending technique in a solvent system, *n*-butanol–ethanol–water (5:2:2). Radiochromatograms were scanned in a radiochromatogram scanner, Actigraph III (Nuclear-Chicago). Albino rats were obtained from Southern Animal Farms, Prattville, Ala. Radioactivity in the tissues was recorded in a G-M counter (Tracerlab, model TGC-2).

Table I—Radioactivity[a] Recovered from Urine and Feces of Rats after Intraperitoneal Administration of Mepazine Methiodide-^{14}C

Time, hr.	Urine, %	Feces, %
12	9.60 ± 1.60	No specimen
24	6.00 ± 0.60	18.20 ± 1.50
36	3.75 ± 0.05	15.90 ± 0.50
48	1.60 ± 0.10	16.65 ± 1.25
60	0.95 ± 0.25	6.25 ± 0.05
72	0.83 ± 0.25	0.50 ± 0.20
84	1.15 ± 1.05	2.20 ± 2.00
96	0.15 ± 0.05	0.20 ± 0.01

[a] Mean percent of the administered activity ± standard error.

hr. for the PMZ-MEI-^{14}C group of animals. An aliquot (1 ml.) of urine specimens was measured in a planchet and dried, and the activity was recorded in a G-M counter. Feces were powdered and dried. A fraction (1 g.) of the specimen was weighed out and extracted three times with 2-ml. portions of methanol. The combined methanol extracts were evaporated to dryness and the activity of the residue measured.

Metabolic Studies—Two groups of rats (five rats in each group) were administered intraperitoneally with 1 g. each of MPZ-MEI-^{14}C and PMZ-MEI-^{14}C, respectively. Pooled urine (20 ml.) from each group of animals was condensed to about 2 ml. An aliquot of 0.5 ml. was placed on Whatman 3 MM paper, and the chromatogram was developed by ascending technique (14) in the solvent system previously mentioned. The chromatogram was scanned in a radiochromatogram scanner, Actigraph III (Nuclear-Chicago), and then sprayed with 50% sulfuric acid to detect nonradioactive metabolites.

Feces collected over a 5-day period from both groups of animals were powdered and extracted successively with ether and methanol in a continuous extraction apparatus. Fatty substances in ether extracts that did not have radioactivity or color reaction with 50% sulfuric acid were discarded. The methanol extracts were combined and reduced to about 1 ml. *in vacuo*. An aliquot of 0.5 ml. was chromatographed and treated as described for the urine specimens.

RESULTS

During the 5-day period, the MPZ-MEI-^{14}C group of rats excreted a total of about 82% of the administered radioactivity with 24% in the urine and 58% in the feces (Table I). In the PMZ-MEI-^{14}C group of rats, fecal excretion accounted for about 59% and urinary excretion represented 12% of the administered activity (Table II). In all cases, nearly 70% of the recovered activity in the urine was excreted in the first 24-hr. period. Feces specimens could not be obtained until after the first 24 hr. from the mepazine group and after 40 hr. for the promethazine group of rats. The recordings of the radiochromatogram scanner indicated only one peak on the chromatograms of the urine specimen from both MPZ-MEI-^{14}C and PMZ-MEI-^{14}C groups of animals; however, 50% sulfuric acid spray revealed two spots (R_f 0.76 and 0.65) on the chromatogram of MPZ-MEI-^{14}C urine. The spot with R_f 0.76, which appeared to be the major metabolite, was identified to be the unchanged MPZ-MEI-^{14}C. The second spot with R_f 0.65, which represented only a small portion of the urinary metabolites, was tentatively identified to be the *N*-demethylated mepazine. The chromatogram of the PMZ-MEI-^{14}C urine exhibited only one spot (R_f 0.76) after 50% sulfuric acid spray. This spot corresponded to the recorded peak on the radiochromatogram scanner. In contrast to the finding of Hansson and Schmitterlöw (13) on the hydroxyethyl chloride salt of promethazine in mice, fecal excretion was the major route of elimination of both quaternary ammonium salts of mepazine and promethazine in the rats. This difference in the route of excretion appears to be due to the different types of quaternary onium derivative and species of animals used in these studies. This type of excretion was similar to that recorded with trifluoperazine methiodide-^{14}C (15).

Table II—Radioactivity[a] Recovered from Urine and Feces of Rats after Intraperitoneal Administration of Promethazine Methiodide-^{14}C

Time, hr.	Urine, %	Feces, %
8	6.42 ± 1.90	—[b]
16	1.66 ± 0.09	—
24	0.97 ± 0.37	—
32	0.71 ± 0.01	
40	0.48 ± 0.04	36.14 ± 1.98
48	0.49 ± 0.12	12.52 ± 3.18
56	0.51 ± 0.01	6.78 ± 0.79
64	0.32 ± 0.10	1.47 ± 0.54
72	0.41 ± 0.04	1.21 ± 0.89
80	0.23 ± 0.01	0.83 ± 0.16

[a] Mean percent of the administered activity ± standard error. [b] No specimen was obtained.

Table III—Distribution of Radioactivity[a] in Tissues of the Rat after Intraperitoneal Administration of Mepazine Methiodide-^{14}C

Organs	Time, hr. 0.5	1	2	4	8
Blood	0.15[b]	0.60	0.92	0.24	0.10
Bone (femur)	0.41	0.53	0.64	1.23	2.00
Brain	—[c]	—	—	—	—
Heart	0.04	0.04	0.15	0.41	0.02
Intestines including contents	4.82	10.13	14.01	7.20	7.92
Kidneys	7.20	8.03	1.21	1.02	0.28
Liver	12.83	10.74	6.50	6.83	7.04
Lungs	0.12	0.36	0.32	0.46	0.08
Muscle	5.25	4.16	2.05	1.17	0.66
Stomach including contents	0.24	0.21	0.28	0.30	0.28
Urine[d]	—[e]	—	—	3.60	4.53
Abdominal washings	8.25	2.23	0.70	0.38	0.24

[a] Including activities of free and bound materials. [b] Percent of the administered activity. [c] Insignificant radioactivity. [d] Collected from bladder. [e] No specimen obtained.

For the activity recording, organs were homogenized and extracted with methanol. Approximately 90% of the activity was extracted in methanol; however, about 10% of the activity appeared to be bound to the protein and was not extracted in methanol.

In the MPZ-MEI-^{14}C group, the radioactivity in the liver and kidneys was found to prevail over other organs 0.5 hr. after the administration (Table III). Biliary excretion took place rapidly, which was reflected by a sharp rise in the intestinal level after 1 hr.

Table IV—Distribution of Radioactivity[a] in Tissues of the Rat after Intraperitoneal Administration of Promethazine Methiodide-^{14}C

Organs	Time, hr. 0.5	1	2	4	8
Blood	0.47[b]	0.68	0.45	0.05	<0.01
Bone (femur)	3.43	1.61	0.27	1.17	0.39
Brain		0.01	0.02	0.03	0.02
Heart	0.07	0.03	0.03	0.02	0.02
Intestines including contents	4.68	8.91	18.06	10.76	5.44
Kidneys	5.65	3.92	1.74	0.74	0.08
Liver	20.19	5.75	9.33	1.80	0.14
Lungs	0.10	0.14	0.09	0.10	0.03
Muscle	2.52	0.68	0.06	0.45	0.34
Spleen	0.46	0.19	1.05	0.03	0.01
Stomach including contents	0.26	0.28	0.61	0.02	0.08
Urine[c]	—[d]	—	—	5.19	54.30
Abdominal washings	7.76	1.19	0.67	0.41	0.25

[a] Including activities of free and bound materials. [b] Percent of the administered activity. [c] Collected from bladder. [d] No specimen obtained.

In the case of PMZ-MEI-^{14}C, the activity in the kidneys was the highest after 1 hr. (Table IV). Then the activity in the intestines of both MPZ-MEI-^{14}C and PMZ-MEI-^{14}C groups reached its peak at 2 and 4 hr. The rapid rate of absorption of these compounds from the injected site was indicated by a rapid decrease of the activity recovered in the abdominal washings.

Blood levels were low but above the detectable level in both groups of animals (Tables III and IV). The brain level of PMZ-MEI-^{14}C became detectable 1 hr. after the administration, but that of MPZ-MEI-^{14}C was below the significant level throughout the entire period of the experiment. MPZ-MEI-^{14}C appeared to have a particular affinity for the bone; activity in the bone started to increase at 0.5 hr. after the administration and reached its peak after 8 hr. (Table III). Since the bone represents an average of 45% of the total body weight of rats, the total activity accumulated in the bone was higher than in most organs except the intestines and liver at 8 hr. A similar finding was recorded with PMZ-MEI-^{14}C, except that the peak level in the bone was reached at 0.5 hr. In the MPZ-MEI-^{14}C group, the liver and muscle levels showed a peak at 0.5 hr. and declined rapidly thereafter. Other organs followed a similar pattern, except bone in which the activity continued to accumulate until a peak level was reached at 8 hr. (Table III).

The LD_{50} of MPZ-MEI-^{14}C and of PMZ-MEI-^{14}C on mice was 75 mg./kg. and 95 mg./kg., respectively, which are higher than that of the corresponding parent compounds, mepazine (115 mg./kg.) and promethazine (130 mg./kg.). These compounds displayed a comparable antimicrobial activity to that of trifluoperazine methiodide on *Escherichia coli* and *Staphylococcus aureus* at a concentration of 1 mcg./ml.

CONCLUSION

The intraperitoneally administered quaternary ammonium salts of both mepazine and promethazine were well absorbed by the rats. The majority of the drugs was accumulated in the liver and excreted in the intestines (Tables III and IV). Peak blood level was observed at 2 hr. for MPZ-MEI-^{14}C and at 1 hr. for PMZ-MEI-^{14}C. Both compounds showed the highest activity in the intestines 2 hr. after the administration. MPZ-MEI-^{14}C seemed to have a particular affinity for bones; the activity in the bone of rats started to accumulate at 0.5 hr. and reached its peak level at 8 hr. The total activity in the bone was higher than in most organs except the liver and intestines. The brain level of PMZ-MEI-^{14}C was above the detectable level at 1 hr.

Urinary excretion of the two compounds was fairly rapid; nearly 70% of the recorded activity in urine was excreted in the first 24-hr. period. Fecal excretion was the major route of the metabolism of these compounds; 58% of the activity of MPZ-MEI-^{14}C and 59% of PMZ-MEI-^{14}C were recovered during the 5-day period. The discrepancy between the results obtained in this study and those of Hansson and Schmitterlöw (13) is evidently due to the different quaternary onium derivatives and species of animals used in these studies.

REFERENCES

(1) E. M. Caffey, Jr., *Amer. J. Psychiat.*, **117,** 713(1961).

(2) A. A. Kurland, T. E. Hanlon, M. H. Tatom, K. Y. Ota, and A. M. Simopoulos, *J. Nerv. Ment. Dis.*, **133,** 1(1961).

(3) A. A. Kurland, M. H. Michaux, T. E. Hanlon, K. Y. Ota, and A. M. Simopoulos, *ibid.*, **134,** 48(1962).

(4) J. L. Bennet and K. A. Kooi, *Arch. Gen. Psychiat.*, **4,** 413 (1961).

(5) F. J. Ayd, Jr., *Ed. Psychiat.*, **1961,** 355.

(6) O. Nieschulz, *Arzneimittel-Forsch.*, **15,** 271(1965).

(7) J. Lancy, *Can. Psychiat. Ass. J.*, **6,** 26(1961).

(8) S. Bogoch, P. C. Belval, K. T. Dussik, and P. C. Conran, *Amer. J. Psychiat.*, **119,** 128(1962).

(9) K. Kurahara, *Seishin Igaku*, **3,** 523(1961).

(10) D. Muller and E. Gladtke, *Psychiat. Neurol. Med. Psychol.*, **14,** 54(1962).

(11) C. L. Huang and J. Z. Yeh, *Int. Congr. Pharm. Sci. 27th*, **1967,** abstract, p. 43.

(12) A. Heyndrickx, A. De Leenheer, and A. M. Massart-Leen, *J. Pharm. Belg.*, **20,** 359(1965).

(13) E. Hansson and C. G. Schmitterlöw, *Arch. Int. Pharmacodyn. Ther.*, **131,** 309(1961).

(14) C. L. Huang, *Int. J. Neuropharmacol.*, **6,** 1(1967).

(15) C. L. Huang and J. Z. Yeh, unpublished data.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 28, 1969, from the *Department of Pharmacology, School of Pharmacy, University of Mississippi, University, MS 38677*

Accepted for publication January 28, 1970.

This investigation was supported by Public Health Service Grant MH-11927 from the National Institute of Mental Health, Bethesda, Md.

The authors thank Warner-Chilcott Laboratories for the supply of mepazine hydrochloride (Pacatal) and Wyeth Laboratories for the supply of promethazine hydrochloride (Phenergan) for this study.

Preparation and *In Vitro* Evaluation of a Sustained-Action Suspension of Dextromethorphan

WILLIAM E. SMITH*, JOHN D. BUEHLER†, and MANFORD J. ROBINSON

Abstract □ A stable suspension having a prolonged *in vitro* release was prepared by synthesizing a poorly water-soluble salt of dextromethorphan, crystallizing this salt as a hydrate, coating the crystals in a solid particle-coating device with a triglyceride fatty acid mixture, and dispersing the coated crystals in an aqueous vehicle. Dramatic differences in physical and release-rate stability were encountered with the same chemical salt when it was coated by different methods and crystallized from different solvent systems.

Keyphrases □ Dextromethorphan—sustained-action suspension □ Sustained action—dextromethorphan suspension □ Suspension—triglyceride fatty acid-coated dextromethorphan salt □ Spray-congealed coating—dextromethorphan crystals □ Release-rate stability—dextromethorphan suspension □ Thermal analysis, differential—dextromethorphan salt

Numerous oral sustained-action products have been marketed in the past 16 years. For most of these products, sustained action has been obtained by coating compressed tablets or large pellets, or by compressing drug in admixture with water-insoluble fillers. Very little data have been presented on the use of coated subsieve-sized particles in the formulation of sustained-action dosage form. Bakan (1) has outlined the use of the coacervation process for preparing sustained-action powders. Several investigators (2–5) have reported on the use of spray-congealing procedures for preparing sustained-action powders.

Coacervation coating and spray-congealing methods have not been widely used to prepare sustained-action products, possibly because these methods require expensive, specialized equipment not commonly utilized by the pharmaceutical industry. A simple, relatively inexpensive, solid particle-coating device (PCD) developed in this laboratory may have greater utility for the preparation of sustained-action dosage forms (6). This report presents an example of subsieve-sized crystals coated in the PCD and use of these coated crystals in the formulation of a stable, liquid, sustained-action suspension of dextromethorphan. It also presents the differences found in physical and release-rate stability which illustrate the importance of certain physical and chemical properties of drug and coating components in the formulation of this dosage form.

EXPERIMENTAL

Materials—A poorly water-soluble form of dextromethorphan was obtained by reacting *d*-methorphan with *o*-(*p*-hydroxybenzoyl) benzoic acid (7). The resulting salt (DMHB) was crystallized from ethanol–chloroform and dimethylformamide–water solvent systems. Mixtures of glyceryl tristearate (GTS)[1] and 12-hydroxystearic acid (12-HSA)[2] or GTS and behenic acid (BA)[3] were used as coating materials. Chloroform and carbon tetrachloride were used as solvents for these coating materials. The aqueous vehicle for the sustained-action powders contained tragacanth, sorbitol, methylcellulose, sodium cyclamate, saccharin, methyl and propyl paraben, sorbic acid, and imitation black currant flavor.

Table I—Composition and Typical Release Rates for Coated DMHB Prepared by Spray Congealing

A.	Theory, %—DMHB = 25.0, 12-HSA = 37.5, GTS = 37.5
	Actual, %—DMHB = 24.4
	Release rate, %[a]—0.5 = 4, 1.5 + 0.5 = 11, 1.5 + 3 = 55, 1.5 + 5.5 = 86
B.	Theory, %—DMHB = 20.0, 12-HSA = 40.0, GTS = 40.0
	Actual, %—DMHB = 19.7
	Release rate, %[a]—0.5 = 4, 1.5 + 0.5 = 13, 1.5 + 3 = 62, 1.5 + 5.5 = 87

[a] First number indicates hours in USP gastric fluid and second, where given, indicates hours in modified USP intestinal fluid.

Table II—Composition and Typical Release Rates for Coated DMHB Crystals Prepared in PCD

A.	Theory, %—DMHB = 33.0, 12-HSA = 33.5, GTS = 33.5
	Actual, %—DMHB = 33.1
	Release rate, %[a]—0.5 = 17, 1.5 + 0.5 = 37, 1.5 + 3 = 70, 1.5 + 5.5 = 78
B.	Theory, %—DMHB = 35.0, BA = 6.5, GTS = 58.5
	Actual, %—DMHB = 32.0
	Release rate, %[a]—0.5 = 19, 1.5 + 0.5 = 27, 1.5 + 3 = 70, 1.5 + 5.5 = 89

[a] First number indicates hours in USP gastric fluid and second, where given, indicates hours in modified USP intestinal fluid.

Equipment and Methodology—Spray-congealed, sustained-action powders were prepared by suspending DMHB crystals in a molten mixture of the coating materials and spraying the resulting suspension in a Niro laboratory model spray drier.[4] Procedures analogous to those described in previous publications were followed (3, 8). DMHB crystals were coated in the PCD, using solvent solutions of the coating materials and techniques generally in accord with those previously described for use with this apparatus (6). A Fisher subsieve size analyzer[5] was used to determine the volume-surface mean diameter (d_{vs}) of DMHB, the spray-congealed powders, and the coated DMHB crystals. Coating uniformity and integrity were checked by microscopic examination of a glycerin–H_2O or mineral oil mull of the sustained-action powders. An L & R ultrasonic bath[6] operating at 73 kc./sec. was used to crystallize the DMHB from the dimethylformamide solvent system. DMHB release was measured using the method described by Souder and Ellenbogen (9), with the exception that the USP intestinal fluid was prefiltered and the undissolved particles containing DMHB were collected on 1.2-μ filter paper.[7]

[1] Hydrofol Glycerides T57L, Ashland Chemical Co., Columbus, Ohio.

[2] Baker Castor Oil Co., Bayonne, N. J.
[3] Hydrofol Acid 560, Ashland Chemical Co., Columbus, Ohio.
[4] Nichols Engineering and Research Corp., New York, N. Y.
[5] Fisher Scientific Co., Pittsburgh, Pa.
[6] L & R Manufacturing Co., Kearny, N. J.
[7] Millipore Corp., Bedford, Mass.

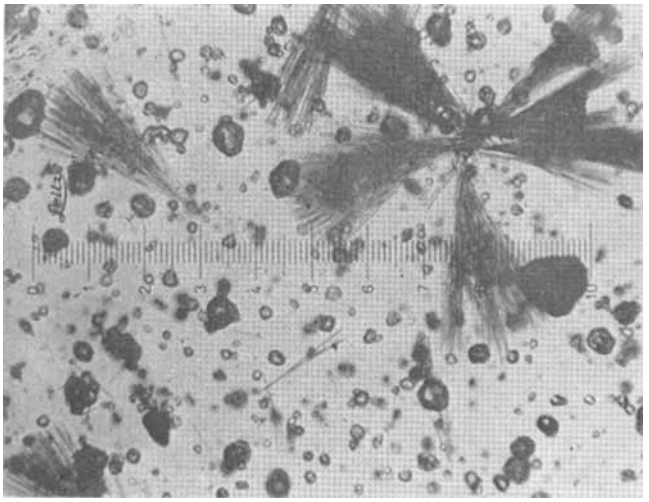


Figure 1—*Crystal growth from DMHB "ethanolate"-coated particles in an aqueous suspension (magnification: 0–1 equals 63.9 μ).*

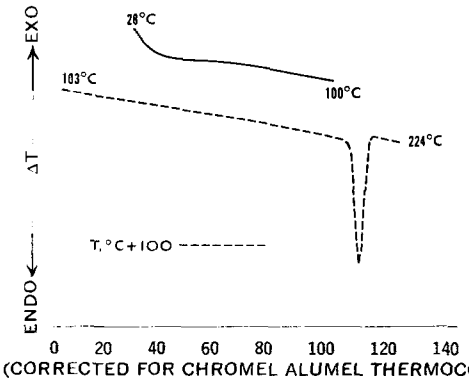


Figure 2—*A typical thermogram for the anhydrous form of DMHB.*

RESULTS AND DISCUSSION

The solubility of DMHB in the aqueous vehicle was about 0.9 mg./ml. at 25°. At a concentration below saturation, uncoated DMHB crystals dissolved completely in USP gastric or intestinal fluid in less than 5 min. At the same concentration, the spray-congealed and the coated crystals released DMHB slowly over time in USP gastric and intestinal fluid. Typical release values and the composition of these powders are given in Tables I and II. The d_{vs} was about 15 μ for the spray-congealed powders and about 30 μ for crystals coated in the PCD. These results suggested that powders prepared by either process should be suitable for the preparation

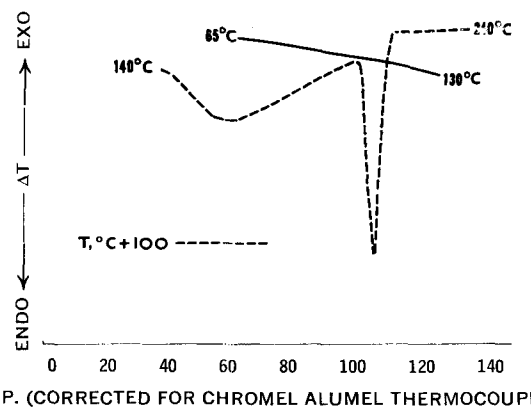


Figure 3—*A typical thermogram for the "ethanolate" form of DMHB.*

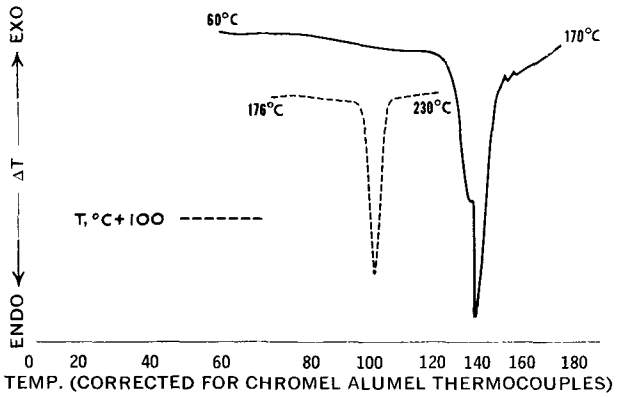


Figure 4—*A typical thermogram for the hydrate form of DMHB.*

of a sustained-action suspension. The particle size was low enough to minimize grittiness and sedimentation propensity. The theoretical density was in a range where the addition of a small amount of sorbitol permitted a match between particle and vehicle density. Only about 10% of the DMHB dose (9.0 mg./ml.) would be needed to saturate the aqueous vehicle, permitting formulation of a suspension with a desirable balance between initial and prolonged release. The *in vitro* release rates of the spray-congealed and the coated DMHB crystals suggested that a suspension of these powders would provide sustained antitussive activity *in vivo*.

An additional requirement for the sustained-action powders is the absence of significant change in chemical potency and release rate over time. This was determined for these formulations by storing them at 37° and under ambient conditions. The results obtained after several months' storage are given in Table III. It is evident that the release rate of the spray-congealed formulation has increased

Table III—Chemical and Release-Rate Stability of DMHB Sustained-Action Powders

Sample	Storage Temp.	Storage Time, month	Assay, %	Release Rate, %[a] Hours 0.5	1.5 + 0.5	1.5 + 3	1.5 + 5
Spray-congealed	Ambient	0	24.4	4	11	55	86
	Ambient	1	24.6	—	15	75	—
	37°	1	24.6	12	30	86	94
	37°	2	24.6	9	40	91	96
Spray-congealed	Ambient	0	19.7	4	13	62	87
	Ambient	1	19.7	—	23	74	—
	37°	1	19.9	9	32	84	92
	37°	3	19.9	7	40	88	94
PCD	Ambient	0	33.1	17	37	70	78
	Ambient	2	34.0	16	34	69	80
	37°	1	34.1	13	34	69	80
	37°	3	34.2	16	36	66	76

[a] First number indicates hours in USP gastric fluid and second, where given, indicates hours in modified USP intestinal fluid.

Table IV—Release-Rate Stability of a Sustained-Action Suspension of DMHB "Ethanolate"

Release Rate, %[a]	After 25° Storage, days 1	4	6
0.5	40%	59%	73%
1.5 + 0.5	52%	68%	77%
1.5 + 3	66%	78%	83%
1.5 + 5.5	79%	89%	—

[a] First number indicates hours in USP gastric fluid and second where given, indicates hours in modified USP intestinal fluid.

markedly, whereas that of the coated crystals has not. Microscopic examination of the spray-congealed powders suggested some change in the crystal form in the GTS–12-HSA matrix. This possibility was confirmed by X-ray diffraction which showed that spray-congealed GTS solidified in the polymorphic α-form. This form is unstable and on storage transformed into the stable β-modification. In contrast, GTS could be obtained in the β-form by spraying from chloroform or carbon tetrachloride. Since there was no other discernible physical change in these powders, it is considered likely that the release-rate increase of the spray-congealed powders was related to polymorphic transition of the GTS. In any event, the changes in release rate of the spray-congealed formulation indicated that only the DMHB crystals coated in the PCD would be acceptable for use in formulating a sustained-action suspension.

DMHB crystallized from the chloroform–alcohol solvent system had a d_{vs} of less than 20 μ, and the shape of the individual particles approached sphericity. The particle size and shape were considered optimal for formulation. However, when these crystals were coated and the resultant powder dispersed in the aqueous vehicle, DMHB underwent marked crystal growth. The effect of this crystal growth on coating integrity is shown in Fig. 1 and the resultant increase in release rate is given in Table IV. Chemical analysis of DMHB indicated that, prior to coating, the crystals were either anhydrous or contained varying amounts of alcohol. In contrast, crystals taken from the aqueous suspension contained 3.5–3.8% H_2O. Typical thermograms obtained by differential thermal analysis for the anhydrate, "ethanolate," and hydrate are given in Figs. 2–4. These results indicate that DMHB crystallized from the chloroform–alcohol solvent system as an "ethanolate" of varying composition (possibly unstable) and, in some instances, was converted to the anhydrate by drying at high temperatures. In aqueous media, these more soluble forms recrystallized in the form of the monohydrate.

It was found that crystals of the monohydrate could be obtained by use of a dimethylformamide–water solvent system, but these crystals were excessively large and acicular in shape. Experimentation with various crystallization techniques established the fact that crystals of a desirable size and shape could be obtained by rapid nucleation through the use of insonation and by slowing crystal growth by careful temperature control. Photomicrographs of the DMHB monohydrate crystals obtained by this procedure appear in Fig. 5. The release rates for an aqueous suspension of coated DMHB monohydrate crystals are listed in Table V. Also given in this table are the release-rate values after 6 months' storage at 37° and 1 year's storage at ambient conditions. Photomicrographs of coated crystals alone and in an aqueous dispersion after 1 year's storage at ambient conditions appear in Figs. 6 and 7. No evidence of change in appearance, potency, or release rate was found for this formulation during this storage time.

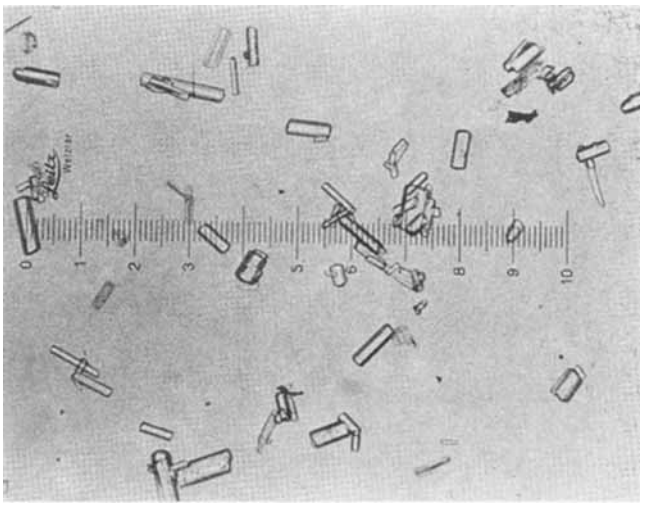


Figure 5—*DMHB crystals obtained from a dimethylformamide–water solvent system (magnification: 0–1 equals 63.9 μ).*

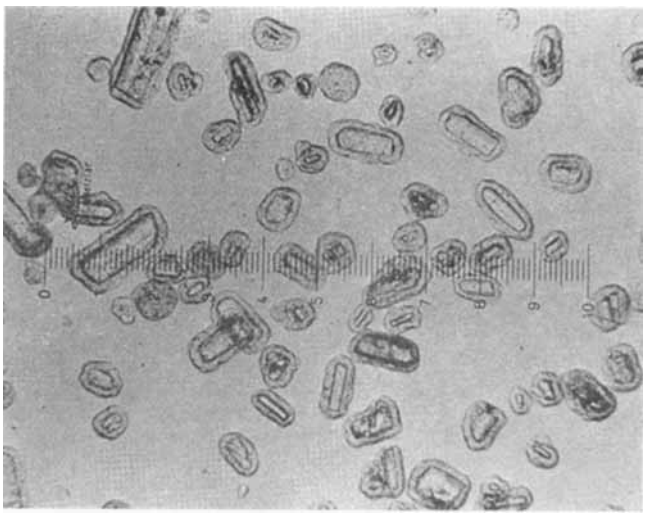


Figure 6—*DMHB crystals coated with a mixture of GTS and BA (magnification: 0–1 equals 63.9 μ).*

Although this formulation was not tested *in vivo* for antitussive activity, the *in vitro* release values suggest that this preparation would release a desirable amount of dextromethorphan for immediate absorption and the remainder would be released for absorption slowly over time.

In this report, dramatically different behavior has been described for the same chemical salt coated by two different methods and crystallized from two different solvent systems. It is likely that the implications of these observations are not only applicable to the formulation of liquid sustained-release dosage forms but also should be considered in the formulation of other dosage forms. This study

Table V—Chemical and Release-Rate Stability of a Sustained-Action Suspension of DMHB Hydrate

Release Rate, %[a]	Original	3 Months RT	3 Months 37°	6 Months RT	6 Months 37°	1 Year, RT
	Assay, mg./ml.					
	8.9	8.9	8.7	9.0	8.8	8.9
0.5	23%	24%	26%	25%	26%	25%
1.5 + 0.5	35%	32%	36%	—	37%	35%
1.5 + 3	70%	71%	71%	73%	81%	80%
1.5 + 5.5	94%	91%	94%	93%	97%	95%
Solubility in vehicle	10–11%	—	—	—	—	—

[a] First number indicates hours in USP gastric fluid and second, where given, indicates hours in modified USP intestinal fluid.

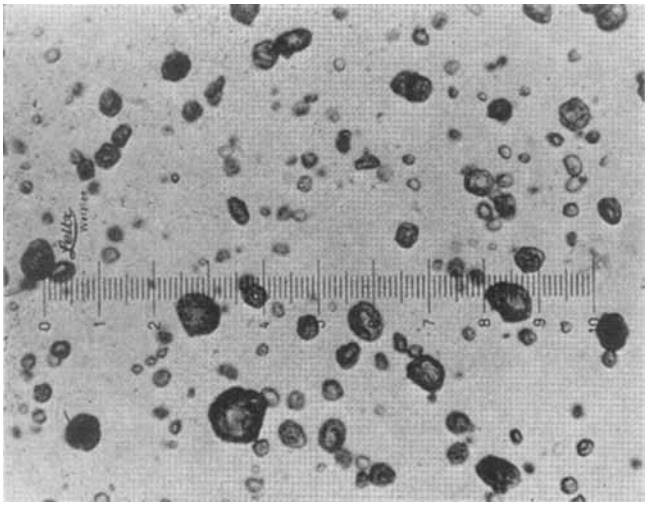


Figure 7—*An aqueous suspension of coated DMHB crystals after 1 year's storage at ambient conditions (magnification: 0–1 equals 63.9 μ).*

illustrates the value of having a choice of materials and processes which can be used to arrive at the best formulation prior to clinical testing. In this instance, the PCD proved to be a useful addition to equipment available in these laboratories for the formulation of a suspension having a prolonged *in vitro* release.

REFERENCES

(1) J. A. Bakan, "Microencapsulation as Applied to Pharmaceutical Products," presented at the Eastern Regional Industrial Pharmaceutical Technology Section, Academy of Pharmaceutical Sciences, Philadelphia, Pa., Oct. 1968.

(2) P. M. John and C. H. Becker, *J. Pharm. Sci.*, **57**, 584(1968).

(3) M. J. Robinson and J. V. Swintosky, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 473(1959).

(4) A. G. Cusimano and C. H. Becker, *J. Pharm. Sci.*, **57**, 1104 (1968).

(5) Y. Raghunatham and C. H. Becker, *ibid.*, **57**, 1748(1968).

(6) M. J. Robinson, G. M. Grass, and R. J. Lantz, *ibid.*, **57**, 1983(1968).

(7) H. C. Caldwell and W. E. Smith, U. S. Pat. 3,381,010.

(8) M. W. Scott, M. J. Robinson, J. F. Pauls, and R. J. Lantz, *J. Pharm. Sci.*, **53**, 670(1964).

(9) J. C. Souder and W. C. Ellenbogen, *Drug Stand.*, **26**, 77 (1958).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 6, 1969, from the *Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101*

Accepted for publication January 27, 1970.

The authors thank Howard Bregman and W. Robert Umbenhauer for their technical assistance, and the analytical chemistry group associated with Edward Bumbier for chemical assay work.

* Present address: Philadelphia College of Pharmacy and Science, Philadelphia, Pa.

† Present address: William H. Rorer Co., Fort Washington, Pa.

Correlation and Prediction of Rates of Alkaline Hydrolysis of Some Benzoate Esters

RICHARD J. WASHKUHN*, SANGSOM REUTRAKUL, and JOSEPH R. ROBINSON

Abstract □ Rate constants have been determined for the alkaline hydrolysis of 36 *p*-substituted alkyl benzoates, and it is shown that these esters can be characterized on the basis of their rates of alkaline hydrolysis. Application of linear free-energy relationships allows calculation of alkaline hydrolysis rate constants for alkyl or aromatic *p*-substituted benzoate esters not included in this study. It is demonstrated that rates can be predicted for esters whether substituent variation is in the acyl or alkyl portion of the molecule by using the conventional Hammett treatment and the more recent alcohol dissociation model.

Keyphrases □ Benzoate esters—alkaline hydrolysis rates □ Alkaline hydrolysis rates—benzoate ester identification □ Linear free-energy relationships—alkaline hydrolysis rate prediction □ UV spectrophotometry—reaction monitoring

Earlier papers in this series (1–4) demonstrated that precise kinetic measurements can be a powerful tool in the identification of organic compounds. In addition, the large number of rate constants generated in these studies, under constant conditions, provides the necessary data for structure–reactivity relationships, mechanistic interpretations, *etc.* The classes of organic compounds previously considered were: alcohols [rates of alkaline hydrolysis of their 3,5-dinitrobenzoate esters (1)], sugars [rates of oxime formation (2)], aliphatic amines [rates of cinnamoylation (3)], and aliphatic esters [rates of alkaline hydrolysis (4)]. The present study demonstrates that rates of alkaline hydrolysis can be used to identify aromatic esters and presents linear free-energy relationships to predict rates of alkaline hydrolysis for esters not included in the study.

EXPERIMENTAL

Chemicals—Fisher certified acetonitrile was used without further purification after it was established that it was spectrally pure (from 220–300 mμ) and that it had no anomalous effect on the rate of alkaline hydrolysis. All other chemicals were either analytical or reagent grade. Water was double distilled from acid permanganate in an all-glass distillation apparatus.

All esters were prepared according to procedures outlined by Shriner *et al.* (5) with slight modification. The acyl chloride was reacted with the appropriate alcohol by heating under reflux for 30 min. The reaction mixture was then taken up in chloroform and extracted with 5% Na_2CO_3 followed by water and finally dried with $MgSO_4$. After removing the chloroform under vacuum, the ester remaining was purified by repeated recrystallization from water or from the alcohol representing the alkyl portion of the ester or, in the case of liquids, by vacuum distillation.

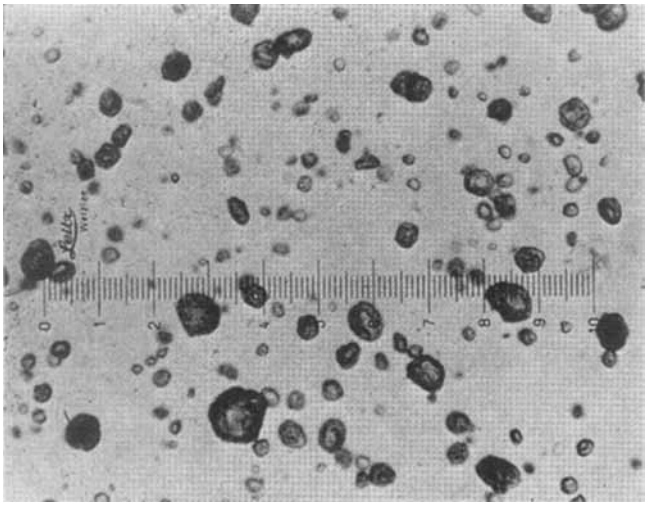


Figure 7—*An aqueous suspension of coated DMHB crystals after 1 year's storage at ambient conditions (magnification: 0–1 equals 63.9 μ).*

illustrates the value of having a choice of materials and processes which can be used to arrive at the best formulation prior to clinical testing. In this instance, the PCD proved to be a useful addition to equipment available in these laboratories for the formulation of a suspension having a prolonged *in vitro* release.

REFERENCES

(1) J. A. Bakan, "Microencapsulation as Applied to Pharmaceutical Products," presented at the Eastern Regional Industrial Pharmaceutical Technology Section, Academy of Pharmaceutical Sciences, Philadelphia, Pa., Oct. 1968.

(2) P. M. John and C. H. Becker, *J. Pharm. Sci.*, **57**, 584(1968).

(3) M. J. Robinson and J. V. Swintosky, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 473(1959).

(4) A. G. Cusimano and C. H. Becker, *J. Pharm. Sci.*, **57**, 1104 (1968).

(5) Y. Raghunatham and C. H. Becker, *ibid.*, **57**, 1748(1968).

(6) M. J. Robinson, G. M. Grass, and R. J. Lantz, *ibid.*, **57**, 1983(1968).

(7) H. C. Caldwell and W. E. Smith, U. S. Pat. 3,381,010.

(8) M. W. Scott, M. J. Robinson, J. F. Pauls, and R. J. Lantz, *J. Pharm. Sci.*, **53**, 670(1964).

(9) J. C. Souder and W. C. Ellenbogen, *Drug Stand.*, **26**, 77 (1958).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 6, 1969, from the *Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101*

Accepted for publication January 27, 1970.

The authors thank Howard Bregman and W. Robert Umbenhauer for their technical assistance, and the analytical chemistry group associated with Edward Bumbier for chemical assay work.

* Present address: Philadelphia College of Pharmacy and Science, Philadelphia, Pa.

† Present address: William H. Rorer Co., Fort Washington, Pa.

Correlation and Prediction of Rates of Alkaline Hydrolysis of Some Benzoate Esters

RICHARD J. WASHKUHN*, SANGSOM REUTRAKUL, and JOSEPH R. ROBINSON

Abstract □ Rate constants have been determined for the alkaline hydrolysis of 36 *p*-substituted alkyl benzoates, and it is shown that these esters can be characterized on the basis of their rates of alkaline hydrolysis. Application of linear free-energy relationships allows calculation of alkaline hydrolysis rate constants for alkyl or aromatic *p*-substituted benzoate esters not included in this study. It is demonstrated that rates can be predicted for esters whether substituent variation is in the acyl or alkyl portion of the molecule by using the conventional Hammett treatment and the more recent alcohol dissociation model.

Keyphrases □ Benzoate esters—alkaline hydrolysis rates □ Alkaline hydrolysis rates—benzoate ester identification □ Linear free-energy relationships—alkaline hydrolysis rate prediction □ UV spectrophotometry—reaction monitoring

Earlier papers in this series (1–4) demonstrated that precise kinetic measurements can be a powerful tool in the identification of organic compounds. In addition, the large number of rate constants generated in these studies, under constant conditions, provides the necessary data for structure–reactivity relationships, mechanistic interpretations, *etc.* The classes of organic compounds previously considered were: alcohols [rates of alkaline hydrolysis of their 3,5-dinitrobenzoate esters (1)], sugars [rates of oxime formation (2)], aliphatic amines [rates of cinnamoylation (3)], and aliphatic esters [rates of alkaline hydrolysis (4)]. The present study demonstrates that rates of alkaline hydrolysis can be used to identify aromatic esters and presents linear free-energy relationships to predict rates of alkaline hydrolysis for esters not included in the study.

EXPERIMENTAL

Chemicals—Fisher certified acetonitrile was used without further purification after it was established that it was spectrally pure (from 220–300 mμ) and that it had no anomalous effect on the rate of alkaline hydrolysis. All other chemicals were either analytical or reagent grade. Water was double distilled from acid permanganate in an all-glass distillation apparatus.

All esters were prepared according to procedures outlined by Shriner *et al.* (5) with slight modification. The acyl chloride was reacted with the appropriate alcohol by heating under reflux for 30 min. The reaction mixture was then taken up in chloroform and extracted with 5% Na_2CO_3 followed by water and finally dried with $MgSO_4$. After removing the chloroform under vacuum, the ester remaining was purified by repeated recrystallization from water or from the alcohol representing the alkyl portion of the ester or, in the case of liquids, by vacuum distillation.

Table I—Second-Order Rate Constants for the Alkaline Hydrolysis of Some Benzoate Esters[a]

Ester	$k \times 10^4$, l./msec.	Number of Determinations	SD[b]
Methyl benzoate	6.08	2	0.05
Ethyl benzoate	1.98	2	0.02
n-Propyl benzoate	1.67	3	0.04
Isopropyl benzoate	0.319	2	0.05
n-Butyl benzoate	1.41	2	0.02
Isobutyl benzoate	1.18	3	0.07
Methyl *p*-nitrobenzoate	275.5	6	6.9
Ethyl *p*-nitrobenzoate	98.8	6	3.9
n-Propyl *p*-nitrobenzoate	76.0	5	1.8
Isopropyl *p*-nitrobenzoate	19.6	4	1.3
n-Butyl *p*-nitrobenzoate	63.4	3	0.8
Isobutyl *p*-nitrobenzoate	60.0	4	2.2
Methyl *p*-chlorobenzoate	19.1	4	0.45
Ethyl *p*-chlorobenzoate	6.51	3	0.39
n-Propyl *p*-chlorobenzoate	5.11	3	0.65
Isopropyl *p*-chlorobenzoate	1.21	2	0.02
n-Butyl *p*-chlorobenzoate	3.49	4	0.29
Isobutyl *p*-chlorobenzoate	3.36	3	0.08
Methyl *p*-methylbenzoate	2.65	4	0.20
Ethyl *p*-methylbenzoate	0.879	2	0.009
n-Propyl *p*-methylbenzoate	0.671	3	0.04
Isopropyl *p*-methylbenzoate	0.200	2	0.02
n-Butyl *p*-methylbenzoate	0.568	2	0.03
Isobutyl *p*-methylbenzoate	0.449	2	0.009
Methyl *p*-fluorobenzoate	12.1	3	0.2
Ethyl *p*-fluorobenzoate	4.05	3	0.08
n-Propyl *p*-fluorobenzoate	2.65	3	0.07
Isopropyl *p*-fluorobenzoate	0.623	3	0.04
n-Butyl *p*-fluorobenzoate	2.44	3	0.06
Isobutyl *p*-fluorobenzoate	1.90	3	0.24
Methyl *p*-cyanobenzoate	195.0	4	2.4
Ethyl *p*-cyanobenzoate	79.7	5	1.50
n-Propyl *p*-cyanobenzoate	52.6	3	0.34
Isopropyl *p*-cyanobenzoate	12.6	5	0.24
n-Butyl *p*-cyanobenzoate	41.9	3	1.16
Isobutyl *p*-cyanobenzoate	39.4	4	1.50

[a] In 50% v/v acetonitrile–0.02 *M* phosphate buffer at 25°. [b] Standard deviation.

Apparatus—pH Measurements and adjustments were made on a Corning model 12 pH meter with an expanded scale using a Beckman type E3 wide-range glass electrode. The pH meter and electrode system was standardized against a phosphate buffer as described by Bates (6).

Water bath temperatures were maintained to 0.1° with Sargent Thermonitor electronic relays. The progress of the reactions was followed on either a Cary model 11 or 14 recording spectrophotometer with thermostated cell compartments.

PROCEDURE

Solvent System—Fifty milliliters of acetonitrile was added to an equal volume of a 0.02 *M* dibasic phosphate buffer, and the resulting solution was brought to the appropriate pH with a few drops of concentrated NaOH solution. The acetonitrile–phosphate buffer solution gives a volume somewhat less than 100 ml. This solution was used for all kinetic determinations.

Continuous Spectrophotometric Procedure—For reactions that had sufficiently rapid rates so that they could be followed directly in the spectrophotometer, the following procedure was used. After equilibrating the solvent system to 25°, 3 ml. of solvent was thoroughly mixed with 50 μl. of a 1×10^{-2} *M* ester in acetonitrile solution in a 1-cm. photometer cell. The reaction progress was monitored by following the disappearance of ester at an appropriate wavelength, benzoates at 242 mμ, *p*-methyl benzoates at 250 mμ, *p*-chlorobenzoates at 254 mμ, *p*-fluorobenzoates at 244 mμ, *p*-cyanobenzoates at 250 mμ, and by the appearance of acid anion at 290 mμ in the *p*-nitrobenzoate case.

Discontinuous Spectrophotometric Procedure—For esters with inconveniently long rates of reaction, the following sampling procedure was used. One hundred milliliters of the buffer solution was prepared and brought to the appropriate pH. Two milliliters of 1×10^{-2} *M* ester in acetonitrile solution was added to the buffer solution, and the resulting mixture was placed in 5-ml. volumetric flasks which were stored in a 25° water bath. At appropriate time intervals the contents of two of the volumetric flasks were used to determine the concentration of remaining ester or acid anion. This sampling procedure was necessary due to the volatility of acetonitrile.

Data Treatment—All experiments were carried out with a large excess of hydroxide ion and thus pseudo-first-order kinetics were observed. Pseudo-first-order rate constants were obtained in the usual manner and were converted to second-order rate constants by dividing by the hydroxide-ion activity.

RESULTS

Second-order rate constants were calculated for the various esters and are presented, together with standard deviations, in Table I. At least two rate determinations were made for each ester, and these were carried out at more than one pH value to ensure that the reactions were first-order in hydroxide ion. The pH range employed for these esters in this solvent system was from 12 to 13.4. Above pH 13.4, phosphate precipitates; below pH 12, the rates are inconveniently slow. The buffer capacity of the solvent system, for concentrations of ester employed in this study, was satisfactory from pH 11 to 13.4.

Correlations of structure and reactivity were made using the Hammett relationship (7)

$$\log \frac{k}{k_0} = \sigma\rho \qquad \text{(Eq. 1)}$$

and a modified form of the Hammett equation (8)

$$\log \frac{k}{k_0} = \phi M \qquad \text{(Eq. 2)}$$

For variation in the acyl portion of the ester molecule, substituent constants (σ values) were obtained from the literature (9) and were plotted against log rate constants.[1] For substituent variation in the alkyl portion of the ester molecule, ϕ values (8) were plotted against log rate constants. Representative plots for both of these treatments are shown in Figs. 1 and 2. Slopes, standard deviations, and correla-

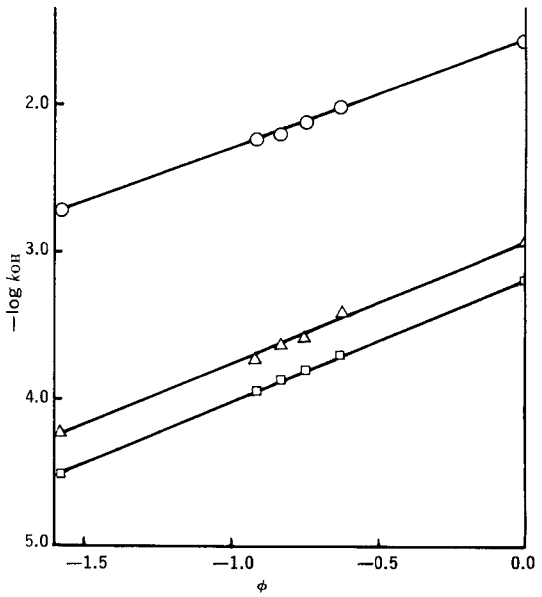


Figure 1—*Plot of the second-order rate constants for alkaline hydrolysis of alkyl-substituted benzoate esters against ϕ values. Key:* ○, p-*nitro benzoates;* △, p-*fluoro benzoates, and* □, *benzoates.*

[1] The substituent constant for the *p*-fluoro group is reported to be 0.06 (9) or 0.17 (10). Although neither value gave good correlations, the 0.06 σ value was used in this study.

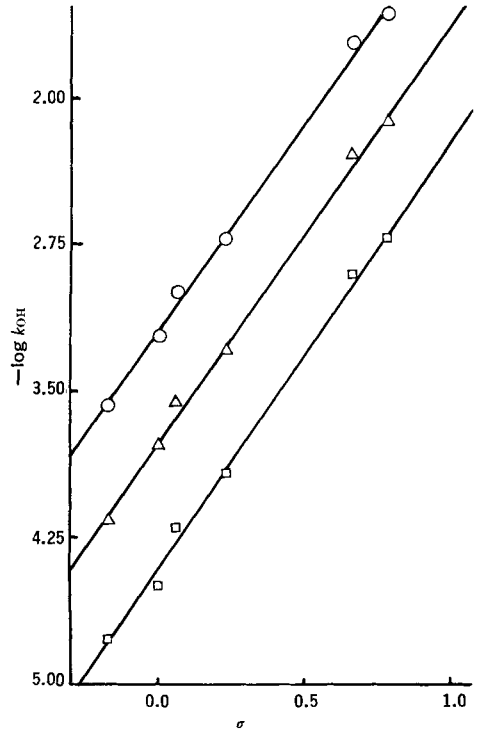


Figure 2—*Plot of the second-order rate constants for alkaline hydrolysis of* p-*substituted alkyl benzoate esters against Hammett's polar substituent constants. Key:* ○, *methyl benzoate esters;* △, n-*propyl benzoate esters, and* □, *isopropyl benzoate esters.*

tion coefficients for these plots and the remaining esters are shown in Table II.

DISCUSSION

It is apparent from Table I that the rate of alkaline hydrolysis is a useful way to identify these aromatic esters. The ratio of the largest to the smallest rate constants for these 36 esters is about 1600; within a given series the constants are sufficiently different so that any ester is easily differentiated from a similar member. It is also clear from the reported rates that for some esters the rates are prohibitively slow, and this is obviously undesirable. However, sufficient rate data are presented to calculate the rates for a large number of esters. For many of these esters, this procedure will be a rapid, sensitive method which will be useful for characterization.

For esters not included in this study, it is possible to calculate the rate of alkaline hydrolysis under the specific conditions of this study (solvent composition and temperature) by employing linear free-energy relationships. For substituent variation in the acyl component of the ester molecule, the Hammett equation has had great success in correlating rates and structural modifications. Table II, Part 2, shows the excellent results that are obtained for the ester data generated in this study, using this procedure. Note that all of the correlation coefficients are better than 0.994, which is excellent. For changes in the alkyl portion of the molecule, the recently reported ϕ values can be correlated with reaction rates. The results of this treatment are shown in Table II, Part 1. The correlation coefficients for this treatment are also excellent. Using both the Hammett relationship, which uses the dissociation of aromatic acids as the model process, and the ϕ values, which are based on the dissociation of alcohols as the model process, it is possible to predict the rate of alkaline hydrolysis for any ester where the σ value (the acyl component) and ϕ value (the alkyl component) have been determined.

Table II—Correlation of Relative Reaction Rates (log k/k_0) with Substituent Constants for Some Aromatic Esters

Ester Series	M^b	SD^c	R^d
Part 1 Substituent Variations in the Alkyl Component[a]			
p-Methyl benzoates	0.772	0.030	0.997
Benzoates	0.816	0.023	0.999
p-Fluoro benzoates	0.832	0.039	0.997
p-Chloro benzoates	0.776	0.051	0.994
p-Cyano benzoates	0.771	0.046	0.995
p-Nitro benzoates	0.734	0.018	0.999
Part 2 Substituent Variations in the Acyl Component[e]			
Ester Series	ρ^f	SD^c	R^d
Methyl benzoates	2.138	0.079	0.996
Ethyl benzoates	2.213	0.093	0.995
Propyl benzoates	2.184	0.049	0.999
Isopropyl benzoates	2.233	0.061	0.998
Butyl benzoates	2.158	0.077	0.996
Isobutyl benzoates	2.243	0.055	0.998

[a] The alkyl substituents for all series in this group were methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, and isobutyl. [b] *M* is the least-squares fitted slope of a log (rate constant) *vs.* ϕ plot. [c] Standard deviation of points from the line. [d] Correlation coefficient for the line. [e] The acyl substituents for all series in this group were *p*-methyl, *p*-hydrogen, *p*-fluoro, *p*-chloro, *p*-cyano, and *p*-nitro. [f] ρ is the least-squares fitted slope of a log (rate constant) *vs.* σ plot.

This is accomplished by simple extrapolation or interpolation of the log rate constant *versus* substituent constant plot.

Other procedures are available to predict rates of hydrolysis for esters when structure variations are made in both the acyl and alkyl portions of the ester molecule (11). These procedures are empirical in nature and have undesirable features associated with them. A thorough discussion of the two procedures, as well as the mechanistic significance of the slopes generated in this study, will be the subject of another publication.

REFERENCES

(1) J. R. Robinson, *Anal. Chem.*, **39**, 1178(1967).

(2) T. J. Mikkelson and J. R. Robinson, *J. Pharm. Sci.*, **57**, 1180(1968).

(3) W. Hong and K. A. Connors, *ibid.*, **57**, 1789(1968).

(4) S. Sun and K. A. Connors, *ibid.*, **58**, 1150(1969).

(5) R. L. Shriner, R. C. Fuson, and D. Y. Curtin, "The Systematic Identification of Organic Compounds," 5th ed., Wiley, New York, N. Y., 1965.

(6) R. G. Bates, "Determination of pH—Theory and Practice," Wiley, New York, N. Y., 1967.

(7) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill, New York, N. Y., 1940, p. 186.

(8) J. R. Robinson and L. E. Matheson, *J. Org. Chem.*, **34**, 3630 (1969).

(9) J. E. Leffler and E. Grunwald, "Rates and Equilibria of Organic Reactions," Wiley, New York, N. Y., 1963, p. 173.

(10) P. R. Wells, *Chem. Rev.*, **63**, 171(1963).

(11) C. K. Hancock and C. P. Falls, *J. Amer. Chem. Soc.*, **83**, 4214(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 10, 1969, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*

Accepted for publication December 16, 1969.

* National Institutes of Health Predoctoral Fellow.

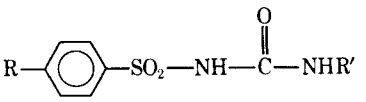
Gas Chromatographic Method for Determination of Tolbutamide and Chlorpropamide

KHALID SABIH and KHAWLA SABIH

Abstract □ Gas-liquid chromatographic methods for the measurement of tolbutamide and chlorpropamide in blood and urine and in pharmaceutical preparations have been developed. In these procedures the drugs were extracted from acidified plasma or urine with chloroform and converted to the corresponding methyl derivative by treatment with dimethylsulfate in the presence of base. The methyl derivatives of these drugs were analyzed on DC-200 coated on diatomaceous earth (Gas Chrom Q). The method has considerable specificity and sensitivity and can measure as little as 0.1 mcg. of these drugs.

Keyphrases □ Tolbutamide in biological fluids—determination □ Chlorpropamide in biological fluids—determination □ Column chromatography—separation, purification □ TLC—identity □ IR spectrophotometry—identity □ GLC—analysis

Tolbutamide (I) and chlorpropamide (II) are sulfonylurea derivatives and are widely used as oral hypoglycemic drugs in the treatment of diabetes mellitus. Few methods are available for the determination of the concentration of these and related drugs in blood or other biological fluids or materials.



I, R = CH_3, R′ = *n*-Bu
II, R = Cl, R′ = *n*-Pr

Mesnard and Crockett (1) described a method for the determination of sulfonylurea derivatives in urine. This method involved hydrolysis of these drugs in the presence of a base to obtain the amine, which in turn was treated with a solution of picric acid to produce a colored material. This color was compared with that obtained with a standard. A colorimetric method was described by Spingler (2), which also involved the hydrolysis of tolbutamide to butyl amine followed by formation of a yellow color by reacting butyl amine with dinitrofluorobenzene. Leal (3) precipitated tolbutamide as its silver salt and identified it by its melting point. Kalinowski and Korzyliski (4) and Voicu (5) described a mercurocoulometric method for tolbutamide determination. A photometric method has been described (6) for the determination of tolbutamide in pharmaceuticals. This method involved treatment of a solution of the drug in dimethylformamide (DMF) with a solution of ascorbic acid in DMF, and absorbance was measured at 530 mμ. Thin-layer chromatographic methods were also described recently (7, 8) using silica gel G and GF. A UV method was also described (9) for the determination of several sulfonylurea derivatives. Some of these methods are not very specific and some are not very sensitive.

The difference in absorption and metabolism rates of these drugs and their success as oral antidiabetics has stimulated the search for a gas chromatographic method to determine these drugs in biological material and in pharmaceutical preparations. This report describes a gas chromatographic method for the determination of tolbutamide and chlorpropamide in blood and urine and in pharmaceutical preparations. This method involves the conversion of these drugs to their methyl derivatives by treatment of their respective sodium or potassium salt with dimethylsulfate.

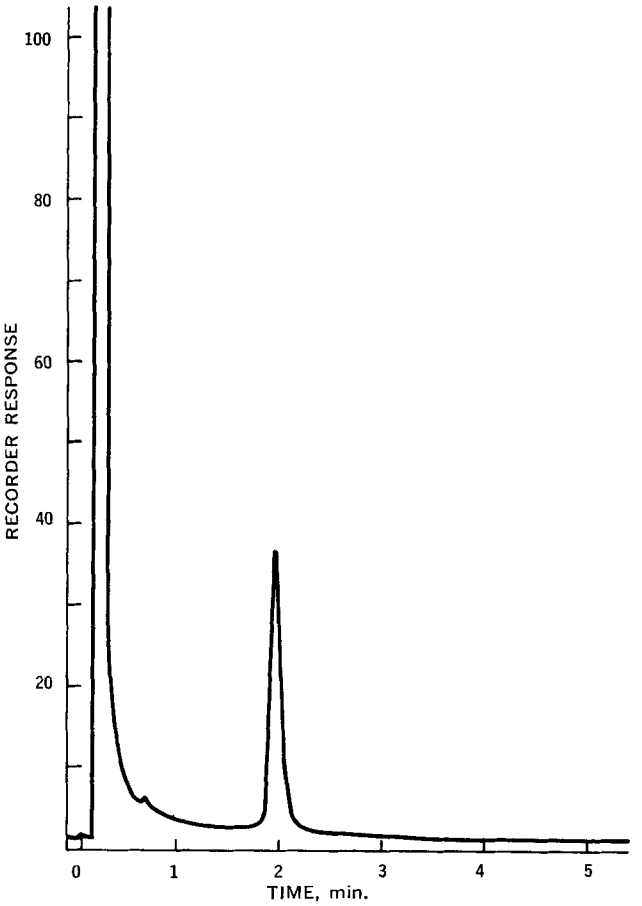


Figure 1—*Typical chromatogram of methylated tolbutamide. The curve represents a 5-μl. injection of 40 mcg. of the drug in 100 μl. chloroform.*

EXPERIMENTAL

Reagents—Tolbutamide (The Upjohn Co., Kalamazoo, Mich.); chlorpropamide (Chas. Pfizer and Co., Inc., New York, N. Y.); and dimethyl sulfate (Matheson, Coleman & Bell, Norwood, Ohio) were used. Chloroform redistilled analytical reagent, methanol analytical reagent, and heptane redistilled were used. Methanolic solution of potassium carbonate was made by mixing 1 ml. of 5–10% aqueous solution of K_2CO_3 with 9 ml. of MeOH. Acetate buffer 0.2 *M*, pH 5.6, was made by mixing 4.8 ml. of AcOH solution (0.2 *M;* 11.55 ml. in 1000 ml. H_2O) and 45.2 ml. of NaOAc solution (0.2 *M;* 16.4 g. in 1000 ml. H_2O), and the mixture was diluted with water to 1000 ml.

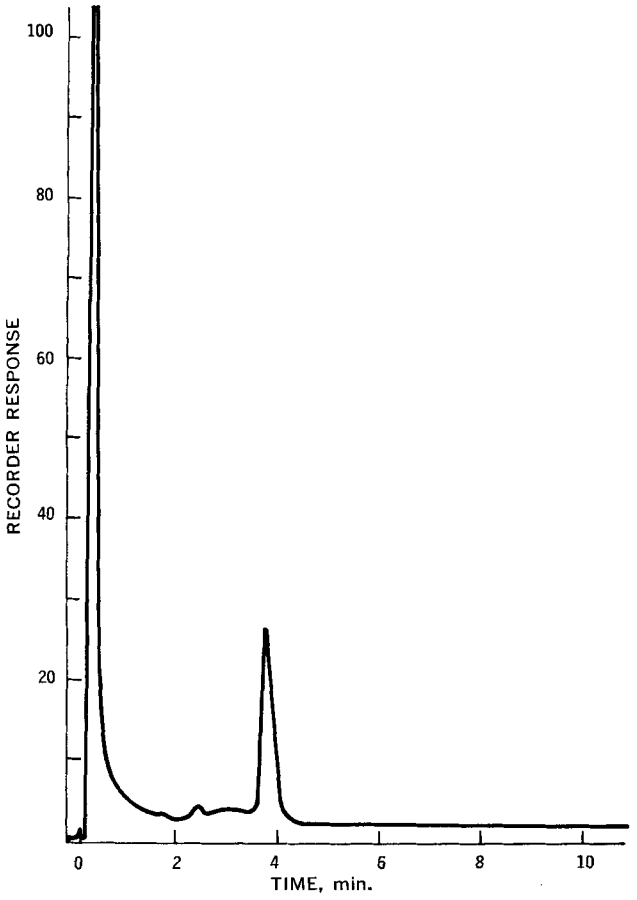


Figure 2—*Typical chromatogram of methylated chlorpropamide. The curve represents a 5-μl. injection of 28 mcg. of the drug in 100 μl. chloroform.*

Apparatus—An F and M model 5755 B gas chromatograph equipped with a flame-ionization detector was used for chromatography. The column was 1.37 m. (4.5 ft.), 0.33 cm. (0.125 in.) stainless steel tubing packed with diatomaceous earth (Gas-Chrom Q), 80–100 mesh, coated with 5% DC-200. The operating temperatures used for tolbutamide were: column, 205–210°; detector, 320°; and injection port, 330°. Helium was used as the carrier gas with a flow rate of 50 ml./min. For analysis of chlorpropamide, column temperature was 180° and the helium flow rate was 40 ml./min.

Procedure—*Standards*—A stock solution of 5 mg. of each sulfonylurea drug in 10 ml. chloroform was prepared. Aliquots containing 0, 10, 20, 40, 60, 80, and 100 mcg. were placed in separate centrifuge tubes; the solvent was removed under a stream of air, and each residue was dissolved in 1 ml. of methanolic potassium carbonate followed by 0.1 ml. of dimethyl sulfate. The resulting mixtures were heated on a water bath at 70° for 5 min. Following removal of the methanol at that temperature by a steady stream of air, 1 ml. 0.2 *M* acetate buffer, pH 5.6, was added and followed by 5 ml. of heptane. Extraction was aided by the use of a vortex mixer. After centrifugation, 4 ml. of the heptane extract from each tube was transferred to a separate 5-ml. flask from which the heptane was evaporated with a stream of air. The resulting residues were dissolved in 100 μl. of chloroform. One- to five-microliter samples were injected onto the column under the conditions cited above. Figures 1 and 2 show typical chromatograms of tolbutamide and chlorpropamide, respectively.

Pharmaceutical Preparations—One tablet of tolbutamide or chlorpropamide was pulverized in a mortar to a fine powder and triturated with 50 ml. ethanol. The mixture was then filtered and the filtrate transferred to a 100-ml. volumetric flask and made to volume with ethanol. Aliquots were placed in separate centrifuge tubes and the ethanol was removed under a stream of air. The residues were then converted to the methyl derivatives and chromatographed as described.

Biological System—A stock solution containing 5 mg. tolbutamide or 5 mg. chlorpropamide in 10 ml. 5% aqueous K_2CO_3 was prepared. The proper volume of either solution was added to plasma or urine in amounts of 0, 2.5, 5, 10, 20, 30, and 40 mcg./ml. Two milliliters of the resulting samples were acidified with 1 ml. of saturated solution of NaH_2PO_4 and then extracted with 10 ml. of chloroform by shaking on a vortex mixer for 2 min. The phases were separated by centrifugation and the aqueous layer was removed by aspiration. Five milliliters of the chloroform extracts was transfered to centrifuge tubes and the solvent was removed under a stream of air. Each residue was treated with Me_2SO_4 and chromatographed as described. One-milliliter plasma samples containing either of the sulfonylurea drugs were acidified with 1 ml. of saturated solution of NaH_2PO_4 and extracted with five volumes of chloroform by shaking carefully for 2 min. Layers were separated by centrifugation (2000 r.p.m. for 5–10 min.); emulsions were broken by stirring with a wooden stick and recentrifuged for another 5–10 min. The aqueous layer was aspirated and four-fifths of the chloroform layer was placed in a second centrifuge tube. Solvent was removed under a stream of air, and the sulfonylurea residues were converted to their respective methyl derivatives and chromatographed as described.

Identification of the Methyl Derivative of Tolbutamide—A solution of 1.0 g. tolbutamide in 30 ml. of 10% water–methanol was placed in a three-necked 150-ml. flask fitted with a reflux condenser and a magnetic stirrer. To this solution was added 2.0 g. of K_2CO_3 followed by 20 ml. of dimethyl sulfate. The reaction was allowed to proceed at 70° by heating on a water bath with stirring for 15 min. The reaction mixture was then cooled and extracted three times with 100-ml. portions of heptane. Removal of heptane under reduced pressure gave 0.9 g. of a semisolid material. The original reaction mixture was then treated with 20 ml. of 0.2 *M* acetate buffer, pH 5.6, and extracted twice with 100-ml. portions of heptane. Removal of heptane under reduced pressure yielded 0.2 g. of a semisolid material. TLC of both extracts using silica gel and chloroform as the moving phase showed only one uniform spot with an R_f value of 0.55. In comparison, tolbutamide did not move under these conditions. The product was purified by column chromatography using silica gel (Merck) and eluted with chloroform. Removal of chloroform under pressure gave 1.0 g. of a colorless oil, which solidified on standing overnight in a cool place, m.p. 33°.

Anal.—Calcd. for $C_{13}H_{20}N_2O_3S$: C, 54.93; H, 7.04; N, 9.85; Found: C, 55.49; H, 7.36; N, 9.96.

Identification of the Methyl Derivative of Chlorpropamide—A solution of 1.0 g. chlorpropamide in 30 ml. of 10% water–methanol was converted to its methyl derivative using the same procedure listed for tolbutamide. Purification of the methyl derivative

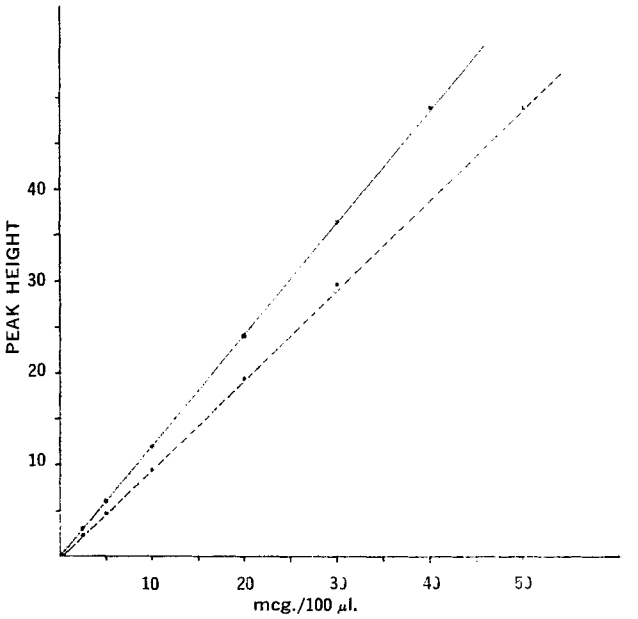


Figure 3—*Relationship between peak height and amount injected. Five microliters injected in each case. Key: ——, tolbutamide; and - - -, chlorpropamide.*

Table I—Recovery of Tolbutamide from Plasma

Amount Added, mcg.	Amount Recovered, mcg.	Recovery, %
5	4.8	96
10	9.8	98
20	19.4	97
30	30.0	100
40	40.8	102
60	57.6	96

was performed by column chromatography using silica gel (Merck) and eluted with chloroform. Removal of the chloroform under reduced pressure gave a colorless oily liquid which did not solidify on standing.

Anal.—Calcd. for $C_{11}H_{15}ClN_2O_3S$: C, 45.44; H, 5.13; N, 9.67; Found: C, 45.89; H, 5.39; N, 9.32.

RESULTS

Attempts to chromatograph tolbutamide and chlorpropamide on four different stationary phases of varying selectivity were not successful. In both cases, two peaks were obtained. The first peak eluted very close to the solvent front and the second peak showed a marked tailing under different temperature and flow-rate settings.

The methyl derivatives of tolbutamide and chlorpropamide were obtained in quantitative yield and were identified by microanalysis and IR spectroscopy. These derivatives were found to be *N*-methyl tolbutamide and *N*-methyl chlorpropamide.

By utilizing conditions cited in the method, the retention time of methylated tolbutamide was 2.0 min. and that of methylated chlorpropamide was 3.8 min. Chromatograms of control samples did not contain any peak with the retention time of the methyl derivative of tolbutamide or chlorpropamide. The retention times of pure methyl derivatives and that prepared from extracted blood or urine were identical. Chromatograms of extracts of control plasma did not contain any peaks with the retention time of either methyl derivative. Addition of increasing amounts of methylated derivatives to extracts of plasma containing the drugs produced an increase in the respective peak height having the retention time of the standard. The relationship between peak height and quantity of the drugs is linear between 1–50 mcg. in both cases as shown in Fig. 3.

To test the reproducibility of the gas chromatographic method, three samples of plasma containing known amounts of the sulfonyl ureas were made. Each sample was divided into four equal parts; the drugs were extracted from each part and converted to the methyl derivatives as described in the method. Gas chromatography of these samples gave the same peak height in each case with a standard error of ±0.52.

In a separate experiment to test the adequacy of the extraction procedure, varying amounts of the drugs were added to plasma samples and the chromatograms of these samples were compared with that of a standard. The recovery ranged between 96–102% of added tolbutamide and 94–100% of added chlorpropamide as shown in Tables I and II, respectively. The extraction method is also applicable to other biological fluids such as urine.

Table II—Recovery of Chlorpropamide from Plasma

Amount Added, mcg.	Amount Recovered, mcg.	Recovery, %
5	4.7	94
10	9.7	97
20	20.0	100
30	28.8	96
40	39.6	94

The methylation procedure of these sulfonylurea drugs was also tested for its completion. Known amounts of these drugs were added to plasma or water and extracted and methylated. The amount methylated was then determined from a standard curve obtained with a pure methylated drug. This showed about 95–100% methylation.

Other commonly used drugs, *e.g.*, phenobarbital, pentobarbital, glutethimide, and diphenylhydantoin, showed no interfering peaks in the same region as methylated tolbutamide or chlorpropamide. These drugs can also be methylated and analyzed using the procedure described in the *Experimental* section.

The amounts of tolbutamide and chlorpropamide were determined using the external standard technique.

DISCUSSION

The presence of two peaks in the chromatograms of unchanged tolbutamide can be attributed to the decomposition of the drug on the column. Methylation of tolbutamide stabilized the molecule and prevented this decomposition. Furthermore, the methylation process was advantageous because it shortened the retention time and reduced the possibility of loss of tolbutamide on the column during chromatography. In the case of chlorpropamide, the molecule decomposed on the column to give a chromatogram of two peaks similar to that of tolbutamide. Methylation of chlorpropamide produced only one symmetrical peak with fairly short retention time.

The IR spectrum of tolbutamide shows, among other bands, a strong absorption band at 1710–1690 $cm.^{-1}$, which is due to the carbonyl (C=O) stretching vibrations. The IR spectrum of methylated tolbutamide shows some differences due to the introduction of the methyl group. However, the spectrum again shows a strong absorption band at 1710–1690 $cm.^{-1}$, which again may be attributed to the presence of a carbonyl group in the molecule. This indicated that the methyl group was introduced on the nitrogen atom rather than the oxygen atom to give the *N*-methyl derivative.

The GLC method described is accurate, sensitive, and specific for the measurements of tolbutamide and chlorpropamide concentration in blood and other biological fluids and can be used in clinical laboratories. The method is also applicable to determine the amount of these drugs in pharmaceutical preparations such as tablets. The method requires approximately 2 hr. for analysis of several samples and the gas chromatographic step takes less than 5 min.

REFERENCES

(1) P. Mesnard and R. Crockett, *Rev. Espan. Fisiol.*, **16,** 163 (1960).

(2) H. Spingler, *Klin. Wochenschr.*, **35,** 533(1957).

(3) A. M. Leal, *Rev. Port. Farm.*, **10,** 197(1960).

(4) K. Kalinowski and R. Korzyliski, *Acta Pol. Pharm.*, **20,** 221(1963).

(5) A. Voicu, *Farmacia*, **10,** 399(1962).

(6) A. Alessandro, R. Emer, and G. Abbondanza, *G. Med. Mil.*, **116,** 827(1966).

(7) K. C. Guven, S. Gecgil, and O. Pekin, *Eczacilik Bul.*, **8,** 158(1966).

(8) M. G. Hutzul and G. F. Wright, *Can. J. Pharm. Sci.*, **3,** 4(1968).

(9) D. L. Smith, T. J. Vecchio, and A. A. Forist, *Metab. Clin. Exp.*, **14,** 229(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 25, 1969, from the *Clinical Pharmacology and Toxicology Center, Department of Pharmacology, University of Kansas Medical Center, Kansas City, KS 66103*

Accepted for publication January 12, 1970.

This work was supported by funds from Public Health Service Grant GM 15956.

Microanalyses were performed by G. Kristiansen, Department of Medicinal Chemistry, University of Kansas, Lawrence, Kan.

Various Species of Sulfathiazole Form I

LATIF S. SHENOUDA

Abstract □ Sulfathiazole crystallized from ethanol, a mixture of ethanol and benzene, a mixture of chloroform and acetone, isopropyl alcohol, or 10% and 1% ammonia solutions gives Form I, which consists of varying proportions of a melting species and another species which undergoes solid–solid transition. This was confirmed both microscopically and by differential scanning calorimetry. Crimping of the samples crystallized from ammonia solutions causes the separation of the peaks representing these species and gives a thermogram identical to that obtained from samples crystallized from alcohol. Grinding appears to destroy the melting species and to have a significant effect on the transition temperature but not on the heat of transition. The calculated average heat of transition, for ground samples crystallized from both ammonia solutions and from alcohol, is 1652 ± 56 cal./mole. IR spectra and X-ray diffraction patterns of the powder did not show any major differences between samples crystallized from either solvent.

Keyphrases □ Sulfathiazole species—Form I □ Grinding, crimping effect—sulfathiazole crystals □ Melting range—sulfathiazole Form I crystals □ Differential scanning calorimetry—identity □ IR spectrophotometry—identity □ X-ray diffractometry—identity

There are conflicting reports as to whether sulfathiazole exists in two or three crystalline forms. Grove and Keenan (1) reported two forms: Forms I and II for which melting points are 175–176° and 201–202.5°, respectively. Milosovich (2), using the solubility method, stated that the transition temperature of Form I is 94.5 ± 2.7° with a heat of transition of 1744 cal./mole. On the other hand, Guillory (3), using differential thermal analysis, reported a transition temperature of 161° and a heat of transition of 1420 ± 40 cal./mole for Form I. Three crystalline forms of sulfathiazole were reported by Miyazaki (4) and Mesley and Houghton (5). These forms are symbolized as α or A (I),[1] β or B (II),[1] and a third form α' or C. These investigators claimed that the third form of sulfathiazole can only be prepared by crystallization from dilute ammonia solution. Form I can be prepared by crystallization from warm alcohol or a mixture of chloroform and acetone, and Form II by crystallization from *n*-propanol at 80°, *sec*-butanol, or by heating Form I at 180°.

Since sulfathiazole has been used extensively as a model drug in research work in recent years, it was found necessary to conduct a further investigation of the sulfathiazole polymorphic system to see whether or not this drug exists in more than two crystalline polymorphs.

EXPERIMENTAL

Materials and Apparatus—Sulfathiazole NF (Mallinckrodt) was used in this investigation. Group I solvents consists of ethanol USP, a mixture of benzene and ethanol (3:1), a mixture of chloroform and acetone (1:3), isopropyl alcohol, and 1% and 10% ammonia solutions. Group II consists of *n*-propanol and *sec*-butanol. Unless otherwise specified, all these solvents were reagent grade.

A DSC-1B (Perkin-Elmer) was employed. Nitrogen, as an effluent gas, was used at a rate of 20 ml./min. Indium and tin were used for calibration of the temperature scale. In this investigation, the former was used as the main calibrant for calculating the heat of fusions and transitions since its heat of fusion is known to be 6.8 cal./g. (3, 6). Since a known weight of indium used in this investigation was supplied by the manufacturer, it was found necessary to check it against a substance whose heat of fusion is well documented. Form II of silver nitrate was used for this purpose. The heat of fusion of Form II of this compound is 2760 cal./mole (7). Areas under the peaks were measured with a planimeter. IR spectra were obtained using both KBr disks and mineral oil mulls. X-ray diffraction patterns, using 200-mesh powder, were obtained using a Siemens X-ray diffraction instrument.[2] Microscopic examinations were done employing a Zeiss polarizing research microscope equipped with a Kofler hot stage.

Table I—Thermal Data of Sulfathiazole Form I Crystallized from Different Solvents Using DSC

Treatment	Solid–Solid Transition Peak, Transition Temp.	Solid–Solid Transition Peak, Maximum Temp.	Melting Species Peak, Maximum Temp.	ΔH, cal./mole
Hot Alcohol in Ice Bath				
Uncrimped and unground	161	170	179	1792 ± 249
Crimped and unground	161	170	177	2058 ± 137
Ground and crimped	141	Broad	—	1637 ± 83
10% Ammonia in Crystallizing Dish				
Uncrimped and unground	169	178	—	1899 ± 131
Crimped and unground	167	174.5	177	1984 ± 82
Ground and crimped	154	164.5	—	1647 ± 49
1% Ammonia in Beaker without Stirring				
Uncrimped and unground	—	—	176.5	2265 ± 369
Crimped and unground	159.5	171	176	1671 ± 236
Ground and crimped	157.0	167.5	—	1673 ± 22

[1] Nomenclature used by other investigators.

[2] The patterns were obtained through the courtesy of Dr. S. C. Mehta at the University of Michigan College of Pharmacy, Ann Arbor, Mich.

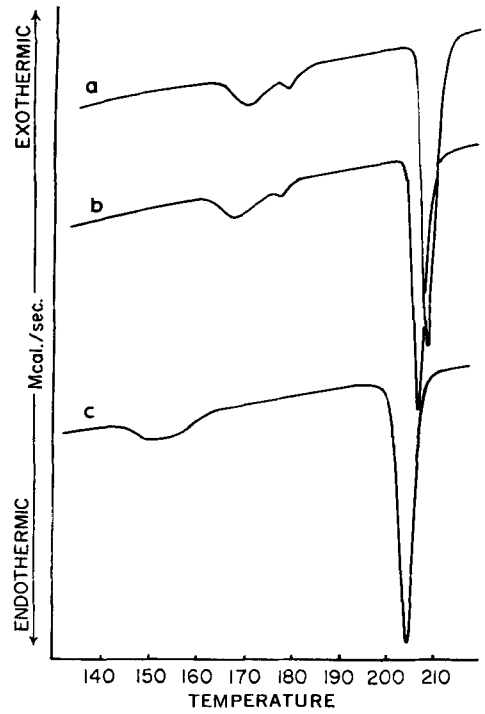


Figure 1—*Thermograms of samples crystallized from alcohol in an ice bath. Key: a, uncrimped and unground; b, crimped and unground; c, ground and crimped; scan speed 40°/min. Peaks appearing below 200° represent the species of Form I and the peak appearing above this temperature represents Form II.*

Preparation of Samples—Sulfathiazole was crystallized from a saturated solution in the various solvents. Sulfathiazole was crystallized from 10% ammonia solution on a watch glass. On the other hand, crystallization from 1% ammonia solution was performed in a beaker without stirring; the scaly crystals were filtered and dried at 100° for 30 min. under vacuum and scanned immediately.

Differential Scanning Calorimetry (DSC)—Samples weighing 1–6 mg. were used. A semimicro balance with a sensitivity of 0.01 mg. was used to weigh all samples. A scanning speed of 40°/min. was employed throughout this study, because a scanning speed of 10°/min. gave broad and irregular transition peaks. Using indium as the calibrant, the calculated heat of fusion of Form II of silver nitrate is 2812 ± 46 cal. mole. This value is comparable to the value reported (7).

Since sulfathiazole does not sublime on heating, it was thought at first to run the crystalline material prepared from different solvents without subjecting them to any stress or strain, *i.e.*, without crimping or grinding. The results obtained were compared with experiments performed on the crimped unground and crimped ground samples. Grinding was done using a mortar and pestle.

RESULTS AND DISCUSSION

Microscopic examination of sulfathiazole crystallized from Group I solvents revealed that these samples consist of two species in various proportions. One species undergoes solid–solid transition in a temperature range of 150–166°, depending on the solvent of crystallization. The second species melts between 170–176° with immediate partial crystallization. On further heating of the samples, complete melting was observed at 198–203°. The latter temperature range is the same melting range for sulfathiazole Form II crystallized from Group II solvents or from warm alcohol and heated to 180°. Crystallization of sulfathiazole from the solvents which yield Form I was done on a glass slide. On microscopic examination of the crystals on a hot stage, it was observed that complete melting occurred at 170–176° without crystallization in all cases. The absence of crystallization from the melt is due to the absence of seed material, as was demonstrated when the two species were present.

Since the two species of sulfathiazole Form I are present regardless of solvent system used and since sulfathiazole crystallized from ammonia presents the point of controversy, it was decided to limit quantitative determination to crystals obtained from 10% and 1% ammonia solutions and to those crystallized from hot alcohol in an ice bath. The DSC results on those samples, subjected to various treatments, are summarized in Table I and their thermograms are shown in Figs. 1–3. Examination of the table and various thermograms in the figures shows that in the region of 140–180°, there are one or two peaks present, depending on the solvent of crystallization and the treatment of the sample. For example, the thermogram for sulfathiazole crystallized from alcohol consists of two peaks regardless of crimping. The first peak represents solid–solid transition and the second represents the melting species. Under the present conditions, *i.e.*, at a scanning speed of 40°/min., it was difficult to calculate the areas under the peak of each species. Slow scanning speed of 10°/min. caused the separation of two peaks, but the peaks were broad and very hard to differentiate from the base line. The total area under the two peaks was measured for a scanning rate of 40°/min., and ΔH was calculated. A large value of ΔH with a very large standard deviation was obtained for both crimped and uncrimped, unground samples. This is an indication of the nonuniformity of samples or, in other words, the samples taken consist of various proportions of the melting species which contribute to this great variation. However, after grinding and crimping the sample, it was found that the peak of the melting species completely disappeared, and only one peak was observed. The calculated heat of transition was found to be 1637 ± 83 cal./mole. On the other hand, uncrimped and unground samples crystallized from ammonia gave one peak on scanning. An exotherm was observed in samples crystallized from 1% ammonia and, in some instances, in samples crystallized from 10% ammonia, indicating some crystallization of the melt. This was confirmed by the microscopic examination, as well as by visual observation in the open pan while scanning. The exotherm was not noticed when the crystallized sample was run after a few days.

After crimping the unground sample, two peaks were obtained similar to those obtained in the case of sulfathiazole crystallized from alcohol. The appearance of two peaks, the first representing solid–solid transition and the second representing melting, may be due to crimping. In the case of samples crystallized from ammonia solutions, crimping caused the separation of the two peaks repre-

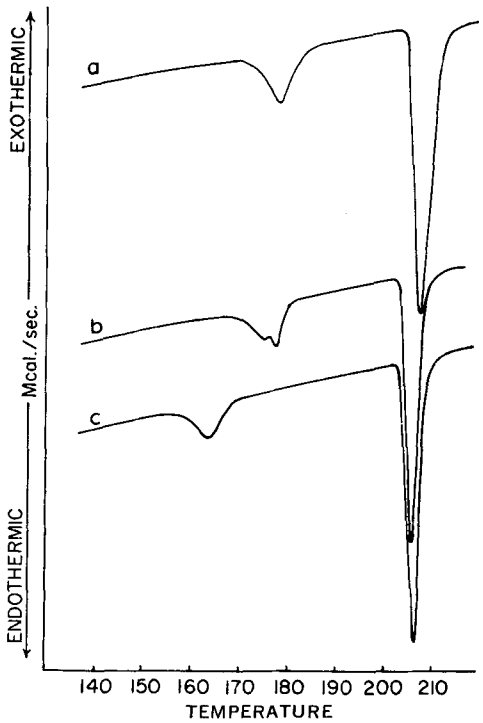


Figure 2—*Thermograms of samples crystallized from 10% ammonia solution in a crystallizing dish. Key: a, uncrimped and unground; b, crimped and unground; c, ground and crimped; scan speed 40°/min. Peaks appearing below 200° represent the species of Form I and the peak appearing above this temperature represents Form II*

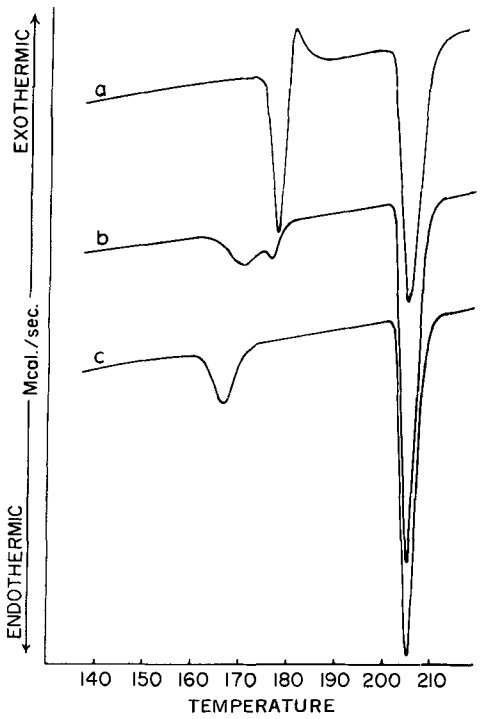


Figure 3—*Thermograms of samples crystallized from 1% ammonia solution in a beaker without stirring. Key: a, uncrimped and unground; b, crimped and unground; c, ground and crimped; scan speed 40°/min. Peaks appearing below 200° represent the species of Form I and the peak appearing above this temperature represents Form II.*

senting the two species. Upon grinding and crimping, the system was apparently stabilized and only one peak was observed. The calculated heats of transition are 1647 ± 49 and 1673 ± 22 cal./mole for both sulfathiazole crystallized from 10% and 1% ammonia, respectively. These values are not significantly different, within experimental errors, from the value obtained when alcohol was used as a crystallizing medium. The values for ΔH obtained from 18 runs gave a calculated average of 1652 ± 56 cal./mole. It appears, therefore, that crimping and grinding have an effect on the thermal behavior of sulfathiazole Form I. Furthermore, grinding caused a shift in transition temperature of about 15 and 20° in the case of sulfathiazole crystallized from ammonia and alcohol, respectively, although the calculated heats of transition are almost the same. This shift in transition temperatures might be due to differences in particle size and change in thermal conductivity of the sample as reported by Guillory (3) and Moustafa and Carless (8). The findings reported here are not different from those reported recently (8). In their report, Moustafa and Carless stated that in no case was melting observed below 200°. The present results, both microscopically and calorimetrically, suggest the presence of a melting species and another that undergoes solid–solid transition. This phenomenon was observed in all cases where Form I was produced and not only in the case of sulfathiazole crystallized from 95% alcohol as reported (8). The same authors reported that vibration milling of Form I crystallized from 95% ethanol resulted in the transition temperature being lowered by 5°. This finding is in agreement with the present observation, although a more dramatic effect was noticed with grinding. Mesley and Houghton (5) reported differences in the IR spectra of sulfathiazole crystallized from dilute ammonia and from alcohol, although no spectra were presented in their report. In the present study, there were no major differences in either IR spectra or X-ray diffraction patterns of both samples when those patterns were compared with already published ones (8, 9). Since grinding is a prerequisite for the preparation of samples tested by these techniques, and since it has been shown to destroy the melting species in this study, differences in the mentioned patterns were not observed.

It is rather misleading to call the melting species of sulfathiazole a third physical form, since the techniques employed in this investigation did not confirm its presence as a separate and pure entity with great differences in physical characteristics. However, the present results do not preclude the existence of a third form of sulfathiazole. It is probable that a third form does exist, although highly unstable on grinding, and this is probably identical to the melting species reported in this investigation.

The peaks representing Form II in all runs performed in this study were evaluated and compared to those obtained from samples crystallized from Group II solvents and from warm alcohol heated at 180°. Large variation was observed in the case of uncrimped and crimped, unground samples. This is probably due to incomplete crystallization of the melting species. However, grinding the sample resulted in a heat of fusion which compares well with that obtained from pure Form II. The calculated average heat of fusion for this form, whether the starting sample was crystallized from ammonia or alcohol, is 6893 ± 185 cal./mole. This value is comparable to the one reported by Moustafa and Carless (8)—*viz.*, 6615 ± 235 cal./mole. The scanning rate was found to have little or no effect on the value of ΔH of Form II. In this study, it was found that there is a difference of about 2% in the value of ΔH of Form II scanned at 10°/min. and 40°/min. This is the same difference observed when the calibrant was scanned at both rates.

REFERENCES

(1) D. C. Grove and G. L. Keenan, *J. Amer. Chem. Soc.*, **63,** 97(1941).

(2) G. Milosovich, *J. Pharm. Sci.*, **53,** 484(1964).

(3) J. K. Guillory, *ibid.*, **56,** 72(1967).

(4) H. Miyazaki, *Jap. J. Pharm. Chem.*, **19,** 133(1947); through *Chem. Abstr.*, **45,** 3559(1951).

(5) R. J. Mesley and E. E. Houghton, *J. Pharm. Pharmacol.*, **19,** 295(1966).

(6) J. Tsau and D. F. R. Gilson, *J. Phys. Chem.*, **72,** 4082(1968).

(7) F. D. Rossini, D. D. Wagman, W. H. Evans, S. Levine, and I. Jaffe, *Nat. Bur. Stand. (U. S.) Circ.*, **500,** 679(1952).

(8) M. A. Moustafa and J. E. Carless, *J. Pharm. Pharmacol.*, **21,** 359(1969).

(9) W. I. Higuchi, P. D. Bernardo, and S. C. Mehta, *J. Pharm. Sci.*, **56,** 200(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 4, 1969, from the *College of Pharmacy, University of Toledo, Toledo, OH 43606*

Accepted for publication December 29, 1969.

The author thanks Dr. Harry B. Kostenbauder and the University of Kentucky, College of Pharmacy, Lexington, Ky., for extending the invitation to perform this study in their own laboratories.

Quantification of the Binding Tendencies of Cholestyramine III: Rates of Adsorption of Conjugated Bile Salt Anions onto Cholestyramine as a Function of Added Inorganic Electrolyte Concentration, Temperature, and Agitation Intensity

WILLIAM H. JOHNS* and THEODORE R. BATES

Abstract ☐ The *in vitro* rate of binding of several conjugated bile salt anions to cholestyramine was studied at 37°, alone and in the presence of varying concentrations of sodium chloride. The kinetic studies were conducted under relatively mild agitation conditions to simulate reasonably the *in vivo* situation existing in the small intestinal lumen. A second-order kinetic model was found to represent the interaction data most suitably. The rate constants for the adsorption process were found to decrease in the presence of increasing concentrations of inorganic electrolyte, the reductive effect being more pronounced with the trihydroxy conjugates than with the dihydroxy derivative. Langmuir affinity constants were found to parallel the apparent second-order rate constants for systems devoid of added inorganic electrolyte. From studies concerning the influence of temperature on the rate of interaction, calculated apparent energies of activation indicated the existence of a substantially lower *energy barrier* to the binding of the dihydroxy glycine-conjugated derivative, glycodeoxycholate, than for its trihydroxy counterpart, the glycocholate anion. Variation in the intensity of agitation at which the reaction was conducted had a more pronounced effect on the rate of uptake of the glycodeoxycholate anion by the resin than on the glycocholate anion. A log–log relationship was found to exist between the second-order interaction rate constant and the speed of agitation. The results obtained from the temperature and agitation studies suggested that the binding of bile salt anions to cholestyramine apparently occurs by means of a diffusion-controlled process.

Keyphrases ☐ Cholestyramine binding—conjugated bile salt anions ☐ Electrolyte-concentration effect—cholestyramine binding ☐ Agitation, temperature effects—anion adsorption, cholestyramine ☐ Rate constants—anion adsorption, cholestyramine ☐ Colorimetric analysis—spectrophotometer

It has been shown that oral administration of the anionic-exchange resin, cholestyramine, results in the lowering of intestinal bile salt concentrations (1) and the interruption of normal enterohepatic circulation of bile salts (2). Cholestyramine also decreases the absorption of dietary cholesterol (3), fatty acids (4), and other materials, both exogenous and naturally occurring in the gastrointestinal tract, which rely on the surface-active properties of bile salts for their efficient intestinal absorption (5, 6). It has been proposed previously that the forces involved in the bile salt anion–cholestyramine interaction are primarily electrostatic in nature and are reinforced by secondary nonelectrostatic forces (7, 8).

The high doses required for efficacy in both clinical (9) and animal studies (10) suggest that cholestyramine is relatively inefficient in its ability to reduce intestinal bile salt levels, even though the capacity expected of such an ion-exchange resin (11) and approached by *in vitro* equilibrium experimentation with cholestyramine (7) would indicate otherwise.

In vivo, the physiologically active glycine- and taurine-conjugated bile salts present in bile are secreted into the lumen of the duodenal segment of the small intestine *via* the common bile duct, where they exist and function as endogenous surfactants in essentially the ionized form (12). Conservation of the secreted bile salts is effectively accomplished by their active reabsorption from the ileal segment of the small intestine (13). Therefore, the resin must interact with bile salt anions during their residence in the duodenal and jejunal regions of the small intestine, prior to their reabsorption from the ileum. If the binding process is inherently slow, or if the presence of physiologic electrolytes or other substances normally present in the intestinal tract tends to retard the rate of adsorption onto the resin, the resin may pass into the large intestine having only been partially utilized, thereby lowering the apparent efficiency of this pharmacologically important adsorbent.

In view of the fact that there would exist two dynamic, competitive reactions in the fluids of the small intestine, that is, the bile salt anion–cholestyramine interaction and the active reabsorption of bile salts from the ileum, it was of interest to investigate the *in vitro* kinetics of the former reaction alone and under the influence of the physiologic electrolyte, sodium chloride. Sodium chloride was previously shown to have an inhibitory effect on the interaction of bile salt anions with cholestyramine, the decrease in apparent capacity being greater for trihydroxy bile salts (*e.g.*, glycocholate and taurocholate) than for dihydroxy derivatives (*e.g.*, glycodeoxycholate) (7). The authors proposed that the electrolyte functioned principally to reduce the charge density on the charged adsorbate and adsorbent species, thereby weakening the electrostatic component of the interaction.

The literature is almost devoid of investigations concerned with the rates of binding of biliary constituents to adsorbents of the ion-exchange type, with the exception of the study of Sawchuk and Nairn (14) on the interaction of the bile pigment, bilirubin, with a series of anionic-exchange resins, including Dowex 1-X2. This work was primarily concerned with the rate of binding as it related to the degree of crosslinkage in the polyvinylstyrene skeleton of the resin polymer and the dependency of the adsorption rate on the particle size of the resin material.

The objectives of the present investigation were to establish and compare the *in vitro* rates of binding of several physiologic, conjugated bile salts to cholesty-

ramine at 37°. Studies of the adsorption-rate process in the presence of varying concentrations of added inorganic electrolyte at 37° were performed in order to appreciate more fully the role these endogenous ions may play in the *in vivo* rates of interaction. The influence of temperature and rate of agitation on the kinetics of the bile salt anion–cholestyramine interaction were also explored to obtain an appreciation for the magnitude of the energy of activation associated with the rate process and to determine whether the adsorption process was film diffusion- or particle diffusion-controlled. Particle diffusion-controlled reactions are rate limited by diffusion of exchangeable ions within the ion-exchange particle itself, whereas film diffusion-controlled exchange is a result of limiting diffusion rates in the diffuse film surrounding the ion-exchange particle.

EXPERIMENTAL

Materials and Methods—The sodium salts of taurocholic,[1] glycocholic,[1] and glycodeoxycholic[1] acids were dried *in vacuo* for at least 48 hr. prior to use. Reagent grade furfuraldehyde,[2] sulfuric and glacial acetic acids, and sodium chloride were used as received. The cholestyramine[3] employed in this investigation was pharmaceutical grade (7).

All binding-rate studies were performed using a 250-ml., three-necked, round-bottom flask maintained at 37 ± 0.1°. Constant agitation of the reaction medium was accomplished by the use of a Cole-Parmer[4] constant torque overhead stirrer (60 ± 1 r.p.m.) equipped with a Teflon-coated three-blade propeller (blade diameter 5.0 cm.) immersed 3.8 cm. into the reaction medium. In all experiments the concentration of adsorbate initially present was maintained at 1.0 millimolar (m*M*)[5] and an equivalent amount of cholestyramine, based on an equivalent weight for the resin of 230, was employed. Each of the binding-rate experiments was performed at least in duplicate, with the rate constants obtained therefrom falling within the limits of experimental error (*i.e.*, a 5% range.)

In the case of the aqueous systems, 57.5 mg. of cholestyramine, dry weight, was allowed contact with 240 ml. of deionized, distilled water at 37° for a period of 0.5 hr. prior to the introduction of a 10-ml. aliquot of a bile salt stock solution. For the studies involving added inorganic electrolyte, identical procedures were followed except that the resin was placed in only 230 ml. of water. Ten-milliliter quantities of concentrated sodium chloride and bile salt stock solutions were simultaneously added to the reaction flask after a half hour.

Samples were withdrawn from the reaction flask at appropriate time intervals using a 1-ml. pipet fitted with a glass wool prefilter; the samples were suitably diluted, if necessary, and spectrophotometrically assayed for the concentration of bile salt remaining free in solution (see *Assay Procedure*).

Since previous work (7) had shown that sodium chloride effectively reduced the capacity of cholestyramine for certain bile salt anions, the concentration of bile salt anion remaining free or unreacted after the reaction had reached completion was determined. Two hundred and fifty-milliliter volumes of a solution containing the same concentration of bile salt, or bile salt and sodium chloride, and the same amount of cholestyramine as that employed in the rate studies were placed into appropriately sealed bottles and shaken at 37° until equilibrium was established. Equilibration was normally established within a 24–48-hr. period. These equilibrium data were utilized in the kinetic analysis of the results obtained in this investigation.

The experimental protocol for those studies designed to establish the role of temperature and agitation intensity on the rate of the bile salt anion–cholestyramine interaction was essentially the same as that described previously. The temperature studies were performed in an aqueous medium at 25, 37, and 47°. The agitation intensity was maintained constant at 60 r.p.m.

The influence of agitation intensity on the reaction rate was studied in pure aqueous medium at a constant temperature of 37° and speeds of agitation of 40, 60, 70, and 100 r.p.m. In these latter studies, the resin was exposed to the medium, which was agitated at 60 r.p.m., for 30 min. After this period, the intensity of agitation was adjusted to the desired level and the bile salt stock solution was added to the system.

Assay Procedure—The concentrations of sodium glycocholate and taurocholate remaining free in solution at the time of sampling were assayed using essentially the same method as reported by Irvin *et al.* (15). The 1-ml. sample, or a similar volume of a suitable dilution thereof, was heated for 0.5 hr. at 65° in the presence of 6.0 ml. of 16 *N* sulfuric acid and 1.0 ml. of a 3% furfuraldehyde solution. Stabilization of the blue color, which developed on heating, was accomplished by the addition of 5.0 ml. of glacial acetic acid. The absorbance (maximum at 660 mμ), which obeyed the Beer's law relationship, was read using a Bausch and Lomb Spectronic-20, equipped with appropriate photomultiplier tube and filter. A solution containing no bile salt and treated in an identical manner served as the blank for these colorimetric determinations. The concentrations of glycodeoxycholate were determined spectrophotometrically as previously described (7).

RESULTS AND DISCUSSION

Theory—The formation of a 1:1 irreversible complex between an anion and an anionic-exchange resin may be expressed by the following equation, the molecularity of which is based on well-established evidence for reactions of ion-exchange resins in general (16, 17):

$$(B^-) + (C^+) \xrightarrow{k} (B\text{-}C) \qquad \text{(Eq. 1)}$$

where (B^-) represents the total concentration (m*M*) of anion present free in solution at any time *t;* (C^+) the total *concentration* (meq.) of ionized binding sites on the anionic-exchange resin unoccupied by anions at any time *t;* (B-C) the concentration (meq.) of anion-resin complex formed at any time *t;* and *k* the second-order rate constant for the reaction. A similar reaction scheme, which assumes that the binding process is virtually irreversible, was suggested by Helfferich (18) as being valid in describing ion-exchange reactions, and has been employed by Sawchuk and Nairn (14) to interpret kinetically the binding between bilirubin and an anionic-exchange resin.

In the bile salt–cholestyramine system under discussion in this paper, Eq. 1 must be modified so as to take account of the binding sites on the anionic-exchange resin that have been previously shown (7) to be unavailable for binding bile salt anions. The concentration of available binding sites on cholestyramine (C^+_a) is therefore equal to the total concentration of sites, (C^+), minus those that are unavailable, (C^+_u). Unless this correction is made, the reaction would appear to be incomplete.

Since a 1:1 stoichiometric relationship exists between the cholestyramine and the bile salt anions, (C^+_a) at any time *t* must equal the concentration of unreacted or free bile salt available for binding to these sites, (B^-_a). Any measurable concentration of bile salt remaining unreacted after the reaction has reached completion is, therefore, a measure of the concentration of bile salt unavailable for binding, (B^-_u), and would equal the concentration of unavailable unoccupied sites, (C^+_u).

The magnitude of (C^+_a) will depend on the accessibility of binding positions on the interior surfaces of the resin bead which, in turn, is a function of the chemical structure of the bile salt anion and thus its ability to assume an optimal orientation for interaction with these sites.

---

[1] Obtained from Calbiochem, Los Angeles, Calif., Grade A.

[2] Obtained from Distillation Products, Inc., Eastman Kodak, Rochester, N. Y.

[3] Supplied by Merck and Co., Inc., Rahway, N. J.

[4] Cole-Parmer Co., Chicago, Ill.

[5] The pH values of 1.0 m*M* aqueous solutions of the sodium salts of taurocholic, glycocholic, and glycodeoxycholic acids, before and after contact with cholestyramine, remained constant at 5.30, 6.80, and 6.60, respectively. These values were insignificantly influenced by the presence or concentration of the inorganic electrolyte used in some of the studies. Based on the pH values obtained and the highest pKa values previously reported for these acids (7), it was determined that the bile acids existed almost completely in the ionized form (*i.e.*, 98.9, 99.6, and 99.8% ionized, respectively).

Table I—Apparent Second-Order Rate Constants Governing the Binding of Conjugated Bile Salts to Cholestyramine at Various Sodium Chloride Concentrations[a]

Bile Salt Anion[b]	Concn. of NaCl, m*M*	Apparent Second-Order Rate Constant, k' (l. mole^{-1} min.$^{-1}$ × 10^{-2})	Langmuir[c] Affinity Constants at 25°, k_1 (l. mole^{-1} × 10^{-4})
Glycocholate	0.00	3.91	0.891
Glycocholate	50.0	1.48	—
Taurocholate	0.00	5.41	1.99
Taurocholate	25.0	3.32	—
Taurocholate	50.0	2.53	—
Taurocholate	75.0	1.98	—
Glycodeoxycholate	0.00	8.61	4.25
Glycodeoxycholate	50.0	8.17	—

[a] Temperature, 37°; agitation intensity, 60 r.p.m. [b] The concentrations of free bile salt remaining unreacted after the reaction had reached completion with respect to the available binding positions on cholestyramine were: glycocholate, at 0 and 50 m*M* concentrations of NaCl, 0.25 and 0.38 m*M*; taurocholate, at 0, 25, 50, and 75 m*M* concentrations of NaCl, 0.21, 0.26, 0.30, and 0.33 m*M*, respectively; and glycodeoxycholate, at 0 and 50 m*M* concentrations of NaCl, 0.01 and 0.05 m*M*. [c] These equilibrium Langmuir constants were obtained from *Reference 7*. The equilibrium binding of bile salt anions to cholestyramine appears to be essentially temperature independent.

The addition of inorganic electrolyte to the bile salt–cholestyramine system has been shown previously to reduce the capacity of the resin to bind bile salt anions (7). The extent of this reduction, which may either result from a competitive reaction between bile salt and electrolyte anions for available, unoccupied positions on the resin or from the ability of the electrolyte to reduce the charge density on the resin, has been found to be not only a function of electrolyte concentration but also of the structure of the bile salt anion. In any event, (C^+_a) will be further reduced below that for the binary bile salt anion–cholestyramine system.

Since the reaction under investigation is bimolecular, and essentially irreversible for the bile salts employed (7), the rate expression for the disappearance of bile salt anion from solution, based only on available binding positions on the resin, may be expressed by the following equation:

$$\frac{-d(B^-_a)}{dt} = k'(B^-_a)(C^+_a) \qquad \text{(Eq. 2)}$$

The experimental design of the binding-rate studies was such that the initial total concentrations of the reactants were equal [*i.e.*, $(B^-)_0 = (C^+)_0$]. A similar equality exists between $(B^-_a)_0$ and $(C^+_a)_0$. Therefore, at any time t the concentration of free bile salt available for binding, (B^-_a), would be equal to the concentration of unreacted available adsorption sites on the resin, (C^+_a). The term (B^-_a) is equivalent to (B^-) minus (B^-_u), or (B^-) minus $(B^-_u)_\infty$, since the concentration of free bile salt unavailable for binding is a constant and therefore independent of time. Substitution of these conditions into Eq. 2, and subsequently integrating the resulting rate expression, yield the apparent irreversible second-order kinetic relationship utilized in the analysis of the bile salt uptake data:

$$\frac{1}{(B^-) - (B^-_u)_\infty} - \frac{1}{(B^-)_0 - (B^-_u)_\infty} = k't \qquad \text{(Eq. 3)}$$

where k' is the apparent second-order rate constant governing the reaction of bile salt anions with *available* binding positions on cholestyramine. The concentration of bile salt remaining unreacted in solution after the reaction has reached completion $[(B^-_u)_\infty]$, under the various experimental conditions, are listed as footnotes to Tables I–III.

Attempts to fit all of the binding-rate data to the well-established integrated rate expression (19) for the reversible reaction, $(B^-) + (C^+) \rightleftarrows$ (B-C), yielded, in a majority of cases, curvilinear rather than the linear plots predicted by this reversible rate expression (19). This finding further supports the use of the apparent irreversible reaction scheme given by Eq. 1.

Effect of Inorganic Electrolyte—The validity of the assumptions made concerning the interactions studied is evidenced by the excellent linearity of the data interpreted by utilization of Eq. 3 (Figs. 1–3). The apparent second-order constants, k', were calculated from the slope of a regression line through the data points subse-

Table II—Effect of Temperature on the Rate of Interaction of Glycine-Conjugated, Bile Salt Anions with Cholestyramine at an Agitation Intensity of 60 r.p.m.

Bile Salt Anion[a]	Temperature	Apparent Second-Order Rate Constant, k' (l. mole^{-1} min.$^{-1}$ × 10^{-2})
Glycocholate	25	2.00
	37	3.91
	47	6.60
Glycodeoxycholate	25	7.89
	37	8.61
	47	10.4

[a] The concentrations of free bile salt remaining unreacted after the reaction had reached completion with respect to the available binding positions on cholestyramine were essentially temperature independent (Table I, Footnote[b]).

Table III—Effect of Agitation Intensity on the Rate of Interaction of Glycine-Conjugated, Bile Salt Anions with Cholestyramine at 37°

Bile Salt Anion[a]	Agitation Intensity, r.p.m.	Apparent Second-Order Rate Constant, k' (l. mole^{-1} min.$^{-1}$ × 10^{-2})
Glycocholate	40	2.99
	60	3.91
	70	4.78
	100	5.91
Glycodeoxycholate	40	5.33
	60	8.61
	70	10.7
	100	14.3

[a] The concentrations of free bile salt remaining unreacted after the reaction had reached completion with respect to the available binding positions on cholestyramine were independent of agitation intensity (Table I, Footnote[b]).

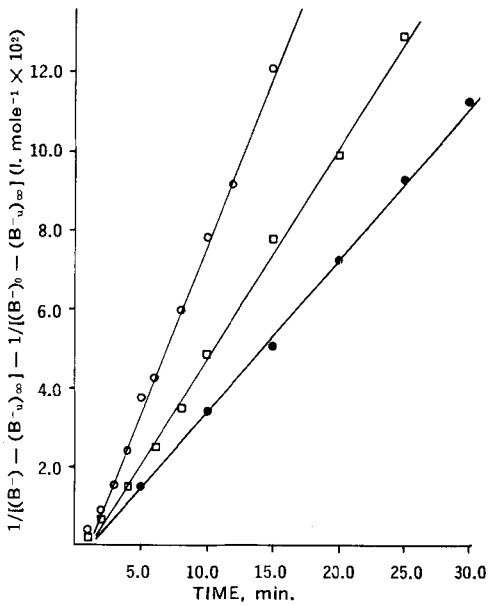


Figure 1—*Rate of binding of conjugated bile salt anions to cholestyramine at 37°. Agitation intensity, 60 r.p.m. Key: glycodeoxycholate* (○), *taurocholate* (□), *and glycocholate* (●).

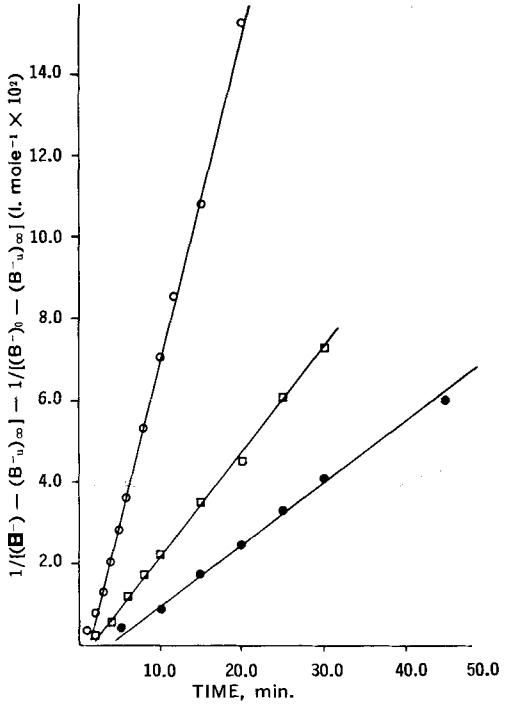


Figure 2—*Rate of binding of conjugated bile salts to cholestyramine in the presence of a 50.0 mM concentration of sodium chloride at 37°. Agitation intensity, 60 r.p.m. Key: glycodeoxycholate* (○), *taurocholate* (□), *and glycocholate* (●).

quent to the initial nonlinear phase. For all of the systems under examination, the apparent lag times (*i.e.*, the extrapolated, least-squares intercepts on the time axis of the linear segments of the plots shown in Figs. 1–3) ranged from 1 to 3 min. As is apparent from an examination of the plots shown in Fig. 1 and the rate-constant data summarized in Table I, the rate constant governing the bile salt–cholestyramine binding process, from aqueous pure medium, is significantly greater for the dihydroxy derivative, glycodeoxycholate, than for the trihydroxy salts investigated. A comparison of the rate-constant data for the two trihydroxy bile salts studied indicates that the taurine-conjugated derivative, taurocholate, is adsorbed onto the resin at a faster rate than the

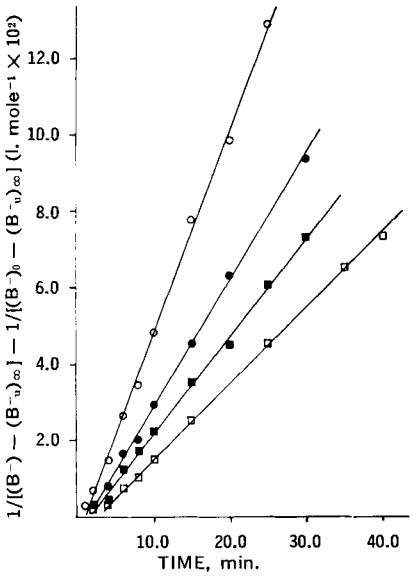


Figure 3—*Rate of binding of taurocholic anion to cholestyramine in the presence of varying concentrations of sodium chloride at 37°. Agitation intensity, 60 r.p.m. Key: 0.00 mM NaCl* (○), *25.0 mM NaCl* (●), *50.0 mM NaCl* (■), *and 75.0 mM NaCl* (□).

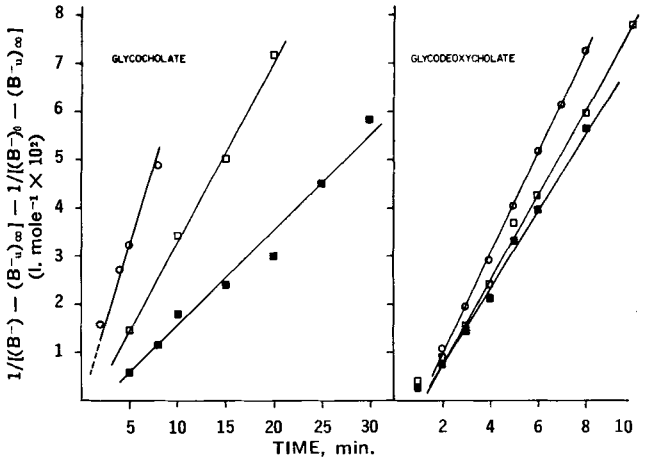


Figure 4—*Effect of temperature on the rate of binding of glycine-conjugated, bile salt anions with cholestyramine at an agitation intensity of 60 r.p.m. Key: 25°* (■), *37°* (□), *and 47°* (○).

glycine-conjugated derivative, glycocholate. Also shown in Table I are the affinity or association constants obtained from a Langmuir interpretation of equilibrium binding data for the bile salts alone (7). The rank order relationship existing between this parameter and the k' values for the binary bile salt–cholestyramine systems suggests that the affinity, which signifies the strength of interaction between adsorbate and adsorbent species, functions as a driving force for the interaction, the rate of binding increasing with increasing interaction strength. This conclusion is in agreement with the statements of Helfferich (20, 21) concerning the role of selectivity in the rate of uptake of simple ions by ion-exchange resins.

The observed rates of interaction in the ternary, bile salt–electrolyte–cholestyramine systems are significantly reduced in comparison to those in the absence of added electrolyte, as is evidenced by a comparison of the appropriate curves presented in Figs. 1 and 2 and the rate constants listed in Table I. The diminution in the rate of adsorption is decidedly more pronounced in the taurocholate- or glycocholate-containing systems than in the glycodeoxycholate-containing system. For example, at an added inorganic electrolyte concentration of 50 mM, there are approximately 53 and 62% decreases in the rates of interaction, respectively, for the former two trihydroxy anions; whereas there is only a 5% reduction in the rate of binding for the latter dihydroxy bile salt anion. These results tend to add supportive evidence to the proposed contributory role of nonelectrostatic interactions in the adsorption process (8),

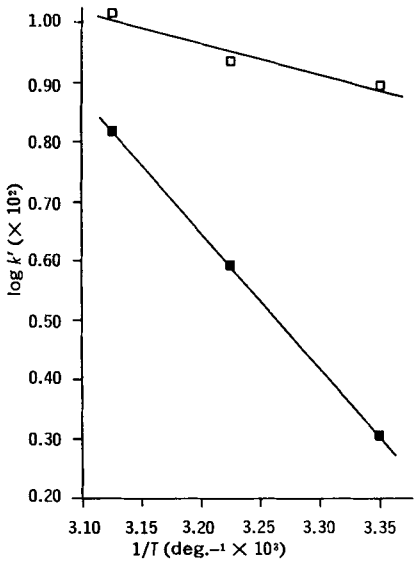


Figure 5—*Arrhenius relationship for glycine-conjugated, bile salt anion–cholestyramine interaction. Key: glycodeoxycholate* (□) *and glycocholate* (■).

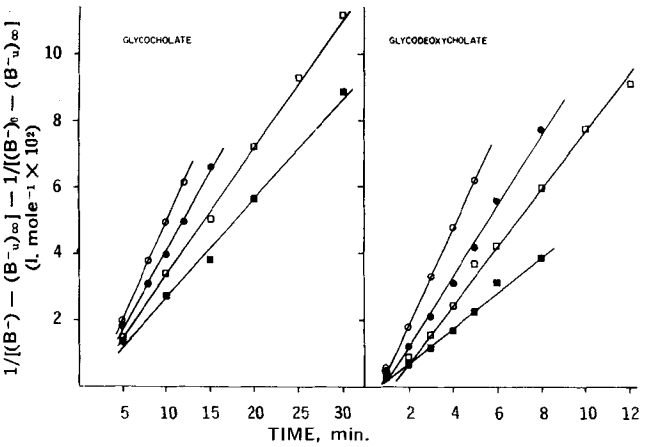


Figure 6—*Effect of agitation intensity on the rate of binding of glycine-conjugated, bile salt anions with cholestyramine at 37°. Key: 100 (○), 70 (●), 60 (□), and 40 (■) r.p.m.*

since electrolytes would be expected to have less of an effect on binding processes which are more nonelectrostatic in nature (*i.e.*, the dihydroxy bile salt–cholestyramine interaction) than on those interactions which are primarily due to an electrostatic mechanism (*i.e.*, trihydroxy bile salt–cholestyramine interactions).

The second-order rate constants appear to decrease with increasing electrolyte concentration, as is clearly exemplified by Fig. 3 and the rate constants listed in Table I for the taurocholate-containing systems. This primary salt effect is most probably due to a reduction in the charge density associated with the two charged reactants produced by the *screening* effect of the added inorganic electrolyte ions. The overall result is a weakening in the electrostatic forces between the two reactants and hence a corresponding decrease in the rate of interaction. A competition between bile salt and added chloride anions for the charged binding positions on cholestyramine may also be operable in the observed reduction in the uptake rates of taurocholate and glycocholate by the resin.

Effect of Temperature—Presented in Fig. 4 and listed in Table II are the results of the studies concerned with the temperature dependency of the initial rate of interaction between bile salt anions and cholestyramine, at a constant agitation intensity of 60 r.p.m. It is readily apparent from an examination of the rate-constant data that both glycocholate and glycodeoxycholate show an increase in their rate of binding with increasing temperature. The 10° temperature coefficient for the reaction was estimated to be approximately 2.1 for the former anion and 1.1 for the latter bile salt anion. These temperature coefficient values are of the order of magnitude similar to that reported for other heterogeneous, diffusion-controlled reactions (23, 24).

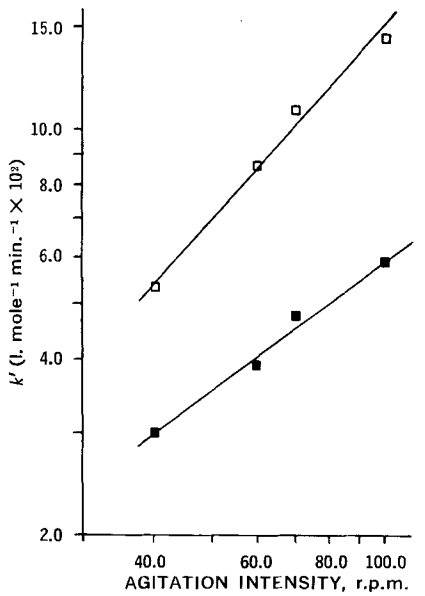


Figure 7—*Log apparent second-order rate constant* versus *log speed of agitation (r.p.m.) for the interaction of glycine-conjugated, bile salt anions with cholestyramine at 37°. Key: glycodeoxycholate (□) and glycocholate (■).*

Apparent energies of activation (E_a) associated with the rates of interaction of glycocholate and glycodeoxycholate with cholestyramine were calculated by means of the Arrhenius equation,

$$\log k' = \log A - \frac{E_a}{2.303R}\frac{1}{T} \qquad \text{(Eq. 4)}$$

in which k' represents the apparent second-order rate constant, A is the frequency factor, E_a is the energy of activation, R is the gas constant (1.987 cal./deg.-mole), and T is the absolute temperature. The temperature data plotted according to Eq. 4 are shown in Fig. 5. The energy of activation for each adsorbate, as determined from the least-squares slope of the linear plots, was found to be +2.39 kcal./mole for the glycodeoxycholate anion and +10.6 kcal./mole for the glycocholate anion. These findings are consistent with those previously obtained in that the dihydroxy bile salt anion, glycodeoxycholate, was suggested to be capable of a higher degree of nonelectrostatic interaction with cholestyramine than was the trihydroxy derivative, glycocholate (8).

Effect of Agitation Intensity—In order to ascertain whether the speed of agitation, to which the reaction medium was subjected, influenced the rate of the bile salt anion-cholestyramine interaction, studies were performed at a constant temperature of 37° and agitation intensities of 40, 60, 70, and 100 r.p.m. The results of these studies are depicted in Fig. 6 and the second-order rate constants, calculated from the slopes of these linear plots, are listed in Table III. The data obtained indicate that the rates of binding of both glycodeoxycholate and glycocholate anions to cholestyramine increase with increasing rate of agitation of the reaction medium, the former anion being more influenced by this parameter than the latter.

A log–log plot of the apparent second-order rate constants *versus* speed of agitation (r.p.m.) is presented in Fig. 7. The excellent linearity of these curves suggests that agitation intensity affects the rate of interaction in accordance with the following empirical relationship:

$$\log k' = b \log S + \log a \qquad \text{(Eq. 5)}$$

where S represents the speed of agitation (r.p.m.), k' is the apparent second-order rate constant governing the interaction, and b and a are constants. This mathematical relationship has been commonly employed to aid in distinguishing the process controlling a heterogeneous reaction rate (25). Generally, if the reaction in question is diffusion controlled, then the value of the constant b should closely approach or be equal to unity, which is in agreement with the Nernst-Brunner film theory (25). The applicability of this equation to bile salt anion–cholestyramine interactions becomes evident if one considers the adsorption process, which in essence involves the removal of adsorbate molecules from solution to essentially an undissolved state, as being the reverse of a dissolution process. The magnitude of the constant b for the two adsorbates was obtained from linear regression slope values of the plots illustrated in Fig. 7. The values of b, so determined, were 0.760 and 1.09 for the glycocholate and glycodeoxycholate anions, respectively, which suggest that the adsorption process is primarily diffusion controlled. It appears, therefore, that the rate of binding of bile salt anions to cholestyramine is dependent on the rate of diffusion of the adsorbate through a stagnant film surrounding the individual resin particle beads which is characteristic of film diffusion-controlled exchange reactions. As a result, as the speed of agitation of the reaction medium is increased, the thickness of this film is reduced and hence there is a potentiation in the rate of interaction. The lower b value, as well as the lower second-order rate constant at any speed of agitation, noted for the glycocholate–cholestyramine interaction as compared to that for the glycodeoxycholate anion can be readily explained based on the fact that trihydroxy bile salt anions are hydrated to a greater extent than dihydroxy derivatives (26). The higher apparent molecular weight of the glycocholate anion results in a lower diffusion coefficient for this anion, thus producing both a decrease in its rate of diffusion through the stag-

nant film as well as a decrease in its rate of interaction with this pharmacologically important anionic exchange resin.

SUMMARY

The present investigation has established, apparently for the first time, that the bile salt anion–cholestyramine reaction occurs by means of apparent second-order kinetics and that the reaction rates are dependent on the chemical structure of the bile salt anion participating in the interaction. Under conditions of mild agitation, it was shown that the reaction rates decrease in the following order: glycodeoxycholate > taurocholate > glycocholate, which parallels the affinity with which they bind to the resin. It is also of physiological interest that the addition of inorganic electrolyte to the system markedly depresses the rate of binding of the taurocholate and glycocholate anions to cholestyramine, which helps to explain why the resin is not as efficient as it should be *in vivo* based on the recommended dosage levels prescribed.

Both an increase in the temperature and the agitation intensity employed in the kinetic studies potentiated the rate of interaction between the glycocholate or glycodeoxycholate anion and cholestyramine. These data suggest that the binding process is most probably film diffusion controlled (27).

Additional kinetic studies are being conducted in this laboratory in order to ascertain the influence of such parameters as resin particle size and the influence of other endogenous substances on this pharmacologically important resin–bile salt interaction.

REFERENCES

(1) M. E. Zanetti and D. M. Tennent, *Proc. Soc. Exp. Biol. Med.*, **112,** 991(1963).

(2) J. B. Carey, Jr., *J. Clin. Invest.*, **40,** 1028(1961).

(3) J. W. Huff, J. L. Gilfillan, and V. M. Hunt, *Proc. Soc. Exp. Biol. Med.*, **114,** 352(1963).

(4) S. A. Hashim, S. S. Bergen, Jr., and T. B. Van Itallie, *ibid.*, **106,** 173(1961).

(5) R. B. Zurier, S. A. Hashim, and T. B. Van Itallie, *Gastroenterology*, **49,** 490(1965).

(6) D. G. Gallo, K. R. Bailey, and A. L. Sheffner, *Proc. Soc. Exp. Biol. Med.*, **120,** 60(1965).

(7) W. H. Johns and T. R. Bates, *J. Pharm. Sci.*, **58,** 179(1969).

(8) *Ibid.*, **59,** 329(1970).

(9) S. S. Bergen, Jr., T. B. Van Itallie, D. M. Tennent, and W. N. Sebrell, *Proc. Soc. Exp. Biol. Med.*, **102,** 679(1959).

(10) G. R. Jansen and M. E. Zanetti, *J. Pharm. Sci.*, **54,** 863 (1965).

(11) "Dowex: Ion Exchange," Dow Chemical Corp., Midland, Mich., 1964, p. 22.

(12) A. F. Hofmann and D. M. Small, *Ann. Rev. Med.*, **18,** 333 (1967).

(13) S. Bergstrom and H. Danielson, in "The Biliary System," W. Taylor, Ed., Blackwell Scientific Publications, Oxford, England, 1965, p. 127.

(14) R. J. Sawchuk and J. G. Nairn, *J. Pharm. Sci.*, **57,** 1896 (1968).

(15) J. L. Irvin, C. G. Johnston, and J. Kopalo, *J. Biol. Chem.*, **153,** 439(1944).

(16) "Dowex: Ion Exchange," Dow Chemical Corp., Midland, Mich., 1964, p. 11.

(17) F. Helfferich, "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, p. 251.

(18) *Ibid.*, p. 286.

(19) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed., Wiley, New York, N. Y., 1961, pp. 18, 187.

(20) F. Helfferich, "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, p. 261.

(21) *Ibid.*, p. 163.

(22) A. Norman, *Acta Chem. Scand.*, **14,** 1295(1960).

(23) L. J. Edwards, *Trans. Faraday Soc.*, **47,** 1191(1951).

(24) M. Abramson and C. V. King, *J. Amer. Chem. Soc.*, **61,** 2290(1939).

(25) D. E. Wurster and P. V. Taylor, *J. Pharm. Sci.*, **54,** 169 (1965).

(26) E. H. Ahrens and L. C. Craig, *J. Biol. Chem.*, **195,** 763 (1952).

(27) F. Helfferich, "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, p. 285.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 25, 1969, from the *Division of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CN 06268*

Accepted for publication February 3, 1970.

Presented before the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This investigation was supported in part by Grant 076 from the University of Connecticut Research Foundation, Storrs, CN 06268

* Predoctoral Fellow, U. S. Public Health Service.

Toxogonin: Blood Levels and Side Effects after Intramuscular Administration in Man

FREDERICK R. SIDELL and WILLIAM A. GROFF

Abstract □ Toxogonin, given intramuscularly to 10 healthy young men in doses of 2.5–10 mg./kg., produced dose-related oxime whole blood levels of 6.3–26.5 mcg./ml. and had a plasma half-time of 82.8 min. Associated side effects included tachycardia, hypertension, and a dose-independent symptom complex consisting of peroral warmth, paresthesia and hypalgesia, and a menthol taste. Some published data on the relative effectiveness of toxogonin and pralidoxime (chloride and methanesulfonate) suggest further inquiry into toxogonin's therapeutic potential may be needed.

Keyphrases □ Toxogonin, intramuscular injection—oxime blood levels □ Plasma half-life—toxogonin □ Dose relation, toxogonin—plasma blood levels □ Urinary excretion—toxogonin □ Side effects—toxogonin

Until about a decade ago the only therapy for poisoning by cholinesterase inhibitors was atropine or another anticholinergic drug plus supportive therapy. In the 1950's the oximes were developed and their usefulness as adjuncts to atropine was established (1, 2).

On the basis of therapeutic potency, relatively low toxicity in man, and other factors, the oxime generally adopted for use is pralidoxime chloride (2-PAMCl;

nant film as well as a decrease in its rate of interaction with this pharmacologically important anionic exchange resin.

SUMMARY

The present investigation has established, apparently for the first time, that the bile salt anion–cholestyramine reaction occurs by means of apparent second-order kinetics and that the reaction rates are dependent on the chemical structure of the bile salt anion participating in the interaction. Under conditions of mild agitation, it was shown that the reaction rates decrease in the following order: glycodeoxycholate > taurocholate > glycocholate, which parallels the affinity with which they bind to the resin. It is also of physiological interest that the addition of inorganic electrolyte to the system markedly depresses the rate of binding of the taurocholate and glycocholate anions to cholestyramine, which helps to explain why the resin is not as efficient as it should be *in vivo* based on the recommended dosage levels prescribed.

Both an increase in the temperature and the agitation intensity employed in the kinetic studies potentiated the rate of interaction between the glycocholate or glycodeoxycholate anion and cholestyramine. These data suggest that the binding process is most probably film diffusion controlled (27).

Additional kinetic studies are being conducted in this laboratory in order to ascertain the influence of such parameters as resin particle size and the influence of other endogenous substances on this pharmacologically important resin–bile salt interaction.

REFERENCES

(1) M. E. Zanetti and D. M. Tennent, *Proc. Soc. Exp. Biol. Med.*, **112,** 991(1963).

(2) J. B. Carey, Jr., *J. Clin. Invest.*, **40,** 1028(1961).

(3) J. W. Huff, J. L. Gilfillan, and V. M. Hunt, *Proc. Soc. Exp. Biol. Med.*, **114,** 352(1963).

(4) S. A. Hashim, S. S. Bergen, Jr., and T. B. Van Itallie, *ibid.*, **106,** 173(1961).

(5) R. B. Zurier, S. A. Hashim, and T. B. Van Itallie, *Gastroenterology*, **49,** 490(1965).

(6) D. G. Gallo, K. R. Bailey, and A. L. Sheffner, *Proc. Soc. Exp. Biol. Med.*, **120,** 60(1965).

(7) W. H. Johns and T. R. Bates, *J. Pharm. Sci.*, **58,** 179(1969).

(8) *Ibid.*, **59,** 329(1970).

(9) S. S. Bergen, Jr., T. B. Van Itallie, D. M. Tennent, and W. N. Sebrell, *Proc. Soc. Exp. Biol. Med.*, **102,** 679(1959).

(10) G. R. Jansen and M. E. Zanetti, *J. Pharm. Sci.*, **54,** 863 (1965).

(11) "Dowex: Ion Exchange," Dow Chemical Corp., Midland, Mich., 1964, p. 22.

(12) A. F. Hofmann and D. M. Small, *Ann. Rev. Med.*, **18,** 333 (1967).

(13) S. Bergstrom and H. Danielson, in "The Biliary System," W. Taylor, Ed., Blackwell Scientific Publications, Oxford, England, 1965, p. 127.

(14) R. J. Sawchuk and J. G. Nairn, *J. Pharm. Sci.*, **57,** 1896 (1968).

(15) J. L. Irvin, C. G. Johnston, and J. Kopalo, *J. Biol. Chem.*, **153,** 439(1944).

(16) "Dowex: Ion Exchange," Dow Chemical Corp., Midland, Mich., 1964, p. 11.

(17) F. Helfferich, "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, p. 251.

(18) *Ibid.*, p. 286.

(19) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed., Wiley, New York, N. Y., 1961, pp. 18, 187.

(20) F. Helfferich, "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, p. 261.

(21) *Ibid.*, p. 163.

(22) A. Norman, *Acta Chem. Scand.*, **14,** 1295(1960).

(23) L. J. Edwards, *Trans. Faraday Soc.*, **47,** 1191(1951).

(24) M. Abramson and C. V. King, *J. Amer. Chem. Soc.*, **61,** 2290(1939).

(25) D. E. Wurster and P. V. Taylor, *J. Pharm. Sci.*, **54,** 169 (1965).

(26) E. H. Ahrens and L. C. Craig, *J. Biol. Chem.*, **195,** 763 (1952).

(27) F. Helfferich, "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, p. 285.

ACKNOWLEDGMENTS AND ADDRESSES

Received August 25, 1969, from the *Division of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CN 06268*

Accepted for publication February 3, 1970.

Presented before the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This investigation was supported in part by Grant 076 from the University of Connecticut Research Foundation, Storrs, CN 06268

* Predoctoral Fellow, U. S. Public Health Service.

Toxogonin: Blood Levels and Side Effects after Intramuscular Administration in Man

FREDERICK R. SIDELL and WILLIAM A. GROFF

Abstract □ Toxogonin, given intramuscularly to 10 healthy young men in doses of 2.5–10 mg./kg., produced dose-related oxime whole blood levels of 6.3–26.5 mcg./ml. and had a plasma half-time of 82.8 min. Associated side effects included tachycardia, hypertension, and a dose-independent symptom complex consisting of peroral warmth, paresthesia and hypalgesia, and a menthol taste. Some published data on the relative effectiveness of toxogonin and pralidoxime (chloride and methanesulfonate) suggest further inquiry into toxogonin's therapeutic potential may be needed.

Keyphrases □ Toxogonin, intramuscular injection—oxime blood levels □ Plasma half-life—toxogonin □ Dose relation, toxogonin—plasma blood levels □ Urinary excretion—toxogonin □ Side effects—toxogonin

Until about a decade ago the only therapy for poisoning by cholinesterase inhibitors was atropine or another anticholinergic drug plus supportive therapy. In the 1950's the oximes were developed and their usefulness as adjuncts to atropine was established (1, 2).

On the basis of therapeutic potency, relatively low toxicity in man, and other factors, the oxime generally adopted for use is pralidoxime chloride (2-PAMCl;

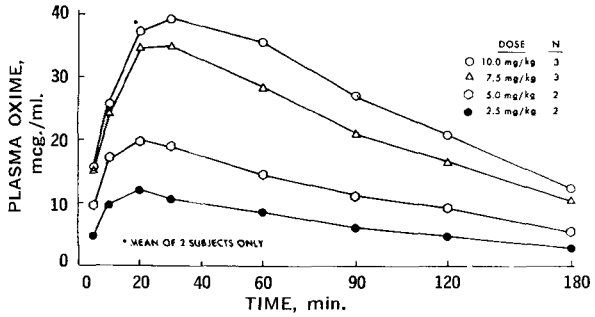


Figure 1—*Toxogonin (i.m.): plasma oxime values* versus *time.*

2-pyridine aldoxime methochloride). However, largely because of the enthusiasm of Engelhard and Erdmann (3, 4), interest has been generated in a newer oxime, toxogonin [LuH6; *N,N'*-oxydimethylene bis(pyridinium-4-aldoxime)dichloride], said to be more effective than pralidoxime chloride. It is similar structurally to TMB-4 [*N,N'*-trimethylene bis(pyridinium-4-aldoxime) dibromide], which has been shown to be more active than pralidoxime chloride therapeutically (5–8) but also more toxic in man (9, 10).

Most reports on the administration of toxogonin to humans would appear to be those of Erdmann and Engelhard. They reported that toxogonin (250 mg., i.v.) was superior to pralidoxime chloride in three cases of E-605 (parathion) poisoning (11), and they studied symptoms and blood levels of oxime in 12 healthy young male volunteers who were given 250 mg. of toxogonin intramuscularly in a 25% solution (12). The purposes of this study were: to extend these latter observations over a wider dose range, to study the blood levels and excretion patterns of this oxime, and to determine what, if any, undesirable side effects would be produced.

SUBJECTS AND METHODS

The subjects were U. S. Army enlisted men, ages 18 to 24 years (average 21 years), who volunteered for this study. Each had a complete medical evaluation including a physical examination, chest X-ray, electrocardiogram, and laboratory tests [hematocrit, total and differential white blood cell count, urinalysis, including microscopic examination, blood urea nitrogen (BUN), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase, bilirubin, serum creatinine, and red blood cell and plasma cholinesterase], and they were found to be free from any abnormality before being accepted into the study. They were told that they would take part in a study of a new and possibly very effective antidote for nerve agent poisoning; the test procedures were described to them, including the necessity of multiple venipunctures. They were told that there might possibly be some symptoms, but these were left unspecified. The only reward for participation was an extra day off.

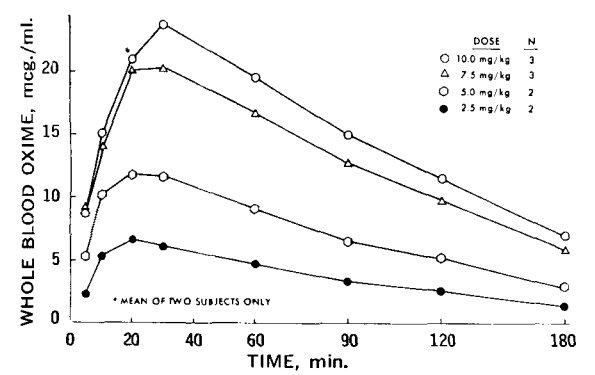


Figure 2—*Toxogonin (i.m.): whole blood oxime levels* versus *time.*

The volunteers slept in the ward the evening before the test. Fluid intake was maintained at a high level prior to and throughout the test. During the hour before the drug was administered, three measurements were recorded of supine blood pressure, heart rate, and pupil size. After appropriate control urine and blood specimens had been taken, the subjects ate a light breakfast.

Except to void, the subjects remained in bed during the first 3 hr. of the test; for the remainder of the 24-hr. test period, they were allowed to be up and around the ward. Blood pressure and heart rate were measured every 15 min. for 2 hr. after administration of the drug. Blood samples were taken at 5, 10, 20, 30, 60, 90, 120, and 180 min. after drug. The subjects were asked to void every 30 min. for 3 hr.; for the next 21 hr., all urine was collected, but no attempt was made to follow a definite schedule.

Whole blood, plasma, and urine were analyzed for oxime content by the method described by Groff and Ellin (13). Blood and plasma were analyzed within minutes of collection; the urine was refrigerated and analyzed several days later. Preliminary studies performed by adding a known amount of toxogonin to urine and making daily determinations of toxogonin content showed that there was insignificant (under 5%) deterioration of the oxime over a 1-week period. The oxime content of red blood cells was also measured and will be the subject of a separate report.

Other blood and urine studies (hematocrit, total and differential white blood cell count, SGOT, alkaline phosphatase, BUN, and urinalysis) were performed by routine analytical methods on samples taken at 24 hr. and 7 days. Red cell and plasma cholinesterase of the three subjects receiving the highest dose was measured in samples taken at 20 min., 30 min. (times of peak oxime levels), and 3 hr. after administration of the oxime by methods previously described (14).

The subjects' average weight was 76 kg. (range 56–93 kg.). The volume of injection ranged from 0.64 to 3.1 ml., and the injection was given into the deltoid muscle with a No. 23 needle. Doses were 2.5 mg./kg. (two subjects), 5.0 mg./kg. (two subjects), 7.5 mg./kg. (three subjects), and 10.0 mg./kg. (three subjects). All doses and blood levels are expressed as the salt of the oxime.

Toxogonin was purchased from E. Merck, Darmstadt, West Germany. Recent studies on the sample used showed that it was over 98% pure by UV spectroanalysis and its LD_{50} [calculated by the method of Bliss (15)] by the intraperitoneal route in 170–200-g. female rats was 109.6 (95% CL: 102.5–117.2) mg./kg. It was dissolved in distilled water to a concentration of 274 mg./ml. and filtered through a Seitz filter. Bacteriological analysis showed it to be sterile.

RESULTS

A. Blood Levels of Oxime—Serial mean plasma and whole blood levels for toxogonin for each dose group are shown in Figs. 1 and 2. Peak blood levels were reached at 20 min. in the two lower dose groups and at 30 min. in the higher dose groups. All groups had plasma values above 5 mcg./ml. by 5 min., and all but the group that received 2.5 mg./kg. maintained a plasma level of about 4 mcg./ml. for over 3 hr. (Because of technical difficulties the analysis of the blood taken at 20 min from one subject who received 10 mg./kg. was not measured; the point is the mean for the other two subjects. The data from this subject were also excluded from dose–response calculations.)

The dose–response relationship, showing the highest plasma value for each subject, is shown in Fig. 3.

The disappearance from plasma of many drugs follows first-order kinetics, $C = C_0e^{-kt}$, where C = concentration at time t; C_0 = apparent concentration at zero time, *i.e.*, the y-intercept; and k = the velocity constant characterizing drug-elimination rate from the blood stream. The half-life ($t_{1/2}$) can be determined most simply by plotting blood levels on a logarithmic scale against time on a linear scale and, if the terminal segment of the plot is linear, calculating from its slope the half-life ($t_{1/2}$) and k value (the rate constant characterizing elimination from the blood stream).

Using the method of least squares, the constants log C_0 and k for the equation

$$\log C = \log C_0 - \frac{kt}{2.303} \qquad \text{(Eq. 1)}$$

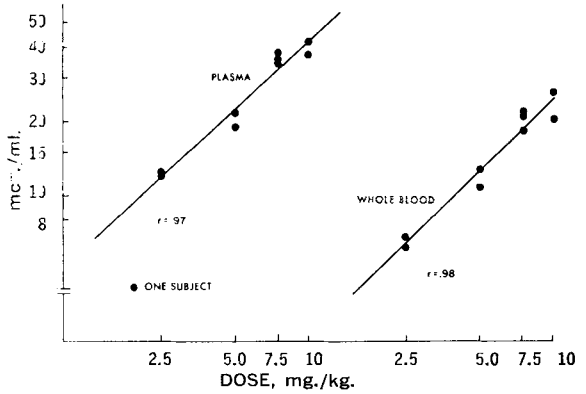


Figure 3—*Toxogonin (i.m.): peak blood levels* versus *dose.*

were computed from each subject's last 4–6 plasma level values. In each case the coefficient of correlation for the fit of the points to the line was greater than 0.99.

In Fig. 4 the data from four typical subjects are plotted in this manner. The average $t_{1/2}$ was 82.8 min. [the mean values by dose groups were 79.4 min. (2.5 mg./kg.), 85.6 min. (5.0 mg./kg.), 82.7 min. (7.5 mg./kg.), and 84.0 min. (10.0 mg./kg.)]; and the average k value was 0.0083 min.$^{-1}$ (by dose groups in min.$^{-1}$: 0.0087 for 2.5 mg./kg., 0.0081 for 5.0 mg./kg., 0.0082 for 7.5 mg./kg., and 0.0083 for 10 mg./kg.).

B. Urinary Excretion—Of the total dose, 84% was excreted into the urine unchanged during the 24-hr. collection period; of this amount, 76% (or 64% of the dose) was excreted in the first 3 hr. (Because of technical problems, data from one subject at the 10-mg./kg. dose could not be used in the urinary analysis.) The percentage of the amount not excreted:

$$\frac{(\text{total amount excreted} - \text{amount excreted by time } t) \times 100}{\text{total amount excreted}}$$

is plotted on a logarithmic scale against time, t, in Fig. 5. Times for 50% excretion for each dose group are 73.0 min. (2.5 mg./kg.), 72.0 min. (5.0 mg./kg.), 110.4 min. (7.5 mg./kg.), and 107.4 min. (10 mg./kg.) and are close to the biological half-life as measured by loss from plasma. This suggests that a large part of the oxime is eliminated rather rapidly by renal mechanisms.

C. Blood Pressure and Heart Rate—All subjects showed a mild to moderate transient increase in blood pressure and heart rate (Figs. 6 and 7). The small number of subjects in each dose group makes exact quantitation of such variables unreliable, but both the systolic and diastolic blood pressures were moderately elevated (11–17 mm. Hg), reaching peak values at about 30 min. after drug administration. The heart rate change was more dose related with an average maximal increase of 8 b.p.m. for 2.5 mg./kg., 15 b.p.m. for 5.0 mg./kg., and 32 b.p.m. for 10 mg./kg. In general the heart rate increased markedly a few minutes after drug administration (data for the lower doses are not shown because heart rates were not measured earlier than 15 min. for the lower dose subjects) and remained elevated for most of the 2-hr. observation period.

D. Other Blood and Urine Studies—No changes were noted in any of the routine blood or urine tests at 24 hr. or 7 days in any subject, nor were there changes in the red blood cell or plasma cholinesterase in the three subjects in whom it was measured.

E. Symptoms—Pain at the site of injection was quite noticeable, particularly with the larger volumes used for the higher doses, and most subjects compared it in intensity to the pain from an injection of plague or Asian influenza vaccine. Within a few minutes the pain seemed to diminish greatly, and by 15 min. most subjects said they did not notice it. A slight residual tenderness of the muscle to percussion was present in all subjects 24 hr. later.

A peculiar symptom complex developed in many subjects. This typically consisted of a generalized warmth, a "hot feeling" over the upper part of the body, within several minutes after the injection. At 5–15 min., this became localized in the face, particularly around the mouth, where it was associated with a "tight feeling" of the skin and muscles of the lower face or forehead and a "numbness" of the circumoral area. Nine subjects (all but one at the highest dose) noted a hot feeling in their throats within 1–3 min. after injection, and three

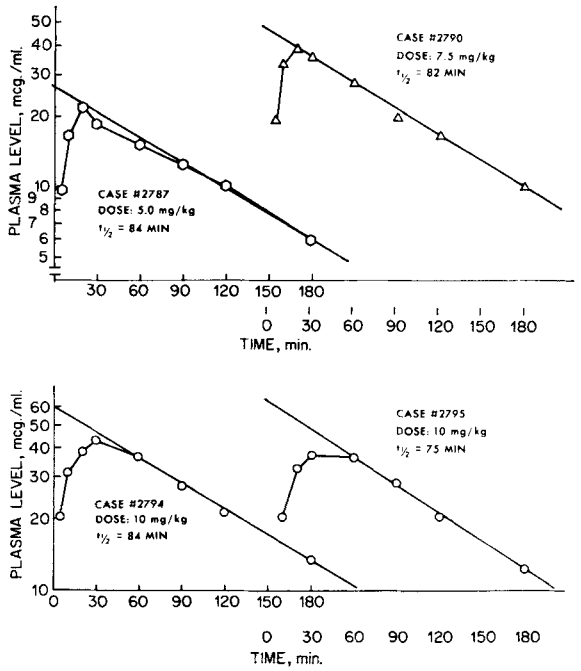


Figure 4—*Toxogonin (i.m.): plasma levels and half-times in four subjects.*

of these men (2.5, 5.0, and 10.0 mg./kg.) also spontaneously identified this as a "menthol" taste. One subject at 2.5 mg./kg., one at 5.0 mg./kg., and one at 10.0 mg./kg. reported a numbness in or around the mouth, together with a definite hypalgesia to pinprick in this area including the anterior tongue and gums (two subjects), which lasted from 3 min. to 6 hr. after injection. No depression of the corneal reflex or muscular weakness in the face was observed. When present, the area of involvement was sharply demarcated. In one, it was from midforehead to just under the chin; in another, it had a definite 5.08–7.62-cm. (2–3-in.) radius around the mouth. Most reported a flushing sensation, but one subject said he felt very hot with

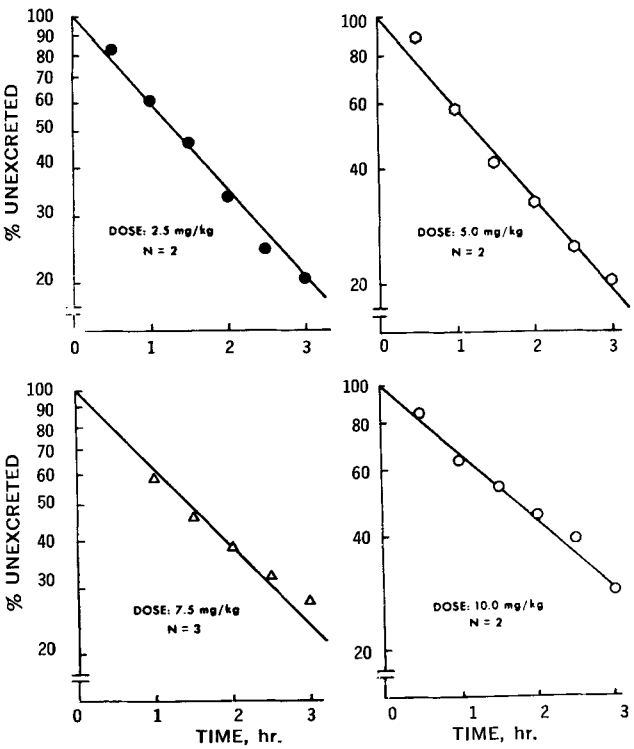


Figure 5—*Urinary excretion of toxogonin: percent unexcreted* versus *time.*

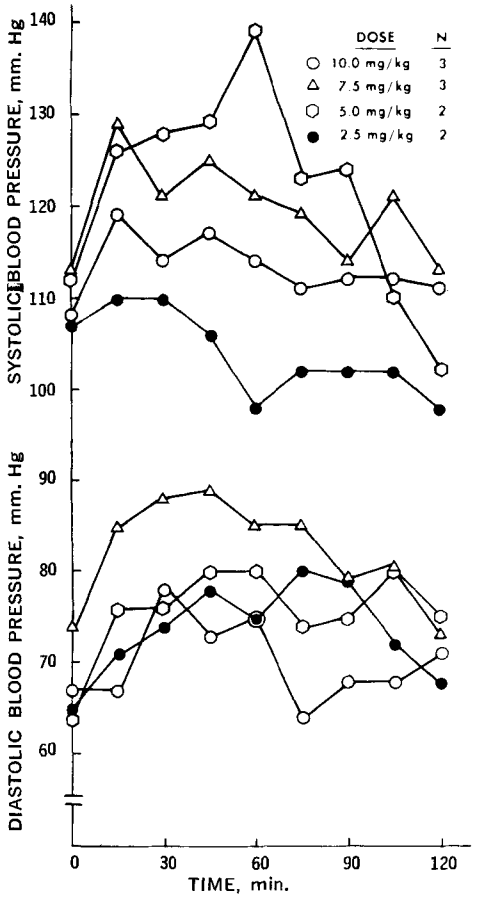


Figure 6—*Toxogonin (i.m.): blood pressure changes* versus *time.*

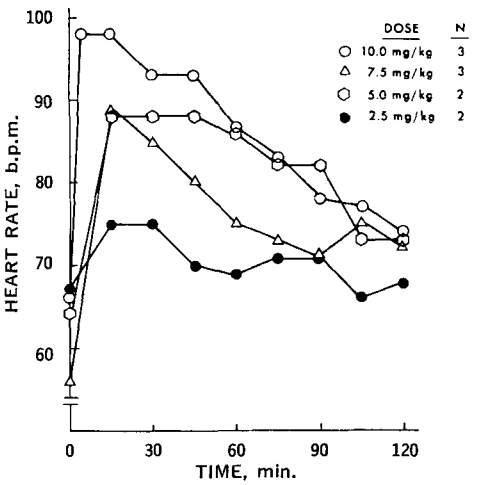


Figure 7—*Toxogonin (i.m.): heart rate changes* versus *time.*

cool flashes. The skin of several subjects was very warm to the touch and appeared slightly pink. Several noted "pins and needles" sensations in their arms and fingers, not associated with hyperventilation. All three subjects at the highest dose reported their eyes "felt like baseballs" with heaviness in the eyeballs or a feeling that "they're enlarging." Only one subject (7.5 mg./kg.) reported nausea and this was very transient. A dry mouth was a unanimous symptom of all subjects in the highest dose groups.

Except for mild paresthesia and, in two cases, mild hypalgesia, all symptoms had subsided by 1–2 hr.

DISCUSSION

In a study of blood levels and urinary excretion of toxogonin after intramuscular administration to 12 healthy young men, Erdmann *et al.* (12) gave an average dose of 3 mg./kg. They noted a peak whole blood oxime level of about 6 mcg./ml. at 30 min., found 52% of the dose in the urine by 2 hr., and reported that their subjects complained of "heat and tension" in the facial area and a menthol-taste sensation in the nasopharynx. In the study reported here, the two subjects who received 2.5 mg./kg. had a peak whole blood level of oxime of 6.6 mcg./ml. at 20 min., and the average excretion for nine subjects was 53% of the dose by 2 hr. The subjects reported the same symptoms and, in addition, had transient neurological findings.

Calesnick *et al.* (10) found that an intramuscular dose of 15 mg./kg. of pralidoxime chloride produced whole blood oxime levels of 8.9 mcg./ml. at 30 min. and 5.7 at 2 hr., levels exceeded by 5.0 mg./kg. of toxogonin at these times, and a dose of 30 mg./kg. produced whole blood levels (14.5 mcg./ml. and 10.4 mcg./ml. at similar times) below those produced by 7.5 mg./kg. of toxogonin. Thus within the ranges studied, three to four times more pralidoxime chloride than toxogonin is needed for a similar blood level.

Pralidoxime chloride in intravenous or intramuscular doses of less than 15 mg./kg. does not elevate the blood pressure in normal recumbant subjects (10, 16, 17). This sympathomimetic property of toxogonin may be of additional therapeutic benefit for the severely poisoned individual.

The cause of the peculiar symptom complex produced by toxogonin is not clear. Because of their "onion skin" pattern, the sensory disturbances of the face resemble those caused by a lesion of the nucleus or descending fibers of the sensory division of the trigeminal nerve in the medulla or upper spinal cord (18), although the production of such a lesion by a drug would be quite rare.

With high doses of pralidoxime chloride (15–30 mg./kg., i.v.), diplopia has been noted[1] (16). The authors also have observed this with pralidoxime chloride (20–30 mg./kg., i.v.) associated with demonstrable weakness of the muscles supplied by the third, fourth, and sixth cranial nerves. It is possible that this side effect is also central in origin.

The usefulness of toxogonin in clinical therapy is unknown, although, as noted, Erdmann and Clarmann (11) felt that toxogonin was more beneficial than pralidoxime.

The relationship of oxime blood levels to therapeutic efficacy is also unknown. Erdmann *et al.* (12), on the basis of *in vitro* studies (3) showing toxogonin to be 30 times more effective than pralidoxime in reactivating organophosphate-inhibited cholinesterase, suggest that the level of 4 mcg./ml., reported to be an effective *in vivo* level of the methanesulfonate salt of pralidoxime (P2S) by Sundwall (19), would be more than adequate. Using identical *in vitro* techniques in a comparative study, they also found that blood concentrations of 0.1–0.2 mcg./ml. reactivated cholinesterase by the same amount as concentrations of 3 mcg./ml. of pralidoxime (a concentration very close to that suggested by Sundwall from *in vivo* work).

However, several studies in which toxogonin (or the chemically similar TMB-4) has been compared with pralidoxime (or P2S, the methanesulfonate salt of pralidoxime) indicate that perhaps this advantage is not quite so great (20, 21).

Both toxogonin and TMB-4 have two oxime groups per molecule and, therefore, on an equimolar basis might be expected to have twice the efficacy of a compound having a single oxime group, such as pralidoxime. (On the other hand, yoked oxime groups might be less effective than the same number of single oxime units.) It has been shown that toxogonin and TMB-4 are roughly equipotent in treating poisoned mice (22), in reactivating blood (mouse) or brain (mouse and rat) cholinesterase inhibited (*in vivo*) by an organophosphate compound (diethyl *p*-nitrophenyl phosphate)[2] (23), or in antagonizing the effects of tabun on the isolated rat phrenic nerve preparation (20).

O'Leary *et al.* (6) showed that 10 mg./kg. (28 μmole/kg.) of TMB-4 with atropine raised the LD_{50} of sarin in rabbits by a factor of 170, whereas 10 mg./kg. (58 μmole/kg.) of pralidoxime chloride raised the LD_{50} by 90, and the same dose of P2S (41 μmole/kg.) raised it by 35. This indicates that, for equimolar amounts, TMB-4 is roughly 3.9 times more effective than pralidoxime chloride and 7.5 times more effective than P2S. Fleisher *et al.* (5) showed that equimolar doses

[1] B. Calesnick, personal communication.
[2] Paraoxon.

of TMB-4 were from 1.4–7.0 times more effective than pralidoxime in raising the LD_{50} of difluorophosphate, sarin, tabun, and tetraethylpyrophosphate in rats.

This latter group of investigators (21) also showed that, in equimolar doses, toxogonin and atropine approximately doubled the LD_{50} of sarin in rats (1.6×) and guinea pigs (2.2×) over that in animals treated with pralidoxime chloride and atropine. When given on an equiweight basis, the two oximes had nearly the same therapeutic activity. LD_{50}'s done by probit analysis [method of Bliss (15)] from data in Table II of the Wolthuis and Cohen report (20) suggest that toxogonin is about 1.7 times more effective therapeutically than P2S against tabun in rats (also receiving atropine) or 2.6 times more effective on an equimolar basis. Heilbronn and Tolagen (22) also showed that in atropinized mice, toxogonin is 4.6 times more effective against sarin and 9.5 times more effective against tabun than P2S when the dose of each oxime is 20% of its LD_{50}. On an equimolar basis, these ratios are approximately doubled to 7.1 and 14.5.

Overall, it would appear that toxogonin may be more effective therapeutically than pralidoxime chloride or P2S, although in two of the three studies where comparisons were made, the therapeutic difference was not marked.

The data in these reports also indicate that in each case, except the guinea pig experiments, the oxime that is more therapeutically potent also has a lower LD_{50} (is more toxic). However, the "therapeutic margin" (potency ratio/LD_{50} ratio) indicates that, in most cases, effectiveness is gained in greater proportion than toxicity, at least when toxogonin and pralidoxime chloride (or P2S) are compared directly.

REFERENCES

(1) H. Kewitz, I. B. Wilson, and D. Nachmansohn, *Arch. Biochem. Biophys.*, **64,** 456(1956).

(2) B. M. Askew, *Brit. J. Pharmacol.*, **12,** 340(1957).

(3) H. Engelhard and W. D. Erdmann, *Klin. Wochenschr.*, **41,** 525(1963).

(4) W. D. Erdmann and H. Engelhard, *Arzneimittel-Forsch.*, **14,** 5(1964).

(5) J. H. Fleisher, H. O. Michel, L. F. Yates, and C. S. Harrison, *J. Pharmacol. Exp. Ther.*, **129,** 31(1960).

(6) J. F. O'Leary, A. M. Kunkel, and A. H. Jones, *ibid.*, **132,** 50(1961).

(7) E. Bay, S. Krop, and L. F. Yates, *Proc. Soc. Exp. Biol. Med.*, **98,** 107(1958).

(8) R. A. Lehman, H. M. Fitch, L. P. Bloch, H. A. Jewell, and M. E. Nicholls, *J. Pharmacol. Exp. Ther.*, **128,** 307(1960).

(9) J. H. Wills, *Fed. Proc.*, **18,** 1020(1959).

(10) B. Calesnick, J. A. Christensen, and M. Richter, *Arch. Environ. Health*, **15,** 599(1967).

(11) W. D. Erdmann and M. V. Clarmann, *Deut. Med. Wochenschr.*, **88,** 2201(1963).

(12) W. D. Erdmann, I. Bosse, and P. Franke, *ibid.*, **90,** 1436 (1965).

(13) W. A. Groff and R. I. Ellin, *Clin. Chem.*, **15,** 72(1969).

(14) W. A. Groff, L. A. Mounter, and V. M. Sim, *Technicon Symposia*, **1,** 498(1966).

(15) C. I. Bliss, "The Statistics of Bioassay," Academic, New York, N. Y., 1952, p. 543.

(16) B. V. Jager and G. N. Stagg, *Bull. Johns Hopkins Hosp.*, **102,** 203(1958).

(17) D. Grob and R. J. Johns, *Amer. J. Med.*, **24,** 497(1958).

(18) S. Locke, "Neurology," Little, Brown and Co., Boston, Mass., 1966, p. 36.

(19) A. Sundwall, *Biochem. Pharmacol.*, **8,** 413(1961).

(20) O. L. Wolthuis and E. M. Cohen, *ibid.*, **16,** 361(1967).

(21) N. C. Thomas, J. H. Fleisher, L. W. Harris, and G. R. Miller, *Fed. Proc.*, **28,** 480(1969).

(22) E. Heilbronn and B. Tolagen, *Biochem. Pharmacol.*, **14,** 73 (1965).

(23) F. Hobbiger and V. Vojvodie, *ibid.*, **15,** 1677(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 2, 1969, from *Clinical Investigation Branch, Clinical Research Department, Medical Research Laboratory, Research Laboratories, Edgewood Arsenal, Edgewood Arsenal, MD 21010*

Accepted for publication January 9, 1970.

The authors thank the nurses and technicians of the Clinical Research Department for the ward care of the subjects; Andris Kaminskis for helping with the laboratory analyses; F. H. Duffy and E. H. Polley for neurological consultation and advice; A. F. Michaelis for the spectroanalysis; J. S. Ketchum, R. I. Ellin, and J. H. Fleisher for critical review of the manuscript; Marion P. Royston for careful editorial assistance; and Norma Vaught for secretarial assistance.

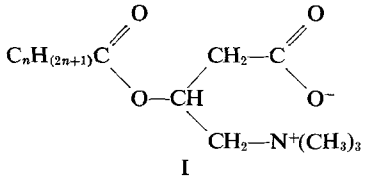
Potentiometric Titration of Monomeric and Micellar Acylcarnitines

SAMUEL H. YALKOWSKY* and GEORGE ZOGRAFI

Abstract □ Carnitine (β-hydroxy-γ-trimethylammonium butyrate) enzymatically combines with fatty acids to facilitate their transport through mitochondrial membranes and thus their metabolism. In view of this fact, a study of the physical-chemical properties of some acylcarnitines was conducted. Potentiometric titrations were carried out at concentrations at which the acylcarnitines are monomeric and micellar. Unlike the monomeric species, the micellar acylcarnitines do not have a constant pK value but rather one that is dependent on the degree of protonation and hence the net positive charge of the micelle. The pK value for the zwitterionic micelle is about 4.85 and decreases linearly with β at low degrees of protonation. The pK of the zwitterionic micelle was taken as the intrinsic pK of a carboxyl group, and pK differences at any value of β were attributed to electrostatic interactions. This permitted calculation of the surface potential of the micelle. The dependence of the observed pK and the calculated surface potential on β and on ionic strength was shown to be qualitatively consistent with the Debye-Hückel theory for a spherical impenetrable particle.

Keyphrases □ Acylcarnitines, monomeric, micellar—potentiometric titration □ CMC—acylcarnitines □ Surface potential—acylcarnitine micelles □ Ionization, micelles—pK value effect □ Potentiometric titration—monomeric, micellar acylcarnitines

Although the coenzyme A derivatives of fatty acids are metabolized through β-oxidation in the mitochondrion, these activated fatty acids cannot enter the mitochondrion from the cytoplasm unless carnitine (β-hydroxy-γ-trimethylammonium butyrate) is present (1–3). At the mitochondrial membrane surface, fatty acyl coenzyme A derivatives are enzymatically combined with carnitine, producing free coenzyme A and fatty acylcarnitine (I).



I

The acylcarnitines pass through the mitochondrial membrane and react with intramitochondrial coenzyme A to form free carnitine and fatty acyl coenzyme A. The intramitochondrial acyl coenzyme A is metabolized *via* the β-oxidation cycle, and the carnitine can pass back across the membrane to react with another extramitochondrial acyl coenzyme A molecule. The fact that several compounds having structures similar to carnitine and acylcarnitine are not able to pass through the mitochondrial membrane suggests that the acylcarnitine structure is specifically required for fatty acid transport (4, 5).

Carnitine and its acylated derivatives (Table I) have three functional groups that can interact with each other and with groups on neighboring molecules. In the case of long-chain acylcarnitines, micelle formation also can occur. These interactions could affect the ionization behavior of such compounds and hence their ability to function in membrane transport. The present study was designed to determine the effects of structural modification and environmental conditions, *e.g.*, ionic strength, pH, and micelle formation, on the ionization behavior of these compounds, with a long-range view of understanding their transport-mediating properties.

EXPERIMENTAL

Materials—The various acylcarnitines used in this study were synthesized by reacting DL-carnitine HCl (Mann Research Laboratories, Inc.) with high purity (99+%) fatty acids (Applied Science Laboratories) according to the method of Ziegler *et al.* (6).

DL-Acetylcarnitine (Otsuka Pharmaceutical Co.), DL-γ-dimethylamino-β-hydroxy butyric acid HCl (norcarnitine) (Riker Laboratories), γ-amino butyric acid (GABA) (Nutritional Biochemicals Corp.), γ-butyrobetaine (GBB), and DL-γ-amino-β-hydroxy butyric acid HCl (Calbiochem) were used.

All physical constants and tests for purity agree well with literature values (6). Plots of surface tension *versus* concentration for the long-chain acylcarnitines show no minima, suggesting the absence of free fatty acid (7).

Determination of Critical Micelle Concentration—The determination of the critical micelle concentration (CMC) is necessary to evaluate the relative contribution of monomeric and micellar species to the titration behavior of the long-chain acylcarnitines. This was accomplished by determining the concentration at which the break in the log concentration *versus* surface tension plot occurs. The surface tension of solutions containing various concentrations of decyl-, lauryl-, myristyl-, and palmitylcarnitine was determined by the drop volume method as described by Weiner and Zografi (7). Table II lists the CMC values for acylcarnitines in their cationic and zwitterionic forms in the presence and absence of 0.20 *M* KCl.

The CMC values shown for octylcarnitine were obtained more conveniently by determining the break in the pH *versus* log concentration plots (8). Values obtained in this manner for other acylcarnitines were in excellent agreement with the results of surface tension measurement.

Table I—Critical Micelle Concentrations of Cationic and Zwitterionic Acylcarnitines in Water and 0.20 *M* KCl at 25°

Compound	Critical Micelle Concentration, moles/l.			
	Cationic Form		Zwitterionic Form	
	H_2O	0.20 *M* KCl	H_2O	0.20 *M* KCl
Octylcarnitine	1.6×10^{-1}	1.0×10^{-1}	1.1×10^{-1}	9.5×10^{-2}
Decylcarnitine	1.5×10^{-2}	7.0×10^{-3}	1.1×10^{-2}	9.0×10^{-3}
Laurylcarnitine	1.5×10^{-3}	7.5×10^{-4}	1.2×10^{-3}	8.5×10^{-4}
Myristylcarnitine	1.5×10^{-4}	9.0×10^{-5}	1.0×10^{-4}	7.5×10^{-5}
Palmitylcarnitine	1.5×10^{-5}	8.5×10^{-6}	—	—

Potentiometric Titration—Potentiometric titrations were carried out using a Beckman 101900 research pH meter equipped with a Beckman 39167 general-purpose glass electrode and a Beckman 19168 silver–silver chloride reference electrode. The instrument was standardized with Beckman pH 4 buffer solution (pH 4.01 at 25°) and calibrated at pH 10.184 by an internal calibration device before and after each titration.

The solutions were prepared by dissolving the undissociated acid in doubly distilled CO_2-free water. The titrant was added in 0.01 mmole or smaller increments by means of an Agla micrometer syringe outfit (Burroughs Wellcome & Co.). The entire titration was run at constant temperature and under nitrogen atmosphere. The apparent ionization constant of zwitterionic compounds such as the acylcarnitines may be defined as:

$$K = \frac{(H^+)(R^\pm)}{(RH^+)} \qquad \text{(Eq. 1)}$$

where $(R^\pm)$ and (RH^+) are the concentrations of the zwitterionic and cationic species, respectively. The logarithmic form of Eq. 1 is, therefore,

$$pK = pH + \log\left(\frac{RH^+}{R^\pm}\right) \qquad \text{(Eq. 2)}$$

If β is defined as the fraction of the total acylcarnitine that is protonated or ionized, then

$$\beta = \frac{(RH^+)}{(R^\pm) + (RH^+)} \qquad \text{(Eq. 3)}$$

and Eq. 2 becomes

$$pK = pH + \log\frac{\beta}{1-\beta} \qquad \text{(Eq. 4)}$$

If the hydrogen- or hydroxyl-ion concentration is not negligible compared to the concentration of acylcarnitine, the condition of electrical neutrality must be considered and these equations must be modified according to a standard method described by Albert and Serjeant (9).

RESULTS

Titration of Monomers—The pK values of 0.01 *M* solutions of carnitine and those acylcarnitines that are monomeric at this concentration are given in Table II. The pK values of other compounds having structures similar to the carnitines are shown for comparison. As expected from Eq. 4, the values are independent of the degree of ionization, β, and of temperature in the range of 20–40°. The pK values obtained at concentrations as high as 0.10 *M* differed only slightly. The pK of acetylcarnitine is increased by 0.1 pK unit in the presence of 0.2 *M* LiCl, NaCl, KCl, KBr, or KI, indicating a relatively small ionic strength effect and no specific effect for these ions.

Effects of Concentration—The pK values of cationic decylcarnitine determined potentiometrically are shown in Fig. 1. It can be seen that the pK is constant below the CMC, indicating that the surfactant behaves as a normal electrolyte at these concentrations. Above the CMC (Fig. 1) the pK is dependent on concentration due to the changing ratio of monomeric to micellar surfactant. After this transition region the pK begins to become constant again, due to the diminishing ratio of the monomeric surfactant to the micellar surfactant. This type of concentration dependence was observed by Ekwall *et al.* for certain cholates (10) and by Tokiwa and Ohki for alkyl amine oxides (11).

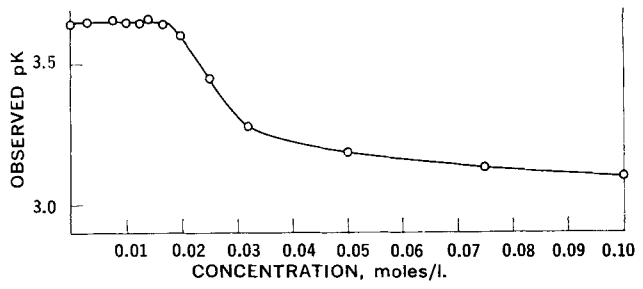


Figure 1—*Effect of micelle formation on the observed pK of decylcarnitine (β = 0.5).*

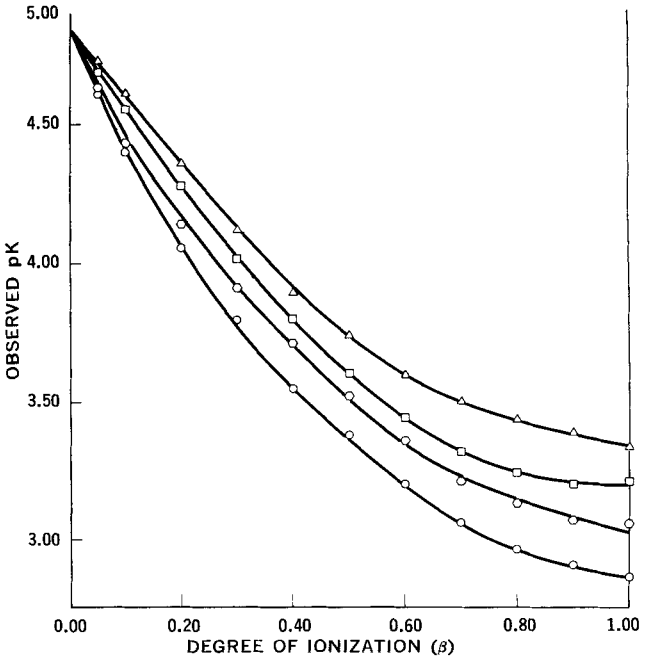


Figure 2—*Titration curves (observed pK* versus β*) for laurylcarnitine at several concentrations of added KCl. Key:* ○, *no salt;* ○, *0.05* M; □, *0.10* M; *and* △, *0.20* M.

Titration of Micelles—At concentrations significantly greater than the CMC (where the contribution of the monomer to the titration curve is negligible) the apparent ionization constant, pK, at any value of β is not independent of β, as is usually the case for monomers. These values, however, are independent of temperature. Figures 2–4 show curves of pK *versus* β for lauryl-, myristyl-, and palmitylcarnitine at several concentrations of added KCl.[1] Values of pK appear to decrease linearly at lower degrees of ionization and extrapolate to a pK at $\beta = 0$ of 4.85 ± 0.03. The change in pK with β is less pronounced at high salt concentrations. Figure 5 contains the titration curves of myristylcarnitine in 0.2 *M* LiCl, NaCl, KCl, KBr, and KI, and indicates that the curves for the three chlorides are identical while those for titration in KBr and KI show significantly higher pK values for each β.

DISCUSSION

The pK values of butyric acid, pentanoic acid, hexanoic acid, and most other aliphatic acids are all about 4.83 ± 0.03. This value may therefore be taken as the intrinsic pK or pG for an aliphatic carboxylic acid. The difference in pK between each compound listed in Table II and 4.83 is proportional to the work required to bring a proton from infinity to the site of the carboxylate group on the molecule. Thus, if the acid also has a positively charged group, work must be done to bring a proton to the molecule, and the pK decreases. If the acid molecule contains a negatively charged group, free energy is lost by the process of bringing a proton to the molecule and the pK increases. The magnitude of this effect is given by the expression (12)

$$pK - pG = 0.434\,\frac{z\epsilon}{D_E RkT} \qquad \text{(Eq. 5)}$$

[1] These curves are also independent of the concentration of acylcarnitine present as long as the ionic strength is not changed. Because of the high CMC of decylcarnitine, titration curves which are free of contributions from monomers present could not be obtained, but significant differences in pK below and above the CMC were noted.

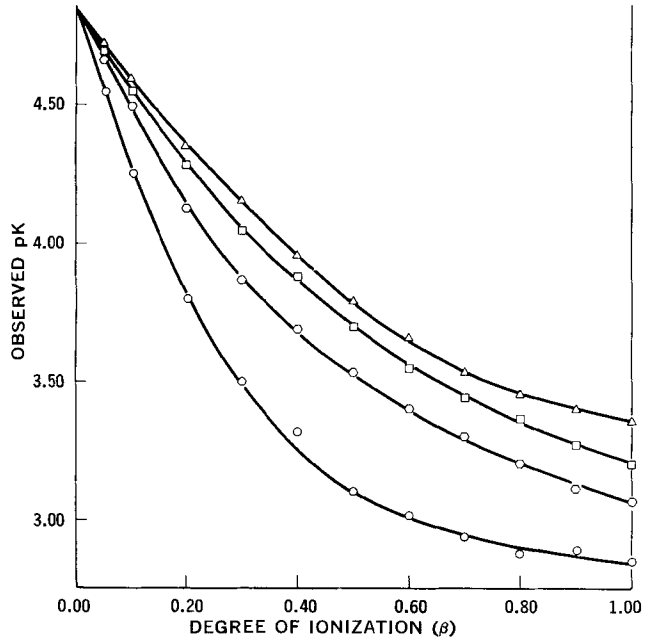


Figure 3—*Titration curves (observed pK* versus *β) for myristylcarnitine at several concentrations of added KCl. Key:* ○, *no salt;* ◯, *0.05* M; □, *0.10* M; *and* △, *0.20* M.

where ϵ is the charge on an electron or carboxylate group; z is the charge of the substituent; R is the distance between the substituent and the carboxyl group; and D_E is the effective dielectric constant separating the substituent and the carboxyl group.

From the data in Table II, it can be seen that carnitine and the acylcarnitines have pK values which are significantly lower than 4.83. These low values are primarily due to the electrostatic interaction between the positively charged nitrogen and the carboxyl group as already discussed. In addition to the above ion–ion interactions, ion–dipole, hydrogen-bonding, and steric interactions involving other groups on the molecule can contribute to the change in the ionization constant of a carboxylic acid. For instance, both the β-hydroxy and β-acyl substituted derivatives have lower pK

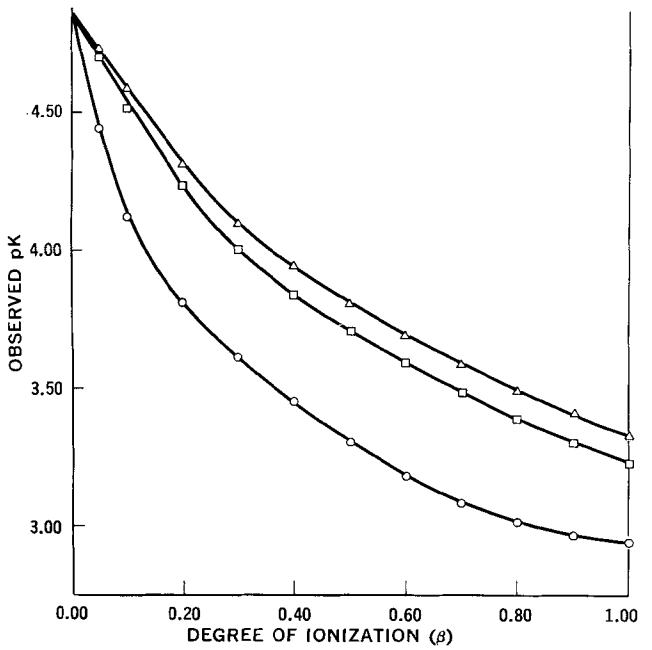


Figure 4—*Titration curves (observed pK* versus *β) for palmitylcarnitine at several concentrations of added KCl. Key:* ○, *no salt;* □, *0.10* M; *and* △, *0.20* M.

Table II—pK Values of Carnitine and Some Related Compounds

Compound	Structure $XCH_2CH(Y)CH_2COOH$ X	Y	pK
Butyric acid	H	H	4.83
Pentanoic acid	CH_3	H	4.80[a]
Hexanoic acid	C_2H_5	H	4.85[a]
GBB	$(CH_3)_3N^+$	H	4.02
GABA	H_3N^+	H	4.01
Carnitine HCl	$(CH_3)_3N^+$	OH	3.80
Norcarnitine HCl	$(CH_3)_2HN^+$	OH	3.81
β-Hydroxy-γ-ammonium butyric acid	H_3N^+	OH	3.80
Acetylcarnitine HCl	$(CH_3)_3N^+$	$C_2H_3O_2$	3.60
Butylcarnitine HCl	$(CH_3)_3N^+$	$C_4H_7O_2$	3.56
Octylcarnitine HCl	$(CH_3)_3N^+$	$C_8H_{15}O_2$	3.60
Decylcarnitine HCl	$(CH_3)_3N^+$	$C_{10}H_{19}O_2$	3.65

[a] Literature values (10).

values than compounds having the same structure except for these β-substituents. The pK values of the four acylcarnitines are nearly the same, indicating that the size of the acyl group has little or no effect on the electrostatic interactions of these compounds. The reason for the difference in pK values between carnitine and its esters (about 0.2 pK unit) is unclear. However, it is likely that the ester carbonyl interacts with the carboxyl group and facilitates dissociation of a proton.

The structures of GBB and GABA are identical except for the substituents on the quaternary nitrogen and, likewise, the structures of carnitine, norcarnitine, and γ-amino-β-hydroxy butyric acid differ only in the number of methyl groups on the nitrogen. The fact that the first two compounds have the same pK value and the remaining three pK values are the same indicates that the electrostatic interaction is independent of the minimum distance with which the quaternary nitrogen and the carboxyl group can approach each other. This interpretation is in agreement with the results of other workers (12, 13), who determined that the carboxyl proton and the quaternary nitrogen of GABA are from 4 to 6 Å apart, *i.e.*, that the molecule is almost fully extended. In view of this the cyclic conformation proposed for acetylcarnitine by Fellman and Fujita (14) seems unlikely.

In the titration of a carboxyl group on a positively charged surface, such as the surface of an acylcarnitine micelle, the observed pK at a particular degree of ionization is dependent upon the surface potential of the micelle, ψ. This relationship is expressed by an equation analogous to Eq. 5, known as the general potentiometric equation for polyelectrolytes (12, 15–18). This equation has been shown to be valid for micelles by Tokiwa and Ohki (19, 20):

$$\mathrm{pK} = \mathrm{pG} + 0.434\,\frac{\epsilon\psi}{kT} \qquad \text{(Eq. 6)}$$

As in Eq. 5 the term pG is related to the work of protonating the carboxyl group in an uncharged environment, *i.e.*, when $\beta = 0$, and the last term in the equation is related to the work required to bring the proton to the micelle surface from infinity. Equation 6 can be rearranged so that ψ is expressed in terms of the difference between the observed and intrinsic pK values:

$$\psi = 2.303\,\frac{kT}{\epsilon}\,(\mathrm{pK} - \mathrm{pG}) \qquad \text{(Eq. 7)}$$

This method of determining the surface potential of a micelle is more convenient than the electrophoretic method (21–23) since it is rapid and requires no equipment other than a pH meter. Since it gives the potential at the plane of the carboxyl groups, it has theoretical advantages over the electrophoretic method in that the value is independent of the thickness of the hydration layer. This method is not limited to the present system, but can be applied to any titratable group solubilized at a micelle surface including those of the surfactant itself.

The values of ψ obtained by applying Eq. 7 to the data shown in Fig. 3 are plotted in Fig. 6. The surface potentials of lauryl-, myristyl-, and palmitylcarnitine at $\beta = 0.1$, 0.5, and 1.0 and at

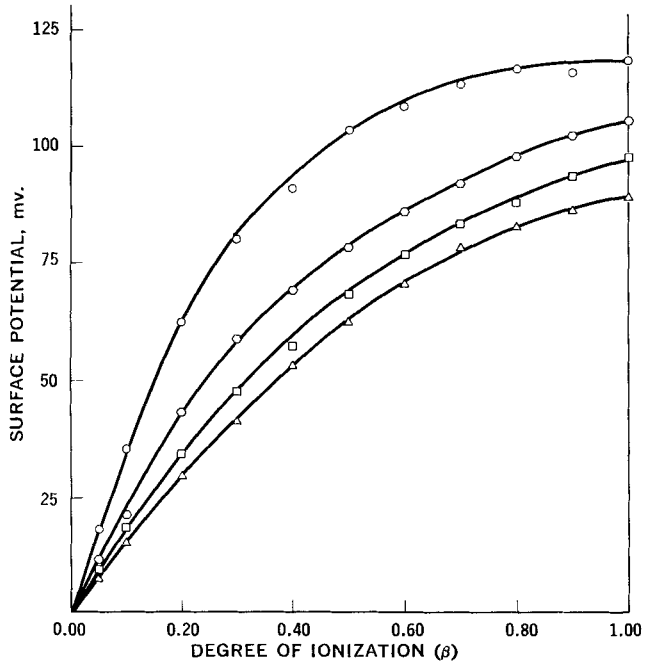


Figure 5—*Plots of surface potential* versus β *for myristylcarnitine at several concentrations of added KCl. Key:* ○, *no salt;* ○, *0.05* M; □, *0.10* M; *and* △, *0.20* M.

several concentrations of added KCl are shown in Table III for comparison. It can be seen from this table that the surface potentials at a given degree of ionization and, ionic strength show no dependence on the length of the aliphatic chain, suggesting that the surface-charge density and, therefore, the surface packing in the micelle is the same for these three compounds.

In order to interpret quantitatively the effect of β and ionic strength on the surface potential and hence the pK, it is necessary to express ψ in terms of measurable parameters such as micelle radius, b, aggregation number, n, and the net charge on the micelle at a given degree of ionization, Z. Unfortunately, this cannot be done analytically for a small spherical particle such as a micelle but it can be approximated; one such approximation is that of Debye-Hückel (12):

$$\psi = \frac{2kT}{\epsilon} wZ \qquad \text{(Eq. 8)}$$

where w, the electrostatic factor, is given by

$$w = \frac{\epsilon^2}{2DkT}\left[\frac{1}{b} - \left(\frac{\kappa}{1+\kappa a}\right)\right] \qquad \text{(Eq. 9)}$$

where a is b plus the average radius of the electrolyte ions,[2] D is the dielectric constant at the micelle surface, and κ is the inverse Debye radius which is given by

$$\kappa = \left(\frac{4\pi\epsilon^2 I}{DkT}\right)^{1/2} \qquad \text{(Eq. 10)}$$

where I is the ionic strength of the solution.

This approximation is valid for impenetrable spherical particles at low surface potential and at low concentrations of 1:1 electrolytes. It is assumed further that the net charge on the particle is spread evenly over the surface. Although this is not the correct physical situation, it has been shown (18, 24, 25) that under these conditions a more rigorous fixed charge model gives almost the same results as a spread charge model. This would be especially true for a micelle in which the individual charges are free to arrange themselves so that they are evenly spaced, *i.e.*, in their lowest energy state. The ability of the cationic and anionic groups of acylcarnitines to arrange themselves in such a manner seems very likely in view of the titration behavior of the monomeric species, which suggested that these groups were far enough apart to interact with neighboring molecules.

[2] Since the distance between charges on the micelle surface is considerably greater than the radius of the electrolyte ions, and since the micelle surface is probably not smooth, it might be more correct to let $a = b$, *i.e.*, to assume that the electrolyte ions behave as point charges.

Table III—Surface Potentials for Acylcarnitines at Several Degrees of Ionization (β) and Several Concentrations of Added Electrolyte

Concentration Added KCl, moles/l.	Surface Potential, mv. Lauryl-carnitine	Myristyl-carnitine	Palmityl-carnitine
	β = **0.10**		
0.00	26.6	29.6	42.6
0.05	24.2	21.9	—
0.10	17.2	17.7	20.1
0.20	14.2	14.8	15.4
	β = **0.50**		
0.00	87.1	102	91.7
0.05	73.7	77.0	—
0.10	68.0	66.8	68.0
0.20	62.2	62.7	62.2
	β = **1.00**		
0.00	117	117	113
0.05	104	104	—
0.10	97.2	97.2	96.0
0.20	89.0	88.7	90.6

If the micelle has an aggregation number of n and a degree of ionization of β, the net charge is given by

$$Z = n\beta \qquad \text{(Eq. 11)}$$

and Eqs. 6 and 8 become, respectively,

$$\text{pK} = \text{pG} + 0.868\, wn\beta \qquad \text{(Eq. 12)}$$

and

$$\psi = \frac{2kT}{\epsilon} wn\beta \qquad \text{(Eq. 13)}$$

From Eqs. 12 and 13, it can be seen that at $\beta = 0$, pK must equal pG, and ψ must become zero. Indeed the value of the experimentally

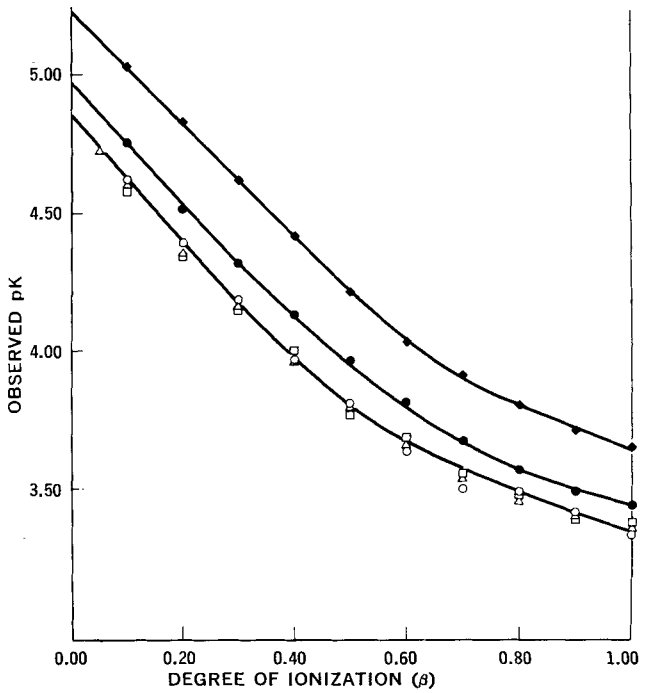


Figure 6—*Effects of specific monovalent ions on the titration curves of myristylcarnitine. Key:* △, *0.20* M *KCl;* ○, *0.20* M *LiCl;* □, *0.20* M *NaCl;* ●, *0.20* M *KBr; and* ◆, *0.20* M *KI.*

determined pG is in excellent agreement with the literature value for aliphatic carboxylic acids. The apparent linearity in the pK *versus* β with ψ *versus* β curves (Figs. 1–4) at low β is expected from Eqs. 12 and 13. The deviation of all of the curves from linearity at high β is probably due to a change in n or w with increasing charge, or more likely to the failure of the Debye-Hückel approximation at high potential (26). Further investigations of the relationship between w, n, and β are now being carried out using other mathematical treatments and experimental measurements of the micelle size (27).

The fact that the pG values of all of the acylcarnitines are independent of concentration of surfactant and added KCl (Figs. 2–4) indicates that neither K^+ nor Cl^- are bound to the zwitterionic micelle. Figure 5 shows that Li^+ and Na^+ also are not bound to the neutral micelle but that Br^- and, to an even greater extent, I^- appear to be bound (28). This order of ion binding to cationic micelles has been noted previously (29). The fact that Cl^-, Br^-, and I^- do not exhibit different effects on the titration of the monomeric acetylcarnitine suggests that these specific ion effects are, indeed, dependent on the presence of the micellar surface.

CONCLUSIONS

The pK values of micellar surfactants such as the acylcarnitines are not constant but change with β, the degree of ionization of the micelle. The difference between the pK value observed at a particular value of β and the intrinsic pK for a carboxyl group has been utilized to determine the surface potential ψ at that value of β.

A model utilizing the Debye-Hückel approximation has been shown to be qualitatively consistent with experimental results.

The ionization constants and surface potentials of lauryl-, myristyl-, and palmitylcarnitine micelles at any given value of β are independent of chain length but are highly dependent on ionic strength. Specific ion effects are observed for Br^- and I^- but not for Li^+, Na^+, K^+, or Cl^-.

The significant effect of surface charges on the ionization equilibria of this particular micellar system suggests the general importance of these considerations for understanding kinetic and adsorption processes involving charged species at charged interfaces such as micelles, monolayers, bilayers, and biological membranes.

REFERENCES

(1) I. B. Fritz, *Advan. Lipid Res.*, **1,** 285(1963).

(2) I. B. Fritz and K. T. N. Yue, *J. Lipid Res.*, **4,** 279(1963).

(3) R. Bressler, R. Katz, and B. Wittels, *Ann. N. Y. Acad. Sci.*, **131,** 207(1965).

(4) I. B. Fritz and S. K. Schultz, *J. Biol. Chem.*, **240,** 2188(1965).

(5) I. B. Fritz, E. Kaplan, and K. T. N. Yue, *Amer. J. Physiol.*, **202,** 117(1962).

(6) H. J. Ziegler, P. Bruchner, and F. Binon, *J. Org. Chem.*, **32,** 3989(1967).

(7) N. D. Weiner and G. Zografi, *J. Pharm. Sci.*, **54,** 436(1965).

(8) A. S. C. Lawrence and M. P. McDonald, *2nd Int. Cong. of Surf. Activity*, **1957,** p. 385.

(9) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Wiley, New York, N. Y., 1962, p. 30.

(10) P. Ekwall, T. Rosendahl, and N. Lofman, *Acta Chem. Scand.*, **11,** 590(1960).

(11) F. Tokiwa and K. Ohki, *Kolloid Z. Z. Polym.*, **223,** 135 (1967).

(12) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic, New York, N. Y., 1958, chaps. 8 and 9.

(13) E. J. Cohn, *Ann. Rev. Biochem.*, **4,** 93(1935).

(14) J. H. Fellman and T. S. Fujita, *Biochem. Biophys. Acta*, **97,** 590(1935).

(15) A. Katchalsky and I. R. Miller, *J. Polymer Sci.*, **13,** 57 (1954).

(16) A. Katchalsky, N. Shavit, and H. Eisenberg, *ibid.*, **13,** 69(1954).

(17) A. Katchalsky, J. Mazur, and P. Spitnik, *ibid.*, **23,** 513 (1957).

(18) C. Tanford, "Physical Chemistry of Macromolecules," Wiley, New York, N. Y., 1967, p. 475.

(19) F. Tokiwa and K. Ohki, *J. Phys. Chem.*, **70,** 3437(1966).

(20) *Ibid.*, **71,** 1824(1967).

(21) F. Tokiwa and K. Ohki, *Kolloid Z. Z. Polym.* **230,** 251 (1968).

(22) F. Tokiwa and K. Ohki, *Bull. Chem. Soc. Jap.*, **42,** 1216 (1968).

(23) D. Stigter and K. J. Mysels, *J. Phys. Chem.*, **59,** 45(1955).

(24) C. Tanford and J. G. Kirkwood, *J. Amer. Chem. Soc.*, **79,** 5333(1957).

(25) *Ibid.*, **79,** 5340(1957).

(26) M. Nagassawa and A. Holtzer, *ibid.*, **86,** 531(1964).

(27) S. H. Yalkowsky, Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1970.

(28) G. Scatchard and E. S. Black, *J. Phys. Colloid. Chem.*, **53,** 88(1949).

(29) W. P. J. Ford, R. H. Ottewill, and H. C. Parreira, *J. Colloid. Interface Sci.*, **21,** 522(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 28, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication January 27, 1970.

The authors thank Dr. Irving Fritz of the Best Institute, Toronto, Canada, for his helpful discussions during the initial phase of this study and Dr. G. Cronhein, Riker Laboratories, for a sample of norcarnitine.

This study was supported in part by the Bristol-Myers Co., Hillside, N. J.; General Research Support Grant FR 05571, National Institutes of Health, and U. S. Public Health Service Training Grant DE 00204, National Institute of Dental Research, National Institutes of Health, Bethesda, Md.

* Present address: The Upjohn Co., Kalamazoo, MI 49001

Placental Transfer of Pentobarbital in the Rat

DAVID P. S. MORK*, WAYNE V. KESSLER, and STANLEY M. SHAW

Abstract □ The placental transfer of pentobarbital and/or metabolites was determined in rats using ^{14}C-labeled pentobarbital. The level of pentobarbital and/or metabolites in fetal blood was greatly influenced by the circulating level of pentobarbital and/or metabolites in the mother. Of the total pentobarbital and/or metabolites transferred, the percentage of unbound pentobarbital in fetal blood plasma was influenced by the pentobarbital dose level administered to the pregnant rat. A greater percentage of total pentobarbital and/or metabolites was present as unbound pentobarbital in fetal blood plasma than in maternal blood plasma. Chronic administration of pentobarbital during gestation decreased sensitivity to the pharmacological effects of further drug administration to the newborn. The changes induced by pretreatment were not permanent.

Keyphrases □ Placental transfer, rat—pentobarbital, metabolites □ Pentobarbital, labeled—fetal blood □ Fetal blood concentration, pentobarbital—maternal blood concentration □ Scintillometry—analysis □ TLC—analysis, radioactivity

Placental transfer of pentobarbital in the human at term was reported by Flowers (1). Transfer was determined by measuring pentobarbital in the arterial and venous blood of the umbilical cord. By observing a decrease in the respiratory rate of the newborn, Dreisbach and Synder (2) determined that pentobarbital crosses the placenta of the rabbit at term. In this investigation, ^{14}C-labeled pentobarbital was employed to determine the placental transfer of pentobarbital and/or metabolites, as well as unbound pentobarbital in the rat. In addition, the influence of chronic pentobarbital administration to the pregnant rat on the sleep time and the blood level of bound and/or unbound pentobarbital required for sleep was studied in the newborn.

EXPERIMENTAL

Animal Care and Mating—Sprague-Dawley[1] albino rats (200–210 g.) were housed individually in screen wire-bottom hanging cages in a controlled light environment. Animals were allowed food[2] and water *ad libitum*. Prior to being used for experimental study, the animals received 5 min. of handling daily for 2 weeks to reduce stress.

For mating, one male was placed with one female at 5:00 p.m. The male was removed at 7:00 a.m. the following morning and a vaginal smear was taken. The slides were fixed in 95% methanol, stained with Giemsa's blood stain,[3] and examined microscopically to determine the estrous cycle of the rat. When sperm were present during estrous, the female was assumed to be mated (3). On Day 18 of gestation, wood shavings were added for bedding to satisfy the nesting instinct.

Labeled Pentobarbital—The labeled compound used was 5-ethyl-5-(1-methylbutyl)barbituric-2-^{14}C acid.[4] It was converted to the sodium salt with 0.01 *N* sodium hydroxide, and the resulting solution was used throughout this study. Chemical purity and radiochemical purity were determined chromatographically using unlabeled authentic pentobarbital and the two solvent systems to be described later. No chemical impurities were found; the radiochemical purity was greater than 99.5%. The specific activity of the labeled pentobarbital injected into rats was 4 μc./mg.

Determination of Radioactivity—Individual tissue samples were placed in tared counting vials containing 1 ml. of a solution of a quaternary ammonium compound,[5] weighed, and dissolved by heating at 60° for 2 hr. with gentle agitation. The vials were cooled; the samples were decolorized with 0.5 ml. of 30% hydrogen peroxide. Glacial acetic acid, 0.5 ml., was added to minimize protein phosphorescence. After 10 min., 15 ml. of XDC scintillator[6] was added to each sample. The samples were counted in a Tri-Carb[7] using the internal standard method (5) to determine the absolute disintegration rates. The maximum counting error was 1%.

Chromatography—TLC plates were prepared with a distilled water slurry of equal parts of two commercial adsorbents.[8] The plates were air-dried, activated in an oven at 100° for 1 hr., and cooled to room temperature prior to spotting. The plates were developed in either a chloroform–acetone (9:1) solvent or a dioxane–benzene–ammonium hydroxide (4:15:1) solvent (6, 7).

Compounds were visually located using UV light. The chromatograms were then sprayed[9] and exposed to Kodak No Screen medical X-ray film[10] for a time period which allowed approximately 10^8 disintegrations/cm.^{2} to occur. The films were developed with Kodak chemicals according to manufacturer's instructions. Labeled compounds on a chromatogram were quantitatively removed for counting by scraping the adsorbent containing the compound from the plate. The adsorbent was placed in a counting vial containing 15 ml. of XDC; colloidal silica[11] was added to form a thixotropic gel. The total radioactivity on the developed plate was summed and considered as 100% of the radioactivity applied. The radioactivity found in a labeled compound was expressed as a percentage of the total applied.

Optimal Time of Sacrifice—Animals were injected intraperitoneally with a 10-mg./kg. dose of labeled pentobarbital on Day 19 of gestation. Day 19 was selected because most compounds have been shown to cross the placental barrier to the greatest extent near the termination of gestation. Groups of animals were sacrificed with ether 1, 3, 6, 12, or 24 hr. following drug administration. Blood samples were obtained from the heart of the mother and fetus. On the average, the blood sample obtained from each fetus weighed approximately 115 mg. From each mother, certain fetuses were removed, weighed, frozen in liquid nitrogen, placed between sheets of plastic, and pulverized. The powdered fetus was thoroughly mixed and a sample was taken and prepared for counting.

The ^{14}C-radioactivity was determined for blood and tissue samples. In this phase of the investigation, no attempt was made to differentiate between pentobarbital and metabolites. The ^{14}C-radioactivity was expressed as micrograms of pentobarbital per gram of sample.

Effect of Dose Level—Pregnant animals were injected intraperitoneally on Day 19 of gestation with a 5- or 25-mg./kg. dose of labeled pentobarbital. The animals were sacrificed 1 hr. after injection, and samples of various tissues were taken. The ^{14}C-radioactivity was expressed as micrograms of pentobarbital per gram of sample. Maternal and fetal blood were also obtained for determina-

[1] Sprague-Dawley Inc., Madison, Wis.
[2] Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.
[3] Matheson, Coleman & Bell, Norwood, Ohio.
[4] Supplied by New England Nuclear, Boston, Mass.
[5] A methanolic solution of Hyamine hydroxide prepared according to the method of Bruno and Christian (4) from Hyamine 10-X crystals, Rohm & Haas, Philadelphia, Pa.
[6] The XDC scintillator was prepared as follows: *p*-xylene, one part; *p*-dioxane, three parts; 2-ethoxyethanol, three parts; naphthalene, 8%; PPO, 1%; and dimethyl POPOP, 0.5%.
[7] Model 3003, Packard Instrument Co., Inc., Downers Grove, Ill.
[8] Adsorbosil P-1 and Adsorbosil 1, Applied Science Laboratories, Inc., State College, Pa.
[9] With Neatan New, Brinkmann Instruments, Inc., Westbury, N. Y.
[10] Eastman Kodak Co., Rochester, N. Y.
[11] Cab-O-Sil, Godfrey L. Cabot, Inc., Boston, Mass.

Table I—Pentobarbital and/or Metabolites in Tissues of Animals Sacrificed at Various Time Intervals following Administration

Time,[b] hr.	No. of Maternal Animals	Pentobarbital and/or Metabolites, mcg./g.[a]			Fetal Blood[d] / Maternal Blood
		Fetal Blood	Fetal Tissue[c]	Maternal Blood	
1	6	4.67 ± 0.56	6.27 ± 1.15	6.39 ± 0.99	0.74 ± 0.06
3	6	5.07 ± 0.54	6.99 ± 0.98	5.93 ± 1.09	0.93 ± 0.14
6	3	3.71 ± 0.42	4.67 ± 0.33	4.34 ± 0.56	0.86 ± 0.01
12	3	1.93 ± 0.21	2.28 ± 0.18	2.51 ± 0.24	0.77 ± 0.05
24	3	0.67 ± 0.15	0.72 ± 0.32	0.77 ± 0.16	0.88 ± 0.09

[a] Mean ± standard error. [b] Time elapsed between injection and sacrifice. [c] Homogeneous sample of a whole fetus. [d] Ratio of amount of pentobarbital and/or metabolites in the fetal blood to amount in the maternal blood.

Table II—Effect of Dose on Transfer of Pentobarbital and/or Metabolites

Dose Level[b]	No. of Maternal Animals	Pentobarbital and/or Metabolites, mcg./g.[a]					Fetal Blood[d] / Maternal Blood
		Fetal Blood	Fetal Tissue[c]	Fetal Liver	Maternal Blood	Maternal Liver	
5	8	3.91 ± 0.24	4.16 ± 0.16	5.96 ± 0.23	4.43 ± 0.18	12.70 ± 0.44	0.89 ± 0.05
25	6	19.92 ± 0.93	22.13 ± 0.81	29.31 ± 0.73	23.98 ± 1.13	63.14 ± 1.48	0.83 ± 0.03

[a] Mean ± standard error. [b] Milligrams of labeled pentobarbital per kilogram of body weight administered on Day 19 of gestation. [c] Homogeneous sample of the whole fetus. [d] Ratio of amount of pentobarbital and/or metabolites in the fetal blood to amount in the maternal blood.

tion of unbound pentobarbital. Each whole-blood sample was centrifuged for 10 min. in a heparinized capillary centrifuge tube[12] which was sealed with a commercial clay.[13] The capillary tube was broken just above the interface between white blood cells and plasma, and the plasma was spotted on a thin-layer plate. Authentic ^{14}C-labeled pentobarbital was also spotted for reference. The plate was developed; labeled compounds were located and quantified according to procedures previously described. The percentage of the total ^{14}C-radioactivity on the chromatogram that represented unbound pentobarbital was determined.

Effect of Pretreatment during Pregnancy on the Sleep Time and Dose Required for Sleeping in the Newborn—Pregnant rats were injected intraperitoneally on Days 11–20 of gestation with 10 mg./kg. of unlabeled pentobarbital. Pregnant control rats received saline solution. The animals were allowed to give birth. Each litter was reduced to six animals to keep maternal care a constant. From each litter, three of the young rats were studied 5 days following birth and the other three were studied 30 days after birth. Rats were weaned 25 days after birth. During the time interval previous to the determination of sleep time, no attempt was made to reduce stress in the 5-day-old animals by daily handling. The 30-day-old rats were handled daily for 10 min. for a period of 6 days prior to sleep studies.

The 5-day-old rats were injected intraperitoneally with 20 mg./kg. of labeled pentobarbital, while the 30-day-old rats received 30 mg./kg. The sleep time was determined by measuring the loss of the righting reflex. Upon return of the righting reflex, blood was removed from the heart to determine the level of bound and/or unbound pentobarbital in the blood which was just below the level required for sleep. Bound and/or unbound pentobarbital was extracted from blood according to the method of Brodie *et al.* (8). This extraction method removes bound and unbound pentobarbital while excluding metabolites of pentobarbital. The petroleum ether layer was added to the XDC scintillator, and ^{14}C-radioactivity was determined. Counting data were expressed as pentobarbital.

Analysis of Results—The Student *t* test (9) was used to test the results of the treatments for significant differences. All results were tested at the 95% level of significance.

RESULTS AND DISCUSSION

Optimal Time of Sacrifice—The results are presented in Table I. Fetal and maternal blood levels of pentobarbital and/or metabolites at 12 and 24 hr. postinjection were significantly different from each other and from the values at 1, 3, and 6 hr. Values at 1, 3, and 6 hr. were not significantly different from each other. In general the measurable level of pentobarbital and/or metabolites in all tissues declined rapidly after 3 hr. One hour after injection was the time selected for sacrifice for the remainder of the study.

For each time interval, the ratio of the fetal blood level of pentobarbital and/or metabolites to the maternal blood level was calculated. No significant difference was found between any of the ratios. The results indicate that by 1 hr. after injection an equilibrium had been established between the pentobarbital and/or metabolite level in the maternal and fetal blood.

Effect of Dose—As may be seen in Table II, an increased dose of pentobarbital administered to the pregnant rat resulted in an increased level of pentobarbital and/or metabolites in all of the tissues studied. The ratios of the amount of pentobarbital and/or metabolites in the fetal blood to the amount in the maternal blood were not significantly different for the two dose levels. Thus, the level of pentobarbital and/or metabolites in the fetal blood was directly proportional to the pentobarbital and/or metabolite level in the maternal blood, regardless of the dose administered to the pregnant rat.

Results of the determination of the unbound pentobarbital level in fetal and maternal blood plasma are shown in Table III. The results are expressed as the percentage of the total ^{14}C-radioactivity on a chromatogram which represented unbound pentobarbital. The data show that a higher percentage of total pentobarbital and/or metabolites was present as unbound pentobarbital in fetal blood plasma than in maternal blood plasma. The relationship was true for both dose levels. The data indicate that unbound pentobarbital transfers through placental membranes more readily than bound pentobarbital and/or metabolites.

There was a significant increase in the percentage of unbound pentobarbital in the maternal and fetal blood when the dose was increased from 5 to 25 mg./kg. As was already shown, an increase in the pentobarbital dose administered to the pregnant rat increased the total pentobarbital and/or metabolites that transferred across the placenta. Of the total compound that transferred, a higher percentage of unbound pentobarbital was present in the fetal blood plasma at the higher dose level than was present at the lower dose level.

Effect of Pretreatment during Pregnancy on the Sleep Time and Pentobarbital Blood Level Required for Sleeping in the Newborn—As may be observed from the results presented in Table IV, there was a significant decrease in sleep time of 5-day-old newborn rats from pretreated mothers compared to 5-day-old newborn rats from control mothers. These results agree with the findings of Hart *et al.* (10) in a study of the stimulation of hepatic microsomal drug metabolism in the newborn and fetal rabbit. The data in Table IV show that a higher blood level of bound and/or unbound pentobarbital was required to induce sleep in the 5-day-old newborn rats from pretreated mothers, which indicated a decreased sensi-

[12] Red Tip Heparinized Capillary Tubes, Division of American Hospital Supply Corp., Evanston, Ill.
[13] Seal-Ease, Clay Adams, Inc., New York, N. Y.

Table III—Unbound Pentobarbital in Fetal and Maternal Blood Plasma

Dose Level[a]	No. of Maternal Animals[b]	Fetal Blood[c]	No. of Maternal Animals[b]	Maternal Blood[c]
5	7	43.21 ± 5.95	6	12.20 ± 3.46
25	6	72.73 ± 3.74	5	28.94 ± 2.58

[a] Milligrams of labeled pentobarbital per kilogram of body weight administered on Day 19 of gestation. [b] Variation in animal number due to difficulty involved in chromatographic techniques. [c] Percent of total ^{14}C-radioactivity which represented unbound pentobarbital. Mean ● standard error.

Table IV—Sleep Time and Pentobarbital Blood Level Required for Sleep[a]

	No. of Maternal Animals	Sleep Time, min.	Pentobarbital Blood Level Required for Sleep, mcg./g. Blood
		Five Day[b]	
Control[c]	6	409.5 ± 4.50[d]	4.05 ± 0.28
Pretreated[e]	6	298.2 ± 5.90	5.23 ± 0.21
		Thirty Day	
Control	5	91.80 ± 10.89	11.93 ± 0.58
Pretreated	6	94.50 ± 2.37	12.19 ± 0.23

[a] Sleep time is expressed as the duration of the loss of the righting reflex. Pentobarbital blood level required for sleep is expressed as the level of bound and/or unbound pentobarbital in the blood, which was necessary for the loss of the righting reflex. [b] Day after birth at which sleep study was conducted. [c] Newborn from mothers treated with saline solution during gestation. [d] Mean ± standard error. [e] Newborn from mothers receiving pentobarbital during gestation.

tivity to the pharmacologic effect of the drug. The 30-day-old pretreated rats exhibited neither a lower sleeping time nor a higher dose required for sleep when compared to 30-day-old rats from control mothers, indicating that in the intervening time interval the effects of pretreatment were not permanent.

SUMMARY

Pentobarbital and/or metabolites reached an equilibrium between maternal and fetal circulation within 1 hr.

An increased dose of pentobarbital administered to the pregnant rat resulted in an increased level of pentobarbital and/or metabolites in all tissues studied. The level of pentobarbital and/or metabolites in fetal blood was greatly influenced by the circulating level of pentobarbital and/or metabolites in the mother. Of the total compound transferred, a higher percentage of unbound pentobarbital was present in the fetal blood plasma at the higher dose level than was observed in animals receiving a lower dose of pentobarbital. A higher percentage of total pentobarbital and/or metabolites was present as unbound pentobarbital in fetal blood plasma than in maternal blood plasma.

The placental transfer of pentobarbital during chronic administration to pregnant rats caused a decreased sensitivity to the pharmacological effects of further drug administration to the newborn. Five-day-old newborn rats from pentobarbital pretreated mothers exhibited a decrease in sleep time, with a higher bound and/or unbound pentobarbital blood level being required for sleep. The changes induced by pretreatment were not permanent and did not exist by the time the rat was 30 days old.

REFERENCES

(1) C. E. Flowers, Jr., *Amer. J. Obstet. Gynecol.*, **78,** 730(1959).

(2) R. Dreisbach and F. F. Synder, *J. Pharmacol. Exp. Ther.*, **79,** 250(1943).

(3) M. X. Zarrow, J. M. Yochim, and J. L. McCarthy, "Experimental Endocrinology—A Sourcebook of Basic Techniques," Academic, New York, N. Y., 1964, p. 37.

(4) G. A. Bruno and J. E. Christian, *Anal. Chem.*, **33,** 1216 (1961).

(5) C. H. Wang and D. L. Willis, "Radiotracer Methodology in Biological Science," Prentice-Hall, Englewood Cliffs, N. J., 1965, p. 294.

(6) A. Weissberger, "Technique of Organic Chemistry," vol. 12, Interscience, New York, N. Y., 1967, p. 504.

(7) P. J. Schechter and L. J. Roth, *J. Pharmacol. Exp. Ther.*, **158,** 164(1967).

(8) B. B. Brodie, J. J. Burns, L. C. Mark, P. A. Lief, E. Bernstein, and E. M. Papper, *ibid.*, **109,** 26(1953).

(9) N. M. Downie and R. W. Heath, "Basic Statistical Methods," 2nd ed., Harper and Row, New York, N. Y., 1965, pp. 138–143.

(10) L. G. Hart, R. H. Adamson, R. L. Dixon, and J. R. Fouts, *J. Pharmacol. Exp. Ther.*, **137,** 103(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 16, 1969, from the *Bionucleonics Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication February 3, 1970.

The investigation was supported in part by the Purdue Research Foundation.

* Department of Biology, Saint Cloud State College, Saint Cloud, MN 56301

Kinetics of Urinary Excretion of D-(−)-Mandelic Acid and Its Homologs I: Mutual Inhibitory Effect of D-(−)-Mandelic Acid and Its Certain Homologs on Their Renal Tubular Secretion in Rats

EDWARD J. RANDINITIS*, MARTIN BARR, HENRY C. WORMSER, and JANARDAN B. NAGWEKAR†

Abstract □ It has been shown from the apparent first-order urinary excretion studies of D-(−)-mandelic acid and certain of its homologs, DL-tropic acid, D-(−)-4-hydroxy-4-phenylbutanoic acid, DL-phenyllactic acid, and D-(−)-benzyllactic acid, that the biological half-lives of the homologs are significantly shorter than that of D-(−)-mandelic acid in rats. Since these compounds, which differ from each other in their content of methylene groups around the carboxyl group, exhibit negligible metabolism and protein binding, low pKa values (3.3–4.7), and low lipid solubility at the physiological pH, and are primarily recovered in the urine in the intact form, they are utilized in these studies as model compounds to study the effect of hydrophobic group(s) on the rate of secretion. These compounds are shown to exert a mutual inhibitory effect on their renal tubular secretion, indicating a common "carrier" mechanism for their secretion. The utilization of these compounds indicated that the addition of methylene group(s) in the vicinity of the carboxyl group of the mandelic acid molecule increased its affinity for the carrier molecules of the renal tubular secretion system in rats.

Keyphrases □ D-(−)-Mandelic acid and homologs—mutual inhibition of urinary excretion □ Urinary excretion—D-(−)-mandelic acid and homologs □ Excretion rates, apparent—D-(−)-mandelic acid and homologs □ ORD—identity □ Polarimetry—identity □ GLC—analysis

Although considerable work has been reported showing the effect of variation of hydrophobic groups within a weakly acidic drug molecule on its rate of transport through kidney slices (1–3), little such work has been carried out *in vivo*. Furthermore, in *in vivo* work, the determination of the effect of hydrophobic variations in the molecule on the rate of urinary excretion and kidney tubular secretion has been shown to be complicated due to the differences of the homologs in their extent of binding to plasma proteins, pKa values, and partition coefficients between a lipid phase and water. The objectives of such studies were to investigate the physicochemical nature of the transport system involved in the kidney tubular secretion of drugs and other chemicals and to gain some insight into the structural specificity of a drug molecule for the transport system. The compounds commonly employed in such studies are *p*-aminohippurate (4), phenylbutazone (5), *p*-aminosalicylic acid (6), salicylic acid (2), and chlorothiazide (7).

From the studies of renal excretion of phenylbutazone analogs (5) and sulfonamide analogs (8), it has been shown that the rate of excretion of most of these compounds is inversely related to their pKa values at the normal slightly acidic urine pH. However, in their inhibitory studies of hippurate transport by phenylbutazone analogs in the kidney slices of dogs and rabbits, Despopoulos *et al.* (9) found a lack of a clearcut relationship between the inhibitory capacity of the phenylbutazone analogs and their pKa values and lipid solubility. Despopoulos and Callahan (10) observed that the reabsorption of certain sulfonamides with pKa values greater than the normal urine pH of 6 to 7 occurs from the renal tubules, and it makes difficult the interpretation of data to correlate the chemical affinity of drug analogs to the renal transport system. This problem becomes further complicated if a drug is largely metabolized and the metabolites interfere with the secretion of the parent compound, as observed with substituted benzoic acids (11).

Rammelkamp and Bradley (12) and Beyer *et al.* (13) were among the first to demonstrate that iodopyracet[1] and *p*-aminohippurate cause an increase in the blood levels of penicillin due to the competition of the inhibitors with penicillin for the carrier of secretory process in the renal tubular membrane. Similar inhibitory effects of probenecid on the urinary excretion of the optical isomers of mandelic acid have been demonstrated in humans by Nagwekar and Kostenbauder (14). Kamienny *et al.* (15) have also shown that, in humans, sulfadiazine, sulfamerazine, sulfamethazine, and (−)-mandelic acid probably share the same renal tubular transport mechanism for secretion. Various possible mechanisms suggested for the inhibition of transport of compounds through renal tubular membranes have been shown to depend on the chemical nature of the substrate and the inhibitor (16). The type of inhibition which is widely studied for the transport of substances across the renal tubular membrane is that in which the substrate and inhibitor probably share the same transport carrier.

The purposes of this project were to study the kinetics of urinary excretion of a model acidic compound and its homologs, which differ from each other in the content of their hydrophobic groups, such as methylene groups, and to determine their biological half-lives and initial excretion rates in the absence and presence of each other in rats. If such compounds are secreted by the same pathway, it may then be expected that they would mutually inhibit the secretion of each other. For reasons described later, D-(−)-mandelic acid is considered as a model compound and is used in the present studies. The compounds used in these studies are listed in Table III. The differences in the initial urinary excretion rates or the biological half-lives that these compounds show are attributed to the differences in the hydrophobic interactions which take place between the compounds and a carrier or carrier system. As described in the next paper

[1] Diodrast, Winthrop Laboratories, New York, N. Y.

in this series (17), an additional objective of this project was to gain some insight into the structural and chemical specificity that the carrier transport system of the renal tubule shows toward anionic drugs by studying the Michaelis-Menten-type kinetics of secretion of these compounds.

EXPERIMENTAL

Materials—D-(−)-Mandelic acid,[2] m.p. 132–133°, $[\alpha]_{25}^{D}$ − 154.2° (c, 1.94, H_2O); DL-tropic acid,[2] m.p. 118-119°; D-(−)-tropic acid, m.p. 123-124°, $[\alpha]_{25}^{D}$ −75° (c, 1.60, H_2O); and L-(+)-tropic acid, m.p. 126–127°, $[\alpha]_{25}^{D}$ + 77.4° (c, 1.94, H_2O), were obtained by the resolution of DL-tropic acid by the method of King (18); DL-phenyllactic acid, m.p. 96–97°; L-(−)-phenyllactic acid, m.p. 122–123°, $[\alpha]_{25}^{D}$ −20.0° (c, 1.89, H_2O); and D-(+)-phenyllactic acid, m.p. 121–122°, $[\alpha]_{25}^{D}$ + 21.2° (c, 2.05, H_2O), were synthesized by the method of Eiduson *et al.* (19) from the corresponding isomers of phenylalanine. D-(−)-Benzyllactic acid, m.p. 113–115°, $[\alpha]_{25}^{D}$ −10.0° (c, 1.50, ethanol), and the sodium salt of D-(−)-4-hydroxy-4-phenylbutanoic acid, $[\alpha]_{25}^{D}$ −12.9° (c, 1.55, H_2O), were obtained on resolving (20, 21) the corresponding racemic acids synthesized by the known procedures (22).

Apparatus—The compounds appearing in the urine of rats were quantitatively analyzed with the aid of an F & M model 810R-19 gas chromatograph equipped with a hydrogen-flame detector. The column employed was a 1.21-m. (4-ft.) long and 0.63-cm. (0.25-in.) o.d. copper tube packed with 80–100 mesh diatomite (Diatoport S) coated with 5% ethylene glycol succinate. Helium was employed as the carrier gas. A Beckman model 72 pH meter equipped with a combination electrode was used for the pH determinations. The specific rotation of the optically active compounds were determined at 25° with the aid of a J & J Fric model 2706 polarimeter using a sodium lamp as the source of light. The optical rotatory dispersion (ORD) curves of the compounds were obtained with the aid of a Cary model 60 recording spectropolarimeter.

Methodology—During the course of these studies, about 30 Sprague-Dawley male rats weighing between 165 and 210 g. were repeatedly used. None of the rats was used more than five times in these studies, and the rest period allowed between the successive use of a rat was at least 1 week. Food was withheld from the animals for 12–18 hr. prior to the administration of the compounds and during urine collection; water was allowed *ad libitum* throughout this period. To induce prompt urination, 5 ml. of water was administered intraperitoneally to each rat 15–20 min. prior to the intravenous administration of the compounds.

The compounds were administered in the dosage range of 150–700 μmole/kg. (5–20 mg./rat) by the intravenous route *via* the tail vein. The compounds were administered as aqueous isotonic solutions of their sodium salts, prepared by the addition of an equivalent amount of sodium hydroxide to an aqueous solution of the acid. In inhibitory urinary excretion studies involving equivalent doses of each compound, an isotonic solution was prepared containing the appropriate dose of the sodium salt of each of the compounds and administered by the i.v. route to the rats. However, in the inhibitory studies involving a larger dose of the inhibitor, the i.p. administration of $1.0–1.7 \times 10^4$ μmole/kg. (300–500 mg./rat) dose of inhibitor was found necessary. A solution containing an appropriate dose of the sodium salt of the inhibitor dissolved in 5 ml. of water was prepared and administered by the i.p. route 15–20 min. prior to the i.v. administration of the substrate compound.

Each rat was placed into a urine-collection cage immediately after the administration of the substrate compound. The urine-collection cage consisted of a 20.32-cm. (8-in.) plastic funnel, two chrome-plated wire baskets, and a 50-ml. graduated cylinder as shown in Fig. 1. A fine wire-mesh screen was fitted at the inner opening of the funnel to prevent rat feces from contaminating the urine. However, the rats rarely defecated during the first 3 hr. of urine collection, during which time most of the compound was recovered. Also, it was demonstrated in the preliminary studies that the excretion of the compounds in the feces was negligible. An animal drinking tube was forced through the wire basket to allow the animal water during the study.

[2] Aldrich Chemical Co., Milwaukee, Wis.

Table I—Conditions Employed for Gas Chromatographic Analysis and Retention Times Observed for the Compounds

Compound	Oven Temp.[a]	He Flow Rate, ml./min.	Retention Time, min.
Methyl mandelate	165°	60	5.8
Methyl tropate	180°	40	8.2
Methyl phenyllactate	180°	40	5.4
Methyl benzyllactate	180°	40	7.4
Phenylbutyrolactone	175°	60	17.0

[a] The injection port and detector temperatures were maintained at 210°.

After the administration of the compound, the rat was observed for 2 hr. in which four to seven urine samples were carefully collected at intervals of 10–30 min. After each passage of urine, the genitalia of the rat were washed with a stream of water from a wash bottle, and the rat was transferred to another urine-collection cage. The cage was thoroughly washed with water, and the urine and washings were brought up to a suitable volume with water. The subsequent urine samples collected over a period of 2–24 hr. were pooled into one sample prior to analysis. The urine samples were analyzed on the same day of collection for the compounds.

Analysis of the Compounds—The urine samples were quantitatively analyzed for intact compounds appearing in the urine by a gas chromatographic method described by Kamienny *et al.* (15) after converting the acids to their methyl esters upon treating with diazomethane. This procedure was employed for the analysis of all the compounds except 4-hydroxy-4-phenylbutanoic acid, which was converted to the corresponding lactone by the addition of enough 5 *N* hydrochloric acid solution to adjust the pH of the urine sample to approximately 2 prior to its ether extraction and gas chromatographic analysis. This was necessary because 4-hydroxy-4-phenylbutanoic acid readily forms a lactone in the strongly acidic medium, and the lactone thus formed can be quantitatively analyzed by gas chromatography. The lactone is readily reconverted to the 4-hydroxy-4-phenylbutanoate ion at pH 6 and above, which usually is the pH range of rat urine. The conditions employed for the gas chromatographic analysis and the retention times observed for the methyl esters of the compounds are described in Table I.

Dissociation Constants, Apparent Partition Coefficients, and Binding of Compounds to Whole Blood (Rat)—The dissociation constants of the compounds were determined using a titrimetric procedure. A 20-mg. quantity of each acid dissolved in 10 ml. of

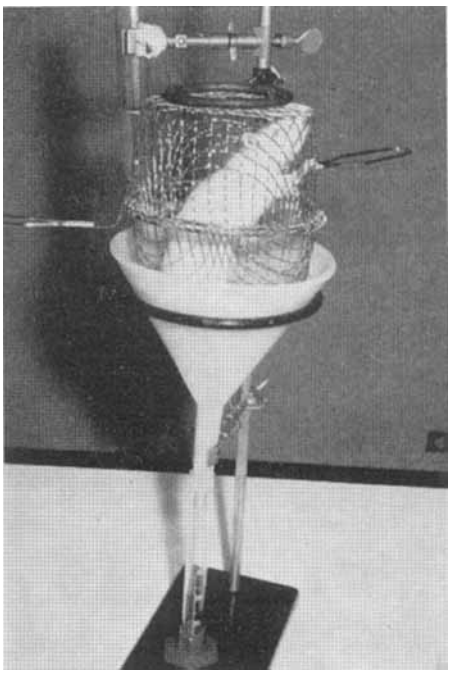


Figure 1—*Urine collection cage employed in the studies.*

Table II—Values of pKa and Partition Coefficient Determined for the Compounds

Compound[a]	pKa (Observed)	Apparent Partition Coefficient $C_{chloroform}/C_{water}$	Apparent Partition Coefficient C_{ether}/C_{water}
D-(−)-Mandelic acid	3.35 (3.36)[b]	[c]	[d]
DL-Tropic acid	4.20 (4.12)[b]	[c]	[d]
DL-Phenyllactic acid	3.80	[c]	[d]
D-(−)-Benzyllactic acid	3.85	<0.01	<0.01
D-(−)-4-Hydroxy-4-phenylbutanoic acid	4.70	0.32	0.35

[a] The assignment of the absolute configuration of the compounds was based on their optical rotatory dispersion curves as explained in the text. [b] Values reported in the literature. [c] The compound was absent in the chloroform layer. [d] The compound was absent in the ether layer.

water was titrated with 0.01 *N* sodium hydroxide solution; the pKa, which is equivalent to the pH at one-half neutralization, was determined.

An apparent partition coefficient was determined between a phosphate buffer at pH 7.0 and ether or chloroform representing the lipid phase. In each case, 5 mg. of the acid was dissolved in 10 ml. of the buffer and shaken with an equal volume of either chloroform or ether. Each phase was analyzed for acid content by the gas chromatographic procedure.

The studies to determine the binding of D-(−)-mandelic acid and each of its homologs by whole blood (rat) were carried out by the equilibrium dialysis procedure. Whole blood (rat) was obtained by decapitation, collecting the blood in a 150-ml. beaker previously coated with 2 ml. of heparin sodium.[3] Four milliliters of freshly collected whole blood was placed into a Visking dialysis bag, sealed, and placed into a 4–5-dr. (15–20 ml.) bottle with a screw cap. Then, 4.0 ml. of an isotonic phosphate buffered (pH 7.4) solution, containing 1.0 mg. of D-(−)-mandelic acid or its homolog, was placed into the bottle; the bottle was sealed and shaken at 37 ± 0.5° for 6 hr. during which the equilibrium for the system was attained. The bottles were removed from the shaker and immediately the liquid outside of the bag was transferred to a 10-ml. beaker and allowed to attain room temperature. These solutions were then analyzed for mandelic acid or its homolog by the gas chromatographic method. Control studies were carried out in which 4 ml. of the phosphate buffer solution was used in place of 4 ml. of blood. Neither D-(−)-mandelic acid nor any of its homologs used in the present study was found to bind to the dialysis membrane.

RESULTS AND DISCUSSION

Selection of a Model Compound and Its Homologs—Recent studies dealing with the transport of ionic and polar substances through biological membranes have pointed to the existence of a carrier system in the membrane which facilitates such transport. According to current theory, there exists in the membrane separate carrier systems responsible for the transport of organic anions and cations through the renal tubular membrane (2, 11, 16, 23, 24). Evidence for the presence of a common carrier system for the active transport of organic anions has been reported (1, 11). Although the carrier system has not yet been isolated, its constituents are suggested to be protein and/or phospholipid in nature. It is also proposed that while the primary linkage between the carrier and substrate compound to be transported occurs through ionic bonds, the secondary linkages result from hydrophobic bonding between the nonpolar portions of the carrier and substrate molecules or from hydrogen bonding between the appropriate portions of the substrate and carrier molecules (1–3). Such secondary bonding forces are expected to become important in relating the affinity of the molecules to the carrier. Therefore, it is considered of interest to determine the initial rate of secretion of compounds which vary slightly in their content of methylene groups around their anionic sites and to relate the differences in their initial secretion rates to the differences in their hydrophobicity in the molecule.

[3] Heparin sodium USP, 1000 units/ml., Testagar and Co., Inc., Detroit, Mich.

The selection of a model compound and its homologs was based on several important properties considered desirable for this study. Among these properties, the most important was that the compounds should be involved in the renal tubular secretory process and be recovered primarily in the urine in the intact form, since this will preclude the possible inhibitory effect of the metabolites on the secretion of the parent compound. The compounds should possess sufficiently low pKa values, so that they will remain in the urine mainly in the ionized form and their passive reabsorption from the renal distal tubule will be minimized. The partition coefficient of the compounds between a lipid phase and water (pH 6–8) should also be as low as possible to prevent their tubular reabsorption. The compounds should exhibit little or no tendency to bind to plasma proteins, since the urinary excretion rate depends on the concentration of the free form of the compound in the blood.

The literature survey indicated that D-(−)-mandelic acid (14, 15) is not metabolized significantly by humans and that DL-tropic acid (25) is not metabolized by rats. Therefore, appropriate preliminary studies carried out in rats indicated that D-(−)-mandelic acid and its homologs (listed in Table II) possess most of the properties described. The recovery of each compound in the unchanged form in the rat urine was found to be 90–100% of the administered i.v. dose. These compounds differ from mandelic acid by the addition of methylene groups in specified positions, in some cases (phenyllactic acid and benzyllactic acid) maintaining the hydroxyl group adjacent to the carboxyl groups as in the mandelic acid molecule, and in others (tropic acid and 4-hydroxy-4-phenylbutanoic acid) separating these groups by the additional methylene groups.

The overall metabolism studies of 3-hydroxy-3-phenylpropanoic, 5-hydroxy-5-phenylpentanoic, 6-hydroxy-6-phenylhexanoic, and 2-hydroxy-5-phenylpentanoic acids, carried out in this laboratory, indicated that these compounds are metabolized by rats to the extent of 30–100% of the administered dose. Therefore, these compounds were considered unsuitable for the proposed studies.

Determination of the Dissociation Constants, Apparent Partition Coefficients of the Compounds, and Their Binding to Whole Blood (Rat)—Values of pKa determined for the compounds used in

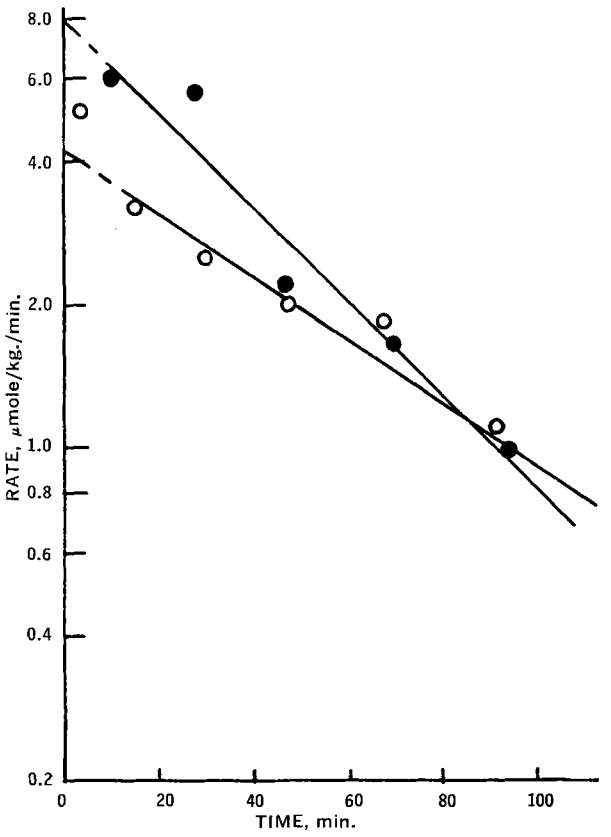


Figure 2—*Apparent first-order urinary excretion of* D-(−)-*mandelic acid following its i.v. administration of 367-μmole/kg. dose in the absence* (●) *and 346-μmole/kg. dose in the presence* (○) *of a 1.2 × 10⁴ μmole/kg. dose of* DL-*tropic acid to rats.*

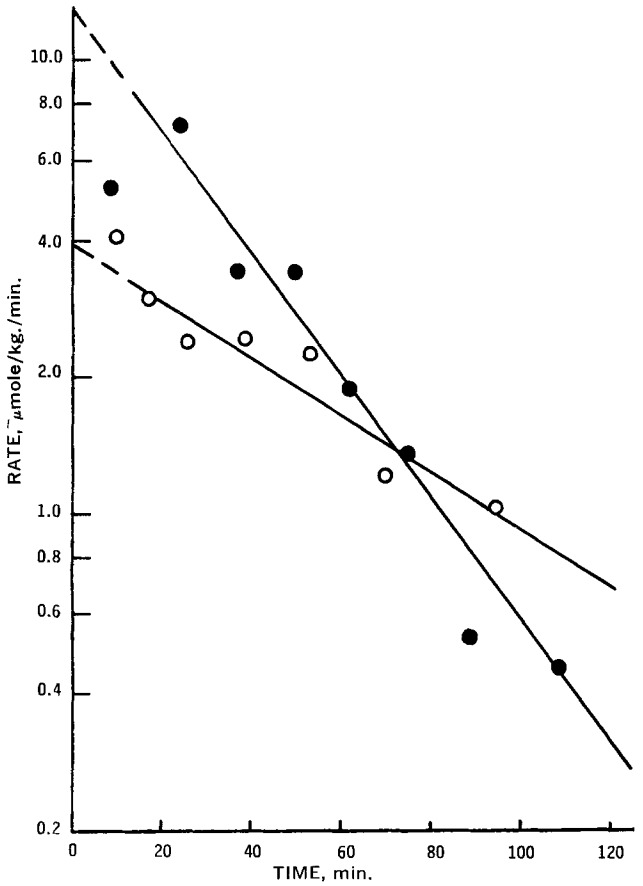


Figure 3—*Apparent first-order urinary excretion of* DL-*tropic acid following its i.v. administration of 410-μmole/kg. dose in the absence* (●) *and 344-μmole/kg. dose in the presence* (○) *of a 1.3 × 10⁴ μmole/kg. i.p. dose of* D-(−)-*mandelic acid to rats.*

the present studies are listed in Table II. These values are found to be in the range of 3.3 to 4.7. Since this range of pKa values is appreciably lower than the range of pH values (6.5–7.5) of the urine of the control rats used in all of the studies, it suggests that these compounds exist in the urine primarily in the ionized form and, therefore, are not likely to be significantly reabsorbed from the urine at the distal renal tubules. It is also evident from the results of the partition studies (Table II) that, except for the slight solubility of 4-hydroxy-4-phenylbutanoic acid, none of the compounds used in these studies is soluble in the lipidlike phase. Therefore, it may be assumed that none of these compounds is subject to significant renal tubular reabsorption from the urine at pH 6.5–7.5.

The results of the binding studies indicated that D-(−)-mandelic acid or any of its homologs used in the present studies is negligibly bound by the whole blood. This was deduced from the fact that, at equilibrium, the concentration of D-(−)-mandelic acid or its homolog in the solution outside of the dialysis bag containing the blood was the same as in the solution outside the dialysis bag of the control dialysis setup. The concentration of 1 mg. of D-(−)-mandelic acid or its homolog per dialysis setup used in the binding studies was based on the expected concentration of the acid in the volume of distribution of a rat weighing 200 g., following the intravenous administration of a 10-mg. dose of the acid. As suggested for humans (26), about 20% of the body weight was assumed to be the volume of distribution for rats. It was further assumed that the concentration of the compound in the blood and in the other possible volumes of distribution in rats is the same. The binding of D-(−)-mandelic acid by rat plasma in this concentration range has been studied and shown to be negligible (27).

Determination of the Biological Half-Life of the Compounds—It has been shown that, in humans, the metabolism and excretion of most drugs follow an apparent first-order process (28). Bray *et al.* (29) have demonstrated that the metabolism of benzaldehyde and toluene to benzoic acid and the urinary excretion of benzoic acid proceed by a pseudo-first-order process in rabbits. The urinary excretion of DL-tropic acid has also been shown to follow pseudo-first-order kinetics in rats (25). Although the urinary excretion of D-(−)-mandelic acid (14, 15) and L-(+)-mandelic acid (14) has been shown to occur by a pseudo-first-order process in humans, such studies have not been reported in rats for D-(−)-mandelic acid; D-(−)-4-hydroxy-4-phenylbutanoic acid; D-, L- or DL-phenyllactic acid; and D-(−)-benzyllactic acid. Therefore, it was necessary to determine if the excretion of D-(−)-mandelic acid and its homologs employed in the present studies follows pseudo-first-order kinetics. After establishing the proper procedures for rat urine collection and quantitative determination of these compounds excreted in the urine, the kinetics of urinary excretion of each compound were studied in rats. Preliminary data obtained in these studies indicated that the urinary excretion of these compounds occurred by a pseudo-first-order process.

Since the compounds used in the kinetic studies were those excreted unchanged in the urine of rats, the elimination of the compounds can be described by the following model:

$$A \xrightarrow{k} Ae$$

where A is the amount of intact compound in the body at any time, Ae is the amount of intact compound excreted in the urine at any time, and k is the apparent first-order rate constant of excretion of the compound. The urinary excretion data obtained for the compound can then be treated according to the following equation (15):

$$\log \frac{\Delta Ae}{\Delta t} = \log kA_0 - \frac{kt}{2.303} \qquad \text{(Eq. 1)}$$

where $\Delta Ae/\Delta t$ is the rate of excretion of the compound at time t, and A_0 is the amount of the compound at zero time.

The log $\Delta Ae/\Delta t$ is plotted against t; from the slope of the straight line, obtained by the method of least squares, the biological half-

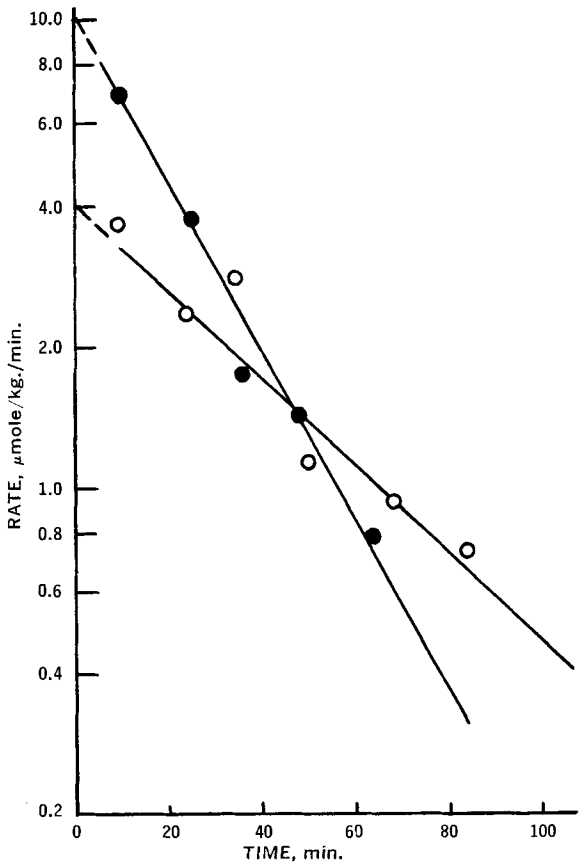


Figure 4—*Apparent first-order urinary excretion of* D-(−)-*4-hydroxy-4-phenylbutanoic acid following its i.v. administration of 277-μmole/kg. dose in the absence* (●) *and 277-μmole/kg. dose in the presence* (○) *of a 1.3 × 10⁴ μmole/kg. i.p. dose of* D-(−)-*mandelic acid to rats.*

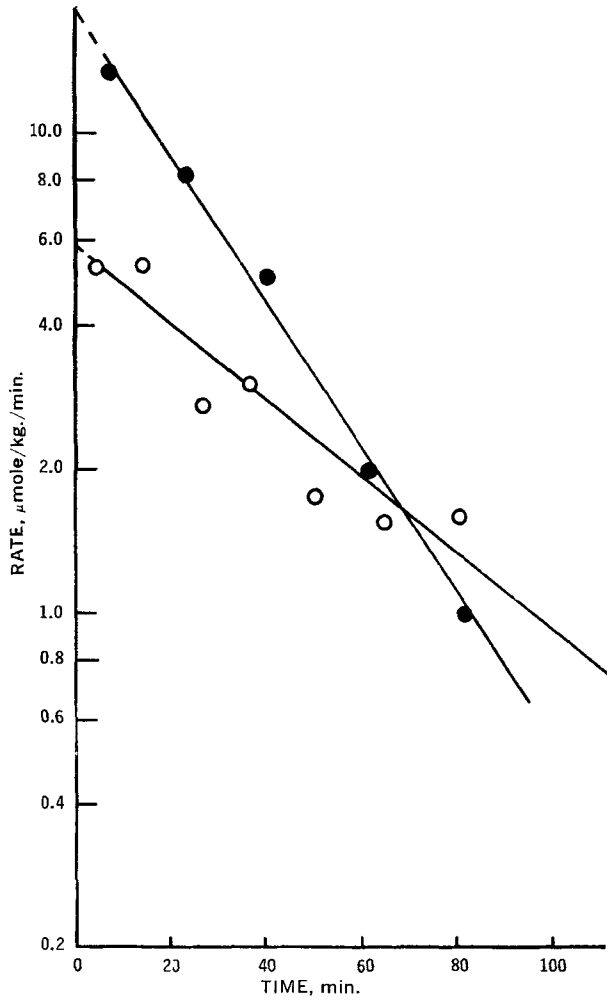


Figure 5—*Apparent first-order urinary excretion of* DL-*phenyllactic acid following its i.v. administration of 486-μmole/kg. dose in the absence* (●) *and 365-μmole/kg. dose in the presence* (○) *of a* 1.3×10^4 *μmole/kg. i.p. dose of* D-(−)-*mandelic acid to rats.*

life of the compound is calculated. The time, t, in such plots represents the midpoints of the urinary collection intervals.

One factor that should be considered in evaluating the excretion rate and biological half-life of compounds is the dose administered to the subject. This is important because the biological half-life of compounds involved in tubular secretion is expected to be longer at the dosage level that saturates the secretory process than at the dosage level that does not saturate this process. Therefore, for the determination of the biological half-life of a compound excreted by an apparent first-order process, its excretion should be studied following the administration of a dose that does not saturate the tubular secretory process. Consequently, the kinetics of urinary excretion of D-(−)-mandelic acid, DL-tropic acid and its optical isomers, D-(−)-4-hydroxy-4-phenylbutanoic acid, DL-phenyllactic acid and its optical isomers, and D-(−)-benzyllactic acid were studied following the intravenous administration of 150–700 μmole/kg. of each compound. The plots of log $\Delta Ae/\Delta t$ *versus* t for these studies were constructed, and from each straight-line plot the biological half-life of the corresponding compound was determined (Table III). The representative plots of log $\Delta Ae/\Delta t$ *versus* t obtained for each of these compounds are shown in Figs. 2–6.

It may be noted from Table III that the values of the biological half-life for DL-tropic acid and each of its optical isomers are similar to each other. Likewise, the values of the biological half-life obtained for DL-phenyllactic acid and each of its optical isomers are similar to each other. This indicated that the renal tubular transport of these compounds by the carrier mechanism is not stereospecific. Therefore, DL-tropic acid and DL-phenyllactic acid, instead of their individual optical isomers, were employed in the subsequent studies. The assignment of the absolute configuration of the compounds used in the study was based on their plain optical rotatory dispersion (ORD) curves as follows. Since the absolute configuration of (−)-mandelic acid is shown to be D-(−)-mandelic acid (30), the ORD curves for the homologs of mandelic acid were obtained over the wavelength range of 600–220 λ, and each curve was compared with that for D-(−)-mandelic acid. It was observed that, except for (−)-phenyllactic acid, the sign and shape of the ORD curves of the levorotatory homologs of mandelic acid were similar to that of D-(−)-mandelic acid. Thus, L-configuration was assigned to (−)-phenyllactic acid and D-configurations to the other levorotatory homologs of mandelic acid. This approach is in accordance with that described by Djerassi (31).

Among the compounds studied, the average biological half-life of D-(−)-mandelic acid is the longest (30 min.) and that of D-(−)-4-hydroxy-4-phenylbutanoic acid is the shortest (16 min.). The average half-life values for DL-phenyllactic acid and D-(−)-benzyllactic acid are not found to be significantly different from each other (20–21 min.). The average half-life for DL-tropic acid was found to be 23 min. (Table III). It is evident that the biological half-lives of the homologs of mandelic acid employed in these studies are significantly shorter than that of D-(−)-mandelic acid. Since the pKa values and the lipid solubility at the physiological pH determined for D-(−)-mandelic acid and its homologs are not significantly different, the differences observed in the values of their biological half-lives may be attributed to the increased hydrophobic interaction between the methylene group(s) of the mandelic acid homolog and a certain hydrophobic portion of the carrier for renal tubular secretion. Such hydrophobic interaction is likely to result in the increased affinity of the homolog for the carrier. In view of the fact that the initial excretion rate is composed of two terms, the initial glomerular filtration rate and the initial secretion rate, this interpretation of the biological half-life data would be acceptable

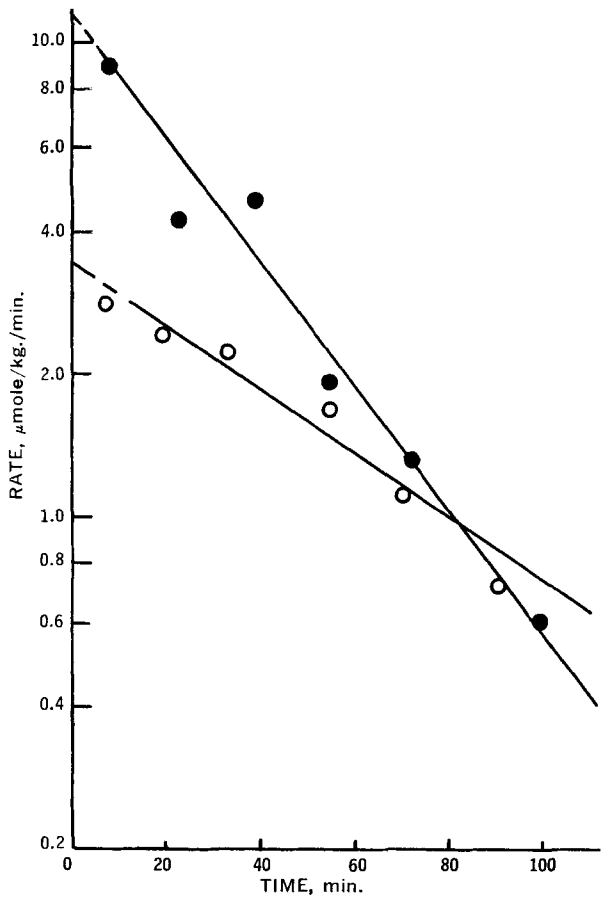


Figure 6—*Apparent first-order urinary excretion of* D-(−)-*benzyllactic acid following its i.v. administration of 346-μmole/kg. dose in the absence* (●) *and 298-μmole/kg. dose in the presence* (○) *of a* 1.3×10^4 *μmole/kg. dose of* D-(−)-*mandelic acid to rats.*

Table III—Summary of the Biological Half-Lives Observed in Rats for D-(−)-Mandelic Acid and Its Homologs[a] in the Absence and Presence of Inhibitor

Compound (Substrate) R = 		Biological Half-Life, min.			
			Inhibitor Present		
		Inhibitor Absent[b]	Equivalent[c] i.v. Dose	$10–15 \times 10^3$ μmole/kg. i.p. Dose	Inhibitor
D-(−)-Mandelic acid	R—CH(OH)—COOH	30 ± 4[d]	30 ± 4[d,e]	46 ± 8	DL-Tropic acid
DL-Tropic acid D-(−)-Tropic acid L-(+)-Tropic acid	$CH_2(OH)$ R—CH—COOH	23 ± 4 24 (2)[f] 25 (4)	28 ± 5	50 ± 10	D-(−)-Mandelic acid
DL-Phenyllactic acid L-(−)-Phenyllactic acid D-(+)-Phenyllactic acid	R—CH_2—CH(OH)—COOH	20 ± 3 21 (2) 20 (2)	26 ± 4	42 ± 5	D-(−)-Mandelic acid
D-(−)-Benzyllactic acid	R—$(CH)_2$—CH(OH)—COOH	21 ± 3	18 ± 2	36 ± 4	D-(−)-Mandelic acid
D-(−)-Hydroxy-4-phenylbutanoic acid	R—CH(OH)—$(CH_2)_2$—COOH	16 ± 2	22 ± 5	26 ± 5	D-(−)-Mandelic acid

[a] I.v. dosage range 150–700 μmole/kg. [b] The biological half-lives of the homologs of D-(−)-mandelic acid are significantly ($p<0.01$) shorter than that of D-(−)-mandelic acid. The biological half-life of D-(−)-4-hydroxy-4-phenylbutanoic acid is significantly shorter than that of DL-tropic acid ($p < 0.01$), DL-phenyllactic acid, and D-(−)-benzyllactic acid ($p < 0.05$). The biological half-lives of DL-phenyllactic acid and D-(−)-benzyllactic acid are not significantly shorter than that of DL-tropic acid. [c] The dose of inhibitor equivalent to the i.v. dose of the substrate compound. [d] Standard deviation was determined from six to eight rat studies. [e] The biological half-life of D-(−)-mandelic acid varied from 30 ± 3 to 33 ± 3 min. in the presence of equivalent i.v. doses of the other homologs of D-(−)-mandelic acid as inhibitors. [f] The values of average biological half-life of the individual optical isomers of tropic acid and phenyllactic acid based on the number of studies indicated in parentheses.

if the initial glomerular filtration rates of D-(−)-mandelic acid and its homologs are shown to be similar at the equimolar dosage levels. The data in support of this interpretation are presented in the subsequent discussion as well as in the next paper of this series (17).

Determination of the Apparent Initial Rate of Excretion—As in the case of biological half-life, a comparison was considered desirable between the apparent initial urinary excretion rates observed for compounds employed in the studies in terms of μmole/kg./min. However, it was realized that, since it is difficult to obtain urine from rats at such short intervals as 1 min. following the intravenous administration of the compound, the determination of the apparent excretion rate of substrate in terms of μmole/kg./min. was not feasible. Therefore, it was decided to determine the apparent initial urinary excretion rate in the above terms from an extrapolation of the straight-line plot of log $\Delta Ae/\Delta t$ *versus* time to zero time. Actually the initial urinary excretion rate should be obtained by an extrapolation of the straight line to the time of 0.5 min., because this would represent the midpoint of the urine collection interval. But this value of the excretion rate was very close to that obtained by an extrapolation to zero time, and the value of the intercept determined upon extrapolation of the straight-line plot to zero time was designated as the apparent initial urinary excretion rate. The apparent initial excretion rates determined in this manner for these compounds are listed in Table IV. It is noted that, in this dosage range, the apparent initial excretion rate of D-(−)-mandelic acid is considerably lower than the apparent initial excretion rate of any of its homologs used in this study. However, the differences in the apparent initial excretion rates of the homologs of D-(−)-mandelic acid are not readily seen in this dosage range (300–400 μmole/kg.) of the compounds.

Mutual Inhibitory Urinary Excretion Studies—To determine if the compounds under study are secreted by the same renal tubular transport mechanism, mutual inhibitory urinary excretion studies were carried out for DL-tropic acid, D-(−)-4-hydroxy-4-phenylbutanoic acid, DL-phenyllactic acid, and D-(−)-benzyllactic acid in the presence of D-(−)-mandelic acid. An isotonic solution containing a dose of 330–500 μmole/kg. of an appropriate homolog of mandelic acid and an equivalent amount of D-(−)-mandelic acid was administered intravenously to the rat; from the apparent first-order urinary excretion data obtained in each study, the biological half-lives of each homolog of mandelic acid and that of D-(−)-mandelic acid were determined (Table III). It is observed

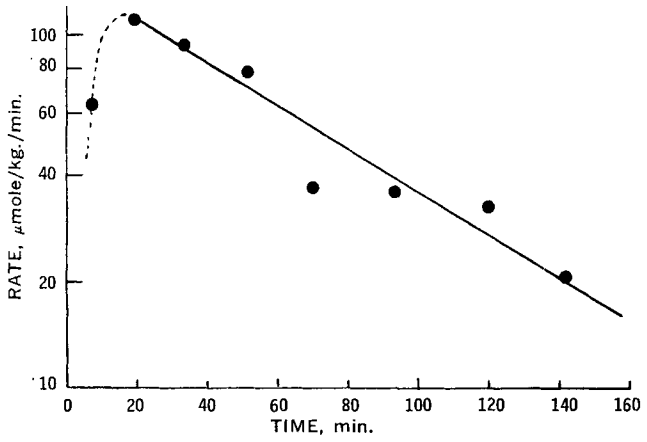


Figure 7—*Urinary excretion of* D-(−)-*mandelic acid observed following its i.p. administration of 1.0* × 10^4 *μmole/kg. dose to rats, indicating that the maximum absorption of the compound occurred in 20 min.*

Table IV—Comparison of Apparent Initial Urinary Excretion Rates Obtained in Rats for D-(−)-Mandelic Acid and Its Homologs[a] in the Absence and Presence of Inhibitor

Compound (Substrate)	Initial Excretion Rate, μmole/kg./min.		
	Inhibitor Absent	Inhibitor Present[b] (i.v. Equivalent[c] Dose per Rat)	Inhibitor Present[b] (i.p. 10–15 × 10^3 μmole/kg.)
D-(−)-Mandelic acid	5.0–8.5	6.1–7.9	3.8–5.8
DL-Tropic acid	11.8–16.4	6.9–9.0	3.5–4.3
DL-Phenyllactic acid	9.2–13.5	7.3–10.7	4.7–5.6
D-(−)-Benzyllactic acid	8.7–15.2	11.9–14.3	3.5–4.5
D-(−)-4-Hydroxy-4-phenylbutanoic acid	10.6–11.9	6.6–11.4	3.9–5.5

[a] I.v. dosage range of 300–400 μmole/kg. [b] DL-Tropic acid was employed as the inhibitor for D-(−)-mandelic acid, and D-(−)-mandelic acid was employed as the inhibitor for the remainder of the compounds. [c] Dose equivalent to that of the i.v. dose of the substrate compound.

from these data that, while the biological half-life of D-(−)-mandelic acid remained unchanged in the presence of each of its homologs, the biological half-life of each homolog, with the exception of D-(−)-benzyllactic acid, increased slightly in the presence of D-(−)-mandelic acid. Consequently, it was expected that there should be a corresponding slight decrease in the apparent initial rate of excretion of each homolog in the presence of D-(−)-mandelic acid. But because of the unavailability of data regarding the initial excretion rate of the compounds at identical doses (μmole/kg.), such a comparison was not possible. However, an attempt is made to compare the range of apparent initial excretion rates observed for the compounds over a dosage range of 300–400 μmole/kg. (Table IV). It can be seen from Table IV that, although there is a slight decrease in the apparent initial rate of excretion of DL-tropic acid and DL-phenyllactic acid, such a decrease in the rate is not observed for D-(−)-4-hydroxy-4-phenylbutanoic acid. Therefore, studies were carried out to determine the effect of higher doses of D-(−)-mandelic acid on the biological half-life and the apparent initial rate of excretion of each of its homologs following the administration of 300–400 μmole/kg. The dose of D-(−)-mandelic acid employed as an inhibitor in the studies was 1.3×10^4 μmole/kg., and it was administered intraperitoneally 15–20 min. prior to the intravenous administration of the substrate homolog. To determine the effect of a large dose of one of the homologs on the biological half-life of D-(−)-mandelic acid, similar studies were performed using DL-tropic acid as the inhibitor. The dose of DL-tropic acid used was 1.5×10^4 μmole/kg. and that of D-(−)-mandelic acid was 330 μmole/kg. The biological half-lives and apparent initial excretion rates determined for these compounds from the apparent first-order urinary excretion data are presented in Tables I and IV, respectively. The typical apparent first-order plots obtained for the compounds are shown in Figs. 2–6. The intraperitoneal administration of the high doses of DL-tropic acid and D-(−)-mandelic acid used as the inhibitor did not produce apparent toxic effects in rats. The rats behaved normally during the experiments as well as a week later when reused in the studies.

The pH of the urine of the rats following the administration of large doses of the inhibitor compounds was found to be in the range of 6.5 to 7.5, which was comparable to the urine pH of the control rats. The 15–20-min. interval between the administration of the inhibitor and the substrate was allowed to permit maximum absorption of the inhibitor, as shown by the studies on the urinary excretion of D-(−)-mandelic acid and DL-tropic acid after their intraperitoneal administration of $1.0–1.5 \times 10^4$ μmole/kg. (Fig. 7).

It is evident from the data presented in Tables I and IV that in the presence of a large dose of the inhibitor, there is a significant increase in the biological half-life and a corresponding decrease in the apparent initial excretion rate of each of the compounds employed in these studies. Therefore, it may be concluded that D-(−)-mandelic acid and its homologs, DL-tropic acid, D-(−)-4-hydroxy-4-phenylbutanoic acid, DL-phenyllactic acid, and D-(−)-benzyllactic acid, are involved in kidney tubular secretion and that they most probably share the same transport system for their secretion.

SUMMARY AND CONCLUSIONS

Studies were carried out in rats to gain some insight into the structural and chemical specificity that the "carrier" molecules of renal tubular transport show toward anionic drugs. D-(−)-Mandelic acid and certain of its homologs, DL-tropic acid, DL-phenyllactic acid, D-(−)-benzyllactic acid, and D-(−)-4-hydroxy-4-phenylbutanoic acid, were selected as the model compounds since they exhibit negligible metabolism and protein binding, low pKa values, and low lipid solubility at the physiological pH, and are primarily recovered in the urine in the unchanged form.

From the apparent first-order urinary excretion studies of these compounds, it was found that the biological half-life of each homolog is significantly shorter than that of D-(−)-mandelic acid in rats.

These compounds are shown to exert a mutual inhibitory effect on their renal tubular secretion in rats, indicating that they share the same carrier mechanism for their secretion.

The utilization of these compounds has demonstrated that the addition of methylene group(s) in the vicinity of the carboxyl group of the mandelic acid molecule increases its affinity for the carrier molecules of the renal transport system in rats.

REFERENCES

(1) A. Despopoulos, *J. Theor. Biol.*, **8,** 163(1965).

(2) I. M. Weiner and G. H. Mudge, *Amer. J. Med.*, **36,** 743 (1964).

(3) A. Essing and J. V. Taggert, *Amer. J. Physiol.*, **199,** 509 (1960).

(4) H. W. Smith, A. L. Finkelstein, B. Crawford, and N. Graber, *J. Clin. Invest.*, **24,** 388(1945).

(5) A. B. Gutman, P. G. Dayton, T. F. Yu, L. Barger, W. Chen, L. E. Secan, and J. J. Burn, *Amer. J. Med.*, **29,** 1017(1960).

(6) K. C. Huang, K. B. Moore, and P. C. Campbell, *Amer. J. Physiol.*, **199,** 5(1960).

(7) A. Essing, *ibid.*, **201,** 303(1961).

(8) S. H. Fisher, L. Troast, A. Waterhouse, and J. A. Shannon, *J. Pharmacol. Exp. Ther.*, **79,** 373(1943).

(9) A. Despopoulos, L. H. Pendergrass, and J. M. Stoeckinger, *Amer. J. Physiol.*, **205,** 489(1963).

(10) A. Despopoulos and P. X. Callahan, *ibid.*, **203,** 19(1962).

(11) I. M. Weiner, K. C. Blanchard, and G. H. Mudge, *ibid.*, **207,** 953(1964).

(12) C. H. Rammelkamp and S. E. Bradley, *Proc. Soc. Exp. Biol. Med.*, **53,** 30(1947).

(13) K. H. Beyer, A. K. Miller, H. F. Russo, E. A. Patch, and W. F. Verway, *Amer. J. Physiol.*, **149,** 355(1947).

(14) J. B. Nagwekar and H. B. Kostenbauder, to be published.

(15) F. M. Kamienny, M. Barr, and J. B. Nagwekar, *J. Pharm. Sci.*, **58,** 1318(1969).

(16) K. H. Beyer, *Pharmacol. Rev.*, **2,** 227(1950).

(17) E. J. Randinitis, M. Barr, and J. B. Nagwekar, *J. Pharm. Sci.*, **59,** 813(1970).

(18) H. King, *J. Chem. Soc.*, **1919,** 115, 476.

(19) S. Eiduson, M. N. Camien, and M. S. Dunn, *Arch. Biochem.*, **29,** 302(1950).

(20) D. Biquard, *Ann. Chim.*, **20,** 132(1933).

(21) H. Thierfelder and E. Schempp, *Hoppe Seyler's Z. Physiol. Chem.*, **114,** 94(1921).

(22) T. Haga, *Nippon Kagaku Zasshi*, **81,** 277(1960).

(23) W. Wilbrandt and T. Rosenberg, *Pharmacol. Rev.*, **13,** 109(1961).

(24) L. Peters, *ibid.*, **12,** 1(1960).

(25) R. E. Gosselin, J. D. Gobocerel, S. C. Kalser, and J. H. Wills, *J. Pharmacol. Exp. Ther.*, **115,** 217(1955).

(26) J. V. Swintosky, E. L. Foltz, A. Bondi, and M. J. Robinson, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 136(1958).

(27) J. Baldridge and J. B. Nagwekar, unpublished data.

(28) E. Nelson, *J. Pharm. Sci.*, **50,** 181(1961).

(29) H. G. Bray, W. V. Thorpe, and K. White, *Biochem. J.*, **61,** 4(1955).

(30) K. Mislow, *J. Amer. Chem. Soc.*, **73,** 3954(1951).

(31) C. Djerassi, "Optical Rotatory Dispersion, Applications to Organic Chemistry," McGraw-Hill, New York, N. Y., 1960, p. 236.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *College of Pharmacy, Wayne State University, Detroit, MI 48202*

Accepted for publication February 4, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Abstracted in part from a dissertation submitted by E. J. Randinitis to the Graduate School, Wayne State University, in partial fulfillment of the Doctor of Philosophy degree requirements.

The authors gratefully acknowledge the assistance of Prof. Stanley Kirshner and Mr. Christy Munir, Chemistry Department, Wayne State University, for obtaining the ORD curves of the compounds used in the study.

* Present address: Product Development Research Laboratories, Parke, Davis & Co., Detroit, MI 48232; American Foundation for Pharmaceutical Education Fellow, 1967–1968.

† To whom all inquiries should be directed.

Kinetics of Urinary Excretion of D-(−)-Mandelic Acid and Its Homologs II: Competitive Inhibitory Effect of D-(−)-Mandelic Acid and DL-Tropic Acid on Their Renal Tubular Secretion in Rats

EDWARD J. RANDINITIS*, MARTIN BARR, and JANARDAN B. NAGWEKAR†

Abstract □ From the pseudo-first-order urinary excretion studies of D-(−)-mandelic acid and DL-tropic acid in rats, data regarding the apparent initial secretion rate *versus* dose (i.v.) are obtained for these compounds and treated according to the Michaelis-Menten kinetics. While the values of maximum apparent initial secretion rate (*Vm*) determined for these compounds are similar, the value of the dose of the substrate required to produce one-half of the maximum apparent initial secretion rate (*Km*) determined for D-(−)-mandelic acid is found to be about twice that determined for DL-tropic acid. Data have been obtained to demonstrate that these compounds competitively inhibit the renal tubular secretion of each other, thereby strongly indicating that these compounds share a common carrier transport system for their secretion. Data obtained in the present studies and those described earlier are utilized to distinguish a certain structural characteristic around the cationic site of the "carrier" molecules of the renal tubular secretion system in rats.

Keyphrases □ D-(−)-Mandelic acid, homologs—urinary excretion □ DL-Tropic acid, D-(−)-mandelic acid—competitive inhibition, urinary excretion □ Structure–excretion rate relationship—D-(−)-mandelic, DL-tropic acids □ Transport system—D-(−)-mandelic acid, DL-tropic acid excretion

The authors (1) have shown that the biological half-lives of DL-tropic acid, DL-phenyllactic acid, D-(−)-benzyllactic acid, and D-(−)-4-hydroxy-4-phenylbutanoic acid, which are homologs of mandelic acid, increased in the presence of D-(−)-mandelic acid in rats. The corresponding decrease in the apparent initial urinary excretion rates of these homologs was also shown to occur in rats in the presence of D-(−)-mandelic acid. The influence of DL-tropic acid on the urinary excretion rate of D-(−)-mandelic acid was also studied. It was demonstrated that the biological half-life of D-(−)-mandelic acid increased and its initial excretion rate decreased in the presence of DL-tropic acid. It was, therefore, suggested that D-(−)-mandelic acid and its previously mentioned homologs share the same transport mechanism for their renal tubular secretion in rats. Since these compounds, which are not significantly metabolized nor bound to the plasma proteins and are excreted entirely in the urine, were found to have different biological half-lives and, at a specific dose, different initial excretion rates, it was further suggested that the common carrier system for renal tubular secretion in rats shows different affinity for these compounds.

Because it is well known that the urinary excretion of most compounds results from a combination of glomerular filtration and tubular secretion, it is conceivable that the compounds used in these studies probably have the same maximum initial secretion rate and initial glomerular filtration rate, if the assumption is correct that these compounds are secreted by the renal tubules of rats by the same carrier mechanism. Therefore, the purposes of the work presented here were to determine the apparent initial glomerular filtration rates and the apparent initial secretion rates of DL-tropic acid and D-(−)-mandelic acid in rats and to analyze the secretion rate data according to Michaelis-Menten kinetics. The additional purpose of the work was to determine if the mutual inhibition caused by these compounds in their renal secretion in rats is competitive or noncompetitive in nature. Since both D-(−)-tropic acid and L-(+)-tropic acid are completely recovered unchanged in the urine of rats and, furthermore, since the biological half-lives of these isomers are found to be similar to each other (1), DL-tropic acid was not resolved into its optical isomers but was used in the racemic form.

EXPERIMENTAL

Materials—D-(−)-Mandelic acid,[1] m.p. 132–133°, $[\alpha]_D^{25}$ −154.2° (c, 1.94, H_2O); and DL-tropic acid,[1] m.p. 118–119°.

Apparatus—The quantitative analysis of mandelic acid and tropic acid appearing in the urine was carried out using an F & M model 810R-19 gas chromatograph by the method described previously (1). A Beckman model 72 pH meter equipped with a combination electrode was used for rat urine pH determinations. The specific rotation of the optically active compound was determined with the aid of J & J Fric model 2706 polarimeter using a sodium lamp as the source of light.

Methodology—The procedure employed for the preparation of the rats and urine collection following the i.v. administration of the compounds was the same as described previously (1). During the course of these studies, approximately 35 Sprague-Dawley male rats weighing between 165 and 215 g. were repeatedly used. None of the rats was used more than five times in these studies; the rest period allowed between the successive use of a rat was at least a week.

To determine the apparent glomerular filtration rate of D-(−)-mandelic acid at dosage levels of 165–330 μmole/kg. (5–10 mg./rat), the appropriate amount of the sodium salt of D-(−)-mandelic acid contained in 0.5 ml. was injected by the i.v. route 15–20 min. after i.p. administration of 15–120 × 10^2 μmole/kg. (50–400 mg./rat) of DL-tropic acid as the sodium salt contained in 5 ml. of water at a pH of 7.0. A similar procedure was followed to determine the apparent glomerular filtration rate of DL-tropic acid at the dosage levels of 150–300 μmole/kg. in the presence of 16–130 × 10^2 μmole/kg. of i.p. administered D-(−)-mandelic acid per rat.

In the studies designed to determine the maximum initial secretion rate (*Vm*) of D-(−)-mandelic acid and its dose required to produce one-half of the maximum initial secretion rate (*Km*), the doses of the sodium salt of D-(−)-mandelic acid in the range of 1.8–21.9 × 10^2 μmole/kg. were dissolved in water (0.5–1.5 ml.) and administered to the rat by the intravenous route. Similar studies were also carried out for DL-tropic acid using the doses of its sodium salt in the range of 1.7–20.0 × 10^2 μmole/kg.

To determine the nature of inhibition of renal tubular secretion of D-(−)-mandelic acid by DL-tropic acid, the initial secretion rates of

[1] Aldrich Chemical Co., Milwaukee, WI 53210

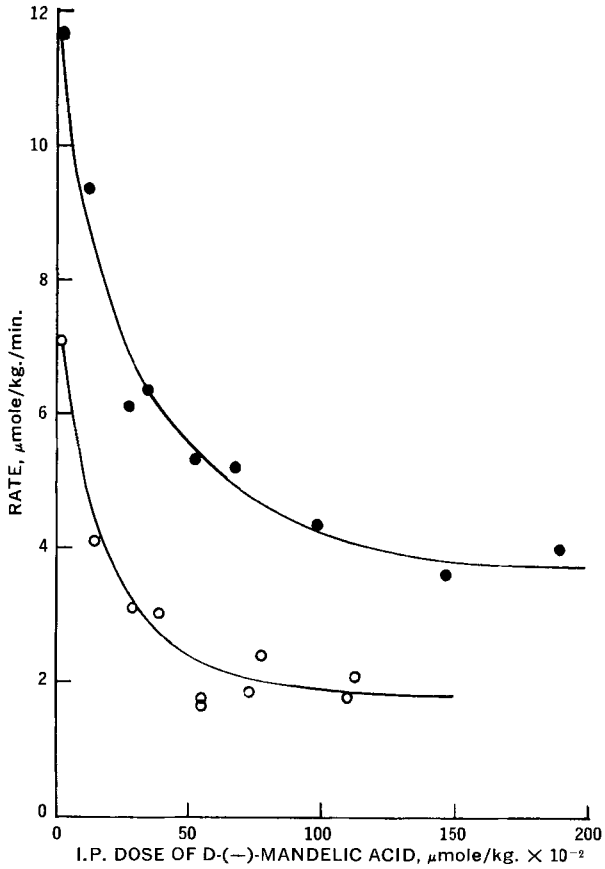


Figure 1—*Determination of the approximate i.p. dose of* D-(−)-*mandelic acid required to obtain a limiting initial excretion rate of* DL-*tropic acid following the administration to rats of its i.v. dose of 170–180 μmole/kg., ○; and 340–350 μmole/kg., ●.*

D-(−)-mandelic acid in the i.v. dosage range of 1.6–8.6 × 10^2 μmole/kg. were determined in the presence of an i.v. dose of 21–26 × 10^2 μmole/kg. (80 mg./rat) DL-tropic acid. Similarly, initial secretion rates for DL-tropic acid in the i.v. dosage range of 4.5–27.4 × 10^2 μmole/kg. were determined in the presence of i.v. doses of 6.0–

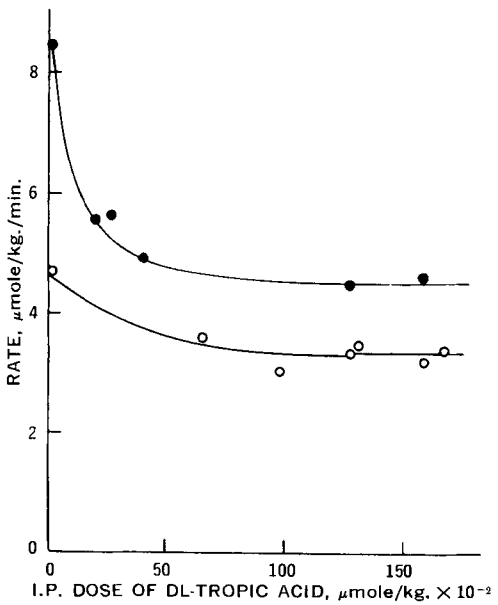


Figure 2—*Determination of the approximate i.p. dose of* DL-*tropic acid required to obtain a limiting initial excretion rate of* D-(−)-*mandelic acid following the administration to rats of its i.v. dose of 170–180 μmole/kg., ○; and 340–350 μmole/kg., ●.*

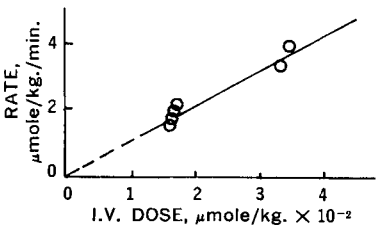


Figure 3—*Relationship between the apparent initial glomerular filtration rate and the i.v. dose for* DL-*tropic acid in rats.*

7.2 × 10^2 μmole/kg. (20 mg./rat), 12–14 × 10^2 μmole/kg. (40 mg./rat), and 24–28 × 10^2 μmole/kg. (80 mg./rat) of D-(−)-mandelic acid. In these studies, DL-tropic acid and D-(−)-mandelic acid were administered by the i.v. route as their sodium salts, and both the substrate and inhibitor compounds were contained in the same i.v. solution.

RESULTS AND DISCUSSION

Determination of the Initial Glomerular Filtration Rate of the Compounds—As mentioned previously, the urinary excretion of most compounds results from a combination of glomerular filtration and renal tubular secretion. Since renal tubular secretion is a saturable process, the reduction can be caused in the excretion rate of a substrate in the presence of an inhibitor that competes for the secretory process. Therefore, it should be possible to inhibit completely the renal tubular secretion of a substrate compound in rats by a simultaneous administration of a large excess of a suitable inhibitor compound. In this event, the initial rate of excretion of the substrate would represent its initial rate of glomerular filtration. It is thus possible to determine the initial rate of glomerular filtration and the initial rate of secretion of a compound at a particular dosage level. Consequently, the initial excretion rates of D-(−)-mandelic acid following i.v. doses of 165–330 μmole/kg. were determined in the presence of increasingly higher doses of DL-tropic acid. The dose of DL-tropic acid, in the range of 12–150 × 10^2 μmole/kg., was administered i.p. about 20 min. prior to the i.v. administration of D-(−)-mandelic acid. In a similar manner, the initial excretion rates of DL-tropic acid following its i.v. doses of 150–300 μmole/kg. were determined in the presence of increasingly higher doses of D-(−)-mandelic acid. The dose of D-(−)-mandelic acid, in the range of 16–130 × 10^2 μmole/kg., was administered i.p. about 20 min. prior to the i.v. administration of DL-tropic acid.

It may be estimated from the uninary excretion data obtained following i.p. administration of 1 × 10^4 μmo'e/kg. of D-(−)-mandelic acid (Fig. 7, *Reference 1*) that, at the 1.65 × 10^4 μmole/kg. dosage level of D-(−)-mandelic acid, the amount of mandelic acid present in the volume of distribution of the rat is approximately 30 times greater than that at the 300 μmole/kg. dosage level of DL-tropic acid and approximately 60 times greater than that at the 150 μmole/kg. dosage level of the acid. At similar large i.p. doses of DL-tropic acid used as the inhibitor of D-(−)-mandelic acid administered at the dosage levels of 165–330 μmole/kg., the amount of DL-tropic acid present in the volume of distribution of the rat would be expected to be in the range described for D-(−)-mandelic acid as the inhibitor. As described previously (1), the apparent initial urinary excretion rates of the compounds were determined by extrapolating the pseudo-first-order plots to zero time. The apparent initial excretion rates obtained from these studies were then plotted against the i.p. dose of the inhibitor as shown in Figs. 1 and 2. It is noted from these plots that an apparently limiting initial excretion rate for each substrate compound is obtained in rats in the presence of 0.9–1.0 × 10^4 μmole/kg. i.p. dose of inhibitor. Since a further decrease in the initial excretion rate was not observed even at higher inhibitor dosage levels, it was evident that at this i.p. dose of inhibitor an apparent saturation of tubular secretion process occurred and the initial excretion rate observed for the substrate was due to the apparent initial glomerular filtration rate. A reasonable linear relationship observed between the apparent initial glomerular filtration rate and the i.v. dose of DL-tropic acid is shown in Fig. 3. It may be seen from the data presented in Fig. 2 that a limiting initial excretion rate (3.4 μmole/kg./min.) obtained for D-(−)-mandelic acid at the dose of 165 μmole/kg. is substantially higher than that expected on the basis of the limiting initial excretion rate (4.5 μmole/kg./min.) obtained at the dose of 330 μmole/kg. Therefore, the initial excretion rate of D-(−)-mandelic acid at the dosage level of 180 μmole/kg. was determined in rats in the presence of a simultaneously administered 3

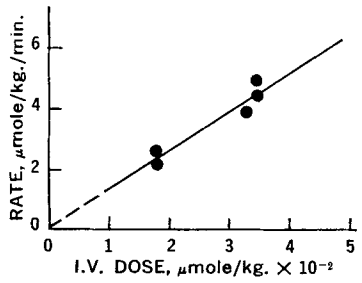


Figure 4—*Relationship between the apparent initial glomerular filtration rate and the i.v. dose for* D-(−)-*mandelic acid in rats.*

$\times 10^3$ μmole/kg. i.v. dose of DL-tropic acid. Both D-(−)-mandelic acid and DL-tropic acid were administered as their sodium salts. The initial excretion rate (2.4 μmole/kg./min.) noted for D-(−)-mandelic acid in this study was of the magnitude expected on the basis of the limiting initial excretion rate obtained at the dose of 330 μmole/kg. Therefore, 2.2 μmole/kg./min. was considered as the apparent glomerular filtration rate at the dose of 165 μmole/kg. of the acid instead of the limiting initial excretion rate noted in Fig. 2. Thus, when these apparent glomerular excretion rates were plotted against the i.v. doses of D-(−)-mandelic acid, a reasonable linear relationship was observed (Fig. 4). These apparent initial glomerular filtration rates observed for D-(−)-mandelic acid are found to be in agreement with those obtained by Khambati and Nagwekar (2) for the acid in the presence of the simultaneously administered 3×10^3 μmole/kg. i.v. dose of DL-tropic acid in rats. Khambati and Nagwekar determined the initial glomerular filtration rates of D-(−)-mandelic acid in the course of determining those for D-(−)-*p*-isopropyl mandelic acid. It seems that the limiting initial excretion rate obtained for D-(−)-mandelic acid at the 165-μmole/kg. dose in Fig. 2 was an experimental artifact. Therefore, the use of the value of the apparent glomerular filtration rate (2.2 μmole/kg./min.) obtained for the acid at the i.v. dosage level of 165 μmole/kg. in the presence of the simultaneously administered 3×10^3 μmole/kg. i.v. dose of DL-tropic acid may be justified in constructing the plot in Fig. 4. It is then theoretically possible to estimate the apparent initial glomerular filtration rate of these acids at higher i.v. doses by the extrapolation of the straight lines shown in Figs. 3 and 4.

Incidentally, since D-(−)-mandelic acid is able to saturate the tubular secretory system upon the administration of an i.p. dose of 1.3×10^4 μmole/kg. in a rat, initial excretion rates reported previously (1) for D-(−)-4-hydroxy-4-phenylbutanoic acid, DL-phenyllactic acid, and D-(−)-benzyllactic acid in the presence of an i.p. dose of 1.3×10^4 μmole/kg. of D-(−)-mandelic acid represent their respective apparent initial glomerular filtration rates, as evidenced by a linear relationship observed in Fig. 5 upon plotting these rates against their i.v. doses. This supports the observation that an i.p. dose of 1.3×10^4 μmole/kg. of D-(−)-mandelic acid serves as an inhibitor of secretion of its homologs and, likewise, an i.p. dose of 1.2×10^4 μmole/kg. of DL-tropic acid serves as an inhibitor of secretion of D-(−)-mandelic acid. The data presented in Fig. 5 also indicate that, in rats, the volume of distribution for all of the compounds used in the study is similar.

Determination of Michaelis-Menten Kinetic Parameters—Since renal tubular secretion represents a saturable system, the transport of molecules through the renal tubular membrane has been described by Michaelis-Menten kinetics (3), and the nature of inhibition of this transport by other compounds has been identified utilizing a Lineweaver-Burk plot technique (4). As in the study of enzyme kinetics, the maximum rate of tubular secretion or transport is generally designated as *Vm* or *Tm*, and the concentration of substrate required to produce one-half of the maximum rate of secretion as *Km*. It is generally expected that for compounds of a homologous series, which are secreted by the same carrier mechanism, the value of *Vm* is the same, but, due to their differences in affinities for the carrier, the value of *Km* for each compound is different. Homologs with a greater affinity for the carrier would be expected to possess a lower value of *Km* (3). In an *in vitro* study of inhibition of uptake of *N'*-methylnicotinamide by kidney slices, Farah *et al.* (4) have applied the Lineweaver-Burk plot technique to demonstrate that organic bases, such as tetraethylammonium, choline, and guanidine, competitively inhibit the uptake of the compound. Cho *et al.* (5) studied the tubular secretion of iodopyracet[2] and *p*-aminohippurate by the renal clearance method in dogs and showed that these compounds competitively inhibit the secretion of each other. To determine the tubular secretion rate, these workers assumed the values of filterability of *p*-aminohippurate and iodopyracet to be 0.92 and 1.00, respectively. The *Vm* value obtained for iodopyracet was 1.6 μmole/kg./min. and that determined for *p*-aminohippurate was 5.0 μmole/kg./min.

[2] Diodrast, Winthrop Laboratories, New York, NY 10016

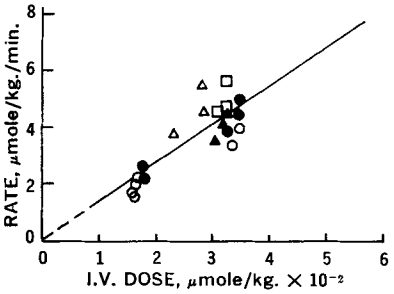


Figure 5—*A plot of the limiting initial excretion rates obtained in rats for* D-(−)-*mandelic acid,* ●; DL-*tropic acid,* ○; D-(−)-4-*hydroxy-4-phenylbutanoic acid,* △; DL-*phenyllactic acid,* □; *and* D-(−)-*benzyllactic acid,* ▲; *following their i.v. administration in the presence of 1.0–1.5* $\times 10^4$ *μmole/kg. of inhibitor. (These data are obtained from Table IV of* Reference 1.)

As applied to secretion kinetics of the present study, the various terms appearing in the following Michaelis-Menten equation (6)

$$v = \frac{Vm\,(S)}{Km + (S)} \qquad \text{(Eq. 1)}$$

are defined as follows. In this equation, v represents the apparent initial secretion rate (μmole/kg./min.), *Vm* the maximum apparent initial secretion rate (μmole/kg./min.), (S) the intravenous dose of the substrate (μmole/kg.), and *Km* the dose of the substrate required to produce one-half of the maximum apparent initial secretion rate (μmole/kg.). From the following Lineweaver-Burk form (7) of Eq. 1,

$$\frac{1}{v} = \frac{Km}{Vm\,(S)} + \frac{1}{Vm} \qquad \text{(Eq. 2)}$$

the values of *Vm* and *Km* are determined from the slope and intercept of the straight line obtained by plotting $1/v$ *versus* $1/(S)$.

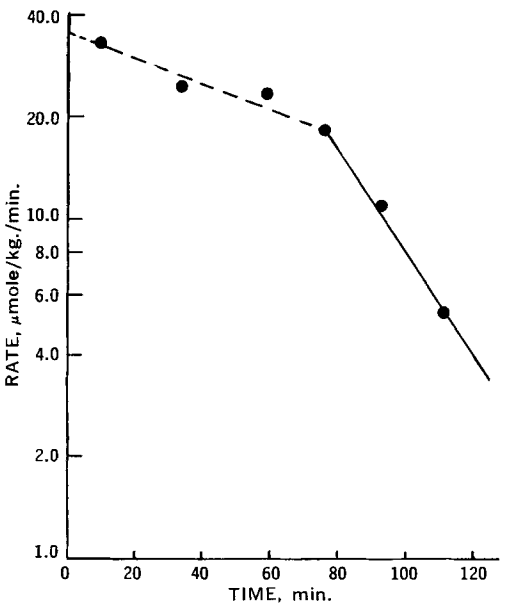


Figure 6—*Urinary excretion data obtained for* D-(−)-*mandelic acid following the i.v. administration of a* 3.37×10^3 *μmole/kg. dose to a rat (indicating the longer biological half-life of the compound during the earlier period being mainly due to an apparent saturation of the secretory process for the compound in the rat).*

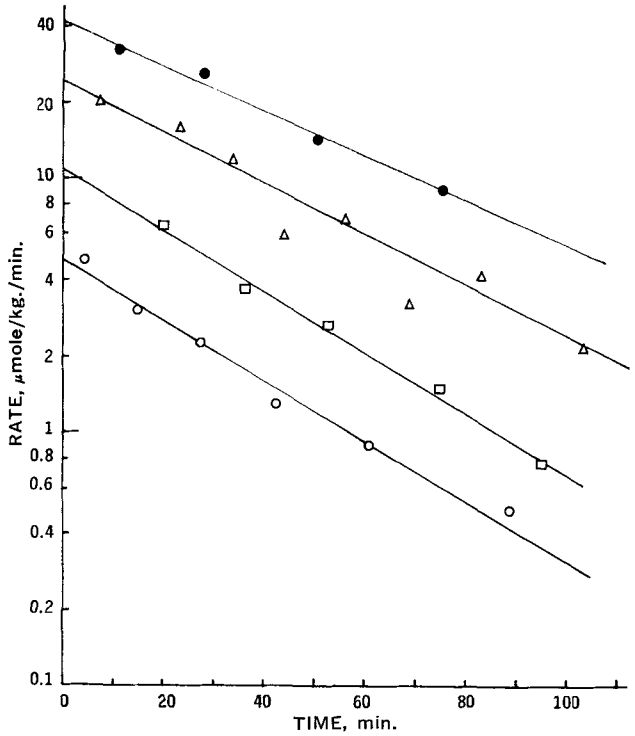


Figure 7—*Apparent first-order urinary excretion data obtained for* D-(−)-*mandelic acid following the i.v. administration of various doses to rats. Key:* ○, *182 μmole/kg.;* □, *533 μmole/kg.;* △, *1169 μmole/kg.; and* ●, *2190 μmole/kg.*

Although the renal clearance method (5) for the *in vivo* determination of Michaelis-Menten kinetic parameters has been used, it was decided to devise a procedure which will make it possible to determine experimentally these parameters without involving a surgical procedure. To apply Michaelis-Menten kinetics to the urinary

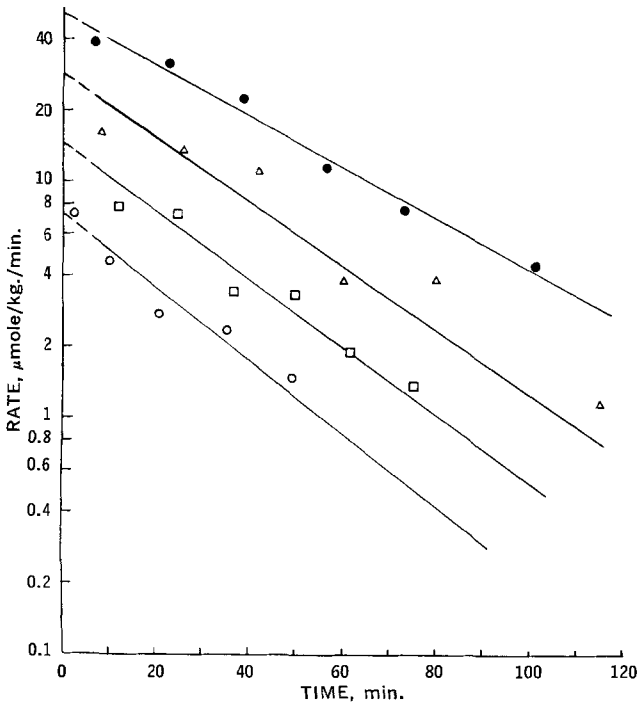


Figure 8—*Apparent first-order urinary excretion data obtained for* DL-*tropic acid following the i.v. administration of various doses to rats. Key:* ○, *167 μmole/kg.;* □, *412 μmole/kg.;* △, *950 μmole/kg.; and* ●, *2110 μmole/kg.*

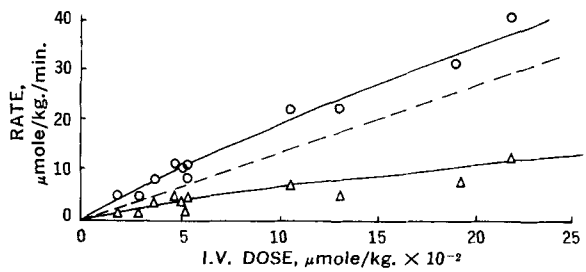


Figure 9—*Apparent initial excretion rates,* ○; *initial glomerular filtration rates,* - - -; *and initial secretion rates,* △; *observed for* D-(−)-*mandelic acid following the i.v. administration of appropriate doses to rats.*

excretion data obtained in the present studies, it was necessary to determine the initial secretion rate of compounds over an appropriate range of dosage levels. The range of doses considered appropriate for a substrate compound in these studies is the range over which not only the urinary excretion of the compounds takes place by a first-order process but also the biological half-life of the substrate remains practically constant. Therefore, the kinetics of urinary excretion of D-(−)-mandelic acid was studied at doses 1.65–4 × 10^2 μmole/kg. It is noted that the biological half-life of D-(−)-mandelic acid remains practically constant (33 min.) over the i.v. dosage range of 1.65–20 × 10^2 μmole/kg. and possibly up to 33 × 10^2 μmole/kg. But at the dosage levels of 33–40 × 10^2 μmole/kg., a slower excretion rate (increased biological half-life) was observed for an hour following the i.v. administration of D-(−)-mandelic acid, which may mainly be due to an apparent saturation of the secretory process of the compound (Fig. 6). Therefore, the dosage range of this acid considered appropriate in this study was 1.65–20 × 10^2 μmole/kg. The results obtained for DL-tropic acid were found to be similar to those obtained for D-(−)-mandelic acid and, therefore, the dosage range of this acid considered suitable was 1.5–20 × 10^2 μmole/kg. The data obtained for the urinary excretion of D-(−)-mandelic acid following the i.v. administration of various doses are presented in Fig. 7. Similarly, the data obtained for the urinary excretion of DL-tropic acid are presented in Fig. 8.

The apparent initial renal tubular secretion rates were determined from the total initial excretion rates at each i.v. dosage level of DL-tropic acid, as well as D-(−)-mandelic acid, by subtracting the apparent initial glomerular filtration rates noted for these compounds from Figs. 3 and 4 at the corresponding dosage levels. The plot of the observed apparent initial excretion rate *versus* dose (i.v.) and the plot of the initial secretion rate *versus* dose (i.v.) are also constructed for these compounds, as shown in Figs. 9 and 10. The extrapolated lines due to the apparent initial glomerular filtration rate *versus* dose for these compounds are included in Figs. 9 and 10. The curvilinear plots obtained for the initial excretion rate data are in accordance with the expectation for compounds which are excreted by both glomerular filtration and tubular secretion (8).

To determine the Michaelis-Menten kinetic parameters, the reciprocal of the initial secretion rate was plotted against the reciprocal of the i.v. dose for both D-(−)-mandelic acid and DL-tropic acid (Fig. 11). From the slope of the straight line obtained by the method

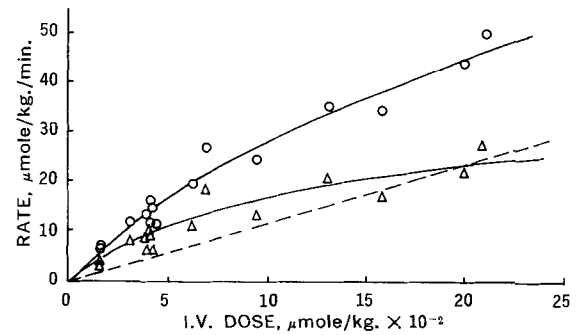


Figure 10—*Apparent initial excretion rates,* ○; *initial glomerular filtration rates,* - - -; *and initial secretion rates,* △; *observed for* DL-*tropic acid following the i.v. administration of appropriate doses to rats.*

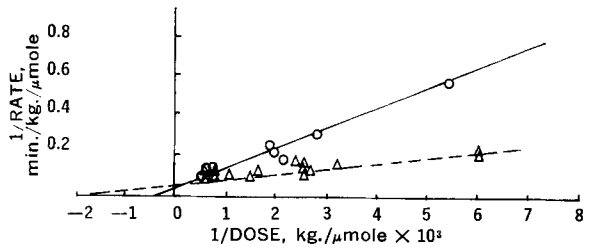


Figure 11—*Lineweaver-Burk plots of the reciprocal of the i.v. dose of* D-(−)-*mandelic acid,* ○; *and* DL-*tropic acid,* △; *against their corresponding reciprocal of the apparent initial secretion rates.*

of least squares and from the *y*-intercept obtained by extrapolation of the straight line, the respective values of *Km* and *Vm* were determined for D-(−)-mandelic acid and for DL-tropic acid. These values are listed in Table I. Values for the maximum secretion rate (*Vm*) obtained for *p*-aminohippurate in rats by the renal clearance method have been reported by several workers (9–11). For the sake of comparison, when these *Vm* values are converted to units of μmole/kg./min., they are found to be 9.27, 14.93, and 18.02 μmole/kg./min. Therefore, in light of such a wide variation observed by other workers in the *Vm* values for *p*-aminohippurate, the *Vm* value of 18.8 μmole/kg./min. obtained for D-(−)-mandelic acid may be considered comparable to the *Vm* value of 20.8 μmole/kg./min. obtained for DL-tropic acid, thus strongly suggesting that the two acids are secreted by the same carrier mechanism present in the renal tubule of the rat. The values of *Km* obtained in these studies for DL-tropic acid (5.2×10^2 μmole/kg.) and D-(−)-mandelic acid (11.1×10^2 μmole/kg.) strongly indicate that the affinity of DL-tropic acid for the renal tubular transport carrier is about twice that of D-(−)-mandelic acid.

Determination of the Nature of Inhibition—To determine whether the mutual inhibition observed for DL-tropic acid and D-(−)-mandelic acid is competitive or noncompetitive in nature, the apparent initial secretion rates of DL-tropic acid at various intravenous dosage levels were determined in the presence of a fixed dose of D-(−)-mandelic acid, and those of D-(−)-mandelic acid at various intravenous dosage levels were determined in the presence of a fixed dose of DL-tropic acid, and analyzed by the Lineweaver-Burk plot method. Accordingly, following the administration of 1.65–26 $\times 10^2$ μmole/kg. doses, apparent initial excretion rates of D-(−)-mandelic acid were determined in the presence of 21–26 $\times 10^2$ μmole/kg. (80 mg./rat) of DL-tropic acid. Similarly, initial excretion rates for DL-tropic acid at dosage levels of 3–24 $\times 10^2$ μmoles/kg. were determined in the presence of 6.0–7.2 $\times 10^2$ μmole/kg. (20 mg./rat), 12–14 $\times 10^2$ μmole/kg. (40 mg./rat), and 24–28 $\times 10^2$ μmole/kg. (80 mg./rat) doses of D-(−)-mandelic acid. From these data, initial secretion rates for the substrates were calculated in the manner described earlier. The reciprocals of the substrate dose were then plotted against the reciprocals of the initial excretion rate of the substrate, as shown in Figs. 12 and 13. The straight-line plots shown for the inhibitory studies involving 20, 40, and 80 mg. of inhibitor were constructed by visual inspection. It becomes evident from the apparent common *y*-intercept in Figs. 12 and 13 that these acids competitively inhibit the renal tubular secretion of each other in rats.

Possible Implications of the Results Obtained in the Studies—Although the *Vm* and *Km* values have not been determined for DL-phenyllactic acid, D-(−)-benzyllactic acid, and D-(−)-4-hydroxy-4-phenylbutanoic acid, it is observed from the inhibitory effect of D-(−)-mandelic acid on the urinary excretion of these compounds that their apparent glomerular filtration rates are similar to those of D-(−)-mandelic acid and DL-tropic acid (Fig. 5). Therefore, it is not unreasonable to conclude that there exists a common carrier system in the renal tubule membrane of rats for the transport of the acids employed in the present study.

Table I—The Michaelis-Menten Kinetic Parameters of Secretion for D-(−)-Mandelic Acid and DL-Tropic Acid in Rats

Compound	Apparent *Vm*,[a] μmole/kg./min.	Apparent *Km*,[a] μmole/kg. $\times 10^{-2}$
D-(−)-Mandelic acid	18.8 ± 3.1[b]	11.1 ± 2.5[b]
DL-Tropic acid	20.8 ± 2.5	5.2 ± 1.8

[a] The values of the apparent *Vm* obtained for the compounds are not significantly different from each other, while the values of the apparent *Km* determined for the compounds are significantly different from each other ($p < 0.01$). [b] Standard deviation for each compound was determined from 11–15 rat studies described in Fig. 11.

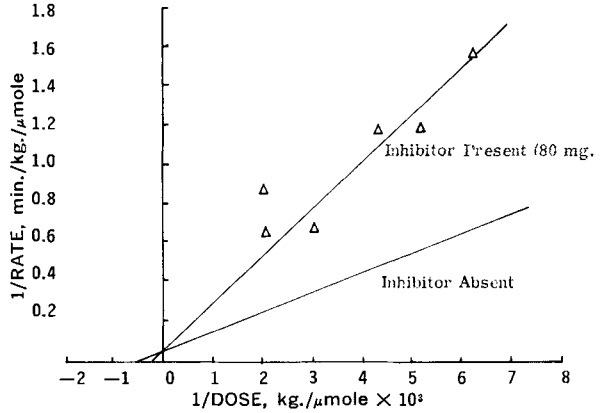


Figure 12—*Lineweaver-Burk plots indicating competitive inhibition of secretion of* D-(−)-*mandelic acid by* DL-*tropic acid in the i.v. dose of 80 mg./rat (21–26 $\times 10^2$ μmole/kg.).*

The additional purpose of this project was to determine the effect of the incorporation of methylene groups in the side chain of the mandelic acid molecules on the rate of secretion, and thereby to gain some insight into the possible chemical nature of the carrier molecule in the vicinity of its positively charged primary binding site. Based on the data obtained in these studies, it seems possible to distinguish certain characteristics of the carrier molecule.

It has been illustrated in the present studies that the incorporation of methylene group(s) in the side chain of the mandelic acid molecule results in a significant decrease (1) in the biological half-life of the compounds due to an increase in the rate of renal tubular secretion. DL-Tropic acid ($t_{1/2}$, 23 ± 4 min.) and DL-phenyllactic acid ($t_{1/2}$, 20 ± 3 min.), each possessing one more methylene group than does mandelic acid, are found to have significantly shorter biological half-lives than that of D-(−)-mandelic acid ($t_{1/2}$, 30 ± 4 min.). Although the D-(−)-4-hydroxy-4-phenylbutanoic acid and D-(−)-benzyllactic acid molecules contain two more methylene groups than does the molecule of mandelic acid, the biological half-life of D-(−)-4-hydroxy-4-phenylbutanoic acid ($t_{1/2}$, 16 ± 2 min.) is significantly shorter than that observed for D-(−)-benzyllactic acid ($t_{1/2}$, 21 ± 3). It was also observed that the biological half-lives obtained for DL-phenyllactic acid and D-(−)-benzyllactic acid are practically the same. These observations indicate that the presence of methylene groups next to the carboxyl group contribute significantly to the affinity of the mandelic acid homolog to the carrier molecule. Since the primary interaction of the substrate molecule with the carrier molecule is expected to be due to electrostatic forces, the further affinity of the homologs of mandelic acid may be attributed to the secondary interaction (12–14) between the hydrophobic groups of the carrier molecule and the homolog. Therefore, it is suggested that the hydrophobic groups, such as methylene groups, should be pres-

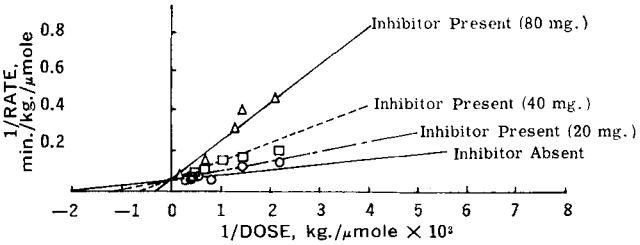


Figure 13—*Lineweaver-Burk plots indicating competitive inhibition of secretion of* DL-*tropic acid by* D-(−)-*mandelic acid in the i.v. doses of* △, *80 mg./rat (36–44 $\times 10^2$ μmole/kg.);* □, *40 mg./rat (12–14 $\times 10^2$ μmole/kg.); and* ○, *20 mg./rat (6.0–7.2 $\times 10^2$ μmole/kg.).*

ent next to the positively charged group or site on the carrier molecule.

An examination of the chemical structures of D-(−)-benzyllactic acid and D-(−)-4-hydroxy-4-phenylbutanoic acid (1) may shed some light on the extent of the hydrophobic group in the vicinity of the cationic site of the carrier molecule. Both of these compounds have the same molecular weight, chemical composition, and absolute configuration. The only respect in which these molecules differ from each other is the position of the methylene groups in relation to the carboxyl group. In a D-(−)-4-hydroxy-4-phenylbutanoic acid molecule, the two methylene groups are present next to the carboxyl group; in a D-(−)-benzyllactic acid molecule, the two methylene groups are separated from the carboxyl group by the carbon containing the hydroxyl group. Since the biological half-life for D-(−)-4-hydroxy-4-phenylbutanoic acid is significantly shorter than that for D-(−)-benzyllactic acid, it suggests that the hydrophobic region in close proximity to the cationic groups of the carrier molecule probably does not extend uninterrupted beyond the hydrophobic region present in close proximity to the carboxyl group of the D-(−)-4-hydroxy-4-phenylbutanoic acid molecule. This inference seems to be supported by the fact that the biological half-lives of D-(−)-benzyllactic acid and DL-phenyllactic acid are practically the same, in spite of the facts that the benzyllactic acid molecule contains one more methylene group than does the phenyllactic acid molecule and the position of the hydroxyl group in relation to the carboxyl group in these molecules is the same.

The comparison of the biological half-life data obtained for D-(−)-mandelic acid, DL-tropic acid, and DL-phenyllactic acid also seems to support this view regarding the hydrophobic region in close proximity to the positively charged site on the carrier molecule. Perhaps the use of 5-hydroxy-5-phenylpentanoic acid would have offered some additional information in this regard, but its extensive metabolism by rats precluded its use in the present studies. However, studies similar to those described here are being carried out with other homologs of D-(−)-mandelic acid to distinguish further characteristics of the carrier molecule.

SUMMARY AND CONCLUSIONS

Using DL-tropic acid and D-(−)-mandelic acid as the mutual competitive inhibitors of their renal tubular secretion, urinary excretion kinetic studies were carried out in rats to determine their initial glomerular filtration rates at various doses without involving a surgical procedure. The initial rate–i.v. dose profiles were obtained for urinary excretion, renal secretion, and glomerular filtration of these compounds.

The Michaelis-Menten kinetic parameters (*Vm* and *Km*) of secretion were determined for each of the two compounds. The values of *Vm* determined for these compounds are found to be similar, but the value of *Km* obtained for D-(−)-mandelic acid is approximately twice that obtained for DL-tropic acid, indicating that the affinity of the former for the renal tubular transport carrier is about one-half that of the latter.

Based on the initial glomerular filtration rate data obtained for D-(−)-mandelic acid and DL-tropic acid in this study and those obtained for DL-phenyllactic acid, D-(−)-benzyllactic acid, and D-(−)-4-hydroxy-4-phenylbutanoic acid in the previous study, it was concluded that the volume of distribution of D-(−)-mandelic acid and its above homologs is similar in rats.

The results of these studies have been utilized to attempt to distinguish certain structural characteristics around the positively charged site of the carrier molecules for renal tubular secretion in rats.

REFERENCES

(1) E. J. Randinitis, M. Barr, H. C. Wormser, and J. B. Nagwekar, *J. Pharm. Sci.*, **59,** 806(1970).

(2) M. Khambati and J. B. Nagwekar, to be published.

(3) H. N. Christensen, "Biological Transport," W. A. Benjamin, New York, N. Y., 1962, pp. 45–51.

(4) A. Farah, M. Frazer, and E. Porter, *J. Pharmacol. Exp. Ther.*, **126,** 202(1959).

(5) K. C. Cho, J. H. Kim, S. K. Hong, and W. C. Lee, *Yonsei Med. J.*, **1,** 25(1960).

(6) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49,** 333(1913).

(7) H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, **56,** 658(1934).

(8) J. G. Wagner, *Drug Intel.*, **2,** 95(1968).

(9) S. M. Friedman, J. R. Polley, and C. L. Friedman, *Amer. J. Physiol.*, **150,** 340(1947).

(10) A. C. Corcoran, G. Musson, R. Reuting, and I. H. Page, *ibid.*, **154,** 170(1948).

(11) A. C. Corcoran and I. H. Page, *Fed. Proc.*, **6,** 91(1947).

(12) A. Despopoulos, *J. Theor. Biol.*, **8,** 163(1965).

(13) I. M. Weiner and G. H. Mudge, *Amer. J. Med.*, **36,** 743 (1964).

(14) A. Essig and J. V. Taggert, *Amer. J. Physiol.*, **199,** 509 (1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *College of Pharmacy, Wayne State University, Detroit, MI 48202*

Accepted for publication February 4, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Abstracted in part from a dissertation submitted by E. J. Randinitis to the Graduate School, Wayne State University, Detroit, in partial fulfillment of the Doctor of Philosophy degree requirements.

* Present address, Product Development Research Laboratories, Parke, Davis & Co., Detroit, MI 48232. American Foundation for Pharmaceutical Education Fellow, 1967–1968.

† To whom all inquiries should be directed.

Effect of Chemical Modification on the Surface Activity of Some Phenothiazine Derivatives

GEORGE ZOGRAFI and MAYANK V. MUNSHI

Abstract □ The ability of some substituted phenothiazines to reduce the surface tension of aqueous solutions has been studied in order to evaluate their hydrophobic behavior. Particular emphasis was placed on utilizing conditions that would allow specific structural effects to be isolated quantitatively and to point out situations where this may be difficult to do. Substitution on the phenothiazine ring enhances surface activity in the order $CF_3 \gg Cl > H$. Changing the position of the chloro group on the ring significantly influences surface activity, the order being 3Cl > 2Cl > 1Cl. The effect on surface activity due to changes in the number of alkyl groups, the degree of branching, and the number of dissociable groups on the alkylamino portion of the molecule also has been evaluated.

Keyphrases □ Phenothiazine derivatives—surface activity □ Surface activity, phenothiazines—chemical modification effect □ Structure, phenothiazines—surface activity relationship □ Concentration, phenothiazines—surface activity

The fact that many drugs exhibit surface-active properties has led to numerous studies concerned with their relative surface and biological activity (1). In particular, for many drugs exhibiting an apparent action at biological membrane surfaces, *e.g.*, local anesthetics (2) and the substituted phenothiazines (3, 4), significant surface activity at a variety of interfaces has been reported. This is not to say that surface activity is solely responsible for pharmacological activity, but rather that it reflects hydrophobic characteristics of a drug molecule, known to influence availability as well as reactivity at a site of action.

In previous reports from this laboratory (5, 6), it has been suggested that measurement of surface-tension change at the air–solution interface is a convenient means of observing the hydrophobic behavior of chlorpromazine without the necessity of introducing more complex hydrophobic phases such as oils, solids, or specific reactants. Such studies have shown that factors tending to promote hydrophobic character, *e.g.*, decreasing the degree of ionization, increasing ionic strength, and the presence of counterions exhibiting some tendency to ion-pair, all increase surface activity, while substances tending to reduce hydrophobic behavior, such as urea and the tetraalkylammonium salts, decrease surface activity.

The present study was designed to evaluate the effect of chemical modification on the surface activity and, hence, hydrophobicity of several phenothiazine derivatives. Consideration was given to a means of choosing solution conditions and a point of reference that could be used for more meaningful quantitative comparisons.

EXPERIMENTAL

Materials—The chemical structure of each phenothiazine studied is given in Table I along with reported pKa values for the protonated species. Chlorpromazine, its 1-chloro, 3-chloro, ethylamino, and butylamino analogs, as well as trimeprazine, prochlorperazine, and trifluoperazine (Smith Kline & French Laboratories), triflupromazine (E. R. Squibb & Sons), and promazine and promethazine (Wyeth Laboratories) were used. All buffer ingredients and inorganic compounds were of reagent grade, while the water used was double distilled.

Surface-Tension Measurement—Surface tension was measured by means of the drop volume method using equipment and procedures described previously (7). All studies were conducted at 25 ± 0.1°. Great care was taken to avoid contact with light because of possible photodecomposition exhibited by the phenothiazines (8). All surface-tension data are expressed in terms of surface pressure, π, which is the difference between the surface tension of the solvent and that of a given solution being measured. Thus as surface tension is reduced, the surface pressure increases.

RESULTS AND DISCUSSION

Considerations in Evaluating Relative Surface Activity—In order to make meaningful comparisons of various drugs, an appropriate set of conditions and a point of reference must be chosen. Two conditions which must be chosen with care are the pH and ionic strength of the solution. Since the phenothiazines can exist as protonated and nonprotonated species, comparisons should be made where only one species is present or where the ratio of one species to the other is constant. Studies with the nonprotonated form of the phenothiazines are extremely difficult because of their very great water insolubility, as well as their tendency to adsorb on all materials with which they come in contact (9). Comparison at a constant ratio of protonated to nonprotonated species also is difficult because there is no assurance that the two species of a phenothiazine, both of which adsorb, will exhibit the same ratio of relative surface activity as that for another derivative. Uncertainties in exact pKa values (Table I) also make this approach difficult. Thus, for comparing such systems, probably it is best to choose a pH value where all drugs studied exist only in the protonated form. Figure 1 demonstrates that one should be at a pH of 5.0 or less to obtain pH independence for phenothiazines having pKa values close to that of chlorpromazine. In the present study, therefore, all experiments were carried out at a pH of 5.0 or less, depending on the pKa of the drugs in question.[1]

The use of a protonated species introduces an electrical contribution to the free energy of adsorption since work is required to overcome the electrical repulsion of those molecules already adsorbed. Increasing the ionic strength would be expected to increase surface activity significantly since these repulsive forces would be screened out by the higher concentration of ions in the vicinity of the surface. Figure 2 shows that such effects for chlorpromazine are significant; and since this was observed for all compounds, ionic strength was maintained at one value throughout the study.

Once standard conditions of pH and ionic strength are chosen for comparing the surface activity of drugs, it is desirable to choose some means of expressing the relative surface activity of each drug. Ideally, comparing the bulk concentration required to produce a given number of adsorbed molecules per unit area would be of value. Ordinarily the number of adsorbed molecules per unit area can be determined by measuring the change in surface tension or surface pressure with bulk solution activity and then applying the Gibbs adsorption equation (16). The major difficulty with this approach is not knowing the thermodynamic activity of the drug in solution.

[1] Previous studies have indicated buffer effects to be possible, presumably due to counterion binding (9). In such studies, however, acetate buffers at concentrations used in the present study were found to have no effect on surface activity so buffer effects were not considered as a variable in this study.

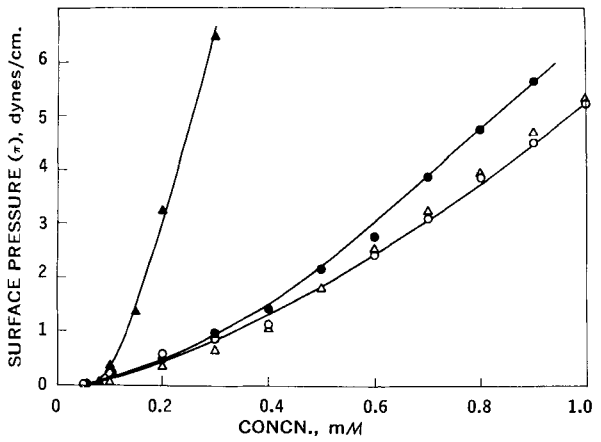


Figure 1—*Plot of surface pressure* versus *molar concentration for chlorpromazine at pH: 4.0* (○), *5.0* (△), *5.6* (●), *and 7.1* (▲) *at 25° and an ionic strength of 0.1.*

This is particularly applicable for the phenothiazines, where complex salt effects and aggregation have been noted (6, 9, 10).

In view of this and the need only to obtain relative surface activity under conditions which are identical except for the structural changes being considered, a much simpler approach can be used. What is done is to compare the bulk concentration of each drug required to produce a given surface pressure and to express this relative to one of the drugs. Solution concentration is kept as low as possible to minimize solution activity effects and, as stated earlier, all other conditions are maintained constant. Such a comparison, therefore, leads to a measure of the free energy change for adsorption relative to the drug chosen as the standard. If the π *versus* concentration plot for the series of drugs is similar in curvature with about the same intercept, one can expect that the ratio of concentrations and hence the free energy change will be independent of the π chosen for comparison. If, however, one merely chooses one arbitrary value without checking the complete curve or at least another surface pressure, results may be quite misleading (11, 12). As an extreme example, it is quite possible that some plots may intersect and give different relative values depending on where comparisons are made. To demonstrate this point, plots up to 5 or 6 dynes/cm. are presented and ratios are calculated at 3 and 5 dynes/cm.

Comparison of the Various Phenothiazine Derivatives—Figures 3 through 7 are presented to compare the surface activity of the various derivatives, each plot demonstrating the effect of one type of structural change. Based upon these results, the ratio of drug concentration required to produce 3 and 5 dynes/cm. surface pressure to the chlorpromazine concentration required to do this has been calculated and is presented in Table I. Values greater than one indicate less surface activity than chlorpromazine, while values less than one indicate a greater surface activity than chlorpromazine. The general agreement at 3 and 5 dynes/cm. seems to suggest that the free energy change for adsorption relative to chlorpromazine is independent of the surface pressure chosen at lower values of surface pressure.

Figures 3 and 7 and the ratios given in Table I illustrate the significant effect of changing the substituent on Position 2 of the

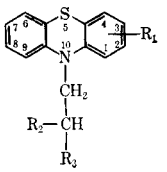
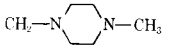
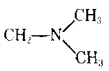
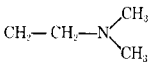
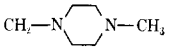
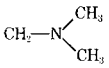
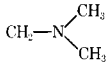
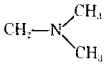
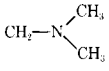
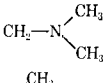


Table I—Phenothiazine Derivatives

Drug	R_1	R_2	R_3	Concn. Ratio, dynes/cm.[a] 3	5	pKa[b]
Trifluoperazine	2—CF_3	H		0.12	0.12	8.1, 8.4
Triflupromazine	2—CF_3	H		0.24	0.24	9.2
Butyl chlorpromazine[c]	2—Cl	H		0.47	0.45	9.7
Prochlorperazine	2—Cl	H		0.58	0.57	8.1, 7.5
3-Chlorpromazine[c]	3—Cl	H		0.62	0.63	9.2
Chlorpromazine	2—Cl	H		1.0	1.0	9.3, 9.2
1-Chlorpromazine[c]	1—Cl	H		1.4	1.6	9.4
Trimeprazine	H	CH_3		1.7	1.8	—
Ethyl chlorpromazine[c]	2—Cl	H		2.2	2.2	8.6
Promazine	H	H		2.5	2.5	9.4, 9.5
Promethazine	H	CH_3		2.7	2.6	9.1

[a] Concentration of drug required to produce a given surface pressure relative to concentration of chlorpromazine required. [b] Values are taken from *References 11* and *13–15*. [c] Actually only analogs of chlorpromazine without a generic name.

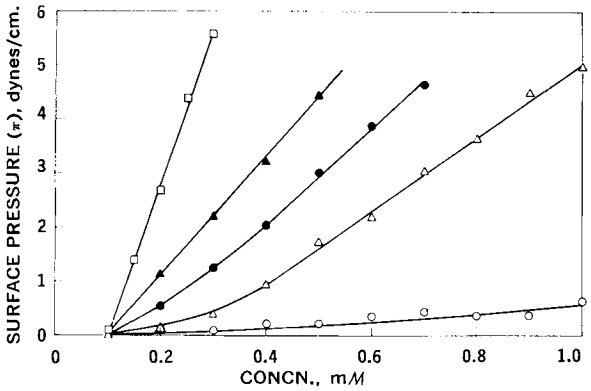


Figure 2—*Plot of surface pressure* versus *molar concentration for chlorpromazine at pH 5.0, 25°, and ionic strength: 0.01* (○), *0.10* (△), *0.15* (●), *0.20* (▲), *and 0.50* (□).

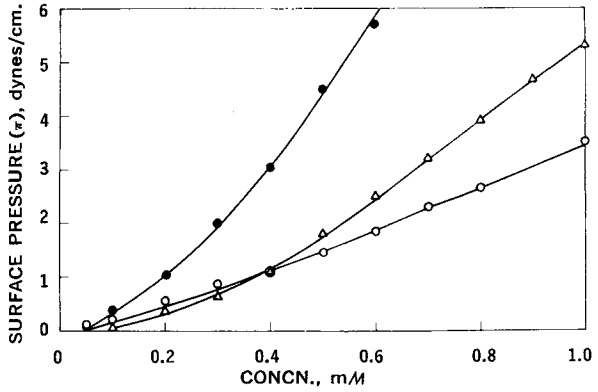


Figure 4—*Plot of surface pressure* versus *molar concentration at pH 5.0, ionic strength 0.1, and 25° for: 1-chlorpromazine* (○), *2-chlorpromazine* (△), *and 3-chlorpromazine* (●).

phenothiazine ring while holding other factors constant. Both compounds containing a —CF_3 group are many times more surface active than those containing a —Cl group, which in turn are more surface active than those containing only —H at that position. Picturing the phenothiazine molecules oriented at the interface with the ring toward the air and the alkylamino group directed toward the bulk aqueous phase, it is not surprising that changes in ring structure change surface activity so significantly. The strong contribution of the ring to the surface activity of the molecule, and hence its hydrophobic properties, is made apparent also by an earlier observation with chlorpromazine sulfoxide (5). Here, the addition of oxygen to the sulfur produces a hydrophilic species with a ratio of surface activity relative to chlorpromazine of about 100. Thus a polar group in the ring essentially eliminates any surface activity. The significant hydrophobic effect of adding the —CF_3 and —Cl group is made apparent further when one sees (Fig. 6) that trimeprazine, with four carbons in the alkylamino portion of the molecule but only —H on the ring, is significantly less surface active than any of the substituted propylamino derivatives which, of course, have only three carbons at that position.

Since the phenothiazine ring is the primary hydrophobic portion of the molecule, the ring position of substituent groups might be expected to have an effect on surface activity. Figure 4 compares chlorpromazine (2-chloro) with its 1-chloro and 3-chloro analogs. This figure and the ratios given in Table I indicate a fairly significant increase in surface activity as the chloro group is moved away from the vicinity of Position 10. It can be shown with molecular models that substitution at Position 1 definitely alters the orientation of the alkylamino group relative to the ring, restricting movement and limiting the number of possible orientations. No such effects are likely with the 2- and 3-chloro derivatives. In addition, intermolecular steric effects which also influence packing in the surface film may contribute to the difference between these compounds, although again little difference between the 2- and 3-chloro derivatives is noted with molecular models. In view of this, the significant increase in surface activity of the 3-chloro derivative may be more related to a change in the electronic structure of the phenothiazine ring. Evaluation with a wider variety of chemical structures would be extremely valuable in elucidating possible steric and electronic effects, but unfortunately the availability of just the right compounds is limited at the present time.

Figures 5–7 illustrate the effect of altering the nature of the alkylamino group at Position 10 of the ring. Comparing the ethyl, propyl, and butyl derivatives illustrates the well-known effect of increasing alkyl chain length on surface activity, an increase of one —CH_2— group giving about a twofold increase in activity. Comparison of the isomers, promazine and promethazine, indicates that the presence of a branched chain reduces surface activity relative to a compound with the same number of carbons. This agrees with the fact that branched chain hydrocarbons generally are less hydrophobic than their straight chain isomers. Examination of Fig. 7 illustrates the influence of adding a piperazine ring onto the propylamino group, namely, an increase in surface activity, presumably due to the extra carbons introduced into the molecule.

A closer analysis of the results with compounds differing at Position 10 demonstrates that great care must be taken in making simple correlations. It is apparent from Table I, for example, that the dissociation constant for the amino group is more sensitive to structural changes at this position. Thus it is especially important to compare these molecules at pH values much lower than the pKa value of the drug having the lowest pKa. Sometimes, as in the case of the piperazine compounds with two dissociable groups, this is not possible and quantitative correlation is made difficult. For example, as seen in Table I, the addition of only one —CH_2— group to chlorpromazine, giving the butyl derivative, produces a more surface-active molecule than prochlorperazine, with more CH_2 groups, because of the second ionized portion of the latter compound. Another situation where this factor apparently is important is when one compares the ratio of trifluoperazine to triflupromazine and that of prochlorperazine to chlorpromazine. If differences in surface activity are due only to the introduction of the same substituent, *i.e.*, the

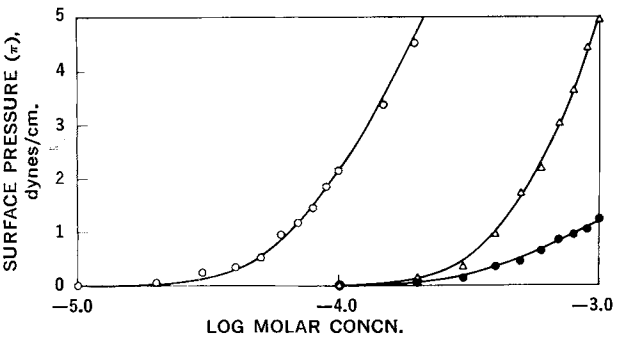


Figure 3—*Plot of surface pressure* versus *log molar concentration at pH 5.0, ionic strength 0.10, and 25° for: triflupromazine* (○), *chlorpromazine* (△), *and promazine* (●).

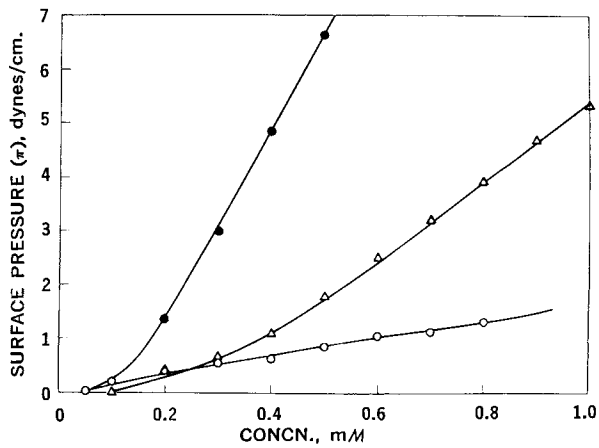


Figure 5—*Plot of surface pressure* versus *molar concentration at pH 5.0, ionic strength 0.1, and 25° for: ethyl chlorpromazine* (○), *propyl chlorpromazine* (△), *and butyl chlorpromazine* (●).

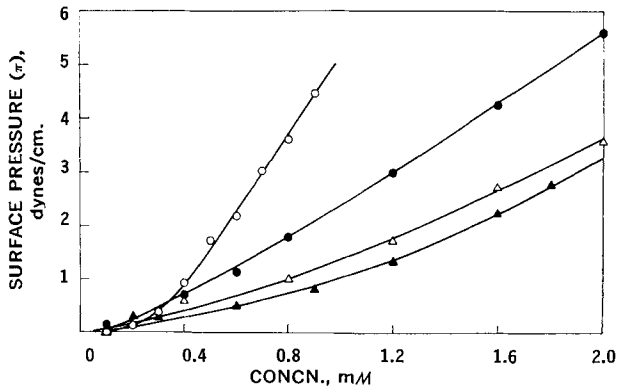


Figure 6—*Plot of surface pressure* versus *molar concentration at pH 5.0, ionic strength 0.1, and 25° for: chlorpromazine* (○), *trimeprazine* (●), *promazine* (△), *and promethazine* (▲).

piperazine ring, these ratios should be the same; however, the value for the CF_3 compounds is 0.50 and that for the Cl compounds is 0.59. What is happening, of course, is that at pH 4.0 all of these compounds are essentially completely protonated at one amino group, but the two piperazine derivatives have different second dissociation constants. Trifluoperazine has a dissociation constant of 3.9 while the value for prochlorperazine is 3.6. Hence at pH 4.0 the piperazine of trifluoperazine probably contributes more to the surface activity because it has introduced a lower degree of ionization to the molecule.

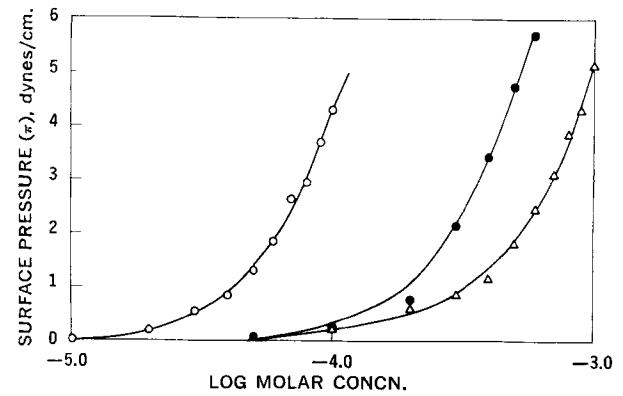


Figure 7—*Plot of surface pressure* versus *log molar concentration at pH 4.0, ionic strength 0.1, and 25° for: trifluoperazine* (○), *prochlorperazine* (●), *and chlorpromazine* (△).

SUMMARY

The relative surface activity of various phenothiazine derivatives has been measured under solution conditions which for the first time allow meaningful comparison of structural effects.

Estimation of the concentration required to produce given changes in surface tension, relative to that produced by chlorpromazine, reveals significant effects due to substitution of H, Cl, and CF_3 groups on the phenothiazine ring. The position of a substituent on the ring has been shown to be another important factor.

The influence of changing the alkylamino group at Position 10 has been discussed in terms of changes in hydrophobicity due to the number and arrangement of alkyl groups. A primary factor to consider also is how substitution influences the dissociation constant(s) of the amino group(s) and hence the degree of ionization at the pH being utilized for comparison.

REFERENCES

(1) A. T. Florence, *Advan. Colloid Interface Sci.*, **2,** 115(1968).

(2) J. C. Skou, *Acta Pharmacol. Toxicol.*, **10,** 305(1954).

(3) P. S. Guth and M. A. Spirtes, *Intern. Rev. Neurobiol.*, **7,** 231(1964).

(4) E. F. Domino, R. A. Hudson, and G. Zografi, in "Drugs Affecting the Central Nervous System," vol. 2, A. Burger, Ed., Marcel Dekker, New York, N. Y., 1968, p. 327.

(5) G. Zografi, D. E. Auslander, and P. E. Lytell, *J. Pharm. Sci.*, **53,** 573(1964).

(6) R. M. Patel and G. Zografi, *ibid.*, **55,** 1345(1966).

(7) N. D. Weiner and G. Zografi, *ibid.*, **54,** 436(1965).

(8) A. Felmeister and C. A. Discher, *ibid.*, **53,** 756(1964).

(9) G. Zografi and I. Zarenda, *Biochem. Pharmacol.*, **1966,** 15.

(10) W. Scholtan and R. Gonnert, *Med. Chem.*, **5,** 314(1956).

(11) A. L. Green, *J. Pharm. Pharmacol.*, **19,** 207(1967).

(12) J. V. Levy, *ibid.*, **20,** 813(1968).

(13) P. B. Marshall, *Brit. J. Pharmacol.*, **10,** 270(1955).

(14) L. G. Chatten and L. E. Harris, *Anal. Chem.*, **34,** 1495(1962).

(15) A. L. Green, *J. Pharm. Pharmacol.*, **19,** 10(1966).

(16) J. W. Gibbs, "The Collected Works of J. W. Gibbs," vol. 1, Longmans, Green, New York, N. Y., 1931, p. 219.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 25, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication December 31, 1969.

Supported by Grant ONB07134 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Md.

Centrally Acting Emetic Agents IV: Synthesis and Chromatographic Methods for Certain Nornuciferine Derivatives

RAYMOND J. VAVREK, JOSEPH G. CANNON*, and ROBERT V. SMITH

Abstract □ *dl*-Nornuciferine has been synthesized by a literature procedure; alkyl groups were introduced on the nitrogen by direct alkylation, reductive alkylation, or by acylation followed by reduction to afford eight *N*-alkylnornuciferine derivatives. Selective cleavage of the ether function at Position 1 of the 1,2-dimethoxyaporphine systems was achieved by use of hydriodic acid in acetic anhydride. As a preliminary to metabolic studies on the products, thin-layer and gas chromatographic methods were devised for qualitative and quantitative analysis of them.

Keyphrases □ Emetic agents—centrally acting □ Nornuciferine derivatives—synthesis □ IR spectrophotometry—identity □ NMR spectroscopy—identity □ TLC—analysis □ GLC—analysis

In a continuing study of aporphine derivatives, which are potential emetic agents (1), a series of 1,2-dimethoxylated aporphine (nornuciferine) derivatives was investigated. Compounds I–XVI (Table I) were prepared for biological evaluation of their emetic effects and for initiation of a study of the metabolic fate of dioxygenated aporphine systems in mammals.

The literature reveals a paucity of information on metabolism or biological fate of aporphine systems in general; Compounds I–VIII have been selected for initial metabolic investigation. In considering possible biotransformations of *N*-substituted nornuciferines, *N*- and/or *O*-dealkylation seem to be plausible reactions (2). Thus, for Compounds II–VIII, three possible metabolite types are available for study: I, an *N*-dealkylated product; IX, an *N*- and *O*-dealkylated system; and X–XVI, *O*-dealkylated products of the parent compounds. Analytical systems were required for separation, identification, and quantitation of each of these substances as a prelude to the proposed metabolic studies.

TLC was envisioned as the qualitative method of choice. Solvents and techniques employed in the separation of closely related alkaloids (3) served as a reference point for the development of systems in this investigation. For quantitative analysis, gas chromatography was considered. Arndt *et al.* (4) chromatographed nornuciferine (I) and nuciferine (II) with an SE-30 column. Since the nuciferine analogs in the present study represent wider structural variation and were expected to possess poorer volatility, a similar but more thermally stable internal phase was sought. OV-17, a silicone polymer with 50% phenyl substitution and a temperature limit of approximately 350°, was used.

RESULTS AND DISCUSSION

dl-Nornuciferine (I), prepared by the method of Weisbach and Douglas (5), was *N*-alkylated directly with an alkyl halide or was *N*-acylated and then reduced with lithium aluminum hydride. *dl*-Nuciferine (II) was prepared by Eschweiler-Clarke methylation of I.

It is noteworthy that the Pschorr cyclization of 1-(2′-aminobenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline to an aporphine system proceeded in approximately twice the percent yield reported for this step in the literature (5). Initial low recovery of products in the lithium aluminum hydride reductions increased significantly by use of a procedure (6, 7) in which the reduction mixture, after quenching with water, was stirred with an ammonium tartrate solution which presumably aids in breaking the aluminum complexes of the amino product.

Attempts were made to cleave the ether links of Compounds I–VII to form 1,2-dihydroxyaporphine systems; hydriodic acid in acetic anhydride (8) was selected as the most promising reagent. Elemental analysis of the ether-cleaved products of I–VIII indicated an incomplete cleavage and suggested that the products were monohydroxy- and monomethoxyaporphines. NMR spectra of all of the ether-cleaved materials demonstrated a singlet (3H) at 3.85–3.89 δ, while the parent dimethoxylated aporphines exhibited signals at 3.82–3.86 δ (3H) and at 3.60–3.62 δ (3H). The higher field shift has been assigned to the methoxyl group at Position 1 (9, 10), on the basis of steric interactions of the 1-methoxyl group with the *peri*-hydrogen at Position 11 and with the methoxyl group at Position 2. These interactions force the methyl portion of the Number 1 methoxyl out of the plane of the aromatic ring and make it more susceptible to the shielding effects of both aromatic rings. This assignment has been corroborated in the nuciferine system itself (11). Kametani and Noguchi (12) have prepared *dl*-*N*-methylcaaverine (1-hydroxy-2-methoxy-*N*-methylaporphine) by an unequivocal route. This compound, which is the free base form of Compound X (Table I), exhibited an NMR signal at 3.86 δ (3H) which was assigned to the protons of the 2-methoxy group. Therefore, the cleavage procedure employed in the present study was selective for the ether function at Position 1 of the 1,2-dimethoxyaporphines, giving a series of 1-hydroxy-2-methoxyaporphines (IX–XVI). There was no indication that isomeric 1-methoxy-2-hydroxyaporphines were formed in any of the cleavage reactions.

The literature contains numerous accounts of selective cleavage of polymethoxy aromatic ethers with sulfuric acid (13, 14) and with hydrohalic acids (15). Bruderer and Brossi's proposal (15) that selective ether cleavage in certain polymethoxylated heterocyclic systems is a function of differences in basicity of the ether oxygens due to electrostatic substituent effects has been rejected by Wilcox and Seager (16) following their kinetic study of similar systems, on the basis that this explanation requires postulating "unprecedently negative *ortho* σ (OCH_3) constants." These latter workers rationalized the greatly enhanced cleavage rates of the central methoxyl group of 1,2,3-trimethoxybenzene derivatives by hydrobromic acid in acetic acid as being due to steric acceleration factors; the greater basicity of the central methoxyl group is due to its being twisted out of the plane of the aromatic ring. In the present case of the apparently exclusive cleavage of the Number 1 methoxyl of 1,2-dimethoxyaporphines, the explanation of Wilcox and Seager (16) may be invoked as a reasonable one. The NMR data cited previously corroborated this type of steric disposition of the Number 1 methoxyl. Catalin models indicate serious nonbonded interactions of the Number 1 methoxyl with the Number 2 methoxyl and with the Number 11 hydrogen, when the Number 1 methoxyl is coplanar with the benzene ring to which it is attached.

X-ray crystallographic studies (17) indicate that the biphenyl portion of aporphine systems is appreciably strained, the angle of twist being 29.9°. This strain increases in aporphines bearing substituents at Positions 1 and/or 11. Based upon the premise that the release of steric strain in the course of a reaction can be a driving force, and upon the proposal (18) that the "packing strain" in di-

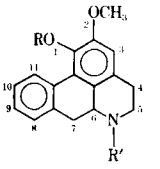


Table I—Nornuciferine Derivatives

No.	R	R′	Method	Yield, %	M.p., Recrystn. Solvent[a]	Molecular Formula	Anal., % Calcd.	Found
I	Me	H	C	88	263°, dec.[b] (M)	$C_{18}H_{20}NO_2Cl$[c]		
II	Me	Me	D	80	261°, dec. (EEt)	$C_{19}H_{22}No_2Cl$[c]	C, 68.78 H, 6.64 N, 4.22	C, 69.15 H, 6.84 N, 4.39
	Me	COMe	E	97	224–225° (M)	$C_{20}H_{21}NO_3$[d]	C, 74.30 H, 6.50 N, 4.33	C, 73.99 H, 6.35 N, 4.31
III	Me	Et	F	85	263°, dec. (E)	$C_{20}H_{24}NO_2Cl$[c,d]	C, 69.46 H, 6.95 N, 4.05	C, 69.71 H, 7.05 N, 4.04
	Me	COEt	E	73	210–211° (M)	$C_{21}H_{23}NO_3$	C, 74.78 H, 6.82 N, 4.15	C, 74.93 H, 6.93 N, 4.20
IV	Me	*n*-Pr	F	70	240°, dec. (E)	$C_{21}H_{26}NO_2Cl$[c]	C, 70.09 H, 7.20 N, 3.89	C, 69.93 H, 7.19 N, 4.01
	Me	CO—⟨	E	88	213–214° (M)	$C_{22}H_{23}NO_3$	C, 75.64 H, 6.59 N, 4.01	C, 75.50 H, 6.67 N, 4.09
V	Me	CH_2—⟨	F	89	244°, dec. (E)	$C_{22}H_{26}NO_2Cl$[c]	C, 71.06 H, 6.99 N, 3.77	C, 70.60 H, 6.93 N, 3.88
VI	Me	CH_2—CH=CH_2	G	61	270°, dec. (E)	$C_{21}H_{24}NO_2Cl$[c]	C, 70.49 H, 6.71 N, 3.92	C, 70.34 H, 6.73 N, 3.90
VII	Me	CH_2—C≡CH	G	86	240°, dec. (E)	$C_{21}H_{22}NO_2Cl$[c]	C, 70.89 H, 6.19 N, 3.94	C, 70.98 H, 6.25 N, 3.98
VIII	Me	CH_2—C_6H_5	H	39	259°, dec. (E)	$C_{25}H_{26}NO_2Cl$[c]	C, 73.62 H, 6.38 N, 3.44	C, 73.72 H, 6.66 N, 3.51
IX	H	H	I	55	248°, dec. (EEt)	$C_{17}H_{18}INO_2$[e]	C, 51.65 H, 4.56 N, 3.54	C, 51.45 H, 4.61 N, 3.59
X	H	Me	I	51	251°, dec. (W)	$C_{18}H_{20}INO_2$[e]	C, 52.85 H, 4.89 N, 3.42	C, 52.63 H, 4.93 N, 3.24
XI	H	Et	I	85	189–190° (EEt)	$C_{19}H_{22}INO_2$[e]	C, 53.89 H, 5.20 N, 3.29	C, 53.76 H, 5.53 N, 3.27
XII	H	*n*-Pr	I	89	184–185° (EWEt)	$C_{20}H_{24}INO_2$[e]	C, 54.69 H, 5.47 N, 3.20	C, 54.14 H, 5.55 N, 3.16
XIII	H	CH_2—⟨	I	72	245°, dec. (E)	$C_{21}H_{24}INO_2$[e]	C, 56.12 H, 5.35 N, 3.12	C, 56.20 H, 5.41 N, 3.51
XIV	H	CH_2—CH=CH_2	I	48	244°, dec. (EEt)	$C_{20}H_{22}INO_2$[e]	C, 55.17 H, 5.06 N, 3.22	C, 54.84 H, 4.97 N, 3.22
XV	H	CH_2—C≡CH	I	79	203°, dec. (EW)	$C_{20}H_{20}INO_2$[e]	C, 55.43 H, 4.62 N, 3.23	C, 55.20 H, 4.91 N, 3.09
XVI	H	CH_2—C_6H_5	I	58	207°, dec. (MEt)	$C_{24}H_{24}INO_2$[e]	C, 59.42 H, 4.95 N, 2.87	C, 59.30 H, 5.09 N, 2.94

[a] M = methanol; EEt = ethanol–ether; E = ethanol; W = water; EWEt = ethanol–water–ether; EW = ethanol–water; MEt = methanol–ether. [b] Weisbach and Douglas (5) give m.p. 262° (dec.). [c] Hydrochloride. [d] Baarschers *et al.* (11) give NMR data for this compound but do not otherwise characterize it or describe its preparation. [e] Hydroiodide.

tert-butyl ether accounts for its ease of cleavage under remarkably mild conditions, it seems reasonable that cleavage of the more strained ether function at Position 1 in the 1,2-dimethoxylated aporphine systems would relieve sufficient strain to account in part for the selectivity encountered. This proposal complements the hypothesis of steric inhibition of coplanarity of the Number 1 methoxyl.

No attempt was made to force the cleavage of the 1-hydroxy-2-methoxyaporphines to the diphenolic systems.

Thin-Layer Chromatography—Separation of the *N*-substituted nornuciferines (II–VIII) from their possible metabolites was attempted with 36 solvent systems; Table II lists the R_f values and detection characteristics of Compounds I–XVI in four solvent systems found suitable [*i.e.*, solvent systems yielding separation of each of the *N*-alkylated nornuciferines (II–VIII) from their *O*- and *N*-dealkylated congeners (I and IX–XVI)]. An advantage of the piperidine-containing systems (Systems 2–4) was that either the hydrohalide salts or free bases could be spotted and after development would reveal the same R_f values. Using Solvent System 1, comparable development without tailing was observed only with the free bases.

Detection was achieved with Dragendorff's reagent (3) and by fluorescence in 254-mμ radiation. With the latter immediately

Table II—Thin-Layer Chromatography

No.	R_f[a] in Solvent Systems[b] 1	2	3	4	Detection, Fluorescence[c]
I	0.16	0.46	0.31	0.45	Blue
II	0.49	0.57	0.54	0.57	Blue
III	0.53	0.62	0.61	0.64	Blue
IV	0.67	0.65	0.64	0.71	Blue
V	0.66	0.65	0.62	0.69	Blue
VI	0.74	0.65	0.63	0.70	Blue
VII	0.75	0.63	0.54	0.68	Yellow
VIII	0.81	0.69	0.64	0.77	Yellow
IX	0.08	0.32	0.17	0.35	Orange
X	0.34	0.47	0.29	0.48	Yellow
XI	0.36	0.51	0.35	0.52	Yellow
XII	0.51	0.53	0.38	0.53	Yellow
XIII	0.51	0.52	0.37	0.53	Yellow
XIV	0.66	0.52	0.33	0.53	Yellow
XV	0.74	0.48	0.31	0.50	Orange
XVI	0.75	0.52	0.38	0.52	Orange

[a] Average of 6 to 15 determinations; average of the percent standard deviation of R_f's of all compounds in Solvent Systems 1–4 = 4.4%. [b] With silica gel G plates, solvent systems employed were: 1, chloroform–methanol (93:7); 2, benzene–piperidine (9:1); 3, cyclohexane–chloroform–piperidine (8:1:1); 4, benzene–ethyl acetate–piperidine (6:3:1). [c] In 254-mμ radiation, after heating at 110° for 30 min.

after development, all compounds appeared as blue-fluorescing spots; with heating, characteristic and more intense orange, yellow, or blue spots were observed. Sensitivities using this technique were improved over Dragendorff's reagent, and the limit of detection was estimated to be less than 1 mcg. for most of the compounds.

Gas Chromatography (GC)—Initial GC attempts with nuciferine (II) utilizing copper columns revealed significant decomposition which was apparently metal-catalyzed and could be obviated by employing a glass column and glass-lined injection port. Good development of all compounds was attained, except for XV which appeared to decompose at all temperatures studied. Retention times, relative to nornuciferine (I), are indicated in Table III. Separation of all *N*-substituted nornuciferines (II–VIII) from their possible *N*- and *O*-dealkylated metabolites (I, IX–XIV, and XVI) was excellent, except in the *N*-ethyl series where *N*-ethylnornuciferine (III) could not be completely resolved from nornuciferine (I).

The satisfactory GC of the nornuciferine derivatives in general and the free phenolic systems (IX–XVI) in particular was somewhat unexpected, because these compounds readily decompose on heating. The successful GC of most of the phenolic aporphines may be due to intramolecular hydrogen bonding between the phenolic hydrogen and the oxygen of the vicinal methoxyl group (19, 20). Quantities analyzed were between 0.5 and 3.0 mcg., and curves of peak height *versus* amount chromatographed were linear for Compounds I–XIV and XVI.

EXPERIMENTAL[1]

***N*-[2-(3,4-Dimethoxyphenyl)ethyl]*o*-nitrophenylacetamide**—*Method A*—This was a modification of the method of Gulland and Haworth (21). A mixture of *o*-nitrophenylacetic acid (100 g.), 400 ml. of thionyl chloride, and 600 ml. of chloroform was refluxed for 3 hr.; then volatile materials were removed under reduced pressure at room temperature. The resulting red oil was added dropwise to a well-stirred and chilled mixture of 50 g. of 3,4-dimethoxyphenethylamine in 200 ml. of ether and a solution of 70 g. of sodium hydroxide in 1 l. of water; the resulting mixture was stirred at room temperature for 1 hr. The solid which separated was collected on a filter and washed several times with water. It was then dissolved in chloroform; this solution was washed with saturated sodium chloride solution, dried over sodium sulfate, and filtered. Removal of solvent from the filtrate gave a brown solid which was taken up in hot chloroform, treated with charcoal, filtered, and concentrated to 250 ml. Upon cooling, 113 g. (79%) of buff-colored crystals was deposited, m.p. 112° [lit. (21) m.p. 109–111°]. An IR spectrum (10% in chloroform) showed a peak at 1665 cm.$^{-1}$ (amide).

Method B—An excess of an ethereal solution of diazomethane was added to a solution of 37 g. of *o*-nitrophenylacetic acid in 200 ml. of ether, and the resulting solution was stirred overnight at room temperature. Removal of the ether left a brown oil which was added to a solution of 73 g. of 3,4-dimethoxyphenethylamine in 300 ml. of benzene, and the resulting mixture was refluxed for 10 hr. The benzene was removed under reduced pressure, and the residual brown solid was recrystallized from methanol to yield 59 g. (78%) of product, m.p. 112°.

***dl*-Nornuciferine (I)**—*Method C*—This was prepared by the method of Weisbach and Douglas (5) and was recrystallized, m.p. 121–125° [lit. (21), (−)-nornuciferine, m.p. 124–125°]. A polymorphic form crystallized from 2-propanol, m.p. 165–166°.

***dl*-Nuciferine Hydrochloride (II)**—*Method D*—Conversion of I to *dl*-nuciferine hydrochloride was by application of the method of Icke and Wisegarver (22). A solution of 3 g. of I, 40 ml. of 88% formic acid, and 40 ml. of 37% aqueous formaldehyde solution was refluxed for 5 hr.; then volatile materials were removed under reduced pressure. The final residue was taken up in 250 ml. of 2% sulfuric acid, the solution was filtered, and the filtrate was treated with an excess of 14% ammonium hydroxide. The resulting mixture was extracted with ether; this extract was washed with saturated sodium chloride solution, dried with sodium sulfate, filtered, and treated with anhydrous hydrogen chloride. The white precipitate which separated was recrystallized (Table I). A portion of the hydrochloride salt of II was treated with sodium hydroxide solution, and the gummy solid resulting was extracted with ether. This extract was washed with saturated sodium chloride solution, dried with sodium sulfate, filtered, and concentrated to deposit large, slightly yellow prisms, m.p. 137–140° [lit. (21) m.p. 136–137°].

***N*-Acylated Nornuciferines**—*Method E*—A solution of a twofold excess of the appropriate acid chloride and 5 g. of nornuciferine (I) in 100 ml. of dry pyridine and 200 ml. of anhydrous benzene was refluxed and stirred for 2 hr. After cooling and filtering, the reaction solution was washed with 200 ml. of water; the water was extracted with benzene which was added to the original organic phase, and the combined organic solutions were washed with water, dried with sodium sulfate, and filtered. The solvent was removed from the filtrate under reduced pressure. The solid residue was recrystallized (Table I).

***N*-Alkylnornuciferines (III–V)**—*Method F*—A suspension of 15.5 mmoles of the appropriate *N*-acylnornuciferine in 250 ml. of purified tetrahydrofuran (distilled from lithium aluminum hydride) was added to a suspension of 5.2 g. of lithium aluminum hydride

Table III—Gas Chromatography

No.	Retention Time[a] Relative to Nornuciferine (I) at Operating Conditions[b] A[c]	B[d]
I	1.00	1.00
II	0.84	—
III	0.94	—
IV	1.11	—
V	1.90	—
VI	1.95	—
VII	2.18	—
VIII	—	4.92
IX	1.63	1.59
X	1.37	—
XI	1.53	—
XII	1.83	—
XIII	3.14	—
XIV	1.95	—
XV[e]	—	—
XVI	—	7.87

[a] Average of 4 to 15 determinations. [b] See *Experimental*. [c] Retention time of nornuciferine (I), 7.8 min. (22 determinations). [d] Retention time of nornuciferine (I), 3.0 min. (4 determinations). [e] Decomposes at all temperatures employed.

[1] Melting points are corrected. IR spectra were recorded on Beckman IR-5A and IR-10 instruments. NMR spectra were obtained with a Varian A-60 instrument; samples were prepared as 10% solutions in DMSO-d_6 using tetramethylsilane as internal standard. GC was performed with a Hewlett-Packard 5750B gas chromatograph equipped with dual-flame ionization detectors.

in 250 ml. of purified tetrahydrofuran, and the resulting mixture was refluxed for 6 hr. Water (10.4 ml.) was carefully added; then 200 ml. of saturated ammonium tartrate solution was added and the mixture was stirred for 1 hr. at room temperature. The layers were separated and the aqueous layer was extracted repeatedly with ether. The combined organic phases were washed with saturated sodium chloride solution, dried with sodium sulfate, and filtered; the filtrate was evaporated under reduced pressure. The residual brown oil was dissolved in dry ether, and this solution was treated with anhydrous hydrogen chloride to yield a white precipitate which was recrystallized (Table I).

***N*-Alkylnornuciferines (VI and VII)**—*Method G*—A mixture of 2 g. of nornuciferine (I), a 0.1 molar excess of the appropriate alkyl bromide, a 0.5 molar excess of sodium bicarbonate, and 25 ml. of anhydrous ethanol was refluxed for 10 hr. The resulting mixture was filtered while hot, and the filtrate was concentrated under reduced pressure to give a brown oil. Treatment of an anhydrous ether solution of this oil with anhydrous hydrogen chloride gave a white precipitate which was recrystallized (Table I).

***dl*-*N*-Benzylnornuciferine (VIII)**—*Method H*—This was prepared from *N*-[2-(3,4-dimethoxyphenyl)ethyl]*o*-nitrophenylacetamide by the method of Weisbach and Douglas (5) and was recrystallized, m.p. 98–99° [lit. (5) 98–99.5°].

Hydroiodides of 1-Hydroxy-2-methoxynoraporphine (IX) and *N*-Alkylated Derivatives (X–XVI)—*Method I*—These were prepared by a modification of the method of Howell and Robertson (24). Nitrogen was passed through a suspension of 3 mmoles of the appropriate 1,2-dimethoxyaporphine (I–VIII) in 7 ml. of acetic anhydride. To this suspension, 4.8 ml. of 57% hydriodic acid (stabilized with 1.4% hypophosphorous acid) was carefully added. The reaction mixture was refluxed in an oil bath of 150° under a stream of nitrogen for 0.5 hr.; then it was permitted to cool to room temperature in a stream of nitrogen. Excess volatile material was removed under reduced pressure, and the solid residue was recrystallized (Table I).

Thin-Layer Chromatography—Silica gel G plates (20 × 20 cm.) were prepared from a slurry containing 3 g. of silica gel G[2] and 7 ml. of water per plate. After air drying, plates were activated at 110° for 1 hr. and kept in a desiccator until used. Developing distance throughout was 10 cm. with the solvent systems listed in Table II. All solvents were reagent grade. Detection was accomplished with Dragendorff's reagent (Thies, Reuther, modified by Vagufalvi) (3) and by fluorescence under 254-mμ radiation, either immediately after development or following heating at 110° for 30 min.

Gas Chromatography—Glass columns [182.9 cm. (6 ft.) × 4 mm. i.d.] were packed with 3% OV-17 on diatomaceous earth,[3] 100/120 mesh. Operating temperatures were: (*a*) injection port, 260°; column, 260°; and detector, 300°; (*b*) injection port, 290°; column, 290°; and detector, 320°. Conditions held constant were: carrier gas (helium), 120 ml./min. (50 psig); hydrogen, 40 ml./min. (13 psig); air, 440 ml./min. (30 psig); attenuation, 8; and range, 10^2. For GC, aqueous solutions of hydrohalide salts were treated with equal volumes of pH 8.0 *tris*-hydrochloric acid buffer (0.2 *M*); free bases were extracted into 1% isoamyl alcohol in *n*-heptane. All compounds except IX could be recovered as the free base by this procedure; extraction of IX required 25% isoamyl alcohol in *n*-heptane. Quantities analyzed were between 0.5–3.0 mcg.; curves of peak height *versus* amount chromatographed were plotted.

[2] Brinkmann Instruments, Westbury, N. Y.
[3] Gas-Chrom Q, Applied Science Laboratories, State College, Pa.

REFERENCES

(1) W. K. Sprenger, J. G. Cannon, B. K. Barman, and A. M. Burkman, *J. Med. Chem.*, **12,** 487(1969).

(2) D. V. Parke, "The Biochemistry of Foreign Compounds," Pergamon Press, New York, N. Y., 1968, p. 34.

(3) D. Waldi, in "Thin-Layer Chromatography, A Laboratory Handbook," E. Stahl, Ed., Academic, New York, N. Y., 1965, p. 279.

(4) R. R. Arndt, W. H. Baarschers, B. Douglas, E. C. Shoop, and J. A. Weisbach, *Chem. Ind. London*, **1963,** 1163.

(5) J. A. Weisbach and B. Douglas, *J. Org. Chem.*, **27,** 3738 (1962).

(6) M. Gates and T. A. Montzka, *J. Med. Chem.*, **7,** 127(1964).

(7) M. V. Koch, J. G. Cannon, and A. M. Burkman, *ibid.*, **11,** 977(1968).

(8) J. P. Brown, N. J. Cartwright, A. Robertson, and W. B. Whalley, *J. Chem. Soc.*, **1949,** 859.

(9) O. E. Edwards and K. L. Handa, *Can. J. Chem.*, **39,** 1801 (1961).

(10) I. C. R. Bick, J. Harley-Mason, N. Sheppard, and M. J. Vernengo, *J. Chem. Soc.*, **1961,** 1896.

(11) W. H. Baarschers, R. R. Arndt, K. Pachler, J. A. Weisbach, and B. Douglas, *ibid.*, **1964,** 4778.

(12) T. Kametani and I. Noguchi, *J. Chem. Soc. C*, **1969,** 502.

(13) F. Bock, *Monatsh. Chem.*, **23,** 1008(1902).

(14) A. Brossi, H. Burien, A. I. Rachlin, and S. Teitel, *J. Org. Chem.*, **32,** 1269(1967).

(15) H. Bruderer and A. Brossi, *Helv. Chim. Acta*, **48,** 1945 (1965).

(16) C. F. Wilcox, Jr., and M. A. Seager, *J. Org. Chem.*, **34,** 2319 (1969).

(17) M. Shamma and W. A. Slusarchyk, *Chem. Rev.*, **64,** 59 (1964).

(18) J. L. E. Erickson and W. H. Ashton, *J. Amer. Chem. Soc.*, **63,** 1769(1941).

(19) H. M. Fales and J. J. Pisano, in "Biomedical Applications of Gas Chromatography," H. A. Szymanski, Ed., Plenum Press, New York, N. Y., 1964, p. 56.

(20) G. J. Kapadia and G. S. Rao, *J. Pharm. Sci.*, **54,** 1817 (1965).

(21) J. M. Gulland and R. D. Haworth, *J. Chem. Soc.*, **1928,** 581.

(22) R. N. Icke and B. B. Wisegarver, in "Organic Synthesis," coll. vol. 3, E. C. Horning, Ed., Wiley, New York, N. Y., 1955, p. 723.

(23) S. M. Kupchan, B. Dasgupta, E. Fujita, and M. L. King, *Tetrahedron*, **19,** 227(1963).

(24) W. N. Howell and A. Robertson, *J. Chem. Soc.*, **1936,** 587.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 15, 1969, from the *Division of Medicinal Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication February 11, 1970.

Abstracted in part from a thesis submitted by Raymond J. Vavrek to the University of Iowa in partial fulfillment of Doctor of Philosophy degree requirements.

This research was supported by Grant NB-04349, National Institute of Neurological Diseases and Blindness, U. S. Public Health Service.

* To whom all correspondence should be addressed.

Oral Dosage Form Design and Its Influence on Dissolution Rates for a Series of Drugs

FOO SONG HOM and JOHN J. MISKEL

Abstract □ The study illustrates the influence oral dosage form design has on dissolution rates for drugs from different chemical and pharmacological classes. Comparative data demonstrate that relatively insoluble drugs, when formulated in soft elastic capsules, are released faster than from commercially available tablets. Faster dissolution from soft elastic capsules is believed to be due to the more rapid dispersion of the active ingredients. Rapid dispersion of medicaments is enhanced by the use of solubilizers and/or surfactants in the formulation design. Soft elastic capsules are recommended for the formulation of low-dose medications, of relatively insoluble drugs, and of drugs where early high-blood level of the drug is indicated.

Keyphrases □ Oral dosage form design effect—dissolution rates □ Capsules, soft elastic, tablets—drug release-rate comparison □ Rotating-bottle apparatus—dissolution testing □ UV spectrophotometry—analysis

In recent years, numerous scientists have demonstrated the direct relationship between proper formulation of a dosage form and the production of a clinically effective drug product. Many correlations have been established between formulation design and therapeutic activity, reporting the interrelation of particle size, dissolution, and absorption. In a review, Nelson (1) indicated that the rate of gastrointestinal absorption of a drug is often a function of the time needed for the drug to dissolve in the fluid at the site of absorption. He pointed out that, in general, the availability for absorption decreases in the order: solution > suspension > powder-filled capsule > compressed tablet > coated tablet. However, changes in this order may occur for various reasons, but they are exceptions. Tannenbaum *et al.* (2) demonstrated that in some cases, through the use of modern pharmaceutical techniques, it is possible to produce a tablet that results in greater drug absorption than a powder-filled capsule. Wagner *et al.* (3), in their study on the effect of dosage form on serum levels of indoxole, observed that the serum level response decreases in the order: emulsion (Lipomul-Oral) ≃ soft gelatin capsule > aqueous suspension > powder-filled capsule. Aguiar *et al.* (4) studied and correlated the effects of deaggregation or dispersion, dissolution, and *in vitro* gut-permeation rates on the chloramphenicol availability from four commercial lots of capsules. Glazko *et al.* (5) observed different absorption rates from capsules containing identical amounts of chloramphenicol from different manufacturers. Similarly, Brice and Hammer (6) observed significant differences in serum antibiotic levels obtained from 16 commercially available lots of oxytetracycline capsules distributed by 13 different suppliers.

The present work is a study of the influence of soft elastic capsule and tablet dosage forms on dissolution rates for a series of chemically and pharmacologically different drugs. While it will be agreed that *in vitro* dissolution rate data do not necessarily reflect *in vivo* absorption and availability, sufficient correlation exists to justify the use of this technique to measure potential dosage form efficiency (7–12). The rotating-bottle method (13–15) is used to compare the dissolution rates of soft elastic capsules and tablets.

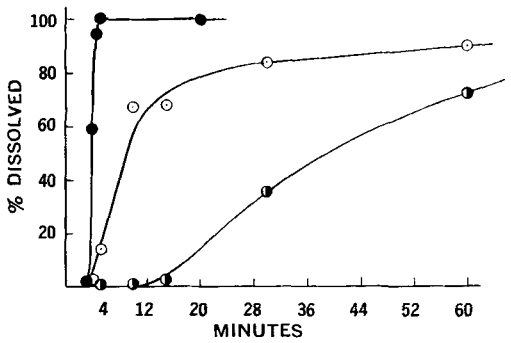


Figure 2—*Dissolution rates of ethinyl estradiol capsule and tablets in simulated gastric fluid T.S. Key: ●, soft elastic capsule; ⊙, Tablet A; and ◑, Tablet B.*

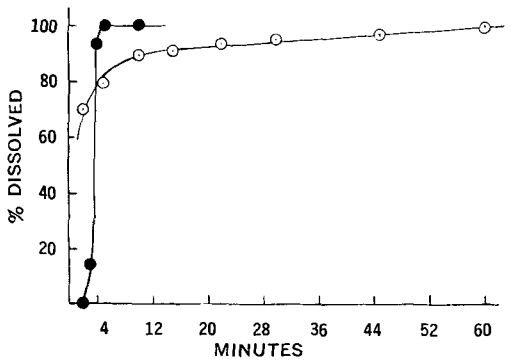


Figure 1—*Dissolution rates of hydrocortisone capsule and tablet in simulated gastric fluid T.S. Key: ●, soft elastic capsule; and ⊙, Tablet A.*

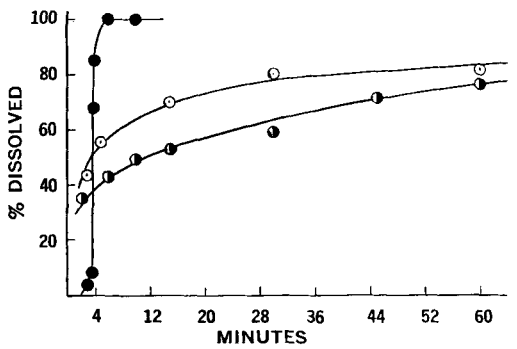


Figure 3—*Dissolution rates of diethylstilbestrol capsule and tablets in simulated gastric fluid T.S. Key: ●, soft elastic capsule; ⊙, Tablet A; and ◑, Tablet B.*

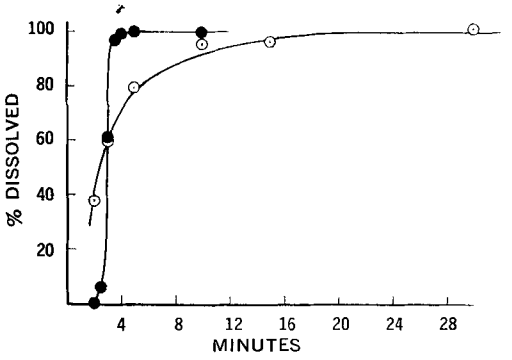


Figure 4—*Dissolution rates of phenobarbital capsule and tablet in simulated gastric fluid T.S. Key: ●, soft elastic capsule; and ⊙, Tablet A.*

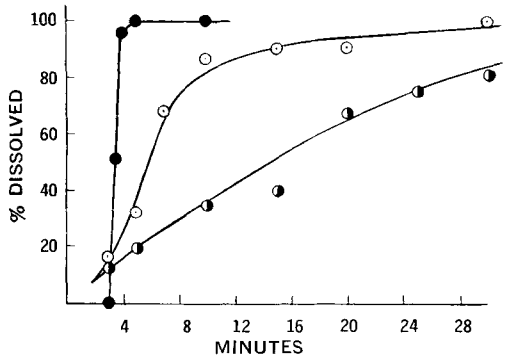


Figure 5—*Dissolution rates of reserpine capsule and tablets in simulated gastric fluid T.S. Key: ●, soft elastic capsule; ⊙, Tablet A; and ◑, Tablet B.*

EXPERIMENTAL

A drug must be formulated in one of several dosage forms, depending on its intended use. In general, a dosage form is designed for rapid and complete absorption of the drug. In some cases a dosage form may be developed for delayed action or for combined effects of quick onset and prolonged action. For rapid and complete absorption of a drug, it is best to formulate in solution. However, this is not always practical, as with very insoluble drugs. In this case the next best form is the suspension dosage form. Modern pharmaceutical technology embraces the use of surfactants and/or solubilizers to enhance the rapidity and maximum absorption of a drug. By nature, many surfactants and solubilizers are liquids at room temperature. Hence, these agents may play multifaceted roles in liquid formulations: as vehicles and interface modifiers. Formulations of a drug containing surfactants and/or solubilizers may be encapsulated conveniently in soft elastic capsules, as demonstrated in the present work which includes examples of relatively insoluble drugs.

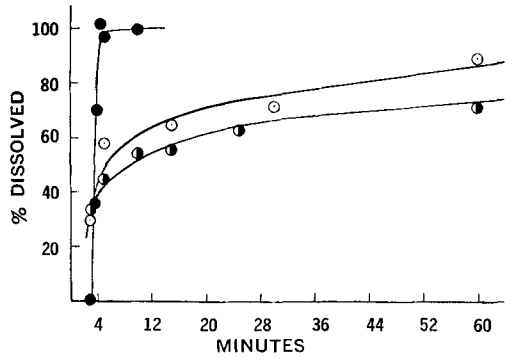


Figure 6—*Dissolution rates of digitoxin capsule and tablets in simulated gastric fluid T.S. Key: ●, soft elastic capsule; ⊙, Tablet A; and ◑, Tablet B.*

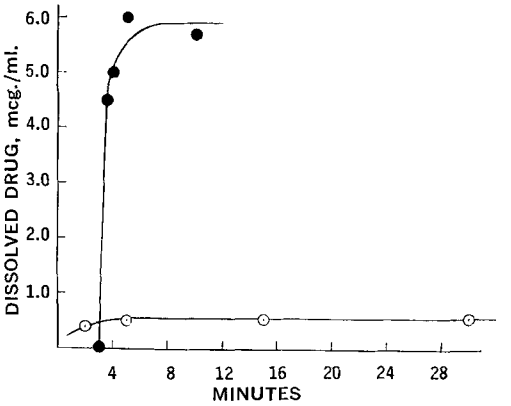


Figure 7—*Dissolution rates of bishydroxycoumarin capsule and tablet in simulated gastric fluid T.S. Key: ●, soft elastic capsule; and ⊙, Tablet A.*

Products Tested—For the purpose of this study the drugs chosen represent a number of chemical and pharmacological classes. The soft elastic capsules were manufactured using the continuous rotary die process. The drugs, where possible, were dissolved in polyethylene glycol 400 USP or suspended in various polyols with 1–3% of a nonionic surfactant. In some cases the dispersion vehicle was a nonionic surfactant or mixture of nonionic surfactants. Commercial capsules and tablets were purchased on the open market. These include "brand name" drugs and their generic counterparts.

Method—The rotating-bottle apparatus (13–15) was used for the dissolution tests in the following manner. Into each of the bottles was placed 50 ml. of simulated gastric fluid T.S. (without pepsin), and the bottles were allowed to come to temperature equilibrium in a water bath at 37.5 ± 0.1°. One tablet or capsule was added to each bottle and rotation begun at about 40 r.p.m. At suitable time intervals, a bottle was removed and the contents filtered immediately through a 0.22-μ Millipore filter in a microsyringe filter holder.[1] An aliquot of the clear filtrate was diluted and analyzed by appropriate USP (16) methods of colorimetry or spectrophotometry. Ethinyl estradiol was analyzed by a special colorimetric method (17). A technique similar to the one published by Burns *et al.* (18) was used to analyze phenylbutazone (λ = 265 mμ, 0.1 *N* NaOH). Similarly the method of UV spectrophotometry was used to analyze sulfadiazine (λ = 309 mμ, 0.1 *N* HCl) and propylthiouracil (λ = 275 mμ, H_2O).

RESULTS AND DISCUSSION

Results, where possible, were plotted as percent of the amount ultimately dissolved so as to remove differences of overages occur-

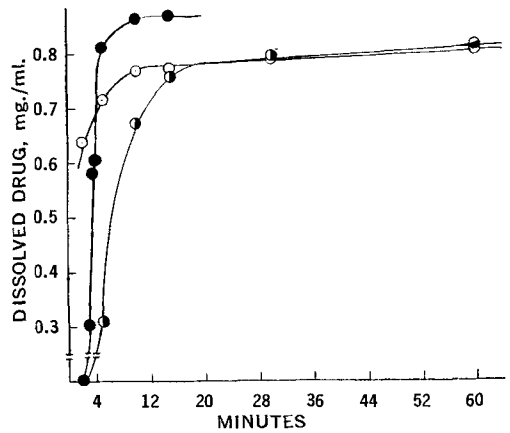


Figure 8—*Dissolution rates of sulfadiazine capsule and tablets in simulated gastric fluid T.S. Key: ●, soft elastic capsule; ⊙, Tablet A; and ◑, Tablet B.*

[1] Cat. No. XX30 025 00, Millipore Corp., Bedford, MA 01730

Table I—Dissolution Time in Minutes for 90% Drug Dissolved in Simulated Gastric Fluid at 37.5°

Drug	Dose, mg./ Capsule or Tablet	Soft Elastic Capsule,[a] min.	Tablet A, min.	Tablet B, min.
Hydrocortisone	5.0	4.0 ± 0.2	12 ± 1	
Ethinyl estradiol	0.05	4.2 ± 0.1	60 ± 2	83 ± 2
Diethylstilbestrol	0.5	4.2 ± 0.1	96 ± 8	96 ± 8
Phenobarbital	32.0	3.1 ± 0.3	9 ± 0.5	
Reserpine	0.25	3.9 ± 0.3	14 ± 2	35 ± 2
Digitoxin	0.2	4.2 ± 0.2	70 ± 8	136 ± 8

[a] Solution of drug encapsulated.

Table II—Amount in Micrograms of Drug Dissolved per Milliliter of Simulated Gastric Fluid at 37.5° after 5 Min. of Dissolution

Drug	Dose, mg./ Capsule or Tablet	Soft Elastic Capsule,[a] mcg./ml.	Tablet A, mcg./ml.	Tablet B, mcg./ml.
Bishydroxycoumarin	25.0	5.9 ± 0.3	0.6 ± 0.1	
Sulfadiazine	500.0	810 ± 5	715 ± 5	310 ± 25
Phenylbutazone	100.0	100 ± 30	1.5 ± 0.5	
Propylthiouracil	50.0	980 ± 20	380 ± 20	250 ± 20

[a] Suspension of drug encapsulated.

ring in different products. With practically insoluble drugs, where only a fraction of the dose is dissolved, the amount of drug dissolved in 1 ml. of fluid was plotted *versus* dissolution time. Completion of dissolution, the saturation point in the case of poorly soluble drugs, was taken as the time on the graph when a smooth curve through the points reached a maximum. Typical curves are shown in Figs. 1–10. Figures 1–6 are plots of percent drug dissolved *versus* time from tablets and from solutions encapsulated in soft elastic capsules. Figures 7–10 are plots of milligrams or micrograms of drug dissolved per milliliter of fluid *versus* time for commercial tablets and for suspensions encapsulated in soft elastic capsules. A cursory observation of Figs. 1–10 is sufficient to conclude that dissolution of the drug from soft elastic capsules is faster than from tablets in simulated gastric fluid T.S. (without pepsin) at 37.5°. However, a quantitative comparison may be made by observing the average time from duplicate runs for dissolution of 90% of the drug, as shown in Table I. Table II lists the average amount in micrograms from duplicate runs of relatively insoluble drugs dissolved per milliliter of fluid after 5 min. of dissolution. An examination of Table I reveals that a drug, when formulated in soft elastic capsules, will have a dissolution rate from 3 to 30 times faster than tablets. It is apparent that the increase in dissolution rate is due to the rapid dispersion of the drug when the capsule splits open. Also, the use of a proper vehicle, solubilizer, and surfactant apparently helps to enhance the dispersion of a relatively insoluble drug. Table II details the average increase, provided by the soft elastic capsule, in the apparent solubility of the drug ranging from 1.1 to 67 times that of tablets reflecting the effects of the vehicle, solubilizer, and surfactant.

The data indicate that it is possible to solubilize many low-dose, relatively insoluble drugs and to encapsulate them in soft elastic capsules of appropriate sizes. For high-dose and very insoluble drugs, it is preferable to encapsulate them as suspensions. With the applications of these two techniques in encapsulation, conceivably one can either singly or collectively use solubilizers and/or surfactants as specially designed vehicles for each drug. The soft elastic capsule is an ideal dosage form for drugs such as the oral contraceptives where submilligram unit doses are common.

An appreciation of the profound influence dosage form design has on dissolution, absorption, and efficacy (1–12) should preclude sole reliance on conventional tablet and powder-filled capsule technology in the development of "new drug" dosage forms. The costs of carrying new drug dosage forms through toxicity studies, clinical trials, and a new drug application procedure make errors in this area disastrous. Solutions and suspensions in soft elastic capsules, as well as tablets and dry-filled capsules, should be investigated in early stages of development employing *in vitro* dissolution studies. Availability as provided by the more promising dosage forms should then be appraised by *in vivo* animal studies.

CONCLUSIONS

The study illustrates the influence of oral dosage form design on dissolution rates for drugs from different chemical and pharma-

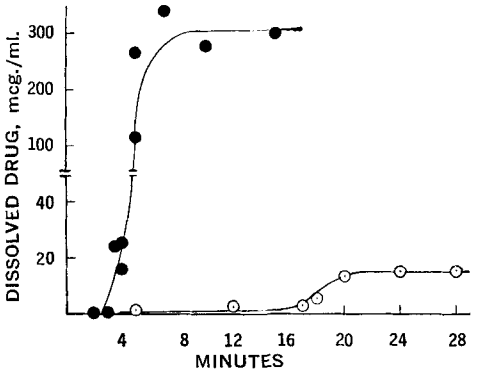


Figure 9—*Dissolution rates of phenylbutazone capsule and tablet in simulated gastric fluid T.S. Key: ●, soft elastic capsule; and ⊙, Tablet A.*

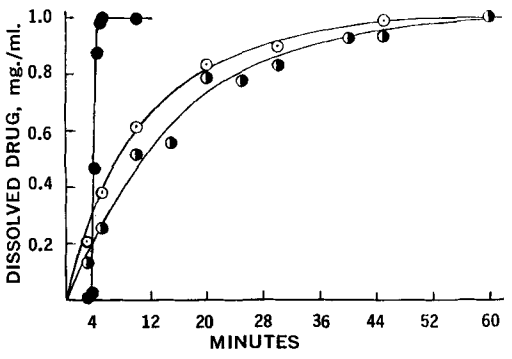


Figure 10—*Dissolution rates of propylthiouracil capsule and tablets in simulated gastric fluid T.S. Key: ●, soft elastic capsule; ⊙, Tablet A; and ◑, Tablet B.*

cological classes. It also suggests avenues that should not be overlooked when investigating new drugs or improving older medications. The results demonstrate that many relatively insoluble drugs may be readily formulated in soft elastic capsules and have faster dissolution rates than tablets in that solutions or suspensions of a drug can be readily encapsulated. Furthermore, surfactants or other compounds may be encapsulated along with the drug so as to enhance its solubility and potential absorption rate. Soft elastic capsules are recommended in the formulations of low-dose medication, of relatively insoluble drugs, and of drugs where early high-blood level of the drug is indicated.

REFERENCES

(1) E. Nelson, *Clin. Pharmacol. Ther.*, **3**, 673(1962).

(2) P. J. Tannenbaum, E. Rosen, T. Flanagan, and A. P. Crosley, Jr., *ibid.*, **9**, 598(1968).

(3) J. Wagner, E. S. Gerard, and D. G. Kaiser, *ibid.*, **7**, 610 (1966).

(4) A. J. Aguiar, L. M. Wheeler, S. Fusari, and J. E. Zelmer, *J. Pharm. Sci.*, **57**, 1844(1968).

(5) A. J. Glazko, A. W. Kinkel, W. C. Alegnani, and E. L. Holmes, *Clin. Pharmacol. Ther.*, **9**, 472(1968).

(6) G. W. Brice and H. F. Hammer, *J. Amer. Med. Ass.*, **208**, 1189(1969).

(7) S. Symchowicz and B. Katchen, *J. Pharm. Sci.*, **57**, 1383 (1968).

(8) W. E. Moore, G. A. Portmann, H. Stander, and E. W. Mc Chesney, *ibid.*, **54**, 36(1965).

(9) B. Calesnick, B. Katchen, and J. Black, *ibid.*, **54**, 1277(1965).

(10) F. A. Campagna, G. Cureton, R. A. Mirigian, and E. Nelson, *ibid.*, **52**, 605(1963).

(11) G. Levy, N. A. Hall, and E. Nelson, *Amer. J. Hosp. Pharm.*, **21**, 402(1964).

(12) G. Levy, *Can. Med. Ass. J.*, **90**, 978(1964).

(13) J. C. Souder and W. C. Ellenbogen, *Drug Stand.*, **26**, 77 (1958).

(14) E. O. Kreuger and E. B. Vliet, *J. Pharm. Sci.*, **51**, 181(1962).

(15) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965; second suppl., 1967, p. 15.

(16) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

(17) L. Chafetz, M. Boudjouk, D. Tsilifonis, and F. S. Hom, *J. Pharm. Sci.*, **57**, 1000(1968).

(18) J. J. Burns, R. K. Rose, T. Chenkin, A. Goldman, A. Schulert, and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, **109**, 346(1953).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 22, 1969, from the *Research and Development Department, R. P. Scherer Corp., Detroit, MI 48213*

Accepted for publication December 11, 1969.

DRUG STANDARDS

Chemical Standardization and Quality Assurance of Whole Crude Coal Tar USP Utilizing GLC Procedures

M. GRUBER, R. KLEIN, and M. FOXX

Abstract □ A procedure has been developed which utilizes gas-liquid chromatographic (GLC) analysis for the chemical standardization of medicinal crude coal tar USP. A similar method is recommended for the determination of coal tar fractions in Liquor Carbonis Detergens (LCD) (coal tar solution USP). Data confirming that LCD and similar "extracts," "fractionates," and "synthetics" cannot be considered as generic, pharmaceutical, or medicinal equivalents of a properly standardized whole crude coal tar are presented.

Keyphrases □ Crude coal tar—analysis □ Coal tar solution—analysis □ Ethanol content, coal tar solution—determination □ GLC—analysis.

Dioscorides, a Greek physician, described nearly 2000 years ago the merits of asphaltic tar in the "Materia Medica" as a treatment for cutaneous disorders (1). The advantages of the empirical use of "tars" were subsequently emphasized by numerous investigators including Brocq (2), White (3), and Goeckerman (4, 5).

In modern times, this medication is widely prescribed for various skin diseases, such as psoriasis and eczema, which are frequently severe and occasionally disabling. In addition, this modality is routinely prescribed for seborrheic dermatitis, occupational and contact dermatitis, dermatophytosis, varicose eczema, chronic and exudative and lichenoid dermatitis, pruritis ani, and various other chronic skin disorders.

Although therapeutic response is often dramatic, the known variability of coal tar composition and consequent inconsistency of clinical results has made this medication the subject of complaint and controversy among dermatologists.

This ancient but fundamental topical drug is virtually devoid of any guardian standards of chemical composition. Consequently, almost any coal tar, regardless of its composition, may satisfy the requirements of current official compendia for crude coal tar. Practically no controls have been established to assure uniformity, potency, safety, and efficacy. It is, therefore, quite evident that the scientific development of far more definitive drug reference standards and methods of analysis for this valuable, but variable, therapeutic agent is mandatory. No proficient effort has been initiated to create an effective method to control the physical and

cological classes. It also suggests avenues that should not be overlooked when investigating new drugs or improving older medications. The results demonstrate that many relatively insoluble drugs may be readily formulated in soft elastic capsules and have faster dissolution rates than tablets in that solutions or suspensions of a drug can be readily encapsulated. Furthermore, surfactants or other compounds may be encapsulated along with the drug so as to enhance its solubility and potential absorption rate. Soft elastic capsules are recommended in the formulations of low-dose medication, of relatively insoluble drugs, and of drugs where early high-blood level of the drug is indicated.

REFERENCES

(1) E. Nelson, *Clin. Pharmacol. Ther.*, **3**, 673(1962).

(2) P. J. Tannenbaum, E. Rosen, T. Flanagan, and A. P. Crosley, Jr., *ibid.*, **9**, 598(1968).

(3) J. Wagner, E. S. Gerard, and D. G. Kaiser, *ibid.*, **7**, 610 (1966).

(4) A. J. Aguiar, L. M. Wheeler, S. Fusari, and J. E. Zelmer, *J. Pharm. Sci.*, **57**, 1844(1968).

(5) A. J. Glazko, A. W. Kinkel, W. C. Alegnani, and E. L. Holmes, *Clin. Pharmacol. Ther.*, **9**, 472(1968).

(6) G. W. Brice and H. F. Hammer, *J. Amer. Med. Ass.*, **208**, 1189(1969).

(7) S. Symchowicz and B. Katchen, *J. Pharm. Sci.*, **57**, 1383 (1968).

(8) W. E. Moore, G. A. Portmann, H. Stander, and E. W. Mc Chesney, *ibid.*, **54**, 36(1965).

(9) B. Calesnick, B. Katchen, and J. Black, *ibid.*, **54**, 1277(1965).

(10) F. A. Campagna, G. Cureton, R. A. Mirigian, and E. Nelson, *ibid.*, **52**, 605(1963).

(11) G. Levy, N. A. Hall, and E. Nelson, *Amer. J. Hosp. Pharm.*, **21**, 402(1964).

(12) G. Levy, *Can. Med. Ass. J.*, **90**, 978(1964).

(13) J. C. Souder and W. C. Ellenbogen, *Drug Stand.*, **26**, 77 (1958).

(14) E. O. Kreuger and E. B. Vliet, *J. Pharm. Sci.*, **51**, 181(1962).

(15) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965; second suppl., 1967, p. 15.

(16) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

(17) L. Chafetz, M. Boudjouk, D. Tsilifonis, and F. S. Hom, *J. Pharm. Sci.*, **57**, 1000(1968).

(18) J. J. Burns, R. K. Rose, T. Chenkin, A. Goldman, A. Schulert, and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, **109**, 346(1953).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 22, 1969, from the *Research and Development Department, R. P. Scherer Corp., Detroit, MI 48213*

Accepted for publication December 11, 1969.

DRUG STANDARDS

Chemical Standardization and Quality Assurance of Whole Crude Coal Tar USP Utilizing GLC Procedures

M. GRUBER, R. KLEIN, and M. FOXX

Abstract □ A procedure has been developed which utilizes gas-liquid chromatographic (GLC) analysis for the chemical standardization of medicinal crude coal tar USP. A similar method is recommended for the determination of coal tar fractions in Liquor Carbonis Detergens (LCD) (coal tar solution USP). Data confirming that LCD and similar "extracts," "fractionates," and "synthetics" cannot be considered as generic, pharmaceutical, or medicinal equivalents of a properly standardized whole crude coal tar are presented.

Keyphrases □ Crude coal tar—analysis □ Coal tar solution—analysis □ Ethanol content, coal tar solution—determination □ GLC—analysis.

Dioscorides, a Greek physician, described nearly 2000 years ago the merits of asphaltic tar in the "Materia Medica" as a treatment for cutaneous disorders (1). The advantages of the empirical use of "tars" were subsequently emphasized by numerous investigators including Brocq (2), White (3), and Goeckerman (4, 5).

In modern times, this medication is widely prescribed for various skin diseases, such as psoriasis and eczema, which are frequently severe and occasionally disabling. In addition, this modality is routinely prescribed for seborrheic dermatitis, occupational and contact dermatitis, dermatophytosis, varicose eczema, chronic and exudative and lichenoid dermatitis, pruritis ani, and various other chronic skin disorders.

Although therapeutic response is often dramatic, the known variability of coal tar composition and consequent inconsistency of clinical results has made this medication the subject of complaint and controversy among dermatologists.

This ancient but fundamental topical drug is virtually devoid of any guardian standards of chemical composition. Consequently, almost any coal tar, regardless of its composition, may satisfy the requirements of current official compendia for crude coal tar. Practically no controls have been established to assure uniformity, potency, safety, and efficacy. It is, therefore, quite evident that the scientific development of far more definitive drug reference standards and methods of analysis for this valuable, but variable, therapeutic agent is mandatory. No proficient effort has been initiated to create an effective method to control the physical and

chemical properties of this medicinal substance with the exception of an exploratory investigation by de Martin and Cyr in 1953 (6).

GENERAL CHARACTERISTICS

Crude coal tar is an extremely complex by-product of the destructive distillation of coal. There are uncontrolled qualitative and quantitative chemical and pharmacological differences, dependent upon the source of raw material, method, and temperature of distillation, shape and size of retorts, and other factors (7). Accordingly, since the medicinal qualities of this material are dependent upon the aggregate effect of its hundreds of discrete components, many of which have never been identified, considerable variations in clinical results are apparently unavoidable. A lack of uniformity and the variations in therapeutic effect of different coal tars have caused concern (8).

All attempts to retain the therapeutic effects, while simultaneously removing the objectionable black color, odor, and staining properties of coal tar, have been futile, although it has been "fractionated," "separated," "extracted," "filtered" and "synthesized" to improve its esthetic appearance. The relatively clear substances which have emerged possess one common deficiency, namely a considerable reduction of pharmacological activity caused by the removal of the "objectionable" tar fractions including pitch, carbon, and asphaltic compounds (9).

Even its mode of action has not been satisfactorily defined, having been variously described as "reducing," "photosensitizing," "irritant," "antiseptic," "antipruritic," "keratoplastic," "antiacanthotic," "vasoconstrictive," "antiparasitic," "antifungal," and "antibacterial."

Coal tar is described in the USP XVII as a "nearly black, viscous liquid, heavier than water, having a characteristic, naphthalene-like odor and a sharp, burning taste" (10). These vague descriptions have only compounded the physician's problem of attempting to obtain consistent and uniform clinical results.

The monograph on LCD (coal tar solution USP) is equally obscure (11). The only quantitative specification is for alcohol concentration. This permits enormous variation in the tar-extract content and the incorporation of such dissimilar constituents as to make this qualification virtually meaningless.

For the past 15 years, the authors' control laboratory has employed, with some success, the methods introduced by de Martin and Cyr (6). These techniques considerably improved quality and uniformity, but were inadequate for assuring a specific, chemically standardized, medicinal "whole" coal tar. During the past 2 years, the authors' have utilized GLC to achieve this goal.[1]

Other attempts have been made to standardize the therapeutic response which physicians could reasonably anticipate from tar by comparing relative bioassays or measurements of the photodynamic action of tar on normal guinea pig skin (12). Photobiological activity as the quality control determinant of tar, manifested by sensitization effects on human, rabbit, guinea pig, or other mammalian skin, is deceiving since this attribute of coal tar is related primarily to its anthracene–acridine content (9). The fluorescence under longwave UV light is easily induced or modified by the mere presence of anthracene and/or acridine in sufficient amounts. Accordingly, UV photosensitization cannot assure a medicinally acceptable grade of raw material since this characteristic bears only a singular relationship to the total properties of coal tar.

Some manufacturers have incorporated surface-active agents with coal tar and equated their antifungal release activities as a measure of their biological effect (13). Anticipated therapeutic performance, as measured by the reference standard of antiseptic properties, is also unpredictable. Coal tars, regardless of composition, demonstrate considerable fungistatic and bacteriostatic activity. These properties, while of value, have never been demonstrated to be primarily responsible for the clinical efficacy of tar. They are simply additional chemical and therapeutic attributes.

A gas chromatographic procedure is presented for the quality control of crude coal tar, as well as LCD (coal tar solution USP).

[1] Zetar (colloidal whole crude coal tar USP), manufactured by Dermik Laboratories, Inc., Syosset, N. Y.

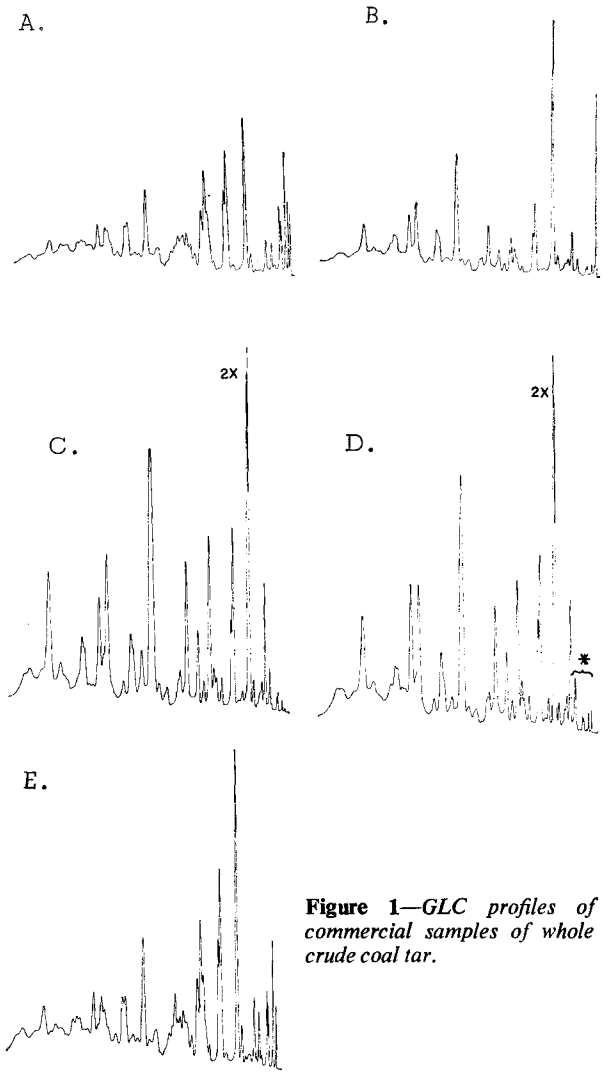


Figure 1—*GLC profiles of commercial samples of whole crude coal tar.*

Comparison of the various samples of each indicates the wide and uncontrolled range of presently admissable materials and demonstrates why consistent clinical results are not possible under the present official specifications defining this important topical drug.

The scientific data and information developed would enable the preparation of a monograph, which would exclude from official recognition any coal tar that does not possess comparable physical characteristics and chemical composition.

EXPERIMENTAL

In order to provide a more selective and specific tar analysis, it was necessary to analyze a number of market samples. These were obtained from various commercial sources and pharmacies, selected at random throughout the U. S. Although all of the samples secured were labeled USP and met present monograph specifications for coal tar and coal tar solution, they did nevertheless demonstrate marked physical differences and profound chemical variations both qualitatively and quantitatively.

Apparatus—A dual column Varian Aerograph model 204B chromatograph equipped with dual flame-ionization detectors was employed for all GLC work. Injection port and detector temperatures were 275 and 280°, respectively. Flow rates of 40 ml. He/min., 30 ml. H_2/min., and 300-ml. filtered air/min. (supplied by two Oscar's Vibrator Air Pumps) were maintained. Specific parameters are listed for each type of sample and analysis in the sections following.

Methods and Procedures—*Crude Coal Tar*—GLC sample size was 0.3 μl. supplied from a Hamilton No. 7101 1-μl. syringe. These

Table I—Solubility and Specific Gravity Data for Illustrated Crude Coal Tar Samples

Sample	CS_2, % Insoluble	CCl_4, % Insoluble	Carbenes, %	Benzol, % Insoluble	Sp. Gr.$_{25°}$
A	12.42	28.42	16.00	17.41	1.2011
B	9.64	16.89	7.25	14.22	1.2131
C	3.42	9.64	6.24	6.42	1.4232
D	3.39	10.40	7.01	8.21	1.4309
E	6.71	14.02	7.31	12.19	1.2247

samples were programmed on a 1.52-m. × 0.32-cm. (5-ft. × 0.125-in.) o.d. stainless steel column packed with 5% SE 30 on 80–100 mesh (chromasorb W, acid washed) from 110 to 255° at 10°/min., and held at the upper limit until no further peaks were recorded.

The variability of the chromatographic portion of crude coal tar was verified using a liquid-solid chromatographic technique on the samples, which involved filling a 22-mm. o.d. chromatographic tube equipped with a stopcock to a depth of 150 mm. with alumina (Grade F-20, Aluminum Corp. of America), and washing with 200 ml. of reagent grade petroleum ether. An accurately weighed 1-g. sample of the crude coal tar was macerated with 5 g. of alumina (prewashed with petroleum ether) and quantitatively transferred to the tube with the aid of 25 ml. of petroleum ether. The sample was eluted using 350 ml. of petroleum ether and the eluate collected at a rate of 4 ml./min. in a tared 400-ml. beaker, evaporated to near dryness on a steam bath under nitrogen, and the remainder of the solvent spontaneously evaporated at room temperature under a stream of nitrogen. The sample was desiccated for 15 min. and weighed. The column was further eluted with 350 ml. of reagent grade ethyl acetate in the same fashion. The residues were in turn run *via* GLC to determine if any significant portion of the petroleum ether eluate was lost in evaporation or any significant amounts of chromatographable material were present in the ethyl acetate fraction.

All other wet tests were performed according to the methods described by de Martin and Cyr (6).

LCD (Coal Tar Solution USP)—*Tar Determination*—GLC samples were 2.50 μl. using a Hamilton No. 7105, 5-μl. syringe for injection, programmed from 100 to 255° at 10°/min., and held at the upper limit using the same column as for coal tar. The initial temperature was decreased to allow for better separation of ethanol from the tar constituents. The percent tar was calculated by comparison of peak heights of naphthalene, methyl naphthalene, and phenanthrene *versus* those obtained from a standard using the same crude coal tar diluted to exactly 20.00% w/v in carbon disulfide A.R., according to the calculation:

$$\% \text{ coal tar} = \frac{Ji}{Js} \times \frac{Vs}{Vi} \times Cs \qquad \text{(Eq. 1)}$$

where Ji is the height of peak j in the sample; Js is the height of peak j in the standard; Vs is the injected volume (μl.) of standard; Vi is the injected volume (μl.) of sample; and Cs is the concentration (% w/v) of tar in CS_2 used as standard.

Ethanol Determination—The column used is 1.52-m. × 0.32-cm. (5-ft. × 0.125-in.) o.d. stainless steel filled with 10% polyethylene glycol[2] on chromasorb W (acid washed) isothermally at 70° with all other parameters as cited previously. The technique involves the use of 20.00% (v/v) acetone A.R./methanol A.R. as internal standard. The height responses were found to be linear between 0.2 μl. and 2.5 μl. of standard. Each analysis is performed using three injections of various volumes between 0.5 and 1.5 μl., both for standard and sample. The ethanol response was plotted *versus* acetone response. Percentage of ethanol is found by determining the appropriate responses for ethanol from the graph in both sample and standard at the same acetone response and employing the following equation:

$$\% \text{ ethanol} = \frac{Ru}{Rs} \times \frac{\text{sp.gr.}_s \times \% Es}{\text{sp.gr.}_u} \qquad \text{(Eq. 2)}$$

where Ru is the response of sample from graph; Rs is the response of standard from graph; sp. gr.$_s$ is the specific gravity of standard ethanol at temperature T; sp. gr.$_u$ is the specific gravity of sample at temperature T; % Es is the percentage of ethanol w/w in standard; *i.e.*, % Es of 95% ethanol = 92.3. The results were checked according to the USP determination for ethanol as stated in the monograph.

Thirty-five random samples, labeled "crude coal tar USP" of exactly 0.3 μl. each were chromatographed under these conditions; five are presented in Fig. 1 for the purpose of illustration. Samples A and B were obtained from pharmacies, C is representative of material used by these laboratories, and D and E are samples from different batches obtained from another pharmaceutical manufacturer.

[2] Carbowax 20M, Union Carbide Corp., New York, N. Y.

Table II—Comparison of Liquid *vs.* Gas Chromatography for the Determination of Chromatographic Portion of Crude Coal Tar

Sample	% Chromatographic *via* GLC		% Chromatographic *via* Liquid Chromatography
	vs. Phenanthrene	*vs.* Naphthalene	
A	12.1	12.4	13.2
B	17.4	17.0	15.9
C	26.2	26.9	25.8
D	27.1	27.0	27.4
E	21.1	20.8	20.2

RESULTS AND DISCUSSION

The discrepancies found among the random samples of coal tar USP, both qualitatively and quantitatively, are enormous. The solubility and specific gravity data presented in Table I corroborate the observations of de Martin and Cyr (6); although in the case of Samples D and E, no positive prediction as to formulation compatibility could be made from the data obtained in wet tests. Predictions based on chromatographic evidence were subsequently substantiated by experimental incorporation into washable and greasy bases.

Crude coal tars A, B, and E yielded coarsely dispersed, inelegant pharmaceuticals. Even crude tar received from the same supplier may vary markedly from lot to lot, yielding different GLC profiles and producing visible physical variations in finished products while conforming to USP specifications. The preparations containing material from Samples A through E were not evaluated clinically. However, clinical trials previously conducted demonstrated that different tars produce significantly different patient responses on treatment of various dermatological conditions.

The evident chromatographic differences were checked on a weight basis by GLC and column chromatography to determine the validity of the measurement technique employed. These results are presented in Table II.

The petroleum ether fraction obtained by column chromatography indicated significant losses of benzene, toluene, xylenes, and pyridine. These, however, comprise less than 5% of the chromatographable portion taken on an area response basis; the overall net loss was considered insignificant. More important, excluding the peaks for xylenes, pyridine, benzene, and toluene (starred peaks in Fig. 1), the chromatographic traces were virtually identical to those obtained from the original samples of crude tar. Quantitative measurements based on naphthalene and phenanthrene responses, when compared to the original, yielded results which approximated those found on a weight basis (Table II). No significant amounts of chromatographable material were evident in the trace of the ethyl acetate fractions. Modifications of this technique are being used in this laboratory as a separation procedure for estimating the quality and quantity of coal tar in various preparations. It is obvious that additional separations must be performed if significant amounts of nonpolar materials, such as mineral oil, are present in the finished formulation. However, for this investigation, it did serve to provide additional quantitative evidence of the extent of composite differences which might be expected.

LCD—Exactly 2.50 μl. of each of the five samples of coal tar solution (Fig. 2) were chromatographed under the conditions described. Because of the large amount of ethanol present, it was anticipated that the sample size would be inconsistent. Included in

Table III—Reproducibility of Peak Heights for LCD Samples (Sample C, Fig. 2)

Peak	Height, mm. Run 1	Height, mm. Run 2	Reproducibility[a]
a	110.0	109.0	±0.5%
b	29.8	33.5	±5.5%
c	33.0	34.5	±1.4%
d	31.0	32.0	±1.6%
e	163.2	165.5	±0.6%
f	67.0	65.5	±1.1%
g	45.8	44.8	±1.2%
h	35.0	38.6	±4.4%

[a] $\bar{X}$ = ±1.6%.

Fig. 2 are replicate runs of Sample C with peaks labeled "a" through "h." Height ratios were taken as a function of Run 2 *versus* Run 1. These data are presented in Table III and indicate an average error of ±1.6%, which was found to be typical and acceptable.

Each of these samples fulfilled the requirement for coal tar solution as directed in USP XVII (*i.e.*, ethanol content between 81% and 86%). Although no quantitation of the coal tar content of these solutions was attempted, inasmuch as the original tar samples were not available, two coal tar solutions were prepared in the laboratory according to the USP procedure using one randomly selected tar sample. Analyses were performed *versus* the original tar diluted to exactly 20.00% w/v in CS_2 *via* GLC using the conditions noted previously. Peaks A, B, and C (naphthalene, methyl naphthalene, and phenanthrene) were used for calculation. The chromatograms are shown in Fig. 3 and the data presented in Table IV. Sample I was estimated to contain 20.29% w/v chromatographable tar and Sample II, 20.46% w/v tar.

Some variations were noted, particularly that the results derived from the phenanthrene peak produced lower, although acceptable, results than either of the other two employed. The authors' suspect that some sorption of phenanthrene occurs on the sand and, from the data, it appears to be approximately 6.5% of the amount present. Since a detailed investigation was not performed regarding this possibility, the authors chose to include no correction and the results are presented as a mean of the determinations from each of the three selected peaks. On an overall basis, no significant sorption of the chromatographable portion of the tar is apparent, thereby presenting a much more valid assay procedure for coal tar solution USP (LCD).

It is important to emphasize that *none of the LCD and "extract" samples actually contain the labeled amount of coal tar (20%).* This figure (20%) is based on the amount of crude coal tar initially added to the ethanol, sand, and polysorbate 80, and not the amount of coal tar remaining in the extract after filtration. For example, Sample I of LCD was analyzed for water content *via* the classical Karl Fischer method and found to contain 6.5%. Combined with the ethanol data (81.39% per USP analysis), this leaves a material balance of 12.1% of which 5.0% is polysorbate 80. (The polysorbate 80 does not, for all practical purposes, ab(ad)sorb on the sand used in the laboratory preparation of LCD.) This leaves *approximately 7% tar "fractions" in the LCD solution*, the bulk of which is chromatographable.

Previous clinical research by Obermayer (9) has demonstrated that no particular fraction or fractions derived from whole crude coal tar yield total clinical results comparable to whole crude coal tar. In fact, certain of the fractions he tested had little or no medicinal value. It is quite evident, therefore, that even a "standardized" LCD constitutes only a fraction of the total composition of crude coal tar and cannot be considered a 20% solution. It similarly cannot be expected to produce comparable and reproducible therapeutic results.

Finally, a check of a simple GLC technique for the determination of ethanol was performed to assure the validity of the findings. Each of the samples was analyzed in duplicate and confirmed by the USP distillation procedure. These data were presented in Table V. All were found to conform, as stated previously, to the USP monograph for coal tar solution (LCD).

As an alternate to polyethylene glycols,[2] the authors have used a column of 1.52-m. × 0.32-cm. (5-ft. × 0.125-in.) o.d. stainless steel, filled with Poropak Q, isothermally at 150° with some success. However, this column becomes inconvenient when several samples of coal tar solution are to be analyzed since the elution of portions of the tar interferes with subsequent injections. Clearing of the column overnight at elevated temperatures was not always sufficient. Since no problem of this type was found with the polyethylene glycols[2] column (overnight purging at 195° was sufficient to clear it of retained chromatographable tar components), this is the column of choice for this procedure.

CONCLUSIONS AND SUMMARY

From the data reported in the literature regarding whole crude coal tar and LCD, certain differences were expected due to the variability of source, conditions of distillation, and modes of collection used in the manufacture of coal tar. This investigation has demonstrated that the situation is far more critical than originally suspected. The combination of previously discussed factors has resulted in an incompletely controlled drug and the indiscriminate manufacture of crude coal tar substitutes which include fractionated

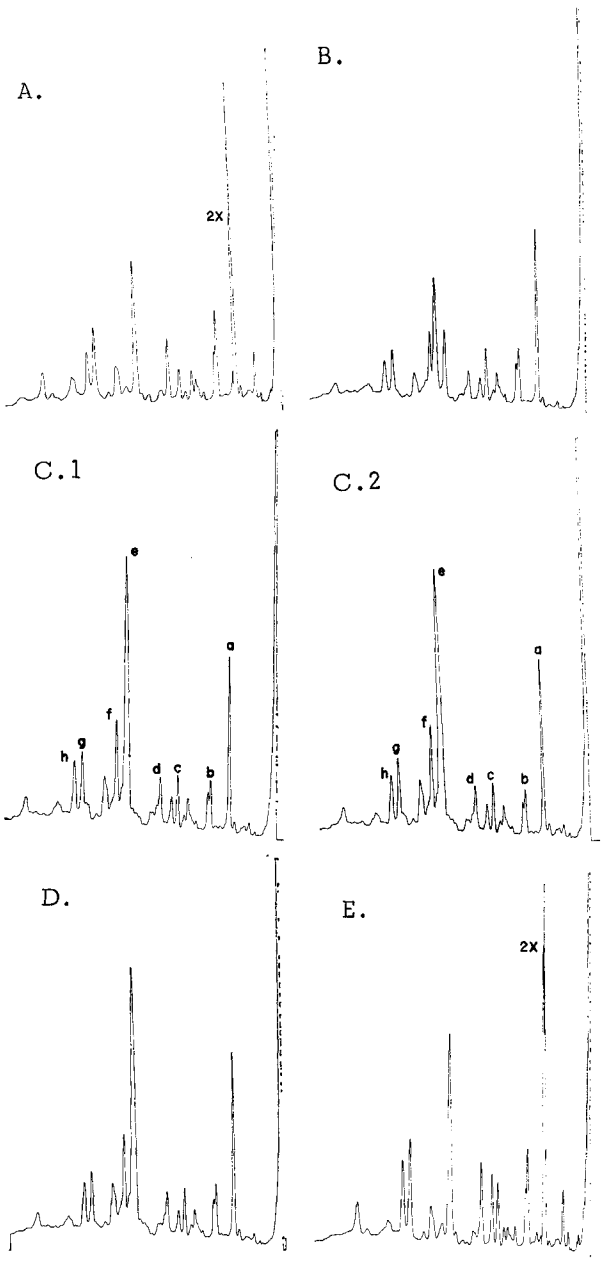


Figure 2—*GLC profiles of commercial samples of LCD.*

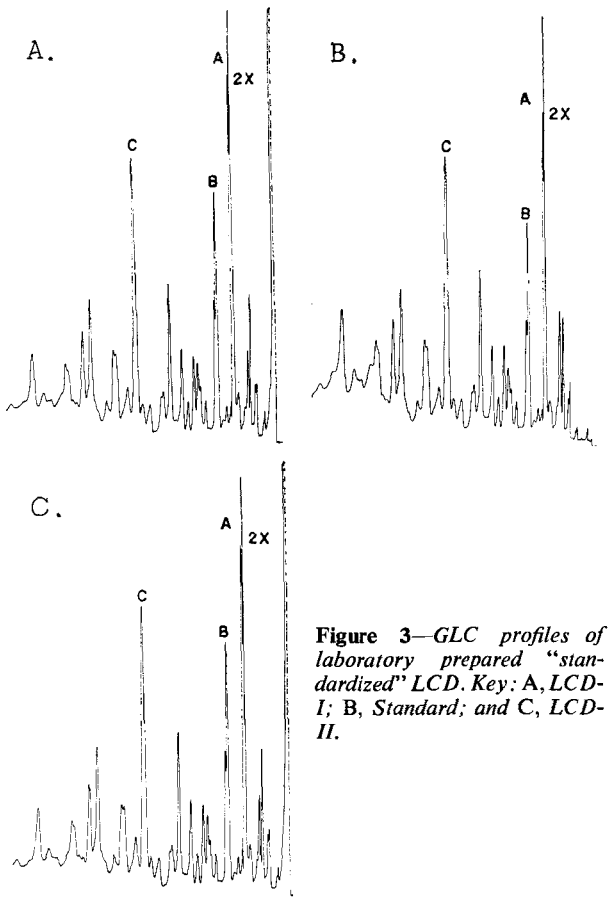


Figure 3—*GLC profiles of laboratory prepared "standardized" LCD. Key:* A, *LCD-I;* B, *Standard; and* C, *LCD-II.*

extracted, filtered, and synthesized liquids, which bear little physical and chemical resemblance or correlation to a therapeutically acceptable grade of crude coal tar.

The authors have presented a new approach to the chemical standardization and analysis of this drug. In addition, an alternate method for determining the ethanol content of coal tar solution USP (LCD) is presented, which is equivalent to, and more rapid than, the procedure specified in the pharmacopeia.

It is reasonable to conclude that by designation of starting raw material, shape and size of retort, destructive distillation temperature, temperature during tar collection, and various other factors, in conjunction with laboratory specifications (including 15% maximum permissible variation in any one of the selected major constituents of the chromatographable portion of the tar, with differential solubility and specific gravity data), one would produce a tar demonstrating little or no inconsistencies in composition from batch to batch. In fact, this GLC technique has been routinely and successfully employed in the authors' laboratory for the past 2 years. It is equally obvious that while GLC standardized crude coal tar may yield a "standardized" LCD, the activity of this or any other "extract" cannot be expected to approach the efficacy obtained from the original whole substance.

This investigation is intended as a beginning, and further research pertaining to a nonchromatographable fraction of crude coal tar, as well as the sorption phenomena which occur in the manufacture of coal tar solutions, is being conducted. Additional analytical methods such as wet analyses and differential solubilities are being explored. These further investigations present interesting possibilities for future reports.

Table IV—Analysis of Laboratory Prepared LCD

Standard		LCD I[a]		LCD II[b]	
V_s = 2.32 μl.		V_i = 2.49 μl.		V_i = 2.51 μl.	
Peak	*Js*	*Ji*	% Tar	*Ji*	% Tar
A	76.2	85.19	20.84	83.11	20.16
B	51.3	59.10	21.47	60.10	21.98
C	66.1	67.10	18.92	68.71	19.21

[a] $\bar{X}$ = 20.41%. Est. = 20.29%. [b] $\bar{X}$ = 20.45 %. Est. = 20.46%.

Table V—Comparison of GLC *vs.* Distillation (USP) Method for the Determination of Ethanol in LCD

Sample	% C_2H_5OH (USP)	% C_2H_5OH(GLC)
A	83.70, 83.61	83.40
B	85.43, 85.59	85.72
C	82.10, 81.89	82.06
D	84.20, 83.96	83.82
E	81.69, 81.88	81.80
I[a]	81.42, 81.36	81.10
II[a]	81.30, 81.56	81.15

[a] Laboratory preparations.

Since whole crude coal tar is not a chemical entity and its total therapeutic effect is dependent upon a myriad of carbonized and volatile constituents, achievement of complete and absolute uniformity of clinical results is improbable. It is apparent, however, that modern, precise analytical instrumentation can be utilized to revise the inadequate USP monograph on coal tar. A GLC tar profile, together with adequate chemical and physical specifications, will assure pharmacists of minimum rather than maximum variations in composition. Consequently, physicians may anticipate greater consistency and uniformity of therapeutic results.

REFERENCES

(1) J. G. Downing and C. W. Bauer, *Arch. Dermatol. Syph.*, **57,** 985(1948).

(2) L. Brocq, *Ann. Dermatol. Syph.*, **10,** 1(1909).

(3) C. J. White, *Arch. Dermatol. Syph.*, **14,** 796(1921).

(4) W. H. Goeckerman, *Northwest Med.*, **24,** 229(1921).

(5) W. H. Goeckerman, *Arch. Dermatol. Syph.*, **24,** 446(1931).

(6) I. N. de Martin and G. N. Cyr, *J. Amer. Pharm. Ass., Sci. Ed.*, **42,** 153(1953).

(7) F. C. Combes, *Arch. Dermatol. Syph.*, **56,** 583(1947).

(8) J. A. Zapotocky, personal communication.

(9) M. E. Obermayer and S. W. Becker, *Arch. Dermatol. Syph.*, **31,** 796(1935).

(10) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 137.

(11) *Ibid.*, p. 138.

(12) R. F. Dickey and E. N. Hesbacher, *Derm. Digest*, **1964,** p. 53.

(13) J. C. King and W. R. Lloyd, *J. Amer. Pharm. Ass., Pract. Pharm. Ed.*, **20,** 88(1959).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 13, 1969, from the *Analytical Research Department, Dermik Laboratories, Inc., Syosset, New York, NY 11791*

Accepted for publication December 29, 1969.

Quantitative Chromatographic Analysis of Methallibure in Animal Feed Mixtures

G. J. KROL, J. F. CARNEY, and B. T. KHO

Abstract □ Methallibure [1-methyl-6-(1-methylallyl)-2,5-dithiobiurea] was separated from the constituents of feed extracts by partition column chromatography. The separation utilized polydextran gel as a stationary phase and a composite organic solvent as a mobile phase. The method requires no special treatment of the solid support and the column can be used for an indeterminate number of elutions. The observed interference of metal ions in the extraction and chromatography step was eliminated by the addition of dithizone to the extracting solvent. The procedure is quantitative and applicable to routine analysis of different commercial field samples. The chromatographic separation was tested for specificity with a thiadiazole derivative, which was prepared from methallibure, and complete separation between the two related structures was observed. The chromatographic column, used in the analysis, yielded approximately 900 theoretical plates.

Keyphrases □ Methallibure—determination □ Animal feed mixtures—methallibure determination □ Column chromatography—separation □ Dithizone—metal-ion interference elimination □ UV spectrophotometry—analysis

A partition chromatographic method for analysis of methallibure, an oestrus regulator for veterinary applications, in the presence of certain animal feed mixtures was reported by Hudson and Pearson (1). The method used diatomaceous earth[1] as a solid support and chloroform, formamide, and *n*-hexane as a two-phase solvent system. However, this procedure is relatively time consuming, since it requires preparation of a new column for every elution and involves relatively large volumes of eluent.

In view of these limitations, an alternative chromatographic method was developed. The method is based on an alkylated polydextran gel[2] solid support and an eluent composed of a mixed organic solvent. The preferential solvation of the gel by the more polar components of the solvent mixture yields a partition effect similar to that observed by Nystrom and Sjovall (2). In contrast to the diatomaceous earth method (1), the liquid partition effect obtained in this method requires no special treatment of the solid support, since the solvation of the gel and elution are carried out with the same mixed solvent system. Furthermore, once the column is packed, it can be used for an indeterminate number of elutions.

During the development phase of this method, a complication was encountered since some feed samples contained significant amounts of metal salts and the dithiobiurea structure of methallibure is a relatively efficient metal-ion complexing group (3, 4). In a number of instances, this situation led to a significant apparent loss in the percent recovery of the methallibure, since a significant fraction of methallibure was complexed by metal ions and eluted in different volume. To avoid this complication, the extraction was carried out in the presence of an excess amount of diphenylthiocarbazone (dithizone), which competes effectively for metal ions with methallibure. Initially, 8-hydroxyquinoline was utilized for this purpose; however, it was subsequently observed that some metal-ion complexes of 8-hydroxyquinoline were eluted in the same volume as methallibure. A complete separation between the methallibure, dithizone, and dithizone metal-ion complexes was obtained.

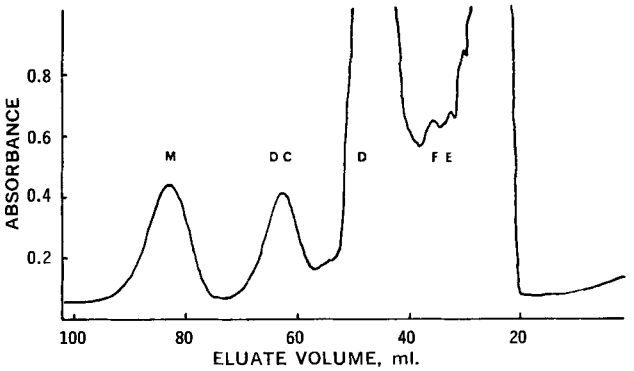


Figure 1—*Separation of methallibure from diphenylthiocarbazone and Feed Mixture B extract (Drago) on polydextran gel (1.2 × 44-cm. column) with isopropanol–chloroform–cyclohexane–acetic acid–water (100:100:130:8:2) solvent. Key: M, methallibure; D, diphenylthiocarbazone; DC, diphenylthiocarbazone complex; and FE, feed extractives.*

EXPERIMENTAL

Equipment—Kontes glass chromaflex columns (or equivalent), 50 × 1.2 cm. (i.d.) and 50 × 5 cm., were used (Kontes, Vineland, N. J.). To prevent introduction of UV-absorbing extractives from the O-ring which is provided with the above columns, the O-rings have been replaced with O-rings improvised from 0.32-cm. (0.125-in.) (o.d.) organic solvent resistant tubing.[3] The sintered-glass disk, which is present in the column, was covered with a nylon mesh (Pharmacia Fine Chemicals Inc., New Market, N. J.). The smaller column was fitted with a 500-ml. separator, the larger with a 2-l. funnel. Each separator had an airtight connection to the column. A Cary model 14 was used for the UV determinations, while Beckman DB was used in the method development to determine the optimum chromatographic solvent and the elution volumes. UV flow cell (1-cm. light path) was obtained from A. H. Thomas Co.

Solvents and Chemicals—Redistilled isopropanol (analytical reagent grade, Mallinckrodt), chloroform, glacial acetic acid, and spectro grade cyclohexane were used. The chromatographic solvent consisted of isopropanol, chloroform, cyclohexane, glacial acetic acid, and water (1.0:1.0:1.3:0.08:0.02 by volume, respectively).

[1] Celite, Johns-Manville, New York, N. Y.
[2] Sephadex LH-20, Pharmacia Fine Chemicals, Inc., New Market, NJ 08854
[3] Acidflex, Technicon Inc., Chauncey, N. Y.

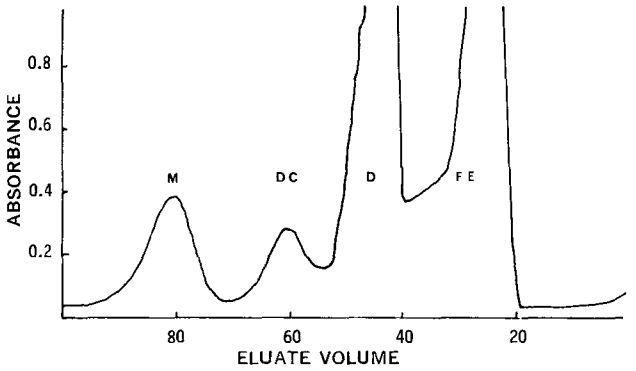


Figure 2—*Separation of methallibure from diphenylthiocarbazone and Feed Mixture C (Kent) on polydextran gel (1.2 × 44-cm. column) with isopropanol–chloroform–cyclohexane–acetic acid–water (100:100:130:8:2) solvent. Key: M, methallibure; D, diphenylthiocarbazone; DC, diphenylthiocarbazone complex; and FE, feed extractives.*

Methallibure was obtained from I.C.I., Pharmaceutical Division, Macclesfield, Cheshire, England.

Since the commercially available dithizone (obtained from either Eastman Organic Chemicals or Fisher Scientific Co.) was found to be insufficiently pure for purposes of this study, it was purified by the following procedure: 250 mg. of dithizone was dissolved in 50 ml. of the chromatographic solvent. The solution was filtered, applied to the larger column (23-cm. bed height), and eluted with 460 ml. of the chromatographic solvent. The preparation of the large column is analogous to the procedure described for the preparation of 50 × 1.2-cm. columns. However, the solvent used for both preparation and elution may be composed of undistilled reagent grade solvents. The purified dithizone fraction, which was contained in the major blue band, was eluted in the 350- to 460-ml. fraction and used without further treatment. The solution prepared previously was stored in the dark at 4° and may be used for several days. The chromatographic column may be used repeatedly for the preparation of more dithizone after washing the column with additional 300 ml. of the solvent between sample applications.

Procedure—During the development phase and the subsequent testing of the method with different commercial feeds, the authors have utilized a Beckman DB flow cell–log recorder system. The spectrophotometer was set at the wavelength of the absorption maximum of methallibure (249 mμ), and the area of the methallibure peak on the recorder scan was found to be proportional to the amount of methallibure. Representative elution patterns are illustrated by Figs. 1–3. However, since the volume which contained the methallibure fraction was found to be quite reproducible, the procedure was modified to a more suitable routine analysis which does not require a spectrophotometer–flow cell system. The following description outlines the procedure for routine analysis.

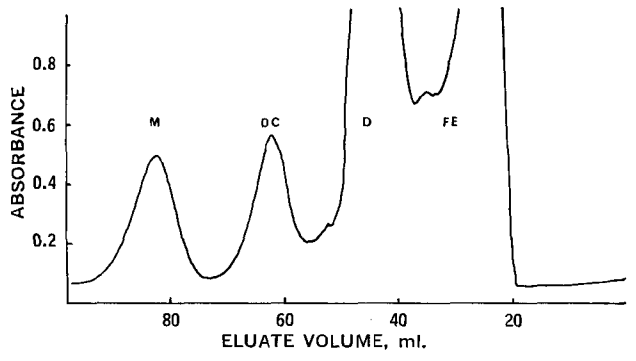


Figure 3—*Separation of methallibure from diphenylthiocarbazone and Feed Mixture A extract (Felco) on polydextran gel (1.2 × 44-cm. column) with isopropanol–chloroform–cyclohexane–acetic acid–water (100:100:130:8:2) solvent. Key: M, methallibure; D, diphenylthiocarbazone; DC, diphenylthiocarbazone complex; and FE, feed extractives.*

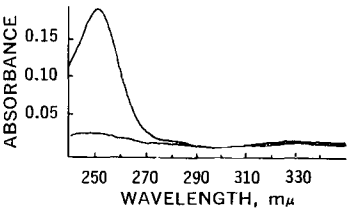


Figure 4—*UV scan of the 25-ml. methallibure and the 4-ml. blank fraction. Origin of the fraction: Feed Mixture B extract (Drago).*

Preparation of Column—Polydextran gel was suspended in the chromatographic solvent (200 ml. of solvent/10 g. of polydextran gel) and the slurry equilibrated by shaking for 2 hr. The gel was allowed to settle, excess solvent decanted, and a fresh portion of solvent added. This procedure was repeated three times. The final slurry was transferred to the column by gravity feed. The column height was 44 cm.

Extraction of Methallibure from Feed—A 40-g. feed sample, containing approximately 2.2 mg. of methallibure, was transferred into a 250-ml. bottle and extracted with 10 ml. of the dithizone solution and 150 ml. of the chromatographic solvent. The extraction was aided by continuous shaking for 5 min. This procedure is applicable to a methallibure premix, such as polyethylene glycol (PEG), which is readily soluble in the chromatographic solvent. If methallibure is present in a methylcellulose premix, which is not soluble in the chromatographic solvent, the extraction could be carried out with a high speed homogenizer (for 15 min. at 40,000 r.p.m.). A VirTis "45" homogenizer is suitable for this purpose. Alternatively, the extraction could also be carried out by shaking with the more polar components of chromatographic solvent (omit cyclohexane). A portion of the extracted feed slurry was filtered through a fine- or medium-porosity sintered-glass funnel to provide a 4-ml. aliquot of clear filtrate. In order to avoid evaporation, the filtration was carried out under positive pressure with the aid of a

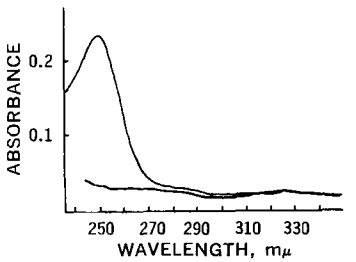


Figure 5—*UV scan of the 25-ml. methallibure and the 4-ml. blank fraction. Origin of the fractions: Feed Mixture A extract (Felco).*

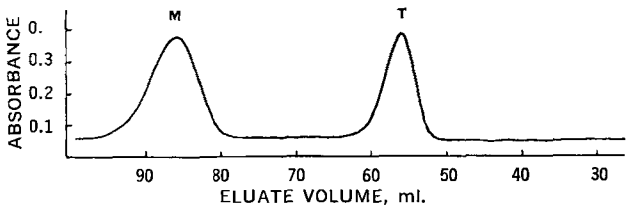
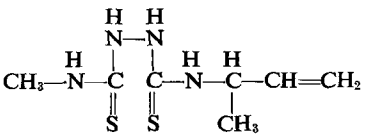
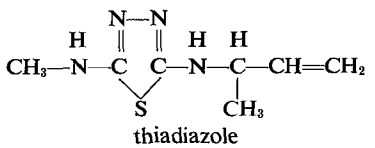


Figure 6—*Separation of methallibure (M) from thiadiazole (T) on polydextran (1.2 × 44-cm. column) with isopropanol–chloroform–cyclohexane–acetic acid–water (100:100:130:8:2) solvent.*



methallibure



thiadiazole

Table I—Observed Recoveries of Methallibure from Feed Samples Spiked with Methylcellulose and PEG Premixes

Feed Mixture	Premix Calculated, mcg.	Premix Observed, mcg.	Premix Recovery, %
	Methylcellulose		
A[a]	29.9	29.8	99.6
		29.6	98.9
	35.0	36.8	105.0
		38.3	109.3
	33.8	32.0	94.7
		33.0	97.7
B[b]	32.2	33.8	104.8
		33.8	104.8
	33.2	33.0	99.4
	35.4	33.5	94.6
		33.0	93.3
C[c]	34.4	34.4	100.0
		34.6	100.6
	32.1	34.8	108.3
		33.0	102.8
	34.4	35.2	102.2
		34.6	100.6
	PEG		
A[a]	35.7	35.4	99.2
		33.3	93.2
	30.5	29.8	97.8
		30.6	100.3
	31.4	32.8	104.4
		31.0	98.8
B[b]	34.2	35.3	103.1
		32.8	95.9
	29.3	28.3	96.6
		29.4	100.3
	31.6	32.4	102.4
		33.8	107.0
C[c]	30.3	30.3	100.0
	33.7	32.4	96.2
		35.1	104.1
	32.5	31.0	95.5
		30.5	93.8

[a] Felco Sow Chunks. [b] Drago, 3 parts ground corn and 1 part Sow Concentrate 853. [c] Kent Hand Feed Sow Mix.

rubber bulb. A 4-ml. aliquot of the filtrate was transferred into a 5-ml. volumetric flask and diluted to 5 ml. with 1 ml. of dithizone solution.

Chromatography—A 3-ml. aliquot of the prepared solution was applied quantitatively to the column. The column was eluted with 102 ml. of the chromatographic solvent at a flow rate of approximately 0.5 ± 0.1 ml./min. (A pressure head was provided by a 300-ml. solvent reservoir in the separator above the column.)

The first 73-ml. fraction, which contained the feed extractives and dithizone (free and chelated by metal ions), was discarded and the next two fractions containing 25 and 4 ml. were collected and retained for quantitation by UV absorption. Both fractions were scanned in the 350–240-mμ range against the chromatographic solvent. Figures 4 and 5 illustrate typical UV scans of the 25- and 4-ml. fractions.

Quantitation—If the UV absorption of the 25-ml. fraction matched (within ±0.005) the UV absorption of the 4-ml. fraction in the 350–290-mμ range, the absorbance (at 249 mμ) of the 4-ml. fraction was subtracted from the absorbance of the 25-ml. fraction and the difference in absorption $(A)_c$ used to calculate the amount of methallibure in the sample. The following formula was used to calculate the amount of methallibure in the 25-ml. fraction.

$$\text{mcg. (of methallibure recovered after chromatography)} = \frac{(A)_c \times \text{vol.}_f}{0.129} \quad \text{(Eq. 1)}$$

$$\text{mcg. (of methallibure per g. of feed)} = (A)_c \times 194 \times {}^5/_3 \quad \text{(Eq. 2)}$$

where $(A)_c$ is $(A_{25} - A_4)$, absorbance of the 25-ml. fraction which contains methallibure corrected for background absorbance as reflected by the 4-ml. fraction; vol._f is the volume of the fraction which contains the methallibure; 0.129 is the extinction factor of methallibure in the chromatographic solvent expressed in ml./mcg. units; and ${}^5/_3$ is the dilution factor of the feed extract.

Table II—Accuracy and Precision of the Methallibure Assay

Feed Mixture	Premix Mean Recovery, %	Premix *SD*	Premix *CV*
	Methylcellulose		
A[a]	100.9	5.3	5.3
B[b]	99.4	5.4	5.5
C[c]	102.4	3.1	3.0
	PEG		
A[a]	99.0	3.6	3.7
B[b]	100.9	4.2	4.2
C[c]	97.9	4.1	4.2

[a] Felco Sow Chunks. [b] Drago, 3 parts ground corn and 1 part Sow Concentrate 853. [c] Kent Hand Feed Sow Mix.

If the absorbance of the 25-ml. fraction in the 350–290-mμ range does not match the absorbance of the 4-ml. fraction, one may determine the amount of methallibure in the 25-ml. fraction by the curvature inversion technique (5, 6). This procedure involves scanning the methallibure fraction (25 ml.) against several reference solutions of methallibure at different concentrations but in the concentration range of the unknown sample. From the curvature at the inflection point (249 mμ, absorption maximum of methallibure), one can determine the concentration of the unknown sample. However, essentially all feed mixtures the authors encountered were analyzed without resorting to the curvature inversion technique. Usually, the magnitude of background absorbance was predictable and equivalent to 10–15% of the total absorbance of the methallibure fraction. The estimation of the background absorbance did not introduce more than a 3% error in calculation of the amount of methallibure.

RESULTS AND DISCUSSION

Three different commercial feed mixtures were spiked with known amounts of PEG and methylcellulose premixes. The samples were analyzed by direct UV determination without the aid of the curvature inversion technique. Tables I and II present the observed recoveries and the statistical analysis of the observed data. It is apparent from Tables I and II that although the standard deviations are relatively large, the observed recovery is essentially complete and independent of the nature of the feed mixture.

The chromatographic separation utilized in this study has also been tested for specificity. A complete separation between methallibure and its oxidation product, thiadiazole, has been observed (Fig. 6). On the basis of the elution volume and peak width, the chromatographic column used in this analysis yielded approximately 900 theoretical plates. This is equivalent to 20 theoretical plates/cm.

A similar partition chromatographic system was applied to the separation of the coccidiostat, nequinate (oxyquinoline structure), from feed mixtures (7). Although the chemical structure of methallibure is unrelated to the structure of nequinate, the chromatographic system is specific for both compounds; each compound was separated from closely related structures. This chromatographic approach was also extended to the quantitative separation of structurally related steroid compounds (8).

REFERENCES

(1) H. E. Hudson and B. Pearson, *Analyst*, **93**, 319(1968).

(2) E. Nystrom and J. Sjovall, *Anal. Biochem.*, **12**, 235(1965).

(3) E. Popper, L. Popa, R. Craiciuneanu, and L. Z. Roman, *Anal. Chem.*, **33**, 184(1961).

(4) N. K. Dutt and A. Dutta Ahmed, *Mikrochim. Acta*, 571 (1961).

(5) C. F. Hiskey, *Anal. Chem.*, **33**, 927(1961).

(6) J. B. Wilkie, *ibid.*, **36**, 896(1964).

(7) G. J. Krol and N. G. Nash, *J. Ass. Offic. Anal. Chem.*, **52**, 1141(1969).

(8) G. J. Krol, R. P. Masserano, J. Carney, and B. T. Kho, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 6, 1969, from the *Analytical Development Department, Ayerst Laboratories Inc., Rouses Point, NY 12979*
Accepted for publication January 12, 1970.

Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors thank Dr. W. Hausmann for his interest and helpful discussions. They also thank N. Nash, P. Cox, and J. Russell for their assistance in the application of this method to large-scale analysis of commercial field samples, and Dr. K. Sestanj of Ayerst, Montreal, for the preparation of the thiadiazole derivative.

Improved Colorimetric Determination of Primary Aromatic Amines with 9-Chloroacridine: Application to Some Local Anesthetics

J. T. STEWART and D. M. LOTTI

Abstract □ Improvement in the stability of the 9-chloroacridine stock solution has been made in the colorimetric method for primary aromatic amines based on the reaction between the acridine and an amine. The improved procedure has been applied to some local anesthetics and local anesthetic mixtures. It has been found to be comparable in sensitivity to other local anesthetic determinations, particularly the popular diazotization-coupling procedures. Quantitative data from several systems reveal that use of this procedure permits the determination of local anesthetics in the presence of various drugs and other local anesthetics. Comparative analyses were performed with the method of Bratton and Marshall on procaine, metabutethamine, and nesacaine hydrochlorides.

Keyphrases □ Amines, primary aromatic—improved analysis □ 9-Chloroacridine solution—stabilization □ Tetrahydrofuran—9-chloroacridine solvent □ Colorimetric analysis—spectrophotometry

A colorimetric method for the determination of small quantities of primary aromatic amines with 9-chloroacridine and its use in the analysis of some sulfonamides have been previously reported by this laboratory (1, 2). Data presented in these papers showed that the sensitivity of the method rivals that of the commonly used diazotization-coupling procedures for primary aromatic amines and sulfonamides. It is necessary for the ethanolic 9-chloroacridine stock solution to be prepared immediately before use since the acridine undergoes rapid ethanolysis in ethanol (3). This fresh solution is permissible to use for approximately 0.5 hr. after preparation.

The objective of this paper is to report an improvement in the existing method by stabilization of the 9-chloroacridine stock solution, and application of the method to the analysis of several local anesthetics and local anesthetic mixtures. A comparative study of this improved technique was made with the procedure of Bratton and Marshall (4).

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with spectrophotometers (Perkin-Elmer, model 202, and Beckman, model DU). Matched cells with a 1-cm. optical path were used.

Reagents and Chemicals—9-Chloroacridine[1] was used as the chromogenic reagent. Powdered samples of propoxycaine hydrochloride,[2] butacaine sulfate,[3] butesin,[3] butethamine hydrochloride,[4] metabutethamine hydrochloride,[4] nesacaine hydrochloride,[5] benzocaine,[1] and procaine hydrochloride[6] were used in the analytical procedure for preparation of standard curves. Piperocaine,[7] lidocaine,[8] and tetracaine[9] hydrochlorides were also used in the analysis. All other chemicals used were the highest grade of the commercially available materials.

Solutions (4×10^{-4} *M*) were prepared by dissolving weighed amounts of benzocaine and butesin in ethanol and the remaining local anesthetics in water. The reagent solution of 9-chloroacridine (4×10^{-4} *M*) was prepared by dissolving a weighed amount in tetrahydrofuran[10] and storing in a light-resistant volumetric flask.[11]

Procedure—One milliliter of an ethanolic or aqueous solution of a local anesthetic (4×10^{-4} *M*) was placed in a 10-ml. volumetric flask. To this was added 1 ml. of a tetrahydrofuran solution of 9-chloroacridine (4×10^{-4} *M*). Then the pH was adjusted to approximately 4 with 10% v/v aqueous hydrochloric acid. The solution was shaken and allowed to sit for 15 min. at room temperature, followed by the addition of ethanol to volume, and absorbance was measured at 435 mμ. Absorbance measurements were corrected for reagent blanks in the procedure.

RESULTS AND DISCUSSION

The colorimetric method for primary aromatic amines using 9-chloroacridine has been improved by using tetrahydrofuran in place of ethanol as solvent to make the acridine stock solution. Data shown in Table I for some local anesthetics reveal that the addition of small amounts of tetrahydrofuran to the analytical procedure does not cause any significant change in the sensitivity. The ethanolic solution was only useful for about 0.5 hr. due to reaction between the acridine and ethanol, and it was a disadvantage to prepare new acridine stock solutions that often. There was a need for a solvent in which dissolution, but no reaction between the acridine and solvent, would occur. Miscellaneous solvents were investigated, but only tetrahydrofuran proved successful in meeting these requirements.

[1] Eastman Chemical Co.
[2] Sterling Drug Co., Rensselaer, N. Y.
[3] Abbott Laboratories, North Chicago, Ill.
[4] Novocol Chemical Manufacturing Co., Inc., Brooklyn, N. Y.
[5] Strasenburgh Laboratories, Rochester, N. Y.
[6] Purocaine Chemical Co., New York, N. Y.
[7] Eli Lilly and Co., Indianapolis, Ind.
[8] Astra Pharmaceutical Products, Worcester, Mass.
[9] Winthrop Laboratories, New York, N. Y.
[10] Mallinckrodt, analytical reagent grade.
[11] Low actinic volumetric flask (Corning No. 55640).

(8) G. J. Krol, R. P. Masserano, J. Carney, and B. T. Kho, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 6, 1969, from the *Analytical Development Department, Ayerst Laboratories Inc., Rouses Point, NY 12979*
Accepted for publication January 12, 1970.

Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors thank Dr. W. Hausmann for his interest and helpful discussions. They also thank N. Nash, P. Cox, and J. Russell for their assistance in the application of this method to large-scale analysis of commercial field samples, and Dr. K. Sestanj of Ayerst, Montreal, for the preparation of the thiadiazole derivative.

Improved Colorimetric Determination of Primary Aromatic Amines with 9-Chloroacridine: Application to Some Local Anesthetics

J. T. STEWART and D. M. LOTTI

Abstract □ Improvement in the stability of the 9-chloroacridine stock solution has been made in the colorimetric method for primary aromatic amines based on the reaction between the acridine and an amine. The improved procedure has been applied to some local anesthetics and local anesthetic mixtures. It has been found to be comparable in sensitivity to other local anesthetic determinations, particularly the popular diazotization-coupling procedures. Quantitative data from several systems reveal that use of this procedure permits the determination of local anesthetics in the presence of various drugs and other local anesthetics. Comparative analyses were performed with the method of Bratton and Marshall on procaine, metabutethamine, and nesacaine hydrochlorides.

Keyphrases □ Amines, primary aromatic—improved analysis □ 9-Chloroacridine solution—stabilization □ Tetrahydrofuran—9-chloroacridine solvent □ Colorimetric analysis—spectrophotometry

A colorimetric method for the determination of small quantities of primary aromatic amines with 9-chloroacridine and its use in the analysis of some sulfonamides have been previously reported by this laboratory (1, 2). Data presented in these papers showed that the sensitivity of the method rivals that of the commonly used diazotization-coupling procedures for primary aromatic amines and sulfonamides. It is necessary for the ethanolic 9-chloroacridine stock solution to be prepared immediately before use since the acridine undergoes rapid ethanolysis in ethanol (3). This fresh solution is permissible to use for approximately 0.5 hr. after preparation.

The objective of this paper is to report an improvement in the existing method by stabilization of the 9-chloroacridine stock solution, and application of the method to the analysis of several local anesthetics and local anesthetic mixtures. A comparative study of this improved technique was made with the procedure of Bratton and Marshall (4).

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with spectrophotometers (Perkin-Elmer, model 202, and Beckman, model DU). Matched cells with a 1-cm. optical path were used.

Reagents and Chemicals—9-Chloroacridine[1] was used as the chromogenic reagent. Powdered samples of propoxycaine hydrochloride,[2] butacaine sulfate,[3] butesin,[3] butethamine hydrochloride,[4] metabutethamine hydrochloride,[4] nesacaine hydrochloride,[5] benzocaine,[1] and procaine hydrochloride[6] were used in the analytical procedure for preparation of standard curves. Piperocaine,[7] lidocaine,[8] and tetracaine[9] hydrochlorides were also used in the analysis. All other chemicals used were the highest grade of the commercially available materials.

Solutions (4×10^{-4} *M*) were prepared by dissolving weighed amounts of benzocaine and butesin in ethanol and the remaining local anesthetics in water. The reagent solution of 9-chloroacridine (4×10^{-4} *M*) was prepared by dissolving a weighed amount in tetrahydrofuran[10] and storing in a light-resistant volumetric flask.[11]

Procedure—One milliliter of an ethanolic or aqueous solution of a local anesthetic (4×10^{-4} *M*) was placed in a 10-ml. volumetric flask. To this was added 1 ml. of a tetrahydrofuran solution of 9-chloroacridine (4×10^{-4} *M*). Then the pH was adjusted to approximately 4 with 10% v/v aqueous hydrochloric acid. The solution was shaken and allowed to sit for 15 min. at room temperature, followed by the addition of ethanol to volume, and absorbance was measured at 435 mμ. Absorbance measurements were corrected for reagent blanks in the procedure.

RESULTS AND DISCUSSION

The colorimetric method for primary aromatic amines using 9-chloroacridine has been improved by using tetrahydrofuran in place of ethanol as solvent to make the acridine stock solution. Data shown in Table I for some local anesthetics reveal that the addition of small amounts of tetrahydrofuran to the analytical procedure does not cause any significant change in the sensitivity. The ethanolic solution was only useful for about 0.5 hr. due to reaction between the acridine and ethanol, and it was a disadvantage to prepare new acridine stock solutions that often. There was a need for a solvent in which dissolution, but no reaction between the acridine and solvent, would occur. Miscellaneous solvents were investigated, but only tetrahydrofuran proved successful in meeting these requirements.

[1] Eastman Chemical Co.
[2] Sterling Drug Co., Rensselaer, N. Y.
[3] Abbott Laboratories, North Chicago, Ill.
[4] Novocol Chemical Manufacturing Co., Inc., Brooklyn, N. Y.
[5] Strasenburgh Laboratories, Rochester, N. Y.
[6] Purocaine Chemical Co., New York, N. Y.
[7] Eli Lilly and Co., Indianapolis, Ind.
[8] Astra Pharmaceutical Products, Worcester, Mass.
[9] Winthrop Laboratories, New York, N. Y.
[10] Mallinckrodt, analytical reagent grade.
[11] Low actinic volumetric flask (Corning No. 55640).

Table I—Comparison of Absorbance Readings of Reaction Products Formed During the Analytical Procedure, with Ethanol and/or Tetrahydrofuran (THF) as Solvent for 9-Chloroacridine

Local Anesthetic	Concn., $M \times 10^{-5}$	Absorbance — Analytical Reaction With Ethanol	Absorbance — Analytical Reaction With THF
Propoxycaine hydrochloride	4.00	0.305	0.310
Butacaine sulfate	4.00	0.325	0.320
Butethamine hydrochloride	4.00	0.305	0.305

There was, however, limited success with *tert*-butyl alcohol. The tetrahydrofuran solution should be stored in a light-resistant volumetric flask, since it has been observed in this laboratory that light caused some decomposition of the 9-chloroacridine, possibly through a free radical reaction (5). Stock solutions of the acridine in tetrahydrofuran have been stable up to 1 month after preparation when properly stored.

tert-Butyl alcohol has some limitations in its employment as a solvent for 9-chloroacridine. The alcohol is very difficult to handle since it freezes at 25.5° (6). The observed reaction time for the analytical procedure is 30–45 min. longer with *tert*-butyl alcohol present than with tetrahydrofuran, and the stock solution of the acridine is stable for only 1 week. It has been possible in this laboratory to overcome the disadvantage of handling due to freezing by insulating the volumetric flask containing the acridine stock solution, but the observed slower reaction time for the analysis and instability of the reagent solution for periods longer than 1 week make tetrahydrofuran the preferred solvent for the acridine.

The improved method using the tetrahydrofuran–acridine solution was then applied to the analysis of some local anesthetics and local anesthetic mixtures. The results indicated that the reaction between local anesthetics containing a primary aromatic amino group and 9-chloroacridine to yield highly colored aminoacridine hydrochlorides can be utilized as a suitable assay procedure for local anesthetics. The absorption curve in the visible spectrum for a typical sample of benzocaine is shown in Fig. 1; the absorption maximum occurs at 435 mμ. Reagent blank readings at this wavelength are very low.

In comparing absorption curves of the colored solutions obtained with equimolar concentrations of the various local anesthetics containing primary aromatic amino groups, it was noted that the curves were almost identical. Compounds such as propoxycaine, butacaine, butesin, butethamine, metabutethamine, and procaine all produce color that absorbs at the same wavelength and with essentially the same intensity as does benzocaine. Absorbance values for these anesthetics were between 0.30–0.35 absorbance units at 435 mμ as exemplified by benzocaine and metabutethamine (Fig. 1).

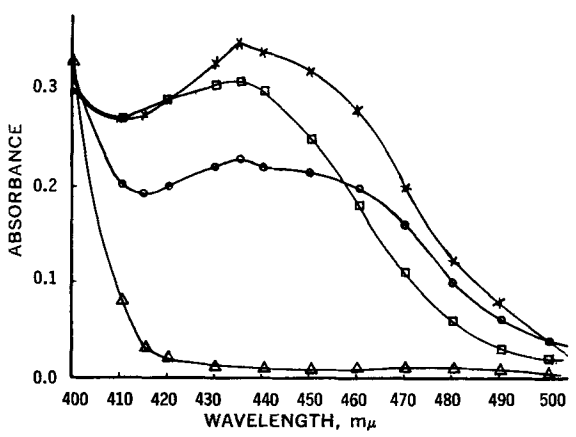


Figure 1—*Visible absorbance curves of aminoacridine derivatives of local anesthetics. Key: ×, benzocaine; □, metabutethamine hydrochloride; ○, nesacaine hydrochloride; and △, reagent blank.*

Table II—Analysis of Known Local Anesthetic Mixtures for Local Anesthetic

Mixture	Components, Concn. of 4.00×10^{-5} M	Local Anesthetic — Found, $M \times 10^{-5}$	Local Anesthetic — Yield, %
I[a]	Procaine hydrochloride Tetracaine hydrochloride	3.976	99.4
II[b]	Propoxycaine hydrochloride Piperocaine hydrochloride	3.960	99.0
III[c]	Butacaine sulfate Lidocaine hydrochloride	3.988	99.7
IV	Benzocaine Benzyl alcohol 8-Hydroxyquinoline	3.992	99.8
V	Butethamine hydrochloride Pentobarbital sodium Aminophylline	3.972	99.3
VI	Butesin Menthol Camphor Isopropyl alcohol	3.988	99.7

[a] Mixture analyzed for procaine content. [b] Mixture analyzed for propoxycaine content. [c] Mixture analyzed for butacaine content.

Structurally all of these compounds have a primary aromatic amino group in the position *para* to the ester linkage except metabutethamine, in which the amino function is *meta* to the ester linkage. Nesacaine shows somewhat diminished intensity values at the same wavelength (Fig. 1), due presumably to a less-than-quantitative yield of reaction with 9-chloroacridine caused by the inductive effect of the chloro group *meta* to the primary amino group in the local anesthetic.

The local anesthetics, piperocaine, lidocaine, and tetracaine, which do not contain a primary aromatic amino group, give no color formation with this procedure.

Standard curves can be prepared by plotting observed absorbance readings *versus* the volumes taken of equimolar concentrations of various local anesthetics. In all cases, Beer's law holds for this system.

Quantitative data from several systems shown in Table II reveal that use of this improved procedure permits the determination of local anesthetics containing a primary aromatic amino group in the presence of other local anesthetic derivatives, such as piperocaine, lidocaine, and tetracaine, and in the presence of other compounds which are found in various combinations with local anesthetics in commercially available products. It was shown from earlier studies that primary, secondary, and tertiary aliphatic amines; secondary and tertiary aromatic amines; heterocycles; and carbonyl-containing compounds also do not interfere with this method (1).

The analytical method is essentially a microprocedure, and sensitivity is in the range of 10^{-5} M of local anesthetic, which makes it comparable to other local anesthetic determinations, particularly the popular diazotization-coupling procedures.

Table III—Determination of 2.00×10^{-5} M Solutions of Procaine, Nesacaine, and Metabutethamine Hydrochlorides by the 9-Chloroacridine Method and the Method of Bratton and Marshall

	9-Chloroacridine Method — Mean % of Concn. Employed	9-Chloroacridine Method — SD of Mean, %	Bratton-Marshall Method — Mean % of Concn. Employed	Bratton-Marshall Method — SD of Mean, %
Procaine hydrochloride	99.30	0.29	99.43	0.28
Nesacaine hydrochloride	99.60	0.17	99.75	0.23
Metabutethamine hydrochloride	99.55	0.23	99.20	0.29

A favorable characteristic of the analysis is that the absorbance of the product formed is stable and does not fade over a 24-hr. period. This is an advantage over the colorimetric method of Bratton and Marshall. In the latter method, absorbance readings must be made within 15 min. after color development, due to precipitation of the azo dyes in the method (7). The 9-chloroacridine method also does not involve diazotization. Thus, it eliminates the need for freshly prepared sodium nitrite and ammonium sulfamate solutions required with the Bratton-Marshall technique. Control of pH is required in both methods.

The improved method of analysis for local anesthetics by the 9-chloroacridine approach was carried out for some representative local anesthetics, and comparative analysis were performed using the colorimetric procedure of Bratton and Marshall. Assays were performed on procaine, nesacaine, and metabutethamine hydrochlorides.

The procedure outlined by Connors was used for the analysis by the Bratton-Marshall method (8).

Four determinations by each method were performed for each local anesthetic. The mean percent of concentration employed and the percent standard deviation of the mean for each local anesthetic are shown in Table III for both methods (9).

REFERENCES

(1) J. T. Stewart, A. B. Ray, and T. D. Shaw, *Anal. Chem.*, **41**, 360(1969).

(2) J. T. Stewart, A. B. Ray, and W. B. Fackler, *J. Pharm. Sci.*, **58**, 1261(1969).

(3) A. Albert, "The Acridines," 2nd ed., St. Martin's Press, New York, N. Y., 1966, p. 254.

(4) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).

(5) C. D. Blanton, University of Georgia, personal communication, 1969.

(6) "Handbook of Chemistry and Physics," R. C. Weast, Ed., 46th ed., The Chemical Rubber Co., Cleveland, Ohio, 1965, p. C 504.

(7) J. P. Dux and C. Rosenblum, *Anal. Chem.*, **21**, 1524(1949).

(8) K. A. Connors, "A Textbook of Pharmaceutical Analysis," Wiley, New York, N. Y., 1967, p. 198.

(9) *Ibid.*, p. 572.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 2, 1969, from the *Analytical Laboratory, Department of Medicinal Chemistry, School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication December 12, 1969.

A portion of this investigation was presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This work was supported in part by the National Science Foundation Undergraduate Research Grant GY-6087.

Qualitative and Quantitative Determination of 1,2- and 1,3-Diglycerides by Nuclear Magnetic Resonance Spectroscopy

R. J. WARREN and J. E. ZAREMBO

Abstract □ An NMR procedure is presented for the qualitative and quantitative analysis of 1,2- and 1,3-diglycerides alone or in combination. The method provides a rapid, accurate quantitative analysis, as well as serving as a specific identification of the two isomers. The determination can be carried out on sample sizes in the range 20–50 mg.

Keyphrases □ 1,2- and 1,3-Diglycerides—determination □ NMR spectroscopy—analysis

One of the more difficult problems in glyceride analysis is the differentiation and quantitative determination of 1,2- and 1,3-diglycerides in the presence of one another. Chemical methods are tedious and time consuming. IR spectra are of little value when trying to determine low percentages of one isomer in a mixture of the two. Near-IR spectroscopy (1) has been used to differentiate the 1,2- and 1,3-diglycerides and might have some value. The major drawbacks to using this technique are the large amounts of sample required for a determination, overlap of absorption bands, and relatively small differences in absorptivity values.

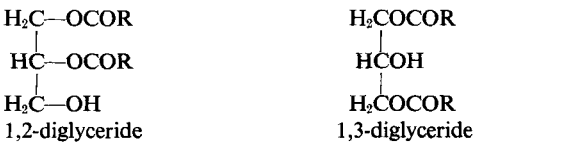
The purpose of this study was to establish the feasibility of using NMR for differentiating between the two isomers and for quantitative analysis of the two isomers.

EXPERIMENTAL

All spectra were recorded on a JEOLCO C60H spectrometer. Deuterated chloroform with 3% $CHCl_3$ added was used as solvent. The spectra were recorded at room temperature at a concentration of 80 mg./ml. Chemical shifts were measured relative to trimethylsilane (TMS).

The 1,2- and 1,3-diglycerides used were 1,2- and 1,3-distearins of reference standard quality.[1]

RESULTS AND DISCUSSION



The NMR spectra of the 1,2- and 1,3-diglyceride isomers differ markedly in the region 220–260 c.p.s. (3.6–4.4 p.p.m.) (Fig. 1). The 1,3-isomer has a singlet absorption at 249 c.p.s. due to the

[1] Supelco and Applied Science Laboratories, State College, PA 16801

A favorable characteristic of the analysis is that the absorbance of the product formed is stable and does not fade over a 24-hr. period. This is an advantage over the colorimetric method of Bratton and Marshall. In the latter method, absorbance readings must be made within 15 min. after color development, due to precipitation of the azo dyes in the method (7). The 9-chloroacridine method also does not involve diazotization. Thus, it eliminates the need for freshly prepared sodium nitrite and ammonium sulfamate solutions required with the Bratton-Marshall technique. Control of pH is required in both methods.

The improved method of analysis for local anesthetics by the 9-chloroacridine approach was carried out for some representative local anesthetics, and comparative analysis were performed using the colorimetric procedure of Bratton and Marshall. Assays were performed on procaine, nesacaine, and metabutethamine hydrochlorides.

The procedure outlined by Connors was used for the analysis by the Bratton-Marshall method (8).

Four determinations by each method were performed for each local anesthetic. The mean percent of concentration employed and the percent standard deviation of the mean for each local anesthetic are shown in Table III for both methods (9).

REFERENCES

(1) J. T. Stewart, A. B. Ray, and T. D. Shaw, *Anal. Chem.*, **41**, 360(1969).

(2) J. T. Stewart, A. B. Ray, and W. B. Fackler, *J. Pharm. Sci.*, **58**, 1261(1969).

(3) A. Albert, "The Acridines," 2nd ed., St. Martin's Press, New York, N. Y., 1966, p. 254.

(4) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).

(5) C. D. Blanton, University of Georgia, personal communication, 1969.

(6) "Handbook of Chemistry and Physics," R. C. Weast, Ed., 46th ed., The Chemical Rubber Co., Cleveland, Ohio, 1965, p. C 504.

(7) J. P. Dux and C. Rosenblum, *Anal. Chem.*, **21**, 1524(1949).

(8) K. A. Connors, "A Textbook of Pharmaceutical Analysis," Wiley, New York, N. Y., 1967, p. 198.

(9) *Ibid.*, p. 572.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 2, 1969, from the *Analytical Laboratory, Department of Medicinal Chemistry, School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication December 12, 1969.

A portion of this investigation was presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This work was supported in part by the National Science Foundation Undergraduate Research Grant GY-6087.

Qualitative and Quantitative Determination of 1,2- and 1,3-Diglycerides by Nuclear Magnetic Resonance Spectroscopy

R. J. WARREN and J. E. ZAREMBO

Abstract □ An NMR procedure is presented for the qualitative and quantitative analysis of 1,2- and 1,3-diglycerides alone or in combination. The method provides a rapid, accurate quantitative analysis, as well as serving as a specific identification of the two isomers. The determination can be carried out on sample sizes in the range 20–50 mg.

Keyphrases □ 1,2- and 1,3-Diglycerides—determination □ NMR spectroscopy—analysis

One of the more difficult problems in glyceride analysis is the differentiation and quantitative determination of 1,2- and 1,3-diglycerides in the presence of one another. Chemical methods are tedious and time consuming. IR spectra are of little value when trying to determine low percentages of one isomer in a mixture of the two. Near-IR spectroscopy (1) has been used to differentiate the 1,2- and 1,3-diglycerides and might have some value. The major drawbacks to using this technique are the large amounts of sample required for a determination, overlap of absorption bands, and relatively small differences in absorptivity values.

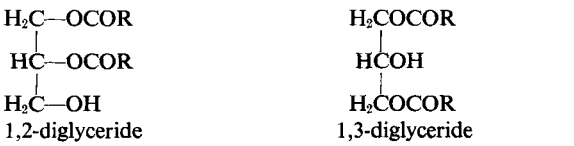
The purpose of this study was to establish the feasibility of using NMR for differentiating between the two isomers and for quantitative analysis of the two isomers.

EXPERIMENTAL

All spectra were recorded on a JEOLCO C60H spectrometer. Deuterated chloroform with 3% $CHCl_3$ added was used as solvent. The spectra were recorded at room temperature at a concentration of 80 mg./ml. Chemical shifts were measured relative to trimethylsilane (TMS).

The 1,2- and 1,3-diglycerides used were 1,2- and 1,3-distearins of reference standard quality.[1]

RESULTS AND DISCUSSION



The NMR spectra of the 1,2- and 1,3-diglyceride isomers differ markedly in the region 220–260 c.p.s. (3.6–4.4 p.p.m.) (Fig. 1). The 1,3-isomer has a singlet absorption at 249 c.p.s. due to the

[1] Supelco and Applied Science Laboratories, State College, PA 16801

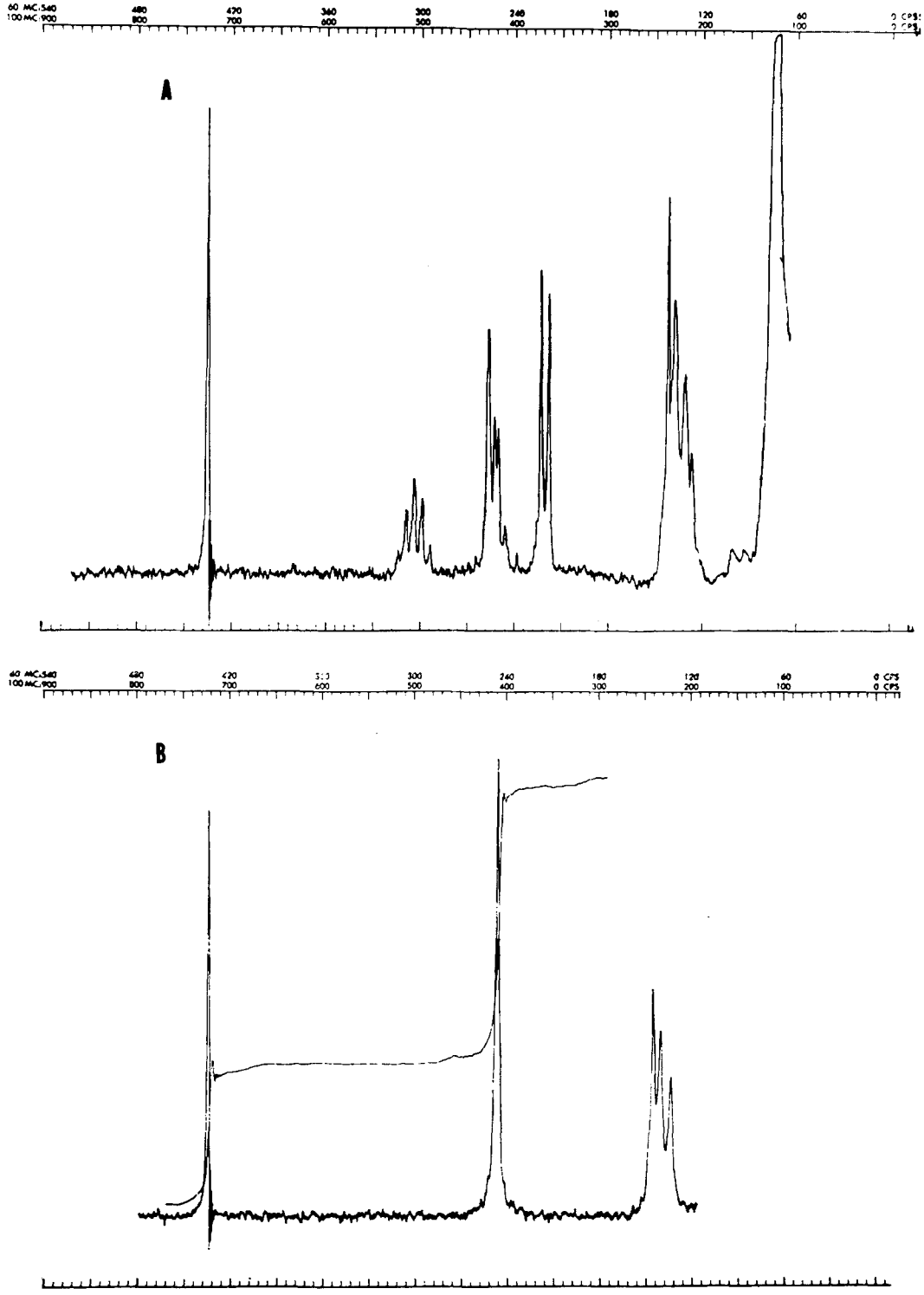


Figure 1—*A, NMR spectra of the 1,2-diglyceride isomer. B, NMR spectra of the 1,3-diglyceride isomer. TMS was used as the internal reference standard.*

five glyceryl protons. The 1,2-isomer has a more complex spectrum consisting of a quartet at 259 c.p.s. from the two mutually non-equivalent 1-protons and a doublet at 225 c.p.s. from the two 3-protons. The quintet of the 2-proton is not used in this analysis. There is no interference from the 1,3-isomer at the first doublet, and this enables one to determine the 1,2-content in a mixture of the two isomers. A series of standards was prepared containing approximately 5, 7.5, 10, and 13 mg. of the 1,2-isomer in 25 mg. of the 1,3-isomer. The materials used for the mixture were reference standard samples. The mixtures were dissolved in $CDCl_3$ containing 3% (v/v) of $CHCl_3$. The single absorption of the $CHCl_3$ at 436 c.p.s. was used as an internal standard in determining the amount of 1,2-isomer present. The NMR spectra of the mixtures were obtained and integrated. Figure 2 shows a spectrum of a representative mixture. The integrated intensity of the 1,2-absorption relative to the internal standard was recorded. The value A 1,2-/A $CHCl_3$ was then plotted *versus* percent of 1,2-diglyceride (Fig. 3). (A 1,2- = integral value of signal at 225 c.p.s. A $CHCl_3$ = integral value of signal at 436 c.p.s.)

Subsequent samples were prepared and run in the same way. By comparison of standards and samples, it was possible to determine the amount of 1,2-isomer present.

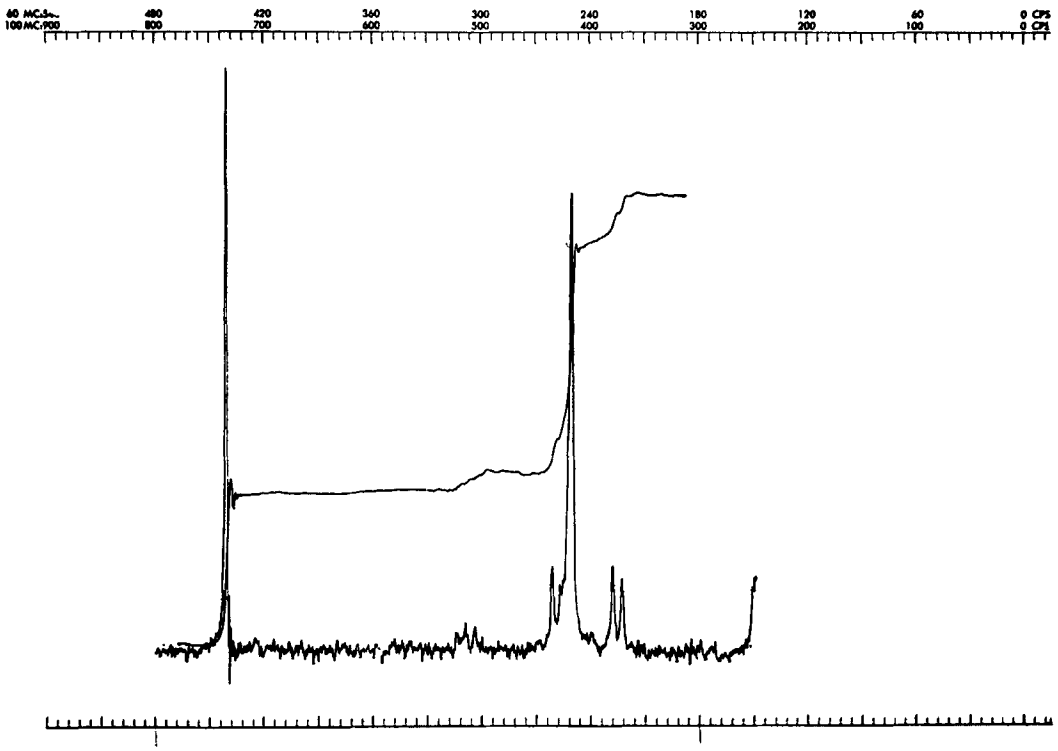


Figure 2—*NMR spectra of a mixture of 1,2- and 1,3-distearin.*

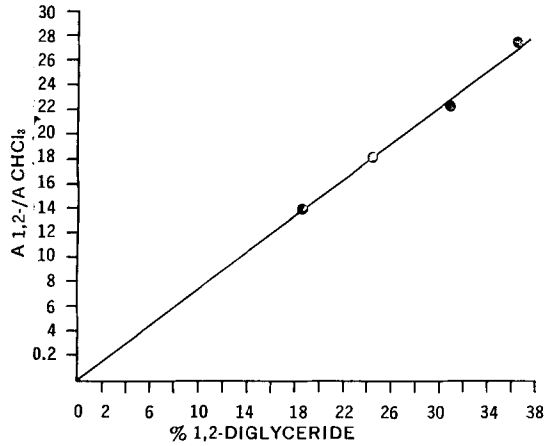


Figure 3—*The value of A 1,2-/A $CHCl_3$ plotted against the percent of 1,2-diglyceride, with A 1,2- = integral value of signal at 225 c.p.s. and A $CHCl_3$ = integral value of signal at 436 c.p.s.*

Some interference could be expected if large amounts of triglycerides were present, but this is an unlikely circumstance. Unsaturated acid moieties would not interfere since they would absorb in the region beyond 275 c.p.s.

The determination can be carried out on samples in the 10–20-mg. range, but the results are best obtained with samples of approximately 50 mg. In addition to the quantitative aspect of the method, the NMR spectra also provide a specific qualitative identification of the isomers.

SUMMARY

A quantitative and qualitative method of analysis for 1,3- and 1,2-diglyceride isomers has been presented. The method can be carried out on sample sizes at the order of 50 mg. The method is fast, accurate, and specific for the two isomers.

REFERENCE

(1) H. Susi, S. G. Morris, T. A. Zell, and W. E. Scott, *J. Amer. Oil Chem. Soc.*, **40**, 329(1963).

ACKNOWLEDGMENT AND ADDRESSES

Received December 15, 1969, from *Smith Kline & French Laboratories, Philadelphia, PA 19101*

Accepted for publication February 9, 1970.

The authors are grateful to Mr. John A. Messina for technical assistance in obtaining the spectra presented here.

Adsorption of Organic Compounds by Commercial Filter Papers and Its Implication on Quantitative–Qualitative Chemical Analysis

WIN LOUNG CHIOU and LLOYD D. SMITH

Abstract □ Eighteen acidic and neutral organic compounds were studied for adsorption onto Filter I (MF-Millipore filter) with a diameter of 17.5 mm. The adsorption after filtration of 3 ml. of solution ranged from zero to almost 100%. The adsorption to Filter II (Whatman filter), however, was much lower and in some cases negligible. The water-soluble or ionic forms of compounds are less adsorbed. The extent of adsorption for each compound might depend on its concentration, filtration rate, volume of solution filtered, and Filter I size used. The adsorption was found to be reversible and could be represented by the Freundlich adsorption isotherm equation. The water-soluble impurities from both types of filters were also studied. Their absorbances below 230 mμ were considerably high. The implication of adsorption and impurities from filters on quantitative–qualitative chemical analysis was discussed.

Keyphrases □ Filter paper adsorption—organic compounds □ Adsorption—organic compounds on filters □ Impurities—filters □ Solubility, organic compounds—filter adsorption □ Filtration rate effect—filter adsorption

The phenomenon of the possible adsorption of organic or inorganic compounds onto polymeric materials such as nylon (1), polyethylene (2), cellulose dialysis membrane, and regular filter paper has been well recognized in the past. Filter pads,[1] made from cellulose esters or similar polymeric materials, have been widely used in both analytical and biological work (3–14). However, the possible adsorption of chemicals to these filters has been generally underestimated or not noticed by research workers using these products. Some of the reasons for unawareness may stem from a statement by the manufacturer in their brochure (15) that the filters will not generally adsorb components from liquid solutions.

The systematic investigation, reported in this article, of the potential effect of these filters on both quantitative and qualitative chemical analysis was begun after an accidental discovery of the surprisingly high adsorption of a water-soluble organic compound after filtration. It was also stimulated by a report of Saad and Higuchi (5) that a discrepancy of 150 to 200% in the solubility of cholesterol was observed after filtering a saturated aqueous suspension through different pore size of the filters. The authors, however, gave no explanation about the possible cause of this difference. Although there are many types of these filters, only Filter I[2] was selected for thorough studies due to its wide use. The adsorption to the Filter II[3] was also studied to a lesser extent for the purpose of comparison.

[1] Millipore filter disks, Millipore Corp., Bedford, Mass.
[2] MF Millipore.

EXPERIMENTAL

Materials—Eighteen acidic or neutral organic compounds were chosen for adsorption studies: *m*-nitrobenzoic acid (Eastman Organic Chemicals); iopanoic acid (Sterling Drug Inc.); hydrocortisone, hydrocortisone acetate, and ethinyl estradiol (The Upjohn Co.); chloramphenicol (Parke, Davis & Co.); benzoic acid (Merck & Co.); phenobarbital (Smith Kline & French Labs.); sodium phenobarbital (Merck & Co.); hexachlorophene (Robinson Laboratory Inc.); salicylic acid (Mallinckrodt Chemical Works); *p*-nitrobenzoic acid (Eastman Organic Chemicals); griseofulvin (McNeil Laboratories, Inc.); warfarin (S. B. Penick & Co.); sodium warfarin (Abbott Laboratories); digitoxin (Eli Lilly & Co.); naphthalene (Matheson Co. Inc.); and spironolactone (Searle & Co.). All the chemicals were reagent grade and were not further purified prior to experiments.

Filter I disks[4] had a prefilter diameter of 17.5 mm. and various pore sizes: 0.025 μ (VS type), 0.22 μ (GS type), and 1.2 μ (RA type). Pyrex microanalysis filter holder (Cat. No. XX10 025 00 from the Millipore Corp.) was used for the support of filtration throughout the experiments. Filter II (W. R. Balston, Ltd., England) employed a round form of disk with the same diameter as Filter I, cut with a pair of scissors and placed into the Millipore filter holder for the filtration study.

Preparation of Solutions—Solutions of relatively water-soluble compounds such as *m*-nitrobenzoic acid, benzoic acid, phenobarbital, sodium phenobarbital, salicylic acid, *p*-nitrobenzoic acid, sodium warfarin, and naphthalene were prepared by dissolving them directly in distilled water or diluted aqueous hydrochloric acid solution. Solutions of relatively water-insoluble compounds such as iopanoic acid, hydrocortisone, chloramphenicol, hydrocortisone acetate, hexachlorophene, griseofulvin, ethinyl estradiol, digitoxin, and spironolactone were prepared by dilution of the concentrated stock solution in 95% ethanol with distilled water. These solutions contained, however, less than 1% ethanol (v/v).

Adsorption Studies—Before carrying out adsorption studies, the filter apparatus without the placement of filter disks was thoroughly cleaned with distilled water. Three milliliters of the distilled water or aqueous hydrochloric acid solution was then passed through the apparatus by the suction of a water aspirator. Possible contamination in the filtrate was checked spectrophotometrically by using a Cary 15 spectrophotometer.

The effect of both single and multiple filtrations on adsorption to the filters was studied. In the single-filtration studies, 3 ml. of aqueous solutions of organic substances was filtered through either the Filter I or II disk. The time of the filtration was kept as constant as possible: about 30 sec. for 0.22-μ Filter I and 1.2-μ Filter I and Filter II, and about 2.5 min. for the 0.025-μ Millipore. The absorbance of the solution, usually at the peak absorption wavelength of each

[3] Whatman filter paper, No. 4.
[4] All Millipore filter disks used in this investigation were purchased from the the Millipore Corp. in 1969. The lot number for 1.2-μ pore size is 1145.

Table I—Absorbances[a] of the Filtrate after Passing 3 ml. of Distilled Water through Different Filter Paper[b]

Wavelength, mμ	Filter I			Filter II
	0.025-μ	0.220-μ	1.20-μ	
205	0.406	0.133	0.238	0.095
210	0.320	0.110	0.217	0.080
220	0.156	0.073	0.137	0.054
230	0.062	0.040	0.034	0.027
240	0.048	0.018	0.004	0.011
250	0.047	0.017	0.013	0.009
260	0.039	0.013	0.025	0.008
270	0.022	0.013	0.043	0.009
280	0.015	0.010	0.055	0.007
290	0.012	0.005	0.019	0.005
300	0.009	0.003	0.012	0.004
310	0.009	—	—	0.003
320	0.009	—	—	0.002

[a] All values are the average of three runs. [b] Area of the filter paper is 2.5 cm.².

compound, was measured before and after the filtration using a Cary 15 spectrophotometer. Within the concentration range studied, it was found that the absorbances of the solutions of all the compounds followed Beer's law.

In the multiple-filtration studies, four aliquots of 3 ml. each of the aqueous solutions were passed successively through the same 0.22-μ Filter I pad; the absorbance of each 3-ml. filtrate was then determined. The effect of the rate of filtration on adsorption was also studied by the single-filtration method filtered over 30 and 300 sec.

It was found that both Filters I and II contain a fairly large amount of water-soluble impurities which can be extracted into the filtrate during the filtration process and show optical absorption at various wavelengths. Therefore, control experiments were performed by passing distilled water or appropriate aqueous vehicles through filter disks for all adsorption studies reported in this communication. The extent of adsorption was estimated after making such blank corrections. All experiments were run at least in duplicate. It must be noted the variation of adsorption from different runs was generally very small and insignificant.

Desorption Studies—In desorption studies, 3 ml. of aqueous solution was passed through 0.22-μ Filter I, the absorbance of the filtrate was measured, and the percent adsorbed was determined. The filter pad was then removed, the apparatus was thoroughly cleaned, and a blank check was run to ensure no contamination. The same pad was replaced in the apparatus, and 3 ml. of distilled water or 0.01 *N* HCl (as in the case of warfarin) passed through the pad. The absorbance of the filtrate was measured and the percent of chemical washed from the filter pad was determined. Additional two or three similar washings were carried out on the same pad, and the extent of desorption was determined each time.

Adsorption Equilibrium Studies—Two 0.22-μ Filter I disks were placed in 15-ml. screw-top culture tubes. Five milliliters of various concentrations of griseofulvin or warfarin was added. The tubes were stirred at 100 strokes/min. in a constant-temperature (29 ± 0.5°) water bath shaker (Eberbach Corp.) for 24 hr. The solutions were drained off the pads and the absorbance at suitable wavelengths was measured. It was found the adsorption equilibrium was reached after 24 hr. of shaking.

RESULTS AND DISCUSSION

Impurities from Filters—As previously noted (5), a significant amount of UV-absorbing impurities could be extracted from Filter I and these interfered with the spectrophotometric assay of the filtrate at 205 mμ during the study of the water solubility of cholesterol. However, it seems, to date, that no quantitative analysis of the impurities has been reported in the literature. The water-soluble impurities from Filter II also appear to be generally ignored.

Table I shows the absorbances from 205 to 320 mμ of the filtrates after passing 3 ml. of distilled water through different filters. It is obvious that the absorbance of extracted contaminants increases with the decrease of the wavelength except for the 1.2-μ Filter I disk which shows a minimum absorbance at 240 mμ. The absorbance of the impurities below 230 mμ is so high that it can result in a serious error in the quantitative–qualitative analysis if not taken into consideration. It must be noted the absorbance of filtrates decreased as the volume of the distilled water filtered increased. This is expected because there is only a maximum amount of impurities present. Therefore, the effect of the contaminants on the spectrophotometric measurements will be greater when working with smaller volumes of solutions. It should also be emphasized that the filtering area used in these studies is only about 2.5 cm.². Since much larger filter paper, especially Filter II type, is usually employed for the filtration purpose, the amount of extracted impurities will be considerable. This investigation does not attempt to determine the exact chemical nature of the impurities, although it has been reported in the past that Filter I might contain 2–3% of its dry weight as a detergent (16). It should be noted that the different spectra of the impurities from various pore sizes of Filter I might indicate that they contained different water-extracted contaminants and/or different quantities of such contaminant.

Adsorption from Single Filtration through Filter I—Table II lists the wavelength used for each compound, their initial concentrations and absorbances, and the percent adsorbed from the first 3 ml. of the filtering solutions by the 0.22- and 0.025-μ Filter I pads. It is quite surprising to find all 18 compounds with a wide range of solubility

Table II—Adsorption of Various Chemicals by Filter I Pads (17.5-mm. Diameter)

Chemicals	Initial Concn., mcg./ml.	Wavelength, mμ	Initial Absorbance	Chemicals Adsorbed, %[a] Pore Size	
				0.22-μ	0.025-μ
m-Nitrobenzoic acid (distd. H_2O)	20	270	0.841	3.2	4.6
Iopanoic acid	10	230	0.675	5.8	0.0[b]
Hydrocortisone	10	247	0.414	6.1	9.6
Chloramphenicol	20	276	0.649	9.9	14.4
Benzoic acid (0.1 *N* HCl)	10	230	0.966	14.2	46.6
Benzoic acid (distd. H_2O)	10	225	0.723	9.1	2.6
Phenobarbital (0.01 *N* HCl)	20	223	0.392	16.9	23.5
Na-Phenobarbital (distd. H_2O)	20	240	0.645	6.2	6.5
Hydrocortisone acetate	10	247	0.395	17.0	35.6
Hexachlorophene	20	314	0.328	17.4	64.7
Salicylic acid (0.1 *N* HCl)	25	303	0.664	21.2	69.5
Salicylic acid (distd. H_2O)	25	302	0.641	9.2	71.2
p-Nitrobenzoic acid (0.1 *N* HCl)	10	264	0.612	25.8	46.6
Griseofulvin	10	295	0.717	28.9	89.2
Ethinyl estradiol	10	210	0.353	37.5	100.0[b]
Warfarin (0.01 *N* HCl)	10	273	0.329	38.6	94.8
Na-Warfarin (distd. H_2O)	20	308	0.803	4.7	0.0[b]
Digitoxin	10	220	0.190	57.9	100.0[b]
Naphthalene	25	276	0.949	86.0	97.7
Spironolactone	10	242	0.435	99.4	60.2

[a] Values obtained after analysis of a 3-ml. filtrate. [b] Approximate value.

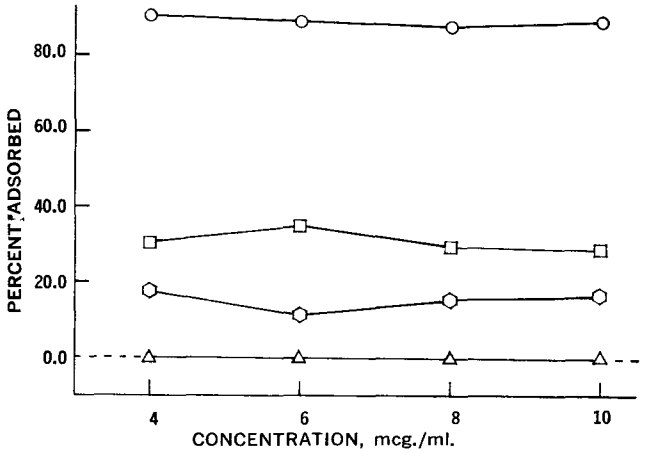


Figure 1—*Percent of griseofulvin adsorbed after passing 3 ml. of solutions through different filter paper. Key: ○, 0.025-μ Filter I; □, 0.22-μ Filter I; ⬡, 1.20-μ Filter I; and △, Filter II.*

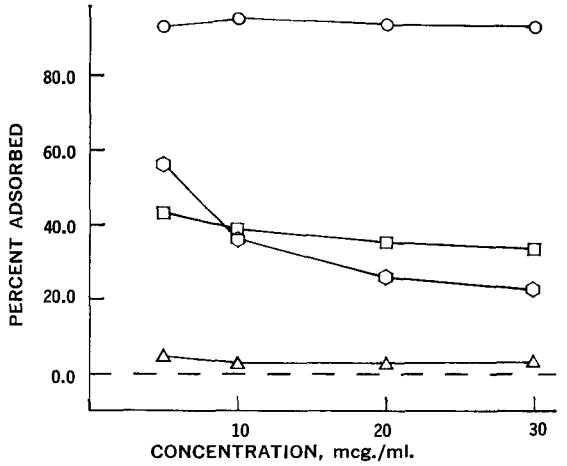


Figure 3—*Percent of warfarin adsorbed after passing 3 ml. of solutions through different filter paper. Key: ○, 0.025-μ Filter I; □, 0.22-μ Filter I; ⬡, 1.20-μ Filter I; and △, Filter II.*

and chemical structure were adsorbed by the 0.22-μ Filter I, ranging from 3.2 to 99.4%. This is contrary to the statement given by the manufacturer. A range of zero to almost 100% adsorption was found for the 0.025-μ Filter I.

From Table II several interesting points seem worth discussing.

1. Adsorption by Filter I probably exists for every organic compound. Hence, one should check this possibility whenever they are intended to be used for quantitative separation. A warning of the possible adsorption should be stated in the brochure by the manufacturer.

2. It is recommended that the adsorption problem should be checked when using other types of Millipore filters (Duralon and Mitex). They were not studied in this investigation.

3. As will be further discussed later, the extent of adsorption seems to relate to the pore size of filters: the smaller the pore size, the more the adsorption. This might be due to the higher specific surface area available for adsorption in the smaller pore size filters. This is demonstrated in the table, where a majority of compounds show higher adsorption by the 0.025-μ Filter I. It is, however, possible that the presence of different amounts or nature of the water-extractable impurities may also affect the degree of adsorption. The effect of the removal of these impurities on adsorption seems worth further study.

4. The water solubility of compounds also appears to relate to the adsorption: the lower the solubility, the stronger the adsorption. This is illustrated by the generally lower adsorption of the more soluble compounds listed at the top of the table (iopanoic acid is an exception). One interesting example is hydrocortisone and hydrocortisone acetate. Both have similar chemical structures. However, hydrocortisone acetate, with much less water solubility, shows greater adsorption.

5. The ionic forms are generally much less adsorbed than their nonionic forms. This is clearly shown by warfarin, phenobarbital, benzoic acid, and salicylic acid. It indicates that the ionic bonding between organic adsorbates and filter adsorbents is not significant in contributing to adsorption. The adsorption between them may mainly be due to hydrophobic and hydrogen bonds.

6. The importance of the van der Waals force in causing adsorption is demonstrated by the high adsorption of naphthalene, a fully conjugated flat hydrocarbon.

7. It should be pointed out that the concentrations of several compounds listed in Table II such as hydrocortisone acetate (17), ethinyl estradiol (17), digitoxin (18), griseofulvin (6), and spironolactone (the authors' preliminary study) are approximately equal to their water solubility. A serious error in the solubility value may be made if one uses these filters and ignores their adsorption problem. It is also possible the discrepancy of the reported solubilities of many compounds in the literature (5, 17) might be attributed to the impurity, contamination and adsorption by the filters. Cholesterol might represent a good example. It is quite startling to find from the literature that this biologically important steroid has an extremely wide range of reported water-solubility values: 0.025 mcg./ml. at 30° from Saad and Higuchi (5); about 2.0 mcg./ml. from the Merck Index (18) and Gemant (19); 52 mcg./ml. from Lange and Amund-

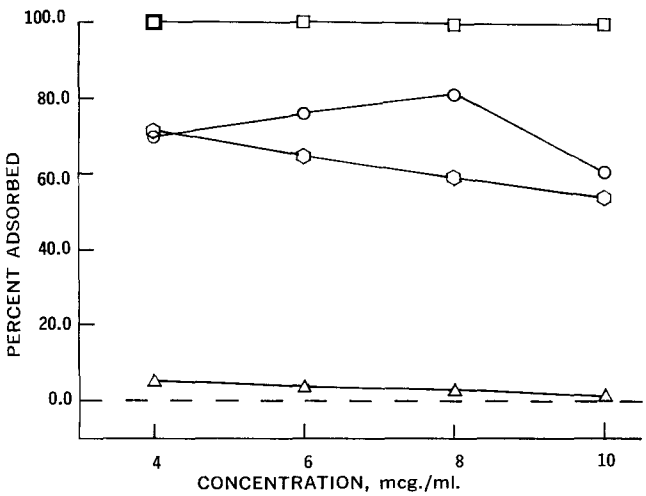


Figure 2—*Percent of spironolactone adsorbed after passing 3 ml. of solutions through different filter paper. Key: □, 0.22-μ Filter I; ○, 0.025-μ Filter I; ⬡, 1.20-μ Filter I; and △, Filter II.*

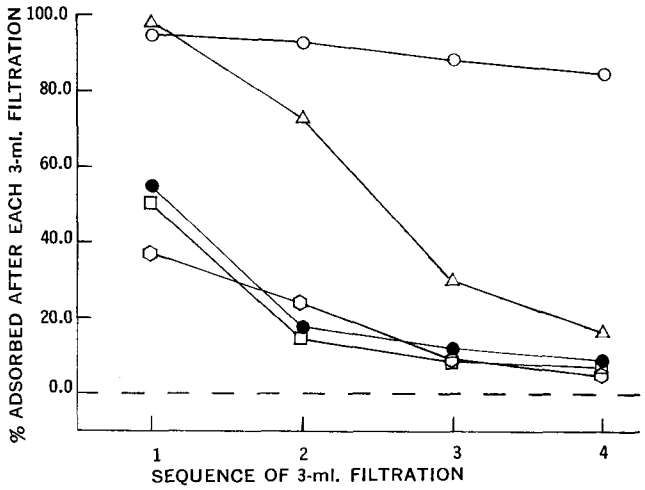


Figure 4—*Adsorption of four chemicals by 0.22-μ Filter I pads after repetitive filtration of 3 ml. aqueous solution. Key: ○, naphthalene (initial concentration, 7.6 mcg./ml.); △, spironolactone (10 mcg./ml.); ●, warfarin (0.01 N HCl, 10 mcg./ml.); □, griseofulvin (5 mcg./ml.); and ⬡, ethinyl estradiol (10 mcg./ml.).*

Table III—Effect of Filtration Rate on Adsorption by Filter I Pad, 0.22-μ Pore Size

Chemicals	% Adsorbed 30 sec.[a]	% Adsorbed 300 sec.[a]
Salicylic acid (0.1 *N* HCl; 25 mcg./ml.)	21.2	27.3
p-Nitrobenzoic acid (0.1 *N* HCl; 10 mcg./ml.)	25.8	46.6
Griseofulvin (distd. H_2O; 10 mcg./ml.)	28.9	29.1
Warfarin (0.01 *N* HCl; 30 mcg./ml.)	33.7	36.6

[a] Filtration time of 3 ml. aqueous solution.

son (20); and 2600 mcg./ml. from the "Handbook of Chemistry and Physics" (21) which cites the value from Dehn (22). Although the solubility of a compound may vary with its particle size, crystalline form, and electric charge of particles (23), it is believed these factors could not explain a 10,000-fold difference.

Adsorption by Filters I and II *versus* Concentrations—The percent of adsorption at different concentrations of three compounds, griseofulvin, spironolactone, and warfarin, by Filter II and three different pore sizes of Filter I is shown in Figs. 1–3. The percent adsorbed was found to vary with the type of filters, Filter I *versus* Filter II, the pore size of Filter I, and finally the concentration of each compound. The decreasing degree of adsorption by the four different filters is generally in the following order: 0.025-μ Filter I > 0.22-μ Filter I > 1.2-μ Filter I > Filter II.

Adsorption from Multiple Filtration through Filter I—The percent of adsorption from the multiple filtration of five compounds, *i.e.*, spironolactone, griseofulvin, ethinyl estradiol, warfarin, and naphthalene, through the 0.22-μ Filter I is shown in Fig. 4. The extent of adsorption of all compounds studied decreased as the volume of filtering solutions increased. This is not unexpected because the adsorption site is usually saturable. The adsorption capacity of Filter I toward the naphthalene molecule is, however, surprisingly high, as evidenced by the least steepness of the slope in Fig. 4, and 85% of naphthalene was still adsorbed in the fourth filtration through the same filter.

Effect of Filtration Rate on Adsorption—From the practical point of view, it is also interesting and important to know whether the filtration rate will affect the adsorption, since different rates may be employed at different occasions by the same or different workers. The results obtained from four compounds after filtering through 0.22-μ Filter I are shown in Table III. A 10-fold difference in the filtration rate did indeed result in a change in the degree of adsorption. The slower the filtration rate, the more was the adsorption. Some compounds like *p*-nitrobenzoic acid and salicylic acid are sensitive to the rate while some are not.

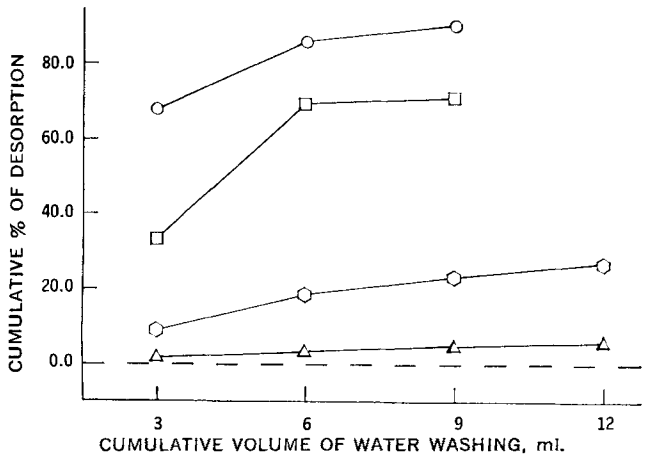


Figure 5—*Cumulative desorption of four chemicals from 0.22-μ Filter I pads after previous filtration of 3 ml. aqueous solution. Key:* ○, *spironolactone (initial filtered concentration, 10 mcg./ml.);* △, *naphthalene (7.6 mcg./ml.);* □, *salicylic acid (H_2O; 25 mcg./ml.); and* ○, *warfarin (0.01 N HCl; 20 mcg./ml.).*

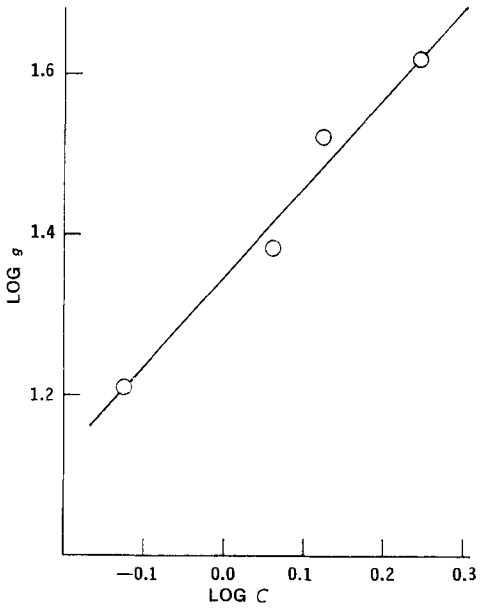


Figure 6—*Freundlich adsorption isotherm of griseofulvin (0.22-μ Filter I).*

Desorption Studies—Desorption experiments on four compounds, *i.e.*, spironolactone, naphthalene, salicylic acid, and warfarin, were run to study further the binding nature of adsorption on Filter I. The results are shown in Fig. 5. The fact that the adsorbed compounds could be washed from the filter indicates a reversible characteristic of the adsorption. The strong van der Waals bonding between the naphthalene and Filter I is once again demonstrated by the least desorption of naphthalene. Only 6% of the adsorbed naphthalene was washed away by 12 ml. of distilled water.

Adsorption Equilibrium—Table IV represents equilibrium adsorption data for griseofulvin and warfarin. The percent adsorbed was found to be almost independent of initial concentration.The plots of Freundlich adsorption isotherms are shown in Figs. 6 and 7 in which q represents micrograms of compounds adsorbed by the two filter disks used for each study, and c, in micrograms per milliliter, is the concentration of the solutions at equilibrium.

SUMMARY

1. Eighteen acidic and neutral organic compounds with a wide range of water solubility and chemical structure were studied for their adsorption to Filters I and II. The adsorption on the 0.22- and 0.025-μ Filter I varied from zero to almost 100% during the single-filtration studies. The adsorption on Filter II was low or negligible.

2. Water-soluble compounds generally showed a lower tendency for adsorption.

Table IV—Data Showing Equilibrium Adsorption of Griseofulvin and Warfarin by 0.22-μ Pore Size Filter I

Chemicals	Initial Concn., mcg./ml.	% Adsorbed
Griseofulvin	4.0	81.2
	6.0	80.7
	8.0	83.5
	10.0	82.5
Warfarin	5.0	99.4
	10.0	98.8
	20.0	98.4
	30.0	98.4

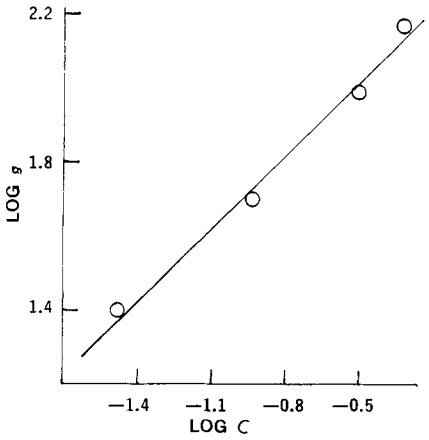


Figure 7—*Freundlich adsorption isotherm of warfarin (0.22-μ Filter I).*

3. Ionic salts were less adsorbed than their neutral forms.

4. The adsorption of a compound might vary with its concentration, volume of the solution filtered, filtration rate, and the pore size.

5. The adsorption was found to be reversible. The adsorption at equilibrium could be represented by Freundlich adsorption isotherm plots.

6. Water-soluble impurities from Filters I and II were studied spectrophotometrically. Their absorbances below 230 mμ were found to be considerably high.

7. The possible effects of adsorption and impurities from filters on the quantitative-qualitative chemical analysis were discussed.

REFERENCES

(1) H. F. Berg, W. L. Guess, and J. Autian, *J. Pharm. Sci.*, **54,** 79(1965).

(2) P. Benes and I. Rajman, *Collect. Czech. Chem. Commun.*, **34,** 1353(1969).

(3) G. Levy and R. H. Reuning, *J. Pharm. Sci.*, **53,** 1471(1964).

(4) G. Levy and T. Tatsuzaqa, *ibid.*, **54,** 1003(1965).

(5) H. Y. Saad and W. I. Higuchi, *ibid.*, **54,** 1205(1965).

(6) A. H. Goldberg, M. Gibaldi, and J. L. Kanig, *ibid.*, **55,** 487(1966).

(7) M. Mayersohn and M. Gibaldi, *ibid.*, **55,** 1323(1966).

(8) Z. Mansour and E. P. Guth, *ibid.*, **57,** 404(1968).

(9) S. L. Lin, J. Menig, and L. Lachman, *ibid.*, **57,** 2143(1968).

(10) S. Feldman and M. Gibaldi, *ibid.*, **58,** 425(1969).

(11) R. A. Anderson and K. J. Morgan, *J. Pharm. Pharmacol.*, **18,** 449(1965).

(12) T. Tachibana and A. Nakamura, *Kolloid-Z.*, **203,** 130(1965).

(13) M. A. England, *Exp. Cell. Res.*, **54,** 222(1969).

(14) M. R. W. Brown, J. A. Farwell, and S. A. Rosenbluth, *Anal. Biochem.*, **27,** 484(1969).

(15) Catalog MF-68, Millipore Corp., Bedford, Mass., p. 25.

(16) R. D. Cahn, *Science*, **155,** 195(1967).

(17) P. Kabasakalian, E. Britt, and M. D. Yudis, *J. Pharm. Sci.*, **55,** 642(1966).

(18) "The Merck Index," 8th ed., Merck and Co., Inc., Rahway, N. J., 1968, pp. 253–364.

(19) A. Gemant, *Life Sci.*, No. **6,** 233(1962).

(20) W. E. Lange and M. E. Amundson, *J. Pharm. Sci.*, **51,** 1102(1962).

(21) "Handbook of Chemistry and Physics," 49th ed., R. C. Weast, Ed., The Chemical Rubber Co., Cleveland, Ohio, 1968–1969, p. c-250.

(22) W. M. Dehn, *J. Amer. Chem. Soc.*, **39,** 1399(1917).

(23) L. F. Knapp, *Trans. Faraday Soc.*, **17,** 457(1922).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 22, 1969, from the *College of Pharmacy, Washington State University, Pullman, WA 99163*

Accepted for publication December 11, 1969.

This investigation was supported in part by NIH Grant FR056866

Polytetrafluoroethylene Coating as a Suppository Mold Releasing Agent

H. W. PUFFER and PAMELA A. BARNETT

Abstract □ The release quality of suppository molds with either smooth or damaged polytetrafluoroethylene coated or uncoated cavity surfaces was compared using four different suppository bases. The release of suppositories from damaged molds was improved by coating the damaged cavity surfaces with polytetrafluoroethylene. Such a coating did not appreciably improve the release characteristics of new molds.

Keyphrases □ Polytetrafluoroethylene coating—suppository mold □ Suppository molds—suppository releasing agent

The fusion or melting-casting process is a popular method in the preparation of suppositories (1). However, sticking of the suppositories to the cavity surface can become a problem, particularly with worn or damaged molds. To obviate this problem, some authorities (2, 3) recommend coating the cavity surfaces of the mold with a lubricant such as mineral oil or green soap tincture. Obviously such a step requires time and is inconvenient. Lubricants used in this manner may also be deposited as a thin film on the surface of the suppository. It would seem best to avoid the use of lubricants entirely.

Similar problems of sticking in industrial molds have been prevented or reduced by using polytetrafluoroethylene[1] (4, 5), an inert plastic homopolymer. Polytetrafluoroethylene has the advantage of imparting a nonstick finish to the mold. This study, therefore, was undertaken to evaluate polytetrafluoroethylene as a mold releasing agent in the fusion method of molding suppositories.

EXPERIMENTAL

Materials—The four molds used in this study were constructed of nickel-plated brass, having outside dimensions of 14.7 × 3 × 3.7 cm. and weighing 1261 g. each. They were of the divided type, aligned by two affixed pins and secured by two thumbscrews. Each mold contained six Wellcombe-shaped cavities having a capacity of 2 g. each. Two of the molds were new and their surfaces were smooth and polished. Two of the molds were badly damaged by severe scratches and chemical erosion. One new mold and one damaged mold were custom coated[2] with polytetrafluoroethylene.

The following suppository bases were used: (*a*) theobroma oil BP,[3] (*b*) suppository base II,[4] (*c*) glycerinated gelatin USP,[5] and (*d*) polyethylene glycol containing polyethylene glycol 1000,[6] 50%, and polyethylene glycol 4000,[6] 50%.

Procedure—The theobroma oil and suppository base II were melted and maintained molten, using a constant-temperature water bath,[7] at a temperature of 36.5 ± 0.5°. The polyethylene glycol base was similarly prepared and used at a temperature of 54.5 ± 0.5°. Glycerinated gelatin base was prepared according to the USP (6) method and maintained molten with the water bath at a temperature of 46.5 ± 0.5°. Each base was prepared and used as a single batch throughout the study.

For each time period (Table I), 10 lots containing six suppositories were formed for each of the possible suppository base mold surface combinations. The molten base was removed from the water bath and poured continuously to form each lot. After pouring, each lot of suppositories was allowed to solidify at room temperature, 17 ± 3°, for the designated time. At the end of the time period, the suppositories were removed from the mold, and the quantity of perfectly formed suppositories released from the mold was counted. After use, the molds were allowed to stand and equilibrate with room temperature before being used again.

Table I—Suppository Release from Polytetrafluoroethylene Coated and Uncoated Molds Using Various Bases

Time Before Unmolding, min.	Condition of Mold[a]	Number of Suppositories Released from Molds[b]			
		Theobroma Oil	Suppository Base II	Glycerinated Gelatin	Polyethylene Glycol
1	N	0	6	17	21
	NP	0	3	20	25
	DP	0	5	19	28
	D	0	0	8	12
2	N	2	12	29	25
	NP	0	12	39	29
	DP	1	13	39	38
	D	0	9	25	21
3	N	18	27	41	44
	NP	10	26	44	37
	DP	8	24	48	40
	D	4	15	20	32
4	N	23	42	46	51
	NP	14	40	49	52
	DP	15	36	50	53
	D	9	22	47	45
6	N	45	43	56	60
	NP	41	44	50	60
	DP	42	42	48	60
	D	25	27	44	60
8	N	55	56	60	60
	NP	45	51	59	60
	DP	44	51	58	57
	D	32	45	52	60
10	N	57	54	60	56
	NP	54	55	60	60
	DP	56	53	60	60
	D	36	47	42	58
12	N	60	57	59	60
	NP	60	60	60	60
	DP	59	60	60	60
	D	42	48	43	56

[a] Key: N, new mold with smooth polished cavity surfaces; NP, new mold with polytetrafluoroethylene-coated cavity surfaces; DP, damaged mold with polytetrafluoroethylene-coated cavity surfaces; D, damaged mold with scratched and eroded cavity surfaces. [b] For each time period, 10 lots containing six suppositories were formed for each of the possible suppository base–mold surface combinations. Each figure represents the quantity of perfectly formed suppositories released from the mold (60 suppositories maximum possible for each figure).

[1] Teflon, E. I. du Pont de Nemours, Inc., Wilmington, Del.

[2] Custom coating applied by Plastic Coatings Ltd., Christchurch 1, New Zealand.

[3] Kempthorne Prosser and Co., Dunedin, New Zealand.

[4] A base composed of special, hardened, fatty alcohols and fats, marketed by Henkel International GMBH, Dusseldorf, Germany.

[5] The gelatin and glycerin used in this base were supplied by Kempthorne Prosser and Co., Dunedin, New Zealand.

[6] Union Carbide Corp., New York, N. Y.

[7] Constant-temperature water bath, type SB 3, manufactured by Grant Instruments (Cambridge) Ltd., Barrington, Cambridge, England.

Table II—Total Suppositories Released from Each Type Mold

Suppository Base	New Mold, Uncoated	New Mold, Polytetrafluoroethylene Coated	Damaged Mold, Polytetrafluoroethylene Coated	Damaged Mold, Uncoated
Theobroma oil	260	224	225	148
Suppository base II	301	291	284	213
Glycerinated gelatin	368	381	382	281
Polyethylene glycol	377	383	396	344
Total	1306	1279	1287	986
% of maximum possible yield[a]	68.0	66.6	67.0	51.4

[a] Maximum possible yield was 1920 suppositories.

RESULTS AND DISCUSSION

Polytetrafluoroethylene was most effective in improving the release of suppositories composed of a fatty base and had little effect on the release of polyethylene glycol suppositories after the first 6 min. (Table I). Coating the molds with polytetrafluoroethylene did not decrease appreciably the time required for the molds to stand before removing the suppositories (Table I). As indicated in Table II, there was little difference in the number of suppositories released from polytetrafluoroethylene-coated molds *versus* the new mold with smooth cavity surfaces. However, the release qualities of the damaged mold that had been coated with polytetrafluoroethylene appeared to be very much improved and approximated that of the new molds (Table II). With the assumption that 12 min. is adequate for complete solidification of the four bases tested, the experimental results indicate an increase of approximately 20%[8] in the yield of suppositories from damaged molds that were polytetrafluoroethylene coated. According to these results, badly damaged molds could be restored by coating their surfaces with polytetrafluoroethylene. However, there would be little advantage in polytetrafluoroethylene coating new molds. The cost of the coating is relatively inexpensive and substantially less than buying a new mold. The coating is easily damaged and, as with new molds, the surfaces should be handled carefully.

[8] Calculated using data from the 12-min. time period, Table I, as follows: damaged molds (D) yield 42+48+43+56 = 189 suppositories, damaged molds polytetrafluoroethylene coated (DP) yield 59+60+60+60 = 239; 239−189 = 50 suppositories; 50/239 = 20.5%.

REFERENCES

(1) I. Setnikar and V. Pietra, *J. Pharm. Sci.*, **58**, 112(1969).

(2) H. Davis, "Bentley's Text-Book of Pharmaceutics," 7th ed., Baillieve, Tindall and Cox, London, England, 1961, p. 567.

(3) L. C. Zopf and S. M. Blaug, in "Remington's Pharmaceutical Sciences," E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965, p. 552.

(4) *J. Teflon*, **9**, 2(1968).

(5) S. Siegel, E. J. Hanus, and J. W. Carr, *J. Pharm. Sci.*, **52**, 604(1963).

(6) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 793.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 25, 1969, from the *Department of Pharmacology and Pharmacy, University of Otago, Dunedin, New Zealand.*

Accepted for publication January 19, 1970.

Gas Chromatographic Determination of *N*-γ-Phenylpropyl-*N*-benzyloxy Acetamide (W-1372) in Blood

J. F. DOUGLAS and J. A. STOCKAGE*

Abstract □ A procedure is described for the determination of *N*-γ-phenylpropyl-*N*-benzyloxy acetamide (W-1372) in plasma or whole blood. W-1372 is extracted from plasma with hexane and measured quantitatively by gas chromatography. The technique is simple, reproducible, and accurate in the range of 1–10 mcg./ml. Dibutyl phthalate is used as the internal standard for quantitation by the relative peak area method.

Keyphrases □ *N*-γ-Phenylpropyl-*N*-benzyloxy acetamide (W-1372)—determination, blood □ Blood—W-1372 drug determination □ GLC—analysis

W-1372 is a new hypolipidemic agent which has been shown by Berger *et al.* (1, 2) to retard atherosclerotic lesions and reduce blood cholesterol in animals maintained on a hypercholesteremic diet. This manuscript describes a procedure for the quantitative determination of W-1372 in blood.

EXPERIMENTAL

Equipment and Reagents—An F and M model 402 dual-column gas chromatograph, equipped with a hydrogen-flame ionization detector and a 1-mv. Minneapolis-Honeywell recorder, was employed. The chromatographic columns used were 1.23-m. × 0.63-cm. (4-ft. × 0.25-in.) glass tubes packed with 3.8% methylvinyl silicone gum rubber (W98) on 80–100 mesh Diataport S. The temperatures used were: column, 200°; injection port, 275°; and detector block, 225°. Carrier gas (helium) flow was 60 ml./min.

Sensitivity settings were range 10 with an attenuation factor of 4×. The reagents were redistilled hexane, redistilled chloroform, and dibutyl phthalate (Eastman). The retention times under these conditions are 4.8 min. for W-1372 and 1.6 min. for dibutyl phthalate (Fig. 1).

Procedure—Plasma or whole blood, 1.0 ml., was extracted with 10 ml. of hexane, and 8.0 ml. of the organic solvent was removed and evaporated to dryness in a stream of nitrogen. For drug concentration greater than 10 mcg./ml., the specimen was diluted with physiological saline to an appropriate volume prior to extraction. The dried sample was dissolved in 0.1 ml. of chloroform containing 1.0 mcg. of dibutyl phthalate, and 2.6 μl. of this solution was injected into the gas chromatograph. The concentration of W-1372

Table I—Recovery of W-1372 Added to Human Plasma

mcg./ml. Added	Recovery, %[a]
2.0	99.2 ± 1.0
5.0	98.0 ± 1.1
10.0	98.3 ± 0.6

[a] Values are the mean of quadruplicate determinations and are given with their standard error.

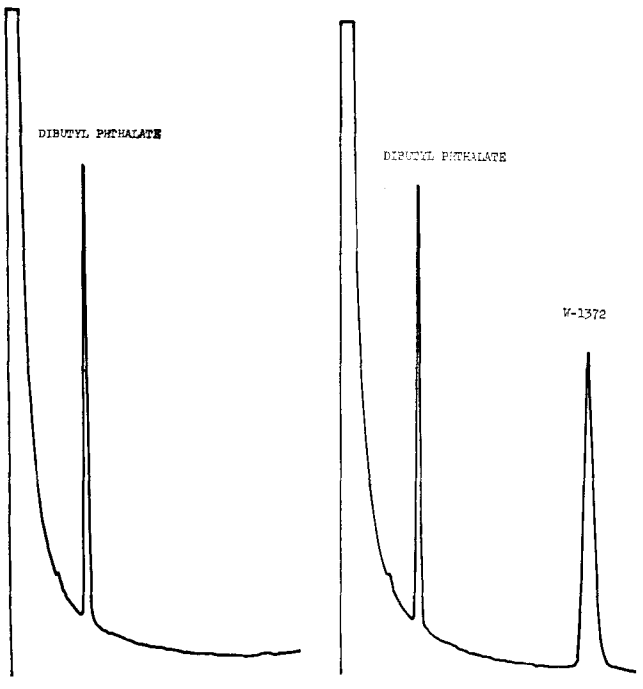


Figure 1—*Gas chromatograms of human plasma treated as described. Left: Chromatogram from normal plasma. Right: Chromatogram from normal plasma with 10 mcg./ml. of W-1372 added.*

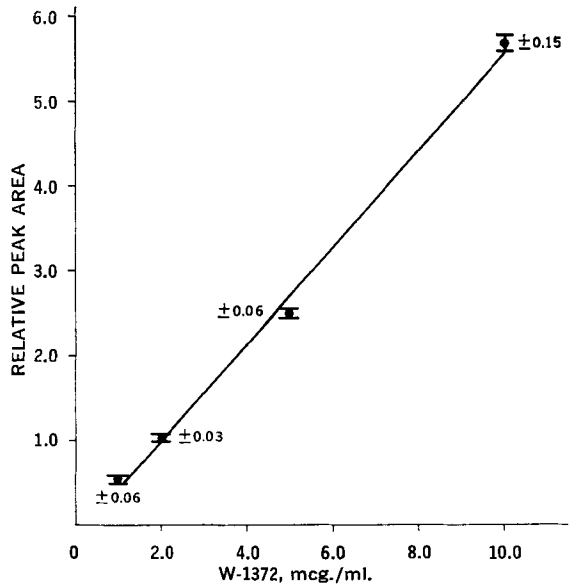


Figure 2—*Relationship between relative peak area and W-1372 concentration in plasma.*

Table II—Blood Concentrations of W-1372 in Several Species after Intravenous Administration[a]

Time, min.	W-1372, mcg./ml.
Dog, 20 mg./kg.	
1	11.9
5	6.1
15	1.1
30	Trace
60	Trace
Squirrel Monkey, 25 mg./kg.	
1	28.9
10	3.9
30	2.0
Cebus Monkey, 25 mg./kg.	
1	9.2
10	3.8
30	Trace

[a] Values are the average of two animals. The amount of drug given is as indicated.

was determined by the relative peak area method, using dibutyl phthalate as the internal standard.

RESULTS AND DISCUSSION

W-1372 can be quantitated gas chromatographically when the relative peak area is used as an index of concentration. The relationship between relative peak area and drug concentration in the range of 1–10 mcg./ml. of plasma is illustrated in Fig. 2. The reproducibility of the procedure, as indicated by the standard error of quintuplicate determinations, is also shown in Fig. 2. The recovery of W-1372 was 98–99%, as shown in Table I.

The extraction procedure effectively separates W-1372 from normal interfering plasma constituents, since determinations in normal plasma of humans (Fig. 1), dogs, or monkeys give little or no blank (<0.1 mcg./ml.). The known major metabolites of W-1372—benzoic acid, hippuric acid, and *N*-γ-phenylpropyl-*N*-benzyloxyamine (3)—do not interfere.

The blood-depletion pattern of W-1372 following intravenous administration was studied in the dog, squirrel monkey, and Cebus monkey. The results (Table II) indicate that the drug is rapidly removed from the blood, with only trace amounts present after 30 min. in the dog and Cebus monkey and a low level present at this time in the squirrel monkey. A previous study (3) has shown that the plasma half-life of radioactivity following oral administration of W-1372-benzyl-^{14}C is 4 hr. in the squirrel monkey and 12 hr. in the dog.

REFERENCES

(1) F. M. Berger, J. F. Douglas, B. J. Ludwig, and S. Margolin, *J. Pharmacol. Exp. Ther.*, **170**, 371(1969).

(2) F. M. Berger and G. G. Lu, *Fed. Proc.*, **28**, 677(1969).

(3) J. Edelson, J. F. Douglas, and B. J. Ludwig, *ibid.*, **28**, 798 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 17, 1969, from *Wallace Laboratories, Division of Carter-Wallace, Inc., Cranbury, NJ 08512*

Accepted for publication January 27, 1970.

* Present address: Wallace and Tiernan, Inc., Cedar Knolls, N. J.

Mechanism of Action of Retinyl Compounds on Wound Healing I: Structural Relationship of Retinyl Compounds and Wound Healing

K. H. LEE and THEODORE G. TONG

Abstract □ Retinol, retinyl acetate, and retinoic acid promote wound healing. Retinoic acid is most active. Beta-carotene is active, while lycopene is inactive; β-ionone is active and α-ionone is inactive. For full activity, the compound should contain a β-ionone ring, a conjugated double-bond side chain, and a terminal carboxylic acid side chain.

Keyphrases □ Retinyl compounds, activity—structural relationship □ Wound healing—retinyl compound effect □ GLC—separation □ NMR spectroscopy—identity

In a previous report, it was shown that local application of retinol, retinyl acetate, or retinoic acid, dissolved in a nonionic base (NIB), promotes skin wound healing (1). Retinoic acid is relatively more effective than retinol or retinyl acetate. The fact that retinol, retinyl acetate, and retinoic acid are all effective when applied locally on the skin wound indicates that the primary alcohol group is not essential in promoting wound healing. It is of interest, therefore, to study the structural relationship of the other part of the retinol molecule for wound-healing activity. The compounds evaluated in this investigation are β-carotene, lycopene, β-ionone, and α-ionone.

Beta-carotene has the same ring structure and conjugated double-bond hydrocarbon side chain as retinol. It should show activity on wound healing as retinol. Lycopene has essentially the same structure as β-carotene but differs from the latter in not having the closed ring structure at either end of the molecule. Beta-ionone, on the other hand, has the same cyclohexene ring but does not have the same length of hydrocarbon side chain as retinol or β-carotene. Alpha-ionone differs from β-ionone by the position of the double bond in the cyclohexene ring and does not conjugate with the side chain. The structure–activity relationships of these compounds to wound healing are discussed.

Table II—Blood Concentrations of W-1372 in Several Species after Intravenous Administration[a]

Time, min.	W-1372, mcg./ml.
Dog, 20 mg./kg.	
1	11.9
5	6.1
15	1.1
30	Trace
60	Trace
Squirrel Monkey, 25 mg./kg.	
1	28.9
10	3.9
30	2.0
Cebus Monkey, 25 mg./kg.	
1	9.2
10	3.8
30	Trace

[a] Values are the average of two animals. The amount of drug given is as indicated.

was determined by the relative peak area method, using dibutyl phthalate as the internal standard.

RESULTS AND DISCUSSION

W-1372 can be quantitated gas chromatographically when the relative peak area is used as an index of concentration. The relationship between relative peak area and drug concentration in the range of 1–10 mcg./ml. of plasma is illustrated in Fig. 2. The reproducibility of the procedure, as indicated by the standard error of quintuplicate determinations, is also shown in Fig. 2. The recovery of W-1372 was 98–99%, as shown in Table I.

The extraction procedure effectively separates W-1372 from normal interfering plasma constituents, since determinations in normal plasma of humans (Fig. 1), dogs, or monkeys give little or no blank (<0.1 mcg./ml.). The known major metabolites of W-1372—benzoic acid, hippuric acid, and *N*-γ-phenylpropyl-*N*-benzyloxyamine (3)—do not interfere.

The blood-depletion pattern of W-1372 following intravenous administration was studied in the dog, squirrel monkey, and Cebus monkey. The results (Table II) indicate that the drug is rapidly removed from the blood, with only trace amounts present after 30 min. in the dog and Cebus monkey and a low level present at this time in the squirrel monkey. A previous study (3) has shown that the plasma half-life of radioactivity following oral administration of W-1372-benzyl-^{14}C is 4 hr. in the squirrel monkey and 12 hr. in the dog.

REFERENCES

(1) F. M. Berger, J. F. Douglas, B. J. Ludwig, and S. Margolin, *J. Pharmacol. Exp. Ther.*, **170**, 371(1969).

(2) F. M. Berger and G. G. Lu, *Fed. Proc.*, **28**, 677(1969).

(3) J. Edelson, J. F. Douglas, and B. J. Ludwig, *ibid.*, **28**, 798 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 17, 1969, from *Wallace Laboratories, Division of Carter-Wallace, Inc., Cranbury, NJ 08512*

Accepted for publication January 27, 1970.

* Present address: Wallace and Tiernan, Inc., Cedar Knolls, N. J.

Mechanism of Action of Retinyl Compounds on Wound Healing I: Structural Relationship of Retinyl Compounds and Wound Healing

K. H. LEE and THEODORE G. TONG

Abstract □ Retinol, retinyl acetate, and retinoic acid promote wound healing. Retinoic acid is most active. Beta-carotene is active, while lycopene is inactive; β-ionone is active and α-ionone is inactive. For full activity, the compound should contain a β-ionone ring, a conjugated double-bond side chain, and a terminal carboxylic acid side chain.

Keyphrases □ Retinyl compounds, activity—structural relationship □ Wound healing—retinyl compound effect □ GLC—separation □ NMR spectroscopy—identity

In a previous report, it was shown that local application of retinol, retinyl acetate, or retinoic acid, dissolved in a nonionic base (NIB), promotes skin wound healing (1). Retinoic acid is relatively more effective than retinol or retinyl acetate. The fact that retinol, retinyl acetate, and retinoic acid are all effective when applied locally on the skin wound indicates that the primary alcohol group is not essential in promoting wound healing. It is of interest, therefore, to study the structural relationship of the other part of the retinol molecule for wound-healing activity. The compounds evaluated in this investigation are β-carotene, lycopene, β-ionone, and α-ionone.

Beta-carotene has the same ring structure and conjugated double-bond hydrocarbon side chain as retinol. It should show activity on wound healing as retinol. Lycopene has essentially the same structure as β-carotene but differs from the latter in not having the closed ring structure at either end of the molecule. Beta-ionone, on the other hand, has the same cyclohexene ring but does not have the same length of hydrocarbon side chain as retinol or β-carotene. Alpha-ionone differs from β-ionone by the position of the double bond in the cyclohexene ring and does not conjugate with the side chain. The structure–activity relationships of these compounds to wound healing are discussed.

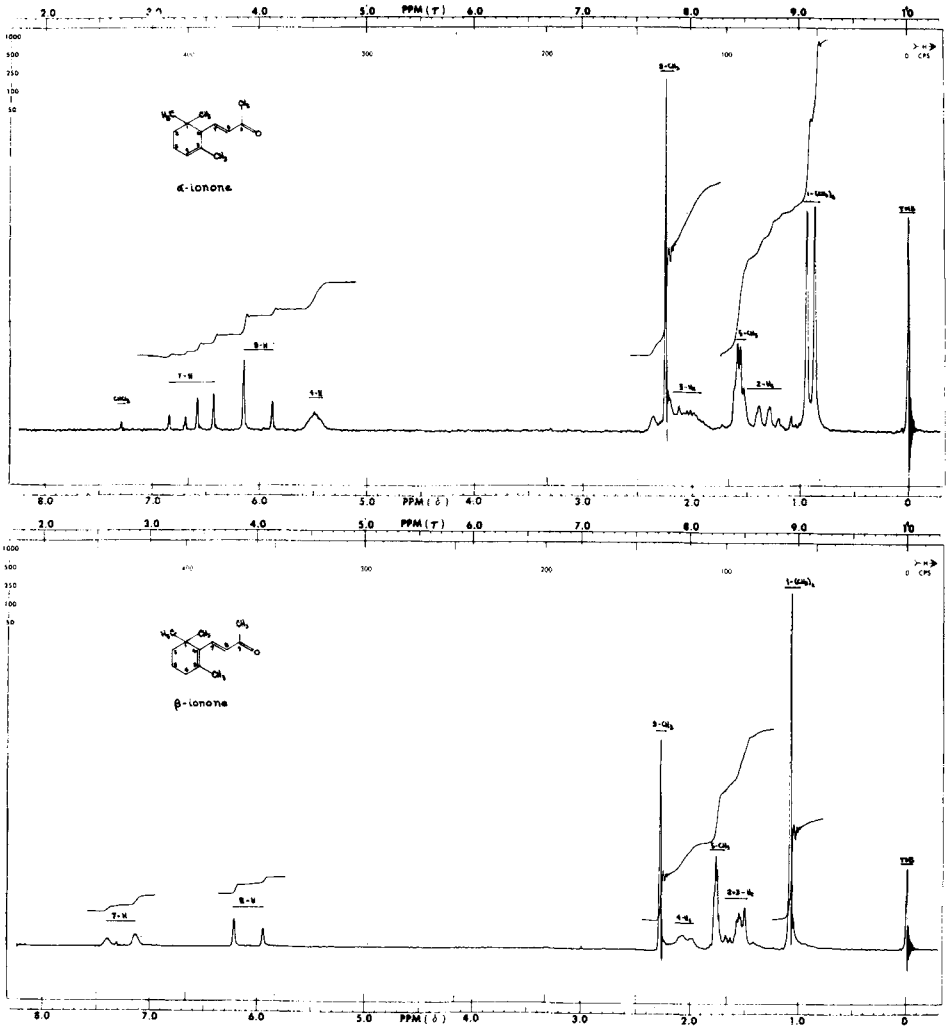


Figure 1—*NMR spectra of α- and β-ionones.*

EXPERIMENTAL

Materials and Chemicals—Synthetic crystalline, β-carotene, Sigma grade, Type 1, and crystalline lycopene, Blakeslea trispora origin, were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium salicylate and salicylic acid, reagent grade, were obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. The prednisone used was a product of The Upjohn Co., Kalamazoo, Mich. Nonionic base and 1% hydrocortisone in NIB were prepared by the Pharmaceutical Technology Laboratory, San Francisco Medical Center, San Francisco, Calif. Beta-ionone (n_D^{20} 1.584) and α-ionone (n_D^{20} 1.5030), 77%, were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Purification of α-Ionone—The purities of the ionones were measured by their spectra (Analytical NMR Spectrometer, model A-60A, Varian Associates, Palo Alto, Calif.). Beta-ionone, as purchased, was a pure preparation, while α-ionone contained 31% of the β-isomer. The isomers were separated by using a preparative gas chromatograph (Varian Aerograph, model 700, Walnut Creek, Calif.). A FFAP column, 3.04 m. × 0.95 cm. (10 ft. × 0.37 in.), at 190° was used. The separation was complete and satisfactory. Both fractions collected were pure as measured by an NMR spectrometer. The NMR spectra are shown in Fig. 1.

Application of NIB Preparations—NIB preparations were applied, with gentle rubbing, directly on the sutured wound right after wounding. The application was repeated, once a day, on the 1st and 2nd days after wounding. For the control, only NIB was applied.

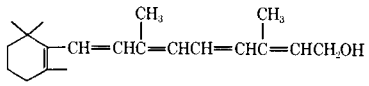
Administration of Drugs—Sodium salicylate, dissolved in a small amount of water and prednisone suspended in corn syrup, was fed to the rats daily for 4 days through a short stomach tube connected to a blunt hypodermic needle attached to a 50-ml. syringe, starting 1 day before operation. The dosage levels for sodium salicylate and prednisone were 50 and 2.5 mg. per rat per day, respectively.

Wound Procedure—Sprague-Dawley male rats, weighing 230–240 g., were anesthetized with ethyl ether in an open mask. The hair on the back was depilated with an electric clipper. One incision, 6 cm. in length, was made through the skin and underlying musculature at a distance about 1.5 cm. from the midline on each side; no ligatures were used. Bleeding usually ceased after a few minutes. The incisions were closed with continuous through-and-through sutures with stitches 0.5 cm. apart. Black silk surgical thread and a curved needle were used. The continuous suture was pulled tight enough to secure good adaptation of the wound edges. The wounds were left undressed.

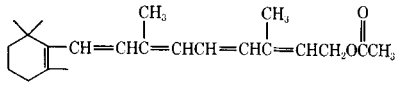
Measurement of Healing—Tensile strength, the force required to open a healing skin wound, was used to measure healing (2). On the 7th day after wounding, the tensile strength of the wound was measured with a simple laboratory-made tensiometer. The tensiometer consisted of a 15.24 × 30.48-cm. (6 × 12-in.) board with one post, 10.16 cm. (4 in.) long, fixed on each side of the long ends. The board was placed at the end of a table. A pulley, with bearing, was mounted on the top of one post. A battery clamp, with 1-cm. clamp width, was tied to the tip of the post without a pulley by a piece of 20-lb. test monofilament fishing line so that the clamp could reach the middle of the board. Another battery clamp was tied to a long piece of fishing line with a 1-l. polyethylene bottle tied to the other end. Before testing, the animal was anesthetized with ethyl ether in an open mask. The sutures of the wound were cut out with a pair of scissors. The animal was then placed on a stack of paper towels on the middle of the board. The amount of the towels could be adjusted so that the wound was on the same level as the tips of the posts. The clamps were then carefully clamped on the skin at the

Table I—Structural Formulas of the Retinyl Compounds Studied

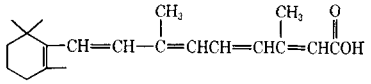
I. Retinol (vitamin A)



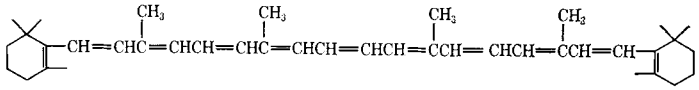
II. Retinyl acetate



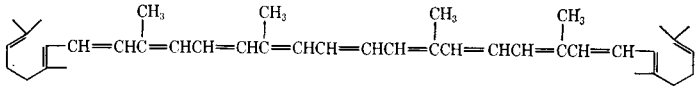
III. Retinoic acid



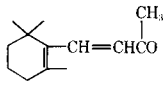
IV. β-Carotene



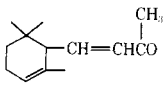
V. Lycopene



VI. β-Ionone



VII. α-Ionone



opposite sides of the wound at a distance 0.5 cm. from the wound. The longer piece of fishing line was placed on the pulley, and the position of the board was adjusted so that the polyethylene bottle was freely hanging in the air. Water was added to the polyethylene bottle at a rapid but constant rate from a large reservoir (20-l. bottle) until the wound began to open. The amount of water in the polyethylene bottle was weighed and considered as the tensile strength of the wound. Two to three determinations were made on each wound. The mean of the determinations made on wounds on both sides of the animal was taken as the tensile strength of the wound.

RESULTS AND DISCUSSION

The structural formulas for the compounds are shown in Table I.

The fact that retinol, retinyl acetate, and retinoic acid are all active in promoting wound healing indicates that the primary alcohol group is not essential (1). Beta-carotene, the natural precursor of retinol, has essentially the same trimethylcyclohexene ring and conjugated double-bond hydrocarbon side chain as retinol (Compound IV, Table I). The results of the effect of β-carotene on skin wound healing in rats are summarized in Table II. The mean tensile strength of the NIB control is 451 ± 9 g. (Group I). The mean tensile strength of Group II animals to which β-carotene in NIB was applied is 514 ± 7 g. The increase in tensile strength as compared with the control is 14%. The mean tensile strength of Group III animals receiving β-carotene in isopropyl myristate (IPM) is 515 ± 9 g., which is 14% higher than that of the control and is essentially the same as that of Group II animals. This also indicates that neither NIB nor IPM affects healing. The results from Group V and Group VII animals have definitely shown that topical application of β-carotene, like retinoic acid, reverses the healing-inhibitory action caused by oral administration of sodium salicylate or prednisone, as shown in Group IV and Group VI (1).

The effects of lycopene and ionones on wound healing are shown in Table III. Lycopene has essentially the same structure as β-carotene, except that it does not have the closed ring structure at either end of the molecule (Compound V, Table I). The mean tensile strength of Group II animals receiving 1% lycopene in NIB topically is 418 ± 5 g. It is clear that lycopene does not promote healing and, instead, it has a mild inhibitory effect.

Beta-ionone has essentially the same trimethyl cyclohexene ring structure as retinol or β-carotene, except that it does not have the same side chain (Compound VI, Table I). The mean tensile strength of the healing wound of animals receiving topical application of 1% β-ionone in NIB is 497 ± 11 g., which is 110% of the control (Group III). Beta-ionone is less active than β-carotene.

On the other hand, α-ionone, the isomer of β-ionone (the only difference being the position of the double bond in the trimethyl cyclohexene ring) does not have any activity in promoting healing. The mean tensile strength of the rats receiving 1% of α-ionone in NIB is 453 ± 6 g. (Group IV). It is interesting to point out the well-known fact that the α-isomer of retinol also does not have any vitamin A activity. The results from the foregoing structural relationship and

Table II—Effect of Topical Application of β-Carotene on Wound Healing

Group	No. of Animals	Drugs Given		Mean Tensile Strength, g.	Percent Control
		Orally	Topically		
I	14	—	NIB	451 ± 9	100
II	16	—	1% β-Carotene in NIB	514 ± 7	114
III	10	—	1% β-Carotene in IPM[a]	515 ± 9	114
IV	8	NaSA	—	358 ± 10	79
V	8	NaSA	1% β-Carotene in IPM	478 ± 15	106
VI	7	Prednisone	—	346 ± 11	77
VII	9	Prednisone	1% β-Carotene in IPM	445 ± 11	99

[a] Isopropyl myristate.

Table III—Effect of Topical Application of Lycopene and Ionones on Wound Healing

Group	No. of Animals	Drugs Applied	Mean Tensile Strength, g. ± *SE*
I	14	NIB Control	451 ± 9
II	8	1% Lycopene in NIB	418 ± 5
III	9	1% β-Ionone in NIB	497 ± 11
IV	8	1% α-Ionone in NIB	453 ± 6

wound-healing promotion activity studies indicate that β-ionone ring and conjugated double-bond hydrocarbon side chain and terminal carboxylic group are responsible for the full healing-promotion activity.

REFERENCES

(1) K. H. Lee and T. G. Tong, *J. Pharm. Sci.*, **58**, 773(1969).
(2) K. H. Lee, *ibid.*, **57**, 1042(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 24, 1969, from the *University of California School of Pharmacy, San Francisco Medical Center, San Francisco, CA 94122*

Accepted for publication December 31, 1969.

This work was supported by research funds from the Academic Senate Committee on Research, University of California, San Francisco Medical Center, San Francisco, CA 94122

The authors wish to thank Mr. Vahn Lewis for gas chromatographic separation of ionones and Mr. Michael R. Spencer for NMR analysis.

Tetrameric Structure and Conformation of Heat-Microaggregated Human Serum Albumin

JOEL KIRSCHBAUM

Abstract □ ^{131}I-labeled heat-microaggregated human serum albumin is a colloid used in a method that measures liver blood flow through a determination of the rate of removal of microaggregates from the circulation. The heat-microaggregated material, either labeled or unlabeled, has a molecular weight (mol. wt.) of 273,000 daltons and a sedimentation coefficient of 8.6 *S*. Since the precursor, human serum albumin, has a molecular weight of 67,000 daltons and a sedimentation coefficient of 4.6 *S*, the microaggregate is a tetramer. The tetramer may be converted to subunits with a molecular weight of 72,000–76,000 daltons and a sedimentation coefficient of 4.7 *S* by the addition of buffered urea, guanidinium chloride, or formamide or by decreasing the pH to 2.2. Immunological studies indicate that this subunit has a different conformation from that of native human serum albumin. Hydrodynamic calculations indicate that the colloid has a particle size between 50 × 50 × 200 Å and 50 × 100 × 100 Å.

Keyphrases □ Albumin, human serum, heat-microaggregated—tetrameric structure, conformation, physical constants □ Human serum albumin, heat-microaggregated—structure, physical constants □ Ultracentrifugation—sedimentation coefficients □ Conformational studies—heat-microaggregated human serum albumin

Heating human serum albumin results, under certain conditions, in the formation of two protein aggregates (1, 2), made clinically useful by subsequent labeling with ^{131}I. One preparation, particulate in nature (2), is known as heat-macroaggregated human serum albumin. The other preparation is known as heat-microaggregated human serum albumin (3). The rate of disappearance of injected colloidal microaggregated particles from the circulation of mammals permits an estimation of the phagocytic capacity of the reticuloendothelial system, because this removal involves principally the Kupffer cells of the liver. Knowledge of phagocytic capacity appears to be useful in determining the extent of such diseases as pneumococcal pneumonia, typhoid fever, and Hodgkin's disease. This paper describes some physical properties of the metabolizable microaggregate, either unlabeled or labeled with ^{131}I.

EXPERIMENTAL

Source of Heat-Microaggregated Human Serum Albumin—The aggregation of human serum albumin (HSA) was performed according to the method of Iio and Wagner (3), as modified from the method of Benacerraf *et al.* (1). HSA at a concentration of 3% protein in 0.9% NaCl adjusted to pH 10 with NaOH was shaken vigorously for 20 min. at 70° and then for 15 min. at 79°. After rapid cooling, the precipitate that formed from the solution was resuspended in 0.1 *M* $NaHCO_3$. After storage, the precipitate dissolved and the solution of heat-microaggregated HSA was diluted to 10 mg./ml.

Sedimentation Coefficients—By the use of a Spinco model E analytical ultracentrifuge, the sedimentation coefficients of ^{131}I-labeled and unlabeled heat-microaggregated HSA at 20° and the viscosity and density of water, $s^0_{20,w}$ (4), were calculated at 29,500, 42,040, and 50,740 r.p.m. with 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer (pH 6.85) as diluent. The viscosity was measured with the aid of either capillary or rotating (5) viscometers. A 10-ml. pycnometer at 20 ± 0.002° (Fisher Isotemp water bath) was used for density measurements. Viscosity and density corrections for aqueous solutions of urea and guanidinium chloride were also obtained from the data of Kawahara and Tanford (6).

Molecular Weights—The molecular weight was determined by two methods. The Archibald approach-to-sedimentation equilibrium method (7) was used with the modification of Engelberg (8) to evaluate the integral of the concentration gradient. The Yphantis meniscus-depletion (9) analyses were performed in a capillary-type double-sector cell at 20° at a speed of 20,410 r.p.m. The cell bottom was layered with FC-43 fluorochemical oil (Beckman Instruments), and 0.03 ml. of 0.1% dialyzed solution was layered over the oil. The solvent was the last dialysate.

Immunological studies utilized rabbit and horse antihuman serum albumin (Hyland Laboratories, Los Angeles, Calif.).

Polyacrylamide gel electrophoresis was performed with the Canalco model 6 apparatus (Canal Industrial Corp., Rockville, Md.).

Table III—Effect of Topical Application of Lycopene and Ionones on Wound Healing

Group	No. of Animals	Drugs Applied	Mean Tensile Strength, g. ± *SE*
I	14	NIB Control	451 ± 9
II	8	1% Lycopene in NIB	418 ± 5
III	9	1% β-Ionone in NIB	497 ± 11
IV	8	1% α-Ionone in NIB	453 ± 6

wound-healing promotion activity studies indicate that β-ionone ring and conjugated double-bond hydrocarbon side chain and terminal carboxylic group are responsible for the full healing-promotion activity.

REFERENCES

(1) K. H. Lee and T. G. Tong, *J. Pharm. Sci.*, **58**, 773(1969).
(2) K. H. Lee, *ibid.*, **57**, 1042(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 24, 1969, from the *University of California School of Pharmacy, San Francisco Medical Center, San Francisco, CA 94122*

Accepted for publication December 31, 1969.

This work was supported by research funds from the Academic Senate Committee on Research, University of California, San Francisco Medical Center, San Francisco, CA 94122

The authors wish to thank Mr. Vahn Lewis for gas chromatographic separation of ionones and Mr. Michael R. Spencer for NMR analysis.

Tetrameric Structure and Conformation of Heat-Microaggregated Human Serum Albumin

JOEL KIRSCHBAUM

Abstract □ ^{131}I-labeled heat-microaggregated human serum albumin is a colloid used in a method that measures liver blood flow through a determination of the rate of removal of microaggregates from the circulation. The heat-microaggregated material, either labeled or unlabeled, has a molecular weight (mol. wt.) of 273,000 daltons and a sedimentation coefficient of 8.6 *S*. Since the precursor, human serum albumin, has a molecular weight of 67,000 daltons and a sedimentation coefficient of 4.6 *S*, the microaggregate is a tetramer. The tetramer may be converted to subunits with a molecular weight of 72,000–76,000 daltons and a sedimentation coefficient of 4.7 *S* by the addition of buffered urea, guanidinium chloride, or formamide or by decreasing the pH to 2.2. Immunological studies indicate that this subunit has a different conformation from that of native human serum albumin. Hydrodynamic calculations indicate that the colloid has a particle size between 50 × 50 × 200 Å and 50 × 100 × 100 Å.

Keyphrases □ Albumin, human serum, heat-microaggregated—tetrameric structure, conformation, physical constants □ Human serum albumin, heat-microaggregated—structure, physical constants □ Ultracentrifugation—sedimentation coefficients □ Conformational studies—heat-microaggregated human serum albumin

Heating human serum albumin results, under certain conditions, in the formation of two protein aggregates (1, 2), made clinically useful by subsequent labeling with ^{131}I. One preparation, particulate in nature (2), is known as heat-macroaggregated human serum albumin. The other preparation is known as heat-microaggregated human serum albumin (3). The rate of disappearance of injected colloidal microaggregated particles from the circulation of mammals permits an estimation of the phagocytic capacity of the reticuloendothelial system, because this removal involves principally the Kupffer cells of the liver. Knowledge of phagocytic capacity appears to be useful in determining the extent of such diseases as pneumococcal pneumonia, typhoid fever, and Hodgkin's disease. This paper describes some physical properties of the metabolizable microaggregate, either unlabeled or labeled with ^{131}I.

EXPERIMENTAL

Source of Heat-Microaggregated Human Serum Albumin—The aggregation of human serum albumin (HSA) was performed according to the method of Iio and Wagner (3), as modified from the method of Benacerraf *et al.* (1). HSA at a concentration of 3% protein in 0.9% NaCl adjusted to pH 10 with NaOH was shaken vigorously for 20 min. at 70° and then for 15 min. at 79°. After rapid cooling, the precipitate that formed from the solution was resuspended in 0.1 *M* $NaHCO_3$. After storage, the precipitate dissolved and the solution of heat-microaggregated HSA was diluted to 10 mg./ml.

Sedimentation Coefficients—By the use of a Spinco model E analytical ultracentrifuge, the sedimentation coefficients of ^{131}I-labeled and unlabeled heat-microaggregated HSA at 20° and the viscosity and density of water, $s^0_{20,w}$ (4), were calculated at 29,500, 42,040, and 50,740 r.p.m. with 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer (pH 6.85) as diluent. The viscosity was measured with the aid of either capillary or rotating (5) viscometers. A 10-ml. pycnometer at 20 ± 0.002° (Fisher Isotemp water bath) was used for density measurements. Viscosity and density corrections for aqueous solutions of urea and guanidinium chloride were also obtained from the data of Kawahara and Tanford (6).

Molecular Weights—The molecular weight was determined by two methods. The Archibald approach-to-sedimentation equilibrium method (7) was used with the modification of Engelberg (8) to evaluate the integral of the concentration gradient. The Yphantis meniscus-depletion (9) analyses were performed in a capillary-type double-sector cell at 20° at a speed of 20,410 r.p.m. The cell bottom was layered with FC-43 fluorochemical oil (Beckman Instruments), and 0.03 ml. of 0.1% dialyzed solution was layered over the oil. The solvent was the last dialysate.

Immunological studies utilized rabbit and horse antihuman serum albumin (Hyland Laboratories, Los Angeles, Calif.).

Polyacrylamide gel electrophoresis was performed with the Canalco model 6 apparatus (Canal Industrial Corp., Rockville, Md.).

Table I—Sedimentation Coefficients of Human Serum Albumin, Heat-Microaggregated Human Serum Albumin, and ^{131}I Heat-Microaggregated Human Serum Albumin in Various Solvents

Solvent	HSA[a]	$s^0_{20,w} \times 10^{13}$ sec. Aggregated HSA[b]	$s^0_{20,w} \times 10^{13}$ sec. ^{131}I-Aggregated HSA[c]
0.2 *M* NaCl–0.02 *M* phosphate buffer, pH 6.85	4.6	8.6	8.5
Buffered 8 *M* urea, pH 6.85	4.0	4.0	4.0
Buffered 8 *M* urea, pH 6.85, followed by dialysis against neutral buffer	4.5	4.7	4.5
Buffered 9 *M* formamide, pH 6.85	4.1	4.0	4.2
Buffered 5 *M* guanidinium chloride, pH 6.85	3.3	3.3	3.2

[a] Human serum albumin. [b] Heat-microaggregated human serum albumin. [c] ^{131}I heat-microaggregated human serum albumin.

RESULTS AND DISCUSSION

Hydrodynamic Properties of the Heat-Microaggregated Human Serum Albumin Molecule—The heat-microaggregated HSA was considered sufficiently pure for hydrodynamic studies, because polyacrylamide gel electrophoresis indicated that approximately 95% of the heat-microaggregated HSA migrated as one component and the sedimentation velocity studies showed only one peak with almost no asymmetry. The sedimentation coefficient of unlabeled heat-microaggregated HSA at 20°, corrected to the viscosity of water and extrapolated to zero concentration, was 8.56 ± 0.04 *S* (*S* = 10^{-13} sec.) between pH 4.7 and 10.2 (Table I). The sedimentation coefficient of the heat-microaggregated ^{131}I-labeled HSA was 8.45 ± 0.08 *S*. The heat-microaggregated preparation was sensitive to small changes in duration of heating; an increase of 5 min. in the heating time at 79° led to the formation of a particle with a molecular weight of 880,000 daltons.

The partial specific volume of heat-microaggregated HSA was experimentally determined (10) to be 0.737 cm.^{3}/g., compared with 0.733–0.736 cm.^{3}/g. for native HSA (11, 12).

According to the Archibald method, approximately 95% of the sedimenting material had a molecular weight of 279,000 ± 10,000 daltons at the meniscus and cell bottom; the remaining 5% possessed an approximate molecular weight of 132,000 ± 9000 daltons, which corresponds to the naturally occurring dimer of HSA found in serum albumin at similar concentrations of 5–8% (12). The molecular weight found by the Yphantis method at 20,410 r.p.m. was 267,000 ± 8000 daltons. Since the molecular weight of HSA is 67,000 (12, 13), four molecules of HSA must have combined to give one molecule of microaggregated HSA. The addition of ^{131}I has no apparent effect on molecular weight or sedimentation coefficient.

The diffusion constant, $D_{20,w}$, was calculated to be 2.84 × 10^{-7} cm.^{2} sec.$^{-1}$ from the Svedberg equation (14), $D_{20,w} = RTs/M(1 - \bar{v}\rho)$, where R is the gas constant, T is the temperature in °K., s is the sedimentation coefficient, M is the molecular weight, $\bar{v}$ is the partial specific volume, and ρ is the density. Using this value for $D_{20,w}$, $f/f_{min.}$ was calculated (15) to be 1.7, where $f = kT/D$ (k is Boltzmann's constant) and $f_{min.} = 6\pi\eta\ (3M\bar{v}/4\pi N)^{1/3}$ (η is the viscosity of solvent and N is Avogadro's number). This indicates that the heat-microaggregated HSA molecule is slightly asymmetric, since HSA is globular with a calculated $f/f_{min.}$ value of 1.3–1.4 (15, 16).

Dissociation of the Aggregate—The apparently mild conditions required for aggregation suggested that dissociation of the aggregate to monomers of HSA was possible. As will be shown later, it was found that although the monomeric molecular weight of the dissociated microaggregate was similar to that of the monomer, the conformation changed. As seen in Table I, the sedimentation coefficients of microaggregated HSA and monomeric HSA dissolved in buffered 8 *M* urea, 9 *M* formamide, or 5 *M* guanidinium chloride were identical within the limits of experimental error (17). These values indicated that heat-microaggregated HSA (mol. wt. 273,000) had dissociated to monomeric HSA. The molecular weight in 8 *M* urea, 9 *M* formamide, and 5 *M* guanidinium chloride was between 72,000 and 76,000 daltons. Ten milligrams microaggregated HSA/milliliter 0.2 *M* glycine–HCl buffer (pH 2.2) showed two components. The $s^0_{20,w}$ values of 4.5 and 9 *S* indicated some breakdown of the aggregate. Aggregated HSA, HSA, or mixtures of both HSA and aggregated HSA all produced schlieren images with one broad peak, indicating not only breakdown of the aggregate but also some denaturation of the monomeric HSA (18). After removal of urea by dialysis, the schlieren peaks still appeared broad, indicating that denaturation of HSA is only partially reversible.

Conformation of Heat-Microaggregated Human Serum Albumin—Although both monomeric HSA and monomeric HSA first dissolved in buffered 8 *M* urea and then dialyzed against H_2O yield strong precipitin bands, both aggregated HSA and urea-dissociated, aggregated HSA (dialyzed subsequently against H_2O and buffer to remove urea) fail to form precipitin bands with rabbit or horse antihuman serum albumin. This failure indicates that the HSA monomer formed from dissociated tetrameric microaggregated HSA has a different conformation from that of human serum albumin.

Since: (*a*) the HSA molecules are globular (15) with a diameter of approximately 50 Å [radius = $(3M\bar{v}/4\pi N)^{1/3}$]; (*b*) the conformation of each of the HSA molecules after heating is still generally spherical, as seen by the sedimentation coefficient of 4.5 *S* found after the addition of urea and subsequent dialysis (Table 1); and (*c*) the aggregate is asymmetric, then the aggregate may consist of either: (*a*) a chain 4 subunits long (200 × 50 × 50 Å); (*b*) a tetragon, two subunits wide and 2 subunits long (100 × 100 × 50 Å), or an intermediate conformation. One forbidden conformation is the symmetrical tetrahedral pyramid.

Binding Forces of the Aggregate—The disaggregation of the tetramer caused by buffered urea, guanidinium chloride, formamide, or low pH indicates that the denaturing agent may be interacting with peptide groups through hydrogen and hydrophobic bonds (19). The aggregate may have been formed by the reverse process, with heat causing the cleavage ("melting") of some interpeptide bonds and the subsequent cooling of the mixture resulting in the partial intertwining of the protein chains from four molecules.

REFERENCES

(1) B. Benacerraf, G. Biozzi, B. N. Halpern, C. Stiffle, and D. Mouton, *Brit. J. Exp. Pathol.*, **38,** 35(1957).

(2) G. V. Taplin, D. E. Johnson, E. K. Dore, and H. S. Kaplan, *J. Nucl. Med.*, **5,** 259(1964).

(3) M. Iio and H. A. Wagner, Jr., *J. Clin. Invest.*, **42,** 417 (1963).

(4) H. K. Schachman, "Ultracentrifugation in Biochemistry," Academic, New York, N. Y., 1959, p. 81.

(5) B. H. Zimm and D. N. Crothers, *Proc. Natl. Acad. Sci., U. S.*, **48,** 905(1962).

(6) K. Kawahara and C. Tanford, *J. Biol. Chem.*, **241,** 3228 (1966).

(7) W. J. Archibald, *J. Phys. Colloid Chem.*, **51,** 1204 (1947).

(8) J. Engelberg, *Anal. Biochem.*, **6,** 530(1963).

(9) D. A. Yphantis, *Biochemistry*, **3,** 297(1964).

(10) H. K. Schachman, *Methods Enzymol.*, **4,** 70(1957).

(11) J. L. Oncley, G. Scatchard, and A. Brown, *J. Phys. Colloid Chem.*, **51,** 184(1947).

(12) G. I. Loeb and H. A. Scheraga, *J. Phys. Chem.*, **60,** 1633 (1956).

(13) T. P. King, D. A. Yphantis, and T. C. Craig, *J. Amer. Chem. Soc.*, **82,** 3355(1960).

(14) T. Svedberg and K. O. Peterson, "The Ultracentrifuge," Oxford University Press, London, England, 1940, p. 5.

(15) C. Tanford, "Physical Chemistry of Macromolecules," Wiley, New York, N. Y., 1963, p. 356.

(16) C. Tanford and J. G. Buzzell, *J. Phys. Chem.*, **60**, 225 (1956).

(17) H. K. Schachman, "Ultracentrifugation in Biochemistry," Academic, New York, N. Y., 1959, p. 82.

(18) H. Neurath, G. R. Cooper, and J. O. Erickson, *J. Biol. Chem.*, **142**, 249(1942).

(19) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p. 323.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 8, 1969, from the *Squibb Institute for Medical Research, New Brunswick, NJ 08903*

Accepted for publication January 27, 1970.

The author thanks Dr. K. Florey for helpful suggestions and criticism, Drs. G. Bruno and D. R. K. Murty for samples of unlabeled and ^{131}I-labeled heat-microaggregated and heat-macroaggregated human serum albumin and for their useful advice, the American National Red Cross for samples of human serum albumin, Mr. O. Kocy for performing the polyacrylamide gel electrophoresis, Dr. R. Millonig for the immunological analyses, and Dr. David Frost for editorial assistance.

Phospholipid Spherules as a Model to Assess Photosensitizing Properties of Drugs

ALVIN FELMEISTER and SHRILEKHA V. TOLAT

Abstract □ The effect of a series of UV irradiated and nonirradiated phenothiazine drugs on the chromate leakage from lipid spherules has been determined. All of the drugs studied increased chromate leakage to varying degrees prior to irradiation. However, only chlorpromazine and prochlorperazine exhibited a marked increase in chromate leakage after irradiation. These effects are discussed in terms of the *in vivo* photosensitizing properties of the drugs.

Keyphrases □ Photosensitizing properties—phenothiazines □ Lipid spherules, chromate leakage—photosensitizing properties, phenothiazines □ Phenothiazines—photosensitizing properties □ Spectrophotometry—analysis

Nonphysiologic photosensitized reactions have been studied extensively since Raab first observed the photosensitized activity of acridine toward paramecium almost 70 years ago (1). During the intervening time, much has been learned about such reactions, although their exact mechanism of action has not been completely elucidated. However, it has been reasonably well established that changes in the permeability of the membrane of cells or cell organelles are often induced by light-irradiated photosensitizing agents (2).

It was the purpose of this investigation, therefore, to develop a physical model that might be used to assess photosensitizing agents by measuring their influence on membrane permeability. The phospholipid spherule model developed by Bangham *et al.* (3) was selected for this purpose. These workers have shown that phospholipids, when permitted to swell in an aqueous salt solution, form salt-containing compartments bounded by bimolecular membranes. These structures (spherules) exhibit permeability characteristics that are quite similar to those of biological membranes. Furthermore, these spherules have been shown by numerous researchers to be useful tools for studying membrane–drug interactions, particularly when changes in permeability are involved (4). Therefore, changes in permeability of these spherules induced by light-irradiated drugs should be a measure of the photosensitizing property of these drugs.

Five phenothiazine derivatives were selected for this initial study. Two of these compounds, chlorpromazine and prochlorperazine, have been shown unequivocally to be photosensitizers (5). The other three compounds, promazine, triflupromazine, and fluphenazine, rarely if ever produce photosensitization (5–7).

MATERIALS AND METHODS

The phenothiazine derivatives were used without further purification. These were chlorpromazine hydrochloride and prochlorperazine hydrochloride (Smith Kline & French Laboratories); promazine hydrochloride (Wyeth Laboratories); and triflupromazine hydrochloride and fluphenazine dihydrochloride (The Squibb Institute for Medical Research).

The lipid spherules were prepared by the method of Bangham *et al.* (3) with slight modification. Briefly, egg lecithin and dicetylphosphate (90 and 10 μmoles, respectively) were dissolved in chloroform and placed in a 50-ml. round-bottom flask. The solvent was removed under reduced pressure using a flash evaporator. Six milliliters of a 0.145 *M* potassium chromate solution was then added to the flask, and the lipid material was permitted to swell for 4 hr. at room temperature. At the end of 4 hr., any chromate ion not trapped within the spherules was removed by dialyzing the dispersion against a 0.145 *M* KCl solution for 18–20 hr. One milliliter of the dialyzed suspension of chromate-containing spherules was transferred to each of five cells. By use of a micrometer syringe, 0.05 ml. of a 1×10^{-2} *M* solution in 0.145 *M* KCl of the

(14) T. Svedberg and K. O. Peterson, "The Ultracentrifuge," Oxford University Press, London, England, 1940, p. 5.

(15) C. Tanford, "Physical Chemistry of Macromolecules," Wiley, New York, N. Y., 1963, p. 356.

(16) C. Tanford and J. G. Buzzell, *J. Phys. Chem.*, **60**, 225 (1956).

(17) H. K. Schachman, "Ultracentrifugation in Biochemistry," Academic, New York, N. Y., 1959, p. 82.

(18) H. Neurath, G. R. Cooper, and J. O. Erickson, *J. Biol. Chem.*, **142**, 249(1942).

(19) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p. 323.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 8, 1969, from the *Squibb Institute for Medical Research, New Brunswick, NJ 08903*

Accepted for publication January 27, 1970.

The author thanks Dr. K. Florey for helpful suggestions and criticism, Drs. G. Bruno and D. R. K. Murty for samples of unlabeled and ^{131}I-labeled heat-microaggregated and heat-macroaggregated human serum albumin and for their useful advice, the American National Red Cross for samples of human serum albumin, Mr. O. Kocy for performing the polyacrylamide gel electrophoresis, Dr. R. Millonig for the immunological analyses, and Dr. David Frost for editorial assistance.

Phospholipid Spherules as a Model to Assess Photosensitizing Properties of Drugs

ALVIN FELMEISTER and SHRILEKHA V. TOLAT

Abstract □ The effect of a series of UV irradiated and nonirradiated phenothiazine drugs on the chromate leakage from lipid spherules has been determined. All of the drugs studied increased chromate leakage to varying degrees prior to irradiation. However, only chlorpromazine and prochlorperazine exhibited a marked increase in chromate leakage after irradiation. These effects are discussed in terms of the *in vivo* photosensitizing properties of the drugs.

Keyphrases □ Photosensitizing properties—phenothiazines □ Lipid spherules, chromate leakage—photosensitizing properties, phenothiazines □ Phenothiazines—photosensitizing properties □ Spectrophotometry—analysis

Nonphysiologic photosensitized reactions have been studied extensively since Raab first observed the photosensitized activity of acridine toward paramecium almost 70 years ago (1). During the intervening time, much has been learned about such reactions, although their exact mechanism of action has not been completely elucidated. However, it has been reasonably well established that changes in the permeability of the membrane of cells or cell organelles are often induced by light-irradiated photosensitizing agents (2).

It was the purpose of this investigation, therefore, to develop a physical model that might be used to assess photosensitizing agents by measuring their influence on membrane permeability. The phospholipid spherule model developed by Bangham *et al.* (3) was selected for this purpose. These workers have shown that phospholipids, when permitted to swell in an aqueous salt solution, form salt-containing compartments bounded by bimolecular membranes. These structures (spherules) exhibit permeability characteristics that are quite similar to those of biological membranes. Furthermore, these spherules have been shown by numerous researchers to be useful tools for studying membrane–drug interactions, particularly when changes in permeability are involved (4). Therefore, changes in permeability of these spherules induced by light-irradiated drugs should be a measure of the photosensitizing property of these drugs.

Five phenothiazine derivatives were selected for this initial study. Two of these compounds, chlorpromazine and prochlorperazine, have been shown unequivocally to be photosensitizers (5). The other three compounds, promazine, triflupromazine, and fluphenazine, rarely if ever produce photosensitization (5–7).

MATERIALS AND METHODS

The phenothiazine derivatives were used without further purification. These were chlorpromazine hydrochloride and prochlorperazine hydrochloride (Smith Kline & French Laboratories); promazine hydrochloride (Wyeth Laboratories); and triflupromazine hydrochloride and fluphenazine dihydrochloride (The Squibb Institute for Medical Research).

The lipid spherules were prepared by the method of Bangham *et al.* (3) with slight modification. Briefly, egg lecithin and dicetylphosphate (90 and 10 μmoles, respectively) were dissolved in chloroform and placed in a 50-ml. round-bottom flask. The solvent was removed under reduced pressure using a flash evaporator. Six milliliters of a 0.145 *M* potassium chromate solution was then added to the flask, and the lipid material was permitted to swell for 4 hr. at room temperature. At the end of 4 hr., any chromate ion not trapped within the spherules was removed by dialyzing the dispersion against a 0.145 *M* KCl solution for 18–20 hr. One milliliter of the dialyzed suspension of chromate-containing spherules was transferred to each of five cells. By use of a micrometer syringe, 0.05 ml. of a 1×10^{-2} *M* solution in 0.145 *M* KCl of the

drug under investigation was added to each of two of these cells (final drug concentration $\approx 5 \times 10^{-4}$ *M*). The same volume of an UV-irradiated solution of the drug was added to two of the remaining cells. An equal volume of 0.145 *M* KCl was added to the remaining cell, which served as the control. After mixing the material in each cell, 1 ml. of the dispersion was transferred from each cell to a corresponding dialyzing sac. The sacs were sealed and then placed in separate test tubes containing 5 ml. of 0.145 *M* KCl. The test tubes were maintained at 37° in a water bath for 30 min. At the end of this time the sacs were removed and the concentration of chromate ion present in the KCl solution was determined spectrophotometrically (absorbance at 380 mμ). The absorbance, which is proportional to the chromate-ion concentration, was used as a measure of leakage of this ion from the spherule (*i.e.*, lipid membrane permeability).

Known concentrations of chromate ion were determined by this procedure in the presence of the irradiated and nonirradiated phenothiazines (5×10^{-4} *M*) to determine whether either the drug itself or any of its photoproducts interfered with the spectrophotometric analysis. The results obtained were not significantly different from those obtained in the absence of the phenothiazines.

Irradiation of Drug Solution—Three milliliters of a 1×10^{-2} *M* solution of the drug was placed in a standard 1-cm. quartz cell. The cell was positioned 4 in. from the center of a "black light" UV lamp, model 16, Eastern Corp., and exposed for 90 min. The wavelengths emitted by this lamp fall between 290 and 400 mμ. The irradiated solution was then transferred, as previously described, to cells containing the spherules.

RESULTS AND DISCUSSION

The effect of both the irradiated and nonirradiated phenothiazine derivatives on the release of chromate ion from the spherules is summarized in Table I.

Preirradiation—All the nonirradiated drugs tested induced considerably more leakage than that observed with the control. This probably is a measure of the ability of these compounds to interact at a lipid–water interface. Similar effects have been observed with these drugs on other model systems such as erythrocytes, platelets (8), and monomolecular films (9). In fact, phenothiazine–membrane interaction has been proposed as one mechanism of the pharmacologic activity of these drugs (10). The greater leakage induced by chlorpromazine and prochlorperazine apparently is a measure of a greater drug–spherule interaction.

Postirradiation—Of the five drugs studied, only chlorpromazine and prochlorperazine showed any significant change in chromate leakage after irradiation. With both of these phenothiazines, the increase in leakage is considerable. The difference between these two compounds, however, is not significant, indicating that on an equal concentration basis, both of these drugs, when irradiated, induce about the same degree of leakage.

The large increase in leakage induced by the UV irradiation of chlorpromazine and prochlorperazine suggests that such irradiation results in the formation of species that are considerably more membrane active than the parent compounds.

It has been reported that chlorpromazine photopolymerizes *via* a free radical formed by the elimination of the chlorine from the 2-position (11). It is reasonable to postulate that prochlorperazine, which also has a chlorine at the 2-position, will polymerize *via* a similar mechanism. The fact that only these chlorine-containing compounds resulted in increased chromate leakage, coupled with the likelihood that only these compounds will polymerize on exposure to UV radiation, suggests that a photopolymer may be the membrane-active species.

Huang *et al.* (11) postulated that the formation of a photopolymer of chlorpromazine could be responsible for some photosensitized reactions observed in individuals maintained on large doses of this drug.

However, the possibility cannot be eliminated that the *N*-oxide and hydroxy derivatives of chlorpromazine, which were also identified by Huang and Sands (12) as photoproducts, may be responsible to some degree for the observed effects. These products, resulting from the UV irradiation of chlorpromazine, have been shown to be more surface active than chlorpromazine itself (13) and thus more likely to penetrate and disrupt phospholipid membranes.

Table I—Release of Chromate from Phospholipid Spherules Induced by UV-Irradiated and Nonirradiated Phenothiazines

Drug Added	—% of Chromate Released[a]— Nonirradiated Mean[b] ± *SEM*	Irradiated Mean[b] ± *SEM*	Increase due to Irradiation, %[a]
Chlorpromazine	370 ± 17	2790 ± 570	86
Prochlorperazine	428 ± 25	2400 ± 220	82
Promazine	180 ± 30	170 ± 22	—
Triflupromazine	220 ± 50	230 ± 55	—
Fluphenazine	220 ± 40	190 ± 40	—

[a] As compared to that released by the control. [b] Calculated from three to five independently obtained values.

CONCLUSION

Weissman *et al.* (14) have pointed out that artificial lipid spherules appear to resemble natural structures (lysosomes, mitochondria, and erythrocytes) in their release of ions after exposure to lytic agents such as lysolecithin, streptolysin S, a nonionic surfactant (Triton X-100), and steroids. Apparently, these effects, in both natural and artificial systems, are the results of changes in permeability induced by the interaction and subsequent rearrangement of the lipid layers by the lytic agents.

It would appear then that the five phenothiazines are lytic at the concentration used in this study (5×10^{-4} *M*), since they all produced a significant increase in chromate leakage. Such a lytic effect was observed by Ahtee and Paasonen (8) with a series of phenothiazine drugs using erythrocytes, although these authors noted that at lower drug concentration, erythrocyte membrane stabilization occurred rather than lysis. Furthermore, irradiated chlorpromazine and prochlorperazine appear to be much more potent lytic agents than any of the nonirradiated compounds, suggesting the formation of new, more membrane-active species by the irradiation.

Thus, the results of this investigation suggest that if either of these latter two drugs accumulate in membranes of cells or cell organelles, even at levels below that required to produce lysis, irradiation could convert them to species with significant lytic activity. Such lytic species in turn could lead to increased cell membrane permeability and subsequent edema and inflammation. In contrast, the lytic potential of the other drugs studied would be expected to remain relatively unchanged after exposure to UV irradiation. Based on this finding, it appears that of these five phenothiazines, only chlorpromazine and prochlorperazine should produce direct photosensitized cutaneous reactions.

The available clinical data generally support this postulation. Both chlorpromazine and prochlorperazine are consistently reported to produce photosensitization (5). In addition, both also are capable of producing this effect in all exposed individuals if a sufficient drug concentration level is achieved. In contrast, based on the available clinical reports, triflupromazine and fluphenazine are essentially nonphotosensitizing (5, 6), and promazine has been reported as a photosensitizer in only one early study (7).

This preliminary study does indicate that lipid spherules are useful models to assess photosensitizing properties of the phenothiazine drugs. Studies with additional known and potential photosensitizers are currently underway to validate the usefulness of this model for other groups of compounds.

REFERENCES

(1) H. F. Blum, "Photodynamic Action and Diseases Caused by Light," Rheinhold, New York, N. Y., 1941, p. 3.

(2) A. C. Allison, I. A. Magnus, and M. R. Young, *Nature*, **209**, 874(1966).

(3) A. D. Bangham, M. M. Standish, and J. C. Watkins, *J. Mol. Biol.*, **13**, 238(1965).

(4) G. Sessa and G. Weissman, *J. Lipid Res.*, **9**, 310(1968).

(5) B. A. Kirshman and H. Beerman, *Amer. J. Med. Sci.*, **248**, 445(1964).

(6) M. M. Cahn, E. J. Levy, and W. L. Hamilton, *Squibb Inst. Med. Res.*, **2**, 208(1967).

(7) W. M. Sams, *J. Amer. Med. Ass.*, **174,** 2043(1960).
(8) L. Ahtee and M. K. Paasonen, *Ann. Med. Exp. Fenn.*, **43,** 101(1965).
(9) A. Felmeister and R. Schaubman, *J. Pharm. Sci.*, **58,** 64 (1969).
(10) P. S. Guth and M. A. Sprites, *Int. Rev. Neurobiol.*, **7,** 231 (1964).
(11) C. L. Huang and F. L. Sands, *J. Pharm. Sci.*, **56,** 259(1967).
(12) C. L. Huang and F. L. Sands, *J. Chromatogr.*, **13,** 246(1964).
(13) A. Felmeister and R. Schaubman, *J. Pharm. Sci.*, **58,** 1232 (1969).
(14) G. Weissman, G. Sessa, and S. Weissman, *Biochem. Pharmacol.*, **15,** 1537(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 13, 1969, from the *College of Pharmacy, Rutgers —The State University, Newark, NJ 07104*

Accepted for publication January 27, 1970.

Abstracted in part from a thesis submitted by S. V. Tolat to the College of Pharmaceutical Sciences, Columbia University, in partial fulfillment of the Master of Science degree requirements.

Dermatitic Effect of Nonionic Surfactants IV: Phospholipid Composition of Normal and Surfactant-Treated Rabbit Skin

MICHAEL MEZEI and AMBROSE K. Y. LEE

Abstract □ Results of the authors' previous experiment indicated that the primary site of action of topically applied surfactants (polysorbate 85 and polyoxyethylene ether 96) is in the epidermal membranes. To elucidate the interaction of surfactants with biological membranes, the composition of epidermal phospholipids and the rate of biosynthesis of major phospholipid components were determined by utilizing thin-layer chromatographic, spectrophotometric, and radiotracer techniques. Results indicated that the major lipid components are cholesterol, lecithin, lysolecithin, phosphatidyl ethanolamine, and sphingomyelin. The treatment with surfactant did not induce any significant change in the phospholipid composition. The biosynthetic and turnover rates of all identified phospholipids, however, were greatly increased (two to four times) in the surfactant-treated skin. Available data suggest that the tested surfactants damaged the epidermal membranes. A role of surfactants in increasing the absorption of medicinal substances was also proposed in view of these results and other reports regarding the effect of surfactants on biological membranes.

Keyphrases □ Phospholipid composition, rabbits—normal, surfactant-treated skin □ Nonionic surfactants—dermatitic effect, rabbits □ TLC—analysis □ Scintillometry—analysis, ^{32}P-incorporation □ Spectrophotometry—analysis

In a multiphase system the molecules of a surface-active agent align and orient themselves at the interface. In a biological system the membranes provide the interface. The concentration of a surfactant in a tissue because of its hydrophilic–lipophilic character is the highest at, or in, the biological membranes; therefore, the site of action of a topically applied surfactant is very likely in the epidermal membranes.

Unfortunately, it is difficult to design experiments to test the action of surfactants on biological membranes. At present, the exact structural configuration of membranes is not defined because of the lack of reliable techniques to study membranes at cellular or molecular level. Recent reviews describe most of the presently accepted theories relating to the structure and function of biological membranes (1–5). Changes in membranes induced by surfactants or any other agents can be studied only by indirect methods. On the basis of the presently accepted concepts of biological membranes, a qualitative and/or quantitative change in lipid composition of a tissue may indicate structural changes and, consequently, functional changes in the membranes. Results of previous investigations (6–8) indicated that the treatment with surfactant preparations induced an increase both in the content and in the biosynthetic rate of epidermal phospholipids, nucleic acids, and acid-soluble material. This increase was explained by the assumption that the surfactants damaged the biological membranes by either rupturing the membranes or replacing certain phospholipid molecules in the continuous phospholipid micelles present in the membranes. Phospholipid molecules present in ruptured membranes and those that are possibly replaced by surfactants were measured along with newly formed molecules during the analysis of skin tissue.

The higher rate of biosynthesis of epidermal phospholipids was explained by the reasoning that it was expected in order to repair the surfactant-damaged membranes or to regenerate membranes. A further step in this project was to find out whether the surface-active agents interact with the membrane as a whole or with only certain components of the membrane. If the surfactants disrupt or damage the membrane as a result of hydrophobic or micellar interactions or by hydrogen bonding and a completely new membrane is regenerated, then the content and the rate of biosynthesis of all phospholipid membrane components will be increased. On the other hand, if the surfactant interacts with certain phospholipid molecules participating in the membrane, *i.e.*, a molecule can replace one phospholipid molecule present in the lipid micelles, one should find that the content and the rate of biosynthesis of those particular phospholipids would be increased more than that of other phospholipid components. The determination of phospholipid composition and the rate of biosynthesis of each phospholipid component in the control and sur-

(7) W. M. Sams, *J. Amer. Med. Ass.*, **174,** 2043(1960).
(8) L. Ahtee and M. K. Paasonen, *Ann. Med. Exp. Fenn.*, **43,** 101(1965).
(9) A. Felmeister and R. Schaubman, *J. Pharm. Sci.*, **58,** 64 (1969).
(10) P. S. Guth and M. A. Sprites, *Int. Rev. Neurobiol.*, **7,** 231 (1964).
(11) C. L. Huang and F. L. Sands, *J. Pharm. Sci.*, **56,** 259(1967).
(12) C. L. Huang and F. L. Sands, *J. Chromatogr.*, **13,** 246(1964).
(13) A. Felmeister and R. Schaubman, *J. Pharm. Sci.*, **58,** 1232 (1969).
(14) G. Weissman, G. Sessa, and S. Weissman, *Biochem. Pharmacol.*, **15,** 1537(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 13, 1969, from the *College of Pharmacy, Rutgers —The State University, Newark, NJ 07104*

Accepted for publication January 27, 1970.

Abstracted in part from a thesis submitted by S. V. Tolat to the College of Pharmaceutical Sciences, Columbia University, in partial fulfillment of the Master of Science degree requirements.

Dermatitic Effect of Nonionic Surfactants IV: Phospholipid Composition of Normal and Surfactant-Treated Rabbit Skin

MICHAEL MEZEI and AMBROSE K. Y. LEE

Abstract □ Results of the authors' previous experiment indicated that the primary site of action of topically applied surfactants (polysorbate 85 and polyoxyethylene ether 96) is in the epidermal membranes. To elucidate the interaction of surfactants with biological membranes, the composition of epidermal phospholipids and the rate of biosynthesis of major phospholipid components were determined by utilizing thin-layer chromatographic, spectrophotometric, and radiotracer techniques. Results indicated that the major lipid components are cholesterol, lecithin, lysolecithin, phosphatidyl ethanolamine, and sphingomyelin. The treatment with surfactant did not induce any significant change in the phospholipid composition. The biosynthetic and turnover rates of all identified phospholipids, however, were greatly increased (two to four times) in the surfactant-treated skin. Available data suggest that the tested surfactants damaged the epidermal membranes. A role of surfactants in increasing the absorption of medicinal substances was also proposed in view of these results and other reports regarding the effect of surfactants on biological membranes.

Keyphrases □ Phospholipid composition, rabbits—normal, surfactant-treated skin □ Nonionic surfactants—dermatitic effect, rabbits □ TLC—analysis □ Scintillometry—analysis, ^{32}P-incorporation □ Spectrophotometry—analysis

In a multiphase system the molecules of a surface-active agent align and orient themselves at the interface. In a biological system the membranes provide the interface. The concentration of a surfactant in a tissue because of its hydrophilic–lipophilic character is the highest at, or in, the biological membranes; therefore, the site of action of a topically applied surfactant is very likely in the epidermal membranes.

Unfortunately, it is difficult to design experiments to test the action of surfactants on biological membranes. At present, the exact structural configuration of membranes is not defined because of the lack of reliable techniques to study membranes at cellular or molecular level. Recent reviews describe most of the presently accepted theories relating to the structure and function of biological membranes (1–5). Changes in membranes induced by surfactants or any other agents can be studied only by indirect methods. On the basis of the presently accepted concepts of biological membranes, a qualitative and/or quantitative change in lipid composition of a tissue may indicate structural changes and, consequently, functional changes in the membranes. Results of previous investigations (6–8) indicated that the treatment with surfactant preparations induced an increase both in the content and in the biosynthetic rate of epidermal phospholipids, nucleic acids, and acid-soluble material. This increase was explained by the assumption that the surfactants damaged the biological membranes by either rupturing the membranes or replacing certain phospholipid molecules in the continuous phospholipid micelles present in the membranes. Phospholipid molecules present in ruptured membranes and those that are possibly replaced by surfactants were measured along with newly formed molecules during the analysis of skin tissue.

The higher rate of biosynthesis of epidermal phospholipids was explained by the reasoning that it was expected in order to repair the surfactant-damaged membranes or to regenerate membranes. A further step in this project was to find out whether the surface-active agents interact with the membrane as a whole or with only certain components of the membrane. If the surfactants disrupt or damage the membrane as a result of hydrophobic or micellar interactions or by hydrogen bonding and a completely new membrane is regenerated, then the content and the rate of biosynthesis of all phospholipid membrane components will be increased. On the other hand, if the surfactant interacts with certain phospholipid molecules participating in the membrane, *i.e.*, a molecule can replace one phospholipid molecule present in the lipid micelles, one should find that the content and the rate of biosynthesis of those particular phospholipids would be increased more than that of other phospholipid components. The determination of phospholipid composition and the rate of biosynthesis of each phospholipid component in the control and sur-

Table I—Relative Percent Composition of Epidermal Phospholipids

Phospholipids	Untreated, mean ± *SD*	Relative Percent[a] Petrolatum, mean ± *SD*	Polysorbate 85, mean ± *SD*	Polyoxyethylene Ether 96, mean ± *SD*
Lecithin	41.6 ± 5.0	41.6 ± 5.2	43.3 ± 4.8	40.2 ± 4.6
Lysolecithin	10.9 ± 4.3	10.4 ± 3.9	10.3 ± 4.6	10.4 ± 4.8
Phosphatidyl ethanolamine	27.1 ± 4.0	27.7 ± 4.6	27.5 ± 3.4	29.2 ± 3.6
Sphingomyelin	20.3 ± 5.1	20.4 ± 2.0	18.8 ± 5.5	20.1 ± 5.4

[a] Results indicated represent the mean value of percentage composition, on the basis of lipid phosphorus, with standard deviation (±*SD*, N = 31). The percentage composition was calculated, in case of each rabbit, from the sum of phosphorus recovered from spots on the thin-layer plate corresponding to the four phospholipids which was accepted as 100%. Epidermal samples were obtained from three groups of rabbits: 21 rabbits were treated for 4 days, five for 2 days, and five for 7 days.

factant-treated skin, as described in this report, was another attempt to test, although indirectly, the site of action of the selected surfactant at the molecular level.

MATERIALS AND METHODS

The selection and the treatment of the experimental animals and the isolation of epidermis and epidermal lipids have been described in the authors' previous reports (6–8). Aliquots of the lipid extract were used for the separation of phospholipids by TLC.

Thin-Layer Chromatography—Thirty grams of silica gel G (E. Merck, A. G., Darmstadt, Germany) was mixed with 63 ml. of 0.01 *M* Na_2CO_3 solution and spread on 20 × 20-cm. glass plates in 0.25-mm. thickness. The plates were dried at room temperature and were activated for 1 hr. at 105° just before use. Aliquots of lipid extracts were applied along with standard solutions of known phospholipids with a microliter syringe as narrow streaks on 2-cm. wide lanes, 1.5 cm. from the bottom of the plate. The plates were subjected to ascending chromatography in a closed glass developing tank, which contained 100 ml. of chloroform–methanol–distilled water, 65:30:5. This solvent was allowed to rise to 15 cm. from the starting line.

The developed chromatograms were examined by various detection methods, such as exposure to iodine vapor, spraying with ammonium molybdate–perchloric acid, rhodamine B, and Dragendorff reagents (9). Individual lipids were identified by comparison with standard lipid applied on the same thin-layer plate. Additional evidence of identity of the various phospholipids was obtained by recent experiment using the Zeiss chromatogram spectrophotometer (10). The identified components, as well as standard known lipids and blank areas, were scraped into glass-stoppered test tubes, and the phospholipids were digested with sulfuric acid and hydrogen peroxide and the phosphorus was determined as described by Keenan *et al.* (11).

Radiotracer Technique—Recent experiments were extended to measure the rate of *in vivo* incorporation of ^{32}P into the epidermal phospholipid components. In this part of the project, rabbits (five in a group) were treated for 2, 4, and 7 days. Twenty-four hours before the end of treatment, approximately 5 mc. inorganic ^{32}P (Na_2HPO_4) was injected intravenously through the ear veins. The experimental procedure for extracting the epidermal lipids was the same as with other rabbits treated for 4 days without the use of an isotope (7). To minimize the error introduced by the TLC technique, the same sample that was obtained from the identified and marked spots was used for both spectrophotometric determination of phosphorus (11) and for the measurement of ^{32}P incorporation. In this way a more reliable specific radioactivity (c.p.m./mcg. lipid-P) could be calculated. Aliquots (3.0 ml.) of the isobutanol layer, which was used for the spectrophotometric determination of phosphorus, were mixed with scintillator solvent (7.0 ml. Bray's solution) composed of 60 g. naphthalene, 20 ml. ethylene glycol, 100 ml. methanol, 4 g. PPO, and 0.2 g. POPOP, and dioxane made up the volume to 1.0 l. The radioactivity was measured by Unilux I liquid scintillation counter (Nuclear Chicago).

RESULTS AND DISCUSSION

Thin-layer chromatographic analyses of epidermal lipid extracts indicated that there are four phospholipids—lecithin, lysolecithin, phosphatidyl ethanolamine, and sphingomyelin—present in rabbit skin in amounts that could be measured and identified. Besides these four components, three other phospholipids were detected but not identified, mainly because of the minute amount present. Table I demonstrates the relative percent composition of the four major epidermal phospholipids of untreated and surfactant-treated rabbit skin. The relative percent composition was calculated from the sum of phosphorus recovered from spots containing these four phospholipids. The unidentified phospholipids, which probably compose 10–20% of the total phospholipid content, were disregarded.

According to the data shown by Table I, there is no statistically significant change in the percent composition of these phospholipids after the treatment with the tested surfactant preparations (10% in white petrolatum). There is, however, a slight increase in lecithin content as well as a slight decrease in sphingomyelin content of samples treated with polysorbate 85 compared with that of untreated skin samples. The distribution of incorporated radioactivity in epidermal phospholipids is shown by Table II. Due to the treatment with polysorbate 85, the content of lecithin seemed to be increased (Table I) but the reverse is true regarding the radioactivity (Table II). The same treatment resulted in a decrease in the content (Table I) and an increase in the radioactivity (Table II) of sphingomyelin. That the treatment with surfactants has an effect, however slight, on the content but more on the rate of biosynthesis of these four phospholipids becomes evident if the results shown in Table III are considered. Table III demonstrates the effect

Table II—Distribution of Incorporated Radioactivity in Epidermal Phospholipids

Phospholipids	Untreated, mean ± *SD*	Relative Percent[a] Petrolatum, mean ± *SD*	Polysorbate 85, mean ± *SD*	Polyoxyethylene Ether 96, mean ± *SD*
Lecithin	50.0 ± 4.7	48.5 ± 5.2	46.9 ± 5.8	43.2 ± 5.8
Lysolecithin	7.5 ± 2.9	7.6 ± 2.1	6.6 ± 1.7	8.6 ± 2.8
Phosphatidyl ethanolamine	28.9 ± 3.2	31.2 ± 3.7	27.6 ± 5.7	29.6 ± 2.8
Sphingomyelin	13.5 ± 4.4	12.8 ± 5.3	18.8 ± 7.2	18.7 ± 5.3

[a] Values represent the mean percentage of counts measured in the four phospholipids recovered from the thin-layer plates. The percentage of radioactivity was calculated, in case of each rabbit, from the sum of counts in the four phospholipids recovered which was accepted as 100%. The mean of these percentages were calculated, in case of each phospholipid component, with standard deviations (±*SD*, N = 15). Epidermal samples were obtained from three groups of rabbits (five in each group) treated for 2, 4, and 7 days, respectively.

Table III—Incorporation of ^{32}P into Epidermal Phospholipids

Phospholipids	Length of Treatment, days	Specific Activity as Percent of Control[a]		
		Petrolatum, mean ± *SD*	Polysorbate 85, mean ± *SD*	Polyoxyethylene Ether 96, mean ± *SD*
Lecithin	2	128 ± 35	194 ± 52	179 ± 52
	4	118 ± 15	202 ± 12	151 ± 6
	7	113 ± 14	196 ± 85	194 ± 60
Lysolecithin	2	142 ± 33	159 ± 29	117 ± 19
	4	104 ± 6	466 ± 69	221 ± 40
	7	103 ± 37	145 ± 22	209 ± 78
Phosphatidyl ethanolamine	2	120 ± 32	155 ± 19	129 ± 23
	4	120 ± 25	211 ± 45	197 ± 27
	7	142 ± 33	222 ± 84	216 ± 27
Sphingomyelin	2	112 ± 8	290 ± 148	226 ± 20
	4	133 ± 30	410 ± 55	341 ± 50
	7	103 ± 16	283 ± 132	269 ± 87

[a] The results are expressed as percent differences of specific activity (c.p.m./mcg. lipid-P) of skin samples treated with the ointment base: white petrolatum, and nonionic surfactants: polysorbate 85 and polyoxyethylene ether 96, as compared with that of the untreated skin, which is accepted as 100%. Values show the mean ± standard deviation (± *SD*, N = 5).

of treatment with surfactant preparations on the rate of incorporation of ^{32}P into epidermal lipid components.

To assess statistical significance of the data, the percentage stimulations of ^{32}P incorporation into the epidermal phospholipid component of each individual rabbit were calculated. These percentage increases were averaged, and then the mean values were calculated with standard deviations. This was necessitated by the variation in the absolute incorporation of ^{32}P into the various epidermal phospholipids of different rabbits. This variation was due not only to individual biological variation but, to a greater extent, to the fact that it was almost impossible to inject intravenously exactly 5.0 mc. ^{32}P. Since the amount of ^{32}P in the circulatory blood and, consequently, at the site of the designated epidermal tissue varied with each rabbit, the variation in the rate of incorporation of ^{32}P into phospholipid components was very large, even within one type of skin. This variation would tend to decrease the statistical significance of any differences that might be observed between the specific activity of a particular phospholipid extracted from the untreated and surfactant-treated skin of one rabbit. The rate of ^{32}P incorporation was expressed as specific activity in counts per minute per phosphorus (c.p.m./mcg.-P) present in the particular phospholipid recovered from the thin-layer plate.

In all cases the incorporation of ^{32}P into phospholipids of the surfactant-treated skin was much greater than that of the untreated skin samples. The treatment with petrolatum also seems to stimulate the incorporation of ^{32}P, but this stimulation is not significant. The highest increase in the rate of ^{32}P incorporation is observed in skin samples treated with polysorbate 85 preparation for 4 days. This increase is more than fourfold in the case of lysolecithin and sphingomyelin and somewhat more than twofold with lecithin and phosphatidyl ethanolamine. Treatment with polysorbate 85 for 2 and 7 days induced a less dramatic increase in the specific activity of each of the four phospholipids. The explanation for the relationship between the length of time of treatment and the extent of increase in the specific activity of the phospholipids is rather complex. Speculations for possible mechanisms of reaction to injury caused by the surfactant at various stages of the treatment are presented elsewhere (8). The treatment with polyoxyethylene ether 96 also induced a considerable increase in the rate of incorporation of ^{32}P into each of the phospholipids. These increases, however, were almost independent of the length of time of treatment.

In most cases the standard deviation is rather large, mainly because of the thin-layer chromatographic technique and partly because of other numerous steps in the experimental procedure. The quantitative recovery of phospholipids from the corresponding spots on the thin-layer plates and the spectrophotometric and radioactive measurement of the phospholipid components in the presence of silica gel are the major factors in the spread of results. In spite of these problems, the effect of the tested surfactants on the rate of biosynthesis of the major epidermal phospholipids is apparent.

Results reported previously (6–8) and data presented herein are clear, although indirect, indications for changes in epidermal membranes as a result of treatment with surfactants. Many investigators (12–20) reported that surfactants in a variety of dosage forms increase the absorption of medicinal substances. In most of these reports (12–20) the role of surfactants in enhancing absorption was explained by the physicochemical properties of the tested surfactants; a surfactant may reduce the disintegration time of a tablet or may enhance the dissolution or diffusion of an active ingredient by various physicochemical phenomena, thereby influencing the rate of absorption of agents administered along with a surface-active agent. These are sound explanations, and no doubt a surfactant can influence absorption on these bases due to its physicochemical properties. The authors would like to propose, on the bases of previous studies (6–8), that the role of a surfactant in enhancing absorption of other agents present in the same product is also due to its physiological properties. As the authors postulated (21), the surfactants may act on the membranes by various possible mechanisms, and this is also a contributing factor for the reported increased rate of absorption. A damaged or regenerating membrane is less of a barrier to penetrating substances than are intact membranes. The question now is whether it is wise to increase the absorption of a substance by damaging the membranes, especially in the case of chronic treatment. The surfactants increase the permeability of membranes, which may lead not only to higher penetration of medicinal substances but, by disturbing the balance of a cell system, also to metabolic disorder.

REFERENCES

(1) V. P. Whittaker, *Brit. Med. Bull.*, **24,** 101(1968).

(2) J. A. Lucy, *ibid.*, **24,** 127(1968).

(3) A. Rothstein, *Ann. Rev. Physiol.*, **30,** 15(1968).

(4) E. D. Korn, *Ann. Rev. Biochem.*, **38,** 263(1969).

(5) D. E. Green and D. H. MacLennan, *BioScience*, **19,** 213 (1969).

(6) M. Mezei and R. W. Sager, *J. Pharm. Sci.*, **56,** 1604(1967).

(7) M. Mezei and G. N. White, *ibid.*, **58,** 1209(1969).

(8) M. Mezei, to be published.

(9) D. Waldi, in "Thin-Layer Chromatography," E. Stahl, Ed., Springer-Verlag, New York, N. Y., 1965.

(10) M. Mezei and A. K. Y. Lee, to be published.

(11) R. W. Keenan, G. Schmidt, and T. Tanaka, *Anal. Biochem.*, **23,** 555(1968).

(12) R. W. Wissler, W. F. Bethard, P. Baker, and H. D. Mori, *Proc. Soc. Exp. Biol. Med.*, **86,** 170(1954).

(13) G. Levy and R. H. Reuning, *J. Pharm. Sci.*, **53,** 1471(1964).

(14) G. Levy, K. E. Miller, and R. H. Reuning, *ibid.*, **55,** 394 (1966).

(15) H. Matsumoto, *Chem. Pharm. Bull.*, **14,** 398(1966).

(16) H. Yamada, T. Ichihashi, F. Kogishi, and R. Yamamoto, *ibid.*, **14,** 786(1966).

(17) K. Kakemi, H. Sezaki, S. Muranishi, and H. Matsui, *ibid.*, **15,** 172(1967).

(18) T. Matsuzawa, H. Fujisawa, K. Aoki, and H. Mima, *ibid.*, **17,** 999(1969).

(19) L. J. Ravin, E. G. Shami, A. Intoccia, E. Rattie, and G. Joseph, *J. Pharm. Sci.*, **58,** 1242(1969).

(20) C. W. Whitworth and E. R. Carter, *ibid.*, **58**, 1285(1969).
(21) M. Mezei, R. W. Sager, W. D. Stewart, and A. L. deRuyter, *ibid.*, **55**, 584(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 3, 1969, from the *College of Pharmacy, Dalhousie University, Halifax, Nova Scotia, Canada.*

Accepted for publication February 4, 1970.

Presented at the Sixteenth Canadian Conference of Pharmaceutical Research, St. John's, Newfoundland, August 9, 1969.

This work was supported by the Medical Research Council (Canada) Grant MA-2983 and the Canadian Foundation for the Advancement of Pharmacy.

The technical assistance of Miss Zenora Rapersad is acknowledged.

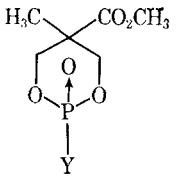
1,3,2-Dioxaphosphorinane 2-Oxides IV: Preparation of Some 2-Substituted-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxides as Potential Antitumor Agents

JOHN H. BILLMAN and GERALD R. ROEHRIG

Abstract □ Twenty-two of the title compounds, in which the substituents are chloro, alkylamino, dialkylamino, arylamino, hydroxy, and amine salts, as well as the pyrophosphate, have been synthesized and submitted for antitumor evaluation.

Keyphrases □ 1,3,2-Dioxaphosphorinane 2-oxides—synthesis □ Antitumor agents—synthesis, 1,3,2-dioxaphosphorinane 2-oxides □ IR spectrophotometry—structure, analysis

Previous work in the authors' laboratory (1) has led to consideration of the dioxaphosphorinane 2-oxides as potential antitumor agents. Synthesis and evaluation of compounds of Type I are described at this time.

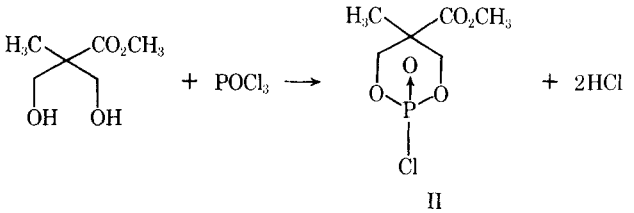


I

Y = —Cl, —NHR, —NR_2, —NHAr, —OH, -amine salts and the pyrophosphate

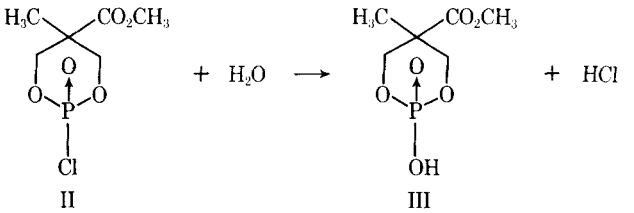
The starting point for the preparation of these compounds was the phosphochloridic acid II, which was prepared according to Scheme I. Compound II can formally be considered as an acid chloride analogous to the more commonly encountered carboxylic acid chlorides; as such, one would expect that it might exhibit many of the same types of reactions as the acyl chlorides. Indeed, many of those reactions have been observed and used to advantage. Schemes II–IV are representative of those used for the preparation of the compounds cited in this paper.

The salts of the acid were prepared for two basic reasons. Since the majority of these compounds are relatively water insoluble, it was thought that a compound with considerably greater water solubility might exhibit a greater degree of antitumor activity. Also, by incorporating amines that exhibit biological activity, one could compare the activity of the salt with that of the free amine. At this point, insufficient data have been returned to clarify either of these points.



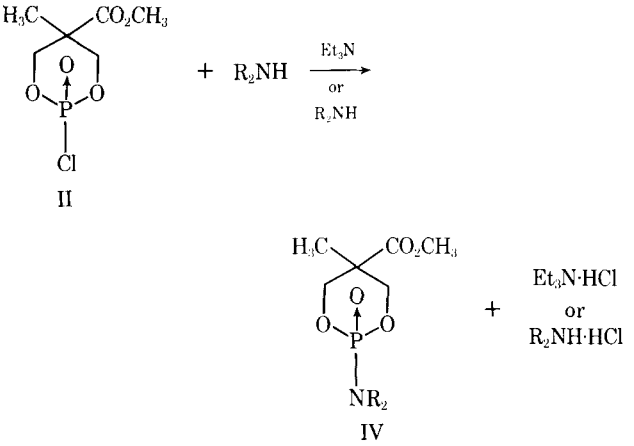
II

Scheme I



II III

Scheme II



II IV

R = alkyl, aryl, or hydrogen

Scheme III

Tables I and II contain the data pertinent to the structures of the compounds under consideration.

(20) C. W. Whitworth and E. R. Carter, *ibid.*, **58**, 1285(1969).
(21) M. Mezei, R. W. Sager, W. D. Stewart, and A. L. deRuyter, *ibid.*, **55**, 584(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 3, 1969, from the *College of Pharmacy, Dalhousie University, Halifax, Nova Scotia, Canada.*

Accepted for publication February 4, 1970.

Presented at the Sixteenth Canadian Conference of Pharmaceutical Research, St. John's, Newfoundland, August 9, 1969.

This work was supported by the Medical Research Council (Canada) Grant MA-2983 and the Canadian Foundation for the Advancement of Pharmacy.

The technical assistance of Miss Zenora Rapersad is acknowledged.

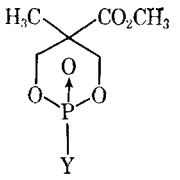
1,3,2-Dioxaphosphorinane 2-Oxides IV: Preparation of Some 2-Substituted-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxides as Potential Antitumor Agents

JOHN H. BILLMAN and GERALD R. ROEHRIG

Abstract □ Twenty-two of the title compounds, in which the substituents are chloro, alkylamino, dialkylamino, arylamino, hydroxy, and amine salts, as well as the pyrophosphate, have been synthesized and submitted for antitumor evaluation.

Keyphrases □ 1,3,2-Dioxaphosphorinane 2-oxides—synthesis □ Antitumor agents—synthesis, 1,3,2-dioxaphosphorinane 2-oxides □ IR spectrophotometry—structure, analysis

Previous work in the authors' laboratory (1) has led to consideration of the dioxaphosphorinane 2-oxides as potential antitumor agents. Synthesis and evaluation of compounds of Type I are described at this time.

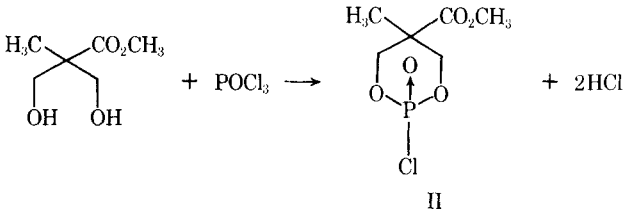


I

Y = —Cl, —NHR, —NR_2, —NHAr, —OH, -amine salts and the pyrophosphate

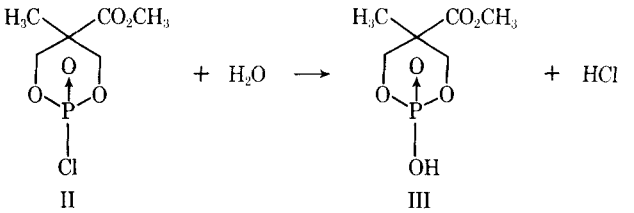
The starting point for the preparation of these compounds was the phosphochloridic acid II, which was prepared according to Scheme I. Compound II can formally be considered as an acid chloride analogous to the more commonly encountered carboxylic acid chlorides; as such, one would expect that it might exhibit many of the same types of reactions as the acyl chlorides. Indeed, many of those reactions have been observed and used to advantage. Schemes II–IV are representative of those used for the preparation of the compounds cited in this paper.

The salts of the acid were prepared for two basic reasons. Since the majority of these compounds are relatively water insoluble, it was thought that a compound with considerably greater water solubility might exhibit a greater degree of antitumor activity. Also, by incorporating amines that exhibit biological activity, one could compare the activity of the salt with that of the free amine. At this point, insufficient data have been returned to clarify either of these points.



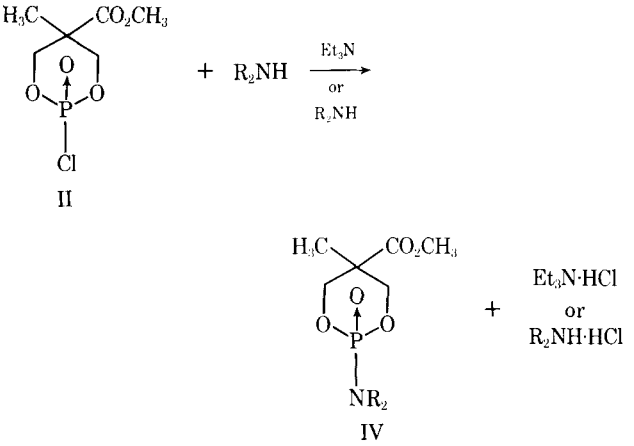
II

Scheme I



II III

Scheme II



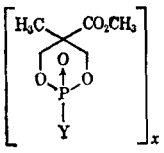
II IV

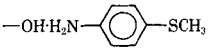
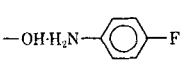
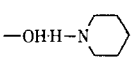
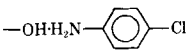
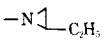
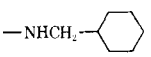
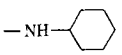
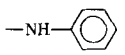
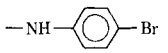
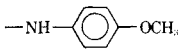
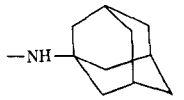
R = alkyl, aryl, or hydrogen

Scheme III

Tables I and II contain the data pertinent to the structures of the compounds under consideration.

Table I—2-Substituted-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxides



Compd. Number	Y	x	Formula	Pure Yield, %	M.p.	Procedure
1	—Cl	1	$C_6H_{10}ClO_5P$	41.3	108–109°	A
2	—OH	1	$C_6H_{11}O_6P$	52.7	182–184°	B
3		1	$C_{13}H_{20}NO_6PS$	79.4	174–176°	C
4		1	$C_{12}H_{17}FNO_6P$	94.5	150–151.5°	C
5		1	$C_{11}H_{22}NO_6P$	54.2	117–121°	C
6		1	$C_{12}H_{17}ClNO_6P$	54.6	135–136.5°	C
7	—O—	2	$C_{12}H_{20}O_{12}P_2$	20.0	186–187°	D
8		1	$C_8H_{14}NO_5P$	33.3	90–91°	E
9		1	$C_9H_{16}NO_5P$	59.0	b.p. 124–128° at 0.1–0.3 Torr.	E
10		1	$C_{10}H_{18}NO_5P$	59.1	47–49°	E
11		1	$C_{11}H_{20}NO_5P$	35.0	112–114°	E
12		1	$C_{13}H_{18}NO_5P$	33.1	100–101°	E
13		1	$C_{12}H_{22}NO_5P$	70.0	167.5–169.5°	E
14		1	$C_{10}H_{18}NO_5P$	50.0	85–86.5°	E
15		1	$C_{12}H_{22}NO_5P$	38.0	125.5–127.5°	E
16		1	$C_{12}H_{16}NO_5P$	49.4	165–166°	F
17		1	$C_{12}H_{15}BrNO_5P$	57.2	158.5–159.5°	F
18		1	$C_{13}H_{18}NO_6P$	56.9	140–141°	F
19		1	$C_{15}H_{26}NO_5P$	29.7	171.5–173°	E
20	—$NH(CH_2)_3NH$—	2	$C_{15}H_{28}N_2O_{10}P_2$	44.4	195–197°	E
21	—$NH(CH_2)_8NH$—	2	$C_{20}H_{38}N_2O_{10}P_2$	26.9	166–167.5°	E
22	—$NH(CH_2)_4NH$—	2	$C_{16}H_{30}N_2O_{10}P_2$	55.6	227.5–228.5°	E

BIOLOGICAL RESULTS

All biological testing was carried out by the Cancer Chemotherapy National Service Center (CCNSC), Bethesda, Md. Compounds I–VI, VIII–X, XII–XIX, XXI, and XXII were tested against L1210 lymphoma over a total dose range of 40–400 mg./kg. and found to be inactive. Compounds II, III, V, VI, X, XIII, and XV–XVIII were tested against Walker 256 carcinosarcoma over a total dose range of 100–400 mg./kg. and found to be inactive.

EXPERIMENTAL[1]

2-Chloro-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxide (II)—*Procedure A*—Phosphorus oxychloride (154.5 g., 1.01 moles) was added in small portions to stirring 2-carbomethoxy-2-methyl-1,3-propanediol (50.0 g., 0.337 mole) in a conical flask fitted with a drying tube. After the exothermic reaction had subsided, the stirring reaction mixture was heated to 90° for 4 hr. The reaction mixture was poured with stirring into 600 ml. of petroleum ether (90–120° b.p.) which had been cooled to 0° in an ice bath. White crystals of the phosphochloridic acid precipitated; they were filtered and washed with anhydrous ether, yielding 31.3 g. or 41.3%.

Anal.—Calcd. for $C_6H_{10}ClO_5P$: C, 31.52; H, 4.41. Found: C, 31.35; H, 4.35.

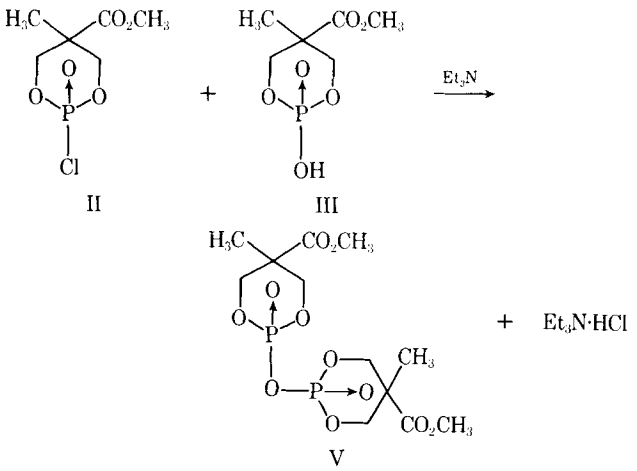
2-Hydroxy-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxide (III)—*Procedure B*—A stirring solution of II (42.50 g., 0.186 mole) in 100 ml. of 95:5 acetone–water was refluxed for 3.5 hr. and the reaction mixture was cooled overnight. The resulting white crystals of III were recrystallized from acetone, yielding 20.6 g. or 52.7%.

Anal.—Calcd. for $C_6H_{11}O_6P$: C, 34.25; H, 5.28. Found: C, 34.30; H, 5.41.

[1] All melting points were determined on a Thomas-Hoover capillary apparatus and are corrected. IR spectra were obtained from a Perkin-Elmer 137 infrared spectrophotometer. Analyses were carried out by Midwest Microlab, Inc., Indianapolis, Ind.

Table II—Analytical Data for 2-Substituted-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxides

Compd. Number	Anal., %: C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found	IR Bands (cm.$^{-1}$): N—H	C=O	P→O	POC	POP	O ‖ CCH$_3$	OH
1	31.52	31.35	4.41	4.35	—	—	—	1735	1225	990	—	1360	—
2	34.25	34.30	5.28	5.41	—	—	—	1725	1245	1000	—	1350	3000–2000
3	—	—	—	—	4.01	3.99	2700–2200	1725	1200	1000	—	1345	—
4	—	—	—	—	4.36	4.40	2700–2100	1725	1200	1000	—	1350	—
5	—	—	—	—	4.74	5.03	3000–2100	1725	1210	1000	—	1375	—
6	—	—	—	—	4.15	3.97	2700–2100	1730	1200	1010	—	1350	—
7	35.83	35.91	5.01	5.16	—	—	—	1725	1235	990	960	1360	—
8	—	—	—	—	5.96	6.04	—	1725	1240	1000	—	1360	—
9	—	—	—	—	5.60	5.33	—	1725	1260	1000	—	1360	—
10	—	—	—	—	5.30	5.30	—	1725	1250	950–1050	—	1360	—
11	—	—	—	—	5.02	4.86	—	1725	1235	995	—	1365	—
12	—	—	—	—	4.68	4.75	3200	1730	1225	1000	—	1365	—
13	—	—	—	—	4.83	4.88	3200	1725	1215	995	—	1350	—
14	—	—	—	—	5.32	5.47	—	1730	1230	1010	—	1350	—
15	—	—	—	—	4.81	4.81	—	1730	1230	1000	—	1350	—
16	—	—	—	—	4.91	4.67	3150	1725	1220	990	—	1350	—
17	—	—	—	—	3.85	3.86	3100	1730	1220	990	—	1350	—
18	—	—	—	—	4.44	4.50	3300	1720	1220	1000	—	1370	—
19	—	—	—	—	4.23	4.23	3310	1725	1225	1000	—	1325	—
20	—	—	—	—	6.11	6.30	3240	1735	1225	1010	—	1325	—
21	—	—	—	—	5.30	5.45	3210	1730	1220	1000	—	1365	—
22	—	—	—	—	5.91	5.96	3200	1730	1230	1000	—	1320	—



Scheme IV

2-Hydroxy-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxide, 4-Methylmercaptoaniline Salt—*Procedure C*—This procedure is representative of those used in preparation of the salts. A solution of 4-methylmercaptoaniline (3.31 g., 23.8 mmoles) in 50 ml. of acetone was added dropwise to a stirring solution of III (5.00 g., 23.8 mmoles) in 25 ml. of acetone, and the resulting mixture was stirred for 1 hr. at room temperature. White crystals of the salt precipitated; they were washed with acetone and dried, yielding 6.60 g. or 79.4%.

Anal.—Calcd. for $C_{13}N_{20}NO_6PS$: N, 4.01. Found: N, 3.99.

Bis(5-carbomethoxy-5-methyl-2-oxo-1,3,2-dioxaphosphorinanyl)-pyrophosphate (V)—*Procedure D*—A solution of III (4.60 g., 21.9 mmoles) in 25 ml. of acetone was added dropwise to a stirring solution of II (5.00 g., 21.9 mmoles) in 25 ml. of acetone at room temperature. An excess of triethylamine in acetone was added to the reaction mixture, which was then stirred for 2 hr. at room temperature. Triethylamine hydrochloride precipitated; the reaction mixture was chilled and filtered. The solvent was removed from the filtrate *in vacuo;* the resulting white crystals of V were recrystallized from ethyl acetate–acetonitrile, yielding 1.75 g. or 20.0%.

Anal.—Calcd. for $C_{12}H_{20}O_{12}P_2$: C, 35.83; H, 5.01. Found: C, 35.91; H, 5.16.

2-Aziridino-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxide—*Procedure E*—This procedure is representative of those used in the preparation of the alkylamides of Type IV. A solution of aziridine (0.94 g., 21.7 mmoles) and an excess of triethylamine in 50 ml. of acetone was added dropwise to a stirring solution of II (5.00 g., 21.9 mmoles) in 25 ml. of acetone at 0°. Triethylamine hydrochloride precipitated immediately. The reaction mixture was filtered after precipitation was complete, and the solvent was evaporated from the filtrate *in vacuo*. The resulting white crystals of the amide were recrystallized from ether, yielding 1.70 g. or 33.3%.

Anal.—Calcd. for $C_8H_{14}NO_5P$: N, 5.96. Found: N, 6.04.

2-(4-Bromoanilino)-5-carbomethoxy-5-methyl-1,3,2-dioxaphosphorinane 2-Oxide—*Procedure F*—This procedure is representative of those used in the preparation of the arylamides of Type IV. A solution of 4-bromoaniline (15.04 g., 87.4 mmoles) in 50 ml. of benzene was refluxed with stirring for 1 hr. in an apparatus equipped with a Dean trap to remove moisture. A solution of II (10.00 g., 43.7 mmoles) in 50 ml. of benzene was added dropwise to the stirring reaction mixture, which was then refluxed for an additional 4 hr. 4-Bromoaniline hydrochloride precipitated; the reaction mixture was filtered while still hot and the filtrate was chilled. The resulting white crystals of the amide were recrystallized from benzene, yielding 9.1 g. or 57.2%.

Anal.—Calcd. for $C_{12}H_{15}BrNO_5P$: N, 3.85. Found: N, 3.86.

REFERENCE

(1) J. H. Billman and R. F. May, *J. Pharm. Sci.*, **57**, 1812(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 24, 1969, from the *Department of Chemistry, Indiana University, Bloomington, IN 47401*

Accepted for publication February 3, 1970.

Presence of Diosgenin in Tissue Cultures of *Dioscorea composita* Hemsl. and Related Species

ATUL R. MEHTA* and E. JOHN STABA

Abstract □ Four species of *Dioscorea* were grown as callus and suspension tissue cultures on revised Murashige and Skoog's tobacco medium and contained diosgenin.

Keyphrases □ Diosgenin, presence—tissue cultures, determination □ *Dioscorea* tissue cultures—diosgenin determination □ TLC—separation □ GLC—determination

A number of *Dioscorea* species (family *Dioscoreaceae*) are cultivated commercially for their tubers which yield diosgenin, an important starting compound for the manufacture of corticosteroidal drugs. Of these, only *D. composita* and *D. deltoidea* have previously been established as tissue cultures. Diosgenin, however, was not detected in the tissue cultures of *D. composita* (1–4) while the presence of diosgenin in the callus and suspension cultures of *D. deltoidea* is reported. In this communication, the authors report the establishment of four species of *Dioscorea* as tissue cultures and their diosgenin content.

EXPERIMENTAL

Tissue Culture—Callus cultures of *D. composita*, *D. deltoidea*, *D. floribunda*, and *D. spiculiflora* were initiated from aseptically germinated seedlings placed in glass vials containing revised Murashige and Skoog's tobacco medium supplemented with 1–3 p.p.m. 2,4-dichlorophenoxyacetic acid (2,4-D). Details of the cultural procedures followed were described previously (5). Yellowish-white to pale-brown callus developed from the seedling within 3–4 weeks, and these were subcultured to fresh agar media every 4 weeks. On low 2,4-D medium (1 p.p.m.), root primordia differentiated from *D. floribunda* callus tissues; therefore, all callus cultures (except *D. deltoidea*) were maintained on a medium containing 3 p.p.m. 2,4-D.

In the present investigation: (*a*) callus tissues which had undergone four subcultures and (*b*) later grown as suspension culture on a reciprocal shaker for 3 weeks were assayed for the presence of diosgenin. Differentiated and undifferentiated tissue cultures of *D. floribunda* were assayed separately for diosgenin.

Analysis—The dried and acid-hydrolyzed tissues were extracted with benzene for TLC (qualitative) (6) or with chloroform for GLC (quantitative). Dried chloroform extracts and standards (diosgenin and cholesterol) were silylated with a Tri-Sil–pyridine mixture (Pierce Chemical Co., Rockford, Ill.) and assayed (Varian Aerograph model 1740 with flame-ionization detector). A 1.52-m. × 0.63-cm. (5-ft. × 0.25-in.) glass column containing 80–100-mesh Varaport 30 coated with 3% OV-17 was used at 275°.

Table I—*D. deltoidea*—Strain I: Diosgenin Content of Suspension Cultures

Age, weeks	G.I.[a]	Medium, pH	Diosgenin, mg.% Dry Weight[b]
2	4.2	6.5	454.3
4	3.7	5.2	225.8
6	3.0	3.2	182.4
8	2.0	6.5	432.8
10	1.8	6.9	302.5

[a] Growth index (G.I.) = final dry weight/initial dry weight. [b] Average for cells obtained from three to five 500-ml. conical flasks containing 100 ml. RT.1 medium.

Table II—*Dioscorea* Tissue Cultures: Diosgenin Content and Growth Index

Species	Callus/ Diosgenin Content[a]	G.I.[b]	Suspension/ Diosgenin Contents[a]	G.I.[b]
D. deltoidea, Strain II	348.5	3.1	223.7	1.9
D. floribunda, undiff.	145.0	3.6	63.5	2.8
D. floribunda, diff.	94.5	3.0	65.0	2.2
D. spiculiflora	90.4	2.8	35.3	1.8
D. composita, Strain I	60.2	3.2	38.1	2.5
D. composita, Strain II	24.5	2.4	50.9	1.7

[a] Diosgenin content expressed as mg.% dry weight of 4-week-old callus tissue or 3-week-old first generation suspension tissue. [b] Growth index (G.I.) = final dry weight/initial dry weight.

RESULTS AND DISCUSSION

The quantitative assays for diosgenin from tissues and suspension cultures of different *Dioscorea* species are summarized in Tables I and II. Of the species examined, maximum diosgenin content was present in tissue cultures of *D. deltoida* and then in decreasing order of diosgenin production by callus tissue cultures in *D. floribunda* (undifferentiated), *D. spiculiflora*, and *D. composita*. Progressive changes in diosgenin content in *D. deltoidea* cell suspensions during the course of culture for 10 weeks were studied (Table I). The diosgenin content is high initially, declines, and again rises to a high level at 8 weeks. Of all the cultural parameters examined, the pH of the medium follows a strikingly similar pattern. However, further experimentation is needed to confirm and explain this observation and why *D. composita* was not reported earlier to contain diosgenin.

It has been suggested (7, 8) that diosgenin is biosynthesized principally in the aerial parts of *Dioscorea* species and is then translocated to the tubers where it is stored. Earlier studies (5, 6) clearly indicated that undifferentiated *D. deltoidea* tissue cultures produced diosgenin, whereas root-differentiated tissue cultures produced very small amounts. In this study, root-differentiated cultures of *D. floribunda* produced significant amounts of diosgenin.

REFERENCES

(1) L. H. Weinstein, L. G. Nickell, H. J. Laurencott, Jr., and W. Tulecke, *Contrib. Boyce Thompson Inst.*, **20,** 239(1959).

(2) W. Tulecke and L. G. Nickell, *Trans. N. Y. Acad. Sci.*, **22,** 196(1960).

(3) L. G. Nickell, *Advan. Appl. Microbiol.*, **4,** 213(1962).

(4) J. D. Ehrhardt, L. Hirth, and G. Ourisson, *Compt. Rend.*, **262,** 809(1966).

(5) B. Kaul and E. J. Staba, *Lloydia*, **31,** 171(1968).

(6) S. J. Stohs, B. Kaul, and E. J. Staba, *Phytochemistry*, **8,** 1679 (1969).

(7) R. D. Bennett, E. Heftmann, W. H. Preston, Jr., and J. R. Haun, *Arch. Biochem. Biophys.*, **103,** 74(1963).

(8) E. A. Baker, J. T. Martin, and A. P. Wilson, *Ann. Appl. Biol.*, **68**, 203(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 19, 1969, from the *Department of Pharmacognosy, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455*

Accepted for publication January 19, 1970.

This work was supported by a grant from the U. S. National Institutes of Health (GM 16241-02) and was reported at the XI International Botanical Congress, Seattle, Wash., Aug. 1969.

The authors thank Dr. Martin and Mr. Cabanillas of the U. S. Department of Agriculture, Federal Experiment Station, Puerto Rico, for the supply of seeds of *Dioscorea* species.

* Department of Botany, University of Baroda, Borada, India.

Role of Hydrophobic Interactions in Enzyme Inhibition by Drugs

ERIC J. LIEN, MEHDI HUSSAIN*, and GEORGE L. TONG†

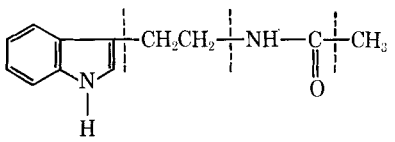
Abstract □ The role of hydrophobic interactions in inhibiting the relatively specific enzymatic reactions of five enzyme systems by series of congeneric drugs has been illustrated by the use of substituent constants and regression analysis. The inhibition of lipoxygenase by alcohols, the inhibition of D-amino acid oxidase by maleimides, and the inhibition of hydroxyindole-*o*-methyltransferase by *N*-acyltryptamines are found to be linearly dependent on the lipohydrophilic character of the inhibitors (log *P* or π). The inhibition of carbonic anhydrase by sulfonamides is found to be linearly dependent on the log *P* and Hammett's σ constant. For monoamine oxidase inhibition by substituted β-carbolines, a parabolic equation of log *P* gives the most significant correlation. The ideal lipohydrophilic character (log P_0) for maximum inhibition is found to be 2.74.

Keyphrases □ Enzyme inhibitory activity—hydrophobic interactions, drugs □ Hydrophobic interactions, drugs—enzyme inhibitory activity □ Physicochemical constants, enzyme inhibition—correlation

In recent years much effort has been focused on elucidation of weak intermolecular forces in biological systems (1), especially on the importance of hydrophobic interactions (2–6). Various experimental methods have been used to estimate the hydrophobic bonding tendency of drug molecules, such as partitioning and chromatographic methods (3, 7). Organic solvents capable of forming hydrogen bonds (*e.g.*, alcohols and esters) appear to give better correlations than hydrocarbons (8). The purpose of this paper is to correlate quantitatively enzyme inhibitory activity with the tendency of hydrophobic interactions of series of drugs, as measured by the partition coefficient of 1-octanol–water. It is hoped that this work may shed some light on the intermolecular forces involved in enzyme inhibition and provide some clues in designing new enzyme inhibitors.

METHOD

The biological data given in Table I are taken from the literature (4, 9–12). The Hammett's sigma constants (σ) are from the compilation of Jaffé (13) unless otherwise stated. The log *P* values are either experimentally determined or calculated from the π constants (14, 15) of Hansch. For example, the log *P* of *N*-acetyltryptamine is calculated as follows:



$$\Sigma\pi = 2.14 + 1.00 - 1.71 + 0.50 = 1.93 = \log P$$

The steric constants, E_s, are taken from Leffler and Grunwald (16). The equations listed in Table II are derived *via* the method of least squares using an IBM 360/65 computer. The inhibition constant K_I, $k' = (k_1/K_I)$, or the concentration of an inhibitor giving 50% inhibition of the enzyme (I_{50}) is converted to the molar basis, and pK_I, log k', or log $1/I_{50}$ is used as a measure of the inhibitory activity.

RESULTS

The equations correlating enzyme inhibition with the physicochemical constants are summarized in Table II, where *n* is the number of data points used in the analysis, *r* is the correlation coefficient, and *s* is the standard deviation.

In the inhibition of lipoxygenase by monohydric alcohols, the relative inhibitory activity is mainly determined by the lipohydrophilic character (log *P*). More than 98% ($r^2 = 0.983$) of the variance in the data can be accounted for by the simple linear equation (Eq. 1*a*). Equation 1*b*, derived by Mitsuda *et al.* (4), gives a slightly lower correlation coefficient, presumably due to the slightly different log *P* values used.

For the carbonic anhydrase inhibition by sulfonamides, by comparing Eq. 2*a* with Eq. 2*b* one can see that the electronic term σ is slightly more important than the log *P* term. The positive coefficient associated with σ indicates that electron-withdrawing groups will increase the inhibitory activity. By using both terms simultaneously, a much better correlation is obtained (Eq. 2*c*). The log *P* term in Eq. 2*c* is significant at 97.5-percentile level, as indicated by an *F*-test ($F_{1,16} = 7.2$; $F_{1,15\ 0.975} = 6.2$).

The π-constant alone gives almost perfect correlation for inhibition of D-amino acid oxidase by *N*-alkylmaleimides (Eqs. 3*b* and 3*c*). By using π and σ terms together, high correlation is obtained for the *N*-aryl as well as *N*-alkyl derivatives. The σ term in Eq. 3*a* is highly significant ($F_{1,5} = 111$; $F_{1,5\ 0.995} = 63.6$).

For the inhibition of hydroxyindole-*o*-methyltransferase by *N*-acyltryptamines, log *P* alone gives fairly good correlation (Eq. 4*a*). By deleting three molecules with deviation greater than 2*s*, a better

(8) E. A. Baker, J. T. Martin, and A. P. Wilson, *Ann. Appl. Biol.*, **68**, 203(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 19, 1969, from the *Department of Pharmacognosy, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455*

Accepted for publication January 19, 1970.

This work was supported by a grant from the U. S. National Institutes of Health (GM 16241-02) and was reported at the XI International Botanical Congress, Seattle, Wash., Aug. 1969.

The authors thank Dr. Martin and Mr. Cabanillas of the U. S. Department of Agriculture, Federal Experiment Station, Puerto Rico, for the supply of seeds of *Dioscorea* species.

* Department of Botany, University of Baroda, Borada, India.

Role of Hydrophobic Interactions in Enzyme Inhibition by Drugs

ERIC J. LIEN, MEHDI HUSSAIN*, and GEORGE L. TONG†

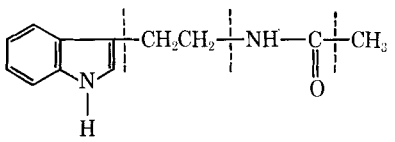
Abstract □ The role of hydrophobic interactions in inhibiting the relatively specific enzymatic reactions of five enzyme systems by series of congeneric drugs has been illustrated by the use of substituent constants and regression analysis. The inhibition of lipoxygenase by alcohols, the inhibition of D-amino acid oxidase by maleimides, and the inhibition of hydroxyindole-*o*-methyltransferase by *N*-acyltryptamines are found to be linearly dependent on the lipohydrophilic character of the inhibitors (log *P* or π). The inhibition of carbonic anhydrase by sulfonamides is found to be linearly dependent on the log *P* and Hammett's σ constant. For monoamine oxidase inhibition by substituted β-carbolines, a parabolic equation of log *P* gives the most significant correlation. The ideal lipohydrophilic character (log P_0) for maximum inhibition is found to be 2.74.

Keyphrases □ Enzyme inhibitory activity—hydrophobic interactions, drugs □ Hydrophobic interactions, drugs—enzyme inhibitory activity □ Physicochemical constants, enzyme inhibition—correlation

In recent years much effort has been focused on elucidation of weak intermolecular forces in biological systems (1), especially on the importance of hydrophobic interactions (2–6). Various experimental methods have been used to estimate the hydrophobic bonding tendency of drug molecules, such as partitioning and chromatographic methods (3, 7). Organic solvents capable of forming hydrogen bonds (*e.g.*, alcohols and esters) appear to give better correlations than hydrocarbons (8). The purpose of this paper is to correlate quantitatively enzyme inhibitory activity with the tendency of hydrophobic interactions of series of drugs, as measured by the partition coefficient of 1-octanol–water. It is hoped that this work may shed some light on the intermolecular forces involved in enzyme inhibition and provide some clues in designing new enzyme inhibitors.

METHOD

The biological data given in Table I are taken from the literature (4, 9–12). The Hammett's sigma constants (σ) are from the compilation of Jaffé (13) unless otherwise stated. The log *P* values are either experimentally determined or calculated from the π constants (14, 15) of Hansch. For example, the log *P* of *N*-acetyltryptamine is calculated as follows:



$$\Sigma\pi = 2.14 + 1.00 - 1.71 + 0.50 = 1.93 = \log P$$

The steric constants, E_s, are taken from Leffler and Grunwald (16). The equations listed in Table II are derived *via* the method of least squares using an IBM 360/65 computer. The inhibition constant K_I, $k' = (k_1/K_I)$, or the concentration of an inhibitor giving 50% inhibition of the enzyme (I_{50}) is converted to the molar basis, and pK_I, log k', or log $1/I_{50}$ is used as a measure of the inhibitory activity.

RESULTS

The equations correlating enzyme inhibition with the physicochemical constants are summarized in Table II, where *n* is the number of data points used in the analysis, *r* is the correlation coefficient, and *s* is the standard deviation.

In the inhibition of lipoxygenase by monohydric alcohols, the relative inhibitory activity is mainly determined by the lipohydrophilic character (log *P*). More than 98% ($r^2 = 0.983$) of the variance in the data can be accounted for by the simple linear equation (Eq. 1*a*). Equation 1*b*, derived by Mitsuda *et al.* (4), gives a slightly lower correlation coefficient, presumably due to the slightly different log *P* values used.

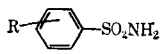
For the carbonic anhydrase inhibition by sulfonamides, by comparing Eq. 2*a* with Eq. 2*b* one can see that the electronic term σ is slightly more important than the log *P* term. The positive coefficient associated with σ indicates that electron-withdrawing groups will increase the inhibitory activity. By using both terms simultaneously, a much better correlation is obtained (Eq. 2*c*). The log *P* term in Eq. 2*c* is significant at 97.5-percentile level, as indicated by an *F*-test ($F_{1,16} = 7.2$; $F_{1,15\ 0.975} = 6.2$).

The π-constant alone gives almost perfect correlation for inhibition of D-amino acid oxidase by *N*-alkylmaleimides (Eqs. 3*b* and 3*c*). By using π and σ terms together, high correlation is obtained for the *N*-aryl as well as *N*-alkyl derivatives. The σ term in Eq. 3*a* is highly significant ($F_{1,5} = 111$; $F_{1,5\ 0.995} = 63.6$).

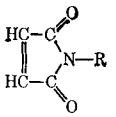
For the inhibition of hydroxyindole-*o*-methyltransferase by *N*-acyltryptamines, log *P* alone gives fairly good correlation (Eq. 4*a*). By deleting three molecules with deviation greater than 2*s*, a better

Table I—Enzymological Data and the Physicochemical Constants Used in Deriving the Equations in Table II

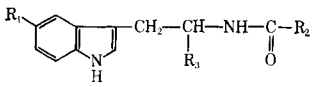
pK_I Calcd.[a]	pK_I Found[b]	R—OH log P[c]	R—
−0.30	−0.18	−0.66	CH_3—
0.18	0.18	−0.16	C_2H_5—
0.46	0.37	0.14	*iso*-C_3H_7—
0.65	0.68	0.34	*n*-C_3H_7—
0.72	0.49	0.41	*tert*-C_4H_9—
0.91	0.86	0.61	*s*-C_4H_9—
0.94	1.13	0.64	*iso*-C_4H_9—
1.13	1.15	0.84	*n*-C_4H_9—
1.39	1.34	1.11	*iso*-C_5H_{11}—
1.61	1.61	1.34	*n*-C_5H_{11}—
2.08	2.10	1.84	*n*-C_6H_{13}—
2.56	2.60	2.34	*n*-C_7H_{15}—



pK_I (log $1/K_I$) Calcd.[d]	pK_I (log $1/K_I$) Found[e]	log P[f]	σ[g]	R—
4.72	4.96	−0.28	−0.59	*p*-CH_3NH—
4.53	4.60	−0.78	−0.66	*p*-NH_2—
5.15	5.30	0.27	−0.27	*p*-CH_3O—
5.38	5.50	0.83	−0.17	*p*-CH_3—
5.47	5.22	0.82	−0.07	*m*-CH_3—
5.39	5.13	0.31	0.00	H—
5.78	5.96	1.01	0.23	*p*-Cl—
5.86	5.96	1.33	0.23	*p*-Br—
5.92	5.92	1.07	0.37	*m*-Cl—
6.07	5.89	−0.06	0.87	*p*-$CH_3C(=O)$—
5.89	6.19	−0.01	0.65	*p*-CN—
6.05	6.12	0.42	0.71	*m*-NO_2—
6.15	6.26	0.55	0.78	*p*-NO_2—
6.22	6.52	1.77	0.50	3,4-Cl_2—
6.44	6.60	1.12	0.94	3-NO_2—4—Cl—
6.80	6.66	1.62	1.20	3-CF_3—4—Cl—
5.45	4.92	0.99	−0.14	2-CH_3—
5.73	5.62	0.90	0.20	2-Cl—
5.82	5.46	0.08	0.55	2-NO_2—



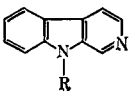
log k' (log k_1/K_I) pH 7.0 Calcd.[h]	log k' (log k_1/K_I) pH 7.0 Found[i]	log k' (log k_1/K_I) pH 7.5 Calcd.[j]	log k' (log k_1/K_I) pH 7.5 Found[i]	π	σ	R—
1.44	1.43	1.89	1.88	1.00	−0.15	C_2H_5—
1.84	1.86	2.27	2.27	2.00	−0.15	*n*-C_4H_9—
2.04	2.04	2.46	2.46	2.50	−0.15	*n*-C_5H_{11}—
2.24	2.23	2.65	2.64	3.00	−0.15[k]	*n*-C_6H_{13}—
2.44	2.45	2.84	2.87	3.50	−0.15[k]	*n*-C_7H_{15}—
2.64	2.61	3.03	3.01	4.00	−0.15[k]	*n*-C_8H_{17}—
2.64	2.51[l]	—	—[m]	2.13	0.01	C_6H_5—
2.72	2.85[l]	—	—[m]	2.32	0.01[k]	4-$(CH_3)_2N$—3,5—$(NO_2)_2$—Ph—



log $1/I_{50}$ Calcd.[n]	log $1/I_{50}$ Found[o]	log P	R_1—	R_2—	R_3—
2.65	2.85	1.93	H—	CH_3—	H—
3.93	3.74	4.06	H—	$C_6H_5CH_2$—	H—
3.63	3.43	3.56	H—	C_6H_5—	H—
4.01	4.21	4.19	F—	$C_6H_5CH_2$—	H—
4.50	4.30	5.00	Br	$C_6H_5CH_2$—	H—
4.02	3.96	4.21	H—	*p*-F—$C_6H_4CH_2$—	H—
4.35	4.17	4.76	H—	*p*-Cl—$C_6H_4CH_2$—	H—
3.68	3.60	3.64	H—	1-Cyclohexenyl—	H—
3.64	3.80	3.57	H—	*o*-F—C_6H_4—	H—
3.71	3.70	3.69	H—	*m*-F—C_6H_4—	H—
3.72	3.47	3.71	H—	*p*-F—C_6H_4—	H—
3.99	4.26	4.15	H—	*o*-Cl—C_6H_4—	H—
4.09	4.00	4.32	H—	*m*-Cl—C_6H_4—	H—
4.05	4.18	4.26	H—	*p*-Cl—C_6H_4—	H—
4.41	4.26	4.85	H—	2,4—$Cl_2C_6H_3$—	H—

Table I—(*Continued*)

log $1/I_{50}$ Calcd.[n]	log $1/I_{50}$ Found[o]	log P	R_1—	R_2—	R_3—
4.51	4.70	5.02	H—	3,4—Cl_2—C_6H_3—	H—
3.75	3.70	3.76	H—	3,4,5—$(CH_3O)_3$—C_6H_2—	H—
3.71	3.77	3.69	F—	C_6H_5—	H—
4.20	4.15	4.50	Br—	C_6H_5—	H—
4.59	4.66	5.15	F—	3,4—Cl_2—C_6H_3—	H—
5.08	5.30	5.96	Br—	3,4—Cl_2—C_6H_3—	H—
4.11	3.57[p]	4.36	H—	$C_6H_5CH_2$—	CH_3—
4.05	3.42[p]	4.26	H—	3,4,5—$(CH_3O)_3$—$C_6H_2CH_2$—	H—
4.54	3.96[p]	5.08	H—	3,5—Cl_2—C_6H_3—	H—



log $1/I_{50}$ Calcd.[q]	log $1/I_{50}$ Found[r]	E_s	log P	σ	R—
4.38	4.54	1.24	2.08	0.00	H—
4.65	5.00	0.00	2.58	−0.17	CH_3—
4.59	4.32	−0.07	3.08	−0.15	C_2H_5—
4.19	3.82	−0.36	3.58	−0.13	n-C_3H_7—
3.45	3.85	−0.39	4.08	−0.16	n-C_4H_9—
2.84	2.74	−0.35	4.38	−0.23	iso-C_5H_{11}—
3.71	3.40	−0.19	1.55	0.06	—CH_2OCH_3
3.68	3.82	−0.47	1.53	0.52	—$COCH_3$
2.10	3.32[s]	−0.39	2.42	−0.13	—$CH_2CH_2CH_2OH$
2.18	3.96[s]	−0.36	1.92	−0.07	—CH_2CH_2OH
2.59	4.47[s]	−0.07	1.42	0.06	—CH_2OH

[a] Calculated from Eq. 1*a* of Table II. [b] From Fig. 2 of *Reference 4*. [c] From *Reference 19*. [d] Calculated from Eq. 2*c* of Table II. [e] From *Reference 9*. [f] From *References 14* and *15*. [g] From *Reference 13*. [h] Calculated from Eq. 3*a* of Table II. [i] From *Reference 10*. [j] Calculated from Eq. 3*c* of Table II. [k] Estimated values. [l] These points are not included in Eq. 3*b* of Table II. [m] Not measured. [n] Calculated from Eq. 4*b* of Table II. [o] From *Reference 11*. [p] These points are not included in Eq. 4*b* of Table II. [q] From *Reference 12*. [r] Calculated from Eq. 5*g* of Table II. [s] These points are not included in Eq. 5*g* of Table II.

Table II—Equations Correlating Enzyme Inhibition with Physicochemical Constants

Enzyme	Inhibitors	Equation	n	r	s	Eq. No.	log P_0, (95% c-l.)
Lipoxygenase	ROH	$pK_I = 0.954 \log P + 0.329$	12	0.992	0.110	1*a*	
		$pK_I = 0.944 \log P + 0.830$	12	0.984	—	1*b*[a]	
Carbonic anhydrase	Sulfonamides	$pK_I = 0.553 \log P + 5.378$	19	0.609	0.492	2*a*	
		$pK_I = 1.026 \sigma + 5.438$	19	0.886	0.288	2*b*	
		$pK_I = 0.259 \log P + 0.886 \sigma + 5.314$	19	0.923	0.247	2*c*	
D-Amino acid oxidase	Maleimides (at pH 7.0)	$\log k' = 0.339 \pi + 4.705 \sigma + 1.745$	8	0.988	0.085	3*a*	
	(at pH 7.5)	$\log k' = 0.395 \pi + 1.051$	6	0.999	0.018	3*b*	
		$\log k' = 0.382 \pi + 1.503$	6	0.999	0.019	3*c*	
Hydroxyindole-*o*-methyltransferase	*N*-Acyltryptamines	$\log 1/I_{50} = 0.561 \log P + 1.590$	24	0.870	0.255	4*a*	
		$\log 1/I_{50} = 0.601 \log P + 1.491$	21	0.948	0.170	4*b*	
Monoamine oxidase	β-Carbolines	$\log 1/I_{50} = 0.675 E_s + 4.017$	11	0.509	0.578	5*a*	
		$\log 1/I_{50} = -0.216 \log P + 4.494$	11	0.355	0.628	5*b*	
		$\log 1/I_{50} = -0.373 (\log P)^2 + 1.907 \log P + 1.864$	11	0.604	0.568	5*c*	2.56 (∞)
		$\log 1/I_{50} = 0.454 E_s - 0.304 (\log P)^2 + 1.564 \log P + 2.283$	11	0.648	0.556	5*d*	2.57 (∞)
		$\log 1/I_{50} = 0.635 E_s + 3.983$	8	0.507	0.649	5*e*	
		$\log 1/I_{50} = -0.215 \log P + 4.550$	8	0.341	0.707	5*f*	
		$\log 1/I_{50} = -0.679 (\log P)^2 + 3.719 \log P - 0.422$	8	0.900	0.360	5*g*	2.74 (2.32–2.98)
		$\log 1/I_{50} = 0.140 E_s - 0.645 (\log P)^2 + 3.544 \log P - 0.227$	8	0.905	0.390	5*h*	2.75 (1.99–3.13)

[a] From *Reference 4*.

correlation is obtained (r = 0.948 for Eq. 4*b*). The authors also explored the role of the electronic parameter. For the 15 molecules with X-Ar as R_2 (Table I), neither the σ of X nor the σ of R_1 gives significant improvement in correlation. Of the three poorly predicted molecules, two may be due to steric hindrance, one with the methyl group as R_3, and the other with the 3,4,5-trimethoxybenzyl group as R_2. The fact that the observed activities of these three compounds are lower than the predicted values supports this argument. The third molecule has the 3,5-dichlorophenyl group as R_2. It is difficult to explain why this is poorly predicted since the one with the 3,4-dichlorophenyl group as R_2 is slightly more active than predicted.

For the inhibition of monoamine oxidase by 9-substituted β-carbolines, Taft's steric constant, E_s, gives somewhat better correlation than log P (Eq. 5*a versus* 5*b*). However, the correlation coefficients are too low to be considered significant. Even when a $(\log P)^2$ term is included, the correlation coefficient is still below 0.70 (Eqs. 5*c* and 5*d*). When three compounds with an alcoholic OH group are excluded, a parabolic equation of log P gives fairly good correlation (Eq. 5*g*). The $(\log P)^2$ term in Eq. 5*g* is significant at the 99-percentile level ($F_{1,5}$ = 18.2; $F_{1,5\ 0.99}$ = 16.3). For the eight compounds without an OH group, neither the linear equation of E_s nor that of log P gives a good correlation (Eqs. 5*e* and 5*f*); the addition of the E_s term to the parabolic equation does not improve the correlation significantly (Eq. 5*h versus* 5*g*, $F_{1,4}$ = 0.21). It is felt that an active function like an OH group may have its own *intrinsic activity* not possessed by the other inert substituents. For example, the H of the OH group might form a hydrogen bond with an atom having unshared electron pair(s). The fact that the activities of the compounds with the OH group are appreciably higher than what are predicted from Eq. 5*g* is in accordance with this explanation. The optimum lipohydrophilic character ($\log P_0$) for the maximum inhibition is derived by setting $(d \log 1/I_{50})/(d \log P) = 0$ (17–19). This is the apex of the parabolic curve. Once this $\log P_0$ is obtained, it may serve as a useful guidepost in designing new inhibitors.

The importance of the hydrophobic interactions for the enzyme inhibition is clearly shown by the good correlations obtained by using log P or π with or without a σ term.

DISCUSSION

From the correlations obtained, it is clear that in the five enzyme systems examined the lipohydrophilic character of the inhibitors plays a very important role in inhibition. The rather nonspecific hydrophobic interactions may be involved in two different ways: (*a*) adsorption and desorption on the macromolecule, since all proteins including enzymes contain 20–45% of amino acids with nonpolar side chains (20), and (*b*) inducing proper fit at the active site or the allosteric site (21) by the association of the nonpolar groups in the presence of water molecules. At present, not enough data are available to differentiate which of these two is more important.

It will be interesting to apply the method used in this study to other systems where drugs exert their activity by enzyme inhibition, such as choline esterase inhibitors and histidine decarboxylase inhibitors.

REFERENCES

(1) J. D. Watson, "Molecular Biology of the Gene," W. A. Benjamin, New York, N. Y., 1965, chap. 4.

(2) G. Némethy, *Angew. Chem.* **6,** 195(1967).

(3) C. Hansch, A. R. Steward, J. Iwasa, and E. Deutsch, *Mol. Pharmacol.*, **1,** 205(1965).

(4) H. Mitsuda, K. Yasumoto, and A. Yamamoto, *Arch. Biochem. Biophys.*, **118,** 664(1967).

(5) Eric J. Lien, "The Role of Hydrophobic Bonding in Fungitoxicity," presented at the Symposium on Fungitoxic Action, the 61st Annual Meeting of American Phytopathological Society, Spokane, Wash., Aug. 1969.

(6) R. Franke, *Mol. Pharmacol.*, **5,** 604(1969).

(7) C. B. C. Boyce and B. V. Milborrow, *Nature*, **208,** 537 (1965).

(8) D. E. Burton, K. Clarkae, and G. W. Gray, *J. Chem. Soc.*, **1964,** 1314.

(9) N. Kakeye, M. Aoki, A. Kamada, and N. Yata, *Chem. Pharm. Bull.*, **17,** 1010(1969).

(10) M. L. Fonda and B. B. Anderson, *J. Biol. Chem.*, **244,** 666 (1969).

(11) B. T. Ho, W. M. McIsaac, and L. W. Tansey, *J. Pharm. Sci.*, **58,** 563(1969).

(12) B. T. Ho, W. M. McIsaac, L. W. Tansey, and K. E. Walker, *ibid.*, **58,** 219(1969).

(13) H. H. Jaffé, *Chem. Rev.*, **53,** 191(1953).

(14) T. Fujita, J. Iwasa, and C. Hansch, *J. Amer. Chem. Soc.*, **86,** 5175(1964).

(15) J. Iwasa, T. Fujita, and C. Hansch, *J. Med. Chem.*, **8,** 150 (1964).

(16) J. E. Leffler and E. Grunwald, "Rates and Equilibria of Organic Reactions," Wiley, New York, N. Y., 1963, p. 228.

(17) C. Hansch, E. J. Lien, and F. Helmer, *Arch. Biochem. Biophys.*, **128,** 319(1968).

(18) E. J. Lien, *Amer. J. Pharm. Educ.*, **33,** 368(1969).

(19) E. J. Lien, *J. Agr. Food Chem.*, **17,** 1265(1969).

(20) W. Kauzmann, "Advance in Protein Chemistry," vol. 1, Academic, New York, N. Y., 1959, p. 1.

(21) D. E. Koshland, Jr., *Cold Spring Harbor Symp. Quant. Biol.*, **28,** 473(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 23, 1969, from the *School of Pharmacy, University of Southern California, University Park, Los Angeles, CA 90007*

Accepted for publication January 12, 1970.

The abstract of this paper was presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

* R. M. and J. L. Converse Fund Fellow.

† NSF Undergraduate Research Participant, Summer 1969, GY-5829.

The authors thank the Computer Science Laboratories, University of Southern California, for computing service.

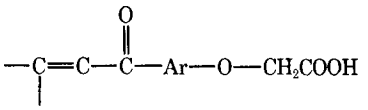
Cyclic Analog of Ethacrynic Acid

M. BRAWNER FLOYD and GEORGE R. ALLEN, Jr.

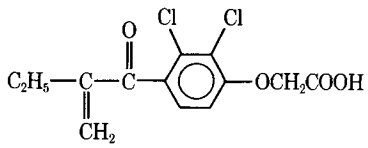
Abstract □ The α,β-unsaturated ketone (4,4-dimethyl-2-ethyl-1[4H]-naphthalenon-6-yl)oxyacetic acid (III), a cyclic analog of ethacrynic acid, has been prepared, and its diuretic activity on oral administration to rats and dogs has been evaluated. No significant activity was observed.

Keyphrases □ Ethacrynic acid cyclic analog—synthesis □ Diuretic activity—ethacrynic acid analog □ UV spectrophotometry—identity

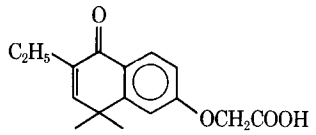
In 1962, Schultz *et al.* (1) disclosed a new class of diuretic agents possessing general structure I. Among the more potent members of this series was ethacrynic acid (II), which has received wide acceptance as a pharmaceutical agent (2). The interesting activity associated with II prompted the authors of this study to explore the preparation of cyclic congeners to assess their effectiveness as diuretic agents; in the present note they describe the preparation of III. Independent of these efforts, Topliss and Konzelman (3) reported recently the preparation and biological properties of the related compounds IV (n = 0,2).



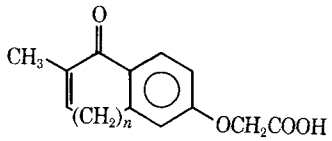
I



II



III

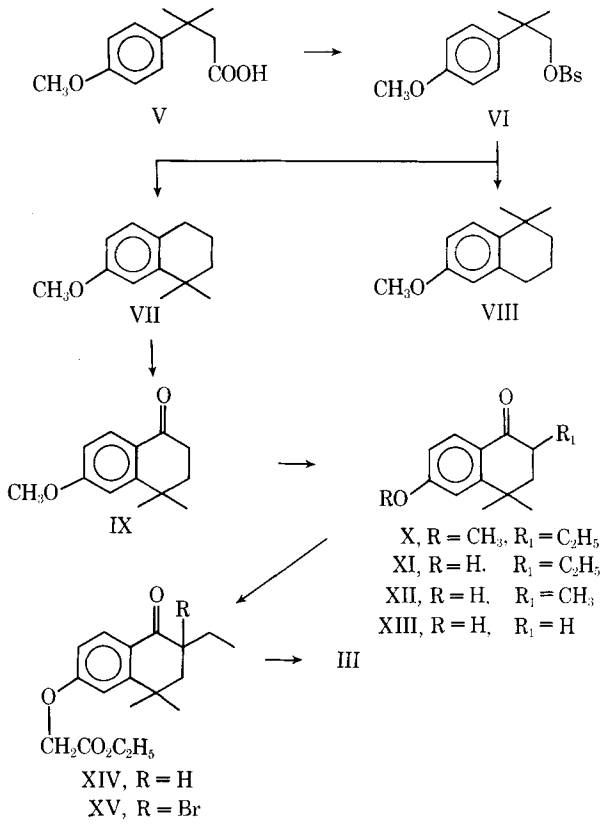


IV

The preparation of III from 4-*p*-anisyl-4-methylpentanoic acid (V) (4) is outlined in Scheme I. Lithium aluminum hydride reduction of V and treatment of the resulting alcohol with *p*-bromobenzenesulfonyl (Bs) chloride afforded the crude brosylate VI. Formolysis of VI has been reported to give the rearranged tetralin VII exclusively in 59% yield along with the formate derived from VI (5). In this study, pyrolysis of VI at 150° gave a 3:2 mixture of tetralins VII and VIII in 74% yield. This result indicates the intervention of a normal ring closure in the absence of solvent. Mild chromic acid oxidation of the tetralin mixture resulted in preferential conversion of VII to tetralone IX. Treatment of IX with methyl magnesium carbonate (6, 7), and ethylation of the resulting magnesium enolate gave the crude 2-ethyltetralone X. Phenol XI was obtained by ether cleavage of the crude alkylation product and chromatographic separation from the phenols XII, derived from methylation of IX, and XIII, derived from unalkylated IX. The genesis of XII may be a consequence of the ability of methyl magnesium carbonate to function as an alkylating agent. Reaction of XI with ethyl bromoacetate and potassium carbonate in refluxing acetone gave the ethyl aryloxyacetate XIV. Finally, bromination of XIV and treatment of the resulting XV with dilute potassium hydroxide effected saponification and dehydrohalogenation to give III.

PHARMACOLOGY

When administered orally to rats at 25 mg./kg., the aryloxyacetic acid III had no significant effect on urinary volume or chloride, sodium, and potassium-ion excretion as determined by the method of Cummings *et al.* (8). Although urine volume was slightly elevated in dogs that had been dosed orally at 5 mg./kg., electrolyte excretion was not increased (9).



X, R = CH_3, R_1 = C_2H_5
XI, R = H, R_1 = C_2H_5
XII, R = H, R_1 = CH_3
XIII, R = H, R_1 = H

XIV, R = H
XV, R = Br

Scheme I

EXPERIMENTAL

General—Melting points are uncorrected and were determined in open capillary tubes on a Mel-Temp apparatus. UV spectra were determined with a Cary recording spectrophotometer in methanol solution.

4-(*p*-Anisyl-4-methylpent-1-yl)-*p*-bromobenzenesulfonate (VI)—To a stirred suspension of lithium aluminum hydride (8.0 g.) in 250 ml. of ether was added a solution of 4-*p*-anisyl-4-methylpentanoic acid (V, 31 g.) in 250 ml. of ether over 1.5 hr. with ice-bath cooling. Following the addition the mixture was refluxed with stirring for 1.5 hr. The crude alcohol (100%) was obtained by hydrolysis with dilute sulfuric acid and solvent removal.

To a stirred solution of the alcohol (27 g.) in 240 ml. of dry pyridine at −15° was added a solution of *p*-bromobenzenesulfonyl chloride (50 g.) in 130 ml. of pyridine over 5 min. The mixture was stirred at 0° for 30 min. and poured into water. The product was extracted with ether; the extracts were washed successively with water, cold 2 *N* HCl, water, saturated $NaHCO_3$, and water. The extract was dried and evaporated to give 53 g. (95%) of crude VI, m.p. 48–55°.

4,4-Dimethyl-6-methoxy-1-tetralone (IX)—The brosylate (51 g.) was heated under argon at 150° for 110 min. The cooled product was treated with saturated $NaHCO_3$ and extracted with light petroleum ether. The solution was washed with water, dried, and evaporated to give an amber liquid which was chromatographed on an alumina column, eluting with hexane and 20:1 hexane–ether. The tetralin fraction so obtained (16.8 g.) analyzed as 60% 1,1-dimethyl-7-methoxytetralin (VII) and 40% isomer, presumably the 6-methoxyisomer VIII, on gas chromatography with a hydrocarbon oil (Apiezon L) column at 200°.

To a solution of the tetralin mixture (16.8 g.) in 85 ml. of glacial acetic acid was added a solution of chromium trioxide (20 g.) in 55 ml. of acetic acid and 10 ml. of water over 70 min. at 15-20° (ice bath). After standing at room temperature for 16 hr., the bulk of the solvent was evaporated; the resulting residue was treated with 5% H_2SO_4. Treatment with ether, filtration through diatomaceous earth (Celite), and further extraction with 1:1 ether–light petroleum ether gave an extract which was washed successively with water, saturated $NaHCO_3$, and water. Distillation of the dried concentrate afforded IX (5.51 g.) as a pale-yellow liquid, b.p. 110–114° (0.14 mm.), λ_{max} 225 mμ (ε11,300); 278 mμ (ε10,900).

Anal.—Calcd. for $C_{13}H_{16}O_2$: C, 76.44; H, 7.90. Found: C, 76.52; H, 7.78.

4,4-Dimethyl-2-ethyl-6-hydroxy-1-tetralone (XI)—A solution of VIII (5.5 g.) in 60 ml. of 2.5 *M* methyl magnesium carbonate in dimethylformamide (7) was heated at 125° for 80 min. After slight cooling, ethyl iodide (42 g.) was added; the resulting mixture was refluxed for 6 hr. The reaction mixture was acidified with 4 *N* HCl and heated on the steam bath for 15 min. The reaction mixture was diluted with water and extracted with ether. The extract was washed with water, dried, and evaporated to give the crude X (6.4 g.) as a dark liquid.

The crude alkylation product (5.6 g.) was added to fused pyridine hydrochloride at 190–200° over 5 min. under argon, and the resulting mixture was stirred for 80 min. The cooled reaction mixture was treated with water and extracted with ether. The extract was washed successively with water, 2 *N* HCl, and water. Phenolic material was extracted into 0.5 *N* NaOH, and the extract was acidified with 4 *N* HCl. Ether extraction afforded a mixture of phenols which were separated by column chromatography on silica gel, eluting with hexane progressively enriched in ethyl acetate. The most mobile component was recrystallized from acetone–hexane, m.p. 128–130°, and consisted of XI (1.76 g.), λ_{max}. 227 mμ (ε11,600); 279 mμ (ε13,400).

Anal.—Calcd. for $C_{14}H_{18}O_2$: C, 77.03; H, 8.31. Found: C, 77.39; H, 8.45.

The methyl derivative XII was recrystallized from acetone–hexane, m.p. 168–173° (50 mg.), λ_{max}. 227 mμ (ε11,900); 276 mμ (ε13,500).

Anal.—Calcd. for $C_{13}H_{16}O_2$: C, 76.44; H, 7.90. Found: C, 76.50; H, 7.99.

Finally, elution gave XIII which was recrystallized from acetone–hexane, m.p. 143–145° (108 mg.), λ_{max}. 228 mμ (ε12,300); 279 mμ (ε14,200).

Anal.—Calcd. for $C_{12}H_{14}O_2$: C, 75.76; H, 7.42. Found: C, 75.73; H, 7.52.

Intermediate fractions contained 800 mg. of an unresolved mixture of XI and XII.

Ethyl (4,4-Dimethyl-2-ethyl-1-tetralon-6-yl)oxyacetate (XIV)—A mixture containing XI (1.73 g.), potassium carbonate (1.22 g.), and ethyl bromoacetate (1.47 g.) in 20 ml. of acetone was refluxed with stirring for 3 hr. The reaction mixture was diluted with water, rendered alkaline with 2.5 *N* NaOH, and extracted with ether. The extract was washed with water, dried, and evaporated to give XIV (2.5 g.) as a pale-yellow oil.

(4,4-Dimethyl-2-ethyl-1[4H]-naphthalenon-6-yl)oxyacetic Acid (III)—To a stirred solution of XIV (2.3 g.) in 35 ml. of chloroform was added a solution of bromine (1.36 g.) in 10 ml. of chloroform over 1.5 hr. at room temperature. After 1 hr. the solution was evaporated to give the crude bromoester XV as an orange oil. This material was dissolved in 300 ml. of methanol containing potassium hydroxide (1.67 g.) and allowed to stand in the dark for 120 hr. The bulk of the methanol was evaporated and the residue was treated with water. Acidification in the cold with 4 *N* HCl and extraction with ether gave a solution of the free acid which was washed with water and dried. Evaporation and recrystallization of the residue from methanol–water gave white crystals of III (1.60 g.), m.p. 149–151°. Another recrystallization gave the analytical sample, m.p. 150–152°, λ_{max}. 238 mμ (ε12,100); 298 mμ (ε10,300).

Anal.—Calcd. for $C_{16}H_{18}O_4$: C, 70.06; H, 6.61. Found: C, 69.80; H, 6.50.

REFERENCES

(1) E. M. Schultz, E. J. Cragoe, Jr., J. B. Bicking, W. A. Bolhofer, and J. M. Sprague, *J. Med. Chem.*, **5,** 660(1962).

(2) W. C. Cutting, "Handbook of Pharmacology," 3rd ed., Appleton-Century-Crofts, New York, N. Y., 1967, p. 192.

(3) J. C. Topliss and L. M. Konzelman, *J. Pharm. Sci.*, **57,** 737 (1968).

(4) S. Mukherjee, *J. Sci. Ind. Res. (India)*, **19B,** 94(1960).

(5) S. Winstein, R. Heck, S. Lapporte, and R. Baird, *Experientia*, **12,** 138(1956).

(6) M. Stiles and H. L. Finkbeiner, *J. Amer. Chem. Soc.*, **85,** 6161(1963).

(7) H. L. Finkbeiner and G. W. Wagner, *J. Org. Chem.*, **28,** 215(1963).

(8) J. L. Cummings, J. D. Haynes, L. M. Lipchuck, and M. A. Ronsberg, *J. Pharmacol. Exp. Ther.*, **128,** 414(1960).

(9) R. Z. Gussin, private communication.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 12, 1969, from the *Process and Preparations Research Section, Lederle Laboratories Division, American Cyanamid Company, Pearl River, NY 10965*

Accepted for publication January 5, 1970.

Preparation and Anticancer Activity of 2-Diethylaminoethyl-*cis*- and -*trans*-methylcyclohexanecarboxylates

ROBERT E. HARMON, PAUL A. MEULMAN, and S. K. GUPTA

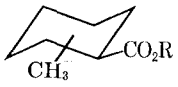
Abstract □ Pure *cis*- and *trans*-methylcyclohexanecarboxylic acids and their 2-diethylaminoethyl esters have been prepared. The latter were isolated as crystalline hydrochloride salts. They were tested for possible anticancer activity.

Keyphrases □ *cis*-, *trans*-Methylcyclohexanecarboxylic acids—synthesis □ 2-Diethylaminoethyl-*cis*-, *trans*-methylcyclohexanecarboxylic esters—synthesis, anticancer activity □ Pharmacological screening—2-diethylaminoethyl-*cis*-, *trans*-methylcyclohexanecarboxylic esters

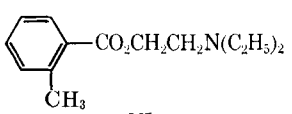
Numerous carboxylic acid esters of tertiary amino alcohols have shown significant pharmacological activity. For instance, the esters of sterically hindered alkyl-substituted benzoic acids have shown anesthetic action (1–5). Similarly, the local anesthetic action of 2-diethylaminoethyl ester hydrochlorides of 1-phenyl and 1-cyclohexanecarboxylic acids is comparable to that of cocaine (6). Investigations of 2-dialkylaminoethyl ester hydrochlorides of various 1-methyl-3-alkyl-cyclohexanecarboxylic acids revealed that these compounds were effective cardiovascular depressants (7). In this paper, the authors report the preparation and anticancer activity of 2-diethylaminoethyl-*cis*- and -*trans*-methylcyclohexanecarboxylate hydrochlorides.

The *cis*- and *trans*-methylcyclohexanecarboxylic acids (I*a*–V*a*) and their 2-diethylaminoethyl esters (I*b*–V*b*), prepared for this investigation, are given in Table I. The three *cis*-methylcyclohexanecarboxylic acids (I*a*, III*a*, and IV*a*) were prepared by the catalytic hydrogenation of the corresponding toluic acids (8, 9). *trans*-2-Methylcyclohexanecarboxylic acid (II*a*) was prepared by heating crotonic acid with 1,3-butadiene in a sealed tube at 175° (10). *trans*-4-Methylcyclohexanecarboxylic acid (V*a*) was prepared by heating the corresponding *cis*-isomer IV*a* in the presence of anhydrous hydrogen chloride (11). However, all attempts to prepare pure *trans*-3-methylcyclohexanecarboxylic acid failed because these procedures gave mixtures of *cis*- and *trans*-isomers which were very difficult to separate. The purity of the acids I*a*–V*a* was established by comparison of boiling points, refractive indexes, and IR spectra with the literature values. The 2-diethylaminoethyl esters of the acids II*a*–V*a* were prepared by the method of Rabjohn (12). This involved heating the potassium salt of the carboxylic acid with 2-chlorotriethylamine in toluene. All attempts to esterify the *cis*-2-methylcyclohexanecarboxylic acid (I*a*) by this method were unsuccessful. Therefore, the acid I*a* was first converted into 2-diethylamino-*o*-toluate (VI). The latter, on catalytic hydrogenation using Adam's catalyst, afforded the 2-diethylaminoethyl-*cis*-2-methylcyclohexanecarboxylate (I*b*). However, all these 2-diethylaminoethyl esters (I*b*–V*b*) were obtained only as oils. Therefore, they were converted into crystalline hydrochloride salts by treating the free esters with hydrogen chloride gas. The hydrochloride salts were characterized by satisfactory elemental analyses and IR and NMR spectroscopy.

Table I—2-Diethylamino-*cis*- and -*trans*-methylcyclohexanecarboxylates



Methyl Group Position	Relative Configuration of the Substituents	Compound
2	*cis*-	I*a*, R = H; I*b*, R = $CH_2CH_2N(C_2H_5)_2$
2	*trans*-	II*a*, R = H; II*b*, R = $CH_2CH_2N(C_2H_5)_2$
3	*cis*-	III*a*, R = H; III*b*, R = $CH_2CH_2N(C_2H_5)_2$
4	*cis*-	IV*a*, R = H; II*b*, R = $CH_2CH_2N(C_2H_5)_2$
4	*trans*-	V*a*, R = H; V*b*, R = $CH_2CH_2N(C_2H_5)_2$



VI

Table II—Anticancer Activity of Compounds I*b*–V*b* and VI

Compd.	Test System[a]	Dose, mg./kg.	Survivors ( ) of ( )		Stage Index
I*b*	SA	125	6	6	0.66
	CA	100	10	10	1.26
	LE	100	6	6	0.94
II*b*	SA	100	6	6	0.93
	CA	200	10	10	0.55
	LE	80	6	6	0.87
	HI	20	6	6	0.50
	HI	20	5	6	0.25
III*b*	SA	250	6	6	0.71
	CA	200	8	10	0.93
	LE	200	6	6	0.88
IV*b*	SA	250	6	6	1.10
	CA	200	10	10	0.53
	CA	200	9	10	0.71
	LE	200	6	6	0.93
V*b*	SA	250	6	6	0.67
	CA	200	10	10	0.83
	LE	200	6	6	0.93
VI	SA	250	6	6	0.96
	CA	200	10	10	1.00
	LE	200	6	6	0.88

[a] SA = sarcoma-180; CA = adenocarcinoma-755; LE = L-1210 lymphoid leukemia; and HI = HS1 human sarcoma (rat, egg).

ANTICANCER ACTIVITY

All the 2-diethylaminoethyl-methylcyclohexanecarboxylates described in this paper were evaluated in the routine mouse tumor

Table III—2-Diethylaminoethyl-methylcyclohexanecarboxylates

Compd.[a]	B.p. (mm. Hg.)	Ref. Index, n_D^{25}	Yield, %	Hydrochloride salt, m.p.	Anal., % Calcd. C	Calcd. H	Found C	Found H
I*b*	116–118° (1.00)	1.4601	70	120–120.5°	60.52	10.16	60.62	10.03
II*b*	114–115° (2.4–2.6)	1.4542	85	117–117.5°	60.52	10.16	60.30	9.96
III*b*	114–114.5° (1.5–1.7)	1.4542	81	100–101°	60.52	10.16	60.59	10.21
IV*b*	115–116° (1.8–1.9)	1.4551	46	123.5–124.5°	60.52	10.16	60.61	10.26
V*b*	118–119° (2.2–2.4)	1.4533	73	154–155°	60.52	10.16	60.76	10.16

[a] All the compounds I*b*–V*b* had empirical formula $C_{14}H_{28}ClNO_2$.

screening of the Cancer Chemotherapy National Service Center, Bethesda, Md. (13). The results are mentioned in Table II. None of the compounds prepared in this investigation showed confirmed anticancer activity. Hence, no correlation could be drawn between an equatorial–equatorial or an axial–equatorial arrangement of the methyl and the diethylaminoethoxycarbonyl groups on the cyclohexane ring of a compound and the anticancer activity of that compound.

EXPERIMENTAL

The melting points were determined with a Thomas-Hoover Unimelt apparatus and are uncorrected. Microanalyses were performed by Galbraith Laboratories Inc., Knoxville, Tenn. The IR spectra were recorded on a Beckman IR-8 spectrophotometer. The NMR spectra were obtained with a Varian A-60 spectrometer.

Preparation of Methylcyclohexanecarboxylic Acids I*a*–V*a*—*cis*-2-Methylcyclohexanecarboxylic acid (I*a*) was prepared by catalytic hydrogenation of *o*-toluic acid by the procedure of MacBeth *et al.* (9) and Cope *et al.* (8). It had b.p. 130–130.5° (10 mm.), n_D^{23} 1.4631 [lit. (9) b.p. 119° (11 mm.), n_D^{20} 1.4644].

trans-2-Methylcyclohexanecarboxylic acid (II*a*) was prepared by heating crotonic acid with 1,3-butadiene in a pressure vessel by the procedure of Diels and Alder (10). It had m.p. 51–52.5° [lit. (14) m.p. 52°].

cis-3-Methylcyclohexanecarboxylic acid (III*a*) was prepared by the catalytic hydrogenation of *m*-toluic acid by the procedure of Darling *et al.* (15). It had b.p. 98–99° (1.3–1.8 mm.), n_D^{25} 1.4561 [lit. (15) b.p. 98–99° (1.2 mm.), n_D^{20} 1.4570].

cis-4-Methylcyclohexanecarboxylic acid (IV*a*) was prepared by the hydrogenation of *p*-toluic acid by the procedure of Delephine and Badoche (11). It had b.p. 96.5–98° (1.25–1.70 mm.), n_D^{25} 1.4581 [lit. (11) b.p. 128–130° (13 mm.), n_D^{16} 1.4605].

trans-4-Methylcyclohexanecarboxylic acid (V*a*) was prepared by heating IV*a* in the presence of hydrogen chloride gas by the procedure of Delephine and Badoche (11). It had m.p. 108–110° [lit. (11) m.p. 111°].

Preparation of 2-Diethylaminoethyl Esters—*2-Diethylaminoethyl-cis-2-methylcyclohexanecarboxylate* (*I*b)—To a solution of *o*-toluoyl chloride (23.8 g., 0.153 mole) in anhydrous ether (50 ml.) was added in small portions, with shaking and cooling, freshly distilled 2-diethylaminoethanol (0.380 mole). The reaction mixture was allowed to stand at room temperature overnight. A saturated solution of sodium carbonate (100 ml.) and ether (50 ml.) was added to the solution. The ether layer was separated and the aqueous layer extracted with ether twice. The combined ether extracts were washed with water, dried (Na_2SO_4), and evaporated to yield an oil. Fractional distillation of the oil afforded 31.0 g. (75%) of 2-diethylaminoethyl-*o*-toluate (VI) as a colorless oil, b.p. 133–135.5° (1.9–2.1 mm.), n_D^{25} 1.5021. A solution of VI (9.8 g., 0.042 mole) in glacial acetic acid (60 ml.) was mixed with platinum oxide (0.31 g.) and hydrogenated at 60 p.s.i. pressure of hydrogen gas. The temperature was maintained at 90° for 5 hr. and at room temperature for 16 hr. The catalyst was removed by filtration and the filtrate evaporated under reduced pressure (100 mm.) to get rid of all the acetic acid. Fractional distillation of the residual oil afforded 7.0 g. (70%) of 2-diethylaminoethyl-*cis*-2-methylcyclohexanecarboxylate (I*b*) as a colorless oil, b.p. 116–118° (1 mm.), n_D^{22} 1.4601.

For the sake of characterization, a small amount of I*b* was converted into its crystalline hydrochloride salt by adding dropwise an ethereal solution of hydrogen chloride to an ethereal solution of I*b* until the solution became acidic. The precipitated hydrochloride salt was filtered and recrystallized from absolute ethanol–ether. It had m.p. 120–120.5°.

General Procedure for the Preparation of Esters II*b*–V*b*—Esterification of the methylcyclohexanecarboxylates II*a*–V*a* was carried out by the method of Rabjohn (12). This procedure involved heating the potassium salt of the carboxylic acid with 2-chlorotriethylamine hydrochloride in toluene during 25–30 hr. Filtration (to remove the precipitated KCl), removal of toluene by evaporation under reduced pressure, and fractional distillation afforded the esters II*b*–V*b* in 70–90% yields. The boiling points, refractive indexes, and yields of the esters II*b*–V*b* are given in Table III. All the esters were converted into crystalline hydrochloride salts by bubbling anhydrous hydrogen chloride gas into an ethereal solution of the ester until the solution turned acidic. The precipitated hydrochloride salts were filtered and recrystallized from absolute ethanol–ether. The melting points and analyses are also given in Table III.

REFERENCES

(1) J. T. Bryan and P. A. Foote, *J. Amer. Pharm. Ass., Sci. Ed.*, **39**, 644(1950).

(2) J. T. Bryan, W. M. Lauter, and P. A. Foote, *ibid.*, **42**, 437 (1953).

(3) L. B. Dale, Jr., and E. Voss, *ibid.*, **42**, 685(1953).

(4) M. B. Moore, *ibid.*, **40**, 388(1951).

(5) N. Rabjohn, J. W. Franabarger, and W. W. Lindstronberg, *J. Org. Chem.*, **20**, 271(1955).

(6) J. Levy and B. Tchoubar, *C. R. Soc. Biol.*, **141**, 257(1947); through *Chem. Abstr.*, **42**, 1351(1947).

(7) M. S. Ziegler and R. M. Herbst, *J. Org. Chem.*, **16**, 920 (1951).

(8) A. C. Cope, A. Fournier, Jr., and H. E. Simmons, Jr., *J. Amer. Chem. Soc.*, **79**, 3905(1957).

(9) A. K. MacBeth, J. A. Mills, and D. H. Simmonds, *J. Chem. Soc.*, **1949**, 1011.

(10) O. Diels and K. Alder, *Ann.*, **470**, 90(1929).

(11) M. Delephine and M. Badoche, *Ann. Chim.*, **17**, 179(1942); through *Chem. Abstr.*, **37**, 5951[4](1943).

(12) N. Rabjohn, U. S. Pat. 2,831,016(1958).

(13) "Protocols for Screening Clinical Agents and Natural Products against Animal Tumors and other Biological Systems," *Cancer Chemother. Rept.*, **25**, 1(1962).

(14) A. Skita, H. Hauber, and R. Schonfelder, *Ann.*, **431**, 1 (1923).

(15) L. H. Darling, A. K. MacBeth, and J. A. Mills, *J. Chem. Soc.*, **1953**, 1364.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 22, 1969, from the *Department of Chemistry, Western Michigan University, Kalamazoo, MI 49001*

Accepted for publication January 19, 1970.

Abstracted in part from a thesis submitted by P. A. Meulman to Western Michigan University in partial fulfillment of Master of Arts degree requirements.

This work was supported by Grant No. CY. 4075-C_2 from the National Institutes of Health.

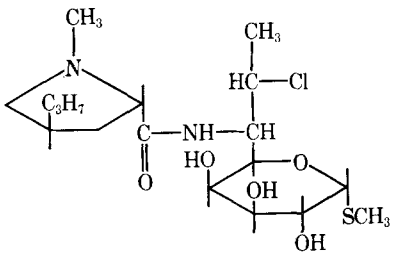
Absorption of Clindamycin from the Buccal Cavity

MILDRED J. TARASZKA

Abstract □ Clindamycin, which is known to be absorbed from the gastrointestinal tract, was absorbed extremely slowly, or possibly not at all, from the buccal cavity at various pH values. This finding indicates that buccal absorption alone cannot be used to predict the gastrointestinal absorption of a compound.

Keyphrases □ Clindamycin—buccal absorption □ Drug absorption, buccal—clindamycin □ GLC—analysis

Recently, several investigators (1–3) have proposed the method of buccal absorption as an example of an *in vivo* model of drug partitioning into, or passive drug transfer through, a lipid membrane. They also proposed the buccal absorption test for predicting the relative absorption and excretion of compounds in biological systems. Since clindamycin (I)[1] is well absorbed from the human gastrointestinal tract (4–6), it was of interest to determine the pH profile for buccal absorption of clindamycin. Clindamycin is an antibiotic which is synthetically derived from lincomycin (7, 8).



clindamycin

I

EXPERIMENTAL

Absorption Studies—The buccal absorption procedure was essentially the same as that used by Beckett and Triggs (1). The subjects were instructed to hold 25 ml. of buffer containing 1 mg. of the base form of the compound in their mouths for a given time interval. During this interval they kept the solution in constant movement with tongue-and-cheek action. At the end of the time interval, the solution was expelled into a 60-ml. Teflon-stoppered, separatory funnel. Immediately the mouth was given a 10-sec. rinse with 10 ml. of deionized water. The water rinse was combined with the previously expelled solution. The amount of compound unabsorbed was measured by extraction and GLC.

Standards were prepared by having the subjects hold plain buffer in their mouths. Various amounts of compound were then added to the expelled solution in the separatory funnels and used as standards.

To ensure that the buccal-absorption methodology was in agreement with that used in the literature, a 1-mg. sample of benzphetamine base in 25 ml. of pH 8.5 phosphate buffer was tested. In two separate experiments, 76 and 79% of the benzphetamine samples were buccally absorbed in 5 min.; these results are in agreement with the data presented by Beckett and Triggs (1).

Assay for Clindamycin—The pH of the buffer solution in the separatory funnel was adjusted to pH 11 with NaOH, and 2 ml. of a water-saturated chloroform solution containing 0.3 mg./ml. of cholesteryl acetate as an internal standard was added. The separatory funnel was shaken vigorously for 1.5 min., and the cloudy chloroform layer collected in a centrifuge tube. The tubes were centrifuged at 10° for 10–15 min. at 12,000 r.p.m. to break the suspension. Approximately 1 ml. of the chloroform layer was transferred to a 2-ml. volumetric flask and evaporated to dryness under a dry stream of air. Then 0.4 ml. of hexamethyldisilazane was added and the flask was shaken to dissolve the residue. One-tenth milliliter of trifluoroacetic acid was added, and the flask was allowed to stand for 1 hr. for complete reaction. Gas chromatography of clindamycin *tris*-trimethylsilyl ether was carried out using glass columns 50.80 cm. × 1.27 cm. (20 in. × 0.5 in.) packed with Gas Chrom Q, 80–100 mesh, and coated with 1% OV-1. The oven temperature was 210° and the carrier gas (He) flow was 60 ml./min.

Assay for Benzphetamine—The assay for benzphetamine is similar to that for clindamycin, except 3 ml. of chloroform solution containing 0.75 μl. nicotine as an internal standard was added to the separatory funnel. The collected chloroform layer was gas chromatographed using glass columns packed with Diaport S, 60–80 mesh, and coated with 6% LAC-728. The oven temperature was 180° and the carrier gas (He) flow was 50 ml./min.

Ionization Constant for Clindamycin—Twenty-milliliter samples of clindamycin (1.5×10^{-3} *M*) were titrated with 0.1 *M* KOH in a radiometer type SBR2/SBU1 titrigraph. A pKa of 7.72 ± 0.04 ($\bar{X} \pm \sigma$) was evaluated from the continuously recorded titration curves at 25°.

Table I—Standard Samples

Weight of Clindamycin, mg.	Peak Area Ratio Clindamycin–Internal Standard
1.0	1.080
0.9	0.898
0.6	0.606
0.4	0.395
0.2	0.224
0.1	0.092

RESULTS AND DISCUSSION

A typical gas chromatogram of clindamycin from buffered saliva solutions is shown in Fig. 1. An impurity extracted from saliva is present (Peak 4 in Fig. 1) but does not interfere with the assay. The 4′-ethyl analog of lincomycin (Peak 2 in Fig. 1) is an antibiotic produced at about the 3% level in the microbiological synthesis of lincomycin (9). Since clindamycin is synthesized from lincomycin, the 4′-ethyl analog of clindamycin was present in small amounts in the clindamycin samples. The total area under Peaks 1, 2, and 3

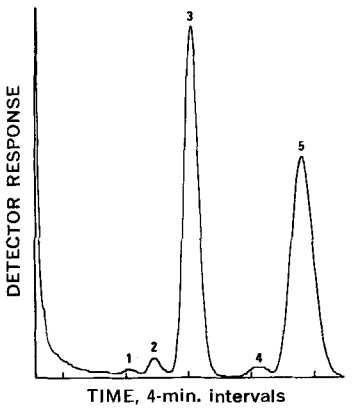


Figure 1—*Typical gas chromatogram of a silanized clindamycin buccal solution. Key: Peak 1, column-thermal degradation product of clindamycin; Peak 2, 4′-ethyl analog of clindamycin; Peak 3, clindamycin; Peak 4, saliva impurity; and Peak 5, internal standard cholesteryl acetate.*

[1] Cleocin, The Upjohn Co., Kalamazoo, Mich.

Table II—Absorption of Clindamycin from the Buccal Cavity at Various pH Values and Time Intervals

Subject	Buffer	pH	Time Interval in Mouth, min.	Absorbed Clindamycin, %
1	Citrate	4.0	5	0
1	Phosphate	7.5	5	2
1	Phosphate	8.5	5	0
1	Phosphate	8.5	15	12
1	Deionized H_2O	6.9[a]	15	6
2	Phosphate	8.5	5	4

[a] pH at end of absorption experiment.

was used to calculate clindamycin in the assay. Table I gives the peak area ratios of clindamycin–internal standard for various known amounts of clindamycin and is typical of the GLC quantitation.

The results of buccal absorption of clindamycin for two subjects are presented in Table II. One milligram of clindamycin base per 25 ml. solution was used for each experiment. Within experimental error, no clindamycin was absorbed buccally in 5 min. at pH 4, 7.5, or 8.5. A small amount of clindamycin may have been absorbed after 15 min. at pH 8.5, or this indicated absorption may have been due to the swallowing of a portion of the solution during the longer time interval. In contrast to the poor buccal absorption, clindamycin is well absorbed from the gastrointestinal tract (4–6).

The data of Bickel and Weder (3) indicated that at pH 7.4 the buccal absorption of imipramine and similar compounds could be related to lipid solubility as measured by partition values. At this pH, imipramine with an apparent partition coefficient (diethylether–water) of 140 was absorbed to the extent of approximately 60%. The true partition coefficient,[2] k, for clindamycin between diethylether and water is 9.8 at 25° (10). The apparent partition coefficient of clindamycin at pH 7.4 was calculated (11) to be 3 from the above k and the pKa. This indicates that the lower lipid solubility of clindamycin relative to imipramine could partially account for the poor buccal absorption of clindamycin. However, it does not explain the difference between the poor buccal absorption and the excellent gastrointestinal absorption of clindamycin. This difference in absorption may be due to the differences in surface area, transport mechanisms, and/or mucous membrane pH between the buccal cavity and the gastrointestinal tract.

[2] The true partition coefficient, k, equals the concentration of unionized species in the organic phase per the concentration of the unionized species in the aqueous phase.

REFERENCES

(1) A. H. Beckett and E. J. Triggs, *J. Pharm. Pharmacol.*, **19,** Suppl. 31S(1967).

(2) A. H. Beckett and A. C. Moffat, *ibid.*, **20,** Suppl. 239S (1968).

(3) M. H. Bickel and H. J. Weder, *ibid.*, **21,** 160(1969).

(4) J. G. Wagner, E. Novak, N. C. Patel, C. G. Chidester, and W. L. Lummis, *Amer. J. Med. Sci.*, **256,** 25(1968).

(5) R. F. McGehee, Jr., C. P. Smith, C. Wilcox, and M. Finland, *ibid.*, **256,** 279(1968).

(6) E. Novak, J. G. Wagner, and D. J. Lamb, to be published.

(7) B. J. Magerlein, R. D. Birkenmeyer, and F. Kagan, *Antimicrob. Ag. Chemother.*, **1966,** 727(1967).

(8) H. Hoekema, B. Bannister, R. D. Birkenmeyer, F. Kagan, B. J. Magerlein, F. A. MacKellar, W. Schroeder, G. Slomp, and R. R. Herr, *J. Amer. Chem. Soc.*, **86,** 4223(1964).

(9) A. D. Argoudelis, J. A. Fox, and T. E. Eble, *Biochemistry*, **4,** 698(1965).

(10) W. Morozowich, to be published.

(11) A. N. Martin, "Physical Pharmacy," Lea and Febiger, Philadelphia, Pa., 1960, pp. 380–388.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 29, 1969, from the *Pharmacy Research Unit, The Upjohn Co., Kalamazoo, MI 49001*

Accepted for publication January 27, 1970.

The author expresses her appreciation to Mr. William Woltersom for his assistance with the assays.

Effect of Polysorbate 80 and Oleic Acid on Drug Absorption from the Rat Intestine

GERHARD LEVY and AINO PERÄLÄ*

Abstract □ Low concentrations of polysorbate 80 and oleic acid, which enhance drug absorption across the external membranes of goldfish, have no apparent effect on the absorption of salicylate, salicylamide, and 4-aminoantipyrine from the *in situ* rat small intestine.

Keyphrases □ Polysorbate 80, oleic acid—drug absorption rate, effects, rat intestine □ Absorption rate, rat intestine—polysorbate 80, oleic acid, effects □ Drugs, absorption—polysorbate 80, oleic acid, effects, rat intestine □ Oleic acid, polysorbate 80—drug absorption rate, rat intestine, effects

Polysorbate 80 and oleic acid enhance the rate of drug absorption by goldfish immersed in drug solutions containing low concentrations of one of these substances (1–4). The purpose of this study was to determine if polysorbate 80 and oleic acid can also increase the absorption rate of certain drugs from the small intestine of the rat.

EXPERIMENTAL

Drug absorption was studied by the *in situ* rat gut technique of Doluisio *et al.* (5) with the following modifications: (*a*) Sprague-Dawley rats (weighing approximately 220 g.) were anesthetized with 1.5 mg. urethan/g. body weight (rather than 1 mg./g.); (*b*) the animals were hydrated immediately after urethan administration by an intraperitoneal injection of 5 ml. normal saline solution; (*c*) the gut was first rinsed with the perfusion solution (5) and then with the drug solution; and (*d*) 7 ml. (rather than 10 ml.) of the drug solution was placed in the intestine for the absorption experiment.

The drug solutions contained 200 or 400 mg.% salicylic acid, 150 or 300 mg.% salicylamide, or 50 or 100 mg.% 4-aminoantipyrine in Sorensen's buffer of pH 6.0. Solutions containing the low concentra-

Table II—Absorption of Clindamycin from the Buccal Cavity at Various pH Values and Time Intervals

Subject	Buffer	pH	Time Interval in Mouth, min.	Absorbed Clindamycin, %
1	Citrate	4.0	5	0
1	Phosphate	7.5	5	2
1	Phosphate	8.5	5	0
1	Phosphate	8.5	15	12
1	Deionized H_2O	6.9[a]	15	6
2	Phosphate	8.5	5	4

[a] pH at end of absorption experiment.

was used to calculate clindamycin in the assay. Table I gives the peak area ratios of clindamycin–internal standard for various known amounts of clindamycin and is typical of the GLC quantitation.

The results of buccal absorption of clindamycin for two subjects are presented in Table II. One milligram of clindamycin base per 25 ml. solution was used for each experiment. Within experimental error, no clindamycin was absorbed buccally in 5 min. at pH 4, 7.5, or 8.5. A small amount of clindamycin may have been absorbed after 15 min. at pH 8.5, or this indicated absorption may have been due to the swallowing of a portion of the solution during the longer time interval. In contrast to the poor buccal absorption, clindamycin is well absorbed from the gastrointestinal tract (4–6).

The data of Bickel and Weder (3) indicated that at pH 7.4 the buccal absorption of imipramine and similar compounds could be related to lipid solubility as measured by partition values. At this pH, imipramine with an apparent partition coefficient (diethylether–water) of 140 was absorbed to the extent of approximately 60%. The true partition coefficient,[2] k, for clindamycin between diethylether and water is 9.8 at 25° (10). The apparent partition coefficient of clindamycin at pH 7.4 was calculated (11) to be 3 from the above k and the pKa. This indicates that the lower lipid solubility of clindamycin relative to imipramine could partially account for the poor buccal absorption of clindamycin. However, it does not explain the difference between the poor buccal absorption and the excellent gastrointestinal absorption of clindamycin. This difference in absorption may be due to the differences in surface area, transport mechanisms, and/or mucous membrane pH between the buccal cavity and the gastrointestinal tract.

[2] The true partition coefficient, k, equals the concentration of unionized species in the organic phase per the concentration of the unionized species in the aqueous phase.

REFERENCES

(1) A. H. Beckett and E. J. Triggs, *J. Pharm. Pharmacol.*, **19,** Suppl. 31S(1967).

(2) A. H. Beckett and A. C. Moffat, *ibid.*, **20,** Suppl. 239S (1968).

(3) M. H. Bickel and H. J. Weder, *ibid.*, **21,** 160(1969).

(4) J. G. Wagner, E. Novak, N. C. Patel, C. G. Chidester, and W. L. Lummis, *Amer. J. Med. Sci.*, **256,** 25(1968).

(5) R. F. McGehee, Jr., C. P. Smith, C. Wilcox, and M. Finland, *ibid.*, **256,** 279(1968).

(6) E. Novak, J. G. Wagner, and D. J. Lamb, to be published.

(7) B. J. Magerlein, R. D. Birkenmeyer, and F. Kagan, *Antimicrob. Ag. Chemother.*, **1966,** 727(1967).

(8) H. Hoekema, B. Bannister, R. D. Birkenmeyer, F. Kagan, B. J. Magerlein, F. A. MacKellar, W. Schroeder, G. Slomp, and R. R. Herr, *J. Amer. Chem. Soc.*, **86,** 4223(1964).

(9) A. D. Argoudelis, J. A. Fox, and T. E. Eble, *Biochemistry*, **4,** 698(1965).

(10) W. Morozowich, to be published.

(11) A. N. Martin, "Physical Pharmacy," Lea and Febiger, Philadelphia, Pa., 1960, pp. 380–388.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 29, 1969, from the *Pharmacy Research Unit, The Upjohn Co., Kalamazoo, MI 49001*

Accepted for publication January 27, 1970.

The author expresses her appreciation to Mr. William Woltersom for his assistance with the assays.

Effect of Polysorbate 80 and Oleic Acid on Drug Absorption from the Rat Intestine

GERHARD LEVY and AINO PERÄLÄ*

Abstract □ Low concentrations of polysorbate 80 and oleic acid, which enhance drug absorption across the external membranes of goldfish, have no apparent effect on the absorption of salicylate, salicylamide, and 4-aminoantipyrine from the *in situ* rat small intestine.

Keyphrases □ Polysorbate 80, oleic acid—drug absorption rate, effects, rat intestine □ Absorption rate, rat intestine—polysorbate 80, oleic acid, effects □ Drugs, absorption—polysorbate 80, oleic acid, effects, rat intestine □ Oleic acid, polysorbate 80—drug absorption rate, rat intestine, effects

Polysorbate 80 and oleic acid enhance the rate of drug absorption by goldfish immersed in drug solutions containing low concentrations of one of these substances (1–4). The purpose of this study was to determine if polysorbate 80 and oleic acid can also increase the absorption rate of certain drugs from the small intestine of the rat.

EXPERIMENTAL

Drug absorption was studied by the *in situ* rat gut technique of Doluisio *et al.* (5) with the following modifications: (*a*) Sprague-Dawley rats (weighing approximately 220 g.) were anesthetized with 1.5 mg. urethan/g. body weight (rather than 1 mg./g.); (*b*) the animals were hydrated immediately after urethan administration by an intraperitoneal injection of 5 ml. normal saline solution; (*c*) the gut was first rinsed with the perfusion solution (5) and then with the drug solution; and (*d*) 7 ml. (rather than 10 ml.) of the drug solution was placed in the intestine for the absorption experiment.

The drug solutions contained 200 or 400 mg.% salicylic acid, 150 or 300 mg.% salicylamide, or 50 or 100 mg.% 4-aminoantipyrine in Sorensen's buffer of pH 6.0. Solutions containing the low concentra-

Table I—Effect of Polysorbate 80 and Oleic Acid on the Intestinal Absorption of Salicylate, Salicylamide, and 4-Aminoantipyrine in Rats

Drug	Concentrations Used, mg./100 ml.	Mean[a] Absorption Half-Life, min. (*SD*)			
		High Conc.	Low Conc.	With Polysorbate 80[b]	With Oleic Acid[c]
Salicylic acid	200; 400	8.2 (0.7)	8.9 (1.7)	8.5 (1.5)	8.6 (2.4)
Salicylamide	150; 300	9.5 (2.9)	9.2 (2.0)	9.5 (2.5)	8.7 (0.7)
4-Aminoantipyrine	50; 100	12.9 (1.4)	13.4 (1.6)	14.0 (0.8)	15.3 (1.9)

[a] Mean of 3 to 5 experiments. [b] The solutions contained the low drug concentration and 0.03% polysorbate 80. [c] The solutions contained the low drug concentration and 0.1% oleic acid.

tions of drug and 0.03% polysorbate 80 or 0.1% oleic acid were also prepared. The concentrations of salicylic acid and salicylamide were determined in suitably diluted and acidified samples by the method of Trinder (6). The concentration of 4-aminoantipyrine was determined by the method of Brun (7), as modified by Levy and Miller (8).

RESULTS AND DISCUSSION

The results of the absorption experiments are summarized in Table I. In agreement with Doluisio *et al.* (5), it was found that the concentration of salicylic acid in the intestinal solution decreased exponentially for at least two half-lives. The absorption half-life of salicylic acid was 8–9 min. in this study, compared to 8 min. reported by Doluisio *et al.* Contrary to the observations of those workers, the authors of this study noted considerable (≃50%) net absorption of water, which was reduced somewhat by hydrating the animals and reducing the volume of the solution in the intestine from 10 to 7 ml.[1]

The absorption of salicylamide from the rat intestine was somewhat slower than that of salicylate (even though the former was essentially nonionized while the latter was almost fully ionized), and the absorption of 4-aminoantipyrine was even slower. Polysorbate 80 and oleic acid had no apparent effect on the absorption of these three drugs, on water absorption, and on the gross appearance of the small intestine. The concentration of oleic acid used in this study was slightly in excess of solubility in order to maintain a reasonably constant concentration during the experiment.

The lack of effect of polysorbate 80 and oleic acid on drug absorption from the small intestine of the rat is in contrast to the pronounced absorption-enhancing effect of similar low concentrations of these substances in goldfish (1–4). The absorption and exsorption rate constants of 4-aminoantipyrine in goldfish are increased almost twofold by 0.01% polysorbate 80 (3). The absorption of barbiturates by goldfish is enhanced appreciably by polysorbate 80 and oleic acid in low concentrations (1, 2, 4). However, while the external membranes of goldfish and the rat intestine have the characteristics of a lipoid barrier and yield qualitatively similar (*i.e.*, the same rank order) results in their respective permeability to a series of drugs with different lipoid–water partition coefficients (9), the two types of membranes do differ in their response to certain additives. Thus, the goldfish membranes are exquisitely sensitive to such substances as sodium lauryl sulfate and salicylic acid (unpublished observations), and become more permeable when exposed to polysorbate 80 and certain other surface-active substances (1–4). On the other hand, the *in situ* rat small intestine appears to be much more resistant to these substances.

[1] In more recent studies in this laboratory, the volume of intestinal solution is maintained essentially constant by adding drug-free solvent at each sampling time. Absorption rate constants obtained under these conditions are somewhat smaller than the rate constants obtained by the method used in this study.

REFERENCES

(1) G. Levy, K. E. Miller, and R. H. Reuning, *J. Pharm. Sci.*, **55**, 394(1966).

(2) G. Levy and J. A. Anello, *ibid.*, **57**, 101(1968).

(3) J. A. Anello and G. Levy, *ibid.*, **58**, 721(1969).

(4) G. Levy and J. A. Anello, *ibid.*, **58**, 494(1969).

(5) J. T. Doluisio, N. F. Billups, L. W. Dittert, E. T. Sugita, and J. V. Swintosky, *ibid.*, **58**, 1196 (1969).

(6) P. Trinder, *Biochem. J.*, **51**, 301(1954).

(7) C. Brun, *J. Lab. Clin. Med.*, **37**, 955(1951).

(8) G. Levy and K. E. Miller, *J. Pharm. Sci.*, **54**, 1319(1965).

(9) *Ibid.*, **53**, 1301(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 22, 1969, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication January 19, 1970.

* Present address: School of Pharmacy, University of Helsinki, Finland.

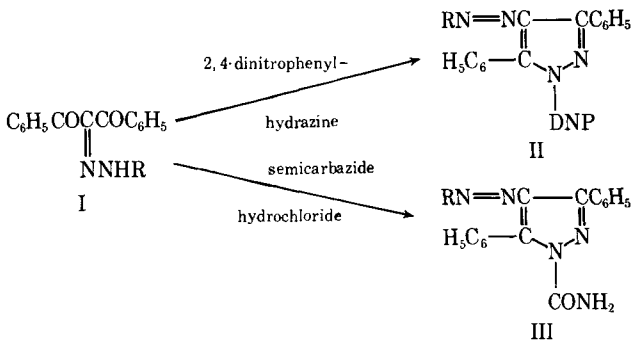
New Compounds: Potential Antidiabetics IV: 1-(2,4-Dinitrophenyl)-3,5-diphenyl-4-arylazopyrazoles and 1-Carbamoyl-3,5-diphenyl-4-arylazopyrazoles

H. G. GARG and PREM PAL SINGH

Abstract □ A series of 1-(2,4-dinitrophenyl)-3,5-diphenyl-4-arylazopyrazoles and 1-carbamoyl-3,5-diphenyl-4-arylazopyrazoles have been synthesised by the condensation of the corresponding 1,3-diphenyl-2-arylhydrazono-1,2,3-propanetriones with 2,4-dinitrophenylhydrazine and semicarbazide hydrochloride, respectively.

Keyphrases □ Antidiabetics—synthesis, pyrazoles, isoxazoles □ 1-(2,4-Dinitrophenyl)-3,5-diphenyl-4-arylazopyrazoles—synthesis, potential hypoglycemics □ 1-Carbamoyl-3,5-diphenyl-4-arylazopyrazoles—synthesis, potential hypoglycemics

Numerous derivatives of pyrazoles and isoxazoles have been prepared for testing their antidiabetic activity (1–4). The compounds 1-phenyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-ones have shown encouraging antidiabetic activity in experimental animals (5). To examine their hypoglycemic activity, a series of 1-(2,4-dinitrophenyl)-3,5-diphenyl-4-arylazopyrazoles and 1-carbamoyl-3,5-diphenyl-4-arylazopyrazoles have been prepared and are reported in the present communication.



R = substituted phenyl
DNP = 2,4-dinitrophenyl

Scheme I

Precursors 1,3-diphenyl-2-arylhydrazono-1,2,3-propanetriones (I) were obtained by coupling diazotized anilines with 1,3-diphenyl-1,3-propanedione (4). 2,4-Dinitrophenylhydrazine and semicarbazide hydrochlo-

Table I—Characteristics of 1-(2,4-Dinitrophenyl)-3,5-diphenyl-4-arylazopyrazoles

No.	R	Yield, %	M.p.	Color	Formula	Anal., % Calcd.	Anal., % Found
1	2-Nitrophenyl	55	208–209°	Red crystals	$C_{27}H_{17}N_7O_6$	N, 18.3	N, 18.0
2	3-Nitrophenyl	65	196–197°	Red crystals	$C_{27}H_{17}N_7O_6$	N, 18.3	N, 18.1
3	3-Chlorophenyl	60	203°	Orange	$C_{27}H_{17}ClN_6O_4$	C, 61.8 H, 3.2 N, 15.4	C, 61.6 H, 3.4 N, 15.0
4	4-Chlorophenyl	75	186°	Orange needles	$C_{27}H_{17}ClN_6O_4$	N, 15.4	N, 15.3
5	2-Methylphenyl	70	228–229°	Orange needles	$C_{28}H_{20}N_6O_4$	N, 16.6	N, 16.4
6	3-Methylphenyl	55	198–199°	Yellow-orange	$C_{28}H_{20}N_6O_4$	N, 16.6	N, 16.3
7	4-Methylphenyl	50	163°	Red crystals	$C_{28}H_{20}N_6O_4$	C, 66.7 H, 4.0 N, 16.6	C, 66.6 H, 4.1 N, 16.1
8	2-Methoxyphenyl	50	233°	Orange-red needles	$C_{28}H_{20}N_6O_5$	N, 16.1	N, 16.0
9	2-Ethoxyphenyl	60	221–222°	Orange needles	$C_{29}H_{22}N_6O_5$	N, 15.7	N, 15.6
10	4-Ethoxyphenyl	65	132°	Pale-yellow needles	$C_{29}H_{22}N_6O_5$	N, 15.7	N, 15.4
11	4-Sulfamoylphenyl	55	132–133°	Orange	$C_{27}H_{19}N_7O_6S$	N, 17.2	N, 17.0
12	2,5-Dichlorophenyl	75	233°	Orange	$C_{27}H_{16}Cl_2N_6O_4$	Cl, 12.7	Cl, 12.4
13	2,5-Dimethylphenyl	60	238–239°	Orange needles	$C_{29}H_{22}N_6O_4$	N, 16.2	N, 16.0
14	2,5-Dimethoxyphenyl	55	242–243°	Dark brown	$C_{29}H_{22}N_6O_6$	N, 15.2	N, 15.0
15	2-Chloro-6-methylphenyl	65	224°	Orange	$C_{28}H_{19}ClN_6O_4$	C, 62.4 H, 3.5 N, 15.6	C, 62.2 H, 3.4 N, 15.1

Table II—Characteristics of 1-Carbamoyl-3,5-diphenyl-4-arylazopyrazoles

No.	R	Yield, %	M.p.	Color	Formula	Anal., % Calcd.	Anal., % Found
1	2-Nitrophenyl	60	193–194°	Orange fibers	$C_{22}H_{16}N_6O_3$	N, 20.3	N, 20.0
2	3-Nitrophenyl	50	265–266°	Orange	$C_{22}H_{16}N_6O_3$	C, 64.1 H, 3.9 N, 20.3	C, 64.1 H, 4.0 N, 19.9
3	2-Methylphenyl	50	192–193°	Orange	$C_{23}H_{19}N_5O$	C, 72.4 H, 4.9 N, 18.3	C, 72.2 H, 4.8 N, 17.9
4	3-Methylphenyl	45	242–243°	Orange fibers	$C_{23}H_{19}N_5O$	N, 18.3	N, 18.2
5	4-Methylphenyl	65	219–220°	Orange	$C_{23}H_{19}N_5O$	N, 18.3	N, 18.0
6	2-Methoxyphenyl	50	180–181°	Orange	$C_{23}H_{19}N_5O_2$	C, 69.5 H, 4.8 N, 17.6	C, 69.4 H, 4.6 N, 17.2
7	3-Methoxyphenyl	45	157°	Red	$C_{23}H_{19}N_5O_2$	N, 17.6	N, 17.5
8	4-Methoxyphenyl	55	168–171°	Orange fibers	$C_{23}H_{19}N_5O_2$	C, 69.5 H, 4.8 N, 17.6	C, 69.6 H, 4.9 N, 17.1
9	2-Ethoxyphenyl	50	163–165°	Orange	$C_{24}H_{21}N_5O_2$	N, 17.0	N, 17.2
10	4-Sulfamoylphenyl	60	250–251°	Orange	$C_{22}H_{18}N_6O_3S$	N, 18.8	N, 18.5
11	2,5-Dichlorophenyl	70	207–209°	Orange	$C_{22}H_{15}Cl_2N_5O$	Cl, 16.2	Cl, 16.0
12	2,6-Dichlorophenyl	75	161–162°	Orange	$C_{22}H_{15}Cl_2N_5O$	Cl, 16.2	Cl, 16.3
13	2,5-Dimethylphenyl	60	186–187°	Orange needles	$C_{24}H_{21}N_5O$	C, 72.9 H, 5.3 N, 17.7	C, 72.8 H, 5.1 N, 17.2

ride react with I to yield 1-(2,4-dinitrophenyl)-3,5-diphenyl-4-arylazopyrazoles (II) (Table I) and 1-carbamoyl-3,5-diphenyl-4-arylazopyrazoles (III) (Table II) congeners, respectively. They are all highly colored crystalline compounds and are soluble in common organic solvents.

EXPERIMENTAL

Melting points were taken with a Kofler hot stage apparatus and are uncorrected.

1,3-Diphenyl-2-arylhydrazono-1,2,3-propanetriones (I)—These were prepared by coupling aryldiazonium salts, prepared from anilines, with 1,3-diphenyl-1,3-propanedione (4).

1-(2,4-Dinitrophenyl)-3,5-diphenyl-4-phenylazopyrazole (II, R = C_6H_5)—To 1,3-diphenyl-2-phenylhydrazono-1,2,3-propanetrione (0.82 g., 0.0025 mole) in glacial acetic acid (25 ml.) was added 2,4-dinitrophenylhydrazine (0.50 g., 0.0025 mole) in ethyl alcohol (15 ml.) containing concentrated sulfuric acid (2 ml.). The mixture was refluxed for 8 hr. On cooling, shining crystals separated out which were recrystallized from alcohol (0.60 g., 50%) as yellow-orange crystals, m.p. 136–137°.

Anal.—Calcd. for $C_{27}H_{18}N_6O_4$: N, 17.1. Found: N, 16.8.

The details of the other 1-(2,4-dinitrophenyl)-3,5-diphenyl-4-arylazopyrazoles which were prepared are given in Table I.

1-Carbamoyl-3,5-diphenyl-4-phenylazopyrazole (III, R = C_6H_5)—A solution of 1,3-diphenyl-2-phenylhydrazono-1,2,3-propanetrione (0.82 g., 0.0025 mole) in alcohol (30 ml.) was treated with an aqueous solution of semicarbazide hydrochloride (0.23 g., 0.0025 mole). This was heated under reflux for 2 hr. On cooling, shining crystals separated out which were recrystallized from alcohol (0.50 g., 65%) as orange fibers, m.p. 215°.

Anal.—Calcd. for $C_{22}H_{17}N_5O$: N, 19.07. Found: N, 18.8.

The details of the other 1-carbamoyl-3,5-diphenyl-4-arylazopyrazoles which were prepared are given in Table II.

REFERENCES

(1) H. G. Garg and P. P. Singh, *J. Med. Chem.*, **11**, 1103(1968).
(2) *Ibid.*, **11**, 1104(1968).
(3) H. G. Garg and P. P. Singh, *J. Chem. Soc. (C)*, **1969**, 1141.
(4) H. G. Garg and P. P. Singh, unpublished data.
(5) H. G. Garg, D.Sc. thesis, Agra University, Agra, India, 1969.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 6, 1969, from the *Department of Chemistry, University of Roorkee, Roorkee, India.*

Accepted for publication January 13, 1970.

The authors thank Dr. Maxwell Gordon, Smith Kline & French Laboratories, Philadelphia, Pa.; Prof. W. U. Malik, Head of Chemistry Department, for providing the necessary facilities for work; and the C.S.I.R., New Delhi for the award of a Junior Research Fellowship (to P.P.S.)

Effect of Mesenteric Blood Flow on Intestinal Drug Absorption

Keyphrases □ Drug absorption, intestinal—mesenteric blood flow effect □ Mesenteric blood flow—drug absorption □ Sulfaethidole half-life—mesenteric blood flow

Sir:

A previously published report from our laboratory indicated that drug absorption rates in rats slowed for several drugs when the animals were fasted for periods beyond 20 hr. (1). These results suggested that some nonspecific phenomenon was occurring during prolonged fasting which affected absorption rates about equally for each drug. Although a multitude of physiological and biochemical changes are known to occur during fasting (1), particular interest is the possibility that intestinal blood flow decreases during periods of inanition. In our previous studies, subjective observation of rat intestine, following prolonged periods of fasting, supported the view that the decreased drug absortion rate was, at least in part, the consequence of reduced intestinal blood perfusion. In the absence of prolonged fasting, the color of the intestines was reddish pink; but after prolonged fasting, the intestines became blanched.

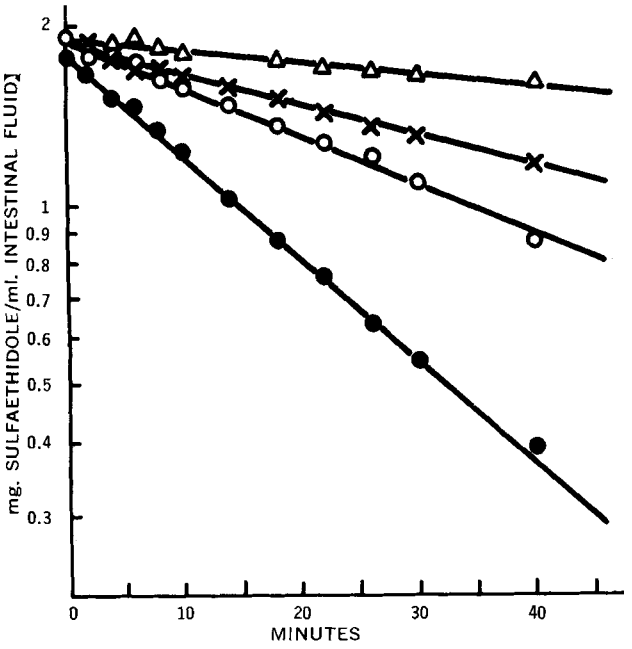


Figure 1—*Semilogarithmic plot of the disappearance of sulfaethidole from the dog intestinal lumen of Dog No. 1 as a function of time for four intestinal blood flow rates. Key:* ●, *100%;* ○, *64%;* ×, *26%; and* △, *0%.*

Table I—Effect of Mesenteric Blood Flow on the Absorption of Sulfaethidole

Dog No.	Approximate Blood Flow Rate, % 100	65	30	0
	Absorption Half-Life, min.			
1	17	37	57	134[a]
2	26	31	76	143[a]
3	26	39	78	122[a]
Mean	23	36	70	133[a]
SD	5.2	4.1	11.6	10.5

[a] Approximate values obtained by extrapolation of disappearance curve.

The possibility that the unusual drug absorption pattern, which was encountered in fasted animals, was the result of an alteration in intestinal blood perfusion led us to investigate the effects of changes in intestinal blood flow on the kinetics of drug absorpion. Three mongrel dogs (8–19 kg.) were anesthetized with allobarbital–urethane, and the small intestine was exposed by midline laparotomy. A segment of the jejunum was cannulated, and approximately 12 luminal samples were withdrawn over a 40-min. period using a double-syringe method for absorption determination (2). The cranial mesenteric artery was exposed from its origin at the aorta to the first branch, a blood flow probe was implanted on the artery, and blood flow was measured by means of an electromagnetic flowmeter (Medicon). Carotid arterial blood pressure, measured by means of a pressure transducer (E & M Linear Core), and cranial mesenteric blood flow were recorded simultaneously on a direct-writing polygraph. Intestinal solutions were buffered at pH 6.0 and warmed to 37° (2).

Initially, serial luminal samples were collected from animals with intestinal blood flow unaltered. The blood supply to the intestines was then gradually diminished by means of an hydraulic occluder located immediately distal to the flow probe. In this way, we determined the effect of reducing the rate of volume flow to approximately 65, 30, and 0% of control values on the absorption profile of sulfaethidole. Sulfaethidole concentrations were determined by the method of Bratton and Marshall (3).

Figure 1 shows the results of a typical experiment using the *in situ* dog intestinal preparation, in which the rate of disappearance of sulfaethidole from the jejunum was followed at several intestinal blood flow rates. Each line represents a separate gut segment, but all four determinations are from the same dog. Half-lives of 17, 37, 56, and 134 (approx.) min. were found for 100, 64, 26 and 0% of control blood flow rate, respectively. In these experiments, the absorption

process obeyed apparent first-order kinetics. The results obtained from three animals are shown in Table I.

Preliminary experimentation indicated that half-lives obtained from different jejunal segments in the same dog did not vary from one another by more than 15%. These results are thus suggestive of a meaningful relationship between intestinal blood perfusion and drug absorption rate. In most instances, a 40–60% reduction in mesenteric blood flow resulted in a dramatic increase in the absorption half-life for sulfaethidole.

Mesenteric circulation is subject to alteration from a wide variety of sources, and some of these will be examined and discussed in a future publication. However, it is obvious that adequate precautions should be taken to assure that significant differences in intestinal blood perfusion rates do not exist among the different animals used in a particular absorption study. Unless such precautions are taken, comparisons of the drug absorption data obtained from the various animals should be viewed with a degree of caution.

Although the data reported herein do confirm that the intestinal drug absorption process is hindered by a decrease in vascular perfusion, additional studies must be designed to quantitate the effects of fasting on intestinal blood flow before ascribing a causative role to this factor in our previous experiments.

(1) J. T. Doluisio, G. H. Tan, N. F. Billups, and L. Diamond, *J. Pharm. Sci.*, **58**, 1200(1969).

(2) J. T. Doluisio, N. F. Billups, L. W. Dittert, E. G. Sugita, and J. V. Swintosky, *ibid.*, **58**, 1196(1969).

(3) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

WILLIAM CROUTHAMEL
JAMES T. DOLUISIO
ROLLEY E. JOHNSON
LOUIS DIAMOND
University of Kentucky
College of Pharmacy
Lexington, KY 40506

Received January 26, 1970.
Accepted for publication March 12, 1970.

Spectrophotometric Analysis of Acetylcholine Levels in Plasma

Keyphrases ☐ Acetylcholine levels, plasma—determination ☐ Plasma—acetylcholine determination ☐ Colorimetric analysis—spectrophotometer

Sir:

The interest in determining acetylcholine levels in biological media before and after angiotensin-II administration parenterally in turtles led to the search for an acceptable method of analysis. The bioassay of isolated guinea pig ileum contractions against known acetylcholine concentrations was initially considered (1), but the sensitivity of the test was proven unacceptable. The bromocresol purple method of Woods (2) and the methyl orange method of Brodie (3, 4) are general methods for determining organic bases and are limited in their usefulness for biological media, which normally contain interfering organic bases.

The bromophenol blue method of Auerbach (5), modified by Mitchell and Clark (6), was chosen because it bypassed this difficulty of interfering bases and increased the sensitivity of the test 10-fold.

The interaction involves the formation of a color complex between anionic bromophenol blue and the quaternary cation, acetylcholine. The reaction occurs in an alkaline medium with the resulting color complex formed being quantitatively extracted, utilizing organic solvents. The advantage of this method is that organic bases and unreacted dye do not interfere with the extraction. Auerbach (5) also has tested 50 tertiary amines with negative results.

Procedure—Three map turtles heparinized with 100 USP units were utilized for the analysis. Each turtle was treated with 20 mg./kg. of physostigmine salicylate 5 min. prior to withdrawal of the blood sample. One milliliter of blood was removed from each turtle and added to 0.5 ml. of a 0.5% physostigmine solution. The resulting mixture was centrifuged for 15 min. at 2000 r.p.m., and a 0.5-ml. plasma sample was used for analysis. Following the addition of a buffer (0.3 g. of K_2HPO_4 and 0.3 g. of Na_2CO_3) to pH 9, the indicator, bromophenol blue, was added in a 0.5-ml. volume (0.08% in 30% K_2HPO_4). Fifteen minutes of shaking with organic solvents (washed ethylene dichloride and 4% isoamyl alcohol) completed the extraction of the dye–acetylcholine complex. The organic phase was read at 600 mμ against an ethylene dichloride–isoamyl alcohol blank because none of the other reagents absorbs. It is mandatory that the analysis take place within a 1-hr. time span because of the rapid fading of the indicator after this period. All absorbances were read on the Coleman (Hitachi 124) double-beam spectrophotometer. The procedure was then repeated following angiotensin-II administration. The differences in absorbance are due to increased acetylcholine levels. This was again repeated, using turtles with both vagi surgically severed. In the turtles with intact vagi, the following values were read: plasma sample plus physostigmine, 0.040, 0.055, and 0.043—mean = 0.046 ± 0.008 *SD;* plasma sample plus physostigmine after administration of angiotensin, 0.395, 0.410, and 0.380—mean = 0.395 ± 0.014 *SD*. The difference in absorbance, 0.349 mμ, is attributed to increased acetylcholine blood levels and corresponds to a concentration of 9.1 mcg./0.5 ml. on the standard curve. When this was repeated in vagotomized turtles, the following values for absorbance were read: plasma sample plus physostigmine, 0.050, 0.040, and 0.045—mean = 0.045 ± 0.004 *SD;* plasma sample plus physostigmine in angiotensin-treated turtles, 0.290, 0.285, and 0.305—mean = 0.293 ± 0.010 *SD*. The difference in absorbance between the two means, 0.248 mμ, is attributed to increased acetylcholine blood levels and corresponds to

process obeyed apparent first-order kinetics. The results obtained from three animals are shown in Table I.

Preliminary experimentation indicated that half-lives obtained from different jejunal segments in the same dog did not vary from one another by more than 15%. These results are thus suggestive of a meaningful relationship between intestinal blood perfusion and drug absorption rate. In most instances, a 40–60% reduction in mesenteric blood flow resulted in a dramatic increase in the absorption half-life for sulfaethidole.

Mesenteric circulation is subject to alteration from a wide variety of sources, and some of these will be examined and discussed in a future publication. However, it is obvious that adequate precautions should be taken to assure that significant differences in intestinal blood perfusion rates do not exist among the different animals used in a particular absorption study. Unless such precautions are taken, comparisons of the drug absorption data obtained from the various animals should be viewed with a degree of caution.

Although the data reported herein do confirm that the intestinal drug absorption process is hindered by a decrease in vascular perfusion, additional studies must be designed to quantitate the effects of fasting on intestinal blood flow before ascribing a causative role to this factor in our previous experiments.

(1) J. T. Doluisio, G. H. Tan, N. F. Billups, and L. Diamond, *J. Pharm. Sci.*, **58**, 1200(1969).

(2) J. T. Doluisio, N. F. Billups, L. W. Dittert, E. G. Sugita, and J. V. Swintosky, *ibid.*, **58**, 1196(1969).

(3) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

WILLIAM CROUTHAMEL
JAMES T. DOLUISIO
ROLLEY E. JOHNSON
LOUIS DIAMOND
University of Kentucky
College of Pharmacy
Lexington, KY 40506

Received January 26, 1970.
Accepted for publication March 12, 1970.

Spectrophotometric Analysis of Acetylcholine Levels in Plasma

Keyphrases ☐ Acetylcholine levels, plasma—determination ☐ Plasma—acetylcholine determination ☐ Colorimetric analysis—spectrophotometer

Sir:

The interest in determining acetylcholine levels in biological media before and after angiotensin-II administration parenterally in turtles led to the search for an acceptable method of analysis. The bioassay of isolated guinea pig ileum contractions against known acetylcholine concentrations was initially considered (1), but the sensitivity of the test was proven unacceptable. The bromocresol purple method of Woods (2) and the methyl orange method of Brodie (3, 4) are general methods for determining organic bases and are limited in their usefulness for biological media, which normally contain interfering organic bases.

The bromophenol blue method of Auerbach (5), modified by Mitchell and Clark (6), was chosen because it bypassed this difficulty of interfering bases and increased the sensitivity of the test 10-fold.

The interaction involves the formation of a color complex between anionic bromophenol blue and the quaternary cation, acetylcholine. The reaction occurs in an alkaline medium with the resulting color complex formed being quantitatively extracted, utilizing organic solvents. The advantage of this method is that organic bases and unreacted dye do not interfere with the extraction. Auerbach (5) also has tested 50 tertiary amines with negative results.

Procedure—Three map turtles heparinized with 100 USP units were utilized for the analysis. Each turtle was treated with 20 mg./kg. of physostigmine salicylate 5 min. prior to withdrawal of the blood sample. One milliliter of blood was removed from each turtle and added to 0.5 ml. of a 0.5% physostigmine solution. The resulting mixture was centrifuged for 15 min. at 2000 r.p.m., and a 0.5-ml. plasma sample was used for analysis. Following the addition of a buffer (0.3 g. of K_2HPO_4 and 0.3 g. of Na_2CO_3) to pH 9, the indicator, bromophenol blue, was added in a 0.5-ml. volume (0.08% in 30% K_2HPO_4). Fifteen minutes of shaking with organic solvents (washed ethylene dichloride and 4% isoamyl alcohol) completed the extraction of the dye–acetylcholine complex. The organic phase was read at 600 mμ against an ethylene dichloride–isoamyl alcohol blank because none of the other reagents absorbs. It is mandatory that the analysis take place within a 1-hr. time span because of the rapid fading of the indicator after this period. All absorbances were read on the Coleman (Hitachi 124) double-beam spectrophotometer. The procedure was then repeated following angiotensin-II administration. The differences in absorbance are due to increased acetylcholine levels. This was again repeated, using turtles with both vagi surgically severed. In the turtles with intact vagi, the following values were read: plasma sample plus physostigmine, 0.040, 0.055, and 0.043—mean = 0.046 ± 0.008 *SD;* plasma sample plus physostigmine after administration of angiotensin, 0.395, 0.410, and 0.380—mean = 0.395 ± 0.014 *SD*. The difference in absorbance, 0.349 mμ, is attributed to increased acetylcholine blood levels and corresponds to a concentration of 9.1 mcg./0.5 ml. on the standard curve. When this was repeated in vagotomized turtles, the following values for absorbance were read: plasma sample plus physostigmine, 0.050, 0.040, and 0.045—mean = 0.045 ± 0.004 *SD;* plasma sample plus physostigmine in angiotensin-treated turtles, 0.290, 0.285, and 0.305—mean = 0.293 ± 0.010 *SD*. The difference in absorbance between the two means, 0.248 mμ, is attributed to increased acetylcholine blood levels and corresponds to

a concentration of 6.0 mcg./0.5 ml. on the standard curve.

Standardization—The experimental values for acetylcholine were standardized against known concentrations of acetylcholine chloride by utilizing a standard curve prepared from the following. A concentration of 20.0 mcg./0.5 ml. gave the following values in three samples: 0.750, 0.760, and 0.769 mμ with a mean of 0.759 mμ; a concentration of 10.0 mcg./0.5 ml. gave the following values in three samples: 0.335, 0.343, and 0.352 with a mean of 0.343 mμ; a concentration of 6.6 mcg./0.5 ml. gave the following: 0.230, 0.243, and 0.250 with a mean of 0.241 mμ; a concentration of 5.0 mcg./0.5 ml. gave the following values: 0.175, 0.187, and 0.199 with a mean of 0.189 mμ; a concentration of 4.0 mcg./0.5 ml. gave the following values: 0.134, 0.145, and 0.156 with a mean of 0.141 mμ.

The results of this analysis support the hypotheses that angiotensin-II administration causes the release of acetylcholine at neuroeffector sites in the turtle plasma and that this release is not dependent upon the presence of intact vagus innervation (7).

(1) C. O. Ward, G. Gianutsos, and M. A. Barletta, unpublished data.
(2) L. A. Woods, J. Cochin, E. J. Fornefeld, F. G. McMahon, and M. H. Seevers, *J. Pharmacol. Exp. Ther.*, **101**, 188(1951).
(3) B. B. Brodie and S. Udenfriend, *J. Biol. Chem.*, **158**, 705 (1945).
(4) B. B. Brodie, S. Udenfriend, and W. Dill, *ibid.*, **168**, 335 (1947).
(5) M. E. Auerbach, *Ind. Eng. Chem. Anal. Ed.*, **15**, 492(1943).
(6) R. Mitchell and B. B. Clark, *Proc. Soc. Exp. Biol. Med.*, **81**, 105(1952).
(7) C. O. Ward, N. Albanese, and M. A. Barletta, *Amer. J. Pharm.*, **141**, 91(1969).

MICHAEL A. BARLETTA
CHARLES O. WARD
St. John's University
College of Pharmacy
Department of Pharmacology
Jamaica, NY 11432

Received October 30, 1969.
Accepted for publication February 10, 1970.

BOOKS

REVIEWS

Pharmaceutical Enzymes and Their Assay. Edited by R. RUYSSEN. Universitaire Pers afd. Uitgoeverij van N. V. Universitaire Boekhandel 12. St.-Amandstraat, Ghent, Belgium, 1969. 151 pp. 15.7 × 24.5 cm. Price $11.00. (*French* and *English*)

In 1961, the Fédération Internationale Pharmaceutique created the Commission for the Standardization of Pharmaceutical Enzymes, the primary mission of which was the establishment of uniform international standards for enzymes having pharmaceutical applications.

The lack of uniformity in the methods used to express the catalytic activity of pharmaceutical enzymes prompted the Commission, as its first resolution, to adopt, insofar as is practicable, an international unit system which defines an enzyme unit as that amount which catalyzes the transformation of 1 micromole of substrate per minute (or where more than one bond of a more complex substrate is attacked, one microequivalent of the group concerned per minute) under well-defined, usually external, optimal conditions. Since a unit system of this kind requires that either new methods be developed or existing methods be modified for the assay of virtually every enzyme, the attention of the Commission has thus far been devoted largely to assay methods and the many problems associated with them.

This book is a hard-cover publication of a symposium held by the Commission at the University of Ghent in May 1968. The selected topics, which are all expertly discussed in this series of seven papers, include: (1) reactions of organofluorophosphate-sensitive enzymes; (2) assay of proteinases; (3) determination of the components of the human plasma fibrinolytic system; (4) action of streptokinase on purified human plasminogen; (5) some aspects of the biochemistry of cellulases and hemicellulases; (6) microbial enzymes and their industrial applications, and (7) assay methods of the F.I.P. Commission on enzymes. Papers 4 and 5, previously mentioned, are written in French; the rest of the book is in English.

The long seventh paper contains an excellent review of the problems encountered and the progress made thus far by the Commission toward its goal. Also discussed are the many requirements which must be met by an assay method if it is to be accepted on an international level. Included herein is a detailed presentation of the Commission's proposed methods of assay for trypsin, chymotrypsin, papain, trypsin-inhibitor, pepsin, pancreatic amylase, pancreatic protease, enterokinase, and pancreatic lipase.

a concentration of 6.0 mcg./0.5 ml. on the standard curve.

Standardization—The experimental values for acetylcholine were standardized against known concentrations of acetylcholine chloride by utilizing a standard curve prepared from the following. A concentration of 20.0 mcg./0.5 ml. gave the following values in three samples: 0.750, 0.760, and 0.769 mμ with a mean of 0.759 mμ; a concentration of 10.0 mcg./0.5 ml. gave the following values in three samples: 0.335, 0.343, and 0.352 with a mean of 0.343 mμ; a concentration of 6.6 mcg./0.5 ml. gave the following: 0.230, 0.243, and 0.250 with a mean of 0.241 mμ; a concentration of 5.0 mcg./0.5 ml. gave the following values: 0.175, 0.187, and 0.199 with a mean of 0.189 mμ; a concentration of 4.0 mcg./0.5 ml. gave the following values: 0.134, 0.145, and 0.156 with a mean of 0.141 mμ.

The results of this analysis support the hypotheses that angiotensin-II administration causes the release of acetylcholine at neuroeffector sites in the turtle plasma and that this release is not dependent upon the presence of intact vagus innervation (7).

(1) C. O. Ward, G. Gianutsos, and M. A. Barletta, unpublished data.
(2) L. A. Woods, J. Cochin, E. J. Fornefeld, F. G. McMahon, and M. H. Seevers, *J. Pharmacol. Exp. Ther.*, **101**, 188(1951).
(3) B. B. Brodie and S. Udenfriend, *J. Biol. Chem.*, **158**, 705 (1945).
(4) B. B. Brodie, S. Udenfriend, and W. Dill, *ibid.*, **168**, 335 (1947).
(5) M. E. Auerbach, *Ind. Eng. Chem. Anal. Ed.*, **15**, 492(1943).
(6) R. Mitchell and B. B. Clark, *Proc. Soc. Exp. Biol. Med.*, **81**, 105(1952).
(7) C. O. Ward, N. Albanese, and M. A. Barletta, *Amer. J. Pharm.*, **141**, 91(1969).

MICHAEL A. BARLETTA
CHARLES O. WARD
St. John's University
College of Pharmacy
Department of Pharmacology
Jamaica, NY 11432

Received October 30, 1969.
Accepted for publication February 10, 1970.

BOOKS

REVIEWS

Pharmaceutical Enzymes and Their Assay. Edited by R. RUYSSEN. Universitaire Pers afd. Uitgoeverij van N. V. Universitaire Boekhandel 12. St.-Amandstraat, Ghent, Belgium, 1969. 151 pp. 15.7 × 24.5 cm. Price $11.00. (*French* and *English*)

In 1961, the Fédération Internationale Pharmaceutique created the Commission for the Standardization of Pharmaceutical Enzymes, the primary mission of which was the establishment of uniform international standards for enzymes having pharmaceutical applications.

The lack of uniformity in the methods used to express the catalytic activity of pharmaceutical enzymes prompted the Commission, as its first resolution, to adopt, insofar as is practicable, an international unit system which defines an enzyme unit as that amount which catalyzes the transformation of 1 micromole of substrate per minute (or where more than one bond of a more complex substrate is attacked, one microequivalent of the group concerned per minute) under well-defined, usually external, optimal conditions. Since a unit system of this kind requires that either new methods be developed or existing methods be modified for the assay of virtually every enzyme, the attention of the Commission has thus far been devoted largely to assay methods and the many problems associated with them.

This book is a hard-cover publication of a symposium held by the Commission at the University of Ghent in May 1968. The selected topics, which are all expertly discussed in this series of seven papers, include: (1) reactions of organofluorophosphate-sensitive enzymes; (2) assay of proteinases; (3) determination of the components of the human plasma fibrinolytic system; (4) action of streptokinase on purified human plasminogen; (5) some aspects of the biochemistry of cellulases and hemicellulases; (6) microbial enzymes and their industrial applications, and (7) assay methods of the F.I.P. Commission on enzymes. Papers 4 and 5, previously mentioned, are written in French; the rest of the book is in English.

The long seventh paper contains an excellent review of the problems encountered and the progress made thus far by the Commission toward its goal. Also discussed are the many requirements which must be met by an assay method if it is to be accepted on an international level. Included herein is a detailed presentation of the Commission's proposed methods of assay for trypsin, chymotrypsin, papain, trypsin-inhibitor, pepsin, pancreatic amylase, pancreatic protease, enterokinase, and pancreatic lipase.

The book is replete with references. Also appended is a list of reference standards (six enzymes and three substrates) available from the Commission.

Reviewed by A. J. Vazakas
Johnson & Johnson
New Brunswick, NJ 08903 ■

British Medical Bulletin: Control of Human Fertility. Vol. 26, No. 1. Edited by G. I. M. SWYER. Medical Department, The British Council, 97 & 99 Park St., London, England, 1970. i + 97 pp. 22 × 28.5 cm. Price $6.50.

This issue contains papers on various aspects of human fertility contributed by a rather impressive list of investigators. This compilation takes a multidisciplinary approach with several papers discussing the relative health hazards of various kinds of contraceptive methods.

The papers are, for the most part, written to put the particular topic into a historical context, present the developments and status of the research, and provide some comments on the future directions to be taken. This particular issue should be of interest not only to scientists actively working within this field, but also to those who are interested in a technical state-of-the-art review.

Staff Review ■

Clinical Toxicology. Second Edition. Edited by C. J. POLSON and R. N. TATTERSALL. J. B. Lippincott Co., Philadelphia, PA 19105, 1969. x + 655 pp. 14.5 × 22.2 cm. Price $18.50.

The growing interest in toxicology in colleges of pharmacy as well as by pharmacists as a whole will require greater attention being given to texts and references on the subject than presently available. Publication of the second edition of the British text "Clinical Toxicology," offers some help to the teacher and student. As a text for American students it suffers from the inclusion of repeated references to medical-legal rules and interpretations which are quite appropriate for British students but extraneous and perhaps confusing to our students. The authors, however, have made it clear that the book is intended primarily for physicians who are in need of general and specific information on the clinical manifestations, treatment, and prognosis of cases of poisoning. In this respect, it can also be valuable to the pharmacist as a source book of clinical toxicology. The book is extremely easy to read and gets directly to the practical aspects of toxicity of compounds and products. Further interest is generated by including illustrative cases of poisoning which helps orient the reader to the effect of a particular substance ingested by a person. The authors do not try to cram volumes of information on a specific compound but include sufficient information which may be of extreme importance in diagnosing and treating a poisoned patient.

"Clinical Toxicology" is divided into two parts. The first and the shorter portion of the book deals with the general considerations of poisoning while the second, and in essence, the bulk of the book, reviews the individual substances generally as separate short chapters. The book can be recommended for pharmacists and physicians but only as an added source of information. As with most texts on clinical toxicology, information on toxicity of newer agents and products must be sought elsewhere. The British book will not replace the present excellent reference source, "Clinical Toxicology of Commercial Products" published by The Williams & Wilkins Company, Baltimore, Md., but may help to supplement portions of it.

Reviewed by John Autian
Materials Science Toxicology Laboratories
College of Pharmacy & College of Dentistry
University of Tennessee Medical Units
Memphis, TN 38103 ■

Review of Biochemistry. By NATHAN H. SLOANE and J. LYNDAL YORK. Macmillan Co., 866 Third Ave., New York, NY 10022, 1969. ix + 278 pp. 18 × 26 cm. Price: $9.95, hardbound; $6.95, paperbound.

This outline review of biochemistry prepared by Nathan H. Sloane, Professor of Biochemistry, University of Tennessee Medical Units, and J. Lyndal York, Associate Professor of Biochemistry, University of Arkansas School of Medicine, is aimed at presenting modern biochemistry in a concise, practical form covering fundamentals applicable to the health professions.

The authors emphasize in the Preface that this book "... is not a text; rather, it can be used to greatest advantage in conjunction with lecture material and assigned reading in textbooks and periodicals."

The outline form which is used throughout the book allows the reader to scan a great deal of information with minimum effort.

The book surveys biochemical methods and physiochemical principles as well as including chapters on the cell, amino acids and peptides, proteins, and enzymes. Three chapters deal with carbohydrate metabolism and chemistry. The metabolism of lipids, amino acids, and nucleic acids is covered. In the concluding chapters, aspects of genetics, hemoglobin, renal function, and vitamins are among the topics discussed.

Staff Review ■

Neurophysiological and Behavioral Aspects of Psychotropic Drugs. Edited by A. G. KARCZMAR and W. P. KOELLA. Charles C Thomas, 301-327 East Lawrence Ave., Springfield, IL 62703, 1969. xviii + 199 pp. 17.5 × 25.5 cm. Price $12.50.

This book is based on reports developed by several study groups of the American College of Neuropsychopharmacology in 1966. These reports were expanded and updated through 1968 before publication.

The work presented in this volume represents the efforts of two particular groups. The first one studied the biological effects of pharmaceutical agents—neurophysiological aspects, with five chapters being devoted to various aspects of that topic including several papers on LSD responses and behavioral changes.

The other major portion of the book contains the reports of the second group which focused on the effects of drugs on chemistry, learning, and memory.

The editors of this book have attempted to compile information from various disciplines and sources related to this topic into one volume which is relatively specific and current.

Staff Review ■

The book is replete with references. Also appended is a list of reference standards (six enzymes and three substrates) available from the Commission.

Reviewed by A. J. Vazakas
Johnson & Johnson
New Brunswick, NJ 08903 ■

British Medical Bulletin: Control of Human Fertility. Vol. 26, No. 1. Edited by G. I. M. SWYER. Medical Department, The British Council, 97 & 99 Park St., London, England, 1970. i + 97 pp. 22 × 28.5 cm. Price $6.50.

This issue contains papers on various aspects of human fertility contributed by a rather impressive list of investigators. This compilation takes a multidisciplinary approach with several papers discussing the relative health hazards of various kinds of contraceptive methods.

The papers are, for the most part, written to put the particular topic into a historical context, present the developments and status of the research, and provide some comments on the future directions to be taken. This particular issue should be of interest not only to scientists actively working within this field, but also to those who are interested in a technical state-of-the-art review.

Staff Review ■

Clinical Toxicology. Second Edition. Edited by C. J. POLSON and R. N. TATTERSALL. J. B. Lippincott Co., Philadelphia, PA 19105, 1969. x + 655 pp. 14.5 × 22.2 cm. Price $18.50.

The growing interest in toxicology in colleges of pharmacy as well as by pharmacists as a whole will require greater attention being given to texts and references on the subject than presently available. Publication of the second edition of the British text "Clinical Toxicology," offers some help to the teacher and student. As a text for American students it suffers from the inclusion of repeated references to medical-legal rules and interpretations which are quite appropriate for British students but extraneous and perhaps confusing to our students. The authors, however, have made it clear that the book is intended primarily for physicians who are in need of general and specific information on the clinical manifestations, treatment, and prognosis of cases of poisoning. In this respect, it can also be valuable to the pharmacist as a source book of clinical toxicology. The book is extremely easy to read and gets directly to the practical aspects of toxicity of compounds and products. Further interest is generated by including illustrative cases of poisoning which helps orient the reader to the effect of a particular substance ingested by a person. The authors do not try to cram volumes of information on a specific compound but include sufficient information which may be of extreme importance in diagnosing and treating a poisoned patient.

"Clinical Toxicology" is divided into two parts. The first and the shorter portion of the book deals with the general considerations of poisoning while the second, and in essence, the bulk of the book, reviews the individual substances generally as separate short chapters. The book can be recommended for pharmacists and physicians but only as an added source of information. As with most texts on clinical toxicology, information on toxicity of newer agents and products must be sought elsewhere. The British book will not replace the present excellent reference source, "Clinical Toxicology of Commercial Products" published by The Williams & Wilkins Company, Baltimore, Md., but may help to supplement portions of it.

Reviewed by John Autian
Materials Science Toxicology Laboratories
College of Pharmacy & College of Dentistry
University of Tennessee Medical Units
Memphis, TN 38103 ■

Review of Biochemistry. By NATHAN H. SLOANE and J. LYNDAL YORK. Macmillan Co., 866 Third Ave., New York, NY 10022, 1969. ix + 278 pp. 18 × 26 cm. Price: $9.95, hardbound; $6.95, paperbound.

This outline review of biochemistry prepared by Nathan H. Sloane, Professor of Biochemistry, University of Tennessee Medical Units, and J. Lyndal York, Associate Professor of Biochemistry, University of Arkansas School of Medicine, is aimed at presenting modern biochemistry in a concise, practical form covering fundamentals applicable to the health professions.

The authors emphasize in the Preface that this book "... is not a text; rather, it can be used to greatest advantage in conjunction with lecture material and assigned reading in textbooks and periodicals."

The outline form which is used throughout the book allows the reader to scan a great deal of information with minimum effort.

The book surveys biochemical methods and physiochemical principles as well as including chapters on the cell, amino acids and peptides, proteins, and enzymes. Three chapters deal with carbohydrate metabolism and chemistry. The metabolism of lipids, amino acids, and nucleic acids is covered. In the concluding chapters, aspects of genetics, hemoglobin, renal function, and vitamins are among the topics discussed.

Staff Review ■

Neurophysiological and Behavioral Aspects of Psychotropic Drugs. Edited by A. G. KARCZMAR and W. P. KOELLA. Charles C Thomas, 301-327 East Lawrence Ave., Springfield, IL 62703, 1969. xviii + 199 pp. 17.5 × 25.5 cm. Price $12.50.

This book is based on reports developed by several study groups of the American College of Neuropsychopharmacology in 1966. These reports were expanded and updated through 1968 before publication.

The work presented in this volume represents the efforts of two particular groups. The first one studied the biological effects of pharmaceutical agents—neurophysiological aspects, with five chapters being devoted to various aspects of that topic including several papers on LSD responses and behavioral changes.

The other major portion of the book contains the reports of the second group which focused on the effects of drugs on chemistry, learning, and memory.

The editors of this book have attempted to compile information from various disciplines and sources related to this topic into one volume which is relatively specific and current.

Staff Review ■

The book is replete with references. Also appended is a list of reference standards (six enzymes and three substrates) available from the Commission.

Reviewed by A. J. Vazakas
Johnson & Johnson
New Brunswick, NJ 08903 ■

British Medical Bulletin: Control of Human Fertility. Vol. 26, No. 1. Edited by G. I. M. SWYER. Medical Department, The British Council, 97 & 99 Park St., London, England, 1970. i + 97 pp. 22 × 28.5 cm. Price $6.50.

This issue contains papers on various aspects of human fertility contributed by a rather impressive list of investigators. This compilation takes a multidisciplinary approach with several papers discussing the relative health hazards of various kinds of contraceptive methods.

The papers are, for the most part, written to put the particular topic into a historical context, present the developments and status of the research, and provide some comments on the future directions to be taken. This particular issue should be of interest not only to scientists actively working within this field, but also to those who are interested in a technical state-of-the-art review.

Staff Review ■

Clinical Toxicology. Second Edition. Edited by C. J. POLSON and R. N. TATTERSALL. J. B. Lippincott Co., Philadelphia, PA 19105, 1969. x + 655 pp. 14.5 × 22.2 cm. Price $18.50.

The growing interest in toxicology in colleges of pharmacy as well as by pharmacists as a whole will require greater attention being given to texts and references on the subject than presently available. Publication of the second edition of the British text "Clinical Toxicology," offers some help to the teacher and student. As a text for American students it suffers from the inclusion of repeated references to medical-legal rules and interpretations which are quite appropriate for British students but extraneous and perhaps confusing to our students. The authors, however, have made it clear that the book is intended primarily for physicians who are in need of general and specific information on the clinical manifestations, treatment, and prognosis of cases of poisoning. In this respect, it can also be valuable to the pharmacist as a source book of clinical toxicology. The book is extremely easy to read and gets directly to the practical aspects of toxicity of compounds and products. Further interest is generated by including illustrative cases of poisoning which helps orient the reader to the effect of a particular substance ingested by a person. The authors do not try to cram volumes of information on a specific compound but include sufficient information which may be of extreme importance in diagnosing and treating a poisoned patient.

"Clinical Toxicology" is divided into two parts. The first and the shorter portion of the book deals with the general considerations of poisoning while the second, and in essence, the bulk of the book, reviews the individual substances generally as separate short chapters. The book can be recommended for pharmacists and physicians but only as an added source of information. As with most texts on clinical toxicology, information on toxicity of newer agents and products must be sought elsewhere. The British book will not replace the present excellent reference source, "Clinical Toxicology of Commercial Products" published by The Williams & Wilkins Company, Baltimore, Md., but may help to supplement portions of it.

Reviewed by John Autian
Materials Science Toxicology Laboratories
College of Pharmacy & College of Dentistry
University of Tennessee Medical Units
Memphis, TN 38103 ■

Review of Biochemistry. By NATHAN H. SLOANE and J. LYNDAL YORK. Macmillan Co., 866 Third Ave., New York, NY 10022, 1969. ix + 278 pp. 18 × 26 cm. Price: $9.95, hardbound; $6.95, paperbound.

This outline review of biochemistry prepared by Nathan H. Sloane, Professor of Biochemistry, University of Tennessee Medical Units, and J. Lyndal York, Associate Professor of Biochemistry, University of Arkansas School of Medicine, is aimed at presenting modern biochemistry in a concise, practical form covering fundamentals applicable to the health professions.

The authors emphasize in the Preface that this book "... is not a text; rather, it can be used to greatest advantage in conjunction with lecture material and assigned reading in textbooks and periodicals."

The outline form which is used throughout the book allows the reader to scan a great deal of information with minimum effort.

The book surveys biochemical methods and physiochemical principles as well as including chapters on the cell, amino acids and peptides, proteins, and enzymes. Three chapters deal with carbohydrate metabolism and chemistry. The metabolism of lipids, amino acids, and nucleic acids is covered. In the concluding chapters, aspects of genetics, hemoglobin, renal function, and vitamins are among the topics discussed.

Staff Review ■

Neurophysiological and Behavioral Aspects of Psychotropic Drugs. Edited by A. G. KARCZMAR and W. P. KOELLA. Charles C Thomas, 301-327 East Lawrence Ave., Springfield, IL 62703, 1969. xviii + 199 pp. 17.5 × 25.5 cm. Price $12.50.

This book is based on reports developed by several study groups of the American College of Neuropsychopharmacology in 1966. These reports were expanded and updated through 1968 before publication.

The work presented in this volume represents the efforts of two particular groups. The first one studied the biological effects of pharmaceutical agents—neurophysiological aspects, with five chapters being devoted to various aspects of that topic including several papers on LSD responses and behavioral changes.

The other major portion of the book contains the reports of the second group which focused on the effects of drugs on chemistry, learning, and memory.

The editors of this book have attempted to compile information from various disciplines and sources related to this topic into one volume which is relatively specific and current.

Staff Review ■

The book is replete with references. Also appended is a list of reference standards (six enzymes and three substrates) available from the Commission.

Reviewed by A. J. Vazakas
Johnson & Johnson
New Brunswick, NJ 08903 ■

British Medical Bulletin: Control of Human Fertility. Vol. 26, No. 1. Edited by G. I. M. SWYER. Medical Department, The British Council, 97 & 99 Park St., London, England, 1970. i + 97 pp. 22 × 28.5 cm. Price $6.50.

This issue contains papers on various aspects of human fertility contributed by a rather impressive list of investigators. This compilation takes a multidisciplinary approach with several papers discussing the relative health hazards of various kinds of contraceptive methods.

The papers are, for the most part, written to put the particular topic into a historical context, present the developments and status of the research, and provide some comments on the future directions to be taken. This particular issue should be of interest not only to scientists actively working within this field, but also to those who are interested in a technical state-of-the-art review.

Staff Review ■

Clinical Toxicology. Second Edition. Edited by C. J. POLSON and R. N. TATTERSALL. J. B. Lippincott Co., Philadelphia, PA 19105, 1969. x + 655 pp. 14.5 × 22.2 cm. Price $18.50.

The growing interest in toxicology in colleges of pharmacy as well as by pharmacists as a whole will require greater attention being given to texts and references on the subject than presently available. Publication of the second edition of the British text "Clinical Toxicology," offers some help to the teacher and student. As a text for American students it suffers from the inclusion of repeated references to medical-legal rules and interpretations which are quite appropriate for British students but extraneous and perhaps confusing to our students. The authors, however, have made it clear that the book is intended primarily for physicians who are in need of general and specific information on the clinical manifestations, treatment, and prognosis of cases of poisoning. In this respect, it can also be valuable to the pharmacist as a source book of clinical toxicology. The book is extremely easy to read and gets directly to the practical aspects of toxicity of compounds and products. Further interest is generated by including illustrative cases of poisoning which helps orient the reader to the effect of a particular substance ingested by a person. The authors do not try to cram volumes of information on a specific compound but include sufficient information which may be of extreme importance in diagnosing and treating a poisoned patient.

"Clinical Toxicology" is divided into two parts. The first and the shorter portion of the book deals with the general considerations of poisoning while the second, and in essence, the bulk of the book, reviews the individual substances generally as separate short chapters. The book can be recommended for pharmacists and physicians but only as an added source of information. As with most texts on clinical toxicology, information on toxicity of newer agents and products must be sought elsewhere. The British book will not replace the present excellent reference source, "Clinical Toxicology of Commercial Products" published by The Williams & Wilkins Company, Baltimore, Md., but may help to supplement portions of it.

Reviewed by John Autian
Materials Science Toxicology Laboratories
College of Pharmacy & College of Dentistry
University of Tennessee Medical Units
Memphis, TN 38103 ■

Review of Biochemistry. By NATHAN H. SLOANE and J. LYNDAL YORK. Macmillan Co., 866 Third Ave., New York, NY 10022, 1969. ix + 278 pp. 18 × 26 cm. Price: $9.95, hardbound; $6.95, paperbound.

This outline review of biochemistry prepared by Nathan H. Sloane, Professor of Biochemistry, University of Tennessee Medical Units, and J. Lyndal York, Associate Professor of Biochemistry, University of Arkansas School of Medicine, is aimed at presenting modern biochemistry in a concise, practical form covering fundamentals applicable to the health professions.

The authors emphasize in the Preface that this book "... is not a text; rather, it can be used to greatest advantage in conjunction with lecture material and assigned reading in textbooks and periodicals."

The outline form which is used throughout the book allows the reader to scan a great deal of information with minimum effort.

The book surveys biochemical methods and physiochemical principles as well as including chapters on the cell, amino acids and peptides, proteins, and enzymes. Three chapters deal with carbohydrate metabolism and chemistry. The metabolism of lipids, amino acids, and nucleic acids is covered. In the concluding chapters, aspects of genetics, hemoglobin, renal function, and vitamins are among the topics discussed.

Staff Review ■

Neurophysiological and Behavioral Aspects of Psychotropic Drugs. Edited by A. G. KARCZMAR and W. P. KOELLA. Charles C Thomas, 301-327 East Lawrence Ave., Springfield, IL 62703, 1969. xviii + 199 pp. 17.5 × 25.5 cm. Price $12.50.

This book is based on reports developed by several study groups of the American College of Neuropsychopharmacology in 1966. These reports were expanded and updated through 1968 before publication.

The work presented in this volume represents the efforts of two particular groups. The first one studied the biological effects of pharmaceutical agents—neurophysiological aspects, with five chapters being devoted to various aspects of that topic including several papers on LSD responses and behavioral changes.

The other major portion of the book contains the reports of the second group which focused on the effects of drugs on chemistry, learning, and memory.

The editors of this book have attempted to compile information from various disciplines and sources related to this topic into one volume which is relatively specific and current.

Staff Review ■

The book is replete with references. Also appended is a list of reference standards (six enzymes and three substrates) available from the Commission.

Reviewed by A. J. Vazakas
Johnson & Johnson
New Brunswick, NJ 08903 ■

British Medical Bulletin: Control of Human Fertility. Vol. 26, No. 1. Edited by G. I. M. SWYER. Medical Department, The British Council, 97 & 99 Park St., London, England, 1970. i + 97 pp. 22 × 28.5 cm. Price $6.50.

This issue contains papers on various aspects of human fertility contributed by a rather impressive list of investigators. This compilation takes a multidisciplinary approach with several papers discussing the relative health hazards of various kinds of contraceptive methods.

The papers are, for the most part, written to put the particular topic into a historical context, present the developments and status of the research, and provide some comments on the future directions to be taken. This particular issue should be of interest not only to scientists actively working within this field, but also to those who are interested in a technical state-of-the-art review.

Staff Review ■

Clinical Toxicology. Second Edition. Edited by C. J. POLSON and R. N. TATTERSALL. J. B. Lippincott Co., Philadelphia, PA 19105, 1969. x + 655 pp. 14.5 × 22.2 cm. Price $18.50.

The growing interest in toxicology in colleges of pharmacy as well as by pharmacists as a whole will require greater attention being given to texts and references on the subject than presently available. Publication of the second edition of the British text "Clinical Toxicology," offers some help to the teacher and student. As a text for American students it suffers from the inclusion of repeated references to medical-legal rules and interpretations which are quite appropriate for British students but extraneous and perhaps confusing to our students. The authors, however, have made it clear that the book is intended primarily for physicians who are in need of general and specific information on the clinical manifestations, treatment, and prognosis of cases of poisoning. In this respect, it can also be valuable to the pharmacist as a source book of clinical toxicology. The book is extremely easy to read and gets directly to the practical aspects of toxicity of compounds and products. Further interest is generated by including illustrative cases of poisoning which helps orient the reader to the effect of a particular substance ingested by a person. The authors do not try to cram volumes of information on a specific compound but include sufficient information which may be of extreme importance in diagnosing and treating a poisoned patient.

"Clinical Toxicology" is divided into two parts. The first and the shorter portion of the book deals with the general considerations of poisoning while the second, and in essence, the bulk of the book, reviews the individual substances generally as separate short chapters. The book can be recommended for pharmacists and physicians but only as an added source of information. As with most texts on clinical toxicology, information on toxicity of newer agents and products must be sought elsewhere. The British book will not replace the present excellent reference source, "Clinical Toxicology of Commercial Products" published by The Williams & Wilkins Company, Baltimore, Md., but may help to supplement portions of it.

Reviewed by John Autian
Materials Science Toxicology Laboratories
College of Pharmacy & College of Dentistry
University of Tennessee Medical Units
Memphis, TN 38103 ■

Review of Biochemistry. By NATHAN H. SLOANE and J. LYNDAL YORK. Macmillan Co., 866 Third Ave., New York, NY 10022, 1969. ix + 278 pp. 18 × 26 cm. Price: $9.95, hardbound; $6.95, paperbound.

This outline review of biochemistry prepared by Nathan H. Sloane, Professor of Biochemistry, University of Tennessee Medical Units, and J. Lyndal York, Associate Professor of Biochemistry, University of Arkansas School of Medicine, is aimed at presenting modern biochemistry in a concise, practical form covering fundamentals applicable to the health professions.

The authors emphasize in the Preface that this book "... is not a text; rather, it can be used to greatest advantage in conjunction with lecture material and assigned reading in textbooks and periodicals."

The outline form which is used throughout the book allows the reader to scan a great deal of information with minimum effort.

The book surveys biochemical methods and physiochemical principles as well as including chapters on the cell, amino acids and peptides, proteins, and enzymes. Three chapters deal with carbohydrate metabolism and chemistry. The metabolism of lipids, amino acids, and nucleic acids is covered. In the concluding chapters, aspects of genetics, hemoglobin, renal function, and vitamins are among the topics discussed.

Staff Review ■

Neurophysiological and Behavioral Aspects of Psychotropic Drugs. Edited by A. G. KARCZMAR and W. P. KOELLA. Charles C Thomas, 301-327 East Lawrence Ave., Springfield, IL 62703, 1969. xviii + 199 pp. 17.5 × 25.5 cm. Price $12.50.

This book is based on reports developed by several study groups of the American College of Neuropsychopharmacology in 1966. These reports were expanded and updated through 1968 before publication.

The work presented in this volume represents the efforts of two particular groups. The first one studied the biological effects of pharmaceutical agents—neurophysiological aspects, with five chapters being devoted to various aspects of that topic including several papers on LSD responses and behavioral changes.

The other major portion of the book contains the reports of the second group which focused on the effects of drugs on chemistry, learning, and memory.

The editors of this book have attempted to compile information from various disciplines and sources related to this topic into one volume which is relatively specific and current.

Staff Review ■

NEW JOURNALS

Current Index to Conference Papers in Life Sciences. By World Meetings Information Center, Harry Baum, Director. CCM Information Corp., 909 Third Ave., New York, NY 10022, 1969. iv + 188 pp. 17 × 25 cm. Price: Single copy, $10; Subscription rate, $90 per year.

This index is one of three recently published; the other two indexing the areas of chemistry and engineering. It is available monthly as a bound copy and weekly on magnetic tape to institutions with computer operated information systems. The index represents an attempt to bring to scientists information presented at professional conferences without waiting one to two years for the proceedings to be published. ■

Emergency Health Services Digest. By U. S. Dept. of Health, Education, and Welfare, Public Health Service, Health Services and Mental Health Administration, Div. of Emergency Health Services, 1969. v + 53 pp. 15 × 23 cm. Price: $0.40 per issue.

As a planned semiannual journal published by the Department of Health, Education, and Welfare, this journal contains summaries of articles published elsewhere. Its purpose is to bring together from various sources information concerning emergency medical and health care procedures. ■

Journal of Texture Studies. An International Journal of Rheology, Psychorheology, Physical and Sensory Testing of Foods and Pharmaceuticals. Edited by P. SHERMAN and A. S. SZCZESNIAK, D. Reidel Publishing Co., P.O. Box 17, Dordrecht, The Netherlands, 1969. 9 + 129 pp. 16 × 24 cm. Price: Single copy, $14; Subscription rate, $45 per year. (*English*)

In the introduction, the editors state that because of the interdisciplinary nature of research in textures of foods and pharmaceuticals, they established this quarterly journal to fill the need of having research papers on texture studies published under one cover. In addition to original research communications, it will also contain abstracts of pertinent papers published elsewhere, book reviews, and notices of meetings. Review articles on different facets of texture studies are also planned. ■

Chemico-Biological Interactions. Edited by G. P. WARWICK, Published by Elsevier Publishing Co., P.O. Box 211, Amsterdam, The Netherlands, 1969. 1 + 126 pp. 18.5 × 26 cm. Price: Subscription rate, Dfl. 90 plus Dfl. 5.40 postage per year. (*English*)

The aim of this bimonthly periodical is to bring together papers concerned with the study of changes brought about by exogenous chemicals in biological systems. An important emphasis will be molecular mechanisms. In addition to research articles, short communications and related review articles will be published. ■

NOTICES

Electrometric Methods. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. viii + 131 pp. 13.5 × 22 cm. Price $7.50.

Spectroscopy. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. vii + 183 pp. 13.5 × 22 cm. Price $8.50.

Chromatography. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. vii + 151 pp. 13.5 × 22 cm. Price $7.50.

WHO Expert Committee on Drug Dependence. 17th Report. By World Health Organization. World Health Organization, Geneva, Switzerland, 1970. i + 31 pp. 15.5 × 24 cm. Price $1.00.

Describing Chemical Engineering Systems. By WILLIAM E. RANZ. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. xx + 248 pp. 21 × 27.5 cm. Price $9.95.

American Drug Index. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. vi + 636 pp. 13.5 × 21 cm. Price $8.50.

Actualites Pharmacologiques. By RENÉ HAZARD, J. CHEYMOL, J. R. BOISSIER, and PAUL LECHAT. Masson & Cie, 120 Boulevard Saint-Germain, Paris, France, 1969. i + 347 pp. 16 × 24 cm. (*French*)

Hagers Handbuch der Pharmazeutischen Praxis. Vol. 2. Edited by P. H. LIST and L. HORHAMMER. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. xxiii + 1271 pp. 17.5 × 25 cm. (*German*).

One-Dimensional Two-Phase Flow. By GRAHAM B. WALLIS. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. xxi + 408 pp. 15.5 × 24 cm. Price $16.50.

Health in a Bottle: Searching for the Drugs That Help. By L. EARLE ARNOW. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. ix + 272 pp. 14 × 21 cm. Price $5.95.

Pharmaceutical Abstracts. Vol. X, No. 4. Edited by HENRY M. BURLAGE. College of Pharmacy, University of Texas, Austin, TX 78712, 1969. 21 × 28 cm. Price $6.00. **FINAL ISSUE.**

Handbook of Experimental Pharmacology. Edited by O. EICHLER, A. FARAH, H. HERKEN, and A. D. WELCH. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. ix + 308 pp. 17 × 25.5 cm. Price $34.10. (*principally in German*)

Experimental Medicine and Surgery. Edited by WILLIAM J. SOUTHWELL and ISIDORE GREENBERG. Brooklyn Medical Press Inc., New York, NY 11216, 1969. i + 244 pp. 17 × 24.5 cm. Price $8.00.

Pharmacological Facts and Figures. Vol. 9. By F. LEMBECK and K. F. SEWING. Translated from the German by H. HELLER and D. R. FERGUSON. Springer-Verlag, 175 Fifth Ave., New York, NY 10010, 1969. viii + 111 pp. 23 × 15 cm. Price $3.00.

NEW JOURNALS

Current Index to Conference Papers in Life Sciences. By World Meetings Information Center, Harry Baum, Director. CCM Information Corp., 909 Third Ave., New York, NY 10022, 1969. iv + 188 pp. 17 × 25 cm. Price: Single copy, $10; Subscription rate, $90 per year.

This index is one of three recently published; the other two indexing the areas of chemistry and engineering. It is available monthly as a bound copy and weekly on magnetic tape to institutions with computer operated information systems. The index represents an attempt to bring to scientists information presented at professional conferences without waiting one to two years for the proceedings to be published. ■

Emergency Health Services Digest. By U. S. Dept. of Health, Education, and Welfare, Public Health Service, Health Services and Mental Health Administration, Div. of Emergency Health Services, 1969. v + 53 pp. 15 × 23 cm. Price: $0.40 per issue.

As a planned semiannual journal published by the Department of Health, Education, and Welfare, this journal contains summaries of articles published elsewhere. Its purpose is to bring together from various sources information concerning emergency medical and health care procedures. ■

Journal of Texture Studies. An International Journal of Rheology, Psychorheology, Physical and Sensory Testing of Foods and Pharmaceuticals. Edited by P. SHERMAN and A. S. SZCZESNIAK, D. Reidel Publishing Co., P.O. Box 17, Dordrecht, The Netherlands, 1969. 9 + 129 pp. 16 × 24 cm. Price: Single copy, $14; Subscription rate, $45 per year. (*English*)

In the introduction, the editors state that because of the interdisciplinary nature of research in textures of foods and pharmaceuticals, they established this quarterly journal to fill the need of having research papers on texture studies published under one cover. In addition to original research communications, it will also contain abstracts of pertinent papers published elsewhere, book reviews, and notices of meetings. Review articles on different facets of texture studies are also planned. ■

Chemico-Biological Interactions. Edited by G. P. WARWICK, Published by Elsevier Publishing Co., P.O. Box 211, Amsterdam, The Netherlands, 1969. 1 + 126 pp. 18.5 × 26 cm. Price: Subscription rate, Dfl. 90 plus Dfl. 5.40 postage per year. (*English*)

The aim of this bimonthly periodical is to bring together papers concerned with the study of changes brought about by exogenous chemicals in biological systems. An important emphasis will be molecular mechanisms. In addition to research articles, short communications and related review articles will be published. ■

NOTICES

Electrometric Methods. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. viii + 131 pp. 13.5 × 22 cm. Price $7.50.

Spectroscopy. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. vii + 183 pp. 13.5 × 22 cm. Price $8.50.

Chromatography. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. vii + 151 pp. 13.5 × 22 cm. Price $7.50.

WHO Expert Committee on Drug Dependence. 17th Report. By World Health Organization. World Health Organization, Geneva, Switzerland, 1970. i + 31 pp. 15.5 × 24 cm. Price $1.00.

Describing Chemical Engineering Systems. By WILLIAM E. RANZ. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. xx + 248 pp. 21 × 27.5 cm. Price $9.95.

American Drug Index. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. vi + 636 pp. 13.5 × 21 cm. Price $8.50.

Actualites Pharmacologiques. By RENÉ HAZARD, J. CHEYMOL, J. R. BOISSIER, and PAUL LECHAT. Masson & Cie, 120 Boulevard Saint-Germain, Paris, France, 1969. i + 347 pp. 16 × 24 cm. (*French*)

Hagers Handbuch der Pharmazeutischen Praxis. Vol. 2. Edited by P. H. LIST and L. HORHAMMER. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. xxiii + 1271 pp. 17.5 × 25 cm. (*German*).

One-Dimensional Two-Phase Flow. By GRAHAM B. WALLIS. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. xxi + 408 pp. 15.5 × 24 cm. Price $16.50.

Health in a Bottle: Searching for the Drugs That Help. By L. EARLE ARNOW. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. ix + 272 pp. 14 × 21 cm. Price $5.95.

Pharmaceutical Abstracts. Vol. X, No. 4. Edited by HENRY M. BURLAGE. College of Pharmacy, University of Texas, Austin, TX 78712, 1969. 21 × 28 cm. Price $6.00. **FINAL ISSUE.**

Handbook of Experimental Pharmacology. Edited by O. EICHLER, A. FARAH, H. HERKEN, and A. D. WELCH. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. ix + 308 pp. 17 × 25.5 cm. Price $34.10. (*principally in German*)

Experimental Medicine and Surgery. Edited by WILLIAM J. SOUTHWELL and ISIDORE GREENBERG. Brooklyn Medical Press Inc., New York, NY 11216, 1969. i + 244 pp. 17 × 24.5 cm. Price $8.00.

Pharmacological Facts and Figures. Vol. 9. By F. LEMBECK and K. F. SEWING. Translated from the German by H. HELLER and D. R. FERGUSON. Springer-Verlag, 175 Fifth Ave., New York, NY 10010, 1969. viii + 111 pp. 23 × 15 cm. Price $3.00.

NEW JOURNALS

Current Index to Conference Papers in Life Sciences. By World Meetings Information Center, Harry Baum, Director. CCM Information Corp., 909 Third Ave., New York, NY 10022, 1969. iv + 188 pp. 17 × 25 cm. Price: Single copy, $10; Subscription rate, $90 per year.

This index is one of three recently published; the other two indexing the areas of chemistry and engineering. It is available monthly as a bound copy and weekly on magnetic tape to institutions with computer operated information systems. The index represents an attempt to bring to scientists information presented at professional conferences without waiting one to two years for the proceedings to be published. ■

Emergency Health Services Digest. By U. S. Dept. of Health, Education, and Welfare, Public Health Service, Health Services and Mental Health Administration, Div. of Emergency Health Services, 1969. v + 53 pp. 15 × 23 cm. Price: $0.40 per issue.

As a planned semiannual journal published by the Department of Health, Education, and Welfare, this journal contains summaries of articles published elsewhere. Its purpose is to bring together from various sources information concerning emergency medical and health care procedures. ■

Journal of Texture Studies. An International Journal of Rheology, Psychorheology, Physical and Sensory Testing of Foods and Pharmaceuticals. Edited by P. SHERMAN and A. S. SZCZESNIAK, D. Reidel Publishing Co., P.O. Box 17, Dordrecht, The Netherlands, 1969. 9 + 129 pp. 16 × 24 cm. Price: Single copy, $14; Subscription rate, $45 per year. (*English*)

In the introduction, the editors state that because of the interdisciplinary nature of research in textures of foods and pharmaceuticals, they established this quarterly journal to fill the need of having research papers on texture studies published under one cover. In addition to original research communications, it will also contain abstracts of pertinent papers published elsewhere, book reviews, and notices of meetings. Review articles on different facets of texture studies are also planned. ■

Chemico-Biological Interactions. Edited by G. P. WARWICK, Published by Elsevier Publishing Co., P.O. Box 211, Amsterdam, The Netherlands, 1969. 1 + 126 pp. 18.5 × 26 cm. Price: Subscription rate, Dfl. 90 plus Dfl. 5.40 postage per year. (*English*)

The aim of this bimonthly periodical is to bring together papers concerned with the study of changes brought about by exogenous chemicals in biological systems. An important emphasis will be molecular mechanisms. In addition to research articles, short communications and related review articles will be published. ■

NOTICES

Electrometric Methods. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. viii + 131 pp. 13.5 × 22 cm. Price $7.50.

Spectroscopy. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. vii + 183 pp. 13.5 × 22 cm. Price $8.50.

Chromatography. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. vii + 151 pp. 13.5 × 22 cm. Price $7.50.

WHO Expert Committee on Drug Dependence. 17th Report. By World Health Organization. World Health Organization, Geneva, Switzerland, 1970. i + 31 pp. 15.5 × 24 cm. Price $1.00.

Describing Chemical Engineering Systems. By WILLIAM E. RANZ. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. xx + 248 pp. 21 × 27.5 cm. Price $9.95.

American Drug Index. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. vi + 636 pp. 13.5 × 21 cm. Price $8.50.

Actualites Pharmacologiques. By RENÉ HAZARD, J. CHEYMOL, J. R. BOISSIER, and PAUL LECHAT. Masson & Cie, 120 Boulevard Saint-Germain, Paris, France, 1969. i + 347 pp. 16 × 24 cm. (*French*)

Hagers Handbuch der Pharmazeutischen Praxis. Vol. 2. Edited by P. H. LIST and L. HORHAMMER. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. xxiii + 1271 pp. 17.5 × 25 cm. (*German*).

One-Dimensional Two-Phase Flow. By GRAHAM B. WALLIS. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. xxi + 408 pp. 15.5 × 24 cm. Price $16.50.

Health in a Bottle: Searching for the Drugs That Help. By L. EARLE ARNOW. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. ix + 272 pp. 14 × 21 cm. Price $5.95.

Pharmaceutical Abstracts. Vol. X, No. 4. Edited by HENRY M. BURLAGE. College of Pharmacy, University of Texas, Austin, TX 78712, 1969. 21 × 28 cm. Price $6.00. **FINAL ISSUE.**

Handbook of Experimental Pharmacology. Edited by O. EICHLER, A. FARAH, H. HERKEN, and A. D. WELCH. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. ix + 308 pp. 17 × 25.5 cm. Price $34.10. (*principally in German*)

Experimental Medicine and Surgery. Edited by WILLIAM J. SOUTHWELL and ISIDORE GREENBERG. Brooklyn Medical Press Inc., New York, NY 11216, 1969. i + 244 pp. 17 × 24.5 cm. Price $8.00.

Pharmacological Facts and Figures. Vol. 9. By F. LEMBECK and K. F. SEWING. Translated from the German by H. HELLER and D. R. FERGUSON. Springer-Verlag, 175 Fifth Ave., New York, NY 10010, 1969. viii + 111 pp. 23 × 15 cm. Price $3.00.

NEW JOURNALS

Current Index to Conference Papers in Life Sciences. By World Meetings Information Center, Harry Baum, Director. CCM Information Corp., 909 Third Ave., New York, NY 10022, 1969. iv + 188 pp. 17 × 25 cm. Price: Single copy, $10; Subscription rate, $90 per year.

This index is one of three recently published; the other two indexing the areas of chemistry and engineering. It is available monthly as a bound copy and weekly on magnetic tape to institutions with computer operated information systems. The index represents an attempt to bring to scientists information presented at professional conferences without waiting one to two years for the proceedings to be published. ■

Emergency Health Services Digest. By U. S. Dept. of Health, Education, and Welfare, Public Health Service, Health Services and Mental Health Administration, Div. of Emergency Health Services, 1969. v + 53 pp. 15 × 23 cm. Price: $0.40 per issue.

As a planned semiannual journal published by the Department of Health, Education, and Welfare, this journal contains summaries of articles published elsewhere. Its purpose is to bring together from various sources information concerning emergency medical and health care procedures. ■

Journal of Texture Studies. An International Journal of Rheology, Psychorheology, Physical and Sensory Testing of Foods and Pharmaceuticals. Edited by P. SHERMAN and A. S. SZCZESNIAK, D. Reidel Publishing Co., P.O. Box 17, Dordrecht, The Netherlands, 1969. 9 + 129 pp. 16 × 24 cm. Price: Single copy, $14; Subscription rate, $45 per year. (*English*)

In the introduction, the editors state that because of the interdisciplinary nature of research in textures of foods and pharmaceuticals, they established this quarterly journal to fill the need of having research papers on texture studies published under one cover. In addition to original research communications, it will also contain abstracts of pertinent papers published elsewhere, book reviews, and notices of meetings. Review articles on different facets of texture studies are also planned. ■

Chemico-Biological Interactions. Edited by G. P. WARWICK, Published by Elsevier Publishing Co., P.O. Box 211, Amsterdam, The Netherlands, 1969. 1 + 126 pp. 18.5 × 26 cm. Price: Subscription rate, Dfl. 90 plus Dfl. 5.40 postage per year. (*English*)

The aim of this bimonthly periodical is to bring together papers concerned with the study of changes brought about by exogenous chemicals in biological systems. An important emphasis will be molecular mechanisms. In addition to research articles, short communications and related review articles will be published. ■

NOTICES

Electrometric Methods. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. viii + 131 pp. 13.5 × 22 cm. Price $7.50.

Spectroscopy. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. vii + 183 pp. 13.5 × 22 cm. Price $8.50.

Chromatography. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. vii + 151 pp. 13.5 × 22 cm. Price $7.50.

WHO Expert Committee on Drug Dependence. 17th Report. By World Health Organization. World Health Organization, Geneva, Switzerland, 1970. i + 31 pp. 15.5 × 24 cm. Price $1.00.

Describing Chemical Engineering Systems. By WILLIAM E. RANZ. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. xx + 248 pp. 21 × 27.5 cm. Price $9.95.

American Drug Index. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. vi + 636 pp. 13.5 × 21 cm. Price $8.50.

Actualites Pharmacologiques. By RENÉ HAZARD, J. CHEYMOL, J. R. BOISSIER, and PAUL LECHAT. Masson & Cie, 120 Boulevard Saint-Germain, Paris, France, 1969. i + 347 pp. 16 × 24 cm. (*French*)

Hagers Handbuch der Pharmazeutischen Praxis. Vol. 2. Edited by P. H. LIST and L. HORHAMMER. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. xxiii + 1271 pp. 17.5 × 25 cm. (*German*).

One-Dimensional Two-Phase Flow. By GRAHAM B. WALLIS. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. xxi + 408 pp. 15.5 × 24 cm. Price $16.50.

Health in a Bottle: Searching for the Drugs That Help. By L. EARLE ARNOW. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. ix + 272 pp. 14 × 21 cm. Price $5.95.

Pharmaceutical Abstracts. Vol. X, No. 4. Edited by HENRY M. BURLAGE. College of Pharmacy, University of Texas, Austin, TX 78712, 1969. 21 × 28 cm. Price $6.00. **FINAL ISSUE.**

Handbook of Experimental Pharmacology. Edited by O. EICHLER, A. FARAH, H. HERKEN, and A. D. WELCH. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. ix + 308 pp. 17 × 25.5 cm. Price $34.10. (*principally in German*)

Experimental Medicine and Surgery. Edited by WILLIAM J. SOUTHWELL and ISIDORE GREENBERG. Brooklyn Medical Press Inc., New York, NY 11216, 1969. i + 244 pp. 17 × 24.5 cm. Price $8.00.

Pharmacological Facts and Figures. Vol. 9. By F. LEMBECK and K. F. SEWING. Translated from the German by H. HELLER and D. R. FERGUSON. Springer-Verlag, 175 Fifth Ave., New York, NY 10010, 1969. viii + 111 pp. 23 × 15 cm. Price $3.00.

NEW JOURNALS

Current Index to Conference Papers in Life Sciences. By World Meetings Information Center, Harry Baum, Director. CCM Information Corp., 909 Third Ave., New York, NY 10022, 1969. iv + 188 pp. 17 × 25 cm. Price: Single copy, $10; Subscription rate, $90 per year.

This index is one of three recently published; the other two indexing the areas of chemistry and engineering. It is available monthly as a bound copy and weekly on magnetic tape to institutions with computer operated information systems. The index represents an attempt to bring to scientists information presented at professional conferences without waiting one to two years for the proceedings to be published. ■

Emergency Health Services Digest. By U. S. Dept. of Health, Education, and Welfare, Public Health Service, Health Services and Mental Health Administration, Div. of Emergency Health Services, 1969. v + 53 pp. 15 × 23 cm. Price: $0.40 per issue.

As a planned semiannual journal published by the Department of Health, Education, and Welfare, this journal contains summaries of articles published elsewhere. Its purpose is to bring together from various sources information concerning emergency medical and health care procedures. ■

Journal of Texture Studies. An International Journal of Rheology, Psychorheology, Physical and Sensory Testing of Foods and Pharmaceuticals. Edited by P. SHERMAN and A. S. SZCZESNIAK, D. Reidel Publishing Co., P.O. Box 17, Dordrecht, The Netherlands, 1969. 9 + 129 pp. 16 × 24 cm. Price: Single copy, $14; Subscription rate, $45 per year. (*English*)

In the introduction, the editors state that because of the interdisciplinary nature of research in textures of foods and pharmaceuticals, they established this quarterly journal to fill the need of having research papers on texture studies published under one cover. In addition to original research communications, it will also contain abstracts of pertinent papers published elsewhere, book reviews, and notices of meetings. Review articles on different facets of texture studies are also planned. ■

Chemico-Biological Interactions. Edited by G. P. WARWICK, Published by Elsevier Publishing Co., P.O. Box 211, Amsterdam, The Netherlands, 1969. 1 + 126 pp. 18.5 × 26 cm. Price: Subscription rate, Dfl. 90 plus Dfl. 5.40 postage per year. (*English*)

The aim of this bimonthly periodical is to bring together papers concerned with the study of changes brought about by exogenous chemicals in biological systems. An important emphasis will be molecular mechanisms. In addition to research articles, short communications and related review articles will be published. ■

NOTICES

Electrometric Methods. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. viii + 131 pp. 13.5 × 22 cm. Price $7.50.

Spectroscopy. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. vii + 183 pp. 13.5 × 22 cm. Price $8.50.

Chromatography. Edited by D. R. BROWNING. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. vii + 151 pp. 13.5 × 22 cm. Price $7.50.

WHO Expert Committee on Drug Dependence. 17th Report. By World Health Organization. World Health Organization, Geneva, Switzerland, 1970. i + 31 pp. 15.5 × 24 cm. Price $1.00.

Describing Chemical Engineering Systems. By WILLIAM E. RANZ. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. xx + 248 pp. 21 × 27.5 cm. Price $9.95.

American Drug Index. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. vi + 636 pp. 13.5 × 21 cm. Price $8.50.

Actualites Pharmacologiques. By RENÉ HAZARD, J. CHEYMOL, J. R. BOISSIER, and PAUL LECHAT. Masson & Cie, 120 Boulevard Saint-Germain, Paris, France, 1969. i + 347 pp. 16 × 24 cm. (*French*)

Hagers Handbuch der Pharmazeutischen Praxis. Vol. 2. Edited by P. H. LIST and L. HORHAMMER. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. xxiii + 1271 pp. 17.5 × 25 cm. (*German*).

One-Dimensional Two-Phase Flow. By GRAHAM B. WALLIS. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1969. xxi + 408 pp. 15.5 × 24 cm. Price $16.50.

Health in a Bottle: Searching for the Drugs That Help. By L. EARLE ARNOW. J. B. Lippincott Co., East Washington Square, Philadelphia, PA 19105, 1970. ix + 272 pp. 14 × 21 cm. Price $5.95.

Pharmaceutical Abstracts. Vol. X, No. 4. Edited by HENRY M. BURLAGE. College of Pharmacy, University of Texas, Austin, TX 78712, 1969. 21 × 28 cm. Price $6.00. **FINAL ISSUE.**

Handbook of Experimental Pharmacology. Edited by O. EICHLER, A. FARAH, H. HERKEN, and A. D. WELCH. Springer-Verlag, Heidelberger Platz 3, West Berlin, Germany, 1969. ix + 308 pp. 17 × 25.5 cm. Price $34.10. (*principally in German*)

Experimental Medicine and Surgery. Edited by WILLIAM J. SOUTHWELL and ISIDORE GREENBERG. Brooklyn Medical Press Inc., New York, NY 11216, 1969. i + 244 pp. 17 × 24.5 cm. Price $8.00.

Pharmacological Facts and Figures. Vol. 9. By F. LEMBECK and K. F. SEWING. Translated from the German by H. HELLER and D. R. FERGUSON. Springer-Verlag, 175 Fifth Ave., New York, NY 10010, 1969. viii + 111 pp. 23 × 15 cm. Price $3.00.

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

JULY 1970
VOLUME 59 NUMBER 7

REVIEW ARTICLE

Pharmaceutical Sciences—1969: Literature Review of Pharmaceutics

EMANUEL J. RUSSO and TERRY L. BENNEY

Keyphrases ☐ Pharmaceutical sciences—1969 literature review ☐ Pharmacy, general—dosage forms, stability, technology ☐ Physical aspects—pharmaceutics ☐ Biopharmaceutics—1969 literature review

This review of the literature represents a comprehensive cross section of the research and development efforts in various selected disciplines of the pharmaceutical sciences. It is the eighth annual survey of the series (1–7). To compile it, numerous periodicals and selected sections of *Chemical Abstracts* were abstracted.

The review was prepared with two purposes in mind. Primarily, it was to provide an opportunity of reviewing the research highlights of the past year in the field of pharmaceutical technology. Secondarily, it was to supply a convenient source of references for anyone interested in the studies carried out in a specific area. The format was slightly altered from that of previous years; tables were included as a new approach to covering articles of preferred interest in specific areas.

GENERAL PHARMACY

A philosophical review article was published describing the effect of pharmaceutical research on science and society in general (8). Several review articles appeared on governmental control of the pharmaceutical industry in various countries (9, 10). These included a description of the origin, development, and application of the National Drug Code in the United States, as well as the quality control procedures for sampling, stability, and packaging required for pharmaceutical products in Turkey. The Food and Drug Adminstration (FDA) outlined its viewpoint on microbiological control of topical and internal preparations where sterility is not a requirement (11). The primary sources of possible contamination were viewed as being the raw materials, water supply, processing operations, equipment and plant facilities, and employees. Microbiological tests on finished products, to be most meaningful, should include tests for *Salmonella*, *Pseudomonas*, and coliforms. Several other interesting papers described the factors responsible for microbiological contamination of cosmetics and proprietaries (12, 13). The authors believed the training of employees and equipment cleanliness to be the most important factors contributing to microbiological contamination. The articles also contained a brief discussion of the numerous known contaminating organisms, and they described methods to control or decrease their levels.

The optimum pH for many commonly used ophthalmic drugs was presented, along with a discussion of preservatives and buffers suitable for use in ophthalmic preparations (14). The use of macromolecules such as methylcellulose and polyvinyl alcohol in preparing artificial tears and lubricants for contact lenses was discussed in detail (15). Clinical tests indicated that these macromolecules are most effective on the corneal epithelium at a concentration of 1.5%. Another paper reported the formulation and method of preparation

Editor's Note: The scope of this article has been limited to a review of the literature in the area of pharmaceutics because reviews of the literature related to other areas of the pharmaceutical sciences are published elsewhere annually.

Table I—References for Some Relatively Nontechnical Discussions Related to Pharmaceuticals

Reference	Topic
18	Review of local anesthetics
19	Review of antihistamines
20	Review of antiepileptic drugs
21	Review of phenothiazine tranquilizers
22	Review of diabetes and hypoglycemic agents
23	Review of antifertility agents
24	Review of over-the-counter (OTC) antacids
25	Review of sleep-aids and other OTC sedatives

of a stable sulfacetamide ophthalmic solution by avoiding heat sterilization or tyndallization during manufacture (16).

The physiology of the nose and the influence of physical and chemical factors on the penetration of medicaments through the nasal mucosa were outlined (17). The effect of vasoconstrictors, wetting agents, and pH on the duration of drug activity was investigated in guinea pigs.

Other papers of interest in general pharmacy are given in Table I.

Preservatives—The water-solubility and surface-tension-lowering properties of a series of homologous quaternary ammonium compounds were reported to be directly related to their antimicrobial effectiveness (26). Other investigators found no direct relationship between disinfectant effects and other properties of a series of amphoteric surfactants (27). Various alcohols were compared for *in vitro* antimicrobial effectiveness; *n*-propanol was the most effective when used alone, while *n*-butanol, *n*-propanol, and ethanol were very effective when combined (28). Dimethylsulfoxide (DMSO) was shown to inhibit completely the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus megaterum* when used in concentrations of 15% or greater (29). Polyethylene glycol 300 reduced the viability of certain spores with or without the presence of heat (30). The ability of certain organisms (mainly *Pseudomonas* species) to degrade parabens and limit their bacteriostatic effectiveness was demonstrated (31). The action of surfactants on the effectiveness of a wide variety of preservative agents was reported in several articles (32–35). In most cases, the effectiveness of the preservative was shown to decrease after a certain critical concentration of the surfactant was exceeded. The effectiveness of chlorobutanol, chlorohexidene, phenylmercuric nitrate, and polymyxin B sulfate appeared to decrease in the presence of methylcellulose and carboxymethylcellulose (36). In the presence of the latter gum, only phenylmercuric nitrate retained its bactericidal activity. A combination of phenylmercuric nitrate and phenylethyl alcohol was observed to be more effective in preserving fluorescein solutions against *Pseudomonas* comtamination than either of the two agents used alone (37).

Several halogenated naphthol derivatives were tested at different levels in liquid pharmaceutical preparations for effectiveness against 20 different organisms (38). All derivatives were active at levels of 20–50 mcg./ml. against most organisms except *P. aerugenosa*, *Proteus vulgaris*, and *Micrococcus caseolyticus*. The loss of phenylmercuric nitrate and phenylethyl alcohol from solution due to their adsorption by the dropper attachments of ophthalmic packages was described (39). The rubber, nylon, and plastic components tested in this study appeared to adsorb phenylmercuric nitrate more than phenylethyl alcohol. The effect of the electrical charge of a preservative on its bactericidal effect in atropine solutions was used to explain why cationic quaternary compounds are so much more effective than anionic preservatives (40).

Basic mathematical models were suggested for calculating the concentration of preservatives in the aqueous phase of emulsion systems (41). The total concentration of the preservative, the oil–water partition coefficient, the oil–water ratio, and the concentration of the emulsifier were all shown to affect the concentration of the preservative in the aqueous phase, which is the controlling factor. The use of phenylmercuric salts to preserve cosmetics and other ointments was the topic of several interesting papers (42–44). The sensitivity to various preservatives commonly used in cosmetics was also investigated (45). Less than 2% of contact allergies were caused by sorbic acid, but group reactivities were noted with various quaternary compounds and the parabens.

Other papers of interest on preservatives are described in Table II.

Flavor, Aroma, and Color—The effect of formulation and chemical modification on the flavor, odor, and color of drugs was reviewed by several authors (54, 55). The effect of the viscosity of pharmaceutical syrups on the bitter taste was studied using narcotine as the test drug (56). The results of this experiment, obtained organoleptically, indicated that the more viscous vehicles are better able to mask bitterness. The steric requirement of sweet-tasting compounds or molecules was described, based on the differences in sweetness observed between D- and L-sugars and amino acids (57). It was proposed that these steric requirements must be met at the chemoreceptor site if the compound is to have a sweet taste.

A method was devised for quantifying perfume formulations for use in a computer so that fragrances can be classified and reproduced (58). Two interesting papers described the stability of aldehyde-type fragrances and the effect of stabilizers on perfumes present in soaps (59, 60). The maximum rate of color change of a drug solution occurs when the solution is irradiated with light of a wavelength near that of the absorption peak

Table II—Additional References on Preservatives

Reference	Topic
46	Review of disinfectants
47	Review of disinfectants
48	Test method for measuring disinfectant stability
49	Review of preservatives useful in cosmetics
50	Review of microbiological control of cosmetic products
51	Review of disinfectants used in soaps and detergents
52	Review of preservatives useful in ophthalmic preparations
53	Effect of heat and preservatives on spores of *B. stearothermophilus*

of the drug being tested (61). The amount of color change was shown to be independent of the volume of the solution and of the concentration of the drug under constant light intensity.

Stability—Changes in the anionic product of water, in polarity of nonaqueous solvents, and in pK were considered to be the three main factors affecting drug stability during antibacterial heat treatment (62). The effect of ultrasonics on the degradation of aspirin and other drugs in mixed solvent systems was investigated (63, 64). These studies indicated that the application of ultrasonic energy to a system undergoing degradation increases the rate of degradation but does not necessarily change the kinetic order of degradation. It was postulated that the rate changes are due to an increased number of molecular collisions as a result of the addition of ultrasonic energy. The effect of light, oxygen, and humidity on the stability of various oils and drug systems was outlined in a series of papers (65–73). Studies on the stability of phenylephrine in aqueous solution indicated that this compound deteriorates more rapidly at an alkaline pH because of the formation of free radicals, followed by chain-type reactions (74). The discoloration of the phenylephrine was found to depend on the pH and the temperature; it is due to the presence of 5-hydroxy-*N*-methylindoxyl formed by oxidative cyclization of the parent compound (75). The racemization of *l*-phenylephrine was shown to occur readily at a pH lower than 2 (76). This racemization is first order with respect to the phenylephrine concentration and is catalyzed by the hydrogen-ion concentration. The decomposition of pilocarpine in aqueous media was shown to occur in a reversible first-order reaction (77, 78). The decomposition is general, acid/base catalyzed, the products showing pH characteristics comparable to those of other lactones. The effects of sterilization and storage on the stability of aqueous solutions of pilocarpine, atropine, and scopolamine also received attention (79). No appreciable decomposition was noted as a result of the storage of solutions that were unbuffered or contained benzoic acid, while almost complete decomposition was noted in solutions containing phosphate buffers. The photolytic decomposition of sulfacetamide was shown to be of zero order and inhibited by the addition of sodium thiosulfate or by lowering the pH and dielectric constant of the vehicle (80).

The stability and mechanism of degradation of therapeutic methane sulfose derivatives were investigated using ^{14}C-labeled materials (81–83). The addition of antioxidants was shown to retard the color formation resulting from the degradation of *p*-aminosalicylic acid solutions (84). Aqueous solutions of acetylsalicylic acid prepared by the addition of polysorbates were shown to be more stable than similar solutions solubilized by the addition of ethanol (85). Stored under varying conditions of heat, light, and moisture, aspirin tablets showed the least amount of decomposition when prepared with tricalcium phosphate and talc (86). Similar tablets prepared with sodium alginate or polyvinyl pyrrolidinone showed the greatest amount of decomposition after storage for 3 months. The stability of cycloserine, determined in solution as well as in tablet dosage forms stored in various containers, was shown to be very poor

Table III—Additional References on Stability

Reference	Topic
105	Review of stability of drugs in solution
106	Review of chemical changes occurring during storage of drugs
107	Decomposition and stabilization of sulfacetamide in drug preparations
108	Instability of atropine and chloramphenicol in aqueous solution
109	Stability of aqueous solutions of amylbarbital (Barbamyl)
110	Decomposition of barbiturates with unsaturated substituents
111	Effect of impurities on stability of *p*-aminosalicylic acid
112	Decomposition of diethylaminoethyl salicylate hydrochloride
113	Decomposition of aminophenazone tablets
114	Stability of amethocaine hydrochloride solutions
115	Stability of phenoxybenzamine in parenteral solutions
116	Stability of galenical digitalis compositions
117	Stability of angiotensinamide in aqueous solutions
118	Stability of bromoform syrup
119	Stability of hydroxymersalyl-^{203}Hg in storage
120	Stability of inulin labeled with ^{131}I
121	Stability of dyes with gelatin
122	Polyurethane degradation in some physiologically active media

in the presence of water or humidity (87–89). The stabilizing effect of antioxidants on procaine hydrochloride solutions was clearly demonstrated by Moraga *et al.* (90). In solutions buffered to pH 7.9, the specific rate constants for chlorothiazide decomposition were observed to decrease with increasing concentrations of organic solvents such as ethanol, propylene glycol, and acetone (91). The effect of micellar binding on the enzymatic hydrolysis of arylsulfate esters was demonstrated by Baxter and Kostenbauder (92); although acid-catalyzed hydrolysis of the aryl sulfate ester was enhanced when the substrate was bound to the surface of the micelle-forming surfactant, the enzyme-catalyzed hydrolysis of this substrate exhibited a marked overall inhibition when the substrate was bound to the same surfactant. Deviations from first-order kinetics were observed at temperatures below $-9°$ for the hydroxyl ion-catalyzed decomposition of hexobarbital (93). Rate increases occurred in frozen solutions that were as much as 42 times the calculated rates in supercooled liquid solutions at the same temperatures. These increases were partially explained by the increased concentration of the reactants which takes place in the liquid regions of the partly frozen solutions. Similar increases in rate of degradation as a result of freezing have been reported for adrenocorticotropic hormone (ACTH) in human plasma (94). The stability of various antibacterial agents in culture media was shown to affect their activity *in vitro* (95). To be effective against bacteria, these drugs, which are hydrazone derivatives, have to release isoniazid upon decomposing in the culture media. The effect of oxidation on the stability of lecithin, Erolan (wool alcohols DAB7), lemon oil, and methoxy mercurate oils was outlined in a series of papers (96–99).

The instability of benzocaine in throat lozenges was reported to be due to the reactivity of the primary amine group (100). Corn syrup, citric acid, and natural cherry flavors, three commonly used excipients of throat

lozenges, were observed to be incompatible with benzocaine. The stabilizing effect of the *ortho*-hydroxyl group on the acid and base hydrolysis of amides was shown in a comparison of salicylamide to benzamide and other *N*-substituted derivatives (101). Benzamide was even less stable in alkaline than in acid media. The stability of the phenolate ions may result from a resonance effect, with consequent resistance to nucleophilic attack by hydroxyl ions.

Studies carried out on dilute prostaglandin solutions indicated that the F series compounds are very stable in an alkaline pH, while those of the E series are rapidly inactivated. In an acid pH, however, the members of the E series are more stable than those of the F series (102). Contact with various types of rubber stoppers was shown to affect the stability of benzalkonium chloride solutions (103). Autoclaving of carbachol solutions buffered at a pH between 5 and 6 had no effect on drug stability, while a similar amount of heat applied to unbuffered solutions caused 5–10% decomposition (104).

Other papers of interest relating to the topic of stability are listed in Table III.

Stability Kinetics—An apparatus for determining the stability of drugs under nonisothermal conditions was described (123). Details were presented for devices that allow programmed automated control of temperature, light, and pressure within the apparatus. The employment of the stirred-flow technique to develop mathematical equations for complex kinetic runs was described by Taylor (124). A computer program for chemical kinetics was used to update kinetic experiments and to correct the laboratory procedure used in these experiments (125).

In a comparative study of three methods for predicting the stability of aspirin and ascorbic acid solutions, the direct application of chemical kinetics at accelerated temperatures and the use of the Arrhenius equation gave predicted shelflife values that coincided with those obtained experimentally after storage at 25° (126). Maulding *et al.* (127) were able to predict the compatibility of aspirin with various tablet excipients, based on the hydrolysis rates of aspirin observed in aqueous suspension of the excipients.

Hydrocortisone hemisuccinate degradation was observed to occur in consecutive first-order reactions, the ester hydrolysis being facilitated by way of an intramolecular attack. The formation of a species devoid of the 17-dihydroxy acetone side chain subsequent to the formation of the steroid alcohol was confirmed in the experiments (128).

The light-catalyzed oxidation rate of chlorpromazine was shown to depend on the pH of the solution (129). The rate of decomposition was observed to be much slower in vehicles whose pH is between 2 and 6 than in more alkaline vehicles. The pH profile for the degradation of hydrochlorothiazide solutions was determined by several investigators (130, 131). The pH of maximum stability for this compound was observed to occur at 2.5. The kinetics of dihydroergotamine methanesulfonate were observed to be first order, the pH of maximum stability occurring at pH 2.5 (132). The heat of activation of this reaction was calculated to be 12–13 kcal./mole. The autoxidation of sorbic acid in citric acid-containing solutions was shown to be accelerated by very low concentrations of the ferric or cupric ions but inhibited by higher concentrations (133). The acid degradation of aldopentoses to furfural and the further degradation of furfural were observed to be first-order functions of the acid and pentose concentrations (134). Although the heat of activation was the same for all of the aldopentoses tested, the order of reactivities did vary. The steric positioning of the hydroxyls in the reactive forms was postulated to be the controlling factor in the rate of reaction. The rate of inversion of sucrose in aqueous solutions of some strongly dissociated acids was investigated by Pethybridge (135). GLC was used to study the kinetics and characterize the acid hydrolysis of acacia polysaccharides (136). The acid degradation products were shown to consist largely of galactopyranose units (present equally as end groups), 3–6 linked branch points, and chain units.

Alkaline hydrolysis was used by Sun and Connors (137) to identify low molecular weight aliphatic esters. They obtained rate constants characteristic of the entire ester molecule. The hydrolysis of monostearin in aqueous media was noted to follow first-order kinetics and to decrease as the pH decreased (138).

Hydrolysis caused by enzyme systems was the topic of several papers. The pH effects of trypsin catalysis of *N*-benzoyl L-alanine methyl ester were thoroughly investigated (139). The kinetic results in acidic pH's suggest that a group, presumably the imidazole function of a histidine residue, is involved in acylation and deacylation, while those in basic solutions suggest that another group, presumably the $-NH_3^+$ function of a terminal isoleucine, controls the activity of the enzyme at different stages of complex formation. Dittert *et al.* (140) found that pseudocholinesterases present in human plasma were primarily responsible for the enzymatic activity of the plasma on the hydrolysis of 4-acetamidophenyl-2,2,2-trichloroethyl carbonate.

The hydrolysis of acylcholines in both acidic and basic solutions was evaluated in the presence of borate buffers (141). In acidic solutions, acylcholines with longer hydrocarbon chains were considerably more stable than those with shorter chains. In basic solutions, acylcholines degraded in a first-order manner below the critical micelle concentration (CMC) but not above. This phenomenon was postulated to be due to the formation of an anionic fatty acid which complexed with the undegraded cationic acylcholine, thus repressing hydrolysis. The effects of pH and buffer concentration on derivation of rate equations for the hydrolysis of ethyl glucuronate in phosphate buffers received attention in a very interesting paper (142). The kinetics of *meso*-inositol hexanicotinate were investigated in acidic solution to determine the gastric stability of this substance as a possible explanation of its absorption from the gastrointestinal tract (143). The compound hydrolyzes 10 times faster than other nicotinic acid esters in acid solution. The kinetics and mechanism of the reaction between hydroxylamine and succinamide or ethyl acetate were thoroughly investigated by Notari (144–146) in a series of papers. The rate of formation of 2-dimethylaminoethanethiol propionate from propionic anhydride

Table IV—Additional References on Stability Kinetics

Reference	Topic
150	Kinetics of esterification of phthalic anhydride with model alcohols
151	Kinetic study of nucleophilic addition of diethyl phosphate to benzylideneanilines
152	General acid catalysis of the dichromate ion
153	Degradation of phenylsulfonyl-5,5-diphenylhydantoins
154	Kinetics of hydrolysis of phosphatidyl choline and lysophosphatidyl choline
155	Hydrolysis of isothiocyanic acid in strongly acidic solution
156	Kinetics of hydrolysis of *N*-benzylidene halogenanilines in aqueous methanol solutions
157	Kinetics of chloropyramine decomposition
158	Review of hydrolysis of barbituric acid derivatives
159	Kinetics of synthesis of hydroxysulfonated fatty acid esters
160	Kinetics of acetylcholinesterase inhibition by atropine
161	Thermodynamics of the hydrolysis of adenosine triphosphate

and 2-dimethylaminoethanethiol was observed to exhibit a pH dependency which could be explained by assuming the thiol anion to be the attacking species (147). Differences observed in the kinetics of propionyl thiocholine iodide and 2-dimethylaminoethanethiol propionate were attributed to the stabilization of the hydrolysis transition state by a protonated nitrogen at a lower pH and by the quaternary nitrogen atom at a higher pH (148). The rapid stop-flow system was utilized to study the kinetics of the oxidation of cystine by 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole (149). The rate constants were determined, and the mechanism of reaction was postulated for this system.

Other kinetic papers of interest are listed in Table IV.

Antibiotic Stability—The kinetics and mechanism of degradation of ampicillin were investigated by Hou and Poole (162). The pH rate profile in buffer solutions showed a minimum at a pH of 4.85, while at zero buffer concentrations the maximum stability was shifted to a pH of 5.85. Similar studies were carried out with regard to hetacillin decomposition (163). It is clear that hetacillin initially decomposes to ampicillin, the decomposition rate being greatest in the presence of phosphate ions and least in the presence of the citrate ion. The mechanisms and rates of catalysis of penicillins by the cupric ion and aminocatechols were investigated by various workers. The cupric ion–penicillin complexation was postulated to occur at a catalytic site which promotes the rapid degradation of the antibiotic in the presence of the metal (164). The catecholamines were observed to catalyze the hydrolysis of penicillin in neutral pH by a mechanism similar to that postulated for several hydrolytic enzymes (165). The antigen which causes penicillin allergy may be formed by aminolysis of the antibiotic *via* a mechanism involving its decomposition in the presence of poly-L-lysine and tris(hydroxymethyl)-aminomethane (166). Subsequent studies indicated that poly-L-lysine catalyzes the hydrolysis of penicillin to penicilloic acid as well as the aminolysis of the antibiotic by tromethamine (167). Differential thermal analysis was used to identify stearic acid as the inactivating component in tablets of sodium dicloxacillin (168). This method also indicated the incompatibility of stearic acid with penicillin G and sodium oxacillin but not with ampicillin. The results were substantiated by chemical analysis after storage of the combinations at accelerated temperatures. Anionic surfactants were observed to increase the stability of penicillin in ophthalmic solutions and ointments, while cationic surfactants did not appear to have such an influence (169).

Chloramphenicol in solution was observed to decompose faster at pH's above 8, particularly when exposed to light (170). The effect of polysorbate 80 (Tween 80) on the acid hydrolysis and discoloration of chloramphenicol was reported in a series of interesting papers (171, 172). The mechanism of solubilization of chloramphenicol by polysorbate indicated that the palisade layers of micelles are responsible for the solubilization and will hinder the degradation of the antibiotic, especially at alkaline pH's. The addition of polysorbate 80, however, was observed to accelerate the color formation of chloramphenicol solutions, primarily because of contamination by trace metals found in the surfactant.

The kinetics of hydrolysis of antimycin A in solution was reported to follow classical consecutive first-order kinetics (173). The addition of ascorbic acid was demonstrated to prevent the oxidative decomposition of tetracycline due to air, light, and the presence of riboflavin (174). The rates and mechanism of catalysis of streptovitacin A were discussed in an interesting paper by Notari and Caida (175). The dehydration of this antibiotic gave rise to anhydrostreptovitacin A, with no evidence of the formation of the phenolic product.

Additional references on antibiotic stability are presented in Table V.

Vitamin Stability—The influence of various additives on the stability of vitamin A was considered in various papers. The aluminum salts of fatty acids were shown to stabilize vitamin A in mixtures with tocopherols and lecithin (180). Proteins, as found in gelatin, enzyme-hydrolyzed casein, and ground nut protein, displayed a protective action for vitamin A, even in the absence of antioxidants (181). Dextrose and sodium benzoate, however, increased the autoxidation of vitamin A under similar conditions. The influence of water on the stability of anhydrovitamin A in ethanol was investigated (182). The stability was shown to be universally proportional to the water content of the ethanolic solutions.

The addition of chelating agents and the purging of oxygen from the vehicle were shown to stabilize thiamine bromide solutions for use in ampuls (183). These ampuls, after sterilization and storage for 2 years at 20°, still met official potency limits. Thiamine hydrochloride tablets were observed to degrade in a pattern in which an apparent equilibrium was reached (184). The amount of thiamine at equilibrium depends on the

Table V—Additional References on Antibiotic Stability

Reference	Topic
176	Review of stability and incompatibilities of various antibiotics
177	Stability of various penicillins in animal foods
178	Shelflife of chloramphenicol solutions
179	Effect of storage on biological activity of streptomycin solutions

Table VI—Additional References on Vitamin Stability

Reference	Topic
204	Review of methods of stabilizing vitamin preparations
205	Degradation of thiamine solutions
206	Effect of sterilization temperatures on vitamin D_3 injections
207	Review of stability of ascorbic acid solutions
208	Compatibility of vitamin K with various drugs

amount of moisture present and exhibits a minimum at a 5.5% moisture content. The model proposed for such a system presumes that the thiamine dissolved in the water adsorbs on the cellulose and that the thiamine present in the monolayer degrades totally, whereas the thiamine in layers beyond the monolayer does not degrade. The kinetics and mechanism of degradation of various thiamine derivatives were outlined in a series of very interesting papers (185–187). The stabilities of these different derivatives were compared with those of thiamine under similar conditions.

Various flavors present in vitamin B_{12} solutions were found to exert a significant influence on their stability (188). Vitamin B_{12} suppositories were reported to be stable when prepared by coating the microgranules containing the vitamin prior to their incorporation into the suppository mass (189).

The kinetics of cupric chloride-catalyzed autoxidation of L-ascorbic acid were found to be related to the polarity of the solvents employed (190). The ionization of ascorbic acid to the ascorbate ion, which is affected by the solvent polarity, was postulated as one of the factors controlling the rate of degradation of the vitamin in solvents of different polarities. The cupric-ion catalysis of ascorbic acid solutions was hindered by 2% sucrose or 0.1% citrate in the presence of commonly used preservatives (191). The action of Complexon III (ethylenediaminetetraacetic acid) on the stability of L-ascorbic acid solutions was demonstrated to be potentiated by the presence of acetic, citric, tartaric, metaphosphoric, oxalic, and hydrochloric acids (192, 193). Ascorbic acid monostearate was shown to have greater stability than free ascorbic acid, especially in alkaline solution (194). The thermal degradation of thiamine and ascorbic acid combinations in aqueous solutions was investigated (195). Thiamine exerts a protective action on ascorbic acid only in aqueous solutions and not in the presence of citrate or phosphate buffers.

The stability of vitamin D_2 in the solid state was the subject of a very thorough investigation by Takahashi and Yamamoto (196–202). In the presence of calcium phosphate and talc, the vitamin was shown to isomerize readily, yielding isocalciferol and isotachysterol. The isomerization was observed to be catalyzed by surface acid on the excipients. Storage of the powders under conditions of high humidity increased the stability of the vitamin, since the surface acidity was decreased by the absorption of moisture. Further investigations indicated that vitamins such as ascorbic acid, thiamine hydrochloride, and pyridoxine hydrochloride, which have a high surface acidity in the dry state, behave in a similar manner.

The effect of polysorbate 80 and sodium lauryl sulfate on the photolytic degradation of flavine mononucleotide was investigated through measurement of the electron-spin resonance of semiquinone, an intermediate degradation product (203). The degradation was observed to follow first-order kinetics in the dark, both additives accelerating the formation of the semiquinone derivative.

Other references pertaining to vitamin stability are given in Table VI.

PHARMACEUTICAL TECHNOLOGY

A series of review articles on the current aspects of pharmaceutics discussed a wide number of topics, including drug release and absorption, drug solubility, preservatives, chemical stability, physical stability, and packaging (209–212). Other articles outlined the general concepts involved in the formulation of products containing corticosteroids or hormones having polypeptide structures (213, 214) and reviewed the possible incompatibilities of these hormones with the excipients present in various dosage forms. Schumacher (215) presented a very thorough review of the bulk-compounding technology utilized in the preparation of liquids, suspensions, emulsions, and ointments. The main trends in pharmaceutical research and development were also aptly reviewed (216).

Parenterals—Possible contamination of parenteral products by the packaging components was the topic of several interesting papers. Contamination with heavy metals from ampul glass as a result of washing was eliminated by an acid rinse followed by a wash with deionized water (217). Zinc ions eluted from rubber stoppers were shown to affect the stability of dextran solutions (218). White rubber stoppers were shown to elute more zinc than black butadiene acrylonitrile rubber stoppers (219). Atomic absorption spectrophotometry was used to detect amounts of silicone removed from disposable plastic syringes by distilled water washes (220).

The factors influencing the turbidity occurring in parenteral solutions containing polysorbate 80 and benzyl alcohol were pointed out (221). The turbidity was determined to be a function of the concentrations of the components and the temperatures to which they are exposed. The use of benzyl benzoate in preparing solutions of steroids in peach oil was described (222). Such oil solutions of hydroxyprogesterone capronate and androstenediol dipropionate did not show the presence of crystals after 1 year of storage at −26°.

A closed system of incorporating nitrogen into vials was found to be much more effective than conventional systems for preventing the degradation of the active agent by aerobic oxidation (223). The advantages and methods employed in the nitrogen flushing of products stored in metal containers were discussed (224).

A new stratified lyophilization technique and an example of the use of this technique in preparing ACTH preparations with prolonged action were described in two interesting papers (225, 226). The presence of isonicotinic acid hydrazide was shown to decrease significantly the potency of streptomycin and *p*-aminosali-

cylic acid after freeze-drying (227). Solutions prepared without the hydrazide but under otherwise similar conditions showed no losses in potency. Injectable solutions of sodium sulfathiazole in a 10% sorbitol solution stored under nitrogen were shown to be stable after storage for 14 months at 37° (228).

Other papers of interest in the category of parenterals are given in Table VII.

Sterility—A very complete review of the different techniques utilized for sterilization was presented by Doyle (233). The advantages of the continuous sterilization technique were presented in two excellent papers (234, 235). Such sterilization is accomplished in towers in which carrier-conveyor baskets filled with containers pass through a succession of zones where the temperature is regulated by water, steam, and air. This method was postulated to be superior to the classic autoclave method.

The efficiency of gaseous sterilization was described by several authors (236, 237). A nonexplosive mixture of ethylene oxide and methyl bromide was shown to be effective against a variety of spores (238). Sterility was achieved in 2–48 hr., depending on the object to be sterilized and the conditions applied. The adsorption and desorption of ethylene oxide on rubber, polyethylene, and polyvinyl chloride objects were investigated (239). It was shown that the adsorbed gas remains as a residual for prolonged periods of time unless desorption is accelerated by heating. Ethylene oxide-sterilized plastic medical devices showed no significant toxicities 24 hr. after sterilization (240). The disinfectant action of peracetic acid and formaldehyde on equipment was also demonstrated in a series of interesting papers (241–243). Complete sterilization of microbes was achieved with a 0.5% peracetic acid solution in 1–30 min.

The bacteriological aspects of sterilization by the use of γ-radiation were reviewed, with emphasis on the mechanism of action and the factors influencing the radiation resistance of bacteria and other microorganisms (244). The specific uses of radiosterilization in pharmaceutics were reviewed by several authors (245, 246). A dose of between 2.5 and 4 mrad sterilized all solutions tested. Physical as well as chemical changes were observed in certain cases, especially when aqueous solutions were irradiated. Similar losses in potency were described after irradiation of aqueous solutions of atropine, morphine, and lidocaine (247). The amount of degradation was increased by an increase in the radiation and by a concentration of oxygen above the irradiated solution. Sterilization of hydrocortisone and chloramphenicol ophthalmic ointments by γ-radiation did not cause any change in the physical or chemical properties of the ointments but did increase the number of free radicals present (248). No changes in characteristics or properties of surgical appliances, especially those of biological origin, were observed after irradiating them with 2.5 mrad (249). This radiation level gave complete sterility of all products tested. Changes induced in fats and glucose solutions as a result of heat sterilization were described in two papers (250, 251). These changes were reportedly caused by the high temperatures required.

Table VII—Additional References on Parenterals

Reference	Topic
229	Chemical resistance tests on Indonesian ampuls
230	Use of homemade detergents in cleaning ampuls
231	pH and resistance of liquids used in injectables
232	Review of incompatibilities of numerous injectable drugs

Methods of sterilizing objects by the use of steam as well as by dipping them into chemical preservative solutions were studied (252, 253). Although these techniques can be used to sterilize Plexiglas objects and polyethylene hypodermic syringes, none is capable of sterilizing rubber stoppers.

Various techniques for sterility testing of pharmaceutical products were described in two review articles (254, 255). The membrane filtration technique was considered the method of choice for determining the bacterial content of liquids. The significance of sample size and sample ratios in sterility testing of drug products was discussed in a very interesting paper (256). To eliminate preservative or drug effects in testing parenteral solutions, the optimum sample size is 10 ml. and the optimum dilution in the culture medium is 1:64. Thioglycollate was deemed an unsatisfactory medium for general sterility testing, especially when only a small amount of contamination is present (257). This medium was felt to be reliable only for the detection of *Proteus* species.

Tablets and Capsules—Some of the controls and testing methods used in tableting were outlined in an excellent article by Sinotte (258). Special consideration was given to the potential for automatic testing and the advantages and disadvantages of automating these processes.

The concepts of comminution and its effects on the properties of powders thus ground were briefly reviewed in several papers (259, 260). The factors affecting the efficiency of grinding during jet milling were studied for the grinding of calcite using a 20-mm. diameter jet mill (261). These experiments evaluated the effect of the feed rate of the solids, the injector nozzle pressure, and the grinding nozzle diameter on the particle-size distribution. Several new techniques for measuring particle size of powders were investigated. An average particle-size-measuring technique was developed, giving standard deviations of approximately 2.5%, based on the observation that the bulk volume of powders needed to form a single layer on an area of fixed size is proportional to the average particle size. This method requires the use of calibration curves for different types of solids and is less applicable to powders and very small nonspherical particles which do not pack reproducibly (262). The use of an attenuated light beam passing through a settling suspension of particles was utilized to develop theoretical curves for determining the size distribution and specific surface of the powder being investigated (263).

The effect of particle shape on the angle of repose, bulk density, and rate of flow through an orifice was investigated using sand (264). In all size ranges in which

there is an increasing departure from the spherical, the angle of repose increases while the bulk density and flowability decrease. The rate of flow of a powder through a circular orifice was reported to be a function of the particle size, bulk density, cohesiveness, tensile strength, and shear index of the powder. Different methods of measuring cohesiveness and the effect of glidants and particle size on cohesiveness of a series of powders were demonstrated (265). In the case of granular solids passing through a circular orifice, differences due to shape, density, porosity, and friction of particles could be eliminated by using the term for bulk density instead of particle density in the flow equation (266). The packing properties of powder particles in centrifugal fields were investigated using calcium carbonate and aluminum oxide powders (267, 268). The results supported the assumption that the bulkiness of a fine powder is the product of the forces of interparticle adhesion and the external forces acting on the individual particle. The use of a shear cell has been proposed to measure the cohesiveness and tensile strength of powder as well as the mechanistic behavior of such powder under shear (269, 270). Some of the processes considered are plastic deformation and structural changes such as consolidation or dilation, blockage to resist continuation of motion, and particle orientation, all of which occur in the powder bed. Utilizing this apparatus, a relative shear force and index of retention value can be defined.

The effect of lubricants and surface-active agents on the flow of powders was evaluated. Studies on a capillary vibroviscosimeter indicated that the addition of surface-active additives lowers the viscosity of a flowing powder. The magnitude of this effect, however, was decreased by increasing the vibration rate (271). The viscosity and the angle of repose increased and the packing density decreased with an increase in temperature above 100°, a level at which moisture has a negligible effect on the adhesiveness of powders (272). Lubricants were observed to reduce the angle of repose of powders and to improve their flow characteristics (273). With the addition of ultrafine solid additives, the adhesive forces of strongly adhesive materials were decreased and those of less adhesive powders were increased (274). These results were interpreted to mean that an ultrafine additive roughens the surface of a powder particle enough to increase its friction coefficient and to decrease its adhesion forces.

Based on a diffusion model, Hogg *et al.* (275) developed a quantitative theory to describe the mixing of particulate systems in which one component is present in trace quantities. The theoretical expressions derived to predict the rates of mixing of identical components, both as spherical and as angular particles, agreed with the data obtained. Three probability distributions were defined governing particle movement within a horizontal drum mixer (276). A relationship for the diffusion coefficient, based on the probability distributions, was derived from Fick's first law. The frequency of collisions resulting in particle movement was determined by the interaction between mixer speed and load. The average distance that a particle travels is affected by mixer load but not by speed. The convective and surface mixing of tracer particles of a granular material over a horizontal blade moving relative to a bed was investigated (277). The horizontal movement is determined by the blade height and immersion and the relative velocity of bed to blade and gravity, while the vertical movement is also affected by the particle diameter. The relationship between sample weight and the coefficient of variation as a mixing index and mixing ratio was examined in a V-shaped mixer, utilizing different mixing ratios (278). In the case of adhesive ingredients such as salicylic acid, the charging point and the size of the diluent were found to have a marked effect on the mixing rate. Such phenomena were believed to be due to differences in adhesive force between the same and different kinds of particles or between the particles and the walls of the mixer.

The physical and mechanical factors affecting the compression of tablets were described in a wide range of papers. The powder segregation that occurs during die filling consists of the finer particles ("fines") filtering through to the bottom of the moving powder mass, where it builds an inner mound of fine rich material on the bottom of the die and an excess of coarser material at the periphery of the die cavity (279). This segregation is lessened when the amount of fines is decreased, the rate of die fill is increased, or the height from which the powder falls is increased. The pressure on the die wall was observed to increase with a decrease in the hardness value of the material being compressed (280). The maximum compression force required for tableting granules was dependent on the granule size, although the energy required for tableting was similar for all sizes (281). The smaller granules were observed to decrease the time of the first compression phase. Experiments with an automated tablet press showed that for the limited range of dimensions applicable to most pharmaceutical tablets, there exists a common linear relation between the applied compaction pressure and the force lost to the die wall per unit area of apparent die wall contact during compression (282). During tablet compression, pressure is believed to be transmitted in two directions: along the compression and along the die wall (283). A model was presented to show the phenomena which occur during repeated compressions; this model illuminates some facts about the internal structure of compression-molded substances and illustrates the need for lubricating agents (284).

The physical and mechanical factors utilized in preparing directly compressed tablets were discussed (285). Modification of the specific gravity of the compressed material was observed to modify its other characteristics. The tableting properties of directly compressible starch, STA Rx 1500 brand of starch, and a material derived from beets were also described (286–289). The fluidity and compressibility of these materials made them especially useful for direct compression. The effects of hydroxypropyl methylcellulose, carboxymethyl dextran, starch dextrans, xylitol, talc, and cyclamic acid on the properties of tablets were discussed by a great many authors (290–302). Polytetrafluoroethylene was successfully used as a lubricant in the compression of powders as well as being bonded to the tips of punches to reduce the adhesion between them and the

tablets (303). The properties of Aerosil (pyrogenic silica) and its use as a glidant were discussed. In some of these studies the addition of Aerosil increased the disintegration time of tablets prepared from inorganic salts (304–306). The crystalline form as well as the granular character of lactose was observed to affect the properties of tablet formulations (307). In a thorough investigation, dicalcium phosphate, calcium sulfate, and magnesium carbonate were compared with glucose and sucrose as diluents in the tableting process, the relative merits being judged by the quality of the finished tablets (308). The optimum tableting pressure for these materials was determined, and the effect of this pressure on the required ejection force and the tablet properties was discussed. Under the experimental conditions, the inorganic salts were clearly superior to glucose and sucrose as diluents. In tablets prepared with polyethylene glycol 4000 as a binder, not only the active ingredient used but also the granulation procedure and the moisture present in the powder mixture were observed to affect the hardness and porosity (309).

The effect of various materials on the disintegration times of tablets was well documented (310–318). In general, additives were placed into three classes: those acting as capillary-forming agents, as in the case of starch; surface-active agents such as Tween 80; and swelling agents such as ultraamylopectin. The latter two types were more effective than starch in lessening the disintegration time of certain hydrophobic materials. The mechanism of tablet disintegration was discussed on the basis of cohesive and adhesive properties of the particles. For a tablet to disintegrate, the dispersion of the particles caused by the penetration of water must overcome the forces binding the particles through cohesion and adhesion (319). The inhibitory effect of magnesium stearate on the penetration of liquids into tablets was found to be roughly proportional to its concentration in the tablets (320). With tablets of sulfamethazine, high-viscosity binders prolonged dissolution and hydrophilic binders shortened it, although both types of binders gave similar disintegration times (321). The dissolution rates in both cases were shown to be directly proportional to the amount of binder added. The particle size of aspirin after disintegration from tablets was shown to be different from that used in preparing the tablets, even in the presence of starch. Large particles were broken, while small particles were observed to be agglomerated and partially aggregated to larger particles, thereby negating the therapeutic advantage of finely divided aspirin (322). The effect of tablet form on physical properties was the subject of an interesting paper (323). It was noted that the disintegration time of tablets was dependent on the surface area, flat tablets disintegrating somewhat more rapidly than convex tablets. The breaking strength of convex tablets, however, was found to be greater than that of flat tablets. Taking all factors into consideration, the authors concluded that strongly convex tablets are often preferable.

The porosity, pore-size distribution, and water permeability of several different coating powders used for subcoating sulfathiazole tablets were evaluated (324). Powders consisting of acacia, sucrose, titanium dioxide, talc, calcium carbonate, and Aerosil formed the coatings that were considered to have the best properties. Exclusion of Aerosil from this powder resulted in the formation of a more compact coat having fewer and smaller pores. Polyvinyl acetal diethylamino acetate was investigated for use in preparing a gastric-soluble protective coating for tablets (325). The effectiveness of this material, which is soluble in a wide variety of organic solvents and aqueous solutions of pH less than 5.8, was determined *in vivo* by obtaining blood levels following the administration of coated triacetyloleandomycin and barium sulfate coated tablets to dogs. Other coating materials evaluated with regard to their properties and physical stability on aging were those consisting of zein, polyvinylpyrrolidinone, sodium carboxymethylcellulose, cellulose acetate phthalate, Polyox WSR 205, and WRS 301 (water-soluble resins) (326–328).

The manufacture, filling, and coating of hard-gelatin capsules were very capably reviewed by Jones (329). The transfer of water vapor through capsules and the experimental equilibrium water constants were determined for encapsulated starch and microcrystalline

Table VIII—Additional References on Tablets and Capsules

Reference	Topic
333	Comparison of methods of determining specific surface of powders
334	Influence of particle size on fluidity of binary mixtures of starch with various pharmaceuticals
335	Comparison of adhesive forces of different pharmaceutical powders by centrifugal method
336	Method proposed for the efficient separation of spherical from nonspherical particles
337	Photographic study of the two-dimensional flow of steel balls
338	Influence of physical properties of particulate solids on the rate of mixing and segregation
339	Comparison of microhardness and elastic modulus of crystalline pharmaceutical materials
340	Significance of formulation on the lower punch pull-down force required in rotary tablet machines
341	Review of formulation variables and tableting factors on weight distribution of tablets
342	Encapsulation of liquid and solid aerosol particles to form dry powders
343	Review of theory and operation of granulation of pharmaceuticals
344	New methods of granule production
345	Effects of granule size on homogeneity of tablet weight
346	Effects of moisture on tablet manufacture
347	Effects of storage on dissolution of aspirin tablets
348	Expansion of compressed starch tablets due to moisture sorption
349	Review of absorbable tablets
350	Determination of the optimum weight of tablets
351	Formulations for soluble tablets for preparing eye drops
352	Formulations for the manufacture of sodium *p*-aminosalicylate tablets
353	Use of urea in preparing tablets of benzalkonium chloride
354	Processing of urea inclusion compounds into tablets
355	Review of various coating materials for use in tablets and capsules
356	Preparation of subcoatings in the manufacture of coated tablets
357	Use of shellac and seed lac as enteric coatings for tablets
358	Review on color coating of tablets
359	Characteristics of Farmoids in sugar coating of drugs
360	Use of synthetic dyes in tablet making
361	Review of polymers useful in preparing capsules soluble in intestinal fluids

cellulose stored in closed containers (330). The equilibrium constants and the estimated values calculated from the sorption isotherms of these substances were observed to be in good agreement. Capsules were evaluated for filling characteristics after being filled with cornstarch or lactose, with or without Aerosil as a glidant, by a semiautomatic ring-filling method. On powders without Aerosil, the coefficient of variation of the dose showed that filling by a screw auger was more accurate than by a flat one. The powders containing Aerosil exhibited maximum deviations between 0.1–2% of the glidant (331). The use of a shellac coating on capsules of pancreatic enzymes to prevent dissolution in gastric fluids was evaluated both *in vitro* and *in vivo* (332). This coating inhibited release of the enzymes for at least 2 hr. in artificial gastric juice, yet the enzymes were released in less than 1 hr. when placed in synthetic duodenal fluid.

Further references of interest in the field of tablets and capsules are given in Table VIII.

Suspensions—The effects of the nature of the suspended solids, surface charge ζ-potential, and shear rate on the properties of dispersed systems were thoroughly reviewed in several excellent papers (362–364). A rotational viscosimeter was used to measure the apparent rheological properties of rapidly settling suspensions (365). Using this data, a generalized correlation between the viscosity of the medium and the viscosity of the suspension was developed to predict the apparent viscosity of such rapidly settling suspensions. Measurements were presented on kaolin suspensions, which confirmed that interaction between the suspended particles, macromolecules of the suspension media, and electrolytes influences the flow behavior and stability of the suspensions (366). The adsorption of ions on the surfaces of suspended particles, by changing their distribution in an electrical field, influences their coagulation, flocculation, and sedimentation characteristics through the formation of electrical double layers. The sedimentation and flocculation of dispersed phases were investigated utilizing suspensions of calcium carbonate (367). The volume of immobilized medium present on the particle surface varied, depending on the kind of suspending medium; the larger the volume of the immobilized medium, the greater was the effect on structural viscosity. In a review of the mechanism of flocculation and floccule sedimentation, it was pointed out that theories applicable to colloidal systems must be modified when paracolloidal particles are present (368). The existence of order in dilute colloidal suspensions due to long-range electrostatic forces was shown by the measurement of diffraction of light caused by such suspensions (369).

The effect of the concentration and the shape and size of the solid on the settling of a suspension was outlined in several papers (370–372). Numerical data obtained by utilizing such a model system compared well with the actual experimental data obtained using aluminum oxide suspended in salt solutions.

The rheology of kaolin suspensions was the topic of many interesting papers. The curves of yield stress *versus* pH were shown to exhibit a maximum for sodium kaolinite at pH 5.75, for aluminum kaolinite at pH 7.95, and for the acid form of kaolinite at pH 7. The maximum appeared to occur at the zero point of charge of the kaolinite edges, which is partly dependent on the ionic environment in which the clay is prepared (373). The kaolin–water system was used to illustrate techniques for determining the electrophoretic mobility of particles in suspension (374, 375). These data were used to show the relation between the salt flocculation value of a suspension and its electrophoretic mobility.

The viscosity of various kaolin suspensions was observed following the addition of a basic aluminum chloride complex (376). After addition of only small amounts of the complex, the clays still showed individual differences; but at higher concentrations, common trends could be distinguished, indicating that the adsorption of the complex masks the individual surface characteristics of the kaolin particles. The negative surface charge of kaolin was shown to increase during oven drying of suspensions of kaolin containing a soluble phosphate (377). The viscosity of certain kaolin–water systems was correlated with the montmorillonite content of kaolin (378). Transition from low viscosity to high viscosity occurred when the montmorillonite content exceeded 5%. Adsorption of hexadecyl sulfate by kaolinite crystals was postulated to occur on edges of the tabular kaolinite crystals, forming a bimolecular layer which causes the edges to become negative and deflocculation to occur (379). The rheological behavior of other clay systems was also extensively investigated. The electrical double-layer theory was used to analyze an idealized clay particle system, with the purpose of describing the mechanical stability in terms of attractive and repulsive forces (380). The general pattern of behavior of the model was shown to be compatible with the behavior of the clay mass. The chemical factors involved in the flocculation of clay slurries were pointed out by Slater *et al.* (381). For a polymer to act as an effective flocculant by the "bridging mechanism," it must have a certain extended configuration in solution which is influenced by pH, ionic strength, and polyvalent counterions, and the polymer must be adsorbed on the mineral by multiple functional groups. In various systems, this attachment may occur by electrostatic attraction, hydrogen bonding, dipole interaction, or even covalent bonding. The use of polyacrylamine and polyvinyl alcohol in flocculating and increasing the structural strength of bentonite suspensions was pointed out (382, 383).

Factors affecting the long-term settling characteristics of clay suspensions were described in an interesting article (384). Temperature and concentration were shown to affect greatly the settling characteristics of the clays tested. Layering formed most readily in aqueous vehicles having a low viscosity and density. A mechanism was postulated for the deflocculation of clays in nonaqueous systems by the addition of amines and amino acetates (385). The partition of the amine between the clay and the organic liquid was measured, and this was considered an important factor in the deflocculating efficiency of the amine.

The effect of surfactants on the stability of suspensions of bentonite, polyethylene, carbon black, and kaolin was the subject of several papers (386–389). In most

Table IX—Additional References on Suspensions

Reference	Topic
396	Condensation stability of dispersed systems
397	Review of factors affecting the stability of pharmaceutical suspensions
398	Review of theory of dielectric dispersion of colloidal particles
399	Review of the formation of dispersed systems and spatial structures of these systems
400	Comparison of techniques of measuring particle size of suspensions using microscopically calibrated glass beads
401	Spatial distribution of particles in a suspension
402	Flow behavior of kaolinite suspensions
403	Use of sucrose esters as suspension stabilizers
404	Electrostatic forces between clay and cations as calculated and inferred from electrical conductivity
405	Thixotropy of clays
406	Influence of monomolecular nonionic agents on the behavior of drug suspensions
407	Effect of surface and particle size of solid phase on sedimentation of talc and zinc oxide suspensions
408	Effect of solid-phase concentration and mixing ratio of a second solid on the sedimentation of talc and zinc oxide suspensions
409	Effect of manufacturing conditions of sulfonamide and chloramphenicol suspensions on their physical properties
410	*In vitro* studies of antacid suspensions
411	Effect of magnetic treatment on sedimentation volume of montmorillonite suspensions
412	Sugar-containing suspensions of chloramphenicol
413	Crystal growth studies involving phase transitions in aqueous suspensions

cases, unstable hydrophilic particles can be stabilized by the addition of surface-active agents. The temperature for optimum stability of these systems, however, was shown to increase as the chain length of the surfactant used was increased. The relation between the extent of dilatancy and colloidal stability of suspensions was studied utilizing polysodium methacrylate as a dispersing agent (390). The colloidal stability of this system was varied by changing the degree of dissociation of the acid groups of the polymer. The extent of adsorption of the dispersant was found to correlate well with the viscosity measurements. The increase in viscosity could result from an increase in flocculation due to shear. The pH of maximum stability and the maximum ζ-potential were observed to occur between 7 and 10 for titanium dioxide suspensions (391). This system has an isoelectric point at pH 3, when surface hydroxyls on the titanium dioxide surface are completely neutralized. Above pH 10 the ζ-potential of this system once again drops, with a resultant decrease in stability. The stability of aluminum hydroxide in normal alcohols and toluene stabilized with surface-active agents was postulated to be due to the double-layer electrostatic repulsion forces on the gel (392). The effect of impurities on the charge of aluminum oxide in aqueous suspensions was also determined (393). The ζ-potential of the less pure oxide was greater, resulting in increased structural characteristics of the suspensions. The impurities on the surface of the aluminum oxide particles were postulated to increase the thickness of the firmly bound layer, consequently increasing the effective particle radius and lowering the amount of dispersive medium.

The effects of ultrasound on the properties of suspended solids were demonstrated in two interesting papers (394, 395). Ultrasonic energy was shown to have a greater effect on larger particles than on smaller particles. A logarithmic relation was demonstrated between the increase in solid surface and the concentration of suspended solids. Increasing the viscosity of the dispersing medium appears to reduce the increase of solid surface, while the addition of surfactant enhances it. The maximum increase occurs in the region of the CMC of the dispersed system. Ultrasonic energy may increase the solid surface by reducing the particle size of some materials and the agglomeration of others.

Other articles of interest relating to suspensions are described in Table IX.

Emulsions—Review articles were published on emulsion theory and practice, recent advances in emulsion processes, and critical factors affecting emulsion systems (414–417). A computer was utilized to solve the fundamental flocculation-rate equations, including the effects of polydispersity and the particle–particle potential energy barrier (418). These calculations were based on the assumption that the only rate-limiting process is the passage of particles over the primary electrical barrier to the flocculation of the suspension. The description of a Coulter counter technique for obtaining size distribution of oil-in-water emulsions was presented (419). Although this method compares well with other measuring techniques when certain precautions are taken, it has the same disadvantage of being rather laborious.

The flow characteristics of oil-in-water liquid petrolatum emulsions were investigated using a cylinder-type viscosimeter (420). The shear rate–shear stress curves showed a hysteresis loop which changed from a dilatant to a thixotropic form in accordance with change in distribution of particle size and state of aggregation of the emulsion. The increase in the fineness of particle size of the dispersed phase was shown to increase the viscosity of the emulsion (421).

The distribution of surfactant between the water and oil phases at the point of immersion was a contributing factor in the type of emulsion formed (422). Placing more surfactant in the aqueous phase was shown to favor the formation of an oil-in-water emulsion. No relation was observed between the rate of coalescence of oil droplets in an oil-in-water emulsion and the interfacial tension (423). Although the stability of the oil drops differs from the so-called "emulsion stability," the rate of coalescence of drops was observed to correlate with such stability. The effect of the hydrophilic-lipophilic balance (HLB) of the emulsifiers on emulsion stability was investigated by a number of workers (424–429). The size of the emulsion droplets changed remarkably with temperature and HLB of emulsifier. The droplets coalesced more readily when close to the phase-inversion temperature, and relatively stable oil-in-water emulsions were obtained when the phase-immersion temperature was 20–65° above the storage temperature of the emulsion. At optimum stability, emulsions are relatively insensitive to changes in HLB values but are quite sensitive to the phase-inversion temperature of the system being investigated.

Table X—Additional References on Emulsions

Reference	Topic
442	Relation between composition of emulsion and its viscosity
443	Continuous oil phase emulsion and inversion
444	Studies of density gradient in certain oil-in-water emulsions using mechanical γ-ray analysis
445	Structure determination of superfat oil–water emulsions
446	Mechanism of emulsification with special reference to solid stabilized emulsions
447	Evaluation of hydrogenated lanolin, a new oil–water emulsifier
448	Characteristics of ethoxy polysiloxane oil emulsions

The stabilization of oil-in-water emulsions by the use of nonionic detergents was investigated in a series of papers by Elworthy and Florence (430–432). The higher stability obtained by increasing the glycol chain length of the emulsifier was not due to a raised surface potential but was ascribed to entropic effects. The rate of coalescence of the emulsions was related to the ζ-potential, surface concentration, and polyoxyethylene chain length. In these systems, the adsorbed film was observed to increase the attractive forces between particles. The apparent CMC determined from the interfacial tension–concentration curves were higher than those obtained at the air–water interface. The effect of cationic detergents on emulsions stabilized by acacia and sodium alginate received attention (433). The ζ-potentials and particle sizes were obtained so that the interaction energies could be calculated for these systems. By utilizing the observed degrees of aggregation, the van der Waals constant was estimated and the binding parameters, numbers of binding sites available, and free energies of absorption were calculated. Emulsions prepared with Carbopol (water-soluble resin) neutralized with hexylamine and 2-ethylhexylamine were noted to give stable emulsions, while other shorter or longer chained amines yielded poorer emulsions (434). These results were rationalized on the basis of polymer confirmation and HLB. The use of lecithin, starch phosphate, gelatin, and other hydrophilic colloids as emulsifiers was evaluated in a series of papers (435–437). The viscosities, ζ-potentials, and stabilities of these emulsions were determined. The temperature effects and the effects of adding electrolytes or hydrophilic colloids to solid, stabilized, kerosene–water emulsions were studied (438). Large quantities of electrolytes were observed to have a deteriorating effect, which was ascribed to chemical changes taking place in the stabilizers. The addition of hydrophilic colloids led to better emulsification and finer dispersion. This behavior was attributed to the formation of a protective layer which hinders coalescence of the oil droplets. The use of mixtures of surfactant fatty alcohol emulsifiers to stabilize oil-in-water emulsions and alter their consistency from fluid to semisolid was described (439). A mechanism involving the formation of a viscoelastic network in the continuous phase was proposed to explain this self-bodying action.

Peterson (440) thoroughly reviewed factors affecting nonaqueous emulsion systems and their stability. Anionic emulsifiers are best suited for the preparation of stable emulsions; glycerin appears best at low emulsifier concentrations. Measurement of particle size after long periods showed that such emulsions have good stability during aging. The factors affecting the formation of microemulsions and their resulting properties were outlined in an interesting paper (441). The presence of excess electrolyte in the aqueous phase was shown to inhibit microemulsification. Microemulsions in systems based on soaps are strongly cation dependent, while those prepared with dodecyl sulfate are independent of the nature of the cation. Changing of the water–emulsifier ratio was observed to have no effect on either the total interfacial area or interfacial stoichiometry of the microemulsion tested.

Other articles related to emulsions are presented in Table X.

Ointments and Creams—Ointment bases were classified according to their properties in several interesting review articles (449, 450). Lipophilic and hydrophilic ointments, the first two classes, were further subdivided according to the chemical composition of the bases. Diphilic ointments, the third class, were divided into those containing emulsifiers and emulsifier-type bases.

Rheological measurements were made on a number of ointment bases over a wide range of temperatures (451, 452). The addition of a small quantity of complex material to a paraffin ointment base was shown to change its behavior from elastic to viscoelastic. These viscoelastic materials were all assessed in creep testing, where fundamental parameters were provided and the rheological behavior was represented by mechanical models. There was a distinct difference between the behavior of creams prepared with ionic and nonionic soaps. Lipophilic emulsifiers, such as wool fat alcohols, were noted to reduce the yield value and thixotropy of petrolatum, but complex emulsifiers had the opposite effect (453, 454). The effect of soaps on the yield value and consistency of oil-in-water emulsion ointments was also carefully evaluated (455). The use of stearic acid–sodium lauryl sulfate combinations in these systems led to the formation of creams, which were actually fluids without a gel structure, while cetyl alcohol–sodium lauryl sulfate or cetyl alcohol–sodium stearate combinations formed ointments with a measurable rigidity. The internal structures of ointments prepared with bentonite, ichthyol ointment prepared from petrolatum and methylcellulose bases, sulfonamide ointments, and zinc paste were evaluated using various rheological testing methods (456–462). In most cases, a thixotropy was observed which changed markedly at higher temperatures, approaching the idealized state. Regeneration of the inner structure was usually observable by 24 hr., although the time varied, depending on the surfactant present in the ointment system.

The use of the newer surfactants and emulsifying agents in the preparation of emulsion ointments was thoroughly reviewed by Goldenberg (463). The effect of various emulsifiers on the emulsion system of creams was also studied (464). This paper described the preparation of an ambiphilic cream system, one that can be diluted with either water or oil and still give a stable emulsion. It also listed the properties of other creams prepared with different emulsion systems. The ad-

vantages of methylcellulose as a base in preparing various ointments was reviewed (465). Although active materials were observed to penetrate the skin to a greater extent in ointments prepared with methylcellulose than in those prepared from other commonly used bases, too great an amount of methylcellulose in an ointment should be avoided, because it may interfere with the healing of wounds by the formation of a film. The use of polyhydric alcohols, sodium lauryl sulfate, cetyl alcohol, decaglycerol esters, sugar esters, and other complex esters in preparing ointments of various types was also considered (466–470). The viscosity and some of the other properties of ointments prepared from these materials were measured and further described in these papers.

A method was demonstrated for measuring the particle size of solid pharmaceuticals present in ointments (471). This technique consists primarily of mixing the ointment with a solvent in which it is soluble and then filtering and measuring the size of the insoluble particles remaining behind. For meaningful results, however, the solvent utilized in this technique must not dissolve the solids present in the ointment. To compare the wettability of ointments by liquids of different surface tensions, the contact-angle kinetics of various ointments were investigated (472). The contact angle was observed to change characteristically with the properties of the ointments and the surface tensions of the liquids being evaluated. The water-absorptive properties of lipophilic bases and the dehydration kinetics of hydrogels were also investigated (473, 474). The absorptive properties of starch, talc, and zinc oxide dispersed in petrolatum were evaluated in terms of absorptive rate and capacity. The effect of sample area and air flow on the kinetics of dehydration of hydrogels was evaluated. The dehydration was observed to proceed in two separate steps, a zero-order followed by a one-third-order rate.

In vitro as well as *in vivo* methods for evaluating ointment bases were reviewed in an interesting article (475). The specific evaluation of drug release from ointment-type dosage forms was described by a number of authors. Skin penetration of methyl nicotinate was observed to occur rapidly from water solutions because of hydration of the skin, but was markedly slower from more viscous vehicles which reduced the hydration of the skin (476). The drug-releasing properties of ointments were demonstrated, using a diffusion membrane prepared from freshly removed skin of the rabbit (477). The ointment being tested was placed on the skin, which was carefully stretched over the opening of a diffusion cell, and the amount of drug that penetrated the membrane was determined in 3 hr. The results obtained by this technique for procaine and pentachlorophenol ointments were compared with those obtained using a cellophane membrane. The use of an agar plate technique to study drug release from ointments was also widely investigated (478–480). In general, the release of antibiotics from emulsion-type ointment bases containing surface-active agents or those of nonoleaginous consistency was greater than that observed from oleaginous bases. The interactions between hexachlorophene, polysorbate 20 (Tween 20), and polyethylene glycol 4000 were demonstrated by the hindered release of the antibacterial agent in the presence of the two surfactants.

Table XI—Additional References on Ointments and Creams

Reference	Topic
487	Review of the physiology of skin and methods of measuring skin moisture
488	Review of the effects of emollient emulsions on the moisture of skin
489	Application of silicone fluids in ointments and their uses
490	Use of magnesium oleate as emulsifier in ointment bases
491	Rate of diffusion of cations from various ointment bases
492	Isotropic gel phases in surfactant–oil–water systems
493	Comparative studies of physical properties of various ointment bases
494	Properties of soft white paraffin

The properties and usual components of transparent gels were carefully reviewed by Hynniman and Lamy (481). A special technique to observe the structural features of such gels utilizing the electron microscope following a freezing step to prevent changes in micelles was reported (482). Such techniques are necessary, since the components of such gels and emulsions are very difficult to observe microscopically. The use of polyvinyl alcohol in preparing transparent gels was discussed, as was the stability of various antibiotics in these gel systems (483–484).

The microbiological contamination of antibiotic ophthalmic ointments was examined (485). It was determined that 8 of the 114 batches tested were contaminated. The disadvantages of chlorohexidine as a preservative in nonstick ointments containing anionic surfactants was pointed out (486). The interaction of this preservative with stearic acid soaps and other anionic surfactants completely eliminates its antimicrobial activity. Further disadvantages of chlorohexidine are its inability to control certain *Pseudomonas* strains and its lack of fungistatic activity.

Other articles of interest relating to ointments and creams are described in Table XI.

Suppositories—The use of new suppository bases consisting of hydrogenated coconut oil and beef fat fractions was described (495). These bases compared favorably with cocoa butter and synthetic lauric acid glyceride bases and were shown to be nonirritating. The characterization of Witepsol, a new synthetic suppository base, was reported (496). This material was observed to contain di- and triglycerides, some monoglycerides, and an unidentifiable strong polar substance. A modified glycerin suppository was proposed, based on its different water absorption, solubility, hardness, and ability to release medication (497). The effects of varying the glycerin, gelatin, and sodium stearate contents of these suppositories were reported. The addition of saponins to oleaginous suppository bases was shown to increase greatly the liberation of triphthazine from them (498).

The release of medication from suppository bases received wide attention during the past year. In an excellent paper, Weiss and Sciarrone (499) described

Table XII—Additional References on Suppositories

References	Topic
510	Use of penicillin in rectal suppositories
511	Use of antioxidants to stabilize nystatin in suppositories
512	Review of uses of pressed suppositories
513	Preparation of two-layer suppositories
514	Preparation of polystratified suppositories
515	Proposed tests for weight variation of suppositories

the diffusion of salicylates across a hydrophobic membrane from cocoa butter to an aqueous layer. The release of the drug at the surface was observed to be controlled by diffusion. However, the transfer across the interface is a function of the partition coefficient; consequently, as the partition coefficient decreased, the release rate appeared to approach a limiting value. The release of chloramphenicol from suppositories prepared from different type bases was determined by an agar plate diffusion method (500). Although all of the bases tested gave similar antibiotic release after 2 hr., the suppositories prepared from polyethylene glycol bases showed a considerably better release after 8 hr. The water solubility of the drug was an important factor in the release from fatty-type suppository bases (501). The addition of viscosity-inducing agents such as Arlacel 161 or aluminum stearate or Aerosil had an inhibiting effect on the release of very soluble drugs but only a slight effect on difficult-to-release drugs. The addition of emulsifiers with increasing HLB values was observed to increase the release of soluble drugs from cocoa butter or Witepsol-type suppository bases (502). Optimum release occurred when the HLB value was greater than 10. The effect of the solubility and particle size of aminophenazone on its release from Witepsol suppositories was evaluated (503). As expected, release was most rapid when the drug was in solution and least when it was incorporated as large agglomerates. The effect of surfactants in increasing the absorption of triphthazine from fatty bases was ascribed to their surface tension-reducing properties (504, 505). Similar results were obtained with regard to the rectal absorption of oxytetracycline (506). Fatty-type suppository bases prepared with oil–water emulsifiers showed the greatest absorption, while similar bases prepared without the emulsifiers gave poor absorption of the antibiotic. Intermediate values were obtained with polyethylene glycol-type bases.

The complexation of anionic dyes with quaternary ammonium salts produces a material capable of coloring fatty base suppositories (507, 508). Such techniques must be utilized, because most common dyes are insoluble in the fatty acid glycerides and behave as pigments. The use of various carotenoid dyes, which are fat and oil soluble, to color fatty-type suppository bases was also presented (509).

Further articles of interest on suppositories are presented in Table XII.

Aerosols—Many papers published during the past year reviewed the principles and components of aerosol systems (516–527). These papers discussed operating principles of aerosols, components such as valves, containers, dispensing mechanisms, typical aerosol formulations, filling techniques and equipment, propellants, and the many uses of aerosol products.

The kinetic theory of aerosols and droplet formation during coalescence were discussed in great detail in several papers (528–532). Equations were derived for the solution effect on droplet growth, generalizing this effect as a function of the mean ionic activity coefficient. Two processes were postulated to occur during liquid coalescence: drainage from the drop and Rayleigh disturbance. Interfacial tension and the viscosity of the dispersed phase were concluded to be contributing factors to drop coalescence.

The advantages of carbon dioxide as an aerosol propellant, especially for foods and cosmetics, were described (533, 534). Mixtures of branched-chain aliphatic hydrocarbons were proposed (535). These propellants, which are odorless, water resistant, noncorrosive, and physiologically inactive, may be used in a wide variety of sprays.

The effect of particle size, surfactants, and propellant on the depth of penetration of propyliodone in dogs was studied (536). Greater depth of penetration into the lungs was obtained with powders in the 0.5–10-μ range, although concentration, ratio of propellants, and type of surfactants were also important factors. A cascade impactor was employed to measure the formulation factors influencing the particle size of dexamethasone phosphate aerosols (537). The aerosol particle was capable of being reduced in size by reducing the size of the drug particle, adding a surfactant, reducing the diameter of the spray orifice, increasing the propellant vapor pressure, and increasing the propellant temperature.

The effects of nonionic emulsifiers, buffers, and HLB values on the stability of emulsions used in aerosol foams were investigated (538). Factors affecting aerosol foam appearance were pointed out in an interesting paper (539). Prevention of aerosol propellant loss to the atmosphere will minimize foam wetness, while an increase in the amount of product ingredients in which the propellant is soluble and a lowering of the viscosity of the system will allow a rapid rise of bubbles and an improvement in the percentage of product that can be extruded as a suitable foam. Unusual aqueous aerosol foams and pearlescent aerosols were reported in other papers (540, 541). These new and unusual aerosal systems may find uses in the near future.

The incompatibilities of the components of the aerosol container with its contents were pointed out in several papers (542, 543). Some incompatibilities were those between the contents and the rubber or resin present in the valves and gaskets. The most serious consequence of such incompatibilities is the loss of propellant. A technique was described to test the swelling effects of aerosol contents on gaskets (544); this closed system equilibrates in 24 hr. and gives reliable results in 2 or 3 days.

Several authors evaluated techniques for sterilizing aerosols (545, 546). Sterile filling, bacterial filtration of the vehicle, γ-radiation, and incorporation of ethylene oxide into the gases were all tested for their effectiveness in producing sterile aerosols. Only the ethylene

oxide method was completely successful in producing a sterile product. The main disadvantage of this technique is its possible toxic effects if the aerosol is to be used in inhalation therapy. Other problems associated with the testing of aerosol products were discussed (547–549). The biggest problems were considered to be sterility of the product, corrosion of components, and chemical decomposition during storage. For determination of the propellants, a pressurized liquid-sampling technique and a gas chromatographic analytical method were proposed (550). Another paper described the use of cloud point titration to determine the propellant content of aerosols (551). As shown by tests on 10 commercially available aerosols, the dispensing efficiency of nonmetered topical spray aerosols is very low (552). The pickup efficiency of these aerosols was observed to decrease with an increase in the target distance or in the temperature of the aerosol and to increase with an increase in the nonvolatile content. The formulation, propellant systems, and storage conditions were shown to affect the uniformity of the dose dispensed by different metered valves, above and beyond the normal variation produced by the valves themselves (553).

The toxicological testing of aerosol products was reviewed from both the standpoint of skin and membrane reactions and the dangers of respiratory retention (554). Also discussed was a quantitative method of evaluating the chilling effect of topical aerosol sprays (555). The drop in temperature was observed to be a function of exposure time and to follow first-order kinetics. The chill index was determined and used to compare the chilling effects of commonly used aerosol propellant systems.

Further papers of interest relating to aerosols are presented in Table XIII.

Table XIII—Additional References on Aerosols

Reference	Topic
556	Freezing of droplets of aqueous solution aerosols
557	Effect of soluble surface-active agents on aqueous aerosols
558	Retention of Freon (fluorocarbon refrigerant) in poly(*N*-cyanoacrylate)-sprayed films

Sustained Release—Several articles were published dealing with the terminology, the biopharmaceutical and technical aspects, and the advantages of sustained-release dosage forms (559–561). Equations for the kinetics of the liberation of drugs from sustained-release tablets were developed (562). These equations, which consisted of simplifications of Higuchi's equation for the case where a solid drug is incorporated in a solid matrix, were developed for low-solubility drugs. A method for measuring the permeability of water vapor through a polymer film by a radioisotope dilution technique was reported (563). This method was shown to be rapid, precise, and sensitive and also applicable to the investigation of other parameters which influence the permeability of water vapor.

The application of various epoxy resins in the preparation of pharmaceutical dosage forms was thoroughly investigated (564–566). The addition of basic and acidic curing agents was observed to influence the solubility of the resins in basic and acidic buffers. The type of resin, size of beads, and concentration of drug used were shown to affect the release of the drug. Hydrocortisone, pentobarbital, and papavarine were used as model drug substances in these experiments. Kornblum (567) reported a spray-drying technique which provides a free-flowing powder with decreased dissolution rate after compression into tablets. Since this technique gives good reproducibility of drug release after tableting, it offers another method of preparing sustained-release products.

The chronology of development of a prolonged-action dosage form of proxyphylline was described in detail (568, 569). *In vivo* results indicated that tablets prepared by mixing the active ingredient with a synthetic lipid prior to tableting (embedded tablets) gave the best clinical results. The use of coated tablets did not give as suitable clinical results. *In vitro* comparison of these same formulations showed the liberation of the proxyphylline from the embedded tablet to be dependent on the particle size, the drug concentration, the pH, and the enzyme activity of the solvent system. The release from the coated tablet was much more rapid and was independent of pH and enzyme activity.

The ability of ethylene glycol monomethacrylate gel to hinder the release of drugs saturated in it was described (570). The *in vivo* plasma salicylate concentrations obtained following the administration of oral gels saturated with sodium salicylate confirmed this property of the gel. The effect of gel particle size on the release of the salicylate was shown. Poly vinyl acetate was used to coat micropearls of ascorbic acid to hinder their release (571). These coated granules slowly released ascorbic acid *in vitro* over 20 hr. in gastric and intestinal fluids. *In vivo*, ascorbic acid blood levels were obtained for periods of up to 20 hr. With tablets of ascorbic acid compressed with amyl sodium polyethylene, the release of the vitamin was shown to be inversely related to the degree of compression (572). This effect was postulated as being due to the differences in microstructure obtained by different forces of compression. In the case of *N,N'*-dibenzylethylenediamine ampicillin, the chemical modification was considered to prolong the release of the active drug (573). When administered intramuscularly once daily, this antibiotic had a bactericidal activity equivalent to orally administered ampicillin trihydrate given four times a day. Subcutaneous injection of zinc protamine glucagon gave a prolonged action compared with zinc glucagon suspensions administered to dogs under similar conditions (574). The differences in duration of action were believed to be caused by differences in the composition of the suspension media used.

Bolton (575) presented a method for calculating the release pattern of sustained-release products when more than 1-hr. release rates must be determined. This method is useful whenever hourly assays, as required by the Wiley method, are undesirable.

Other articles of interest related to sustained release are given in Table XIV.

Cosmetics—The trends in cosmetics and toiletries were well reviewed in several articles covering new cosmetic materials, testing methods, and types of prepa-

Table XIV—Additional References on Sustained-Release Preparations

Reference	Topic
576	Preparation of sustained-release oxytetracycline micropills
577	Apparatus for determining release from prolonged-activity solid dosage forms
578	Long-acting tablets containing soluble drugs incorporated into hardened castor oil
579	Use of carboxypolymethylene as an excipient in slow-release tablets
580	Interaction of cellulose acetate phthalate with organic and inorganic cations to form a film soluble in intestinal fluid but insoluble in gastric fluid
581	Application of solid coatings by pressure to hinder drug release
582	Comparison of regular- and slow-release ± 1-(4-hydroxyphenyl)-1-hydroxy-2-butylaminoethane sulfate (Vasculan) tablets

rations useful in cosmetics (583–586). The use of silicones in shampoos, hair sprays, hand lotions, aerosols, quick-breaking foams, toothpastes, and shaving creams was discussed (587, 588). Formulations were given for the types of silicone preparations used in these various products. The properties and uses of fatty acid lactylates were pointed out in a series of interesting articles (589, 590). The use of these materials in deodorant sprays, hair preparations, and shampoos was described. Brandau (591) discussed the various types of surfactants currently used in cosmetic preparations and presented formulations for shampoos, lotions, gels, and skin cleansers utilizing such surfactants. The foaming and surface-tension changes induced by several fatty acid sulfate ethers were described (592). Irritation studies of these materials in the rabbit eye indicated their lack of irritability and possible usefulness in shampoos. Materials which may be used as emollients were broken down into four general classes: hydrocarbons, vegetable fats, animal fats, and fatty alcohols. The properties and advantages of each group were discussed in an interesting review (593).

A series of articles pointed out the use of various materials, such as hexadecyl alcohol, metallic stearates, and vitamins in cosmetics (594–596). The properties of each of these materials, as well as typical cosmetic formulations, were described for each class of compounds. The use of zinc salts in cosmetics and as astringents was also discussed (597). The chemistry and biology of these compounds were thoroughly studied. Blake reviewed the application of radioisotopes in cosmetic research (598). The application and detection of such compounds were covered in detail. The use of tallow and coconut fatty acids to prepare soaps was discussed (599). The soaps were evaluated for their ability to dissolve, slush, lather, and swell. The effects of different ratios of these materials on the properties of the soaps were described.

In a series of articles (600–604), Gucklhorn fully outlined the advantages and disadvantages of the various preservatives used in cosmetic preparations. The bacteriologic spectra and reactions of the parabens, halogenated salicylonilides, organic mercurials, essential oils, sorbates, and other commonly used preservatives were discussed. The antibacterial and antimycotic activities of undecylenic acid and mono- and diethanolamine were determined against a wide variety of organisms (605). Since all of these materials exhibit some antibacterial activity, formulations containing them were presented. In view of the nutritive properties of cosmetics, they are best prepared using bactericidal rather than bacteriostatic preservatives. The bactericidal levels of certain commonly used preservatives were described (606). UV light at 253 μ was utilized to purify water prior to its use in preparing cosmetics and pharmaceuticals (607). This UV method of sterilization was shown to be completely effective if carried out properly.

The use of the scanning electron microscope in cosmetic research was described (608). This instrument, which scans the topography of skin and nails, may be used to investigate the effects of cosmetics on skin. Various viscosimeters and their application in studying the rheology of cosmetics were also discussed (609).

Review articles on hair preparations and shampoos described typical formulations and laboratory techniques (610, 611). Amphoteric surfactants were described which give additional hair-conditioning properties to shampoos (612). The perfuming of soaps and a technique for determining the minimum odor intensity were discussed in several other interesting articles (613–615).

Packaging—During the year, many articles were published covering the use of plastics in pharmacy and pharmaceutical packaging. Varsano and Gilbert (616–618) had a series describing the effects of preservatives, pH, and temperature on plastic packaging materials. The biological effects of plastics and of drug–plastic interactions, along with newer packaging innovations involving plastics, were also reviewed. The properties of polystyrene, polypropylene, polyvinyl chloride, vinyl resins, and other general considerations relating to the use of these materials in pharmaceutical packaging were discussed (619–622). The interaction between silver nitrate solutions and polyethylene containers from various sources was investigated (623). Storage of these solutions in plastic containers resulted in the precipitation of silver particles and an increase in pH. Preservatives containing free hydroxyl groups were prone to adsorption by polyvinyl chloride containers (624). The solute composition and pH of the solute were also shown to be contributing factors. The stability of fatty oils in plastic containers, a measure of their oxygen permeability, was investigated (625). Hot, humid storage conditions were shown to cause clouding of neutral oils stored in plastic containers. Comparisons of various plastic and glass containers with regard to influence of pH, oxygen permeability, and catalytic action of the surface were ably made by Houta and Lenpin (626). The influence of plastic materials on the pH of water is nearly negligible compared with that of glass. The permeability of plastic material to oxygen has no substantial effect on the stability of easily oxidizable substances, although certain plastics are affected by strong oxidizing agents. The alkalinity of glass containers was the subject of several detailed articles (627–629). The importance of this alkalinity on liquid pharmaceuticals was pointed

out. The ions eluted from different types of glass following acidic or basic attack at 121° were also described.

The protective properties of packaging materials, the properties of rubber, its permeability to water, the permeability of glass to light, and the resistance of aluminum ointment tubes to corrosion were discussed (630).

The effects of sterilization on leaching from stoppers composed of natural rubber, IR-25, butyl rubber, POV-30, and polycarbonates were investigated (631). None of the material leached from these stoppers affected the medicinal or physicochemical properties of the solutions being sterilized. The use of polyethylene or cellophane liners over rubber closures of multiple-dose injection vials to protect the solutions was investigated (632). Although this technique was successful in protecting ascorbic acid or sodium sulfadiazine solutions, it was unsuccessful in protecting phenol solutions from the rubber stoppers.

EQUIPMENT

A thorough review of pharmaceutical engineering was presented in several articles (633–636). The engineering aspects and the equipment used in drying, analyzing particle size, and mixing of solids and liquids were described in these reviews. A comprehensive review of methods of mixing and of mixers utilized in the mixing of liquids was presented by Skidmore (637). The advantages and disadvantages of each system were pointed out in this excellent paper. An apparatus for adding suspensions or solids to reaction mixtures was evaluated (638). Wray (639) presented an excellent account of the uses of an instrumented rotary tablet machine. The effects of compression forces on tablet hardness, disintegration time, and tablet weight were given. The characteristics of good and bad formulations, as determined by the instrumented tablet machine, were described. The advantages and disadvantages of new tableting equipment were pointed out (640). The Industrial Pharmaceutical Technology Section (APhA Academy of Pharmaceutical Sciences) standards for tableting tools and the proposed inspection program for such tools were detailed (641).

A description of the concept of laminar flow and the equipment necessary for such systems was presented (642). The effectiveness and limitations of horizontal and vertical laminar flow systems were shown. The equipment used for color measuring and matching was described (643). The use of spectrophotometers for reflectance data and the tristimulus colorimeter to take into account the light source and color sensitivity of the eye was reviewed.

Filling equipment for tablets and ampuls was described in detail in two papers (644, 645). The ampul-filling equipment utilized an automatic magnetic logic setup which gave very reproducible filling volumes.

PHYSICAL PHARMACY

Yang (646), in studying polymorphism in sulfonamides, suggested that the *para*-amino group, the acidic N^1-hydrogen atom, and the oxygen of the sulfonamide group are implicated in the various hydrogen-bonding arrangements that distinguish one polymorphic form from another. Functional groups attached to the N^1-position which serve as electron-withdrawing or electron-furnishing substituents apparently influence the strength of the hydrogen bonds formed and hence the tendency of compounds to exist in more than one crystal form. Through IR spectral studies, the physiological activity of the α-form of chloramphenicol palmitate was shown to be due to the weak intermolecular hydrogen bonding of the alcoholic hydroxyl groups. The β-form of this compound showed a very strong intermolecular hydrogen bonding of the alcoholic hydroxyl groups and is said to be physiologically inactive (647). Haleblian and McCrone (648) reviewed the applications of polymorphism in the pharmaceutical industry. Their publication discussed the preparation of physically stable dosage forms, the possible differences in chemical stability with various polymorphs, the absorption of compounds exhibiting polymorphism, the tableting of polymorphic compounds, and methods of studying polymorphism. Rosenstein and Lamy (649) discussed the pharmaceutical aspects of polymorphism and suggested that the more energetic polymorphic form of a drug may be considered the form of choice in dosage form development.

Using an electrostatic model, Ladd and Lee (650) suggested that the process of hydrate formation takes place in two stages: (*a*) expansion of the anhydrous crystal and accommodation of gaseous water molecules, and (*b*) water–ion interaction. The maximum hydration of dimethylsulfoxide (DMSO) in aqueous solution at 25° was investigated by the deviation of experimental values of fluidity, dielectric constant, refractive index, density, and molar refraction from values calculated by linear interpolation between the values of the two pure liquids as a function of mole fraction. The maximum deviation in fluidity and dielectric constant occurred at the ratio of 3 water molecules per DMSO molecule, which was the maximum hydration (651).

The application of calorimetry to simultaneous determination of equilibrium constants and enthalpy changes for reactions in solution was discussed, and data were presented supporting a more optimistic evaluation of the calorimetric method (652). A calorimetric method was also employed to study proton ionization from aqueous solutions of protonated amines. Resulting enthalpy values were combined with pK values to calculate entropy values. The effect of hydrocarbon chain length and branching on entropy and enthalpy values for proton ionization from primary and secondary aliphatic protonated amines was described by a simple linear equation (653). Michaelis and Higuchi (654) demonstrated the influence of temperature on the distribution ratio of pharmaceutical ammonium species paired with various anions and between aqueous and organic phases, and they determined the thermodynamic parameters associated with these extractive processes. These authors concluded that, unlike transfer of uncharged solute molecules, ion-pair transfers involving inorganic anions appear to be largely entropically controlled. The process of movement from water to chloroform of dextromethorphanium halides, for

example, involved a material increase in ordering. Less polar organic phases tended to lead to less negative entropic changes.

Based on the principle of the higher kinetic energy of water particles in Brownian movement relative to the kinetic energy of particles dissolved in water, Wolkowski (655) presented a new hypothesis for the osmotic mechanism; it explains the principle of water penetration from lower pressures to the higher pressures which exist in solutions of higher concentration. Kostenbauder *et al.* (656) described the unique permeability characteristics of nylon film which make it a useful membrane for conducting dialysis studies or separations that are not readily accomplished with the semipermeable membranes more commonly employed for these purposes. The authors indicated that the permeability of nylon to drugs is not that exhibited by a porous membrane nor that of many nonporous membranes. Nylon is relatively impermeable to small molecules and ions such as water, urea, and sodium chloride; but many less polar, high-molecular weight nonionized species, as well as such ionic compounds as cetyl-, dodecyl-, and ethylpyridinium bromides and sodium naphthalene sulfonate, diffuse readily through nylon films (656). The diffusion rate of sennoside A through a cellulose membrane into water increased, irrespective of the temperature conditions under which diffusion took place, when the membrane was irradiated with ultrasound. These results were consistent with the hypothesis of boundary-layer disruption at the phase interface (657). Rushing discussed the osmotic properties of aqueous solutions of certain divalent salts of disulfonic acids (658).

Repta and Higuchi (659) described the preparation and chemistry of an unsymmetrical monomolecular crystalline anhydride of citric acid, and they suggested possible uses of this compound in pharmaceutical formulations.

Dissolution—Dissolution testing assumed increasing importance during the past year. Studies of three different lots of diazepam tablets revealed no correlation between the rate of disintegration and the rate of dissolution. Increased stirring rate and solvent volume, however, did accelerate the dissolution rate, as did the addition of polysorbate (Tween) (660). Tawashi (661) reviewed the current concepts of crystal dissolution and how dissolution rates and release patterns of drugs are related to such parameters as the cohesive forces in the crystal, which in turn are affected by crystal imperfections or defects. These defects explain some of the discrepancies between experimental and calculated yield strengths. Crystal defects included grain boundaries and cracks, lattice flaws, vacant sites, and foreign atoms outside the regular geometric pattern of the crystal. Other important factors noted in the review were crystal polymorphism, crystal impurities, dosage form additives, processing, and compaction, all of which may act to alter the dissolution rate of the dosage form.

In studying dissolution rates of high-energy polyvinyl pyrrolidone (PVP)–sulfathiazole coprecipitates, Simonelli *et al.* (662) demonstrated the apparent solubility and rate of solution of sulfathiazole from compressed tablets containing PVP to be greatly increased if sulfathiazole was previously coprecipitated with PVP.

Table XV—Additional Dissolution Studies

Reference	Topic
672	Proposed model for analyzing simultaneous phase change dissolution phenomena using *p*-hydroxybenzoic acid and phenobarbital, which change to the respective hydrates during dissolution
673	Minimizing loss of effective surface area during *in vitro* dissolution of sustained-release cellulose acetate phthalate pellets suspended in a turbulent flow field
674	Dissolution of dicoumarol tablets of different crystal properties
675	Parameters of theoretical importance in drug dissolution technology
676	Effect of the nonionic surfactant cetomacrogol on the rate of solution of powdered griseofulvin in water
677	A modified USP disintegration apparatus for use in measuring the dissolution rate of chloramphenicol capsules
678	An automated apparatus for measuring the dissolution rate of solid dosage forms at constant volume under sink conditions maintained by continuous elimination of the solution and replacement with fresh buffer
679	Dialysis method for determining the effects of compression forces and tablet additives on the dissolution rate of sparingly soluble dosage forms
680	Review of the history of dissolution studies and the methods used
681	Dissolution apparatus in which agitation is provided by a flow of liquid maintained by a peristaltic pump
682	Review of the current methods employed in evaluating the rate of drug release from solid dosage forms, and a description of a new apparatus employed for the determination of dissolution rates
683	Influence of polymer molecular weight and solvent pH on solvent penetration and polymer swelling in compressed disks of a series of polymer-free acids derived from ethylene maleic anhydride resins

The increase noted was found to be a function of the chain length of the PVP used as a coprecipitate and the sulfathiazole-to-PVP weight ratio of the coprecipitate powder mixture used to compress the tablet. Similarly, Bates (663) showed that a 1:6, reserpine–PVP, coprecipitate had a 200-fold increase in dissolution rate over that for pure reserpine. This was due to the reduced particle size and, consequently, to the increased surface area of the reserpine in the coprecipitate.

The relative rates of dissolution and solubilities of three polymorphic forms of chloramphenicol palmitate in a 35% tertiary butanol–water mixture and two polymorphs of mefenamic acid in dodecyl alcohol were measured. The thermodynamic relationships involving the transition of the metastable polymorphs to the stable one were examined; the significance of the energy differences between the polymorphs and their absorption, as reflected by blood levels in humans, were discussed (664).

An IR-attenuated total reflectance spectrophotometric technique was employed to demonstrate the surface reversion of methyl prednisolone Polymorph II to Polymorph I in the presence of water. The process was shown to occur rapidly enough to account for the slower than expected dissolution rates of nondisintegrating pellets of Form II in water. The unusual effects of agitation upon the dissolution rate of Form II pellets were thus explained (665).

A marked increase of dissolution rates and attainment of supersaturation of griseofulvin were found when the drug was dispersed by fusion or solvent methods in

matrices of polyethylene glycol (PEG) polymers, pentaerythritol, pentaerythrityl tetraacetate, or citric acid. Although the exact physical nature of the dispersion systems was not determined, it was suggested that some griseofulvin is molecularly and/or colloidally dispersed in the PEG polymers due to their highly viscous and supercooling effect, which would retard the nucleation and growth of griseofulvin precipitation during the solidification process. Pentaerythritol and pentaerythrityl tetraacetate are believed to form limited or completely solid solutions with griseofulvin. The griseofulvin–citric acid mixture forms a glass, and the resultant glass solution may represent a new class or physical modification of drugs exhibiting a strikingly fast dissolution rate of griseofulvin (666).

A column-type method was described for the assessment of the dissolution behavior of solid dosage forms. The method, which is based on the mass transfer between solid and liquid phase in an exchange column, was shown to avoid certain disadvantages of the commonly used beaker methods employing fixed liquid volumes. Because of its reproducibility and the absence of arbitrary external parameters, the method appears to be useful for a meaningful study of dissolution kinetics (667).

Two samples of commercial aspirin with no crystallographic or solubility differences did, however, exhibit different thermodynamic activities, as determined by the effects of agitation and temperature on intrinsic dissolution rates. The metastable form was capable of rapid reversion to a more stable form, depending on the conditions of study (668).

Wagner (669) showed that, under sink conditions, the percent dissolved value at time t may simply be equivalent to the percent surface area generated to that time; if this is so, the percent dissolved–time data may best be described by a distribution function, and the parameters of the distribution may best be employed to describe the data. Simulated percent dissolved–time data, generated by means of the logarithmic normal distribution function, were shown to yield apparent first-order plots. Hence, apparent first-order kinetics, derived from *in vitro* dissolution tests on conventional tablets and capsules, may be artifacts in some cases. In the special case when surface area of drug available for dissolution decreases exponentially with time after some lag time, t_0, first-order kinetics appear applicable to the dissolution data. The new method of examining dissolution-rate data is capable of providing characterizing parameters of greater potential utility than conventional treatments used previously. Gibaldi *et al.* (670) showed that dissolution from constant-surface pellets into micellar solutions followed first-order kinetics in agreement with theory. A method for determining the apparent zero-order rate constant for dissolution from constant-surface pellets, which does not require maintenance of sink conditions, was also suggested.

Low (premicellar) concentrations of polyoxyethylene lauryl ether and lysolecithin markedly enhanced the dissolution rate of salicylic acid powder, while pepsin and gastric mucin were without effect. The same surfactants enhanced the dissolution rate of aspirin from a tablet dosage form but were without effect on the dissolution rate of the drug from a capsule dosage form. Good correlation was observed between the surface tension of the polyoxyethylene lauryl ether solutions and the dissolution rates of aspirin from the tablet dosage form in these media. The authors discussed the relevance of these data to design of *in vitro* dissolution tests (671).

Additional studies on dissolution are provided in Table XV.

Table XVI describes references on the measurement of particle size.

Table XVI—Additional Studies on Measurement of Particle Size

Reference	Topic
684	Review of the advantages and disadvantages of methods employed for determining the distribution of particle size
685	Millipore's combination microscope, TV camera, and computer for the determination of particle size
686	Determination of particle size of water and salt solution aerosols by the dye film technique

Additional references on physical pharmacy are provided in Table XVII.

Solubility—Paruta (699) in determining the solubilities of methyl-, ethyl-, propyl-, and butylparabens in a series of normal alcohols, noted that these compounds have a dielectric requirement of about 14 with another postulated to exist at about 30. These compounds exhibited a good parallel solubility in the various alcohols, indicating a similarity of interactions in the dissolution

Table XVII—Additional Studies on Physical Pharmacy

Reference	Topic
687	Two FORTRAN programs for the IBM 1620 computer which permit determination of the pH of weak acids and bases and salts of these species in aqueous solution at 25°
688	Limitation on the validity of the additivity of molar attraction constants on a functional group basis
689	Dissociation kinetics of methyl red in dilute aqueous solution
690	Acid dissociation constants of barbiturates as determined by pH measurement
691	Dissociation of aminomethanesulfonates in aqueous solution; role in the complex equilibria of neutral solutions of sodium colistimethate
692	Correlation of percentage composition of hydrochloric, perchloric, phosphoric, and sulfuric acids in aqueous solution with molarity, molality, activity coefficient, water activity, and Hammett acidity function
693	Occurrence of complete erythrocyte hemolysis in water–amide solutions and prevention with sodium chloride at low amide concentrations
694	Sodium chloride equivalents and freezing point depressions at various aqueous solution concentrations of 30 different medicinal substances, with isosmotic concentrations of the soluble materials
695	Lithium and sodium chlorates in water and in water–dioxane solvents: the higher solvation of lithium chlorate, as determined using a vapor pressure method, and the major role of dioxane in the solvation of both electrolytes
696	Sodium and lithium chlorates in water: diffusion coefficients and hydration numbers
697	Review of the principles and concepts of ionic motion
698	Isotonic buffer mixtures of pharmaceutical interest: tonicity and pH at 37°; discrepancies between freezing-point depression and vapor-pressure osmometer data

Table XVIII—Additional Studies of Solubilities

Reference	Topic
710	Measurement of dissolved oxygen in water–glycol mixtures in which the glycol has either a hydrophilic group or both a hydrophilic and hydrophobic group
711	Effect of temperature on the solubility and rheology of an ascorbic acid–water–polysorbate system
712	Solubilization of *p*-dimethylamino anil of phenylglyoxal nitrile by cationic surfactants, and effect of electrolytes on micelle formation
713	Review of methods for dissolving insoluble drugs
714	Linear increase in chloramphenicol solubility with concentration of solubilizer once the CMC is reached
715	Review of the solubilization of drugs
716	Use of Tweens (polysorbates) to increase the extraction of rose oil from the rose flower without altering the quality of the oil
717	Solubilization of sulfamethoxypyridazine using a mixture of polyethylene glycol 300, water, alcohol, and piperazine
718	Review of pharmaceutical solvents and solubilizers
719	Increase in aqueous solubility of various drugs by use of polyethylene glycol 400
720	Relationship between the CMC of the surfactant and its ability to solubilize benzyl alcohol or naphthalene
721	Effect of water hardness on the solubilizing activity of linear alkylbenzenesulfonates
722	Formation of micelles by aerosol OT in all nonaqueous solvents studied except methanol, as determined by ultracentrifugation, light scattering, and viscometric techniques
723	Review of the theory of solubilization and stabilization of drugs
724	Increase in solubility and bacteriostatic activity of salicylic acid by use of Tweens (polysorbates), Spans (surface-active agents), polyethylene glycols, propylene glycol, and various mixtures of these nonionics
725	Solubilization of camphor using a pentaerythritol oleate polyethylene glycol ether
726	Review of solubilization and its use in pharmacy
727	Solubilization of water by oil-soluble anionic surfactants: micellar interaction between water and cation of surfactant
728	Solubilization of water by oil-soluble cationic surfactants: ion-dipole interaction between water molecule and halogen anion

process. In aqueous solutions of dioxane, these same *n*-alkyl parabens had a dielectric requirement of about 8–10. Two-phase systems were found for certain of these paraben derivatives over a given composition range of the binary solvent mixture, suggesting the production of a solvate between the paraben and a fixed composition of the binary mixture, thus forming a biphasic system in equilibrium (700). Similar conclusions relative to solvate formation were drawn from an investigation of the solubilities of *n*-alkyl parabens in binary mixtures of ethanol and water. With the latter solvent system, a dielectric requirement of 29–32 was suggested for *n*-alkyl parabens (701).

Several interesting papers dealing with micellar solubilization were published in the past year. Oil-soluble surfactants were studied as solubilizing agents for water-soluble food dyes in the solvent perchloroethylene. In the absence of water, no solubilization of dye occurred; but above a minimum water-to-surfactant ratio, the dye was soluble to a measurable degree. The author concluded that the CMC decreases linearly with an increasing water-to-surfactant ratio (702). The solubility of testosterone, methyltestosterone, and testosterone propionate in aqueous solutions of ethoxylated cholesterol was determined and found to be greatest for the propionate derivative. Based on the UV spectral characteristics of the three steroids in aqueous solutions of the surfactant, which were similar to those in various polyethylene glycols, the mechanism of solubilization was reported to involve association of the steroid with the polyoxyethylene portion of the surfactant (703). Wan and Hwang (704) showed that the solubilities of a series of alkyl gallates in water and in cetomacrogol solution increased with a decrease in alkyl chain length. The solubilities of the short-chain gallates in benzaldehyde increased, while those of the long-chain gallates decreased with chain length. The comparative antioxidant efficiency in solubilized systems appeared to be related to the distribution ratios, and that in emulsified systems to the solubility of the antioxidant in the aldehyde. Thakkar and Hall (705) attempted to explain the anomolous initial supersaturation observed in solubilized systems of testosterone and concluded that the behavior was related to conversion of the anhydrous crystal form to a hydrate crystal form. In studying the solution behavior of the anhydrous form, the authors noted that the peak solubility time in surfactant solution was either increased or decreased relative to water, depending on the concentration and type of surfactant used.

Using ultracentrifugation and viscometric techniques, the behavior and state of aggregation of barium dinonylnaphthalene sulfonate in toluene, toluene–methanol, and methanol were studied, and methanol was shown to diminish the size of sulfonate and carboxylate micelles in nonaqueous solutions (706).

Phenobarbital USP, when crystallized slowly at room temperature from 50% aqueous acetone, dissolved faster in single crystal as well as in powder and tablet form than did an anhydrous form of phenobarbital crystallized in a similar manner from a saturated solution of 85% ethanol. With heat or upon storage in a dry atmosphere, the hydrated crystal form was converted to the anhydrous crystal form (707).

Guess and Jones (708) showed that the degree of solubility of ethylene oxide in plasticizers is a function of the chain length of the plasticizer hydrocarbon moiety. They concluded that the degree of polarity of the plasticizer might, therefore, be a great factor in controlling the solubilization.

The solubility of free modified cellulose polymer films was found to be decreased in the presence of certain FD&C and D&C red dyes. These same dye-containing polymers, when employed as coatings on riboflavin tablets, retarded the tablet disintegration time and the riboflavin dissolution rate. Riboflavin urinary excretion studies confirmed that the dyes may adversely affect *in vivo* product performance (709).

Additional studies of solubility are listed in Table XVIII.

Complexation—Higuchi *et al.* (729) reported a study of some of the methods available for the experimental measurement of hydrogen-bonding interactions in the formation of complexes and the means of determining the association (equilibrium) constants from the experimental results. An attempt was made to develop suitable methods for the quantitative analysis of hydrogen-bond-

ing data so that useful estimates of association constants could be made *a priori*. The study also showed the effect of a nonaqueous solvent on the value of the association constant, and a method was provided whereby an estimate of solvent interaction could be calculated. A critical evaluation of the utility of thermal methods for the detection of possible interactions between the solid components of pharmaceuticals was reported. Phase diagrams were constructed for a number of binary systems. Using thermal methods, interactions were detected with deoxycholic acid–menadione, quinine–phenobarbital, theophylline–phenobarbital, and caffeine-phenobarbital systems(730). Evidence for the interaction of procaine and procaine amide with ATP in aqueous solution was found by means of optical rotation and NMR measurement. The aromatic components of drug and nucleotide molecules were shown to associate through hydrophobic bonding with vertical stack formation rather than through horizontal bonding (731). A simple and general procedure for calculating the statistical factors of mixed ligand complexes was presented. It was noted, for example, that the formation of a mixed complex, MAB, from a metal ion, M, in the presence of equal concentrations of Ligands A and B, is always favored, on a statistical basis, over the formation of MA_2 or MB_2. Thus, there is an enhanced probability of bringing together two different ligands, or a small substrate molecule and an enzyme, *via* a metal ion—a fact which may have great biological implications, particularly as regards the multimetal–multiligand systems in biological fluids (732). Spectroscopic studies on complexation between caffeine and benzoic acids were reported. The relation between the free energy change and the pKa for benzoates suggested that direct electrostatic forces between the carboxyl group in the benzoate molecules and the nitrogen of the 7-position in the caffeine molecule play a dominant role in the complexations (733). The physical and chemical properties of complexes of mono-, di-, and triethanolamine with bentonite, kaolin, and other clays were reported. A triethanolamine–bentonite complex having optimum swelling characteristics at pH 7 gave good stabilization of aqueous suspension. Electrolyte concentrations greater than 1% decreased the stabilization effect of the complex (734). Polli and Frost (735) investigated the role of PVP as a stabilizer for hexylresorcinol in a compressed tablet. While the presence of PVP was shown to be responsible for the color stability of hexylresorcinol, the antimicrobial activity of hexylresorcinol was found to be reduced in the presence of PVP. This reduction in activity was apparently due to the molecular interaction between hexylresorcinol and PVP, and indicates the need for biological evaluation of complexes whenever their formation is suspected. The apparent stability constant of the salicylic acid–caffeine complex was studied in a medium of varying polarity. The salicylic acid and caffeine interacted very strongly in the nonpolar solvent, presumably by hydrogen bonding, but their interaction was minimal in the moderately polar solvents. Because the complexes differ in the organic and aqueous phases, they are not likely to penetrate the phase boundary when the caffeine and salicylic acid are allowed to partition between these phases (736). By this means, Krivis and Rabb (737) reevaluated the Cu–isonicotinic hydrazide system and obtained evidence for the formation of a Cu (I) species rather than a Cu (II) species. The reduction of Cu (II) to Cu (I) and the subsequent formation of an isonicotinic hydrazide complex with the latter ion may be the critical reaction responsible for the therapeutic efficacy of isonicotinic hydrazide. The stability constants and the enthalpies of formation of complexes of caffeine and sodium salts of aromatic acids showed that the stability of the complex is the most important factor in the solubilization of caffeine by these salts (738). The interactions of ergotamine tartrate and caffeine between pH 1 and 6.65 demonstrated that there are marked changes in the solubility of the alkaloid with increased caffeine concentration. Caffeine enhanced the dissolution rate of ergotamine tartrate by a factor of 3 at gastric pH (739). The degree of sorption of chlorobutanol-^{14}C by polyamide and polyethylene was determined using scintillation spectroscopy. The magnitude and rate of sorption were measured, as well as the standard chemical potentials, heats of sorption, and standard entropy values (740).

Additional references on complexation are provided in Table XIX.

Surface Phenomena—To aid the reader in locating areas of specific interest, the authors of this review have subdivided the various publications dealing with surface phenomena; however, because of the obvious overlap in subject matter, the reader who needs a thorough review is advised to consider the entire section.

Interface Studies—The absorption of cellulosic ethers at lipid–liquid/water interfaces was studied in order to clarify the role of polymers in depressing the rate of drug transfer. Carboxymethylcellulose lowered the interfacial tension only when the lipid was nonpolar and the aqueous phase was acidic. Hydroxypropylcellulose markedly depressed the interfacial tension regardless of the polarity of the lipid phase or the pH of the aqueous phase (777). Air–water interface adsorption studies were conducted using seven members of a homologous series of *N*-alkyl betaine zwitterionic amphiphiles. The standard free energies of adsorption were calculated and resolved into separate contributions from polar head groups and methylene groups in the alkyl chain. The data were compared with previously published data for the same compounds undergoing micellization (778). A similar study was made of the properties of monolayers of normal alkyl betaines at the air–water interface. Condensation of the films at pH 4.5–6 was ascribed to the formation of the zwitterions, but there was no indication that an anionic species is produced on alkaline substrates. The entropies and enthalpies of spreading suggest that there is intramolecular neutralization of charges between N^+ and COO^- groups in each molecule (779). The adsorption of cetyltrimethylammonium bromide at different concentrations of the liquid paraffin–water interface was measured by an interfacial tension-lowering technique as well as by an emulsion technique. The experimental data on adsorption appeared to fit both the Freundlich and Langmuir adsorption isotherms (780). On the basis of previous work on interface potentials in amino acid solutions, a structural scheme of interactions between nonpolar radicals and water molecules was sug-

Table XIX—Additional Studies on Complexation

Reference	Topic
741	Zein as a model substance in studying the interactions of proteins and detergents
742	The adsorption of cyanocobalamin on talc in the presence of pyridoxine and thiamine, and the use of talc as a lubricant in tablets of these vitamins
743	The employment of simulated absorbance data to demonstrate the effects of 2:1 molecular complexes on formation constants and absorptivities calculated for 1:1 donor–acceptor interactions
744	Decrease of urotropine–water interaction with increase of the ionic radius of the alkali ion, suggesting its hydration
745	Spectroscopic studies of the triethylamine–iodine systems showing the advantages of the Benesi-Hildebrand method for the determination of equilibrium constants
746	*In vitro* binding of neomycin and its analogs by fatty acids, showing the marked loss of neomycin activity when the interaction results in the formation of a precipitate
747	The effect of magnesium ions on an ascorbic acid monostearate–nicotinamide complex
748	Formation of a 1:1 association compound by chlorpromazine and ninhydrin, as determined by a spectrophotometric method
749	Unique spatial orientation of flufenamic acid and other *N*-arylanthranilates with respect to serum albumin, even when the drugs are bound to the same site
750	Increase in solubility of aromatic hydrocarbons in water by complexation with caffeine
751	Interaction between salts of primary aliphatic amines and hydrophilic organic colloids like sodium carboxymethylcellulose dependence on pH, ionic strength, and chain length of the amine
752	Interactions between colloidal silicic acid containing a sorbed water layer and drugs such as 8-hydroxyquinoline sulfate and quaternary ammonium compounds
753	Spectrophotometric studies on the interaction of acacia and sodium alginate with certain preservatives such as parabens, benzoic acid, salicylic acid, and sorbic acid
754	Interaction between some thiamine derivatives and styrene–maleic anhydride copolymer
755	Polyvinyl alcohol was shown to solubilize benzocaine, phenobarbital, and thymol through complexation by polyvinyl alcohol
756	Single crystal X-ray diffraction methods for obtaining the crystalline and molecular structure of a 1:1 association complex between 5-chlorosalicylic acid and theophylline
757	The formation of an acridine–triphenylmethane dye complex as a possible explanation for the therapeutic interference by such dyes with the action of the acridines
758	The relationship of the anti-inflammatory activity of ethacrynic acid and *n*-ethylmaleimide to the binding properties of their sulfhydryl groups
759	The role of complex formation with water-soluble steroids in the increased *in vitro* activity of water-insoluble polyene-type antibiotics
760	The role of van der Waals-type forces and charge-transfer forces in the strength of a PVP–iodine complex, as determined by a spectroscopic method
761	Improvement in color stability of aqueous sodium erythrosin solution over a broad pH range as a result of complexation with PVP
762	Interaction of thiodiphenylamine with sodium and copper chloride and sulfate solutions
763	Binding of enzyme inducers to histones and nucleic acids
764	Computer program for calculation of stability constants of metal complexes of amino acids and penicillin derivatives from pH values, using known relations
765	X-ray diffraction study of the structure of polymorphism of protein–lipid–water phases
766	Increase in serum vitamin B_{12} binding capacity in women receiving oral contraceptives
767	Possible inactivation of carbocaine and lidocaine by complexation with phospholipids
768	Binding of calcium, sodium, and potassium ions to acid polysaccharides
769	The influence of 36 different drugs on the binding of promazine to bovine serum albumin
770	The predominantly zwitterionic form of aqueous pyridine carboxylic acids and hydroxypyridines as a cause of their lesser complexing tendencies toward 8-methoxycaffeine as compared with corresponding benzene derivatives
771	A solubility technique used to demonstrate the forces involved and the differences in interaction between β-cyclodextrin and 11 pharmaceuticals in aqueous solution
772	Apparent association constants used to estimate the binding of thyroxine to proteins
773	Preferential complexation as the mechanism whereby certain barbiturates prevent the association of riboflavin and adenine derivatives in chloroform solution
774	Increase in the solubility of rutin and quercetin as a result of complex formation with starch
775	Description of the kinetics of intramolecular hydrogen bonding in methyl and ethyl salicylaldehyde by means of an ultrasonic absorption method
776	Review of the binding forces between molecules and an introduction to the theory of adhesion

gested. The aqueous molecules are spontaneously oriented at the interface, with nonpolar substances forming a maximally dense monomolecular layer. This layer forms a basis on which other layers can be formed by hydrogen bonding (781). An apparatus and technique to obtain accurate surface pressure measurements were described, and a single valid surface pressure area per molecule curve was constructed for an insoluble cationic film spread from pure water onto a substrate of sodium chloride solution. With this curve and the results obtained by a crystal spreading method, it was shown that the old criteria for assessing the reliability of the π times area results for an expanded film are invalid and that the spreading of solvents is a major source of error (782).

Additional studies on interface phenomena are described in Table XX.

Surface Tension Studies—Zettlemoyer and Rao (800) reported that the DuNuoy procedure for measurement of surface tension of anionic sodium α-sulfo fatty acid ester surfactants did not give reproducible values, thus indicating that the equilibrium surface tension is too variable for a given solution using this testing procedure. The surface thermodynamic properties of a series of alcohols (C_6–C_{18}), cellosolves, and carbitols were derived from surface tension measurements at various temperatures. A systematic variation of thermodynamic properties with respect to chain length and change in temperature was observed with the alcohols and cellosolves (801). The surface tension of pure liquids was shown to depend on the miscibility of the phases and the orientation of the molecules at the interface. As a function of temperature, the surface tension isobars were concave at elevated pressures but were straight lines at lower vapor pressures. The temperature coefficient of surface tension is affected by the decrease in surface tension resulting from an increase in the saturated vapor pressure and by

Table XX—Additional Studies on Interface Phenomena

Reference	Topic
783	The adsorption of surface-active agents at a liquid–liquid interface
784	Spreading at the solid–oil–water interface
785	Properties of interfacial films of colloidal electrolyte–protein complexes
786	Effects on surface pressure and surface potential of the addition of glycerol to the aqueous substrates of various monomolecular films at the air–water interface
787	Review of the practical application of the monomolecular films formed at air–water interfaces
788	The kinetics of adsorption at the oil–water interface as determined using a suspended-drop tensiometer
789	Ionized monolayers of surfactant at oil–water interfaces
790	Measurement of the attractive forces at liquid–solid interfaces
791	Surface pressure relaxation and hysteresis in stearic acid monolayers at air–water interfaces
792	Review of the intermolecular and interatomic forces at interfaces
793	Determination of the interfacial surface during mechanical mixing of immiscible liquids
794	Review of the work on the properties of water-insoluble soap films at surfaces and interfaces
795	Measurement of the adsorption of sodium dodecyl sulfate at toluene–water interfaces using an interfacial tension-lowering method
796	Review of the discrepancies in the data reported in the literature on solid–liquid interfaces
797	Measurement of electrocapillary phenomena at oil–water interfaces, including the interaction between surfactants and dyes
798	The effect of lysolecithin on lecithin monolayers at air–water interfaces
799	The adsorption of stearic acid on silica and alumina and of decanoic acid on magnesia

the increase in the mobility of the liquid phase molecules with increasing temperature (802). A method was described for substituting a dynamic measurement of surface tension in aqueous solution for the usual static measurements. The dyanmic method shows a time-dependent accumulation of surfactants and a change in the surfaces prior to their reaching equilibrium (803). The influence of temperature and added electrolytes on the dynamic surface tension of sodium dodecyl sulfate solutions was examined by an oscillating jet method. An increase in temperature increased the initial rate of surface tension lowering but had little subsequent effect. Addition of electrolytes increased the subsequent rate but had little effect on the initial rate (804).

Additional references on surface tension studies are provided in Table XXI.

Wetting and Contact Angle Studies—The minimum depth of free energy corresponding to an equilibrium in thin liquid films was determined by measuring the contact angle between the film and the bulk solution. The significance of this angle and its relationship to conventional contact angles were discussed and an experimental method for measuring it was described. Results were also provided for solutions of sodium dodecyl sulfate containing sodium chloride (821). Shafrin and Zisman (822) reported on a new class of surfactant compounds designed to adsorb on solids to improve adhesion of liquids, resins, and protective coatings. Shortening the aliphatic chain from chlorphenyldodecanoic to chlorphenylacetic acid influenced the wettability properties but also permitted use of solvents such as water. A technique was developed for measurement of contact angle in the transition zone between very thin and thick liquid films, based on the Wilhelmy plate technique for measuring surface tensions of liquids. Results were obtained at 25° for films stabilized with sodium dodecyl sulfate in varying amounts of sodium chloride (823). Equations were derived which described the thermocapillary flow of liquid films spread on hydrophilic surfaces and consisting of two layers having different rheological properties. The layer adhering to the solid surface was presumed to exhibit viscoelasticity and the top layer to exhibit Newtonian flow patterns. Dimensionless velocity gradients were established according to layer thickness (824). A theoretical formula was presented for the spreading of a spherical droplet on a smooth rigid surface, based on the assumption that spreading is impelled by surface tensions at the interface and retarded by viscous flow of the droplet. Equations were also derived to predict the rate of spreading and change of contact angle with time (825).

Additional references on wetting and contact angle studies are provided in Table XXII.

Micelle Studies—Frank and Zografi (832) stated that di-(2-ethylhexyl)sodium sulfosuccinate, when dissolved in various hydrocarbon solvents, was capable of solubilizing large amounts of water, whereas closely related compounds such as the di-*n*-octyl and di-*n*-hexyl derivatives exhibited negligible solubilizing capacity. Using light-scattering techniques, the authors measured the micelle size of these three compounds in *n*-octane and

Table XXI—Additional Studies on Surface Tension

Reference	Topic
805	Review of the classification and testing of wetting agents
806	The effect of purity on the surface tension behavior of a homogeneous nonionic detergent
807	The suggested absence of significant inflections in the variation of the surface tension of pure water with temperature
808	Surface tensions of solutions of polydimethylsiloxanes in toluene and tetrachloroethylene at room temperature
809	Equations showing the relationship between surface tension and surfactant concentrations and the effect of temperature on this relationship
810	The variation of surface activity with concentration of surfactant and CMC for cetylpicolinium compounds
811	Mathematical equations for formulating the force between two spheres in contact due to the presence of a pendular ring of liquid
812	The surface tension and viscosity of liquids according to the transient state theory of liquids
813	Review of surface tension theory
814	Review of surface tension
815	Review of the surface tension of liquids in meniscuses with small radii of curvature
816	Dependence of surface tension on surfactant concentration
817	Mathematical equation for the surface tension of water–ethanol–methanol solutions
818	Relation of the purity of sodium dodecyl sulfate to surface tension equilibrium time
819	Calculated thickness of the surface layer of liquids, based on thermodynamics and the theory of Brillouin, which relates surface tension to layer thickness
820	Changes in surface tension during mixing of different types of disperse systems

Table XXII—Additional Studies on Wetting and Contact Angle Measurement

Reference	Topic
826	A light interference measurement technique for determining small contact angles between liquids and solids
827	Relation between the wettability of a paraffin surface by an aqueous solution of sodium stearate and the adsorption of sodium stearate onto the solid paraffin surface
828	Use of three criteria to assess the wetting effect of similar anionic surface-active agents on cellulose
829	Description of wetting on a molecular basis by direct calculation of contact angles from intermolecular forces
830	Review of the determination of wettability by use of liquid contact angles
831	The mechanism of spreading of a drop on a smooth solid surface and the role of surface viscosity in the dynamics of this process

found large differences in micelle weight between di-(2-ethylhexyl)sodium sulfosuccinate and the other compounds in *n*-octane and suggested a significant role for the 2-ethyl side chain. The active participation of water in the organization of these micelles was suggested by significant changes in micellar weight well in excess of that accounted for by the added amount of water. The influence of different hydrocarbon solvents on micelle weight was also found to be quite significant. The effects of a series of nonionic surfactants on micellization of an anionic surfactant was studied as a function of mole ratio and polyoxyethylene chain length. Changes in the degree of association of the anionic surfactant brought about by its incorporation in the mixed micelles was also studied. The degree of ionic dissociation of ionic surfactant in mixed micelles increases as the proportion of a nonionic material increases and as the polyoxyethylene chain is lengthened. At low concentrations, specific conductances are smaller for mixed solutions than for anionic solutions alone, whereas at higher concentrations, mixtures have greater conductance. These results are explained by the degree of ionic dissociation and the mobility of the mixed micelles (833). Micellar weights were determined from mixtures of nonionic and anionic surfactants. The degree of association was less than that calculated, showing the existence of mixed micelles. The increase in micellar weight with rising temperature, characteristic of nonionics, is suppressed by adding anionic surfactants. The micellar weight and the degree of association increased in the presence of sodium chloride (834). Schott (835) noted a remarkable resemblance in the shape, compactness, degree of hydration, and intrinsic viscosity of solutions of globular proteins near their isoelectric point and of nonionic detergent micelles. The polarographic micelle point values of nonionic surfactants were determined by a polarographic maximum suppression method in the presence of electrolytes. These values did not compare well with the CMC's obtained by other methods, which were always higher. These differences were explained as due to the presence of ions which influence the water structure, causing a lowering of the CMC value (836). The decrease in the CMC caused by electrolytes was interpreted in terms of a salting-out mechanism, and evidence was offered in support of the contention that micelles of polyoxyethylated nonionic detergents have a weak positive charge. The effects of urea and formamide on CMC values indicate that hydrophobic bonding is lessened in their presence, with a resulting increase in the CMC (837). Sodium and potassium caprate, laurate, and myristate solutions, both below and above the CMC, were titrated with HCl. By simultaneous monitoring of hydrogen-ion and potassium-ion activity during the course of the titration of micellar laurate solutions, the authors were led to conclude that hydrogen ion competes with potassium ion at the negatively charged micelle–solution interface (838). Keymer (839) noted the unclear relationship that exists between CMC and molecular size or structure, pointing out that the CMC values of anionic substances decrease with an increasing ratio of hydrophilic to hydrophobic portions of the molecule, but that the CMC values of amphoteric, nonionic, and free acid molecules behave in a reverse manner. The author stated that as the aqueous solubility increases, the formation of micelles is reduced and the CMC increases, and if the solubility approaches infinity, no micelle formation is possible. Wan and Poon (840) studied the effect of salts on the surface interfacial tension and CMC of surfactants and noted that all the salts used produced shifts in the CMC to lower concentrations and reduced the surface interfacial tensions of air–surfactant solution and liquid paraffin–surfactant solution. No appreciable difference was observed when air was substituted for liquid paraffin as the upper phase, indicating that the hydrocarbon layer exerts no pressure effects. Shifts in the CMC were related to the valency of the gegenion, with a divalent gegenion producing a shift much greater than a monovalent gegenion. The CMC of cetomacrogol 1000 was practically unaffected by the addition of salt, and the extent of interfacial tension reduction was small with respect to salt concentration when compared with corresponding systems containing ionic surfactants.

Table XXIII provides additional references on micelle studies.

Adsorption Studies—The adsorption of cyanocobalamin on talc is remarkably repressed by polyvinylpyrrolidone (PVP); this phenomenon is due to the preferential adsorption of PVP by talc and not to any direct interaction between PVP and cyanocobalamin (874). Study of the sorption characteristics of cationic surface-active agents by a polyamide (nylon 6,6) showed that maximum sorption occurs in the region of the CMC of the surface-active agent. It was theorized that the hydrophobic moiety of benzalkonium chloride, a cation, interacts with the nonspecific sites in the hydrocarbon units of the polyamide (875). The surface behavior of aqueous dispersions of cholesterol was studied by microelectrophoresis and the adsorption of radiolabeled surface-active agents. Although bile salts were not greatly adsorbed on cholesterol, they did retard its crystal growth, apparently because their nuclear structure permits adsorption at those sites governing the rate of crystal growth but is too rigid to permit general adsorption at all sites and surfaces (876). The equation of state of an adsorbed film was used to derive both a more general equation of equilibrium and an analytical

Table XXIII—Additional Studies on Micelles

Reference	Topic
841	Hydrophobic and electrostatic interactions in ionic micelles, with particular reference to the problems of calculating the contribution of the monomer to the free energy
842	Determining the thermodynamics of micellization of some zwitterionic *N*-alkyl betaines using light-scattering techniques
843	Influence of structure, concentration, counterion concentration, pH, and temperature on the size and structure of bile salt micelles
844	Effect of temperature on the CMC of solutions of certain surface-active *N*-alkylpyridinium halides
845	Proposed model for treating the micelle of an ionic agent as a charged phase
846	Relationship between log CMC of certain acylcholines and the number of carbon atoms in the hydrocarbon chain
847	Effect of urea and amides on the micelle formation of anionic and cationic soaps
848	Micelle structure by fluorine magnetic resonance, with particular reference to the effect of organic additives on sodium 12,12,12-trifluorododecyl sulfate solutions
849	Employment of a fluorescein dye in a spectrophotometric determination of the CMC of some alkyldimethylbenzylammonium chorides, with varying results relative to surface tension measurements of the CMC
850	Claim for the amphiphilic nature of k-casein as the basis for its ability to stabilize micelles against aggregation
851	The reaction kinetics in certain micellar systems
852	The influence of hydrophobic hydration on the conductance and viscosity of *n*-alkylamine hydrobromides in water at 25° both above and below the CMC
853	Description of two types of micelle formation of ionically associated colloids in organic solvents in which the micelle core is either hydrocarbon or aqueous, depending on the solvents used
854	Solubilizing effect of binary systems of ionic surface-active agents, ascribed to the formation of mixed micelles with oleophilic properties different from those of the original components
855	The thermodynamics of micelle formation
856	Review of the interaction between nonionic surface-active agents and water, with particular emphasis on the phase behavior and the formation and thermodynamic properties of micellar solutions
857	Estimation of the degree of polydipersity of macromolecules in solution by comparing the weight average molecular weight value with the number average molecular weight value
858	The effect of sodium chloride on the micellar properties of anionic–nonionic detergents in aqueous solution
859	A light-scattering temperature-jump technique for assessing the kinetics of sodium lauryl sulfate micelle dissociation
860	Use of a stopped-flow conductance apparatus for measuring the rate of breakdown of micelles of anionic and cationic surface-active agents in solution
861	Dye solubilization and light-scattering methods for determining the CMC of sodium cholate
862	The influence of CMC on the detergent action of sodium dodecyl sulfate
863	The interaction between disperse dyes and surface-active agents below the CMC of the surfactant
864	Validity of an NMR method for the determination of the CMC of high molecular weight fatty acids and the lauryl ammonium salts of these acids in sulfuric acid and in carbon tetrachloride
865	The micellar properties of disodium monoalkyl phosphates in aqueous solutions, and the comparatively large aggregation numbers of these salts, reflecting the two dissociable groups characteristic of alkyl phosphate anions
866	The colloidal properties of mixed solutions of anionic and cationic surfactants: discrepancies between the molar concentrations obtained by the vapor pressure osmometer and the actual molar concentration, due to the formation of aggregates of several molecules at low concentration and the increase in degree of dissociation of the micelles at high concentration
867	Demonstration of an increase in the charge on the polyoxyethylene sulfate-type surfactant micelle with increasing oxyethylene content and a decrease in the degree of solubilization
868	The electrophoretic behavior of micelles of a polyether sulfate-type surfactant
869	Estimation of the micellar molecular weights of mixed surfactants using a gel filtration technique, with the observation of a linear relationship between log micellar weight and relative retention volumes of micelles at various ratios in the presence and absence of 0.1 *M* sodium chloride
870	Determination of the degree of ionic dissociation of mixed micelles in aqueous solutions of cationic and nonionic surfactants
871	The thermodynamics of micellar solutions: possible insight provided by examining the distribution ratios for the components of two equilibrium phases of certain systems of micellar solutions
872	The surface and micellar properties of long-chain nonionic surfactants
873	The aggregation of surface-active molecules to form micelles, as shown by a multiple equilibrium model which considers changes in the distribution of micelle aggregation numbers with concentration

expression for the heat of adsorption as a function of the amount adsorbed. The van der Waals equation was used to show that forces of molecular interaction on the surface of the adsorbent are generally repulsive and account for variation in the adsorption of heat with the amount adsorbed (877). Adsorption isotherms were obtained from the adsorption of methylene blue by carbon black, indicating that dye adsorption increases with increasing pH until the amount of dye adsorbed becomes constant between pH 7 and 9 (878). Based on simplified models of an adsorption system, equations were derived for estimating the role of various factors in adsorption. The relative surface activity of adsorbates which adsorb at a smooth adsorbent–liquid interface was estimated as a function of the state of distribution of molecules before adsorption, the structure of adsorption layers, the size and shape of adsorbate molecules, the orientation of adsorbate molecules in the adsorption layer, the interaction energy of adsorbate with solvent and adsorbent, and the interaction energy between adsorbate molecules (879). When large organic molecules diffuse through a multiphase matrix containing water, dispersed water-insoluble solvents, proteins, etc., they become partially immobilized by adsorption, the overall effect being a decrease in the apparent coefficient of diffusion. To differentiate the effects of sorption and free diffusion, a model was proposed which assumes a rapid, dynamic equilibrium between the diffusible and sorbed species. Fick's law is assumed to apply for the diffusing species, while the sorbed species is assumed to be immobile. Results show good agreement between the measured rate and that predicted by the model (880).

Additional references on adsorption studies are provided in Table XXIV.

Table XXIV—Additional Studies on Adsorption

Reference	Topic
881	Cation-dipole interactions in clay-organic complexes, showing that such interactions play an important role in the process of adsorption
882	The greater viscosity and higher structure of water when adsorbed onto kaolin than onto bentonite
883	History of formation and methods used to study the physical properties of natural adsorbents
884	Adsorption isotherms for dinonyl phthalate adsorbed from toluene solution by bentonite and kaolin
885	The *in vitro* resorption of sulfonamides from bentonite derivatives when the latter are incorporated into emulsion and fatty acid ointment-type bases
886	Employment of an IR method to determine the nature of water adsorbed on Wyoming bentonite
887	Interaction of water molecules with various montmorillonite surfaces
888	Adsorption of pigments from nonaqueous solutions
889	The binding of calcium and potassium ions to some polyuronides and monouronates
890	Comparison of the calculated and experimental adsorption isotherms of organic nonelectrolytes adsorbed from aqueous solution
891	Cyclic organic compounds that are adsorbed more strongly than other compounds by activated carbons
892	Employment of the Gibbs-Helmholtz equation to calculate the heat of wetting of charcoal by methanol
893	The effect of pH on the amount of phosphate ion adsorbed by fresh and aged boehmite, and the lack of such effect at very low phosphate-ion concentrations
894	Types of forces involved in particle interaction and particle–water interaction in clay–water systems
895	The cation exchange between the mobile metal cations of bentonite and some alkaloids, amino acids, sulfonamides, and quaternary ammonium and pyridinium salts
896	Adsorption isotherms of dodecyl sulfate and dodecyl amine acetate adsorbed from monazite–water systems
897	Review of crystal structure of amorphous and crystalline materials and the effect of free valences on surface adsorption
898	Reported on the reactions of phosphate with aluminum and Wyoming bentonite
899	Survey of current problems in the interpretation of data on adsorption from solution
900	The physicochemical and adsorption properties of D-cycloserine: adsorption by a strongly cross-linked sulfonated cationic resin
901	The adsorption of heavy metal cations by hectorite and its accompaniment by the removal from solution of silicic acid released by clay dissolution
902	The influence of adsorbed alkylammonium ions on the water sorption and swelling of sodium and calcium montmorillonite; decrease in the water uptake of the sodium salt, accompanied by extensive crystal swelling, with increase in the ratio of exchangeable alkyl ammonium to ammonium ions
903	Measurement of the electrophoretic mobilities of carbon black, titanium dioxide, ferric oxide, and bentonite particles in solutions of a series of sodium polyphosphates and metaphosphates
904	The static adsorption of a water-soluble polymer on natural sorbents
905	Ability of hydroxylated silica surfaces to carry two distinct types of surface hydroxyl sites
906	A multilayer theory for adsorption from solutions composed of molecules of different size
907	A parallel layer model for describing the thermodynamics of adsorption from polymer solutions
908	A phosphate-adsorption phenomenon in kaolin clays associated with exchangeable hydroxyl groups in the clay crystal
909	Adsorbed molecules which may either be localized or move freely along the adsorbing surface
910	Report on the interaction of clay–water systems as affected by hydrous aluminum oxide films
911	Classification of water vapor sorption isotherms of various solids with respect to the mechanism of water binding, chemical composition, and physical structure of the sorbents
912	The adsorption of carbon dioxide by alumina using IR and isotherm measurements
913	The acid character of montmorillonite as shown by titration curves in water and some nonaqueous solvents; the presence of aluminum ions at the edges of the clay crystal as an explanation of their weakly acidic character
914	A statistical theory of adsorption based on the reverse expansion method
915	Review of the progress of adsorption studies
916	Some of the essential features of adsorption isotherms of binary liquid solutions of low molecular weight nonelectrolytes at the liquid–vapor and solid–liquid interfaces
917	Correlation of the adsorption affinity of organic substances with their acid–base properties; the contribution of steric factors and molecular polarizability
918	Equation for the adsorption of two different adsorbates on the same adsorbent under different conditions
919	Use of the reactivity of magnesium- and calcium-saturated montmorillonite surfaces for studying the adsorption of organic amines and pyridines
920	An equilibrium theory of the kaolinite–water system at low moisture contents, with some remarks concerning adsorption hysteresis
921	The influence of a presorbed anionic surfactant on the sorption of a cationic surfactant by hair
922	The evaporation resistance and interaction of (polymethylvinyl ether/maleic anhydride) with plasticizers

Surface Area and Porosity Studies—Giles and Trivedi (923) described a rapid semimicro method for the determination of the specific surface of solids by dye adsorption. The method utilizes known volumes of solutions of rated concentrations, with which the solid is shaken and the adsorbed weight determined by analysis. From these data the adsorption isotherm is plotted, the level of the plateau representing the amount of dye in a complete monolayer on the surface. The authors have successfully used microporous silica, alumina, and graphite as solid adsorbents. The theory of multilayer adsorption on solid adsorbents was statistically and mechanically formulated by taking into account the lateral attractions between adsorbate models. The theory was generalized to a mobile adsorption on flat, homogeneous surfaces (924). Henson and Hunter (925) presented criteria for determining the best setting of relative pressures at which to conduct adsorption determinations so that a good estimation of the capacity and surface area of the monolayer can be obtained. Experiments were selected by maximizing a function of partial derivatives of equations at specific values of the parameters. *p*-Nitrophenol in water or in benzene solution and several dyes were used to measure the apparent specific surface of porous charcoals, silicas, and alumina by adsorption. The results reveal a relation between the apparent surface and pore size distribution (926). An apparatus was developed for B.E.T.-type adsorption measurements for surface area determinations. The apparatus is purported to expedite the measuring process and the evaluation of the resulting data (927). An air-permeability method compared favorably with the B.E.T. method for determining specific surface areas, owing to the smaller surface microporosity, but the calculated average particle diameters only

roughly coincided with those from microscopic measurements (928). Conductometric titration of clay suspensions with dodecylamine hydrochloride solutions was presented as a rapid method for the determination of surface area of clay particles suspended in aqueous media (929). A critical examination of the different methods proposed for the measurement of surface area of solids led to the development of a differential volumetric measurement technique (930).

Diffuse (Electrical) Double-Layer Studies—Jones (931) reported on the relation between surface charge density and the double-layer potential of soap films. With the aid of the Gouy-Chapman theory of double-layer potential, surface charge density, and electrolyte concentration in liquid films, it was shown to be more realistic for systems to behave as though characterized by constant surface charge density than by constant potential. Conway and Gordon (932) directed attention to some of the problems arising in the treatment of the double layer which can usefully be considered in light of similar or related problems regarding ionic solutions. They provided a table which compares the problems observed under conditions of ionic equilibrium with those observed in double-layer systems. Becher (933) published an excellent review on the theory of the electrical double layer, including a complete solution of the case of the diffuse double layer for an infinite flat plane. The effect of adsorption, ionic strength, and pH on the potential of the diffuse electric layer was described (934), along with an evaluation of the electrical double layer on silica in the presence of bivalent counterions such as magnesium, calcium, and barium (935). Electrophoretic measurements were made of micelles of dimethyldodecylamine oxide and betadodecylaminopropionic acid in 0.1 *M* sodium chloride solutions of varying pH. The electrical double layer around the micelle at different pH values was then constructed according to the Stern theory (936).

Foam Studies—The effect of electrolytes on the foam stability of aqueous solutions of nonionic surfactants was discussed (937). A study was made of the relation between the initial foam height, as measured by the Ross-Miles test, and such factors as concentration of the surfactant, CMC of the solute, surface tension of the solution, surface area of the foam, and the work involved in the production of the foam surface (938). Ranny (939) reported that optimum foam volume and stability were achieved with surfactants having long, straight carbon chains with the hydrophilic groups at one end. Shifting of the hydrophilic groups toward the middle of the chain decreased the stability of the foam.

General Studies on Colloids, Gels, and Sols—A silver iodide sol, when coagulated by aluminum sulfate in solutions acidified with sulfuric acid, showed an increase in the critical coagulation concentration with a decrease in the pH. This result, which was attributed to the formation of an $AlSO_4^+$ complex, indicated that the antagonistic effect observed when coagulation is carried out with pairs of electrolytes may be entirely due to counterion complexing (940). Napper (941) considered the stabilization of colloids by nonionic hydrophilic macromolecules to be steric. The stability of ion-stabilized colloids in the presence of surfactants was reported; according to this concept, the stability of the sol depends on the recession of the electrical double layer and the thickening of the adsorbed film of soap on the particles, a process which lowers the molecular forces between them (942). Dukhin and Stoilov (943) critically reviewed the three most frequently used methods for determining the permanent dipole moment of anisodiametric colloid particles. A method was reported for modifying the texture of aluminum phosphate gel by thermal treatment (944), and a description was given of a procedure for incorporating oils into transparent gels without the development of haze during storage (945). By studying the colloidal stability of silver iodide at levels far above the coagulation concentration, it was shown that sols do not restabilize at salt concentrations up to 3 *M*, even though a repulsive force can exist at such levels (946). Current views on the stability of colloidal solutions were discussed, along with approaches to stabilizing these systems (947).

Studies on General Properties of Surface-Active Agents—A comparison of HLB values according to two prevailing systems was made for two classes of nonionic surfactants, namely, the ethylene oxide adducts of *n*-dodecanol and of branched nonylphenol with increasing degrees of polyoxyethylation. The two systems were shown to differ fundamentally in that only one treats the HLB value as constitutive and additive. For both HLB systems, simple relationships were found between the HLB values of each class of surfactants and their critical micelle concentrations. These relationships have different forms for the two systems and, within the same system, different numerical values for the two classes of surfactants (948). Schott (949) compared the cloud points of 165 nonionic surfactants, based on their calculated HLB values. Increasing length of the polyoxyethylene moiety increased the HLB and cloud points. The equations for calculating the HLB, which had been derived from emulsification experiments, contained the weight-percentage of polyoxyethylene as the sole variable characterizing the surfactant. Therefore, the calculated HLB was not affected by the surfactant characteristics, which largely govern the values of cloud point, CMC, and interfacial tension. Additional HLB measurements would be needed to determine whether all experimental HLB values are really independent of the structure of the surfactant molecules, as proposed. The properties of sodium alkane (C_{13} to C_{19}) sulfonates were determined and shown to be highly dependent on their molecular weight (950). While studying the turbidity point of nonionic surfactants, it was noted that the length of the hydrophobic alkyl group influences the cloud point, contrary to the dehydration theory, which assumes clouding to be due to the hydrated ether groups of the surfactant (951). A change was observed in the physical and chemical properties of surface-active semicolloid solutions after magnetic field treatment. Concentration of surfactant above the CMC, by destroying micelles, caused an increase in the amount of the ionic form of the surfactant and, consequently, an increase in conductance and decrease in surface tension (952). Felmeister and Schaubman (953) reported on studies in which a monomolecular film of dipalmitoyl lecithin was spread on an aqueous phase into which a

Table XXV—Additional Studies on General Properties of Surface-Active Agents

Reference	Topic
957	The solubilization of a three-component system of liquid paraffin, water, and nonionic surfactant, showing that structure and number of ethylene oxide molecules affect the solubilizing power of the surfactant
958	Interaction between *p*-aminoazobenzene and anionic surfactants
959	The influence of *N*-methylacetamide and urea on the properties of surfactants in aqueous solutions
960	The effect of alkyl-chain structure on the physicochemical properties of sulfate-type and ether sulfate-type surfactants
961	The surfactant properties of hydroxylated sulfonated esters of various fatty acids, showing the generally superior properties of the unsaturated acids relative to the saturated fatty acids
962	The coalescence of a liquid drop at the liquid–liquid interface, and the effect of surface-active agents
963	The rate of formation of a monolayer on a drop of water from the vapor and dispersed phases of surface-active agents
964	The structure–activity relationships of nonionic and anionic surfactants
965	Biodegradability of alkyl benzene sulfonates and alkyl sulfates
966	Hydrolytic stability of ester-type nonionic surfactants; the near-constancy of log k between pH 4 and 6 and its proportionality to hydrogen-ion concentration or hydroxyl-ion concentration outside these pH limits
967	Criteria for surface-active agents which have emulsifying capacity
968	The concept of permeability number to define the permeation character of "intestinosolvent" coating materials
969	Synthesis and study of the properties of polyethylene glycol ethers of C_9 to C_{18} fatty alcohols
970	The composition and elasticity of thin-liquid films drawn from solutions containing sodium decanesulfonate and varying amounts of dodecanol pentaethyleneglycol ether
971	Review of the trends in types and uses of soaps
972	Basic values and typical properties of various surfactants and detergents

photosensitive phenothiazine drug was dissolved. The system was then exposed to UV radiation, and the resultant changes indicated that substitution in the 2-position of the phenothiazine nucleus is critical in the photosensitized interaction. In a similar study, the same authors proposed a relationship between the increase in surface activity induced by irradiating a drug and its *in vivo* photosensitizing properties (954). ζ-Potential measurements were made of plasma, bile, and solutions of albumin, calcium, and sodium chloride in order to evaluate their effect on the net surface charge of suspended particles (955). To test the validity of ζ-potential as a determining factor in the stability of colloidal systems, and the possibility that electrolytes might change the electrophoretic properties of colloid particles by their influence on ζ-potential and solution ionic strength, the electrophoretic mobility of suspensions of solid emulsion particles of paraffin with stearic acid, using petroleum spirit as a spreading solvent, was studied as a function of salt concentration. The electrophoretic mobilities were strongly influenced by the nature of the counterion, making it necessary to modify the equations expressing the effect of decrease of repulsion potential attributed to a hydration barrier (956).

Additional studies on general properties of surface-active agents are provided in Table XXV.

Crystallization—Lin and Lachman (973) reported that different crystal forms were obtained on dissolution and recrystallization of a new antihypertensive agent, as determined by X-ray diffraction, IR, and photomicrographs. A method was developed, based on dissolution rates, for estimating the ratio of crystalline drug to that dispersed at the molecular level within a carrier. Under appropriately chosen conditions in the two diverse systems, the dissolution rate of the drug was linearly related to its degree of crystallinity (974). Methods of preparation and characterization of two crystal forms of sulfathiazole, using differential scanning calorimetry and IR spectroscopy, were presented. The transition temperature depended on the rate of heating and the sample source. Interconversion of the crystal forms under different conditions, such as heating and suspension in water, was described. An assay procedure was devised, based on the area of the thermal transition peak of Form I, and results of analysis of synthetic mixtures of Forms I and II were given (975). To obtain basic data on crystallization of the molecular compound of aminopyrine and baribital from aqueous solution, the diagram of the ternary system aminopyrine–barbital–water was studied in the temperature range 0–98°. The molecular compound was best obtained by using an excess of aminopyrine and by crystallizing from concentrated solution at high temperature (976). Marshall and Nancollas (977) investigated the kinetics of crystal growth of dicalcium phosphate dihydrate and showed that, after a brief initial surge, the rate of growth of crystals follows a second-order rate, with respect to calcium and biphosphate concentration, over a wide range of calcium and phosphate concentration, suggesting a predominantly surface-reaction controlled process.

Similarly, the kinetics of calcium sulfate precipitation from aqueous supersaturated solutions containing gelatin or sodium carboxymethylcellulose were studied. The rate of precipitation was shown to depend upon the rate of conglomeration of the calcium sulfate colloidal particles present in the system. Gelatin and sodium carboxymethylcellulose concentration and pH all affect the kinetics. Above the isoelectric point, gelatin slows down precipitation, but below this point the precipitation is accelerated. The negatively charged carboxymethylcellulose stabilized the system and was more effective than gelatin in this regard (978). Phenobarbital can be freed of impurities, especially occluded mother liquor, by controlled crystallization (979). The aging of an aqueous suspension of amorphous aluminum hydroxide was followed in a simple dilatometer. Initially, an increase in volume was observed, and the simultaneous development of pseudoboehmite was revealed by X-ray diffraction. The subsequent formation of a trihydroxide was accompanied by a decrease in volume. Rheological measurements showed that the formation of pseudoboehmite through a condensation polymerization process was accompanied by gelation. The gel structure could eventually hinder the growth of pseudoboehmite particles, with a consequent discontinuity in the kinetics of the process (980).

Rheology—Rheological studies of triethanolamine–bentonite gels revealed their typical thixotropic characteristics (981). By postulating that thermodynamic transportation coefficients may be related to the friction that occurs in viscous flow, a relation between the viscosity and the intradiffusion coefficient was obtained for multicomponent systems (982). Several proposed mechanisms of thixotropic behavior of montmorillonite–water systems were evaluated and the effects of temperature, reshearing, concentration, and storage time on these systems were studied by NMR and rheological measurements. The specific parameters used were the change in line width of the NMR spectrum and the static yield values obtained from the rheograms. The results were in complete agreement with the theory that the colloidal particles, upon contact, adhere to form a spacious matrix resembling a house of cards (983).

Additional studies on rheology are provided in Table XXVI.

Table XXVI—Additional Studies on Rheology

Reference	Topic
984	Optimum method and suggested criteria for evaluating the thixotropy of macromolecular gels
985	Rheological characteristics and sedimentation rates of kaolinite suspensions
986	Use of ointments and pastes to determine static lower plastic-flow limits with the extensometer balance
987	Characteristics of kaolin suspensions, which are Newtonian at low concentrations, pseudoplastic at medium concentrations, and plastic at high concentrations, with thixotropy appearing at the yield point
988	Preparation of microemulsions of Spans (surface-active agents) and Tweens (polysorbates) in benzene and water and determination of their viscosities
989	Determination of the rheological properties of foam stabilizers with a canal viscometer which provides absolute values of surface shear viscosity and yield strength
990	Determination of the physicochemical properties of gels by rheological and thermal analysis
991	Comparison of the rheological properties of bentonite-based salves with petrolatum-based salves by means of a pendulum consistometer
992	Flow properties and hysteresis behavior of oily gelatinous preparations containing liquid paraffin, stearic acid, polyethylene glycols, petrolatum, stearyl alcohol, paraffin wax, and palmitic acid
993	The rheological behavior of kaolinitic clay in the presence of sodium salts of organic acids
994	The rheological characteristics of the kaolin–polyelectrolyte interaction
995	The influence of complex anions on the rheological properties of kaolinitic clay

PHARMACEUTICAL ASPECTS

Radiopharmaceuticals—Spencer *et al.* (996) demonstrated that, in man, aluminum phosphate gel reduces the absorption of radiostrontium by 85% and the absorption of radiocalcium by only 38%. Vertua (997) reviewed the theoretical concepts and applications of radioisotopes in pharmacology. Another article reviewed the compounds which protect against radiation and discussed possible mechanisms (998). Another described radioactive medicinal substances (999).

Antibiotics—In reviewing the chemical aspects of penicillin allergy, Schwartz (1000) discussed the biochemical basis of drug allergy and the antigenic determinants of penicillin allergy and penicillin metabolism. He concluded that more work should be directed toward defining degradation products, their rate of formation, and their ability to react with protein. Benzyl penicillin and 6-APA were shown to contain small but significant amounts of high molecular weight protein impurities attached to penicilloyl groups. These impurities stimulate formation of the antibodies with penicilloyl specificity that may be responsible for penicillin allergies (1001). The amphoteric penicillins, ampicillin and cyclacillin, possess properties similar to the alicyclacillinphatic amino acids. At a pH equal to the isoelectric point, they exist essentially as zwitterions, and in this form are most stable and least soluble in water. The aqueous solubility of ampicillin changes only slightly with a change in ionic strength, unless a nonpolar solvent is added. In water at 25° the carboxyl groups of all penicillins appear to have the same pK_1, while the amino groups of the amphoteric penicillins vary in the pK_2 values over a wide range, probably being influenced by the adjacent side-chain groups. A change in the dielectric constant affects the pK_1 more than the pK_2, while a change in temperature does the opposite (1002). Cyclacillin (WY-4508) was shown to have more selective *in vitro* activity than ampicillin against a variety of Gram-positive and Gram-negative organisms. Rapid absorption follows oral administration of this new antibiotic, with 44% renal excretion in 24 hr. (1003). Lincomycin-2-phosphate was inactive in a plate antibacterial assay using *Sarcina lutea*, although *in vivo* the phosphate ester is as active as the parent compound, lincomycin, in mice infected with *Staphylococcus aureus*. The ester gave slightly higher blood levels than the parent compound upon oral adminstration to dogs. In addition, the ester has a less bitter taste than the parent compound (1004). Compared with tetracycline, doxycycline has equal or better *in vitro* activity against a wide range of organisms. A greater lipid solubility and a greater degree of binding to serum protein was also noted for doxycycline (1005). The bactericidal activity of various deoxystreptamine antibiotics was tested against a large number of organisms *in vitro* and in mice. Good correlation was seen between the *in vivo* and *in vitro* activity (1006). Fifteen salts of erythromycin were prepared and their relative water solubilities and bitterness levels measured. The water solubilities were found to be related to the size of the alkyl group attached to the acid. The level of bitterness, however, was related not only to the size of the alkyl group but also to the stability of the salt, which was shown to be a function of the strength of the acid used to prepare it. The least bitter salt was the stearyl sulfate (1007). As shown by experiments using two antibiotics against *Escherichia coli* in the guinea pig, bacterial kinetics can be divided into a bacteriostatic phase, a rapid bactericidal phase, and a slow bactericidal phase. At high antibiotic levels the first phase slows, while the second phase intensifies. The same results were obtained *in vivo* and *in vitro* (1008). Cephalexin and cephaloglycine were tested for activity against a large number of pathogens *in vitro*. Both antibiotics were rapidly ab-

Table XXVII—Additional Studies on Antibiotics

Reference	Topic
1017	Relation between bacterial production of penicillinase and sensitivity to Penbritin (ampicillin)
1018	Emergence of *Pseudomonas aeruginosa* strains highly resistant to carbenicillin
1019	Greater effectiveness of colistin and polymyxin B than gentamicin against certain strains of *P. aeruginosa*
1020	Increase in resistance of *Bacillus subtilis* strains to oxytetracycline as the cause of its increased resistance to tetracycline, chlortetracycline, and penicillin
1021	Review of bacterial resistance to penicillins and cephalosporins
1022	Review of the physicochemical aspects of chelation and the role of chelation in antibiotic actions
1023	Review of the chemistry and production of antibiotics by several *Bacillus* and *Streptomyces* species
1024	Review of the use of polyelectrolytes as flocculants in fermentation broths of antibiotics, including mathematical equations for adsorption of flocculants and filtration of flocculated suspensions
1025	The mechanism of action of certain antibiotics in relation to protein synthesis
1026	Review of the use of chloramphenicol in ophthalmology
1027	Review of tyrothricin, gramicidin, bacitracin, polymyxin, colistin, and viomycin
1028	Review of the progress in antibiotics between 1945 and 1965
1029	Review of penicillins
1030	Suggested use of a blue dye to assure that a seed layer containing the test organism has been added to a base layer in microbiological assays by agar plate methods
1031	Investigation of the *in vivo* concentration and comparative *in vitro* sensitivity of some strains of staphylococci to dicloxacillin
1032	Report on the *in vitro* and *in vivo* activity of oxolinic acid, noting that its spectrum and primary activity against Gram-negative bacteria are similar to those of nalidixic acid
1033	The antituberculotic activity of tuberactin
1034	Correlation of the *in vitro* activity of gentamicin with that of other antibiotics
1035	Effect of chemical structure on polypeptide synthesis and miscoding activity of antibiotics
1036	Halomicin, a new micromonospora-produced antibiotic
1037	Limited activity of carbenicillin against resistant staphylococci and its special effectiveness against Gram-negative bacteria such as *Pseudomonas* and *Proteus* species
1038	Human pharmacodynamic studies with rifamycin and its role in interference with the bilirubin cycle
1039	Bacteriologic properties of rifamycin
1040	Antimicrobial activity and pharmcological behavior of cephaloglycine
1041	High *in vitro* antibacterial activity of furazolium chloride
1042	Antibacterial activities of a number of penicillin amide derivatives against penicillin-sensitive and penicillin-resistant organisms
1043	Comparative *in vivo* antibacterial activity of benzylpenicillin and penicillin dipeptides
1044	Review of the spectrum and activity of lincomycin
1045	Resistance of all *Clostridium perfringens* strains to therapeutic doses of various antibiotics
1046	Susceptibility of staphyloccci to new antimicrobial agents
1047	Comparative *in vitro* antibacterial activities of 7-chloro-7-deoxylincomycin, lincomycin, and erythromycin
1048	Lincomycin and clinimycin: comparative absorption, excretion, and antibacterial activity *in vitro;* the superior absorption properties of clinimycin
1049	Demonstration of synergy by competitive inhibition of β-lactamase in *P. aeruginosa*, using various combinations of benzylpenicillin, methicillin, and cloxacillin
1050	Therapeutic synergistic activity of ampicillin and cloxacillin, and the protective effect of cloxacillin on enzymic degradation of ampicillin by penicillinase
1051	Doxycycline, an antibiotic which resembles tetracycline in antibacterial spectrum but which is two to four times more potent *in vitro* and more stable to pH change
1052	Synthesis and *in vitro* fungistatic activity of some *N*-substituted amides and amine salts of sorbic acid

sorbed and excreted in the urine, with the absorption being delayed by food. Serum levels were shown to be higher when the agents were administered with probenecid (1009). The bactericidal action of chloramphenicol and streptomycin was tested on sensitive strains of *E. coli* and *S. aureus*. With a short contact time, chloramphenicol was completely inactive, but as the contact time increased so did its bactericidal activity. Streptomycin had a strong bactericidal action (1010). The plasma concentration curve for bamifylline after oral adminstration was similar to that obtained after i.v. administration of the same dose. The drug is rapidly excreted in the urine and follows two metabolic routes of degradation (1011). Koyama *et al.* (1012) described the configuration of viomycidine, as determined by X-ray diffraction. A review of various tetracyclines was published, including their absorption, excretion, distribution in the body, and daily dosage (1013). The apparent partition coefficients between *n*-octyl alcohol and aqueous buffers were determined for several tetracyclines. Using microscopic dissociation constants for tetracycline, the relative amounts of each microscopic ionic form of tetracycline theoretically present at each pH were calculated. The zwitterionic form, which was present in the highest concentration in the pH range from 4 to 7, appeared to be the most lipid-soluble form, its reduced polarity possibly resulting from an intramolecular type of ion-pair formation. The possible relationships between the biological activity of the various tetracycline analogs and their pH–octanol solubility profiles were discussed (1014). Carbenicillin-resistant variants obtained from each of eight strains of *Pseudomonas aeruginosa* were tested by growing inoculum on agar plates containing carbenicillin. The resistant variants resembled the parent strains in cultural appearance, pigment production, and virulence for mice (1015). The surface tension of 21 antibiotic compounds was correlated with their antibacterial activity *in vitro*. It was noted that the compounds with the lowest surface tension had the highest antibacterial activity (1016).

Additional studies on antibiotics are listed in Table XXVII.

BIOPHARMACEUTICS

A thorough review of biopharmaceutics was presented by Garrett and Araujo (1053). Also published during the year were reviews on drug–response evaluations, empirical equations for correlating the biological efficiency of organic compounds, and test models for

evaluating the chemotherapeutic effectiveness of drugs (1054–1056). Drug absorption, distribution, and excretion were reviewed, and, in particular, the metabolic fate of chlorpropamide, diphenylhydantoin, and hetacillin (1057–1061). The clinical effectiveness of hetacillin was shown to be due to its conversion to ampicillin (1062).

Reports were given describing the species differences in the metabolism of diazepam, apomorphine, sulfisomidine and sulfamethomidine, sulfadimethoxine, and phenacetylurea, and pharmacological response in general was discussed (1063–1071). Also studied were the effects of species differences on the binding of drugs to plasma protein (1072–1074). The effects of age and sex on drug metabolism were reported (1075–1077).

Effects of Physicochemical Properties—The various physical and chemical factors affecting drug absorption, availability, and therapeutic response in general were reviewed (1078–1083).

Two polymorphic forms of aspirin were prepared by Tawashi (1084) and shown to be thermodynamically different, based on their differential thermal analysis, thermogravimetric analysis, and dissolution rates. Form II, the most thermodynamically unstable, gave blood serum salicylate levels 70% higher than Form I for the same time period. The urinary excretion of ethylamphetamine and its metabolite, amphetamine, was studied in man after oral administration of the (+), (−), and (±) isomers of ethylamphetamine hydrochloride. The rate of excretion of these amines is dependent on the pH of the urine. At acid values, the (+) isomer is metabolized faster and to a greater extent than the (−) isomer, which is excreted mostly unchanged (1085). Similar results were shown for the influence of urinary pH on the rate of excretion of *l*-adamantanamine (1086). Partition coefficients in *n*-heptane–sodium hydroxide or HCl were determined for a series of amines and acids, and a linear relation was found between the chain length and the log of the partition coefficient. Alkyl chain length was linear with buccal absorption of certain amphetamines and fenfluramines when they were 1% nonionized. Similarly, there was a linear relation between the logs of the partition coefficients and buccal absorption of the amines and acids when these compounds were 1 and 10% nonionized. When the amines and the acids had similar partition coefficients, their buccal absorption was similar over a pH range of 4 to 9. *n*-Heptane was considered equivalent in solvent properties to the buccal lipid membrane for the compounds used in the test (1087).

The phenomenon of displacing one drug from a plasma-binding site with another drug continues to be of interest. Solomon (1088) advised that concurrent therapy with two compounds which bind to protein should always be undertaken with caution, since many drugs appear to compete for a common binding site. Data on the extent of binding of a drug to albumin and its rate of metabolism in man are of great value in predicting whether it will displace other compounds from binding sites on albumin. The effect of caffeine on the gastric absorption of nonabsorbable drugs such as sulfathiazole and *p*-aminobenzoic acid was studied in rabbits. While caffeine enhanced the gastric absorption of *p*-aminobenzoic acid, it had no effect on the rate of absorption of sulfathiazole, probably because of the negligible complex this compound forms with it at gastric pH (1089). Similar studies involving the competition between digitoxin and other drugs in their interaction with serum proteins were published (1090–1092).

The affinity of certain drugs for plasma protein and the resulting effect of this binding on drug absorption were extensively studied. Jusko and Levy (1093) indicated that the interaction between riboflavin and albumin is nonionic, but that electrostatic forces contribute appreciably to the binding of riboflavin-5-phosphate to albumin. Salicylates, when added to saliva, are bound to the saliva proteins to the extent of 35–50% after a contact time of 0.5–2 hr. at 37° (1094). The partitioning of bishydroxycoumarin from rat plasma to an organic solvent phase was found to decrease with increasing drug concentration to a minimum value and then to increase as the concentration was further increased. The same type of profile was observed in the partitioning of the drug from rat plasma to the liver, both *in vitro* and *in vivo*. These results demonstrate the unusual concentration dependence of the plasma protein binding of bishydroxycoumarin, the pronounced effect of the binding on the distribution of the drug, and the effect of the distribution on elimination (1095). The markedly greater affinity of digitoxin than digoxin for serum albumin is reflected in the higher plasma concentrations, lower rate of urinary excretion, and longer half-time of digitoxin when the compounds are administered to man (1096).

Additional studies describing the influence of physicochemical properties on drug absorption are provided in Table XXVIII.

Effects of Formulation—Schneller (1144), in describing the hazard of therapeutic nonequivalency of drug products, cited published examples whereby a given chemical entity showed different blood levels depending on the dosage formulation. He concluded that every manufacturer, before distributing any new or modified product, should be obligated to perform tests which are appropriate and sufficient to demonstrate the clinical safety and efficacy claimed for it. In the absence of such tests, it cannot be assumed that the product will prove clinically acceptable simply because an apparently identical product has already been marketed. Schamberg's (1145) review indicated that different drug formulations containing the same kinds and amounts of active ingredients may differ appreciably in their effect and cannot be considered therapeutically equivalent. In a similar vein, it was suggested that attempts be made to establish the therapeutic availability of dosage forms used in clinical trials and that outlined details of the formulations utilized be included in published reports of clinical comparisons of drugs (1146). Another review article described the importance of dissolution rates and the pitfalls of dissolution methods for chemical substances as well as drug products (1147). The solubilities and absorption rates of the active compounds in pharmaceuticals are known to be affected by the technological factors of manufacturing (1148). The fast, medium, and slow *in vitro* dissolution rates of three

Table XXVIII—Additional Studies of the Influence of Physicochemical Properties on Drug Absorption

Reference	Topic
1097	Direct pH dependence of benactyzine absorption and lack of pH dependence of procaine amide absorption from isolated rat ileum
1098	Charge density and superdelocalization: suggested relationship to partition coefficients and maximum biological response
1099	Extent of molecular interaction between carbazochrome or nitrotinamide and various other drugs; effect of the difference between the rate of absorption of the drug and that of the complexing drug on the absorption of the complex
1100	Enhancement in absorption of certain drugs by complex formation
1101	Investigation of the relationship between the excretion ratio (saliva/blood level) of various sulfa drugs and their binding rate with plasma protein
1102	The low level of carbenicillin and its failure to influence bactericidal activity
1103	Correlation between the absorption of barbituric acid derivatives from the small rat intestine and their binding to the mucosa
1104	Relationship between the lipid solubility, tissue binding, and metabolism of xanthine derivatives and their passage into the brain and the cerebrospinal fluid
1105	The unchanged activity of phenylbutazone in the presence of serum and the sharp decrease in serum activity produced by indomethacin and deoxycholic acid
1106	Significant reduction of coronary dilational properties of dipyrimidole by binding to human plasma
1107	Increase in albumin concentration of promazine by binding to bovine serum albumin
1108	Antibiotic binding to albumin, as reflected by decrease in activity in the presence of this protein
1109	Sex differences in binding of pentobarbital to plasma: the more extensive plasma binding capacity in the female than the male rat, despite similar fractions of drug bound in either sex
1110	Effect of structure and added electrolytes on the binding of unconjugated and conjugated bile salt anions to cholestyramine
1111	Appreciable binding of betamethasone, dexamethasone, and cortisol to cow, dog, and rat plasma protein
1112	Report on the binding capacity of serum protein for cardiac glycosides, especially pengitoxin
1113	Binding of various tricyclic antidepressants to human plasma, and the effects of other drugs thereon
1114	Possible mechanism for the loose binding of iron to protein: a molecular bridge susceptible to irreversible cleavage by EDTA–acetate buffer
1115	Optical methods of describing the interaction of phenylbutazone and its analog with human serum albumin
1116	Comprehensive table of number of sites and binding constants for complex formation between bovine serum albumin and aliphatic sulfates and sulfonates, aromatic sulfonates, naphtholates, and phenolates, generalized as to the relationship between ligand properties and free energy of binding
1117	Effect of pH on vitamin B_{12} binding capacity of the intrinsic factor
1118	Effect of plasma binding of radioactive iodine pharmaceuticals on their renal clearance
1119	The binding of salicylates to serum proteins
1120	Effect of salicylate concentration on the binding to bovine serum albumin at pH 7.4
1121	Binding of taurinophenetidine to rabbit serum protein *in vivo* and *in vitro*
1122	Possible inverse relationship between the number of albumin-bound molecules of salicylic acid and sulfanilamide and the molar concentration of the albumin
1123	Evidence to support the hypothesis that tryptamine and its relatives bind to nucleic acids mainly by intercalation, similar to the binding of LSD to DNA
1124	Inhibition of the proteolytic activity of trypsin by its binding to organic mercury compounds
1125	Suggested existence of a hydrogen bond of the type O—H···S in barium thiosulfate monohydrate and the importance of such a bond to the consideration of hydrogen bonding in biological systems
1126	Similarity of the *in vitro* transacetylation between aspirin and human albumin to that which occurs *in vivo*
1127	Linear relationship between the percent buccal absorption and alkyl chain length of a series of *p-n*-alkyl phenylacetic acids
1128	Increase in antibacterial activity of fluorophenols with increase in the number of substituent fluorine atoms
1129	Relation between structure and antimicrobial activity of aminosteroids
1130	Steric parameters used to describe the structure–activity relationships for certain monoamine oxidase inhibitors and antihistamines
1131, 1132	Structure–activity relationships of sulfonamide carbonic anhydrase inhibitors
1133	Quantification and prediction of the biological activity of *meta*- and *para*-substituted *N*-phenylsulfanilamides by microbialkinetics
1134	Multiple-parameter approaches to structure–activity relationships
1135	Homolytic constants that give a better structure–activity correlation for chloramphenicol derivatives than either the usual Hammett constant or polarizability constants
1136	The use of substituent constants and regression analysis in the study of structure–activity relationships
1137	Comparison of the parameters currently used in the study of structure–activity relationships
1138	Increase in anti-inflammatory activity of a number of carboxylic acid derivatives of phenothiazine by substitution of an acetic group on the nucleus, particularly at Position 2
1139	Suggested essential dependence of partition coefficient and biological activity of substituted benzene derivatives on molecular electronic conditions
1140	Contribution of the phytyl side chain of *dl*-α-tocopherol to its biological activity in rabbits and its enhancement by the presence of an intact 5-methyl group
1141	Review of the significance and limitations of various biological drug parameters used for studying structure–activity relationships
1142	Influence of α- and β-methylation of the ethylenic group in the procaine molecule on its physical and chemical properties
1143	Relationship between chemical structure and activity in a series of halosubstituted 4-quinazolones

sulfamethazine tablet formulations were correlated with the *in vivo* blood level data. A significant statistical difference existed for areas under the blood level curves and for maximum blood concentration of sulfamethazine when the fast-dissolving formulation was compared with the slow-dissolving formulation (1149). Using *in vitro* techniques, the penetration of ^{14}C-labeled fluocinolone acetonide and its acetate ester through human skin at 37° was examined with vehicle mixtures of isopropanol and isopropyl myristate or propylene glycol. Little penetration was found with either of the nonvolatile solvents. As the formulation was changed to include increasing amounts of a volatile component, however, the penetration was increased up to 8 to 10 times. Precipitation of steroid prevented greater increases (1150). Results were presented which showed that two oral dosage forms of nitrofurantoin, microcrystalline drug in a tablet and macrocrystalline drug in

Table XXIX—Additional Studies on the Effects of Formulation on Drug Availability

Reference	Topic
1158	Correlation between *in vitro* release of labeled dexamethasone from nonaqueous vehicles and its *in vivo* penetration
1159	Addition of antacids to aspirin tablets as a method of reducing injury to the gastrointestinal mucosa by shortening the time of contact without simultaneously increasing the area of contact with drug
1160	Prevention or significant reduction of aspirin-induced occult gastrointestinal blood loss by use of sufficiently buffered solutions
1161	Formulation factors affecting blood concentration of *p*-aminosalicylic acid
1162	Review of the adjuvant effects, particle size, and form of drug for optimum resorption
1163	Effect of vehicles on percutaneous absorption of fatty acid esters of pyridoxine: enzymatic hydrolysis during permeation through the skin as the rate-limiting process for percutaneous absorption of pyridoxine 3,4-dioctanoate
1164	Review of recent developments regarding the influence of pharmaceutical formulations on therapeutic effects
1165	Essentially equal availability of six generic and brand name formulations of isoniazid
1166	Superiority of nonionized to ionized species of drugs in absorption from degenerated intestinal mucosa, except those which participate in an ionic interaction with surfactants
1167	Unsuitability of the capsule dosage form of triamterene as an alternative to the tablet dosage form
1168	Intestinal absorption of heparin: facilitation by sulfated or sulfonated surfactants
1169	Rectal absorption of pharmaceutical amines: enhancement with sodium lauryl sulfate and saccharinate anions
1170	Increased absorption of salicylic acid–polysorbate solutions by the frog, due apparently to complexation and possibly to lowered surface tension
1171	Effect of nonionic surfactants on absorption of enduracidin from muscle
1172	Polysorbate 80-induced increase in the solubility and the *in vivo* absorption of an experimental compound, SKF 33134-A, in the rat: unsuitability of urinary excretion measurements for indicating the degree of absorption
1173	Sodium taurodeoxycholate and EDTA: different mechanisms of altering membrane structure and permeability in the rat, as shown by difference in transfer of salicylamide and salicylate across the everted small intestine
1174	Increase in permeability of the everted intestine to salicylate with increase in concentration of surfactant, sodium taurodeoxycholate
1175	Evidence demonstrating that the absorption of 4-aminoantipyrine is increased in the presence of sodium taurodeoxycholate and involves a passive process whether the bile salt is present or not
1176	Increase in salicylic acid resorption with increasing concentration of surfactant from various types of ointment bases
1177	Lack of relationship between the concentration and solubility of hydrocortisone acetate in liquid vehicles and its availability to human excised skin, as shown by the effect of nonionic surface-active agents on its release
1178	Inferiority of a premarketed formulation of chloramphenical (Amphicol) to chloramphenicol (Chloromycetin) in blood levels and urinary excretion
1179	Faster absorption of solutions of theophylline than equivalent amounts of the drug administered in capsules
1180	Liberation of active substances from soft gelatin capsules *in vitro* and *in vivo*
1181	Importance of drug particle size to biological activity
1182	The occlusion potential of various ointment vehicles on percutaneous absorption
1183	Absorption of drugs from various-type suppository bases
1184	Equivalent absorption of diazepam in capsule and tablet form
1185	Doubling the absorption of obidoxime from the rat intestine by use of 1% EDTA
1186	Factors affecting drug transfer in the presence of macromolecules: viscosity as the principal factor in transfer of sodium carboxymethylcellulose and hydroxypropylcellulose, except in the presence of mucin, when drug interaction and interfacial tension also appear to be involved
1187	Effect of antacids on the *in vitro* and *in vivo* absorption of ethionamide and prothionamide in various tablet and capsule dosage forms

a capsule, were both well absorbed. However, differences were observed in the urinary recoveries and in the urinary excretion patterns between these dosage forms, which suggested a slower rate of absorption for the macrocrystals than for the microcrystals (1151). The *in vivo* absorption and *in vitro* dissolution characteristics of a commercial suspension, a commercial tablet, and an experimental tablet formulation of salicylamide were compared. The absorption of this drug was shown to be dissolution rate-dependent, and the initial *in vitro* dissolution rate in 0.1 *N* HCl correlated well with the initial absorption rates of the test dosage forms in human subjects (1152). Studies were undertaken to define the effects of the emulsion components on the absorption of heparin, as measured by clearing factor activity, and to determine the optimum composition of the emulsion. Data suggested that heparin absorption is directly related to, and may vary with, the particle size and total surface area of the oil droplets, but that the relationships presented may be unique for the particular surfactant and oil chosen for study (1153). Low concentrations of polysorbate 80 in the water significantly increased the absorption- and exsorption-rate constants of 4-aminoantipyrine in goldfish. It was concluded that polysorbate 80 enhances the transfer of the drug by a direct effect on the biologic membranes and not by interacting with it (1154). In an attempt to develop an oral dosage form of aminoxafen which would produce prolonged, stable plasma levels of total drug, an arbitrary set of *in vitro* dissolution conditions were chosen which correlated well with *in vivo* absorption rates. A one-compartment open model was used to describe the system (1155). The enhanced absorption of dextromethorphan from trichloroacetate buffers appears to be due to the increased surface activity of the dextromethorphan rather than to ion-pair formation. However, thiopental was more strongly absorbed from trichloroacetate solution than from physiologic sodium chloride solution, apparently because of the preferential binding of serum protein with trichloroacetate rather than thiopental, thus leaving more unbound thiopental available for absorption. Dialysis experiments showed the binding of 55% of the thiopental to human albumin in the presence of physiologic phosphate buffer and only 22% in the presence of trichloroacetate solution (1156). The rate of dissolution of one investigational compound

Table XXX—Additional Studies on Absorption Control and Alteration

Reference	Topic
1193	Method for estimating the biologic half-life of tetracyclines from steady-state serum level data plotted semilogarithmically against time
1194	Review of drug interactions
1195	Mechanisms of drug interactions
1196	The comparative pharmacodynamic activity of single and divided doses of benzphetamine hydrochloride
1197	Enhancement in activity of orally administered reserpine–cholanic acid coprecipitates due to reduced particle size of reserpine in the coprecipitate
1198	Theories dealing with the mathematical interpretation of competitive actions of drugs on isolated tissues
1199	Method for estimating individual drug-dosage regimens
1200	Alternative approaches in the choice of experimental designs for estimating effective doses when there is some curvature in the dose–response relationship but a linear approximation is still used
1201, 1202	Review of drug interactions according to therapeutic activity
1203	Block of renal tubular secretion of sulfinpyrazone by probenecid and its lack of significant influence on the ability of sulfinpyrazone to increase urate excretion
1204	Inhibition by dicoumarol of the intestinal absorption of D-glucose and its enhancement of the absorption of L-arabinose
1205	Increased rate of conversion of diphenylhydantoin to *p*-hydroxylphenylhydantoin in the liver as a possible reason for the increased rate of disappearance of i.v. doses after pretreatment with phenobarbital
1206	Alteration in metabolism and distribution of methotrexate by neomycin and sulfathiazole so that excretion by the intestinal route is significantly enhanced
1207	Thyroxin-accelerated and insulin-delayed absorption of isoniazid from the gastrointestinal tract
1208	Effect on membrane of chymotrypsin permeability and binding to serum proteins in enhancing the absorption of penicillin G
1209	Chymotrypsin enhancement of tetracycline blood levels
1210	Heparin-induced increase in absorption of chlortetracycline
1211	Review of the biological reactions to drugs
1212	Reduction of the serum salicylate concentration of aspirin to 50% by administration of activated charcoal
1213	Relative *in vitro* absorption by activated charcoal of a wide variety of drugs found in the home
1214	Effect of drugs on the rate of disappearance of amphetamine in rats
1215	Effect of simultaneous administration of other pharmaceuticals on the competitive inhibition of biliary excretion of antibiotics and sulfonamides
1216	The dog as an experimental model for studying interaction of drugs with bishydroxycoumarin
1217	Retardation of wound healing by oral or topical administration of sodium salicylate, prednisone, or hydrocortisone and its reversal by local application of retinoic acid
1218	Influence of cholestyramine on thyroxine absorption
1219	Effect of caffeine on the absorption of salicylic acid derivatives from the small intestine of the rat
1220	Review of various factors in the transfer of drugs across the placenta
1221	Determination of plasma salicylate levels after administration of acetylsalicylic acid and a combination of acetylsalicylic acid with ascorbic acid, which greatly increases salicylate absorption
1222	Marked increase in renal excretion of the monomethylated tricyclic antidepressants as sole clinical result of changing the acidity of the urine
1223	Possible difference in metabolism or excretion of warfarin and vitamin K, as suggested by the marked individual variation in ability to antagonize the anticoagulant effect after simultaneous administration of both drugs
1224	Therapeutic importance of the ability of fatty acids and other drugs to interfere in the binding with albumin
1225	A mathematical treatment of two-point attachment between drug and receptor site
1226	Possible role of phenylbutazone in masking the influence of methandrostenolone on plasma levels of oxyphenbutazone by displacing the steroid from binding sites on plasma protein
1227	Salicylate-induced release of L-tryptophan from its binding sites on human serum protein
1228	Phenylbutazone displacement of sulphormethoxine from its protein binding site

from tablet and capsule formulations could not be related to its *in vivo* absorption in dogs. The rate of partitioning of the drug into organic solvents and its absorption in goldfish and through human buccal membrane depended on the pH of the solution. Absorption was shown to increase with a decrease in the degree of ionization. In man, however, the dissociation constant and the elimination rate of the drug are such that high blood levels are unlikely to occur after oral administration (1157).

Additional studies of the effects of formulation on drug availability are provided in Table XXIX.

Absorption Control and Alteration—The hypothesis that the formation of ion pairs and their transport across the lipid barrier are important mechanisms for drug absorption was examined, using a quaternary ammonium compound, isopropamide, as the cationic component and trichloroacetate as the anionic component of an ion pair. *In vitro*, ion pairing greatly increased organic solubility, while in mice, both the rate and the efficiency of oral absorption of isopropamide were increased when it was administered with an excess of trichloroacetate (1188). The extent of metabolism of the (−) isomer of mandelic acid is not significantly altered in the presence of sulfadiazine, sulfamethazine, or sulfamerazine, and this fact was utilized to calculate the ratio of the rate constants of the overall elimination of mandelic acid in the presence and absence of the sulfa drugs, as a measure of their inhibitory effects on its urinary excretion. It was concluded that the three sulfonamides probably share the same renal tubular transport system for their secretions in humans (1189). Chloramphenicol was shown to retard the biotransformation of tolbutamide, diphenylhydantoin, and dicoumarol in man, resulting in an increase in the half-life values of these compounds in the blood after its adminstration. A case of chloramphenicol-induced hypoglycemic collapse in a tolbutamide-treated patient was reported (1190). To design a dosage form, especially a sustained-release type, of amphetamine or other drugs which have an approximate 12-hr. half-life in blood and urine, it was recommended that blood studies be conducted to define the pharmacokinetic parameters necessary for calculating the initial and maintenance doses, and that excretion data be used mainly to confirm the half-life of amphetamine beyond the levels which are conveniently measurable in the blood. Blood data are more meaningful, since urine levels of amphetamines are highly sensitive to the urinary pH, re-

Table XXXI—Additional Studies on the Mechanism of Absorption

Reference	Topic
1240	Dependency of molecular penetration of spore wall and consequent toxicity on compatibility between geometry and charge distribution of the molecule and that around the periphery of the hole in the wall
1241	Concentration and dissociation gradient between the aqueous phases of a three-phase system as decisive factors in the absorption and rate of dissolution of drug
1242	Interfacial barriers in interphase transport: the retardation of the transport of diethylphthalate across the hexadecane–water interface by an adsorbed gelatin film
1243	Solubility diffusion as the most likely mechanism of water permeation through lipid bilayer membranes
1244	Suggested model for describing the nonmediated transfer of nonelectrolytes in terms of diffusion in homogeneous networks
1245	Review of the mechanisms of absorption, distribution, and elimination of drugs, with reference to methods of modifying these mechanisms by inducing changes in acid–base balance
1246	Evidence indicating that salicylates retard wound healing by inhibiting mucopolysaccharides synthesis
1247	Passive diffusion mechanism for absorption of dextromethorphan from the rat's stomach as a protonated species
1248	Confirmation of absorption of *S*-benzoylthiamine *O*-monophosphate into the blood after its dephosphorylation at the mucosal surface of the intestine
1249	Review of phospholipid cell-membrane models
1250	Data presented to support the hypothesis that benzylpenicillin is actively transported from the cerebrospinal fluid to the blood
1251	Review of the mechanisms by which cardiac glycosides are absorbed through the intestinal wall
1252	Rate of absorption of secobarbital in goldfish and the relationship between absorption-enhancing effect and surface tension (or concentration) and the type of surfactant used
1253	Three possible modes of interaction between membranes and surfactants incorporated into ointment bases: rupture of membrane, replacement of certain phospholipids present in the lipid micelles, and induction of configurational changes in the micelles
1254	Experimental results interpreted as an indication that permeability changes of nerve membranes are mediated by migration of hydrogen ions
1255	Suggested formation of a hydrogen-bonded complex between drug and acceptor group on the neural membrane as a mechanism in the action of local anesthetics

sulting in kidney reabsorption changes and a consequent wide variation in urinary excretion rates. While control of pH compensates for the variation, it is impractical for large-scale clinical studies (1191). The ability of methocarbamol to potentiate aspirin or morphine might be due to the competition between it and aspirin or morphine for glucuronide formation, with consequent enhancement in the aspirin or morphine blood levels (1192).

Additional studies on absorption control and alteration are listed in Table XXX.

Absorption Mechanism—A model was presented which can describe the overall transfer of a drug across biologic membranes in the presence of a complexing agent. This model was applied to the transfer of salicylamide across a cannulated everted rat intestine in the presence of caffeine. The intestinal transfer rate constant of the salicylamide–caffeine complex was found to be considerably lower than that of salicylamide but essentially the same as that of caffeine (1229). Some fundamentals of micelle formation and of solubilization of water-insoluble substances through micelles were reviewed. The accelerating effect of micellization on rate of solubilization and of transport of solubilizate through bulk liquid was considered. Transport across the membrane is accelerated whether or not micelles are effective within it (1230). Wagner (1231) presented equations to demonstrate the mechanism of gastrointestinal absorption, with special attention to the permeability coefficients for both ionized and nonionized species. The *in vitro* transfer of drugs from a buffered aqueous phase through a barrier consisting of a lipid liquid into another buffered aqueous phase was suggested as simulating the process involved in gastrointestinal absorption, *i.e.*, the partitioning of a drug between the gastrointestinal fluid and the lipoidal membrane and the plasma (1232). For studying the oil-water interface transport of drugs, a two-phase model was investigated. When the oil-water partition coefficient is large, the transport is aqueous diffusion-controlled and the first-order behavior is followed in the aqueous phase with time. Deviation from first-order behavior occurs when the partition coefficient is low, when the diffusion coefficient in the oil is low, when the diffusion coefficient in the aqueous phase is large, or when the thickness of the aqueous diffusion layer is small. These results may be useful in the design and interpretation of both *in vitro* and *in vivo* data on drug transport (1233). Similar investigations involving two-phase methods for investigation of the interphase transport of drugs were reported (1234, 1235). Wagner reviewed the transport of drugs through membranes and barriers other than the gastrointestinal tract, including buccal absorption as well as excretion by three different glands (1236). DMSO, in combination with sodium chloride, decreases skin electrical conductivity, probably by reducing skin resistance and facilitating the absorption of the electrolyte simultaneously with its own absorption (1237). With chlorpromazine, whatever the mechanism of absorption, the final therapeutic effect was shown to depend on the free radical form (1238). Using tritiated digoxin in rats, it was concluded that digoxin is absorbed by a passive nonsaturable transport process not dependent on metabolic energy (1239).

Additional references on the mechanism of absorption are provided in Table XXXI.

Kinetic Studies—Wagner (1256) reviewed certain aspects of pharmacokinetics and biopharmaceutics in relation to drug activity, considering such factors as route of administration, dose and dosage regimen, absorption, distribution, metabolism and excretion, effects of disease, and drug–drug interactions. The dose-dependent elimination of bishydroxycoumarin, known to occur in man and monkeys, can also be observed with warfarin when sufficiently high doses of the latter are administered. This effect is not seen clinically, perhaps, because the therapeutic dose range of warfarin is much lower than that of bishydroxycoumarin. The results suggest that the two coumarin anticoagulants are subject to the same major biotransformation pathways

Table XXXII—Additional Studies on Pharmacokinetics

Reference	Topic
1270	Characterization of the kinetics of salicyluric acid formation from salicylate by considering the formation process as the rate-limiting step in the excretion of salicyluric acid after salicylate administration
1271	Review of the relation between dosage forms, dosage regimens, and pharmacokinetics
1272	Description of a linear relationship between logarithm of warfarin concentration in the plasma at a given time and the pharmacologic effect at that time, and the decline in pharmacologic effect at a constant rate following cessation of therapy
1273	Calculation of the theoretical optimum dosage regimen for antituberculosis drugs
1274	Pharmacokinetics of large doses of penicillin
1275, 1276	Pharmacokinetics of two sulfonamides in children during the 1st year of life
1277	Pharmacokinetics of clindamycin
1278	Pharmacokinetics of streptokinase
1279	Pharmacokinetics of Fanasil (sulfaorthodimethoxine)
1280	Description of the kinetics of salicylic acid and the formation of gentisic acid, with the suggestion that the elimination of salicylic acid cannot be entirely described by first-order kinetics
1281	Toxicological, chemotherapeutic, and pharmacokinetic data for sulphormethoxine and other sulfonamides in animals and man
1282	The kinetics of 2-propanol and acetone in dogs and rats
1283	Postulation that the dose-dependent disposition kinetics of griseofulvin might be attributed to changes in the tissue distribution rather than to changes in the intrinsic metabolic activity
1284	Demonstration of the pharmacokinetics of 2-sulfanilamido-3-methoxypyrazine in children, including the elimination, intestinal absorption, distribution, and dosage
1285	Review of the pharmacokinetics of antibiotics in animals
1286	An equation for turnover time of goldfish as a function of concentration of ethanol, with a theoretical derivation based on a combination of occupation and rate-receptor theories
1287	Problems in data collection in analysis in human pharmacokinetics
1288	The value of the buccal absorption test for interpreting or predicting pharmacokinetic behavior of a drug such as imipramine and its metabolites
1289	Pharmacokinetics of peruvoside compared in man and dog
1290	Pharmacokinetics of ouabain, digitoxin, and peruvoside in the guinea pig
1291	Metabolism and pharmacokinetics of rifampicin in animals and humans
1292	Pharmacokinetics of kanamycin
1293	Kinetics of isoxazolylpenicillins
1294	Metabolism and pharmacokinetics of medazepam
1295	Review of pharmacokinetic principles
1296	Indications that computer analysis of pharmacokinetic data is a valuable and indispensable aid in the evaluation of experimental chemotherapy
1297	Some pharmacokinetic aspects of doxycycline metabolism in man
1298	A simple dilution analog computer for simulation of drug distribution processes: its uses in teaching and visualizing tandem first-order reactions, such as pharmacokinetic models
1299	Suggestion that linear models, used for compartmental analysis, can also be employed to study interactions between drugs and their receptors and to evaluate the kinetics of the pharmacologic response
1300	Employment of a digital computer for solution of pharmacological problems involving nonfirst-order models of drug metabolism
1301	Employment of a digital computer for statistical treatment of the data on a two-compartment model of the disappearance of ethoxybenzamide from plasma
1302	Use of apparent rate constants obtained by analog computer analysis of plasma and dialysate curves to compare a variety of compounds for their effect on the peritoneal dialysis of salicylate
1303	Description of dose-dependent effects in pharmacokinetics, indicating that studies should be done at more than one dose level, since neither humans nor animals have unlimited capacity to metabolize drugs

(1257). A model was presented which can be used to obtain the pharmacokinetic parameters of the two-compartment open system of drugs which are too poorly soluble or too irritating to be adminstered by rapid intravenous injection. Experimentally, the method involves administering the drug by a constant-rate intravenous infusion until the attainment of infusion equilibrium, and determining the plasma concentrations of drug in the postinfusion period. The approach was applied to literature data and resulted in the evaluation of the two-compartment pharmacokinetics of oxacillin (1258). The apparent volume of distribution at the steady state in a two-compartment open system cannot be used to relate the drug concentration in the plasma to the amount of drug in the body, except at the one point in time when the rate of change of the amount of drug in the peripheral compartment is zero. A new concept of apparent volume of distribution, introduced for the pharmacokinetic analysis of the three-compartment open system, was applied to the two-compartment open system (1259). A graphic method was described for estimating the absorption half-life from the time of the peak level following the extravascular administration of a drug. The method is useful for drug products having absorption rates five or more times faster than their elimination rates (1260). The pharmacokinetics of drug distribution was evaluated for two types of drug administration—*viz.*, constant-rate intravenous infusion and instantaneous intravenous injection. Both modes of administration eventually result in a constant tissue compartment–central compartment distribution ratio of drug. However, the distribution ratios are not equivalent at pseudodistribution equilibrium and at infusion equilibrium. Consequently, at equivalent plasma concentrations, more drug will be in the tissue compartment during pseudodistribution equilibrium than during infusion equilibrium, although the total amount which will enter the tissue compartment is independent of the mode of administration. These findings may have important implications for drug distribution studies and with respect to the relative effectiveness of continuous and intermittent drug administration (1261). The pharmacokinetic analysis of drug concentration in the plasma *versus* time data, achieved by use of multicompartment models, made it possible not only to examine the relationship between drug concentration in the plasma and the intensity of the pharmacologic effect, but also to assess the relationship between pharmacologic effects at the relative drug levels in other apparent compartments of the body (1262). A three-compartment open system was proposed to explain the influence of route of administration

on the area under the plasma concentration–time curve. Computer analysis of the model, using estimated pharmacokinetic parameters, provided a successful prediction of the relative area under the plasma concentration–time curves after oral and intravenous administration of aspirin in man. After intravenous administration, the proposed model yielded a curve which may be described adequately by a biexponential equation (1263). Gladtke (1264) determined the elimination half-life of phenylbutazone after intravenous administration in children to be 21 hr. After oral administration, 85% of the dose entered the blood within 8 hr. In patients with liver disease, the half-life of meprobamate in the blood was shown to increase to 24.3 hr. from the 12.6 hr. observed in the normal patient. In drug addicts it was lowered to 4.5 hr. (1265). Benet and Ronfeld (1266) reviewed the use of different types of volume terms in pharmacokinetic equations. Amsel and Levy (1267), in a pharmacokinetic study of the simultaneous conjugation of benzoic and salicylic acids with glycine, found that in man the availability of glycine is rate-limited by the formation of hippuric acid but not of salicylic acid. Apparently the inhibitory effect of benzoic acid on the formation of salicylic acid is not due to competition for glycine but involves another phase in the biotransformation process. In a study describing the pharmacokinetics of sulfonamides in patients with cirrhosis of the liver, it was noted that the half-life of sulfamethoxypyridazine was not sufficiently different from the control, while the half-life of sulfadimethoxine was significantly diminished as a result of the increased binding of the drug by plasma proteins (1268). Data generated with a two-compartment open model were analyzed according to the one-compartment open model, in an attempt to use single-dose blood level data to predict blood levels after multiple doses (1269).

Additional studies on pharmacokinetics are listed in Table XXXII.

Drug Absorption—Using a model consisting of a thermostated upper and lower chamber fabricated from methyl methacrylate and having various membranes sandwiched between the chambers, Aguiar and Weiner (1304) showed the effect of varying the concentration of surfactants and of propylene glycol on the permeation of chloramphenicol through the barriers. The authors measured the activation energies for permeation and diffusion of this drug through a filter membrane saturated with peanut oil and also those for its permeation through hairless mice skins, at the same time obtaining an estimation of the partition coefficients. The law of corresponding areas was used to assess the percent absorption of a drug, by comparing the relative areas under the plasma concentration–time curves after oral and intravenous administration. If any metabolism occurred in the gut wall or liver, the areas under the curves would not be similar. This was verified using aspirin in dogs (1305). Chiou (1306) concluded that it is almost impossible to obtain an empty stomach in the rabbit by fasting the animal, since the fasting state markedly prolongs the stomach emptying time; thus the rabbit is not a useful animal in which to study drug absorption. A method was reported for studying *in situ* the gastrointestinal absorption of drugs from isolated gut segments of the anesthetized rat. Disappearance of the drugs from the lumen of the small intestine followed apparent first-order kinetics. However, the observed absorption rates were much faster than those normally observed in *in situ* intestinal preparations (1307). The effects of fasting on the intestinal absorption profiles of salicylic acid, barbital, haloperidol, and chlorpromazine were studied in anesthetized rats. The *in situ* technique employed in the

Table XXXIII—Additional Studies on Drug Absorption

Reference	Topic
1313	Review of percutaneous absorption of medicinal agents
1314	A new method for calculating ionophoretic permeability of the skin
1315	Decrease in sweating ability of the skin as cause of a decrease in blanching and presumably a decrease in percutaneous absorption of steroid
1316	Occurrence of percutaneous absorption of steroids and other large molecules *via* appendages and through the unbroken stratum corneum
1317	Relative magnitude of percutaneous absorption of various ^{14}C-labeled steroids as shown by measuring urinary excretion
1318	Review of the cutaneous penetration of dimethyl sulfoxide
1319	Effect of skin conditions on susceptibility of topical tolnaftate penetration
1320	Demonstration that an increase in the perfusion flow rate significantly increases the penetration rate of some compounds, suggesting that data obtained *in vitro* may be more meaningful when ideal flow rates are determined and validated with *in vivo* data
1321	Effect of essential oils on drug absorption
1322	Review of the mathematical treatments used to describe the various parameters involved in drug absorption
1323	Review of drug schedules and drug combinations as factors influencing absorption and efficacy
1324	Review of the relationship between receptor structure and pharmacological activity
1325	Comparability of doxycycline plasma concentrations achieved orally and after i.v. injection in man
1326	Intestinal absorption of six tritium-labeled digitalis glycosides
1327	Intestinal absorption of cardiac glycosides *in vitro* and *in vivo*
1328	Significantly higher serum and urine levels obtained with oxolinic acid when administered with food
1329	Absorption of pyrazinamide in man
1330	Correlation of the analgesic effect of aspirin with the blood concentration of salicylic acid, suggesting that aspirin probably exerts its effect through its hydrolytic product, salicylic acid
1331	Demonstration that isonicotinic acid derivatives are absorbed from the skeletal muscle of the rat, their absorption being proportional to the amounts remaining at the injection site and to both their molecular weights and partition coefficients
1332	Gastrointestinal absorption of 2-pyridine aldoxime methiodide and its derivatives
1333	Blood levels of 2-pyridine aldoxime methochloride and symptoms in humans after single and multiple oral doses
1334	Relative absorption of tetracycline and penicillin G after rectal and oral administration in aqueous solution
1335	Independence of the absorption of guanidine and the dose used for clinical control of hypertension
1336	Use of a quaternary ammonium dye to describe biochemical and morphological correlations of intestinal absorption
1337	Colonic absorption of thiamine
1338	Influence of blood flow on the absorption of drugs from the jejunum of the rat

study yielded absorption rate constants which were realistic and comparable to those observed following oral drug administration. Although apparent deviation in absorption patterns occurred when fasting periods were less than 20 hr., with longer periods the absorption rates were found to decrease significantly, the decrease being dependent on the duration of the fasting period. The unusual drug absorption patterns noted in these studies might be accounted for by one or more of the various physiological and/or biochemical changes which occur within an organism subjected to conditions of prolonged fasting (1308). When prolonged administration of salicylates in amounts comparable to those used in acute rheumatic fever was used, plasma salicylurate formation occurred at an essentially constant rate practically independent of the amount of salicylate in the body, thus demonstrating the capacity-limited formation of salicylurate during the prolonged administration of aspirin (1309). The decline in plasma concentration of dextroamphetamine was more rapid under controlled acidic conditions than under conditions of fluctuating urinary pH. The apparent rate of urinary excretion of amphetamine was proportional to its plasma concentration only under controlled acidic urinary conditions. Under acid conditions, amphetamine was cleared from blood more rapidly than could be accounted for by glomerular filtration, but when urinary pH fluctuated, its clearance could be accounted for by this route (1310). By using crystalline folic acid to study intestinal absorption in rats, it was noted that when increasing doses are introduced into the jejunum the percent of drug absorption decreases, suggesting an active transport mechanism. Absorption from the ileum remains constant with increasing dose, suggesting passive diffusion (1311). Comparative urinary excretion studies after the oral administration of 4′-chloro-2-ethylaminopropiophenone in a sustained-release form and in single or divided doses indicated that when kidney tubular reabsorption is minimized, the biological availability of the drug can be followed by examining the excretion of either the unchanged drug or the metabolites that quickly and directly form from the administered drug (1312).

Additional references on drug absorption are provided in Table XXXIII.

REFERENCES[1]

(1) W. McKeehan, *J. Pharm. Sci.*, **52,** 613(1963).
(2) W. McKeehan and L. Lappas, *ibid.*, **53,** 695(1964).
(3) L. Lappas and R. Dempski, *ibid.*, **54,** 931(1965).
(4) R. Dempski and G. Polli, *ibid.*, **55,** 645(1966).
(5) G. Polli and L. Ravin, *ibid.*, **56,** 781(1967).
(6) L. Ravin and P. Bernardo, *ibid.*, **57,** 1075(1968).
(7) P. Bernardo and E. Russo, *ibid.*, **58,** 777(1969).
(8) L. Lasagna, *Science*, **166,** 1227(1969).
(9) M. Slavin, *J. Amer. Pharm. Ass.*, **NS9,** 460(1969).
(10) R. Gulen, *Kim. Sanayi*, **16,** 5(1968); through *Chem. Abstr.*, **70,** 22858h(1969).
(11) K. Lennington, *Drug Cosmet. Ind.*, **104,** 44(1969).
(12) J. Gain, *ibid.*, **104,** 48(1969).
(13) C. Frank. *Amer. Perfum. Cosmet.*, **84,** 49(1969).
(14) K. Baeschlin and J. Etter, *Schweiz. Apoth.-Ztg.*, **106,** 935 (1968); through *Chem. Abstr.*, **70,** 109148a(1969).
(15) C. Fica, *Farmacia (Bucharest)*, **17,** 347(1969); through *Chem. Abstr.*, **71,** 128657c(1969).
(16) K. Usiekniewicz and I. Reszka, *Farm. Pol.*, **25,** 329(1969); through *Chem. Abstr.*, **71,** 84516z(1969).
(17) A. Djiane, *Prod. Probl. Pharm.*, **24,** 269(1969); through *Chem. Abstr.*, **71,** 64038s(1969).
(18) H. Evans, *Australas. J. Pharm.*, **49,** 575(1968); through *Chem. Abstr.*, **70,** 1973b(1969).
(19) J. Watt, *Can. Pharm. J.*, **102,** 275(1969).
(20) D. Mills, *ibid.*, **102,** 133(1969).
(21) *Ibid.*, **102,** 40(1969).
(22) J. Parks, *ibid.*, **102,** 184(1969).
(23) B. Tuttle, *ibid.*, **102,** 102(1969).
(24) R. Penna, *ibid.*, **102,** 15(1969).
(25) C. Erickson, *ibid.*, **102,** 44(1969).
(26) W. Cox, *Chem. Phys. Appl. Surface Active Subst., Proc. Int. Cong. 4th*, **1,** 79(1964); through *Chem. Abstr.*, **70,** 89066s (1969).
(27) Y. Yamada, *Shika Igaku*, **31,** 506(1968); through *Chem. Abstr.*, **71,** 6508j(1969).
(28) C. Szeremi, *Amer. Perfum. Cosmet.*, **84,** 37(1969).
(29) H. Ansel, W. Norred, and I. Roth, *J. Pharm. Sci.*, **58,** 836(1969).
(30) R. Robison and M. Weinswig, *ibid.*, **58,** 275(1969).
(31) E. Beveridge and A. Hart, *Pharm. J.*, **202,** 730(1969).
(32) G. Schuster and H. Modde, *Fette, Seifen, Anstrichm.*, **71,** 394(1969); through *Chem. Abstr.*, **71,** 40625e(1969).
(33) G. Schuster and H. Modde, *Amer. Perfum. Cosmet.*, **84,** 37(1969).
(34) L. Adam, Z. Csath-Stincel, E. Szantho, E. Peteanu, and L. Domokos, *Rev. Med. (Tirgu-Mures)*, **15,** 11(1969); through *Chem. Abstr.*, **71,** 84515y(1969).
(35) G. Henderson and J. Newton, *Pharm. Acta Helv.*, **44,** 129(1969); through *Chem. Abstr.*, **70,** 99589d(1969).
(36) R. Yousef, *Alexandria Med. J.*, **13,** 133(1967); through *Chem. Abstr.*, **70,** 71033u(1969).
(37) R. Richards, P. Suwanprakorn, S. Neawbanij, and N. Surasdikul, *J. Pharm. Pharmacol.*, **21,** 681(1969).
(38) M. Baichwal and A. Phadnis, *Indian J. Pharm.*, **31,** 19 (1969); through *Chem. Abstr.*, **71,** 64062v(1969).
(39) K. Christensen and E. Dauv, *J. Mond. Pharm.*, **12,** 5 (1969); through *Chem. Abstr.*, **71,** 64058y(1969).
(40) M. Zafir, *Folia Pharm. (Istanbul)*, **5,** 801(1968); through *Chem. Abstr.*, **70,** 71046a(1969).
(41) H. Bean, G. Konning, and S. Malcolm, *J. Pharm. Pharmacol.*, **21,** 173S(1969).
(42) W. Eckardt, *Fette, Seifen, Anstrichm.*, **71,** 237(1969); through *Chem. Abstr.*, **71,** 33340e(1969).
(43) L. Ivanova and T. Kondrat'eva, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 181(1968); through *Chem. Abstr.*, **71,** 94716d(1969).
(44) I. Glucklhorn, *Amer. Perfum. Cosmet.*, **84,** 49(1969).
(45) F. Klaschka, *Fette, Seifen, Anstrichm.*, **7,** 887(1968); through *Chem. Abstr.*, **70,** 46111k(1969).
(46) A. Walters, *Mfg. Chem. Aerosol News*, **40,** 37(1969).
(47) H. Bean, *Rep. Progr. Appl. Chem.*, **52,** 472(1967); through *Chem. Abstr.*, **70,** 60752b(1969).
(48) I. Maurer, *Pharm. J.*, **203,** 529(1969).
(49) G. Schuster, *Dragoco Rep. Ger. Ed.*, **16,** 72(1969); through *Chem. Abstr.*, **71,** 42145x(1969).
(50) A. Dunningan, *Proc. Joint Conf. Cosmet. Sci.*, **1968,** 179; through *Chem. Abstr.*, **70,** 109085c(1969).
(51) K. Bergwein, *Kosmet.-Parfum-Drogen Rundsch.*, **16,** 27(1969); through *Chem. Abstr.*, **71,** 40608b(1969).
(52) T. Kondrat'eva, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 143(1968); through *Chem. Abstr.*, **71,** 94719g(1969).
(53) R. Anderson, *J. Hosp. Pharm.*, **26,** 48(1969).
(54) M. Traisnel, *Farmaco, Ed. Prat.*, **24,** 523(1969); through *Chem. Abstr.*, **71,** 116492g(1969).
(55) O. Yalcindag, *Kim. Sanayi*, **16,** 45(1968); through *Chem. Abstr.*, **70,** 31663t(1969).
(56) I. Totev, M. Boyadzhieva, and I. Isaev, *Farmatsiya (Sofia)*, **19,** 20(1969); through *Chem. Abstr.*, **71,** 64071x(1969).
(57) R. Shallenberger, T. Acree, and C. Lee, *Nature*, **221,** 555 (1969).

[1] All references to *Chemical Abstracts* in this article give abstract numbers instead of page numbers.

(58) R. Randebrock, *Amer. Perfum. Cosmet.*, **84,** 59(1969).
(59) L. Kheifits and L. Shulov, *Maslo-Zhir. Prom.*, **35,** 27(1969); through *Chem. Abstr.*, **71,** 6467v(1969).
(60) M. Grudev and T. Petkova, *Maslo-Sapunena Prom.*, **4,** 1(1968); through *Chem. Abstr.*, **70,** 50421d(1969).
(61) N. Ikeda, *Nara Igaku Zasshi*, **19,** 566(1968); through *Chem. Abstr.*, **70,** 50446r(1969).
(62) P. Speiser, *Pharm. Acta Helv.*, **43,** 693(1968); through *Chem. Abstr.*, **70,** 14376j(1969).
(63) T. Needham, Jr., and R. Gerraughty, *J. Pharm. Sci.*, **58,** 62(1969).
(64) N. Tuchel, *Farmacia (Bucharest)*, **17,** 201(1969); through *Chem. Abstr.*, **71,** 42229c(1969).
(65) G. Schenck, *Deut. Apoth.-Ztg.*, **107,** 1516(1967); through *Chem. Abstr.*, **70,** 60766j(1969).
(66) M. Schorderet, *Pharm. Acta Helv.*, **44,** 13(1969); through *Chem. Abstr.*, **70,** 60776n(1969).
(67) L. Wan and C. Hwang, *J. Pharm. Sci.*, **58,** 262(1969).
(68) K. Tanaka and M. Kurebayashi, *Eiyo To Shokuryo*, **22,** 112(1969); through *Chem. Abstr.*, **71,** 94714b(1969).
(69) H. Mital, *Pharm. Acta Helv.*, **44,** 170(1969); through *Chem. Abstr.*, **70,** 99575w(1969).
(70) F. El Khawas and H. Von Czetsch-Lindenwald, *Sci. Pharm.*, **37,** 30(1969); through *Chem. Abstr.*, **70,** 118043q(1969).
(71) L. Rockland, *Food Technol.*, **23,** 11(1969).
(72) E. Nuernberg, *Pharm. Ztg.*, **114,** 128(1969); through *Chem. Abstr.*, **71,** 42264k(1969).
(73) E. Nuernberg and H. Mueller, *Deut. Apoth.-Ztg.*, **109,** 89(1969); through *Chem. Abstr.*, **70,** 109132r(1969).
(74) H. El-Shibini, N. Daabis, and M. Motawi, *Arzneim.-Forsch.*, **19,** 676(1969); through *Chem. Abstr.*, **71,** 53529b(1969).
(75) *Ibid.*, **19,** 828(1969); through *Chem. Abstr.*, **71,** 64063w (1969).
(76) *Ibid.*, **19,** 1613(1969); through *Chem. Abstr.*, **71,** 116505p (1969).
(77) K. Baeschlin and J. Etter, *Pharm. Acta Helv.*, **44,** 339 (1969); through *Chem. Abstr.*, **71,** 33366t(1969).
(78) *Ibid.*, **44,** 348(1969); through *Chem. Abstr.*, **71,** 33363q (1969).
(79) Y. Zelikson, *Farmatsiya (Moscow)*, **18,** 23(1969); through *Chem. Abstr.*, **71,** 33365s(1969).
(80) E. Pandula, I. Racz, and Z. Pajor, *Pharmazie*, **24,** 155 (1969); through *Chem. Abstr.*, **71,** 42231x(1969).
(81) J. Chanal, R. Marignan, and J. Coste, *Trav. Soc. Pharm. Montpellier*, **29,** 53(1969); through *Chem. Abstr.*, **71,** 105167d (1969).
(82) *Ibid.*, **29,** 61(1969); through *Chem. Abstr.*, **71,** 105168e (1969).
(83) *Ibid.*, **29,** 67(1969); through *Chem. Abstr.*, **71,** 105169f (1969).
(84) R. Lacroix and J. Zigliara, *ibid.*, **28,** 183(1968); through *Chem. Abstr.*, **70,** 109147z(1969).
(85) S. Timar Spinad, *An. Fac. Quim. Farm., Univ. Chile*, **19,** 120(1967); through *Chem. Abstr.*, **70,** 76393w(1969).
(86) H. Delonca, A. Puech, G. Segura, and J. Youakim, *J. Pharm. Belg.*, **24,** 243(1969); through *Chem. Abstr.*, **71,** 105171a (1969).
(87) P. Jonsson and B. Turesson, *Svensk Farm. Tidskr.*, **72,** 963(1968); through *Chem. Abstr.*, **70,** 71032t(1969).
(88) N. Isobe and S. Seki, *Iryo*, **22,** 1201(1968); through *Chem. Abstr.*, **70,** 60769n(1969).
(89) K. Rao, E. Nageswara, C. Evans, S. Kailasam, S. Radhakrishna, P. Somasundaram, H. Scott, S. Subbammax, and S. Tripathy, *Indian J. Med. Res.*, **57,** 546(1969); through *Chem. Abstr.*, **71,** 6505f(1969).
(90) E. Moraga, B. Aranguiz, and R. Belmar, *Rev. Real Acad. Cienc. Exactas, Fis. Natur. Madrid*, **62,** 851(1968); through *Chem. Abstr.*, **71,** 42220t(1969).
(91) T. Yamana, Y. Mizukami, and A. Tsuji, *Yakugaku Zasshi*, **89,** 422(1969); through *Chem. Abstr.*, **71,** 24714c(1969).
(92) T. Baxter and H. Kostenbauder, *J. Pharm. Sci.*, **58,** 33 (1969).
(93) S. Larsen and V. Jensen, *Dansk. Tidsskr. Farm.*, **43,** 47 (1969); through *Chem. Abstr.*, **71,** 42230w(1969).
(94) H. Imura, H. Matsuyama, S. Matsukura, T. Miyake, and M. Fukase, *Proc. Asia Oceania Congr. Endocrinol., 3rd*, **3,** 393(1967); through *Chem. Abstr.*, **70,** 60772h(1969).
(95) K. Kakemi, H. Sezaki, K. Iwamoto, and Y. Sano, *Chem. Pharm. Bull.*, **17,** 1623(1969).
(96) A. Popov and N. Yanishlieva, *Dokl. Bolg. Akad. Nauk*, **22,** 411(1969); through *Chem. Abstr.*, **71,** 62478m(1969).
(97) L. Klotz and S. Rehfeld, *Pharmazie*, **23,** 496(1968); through *Chem. Abstr.*, **70,** 22879r(1969).
(98) I. Waginaire, *Bull. Tech. Gattefosse SFPA*, **63,** 58(1968); through *Chem. Abstr.*, **70,** 6511j(1969).
(99) L. Martonfi, L. Fulop, C. Szantho, I. Vereph, A. Gyeresi, and L. Domokos, *Rev. Med. (Tirgu-Mures)*, **15,** 5(1969); through *Chem. Abstr.*, **71,** 84503t(1969).
(100) P. Kabasakalian, G. Cannon, and G. Pinchuk, *J. Pharm. Sci.*, **58,** 45(1969).
(101) K. Koshy, *ibid.*, **58,** 560(1969).
(102) S. Karim, J. Devlin, and K. Hillier, *Eur. J. Pharmacol.*, **4,** 416(1968); through *Chem. Abstr.*, **70,** 50448t(1969).
(103) J. Fokkens and E. Burman, *Pharm. Weekbl.*, **104,** 177 (1969); through *Chem. Abstr.*, **70,** 90714h(1969).
(104) P. Lundgren, *Acta Pharm. Suecica*, **6,** 299(1969); through *Chem. Abstr.*, **71,** 105174d(1969).
(105) K. Ikeda, *Nagoya Shiritsu Daigaku Yakugakubu Kenkyu Nempo*, **16,** 1(1968); through *Chem. Abstr.*, **70,** 71020n(1969).
(106) P. Braeckman, *Pharm. Tijdschr. Belg.*, **45,** 93(1968); through *Chem. Abstr.*, **70,** 71017s(1969).
(107) E. Pandula, I. Racz, and Z. Pajor, *Gyogyszereszet*, **13,** 208(1969); through *Chem. Abstr.*, **71,** 116507r(1969).
(108) H. Tarrerias, *Bull. Tech. Gattefosse SFPA*, **63,** 19(1968); through *Chem. Abstr.*, **70,** 6532s(1969).
(109) L. Rapaport, *Farmatsiya (Moscow)*, **17,** 34(1968); through *Chem. Abstr.*, **70,** 14367g(1969).
(110) J. Derlikowski, A. Narbutt-Mering, and W. Weglowska, *Acta Pol. Pharm.*, **25,** 419(1968); through *Chem. Abstr.*, **70,** 60774k (1969).
(111) J. Krepinsky and J. Stiborova, *Cesk. Farm.*, **17,** 349 (1968); through *Chem. Abstr.*, **70,** 32877p(1969).
(112) I. Grabowska, *Acta Pol. Pharm.*, **25,** 513(1968); through *Chem. Abstr.*, **70,** 60777p(1969).
(113) L. Wendt, Z. Olszewski, and H. Bujar, *Farm. Pol.*, **25,** 237(1969); through *Chem. Abstr.*, **71,** 74008w(1969).
(114) N. Harb, *J. Hosp. Pharm.*, **26,** 44(1969).
(115) M. Nedergaard and M. Collado, *Pharm. Acta Helv.*, **44,** 541(1969); through *Chem. Abstr.*, **71,** 105175e(1969).
(116) W. Hauser, T. Kartnig, and G. Verdino, *Sci. Pharm.*, **36,** 7(1968); through *Chem. Abstr.*, **70,** 60767k(1969).
(117) M. Thymann, *Dansk. Tidsskr. Farm.*, **42,** 225(1968); through *Chem. Abstr.*, **70,** 60778q(1969).
(118) R. Van Severen, *Pharm. Tijdschr. Belg.*, **46,** 140(1969); through *Chem. Abstr.*, **71,** 84502s(1969).
(119) A. Kubat, *Isotopenpraxis*, **3,** 240(1967); through *Chem. Abstr.*, **70,** 109136v(1969).
(120) K. Buchali, H. Herzmann, D. Strangfeld, and G. Schneider, *ibid.*, **3,** 229(1967); through *Chem. Abstr.*, **71,** 33368v (1969).
(121) J. Torrado Valeiras, *Ann. Real Acad. Farm.*, **32,** 353 (1966); through *Chem. Abstr.*, **71,** 74006u(1969).
(122) T. Lipatova and R. Veselovskii, *Vysokomol. Soedin., Ser. A*, **11,** 1459(1969); through *Chem. Abstr.*, **71,** 105158b(1969).
(123) I. Rizescu and M. Sterescu, *Rev. Chim. (Bucharest)*, **19,** 733(1968); through *Chem. Abstr.*, **70,** 118042p(1969).
(124) J. Taylor, *J. Chem. Educ.*, **46,** 742(1969).
(125) B. Musulin, *ibid.*, **46,** 109(1969).
(126) C. Lingsch Wuensch, I. Moran, and A. Arancibia, *Farmaco, Ed. Prat.*, **24,** 562(1969); through *Chem. Abstr.*, **71,** 116506q(1969).
(127) H. Maulding, M. Zoglio, F. Pigois, and M. Wagner, *J. Pharm. Sci.*, **58,** 1359(1969).
(128) J. Mauger, A. Paruta, and R. Gerraughty, *ibid.*, **58,** 574 (1969).
(129) Z. Estes, *Mil. Med.*, **134,** 59(1969); through *Chem. Abstr.*, **70,** 60775m(1969).
(130) J. Mollica, C. Rehm, and J. Smith, *J. Pharm. Sci.*, **58,** 635(1969).
(131) T. Yamana, Y. Mizukami, A. Tsuji, and F. Ichimura, *Yakugaku Zasshi*, **89,** 740(1969).
(132) R. Adamski, J. Lutomski, W. Turowska, and H. Speichert, *Farm. Pol.*, **24,** 937(1968); through *Chem. Abstr.*, **70,** 118046t (1969).

(133) L. Pekkarinen, *Z. Lebensm.-Unters. Forsch.*, **139,** 23 (1968); through *Chem. Abstr.*, **70,** 71036x(1969).
(134) E. Garrett and B. Dvorchik, *J. Pharm. Sci.*, **58,** 813(1969).
(135) A. Pethybridge, *J. Chem. Soc., A*, **1969,** 1345.
(136) P. Bekker, S. Churms, A. Stephen, and G. Woolard, *Tetrahedron*, **25,** 3359(1969).
(137) S. Sun and K. Connors, *J. Pharm. Sci.*, **58,** 1150(1969).
(138) N. Armstrong, *J. Soc. Cosmet. Chem.*, **19,** 707(1968); through *Chem. Abstr.*, **70,** 6516q(1969).
(139) H. Kasserra and K. Laidler, *Can. J. Chem.*, **47,** 4021(1969).
(140) L. Dittert, G. Irwin, E. Rattie, C. Chong, and J. Swintosky, *J. Pharm. Sci.*, **58,** 557(1969).
(141) H. Nogami and Y. Kanakubo, *Chem. Pharm. Bull.*, **17,** 2013(1969).
(142) T. Yamana, Y. Mizukami, and F. Ichimura, *Yakugaku Zasshi*, **89,** 1338(1969).
(143) T. Suzuki and Y. Tanimura, *Chem. Pharm. Bull.*, **17,** 1422(1969).
(144) R. Notari and J. Munson, *J. Pharm. Sci.*, **58,** 1060(1969).
(145) R. Notari, *ibid.*, **58,** 1064(1969).
(146) *Ibid.*, **58,** 1069(1969).
(147) A. Hussain and P. Schurman, *ibid.*, **58,** 684(1969).
(148) *Ibid.*, **58,** 687(1969).
(149) W. Wolf and T. Wu, *ibid.*, **58,** 491(1969).
(150) A. Vander Zeeuw, *Chem. Ind. (London)*, **29,** 978(1969).
(151) W. Henderson and W. Shelver, *J. Pharm. Sci.*, **58,** 106 (1969).
(152) P. Baharad, B. Perlmutter-Hayman, and M. Wolff, *J. Phys. Chem.*, **73,** 4391(1969).
(153) A. Kobayashi and S. Umemoto, *Yakugaku Zasshi*, **89,** 1549(1969).
(154) P. Marriott, *J. Pharm. Pharmacol.*, **21,** 137(1969).
(155) T. Morgan, E. Phillips, and G. Stedman, *J. Chem. Soc., A*, **16,** 2318(1969).
(156) I. Bellobono and G. Favini, *Tetrahedron*, **25,** 57(1969).
(157) E. Pawelczyk and M. Zajac, *Diss. Pharm. Pharmacol.*, **20,** 641(1968); through *Chem. Abstr.*, **70,** 80814b(1969).
(158) J. Bojarski, *Wiad. Chem.*, **23,** 399(1969); through *Chem. Abstr.*, **71,** 64033m(1969).
(159) R. Mukherjea and A. Gupta, *Fette, Seifen, Anstrichm.*, **71,** 53(1969); through *Chem. Abstr.*, **71,** 62482h(1969).
(160) L. Kamaric, *Iugoslav. Physiol. Pharmacol. Acta, Suppl.*, **1968,** 41; through *Chem. Abstr.*, **71,** 47975c(1969).
(161) R. Alberty, *J. Chem. Educ.*, **46,** 713(1969).
(162) J. Hou and J. Poole, *J. Pharm. Sci.*, **58,** 447(1969).
(163) F. Saccani and F. Pansera, *Boll. Chim. Farm.*, **107,** 640 (1968); through *Chem. Abstr.*, **70,** 60770f(1969).
(164) W. Cressman, E. Sugita, J. Doluisio, and P. Niebergall, *J. Pharm. Sci.*, **58,** 1471(1969).
(165) R. Kinget and M. Schwartz, *ibid.*, **58,** 1102(1969).
(166) M. Schwartz, *J. Med. Chem.*, **12,** 36(1969).
(167) M. Schwartz and A. Delduce, *J. Pharm. Sci.*, **58,** 1137 (1969).
(168) H. Jacobson and G. Reier, *ibid.*, **58,** 631(1969).
(169) I. Gurevich, S. Stroev, L. Mashkova, and V. Gorlova, *Tr. Leningrad Khim.-Farm. Inst.*, **22,** 167(1967); through *Chem. Abstr.*, **70,** 50450n(1969).
(170) M. Kostolowska, L. Krowczynski, and Z. Kuzniak, *Acta Pol. Pharm.*, **25,** 519(1968); through *Chem. Abstr.*, **70,** 60784p (1969).
(171) N. Dabbis, H. El-Shibini, and M. Motawi, *Pharmazie*, **23,** 491(1968); through *Chem. Abstr.*, **70,** 22881k(1969).
(172) N. Dabbis, H. El-Shibini, and A. Hussein, *ibid.*, **23,** 573(1968); through *Chem. Abstr.*, **70,** 22876n(1969).
(173) A. Hussain, *J. Pharm. Sci.*, **58,** 316(1969).
(174) L. Leeson and J. Weidenheimer, *ibid.*, **58,** 355(1969).
(175) R. Notari and S. Caida, *ibid.*, **58,** 1203(1969).
(176) R. Wornick, *Proc. Annu. Pfizer Res. Conf. 15th*, **1967,** 54, through *Chem. Abstr.*, **70,** 40615f(1969).
(177) C. Wornick and O. Kuhn, *J. Agr. Food Chem.*, **17,** 318 (1969); through *Chem. Abstr.*, **70,** 109134t(1969).
(178) B. Olms and W. Fuertig, *Pharm. Prax. (Berlin)*, **1968,** 257; through *Chem. Abstr.*, **70,** 22882m(1969).
(179) J. Jeske, K. Stochla, E. Ranc-Bukowska, B. Sar-Zagorowska, and K. Misicicka-Grodner, *Farm. Pol.*, **24,** 465(1968); through *Chem. Abstr.*, **70,** 22878q(1969).
(180) S. Bhattacharya, *J. Inst. Chem. (Calcutta)*, **41,** 33(1969); through *Chem. Abstr.*, **70,** 80803x(1969).
(181) *Ibid.*, **41,** 110(1969); through *Chem. Abstr.*, **71,** 73999h (1969).
(182) T. Ammo, M. Washitake, Y. Takashima, and S. Sato, *Bitamin*, **38,** 427(1968); through *Chem. Abstr.*, **70,** 31651n(1969).
(183) D. Ruzhitskii, Y. Bartkov, and B. Matviikiv, *Farm. Zh. (Kiev)*, **24,** 42(1969); through *Chem. Abstr.*, **71,** 42216w(1969).
(184) J. Carstensen, M. Osadca, and S. Rubin, *J. Pharm. Sci.*, **58,** 549(1969).
(185) K. Inazu, S. Sunagawa, and R. Yamamoto, *Yakugaku Zasshi*, **89,** 320(1969).
(186) *Ibid.*, **89,** 331(1969).
(187) K. Inazu, M. Inoue, and R. Yamamoto, *Bitamin*, **39,** 119(1969); through *Chem. Abstr.*, **70,** 71035w(1969).
(188) B. Trivedi and P. Kapadia, *Indian J. Pharm.*, **31,** 60 (1969); through *Chem. Abstr.*, **71,** 128051w(1969).
(189) L. Grippa, *Farmacista*, **20,** 189(1969); through *Chem. Abstr.*, **71,** 33369w(1969).
(190) Y. Ogata and Y. Kosugi, *Tetrahedron*, **25,** 1055(1969).
(191) M. Kagami, *Fukushima Daigaku Kokakubu Hokoku*, **68** (1967); through *Chem. Abstr.*, **70,** 31647r(1969).
(192) V. Rajkovic, J. Dordevic, and J. Banjac, *Glas. Hem. Drus., Beograd*, **31,** 351(1966); through *Chem. Abstr.*, **70,** 31648s(1969).
(193) V. Rajkovic, J. Banjac, and J. Dordevic, *ibid.*, **31,** 461 (1968); through *Chem. Abstr.*, **70,** 31649t(1969).
(194) Y. Kato and A. Hatano, *Bitamin*, **40,** 149(1969); through *Chem. Abstr.*, **71,** 105170z(1969).
(195) M. Sabri, P. George, and V. Rao, *Indian J. Technol.*, **6,** 272(1968); through *Chem. Abstr.*, **70,** 31658v(1969).
(196) T. Takahashi and R. Yamamoto, *Yakugaku Zasshi*, **89,** 909(1969).
(197) *Ibid.*, **89,** 914(1969).
(198) *Ibid.*, **89,** 919(1969).
(199) *Ibid.*, **89,** 925(1969).
(200) *Ibid.*, **89,** 933(1969).
(201) *Ibid.*, **89,** 938(1969).
(202) *Ibid.*, **89,** 943(1969).
(203) C. Kowarski, *J. Pharm. Sci.*, **58,** 360(1969).
(204) E. Nuernberg, *Deut. Apoth.-Ztg.*, **109,** 1103(1969); through *Chem. Abstr.*, **71,** 116493h(1969).
(205) R. Huettenrauch, *Pharmazie*, **24,** 111(1969); through *Chem. Abstr.*, **70,** 118044r(1969).
(206) H. Nerlo and W. Palak, *Acta Pol. Pharm.*, **25,** 435(1968); through *Chem. Abstr.*, **70,** 71031s(1969).
(207) M. Struhar, B. Facak, and M. Mandak, *Cesk. Farm.*, **17,** 306(1968); through *Chem. Abstr.*, **70,** 14346z(1969).
(208) N. Taniguchi, *Iryo*, **23,** 144(1969); through *Chem. Abstr.*, **71,** 15981k(1969).
(209) J. Murray, *Pharm. J.*, **202,** 96(1969).
(210) R. McBride, *ibid.*, **202,** 445(1969).
(211) R. McBride, J. Murray, and G. Smith, *ibid.*, **203,** 71 (1969).
(212) *Ibid.*, **203,** 478(1969).
(213) L. Krowczynski, *Farm. Pol.*, **24,** 251(1968); through *Chem. Abstr.*, **70,** 60756f(1969).
(214) J. Polderman, *J. Mond. Pharm.*, **11,** 327(1968); through *Chem. Abstr.*, **70,** 80816d(1969).
(215) G. Schumacher, *Amer. J. Hosp. Pharm.*, **26,** 71(1969).
(216) P. Szentmiklosi, *Gyogyszereszet*, **13,** 108(1969); through *Chem. Abstr.*, **71,** 42189q(1969).
(217) M. Kassem, A. Kassem, and H. Ammar, *Pharm. Acta Helv.*, **44,** 535(1969); through *Chem. Abstr.*, **71,** 105192h(1969).
(218) J. Detak, J. Hejlek, J. Janat, and L. Jinora, *Sci. Pharm.*, **2,** 439(1965); through *Chem. Abstr.*, **70,** 50451p(1969).
(219) M. Tsuruoka, Y. Nakao, and M. Horioka, *Kyushu Yakugakkai Kaiho*, **22,** 17(1968); through *Chem. Abstr.*, **71,** 6521h (1969).
(220) J. Miller, J. Helprin, and J. Finlayson, *J. Pharm. Sci.*, **58,** 455(1969).
(221) J. Vasani and R. King, *Amer. J. Pharm.*, **141,** 177(1969).
(222) L. Volkovinskaya, T. Fabrichnaya, and A. Pozharskaya, *Khim.-Farm. Zh.*, **2,** 60(1968); through *Chem. Abstr.*, **70,** 71052z (1969).
(223) M. Brown and L. Leeson, *J. Pharm. Sci.*, **58,** 242(1969).

(224) A. Kleniewski, *Prod. Probl. Pharm.*, **24,** 144(1969); through *Chem. Abstr.*, **71,** 6520g(1969).
(225) E. Onur and O. Yalcindag, *Kim. Sanayi*, **15,** 215(1967); through *Chem. Abstr.*, **70,** 22860c(1969).
(226) V. Pilea, E. Popa-Burca, and R. Paiusan, *Cesk. Farm.*, **18,** 146(1969); through *Chem. Abstr.*, **71,** 42227a(1969).
(227) I. Soekadarova, *Rozhl. Tuberk.*, **29,** 214(1969); through *Chem. Abstr.*, **71,** 128647z(1969).
(228) H. Nerlo and M. Wiktorowicz, *Farm. Pol.*, **24,** 673 (1968); through *Chem. Abstr.*, **70,** 118057x(1969).
(229) B. Logawa and A. Jusman, *Suara Pharm.*, **11,** 151(1968); through *Chem. Abstr.*, **71,** 116519w(1969).
(230) A. Stawowczyk and L. Krowczynski, *Farm. Pol.*, **24,** 587(1968); through *Chem. Abstr.*, **70,** 118054u(1969).
(231) G. Bouyssoux and C. Courty, *Bull. Trav. Soc. Pharm. Lyon*, **13,** 115(1968); through *Chem. Abstr.*, **71,** 94724e(1969).
(232) F. Koechel, *Krankenhaus-Apoth.*, **17,** 1(1967); through *Chem. Abstr.*, **71,** 64065y(1969).
(233) J. Doyle, *Kirk-Othmer Encycl. Chem. Technol., 2nd Ed.*, **18,** 805(1969); through *Chem. Abstr.*, **71,** 128608n(1969).
(234) W. Lhoest, *Ind. Chim. Belge*, **32,** 277(1967); through *Chem. Abstr.*, **70,** 71045z(1969).
(235) K. Ruig, *Drug Cosmet. Ind.*, **104,** 64(1969).
(236) C. Phillips, *U. S. Clearinghouse Fed. Sci. Tech. Inform. AD-671373*, **1968,** 17; through *Chem. Abstr.*, **70,** 40617h(1969).
(237) J. Brewer and G. Keller, *Radiosteril. Med. Prod. Recom. Code Pract., Proc. Symp. Results Panel Meet.*, **1966,** 311; through *Chem. Abstr.*, **70,** 31661r(1969).
(238) V. Vashkov and A. Prishchep, *Life Sci. Space Res.*, **5,** 44(1966); through *Chem. Abstr.*, **70,** 6535v(1969).
(239) D. Gunther, *Amer. J. Hosp. Pharm.*, **26,** 45(1969).
(240) R. O'Leary, W. Watkins, and W. Guess, *J. Pharm. Sci.*, **58,** 1007(1969).
(241) V. Milojkovic, *Mikrobiologija*, **6,** 145(1969); through *Chem. Abstr.*, **71,** 128664c(1969).
(242) R. Velecky and Z. Harna, *Cesk. Hyg.*, **14,** 170(1969); through *Chem. Abstr.*, **71,** 128663b(1969).
(243) H. Weiss, *Pharm. Zentralh.*, **108,** 533(1969); through *Chem. Abstr.*, **71,** 128662a(1969).
(244) T. Bartha and E. Mero, *Egeszsegtudomany*, **13,** 78(1969); through *Chem. Abstr.*, **71,** 42180e(1969).
(245) R. Seidl, Z. Opletal, J. Blazek, and P. Cerny, *Pharmazie*, **24,** 48(1969); through *Chem. Abstr.*, **70,** 109141t(1969).
(246) G. Hortobagyi, G. Hangay, B. Lukats, G. Muranyi, and A. Zarandy, *Radiosteril. Med. Prod. Recom. Code Pract., Proc. Symp. Results Panel Meet.*, **1966,** 25; through *Chem. Abstr.*, **70,** 31660q(1969).
(247) E. Pandula, E. Farkas, and I. Racz, *ibid.*, **1966,** 83; through *Chem. Abstr.*, **70,** 14388q(1969).
(248) G. Hangay, G. Hortobagyi, and G. Muranyi, *ibid.*, **1966,** 55; through *Chem. Abstr.*, **70,** 14389r(1969).
(249) J. Schnell, *Nucl. Sci. Abstr.*, **23,** 24776(1969); through *Chem. Abstr.*, **71,** 105177g(1969).
(250) J. Masiakowski, *Farm. Pol.*, **24,** 453(1968); through *Chem. Abstr.*, **70,** 31659w(1969).
(251) W. Kedzia, Z. Muszynski, and K. Muszynska, *ibid.*, **24,** 354(1968); through *Chem. Abstr.*, **70,** 50449u(1969).
(252) A. Sirota, *Izobret. Prom. Obraztsy Tovarnye Znaki*, **45,** 65(1968); through *Chem. Abstr.*, **70,** 31688e(1969).
(253) A. Stawowczyk, L. Krowczynski, and A. Sokol, *Farm. Pol.*, **24,** 749(1968); through *Chem. Abstr.*, **71,** 42243c(1969).
(254) B. Trasen, *Drug Cosmet. Ind.*, **105,** 52(1969).
(255) F. Bowman, *J. Pharm. Sci.*, **58,** 1301(1969).
(256) N. Stoichev, *Pharmazie*, **24,** 475(1969); through *Chem. Abstr.*, **71,** 116517u(1969).
(257) S. Kucharski, *Farm. Pol.*, **25,** 267(1969); through *Chem. Abstr.*, **71,** 94720a(1969).
(258) L. Sinotte, *Drug Cosmet. Ind.*, **106,** 47(1969).
(259) E. Smith, *Mfg. Chem. Aerosol News*, **40,** 26(1969).
(260) M. Peters, *Aust. Chem. Process. Eng.*, **22,** 22(1969); through *Chem. Abstr.*, **71,** 116495k(1969).
(261) M. Ramanujam and D. Venkateswarlu, *Powder Technol.*, **3,** 92(1969).
(262) F. Cartan and G. Curtis, *Anal. Chem.*, **41,** 1719(1969).
(263) T. Allen, *Powder Technol.*, **2,** 133(1969).
(264) K. Ridgway and R. Rupp, *J. Pharm. Pharmacol.*, **21,** 30S(1969).
(265) N. Pilpel, *Endeavour*, **28,** 73(1969); through *Chem. Abstr.*, **71,** 25015n(1969).
(266) C. Harwood and N. Pilpel, *J. Pharm. Pharmacol.*, **21,** 721(1969).
(267) A. Otsuka, H. Sunada, and K. Danjo, *Yakugaku Zasshi*, **89,** 1013(1969).
(268) A. Otsuka, H. Sunada, and K. Danjo, *Zairyo*, **18,** 509 (1969); through *Chem. Abstr.*, **71,** 64057x(1969).
(269) N. Pilpel, *Mfg. Chem. Aerosol News*, **40,** 29(1969).
(270) E. Hiestand and C. Wilcox, *J. Pharm. Sci.*, **58,** 1403 (1969).
(271) N. Ur'ev, N. Mikhailov, and P. Rebinder, *Dokl. Akad. Nauk SSSR*, **184,** 665(1969); through *Chem. Abstr.*, **70,** 81279t (1969).
(272) M. Nishino, M. Arakawa, and E. Suito, *Zairyo*, **18,** 535(1969); through *Chem. Abstr.*, **71,** 64521u(1969).
(273) E. Yumioka, I. Koda, and H. Makita, *Yakuzaigaku*, **26,** 127(1966); through *Chem. Abstr.*, **70,** 6537x(1969).
(274) G. Jimbo, S. Asakawa, N. Soga, S. Hatano, and T. Uchiyama, *Zairyo*, **18,** 541(1969); through *Chem. Abstr.*, **71,** 64476h(1969).
(275) R. Hogg, G. Mempel, and D. Fuerstenau, *Powder Technol.*, **2,** 223(1969).
(276) D. Cahn and D. Fuerstenau, *ibid.*, **2,** 215(1969).
(277) J. Bridgwater, D. Bagster, S. Chen, and J. Hallam, *ibid.*, **2,** 198(1969).
(278) J. Okada, Y. Matsuda, and Y. Matsuda, *Yakugaku Zasshi*, **89,** 1562(1969).
(279) L. Lawrence and J. Beddow, *Powder Technol.*, **2,** 253 (1969).
(280) K. Ridgway, J. Glasby, and P. Rosser, *J. Pharm. Pharmacol.*, **21,** 24S(1969).
(281) H. Heins, W. Ott, and C. Fuehrer, *Pharm. Ind.*, **31,** 155 (1969); through *Chem. Abstr.*, **71,** 42247g(1969).
(282) J. Rees and E. Shotton, *J. Pharm. Pharmacol.*, **21,** 731 (1969).
(283) S. Bessho, S. Tomioka, and S. Ito, *Yakugaku Zasshi*, **89,** 599(1969).
(284) *Ibid.*, **89,** 1130(1969).
(285) C. Leoveanu, N. Zaharia, V. Pilea, and M. Pascu, *Ind. Chim. Belge*, **32,** 321(1967); through *Chem. Abstr.*, **70,** 90717m (1969).
(286) K. Manudhane, A. Contractor, H. Kim, and R. Shangraw, *J. Pharm. Sci.*, **58,** 616(1969).
(287) K. Manudhane, *Diss. Abstr.*, **30,** 1777B(1969).
(288) Z. Sykulska, *Herba Pol.*, **14,** 155(1968); through *Chem. Abstr.*, **70,** 109142u(1969).
(289) *Ibid.*, **14,** 163(1968); through *Chem. Abstr.*, **70,** 109143v (1969).
(290) Y. Borisenko, E. Borzunov, R. Obukhova, and V. Dekhtyarenko, *Farm. Zh. (Kiev)*, **24,** 51(1969); through *Chem. Abstr.*, **71,** 42248h(1969).
(291) L. Chwialkowska and L. Krowczynski, *Acta Pol. Pharm.*, **25,** 583(1968); through *Chem. Abstr.*, **70,** 60748e(1969).
(292) *Ibid.*, **26,** 45 (1969); through *Chem. Abstr.*, **70,** 109138x (1969).
(293) *Ibid.*, **26,** 51(1969); through *Chem. Abstr.*, **70,** 109144w (1969).
(294) B. Reich and F. Gstirner, *Arch. Pharm. (Weinheim)*, **301,** 830(1968); through *Chem. Abstr.*, **70,** 71050x(1969).
(295) R. Huettenrauch, U. Zahn, and R. Ditscher, *Pharm. Zentralh.*, **107,** 820(1968); through *Chem. Abstr.*, **70,** 50454s(1969).
(296) V. Marker, E. Shtyrkova, and D. Bernatonis, *Sakh. Prom.*, **42,** 50(1968); through *Chem. Abstr.*, **70,** 40618j(1969).
(297) J. Maly, J. Rak, and M. Chalabala, *Cesk. Farm.*, **17,** 486(1968); through *Chem. Abstr.*, **70,** 99594b(1969).
(298) L. Vrublevskaya and D. Kollerov, *Tr. Metrol. Inst. SSSR*, **96,** 5(1968); through *Chem. Abstr.*, **70,** 31667x(1969).
(299) B. Selmeczi and G. Kedvessy, *Pharm. Ind.*, **31,** 564(1969); through *Chem. Abstr.*, **71,** 128672d(1969).
(300) D. Bernatonis and Y. Blagovidova, *Tr. I [Pervogo] Moskv. Med. Inst.*, **61,** 128(1968); through *Chem. Abstr.*, **71,** 105186j (1969).
(301) H. Bervenmark, *Acta Pharm. Suecica*, **5,** 579(1968); through *Chem. Abstr.*, **70,** 109139y(1969).
(302) I. Cruceanu, *Farmacia (Bucharest)*, **17,** 197(1969); through *Chem. Abstr.*, **71,** 42188p(1969).

(303) O. Alpar, J. Deer, J. Hersey, and E. Shotton, *J. Pharm. Pharmacol.*, **21,** 6S(1969).

(304) A. David, G. Horvath, G. Liptay, and E. Orban, *Pharm. Ind.*, **30,** 819(1968); through *Chem. Abstr.*, **70,** 118064x(1969).

(305) M. Alyushin and M. Astrakhanova, *Farmatsiya (Moscow)*, **17,** 73(1968); through *Chem. Abstr.*, **70,** 60757g(1969).

(306) B. Selmeczi and G. Kedvessy, *Acta Pharm. Hung.*, **38,** 344(1968); through *Chem. Abstr.*, **70,** 14395q(1969).

(307) F. Jaminet, *Bull. Tech. Gattefosse SFPA*, **63,** 63(1968); through *Chem. Abstr.*, **70,** 6536w(1969).

(308) V. Gandel, *Khim.-Farm. Zh.*, **3,** 51(1969); through *Chem. Abstr.*, **70,** 109146y(1969).

(309) E. Kristoffersson and V. Krogerus, *Farm. Aikak.*, **78,** 54(1969); through *Chem. Abstr.*, **71,** 42251d(1969).

(310) B. Chodkowska-Granicka and L. Krowczynski, *Acta Pol. Pharm.*, **25,** 447(1968); through *Chem. Abstr.*, **70,** 71049d (1969).

(311) *Ibid.*, **25,** 527(1968); through *Chem. Abstr.*, **70,** 60786r (1969).

(312) M. Kata, *Gyogyszereszet*, **11,** 420(1967); through *Chem. Abstr.*, **70,** 22892q(1969).

(313) L. Krowczynski, K. Kolarski, and K. Swoboda, *Acta Pol. Pharm.*, **25,** 441(1968); through *Chem. Abstr.*, **70,** 71051y (1969).

(314) D. Bernatonis and Y. Blagovidova, *Farmatsiya (Moscow)*, **18,** 27(1969); through *Chem. Abstr.*, **71,** 42252e(1969).

(315) D. Bernatonis, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 132 (1968); through *Chem. Abstr.*, **71,** 105184g(1969).

(316) B. Chodkowska-Granicka and L. Krowczynski, *Acta Pol. Pharm.*, **25,** 299(1968); through *Chem. Abstr.*, **70,** 31665v (1969).

(317) D. Ward, L. Lathrop, and M. Lynch, *J. Pharm. Sci.*, **58,** 1464(1969).

(318) E. Borzunov and S. Shevchenko, *Farmatsiya (Moscow)*, **18,** 20(1969); through *Chem. Abstr.*, **71,** 33385y(1969).

(319) H. Nogami, T. Nagai, E. Fukuoka, and T. Sonobe, *Chem. Pharm. Bull.*, **17,** 1450(1969).

(320) D. Ganderton, *J. Pharm. Pharmacol.*, **21,** 9S(1969).

(321) L. Krowczynski and T. Stozek, *Diss. Pharm. Pharmacol.*, **20,** 665(1968); through *Chem. Abstr.*, **70,** 80820a(1969).

(322) M. Van Ooteghem, R. Boni, and H. Herbots, *Cesk. Farm.*, **18,** 122(1969); through *Chem. Abstr.*, **71,** 42249j(1969).

(323) B. Selmeczi and J. Lenart, *Pharmazie*, **23,** 577(1968); through *Chem. Abstr.*, **70,** 22890n(1969).

(324) B. Reich and F. Gstirner, *Pharm. Ind.*, **31,** 559(1969); through *Chem. Abstr.*, **71,** 128665d(1969).

(325) H. Negoro, E. Awada, Y. Ikegami, S. Akagi, and K. Matsui, *Ind. Chim. Belge*, **3,** 241(1967); through *Chem. Abstr.*, **70,** 40619k(1969).

(326) P. Constantino and A. Maris, *Rev. Port. Farm.*, **19,** 1(1969); through *Chem. Abstr.*, **71,** 42245e(1969).

(327) E. Selles Flores, *An. Real Acad. Farm.*, **32,** 489(1966); through *Chem. Abstr.*, **71,** 42250c(1969).

(328) K. Lehmann and D. Dreher, *Pharm. Ind.*, **31,** 409(1969); through *Chem. Abstr.*, **71,** 116521r(1969).

(329) B. Jones, *Mfg. Chem. Aerosol News*, **40,** 25(1969).

(330) K. Ito, S. Kaga, and Y. Takeya, *Chem. Pharm. Bull.*, **17,** 1134(1969).

(331) K. Ito, M. Hitomi, S. Kaga, and Y. Takeya, *ibid.*, **17,** 1138(1969).

(332) G. Cordes, *Pharm. Ind.*, **31,** 328(1969); through *Chem. Abstr.*, **71,** 64072y(1969).

(333) M. Dudkevich, S. Larinov, and N. Tsyurupa, *Tr. Mosk. Khim.-Tekhnol. Inst.*, **58,** 212(1968); through *Chem. Abstr.*, **70,** 11836d(1969).

(334) A. Ikekawa and N. Kaneniwa, *Chem. Pharm. Bull.*, **17,** 827(1969).

(335) J. Okada, Y. Matsuda, and Y. Fukumori, *Yakugaku Zasshi*, **89,** 1539(1969).

(336) G. Riley, *Powder Technol.*, **2,** 315(1969).

(337) T. Oweberg, R. McDonald, and R. Traninor, Jr., *ibid.*, **3,** 56(1969).

(338) M. Pramoda, *Diss. Abstr.*, **30,** 1612B(1969).

(339) K. Ridgway, E. Shotton, and J. Glasby, *J. Pharm. Pharmacol.*, **21,** 19S(1969).

(340) F. Goodhart, G. Mayorga, and F. Ninger, *J. Pharm. Sci.*, **58,** 248(1969).

(341) S. Bessho, S. Tomioka, and S. Ito, *Yakugaku Zasshi*, **89,** 1553(1969).

(342) G. Langer and G. Yamate, *J. Colloid Interface Sci.*, **29,** 450(1969).

(343) N. Pilpel, *Chem. Process Eng.*, **1969,** 67; through *Chem. Abstr.*, **71,** 73965u(1969).

(344) C. Gandel, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 401 (1968); through *Chem. Abstr.*, **71,** 105183f(1969).

(345) H. Delonca, A. Puech, Y. Youakim, and G. Segura, *Pharm. Acta Helv.*, **44,** 464(1969).

(346) R. Griffiths, *Mfg. Chem. Aerosol News*, **40,** 29(1969).

(347) A. Puech, J. Serrano, and C. Serrano, *J. Pharm. Belg.*, **23,** 445(1968); through *Chem. Abstr.*, **70,** 71043x(1969).

(348) A. Otsuka, T. Wakimoto, and A. Takeda, *Zairyo*, **18,** 484(1969); through *Chem. Abstr.*, **71,** 74007v(1969).

(349) E. Kristoffersson, *Farm. Aikak.*, **77,** 248(1968); through *Chem. Abstr.*, **70,** 99564s(1969).

(350) V. Gandel, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 158 (1968); through *Chem. Abstr.*, **71,** 94723d(1969).

(351) T. Smazynski and L. Krowczynski, *Acta Pol. Pharm.*, **25,** 291(1968); through *Chem. Abstr.*, **70,** 31654r(1969).

(352) Y. Shumakov, *Farm. Zh. (Kiev)*, **24,** 61(1969); through *Chem. Abstr.*, **71,** 53537c(1969).

(353) D. Cadwallader and Q. Islam, *J. Pharm. Sci.*, **58,** 238(1969).

(354) K. Froemming, *Deut. Apoth.-Ztg.*, **109,** 309(1969); through *Chem. Abstr.*, **70,** 109145x(1969).

(355) L. Efimova and S. Minina, *Khim.-Farm. Zh.*, **3,** 55(1969); through *Chem. Abstr.*, **71,** 128602f(1969).

(356) E. Pandula and B. Toth, *Gyogyszereszet*, **13,** 15(1969); through *Chem. Abstr.*, **71,** 33384x(1969).

(357) L. Efimova and S. Minina, *Farmatsiya (Moscow)*, **18,** 16(1969); through *Chem. Abstr.*, **71,** 116522s(1969).

(358) Anonymous, *Drug Cosmet. Ind.*, **105,** 63(1969).

(359) L. Carletti and S. Rigamonti, *Boll. Chim. Farm.*, **107,** 579(1968); through *Chem. Abstr.*, **70,** 22889u(1969).

(360) P. Pashnev and R. Safiulin, *Farm. Zh. (Kiev)*, **24,** 57 (1969); through *Chem. Abstr.*, **71,** 53536b(1969).

(361) K. Bolewski and B. Rychly, *Farm. Pol.*, **24,** 461(1968); through *Chem. Abstr.*, **70,** 60750z(1969).

(362) G. Schumacher, *Amer. J. Hosp. Pharm.*, **26,** 548(1969).

(363) B. Ecanow, B. Gold, and C. Ecanow, *Amer. Perfum. Cosmet.*, **84,** 27(1969).

(364) S. Rigamonti and G. Rugginenti, *Farmaco, Ed. Prat.*, **24,** 445(1969); through *Chem. Abstr.*, **71,** 73967w(1969).

(365) N. Purohit and A. Roy, *Trans. Indian Inst. Chem. Eng.*, **1968,** 60; through *Chem. Abstr.*, **70,** 71346y(1969).

(366) H. Kaes and R. Brueckner, *Kolloid Z.-Z. Polym.*, **230,** 240(1969); through *Chem. Abstr.*, **71,** 6810b(1969).

(367) M. Aoki, A. Kamada, H. Fukuchi, and Y. Sumiyama, *Chem. Pharm. Bull.*, **17,** 1444(1969).

(368) B. Ecanow, B. Gold, R. Levinson, H. Takruri, and W. Stanaszek, *Amer. Perfum. Cosmet.*, **84,** 30(1969).

(369) P. Hiltner and I. Krieger, *J. Phys. Chem.*, **73,** 2386(1969).

(370) A. Singh and K. Roychdhury, *Chem. Sci.*, **24,** 1185(1969); through *Chem. Abstr.*, **71,** 54036a(1969).

(371) Y. Mori and A. Yoshizawa, *Kagaku Kogaku*, **32,** 1233 (1968); through *Chem. Abstr.*, **70,** 118468g(1969).

(372) B. Kaye, *Powder Technol.*, **3,** 78(1969).

(373) A. Flegmann, J. Goodwin, and R. Ottewill, *Proc. Brit. Ceram. Soc.*, **13,** 31(1969); through *Chem. Abstr.*, **71,** 84936m(1969).

(374) O. Korob and R. McCartney, *J. Aust. Ceram. Soc.*, **2,** 20(1966); through *Chem. Abstr.*, **71,** 105671p(1969).

(375) J. Olivier and P. Bennett, *Clay Miner.*, **15,** 345(1966); through *Chem. Abstr.*, **71,** 95312f(1969).

(376) E. McCartney and L. Yeo, *J. Aust. Ceram. Soc.*, **1,** 13 (1965); through *Chem. Abstr.*, **71,** 74373m(1969).

(377) B. Bar-Yosef, U. Kafkafi, and N. Lahav, *Soil Sci. Soc. Amer., Proc.*, **33,** 672(1969); through *Chem. Abstr.*, **71,** 129091a (1969).

(378) J. Iannicelli and N. Millman, *Clay Miner.*, **14,** 347(1964); through *Chem. Abstr.*, **71,** 95218e(1969).

(379) A. Flegmann and R. Ottewill, *Chem. Phys. Appl. Surface Active Subst., Proc. Int. Congr., 4th*, **2,** 1271(1964); through *Chem. Abstr.*, **71,** 129078b(1969).

(380) E. Geuze and P. Rebull, *Clay Miner.*, **14,** 103(1964); through *Chem. Abstr.*, **71,** 95211x(1969).

(381) R. Slater, J. Clark, and J. Kitchener, *Proc. Brit. Ceram. Soc.*, **13,** 1(1969); through *Chem. Abstr.*, **71,** 84930e(1969).
(382) L. Kuznetsona and N. Serb-Serbina, *Kolloid Zh.*, **30,** 853(1968); through *Chem. Abstr.*, **70,** 50927y(1969).
(383) L. Prilipko and O. Kurilenko, *Pishch. Prom. (Kiev)*, **5,** 93(1967); through *Chem. Abstr.*, **70,** 41099c(1969).
(384) T. Tobias and A. Ruotsala, *Clay Miner.*, **13,** 395(1964); through *Chem. Abstr.*, **71,** 84934j(1969).
(385) R. Smith and W. Worrall, *Proc. Brit. Ceram. Soc.*, **13,** 57(1969); through *Chem. Abstr.*, **71,** 84937n(1969).
(386) K. Shinoda, N. Moriyama, and K. Hattori, *J. Colloid Interface Sci.*, **30,** 270(1969).
(387) V. Enal'ev, R. Kucher, B. Lapan, and P. Gurenko, *Kolloid Zh.*, **31,** 53(1969); through *Chem. Abstr.*, **70,** 91113y(1969).
(388) N. Moriyama, K. Hattori, and K. Shinoda, *Nippon Kagaku Zasshi*, **90,** 35(1969); through *Chem. Abstr.*, **70,** 81282p (1969).
(389) H. Lange, *Staub*, **28,** 493(1968); through *Chem. Abstr.*, **70,** 71345x(1969).
(390) R. Morgan, *Trans. Soc. Rheol.*, **12,** 511(1968); through *Chem. Abstr.*, **70,** 81275p(1969).
(391) A. Samokhin, V. Maksimov, and E. Aleksandrova, *Tr. Mosk. Khim.-Tekhnol. Inst.*, **58,** 219(1968); through *Chem. Abstr.*, **70,** 118466e(1969).
(392) L. Romo, *Chem. Phys. Appl. Surface Active Subst., Proc. Int. Congr., 4th*, **2,** 1279(1964); through *Chem. Abstr.*, **71,** 129172c(1969).
(393) A. Karaulov and G. Gnatyuk, *Sb. Nauch. Tr., Ukr. Nauch.-Issled. Inst. Ogneuporov*, **10,** 22(1969); through *Chem. Abstr.*, **71,** 95304e(1969).
(394) W. Suess and H. Hanke, *Pharmazie*, **24,** 211(1969); through *Chem. Abstr.*, **71,** 105677v(1969).
(395) N. Kruglitskii, V. Simurov, and S. Barshchevskaya, *Izv. Akad. Nauk SSSR, Neorg. Mater*, **5,** 943(1969); through *Chem. Abstr.*, **71,** 95307h(1969).
(396) V. Mishin and V. Popkov, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 170(1968); through *Chem. Abstr.*, **71,** 105670n(1969).
(397) E. Savopol, D. Serbanescu, C. Illiescu, and C. Fica, *Farmacia (Bucharest)*, **16,** 675(1968); through *Chem. Abstr.*, **70,** 31630e(1969).
(398) R. Alwitt, *J. Phys. Chem.*, **73,** 1052(1969).
(399) P. Rebinder, *Sovrem. Probl. Fiz. Khim.*, **3,** 334(1968); through *Chem. Abstr.*, **71,** 74335a(1969).
(400) C. Hunt and A. Woolf, *Powder Technol.*, **3,** 9(1969).
(401) L. Leek, P. Deshpande, and J. Couper, *Ind. Eng. Fundam.*, **8,** 540(1969); through *Chem. Abstr.*, **71,** 54031v(1969).
(402) R. Hunter, *Proc. Roy. Aust. Chem. Inst.*, **36,** 168(1969); through *Chem. Abstr.*, **71,** 95096p(1969).
(403) K. Duckova, Z. Gruntova and M. Mandak, *Cesk. Farm.*, **18,** 73(1969); through *Chem. Abstr.*, **71,** 128650v(1969).
(404) I. Shainberg and W. Kemper, *Clay Miner.*, **14,** 117(1964); through *Chem. Abstr.*, **71,** 95219f(1969).
(405) G. Titeica, *Dari Seama Sedintelor, Com. Stat Geol., Repub. Soc. Rom.*, **53,** 341(1968); through *Chem. Abstr.*, **71,** 84935k (1969).
(406) P. Ionescu-Stoian, E. Savopol, and D. Serbanescu, *Farmacia (Bucharest)*, **17,** 11(1969); through *Chem. Abstr.*, **70,** 80818f(1969).
(407) P. Rohdewald, *Sci. Pharm.*, **37,** 195(1969); through *Chem. Abstr.*, **71,** 128675g(1969).
(408) *Ibid.*, **37,** 215(1969); through *Chem. Abstr.*, **71,** 128676h (1969).
(409) A. Moes, *Mem. Acad. Roy. Med. Belg.*, **5,** 797(1969); through *Chem. Abstr.*, **71,** 94718f(1969).
(410) J. Van Sonsbeek, *Pharm. Weekbl.*, **104,** 825(1969); through *Chem. Abstr.*, **71,** 79429u(1969).
(411) V. Minenko, N. Vdovenko, and I. Zurabov, *Ukr. Khim. Zh.*, **35,** 162(1969); through *Chem. Abstr.*, **71,** 6828p(1969).
(412) H. Nerlo and S. Umer, *Farm. Pol.*, **24,** 793(1968); through *Chem. Abstr.*, **71,** 42244d(1969).
(413) J. Pearson and G. Varney, *J. Pharm. Pharmacol.*, **21,** 60S(1969).
(414) A. Alexander, *Amer. Perfum. Cosmet.*, **84,** 27(1969).
(415) G. Rimlinger, *Fr. Ses Parfums*, **11,** 233(1968); through *Chem. Abstr.*, **70,** 6508p(1969).
(416) H. Maso and L. Conrad, *Amer. Perfum. Cosmet.*, **84,** 53(1969).
(417) F. Lachampt and R. Vila, *Parfum., Cosmet. Savons*, **12,** 239(1969); through *Chem. Abstr.*, **71,** 84832z(1969).
(418) A. Suzuki, N. Ho, and W. Higuchi, *J. Colloid Interface Sci.*, **29,** 552(1969).
(419) P. Walstra and H. Oortwijn, *ibid.*, **29,** 424(1969).
(420) K. Morosawa, K. Mimata, and T. Mitsui, *Nippon Kagaku Zasshi*, **90,** 352(1969); through *Chem. Abstr.*, **71,** 42623b(1969).
(421) L. Mukerjee and R. Sharma, *Indian J. Appl. Chem.*, **31** 164(1968); through *Chem. Abstr.*, **71,** 53899x(1969).
(422) T. Lin and J. Lambrechts, *J. Soc. Cosmet. Chem.*, **20,** 185(1969).
(423) S. Ishida, T. Sonoda, and T. Yoshida, *Yukagaku*, **17,** 562(1968); through *Chem. Abstr.*, **70,** 23127f(1969).
(424) K. Shinoda and H. Saito, *J. Colloid Interface Sci.*, **30,** 258(1969).
(425) M. Seiller, C. Arguillere, P. David, F. Puisieux, and A. LeHir, *Ann. Pharm. Fr.*, **26,** 557(1968); through *Chem. Abstr.*, **70,** 40622f(1969).
(426) H. Rahm, M. Soliva, and P. Speiser, *Pharm. Acta Helv.*, **44,** 356(1969); through *Chem. Abstr.*, **71,** 105579q(1969).
(427) P. Blonchard, *Parfum., Cosmet. Savons*, **12,** 82(1969); through *Chem. Abstr.*, **70,** 118383a(1969).
(428) H. Rahm, M. Soliva, and P. Speiser, *Pharm. Acta Helv.*, **44,** 1(1969); through *Chem. Abstr.*, **70,** 60751a(1969).
(429) G. Rimlinger, *Amer. Perfum. Cosmet.*, **84,** 31(1969).
(430) P. Elworthy and A. Florence, *J. Pharm. Pharmacol.*, **21,** 70S(1969).
(431) *Ibid.*, **21,** 79S(1969).
(432) *Ibid.*, **21,** 72(1969).
(433) C. Prakash and S. Srivastava, *Bull. Chem. Soc. Jap.*, **42,** 1787(1969); through *Chem. Abstr.*, **71,** 72225r(1969).
(434) N. Weiner, A. Shah, J. Kanig, and A. Felmeister, *J. Soc. Cosmet. Chem.*, **20,** 215(1969).
(435) T. Sastry and S. Srivastava, *Indian J. Chem.*, **6,** 728(1968); through *Chem. Abstr.*, **70,** 81178j(1969).
(436) N. Savostikova, Y. Rybakova, and A. Luk'yanov, *Maslo-Zhir. Prom.*, **35,** 28(1969); through *Chem. Abstr.*, **71,** 84831y(1969).
(437) N. Sverdlova and O. Grigorov, *Vestn. Leningrad. Univ., Fiz. Khim.*, **23,** 133(1968); through *Chem. Abstr.*, **70,** 23126e (1969).
(438) L. Mukerjee and O. Gupta, *Indian J. Appl. Chem.*, **31,** 223(1968); through *Chem. Abstr.*, **71,** 53898w(1969).
(439) B. Barry, *J. Pharm. Pharmacol.*, **21,** 533(1969).
(440) R. Petersen, *Umschau*, **69,** 85(1969); through *Chem. Abstr.*, **71,** 74369q(1969).
(441) H. Rosano, R. Peiser, and A. Eydt, *Rev. Fr. Corps Gras*, **16,** 249(1969); through *Chem. Abstr.*, **71,** 74371j(1969).
(442) G. Rimlinger, *Bull. Tech. Gattefosse SFPA*, **1968,** 65; through *Chem. Abstr.*, **71,** 95137c(1969).
(443) G. Rimlinger, *Fr. Ses Parfums*, **12,** 57(1969); through *Chem. Abstr.*, **71,** 53462z(1969).
(444) W. Parsons and D. Dondero, *J. Pharm. Sci.*, **58,** 1453 (1969).
(445) G. Richter and H. Kadner, *Pharmazie*, **24,** 398(1969); through *Chem. Abstr.*, **71,** 128674f(1969).
(446) S. Jain and S. Srivastava, *Bull. Chem. Soc. Jap.*, **42,** 1171 (1969); through *Chem. Abstr.*, **71,** 105578p(1969).
(447) V. Castillo and F. Siegel, *Amer. Perfum. Cosmet.*, **84,** 41 (1969).
(448) A. Fiebig, K. Ludwikowska, A. Radecki, and R. Piekos, *Przem. Chem.*, **48,** 216(1969); through *Chem. Abstr.*, **71,** 42219z (1969).
(449) G. Mikhailova, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 168(1968); through *Chem. Abstr.*, **71,** 84530z(1969).
(450) Y. Blagovidova and V. Gretskii, *ibid.*, **61,** 139(1968); through *Chem. Abstr.*, **71,** 94728j(1969).
(451) S. Davis, *J. Pharm. Sci.*, **58,** 412(1969).
(452) *Ibid.*, **58,** 418(1969).
(453) I. Eros and G. Kedvessy, *Acta Pharm. Hung.*, **39,** 164 (1969); through *Chem. Abstr.*, **71,** 73996e(1969).
(454) I. Eros and G. Kedvessy, *Sci. Pharm.*, **37,** 2(1969); through *Chem. Abstr.*, **70,** 118058y(1969).
(455) K. Muenzel and K. Berneis, *ibid.*, **37,** 55(1969); through *Chem. Abstr.*, **70,** 118065y(1969).
(456) M. Zhakova, *Tr. Perm. Farm. Inst.*, **2,** 113(1967); through *Chem. Abstr.*, **70,** 118061u(1969).

(457) O. Ryaposova, *ibid.*, **2,** 107(1967); through *Chem. Abstr.*, **70,** 118059z(1969).

(458) G. Tsagareishvili and G. Bashura, *Soobshch. Akad. Nauk Gruz. SSR*, **53,** 357(1969); through *Chem. Abstr.*, **71,** 53543b (1969).

(459) O. Ponomareva, A. Nosenkova, and L. Vyshenskaya, *Tr. Perm. Farm. Inst.*, **2,** 111(1967); through *Chem. Abstr.*, **70,** 118060t(1969).

(460) V. Gretskii, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 112 (1968); through *Chem. Abstr.*, **71,** 105172b(1969).

(461) K. Khristov, M. Gluzman, and I. Levitskaya, *Farmatsiya (Sofia)*, **19,** 15(1969); through *Chem. Abstr.*, **71,** 64066z(1969).

(462) M. Mandak, J. Kucera, and V. Veber, *Pharm. Tijdschr. Belg.*, **46,** 114(1969); through *Chem. Abstr.*, **71,** 53539e(1969).

(463) R. Goldenberg, *Drug Cosmet. Ind.*, **104,** 54(1969).

(464) E. Nuernberg, *Fette, Seifen, Anstrichm.*, **71,** 386(1969); through *Chem. Abstr.*, **71,** 24717f(1969).

(465) O. Ponomareva and I. Masetkin, *Tr. Perm. Farm. Inst.*, **2,** 129(1967); through *Chem. Abstr.*, **70,** 118063w(1969).

(466) Z. Gruntova, M. Mandak, and K. Duckova, *Farm. Obz.*, **36,** 499(1967); through *Chem. Abstr.*, **70,** 40621e(1969).

(467) Z. Gruntova, M. Mandak, and K. Duckova, *ibid.*, **36,** 433(1967); through *Chem. Abstr.*, **70,** 14397z(1969).

(468) J. Kucera and M. Mandak, *Cesk. Farm.*, **18,** 22(1969); through *Chem. Abstr.*, **71,** 33380t(1969).

(469) G. Gryadunova, *Farmatsiya (Moscow)*, **17,** 18(1968); through *Chem. Abstr.*, **70,** 6533t(1969).

(470) H. Wasiak and A. Keresztes, *Acta Pharm. Hung.*, **39,** 110(1969); through *Chem. Abstr.*, **71,** 42224x(1969).

(471) M. Islamgulov and Z. Dzhamilova, *Med. Zh. Uzb.*, **7,** 47(1968); through *Chem. Abstr.*, **71,** 42235b(1969).

(472) M. Melichar, *Sci. Pharm.*, **2,** 337(1965); through *Chem. Abstr.*, **70,** 22893r(1969).

(473) E. Sumner, W. Poole, D. Entrekin, and A. Ike, *J. Pharm. Sci.*, **58,** 83(1969).

(474) J. Virgili, L. Garzon, and J. Polanco, *An. Quim.*, **64,** 801 (1968); through *Chem. Abstr.*, **70,** 81194m(1969).

(475) M. Mandak, J. Kucera, and V. Veber, *Farmatsiya (Moscow)*, **18,** 73(1969); through *Chem. Abstr.*, **71,** 24706b(1969).

(476) B. Baker, R. Fountain, J. Hadgraft, and I. Sarkany, *Brit. J. Dermatol.*, **81,** 60(1969); through *Chem. Abstr.*, **70,** 118037r (1969).

(477) J. Sallai and A. Mayer, *Pharmazie*, **24,** 464(1969); through *Chem. Abstr.*, **71,** 116520q(1969).

(478) J. Kucera, M. Mandak, and D. Mlynarcik, *Sci. Pharm.*, **36,** 242(1968); through *Chem. Abstr.*, **70,** 71056d(1969).

(479) A. Mayer and G. Kedvessy, *Pharm. Ind.*, **31,** 323(1969); through *Chem. Abstr.*, **71,** 105181d(1969).

(480) N. Billups, *Can. J. Pharm. Sci.*, **4,** 11(1969).

(481) C. Hynniman and P. Lamy, *Drug Cosmet. Ind.*, **105,** 40 (1969).

(482) F. Gstirner, D. Kottenberg, and A. Maas, *Arch. Pharm. (Weinheim)*, **302,** 340(1969); through *Chem. Abstr.*, **71,** 105166c (1969).

(483) G. Morozova, N. Solun, L. Martynov, and E. Korchagina, *Protsessy Geleobrazovaniya*, **1968,** 86; through *Chem. Abstr.*, **71,** 24718g(1969).

(484) S. Negoita, D. Zubcov, E. Gherghiniu, C. Popescu, S. Balliu, S. Coroi, and S. Boteanu, *Farmacia (Bucharest)*, **17,** 215 (1969); through *Chem. Abstr.*, **71,** 42262h(1969).

(485) F. Bowman, *J. Pharm. Sci.*, **58,** 277(1969).

(486) A. Launchbury, *Lancet*, **II,** 1008(1969).

(487) B. Idson, *Drug Cosmet. Ind.*, **104,** 44(1969).

(488) *Ibid.*, **105,** 48(1969).

(489) T. Higuchi and T. Tanimoto, *Kyushu Yakugakkai Kaiho*, **22,** 27(1968); through *Chem. Abstr.*, **71,** 33357r(1969).

(490) G. Gryadunova, Y. Boyazitov, and A. Konstantinov, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 115(1968); through *Chem. Abstr.*, **71,** 105189n(1969).

(491) V. Gretskii, *ibid.*, **61,** 160(1968); through *Chem. Abstr.*, **71,** 105180c(1969).

(492) H. Hoffmann and E. Paulus, *Fette, Seifen, Anstrichm.*, **71,** 399(1969); through *Chem. Abstr.*, **71,** 24693v(1969).

(493) J. Sobotka-Wierzbowicz and H. Ludwicki, *Farm. Pol.*, **25,** 241(1969); through *Chem. Abstr.*, **71,** 73974w(1969).

(494) A. Longworth and J. French, *J. Pharm. Pharmacol.*, **21,** 1S(1969).

(495) A. Schmidt, I. Spiridonova, G. Balyabina, and I. Yusupova, *Farmatsiya (Moscow)*, **17,** 29(1968); through *Chem. Abstr.*, **70,** 14396r(1969).

(496) K. Stefanov and B. Bochev, *Farmatsiya (Sofia)*, **18,** 1(1968); through *Chem. Abstr.*, **71,** 6519p(1969).

(497) E. Maggiorelli, E. Magonio, and F. Trabucchi, *Boll. Chim. Farm.*, **107,** 649(1968); through *Chem. Abstr.*, **70,** 60788t (1969).

(498) T. Sidorovich and V. Malakhova, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 179(1968); through *Chem. Abstr.*, **71,** 94727h(1969).

(499) A. Weiss and B. Sciarrone, *J. Pharm. Sci.*, **58,** 980(1969).

(500) R. Yousef and A. Ghobashy, *Dansk. Tidsskr. Farm.*, **43,** 31(1969); through *Chem. Abstr.*, **71,** 33360m(1969).

(501) R. Voigt and G. Falk, *Pharmazie*, **23,** 709(1968); through *Chem. Abstr.*, **70,** 80819g(1969).

(502) M. Kata, *ibid.*, **24,** 395(1969); through *Chem. Abstr.*, **71,** 128667f(1969).

(503) M. Kata and G. Kedvessy, *ibid.*, **23,** 667(1968); through *Chem. Abstr.*, **70,** 50453r(1969).

(504) I. Azhgikhin, V. Malakhova, and E. Grosfel'd, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 174(1968); through *Chem. Abstr.*, **71,** 105179j(1969).

(505) I. Azhgikhin, R. Bobylev, and V. Gandel, *ibid.*, **61,** 136 (1968); through *Chem. Abstr.*, **71,** 105193j(1969).

(506) R. Huettenrauch and R. Keil, *Pharmazie*, **24,** 159(1969); through *Chem. Abstr.*, **71,** 64077d(1969).

(507) P. Balatre, A. Guyot-Hermann, J. Guyot, and M. Traisnel, *Prod. Probl. Pharm.*, **23,** 531(1968); through *Chem. Abstr.*, **70,** 80824e(1969).

(508) P. Balatre, A. Guyot-Hermann, J. Guyot, and M. Traisnel, *Bull. Soc. Pharm. Lille*, **1,** 45(1969); through *Chem. Abstr.*, **71,** 15990n(1969).

(509) K. Muenzel and W. Fueller, *Pharm. Acta Helv.*, **44,** 208 (1969); through *Chem. Abstr.*, **71,** 42254g(1969).

(510) M. Yashkul, I. Azhgikhin, and B. Zudin, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 122(1968); through *Chem. Abstr.*, **71,** 105190r (1969).

(511) T. Skvirskaya, G. Naumchik, A. Pavlova, and M. Ugleva, *Farmatsiya (Moscow)*, **18,** 13(1969); through *Chem. Abstr.*, **71,** 128652x(1969).

(512) V. Gandel, I. Azhgikhin, and D. Bernatonis, *Tr. 1 [Pervogo] Mosk. Med. Inst.*, **61,** 155(1968); through *Chem. Abstr.*, **71,** 84529f(1969).

(513) N. Sharova, B. Dashevskaya, and M. Gluzman, *Farmatsiya (Moscow)*, **18,** 21(1969); through *Chem. Abstr.*, **71,** 15991p (1969).

(514) G. Zoni and V. Lazzeretti, *Farmaco, Ed. Prat.*, **24,** 355 (1969); through *Chem. Abstr.*, **71,** 42257k(1969).

(515) I. Setnikar and V. Pietra, *J. Pharm. Sci.*, **58,** 112(1969).

(516) P. Sgaramella, *Amer. Perfum. Cosmet.*, **84,** 95(1969).

(517) R. Lehne and J. Hublard, *ibid.*, **84,** 69(1969).

(518) M. Blaine, *Prod. Probl. Pharm.*, **24,** 119(1969); through *Chem. Abstr.*, **71,** 6482w(1969).

(519) N. Fuks, *Kolloid. Zh.*, **31,** 471(1969); through *Chem. Abstr.*, **71,** 74334z(1969).

(520) A. Parisse, *Drug Cosmet. Ind.*, **105,** 40(1969).

(521) D. Zucco, *Mfg. Chem. Aerosol News*, **40,** 60(1969).

(522) J. Sciarra, *Drug Cosmet. Ind.*, **105,** 46(1969).

(523) A. Iannacone, *Amer. Perfum. Cosmet.*, **84,** 107(1969).

(524) E. Graham, *ibid.*, **84,** 53(1969).

(525) H. Kuebler, *ibid.*, **84,** 57(1969).

(526) J. Sciarra and B. Sciarra, *ibid.*, **84,** 77(1969).

(527) G. Holzner, *Parfum. Cosmet. Savons*, **11,** 528(1968); through *Chem. Abstr.*, **70,** 90698f(1969).

(528) G. Hidy, *Surface Colloid Sci.*, **2,** 213(1969).

(529) N. Nix, *Staub*, **29,** 188(1969); through *Chem. Abstr.*, **71,** 84825z(1969).

(530) R. Low, *J. Atmos. Sci.*, **26,** 608(1969); through *Chem. Abstr.*, **71,** 24958k(1969).

(531) R. Ford and C. Furmidge, *Advan. Chem. Ser.*, **86,** 155 (1967); through *Chem. Abstr.*, **70,** 91073k(1969).

(532) A. Brown and C. Hanson, *Solvent Extr. Chem., Proc. Int. Conf.*, **1966,** 522; through *Chem. Abstr.*, **70,** 6775y(1969).

(533) L. Tobler, *Kosmet.-Parfum-Drogen Rundsch.*, **16,** 89(1969); through *Chem. Abstr.*, **71,** 128587e(1969).

(534) D. Hoffmann, *Soap Perfum. Cosmet.*, **42,** 298(1969); through *Chem. Abstr.*, **71,** 33336h(1969).

(535) A. Kluge, *Kosmetik*, **42,** 299(1969); through *Chem. Abstr.*, **71,** 33343h(1969).
(536) S. Blaug, H. Fischer, and A. Karig, *Amer. J. Hosp. Pharm.*, **26,** 210(1969).
(537) G. Polli, W. Grim, F. Bacher, and M. Yunker, *J. Pharm. Sci.*, **58,** 484(1969).
(538) D. Schiefer and W. Henderson, *Amer. J. Hosp. Pharm.*, **26,** 114(1969).
(539) H. Mace and C. Carrion, *J. Soc. Cosmet. Chem.*, **20,** 511(1969).
(540) P. Sanders, *Amer. Perfum. Cosmet.*, **84,** 111(1969).
(541) P. Sanders, *J. Soc. Cosmet. Chem.*, **20,** 577(1969).
(542) K. Bergwein, *Kosmet.-Perfum-Drogen Rundsch.*, **15,** 130(1968); through *Chem. Abstr.*, **70,** 99553n(1969).
(543) F. Stanave, J. Ward, and H. Hotchins, *Amer. Perfum. Cosmet.*, **84,** 81(1969).
(544) J. Coley and J. Howard, *Mfg. Chem. Aerosol News*, **40,** 56(1969).
(545) B. Joyner, *ibid.*, **40,** 63(1969).
(546) S. Kalfayan, R. Silver, and B. Campbell, *Sci. Tech. Aerosp. Rep.*, **6,** 4215(1968); through *Chem. Abstr.*, **71,** 84522y(1969).
(547) K. Schrader, *Dragoco Rep. Ger. Ed.*, **16,** 95(1969); through *Chem. Abstr.*, **71,** 73947q(1969).
(548) J. Sciarra, *Givaudanian*, **3,** 7(1969); through *Chem. Abstr.*, **71,** 42163b(1969).
(549) D. Parker, *Drug Cosmet. Ind.*, **105,** 54(1969).
(550) R. Cannizzaro and D. Lewis, *J. Soc. Cosmet. Chem.*, **20,** 353(1969).
(551) R. Werner and R. Scott, *Drug Cosmet. Ind.*, **104,** 68 (1969).
(552) P. Kabasakalian, *J. Pharm. Sci.*, **58,** 245(1969).
(553) A. Contractor, R. Shangraw, and M. Richman, *Drug Cosmet. Ind.*, **105,** 44(1969).
(554) P. Giovacchini, L. Hardy, and D. O'Brien, *Amer. Perfum. Cosmet.*, **84,** 63(1969).
(555) A. Kulkarni, P. Carrigan, J. Ward, and H. Hutchins, *J. Pharm. Sci.*, **58,** 481(1969).
(556) Y. Uzu and I. Sano, *Bull. Chem. Soc. Jap.*, **42,** 1807 (1969); through *Chem. Abstr.*, **71,** 6446y(1969).
(557) E. Germet and L. Rozentsvaig, *Vop. Ispareniya, Goreniya Gazov. Din. Dispersnykh Sist., Master. Vses. Nauch. Konf., 4th, 5th*, 226 (1966); through *Chem. Abstr.*, **71,** 64572m(1969).
(558) F. Leonard, J. Lusardi, R. Kulker, and C. Wade, *J. Biomed. Mater. Res.*, **3,** 267(1969); through *Chem. Abstr.*, **71,** 53542a(1969).
(559) G. Nairn, *Can. Pharm. J.*, **102,** 336(1969).
(560) L. Tomasini and D. Naplatanova, *Farmatsiya (Sofia)*, **19,** 62(1969); through *Chem. Abstr.*, **71,** 73971t(1969).
(561) P. Speiser, *Sci. Pharm.*, **37,** 14(1969); through *Chem. Abstr.*, **70,** 118007f(1969).
(562) N. Bonciocat, T. Postelnicu, and P. Ban, *Farmacia (Bucharest)*, **17,** 29(1969); through *Chem. Abstr.*, **70,** 80823d(1969).
(563) E. Matsui, *U.S. Govt. Res. Develop. Rep.*, **69,** 98(1969); through *Chem. Abstr.*, **71,** 84835c(1969).
(564) S. Khanna and P. Speiser, *J. Pharm. Sci.*, **58,** 1114 (1969).
(565) S. Khanna, M. Soliva, and P. Speiser, *ibid.*, **58,** 1385 (1969).
(566) P. Speiser, *Farm. Aikak.*, **77,** 116(1968); through *Chem. Abstr.*, **71,** 6518n(1969).
(567) S. Kornblum, *J. Pharm. Sci.*, **58,** 125(1969).
(568) W. Ritschel and R. Clotten, *Arzneim.-Forsch.*, **19,** 221 (1969); through *Chem. Abstr.*, **70,** 104925s(1969).
(569) W. Ritschel and R. Clotten, *ibid.*, **19,** 347(1969); through *Chem. Abstr.*, **70,** 99576x(1969).
(570) L. Zathurecky, H. Raskova, M. Rochova, V. Krupa, O. Wichterle, R. Chromecek, and I. Gavrilova, *ibid.*, **19,** 951(1969); through *Chem. Abstr.*, **71,** 79469g(1969).
(571) O. Schettino and M. La Rotonda, *Boll. Soc. Ital. Biol. Sper.*, **44,** 1915(1968); through *Chem. Abstr.*, **71,** 24715d(1969).
(572) P. Ionescu-Stoian and A. Moldovan, *Farmacia (Bucharest)*, **16,** 601(1968); through *Chem. Abstr.*, **70,** 22891p(1969).
(573) E. Lazareva, L. Kovaleva, V. Vasil'ev, and P. Braginskaya, *Antibiotiki (Moscow)*, **14,** 813(1969); through *Chem. Abstr.*, **71,** 111183x(1969).
(574) F. Trading, P. Nielsen, M. Pingel, and A. Voelund, *Eur. J. Pharmacol.*, **7,** 206(1969); through *Chem. Abstr.*, **71,** 111234q (1969).
(575) S. Bolton, *J. Pharm. Sci.*, **58,** 1287(1969).
(576) E. Pandula, S. Tarjanyi, A. Csontos, and I. Nagy, *Acta Pharm. Hung.*, **38,** 400(1968); through *Chem. Abstr.*, **70,** 22886r (1969).
(577) M. Chalabala, L. Starha, and J. Maly, *Sci. Pharm.*, **37,** 50(1969); through *Chem. Abstr.*, **70,** 118055v(1969).
(578) L. Starha and M. Chalabala, *Cesk. Farm.*, **18,** 80(1969); through *Chem. Abstr.*, **71,** 128666e(1969).
(579) N. Iribarren, S. Gagliardo, and G. Dalesio, *Rev. Farm. (Buenos Aires)*, **111,** 7(1969); through *Chem. Abstr.*, **71,** 64073z (1969).
(580) Y. Borisenko and I. Kovalev, *Khim.-Farm. Zh.*, **3,** 37 (1969); through *Chem. Abstr.*, **71,** 33359t(1969).
(581) Y. Borisenko, R. Obukhova, and R. Safiulin, *Farmatsiya (Moscow)*, **18,** 17(1969); through *Chem. Abstr.*, **71,** 6510d(1969).
(582) P. Danneberg, R. Hahn, and E. Haase, *Arzneim.-Forsch.*, **19,** 641(1969); through *Chem. Abstr.*, **71,** 29211w(1969).
(583) H. deNavarre, *Amer. Perfum. Cosmet.*, **84,** 47(1969).
(584) P. Alexander, *Mfg. Chem. Aerosol News*, **40,** 31(1969).
(585) E. Green, *Rep. Progr. Appl. Chem.*, **52,** 203(1967); through *Chem. Abstr.*, **70,** 50408e(1969).
(586) G. Pratt, *Amer. Perfum. Cosmet.*, **84,** 41(1969).
(587) F. Saunders, *Mfg. Chem. Aerosol News*, **40,** 27(1969).
(588) R. Thimineur, *Drug Cosmet. Ind.*, **106,** 44(1969).
(589) L. Osipow, D. Marra, and N. Resnansky, *ibid.*, **104,** 56 (1969).
(590) *Ibid.*, **104,** 56(1969).
(591) R. Brandau, *Deterg. Age*, **5,** 56(1968); through *Chem. Abstr.*, **70,** 99539n(1969).
(592) F. Gohlke and H. Bergerhausen, *Parfum. Cosmet. Savons*, **12,** 143(1969); through *Chem. Abstr.*, **71,** 14418v(1969).
(593) B. Idson, *Drug Cosmet. Ind.*, **105,** 48(1969).
(594) M. Coopersmith and R. McParland, *ibid.*, **104,** 44(1969).
(595) G. Barker and D. Eckert, *ibid.*, **104,** 44(1969).
(596) D. Ragusa, *Corr. Farm.*, **23,** 199(1968); through *Chem. Abstr.*, **70,** 85864c(1969).
(597) F. Fryer, *Soap Perfum. Cosmet.*, **42,** 343(1969); through *Chem. Abstr.*, **71,** 53442t(1969).
(598) M. Blake, *Amer. Perfum. Cosmet.*, **84,** 35(1969); through *Chem. Abstr.*, **71,** 73935j(1969).
(599) M. Ginn, R. Steinhauer, I. Liebman, and E. Jungermann, *J. Amer. Oil Chem. Soc.*, **45,** 666(1968); through *Chem. Abstr.*, **70,** 5350u(1969).
(600) I. Gucklhorn, *Mfg. Chem. Aerosol News*, **40,** 23(1969).
(601) *Ibid.*, **40,** 71(1969).
(602) *Ibid.*, **40,** 33(1969).
(603) *Ibid.*, **40,** 33(1969).
(604) *Ibid.*, **40,** 35(1969).
(605) P. Fresnel, *Riv. Ital. Sostanze Grass.*, **46,** 327(1969); through *Chem. Abstr.*, **71,** 105131n(1969).
(606) G. Sykes and R. Smart, *Amer. Perfum. Cosmet.*, **84,** 45(1969).
(607) G. Ellner, *Drug Cosmet. Ind.*, **104,** 54(1969).
(608) L. Wolfram, *Amer. Perfum. Cosmet.*, **84,** 29(1969).
(609) P. Velon, *Parfum. Cosmet. Savons*, **12,** 137(1969); through *Chem. Abstr.*, **71,** 15960c(1969).
(610) T. Ito and S. Hayashi, *Yukagaku*, **18,** 364(1969); through *Chem. Abstr.*, **71,** 94676r(1969).
(611) G. Sperandio, *Can. Pharm. J.*, **102,** 109(1969).
(612) A. Shansky, *Amer. Perfum. Cosmet.*, **84,** 47(1969).
(613) M. Bierre, *Soap Perfum. Cosmet.*, **42,** 351(1969); through *Chem. Abstr.*, **71,** 53440r(1969).
(614) D. Anonis, *Drug Cosmet. Ind.*, **105,** 50(1969).
(615) L. Appell, *Amer. Perfum. Cosmet.*, **84,** 45(1969).
(616) J. Varsano and S. Gilbert, *Drug Cosmet. Ind.*, **104,** 88 (1969).
(617) *Ibid.*, **104,** 72(1969).
(618) *Ibid.*, **104,** 98(1969).
(619) P. Smith, *Chem. Drug.*, **191,** 56(1969); through *Chem. Abstr.*, **71,** 94729k(1969).
(620) J. Estevez, *ibid.*, **192,** 242(1969); through *Chem. Abstr.*, **71,** 128678k(1969).
(621) M. Busse and D. Hughes, *Pharm. J.*, **203,** 338(1969).
(622) A. Szymanska, K. Lenartowicz, W. Zalewski, and D. Jastalska, *Acta Pol. Pharm.*, **26,** 75(1969); through *Chem. Abstr.*, **71,**

53545d(1969).
(623) S. Bartkowicz, *Farm. Pol.*, **25,** 21(1969); through *Chem. Abstr.*, **71,** 33372s(1969).
(624) H. Jaspersen, *Schweiz. Apoth.-Ztg.*, **107,** 160(1969); through *Chem. Abstr.*, **71,** 15987s(1969).
(625) F. Neuwald and D. Scheel, *Pharm. Ind.*, **30,** 754(1968); through *Chem. Abstr.*, **71,** 15993r(1969).
(626) H. Houta and K. Leupin, *Pharm. Acta Helv.*, **44,** 336 (1969).
(627) S. Kim and K. Lee, *Yakhak Hoeji*, **12,** 35(1968); through *Chem. Abstr.*, **71,** 33386z(1969).
(628) E. Mecarelli, G. Brauchi, and L. Bersellini, *Farmaco, Ed. Prat.*, **24,** 576(1969); through *Chem. Abstr.*, **71,** 116523t(1969).
(629) C. Moretti, *Mostra Int. Ind. Conserve Aliment., Congr.*, **18,** 59(1963); through *Chem. Abstr.*, **71,** 128677j(1969).
(630) F. Jaminet, *Prod. Probl. Pharm.*, **23,** 375(1968); through *Chem. Abstr.*, **70,** 14338y(1969).
(631) V. Mironova, *Farmatsiya (Moscow)*, **18,** 31(1969); through *Chem. Abstr.*, **71,** 64036p(1969).
(632) B. Logawa and O. Khong, *Suara Pharm.*, **11,** 154(1968); through *Chem. Abstr.*, **71,** 84532b(1969).
(633) H. Fowler, *Mfg. Chem. Aerosol News*, **40,** 29(1969).
(634) H. Fowler, *ibid.*, **40,** 33(1969).
(635) O. Adica, *Kagaku Hogaku*, **33,** 22(1969); through *Chem. Abstr.*, **70,** 109100k(1969).
(636) E. Awada, *ibid.*, **33,** 28(1969); through *Chem. Abstr.*, **70,** 109107m(1969).
(637) J. Skidmore, *Amer. Perfum. Cosmet.*, **84,** 31(1969).
(638) A. DeGroot and K. Hovius, *J. Chem. Educ.*, **46,** 498 (1969).
(639) P. Wray, *Drug Cosmet. Ind.*, **106,** 58(1969).
(640) C. Swartz, *ibid.*, **106,** 51(1969).
(641) C. Swartz, *J. Pharm. Sci.*, **58,** 473(1969).
(642) P. Lamy, *Drug Cosmet. Ind.*, **104,** 51(1969).
(643) W. Braun, *Amer. Perfum. Cosmet.*, **84,** 43(1969).
(644) Anonymous, *Drug Cosmet. Ind.*, **104,** 112(1969).
(645) M. Seletskii and A. Belogolovskii, *Khim.-Farm. Zh.*, **3,** 56(1969); through *Chem. Abstr.*, **70,** 109149b(1969).
(646) S. Yang, *Diss. Abstr.*, **30,** 585B(1969).
(647) L. Borka and K. Backe-Hansen, *Acta Pharm. Suecica*, **5,** 525(1968); through *Chem. Abstr.*, **70,** 55920a(1969).
(648) J. Haleblian and W. McCrone, *J. Pharm. Sci.*, **58,** 911 (1969).
(649) S. Rosenstein and P. Lamy, *Amer. J. Hosp. Pharm.*, **26,** 598(1969).
(650) M. Ladd and W. Lee, *J. Phys. Chem.*, **73,** 2033(1969).
(651) H. Schott, *J. Pharm. Sci.*, **58,** 946(1969).
(652) J. Christensen, J. Rytting, and R. Izatt, *J. Chem. Soc., A*, **1969,** 861.
(653) J. Christensen, R. Izatt, D. Wrathall, and L. Hansen, *ibid.*, **8,** 1212(1969).
(654) A. Michaelis and T. Higuchi, *J. Pharm. Sci.*, **58,** 201 (1969).
(655) B. Wolkowski, *Przem. Ferment. Rolny*, **12,** 29(1968); through *Chem. Abstr.*, **70,** 6818q(1969).
(656) H. Kostenbauder, H. Boxenbaum, and P. DeLuca, *J. Pharm. Sci.*, **58,** 753(1969).
(657) R. Woodford and J. Morrison, *J. Pharm. Pharmacol.*, **21,** 602(1969).
(658) C. Rushing, *Diss. Abstr., B*, **29,** 3706(1969).
(659) A. Repta and T. Higuchi, *J. Pharm. Sci.*, **58,** 1110(1969).
(660) O. Wulff, *Farm. Revy*, **68,** 97(1969); through *Chem. Abstr.*, **71,** 15989u(1969).
(661) R. Tawashi, *J. Mond. Pharm.*, **11,** 371(1968); through *Chem. Abstr.*, **71,** 64030h(1969).
(662) A. Simonelli, S. Mehta, and W. Higuchi, *J. Pharm. Sci.*, **58,** 538(1969).
(663) T. Bates, *J. Pharm. Pharmacol.*, **21,** 710(1969).
(664) A. Aguiar and J. Zelmer, *J. Pharm. Sci.*, **58,** 983(1969).
(665) W. Higuchi, W. Hamlin, and S. Mehta, *ibid.*, **58,** 1145 (1969).
(666) W. Chiou and S. Riegelman, *ibid.*, **58,** 1505(1969).
(667) F. Langenbucher, *ibid.*, **58,** 1265(1969).
(668) A. Mitchell and D. Saville, *J. Pharm. Pharmacol.*, **21,** 28 (1969).
(669) J. Wagner, *J. Pharm. Sci.*, **58,** 1253(1969).
(670) M. Gibaldi, S. Feldman, and N. Weiner, *ibid*, **58,** 132 (1969).
(671) H. Weintraub and M. Gibaldi, *ibid.*, **58,** 1368(1969).
(672) H. Nogami, T. Nagai, and T. Yotsuyanagi, *Chem. Pharm. Bull.*, **17,** 499(1969).
(673) E. Rippie and J. Johnson, *J. Pharm. Sci.*, **58,** 428(1969).
(674) P. Reimer, *Arch. Pharm. Chemi*, **76,** 638(1969); through *Chem. Abstr.*, **71,** 105182e(1969).
(675) E. Pandula and I. Racz, *Gyogyszereszet*, **13,** 161(1969); through *Chem. Abstr.*, **71,** 74001s(1969).
(676) P. Elworthy and F. Lipscomb, *J. Pharm. Pharmacol*, **21,** 273(1969).
(677) R. Withey and C. Mainville, *J. Pharm. Sci.*, **58,** 1120 (1969).
(678) A. Richter, B. Myhre, and S. Khanna, *J. Pharm. Pharmacol.*, **21,** 409(1969).
(679) J. Hersey and R. Barzilay, *ibid.*, **21,** 65(1969).
(680) J. Hersey, *Mfg. Chem. Aerosol News*, **40,** 32(1969).
(681) K. Marshall and D. Brook, *J. Pharm. Pharmacol.*, **21,** 790(1969).
(682) D. Baun and C. Walker, *J. Pharm. Sci.*, **58,** 611(1969).
(683) A. Heyd, D. Kildsig, and G. Banker, *ibid.*, **58,** 586(1969).
(684) A. Muta, *Kagaku To Kogyo (Tokyo)*, **27,** 315(1968); through *Chem. Abstr.*, **70,** 14669g(1969).
(685) Anonymous, *Drug Cosmet. Ind.*, **105,** 53(1969).
(686) I. Sano and Y. Ueno, *Nippon Kagaku Zasshi*, **89,** 1022 (1968); through *Chem. Abstr.*, **70,** 61364p(1969).
(687) G. Schlessinger, *J. Chem. Educ.*, **46,** 680(1969).
(688) J. Ostrenga, *J. Pharm. Sci.*, **58,** 1281(1969).
(689) L. Holmes, A. Silzars, D. Cole, L. Rich, and E. Eyring, *J. Phys. Chem.*, **73,** 737(1969).
(690) D. Doornbos and R. DeZeeuw, *Pharm. Weekbl.*, **104,** 233(1969); through *Chem. Abstr.*, **70,** 99588c(1969).
(691) F. McMillan and I. Pattison, *J. Pharm. Sci.*, **58,** 730 (1969).
(692) C. O'Connor, *J. Chem. Educ.*, **46,** 686(1969).
(693) D. Cadwallader and J. Phillips, *J. Pharm. Sci.*, **58,** 1220 (1969).
(694) W. Fassett, T. Fuller, and E. Hammarlund, *ibid.*, **58,** 1540 (1969).
(695) A. Campbell and B. Oliver, *Can. J. Chem.*, **47,** 2671 (1969).
(696) *Ibid.*, **47,** 2681(1969).
(697) L. Onsager, *Science*, **166,** 1359(1969).
(698) A. Cutie and B. Sciarrone, *J. Pharm. Sci.*, **58,** 990(1969).
(699) A. Paruta, *ibid.*, **58,** 216(1969).
(700) *Ibid.*, **58,** 204(1969).
(701) *Ibid.*, **58,** 364(1969).
(702) M. Wentz, W. Smith, and A. Martin, *J. Colloid Interface Sci.*, **29,** 36(1969).
(703) A. Thakkar and P. Kuehn, *J. Pharm. Sci.*, **58,** 850(1969).
(704) L. Wan and C. Hwang, *ibid.*, **58,** 889(1969).
(705) A. Thakkar and N. Hall, *ibid.*, **58,** 68(1969).
(706) A. Fryar and S. Kaufman, *J. Colloid Interface Sci.*, **29,** 444(1969).
(707) S. Chopra and R. Tawashi, *Pharm. Ind.*, **31,** 489(1969); through *Chem. Abstr.*, **71,** 116512p(1969).
(708) W. Guess and A. Jones, *Amer. J. Hosp. Pharm.*, **26,** 180 (1969).
(709) E. Prillig, *J. Pharm. Sci.*, **58,** 1245(1969).
(710) Y. Kato, K. Izumi, Y. Sakaniwa, H. Shioyama, and H. Aoki, *Yakugaku Zasshi*, **89,** 1297(1969).
(711) J. Nixon and B. Chawla, *J. Pharm. Pharmacol.*, **21,** 79 (1969).
(712) W. Malik and A. Jain, *J. Indian Chem. Soc.*, **46,** 631 (1969); through *Chem. Abstr.*, **71,** 82909f(1969).
(713) S. Mirkovic, *Arch. Farm.*, **18,** 181(1968); through *Chem. Abstr.*, **70,** 71018t(1969).
(714) A. Malmio, *Farm. Aikak.*, **77,** 244(1968); through *Chem. Abstr.*, **70,** 99581v(1969).
(715) E. Minkov, *Farmatsiya (Sofia)*, **19,** 60(1969); through *Chem. Abstr.*, **71,** 128610g(1969).
(716) E. Minkov and T. Trandafilov, *Pharmazie*, **24,** 327(1969); through *Chem. Abstr.*, **71,** 84445a(1969).
(717) R. Lombardi, *Rev. Farm. (Buenos Aires)*, **110,** 154(1968); through *Chem. Abstr.*, **70,** 60780j(1969).
(718) B. Guillot, *Lyon Pharm.*, **19,** 307(1968); through *Chem.*

Abstr., **70,** 50434k(1969).

(719) V. Gusyakov, N. Likholet, and I. Kutna, *Farm. Zh. (Kiev)*, **23,** 56(1968); through *Chem. Abstr.*, **70,** 109137w(1969).

(720) P. Demchenko and B. Shapoval, *Fiz.-Khim. Mekh. Liofil'nost Dispersnykh Sist.*, **1968,** 181; through *Chem. Abstr.*, **71,** 31646k(1969).

(721) H. Arai and K. Yoshizaki, *J. Amer. Oil Chem. Soc.*, **46,** 544(1969); through *Chem. Abstr.*, **71,** 103444t(1969).

(722) J. Peri, *J. Colloid. Interface Sci.*, **29,** 6(1969).

(723) L. Krasnec, *Sci. Pharm.*, **2,** 429(1965); through *Chem. Abstr.*, **70,** 50432h(1969).

(724) A. Popovici, E. Peteanu, and M. Peter, *Farmacia (Bucharest)*, **17,** 415(1969); through *Chem. Abstr.*, **71,** 128655a(1969).

(725) R. Popova, *Tr. I [Pervogo] Mosk. Med. Inst.*, **61,** 164 (1968); through *Chem. Abstr.*, **71,** 84520w(1969).

(726) R. Popova, *Tr. Perm. Farm. Inst.*, **2,** 121(1967); through *Chem. Abstr.*, **70,** 118009h(1969).

(727) A. Kitahara, K. Watanabe, K. Konno, and T. Ishikawa, *J. Colloid Interface Sci.*, **29,** 48(1969).

(728) A. Kitahara and K. Konno, *ibid.*, **29,** 1(1969).

(729) T. Higuchi, J. Richards, S. Davis, A. Kamada, J. Hou, M. Nakano, N. Nakano, and I. Pitman, *J. Pharm. Sci.*, **58,** 661 (1969).

(730) J. Guillory, S. Hwang, and J. Lach, *ibid.*, **58,** 301(1969).

(731) P. Thyrum, R. Luchi, and H. Conn, *J. Pharmacol. Exp. Ther.*, **164,** 239(1968); through *Chem. Abstr.*, **70,** 2091z(1969).

(732) V. Sharma and J. Schubert, *J. Chem. Educ.*, **46,** 506(1969).

(733) I. Moriguchi, M. Nayuki, and N. Kaneniwa, *Chem. Pharm. Bull.*, **17,** 1339(1969).

(734) O. Lekhan, D. Salo, and G. Eashura, *Farm. Zh. (Kiev)*, **23,** 55(1968); through *Chem. Abstr.*, **70,** 14370c(1969).

(735) G. Polli and B. Frost, *J. Pharm. Sci.*, **58,** 1543(1969).

(736) K. Kakemi, H. Sezaki, E. Suzuki, and M. Nakano, *Chem. Pharm. Bull.*, **17,** 212(1969).

(737) A. Krivis and J. Rabb, *Science*, **164,** 1064(1969).

(738) P. Rohdewald and M. Baumeister, *J. Pharm. Pharmacol.*, **21,** 867(1969).

(739) M. Zoglio, H. Maulding, Jr., and J. Windheuser, *J. Pharm. Sci.*, **58,** 222(1969).

(740) R. Schoenwald and P. Belcastro, *ibid.*, **58,** 930(1969).

(741) M. Schwuger, *Kolloid-Z. Z. Polym.*, **233,** 898(1969); through *Chem. Abstr.*, **71,** 103445u(1969).

(742) I. Moriguchi and N. Kaneniwa, *Chem. Pharm. Bull.*, **17,** 394(1969).

(743) C. Thompson, Jr., *Can. J. Chem.*, **47,** 2605(1969).

(744) C. Botre, A. Memoli, C. Del Vecchio-Blanco, F. Alhaique, and M. Marchetti, *Farmaco, Ed. Sci.*, **24,** 384(1969); through *Chem. Abstr.*, **71,** 42241a(1969).

(745) I. Moriguchi and N. Kaneniwa, *Chem. Pharm. Bull.*, **17,** 2173(1969).

(746) R. Lacey, *J. Clin. Pathol.*, **21,** 564(1968); through *Chem. Abstr.*, **70,** 10140e(1969).

(747) A. Hatano, *Bitamin*, **39,** 399(1969); through *Chem. Abstr.*, **71,** 42238e(1969).

(748) M. Gasco, *Farmaco, Ed. Prat.*, **24,** 137(1969); through *Chem. Abstr.*, **70,** 99586a(1969).

(749) C. Chignell, *Mol. Pharmacol.*, **5,** 455(1969); through *Chem. Abstr.*, **71,** 100065j(1969).

(750) J. Eisenbrand and K. Baumann, *Z. Lebensm.-Unters.-Forsch.* **140,** 210(1969); through *Chem. Abstr.*, **71,** 74003r(1969).

(751) M. Pavlyuchenko and E. Litvinenko, *Dokl. Akad. Nauk Beloruss. SSR*, **12,** 1006(1968); through *Chem. Abstr.*, **70,** 50929a (1969).

(752) K. Thoma, E. Ullmann, and E. Wolferseder, *Sci. Pharm.*, **2,** 325(1965); through *Chem. Abstr.*, **70,** 50457(1969).

(753) C. Woo, S. Kim, and S. Min, *Yakhak Hoeji*, **11,** 27(1967); through *Chem. Abstr.*, **71,** 53530v(1969).

(754) K. Shima, K. Mizojiri, S. Morita, and R. Yamamoto, *Yakugaku Zasshi*, **89,** 1587(1969).

(755) W. Suess, R. Huettenrauch, and U. Schmeiss, *Pharmazie*, **24,** 424(1969); through *Chem. Abstr.*, **71,** 116515s(1969).

(756) E. Shefter, *J. Pharm. Sci.*, **58,** 710(1969).

(757) N. Sharpless and C. Greenblatt, *Exp. Parasitol.*, **24,** 205 (1969); through *Chem. Abstr.*, **70,** 113549z(1969).

(758) A. Oronsky, L. Triner, O. Steinsland, and G. Nahas, *Nature*, **223,** 619(1969).

(759) M. Muftic, *Quart. J. Crude Drug Res.*, **8,** 1274(1968); through *Chem. Abstr.*, **70,** 22885q(1969).

(760) I. Moriguchi, Y. Araki, and N. Kaneniwa, *Chem. Pharm. Bull.*, **17,** 2088(1969).

(761) J. Anderson and G. Boyce, *J. Pharm. Sci.*, **58,** 1425(1969).

(762) L. Druzhinina and S. Beskov, *Uch. Zap. Mosk. Obl. Pedagog. Inst.*, **193,** 245(1968); through *Chem. Abstr.*, **71,** 128659e (1969).

(763) R. Daly, *Diss. Abstr. B*, **29,** 1112(1968); through *Chem. Abstr.*, **70,** 18592z(1969).

(764) D. Doornbos, *Pharm. Weekbl.*, **103,** 1213(1968); through *Chem. Abstr.*, **70,** 14369j(1969).

(765) T. Gulik-Krzywicki, E. Schecter, and V. Luzzati, *Nature*, **223,** 1116(1969).

(766) J. Bianchine, B. Bonnlander, P. Macaraeg, Jr., R. Hersey, J. Bianchine, and P. McIntyre, *J. Clin. Endocrinol. Metab.*, **29,** 1425(1969).

(767) A. Boninsegna and F. Zucchi, *Minerva Anestesiol.*, **35,** 583(1969); through *Chem. Abstr.*, **71,** 79562g(1969).

(768) T. Brunelle, *Diss. Abstr.*, **30,** 483(1969).

(769) J. Franz, E. Jaehnchen, and J. Krieglstein, *Naunyn-Schmiedebergs Arch. Pharmakol.*, **264,** 462(1969); through *Chem. Abstr.*, **71,** 122051d(1969).

(770) K. Kakemi, H. Sezaki, M. Nakano, and K. Ohsuga, *J. Pharm. Sci.*, **58,** 699(1969).

(771) K. Koizumi, J. Tatsumi, M. Ohae, H. Kumagai, and T. Hayata, *Yakugaku Zasshi*, **89,** 1594(1969).

(772) P. Keane, P. Pegg, and E. Johnson, *J. Clin. Endocrinol. Metab.*, **29,** 1126(1969).

(773) Y. Kyogoku and B. Yu, *Bull. Chem. Soc. Jap.*, **42,** 1387 (1969); through *Chem. Abstr.*, **71,** 33375v(1969).

(774) K. Takaishi and Y. Torii, *Yakugaku Zasshi*, **89,** 538 (1969).

(775) T. Yasunaga, N. Tatsumoto, H. Inoue, and M. Miura, *J. Phys. Chem.*, **73,** 477(1969).

(776) M. Imoto, *Nippon Secchaku Kyokai Shi*, **5,** 74(1969); through *Chem. Abstr.*, **71,** 105563e(1969).

(777) L. Block and P. Lamy, *Kolloid-Z. Z. Polym.*, **27,** 164 (1968); through *Chem. Abstr.*, **70,** 6530q(1969).

(778) J. Swarbrick, *J. Pharm. Sci.*, **58,** 147(1969).

(779) N. Evans and N. Pilpel, *ibid.*, **58,** 1228(1969).

(780) L. Kundu and B. Ghosh, *J. Indian Chem. Soc.*, **46,** 39 (1969); through *Chem. Abstr.*, **70,** 81168f(1969).

(781) V. Pchelin, *Kolloid Zh.*, **31,** 153(1969); through *Chem. Abstr.*, **71,** 6763p(1969).

(782) J. Mingins, N. Owen, and D. Iles, *J. Phys. Chem.*, **73,** 2118(1969).

(783) E. Malakhova and A. Abramzon, *Dokl. Akad. Nauk SSSR*, **180,** 1157(1968); through *Chem. Abstr.*, **70,** 23125d(1969).

(784) J. Lavelle, *Diss. Abstr. B.*, **29,** 4129(1969).

(785) I. Elei, *Advan. Chem. Ser.*, **84,** 149(1968); through *Chem. Abstr.*, **70,** 23124c(1969).

(786) D. Cadenhead and R. Demchak, *Biochim. Biophys. Acta*, **176,** 849(1969); through *Chem. Abstr.*, **71,** 95113s(1969).

(787) D. Cadenhead, *Ind. Eng. Chem.*, **61,** 22(1969).

(788) J. Baret, *Kolloid-Z. Z. Polym.*, **233,** 971(1969); through *Chem. Abstr.*, **71,** 129042k(1969).

(789) J. Brooks and B. Pethica, *Chem. Phys. Appl. Surface Active Subst., Proc. Int. Congr., 4th*, **2,** 191(1964); through *Chem. Abstr.*, **71,** 129043m(1969).

(790) F. Fowkes, *ibid.*, **2,** 309(1964); through *Chem. Abstr.*, **71,** 129079c(1969).

(791) J. Munden, D. Blois, and J. Swarbrick, *J. Pharm. Sci.*, **58,** 1308(1969).

(792) F. Fowkes, *Surfaces Interfaces I, Proc. Sagamore Army Mater. Res. Conf., 13th*, **1966,** 197; through *Chem. Abstr.*, **70,** 23097w(1969).

(793) I. Pavlushenko, Y. Averbukh, and N. Kostin, *Zh. Prikl. Khim. (Leningrad)*, **42,** 1085(1969); through *Chem. Abstr.*, **71,** 64452x(1969).

(794) N. Pilpel, *Advan. Colloid Interface Sci.*, **2,** 261(1969); through *Chem. Abstr.*, **70,** 91059k(1969).

(795) L. Kundu and B. Ghosh, *J. Indian Chem. Soc.*, **46,** 462 (1969); through *Chem. Abstr.*, **71,** 53961m(1969).

(796) W. Zisman, *Advan. Chem. Ser.*, **87,** 1(1968); through *Chem. Abstr.*, **70,** 31818x(1969).

(797) A. Watanabe, H. Tamai, M. Matsumoto, and R. Gotoh,

Nippon Kagaku Zasshi, **90,** 738(1969); through *Chem. Abstr.*, **71,** 129048s(1969).

(798) G. Torosian and A. Lemberger, *J. Pharm. Sci.*, **58,** 864 (1969).

(799) M. Hasegawa and M. Low, *J. Colloid Interface Sci.*, **30,** 378(1969).

(800) A. Zettlemoyer and V. Rao, *ibid.*, **29,** 172(1969).

(801) S. Katti and S. Pathak, *J. Chem. Eng. Data*, **14,** 73(1969); through *Chem. Abstr.*, **70,** 91071h(1969).

(802) A. Abramson and Y. Moiseeva, *Kolloid Zh.*, **30,** 795 (1968); through *Chem. Abstr.*, **70,** 40953q(1969).

(803) C. Fuehrer and J. Ghadially, *Pharm. Acta Helv.*, **43,** 653 (1968); through *Chem. Abstr.*, **70,** 12841h(1969).

(804) D. Owens, *J. Colloid Interface Sci.*, **29,** 496(1969).

(805) W. Langmann, *Chemiefasern Text.-Anwendungstech.*, **19,** 363(1969); through *Chem. Abstr.*, **71,** 40605y(1969).

(806) P. Elworthy, D. Gyane, and C. Macfarlane, *J. Pharm. Pharmacol.*, **21,** 617(1969).

(807) G. Gittens, *J. Colloid Interface Sci.*, **30,** 406(1969).

(808) G. Gaines, *J. Phys. Chem.*, **73,** 3143(1969).

(809) D. Ekserova, A. Sheludko, and M. Zakharieva, *God. Sofii. Univ., Khim. Fak.*, **61,** 383(1966–1967); through *Chem. Abstr.*, **71,** 64451w(1969).

(810) D. Ceacareanu and M. Olteanu, *An. Univ. Bucuresti, Ser. Stiint. Nautr., Chim.*, **16,** 49(1967); through *Chem. Abstr.*, **70,** 41051f(1969).

(811) R. Picknett, *J. Colloid Interface Sci.*, **29,** 173(1969).

(812) H. Pak, *Daehan Hwahak Hwoejee*, **12,** 18(1968); through *Chem. Abstr.*, **70,** 50842s(1969).

(813) J. Padday, *Surface Colloid Sci.*, **1,** 39(1969); through *Chem. Abstr.*, **71,** 129033h(1969).

(814) A. Iguchi, *Kagaku Kogaku*, **32,** 839(1968); through *Chem. Abstr.*, **70,** 23093s(1969).

(815) R. Kashcheeva, *Mater. Nauch. Konf. Aspir., Rostov-Na-Donu Gos. Univ., 7th, 8th*, **1967;** through *Chem. Abstr.*, **71,** 6744h (1969).

(816) R. Keymer, *Pharm. Ind.*, **31,** 152(1969); through *Chem. Abstr.*, **71,** 84819a(1969).

(817) S. Kharin, A. Kniga, and G. Sorokina, *Izv. Vyssh. Ucheb. Zaved., Khim. Khim. Tekhnol.*, **11,** 1341(1968); through *Chem. Abstr.*, **71,** 24955g(1969).

(818) J. Kloubek and A. Neumann, *Tenside*, **6,** 4(1969); through *Chem. Abstr.*, **71,** 95112r(1969).

(819) M. Ramanadham, *Indian J. Pure Appl. Phys.*, **6,** 517A (1968); through *Chem. Abstr.*, **70,** 6774x(1969).

(820) Z. Romanova and N. Baramboim, *Kolloid. Zh.*, **31,** 573 (1969); through *Chem. Abstr.*, **71,** 105675t(1969).

(821) F. Huisman and K. Mysels, *J. Phys. Chem.*, **73,** 489(1969).

(822) E. Shafrin and W. Zisman, *Advan. Chem. Ser.*, **87,** 20 (1968); through *Chem. Abstr.*, **70,** 39109n(1969).

(823) A. Prins, *J. Colloid Interface Sci.*, **29,** 177(1969).

(824) P. Zolotarev and N. Churaev, *Kolloid Zh.*, **31,** 220(1969); through *Chem. Abstr.*, **71,** 6761m(1969).

(825) T. Yin, *J. Phys. Chem.*, **73,** 2413(1969).

(826) F. Seemann and P. Sliwinski, *Z. Phys. Chem. (Frankfurt au Main)*, **63,** 202(1969); through *Chem. Abstr.*, **71,** 24994u(1969).

(827) T. Seimiya, S. Saito, and T. Sasaki, *J. Colloid Interface Sci.*, **30,** 153(1969); through *Chem. Abstr.*, **71,** 24977n(1969).

(828) M. Gara and G. Szatlmayer, *Kolor. Ert.*, **11,** 179(1969); through *Chem. Absir.*, **71,** 116825t(1969).

(829) J. Bikerman, *Amer. Chem. Soc., Div. Org. Coatings Plast. Chem., Preprints*, **27,** 105(1967); through *Chem. Abstr.*, **70,** 99902a(1969).

(830) R. Johnson, Jr., and R. Dettre, *Surface Colloid Sci.*, **2,** 85(1969); through *Chem. Abstr.*, **71,** 105562D(1969).

(831) E. Ruckenstein, *Chem. Eng. Sci.*, **24,** 1223(1969); through *Chem. Abstr.*, **71,** 74449r(1969).

(832) S. Frank and G. Zografi, *J. Pharm. Sci.*, **58,** 993(1969).

(833) F. Tokiwa and N. Moriyama, *J. Colloid Interface Sci.*, **30,** 338(1969).

(834) T. Solov'eva, L. Eremina, and R. Panich, *Kolloid Zh.*, **31,** 285(1969); through *Chem. Abstr.*, **71,** 14419w(1969).

(835) H. Schott, *J. Amer. Oil Chem. Soc.*, **45,** 823(1968); through *Chem. Abstr.*, **70,** 12835j(1969).

(836) W. Malik and P. Chand, *ibid.*, **46,** 285(1969).

(837) W. Malik and S. Saleem, *ibid.*, **45,** 670(1968); through *Chem. Abstr.*, **70,** 5356a(1969).

(838) M. Feinstein and H. Rosano, *J. Phys. Chem.*, **73,** 601 (1969).

(839) R. Keymer, *Pharm. Ind.*, **30,** 817(1968); through *Chem. Abstr.*, **71,** 4757r(1969).

(840) L. Wan and P. Poon, *J. Pharm. Sci.*, **58,** 1562(1969).

(841) P. Mukerjee, *J. Phys. Chem.*, **73,** 2054(1969).

(842) J. Swarbrick and J. Daruwala, *ibid.*, **73,** 2627(1969).

(843) D. Small, *Advan. Chem. Ser.*, **84,** 31(1968); through *Chem. Abstr.*, **70,** 23205e(1969).

(844) J. Stead and H. Taylor, *J. Colloid Interface Sci.*, **30,** 482 (1969).

(845) K. Shinoda, *Chem. Phys. Appl. Surface Active Subst., Proc. Int. Congr. 4th.*, **2,** 527(1964); through *Chem. Abstr.*, **71,** 129039q(1969).

(846) H. Nogami and Y. Kanakubo, *Chem. Pharm. Bull.*, **17,** 2008(1969).

(847) W. Malik and S. Verma, *Kolloid-Z. Z. Polym.*, **233,** 985(1969); through *Chem. Abstr.*, **71,** 114457a(1969).

(848) N. Muller and T. Johnson, *J. Phys. Chem.*, **73,** 2042(1969).

(849) J. Ledbetter and J. Bowen, *Anal. Chem.*, **41,** 1345(1969); through *Chem. Abstr.*, **71,** 64574p(1969).

(850) R. Hill and R. Wake, *Nature*, **221,** 635(1969).

(851) R. Dunlap, *Diss. Abstr. B*, **29,** 3673(1969).

(852) J. Desnoyers, M. Arel, and P. Leduc, *Can. J. Chem.*, **47,** 547(1969).

(853) P. Ekwall, *J. Colloid Interface Sci.*, **29,** 16(1969).

(854) P. Demchenko and B. Shapoval, *Kolloid. Zh.*, **31,** 345 (1969); through *Chem. Abstr.*, **71,** 82907d(1969).

(855) J. Corkill, J. Goodman, and J. Tate, *Hydrogen-Bonded Solvent Syst., Proc. Symp.*, **1968,** 181; through *Chem. Abstr.*, **70,** 118469h(1969).

(856) J. Corkill and J. Goodman, *Advan. Colloid Interface Sci.*, **2,** 297(1969); through *Chem. Abstr.*, **70,** 99889b(1969).

(857) D. Attwood, P. Elworthy, and S. Kayne, *J. Pharm. Pharmacol.*, **21,** 619(1969).

(858) D. Attwood, *Kolloid-Z. Z. Polym.*, **232,** 788(1969); through *Chem. Abstr.*, **71,** 82906c(1969).

(859) B. Bennion, L. Tong, L. Holmes, and E. Eyring, *J. Phys. Chem.*, **73,** 3288(1969).

(860) M. Jaycock and R. Ottewill, *Chem. Phys. Appl. Surface Active Subst., Proc. Int. Congr. 4th*, **2,** 545(1964); through *Chem. Abstr.*, **71,** 129040h(1969).

(861) K. Juna and T. Sugano, *Nippon Kagaku Zasshi*, **90,** 463 (1969); through *Chem. Abstr.*, **71,** 105679x(1969).

(862) C. Kurzendoerfer and H. Lange, *Fette, Seifen, Anstrichm.*, **71,** 561(1969); through *Chem. Abstr.*, **71,** 92949lh(1969).

(863) S. Kuroiwa and S. Ogasawara, *Sen-i Gakkaishi*, **24,** 536 (1968); through *Chem. Abstr.*, **71,** 42621z(1969).

(864) J. Yan and M. Palmer, *J. Colloid Interface Sci.*, **30,** 177 (1969).

(865) T. Tahara, I. Satake, and R. Matuura, *Bull. Chem. Soc. Jap.*, **42,** 1201(1969); through *Chem. Abstr.*, **73,** 31641e(1969).

(866) F. Tokiwa, N. Moriyama, and H. Sugihara, *Nippon Kagaku Zasshi*, **90,** 673(1969); through *Chem. Abstr.* **71,** 10566r (1969).

(867) F. Tokiwa, *J. Phys. Chem.*, **72,** 4331(1968).

(868) F. Tokiwa and K. Ohki, *Bull. Chem. Soc. Jap.*, **42,** 1216 (1969); through *Chem. Abstr.*, **71,** 31642f(1969).

(869) F. Tokiwa, K. Ohki, and I. Kokubo, *ibid.*, **42,** 575(1969); through *Chem. Abstr.*, **70,** 89075u(1969).

(870) F. Tokiwa, N. Moriyama, and H. Sugihara, *Nippon Kagaku Zasshi*, **90,** 454(1969); through *Chem. Abstr.*, **71,** 95300a (1969).

(871) W. Tosch, S. Jones, and A. Adamson, *J. Colloid Interface Sci.*, **31,** 297(1969).

(872) H. Schott, *J. Pharm. Sci.*, **58,** 1521(1969).

(873) J. Corkill, J. Goodman, T. Walker, and J. Wyer, *Proc. Roy. Soc., Ser. A*, **312,** 243(1969); through *Chem. Abstr.*, **71,** 54032w(1969).

(874) I. Moriguchi and N. Kaneniwa, *Chem. Pharm. Bull.*, **17,** 961(1969).

(875) D. Powell, J. Nemathollahi, W. Guess, and J. Autian, *J. Pharm. Sci.*, **58,** 842(1969).

(876) D. Mufson, *Diss. Abstr.*, **30,** 145B(1969).

(877) J. Gibert, H. Angelino, and J. Besombes-Vailhe, *Chim. Ind. Genie Chim.*, **100,** 1424(1968); through *Chem. Abstr.*, **70,**

81204q(1969).
(878) J. Abram, *Chem. Ind. (London)*, **42,** 1557(1969).
(879) E. Weronski, *Electrochim. Acta*, **14,** 231(1969); through *Chem. Abstr.*, **70,** 81221t(1969).
(880) K. Liang and L. Tong, *J. Phys. Chem.*, **73,** 3125(1969).
(881) K. Bissada, W. Johns, and F. Cheng, *Clay Miner.*, **7,** 155 (1967); through *Chem. Abstr.*, **71,** 25007m(1969).
(882) A. Blackmore, *Aust. J. Sci.*, **31,** 402(1969); through *Chem. Abstr.*, **71,** 64492k(1969).
(883) V. Bykov, *Prir. Sorbenty, Sb. Dokl. Sess. Nauch. Sov. Sin.; Izuch. Primen. Adsorbentov Akad. Nauk SSSR*, **1966,** 77; through *Chem. Abstr.*, **70,** 41012u(1969).
(884) V. Ezdakov, *Kolloid Zh.*, **31,** 513(1969); through *Chem. Abstr.*, **71,** 105615y(1969).
(885) G. Tsagareishvili, *Soobshch. Akad. Nauk Gruz. SSR*, **53,** 121(1969); through *Chem. Abstr.*, **71,** 24716e(1969).
(886) P. Joergensen, *Geol. Foeren. Stockholm Foerh.*, **90,** 213 (1968); through *Chem. Abstr.*, **70,** 41039h(1969).
(887) J. Kijne, *Soil Sci. Soc. Amer., Proc.*, **33,** 539(1969); through *Chem. Abstr.*, **71,** 74419f(1969).
(888) A. Kitahara, *Shikizai Kyokaishi*, **42,** 181(1969); through *Chem. Abstr.*, **71,** 74364j(1969).
(889) R. Koh, I. Furda, A. Haug, and O. Smidsroed, *Acta Chem. Scand.*, **22,** 3098(1968); through *Chem. Abstr.*, **70,** 81248g(1969).
(890) A. Koganovskii and T. Levchenko, *Kolloid Zh.*, **31,** 384(1969); through *Chem. Abstr.*, **71,** 74422b(1969).
(891) I. Korshunov, O. Kolmakov, N. Novotorov, N. Chernorukov, Z. Vestrova, and N. Gusarskaya, *Tr. Khim. Tekhnol.*, **1967,** 21; through *Chem. Abstr.*, **70,** 81184h(1969).
(892) R. Razouk, F. Saleeb, and F. Said, *J. Chem. UAR*, **11,** 207(1968); through *Chem. Abstr.*, **70,** 118436v(1969).
(893) N. Rajendran and S. Larsen, *Chem. Ind. (London)*, **44,** 1591(1969).
(894) H. Van Olphen, *Tappi*, **51,** 145A(1968); through *Chem. Abstr.*, **70,** 31856h(1969).
(895) H. Vogt and G. Beck, *Pharm. Ind.*, **30,** 694(1968); through *Chem. Abstr.*, **70,** 71041v(1969).
(896) K. Viswanathan, *India At. Energy Comm., Bhabha At. Res. Cent. BARC*, **1968,** 385; through *Chem. Abstr.*, **71,** 129090z (1969).
(897) O. Radczewski, *Staub*, **28,** 435(1968); through *Chem. Abstr.*, **70,** 71265w(1969).
(898) M. Webber and J. Clark, *Can. J. Soil Sci.*, **49,** 231(1969); through *Chem. Abstr.*, **71,** 25001e(1969).
(899) E. Wright, *Chem. Ind. (London)*, **21,** 682(1969); through *Chem. Abstr.*, **71,** 33688z(1969).
(900) L. Yakhontova, B. Bruns, V. Kartseva, S. Kobzieva, and, N. Perevozskaya. *Antibiotiki*, **14,** 205(1969); through *Chem. Abstr.*, **70,** 118040m(1969).
(901) K. Tiller, *Clay Miner.*, **7,** 409(1968); through *Chem. Abstr.*, **71,** 116860a(1969).
(902) B. Theng, D. Greenland, and J. Quirk, *ibid.*, **7,** 271(1968); through *Chem. Abstr.*, **71,** 74418e(1969).
(903) F. Tokiwa and T. Imamura, *J. Amer. Oil Chem. Soc.*, **46,** 280(1969); through *Chem. Abstr.*, **71,** 95316k(1969).
(904) E. Aripov, K. Saidov, B. Nuriev, and K. Akhmedov, *Uzb. Khim. Zh.*, **12,** 26(1968); through *Chem. Abstr.*, **70,** 91096v (1969).
(905) C. Armistead, A. Tyler, F. Hambleton, S. Mitchell, and J. Hockey, *J. Phys. Chem.*, **73,** 3947(1969).
(906) S. Ash, D. Everett, and G. Findenegg, *Trans. Faraday Soc.*, **64,** 2645(1968); through *Chem. Abstr.*, **70,** 14691h(1969).
(907) *Ibid.*, **64,** 2639(1968); through *Chem. Abstr.*, **70,** 14698r (1969).
(908) P. Burns and M. Salomon, *Proc. Nat. Shellfish Ass.*, 121(1968); through *Chem. Abstr.*, **71,** 64499t(1969).
(909) J. De Boer, *Mol. Processes Solid Surfaces, Battelle Inst. Mater. Sci. Colloq., 3rd*, **1968,** 3; through *Chem. Abstr.*, **71,** 116835w (1969).
(910) B. Davey and P. Low, *Trans., Int. Congr. Soil Sci., 9th*, **1,** 607(1968); through *Chem. Abstr.*, **71,** 129047r(1969).
(911) S. Gal, *Chimia*, **22,** 409(1968); through *Chem. Abstr.*, **70,** 31820s(1969).
(912) S. Gregg and J. Ramsay, *J. Phys. Chem.*, **73,** 1243(1969).
(913) R. Mitra and B. Kapoor, *Soil Sci.*, **108,** 11(1969); through *Chem. Abstr.*, **71,** 64475g(1969).
(914) G. Martynov, *God. Sofii, Univ., Khim. Fak.* **61,** 341(1966–1967); through *Chem. Abstr.*, **71,** 84876s(1969).
(915) J. Nemeth, *Magy. Kem. Lapja*, **23,** 640(1968); through *Chem. Abstr.*, **70,** 23094t(1969).
(916) G. Schay, *Surface Colloid Sci.*, **2,** 155(1969); through *Chem. Abstr.*, **71,** 116815g(1969).
(917) E. Soczewinski and W. Golkiewicz, *Chem. Anal. (Warsaw)*, **14,** 465(1969); through *Chem. Abstr.*, **71,** 74438m(1969).
(918) S. Sabirov, *Izv. Akad. Nauk Uzb. SSR, Ser. Tekh. Nauk*, **13,** 65(1969); through *Chem. Abstr.*, **71,** 53894s(1969).
(919) A. Swoboda and G. Kunze, *Soil Sci. Soc. Amer., Proc.*, **32,** 806(1968); through *Chem. Abstr.*, **70,** 61431h(1969).
(920) G. Sposito and K. Babcock, *Clay Miner.*, **14,** 133(1964); through *Chem. Abstr.*, **71,** 95223c(1969).
(921) C. Robbins, G. Scott, and J. Barnhurst, *Text. Res. J.*, **38,** 1197(1968); through *Chem. Abstr.*, **70,** 50418h(1969).
(922) J. Zatz, N. Weiner, and M. Gibaldi, *J. Pharm. Sci.*, **58,** 1493(1969).
(923) C. Giles and A. Trivedi, *Chem. Ind. (London)*, **40,** 1426 (1969).
(924) J. Horiuti and C. Lin, *J. Res. Inst. Catal., Hokkaido Univ.*, **16,** 411(1968); through *Chem. Abstr.*, **70,** 81193k(1969).
(925) T. Henson and W. Hunter, *J. Catal.*, **14,** 197(1969); through *Chem. Abstr.*, **71,** 16196v(1969).
(926) C. Giles, *Trans. Faraday Soc.*, **65,** 1943(1969); through *Chem. Abstr.*, **71,** 42648p(1969).
(927) R. Sieh, *Staub*, **28,** 480(1968); through *Chem. Abstr.*, **70,** 71278c(1969).
(928) G. Poluektova and K. Gorkovenko, *Zh. Prikl. Khim. (Leningrad)*, **41,** 2005(1968); through *Chem. Abstr.*, **70,** 31825x (1969).
(929) G. Kalb and R. Curry, *Clay Miner.*, **17,** 47(1969); through *Chem. Abstr.*, **71,** 105672q(1969).
(930) B. Thuret, *Rev. Mater. Constr. Trav. Publics*, **641,** 41 (1969); through *Chem. Abstr.*, **71,** 64455a(1969).
(931) M. Jones, *Kolloid-Z. Z. Polym.*, **230,** 236(1969); through *Chem. Abstr.*, **71,** 6756p(1969).
(932) B. Conway and L. Gordon, *J. Phys. Chem.*, **73,** 3523 (1969).
(933) P. Becher, *J. Paint Technol.*, **41,** 523(1969); through *Chem. Abstr.*, **71,** 116895r(1969).
(934) D. Ekserova, *Kolloid-Z. Z. Polym.*, **232,** 703(1969); through *Chem. Abstr.*, **71,** 74416c(1969).
(935) T. Tadros and J. Lyklema, *J. Electroanal. Chem. Interfacial Electrochem.*, **22,** 1(1969); through *Chem. Abstr.*, **71,** 64482g (1969).
(936) F. Tokiwa and K. Ohki, *Bull. Chem. Soc. Jap.*, **41,** 2828 (1968); through *Chem. Abstr.*, **70,** 91122a(1969).
(937) K. Kinoshita and T. Yamanaka, *Asahi Garasu Kogyo Gijutsu Shoreikai Kenkyu Hokoku*, **14,** 321(1968); through *Chem. Abstr.*, **71,** 114475e(1969).
(938) M. Rosen and J. Solash, *J. Amer. Oil Chem. Soc.*, **46,** 399(1969); through *Chem. Abstr.*, **71,** 64439y(1969).
(939) M. Ranny, *Prum. Potravin.*, **20,** 101(1969); through *Chem. Abstr.*, **71,** 23149k(1969).
(940) L. Stryker and E. Matijevic, *J. Phys. Chem.*, **73,** 1484 (1969).
(941) D. Napper, *J. Colloid Interface Sci.*, **29,** 168(1969).
(942) G. Martynov and D. Lychnikov, *Issled. Obl. Poverkh. Sil., Sb. Dokl Konf. 3rd*, **1966,** 256; through *Chem. Abstr.*, **70,** 14687m(1969).
(943) S. Dukhin and S. Stoilov, *God. Sofii, Univ., Khim. Fak*, **60,** 81(1965–1966); through *Chem. Abstr.*, **70,** 6771u(1969).
(944) A. Mata-Arjona, M. Alario-Franco, and D. Escolar-Mendez, *An. Quim*, **64,** 667(1968); through *Chem. Abstr.*, **70,** 6828t(1969).
(945) A. Shansky, *Amer. Perfum. Cosmet.*, **84,** 31(1969).
(946) G. Fleer and J. Lyklema, *J. Colloid Interface Sci.*, **29,** 170 (1969).
(947) V. Barboi, Y. Glazman, and G. Fuks, *Kolloid Zh.*, **30,** 804(1968); through *Chem. Abstr.*, **70,** 41047j(1969).
(948) H. Schott, *J. Pharm. Sci.*, **58,** 1131(1969).
(949) *Ibid.*, **58,** 1449(1969).
(950) J. McAlteer and L. Kinnard, *Chem. Phys. Appl. Surface Active Subst.; Proc. Int. Congr. 4th*, **1,** 127(1964); through *Chem. Abstr.*, **70,** 79392f(1969).
(951) H. Lange, *Fette, Seifen, Anstrichm.*, **70,** 748(1968); through *Chem. Abstr.*, **70,** 12846p(1969).
(952) N. Grebnev, V. Klassen, and L. Stefanovskaya, *Kolloid*

Zh., **31,** 42(1969); through *Chem. Abstr.*, **71,** 6758r(1969).

(953) A. Felmeister and R. Schaubman, *J. Pharm. Sci.*, **58,** 64 (1969).

(954) *Ibid.*, **58,** 1232(1969).

(955) B. Gardner, *J. Lab. Clin. Med.*, **73,** 202(1969).

(956) L. Gargallo, A. Sepulveda, and J. Goldfarb, *Kolloid-Z. Z. Polym.*, **229,** 51(1969); through *Chem. Abstr.*, **70,** 81289w (1969).

(957) T. Mitsui and Y. Machida, *J. Soc. Cosmet. Chem.*, **20,** 199(1969).

(958) T. Shibusawa, *Sen-i Gakkaishi*, **25,** 107(1969); through *Chem. Abstr.*, **71,** 126314q(1969).

(959) M. Schwuger, *Kolloid-Z. Z. Polym.*, **232,** 775(1969); through *Chem. Abstr.*, **71,** 82908e(1969).

(960) K. Ohki and F. Tokiwa, *Nippon Kagaku Zasshi*, **90,** 760(1969); through *Chem. Abstr.*, **71,** 103446v(1969).

(961) R. Mukherjea and A. Gupta, *Fette, Seifen, Anstrichm.*, **70,** 876(1968); through *Chem. Abstr.*, **70,** 39105h(1969).

(962) S. Hartland, *Trans. Inst. Chem., Eng.*, **46,** T275(1968); through *Chem. Abstr.*, **70,** 91065j(1969).

(963) L. Leonov and P. Prokhorov, *Vop. Ispareniya, Goreniya Gazov. Din. Dispersnykh Sist., Mater. Vses. Nauch. Konf., 4th, 5th,* **1966,** 192; through *Chem. Abstr.*, **71,** 74345d(1969).

(964) E. Goette, *Fette, Seifen, Anstrichm.*, **71,** 219(1969); through *Chem. Abstr.*, **71,** 40606z(1969).

(965) M. Crauland, A. Courtier, and J. Bolle, *Chem. Phys. Appl. Surface Active Subst., Proc. Int. Congr., 4th,* **1,** 93(1964); through *Chem. Abstr.*, **70,** 89063p(1969).

(966) K. Aoki, H. Fujisawa, and J. Ise, *Ann. Rep. Takeda Res. Lab.*, **27,** 172(1968); through *Chem. Abstr.*, **70,** 59145z(1969).

(967) A. Abramzon and Z. Slavina, *Dokl. Akad. Nauk SSSR*, **186,** 116(1969); through *Chem. Abstr.*, **71,** 64545e(1969).

(968) E. Pandula and B. Kovacs, *Acta Pharm. Hung.*, **38,** 350(1968); through *Chem. Abstr.*, **70,** 14392m(1969).

(969) F. Popescu, *Rev. Chim. (Bucharest)*, **19,** 503(1968); through *Chem. Abstr.*, **70,** 48887s(1969).

(970) A. Prins and M. Tempel, *J. Phys. Chem.*, **73,** 2828(1969).

(971) L. Raphael, *Mfg. Chem. Aerosol News*, **40,** 36(1969).

(972) M. Ranny, *Prum. Potravin.*, **20,** 271(1969); through *Chem. Abstr.*, **71,** 103450s(1969).

(973) S. Lin and L. Lachman, *J. Pharm. Sci.*, **58,** 377(1969).

(974) D. Allen and K. Kwan, *ibid.*, **58,** 1190(1969).

(975) M. Moustafa and J. Carless, *J. Pharm. Pharmacol.*, **21,** 359 (1969).

(976) S. Kiryu and S. Iguchi, *Yakugaku Zasshi*, **89,** 707(1969).

(977) R. Marshall and G. Nancollas, *J. Phys. Chem.*, **73,** 3838 (1969).

(978) O. Neiman and M. Belikova, *Kolloid Zh.*, **30,** 863(1968); through *Chem. Abstr.*, **70,** 50849z(1969).

(979) A. Altykis, T. Filippos'yants, R. Kuznetsova, E. Kuz'mina, B. Petreikova, E. Kurova, and N. Shcherbakov, *Khim.-Farm. Zh.*, **3,** 43(1969); through *Chem. Abstr.*, **71,** 84500q(1969).

(980) D. Aldcroft and G. Bye, *Proc. Brit. Ceram. Soc.*, **13,** 125(1969); through *Chem. Abstr.*, **71,** 95286a(1969).

(981) O. Lekhan, G. Bashura, and D. Salo, *Farm. Zh. (Kiev)*, **23,** 61(1968); through *Chem. Abstr.*, **70,** 50445q(1969).

(982) J. Albright, *J. Phys. Chem.*, **73,** 1280(1969).

(983) A. Kibbe and O. Araujo, *J. Pharm. Sci.*, **58,** 714(1969).

(984) L. Djakovic, P. Dokic, and S. Nesic, *Tehnika (Belgrade)*, **23,** 2143(1968); through *Chem. Abstr.*, **71,** 105661k(1969).

(985) M. Gilde-Farkas and F. Szanto, *Foldt. Kozl.*, **99,** 60 (1969); through *Chem. Abstr.*, **71,** 74442h(1969).

(986) R. Huettenrauch, W. Suess, and U. Schmeiss, *Pharmazie*, **24,** 53(1969); through *Chem. Abstr.*, **70,** 109150u(1969).

(987) Z. Csuros, J. Bozzoy, and B. Zsoldos, *Period. Polytech., Chem. Eng.*, **12,** 381(1969); through *Chem. Abstr.*, **71,** 64531x(1969).

(988) S. Matsumoto and P. Sherman, *J. Colloid Interface Sci.*, **30,** 525(1969).

(989) R. Mannheimer, *AIChEJ.*, **15,** 88(1969); through *Chem. Abstr.*, **70,** 59147b(1969).

(990) B. Allan, *Diss. Abstr. B.*, **29,** 3694(1969); through *Chem. Abstr.*, **71,** 64547g(1969).

(991) M. Zhakova, *Tr. Perm. Farm. Inst., No. 2,* **1967,** 117; through *Chem. Abstr.*, **70,** 118062v(1969).

(992) M. Takehara and T. Nakagawa, *Nippon Kagaku Zasshi*, **90,** 775(1969); through *Chem. Abstr.*, **71,** 129154y(1969).

(993) N. Mitra, G. Surana, and R. Datta, *Indian J. Appl. Chem.*, **31,** 167(1968); through *Chem. Abstr.*, **71,** 74364g(1969).

(994) N. Mitra, S. Samanta, and P. Bhaumik, *J. Inst. Chem., Calcutta*, **41,** 142(1969); through *Chem. Abstr.*, **71,** 105660j(1969).

(995) N. Mitra and A. Biswas, *Indian Ceram.*, **13,** 143(1968); through *Chem. Abstr.*, **71,** 129076z(1969).

(996) H. Spencer, I. Lewin, M. Belcher, and J. Samachson, *Int. J. Appl. Radiat. Isotop.*, **20,** 507(1969).

(997) R. Vertua, *Boll. Chim. Farm.*, **107,** 461(1968); through *Chem. Abstr.*, **70,** 10061e(1969).

(998) W. Foye, *J. Pharm. Sci.*, **58,** 283(1969).

(999) V. Gusyakov and L. Voloshina, *Farm. Zh. (Kiev)*, **23,** 32(1968); through *Chem. Abstr.*, **70,** 50433j(1969).

(1000) M. Schwartz, *J. Pharm. Sci.*, **58,** 643(1969).

(1001) R. Weston, *Antimicrob. Agents Chemother.*, **1967,** 553; through *Chem. Abstr.*, **70,** 189(1969).

(1002) J. Hou and J. Poole, *J. Pharm. Sci.*, **58,** 1510(1969).

(1003) S. Rosenman, L. Weber, G. Owen and G. Warren, *Antimicrob. Agents Chemother.*, **1967,** 590; through *Chem. Abstr.*, **70,** 10177x(1969).

(1004) W. Morozowich, D. Lamb, H. Karnes, F. Mackellar, C. Lewis, K. Stern, and E. Rowe, *J. Pharm. Sci.*, **58,** 1485(1969).

(1005) G. Williamson, *Chim. Ther.*, **4,** 53(1969); through *Chem. Abstr.*, **71,** 37247c(1969).

(1006) F. Vymola and Z. Jedlickova, *J. Hyg., Epidemiol., Microbiol., Immunol.*, **12,** 244(1968); through *Chem. Abstr.*, **71,** 1969a(1969).

(1007) P. Jones, E. Rowley, A. Weiss, D. Bishop, and A. Chun, *J. Pharm. Sci.*, **58,** 337(1969).

(1008) E. Yourassowsky, M. Trap, and R. Monsieur, *Int. Congr. Chemother., Proc. 5th,* **1,** 231(1967); through *Chem. Abstr.*, **70,** 45903h(1969).

(1009) P. Braun, J. Tillotson, D. Wilcox, and M. Finland, *Appl. Microbiol.*, **16,** 1684(1968); through *Chem. Abstr.*, **70,** 10080k (1969).

(1010) T. Kainuma, *Niigata Igakkai Zasshi*, **82,** 672(1968); through *Chem. Abstr.*, **70,** 95161k(1969).

(1011) L. Dodion, P. Dusart, and P. Temmerman, *Arzneim.-Forsch.*, **19,** 785(1969); through *Chem. Abstr.*, **71,** 37236y(1969).

(1012) G. Koyama, H. Nakamura, S. Omoto, T. Takita, and K. Maeda, *J. Antibiotics (Tokyo)*, **22,** 34(1969).

(1013) M. Cairella, *Clin. Ter.*, **47,** 415(1968); through *Chem. Abstr.*, **71,** 6479a(1969).

(1014) J. Coliaizzi and P. Klink, *J. Pharm. Sci.*, **58,** 1184(1969).

(1015) S. Bell and D. Smith, *Lancet*, **I,** 753(1969).

(1016) N. Elinov and E. Mordvinova, *Tr. Leningrad, Khim.-Farm. Inst.*, **1967,** 138; through *Chem. Abstr.*, **70,** 76237y(1969).

(1017) B. Bandur and S. Mrsevic, *Mikrobiologija*, **6,** 65(1969); through *Chem. Abstr.*, **71,** 122048h(1969).

(1018) E. Lowbury, A. Kidson, H. Lilly, G. Ayliffe, and R. Jones, *Lancet*, **II,** 448(1969).

(1019) T. Bergan, *Nord. Med.*, **81,** 73(1969); through *Chem. Abstr.*, **71,** 1868s(1969).

(1020) M. Ho'jevac, *Mikrobiologija*, **4,** 277(1967); through *Chem. Abstr.*, **70,** 95157p(1969).

(1021) J. Smith, J. Hamilton-Miller, and R. Knox, *J. Pharm. Pharmacol.*, **21,** 337(1969).

(1022) B. Penicaut, *Bull. Soc. Pharm. Bordeaux*, **107,** 179(1968); through *Chem. Abstr.*, **71,** 6477y(1969).

(1023) S. Wilkinson, *Kirk-Othmer Encycl. Chem. Technol., 2nd Ed.*, **16,** 306(1968); through *Chem. Abstr.*, **70,** 80797y(1969).

(1024) L. Vernikova and S. Zhukovskaya, *Antibiotiki*, **13,** 982 (1968); through *Chem. Abstr.*, **70,** 40607e(1969).

(1025) S. Umezawa, *Kagaku (Kyoto)*, **21,** 1118(1966); through *Chem. Abstr.*, **70,** 1986h(1969).

(1026) P. Ellis, *Surv. Ophthalmol.*, **13,** 357(1968); through *Chem. Abstr.*, **70,** 95067j(1969).

(1027) C. Bogentoft, *Farm. Revy*, **67,** 749(1968); through *Chem. Abstr.*, **70,** 80793u(1969).

(1028) J. Berdy and K. Magyar, *Process Biochem.*, **3,** 45(1968); through *Chem. Abstr.*, **70,** 14342v(1969).

(1029) P. Hoeprich, *Calif. Med.*, **109,** 301(1968); through *Chem. Abstr.*, **70,** 36164r(1969).

(1030) S. Pathak and D. Reese, *J. Pharm. Sci.*, **58,** 505(1969).

(1031) F. Caruntu, *Viata Med.*, **15,** 1279(1968); through *Chem. Abstr.*, **70,** 104829p(1969).

(1032) F. Turner, S. Ringel, J. Martin, P. Storino, J. Daly, and

B. Schwartz, *Antimicrob. Agents Chemother.*, **1967,** 475; through *Chem. Abstr.*, **70,** 2122k(1969).

(1033) M. Toyohara, *Kekkaku*, **43,** 245(1968); through *Chem. Abstr.*, **70,** 10264y(1969).

(1034) F. Vymola and Z. Jedlickova, *Int. Congr. Chemother., Proc. 5th*, **1,** 79(1967); through *Chem. Abstr.*, **70,** 45901f(1969).

(1035) Y. Yamada, M. Kojima, and H. Umezawa, *Jap. J. Med. Sci. Biol.*, **21,** 223(1968); through *Chem. Abstr.*, **70,** 2024e(1969).

(1036) M. Weinstein, G. Luedemann, E. Oden, and G. Wagman, *Antimicrob. Agents Chemother.*, **1967,** 435; through *Chem. Abstr.*, **70,** 2121j(1969).

(1037) G. Rolinson and R. Sutherland, *ibid.*, **1967,** 609; through *Chem. Abstr.*, **70,** 10109b(1969).

(1038) H. Cohn, *J. Clin. Pharmacol. J. New Drugs*, **9,** 118(1969).

(1039) C. Kunin, D. Brandt, and H. Wood, *J. Infec. Dis.*, **119,** 132(1969); through *Chem. Abstr.*, **71,** 1858p(1969).

(1040) J. Pitt, R. Siasoco, K. Kaplan, and L. Weinstein, *Antimicrob. Agents Chemother.*, **1967,** 630; through *Chem. Abstr.*, **70,** 2125p(1969).

(1041) R. Freedman and R. Chamberlain, *ibid.*, **1967,** 502; through *Chem. Abstr.*, **70,** 2043k(1969).

(1042) L. Brown, W. Zygmunt, and H. Stavely, *Appl. Microbiol.*, **17,** 339(1969); through *Chem. Abstr.*, **70,** 95188z(1969).

(1043) G. Beskid and E. Grunberg, *Med. Exp.*, **18,** 327(1968); through *Chem. Abstr.*, **71,** 37394y(1969).

(1044) A. Chernomordik, *Antibiotiki*, **13,** 1130(1968); through *Chem. Abstr.*, **70,** 55873n(1969).

(1045) Z. Csukas and M. Fuzi, *Orv. Hetil.*, **109,** 2267(1968); through *Chem. Abstr.*, **70,** 95164p(1969).

(1046) P. Hoeprich, *Antimicrob. Agents Chemother.*, **1967,** 697; through *Chem. Abstr.*, **70,** 10112x(1969).

(1047) D. Garrison, R. De Haan, and J. Lawson, *ibid.*, **1967,** 397; through *Chem. Abstr.*, **70,** 2041h(1969).

(1048) R. McGehee, C. Smith, C. Wilcox, and M. Finland, *Amer. J. Med. Sci.*, **256,** 279(1968); through *Chem. Abstr.*, **70,** 10310k(1969).

(1049) L. Sabath, *Antimicrob. Agents Chemother.*, **1967,** 210; through *Chem. Abstr.*, **70,** 18604e(1969).

(1050) M. Nishida, Y. Mine, and S. Kuwahara, *J. Antibiot. (Tokyo)*, **22,** 144(1969).

(1051) S. Nakazawa, H. Ono, M. Kobayashi, and H. Komatsu, *Nippon Kagaku Ryohogakukai Zasshi*, **17,** 123(1969); through *Chem. Abstr.*, **71,** 1867r(1969).

(1052) L. Morgan, D. Cronk, and R. Knott, *J. Pharm. Sci.*, **58,** 942(1969).

(1053) E. Garrett and O. Araujo, *Annu. Rep. Med. Chem.*, **1967,** 337; through *Chem. Abstr.*, **70,** 27306m(1969).

(1054) W. Kalow, *Rep. Ross Pediat. Res. Conf.*, **58,** 48(1968); through *Chem. Abstr.*, **70,** 66430s(1969).

(1055) R. Zahradnik and K. Bocek, *Proc. Int. Pharmacol. Meet., 3rd*, **7,** 127(1966); through *Chem. Abstr.*, **70,** 18546n(1969).

(1056) A. Goldin, S. Vadlamudi, I. Kline, M. Gang, and J. Venditti, *Int. Congr. Chemother., Proc., 5th*, **3,** 333(1967); through *Chem. Abstr.*, **70,** 18539n(1969).

(1057) R. Long, *Pharm. J.*, **202,** 543(1969).

(1058) J. Burns, *Clin. Pharmacol. Ther.*, **10,** 607(1969); through *Chem. Abstr.*, **71,** 121943j(1969).

(1059) D. Stuart, *Pharm. Index*, **10,** 4(1968); through *Chem. Abstr.*, **70,** 1992g(1969).

(1060) P. Brotherton, P. Grieveson, and C. McMartin, *Clin. Pharmacol. Therap.*, **10,** 505(1969).

(1061) N. Gerber and K. Arnold, *J. Pharmacol. Exp. Ther.*, **167,** 77(1969); through *Chem., Abstr.*, **70,** 113534r(1969).

(1062) D. Brown, D. Hannan, and P. Langley, *Toxicol. Appl. Pharmacol.*, **5,** 136(1969); through *Chem. Abstr.*, **71,** 79378b(1969).

(1063) F. Marcucci, A. Guaitani, J. Kvetina, E. Mussini, and S. Garattini, *Eur. J. Pharmacol.*, **4,** 467(1968); through *Chem. Abstr.*, **70,** 55911y(1969).

(1064) I. Lapin and M. Samsonova, *Byull. Eksp. Biol. Med.*, **66,** 63(1968); through *Chem. Abstr.*, **70,** 45970c(1969).

(1065) J. Bridges, S. Walker, and R. Williams, *Biochem. J.*, **111,** 173(1969); through *Chem. Abstr.*, **70,** 56217p(1969).

(1066) J. Bridges, M. Kibby, S. Walker, and R. Williams, *ibid.*, **109,** 851(1968); through *Chem. Abstr.*, **70,** 10083p(1969).

(1067) K. Tatsumi, S. Yoshihara, C. Yamato, H. Yoshimura, and H. Tsukamoto, *Chem. Pharm. Bull.*, **17,** 1629(1969).

(1068) T. Fox, *Symp. Ser. Immunobiol. Stand.*, **5,** 133(1967); through *Chem. Abstr.*, **70,** 95068k(1969).

(1069) B. Brodie and W. Reid, *Am. J. Pharm.*, **141,** 21(1969).

(1070) K. Beyer, *Importance Fundam. Princ. Drug Eval., Proc. Symp.*, **1968,** 473; through *Chem. Abstr.*, **71,** 11391v(1969).

(1071) J. Gillette, *Rep. Ross Pediat. Res. Conf.*, **58,** 43(1968); through *Chem. Abstr.*, **70,** 66462d(1969).

(1072) J. Kucera and F. Bullock, *J. Pharm. Pharmacol.*, **21,** 293 (1969).

(1073) D. Witiak, T. Sokolosky, M. Whitehouse, and F. Hermann, *J. Med. Chem.*, **12,** 754(1969).

(1074) D. Witiak and M. Whitehouse, *Biochem. Pharmacol.*, **18,** 971(1969); through *Chem. Abstr.*, **71,** 20649n(1969).

(1075) R. Kato and A. Takanaka, *Jap. J. Pharmacol.*, **18,** 389 (1968); through *Chem. Abstr.*, **70,** 45854t(1969).

(1076) F. Keating, J. Jones, L. Elveback, and R. Randall, *J. Lab. Cun. Med.*, **73,** 825(1969).

(1077) L. Buchel, J. Absil, L. Liblau, and V. Simone, *Arch. Sci. Physiol.*, **23,** 109(1969); through *Chem. Abstr.*, **70,** 95083m (1969).

(1078) E. Garrett, *J. Amer. Pharm. Ass., NS*, **9,** 110(1969).

(1079) P. Lamy, *Pharm. Times*, **35,** 32(1969).

(1080) G. Levy, *Proc. Int. Pharmacol. Meet., 3rd*, **7,** 33(1966) (Pub. 1968); through *Chem. Abstr.*, **70,** 18544k(1969).

(1081) A. Arancibia, *Farmaco, Ed. Prat.*, **24,** 375(1969); through *Chem. Abstr.*, **71,** 79326h(1969).

(1082) O. Belozerova, *Khim.-Farm. Zh.*, **3,** 34(1969); through *Chem. Abstr.*, **71,** 42185k(1969).

(1083) C. Barrett, *J. Soc. Cosmet. Chem.*, **20,** 487(1969); through *Chem. Abstr.*, **71,** 94677s(1969).

(1084) R. Tawashi, *J. Pharm. Pharmacol.*, **21,** 701(1969).

(1085) A. Beckett, L. Brookes, and E. Shenoy, *ibid.*, **21,** 151S (1969).

(1086) H. Geuens and R. Stephens, *Int. Congr. Chemother., Proc., 5th*, **2,** 703(1967); through *Chem. Abstr.*, **70,** 27555s(1969).

(1087) A. Beckett and A. Moffat, *J. Pharm. Pharmacol.*, **21,** 144S(1969).

(1088) H. Solomon, *Develop. Saf. More Eff. Drugs, Symp.*, **1967,** 49; through *Chem. Abstr.*, **71,** 111389u(1969).

(1089) S. Goto, O. Tsuzuki, and S. Iguchi, *Chem. Pharm. Bull.*, **17,** 837(1969).

(1090) E. Genazzani, R. Di Carlo, and G. Pagnini, *Atti Soc. Ital. Sci. Vet.*, **22,** 265(1968); through *Chem. Abstr.*, **71,** 111098y (1969).

(1091) E. Genazzani, C. Granato, and G. Pagnini, *ibid.*, **22,** 269 (1968); through *Chem. Abstr.*, **71,** 111099z(1969).

(1092) E. Genazzani, R. Di Carlo, G. Pagnini, C. Granato, and R. Santamaria, *ibid.*, **22,** 273(1968); through *Chem. Abstr.*, **71,** 111100t(1969).

(1093) W. Jusko and G. Levy, *J. Pharm. Sci.*, **58,** 58(1969).

(1094) W. Lange, D. Floriddia, and F. Pruyn, *ibid.*, **58,** 771 (1969).

(1095) G. Levy and R. Nagashima, *ibid.*, **58,** 1001(1969).

(1096) D. Lukas and A. De Martino, *J. Clin. Invest.*, **48,** 1041 (1969); through *Chem. Abstr.*, **71,** 79367x(1969).

(1097) K. Jakimowska, *Diss. Pharm. Pharmacol.*, **20,** 397(1968); through *Chem. Abstr.*, **70,** 2193j(1969).

(1098) K. Rogers and A. Cammarata, *J. Med. Chem.*, **12,** 692 (1969).

(1099) I. Sugimoto, *Chem. Pharm. Bull.*, **17,** 1377(1969).

(1100) *Ibid.*, **17,** 994(1969).

(1101) T. Ito, *Shika Igaku*, **31,** 145(1968); through *Chem. Abstr.*, **70,** 18589d(1969).

(1102) C. Smith and M. Finland, *Appl. Microbiol.*, **16,** 1753 (1968); through *Chem. Abstr.*, **70,** 10081m(1969).

(1103) K. Kakemi, T. Arita, R. Hori, R. Konishi, and K. Nishimura, *Chem. Pharm. Bull.*, **17,** 248(1969).

(1104) R. Hess, H. Teschemacher, and A. Herz, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **261,** 469(1968); through *Chem. Abstr.*, **70,** 18784p(1969).

(1105) B. Catanese, R. Lisciani, and D. Piccinelli, *Biochem. Pharmacol.*, **18,** 1707(1969); through *Chem. Abstr.*, **71,** 69075m (1969).

(1106) W. Kuebler, H. Bretschneider, and P. Spieckermann, *Klin. Wochenschr.*, **47,** 108(1969); through *Chem. Abstr.*, **70,** 66731r (1969).

(1107) J. Krieglstein, *Naunyn-Schmiedebergs Arch. Pharmacol.*

Exp. Pathol., **262,** 474(1969); through *Chem. Abstr.*, **70,** 104828n (1969).

(1108) R. Joos and W. Hall, *J. Pharmacol. Exp. Ther.*, **166,** 113(1969); through *Chem. Abstr.*, **70,** 66468k(1969).

(1109) H. Kitagawa and H. Kadosawa, *Yakugaku Zasshi*, **89,** 1412(1969).

(1110) W. Johns and T. Bates, *J. Pharm. Sci.*, **58,** 179(1969).

(1111) E. Peets, M. Staub, and S. Symchowicz, *Biochem. Pharmacol.*, **18,** 1655(1969).

(1112) R. Emmrich, J. Wagner, and E. Axthelm, *Muenchen. Med. Wochenschr.*, **111,** 401(1969); through *Chem. Abstr.*, **70,** 85897r(1969).

(1113) O. Borga, D. Azarnoff, G. Forshell, and F. Sjoquist, *Biochem. Pharmacol.*, **18,** 2135(1969).

(1114) L. Anghileri, *Int. Z. Klin. Pharmakol. Ther. Toxikol.*, **1,** 523(1968); through *Chem. Abstr.*, **70,** 46028p(1969).

(1115) C. Chignell, *Mol. Pharmacol.*, **5,** 244(1969); through *Chem. Abstr.*, **70,** 113818m(1969).

(1116) J. Cassel, *Diss. Abstr.*, **29,** 2731B(1969).

(1117) L. Goldberg and H. Fundenberg, *J. Lab. Clin. Med.*, **73,** 469(1969).

(1118) F. Maher and W. Tauxe, *J. Amer. Med. Ass.*, **207,** 97 (1969); through *Chem. Abstr.*, **70,** 56235t(1969).

(1119) J. McArthur and M. Smith, *J. Pharm. Pharmacol.*, **21,** 589(1969).

(1120) J. McArthur and M. Smith, *F.E.B.S. Lett.*, **1,** 332(1968); through *Chem. Abstr.*, **70,** 55961q(1969).

(1121) S. Naito and K. Fukui, *J. Pharm. Sci.*, **58,** 1217(1969).

(1122) J. Crawford and H. Hooi, *Brit. J. Anesth.*, **40,** 825(1968); through *Chem. Abstr.*, **70,** 66709q(1969).

(1123) J. Smythies and F. Antun, *Nature*, **223,** 1061(1969).

(1124) C. Kreke, *J. Pharm. Sci.*, **58,** 457(1969).

(1125) L. Manojlović-Muir, *Nature*, **224,** 686(1969).

(1126) D. Hawkins, R. Pinckard, I. Crawford, and R. Farr, *J. Clin. Invest.*, **48,** 536(1969); through *Chem. Abstr.*, **70,** 105080z (1969).

(1127) A. Beckett and A. Moffat, *J. Pharm. Pharmacol.*, **21,** 139S (1969).

(1128) R. Pinney and V. Walters, *ibid.*, **21,** 415(1969).

(1129) K. Yagishita and Y. Nagai, *Yakugaku Zasshi*, **89,** 1152 (1969).

(1130) E. Kutter and C. Hansch, *J. Med. Chem.*, **12,** 647(1969).

(1131) N. Kakeya, N. Yata, A. Kamada, and M. Aoki, *Chem. Pharm. Bull.*, **17,** 2000(1969).

(1132) N. Kakeya, M. Aoki, A. Kamada, and N. Yata, *ibid.*, **17,** 1010(1969).

(1133) E. Garrett, J. Mielck, J. Seydel, and H. Kessler, *J. Med. Chem.*, **12,** 740(1969).

(1134) C. Hansch, *Proc. Int. Pharmacol. Meet., 3rd*, **7,** 141 (1966); through *Chem. Abstr.*, **70,** 18567v(1969).

(1135) C. Hansch, E. Kutter, and A. Leo, *J. Med. Chem.*, **12,** 746(1969).

(1136) E. Lien, *Amer. J. Pharm. Educ.*, **33,** 368(1969).

(1137) A. Leo, C. Hansch, and C. Church, *J. Med. Chem.*, **12,** 766(1969).

(1138) M. Messer, D. Farge, J. Guyonnet, C. Jeanmart, and L. Julou, *Arzneim.-Forsch.*, **19,** 1193(1969); through *Chem. Abstr.*, **71,** 111191y(1969).

(1139) V. Sasaki and M. Suzuki, *Chem. Pharm. Bull.*, **17,** 1569 (1969); through *Chem. Abstr.*, **71,** 116501j(1969).

(1140) W. Skinner, R. Parkhurst, and J. Scholler, *J. Med. Chem.*, **12,** 64(1969).

(1141) J. Van Rossum, *Proc. Int. Pharmacol. Meet., 3rd*, **7,** 237(1966); through *Chem. Abstr.*, **70,** 66429y(1969).

(1142) J. Buechi, J. Doulakas, and X. Perlia, *Arzneim.-Forsch.*, **19,** 578(1969); through *Chem. Abstr.*, **71,** 20635e(1969).

(1143) V. Grishina, *Tr. Perm. Farm. Inst.*, **1967,** 9; through *Chem. Abstr.*, **71,** 20638h(1969).

(1144) G. Schneller, *J. Amer. Pharm. Ass.*, **NS9,** 455(1969).

(1145) I. Schamberg, *J. Clin. Pharmacol. J. New Drugs*, **9,** 205 (1969).

(1146) D. Freestone, *Lancet*, **I,** 98(1969).

(1147) A. Marcus, *Amer. J. Pharm.*, **141,** 29(1969).

(1148) J. Sjogren, *Oesterr. Apoth. Ztg.*, **23,** 53(1969); through *Chem. Abstr.*, **71,** 15975m(1969).

(1149) M. Taraszka and R. Delor, *J. Pharm. Sci.*, **58,** 207(1969).

(1150) M. Coldman, B. Poulsen, and T. Higuchi, *ibid.*, **58,** 1098 (1969).

(1151) J. Conklin, R. Sobers, and D. Wagner, *ibid.*, **58,** 1365 (1969).

(1152) T. Bates, D. Lambert, and W. Johns, *ibid.*, **58,** 1468 (1969).

(1153) R. Engel and S. Riggi, *ibid.*, **58,** 1372(1969).

(1154) J. Anello and G. Levy, *ibid.*, **58,** 721(1969).

(1155) W. Cressman, C. Janicki, P. Johnson, J. Doluisio, and G. Braum, *ibid.*, **58,** 1516(1969).

(1156) E. Mroszczak, J. Vallner, and J. Perrin, *ibid.*, **58,** 1567 (1969).

(1157) T. Flanagan, R. Broad, M. Rubinstein, and A. Longworth, *J. Pharm. Pharmacol.*, **21,** 129S(1969).

(1158) R. Dempski, J. Portnoff, and A. Wase, *J. Pharm. Sci.*, **58,** 579(1969).

(1159) J. Leonards and G. Levy, *ibid.*, **58,** 1277(1969).

(1160) J. Leonards and G. Levy, *Clin. Pharmacol. Ther.*, **10,** 571(1969).

(1161) E. Tala, *Farm. Aikak.*, **78,** 38(1969); through *Chem. Abstr.*, **71,** 47898e(1969).

(1162) F. Jaminet, *Pharm. Acta Helv.*, **44,** 261(1969); through *Chem. Abstr.*, **71,** 37228x(1969).

(1163) N. Mizuno, M. Aoki, A. Kamada, and N. Yata, *Bitamin*, **39,** 26(1969); through *Chem. Abstr.*, **70,** 56045f(1969).

(1164) T. Hoeyland, *Medd. Nor. Farm. Selsk.*, **30,** 185(1968); through *Chem. Abstr.*, **71,** 6480u(1969).

(1165) R. Gelber, P. Jacobsen, and L. Levy, *Clin. Pharmacol. Ther.*, **10,** 841(1969).

(1166) M. Aoki, A. Kamada, N. Yata, K. Kishimoto, C. Mugino, H. Sakaguchi, K. Tanabe, and I. Mimura, *Chem. Pharm. Bull.*, **17,** 1109(1969).

(1167) E. Rosen, P. Tannenbaum, A. Crosley, and T. Flanagan, *Develop. Saf. More Eff. Drugs, Symp.*, **1967,** 111; through *Chem. Abstr.*, **71,** 128604h(1969).

(1168) R. Engel and S. Riggi, *J. Pharm. Sci.*, **58,** 706(1969).

(1169) K. Kakemi, H. Sezaki, S. Muranishi, and Y. Tsujimura, *Chem. Pharm. Bull.*, **17,** 1650(1969).

(1170) C. Whitworth and E. Carter, *J. Pharm. Sci.*, **58,** 1285 (1969).

(1171) T. Matsuzawa, H. Fujisawa, K. Aoki, and H. Mima, *Chem. Pharm. Bull.*, **17,** 999(1969).

(1172) L. Ravin, E. Shami, A. Intoccia, E. Rattie, and G. Joseph, *J. Pharm. Sci.*, **58,** 1242(1969).

(1173) S. Feldman and M. Gibaldi, *ibid.*, **58,** 967(1969).

(1174) *Ibid.*, **58,** 425(1969).

(1175) C. Nightingale, R. Wynn, and M. Gibaldi, *ibid.*, **58,** 1005(1969).

(1176) G. Bashura, M. Gluzman, and E. Labunskii, *Farm. Zh. (Kiev)*, **24,** 40(1969); through *Chem. Abstr.*, **70,** 90712f(1969).

(1177) H. Shinkai and I. Tanaka, *Yakugaka Zasshi*, **89,** 1283 (1969).

(1178) P. Bartelloni, *Amer. J. Med. Sci.*, **258,** 203(1969); through *Chem. Abstr.*, **71,** 100032w(1969).

(1179) K. Bulow, *Pharmacol. Clin.*, **1,** 156(1969); through *Chem. Abstr.*, **71,** 89958e(1969).

(1180) T. Eckert, *Arzneim.-Forsch.*, **19,** 821(1969); through *Chem. Abstr.*, **71,** 53534z(1969).

(1181) W. Reetz, *Pharm. Acta Helv.*, **44,** 238(1969); through *Chem. Abstr.*, **71,** 42242b(1969).

(1182) H. Baker, *J. Soc. Cosmet. Chem.*, **20,** 239(1969).

(1183) T. Matsuzaki and E. Noda, *Yakuzaigaku*, **26,** 109(1966); through *Chem. Abstr.*, **70,** 2363q(1969).

(1184) A. Virala and J. Cano, *Therapie*, **23,** 775(1968); through *Chem. Abstr.*, **70,** 27574x(1969).

(1185) W. Erdmann and S. Okonek, *Arch. Toxikol.*, **24,** 91 (1969); through *Chem. Abstr.*, **70,** 56022w(1969).

(1186) L. Block and P. Lamy, *Pharm. Acta Helv.*, **44,** 44(1969); through *Chem. Abstr.*, **70,** 66746z(1969).

(1187) M. Mattila, R. Koskinen, and S. Takki, *Ann. Med. Intern. Fenn.*, **57,** 75(1968); through *Chem. Abstr.*, **70,** 27345y(1969).

(1188) G. Irwin, H. Kostenbauder, L. Dittert, R. Staples, A. Misher, and J. Swintosky, *J. Pharm. Sci.*, **58,** 313(1969).

(1189) F. Kamienny, M. Barr, and J. Nagwekar, *ibid.*, **58,** 1318 (1969).

(1190) L. Christensen and L. Skovsted, *Lancet*, **II,** 1397(1969).

(1191) M. Rowland, *J. Pharm. Sci.*, **58,** 508(1969).

(1192) M. Fennessy and C. Raper, *J. Pharm. Pharmacol.*, **21,** 873(1969).
(1193) J. Doluisio and L. Dittert, *Clin. Pharmacol. Ther.*, **10,** 690(1969).
(1194) L. Block and P. Lamy, *J. Amer. Pharm. Ass.*, **NS9,** 202 (1969).
(1195) D. Hussar, *ibid.*, **NS9,** 208(1969).
(1196) C. Schlagel and E. Sanborn, *J. Pharm. Sci.*, **58,** 1453 (1969).
(1197) R. Stoll, T. Bates, K. Nieforth, and J. Swarbrick, *ibid.*, **58,** 1457(1969).
(1198) A. Kaumann, *Cienc. Invest.*, **24,** 262(1968); through *Chem. Abstr.*, **70,** 76118k(1969).
(1199) J. Orr, J. Shimmins, and C. Speirs, *Lancet*, **II,** 771(1969).
(1200) R. Ott and R. Myers, *Clin. Pharmacol. Ther.*, **10,** 207 (1969).
(1201) A. Zupko, *Pharm. Times*, **35,** 33(1969).
(1202) *Ibid.*, **35,** 38(1969).
(1203) J. Perel, P. Dayton, M. Snell, T. Yu, and A. Gutman, *Clin. Pharmacol. Ther.*, **10,** 834(1969).
(1204) F. Ponz and M. Lluch, *Rev. Espan. Fisiol.*, **24,** 203(1968); through *Chem. Abstr.*, **70,** 18802t(1969).
(1205) M. Kristensen, H. Moelholm, and L. Skovsted, *Acta Med. Scand.*, **185,** 347(1969); through *Chem. Abstr.*, **71,** 1814w (1969).
(1206) D. Zaharko, H. Bruckner, and V. Oliverio, *Science*, **166,** 887(1969).
(1207) V. Temmere, *Prob. Tuberk.*, **11,** 83(1967); through *Chem. Abstr.*, **70,** 85899t(1969).
(1208) A. Wohlman, M. Syed, and M. Ronchi, *Can. J. Physiol. Pharmacol.*, **46,** 815(1968); through *Chem. Abstr.*, **70,** 2171a(1969).
(1209) H. Takizawa, M. Niwayama, and K. Abe, *Niigata Igakkai Zasshi*, **82,** 791(1968); through *Chem. Abstr.*, **70,** 95361a(1969).
(1210) E. Brodskii, *Antibiotiki*, **14,** 365(1969); through *Chem. Abstr.*, **71,** 1893w(1969).
(1211) E. Ariens, *Pharm. Weekbl.*, **104,** 153(1969); through *Chem. Abstr.*, **70,** 76122g(1969).
(1212) W. Decker, R. Shpall, D. Corby, H. Combs, and C. Payne, *Clin. Pharmacol. Ther.*, **10,** 710(1969).
(1213) W. Decker, H. Combs, and D. Corby, *Toxicol. Appl. Pharmacol.*, **13,** 454(1968); through *Chem. Abstr.*, **70,** 2002w(1969).
(1214) A. Groppetti and E. Costa, *Int. J. Neuropharmacol.*, **8,** 209(1969); through *Chem. Abstr.*, **71,** 29109u(1969).
(1215) G. Hitzenberger, *Verh. Deut. Ges. Inn. Med.*, **74,** 1145 (1968); through *Chem. Abstr.*, **71,** 29193s(1969).
(1216) R. Welch, Y. Harrison, A. Conney, and J. Burns, *Clin. Pharmacol. Ther.*, **10,** 817(1969).
(1217) K. Lee and T. Tong, *J. Pharm. Sci.*, **58,** 773(1969).
(1218) R. Northcutt, J. Stiel, J. Hollifield, and E. Stant, *J. Amer. Med. Ass.*, **208,** 1857(1969); through *Chem. Abstr.*, **71,** 29231c(1969).
(1219) H. Niwa and N. Hikichi, *Tohoku Yakka Daigaku Kenkyu Nempo*, **1968,** 1; through *Chem. Abstr.*, **71,** 111395t(1969).
(1220) R. Long, *Proc. Roy. Soc. Med.*, **62,** 318(1969); through *Chem. Abstr.*, **71,** 47856q(1969).
(1221) H. Staudacher and H. Mueller, *Arzneim.-Forsch.*, **19,** 1612(1969); through *Chem. Abstr.*, **71,** 111065k(1969).
(1222) F. Sjoqvist, F. Berglund, O. Borga, W. Hammer, S. Andersson, and C. Thorstrand, *Clin. Pharmacol. Ther.*, **10,** 826 (1969).
(1223) P. Zieve and H. Solomon, *J. Lab. Clin. Med.*, **73,** 103 (1969).
(1224) E. Genazzani, *Int. Congr. Chemother., Proc. 5th*, **4,** 187 (1967); through *Chem. Abstr.*, **70,** 45902g(1969).
(1225) A. Gero, *Proc. Int. Pharmacol. Meet., 3rd*, **7,** 261(1966) (Pub. 1968); through *Chem. Abstr.*, **70,** 27371d(1969).
(1226) E. Hvidberg, P. Dayton, J. Read, and C. Wilson, *Proc. Soc. Exp. Biol. Med.*, **129,** 438(1968); through *Chem. Abstr.*, **70,** 27364d(1969).
(1227) J. McArthur and P. Dawkins, *J. Pharm. Pharmacol.*, **21,** 744(1969).
(1228) E. McQueen, *Brit. J. Pharmacol.*, **36,** 29(1969); through *Chem. Abstr.*, **71,** 20658q(1969).
(1229) R. Reuning and G. Levy, *J. Pharm. Sci.*, **58,** 79(1969).
(1230) K. Mysels, *Advan. Chem. Ser.*, **86,** 24(1967); through *Chem. Abstr.*, **70,** 91125d(1969).
(1231) J. Wagner, *Drug Intel.*, **2,** 244(1968); through *Chem. Abstr.*, **70,** 1981c(1969).
(1232) M. Kitler, *Diss. Abstr.*, **29,** 2559B(1969).
(1233) S. Howard, M. Farvar, A. Suzuki, and W. Higuchi, *J. Pharm. Sci.*, **58,** 1325(1969).
(1234) S. Howard, A. Suzuki, M. Farvar, and W. Higuchi, *ibid.*, **58,** 1330(1969).
(1235) A. Goldberg and W. Higuchi, *ibid.*, **58,** 1341(1969).
(1236) J. Wagner, *Drug Intel.*, **2,** 294(1968); through *Chem. Abstr.*, **70,** 95060b(1969).
(1237) S. Turco and A. Canada, *Amer. J. Hosp. Pharm.*, **26,** 120 (1969).
(1238) C. Gooley, H. Keyzer, and F. Setchell, *Nature*, **223,** 80 (1969).
(1239) J. Caldwell, J. Martin, S. Dutta, and N. Greenberger, *Amer. J. Physiol.*, **217,** 1747(1969).
(1240) H. Gershon, M. McNeil, and Y. Hinds, *J. Med. Chem.*, **12,** 1115(1969).
(1241) H. Dibbern, *Arzneim.-Forsch.*, **19,** 1140(1969); through *Chem. Abstr.*, **71,** 100073k(1969).
(1242) A. Ghanem, W. Higuchi, and A. Simonelli, *J. Pharm. Sci.*, **58,** 165(1969).
(1243) H. Price, *Diss. Abstr.*, **30,** 493B(1969).
(1244) W. Lieb and W. Stein, *Nature*, **224,** 240(1969).
(1245) M. Maritano, P. Musto, and C. Barile, *Minerva Anestesiol.*, **34,** 1526(1968); through *Chem. Abstr.*, **71,** 1796s(1969).
(1246) K. Lee and M. Spencer, *J. Pharm. Sci.*, **58,** 464(1969).
(1247) G. Fiese and J. Perrin, *ibid.*, **58,** 599(1969).
(1248) H. Shindo, K. Okamoto, E. Nakajima, and I. Takahashi, *Bitamin*, **39,** 310(1969); through *Chem. Abstr.*, **71,** 20625b(1969).
(1249) J. Castleden, *J. Pharm. Sci.*, **58,** 149(1969).
(1250) R. Dixon, E. Owens, and D. Rall, *ibid.*, **58,** 1106(1969).
(1251) F. Lauterbach, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **263,** 26(1969); through *Chem. Abstr.*, **71,** 20604u (1969).
(1252) G. Levy and J. Anello, *J. Pharm. Sci.*, **58,** 494(1969).
(1253) M. Mezei and G. White, *ibid.*, **58,** 1209(1969).
(1254) W. Stephens, *Nature*, **224,** 547(1969).
(1255) M. Sax and J. Pletcher, *Science*, **166,** 1546(1969).
(1256) J. Wagner, *Amer. J. Pharm.*, **141,** 5(1969).
(1257) R. Nagashima and G. Levy, *J. Pharm. Sci.*, **58,** 845(1969).
(1258) M. Gibaldi, *ibid.*, **58,** 1133(1969).
(1259) M. Gibaldi, R. Nagashima, and G. Levy, *ibid.*, **58,** 193 (1969).
(1260) J. Swintosky, L. Dittert, and J. Doluisio, *Amer. J. Hosp. Pharm.*, **26,** 519(1969).
(1261) M. Gibaldi, *J. Pharm. Sci.*, **58,** 327(1969).
(1262) G. Levy, M. Gibaldi, and W. Jusko, *ibid.*, **58,** 422(1969).
(1263) M. Gibaldi and S. Feldman, *ibid.*, **58,** 1477(1969).
(1264) E. Gladtke, *Farmaco, Ed. Sci.*, **23,** 897(1968); through *Chem. Abstr.*, **70,** 10092r(1969).
(1265) H. Held and H. Von Oldershausen, *Klin. Wochenschr.*, **1969,** 78; through *Chem. Abstr.*, **70,** 56240r(1969).
(1266) L. Benet and R. Ronfeld, *J. Pharm. Sci.*, **58,** 639(1969).
(1267) L. Amsel and G. Levy, *ibid.*, **58,** 321(1969).
(1268) H. Held, L. Von Berger, and H. Von Oldershausen, *Verh. Deut. Ges. Inn. Med.*, **74,** 1140(1968); through *Chem. Abstr.*, **71,** 37255d(1969).
(1269) J. Wagner and C. Metzler, *J. Pharm. Sci.*, **58,** 87(1969).
(1270) G. Levy, L. Amsel, and H. Elliott, *ibid.*, **58,** 827(1969).
(1271) J. Wagner, *Drug Intel.*, **3,** 21(1969); through *Chem. Abstr.*, **70,** 105103j(1969).
(1272) R. Nagashima, R. O'Reilly, and G. Levy, *Clin. Pharmacol. Ther.*, **10,** 22(1969).
(1273) E. Krueger-Thiemer, *Int. Congr. Chemother. Proc. 5th*, **6,** 445(1967); through *Chem. Abstr.*, **70,** 2399f(1969).
(1274) E. Rudzit, *Antibiotiki (Moscow)*, **14,** 725(1969); through *Chem. Abstr.*, **71,** 100121z(1969).
(1275) B. Krauer, *Int. Z. Klin. Pharmakol. Ther. Toxikol.*, **2,** 217(1969); through *Chem. Abstr.*, **71,** 100317t(1969).
(1276) B. Krauer, P. Spring, L. Dettli, and F. Frueh, *Pharmacol. Clin.*, **1,** 47(1968); through *Chem. Abstr.*, **70,** 27363c(1969).
(1277) O. Kitamoto, K. Fukaya, and G. Tomori, *Nippon Kagaku Ryohogakukai Zasshi*, **17,** 822(1969); through *Chem. Abstr.*, **71,** 69223h(1969).
(1278) G. Pfeifer, F. Doerr, and K. Brod, *Klin. Wochenschr.*, **47,** 482(1969); through *Chem. Abstr.*, **71,** 11677t(1969).

(1279) P. Buenger, *Infektionskr., Int. Kongr. Infectionskr., Verhandlungsber 4th*, **1966**, 858; through *Chem. Abstr.*, **71**, 11675r (1969).

(1280) D. Boreham and B. Martin, *Brit. J. Pharmacol.*, **37**, 294 (1969); through *Chem. Abstr.*, **71**, 121980u(1969).

(1281) E. Boehni, B. Fust, J. Rieder, K. Schaerer, and L. Havas, *Chemotherapy*, **14**, 195(1969); through *Chem. Abstr.*, **71**, 79662q (1969).

(1282) U. Abshagen and N. Rietbrock, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **264**, 110(1969); through *Chem. Abstr.*, **71**, 89887f(1969).

(1283) W. Chiou and S. Riegelman, *J. Pharm. Sci.*, **58**, 1500 (1969).

(1284) F. Dost and E. Gladtke, *Arzneim.-Forsch.*, **19**, 1304 (1969); through *Chem. Abstr.*, **71**, 111354d(1969).

(1285) M. Debackere, *Diergeneesk. Memo.*, **15**, 160(1968); through *Chem. Abstr.*, **71**, 28886h(1969).

(1286) A. Disanto and J. Wagner, *J. Pharm. Sci.*, **58**, 1077 (1969).

(1287) W. McCann, *Drug Inform. Bull.*, **3**, 52(1969).

(1288) M. Bickel and H. Weder, *J. Pharm. Pharmacol.*, **21**, 160 (1969).

(1289) H. Hundeshagen, U. Zeidler, H. Fabel, A. Garbe, and H. Nowak, *Arzneim.-Forsch.*, **18**, 1592(1968); through *Chem. Abstr.*, **70**, 55899a(1969).

(1290) A. Garbe and H. Nowak, *ibid.*, **18**, 1597(1968); through *Chem. Abstr.*, **70**, 55900u(1969).

(1291) H. Myer-Brunot, K. Schmid, and H. Keberle, *Int. Congr. Chemother., Proc. 5th*, **1**, 763(1967); through *Chem. Abstr.*, **70**, 2386z(1969).

(1292) B. Orme and R. Cutler, *Clin. Pharmacol. Ther.*, **10**, 543 (1969).

(1293) Z. Modr, K. Dvoracek, and A. Necaskova, *Rev. Czech. Med.*, **15**, 7(1969); through *Chem. Abstr.*, **71**, 89973f(1969).

(1294) J. Rieder and G. Rentsch, *Arzneim.-Forsch.*, **18**, 1545 (1968); through *Chem. Abstr.*, **70**, 55898z(1969).

(1295) L. Dettli and P. Spring, *Proc. Int. Pharmacol. Meet., 3rd*, **7**, 5(1966); through *Chem. Abstr.*, **70**, 18543j(1969).

(1296) W. Wagner, *Int. Congr. Chemother., Proc. 5th*, **4**, 93(1967); through *Chem. Abstr.*, **70**, 36415y(1969).

(1297) M. Von Wittenau, *Chemotherapy*, **13**, 41(1968); through *Chem. Abstr.*, **70**, 18535h(1969).

(1298) E. Rowe and W. Morozowich, *J. Pharm. Sci.*, **58**, 1375 (1969).

(1299) G. Segre, *Farmaco, Ed. Sci.*, **23**, 907(1968); through *Chem. Abstr.*, **70**, 10232m(1969).

(1300) E. Krueger-Thiemer and R. Levine, *Arzneim.-Forsch.*, **18**, 1575(1968); through *Chem. Abstr.*, **70**, 66448d(1969).

(1301) H. Nogami, M. Hanano, S. Awazu, and H. Moon, *Chem. Pharm. Bull.*, **17**, 2097(1969).

(1302) A. Mattocks, *J. Pharm. Sci.*, **58**, 595(1969).

(1303) G. Levy, *Importance Fundam. Princ. Drug Eval., Proc. Symp.*, **1968**, 141; through *Chem. Abstr.*, **71**, 11418j(1969).

(1304) A. Aguiar and M.Weiner, *J. Pharm. Sci.*, **58**, 210(1969).

(1305) P. Harris and S. Riegelman, *ibid.*, **58**, 71(1969).

(1306) W. Chiou, S. Riegelman, and J. Amberg, *Chem. Pharm. Bull.*, **17**, 2170(1969).

(1307) J. Doluisio, N. Billups, L. Dittert, E. Sugita, and J. Swintowsky, *J. Pharm. Sci.*, **58**, 1196(1969).

(1308) J. Doluisio, G. Tan, N. Billups, and L. Diamond, *ibid.*, **58**, 1200(1969).

(1309) G. Levy, A. Vogel, and L. Amsel, *ibid.*, **58**, 503(1969).

(1310) A. Beckett, J. Salmon, and M. Mitchard, *J. Pharm. Pharmacol.*, **21**, 251(1969).

(1311) G. Hepner, *Brit. J. Haematol.*, **16**, 241(1969); through *Chem. Abstr.*, **71**, 11519t(1969).

(1312) A. Beckett and R. Hossie, *J. Pharm. Pharmacol.*, **21**, 157S(1969).

(1313) I. Krushkov, *Dermatol. Venerol. (Sofia)*, **7**, 163(1968); through *Chem. Abstr.*, **70**, 76119m(1969).

(1314) V. Ulashchik, *Zdravookhr. Beloruss.*, **15**, 41(1969); through *Chem. Abstr.*, **71**, 48268(1969).

(1315) L. Solomon and A. Altman, *J. Invest. Dermatol.*, **53**, 454 (1969).

(1316) R. Scheuplein, I. Blank, G. Brauner, and D. MacFarlane, *ibid.*, **52**, 63(1969).

(1317) R. Feldmann and H. Maibach, *ibid.*, **52**, 89(1969).

(1318) J. Morelle, *Arch. Biochim. Cosmetol.*, **11**, 18(1969); through *Chem. Abstr.*, **70**, 18533f(1969).

(1319) B. Jaskoski and R. Betti, *J. Amer. Pharm. Ass.*, **NS9**, 224 (1969).

(1320) W. Crutcher and H. Maibach, *J. Invest. Dermatol.*, **53**, 264(1969).

(1321) A. Jori, A. Bianchetti, and P. Prestini, *Biochem. Pharmacol.*, **18**, 2081(1969); through *Chem. Abstr.*, **71**, 111084r(1969).

(1322) V. Pliska, *Proc. Int. Pharmacol. Meet., 3rd*, **7**, 115(1966); through *Chem. Abstr.*, **70**, 18566u(1969).

(1323) T. Hall, *Int. Congr. Chemother., Proc. 5th*, **7**, 9(1967); through *Chem. Abstr.*, **71**, 28889m(1969).

(1324) P. Waser, *Arzneim.-Forsch.*, **19**, 260(1969); through *Chem. Abstr.*, **70**, 104769u(1969).

(1325) M. Von Wittenau, *Int. Z. Klin. Pharmakol. Ther. Toxikol.*, **2**, 52(1969); through *Chem. Abstr.*, **71**, 29229h(1969).

(1326) N. Greenberger, R. MacDermott, J. Martin, and S. Dutta, *J. Pharmacol. Exp. Ther.*, **167**, 265(1969); through *Chem. Abstr.*, **71**, 11578m(1969).

(1327) W. Forth, E. Furukawa, W. Rummel, and H. Andres, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **262**, 53(1969); through *Chem. Abstr.*, **70**, 55915c(1969).

(1328) S. Ringel, F. Turner, S. Roemer, J. Daly, R. Zlatanoff, and B. Schwartz, *Antimicrob. Agents Chemother.*, **1967**, 486; through *Chem. Abstr.*, **70**, 2123m(1969).

(1329) G. Ellard, *Int. Congr. Chemother., Proc. 5th*, **2**, 607(1967); through *Chem. Abstr.*, **70**, 27554r(1969).

(1330) S. Ljungberg, U. Otto, and L. Paalzow, *Acta Pharm. Suecica*, **5**, 489(1968); through *Chem. Abstr.*, **70**, 27427b(1969).

(1331) K. Kakemi, H. Sezaki, K. Okumura, and S. Ashida, *Chem. Pharm. Bull.*, **17**, 1322(1969).

(1332) K. Kakemi, H. Sezaki, and T. Kondo, *ibid.*, **17**, 1864 (1969).

(1333) F. Sidell, W. Groff, and R. Ellin, *J. Pharm. Sci.*, **58**, 1093 (1969).

(1334) J. Wagner, L. Leslie, and R. Gove, *Int. Z. Klin. Pharmakol. Ther. Toxikol.*, **2**, 44(1969); through *Chem. Abstr.*, **71**, 29228g (1969).

(1335) K. Rahn and L. Goldberg, *Clin. Pharmacol. Ther.*, **10**, 858(1969).

(1336) P. Kornguth, *Diss. Abstr.*, **29**, 260B(1969).

(1337) H. Kasper, *Proc. Int. Congr. Nutr., 7th*, **5**, 104(1966); through *Chem. Abstr.*, **70**, 18844h(1969).

(1338) H. Ochsenfahrt, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **264**, 55(1969); through *Chem. Abstr.*, **71**, 89885d(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Pharmacy Research and Development Division, Wyeth Laboratories, Inc., Radnor, PA 19101*

Oral Absorption of Griseofulvin in Dogs: Increased Absorption *via* Solid Dispersion in Polyethylene Glycol 6000

WIN LOUNG CHIOU* and S. RIEGELMAN

Abstract □ Four different dosage forms of griseofulvin were administered orally to dogs: (*a*) griseofulvin in the solution of polyethylene glycol (PEG) 400; (*b*) griseofulvin dispersed in PEG 6000 (1:9 w/w) prepared by the fusion method and administered in capsule form; (*c*) a commercial tablet of micronized griseofulvin; and (*d*) a commercial capsule of micronized griseofulvin. The drug can be followed in the blood for 8–12 hr. after a 250- or 500-mg. dose. However, the blood data can lead to a false conclusion as to the degree and duration of the absorption process. This is due to fast metabolism ($t_{1/2}$ = 40–50 min.), to absorption being a dissolution rate-limited process, and finally to an assay that is insufficient to follow the total time course of the drug in the body. In contrast, the urinary excretion data for 6-demethylgriseofulvin (6-DMG) yield convincing evidence for prolonged absorption of griseofulvin for over 30 hr. for the commercial preparations. By comparison of the percent 6-DMG excreted to that obtained after i.v. administration, absorption was found to be complete for the solution form, 88% for the PEG dispersion, 45% for the commercial capsule, and 33% for the commercial tablet.

Keyphrases □ Griseofulvin dosage forms, oral—absorption, dogs □ Polyethylene glycol 6000 effect—griseofulvin absorption □ Dissolution rates—griseofulvin dosage forms □ Absorption, dissolution rates—correlation □ Urinary excretion—griseofulvin metabolites

Sekiguchi and Obi (1) were the first to apply the principle of solid dispersions utilizing a water-soluble carrier as a matrix for a poorly soluble drug to increase the rate of dissolution and oral absorption. They proposed the formation of a eutectic mixture of a poorly water-soluble drug with a physiologically inert, readily soluble carrier. Goldberg *et al.* (2) later suggested the formation of solid solutions (mixed crystals) rather than eutectic mixtures to obtain faster dissolution and absorption rates. Recently, Chiou (3) proposed, from the theoretical standpoint, that a poorly soluble or insoluble drug can achieve the fastest rates of dissolution and absorption when dispersed in a glass solution of a water-soluble carrier. Upon exposure to aqueous fluids, the active drug will be released in a state of fine particles (eutectic mixture) or single molecule (solid or glass solution). The enhancement of *in vitro* dissolution rates has been shown with solid dispersions containing chloramphenicol (4, 5), griseofulvin (Gris) (6, 7), and reserpine (8). However, the *in vivo* investigation of such systems has been limited. Only sulfathiazole (1), chloramphenicol (4), and reserpine (9) have been studied. One major and commonly used approach to enhance oral absorption is micronization. Yet, no study has been reported to compare the absorption of a drug in micronized form and in a solid dispersion form. Therefore, the clinical and practical value of the solid dispersion approach has not been fully established. The main objective of this communication is to compare quantitatively the absorption characteristics of a water-insoluble antibiotic, Gris, in dogs. The absorption properties of these systems in man will be reported in a future article.

In another communication (10), the authors reported that the dissolution rate of Gris was increased considerably when dispersed in the carriers of polyethylene glycol (PEG) 4000, 6000, and 20,000; citric acid; pentaerythritol; and pentaerythrityl tetraacetate. The results obtained after i.v. studies of Gris and its metabolite, 6-demethylgriseofulvin (6-DMG), in the same dogs have also been previously reported (11). The PEG 6000 was selected as a model carrier to test in dogs.

EXPERIMENTAL

Dosage Forms—Four different dosage forms were used for oral studies: 250 mg. of Gris dissolved in 50 ml. of PEG 400; 250 mg. of Gris dispersed in 2250 mg. of PEG 6000 (250 mg. 10% Gris–PEG 6000) by the melting method (10) and packed loosely into five gelatin capsules (size 00); a commercial tablet containing 500 mg. of micronized Gris; and a commercial capsule containing 250 mg. of micronized Gris.

Protocol in Dog Studies—Male, mongrel, conditioned, unanesthetized dogs weighing 19–22 kg. were used throughout the studies. Dogs were fasted with water *ad libitum* for 16 to 18 hr. prior to experiments. The Gris in the solution form was administered through a stomach tube followed by 30 ml. of 50% aqueous PEG 400 solution to rinse the syringe and the stomach tube. The solid dosage forms, mixed with a small amount of ground meat, were swallowed quickly by the dogs. One hundred fifty milliliters of lukewarm tap water was then given through the stomach tube. Dogs were observed for at least 2 hr. to make sure that there was no vomiting of the drug. Food was withdrawn for 8 hr. after administration, while water was freely available.

Blood samples (5 ml.) were drawn at 0, 1, 2, 3, 5, 8, and occasionally up to 12 hr. after drug administration from a cephalic vein with a 22-gauge disposable needle attached to a 6-ml. syringe. For the solution dosage form, additional samples were taken at 15 and 30 min. Urine samples were taken at 0, 2, 5, 8, 30, and 48 hr. through a urethral catheter. After the initial withdrawal of urine, at least 20 ml. of saline was used to wash the bladder. Both the urine and the washing were combined together for the assay. Occasionally, only blood or urine samples were taken.

Table I—Plasma Data (mcg./ml.) after Oral Administration of Various Dosage Forms of Gris to Dogs

Dosage Forms	Expts.	Time 15 min.	30 min.	1 hr.	2 hr.	3 hr.	5 hr.	8 hr.	Area, min. mcg./ml.
250 mg. *in solution*	F-5	1.00	0.92	2.12	1.23	0.55	0.17	0.00	280
	G-13	2.52	2.08	2.46	1.63	0.95	0.32	0.08	434
	H-3	1.56	3.43	3.25	2.20	1.44	0.71	0.21	647
	K-1	2.19	2.25	2.73	2.34	1.82	0.82	0.26	658
	Av.	1.82±	2.17±	2.64±	1.85±	1.19	0.51	0.14±	505±
	± *SEM*	0.34	0.51	0.24	0.26	0.28	0.15	0.06	91
250 mg. dispersed in PEG 6000 (capsule)	H-2			0.35	1.33	1.50	0.66	0.22	345
	H-5			0.94	1.44	1.28	0.58	0.19	367
	H-6			0.34	0.59	0.77	0.32	0.13	185
	G-2			1.20	1.71	2.87	2.16	0.80	830
	G-19			0.62	1.08	1.17	0.95	0.39	445
	K-2			0.12	0.40	0.65	1.27	0.61	335
	K-19			0.08	0.28	0.70	0.56	0.53	217
	Av.			0.55±	0.98±	1.24±	0.97±	0.41±	389±
	± *SEM*			0.18	0.21	0.34	0.27	0.09	81
500 mg. micronized (tablet)	H-1			0.10	1.60	1.97	1.01	0.40	466
	H-4			0.14	0.25	0.31	0.34	0.10	112
	H-14			0.19	0.38	0.59	0.31	0.12	145
	G-1			0.88	1.27	1.73	1.04	0.34	471
	G-4			0.33	0.50	0.53	0.18	0.04	129
	G-14			0.05	0.12	0.11	0.13	0.06	46
	G-15			0.41	0.48	0.53	0.20	0.05	137
	K-15			0.17	0.31	0.36	0.29	0.10	115
	Av.			0.28±	0.61±	0.77±	0.44±	0.15±	203±
	± *SEM*			0.10	0.19	0.24	0.13	0.05	57.4
250 mg. micronized (capsule)	D-2			0.20	0.24	0.21	0.12	0.06	69
	D-6			0.41	0.22	0.25	0.17	0.14	112
	F-12			0.10	0.32	0.36	0.07	0.00	68
	G-12			0.13	0.26	0.54	0.26	0.07	118
	H-12			0.11	0.34	0.23	0.10	0.03	67
	H-13			0.18	0.35	0.43	0.22	0.07	107
	H-15			0.19	0.30	0.37	0.17	0.06	95
	Av.			0.19±	0.29±	0.34±	0.16±	0.06±	91±
	± *SEM*			0.04	0.02	0.04	0.02	0.02	6.7

Samples of urine and plasma obtained after separation from red cells by centrifugation were stored at 4° until required for analysis. The spectrophotofluorometric assay of Gris in the plasma (12) and the UV assay of 6-DMG in the urine (13) were conducted as previously described. An interval of at least 1 week separated each experiment.

***In Vitro* Dissolution Rate Studies**—Dissolution rate studies of 125 mg. of Gris in three solid dosage forms were run in 18 l. of simulated intestinal fluid (14) at 36.7 ± 0.05° in an apparatus previously described (10). For dissolution studies, dispersed Gris was filled in two gelatin capsules (size 00), an equivalent amount of powder from the commercial capsule was refilled in a size 1 gelatin capsule, and a single mass was cut from the commercial tablet. Dissolution was studied using the USP disintegration apparatus (Scientific Glass Apparatus Co., Bloomfield, N. J.). However, a single cylindrical covered basket, diameter 2.8 cm. and height 5.6 cm., made up of an 8-mesh stainless steel screen, was used instead of the conventional basket-rack assembly, because Gris–PEG preparation was found to stick to the plastic wall in the standard basket.

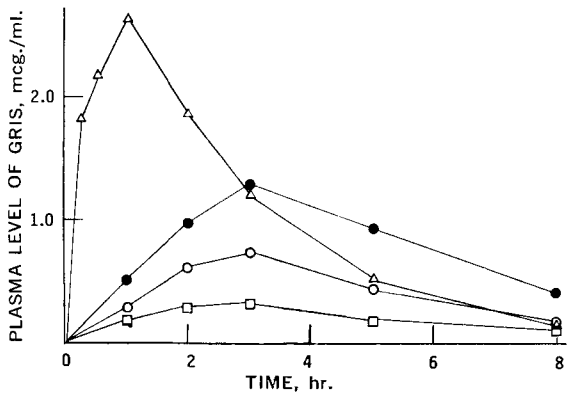


Figure 1—*Mean plasma levels of Gris after oral administration of various dosage forms to dogs. Key: △, 250 mg. in solution; ●, 250 mg. dispersed in PEG 6000; ○, commercial tablet of 500 mg. micronized Gris; and □, commercial capsule of 250 mg. micronized Gris.*

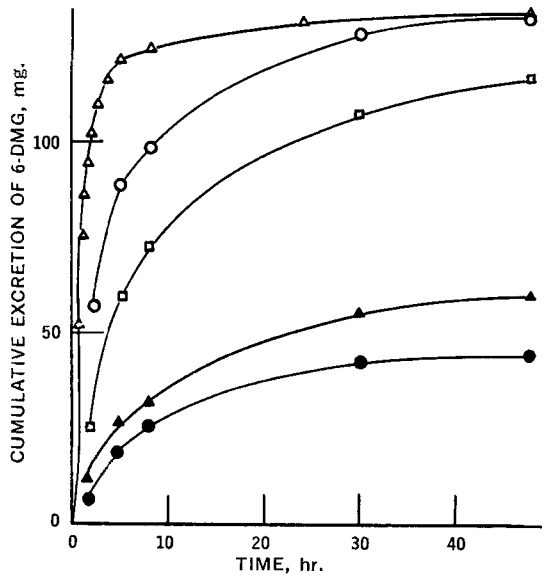


Figure 2—*Average cumulative excretion of 6-DMG after oral and i.v. doses of Gris. Key: △, i.v. Gris; ○, Gris in solution; □, Gris dispersed in PEG 6000; ▲, commercial capsule of micronized Gris; and ●, commercial tablet of micronized Gris. (All data corrected for 250-mg. dose.)*

RESULTS AND DISCUSSION

Plasma Levels of Gris after Oral Administration—The plasma concentration data after oral administrations of four different dosage forms of Gris are shown in Table I and their average values are plotted in Fig. 1.

Although Gris has long been used for antifungal therapy in dogs (15), its oral absorption has not thus far been reported. The recommended dose of 5 mg./lb./day in dogs is the same as that used in man. The results reported from this laboratory (11, 16) indicated that the metabolic rate of Gris in dogs may be 20–30 times faster than that in man. Therefore, it is not surprising that very low blood levels of Gris were obtained from 250 mg. of commercial capsule (Table I) administered to dogs weighing 45 lb. (approximately 5 mg./lb.) as compared with that obtained from man (12). The average peak level was found to be about 0.3 mcg./ml., and the concentration after 8 hr. of administration was almost negligible. Furthermore, even the blood level from the solution dosage form was very low after 8 hr. of administration. It has been reported that the minimal plasma concentration of Gris required for the effective therapy in man is about 1 mcg./ml. (17). Hence, it is highly doubtful that the presently recommended dose in dogs is adequate. Further study of this aspect should be pursued.

Peaks of plasma level of Gris from three solid formulations were all reached at about 3 hr. after administration as found in man (12), cats (18), and rats (19). The absorption from the solution form was much faster, as expected. The peak plasma levels after administration of the Gris–PEG solution were in the range of 15–30 min. in Dogs G and H.

The absorption from the commercially available micronized preparations appears to be quite incomplete. The average peak levels from these preparations are about 25 to 29% of that from Gris dispersed in PEG 6000 on the basis of equal amount of Gris administered.

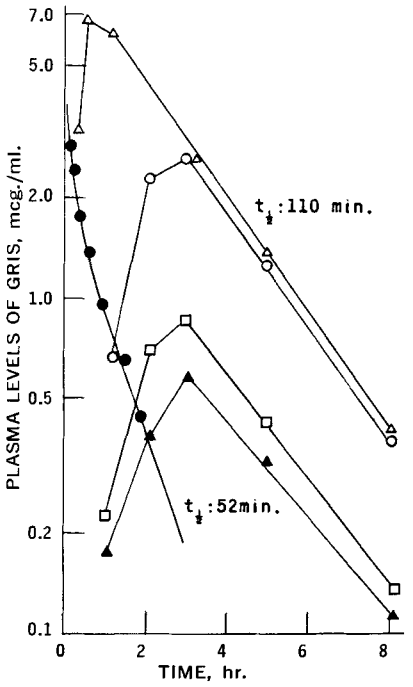


Figure 3—*Plasma concentration of Gris after administration of various dosage forms to Dog H (oral doses all corrected to 50 mg. Gris). Key: △, Gris in solution (H-3); ○, Gris dispersed in PEG 6000 (H-6); □, commercial capsule of micronized Gris (H-13); ▲, commercial tablet of micronized Gris (H-14); and ●, 50 mg. Gris i.v. (H-9).*

Table II—Cumulative 6-DMG Urinary Excretion Data (mg. of 6-DMG) after Oral Administration of Various Dosage Forms of Gris to Dogs

Dosage Forms	Expts.	Time 2 hr.	5 hr.	8 hr.	30 hr.	48 hr.
250 mg. in solution	F-5	54.0	82.2	90.7	114.3	128.3
	G-13	59.6	89.6	97.2	122.4	130.4
	H-3	55.0	91.6	103.0	—	132.8
	K-5	—	—	—	—	—
	Av. ± *SEM*	56.2 ± 1.72	87.8 ± 2.8	97 ± 3.5	118 ± 4.0	130.5 ± 1.3
250 mg. dispersed in PEG 6000 (capsule)	F-6	16.3	54.7	69.0	97.1	109.8
	F-9	28.8	63.5	81.7	103.1	113.3
	G-2	17.0	53.6	71.3	108.0	119.0
	G-19	19.7	49.6	65.4	108.6	123.8
	H-2	15.9	44.8	56.2	88.3	96.3
	H-6	27.4	71.7	81.4	117.2	124.2
	H-5	46.5	72.7	83.4	117.0	125.0
	Av. ± *SEM*	24.5 ± 4.2	58.7 ± 4.1	72.6 ± 3.8	105.6 ± 3.9	115.9 ± 3.9
500 mg. micronized (tablet)	H-1	11.8	64.2	80.8	103.3	106.4
	H-4	10.0	29.4	39.2	72.5	75.8
	H-14	9.0	30.9	40.2	63.5	67.1
	H-8	—	—	37.4	68.1	71.0
	G-1	14.3	49.9	66.1	—	—
	G-4	14.4	32.6	37.6	62.1	75.0
	G-8	—	—	46.3	89.5	98.2
	G-14	6.8	20.0	26.3	52.1	63.0
	G-15	17.8	46.9	61.3	119.8	136.8
	F-8	—	—	66.5	122.5	138.7
	F-14	—	—	—	—	85.6
	K-15	—	—	—	—	37.8
	Av. ± *SEM*	12.0 ± 1.4	39.1 ± 5.7	50.2 ± 5.5	83.7 ± 8.7	87 ± 9.3
250 mg. micronized (capsule)	F-2	5.2	20.6	24.3	72.2	75.8
	G-12	6.0	25.1	30.6	60.4	72.4
	H-12	10.3	21.1	24.6	36.3	37.0
	H-13	15.7	38.9	45.5	59.6	68.9
	H-15	7.8	20.8	25.8	40.0	43.5
	Av. ± *SEM*	9.0 ± 1.9	25.3 ± 3.5	30.2 ± 4.0	53.7 ± 6.7	59.5 ± 8.0

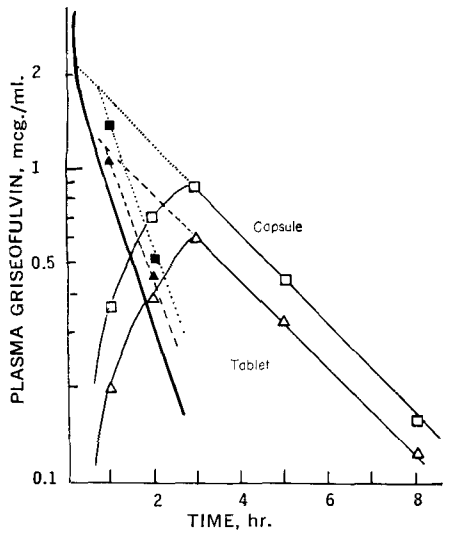


Figure 4—*Replot of the data shown in Fig. 3 on 500 mg. Gris administered to Dog H (see Fig. 3 for details). Extrapolation difference curves (solid circles and triangles) indicate the faster of the two rate processes.*

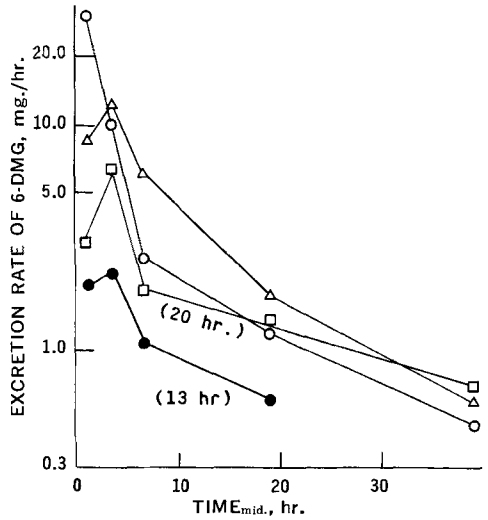


Figure 6—*6-DMG excretion rate plots after oral administration of various dosage forms to Dog G (all data corrected to 500-mg. dose). Key: ○, Gris in solution (G-13); △, Gris dispersed in PEG 6000 (G-2); □, commercial capsule of micronized Gris (G-12); and ●, commercial tablet of micronized Gris (G-14).*

The solution form of Gris might be presumed to be more completely absorbed and could be used as a standard for comparison of the dosage forms. By comparison with the area under the plasma concentration–time curve in 8 hr. from the solution form, the availability is 77% for Gris dispersed in PEG 6000, 26% for the commercial tablet, and 18% for the commercial capsule. There is, however, a fallacy in this method of comparison, since it was reported in a previous communication (11) that the elimination characteristics of Gris after i.v. administration in some dogs followed dose-dependent kinetics. Therefore, it seems invalid to compare the availability on the basis of the blood area. However, as reported in the previous communication (11), the total urinary excretion of 6-DMG, its major metabolite, was found to be almost constant for every dog studied and to be independent of doses in dogs exhibiting dose-dependent kinetics of Gris. Therefore, it was concluded that the total 6-DMG excretion in the urine can be used to evaluate the extent of drug absorption.

The absorption of micronized Gris preparation in man has been shown in this laboratory to continue for more than 30 or 40 hr.

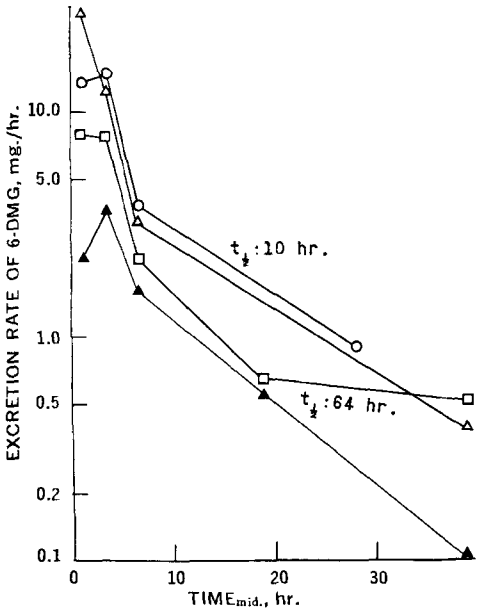


Figure 5—*6-DMG excretion rate plots after oral administration of various dosage forms to Dog H (see Fig. 3 for details). The half-life of 6-DMG excretion from i.v. dose to Dog H is 4.8 hr.*

(12). However, after the first 10 hr., the rate of absorption is much reduced. This may also take place in dogs; the drug concentration might fall below the level of spectrophotofluorometric assay sensitivity after 8 or 12 hr. postadministration, even though the drug is continuing to be absorbed. The authors, therefore, may not be able to follow the drug long enough in the blood to detect the total absorption process. This will be discussed further.

Urinary Excretion of 6-DMG after Oral Administration—Two urinary metabolites, 6-DMG and 6-DMG glucuronide, were previously identified in this laboratory after an i.v. dose of Gris to dogs (16). The excretion of 6-DMG glucuronide was found to contribute only insignificantly to the metabolic process either after i.v. or oral administration of Gris (11).

The excretion of 6-DMG after administration of four different dosage forms is shown in Table II. The average cumulative data for 6-DMG from i.v. (11) and oral doses are shown in Fig. 2. The percent of the total excretion of 6-DMG in 48 hr. from four dogs was surprisingly constant with an average of 55.1%, which is virtually identical to that obtained from i.v. dose, 56.4% (11), and indicates complete absorption from Gris in solution form. Using the amount of metabolite excretion in 48 hr. after the i.v. doses, the metabolite recovery is 88% from the Gris dispersed in PEG 6000, 45% from the commercial capsule, and 33% from the commercial tablet. Therefore, it is evident that the insoluble Gris dispersed in a water-soluble matrix of PEG 6000 does indeed result in a faster and more complete absorption than micronized products. Of additional importance is the fact that the absorption appears to be more consistent from the PEG dispersion.

There appear to be many potential physical and chemical advantages of a solid dispersion prepared with water-soluble carriers. The carriers will obviously be chosen on their ability to dissolve the required amounts of the drug in the solid dispersion. As such, it may show an increased tendency to produce supersaturated solution, fast dissolution rate, and good wetting and dispersion on exposure to the aqueous fluids. Although the micronization of insoluble drugs can increase the specific surface area and thereby increase the dissolution rate, this potential advantage may be easily lost due to the aggregation or agglomeration during the formulation or processing, which may not be overcome by adequate wetting in the biological fluids during the absorption process. It appears that the physicochemical advantages intrinsic in the solid dispersion form markedly influence the biological availability of the Gris in dogs. It is hoped that the approach of solid dispersion can also be applied to other insoluble drugs.

Long Absorption Time of Gris in Dogs—An interesting absorption phenomenon was frequently observed in dogs. Typical plasma concentration curves of Gris after administration of different dosage forms to a dog are shown as semilog plots in Fig. 3. One

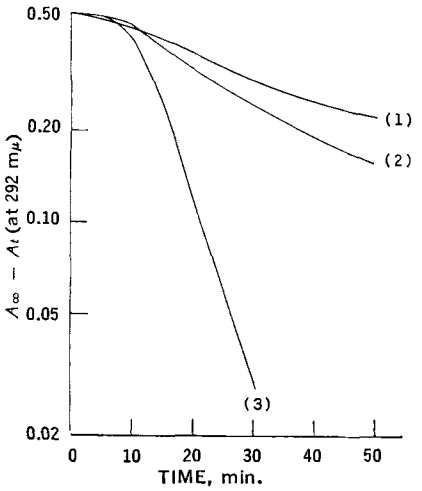


Figure 7—*Gris dissolution rate data (amount remaining to be dissolved) from 125 mg. in 18 l. of simulated intestinal fluid at 36.7°. Key:* (1), *commercial capsule;* (2), *commercial tablet; and* (3), *capsule of Gris dispersed in PEG 6000.*

certainly would wonder why four different preparations with different dissolution rates will have the same postpeak disappearance curve with a half-life of about 2 hr. (even up to 12 hr. postadministration). This would usually be defined as the elimination half-life of the drug.

The elimination half-life ($t_{1/2}$ = 52 min.) in this dog after i.v. dose was found to be dose-independent (Fig. 1 in *Reference 11*). Therefore, it indicates that the postpeak curve is primarily due to the slower process, the absorption process, which continued for at least 8 hr. Further evidence for this contention is seen in Fig. 4 in which some of the data from the previous figure have been redrawn. The linear portions of the postpeak curves for the capsule and tablet experiments have been extrapolated back to zero time, and the method of extrapolation difference has been used to estimate the rate constant for the faster of the two competing processes which primarily contribute to the rising portion of the blood curves. The extrapolation difference values led to the dashed lines representing the contribution from this first-order rate process. These lines show essentially the same slope as found after i.v. administration of Gris (shown as the heavy solid line). This interpretation would require the definition of the postpeak curve as indicative of the apparent absorption rate of the drug with a half-life of approximately 2 hr. Figure 5 includes plots of the 6-DMG excretion rate for the same experiments. The slopes of these curves during the same time interval are compatible with the previous interpretation of the blood data. This unusual pattern of absorption is similar to that of the oral administration of spironolactone (a drug of similar insolubility to Gris) to man (21) and was left unexplained by Levy (22).

The prolonged slow absorption of Gris can be shown by review of the urinary excretion rate data for the metabolite of Gris which are found in Table II and Figs. 5 and 6. If the absorption process stops at 8 hr., the excretion rate should be the same as that of the slow phase of excretion after i.v. dose of Gris ($t_{1/2}$ = 3–4.8 hr. in the two dogs shown). However, it is clear from Figs. 5 and 6 that the apparent excretion rate is much slower. Indeed, the half-life of the processes contributing to the postpeak curves 8 or more hr. after administration of the drug ranges from 10 to 20 hr. The authors interpret this to mean that the absorption process is the rate-limiting step and continues for more than 30 hr. This finding is in agreement with the report of Gris absorption in man from this laboratory (12).

The urinary excretion method described in this communication can serve as a convenient means of screening dosage form effects on a drug which is dissolution rate limited on the absorption. Thus, even though the blood data are of limited value due to slow absorption followed by rapid metabolism, the urinary excretion of the metabolite continues long after the drug is undetectable in the blood.

Correlation between the Absorption and the Dissolution Rate—Dissolution rates of Gris in three solid dosage forms in the 18 l. of simulated intestinal fluid are shown in Fig. 7. An interesting correlation appears to exist between the *in vitro* dissolution rate data and the *in vivo* data discussed; namely, the cumulative urinary excretion of 6-DMG in 48 hr. was found to correlate linearly with the logarithm of percent of Gris dissolved in 25 min. in the simulated intestinal fluid. This is shown in Fig. 8. It should be noted that similar correlation between the area under the blood concentration–time curve with the specific surface area of Gris powder (23) and the dissolution rates (24, 25) of Gris dosage forms in the simulated intestinal fluid have been reported in the past. The amount of data available to include in this plot is very minimal; it merely serves once again to point out *in vivo–in vitro* correlations can be established.

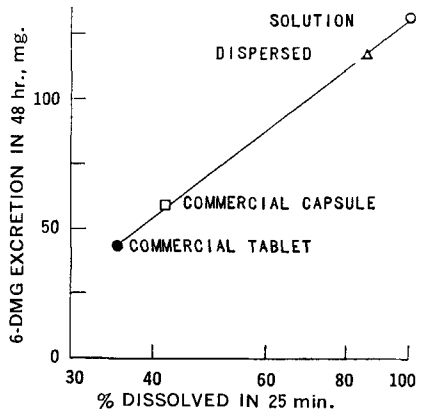


Figure 8—*Correlation between dissolution rate and total 6-DMG excretion in 48 hours.*

REFERENCES

(1) K. Sekiguchi and N. Obi, *Chem. Pharm. Bull.*, **9**, 866(1961).
(2) A. H. Goldberg, M. Gibaldi, and J. L. Kanig, *J. Pharm. Sci.*, **54**, 1145(1965).
(3) W. L. Chiou, Ph.D. dissertation, University of California, 1969.
(4) K. Sekiguchi, N. Obi, and Y. Ueda, *Chem. Pharm. Bull.*, **12**, 134(1964).
(5) A. H. Goldberg, M. Gibaldi, J. L. Kanig, and M. Mayersohn, *J. Pharm. Sci.*, **55**, 581(1966).
(6) A. H. Goldberg, M. Gibaldi, and J. L. Kanig, *ibid.*, **55**, 487(1966).
(7) M. Mayersohn and M. Gibaldi, *ibid.*, **55**, 1324(1966).
(8) M. Gibaldi, S. Feldman, and T. R. Bates, *ibid.*, **57**, 708 (1968).
(9) M. Malone, H. I. Hochman, and K. A. Nieforth, *ibid.*, **55**, 972(1966).
(10) W. L. Chiou and S. Riegelman, to be published.
(11) *Ibid.*, to be published.
(12) M. Rowland, S. Riegelman, and W. L. Epstein, *J. Pharm. Sci.*, **57**, 984(1968).
(13) M. Rowland and S. Riegelman, to be published.
(14) B. Katchen and S. Symchowicz, *J. Pharm. Sci.*, **56**, 1108 (1967).
(15) G. Muller, *Vet. Med.*, **59**, 846(1959).
(16) P. A. Harris and S. Riegelman, *J. Pharm. Sci.*, **58**, 93(1969).
(17) E. J. Grin and M. Denic, *Acta Med. Iugoslav.*, **19**, 53(1965).
(18) C. Bedford, D. Busfield, K. J. Child, I. MacGregor, P. Sutherland, and E. G. Tomich, *Arch. Dermatol.*, **81**, 735(1960).
(19) M. Kramal, J. Dubuc, and D. Beall, *Can. J. Biochem. Physiol.*, **40**, 1449(1962).
(20) P. A. Harris, Ph.D. dissertation, University of California, 1968.
(21) G. Bauer, P. Rieckman, and W. Schaumann, *Arzneim.-Forsch.*, **12**, 487(1962).
(22) G. Levy, *Amer. J. Pharm.*, **135**, 78(1963).
(23) R. M. Atkinson, C. Bedford, K. J. Child, and E. G. Tomich, *Antibiotic Chemother.*, **12**, 232(1962).
(24) B. Katchen and S. Symchowicz, *J. Pharm. Sci.*, **56**, 1108 (1967).
(25) S. Symchowicz and B. Katchen, *ibid.*, **57**, 1383(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *University of California School of Pharmacy, San Francisco Medical Center, San Francisco, CA 94112*

Accepted for publication February 3, 1970.

Abstracted from the dissertation submitted by W. L. Chiou to the Graduate Division, University of California, in partial fulfillment of the Ph.D. degree requirements.

Supported in part by a research grant from the Research Committee, San Francisco Academic Senate, University of California.

The information disclosed in this paper is proprietary to the Regents of University of California.

* Present address: Washington State University, College of Pharmacy, Pullman, WA 99163

Synthesis of Some Glycidic Hydrazides and Amides as Potential Psychotropic Agents and Anticholinergic Agents

REYNALDO V. SAENZ*, ROBERT G. BROWN, E. I. ISAACSON†, and JAIME N. DELGADO

Abstract □ A series of glycidic hydrazides and amides was prepared by hydrazinolysis or aminolysis of glycidic esters obtained *via* a modified Darzens condensation. The hydrazides thus obtained were subjected to suitable acylating or alkylating reagents to obtain *N*-substituted hydrazides. The results of a preliminary pharmacological evaluation are summarized. The synthesized compounds were tested for their ability to reverse reserpine hypothermia in mice. Compounds synthesized as potential anticholinergics were evaluated for their spasmolytic activity using isolated rabbit ileum.

Keyphrases □ Glycidic hydrazides, amides—synthesis □ Hydrazides, glycidic—synthesis □ Amides, glycidic—synthesis □ IR spectrophotometry—identity □ Pharmacological screening—glycidic hydrazides, amides

Appropriately substituted hydrazides and amides have been of interest to the medicinal chemist for various reasons. Monoamine oxidase-inhibitory hydrazides have proved to possess dynamic pharmacological properties (*i.e.*, antitubercular and/or antidepressant properties). Amides that are analogous to bioactive esters (*e.g.*, procaine *versus* procainamide) offer models for structure–activity analysis on the basis of the predicted greater metabolic stability of amides due to their greater resonance stabilization.

Since the epoxide group affects the physicochemical properties (*e.g.*, lipid–water partition coefficient) of a compound, this function might also influence pharmacologic properties. Hence, it was decided to prepare α,β-epoxy hydrazides and amides for pharmacologic evaluation and to provide a basis for the study of the effect of the epoxide moiety on bioactivity.

Literature reports and reviews (1–4) substantiate the utility and applications of the Darzens glycidic ester condensation. Consequently, this reaction was applied to the synthesis of α,β-epoxy esters which, when exposed to hydrazinolysis and/or aminolysis, yield potentially active hydrazides and amides.

α,β-Epoxy amides and hydrazides have been of interest to the authors and others (5) as potential therapeutic agents. In appropriately substituted glycidic hydrazides, the presence of the epoxide function may affect the distribution of compounds possessing monoamine oxidase-inhibiting pharmacophores. In addition, the epoxide function may affect the susceptibility of such compounds to metabolic degradation. Conceivably, the drug–receptor interaction would be influenced as well, depending upon the degree of hydrolysis of the hydrazide linkage. Zeller (6) and others (7) have postulated that certain substituents play an important function in the bioactivity of appropriately substituted hydrazine derivatives. Several basic structural features appear to be associated with optimal monoamine oxidase inhibitory activity. These can be summarized as follows: (*a*) there should be at least one alkyl substituent on the hydrazine moiety, and (*b*) *N*-1 alkylation and *N*-2 acylation yield compounds with increased activity and decreased toxicity. On the other hand, disubstitution of either hydrazine nitrogen leads to inactive compounds. The alkyl and aralkyl substituents contribute to electronic, steric, and hydrophobic factors involved in the inhibitor–enzyme interaction, whereas the acyl moiety has been implicated as affecting the distribution and should be of such a nature that it can be easily hydrolyzed (8). Accordingly, the choice of the acyl moiety presumably is important with regard to tissue selectivity and susceptibility to metabolic cleavage to yield the postulated active moiety.

Since this work involves the synthesis of selected α,β-epoxy hydrazides as potential psychotropic agents, it is fundamental to the study of the effect of the epoxide function on absorption, distribution, and metabolic fate of hydrazine derivatives.

Additionally, glycidic amides were prepared modeled after classical anticholinergic agents. Such compounds possessing the α,β-epoxy amide function, in addition to the potential ammonium group and appropriately positioned bulky, semirigid, and hydrophobic moieties, should exhibit anticholinergic spasmolytic activity; those without a potential ammonium function should be less active. Accordingly, it was decided to compare Compound X with the classic anticholinergic atropine as well as with the analogous amides: Compounds VII and VIII, which do not possess potential ammonium functions. The epoxide cycle should affect distribution as well as the receptor interaction; hence, α,β-epoxy amides were chosen for this study.

The compounds described herein were prepared by the application of the following reactions: Darzens

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *University of California School of Pharmacy, San Francisco Medical Center, San Francisco, CA 94112*

Accepted for publication February 3, 1970.

Abstracted from the dissertation submitted by W. L. Chiou to the Graduate Division, University of California, in partial fulfillment of the Ph.D. degree requirements.

Supported in part by a research grant from the Research Committee, San Francisco Academic Senate, University of California.

The information disclosed in this paper is proprietary to the Regents of University of California.

* Present address: Washington State University, College of Pharmacy, Pullman, WA 99163

Synthesis of Some Glycidic Hydrazides and Amides as Potential Psychotropic Agents and Anticholinergic Agents

REYNALDO V. SAENZ*, ROBERT G. BROWN, E. I. ISAACSON†, and JAIME N. DELGADO

Abstract □ A series of glycidic hydrazides and amides was prepared by hydrazinolysis or aminolysis of glycidic esters obtained *via* a modified Darzens condensation. The hydrazides thus obtained were subjected to suitable acylating or alkylating reagents to obtain *N*-substituted hydrazides. The results of a preliminary pharmacological evaluation are summarized. The synthesized compounds were tested for their ability to reverse reserpine hypothermia in mice. Compounds synthesized as potential anticholinergics were evaluated for their spasmolytic activity using isolated rabbit ileum.

Keyphrases □ Glycidic hydrazides, amides—synthesis □ Hydrazides, glycidic—synthesis □ Amides, glycidic—synthesis □ IR spectrophotometry—identity □ Pharmacological screening—glycidic hydrazides, amides

Appropriately substituted hydrazides and amides have been of interest to the medicinal chemist for various reasons. Monoamine oxidase-inhibitory hydrazides have proved to possess dynamic pharmacological properties (*i.e.*, antitubercular and/or antidepressant properties). Amides that are analogous to bioactive esters (*e.g.*, procaine *versus* procainamide) offer models for structure–activity analysis on the basis of the predicted greater metabolic stability of amides due to their greater resonance stabilization.

Since the epoxide group affects the physicochemical properties (*e.g.*, lipid–water partition coefficient) of a compound, this function might also influence pharmacologic properties. Hence, it was decided to prepare α,β-epoxy hydrazides and amides for pharmacologic evaluation and to provide a basis for the study of the effect of the epoxide moiety on bioactivity.

Literature reports and reviews (1–4) substantiate the utility and applications of the Darzens glycidic ester condensation. Consequently, this reaction was applied to the synthesis of α,β-epoxy esters which, when exposed to hydrazinolysis and/or aminolysis, yield potentially active hydrazides and amides.

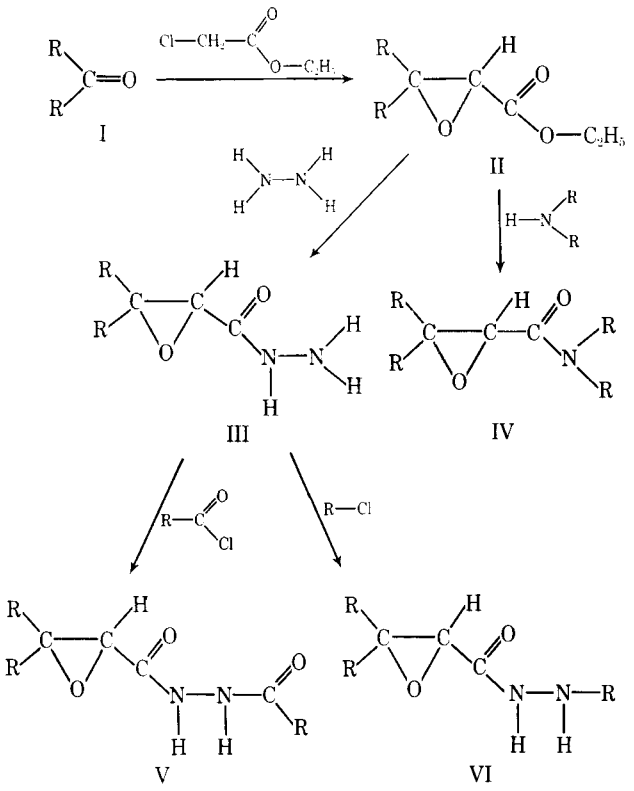
α,β-Epoxy amides and hydrazides have been of interest to the authors and others (5) as potential therapeutic agents. In appropriately substituted glycidic hydrazides, the presence of the epoxide function may affect the distribution of compounds possessing monoamine oxidase-inhibiting pharmacophores. In addition, the epoxide function may affect the susceptibility of such compounds to metabolic degradation. Conceivably, the drug–receptor interaction would be influenced as well, depending upon the degree of hydrolysis of the hydrazide linkage. Zeller (6) and others (7) have postulated that certain substituents play an important function in the bioactivity of appropriately substituted hydrazine derivatives. Several basic structural features appear to be associated with optimal monoamine oxidase inhibitory activity. These can be summarized as follows: (*a*) there should be at least one alkyl substituent on the hydrazine moiety, and (*b*) *N*-1 alkylation and *N*-2 acylation yield compounds with increased activity and decreased toxicity. On the other hand, disubstitution of either hydrazine nitrogen leads to inactive compounds. The alkyl and aralkyl substituents contribute to electronic, steric, and hydrophobic factors involved in the inhibitor–enzyme interaction, whereas the acyl moiety has been implicated as affecting the distribution and should be of such a nature that it can be easily hydrolyzed (8). Accordingly, the choice of the acyl moiety presumably is important with regard to tissue selectivity and susceptibility to metabolic cleavage to yield the postulated active moiety.

Since this work involves the synthesis of selected α,β-epoxy hydrazides as potential psychotropic agents, it is fundamental to the study of the effect of the epoxide function on absorption, distribution, and metabolic fate of hydrazine derivatives.

Additionally, glycidic amides were prepared modeled after classical anticholinergic agents. Such compounds possessing the α,β-epoxy amide function, in addition to the potential ammonium group and appropriately positioned bulky, semirigid, and hydrophobic moieties, should exhibit anticholinergic spasmolytic activity; those without a potential ammonium function should be less active. Accordingly, it was decided to compare Compound X with the classic anticholinergic atropine as well as with the analogous amides: Compounds VII and VIII, which do not possess potential ammonium functions. The epoxide cycle should affect distribution as well as the receptor interaction; hence, α,β-epoxy amides were chosen for this study.

The compounds described herein were prepared by the application of the following reactions: Darzens

condensation, hydrazinolysis, aminolysis, *N*-acylation, and *N*-benzylation.



Scheme I

The carbonyl compounds (I) (Scheme I) employed as starting materials included pyridine-3-carboxaldehyde, pyridine-4-carboxaldehyde, diphenylacetaldehyde, benzophenone, dibenzosuberone, and xanthone. The Darzens glycidic ester product (II) of each of the foregoing carbonyl compounds was subjected to aminolysis (IV) and/or hydrazinolysis (III). The hydrazides (III) obtained then were treated with either benzyl chloride, benzoyl chloride, or 3,4,5-trimethoxybenzoyl chloride to prepare the substituted hydrazide derivatives (V and VI). The compounds prepared, together with their respective melting points and analytical data, are listed in Table I.

EXPERIMENTAL[1]

Preparation of Esters—The glycidic esters, starting materials for the synthesis of the glycidic hydrazides and amides, were prepared according to the method of Omodt and Gisvold (3) with suitable modifications. The yield was better from pyridine-3-carboxaldehyde (60–70%) than from pyridine-4-carboxaldehyde (40–50%). Additionally, among the ketones, dibenzosuberone gave higher yields (40–50%) of the glycidic ester than did xanthone (20–30%). IR spectrophotometry was utilized in the characterization of the esters described. The IR spectra showed characteristic peaks for the α,β-epoxy ester function: 1720 cm.$^{-1}$ ($\rangle$C=O); 1260 and 880 cm.$^{-1}$ (epoxide).

Ethyl 3-(4-Pyridyl)glycidate—Pyridine-4-carboxaldehyde (10.7 g.; 0.1 mole) and ethyl chloroacetate (12.6 g.; 0.1 mole) dissolved in anhydrous ether (150 ml.) were placed in a 3-neck, round-bottom flask equipped with mechanical stirrer, reflux condenser, calcium chloride drying tube, nitrogen inlet, and dropping funnel. Sodium hydride (4.8 g.; 0.1 mole of a 51% mineral oil dispersion) was added slowly to prevent excessive effervescence. Ethanol (4.8 g.; 0.1 mole) was added dropwise while cooling the solution to 0–10°. The mixture was stirred for 18 hr. while it was allowed to come to room temperature. All the while, nitrogen was passed through the reaction mixture. At this time the ether was removed under reduced pressure and replaced with an equal volume of chloroform. The mixture was then refluxed for 4 hr. Water (50 ml.) was added. The resulting two layers were separated. The chloroform layer was washed with a saturated solution of sodium chloride (three 50-ml. portions), a saturated solution of sodium bicarbonate (three 50-ml. portions), and a saturated solution of sodium bisulfite (three 50-ml. portions), and it was dried over anhydrous sodium sulfate. After filtering, the solvent was removed using a Rinco rotatory evaporator. The residue which resulted was characterized as the picrate salt, m.p. 101–102°.

Anal.—Calcd. for $C_{16}H_{14}N_4O_{10}$: C, 45.50; H, 3.31. Found: C, 45.54; H, 3.42.

Ethyl 3-(3-Pyridyl)glycidate—Pyridine-3-carboxaldehyde (10.7 g.; 0.1 mole) and ethyl chloroacetate (15.9 g.; 0.13 mole) in ethanol (4.6 g.; 0.1 mole) were added *via* two dropping funnels to a toluene dispersion of sodium hydride (4.8 g.; 0.1 mole) under nitrogen and cooled to 0–10°. The reaction mixture was allowed to come to room temperature and stirred for a period of 12 hr. Glacial acetic acid (3 ml.) and water (150 ml.) were added. The aqueous layer was separated and washed with ether, and the ether extracts were combined with the organic layer. The combined organic portions were washed as before, and the solvents were removed under reduced pressure. The crude epoxy ester was chromatographed on a silicic acid column developed with ether. The oil which was isolated was identified on the basis of its elemental analysis.

Anal.—Calcd. for $C_{10}H_{11}NO_3$: C, 62.15; H, 5.73. Found: C, 61.98; H, 5.95.

Ethyl 3,3-Diphenylglycidate—Benzophenone (18.2 g.; 0.1 mole) was dissolved in anhydrous ether (100 ml.) and placed in a 3-neck flask equipped as previously described. Ethyl chloroacetate (12.6 g.; 0.1 mole), dissolved in anhydrous ether (50 ml.), was added. While cooling the solution to 0–10°, freshly prepared sodium ethoxide (6.8 g.; 0.1 mole) was dissolved in anhydrous ether (150 ml.) and slowly added to prevent excessive effervescence. Stirring was continued for 18 hr. The ether was removed and replaced with an equivalent amount of anhydrous chloroform, and the mixture was refluxed for 18 hr. Glacial acetic acid (3 ml.) and water (150 ml.) were added cautiously. The organic layer was removed and washed as before and was concentrated under reduced pressure. The concentrate was distilled under reduced pressure; the product, distilling at 120–128° at 5 mm., solidified at room temperature and had a melting point of 46°.

Anal.—Calcd. for $C_{17}H_{16}O_3$: C, 76.10; H, 6.01. Found: C, 75.76; H, 5.92.

Ethyl 3-Xanthylglycidate—Ethyl 3-xanthylglycidate was synthesized in a manner analogous to that used for ethyl 3,3-diphenylglycidate, using xanthone and ethyl chloroacetate as the starting materials. On concentration of the organic layer, the residual material was characterized in the form of Derivatives IX and X, Table I.

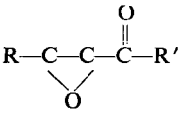
Ethyl 3-Dibenzosuberylglycidate—Ethyl 3-dibenzosuberylglycidate also was prepared according to the foregoing method, using ethyl chloroacetate and dibenzosuberone. Chromatography (silicic acid column) was used to purify the product.

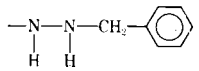
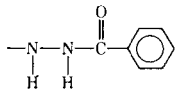
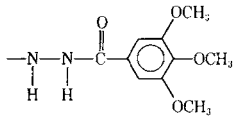
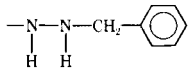
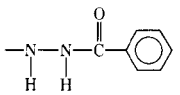
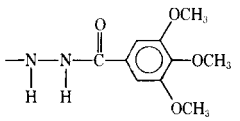
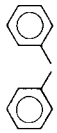
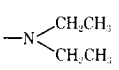
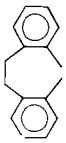
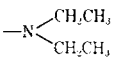
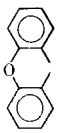
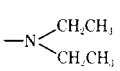
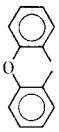
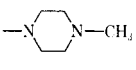
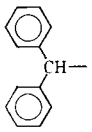
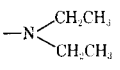
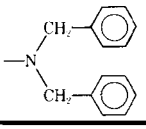
Anal.—Calcd. for $C_{19}H_{18}O_3$: C, 77.58; H, 6.16. Found: C, 77.86; H, 5.94.

Ethyl 3,3-Diphenylmethylglycidate—Ethyl 3,3-diphenylmethylglycidate was synthesized as previously described, employing diphenylacetaldehyde and ethyl chloroacetate. The product was purified by column chromatography on silicic acid and was characterized in the form of the corresponding *N,N*-diethylamide derivative (Compound XI, Table I).

[1] Reported melting points are uncorrected. A Thomas-Hoover Unimelt apparatus was used for the melting point determinations. Elemental analyses were conducted by the Schwarzkopf Micro-analytical Laboratory, Woodside, N. Y. IR spectral analyses were conducted with a Beckman IR-8 spectrophotometer.

Table I—Elemental Analysis for the Synthesized Compounds



Compd. No.	R	R′	Method of Purification	M.p.	Anal., % C Calcd.	C Found	H Calcd.	H Found
I			Alcohol–ether	Semisolid	66.90	66.77	5.57	5.38
II			Ethanol–water	195–196°	63.63	63.87	4.61	4.48
III			Ethanol	160–162°	57.90	57.43	5.10	4.93
IV			Isopropyl ether–ethanol	178–179°	66.90	67.21	5.61	6.02
V			Water–ethanol	187–189°	63.59	63.21	4.63	4.89
VI			Anhydrous ether	160–161°	57.91	58.24	5.09	4.87
VII			Ether	Oil	77.26	77.82	7.16	6.99
VIII			Chromatographed on silica	Oil	78.47	78.80	7.21	6.97
IX			Chloroform–ethanol	196–198°	73.76	74.00	6.19	6.38
X			Chromatographed on silica	Oil	71.41	70.98	5.99	6.18
XI			Chromatographed on silica	Semisolid	77.63	77.82	7.49	6.99
XII			Chromatographed on silica	Oil	76.72	77.05	5.85	5.43

Synthesis of α,β-Epoxy Hydrazides—The hydrazides were prepared by hydrazinolysis of the esters, and the yields were in the range of 25–30% of theory. IR spectral analyses were conducted on representative compounds, and the data substantiated the structures in Table I. The IR spectra showed characteristic peaks: 1600 cm.$^{-1}$ ($\rangle$C=O); 1260 and 880 cm.$^{-1}$ (epoxide).

1-[3-(4-Pyridyl)glycidyl]hydrazine—Ethyl 4-pyridylglycidate (4.8 g.; 0.025 mole) was dissolved in anhydrous benzene (80 ml.) and ethanol (15 ml.) and placed in a flask equipped with a Dean-

Stark separator. Hydrazine (anhydrous 95%) (0.8 g.; 0.025 mole) was added dropwise while stirring. After all of the hydrazine was added, the mixture was refluxed for 4 hr. At this time, most of the alcohol had been collected in the Dean-Stark separator, denoting approximate completion of the hydrazinolysis. The solvent was removed under reduced pressure, and the semisolid remaining was cooled to induce crystallization. The product was dried *in vacuo* and characterized.

Anal.—Calcd. for $C_8H_9N_3O_2$: C, 53.63; H, 5.06. Found: C, 53.97; H, 4.97.

1-[3-(3-Pyridyl)glycidyl]hydrazine—A procedure analogous to that employed for the 4-pyridyl isomer was used to synthesize and characterize the compound.

Anal.—Calcd. for $C_8H_9N_3O_2$: C, 53.62; H, 5.06. Found: C, 53.92; H, 5.54.

1-[3-(4-Pyridyl)glycidyl]-2-benzyl Hydrazine (Compound IV, Table I)—1-[3-(4-Pyridyl)glycidyl]hydrazine (2.2 g.; 0.0125 mole) was dissolved in anhydrous ether (40 ml.) and, while stirring, benzyl chloride (1.42 g.; 0.0125 mole) was slowly added. Additional ether (20 ml.) and 5% aqueous sodium hydroxide solution (10 ml.) were added to the mixture. The ether layer was separated, dried, and concentrated under reduced pressure. The resulting product was purified *via* crystallization from ethanol–isopropyl ether.

1-[3-(3-Pyridyl)glycidyl]-2-benzyl Hydrazine (Compound I, Table I)—The corresponding 3-pyridyl analog was prepared similarly to the 4-pyridyl isomer.

N-1-[3-(4-Pyridyl)glycidyl]-N-2-(3,4,5-trimethoxybenzoyl)hydrazine (Compound VI, Table I)—3-(4-Pyridyl)glycidyl hydrazine (4.4 g.; 0.025 mole) was dissolved in anhydrous benzene (60 ml.) with the aid of ethanol (10 ml.). Pyridine (2 ml.) was added. 3,4,5-Trimethoxybenzoyl chloride (3.7 g.; 0.025 mole) was added, and the mixture was refluxed for 6 hr. At this time, water (15 ml.) was added to dissolve the salt formed. The nonaqueous layer was separated, washed with a saturated solution of sodium bicarbonate, dried, and concentrated under reduced pressure. The resulting product was recrystallized from anhydrous ether.

N-1-[3-(3-Pyridyl)glycidyl]-N-2-(3,4,5-trimethoxybenzoyl)hydrazine (Compound III, Table I)—This compound was prepared in a manner similar to that used for the 4-pyridyl isomer.

1-[3-(4-Pyridyl)glycidyl]-2-benzoyl Hydrazine (Compound V, Table I)—3-(4-Pyridyl)glycidyl hydrazine (5 g.; 0.028 mole) was dissolved in anhydrous pyridine (30 ml.) and anhydrous benzene (60 ml.). The benzoyl chloride (5 g.; 0.036 mole) was added as quickly as possible. The resulting mixture was refluxed for 6 hr. and then poured into 250 ml. of water. The aqueous layer was removed and washed with benzene. The combined organic solutions were washed with 5% aqueous sodium bicarbonate solution and dried. After filtering, the solution was concentrated under reduced pressure. The product was isolated by crystallization from a water–ethanol mixture and characterized.

1-[3-(3-Pyridyl)glycidyl]-2-benzoyl Hydrazine (Compound II, Table I)—This compound was prepared according to the procedure described for the 4-pyridyl analog.

Synthesis of α,β-Epoxy Amides—*α,β-Epoxy* N,N-*Diethyl Amides*—The compounds, together with their respective melting points and/or analytical data, are listed in Table I. Compounds VII–XII were prepared by treating the respective glycidic esters with diethylamine in a procedure according to that given for *N,N*-diethyl-3,3-diphenylmethyl glycidamide. The yields obtained ranged between 30–35%. IR spectral analyses were conducted on representative compounds, and the data substantiated the structures in Table I. The IR spectra showed characteristic peaks: 1650 cm.$^{-1}$ ($\rangle$C=O); 1260 and 880 cm.$^{-1}$ (epoxide).

N,N-Diethyl 3,3-Diphenylmethylglycidamide (Compound XI, Table I)—Ethyl 3,3-diphenylmethylglycidate (7.0 g.; 0.025 mole) was dissolved in anhydrous benzene (75 ml.) with the aid of anhydrous ethanol (15 ml.). Diethylamine (2.0 g.; 0.027 mole) was slowly added. The mixture was refluxed for 6 hr. At this time, it was cooled and a methyl halide was added to remove unreacted amine. The filtered solution was concentrated under reduced pressure. The oil which resulted was chromatographed on silica.

N-3-Xanthylglycidyl-N-methylpiperazine (Compound X, Table I)—Ethyl 3-xanthylglycidate (3.5 g.; 0.0126 mole) was dissolved in anhydrous benzene (80 ml.) with the aid of anhydrous ethanol (10 ml.) and heat. *N*-Methylpiperazine (1.3 g.; 0.0125 mole) was added, and the mixture was refluxed for 4 hr. The *N*-methylpiperazyl derivative was separated from the reaction mixture after concentration under reduced pressure by vacuum distillation The methyl bromide derivative was prepared by conventional methods.

PHARMACOLOGIC EVALUATION

This pharmacologic evaluation was undertaken to obtain a preliminary indication of some structure–activity relationships among the compounds synthesized. Considering that some of these compounds were synthesized as potential monoamine oxidase inhibitors and others as anticholinergic spasmolytics, it was decided to test for these bioactivities according to the following methods. Compounds I through XII were tested for their ability to reverse reserpine hypothermia in mice. Compounds VII, VIII, and X were evaluated for their spasmolytic activity.

Reversal of Reserpine Hypothermia—*Method*—The administration of reserpine to normal animals usually results in a gradual depression of the central and peripheral sympathetic systems and is characterized by conditions such as sedation, ptosis, hypotension, bradycardia, and hypothermia (9).

Pretreatment with a monoamine oxidase inhibitor results in a reversal of the reserpine effects. Symptoms of excitation, exophthalamus, piloerection, mydriasis, and hyperthermia are observed (10).

The method of Garrattini *et al.* (11) was adopted for the estimation of the effect of the test compounds on reserpine-induced hypothermia.

Although only the hydrazides were prepared as potential monoamine oxidase inhibitors, it was decided also to evaluate the amides of the series.

Young male Swiss-Webster mice weighing between 20 and 35 g. were used throughout. The animals were given free access to food and water before and during the evaluation. The testing was scheduled so that no mouse was used more often than once a week.

Prior to the administration of any drug, the rectal temperature of each mouse was determined using a telethermometer with rectal probe. The rectal temperature then was measured at 4, 6, and 24 hr. after the intraperitoneal administration of reserpine, 2.5 mg./kg. One week later, the same group of mice was given a dose of the synthesized drug intraperitoneally, followed by intraperitoneal administration of reserpine 4 hr. later. The ability of the compound to reverse the reserpine-induced hypothermia 4, 6, and 24 hr. after the reserpine injection was noted.

Doses of the individual compounds were isomolar with respect to the standard: iproniazid. Most of the drugs were sufficiently soluble to be given either as a solution or as a stable suspension in propylene glycol.

Results—The experimental results are listed in Table II.

Discussion of Results—The pharmacologic data reveal bioactivity differences among the synthesized compounds in the test method utilized. Some of the more significant may be summarized. Among hydrazine derivatives, the 3-pyridyl series is less active than the 4-pyridyl. Also, among hydrazine derivatives, *N*-benzyl derivatives tend to be more active than the corresponding diacyl derivatives, but a significant exception is found in the case of the trimethoxybenzoyl derivatives which are more active than the benzyl derivatives. Actually, highest activity is found among the trimethoxybenzoyl derivatives. The hydrazine derivatives tend to be more potent than the amides, but some exhibit significant potency in the test.

On the basis of probable metabolic alterations, the bioactivity of the diacylhydrazines *in vivo* could be dependent upon three species. These are: the intact diacyl hydrazine, which should possess little activity; the monoacyl hydrazine, which might possess some activity; and hydrazine which should possess little activity, all to the extent that *in vitro* structure–activity relationships apply. In analogous fashion, the bioactivity of the alkyl acyl hydrazines would be dependent upon two species: the alkyl acyl hydrazine and the alkyl hydrazine. Again, to the extent that *in vitro* structure–activity relationships relate to activity in the intact animal, both of these might possess significant activity. In the case of amides, the likely species would be the amides and the amines. Significant inhibitory potency associated with either of these would not be expected. On the basis of the foregoing, the activity of the compounds synthesized and evaluated would be amides, diacyl hydrazines, and acyl alkyl hydrazines, in increasing order.

As the summary of the results indicates, the results anticipated are, in a general way, obtained, but there are important exceptions

Table II—Effect of the Synthesized Compounds on Body Temperature

	Body Temperature (°F.)			
	0 hr.	4 hr.	6 hr.	24 hr.
Control with known drug				
Reserpine[a]	100	87(2.7)[b]	84(2.2)[b]	85(3.3)[b]
Reserpine & iproniazid[c]	102	93(2.7)[d]	90(2.6)[d]	92(2.5)[d]
I 1-[3-(3-Pyridyl)glycidyl]-2-benzyl hydrazine: (significantly active)				
Reserpine[a]	100	87.1(3.36)	76.6(2.47)	89.0(1.6)
Reserpine & exp. drug[c]	102	91.3(3.43)	89.5(1.76)[d]	87.1(2.17)
II 1-[3-(3-Pyridyl)glycidyl]-2-benzoyl hydrazine: (significantly active)				
Reserpine	101.1	88.7(1.99)	74.2(1.49)	83.1(2.61)
Reserpine & exp. drug	99	88.0(2.5)	84.2(1.92)[d]	84.6(1.48)
III *N*-1-[3-(3-Pyridyl)glycidyl]-*N*-2-(3,4,5-trimethoxybenzoyl)hydrazine: (signif.)				
Reserpine	103	88.1(2.56)	69.8(2.32)	84.2(2.99)
Reserpine & exp. drug	102	89.7(1.58)	89.5(2.7)[d]	89.6(2.26)[d]
IV 1-[3-(4-Pyridyl)glycidyl]-2-benzyl hydrazine: (significantly active)				
Reserpine	100	91(1.7)	81(3.1)	87(3.2)
Reserpine & exp. drug	100	96(0.95)[a]	91(1.5)[a]	89(2.0)
V 1-[3-(4-Pyridyl)glycidyl]-2-benzoyl hydrazine: (significantly active)				
Reserpine	100.6	90.2(2.35)	80.6(3.08)	86.8(3.33)
Reserpine & exp. drug	100.6	84.3(2.62)[d]	80.3(1.97)	83.4(3.09)
VI *N*-1-[3-(4-Pyridyl)glycidyl]-*N*-2-(3,4,5-trimethoxybenzoyl)hydrazine: (signif.)				
Reserpine	101	88.5(1.95)	70.3(2.06)	85.2(3.67)
Reserpine & exp. drug	100	85.5(2.97)[d]	83.8(2.89)[a]	85.4(2.07)
VII *N*,*N*-Diethyl-3,3-diphenylglycidamide:				
Reserpine	101	89.8(3.1)	83.2(2.94)	84.8(1.85)
Reserpine & exp. drug	101	89.3(2.65)	85.3(2.55)	87.6(2.76)
VIII *N*,*N*-Diethyl-3-dibenzosuberylglycidamide: (significantly active)				
Reserpine	100	84.8(2.21)	84.4(1.79)	80.2(1.85)
Reserpine & exp. drug	100	95.4(1.67)[d]	94.1(1.69)[d]	88.2(1.72)[d]
IX *N*,*N*-Diethylxanthylglycidamide: (significantly active)				
Reserpine	101	89.3(2.83)	80.0(3.48)	85.1(2.71)
Reserpine & exp. drug	100.6	88.1(2.5)	87.0(3.02)[d]	86.8(2.99)
X *N*-3-Xanthylglycidyl-*N*-methylpiperazine:				
Reserpine	102	93(2.3)	91(2.4)	93(2.8)
Reserpine & exp. drug	102	94(1.8)	93(2.1)	95(1.7)
XI *N*,*N*-Diethyl 3,3-diphenylmethylglycidamide:				
Reserpine	100	91.6(1.7)	90(2.3)	94(2.7)
Reserpine & exp. drug	100	93(1.7)	90(2.3)	93(2.6)
XII *N*,*N*-Dibenzyl-3-(4-pyridyl)glycidamide:				
Reserpine	101	89.0(2.69)	88.8(1.87)	88.2(1.92)
Reserpine & exp. drug	101	88.0(2.69)	88.2(2.33)	89.8(2.09)

[a] Induced rectal temperature depression with intraperitoneal reserpine, 2.5 mg./kg., in male Swiss-Webster mice. [b] Standard deviation in parentheses. [c] Reversal of reserpine-induced rectal hypothermia in male Swiss-Webster mice; both drugs were administered intraperitoneally. [d] Significantly active; $p = 0.01$.

such as the high activity of the trimethoxybenzoyl derivatives and the amides. In this connection, it is well recognized that there is not necessarily a direct relationship between *in vitro* potency and activity *in vivo*. Additionally, it has been demonstrated that imipramine can reverse reserpine-induced hypothermia, probably through some other mechanism than inhibition of monoamine oxidase. The possible operation of these factors might account for the deviations from anticipated results. The possible operation of these factors throughout the series also precludes a precise delineation of structural features associated with activity. Through further biological evaluations and the synthesis of related compounds, it is hoped that this more precise delineation can be made.

Spasmolytic Activity—*Method*—The method for the determination of spasmolytic activity was adapted from the method of Miller *et al.* (12). The method is simple and convenient and allows potency estimates of a new compound against an established reference drug such as atropine. The method adapted here was the ability of the synthesized compound to reduce the spasm induced by a spasmogen on an isolated rabbit ileum *versus* the ability of the atropine to reduce the spasm of the spasmogen in the rabbit ileum in a set amount of time.

A Tyrode's solution of the following composition in grams per liter of purified water was used as the bath fluid:

Sodium chloride	9.0
Potassium chloride	0.2
Calcium chloride	0.2
Magnesium chloride	0.1
Sodium bicarbonate	1.0
Sodium acid phosphate	0.05
Glucose	1.0

The smooth muscle employed as test tissue was a strip of ileum 1–2 cm. in length, quickly removed from a recently sacrificed rabbit. The strip was suspended in the 10-ml. chamber filled with Tyrode's solution maintained at 37°. The muscle contractions were amplified and recorded on an E. and M. physiograph through a myograph attachment.

The remaining smooth muscle not being used was placed in Tyrode's solution suitably aerated and kept at constant temperature at 37° for subsequent pharmacological evaluation.

Test samples of freshly prepared solutions of the glycidyl compounds were prepared in concentrations of 1.4×10^{-5} m*M*. An aqueous solution of atropine sulfate, 1.4×10^{-6} m*M*, was used as the standard spasmolytic agent throughout the study.

An aqueous solution of mecholyl chloride was used as the standard spasmogen in millimolar quantities (1.3 m*M*).

A glass cylinder of 10-ml. capacity, equipped with an air inlet and a drainage arrangement maintained in a constant-temperature bath, was used throughout. The muscle chamber was equipped with an aerating tube which effectively aerated the muscle strip and efficiently mixed the test drugs.

As soon as the suspended strips had recovered from the contraction caused by the operative manipulations, the testing was begun. The dose of the spasmogen was administered to produce virtually maximum contraction. The spasmolytic agent was introduced without washing out the spasmogen. The degree of relaxation produced in the first 2 min. was noted. Following the observation the drug containing Tyrode's solution was flushed out and replaced with fresh solution. When the strips had relaxed to the former tonus level, they were ready for another trial.

Results—The pharmacological data on the spasmolytic activity of Compounds VII, VIII, and X are represented in Table III,

Table III—Summary of Data Showing Concentration–Response Relationships for the Spasmolytic Effects of Compounds VII, VIII, and X and Atropine Sulfate Against Methacholine Chloride-Induced Spasm in Excised Rabbit Ileum

	Spasmolytic Agent			
	Compound X	Compound VIII	Compound VII	Atropine Sulfate
Ml. added to 10-ml. muscle bath	0.8 ml.	2.0 ml.	2.0 ml., 3.0 ml.	0.2 ml.
Concentration in muscle bath	1.15×10^{-4}	2.88×10^{-4}	2.88 and 4.32×10^{-4}	2.88×10^{-6}
Response: No. positive/No. tried	7/10	6/10	0/10 1/10	8/10
Relative potency	2.5	1.0	0	1×10^{2}

noting concentration in millimolar quantities as well as relative potency when compared to atropine sulfate.

Discussion of Results—Examination of Compound X reveals that it is about 2.5 times as active as Compound VIII. Compound VII was found to be inactive in the ability to reduce the spasms induced by mecholyl. While spasmolytic activity is present in Compounds X and VIII, the activity present is only about 1/40th and 1/100th, respectively, that of the standard spasmolytic agent, atropine sulfate.

REFERENCES

(1) M. S. Newman and B. J. Magerlein, in "Organic Reactions," Vol. V, Wiley, New York, N. Y., 1949, p. 413.

(2) H. O. House, "Modern Synthetic Reactions," W. A. Benjamin, New York, N. Y., 1965, p. 240.

(3) G. Omodt and O. Gisvold, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 153(1960).

(4) A. J. Speciale and H. W. Frazier, *J. Org. Chem.*, **26**, 3176 (1961).

(5) L. H. Schlager, *Arch. Pharm.*, **296**, 217(1963).

(6) E. A. Zeller, *J. Biol. Chem.*, **234**, 389(1959).

(7) J. A. Carbon, W. P. Burkard, and E. A. Zeller, *Helv. Chim. Acta*, **41**, 1883(1958).

(8) J. H. Biel, in "Molecular Modification in Drug Design," American Chemical Society, Washington, D. C., 1964, p. 124.

(9) J. H. Biel, in "Psychopharmacological Agents," M. Gordon, Ed., Academic, New York, N. Y., 1964, p. 384.

(10) R. E. Taylor, *J. Pharmacol. Exp. Ther.*, **138**, 201(1962).

(11) S. Garrattini, A. Giachetti, A. Jori, L. Pieri, and L. Valzelli, *J. Pharm. Pharmacol.*, **14**, 509(1962).

(12) L. C. Miller, T. J. Becker, and M. L. Tainter, *J. Pharmacol. Exp. Ther.*, **92**, 260(1948).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 13, 1969, from the *College of Pharmacy, University of Texas at Austin, Austin, TX 78712*

Accepted for publication February 18, 1970.

* Present address: School of Pharmacy, Northeast Louisiana State College, Monroe, LA 71201

† Present address: College of Pharmacy, Idaho State University, Pocatello, ID 83201

Dissolution of Macromolecules II: Dissolution of an Ethylene–Maleic Acid Copolymer

ALLEN HEYD*, DANE O. KILDSIG, and GILBERT S. BANKER

Abstract □ Factors influencing the dissolution of an ethylene–maleic acid copolymer have been studied. Polymer swelling, hydrated layer thickness, and solvent pH were shown to influence the dissolution of the polymer. Linear dissolution rates were observed following an initial induction period. Hydrated layer thickness was found to be a controlling factor in the dissolution process. An immersion refractometry method was employed to measure aqueous polymer concentrations during dissolution.

Keyphrases □ Ethylene–maleic acid copolymer—dissolution □ Tablets, ethylene–maleic acid copolymer—dissolution study □ Dissolution test apparatus—diagram □ Refractometry, immersion—polymer determination

Polymeric materials are widely used in many pharmaceutical systems. In systems utilizing polymer films and particularly in dosage forms in which the polymer is compressed in tablets to produce controlled drug release, the dissolution of the polymer is an important parameter. Polymer systems are frequently sought which, based on their dissolution properties, will provide a particular type of drug release.

Previous investigators of polymer dissolution have studied the dissolution of polystyrene in organic solvents (1–3). However, a detailed investigation of the dissolution in aqueous solvents of polymers having reactive functional groups has not been reported. In an earlier report the surface phenomena associated with the dissolution of such polymers were described (4). The present investigation reports the dissolution of an ethylene–maleic acid (dicarboxylic acid) copolymer as affected by these surface parameters.

EXPERIMENTAL

The ethylene–maleic acid copolymer, referred to as EMA-22, and the polymer tablets used in this study were identical to those used in a previous investigation (4). The measurement of polymer swelling, solvent penetration, and hydrated layer thickness was also identical to that of the earlier study (4).

The dissolution apparatus employed for the study of polymer dissolution is shown in Figs. 1–3. Figure 1 describes the sample holder; Fig. 2 shows the dimensions of the Plexiglas dissolution cell. Figure 3 is a schematic representation of the dissolution unit and the component parts that made up the entire system. The lip of the

Table III—Summary of Data Showing Concentration–Response Relationships for the Spasmolytic Effects of Compounds VII, VIII, and X and Atropine Sulfate Against Methacholine Chloride-Induced Spasm in Excised Rabbit Ileum

	Spasmolytic Agent			
	Compound X	Compound VIII	Compound VII	Atropine Sulfate
Ml. added to 10-ml. muscle bath	0.8 ml.	2.0 ml.	2.0 ml., 3.0 ml.	0.2 ml.
Concentration in muscle bath	1.15×10^{-4}	2.88×10^{-4}	2.88 and 4.32×10^{-4}	2.88×10^{-6}
Response: No. positive/No. tried	7/10	6/10	0/10 1/10	8/10
Relative potency	2.5	1.0	0	1×10^{2}

noting concentration in millimolar quantities as well as relative potency when compared to atropine sulfate.

Discussion of Results—Examination of Compound X reveals that it is about 2.5 times as active as Compound VIII. Compound VII was found to be inactive in the ability to reduce the spasms induced by mecholyl. While spasmolytic activity is present in Compounds X and VIII, the activity present is only about 1/40th and 1/100th, respectively, that of the standard spasmolytic agent, atropine sulfate.

REFERENCES

(1) M. S. Newman and B. J. Magerlein, in "Organic Reactions," Vol. V, Wiley, New York, N. Y., 1949, p. 413.

(2) H. O. House, "Modern Synthetic Reactions," W. A. Benjamin, New York, N. Y., 1965, p. 240.

(3) G. Omodt and O. Gisvold, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 153(1960).

(4) A. J. Speciale and H. W. Frazier, *J. Org. Chem.*, **26**, 3176 (1961).

(5) L. H. Schlager, *Arch. Pharm.*, **296**, 217(1963).

(6) E. A. Zeller, *J. Biol. Chem.*, **234**, 389(1959).

(7) J. A. Carbon, W. P. Burkard, and E. A. Zeller, *Helv. Chim. Acta*, **41**, 1883(1958).

(8) J. H. Biel, in "Molecular Modification in Drug Design," American Chemical Society, Washington, D. C., 1964, p. 124.

(9) J. H. Biel, in "Psychopharmacological Agents," M. Gordon, Ed., Academic, New York, N. Y., 1964, p. 384.

(10) R. E. Taylor, *J. Pharmacol. Exp. Ther.*, **138**, 201(1962).

(11) S. Garrattini, A. Giachetti, A. Jori, L. Pieri, and L. Valzelli, *J. Pharm. Pharmacol.*, **14**, 509(1962).

(12) L. C. Miller, T. J. Becker, and M. L. Tainter, *J. Pharmacol. Exp. Ther.*, **92**, 260(1948).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 13, 1969, from the *College of Pharmacy, University of Texas at Austin, Austin, TX 78712*

Accepted for publication February 18, 1970.

* Present address: School of Pharmacy, Northeast Louisiana State College, Monroe, LA 71201

† Present address: College of Pharmacy, Idaho State University, Pocatello, ID 83201

Dissolution of Macromolecules II: Dissolution of an Ethylene–Maleic Acid Copolymer

ALLEN HEYD*, DANE O. KILDSIG, and GILBERT S. BANKER

Abstract □ Factors influencing the dissolution of an ethylene–maleic acid copolymer have been studied. Polymer swelling, hydrated layer thickness, and solvent pH were shown to influence the dissolution of the polymer. Linear dissolution rates were observed following an initial induction period. Hydrated layer thickness was found to be a controlling factor in the dissolution process. An immersion refractometry method was employed to measure aqueous polymer concentrations during dissolution.

Keyphrases □ Ethylene–maleic acid copolymer—dissolution □ Tablets, ethylene–maleic acid copolymer—dissolution study □ Dissolution test apparatus—diagram □ Refractometry, immersion—polymer determination

Polymeric materials are widely used in many pharmaceutical systems. In systems utilizing polymer films and particularly in dosage forms in which the polymer is compressed in tablets to produce controlled drug release, the dissolution of the polymer is an important parameter. Polymer systems are frequently sought which, based on their dissolution properties, will provide a particular type of drug release.

Previous investigators of polymer dissolution have studied the dissolution of polystyrene in organic solvents (1–3). However, a detailed investigation of the dissolution in aqueous solvents of polymers having reactive functional groups has not been reported. In an earlier report the surface phenomena associated with the dissolution of such polymers were described (4). The present investigation reports the dissolution of an ethylene–maleic acid (dicarboxylic acid) copolymer as affected by these surface parameters.

EXPERIMENTAL

The ethylene–maleic acid copolymer, referred to as EMA-22, and the polymer tablets used in this study were identical to those used in a previous investigation (4). The measurement of polymer swelling, solvent penetration, and hydrated layer thickness was also identical to that of the earlier study (4).

The dissolution apparatus employed for the study of polymer dissolution is shown in Figs. 1–3. Figure 1 describes the sample holder; Fig. 2 shows the dimensions of the Plexiglas dissolution cell. Figure 3 is a schematic representation of the dissolution unit and the component parts that made up the entire system. The lip of the

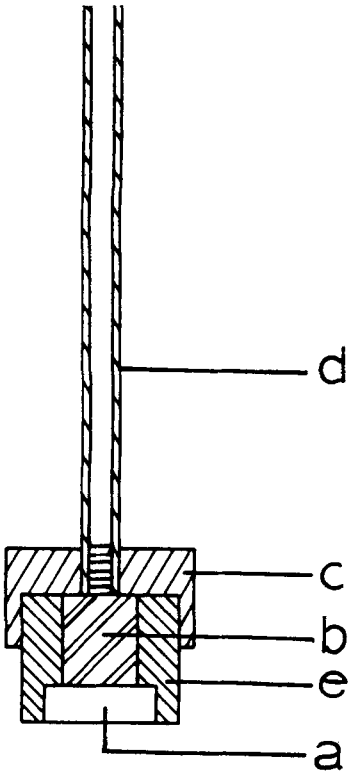


Figure 1—*Sample holder. Key: a, compressed disk cavity; b, plunger; c, die housing; d, steel shaft; and e, 1.12-cm. (0.44-in.) die.*

die housing of the sample holder (Fig. 1) was placed on the edge of the dissolution cell (Fig. 2), which provided constant sample geometry for each dissolution test. The plunger of the sample holder facilitated exact positioning of the compressed disk. The shaft of the sample holder, which permitted positioning of the holder in the dissolution apparatus, extended through the housing of the holder and was threaded into the plunger (Fig. 1).

A Bausch & Lomb immersion refractometer,[1] utilizing prism A, was used to follow the increase in polymer concentration with time. Standard curves of refractometer reading *versus* polymer concentration were linear over the concentration range used, 0 to 4 mg./ml. The precision of the refractometer method was excellent; identical

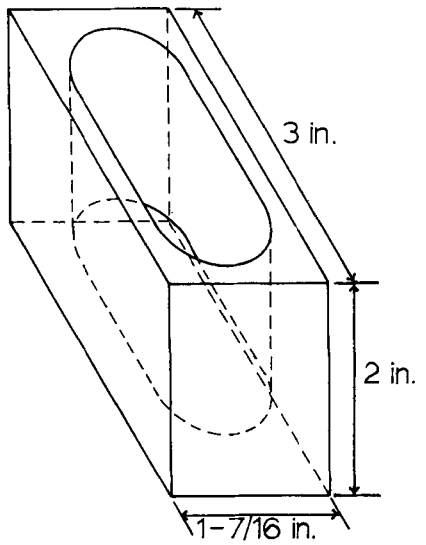


Figure 2—*Plexiglas dissolution cell. The external dimensions are indicated. The inside dimensions are 6.99-cm. (2.75-in.) total length, 2.87 cm. (1.13 in.) wide, and 3.66 cm. (1.44 in.) deep with the ends having a 1.42-cm. (0.56-in.) radius of curvature.*

[1] Bausch & Lomb Optical Co., model DB 502.

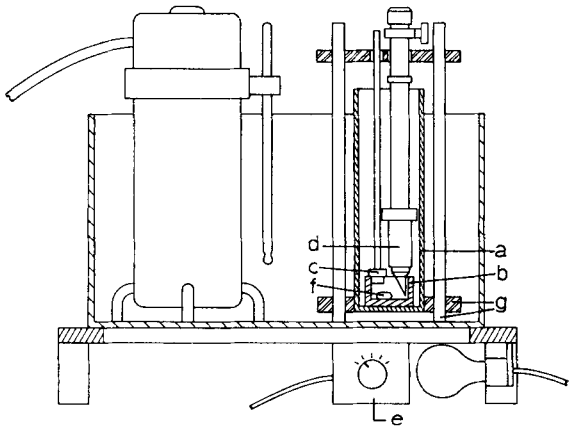


Figure 3—*Dissolution apparatus. Key: a, glass cylinder; b, dissolution cell; c, sample holder; d, immersion refractometer; e, magnetic stirrer; f, stirring bar; and g, support.*

readings on the refractometer scale were obtained for each replicate sample of each polymer solution.

In conducting the dissolution test, a 1-g. compressed tablet of EMA-22 free acid was positioned in the cavity of the sample holder so that 0.05 cm. of the tablet protruded. The dissolution cell was filled with 60 ml. of solvent, which was allowed to equilibrate to 30 ± 0.1°. This volume was sufficient to cover the exposed tablet after the sample holder and the refractometer were precisely positioned in the dissolution cell. A magnetic stirrer unit drove a 1.27-cm. (0.5-in.) stirring bar inside the dissolution cell at 130 r.p.m. Kolthoff's alkaline borate buffer (5) was used to prepare pH 7.4 and 9.4 buffers. The buffers from pH 1.2 to 6.2 were prepared from standard solutions as described by the USP (6). The immersion refractometry method and the technique and apparatus described permitted the dissolution process to be continuously followed without disturbing the system.

RESULTS AND DISCUSSION

The dissolution of EMA-22 in distilled water is shown in Fig. 4. The dissolution rate was linear following an initial induction period.

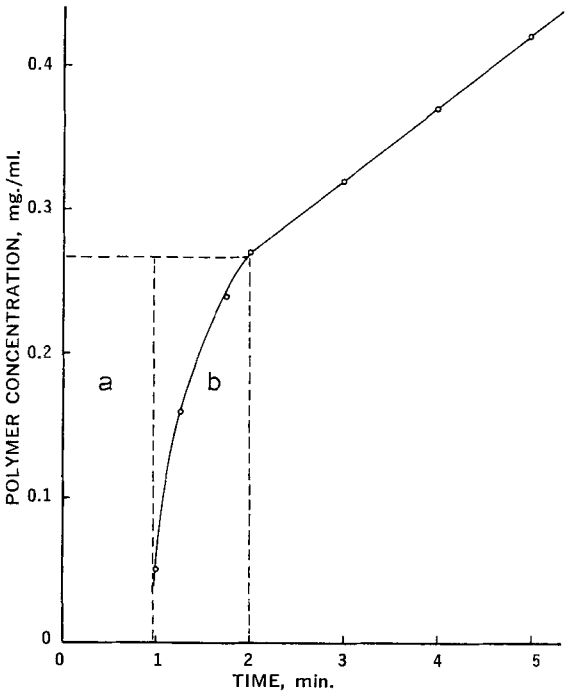


Figure 4—*Linear dissolution rate with initial induction period. Key: a, lag time; b, stabilization period; and a + b, induction period.*

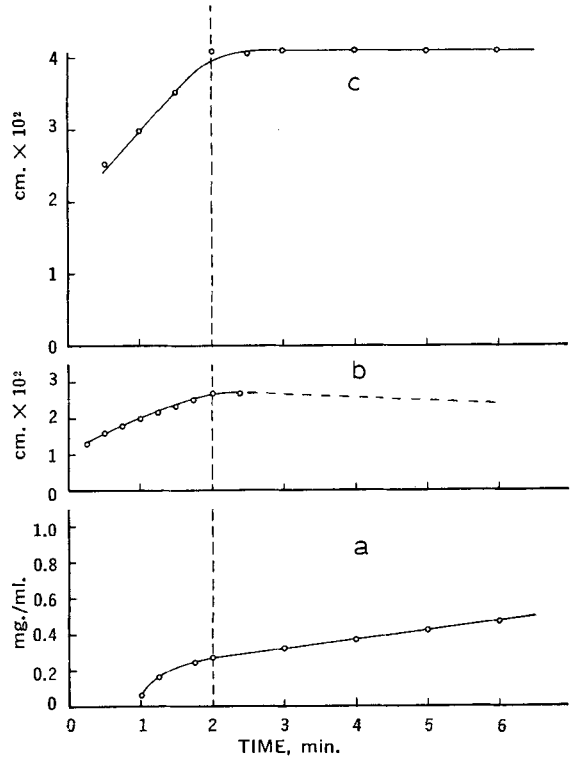


Figure 5—*Correlation between induction period, a, swelling time, b, and hydrated layer formation, c.*

The induction period consisted of a lag time and a stabilization period (Fig. 4). The lag time, or period before measurable concentration appears in solution, is attributed to the time required for initial solvent penetration and initial polymer swelling. During the initial swelling process (0–0.5 min.) in which the largest extent of swelling was achieved (4), little or no polymer dissolved. During the stabilization period, the dissolution rate continuously decreased as the polymer achieved maximum swelling. Also during the stabilization period, maximum hydrated layer thickness was achieved after which the dissolution rate was linear. The correlation between hydrated layer formation, swelling time, and the dissolution induction period is shown in Fig. 5. The establishment of both an equilibrium hydrated layer thickness and maximum swelling was found generally to correlate to the induction period required for achievement of a constant dissolution rate.

The polymer–solvent interaction is responsible for the expansion of the linear chain polymer and the subsequent formation of a gel structure. As the EMA-22 polymer is a dicarboxylic acid, the solvent pH would be expected to influence greatly the dissolution of the polymer (Fig. 6). Contrary to the dissolution of micromolecular weak acids, the equilibrium dissolution rate of the polymer weak acid decreases with increasing pH (Fig. 6). However, the dissolution rates reported in Fig. 6 are the equilibrium values obtained from the linear portion of the curve. The initial dissolution rates did increase with increasing pH but were quickly affected (within 0.5–2 min.) by the swelling phenomena and subsequent hydrated layer

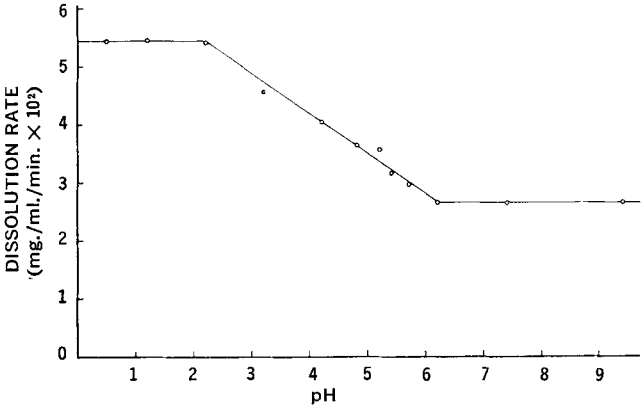


Figure 6—*Decrease in equilibrium dissolution rate with increasing pH.*

formation. Thus the swollen hydrated layer remains the determining factor in the dissolution of the EMA polymer following swelling equilibrium.

SUMMARY

The dissolution of an ethylene–maleic acid copolymer was investigated using an immersion refractometer for polymer analysis. An induction period was observed consisting of a lag time and a stabilization period, during which the dissolution rate was continuously decreasing until a constant rate was obtained. The solvent pH was found to influence the dissolution of the polymer through its effect on polymer swelling and hydrated layer thickness. These two parameters, swelling and hydrated layer thickness, were primarily responsible for the dissolution properties of the polymer. The refractometer method was adequately sensitive for following polymer dissolution and allowed a continuous determination of polymer concentration without sampling.

REFERENCES

(1) K. Ueberreiter and F. Asmussen, *J. Polym. Sci.*, **57**, 187 (1962).

(2) F. Asmussen and K. Ueberreiter, *ibid.*, **57**, 199(1962).

(3) K. Ueberreiter and F. Asmussen, *Makromol. Chem.*, **44–46**, 324(1961).

(4) A. Heyd, D. Kildsig, and G. Banker, *J. Pharm. Sci.*, **58**, 586(1969).

(5) I. M. Kolthoff, *J. Biol. Chem.*, **63**, 135(1965).

(6) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 913.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 28, 1969, from the *Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication January 27, 1970.

* Fellow, American Foundation for Pharmaceutical Education. Present address, University of Connecticut, Storrs, Conn.

Pharmacological Evaluation of Seizures Induced by Electrical Stimulation of the Hippocampus

PENG N. YEOH and HAROLD H. WOLF

Abstract □ A method to induce minimal seizures in unrestrained rats *via* bipolar electrodes implanted in the right dorsal hippocampus has been described. The threshold for such seizures is reproducible, stable over time, and elevated by trimethadione and high doses of diphenylhydantoin. Propranolol and pronethalol also raise seizure threshold, but MJ1999 and D(−)- and L(+)-INPEA are ineffective. The adrenergic agents do not seem to alter seizure threshold by their ability to block β-receptors.

Keyphrases □ Hippocampal stimulation—bipolar electrodes □ Convulsive seizures—electrical stimulation, hippocampus □ Electrically produced seizures—convulsive threshold □ Anticonvulsants effect—electrically produced seizures

Several techniques to produce minimal seizures experimentally have been reported (1). Some involve the application of electric current *via* corneal electrodes to restrained animals, while others employ such chemicals as hexafluorodiethylether and metrazol. The former method is less than ideal, since brief restraint lowers seizure threshold (2), while the latter methods are subject to criticism of possible drug–drug interaction when used in drug studies designed to elucidate seizure mechanisms.

One objective of this study was to develop a method to induce minimal seizures electrically in unrestrained rats. Ideally, such seizures would be easily induced, stable, and reproducible over a long time.

A second objective of this study was to determine whether such seizures would respond to anticonvulsants such as diphenylhydantoin and trimethadione in a manner similar to that observed with other experimentally induced seizures (3–5).

At present, the mechanisms responsible for seizure expression are still not fully known. However, there is much evidence indicating an involvement of catecholamines. For example, Schlesinger *et al.* (6) and Scudder *et al.* (7) reported that mice with higher than normal susceptibility to seizures had lower catecholamine levels. Other studies have shown that reserpine, tetrabenazine, and more selective catecholamine depletors increased seizure susceptibility; while treatment with the catecholamine precursor, L-dopa, in the presence of iproniazid inhibited seizure activity (8–10). In general, the existing evidence seems to relate low catecholamine levels with a high susceptibility to seizure expression.

Some earlier experiments in this laboratory (11) demonstrated that β-adrenergic blocking agents, *e.g.*, propranolol and pronethalol, elevated thresholds in mice to low-frequency electroshock (l. f. ES). In addition, pronethalol protected susceptible mice from audiogenic seizures. Thus, a third objective was to pursue further the effect of selected β-adrenergic blocking agents on convulsive activity by evaluating their ability to modify threshold for electrically induced hippocampal seizures.

EXPERIMENTAL

The drugs studied were diphenylhydantoin, trimethadione, and the adrenergic agents propranolol [1-isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride], pronethalol [D,L-1-(2′-naphthyl)-2-isopropylaminoethanol hydrochloride], D(−)- and L(+)-INPEA [D(−)- and L(+)-1-(4′-nitrophenyl)-2-isopropylaminoethanol hydrochloride], and MJ1999 [4′-(2-isopropylamino-1-hydroxyethyl)methanesulfonanilide]. Except for L(+)-INPEA, all these compounds are well-established peripheral β-adrenergic blocking agents (12).

The experimental animals employed were male Wistar albino rats (200–300 g.).[1] Under pentobarbital anesthesia (45 mg./kg.), these rats were stereotaxically implanted with stainless steel (0.3-mm. diameter) bipolar electrodes (MS 303-018″-312″-SS-010″)[2] in the right dorsal hippocampus (2.59 mm. lateral to saggital zero; 3.80 mm. anterior to frontal zero, and 2.50 mm. below the brain surface) according to the rat atlas of Konig and Klippel (13). The hippocampus was chosen because of its low threshold to seizure expression.

Following surgery, the rats were housed individually in plastic cages (16 × 25 × 25 cm.) with free access to food and water. At least 1 week later, each rat was transferred to a testing chamber (30 × 30 × 50 cm.) equipped with a oneway mirror. The implanted electrodes were then connected to a Grass S4 stimulator in series with a capacitor (to maintain constant waveform), a timer key (set for 6 sec.), and an external resistor (100 ohm). A current passing through the resistor was measured by an oscilloscope and reflected that passing through the electrode. With the key opened, a resistor box was placed in series with the stimulator to measure rat resistance.

In this environment, the rat was able to move about freely. After 5-min. adaptation in the experimental box, the rat was subjected to a series of electrical stimulations of increasing intensities until a minimal seizure was seen.

The stimulus consisted of a train of 0.2-msec. biphasic pulses, 6 sec. in duration with a frequency of 60 c.p.s. A 2-min. interval separated each stimulus. To minimize the total number of stimuli administered to any rat, the following schedule was employed. A starting current intensity of 150 μamp. was increased in steps of 50 μamp. At intensities of 600 μamp., the increment was raised by a factor of 4; above 1000 μamp., it was raised 10 times the original value. The maximum current intensity administered was 3000 μamp.

A seizure was defined as the presence of readily observed jaw chopping and/or myoclonic jerks, and the current intensity that just produced such symptoms was defined as the seizure threshold. In drug studies, rats that did not exhibit seizures with the maximum current were assigned seizure thresholds of 3000 μamp. for statistical computations.

The rats were eventually sacrified with pentobarbital and perfused with saline, followed by 10% formalin, *via* the right ventricle. Serial collodion sections (30 μ) were stained by the technique of Kluver and Barrera (14) and microscopically examined to localize the electrode tract as well as to evaluate possible tissue damage.

All drug injections were made intraperitoneally. Control animals received the drug vehicle.

To determine appropriate doses and the duration of drug activity, pilot studies were conducted based on drug-induced neurotoxicity.

[1] Greenacres Laboratories, Amelia, Ohio.
[2] Plastic Products Co., Roanoke, Va.

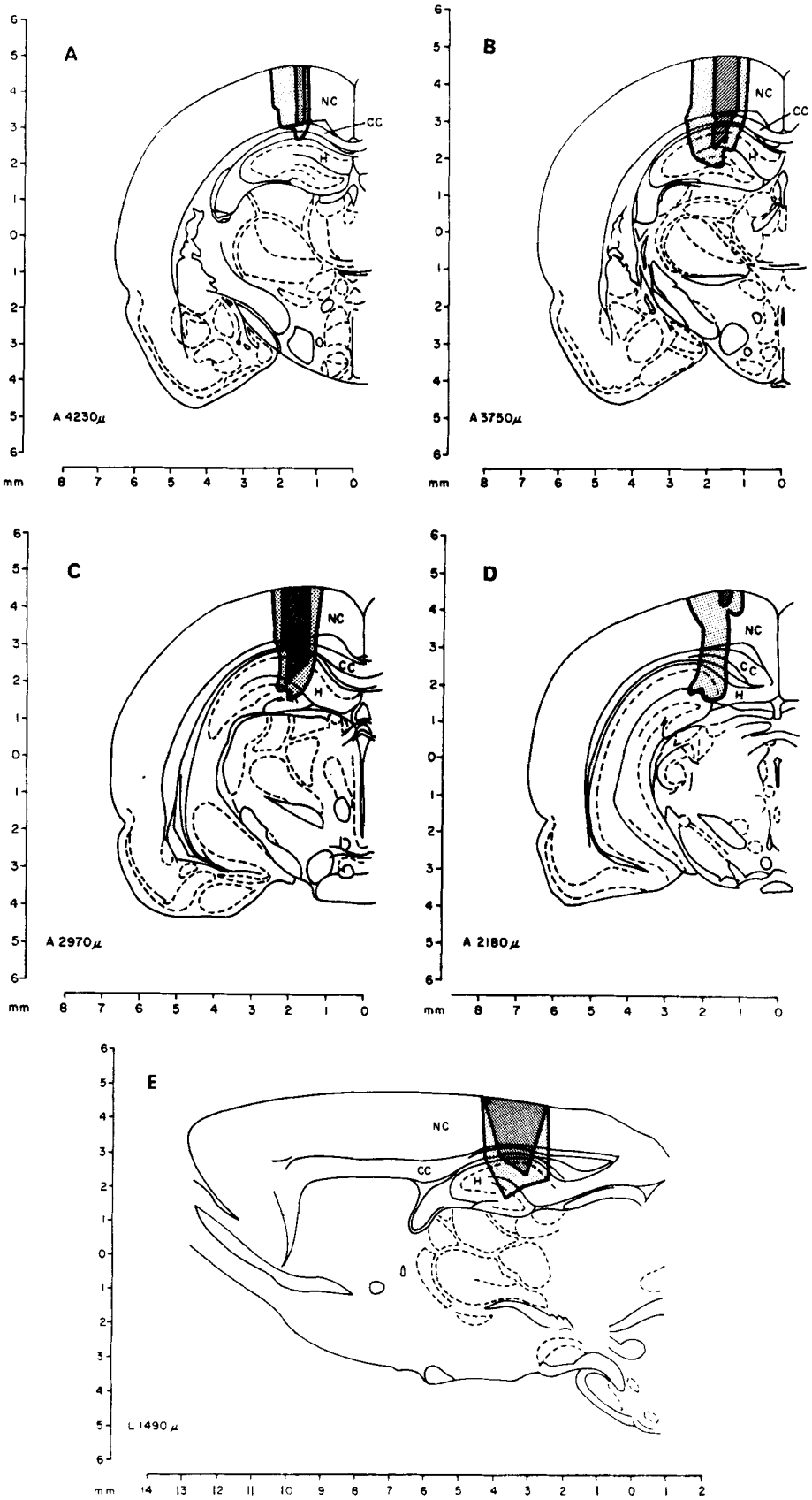


Figure 1—*Minimal (▒) and maximal (:::) extent of the lesions projected on frontal sections (A–D) and saggital section (E) at the stated distances from frontal and saggital zeroes, respectively. Key: NC, neocortex; CC, corpus callosum; and H, hippocampus.*

Table I—Stability of Rat Resistance

Min. between Test	Mean Resistance (kohm) Initial	Final	Difference Mean
2	12.51	11.93	0.58[a]
15	13.05	13.62	0.57[a]
20	12.80	12.94	0.14
30	12.91	14.09	1.18[a]
1440	13.39	13.61	0.22[a]

[a] $p < 0.05$.

This was defined as the failure of a rat to stay on a rotating rod (6 r.p.m.) for 1 min., given three trials. Drug-induced neurotoxicity in 50% of the rats (TD_{50}) with 95% confidence limits was calculated using the method of Litchfield and Wilcoxon (15).

In the evaluation of drug effects on seizure threshold, groups of 6–10 rats (average of 8) with a predetermined stable seizure threshold (SST) were employed. Half of these received the drug and the other half received the drug vehicle. Seizure threshold after drug treatment (DST) and control seizure threshold (CST) were determined at the time of peak neurotoxic effect of the drug. A crossover design with a 7-day interval was employed, so each rat served as its own control. Threshold ratios, *i.e.*, DST/SST and CST/SST, with 95% confidence limits were calculated by the method described by Goldstein (16).

RESULTS AND DISCUSSION

The lesion produced by electrode implantation was reconstructed with the aid of the atlas of Konig and Klippel (13) and is shown in Fig. 1. Tissue damage extended from about 2.2 to 4.2 mm. anterior to frontal zero and from 1.1 to 2.5 mm. lateral–saggital zero. Only the neocortex, the corpus callosum, and the dorsal hippocampus in this region were affected. In all the animals studied, the electrode tip lay in the right dorsal hippocampus between 3.7 and 3.8 mm. anterior to frontal zero, but it was 1.1–2.0 mm. lateral to saggital zero. This could be due to the different strain of rat used in this study compared to those employed for the construction of the Konig and Klippel atlas.

The stability of rat resistance over time was evaluated; these data, presented in Table I, were analyzed by a paired comparison *t* test.

Over time intervals ranging from 2 to 1440 min., rat resistance varied significantly in all but one test. These results emphasize the importance of taking the instantaneous rat resistance into consideration in any measurement of current intensity passing through the brain. Therefore, in all subsequent studies, rat resistance was measured during every stimulus, and the actual current intensity passing through the electrodes was thus recorded.

The stability of hippocampal seizure threshold over time is presented in Fig. 2. The vertical bars denote 95% confidence limits, and any point where a bar does not cross 1.0 indicates a significant change in seizure threshold. These data indicate that hippocampal seizure threshold remained essentially stable over time intervals ranging from 10 to 60 min. between determinations. Additional studies with injection of saline between tests also produced no apparent change in threshold over the same time intervals.

The results obtained in the neurotoxicity studies are represented in Table II. The time of peak effect varied from 10 min. for trimethadione to 60 min. for diphenylhydantoin. The remaining compounds exhibited peak activity between 15 and 30 min. As would be expected from previous studies (4, 5), the neurotoxicity potency of trimethadione was quite low compared to that of the other drugs tested. Complete toxicity studies for MJ1999 were not conducted, due to the limited amount of drug available and its relative nontoxicity. No toxic effect was seen with 8×10^{-1} mmoles/kg. The choice of 30 min. as the time of peak activity for this agent was based on observable gross central nervous system depression and on previous work (17).

Table II—Neurotoxicity of Drugs

Drug	Time of Peak Effect, min.	$TD_{50} \times 10^{-1}$, mmoles/kg.
Diphenylhydantoin	60	8.30 (5.90–11.60)[a]
Trimethadione	10	32.80 (29.50–33.40)
Propranolol	15	1.11 (1.04–1.18)
Pronethalol	15	1.23 (0.99–1.52)
MJ1999	30	>8.0
D(−)-INPEA	20	2.06 (1.35–3.15)
L(+)-INPEA	20	2.08 (1.51–2.87)

[a] Figures in parentheses represent 95% confidence limits.

The effect of diphenylhydantoin on seizure threshold is demonstrated in Fig. 3. Control solution did not alter seizure threshold significantly in this or any subsequent studies. At doses of 0.5 and 1.0×10^{-1} mmoles/kg., diphenylhydantoin did not have a significant effect; a higher dose of 2.5×10^{-1} mmoles/kg. produced a slight but significant elevation of threshold. These results agree with those reported for this compound on other types of experimentally induced minimal seizures, since low doses have no effect on thresholds for minimal electroshock, metrazol, and l.f. ES seizures, while higher doses have been shown to increase l.f. ES seizure threshold (4, 5, 18). Diphenylhydantoin also raises the convulsive threshold of the motor cortex in the monkey (19).

Several workers who measured electrical afterdischarges evoked by stimulation of specific brain areas in monkeys, cats, and rabbits (19–21) generally observed an increase in threshold and shorter discharge duration in the motor cortex, hippocampus, thalamus, and septal area following diphenylhydantoin treatment. However, the compound has been reported to produce no effect on cortical or hippocampal afterdischarges in the rabbit (22), although transcortical spread of abnormal activity from chronic epileptogenic focus in the visual cortex (rabbit) can be suppressed by this agent (23). In view of the wide variation in the animal species employed, the dose, the route of drug administration, the electrical stimulus, and the brain regions stimulated, it is difficult to correlate these data with the present findings. However, diphenylhydantoin is known to have a nonspecific stabilizing action on excitable membranes (24). This is thought to result from a more efficient extrusion of sodium ions from brain cells, probably by stimulation of the metabolic sodium pump (25–27). This activity may account for the slight elevation of seizure threshold induced by the high dose of diphenylhydantoin.

Similar data obtained with trimethadione are presented in Fig. 4. All four doses raised threshold significantly, and an apparent dose–response relationship was seen. The efficacy of trimethadione

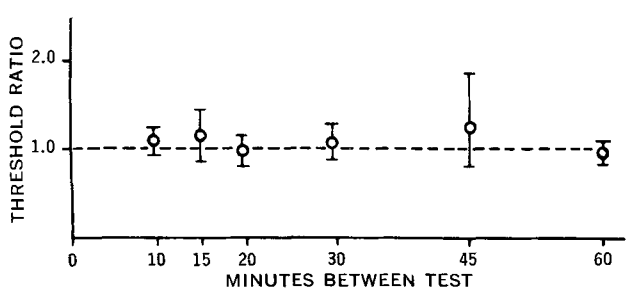


Figure 2—*Stability of hippocampal seizure threshold over time. Threshold ratio was determined by dividing the final seizure threshold by the initial seizure threshold* (n = 6).

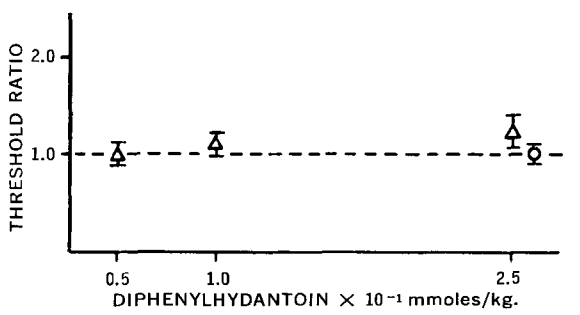


Figure 3—*Effect of diphenylhydantoin on hippocampal seizure threshold* (n = 6). *Key:* ○, *propylene glycol; and* △, *diphenylhydantoin.*

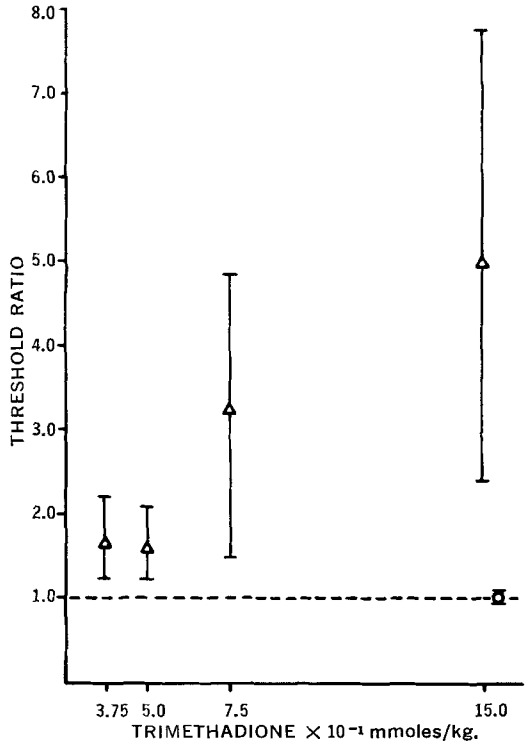


Figure 4—*Effect of trimethadione on hippocampal seizure threshold* (n = *10*). *Key:* ○, *propylene glycol; and* △, *trimethadione.*

in this procedure was further emphasized by the absence of seizures in several animals at the maximum stimulus employed. Again, these data reflect anticipated results, because trimethadione is known to elevate minimal seizure threshold as measured by other techniques (4, 5).

Schallek and Kuehn (20) found trimethadione to be superior to diphenylhydantoin in increasing seizure thresholds at cortical and other brain sites. Furthermore, thresholds to electrical afterdischarges in the motor cortex and the thalamus are elevated, while the duration of these afterdischarges is reduced in several species subsequent to treatment with the drug (19–22). Morrell *et al.* (23) observed that trimethadione not only suppresses chronic epileptogenic foci but also depresses the projection of seizure activity from cortical foci to the thalamus and to the contralateral side. This

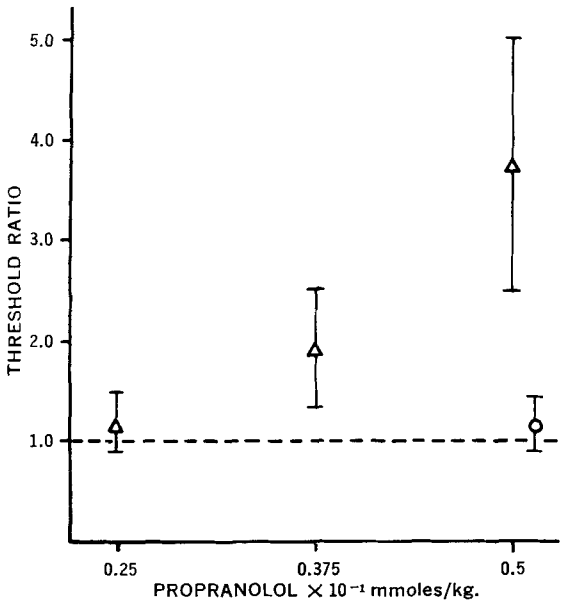


Figure 5—*Effect of propranolol on hippocampal seizure threshold* (n = 9). *Key:* ○, *saline; and* △, *propranolol.*

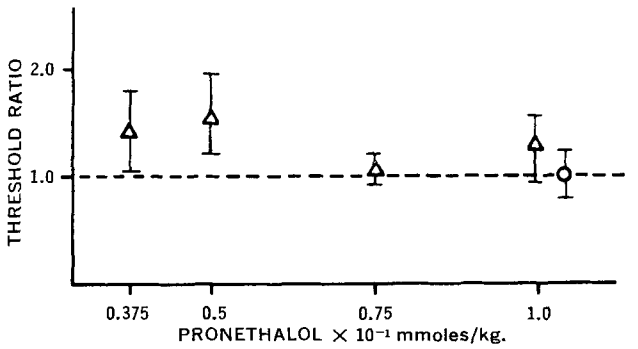


Figure 6—*Effect of pronethalol on hippocampal seizure threshold* (n = *10*). *Key:* ○, *saline; and* △, *pronethalol.*

action may well be due to the ability of the drug to intensify the depression of synaptic transmission following each transmitted volley of impulses (28). Such an action would prevent the build-up and maintenance of an oscillating system between the thalamus and the cortex, postulated to be involved in the precipitation of minimal seizures (26, 29, 30). Thus, the ability of trimethadione to increase seizure threshold in this study may reflect this suppression of showers of impulses at central synapses.

The effects seen with a highly potent β-adrenergic blocking agent, propranolol (31, 32), are illustrated in Fig. 5. At doses higher than 0.25 × 10^{-1} mmoles/kg., it elevated seizure threshold, and again there was an apparent dose–response relationship. An increase in seizure threshold of about 275% was seen with the highest dose employed. At this dose level, several rats did not exhibit seizures even when the maximum stimulus was administered. Since propranolol is known to have central depressant activities, the effect of pronethalol, a less potent β-adrenergic blocking agent with some central stimulant properties, was studied in an attempt to separate effects due to nonspecific central depression from β-blockade. The results are shown in Fig. 6. At low doses, pronethalol increased seizure threshold significantly. A 50% elevation was observed with 0.5 × 10^{-1} mmoles/kg. This is about one-fifth the effect seen with the same dose of propranolol. However, higher doses (in the region of $^1/_2$ TD_{50}) produced no significant alteration of seizure threshold. This could be a reflection of the fact that high doses of pronethalol are known to induce convulsions (33).

Both propranolol and pronethalol have significant local anesthetic properties (34). Thus, the threshold-elevating effects of these two compounds may be related more to this aspect of their activity than to their β-receptor blocking properties. To test this, two blocking agents, MJ1999 and INPEA, reported to be relatively selective as β-adrenergic blocking agents and to possess no local anesthetic action (17, 35), were examined. The results obtained with MJ1999 are seen in Fig. 7. Even with 5 times the effective dose of propranolol, no significant change in seizure threshold was seen.

Similar data obtained with INPEA are presented in Fig. 8. If β-adrenergic blockade was indeed responsible for the elevation of seizure threshold exhibited by propranolol and pronethalol, marked differences between the effects of these two isomers of INPEA would be expected, since only the D(−)-isomer has been reported to be active as a β-adrenergic blocking agent (12). However, both isomers were equally ineffective in changing seizure

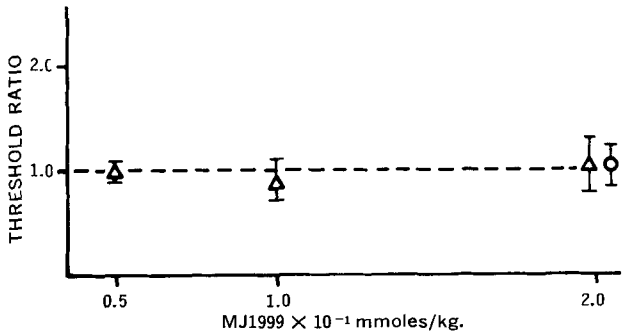


Figure 7—*Effect of MJ1999 on hippocampal seizure threshold* (n = *8*). *Key:* ○, *saline; and* △, *MJ1999.*

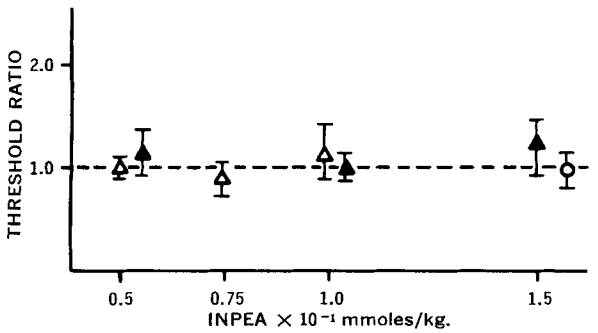


Figure 8—*Effect of INPEA on hippocampal seizure threshold* (n = 9). *Key:* ○, *saline;* △, D(−)-*INPEA; and* ▲, L(+)-*INPEA.*

threshold, even at doses 4 times greater than the effective dose of propranolol.

Thus, although these data do not explain the mechanism whereby propranolol and pronethalol elevate hippocampal seizure threshold, they do provide evidence that the alteration in seizure susceptibility produced by these agents is not causally related to blockade of β-receptors.

CONCLUSION

The threshold of seizures induced by electrical stimulation of the hippocampus in unrestrained rats has been shown to be stable and reproducible over time. Furthermore, it is modified by typical anticonvulsants in a manner similar to that observed with other types of experimentally induced minimal seizures.

Both propranolol and pronethalol increased seizure threshold. These findings are consistent with those seen in earlier studies with l.f. ES and audiogenic seizures (11). However, other β-adrenergic blocking drugs do not have a threshold-elevating effect. Murmann *et al.* (36) have reported essentially similar results employing different seizure-inducing techniques. They found that propranolol and pronethalol reduced susceptibility of animals to maximal metrazol and maximal electroshock seizures, but reported L(+)- and D(−)-INPEA to be ineffective. MJ1999 has also been demonstrated by Chen *et al.* (10) and Lish *et al.* (17) to be incapable of altering both maximal and minimal electroshock seizures.

The results of the latter portion of this study largely substantiate the hypothesis of Leszkovszky and Tardos (37) and Murmann *et al.* (36) that certain β-adrenergic blocking compounds affect seizure expression by mechanisms other than β-blockade.

However, as stated previously, evidence supporting a catecholamine influence on seizure expression exists in the literature. Moreover, α-adrenergic blocking agents, phenoxybenzamine and phentolamine, have been reported to increase seizure susceptibility (10). In view of this, an investigation of the effects of α-adrenergic blocking agents on seizure threshold is currently in progress and should provide useful information.

REFERENCES

(1) J. G. Millichap, *Physiol. Pharmacol.*, **2,** 97(1965).

(2) E. A. Swinyard, N. R. Radhakrishnan, and L. S. Goodman, *J. Pharmacol. Exp. Ther.*, **138,** 337(1962).

(3) J. E. P. Toman and L. S. Goodman, *Physiol. Rev.*, **28,** 409 (1948).

(4) E. A. Swinyard, W. C. Brown, and L. S. Goodman, *J. Pharmacol. Exp. Ther.*, **106,** 319(1952).

(5) W. C. Brown, D. O. Schiffman, E. A. Swinyard, and L. S. Goodman, *ibid.*, **107,** 273(1953).

(6) K. Schlesinger, W. Boggan, and D. X. Freedman, *Life Sci.*, **4,** 2345(1965).

(7) C. L. Scudder, A. G. Karczmar, G. M. Everett, J. E. Gibson, and M. Rifkin, *Int. J. Neuropharmacol.*, **5,** 343(1966).

(8) E. H. Jenny, *Fed. Proc.*, **13,** 370(1954).

(9) J. P. Cooke, *Texas J. Sci.*, **13,** 235(1961).

(10) G. Chen, C. R. Ensor, and B. Bohner, *Arch. Int. Pharmacodyn.*, **172,** 183(1968).

(11) P. N. Yeoh and H. H. Wolf, *J. Pharm. Sci.*, **57,** 340(1968).

(12) L. Almirante and W. Murmann, *J. Med. Chem.*, **9,** 650 (1966).

(13) J. F. R. Konig and R. A. Klippel, "The Rat Brain," 1st ed., Williams and Wilkins, Baltimore, Md., 1963.

(14) H. Kluver and E. Barrera, *J. Neuropath. Exp. Neurol.*, **12,** 400(1953).

(15) J. T. Litchfield and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96,** 99(1949).

(16) A. Goldstein, "Biostatistics," Macmillan, New York, N. Y., 1964, p. 184.

(17) P. M. Lish, J. H. Weikel, and K. W. Dungan, *J. Pharmacol. Exp. Ther.*, **149,** 161(1965).

(18) H. H. Wolf, E. A. Swinyard, and L. S. Goodman, *J. Pharm. Sci.*, **51,** 74(1962).

(19) J. M. R. Delgado and L. Mihailovic, *Ann. N. Y. Acad. Sci.*, **64,** 644(1956).

(20) W. Schallek and A. Kuehn, *Proc. Soc. Exp. Biol. Med.*, **112,** 813(1963).

(21) R. R. J. Strobos and E. V. Spudis, *Arch. Neurol.*, **2,** 399 (1960).

(22) H. Gangloff and M. Monnier, *Electroencephalog. Clin. Neurophysiol.*, **9,** 43(1957).

(23) F. Morrell, W. Bradley, and M. Ptashne, *Neurology*, **9,** 492(1959).

(24) J. E. P. Toman and J. D. Taylor, *Epilepsia*, **1** (3rd series), 31(1952).

(25) D. M. Woodbury, *J. Pharmacol. Exp. Ther.*, **115,** 74(1955).

(26) J. E. P. Toman, in "The Pharmacological Basis of Therapeutics," 3rd ed., L. S. Goodman and A. Gilman, Macmillan, New York, N. Y., 1965, p. 215.

(27) D. W. Esplin and B. Zablocka, *Epilepsia*, **10,** 193(1969).

(28) D. W. Esplin and E. M. Curto, *J. Pharmacol. Exp. Ther.*, **121,** 457(1957).

(29) A. Kreindler, *Progr. Brain Res.*, **19,** 160(1965).

(30) A. Vernadakis and D. M. Woodbury, *Epilepsia*, **10,** 163 (1969).

(31) J. W. Black, A. F. Crother, R. G. Shanks, L. H. Smith, and A. C. Dornhorst, *Lancet*, **I,** 1080(1964).

(32) C. Raper and J. Wale, *Europ. J. Pharmacol.*, **3,** 279(1968).

(33) J. W. Black and J. S. Stephenson, *Lancet*, **II,** 311(1962).

(34) A. Morales-Aguilers and E. M. Vaughan Williams, *Brit. J. Pharmacol.*, **24,** 332(1965).

(35) P. Somani and B. K. B. Lum, *J. Pharmacol. Exp. Ther.*, **147,** 194(1965).

(36) W. Murmann, L. Almirante, and M. Saccani-Guelfi, *J. Pharm. Pharmacol.*, **18,** 317(1966).

(37) G. Leszkovszky and L. Tardos, *ibid.*, **17,** 518(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 2, 1969, from the *Division of Pharmacology, College of Pharmacy, Ohio State University, Columbus, OH 43210*

Accepted for publication March 4, 1970.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Supported by U. S. Public Health Service Grant No. MH-07397-06.

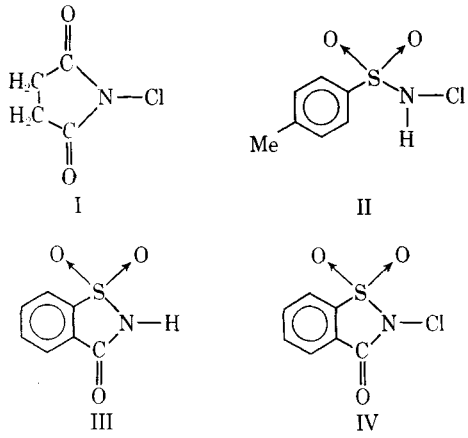
N-Chlorosaccharin as a Possible Chlorinating Reagent: Structure, Chlorine Potential, and Stability in Water and Organic Solvents

HAN-SON DAWN*, IAN H. PITMAN, TAKERU HIGUCHI†, and STEPHEN YOUNG*

Abstract □ *N*-Chlorosaccharin is shown by its low chlorine potential (pK_{cp} = 4.85 at 25°) to be a stronger chlorinating reagent in water than the commonly used chloramine-T or *N*-chlorosuccinimide. Its possible usefulness as a detoxifying or chlorinating reagent was further indicated by its solubility and stability in a variety of solvents. The mechanism of a reaction in which the imide bond of *N*-chlorosaccharin was cleaved in aqueous hypochlorite solution was also investigated.

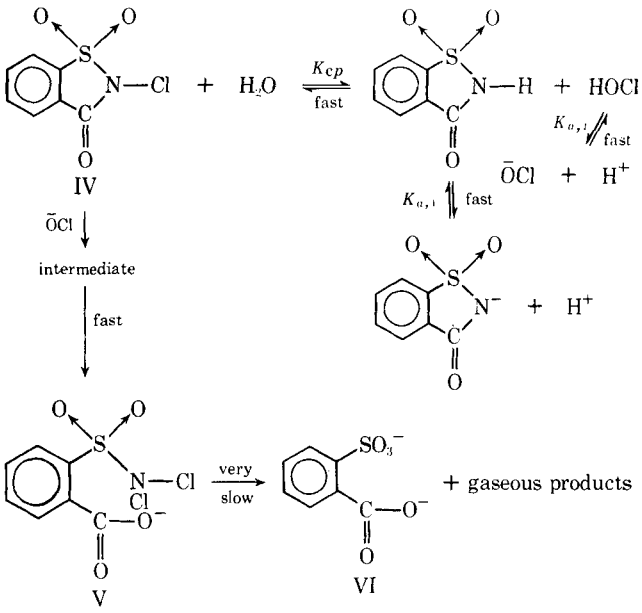
Keyphrases □ *N*-Chlorosaccharin—synthesis □ Chlorine potential—*N*-chlorosaccharin □ Hypochlorite solution—*N*-chlorosaccharin degradation □ IR spectrophotometry—identity □ UV spectrophotometry—identity □ Iodometric titration—analysis

N-Chloro imides [*e.g.*, *N*-chlorosuccinimide (I)] (1) and sulfonamides [*e.g.*, chloramine-T (II)] (2) are widely used as chlorinating and oxidizing reagents. The chlorinating power of these molecules, expressed as their chlorine potential, pK_{cp},[1] has been shown (3) to be related to the acidity of their nonchlorinated conjugates, with the stronger chlorinating agent being derived from the stronger acid. Thus, because saccharin (III) is a stronger acid (pKa = 1.31) (4) than succinimide (pKa = 9.62) (5) or *p*-tolylsulfonamide (pKa = 10.3) (6), it was expected that *N*-chlorosaccharin (IV) would be an even stronger chlorinating reagent than I or II.



I II III IV

When attempting to determine the K_{cp} value of IV, it was found that other reactions were occurring beside the transfer of positive chlorine between IV and water. These included acid dissociation of saccharin, cleavage of the imide bond of IV by the hypochlorite ion, formation of *N*,*N*-dichloro-*o*-sulfamylbenzoic acid (V) (and possibly the monochloro derivative), and slow decomposition of V to yield *o*-carboxylbenzenesulfonic acid (VI) and gaseous compounds. The overall reaction scheme is believed to be as shown in Scheme I.



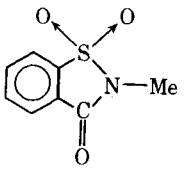
Scheme I

In this communication, evidence is reported for this reaction scheme together with values of the equilibrium constant, K_{cp}, and second-order rate constant, k_1. Approximate values of the solubility of IV in carbon tetrachloride, ethyl acetate, acetone, 1,4-dioxane, and chloroform were also determined, and the stability of its solutions in these solvents and methanol was investigated.

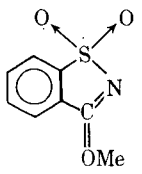
STRUCTURE AND SOLUBILITY OF *N*-CHLOROSACCHARIN

A chlorinated derivative of saccharin was precipitated (7) as a white crystalline powder (m.p. 148–150°) when chlorine gas was passed into cold aqueous solutions of saccharin. This substance was confirmed to be *N*-chlorosaccharin by elemental analysis, iodometric estimation of its positive chlorine content (one equivalent of positive chlorine per molecule), and from a comparison of its IR spectrum to those of saccharin (III), *N*-methylsaccharin (VII), and *o*-methylsaccharin (VIII). The conclusion that the chlorine in the molecule had substituted the imide proton of saccharin was deduced from the facts that: (*a*) the substance liberated positive chlorine in water (this would not be expected if the chlorine atom was substituted in the benzene ring), and (*b*) its IR spectrum contained no peaks above 3000 cm.$^{-1}$ (*i.e.*, in the N—H and O—H stretching region). The conclusion that it was *N*-chlorosaccharin and not the isomeric *o*-chloro derivative came from the observation that its IR spectrum contained a strong peak in the carbonyl stretching fre-

[1] For a chloro compound, R_2NCl, $pK_{cp} = -\log_{10} K_{cp}$, where K_{cp} is the equilibrium constant for the reaction $R_2NCl + H_2O \rightleftharpoons R_2NH + HOCl$.



VII



VIII

quency range (at 1750 cm.$^{-1}$). A similar carbonyl stretching peak was present in the spectra of saccharin (II) and *N*-methylsaccharin (1750 cm.$^{-1}$), but *o*-methylsaccharin did not contain any significant peaks between 1625 and 2000 cm.$^{-1}$. *o*-Chlorosaccharin would have been expected to have similar spectral characteristics to this latter compound (VIII).

Although *N*-chlorosaccharin had very low solubility in water (estimated solubility 0.1 g./l. at 25°), it was considerably more soluble in organic solvents as shown in Table I. UV spectral analysis and iodometric titration indicated that the *N*-chlorosaccharin content of the solutions in Table I did not change more than 3% during 3 hr. at 25°.

Chlorine Potential of *N*-Chlorosaccharin—A substance with a UV spectrum closely resembling that of *N*-chlorosaccharin was rapidly (complete within 30 sec.) formed when a solution of saccharin (1.16×10^{-4} *M*) in 6.8 *N* sulfuric acid was mixed with an equal amount of aqueous hypochlorous acid (1.34×10^{-3} *M*). When similar mixtures were made at higher pH values (in monochloroacetic acid–sodium monochloroacetate buffers), the spectra which were rapidly generated resembled those of mixtures of saccharin, sodium saccharin, and *N*-chlorosaccharin. These rapidly generated spectra did not change more than 3% during 3 min. at pH values below 3.8. They did, however, change appreciably over longer periods because of reactions that will be shown to involve cleavage of the imide bond of *N*-chlorosaccharin followed by slow decomposition of the ring-opened intermediate. Similar spectral behavior was observed when 0.5-ml. aliquots of a solution of *N*-chlorosaccharin in ethyl acetate (9.72×10^{-3} *M*) were mixed with 20 ml. of solutions of hypochlorous acid in various buffers. *N*-Chlorosaccharin was added as its ethyl acetate solution because of its slow rate of dissolution in water.

These initial rapid reactions are believed to be due to the establishment of the equilibria:

$$SH + HOCl \underset{}{\overset{1/K_{cp}}{\rightleftharpoons}} SCl + H_2O \qquad \text{(Eq. 1)}$$

$$SH \overset{K_{a,1}}{\rightleftharpoons} S^- + H^+ \qquad \text{(Eq. 2)}$$

where SH and S^- are the saccharin and saccharin anion, respectively; SCl is *N*-chlorosaccharin; and $K_{cp} = [SH][HOCl]/[SCl]$. The value of K_{cp} was calculated from spectrophotometric measurements of equilibrium concentrations of reactants and products following mixing of aqueous solutions of saccharin with buffered solutions of hypochlorous acid or of ethyl acetate solutions of *N*-chlorosaccharin with buffer solutions. Results from several series of experiments are included in Table II.

The mean value for K_{cp} in water was 1.4×10^{-5} *M* at 25° (excluding the K_{cp} values calculated from Experiments 6 and 7 where 2.4% ethyl acetate was present).

Thus, *N*-chlorosaccharin has a lower chlorine potential ($pK_{cp} = -\log K_{cp} = 4.85$) than *N*-chlorosuccinimide (8) ($pK_{cp} = 7.91$) and chloramine-T (8) ($pK_{cp} = 7.77$). Its value is very close to that which would be predicted (3) (5.27) on the basis that *N*-chlorosaccharin is a complex between a polarizable nitrogen containing anion (S^-) and the Lewis acid (Cl^+). For such a case the chlorine potential would be

Table I—Solubility of *N*-Chlorosaccharin at 25°

Solvent	Solubility, g./l.$^{-1}$
Carbon tetrachloride	4.3
Ethyl acetate	81.1
Chloroform	112.0
Acetone	173.0
1,4-Dioxane	287.0

Table II—Hydrolytic Constant,[a] K_{cp}, for *N*-Chlorosaccharin at 25°

Exp. No.	$[HOCl]_{added}$, *M*	$[Saccharin]_{added}$, *M*	$[SCl]_{added}$, *M*	$K_{cp} \times 10^5$ *M*
1	7.23	1.81	—	1.4
2	10.6	1.77	—	1.4
3	13.8	1.73	—	1.3
4	9.55	3.54	—	1.3
5	4.66	2.72	—	1.4
6[b]	—	0.379	1.58	1.8
7[b]	—	0.948	1.74	2.0

[a] Calculated as described in *Experimental* section. [b] Solutions contain 2.4 vol.-% ethyl acetate.

expected to be related to the pKa of saccharin by the identity $pK_{cp} = 0.28\ pKa + 4.90$.

Degradation of *N*-Chlorosaccharin in Aqueous Hypochlorite Solution—The previously mentioned changes in the UV spectrum of an equilibrium mixture of saccharin, saccharin anion, and *N*-chlorosaccharin increased in rate when the pH of the solution was raised or when the concentration of hypochlorous acid was increased. These changes are believed to be due to a relatively fast cleavage of the imide bond of *N*-chlorosaccharin followed by a slower decomposition of the chlorinated *o*-sulfamylbenzoic acid. Results in Fig. 1 show the change in UV absorbance at 282 mμ in a 2-cm. cell plotted against time following the mixing of a solution of saccharin (5.60×10^{-4} *M*) in an acetic acid–sodium acetate buffer at pH 3.9 with an equal volume of aqueous hypochlorous acid (1.05×10^{-2} *M*). After the reaction had proceeded for longer than 40 min., unidentified gases were evolved. The UV spectrum, which was measured 3 min. after mixing the reactants, is shown in Fig. 2 to be almost identical to that obtained 3 min. after mixing equal volumes of solutions of *o*-sulfamylbenzoic acid (6.00×10^{-4} *M*) and hypochlorous acid (1.37×10^{-2} *M*).

The reactions occurring in the hypochlorous acid–saccharin system could be quenched by adding drawn samples to excess sodium bisulfite to reduce the positive chlorine. When the drawn samples were added to sodium bisulfite at progressively longer time intervals after the reactants had been mixed, and the solution was placed on a silicic acid–sulfuric acid column, saccharin and *o*-sulfamylbenzoic acid (identified by their UV spectra in chloroform and their retention time on the column as compared to authentic materials) were eluted with a chloroform–butanol mixture. The amount of saccharin obtained decreased, and the amount of *o*-sulfamylbenzoic acid increased initially and then decreased as the time elapsed before the addition to sodium bisulfite increased. Results of the amounts of saccharin and *o*-sulfamylbenzoic acid obtained are plotted as a function of time before addition to sodium bisulfite in Fig. 3. *o*-Carboxylbenzenesulfonic acid, which was also expected to be a product of the degradation of *N*-chlorosaccharin, could not be eluted from the column with chloroform–butanol mixtures because of its high acidity. It was, however, eluted with aqueous phosphate buffer after the saccharin and *o*-sulfamylbenzoic acid had been removed. It was identified by the similarity of its UV spectra in 1 *M* HCl and 0.1 *M* NaOH to that of authentic material. Thus, the changes in UV absorbance and product analysis are consistent with the proposed reaction scheme for degradation of *N*-chlorosaccharin.

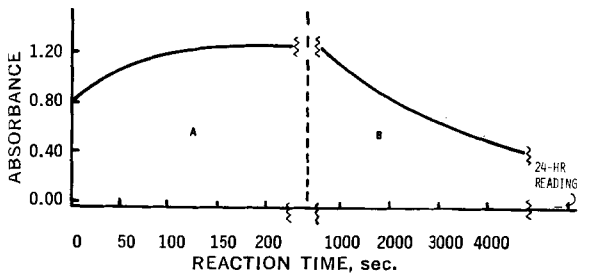


Figure 1—*Observed change in absorbance at 282 mμ in a 2-cm. cell containing initially 5.25×10^{-3} M HOCl and 2.8×10^{-4} M saccharin in pH 3.9, 0.03 M acetate buffer at 25°.*

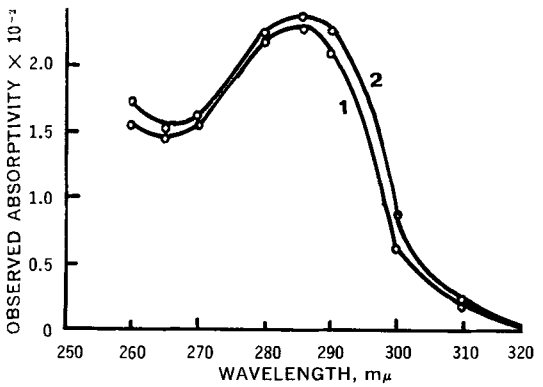


Figure 2—*UV spectra:* (1) *Mixture of saccharin and hypochlorous acid in 3 × 10⁻² M acetate buffer after 3 min. (with same concentration of hypochlorous acid as reference), pH 4.9.* (2) *Mixture of hypochlorous acid and o-sulfamyl benzoic acid (with same concentration of hypochlorous acid as reference), pH 3.30. Observed absorptivities were calculated by dividing absorbances by [saccharin]added or [o-sulfamyl benzoic acid] and the pathlength of the cell.*

Kinetic data for the ring-cleavage reaction were obtained from measurements of changes in UV absorbance at 282 mμ following addition of 1 ml. of solutions of saccharin ($1.74 - 2.18 \times 10^{-3}$ *M*) and 1 ml. hypochlorous acid ($5.78 - 2.88 \times 10^{-2}$ *M*) to 4.6 ml. acetate buffer of ionic strength, I, 0.5 *M*. Under these conditions (where the initial concentration of hypochlorous acid, $[HOCl]_{added}$, was greatly in excess of the added concentration of saccharin, $[SH]_{added}$), the ring-cleavage reaction was pseudo-first-order; a pseudo-first-order rate constant, k_1', was calculated by taking the maximum absorbance as the absorbance of the pure product. Because it is not known whether this product was *N,N*-dichloro-*o*-sulfamylbenzoic acid, *N*-chloro-*o*-sulfamylbenzoic acid, or an isomer of these, an unspecified intermediate was included in the reaction scheme. However, it is believed that irreversible ring cleavage was the rate-determining step in the reaction. On this basis, the rate equation for consumption of total saccharin species, SH_{total} $\{[SH_{total}] = [SH] + [S^-] + [SCl]\}$ would be:

$$\text{rate} = \frac{k_1 K_{a,2} [HOCl]_{added}^2 [SH_{total}]}{\{[H^+][HOCl]_{added} + K_{a,1} K_{cp} + K_{cp} [H^+]\}} \quad \text{(Eq. 3)}$$

where $K_{a,1}$ and $K_{a,2}$ are the acid dissociation constants of saccharin and hypochlorous acid ($K_{a,2} = 2.82 \times 10^{-8}$) (9). Thus the second-order rate constant would be related to the pseudo-first-order rate constant k_1' at different pH values and added hypochlorous acid concentrations by the identity

$$k_1 = k_1' \frac{[H^+][HOCl]_{added} + K_{a,1} K_{cp} + K_{cp} [H^+]}{K_{a,2} [HOCl]_{added}^2} \quad \text{(Eq. 4)}$$

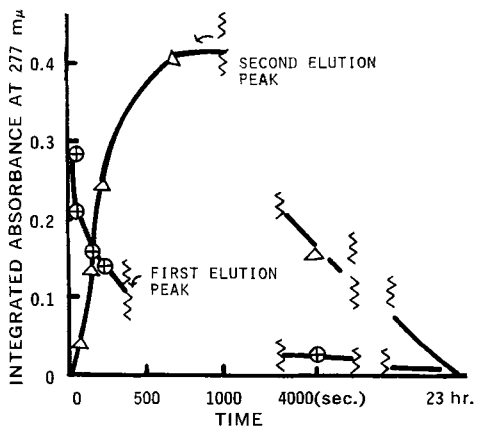


Figure 3—*Integrated absorbance areas under the first and second elution peaks obtained at various reaction times for mixture of hypochlorous acid and saccharin. The system initially contained 3.90 × 10⁻³ M hypochlorous acid and 4.85 × 10⁻⁴ M saccharin, pH 3.4.*

Table III—Rate Constants for Cleavage of Imide Bond of *N*-Chlorosaccharin

pH	$[Saccharin]_{added} \times 10^4$ *M*	$[HOCl]_{added} \times 10^3$ *M*	$10^2 k_1'$ Sec.$^{-1}$	$10^{-5} k_1$ M^{-1} Sec.$^{-1}$
5.4	3.30	7.28	3.63	1.9
	3.30	5.82	2.32	1.8
	3.30	5.09	1.98	2.0
	3.30	4.36	1.52	2.1
4.9	3.30	7.28	3.30	1.6
	3.30	5.82	2.32	1.7
	3.30	4.36	1.35	1.8
	2.64	5.82	2.24	1.7
	2.64	8.74	4.88	1.7
4.3	3.30	7.28	2.77	1.6
	3.30	5.82	1.79	1.6
	3.30	5.09	1.63	1.8
	3.30	4.36	1.26	1.9
Av.				1.8 ± 0.2

Values of k_1 calculated in this way are included in Table III.

The consistency of values of k_1 in Table III is strong evidence in support of the proposed reaction scheme. The kinetics of degradation of the chlorinated *o*-sulfamylbenzoic acid were not determined.

The overall reaction scheme is essentially the same as that proposed by Chattaway (7) to account for the degradation of *N*-chlorosaccharin in solutions of caustic alkalis.

Stability of *N*-Chlorosaccharin in Methanol—Methanolysis of *N*-chlorosaccharin to saccharin and methyl hypochlorite was a more favorable reaction than hydrolysis to saccharin and hypochlorous acid. Thus, when *N*-chlorosaccharin was dissolved in anhydrous methanol, it was quantitatively converted to saccharin and methyl hypochlorite by the following reaction:

$$SCl + MeOH \rightarrow SH + MeOCl \quad \text{(Eq. 5)}$$

When this reaction was complete (after 20 min.), the titer of iodine against positive chlorine was within 6% of theoretical, indicating that the reaction mixture was still a potential chlorinating system.

The rate of consumption of *N*-chlorosaccharin and the rate of formation of saccharin were pseudo-first-order reactions; from measurements of changes in concentration with time, a pseudo-first-order rate constant, k_m^1, with a value 4.1×10^{-3} sec.$^{-1}$ at 25° was calculated. When known amounts of water were added to the methanol, the rate of conversion of *N*-chlorosaccharin to saccharin increased linearly as shown in Fig. 4. The catalytic rate constant for water was calculated from this plot to be 10^{-2} M^{-1} sec.$^{-1}$. No further studies were undertaken to determine the mechanism of this reaction.

The chemical literature contains what the authors believe to be erroneous references (2, 9) to a reaction between *N*-chlorosaccharin and methanol to yield *N*-methylsaccharin. Although these references are apparently based on studies of Remsen and Dohme (10), the original work makes no mention of this reaction and treats instead the reaction between methanol and the product formed by reaction

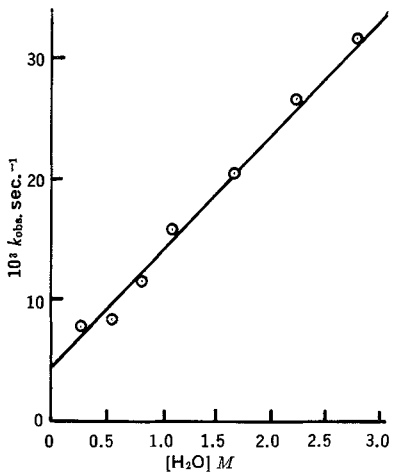


Figure 4—*Plot of* $k_{obs.}$ *against concentration of water in methanol for reaction of* N*-chlorosaccharin in aqueous methanol.*

Table IV—Determination of Hydrolytic Constant, K_{cp}, at 25° under the Condition where $[HOCl]_{added}$ Was Much Greater than $[SH]_{added}$[a]

pH	A_0	A	L[b]	Slope × 10^{-4}	Intercept × 10^{-4}	K_{cp} × 10^5 M	ϵ^{270}_{SCl}
2.30	0.611	0.950	3.47×10^3				
2.37	0.613	0.939	3.81×10^3				
2.48	0.616	0.925	4.62×10^3				
2.69	0.619	0.819	6.79×10^3	−7.22	7.19	1.38	2652
2.94	0.622	0.857	1.00×10^4				
3.34	0.624	0.774	1.55×10^4				
3.67	0.625	0.774	2.03×10^4				

[a] $[SH]_{added} = 1.77 \times 10^{-4}$ M, $[HOCl]_{added} = 1.06 \times 10^{-3}$ M. Buffer concentration = 0.04 M (monochloroacetate). Absorbance measured at 270 mμ in a 2-cm. cell. [b] $L = [(A - A_0)(K_{a,1} + [H]^+)]/([H^+][HOCl]_{added})$.

of saccharin and phosphorous pentachloride at 70–75°. This latter product is presumably pseudosaccharin chloride (11, 12) and not chlorosaccharin.

CONCLUSION

N-Chlorosaccharin is likely to have only limited usefulness as a chlorinating agent in water because of its poor solubility and slow rate of dissolution. On the other hand, it is readily soluble in several organic solvents and, because it readily releases its positive chlorine in water, it is expected to be a good chlorinating agent in these solvents. The reactions of *N*-chlorosaccharin in aqueous hypochlorite solution or methanol do not reduce the availability of positive chlorine appreciably and these solutions also should be useful chlorinating systems.

EXPERIMENTAL

Reagents and Equipment—All reagents used were of the highest grade commonly available and were normally subjected to further purification before use. Saccharin (Aldrich) was recrystallized from acetone (m.p. 228–229°). *N*-Chlorosaccharin was prepared and purified according to Chattaway (7). The purity of the recrystallized product (m.p. 148–150°), determined iodometrically, was 98% based on active chlorine content. Hypochlorous acid was prepared essentially according to Higuchi and Hasegawa (13). Commercially available bleaching solution (containing 5% sodium hypochlorite) was acidified with boric acid and distilled under vacuum at 50°. The distillate was redistilled under vacuum at the same temperature. A diluted solution of the second distillate was used in the reactions. The diluted solutions fell in the concentration range of 2×10^{-2} to 4×10^{-2} M. Monochloroacetic acid (J. T. Baker, A. R. grade) was recrystallized from benzene before use. Water used throughout this study was finally distilled from acid permanganate to remove possible volatile nitrogenous contaminants.

Table V—Determination of Hydrolytic Constant, K_{cp}, at 25° in Water under the Condition where $[HOCl]_{added}$ Was Not Much Greater than $[SH]_{added}$[a]

pH	A_0	A	A_∞	K_{cp} × 10^5 M
2.30	0.861	1.245	1.453	1.5
2.40	0.866	1.236	1.453	1.3
2.47	0.868	1.217	1.453	1.4
2.59	0.872	1.187	1.453	1.4
2.81	0.867	1.140	1.453	1.3
3.06	0.880	1.070	1.453	1.4
3.20	0.881	1.032	1.453	1.4
3.30	0.882	1.015	1.453	1.4
3.47	0.882	0.976	1.453	1.5
			Av.	1.4 ± 0.1

[a] $[SH]_{added} = 2.72 \times 10^{-4}$ M, $[HOCl]_{added} = 4.66 \times 10^{-4}$ M. Buffer concentration = 0.04 M (monochloroacetate). Absorbances were measured at 270 mμ in a 2-cm. cell.

Table VI—Determination of Hydrolytic Constant, K_{cp}, at 25° under the Condition where *N*-Chlorosaccharin Was Added to Buffered Solutions of Saccharin[a]

pH	A_0	A	A_∞	K_{cp}[b] × 10^5 M
2.29	0.604	0.760	1.028	1.9
2.33	0.606	0.753	1.028	2.0
2.47	0.611	0.751	1.028	1.6
2.70	0.617	0.707	1.028	2.1
3.02	0.621	0.683	1.028	1.8
3.30	0.623	0.671	1.028	1.4
3.46	0.624	0.658	1.028	1.5
3.57	0.624	0.646	1.028	2.0
			Av.	1.8 ± 0.3

[a] $[SCl]_{added} = 1.58 \times 10^{-4}$ M, $[SH]_{added} = 3.79 \times 10^{-5}$ M. Buffer concentration = 0.05 M (monochloroacetate). All solutions contained 2.4 vol.-% of ethyl acetate. Absorbances were measured at 270 mμ in a 2-cm. cell. [b] Using Eq. 9 and 10 to calculate K_{cp}, the values 1.96×10^{-4} M (added concentration of SCl + added concentration of SH) and 1.58×10^{-4} M (added concentration of SCl) were used for the terms "$[SH]_{added}$" and "$[HOCl]_{added}$."

Methanol (Allied Chemical, reagent grade) was dried according to Vogel (14). *o*-Methylsaccharin (VIII) was prepared by following the procedure used by Meadow and Reid (12) (m.p. 180–181°). *N*-Methylsaccharin (VII) was synthesized according to Brackett (15) (m.p. 131–132°). 1,4-Dioxane (Allied Chemical, reagent grade) was purified according to Vogel (14).

Cary 11, 14, or 15 recording spectrophotometers, which were thermostated at 25.0 ± 0.2° by circulating water, were used to measure absorbances. pH values were measured using a Corning 12 research pH meter.

CALCULATIONS AND PROCEDURES

Solubility of *N*-Chlorosaccharin in Organic Solvents—Solubility was estimated by measuring the maximum concentration of *N*-chlorosaccharin that could be dissolved by stirring with the solvent in a sealed vessel at 25°. Samples of solution were removed every 15 min. and analyzed by UV spectrophotometry and iodometric titration for *N*-chlorosaccharin and positive chlorine, respectively. When the concentration of the solution in the presence of undissolved crystals did not change during 45 min., it was assumed to be saturated.

Acid Dissociation Constant and Molar Absorptivity of Saccharin—Because saccharin is a strong acid, the absorbance of the neutral molecule in water was difficult to measure. Its acid dissociation constant, $K_{a,1}$, could not be calculated directly by using the relationship:

$$K_{a,1} = \frac{(A - A_{SH})\,[H^+]}{(A_S^- - A)} \qquad \text{(Eq. 6)}$$

where A is the absorbance of an equilibrium solution of saccharin and saccharin anion, and A_{SH} and A_S^- are the absorbances of the same solution at pH values where 99% of the saccharin is in the form of its neutral molecule and anion, respectively. Although the value of A_{SH} could not be measured, values of A_S^-, A (both in a 5-cm. cell at 270 mμ) and $[H^+]$ were measured for the equilibrium solutions formed when 1-ml. aliquots of aqueous solutions of saccharin (3.80×10^{-3} M) were added to 20 ml. of solutions of HCl and KCl (total concentration 2×10^{-1} M) at 25°. Plots of $(A_S^- - A)/[H^+]$ against A gave a straight line with a slope of 20.4 and an intercept on the Y-axis when $A = O$ of 21.7. From Eq. 6 it can be seen that the slope of this line and the intercept of the Y-axis are related to $K_{a,1}$ and the molar absorptivity of saccharin, ϵ^{270}_{SH}, by the identities

$$K_{a,1} = \frac{1}{\text{slope}} \quad \text{and} \quad \epsilon^{270}_{SH} = -\frac{\text{intercept}}{\text{slope} \times [SH]_{added} b} \qquad \text{(Eq. 7)}$$

where b is the pathlength of the spectrophotometer cell. From results of the experiment, values of $K_{a,1} = 4.91 \times 10^{-2}$ (lit. 2.5×10^{-2} at 18°) (16) and $\epsilon^{270}_{SH} = 1119$ in water at 25.0 ± 0.2° were calculated.

Determination of the Hydrolytic Constant, K_{cp} Value, for *N*-Chlorosaccharin—The K_{cp} value was calculated from the ab-

sorbance, A, of an equilibrium solution of saccharin (SH), saccharin anion (S^-), *N*-chlorosaccharin (SCl), and hypochlorous acid by using the equation:

$$\frac{(A - A_0)(K_{a,1} + [H^+])}{[H^+]\{[HOCl]_{added} - [(A - A_0)/(A_\infty - A_0)][SH]_{added}\}} = \frac{A_\infty}{K_{cp}} - \frac{A}{K_{cp}} \quad \text{(Eq. 8)}$$

A_0 and A_∞ were the absorbances of the solution under conditions where >99% of the added saccharin was in the form of saccharin plus saccharin anion and *N*-chlorosaccharin, respectively, and $K_{a,1}$ was the acid dissociation constant of saccharin. Values of A_0, A, and $[H^+]$ could be measured directly, but values of A_∞ could not be because of the difficulty of working at pH values where the saccharin would not be dissociated to an appreciable extent. Also, A_∞ could not be calculated from the identity

$$A_\infty = \epsilon_{SCl}[SH]_{added}\,b + \epsilon_{HOCl}([HOCl]_{added} - [SH]_{added})b \quad \text{(Eq. 9)}$$

until a sufficiently accurate value of the molar absorptivity of *N*-chlorosaccharin, ϵ_{SCl}, was available.

Inspection of Eq. 8 shows that when $[HOCl]_{added}$ is much larger than $[SH]_{added}$ and at a wavelength where $\epsilon_{SCl} > \epsilon_{SH}$ or ϵ_{S^-} a plot of

$$\frac{(A - A_0)(K_{a,1} + [H^+])}{[H^+][HOCl]_{added}}$$

against A at different pH values would give a straight line from which values of K_{cp} [= − (1/slope)] and A_∞ [= (−intercept/slope)] could be calculated. A value of ϵ_{SCl} could then be calculated from this A_∞ value and used to calculate A_∞ values for subsequent experiments where $[HOCl]_{added}$ was not much greater than $[SH]_{added}$. This was the method used to compute K_{cp} values. Typical sets of results for experiments carried out under conditions where: (*a*) $[HOCl]_{added} \gg [SH]_{added}$; (*b*) $[HOCl]_{added}$ was not much greater than $[SH]_{added}$, and (*c*) *N*-chlorosaccharin was added to buffered solution of saccharin are shown in Tables IV, V, and VI, respectively.

Determination of the Rate Constant for the Cleavage of Imide Bond of *N*-Chlorosaccharin—A saccharin stock solution, acetate buffer solutions (0.5 *M* with ionic strength adjusted to 0.5 by adding sodium sulfate) of different pH values, and hypochlorous acid of various concentrations were prepared and brought to 25°. Then 4.6 ml. of buffer solution of desired acidity was mixed with 1.0 ml. of saccharin solution in a 2-cm. cell. Into this cell 1.0 ml. of hypochlorous acid of desired concentration was injected. The cell was quickly shaken and placed in a spectrophotometer. The change of absorbance at 282 mμ was then recorded. At the end of the fast reaction (after the absorbance had passed its maximum reading), the pH of the mixture was determined.

Chromatographic Separation of the Components of the Reaction Mixture of Saccharin and Hypochlorous Acid—A partition column was prepared as follows: 20 ml. of 2 *N* sulfuric acid was added to 20 g. of silicic acid and mixed well. A slurry was made with 40 ml. of chloroform and packed into a glass column (50 cm. long, 2 cm. in diameter) containing a plug of glass wool and having a Teflon stopcock.

A 10-ml. portion of the reaction mixture was added to 2 ml. of 4 *N* sulfuric acid which contained sufficient sodium bisulfite to quench the reaction by reducing the positive chlorine and, consequently, *N*-chlorospecies were converted into their conjugate nonchlorinated derivatives. Five milliliters of the quenched reaction mixture was then chromatographed according to the following procedure: 5 g of silicic acid was added to the mixture and a slurry was made with 10 ml. of chloroform. The slurry was then packed on the top of the column.

Saccharin was eluted with 100 ml. of 2% butanol in chloroform and *o*-sulfamyl benzoic acid with 100 ml. of 6% butanol in chloroform. The integrated absorbance was obtained by adding the absorbances of all fractions (10 ml. eluate in each fraction) that contained the same component.

Alcoholysis of *N*-Chlorosaccharin to Saccharin in Methanol—A stock solution of *N*-chlorosaccharin was made up in ethyl acetate (4.00×10^{-2} *M*). Then 0.02 ml. of this solution was injected into a 1-cm. stoppered cell containing 2.0 ml. of methanol or aqueous methanol. The disappearance of *N*-chlorosaccharin was then followed spectrophotometrically at 276 mμ.

REFERENCES

(1) F. L. Lambert, W. D. Ellis, and R. J. Parry, *J. Org. Chem.*, **30,** 304(1965); L. F. Fieser, and M. Fieser, "Reagents for Organic Synthesis," Wiley, New York, N. Y., 1967.

(2) C. M. Suter, "The Organic Chemistry of Sulfur," Wiley, New York, N. Y., 1944.

(3) I. H. Pitman, H. Dawn, A. A. Hussain, and T. Higuchi, *J. Chem. Soc. (B)*, **1969,** 1230.

(4) Unpublished data.

(5) H. F. Walton and A. A. Shilt, *J. Amer. Chem. Soc.*, **74,** 4995(1952).

(6) T. Higuchi, K. Ikeda, and A. A. Hussain, *J. Chem. Soc. (B)*, **1967,** 546.

(7) F. D. Chattaway, *J. Chem. Soc.*, **87,** 1882(1905).

(8) T. Higuchi, A. A. Hussain, and I. H. Pitman, *J. Chem. Soc. (B)*, **1969,** 626.

(9) S. S. Verma and R. C. Srivastava, *Indian J. Chem.*, **4,** 445 (1966).

(10) I. Remsen and A. R. L. Dohme, *Amer. Chem. J.*, **11,** 345 (1889).

(11) J. A. Jesurun, *Ber.*, **26,** 2286(1893).

(12) J. R. Meadow and E. E. Reid, *J. Amer. Chem. Soc.*, **65,** 457(1943).

(13) T. Higuchi and J. Hasegawa, *J. Phys. Chem.*, **69,** 796 (1965).

(14) A. I. Vogel, "Practical Organic Chemistry," 3rd ed., Wiley, New York, N. Y., 1962.

(15) R. N. Brackett, *Amer. Chem. J.*, **9,** 407(1887).

(16) I. M. Kolthoff, *Rec. Trav. Chim. Pays-Bas*, **44,** 629(1925).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 18, 1969, from the *Pharmaceutical Chemistry Laboratory, School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication February 5, 1970.

Presented before the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This work was supported in part by Department of the Army, Contract DAAA-15-67-C-0638.

* Present address: Wyeth Laboratories Inc., Research and Development Dept., Philadelphia, PA 19101

† To whom all correspondence should be addressed.

Normal and Promoted Gastrointestinal Absorption of Water-Soluble Substances I: Induced Rapidly Reversible Hyperabsorptive State in the Canine Fundic Stomach Pouch

W. W. DAVIS, R. R. PFEIFFER, and J. F. QUAY

Abstract □ Certain surface-active agents placed in a Thomas canine fundic pouch in buffered solutions influence the absorption of soluble drugs (antibiotics). Such agents induce a rapidly reversible hyperabsorptive state of the organ, resulting in blood levels of absorbed drug many times greater than control values. Effective surface-active agents may be nonionic, anionic, or zwitterionic. The influence of the surface-active agent is upon the organ and not upon the drug, as evidenced by the efficacy of the absorption promoter when it is employed and removed before the drug is introduced.

Keyphrases □ Absorption, antibiotics—surfactant effect □ Surfactant effect—antibiotic absorption □ Gastric fundus pouch, dog—absorption, antibiotics □ Plasma levels—antibiotics □ Microbiological analysis—plasma, antibiotic concentration

Detergents and bile salts interfere with the normal barrier to the movements of Na^+, K^+, and H^+ in the gastric mucosa (1). Many other reports in the literature describe examples of surface-active agents having a marked effect, either positive or negative, on passage of molecules through physiologic barriers (2–8).

Most of these observations, however, deal with translocation of substances under conditions complicated by insolubility of the compound (6), involvement of active transport processes (7, 8), or interaction of the surface-active agent with the penetrating species (4, 5). These complications prevent formulation of conclusions concerning the general nature of the influence of surfactants on the absorption of drugs from the gastrointestinal tract.

This and subsequent reports present observations regarding the general nature of the effects of surfactants on the absorption of drugs from the gastrointestinal tract. By restricting these studies to soluble drugs, the authors avoid variability due to differences in wetting, solubilization, and dispersion. In this initial study, the gastric fundic mucosa was used as a test membrane to confine the observations to a relatively simple system. By sometimes exposing the tissue first to surfactant and subsequently to the drug, the authors were able to separate the effects of surfactant–membrane interactions from those of surfactant–drug interactions.

Parenteral antibiotics were chosen for specific study because this class of drugs is readily detected in the blood by known quantitative methods. These compounds, furthermore, are ordinarily not well absorbed from the gastrointestinal tract. Their absorption in significant amounts would therefore be easier to demonstrate and be of greater practical interest than if the enhanced absorption of an already well-absorbed drug was the subject of study.

EXPERIMENTAL

Dogs were surgically prepared with Thomas-type pouches of the gastric fundus several months prior to the experimental period. Catheter access to the pouch permitted ready introduction and removal of solutions containing conditioning agents and drugs, either together or separately.

Dogs were fasted overnight before use so that observed gastric acid secretion was minimal. The secretory state, in addition to causing undesirable fluctuations in the pouch contents, was observed to oppose absorption where such absorption had been previously observed in the absence of secretion. In the early experiments, whenever the pouch contents were observed to be acidic, the animal was made nonsecreting by giving it 3 mg. of an anticholinergic drug[1] orally 30 min. before the experiment. This treatment had no apparent effect on either control or experimental results. To prevent the occasional inconsistencies attributable to the secretory state and to reduce the number of experimental variables, the treatment was subsequently made routine.

Solutions of sodium cephalothin[2] in pH 7.0 sodium phosphate buffer, with varying amounts of POE-24-cholesterol ether,[3] were placed in the pouch of a dog and replaced at approximately 40-min. intervals with a fresh solution. Heparinized samples of peripheral venous blood, removed periodically by venipuncture, were centrifuged; the plasma was assayed for microbiological activity of cephalothin by standard disk-plate tests.[4]

RESULTS AND DISCUSSION

Absorption of cephalothin from the gastric pouch was promoted by the presence of POE-24-cholesteryl ether. The plasma antibiotic level achieved was dose responsive to the pouch concentration of this nonionic surface-active agent (Fig. 1*a*) at constant antibiotic concentration.

Absorption of cephalothin from the gastric pouch under the same conditions was also promoted by many, but not all, other surface-active agents (Table I). The absorption of cephalothin was dose responsive to the concentration of coadministered sodium lauryl sulfate (Fig. 1*b*), but with the limitations to be discussed.

The absorption of cephaloridine,[5] an antibiotic related to cephalothin but amphoteric in nature, was also promoted by the presence of POE-20-oleyl ether[6]; the extent of absorption was again dependent on surfactant concentration (Fig. 1*c*).

The relationships between surfactant concentration and rate of drug absorption in these experiments illustrate an important aspect of the action of the surface-active agents. The critical micelle concentration (CMC) of sodium lauryl sulfate under comparable conditions is about 0.035% (12, 13), corresponding to the concentration at which the absorption response to increased surfactant concentra-

[1] α- and β-DL(1-Methyl-3-pyrrolidinyl)-α-phenyl-α-(2-thienyl)glycolate methyl bromide. Compound X in *Reference 9*.

[2] 2-(Thiophene acetamido)cephalosporanic acid (10).

[3] Solulan C-24, American Cholesterol Products, Inc., Edison, N. J. POE is used as an abbreviation for "polyoxyethylene."

[4] Cephalothin, methicillin, streptomycin, and penicillin G were assayed against *Bacillus subtilis* ATCC 6633. Cephaloridine and tylosin were assayed against *Sarcina lutea* PC1-1001-FDA. Tetracycline was assayed against *Bacillus megatherium*.

[5] 7-[α-(2-Thiophene)acetamido]-3-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine (11).

[6] Brij-98, Atlas Chemical Industries, Wilmington, Del.

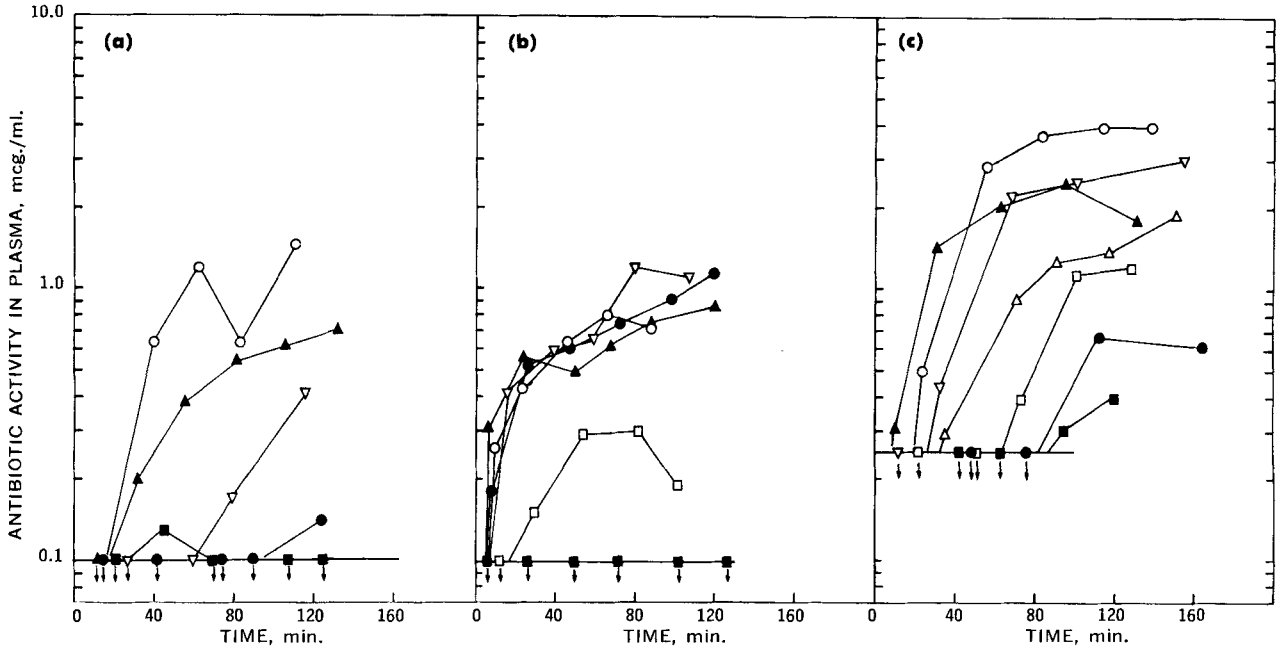


Figure 1—*Effect of surfactant concentration on the absorption of cephalosporin antibiotics from the dog fundic pouch. Pouch contents: 25 ml. 1% antibiotic and indicated concentration of surfactant, dissolved in isosmolar sodium phosphate buffer, pH 7.0. The horizontal lines are drawn at the minimum detection limit of the assay. Key: (a) Absorption of cephalothin promoted by POE-24-cholesteryl ether; surfactant concentration, w/v: ■, 0.0%; ●, 0.025%; ▽, 0.05%; ▲, 0.1%; and ○, 0.5%. (b) Absorption of cephalothin promoted by sodium lauryl sulfate; surfactant concentration, w/v: ■, 0.0%; □, 0.025%; ●, 0.05%; ▽, 0.2%; ▲, 0.4%; and ○, 0.8%. (c) Absorption of cephaloridine promoted by POE-20-oleyl ether; surfactant concentration, w/v: ■, 0.0%; ●, 0.15%; □, 0.031%; △, 0.062%; ▽, 0.125%; ▲, 0.25%; and ○, 0.5%.*

tion terminates. Therefore, the absorptive response to surfactant concentration develops only throughout the range in which the concentration of molecularly dispersed sodium lauryl sulfate is increasing. The absorptive response to nonionic surfactants (Figs. 1*a* and 1*c*) occurs over a wider range of surfactant concentration, in keeping with the continued increase in concentration of the molecularly dispersed species above the CMC which is a characteristic of nonionic surfactants. This dose response to molecularly dispersed surfactant is in contrast to the ability of surfactants to solubilize water-insoluble substances, which depends on and is proportional to the amount of surfactant in the micellar phase.

To characterize the onset of the hyperabsorptive state as a result specifically of contact of the stomach with the absorption promoter, POE-24-cholesteryl ether in buffered solution was placed in the pouch for periods from 5 to 60 min. and then removed. The pouch was rinsed several times with buffer (requiring about 5 min.), and then a solution of cephalothin (in buffer) without surfactant was introduced into the pouch. The resulting plasma levels of antibiotic activity rose more rapidly and to higher peak values as the duration of pretreatment with promoter was increased, demonstrating the gradual appearance of the hyperabsorptive state of the pouch with increasing time of exposure to the surfac-

Table I—Effect of Various Promoters on the Absorption of Several Antibiotics from the Dog Fundic Pouch

Antibiotic	Promoter	pH of Buffered Pouch Contents	Peak Plasma Level of Drug, mcg./ml.[a] Without Promoter[b]	With Promoter[c]
Methicillin	POE-24-cholesteryl ether	7.0	0.50	1.60
Streptomycin[d]	POE-24-cholesteryl ether	7.0	2.3	10.0
Streptomycin[d]	POE-24-cholesteryl ether	3.0	0.20	2.80
Tetracycline	POE-24-cholesteryl ether	2.3[e]	0.25	2.30
Penicillin G	POE-24-cholesteryl ether	7.0	0.39	2.45
Tylosin[d]	POE-24-cholesteryl ether	7.0	0.10	1.00
Cephaloridine	POE-24-cholesteryl ether	7.0	0.26	2.10
Cephaloridine	POE-32-cholesteryl ether[f]	7.0	0.26	3.00
Cephaloridine	POE-20-cetyl ether[g,h]	7.0	0.26	9.40
Cephaloridine	POE-10-stearyl ether[g,i]	7.0	0.26	3.85
Cephaloridine	POE-20-stearyl ether[g,j,k]	7.0	0.26	2.50
Cephaloridine	POE-20-oleyl ether	7.0	0.26	5.40
Cephaloridine	3-Carboxy-*N*-(*n*-tetradecyl)pyridinium·HCl[l]	7.0	0.26	2.75
Cephaloridine	Sodium lauryl sulfate[m]	7.0	0.26	1.65
Cephaloridine	Sodium lauryl sulfate[m]	4.0[n]	0.20	2.60
Cephalothin	POE-20-oleyl ether	7.0	0.10	2.40
Cephalothin	POE-2-stearyl ether[g,o]	7.0	0.10	0.14
Cephalothin	POE-X-stearyl ether[g,p]	7.0	0.10	0.20
Cephalothin	Polysorbate-40	7.0	0.10	0.22

[a] The results are the highest value observed during 120 min. following administration to a single dog with and without promoter. [b] Pouch contents: 25 ml. 1% antibiotic in isosmolar sodium phosphate, pH 7.0, except as noted. [c] Pouch contents: 25 ml. 1% antibiotic, 0.5% promoter, in isosmolar sodium phosphate, except as noted. [d] Concentration 2%. [e] Isosmolar citric acid–HCl. [f] Solulan C-32, American Cholesterol Products, Inc., Edison, N. J. [g] Atlas Chemical Industries, Wilmington, Del. [h] Brij-58. [i] Brii-76. [j] Brij-78. [k] Concentration 0.25%. [l] *Reference 16*. [m] Concentration 0.20%. [n] Isosmolar citrate–phosphate (sodium) [o] Brij-72. [p] Myrj 51.

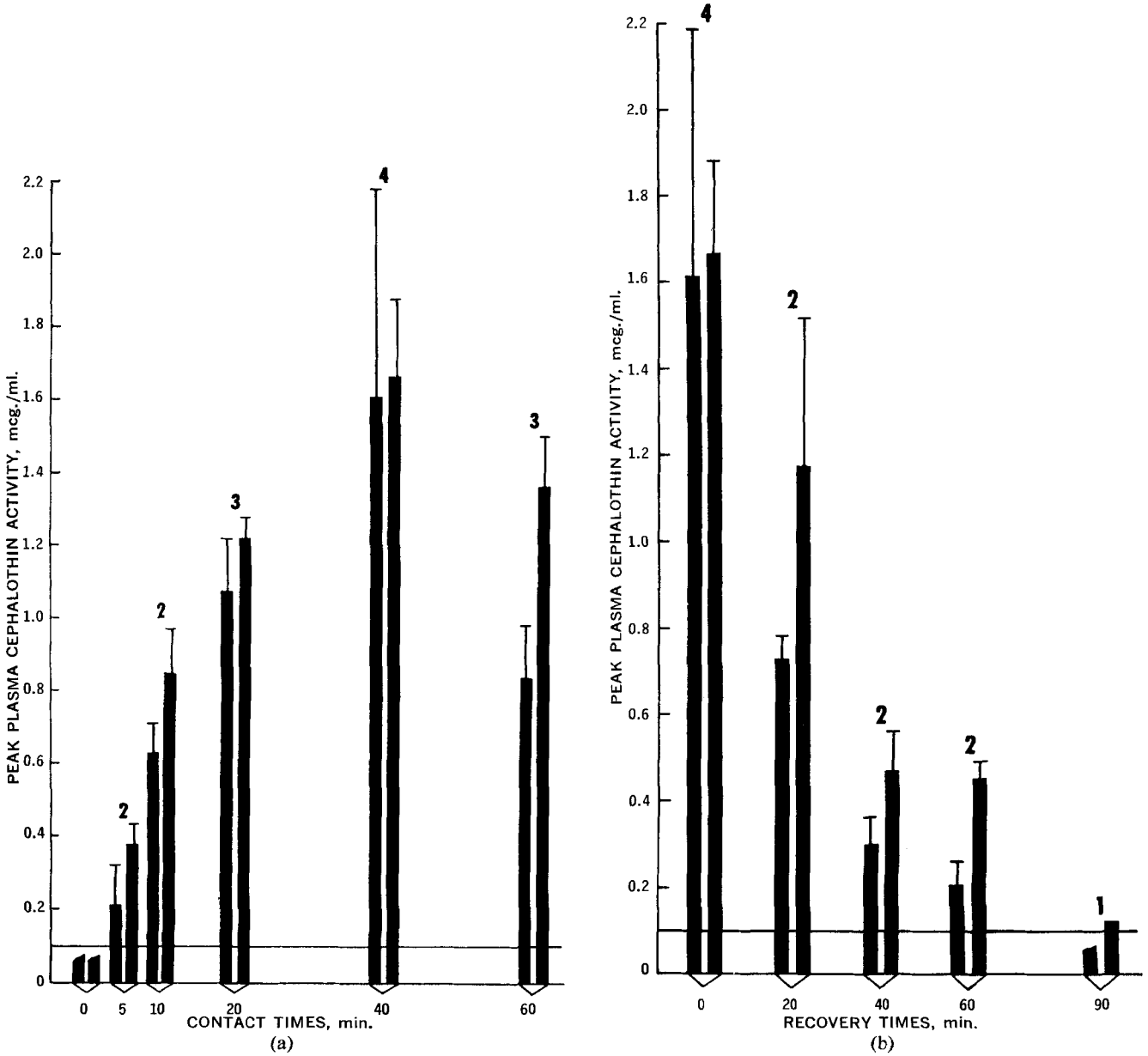


Figure 2—(*a*) *Effect of duration of contact between POE-24-cholesteryl ether solution and the dog fundic pouch upon the subsequent absorption of cephalothin. Sequence of pouch contents: (1) 25 ml. 0.5% POE-24-cholesteryl ether in isosmolar sodium phosphate buffer, pH 7.0, for the indicated time of contact; (2) three rinses with 25 ml. buffer; (3) 25 ml. 1% cephalothin in isosmolar sodium phosphate buffer, pH 7.0 (without POE-24-cholesteryl ether), for 120 min. Plasma activity was followed for 120 min. Each point is the mean ± SEM of the maximum levels from the number of experiments shown in bold numerals performed on each of two 21-kg. dogs. The horizontal line is the minimum detection limit of the assay.* (*b*) *Effect of recovery time on the hyperabsorptive state of the dog fundic pouch, the absorption of cephalothin after various time intervals between removal of POE-24-cholesteryl ether and introduction of cephalothin. Sequence of pouch contents: (1) 25 ml. 0.5% POE-24-cholesteryl ether in isosmolar sodium phosphate buffer, pH 7.0, for 40 min.; (2) three rinses with 25 ml. buffer; (3) pouch empty for indicated time; (4) 25 ml. 1% cephalothin in isosmolar sodium phosphate buffer, pH 7.0 (without POE-24-cholesteryl ether), for 120 min. Plasma activity was followed for 120 min. Each point is the mean ± SEM of the maximum levels from the number of experiments shown in bold numerals performed on each of two 21-kg. dogs. The horizontal line is the minimum detection limit of the assay.*

tant. The effect increased with time of exposure to this promoter at this concentration up to about 40 min., as seen in the plot of peak antibiotic plasma levels against time of contact (Fig. 2*a*). This graph also shows that as little as 5 min. of contact with 0.5% POE-24-cholesteryl ether solution resulted in some promoted absorption.

To quantitate the rate at which the fundic pouch loses the hyperabsorptive state after removal of the surface-active solution, 0.5% buffered POE-24-cholesteryl ether solutions were maintained in the pouch for 40 min. and then rinsed out with buffer. Then a time was permitted to elapse before the cephalothin solution without surfactant was introduced (Fig. 2*b*). As the elapsed time between removal of promoter and introduction of antibiotic increased, the maximum resulting blood levels declined rapidly. The peak plasma level obtained fell to one-half when the elapsed time was 25 min., and it fell below the detection limit when the elapsed time was 2 hr.

This rapid recovery of normal absorptiveness, suggesting the rapid restoration of a normal barrier to diffusion, is in contrast to the persistent hyperpermeability associated with damage to the gastric mucosa caused by fatty acids and acetylsalicylic acid at low pH, as reported by Davenport (14).

In addition to the observation that peak plasma levels of drug reach lower levels as the recovery time increases, other observations provide evidence of the rapid reversal of the hyperabsorptive state and the return to a normal absorptive state after removal of the promoter. When the drug is placed without promoter in the preconditioned pouch, blood levels decline rapidly after an initial

sharp rise, implying that absorption continues only briefly in the absence of promoter in the pouch contents.

The complete reversibility of this induced hyperabsorptive state and the absence of permanent damage in the Thomas pouch dogs used for almost daily experiments are indicated by the fact that they lost neither their characteristic control response nor their reactivity to promoters over the course of several years.

These experiments clearly show that the surface-active agent acts on the organ rather than on the drug. The effect of surface-active agents in rendering the stomach hyperabsorptive toward water-soluble drugs is thus distinguished from the reported effects of similar agents on the absorption of poorly soluble drugs, which is commonly attributed to improved physical dispersion or rate of dissolution.

All agents that the authors found to possess the ability to promote gastric absorption of drugs are surface-active agents, but not all surface-active agents are effective at the concentrations employed.

The phenomenon of promoted absorption is not limited by the specific molecular nature or charge of the soluble drug (Table I). It also can be observed at low pH and is seen in presence of citrate or phosphate buffers of a range of concentrations and with isotonic sucrose when these solutions are used to dissolve the drug and promoter.

The effectiveness of the surfactants in promoting gastric absorption of cephalothin is poorly correlated with the hydrophile–lipophile balance of the surface-active agent and is not proportionate to the ability of the agent to lower the surface tension at an air–water interface. A general correlation was observed, however, between the effectiveness of a surface-active agent in promoting gastric absorption and its ability to lyse erythrocytes *in vitro*. This lytic action, to which erythrocytes are notoriously sensitive, is generally regarded as due to the accumulation of the surfactant molecules in the lipid membrane of the cell wall in a manner that causes loss of functional or physical integrity of the membrane.

A high degree of order on a monomolecular or bimolecular leaflet level (15) is generally pictured as having an essential role in the maintenance of a barrier to passive diffusion across cell walls. The reversible changes in gastric absorption reported in the present paper are, the authors believe, the consequences of reversible alterations of this highly ordered barrier that accompany the addition or removal of surfactant as described in these experiments.

The hyperabsorptive state of the stomach epithelium is thus regarded as one in which interference with the diffusion barrier permits increased absorption of a wide variety of water-soluble substances placed in the organ. However, the net absorption (or excretion) of a substance by active transport processes may simultaneously suffer from this reduction of the barrier to free diffusion, since the barrier is presumably operative in both directions and lowering it would lend advantage to the flux opposite to the active transport. Indeed, some compounds used here as promoters have elsewhere been found to inhibit active transport processes (8, 17).

In this study, convenience of measurement has prejudiced the choice of drugs to antibiotics, and the authors have dealt with absorption from the dog fundic pouch only. The observations of promoted absorption of other drugs in the intact gastrointestinal tract, as well as in ligated segments of the gastrointestinal tract, of the dog and other animal species have confirmed the more general implications of this work to drug absorption.[7] Such studies will be reported in following communications.

REFERENCES

(1) H. W. Davenport, *Gastroenterology*, **54,** 175(1968).

(2) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **58,** 425(1969).

(3) G. Levy and J. A. Anello, *ibid.*, **57,** 101(1968).

(4) P. M. Lish and J. H. Weikel, Jr., *Toxicol. Appl. Pharmacol.*, **1,** 501(1959).

(5) S. Riegelman and W. Crowell, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 123(1958).

(6) J. R. Marvel, D. A. Schlichting, C. Denton, E. S. Levy, and M. M. Cahn, *J. Invest. Dermatol.*, **42,** 197(1964).

(7) J. A. Nissim, *Brit. J. Pharmacol.*, **24,** 205(1965).

(8) C. B. Taylor, *J. Physiol. (London)*, **165,** 199(1963).

(9) C. W. Ryan and C. Ainsworth, *J. Org. Chem.*, **27,** 2901 (1962).

(10) R. R. Chauvette, E. H. Flynn, B. G. Jackson, E. R. Lavagnino, R. B. Morin, R. A. Mueller, R. P. Pioch, R. W. Roeske, C. W. Ryan, J. L. Spencer, and E. M. Van Heyningen, *Antimicrob. Ag. Chemother.*, **1962,** 687.

(11) J. L. Spencer, F. Y. Siu, B. G. Jackson, H. M. Higgins, and E. H. Flynn, *J. Org. Chem.*, **32,** 500(1967).

(12) M. L. Corrin and W. D. Harkins, *J. Amer. Chem. Soc.*, **69,** 683(1947).

(13) B. D. Flockhart and A. R. Ubbelohde, *J. Colloid Sci.*, **8,** 428(1953).

(14) H. W. Davenport, *Gastroenterology*, **46,** 245(1964).

(15) W. D. Stein, "The Movement of Molecules Across Cell Membranes," Academic, New York, N. Y., 1967, chap. 1.

(16) C. Ainsworth, D. N. Benslay, J. Davenport, J. L. Hudson, D. Kau, T. M. Lin, and R. R. Pfeiffer, *J. Med. Chem.*, **10,** 158 (1967).

(17) J. A. Nissim, *Nature*, **204,** 148(1964).

(18) J. A. Anello and G. Levy, *J. Pharm. Sci.*, **58,** 721(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 1, 1969, from the *Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46206*

Accepted for publication February 18, 1970.

The authors thank Mr. W. E. Wick and Miss Stanis Stroy for the microbiological assays, and Dr. T. M. Lin and Mr. D. N. Benslay for the surgical preparation of animals.

[7] Anello and Levy (18) have drawn similar conclusions regarding the effect of polysorbate-80 on the permeability of the absorptive membranes of the goldfish.

Effects of a Tranquilizer and Two Antidepressants on Learned and Unlearned Behaviors

HARRY M. GEYER, III, NATHAN WATZMAN*, and JOSEPH P. BUCKLEY

Abstract □ The dose–response effects of chlorpromazine, imipramine, and thiazesim were investigated on unlearned behaviors (spontaneous motor activity, eating, drinking, mouse-killing, self-grooming, and forced motor activity) and learned behavior using the rat pole-climbing unit. Three or four doses of each drug were used in the study of each parameter, and ED_{50} values were calculated from the generated log dose–response line. Ratios of the forced motor activity ED_{50} divided by the ED_{50}'s of the various behavioral tests were used to determine whether the blockades of the behavioral parameters occurred at debilitating or nondebilitating doses. The tranquilizer, chlorpromazine, required a debilitating dose to block four of the five unlearned behaviors. The antidepressant, imipramine, disrupted three of these at nondebilitating doses; the antidepressant, thiazesim, blocked all unlearned behaviors at nondebilitating doses. All compounds required debilitating doses to block the learned behavior, a conditioned avoidance response. The results generally support the hypothesis that antidepressants selectively block unlearned behaviors which are not blocked by tranquilizers until debilitating doses are used.

Keyphrases □ Tranquilizer, antidepressant effects—learned, unlearned behavior □ Chlorpromazine, imipramine, thiazesim—comparative effects, learned, unlearned behavior □ Behavioral response, learned, unlearned—antidepressants, tranquilizer effects

Although two major classes of psychotherapeutic agents, tranquilizers and antidepressants, are quite different in their clinical applications, often the assignment of a compound into either of these classifications must await clinical evaluation since the difference is not readily assessible by preclinical animal testing. Tranquilizers and antidepressants have been reported to have qualitatively similar electroencephalographic effects (1–4). Herr *et al.* (5) reported the lack of qualitative differences when these compounds were compared in various toxicological and behavioral studies. Horovitz *et al.* (6) reported a possible method for a preclinical differentiation. These authors reported that antidepressants, but not tranquilizers, given to rats at nondebilitating doses had a blocking action on mouse-killing, an unlearned behavior described by Karli (7). Horovitz *et al.* (6) also reported that this difference between tranquilizers and antidepressants was not found in the comparison of drug effects on a learned avoidance response.

The present study was an attempt to determine if other nonlearned behaviors in rats show the "selective" blocking action by antidepressants.

MATERIALS AND METHODS

Subjects—The subjects were Sprague-Dawley male rats (519) and female rats (82) weighing 200–350 g. The subjects were maintained in individual wire-mesh cages on a 12-hr. light-dark cycle at ambient temperatures between 23.9 and 25.5° (75 and 78°F). Purina laboratory chow and tap water were available *ad libitum* for all subjects except those used in the studies of food and water consumption. All subjects were used only once except in the study of muricide. Maternal behavior and muricide were tested in the light portion of their light-dark cycle. All other studies were performed during the dark portion of the cycle; in the testing of self-grooming and forced motor activity, the subjects were illuminated by two 1.5-m. (5-ft.) fluorescent red light bars approximately 0.9 m. (3 ft.) from the cage and between the investigator and the subjects.

Drugs—The prototype tranquilizer selected was chlorpromazine hydrochloride, and the antidepressants were imipramine hydrochloride and thiazesim hydrochloride. All drugs were dissolved in distilled water, with concentrations adjusted to enable intraperitoneal injection in volumes of 1 ml./250 g. of body weight. The various testing procedures were performed during peak drug effect which occurred 30 min. after chlorpromazine, 40 min. after imipramine, and 30 min. after thiazesim administration (8). The saline subjects (0.9% NaCl) were tested 30 min. after saline administration. The doses used were as follows: chlorpromazine, 1, 2, and 4 mg./kg.; imipramine, 8, 16, and 32 mg./kg.; and thiazesim, 10, 20, and 40 mg./kg., i.p. In the experiments with self-grooming, mouse-killing, and conditioned avoidance response, an additional dose of chlorpromazine, 8 mg./kg., was included. In the study of water consumption, an additional dose of 5 mg./kg. of thiazesim was included, and imipramine was used in doses of 4, 8, and 16 mg./kg.

Forced Motor Activity (FMA)—Forced motor activity was evaluated by the use of a revolving wooden rod, 5.08 cm. (2 in.) in diameter, as described by Watzman *et al.* (9). The rod first revolved at 7.8 r.p.m., and the speed was increased by 4.5 r.p.m. every 30 sec. The amount of time the subjects remained on the rod was used as the measure of motor activity. The subjects, 81 male rats, were given five consecutive training trials both in the morning and afternoon of the 1st day, four consecutive trials in both the morning and afternoon of the 2nd day, and three consecutive trials on the morning of the 3rd day. The rotarod performance was found in preliminary trials to be stable by the 3rd day. The drugs and saline were administered in the afternoon of the 3rd day, and the subjects were given three consecutive test trials at the time of peak drug effect. The test trials were averaged for each subject, and the groups receiving the experimental compounds were compared with the saline controls.

Spontaneous Motor Activity (SMA)—Spontaneous motor activity was tested in four circular, 6-beam photocell activity cages (Actophotometer, Metro Industries, Inc., New York, N. Y.), using a single subject per cage. The drugs, doses, saline controls, and cages were arranged in a modified factorial design. The testing was performed on 3 consecutive days. There were three experimental subjects and one saline control at each testing session, and each drug–dose group was compared with its appropriate saline controls. The subjects, 95 male rats, were introduced at the time of peak drug effect; their activity counts were recorded at the end of 30 min.

Food Consumption—In this study, 68 male rats were deprived of food for 24 hr. but water was available *ad libitum*. At the end of the deprivation period, the subjects were treated with drugs or saline and, at the time of the peak effect, were given a preweighed amount of Purina laboratory pellets. At the end of 1 hr., the remaining pellets and spillage were collected and weighed. The recorded data were both the weight eaten and the weight of the subject just prior to testing.

Water Consumption—The subjects used in the study of drinking (75 male rats) were deprived of water for 3 days and then placed at the time of peak drug effect in a compartment, 25.4 × 26.6 × 27.9 cm. (10 × 10.5 × 11 in.), which had a drinking spout attached to a drinkometer apparatus. The number of licks recorded in 30 min. was used as the measure of water consumption.

Maternal Behavior—Maternal behavior was studied 1 to 4 days postparturition. The subjects (82 female rats) were removed from their home cages momentarily, the wood floors were cleared, and a new supply of the nesting material and all pups were deposited on

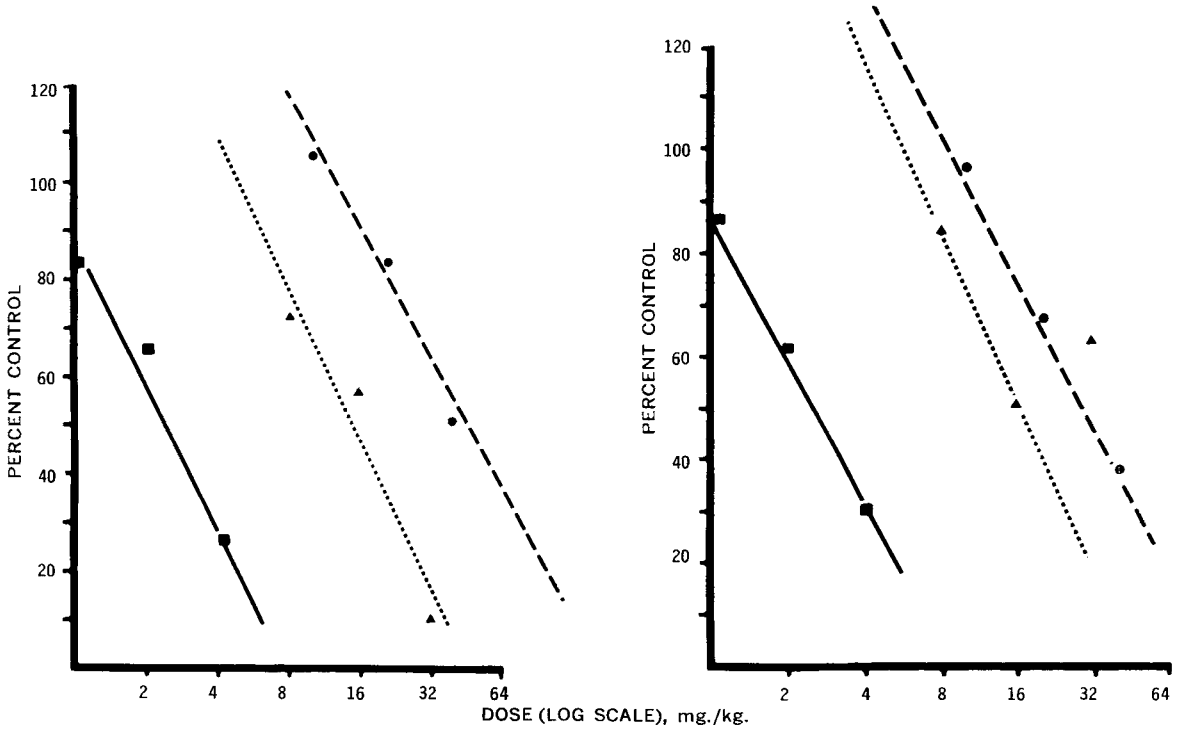


Figure 1—*Effects of chlorpromazine, imipramine, and thiazesim on spontaneous (right side) and forced motor activity (left side). Key:* ■—, *chlorpromazine;* ▲. . ., *imipramine; and* ●- - -, *thiazesim.*

the side of the cage opposite the previously noted nesting site. The subjects were then returned to their cages and rated on the 5-point scale of 0, 1, 2, 3, and 4 according to approximate percents of the nesting material retrieved to the nesting site: 0, 25, 50, 75, and 100%. Simultaneously, the subjects were scored according to the percent of young retrieved. Each session was 8 min. in duration; 24 hr. later the testing procedure was repeated at the time of peak drug action. The experimental subjects were compared with saline controls, and the preliminary trial (saline injection) was used to eliminate the few subjects not completing both nest-building and young-retrieval within 4 min.

Mouse-Killing—An albino mouse, 25 to 30 g., was introduced in the rat's home cage for 5 min.; if the rat killed the mouse, it was retested at least once a day for 5 days. The latter tests were 30 min. after saline injection, and a 60-sec. time limit was imposed. Only those rats (36 males) that killed in all of the predrug trials were used in this study. The animals received drug injections and were tested on four to six occasions. There were always at least 1 week and one saline test between drug administrations. The total number killing within 60 sec. was recorded for each drug and dose.

Self-Grooming—The 84 male rats used in this study were immersed in a sample of rat urine to wet the forepaws, hind paws, and undersides. They were immediately returned to their home cages, and the total time spent grooming was measured over a 5-min. period. Twenty-four hours later, the subjects were weighed and injected and the same procedure was repeated. The time spent grooming each day was recorded.

Conditioned Avoidance Response (CAR)—This was studied in automated pole-climbing units (25.4 × 26.6 × 35.5 cm.) (10 × 10.5 × 14 in.) (10). A 15-sec. tone was followed by an electrical shock (300 v., 2 mamp.) from the grid floor with a maximum duration of 45 sec. The tone was continued through the shock period, and this was followed by a 2-min. intertrial period. Pole-climb latencies during tone alone (shock avoidance) and during the shock cycle (shock escape) were recorded on a pen polygraph (Lehigh Valley Electronics No. 1321-4).

The subjects, 80 male rats, were trained to a minimum of 75% avoidance in three to five 1-hr. sessions. The training sessions were once daily on consecutive days, and the animals were retested at peak drug effect 24 hr. after the training session in which they reached the 75% avoidance criterion. The number of avoidances made by the drugged subjects were compared with the appropriate saline controls.

RESULTS

Forced Motor Activity (FMA)—All drugs produced dose-dependent decrease in motor performance, and chlorpromazine and imipramine produced a decrement exceeding 50%. Thiazesim did not quite reach the 50% level at the highest dose, 40 mg./kg. (Fig. 1). This was the maximum dose used because pilot studies indicated that both 50 and 60 mg./kg. could produce convulsions. The data were calculated and presented as the percent of saline scores.

Spontaneous Motor Activity (SMA)—The SMA data (Fig. 1) depict the drug actions as dose-dependent decreases in activity and are reasonably straight lines for chlorpromazine and thiazesim. The imipramine effects appear biphasic in this and in several other measures in this study. This biphasic action will be discussed later. The ED_{50} value for imipramine was calculated with only the low and medium doses because the latter approaches the 50% level (51.8%).

Food Consumption—The data on this parameter produced a fairly linear dose-dependent decrease in food consumption by each drug (Fig. 2). The results depicted are percent control values and are calculated as the amount eaten, divided by the subject's weight. The subject's weights did not differ significantly between groups; however, the within-group weights varied up to 100 g. For this reason the results were weighted in relation to subject's size. This weighting of scores assumed that larger subjects would eat more, even under the experimental conditions. This was substantiated by the overall mean Pearson correlation coefficient of +0.48 for the weight of food eaten per gram of body weight.

Water Consumption—The inhibitory effects on water consumption produced by the three compounds are summarized in Fig. 2. Although the linearity of chlorpromazine and thiazesim results is clear, imipramine again shows a biphasic response. The two low doses, one on either side of the 50% level, were used to calculate the ED_{50} value.

Maternal Behavior—The results summarized in Fig. 3 failed to show a clear log dose–response relationship in most cases. Although rough approximations of the respective ED_{50} values do agree with the proposed hypothesis, the lack of clear dose–response relationships is considered ample reason for deletion of these parameters from further considerations. The cause of this discrepancy is not clear, although there is the possibility that the home cages [20.3 × 21.6 × 27.9 cm. (8 × 8.5 × 11 in.)] may not have been of sufficient size to stimulate the maternal behaviors tested. That is, pups,

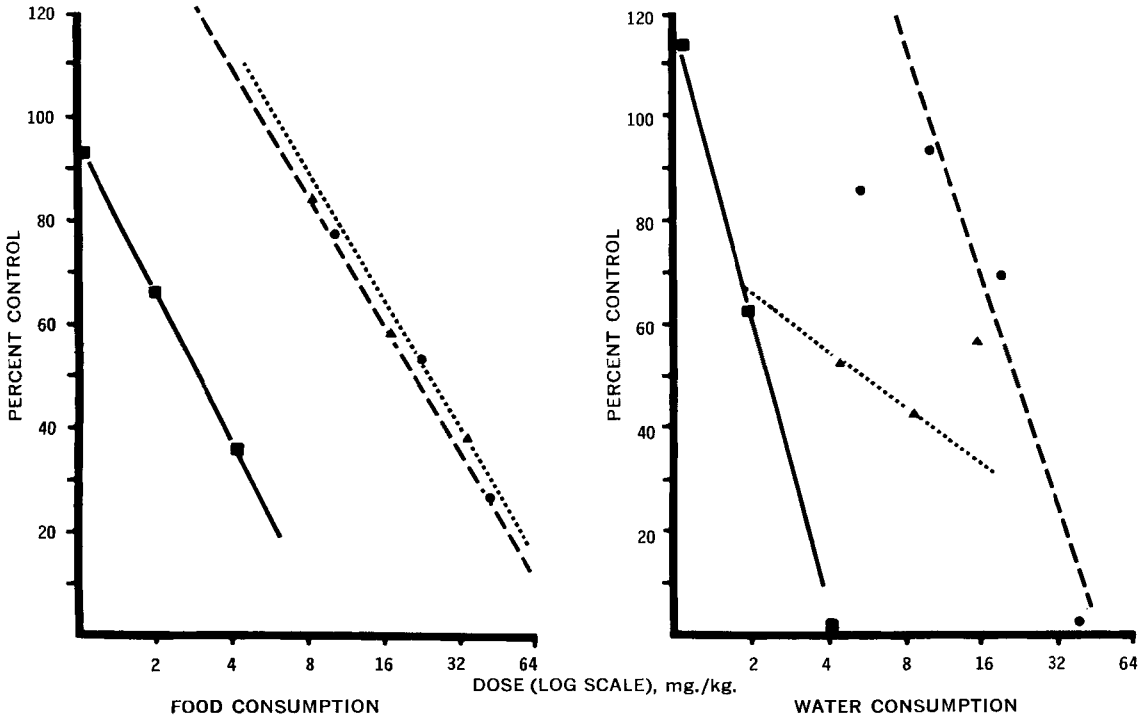


Figure 2—*Effects of chlorpromazine, imipramine, and thiazesim on food and water consumption. Key:* ■—, *chlorpromazine;* ▲. . ., *imipramine; and* ●- - -, *thiazesim.*

8 to 10 in. away in the home cage, may have been an inadequate stimulus to induce retrieval behavior in some of the animals.

Mouse-Killing—The data summarized in Fig. 4 indicate that the drugs produced a linear log dose–response relationship. The ability of chlorpromazine to block muricide, even at the high dose (8 mg./kg.), is, however, open to question. The test sequences were only 60 sec. and, at the highest dose, chlorpromazine appeared to induce the expected sedation. To clarify this, after the end of their test sequence, some sedated rats were manually jostled and the mice were immediately killed by the aroused rat. This was found in eight of the nine subjects receiving 8 mg./kg. of chlorpromazine, although the same procedure did not induce killing when used with six of the subjects receiving 32 mg./kg. of imipramine or six of the subjects receiving 40 mg./kg. of thiazesim. The tranquilizer, therefore, appeared to block mouse-killing mainly due to its sedative action.

Self-Grooming—The results depicted in Fig. 4 show the log dose–response relationship for the compounds investigated. Of interest is the evident increase in grooming induced by 2 mg./kg. of chlorpromazine, although this difference was short of statistical sig-

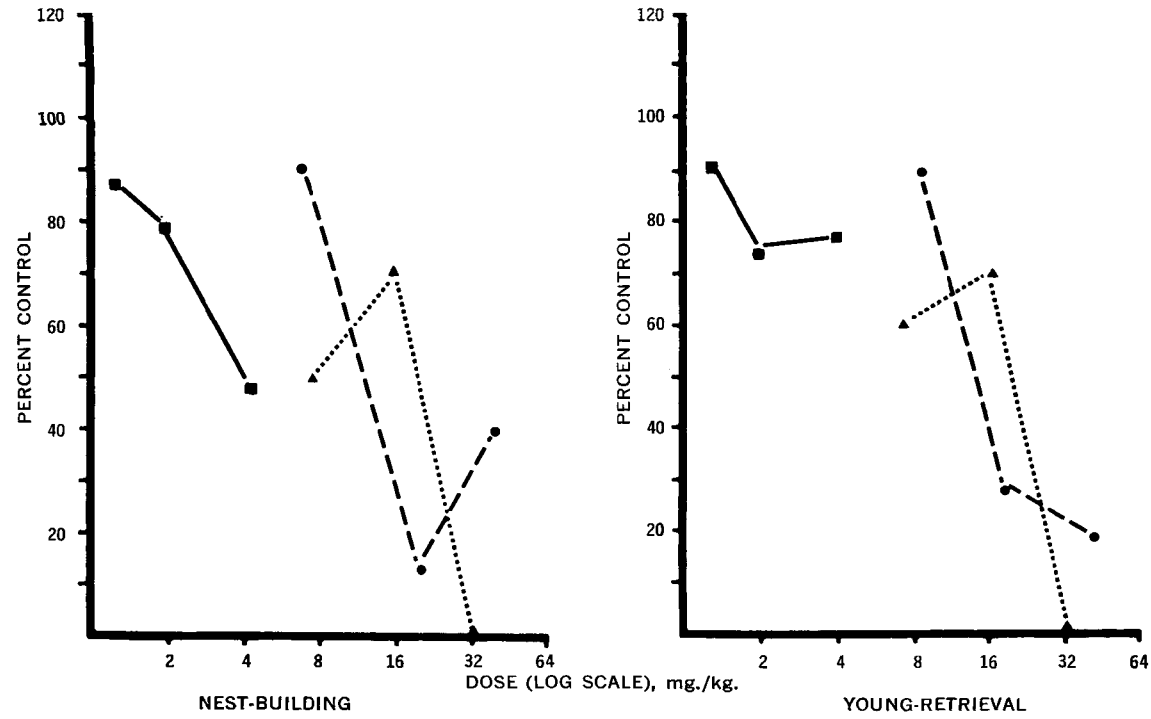


Figure 3—*Effects of chlorpromazine, imipramine, and thiazesim on nest-building and young-retrieval. Key:* ■—, *chlorpromazine;* ▲. . ., *imipramine; and* ●- - -, *thiazesim.*

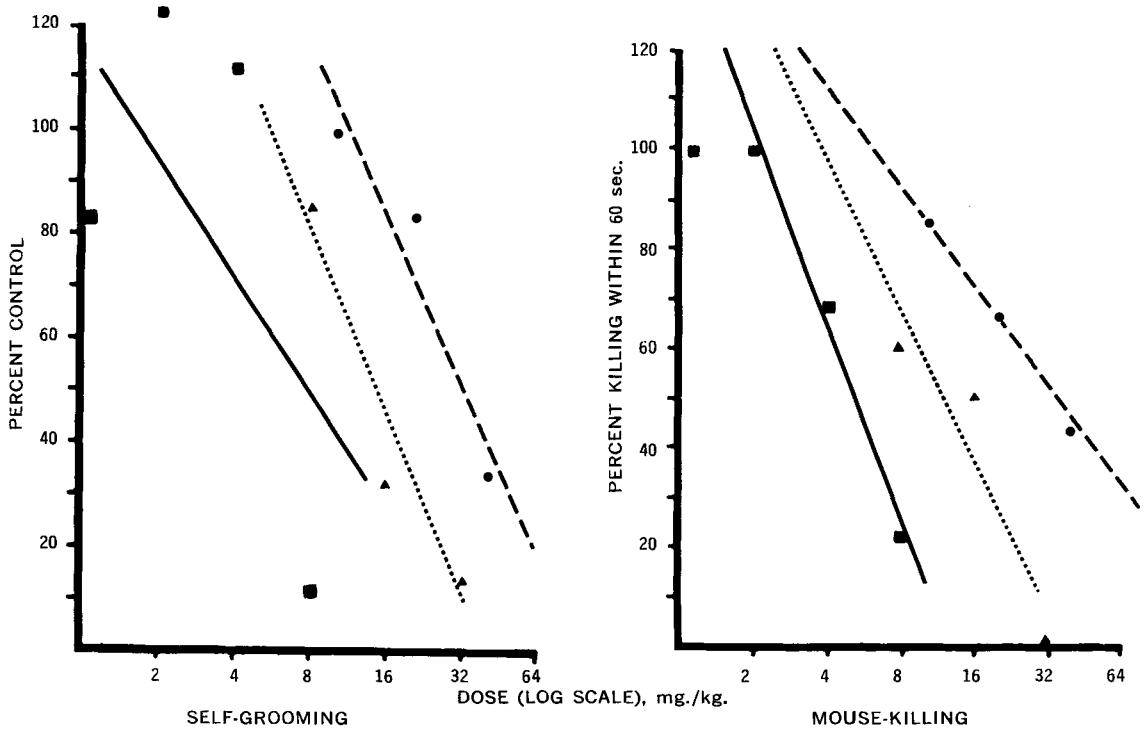


Figure 4—*Effects of chlorpromazine, imipramine, and thiazesim on self-grooming and mouse-killing in the rat. Key:* ■—, *chlorpromazine;* ▲. . ., *imipramine; and* ●- - -, *thiazesim.*

nificance ($p > 0.05$). These results are in agreement with the report of Silverman (11), which gave data indicating that low doses of chlorpromazine significantly increase unstimulated self-washing of the face and forepaws. An increase in any behavior after chlorpromazine administration is of interest because of this compound's general depressant action.

Conditioned Avoidance Response (CAR)—The results shown in Fig. 5 are for the most part in agreement with those reported by Horovitz *et al.* (6), although these investigators found imipramine to be more potent in CAR disruption. This causes no change in direction of the ratio values employed by these investigators, only an insignificant change in magnitude.

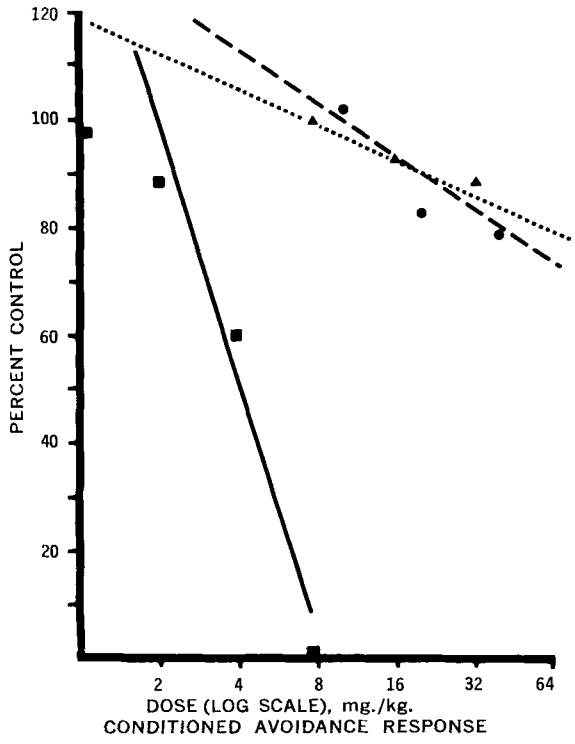


Figure 5—*Effects of chlorpromazine, imipramine, and thiazesim on the rat pole-climbing conditioned avoidance response. Key:* ■—, *chlorpromazine;* ▲. . ., *imipramine; and* ●- - -, *thiazesim.*

ED_{50} Values—The ED_{50} values were calculated from the best fitting straight lines as established by the method of least squares (Table I). Exceptions to this method of analysis were the aforementioned cases involving the biphasic imipramine action in SMA and water consumption. In these cases, the line between the lower doses transversed or approached the 50% level, and the ED_{50} was calculated from this line. The ED_{50} for the thiazesim effect on drinking was calculated after deletion of the lowest of the four doses.

Ratio Values—The ratio values presented in Table II are the ED_{50} values of the experimental compounds in the FMA divided by their respective ED_{50} values in the behavioral parameters investigated. The ratio values for CAR are listed as <1.0, since the ED_{50}'s for impramine and thiazesim were greater than the maximum doses used. Ratio values greater than unity are, therefore, indicative of a "selective" blocking action, a disruption of specific behaviors at nondebilitating doses. Ratio values less than 1 show that the behavior was blocked by debilitating doses.

DISCUSSION

The biphasic aspects of the imipramine action were troublesome in analyzing the data. The possibility of multiple experimental

Table I—Drug Effects on Learned and Unlearned Behavior

Behavioral Parameter	ED_{50} Values, mg./kg., i.p.		
	Chlorpromazine	Imipramine	Thiazesim
Forced motor activity	2.48	15.10	43.89
Spontaneous motor activity	2.51	16.68	29.88
Food consumption	2.88	21.52	20.56
Water consumption	2.26	5.09	21.33
Self-grooming	7.71	14.13	32.10
Mouse-killing	5.10	11.76	33.30
Conditioned avoidance response	4.08	>32.00	>40.00

Table II—Ratio Values for Experimental Compounds[a]

Behavioral Parameter	Chlorpromazine	Imipramine	Thiazesim
Spontaneous motor activity	0.98	0.92	1.47
Food consumption	0.86	0.70	2.14
Water consumption	1.09	2.96	2.06
Grooming	0.32	1.07	1.37
Mouse-killing	0.49	1.28	1.32
Conditioned avoidance response	<1.0	<1.0	<1.0

[a] ED_{50} forced motor activity/ED_{50} behavior parameter.

errors was not considered likely; as in the study of SMA, eight subjects were used at each dose, including saline controls, and a prior pilot study measuring SMA with two subjects per activity cage yielded similar results. A close examination of the literature revealed that this biphasic effect is not uncommon. Furgiuele *et al.* (12) reported a biphasic imipramine response in SMA testing that was parallel to that observed in the present experiment. The biphasic imipramine action has also been reported on bulbocapnine and paraldehyde depression of motor control (13, 14). Osborne and Sigg (15) and Schaeppi (16) reported that the pressor responses to epinephrine and norepinephrine injections were potentiated by low doses of imipramine and blocked by high doses. This biphasic imipramine action in the adrenergic system may be related to its effect on drinking, since Grossman (17) and Hutchinson and Renfrew (18) have reported that adrenergic stimulation in several areas of the brain can greatly modify drinking behavior. In addition, Furgiuele *et al.* (12) also reported a biphasic imipramine effect on SMA increased by a compound with adrenergic activity. The work of Thoenen *et al.* (19) may well explain the biphasic imipramine action. These investigators studied the perfused cat spleen and induced contractions by stimulation of the postganglionic splenic nerve. Their data indicate that imipramine, in low doses, augments adrenergic responses by inhibiting reuptake of the transmitter; in high doses the adrenergic response is inhibited in a manner resembling α-adrenergic blockage. Although extrapolation of data from actions on isolated tissue to the behavioral responses of the whole organism is hazardous at best, the wealth of information relating imipramine to adrenergic activity, the reports of biphasic imipramine actions, and the desire for explanations of obtained results all make the extrapolation possible.

The present study provides evidence that there is a qualitative difference between the effects of a tranquilizer and antidepressant compounds on unlearned behaviors. The antidepressants appear to block selectively unlearned behaviors which were blocked by chlorpromazine only at debilitating doses. The ratio values (Table II) show that 12 of the 15 pairings of the three experimental compounds could have been predicted on this basis. As discussed previously, the biphasic imipramine action in drinking and SMA did create some problem in the determination of the ED_{50} values. It should be noted that these two parameters are the only ones that did not show an imipramine block at nondebilitating doses. The use of only one tranquilizer and two clinically classified antidepressants does make generalizing the results to include the two classes quite speculative. However, Horovitz (20) reported at least six tranquilizers and six antidepressants had the ratio values of FMA ED_{50}/muricide ED_{50} characteristic of their respective classes, and this suggests that the generalization may be warranted.

REFERENCES

(1) H. Himwich, A. Morillo, and W. G. Steiner, *J. Neuropsychiat.*, **3**, S15(1962).

(2) W. Schallek, A. Kuehn, and N. Jew, *Ann. N. Y. Acad. Sci.*, **96**, 303(1962).

(3) W. G. Steiner and H. E. Himwich, *J. Nerv. Ment. Dis.*, **137**, 277(1963).

(4) G. Stille and A. Sayer, *Int. J. Neuropharmacol.*, **3**, 605 (1964).

(5) F. Herr, J. Stewart, and M. Charest, *Arch. Int. Pharmacodyn.*, **134**, 328(1961).

(6) Z. P. Horovitz, J. J. Piala, J. P. High, J. C. Burke, and R. C. Leaf, *Int. J. Neuropharmacol.*, **5**, 405(1966).

(7) P. Karli, *Behavior*, **10**, 81(1956).

(8) Z. P. Horovitz, personal communication, 1967.

(9) N. Watzman, H. Barry, III, J. P. Buckley, and W. J. Kinnard, Jr., *J. Pharm. Sci.*, **53**, 1429(1964).

(10) M. D. G. Aceto, W. J. Kinnard, Jr., and J. P. Buckley, *Arch. Int. Pharmacodyn.*, **144**, 214(1963).

(11) A. P. Silverman, *Brit. J. Pharmacol.*, **24**, 579(1965).

(12) A. R. Furgiuele, M. H. Aumente, and Z. P. Horovitz, *Arch. Int. Pharmacodyn.*, **151**, 170(1964).

(13) R. Domenjoz and W. Theobald, *ibid.*, **120**, 450(1959).

(14) G. Vogel and L. Ther, *Arzneim.-Forsch.*, **13**, 779(1963).

(15) M. Osborne and E. B. Sigg, *Arch. Int. Pharmacodyn.*, **129**, 273(1960).

(16) U. Schaeppi, *Helv. Physiol. Pharmacol. Acta*, **18**, 545(1960).

(17) S. P. Grossman, *J. Comp. Physiol. Psychol.*, **57**, 29(1964).

(18) R. R. Hutchinson and J. W. Renfrew, *ibid.*, **63**, 408(1964).

(19) H. Thoenen, A. Hurlimann, and W. Haefely, *J. Pharmacol. Exp. Ther.*, **144**, 405(1964).

(20) Z. P. Horovitz, in "International Symposium on Antidepressant Drugs," S. Garrattini and M. N. G. Dukes, Eds., Excerpta Medica Foundation, New York, N. Y., 1966, pp. 121–129.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 23, 1969, from the *Department of Pharmacology, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213*

Accepted for publication February 24, 1970.

This investigation was supported by General Research Support Grant, FR-05455, from the National Institute of General Medical Sciences.

* Present address: Division of Research Grants, National Institutes of Health, Bethesda, MD 20014

Laboratory Evaluation of Aggressive Behavior of the Grasshopper Mouse (*Onychomys*)

HENRY F. COLE* and HAROLD H. WOLF

Abstract ☐ The genetically predisposed aggressive behavior of two species of grasshopper mouse, *Onychomys leucogaster* and *O. torridus*, was evaluated in an experimental paradigm involving isolation-induced aggression. *O. leucogaster* exhibited considerably more aggression than did *O. torridus*. When aggression was provoked in *Onychomys* by isolation, the behavior could not be suppressed by pentobarbital nor by chlordiazepoxide. Indeed, chlordiazepoxide increased the amount of time spent fighting. Chlorpromazine was able to depress fighting behavior in *torridus* but not in *leucogaster*. The results obtained support the contention that an animal's behavioral predisposition may markedly alter the organism's response to drugs.

Keyphrases ☐ Grasshopper mouse—aggressive behavior ☐ Aggression—genetic predisposition, mice ☐ Chlorpromazine HCl, chlordiazepoxide, Na pentobarbital effect—aggression ☐ Genetic predisposition effect—pharmacological tests

The effects of tranquilizers and other central nervous system depressants on the aggressive behavior of albino mice have been studied by many investigators (1–7). Such investigations reveal that aggressive behavior of most albino mice is mild and relatively unstable. This is not surprising because albino mice have been bred for hundreds of generations for docility and tolerance to high-population densities. Moreover, in laboratory colonies, two major causes of aggression, competition for food and competition for territory, are removed. Under these conditions, a high level of aggressiveness becomes an undesirable trait, to be removed from the population.

In view of the relative docility of albino mice, the authors felt that an aggressive animal might serve as a more appropriate subject for studies on aggression. One such animal is the northern grasshopper mouse, *Onychomys leucogaster*. These grasshopper mice are predatory and carnivorous. In their natural habitat, they stalk and quickly kill other small rodents by gnawing into the base of the victim's brain (8). Ingles (9) estimates that small mammals and insects form 90% of *Onychomys*' natural diet. In the laboratory, the fighting behavior of *O. leucogaster* is readily elicited, quite stable, and easily quantified (10). When given the opportunity, *leucogaster* will quickly and vigorously attack a victim mouse. When given regular opportunities to fight, they may become so adept at expressing this behavior that the victim is killed as quickly as 20 sec. after its exposure to the aggressor (10).

The highly stereotyped and invariable method of killing by gnawing through the base of the skull exhibited by *leucogaster*, as well as the ease with which the behavior is elicited, even in naive subjects several generations removed from the wild, strongly suggests that this behavior is primarily genetically determined rather than learned. Therefore, the authors thought it worthwhile to investigate the genetically predisposed aggressive behavior of these grasshopper mice in an experimental paradigm involving isolation-induced aggression.

Members of a second species, *O. torridus*, the southern grasshopper mouse, were also included in this investigation. Because the authors are unaware of any studies concerned with the behavior of *torridus* under laboratory conditions, they felt valuable behavioral data could be provided by the inclusion of this species.

EXPERIMENTAL

A commonly employed method of producing aggressive behavior in albino mice is to subject them to extended periods of social isolation. Since such isolation-induced aggression has been frequently employed in drug studies (1, 2, 4, 6), the authors also utilized this method in their investigations of the aggressive behavior of *Onychomys*.

The *O. leucogaster* ranged in weight between 24 and 41 g., were derived from a colony previously maintained at the University of Utah College of Pharmacy, and were approximately 6 to 10 generations removed from the wild. The *O. torridus* were first-generation offspring of mice trapped by The Pet Corral, Tucson, Arizona, and weighed 20–32 g. *Onychomys* were segregated according to sex and were housed in groups of at most three mice to a standard 43 × 25 × 12-cm. plastic cage. When it became necessary to employ female subjects, care was taken to ensure that pregnant females were excluded. Purina Lab Chow and water were continually available in the cages. This diet was periodically supplemented with sunflower seeds, wheat, or small quantities of canned dog or cat food. Results were statistically analyzed by means of the following specific procedures; except where otherwise indicated, differences were considered to be significant at $p < 0.05$.

The drugs employed in these studies, chlordiazepoxide HCl (CDP), chlorpromazine HCl (CPZ), and sodium pentobarbital (PTB), were administered intraperitoneally in aqueous solution in such concentration that 1 ml./100 g. body weight contained the appropriate dose. Dosage and time of peak drug effect (TPE) were based on a roller-rod test slightly modified from that previously described (11) in that a speed of 4 r.p.m. was employed. The time at which the greatest number of mice were unable to remain on the rod for at least 30 sec. in any one of three trials (minimal neurological deficit) was taken as the time of peak drug effect (TPE). Employing this TPE, the dose of each drug producing evidence of minimal neurological deficit in 50% of the mice (TD_{50}) was calculated by the method of Litchfield and Wilcoxon (12). All further drug-behavior studies were carried out at the TPE using a dose level of $^1/_2$ TD_{50}. The TPE's and TD_{50}'s for the drugs employed are presented in Table I.

Four male and two female adult *leucogaster* and five male and two female adult *torridus* were caged individually and then tested at intervals of every 2 or 3 days until they would consistently attack a small albino victim mouse introduced into the aggressor's cage for a 5-min. period. Isolation periods as short as 3 or 4 days were usually sufficient to evoke this behavior in *leucogaster*, whereas *torridus* required as much as 2 to 3 weeks of isolation. When attack behavior was consistently displayed by *Onychomys*, the drug studies were initiated. Mice were randomly assigned to receive each of four treatments (control, CPZ, CDP, and PTB) in a unique sequence. Mice were injected with the appropriate treatment; at the TPE listed in Table I, a small (10–14 g.) victim albino mouse was placed in the cage of the *Onychomys* for a maximum of 5 min. The latency to the first attack by the aggressor and total amount of time spent engaged in aggressive activity were recorded. Mice not observed to attack the

Table I—Median Toxic Doses and Times of Peak Drug Effect

Species	CPZ		CDP		PTB	
	TD_{50}[a]	TPE[b]	TD_{50}	TPE	TD_{50}	TPE
O. leucogaster	10.0 (6.8–14.6)[c]	60	54.0 (31.8–91.8)	20	22.0 (17.6–27.5)	5
O. torridus	10.2 (5.7–18.4)	60	29.5 (18.4–47.2)	5	14.0 (9.5–20.6)	10

[a] Median toxic dose, mg./kg. [b] Time of peak drug effect, min. [c] 95% fiducial limits.

victim within the 5-min. test period were assigned a latency of 300 and a fighting duration of 0 sec. If a victim was killed before the end of the 5-min. test period, the time was noted and the victim was removed immediately.

Mice were given one of the four treatments no more than once every 5 days. On the day immediately preceding that on which a treatment (drug or control) was administered, each mouse was injected with distilled water and tested at the TPE of the treatment to be given the following day. Thus, each mouse received five control (distilled water) treatments over the duration of the study. This paradigm allowed each treatment to be compared to the immediately preceding control treatment and, furthermore, allowed an evaluation of the stability of the behavior with respect to time and exposure to the fighting situation. Moreover, this schedule permitted each animal to serve as its own control.

The results, unless otherwise indicated, were analyzed with the Wilcoxon matched-pairs signed-ranks test or with the Friedman two-way analysis of variance (13).

RESULTS

The effects of $^1/_2$ TD_{50} of chlorpromazine, chlordiazepoxide, and pentobarbital and of the combined control treatments on the amount of time spent engaged in aggressive activity after isolation are displayed in Figs. 1 and 2 for *leucogaster* and *torridus*, respectively. None of the drugs significantly depressed fighting time in *leucogaster*. Indeed, CDP significantly increased the duration of fighting in this species. The fighting time of *torridus* was also slightly, but significantly, prolonged by this drug, while CPZ significantly decreased the duration of fighting time of *torridus*.

The time that the victims survived on exposure to either species of *Onychomys* after the various treatments is tabulated in Table II. None of the drugs significantly altered the time either species required to kill a victim. However, as Table III reveals, CPZ did significantly increase the latency of *torridus* to attack a victim.

A statistical analysis of differences in performance between each of the five control treatments for either species revealed no significant changes in latency to attack, survival time of the victim, or fighting time. Thus, levels of aggression remained constant over the entire experimental period. Moreover, a given treatment had no appreciable lasting effect on subsequent fighting performance.

A Mann-Whitney "U" test comparison (13) of control performances of *torridus* and those of *leucogaster* revealed that *torridus* exhibited significantly longer latencies to attack and significantly shorter fighting durations than did similarly treated *leucogaster*. Although no significant difference was found between the survival times of *leucogaster's* and *torridus's* victims, *leucogaster* did kill a significantly ($p < 0.01$) greater number of their victims (58%) than did *torridus* (40%) as determined by the Fisher exact probability test (13).

DISCUSSION

Under the conditions employed in these studies, the *torridus* appeared to be somewhat less aggressive than *leucogaster*. In the isolated subjects, *torridus* displayed longer latencies to attack, shorter fighting times, and made fewer kills than did *leucogaster*. Moreover, in an unpublished study conducted in this laboratory, it was observed that a conditioned aggressive response which was readily acquired by *leucogaster* could not be reliably reproduced in *torridus*. Studies to date from this laboratory have not provided an apparent explanation for the quantitative discrepancy in aggression exhibited by the two species of *Onychomys*, and the authors are unaware of any studies comparing the aggressive behavior of these two mice either in the laboratory or the field. Nevertheless, it should be emphasized that both species of *Onychomys*, especially when

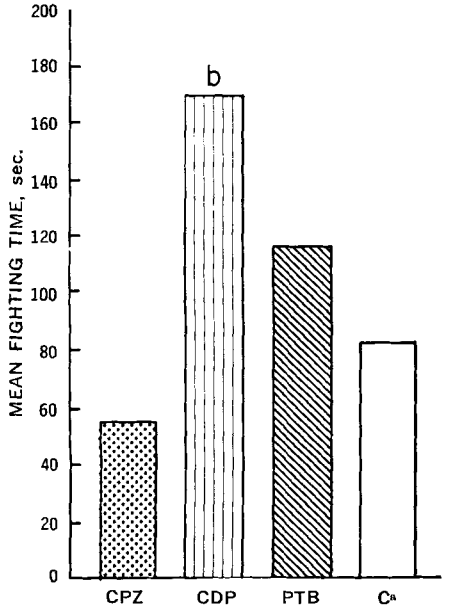


Figure 1—*Effects of drugs on fighting time of* O. leucogaster. *Key: a, combined control treatments; and b, significant drug effect* ($p < 0.05$).

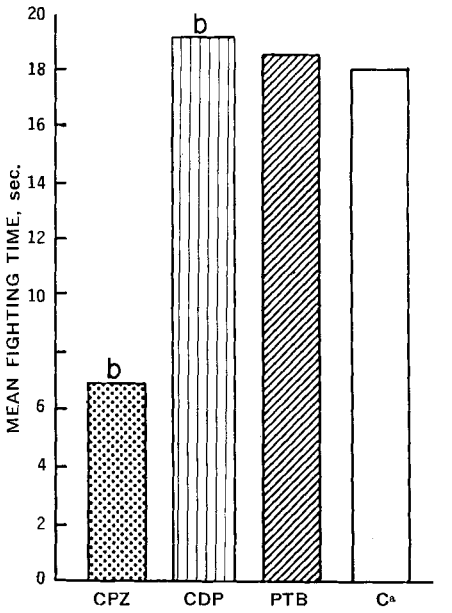


Figure 2—*Effects of drugs on fighting time of* O. torridus. *Key: a, combined control treatments; and b, significant drug effect* ($p < 0.05$).

Table II—Mean Survival Times of Victim Mice, Given in Seconds

Aggressor	CPZ	CDP	PTB	Control
O. leucogaster	156	190	151	178
O. torridus	250	199	146	219

Table III—Mean Latencies to Attack Victim Mice, Given in Seconds

Aggressor	CPZ	CDP	PTB	Control
O. leucogaster	11.0	6.7	5.2	7.2
O. torridus	121.9[a]	34.4	51.7	46.1

[a] Significantly longer than control latencies ($p < 0.05$).

isolated, were found to display a markedly greater degree of aggressive behavior than would albino subjects under similar conditions.

To evoke aggressive behavior in albino subjects, weanlings are typically isolated for periods of a month or longer (1, 7, 14). In contrast, the genetically predisposed aggressive behavior of *torridus* could be evoked in half this time and *leucogaster* would consistently attack victim mice after periods of isolation as short as 3 or 4 days. Moreover, less than 70% of a population of albino mice subjected to extended periods of social isolation can be expected to display even a minimal amount of aggressive behavior (1, 4, 7), while each of the *Onychomys* vigorously and consistently attacked the victim mice after the isolation period. Furthermore, an analysis of the latencies to attack, fighting times, and killing times for each of the five control treatments which every *Onychomys* received revealed no change for either species over the entire course of this study (eight opportunities to display aggressive behavior). In contrast, in a similar experimental paradigm, half of a population of albino mice failed to display a minimal amount of aggressive behavior after as few as six exposures to a victim (1). It should be mentioned also that while albino mice are commonly isolated while relatively young, the *Onychomys* employed in these studies were placed in isolation as adults, a time when isolation is much less effective in producing aggression (14).

The aggressive behavior of albino mice is characterized by sparring and nipping, components of mild rather than severe fighting. The injury to the victim is only superficial and the authors have never observed an albino aggressor kill a victim, even though exposure periods of up to 15 min. were employed (1). On the other hand, *torridus* and *leucogaster* killed 40 and 58%, respectively, of their victims.

Differences between the aggressive behavior of isolated albino mice and *Onychomys* are also apparent with regard to drug effects. For example, CPZ has been shown to depress the isolation-induced aggressive behavior of albino mice in small, nontoxic doses (1–3, 6). In contrast, a dose of CPZ as large as $1/2$ TD_{50} was ineffective in suppressing significantly the isolation-induced aggressive behavior of *leucogaster*. Although the less aggressive *torridus* did respond to CPZ, this effect might be at least partially due to a nonselective depression of the central nervous system, since at this dose the animals appeared to be somewhat sedated. Clark (10) also found the aggressive behavior of *leucogaster* to be quite resistant to suppression by this drug. He further reported that aggression was increased on the day following CPZ treatment. However, the experiments reported here were not designed to investigate such late alterations of behavior.

CDP has been reported to possess unique taming properties (15) and to be effective in calming aggressive or vicious albino mice and rats, dogs, cats, and monkeys (5, 15). The authors have found it effective in suppressing the isolation-induced aggressive behavior in albino mice in doses as low as $1/8$ TD_{50} (1). However, in both *leucogaster* and *torridus*, a much higher dose of CDP was not only ineffective in suppressing aggression, but indeed significantly increased the amount of time the aggressor spent engaged in fighting activities. This increase in fighting time could not be attributed to a decrease in fighting efficiency due to neurotoxicity, since both the latency to attack and survival time of the victims remained essentially unchanged.

Pentobarbital was not found to alter significantly the aggressive behavior of either species of *Onychomys*. Similarly, this nonselective central depressant is ineffective in altering the aggressive behavior of albino mice except in severely debilitating doses (1, 3, 4, 6).

It should be reemphasized that *Onychomys* are predatory rodents. In their natural habitat, survival probably depends in large part on the ability of these mice to track and kill victims for food. Moreover, this behavior appears to be genetically predetermined since individuals several generations removed from the wild will readily attack and kill victims in their typical stereotyped pattern. Taken in light of these factors, the experimental results reported here demonstrate how markedly the aggression of albino subjects differs from that of *Onychomys*. The relatively mild, difficult to elicit, and unstable aggressive behavior of albino subjects contrasts sharply with the genetically predisposed, predatory nature of *Onychomys*. Thus, the results the authors have reported support and extend their earlier contention (16) that the genetic predisposition underlying an animal's behavior may qualitatively and quantitatively influence the results obtained in pharmacological and behavioral investigations and must not be ignored.

REFERENCES

(1) H. F. Cole and H. H. Wolf, *Psychopharmacologia*, **8,** 389 (1966).

(2) J. P. DaVanzo, M. Daugherty, R. Ruckhart, and L. Kang, *ibid.*, **9,** 210(1966).

(3) W. D. Gray, A. C. Osterberg, and C. E. Rauh, *Arch. Int. Pharmacodyn. Ther.*, **134,** 198(1961).

(4) P. A. Janssen, A. H. Jageneau, and C. J. E. Niemegeers, *J. Pharmacol. Exp. Ther.*, **129,** 471(1960).

(5) L. O. Randall, *Dis. Nerv. Syst.*, **22,** Suppl. 2, 7(1961).

(6) H. C. Y. Yen, R. L. Stanger, and N. Millman, *J. Pharmacol. Exp. Ther.*, **122,** 85A(1958).

(7) H. C. Y. Yen, R. L. Stanger, and N. Millman, *Arch. Int. Pharmacodyn. Ther.*, **123,** 179(1959).

(8) V. V. Baily and C. C. Sperry, *U. S. Dep. Agr. Tech. Bull.*, **145,** 1(1929).

(9) L. G. Ingles, "Mammals of the Pacific States," Stanford University Press, Stanford, Calif., 1965.

(10) L. D. Clark, *Amer. J. Psychiat.*, **119,** 336(1962).

(11) T. R. Rudy and H. H. Wolf, *J. Pharmacol. Exp. Ther.*, **156,** 397(1967).

(12) J. T. Litchfield, Jr., and F. Wilcoxon, *ibid.*, **96,** 99(1949).

(13) S. Siegel, "Nonparametric Statistics for the Behavioral Sciences," McGraw-Hill, New York, N. Y., 1956.

(14) J. P. Scott and E. Fredericson, *Physiol. Zool.*, **24,** 273(1951).

(15) L. O. Randall, W. Schallek, G. A. Heise, E. F. Keith, and R. E. Bagdon, *J. Pharmacol. Exp. Ther.*, **129,** 163(1960).

(16) H. H. Wolf, E. A. Swinyard, and L. D. Clark, *Psychopharmacologia*, **3,** 438(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 12, 1969, from the *Division of Pharmacology, College of Pharmacy, Ohio State University, Columbus, OH 43210*

Accepted for publication January 28, 1970.

Abstracted in part from a thesis presented by H. F. C. in partial fulfillment of Doctor of Philosophy degree requirements.

This investigation was supported in part by grants from the National Institute of Mental Health (MH-07397-05 and 2-F1-MH-23, 869-05), U. S. Public Health Service.

Preliminary report: *Fed. Proc.* **27,** 438(1968).

* Present address: College of Pharmacy, University of Missouri, Kansas City, MO 64110

Polymorphism in Sulfanilamide-d_4

HWAING OU LIN and J. KEITH GUILLORY

Abstract □ Thermal behaviors of four crystalline forms of sulfanilamide and of sulfanilamide-d_4 have been examined. Heats of transition and fusion have been determined. The deuterated modifications exhibit smaller heats of transition and heats of fusion than the corresponding undeuterated forms. There is, however, a difference in the magnitude of this decrease in the case of the heats of transition.

Keyphrases □ Sulfanilamide-d_4—polymorphism □ Polymorphic forms—sulfanilamide-d_4 □ Differential thermal analysis—identity □ IR spectrophotometry—identity □ NMR spectroscopy—identity

Polymorphic forms of the same compound exhibit a number of divergent physical properties including crystal structure, rate of dissolution, density, solubility, and refractive index. Differences in dissolution rate have been noted in such widely used drugs as aspirin (1) and chloramphenicol palmitate (2). These differences in solution properties can be expected to result in variations in therapeutic blood levels and therapeutic efficacy.

In spite of the fact that polymorphism is a widespread phenomenon among organic compounds, in general, and among pharmaceuticals, in particular, very little progress has been made toward an understanding of the factors that permit some compounds to exhibit and prevent others from exhibiting polymorphism. The availability of commercial instrumentation which makes thermodynamic data concerning heats of transition and fusion more readily accessible encouraged the authors to study a group of structurally related compounds, the sulfonamides (3). Polymorphism has been detected in some sulfonamides but not in others.

One sulfonamide that has been studied extensively is sulfanilamide. The existence of polymorphic forms of the compound was first reported by Zyp in 1938 (4). He observed that sulfanilamide appeared in several crystal forms when crystallized from a drop of water and examined under the microscope. Watanabe (5, 6) conducted an X-ray diffraction study of sulfanilamide crystallized from various solvents, and he determined that the compound could crystallize in at least three polymorphic modifications designated as α-, β-, and γ-forms. Yakowitz (7), in his report, confirmed Watanabe's discovery that sulfanilamide is polymorphic, with at least three phases, and reported refractive indexes and heats of solution of these forms. McLachlan (8), in his book on X-ray crystal structure, mentions four polymorphic forms of sulfanilamide, designated as β-, γ-, δ-, and ϵ-forms.

The techniques of neutron diffraction and X-ray diffraction have been employed in the elucidation of the crystal structures of some polymorphic forms of sulfanilamide. O'Connor and Maslen have determined the structures of α-sulfanilamide (9) and of β-sulfanilamide (10), and Alleaume and Decap have determined the structures of β- and of γ-sulfanilamide (11–14). The present investigation was undertaken to obtain thermodynamic data on heats of transition and fusion of sulfanilamide and sulfanilamide-d_4.

EXPERIMENTAL

Materials—Sulfanilamide-d_4 was prepared by repeated recrystallization of sulfanilamide from monodeuteroethanol, monodeuteromethanol, or deuterium oxide. The progressive exchange of D for H in the amino and amide groups of sulfanilamide was observed in the IR spectrum of the material. After five or six recrystallizations, the exchange was virtually complete. The isotopic purity of sulfanilamide-d_4 was further confirmed by NMR spectroscopy using dimethyl-d_6-sulfoxide as the solvent. Monodeuteromethanol and monodeuteroethanol were prepared by the method described by Greive and Sporek (15). Monodeuterobutanol was obtained by repeated isotopic exchange of *n*-butanol with deuterium oxide (Merck, AG, 99.75% isotopic purity) in the presence of deuterium chloride as the catalyst. Acetone-d_6 (Mallinckrodt) had a stated isotopic purity of not less than 99.5%.

Methods and Instrumentation—Polymorphic forms of sulfanilamide and sulfanilamide-d_4 were prepared by recrystallization from appropriate solvents. Distilled water, ethanol, methanol, butanol, and acetone were used as solvents in the recrystallization of sulfanilamide, while deuterium oxide, monodeuteroethanol, monodeuteromethanol, monodeuterobutanol, and acetone-d_6 were used in the recrystallization of sulfanilamide-d_4, which had been prepared as described. The α-, β-, and γ-forms of sulfanilamide were obtained by methods described by Watanabe and Kamio (5). The δ-form was obtained by recrystallization of sulfanilamide from hot, saturated butyl alcohol. Deuterated solvents, analogous to those used for obtaining the four forms of sulfanilamide, were employed in the preparation of the four deuterated modifications.

The Du Pont 900 differential thermal analyzer (DTA), equipped with a standard cell, was employed to detect polymorphic transitions and melting in the various forms of sulfanilamide and sulfanilamide-d_4. Thermograms, plots of differential temperature as a function of the sample temperature, were obtained. In each case the sample was heated from room temperature to a temperature several degrees above fusion, at a uniform rate of 10°/min. Samples of approximately 2 mg. were employed in ordinary glass capillary tubes, 2 mm. in diameter. Nitrogen gas was flushed through the standard cell during the heating procedure to minimize oxidative decomposition. The reference used in these experiments was glass beads. Temperatures of phase transitions were obtained from the thermograms and were corrected for nonlinear temperature response of the chromel/alumel thermocouple.

Quantitative measurements of the heats of transition and fusion of the compounds were obtained using the Du Pont 900 differential thermal analyzer equipped with a calorimeter cell. While the Du Pont standard cell provides useful qualitative information concerning polymorphic transitions, the geometry of the cell precludes its use for quantitative determinations of heats of transition and fusion. The calorimeter cell was designed for this purpose. Samples of approximately 5 mg. were employed in measurements of heats of fusion, and samples of approximately 7 mg. were employed in measurements of heats of transition. These were weighed on a Cahn electrobalance to the nearest 0.002 mg. The samples were placed in cups fashioned from aluminum foil, and the cups were fitted into the silver sample holder of the calorimeter cell in such a way as to obtain good contact with the bottom of the sample holder. Neither aluminum liner nor reference material was used in the reference cup. Thermograms incorporating the temperature ranges of interest were recorded using the chart recorder of the

Table I—Thermal Behaviors of Sulfanilamide and Sulfanilamide-d_4

Polymorphic Forms	Solvents Used for Recrystallization	Phase Transition Temperature	Heat of Transition, cal./mole × 10^{-2}	Fusion Temperature	Heat of Fusion, cal./mole × 10^{-3}
Form I (α-form)	Ethanol, water	108°	3.58 ± 0.15	166°	5.22 ± 0.07
	Monodeuteroethanol, heavy water	101–105°	3.45 ± 0.02	165°	5.09 ± 0.05
Form II (β-form)	Ethanol, water, methanol, acetone	131–141°	3.47 ± 0.16	166°	5.32 ± 0.03
	Monodeuteroethanol, monodeuteromethanol, heavy water, acetone-d_6	120–122°	2.66 ± 0.02	165°	5.04 ± 0.05
Form III (γ-form)	*n*-Pentanol			166°	5.24 ± 0.05
	Monodeuteropentanol			165°	5.07 ± 0.05
Form IV (δ-form)	*n*-Butanol	108°	3.86 ± 0.07	166°	5.27 ± 0.05
	Monodeuterobutanol	105°	3.60 ± 0.05	165°	5.04 ± 0.03

DTA instrument. The areas of the transition and fusion peaks were obtained by drawing a line from the point at which the thermogram first departed from the baseline to the point at which the baseline was reestablished. Area measurements were performed using a polar planimeter (Keuffel & Esser No. 62005). The area of the peaks obtained in DTA calorimetry is proportional to the heat of transition, and the value of ΔH can be obtained by calculation based on the use of a calibration curve. This curve is constructed from data on the areas of the fusion peaks of materials with known heats of fusion, including gallium, tin, indium, and zinc. The procedure is described in more detail in a paper by Guillory (16). The calibration was found to be accurate to within 2.1% when checked with samples of silver nitrate and benzoic acid. Heats of transition and fusion reported in Table I are average values of six or more determinations. The average deviations from the mean for these measurements are also given.

IR spectra of crystals of sulfanilamide and sulfanilamide-d_4 were taken as potassium bromide pellets. The pellets were prepared using a Beckman potassium bromide dye and a Pasadena Hydraulics, Inc., press at a pressure of 20 tons. The sample–potassium bromide ratio employed was 1:200. Some samples were obtained using the Wilks Mini-Press with a sample–potassium bromide ratio of 0.7:120. Both methods resulted in identical IR patterns. Spectra were obtained on a Beckman IR-10 IR spectrophotometer. The instrument employs two precision replica gratings in a single monochromator. The first operates from 4000 to 600 $cm.^{-1}$, and the second grating operates from 650 to 300 $cm.^{-1}$. Polystyrene film was used to calibrate the wavelength axis.

As an independent check, observations of phase transitions were carried out on the Koefler micro hot stage. A small amount of sample was placed between a slide and cover glass, and the slide was placed on the hot stage. The sample was observed through a microscope as it was heated at a constant rate (10°/min.) up to fusion.

X-ray diffractograms of the polymorphic forms were recorded by the powder diffraction method.[1] These diffractograms are reproduced elsewhere (17).

RESULTS AND DISCUSSION

Four polymorphic modifications of sulfanilamide and sulfanilamide-d_4 were obtained by recrystallization from the solvents listed in Table I. These modifications were subjected to DTA, IR spectroscopy, and DTA calorimetry. Forms I, II, and III of undeuterated sulfanilamide are analogous to the α-, β-, and γ-forms of Watanabe and Kamio (5). The deuterated polymorphic forms are named by analogy to the corresponding undeuterated forms.

The commercially available form of sulfanilamide is the β-form. The three other forms of sulfanilamide also remain stable at room temperature. DTA reveals that the α-, β-, and δ-forms are transformed to the γ-form prior to melting. The γ-form, then, is the only polymorph that melts without undergoing a polymorphic transformation. The deuterated forms of these four polymorphs behave in a precisely analogous fashion.

[1] National Spectrographic Laboratories, Inc., Cleveland, Ohio.

In 1961, Inoue and Saito (18) reported the use of DTA in the study of polymorphic transitions occurring in sulfanilamide. Two thermograms appear in this paper, and an explanation of the thermograms is given by the authors. Two transition peaks are observed in the thermogram of what the authors refer to as the α-form of sulfanilamide. They associate these two peaks with transformations from the α- to the β-form and from the β- to the γ-form, respectively. The latter peak is substantially smaller in area than the former. In the second thermogram, the transition peak is identified as being associated with the β- to γ-transition.

In the present investigation (Figs. 1 and 2), repeated experiments have failed to produce two transition peaks when the α-form is heated from room temperature to fusion. An examination of the data obtained from DTA calorimetry (Table I) reveals that the magnitude of the heat of transition in the case of these two forms is virtually the same. If the α-form did indeed transform to the β-modification at 108°, one would expect to find a transition peak of approximately the same area at 131–141°, the transition temperature range of the β-form. The fact that Inoue and Saito (18) found only a small peak in this range indicates that their sample of the α-form was contaminated with some β-form. The authors of this study believe

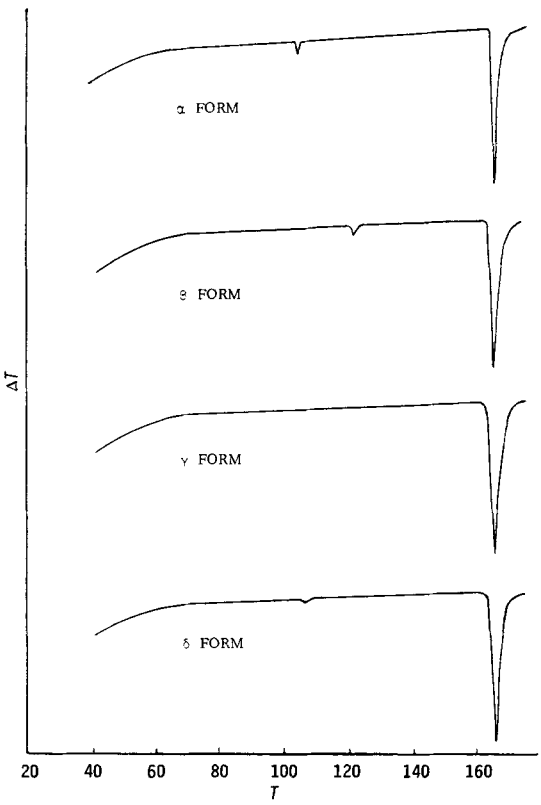


Figure 1—*DTA thermograms of four polymorphic forms of sulfanilamide.*

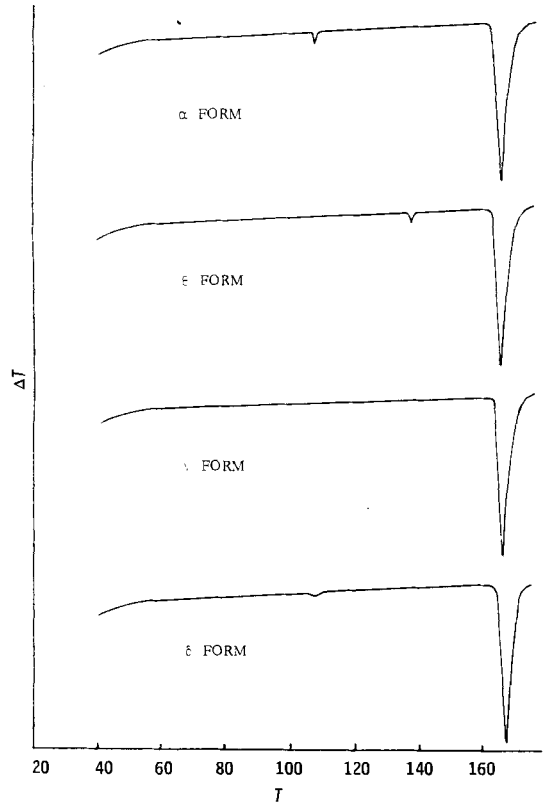


Figure 2—*DTA thermograms of four polymorphic forms of sulfanilamide-d_4.*

that Watanabe and Kamio (5), who reported from thermomicroscopic observations a transition from α to β occurring at temperatures near 90°, made a similar error. According to their account, when suction was used to dry crystals of the α-form, they were converted to the β-form. The authors have found that a similar transition occurs on trituration of the α-modification. Inoue and Saito (18) do not describe the δ- to γ-transition which was detected in this study, nor did they estimate the heats of transition of the polymorphic changes.

Data obtained from DTA calorimetry are listed in Table I. Heats of transition are reported for the α-, β-, and δ-forms. These three forms are converted to the γ-form, which melts without undergoing polymorphic transition. Since three forms are converted to the γ-form prior to fusion, all four forms are expected to exhibit the same heat of fusion. This is found to be true, within the limits of experimental error, for both the nondeuterated and the deuterated forms.

An examination of the heats of transition for the three polymorphs which undergo transition reveals that the δ-form requires the greatest amount of energy to affect its transition to the γ-form. The α- and β-modifications apparently have similar internal energies. This may explain why conversion from the α-form to the β-form occurs so readily. Simple trituration will bring about this change (although heating from room temperature to fusion will not, as pointed out previously).

Hydrogen bonding plays an important role in the crystalline structure of sulfanilamide. Apparently the two hydrogens of each amide group are engaged in NH---O bonding with oxygens on two adjacent sulfanilamide molecules. Similarly, the two hydrogens on each amine function participate in similar hydrogen-bonding interactions. It is interesting to note that X-ray diffraction studies (9–14) suggest that the hydrogen bonds in the α- and β-forms are, on the average, very nearly the same length, but those of the β-modification are slightly longer. This observation is in line with the heats of transition determined in this study (*i.e.*, higher heat of transition for the form with shorter bonds).

The γ-form, which does not exhibit a polymorphic transition, forms hydrogen bonds that are, on the average, somewhat longer than those of the α- and β-forms. Data are not available for the lengths of the hydrogen bonds in the δ-form, but results of this investigation suggest that, on the average, the hydrogen bond lengths in this form will be considerably shorter than those of the α- and β-forms.

The heat of fusion of the deuterated γ-form is approximately 5% less than that of the undeuterated modification, although the melting point is decreased only about 1° by deuteration. Substitution of deuterium for hydrogen apparently lengthens the hydrogen bonds in the crystal lattice (19), and this accounts for the lower heat of fusion.

In the case of the three modifications that exhibit phase transitions prior to fusion, deuteration diminishes the magnitude of the energy required for conversion to the γ-form. The degree of reduction of the heat of transition is approximately the same for the α- and δ-forms as the reduction in the heat of fusion measurements. The β-form, however, shows anomalous behavior, with a reduction in heat of transition of approximately 25%. Apparently, isotopic substitution affects the bond lengths and bond strengths of the hydrogen bonds of these crystals to a different degree.

When the X-ray diffraction patterns obtained for the four modifications of sulfanilamide and sulfanilamide-d_4 are compared (17), it is seen that the crystal structures appear to have similar molecular arrangements in the case of the deuterated and undeuterated β- and δ-forms. There are, however, significant differences in the molecular arrangements in the α- and γ-forms following deuteration. This implies that, at least in some cases, substitution of deuterium for hydrogen in sulfanilamide does more than simply expand the crystal lattice. Deuteration may, or may not, affect the lattice structure.

Figures 3 and 4 show IR spectra of sulfanilamide and sulfanilamide-d_4, respectively. In Fig. 3 it can be seen that the spectra for the α- and β-forms are virtually identical. In preparing samples for IR analysis, grinding and pressing operations are employed which can be expected to bring about a transformation from the α-form to the β-form. Therefore, the first two spectra shown are both of the β-modification. A comparison of the spectra for the deuterated sulfanilamides reveals that three of the forms exhibit essentially identical spectra. Ito and Sekiguchi (20), in a paper on the formation of a molecular compound of sulfanilamide and sulfathiazole, reported that they had examined three deuterated forms of sul-

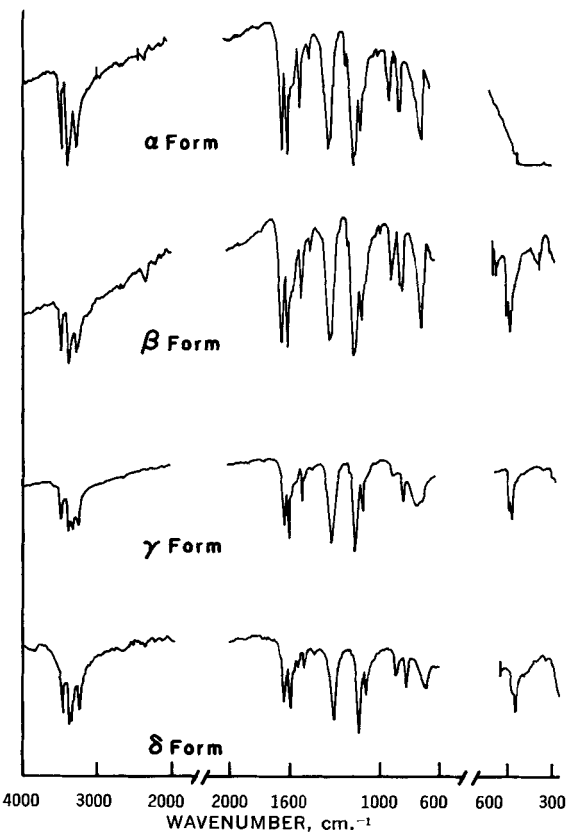


Figure 3—*IR spectra of four polymorphic forms of sulfanilamide.*

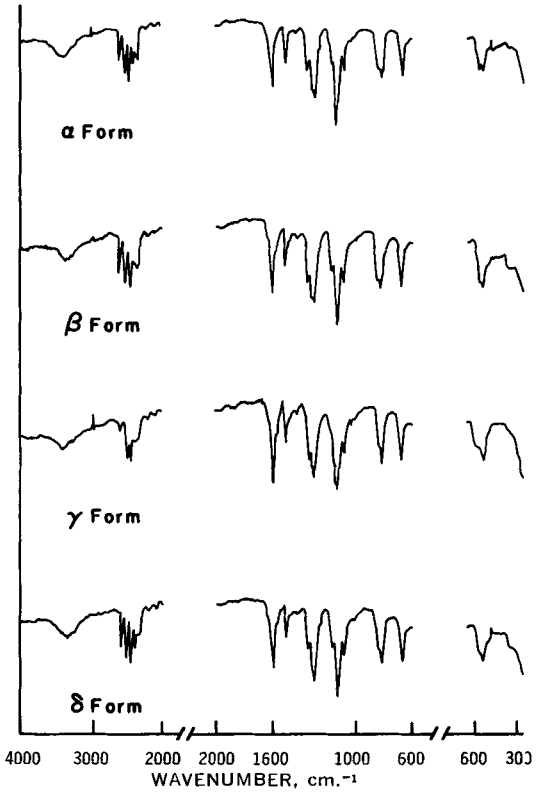


Figure 4—*IR spectra of four polymorphic forms of sulfanilamide-d_4.*

fanilamide and found that: "spectral differences between the three polymorphic forms . . . (were) comparatively few." In the present investigation, minor differences in the spectra were noted in the $-NH_2$ stretching regions but not in the $-ND_2$ stretching regions. Similar differences can be seen in spectra published by Novak *et al.* (21).

The study of polymorphism in sulfanilamide is complicated by the large number of possible hydrogen bonding sites in the molecule; by the formation of hydrogen bonds of various lengths; by the fact that in some forms the unit cell is made up of eight, and in others, of four molecules; and by the fact that the crystal structure of the δ-form has not yet been elucidated. Additional information is required before the mechanisms of the polymorphic transitions occurring in this compound can be understood.

REFERENCES

(1) R. Tawashi, *Science*, **160,** 76(1968).
(2) A. J. Aguiar, *Drug Inform. Bull.*, **3,** 17(1969).
(3) S. S. Yang, "Polymorphism in Sulfonamides," Ph.D. thesis, University of Iowa, Iowa City, Iowa, 1969.
(4) C. V. Zyp, *Pharm. Weekbl.*, **75,** 585(1938).
(5) A. Watanabe and H. Kamio, *J. Pharm. Soc. Jap.*, **62,** 501 (1942).
(6) A. Watanabe, *Naturwissenschaften*, **29,** 116(1941).
(7) M. L. Yakowitz, *J. Ass. Offic. Agr. Chem.*, **31,** 656(1948).
(8) D. McLachlan, "X-ray Crystal Structure," McGraw-Hill, New York, N. Y., 1957, p. 138.
(9) B. H. O'Connor and E. N. Maslen, *Acta Crystallogr.*, **18,** 363(1965).
(10) A. M. O'Connell and E. N. Maslen, *ibid.*, **22,** 134(1967).
(11) M. Alleaume and J. Decap, *ibid.*, **19,** 934(1965).
(12) *Ibid.*, **18,** 731(1965).
(13) M. Alleaume and J. Decap, *C. R. Acad. Sci. Paris*, **261,** 4111(1965).
(14) *Ibid.*, **259,** 3265(1964).
(15) W. H. Greive and K. F. Sporek, *J. Chem. Educ.*, **43,** 381 (1966).
(16) J. K. Guillory, *J. Pharm. Sci.*, **56,** 72(1967).
(17) Hwaing Ou Lin, "Polymorphism in Sulfanilamide-D_4," M.S. thesis, University of Iowa, Iowa City, Iowa, 1968, p. 104.
(18) M. Inoue and T. Saito, *J. Pharm. Soc. Jap.*, **81,** 615(1961).
(19) W. C. Hamilton and J. A. Ibers, "Hydrogen Bonding in Solids," W. A. Benjamin, New York, N. Y., 1968, p. 104.
(20) K. Ito and K. Sekiguchi, *Chem. Pharm. Bull.*, **15,** 420(1967).
(21) A. Novak, J. Lascombe, and M. L. Josien, *J. Physiol. Paris Suppl.*, **5–6,** 38(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 5, 1969, from the *College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication February 6, 1970.

Abstracted in part from a thesis submitted by Hwaing Ou Lin to the Graduate College, University of Iowa, in partial fulfillment of Master of Science degree requirements.

This research was supported by Grant Al 07572 from the U. S. Public Health Service, National Institutes of Health, Bethesda, MD 20014

Distribution, Excretion, and Metabolism of ^{14}C-Labeled Quaternary Ammonium Salt of Perphenazine in Rats

C. L. HUANG, G. M. MIR, and J. Z. YEH

Abstract □ Urinary excretion was the major route of excretion of intraperitoneally administered perphenazine dimethiodide-^{14}C in rats. Peak blood level was observed at 0.5 hr. after the administration of the compound, and brain level was above the detectable level at the same period of time. Antimicrobial activities were demonstrated. The toxicity of quaternary ammonium salt of perphenazine on mice is higher than perphenazine.

Keyphrases □ Perphenazine dimethiodide-^{14}C—synthesis □ Distribution, metabolism, excretion—perphenazine dimethiodide-^{14}C □ Antimicrobial activity—perphenazine dimethiodide-^{14}C □ UV spectrophotometry—analysis □ Paper chromatography—radio scanning

Perphenazine is a phenothiazine derivative which differs chemically from prochlorperazine only with respect to the substitution of a hydroxyethyl group for the methyl group of the latter drug. Perphenazine is approximately twice as potent dosagewise as prochlorperazine and exhibits clinical effects and side actions similar to those of chlorpromazine (1–3).

Symchowicz *et al.* (4, 5) have reported the tissue distribution of ^{35}S-perphenazine in rats after the subcutaneous injection of 0.3 mg./kg. of the compound. High concentrations were found in the lungs, adrenals, liver, kidneys, spleen, and pituitary, while the brain had only a residual activity. Blood levels were consistently low. High levels of radioactivity were found in the pituitary gland after 48 hr. and remained relatively high even after 6 days. Over 80% of the administered activity was excreted during the 24-hr. period, of which 64% was found in the feces and only 16% in the urine.

Huang and Kurland (6), who focused their attention on identification and quantitative determination of the major metabolites of perphenazine, found that unlike chlorpromazine (7, 8) the rate of excretion of perphenazine metabolites was low; approximately 44% of the administered dose was recovered in man during a 7-day period. Perphenazine glucuronides were found to be the major metabolites, representing approximately 69% of the total urinary metabolites. Perphenazine sulfoxide was found to be approximately 13% of the administered dose, and the unchanged perphenazine was usually undetectable.

It has been demonstrated that toxic properties of phenothiazine neuroleptics do not diminish by quaternization of the side-chain nitrogen but rather increase in most cases (trifluoperazine, mepazine, and promethazine). The excretion of the majority of the administered activity of quaternary derivative of mepazine, promethazine (9), and trifluoperazine (10) was consistent with predominantly fecal excretion.

Absorption of these compounds from the intraperitoneal cavity was rapid, which is reflected in a rapid increase of blood levels in rats in 0.5 hr. Brain levels were generally low and were either trace or insignificant against the background activity. All these compounds seemed to have a particular affinity for the bone, since significant activities were recorded in the bone after 5 days. No radioactivity was found in the calcium carbonate formed from the carbon dioxide collected in the expired air from the animals placed in metabolic jars, which indicated that demethylation of these quaternary compounds did not occur *in vivo*. Antimicrobial activities were also demonstrated. High intestinal activity appeared to be due to the secretory process of these drugs *via* bile. As indicated in a previous communication (10), trifluoperazine, one of the potent phenothiazine neuroleptics with a piperazinyl side chain, is excreted unchanged. It would be of interest to see how a compound of this series with a primary alcohol function on the side chain would behave *in vivo*. In this report, distribution and elimination of perphenazine dimethiodide-^{14}C in rats are described.

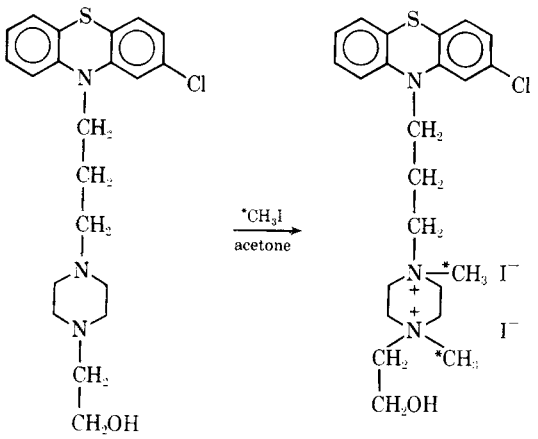
METHODS[1]

Synthesis of Perphenazine Dimethiodide (PPZ-DMEI)—Perphenazine dihydrochloride (0.53 g., 1.1 mmoles) was dissolved in water. The solution was adjusted to pH 10 and extracted several times with ether. The combined ether extracts were dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The oil (0.42 g., 0.97 mmole) remaining in the container was dissolved in 10 ml. of acetone, and methyl iodide (0.71 g., 5 mmoles) was added. The mixture was left at room temperature for 1 hr., and about 10 ml. of anhydrous ether was added to precipitate the quaternary ammonium salt. The precipitate was collected and recrystallized from methanol–ether to yield 0.36 g. (50%) of a white crystalline powder with m.p. 207–209°; $\lambda_{max.}$, 218 and 256 mμ; R_f = 0.50.

Anal.—Calcd. for $C_{23}H_{32}ClIN_3OS$: C, 40.12; H, 4.65; N, 6.10; S 4.65. Found: C, 40.38; H, 4.76; N, 6.11; S, 4.87.

Synthesis of Perphenazine Dimethiodide-^{14}C (PPZ-DMEI-^{14}C)—The free perphenazine base (121 mg., 0.3 mmole) was dissolved in 5 ml. of acetone, and 85.5 mg. (0.6 mmole) of ^{14}C-methyl iodide (11.7 μc./mg. in 5 ml. of acetone) was added. About 300 mg. of unlabeled methyl iodide was added as a carrier. The container was stoppered and left standing at room temperature for 24 hr. Then ether was added to precipitate the product. The crude material was recrystallized from ethanol–ether to yield 104 mg. (50%) of the final product with m.p. 207–209° and specific activity 3.27 μc./mg. Mixed melting point with PPZ-DMEI above did not show depression (207–209°). The authenticity and radiochemical purity of the product were established by paper chromatography coupled with a radiochromatogram scanner, Actigraph III. The physical properties of this compound were found to be identical with PPZ-DMEI. The scheme of the synthesis of this compound is shown in Scheme I.

[1] Melting points were taken in a Fisher-Johns apparatus and were corrected. UV absorption spectra were recorded on a Perkin-Elmer model 202 spectrophotometer. Elemental analysis was performed by Galbraith Laboratories. Paper chromatograms were developed in a solvent system, *n*-butanol–ethanol–water (5:2:2), and in the case of radiochromatogram, an Actigraph III (Nuclear-Chicago) was used to record the activity. Radioactivity in the tissues was recorded in a G-M counter (Tracerlab, model TGC-2), with the efficiency of 9%. All of the recordings were carried out at a constant geometry, and corrections for self-absorption were made for the thick-layer preparations.



Scheme I—Synthesis of Perphenazine Dimethiodide-^{14}C

Tissue Distribution Studies—PPZ-DMEI-^{14}C (1 mg.) was dissolved in 1 ml. of water and injected intraperitoneally into five Holtzman rats (one rat for each time period) weighing 175–250 g. The animals were sacrificed at various intervals of 0.5, 1, 2, 4, and 8 hr. after the injection. The liver, kidneys, spleen, heart, lungs, intestines, stomach, muscle, bone, and brain were isolated, rinsed with normal saline solution, and briefly dried, and their weights were recorded. The whole organ (except blood and bone) was homogenized with water 10 times its weight. An aliquot (0.5 ml.) was measured and evaporated to dryness in a planchet and the activity of the residue was recorded. The blood specimen (0.1 ml.) was measured and dried directly in a planchet, and a portion (0.1 g.) of the bone (femur, dried and ground to a powder) was placed in a planchet to record the activity.

Urinary and Fecal Excretion Studies—One milliliter of the aqueous solution of PPZ-DMEI-^{14}C (1 mg./ml.) was injected intraperitoneally into four Holtzman rats weighing 175–250 g. The animals were maintained in metabolic cages with free access to food and water. The urine and feces were collected separately every 8 hr. An aliquot (0.5 ml.) of the urine specimens was measured in a planchet and dried for recording activity. Since it was a thin-layer preparation, no correction for self-absorption was required. The feces were dried and powdered. A fraction (0.1 g.) was weighed out in a planchet to record the activity. Corrections for self-absorption were made for the preparation.

Biliary Excretion Studies—Holtzman rats weighing about 300 g. were anesthetized by a subcutaneous administration of 70 mg./kg. of pentobarbital sodium. An incision was made on the abdominal wall, and the common bile duct was cannulated with a polyethylene tube, PE50 (Clay-Adams). About 1 ml. of the test solution (1 mg./ml.) was placed directly into the intraperitoneal cavity and the incision was closed. Bile specimens were collected at the intervals of 0.5, 1, and 2 hr. All animals were sacrificed at the end of the 2-hr. period; intestines and urine in the bladder were collected and the activities were recorded.

Metabolic Studies—To study the *N*-demethylation *in vivo*, two rats were injected intraperitoneally with PPZ-DMEI-^{14}C and placed in a large metabolic jar with a constant flow of air to remove carbon dioxide produced by the animals. The air was passed through a gas washing bottle containing 40% sodium hydroxide solution. The carbon dioxide collected in the gas washing bottle was precipitated by adding 50% calcium chloride solution; the calcium carbonate thus formed was collected and dried and the activity was recorded.

Paper chromatographic technique was used to determine metabolites of PPZ-DMEI-^{14}C in urine and feces. Pooled urine (10 ml.) from three rats after the intraperitoneal administration of PPZ-DMEI-^{14}C was condensed to 1 ml. and centrifuged to remove solid precipitate. An aliquot of 0.5 ml. of the supernatant liquid was placed linearly on Whatman 3 MM paper, and the chromatogram was developed in the solvent system previously mentioned. A reference PPZ-DMEI-^{14}C was used as a control. The chromatogram was scanned in a radiochromatogram scanner, Actigraph III, and the R_f values of the corresponding spots were calculated.

Feces collected over a 3-day period after the administration of PPZ-DMEI-^{14}C were extracted with ether in a continuous extraction apparatus for 2 days to remove fatty substances. The ether extracts which did not show radioactivity nor color reaction with 50% sulfuric acid were discarded. The residue in the continuous extraction apparatus extractor was extracted with methanol for 3 days. The methanol extracts were evaporated to 1–2 ml. under reduced pressure, and an aliquot of 0.1 ml. was chromatographed on Whatman 3 MM paper and treated in the same manner described for the analysis of urinary metabolites.

Table I—Recovery of Radioactivity[a] from Urine and Feces of Four Rats after Intraperitoneal Administration of 1 mg. (1 ml.) of Aqueous Solution of Perphenazine Dimethiodide-^{14}C

Time, hr.	Urine, %	Feces, %
8	13.0 ± 4.05	0.03 ± 0.00
16	14.0 ± 3.01	0.03 ± 0.00
24	3.4 ± 0.60	0.48 ± 0.04
32	2.5 ± 0.21	9.12 ± 2.32
40	1.9 ± 0.33	0.60 ± 0.05
48	1.5 ± 0.20	0.44 ± 0.25
56	1.1 ± 0.07	0.20 ± 0.08
64	1.1 ± 0.10	0.12 ± 0.06
88	0.5 ± 0.85	2.4 ± 0.22
112	0.4 ± 0.01	0.96 ± 0.31

[a] Mean percent of the administered activity ± standard error.

Acute Toxicities—A series of dilutions of aqueous solution of PPZ-DMEI was administered intraperitoneally to a group of 10 male albino mice (Southern Animal Farms, Prattville, Ala.). Each animal was placed in an individual cage with free access to water. Acute toxicity was observed and the mortality recorded. LD_{50} was calculated according to the method of Litchfield and Wilcoxon (11).

Antimicrobial Activities—Antibacterial and antifungal activities of PPZ-DMEI were tested by using a diffusion method in which a short glass cylinder containing 0.3 ml. of the test solution was placed on a solid culture medium which was previously seeded with a test organism. After incubation at 37° for an optimum growth period for the organism, the diameter of the clear zone surrounding the glass cylinder was taken as the measure of the inhibitory effect of the drug against the organism. In this test, *Staphylococcus aureus* and *Escherichia coli* were chosen to represent a Gram-positive microorganism and a Gram-negative microorganism, respectively. Brain heart infusion agar (Baltimore Biological Laboratory, Baltimore, Md.) was used as the culture medium.

Saccharomyces carlbergensis and *Aspergillus niger* grown on Sabouraud dextrose agar medium were used in the test for antifungal activity.

RESULTS

In contrast to the excretion pattern of trifluoperazine methiodide-^{14}C, the major route of excretion of PPZ-DMEI-^{14}C was

Table II—Distribution of Radioactivity in Tissues of the Rat after Intraperitoneal Administration of Perphenazine Dimethiodide-^{14}C[a]

Organs	Time after Administration, hr. 0.5	1	2	4	8
Blood	19.84	13.84	12.54	6.64	0.64
Bone	2.42	4.21	6.06	6.20	6.70
Brain	0.08	0.08	0.14	0.11	0.15
Muscle	4.78	2.71	2.49	1.31	0.71
Lungs	0.71	0.59	0.65	0.56	0.25
Heart	0.20	0.14	0.13	0.08	0.02
Kidneys	6.13	12.18	14.53	20.93	19.93
Spleen	0.77	0.31	0.37	0.26	0.29
Stomach	1.16	0.44	0.51	0.31	0.35
Intestines	6.46	5.22	5.66	5.54	4.76
Liver	4.76	5.86	5.68	7.25	6.28
Urine	2.84	8.34	15.86	16.00	17.99
Abdominal washings	3.53	1.03	0.50	0.31	0.05
Total	53.60	54.95	65.12	65.50	58.12

[a] Expressed in terms of percent of the administered activity.

Table III—Distribution of Radioactivity in Tissues of the Rat after Intraperitoneal Administration of Perphenazine Dimethiodide-^{14}C[a]

Organs	Time after Administration, hr. 0.5	1	2	4	8
Blood	198.40	138.40	125.40	66.40	6.40
Bone	4.95	8.61	12.40	12.69	13.74
Brain	10.00	11.77	15.31	15.25	17.53
Muscle	21.72	12.40	11.31	5.96	3.24
Lungs	108.54	101.28	87.50	105.89	35.96
Heart	55.00	41.20	32.50	23.77	5.34
Kidneys	670.46	1475.90	1589.22	1978.83	1800.45
Spleen	224.58	91.22	92.50	90.13	90.38
Stomach	24.45	21.07	49.58	20.15	9.09
Intestines	94.20	69.37	83.94	115.02	72.36
Liver	117.32	138.75	132.53	173.33	167.76

[a] Expressed in terms of percent of specific activity of organ (c.p.m./g. of wet weight) against specific activity of whole body (administered activity/g. of body weight).

consistent with predominantly urinary excretion as indicated by the cumulative excretion data shown in Table I. About 55% of the administered activity was recovered during the 112-hr. period, of which 41% was found in the urine and only 14% in the feces. In all cases, urinary excretion was rapid; nearly 70% of the recovered activity appeared in the urine during the first 16-hr. period. However, fecal excretion was somewhat slow; that is, only a small amount of feces specimen was collected within the first 24-hr. period and a large amount of specimen was obtained between 24 and 32 hr. The slow fecal excretion was apparently due to the decreased intestinal motility associated with the sedative effect of the drug. The ratio of the activity between the urine and feces was 2.8:1.

The distribution pattern of radioactivity of this compound is presented in Table II. Radioactivity in the kidneys and liver prevailed over other organs 0.5 hr. after the administration. Rapid absorption of the compound was indicated by a rapid decline in the activity of the recovered abdominal washings, and it was further reflected by the high blood level with a peak at 0.5 hr. after the injection. The blood level decreased thereafter; however, the activity remained above the detectable level after 8 hr. The kidney level was almost parallel to the urinary level, which started to increase at 2 hr. and reached its peak at 4 hr. An average of 6% of the administered activity was found in the liver and 5% in the intestines during the 8-hr. period. The brain level was low but above the significant level. Activity in the brain accumulated slowly but steadily, reaching its peak at 8 hr. In other organs, the radioactivity was in the descending order of stomach, spleen, lungs, and heart during the 8-hr. period.

The overall recovery of the administered activity was between 54 and 66% in these animals. A considerable amount of the material apparently was retained in the fat and skin of the animals, because an average of 4.2 and 9.3% of the injected activity was found in the fat and skin tissues, respectively (digested with hyamine hydroxide and dried in a planchet or activity recording), of two rats (used in the excretion study) 6 days after the administration of PPZ-DMEI-^{14}C.

In contrast to chlorpromazine methiodide-^{14}C, only a low radioactivity (0.45% of the injected dose) of PPZ-DMEI-^{14}C was recovered in the bile collected from the common bile duct at 8 hr. This may be due to a weak affinity of this compound for the hepatic transport mechanism, because a compound which is not actively taken up by liver cells is not actively secreted into the bile through the liver. Activity in the intestinal content from the bile duct-cannulated animals was low (4%) but above the significant level, which suggested an active secretion of the compound to the intestinal lumen by the glands of the intestinal mucosa. Urinary excretion during the same period of time was 8% of the administered activity.

When these data were interpreted in terms of percent per unit wet weight of organs or tissues, a remarkable change in the ratio of activity between the organ was observed. The relative specific activity in the brain was higher than that in the muscles 2 hr. after the administration of the compound (Table III).

A comparative rate of biliary, intestinal, and urinary excretion of PPZ-DMEI-^{14}C and chlorpromazine methiodide-^{14}C in the rats 2 hr. after the intraperitoneal administration of these compounds is shown in Table IV.

The calcium carbonate, obtained by collecting the carbon dioxide produced by the rat after PPZ-DMEI-^{14}C administration, did not show a significant activity. This indicated that *N*-demethylation and degradation of this compound did not occur *in vivo*. However, this does not rule out the possibility that *N*-methyl exchange with neuroamines such as catecholamines might have occurred *in vivo*.

Paper chromatographic technique coupled with radiochromatogram scanner revealed that there were at least three metabolites present in the urine. One of the substances with R_f 0.50 was identified to be the unchanged PPZ-DMEI-^{14}C. A trace of the metabolite with R_f 0.66 was found to be its sulfoxide by cochromatography with an authentic perphenazine sulfoxide. Fecal metabolites are currently under investigation.

When LD_{50} (18.2 mg./kg.) was administered, the principal signs of toxicity were clonic and tonic convulsions; respiratory depression occurred between 5 and 30 min. after the intraperitoneal injection. At lower doses, respiratory depression as well as cyanosis was observed. Dosagewise, PPZ-DMEI-^{14}C (LD_{50}, 18.2 mg./kg., i.p.) appears to be more toxic than its parent compound, perphenazine (LD_{50}, 64 mg./kg., i.p.). On a molar basis, PPZ-DMEI-^{14}C has a higher toxicity than the quaternary methiodide of its analog, such as chlorpromazine and trifluoperazine.

PPZ-DMEI-^{14}C showed an inhibitory effect against the Gram-positive bacteria, *Escherichia coli*, at 10 γ/ml. No remarkable effect was observed on the yeast and fungus tested.

CONCLUSION

Intraperitoneally administered perphenazine dimethiodide-^{14}C was well absorbed by the rats. The majority (40%) of the drug was accumulated in the kidneys and was excreted in the urine; only 14% of the drug was excreted by the intestines. The ratio of the urinary to fecal excretion was 2.8:1. Peak blood level was observed 0.5 hr. after the administration of the drug. Perphenazine dimethiodide-^{14}C seemed to have a particular affinity for the bone. The activity in the bone started to rise in 0.5 hr. and reached its peak level in 1 hr. Brain level was low but above the detectable level at 0.5 hr.

Excretion of the activity of perphenazine dimethiodide-^{14}C was fairly rapid; almost 70% of the activity excreted in urine and feces was recovered in the first 32-hr. period. The quaternary ammonium salt of this compound has a toxicity higher than the parent compound, perphenazine. Antibacterial activities against Gram-positive and Gram-negative bacteria were demonstrated.

REFERENCES

(1) E. A. Swinyard and S. C. Harvey, in "Remington's Pharmaceutical Sciences," 13th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965, p. 1168.

(2) A. A. Kurland, T. E. Hanlon, M. H. Tatum, and A. M. Simapoulos, *J. Nerv. Ment. Dis.*, **132**, 61(1961).

(3) A. A. Kurland and C. L. Huang, *Psychopharmacol. Serv. Cent. Bull.*, **2**, 84(1962).

Table IV—PPZ-DMEI-^{14}C and Chlorpromazine Methiodide-^{14}C Excretion

	Bile	Intestines	Urine
Perphenazine dimethiodide-^{14}C	0.45 ± 0.02[a]	4.10 ± 0.24	8.55 ± 1.10
Chlorpromazine methiodide-^{14}C	29.99 ± 5.88	10.09 ± 1.67	4.10 ± 0.94

[a] Percent of the administered activity ± *SE*.

(4) S. Symchowicz, W. D. Peckham, and P. L. Perlman, *Biochem. Pharmacol.*, **11**, 417(1962).
(5) S. Symchowicz, W. D. Peckham, C. A. Kordubo, and P. L. Perlman, *ibid.*, **11**, 499(1962).
(6) C. L. Huang and A. A. Kurland, *Arch. Gen. Psychiat.*, **10**, 639(1964).
(7) C. L. Huang and A. A. Kurland, *Amer. J. Psychiat.*, **118**, 428(1961).
(8) C. L. Huang and A. A. Kurland, *Arch. Gen. Psychiat.*, **8**, 301(1963).
(9) C. L. Huang, J. Z. Yeh, and S. Y. Hsu, to be published.
(10) C. L. Huang and J. Z. Yeh, to be published.
(11) J. T. Litchfield, Jr., and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96**, 99(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 24, 1969, from the *School of Pharmacy, University of Mississippi, University, MS 38677*
Accepted for publication February 4, 1970.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami meeting, May 1968.

Dissolution of Slightly Soluble Powders under Sink Conditions I: Development of an Apparatus and Dissolution Studies of Salicylic Acid Powders

ISMAT ULLAH* and DONALD E. CADWALLADER†

Abstract □ A three-compartment apparatus was developed for dissolution studies of slightly soluble powders under sink conditions. The apparatus was designed to accommodate up to three phases to provide sink conditions. The apparatus could accommodate a barrier in the dissolution medium to prevent floating powders from entering and dissolving directly into the sink phase. Dissolution studies were conducted with several particle size grades of salicylic acid under nonsink as well as sink conditions. Effects of the rate of agitation and methods of introducing samples (dry or wetted suspensions) were also investigated. The data indicated that under diffusion-controlled rate of agitation, using the appropriate placement of propellers, it was possible to establish a rank order for *in vitro* dissolution times of different particle size salicylic acid powders.

Keyphrases □ Powders, slightly soluble—dissolution, sink conditions □ Particle-size effect—powder dissolution □ Diagram—powder dissolution apparatus □ UV spectrophotometry—analysis

In recent years, much attention has been focused on the problem of drug availability from solid dosage forms. The importance of *in vitro* dissolution-rate studies for solid dosage forms in determining the drug availability has been recognized (1–5), but it is now generally accepted that the *in vitro* results should be correlated to some physiologic parameter. It has been shown that unless appropriate sink conditions are maintained in certain cases, *in vitro* dissolution studies might bear little relationship to *in vivo* dissolution results (6).

Recently, several methods have been developed (3–17) to study the *in vitro* dissolution rates of drugs from solid dosage forms; however, most of these methods lack sink conditions. Only a few methods (6, 15) have been reported for dissolution studies under sink conditions, but these are not suitable for powders. Due to flotation and flocculation of slightly soluble powder, the determination of a rank order in the dissolution rates of slightly soluble powders is a problem even under nonsink conditions. Unless these floating floccules could be broken up, and the powders distributed in such a way that the relative surface areas of different particle-size powders would be available for dissolution, a rank order in the dissolution rates would not be possible. Finholt *et al.* (18, 19) encountered these problems in their attempt to study the effect of particle size on dissolution rates and while comparing the dissolution rates of powders with granules and tablets. Lin *et al.* (20) also found similar problems in the rank order in the dissolution rates of different particle-size powders.

Because certain properties of drug powders play an important part in their dissolution rates from the dosage forms, it is important that the dissolution behavior of powders be studied. An apparatus, which could give a rank order in the *in vitro* dissolution rates of powders and could also accommodate sink phases, would be of value in the development and evaluation of dosage forms where control of certain powder characteristics is important.

The objectives of this investigation were to develop an apparatus which could be used to carry out dissolution studies of slightly soluble powders under sink conditions, and to demonstrate the utility of this apparatus by obtaining an appropriate rank order in the dissolution rates of different particle-size powders of a model drug.

EXPERIMENTAL

Chemicals and Materials —The salicylic acid[1] used was USP grade. The different particle-size grades were obtained by sieving twice through Ro-Tap testing sieve shaker, using U. S. standard sieves. Isopropyl myristate[2] and polysorbate 80[3] were used. All other chemicals were reagent or certified ACS grade.

[1] Fisher Scientific Co.
[2] S. B. Penick, New York, NY 10008
[3] Atlas Chemical Industries, Inc., Wilmington, DE 19899

(4) S. Symchowicz, W. D. Peckham, and P. L. Perlman, *Biochem. Pharmacol.*, **11**, 417(1962).
(5) S. Symchowicz, W. D. Peckham, C. A. Kordubo, and P. L. Perlman, *ibid.*, **11**, 499(1962).
(6) C. L. Huang and A. A. Kurland, *Arch. Gen. Psychiat.*, **10**, 639(1964).
(7) C. L. Huang and A. A. Kurland, *Amer. J. Psychiat.*, **118**, 428(1961).
(8) C. L. Huang and A. A. Kurland, *Arch. Gen. Psychiat.*, **8**, 301(1963).
(9) C. L. Huang, J. Z. Yeh, and S. Y. Hsu, to be published.
(10) C. L. Huang and J. Z. Yeh, to be published.
(11) J. T. Litchfield, Jr., and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96**, 99(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 24, 1969, from the *School of Pharmacy, University of Mississippi, University, MS 38677*
Accepted for publication February 4, 1970.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami meeting, May 1968.

Dissolution of Slightly Soluble Powders under Sink Conditions I: Development of an Apparatus and Dissolution Studies of Salicylic Acid Powders

ISMAT ULLAH* and DONALD E. CADWALLADER†

Abstract □ A three-compartment apparatus was developed for dissolution studies of slightly soluble powders under sink conditions. The apparatus was designed to accommodate up to three phases to provide sink conditions. The apparatus could accommodate a barrier in the dissolution medium to prevent floating powders from entering and dissolving directly into the sink phase. Dissolution studies were conducted with several particle size grades of salicylic acid under nonsink as well as sink conditions. Effects of the rate of agitation and methods of introducing samples (dry or wetted suspensions) were also investigated. The data indicated that under diffusion-controlled rate of agitation, using the appropriate placement of propellers, it was possible to establish a rank order for *in vitro* dissolution times of different particle size salicylic acid powders.

Keyphrases □ Powders, slightly soluble—dissolution, sink conditions □ Particle-size effect—powder dissolution □ Diagram—powder dissolution apparatus □ UV spectrophotometry—analysis

In recent years, much attention has been focused on the problem of drug availability from solid dosage forms. The importance of *in vitro* dissolution-rate studies for solid dosage forms in determining the drug availability has been recognized (1–5), but it is now generally accepted that the *in vitro* results should be correlated to some physiologic parameter. It has been shown that unless appropriate sink conditions are maintained in certain cases, *in vitro* dissolution studies might bear little relationship to *in vivo* dissolution results (6).

Recently, several methods have been developed (3–17) to study the *in vitro* dissolution rates of drugs from solid dosage forms; however, most of these methods lack sink conditions. Only a few methods (6, 15) have been reported for dissolution studies under sink conditions, but these are not suitable for powders. Due to flotation and flocculation of slightly soluble powder, the determination of a rank order in the dissolution rates of slightly soluble powders is a problem even under nonsink conditions. Unless these floating floccules could be broken up, and the powders distributed in such a way that the relative surface areas of different particle-size powders would be available for dissolution, a rank order in the dissolution rates would not be possible. Finholt *et al.* (18, 19) encountered these problems in their attempt to study the effect of particle size on dissolution rates and while comparing the dissolution rates of powders with granules and tablets. Lin *et al.* (20) also found similar problems in the rank order in the dissolution rates of different particle-size powders.

Because certain properties of drug powders play an important part in their dissolution rates from the dosage forms, it is important that the dissolution behavior of powders be studied. An apparatus, which could give a rank order in the *in vitro* dissolution rates of powders and could also accommodate sink phases, would be of value in the development and evaluation of dosage forms where control of certain powder characteristics is important.

The objectives of this investigation were to develop an apparatus which could be used to carry out dissolution studies of slightly soluble powders under sink conditions, and to demonstrate the utility of this apparatus by obtaining an appropriate rank order in the dissolution rates of different particle-size powders of a model drug.

EXPERIMENTAL

Chemicals and Materials —The salicylic acid[1] used was USP grade. The different particle-size grades were obtained by sieving twice through Ro-Tap testing sieve shaker, using U. S. standard sieves. Isopropyl myristate[2] and polysorbate 80[3] were used. All other chemicals were reagent or certified ACS grade.

[1] Fisher Scientific Co.
[2] S. B. Penick, New York, NY 10008
[3] Atlas Chemical Industries, Inc., Wilmington, DE 19899

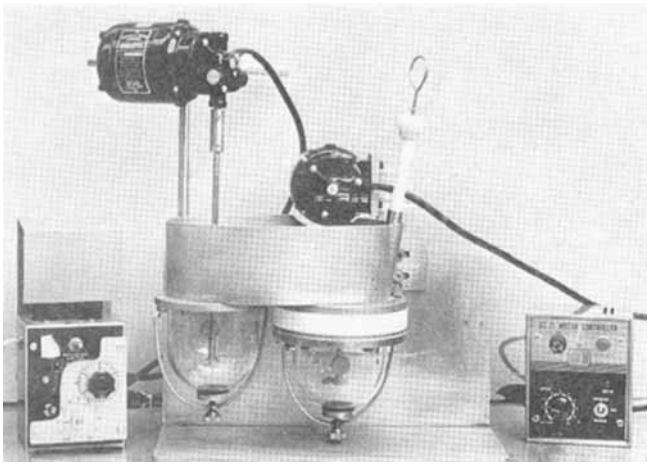


Figure 1—*Dissolution apparatus.*

Apparatus—The dissolution apparatus developed for this study is illustrated in Fig. 1, a schematic diagram of the assembly is shown in Fig. 2, and the dismantled apparatus is given in Fig. 3. The basic parts of the apparatus consist of a main frame; two glass bowls; a pair of Teflon gaskets; a screen or a filter membrane-holding frame; two controlled speed stirrers with stirring shafts, propellers, and impellers; and a sample injector.

The main frame was fabricated from stainless steel. When the glass bowls were clamped to the main frame, the apparatus consisted of three compartments. The main frame served as the compartment (Compartment C) for an organic sink phase.

The glass bowls were 9.0 cm. in diameter with round bottoms. The bowl for Compartment A was 7.3-cm. deep, and the bowl for Compartment B was 9.1-cm. deep.

The Teflon gaskets had 9.1-cm. open diameter. One gasket was 2.0-cm. thick, while the other was 0.16 cm. The screen-holding frame, to which a screen was secured, was 0.3-cm. thick, had four support arms, and had a central hole for a stirrer shaft. As seen in Fig. 3, this screen-holding frame was combined with the sample entrance tube through which samples being studied could be introduced directly into the dissolution medium. To prevent injecting air below the screen when introducing the samples, a side tube was affixed to the sample entrance tube to vent the air. The level of this air vent tube corresponded to the level of the liquid in the apparatus. A 200-mesh screen[4] was used as the barrier throughout these studies. This screen was selected on the basis of transport and equilibrium studies carried out using various membranes and screens as barriers. The results of these studies will be reported in a forthcoming paper.

Two propellers were attached to the left-hand stirrer shaft. A three-blade propeller, 1.9 cm. in diameter, was positioned 2.5 cm. from the bottom of the glass bowl in Compartment B. A three-blade turbine impeller, 2.6 cm. in diameter, was used in the organic phase and positioned 0.9 cm. above the interface in Compartment B.

Two, three, or four propellers were attached to the right-hand stirrer shaft. A three-blade propeller, 1.9 cm. in diameter, was placed 2.2 cm. from the bottom of the glass bowl in Compartment A. A three-blade turbine impeller, 3.0 cm. in diameter, was attached 0.2 cm. above the screen. Other additional impellers were used, depending on the particular experimental conditions, and will be described in detail under other headings.

The sample injector is shown in Fig. 4 and consists of a syringe-like assembly made of Teflon. A Teflon piston was attached to a stainless steel rod, which could be unscrewed to clean the assembly. A Teflon disk or Whatman No. 1 filter paper disk was used to cover the tip of the injector assembly. The fitting of these disks was such that they could be ejected with a slight pressure but would remain intact while the samples were being introduced.

Dissolution of Salicylic Acid Powders under Nonsink Conditions—These experiments were carried out in Compartment A with the apparatus immersed in a 37 ± 0.5° water bath. A 600.0-ml. volume of 0.2 *M* Clark-Lubs pH 2.0 buffer containing 0.05% polysorbate 80 (dissolution medium), which was previously equilibrated[5] at 37.0°, was poured into Compartment A through the sample entrance tube with the help of a separator[6] (there was approximately 145 ml. of dissolution medium above the screen). The stirrer, which was previously set at 55 r.p.m., was started. One gram of salicylic acid powder of desired mesh size was placed in the sample injector. The closed end of the injector was gently tapped three times on a firm surface to give uniform packing. The piston rod was pushed to bring the powder to the tip of the injector, and a Whatman No. 1 filter paper disk was placed over the opening. The sample injector was then introduced into the aqueous phase through the sample entrance tube with the lower tip of the injector placed 4.5 cm. above the bottom of the glass bowl; the sample was then slowly injected into the aqueous phase over a 30-sec. period. One-milliliter samples were removed from the solution above the screen at appropriate intervals. The samples were diluted with pH 2.0 buffer and assayed spectrophotometrically at 302 mμ using a Beckman DU-2 spectrophotometer. A volume of the buffer used for dissolution (37.0°) equal to the sample volume removed was replaced immediately after each sample was taken. Using this method, the effect of particle size on dissolution of salicylic acid was investigated.

Dissolution of Salicylic Acid Powders under Sink Conditions—Three different methods were used for these experiments. In Compartment A, three propellers were used for Methods A and B while four propellers were used for Method C. The third propeller was a three-blade turbine-type impeller, 2.2 cm. in diameter, and was placed 0.6 cm. above the interface. The fourth propeller was also a three-blade turbine-type impeller. This impeller was 4.3 cm. in diameter and was attached 0.1 cm. below the screen-holding frame.

Method A—A 600.0-ml. quantity of dissolution medium was poured into Compartment A as described earlier. A 480.0-ml. volume of 0.2 *M* Clark-Lubs pH 7.4 buffer was used in Compartment B, while 300.0 ml. of isopropyl myristate was placed above the two aqueous phases. The stirrers were started and the three phases allowed to equilibrate for 45 min. The stirrer for Compartment B was adjusted to 100 r.p.m., while the Compartment A stirrer was varied for different experiments as indicated in the particular experiment. One gram of salicylic acid powder of desired particle size was packed and introduced into the dissolution medium as described earlier. One-milliliter samples were removed from each phase[7] at appropriate intervals, and the sample volume was replaced immediately with the appropriate solvent. The samples from Compartments A, B, and C were diluted, respectively, with pH 2.0 buffer, pH 7.4 buffer, and isopropyl myristate and assayed spectrophotometrically at 302, 297, and 306 mμ using a Beckman DU-2 spectrophotometer. Using this method, the effects of various particle sizes and two Compartment A agitation rates (55 and 100 r.p.m.) on the dissolution rate of salicylic acid powder were studied.

Method B—These experiments were carried out as described under Method A except that the samples were introduced as suspensions. A suspension of 1.0 g. of salicylic acid powder and 1.5 ml. of the dissolution medium was made in the sample injector by stirring with a melting point capillary tube. The piston rod was moved to bring the suspension level up to the tip of the injector, and a Teflon disk was placed over the opening. The samples were then shaken vigorously before introducing them into the dissolution medium in Compartment A. The suspensions were slowly injected into the dissolution medium over a 30-sec. period, and samples from each phase were removed at appropriate intervals and assayed spectrophotometrically as described earlier.

Method C—With this method the experiments were carried out as described under Method A, with the exception that an additional three-blade impeller, as described earlier, was attached to the stirrer shaft below the screen in Compartment A. An agitation rate of 30 r.p.m. in Compartment A was used in these experiments.

[4] This screen was obtained from Cambridge Wire Cloth Co. The screen had twilled weave, 68.6-μ pore size and was made from a 58.42-μ diameter wire.

[5] This was necessary so that dissolved air in the buffer would not be evolved and block the screen.

[6] The separator had a 20.0-cm. long stem which, when introduced through the sample entrance tube, would rest on the bottom of the glass bowl. There was no formation of foam below the screen when the dissolution medium was introduced in this manner.

[7] The samples from aqueous phases were removed by dipping pipets into the respective phases through the organic phase. The sides of these pipets were wiped off before delivering the samples.

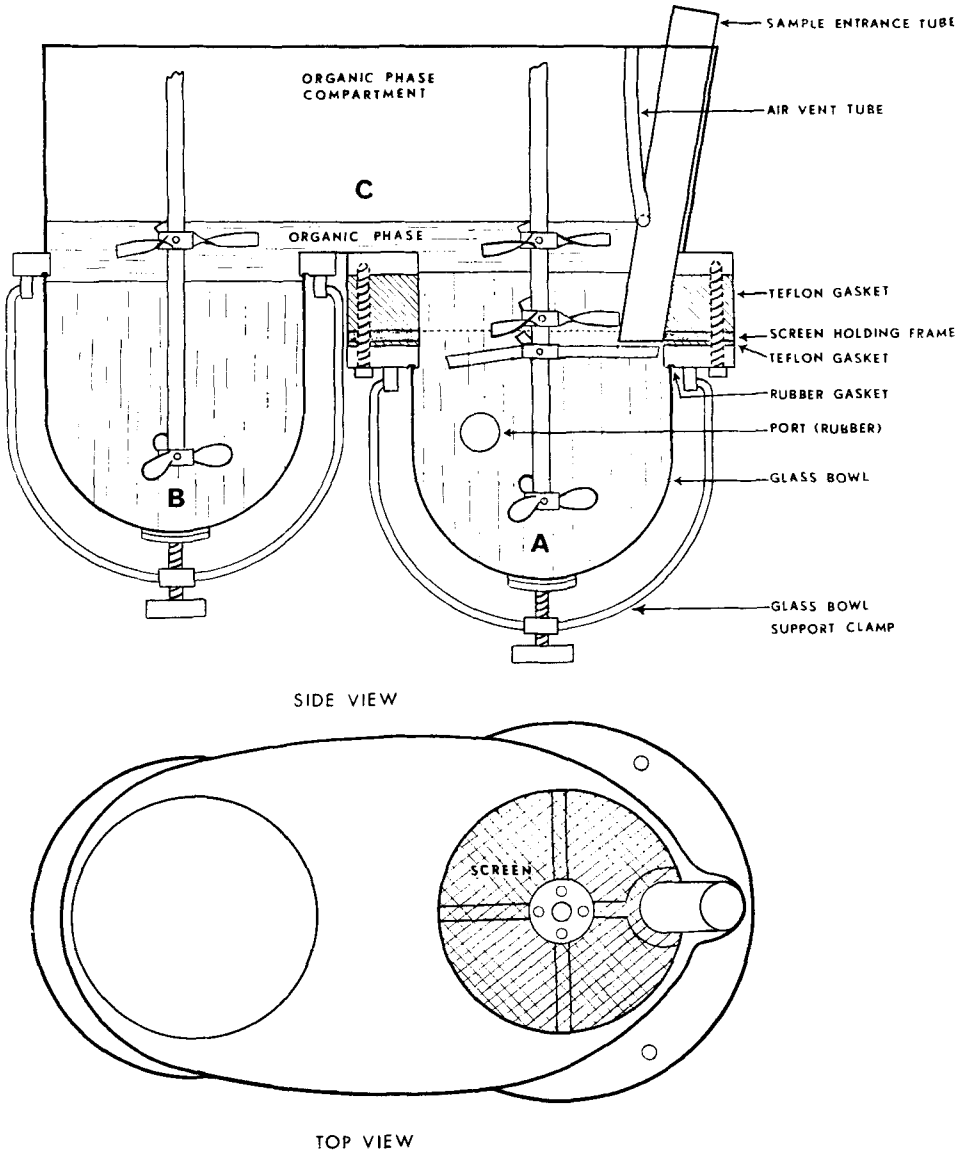


Figure 2—*Schematic diagram of the dissolution apparatus.*

The sample injector was positioned just below the lower edge of the screen-holding frame, so the lower impeller would scrape off the powder sample as it was slowly introduced into the dissolution medium over a 30-sec. period.

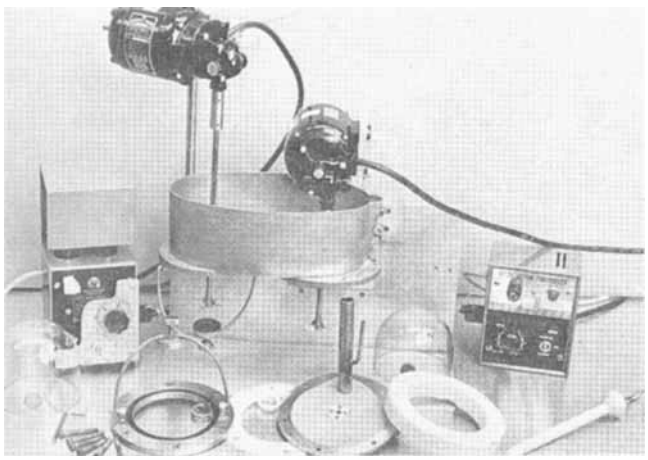


Figure 3—*Dissolution apparatus dismantled.*

RESULTS AND DISCUSSION

Dissolution of Salicylic Acid Powders under Nonsink Conditions—The apparatus used for these studies, as described under *Experimental*, was essentially a modified form of Levy's beaker stirrer assembly. Using this apparatus, the dissolution studies of different particle size salicylic acid powders under nonsink conditions (Fig. 5) show a good rank order for the dissolution rates of medium particle size range powders (40/60, 60/80, and 100/120). The method however, fails to give relative dissolution rates for coarser (20/30) or finer (120/140) powders. Figure 5 shows that after about an hour, there is a higher dissolution rate for 20/30-mesh powder as compared to 40/60 mesh. Similar discrepancies have been reported by other workers (18–20). This might be due to the packing characteristics of different particle-size powders. The 20/30-mesh powder, being coarser, may not pack as tightly as the 40/60-mesh powder and thus might allow a greater movement of the dissolution medium through its particles, resulting in a higher dissolution rate. It was

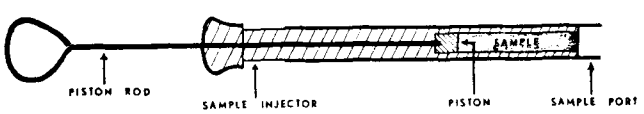


Figure 4—*Sample injector.*

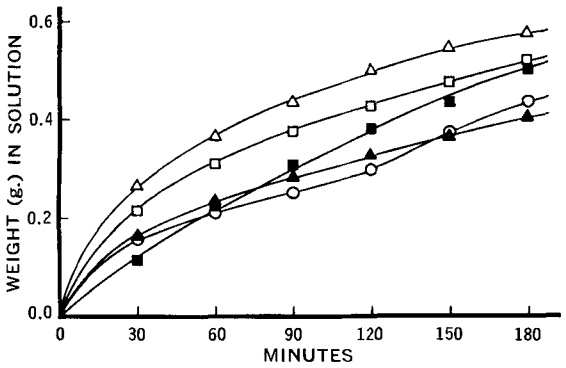


Figure 5—*Effect of particle size on dissolution of salicylic acid under nonsink conditions at 55 r.p.m. and 37°. Key: ○, 120/140 mesh; △, 100/120 mesh; □, 60/80 mesh; ▲, 40/60 mesh; and ■, 20/30 mesh.*

observed that a small portion of the 40/60-mesh powder was dispersed over a larger surface area at the bottom of the vessel than the 20/30-mesh powder, and this could be the reason for higher initial dissolution rates for the finer powder.

Figure 5 also shows a lower than expected dissolution rate for 120/140-mesh powder. This lower dissolution rate is due to the greater tendency of this size powder to agglomerate because of greater surface energy and electrostatic charges (21). In these studies, the powder remained mainly as a single large mass against the bottom of the screen, which resulted in a much lower effective surface area for dissolution. This mass slowly, but not uniformly, disintegrated during the dissolution period. After about 2 hr., there was a slight increase in the dissolution rate due to the breakup of the floating mass into smaller particles.

The flotation problem could still be seen with fine powders, even though the dissolution medium used in the studies contained 0.05% polysorbate 80. In these studies, 20/30 and 40/60-mesh powders sank rapidly; but 60/80-mesh powder floated for about 2 min., 100/120-mesh powder floated for about 20 min., and 120/140-mesh powder remained floating during a 3-hr. experimental run. The preliminary experiments showed that in the absence of polysorbate 80, the flotation problem was much greater even in relatively much coarser powders, and a 120/140-mesh powder did not completely disintegrate and sink even after 24 hr. under similar experimental conditions. The presence of the surfactant in the gastrointestinal contents (22–24) and its use in the dissolution studies have been shown (24). In these studies, the presence of surfactant in the dissolution medium does not completely solve the flotation problem; however, it does substantially decrease its magnitude.

Dissolution of Salicylic Acid Powders under Sink Conditions—The studies under sink conditions show that the dissolution rates (Fig. 6) are generally higher than those under nonsink conditions (Fig. 5). However, a close comparison shows that the differences are small. This is due to the fact that after 3 hr. the dissolution medium is never more than 35% saturated for any mesh size salicylic acid powder, even under nonsink conditions.

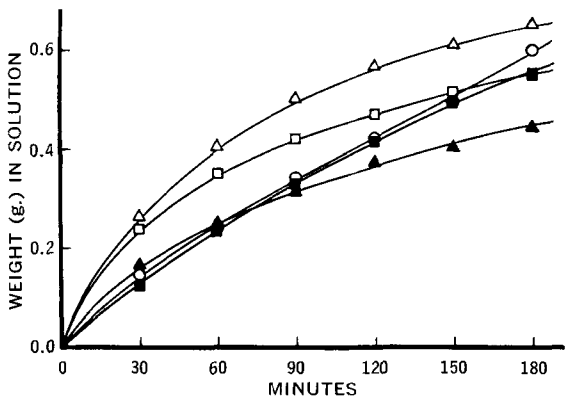


Figure 6—*Effect of particle size on dissolution of salicylic acid under sink conditions at 55 r.p.m. and 37°. Key: ○, 120/140 mesh; △, 100/120 mesh; □, 60/80 mesh; ▲, 40/60 mesh; and ■, 20/30 mesh.*

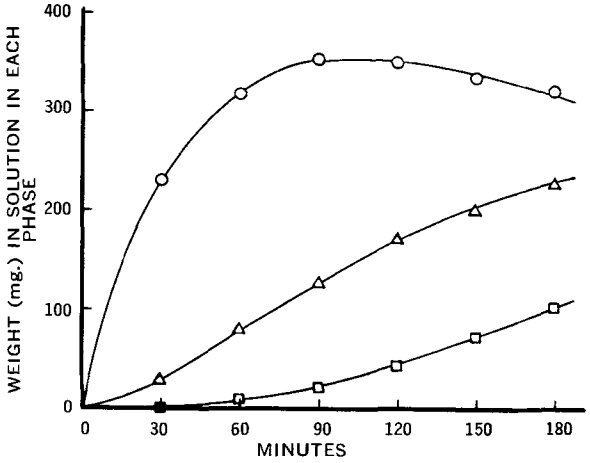


Figure 7—*Dissolution and partitioning of 100/120-mesh salicylic acid powder using Method A at 55 r.p.m. and 37°. Key: ○, pH 2.0 buffer; △, isopropyl myristate; and □, pH 7.4 buffer.*

It is apparent from these studies that the failure to obtain a rank order in the dissolution rates of salicylic acid powders under nonsink conditions was not due to the lack of sink conditions but to the packing, agglomerating, and floating characteristics of the powders. Under these circumstances, the presence of a sink condition would not be expected to make a qualitative change in the dissolution results. The method, however, does demonstrate the technique for using sink conditions and the necessity for a screen barrier to prevent the floating powders from entering directly in the organic phase under sink conditions. The experiments also did not indicate any emulsification at the interface.

Figure 7 shows the partitioning of 100/120-mesh salicylic acid powder as the dissolution takes place. The development of concentration in the pH 7.4 buffer seems to be a little slow and could possibly be increased by using a higher pH buffer; however, no such attempt was made because the purpose of these studies was mainly to demonstrate the use of a three-phase sink system and not necessarily to develop a perfect sink system which, of course, would be different from product to product.

Dissolution studies at 100 r.p.m. under sink conditions (Fig. 8) show that, at this rate of agitation, it is possible to have a large rank order difference between coarser powders (20/30 and 40/60). This could be due to a greater distribution of 40/60-mesh powder under the force of agitation. Figure 8, however, shows a decrease in the difference of the dissolution rates of the finer (60/80 and 100/120) powders. This could be due to the fact that, at this rate of agitation,

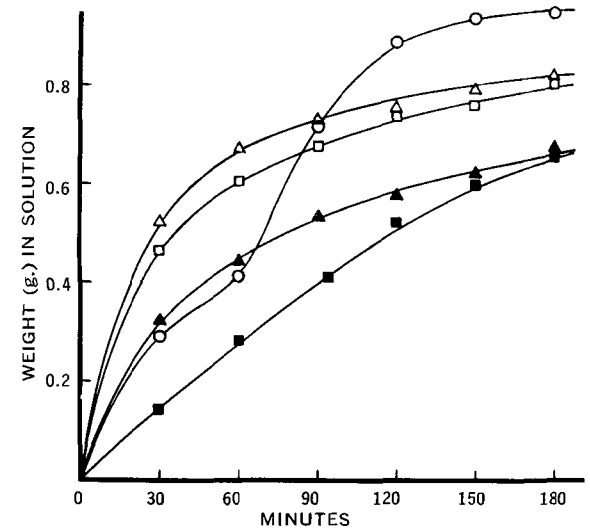


Figure 8—*Effect of particle size on dissolution of salicylic acid under sink conditions at 100 r.p.m. and 37°. Key: ○, 120/140 mesh; △, 100/120 mesh; □, 60/80 mesh; ▲, 40/60 mesh; and ■, 20/30 mesh.*

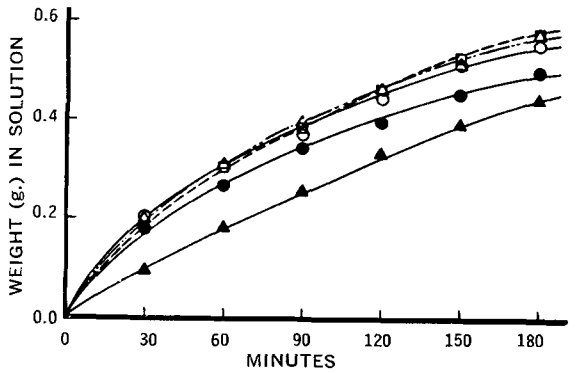


Figure 9—*Effect of particle size on dissolution of salicylic acid under sink conditions using suspension samples at 55 r.p.m. and 37°. Key: ○, 200/230 mesh; △, 120/140 mesh; □, 60/80 mesh; ●, 40/60 mesh; and ▲, 20/30 mesh.*

the finer powders cannot remain distributed on the lower sides and bottom of the vessel but are forced into the central vortex, where there is less surface area exposed to the dissolution medium. The 120/140-mesh powder shows an initial lower dissolution rate even at this high rate of agitation. The floating mass is wetted and completely disintegrated after about an hour, resulting in an increased dissolution rate. Although the flotation of powders was partially reduced with the increase in the rate of agitation, the problem of powder distribution was aggravated due to vortexing; the rank order problem was not solved by this experimental method.

Dissolution of Salicylic Acid Powders Using Suspension Samples—Since the problem of rank order in the dissolution rates could be attributed mainly to flotation, flocculation, and poor distribution, it was thought that if prewetted powder samples could be properly distributed, a rank order might be possible. Dissolution studies using samples as wetted suspensions (Method B) did not show any significant difference in the dissolution profiles of powders having particle sizes higher than 40/60 mesh (Fig. 9). A rapid settling of the powders was observed and there was no flotation and flocculation. Due to rapid settling of the powders, however, it appeared that the particles could not be distributed well enough to expose relatively different surface areas; different dissolution rates for different particle size powders above 40/60 mesh did not occur.

A similar phenomenon was observed by Finholt *et al.* (18), who could not differentiate dissolution rates of different particle size acetylsalicylic acid powders in 0.1 *N* HCl containing 0.2% polysorbate 80. These workers mentioned the possibility of a complex formation between acetylsalicylic acid and polysorbate 80 as

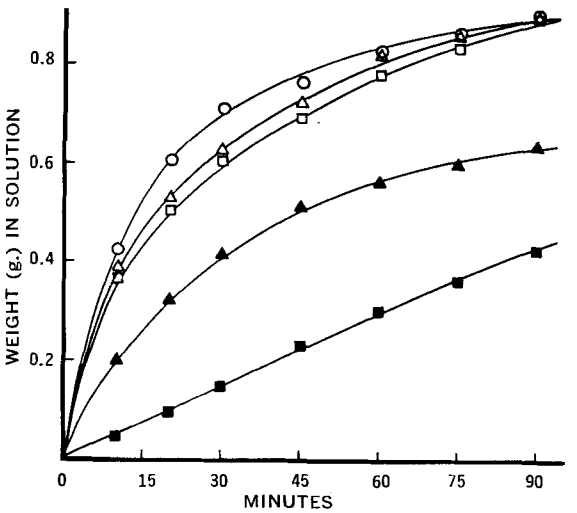


Figure 10—*Effect of particle size on dissolution of salicylic acid under sink conditions using four propellers at 30 r.p.m. and 37°. Key: ○, 200/230 mesh; △, 120/140 mesh; □, 60/80 mesh; ▲, 40/60 mesh; and ■, 20/30 mesh.*

Table I—Reproducibility of Dissolution Method A for the Dissolution of 100/120-Mesh Salicylic Acid Powder at 55 r.p.m. and 37°

Dissolution Time, min.	Mean *Weight*[a] (mg.) in Solution	*SD* ± mg.	Percent *SD*
30	262.9	31.1	11.82
60	406.1	59.7	14.70
90	504.3	86.7	17.39
120	567.3	85.5	15.05
150	610.5	89.7	14.69
180	652.8	92.7	14.20
		Mean =	14.65

[a] Each value is an average of six experiments.

being responsible for this unusual behavior. They did not introduce the samples as suspensions, but the powders were spread over the dissolution medium. An alternative explanation for their observation might be that the powders were wetted rapidly and, under the slow rate of agitation used, were not distributed enough in the dissolution vessel to give relatively different surface areas for dissolution.

In the present studies, a lower concentration of polysorbate 80 (0.05%) was used. This low concentration is not enough to complex a major fraction of the sample used and probably does not explain the results. Since good differentiation in the dissolution rates of some powders was achieved using the same concentration (0.05%) of polysorbate 80 (Figs. 5 and 6), it would appear that some other factor is responsible for the lack of differentiation in this case. The most likely factor that would explain the lack of differentiation is the rapid settling of the powders. Figure 9 also shows relatively lower dissolution rates as compared to Fig. 6. This difference can also be explained on the basis of rapid settling and poor distribution of the powders.

Dissolution of Salicylic Acid Powders under Sink Conditions Using Additional Lower Impeller—The preceding studies on dissolution of salicylic acid powders showed that the apparatus was adequate for dissolution studies under sink conditions; however, a rank order in the dissolution rates of different particle size powders could not be obtained. The failure was probably due to a lack of mechanism to wet and disintegrate rapidly the large floating powder masses (in the case of fine powders) and to inadequate distribution of the particles in the dissolution medium. An increase of rate of agitation or prewetting of the samples also did not overcome the problems. Consideration of the previous results indicated that an additional lower impeller just below the barrier might solve these problems. A rate of agitation of 30 r.p.m. was selected on the basis of preliminary equilibrium and dissolution studies using the additional lower impeller.

Dissolution profiles of various particle size salicylic acid powders, using an additional lower impeller (Method C), are shown in Fig. 10. The profiles show a good rank order for the different particle size powders studied. The dissolution profiles do not give conventional linear rate order plots, but this is not surprising since a multiparticulate system is very complex.

Table II—Reproducibility of Dissolution Method C for the Dissolution of 120/140-Mesh Salicylic Acid Powder at 30 r.p.m. and 37°

Dissolution Time, min.	Mean Weight[a] (mg.) in Solution	*SD* ± mg.	Percent *SD*
10	392.1	9.5	2.42
20	538.0	8.0	1.48
30	632.0	15.2	2.40
45	730.8	19.6	2.68
60	828.3	26.7	3.22
75	863.0	16.8	1.94
90	891.7	18.4	2.06
		Mean =	2.31

[a] Each value is an average of six experiments.

Since these studies show a rank order for the dissolution of different particle-size powders and the studies were conducted under sink conditions using a diffusion-controlled rate of agitation, it might be possible to correlate such studies with some physiological parameter.

Since the main difference in the dissolution Methods A and C was the presence of an additional lower impeller in the latter method, the achievement of the rank order in the dissolution rates of different particle-size powders can be attributed to the effect of this impeller. This impeller runs very close (0.1 cm.) to the lower end of the sample entrance tube where it scrapes off the powders as they are introduced into the dissolution medium and distributes them evenly on the bottom and sides of the dissolution vessel. In this way, even coarser powders are not allowed to drop to the bottom in the form of small piles but are distributed relatively more widely, resulting in higher dissolution rates as compared to the rates of Method A (Figs. 5 and 6). Since the smaller particle-size powders require relatively longer times for settling, they are distributed differently and relatively more widely by the action of the lower impeller. Another very important effect of the lower impeller on very fine powders (120/140 and smaller), which tend to float, is the slight impact it has on the floating masses due to its proximity (upper edge of this impeller is 0.4 cm. below the screen). Under this gentle impact, the floating masses were rapidly disintegrated and settled evenly on the lower sides of the dissolution vessel.

Figure 10 shows that after about 80% of the sample weight has been dissolved, the dissolution profiles of finer powders converge. This could be due to the fact that in the case of very fine powders (120/140 and 200/230), some glomerates are not completely disintegrated before settling on the bottom portion of the dissolution vessel. After the major portion of the powder has been dissolved, these small glomerates remain at the bottom of the vessel and their surface area is about the same as the large-size powders. However, this does not seem to be a serious problem since it occurs after a major portion (above 80%) of the sample has been dissolved.

Although a fine powder (120/140 mesh) was used for dissolution studies with Method C as compared to a relatively coarser powder (100/120 mesh) for Method A, the comparison of the data in Tables I and II shows that Method C with an additional lower impeller is much more reproducible as compared to Method A which does not have this lower impeller. The mean percent standard deviation is more than 6 times greater than for Method C. Student's t test conducted on percent standard deviation gave the value of 15.95, which shows that Method C is significantly better than Method A even at 99.9% confidence limits. This can be explained on the basis that in the case of Method C, the lower impeller distributes the powders uniformly each time, while in its absence (Method A), the powders disintegrate and distribute in a haphazard and nonuniform fashion.

REFERENCES

(1) L. J. Edwards, *Trans. Faraday Soc.*, **47,** 1911(1951).

(2) E. L. Parrott, D. E. Wurster, and T. Higuchi. *J. Amer. Pharm. Ass., Sci. Ed.*, **44,** 269(1955).

(3) E. Nelson, *ibid.*, **47,** 297(1958).

(4) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262,** 1058 (1960).

(5) G. Levy and B. A. Sahli, *J. Pharm. Sci.*, **51,** 58(1962).

(6) M. Gibaldi and S. Feldman, *ibid.*, **56,** 1238(1967).

(7) L. C. Schroeter and J. G. Wagner, *ibid.*, **51,** 957(1962).

(8) P. J. Niebergall and J. E. Goyan, *ibid.*, **52,** 29(1963).

(9) E. Shefter and T. Higuchi, *ibid.*, **52,** 781(1963).

(10) G. Levy, *ibid.*, **52,** 1039(1963).

(11) G. Milosovich, *ibid.*, **53,** 484(1964).

(12) A. H. Goldberg, M. Gibaldi, J. L. Kanig, and J. Shanker, *ibid.*, **54,** 1723(1965).

(13) B. Calesnick, B. Katchen, and J. Black, *ibid.*, **54,** 1277 (1965).

(14) E. Marlow and R. P. Shangraw, *ibid.*, **56,** 498(1967).

(15) P. J. Niebergall, M. Y. Patil, and E. T. Sugita, *ibid.*, **56,** 943(1967).

(16) M. Pernarowski, W. Woo, and O. Searl, *ibid.*, **57,** 1419(1968).

(17) National Formulary Committee, personal communication, Dec. 1968.

(18) P. Finholt, H. Kristiansen, O. C. Schmidt, and K. Wold, *Medd. Nor. Farm. Selsk.*, **28,** 17(1966).

(19) P. Finholt, R. H. Pedersen, S. Solvang, and K. Wold, *ibid.*, **28,** 238(1966).

(20) S. L. Lin, J. Menig, and L. Lachman, *J. Pharm. Sci.*, **57,** 2143(1968).

(21) J. H. Fincher, *ibid.*, **57,** 1825(1968).

(22) B. Borgstrom, *Acta Chem. Scand.*, **11,** 749(1957).

(23) B. Borgstrom, A. Dahlquist, G. Lundh, and J. Sjovall, *J. Clin. Invest.*, **36,** 1521(1957).

(24) P. Finholt and S. Solvang, *J. Pharm. Sci.*, **57,** 1322(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 6, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication March 5, 1970.

Abstracted in part from a dissertation submitted by Ismat Ullah to the Graduate School, University of Georgia, in partial fulfillment of Doctor of Philosophy degree requirements.

The authors wish to acknowledge the valuable suggestions of James Price, John Poole, and George Irwin.

* Present address: Pharmaceutical Pilot Plant, Wyeth Laboratories, Inc., P.O. Box 861, Paoli, PA 19301

† To whom inquiries should be sent.

Release, Uptake, and Permeation Behavior of Salicylic Acid in Ointment Bases

MASAHIRO NAKANO* and NAGIN K. PATEL†

Abstract □ *In vitro* release of salicylic acid from ointments was investigated by a diffusion technique employing a silicone rubber membrane. The advantages of this membrane over a cellophane membrane are discussed. Release of salicylic acid from five ointment bases indicated that emulsion-type ointment bases are superior to oleaginous and polyethylene glycol ointment bases. Uptake of salicylic acid by polyethylene glycol ointment from the solution was much faster than that by others, indicating a fairly strong affinity of this drug for the polyethylene glycol base. The permeation of salicylic acid from the aqueous solution through ointment bases to another aqueous solution was studied with a three-compartment diffusion cell to examine the relative importance of the factors involved in the process. Dimethyl sulfoxide and dimethylacetamide facilitated the release of salicylic acid from the ointments. Di-*n*-butylpropionamide was found to increase both the release of salicylic acid from the ointment and permeation through the ointment base. The *in vitro* release pattern from various bases is in agreement with the *in vivo* data reported in the literature.

Keyphrases □ Ointment bases—salicylic acid □ Salicylic acid in ointment—release, uptake, permeation □ Release rates—salicylic acid, ointment bases □ Silicone rubber membrane—salicylic acid transfer □ UV spectrophotometry—analysis

Physiological availability of a topically applied drug depends on both the rate of release from the vehicle and the permeability through the skin. The former is physicochemical in nature, whereas the latter may be called physiological. The role of the physicochemical factor in the overall availability of a drug has not been fully understood. To explore this point, the behavior of a drug in ointment bases was examined with a diffusion cell and a silicone rubber membrane instead of the commonly used cellophane membranes (1, 2). Since an *in vitro* study using an excised skin (3) and a number of *in vivo* studies (4–6) deal with salicylic acid, this agent was selected for the present study.

Some of the special features and objectives of the present work are as follows. (*a*) The feasibility of the use of silicone rubber membranes in availability studies *in vitro* was investigated. (*b*) The very slow rate of release of salicylic acid from polyethylene glycol ointment prompted the authors to investigate the affinity or interactive nature of the drug for the ointment base by measuring the uptake of the drug from the solution by the ointment base. (*c*) To ascertain whether the diffusivity of the drug through the ointment or the solubility of the drug in the ointment base (7) plays the major role in the release characteristics of salicylic acid, the rate of permeation[1] of the drug from the solution to another solution through an ointment-base layer was determined with the use of a three-compartment cell. (*d*)

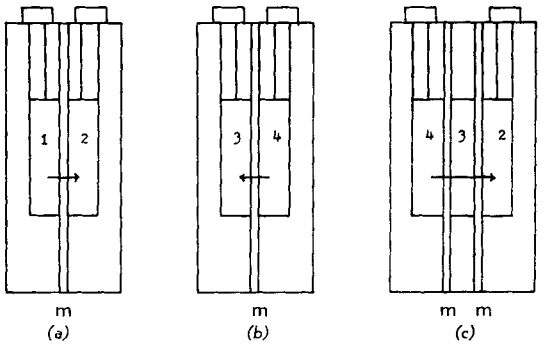


Figure 1—*Diagrams of apparatus used in:* (a) *release study*, (*b*) *uptake study, and* (c) *permeation study. Key:* **1.** *salicylic acid incorporated in an ointment base;* **2.** *sodium hydroxide solution;* **3.** *ointment base;* **4.** *salicylic acid solution; and* m, *dimethyl polysiloxane membrane. An arrow indicates direction of transfer of salicylic acid.*

The effect of dimethyl sulfoxide and two amides on the release and permeation of the drug was also investigated, since these organic solvents have received considerable attention with regard to their effect on membrane permeability (8).

EXPERIMENTAL

Materials—Reagent grade salicylic acid[2] was used throughout. Dimethyl polysiloxane[3] sheeting in a labeled thickness of 5 mil was used. Dimethyl sulfoxide,[4] *N,N*-dimethylacetamide,[5] and *N,N*-di-*n*-butylpropionamide[5] were used without further purification. Polyethylene glycol ointment, hydrophilic petrolatum, and hydrophilic ointment were prepared according to USP XVII. Water-in-oil and oil-in-water bases were prepared according to the method of Whitworth (9).

Apparatus—The polymethyl methacrylate[6] diffusion cell described by Patel and Foss (11) was used. The diameter of the available area for diffusion was 34 mm. A three-compartment cell designed for permeation studies consisted of the mentioned diffusion components and a thin plastic[6] plate (1.8 mm. in thickness) with a central opening, 34 mm. in diameter.

Procedure—Finely powdered salicylic acid (final concentration = 3%) was levigated with 10 drops of liquid petrolatum and incorporated into an ointment base by means of a mortar and pestle. Organic solvent (final concentration = 5%) was blended with the ointment base using a mortar and pestle prior to incorporation of the drug.

Three types of experiments performed in the present investigation: (*a*) release, (*b*) uptake, and (*c*) permeation, are illustrated diagrammatically (not to scale) in Fig. 1 and the procedure is given for each experiment.

Release from Ointment—One compartment of the diffusion cell was filled with an ointment and the excess was removed with the edge of a spatula to produce an even surface. The silicone rubber

[1] In this communication the following terminology is used. Diffusion is designated as the transport of a material within a medium, whereas permeation is defined as the transport of a material from one aqueous medium to another aqueous medium through a semisolid phase which separates the two aqueous media, since in the latter both diffusion and partition take place.

[2] General Chemical Division, Allied Chemical Corp., New York, N. Y.

[3] Supplied by Medical Products Division, Dow Corning Corp., Midland, Mich.

[4] Chemical Manufacturing Division, Fisher Scientific Co., Fair Lawn, N. J.

[5] Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y.

[6] Plexiglas, Rohm & Haas Co., Philadelphia, Pa. This material has recently been shown to absorb some nonelectrolytes from the solution (10). Salicylic acid may also be absorbed to a certain extent.

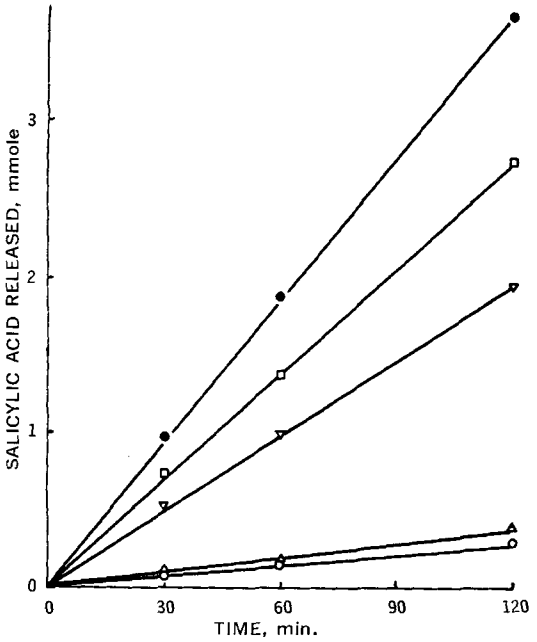


Figure 2—*Release of salicylic acid from ointments at 30°. Key: ●, oil-in-water ointment; □, hydrophilic ointment; ▽, water-in-oil ointment; △, hydrophilic petrolatum; and ○, polyethylene glycol ointment.*

membrane was then placed on it and carefully pressed to ensure complete contact of the membrane with the ointment. The other compartment of the cell was then placed on the membrane. The cell was assembled and tightly secured with bolts and wing nuts. The unit was brought up to a temperature of 30° by keeping it in a constant-temperature incubator.[7] A 10-ml. portion of prewarmed 0.01 *N* NaOH was placed in the empty compartment. Sodium hydroxide was added to maintain the effective concentration of permeable species to a value of zero in solution. The entire assembly was agitated on a shaker[8] in the incubator at a constant temperature of 30°. A 0.5-ml. aliquot of the aqueous solution was withdrawn at a definite interval and diluted to 10 ml. with 0.02 *N* HCl to suppress the dissociation of salicylic acid. The concentration of salicylic acid was determined spectrophotometrically[9] at 302 mμ.

Uptake by Ointment Base—The general procedure is the same as just described with the exception that the ointment base was packed in one compartment and a 10-ml. portion of a 10 m*M* salicylic acid solution in 0.01 *N* HCl was placed in the opposite compartment. Decrease in the salicylic acid content of the aqueous phase was determined spectrophotometrically.

Permeation through Ointment Base—The plastic plate with a circular opening was placed on the silicone rubber membrane and the opening was filled with an ointment base. The second membrane was then placed on it and pressed down to ensure complete contact with the ointment base. This unit was then placed between the two halves of the diffusion cell and the cell was assembled. The cell was brought up to 30° by placing it in the incubator. A 10-ml. portion of prewarmed 0.01 *N* NaOH was placed in one compartment, and an equal amount of prewarmed 10 m*M* salicylic acid in 0.01 *N* HCl was pipeted into the opposite compartment. The cell was agitated at a constant temperature; the increase in the drug concentration of the alkaline solution at definite time intervals was followed spectrophotometrically.

RESULTS

Release of Salicylic Acid from Ointments—The release characteristic of salicylic acid from various ointment bases over a 2-hr. period is illustrated in Fig. 2. The increase in the drug concentration of the aqueous phase at varying time intervals was used to assess the rate of drug release from the ointments. The emulsion-type ointments were superior to either hydrophilic petrolatum or polyethylene glycol ointment bases in salicylic acid release. Among the emulsion-type bases investigated, the oil-in-water type bases gave a better release than the water-in-oil type. Although not shown in the figure, the rate of release of salicylic acid from white petrolatum was slower than that from hydrophilic petrolatum; whereas the drug release from Base II, a polyethylene glycol base containing cetyl alcohol (12), was comparable to that from the polyethylene glycol base. Results are in agreement with those published in the literature (4) with the exception of the release data from polyethylene glycol bases, which are in direct contrast with those reported earlier (13). A possible explanation is offered under *Discussion*. Three official ointment bases, *i.e.*, hydrophilic ointment (an emulsion base), hydrophilic petrolatum (an absorption base), and polyethylene glycol base (a water-soluble base), were further examined in the uptake and permeation studies.

Uptake of Salicylic Acid by Ointment Bases—The drug uptake from aqueous solution by the ointment bases, separated by the silicone rubber membrane, was measured to evaluate the relative affinity of the drug for the bases. The data for this part of the study are plotted in Fig. 3. The figure shows that only 8% of the drug remained in solution after a 24-hr. period in the case of polyethylene glycol base, thus demonstrating a comparatively rapid rate of uptake by this base. The uptake by hydrophilic ointment was fairly rapid, whereas it was very slow for hydrophilic petrolatum.

Permeation of Salicylic Acid through Ointment Bases—Figure 4 illustrates the permeation profile of salicylic acid from the acidic solution to the alkaline solution through various ointment bases. Here the drug concentration of the alkaline solution is plotted as a function of time. The permeation of salicylic acid through hydrophilic ointment was comparatively rapid, whereas very little drug permeated through polyethylene glycol base within a 10-hr. period, with hydrophilic petrolatum as an intermediate. Even though only a small fraction of salicylic acid permeated through polyethylene glycol base into the alkaline solution, there was a large decrease in the drug content of the acid solution, thus indicating a significant accumulation of salicylic acid in the base.

Effects of Some Organic Solvents on the Release and Permeation—The data on the influence of dimethyl sulfoxide and two amides for the release of salicylic acid from hydrophilic petrolatum are presented in Fig. 5. The rate of drug release was dependent upon the nature of the organic solvent. Di-*n*-butylpropionamide exhibited the greatest enhancing effect, followed by dimethylacetamide and dimethyl sulfoxide in decreasing order. Similar enhancing effects of these solvents were observed with polyethylene glycol and hydrophilic ointments.

Figure 6 illustrates the effects of the solvents on the rate of drug permeation through hydrophilic petrolatum. Permeation through the vehicle was accelerated in the presence of di-*n*-butylpropionamide, but the effect due to dimethylacetamide was not very pronounced. Polyethylene glycol ointment showed a similar behavior with the solvents; however, the magnitude of acceleration was less

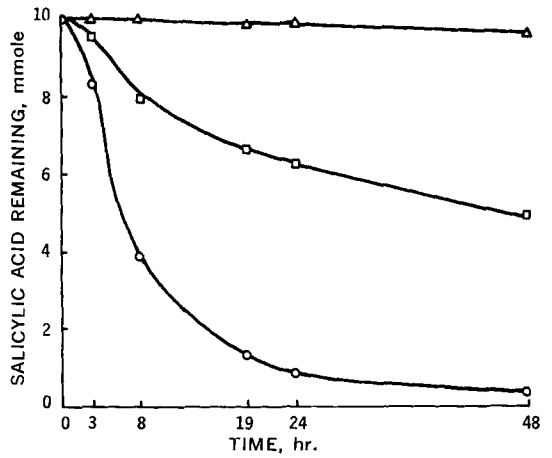


Figure 3—*Uptake of salicylic acid by ointment bases at 30°. Key: △, hydrophilic petrolatum; □, hydrophilic ointment; and ○, polyethylene glycol ointment.*

[7] Model 82, Fisher Scientific Co., Pittsburgh, Pa.
[8] Lab-Tek aliquot shaker, Ames Lab-Tek, Inc., Westmont, Ill.
[9] Model DB, Beckman Instruments, Inc., Fullerton, Calif.

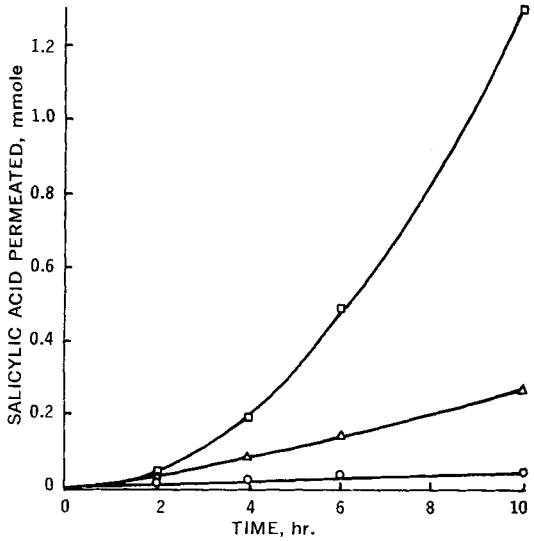


Figure 4—*Permeation of salicylic acid through ointment bases at 30°. Key: □, hydrophilic ointment; △, hydrophilic petrolatum; and ○, polyethylene glycol ointment.*

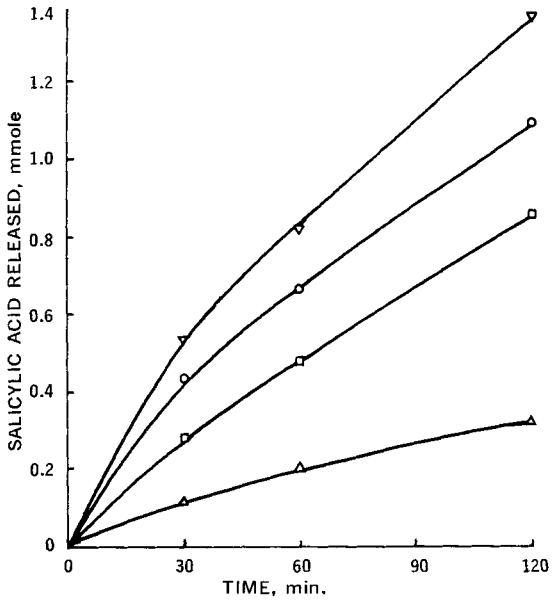


Figure 5—*Effect of dimethyl sulfoxide and amides on the rate of release of salicylic acid from hydrophilic petrolatum at 30°. Key: ▽, with di-n-butylpropionamide; ○, with dimethylacetamide; □, with dimethyl sulfoxide; and △, without organic solvent.*

marked. These solvents had little effect on the permeation of the drug through hydrophilic ointment.

DISCUSSION

As illustrated in Fig. 2, the release characteristics of salicylic acid from five physically different ointment bases through a silicone rubber membrane over a period of 2 hr., indicate that the membrane of a thickness of 5 mil provides reasonably fast rates of release to be of practical use for investigations of release of drugs from ointments. The linearity of the plots appears to indicate that the release of salicylic acid follows apparent zero-order kinetics, although this is not always the case (Fig. 5). The release pattern obtained using a silicone rubber membrane is in agreement with the *in vivo* data of Stolar *et al.* (4). Their ranking of the ointment bases for percutaneous absorption of salicylic acid was: hydrophilic ointment > hydrophilic petrolatum > polyethylene glycol.

Billups and Patel reported that cellophane membrane gave unusually rapid release of salicylic acid from the polyethylene glycol–cetyl alcohol base (Base II), although it was satisfactory for emulsion- and oleaginous-type bases (13). This is in direct contrast to the poor percutaneous absorption of salicylic acid from polyethylene glycol base (4). Cellophane membrane is freely permeable to water. In the case of an ointment base with high affinity for water, such as polyethylene glycol, the base attracts water, thus forming a solution and thereafter allowing the drug to permeate from the aqueous solution rather than from the base.

The mechanism of transfer of salicylic acid through the silicone rubber membrane is possibly governed by the partitioning of the drug into and diffusion through the membrane (14). The partition coefficient of the drug between the membrane and the ointment base thus plays an important role. In overall availability, this factor may be desirable because of the similarities in the physical properties of the membrane with those of skin. The skin barrier is generally considered to be lipoid in nature, although the exact mechanism of percutaneous absorption is not fully understood (15). Silicone rubber membranes are known to be permeable to nonionic drug molecules; and because of their lipidlike properties, they are claimed to be of value in the investigation of the drug transport through lipoid barriers (14, 16, 17). Based on the agreement of *in vitro* release pattern with *in vivo* data (4), a silicone membrane appears to be ideal for investigating drug release from diverse bases.

The very slow release of salicylic acid from polyethylene glycol ointment observed in this study was also noted by Loveday using excised pig skin (3) and by Stolar *et al.* from the measurement of the blood level in the rabbit (4). This slow release is most likely due to the affinity of salicylic acid for polyethylene glycol. This was demonstrated by the fairly rapid uptake of salicylic acid by the polyethylene glycol base from the solution (Fig. 3) and the drug accumulation within the ointment base in the permeation studies (Fig. 4). Hydroxybenzoic acids and phenols form molecular complexes with polyethylene glycol (18, 19) through hydrogen bonding. Due to complexation, salicylic acid is held up by the vehicle, thus retarding its release. Drugs which do not complex with polyethylene glycol would be expected to show better drug release than the acid. Loveday observed superior release of methyl salicylate from polyethylene glycol ointment (3). Methyl salicylate would not be expected to complex with polyethylene glycol because of the lack of the carboxylic acid group and involvement of the hydroxy group in intramolecular hydrogen bonding.

For diffusion to occur, the medicinals should be dissolved in the ointment base; this factor is of considerable importance for poorly soluble drugs. The three-compartment cell was used to examine the permeability characteristics of the drug in each ointment base without complications from the difference in the solubilities of the drug in various bases. In this experiment the initial concentration of salicylic acid in one compartment was kept constant, and the drug was allowed to permeate through varied bases into another

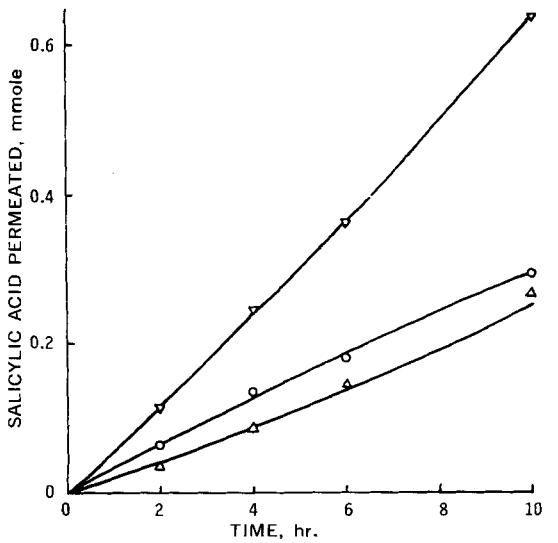


Figure 6—*Effect of amides on the rate of permeation of salicylic acid through hydrophilic petrolatum. Key: ▽, with di-n-butylpropionamide; ○, with dimethylacetamide; and △, without amide.*

compartment. Under these conditions the partition coefficient of the drug between the ointment base and membrane, as well as its diffusivity in the ointment base, would influence the rate of permeation and consequently the rate of release. The overall similarity of the release and permeation profile of the drug from hydrophilic ointment, hydrophilic petrolatum, and polyethylene glycol indicates that the solubility factor is not of overwhelming importance and the contribution from other factors, such as the diffusivity of the drug in the ointment base and possibly the partition characteristics of the drug between the base and the membrane, also have a significant bearing upon the release characteristics of the drug.

In the absence of specific interaction between the drug and the ointment base, as was the case with salicylic acid and polyethylene glycol ointment base, the base that takes up the drug fast appears to release the drug fast (Figs. 2 and 3). The rate of uptake of the acid by the ointment base from a solution through the silicone rubber membrane depends upon both the partition coefficient between the ointment base and the membrane and the diffusivity through the ointment base. Diffusivity through the ointment base may be the rate-limiting factor for ointments with poor drug release. This can be explained on the basis of uptake data for hydrophilic ointment and hydrophilic petrolatum. The greater uptake of drug by hydrophilic ointment than by hydrophilic petrolatum can be related to faster diffusion of salicylic acid through hydrophilic ointment. Water, being a continuous phase in hydrophilic ointment, is more mobile than the continuous oil phase of hydrophilic petrolatum and this, in turn, would favor diffusion of salicylic acid through hydrophilic ointment. The partition factor, on the other hand, should have a favorable effect upon hydrophilic petrolatum over hydrophilic ointment, since salicylic acid is roughly five times more soluble in oils and fats than in water (20).

Accelerated release of salicylic acid from white petrolatum and water-in-oil-type emulsion base in the presence of organic solvents has been reported by Whitworth (9). The results of the present study showed that the *in vitro* release of salicylic acid from hydrophilic petrolatum, polyethylene glycol base, and hydrophilic ointment was also increased in the presence of dimethyl sulfoxide and the amides. The reported increased percutaneous absorption of salicylic acid in the rabbit in the presence of dimethyl sulfoxide (21) may be attributed to the increased drug release from the ointment (Fig. 5) rather than merely to the change in the skin permeability by the sulfoxide. The increased solubility of salicylic acid in the ointment base containing organic solvents would be expected to increase the rate of release. The reason for attributing the increased release of salicylic acid mainly to the increased solubility of the drug in the base, rather than to change in membrane permeability, comes from the fact that the effect of dimethyl sulfoxide and dimethylacetamide on the rate of permeation was not so pronounced as that on the release rate. In addition, these two organic solvents have been shown not to partition to an appreciable extent into the silicone rubber membrane (22). These solvents, therefore, may not have a significant influence on the silicone rubber membrane itself, although the possibility of this effect may exist with biological membranes. The greater enhancing effect of di-*n*-butylpropionamide compared to dimethylacetamide may be due in part to the greater solubility of the former in the membrane. Di-*n*-butylpropionamide partitions into the membrane from the ointment, and it can form hydrogen bonds with salicylic acid which, in turn, facilitates the transfer of salicylic acid from the ointment into the membrane. This interaction is expected to accelerate the permeation of salicylic acid (22).

REFERENCES

(1) N. F. Billups and R. W. Sager, *Amer. J. Pharm.*, **137,** 57 (1965).

(2) J. M. Howze and N. F. Billups, *ibid.*, **138,** 193(1966).

(3) D. E. Loveday, *J. Soc. Cosmet. Chem.*, **12,** 224(1961).

(4) M. E. Stolar, G. V. Rossi, and M. Barr, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 144(1960).

(5) V. F. Cotty, J. Skerpac, H. M. Ederma, and F. Zurzola, *J. Soc. Cosmet. Chem.*, **11,** 97(1960).

(6) F. Gstirner and R. Elsner, *Arzneim.-Forsch.*, **14,** 281(1964).

(7) T. Higuchi, *J. Soc. Cosmet. Chem.*, **11,** 85(1960).

(8) H. Baker, *ibid.*, **20,** 239(1969).

(9) C. W. Whitworth, *J. Pharm. Sci.*, **57,** 1540(1968).

(10) N. Nagabhushan, R. T. Coutts, and N. K. Patel, *Can. J. Pharm. Sci.*, **4,** 79(1969).

(11) N. K. Patel and N. E. Foss, *J. Pharm. Sci.*, **53,** 94(1964).

(12) L. C. Zopf and S. M. Blaug, in "Remington's Pharmaceutical Sciences," 13th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965, p. 533.

(13) N. F. Billups and N. K. Patel, *Amer. J. Pharm. Educ.*, **1970,** 34.

(14) E. R. Garrett and P. B. Chemburkar, *J. Pharm. Sci.*, **57,** 949(1968).

(15) M. Barr, *ibid.*, **51,** 395(1962).

(16) E. R. Garrett and P. B. Chemburkar, *ibid.*, **57,** 944(1968).

(17) *Ibid.*, **57,** 1401(1968).

(18) T. Higuchi and J. L. Lach, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 465(1954).

(19) D. Guttman and T. Higuchi, *ibid.*, **45,** 659(1956).

(20) "The Merck Index," 8th ed., P. G. Stecher, Ed., Merck & Co., Rahway, N. J., 1968, p. 930.

(21) J. M. Stelzer, Jr., J. L. Colaizzi, and P. J. Wurdack, *J. Pharm. Sci.*, **57,** 1732(1968).

(22) M. Nakano and N. K. Patel, *ibid.*, **59,** 77(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 28, 1969, from the *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada* and *Research Laboratories, Food and Drug Directorate, Ottawa 3, Ontario, Canada.*

Accepted for publication February 3, 1970.

This work was supported in part by the University of Alberta Postdoctoral Fellowship (to M. N.), Research Grant GR-766 from the University of Alberta General Research Funds, and a research grant from Smith Kline & French Inter-American Corp. (Canada).

The authors thank Miss J. M. Romanowski for her technical assistance.

* Present address: Pharmaceutical Chemistry Division, Research Laboratories, Food and Drug Directorate, Tunney's Pasture, Ottawa 3, Ontario, Canada.

† Present address: Research Laboratories, Frank W. Horner Limited, Montreal 307, Quebec, Canada.

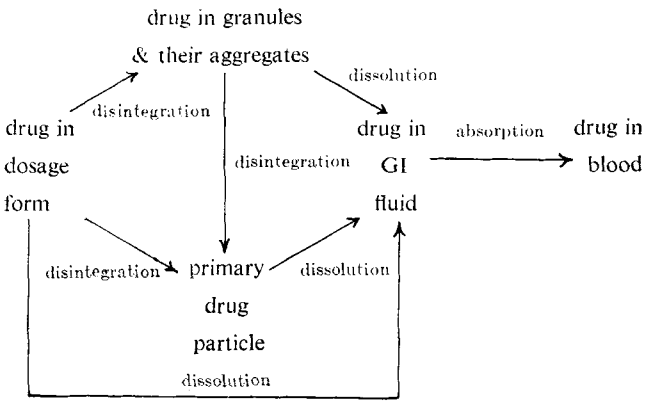
Comparative Evaluation of Various Dissolution Apparatus for Capsule Dosage Forms

SONG-LING LIN, JOHANNE MENIG, and CHARLES J. SWARTZ

Abstract □ The dissolution profiles of experimental diuretic and antidiabetic compounds in capsules were investigated by bead, plate, blade, holder, basket, disintegration time, and proposed USP dissolution methods. The apparent initial dissolution rate and the extent of drug released within the testing period were used as parameters in comparative evaluation of these seven methods. The influence of the size of the stirrer, the volume of dissolution medium, and the size of screen cloths for making the basket on the dissolution profile was studied and discussed. The Reynolds number of the fluid is introduced to explain the influence of the size of the stirrer on the dissolution rate. Due to the small screen (40 mesh) employed in the proposed USP dissolution method, the visual observation of the behavior of the capsule is impaired, and the chance of clogging the screen by the granules is greater than the self-designed (8 mesh) basket used in the basket method. The dissolution profiles obtained with the disintegration time method provide the fastest rate and the greatest extent of dissolution for both experiemental diuretic and antidiabetic compounds.

Keyphrases □ Capsule dosage forms—dissolution apparatus □ Dissolution apparatus, comparison, evaluation—capsule dosage forms □ Stirring rate effect—capsule dissolution □ UV spectrophotometry—analysis

The majority of drugs today are formulated and marketed as tablets and capsules. For slightly and poorly water-soluble compounds, the effective absorption process is generally controlled by the disintegration time of the dosage form and the rate and extent of the subsequent dissolution at which the drug goes into and remains in the solution state. Thus, for a drug contained in a solid dosage form to be absorbed, the following illustrated sequence must occur (Scheme I):



Scheme I

The usefulness and importance of developing suitable dissolution apparatus and procedures in product research and development have been recognized for several years. In the product research area, dissolution testing is commonly employed as a means of evaluating and selecting the crystalline or amorphous forms, polymorphic or solvate forms, or the eutectic or complex forms of the compound. On the other hand, dissolution testing is generally accepted in the product development area for studying the drug-release patterns of the conceptual dosage forms, for investigating the formulation and process factors in the development of dosage forms, and for facilitating the selection of certain preliminary formulations for preclinical *in vivo* efficacy studies of the formulation. In general, when the correlations between the *in vitro* dissolution data and the *in vivo* clinical or pharmacokinetic evidence have been established, the dissolution method may be incorporated in the specification of the dosage form as a quality control tool. Therefore, the standardization of test apparatus and methodology is extremely important in the evolution of drug standards, and proper comparative evaluations play a significant role in such standardization.

Dissolution characteristics of solid dosage forms such as tablets or capsules may be determined by various apparatus employing various dissolution media, agitation intensities, and sampling methods for assaying the drug content in solution. Many *in vitro* techniques appear in the literature for the evaluation of drug-release patterns from solid dosage forms (1–24). The applicability and reliability of these apparatus and procedures are, in general, more suited for tablets than for capsules. It is the purpose of this article to evaluate comparatively the dissolution profiles attainable by various devices which are shown to be practical and reproducible for investigating the release pattern of the compound from the capsule.

EXPERIMENTAL

Materials—The diuretic compound was dried at 100° for 8 hr. The chemical identity was confirmed by elemental analysis and IR spectra. The moisture content, as determined by the Karl Fischer method, was about 1%. Analysis by a nonaqueous titrimetric method showed a purity of better than 99% which was substantiated by TLC analysis. The equilibrium solubility at 37° in phosphate buffer at pH 7.3 was about 1 mg./ml. Using U. S. standard sieves, the fraction of the diuretic compound passing through 40 mesh but retained in 100 mesh was collected and used throughout this investigation.

The antidiabetic compound was dried at 80° under vacuum for 12 hr. The moisture content, as determined by the Karl Fischer method, was negligible. The chemical purity was found to be better than 99.5%. The equilibrium solubility at 37° in double-distilled water was approximately 2%. The samples were sieved through U.S. standard sieves, and fractions of 20/100-mesh particles were collected for use in this investigation.

Determination of Dissolution Profile—Approximately 100 mg. of the sieved sample of the diuretic or antidiabetic compound was accurately weighed and carefully introduced into No. 1 clear gelatin capsules with as little compaction as possible. The capsule was then placed in one of the several devices, as depicted in Fig. 1, for obtaining the dissolution profile. The devices or apparatus employed are as follows: (*a*) The capsule is weighted down to the

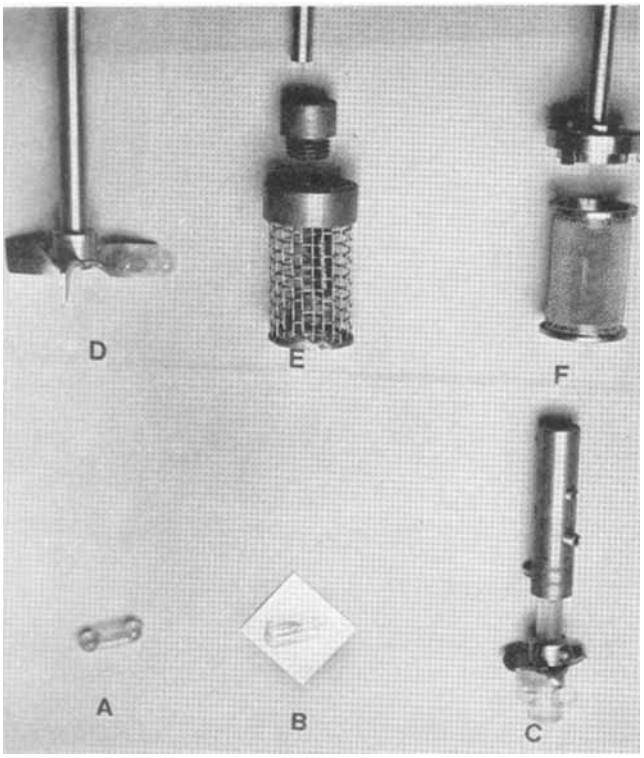


Figure 1—*Photograph illustrating the various devices employed for the investigation of capsule dissolution profile. Key: A, bead method; B, plate method; C, holder method with small stirrer; D, blade method with large stirrer; E, basket method with large stirrer; and F, proposed USP dissolution method.*

bottom of the dissolution flask by inserting two glass beads at both ends of the capsule (bead method) prior to the filling of the drug into the capsule. (*b*) The capsule sinks to the bottom of the dissolution flask by affixing the capsule to a 2 × 2-cm. stainless steel plate (plate method) with the aid of water-repellant glue. (*c*) The capsule is affixed to one of the three blades of the stirrer (blade method) with the same water-repellant glue. (*d*) The capsule is positioned into the orifice of a plastic capsule holder (holder method) specifically designed for a No. 1 gelatin capsule. (*e*) The capsule is placed in a self-fabricated basket (basket method) made from a stainless steel metal screen. (*f*) The capsule is placed in the USP proposed basket (proposed USP method) designed to be one of the official dissolution testing apparatus in the future.

The dissolution studies were performed using 300 ml. of dissolution medium at 37° in a pyrex beaker of 800-ml. capacity. The bottom face of the beaker was converted into a convex shape. The agitation of the dissolution medium was achieved by an overhead stirrer operated at 60 r.p.m. and placed at the center of the beaker. The agitation mechanism and the volume of dissolution fluid employed for each method are indicated in Table I. In all cases, the top surface of the agitation mechanism, such as the stirrer or basket, was immersed 2 cm. below the surface of the dissolution medium. The dissolution media employed for the diuretic and antidiabetic compounds were phosphate buffer at pH 7.3 and double-distilled water, respectively. At zero time, the capsule was introduced into the dissolution medium maintained at 37°. At prescribed time intervals, sample aliquots were withdrawn and replaced immediately with the same volume of fresh medium maintained at 37°. The aliquot was immediately filtered through 0.45-μ pore size Millipore filter paper. The clear filtrate was properly diluted with an appropriate solvent and assayed spectrophotometrically at 282 mμ for the diuretic compound and 347 mμ for the antidiabetic compound. Beer's law curves were constructed previously in the solvent systems employing 50% aqueous methanol and distilled water. A cumulative correction was made to account for the previously removed samples in determining the total amount of drug dissolved at any specific time. An average of at least three determinations was performed for each method investigated in this study. The gelatin capsule in the diluted sample aliquots was found not to interfere with the spectrophotometric assay at the aforementioned wavelengths.

RESULTS AND DISCUSSION

The experimental conditions employed to obtain dissolution profiles, together with their apparent agitation mechanism, are summarized in Table I. Numbers appearing in the first column of Table I correspond to the number designated for the curves in Figs. 2–6. Whenever the device investigated was suitable for studying the effect of the size of the stirrer on the release rate of drug from the capsule, two kinds of three-bladed stirrers were employed. The small stirrer used was 2.0 cm. in diameter and the angle of the blade to the horizontal plane was 45°, whereas the large stirrer employed was 4.3 cm. and the blade was perpendicular to the horizontal plane. It was suggested by Levy (15) that, using a stirring rate of 30 to 60 r.p.m., the agitation intensity is sufficient to obtain a homogeneous solution for sampling purposes yet low enough to preserve the microenvironment of the tablet being tested. Consequently, the constant stirring rate of 60 r.p.m. was employed throughout the study unless otherwise specified.

The plot in Fig. 2 depicts the dissolution profiles of the diuretic and antidiabetic compounds, using the bead method with the small and large stirrers. It is evident from the plot that a lag time does exist prior to the dissolution of drug from its encapsulated form into the dissolution medium. The existence of the lag time is explained by the dissolution of gelatin capsules prior to the leaching or releasing of the drug from the capsule. The lag time is about 5 min. for gelatin capsules under the experimental conditions employed. In general, the capsule breaks initially from both ends that are in contact with the glass beads, and this is soon followed by the melting of gelatin from the middle portion of the capsule. Comparison of the dissolution profiles of the two compounds investigated shows that the higher the aqueous solubility of the compound, the faster the initial rate of dissolution and the greater the extent of drug released. This finding is in accord with the Noyes-Whitney equation.

Although the stirring speed was kept constant at 60 r.p.m., it is conceivable that the larger the diameter of the stirrer, the greater the driving force and the better the efficiency of the dissolution fluid impacting on the capsule to release the drug. The increased dissolution rate could be correlated qualitatively to the Reynolds number, a dimensionless group of importance in fluid dynamics.

Table I—Experimental Conditions for the Dissolution Profile Study

Curve Ref.[a]	Method[b]	Agitation Mechanism	Volume of Dissolution Medium in ml.
1	Bead method	Small stirrer[c]	300
2	Bead method	Large stirrer[d]	300
3	Plate method	Small stirrer	300
4	Plate method	Large stirrer	300
5	Blade method	Small stirrer	300
6	Blade method	Large stirrer	300
7	Holder method	Small stirrer	300
8	Holder method	Large stirrer	300
9	Basket method	Basket alone	300
10	Basket method	Basket alone	600
11	Basket method	Basket & small stirrer	600
12	Basket method	Basket & large stirrer	600
13	D.T. method	D.T. rack assembly with disk	700
14	Proposed USP method	USP basket	300
15	Proposed USP method	USP basket	600
16	Proposed USP method	USP basket & large stirrer	600

[a] Numbers correspond to curves in Figs. 2–6. [b] See text for details. [c] The small stirrer employed is 2.0 cm. in diameter and the angle of the blade to the horizontal plane is 45°. [d] The larger stirrer employed is 4.3 cm. in diameter and the angle of the blade to the horizontal plane is 90°.

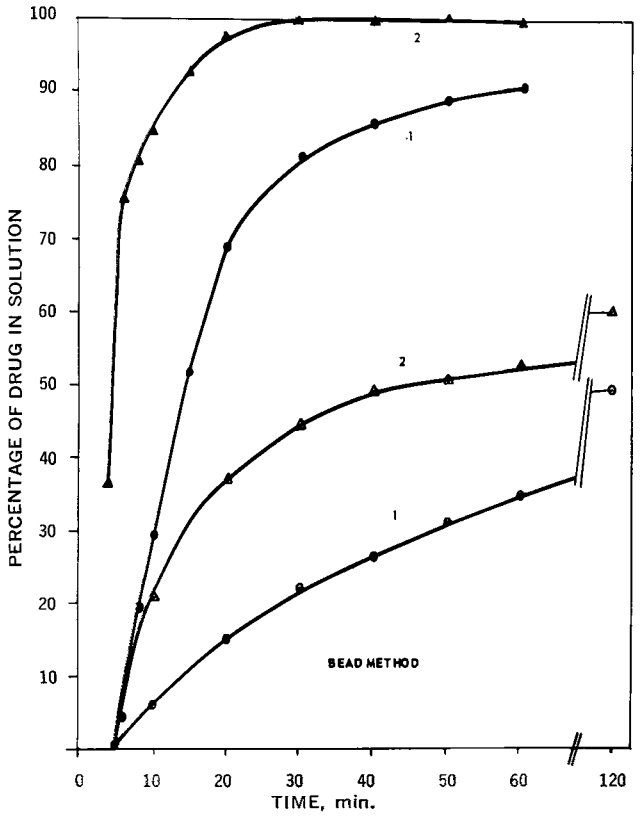


Figure 2—*Dissolution profiles of the diuretic (open symbols) and antidiabetic (solid symbols) compounds by bead method at 60 r.p.m. Key: 1, small stirrer; and 2, large stirrer.*

The Reynolds number (N_R) of the fluid having been agitated at constant speed (r) is related to the diameter of the stirrer (D) through the following equations:

$$N_R = kD^2 \qquad \text{(Eq. 1)}$$

$$k = \frac{r\rho}{\eta} \qquad \text{(Eq. 2)}$$

where ρ and η represent the density and viscosity of the fluid, respectively. Assuming that the ρ and η of the dissolution fluid are macroscopically independent of stirrer dimension, k will remain as a constant when the fluid is stirred at constant speed, r, Then, it follows from Eq. 1 that the ratio of Reynolds numbers obtained with larger *versus* smaller stirrers employed in this study are:

$$\frac{N_R^1}{N_R^s} = \frac{D_1^2}{D_s^2} = \frac{(4.3)^2}{(2.0)^2} = 4.6 \qquad \text{(Eq. 3)}$$

Therefore, as expected, the larger the stirrer size, the greater are the initial dissolution rate and the extent of the dissolution of the diuretic and antidiabetic compounds (Fig. 2). For the diuretic compound, the initial dissolution rate is increased about threefold, and the extent of dissolution within a 2-hr. period is approximately doubled when the smaller stirrer (Curve 1, open symbol, Fig. 1) is replaced with the large stirrer (Curve 2, open symbol, Fig. 1). Quantitative study of the effect of N_R on dissolution rate is in progress (25).

In the plate method, the capsule is affixed to a 2 × 2-cm. stainless steel plate in such a way that the contact area between the capsule and the metal plate is minimized by applying as little as possible of the water-repellant glue. The dissolution data obtained with the plate method are illustrated in Fig. 3 for the diuretic compound (open symbols) and the antidiabetic compound (solid symbols). It is clearly demonstrated again that the rate and extent of dissolution are increased with the intensity of agitation and the size of the stirrer. The general characteristics of these four curves of Fig. 3 are very similar to the corresponding curves illustrated in Fig. 2.

For the diuretic compounds (the lower two curves of Figs. 2 and 3), the apparent initial rate and the extent of dissolution are slightly greater for the bead method than for the plate method when small stirrers are employed; the similar apparent initial rate but a greater extent of dissolution is observed for the bead method than for the plate method when a large stirrer is used. The situation is somewhat different for the antidiabetic compound (the upper two curves of Fig. 3). Using a small stirrer, the identical apparent initial release rate is observed and the extent of dissolution is found to be slightly greater for the plate method than for the bead method when a larger stirrer is employed.

The data obtained with the blade method and the holder method are summarized together as depicted in Fig. 4. For the antidiabetic compound, the dissolution profiles obtained with either large or small stirrer using either blade or holder method are nearly superimposable. Therefore, the resulting dissolution profiles are illustrated representatively by the upper curve of Fig. 4. The effect of the size of the stirrer on the dissolution profile of the antidiabetic compound was not discernible for both the blade method and the holder method. This finding differs from that obtained with either the bead method or the plate method, in which the rate as well as the extent of dissolution is enhanced with increasing agitation intensity by replacing the small stirrer with the large stirrer.

The dissolution profile of the diuretic compound is not affected by the size of the stirrer used in the blade method (Curves 5 and 6, open symbol, Fig. 4) but is slightly altered in the holder method (Curves 7 and 8, open symbol, Fig. 4). Comparison of dissolution profiles of the diuretic compound in Figs. 2–4 indicated that, using a small stirrer, the apparent initial dissolution rate and the extent of drug release are decreased in the following order: blade method > holder method > bead method > plate method. However, no significant difference in the apparent initial dissolution rate is observed when the large stirrer is employed among the blade, holder, bead, and plate methods. It is interesting to note that the scattering of the dissolution profiles of the diuretic compound obtained among blade, holder, plate, and bead devices is greatly minimized by substituting the small stirrer with the large stirrer. It appears that the larger the stirrer operating at the same rotating

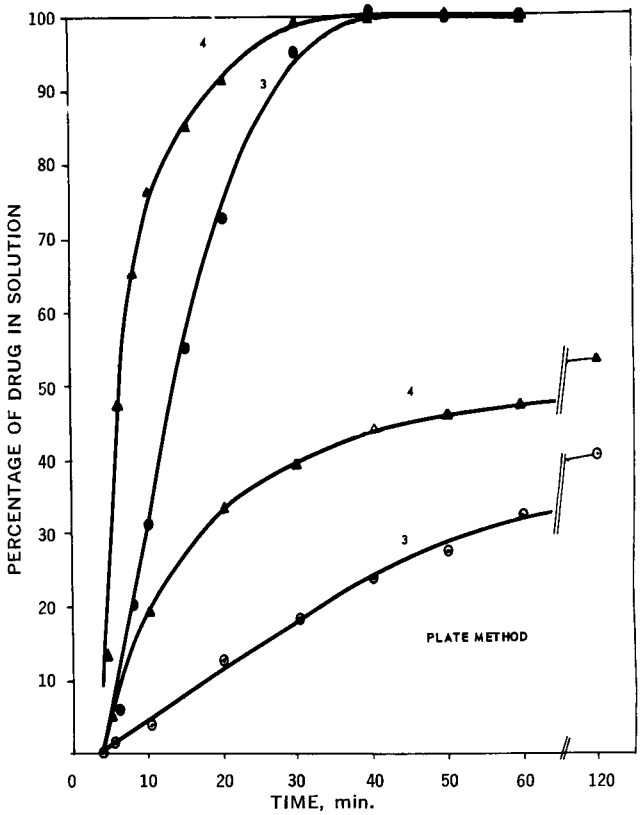


Figure 3—*Dissolution profiles of the diuretic (open symbols) and antidiabetic (solid symbols) compounds by plate method at 60 r.p.m. Key: 3, small stirrer; and 4, large stirrer.*

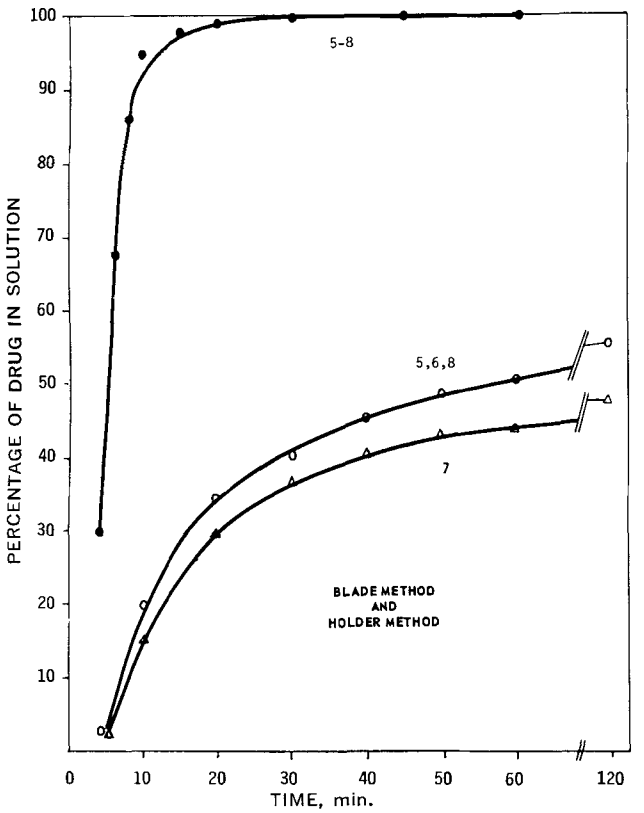


Figure 4—*Dissolution profiles of the diuretic (open symbols) and antidiabetic (solid symbols) compounds by blade (Curves 5 and 6) and holder (Curves 7 and 8) methods at 60 r.p.m. Key: 5 and 7, small stirrer; and 6 and 8, large stirrer.*

speed, the smaller variation of dissolution profiles is obtained among various devices. It was found in preliminary experiments with the blade methods that the difference in dissolution profiles of the diuretic compound is negligible when the capsule investigated is affixed at the bottom of the stirring shaft, at the face of the blade opposite to the direction of the flow, or at the face of the blade facing the same direction of the flow as shown in Fig. 1.

With blade or holder method, the majority of the drug is fairly well dispersed in the testing fluid at 60 r.p.m., although a small fraction of drug falls to the bottom of the dissolution vessel. The observation of the relative degree of drug dispersion in the dissolution fluid, together with the determination of dissolution profile, could be employed as a fast and useful means for screening the dosage forms. In general, if the large particulate agglomerates are visually observed to swell or sink in the dissolution flask, an unsatisfactory dissolution profile is expected. The poor dispersion effect may be taken as a possible early warning of drug-availability problems from the absorption viewpoint.

One common characteristic among the blade, holder, plate, and bead methods is that a certain segment of the capsule is physically in contact with supporting devices such as the stirring blade, plastic holder, metal plate, or glass bead, respectively. To prevent the physical contact and to alleviate the common problem of floating of the capsule on the surface of the dissolution medium, a self-designed basket was employed. The basket is labeled E in Fig. 1. The basket consists of a stainless steel cylinder 4.5 cm. in height and 2.3 cm. in diameter. The sides and bottom of the basket are No. 8 mesh stainless steel cloth. The bottom wire is welded together. The wire at the top of the cylinder is welded to a stainless steel ring which, in turn, is welded to an inverted T-shaped stainless steel attachment. The top of this cylindrical assembly has an orifice to be affixed to the bottom tip of the stirring shaft.

With this cylindrical basket, the dissolution profiles of the diuretic and the antidiabetic compounds were investigated. The results obtained are illustrated as Curves 9–12 in Fig. 5. When the agitation of dissolution fluid is provided by the rotational movement of the basket alone, the dissolution profile of the diuretic compound (Curve 9, open symbol, Fig. 5) closely resembles the profiles obtained with the plate—large stirrer (Curve 4, Fig. 3), holder—large stirrer (Curve 8, Fig. 3), and blade—small or large stirrer (Curves 5 and 6, Fig. 4) systems.

For evaluating the influence of the stirrer on the dissolution profile of the diuretic compound, it is necessary to increase the volume of dissolution fluid to accommodate the stirring assembly. As the volume is doubled from 300 to 600 ml. (Curves 9 and 10, Fig. 5), the rate and the extent of dissolution are enhanced when the basket alone is rotated at 60 r.p.m. to supply the agitation. By keeping the volume of dissolution fluid at 600 ml., the dissolution profiles (Curves 10–12, Fig. 5) show that the apparent initial dissolution rate and the extent of drug released are increased with the increase of the stirrer dimension.

Since the disintegration time (D.T.) apparatus, as described in USP XVII, is commonly employed in assessing the dissolution profile of a solid dosage form, this apparatus was incorporated in this investigation with the following slight modifications: a round-bottom dissolution flask of 800-ml. capacity was employed and one capsule was introduced to any one of the six compartments of the basket rack assembly. The assembly, agitated at 30 c.p.m., was allowed to descend to 1 cm. from the bottom of the dissolution flask on the downward stroke. The disk was used, and the volume of dissolution fluid was 700 ml. The data obtained for the diuretic compound are shown as Curve 13 (open symbol) in Figs. 5 and 6. The extent of the diuretic compound released within the testing period of 1 hr. is greatly enhanced with the D.T. method as compared with the basket, blade, holder, plate, or bead methods. This may be attributed to the stronger turbulent flow and the greater impacting force exerted on the capsule and the particulate aggregates by the D.T. method. Therefore, the percentages of drug released within 5 and 10 min. are already at the level of about 33 and 50%, respectively. However, the shape of the dissolution profile of the diuretic compound obtained by the D.T. method is similar to the following systems: (*a*) bead—large stirrer system (Curve 2, Fig. 2); (*b*) plate—large stirrer system (Curve 4, Fig. 3); (*c*) blade—small or large stirrer system (Curves 5 and 6, Fig. 4); (*d*) holder—large stirrer system (Curve 8, Fig. 4); and (*e*) basket—no stirrer system (Curve 9, Fig. 5).

The dissolution profiles obtained for the antidiabetic compound are depicted in Fig. 5 as circular and inverted-triangular solid symbols for basket and D.T. methods, respectively. It appears that the change of the volume of the dissolution fluid and the addition

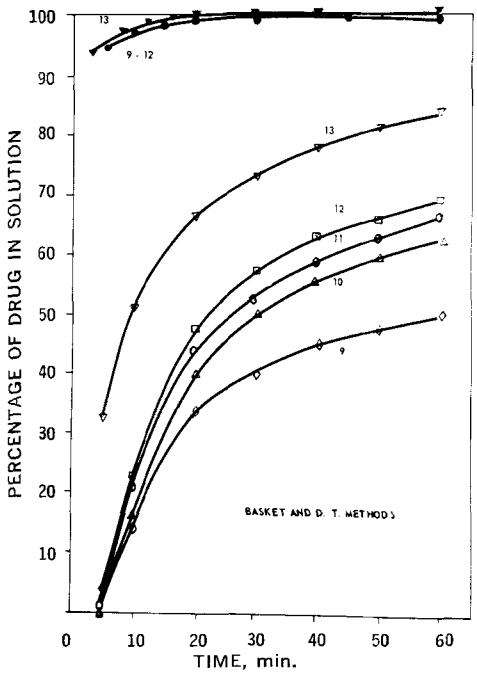


Figure 5—*Dissolution profiles of the diuretic (open symbols) and antidiabetic (solid symbols) compounds by basket (Curves 9–12) method at 60 r.p.m. and D.T. (Curve 13) method at 30 c.p.m. See Table I for detailed experimental conditions.*

of a small or large stirrer to the basket have not caused any detectable alteration in the dissolution profile of the antidiabetic compound. Consequently, the results are plotted collectively as solid circles of Curves 9–12 of Fig. 5. With the D.T. method, the dissolution profile is nearly superimposable to that obtained by the basket method.

Toward the completion of this investigation, a similar basket as was employed in this study was advocated by the USP as a tentative apparatus for investigating the dissolution of tablet and capsule dosage forms. Therefore, the suggested USP basket was purchased and incorporated in this study. The proposed USP apparatus (F in Fig. 1) consists of a stainless steel basket 3.6 cm. high and 2.5 cm. in diameter. The sides and bottom of the cylinder are 40 mesh stainless steel. The wire is welded to two stainless steel rings, top and bottom, and joined by welding at the seam. A stainless steel rod 30 cm. long with a 2.5-cm. plate and three spring clips are used to hold the basket (26). The results obtained with the proposed USP dissolution apparatus are shown in Fig. 6 for the diuretic (Curves 14–16, open symbols) and antidiabetic (Curves 14–16, closed symbols) compounds. For easy comparison, the dissolution profiles of both compounds obtained with the D.T. method are reproduced in Fig. 6.

For the diuretic compound, the initial dissolution rate and the extent of the drug released are enhanced by the increasing volume of dissolution medium (Curves 14 and 15) when the stirring mechanism is kept constant and by the use of additional stirrers (Curves 15 and 16) when the volume of dissolution medium is kept constant. This is in accord with the trend obtained with the basket method. The major difference between the basket method and USP dissolution apparatus is the stainless steel screen cloths used to fabricate the basket; USP apparatus uses 40-mesh screen, whereas 8-mesh screen is employed for the self-designed basket. When the basket method was replaced with the USP apparatus in determining the dissolution profile of the diuretic compound, no discernible difference was observed when the volume of dissolution medium was 300 ml. (Curve 14, Fig. 6, *versus* Curve 9, Fig. 5), whereas slight enhancement of the initial dissolution rate was observed when the volume was 600 ml. (Curve 15, Fig. 6, *versus* Curve 10, Fig. 5; and Curve 16, Fig. 6, *versus* Curve 12, Fig. 5).

The increase in the initial dissolution rate by substituting the basket method with the USP apparatus may be attributed to the higher agitation intensity of the smaller mesh screen used in the USP apparatus. With the larger screen used in the basket method, large drug particles and their agglomerates were observed to pass through the screen orifice and fall to the bottom of the dissolution flask after the dissolution of the capsule; the small screen employed in the USP method was observed to provide a mechanical sieve action which forced the drug particles and their aggregates through the small orifice of the screen as a fine dispersion, with lesser amounts of particulate matter swelling and remaining at the bottom of the dissolution vessel. However, due to the small orifice of the screen, the visual observation of the behavior of the capsule within the basket of the USP dissolution apparatus is impaired and not as convenient as when an 8-mesh screen was employed as in the basket method. It appears that a compromise lies in adopting a suitable screen cloth, between 8 and 40 mesh, to provide the convenience of visual observation of the capsule behavior in the basket as well as the prevention of the falling and accumulating of large drug particles and their aggregates at the bottom of the dissolution flask.

As shown in Figs. 5 and 6, the dissolution profiles obtained with the D.T. method provide the fastest rate and greatest extent of dissolution for both diuretic and antidiabetic compounds among the various devices employed in this investigation. It is sufficient to say that the turbulent flow created by D.T. apparatus operating vertically at 30 c.p.m. is stronger in agitation intensity than any other devices employing unidirectional convection and turbulence at 60 r.p.m. The disadvantage of the strong agitation intensity of the D.T. method is that the mild difference in the dissolution characteristics of several formulations to be screened may not be revealed explicitly.

SUMMARY AND CONCLUSION

The dissolution profiles of experimental antidiabetic and diuretic compounds in capsules were investigated by bead, plate, blade, holder, basket, disintegration time, and proposed USP dissolution methods. These methods are capable of preventing the common problem of the floating of capsules on the surface of dissolution fluid. The evaluation of drug-release patterns by these seven methods was compared from the standpoint of apparent initial dissolution rate, extent of dissolution, and reproducibility within the period of 1 to 2 hr. at 60 r.p.m.

At the constant stirring rate of 60 r.p.m., the initial rate and the extent of dissolution are enhanced with increasing stirrer dimension. It was found that the larger the size of the stirrer, the smaller was the variation of dissolution profiles obtained among various dissolution devices. The observation of the degree of the dispersion of drug in the dissolution media can be used as a visual method to predict the dissolution patterns during dosage form development work. For hydrophobic compounds, the better the dispersion and the lesser the amount of drug remaining or swelling in the bottom of the flask, the faster was the rate and the greater was the extent of dissolution.

For the easy visualization and comparison of the dissolution profiles obtained with the seven methods employed in this investigation, the time needed for dissolving the 25, 50, 75, and 90% of the total drug from the capsule was obtained from Figs. 2–6 and summarized in Table II. It is clearly indicated that the D.T. method provides the fastest rate and the greatest extent of dissolution for both diuretic and antidiabetic compounds among various devices employed in this investigation. This finding is attributed to the stronger turbulent flow and the greater impacting force exerted on the capsule and the particulate aggregates by D.T. apparatus operating vertically at 30 c.p.m. than that obtained by other devices employing unidirectional convection and turbulence at 60 r.p.m.

Although the reproducibility of the dissolution profiles obtained with the seven dissolution methods for capsules is satisfactory and comparable, the authors prefer the simplicity, convenience, and versatility of the basket method and the proposed USP method. However, due to the small screen (40 mesh) used in the basket of the USP method, the visual observation of the behavior of the capsule in the USP method is impaired and the chance of clogging the screen by the granules is greater than with the self-designed (8-mesh screen) basket used in the basket method. For providing the convenient visual observation of the behavior of the capsule in the basket and for preventing the fall and accumulation of large drug particles and their aggregates at the bottom of the dissolution flask,

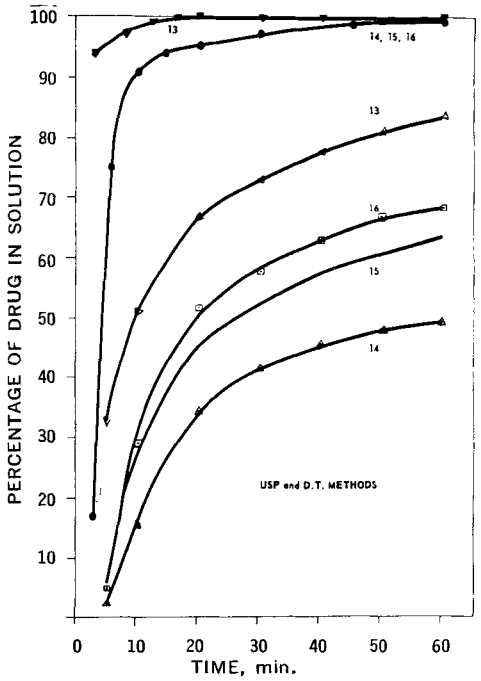


Figure 6—*Dissolution profiles of the diuretic (open symbols) and antidiabetic (solid symbols) compounds by proposed USP method (Curves 14–16) at 60 r.p.m. and D.T. (Curve 13) method at 30 c.p.m. See Table I for detailed experimental conditions.*

Table II—Dissolution Characteristics of Diuretic and Antidiabetic Compounds Investigated by Various Dissolution Apparatus

Expt. Cond.[a]	Time at Which the Indicated Fraction of Drug Dissolved, min. — Diuretic Compd. $t_{25\%}$	$t_{50\%}$	$t_{75\%}$	$t_{90\%}$	Antidiabetic Compd. $t_{25\%}$	$t_{50\%}$	$t_{75\%}$	$t_{90\%}$
1	36.5	120	—[b]	—	9	15	24	55
2	11.5	44	—	—	<4	4.5	6	12.5
3	42	—	—	—	9	13.5	18.5	27
4	14	50	—	—	5	6.5	9.0	18.5
5	13	57	—	—	<4	5	6	8.5
6	12.5	58	—	—	<4	5	6	8.5
7	16	135	—	—	<4	5	6	8.5
8	13	57	—	—	<4	5	6	8.5
9	15.5	60	—	—	<4	<5	<5	<5
10	13	30	—	—	<4	<5	<5	<5
11	11.5	26	—	—	<4	<5	<5	<5
12	10.5	22	—	—	<4	<5	<5	<5
13	4	10	33	88	<4	<5	<5	<5
14	14	60	—	—	<4	<5	5	10
15	9.5	27	—	—	<4	<5	5	10
16	8.5	20	—	—	<4	<5	5	10

[a] Numbers correspond to curve reference in Table I. [b] Not reached within experimental period of time.

the compromise should be made to employ a basket fabricated with the screen cloths of between 8 and 40 mesh.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 919.

(2) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1963, p. 1157.

(3) N. C. Chaudhry and L. Saunders, *J. Pharm. Pharmacol.*, **8,** 975(1956).

(4) R. H. Blythe, *Drug Stand.*, **26,** 1(1958).

(5) D. G. Campbell and J. G. Theivagt, *ibid.*, **26,** 73(1958).

(6) E. J. Nelson, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 297(1958).

(7) J. C. Souder and W. C. Ellenbogen, *Drug Stand.*, **26,** 77 (1958).

(8) W. R. Wensley, L. N. Elowe, and G. C. Walker, *Can. Pharm. J.*, **92,** 141(1959).

(9) E. B. Vleit, *Drug Stand.*, **27,** 97(1959).

(10) R. A. Nash and A. D. Marcus, *ibid.*, **26,** 77(1958).

(11) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262,** 1053 (1960).

(12) D. Kadar and G. C. Walker, *Bull. Ont. Coll. Pharm.*, **10,** 44(1961).

(13) L. C. Schroeter and J. G. Wagner, *J. Pharm. Sci.*, **51,** 957 (1962).

(14) *Ibid.*, **52,** 811(1963).

(15) G. Levy, *J. Pharm. Sci.*, **52,** 1039(1963).

(16) G. Milosovich, *ibid.*, **53,** 484(1964).

(17) M. S. Vora, A. J. Zimmer, and P. V. Maney, *ibid.*, **53,** 487 (1964).

(18) J. Lazarus, M. Pagliery, and L. Lachman, *ibid.*, **53,** 798 (1964).

(19) L. L. Kaplan, *ibid.*, **54,** 457(1965).

(20) G. Levy and L. E. Hollister, *ibid.*, **54,** 1121(1965).

(21) D. Cook, H. S. Chang, and C. A. Mainville, *Can. J. Pharm. Sci.*, **1,** 69(1966).

(22) E. Marlowe and R. F. Shangraw, *J. Pharm. Sci.*, **56,** 498 (1967).

(23) R. A. Castello, G. Jellinek, J. M. Konieczny, K. C. Kwan, and R. O. Toberman, *ibid.*, **57,** 485(1968).

(24) M. Pernarowski, W. Woo, and R. O. Searl, *ibid.*, **57,** 1419 (1968).

(25) K. Lin and A. Rankell, to be published.

(26) W. Mader, private communication.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 17, 1969, from *Pharmacy Research and Development Division, Development and Control Department, Ciba Pharmaceutical Co., Summit, NJ 07901*

Accepted for publication February 18, 1970.

Effect of Nonionic Surfactants on the Transport of Testosterone across a Cellulose Acetate Membrane

P. M. SHORT*, E. T. ABBS†, and C. T. RHODES

Abstract □ Solubilization of testosterone by a series of three alkylpolyoxyethylene surfactants at 37° has been examined as a function of surfactant concentration. The effect of these surfactants has also been investigated upon the diffusion of testosterone through cellulose acetate membranes. Diffusion coefficients were calculated using a method that allows the measurements to be completed in a very short time. Possible mechanisms by which surfactants may affect drug transport are discussed. In all cases examined, the surfactants reduced the diffusion coefficient of testosterone.

Keyphrases □ Testosterone transport—cellulose membrane □ Surfactants effect—testosterone transport, cellulose membrane □ Solubilization, testosterone—surfactants □ Diffusion coefficient—testosterone

The effect of surfactants upon the biological availability and pharmacological activity of drugs has attracted the attention of a considerable number of researchers in recent years (1).

In the present work, the authors report an investigation of the solubilization of testosterone by three alkylpolyoxyethylene surfactants. The effect of these surfactants upon the diffusion of testosterone across a cellulose acetate membrane has been studied. The work was carried out in a simple closed system which may be more similar to physiological conditions than the use of a "sink." The authors utilized a method for the evaluation of membrane diffusion coefficients that is rapid and avoids the problems of back diffusion.

EXPERIMENTAL

Materials—Three *n*-alkylpolyoxyethylene surfactants of the general formula $C_{16}OE_nOH$ (A30, A45, and A60) were used.[1] The mean molecular weights of these compounds were estimated by NMR spectroscopy as previously described (2, 3). Testosterone,[2] m.p. 155.5–156.0° [lit. (4) 152–156°], glass-distilled water, spectral quality ethanol, and cellulose acetate membranes[3] were also used.

Testosterone Assay—Testosterone was assayed in 50% ethanol by UV spectrometry at 245 mμ. The molar absorptivity for testosterone at this wavelength was found to be 1.61×10^4, obeying the Beer-Lambert law. Surfactant solutions were used as blanks when required.

Solubility Determinations—An aqueous suspension of testosterone, plus the appropriate amount of surfactant, was stirred for 1 week at 37 ± 0.1° until equilibrium had been reached. Samples were filtered twice through 0.22-μm. membrane filters[4] and assayed spectrophotometrically.

Determination of Diffusion Coefficients—The apparatus used was similar to that described by Humphreys and Rhodes (3). The Perspex donor and recipient cells were separated by the membrane and stirring was effected by bar magnets activated by immersible control units. The temperature was controlled at 37 ± 0.1°. Preliminary tests showed that the surfactant did not cross the membrane, although small traces of nonsurface-active impurities did. Blank solutions, not containing drug, were therefore used as reference in the spectrophotometric assay.

In all the diffusion results, from which diffusion coefficients, D values, were calculated, the total initial concentration of steroid, $[D_w] + [D_m]$, in the donor cell was 6.93×10^{-5} M.

The thickness of the membrane, L, used in the diffusion studies was obtained from replicate determinations made using two micrometer screw gauges; the L value for the wet membrane was 6.4×10^{-5} m.

Results were calculated using an Elliott 803 digital computer.

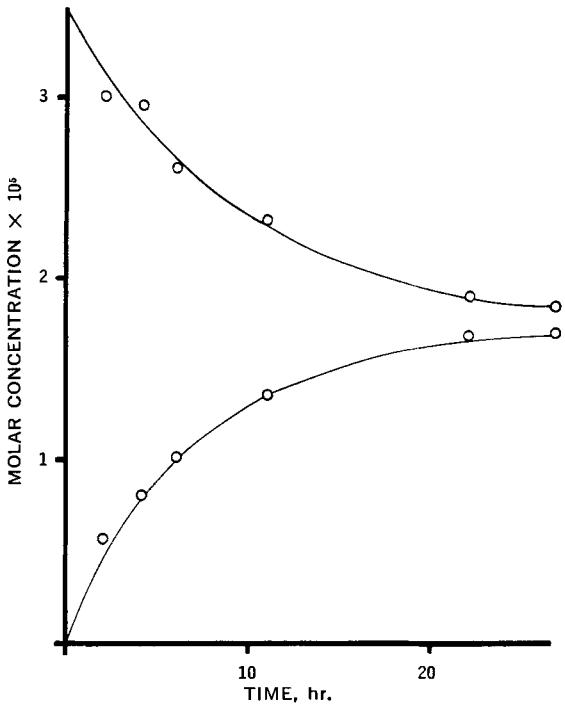


Figure 1—*Testosterone concentration in donor (upper curve) and recipient (lower curve) cells as a function of time.*

THEORY

A typical set of full-term diffusion study results showing the concentration of drug in both the donor and recipient cells as a function of time is shown in Fig. 1. It is possible, using equations based on first-order kinetic assumptions, to determine transport rates from such data. Although such determinations can be most useful, they are subject to several limitations. The transport process is often inconveniently lengthy. Also, back diffusion of drug can complicate the estimation of diffusion coefficients.

Rogers *et al.* (5) derived equations which overcome the difficulties outlined above. They investigated the diffusion of helium across glass and obtained the following equation:

$$(dp/dt) = (2A/V)SP_1\,(D/\pi t)^{0.5} \sum_{m=0}^{\infty} \exp[-(L^2/4Dt) \times (2m+1)^2] \quad \text{(Eq. 1)}$$

[1] Glover's Ltd., Leeds, England.
[2] Steraloids Ltd., Croydon, England.
[3] Visking, Scientific Instruments, Chichester, England.
[4] Millipore Ltd., Middlesex, England.

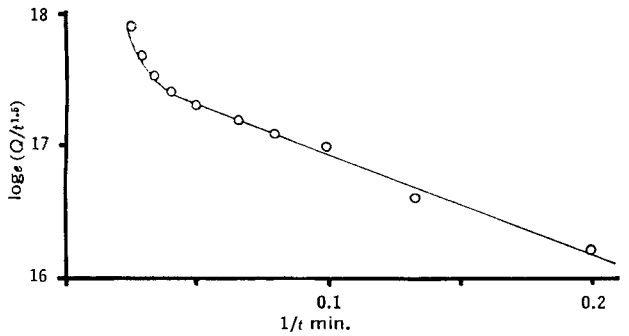


Figure 2—*Plot for the determination of diffusion coefficient.*

where P represents the pressure, t the time, A the area of the membrane, L the membrane thickness, P_1 the pressure on the donor side of the membrane, S the solubility coefficient, and D the diffusion coefficient. This equation is derived from Fick's law and includes the assumptions that D is independent of time and pressure (*i.e.*, concentration for a solution). However, measurements are made over a very short time period and the D determined is that for when $t \to 0$. Thus, these assumptions are unlikely to be of great practical significance for small concentration changes.

By integrating the original equation of Rogers *et al.*, Eq. 2 is obtained, in which the diffusion coefficient is related to the amount of drug diffused:

$$\frac{S_0 Q}{S_i C_0 L} = \frac{8}{\sqrt{\pi}}\left(\frac{Dt}{L^2}\right)^{1.5} \exp -L^2/4Dt\left[1 - \frac{6Dt}{L^2} + 60\left(\frac{Dt}{L^2}\right)^2 + \cdots - \frac{1}{9}\left\{1 - \left(\frac{6Dt}{9L^2}\right) + 60\left(\frac{Dt}{9L^2}\right) - \cdots^2\right\} \exp -2L^2/Dt\right] \quad \text{(Eq. 2)}$$

where S_0 is the solubility of drug in the solvent, S_i is the solubility of drug in the membrane, C_0 is the concentration of drug in the solvent, Q is the amount of drug diffused across the membrane, of length L, t is the time, and D is the diffusion coefficient.

Not all the terms of the integration are shown in Eq. 2 but, because of the inverted placement of t in the exponentials, this series converges most rapidly for very small values of t rather than for large values. After taking logarithms of both sides, Eq. 3 is obtained:

$$\log_e\left(\frac{Q}{t^{1.5}}\right) = \log_e\left(\frac{8C_0S_i}{\sqrt{\pi}\,L^2S_0}\right) + \frac{3}{2}\log_e D - \frac{L^2}{4D}\cdot\frac{1}{t} \quad \text{(Eq. 3)}$$

By plotting $\log_e (Q/t^{1.5})$ as a function of reciprocal time, a straight line is obtained (Fig. 2). From the slope of the line the diffusion coefficient, D, may be obtained using Eq. 4:

$$\text{slope} = -L^2/4D \quad \text{(Eq. 4)}$$

When accurate values of S_i are available, high precision values of D may be calculated by use of an iterative technique (Fig. 3). In the work reported in this paper, however, D has been calculated directly from Eq 4.

There are limitations at either end of the straight line obtained by use of Eq. 3. At the beginning of the transport process, concentrations of drug in the recipient cell are very low and errors in their analytical estimation are thus relatively high. Later when the concentration of drug in the recipient cell exceeds a critical value, back diffusion occurs and the graph starts to curve. However, results on the linear portion of the graph always returned correlation coefficients approaching unity; for example, a typical set of results of five readings gave a correlation coefficient of 0.990.

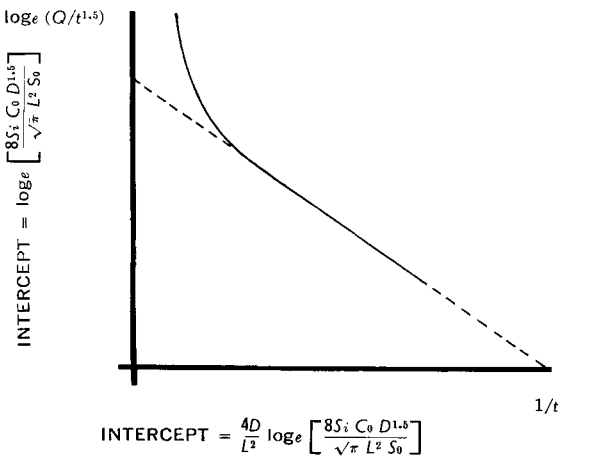


Figure 3—*Plot for the determination of high precision diffusion coefficient.*

RESULTS AND DISCUSSION

The results of the solubilization study are shown in Fig. 4. In all cases the relationship between steroid solubility and surfactant concentration was linear. This type of solubilization isotherm is indicative of micellar solubilization governed by a distribution equation (6). Distribution coefficients for testosterone in the solutions of the three surfactants have been calculated by the method of Humphreys and Rhodes (3) (Table I). When the extent of solubilization is calculated on a molar rather than a percent weight basis, the solubilization efficiency increases with chain length. This finding indicates that the solubilized steroid may be primarily located in the polyoxyethylene exterior of the micelle. Spectroscopic studies of the solubilization of several steroids have led to a similar conclusion (7).

In any aqueous isotopic surfactant solution containing a drug, the following equilibrium will exist:

$$[D_w] \rightleftharpoons [D_m] \quad \text{(Eq. 5)}$$

where D_w represents the free and D_m the bound or micellar drug. In the transport of a drug from a surfactant solution across a membrane the bound or micellar drug is not normally involved. Passive transport of drug across a membrane is a function of the concentration (or more accurately, activity) gradient of free drug across the membrane, and reduction in the value of $[D_w]$ will tend to reduce drug transport. (In those cases in which pinocytosis of micellar drug can occur the situation will, of course, be more complex.)

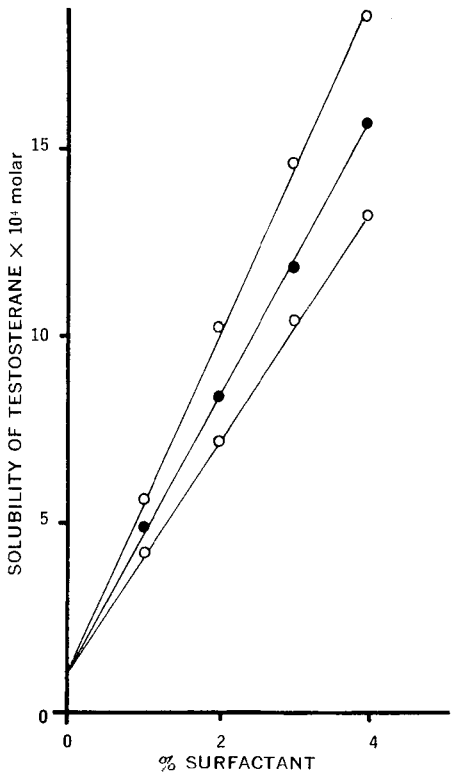


Figure 4—*Testosterone solubilization at 37° as a function of percentage n-alkyl polyoxyethylene concentration. Key: Top line, A30; middle line, A45; and bottom line, A60.*

Table I—Diffusion and Micellar Partition Coefficients for Testosterone in *n*-Alkylpolyoxyethylene Surfactant Solutions at 37°

Surfactant	No. of Ethylene Oxide Groups Estimated by NMR	K_d	Diffusion Coefficient $\times 10^7$ cm.^{2} min.$^{-1}$
—	—	—	18.80
A30	34	476	7.41
A45	62	397	10.18
A60	88	333	11.58

There are several recent reports exemplifying the reduction in the antimicrobial action of drugs caused by surfactants. However, it is apparent that other effects besides reductions in $[D_w]$ are also operative (8, 9).

The second mechanism by which surfactants can alter the transport of drugs from solution across a membrane is by modifying the aqueous diffusion coefficient of the drug. Recent work by Bloor *et al.* has shown that surfactants can increase or decrease such values (10, 11). Thus, in systems where diffusion of drug to the membrane surface is a rate-limiting factor the presence of surfactant could increase or decrease the overall transport rate. It is also possible, though rather unlikely, that monomeric surfactant might reduce the aqueous activity coefficient of the drug, the diminished activity gradient resulting in slower diffusion.

In those cases where adsorption of drug upon the membrane surface is a necessary prerequisite to membrane passage, the presence of surfactant may have further influence. Reduction of surface tension at the interface is likely to have a generalized depressant effect upon the adsorption of all species. The possibility of competition between monomeric surfactant and drug for adsorption sites also exists. However, since it is feasible that a surfactant–drug complex, such as mixed micelle, might also be adsorbed, presence of surfactant could increase or decrease the amount of drug adsorbed. The overall effect will depend upon the relative concentrations of the various species present and the values of their free energies of sorption for the membrane involved. For those substances for which active transport systems exist in a biological membrane, there is a possibility of specific interference by the surfactant with this process. There are also a number of reports which may indicate that surfactants can have direct effects on membrane permeability (12–14). Such effects could be caused by partial defatting or interaction between the surfactant and protein or phospholipid (15).

Because surfactants may affect drug transport in so many ways, there are great advantages in using initially simple *in vitro* studies which can be designed so as to allow the different mechanisms to be distinguished. Such work, of course, can not be regarded as a substitute for *in vivo* evaluation.

Diffusion coefficients of testosterone in distilled water and in aqueous 1% w/v solutions of the three surfactants, increasing in HLB value, are shown in Table I, and the effect of surfactant concentration upon the testosterone diffusion coefficient is recorded in Table II. These results show that the higher the K_d value, *i.e.*, the more the equilibrium shown in Eq. 5 is in favor of the micellar pseudophase, the greater the reduction in diffusion coefficient. Plaxco *et al.* observed similar effects with ethylene oxide chain length, and thus HLB, in investigations of drug release from suppositories (16).

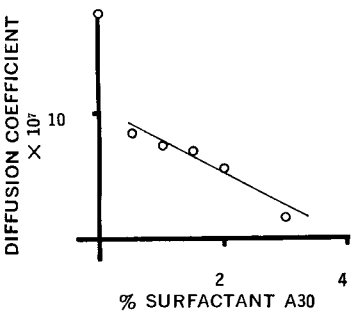


Figure 5—*Effect of surfactant (A30) concentration on testosterone diffusion coefficient.*

Table II—Diffusion Coefficients for Testosterone as a Function of Surfactant (A30) Concentration at 37°

Surfactant Concentration, % w/v	Diffusion Coefficient $\times 10^7$ cm.^{2} min.$^{-1}$
0.5	8.44
1.0	7.41
1.5	6.93
2.0	5.54
3.0	1.56

Figure 5 shows the effect of the concentration of surfactant upon diffusion coefficient. The value of the diffusion coefficient determined in the presence of surfactant decreases linearly with increase in surfactant concentration.

Extrapolation, to zero surfactant concentration, of the relationship between finite surfactant concentration and diffusion coefficient yields a value substantially different to the diffusion coefficient of testosterone determined in distilled water. It is highly improbable that this finding is due to experimental error. Since the surfactant solutions used in this investigation were stirred during the membrane diffusion measurements, this effect cannot be attributed to modification of the aqueous diffusion of the steroid. This change must therefore be attributed to some effect of the surfactant upon the membrane. It seems likely that D changes rapidly at or about the CMC (critical micelle concentration). It has been established that there is no permanent interference with the integrity of the membrane. A membrane, which had been used for the study of the effect of surfactant upon the steroid membrane transport, when thoroughly washed, behaved normally with respect to the steroid diffusion. It is suggested that this depressant effect on steroid transport is probably due either to a generalized inhibition of adsorption at the membrane–solution interface or competition between the monomeric surfactant and steroid for adsorption sites upon the membrane. Further studies of the effect of surfactants upon the transport of drugs across membranes will be published shortly.

REFERENCES

(1) P. H. Elworthy, A. T. Florence, and C. B. Mcfarlane, "Solubilization by Surface-Active Agents," Chapman and Hall, London, England, 1968.

(2) C. T. Rhodes, *Can. J. Pharm. Sci.*, **2**, 16(1967).

(3) K. J. Humphreys and C. T. Rhodes, *J. Pharm. Sci.*, **57**, 79 (1968).

(4) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1968.

(5) W. A. Rogers, R. S. Buritz, and D. Alpert, *J. Appl. Phys.*, **25**, 868(1954).

(6) J. L. Moilliet, B. Collie, and W. Black, "Surface Activity," 2nd ed., E. and F. Span Ltd., London, England, 1961.

(7) A. L. Thakkar and P. B. Kuehn, *J. Pharm. Sci.*, **58**, 850 (1969).

(8) R. A. Anderson and K. J. Morgan, *J. Pharm. Pharmacol.*, **18**, 449(1966).

(9) K. J. Humphreys, G. Richardson, and C. T. Rhodes, *ibid.*, **20**, 4S, (1968).

(10) J. Bloor, J. M. Morrison, and C. T. Rhodes, to be published.

(11) J. Bloor and J. M. Morrison, to be published.

(12) S. C. Penzotti and A. M. Mattocks, *J. Pharm. Sci.*, **57**, 1192 (1968).

(13) S. Feldman and M. Gibaldi, *ibid.*, **58**, 425(1969).

(14) J. A. Anello and G. Levy, *ibid.*, **58**, 721(1969).

(15) W. D. Seufert, *Nature*, **207**, 174(1965).

(16) J. M. Plaxco, C. B. Free, and C. R. Rowland, *J. Pharm. Sci.*, **56**, 809(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 3, 1969, from the *Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver 8, Canada.*

Accepted for publication January 19, 1970.
Presented to the International Pharmaceutical Conference, London, England, 1969.
The authors thank Dr. R. C. Jenkins, Portsmouth Polytechnic, for most valuable mathematical advice during the course of this work. The Medical Research Council of Canada is thanked for the Visiting Scientist Award made for the academic year 1969–1970 to C. T. R.

* Present address: Portsmouth Polytechnic, Portsmouth, Hampshire, England, to whom all correspondence should be addressed.
† Present address: Portsmouth Polytechnic, Portsmouth, Hampshire, England.

Hydrogenation of Substituted Isoquinolines over Nickel Catalyst II: Effects of Pressure and Temperature on the Hydrogenation of 5-Hydroxy-2-alkylisoquinolinium Salts

IAN W. MATHISON, WILEY L. FOWLER, Jr., and KATHLEEN C. FOWLER

Abstract □ A study of the effect of high pressure and high temperature on the nickel-catalyzed hydrogenation of 5-hydroxy-2-ethylisoquinolinium salt is described. The effects of these parameters on the yield and stereochemistry of the 5-hydroxy-2-ethyldecahydroisoquinolines produced are discussed. Comparisons of these data with those from analogous hydrogenations of the 5-nitro-2-methylisoquinolinium salt are included.

Keyphrases □ 5-Hydroxy-2-alkylisoquinolinium salts—hydrogenation □ Hydrogenation, isoquinolinium salts—temperature, pressure effect □ Vapor phase chromatography—analysis □ GLC—analysis □ IR spectrophotometry—structure

In a continuing study of the stereochemistry of variously substituted, fully reduced isoquinolines possessing pharmacological activity (1, 2), the authors have been recently interested in the hydrogenation of 5-substituted isoquinolines at high temperature and high pressure over Raney nickel catalyst (3). Their initial attention was directed toward the hydrogenation of a 5-nitroisoquinolinium salt in which they were able to demonstrate that increases in temperature were effective in inducing changes in the specificity of the hydrogenation while increases in pressure played little or no role in determining the stereochemistry of the desired 5-aminodecahydroisoquinolines produced. The results in regard to the effects of temperature were not totally unanticipated (4); however, an unexpected result was that increases in temperature initially resulted in increased specificity of hydrogenation up to a certain point which was then followed by a more randomized reduction. It was of significance that the *cis* ring junction decahydroisoquinoline was the heavily favored isomer produced (approximately 13:1 to 2:1, depending on conditions) (5, 6). The authors demonstrated that hydrogenolysis occurred to a significant extent at temperatures of 200° and above, while at 160° and 1500 p.s.i. an optimum yield of 71% of the desired 5-aminodecahydroisoquinolines was produced (*cis*:*trans*, 7.7:1). In view of the reported pharmacological activity of derivatives of 5-hydroxy-2-alkyldecahydroisoquinolines (2, 7) and the need for a rapid, efficient synthesis of these compounds, the authors wish to report the effects of increased pressure and temperature on the one-stage hydrogenation of 5-hydroxy-2-ethylisoquinolinium *p*-toluenesulfonate over W7 Raney nickel catalyst. The effects of these parameters on the stereochemistry of the hydrogenation and yields of the 5-hydroxy-2-ethyldecahydroisoquinolines produced will be discussed and comparisons will be drawn with the previously reported study (3).

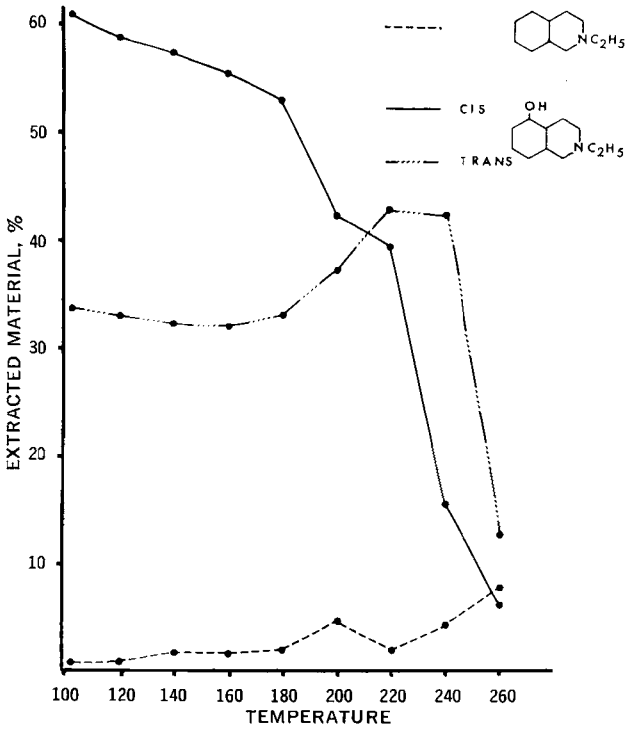


Figure 1—*Effect of temperature on reaction products at 2000 p.s.i.*

EXPERIMENTAL

The melting point is corrected. Analyses were run by Galbraith Laboratories, Knoxville, Tenn. Vapor phase chromatograms were recorded on a Varian Aerograph model 700 Autoprep chromatograph. Chromatographic peak areas were determined using a Dietzgen model D-1803-8 planimeter.

Accepted for publication January 19, 1970.
Presented to the International Pharmaceutical Conference, London, England, 1969.
The authors thank Dr. R. C. Jenkins, Portsmouth Polytechnic, for most valuable mathematical advice during the course of this work. The Medical Research Council of Canada is thanked for the Visiting Scientist Award made for the academic year 1969–1970 to C. T. R.

* Present address: Portsmouth Polytechnic, Portsmouth, Hampshire, England, to whom all correspondence should be addressed.
† Present address: Portsmouth Polytechnic, Portsmouth, Hampshire, England.

Hydrogenation of Substituted Isoquinolines over Nickel Catalyst II: Effects of Pressure and Temperature on the Hydrogenation of 5-Hydroxy-2-alkylisoquinolinium Salts

IAN W. MATHISON, WILEY L. FOWLER, Jr., and KATHLEEN C. FOWLER

Abstract □ A study of the effect of high pressure and high temperature on the nickel-catalyzed hydrogenation of 5-hydroxy-2-ethylisoquinolinium salt is described. The effects of these parameters on the yield and stereochemistry of the 5-hydroxy-2-ethyldecahydroisoquinolines produced are discussed. Comparisons of these data with those from analogous hydrogenations of the 5-nitro-2-methylisoquinolinium salt are included.

Keyphrases □ 5-Hydroxy-2-alkylisoquinolinium salts—hydrogenation □ Hydrogenation, isoquinolinium salts—temperature, pressure effect □ Vapor phase chromatography—analysis □ GLC—analysis □ IR spectrophotometry—structure

In a continuing study of the stereochemistry of variously substituted, fully reduced isoquinolines possessing pharmacological activity (1, 2), the authors have been recently interested in the hydrogenation of 5-substituted isoquinolines at high temperature and high pressure over Raney nickel catalyst (3). Their initial attention was directed toward the hydrogenation of a 5-nitroisoquinolinium salt in which they were able to demonstrate that increases in temperature were effective in inducing changes in the specificity of the hydrogenation while increases in pressure played little or no role in determining the stereochemistry of the desired 5-aminodecahydroisoquinolines produced. The results in regard to the effects of temperature were not totally unanticipated (4); however, an unexpected result was that increases in temperature initially resulted in increased specificity of hydrogenation up to a certain point which was then followed by a more randomized reduction. It was of significance that the *cis* ring junction decahydroisoquinoline was the heavily favored isomer produced (approximately 13:1 to 2:1, depending on conditions) (5, 6). The authors demonstrated that hydrogenolysis occurred to a significant extent at temperatures of 200° and above, while at 160° and 1500 p.s.i. an optimum yield of 71% of the desired 5-aminodecahydroisoquinolines was produced (*cis*:*trans*, 7.7:1). In view of the reported pharmacological activity of derivatives of 5-hydroxy-2-alkyldecahydroisoquinolines (2, 7) and the need for a rapid, efficient synthesis of these compounds, the authors wish to report the effects of increased pressure and temperature on the one-stage hydrogenation of 5-hydroxy-2-ethylisoquinolinium *p*-toluenesulfonate over W7 Raney nickel catalyst. The effects of these parameters on the stereochemistry of the hydrogenation and yields of the 5-hydroxy-2-ethyldecahydroisoquinolines produced will be discussed and comparisons will be drawn with the previously reported study (3).

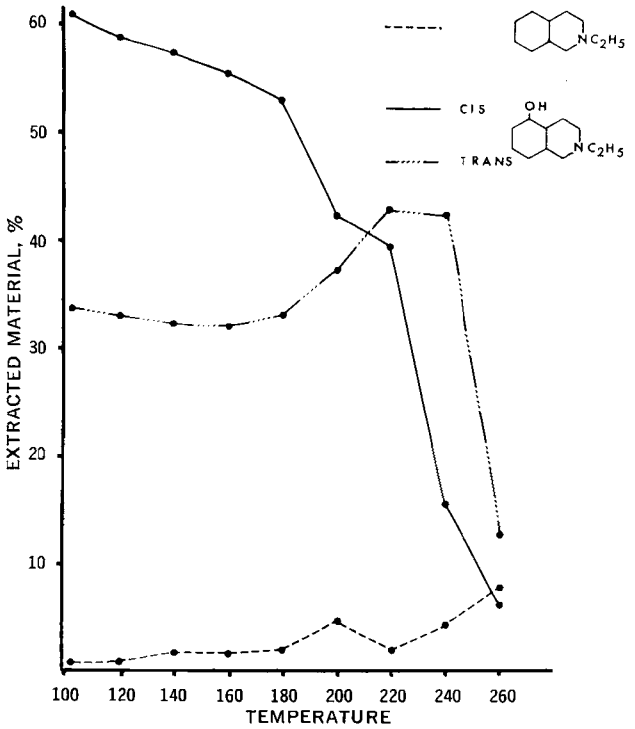


Figure 1—*Effect of temperature on reaction products at 2000 p.s.i.*

EXPERIMENTAL

The melting point is corrected. Analyses were run by Galbraith Laboratories, Knoxville, Tenn. Vapor phase chromatograms were recorded on a Varian Aerograph model 700 Autoprep chromatograph. Chromatographic peak areas were determined using a Dietzgen model D-1803-8 planimeter.

5-Hydroxy-2-ethylisoquinolinium *p*-Toluenesulfonate (I)—5-Hydroxyisoquinoline (25 g., 0.17 mole) and ethyl *p*-toluenesulfonate (35.5 g., 0.18 mole) were dissolved in dimethylformamide (100 ml.) and allowed to stand at room temperature for 72 hr. The precipitated crystals (50 g., 83%) of 5-hydroxy-2-ethylisoquinolinium *p*-toluenesulfonate were recrystallized from methanol to yield yellow needles, m.p. 212–213°.

Anal.—Calcd. for $C_{18}H_{19}NO_4S$: C, 62.59; H, 5.54; N, 4.06; S, 9.28. Found: C, 62.74; H, 5.67; N, 4.05; S, 9.57.

The IR spectrum (KBr) was consistent with the proposed structure (3200 cm.$^{-1}$; 1230 cm.$^{-1}$, —OH).

HYDROGENATION PROCEDURE

The catalytic reduction of I (10 g.) was carried out in methanol (150 ml.) over W7 Raney nickel catalyst (8) (3.0 g.) in a Parr series 4000 hydrogenator under varying conditions of temperature and pressure. The temperature was carefully controlled using a Honeywell temperature controller No. 4811. Standardization of each hydrogenation experiment regarding time of heating and agitation has been previously described in detail (3). At the termination of the reduction, the hydrogenated solution was removed from the bomb and the exhausted catalyst was filtered using diatomaceous earth (Celite) as a filtering aid. The methanol filtrate was evaporated on the rotary evaporator to yield a pale-yellow viscous oil which was suspended in water, made strongly alkaline with sodium hydroxide, and extracted with ether. The dried ether extract was then distilled to yield a yellow oil. This oil was assayed gas chromatographically on a 6.09 m. × 0.95 cm. (20 ft. × 0.375 in.) SE 30 (30%) on Chromosorb W column at 215° using a thermal conductivity detector at a gas (helium) flow rate of 100 ml./min. Authentic samples, which were used for identification of the chromatographic peaks of the reaction product mixture, included:

1. *cis*-5,9,10-*H*-5-Hydroxy-2-ethyldecahydroisoquinoline (2). Retention time = 28.4 min.
2. *trans*-9,10-*trans*-5-*H*-5-Hydroxy-2-ethyldecahydroisoquinoline.[1] Retention time = 26.8 min.
3. *cis*-2-Ethyldecahydroisoquinoline.[2] Retention time = 14.3 min.
4. *trans*-2-Ethyldecahydroisoquinoline.[2] Retention time = 13.4 min.

RESULTS AND DISCUSSION

The average areas under each identified peak of duplicate gas chromatograms of at least two separate hydrogenations were plotted and Figs. 1–6 were obtained.

Effect of Temperature at Constant Pressure

The study was approached by an initial examination of the influence of temperature on the reduction of 5-hydroxy-2-ethylisoquinolinium *p*-toluenesulfonate. An initial bomb pressure of 2000 p.s.i. was utilized, since it allowed comparisons with the previous investigation (3). Examination of Figs. 1–3 shows clearly the overall effects of temperature on the hydrogenation. As anticipated, increases in temperature resulted initially in an increase in yield of the desired 5-hydroxy-2-ethyldecahydroisoquinolines (Fig. 3); this was followed by a decreased yield concurrently with increased hydrogenolysis (Figs. 1 and 3). It was apparent that the optimum temperature was in the region of 200°, a significantly higher temperature than that for the corresponding nitroisoquinolinium salt (3). The influence of temperature on the stereochemistry of the products yielded some interesting results (Figs. 1 and 2). It is evident (Fig. 1) that increased temperature resulted in increased yields of the *trans* ring junction product accompanied by a comparable decrease in the *cis* product. This holds up to 200–220°, at which point marked decreases in both isomers occur, primarily due to hydrogenolysis and decomposition. This trend, while evident

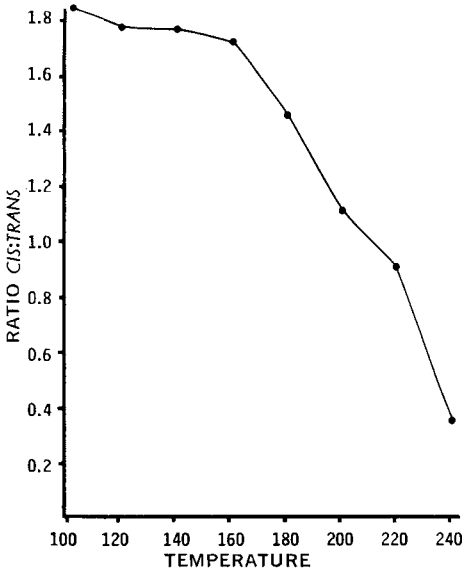


Figure 2—*Effect of temperature on the stereochemistry at 2000 p.s.i.*

during the reduction of the nitro analog (3), was not nearly so dramatic as in the present study on the hydrogenation of the hydroxy compound. Indeed, at 240° and above, the authors were able to produce predominantly the *trans* isomer, a situation never realized during the reduction of the nitro compound. More interesting were the data obtained regarding isomer ratios as temperature was increased (Fig. 2). At 100°, a favored *cis* ring junction hydrogenation occurred. As the temperature was increased to 200°, a more randomized hydrogenation resulted where the *cis*:*trans* ratio was 1:1. However, beyond 200° the randomization of hydrogenation was decreased which resulted in a *cis*:*trans* ratio of 0.8, *i.e.*, a reduction favoring the *trans* isomer. While it is acknowledged that increased temperature favors a more randomized hydrogenation (4), the authors are unaware of temperature increases resulting in increased randomization followed by a decrease in the randomization. Additionally, it was unanticipated that such a marked reversal of the favored stereochemistry would result. No comparable data were obtained during the reduction of the nitroisoquinoline (3). It is also pertinent that in the reduction of the nitro compound the *cis*-5-aminodecahydroisoquinoline was the very heavily favored product (*cis*:*trans* ratio of 14:1 to 2:1 depending on the temperature), while in the present study with the hydroxy compound the *cis* isomer was the most favored at best at isomer ratios of only 2:1.

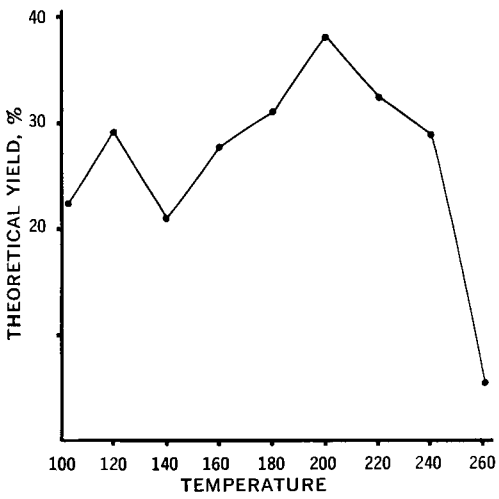


Figure 3—*Effect of temperature on yield of 5-hydroxy-2-ethyldecahydroisoquinolines at 2000 p.s.i.*

[1] Prepared from *trans*-9,10-*trans*-5-*H*-5-hydroxydecahydroisoquinoline using essentially the method of Blicke and Monroe (9) as outlined by Mathison *et al.* (2) for the corresponding *cis*-5,9,10-*H*-5-hydroxy-2-ethyldecahydroisoquinoline.

[2] Prepared by the catalytic hydrogenation of 2-ethylisoquinolinium *p*-toluenesulfonate using the general procedure outlined by Witkop (10).

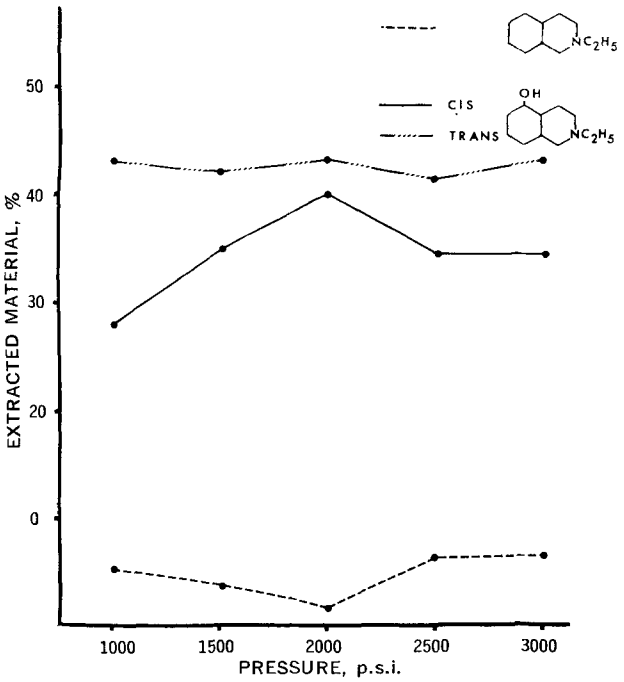


Figure 4—*Effect of pressure on reaction products at 220°.*

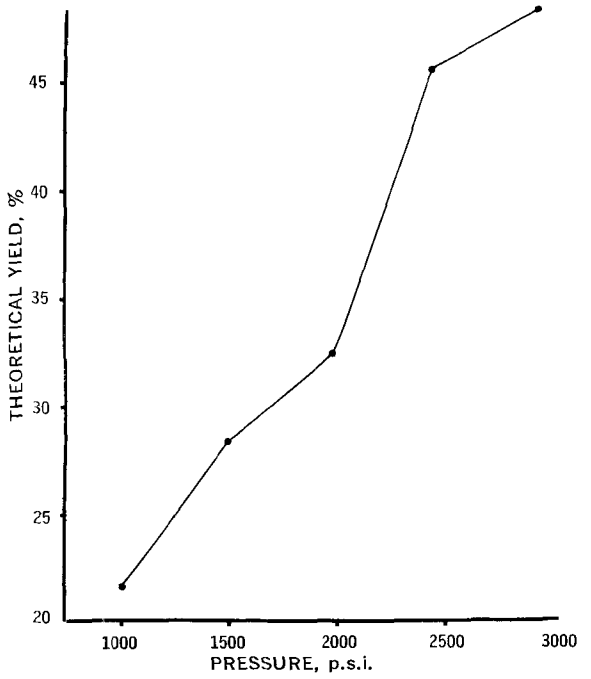


Figure 6—*Effect of pressure on yield of 5-hydroxy-2-ethyldecahydroisoquinolines at 220°.*

Effect of Pressure at Constant Temperature

In view of the superior yield and nearly equal isomer ratio, it was decided to examine the effects of pressure at 220°. The results are summarized in Figs. 4–6. Figure 4 shows clearly that the production of the *trans* ring junction isomer is virtually unaffected by increases in pressure. On the other hand, the amount of the *cis* analog produced undergoes marked changes during a similar pressure increase. Increased pressure up to 2000 p.s.i. results in increased quantities of *cis*-5,9,10-*H*-5-hydroxy-2-ethyldecahydroisoquinoline, while pressure increases beyond this point result in decreased product. The correlation between the amount of *cis* isomer produced and the amounts of hydrogenolyzed products is of interest. As the amount of the *cis* analog increases, the hydrogenolysis products decrease and vice versa. The summation of the amounts of *cis* product and the hydrogenolysis products remains at a figure of approximately 39% of extracted material throughout the pressure range 1500–3000 p.s.i. These data strongly suggest that the *cis* isomer is more readily hydrogenolyzed than the *trans* analog, a finding compatible with the data obtained during the temperature study on the hydrogenation of the nitro and hydroxy compounds. The authors were unable to demonstrate this trend in the analogous pressure experiments on the nitro compound (3). The results regarding the stereochemistry of the hydrogenation (Fig. 5) were comparable with the data obtained for the nitro compound. Increased pressure results in decreased specificity of hydrogenation up to a point (approximately 2000 p.s.i.); this is then followed by increased specificity. This finding is in contrast to the anticipated results (11). As pressure increases, hydrogen availability increases, and processes requiring hydrogen would be accelerated. One would expect, therefore, that greater *cis*:*trans* ratios would result since desorption from the catalyst surface would be less likely to occur prior to completion of the hydrogenation. The results concur with this rationale up to 2000 p.s.i; however, beyond this point the data contradict this theory. The authors can only assume that pressures beyond 2000 p.s.i. in some way perturb the adsorption of the compound to the catalyst prior to completion of the reduction which results in desorption and, therefore, decreased specificity of hydrogenation. As anticipated, pressure was more effective than temperature in inducing higher yields of the desired 5-hydroxydecahydroisoquinolines. Superior yields, however, were obtained with the nitro compound at increased pressures (3).

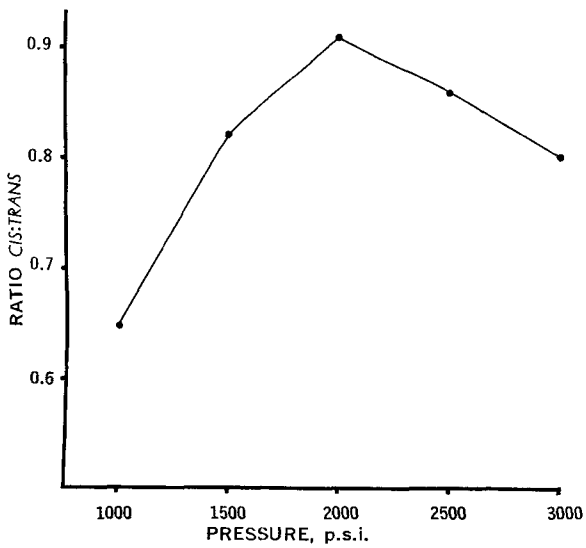


Figure 5—*Effect of pressure on stereochemistry at 220°.*

The differences in stereochemistry observed between the hydrogenations carried out on the 5-nitro-2-alkylisoquinolinium salts (3) and the present study on the hydroxy compounds may be related to the basicity of the products, the aminodecahydroisoquinolines produced in the former investigations being stronger bases than the hydroxydecahydroisoquinolines produced in this study. It is well established (12) that pH is an important factor in determining the steric course of catalytic reductions.

REFERENCES

(1) I. W. Mathison and R. C. Gueldner, *J. Org. Chem.*, **33**, 2510(1968).

(2) I. W. Mathison, R. C. Gueldner, J. W. Lawson, S. J. Fowler, and E. R. Peters, *J. Med. Chem.*, **11**, 997(1968).

(3) I. W. Mathison and W. L. Fowler, Jr., *J. Pharm. Sci.*, **58**, 1238(1969).

(4) P. N. Rylander, "Catalytic Hydrogenation Over Platinum Metals," Academic, New York, N. Y., 1967, pp. 35–56.

(5) R. P. Linstead and P. Levine, *J. Amer. Chem. Soc.*, **64**, 2022 (1942).

(6) B. Witkop, *ibid.*, **71**, 2559(1949).

(7) I. W. Mathison and J. W. Lawson, *Chim. Ther.*, **3**, 468 (1968).
(8) H. R. Billica and H. Adkins, in "Organic Syntheses Collective Volume III," E. C. Horning, Ed., Wiley, New York, N. Y., 1955, pp. 176–180.
(9) F. F. Blicke and E. Monroe, *J. Amer. Chem. Soc.*, **61**, 91 (1939).
(10) B. Witkop, *ibid.*, **70**, 2617(1948).
(11) S. Siegel, F. V. Smith, B. Dmuchovsky, D. Dubbell, and W. Halpern, *ibid.*, **84**, 3136(1962).
(12) R. L. Augustine, "Catalytic Hydrogenation," Dekker, New York, N. Y., 1965, pp. 46–49.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 27, 1969, from the *Marion Chemical Research Laboratory, Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee Medical Units, Memphis, TN 38103*

Accepted for publication January 16, 1970.

Presented at the Second International Congress of Heterocyclic Chemistry, Montpellier, France, July 1969.

The authors thank Marion Laboratories, Inc., Kansas City, Mo., for their continued generous support of this project.

Influence of Phase Equilibria on Properties of Emulsions

S. FRIBERG and L. MANDELL

Abstract □ An investigation was made concerning the phase equilibria in water–oil–emulsifier systems. The results have shown the presence of liquid crystalline phases and the pronounced influence of these on the properties of emulsions. The pronounced solubilizing power of emulsifier micelles in the oil phase, the changed stability due to these, and the drastic changes of volume ratios, water to oil, with increasing emulsifier concentration which this gives rise to, have been investigated.

Keyphrases □ Emulsions—phase equilibria effect □ Phase equilibria—w/o emulsions □ Stability, emulsions—emulsifier concentration □ Liquid crystalline phase—emulsions

The complicated behavior of emulsions has been treated in numerous articles and reviews, due to the importance of such systems in chemical technology and in a vast number of systems of biological origin. In his bibliography, Becher (1) dealt with the different factors that had been found responsible for the behavior of emulsions. Davies discussed the stability of emulsions based on a collision theory (2). Sherman has made important contributions concerning the general properties and rheological behavior of emulsions (3). Recently, Shinoda has introduced the PIT value (4), which denotes the phase inversion temperature. Shinoda assumes this temperature to be more useful than the well-known HLB value in the prediction of the behavior of emulsions from the properties of the emulsifiers.

The HLB value is useful to a very high degree; its success in practical emulsion preparation has shown this beyond doubt, but its shortcomings are obvious. As an example, Sherman (5) pointed out the inversion of emulsions when the amount of emulsifier is increased, and Davies (2) showed the difference in emulsion behavior when the oil phase was changed from benzene to petroleum ether. Neither of these examples can be explained by the HLB value of the emulsifier.

Preliminary results on the influence of phase equilibria on the properties of emulsions showed a sudden increase in stability in the presence of a mesomorphous phase (6). Further investigations (7) showed a pronounced change in phase equilibria when the aromatic *p*-xylene in the oil phase was replaced by hexadecane. These results gave a tentative explanation to the problem stated by Davies (2). The rather complicated rheological behavior of emulsions when the emulsifier concentration was changed could also be satisfactorily explained by interpretation of phase equilibria (8). Since these results appeared to be promising, it was considered that further investigations of phase equilibria in the water–emulsifier–oil systems could be of general value.

Emulsions of w/o type are considerably more difficult to treat theoretically due to the low electric field strength in the continuous medium. Taking this into consideration, the authors chose a set of emulsifiers of the w/o type and determined the phase equilibria of the systems.

EXPERIMENTAL

Materials—The water was twice distilled. The nonylphenol diethyleneglycol ether was of commercial origin (Berol AB, Sweden), which was purified from polyglycols; other impurities are less than 0.01%. Other chemicals used were: octylamine (puriss. gas chromatographic >99%) and *p*-xylene (puriss. gas chromatographic >99.5%) (Fluka A.G., Switzerland); monocapryline (synthesized at the Institute of Medical Biochemistry, University of Gothenburg, Sweden); tricapryline (Eastman Distillation Products Industries); and lecithin, prepared from egg yolk according to a simplified method (9).

Phase Equilibria—The samples for investigation of the phase equilibria in the three-component systems given in Fig. 1 (A–D) were weighed directly into glass ampuls which were sealed. The samples were heated to homogeneity, slowly cooled to 20° under agitation, and allowed to stand at this temperature. The different phases were separated by ultracentrifugation and identified by visual observation under a polarizing microscope or by X-ray methods according to previous work on phase equilibria in ternary systems (10, 11).

Emulsion Preparation and Properties—The components were weighed into ampuls which were treated in an ultrasonic device at 20° for 1 min. followed by vacuum treatment to remove air. This was repeated five times.

The nature of the emulsion was determined by visual observation through a microscope of the spread of oil- or water-soluble dyes (Sudan III and Brilliant Blue F.C.F.). The emulsions with an

(7) I. W. Mathison and J. W. Lawson, *Chim. Ther.*, **3**, 468 (1968).
(8) H. R. Billica and H. Adkins, in "Organic Syntheses Collective Volume III," E. C. Horning, Ed., Wiley, New York, N. Y., 1955, pp. 176–180.
(9) F. F. Blicke and E. Monroe, *J. Amer. Chem. Soc.*, **61**, 91 (1939).
(10) B. Witkop, *ibid.*, **70**, 2617(1948).
(11) S. Siegel, F. V. Smith, B. Dmuchovsky, D. Dubbell, and W. Halpern, *ibid.*, **84**, 3136(1962).
(12) R. L. Augustine, "Catalytic Hydrogenation," Dekker, New York, N. Y., 1965, pp. 46–49.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 27, 1969, from the *Marion Chemical Research Laboratory, Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee Medical Units, Memphis, TN 38103*

Accepted for publication January 16, 1970.

Presented at the Second International Congress of Heterocyclic Chemistry, Montpellier, France, July 1969.

The authors thank Marion Laboratories, Inc., Kansas City, Mo., for their continued generous support of this project.

Influence of Phase Equilibria on Properties of Emulsions

S. FRIBERG and L. MANDELL

Abstract □ An investigation was made concerning the phase equilibria in water–oil–emulsifier systems. The results have shown the presence of liquid crystalline phases and the pronounced influence of these on the properties of emulsions. The pronounced solubilizing power of emulsifier micelles in the oil phase, the changed stability due to these, and the drastic changes of volume ratios, water to oil, with increasing emulsifier concentration which this gives rise to, have been investigated.

Keyphrases □ Emulsions—phase equilibria effect □ Phase equilibria—w/o emulsions □ Stability, emulsions—emulsifier concentration □ Liquid crystalline phase—emulsions

The complicated behavior of emulsions has been treated in numerous articles and reviews, due to the importance of such systems in chemical technology and in a vast number of systems of biological origin. In his bibliography, Becher (1) dealt with the different factors that had been found responsible for the behavior of emulsions. Davies discussed the stability of emulsions based on a collision theory (2). Sherman has made important contributions concerning the general properties and rheological behavior of emulsions (3). Recently, Shinoda has introduced the PIT value (4), which denotes the phase inversion temperature. Shinoda assumes this temperature to be more useful than the well-known HLB value in the prediction of the behavior of emulsions from the properties of the emulsifiers.

The HLB value is useful to a very high degree; its success in practical emulsion preparation has shown this beyond doubt, but its shortcomings are obvious. As an example, Sherman (5) pointed out the inversion of emulsions when the amount of emulsifier is increased, and Davies (2) showed the difference in emulsion behavior when the oil phase was changed from benzene to petroleum ether. Neither of these examples can be explained by the HLB value of the emulsifier.

Preliminary results on the influence of phase equilibria on the properties of emulsions showed a sudden increase in stability in the presence of a mesomorphous phase (6). Further investigations (7) showed a pronounced change in phase equilibria when the aromatic *p*-xylene in the oil phase was replaced by hexadecane. These results gave a tentative explanation to the problem stated by Davies (2). The rather complicated rheological behavior of emulsions when the emulsifier concentration was changed could also be satisfactorily explained by interpretation of phase equilibria (8). Since these results appeared to be promising, it was considered that further investigations of phase equilibria in the water–emulsifier–oil systems could be of general value.

Emulsions of w/o type are considerably more difficult to treat theoretically due to the low electric field strength in the continuous medium. Taking this into consideration, the authors chose a set of emulsifiers of the w/o type and determined the phase equilibria of the systems.

EXPERIMENTAL

Materials—The water was twice distilled. The nonylphenol diethyleneglycol ether was of commercial origin (Berol AB, Sweden), which was purified from polyglycols; other impurities are less than 0.01%. Other chemicals used were: octylamine (puriss. gas chromatographic >99%) and *p*-xylene (puriss. gas chromatographic >99.5%) (Fluka A.G., Switzerland); monocapryline (synthesized at the Institute of Medical Biochemistry, University of Gothenburg, Sweden); tricapryline (Eastman Distillation Products Industries); and lecithin, prepared from egg yolk according to a simplified method (9).

Phase Equilibria—The samples for investigation of the phase equilibria in the three-component systems given in Fig. 1 (A–D) were weighed directly into glass ampuls which were sealed. The samples were heated to homogeneity, slowly cooled to 20° under agitation, and allowed to stand at this temperature. The different phases were separated by ultracentrifugation and identified by visual observation under a polarizing microscope or by X-ray methods according to previous work on phase equilibria in ternary systems (10, 11).

Emulsion Preparation and Properties—The components were weighed into ampuls which were treated in an ultrasonic device at 20° for 1 min. followed by vacuum treatment to remove air. This was repeated five times.

The nature of the emulsion was determined by visual observation through a microscope of the spread of oil- or water-soluble dyes (Sudan III and Brilliant Blue F.C.F.). The emulsions with an

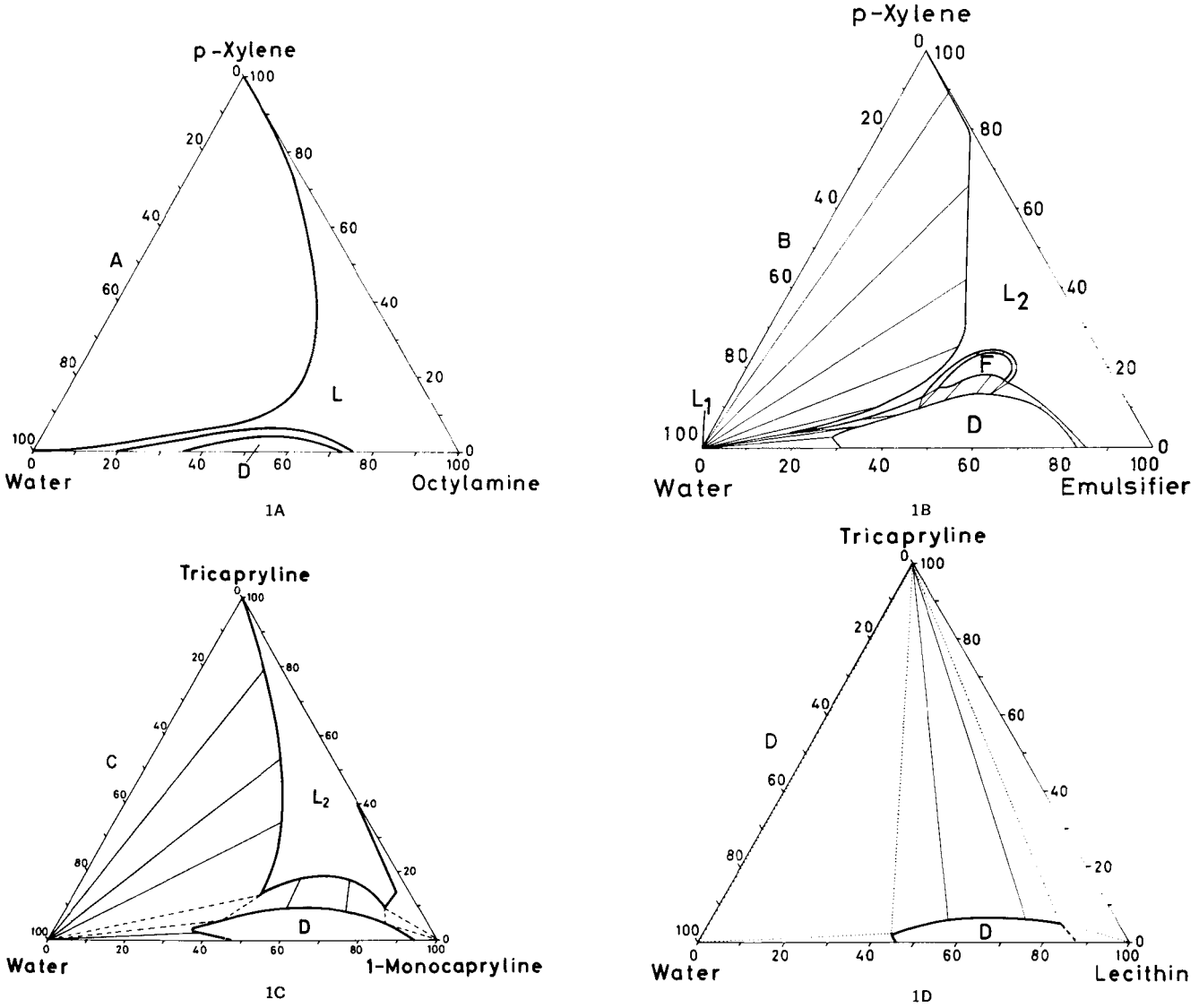


Figure 1—*Phase equilibria in water–oil–emulsifier systems at 20°. Key: A, water–p-xylene–octylamine; B, water–p-xylene–nonylphenol diethyleneglycol ether; C, water–tricapryline–monocapryline; and D, water–tricapryline–egg lecithin.*

amine as emulsifier were too unstable for such a procedure. In that case the emulsion was allowed to settle, and the nature of the two developing layers was determined. This eased the determination of the emulsion type when one of the layers—the emulsion—was emulsified in the other, since a change from water to oil of the continuous phase gave indications of a double emulsion, which could later be confirmed by direct observation in the microscope.

The estimation of the emulsion stability was performed in a rough way by judging the sedimentation rate from photographs. With the pronounced differences of stability characteristic of these systems, the method was considered to be satisfactory. The emulsions containing an amine as stabilizer were too unstable to be determined in this way. In that case the time for clarification of the phases was used as a measure of the stability.

The emulsions were prepared in several series. In each series the ratio of water to oil was kept constant while the amount of emulsifier was varied. The total compositions of the emulsions are found in Tables I and II.

Table I—Total Composition of Water–Tricapryline Emulsions with Monocapryline as Emulsifier

Series	Ratio, % Water / % Tricapryline	Monocapryline Sample No. 1	2	3	4
I	95/5	9.5	19.2	32.1	
II	90/10	9.1	18.9	31.0	
III	80/20	8.2	16.7	28.7	
IV	60/40	6.3	13.1	23.1	36.6
V	40/60	4.2	16.8	27.9	40.5
VI	20/80	2.3	9.1	16.2	25.5

RESULTS

Phase Equilibria—Figures 1A–D contain diagrams of the phase equilibria in the four systems of this investigation. The conditions in the tentative three-phase areas to the right in the diagram (Fig. 1D) have not been examined in detail, since they are of no value to the present investigation.

The diagrams (Figs. 1A–C) are all of the same general shape with a pronounced solution area where the oil and the emulsifier solubilize water. Xylene and octylamine solubilize water to such a high extent that a continuous solubility area connecting all three components is formed. The solubilizing power decreases in the order A to C in the diagrams; in the water–tricapryline–egg lecithin system, too little mutual solubility is found to be observable in the diagram. The solubilization only takes place in the form of a liquid crystalline phase with a layer structure (denoted D in the figure). This phase is also found in all other diagrams and is always formed by water and the emulsifier with the oil solubilized to a few percent.

Emulsion Type—Figure 2 shows the emulsion type in the different parts of the region containing two liquid phases in the equilibrium. The system containing lecithin has not been included, since the corresponding two-phase area of that system is too small to be investigated conveniently.

The octylamine gives o/w emulsions in the water-rich part. In the remaining part of the two-phase area, it forms emulsions of the o/w/o type, which, however, are unstable and separate into two emulsions within a short time (Fig. 2A). Diglycol ether forms w/o emulsions when the emulsifier is associated in micelles in the *p*-xylene phase and when the ratio of *p*-xylene to water exceeds 0.70–0.75 (Fig. 2B). Monocapryline forms w/o emulsions in the whole area except where the water–tricapryline ratio is too high to permit the formation of a w/o emulsion (Fig. 2C).

Emulsion Stability—The stability of the system with diethyleneglycol ether as an emulsifier has previously been investigated and reported (12), and the system containing egg lecithin will not be treated here, since the solution areas of this are too small to be investigated conveniently.

The emulsions containing octylamine are considerably less stable than the other emulsions (Table II). The comparison between the stability of different emulsions in this system was consequently not done by means of photographing the emulsions, but it was made by measuring the time required for the upper phase to form a clear solution.

The upper layer is oil-continuous, and it is striking to compare (Table II) how the time for the clarification of this layer is reduced when the amount of emulsifier is increased. This tendency is most pronounced when the emulsion contains high amounts of *p*-xylene. On the other hand, the emulsions formed by the liquid crystalline phase and the solution were stable to a very high degree.

The emulsions containing water and tricapryline and with monocapryline as an emulsifier show two distinct regions of different behavior. At first, Series I and II contain the liquid crystalline phase in Sample 3 (Table I and Fig. 1C). This emulsion is by far the most stable that can be observed in Fig. 3. Only Series II is shown for spatial reasons. Sample 2, containing more emulsifier than Sample 1, is less stable.

This phenomenon of decreasing stability with increasing emulsifier concentration can be observed in all the emulsions belonging to the second region, the large two-phase area, in Fig. 1C. Only Series V is shown here as an example because of space limitations.

These results imply an optimum addition of monocapryline to obtain maximal stability. This has, however, not been investigated at present.

Table II—Stability of Water–*p*-Xylene Emulsions, with Octylamine as Emulsifier

Ratio, % Water / % *p*-Xylene	Octylamine	Time Required for Formation of a Clear Top Layer, min.	Time Required for Clarification of the Whole Sample, min.
20/80	8.0	120	170
20/80	17.0	19	35
20/80	35.0	4.5	8
40/60	6.0	120	325
40/60	13.0	55	220
40/60	29.0	3	220
40/60	47.0	3.5	220
60/40	4.5	140	200
60/40	9.5	37	200
60/40	22.0	3	200
60/40	38.0	3	200
80/20	5.0	90	190
80/20	13.5	175	190
80/20	24.0	54	190
80/20	40.0	5	190
90/10	2.0	160	
90/10	8.5	160	1100
90/10	14.5	160	
90/10	26.0	145	1100

DISCUSSION

The results have shown two factors governing the stability of emulsions. The first one is the presence of a liquid crystalline phase of the layer type which will increase the stability of the emulsion. This is well shown by Series II in Fig. 3 where Sample 3 contains the liquid crystalline phase. The same trend has been shown for other systems (6, 12). The two-phase area between water and the liquid crystalline phase containing only monoglyceride and water was treated by Larsson (13), who also gave suggestions for the structure of such a dispersion in water.

The second factor is the presence of micelles. From the present investigations and from earlier studies (12), it appears that the emulsion will have a reduced stability when the concentration of the emulsifier is in excess of the critical micelle concentration. This is especially pronounced in the case of octylamine, *p*-xylene, and water where the increased emulsifier concentration gives a reduction of emulsion stability (Table II). The reason for this is not completely clear at present but the following explanation may serve as a hypothesis.

When two emulsified drops approach each other, a temporary oversaturation will be established in the region between the surfaces of the drops owing to the adsorbed emulsifier. This temporarily increased concentration may give rise to the formation of

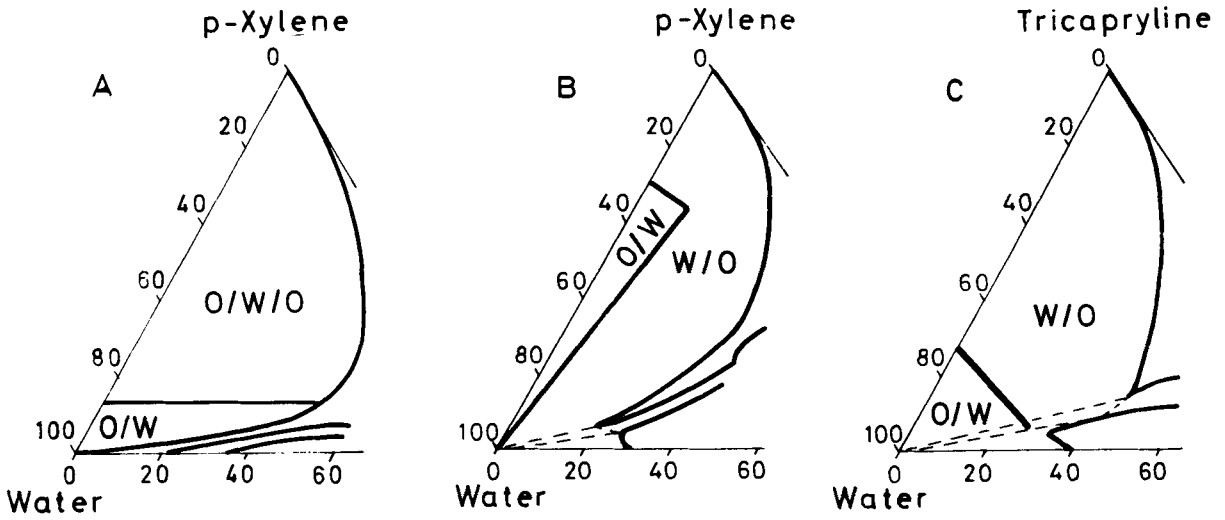


Figure 2—*Emulsion type in different parts of the two-phase areas. Key: A, water–*p*-xylene–octylamine; B, water–*p*-xylene–nonylphenol diethyleneglycol ether; and C, water–tricapryline–monocapryline.*

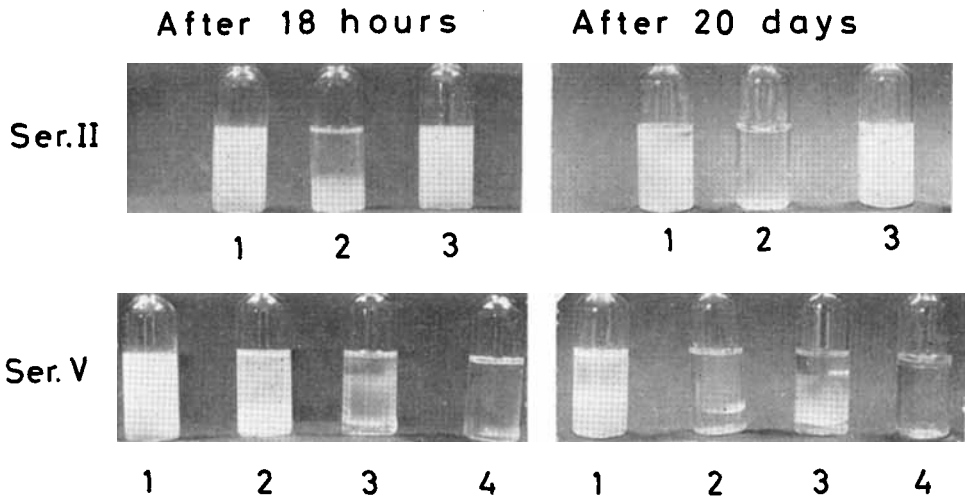


Figure 3—*The behavior of emulsions in series where the ratios of water to tricapryline are constant and the amount of monocapryline is increased. The composition of the samples is given in Table I.*

micelles in the continuous phase, or micelle formation also can take place in the dispersed phase in some cases. It appears likely that the formation of micelles will mean a source of decreased order between the surfaces and, consequently, the resistance to coalescence will be reduced. If the increased concentration, on the other hand, gives rise to an ordered layer structure of the liquid crystalline type, the temporary overconcentration between the surfaces will not imply a disorder phenomenon. On the contrary, a several layer structure can be formed containing more ordered structures.

This hypothesis is not contradicted by any of the results—they all support it. The water–amine–*p*-xylene system, which can form micelles both in the aqueous and *p*-xylene phase, gives rise to a very unstable emulsion when the concentration of emulsifier is increased. At high amine to *p*-xylene ratios, where the solution changes from *p*-xylene to water continuous, the stability of the emulsion is very low. For the same ratio of *p*-xylene to amine but with the water content reduced to such a degree that the liquid crystalline phase is present, the stability is increased in the most pronounced way.

The marked increase of the water-solubilizing power of the oil phase at certain ratios (oil–emulsifier), which can be observed in Figs. 1A–C, is also an important factor. The solubilizing implies that the volume ratio, water to oil, can be most drastically changed when the concentration of emulsifier is increased. This means that the changed concentration of emulsifier—in some cases due to the evaporation of the solvents—can cause an inversion of the emulsion due to volume changes under certain conditions.

CONCLUSIONS

The investigations have shown how the properties of emulsions can be related to the phase equilibria in water–oil–emulsifier systems. They have confirmed the hypothesis about the pronounced influence of the liquid crystalline phases present on the properties of the emulsion but have also pointed to the influence of micelles in the systems. Both of these factors have been neglected in earlier studies of the behavior of emulsions except one preliminary study (14).

The results have also made evident the pronounced solubilization of water in the oil phase and shown how this fact can cause drastic changes in the volume ratios of the two liquid phases.

REFERENCES

(1) P. Becher, "Emulsions, Theory and Practice," Reinhold, New York, N. Y., 1965.

(2) J. T. Davies, *Recent Progr. Surface Sci.*, **2**, 129(1964).

(3) "Emulsion Science," P. Sherman, Ed., Academic, London, England, 1968.

(4) K. Shinoda, *J. Colloid Interface Sci.*, **30**, 258(1969).

(5) P. Sherman, *J. Soc. Cosmet. Chem.*, **16**, 465(1965).

(6) S. Friberg, L. Mandell, and M. Larsson, *J. Colloid Interface Sci.*, **29**, 155(1969).

(7) S. Friberg, L. Mandell, and K. Fontell, *Acta Chem. Scand.*, **23**, 1055(1969).

(8) S. Friberg and P. Solyom, *Kolloid Z.*, **236**, 173(1970).

(9) W. S. Singleton, M. S. Gray, M. L. Brown, and J. D. White, *J. Amer. Oil Chem. Soc.*, **42**, 53(1965).

(10) L. Mandell and P. Ekwall, *Acta Polytech. Scand. Chem.*, **74**, I(1968).

(11) K. Fontell, L. Mandell, H. Lehtinen, and P. Ekwall, *ibid.*, **74**, III(1968).

(12) S. Friberg and L. Mandell, to be published.

(13) K. Larsson, *Z. Phys. Chem.*, **56**, 173(1967).

(14) F. Lachampt and R. M. Vila, *Amer. Perfum. Cosmet.*, **82**, 29(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 22, 1969, from the *Swedish Institute for Surface Chemistry, Drottn. Kvist. Väg. 45–47, Stockholm, Sweden.*

Accepted for publication February 24, 1970.

The authors thank Mr. Bengt Jernström, Miss Solveig Sundberg, and Miss Kerstin Lundin for valuable technical assistance.

The investigation has received support from the Swedish Board for Technical Development.

Rapid Determination of Aluminum in Pharmaceutical Dosage Forms by Neutron Activation

JOSEPH P. F. LAMBERT* and MICHEL MARGOSIS

Abstract □ A rapid, efficient, and highly accurate assay by neutron-activation analysis is presented for aluminum content as a function of the aluminum compounds contained in complex antibiotic formulations. The assay requires no chemical isolation or cleanup. The relatively short half-life of ^{28}Al allows rapid analysis, and the technique favors the detection of aluminum over possible interferences by longer lived radionuclides.

Keyphrases □ Aluminum in dosage forms—determination □ Oleaginous products—aluminum determination □ Neutron-activation analysis—aluminum

Aluminum monostearate (AMS) is incorporated as a thixotropic gelling agent in oleaginous pharmaceutical formulations. Many of these products are intended for use as intramammary infusions for the treatment of mastitis in dairy animals. These often comprise assorted mixtures of antibiotics (procaine penicillin G, neomycin sulfate, dihydrostreptomycin sulfate, polymyxin B sulfate, and sodium novobiocin), sulfonamides, corticosteroids, and various preservatives. Milk-out studies indicate that prolonged antibiotic residues in milk may be associated with certain formulations containing AMS, with greater prolongation being correlated with higher soap content.

The product known as sterile procaine penicillin G with aluminum stearate suspension is used in human medicine as a depot preparation to obtain prolonged duration of penicillin in the blood following a single intramuscular injection. Aluminum chlorohydroxide is a drug possessing astringent properties commonly used in antiperspirant formulations.

The analysis of aluminum salts in pharmaceutical dosage forms is usually performed titrimetrically with EDTA or gravimetrically as Al_2O_3 after combustion (USP, NF). These methods are often cumbersome, inefficient, tedious, occasionally erroneous, and generally lacking in specificity.

Well over 100 applications of neutron activation for the analysis of aluminum have been reported (1) but none in the field of pharmaceutical dosage forms. The technique reported here should be most valuable to drug chemists because of its specificity, accuracy, and practical application at the common dosage concentration. The reaction of thermal neutrons with aluminum, $^{27}Al(n,\gamma)^{28}Al$, is favorable, and the resulting radioactive nuclide has a half-life of 2.3 min. and a γ-ray energy of 1.78 Mev. A rapid recording of the γ-ray spectrum at the reactor site is dictated by the relatively short half-life of ^{28}Al. The short irradiation enhances the discrimination against longer lived radionuclides and enables the rapid analysis for aluminum of a large volume of samples.

Interferences from fast neutrons reacting with silicon or phosphorus and from thermal neutrons reacting with magnesium (2) are minimized, since the analysis involves relatively large quantities of aluminum as compared with the amounts of the interfering elements, if at all present. Gamma-ray spectral interferences are also minimal, since the matrix is essentially organic, time of irradiation is short, and the γ-ray energy for aluminum is relatively high. Neutron-activation analysis is rendered even more efficient in this case, because there is no necessity to separate and isolate the aluminum in any fashion from these intractable formulations. This study covers mainly a variety of oleaginous commercial products containing antibiotics, but the method is readily applicable to any aluminum-containing drug.

EXPERIMENTAL

Irradiation Containers—The 0.40-dr. (1 ml.), capped polyethylene vials[1] were cleaned with 1:1 HNO_3, rinsed with distilled water, and dried with acetone or methanol.

Standards—A stock solution of aluminum standard was prepared by dissolving 12.3534 g. of reagent grade $Al_2(SO_4)_3 \cdot 18H_2O$ in distilled water and diluting to 1 l. in a volumetric flask. A secondary standard was prepared by dissolving 0.0458 g. of aluminum wire (grade 1100) in dilute H_2SO_4 and diluting to 100 ml. in a volumetric flask. Aliquots of 1.00 ml. were transferred to the polyethylene vials.

Samples—About 1 g. of the oily product was accurately weighed into a polyethylene vial. Antiperspirants were prepared by diluting an accurately weighed sample of about 1 g. to 25 ml. with distilled water and transferring a 1.00-ml. aliquot to a polyethylene vial.

Apparatus—All samples were irradiated in either an exposure tube (thermal neutron flux of approximately $10^{13} n/cm.^2$ sec.) or a pneumatic tube facility (thermal neutron flux of approximately $10^{12} n/cm.^2$ sec.) at the 1-Mw. nuclear reactor of the Naval Research Laboratory, Washington, D. C. The γ-ray spectra were obtained with a 1024-channel pulse height analyzer,[2] using a 7.62 × 7.62-cm.(3 × 3-in.) NaI(Tl) detector.[3] The readout equipment consisted of a teletype printer and an X-Y recorder.[4] The samples were mounted approximately 10 cm. from the detector, which is housed in a large steel shield. This detector, which was covered with a 1-cm. thick plastic β-absorber, has a resolution of 8.0% at 662 kev. The pulse height analyzer was divided into four 256 channels, so four independent counts could be recorded. The gain control of the analyzer was set at 10 kev./channel over the 256 channels.

Procedure—Three samples and one standard were packaged into an irradiation bucket and lowered into the exposure tube for a 60-sec. irradiation, or one sample and one standard were packaged into

[1] Chemical Rubber Co., Cleveland, Ohio.
[2] Northern Scientific, Madison, Wis.
[3] The Harshaw Chemical Co., Cleveland, OH 44106
[4] Mosley model 7590.

Table I—Neutron-Activation Analysis of Aluminum in AMS

Sample	Al Found, %	AMS (w/w), % Tech.[a]	AMS (w/w), % USP[b]	Expected AMS[c] (w/v), %
1	0.01	—	—	None
2	0.01	—	—	None
3	0.003	—	—	None
4	0.003	—	—	None
5	0.001	—	—	None
6	0.001	—	—	None
7	0.01	—	—	None
8	0.01	—	—	None
9	0.02	—	—	None
10	0.104	2.34	1.31	2
10A[d]	0.0916	2.06	1.15	2
11	0.119	2.68	1.50	2
12	0.0955	2.15	1.20	2
12A	0.102	2.30	1.28	2
13	0.0950	2.14	1.20	2
14	0.0734	1.65	0.92	1.78[e]
15	0.107	2.11	1.35	1.49[e,f]
15A	0.101	2.27	1.27	1.49[e,f]
16	0.0911	2.05	1.15	—
17	0.0872	1.96	1.10	—
18	0.0985	2.22	1.24	1.47[e,f]
18A	0.105	2.36	1.32	1.47[e,f]
19	0.136	3.06	1.71	2.00[e]
19A	0.143	3.22	1.80	2.00[e]
20	0.123	2.77	1.55	2.00[e]
20A	0.122	2.75	1.53	2.00[e]
21	0.126	2.84	1.59	2.00[e]
21A	0.135	3.04	1.70	2.00[e]
22	0.173	3.89	2.18	3.60
22A	0.150	3.38	1.89	3.60
23	0.138	3.11	1.74	3.60
23A	0.135	3.04	1.70	3.60
24	0.131	2.95	1.65	3.60
24A	0.148	3.33	1.86	3.60
25	0.147	3.31	1.85	3.60
25A	0.153	3.44	1.92	3.60
26	0.127	2.86	1.60	2
27	0.148	3.33	1.86	2
28	0.133	2.99	1.67	2
28A	0.137	3.08	1.72	2
29	0.137	3.08	1.72	2
30	0.161	3.62	2.03	2.12
31	0.146	3.29	1.84	2.12
32	0.170	3.83	2.14	1.94
33	0.187	4.21	2.35	3
34	0.208	4.68	2.62	—
34A	0.186	4.19	2.34	—
35	0.232	5.22	2.92	6.00
36	0.189	4.25	2.38	3
37	0.220	4.95	2.77	6.00
37A	0.206	4.64	2.59	6.00
38	0.249	5.60	3.13	—
38A	0.239	5.38	3.01	—
39	0.298	6.71	3.75	6.60
39A	0.280	6.30	3.52	6.60

[a] Based on technical grade containing 4.45% Al by neutron-activation analysis. [b] Based on USP grade containing 7.95% Al. [c] Actual or labeled amount on weight/volume basis. [d] Samples labeled A are duplicates. [e] Weight/weight basis. [f] Official drug.

a pneumatic tube shuttle and irradiated for 60 sec. in the pneumatic tube facility.

After a group irradiation, the standard was counted for 1 min. at a decay time of 10 min., whereas the three samples were counted individually for 1 min. at suitable decay times based on the indicated dead time of the pulse height analyzer.

The direct method of quantitation involves taking one count for each irradiated specimen at a given time, whereas the differential method requires a count of two different times for each specimen. The data were analyzed by the Covell method (3) for the photopeak area at 1.78 Mev., and the activity (net count summation) was adjusted to the time equivalent to the end of irradiation, using a calculated decay factor, according to equation

$$A_0 = A_1/e^{-\lambda t} \qquad \text{(Eq. 1)}$$

where A_0 is the activity of specimen at time equivalent to the end of irradiation; A_1 is the activity of specimen at time count was made; λ is specific decay constant for nuclide; and t is equivalent time of count. When a sample and a standard are treated identically in every respect, the activity to aluminum content ratio is directly comparable.

Table II—Neutron-Activation Analysis of Aluminum in Products Containing Different Aluminum Salts

Salt	Al Found, %	Expected, %	% of Expected
Tristearate	0.0630	0.07078	89.0
Tristearate	0.0644	0.07078	91.0
Chlorohydroxide	4.50	4.79	93.9
Chlorohydroxide	4.95	5.03	98.4
Chlorohydroxide	4.86	5.03	96.6
Chlorohydroxide	5.00	5.03	99.4
Chlorohydroxide	5.00	5.03	99.4

The differential method subtracts the sample activity at two given times, t_1, t_2, and A_0 is determined as follows:

$$A_1 - A_2 = A_0(e^{-\lambda t_1} - e^{-\lambda t_2}) \qquad \text{(Eq. 2)}$$

$$\therefore A_0 = \Delta A/(e^{-\lambda t_1} - e^{-\lambda t_2}) \qquad \text{(Eq. 3)}$$

The relationship between activity and aluminum content of the sample is again compared to that of the standard.

The data obtained for all samples are based on total aluminum content, regardless of source, and physicochemical state of the specimen.

RESULTS

Table I lists results obtained for 39 different samples of oleaginous drug formulations from various manufacturers. Column 2 lists the actual amount of aluminum found; the values in Column 3 are calculated to the monostearate salt based on the technical grade (Table IV); and Column 4 lists the values calculated on the basis of the USP grade for comparison to the actual or labeled amounts as listed in Column 5. Table II likewise lists results for aluminum salts found in one otic and several antiperspirant formulations. The results obtained from various standard samples, using the pneumatic tube facility, are listed in Table III. Table IV shows results from several determinations of a sample of aluminum monostearate. Table V lists the results from four identical irradiations of the secondary standard, using the pneumatic tube facility.

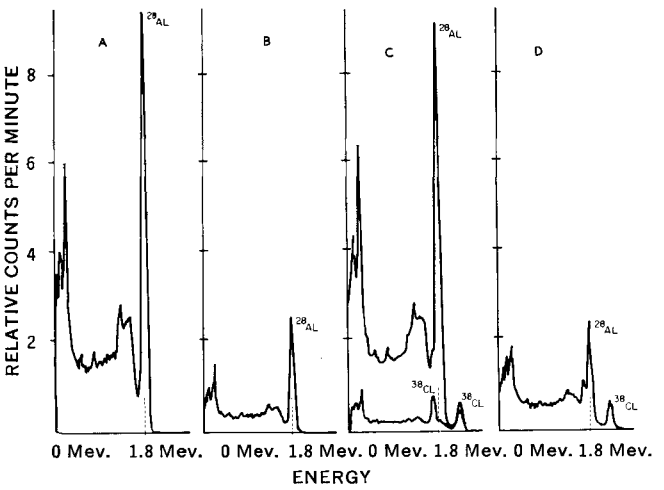


Figure 1—*Typical γ-ray spectra for an aluminum standard and a mastitis drug sample after a 1-min. irradiation in a flux of* 10^{12} *n/cm.*^{2} *sec. Key: A, aluminum standard counted after 3-min. decay period; B, aluminum standard counted after 8-min. decay period; C, drug counted after 5-min. (upper) and 20-min. (lower) decay period; and D, drug counted after 10-min. decay period.*

Table III—Recovery of Aluminum from Materials Used as Standards

Sample	Weight, mg.	Al Found, mg.	Al Expected, mg.	Recovery, %[a]
1100 wire, solution	0.4576, mg./ml.	0.458, mg./ml.	0.4576, mg./ml.	100
1100 wire, solid	5.00	5.04	5.00	101
99.9% Al + 0.1% Au wire	1.11	1.08	1.11	97.3
SRM-87a Al–Si alloy[b]	1.77	1.79	1.61	111
Al monostearate, in oil	27.7	1.22	2.20[c]	55.5
Al monostearate, powder	13.7	0.6	1.09[c]	59
Al_2O_3 reagent grade	24.9	8.53	13.2[d]	64.7

[a] Ratio of Al determined by neutron activation to that calculated from sample weight. [b] SRM-87a Al–Si alloy is an NBS reference standard containing 91.0% Al. [c] Calculated on basis of USP value of 7.95% Al. [d] Sample assumed to be anhydrous, but was in fact the trihydrate; actually 99.2% recovery as $Al_2O_3 \cdot 3H_2O$.

Figure 1 shows examples of typical γ-ray spectra for an aluminum standard and a drug.

DISCUSSION

Sterile procaine penicillin G with aluminum stearate suspension is an official USP drug containing 2% of aluminum monostearate. The soap, however, usually contains mixtures of stearates, palmitates, oleates, and free or loosely combined fatty acids, as well as hydrates and basic soaps (4). The aluminum content of AMS (USP) is equivalent to 14.5–16.0% of Al_2O_3 obtained by combustion, whereas the technical grade salt may yield around 10% Al_2O_3.

The results in Table I may then be used as a presumptive test, if necessary, to determine the grade of soap used in the formulation for control and regulatory purposes. In the first grouping, there was no ^{28}Al photopeak detected, and an upper limit value was computed from the counts. Considering an approximate 10% variation in density of the samples in comparing percent weight/weight against percent weight/volume, it is noted that most samples fall into one or the other grade. The standard deviation for samples listed in Table I approached 10%. When samples were handled individually, the standard deviation was lower than 3%; however, the rate of sample handling decreased about threefold.

The precision is also affected by the background, which may occasionally show other isotopes to be present in substantial amounts. For instance, peaks due to sodium, chlorine, and cobalt have been detected in many of these samples. The presence of chlorine (Fig. 1) and/or sodium contributes to the ^{28}Al γ-ray spectrum peak area. The Covell method or the differential technique of data analysis is used to correct for these spectral interferences. These corrections somewhat limit the overall precision of neutron-activation analysis for aluminum.

To verify the results in Table III, the AMS bulk and the aluminum oxide standard were subjected to combustion. The residue on ignition values of 9.4 and 67%, respectively, confirmed the results from neutron-activation analysis and verified the accuracy of the method. The sample of AMS was indeed of a technical grade; the aluminum oxide was indeed a trihydrate. The only outlying result in Table III is 111% recovery for the standard reference material.

Table IV—Determination of Aluminum in a Batch of AMS

Sample[a]	Weight, mg.	Al Found, mg.	Al, %
1A	23.90	1.07	4.48
1B	23.90	1.05	4.39
1C	23.90	1.077	4.51
2	13.71	0.635	4.63
3	20.25	0.885	4.37
4A	27.7	1.25	4.51
4B	27.7	1.24	4.48
4C	27.7	1.194	4.31
5	37.2	1.630	4.38

Av. = 4.45%
SD = 0.031 (0.7%)

[a] Samples 1 and 4 were irradiated and counted three times each.

Table V—Precision in Relative Counts in Irradiations

Identification[a]	Relative Net Counts
Aliquot 1	9409
	9295
	9473
Aliquot 2	9597
	9615
Av.	9478
SD	119 (1.26%)

[a] The same sample was irradiated more than once over a period of 2 days. Both aliquots are from the same stock solution. A blank stock solution contained less than 0.002 mg. of Al.

However, this was not unexpected since the geometry of the sample was different from that of the primary standard, and the sample was small.

The secondary standard was handled individually; the results listed in Table V indicate that the relative counts from different irradiations are reproducible, showing a precision of better than 3%. Comparisons of values also indicate excellent accuracy.

REFERENCES

(1) "Activation Analysis: A Bibliography," G. J. Lutz, R. J. Boreni, A. S. Maddock, and W. W. Meinke, Eds., Superintendent of Documents, NBS T.N. 467, Washington, D. C., 1968.

(2) R. C. Koch, "Activation Analysis Handbook," Academic, New York, N. Y., 1960, pp. 48–49.

(3) D. F. Covell, *Anal. Chem.*, **31**, 1785(1959).

(4) "Fatty Acids," K. S. Markley, Ed., Interscience, New York, N. Y., 1961.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 29, 1969, from the *Division of Food Chemistry and Technology and the Division of Pharmaceutical Sciences, National Center for Antibiotics and Insulin Analysis, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, DC 20204*

Accepted for publication February 11, 1970.

* Present address: Nuclear Engineering Department, North Carolina State University, Raleigh, NC 27607

Alternatives to the *National Formulary* Procedure for Detecting Nitrobenzene in Benzaldehyde

JOHN H. BLOCK*, MATHEWS A. NUNES†, and RICHARD O. MUHLHAUSER

Abstract □ A TLC technique has been developed which can be used to detect microgram quantities of nitrobenzene in benzaldehyde. This method, with its sensitivity and simplicity, provides a great advantage over the current detection procedure in the NF XII. A color complex reaction utilizing sodium pentacyanoamine ferroate and a spot test procedure on filter paper were also investigated, and each of these offers specific advantages over the current NF procedure. A mechanism for formation of the color complex is postulated.

Keyphrases □ Nitrobenzene detection—benzaldehyde □ TLC—analysis □ UV light—TLC spot visualization □ Colorimetric analysis □ Pentacyanoamine ferroate—color reagent □ Spot testing—nitrobenzene in benzaldehyde

Benzaldehyde has been in the official compendia since 1905 when it became a part of the *United States Pharmacopeia VIII*. In 1936 benzaldehyde was deleted from the USP and became official in the *National Formulary VI*. In 1916, a test for the detection of nitrobenzene became a part of the monograph, and this same test is still in the present revision. The test for nitrobenzene is included because it has an odor similar to benzaldehyde but is extremely toxic, having a lethal dose of 5–50 mg./kg. in man (1). Nitrobenzene is toxic by all routes and is capable of causing methemoglobinemia and destruction of red blood cells (2). The official test involves reduction of nitrobenzene to aniline and then oxidation with potassium dichromate to give a purple color if nitrobenzene is present in the sample (3). A problem which is often encountered is the formation of a precipitate that may make detection of the color difficult if not impossible. Furthermore, since the test is run by different technicians, results may be interpreted differently.

Aromatic nitro compounds have been detected by spot tests (4) and by formation of a colored complex (5). Interpretation of these results, however, is quite difficult for small amounts of nitrobenzene, and the procedures involve many steps. A spectrophotometric procedure was developed in 1949 by Glazko *et al.* (6); although quantitative results are obtained, the procedure is quite involved and lengthy.

To overcome problems associated with interpretation of results and complicated procedures, this paper reports and evaluates three different procedures for the detection of nitrobenzene in benzaldehyde.

EXPERIMENTAL

Reagents—The following chemicals and reagents were used without further purification: benzaldehyde NF and nitrobenzene (Matheson, Coleman & Bell), sodium fluorescein (Eastman Organic Chemicals), sodium pentacyanoamine ferroate (K & K Laboratories), and silica gel G and silica gel HF (Brinkmann Instruments, Inc.). Visualization was made by means of a General Electric purple-X 250 w. UV light.

Table I—Reagents and Results Obtained by Color Complexation Procedure

Benzaldehyde	Neg.	Butylamine	Neg.
Nitrobenzene	Pos.	Dimethylamine	Neg.
Aniline[a]	Pos.	Ethylbenzene	Neg.
N-Methylaniline	Neg.	Xylene	Neg.
N,N-Diethylaniline	Neg.	2-Methylnaphthalene	Neg.
N-Benzylisopropylamine	Neg.	Phenol	Neg.
N,N-Dibenzylaniline	Neg.	Anisole	Neg.
p-Aminobenzoic acid	Pos.	*o*-Anisidine	Pos.

[a] A positive result was obtained also without the reduction step.

NF Procedure—Solutions analyzed by the NF XII procedure were nitrobenzene, benzaldehyde, and nitrobenzene in benzaldehyde 5:100 and 1:100. (All dilutions are by volume.)

Spot Tests (4)—Samples analyzed were nitrobenzene, benzaldehyde, and nitrobenzene in benzaldehyde 1:100 and 1:1000. Two drops of each solution was placed on a piece of filter paper, and 1–2 drops of 10% stannous chloride in ethanol saturated with hydrogen chloride gas was added. After absorption, 1 drop of 35% cuprous chloride in concentrated hydrochloric acid was added and allowed to be absorbed. Six drops of pyridine was added and a green color developed. Two drops of carbon tetrachloride was then added and the characteristic colors formed in 10–30 sec.

Color Complex (5)—Samples analyzed were nitrobenzene, benzaldehyde, and nitrobenzene in benzaldehyde 1:100, 1:1000, and 1:10,000. One-tenth milliliter of the solution to be analyzed was dissolved in 3 ml. of ethanol, previously heated on a hot water bath, in a 10-cm. (4-in.) test tube. Six drops of 10% calcium chloride solution and about 50 mg. of zinc dust were added, and the solution was heated to boiling in a hot water bath. The excess zinc was removed by filtration, and the cooled filtrate was treated with 1 drop of a 1% solution of sodium pentacyanoamine ferroate. A colored flocculent precipitate formed in about 1 min., the time required depending upon the amount of nitrobenzene present. Best results were obtained when the solution was not stirred. Dilute samples required 2–3 hr. if the solution was stirred after the addition of the sodium pentacyanoamine ferroate solution.

Additional samples were also tested to learn something about the nature of the complex formed. The samples used and results appear in Table I.

Thin-Layer Chromatography—TLC was carried out on 5 × 20-cm. chromatoplates prepared according to Stahl, utilizing silica gel G, with 0.04% sodium fluorescein, and silica gel HF. Eight solvent systems were investigated (7–9) (Table II); however, only the visualization technique of Berei and Vasaros (7) was employed.

The other adsorbent was investigated with the 2.5% acetone in benzene solvent system.

Samples analyzed were nitrobenzene, benzaldehyde, and nitrobenzene in benzaldehyde 1:100, 1:1000, and 1:10,000. In all of the initial chromatograms, normal spotting procedures using a capillary tube were employed. The sensitivity of this procedure was also evaluated; for this test, 10 μl. of the solution was spotted *via* a calibrated micropipet.

RESULTS AND DISCUSSION

NF Procedure—The 1:100 solution gave a negative result for nitrobenzene using this procedure. The 5:100 solution did give positive results; however, another analyst may well have judged the results to be negative when compared with a control sample.

Spot Tests—The pure sample of benzaldehyde gave a predominantly blue spot, and the nitrobenzene produced a definite red-orange color. However, problems were encountered in trying to

Table II—Solvent Systems Investigated Using Silica Gel G with 0.04% Sodium Fluorescein

Benzene–methanol–acetic acid, 45:8:4
Petroleum ether–ethyl acetate, 4:1
Diethyl ether
Acetone (2.5%) in benzene
n-Hexane–diisopropyl ether, 6:4
Cyclohexane–diethyl ether–diisopropyl ether, 2:1:1
Cyclohexane–diethyl ether, 2:1 with 10% silicone oil
Benzene–ethyl acetate, 5:1

distinguish the characteristic orange color produced by the nitrobenzene from the yellowish discoloration of the filter paper due to the other chemicals. The 1:100 dilution was definitely positive; but with the 1:1000 dilution, it was not possible to determine whether the test was positive when compared with a control sample.

Color Complex—In this test, pure benzaldehyde produced a yellow precipitate whereas pure nitrobenzene produced a deep-purple precipitate. As the amount of nitrobenzene in the sample was reduced, the intensity of the color faded through blue to a blue-gray color for the 1:1000 dilution. No bluish hue was distinguishable for the 1:10,000 dilution.

The basis of these tests is the reduction of nitrobenzene and subsequent reaction with an appropriate reagent to produce a characteristic color. In the NF XII procedure the reduction is carried out with zinc and sulfuric acid, followed by the addition of potassium dichromate to yield a blue-green colored product if nitrobenzene was originally present. In the spot test procedure, stannous chloride and HCl reduce the nitro group to the primary aromatic amine. The color formed is most likely due to the formation of a complex between the amine and the cuprous ion (4).

In the colored-complex procedure, reduction is accomplished with zinc dust and the calcium chloride solution. The color formed is probably due to the formation of a complex between the reduced nitro group and the $Fe(CN)_5^{-3}$ moiety of the sodium pentacyanoamine ferroate, $Na_3[Fe(CN)_5NH_3]$. Two possibilities exist: (*a*) that there is a replacement of the NH_3 ligand by the $—NH_2$ portion of the aromatic amine or (*b*) that a charge transfer complex exists between iron and π electrons of the phenyl ring (10). On the basis of data in Table I, it seems reasonable to postulate that the complexation consists of a replacement of NH_3 ligand by the primary aromatic amine. If a charge transfer complex formed, all the aromatic compounds with strongly electron-donating groups would have been expected to form the complex. The data also indicate that a primary aromatic amine is necessary for complexation. The differences in inductive effects of aniline and *N*-methylaniline are so small that a charge transfer complex should have been formed with both. The methyl group of *N*-methylaniline, in addition to providing steric hindrance for ligand replacement, prevents overlap of the unshared *p*-orbital electrons of the nitrogen with the π electrons of the phenyl ring. It is this delocalization of electrons that is probably of primary importance in color change, as pointed out by the fact that the aliphatic amines tested did not delocalize and gave negative results. It would seem that phenol, which also has an unshared pair of electrons, should also form the complex. However, Basolo and Johnson have pointed out that the greater the base strength of a ligand, the greater the stability of the complex formed (11). Thus, aniline, which has a greater basicity than phenol, will form a more stable complex.

Thin-Layer Chromatography—Of the eight solvent systems examined, none gave better separation than that of 2.5% acetone in benzene. The solvent systems of benzene–methanol–acetic acid and of diethyl ether provided very poor separation, and the other systems used gave results similar to the 2.5% acetone in benzene system. This acetone–benzene system had the added advantage of being one of the faster systems, requiring only about 40 min. for the solvent front to travel 15 cm.

Using the silica gel G with 0.04% sodium fluorescein, the 1:100 dilution showed a red-violet band (R_f 0.93) which appeared purple under UV light. This band was not visible without irradiation at 1:1000 but was clearly seen with the aid of UV light. At a dilution of 1:10,000, this band was barely visible even with UV light, and the limit of the test was taken to be the 1:1000 dilution. Pure samples were also spotted for comparison, and the appearance of the chromatograms is shown in Fig. 1. Since aldehydes are easily

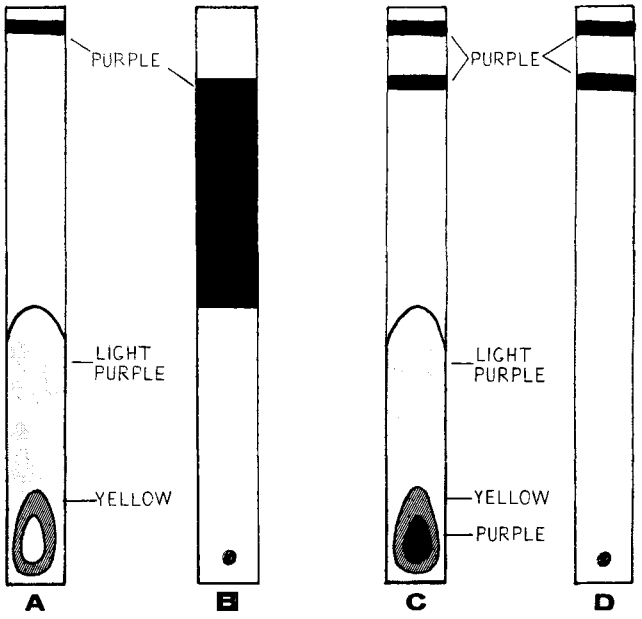


Figure 1—*Thin-layer chromatograms. Solvent system: 2.5% acetone in benzene. Plates: A, B, and C, silica gel G with 0.04% sodium fluorescein; D, silica gel HF. Key: A, benzaldehyde; B, nitrobenzene; and C and D, nitrobenzene in benzaldehyde, 1:1000.*

oxidized and possibly reduced, benzyl alcohol and benzoic acid were also chromatogramed and found not to interfere.

The silica gel HF showed the same sensitivity as the silica gel G but had the advantage of not showing the spots at the lower end of the plate which are attributed to the benzaldehyde. Using silica gel G and spraying with 0.04% sodium fluorescein failed to differentiate between the nitrobenzene and the benzaldehyde.[1]

Unlike the previous procedures, TLC does not require the reduction of nitrobenzene. Thus, it is possible to detect smaller amounts of nitrobenzene, since the other procedures are sensitive to only that portion of the nitrobenzene that has been reduced.

SUMMARY

A TLC technique has been developed which can be used to detect small amounts of nitrobenzene. The estimated limit of this test is 10^{-5} ml. or 12 micrograms of nitrobenzene. This test is 100 times as sensitive as the color complex procedure. In addition, there are fewer mechanical steps involved and, therefore, less chance of error when compared with the NF procedure.

The spot test procedure has approximately the same sensitivity as the NF procedure, but it takes much less time. It requires only about 5 min. as compared to about 1.5 hr. for the monograph procedure.

The color complex reaction is much more sensitive than either the NF or the spot test procedure. Nitrobenzene was detectable in 0.1 ml. of the 1:100 dilution which corresponded to 1.2 mg. of nitrobenzene. This procedure is also quite simple and requires less than 0.5 hr. total.

On the basis of this investigation, it can be stated that the color complex and the TLC procedures are superior to the present NF procedure, both from the standpoint of sensitivity and time required for routine use. The thin-layer procedure is the simpler and more sensitive of the two and could presumably be adapted to obtain quantitative results.

REFERENCES

(1) M. N. Gleason, R. E. Gosselin, and H. C. Hodge, "Clinical Toxicology of Commercial Products," Williams and Wilkins,

[1] Two commercial precoated silica gel products with fluorescent indicator failed to show spots with or without UV light.

Baltimore, Md., 1963, sec. II, inside front cover, and p. 85.
(2) S. Locket, "Clinical Toxicology," C. V. Mosby, St. Louis, Mo., 1957, p. 505.
(3) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 48.
(4) Y. D. Mogilyanskii, *Tr. Kom., Anal. Khim., Akad. Nauk SSSR*, **13**, 418(1963); through *Chem. Abstr.*, **60**, 1901d(1964).
(5) F. Feigl, "Spot Tests in Organic Analysis," 7th ed., Elsevier, New York, N. Y., 1966, p. 297.
(6) A. J. Glazko, L. M. Wolf, and W. A. Dill, *Arch. Biochem.*, **23**, 411(1949).
(7) K. Berei and L. Vasaros, *J. Chromatogr.*, **26**, 301(1967).
(8) K. Randerath, "Thin Layer Chromatography," Academic, New York, N. Y., 1963, p. 179.
(9) L. Frishbein, *J. Chromatogr.*, **27**, 368(1967).
(10) J. Nibler, personal communication.
(11) F. Basolo and R. Johnson, "Coordination Chemistry," W. A. Benjamin, New York, N. Y., 1964, p. 125.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 2, 1969, from the *Departments of Pharmaceutical Chemistry and Pharmaceutical Sciences, School of Pharmacy, Oregon State University, Corvallis, OR 97331*

Accepted for publication January 19, 1970.

* To whom all correspondence should be addressed.

† Present address: School of Pharmacy, University of California Medical Center, San Francisco, CA 94122

Spectrophotometric Determination of Chlorpromazine in Pharmaceutical Dosage Forms

B. S. R. MURTY and R. M. BAXTER

Abstract □ A rapid and convenient spectrophotometric method for the determination of chlorpromazine hydrochloride and its unit dosage forms is described. Extensive separation and extraction of the active ingredient are not required. The microsensitive color response (λ_{max}, 520 mμ) with Van Urk's reagent is the basis of the analytical technique. The results are reproducible.

Keyphrases □ Chlorpromazine dosage forms—analysis □ Colorimetric analysis—spectrophotometry □ Van Urk's reagent—color formation

Chlorpromazine hydrochloride (CPZH), a phenothiazine derivative, is a widely used psychopharmacological agent. Chlorpromazine hydrochloride and some of its unit dosage forms are official in BP 1968 (1), USP XVII (2), and Ph.I. (3). A number of gravimetric, titrimetric, opticometric, electrometric, and chromatographic methods for the quantitative determination of phenothiazines have been reported in the literature, each one claiming individual advantages. These have been reviewed by Blazek (4), Blazek *et al.* (5), and Gyenes (6). Blake and Agarwal (7) recently reported a photometric titration of phenothiazines with ceric sulfate. The current pharmacopeias recognized nonaqueous titrimetry for determining the drug, while the unit dosage forms containing the drug—*viz.*, tablets, injections, *etc.*, are determined by methods based on the UV absorption properties of the phenothiazine base. However, these methods involve a series of extractions of the active ingredient from the unit dosage forms.

The authors have observed that a color ($\lambda_{max.}$, 520 mμ) results when CPZH is treated with Van Urk's reagent. The color-producing reaction with phenothiazines has not been previously reported in the literature. This investigation is primarily directed toward the evaluation of the observed novel reaction as a quantitative measure of chlorpromazine in its various dosage forms.

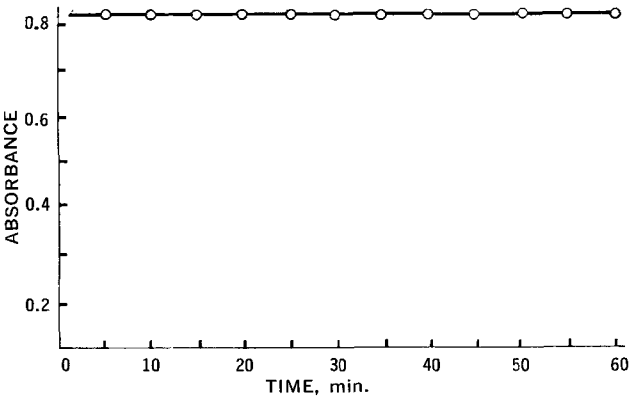


Figure 1—*Effect of time on the stability of color with Van Urk's reagent and CPZH.*

EXPERIMENTAL

Instrumentation—Beckman DU spectrophotometer (1-cm. cell) was used.

Materials—Van Urk's reagent, BP 1968 (8), was used. Chlorpromazine hydrochloride and the various dosage forms were obtained from commercial sources. All reagents were analytical grade. Glass-distilled water was used throughout this work.

Standard Reference Solution—Chlorpromazine hydrochloride (50 mg.), previously dried, in distilled water (250 ml.) was used.

Sample Preparation—*Tablets*—Twenty tablets were weighed and reduced to a fine powder. An accurately weighed portion of the powder, equivalent to 50 mg. of the drug, was transferred to a 250-ml. volumetric flask. The flask was shaken thoroughly for 10–15 min. after adding 100 ml. of water and was made to volume. The contents of the flask were filtered.

Injections—An equivalent volume, representing 50 mg. of the drug, was measured and diluted with water to 250 ml.

Suppositories—Suppositories representing 100 mg. of the drug were placed in a 500-ml. volumetric flask and melted by heating on a

Baltimore, Md., 1963, sec. II, inside front cover, and p. 85.
(2) S. Locket, "Clinical Toxicology," C. V. Mosby, St. Louis, Mo., 1957, p. 505.
(3) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 48.
(4) Y. D. Mogilyanskii, *Tr. Kom., Anal. Khim., Akad. Nauk SSSR*, **13**, 418(1963); through *Chem. Abstr.*, **60**, 1901d(1964).
(5) F. Feigl, "Spot Tests in Organic Analysis," 7th ed., Elsevier, New York, N. Y., 1966, p. 297.
(6) A. J. Glazko, L. M. Wolf, and W. A. Dill, *Arch. Biochem.*, **23**, 411(1949).
(7) K. Berei and L. Vasaros, *J. Chromatogr.*, **26**, 301(1967).
(8) K. Randerath, "Thin Layer Chromatography," Academic, New York, N. Y., 1963, p. 179.
(9) L. Frishbein, *J. Chromatogr.*, **27**, 368(1967).
(10) J. Nibler, personal communication.
(11) F. Basolo and R. Johnson, "Coordination Chemistry," W. A. Benjamin, New York, N. Y., 1964, p. 125.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 2, 1969, from the *Departments of Pharmaceutical Chemistry and Pharmaceutical Sciences, School of Pharmacy, Oregon State University, Corvallis, OR 97331*

Accepted for publication January 19, 1970.

* To whom all correspondence should be addressed.

† Present address: School of Pharmacy, University of California Medical Center, San Francisco, CA 94122

Spectrophotometric Determination of Chlorpromazine in Pharmaceutical Dosage Forms

B. S. R. MURTY and R. M. BAXTER

Abstract □ A rapid and convenient spectrophotometric method for the determination of chlorpromazine hydrochloride and its unit dosage forms is described. Extensive separation and extraction of the active ingredient are not required. The microsensitive color response (λ_{max}, 520 mμ) with Van Urk's reagent is the basis of the analytical technique. The results are reproducible.

Keyphrases □ Chlorpromazine dosage forms—analysis □ Colorimetric analysis—spectrophotometry □ Van Urk's reagent—color formation

Chlorpromazine hydrochloride (CPZH), a phenothiazine derivative, is a widely used psychopharmacological agent. Chlorpromazine hydrochloride and some of its unit dosage forms are official in BP 1968 (1), USP XVII (2), and Ph.I. (3). A number of gravimetric, titrimetric, opticometric, electrometric, and chromatographic methods for the quantitative determination of phenothiazines have been reported in the literature, each one claiming individual advantages. These have been reviewed by Blazek (4), Blazek *et al.* (5), and Gyenes (6). Blake and Agarwal (7) recently reported a photometric titration of phenothiazines with ceric sulfate. The current pharmacopeias recognized nonaqueous titrimetry for determining the drug, while the unit dosage forms containing the drug—*viz.*, tablets, injections, *etc.*, are determined by methods based on the UV absorption properties of the phenothiazine base. However, these methods involve a series of extractions of the active ingredient from the unit dosage forms.

The authors have observed that a color ($\lambda_{max.}$, 520 mμ) results when CPZH is treated with Van Urk's reagent. The color-producing reaction with phenothiazines has not been previously reported in the literature. This investigation is primarily directed toward the evaluation of the observed novel reaction as a quantitative measure of chlorpromazine in its various dosage forms.

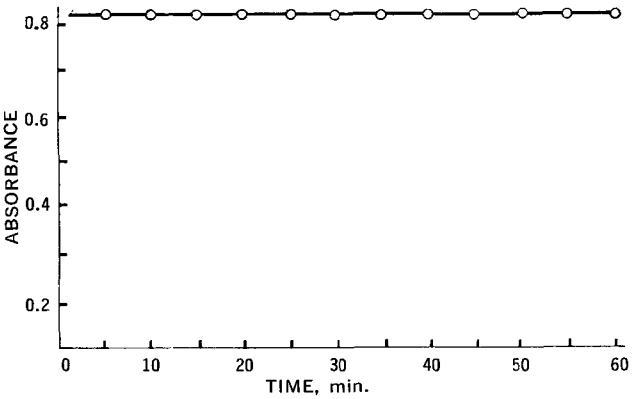


Figure 1—*Effect of time on the stability of color with Van Urk's reagent and CPZH.*

EXPERIMENTAL

Instrumentation—Beckman DU spectrophotometer (1-cm. cell) was used.

Materials—Van Urk's reagent, BP 1968 (8), was used. Chlorpromazine hydrochloride and the various dosage forms were obtained from commercial sources. All reagents were analytical grade. Glass-distilled water was used throughout this work.

Standard Reference Solution—Chlorpromazine hydrochloride (50 mg.), previously dried, in distilled water (250 ml.) was used.

Sample Preparation—*Tablets*—Twenty tablets were weighed and reduced to a fine powder. An accurately weighed portion of the powder, equivalent to 50 mg. of the drug, was transferred to a 250-ml. volumetric flask. The flask was shaken thoroughly for 10–15 min. after adding 100 ml. of water and was made to volume. The contents of the flask were filtered.

Injections—An equivalent volume, representing 50 mg. of the drug, was measured and diluted with water to 250 ml.

Suppositories—Suppositories representing 100 mg. of the drug were placed in a 500-ml. volumetric flask and melted by heating on a

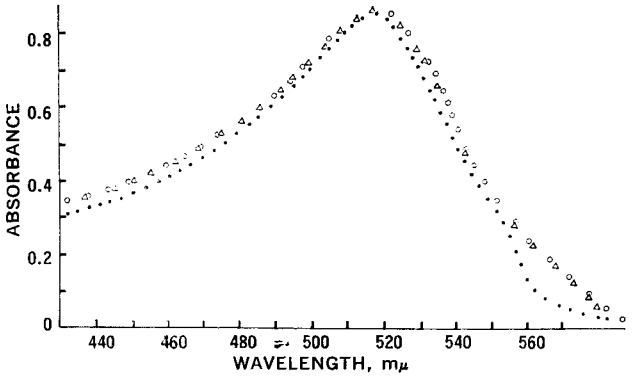


Figure 2—*Effect of water-soluble inert diluents on the absorption maxima. Key: . . ., CPZH and lactose and sucrose; ○, CPZH and acacia or tragacanth; and △, CPZH.*

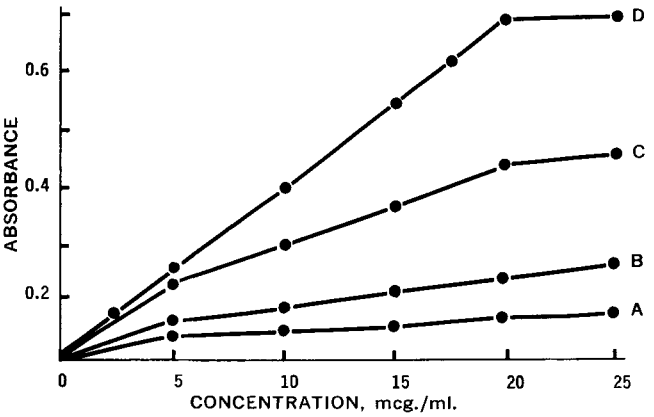


Figure 3—*Effect of volume of Van Urk's reagent on absorbance. Key: volume of reagent: A = 1 ml.; B = 2 ml.; C = 3 ml.; and D = 4 ml.*

steam bath. Hot water (100 ml.) was added; the solution was shaken and finally made to volume with water. Following filtration, the filtrate was used for the assay.

Syrup—A volume of syrup representing 50 mg. of the drug was diluted with water to 250 ml.

Method—A 0.1–0.5-ml. aliquot of the sample solution, prepared as previously described, was treated with 4 ml. of Van Urk's reagent, shaken, and made to a known volume with water. The intensities of the colors developed by the sample solution and by the chlorpromazine standard, treated simultaneously with the reagent, were measured at 520 mμ against the reagent blank.

RESULTS AND DISCUSSION

The color produced in the reaction is stable for at least 60 min. (Fig. 1); the presence of 0.5 mg. of the commonly used inert diluents —acacia, tragacanth, lactose, and sucrose—did not interfere (Fig. 2). The most suitable slope of the calibration curve and the maximum color intensity were obtained with the use of 4 ml. of the reagent with 0.1–1.0 ml. of the standard solution in a total volume of 10 ml. (Fig. 3). For a constant volume of Van Urk's reagent, an increase in the concentration of CPZH up to 20 mcg./ml. produced an increase in color intensity. Color generation under standard conditions obeyed Beer's law between 5–20 mcg./ml. (0.165–0.7 absorbance, respectively, at 520 mμ) (Fig. 3). The mean absorbance of 10 replicates from one solution to another was noted. The standard deviation was found to be ± 0.005.

The data in Table I indicate that quantitative recoveries were obtained for the unit dosage forms and that the results were in good agreement with the official methods. The reported procedure has the advantage that it does not require the preliminary extraction of the active ingredient. The effect of decomposition products on the recovery of the parent compound has not been evaluated. The procedure allows for a simple, rapid, and accurate determination of small quantities of chlorpromazine.

Table I—Analysis of Unit Dosages Containing CPZH

Dosage Form[a]	Labeled Amount per Unit, mg.	Method BP 1968, % R[b]	Method USP XVII, % R	Proposed Method, % R
Tablet	50.00	101.00[c]	102.00[c]	102.00 ± 0.4[d]
Injection	27.90	99.30	102.10	102.10 ± 0.4
Syrup	5.58	98.60	98.60	98.60 ± 1.0
Suppositories	111.60	Not official	98.80	98.60 ± 0.6
Control CPZH	50.00	100.40	100.40	100.40 ± 0.2

[a] Dosage forms analyzed for two different labeled amounts; one only is given. [b] % R represents the percentage recovery. [c] Average of at least three determinations by official method. [d] Standard deviation based on at least four determinations.

REFERENCES

(1) "British Pharmacopoeia," The Pharmaceutical Press, W. C. 1, London, England, 1968, pp. 209–212.

(2) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 128–130.

(3) "Pharmacopoeia Internationalis," 2nd ed., World Health Organization, Geneva, Switzerland, 1967, pp. 122–123.

(4) J. Blazek, *Pharmazie*, **22**, 129(1967).

(5) J. Blazek, V. Spinkova, and E. Steijakal, *An. Farm. Hosp.*, **10**, 7(1967).

(6) I. Gyenes, "Titration in Non-aqueous Media," D. Van Nostrand, Princeton, N. J., 1967, pp. 327–328, 356–361.

(7) M. I. Blake and S. P. Agarwal, *J. Pharm. Sci.*, **58**, 1011(1969).

(8) "British Pharmacopoeia," The Pharmaceutical Press, W. C. 1, London, England, 1968, p. 1121.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 5, 1969, from the *Faculty of Pharmacy, University of Toronto, Toronto 181, Ontario, Canada.*

Accepted for publication February 17, 1970.

Design and Evaluation of a Pressure Attachment for a Rotational Rheometer

KAKUBHAI R. M. VORA*, LARRY L. AUGSBURGER, and RALPH F. SHANGRAW

Abstract □ The problems associated with the measurement of the rheological properties of pressurized aerosol concentrates consisting of emulsions and/or micellar solutions are discussed. A pressure attachment for a commercially available rheometer is described, and data are presented for simple soap formulations. Pressurized concentrates exhibited pseudoplastic properties which could not always be correlated with the flow properties of resultant foams.

Keyphrases □ Rheometer, rotational—aerosol rheological properties □ Pressure attachment, rheometer—design, evaluation □ Diagram—pressure attachment, rheometer □ Viscosity-increasing additives—aerosol foam effect

A major problem, which has plagued the formulators of "aerosol" foams and other products utilizing high-consistency concentrates, has been the lack of a suitable and convenient means to evaluate their rheological properties. Most pharmaceutical and cosmetic foams utilize a pressurized concentrate in which the insoluble propellant is emulsified or micellized into the internal phase of an oil-in-water system by means of a suitable surfactant. Evaluation of the flow properties of the concentrate prior to the addition of propellant is of limited usefulness, because it does not reflect the influence of the internal phase. The fact that the concentrate systems are almost always non-Newtonian complicates the situation even further. The rheology of these pressurized concentrates plays an important role in the flow of material in the dip tube and valve assembly and influences drainage from the inside of the can during product use.

Although the importance of determining rheological characteristics of the non-Newtonian concentrates under pressure has been well recognized, very few reports are available in the literature. A preliminary report on the pressure–viscosity–time factors in dispensing of liquid aerosols was published by Mina in 1959 (1). The author measured the delivery rate of dimethyl silicone oils using a Precision Valve (3 × 0.040/0.080) (Precision Valve Corp., Yonkers, N. Y.) at different pressures. By the use of suitable blending of different hydrocarbons and halocarbon propellants, the author was able to achieve a range of pressure from 10 to 100 psig. inside the sealed container. Viscosity determinations were made with an Ultra-Viscoson viscometer (Bendix Research Corp., Towson, Md.), utilizing a special twin-top adapter on the container to accommodate the ultrasonic probe as well as a normal valve. The author concluded that viscosity is significantly lowered under pressure as the pressure is increased, and this phenomenon is reversible upon removal of the added pressure.

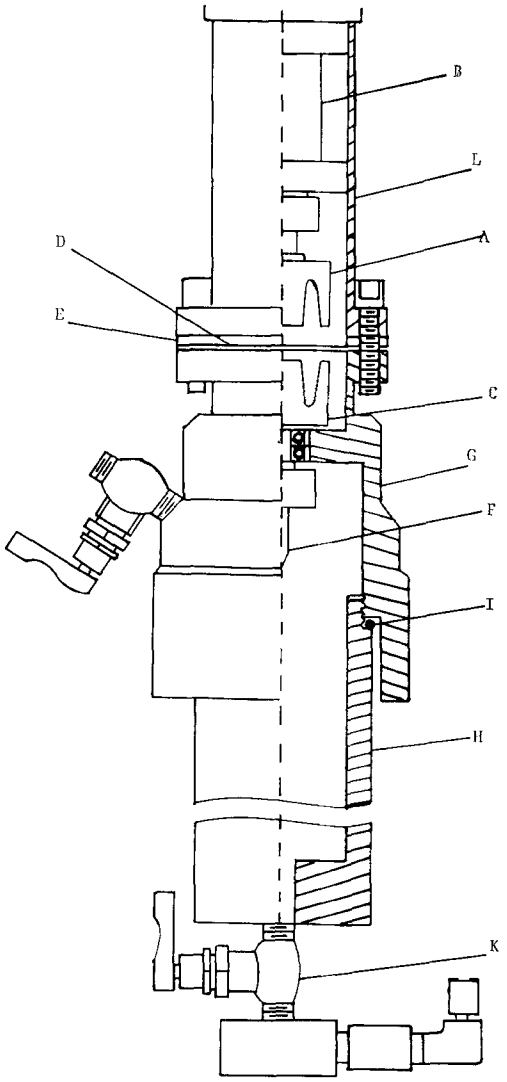


Figure 1—*A schematic diagram of the pressure assembly for the Rotovisco viscometer.*

Cohen *et al.* have described the use of the Brookefield viscometer (Brookefield Engineering Laboratory, Stoughton, Mass.) for measuring viscosity at low rates of shear, supplemented by use of the Severs extrusion rheometer model A-100 (Castor Laboratory Equipment Co., Pittsburgh, Pa.) for high shear measure-

Figure 2—*Assembled pressure attachment.*

ments (2, 3). A series of aerosol valves were calibrated in the Severs extrusion rheometer for pressures varying from 10 to 100 psig., and the rheological characterization method was demonstrated by the use of polymer "solutions" as well as a group of four commercial pressure-packaged toothpastes.

A qualitative method of evaluating pressurized emulsion systems was reported by Strickland (4). He compared different emulsions under pressure by measuring the time required for a 5-mm. diameter glass bead to fall through the emulsion when the transparent bottle was inverted. Sanders reported a method for evaluating the approximate viscosity of pressurized emulsions by inverting bottles slowly and noting the flow characteristics (5).

Augsburger made use of the Rotovisco viscometer with a modified pressure chamber assembly to accommodate the emulsion concentrate under pressure (6). Although Augsburger concluded that further modifications were necessary in the design of the pressure assembly, he was able to carry out rheological measurements of soap concentrates under pressure. He found that at a 5% (w/w) concentration of soap, propellant was solubilized in the soap solution, giving lower viscosity values; at higher concentrations of soap the propellant was emulsified, giving higher viscosity values (6).

DESIGN AND CONSTRUCTION OF PRESSURIZED RHEOMETER

Rotational viscometers offer a means of transferring the standard model of Newtonian flow in a plane to a closed continuous system in which the liquid is trapped between the surfaces of a cup and a bob. By rotating either surface at a fixed rate and measuring the viscous drag transferred through the liquid (or on the rotating member itself), the two necessary parameters of viscosity—rate of shear, which is proportional to r.p.m., and shear stress, which is proportional to viscous drag—can be measured. Rotational viscometers are particularly useful in measuring non-Newtonian systems, because rates of shear can be varied and relationships between rate of shear and shear stress within a particular system can be established. These are most often described as flow curves.

The Haake Rotovisco rotational viscometer (Polyscience Corp., Evanston, Ill.), was employed in this study to determine the viscosity of the liquid concentrates as well as the rheological properties of the foams produced by the pressurized emulsion systems. The measuring head of the Rotovisco viscometer contains two readily interchangeable torsion springs, one of 50-g. cm. capacity for low-viscosity fluids and the other of 500-g. cm. capacity for medium-viscosity fluids. The use of the 50 head allows for expansion of lower stress readings over the entire dial range, giving greater accuracy to low-viscosity readings.

The pressure assembly previously utilized by Augsburger in his preliminary work on pressure rheometry was used in the current study, with some modifications. This assembly involves the use of a "driving" magnet, which is attached to the spindle of the viscometer in the same fashion that a bob is normally attached. On rotation of the spindle, this magnet drives a second magnet situated across a seal within a pressure chamber. A bob attached to the driven magnet inside the sealed chamber is held concentrically in the cup by means of a pair of ball bearings.

A schematic diagram of the pressure assembly is presented in Fig. 1. A magnet (A), attached to the spindle of the Rotovisco measuring head (B), drives a magnet (C) across the pressure seal plate (D). A spacer (E) assures that the driving magnet will not contact the pressure plate. Previous experience had shown that, under pressure, the pressure plate tends to become convex in the direction of the driving magnet, and that the clearance originally allowed for this magnet was insufficient. The driven magnet, tapped onto a standard Rotovisco spindle (F), rides on two Fafnir No. F4DD bearings (The Fafnir Bearing Co., New Britain, Conn.). The pressure chamber housing (G) is threaded to accommodate the sample cup (H). The dimensions of the cup and its relationship to the spindle are identical to that of the standard Rotovisco assembly. Consequently, all instrument constants remain the same for this attachment. An O-ring (I), at the base of the threads of the cup, seats itself against a ledge cut into the inner surface of the housing. By virtue of this O-ring, only minimal hand-tightening of the cup is required to make a pressuretight seal. Hoke No. 450 toggle valves (Hoke, Inc., Cresskill, N. J.) are tapped into both the cup and the housing. The valve (K) in the cup was used to fill the chamber with the pressurized emulsions using a transfer actuator. The sample was removed from the same valve, because this minimizes internal foaming into the bearings when the pressure is released. The sleeve (L) is designed to fit over the lower portion of the Rotovisco measuring head where it is fastened with two thumb screws. While the housing was machined from brass, the cup was machined from plastic. A photograph of the pressure assembly is shown in Fig. 2.

Before proceeding further, it might be appropriate to summarize the difficulties encountered in the construction and operation of the pressure rheometer as follows:

(1) *Sealing of pressure chamber:* difficulty was solved by means of a Teflon spacer seal next to the brass plate and an O-ring at the base of the threads of the cup.

(2) *Concentricity of bob in the cup:* two bearings instead of one bearing were used to support the drive spindle more rigidly.

(3) *Introduction of samples into the cup:* as pressurized soap concentrates were transferred, they would foam into the cup. The foam

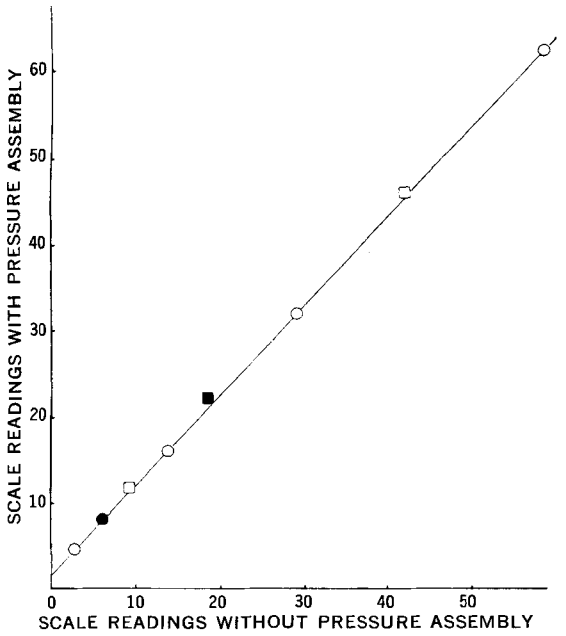


Figure 3—*Relationship of shearing stress with and without pressure assembly, obtained with various standard viscosity liquids. Key: shear rate, sec.*$^{-1}$*: ○, 9.2; ●, 18.4; □, 27.6; and ■, 55.2.*

would occupy the gap between the cup and the bob, giving abnormally high readings. This difficulty was solved as follows: (*a*) prepressurizing the cup before loading the sample; and (*b*) machining the cup out of clear plastic so that the sample could be observed and determinations disregarded if foaming occurred.

(4) *Slippage on the surface of the bob:* the use of the MVII-P profiled bob (with serrated surface) minimized the slippage encountered with high-viscosity systems.

A major problem was associated with the small but ever present additional frictional drag on the measuring head produced by the magnetic clutch and bearings. Standard viscosity liquids were measured and shear stress values at various rates of shear were determined, both with and without the pressure assembly. A relationship was found to exist between the stress values with and without the pressure assembly. As can be seen from Fig. 3, this relationship proved to be linear. The slope of the straight line is 0.95, which means that actual stress values were 95% of those observed with the pressure assembly.

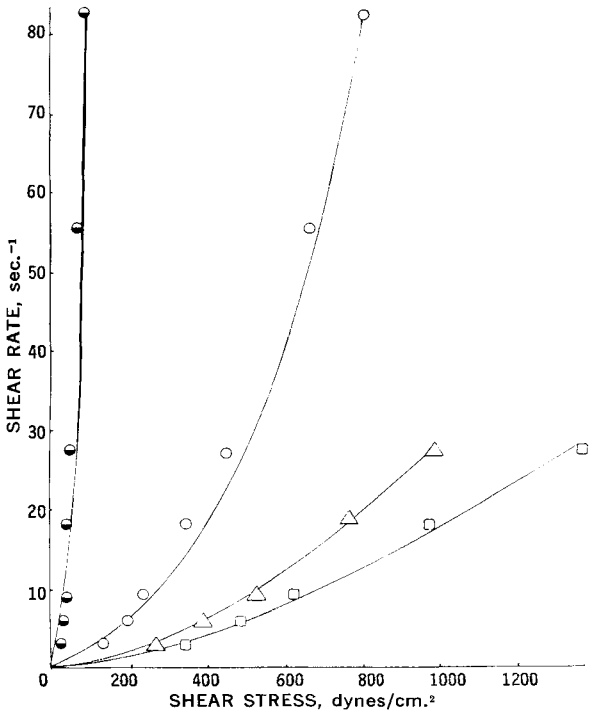


Figure 4—*Pressurized emulsion flow curves—the effect of methylcellulose on the rheology of formulations containing 10% (w/w) soap. Key: ◒, control soap formulation; ○, 1.0% methylcellulose; △, 1.5% methylcellulose; and □, 2.0% methylcellulose.*

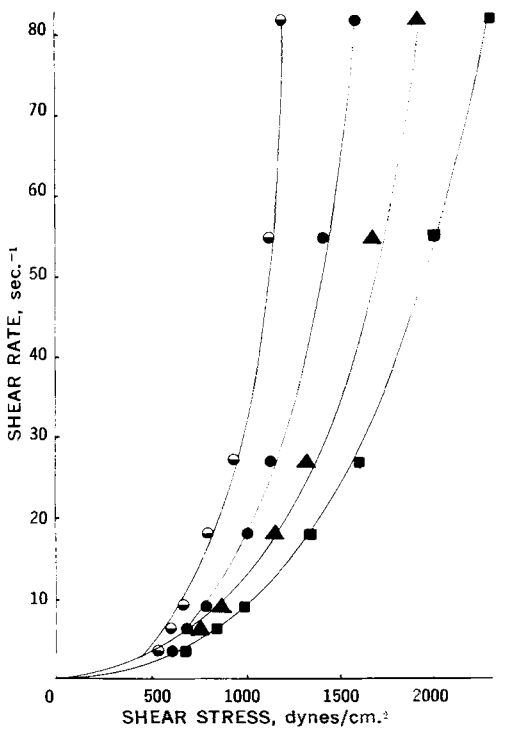


Figure 5—*Pressurized foam flow curves—the effect of methylcellulose on the rheology of high consistency soap foams extruded from formulations containing 10% (w/w) soap. Key: ◒, control soap foam; ●, 1.0% methylcellulose; ▲, 1.5% methylcellulose; and ■, 2.0% methylcellulose.*

EXPERIMENTAL

Pressurized Packaging—Soap concentrates were weighed into "6-ounce" standard coated cans[1] which had been previously purged with a few drops of propellant, and Precision 0.020/0.080 valves (Precision Valve Corp., Yonkers, N. Y.) were crimped into place. Dip tubes were omitted to facilitate the filling of test vessels. The propellant was filled into the test containers by weight. The finished products were shaken vigorously and placed in a 50° water bath for 20 min. to test for leakage and can distortion. After removal from the water bath, the cans were allowed to remain at room temperature for 3 days prior to testing; all tests were completed within 2 weeks of preparation to minimize any possible aging effects.

Foam Rheology—Foam rheology was carried out after the manner of Richman (7) using the Haake Rotovisco viscometer and the MVII-P profiled bob and cup system. After shaking the pressurized containers for 15 sec., the foam was introduced directly into the cup through a 12.7-cm. (5-in.) extension of 0.64-cm. (0.25-in.) polyethylene tubing attached to Precision foam actuator SP-115. The cup was filled slowly from the bottom up, and shearing was begun 1 min. after the cup was filled. Since dip tubes were not employed, pressurized containers were inverted during actuation. All measurements were made at 28 ± 0.5°.

Basic Soap Formulation—Richman reported rheological measurements of soap solutions at atmospheric pressure and observed the dilitant nature of the flow curves at high-shear rates in the region of about 500 sec.$^{-1}$ (7). An experiment was designed to measure the

[1] Spra-tainer, Crown Can Co., Philadelphia, Pa.

Table I—Relationship of Shear Rate to Shear Stress in a Basic Soap Formulation Consisting of 10% (w/w) Soap and 10% (w/w) Propellant Blend 12/114 (57:43)

Shear Rate, sec.$^{-1}$	Fine Scale Value (Viscometer Reading) Sample 1	Sample 2	Mean	Normal Scale Value[a]	Scale Value × Pressure Factor[b]	Stress Value, dynes/cm.^{2} × 10^{-3}	Foam Stress Value,[c] dynes/cm.^{2} × 10^{-3}
3.0	1.2	1.1	1.15	0.29	0.28	0.011	0.57
6.1	1.6	1.7	1.65	0.41	0.39	0.015	0.61
9.2	1.9	1.9	1.90	0.48	0.46	0.018	0.68
18.4	2.5	2.6	2.55	0.65	0.61	0.024	0.79
27.6	3.9	4.0	3.95	0.99	0.94	0.037	0.92
55.2	5.4	5.6	5.50	1.37	1.30	0.051	1.08
82.8	8.2	8.4	8.30	2.07	1.97	0.077	1.17

[a] Normal scale value is one-fourth of fine scale reading. [b] Pressure factor = 0.95, from Fig. 3 (a factor to convert scale values with pressure assembly into equivalent values without pressure assembly). [c] Values for the foam extruded from the same basic formulation presented in this table for comparison and reference in the following sections.

shear stress values under pressure of the following basic soap formulation:

	(w/w)	
Soap concentrate	10%	
Stearic acid ..... 5 parts		
Coconut oil fatty acids ..... 3 parts		
Triethanolamine ..... 5 parts		..... 90%
Additive	(−%)	
Distilled water *q.s. ad.*	100%	
Propellant blend 12/114 (57:43)		..... 10%

No additives were employed in the control formulation. Samples were prepared in duplicate, and the rheological measurements were carried out at 28 ± 0.5°. Shear stress values obtained at varying rates of shear are presented in Table I. The flow curve appears in Fig. 4 as the control soap formulation.

Effect of Additives—Once the basic rheology of a simple pressurized concentrate was determined, the effects of viscosity-increasing additives were studied. Methylcellulose (4000 cps.) was selected for its known property of increasing the viscosity of aqueous soap solutions; three different concentrations (1.0, 1.5, and 2.0% w/w) were employed. The flow curves obtained from these systems are presented in Fig. 4. To compare the effect of this additive on the rheology of foams produced from these same formulations, rheological measurements were carried out on the corresponding foams and the results are shown in Fig. 5. It becomes obvious upon examination of these data that the sequence of flow curves remains the same in both the concentrate and foam. In a subsequent experiment, soap solutions were prepared containing different additives known to increase the consistency of aerosol foams. While glycerin was selected for its Newtonian property, hydroxyethylcellulose and a commercial hydrocolloid[2] were selected for their non-Newtonian and pseudoplastic properties in aqueous solutions. Flow curves of the emulsion concentrates with various additives are presented in Fig. 6, and flow curves of the corresponding resultant foams are shown in Fig. 7. The sequence of flow curves is not the same in both figures. The pressurized emulsion containing the hydrocolloid gave higher shear stress values than the one containing hydroxyethylcellulose, whereas this order was reversed in the case of pressurized foams. Thus, it can be noted that the agents that affect bulk viscosity

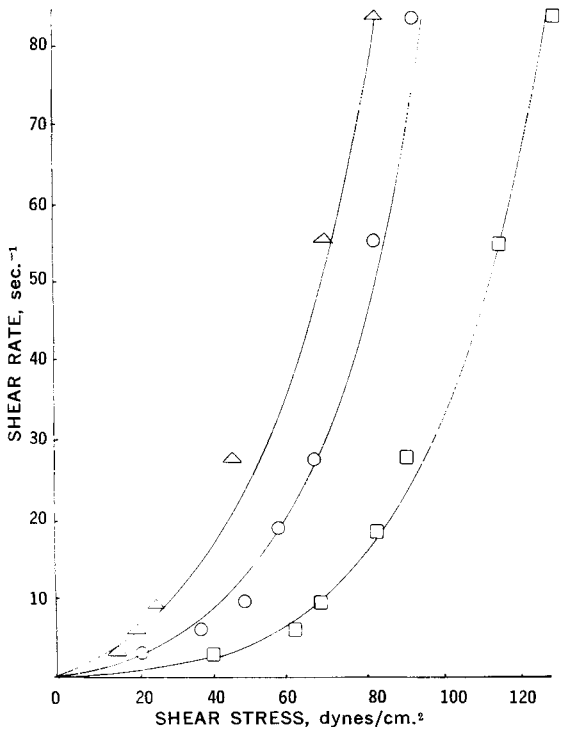


Figure 6—*Pressurized emulsion flow curves—the effect of additives on formulations containing 10% (w/w) soap. Key: △, 0.125% hydroxyethylcellulose; ○, 0.125% commercial hydrocolloid; and □, 40% glycerin.*

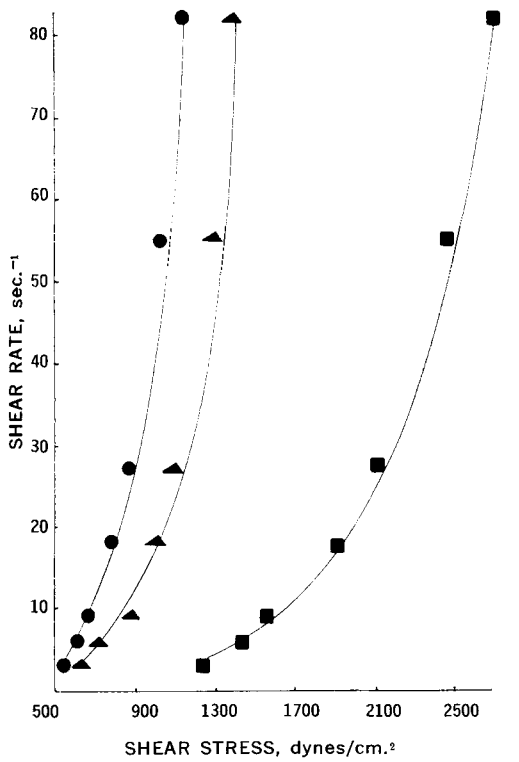


Figure 7—*Pressurized foam flow curves—the effect of additives on formulations containing 10% (w/w) soap. Key: ●, 0.125% commercial hydrocolloid; ▲, 0.125% hydroxyethylcellulose; and ■, 40% glycerin.*

[2] Polyhall-295^{R1}, a water-soluble nonionic polyacrylamide hydrocolloid of high molecular weight, Stein, Hall and Co., Inc., New York, N. Y.

of a concentrate may not affect the viscosity of the resulting foam in exactly the same manner. Surface viscosity and surface area are dominant factors in the viscosity of the pressurized foam.

The pressure rheometer can be a useful tool for studying aerosol concentrates containing propellants. Since foam-producing systems are generally emulsions or micellized solutions, determining the rheological properties of concentrates before pressurization is of limited value. Commercial pressure rheometers have become available since this research was begun. However, the magnetic clutch unit (introduced by the Polyscience Corp., Evanston, Ill.) for use with the Rotovisco viscometer requires a sample size of approximately 1 l., which makes it unattractive for product-development work. The Viscoclav MA attachment to the Contraves rheometer (Olkon Corp., Stanford, Conn.), which also utilizes a magnetically driven bob, employs a much smaller (10 ml.) sample.

It is important that cosmetic and pharmaceutical aerosol chemists investigate their pressurized systems using techniques such as those described in this paper if meaningful rheological data are to be generated.

SUMMARY AND CONCLUSIONS

The design of a device for modifying a commercially available rotational viscometer to measure the rheological properties of fluids under pressure is described.

Data presented for simple aerosol concentrate soap formulations revealed pseudoplastic properties which could not always be correlated with the flow properties of the resultant foams. This lack of correlation may be attributable to the role that surface viscosity (rather than bulk viscosity) plays in determining foam consistency.

REFERENCES

(1) F. A. Mina, *Soap Chem. Spec.*, **35**, 121(1959).

(2) L. Cohen, R. J. Meyer, and J. S. Wolff, *ibid.*, **35**, 71(1959).

(3) *Ibid.*, **35**, 77(1959).

(4) W. A. Strickland, Jr., presented to the Industrial Pharmacy Section, APhA Academy of Pharmaceutical Sciences, Dallas meeting, 1966.

(5) P. A. Sanders, *J. Soc. Cosmet. Chem.*, **17**, 801(1966).

(6) L. L. Augsburger, Ph.D. thesis, University of Maryland, Baltimore, Md., 1967.

(7) M. D. Richman, Ph.D. thesis, University of Maryland, Baltimore, Md., 1966.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 16, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Maryland, Baltimore, MD 21201*

Accepted for publication February 26, 1970.

Abstracted from a thesis submitted by K. R. M. Vora to the Graduate School, University of Maryland, Baltimore, MD 21201, in partial fulfillment of the Doctor of Philosophy degree requirements.

* Present address: Pharmaceutical Research and Development Department, Chas. Pfizer & Co., Inc., 630 Flushing Ave., Brooklyn, NY 11206

Titanium Dioxide Lakes I: Prepared from Certified Water-Soluble Dyes and Employed in Color-Coating Tablets

SAUL S. KORNBLUM and BENITO LOPEZ*

Abstract □ Titanium dioxide, ranging in particle size from 0.005 to 0.040 μ, has been investigated as an adsorbate in the manufacture of lake dyes from certified water-soluble dyes. The manufacturing technique for the titanium dioxide lakes and the pharmaceutical application in color-coating tablets have been demonstrated and discussed in conjunction with automatic spray coating.

Keyphrases □ Titanium dioxide lakes—preparation □ Lakes, water-soluble dyes—titanium dioxide adsorbate □ Spray drying—titanium dioxide lake preparation □ Color coating, tablets—titanium dioxide lakes

Color lakes, prepared in liquid suspension, have been extensively employed pharmaceutically for coloring sugar-coated tablets. There are advantages for using the color lakes rather than the water-soluble dyes, which are well known to the coating expert. These advantages consist of: significant time reduction for the color-coating operation, greater color stability, and greater color uniformity for consecutive batches (1). The majority of color lakes are manufactured by employing alumina as the adsorbate material interacted with various dyes. Commercial lakes are available as micronized powders that render better color dispersion in liquid suspensions than if not so treated.

Color-concentrate suspensions prepared from color lakes are commercially available and are extensively used by the pharmaceutical industry for coloring sugar-coated tablets.

The purpose of this study was to determine the acceptability of titanium dioxide as an adsorbate material for color lake manufacture from water-soluble dyes. The titanium dioxide selected is referred to as "fumed" and possesses a submicron particle size The ultimate goal of this project was to prepare extemporaneously color lakes for tablet coating.

EXPERIMENTAL

Materials—Fumed titanium dioxide[1] was selected for study as an adsorbate in the manufacture of color lakes because it possesses a suitable particle-size range from 0.005 to 0.040 μ. Titanium dioxide USP differs significantly from the fumed material with

[1] Titanium Dioxide P-25, DeGussa, Inc., Kearny, N. J.; Cab-O-Ti, Cabot Corp., Boston, Mass.

of a concentrate may not affect the viscosity of the resulting foam in exactly the same manner. Surface viscosity and surface area are dominant factors in the viscosity of the pressurized foam.

The pressure rheometer can be a useful tool for studying aerosol concentrates containing propellants. Since foam-producing systems are generally emulsions or micellized solutions, determining the rheological properties of concentrates before pressurization is of limited value. Commercial pressure rheometers have become available since this research was begun. However, the magnetic clutch unit (introduced by the Polyscience Corp., Evanston, Ill.) for use with the Rotovisco viscometer requires a sample size of approximately 1 l., which makes it unattractive for product-development work. The Viscoclav MA attachment to the Contraves rheometer (Olkon Corp., Stanford, Conn.), which also utilizes a magnetically driven bob, employs a much smaller (10 ml.) sample.

It is important that cosmetic and pharmaceutical aerosol chemists investigate their pressurized systems using techniques such as those described in this paper if meaningful rheological data are to be generated.

SUMMARY AND CONCLUSIONS

The design of a device for modifying a commercially available rotational viscometer to measure the rheological properties of fluids under pressure is described.

Data presented for simple aerosol concentrate soap formulations revealed pseudoplastic properties which could not always be correlated with the flow properties of the resultant foams. This lack of correlation may be attributable to the role that surface viscosity (rather than bulk viscosity) plays in determining foam consistency.

REFERENCES

(1) F. A. Mina, *Soap Chem. Spec.*, **35**, 121(1959).

(2) L. Cohen, R. J. Meyer, and J. S. Wolff, *ibid.*, **35**, 71(1959).

(3) *Ibid.*, **35**, 77(1959).

(4) W. A. Strickland, Jr., presented to the Industrial Pharmacy Section, APhA Academy of Pharmaceutical Sciences, Dallas meeting, 1966.

(5) P. A. Sanders, *J. Soc. Cosmet. Chem.*, **17**, 801(1966).

(6) L. L. Augsburger, Ph.D. thesis, University of Maryland, Baltimore, Md., 1967.

(7) M. D. Richman, Ph.D. thesis, University of Maryland, Baltimore, Md., 1966.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 16, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Maryland, Baltimore, MD 21201*

Accepted for publication February 26, 1970.

Abstracted from a thesis submitted by K. R. M. Vora to the Graduate School, University of Maryland, Baltimore, MD 21201, in partial fulfillment of the Doctor of Philosophy degree requirements.

* Present address: Pharmaceutical Research and Development Department, Chas. Pfizer & Co., Inc., 630 Flushing Ave., Brooklyn, NY 11206

Titanium Dioxide Lakes I: Prepared from Certified Water-Soluble Dyes and Employed in Color-Coating Tablets

SAUL S. KORNBLUM and BENITO LOPEZ*

Abstract □ Titanium dioxide, ranging in particle size from 0.005 to 0.040 μ, has been investigated as an adsorbate in the manufacture of lake dyes from certified water-soluble dyes. The manufacturing technique for the titanium dioxide lakes and the pharmaceutical application in color-coating tablets have been demonstrated and discussed in conjunction with automatic spray coating.

Keyphrases □ Titanium dioxide lakes—preparation □ Lakes, water-soluble dyes—titanium dioxide adsorbate □ Spray drying—titanium dioxide lake preparation □ Color coating, tablets—titanium dioxide lakes

Color lakes, prepared in liquid suspension, have been extensively employed pharmaceutically for coloring sugar-coated tablets. There are advantages for using the color lakes rather than the water-soluble dyes, which are well known to the coating expert. These advantages consist of: significant time reduction for the color-coating operation, greater color stability, and greater color uniformity for consecutive batches (1). The majority of color lakes are manufactured by employing alumina as the adsorbate material interacted with various dyes. Commercial lakes are available as micronized powders that render better color dispersion in liquid suspensions than if not so treated.

Color-concentrate suspensions prepared from color lakes are commercially available and are extensively used by the pharmaceutical industry for coloring sugar-coated tablets.

The purpose of this study was to determine the acceptability of titanium dioxide as an adsorbate material for color lake manufacture from water-soluble dyes. The titanium dioxide selected is referred to as "fumed" and possesses a submicron particle size The ultimate goal of this project was to prepare extemporaneously color lakes for tablet coating.

EXPERIMENTAL

Materials—Fumed titanium dioxide[1] was selected for study as an adsorbate in the manufacture of color lakes because it possesses a suitable particle-size range from 0.005 to 0.040 μ. Titanium dioxide USP differs significantly from the fumed material with

[1] Titanium Dioxide P-25, DeGussa, Inc., Kearny, N. J.; Cab-O-Ti, Cabot Corp., Boston, Mass.

regard to the particle-size range and the quantity of soluble titanium salts. Commercial titanium dioxide USP, which is used as an opaquing agent with soluble and insoluble dyes in tablet coating, is unsuitable for this purpose (2). The imbalance of electrostatic charges on the surface and particle-size dimensions of the titanium dioxide USP causes agglomerates to form which prevent significant physical or chemical surface interaction with the dyes. The water-soluble dyes employed in this project were: F.D. & C. Red No. 3, F.D. & C. Blue No. 1, F.D. & C. Yellow No. 5, F.D. & C. Violet No. 1, and F.D. & C. Green No. 3. In addition, surface-active agents, *i.e.*, polyoxypropylenepolyoxyethylene condensates;[2] binding agents, *i.e.*, partially hydrolyzed polyvinylacetate;[3] and acidifying agents, *i.e.*, hydrochloric, acetic, tannic, and citric acids, were also employed in the manufacture of the color lakes.

Color Lakes Composition and Method of Manufacture—The following color lakes were prepared with modification of the composition as an indication of the versatility of this method for preparing color lakes. Numerous lakes have been prepared; however, only one example of each modification is described in the text. The component quantities indicated are representative of 1 kg. of finished color lake.

Type 1	Percent by Weight
Fumed titanium dioxide P-25	96
F.D. & C. Violet No. 1	4

The F.D. & C. Violet No. 1 was dissolved in 500 ml. of distilled water. The fumed titanium dioxide was dispersed in 2000 ml. of water and maintained in a state of agitation employing a Kady colloid mill[4] with a rotor of 12,000 r.p.m. The dye solution was added to the titanium slurry and milled for 30 min. The resultant color slurry was filter pressed and washed with distilled water to remove soluble titanium salts. The slurry was then vacuum dried and stored in suitable glass containers.

Type 2	Percent by Weight
Fumed titanium dioxide P-25	75
F.D. & C. Red No. 3	20
Surface-active agent (Pluronic F-68)	5

The F.D. & C. Red No. 3 was dissolved in 2 l. of distilled water. The fumed titanium dioxide was dispersed in 700 ml. of distilled water and maintained in a state of agitation by employing a Kady colloid mill. The dye solution was added to the titanium slurry and milled for 30 min. The surface-active agent (Pluronic F-68) in 1 l. of distilled water was added to the color slurry. The color slurry and 1% acetic acid–ethanolic solution was then simultaneously pumped in equal portions into a Nerco-Niro portable laboratory spray dryer[5] employing a Sigmamotor pump.[6] The heated chamber of the spray dryer was maintained at 175° inlet and 75° outlet temperatures. The color lake obtained was then vacuum dried for 6–8 hr. at 45° and stored in a suitable glass container.

Type 3	Percent by Weight
Fumed titanium dioxide P-25	89.5
F.D. & C. Blue No. 1	5.0
Surface-active agent (Pluronic F-68)	0.5
Partially hydrolyzed polyvinylacetate	5.0

The F.D. & C. Blue No. 1 was dissolved in 2 l. of distilled water. The fumed titanium dioxide was dispersed in 900 ml. of distilled water and maintained in a state of agitation by employing a Kady colloid mill. The dye solution was added to the titanium slurry and milled for 30 min. Then the partially hydrolyzed polyvinylacetate in 1 l. of hot distilled water was added to the color slurry. The surface-active agent (Pluronic F-68) was dissolved in 300 ml. of distilled water and was added with thorough agitation. The color slurry was then spray dried in the same manner as described in Type 2; however, the acetic acid–ethanolic solution was eliminated from the procedure.

Fumed alumina[7] was also employed in exactly the same manner as with Types 1, 2, and 3, which gave similar results as obtained with the fumed titanium dioxide.

Color-Coating Suspension—Fumed titanium dioxide lakes were employed to prepare coating suspensions that were applied to sized or smoothed tablets. As outlined in Method 2, extemporaneous preparation of the color lake with a sucrose syrup (71% w/w) was used to obtain a color suspension for pan-coating tablets. The composition of the color suspensions and two procedures for manufacture are outlined as Methods 1 and 2.

Method 1	Per 3 l.
Titanium dioxide color lake described as Type 2	60.0 g.
Polyvinylpyrrolidone	15.0 g.
Sucrose syrup 71% w/w to	3.0 l.

Sixty grams of color lake described as Type 2 was dispersed in 2.8 l. of sucrose syrup (71% w/w) in which 15 g. of polyvinylpyrrolidone was dissolved. The resulting suspension was milled, employing the Kady colloid mill for 30 min. The color suspension was brought to volume, and was then suitable for application to smooth-coated tablets.

Method 2	Per 10 kg.
Fumed titanium dioxide	0.100 kg.
F.D. & C. Violet No. 1	0.004 kg.
F.D. & C. Green No. 3	0.001 kg.
F.C. & C. Yellow No. 5	0.015 kg.
Titanium dioxide USP	0.100 kg.
Distilled water	2.000 kg.
Sucrose, granular	5.000 kg.
Sucrose syrup 71% w/w *q.s. ad.*	10.000 kg.

The F.D. & C. dyes were dissolved in 1 l. of distilled water. The fumed titanium dioxide was dispersed in 1 l. of sucrose syrup (71% w/w). The liquids were then placed in the Kady colloid mill and milled for 1 hr. Five kilograms of sucrose was added to the Kady mill vessel and milled until solution was obtained. The titanium dioxide USP was mechanically milled in 1 l. of distilled water and then was added to the color lake suspension. The resulting color suspension was brought to volume with sucrose syrup (71% w/w) and milled an additional 15 min.

The resulting color suspension was then applied to 25 kg. of smoothed tablets which were contained in a rotating coating pan. The tablets employed in this project had a 180-mg. core weight and 250-mg. smooth-coat weight. The tablet cores were 8 mm. in diameter and had a deep concave shape. The coating pan employed was the Manesty CP-2 which has a 91.44-cm. (36-in.) diameter. The coating pan was equipped with four baffles as described by Lachman (3). The color-coating suspension was sprayed, using an apparatus similar to that described by Steinberg (4). A DeVilbiss

Table I—Pertinent Operational Details of the Automatic Color-Coating Operation Employing the Titanium Color Lakes

Coating pan speed	26 r.p.m.
Air inlet temperature	55°, 150 c.f.m.
Air exhaust temperature	35°, 200 c.f.m.
Liquid nozzle (DeVilbiss)	AV 1115-D-S
Air nozzle (DeVilbiss)	491-D
Color-coating suspension temperature	55°
Liquid pressure	30 p.s.i.
Air pressure	20 p.s.i.
Spraying time	30 sec.
Dwell time	4 min.
Drying time	6.5 min.
Color coat weight	25–35 mg./tablet
Number of applications	8–12
Color coating time	100–120 min.

[2] Pluronic F-68, Wyandotte Chemical Corp., Wyandotte, Mich.
[3] E. I. du Pont de Nemours & Co., Wilmington, Del.
[4] Kinetic Dispersion Corp., Buffalo, N. Y.
[5] Nichols Engineering & Research Corp., New York, N. Y.
[6] Sigmamotor, Inc., Middleport, N. Y.
[7] Aluminum Oxide C, DeGussa Inc., New York, N. Y.; Alon C, Cabot Corp., Boston, Mass.

automatic spray gun (AGB504) equipped with an internal mixing nozzle was employed in this study. Pertinent parameters of the automatic color-coating operation are outlined in Table I.

Color Fastness of the Color Lakes—The permanency of the color lakes prepared from fumed titanium dioxide and the soluble dyes was tested using a centrifuge technique. One gram of color lake was suspended in 30 ml. of water and submitted to centrifugal force at 5000 r.p.m. for 5 min. The supernatant liquid was poured off and observed for color-leaching tendencies. The same color lake was resuspended in 30 ml. of water and centrifuged. In most cases, negligible transfer of the dye to the aqueous media occurred during the first step but was absent after the second treatment.

Dispersibility of the Color Lakes—The dispersibility of the color lakes when in an aqueous suspension was determined by employing sedimentation-rate analysis. Commercially available lakes were used as a control for this study. Two grams of color lake was suspended in 100 ml. water and placed in a 100-ml. graduated cylinder. The commercial lakes settled out about five times as rapidly as those prepared and indicated poor dispersibility. The color lake particles containing fumed titanium had greater resistance to the force of gravity, which was evidenced by its flocculated state

RESULTS AND DISCUSSION

Fumed titanium dioxide was employed in this investigation as an adsorbate for color lakes. The application results from a physicochemical reaction of the submicron material with water-soluble dyes. This results from the strong attractive forces existing between the positive-charged fumed inorganic dioxide and the negative-charged dye particles. The fumed material ranges in particle size from 0.005 to 0.040 μ, and thus it possesses a surface area (B.E.T. method) from 35 to 100 m.^{2}/g. (5). The absence of permanent interaction between titanium dioxide USP and dyes without permanency of lake formation is well known to the dye chemist (6).

The traditional method for color lake formation involves the coprecipitation of a soluble dye with a selected, hydrated, inorganic oxide or freshly precipitated salt such as calcium sulfate or barium sulfate. This method is not applicable for titanium dioxide lake formation from the USP grade material since it does not have the necessary physical characteristics of the traditional lake adsorbates. Titanium dioxide USP used for pigmentary purposes results in particle aggregation and a deficient pigment having less than optimum dispersibility and decreased properties, *i.e.*, tinting strength. This causes the titanium pigments to possess the following undesirable qualities: poor dispersibility, aeration, and fast settling, which interfere with excellence in their pharmaceutical use. The titanium dioxide lakes which were prepared from the fumed material resulted in a lake particle having optimum pigmentary properties, notably tinting strength, and demonstrated improved dispersibility.

As a result of fumed titanium dioxide's smaller particle size as compared with the USP grade, when it was dispersed in an aqueous medium it appeared relatively transparent and exhibited a much lower tinting strength.

When the fumed titanium dioxide was dispersed in an aqueous medium, it showed an isoelectric point at pH 6.5. Fumed titanium dioxide differs from the regular opacifying grades, because it possesses a substantial positive surface charge which causes it to interact exceptionally well with negatively charged particles, *i.e.*, soluble dyes. Acidification of the titanium dioxide–dye slurry when preparing the color lake permitted greater dye inclusion, since the potential of the titanium dioxide is higher at a low pH. The isoelectric point should be avoided in lake formation because it would provide the lowest potential. The acidified dyes are poorly soluble in water, and the color lake formed was evaluated as more permanent. An important aspect of the titanium lakes is that strong acids such as hydrochloric and acetic may be employed to fix the dye without causing the adsorbate to dissolve, as would occur with alumina. When a particular acid was varied in the treatment of the dye adsorbate, different shades of a specific color were obtained.

The spray-dried titanium lakes had a particle-size range similar to those obtained by a micronizing process and fall in a range from 1 to 10 μ as demonstrated by photomicroscopy. Excellent dispersibility of these color lakes in water was observed when compared with the commercially available color lakes prepared by jet-milled micronization.

An opaque gel structure formed during the extension of the dye on the fumed titanium dioxide. Addition of the dye caused a thickening of the fumed titanium slurry almost instantly, provided that the amount of dye was either in excess or sufficient to saturate the dye-adsorbing capacity of the fumed material. When the amount of titanium dioxide in the slurry was about 1% w/w and the dye about 1–2% w/w, a change occurred, a shifting from a colloidal suspension to a dense precipitate at the saturation point. If the dye saturation point was just reached, the lake obtained was easily separated by centrifugation; however, those slurries with a deficiency or excess of dye behaved like colloidal suspensions, and it was extremely difficult to separate the lakes from the dispersion medium by centrifugation. This phenomenon varied with the chemical structure of the dye considered.

The method of choice in the manufacture of the titanium lakes was the employment of the spray-drying technique which included preacidification before atomization into the spray-drying chamber. The inlet and outlet temperatures of the spray dryer were based on the composition of the dispersion media. The viscosity of the slurry prior to spray drying was decreased by using either inorganic or organic acids. If strong acids were employed, it was imperative that they were volatile at the inlet temperature of the spray dryer. The color fastness of these titanium dioxide lakes prepared by spray drying exhibited exceptional permanency of the dye–adsorbate interaction.

The color lakes prepared in this study were suspended in sucrose syrup (71% w/w), and the certified dye content, in certain cases, was utilized at 0.4% w/v. The color suspension was then applied to rounded, rotating tablets, and the tablets required about 8–12 applications to be considered acceptable from a color and physical appearance standpoint. The coating suspension permitted a reduction of the color-coating operation when compared with conventional dye solutions. The advantages of this process for coating tablets are: uniform color dispersion, consecutive batch reproducibility, extemporaneous preparation of color lakes, and the necessity of only 8–12 applications of the color suspension to obtain maximum color tone.

REFERENCES

(1) A. E. Nicholson, U. S. pat. 2,925,365, Feb. 1960.

(2) C. W. Baker, U. S. pat. 3,185,626, May 1965.

(3) L. Lachman, *Mfg. Chem. Aerosol News*, **37**, 35(1966).

(4) G. Steinberg, *Pharm. Ind.*, **26**, 91(1964).

(5) DeGussa, Inc., Technical Bulletin No. 3001, New York, N. Y., 1968.

(6) R. Roberts, personal communication.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 22, 1969, from the *Pharmacy Research and Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication February 4, 1970.

The technical assistance of J. Pyckowski and T. Knowlton is gratefully acknowledged.

* Present address: Foremost-McKesson Inc., International Division, New York, NY 10017

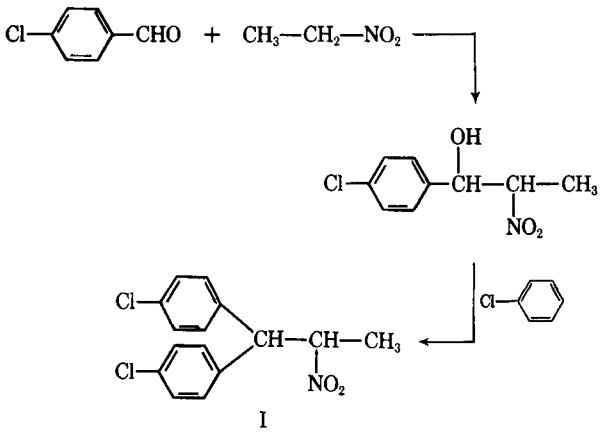
Synthesis of Radiolabeled 1,1-Bis(*p*-chlorophenyl)-2-nitropropane

RALPH H. JARBOE, Jr.*, JOHN B. DATA, and JOHN E. CHRISTIAN

Abstract □ Radiolabeled 1,1-bis(*p*-chlorophenyl)-2-nitropropane was synthesized, and its purity was established by TLC. Autoradiograms of labeled and unlabeled products were compared for authenticity. The specific activity was determined.

Keyphrases □ 1,1-Bis(*p*-chlorophenyl)-2-nitropropane—synthesis, specific activity □ ^{14}C-Labeling—specific activity, 1,1-bis(*p*-chlorophenyl-2-nitropropane □ Liquid scintillation spectrometry—specific activity □ TLC—analysis

The primary objectives of this work were to devise a practical synthesis and to establish the purity of radiolabeled 1,1-bis(*p*-chlorophenyl)-2-nitropropane (I) for the purpose of subjecting the compound to distribution and metabolic studies in its use as an insecticide. The preparation of I is basically an adaptation of the method described by Hass *et al.* (1):



I

Scheme I

The desired compound (I) was obtained in 32.5% yield. Purity of the product was established by TLC, using two solvent systems and a commercial adsorbent.[1] Autoradiograms of the labeled product were compared to an authentic, unlabeled sample. The pure product had a specific activity of 13.85 ± 0.11 μc./mmole.

EXPERIMENTAL

1-*p*-Chlorophenyl-2-nitro-1-propanol—Fifty-six grams (0.4 mole) of *p*-chlorobenzaldehyde was stirred mechanically for 3 hr. with 200 ml. of water containing 50.0 g. (0.475 mole) sodium bisulfite. Thirty-three grams (0.44 mole) nitroethane, previously dissolved in 80 ml. of 6.25 *N* NaOH at 8–13°, was added in small portions to the stirred, thick, white suspension of bisulfite addition product. As the reaction proceeded, the magma dissolved and a flocculent precipitate formed. Stirring was continued for 18 hr. after the last addition of the alkaline nitroethane solution.

The reaction mixture was transferred to a separator, the yellow organic layer which settled to the bottom was removed, and the milky aqueous layer was extracted three times with 25-ml. portions of ether to remove additional organic material. The combined organic substances were extracted with 50-ml. portions of 10% aqueous sodium bisulfite solution until all unreacted *p*-chlorobenzaldehyde had been removed. The ethereal solution was dried with anhydrous sodium sulfate for 2 hr. and filtered; the solvent was removed under reduced pressure to give 70.0 g. of light-yellow oil. Fractionation of the crude product gave 31.0 g. (36.1%) of highly viscous yellow oil which distilled at 145–147° at 0.2 mm.

1,1-Bis(*p*-chlorophenyl)-2-nitropropane-^{14}C—To 25 ml. of 2% fuming sulfuric acid, maintained at 10° and mechanically stirred, was added 57 mg. (0.50 mmole) of chlorobenzene-^{14}C (2.98 mc./mmole)[2] and 3.8 ml. of unlabeled chlorobenzene used to rinse the shipping vial. When the mixture had reached bath temperature, 8.78 g. (0.041 mole) of 1-*p*-chlorophenyl-2-nitro-1-propanol was added dropwise. The cooling bath was removed; the reaction mixture was stirred for an additional 1.5 hr., poured onto 100 g. cracked ice, and allowed to stand for 2 hr. The product was extracted exhaustively with ether, and the combined ether extracts were washed successively with 50-ml. portions of 5% sodium bicarbonate. 10% sodium bisulfite, and water. The ether was removed under reduced pressure, the oily residue was steam distilled, and the product remaining in the distillation flask was dissolved in ether.

The ethereal solution was dried with anhydrous sodium sulfate for 2 hr. and filtered. The ether was removed under reduced pressure, and the viscous oil was poured onto a watch glass to solidify overnight. About 2 ml. absolute ethanol was added to the original flask to dissolve residual material. The alcoholic solution and the solidified material were combined, crystallized from hot ethanol, and twice recrystallized from ethanol to obtain 4.1276 g. (32.5%) of a white, crystalline product.

TLC—Microscope slides coated with commercial adsorbent were placed vertically in individual, small, square, amber bottles containing 10 ml. of the desired developing solvent. The bottles were capped and slides were equilibrated overnight. Five microliters of the sample, in methanol, was pipeted onto each slide about 1 cm. above the solvent level. The slides were developed for about 10 min., air dried, exposed to UV radiation to locate I, and sprayed with a plastic preservative if subsequent autoradiography was intended. Repeated runs with authentic samples of I gave an R_f value of 0.48 in hexane–ethyl acetate (9:1) and 0.30 in hexane–acetic acid (9:1).

Autoradiography—For autoradiography, separate solutions of the authentic unlabeled product and of the labeled product containing 10 mg./ml. were prepared in methanol. Five-microliter samples of the authentic and labeled products were chromatographed in parallel on 20 × 20-cm. commercial adsorbent thin-layer plates, using both of the solvent systems described previously. In both solvent systems, UV irradiation of the plates disclosed identical R_f values for authentic and unlabeled samples. In neither solvent was there evidence of impurities. The plates were sprayed with plastic preservative to prevent sloughing, exposed for 6 days to film, developed, and fixed.[3] For both solvent systems, a single radioactive

[1] Adsorbosil P-1, Allied Science Laboratories Inc., State College, Pa.

[2] Uniformly labeled.

[3] Eastman No Screen medical X-ray film. Developed and fixed in Eastman Kodak liquid developer and liquid fixer, respectively.

spot, corresponding to the position of the authentic unlabeled sample, was obtained. There was no tailing in the hexane–ethyl acetate system, but there was a small amount of tailing in the hexane–acetic acid system.

Determination of Specific Activity—A 10 mg./ml. solution of 1,1-bis(*p*-chlorophenyl)-2-nitropropane-^{14}C in methanol was used to determine the specific activity of the compound.

Five samples were prepared by pipeting 100 μl. of the solution into five scintillation vials containing a liquid scintillation cocktail composed of 0.14% PPO (2,5-diphenyloxazole) and 0.01% dimethyl POPOP [1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene] in equal volumes each of toluene and 2-ethoxyethanol. These samples were cooled in a Packard TRI CARB liquid-scintillation spectrometer, model 3003, for 0.5 hr. The window width of 50–100° was chosen. Utilizing an internal standard of toluene-^{14}C (toluene-^{14}C, 5.01×10^5 dis./min./ml., R-21 Tracerlab), the observed counts per minute were converted to disintegrations per minute and corrected for quenching and counter efficiency. A specific activity of 13.85 ± 0.11 μc./mmole was obtained.

REFERENCE

(1) H. B. Hass, M. B. Neher, and R. T. Blickenstaff, *Ind. Eng. Chem.*, **43**, 2875(1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from the *Departments of Bionucleonics and Medicinal Chemistry, Purdue University, Lafayette, IN 47907*

Accepted for publication February 9, 1970.

This research was conducted under the auspices of the Institute for Environmental Health, Purdue University.

* Present address: Radiopharmaceutical Research, Abbott Laboratories, North Chicago, Ill.

Development of Tolerance to Pentobarbital

JASBIR M. SINGH, BRUCE FIEGENSCHUE, and CARL SCHEXNAYDRE

Abstract □ Tolerance to pentobarbital developed 4 hr. after the first injection and reached a peak at 17–22 hr., after which it decreased to nonsignificant levels by 48 hr. The duration and frequency of administration of pentobarbital affected the degree of development of tolerance to pentobarbital. PTI (Percentage Tolerance Index) decreased progressively with the increase in the number of injections. When four injections were given within the span of 28 hr., the animals showed a greater degree of tolerance on the third injection which was administered 24 hr. after the initial injection. Tolerance was also present on the fourth injection but to a lesser degree when compared with the third injection. The experimental data from this study suggest that tolerance to pentobarbital does develop and is the result of the pentobarbital stimulating its own metabolizing enzyme.

Keyphrases □ Pentobarbital—tolerance development, rats □ Drug tolerance—pentobarbital, rats □ Tolerance, pentobarbital—rats

Animals can be shown to develop tolerance to barbiturates, pentobarbital and thiopental (1). Gruber and Keyser found that dogs tend to become tolerant to a variety of barbiturates (2). As a criterion of tolerance, they used the reduction of the sleeping time elicited by the same dose after it had been repeated several times. According to Goodman and Gilman, tolerance to barbiturates has developed when, after repeated administration, a given dose produces a decreasing effect or, conversely, when increasingly larger doses must be given to obtain true barbiturate effects obtained with the original dose (3). Tatum *et al.* provide the classic definition of tolerance as "a phenomenon characterized by the fact that more drug must be used to produce equivalent effects" (4). Jaffe and Sharpless have shown that some degree of physical dependence can be produced in as little as 20 hr. after pentobarbital administration (5). Singh has also shown that tolerance to pentobarbital and thiopental is developed within 24 hr. (1). The purpose of this paper is to report that: (*a*) a certain time lapse occurs before the tolerance is developed, and (*b*) this developed tolerance reaches a peak and then declines.

EXPERIMENTAL

Female albino rats, random by breed (Caeserian Drive One) and weighing 125–175 g., were used. Pentobarbital sodium was dissolved in distilled water. The volume of each injection was kept constant, *i.e.*, 1 ml./kg. All injections of pentobarbital (25 mg./kg.) were given intraperitoneally. The sleeping time (difference between loss of righting reflex and regain in righting reflex in minutes) was determined.

Animals were divided into five major groups:

Group 1—Pentobarbital was administered to 34 animals at zero time and at an interval of 24 hr.

Group 2—Eighteen subgroups were composed of 10 to 15 animals each. In these subgroups, the first injection was given at zero time and then the second injection was given at intervals of 2, 3, 4, 7, 9, 13, 17, 18, 19, 20, 21, 21, 22.5, 22.5, 24, 48, 72, and 168 hr. Some experiments (21 and 22.5 hr.) were duplicated.

Group 3—Pentobarbital, 25 mg./kg., was administered daily to 10 animals at intervals of 24 hr. for 16 days.

Group 4—Three subgroups were comprised of 10 to 15 animals each. To each subgroup, pentobarbital, 25 mg./kg., was administered at intervals of 0, 2, 24, 26; 0, 3, 24, 27; and 0, 4, 24, 28 hr.

Group 5—Clinical signs, *i.e.*, water consumption, urine output, food consumption, and growth, were observed in animals that had developed tolerance to pentobarbital, 25 mg./kg. Each animal was housed separately in a metabolism cage, and initial observations on each animal were taken. Each animal was given two injections of pentobarbital after the initial observations. Then the animals were allowed to recover on the third and fourth day. Statistical methods used were those of Snedecor (6).

Because most work on the development of tolerance to barbiturates has been done on male rats, guinea pigs, mice, and dogs, in this project the authors decided to study tolerance to pentobarbital in female white rats. Percentage tolerance index (PTI) was computed as follows (1):

$$\frac{\text{hypnotic effect of first injection}}{\text{hypnotic effect of second injection}} \times 100$$

If PTI is unity, *i.e.*, 100%, it indicates no tolerance. PTI greater or less than unity indicates tolerance or cumulative effect. Before

spot, corresponding to the position of the authentic unlabeled sample, was obtained. There was no tailing in the hexane–ethyl acetate system, but there was a small amount of tailing in the hexane–acetic acid system.

Determination of Specific Activity—A 10 mg./ml. solution of 1,1-bis(*p*-chlorophenyl)-2-nitropropane-^{14}C in methanol was used to determine the specific activity of the compound.

Five samples were prepared by pipeting 100 μl. of the solution into five scintillation vials containing a liquid scintillation cocktail composed of 0.14% PPO (2,5-diphenyloxazole) and 0.01% dimethyl POPOP [1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene] in equal volumes each of toluene and 2-ethoxyethanol. These samples were cooled in a Packard TRI CARB liquid-scintillation spectrometer, model 3003, for 0.5 hr. The window width of 50–100° was chosen. Utilizing an internal standard of toluene-^{14}C (toluene-^{14}C, 5.01×10^5 dis./min./ml., R-21 Tracerlab), the observed counts per minute were converted to disintegrations per minute and corrected for quenching and counter efficiency. A specific activity of 13.85 ± 0.11 μc./mmole was obtained.

REFERENCE

(1) H. B. Hass, M. B. Neher, and R. T. Blickenstaff, *Ind. Eng. Chem.*, **43**, 2875(1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from the *Departments of Bionucleonics and Medicinal Chemistry, Purdue University, Lafayette, IN 47907*

Accepted for publication February 9, 1970.

This research was conducted under the auspices of the Institute for Environmental Health, Purdue University.

* Present address: Radiopharmaceutical Research, Abbott Laboratories, North Chicago, Ill.

Development of Tolerance to Pentobarbital

JASBIR M. SINGH, BRUCE FIEGENSCHUE, and CARL SCHEXNAYDRE

Abstract □ Tolerance to pentobarbital developed 4 hr. after the first injection and reached a peak at 17–22 hr., after which it decreased to nonsignificant levels by 48 hr. The duration and frequency of administration of pentobarbital affected the degree of development of tolerance to pentobarbital. PTI (Percentage Tolerance Index) decreased progressively with the increase in the number of injections. When four injections were given within the span of 28 hr., the animals showed a greater degree of tolerance on the third injection which was administered 24 hr. after the initial injection. Tolerance was also present on the fourth injection but to a lesser degree when compared with the third injection. The experimental data from this study suggest that tolerance to pentobarbital does develop and is the result of the pentobarbital stimulating its own metabolizing enzyme.

Keyphrases □ Pentobarbital—tolerance development, rats □ Drug tolerance—pentobarbital, rats □ Tolerance, pentobarbital—rats

Animals can be shown to develop tolerance to barbiturates, pentobarbital and thiopental (1). Gruber and Keyser found that dogs tend to become tolerant to a variety of barbiturates (2). As a criterion of tolerance, they used the reduction of the sleeping time elicited by the same dose after it had been repeated several times. According to Goodman and Gilman, tolerance to barbiturates has developed when, after repeated administration, a given dose produces a decreasing effect or, conversely, when increasingly larger doses must be given to obtain true barbiturate effects obtained with the original dose (3). Tatum *et al.* provide the classic definition of tolerance as "a phenomenon characterized by the fact that more drug must be used to produce equivalent effects" (4). Jaffe and Sharpless have shown that some degree of physical dependence can be produced in as little as 20 hr. after pentobarbital administration (5). Singh has also shown that tolerance to pentobarbital and thiopental is developed within 24 hr. (1). The purpose of this paper is to report that: (*a*) a certain time lapse occurs before the tolerance is developed, and (*b*) this developed tolerance reaches a peak and then declines.

EXPERIMENTAL

Female albino rats, random by breed (Caeserian Drive One) and weighing 125–175 g., were used. Pentobarbital sodium was dissolved in distilled water. The volume of each injection was kept constant, *i.e.*, 1 ml./kg. All injections of pentobarbital (25 mg./kg.) were given intraperitoneally. The sleeping time (difference between loss of righting reflex and regain in righting reflex in minutes) was determined.

Animals were divided into five major groups:

Group 1—Pentobarbital was administered to 34 animals at zero time and at an interval of 24 hr.

Group 2—Eighteen subgroups were composed of 10 to 15 animals each. In these subgroups, the first injection was given at zero time and then the second injection was given at intervals of 2, 3, 4, 7, 9, 13, 17, 18, 19, 20, 21, 21, 22.5, 22.5, 24, 48, 72, and 168 hr. Some experiments (21 and 22.5 hr.) were duplicated.

Group 3—Pentobarbital, 25 mg./kg., was administered daily to 10 animals at intervals of 24 hr. for 16 days.

Group 4—Three subgroups were comprised of 10 to 15 animals each. To each subgroup, pentobarbital, 25 mg./kg., was administered at intervals of 0, 2, 24, 26; 0, 3, 24, 27; and 0, 4, 24, 28 hr.

Group 5—Clinical signs, *i.e.*, water consumption, urine output, food consumption, and growth, were observed in animals that had developed tolerance to pentobarbital, 25 mg./kg. Each animal was housed separately in a metabolism cage, and initial observations on each animal were taken. Each animal was given two injections of pentobarbital after the initial observations. Then the animals were allowed to recover on the third and fourth day. Statistical methods used were those of Snedecor (6).

Because most work on the development of tolerance to barbiturates has been done on male rats, guinea pigs, mice, and dogs, in this project the authors decided to study tolerance to pentobarbital in female white rats. Percentage tolerance index (PTI) was computed as follows (1):

$$\frac{\text{hypnotic effect of first injection}}{\text{hypnotic effect of second injection}} \times 100$$

If PTI is unity, *i.e.*, 100%, it indicates no tolerance. PTI greater or less than unity indicates tolerance or cumulative effect. Before

Table I—Effect of Pentobarbital (First and Second Injections) on Sleeping Time (S.T.)

Mean S.T. of First Injection, min. (A)	Interval of Second Injection, hr.	Mean S.T. of Second Injection, min. (B)	Difference in S.T.	% Change between A and B
102 (10)[a]	2	140 (10)	+38[b]	+37.3[c]
100 (11)	3	112 (11)	+12	+12.0
88 (12)	4	72 (12)	−16	−18.2[c]
67 (12)	7	53 (12)	−14	−20.9[c]
63 (12)	9	50 (12)	−13	−20.6[c]
57 (13)	13	24 (13)	−33	−57.8[c]
47 (10)	17	15 (10)	−32	−68.0[c]
62 (14)	18	18 (14)	−44	−70.9[c]
70 (14)	19	16 (14)	−54	−77.2[c]
57 (12)	20	12 (12)	−45	−78.9[c]
64 (12)	21	15 (12)	−49	−76.6[c]
55 (12)	21	14 (12)	−41	−74.5[c]
64 (12)	22.5	20 (12)	−44	−68.7[c]
73 (12)	22.5	23 (12)	−50	−68.5[c]
57 (14)	24	24 (14)	−33	−57.8[c]
88 (15)	48	72 (15)	−16	−18.2[c]
88 (10)	72	80 (10)	−8	−9.1
70 (10)	168	64 (10)	+1	+1.4

[a] Sample size is given in parentheses. [b] (+) or (−) indicates increase or decrease in S.T. [c] $p < 0.05$.

computing PTI, the data of the first and second injections were analyzed by means of differences (6).

RESULTS

Group 1—Significant tolerance ($p < 0.05$) was developed in female albino rats when the second injection of pentobarbital was administered at an interval of 24 hr. after the initial injection.

Group 2—Effect of pentobarbital (first and second injections) on the sleeping time is computed from Table I and given in Fig. 1. Significant cumulative hypnotic effect was present during the first 3 hr. The animals began to show tolerance after 4 hr., reaching a peak between 17 and 22 hr., then declining and was not evident at or after 48 hr.

Group 3—Table II shows the effect of continuous administration of pentobarbital on the development of tolerance. In this group, four injections of pentobarbital were given to the same animals at different time intervals. No tolerance was exhibited at 2 and 3 hr. However, tolerance was present if the third and fourth injections were given at 0, 2, 24, 26, 0, 3, 24,27; and 0, 4, 24, 28 hr. Comparison is made between the first two injections (0, 2; 0, 3; 0, 4) and the last two injections (24, 26; 24, 27; 24, 28). Significant ($p < 0.05$) tolerance is developed at and after 24 hr. Significant ($p < 0.05$) tolerance was present when the fourth injection was given at 26, 27, and 28 hr. following the initial injection. However, the degree of developed tolerance was significantly ($p < 0.05$) less when compared with the third injection.

Group 4—The effect of continuous administration of pentobarbital daily for 16 days is shown in Table III. Significant ($p < 0.05$) tolerance was developed after 24 hr. PTI decreased progressively with the increase in the number of injections.

Group 5—Clinical signs, *i.e.*, urine output, water consumption, food consumption, and weight, were not affected during the development of tolerance to pentobarbital. However, on the 3rd day, 24 hr. after the second injection, a significant ($p < 0.05$) increase in water consumption and urine output was observed; food consumption was not altered over the period of 5 days. Weight remained constant in the treated rats. However, a significant ($p < 0.05$) increase in weight was observed in the control animals.

DISCUSSION

The hypothesis of the authors was that the development of tolerance to pentobarbital can be influenced by interval and frequency of administration of the drug, duration of administration of the drug, and metabolism in the liver.

Interval and Frequency of Administration of Drug—Singh has shown that tolerance to barbiturates, pentobarbital, and thiopental

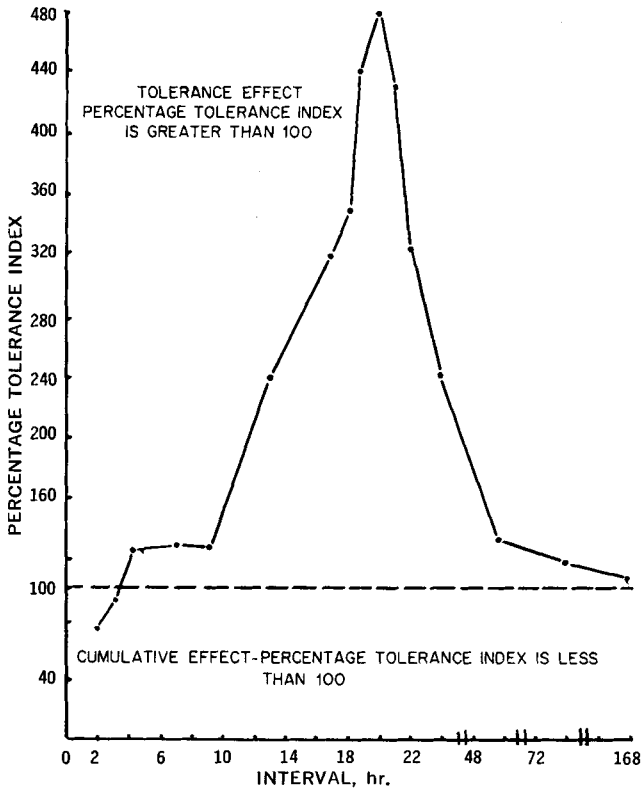


Figure 1—*Effect of pentobarbital, 25 mg./kg., on the percentage tolerance index of female albino rats.*

is developed in 24 hr. (1). Results of the present experiment also indicate that a minimum of 4 hr. is necessary for the tolerance to be induced (Table I). Maximum tolerance is induced in an animal if the second injection is given between 17 and 22 hr.; then it declines. Nonsignificant tolerance is present after 48 hr. In less than 4 hr., animals showed cumulative effect instead of tolerance.

Duration of Administration of Drug—Duration of administration of pentobarbital will also affect the degree of development of tolerance to pentobarbital (Table III). PTI was greater on the second and subsequent administrations. In these experiments, all the injections were given daily for 16 days. On the other hand, when four injections were given within the span of 28 hr., animals showed a greater degree of tolerance on the third injection administered at 24 hr. after the initial injection (Table II). Tolerance was also present on the fourth injection but to a lesser degree when compared with the third injection.

Metabolism in the Liver—The liver plays an important role in the duration of action of barbiturates (3). Duration of action of barbiturates is decreased once tolerance is developed to barbiturates (Tables II and III). Anesthesia induced by barbiturates can be

Table II—Effect of Continuous Administration of Pentobarbital (25 mg./kg.) on Development of Tolerance[a]

Subgroups		Intervals, hr. 0	2	24	26
1	S.T.	76.4 (10)	123.0 (10)	27.1 (10)	59.4 (10)
	PTI[b]		62.0[c]	281.9[c]	128.6[c]
		0	3	24	28
2	S.T.	87.9 (10)	105.5 (10)	21.1 (10)	41.8 (10)
	PTI		83.3[c]	416.5[c]	210.0[c]
		0	4	24	28
3	S.T.	78.9 (10)	52.3 (10)	27.2 (10)	33.5 (10)
	PTI		150.8[c]	290.0[c]	235.5[c]

[a] $N = 10$. The same animals are used in each group. [b] PTI = percentage tolerance index. [c] $p < 0.05$.

Table III—Effect of Continuous Administration of Pentobarbital (25 mg./kg. i.p.) on Development of Tolerance

No. Injections	Injection Interval, Days[a]	Mean S.T., min.[b]	Tolerance Index, %
1	1	46.4 (8)	
2	2	26.7 (8)	173.0[c]
3–7	3–7	—	
8	8	36.0 (8)	128[c]
9	9	31.8 (8)	145[c]
10–14	10–14	—	
15	15	38.2 (8)	121[c]
16	16	41.6 (8)	111

[a] Injections were given daily to the same animals for 16 days. [b] The sleeping time was determined on 1, 2, 8, 9, 15, and 16 days. Sample size is given in parentheses. [c] $p < 0.05$.

prolonged by hepatic injury (7). Plaa *et al.* found a positive relationship between the doses of several hepatotoxins and the prolongation of pentobarbital sleeping time (7). Singh and Boyd found that tannic acid, which causes centrilobular liver necrosis, prolonged the sleeping time of thiopental (8). This effect became evident at 72 hr. after the administration of tannic acid. Balazs and Grice also reported that administration of carbon tetrachloride can prolong the sleeping time of pentobarbital (9).

Pentobarbital is metabolized in the liver (3). Conney *et al.* have shown that pentobarbital is an enzyme inducer (10). One explanation of the development of tolerance to pentobarbital is that pentobarbital possibly stimulates its own metabolizing enzyme and thus results in increased pentobarbital metabolism on repeated administration and decreased sleeping time (Tables I–III). This leads the authors to suggest that in the development of tolerance to pentobarbital, the liver possibly is involved.

Clinical Signs—During the development of tolerance to pentobarbital, neither water consumption nor urine excretion was affected. However, on the day after the tolerance had been induced, a significant increase ($p < 0.05$) was noticeable in water consumption and urine excretion. Singh reported similar clinical findings with thiopental also (11).

REFERENCES

(1) J. M. Singh, *Toxicol. Appl. Pharmacol.*, **11**, 320(1968).

(2) C. M. Gruber and G. F. Keyser, *J. Pharmacol. Exp. Ther.*, **86**, 186(1946).

(3) L. S. Goodman and A. Gilman, "The Pharmacological Basis of Therapeutics," Macmillan, New York, N. Y., 1965, p. 285.

(4) A. L. Tatum, M. H. Seevers, and K. H. Collins, *J. Pharmacol. Exp. Ther.*, **132**, 202(1961).

(5) J. H. Jaffe and S. K. Sharpless, *ibid.*, **150**, 140(1965).

(6) G. W. Snedecor, "Statistical Methods," Iowa State College Press, Ames, Iowa, 1957, p. 97.

(7) G. L. Plaa, E. A. Evans, and G. H. Hine, *J. Pharmacol. Exp. Ther.*, **123**, 224(1958).

(8) J. M. Singh and E. M. Boyd, *Can. Med. Ass. J.*, **95**, 558 (1962).

(9) T. Balazs and H. C. Grice, *Toxicol. Appl. Pharmacol.*, **5**, 387(1963).

(10) A. H. Conney, I. A. Michaelson, and J. J. Burns, *J. Pharmacol. Exp. Ther.*, **132**, 202(1961).

(11) J. M. Singh, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 27, 1969, from the *Department of Pharmacology, College of Pharmacy, Xavier University, New Orleans, LA 70125*

Accepted for publication January 19, 1970.

Presented to the Biochemical and Pharmacology Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors thank Drs. C. J. Kelly and Joseph P. Buckley.

Sterility Testing of Insulin by Membrane Filtration: A Collaborative Study

MIRIAM P. CALHOUN, MACK WHITE, and FRANCES W. BOWMAN

Abstract □ A membrane filtration procedure was devised for testing the sterility of insulin zinc suspensions solubilized in ascorbic acid diluting fluid. A collaborative study showed that the filtration procedure afforded significant improvements over the direct method of sterility testing.

Keyphrases □ Insulin, sterility—membrane filtration □ Membrane filtration—sterility testing, insulin zinc suspensions □ Sterility testing—insulin, membrane filtration □ Collaborative study—membrane filtration, insulin sterility

In 1941 the Federal Food, Drug, and Cosmetic Act was amended to establish a certification service to ensure the safety and efficacy of insulin-containing drugs by testing each lot prior to distribution. The official analytical methods and standards of quality and purity are described in the USP (1), the NF (2), and the *Code of Federal Regulations* (3). Since insulin must be administered by parenteral injection, these official compendia require all insulin preparations to be sterile.

The official USP XVII/NF XII method for the sterility testing of insulin requires that 20 containers from each "filling operation" be tested in thioglycollate broth for detecting bacteria and in Sabouraud fluid medium for molds and yeasts. The solution or suspension of insulin is transferred with a sterile syringe and needle directly to tubes of media. Tubes containing thioglycollate medium are incubated for not less than 7 days at 30–32° and those containing fluid Sabouraud for not less than 10 days at 22–25°. After incubation the media are examined for the presence or absence of microbial growth.

The principal objection to the USP XVII/NF XII method is that a precipitate is formed by the insulin suspensions in the culture media. Macroscopically the precipitate is indistinguishable from microbial growth;

Table III—Effect of Continuous Administration of Pentobarbital (25 mg./kg. i.p.) on Development of Tolerance

No. Injections	Injection Interval, Days[a]	Mean S.T., min.[b]	Tolerance Index, %
1	1	46.4 (8)	
2	2	26.7 (8)	173.0[c]
3–7	3–7	—	
8	8	36.0 (8)	128[c]
9	9	31.8 (8)	145[c]
10–14	10–14	—	
15	15	38.2 (8)	121[c]
16	16	41.6 (8)	111

[a] Injections were given daily to the same animals for 16 days. [b] The sleeping time was determined on 1, 2, 8, 9, 15, and 16 days. Sample size is given in parentheses. [c] $p < 0.05$.

prolonged by hepatic injury (7). Plaa *et al.* found a positive relationship between the doses of several hepatotoxins and the prolongation of pentobarbital sleeping time (7). Singh and Boyd found that tannic acid, which causes centrilobular liver necrosis, prolonged the sleeping time of thiopental (8). This effect became evident at 72 hr. after the administration of tannic acid. Balazs and Grice also reported that administration of carbon tetrachloride can prolong the sleeping time of pentobarbital (9).

Pentobarbital is metabolized in the liver (3). Conney *et al.* have shown that pentobarbital is an enzyme inducer (10). One explanation of the development of tolerance to pentobarbital is that pentobarbital possibly stimulates its own metabolizing enzyme and thus results in increased pentobarbital metabolism on repeated administration and decreased sleeping time (Tables I–III). This leads the authors to suggest that in the development of tolerance to pentobarbital, the liver possibly is involved.

Clinical Signs—During the development of tolerance to pentobarbital, neither water consumption nor urine excretion was affected. However, on the day after the tolerance had been induced, a significant increase ($p < 0.05$) was noticeable in water consumption and urine excretion. Singh reported similar clinical findings with thiopental also (11).

REFERENCES

(1) J. M. Singh, *Toxicol. Appl. Pharmacol.*, **11**, 320(1968).

(2) C. M. Gruber and G. F. Keyser, *J. Pharmacol. Exp. Ther.*, **86**, 186(1946).

(3) L. S. Goodman and A. Gilman, "The Pharmacological Basis of Therapeutics," Macmillan, New York, N. Y., 1965, p. 285.

(4) A. L. Tatum, M. H. Seevers, and K. H. Collins, *J. Pharmacol. Exp. Ther.*, **132**, 202(1961).

(5) J. H. Jaffe and S. K. Sharpless, *ibid.*, **150**, 140(1965).

(6) G. W. Snedecor, "Statistical Methods," Iowa State College Press, Ames, Iowa, 1957, p. 97.

(7) G. L. Plaa, E. A. Evans, and G. H. Hine, *J. Pharmacol. Exp. Ther.*, **123**, 224(1958).

(8) J. M. Singh and E. M. Boyd, *Can. Med. Ass. J.*, **95**, 558 (1962).

(9) T. Balazs and H. C. Grice, *Toxicol. Appl. Pharmacol.*, **5**, 387(1963).

(10) A. H. Conney, I. A. Michaelson, and J. J. Burns, *J. Pharmacol. Exp. Ther.*, **132**, 202(1961).

(11) J. M. Singh, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 27, 1969, from the *Department of Pharmacology, College of Pharmacy, Xavier University, New Orleans, LA 70125*

Accepted for publication January 19, 1970.

Presented to the Biochemical and Pharmacology Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors thank Drs. C. J. Kelly and Joseph P. Buckley.

Sterility Testing of Insulin by Membrane Filtration: A Collaborative Study

MIRIAM P. CALHOUN, MACK WHITE, and FRANCES W. BOWMAN

Abstract □ A membrane filtration procedure was devised for testing the sterility of insulin zinc suspensions solubilized in ascorbic acid diluting fluid. A collaborative study showed that the filtration procedure afforded significant improvements over the direct method of sterility testing.

Keyphrases □ Insulin, sterility—membrane filtration □ Membrane filtration—sterility testing, insulin zinc suspensions □ Sterility testing—insulin, membrane filtration □ Collaborative study—membrane filtration, insulin sterility

In 1941 the Federal Food, Drug, and Cosmetic Act was amended to establish a certification service to ensure the safety and efficacy of insulin-containing drugs by testing each lot prior to distribution. The official analytical methods and standards of quality and purity are described in the USP (1), the NF (2), and the *Code of Federal Regulations* (3). Since insulin must be administered by parenteral injection, these official compendia require all insulin preparations to be sterile.

The official USP XVII/NF XII method for the sterility testing of insulin requires that 20 containers from each "filling operation" be tested in thioglycollate broth for detecting bacteria and in Sabouraud fluid medium for molds and yeasts. The solution or suspension of insulin is transferred with a sterile syringe and needle directly to tubes of media. Tubes containing thioglycollate medium are incubated for not less than 7 days at 30–32° and those containing fluid Sabouraud for not less than 10 days at 22–25°. After incubation the media are examined for the presence or absence of microbial growth.

The principal objection to the USP XVII/NF XII method is that a precipitate is formed by the insulin suspensions in the culture media. Macroscopically the precipitate is indistinguishable from microbial growth;

Table I—Recovery of Microorganisms from Insulin Zinc Suspension Preserved with 0.1% Methylparaben Tested at 0 Time and after 7 Days at 25°[a]

Microorganisms	Estimated No. Cells/ml.	Insulin				0.1% Peptone Solution			
		0 Time		7 Days		0 Time		7 Days	
		USP	MF	USP	MF	USP	MF	USP	MF
Saccharomyces cerevisiae ATCC 9763	15	+	+	+	+	+	+	+	+
Aspergillus niger ATCC 6275 (spores)	20	+	+	+	+	+	+	+	+
Bacillus subtilis ATCC 6633 (spores)	10	+	+	+	+	+	+	+	+
Bacillus circulans PCI 260 (spores)	10	+	+	+	+	+	+	+	+
Corynebacterium acnes PCI 1502	20	+	+	−	−	+	+	+	+
Pseudomonas aeruginosa ATCC 9027	15	+	+	−	−	+	+	+	+
Salmonella sp. PCI 431	10	+	+	−	−	+	+	+	+
Staphylococcus aureus ATCC 6538P	10	+	+	−	−	+	+	+	+

[a] MF = Membrane filtration method; USP = direct method; − = no growth; + = growth.

therefore, subcultures are necessary to determine whether viable microorganisms are present. Because the additional work required for these subcultures is time-consuming and expensive and could lead to false positive results due to adventitious contamination, a method was sought to improve the test.

EXPERIMENTAL

The membrane filtration sterility test method described for antibiotics by Bowman (4) is easily applied to insulin solutions, because they filter readily and leave no residue on the membrane. However, the suspensions (*e.g.*, protamine zinc insulin suspension, isophane insulin suspension, and insulin zinc suspension) are not amenable to the filtration sterility test, because the crystals are insoluble in the peptone diluting fluid used in the procedure.

Solubilization Studies—Because insulin suspensions presented problems in filtering, various procedures were tried for solubilizing them. When the pH was lowered from the normal of approximately 7.2 to 3.2 ± 0.2, the suspended material immediately dissolved at room temperature and the resultant solutions could then be filtered quickly, without leaving any visible residue on the membrane. However, when 1 *N* hydrochloric acid was used to lower the pH, it was toxic to some vegetative organisms.

Preliminary investigation indicated that ascorbic acid would be suitable for solubilizing insulin suspensions without adversely affecting the viability of microorganisms that might be present. A 1% ascorbic acid solution (AA diluting fluid) was prepared in 0.1% peptone (w/v) solution (pH 3.2 ± 0.2). Protamine zinc insulin suspensions were solubilized immediately and insulin zinc suspensions were solubilized in approximately 1 min. with the AA diluting fluid. The membranes were assayed by a radioimmuno assay for residual insulin (5). Less than 1 unit of insulin was retained by a membrane used to filter 1600 units of insulin (6).

Survival Studies—To assess the ability of organisms to survive in insulin suspensions, survival studies were performed. Vials of sterile insulin containing 0.1% methylparaben as a preservative were inoculated with vegetative bacteria or yeasts or with spores of bacteria or molds. Vials containing peptone water were used as controls. All were tested for sterility by the USP/NF method and by the proposed membrane filtration test at zero time and after 1 week at room temperature. Growth of the added microorganisms was ob-

Table II—Results of a Collaborative Study Comparing the Direct Method (DM) to the Membrane Filtration Method (MF) for the Sterility Testing of Insulin Suspensions (80 units/ml.)[a,b]

Test Organisms	Collaborator No.	Insulin								0.1% Peptone Solution Controls							
		1–5 CPU				50–100 CPU				1–5 CPU				50–100 CPU			
		DM		MF		DM		MF		DM		MF		DM		MF	
		FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD
Salmonella sp. PCI 431	1	Neg.	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	2	7	7	7	Neg.	7	7	7	7	7	7	7	7	7	7	7	7
Bacillus subtilis ATCC 6633 (spores)	1	Neg.	7	Neg.	7	7	7	7	7	7	7	Neg.	7	7	7	7	7
	2	Neg.	7	7	7	7	7	Neg.	7	Neg.	7	Neg.	7	7	7	Neg.	7
Staphylococcus aureus ATCC 6538P	1	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	2	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Aspergillus niger ATCC 6275 (spores)	1	10	Neg.	7	7	10	7	7	7	7	Neg.	Neg.	7	7	7	7	7
	2	10	10	Neg.	7	7	10	7	7	7	10	Neg.	7	7	7	7	7
Bacillus circulans PCI 260 (spores)	1	7	Neg.	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	2	7	Neg.	7	Neg.	7	10	7	7	7	10	Neg.	Neg.	7	10	7	7
Bacillus sp. PCI 261 (spores)	1	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	2	7	7	7	10	7	7	7	7	Neg.	7	Neg.	Neg.	7	7	7	7
Saccharomyces cerevisiae[c] ATCC 9763	1	7	Neg.	7	7	7	Neg.	7	7	7	Neg.	7	7	7	Neg.	7	7
	2	Neg.	7	7	7	7	7	7	7	Neg.	7	10	7	7	7	7	7
Candida albicans[c] ATCC 10231	1	14	Neg.	7	7	14	14	7	7	7	Neg.	7	7	7	14	7	7
	2	Neg.	7	7	7	7	7	7	7	Neg.	7	7	7	7	7	7	7

[a] Results are given as the maximum number of days incubation required for recovery of microorganisms. [b] Definitions and conditions as follows: CPU, colony-producing units; FTM, fluid thioglycollate medium incubated at 32–35°; SBCD, soybean-casein digest medium incubated at 22–25°. [c] Subcultures were necessary to determine whether viable microorganisms were present or absent when the direct method was used.

tained from all the suspensions at zero time. As shown in Table I, only the yeast and the sporing microorganisms survived in insulin for 7 days. In a similar study on globin insulin preserved with phenol, Sykes and Hooper (7) found that vegetative bacteria were killed by components other than preservatives (such as protamine) while spores of bacteria and molds survived.

The results obtained in these survival studies indicated that a filtration sterility test could be applied to insulin suspension and solutions. After some preliminary studies in collaboration with two manufacturers of insulin, it was decided that a definitive collaborative study should be conducted to establish the validity of the filtration method before proposing its inclusion in the USP and NF.

Collaborative Study on the Sterility Testing of Insulin—The procedure used was essentially that used for antibiotics (8) except that a separate membrane was used for each medium, soybean-casein digest broth was substituted for liquid Sabouraud medium, and the membrane was washed only once with 0.1% peptone (w/v) solution. These changes were based on the understanding that USP XVIII (9) and NF XIII (10) will include a similar membrane filtration sterility test as an alternative test for sterile preparations that are amenable to filtration.

Cultures of the eight microorganisms used in the study were supplied by the authors. Dilutions of inocula were selected to simulate low levels of contamination that might possibly be encountered in a contaminated insulin preparation.

The following directions (11) were followed by the two collaborating laboratories: aseptically add 39 ml. of insulin suspension preserved with 0.1% methylparaben to each of 80 sterile 60-ml. (2-oz.) prescription bottles. To each of another 80 bottles, add 39 ml. of 0.1% peptone solution. Prepare a dilution of one culture to yield five cells per ml., then aseptically add 1 ml. of this dilution to each of five bottles of insulin suspension and to five bottles of the control peptone solution. Prepare another dilution of the same culture to yield approximately 100 cells per ml., and add 1 ml. to each of another five bottles of insulin suspension and of peptone solution, respectively.

Perform a USP XVII/NF XII direct sterility test on the contents of two of the bottles of each dilution of microorganisms. From one bottle transfer 2-ml. portions to each of 20 tubes containing 40 ml. of thioglycollate medium and from the other bottle transfer 2 ml. to each of 20 tubes containing 40 ml. of soybean-casein digest medium.

Perform the proposed membrane filtration test, described here and in USP XVIII. using two more bottles of each dilution of microorganisms in the test fluids. These two bottles simulate the two pooled vessels (prior to filtration) to be described in the USP XVIII method.

As a control on the inoculum, filter the contents of the remaining bottle through a membrane filter; but instead of transferring the membrane to fluid media, place it on the surface of a soybean-casein digest agar plate.

Read all tests after incubation at the appropriate temperature for 7, 10, and 14 days.

Repeat this procedure for each of the other seven microorganisms.

Thus, for each microorganism, there is a total of 10 bottles for each testing fluid, five containing 5 cells per bottle and five containing 100 cells per bottle.

Proposed Membrane Filtration Method—Aseptically transfer the required volumes from each of 20 containers either directly to each of two sterile filter funnels or to each of two sterile flasks for pooling prior to transfer. [For required volume see the table in the chapter on Sterility Tests (9) in USP XVIII which describes the ratio of product to container content.] If the sample is a suspension, solubilize it by pouring the pooled insulin from a flask into 200–400 ml. of freshly prepared 1.0% ascorbic acid USP in 0.1% (w/v) peptone solution. (If the sample is a solution, omit this solubilization step.) After solubilization is complete, filter the solution through a bacteriological membrane filter. All air entering the system must be passed through air filters capable of removing microorganisms. Filter one 100-ml. portion of 0.1% peptone solution through the membrane to remove residual insulin. Transfer the entire membrane to a sterile 38 × 200-mm. (outside dimensions) test tube containing approximately 90 ml. of sterile thioglycollate medium. Repeat this procedure for the pooled insulin in the second flask, and transfer the second membrane to a tube containing approximately 90 ml. of fluid soybean-casein digest medium. Incubate the tubes for 7 days at 32 and 25°, respectively, and then examine for macroscopic evidence of growth, *i.e.*, the development of turbidity. The batch meets the requirements of the test if growth is absent in all tubes. If growth is observed in any tube, the lot fails to meet the requirements of the test for sterility, unless it can be demonstrated by retests or by other means that the test was invalid for causes unrelated to the product.

RESULTS AND CONCLUSIONS

As shown in Table II, both the high and low levels of microorganisms were recovered by the USP XVII/NF XII direct method and by the proposed membrane method. The inoculum control proved that the added test microorganisms were within the desired range of either 100 cells or 5 cells per 40 ml. of the sample. In each individual test by membrane filtration the microorganisms grew in one of the two media within 7 days. However, the USP/NF direct method required 10 days of incubation for the visual detection of *Aspergillus niger*, and the growth of *Saccharomyces cerevisiae* and *Candida albicans* could be determined only by subculture. Both collaborators agreed that the proposed membrane filtration method using AA diluting fluid to solubilize zinc insulin suspension is acceptable for sterility testing. Since USP XVIII (9) and NF XIII (10) are expected to recognize both the direct and the membrane filtration methods, either may be acceptable for the sterility testing of insulin after these compendia become official. However, several advantages accrue from the use of the filtration test. The incubation time is shortened from 14 to 7 days, the necessity for subculturing turbid broth tubes to determine the presence or absence of microbial growth is eliminated, and the volume of medium required for testing is drastically reduced.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 829.

(2) "National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 497.

(3) "Code of Federal Regulations," Title 21, Part 164.15.

(4) F. W. Bowman, *J. Pharm. Sci.*, **55**, 818(1966).

(5) G. W. Probst, W. F. Brown, and H. J. Henry, *ibid.*, **55**, 1408 (1966).

(6) R. Amos, personal communication.

(7) G. Sykes and M. Hooper, *J. Pharm. Pharmacol.*, **6**, 552 (1954).

(8) "Code of Federal Regulations," Title 21, Part 141.2.

(9) "United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 853.

(10) "First Supplement to NF XIII," Mack Publishing Co., Easton, Pa., 1970, p. 1033.

(11) "Protocol for Collaborative Study of Proposed Sterility Test of Insulin by Membrane Filtration," National Center for Antibiotics and Insulin Analysis, Washington, DC 20204, April 1, 1969 (available upon request).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 25, 1969, from the *Division of Pharmaceutical Sciences, National Center for Antibiotics and Insulin Analysis, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, DC 20204*

Accepted for publication February 3, 1970.

Simultaneous TLC Separation of Khellin and Visnagin and Their Assay in *Ammi visnaga* Fruits, Extracts, and Formulations

M. S. KARAWYA*, M. A. EL-KIEY, A. SINA, and G. NOUR†

Abstract □ A rapid and sensitive two-dimensional TLC–colorimetric method for the simultaneous determination of khellin and visnagin in *Ammi visnaga* fruits, extracts, and formulations is presented. Horstmann's *m*-dinitrobenzene colorimetric method was studied; a modified method, ensuring stability of the produced color and strict adherence to Beer's law over a wide range of concentration (5–150 mcg.), is proposed.

Keyphrases □ Khellin—colorimetric determination, *m*-dinitrobenzene □ Visnagin—colorimetric determination, *m*-dinitrobenzene □ Column chromatography—separation □ TLC—separation, determination □ UV spectrometry—analysis

In 1956, Schönberg and Sidky (1) described a color test for 2-methylchromones with *m*-dinitrobenzene, in the presence of alkali, whereby a deep-violet color was produced. On the basis of this color test, Horstmann (2) later devised a quantitative method for estimating khellin and visnagin in *Ammi visnaga* fruits. He allowed the dry chromone derivative to react with *m*-dinitrobenzene ethanolic solution in the presence of ethanolic potassium hydroxide; he measured the developed dark-blue color at 570 mμ. However, Horstmann stated that the color was unstable and disappeared rather quickly. The authors of this article confirmed the instability of the produced color, and they found that the linear relationship between absorbance and concentration was poor in the range of concentration (10–70 mcg.) described by Horstmann, especially with visnagin. In addition, the ethanolic potassium hydroxide solution (12%) was unstable. It changed rapidly to a reddish-brown color, thus giving interference due to background absorption. Consequently, an attempt was made to improve the *m*-dinitrobenzene colorimetric method so as to obtain a stable, sensitive, and measurable color. It was hoped that this color could be used in a new TLC–colorimetric assay of khellin and visnagin in fruits, extracts, and pharmaceutical formulations of *Ammi visnaga*.

EXPERIMENTAL

Reagents and Apparatus—The following were used: authentic samples of khellin, visnagin, and other known constituents of *Ammi visnaga* fruits; *m*-dinitrobenzene solution, 2% w/v in ethanol; potassium hydroxide solution, 50% in water; sodium chloride solution, 10% in 25% ethanol; isobutanol–ether mixture (1:1); ethanol (aldehyde free); methanol; chloroform; aluminium oxide for adsorption chromatography (British Drug House); silica gel G (Rhône Poulenc); Beckman DU spectrophotometer; and Desaga TLC equipment.

Analytical grade reagents were used whenever possible.

Color Reaction—The factors affecting color development and stability due to the action of *m*-dinitrobenzene and potassium hydroxide on khellin or visnagin were investigated. These factors were: (*a*) temperature, (*b*) time, (*c*) medium of reaction, and (*d*) concentration of reactants. The following experimental conditions for the colorimetric estimation of khellin or visnagin were found to be optimum.

For a concentration range of 5–150 mcg. of either khellin or visnagin in 1.5 ml. of an ethanolic solution, 0.5 ml. of 2% ethanolic *m*-dinitrobenzene and 0.5 ml. of 50% aqueous potassium hydroxide were adequate, giving a final ethanolic strength of about 75% in the medium. The color was obtained best at 20 ± 2°, 30 min. after the addition of the reagents. The stability of the formed color was improved by its extraction with an isobutanol–ether mixture (1:1) in the presence of sodium chloride (10%) in ethanol (25%). The absorbance of the colored organic layer was measured at 570 mμ within 30 min. after the addition of the sodium chloride solution.

Standard Curve—These same conditions were employed, and absorbance–concentration curves for khellin and for visnagin were plotted using the following method. Introduce, from a 0.01% ethanolic solution of either khellin or visnagin, variable concentrations (5–150 mcg.) into dry, glass-stoppered 15-ml. test tubes and complete to 1.5 ml. with ethanol. To each tube add 0.5 ml. of *m*-dinitrobenzene solution and 0.5 ml. of potassium hydroxide solution. Mix gently and maintain the tube at 20 ± 2° for 30 min. Then add 5 ml. of sodium chloride solution and 5 ml. of isobutanol–ether (1:1). Stopper the tube, shake it for about 10 sec., and allow the phases to separate completely. Pipet a portion of the clear bluish-violet upper layer into a 1-cm. silica cell; measure the absorbance of the color at 570 mμ against a blank within 30 min. after the addition of the sodium chloride solution.

The amount of khellin or visnagin in a solution of unknown concentration can be deduced from the respective standard curve or simply by adaptation of a *K* factor:

$$K_{\text{khellin}} = \frac{\text{khellin (mcg.)}}{A_{570}} = 85.1 \qquad \text{(Eq. 1)}$$

$$K_{\text{visnagin}} = \frac{\text{visnagin (mcg.)}}{A_{570}} = 95.4 \qquad \text{(Eq. 2)}$$

where A_{570} is the absorbance value of the respective concentration at 570 mμ. These *K* values (average of six concentrations) of khellin and visnagin must be ascertained according to prevailing experimental conditions.

Quantitative TLC Recovery of Khellin and Visnagin from Their Mixtures—Silica gel G plates[1] (20 × 20 cm. and 0.25 mm. thick) were spotted with ethanolic solutions of both khellin and visnagin corresponding to a concentration range of 20–120 mcg. for each. Spotting was done, with an Agla micrometer, at the corner of each plate 2 cm. away from the lower edge and the side edge. The plates were developed with ethyl acetate until the solvent front ascended about 15 cm. The developed plates were allowed to dry and then were redeveloped with the same solvent to 15 cm. at a right angle to the former direction. The plates were then dried and examined under UV light. The well-separated yellowish-brown and greenish-blue fluorescent areas corresponding to khellin and visnagin, respectively, were removed quantitatively by means of a vacuum zone extractor and eluted with 5 ml. of chloroform. Simultaneously, similar blank areas of the silica gel G layer, free from any constituent, were treated in the same way. The chloroform was evaporated on a water bath; the residues, after cooling, were dissolved in 1.5 ml. of ethanol. The ethanolic solution was then treated with the color-developing rea-

[1] Before the layer was spread, the silica gel G was washed three times with three parts of boiling methanol and dried.

Table I—Recovery of Khellin and Visnagin from Their Mixtures by Silica Gel G Plates

Added, mcg.	Recovered, mcg.	Recovery, %
	Khellin	
20	19.0	95.0
40	39.2	98.0
60	58.5	95.0
80	77.5	96.1
100	96.5	96.5
120	118.2	98.5
	Visnagin	
120	119.0	99.1
100	98.0	98.0
80	80.1	100.1
60	59.5	96.1
40	41.4	103.9
20	19.4	97.0

Table II—Analysis of Khellin and Visnagin[a] in Samples of *Ammi visnaga* Fruits and Extracts

Khellin, %	Visnagin, %
Fruits	
1.078	0.761
1.168	0.667
1.221	0.718
1.070	0.629
1.260	0.650
Extracts	
0.599	0.372
0.627	0.392
0.601	0.437
0.559	0.417
0.530	0.401

[a] Results represent the average of at least three assays.

gents, and the color was measured following the method previously outlined. Results were deduced from previously constructed standard curves of reference khellin and of reference visnagin recovered from silica gel G plates (Table I).

Determination of Khellin and Visnagin in the Fruits and Extracts of *Ammi Visnaga* by the TLC-Colorimetric Method—*In Fruits*—Transfer 0.5 g. of powdered *Ammi visnaga* fruits to the top of 2 g. of acid alumina in the thimble of a small continuous extraction apparatus. Exhaust the powder with chloroform (Exhaustion is tested by evaporating a few milliliters of the extract to dryness and then adding a few drops each of ethanol, *m*-dinitrobenzene solution, and potassium hydroxide solution. No violet color should be obtained.) Evaporate the extract to dryness, dissolve the residue in chloroform, adjust the volume to 1.0 ml. with chloroform in a volumetric flask, and mix well. Apply aliquots of the chloroform solution containing 30–100 mcg. each of khellin and visnagin (usually about 10-30 μl.) to the corner of a silica gel G plate and proceed as described under the preceding method. See Table II.

In Extracts—In the case of the liquid extract of *Ammi visnaga* fruits (Egyptian Pharmacopoeia 1963), an aliquot expected to contain 30–100 mcg. each of khellin and visnagin (corresponding to about 10–30 μl.) was applied directly to the plate and treated as previously described. See Table II.

Determination of Khellin in Pharmaceutical Formulations—*Simple Khellin Formulations*—Lynamine and Lynamine Forte tablets,[2] containing 20 and 100 mg. of khellin, respectively, were used (Table III).

Weigh and powder 20 tablets. To an amount of the powder equivalent to 40 mg. of khellin, add 40 ml. of ethanol and stir with a magnetic stirrer, equipped with a heating device, for 30 min. Filter the supernatant alcoholic solution through dry filter paper (Whatman No. 2) into a dry 100-ml. volumetric flask. Repeat extraction of the residue with 3 × 10 ml. of ethanol, filtering each time through the same filter into the same receiver. Wash, cool to room temperature, and adjust to volume with ethanol. Mix well. Dilute 10 ml. of this solution to 100 ml. with ethanol. From this dilution, pipet off 1.5 ml. (equivalent to 60 mcg. of khellin) into a dry, glass-stoppered test tube. Proceed with the *m*-dinitrobenzene colorimetric method described in the *Standard Curve* section, starting with the words, "add 0.5 ml. of *m*-dinitrobenzene solution. . . ."

Complex Khellin Formulations—Glucolynamine injections[2] were used. Each 10 ml. of the injection solution contained: khellin, 30 mg.; theophylline, 150 mg.; and glucose, 2000 mg.

Column Chromatographic Method—Measure accurately 10 ml. of the injection solution in a separator. Dilute with 30 ml. of water and extract with 4 × 20 ml. of chloroform. Filter the combined chloroform extracts, through a small layer of anhydrous sodium sulfate previously washed with chloroform, into a 100-ml. volumetric flask. Wash the filter, and adjust to volume with chloroform. Mix well. Evaporate 10 ml. of the chloroform solution (equivalent to 3 mg. of khellin) to dryness. Dissolve the residue in 2 ml. of chloroform and transfer to the top of a previously prepared 17-g. aluminium oxide column (1.4-cm. diameter). Carry out the chromatographic process in a dark cabinet equipped with UV light. Wash the column with chloroform containing 2% methanol. Reject the first nonfluorescent 8–10 ml. of effluent. Collect the yellowish-green fluorescent khellin zone into a 50-ml. volumetric flask until the eluate is free from any fluorescence. Bring to volume with chloroform and mix well. One milliliter of this solution is equivalent to 60 mcg. of khellin.

Pipet off 1 ml. of the chloroform extract, evaporate to dryness, dissolve the cooled residue in 1.5 ml. of ethanol, and complete the assay as previously described.

TLC Method—Spot directly a 20-μl. aliquot of the injection solution (equivalent to 60 mcg. of khellin) on a silica gel G (methanol-washed) plate and develop the plate with the chloroform–ethanol (98.5:1.5) solvent system (3) for a distance of 15 cm.

Dry the plate in air, elute khellin from the respective spot area, and proceed as previously described.

RESULTS AND DISCUSSION

During this investigation of factors affecting the development and stability of the studied color reaction, the authors found that the test could be conducted in ethanolic solutions of khellin or visnagin instead of the dried residues of the chromone substances mentioned by Horstmann (2). Accordingly, the evaporation step of the ethanolic solutions of the chromones could be avoided. The direct conduction of the reaction was possible on ethanolic solutions of khellin prepared by extraction or simple dilution of pharmaceutical preparations, *e.g.*, tablets and ampuls.

The appropriate concentration of *m*-dinitrobenzene was found to be 2% ethanolic solution as described by Horstmann (2). Lower concentrations, *e.g.*, 1%, gave lower intensities of the developed color, while higher concentrations were unsuitable because crystals separated on standing. The 2% ethanolic solution was reasonably stable when kept at room temperature (25–30°) for about 2 weeks.

The color produced by reacting khellin or visnagin with *m*-dinitrobenzene in 95% ethanol was more intense than with the 50% ethanolic medium. However, the bluish-violet color obtained in 95% ethanol, as described by Horstmann, was highly unstable and thus difficult to measure. The reddish-brown color obtained in the 50% ethanolic medium had a different wavelength of maximam absorption (480 mμ) and did not obey Beer's law. On further trials, 75% ethanol was found to be a suitable medium for obtaining a sensitive and stable color. This was achieved by the addition of 0.5 ml. of 50% aqueous potassium hydroxide solution to a mixture of ethanolic solution of the sample (1.5 ml.) and 2% *m*-dinitrobenzene solution in 95% ethanol (0.5 ml.).

The optimum temperature for this color reaction was found to be 20 ± 2°. Raising the temperature to 30 or 40° caused a diminution in the color intensity; the absorbances decreased by 20 and 15% at 30° for khellin and visnagin, respectively, although linearity between concentration and absorbance was still maintained.

The time necessary for maximum color intensity was found to be 30–40 min. Dilution of the reaction mixture after full development of the color with 50% ethanol, as described by Horstmann, led to a rapid fading of the color and diminution of the absorbance values. To increase the stability and sensitivity of the formed color after full development, the authors attempted its isolation by extraction of the reaction mixture with suitable organic solvents—*viz.*,

[2] Memphis Chemical Co., Cairo, U.A.R.

Table III—Analysis of Pharmaceutical Formulations of Khellin[a]

Samples	Labeled Khellin Content, mg.	Khellin Found, mg.	Recovery, %
Lynamine tablets	20	20.3	101.5
Lynamine Forte tablets	100	100.3	100.3
Glucolynamine injection	30		
Column		29.44	98.1
TLC		29.98	99.9

[a] Results represent the average of at least three assays.

chloroform, ether, isopropyl ether, and isobutanol. The color could not be extracted with less polar solvents such as *n*-hexane, cyclohexane, and benzene. With chloroform and isopropyl ether, the clarity of the colored solution was impaired by the presence of interfering opalescence which could not be removed by either filtration or centrifugation. Ether gave a clear solution which was stable for about 110 min., but its volatility rendered it unsuitable for spectrophotometric measurements. Extraction of the colored product with isobutanol resulted in a clear solution, but the color was unstable. An isobutanol–ether mixture (1:1) proved to be the most suitable solvent, being less volatile and giving sharp and quick separation of the phases during extraction of the developed color, which was aided by the addition of 10% sodium chloride in 25% ethanol. The separated organic layer was clear and suitable for direct spectrophotometric measurement. The color thus obtained was found to be stable for at least 45 min. and obeyed Beer's law over a concentration range of 5–150 mcg. for either khellin or visnagin, which is a much wider range than that described by Horstmann (10–70 mcg.).

3,5-Dinitrobenzoic acid was also tried as a color-developing reagent, using similar conditions as described for the *m*-dinitrobenzene method. It gave a bluish-violet colored product with khellin and visnagin and was also soluble in an isobutanol–ether mixture (1:1).

Khellol and khellol-glucoside reacted also with *m*-dinitrobenzene and 3,5-dinitrobenzoic acid in the same manner as did khellin and visnagin, although their colored products with 3,5-dinitrobenzoic acid were not extractable with the organic solvent mixture, isobutanol–ether (1:1).

Extraction of the active principles from the powdered fruits was carried out by using chloroform according to a previously described method (4). Horstmann used boiling water, a nonselective solvent leading to the extraction of water-soluble impurities, which necessitated a preliminary purification of the aqueous extract before paper chromatography. The presence of acid aluminium oxide during the extraction step was beneficial; it retained most of the extractive matter other than the chromone constituents (4). In the proposed method, the chloroformic extract was chromatographed (TLC) directly without any need for preliminary purification.

Horstmann effected the separation of khellin and visnagin from *Ammi visnaga* fruit extracts by paper chromatography using filter paper impregnated with polyamide and water-saturated butanol as the developing solvent. In view of the well-known advantages, especially rapidity, of TLC over paper chromatography, the authors of this study resorted to the former technique. Successful separation of khellin and visnagin from the other constituents of *Ammi visnaga* fruits has been achieved on silica gel G plates (3). Contrary to previous findings (5, 6), the authors were not able to achieve complete separation of khellin from visnagin using the solvent systems described. Solvent systems other than those reported in the literature, *e.g.*, chloroform–ethanol–methanol (97:1:2), chloroform–methanol–formamide (90:5:5), and chloroform–methanol–water (90:9:1), were tried and found unsatisfactory. However, two-dimensional TLC on silica gel G plates (Fig. 1), using ethyl acetate as the developing system, offered a better separation of khellin from visnagin than the unidimensional multiple-run technique. This method was used in the quantitative recovery of these two constituents and their subsequent colorimetric estimation. The results cited in Table I indicate the efficient recovery of khellin (95–98.5%) and visnagin (96.1–103.5%). The ratio of khellin content to visnagin content in the examined fruits and extracts of *Ammi visnaga* was found to range from 1.4:1 to 1.9:1 and from 1.3:1 to 1.6:1, respectively.

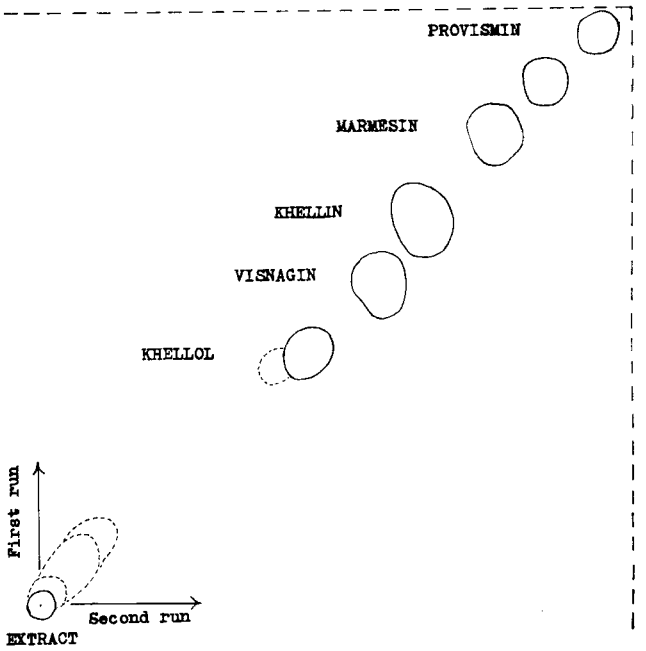


Figure 1—*Two-dimensional TLC using ethyl acetate as the developing system.*

Quantitative extraction of khellin from tablets was achieved efficiently by hot ethanol. The *m*-dinitrobenzene colorimetric method could then be applied directly to the suitably diluted ethanolic extract. On the other hand, direct application of this color reaction to complex khellin formulations (*e.g.*, glucolynamine injection) after suitable dilution with ethanol (1:100) was unsuccessful. The results obtained were about 70% of the labeled khellin concentration. Thus the separation of khellin from the other interfering ingredients (theophylline and glucose) was deemed necessary. This was achieved by either chromatographing the chloroform extract on aluminium oxide or recovery of khellin by TLC prior to its colorimetric determination.

REFERENCES

(1) A. Schönberg and M. M. Sidky, *J. Org. Chem.*, **21**, 476(1956).

(2) C. Horstmann, *Kulturpflanze*, **10**, 132(1962); through *Chem. Abstr.*, **59**, 6708*b*(1963).

(3) L. Hörhammer and H. Wagner, *Deut. Apoth.-Ztg.*, **102**, 733 (1962).

(4) M. S. Karawya, A. Sina, and G. Nour, to be published.

(5) S. I. Balbaa, A. Y. Zaki, and S. M. Abdel-Wahab, *Planta Med.*, **16**, 329(1968).

(6) M. M. Badawi and M. B. E. Fayez, *ibid.*, **15**, 140(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 10, 1969, from the *Quality Control Department, Chemical Industries Development Laboratories (CID), Giza, U.A.R.*, and the *Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, U.A.R.*

Accepted for publication January 14, 1970.

* Present address: Faculty of Pharmacy, Kasrel Aini, Cairo, U.A.R.

† Present address: Chemical Industries Development (CID), Giza, U.A.R.

Inhibitory Properties of Aminoalkylsuccinimides on Isolated Horse Serum Butyrylcholinesterase: *N*-Methyl-2-phenyl-2-(*tert*-aminoalkyl)succinimides

EDWARD O. MAGARIAN, SAMUEL TERRY CHRISTIAN*, and HARRY C. CLEMSON†

Abstract □ The butyrylcholinesterase inhibitory activities of several *N*-methyl-2-phenyl-2-(*tert*-aminoalkyl)succinimides were evaluated. The derivatives, all of which showed a mixed type of inhibition, were more potent as competitive than noncompetitive inhibitors. The results are discussed in terms of a possible mechanism for the formation of enzyme-inhibitor complexes.

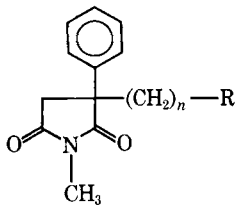
Keyphrases □ *N*-Methyl-2-phenyl-2-(*tert*-aminoalkyl)succinimides—inhibition, butyrylcholinesterase □ Butyrylcholinesterase inhibition—aminoalkylsuccinimides □ TLC—analysis

The effectiveness of compounds containing the functional moiety N—C—C—CO—N (aminopropionamide) as inhibitors of serum cholinesterase (acylcholine acylhydrolase, E.C. 3.1.1.8) has been demonstrated (1–6). Several structural parameters have been investigated for possible correlations with potency of enzyme inhibition. For example, Lasslo *et al.* (3) found a direct relationship between the length of the alkyl chain in 3-(1-alkylpiperidyl)carboxamides and their ability to inhibit plasma cholinesterase (PChE), and Beasley and Williford (5) observed that the introduction of aralkyl groups between the two ring nitrogen atoms in bis(3-piperidinecarboxamides) increases the inhibitory potency of these compounds.

The influence of amino substituents on the rates of hydrolysis of certain pseudocholinesterase substrates has also been investigated. Holmstedt and Sjoqvist (7) reported that the introduction of an amino group in butyrylcholine, the optimum substrate for pseudocholinesterase, to give γ-aminobutyrylcholine strongly inhibits its hydrolysis by this enzyme. Beckett *et al.* (8) reported that benzoylcholine has a greater affinity than butyrylcholine for PChE, even though it is hydrolyzed at only 15% of the rate of butyrylcholine. Prompted by the reported effect of the amino group in γ-aminobutyrylcholine, they evaluated *p*-aminobenzoylcholine and found that it not only has a greater affinity for the enzyme than does benzoylcholine, but it is a more potent inhibitor of the hydrolysis of butyrylcholine and acetylcholine.

Recently, the preparation of several *tert*-aminoalkylsuccinimides (Structure I) was reported (9). These compounds embody structural features resembling not only those in the 3-piperidinecarboxamides but also those in γ-aminobutyrylcholine and *p*-aminobenzoylcholine.

Interest in the influence of amino substituents on cholinesterase-inhibitor interactions prompted the authors to investigate the cholinesterase-inhibitory properties of these succinimides. In the present report, some preliminary data are presented and discussed.



R = *tert*-amino group
n = 1 or 2

I

MATERIALS AND METHODS

All reagents and derivatives used in this study were of analytically pure grade or equivalent. These include acetylcholine chloride[1] and horse serum butyrylcholinesterase (acylcholine acylhydrolase, E.C. 3.1.1.8) in the form of a lyophilized powder.[2] The succinimide derivatives were synthesized according to the procedures of Clemson *et al.* (9).

All the inhibitors were shown to be pure by TLC on silica gel, using various concentrations of absolute ethanol in benzene as the developing solvents (Table I).

Enzyme rate measurements were carried out potentiometrically with butyrylcholinesterase using a Radiometer automatic titrator (Type TTTlc) equipped with a recorder (SBR2c) and a syringe buret unit (SBU1a). Enzyme initial-velocity measurements were recorded at pH 7.40 ± 0.05 in a 25-ml. thermostated vessel at 27.0 ± 0.10°. All reactions were run under a nitrogen atmosphere using a combination glass–calomel electrode (GK2026c), mechanical stirring, and 0.01 *N* sodium hydroxide as the titrant. The titration procedure is essentially the method developed by Stein and Laidler (10) in their studies on the kinetics of α-chymotrypsin. The reaction mixtures were 0.04 *M* in magnesium chloride and 0.01 *M* in sodium chloride and contained substrate, inhibitor, and 0.11 mg. of enzyme.[3] The reaction mixtures, both control and with inhibitors, were preincubated for 5 min. at 27° prior to the initiation of the reaction by the addition of substrate. This preincubation time did not affect the initial rate measurement under the experimental conditions. Six substrate concentrations ranging from 7.7×10^{-3} *M* to 2.2×10^{-3} *M* (each differing by 1.1×10^{-3} *M*) were employed. The total reaction volume was 15 ml. At least two or more inhibitor concentrations were used, and the resulting data were plotted according to the method of Lineweaver and Burk (11). Data on the inhibitor dissociation constants were calculated from these plots (Fig. 1) according to a method described by Krupka (12). The final competitive (K_I) and noncompetitive (K_I') inhibitor dissociation constants are averages of at least two corresponding and independent determinations.

RESULTS AND DISCUSSION

The cholinesterase inhibitory potencies of the title compounds are shown in Table I. A typical plot used in determining the competitive (K_I) and noncompetitive (K_I') inhibitor dissociation constants for one of the inhibitors appears in Fig. 1. All of the com-

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Worthington Biochemical Corp., Harrison, N. J.
[3] The specific activity of the butyrylcholinesterase was found to be approximately 6.3 units/mg., where the activity is expressed as μmole of acetylcholine hydrolyzed/min./mg. of protein at 27° and pH 7.4.

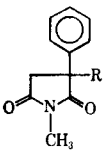


Table I—Inhibition of Isolated Horse Serum Cholinesterase by Succinimides

Compound	R	Absolute EtOH in Benzene[a]	Competitive Inhibitor Dissociation Constant (K_I)	Noncompetitive Inhibitor Dissociation Constant (K_I')
1	$-CH_2-N(CH_3)_2 \cdot HCl$	2%	1.57×10^{-4}	8.53×10^{-4}
2	$-CH_2-N(C_2H_5)_2 \cdot HCl$	2%	1.66×10^{-4}	7.31×10^{-4}
3	$-CH_2-N$ (pyrrolidine) $\cdot HCl$	2%	2.41×10^{-4}	1.25×10^{-3}
4	$-CH_2-N$ (piperidine) $\cdot HCl$	1%	3.00×10^{-5}	2.01×10^{-4}
5	$-CH_2-N$ (4-methylpiperazine) $\cdot 2HCl$	25%	1.11×10^{-4}	7.05×10^{-4}
6	$-CH_2-CH_2-N(CH_3)_2 \cdot HCl$	25%	4.44×10^{-4}	2.40×10^{-3}
7	$-CH_2-CH_2-N(C_2H_5)_2 \cdot HCl$	50%	2.44×10^{-5}	6.77×10^{-4}
8	$-CH_2-CH_2-N$ (pyrrolidine) $\cdot HCl$	25%	1.69×10^{-4}	6.36×10^{-4}
9	$-CH_2-CH_2-N$ (piperidine) $\cdot HCl$	10%	1.35×10^{-4}	7.97×10^{-4}
10	$-CH_2-CH_2-N$ (4-methylpiperazine) $\cdot 2HCl$	50%	1.07×10^{-4}	6.32×10^{-4}
11	$-H$	50%	1.45×10^{-3}	5.43×10^{-3}

[a] As an index of purity, all compounds were chromatographed on Eastman Silica Gel Chromagram Sheets (6061), using solutions of absolute ethanol in benzene as developing solvents.

pounds in this study displayed a mixed type of inhibition (13), thus indicating the presence of a competitive and a noncompetitive component. This suggests that the inhibitors are binding both at the active site (competitive) and at a peripheral site (noncompetitive).

The aminoalkyl derivatives of *N*-methyl-2-phenylsuccinimide are significantly more potent inhibitors of butyrylcholinesterase than is the parent compound (Table I). This is reflected by their lower competitive and noncompetitive inhibitor dissociation constants. However, it should be noted that these amino derivatives are more effective as competitive than as noncompetitive inhibitors. Discussion of the data will be limited to the competitive component, since it is this component that directly interferes with the binding of substrate.

It is apparent from Table I that there is no correlation between potency of inhibition and length of alkyl chain between the carbonyl carbon and the tertiary nitrogen. Indeed, the lengths of the chains in Compounds 4 and 7, the most potent inhibitors of the series, differ by one methylene group. This is in contrast to the observed effect in the piperidinecarboxamide series. The 4-piperidinecarboxamides, which contain three carbon atoms between the carbonyl carbon and the nitrogen atom, are less potent inhibitors than their 3-isomers, which contain only two (4). In the succinimide series, the aminoalkyl side chain is flexible and may, therefore, assume various conformations to accommodate the steric requirements of the active site on the enzyme. However, this is not possible in the piperidinecarboxamides since the aminoalkyl group, being part of the piperidine ring, is held in a rigid conformation.

From the results reported by Holmstedt and Sjoqvist (7), it would appear that the introduction of an amino group in butyrylcholine to give γ-aminobutyrylcholine induces a change in its mode of binding to butyrylcholinesterase. This modification of the substrate molecule may result in an improper orientation of the ester group at the esteratic site and, thereby, produce a reduction in the rate of hydrolysis. To illustrate this hypothesis, one may assume that the conformation of butyrylcholine in Fig. 2a is required for maximum hydrolysis. Then, as a consequence of an interaction between the amino group and a complementary group at Site A (A′, Fig. 2b), γ-aminobutyrylcholine may assume a slightly different conformation. The resulting conformational change of the ester moiety at the esteratic site (Site B) may be unfavorable for maximum hydrolysis. This effect is analogous to the apparent disturbance of an "ideal fit" between the enzyme and substrate by α- and β-methyl groups in butyrylcholine (8).

From all indications, binding of benzoylcholine and *p*-aminobenzoylcholine to the enzyme is similar to that for butyrylcholine and γ-aminobutyrylcholine (8). Benzoylcholine has a greater affinity but a lower hydrolytic rate than does butyrylcholine. The hydrophobic interactions of the phenyl ring of benzoylcholine with Area A (Fig. 2) would explain this increase in binding energy and would also tend to explain the decreased rate of hydrolysis due to an accompanying change of conformation of the ester group at Site B (esteratic site). The additional binding energy reported by Beckett *et al.* (8) in *p*-aminobenzoylcholine strongly suggests that the amino substituent further contributes to the affinity of the molecule for the enzyme by bonding with a complementary group at Site A.

It would seem probable that the interaction between the aromatic amino substituent in *p*-aminobenzoylcholine and a complementary

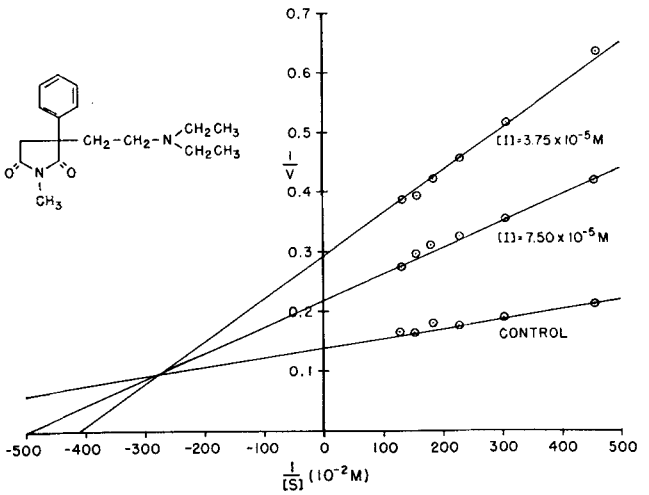


Figure 1—*Mixed inhibition of horse serum cholinesterase (butyrylcholinesterase) by* N*-methyl-2-phenyl-2-(2-diethylaminoethyl)succinimide (Compound 7, Table I). Abscissa, reciprocal molar acetylcholine chloride concentration; ordinate, reciprocal enzyme initial velocity. Velocity is expressed in μmoles of acetylcholine hydrolyzed/min./mg. of protein.*

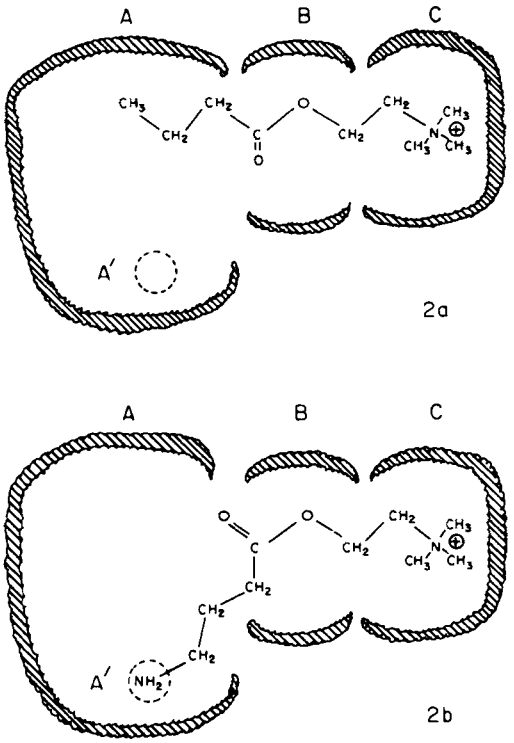


Figure 2—*Concept of differences in conformations of butyrylcholine (2a) and γ-aminobutyrylcholine (2b) at the substrate binding site of butyrylcholinesterase. Key: A = nonpolar area; A' = complementary area in A which interacts with amino group; B = esteratic site; and C = "anionic" site.*

group on the enzyme is not ionic in nature. Esters of *p*-aminobenzoic acid are weakly basic, *e.g.*, pK_b of ethyl *p*-aminobenzoate is 10.84 (14) and, consequently, the existence of the conjugate acid of *p*-aminobenzoylcholine at physiological pH would be negligible. Thus, it would appear that hydrogen bonding or dipolar interactions are more likely involved. However, in the case of γ-aminobutyrylcholine, its conjugate acid would be quite significant at physiological pH and could, therefore, enter into H-bond or ion dipole formation at Site A.

The mode of competitive binding of *N*-methyl-2-phenylsuccinimide (Compound 11, Table I) and its derivatives may be visualized as being analogous to those for butyrylcholine, benzoylcholine, and their amino derivatives. In this case, however, the imide function is pictured as complexing with the esteratic site. The interaction of amide moieties with esteratic sites of cholinesterases has already been proposed by several investigators (4, 6, 15). The aromatic ring, like that in benzoylcholine, may be involved in hydrophobic interactions with Site A and may, thereby, exert a directing influence in the formation of the enzyme-inhibitor complex. The increase in binding energies of the aminoalkylsuccinimide derivatives may be attributable to an additional interaction between the amino substituents and an appropriate group at Site A.

The data presented in this report on the inhibitory properties of some *tert*-aminoalkylsuccinimides are consistent with the hypothesis advanced to explain the interactions of γ-aminobutyrylcholine and *p*-aminobenzoylcholine with butyrylcholinesterase. From a study on the inhibitory properties of a series of 1-substituted 3-piperidinecarboxamides, Beasley *et al.* (6) offered an alternate explanation for the interaction of amino groups with the active site of butyrylcholinesterase. They suggested that the amide function binds at the esteratic site while the nitrogen atom of the piperidine ring binds at the "anionic" site (Site C, Fig. 2). There is no definitive evidence at the present time that discounts either of these hypotheses. Perhaps both modes of binding are operative. The real significance of these proposed mechanisms must await further experimentation.

REFERENCES

(1) A. Lasslo, P. D. Waller, A. L. Meyer, and B. V. Rama Sastry, *J. Med. Pharm. Chem.*, **2**, 617(1960).

(2) A. Lasslo, P. D. Waller, and G. J. Epperson, *J. Med. Chem.*, **6**, 26(1963).

(3) A. Lasslo, J. G. Beasley, G. G. Nelms, and G. J. Epperson, *ibid.*, **6**, 811(1963).

(4) J. G. Beasley, R. P. Quintana, and G. G. Nelms, *ibid.*, **7**, 698(1964).

(5) J. G. Beasley and L. L. Williford, *ibid.*, **10**, 76(1967).

(6) J. G. Beasley, S. T. Christian, W. R. Smithfield, and L. L. Williford, *ibid.*, **10**, 1003(1967).

(7) B. Holmstedt and F. Sjoqvist, *Biochem. Pharmacol.*, **3**, 297(1960).

(8) A. H. Beckett, M. Mitchard, and J. W. Clitherow, *ibid.*, **17**, 1601(1968).

(9) H. C. Clemson, E. O. Magarian, G. C. Fuller, and R. O. Langner, *J. Pharm. Sci.*, **57**, 384(1968).

(10) B. R. Stein and K. J. Laidler, *Can. J. Chem.*, **37**, 1272 (1959).

(11) H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, **56**, 658 (1934).

(12) R. M. Krupka, *Biochemistry*, **5**, 1983(1966).

(13) J. L. Webb, "Enzyme and Metabolic Inhibitors," vol. I, Academic, New York, N. Y., 1963, pp. 54–55, 160–164.

(14) M. E. Krahl, A. K. Keltch, and B. H. A. Clowes, *J. Pharmacol.*, **68**, 330(1940).

(15) W. P. Purcell, J. G. Beasley, R. P. Quintana, and J. A. Singer, *J. Med. Chem.*, **9**, 297(1966).

ACKNOWLEDGMENT AND ADDRESSES

Received December 22, 1969, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, University of Kentucky, Lexington, KY 40506*

Accepted for publication February 24, 1970.

This investigation was supported in part by the University of Kentucky Research Foundation (Grant 6800135).

The authors gratefully acknowledge the technical assistance of Myra Osborne Heintz.

* Present address: National Institute of Mental Health, Addiction Research Center, Lexington, KY 40507

† Present address: Northeastern University, College of Pharmacy, Boston, MA 02115

Antimalarial Properties of a Variety of Substituted *p*-Sulfamoylphenylazo Compounds

R. E. HARMON, B. L. GELLER, S. K. GUPTA, M. H. HERBERT, and D. CHITHARANJAN

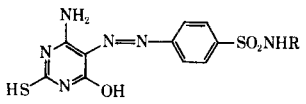
Abstract □ A wide variety of substituted *p*-sulfamoylphenylazo compounds were prepared, and their activity against *Plasmodium berghei* in mice was studied. A study of the effects of substitution in the sulfamoyl group revealed that substitution by pyrimidine, methylpyrimidine, and hexylresorcinol greatly increased the antimalarial activity, while substitution by thiazole, methylthiazole, and 2-methyl-3,4-thiadiazole did not lead to a comparable increase in activity. The least activity was seen in compounds with 2-pyridyl as the substituent. Coupling compounds derived from sulfa drugs, hexylresorcinol, and 1,3-dimethyl-6-aminouracil were either active or curative at lower doses than the remainder of the compounds evaluated. With pyrimidine as the substituent, an amino group in position 4 or 5 was necessary for antimalarial activity. It was also found that coplanarity is not an essential structural requirement for antimalarial activity.

Keyphrases □ *p*-Sulfamoylphenylazo compounds, substituted—antimalarial properties evaluated □ Antimalarials, evaluation—*p*-sulfamoylphenylazo compounds □ UV spectrophotometry—identification, structure □ IR spectrophotometry—identification, structure

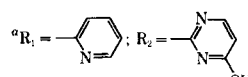
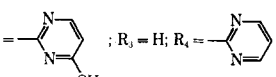
Since Lythgoe *et al.* (1) found that azo coupling occurs in the 5-position of the pyrimidine ring, various substituted 5-arylazopyrimidines have been synthesized and their mode of action in various biological systems studied (2). It was found that at least one amino group adjacent to the arylazo link is necessary for optimum activity and that the aryl group should be unsubstituted or contain electron-releasing substituents for maximum biological activity (3–5). The inhibitory actions of 5-phenylazo-2,4,6-triaminopyrimidine and 5-phenylazo-2,4-diamino-6-hydroxypyrimidine were unaffected by most bases and nucleotides involved in nucleic acid synthesis. This finding, in conjunction with the findings of Roy-Burman and Sen (5) that the inhibitory effects of arylazopyrimidines in the *Streptococcus faecalis* (ATCC 8043) system could be more efficiently reversed by 5-formyltetrahydrofolic acid than by folic acid itself, would seem to indicate that arylazopyrimidines may act as folic acid antagonists and interfere with the enzymatic conversion of folic acid to 5-formyltetrahydrofolic acid. Recent work by Hampshire *et al.* (6) on the inhibitory effects of 5-arylazo-2,4,6-triaminopyrimidines on folic acid reductase from rat liver indicates that these compounds exhibit a wide range of activities, depending on the aryl substituent. There is a strong indication that some sulfonylphenylazo compounds have a high synergistic effect when used in combination therapy (7).

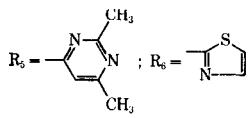
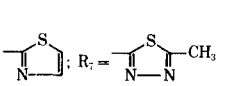
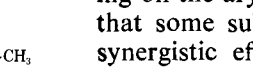
The reactions of various diazotized sulfa drugs with hexylresorcinol, 2,6-diaminopyridine, 3-phenylazo-2,6-diaminopyridine, 1-phenyl-3-methyl-5-pyrazolone, 1-phenyl-3-carbethoxy-5-pyrazolone, and several substituted pyrimidines were studied as an extension of the authors' earlier investigation (2) of the antimalarial

Table I—Activity Data of Substituted *p*-Sulfamoylphenylazo Compounds



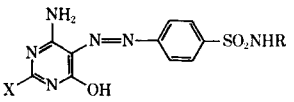
R[a]	M.p.	UV Data mμ	$\epsilon_{max.}$ (10^4)	Dose, mg./kg.	IST[b]	Activity in *P. berghei*[c]
R_1	300°	400	1.21	640	18.0	++
		286	10.1			
		240	14.1			
R_4	221–227°	392	3.56	160	15.8	++
		285	5.70	640	29.0	++++
		240	11.1			

[a] R_1 =  ; R_2 =  ; R_3 = H; R_4 =  ;

R_5 =  ; R_6 =  ; R_7 = 

[b] Increase in (mean survival time of the treated group minus mean survival time of the control group) mean survival time of control mice (M.S.T.) was 6 days. [c] + = 100% increase in survival time, 6.0 ± 0.5 days; ++ = greater than 100% increase in survival time; +++ = greater than 100% increase in survival time but not curative; ++++ = curative, less than 30-days survival. See *Reference 8* for procedures used in evaluating compounds for antimalarial activity.

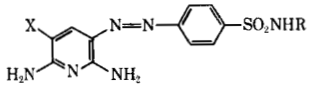
Table II—Activity Data of Substituted *p*-Sulfamoylphenylazo Compounds



R[a]	X	M.p.	UV Data mμ	$\epsilon_{max.}$ (10^4)	Dose, mg./kg.	IST[b]	Activity in *P. berghei*[c]
R_1	NH_2	268–270°	392	7.1	320	20.6	++
			288	1.54	640	21.8	++
			235	7.4			
R_2	NH_2	248–250°	394	2.9	160	13.0	+
			264	7.5	640	22.0	+++
R_5	NH_2	250°	395	7.8	80	13.2	+
			250	7.8	160	12.8	+
					640	21.0	++
R_6	OH	>300°	398	10.93	320	13.8	+
			255	7.01	640	16.8	++
R_3	SCH_3	283–285°	398	11.52	640	15.4	++
			255	6.21			
R_1	SCH_3	200°	397	7.52	80	12.8	+
			247	8.52	160	11.2	+
					640	22.0	+++
R_7	SCH_3	225°	399	10.89	640	16.2	++
			253	7.38			

[a,b,c] See Table I.

Table III—Activity Data of Substituted *p*-Sulfamoylphenylazo Compounds



R[a]	X	M.p.	UV Data mμ	UV Data $\epsilon_{max.}$ (10^4)	Dose, mg./kg.	IST[b]	Activity in *P. berghei*[c]
R_1	H	237–239°	449	9.96	40	13.2	+
			284	5.53	160	15.8	++
			220	6.42	640	19.0	++
R_2	H	225–235°	451	8.06	40	18.8	++
			272	4.30	160	27.2	+++
			240	6.22			
R_5	H	251–257°	499	7.80	160	14.6	++
			273	5.61	640	17.6	++
			247	4.52			
R_6	H	150°	451	9.75	160	15.2	++
			272	6.00	640	33.0	+++
R_7	H	263–266°	453	8.19	640	16.0	++
			270	4.68			
R_2	N_2Ph	221–223°	472	17.57	160	12.4	+
			310	4.29	320	14.0	++
			263	8.00	640	20.8	++
R_4	N_2Ph	215–219°	428	6.92	160	16.4	++
			240	6.63	320	22.7	+++
					640	34.0	++++
R_6	N_2Ph	222–227°	428	7.09	640	17.2	++
			276	4.02			

[a,b,c] See Table I.

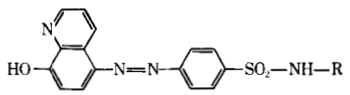
and anticancer properties of arylazopyrimidines. The structures and activities of the compounds are shown in Tables I–VIII.

EXPERIMENTAL

All melting points were determined using a Thomas-Hoover Unimelt apparatus. All the compounds melted with decomposition at or around the temperatures indicated in the tables. The UV spectra were determined by dissolving 10 mg. of the compound in 500 ml. of 1% sodium hydroxide solution. Spectrophotometers (Beckman model DB and Cary model 14) were used to determine the UV spectra. The IR spectra were obtained from mineral oil (Nujol) mulls on a Beckman IR-8 spectrophotometer.

The general procedure for the preparation of substituted *p*-sulfamoylphenylazo compounds was as follows. All the compounds were synthesized using procedures analogous to those previously reported in the literature (2). In a typical preparatory method, 0.05 mole of the sulfonamide drug was dissolved in 100 ml. of 3 *N* HCl. On cooling to −5°, the amine hydrochloride was precipitated. It was diazotized by adding 0.05 mole of $NaNO_2$ in 25 ml. of H_2O. The temperature was maintained at 0°. The pyrimidine (0.05 mole) was dissolved in 3 *N* HCl and cooled to −5°; the solution of the diazonium salt was added to it slowly and with stirring. The addition took about 20 min. The temperature was maintained at 10° for 1 hr. and then at room temperature for 12 hr. A thick slurry containing the *p*-sulfamoylphenylazo compound as a bright-yellow or orange solid formed. The azo compound was filtered, washed with 95% EtOH, and recrystallized from boiling 2-ethoxyethanol. The compounds were analyzed for C, H, and N and were within the normal limits.

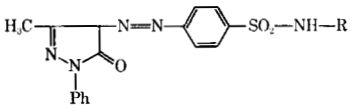
Table IV—Activity Data of Substituted *p*-Sulfamoylphenylazo Compounds



R[a]	M.p.	UV Data mμ	UV Data $\epsilon_{max.}$ (10^4)	Dose, mg./kg.	IST[b]	Activity in *P. berghei*[c]
R_1	220–224°	505	9.88	160	17.4	++
		248	10.94	640	27.3	+++
R_4	239–246°	504	10.15	160	22.3	+++
		243	11.53	640	27.0	++
R_2	200°	504	9.16	160	17.8	++
		249	8.48	640	27.3	+++
R_5	223–225°	508	11.98	160	22.0	+++
		253	10.42	640	28.0	+++
R_6	150°	508	4.52	160	17.2	++
		257	4.85	640	20.7	++
R_7	265–269°	503	5.20	640	24.0	+++
		439	4.77			
		236	7.58			

[a,b,c] See Table I.

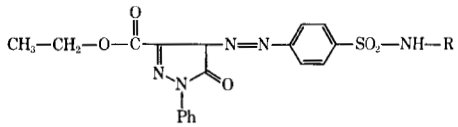
Table V—Activity Data of Substituted *p*-Sulfamoylphenylazo Compounds



R[a]	M.p.	UV Data mμ	UV Data $\epsilon_{max.}$ (10^4)	Dose, mg./kg.	IST[b]	Activity in *P. berghei*[c]
R_1	125–129°	400	7.29	80	15.6	++
		244	11.46	160	21.3	++
				320	23.0	+++
R_4	233–237°	405	6.18	160	26.3	+++
		241	10.70	640	22.0	+++
R_2	144–150°	409	7.09	40	12.6	+
		243	11.49	640	23.7	+++
R_5	273–278°	407	10.28	80	13.8	+
		246	12.41	160	15.8	++
				640	22.0	+++
R_6	180°	405	7.74	320	12.8	+
		249	9.24	640	18.5	++
R_7	147–150°	405	7.73	320	13.0	+
		250	7.55	640	22.0	+++

[a,b,c] See Table I.

Table VI—Activity Data of Substituted *p*-Sulfamoylphenylazo Compounds



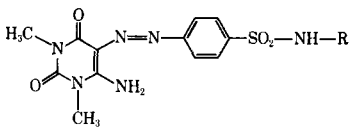
R[a]	M.p.	UV Data mμ	UV Data $\epsilon_{max.}$ (10^4)	Dose, mg./kg.	IST[b]	Activity in *P. berghei*[c]
R_3	200–208°	398	5.39	640	18.4	++
		248	8.63			
R_1	170–174°	408	2.66	640	21.8	++
		240	10.73			
R_4	218–222°	405	3.06	160	16.4	++
		241	6.61	640	27.0	+++
R_2	141–149°	410	3.55	160	12.4	+
		240	11.56	640	21.8	++
R_5	199–203°	410	7.61	160	16.0	++
		248	9.59	640	24.0	+++
R_6	170°	408	3.39	640	21.2	++
		254	8.17			

[a,b,c] See Table I.

RESULTS AND DISCUSSION

IR Spectra—The IR spectra were not very useful in characterizing the azo linkage in arylazopyrimidines, since its absorption was ob-

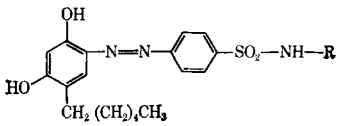
Table VII—Activity Data of Substituted *p*-Sulfamoylphenylazo Compounds



R[a]	M.p.	UV Data mμ	$\epsilon_{max.}$ (10^4)	Dose, mg./kg.	IST[b]	Activity in *P. berghei*[c]
R_1	235°	404	6.91	160	14.6	++
		243	9.93	640	24.0	+++
R_4	263–266°	404	5.91	20	13.2	+
		241	10.35	160	32.3	++++
R_2				40	13.2	+
	287–289°	409	5.56	160	18.0	++
		243	8.38	640	30.0	++++
R_5	266–269°	399	7.59	40	13.2	+
		248	9.09	160	22.0	+++
				320	35.0	++++
				640	36.0	++++

[a,b,c] See Table I.

Table VIII—Activity Data of Substituted *p*-Sulfamoylphenylazo Compounds



R[a]	M.p.	UV Data mμ	$\epsilon_{max.}$ (10^4)	Dose, mg./kg.	IST[b]	Activity in *P. berghei*[c]
R_1	235°	404	6.91	160	14.6	++
		243	9.93	640	24.0	+++
R_4	263–266°	404	5.91	20	13.2	+
		241	10.35	160	32.3	++++
R_2				40	13.2	+
	287–289°	409	5.56	160	18.0	++
		243	8.38	640	30.0	++++
R_5	266–269°	399	7.59	40	13.2	+
		248	9.09	160	22.0	+++
				320	35.0	++++
				640	36.0	++++

[a,b,c] See Table I.

scured by the strong absorption of the pyrimidine ring at 1600 $cm.^{-1}$. The absence of hydroxyl absorptions indicates that these compounds might exist in the tautomeric form (9, 10). In addition, the spectra showed medium to strong absorption bands characteristic of the pyrimidine and benzene rings at 1575 and 1625 $cm.^{-1}$, respectively; strong absorption bands at 1159 and 1320 $cm.^{-1}$ (—SO_2NH when present); medium to strong bands at 3100–3300 $cm.^{-1}$ (NH_2); and medium bands at 800–869 $cm.^{-1}$ (*p*-substituted benzene). These assignments are consistent with the assignments made by Bellamy (11) and Rao (12) for analogous compounds.

UV Spectra—Most of the substituted *p*-sulfamoylphenylazo compounds reported in Tables I–VIII were insoluble in common organic solvents such as ethanol, methanol, chloroform, and carbon tetrachloride. Therefore, they were dissolved in a 1% sodium hydroxide solution for determining the UV spectra. The UV spectral data for all the compounds are listed in Tables I–VIII. A majority of the compounds had two main UV absorption maxima around 400 and 250 mμ. In some cases the absorption maxima were shifted to about 500 and 300 mμ. But it was not possible to draw any definite conclusions about the structures and their UV absorptions.

Antimalarial Activity—All the compounds mentioned in this manuscript were tested for biological activity.[1] The effects of substitution on the antimalarial activity of a series of substituted *p*-sulfamoylphenylazo compounds are shown in Tables I–VIII. A study of the effects of substitution in the sulfamoyl group revealed an interesting pattern of activity. In general, substitution by pyrimidines and methylpyrimidines greatly increased the antimalarial activity, while substitution by thiazole, methylthiazole, and 2-methyl-3,4-thiadiazole did not lead to a comparable increase in activity. The lowest activity was observed in compounds with 2-pyridyl as the substituent.

The greatest activity was observed in the sulfamoyl compounds derived from 3-phenylazo-2,6-diaminopyrimidines (Table III). The next order of activity was seen in compounds derived from hexylresorcinol and 1-phenyl-3-methyl-5-pyrazolone. Coupling compounds derived from sulfa drugs, hexylresorcinol, and 1,3-dimethyl-6-aminouracil were either active or curative at lower doses than the other compounds evaluated. The substituent on the sulfamoyl group did not appear to exert a consistent effect on the antimalarial activity in changing from one group to the other. In the case of the pyrimidines, it has been observed that an amino group in position 4 or 5 is necessary for antimalarial activity.

It has been shown that substitution by an alkyl or aryl group in the 6-position of a 5-phenylazopyrimidine gives rise to a nonplanar configuration of the pyrimidine and benzene rings (13). Since 2,4-diamino-5-(2-chlorophenyl)pyrimidine is an antimalarial of relatively low potency (13), lack of coplanarity is not the sole requirement for antimalarial activity. This is clearly substantiated by the observation that coupling compounds derived from 1-phenyl-3-methyl-5-pyrazolone and 1-phenyl-3-carbethoxy-5-pyrazolone were found to have significant, if not curative, antimalarial activity. These compounds probably are noncoplanar since the carbon-to-nitrogen bond distance is shorter than the carbon-to-carbon bond distance. Osdene *et al.* (8) essentially brought forth the same point of view in their work on the antimalarial activity of 2,4,7-triamino-6-*ortho*-substituted arylpteridines.

[1] Tests were performed at Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC 20012

REFERENCES

(1) B. Lythgoe, A. R. Todd, and A. Topham, *J. Chem. Soc.*, **1944**, 315.

(2) R. E. Harmon, F. E. Dutton, and H. D. Warren, *J. Med. Chem.*, **11**, 627(1968).

(3) E. J. Modest, H. N. Schlein, and G. E. Foley, *J. Pharm. Pharmacol.*, **9**, 68(1957).

(4) K. Tanaka, K. Kaziwara, Y. Aramaki, and M. Kawashina, *Gann.*, **47**, 401(1956).

(5) P. Roy-Burman and D. Sen, *Biochem. Pharmacol.*, **13**, 1437(1964).

(6) J. Hampshire, P. Hebborn, A. M. Triggle, D. J. Triggle, and S. Vickers, *J. Med. Chem.*, **8**, 745(1965).

(7) S. Hibino, *Cancer Chemother. Rep.*, **13**, 141(1961).

(8) T. S. Osdene, P. B. Russell, and L. Rane, *J. Med. Chem.*, **10**, 431(1967).

(9) L. N. Short and H. W. Thompson, *J. Chem. Soc.*, **1952**, 168.

(10) D. J. Brown and L. N. Short, *ibid.*, **1953**, 331.

(11) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," Wiley, New York, N. Y., 1958.

(12) C. N. R. Rao, "Chemical Applications of Infrared Spectroscopy," Academic, New York, N. Y., 1964.

(13) P. B. Russel, *J. Chem. Soc.*, **1954**, 2951.

(14) E. A. Falco, L. G. Goodwin, G. H. Hitchings, I. M. Rollo, and P. B. Russell, *Brit. J. Pharmacol.*, **6**, 185(1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1969, from the *Department of Chemistry, Western Michigan University, Kalamazoo, MI 49001*

Accepted for publication February 27, 1970.

This work was supported by Grant CA-06140 from the National Cancer Institute. The authors are grateful to Walter Reed Army Institute of Research for biological testing of the compounds described in this paper.

Dissolution Behavior of Commercial Tablets Extemporaneously Converted to Capsules

PRAFUL T. SHAH and WILLIS E. MOORE*

Abstract □ While conducting double-blind clinical trials, it is a common practice to convert a commercial tablet dosage form to a capsule so that it may serve as a control drug for the new drug under test. This report will show that when this was done, the *in vitro* dissolution times of drugs from the capsule dosage forms were strikingly prolonged. Various capsule adjuvants were examined, attempting to decrease dissolution times of drugs from capsules. Of the series tested, lyophilized glycine had the best positive effect in shortening the dissolution times of all drugs examined.

Keyphrases □ Dissolution behavior—commercial tablets converted to capsules □ Dissolution rates—effect of starch □ Starch—effect on dissolution

During both single- and double-blind clinical studies, a commercial tablet dosage form is frequently altered to a capsule dosage form (1). The alteration to a capsule from a tablet is usually accomplished by grinding the commercial tablet to a granular or fine powder and then adding a sufficient amount of a physiologically inert capsule adjuvant to prepare extemporaneously a capsule dosage form which is identical in appearance to the research drug.

It was of interest to study any effect on the dissolution process brought about by the conversion of commercial tablet dosage forms to extemporaneously prepared capsule dosage forms. Further, it was of interest to observe the overall effect of certain capsule adjuvants on the dissolution process with the objective of perhaps decreasing the dissolution times of drugs from a capsule formulation.

A drug's dissolution rate and *in vivo* physiological availability have been the subjects of extensive research in the pharmaceutical sciences (2–6). In a recent paper, Withey and Mainville (7) referred to various methods and apparatus for following dissolution rates. They also pointed out some important requirements for an adequate dissolution-rate test.

For this preliminary investigation, the dissolution characteristics were measured by a procedure that involved the use of a modified capsule holder designed by Paikoff and Drumm (8). For comparative purposes, T_{90} values (time required for 90% to dissolve) were obtained for three chemically different types of drugs: (*a*) acetylsalicylic acid,[1] (*b*) diphenhydramine,[2] and (*c*) meprobamate.[3]

EXPERIMENTAL

Equipment—The capsule holder-stirrer had two glass blades with a platinum wire loop attached at the bottom to hold various size capsules. The stirrer was attached to a Heller stirrer (model No. GT 21).

Procedure—A 500-ml. quantity of dissolution medium (deionized water) in an 800-ml. beaker was maintained at 37 ± 0.5° in a temperature-controlled bath. The dosage form was introduced into the medium by attaching it to the stirrer loop and then inserting the assembled unit into the beaker at a controlled height from the bottom. Constant preselected rotational speeds of 60–200 r.p.m. were used for each of the three different drugs. Aliquots for assay were withdrawn with a sampling pipet at appropriate time intervals. After each sample removal, fresh medium was added back to the dissolution test beaker to maintain a constant volume.

The methodology described was suitable for capsules and tablets of acetylsalicylic acid and for diphenhydramine, but it had to be modified for the testing of meprobamate tablets and capsules to prevent flotation of capsule fragments and larger aggregates. A stainless steel wire net (20 mesh) resting on the bottom of the dissolution test beaker was used to hold the tablets and capsules of meprobamate. The glass stirrer without the loop was used.

There was no intention of comparing the dissolution characteristics between drugs. What was examined was the dissolution characteristics of commercial tablets and those tablets after conversion to a capsule dosage form, using the equivalent methodology for both forms.

The acetylsalicylic acid samples were hydrolyzed, and the absorbance of salicylic acid was measured at 294 mμ with the Beckman DK-2A (9). Diphenhydramine was assayed by two methods, one a conductometric method in the absence of glycine and the other a spectrophotometric method using a wavelength of 277 mμ. The meprobamate was assayed according to the method of Maggiorelli (10) with a modification that included using an acetic anhydride and glacial acetic acid mixture to dissolve the evaporated residue from the chloroform–carbon tetrachloride (1:1 v/v) extract (11).

Preparation of Capsules—The capsules were prepared from uncoated commercial tablets (standard compressed tablets) by grinding the tablets to a powder (through 40 mesh) with a mortar and pestle. Sufficient amounts of various capsule adjuvants were added to the powder and mixed well, and the diluted powder was packed into an appropriately sized hard gelatin capsule (Table I).

RESULTS AND DISCUSSION

The conversion of a tablet to a capsule dosage form did bring about a marked change in the *in vitro* dissolution times of all three drugs studied (Tables II–V). Without exception, the capsule dosage form containing starch took a much longer time to release 90% of its contents than did the tablet dosage form. This phenomenon, since it was observed with three different chemical types of drugs, should be more extensively studied and the results called to the attention of designers of clinical trials because of its frequency of occurrence in blind studies. Slowing down the dissolution rate of a commercial drug used as a control in a clinical trial could severely bias the results of such trials.

The materials used as diluents to prepare the capsules (starch, urea, and various forms of glycine, Table I) had varying effects upon the dissolution times. The lyophilized form of glycine had outstandingly beneficial effects upon the dissolution times of all three chemical types of drugs (Tables II–V).

The data reported in Tables II–V are abstracted from the total experimental data developed in this study. To conserve space, data falling into sampling intervals not covered by the table have been omitted. For example, Product D, Table II, had the following experimental sampling intervals in minutes: 3, 6, 10, 12, 15, 18, and 23. Since this inclusive table has only Columns 10 and 15, the data at only these matching times are included. The minimum number of

[1] Bayer Aspirin, Glenbrook Laboratories, New York, N. Y.
[2] Dramamine, G. D. Searle & Co., Chicago, Ill.
[3] Miltown, Wallace Laboratories, Cranbury, N. J.

Table I—Formulations of Various Products

Products[a]	Weights of, g. Five Tablets	Starch Potato	Urea	Lyophilized Urea	Glycine	Lyophilized Glycine	Ball Milled Glycine	Average Weight of Mixture in Capsules, g.[b]
Diphenhydramine								
B-1	1.3494	1.0053	—	—	—	—	—	0.4735 (2)
C	1.3341	—	0.5015	—	—	—	—	0.4547 (2)
D	1.3408	—	—	1.0142	—	—	—	0.3609 (2)
B-2	1.3376	1.0000	—	—	—	—	—	0.5961 (1)
E	1.3133	—	—	—	1.0000	—	—	0.6052 (1)
F	1.3390	—	—	—	—	1.0020	—	0.5603 (1)
Acetylsalicylic acid								
B	2.0262	1.0000	—	—	—	—	—	0.6456 (0)
C	2.0202	—	1.0057	—	—	—	—	0.6717 (0)
E	2.0322	—	—	—	1.2790	—	—	0.6552 (0)
F	2.0514	—	—	—	—	1.0000	—	0.5312 (0)
Meprobamate								
B	2.3811	1.0000	—	—	—	—	—	0.6115 (0)
E	2.4034	—	—	—	1.0030	—	—	0.6201 (0)
F	2.3965	—	—	—	—	1.0047	—	0.6367 (0)
G	2.3923	—	—	—	—	—	1.0012	0.5744 (0)

[a] Product A consists of commercial tablets. [b] Number in parenthesis indicates size of capsules.

sampling intervals for any product was 4, the maximum was 13, and the average was 6 data points. The data reported are the averages of three experimental dissolution studies on each product.

After each dissolution test, orders were estimated as either an apparent first- or zero-order process after the various plots had linearized following a short, but variable, lag time. Least-squares regression lines were calculated; from the appropriate values for the equation, the T_{90} values were calculated. No special significance is claimed for any of the apparent first or zero orders reported.

Wagner (12) has suggested that apparent dissolution-rate orders are artifacts at best. This technique was used only to obtain a value to serve as a basis for the comparison of the dissolution behavior of a tablet and its extemporaneously prepared capsule.

Starch is frequently used as a disintegrating or a wet binding agent in tablet formulations. By identity test, each of the three commercial tablet formulations contained starch in varying amounts. When additional starch was added to the ground-up tablet, it did not visibly show any disintegrant or dispersing effect upon the

Table II—Comparison of Dissolution-Rate Data of Diphenhydramine

Product[a]	Percent Remaining Undissolved,[b] min. 0	5	10	15	20	30	40	50	T_{90}, min.
A	100	78.80	52.80	32.80	—[c]	—	—	—	28.40[d]
B-1	100	—	—	63.24	—	36.71	23.93	14.77	57.80[d]
C	100	—	71.20	54.11	—	23.49	—	—	40.49[d]
D	100	—	77.24	59.04	—	—	—	—	25.37[e]

[a] See Table I for formulation. [b] Each point is the average of three determinations. [c] —, no experimental value at this time. [d] Apparent first-order rate process. [e] Apparent zero-order rate process.

Table III—Comparison of Dissolution-Rate Data of Diphenhydramine with Glycine

Product[a]	Percent Remaining Undissolved,[b] min. 0	5	10	15	20	30	40	50	T_{90}, min.
A	100	79.48	—[c]	37.04	—	—	—	—	31.15[d]
B-2	100	—	—	—	60.91	48.85	38.12	28.14	91.74[d]
E	100	—	—	—	—	46.40	29.79	—	54.00[e]
F	100	—	—	—	58.73	37.00	—	—	37.68[e]

[a] See Table I for formulation. [b] Each point is the average of three determinations. [c] —, no experimental value at this time. [d] Apparent first-order rate process. [e] Apparent zero-order rate process.

Table IV—Comparison of Dissolution-Rate Data of Acetylsalicylic Acid

Product[a]	Percent Remaining Undissolved,[b] min. 0	5	10	15	20	30	40	50	T_{90},[c] min.
A	100	74.31	57.67	49.21	42.76	34.00	24.72	—	84.03
B	100	88.14	77.41	—[d]	60.57	47.85	37.27	32.27	111.11
C	100	—	58.75	33.11	—	—	—	—	39.26
E	100	—	75.83	63.88	53.22	40.28	30.42	22.43	75.64
F	100	—	67.85	46.45	28.25	—	—	—	28.22

[a] See Table I for formulation. [b] Each point is the average of three determinations. [c] Apparent first-order rate process. [d] —, no experimental value at this time.

Table V—Comparison of Dissolution-Rate Data of Meprobamate

Product[a]	Percent Remaining Undissolved,[b] min. 0	10	20	30	40	50	60	75	T_{90},[c] min.
A	100	—[d]	10.01	—	—	—	—	—	20.53
B	100	75.53	—	—	54.60	—	—	40.68	284.09
E	100	69.45	58.91	52.36	44.52	—	38.41	31.36	196.08
F	100	79.23	72.69	62.04	50.23	—	—	—	124.38
G	100	84.36	72.42	53.96	42.68	33.65	28.01	—	100.00

[a] See Table I for formulation. [b] Each point is the average of three determinations. [c] Apparent first-order rate process. [d] —, no experimental value at this time.

capsule formulation.

This observation leads one to believe that the primary granules formed from the normal disintegration of a tablet in an aqueous medium differ in some physical manner from the granules obtained by grinding up a tablet.

REFERENCES

(1) "Clinical Testing of New Drugs," A. D. Herrick and M. Cattell, Eds., Revere, New York, N. Y., 1965.

(2) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262,** 1053 (1960).

(3) G. Levy and R. H. Gumtov, *J. Pharm. Sci.*, **52,** 1139(1963).

(4) R. A. Castello, G. Jellinek, J. M. Konieczny, K. C. Kwan, and R. O. Toberman, *ibid.*, **57,** 485(1968).

(5) A. J. Glazko, A. W. Kinkel, W. C. Alegnani, and E. L. Holmes, *Clin. Pharmacol. Ther.*, **9,** 472(1968).

(6) P. J. Tannenbaum, E. Rosen, T. Flanagan, and A. P. Crosley, *ibid.*, **9,** 598(1968).

(7) R. J. Withey and C. A. Mainville, *J. Pharm. Sci.*, **58,** 1120 (1969).

(8) M. Paikoff and G. Drumm, *ibid.*, **54,** 1963(1965).

(9) G. Machek, *Sci. Pharm.*, **29,** 73(1961).

(10) E. Maggiorelli, *Farm. (Pavia) Ed. Pract.*, **13,** 656(1958); through *Chem. Abstr.*, **53,** 8540a(1959).

(11) A. Hoffman and B. Ludwig, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 740(1959).

(12) J. G. Wagner, *J. Pharm. Sci.*, **58,** 1253(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 5, 1970, from the *College of Pharmacy, Wayne State University, Detroit, MI 48202*

Accepted for publication March 9, 1970.

Abstracted in part from a thesis submitted by P. T. S. to the College of Pharmacy, Wayne State University, in partial fulfillment of Master of Science degree requirements.

* To whom all inquiries should be directed.

Mechanism of Action of Salicylates VIII: Effect of Topical Application of Retinoic Acid on Wound-Healing Retardation Action of a Few Anti-Inflammatory Agents

K. H. LEE and THEODORE G. TONG

Abstract □ Oral administration of phenylbutazone, oxyphenbutazone, mefenamic acid, flufenamic acid, or indomethacin, like salicylates or corticosteroids, retards skin wound healing in rats. The healing inhibitory action of any one of these anti-inflammatory agents can be reversed by local application of retinoic acid.

Keyphrases □ Retinoic acid, topical application—inhibition of wound-healing retardation of anti-inflammatory agents □ Anti-inflammatory agents, wound-healing retardation—retinoic acid inhibition □ Salicylates, wound-healing mechanism—analogy to other anti-inflammatory agents

Recently, it has been shown that oral administration of acetylsalicylic acid, sodium salicylate, or prednisone and topical application of salicylic acid or hydrocortisone in nonionic bases (NIB) retard skin wound healing in rats (1–3). The inhibitory action of these agents is, at least, partially attributed to their anti-inflammatory action since inflammation is an essential feature in healing. Salicylates also inhibit mucopolysaccharide synthesis, which is also an essential feature in wound healing (2). Intraperitoneal injection of retinol or topical application of retinoic acid can reverse the inhibitory action of these anti-inflammatory agents (2, 3).

Phenylbutazone, oxyphenbutazone, mefenamic acid, flufenamic acid, and indomethacin are a few well-known anti-inflammatory agents. These agents, like salicylates, inhibit mucopolysaccharide synthesis (4). In the present study, it was found that all of these anti-inflammatory agents also retard wound healing, and topical application of retinoic acid can reverse the inhibitory action of these agents.

Table V—Comparison of Dissolution-Rate Data of Meprobamate

Product[a]	Percent Remaining Undissolved,[b] min. 0	10	20	30	40	50	60	75	T_{90},[c] min.
A	100	—[d]	10.01	—	—	—	—	—	20.53
B	100	75.53	—	—	54.60	—	—	40.68	284.09
E	100	69.45	58.91	52.36	44.52	—	38.41	31.36	196.08
F	100	79.23	72.69	62.04	50.23	—	—	—	124.38
G	100	84.36	72.42	53.96	42.68	33.65	28.01	—	100.00

[a] See Table I for formulation. [b] Each point is the average of three determinations. [c] Apparent first-order rate process. [d] —, no experimental value at this time.

capsule formulation.

This observation leads one to believe that the primary granules formed from the normal disintegration of a tablet in an aqueous medium differ in some physical manner from the granules obtained by grinding up a tablet.

REFERENCES

(1) "Clinical Testing of New Drugs," A. D. Herrick and M. Cattell, Eds., Revere, New York, N. Y., 1965.

(2) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262,** 1053 (1960).

(3) G. Levy and R. H. Gumtov, *J. Pharm. Sci.*, **52,** 1139(1963).

(4) R. A. Castello, G. Jellinek, J. M. Konieczny, K. C. Kwan, and R. O. Toberman, *ibid.*, **57,** 485(1968).

(5) A. J. Glazko, A. W. Kinkel, W. C. Alegnani, and E. L. Holmes, *Clin. Pharmacol. Ther.*, **9,** 472(1968).

(6) P. J. Tannenbaum, E. Rosen, T. Flanagan, and A. P. Crosley, *ibid.*, **9,** 598(1968).

(7) R. J. Withey and C. A. Mainville, *J. Pharm. Sci.*, **58,** 1120 (1969).

(8) M. Paikoff and G. Drumm, *ibid.*, **54,** 1963(1965).

(9) G. Machek, *Sci. Pharm.*, **29,** 73(1961).

(10) E. Maggiorelli, *Farm. (Pavia) Ed. Pract.*, **13,** 656(1958); through *Chem. Abstr.*, **53,** 8540a(1959).

(11) A. Hoffman and B. Ludwig, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 740(1959).

(12) J. G. Wagner, *J. Pharm. Sci.*, **58,** 1253(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 5, 1970, from the *College of Pharmacy, Wayne State University, Detroit, MI 48202*

Accepted for publication March 9, 1970.

Abstracted in part from a thesis submitted by P. T. S. to the College of Pharmacy, Wayne State University, in partial fulfillment of Master of Science degree requirements.

* To whom all inquiries should be directed.

Mechanism of Action of Salicylates VIII: Effect of Topical Application of Retinoic Acid on Wound-Healing Retardation Action of a Few Anti-Inflammatory Agents

K. H. LEE and THEODORE G. TONG

Abstract □ Oral administration of phenylbutazone, oxyphenbutazone, mefenamic acid, flufenamic acid, or indomethacin, like salicylates or corticosteroids, retards skin wound healing in rats. The healing inhibitory action of any one of these anti-inflammatory agents can be reversed by local application of retinoic acid.

Keyphrases □ Retinoic acid, topical application—inhibition of wound-healing retardation of anti-inflammatory agents □ Anti-inflammatory agents, wound-healing retardation—retinoic acid inhibition □ Salicylates, wound-healing mechanism—analogy to other anti-inflammatory agents

Recently, it has been shown that oral administration of acetylsalicylic acid, sodium salicylate, or prednisone and topical application of salicylic acid or hydrocortisone in nonionic bases (NIB) retard skin wound healing in rats (1–3). The inhibitory action of these agents is, at least, partially attributed to their anti-inflammatory action since inflammation is an essential feature in healing. Salicylates also inhibit mucopolysaccharide synthesis, which is also an essential feature in wound healing (2). Intraperitoneal injection of retinol or topical application of retinoic acid can reverse the inhibitory action of these anti-inflammatory agents (2, 3).

Phenylbutazone, oxyphenbutazone, mefenamic acid, flufenamic acid, and indomethacin are a few well-known anti-inflammatory agents. These agents, like salicylates, inhibit mucopolysaccharide synthesis (4). In the present study, it was found that all of these anti-inflammatory agents also retard wound healing, and topical application of retinoic acid can reverse the inhibitory action of these agents.

Table I—Retinoic Acid and Healing Retardation Action of a Few Anti-Inflammatory Agents[a]

Group	No. of Animals	Drugs Given		Mean Tensile Strength, g.	Percent Control
		Orally	Topically		
I	14	—	NIB	451 ± 9	100
II	9	Indomethacin, 4 mg./kg.	NIB	374 ± 9	83
III	14	Indomethacin, 4 mg./kg.	1% retinoic acid in NIB	433 ± 8	96
IV	7	Mefenamic acid, 20 mg./kg.	NIB	424 ± 9	94
V	16	Mefenamic acid, 40 mg./kg.	NIB	407 ± 7	90
VI	6	Mefenamic acid, 40 mg./kg.	1% retinoic acid in NIB	456 ± 10	101
VII	8	Flufenamic acid, 40 mg./kg.	NIB	398 ± 10	88
VIII	6	Flufenamic acid, 40 mg./kg.	1% retinoic acid in NIB	427 ± 7	95
IX	7	Phenylbutazone, 100 mg./kg.	NIB	403 ± 4	89
X	7	Phenylbutazone, 100 mg./kg.	1% retinoic acid in NIB	427 ± 9	95
XI	7	Oxyphenbutazone, 100 mg./kg.	NIB	385 ± 8	85
XII	6	Oxyphenbutazone, 100 mg./kg.	1% retinoic acid in NIB	439 ± 10	97

[a] The difference between the experiments and the controls is highly significant ($p < 0.01$, Student's t test) except Group IV animals receiving only 20 mg. of mefenamic acid/kg. ($p < 0.10$).

EXPERIMENTAL

Material and Drugs—The following materials and drugs were used in this study: retinoic acid,[1] phenylbutazone,[2] oxyphenbutazone,[2] mefenamic acid,[3] flufenamic acid,[3] indomethacin,[4] and NIB.[5] The strength of retinoic acid used was 1% in NIB. Methylcellulose USP[6] (400 cps.) was also used.

Administration of Drugs—All drugs were fed to rats daily for 4 days through a short stomach tube (PE 160) connected to a blunt hypodermic needle (No. 17) attached to a 50-ml. syringe, starting 1 day before operation. Each drug was suspended in a 0.5% methylcellulose solution. One milliliter of the suspension was fed to each rat.

Application of Retinoic Acid in NIB—Retinoic acid in NIB was applied with gentle rubbing directly on the sutured wound right after wounding. The application was repeated, once a day, on the 1st and 2nd days of wounding. For the control, only NIB was applied.

Wound Procedure—Sprague-Dawley male rats, weighing 230–240 g., were anesthetized with ethyl ether in an open mask. The hair on the back was depilated with an electric clipper. One incision, 6 cm. in length, was made through the skin and cutaneous muscles at a distance about 1.5 cm. from the midline on each side. No ligatures were used. Bleeding usually ceased after a few minutes. The incisions were closed with continuous through-and-through sutures with stitches 0.5 cm. apart. Black silk surgical thread (No. 3–0) and a curved needle (No. 19) were used. The continuous suture was pulled tight enough to secure good adaptation of the wound edges. The wounds were left undressed.

Measurement of Healing—Tensile strength, the force required to open a healing skin wound, was used to measure healing. On the 7th day after wounding, the tensile strength of the wound was measured with a simple laboratory-made tensiometer as described previously (1).

[1] All *trans*, Sigma grade, type XX, a crystalline synthetic compound obtained from Sigma Chemical Co., St. Louis, Mo.
[2] Supplied by Dr. T. A. Terzakis, Geigy Pharmaceuticals, Division of Geigy Chemical Corp., Ardsley, N. Y.
[3] Supplied by Dr. C. V. Winder, Research Laboratories, Parke, Davis and Co., Ann Arbor, Mich.
[4] Supplied by Dr. W. B. Gall, Research Laboratories, Merck Sharp and Dohme, Division of Merck and Co., Inc., Rahway, N. J.
[5] Prepared by Pharmaceutical Technology Laboratory, San Francisco Medical Center, San Francisco, Calif.
[6] Dow Chemical Co., Midland, Mich.

RESULTS AND DISCUSSION

The results of the effect of phenylbutazone, oxyphenbutazone, mefenamic acid, flufenamic acid, and indomethacin on skin wound healing in rats are summarized in Table I. The mean tensile strength of the healing wound of the control animals from Group I was 451 ± 9 g. These animals received only topical application of NIB. Group II animals received 4 mg. of indomethacin/kg. of body weight orally, and the mean tensile strength of the healing wound was reduced to 374 ± 9 g., which is 83% of the control. Group III animals were treated the same way as Group II except that 1% retinoic acid in NIB was applied to the wound once a day during the first 3 days of wounding. The mean tensile strength of Group III animals was increased to 433 ± 8 g. Group IV animals received 20 mg. mefenamic acid/kg. of body weight orally; the mean tensile strength of these animals was 424 ± 9 g., which is not appreciably less than the control. An increased dosage of 40 mg. of mefenamic acid/kg. of body weight was used to feed Group V animals. The mean tensile strength of Group V animals was significantly reduced to 407 ± 7 g. Group VI animals received the same treatment as Group V animals except that retinoic acid was topically applied to the wound as described for Group III animals. The mean tensile strength of Group VI animals was increased to 436 ± 10 g. Group VII animals received 40 mg. of flufenamic acid/kg. of body weight, and the mean tensile strength was reduced to 398 ± 10 g. The reversal of wound-healing retardation action of flufenamic acid by local application of retinoic acid was demonstrated by the increase of mean tensile strength in Group VIII animals. Group IX animals received 100 mg. of phenylbutazone, and Group XI animals received a dosage of 100 mg. of oxyphenbutazone. The mean tensile strengths of Groups IX and XI were reduced to 403 ± 4 and 385 ± 8 g., respectively. Groups X and XII demonstrated that the local application of retinoic acid reverses the wound-healing inhibitory action of phenylbutazone and oxyphenbutazone.

Inflammation and mucopolysaccharide synthesis are the two known essential features in wound healing. Phenylbutazone, oxyphenbutazone, mefenamic acid, flufenamic acid, and indomethacin, like salicylates, prednisone, and hydrocortisone, are anti-inflammatory agents. They are also potent inhibitors to mucopolysaccharide synthesis at the site where uridine-5′-diphosphoglucose is oxidized to UDPGA, as reported recently (5). These anti-inflammatory agents, therefore, retard wound healing also by their anti-inflammation activity and their inhibitory action on mucopolysaccharide synthesis.

SUMMARY

The healing retardation action of anti-inflammatory agents, phenylbutazone, oxyphenbutazone, mefenamic acid, flufenamic acid, and indomethacin, has been demonstrated in rats.

Topical application of retinoic acid can reverse the healing inhibitory action of these anti-inflammatory agents.

The mechanism of action of these agents has been discussed.

REFERENCES

(1) K. H. Lee, *J. Pharm. Sci.*, **57**, 1042(1968).

(2) *Ibid.*, **57**, 1238(1968).

(3) K. H. Lee and T. G. Tong, *J. Pharm. Sci.*, **58**, 773(1969).

(4) K. H. Lee and M. R. Spencer, *ibid.*, **58**, 464(1969).

(5) *Ibid.*, **58**, 1152(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 10, 1969, from the *Department of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122*

Accepted for publication March 3, 1970.

This work was supported by research funds from The Academic Senate Committee on Research, University of California, San Francisco Medical Center, San Francisco, CA 94122

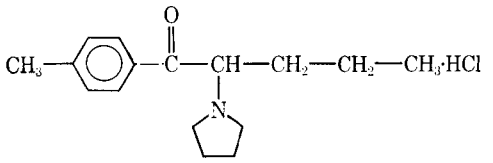
New Compounds: Some Potential Chemotherapeutic Agents Derived from Aralkyl Ketones

PYARE PARIMOO* and W. LEWIS NOBLES†

Abstract □ The Mannich reaction has been successfully applied to some aralkyl ketones, valerophenone and caprophenone, and their substituted derivatives in efforts to find efficient agents to be screened for possible CNS stimulant, analgesic, or antispasmodic activity. The aminoketones (Mannich bases) were converted to the γ-amino secondary alcohols by treatment with sodium borohydride. The synthesis of a series of γ-amino tertiary alcohols was achieved by the application of the Grignard reaction to the corresponding Mannich bases. The last section of the study involved the preparation of γ-amino alkyl esters from the corresponding secondary and tertiary alcohols.

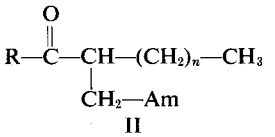
Keyphrases □ Chemotherapeutic agents—aralkyl ketone derivatives, synthesis □ Aralkyl ketone derivatives—synthesis, structure–activity relationships □ Mannich reaction—synthesis □ Grignard reaction—synthesis

The significant action of pyrovalerone hydrochloride (I) as a CNS stimulant has been reported (1):



I

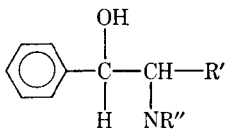
In view of this activity, the authors undertook the synthesis and study of aminoketones having the following structure (II):



II

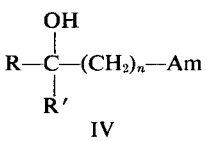
Am = substituted amino group
R = aryl group or substituted aryl group
n = 2,3

To investigate a possible structure–activity relationship, a number of analogs and derivatives were prepared. Recorded in the literature (2–5) are numerous ketonic Mannich bases prepared for pharmacological testing, such as antispasmodic, analgesic, local anesthetic, or chemotherapeutic agents. From the correlation of the chemical structure of these ketones with their antispasmodic activities, the following conclusions were drawn: (*a*) Activity is enhanced by the introduction of a phenyl group into the α-position of the propiophenones. (*b*) The piperidyl group was the most active amino group, while the morpholino group was the least active amino moiety. (*c*) Simple substituents in the *p*-position of the aromatic rings of the propiophenones decreased activity (5). Since some of the structural modifications in these various ketones had an effect on the physiological activity, the transformation of the ketones to the corresponding alcohols might possibly have a greater effect; also the amino alcohols are generally much more stable than the corresponding ketones (6). Lutz *et al.* (7) have reported the preparation and screening of 184 amino alcohols against avian malaria. The general structure (III) of the secondary alcohols is represented as follows:



III

These compounds included examples of 50 variations in the benzene nucleus and over 60 variations in the *N*,*N*-dialkyl groups on the nitrogen. The change in activity with variation in chemical structure led Denton *et al.* (8) to prepare more than 100 γ-amino tertiary alcohols; a majority of them have exhibited pharmacological activity. The general structure (IV) of the amino tertiary alcohols prepared by these workers is reported as follows:



IV

SUMMARY

The healing retardation action of anti-inflammatory agents, phenylbutazone, oxyphenbutazone, mefenamic acid, flufenamic acid, and indomethacin, has been demonstrated in rats.

Topical application of retinoic acid can reverse the healing inhibitory action of these anti-inflammatory agents.

The mechanism of action of these agents has been discussed.

REFERENCES

(1) K. H. Lee, *J. Pharm. Sci.*, **57**, 1042(1968).

(2) *Ibid.*, **57**, 1238(1968).

(3) K. H. Lee and T. G. Tong, *J. Pharm. Sci.*, **58**, 773(1969).

(4) K. H. Lee and M. R. Spencer, *ibid.*, **58**, 464(1969).

(5) *Ibid.*, **58**, 1152(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 10, 1969, from the *Department of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122*

Accepted for publication March 3, 1970.

This work was supported by research funds from The Academic Senate Committee on Research, University of California, San Francisco Medical Center, San Francisco, CA 94122

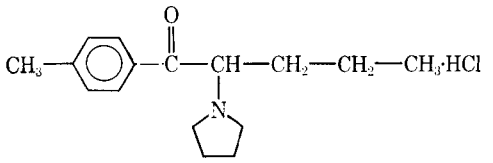
New Compounds: Some Potential Chemotherapeutic Agents Derived from Aralkyl Ketones

PYARE PARIMOO* and W. LEWIS NOBLES†

Abstract □ The Mannich reaction has been successfully applied to some aralkyl ketones, valerophenone and caprophenone, and their substituted derivatives in efforts to find efficient agents to be screened for possible CNS stimulant, analgesic, or antispasmodic activity. The aminoketones (Mannich bases) were converted to the γ-amino secondary alcohols by treatment with sodium borohydride. The synthesis of a series of γ-amino tertiary alcohols was achieved by the application of the Grignard reaction to the corresponding Mannich bases. The last section of the study involved the preparation of γ-amino alkyl esters from the corresponding secondary and tertiary alcohols.

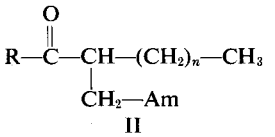
Keyphrases □ Chemotherapeutic agents—aralkyl ketone derivatives, synthesis □ Aralkyl ketone derivatives—synthesis, structure–activity relationships □ Mannich reaction—synthesis □ Grignard reaction—synthesis

The significant action of pyrovalerone hydrochloride (I) as a CNS stimulant has been reported (1):



I

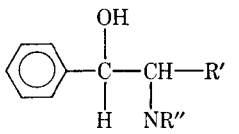
In view of this activity, the authors undertook the synthesis and study of aminoketones having the following structure (II):



II

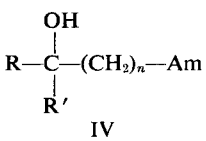
Am = substituted amino group
R = aryl group or substituted aryl group
n = 2,3

To investigate a possible structure–activity relationship, a number of analogs and derivatives were prepared. Recorded in the literature (2–5) are numerous ketonic Mannich bases prepared for pharmacological testing, such as antispasmodic, analgesic, local anesthetic, or chemotherapeutic agents. From the correlation of the chemical structure of these ketones with their antispasmodic activities, the following conclusions were drawn: (*a*) Activity is enhanced by the introduction of a phenyl group into the α-position of the propiophenones. (*b*) The piperidyl group was the most active amino group, while the morpholino group was the least active amino moiety. (*c*) Simple substituents in the *p*-position of the aromatic rings of the propiophenones decreased activity (5). Since some of the structural modifications in these various ketones had an effect on the physiological activity, the transformation of the ketones to the corresponding alcohols might possibly have a greater effect; also the amino alcohols are generally much more stable than the corresponding ketones (6). Lutz *et al.* (7) have reported the preparation and screening of 184 amino alcohols against avian malaria. The general structure (III) of the secondary alcohols is represented as follows:



III

These compounds included examples of 50 variations in the benzene nucleus and over 60 variations in the *N*,*N*-dialkyl groups on the nitrogen. The change in activity with variation in chemical structure led Denton *et al.* (8) to prepare more than 100 γ-amino tertiary alcohols; a majority of them have exhibited pharmacological activity. The general structure (IV) of the amino tertiary alcohols prepared by these workers is reported as follows:



IV

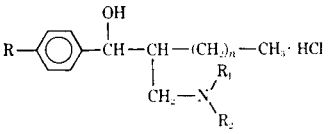


Table I—γ-Amino Secondary Alcohols (Hydrochloride Salts)

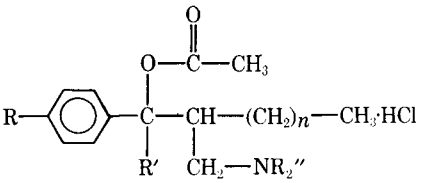
No.[a]	R	NR_1R_2	n	Yield, %	M.p.[b]	Formula	Anal.[c] Calcd.	Anal.[c] Found
1	Hydrogen	1-Methyl piperazino	2	75	233–235°	$C_{17}H_{30}Cl_2N_2O$	C, 56.97 H, 8.71 N, 7.81	C, 56.87 H, 8.79 N, 7.48
2	Methyl	Morpholino	2	77	175°	$C_{17}H_{28}ClNO_2$	C, 65.05 H, 8.98 N, 4.46	C, 64.71 H, 9.08 N, 4.58
3	Methyl	1-Methyl piperazino	3	59	235°	$C_{19}H_{34}ClN_2O$	C, 60.19 H, 9.07 N, 7.42	C, 59.95 H, 9.10 N, 7.15
4	Methoxy	Piperidino	2	77	193°	$C_{18}H_{30}ClNO_2$	C, 65.92 H, 9.22 N, 4.27	C, 65.93 H, 9.08 N, 4.43
5	Hydrogen	Dimethyl amino	2	52	168°	$C_{16}H_{24}ClNO$	C, 65.23 H, 9.38 N, 5.42	C, 64.92 H, 9.53 N, 5.66
6	Methoxy	Morpholino	2	66	190°	$C_{17}H_{28}ClNO_3$	C, 61.89 H, 8.55 N, 4.24	C, 61.61 H, 8.48 N, 4.36

[a] All the γ-amino secondary alcohols in this table were recrystallized from ethanol or ethanol–ethyl acetate solution. [b] All melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Alfred Bernhardt, 433, Mulheim (Ruhr), West Germany.

where R is usually aryl; R′ is alkyl, cycloalkyl, or aryl; and the amino group is, most commonly, dimethyl amino, pyrrolidino, piperidino, and morpholino.

Compounds in which $n = 1$ or 3 usually showed reduced antispasmodic activity. The compound trihexyphenidyl hydrochloride (Artane, Lederle Laboratories), prepared by Cunningham (9), was found to be a potent antispasmodic. The pyrrolidine homolog (Kemadrin, Burroughs Wellcome & Co.) was prepared by an analogous procedure. In large doses, these compounds stimulated the CNS in a manner similar to that of atropine.

A number of esters of 4-dialkyl amino-1,2-diphenylbutanol have been reported to possess a high order of analgesic activity in animals (10). One of these isomers, dextropropoxyphene (Darvon, Eli Lilly and Co.), has been found to be an effective analgesic in human beings (11). In view of these studies, the authors decided to prepare some related open-chain esters. The general structure (V) of the aminoalkyl esters is represented as follows:



V

DISCUSSION

The first group of compounds described in this research is related to the CNS stimulant pyrovalerone hydrochloride, Compound I; the structures described in Tables I–III represent, in a general way, the variations applied to the antispasmodic and analgesic agents. In any event, the analogy of pyrovalerone hydrochloride to Type II compounds does not seem remote. Upon inspection of molecular models, certain points of resemblance stand out and the spatial similarity of pyrovalerone hydrochloride becomes

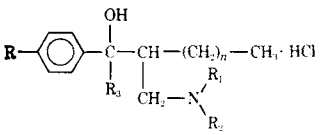


Table II—γ-Amino Tertiary Alcohols (Hydrochloride Salts)

No.[a]	R	NR_1R_2	R_3	n	Yield, %	M.p.[b]	Formula	Anal.[c] Calcd.	Anal.[c] Found
1	Hydrogen	1-Methyl piperazino	Methyl	2	42	220°	$C_{18}H_{32}Cl_2N_2O$	C, 59.49 H, 8.88	C, 59.27 H, 9.24
2	Methyl	Pyrrolidino	Ethyl	2	50	205°	$C_{19}H_{32}ClNO$	C, 66.91 H, 9.89 N, 4.29	C, 66.82 H, 9.92 N, 4.33
3	Hydrogen	Pyrrolidino	Ethyl	2	50	203°	$C_{18}H_{30}ClNO$	C, 69.41 H, 9.71 N, 4.49	C, 69.27 H, 9.75 N, 4.27
4	Methyl	Dimethyl amino	Benzyl	2	21.4	243°	$C_{22}H_{32}ClNO$	C, 73.12 H, 8.64 N, 3.84	C, 72.84 H, 8.98 N, 3.96

[a] All the γ-amino tertiary alcohols in this table were recrystallized from ethanol–ethyl acetate, methanol–ethyl acetate, and benzene solutions. [b] All the melting points are uncorrected. [c] The carbon, hydrogen, nitrogen analyses are through the courtesy of Dr. Alfred Bernhardt, 433, Mulheim (Ruhr), West Germany.

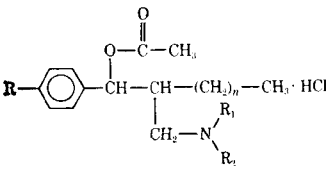


Table III—γ-Aminoalkyl Esters (Hydrochloride Salts)

No.[a]	R	NR_1R_2	*n*	Yield, %	M.p.[b]	Formula	Anal.[c] Calcd.	Anal.[c] Found
1	Hydrogen	Dimethyl amino	2	71	237.5°	$C_{16}H_{26}ClNO$	C, 64.04 H, 8.73 N, 4.60	C, 63.71 H, 8.54 N, 4.86
2	Hydrogen	1-Methyl piperazino	2	40	220°	$C_{19}H_{32}Cl_2N_2O_2$	C, 57.11 H, 8.07 N, 7.01	C, 56.83 H, 8.35 N, 7.02
3	Methyl	1-Methyl piperazino	2	75	208°	$C_{20}H_{34}Cl_2N_2O_2$	C, 56.86 H, 8.35 N, 6.63	C, 56.63 H, 8.47 N, 6.42
4	Methyl	Morpholino	3	55	135–136°	$C_{20}H_{32}ClNO_3$	C, 64.77 H, 8.94 N, 3.77	C, 64.70 H, 8.56 N, 4.01
5	Methyl	1-Methyl piperazino	3	48	193–194°	$C_{21}H_{36}Cl_2N_2O_2$	C, 55.57 H, 8.44 N, 6.17	C, 55.56 H, 8.81 N, 6.42

[a] All the γ-aminoalkyl esters in this table were recrystallized from methanol–ethyl acetate and ethanol–ethyl acetate solutions. [b] All melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Alfred Bernhardt, 433, Mulheim (Ruhr), West Germany.

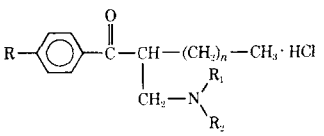


Table IV—β-Amino Ketones (Hydrochloride Salts)

No.[a]	R	NR_1R_2	*n*	Method	Yield, %	M.p.[b]	Formula	Anal.[c] Calcd.	Anal.[c] Found
1	Hydrogen	Dimethyl amino	2	A	22	159–160°	$C_{14}H_{22}ClNO$	C, 63.50 H, 8.74	C, 63.62 H, 8.84
2	Hydrogen	Pyrrolidino	2	B	21	139–140°	$C_{16}H_{24}ClNO$	C, 68.86 H, 8.54 N, 4.96	C, 69.03 H, 8.66 N, 4.92
3	Hydrogen	1-Methyl piperazino	2	B	40	198–200°	$C_{17}H_{28}Cl_2NO$	C, 58.78 H, 8.11 N, 8.11	C, 58.77 H, 8.27 N, 8.46
4	Methyl	Morpholino	2	A,B	55	170–171°	$C_{17}H_{26}ClNO_2$	C, 65.42 H, 8.40 N, 4.50	C, 65.26 H, 8.61 N, 4.68
5	Methyl	Piperidino	2	A,B	22	158–160°	$C_{18}H_{28}ClNO$	C, 67.76 H, 8.85 N, 4.38	C, 67.78 H, 9.04 N, 4.30
6	Methyl	Pyrrolidino	2	B	20	178–180°	$C_{17}H_{26}ClNO$	C, 66.97 H, 8.46 N, 4.59	C, 67.24 H, 8.85 N, 4.82
7	Methyl	1-Methyl piperazino	2	B	48	191–193°	$C_{18}H_{30}Cl_2N_2O$	C, 56.70 H, 8.82 N, 7.71	C, 56.75 H, 8.70 N, 7.79
8	Hydrogen	Dimethyl amino	3	A	11.5	130–131°	$C_{15}H_{24}ClNO$	C, 66.31 H, 8.96 N, 5.18	C, 66.62 H, 8.84 N, 5.34
9	Methyl	Dimethyl amino	3	A	50	142–143°	$C_{16}H_{26}ClNO$	C, 66.70 H, 9.18 N, 4.82	C, 66.71 H, 9.34 N, 5.22
10	Methyl	Morpholino	3	B	76	170°	$C_{18}H_{28}ClNO_2$	C, 66.34 H, 8.66 N, 4.29	C, 66.11 H, 8.49 N, 4.70
11	Methyl	1-Methyl piperazino	3	B	34	188–190°	$C_{19}H_{32}Cl_2N_2O$	C, 58.08 H, 8.71 N, 7.41	C, 58.27 H, 8.72 N, 7.11
12	Hydroxy	Morpholino	2	B	66	180°	$C_{16}H_{24}ClNO_3$	C, 58.90 H, 7.73 N, 4.30	C, 59.03 H, 7.78 N, 4.73
13	Hydroxy	Piperidino	2	B	65	153–154°	$C_{17}H_{20}ClNO_2$	C, 65.26 H, 9.93 N, 4.47	C, 64.86 H, 8.69 N, 4.79

[a] All the Mannich bases in this table were recrystallized from ethanol–ethyl acetate, ethanol–ether, and benzene solutions. [b] All the melting points are uncorrected. [c] The carbon, hydrogen, nitrogen analyses are through the courtesy of Dr. Alfred Bernhardt, 433, Mulheim (Ruhr), West Germany.

apparent. 2-(1-Pyrrolidino methyl)-4′-methyl valerophenone hydrochloride (Compound 6, Table IV) bears a strong resemblance to pyrovalerone hydrochloride. A practical variation of this compound in which the methyl group on the aromatic nucleus was replaced by a methoxy group and also the introduction of an acetoxy methyl in place of the carbonyl group in the alkyl chain has been devised. The acetoxy group may help in favorably altering the distribution of the compound in the animal body and also in increasing the concentrations in the brain. Moreover, such a change as the replacement of the pyrrolidino group by the morpholino group may be effective for the increased activity, possibly because of a certain attraction between the cation head and the unshared pair of electrons on oxygen. The reduction of the keto group to the carbinols may show parallel results, the latter being more stable to the biological reactions.

Aralkyl ketones (valerophenone and caprophenone) and their substituted derivatives were prepared as indicated in the *Experimental* section. The Mannich bases and the corresponding secondary and tertiary alcohols were obtained by modifying the methods in the literature (12–14). The preparation of aminoalkyl esters was patterned after the work of Burckhalter and Johnson (15) and that of Pohland and Sullivan (10). A number of procedures (15–17) and their modifications were attempted to bring about the successful esterification of the tertiary amino alcohols. Each attempt met with failure. An examination of the IR spectrum of each reaction product did not reveal the presence of the characteristic alkyl ester absorption in the region of 1735–1750 $cm.^{-1}$, indicating no reaction had taken place. Similar difficulties in obtaining the acyl derivatives of tertiary amino alcohols have been recorded (13, 18), one of the possible explanations for the failure to bring about this esterification being steric inhibition.

EXPERIMENTAL

Aralkyl ketones were prepared according to the methods described in the literature (19–21).

***p*-Methyl Caprophenone**—Eighty grams (0.06 mole) of *n*-hexanoyl chloride was gradually added to a mixture of 184.0 g. (2.0 moles) of dry toluene and the 131.0 g. (1.0 mole) of powdered anhydrous aluminum chloride. The reaction mixture was stirred for 12 hr. at room temperature and thereafter refluxed for 3 hr. The dark-colored reaction mixture was cooled and then decomposed in a solution of ice and concentrated HCl. The organic layer was separated and washed with 10% NaOH and water, respectively. The product was dried over anhydrous $MgSO_4$ and distilled at 280°. Redistillation yielded 70.0 g. (40%) of a clear liquid, b.p. 280°.

Anal.—Calcd. for $C_{13}H_{18}O$: C, 82.01; H, 9.58. Found: C, 82.13; H, 9.41.

Mannich Bases—Three methods were investigated; Procedure A, as will be noted, was the general method for the preparation of the compounds in Table IV.

2-(1-Pyrrolidinomethyl)-valerophenone Hydrochloride—*Method A*—A solution of 3.35 g. (0.05 mole) of pyrrolidine in 50 ml. of absolute alcohol was cooled and treated with concentrated HCl until the final solution was acidic. Valerophenone (8.10 g., 0.05 mole) and paraformaldehyde (1.80 g., 0.05 mole) were then added and the reaction mixture was refluxed for 8 hr. Two further additions (0.90 g., 0.03 mole) of paraformaldehyde were also made. The solvent was distilled *in vacuo*, and the residue was treated with 30 ml. of water and extracted with ether. The ether portion was discarded. The aqueous layer was made alkaline with 30% NH_4OH and ether extracted. The ether portion was washed with water, dried over anhydrous $MgSO_4$, and treated with gaseous HCl, producing an oil. The oil solidified after refrigeration for 48 hr. Recrystallization from benzene yielded 3.0 g. (21%) of white crystals, m.p. 139–140°.

γ-Amino Secondary Alcohols—2-(1-Morpholinomethyl)-1-(4-methylphenyl)-1-pentan-1-ol Hydrochloride—A solution of 1.10 g. (0.03 mole) of sodium borohydride in 100 ml. of 50% MeOH was cooled to 20°. To this was added gradually a solution of 8.25 g. (0.03 mole) of 2-(1-morpholinomethyl)-4-methylvalerophenone in 50% MeOH with stirring for several hours. The temperature was then raised to 50–60° to decompose excess sodium borohydride. The solvent was distilled *in vacuo*. The residue was dissolved in ether and washed with 50-ml. portions of water. The ether portion was dried ($MgSO_4$) and treated with gaseous HCl, producing a solid product. Recrystallization of HCl salt from ethanol–ethyl acetate mixture yielded 7.0 g. (77%) of product, m.p. 175°.

γ-Amino Tertiary Alcohols—2-(1-Piperidinomethyl)-1,1-Diphenylhexan-1-ol Hydrochloride—This preparation was chosen as a representative of all the Grignard reactions reported in this research. To a solution of 0.06 mole of phenyl magnesium bromide in 100 ml. dry ether was gradually added 5.36 g. (0.02 mole) of 2-(1-piperidinomethyl)caprophenone. The reaction mixture was stirred overnight. The mixture was then decomposed by the dropwise addition of a saturated ammonium chloride solution; the temperature was regulated with an ice bath. The ether solution was decanted from the granular material, and the solid material was washed with 50 ml. of ether. The combined ether solutions were dried ($MgSO_4$) and treated with gaseous HCl, producing a sticky mass which solidified after refrigeration for 24 hr. Recrystallization from methanol–ethyl acetate mixture yielded 3.5 g. (42%) of product, m.p. 210–211°.

REFERENCES

(1) *J. Amer. Pharm. Ass.*, **NS4**, 98(1964).

(2) C. Mannich and D. Lammering, *Ber.*, **55**, 3510(1922).

(3) W. Wilson and L. Y. Kyi, *J. Chem. Soc.*, **1952**, 1321.

(4) F. Mercier and M. R. Sectier, *J. Physiol. Paris*, **45**, 186 (1954); through *Chem. Abstr.*, **47**, 11527(1953).

(5) J. J. Denton, R. J. Turner, W. B. Neier, V. A. Lawson, and H. P. Schedl, *J. Amer. Chem. Soc.*, **71**, 2048(1949).

(6) J. J. Denton, V. A. Lawson, W. B. Neier, and R. J. Turner, *ibid.*, **71**, 2050(1949).

(7) H. R. Lutz, *J. Org. Chem.*, **12**, 617(1947).

(8) J. J. Denton, W. B. Neier, and V. A. Lawson, *J. Amer. Chem. Soc.*, **71**, 2053(1949).

(9) R. W. Cunningham, *J. Pharmacol. Exp. Ther.*, **96**, 151 (1949).

(10) A. Pohland and J. R. Sullivan, *J. Amer. Chem. Soc.*, **75**, 4458(1953).

(11) *Ibid.*, **77**, 3400(1955).

(12) J. B. Wright and E. H. Lincoln, *J. Amer. Chem. Soc.*, **74**, 6301(1952).

(13) F. G. Rogers and W. L. Nobles, *J. Amer. Pharm. Ass., Sci. Ed.*, **51**, 273(1962).

(14) A. Pohland and J. R. Sullivan, *J. Amer. Chem. Soc.*, **79**, 1442(1957).

(15) J. H. Burckhalter and S. H. Johnson, *ibid.*, **73**, 4827(1951).

(16) A. Ziering and J. Lee, *J. Org. Chem.*, **12**, 911(1947).

(17) A. C. Kjaer and P. V. Petersen, *Acta Chem. Scand.*, **5**, 1145 (1951); through *Chem. Abstr.*, **46**, 5010(1952).

(18) C. D. Blanton, "Some Studies in Chemistry of 3-Azabicyclo-[3,2,2]-nonane" (dissertation), University of Mississippi, University, Miss., 1963, p. 118.

(19) M. Sulzbachar and E. Bergman, *J. Org. Chem.*, **13**, 303 (1948).

(20) R. H. Sydner and R. H. Beilfuss, *J. Amer. Chem. Soc.*, **75**, 4923(1953).

(21) S. C. Gibson and B. Levine, *J. Chem. Soc.*, **1931**, 2388.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 18, 1969, from the *Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677*

Accepted for publication February 4, 1970.

Abstracted in part from a thesis submitted to the Graduate School, University of Mississippi, by Pyare Parimoo in partial fulfillment of the Master of Science degree requirements in pharmaceutical chemistry.

* Present address: University of Missouri, Kansas City, Mo.

† Present address: Mississippi College, Clinton, MS 39056

New Compounds: Structural Analogs Related to Asarone and Mescaline

B. S. R. MURTY and R. M. BAXTER

Abstract □ The synthesis and spectral data of a series of trimethoxyphenyl derivatives related to asarone and mescaline are reported.

Keyphrases □ Asarone, mescaline trimethoxyphenyl derivatives—synthesis □ Mescaline, asarone trimethoxyphenyl derivatives—synthesis □ TLC—identification □ UV spectrophotometry—structure, analysis □ IR spectrophotometry—structure, analysis □ NMR spectrophotometry—structure, analysis

Various types of biological activity have been reported for trimethoxyphenyl derivatives. Initially, knowledge regarding the pharmacological activity in trimethoxybenzene derivatives came with the isolation and determination of the chemical structure of mescaline, a potent hallucinogenic agent. Dandiya and Menon (1–3) demonstrated the promising tranquillizing properties of asarone, an active principle isolated from *Acorus calamus* by Baxter *et al.* (4, 5). Although asarone (2,4,5-trimethoxy-1-propenyl benzene) and mescaline (3,4,5-trimethoxy-β-phenethylamine) exhibit a structural resemblance, they manifest more or less opposite pharmacological actions. This led the authors to synthesize a series of compounds in an attempt to establish the structural characteristics that influence the pharmacological activity toward one type or the other. The synthesis and spectral data for one series of compounds are reported. The results of pharmacological evaluation and the associated structure–activity relationships will be communicated subsequently.

EXPERIMENTAL

Synthesis—The carbonyl compound (1 mole) was added to ethylmagnesium bromide (1.6 moles) in tetrahydrofuran (THF) in a flask fitted with a reflux condenser and stirring arrangement. The addition products formed were isolated and purified by standard procedures. The physical and spectral data for six compounds are presented in Tables I and II.

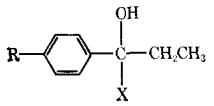


Table I—Trimethoxyphenyl Derivatives

No.	R	X	Yield, %	B.p.	mm.	Formula	Anal., % Calcd.		Anal., % Found	
1	3,4,5-Trimethoxy	H	60	120–124°	5	$C_{12}H_{18}O_4$	C, 63.71	H, 7.96	C, 63.72	H, 8.25
2	2,4,5-Trimethoxy	H	64	—	[a]	$C_{12}H_{18}O_4$	C, 63.71	H, 7.96	C, 63.67	H, 8.22
3	2,4,6-Trimethoxy	H	60	118–120°	5	$C_{12}H_{18}O_4$	C, 63.71	H, 7.96	C, 64.13	H, 7.87
4	2,4,5-Trimethoxy	Phenyl	40	—	[b]	$C_{18}H_{22}O_4$	C, 71.52	H, 7.25	C, 71.20	H, 7.23
5	3,4,5-Trimethoxy	Methyl	35	118–120°	5	$C_{13}H_{20}O_4$	C, 65.00	H, 8.33	C, 64.68	H, 8.39
6	2,4,6-Trimethoxy	Phenyl	36	—	[c]	$C_{18}H_{22}O_4$	C, 71.52	H, 7.25	C, 71.38	H, 7.34

[a] M.p. 70–72°. [b] M.p. 85–87°. [c] M.p. 84–86°. Recrystallizations from benzene–petroleum ether, 60–80.

Table II—TLC and Spectral Data of the Compounds of Table I

No.	Van Urk's Color Response SM[a]	Van Urk's Color Response P[a]	UV Absorbance Maxima, mμ ($\epsilon_{max.}$)	IR —C—OH	IR Aromatic C=C (Micron)	NMR Chemical Shifts, δ[b]
1	Yellow (0.82)	Violet (0.76)	265 (7900)	2.75	6.3	a-0.83(3H,t); b-1.68(2H,m); c-4.40(H,t); d and e-3.73(10H,s); f-6.52(2H,s)
2	Yellow (0.72)	Gr. Black (0.76)	290 (7340)	2.80	6.3	a-0.92(3H,t); b-1.70(2H,m); c-4.82(H,t); d-2.95(H,s); e-3.82(9H,um); f-6.52(H,s); f-6.95(H,s)
3	Yellow (0.65)	Br. Red (0.70)	270 (7190)	2.75	6.2	a-0.88(3H,t); b-1.78(2H,m); c-4.88(H,t); d and e-3.70(10H,s); f-6.12(2H,s)
4	Orange Yellow (0.78)	Gr. Black (0.76)	290 (10,408)	2.80	6.3	a-0.90(3H,t); b-2.15(2H,q); c-7.25(5H,s); d-4.42(H,s); e-3.41(3H,s); e-3.85(6H,s); f-6.50(H,s); f-7.05(H,s)
5	Yellow (0.70)	Br. Red (0.82)	255 (8000)	2.75	6.2	a-0.80(3H,t); b-1.70(2H,q); c-1.50(3H,s); d-3.05(H,s); e-3.80(9H,s); f-6.70(2H,s)
6	Yellow (0.70)	Br. Red (0.76)	250 (10,470)	2.75	6.3	a-0.98(3H,t); b-2.60(2H,q); c-7.28(5H,s); d and e-3.70(10H, um); f-6.18(2H,s)

[a] The values in the parentheses represent the R_f values using *n*-butanol–acetic acid–water (12:5:5); SM = starting material; P = product. [b] s, singlet; t, triplet; q, quartet; m, multiplet; um, unresolvable multiplet.

a = —C(CH₃)— , b = —C(H)—C—C— , c = —C(X)—C— , d = —OH, e = —OCH₃, f = Aromatic.

X = H, CH_3, C_6H_5

Thin-Layer Chromatography—Thin-layer plates (5 × 20 cm.) made of silica gel G were used. Van Urk's reagent (6) was used as a spray reagent to differentiate the starting material and product by a varied color response.

Spectra—UV spectra were obtained on a Beckman DK 2 spectrophotometer (1-cm. cell) in methylene chloride as solvent. IR spectra were determined on a Beckman IR 8 spectrophotometer in potassium bromide pellets and methylene chloride liquid films. NMR spectra were determined on a Varian 60 MC spectrophotometer using deuterochloroform as the solvent and tetramethylsilane as the internal standard.

REFERENCES

(1) P. C. Dandiya and M. K. Menon, *Brit. J. Pharmacol.*, **20,** 436(1963).

(2) P. C. Dandiya and M. K. Menon, *J. Pharmacol. Exp. Ther.*, **145,** 42(1964).

(3) P. C. Dandiya and M. K. Menon, *Life Sci.*, **4,** 1635(1965).

(4) R. M. Baxter, P. C. Dandiya, S. I. Kandel, A. Okany, and G. C. Walker, *Nature*, **185,** 466(1960).

(5) R. M. Baxter, M. C. Fan, and S. I. Kandel, *Can. J. Chem.*, **40,** 154(1962).

(6) "British Pharmacopoeia," The Pharmaceutical Press, W. C. 1, London, England, 1968, p. 1121.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 5, 1969, from the *Faculty of Pharmacy, University of Toronto, Toronto 181, Canada.*

Accepted for publication February 4, 1970.

New Compounds: Demethylated Methocarbamol

C. M. DARLING* and E. K. ROSE

Abstract □ Two isomeric monocarbamates of 3-(*o*-benzyloxyphenoxy)-1,2-dihydroxypropane and 3-(*o*-hydroxyphenoxy)-1,2-dihydroxypropane were synthesized. The structural assignments are supported by spectral data.

Keyphrases □ Demethylated methocarbamol derivatives—synthesis, structure determination □ Methocarbamol metabolites—synthesis, structure determination □ NMR spectroscopy—structure, identification

Baizer *et al.* (1) have presented rigorous proof of the structure of some isomeric monocarbamates of 1,2-dihydroxy-3-aryloxypropanes by independent unequivocal synthesis. In a later paper, Swidinsky *et al.* (2) reported the preparation of two isomeric monocarbamates of 3-(*o*-hydroxyphenoxy)-1,2-dihydroxypropane, Compounds III and IV, by catalytic debenzylation of Compounds I and II. However, their tentative structural assignments differ from those reported here.

This work was prompted by a need for one of the metabolites (Compound III) of methocarbamol.[1] It is shown that the isomeric monocarbamates can be identified by spectral data. The results of a single run indicate that the major product of the reaction of ammonia with the cyclic carbonate, 4-(*o*-benzyloxyphenoxymethyl)-1,3-dioxolone-2, is a primary carbamate (Compound I) and the minor product is a secondary carbamate (Compound II). The isomeric compounds and their melting-point values are shown in Table I.

The NMR spectra of the isomeric pairs (I, II and III, IV) taken in dimethyl sulfoxide-d_6 exhibit significant and distinguishing differences. Compound II clearly must have the secondary carbamate structure as shown by the splitting of the primary hydroxyl proton into a triplet (δ 4.90; J = 5.5 cps.)[2] by the adjacent methylene group, rather than a doublet as expected for a secondary hydroxyl proton. In addition, the methylene protons, CH_2OH, adjacent to the hydroxyl group are coupled by approximately the same coupling constant (5.5 cps.) to the OH proton and the adjacent methine hydrogen, giving rise to a triplet (δ 3.67) which collapses to a doublet on deuteration.

The isomeric Compound I in dimethyl sulfoxide-d_6 shows only a single unsplit hydroxyl peak[3] with all five aliphatic hydrogens appearing under a broad distorted doublet centered at δ 4.1. Acetylation of the hydroxyl group, however, shifts the secondary methine absorption approximately 1.3 p.p.m. down-field,[4] supporting the primary carbamate structure for Compound I.

The NMR structural assignments are supported by the fact that the comparable features of the spectra of I and II are nearly identical to those of the related analogs of known structure (1), 3-(*o*-methoxyphenoxy)-2-hydroxy-1-propyl carbamate and 3-(*o*-methoxyphenoxy)-1-hydroxy-2-propyl carbamate, respectively.

EXPERIMENTAL[5]

The method of Swidinsky *et al.* (2) was used for the preparation of 4-(*o*-benzyloxyphenoxymethyl)-1,3-dioxolone-2.

3-(*o*-Benzyloxyphenoxy)-2-hydroxy-1-propyl Carbamate (I)—While maintaining a reaction temperature below 40°, a suspension of 4-(*o*-benzyloxyphenoxymethyl)-1,3-dioxolone-2 (74 g., 0.25 mole) in isopropyl alcohol (800 ml.) was saturated with ammonia. The mixture was allowed to stand at ambient temperatures for 24 hr.

[1] Methocarbamol is marketed as Robaxin by A. H. Robins Co., Inc., Richmond, Va.

[2] The OH absorption is superimposed on the methine multiplet but is clearly distinguishable and easily removed by deuteration.

[3] Evidently the exchange rate is too great in this case for splitting to be seen.

[4] This comparison was made using $CDCl_3$ as the solvent for both the acetylated and nonacetylated samples.

[5] Melting points are corrected. Elemental analyses were performed by the Analytical Department, Research Laboratories, A. H. Robins Co., Inc., Richmond, Va.

Thin-Layer Chromatography—Thin-layer plates (5 × 20 cm.) made of silica gel G were used. Van Urk's reagent (6) was used as a spray reagent to differentiate the starting material and product by a varied color response.

Spectra—UV spectra were obtained on a Beckman DK 2 spectrophotometer (1-cm. cell) in methylene chloride as solvent. IR spectra were determined on a Beckman IR 8 spectrophotometer in potassium bromide pellets and methylene chloride liquid films. NMR spectra were determined on a Varian 60 MC spectrophotometer using deuterochloroform as the solvent and tetramethylsilane as the internal standard.

REFERENCES

(1) P. C. Dandiya and M. K. Menon, *Brit. J. Pharmacol.*, **20,** 436(1963).

(2) P. C. Dandiya and M. K. Menon, *J. Pharmacol. Exp. Ther.*, **145,** 42(1964).

(3) P. C. Dandiya and M. K. Menon, *Life Sci.*, **4,** 1635(1965).

(4) R. M. Baxter, P. C. Dandiya, S. I. Kandel, A. Okany, and G. C. Walker, *Nature*, **185,** 466(1960).

(5) R. M. Baxter, M. C. Fan, and S. I. Kandel, *Can. J. Chem.*, **40,** 154(1962).

(6) "British Pharmacopoeia," The Pharmaceutical Press, W. C. 1, London, England, 1968, p. 1121.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 5, 1969, from the *Faculty of Pharmacy, University of Toronto, Toronto 181, Canada.*

Accepted for publication February 4, 1970.

New Compounds: Demethylated Methocarbamol

C. M. DARLING* and E. K. ROSE

Abstract □ Two isomeric monocarbamates of 3-(*o*-benzyloxyphenoxy)-1,2-dihydroxypropane and 3-(*o*-hydroxyphenoxy)-1,2-dihydroxypropane were synthesized. The structural assignments are supported by spectral data.

Keyphrases □ Demethylated methocarbamol derivatives—synthesis, structure determination □ Methocarbamol metabolites—synthesis, structure determination □ NMR spectroscopy—structure, identification

Baizer *et al.* (1) have presented rigorous proof of the structure of some isomeric monocarbamates of 1,2-dihydroxy-3-aryloxypropanes by independent unequivocal synthesis. In a later paper, Swidinsky *et al.* (2) reported the preparation of two isomeric monocarbamates of 3-(*o*-hydroxyphenoxy)-1,2-dihydroxypropane, Compounds III and IV, by catalytic debenzylation of Compounds I and II. However, their tentative structural assignments differ from those reported here.

This work was prompted by a need for one of the metabolites (Compound III) of methocarbamol.[1] It is shown that the isomeric monocarbamates can be identified by spectral data. The results of a single run indicate that the major product of the reaction of ammonia with the cyclic carbonate, 4-(*o*-benzyloxyphenoxymethyl)-1,3-dioxolone-2, is a primary carbamate (Compound I) and the minor product is a secondary carbamate (Compound II). The isomeric compounds and their melting-point values are shown in Table I.

The NMR spectra of the isomeric pairs (I, II and III, IV) taken in dimethyl sulfoxide-d_6 exhibit significant and distinguishing differences. Compound II clearly must have the secondary carbamate structure as shown by the splitting of the primary hydroxyl proton into a triplet (δ 4.90; J = 5.5 cps.)[2] by the adjacent methylene group, rather than a doublet as expected for a secondary hydroxyl proton. In addition, the methylene protons, CH_2OH, adjacent to the hydroxyl group are coupled by approximately the same coupling constant (5.5 cps.) to the OH proton and the adjacent methine hydrogen, giving rise to a triplet (δ 3.67) which collapses to a doublet on deuteration.

The isomeric Compound I in dimethyl sulfoxide-d_6 shows only a single unsplit hydroxyl peak[3] with all five aliphatic hydrogens appearing under a broad distorted doublet centered at δ 4.1. Acetylation of the hydroxyl group, however, shifts the secondary methine absorption approximately 1.3 p.p.m. down-field,[4] supporting the primary carbamate structure for Compound I.

The NMR structural assignments are supported by the fact that the comparable features of the spectra of I and II are nearly identical to those of the related analogs of known structure (1), 3-(*o*-methoxyphenoxy)-2-hydroxy-1-propyl carbamate and 3-(*o*-methoxyphenoxy)-1-hydroxy-2-propyl carbamate, respectively.

EXPERIMENTAL[5]

The method of Swidinsky *et al.* (2) was used for the preparation of 4-(*o*-benzyloxyphenoxymethyl)-1,3-dioxolone-2.

3-(*o*-Benzyloxyphenoxy)-2-hydroxy-1-propyl Carbamate (I)—While maintaining a reaction temperature below 40°, a suspension of 4-(*o*-benzyloxyphenoxymethyl)-1,3-dioxolone-2 (74 g., 0.25 mole) in isopropyl alcohol (800 ml.) was saturated with ammonia. The mixture was allowed to stand at ambient temperatures for 24 hr.

[1] Methocarbamol is marketed as Robaxin by A. H. Robins Co., Inc., Richmond, Va.

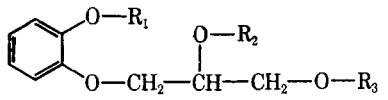
[2] The OH absorption is superimposed on the methine multiplet but is clearly distinguishable and easily removed by deuteration.

[3] Evidently the exchange rate is too great in this case for splitting to be seen.

[4] This comparison was made using $CDCl_3$ as the solvent for both the acetylated and nonacetylated samples.

[5] Melting points are corrected. Elemental analyses were performed by the Analytical Department, Research Laboratories, A. H. Robins Co., Inc., Richmond, Va.

Table I—Isomeric Monocarbamates



No.	R_1	R_2	R_3	M.p.	Anal., % Calcd.	Found
I	$-CH_2C_6H_5$	—H	$-CONH_2$	85–88° (86.6–87.6°[a])	C, 64.34 H, 6.03 N, 4.41	C, 64.17 H, 6.01 N, 4.42
II	$-CH_2C_6H_5$	$-CONH_2$	—H	100–102.5° (73.6–74.8°[b])	C, 64.34 H, 6.03 N, 4.41	C, 64.21 H, 5.97 N, 4.43
III	—H	—H	$-CONH_2$	124–128° (125–126°[c])	C, 52.86 H, 5.77 N, 6.16	C, 52.68 H, 5.81 N, 6.07
IV	—H	$-CONH_2$	—H	94–100° (116–118°[d])	C, 52.86 H, 5.77 N, 6.16	C, 52.69 H, 5.71 N, 6.16
V	$-CH_2C_6H_5$	$-COCH_3$	$-CONH_2$	60–67°	C, 63.50 H, 5.89 N, 3.90	C, 63.12 H, 5.85 N, 3.79

[a] This melting point was reported (2) for Structure II. [b] This melting point was reported (2) for Structure I. [c] This melting point was reported (2) for Structure IV. [d] This melting point was reported (2) for Structure III.

and concentrated *in vacuo*. The residue was recrystallized from ethyl acetate with the aid of activated charcoal, yield 49 g. (61.5%).

A sample of the acetate derivative, Compound V, was prepared for NMR analysis by refluxing a mixture of 0.5 g. of I, 20 ml. of acetic anhydride, and 0.5 g. of sodium acetate for 1 hr. After workup, the product was recrystallized from isopropyl ether, yield 0.3 g.

3-(*o*-Benzyloxyphenoxy)-1-hydroxy-2-propyl Carbamate (II)—The filtrate from the preparation of Compound I was concentrated to about two-thirds volume and refrigerated. The crystals were filtered and recrystallized twice from ethyl acetate, 3.5 g. (4.5%). TLC (eluted with 10% methanol in chloroform) indicated a single component different from Compound I.

3-(*o*-Hydroxyphenoxy)-2-hydroxypropyl Carbamate (III)—A solution of Compound I (12.6 g., 0.04 mole) in absolute ethanol (200 ml.) containing about 1 g. of palladium-on-charcoal (10%) was subjected to Parr hydrogenation at ambient temperature. The theoretical amount of hydrogen was absorbed during 10 min. The mixture was filtered and the filtrate was concentrated *in vacuo*. The residue was recrystallized twice from ethyl acetate, yield 2.8 g. (31%).

1-Hydroxymethyl-2-(*o*-hydroxyphenoxy)ethyl Carbamate (IV)—The procedure followed was the same as that with Compound III. The product from 2.7 g. of Compound II was recrystallized twice from ethyl acetate, yield 0.2 g. (11%).

REFERENCES

(1) M. M. Baizer, J. R. Clark, and J. Swidinsky, *J. Org. Chem.*, **22**, 1595(1957).

(2) J. Swidinsky, J. Kervenski, and B. B. Brown, *J. Pharm. Sci.*, **52**, 955(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 23, 1970, from the *Research Laboratories, A. H. Robins Company, Inc., Richmond, VA 23220*

Accepted for publication March 16, 1970.

The authors wish to thank Dr. W. J. Welstead, Jr., and Mr. Ashby Johnson, A. H. Robins Company, for assistance in the interpretation of the NMR data.

* To whom inquiries should be directed. Present address: School of Pharmacy, Auburn University, Auburn, AL 36830

Unequivocal Distinction between Betamethasone and Dexamethasone by Mass Spectrometry

Keyphrases ☐ Dexamethasone, betamethasone—distinction, determination ☐ Mass spectrometry—identity

Sir:

In a review on stereoisomeric effects on mass spectra, Meyerson and Weitkamp (1) state that mass spectra of stereoisomers are, in general, qualitatively and quantitatively similar. Reference is made therein to work that demonstrates differences between the mass spectra of epimeric pairs of secondary and tertiary steroid alcohols. More recently, Grostic and Rinehart (2) have distinguished between epimeric 11-hydroxyprogesterones by mass spectrometry.

We wish to report that mass spectrometry provides an unequivocal method of distinguishing between the epimeric pair of synthetic corticosteroids betamethasone (9α-fluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-dien-3,20-dione) and dexamethasone (9α-fluoro-11β, 17α,21-trihydroxy-16α-methylpregna-1,4-dien-3,20-dione). The difference is large enough to be of considerable value in forensic analysis. The spectra, which will be discussed fully at a later date in combination with spectra of related compounds, are shown (Fig. 1 = betamethasone, Fig. 2 = dexamethasone). The principal distinguishing feature is the peak at *m/e* 343, which corresponds to a loss of 49 mass units. In the dexamethasone spectrum, this peak is the next most prominent after the base peak (*m/e* 122); whereas in the spectrum of betamethasone, it is relatively insignificant.[1]

The peak at *m/e* 343 (M − 49) almost certainly arises from loss of water from the D-ring followed by cleavage of the 20,21-bond. Dexamethasone has a *trans*-diaxial arrangement of the 16β-hydrogen and 17α-hydroxyl, which favors dehydration. In betamethasone, there is no hydrogen *trans*-diaxially oriented to the 17α-hydroxyl. An analogous situation has been reported by Zaretskii *et al.* (3).

The high sensitivity of mass spectrometers means that this distinction may be carried out with a very limited amount of material, particularly in view of the relative abundance of the M − 49 peak in the spectrum of dexamethasone. There is, in addition, the advantage

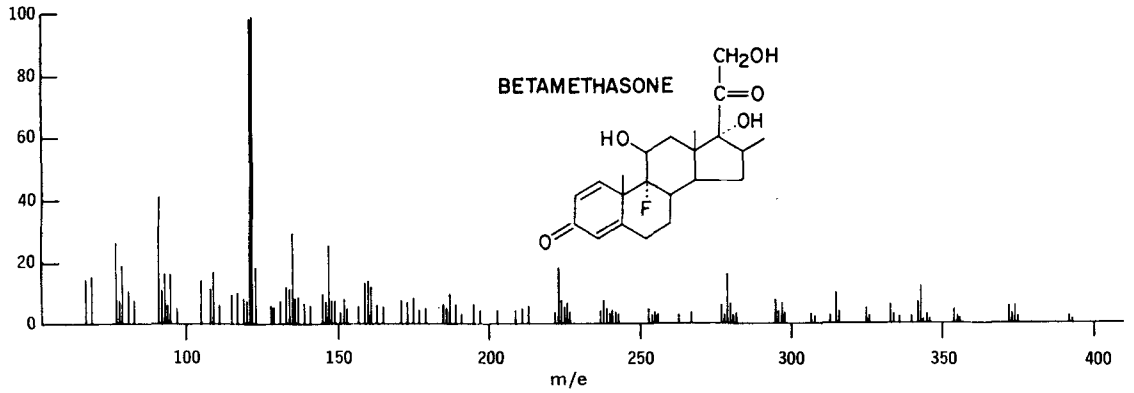


Figure 1—*Mass spectrum of betamethasone.*

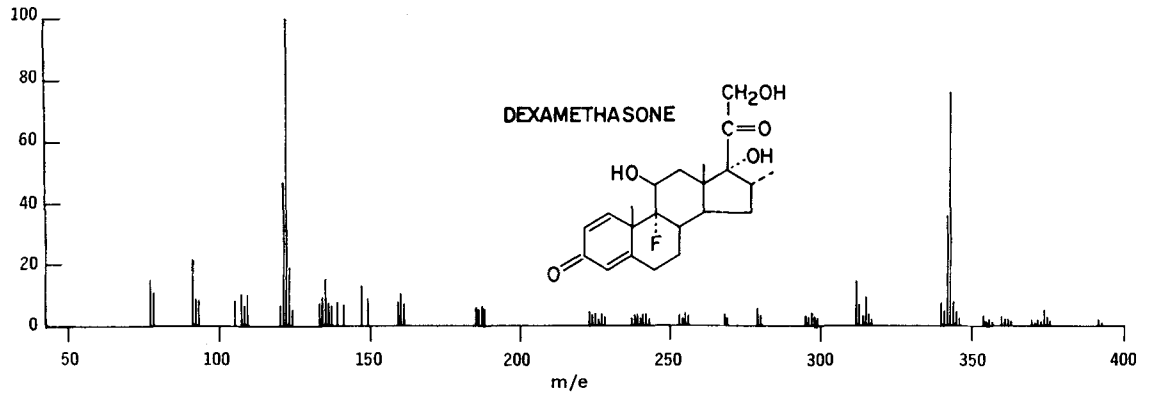


Figure 2—*Mass spectrum of dexamethasone.*

[1] Mass spectra were recorded on a Hitachi-Perkin-Elmer model RMU-6D with an ionization voltage of 70 ev. The compounds were introduced directly into the ion chamber.

for analytical purposes that the molecular weight of the substance is indicated.

(1) S. Meyerson and A. W. Weitkamp, *Org. Mass Spectrom.*, **1**, 659(1968).

(2) M. F. Grostic and K. L. Rinehart, *J. Org. Chem.*, **33**, 1740 (1968).

(3) V. I. Zaretskii, N. S. Wulfson, V. G. Zaikin, V. N. Leonov, and I. V. Torgov, *Tetrahedron*, **24**, 2339(1968).

B. A. LODGE
P. TOFT

Pharmaceutical Chemistry Division
Food and Drug Directorate
Department of National Health and Welfare
Ottawa 3, Ontario
Canada

Received December 12, 1969.

Accepted for publication February 19, 1970.

We are grateful to Dr. J. L. Holmes (University of Ottawa) for the determination of the mass spectra.

Audiosensitization: Potential Screening Method for Drugs Affecting the CNS

Keyphrases □ CNS active drugs—screening method □ Stress, sound induced—seizures

Sir:

With the advent of new types of CNS active drugs, new screening tests with predictive association for subtle drug effects are needed. The phenomenon of audioconditioned convulsions (1, 2) affords unique potential as such a screen.

The exaggerated and abnormal responses of psychiatric patients to auditory stimuli (3) prompted the use of audiogenic seizures in genetically susceptible strains of mice as an analogous reaction pattern for CNS drug research (4, 5). The present report suggests the use of audioconditioning and the subsequent susceptibility to sound-induced seizures as a simple and more informative analogy. Both analogies are based on the hypothesis that stress-induced neurosis can be measured by quantal observations of CNS hyperexcitability in response to a specific triggering mechanism. The use of audioconditioning has the advantage of offering the induction of stress susceptibility, as well as the stress-induced crisis for drug modification and study.

Susceptibility to sound-induced seizures can be conditioned in "sound-resistant" strains of mice by a short period (30 sec.) of auditory stimulation at a critical early age (1, 2). In mice, early experiments with the classical conditioning method of physiology showed that pretest exposure to sound elevates or reduces seizure threshold, depending upon the temporal parameters of treatment (6). In such reports, however, the durations of both the conditioning stimulus and the condition–test interval have been short, generally only a few seconds. The audioconditioned convulsions described here are inherently similar, but the condition–test interval is much longer and is measured in days.

Sound-resistant mice [*e.g.*, CAW:CF-1 (SW)][1] display an auricular startle upon initial sound exposure (audioconditioning), but less than 5% convulse (2). The sound source is a 6.3-cm. doorbell which produces approximately 95 db. (relative to 0.0002 dyne/cm.^{2}) within a glass testing chamber, 25 cm. in diameter by 15 cm. deep. If conditioned at the optimally sensitive age (18–20 days for CF-1), virtually all mice will convulse upon the second (test) sound exposure 2–3 days later. The initial conditioning stimulation is absolutely essential for the genesis of convulsions.

The typical seizure in such sensitized animals consists of a sudden burst of wild running, followed by clonic and then tonic convulsions. Less severe seizures terminate after running or clonus. Estimates of seizure severity can be derived from latency and duration times as well as from seizure pattern (2, 7). The following experimental factors affect these parameters (2, 7, 8):

1. The interval between conditioning and testing is critical. Maximal clonic–tonic convulsions characterize seizures after a 2- or 3-day condition–test interval; with a 1- or 5-day interval, only clonus is seen.

2. Repeated auditory stimulation prior to the development of convulsibility makes mice temporarily refractory to seizure, and prolongation of the initial conditioning sound (over 6 hr.) imparts permanent seizure resistance without causing deafness. Once an animal experiences a convulsion, however, seizure susceptibility persists for several weeks. This indicates that audiosensitization and seizure susceptibility are mediated by separate mechanisms.

3. The tonal characteristics of the sound stimulus are equally or more important than the intensity. Although reproducibility is excellent, it is necessary to bioassay each bell periodically. After extensive use, a bell may no longer induce maximal seizures, despite no alteration in intensity.

4. Genetic and environmental factors must be controlled. Noises in the animal quarters, such as the clatter of metal garbage cans, have profound influence. The critical age for sensitization and the optimum condition–test interval vary from strain to strain. CF-1 mice have a high incidence of maximal seizures, a short duration of audiosensitivity, and a low death risk.

When these experimental factors are controlled, seizures of predictable incidence, severity, and latencies are produced (2).

Theoretically, pharmacologic alteration of audioconditioned seizures should be afforded by: (*a*) drugs that impair hearing or otherwise interfere with input of the sound stimulus; (*b*) drugs that block central perception of the stimulus; (*c*) drugs that inhibit or enhance the slow process of sensitivity development; (*d*) drugs that block the effect of intertrial stress; and (*e*) drugs that modify the mechanism of seizure production. The novel interest in audioconditioning as a screen will be for drugs that alter the development of sensitization (*b* and *c* as previously mentioned). For these drugs, this screen is unique because the potential drug need not be present at the time the animals are challenged for a test response. Thus, the prosensitizing

[1] Carworth Farms, New City, N. Y.

for analytical purposes that the molecular weight of the substance is indicated.

(1) S. Meyerson and A. W. Weitkamp, *Org. Mass Spectrom.*, **1**, 659(1968).

(2) M. F. Grostic and K. L. Rinehart, *J. Org. Chem.*, **33**, 1740 (1968).

(3) V. I. Zaretskii, N. S. Wulfson, V. G. Zaikin, V. N. Leonov, and I. V. Torgov, *Tetrahedron*, **24**, 2339(1968).

B. A. LODGE
P. TOFT

Pharmaceutical Chemistry Division
Food and Drug Directorate
Department of National Health and Welfare
Ottawa 3, Ontario
Canada

Received December 12, 1969.

Accepted for publication February 19, 1970.

We are grateful to Dr. J. L. Holmes (University of Ottawa) for the determination of the mass spectra.

Audiosensitization: Potential Screening Method for Drugs Affecting the CNS

Keyphrases □ CNS active drugs—screening method □ Stress, sound induced—seizures

Sir:

With the advent of new types of CNS active drugs, new screening tests with predictive association for subtle drug effects are needed. The phenomenon of audioconditioned convulsions (1, 2) affords unique potential as such a screen.

The exaggerated and abnormal responses of psychiatric patients to auditory stimuli (3) prompted the use of audiogenic seizures in genetically susceptible strains of mice as an analogous reaction pattern for CNS drug research (4, 5). The present report suggests the use of audioconditioning and the subsequent susceptibility to sound-induced seizures as a simple and more informative analogy. Both analogies are based on the hypothesis that stress-induced neurosis can be measured by quantal observations of CNS hyperexcitability in response to a specific triggering mechanism. The use of audioconditioning has the advantage of offering the induction of stress susceptibility, as well as the stress-induced crisis for drug modification and study.

Susceptibility to sound-induced seizures can be conditioned in "sound-resistant" strains of mice by a short period (30 sec.) of auditory stimulation at a critical early age (1, 2). In mice, early experiments with the classical conditioning method of physiology showed that pretest exposure to sound elevates or reduces seizure threshold, depending upon the temporal parameters of treatment (6). In such reports, however, the durations of both the conditioning stimulus and the condition–test interval have been short, generally only a few seconds. The audioconditioned convulsions described here are inherently similar, but the condition–test interval is much longer and is measured in days.

Sound-resistant mice [*e.g.*, CAW:CF-1 (SW)][1] display an auricular startle upon initial sound exposure (audioconditioning), but less than 5% convulse (2). The sound source is a 6.3-cm. doorbell which produces approximately 95 db. (relative to 0.0002 dyne/cm.^{2}) within a glass testing chamber, 25 cm. in diameter by 15 cm. deep. If conditioned at the optimally sensitive age (18–20 days for CF-1), virtually all mice will convulse upon the second (test) sound exposure 2–3 days later. The initial conditioning stimulation is absolutely essential for the genesis of convulsions.

The typical seizure in such sensitized animals consists of a sudden burst of wild running, followed by clonic and then tonic convulsions. Less severe seizures terminate after running or clonus. Estimates of seizure severity can be derived from latency and duration times as well as from seizure pattern (2, 7). The following experimental factors affect these parameters (2, 7, 8):

1. The interval between conditioning and testing is critical. Maximal clonic–tonic convulsions characterize seizures after a 2- or 3-day condition–test interval; with a 1- or 5-day interval, only clonus is seen.

2. Repeated auditory stimulation prior to the development of convulsibility makes mice temporarily refractory to seizure, and prolongation of the initial conditioning sound (over 6 hr.) imparts permanent seizure resistance without causing deafness. Once an animal experiences a convulsion, however, seizure susceptibility persists for several weeks. This indicates that audiosensitization and seizure susceptibility are mediated by separate mechanisms.

3. The tonal characteristics of the sound stimulus are equally or more important than the intensity. Although reproducibility is excellent, it is necessary to bioassay each bell periodically. After extensive use, a bell may no longer induce maximal seizures, despite no alteration in intensity.

4. Genetic and environmental factors must be controlled. Noises in the animal quarters, such as the clatter of metal garbage cans, have profound influence. The critical age for sensitization and the optimum condition–test interval vary from strain to strain. CF-1 mice have a high incidence of maximal seizures, a short duration of audiosensitivity, and a low death risk.

When these experimental factors are controlled, seizures of predictable incidence, severity, and latencies are produced (2).

Theoretically, pharmacologic alteration of audioconditioned seizures should be afforded by: (*a*) drugs that impair hearing or otherwise interfere with input of the sound stimulus; (*b*) drugs that block central perception of the stimulus; (*c*) drugs that inhibit or enhance the slow process of sensitivity development; (*d*) drugs that block the effect of intertrial stress; and (*e*) drugs that modify the mechanism of seizure production. The novel interest in audioconditioning as a screen will be for drugs that alter the development of sensitization (*b* and *c* as previously mentioned). For these drugs, this screen is unique because the potential drug need not be present at the time the animals are challenged for a test response. Thus, the prosensitizing

[1] Carworth Farms, New City, N. Y.

or antisensitizing effects of the drug can be isolated from any sedative–anticonvulsant action it may have.

Physical impairment of hearing (*e.g.*, glycerin in the ear) during conditioning reduces the incidence of sensitization (and subsequent seizures); impairment at the time of testing reduces seizure severity. When hearing is impaired on both occasions, the two effects are combined (7). The latter should be the case when ototoxic drugs are tested with this screen. Transient and permanent effects can be partitioned by subjecting the mice to a third sound exposure 3 days after the test exposure.

A drug that blocks central perception of the sound stimulus should be detected by its ability to prevent sensitization when present during conditioning, but not when administered immediately afterward. Prototypes of many pharmacologic classes have been tested, but no drug has been found that effectively blocks audioconditioning at nontoxic doses. Latency changes and a decreased incidence of maximal seizures can be observed. However, because of the reduced metabolic and excretory potential of young mice, it is difficult to determine whether these are due to impairment of audioconditioning or to residual drug effects on seizure response. The latter remains a possibility even after the 2- or 3-day condition–test interval. Phenobarbital and diphenylhydantoin have been shown to be proconvulsant (rather than anticonvulsant) 2 days after their administration (8).

It is interesting to note that ether and pentobarbital anesthesias do not block central perception of the stimulus. Their presence during conditioning does not prevent sensitization but rather appears to enhance its development and to counteract the antisensitization effects of unilateral ear blockade (7).

In contrast to drugs that only inhibit or enhance sensitization, we recently have discovered that high doses of atropine sulfate (25 mg./kg., i.p.) completely block the development of sensitization. While this admittedly is an extremely high dosage, it is the first indication that audioconditioning can be prevented by drugs. Furthermore, since it is an ED_{100} dose, it is likely that a lower dose range can be found.

A drug that alters postconditioning development of sensitization would be detected by its ability to inhibit or enhance sensitization when administered after the conditioning stimulus. Several drugs of this type have been observed in our laboratory (8). Low doses of edrophonium (1–2 mg./kg., i.p.) inhibit sensitization when administered 30 min. after conditioning. When tested 2 days later, seizure incidence and severity are reduced and latencies are prolonged. The effect is similar but less striking when edrophonium is given before conditioning. This perhaps is explained by the fact that the postconditioning duration of drug action is shorter in this case. When edrophonium-treated mice are challenged at a 3-day condition–test interval, the usual seizures are elicited, indicating that this drug slows rather than blocks the development of sensitization.

A multitude of drugs promote or inhibit the seizure response when present at the test exposure to sound. In general, convulsants promote seizures, whereas anticonvulsants, sedatives, and tranquilizers inhibit their onset and severity (8). These drugs produce similar effects on audiogenic seizures in genetically susceptible strains of mice.

The advantages of audioconditioned seizures as a screening method lie in several areas. As a biomodel of stress-induced neurosis, it does not require the use of genetically susceptible strains, special diets, chemicals, or surgical manipulation. The test response is a quick quantal observation which can be easily assessed by a technician. Also, since it involves the use of immature animals, this screen may have special predictive value for drugs to treat neurologic diseases of children.

It is our conclusion that audioconditioning should serve as an important experimental approach to the study of neural hyperexcitability and as a useful screening method for drugs affecting the CNS.

(1) W. B. Iturrian and G. B. Fink, *Fed. Proc.*, **26**, 736(1967).
(2) W. B. Iturrian and G. B. Fink, *Develop. Psychobiol.*, **1**, 230 (1968).
(3) R. B. Malmo, *Trans. N. Y. Acad. Sci.*, **18**, 545(1956).
(4) N. P. Plotnikoff, *Arch. Int. Pharmacodyn. Ther.*, **116**, 130 (1958).
(5) G. B. Fink and E. A. Swinyard, *J. Pharmacol. Exp. Ther.*, **127**, 318(1959).
(6) W. Bevan, *Psychol. Bull.*, **52**, 473(1955).
(7) W. B. Iturrian and H. D. Johnson, *Pharmacologist*, **11**, 248 (1969).
(8) W. B. Iturrian, to be published.

W. B. Iturrian
H. D. Johnson
Dept. of Pharmacology
School of Pharmacy
University of Georgia
Athens, GA 30601

Received February 2, 1970.
Accepted for publication March 9, 1970.
A preliminary report was presented at the AAAS meeting, Pharmaceutical Sciences section, Boston, Mass., 1969.

Binding of Salicylate to Crystalline Bovine Serum Albumin and to Fraction V Bovine Serum Albumin

Keyphrases ☐ Salicylate binding—bovine serum albumin ☐ Bovine serum albumin, crystalline, Fraction V—salicylate binding comparison ☐ Equilibrium dialysis—bovine serum albumin salicylate binding

Sir:

In the course of developing analytical methods for protein-binding studies, we have routinely used Fraction V bovine serum albumin (BSA) rather than the more costly crystalline BSA in preliminary work. For a drug such as sulfaethidole (SETD), there appears to be little difference in binding of the drug to Fraction V BSA and to crystalline BSA; a survey of references cited in a recent review (1) indicates that many workers have

or antisensitizing effects of the drug can be isolated from any sedative–anticonvulsant action it may have.

Physical impairment of hearing (*e.g.*, glycerin in the ear) during conditioning reduces the incidence of sensitization (and subsequent seizures); impairment at the time of testing reduces seizure severity. When hearing is impaired on both occasions, the two effects are combined (7). The latter should be the case when ototoxic drugs are tested with this screen. Transient and permanent effects can be partitioned by subjecting the mice to a third sound exposure 3 days after the test exposure.

A drug that blocks central perception of the sound stimulus should be detected by its ability to prevent sensitization when present during conditioning, but not when administered immediately afterward. Prototypes of many pharmacologic classes have been tested, but no drug has been found that effectively blocks audioconditioning at nontoxic doses. Latency changes and a decreased incidence of maximal seizures can be observed. However, because of the reduced metabolic and excretory potential of young mice, it is difficult to determine whether these are due to impairment of audioconditioning or to residual drug effects on seizure response. The latter remains a possibility even after the 2- or 3-day condition–test interval. Phenobarbital and diphenylhydantoin have been shown to be proconvulsant (rather than anticonvulsant) 2 days after their administration (8).

It is interesting to note that ether and pentobarbital anesthesias do not block central perception of the stimulus. Their presence during conditioning does not prevent sensitization but rather appears to enhance its development and to counteract the antisensitization effects of unilateral ear blockade (7).

In contrast to drugs that only inhibit or enhance sensitization, we recently have discovered that high doses of atropine sulfate (25 mg./kg., i.p.) completely block the development of sensitization. While this admittedly is an extremely high dosage, it is the first indication that audioconditioning can be prevented by drugs. Furthermore, since it is an ED_{100} dose, it is likely that a lower dose range can be found.

A drug that alters postconditioning development of sensitization would be detected by its ability to inhibit or enhance sensitization when administered after the conditioning stimulus. Several drugs of this type have been observed in our laboratory (8). Low doses of edrophonium (1–2 mg./kg., i.p.) inhibit sensitization when administered 30 min. after conditioning. When tested 2 days later, seizure incidence and severity are reduced and latencies are prolonged. The effect is similar but less striking when edrophonium is given before conditioning. This perhaps is explained by the fact that the postconditioning duration of drug action is shorter in this case. When edrophonium-treated mice are challenged at a 3-day condition–test interval, the usual seizures are elicited, indicating that this drug slows rather than blocks the development of sensitization.

A multitude of drugs promote or inhibit the seizure response when present at the test exposure to sound. In general, convulsants promote seizures, whereas anticonvulsants, sedatives, and tranquilizers inhibit their onset and severity (8). These drugs produce similar effects on audiogenic seizures in genetically susceptible strains of mice.

The advantages of audioconditioned seizures as a screening method lie in several areas. As a biomodel of stress-induced neurosis, it does not require the use of genetically susceptible strains, special diets, chemicals, or surgical manipulation. The test response is a quick quantal observation which can be easily assessed by a technician. Also, since it involves the use of immature animals, this screen may have special predictive value for drugs to treat neurologic diseases of children.

It is our conclusion that audioconditioning should serve as an important experimental approach to the study of neural hyperexcitability and as a useful screening method for drugs affecting the CNS.

(1) W. B. Iturrian and G. B. Fink, *Fed. Proc.*, **26**, 736(1967).
(2) W. B. Iturrian and G. B. Fink, *Develop. Psychobiol.*, **1**, 230 (1968).
(3) R. B. Malmo, *Trans. N. Y. Acad. Sci.*, **18**, 545(1956).
(4) N. P. Plotnikoff, *Arch. Int. Pharmacodyn. Ther.*, **116**, 130 (1958).
(5) G. B. Fink and E. A. Swinyard, *J. Pharmacol. Exp. Ther.*, **127**, 318(1959).
(6) W. Bevan, *Psychol. Bull.*, **52**, 473(1955).
(7) W. B. Iturrian and H. D. Johnson, *Pharmacologist*, **11**, 248 (1969).
(8) W. B. Iturrian, to be published.

W. B. Iturrian
H. D. Johnson
Dept. of Pharmacology
School of Pharmacy
University of Georgia
Athens, GA 30601

Received February 2, 1970.
Accepted for publication March 9, 1970.
A preliminary report was presented at the AAAS meeting, Pharmaceutical Sciences section, Boston, Mass., 1969.

Binding of Salicylate to Crystalline Bovine Serum Albumin and to Fraction V Bovine Serum Albumin

Keyphrases ☐ Salicylate binding—bovine serum albumin ☐ Bovine serum albumin, crystalline, Fraction V—salicylate binding comparison ☐ Equilibrium dialysis—bovine serum albumin salicylate binding

Sir:

In the course of developing analytical methods for protein-binding studies, we have routinely used Fraction V bovine serum albumin (BSA) rather than the more costly crystalline BSA in preliminary work. For a drug such as sulfaethidole (SETD), there appears to be little difference in binding of the drug to Fraction V BSA and to crystalline BSA; a survey of references cited in a recent review (1) indicates that many workers have

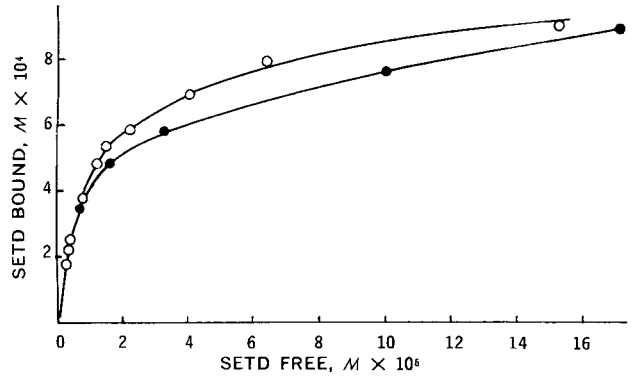


Figure 1—*Binding of SETD to 4.55% crystalline (●) and Fraction V (○) BSA, pH 7.4, 37°.*

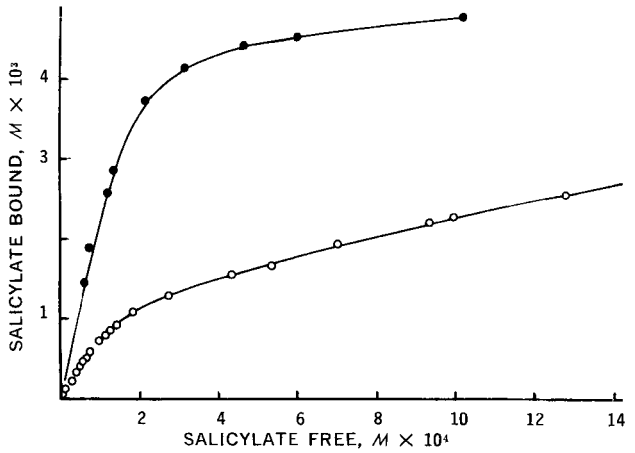


Figure 2—*Binding of salicylate to 4.55% crystalline (●) and Fraction V (○) BSA, pH 7.4, 37°.*

utilized Fraction V BSA to study drug binding. In studies with salicylate, however, we observed a marked difference in binding to these two BSA fractions. There have been reports of striking species differences in serum albumin binding of drugs (2–5), but the potential erroneous predictions which might arise from the assumption that if one drug exhibits similar binding to both crystalline and Fraction V BSA other drugs will also exhibit similar binding to both fractions have not been emphasized.

As shown in Fig. 1, SETD exhibits little difference in binding to Fraction V and crystalline BSA. Figure 2, however, illustrates considerably greater binding of salicylate to crystalline BSA than to Fraction V BSA. For example, at a total salicylate concentration of 47×10^{-4} *M*, 91% is bound in the presence of 4.55% crystalline BSA, while only 63% is bound by 4.55% Fraction V BSA.

A further and striking difference between the crystalline and Fraction V BSA is that 10 mg.% SETD, an agent that competes for binding sites with salicylate, and an agent that is effectively displaced from serum albumin by salicylate (6), greatly enhances the binding of salicylate to Fraction V BSA, while it exhibits the expected displacement of salicylate from binding to crystalline BSA and pooled human plasma (7). As illustrated in Fig. 3, at total salicylate concentrations less than about 6×10^{-4} *M* (9.6 mg.% sodium salicylate), there is displacement of salicylate upon addition of 10 mg.% SETD. But at higher salicylate concentrations, there is markedly enhanced binding of salicylate to the protein in the presence of 10 mg.% SETD.

It is apparent that Fraction V and crystalline BSA differ in such a manner that the binding of SETD is not

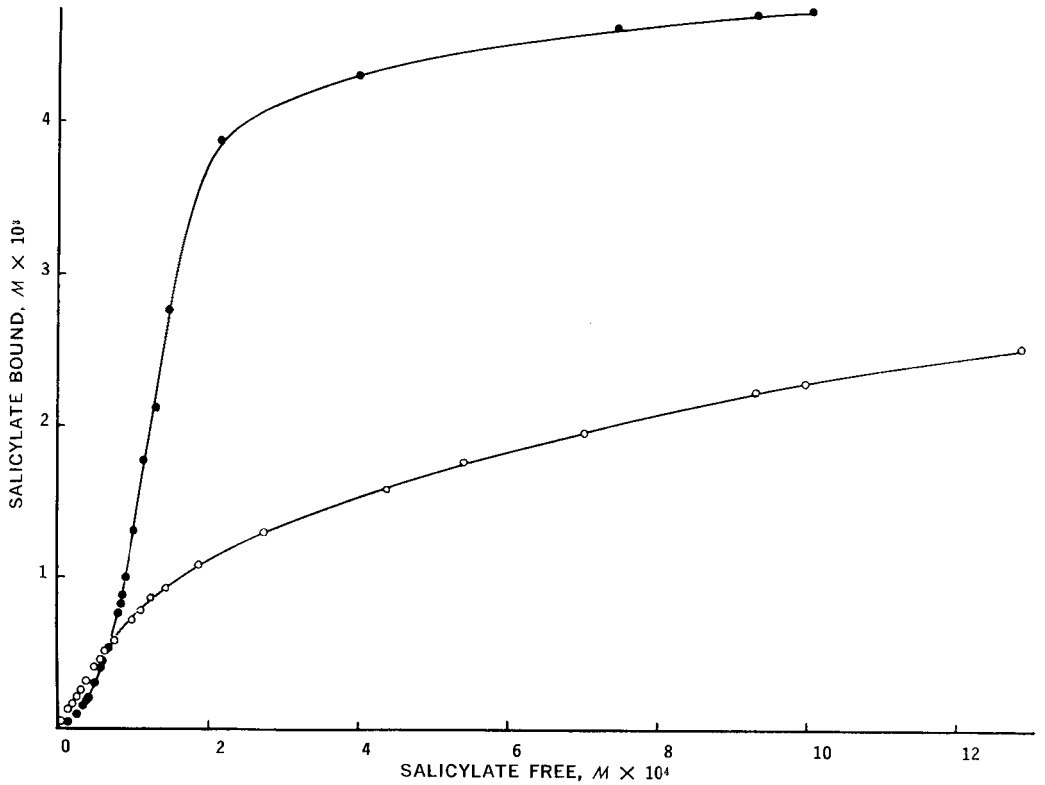


Figure 3—*Binding of salicylate to 4.55% Fraction V BSA (○) and the effect of 10 mg.% SETD on binding of salicylate (●), pH 7.4, 37°.*

greatly affected, but the binding of salicylate to Fraction V is greatly diminished. In Fraction V BSA, all of the salicylate binding sites may not be available or readily accessible to the drug. Upon addition of 10 mg.% SETD, however, there may be a subtle conformational change induced by the binding of SETD such that the Fraction V resembles the crystalline BSA in affinity for salicylate. Further studies in progress are directed toward exploration of the nature of the conformational change and its influence on the number of salicylate binding sites and the respective affinity constants.

Binding data were obtained by equilibrium dialysis for 12 hr. at 37°, in pH 7.4, 0.054 *M* phosphate buffer made isotonic with sodium chloride. Both inside and outside solutions were assayed for drug content, utilizing the Bratton-Marshall procedure for SETD (8), and both UV spectrophotometric analysis and ^{14}C were used for determination of salicylate.

(1) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968).

(2) J. A. Sturman and M. J. H. Smith, *J. Pharm. Pharmacol.*, **19**, 621(1967).

(3) J. L. Kucera and F. J. Bullock, *ibid.*, **21**, 293(1969).

(4) D. T. Witiak and M. W. Whitehouse, *Biochem. Pharmacol.*, **18**, 971(1969).

(5) D. T. Witiak, T. D. Sokoloski, M. W. Whitehouse, and F. Hermann, *J. Med. Chem.*, **12**, 754(1969).

(6) A. H. Anton, *J. Pharmacol. Exp. Ther.*, **134**, 291(1961).

(7) H. B. Kostenbauder and M. J. Jawad, unpublished data.

(8) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

H. B. Kostenbauder
S. M. Bahal
M. J. Jawad

College of Pharmacy
University of Kentucky
Lexington, KY 40506

Received December 22, 1969.

Accepted for publication February 13, 1970.

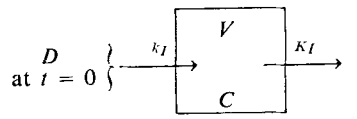
Supported in part by Research Grant GM-17060-01 from the National Institute of General Medical Sciences, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

"Absorption Rate Constants" Calculated According to the One-Compartment Open Model with First-Order Absorption: Implications in *In Vivo–In Vitro* Correlations

Keyphrases □ "Absorption rate constants"—one-compartment open model □ *In vivo–in vitro* correlations, "absorption rate constants"—calculation method effect

Sir:

Plasma or serum concentrations of unchanged drug observed following oral administration of single doses of a drug frequently are readily fit by the one-compartment open model with first-order absorption (Model I). Wagner and Metzler (1) cited much of the literature on this fitting; such fitting leads to Eq. 1:



Model I

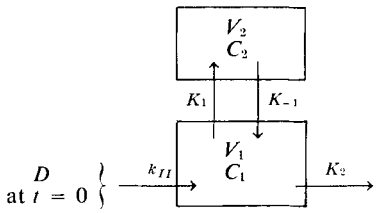
$$C = C^{\circ}\left(\frac{k_I}{k_I - K_I}\right)(e^{-K_I t} - e^{-k_I t}) \qquad \text{(Eq. 1)}$$

Even when simulated data are generated by application of Eqs. 2 and 2*a*,

$$C_1 = \frac{k_{II}D}{V_1}\left[\left\{\frac{K_{-1} - \alpha}{(k_{II} - \alpha)(\beta - \alpha)}\right\}e^{-\alpha t} + \left\{\frac{K_{-1} - \beta}{(k_{II} - \beta)(\alpha - \beta)}\right\}e^{-\beta t} + \left\{\frac{K_{-1} - k_{II}}{(\alpha - k_{II})(\beta - k_{II})}\right\}e^{-k_{II}t}\right] \qquad \text{(Eq. 2)}$$

$$\alpha,\beta = \tfrac{1}{2}\left[(K_1 + K_{-1} + K_2) \pm \sqrt{(K_1 + K_{-1} + K_2)^2 - 4K_{-1}K_2}\right] \qquad \text{(Eq. 2a)}$$

which are appropriate to the two-compartment open model with first-order absorption (Model II), most sets of C_1,t data can be fitted with two exponential terms (Eq. 1) rather than three exponential terms (Eq. 2). With real plasma or serum concentration data observed following oral administration, the same situation exists; however, in some cases, intravenous administration of the same drug tends to dictate the two-compartment open model.



Model II

When correlating "absorption rate constants" derived from plasma concentrations measured in man with *in vitro* rates of drug dissolution from dosage forms, one is most interested in relative values or ratios and not with absolute individual values.

The data in Table I are taken from Wagner and Metzler (1). When apparent "rate constants for absorptions," k_I, were estimated by nonlinear least-squares estimation, by applying Eq. 1 to data generated with Eqs. 2 and 2*a*, the absolute values of k_I deviated from the k_{II} values (either 0.5 or 2.0 hr.$^{-1}$), but the ratios of the k_I values were very close to the ratio of the true k_{II} values (namely, 4.0) when $8 \geq V_1/V_2 = K_{-1}/K_1 \geq 1$. It was previously shown (1) that where Model II was elaborated from actual plasma or serum level data, the ratio of parameters was within the limits shown in Table I.

This observation may help the dilemma of the biopharmaceutical and pharmacokinetic scientist who frequently can fit plasma or serum concentration data, obtained following oral administration, with Eq.

greatly affected, but the binding of salicylate to Fraction V is greatly diminished. In Fraction V BSA, all of the salicylate binding sites may not be available or readily accessible to the drug. Upon addition of 10 mg.% SETD, however, there may be a subtle conformational change induced by the binding of SETD such that the Fraction V resembles the crystalline BSA in affinity for salicylate. Further studies in progress are directed toward exploration of the nature of the conformational change and its influence on the number of salicylate binding sites and the respective affinity constants.

Binding data were obtained by equilibrium dialysis for 12 hr. at 37°, in pH 7.4, 0.054 *M* phosphate buffer made isotonic with sodium chloride. Both inside and outside solutions were assayed for drug content, utilizing the Bratton-Marshall procedure for SETD (8), and both UV spectrophotometric analysis and ^{14}C were used for determination of salicylate.

(1) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968).

(2) J. A. Sturman and M. J. H. Smith, *J. Pharm. Pharmacol.*, **19**, 621(1967).

(3) J. L. Kucera and F. J. Bullock, *ibid.*, **21**, 293(1969).

(4) D. T. Witiak and M. W. Whitehouse, *Biochem. Pharmacol.*, **18**, 971(1969).

(5) D. T. Witiak, T. D. Sokoloski, M. W. Whitehouse, and F. Hermann, *J. Med. Chem.*, **12**, 754(1969).

(6) A. H. Anton, *J. Pharmacol. Exp. Ther.*, **134**, 291(1961).

(7) H. B. Kostenbauder and M. J. Jawad, unpublished data.

(8) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

H. B. Kostenbauder
S. M. Bahal
M. J. Jawad

College of Pharmacy
University of Kentucky
Lexington, KY 40506

Received December 22, 1969.

Accepted for publication February 13, 1970.

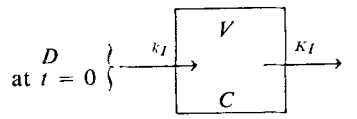
Supported in part by Research Grant GM-17060-01 from the National Institute of General Medical Sciences, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

"Absorption Rate Constants" Calculated According to the One-Compartment Open Model with First-Order Absorption: Implications in *In Vivo–In Vitro* Correlations

Keyphrases □ "Absorption rate constants"—one-compartment open model □ *In vivo–in vitro* correlations, "absorption rate constants"—calculation method effect

Sir:

Plasma or serum concentrations of unchanged drug observed following oral administration of single doses of a drug frequently are readily fit by the one-compartment open model with first-order absorption (Model I). Wagner and Metzler (1) cited much of the literature on this fitting; such fitting leads to Eq. 1:



Model I

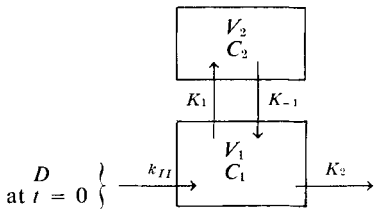
$$C = C^{\circ}\left(\frac{k_I}{k_I - K_I}\right)(e^{-K_I t} - e^{-k_I t}) \qquad \text{(Eq. 1)}$$

Even when simulated data are generated by application of Eqs. 2 and 2*a*,

$$C_1 = \frac{k_{II}D}{V_1}\left[\left\{\frac{K_{-1} - \alpha}{(k_{II} - \alpha)(\beta - \alpha)}\right\}e^{-\alpha t} + \left\{\frac{K_{-1} - \beta}{(k_{II} - \beta)(\alpha - \beta)}\right\}e^{-\beta t} + \left\{\frac{K_{-1} - k_{II}}{(\alpha - k_{II})(\beta - k_{II})}\right\}e^{-k_{II}t}\right] \qquad \text{(Eq. 2)}$$

$$\alpha,\beta = \tfrac{1}{2}\left[(K_1 + K_{-1} + K_2) \pm \sqrt{(K_1 + K_{-1} + K_2)^2 - 4K_{-1}K_2}\right] \qquad \text{(Eq. 2a)}$$

which are appropriate to the two-compartment open model with first-order absorption (Model II), most sets of C_1,t data can be fitted with two exponential terms (Eq. 1) rather than three exponential terms (Eq. 2). With real plasma or serum concentration data observed following oral administration, the same situation exists; however, in some cases, intravenous administration of the same drug tends to dictate the two-compartment open model.



Model II

When correlating "absorption rate constants" derived from plasma concentrations measured in man with *in vitro* rates of drug dissolution from dosage forms, one is most interested in relative values or ratios and not with absolute individual values.

The data in Table I are taken from Wagner and Metzler (1). When apparent "rate constants for absorptions," k_I, were estimated by nonlinear least-squares estimation, by applying Eq. 1 to data generated with Eqs. 2 and 2*a*, the absolute values of k_I deviated from the k_{II} values (either 0.5 or 2.0 hr.$^{-1}$), but the ratios of the k_I values were very close to the ratio of the true k_{II} values (namely, 4.0) when $8 \geq V_1/V_2 = K_{-1}/K_1 \geq 1$. It was previously shown (1) that where Model II was elaborated from actual plasma or serum level data, the ratio of parameters was within the limits shown in Table I.

This observation may help the dilemma of the biopharmaceutical and pharmacokinetic scientist who frequently can fit plasma or serum concentration data, obtained following oral administration, with Eq.

Table I—Parameter Ratios Used to Calculate C_1 Values of Model II and k_I Values and their Ratios Obtained by Fitting the C_1,t Values to Model I[a]

Set No.	$V_1/V_2 = K_{-1}/K_1$	K_1/K_2	k_I when $k_{II} = 0.5$	k_I when $k_{II} = 2.0$	Ratio of k_I's
1	8	0.1	0.529	2.07	3.91
2	8	1.0	0.569	2.31	4.06
3	8	2.0	0.543	2.24	4.13
4	8	10.	0.510	2.06	4.04
5	8	100.	0.501	2.005	4.00
6	2	0.1	0.513	2.03	3.96
7	2	1.0	0.763	2.78	3.64
8	2	2.0	0.806	3.19	3.96
9	2	10.	0.609	2.80	4.60
10	2	100.	0.510	2.06	4.04
11	1	0.1	0.507	2.02	3.98
12	1	1.0	0.790	2.71	3.43
13	1	2.0	1.005	3.56	3.54
14	1	10.	0.840	4.43	5.27
15	1	100.	0.530	2.21	4.17
					Av. 4.05

For Model II, $K_2 = 0.15$, $D = 1{,}000{,}000$, and $V_1 = 5000$ for each set. Note that the ratio of k_{II} values is 4.0. A typographical error in Table II of *Reference 1* gave $K_2 = 0.015$ instead of the actual $K_2 = 0.15$.

1 but not with Eq. 2, even though other data, obtained following intravenous administration, provide strong evidence for Model II or a more complicated model. If he wishes to correlate the apparent "absorption rate constants" derived from application of Model I and Eq. 1, he may be reasonably safe if he utilizes the ratios of the "absorption rate constants" and correlates these with ratios of rates of dissolution or times to dissolve a given percentage of drug derived from an *in vitro* test. I have been quite successful in doing this and will report the details in a future publication.

(1) J. G. Wagner and C. M. Metzler, *J. Pharm. Sci.*, **58**, 87(1969).

JOHN G. WAGNER
College of Pharmacy and Pharmacy Service
University Hospital
University of Michigan
Ann Arbor, MI 48104

Received October 30, 1969.
Accepted for publication March 23, 1970.

BOOKS

REVIEWS

Human Ecology and Public Health. 4th Edition. Edited by EDWIN D. KILBOURNE and WILSON G. SMILLIE. Macmillan, New York, N. Y., 1969. xii + 462 pp. 18 × 26 cm. Price $11.95.

Fairly priced, handsomely printed, sympathetically edited, well illustrated (49 tables; 75 figures), and eminently readable, *Human Ecology and Public Health* is a useful reference for pharmaceutical scientists.

This fourth edition of a work previously entitled *Preventive Medicine and Public Health* relates public health principles to the broader concepts of human ecology. The book is divided into three main divisions—Human Ecology and Human Disease, Public Health Problems and Practice, and the Administration of Health Services; the divisions are subdivided into fifteen chapters. The contributing authors' credentials are excellent; their efforts match their credentials. The references at the conclusion of the chapters are quite comprehensive.

The senior editor describes ecology as an "in" word, and admits to using it with some trepidation. Some examples out of context bear out this concern—on page 85, we learn that "nuclear energy has been harnessed to provide electrical power without polluting the air or depleting natural resources," while on page 90 we are told that the introduction of nuclear reaction power plants has expanded the need for water cooling, "further aggravating the problem" of thermal pollution that alters the life support process of our lakes and streams. We learn of the problems caused by the introduction of synthetic hydrocarbon detergents and are told that the problem was remedied by newly developed biodegradables. No discussion follows about the potential of overloading of surface waters with phosphates, thus upsetting another ecological balance.

Despite these brief lapses from a balanced presentation of bioecology, the book has great merit. Major problem areas—the population explosion, pollution, automobile accidents, and inner-city tensions—are treated with great objectivity. One almost wishes for a touch of the urgent tones of a Commoner (see his *Science and Survival*, for example) when the specific problem of pesticides is considered in Chapter 4, although the purpose of this work does not call on urgency as a teaching device.

Of special note for pharmaceutical scientists are Chapters 2, 3, and 10 which deal, respectively, with Genetic Determinants of Health and Disease; Genetic Interactions of Man and Microbes; and Approaches to the Control of Human Infection. The first two chapters in the division—The Administration of Health Services—are an excellent introduction for the first-time reader and are a

Table I—Parameter Ratios Used to Calculate C_1 Values of Model II and k_I Values and their Ratios Obtained by Fitting the C_1,t Values to Model I[a]

Set No.	$V_1/V_2 = K_{-1}/K_1$	K_1/K_2	k_I when $k_{II} = 0.5$	k_I when $k_{II} = 2.0$	Ratio of k_I's
1	8	0.1	0.529	2.07	3.91
2	8	1.0	0.569	2.31	4.06
3	8	2.0	0.543	2.24	4.13
4	8	10.	0.510	2.06	4.04
5	8	100.	0.501	2.005	4.00
6	2	0.1	0.513	2.03	3.96
7	2	1.0	0.763	2.78	3.64
8	2	2.0	0.806	3.19	3.96
9	2	10.	0.609	2.80	4.60
10	2	100.	0.510	2.06	4.04
11	1	0.1	0.507	2.02	3.98
12	1	1.0	0.790	2.71	3.43
13	1	2.0	1.005	3.56	3.54
14	1	10.	0.840	4.43	5.27
15	1	100.	0.530	2.21	4.17
					Av. 4.05

For Model II, $K_2 = 0.15$, $D = 1{,}000{,}000$, and $V_1 = 5000$ for each set. Note that the ratio of k_{II} values is 4.0. A typographical error in Table II of *Reference 1* gave $K_2 = 0.015$ instead of the actual $K_2 = 0.15$.

1 but not with Eq. 2, even though other data, obtained following intravenous administration, provide strong evidence for Model II or a more complicated model. If he wishes to correlate the apparent "absorption rate constants" derived from application of Model I and Eq. 1, he may be reasonably safe if he utilizes the ratios of the "absorption rate constants" and correlates these with ratios of rates of dissolution or times to dissolve a given percentage of drug derived from an *in vitro* test. I have been quite successful in doing this and will report the details in a future publication.

(1) J. G. Wagner and C. M. Metzler, *J. Pharm. Sci.*, **58**, 87(1969).

JOHN G. WAGNER
College of Pharmacy and Pharmacy Service
University Hospital
University of Michigan
Ann Arbor, MI 48104

Received October 30, 1969.
Accepted for publication March 23, 1970.

BOOKS

REVIEWS

Human Ecology and Public Health. 4th Edition. Edited by EDWIN D. KILBOURNE and WILSON G. SMILLIE. Macmillan, New York, N. Y., 1969. xii + 462 pp. 18 × 26 cm. Price $11.95.

Fairly priced, handsomely printed, sympathetically edited, well illustrated (49 tables; 75 figures), and eminently readable, *Human Ecology and Public Health* is a useful reference for pharmaceutical scientists.

This fourth edition of a work previously entitled *Preventive Medicine and Public Health* relates public health principles to the broader concepts of human ecology. The book is divided into three main divisions—Human Ecology and Human Disease, Public Health Problems and Practice, and the Administration of Health Services; the divisions are subdivided into fifteen chapters. The contributing authors' credentials are excellent; their efforts match their credentials. The references at the conclusion of the chapters are quite comprehensive.

The senior editor describes ecology as an "in" word, and admits to using it with some trepidation. Some examples out of context bear out this concern—on page 85, we learn that "nuclear energy has been harnessed to provide electrical power without polluting the air or depleting natural resources," while on page 90 we are told that the introduction of nuclear reaction power plants has expanded the need for water cooling, "further aggravating the problem" of thermal pollution that alters the life support process of our lakes and streams. We learn of the problems caused by the introduction of synthetic hydrocarbon detergents and are told that the problem was remedied by newly developed biodegradables. No discussion follows about the potential of overloading of surface waters with phosphates, thus upsetting another ecological balance.

Despite these brief lapses from a balanced presentation of bioecology, the book has great merit. Major problem areas—the population explosion, pollution, automobile accidents, and inner-city tensions—are treated with great objectivity. One almost wishes for a touch of the urgent tones of a Commoner (see his *Science and Survival*, for example) when the specific problem of pesticides is considered in Chapter 4, although the purpose of this work does not call on urgency as a teaching device.

Of special note for pharmaceutical scientists are Chapters 2, 3, and 10 which deal, respectively, with Genetic Determinants of Health and Disease; Genetic Interactions of Man and Microbes; and Approaches to the Control of Human Infection. The first two chapters in the division—The Administration of Health Services—are an excellent introduction for the first-time reader and are a

fine reference source for the more experienced student of public health management.

Considering the individual and society generally, the state of our ecologic environment, and our artificial environment and the interrelationships of each and all of these to disease, the book is eminently successful and well worth study by the pharmaceutical scientist concerned with human ecology and public health.

Reviewed by William L. Blockstein
Health Sciences Unit
University Extension
University of Wisconsin
Madison, WI 53706 ■

New Dimensions in Legal and Ethical Concepts for Human Research Vol. 169, art. 2. Consulting Editor, IRVING LADIMER, New York Academy of Sciences, 2 East 63rd St., New York, NY 10021, 1970. pp. 297–593. 15 × 23 cm. Price $23.00.

The papers in this volume are drawn from a conference of the same name held by the New York Academy of Sciences from May 19 to 21, 1969.

The conference was divided into six sections each of which included the presentation of papers and panel discussions. The sections included were Ethical and Legal Base Lines for Professions and Community; Special Problems of Medical Disciplines; Special Problems of Related Professions; Experience in Design, Conduct, and Evaluation of Research; Professional Controls—Internal and External; and Social Responsibility through Communication.

This conference on "New Dimensions in Legal and Ethical Concepts for Human Research" was convened to enable representatives of major disciplines, mainly medical and legal fields, to present their experience and recommendations for meeting current and anticipated problems of experimentation on and with human beings.

These topics have relevancy to the recent activity in the areas of organ and tissue transplants. Work in these areas suggests that technology can surmount virtually all impediments, but this capacity may have to be curbed by social, ethical, legal, and religious strictures in order to achieve professional and community support.

Of particular interest to the pharmaceutical scientist are the papers on "Control and Surveillance of Investigational Drugs" by Herbert S. Carlin and Ronald T. Turnbull, "Conducting Investigational Drug Studies for Industry" by Kenneth G. Kohlstaedt, and "Drug Evaluation Problems in Academic and Other Contexts" by Louis Lasagna.

Staff Review ■

Parenteral Dosage Forms. By CAROLYN G. HALL and KENNETH E. AVIS. Parenteral Drug Association, Inc., Philadelphia, PA 19107, 1969. vi + 262 pp. 22 × 28 cm. Price $7.50.

This comprehensive annotated bibliography of the literature pertaining to parenteral dosage forms has been prepared by Mrs. Carolyn G. Hall and Dr. Kenneth E. Avis, Department of Pharmaceutics, University of Tennessee, College of Pharmacy.

It covers the period 1959 to 1963 and contains approximately 950 entries. The book is arranged topically with a complete author index. The period just prior to the inception of *International Pharmaceutical Abstracts* was chosen for the first of what is anticipated will be a series of bibliographies because the authors' felt that no coverage of this important period was available.

Staff Review ■

Clinical Pharmacy Handbook. By HUGH F. KABAT. Lea and Febiger, Washington Square, Philadelphia, PA 19106, 1969. v + 108 pp. + 70 workbook style tear-out pp. 21.5 × 27.5 cm. Price $6.50 paperbound.

Portions of this volume were originally presented as course material to the senior students at the University of Minnesota College of Pharmacy. The first three chapters deal with course objectives, a course introduction, and notes on the pharmacist–patient relationship as viewed by the author. Some later chapters involve a collection of common hospital abbreviations and meanings, drug interaction tables, a list of sources of drug information, and a workbook section that affords the pharmacy student the opportunity to investigate the physical, chemical, and pharmacologic properties of any drug by means of charts that must be completed and questions that must be answered and referenced. Approximately one-half of the book is devoted to forms concerning general patient information, the clinical status of the patient, patient progress, and laboratory results. These forms are to be filled in by the pharmacy student as soon as the appropriate information becomes available.

The author indicates in the preface that this text is intended for use by students and for "any pharmacist venturing into the clinical setting." Those others not initiated into a clinically oriented pharmacy practice may find this book to be of some value because some important aspects of clinical pharmacy are presented. For example, the drug interaction tables and the chart on drug-induced modifications of laboratory tests are valuable pieces of literature and the collection of common hospital abbreviations and meanings is a step in dispelling the "language barrier" that, at first, exists between the medical staff and the new pharmacist practitioner.

Those educators thinking of initiating a clinical pharmacy course may also find this book of value because it does offer some basic "patient following" forms that were adapted from forms now in use at other hospitals with a clinical pharmacy service. The book also presents some basic philosophy on the pharmacist–patient relationship and some basic operating rules for the student and clinical instructor while in a patient-care area.

Any pharmacist or pharmacy student who has had any exposure to a clinically oriented pharmacy practice will find this book extremely fundamental and perhaps too course-oriented to be of any great value. The material in the book, for the most part, has already been published in one journal or another. The author has simply compiled such pieces of literature as Dr. Edward Hartshorn's drug interaction tables as they appeared in *Drug Intelligence* and the tables on the drug-induced modifications of laboratory values as they were published in the *American Journal of Hospital Pharmacy*. Any pharmacist entertaining thoughts of a clinical practice should already have well in hand the material that is presented in this book.

Reviewed by Richard de Leon
School of Pharmacy
University of California
San Francisco, CA 94122 ■

Biologically Active Amines Found in Man. By FRANZ FRANZEN and KURT EYSELL. Pergamon Press, Maxwell House, Fairview Parks, Elmsford, NY 10523, 1969. vii + 244 pp. 23 × 16 cm. Price $13.50.

As stated by the authors, there has not been an extensive survey of the field of "biogenic amines" since 1951, although during this time there has been a considerable expansion of our knowledge of these compounds.

In 128 pages of text, the authors, who are apparently clinicians, discuss numerous aspects of these amines derived from decarboxylation of alpha amino acids. In separate chapters the biochemistry, pharmacology, and pathophysiological significance of biologically active amines are discussed. This *Handbuch* is unique as a comprehensive survey of this subject as related to clinical medicine. The

fine reference source for the more experienced student of public health management.

Considering the individual and society generally, the state of our ecologic environment, and our artificial environment and the interrelationships of each and all of these to disease, the book is eminently successful and well worth study by the pharmaceutical scientist concerned with human ecology and public health.

Reviewed by William L. Blockstein
Health Sciences Unit
University Extension
University of Wisconsin
Madison, WI 53706 ■

New Dimensions in Legal and Ethical Concepts for Human Research Vol. 169, art. 2. Consulting Editor, IRVING LADIMER, New York Academy of Sciences, 2 East 63rd St., New York, NY 10021, 1970. pp. 297–593. 15 × 23 cm. Price $23.00.

The papers in this volume are drawn from a conference of the same name held by the New York Academy of Sciences from May 19 to 21, 1969.

The conference was divided into six sections each of which included the presentation of papers and panel discussions. The sections included were Ethical and Legal Base Lines for Professions and Community; Special Problems of Medical Disciplines; Special Problems of Related Professions; Experience in Design, Conduct, and Evaluation of Research; Professional Controls—Internal and External; and Social Responsibility through Communication.

This conference on "New Dimensions in Legal and Ethical Concepts for Human Research" was convened to enable representatives of major disciplines, mainly medical and legal fields, to present their experience and recommendations for meeting current and anticipated problems of experimentation on and with human beings.

These topics have relevancy to the recent activity in the areas of organ and tissue transplants. Work in these areas suggests that technology can surmount virtually all impediments, but this capacity may have to be curbed by social, ethical, legal, and religious strictures in order to achieve professional and community support.

Of particular interest to the pharmaceutical scientist are the papers on "Control and Surveillance of Investigational Drugs" by Herbert S. Carlin and Ronald T. Turnbull, "Conducting Investigational Drug Studies for Industry" by Kenneth G. Kohlstaedt, and "Drug Evaluation Problems in Academic and Other Contexts" by Louis Lasagna.

Staff Review ■

Parenteral Dosage Forms. By CAROLYN G. HALL and KENNETH E. AVIS. Parenteral Drug Association, Inc., Philadelphia, PA 19107, 1969. vi + 262 pp. 22 × 28 cm. Price $7.50.

This comprehensive annotated bibliography of the literature pertaining to parenteral dosage forms has been prepared by Mrs. Carolyn G. Hall and Dr. Kenneth E. Avis, Department of Pharmaceutics, University of Tennessee, College of Pharmacy.

It covers the period 1959 to 1963 and contains approximately 950 entries. The book is arranged topically with a complete author index. The period just prior to the inception of *International Pharmaceutical Abstracts* was chosen for the first of what is anticipated will be a series of bibliographies because the authors' felt that no coverage of this important period was available.

Staff Review ■

Clinical Pharmacy Handbook. By HUGH F. KABAT. Lea and Febiger, Washington Square, Philadelphia, PA 19106, 1969. v + 108 pp. + 70 workbook style tear-out pp. 21.5 × 27.5 cm. Price $6.50 paperbound.

Portions of this volume were originally presented as course material to the senior students at the University of Minnesota College of Pharmacy. The first three chapters deal with course objectives, a course introduction, and notes on the pharmacist–patient relationship as viewed by the author. Some later chapters involve a collection of common hospital abbreviations and meanings, drug interaction tables, a list of sources of drug information, and a workbook section that affords the pharmacy student the opportunity to investigate the physical, chemical, and pharmacologic properties of any drug by means of charts that must be completed and questions that must be answered and referenced. Approximately one-half of the book is devoted to forms concerning general patient information, the clinical status of the patient, patient progress, and laboratory results. These forms are to be filled in by the pharmacy student as soon as the appropriate information becomes available.

The author indicates in the preface that this text is intended for use by students and for "any pharmacist venturing into the clinical setting." Those others not initiated into a clinically oriented pharmacy practice may find this book to be of some value because some important aspects of clinical pharmacy are presented. For example, the drug interaction tables and the chart on drug-induced modifications of laboratory tests are valuable pieces of literature and the collection of common hospital abbreviations and meanings is a step in dispelling the "language barrier" that, at first, exists between the medical staff and the new pharmacist practitioner.

Those educators thinking of initiating a clinical pharmacy course may also find this book of value because it does offer some basic "patient following" forms that were adapted from forms now in use at other hospitals with a clinical pharmacy service. The book also presents some basic philosophy on the pharmacist–patient relationship and some basic operating rules for the student and clinical instructor while in a patient-care area.

Any pharmacist or pharmacy student who has had any exposure to a clinically oriented pharmacy practice will find this book extremely fundamental and perhaps too course-oriented to be of any great value. The material in the book, for the most part, has already been published in one journal or another. The author has simply compiled such pieces of literature as Dr. Edward Hartshorn's drug interaction tables as they appeared in *Drug Intelligence* and the tables on the drug-induced modifications of laboratory values as they were published in the *American Journal of Hospital Pharmacy*. Any pharmacist entertaining thoughts of a clinical practice should already have well in hand the material that is presented in this book.

Reviewed by Richard de Leon
School of Pharmacy
University of California
San Francisco, CA 94122 ■

Biologically Active Amines Found in Man. By FRANZ FRANZEN and KURT EYSELL. Pergamon Press, Maxwell House, Fairview Parks, Elmsford, NY 10523, 1969. vii + 244 pp. 23 × 16 cm. Price $13.50.

As stated by the authors, there has not been an extensive survey of the field of "biogenic amines" since 1951, although during this time there has been a considerable expansion of our knowledge of these compounds.

In 128 pages of text, the authors, who are apparently clinicians, discuss numerous aspects of these amines derived from decarboxylation of alpha amino acids. In separate chapters the biochemistry, pharmacology, and pathophysiological significance of biologically active amines are discussed. This *Handbuch* is unique as a comprehensive survey of this subject as related to clinical medicine. The

fine reference source for the more experienced student of public health management.

Considering the individual and society generally, the state of our ecologic environment, and our artificial environment and the interrelationships of each and all of these to disease, the book is eminently successful and well worth study by the pharmaceutical scientist concerned with human ecology and public health.

Reviewed by William L. Blockstein
Health Sciences Unit
University Extension
University of Wisconsin
Madison, WI 53706 ■

New Dimensions in Legal and Ethical Concepts for Human Research Vol. 169, art. 2. Consulting Editor, IRVING LADIMER, New York Academy of Sciences, 2 East 63rd St., New York, NY 10021, 1970. pp. 297–593. 15 × 23 cm. Price $23.00.

The papers in this volume are drawn from a conference of the same name held by the New York Academy of Sciences from May 19 to 21, 1969.

The conference was divided into six sections each of which included the presentation of papers and panel discussions. The sections included were Ethical and Legal Base Lines for Professions and Community; Special Problems of Medical Disciplines; Special Problems of Related Professions; Experience in Design, Conduct, and Evaluation of Research; Professional Controls—Internal and External; and Social Responsibility through Communication.

This conference on "New Dimensions in Legal and Ethical Concepts for Human Research" was convened to enable representatives of major disciplines, mainly medical and legal fields, to present their experience and recommendations for meeting current and anticipated problems of experimentation on and with human beings.

These topics have relevancy to the recent activity in the areas of organ and tissue transplants. Work in these areas suggests that technology can surmount virtually all impediments, but this capacity may have to be curbed by social, ethical, legal, and religious strictures in order to achieve professional and community support.

Of particular interest to the pharmaceutical scientist are the papers on "Control and Surveillance of Investigational Drugs" by Herbert S. Carlin and Ronald T. Turnbull, "Conducting Investigational Drug Studies for Industry" by Kenneth G. Kohlstaedt, and "Drug Evaluation Problems in Academic and Other Contexts" by Louis Lasagna.

Staff Review ■

Parenteral Dosage Forms. By CAROLYN G. HALL and KENNETH E. AVIS. Parenteral Drug Association, Inc., Philadelphia, PA 19107, 1969. vi + 262 pp. 22 × 28 cm. Price $7.50.

This comprehensive annotated bibliography of the literature pertaining to parenteral dosage forms has been prepared by Mrs. Carolyn G. Hall and Dr. Kenneth E. Avis, Department of Pharmaceutics, University of Tennessee, College of Pharmacy.

It covers the period 1959 to 1963 and contains approximately 950 entries. The book is arranged topically with a complete author index. The period just prior to the inception of *International Pharmaceutical Abstracts* was chosen for the first of what is anticipated will be a series of bibliographies because the authors' felt that no coverage of this important period was available.

Staff Review ■

Clinical Pharmacy Handbook. By HUGH F. KABAT. Lea and Febiger, Washington Square, Philadelphia, PA 19106, 1969. v + 108 pp. + 70 workbook style tear-out pp. 21.5 × 27.5 cm. Price $6.50 paperbound.

Portions of this volume were originally presented as course material to the senior students at the University of Minnesota College of Pharmacy. The first three chapters deal with course objectives, a course introduction, and notes on the pharmacist–patient relationship as viewed by the author. Some later chapters involve a collection of common hospital abbreviations and meanings, drug interaction tables, a list of sources of drug information, and a workbook section that affords the pharmacy student the opportunity to investigate the physical, chemical, and pharmacologic properties of any drug by means of charts that must be completed and questions that must be answered and referenced. Approximately one-half of the book is devoted to forms concerning general patient information, the clinical status of the patient, patient progress, and laboratory results. These forms are to be filled in by the pharmacy student as soon as the appropriate information becomes available.

The author indicates in the preface that this text is intended for use by students and for "any pharmacist venturing into the clinical setting." Those others not initiated into a clinically oriented pharmacy practice may find this book to be of some value because some important aspects of clinical pharmacy are presented. For example, the drug interaction tables and the chart on drug-induced modifications of laboratory tests are valuable pieces of literature and the collection of common hospital abbreviations and meanings is a step in dispelling the "language barrier" that, at first, exists between the medical staff and the new pharmacist practitioner.

Those educators thinking of initiating a clinical pharmacy course may also find this book of value because it does offer some basic "patient following" forms that were adapted from forms now in use at other hospitals with a clinical pharmacy service. The book also presents some basic philosophy on the pharmacist–patient relationship and some basic operating rules for the student and clinical instructor while in a patient-care area.

Any pharmacist or pharmacy student who has had any exposure to a clinically oriented pharmacy practice will find this book extremely fundamental and perhaps too course-oriented to be of any great value. The material in the book, for the most part, has already been published in one journal or another. The author has simply compiled such pieces of literature as Dr. Edward Hartshorn's drug interaction tables as they appeared in *Drug Intelligence* and the tables on the drug-induced modifications of laboratory values as they were published in the *American Journal of Hospital Pharmacy*. Any pharmacist entertaining thoughts of a clinical practice should already have well in hand the material that is presented in this book.

Reviewed by Richard de Leon
School of Pharmacy
University of California
San Francisco, CA 94122 ■

Biologically Active Amines Found in Man. By FRANZ FRANZEN and KURT EYSELL. Pergamon Press, Maxwell House, Fairview Parks, Elmsford, NY 10523, 1969. vii + 244 pp. 23 × 16 cm. Price $13.50.

As stated by the authors, there has not been an extensive survey of the field of "biogenic amines" since 1951, although during this time there has been a considerable expansion of our knowledge of these compounds.

In 128 pages of text, the authors, who are apparently clinicians, discuss numerous aspects of these amines derived from decarboxylation of alpha amino acids. In separate chapters the biochemistry, pharmacology, and pathophysiological significance of biologically active amines are discussed. This *Handbuch* is unique as a comprehensive survey of this subject as related to clinical medicine. The

fine reference source for the more experienced student of public health management.

Considering the individual and society generally, the state of our ecologic environment, and our artificial environment and the interrelationships of each and all of these to disease, the book is eminently successful and well worth study by the pharmaceutical scientist concerned with human ecology and public health.

Reviewed by William L. Blockstein
Health Sciences Unit
University Extension
University of Wisconsin
Madison, WI 53706 ■

New Dimensions in Legal and Ethical Concepts for Human Research Vol. 169, art. 2. Consulting Editor, IRVING LADIMER, New York Academy of Sciences, 2 East 63rd St., New York, NY 10021, 1970. pp. 297–593. 15 × 23 cm. Price $23.00.

The papers in this volume are drawn from a conference of the same name held by the New York Academy of Sciences from May 19 to 21, 1969.

The conference was divided into six sections each of which included the presentation of papers and panel discussions. The sections included were Ethical and Legal Base Lines for Professions and Community; Special Problems of Medical Disciplines; Special Problems of Related Professions; Experience in Design, Conduct, and Evaluation of Research; Professional Controls—Internal and External; and Social Responsibility through Communication.

This conference on "New Dimensions in Legal and Ethical Concepts for Human Research" was convened to enable representatives of major disciplines, mainly medical and legal fields, to present their experience and recommendations for meeting current and anticipated problems of experimentation on and with human beings.

These topics have relevancy to the recent activity in the areas of organ and tissue transplants. Work in these areas suggests that technology can surmount virtually all impediments, but this capacity may have to be curbed by social, ethical, legal, and religious strictures in order to achieve professional and community support.

Of particular interest to the pharmaceutical scientist are the papers on "Control and Surveillance of Investigational Drugs" by Herbert S. Carlin and Ronald T. Turnbull, "Conducting Investigational Drug Studies for Industry" by Kenneth G. Kohlstaedt, and "Drug Evaluation Problems in Academic and Other Contexts" by Louis Lasagna.

Staff Review ■

Parenteral Dosage Forms. By CAROLYN G. HALL and KENNETH E. AVIS. Parenteral Drug Association, Inc., Philadelphia, PA 19107, 1969. vi + 262 pp. 22 × 28 cm. Price $7.50.

This comprehensive annotated bibliography of the literature pertaining to parenteral dosage forms has been prepared by Mrs. Carolyn G. Hall and Dr. Kenneth E. Avis, Department of Pharmaceutics, University of Tennessee, College of Pharmacy.

It covers the period 1959 to 1963 and contains approximately 950 entries. The book is arranged topically with a complete author index. The period just prior to the inception of *International Pharmaceutical Abstracts* was chosen for the first of what is anticipated will be a series of bibliographies because the authors' felt that no coverage of this important period was available.

Staff Review ■

Clinical Pharmacy Handbook. By HUGH F. KABAT. Lea and Febiger, Washington Square, Philadelphia, PA 19106, 1969. v + 108 pp. + 70 workbook style tear-out pp. 21.5 × 27.5 cm. Price $6.50 paperbound.

Portions of this volume were originally presented as course material to the senior students at the University of Minnesota College of Pharmacy. The first three chapters deal with course objectives, a course introduction, and notes on the pharmacist–patient relationship as viewed by the author. Some later chapters involve a collection of common hospital abbreviations and meanings, drug interaction tables, a list of sources of drug information, and a workbook section that affords the pharmacy student the opportunity to investigate the physical, chemical, and pharmacologic properties of any drug by means of charts that must be completed and questions that must be answered and referenced. Approximately one-half of the book is devoted to forms concerning general patient information, the clinical status of the patient, patient progress, and laboratory results. These forms are to be filled in by the pharmacy student as soon as the appropriate information becomes available.

The author indicates in the preface that this text is intended for use by students and for "any pharmacist venturing into the clinical setting." Those others not initiated into a clinically oriented pharmacy practice may find this book to be of some value because some important aspects of clinical pharmacy are presented. For example, the drug interaction tables and the chart on drug-induced modifications of laboratory tests are valuable pieces of literature and the collection of common hospital abbreviations and meanings is a step in dispelling the "language barrier" that, at first, exists between the medical staff and the new pharmacist practitioner.

Those educators thinking of initiating a clinical pharmacy course may also find this book of value because it does offer some basic "patient following" forms that were adapted from forms now in use at other hospitals with a clinical pharmacy service. The book also presents some basic philosophy on the pharmacist–patient relationship and some basic operating rules for the student and clinical instructor while in a patient-care area.

Any pharmacist or pharmacy student who has had any exposure to a clinically oriented pharmacy practice will find this book extremely fundamental and perhaps too course-oriented to be of any great value. The material in the book, for the most part, has already been published in one journal or another. The author has simply compiled such pieces of literature as Dr. Edward Hartshorn's drug interaction tables as they appeared in *Drug Intelligence* and the tables on the drug-induced modifications of laboratory values as they were published in the *American Journal of Hospital Pharmacy*. Any pharmacist entertaining thoughts of a clinical practice should already have well in hand the material that is presented in this book.

Reviewed by Richard de Leon
School of Pharmacy
University of California
San Francisco, CA 94122 ■

Biologically Active Amines Found in Man. By FRANZ FRANZEN and KURT EYSELL. Pergamon Press, Maxwell House, Fairview Parks, Elmsford, NY 10523, 1969. vii + 244 pp. 23 × 16 cm. Price $13.50.

As stated by the authors, there has not been an extensive survey of the field of "biogenic amines" since 1951, although during this time there has been a considerable expansion of our knowledge of these compounds.

In 128 pages of text, the authors, who are apparently clinicians, discuss numerous aspects of these amines derived from decarboxylation of alpha amino acids. In separate chapters the biochemistry, pharmacology, and pathophysiological significance of biologically active amines are discussed. This *Handbuch* is unique as a comprehensive survey of this subject as related to clinical medicine. The

fine reference source for the more experienced student of public health management.

Considering the individual and society generally, the state of our ecologic environment, and our artificial environment and the interrelationships of each and all of these to disease, the book is eminently successful and well worth study by the pharmaceutical scientist concerned with human ecology and public health.

Reviewed by William L. Blockstein
Health Sciences Unit
University Extension
University of Wisconsin
Madison, WI 53706 ■

New Dimensions in Legal and Ethical Concepts for Human Research Vol. 169, art. 2. Consulting Editor, IRVING LADIMER, New York Academy of Sciences, 2 East 63rd St., New York, NY 10021, 1970. pp. 297–593. 15 × 23 cm. Price $23.00.

The papers in this volume are drawn from a conference of the same name held by the New York Academy of Sciences from May 19 to 21, 1969.

The conference was divided into six sections each of which included the presentation of papers and panel discussions. The sections included were Ethical and Legal Base Lines for Professions and Community; Special Problems of Medical Disciplines; Special Problems of Related Professions; Experience in Design, Conduct, and Evaluation of Research; Professional Controls—Internal and External; and Social Responsibility through Communication.

This conference on "New Dimensions in Legal and Ethical Concepts for Human Research" was convened to enable representatives of major disciplines, mainly medical and legal fields, to present their experience and recommendations for meeting current and anticipated problems of experimentation on and with human beings.

These topics have relevancy to the recent activity in the areas of organ and tissue transplants. Work in these areas suggests that technology can surmount virtually all impediments, but this capacity may have to be curbed by social, ethical, legal, and religious strictures in order to achieve professional and community support.

Of particular interest to the pharmaceutical scientist are the papers on "Control and Surveillance of Investigational Drugs" by Herbert S. Carlin and Ronald T. Turnbull, "Conducting Investigational Drug Studies for Industry" by Kenneth G. Kohlstaedt, and "Drug Evaluation Problems in Academic and Other Contexts" by Louis Lasagna.

Staff Review ■

Parenteral Dosage Forms. By CAROLYN G. HALL and KENNETH E. AVIS. Parenteral Drug Association, Inc., Philadelphia, PA 19107, 1969. vi + 262 pp. 22 × 28 cm. Price $7.50.

This comprehensive annotated bibliography of the literature pertaining to parenteral dosage forms has been prepared by Mrs. Carolyn G. Hall and Dr. Kenneth E. Avis, Department of Pharmaceutics, University of Tennessee, College of Pharmacy.

It covers the period 1959 to 1963 and contains approximately 950 entries. The book is arranged topically with a complete author index. The period just prior to the inception of *International Pharmaceutical Abstracts* was chosen for the first of what is anticipated will be a series of bibliographies because the authors' felt that no coverage of this important period was available.

Staff Review ■

Clinical Pharmacy Handbook. By HUGH F. KABAT. Lea and Febiger, Washington Square, Philadelphia, PA 19106, 1969. v + 108 pp. + 70 workbook style tear-out pp. 21.5 × 27.5 cm. Price $6.50 paperbound.

Portions of this volume were originally presented as course material to the senior students at the University of Minnesota College of Pharmacy. The first three chapters deal with course objectives, a course introduction, and notes on the pharmacist–patient relationship as viewed by the author. Some later chapters involve a collection of common hospital abbreviations and meanings, drug interaction tables, a list of sources of drug information, and a workbook section that affords the pharmacy student the opportunity to investigate the physical, chemical, and pharmacologic properties of any drug by means of charts that must be completed and questions that must be answered and referenced. Approximately one-half of the book is devoted to forms concerning general patient information, the clinical status of the patient, patient progress, and laboratory results. These forms are to be filled in by the pharmacy student as soon as the appropriate information becomes available.

The author indicates in the preface that this text is intended for use by students and for "any pharmacist venturing into the clinical setting." Those others not initiated into a clinically oriented pharmacy practice may find this book to be of some value because some important aspects of clinical pharmacy are presented. For example, the drug interaction tables and the chart on drug-induced modifications of laboratory tests are valuable pieces of literature and the collection of common hospital abbreviations and meanings is a step in dispelling the "language barrier" that, at first, exists between the medical staff and the new pharmacist practitioner.

Those educators thinking of initiating a clinical pharmacy course may also find this book of value because it does offer some basic "patient following" forms that were adapted from forms now in use at other hospitals with a clinical pharmacy service. The book also presents some basic philosophy on the pharmacist–patient relationship and some basic operating rules for the student and clinical instructor while in a patient-care area.

Any pharmacist or pharmacy student who has had any exposure to a clinically oriented pharmacy practice will find this book extremely fundamental and perhaps too course-oriented to be of any great value. The material in the book, for the most part, has already been published in one journal or another. The author has simply compiled such pieces of literature as Dr. Edward Hartshorn's drug interaction tables as they appeared in *Drug Intelligence* and the tables on the drug-induced modifications of laboratory values as they were published in the *American Journal of Hospital Pharmacy*. Any pharmacist entertaining thoughts of a clinical practice should already have well in hand the material that is presented in this book.

Reviewed by Richard de Leon
School of Pharmacy
University of California
San Francisco, CA 94122 ■

Biologically Active Amines Found in Man. By FRANZ FRANZEN and KURT EYSELL. Pergamon Press, Maxwell House, Fairview Parks, Elmsford, NY 10523, 1969. vii + 244 pp. 23 × 16 cm. Price $13.50.

As stated by the authors, there has not been an extensive survey of the field of "biogenic amines" since 1951, although during this time there has been a considerable expansion of our knowledge of these compounds.

In 128 pages of text, the authors, who are apparently clinicians, discuss numerous aspects of these amines derived from decarboxylation of alpha amino acids. In separate chapters the biochemistry, pharmacology, and pathophysiological significance of biologically active amines are discussed. This *Handbuch* is unique as a comprehensive survey of this subject as related to clinical medicine. The

sections on renal implications and tumor-secreted active amines are especially lucid. The authors cite 1890 references without being overly verbose. The book contains several useful tables of important data such as amine concentrations in blood, urine, and tissues in both normal and pathologic conditions; concentrations of dopamine, norepinephrine, serotonin, and other amines in 41 areas of postmortem brain; and the influence of a variety of amines on the formation and excretion of urine in various animals and man.

This book should prove a useful addition to the library of researchers and clinicians in the fields of autonomic pharmacology and endocrine pathophysiology involving altered secretion or levels of these amines. The discussion is not particularly instructive although it is comprehensive and thorough in reviewing the available literature.

Reviewed by David A. Blake
School of Pharmacy
University of Maryland
Baltimore, MD 21201 ■

The National Formulary, Thirteenth Edition. Prepared by the National Formulary Board with the approval of the Board of Trustees, by the authority of the American Pharmaceutical Association. The American Pharmaceutical Association, 1970. Distributed by Mack Publishing Co., Easton, PA 18042. lxxiv + 1012 pp. 14.5 × 23 cm. Price $15.00 (Domestic and Foreign).

The publication of the National Formulary, or any other comparable source for methods or standards, is often viewed as an event in itself, rather than the climax of a long series of procedures. In reality, any book of this nature is the end product of much research, fact finding, study, and evaluation. National Formulary, Thirteenth Edition, represents the results of a five-year revision program carried on by the American Pharmaceutical Association, involving more than 500 persons from the fields of medicine, pharmacy, and chemistry.

Certainly, one of the most important considerations—and the most apparent in the preparation of a new NF edition, is the selection of drugs and dosage forms to be recognized and the preparation of monographs for those chosen. Of the 992 officially recognized articles in NF XIII, 411 are newly admitted. Two hundred twenty-one drugs, recognized in NF XII, have been dropped in going to the new edition.

But a second aspect of the NF, which in certain respects has even more far-reaching consequences, is the application of new techniques and methods and the establishment of new criteria by which to ensure the quality and purity of pharmaceuticals. It is this aspect of the NF revision program which probably reflects to a greater extent the rapid changes and advances in technological capabilities and quality control methodology.

In pursuit of its fundamental purpose—to provide standards and specifications which can be used to evaluate the quality of pharmaceuticals—the NF has fostered the study of factors bearing on drug availability. As a result, NF XIII draws upon and utilizes the many recent developments in drug analysis and methodology in providing new tests, new specifications, and new standards.

Of the 80 General Tests chapters in NF XIII, 16 are new; those carried over from NF XII have been revised and updated to reflect more adequately current needs and capacities.

Among the new General Tests chapters, those on X-ray diffraction and dissolution test are important examples. The first provides a technique enabling the NF Board to specify in these drug substance monographs where polymorphism of the crystal may be a problem affecting bioavailability that the article must conform to a specified X-ray diffraction pattern, while dissolution test specifications provide an objective means of determining dissolution characteristics of a solid dosage form. The inclusion of these chapters and specifications in pertinent individual monographs represents an initial effort by the NF to establish specifications where appropriate both for the active ingredient and the final dosage form, to serve as an index of drug quality from the standpoint of expected biological availability.

The aerosol dosage form is recognized officially for the first time in NF XIII through inclusion of monographs for several therapeutically important aerosol preparations. Among the procedures used to establish standards for aerosol products are leak testing, delivery rate, and pressure testing. NF XIII provides standards and specifications for four aerosol propellants in addition to monographs for six individual aerosol dosage forms.

The rise of consumerism on all levels and the increasing consumer awareness and sophistication concerning drugs—in part due to the drug efficacy study, the hearings on oral contraceptives, the cyclamate controversy, concern over misuse of drugs, and rising medical costs in general—have resulted in increased pressure on the official compendia to keep abreast of trends and developments in the scientific areas. In response to this challenge, the NF revision program is to be put on an even more continuous basis with the first supplement having a scheduled official date of September 1, 1970, coinciding with NF XIII.

In accordance with the objectives of a more continuous revision program, additional NF XIII supplements are planned at regular intervals throughout the revision period.

Staff Review ■

sections on renal implications and tumor-secreted active amines are especially lucid. The authors cite 1890 references without being overly verbose. The book contains several useful tables of important data such as amine concentrations in blood, urine, and tissues in both normal and pathologic conditions; concentrations of dopamine, norepinephrine, serotonin, and other amines in 41 areas of postmortem brain; and the influence of a variety of amines on the formation and excretion of urine in various animals and man.

This book should prove a useful addition to the library of researchers and clinicians in the fields of autonomic pharmacology and endocrine pathophysiology involving altered secretion or levels of these amines. The discussion is not particularly instructive although it is comprehensive and thorough in reviewing the available literature.

Reviewed by David A. Blake
School of Pharmacy
University of Maryland
Baltimore, MD 21201 ■

The National Formulary, Thirteenth Edition. Prepared by the National Formulary Board with the approval of the Board of Trustees, by the authority of the American Pharmaceutical Association. The American Pharmaceutical Association, 1970. Distributed by Mack Publishing Co., Easton, PA 18042. lxxiv + 1012 pp. 14.5 × 23 cm. Price $15.00 (Domestic and Foreign).

The publication of the National Formulary, or any other comparable source for methods or standards, is often viewed as an event in itself, rather than the climax of a long series of procedures. In reality, any book of this nature is the end product of much research, fact finding, study, and evaluation. National Formulary, Thirteenth Edition, represents the results of a five-year revision program carried on by the American Pharmaceutical Association, involving more than 500 persons from the fields of medicine, pharmacy, and chemistry.

Certainly, one of the most important considerations—and the most apparent in the preparation of a new NF edition, is the selection of drugs and dosage forms to be recognized and the preparation of monographs for those chosen. Of the 992 officially recognized articles in NF XIII, 411 are newly admitted. Two hundred twenty-one drugs, recognized in NF XII, have been dropped in going to the new edition.

But a second aspect of the NF, which in certain respects has even more far-reaching consequences, is the application of new techniques and methods and the establishment of new criteria by which to ensure the quality and purity of pharmaceuticals. It is this aspect of the NF revision program which probably reflects to a greater extent the rapid changes and advances in technological capabilities and quality control methodology.

In pursuit of its fundamental purpose—to provide standards and specifications which can be used to evaluate the quality of pharmaceuticals—the NF has fostered the study of factors bearing on drug availability. As a result, NF XIII draws upon and utilizes the many recent developments in drug analysis and methodology in providing new tests, new specifications, and new standards.

Of the 80 General Tests chapters in NF XIII, 16 are new; those carried over from NF XII have been revised and updated to reflect more adequately current needs and capacities.

Among the new General Tests chapters, those on X-ray diffraction and dissolution test are important examples. The first provides a technique enabling the NF Board to specify in these drug substance monographs where polymorphism of the crystal may be a problem affecting bioavailability that the article must conform to a specified X-ray diffraction pattern, while dissolution test specifications provide an objective means of determining dissolution characteristics of a solid dosage form. The inclusion of these chapters and specifications in pertinent individual monographs represents an initial effort by the NF to establish specifications where appropriate both for the active ingredient and the final dosage form, to serve as an index of drug quality from the standpoint of expected biological availability.

The aerosol dosage form is recognized officially for the first time in NF XIII through inclusion of monographs for several therapeutically important aerosol preparations. Among the procedures used to establish standards for aerosol products are leak testing, delivery rate, and pressure testing. NF XIII provides standards and specifications for four aerosol propellants in addition to monographs for six individual aerosol dosage forms.

The rise of consumerism on all levels and the increasing consumer awareness and sophistication concerning drugs—in part due to the drug efficacy study, the hearings on oral contraceptives, the cyclamate controversy, concern over misuse of drugs, and rising medical costs in general—have resulted in increased pressure on the official compendia to keep abreast of trends and developments in the scientific areas. In response to this challenge, the NF revision program is to be put on an even more continuous basis with the first supplement having a scheduled official date of September 1, 1970, coinciding with NF XIII.

In accordance with the objectives of a more continuous revision program, additional NF XIII supplements are planned at regular intervals throughout the revision period.

Staff Review ■

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

AUGUST 1970
VOLUME 59 NUMBER 8

REVIEW ARTICLE

Determination of the Decomposition of Aspirin

CLARK A. KELLY

Keyphrases ☐ Aspirin decomposition—determination ☐ Decomposition products—aspirin ☐ Hydrolysis, aspirin—mechanism, kinetics, pH effect ☐ Analytical methods—salicylic acid in aspirin and aspirin products ☐ Stability—aspirin and dosage forms

CONTENTS

February 27, 1917, the day U. S. Patent No. 644,077 expired, a whole new vista opened to the drug industry. What was, and is, the best seller of all times could easily be classed as the wonder drug of all ages. From the latest available figures of the U. S. Tariff Commission,[1] there would be over 200 tablets available for every man, woman, and child in the United States if all the U. S. yearly production was made into tablets containing 325 mg. (5 gr.) of acetylsalicylic acid, more popularly known as aspirin.

Aspirin has the unique standing in the medical world of still being the most widely used drug, even with the advent of modern, highly potent therapeutic agents. Aspirin has superior qualities as an antipyretic and as a general analgesic, but more specifically in the relief of headaches, muscular pain, postoperative and traumatic pain, postpartum pain, dysmenorrhea, malignancy, colds and respiratory diseases, rheumatoid arthritis, acute rheumatic fever, and in the field of dental analgesia.

With such a versatile drug, it was obvious that aspirin would be combined with other drugs with the result that a more potent and effective preparation would result. It is the purpose of this review to emphasize the plight that continually plagues the analytical chemist in his constant search for a truly reliable method of measuring the decomposition of aspirin in the presence of other drugs or compounds. What makes this problem even more acute is that aspirin is highly selective of the type of compounds it intimately associates with. In fact, if aspirin acquires even a trace of moisture, it begins to fall to pieces. It thus becomes a problem of product development to finalize a stable formulation that will withstand a "normal" shelflife under all types of adverse conditions such as humidity, temperature, and interreactions with other components, even in the solid state.

DETERMINATION OF DECOMPOSITION OF ASPIRIN

When aspirin was first introduced as a drug, controversies ensued almost instantaneously as to how one could characterize truly good aspirin. Some became experts on detecting trace amounts of acetic acid and so classed the elegance of the aspirin accordingly. Even in those early days, the advertising agencies made the most

[1] From 1968 preliminary report: 31,248,000 pounds of aspirin by U. S. production.

of this purely subjective classification. Very few papers have been presented on the quantitative determination of acetic acid as a decomposition product of aspirin, chiefly because of the known volatility of acetic acid. Unless the original container was completely airtight, one would be measuring only the residual acetic acid, which would not be representative of the total acetic acid formed by the decomposition of the aspirin. General methods evolved in which dry air was passed over and through a thin layer of the finely powdered sample. The acetic acid vapor was trapped in the water and then titrated with very dilute sodium hydroxide. A simpler approach utilized a Conway micro diffusion cell. The more refined approach involved GLC.

It is interesting to note that visually the presence of any whiskers (very thin elongated crystals of salicylic acid) observed on the surface of a solid product containing aspirin is definitely an indication that some of the aspirin has decomposed and that the resulting salicylic acid has sublimed through the solid material. Here, again, if the container is not airtight, the possibility exists whereby the released salicylic acid, through sublimation, would leave the sample area and so not be measured. This crucial point, on the sublimation of salicylic acid, will be discussed more thoroughly in this review. As with most subjective tests, the evaluation of solid aspirin products by the appearance of salicylic acid whiskers is limited. Time is required for this sublimation to take place, so one would not normally apply it to fresh products. Therefore, one could actually have a poorly made aspirin tablet which, on the surface, showed no whiskers but internally had a high content of salicylic acid.

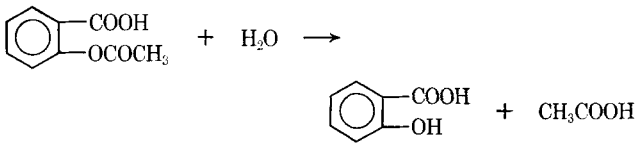
It is the intent of this review, therefore, to pursue a quantitative approach in the determination of the amount of decomposition of aspirin through the presence of salicylic acid rather than acetic acid.

No biological samples, such as blood, containing aspirin will be discussed in this review. Nor will this review include salts of aspirin, such as aluminum aspirin, solutions, or aspirin suspensions. The stability of aspirin in all these cases is definitely limited. The presence of any moisture (with the salts of aspirin, the water of hydration) results in the hydrolysis of aspirin at such a rate that the given formulation does not have a practical or useful shelflife. There is, however, a definite need in the field of pediatrics and geriatrics for a stable liquid formulation of aspirin, because there is no easier or simplier way to give a medicinal than by mouth to infants or to the feeble.

Even limiting this review just to solid preparations leaves a great deal confronting the analyst. A step-by-step evaluation of the approaches published in the scientific literature will be presented. One will readily see the tremendous need for a simple, reliable, universal test for the decomposition of aspirin which can be applied easily and rapidly to a completely unknown preparation containing aspirin.

HYDROLYSIS STUDIES

Decomposition of aspirin results from hydrolysis of the ester group, with the end products being acetic acid and salicylic acid. The oversimplified reaction for the hydrolysis of aspirin is presented only at this time, so one may visualize the overall picture of the decomposition of aspirin (Scheme I).



Scheme I

Those who have made a thorough study of the hydrolysis of aspirin under various well-controlled and stated conditions report that the reaction is very complex. Judged by the number of papers on this subject alone, the reaction is also highly controversial.

The first publication on the hydrolysis of aspirin in water was reported by Rath (1) who conducted his work at an extremely high temperature (100°). As was later found, the hydrolysis of aspirin is very sensitive to temperature changes, even near room temperature. The rate of hydrolysis was determined by titrating the total acidity at stated time intervals. The calculated values indicated a monomolecular reaction. Tsakalotos and Horsch (2, 3) also followed the hydrolysis of aspirin but at more reasonable temperatures (20, 50, and 60°). It took about 100 days to effect complete hydrolysis of aspirin in water at room temperature. Hydrogen ion was found to accelerate the hydrolysis rate, hydrochloric acid being more effective than sulfuric acid. Acetic acid and citric acid caused an initial increase in the rate of hydrolysis; but as the days passed, a decrease in the rate of hydrolysis was noted. The unfounded explanation given by these authors for this slowdown was that the salicylic acid produced was being acylated.

Wolf (4) substantiated the hydrogen-ion effect on the hydrolysis of aspirin by showing that the velocity constant in an acid medium (diluted hydrochloric acid) doubled over that of just water

Aspirin was solubilized in water at room temperature by Morton (5) with the aid of potassium and sodium citrates and acetates. The degree of hydrolysis was followed by titrating the samples with standard alkali at stated time intervals. The rate of hydrolysis was reported to be independent of not only the concentration of the aspirin but also of the solubilizing salt concentration.

Saponification (alkaline hydrolysis) of aspirin was reported by La Mer and Greenspan (6) at 25.000 ± 0.005°. The reaction was stopped by making the given sample (not an aliquot of the bulk solution as is the usual approach in hydrolysis studies) acidic with standard sulfuric acid. The excess acid was then titrated with 0.02 *M* sodium hydroxide. It is assumed that this back titration was conducted immediately; otherwise, hydrolysis of aspirin in the strongly acidic medium would become an unwanted factor in the calculation of the saponification rate. This study showed that aspirin underwent a simple ionic bimolecular reaction with sodium hydroxide in aqueous solution. Thus, a second-order rate constant was calculated.

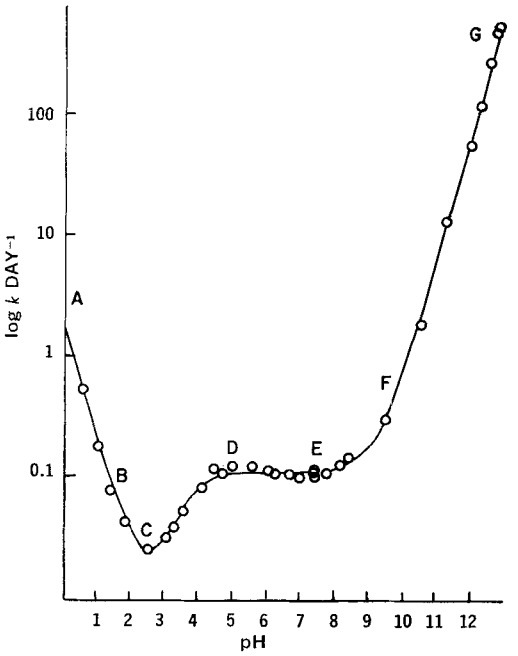


Figure 1—*pH–rate profile for hydrolysis of aspirin.* [*Reprinted, with permission, from L. J. Edwards*, Trans. Faraday Soc., **46**, *723(1950)*.]

Continuing this same approach, Sturtevant (7) analyzed the rate of saponification of aspirin at 35° calorimetrically with the aid of a thermocouple. His conclusion is not often seen in print other than by authors commenting about the previous contributions in relationship to their own work: "The results of these experiments have shown, however, that it would be very difficult to get accurate heat data on these reactions, and it has, therefore, not been considered worthwhile to carry the measurements any further."

A complete and thorough kinetic study of the factors involved in the hydrolysis of aspirin in dilute solution (3×10^{-3} *M*) was conducted by Edwards (8, 9). Using a UV spectrophotometric method for simultaneous determination of aspirin and salicylic acid, he observed the rate of decomposition to be first order at a fixed pH value (between pH 0.53 and 12.77) and constant ionic strength at 17°. Figure 1 depicts the relationship between velocity (rate) constant and pH. This curve was subject to only slight alteration with change in ionic strength. Temperature dependence of this aspirin reaction was studied between 10 and 50°. The pH–rate profile was of the same shape for every temperature, with displacement upward with increasing temperature. This plot of log *k* against pH helps to show visually that the hydrolysis was catalyzed appreciably by hydrogen ion (section AB of figure) and very strongly by hydroxyl ion (section FG of figure). Over the pH range 5–8 (section DE of figure), the rate was constant; in the pH range 2–3 (section C of figure), there was a pronounced minimum rate where the reaction velocity dropped to less than a quarter of the stationary value (DE) which is usually taken to represent the "spontaneous reaction." Edwards explained the relationship between the rate constant and pH on the assumption that the hydrolysis of aspirin may take place *via* the six simultaneous reactions shown in Scheme II.

Table I—Comparison of Hydrolysis Rates of Aspirin in Various Media

Investigator	*k*, Day^{-1}	Medium
Rath (1)	4.35×10^{-2}	Water
Edwards (8)	4.1×10^{-2}	Water
La Mer and Greenspan (6)	7.05×10^{3}	Sodium hydroxide
Sturtevant (7)	7.2×10^{3}	Sodium hydroxide
Edwards (8)	7.50×10^{3}	Sodium hydroxide
Morton (5)	0.103	Potassium citrate buffer about pH 7
Edwards (8)	0.117	pH 7

Through many relationships involving these six equations, the observed unimolecular (first-order) velocity constant could be expressed as a function of the six second-order constants (Eq. 1):

$$k = \frac{k_1 C_H + k_2 C_{H_2O} + k_3 C_{OH}}{1 + K/C_H} + \frac{k_4 C_H + k_5 C_{H_2O} + k_6 C_{OH}}{1 + C_H/K} \qquad \text{(Eq. 1)}$$

When each of the six components of *k* was plotted on a pH–log *k* diagram, four types of curves were obtained. The combination of these four individual curves into one overall curve resulted in a single final curve similar to that depicted in Fig. 1, including the previously inexplicable minimum (C in figure), which Edwards calculated as being at pH 2.44. (The observed minimum was at pH 2.5.)

A comparison of the results of this work (Table I) with those obtained by previous investigators showed good agreement in the rate constant when expressed in the same terminology and conditions.

The hydrolysis of aspirin was accounted for over the whole pH range by considering all the possible bimolecular reactions between the five species present in the equilibrium as: $2H_2O \rightleftarrows H_3O^+ + OH^-$ on the one hand and the equilibrium: $CH_3COOC_6H_4COOH \rightleftarrows CH_3COOC_6H_4COO^- + H^+$ on the other hand.

The mechanism of intramolecular catalysis of the hy-

	k, Day^{-1}
$CH_3COOC_6H_4COOH + H_3O^+ \xrightarrow{k_1} HOC_6H_4COOH + CH_3COOH + H^+$	1.9
$CH_3COOC_6H_4COOH + H_2O \xrightarrow{k_2} HOC_6H_4COOH + CH_3COOH$	$\gg 10^{-6}$
$CH_3COOC_6H_4COOH + OH^- \xrightarrow{k_3} HOC_6H_4COOH + CH_3COO^-$ *or* $HOC_6H_4COO^- + CH_3COOH$	$\ll 10^{6}$ and probably $< 10^{3}$
$CH_3COOC_6H_4COO^- + H_3O^+ \xrightarrow{k_4} HOC_6H_4COOH + CH_3COOH$	46.7
$CH_3COOC_6H_4COO^- + H_2O \xrightarrow{k_5} HOC_6H_4COOH + CH_3COO^-$ *or* $HOC_6H_4COO^- + CH_3COOH$	2.11×10^{-3}
$CH_3COOC_6H_4COO^- + OH^- \xrightarrow{k_6} HOC_6H_4COO^- + CH_3COO^-$	7.5×10^{3}

Scheme II

drolysis of aspirin in the pH region of 5–8 led Davidson and Auerbach (10) to investigate the behavior of aspirin in nonaqueous media. In the presence of dissolved base, aspirin possessed acid anhydride properties and functioned as an effective acetylating agent. They postulated the existence of a cyclic intermediate which results from the intramolecular nucleophilic attack by the ionized carboxyl group on the ester carbonyl. They also postulated that this base-catalyzed isomerization was the rate-controlling step for the hydrolysis of aspirin in the pH 5–8 range. This work was done with organic solvents, and there was no evidence for reactions of this type in aqueous solution.

Ferroni and Baistrocchi (11) measured the rate of hydrolysis of aspirin by determining the liberated salicylic acid fluorometrically. They reported that the reaction followed first-order kinetics and the rate constant was evaluated as 0.0122 $hr.^{-1}$ at 18.5° and pH 9.42.

Hydrolysis rate constants for aspirin, reported by Miyamoto *et al.* (12), agreed with the k_5 rate constant of Edwards (8): 2.625×10^{-3} at 24° and 6.909×10^{-3} at 37°. A general comment was made that aspirin hydrolyzed more quickly in simulated intestinal fluid than in simulated gastric fluid.

Garrett (13), extending the work of Edwards (8) to include a number of acyl salicylates in a very complete investigation, looked more thoroughly into the pH–rate profile of aspirin hydrolysis, particularly in the pH 4–8 range. Edwards' own demonstration that the hydrolysis was not catalyzed by acetate ion (varied from 0.005 to 0.3 *M*) was not consistent with the mechanism involving an attack by a water molecule on the aspirin anion, because the acetate ion is a considerably more powerful nucleophile than water. Garrett's work pointed rather to intramolecular nucleophilic catalysis by the ionized carboxyl group. Even though the carboxylate ion is an unfavorable case from the point of view of nucleophilicity, it apparently participates catalytically in a number of intramolecular catalyses of esters. The hydrolysis of aspirin may be regarded as a classical example. On increasing the alcohol concentration greatly in the pH-independent region of the pH–rate profile, a very unexpected increase was found.

In this light, alcohol would have to be considered a more active nucleophile than water. To clarify this anomalous enhancement of "spontaneous" hydrolysis with increasing alcohol content (0–60%), Garrett made a thorough study. The addition of alcohol to the solvent increased the rate of solvolysis; ethyl acetate was a resulting product. He ruled out the possibility that the rate increase was a generalized solvent effect by showing that the addition of dioxane had very little effect on the rate of hydrolysis of aspirin. He tried to explain his results by proposing a mechanism involving nucleophilic attack by alcohol on the tetrahedral carbon atom of an intermediate compound. This explanation has not been generally accepted. Nevertheless, the demonstration that the addition of alcohol increased the rate of solvolysis did suggest strongly that the question of the involvement of a molecule of solvent in the transition stage ought to be studied.

Using the same four acyl esters of salicylic acid as in the mentioned studies, Garrett (14) studied the stability of their saturated solutions. Prediction of stability in such solutions was made from separate studies of solubility rates and homogeneous rates on dilute solutions of these esters, since solvolytic degradation was a function of these two rates. This study did show that aspirin was the least stable of the esters studied.

Okano and Kojima (15) investigated the effect of salicylic acid upon the rate of aspirin decomposition in solution. They observed deceleration of the aspirin hydrolysis between pH 2.2 and 7 with increasing amounts of salicylic acid being added to the medium. Below pH 2.2 the opposite effect was noted. These studies were conducted for 10 days at 35°, or 8 hr. at 50°, and the rate constants were calculated. The reversal effect of the salicylic acid on the hydrolysis of the aspirin is in the vicinity of the minimum shown by Edwards' (8) pH–rate profile of aspirin hydrolysis.

The hydrolysis of aspirin at pH 6 in water containing 4.3 atom % of ^{18}O produced, after 22 hr. refluxing, salicylic acid containing 6% of the excess ^{18}O in the water. This result was in agreement with the theoretical prediction made by Bender *et al.* (16) and gave backing to the hydrolysis mechanism of aspirin postulated by Garrett (14) and others. This involved an intramolecular attack of the carboxylate ion on the carbonyl carbon atom of the ester to produce acetylsalicyl anhydride, which subsequently hydrolyzes rapidly to produce acetate and salicylate ion, or alternatively that the addition of the carboxylate ion to the carbonyl group of the ester is followed by some reaction with water leading to the same products. It can be calculated from the relative rates of hydrolysis of ethyl acetate and ethyl salicylate that the reaction producing salicylic acid-^{18}O should occur to the extent of 2.5%. The 6% observed was considered to be reasonable and consistent with the postulated reaction in which water was involved.

Using a BPC mixture of aspirin, James (17) investigated the kinetics of the hydrolysis of aspirin from aqueous suspension by comparative, rather than quantitative, means. As long as there was a good excess of aspirin suspension present at the different temperatures, the hydrolysis rate was zero order. Thus, the more concentrated the suspension, the more stable was the aspirin. After 62 days at room temperature, suspensions of 3.3, 6.5, and 13.0% aspirin showed the following percent of intact aspirin remaining: 90, 94, and 97%, respectively. This follows, as the hydrolysis rate depends on the amount of aspirin in solution. Hence, suspensions show a low degree of hydrolysis relative to the total amount of aspirin in suspension.

While James used a titration procedure to follow the rate of hydrolysis in his study, Blaug and Wesolowski (18) used a more refined UV procedure in a pH 3 buffer. This pH was selected because it is the pH of a saturated solution of aspirin (approximately 4 g./l.). The effect of the following additives (calcium gluconate, glycerin, *N*-methyl-2-pyrrolidone, polyethylene glycol 6000,[2] polyvinylpyrrolidone, salicylic acid, sorbitol, pH 3.0 buffer, and water) on the stability of aspirin suspensions (6.5% of 100-mesh and 13% of 60-mesh aspirin) was followed

[2] Carbowax 6000, Union Carbide Corp.

by measuring the salicylic acid content at 298 mμ. The thermodynamic values reported in the paper were calculated from the data obtained with these various suspensions. The suspensions (in duplicate) were stored in a 50 ± 0.5° mechanical shaker for 24 hr. Samples were removed from the suspensions at hourly intervals for assay. Calcium gluconate accelerated the hydrolysis by increasing the pH of the medium, while the *N*-methyl-2-pyrrolidone or glycerin enhanced the solubility of the aspirin, thus increasing the hydrolysis. The presence of saturated salicylic acid did not affect the hydrolysis rate. The most promising additives were polyethylene glycol 6000 and polyvinylpyrrolidone, but physically they were unsatisfactory at this temperature as they formed gummy, insoluble masses of the suspension. Only sorbitol showed any potential stabilizing effect on the aspirin suspension.

In continuing his studies on the effect of alcohol on the hydrolysis rate of aspirin, Garrett (19) synthesized the mixed anhydride of aspirin and acetic acid to help establish his mechanism for the hydrolysis reaction. This compound did not form ethyl acetate as expected in the alcohol medium, so a logical explanation regarding the experimental evidence was still lacking.

Further studies with deuterium oxide solvent isotope effects in the nucleophilic reactions of phenylesters were reported by Bender *et al.* (20). One of the continuing problems associated with the hydrolytic reactions of carboxylic acid derivatives is to distinguish between nucleophilic and general basic catalysis of hydrolysis. The former involves the attack of a nucleophile upon a substrate, leading to the formation of an unstable intermediate which spontaneously breaks down to give the product and regenerates the catalytic entity. The latter catalysis involves the attack of a general base on the substrate removing a proton in a rate-determining stage. Either of these two processes may be carried out by a given substance which, by definition, is at one and the same time both a nucleophile and a general base. The deuterium oxide solvent isotope effect has been used to distinguish between these two possibilities.

The aspirin hydrolysis has been shown, on the basis of kinetic and isotopic experiments, to involve an intramolecular nucleophilic-catalyzed hydrolysis involving an anhydride intermediate. For the purpose of calculation, it has been assumed that the transition state of the reaction was one in which the carboxylate ion has been added to the carbonyl group of the ester, forming a tetrahedral addition intermediate. The formation of this intermediate is, in general, the slow step in the nucleophilic reactions of carboxylic acid derivatives. The authors concluded that the use of deuterium oxide solvent isotope effects as a criterion to distinguish between general base- and nucleophilic-catalyzed reactions was ambiguous; but when applied in a restricted sense, it may be empirically rewarding.

Nogami *et al.* (21) examined the effect of cationic (cetylethyldimethyl ammonium bromide and benzalkonium chloride), anionic (sodium lauryl sulfate), and nonionic (polyoxyethylene lauryl ether) surfactants on the suppression of the hydrolysis of aspirin which exists in anionic and undissociated forms in aqueous solution. The decomposition-rate constants in the buffer solutions (pH 1–7.5) were obtained, with or without the surfactant, and compared. Samples were kept at 37 ± 0.1°, with aliquots being removed at given intervals and assayed for salicylic acid with a ferric nitrate reagent. The color was determined spectrophotometrically at 530 mμ.

The hydrolysis of aspirin was found to follow a pseudo-first-order reaction in the media studied. In the pH 5–7.5 range, aspirin was chiefly in the anionic form. Due to electrostatic attraction, it formed a complex with the cationic surfactant which moved into micelles composed of excess surfactant. Thus, the hydrolysis of aspirin in this pH region was suppressed only by a cationic surfactant. In the pH 1–5 range, all the surfactants suppressed the hydrolysis of aspirin. Because the undissociated aspirin existed in this region, it moved into micelles and was less hydrolyzable. Near pH 1, only the anionic surfactant lost its effect on suppressing the hydrolysis of aspirin. This was explained by the promoting effect of sodium lauryl sulfate on the hydrolysis of aspirin, due to the attracted hydrogen ion on the micelle environment competing with the suppressing effect of the solubilization. Even though this report shows suppression of the hydrolysis of unionized aspirin by all the surfactants and the suppression of the anionic form by cationic surfactants, it by no means implies that these solutions could be used as a stable pharmaceutical formulation.

Nelander (22) reported the heat of hydrolysis of aspirin at 25° by a calorimetric procedure. The $-\Delta H$ (kcal./mole) for aspirin was 25.39 ± 0.03 in 0.8 *N* sodium hydroxide in water–alcohol, 2:3. The heat of solution in aqueous tromethamine solution, ionic strength 0.1, initial pH 8.05, for aspirin was 5.72 ± 0.08 kcal./mole.

The first application of ultrasonic energy in accelerated drug stability studies was published by Mario and Gerraughty (23). Duplicate samples of aspirin in the given buffer (pH 2.00, 4.00, or 5.95) were put in two constant-temperature baths at 21, 25, 35, and 45°, one with ultrasonic energy and the other (control) without. Aliquots from both baths were taken at stated time intervals and assayed for salicylic acid at 302 mμ; the content was calculated from standard curves of salicylic acid in the same buffer. Aspirin runs were done at two different concentration levels. The agreement of duplicate runs was good and indicated that the experimental technique was reproducible.

Pseudo-first-order rates were found at all pH values and with varying temperatures, both with and without ultrasonic energy. The hydrolysis rate constant, k, was calculated. The Arrhenius relationship was followed in all cases, and the heat of activation of the hydrolytic degradation of aspirin was not changed by the introduction of the ultrasonic energy. The increase in the rate found with the ultrasonic samples was equivalent to increasing the reaction mixture temperature within the range of 1.8–2.9°. This range was consistent, regardless of the pH or temperature used. Although the effects of ultrasonic energy were not startling on increasing hydrolysis, these studies did show the potential of this new technique, particularly with heat labile ingredients.

Table II—Apparent Zero-Order Rate Constants of Salicylic Acid at Constant pH

Aspirin plus Lubricant	*k*, mg. FSA/hr.	pH
None	0.123	2.60
Stearic acid	0.133	2.62
Hydrogenated vegetable oil	0.123	2.68
Talc	0.133	2.71
Aluminum stearate	0.281	3.16
Calcium stearate	0.986	3.75
Magnesium stearate	1.314	4.14

Needham and Gerraughty (24) pursued further the hydrolysis of aspirin in mixed solvent systems by ultrasonic energy. The solvent systems were: alcohol–water, 10, 30, 50, and 70%; ether–water, 3 and 5%; and ethylene glycol–water, 5, 10, 30, and 50%. The pH for all of the media was kept at 3.67. Since the thermal energy (use of dual constant-temperature baths) was kept constant for both ultrasonified and control systems, it was apparent that the ultrasonic energy was responsible for the increase in the kinetic rates. With the ethylene glycol–water system, as the concentration ratio was increased, the subsequent increase in viscosity apparently reduced the movement of molecules caused by the ultrasonic vibration, as shown by the smaller rate constants for the hydrolysis of aspirin.

In studying the interaction of aspirin with urea, Santopadre and Bolton (25) shook saturated solutions of aspirin in water at 30° with known varying amounts of urea (0 through 10 *M*) for 5 hr. Kinetic studies were made at pH 2.0, 2.5, 2.75, 3.0, and 3.5 at 30 ± 0.2°. First-order rate constants were calculated. Urea increased the rate of hydrolysis below pH 2.75 and decreased the rate of hydrolysis at pH values greater than 2.75. It is interesting to note that this "crossover" occurs at a pH corresponding to the pH of maximum stability, as reported by Edwards (8). This pH may thus represent a point where the hydrolysis mechanism changes, and this could provide an explanation for the change in the effect of urea.

Murthy and Rippie (26) studied the hydrolysis of aspirin in the presence of polysorbate 80. Saturated solutions of aspirin at 30 ± 0.1° were prepared in 0, 1, 2.5, and 4% solutions of polysorbate 80 at the following pH's: 2.63, 3.63, 4.10, 4.21, and 4.43. Kinetic studies were carried out on suspensions, saturated solutions, and half-saturated solutions for 48 hr. at 30 ± 0.1°. Samples were removed at stated times and assayed by the UV method described by Edwards (8).

With the suspensions, the observed increase in degradation-rate constants (pseudo-zero-order) with added polysorbate 80 was due to the instability of undissociated aspirin in the micellar phase. With the homogeneous solutions, the rate of hydrolysis of aspirin in the polysorbate micelles, while lower than in the aqueous phase, was not negligible. The solubility determinations in the various media showed the absence of dissociated aspirin in the micellar pseudophase of the polysorbate 80 solutions.

Hydrolysis of solubilized aspirin in the presence of the nonionic surfactant, cetomacrogol, was studied by Mitchell and Broadhead (27). All the studies were conducted at 37 ± 0.1° in the pH range of 1–7 on solutions of aspirin with cetomacrogol concentrations of 0.1 through 0.07 *M*.

The hydrolysis of aspirin proceeds as a first-order reaction, both in aqueous buffer and in buffered cetomacrogol solutions. Reaction rate constants were determined. At the pH of maximum stability, pH 2.27, where aspirin exists largely in the unionized form, the half-life increased with cetomacrogol concentration. In 0.07 *M* cetomacrogol, the half-life was approximately twice that in the control buffer. In the plateau region where aspirin is largely ionized, the rate of hydrolysis was independent of cetomacrogol concentration.

Kornblum and Zoglio (28) evaluated the commonly used tablet lubricants as to their effect on the stability of aspirin. Suspensions of aspirin with the various lubricants (talc, hydrogenated vegetable oil,[3] stearic acid, aluminum stearate, calcium stearate, and magnesium stearate) were prepared. The lubricants were also in excess to ensure saturation through the experiments.

The suspensions were maintained at 30°, with appropriate aliquots withdrawn at various time intervals for pH and salicylic acid determination by adding ferric chloride and reading at 540 mμ in a spectrophotometer. From the kinetic studies of these suspensions, the reaction rate appeared to be of zero order. The pH remained relatively constant through the 30-hr. study for the given suspension.

The results are summarized in Table II.

With both calcium and magnesium stearates, the rate of decomposition of aspirin was due to more than just the increase in pH. The authors showed that this increase was due to the high solubility of calcium and magnesium aspirin which were formed in these suspensions. The mechanism primarily involves a reaction of the alkali cation with aspirin in a solution to form a salt of aspirin which, in the presence of solvated aspirin, comprises a buffer system at a pH detrimental to the stability of aspirin.

Reduction of the water content in the aspirin–calcium stearate suspension was done to approach that found in a solid dosage form, the ultimate aim being the achievement of reproducible data which would permit subsequent extrapolation to the tablet or capsule dosage form. A major conclusion from this interesting study is that stearate salts should be avoided as tablet lubricants in preparing aspirin formulations.

In their study on salicylic acid sublimation, Gore *et al.* (29) determined the hydrolysis rates for aspirin at temperatures ranging from 17.2 to 30.2 ± 0.1° at pH 7.4 and reading the resulting salicylic acid at 296.5 mμ. Their data are summarized in Table III. These values are in close agreement with those reported by Morton (5) and Edwards (8).

It is only by pure coincidence, but certainly quite appropriate, that the last papers dealing with hydrolysis of aspirin in this review clarified the situation immensely. In an attempt to circumvent the problems raised by the kinetic equivalence of the several possible mechanisms, Fersht and Kirby (30, 31) looked first at

[3] Sterotex, Capitol City Products, Columbus, Ohio.

Table III—Rate Constants for the Hydrolysis of Aspirin in pH 7.40 Buffer Solution at Various Temperatures

Temperature	k, Day^{-1}
17.2	0.0937
21.3	0.1506
25.5	0.2067
30.2	0.3429

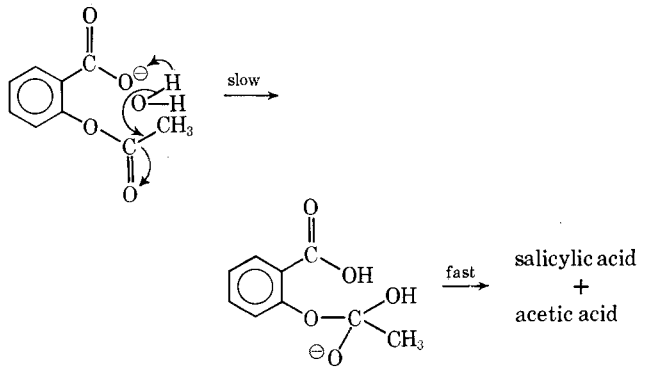
the reactivity toward hydrolysis of a series of substituted aspirins. The results suggested, unambiguously, that the most likely mechanism for the hydrolysis of aspirin was one in which the carboxylate group acts not as a nucleophile but as a general base.

The rate of hydrolysis of aspirin was measured in these studies by following the initial rate of release of salicylate at the isosbestic point, 298.5 mμ, and at 39.0 ± 0.03°. The ionic strength was maintained at 1.0 with added potassium chloride. It was this fact that showed why Edwards (8) failed to detect catalysis by acetate ion, because the ionic strength was not kept constant in his experiments. Fersht and Kirby (30, 31) found that the small acceleration due to the addition of a given concentration of acetate was almost exactly equal to the opposite effect of the increase in ionic strength. If Edwards had only known this fact, there no doubt would have been fewer controversial papers dealing with the mechanism of the hydrolysis of aspirin.

The pH–rate profile for aspirin hydrolysis, measured by Edwards (8), shows that the transition state for hydrolysis in the pH-independent region involves the aspirin anion, either alone in a unimolecular reaction or together with one or more molecules of solvent. Three mechanisms were consistent with this kinetic result for intramolecular catalysis of the hydrolysis of aspirin by the carboxyl group:

1. A unimolecular process in which the carboxylate group acts as a nucleophile. There was no longer any evidence that specifically supported the nucleophilic mechanism. It was not consistent with the effect of substituents on the reaction, and there were several indications that the rate-determining step was not a unimolecular process.

2. A general acid catalysis of the attack of hydroxide ion by the undissociated carboxylic acid group. This mechanism was rejected because intermolecular general acid catalysis by the carboxy group of aspirin should be observed for attack by acetate as well as by the hydroxide ion.



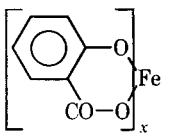
Scheme III—Mechanism of hydrolysis of aspirin as a classical general base catalysis

3. A general base catalysis of the attack of a water molecule by the carboxylate anion. There seems little doubt that the intermolecular reaction of acetate with the aspirin anion represents general base catalysis. There is even less doubt that intramolecular catalysis of hydrolysis by the carboxylate group of aspirin involves the same mechanism as the intermolecular reaction with acetate ion.

In actuality, it seems probable that the aspirin reaction lies close to the borderline between nucleophilic and general base catalysis (Scheme III).

DETERMINATION OF SALICYLIC ACID IN ASPIRIN AND ASPIRIN PRODUCTS WITH FERRIC IRON

Until 1965, the most prominent method of determining salicylic acid in aspirin, or products containing aspirin, was the reaction with ferric iron under a variety of conditions. The complex produced on mixing ferric ion and salicylic acid (a bidentate ligand) results in the formation of a series of intensely colored metal chelates having ligand–ferric-ion ratios of 1:1, 2:1, and 3:1. The formation of the cyclic chelate structure (I) involves the displacement of the weakly acidic, phenolic hydrogen by the metal, resulting in the formation of a six-membered ring by coordination of the metal through the phenolate and carboxylate groups of salicylic acid:



I

In the first of these publications, in 1911 (32), the sample containing aspirin was shaken with water or alcohol and filtered; then one drop of ferric chloride solution was added. On standing, the color changed from reddish to dark violet. This, in essence, was the beginning of the official compendia tests for salicylic acid in aspirin. It was noted very early by Melzer (33) that the presence of either sodium phosphate, tartaric acid, or borax masked the iron–salicylic acid color reaction. The useful suggestion was made that the tablets first be extracted with ether, since only the aspirin and salicylic acid would be extracted; thus a simple separation from the interfering compounds was easily accomplished. This immediately brings to mind that it would be easy to obtain false negative tests for salicylic acid if such a compound was incorporated in the aspirin tablet and no prior separation were made. Before the year was over, Linke (32) had refined the method for determining free salicylic acid in aspirin tablets by comparing the resulting iron–salicylate color to a series of salicylic acid standards. As little as 1 mcg. salicylic acid/ml. could be detected.

A limit test of 0.1% salicylic acid in aspirin was described by Leech (34), using a small volume of alcohol to dissolve the aspirin completely and then diluting with water before the addition of the ferric chloride reagent. This test became the basis for USP procedure when aspirin became official in 1926. It was emphasized that the standard salicylic acid tube should contain the same

amount of alcohol as the sample, because the final color was affected (decreased) by the presence of alcohol. Jones (35) stated that for tablets the limit test should be 0.15% salicylic acid and that there should be no turbidity in the final solutions.

Rather than compare the salicylic acid extract from aspirin, as described, Dahm (36) prepared a permanent color series containing various amounts of cobalt chloride dihydrate. After standardization with known quantities of salicylic acid, one could compare directly, under the stated conditions, aspirin extracts and "read" the percent free salicylic acid (FSA) directly. As these cobalt colors were of a permanent nature, it would save the analyst preparing a fresh salicylic acid reference standard.

Comments about the BP salicylic acid test made by Nutter-Smith (37, 38) brought forth that the official test was not effective below 0.04% salicylic acid, because the ferric chloride reagent was the limiting factor due to its own color. Using ferric ammonium sulfate corrected this situation. The presence of tartaric or citric acid in tablets (1%) has been shown to mask the presence of 0.2% salicylic acid. Thus, if one obtains a negative test for the FSA in unknown aspirin tablets, proper steps should be taken to separate the salicylic acid from the interfering material. Ruddiman (39, 40) added oxalic and tannic acids as masking agents of the iron–salicylate test and commented that sodium phosphate and borax did not interfere with the iron–salicylate test as had been believed. Incompatibility of aspirin with many drugs was proven by Snidow and Langenhan (41) using ferric alum reagent qualitatively.

Valentin and Lieber (42) showed that if ether was used to extract aspirin and salicylic acid from other materials, the evaporation step must be done with care, because too much heat results in high FSA values. They suggested chilling the ether and passing a stream of air over the solution to expedite the evaporation.

The use of a spectrophotometer in the determination of FSA was introduced by Hoffman (43) in 1929. Since the tablets being analyzed contained magnesium oxide, 4 *N* sulfuric acid was added during the grinding of the tablets. This was added to prevent hydrolysis (although not stated, it would also free any salicylic acid which might have been combined with magnesium ion) of the aspirin before the salicylic acid was extracted with a 1:1 mixture of ether and pentane. After evaporation of the clear extract, alcohol was added to dissolve the salicylic acid, and the iron reagent (ferric chloride) was added. This color was then compared with a standard series in the spectrophotometer.

Chloroform was introduced as a direct extractant of aspirin by Hitchens (44). This chloroform extract was shaken with 2% sodium bicarbonate aqueous solution to remove the aspirin (and salicylic acid). This, in turn, was made acidic with hydrochloric acid and extracted quantitatively with ethyl acetate. The ethyl acetate extract was evaporated under reduced pressure in a water bath maintained between 40–45°. The residue was dissolved in alcohol and diluted with water; ferric ammonium sulfate solution was then added. The resulting color was compared with standards of salicylic acid in the same medium. Because hydrolysis does take place in alkaline medium, Hitchens showed that, at 20° for 1 hr. in the sodium bicarbonate solution, about 0.25% of the aspirin was hydrolyzed. At 30°, about 0.35% of the aspirin was hydrolyzed. Since the procedure described took less than 20 min. in the alkaline medium, the error caused by this alkaline hydrolysis was called negligible. With standard runs of aspirin USP, the FSA content was less than 0.15% by this extraction procedure. With various mixes and commerical tablets, the FSA found was never over 0.2%. The purpose of this extraction procedure was to isolate quantitatively the aspirin from compounds such as acetphenetidin, caffeine, acetanilid, antipyrine, amidopyrine, and phenylsalicylate.

In 1937, Banchetti (45) made a critical evaluation of many of the pharmacopeias in regard to their FSA tests which used various ferric iron reagents after extraction of the aspirin and salicylic acid. It was pointed out that the tests should be done at the lowest practical temperature and as rapidly as possible to minimize hydrolysis of the aspirin while conducting the procedure. If evaporation of a solvent extract is required, it should be done with as little heat as possible to avoid excessive FSA values.

The first reported humidity- and temperature-controlled experiments with aspirin tablets in different packagings was conducted by Canback (46). Tablets were stored in wood boxes, tins, impregnated paper, and glass bottles at 20 ± 0.2° for 1 year at various humidity stations (0, 19, 44, 59, 75, and 100% relative humidity). Using 2.5 *M* acid to acidify the pulverized powder, the aspirin and salicylic acid were extracted with a 1:1 mixture of ether and petroleum ether. An aliquot was evaporated, and the salicylic content was determined by dissolving the residue with diluted alcohol. Ferric chloride solution was added, and the resulting color was read in a colorimeter after standing 15 min. After a year at the various humidity stations, the aspirin tablets stored in glass showed very little change in FSA content. The other packagings were greatly inferior with the wooden one being the poorest in regard to aspirin stability. The higher the humidity, the larger and quicker the FSA values increased (other than in the glass bottles where little change was found at any of the humidity stations).

Using a Duboscq colorimeter, Tsuzuki and Sawada (47) measured the amount of FSA produced after aspirin had been heated at 110 and 128°. The heated sample (after 5–35 min. at the stated temperature) was dissolved in methanol, the ferric chloride reagent was added, and the solution was compared to standards. The relationship of increased FSA with a corresponding lowering of the melting point of the aspirin was shown.

Pankratz and Bandelin (48) made a systematic and comprehensive study of the optimum conditions for the reaction of ferric iron and salicylic acid and its reproducibility. Maximum absorption of the ferric–salicylate complex in a nearly aqueous medium was at 525 mμ. This complex was very sensitive to pH changes. On studying pH effect at one pH unit increments from 1.0 through 9.0, the maximum color was found between 3.5 and 8.0. Above pH 6.5 the color faded rapidly, so the useful pH range was between pH 4.0 and 6.0. This emphasizes the point that unless the pH of the sample and of the standard series are close, the equivalent

Table IV—Percent Decomposition of Aspirin of Varying Particle Size after 6 Months[a] at 37°

Crystal Size, mesh	Relative Humidity, % 42	59	84
20–50	0.07	0.08	0.16
50–100	0.08	0.09	0.21
100–200	0.08	0.10	0.59

[a] Reprinted, with permission, from R. Yamamoto and T. Takahashi, *Ann. Rep. Shionogi Research Lab.*, 3, 112(1953).

amount of color will not result. One variable, the alcohol concentration, was not controlled. But at the levels used, it apparently did not affect the linearity of the color to concentration. With such a study, one would have expected a comparison of the different ferric salts, particularly those which have been used in the past such as ferric chloride and ferric ammonium sulfate. Instead, this is the first paper in which ferric nitrate was used.

A study involving just pure aspirin, by Yamamoto and Takahashi (49), answered many questions which arise when one seriously wonders under what conditions aspirin is stable or the most stable. The effect of the particle size of the aspirin on its decomposition rate was studied at 37° over a storage period of 6 months at three controlled humidities. The data in Table IV indicate that the finer the crystals and the higher the relative humidity, the more aspirin hydrolyzed. One may also conclude that under 60% relative humidity at 37°, the percent decomposition of aspirin did not change with particle size. With today's use of micronized aspirin, this is a valuable fact.

In studies at 60° for a total of 25 hr. at 30, 60, 80, and 100% humidities, decomposition was linear with time, and the linearity seemed proportional to the vapor pressure because the slope of each line increased with an increase in vapor pressure. Although the FSA content never exceeded 0.04%, this study showed that an increase in humidity did result in an increase in the decomposition rate.

At 90° and at low humidity, the authors found that, after a total of 12 hr., aspirin decomposition was linear with time but had not surpassed 0.1%. At 120° (low humidity), over 15% of the aspirin was decomposed within 5 hr., and the rate of decomposition was no longer linear with time. This study emphasized the fact that temperature increases alone accelerated the decomposition of aspirin.

Grinding of aspirin for 15–120 sec. did not increase appreciably the percent decomposition. On repeated compression (three times) of aspirin, the percent decomposition was found not to have increased appreciably.

In another paper dealing with the stability of aspirin when mixed with other compounds, Yamamoto and Takahashi (50) found that such mixtures should be stored at the lowest practical humidity to prevent excessive decomposition of the aspirin (Table V).

Table V—Stability of Aspirin in Various Powder Mixtures at 37° for 15 Days[a]

Compound Mixed with Aspirin	Ratio of Aspirin to Compound	Percent Loss of Aspirin at 42% Relative Humidity	84% Relative Humidity
Antipyrine	10:3	1.2	21.1
Aminopyrine	10:3	8.2	33.2
Hexamine	10:3	56.4	83.4
Ethyl aminobenzoate	10:3	32.6	60.8
Caffeine	10:3	0.01	0.08
Zinc sulfate	10:3	0.02	0.03
Sodium benzoate	10:3	3.2	80.2
Sodium salicylate	100:5	—	26.3
Calcium glycerophosphate	10:3	0.4	4.2
Pheniramine maleate	100:3	—	6.0
Phenindamine tartrate	100:3	—	6.4

[a] Reprinted, with permission, from R. Yamamoto and T. Takahashi *Ann. Rep. Shionogi Research Lab.*, 4, 79(1954).

Table VI—Effect of Ethylenediamine Salts on the Stability of Aspirin[a]

Salt of Ethylenediamine	k_1	Loss of Aspirin, %
Hydrochloride	—	0.6
Maleate	1.0×10^{-2}	3.1
Succinate	6.4×10^{-5}	10.5

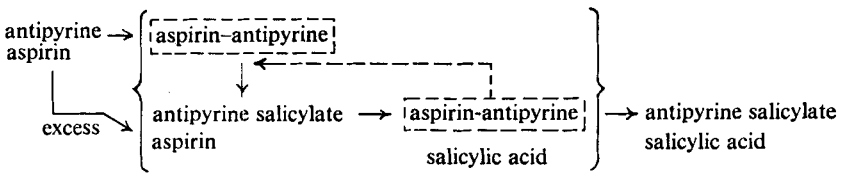
[a] Reprinted, with permission, from R. Yamamoto and T. Takahashi, *Ann. Rep. Shionogi Research Lab.*, 4, 79(1954).

In a comparison of aspirin mixtures with various salts of ethylenediamine, the importance of acid strength on the decomposition of aspirin was shown (Table VI). This study was conducted at 37° and 84% relative humidity for 15 days with a 100:3 ratio of aspirin to ethylenediamine salt.

Further investigation of a 10:1 molar ratio of aspirin–antipyrine mixture stored at 37° and 84% relative humidity for 2 months produced salicylic acid and antipyrine salicylate. The mechanism proposed for this interaction is given in Scheme IV.

Scheme IV was explained in the following manner. First there was an acid–base reaction between aspirin and antipyrine; but because this salt was highly unstable, it decomposed rapidly to antipyrine salicylate. Since aspirin and salicylic acid have approximately the same acidity, the excess aspirin present was capable of reacting with the antipyrine salicylate, releasing salicylic acid and regenerating the unstable aspirin–antipyrine salt which, in turn, started the cycle again.

A similar reaction cycle was proposed for the decomposition of aspirin with pheniramine maleate (amine salt of a weak acid), while no reaction took place with pheniramine hydrochloride (amine salt of a strong acid)



Scheme IV

Table VII—Explanation of Symbols Used in Table VIII

Variable	High Level %	High Level Symbol in Table	Low Level %	Low Level Symbol in Table
Lubricants				
Glycerin monostearate	2	L	0.5	l
Magnesium stearate	2	L	0.5	l
Talc	4	L	1.0	l
Calcium stearate	2	L	0.5	l
Stearic acid	2	L	0.5	l
Mineral oil	4	L	1.0	l
Talc–mineral oil, 1:1	4	L	1.0	l
Pressure	Highest possible	P	Lowest possible	p
Moisture[a]	1.64	M	0.098	m
Aspirin	14-Mesh granules of 10% starch–aspirin granulation	A	40-Mesh crystals	a

[a] Refers to moisture content of the phenacetin–caffeine granulation only.

because aspirin was not capable of displacing the hydrochloric acid.

Without the aid of the computer age, Ribeiro *et al.* (51) undertook a massive, well-executed study of variables in the manufacturing of a stable APC tablet (aspirin, phenacetin, and caffeine). To study the probable causes of the decomposition of aspirin, a factorial experiment was set up testing all combinations of lubricants at two levels, pressure at two levels, moisture at two levels, and two types of aspirin; the entire series was repeated for each of the seven different lubricants (a $2^4 \times 7$ factorial experiment). These variables were selected because they seemed to be the most probable causes of aspirin breakdown in APC tablets. An Association of Official Agriculture Chemists (AOAC) (6th edition) procedure for salicylic acid was modified by using absolute alcohol to extract the salicylic acid from the pulverized tablets and to develop the final iron–salicylate color in a 35% alcohol medium. A spectrophotometer was used to read the resulting color at 537 mμ. Concentrations of salicylic acid were obtained from a standard curve of salicylic acid prepared exactly as the samples. These salicylic acid values were converted to aspirin values and reported in this paper as percent of aspirin content that had decomposed.

Before presenting the data in tabular form, a brief explanation of all the symbols used in the table is essential for proper interpretation of the percent decomposition of aspirin in the tablets after being stored in loosely capped bottles at 45° for 27 days. Tables VII and VIII summarize the results.

Interpretation of these results indicated that more stable combinations were possible if the lubricants used were talc, talc plus mineral oil, mineral oil, or glycerin monostearate rather than stearic acid, magnesium stearate, or calcium stearate. Compression pressure levels showed no effect. Moisture levels were not different enough to be consequential. The crystalline aspirin was superior to the starch–aspirin granulation.

Since difficulties arose in applying the BP colorimetric test for the FSA, Edwards *et al.* (52) initiated an investigation of the kinetics of aspirin hydrolysis and of conditions affecting the formation and stability of the ferric–salicylate complex. Time, as already well known, was an important factor, because aspirin hydrolyzes continually once it is in a given solvent (here alcohol and later water). The need for fast filtration was obvious. Higher temperature expedited the hydrolysis of aspirin, so the lowest practical temperature should be used throughout the test. Constant pH for the test was a

Table VIII—Decomposition of Aspirin after 27 Days at 45° under the Stated Conditions Described in Table VII

Combination	% Aspirin Decomposed[a] Glycerin Monostearate	Magnesium Stearate	Talc	Calcium Stearate	Stearic Acid	Mineral Oil	Talc plus Mineral Oil
MPAL	1.17	8.67	1.10	20.95	8.03	4.11	2.97
MpAL	1.76	8.31	0.97	19.65	8.54	4.59	1.33
MPaL	0.67	7.06	0.55	21.21	0.98	0.99	0.30
Mpal	0.36	2.04	0.00	6.39	0.69	0.39	0.52
MPal	0.33	1.21	0.00	6.95	0.84	0.31	0.22
MpAl	1.63	3.20	2.76	6.20	3.48	4.70	0.87
MpaL	1.05	6.26	0.00	19.54	2.00	0.99	0.75
MPAl	0.99	2.28	2.21	3.84	1.27	2.40	1.16
mPAL	8.56	6.84	1.41	16.04	7.67	1.18	2.66
mpAL	8.59	7.71	2.65	18.35	12.51	2.03	0.58
mPaL	0.96	15.99	0.54	18.54	3.95	0.92	0.87
mpal	0.80	3.39	0.55	3.53	0.44	0.45	0.97
mPal	0.53	3.03	0.48	4.45	1.28	0.90	0.70
mpAl	0.82	2.45	0.49	8.09	2.09	0.68	0.63
mpaL	0.45	15.26	0.73	19.79	4.14	0.93	0.98
mPAl	1.00	3.22	0.73	6.31	3.84	1.06	0.16

[a] The freshly compressed tablets showed negligible decomposed aspirin.

must because the rates of hydrolysis of aspirin vary appreciably with pH. The intensity of the ferric–salicylate complex depends greatly on the pH of the medium. The maximum intensity was obtained between pH 2.5 and 3.5 and was best maintained using an acetic acid–ammonium monochloroacetate buffer. Below or above this pH range the intensity of the color decreased rapidly (intensity at pH 3 was over five times as strong as that at pH 1.5).

As the BP method for FSA is a limit test, a quantitative procedure was developed utilizing the maximum conditions described. If no known interfering materials were present, the aspirin and salicylic acid were dissolved in absolute ethanol diluted with water, all at 25°, and maintained by a thermostated water bath. The time (T_0) was noted when the buffer and ferric ammonium sulfate solution were added and mixed. The volume of the solution was made up to the mark with water, mixed, and kept at 25° for about 10 min. An aliquot was filtered, transferred into an absorption cell, and read at 530 mμ against a reagent blank; the time (T_1) was noted. At least three aliquots were withdrawn at intervals of not less than 10 min. apart. From these values (T_1, T_2, and T_3), a value for the extinction coefficient at zero time (T_0) was obtained by extrapolation. From a standard salicylic acid curve, the amount of salicylic acid at T_0 was thus obtained.

If interfering matter such as phosphate or citrate was present, the aspirin and salicylic acid were extracted from the powdered sample in a separator with benzene. The filtered and pooled benzene was then extracted with small volumes of a solution of the buffer and ferric ammonium sulfate reagent, until an aliquot showed no further coloration in the aqueous extract. The pooled aqueous solution was made to volume with the remaining buffered ferric ammonium sulfate solution and filtered, if necessary, before reading at 530 mμ. The FSA was calculated from a standard salicylic acid curve as before, but without having to calculate a T_0 value; the intact aspirin remains in the benzene layer, so no hydrolysis should be taking place in the aqueous layer.

It is of interest to note that the pH of the aqueous solution (2.95 ± 0.05) read at 530 mμ for both procedures was near the minimum of the hydrolysis rate of aspirin in regard to its pH–rate profile. On comparing the FSA values obtained by using these proposed procedures with those of the BP on several commercial products, the proposed procedure consistently found more FSA, which again raises doubt about the sensitivity of the official BP test for FSA.

Quite independently, Strode *et al.* (53) conducted a systematic study similar to Edwards, only this study involved modifying the USP XV free salicylic test from a limit test to a sensitive, reproducible procedure which was applicable in FSA testing not greater than 0.25%. Hydrolysis curves were constructed from transmittance measurements made at timed intervals on thermostated solutions of aspirin and ferric alum. Under the conditions of the spectrophotometric method, these curves indicated that salicylic acid increased at the rate of 0.0028%/min. at 20°, 0.0036%/min. at 25°, and 0.0054%/min. at 30°. This definitely emphasizes the need for reasonably close temperature control. To obtain reliable readings, 100-mm. cells instead of the usual 10-mm. cells were used. The ferric alum solution in 0.01 *N* hydrochloric acid was kept refrigerated and prepared fresh each week. The calibration standards were prepared so that they would be at the same pH and essentially of the same composition as the sample solutions being measured. This was accomplished by adding an aliquot of freshly prepared aspirin solution in alcohol (SD 30) and water at 25° to individual standard increments of salicylic acid, and noting the time between addition of the ferric alum solution and the reading of final solution (this should be within 5 min.). Through a simplified calculation, a correction for the hydrolysis of the aspirin during this short time interval may be applied. Thus, this is the first time where the salicylic standards contained essentially the same amount of aspirin as the samples being assayed. With colorless aspirin solutions or those from green-tinted formulations, the final iron–salicylate color was read at 515 mμ while the pink-tinted formulations were read at 575 mμ. Appropriate standard curves were run at these given wavelengths. Within the range of concentration measured, the precision and accuracy of this method were within 0.005% salicylic acid at the 95% confidence level.

They also developed a rapid visual method using matched Nessler tubes and a series of salicylic acid standards, which were prepared exactly as the sample in regard to pH, alcohol (SD 30) content, and ferric alum solution. These standard solutions were stable for 2 weeks. The aspirin sample (colorless for this test) was dissolved in alcohol (SD 30), the appropriate aliquot diluted with water cooled to 10°, then treated with the ferric alum solution, and compared within 30 sec. to the standard series of salicylic acid. The salicylic acid content was estimated visually to the nearest 10 mcg. of salicylic acid. By conducting this comparison test so rapidly and at 10°, the hydrolysis error appeared to be within experimental error.

In a thesis and later a publication, Leeson (54) and Leeson and Mattocks (55) made a very thorough study of the decomposition of aspirin in the solid state, utilizing a modification of the AOAC 6th edition procedure which improved the accuracy and sensitivity of the measurements. The aspirin and salicylic acid were dissolved in absolute alcohol. The final color of a given aliquot, which was developed in a 50% alcohol medium, was read in a spectrophotometer at 532 mμ (a slit width of 0.02 mm.) along with a series of salicylic standards treated exactly as the samples.

The step in which the sample or standard salicylic acid aliquot is diluted to exactly 50 ml. with absolute alcohol is an extremely important one. The original procedure consisted of adding the given aliquot to 50 ml. of absolute alcohol, but since the size of the aliquot varied, the concentration of alcohol in the final dilution was not constant. To determine the effect of alcohol concentration on the iron–salicylate color, three different concentrations of salicylic acid were made. They were read over a varying range of alcohol from 5 through 85% alcohol at increasing increments of 10% alcohol. It was readily concluded that the final concentration of alcohol had a significant effect on color intensity. With

Table IX—Stability of Various Aspirin–Antacid Mixtures (2:1)

Antacid	Over 1% FSA Stored for Stated Weeks at RT	Over 1% FSA Stored for Stated Weeks at 37.5°	% FSA after 1 Year at RT	% FSA after 1 Year at 37.5°
Dihydroxy aluminum aminoacetate	52+	52+	0.65	0.70
Calcium gluconate	52+	52+	0.80	0.78
Calcium carbonate	28	8	4.4	11.3
Aluminum hydroxide dried gel	12	4	3.9	6.9
Magnesium carbonate	12	2	11.0	42.9
Magnesium oxide	4	2	18.0	24.0
Magnesium hydroxide	2	2	19.5	38.6
Calcium lactate pentahydrate	36	6	71.0	100.0[a]
Magnesium trisilicate	4	2	100.0	100.0[b]
Dibasic sodium phosphate, anhydrous	16	2	100.0[b]	100.0[c]
Sodium bicarbonate	4	2	100.0[b]	100.0[d]

[a] Within 48 weeks. [b] Within 44 weeks. [c] Within 40 weeks. [d] Within 28 weeks.

the lowest salicylic acid concentration, alcohol content of 30 ml. instead of 25 ml. could introduce an error of 6.7% in FSA value. This error increases greatly with higher concentration of alcohol. For this reason, although a worker may select any alcohol volume desired, he must keep it constant throughout the study for both the samples and standards.

Under anhydrous conditions in sealed ampuls, aspirin (100–140-mesh) with and without calcium stearate (a lubricant in aspirin tablets which has been shown to expedite the decomposition of aspirin) were stored at 35, 45, 60, 80, 100, and 110°. Samples were removed at various time intervals over a period of 50 days and assayed for FSA content. Samples of aspirin alone showed little or no decomposition at 80° or below, while those with calcium stearate decomposed within 2 days to the extent of about 1% FSA and then remained near this level during the remainder of the study. At both 100 and 110°, with or without calcium stearate, the aspirin showed about 2% FSA in 5 days and then decreased gradually with time. As these samples both melted and changed color, it was not known whether the formation of a polymolecular salicylide accounted for the decrease in salicylic acid.

From these studies, it was believed that the small amount of decomposition found could have been caused by traces of moisture, which contaminated the dry aspirin during the filling and sealing of the ampuls. The amount of water necessary to account for the decomposition observed was approximately 10^{-5} moles. The conclusion was thus reached that below 80°, the decomposition of aspirin in the absence of moisture was of minor importance.

Consequently, the role of humidity, or more specifically vapor pressure, on the decomposition of aspirin was studied at various temperatures (50, 60, 70, and 80°) with vapor pressures varying from 46 through 232.5 mm. At various time intervals over a period of nearly 1 year, samples were taken from the given humidistats and assayed for FSA content. Decomposition was noted at all stations. The amount depended on the length of time in the given humidistat, temperature, and vapor pressure. The higher the temperature and vapor pressure, the more rapid was the decomposition.

Tablets containing aspirin, starch, and talc (washed and unwashed) were prepared and studied under similar conditions as the aspirin crystals. The effect of washed talc on the stability of the aspirin was not appreciably different from the talc. The complications arose at various humidistats in that the tablets would liquefy, particularly at the 80° stations (but not below 60°), once the salicylic acid content reached a critical level. Once liquefaction occurred, the salicylic acid content decreased sharply and the study with that humidistat was discontinued. Along with previous workers' conclusions, Leeson showed that the compression into tablets did not change the mechanism of decomposition.

The widespread use of aspirin in combination with various antacid compounds as buffering agents led Bandelin and Malesh (56) to study the stability of aspirin with 11 commonly used antacid compounds. Using a modification of the method of Pankratz and Bandelin (48), previously discussed, the FSA content of powder mixtures of two parts aspirin to one part antacid powder, after being stored at room temperature or at 37.5° for periods of time up to 1 year, were assayed at stated time intervals. The antacid powders were used directly from the commercial container so they were not pretreated or dried in any way before using. Table IX summarizes their results.

Both dihydroxy aluminum aminoacetate and calcium gluconate were definitely superior to the other antacids studied in regard to "available" FSA. The word available is used with the FSA reported in that the mixture assayed was extracted directly with acetone. It was not shown, or stated, if aluminum, calcium, or magnesium salicylate was formed during the decomposition of the aspirin, or if the acetone would dissolve these salts. Further, if they did dissolve, would the ferric iron replace the cation in the 50% acetone medium in which the iron–salicylate color was developed?

The unusual was done by Wirth (57) in that he followed the USP XV procedure for FSA *without* any modifications when assaying APC tablets. The FSA values reported were acceptable.

Using the procedure of Ribeiro *et al.* (51), Kral *et al.* (58) studied various mixtures of drugs commonly given with aspirin. Samples of the various mixes were kept at four different stations: room temperature, anhydrous state at room temperature, 97% relative humidity at room temperature, and 37° for periods up to 6 months. The individual mixtures of aspirin with phenacetin, caffeine, phenobarbital, dextrose, sucrose, or lactose were classed as being stable, while those with

Table X—Effect of Amphetamine Salts on the Stability of Aspirin

Amphetamine Salt	pK of Parent Acid	
Picrate	0.38	Increase in accelerating decomposition of aspirin
Acid oxalate	1.19	
Sulfate	1.92[a]	
Acid maleate	2.00	
Acid tartrate	3.02	

[a] Second dissociation constant.

antipyrine, amidopyrine, and quinine hydrochloride decomposed slightly. Mixtures of sodium bicarbonate, hexamethylenetetramine, and caffeine sodium benzoate decomposed rapidly.

In his thesis, Lippmann (59) used the colorimetric procedure developed by Leeson (54), but substituted 95% alcohol for absolute alcohol in his studies of aspirin decomposition with various amphetamine salts. Mixtures of 100 parts of aspirin (50–70-mesh) and 2.935 parts of amphetamine salt as base (60-mesh) were stored in humidity cabinets at 73° and 67% relative humidity. Amphetamine salts used were sulfate, diphenylacetate, *p*-aminobenzoate, 2-naphthoate, phthalate, and picrate. The phthalate salt increased the rate of decomposition of the aspirin the greatest; the order was as follows: phthalate > diphenylacetate = *p*-aminobenzoate = 2-naphthoate > picrate > sulfate.

A relationship between pK of the parent acid in the amphetamine salt and rate of decomposition of aspirin (Table X) correlated well with the acid strength discussed by Yamamoto and Takahashi (50).

Four different tablets of aspirin were manufactured by Nazareth and Huyck (60) to study the effect of calcium salts on the stability of aspirin. The tablets were stored at 9–12, 25–30, and 45° for a period of 8 weeks. Samples were removed weekly and assayed for salicylic acid by essentially the method of Pankratz and Bandelin (48), reading the final color in a 20% alcohol medium. Table XI summarizes their work.

It was noticed that when the percent FSA was over 6, needle-shaped crystals (whiskers) of salicylic acid appeared on the sides and neck of the container. This study showed that aspirin was unstable in the presence of either calcium carbonate or calcium succinate. This was in agreement with Yamamoto and Takahashi (50), who found that the presence of a salt of a weak acid accelerates the decomposition of aspirin.

Continuing the same type of study, Nazareth and Huyck (61) studied the stability of aspirin in four differently manufactured APC tablets. The tablets were stored for 5 weeks and samples removed weekly for the FSA assay.

Only Tablet A in Table XII showed a rapid decomposition of aspirin. It was the only tablet containing magnesium stearate as a lubricant.

As DeMarco and Marcus (62) did not require the sensitiveness described by Leeson (54), they modified the iron reagent to account for larger amounts of salicylic acid and still adhere to Beer's law (Table XIII).

Reagent No. 1 was used by Leeson and was shown to be very sensitive to the alcohol concentration. Reagent No. 4 was recommended, as it was not only insensitive

Table XI—Stability of Various Aspirin Tablets

Tablet	Weeks of Storage before 0.5% FSA Found at 9–12°	25–30°	45°	% FSA after 8 Weeks at 9–12°	25–30°	45°
I. Aspirin	8+	8+	7	0.3	0.4	0.5
II. Aspirin + calcium carbonate	7	2	1	0.5	1.2	14.0
III. Aspirin + calcium succinate	5	1	1	0.6	1.0	88.5
IV. Aspirin + calcium carbonate and succinate	4	1	1	0.6	1.2	96.7

to alcohol concentration changes, but the intensity of the iron–salicylate complex was greatly increased. Reagent No. 7 emphasized the need for acid in the color development medium.

Okano *et al.* (63) conducted a factorial experiment ($2^8 \times 4$) like Ribeiro *et al.* (51) and obtained essentially the same conclusions. Of the lubricants, talc, edible oil, and stearic acid were better than magnesium stearate or calcium stearate at 56°. Room relative humidity caused less decomposition than 84% relative humidity. Storage temperature at 45° caused more aspirin decomposition than room temperature (10–20°). Presence of lactose or diphenylpyraline hydrochloride resulted in an increase in aspirin decomposition. Little effect was noticed on the stability of aspirin, with or without starch, whether it was prism or needle form. The number of times the tablets were compressed at different pressures did not affect the aspirin.

In continuing their studies on the stability of aspirin with other drugs or compounds, Patel and Huyck (64) manufactured aspirin tablets with and without aluminum hydroxide dried gel USP. The tablets were stored for nine weeks and samples removed weekly for FSA assay as previously described.

The results in Table XIV are in agreement with the work reported by Bandelin and Malesh (56).

In two papers, Grabowska (65, 66) reported on the stability of aspirin with other drugs as powders or tablets after a year's storage. No decomposition of aspirin was reported when mixed with caffeine, quinine sulfate, codeine phosphate, phenobarbital, phenacetin, carbromal, urea, or *p*-aminobenzoic acid.

Slight decomposition was reported with quinine hydrochloride or sodium benzoate. The presence of sodium phenobarbital, codeine base, or caffeine sodium benzoate greatly accelerated the decomposition of the aspirin. Four stabilizers, magnesium oxide, aluminum hydroxide, calcium carbonate, and calcium gluconate, were mixed individually with aspirin, sodium phenobarbital, and caffeine mix, and aspirin, caffeine, and so-

Table XII—Stability of Various APC Tablets

Tablet	% FSA after 5 Weeks at 9–12°	25–30°	45°
A	0.6	1.2	16.0
B	0.1	0.2	1.0
C	0.1	0.2	0.6
D	0.1	0.2	1.3

Table XIII—Effect of Iron Reagent and Percent Alcohol on the Iron–Salicylate Color

Iron Reagent	ml. Iron Reagent Added/100 ml. Solution	% Alcohol in Final Solution	Adherence to Beer's Law at 532 mμ; mcg. Salicylic Acid/ml.
1. 2% Ferric ammonium sulfate in 0.125 *N* hydrochloric acid	2	50	10–20
2. Same as No. 1	5	50	10–60
3. Same as No. 1	5	10	10–80
4. 1% Ferric chloride in 0.1 *N* hydrochloric acid	5	50	10–80
5. Same as No. 4	5	10	10–80
6. 0.5% Ferric chloride in 0.1 *N* hydrochloric acid	2	10	10–50
7. 0.5% Ferric chloride, no acid	2	10	None

dium benzoate mix. Magnesium oxide was best with the former mix, while aluminum hydroxide, calcium carbonate, or calcium gluconate was effective in stabilizing the aspirin in the latter formulation.

Control methods used in the Australian pharmaceutical industry for FSA in aspirin formulations were described by Green (67). The method of Strode *et al.* (53) was used in determining the FSA content in aspirin and aspirin–starch formulations. The results were in line with the BP FSA limits. If the formulation contained magnesium hydroxide, it was necessary to release the salicylic acid from its magnesium salt by first adding an aqueous acid and extracting the salicylic acid immediately with 1:1 pentane–ether solution. After evaporation of the mixed solvent the residue was dissolved in alcohol and assayed.

The effect of selected USP talcs on the stability of aspirin in tablets was reported by Gold and Campbell (68), utilizing a direct benzene extraction of the pulverized tablets. After vigorous shaking and centrifuging until clear, an aliquot was shaken with a ferric ammonium sulfate reagent. The clear aqueous layer, after centrifuging, was read in a colorimeter at 515 mμ. FSA content was calculated from a standard series of salicylic acid treated as the sample. Three series of tablets were prepared. The first used the talc, as is; the second used acid-washed talc. The third series used the best of the four talcs, Talc A, plus known amounts of various impurities (aluminum silicate, red iron oxide, calcium silicate, and calcium carbonate which were mixed individually with the talc to prepare the aspirin tablets).

The data indicated that the four USP talcs were different in regard to FSA formation after being stored at 40° and 90% relative humidity for 12 weeks (FSA varied from 0.8 to 25.8%). The decomposition, however, did not appear to be directly related to the pH of the talc. The acid washing of the talc before use did improve the aspirin stability greatly, particularly with Talc C. Of the added impurities to Talc A, the presence of aluminum silicate or red iron oxide did not significantly affect the stability of the aspirin, while both the calcium salts (carbonate being the worst offender) influenced appreciably the rate of decomposition of the aspirin.

Table XIV—Aspirin Dosage Form Stability

Tablet	% FSA after 9 Weeks at 10°	% FSA after 9 Weeks at RT	% FSA after 9 Weeks at 37.5°
Aspirin	0.13	0.13	0.16
Aspirin and aluminum hydroxide dried gel	1.16	2.04	3.30

Though the main interest of Troup and Mitchner (69) was on degradation of phenylephrine hydrochloride in tablet formulations containing aspirin, they did assay for FSA according to the Gold and Campbell (68) procedure. For the degradation of phenylephrine in aspirin-containing tablets to occur, the breakdown of the aspirin was prerequisite. This was accelerated greatly by the presence of magnesium stearate as a lubricant in the manufacturing of the tablets. For any one formulation held at elevated temperature (usually 70°), the increase in salicylic acid content plotted against the decrease in phenylephrine content gave a linear relationship.

The effect of four granulating solvents in the manufacturing of aspirin tablets was reported by Trose and Danz (70). Granulations prepared with 95 and 70% alcohol or spiritus gelatine did not increase the decomposition of the aspirin, while the use of 5% gelatin mucilage did. The FSA content was found by extracting the pulverized sample with alcohol, filtering, diluting with water, adding ferric chloride reagent, and reading the resulting color at 530 mμ in a colorimeter. If phosphates are present in the formulation, a preliminary extraction and evaporation must be made with 1:1 ether–petroleum ether mixture.

Jaminet and Evrard (71) evaluated the effectiveness of precirol (a glyceryl palmitostearate), a binder lubricant, in the manufacturing of aspirin tablets. After 3 months at 50° and 80% relative humidity, no FSA was reported from the tablets using this substance. The FSA was determined in the pulverized tablets by first extracting with 1:1 ether–petroleum ether solution, filtering, evaporating, dissolving the residue in alcohol, diluting with water, and adding a ferric ammonium sulfate reagent. The solutions were read at 520 mμ.

The effects of humidity on aspirin, on its mixtures and tablets, with five different fillers were reported by Wisniewski and Piasecka (72). The FSA was determined by dissolving the aspirin in alcohol, filtering if

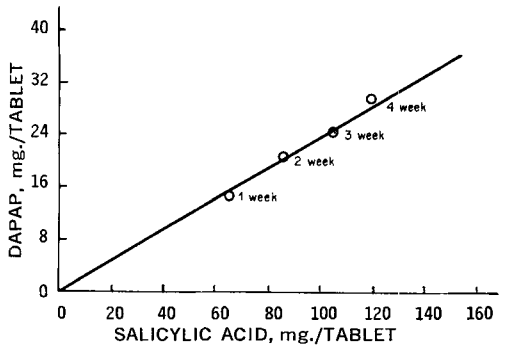


Figure 2—*Relationship between the rate of formation of salicylic acid and of DAPAP.*

required, and adding an aliquot to a Nessler cylinder containing water and the ferric ammonium sulfate reagent. These were compared with the prepared salicylic acid standard solutions. The samples were stored over a period of 4 months at 93, 58, and 20% relative humidity along with changing humidity conditions in the laboratory. The decomposition at 93% relative humidity took place in the following decreasing order of filler used: magnesium oxide > magnesium carbonate > calcium carbonate > magnesium stearate > no filler. This same decomposition order existed at 58 and 20% relative humidity, along with ambient conditions. It is one of the few times that magnesium stearate has not led the list in degree of decomposition of aspirin. During these studies, it was shown that alcohol concentration affected the final color, as did varying amounts of acetic acid. None of the fillers used altered the final color.

In a paper similar to that of degradation of phenylephrine (69), Koshy *et al.* (73) reported on the acetylation of acetaminophen (APAP) in tablet formulations containing aspirin. With tablets stored at 50° for a period of 6 weeks, the formation of diacetyl-*p*-aminophenol (DAPAP) indicated a linear relationship between the rate of formation of salicylic acid through a 4-week period (Fig. 2). The same trend existed in various samples stored at ambient conditions, although at lower salicylic acid and DAPAP levels. Commercial tablets of unknown age, purchased from retail outlets and containing both APAP and aspirin, showed this same relationship of aspirin decomposition and formation of DAPAP. As one of these lots showed high DAPAP, a study was conducted at 50° for 4 weeks on the effect of stearic acid in one and magnesium stearate in the other. The magnesium stearate mix produced nearly 1000 times as much DAPAP as the stearic acid or regular mix.

Continuing their study on the stability of aspirin, Jaminet and Louis (74) made tablets with various lubricants. On assaying these tablets after being stored 6 months at 50° and 80% relative humidity, the lubricants may be listed in order of increased amounts of salicylic acid being found. Magnesium stearate was the poorest lubricant in that nearly 90% of the aspirin was decomposed, the next being polyethylene glycol 6000 (about 15% being decomposed) > glycerol monostearate II > stearic acid > stearyl alcohol > glycerol monostearate I > precirol = geleol. The last two lubricants were the best in protecting the aspirin from decomposing under these conditions.

Table XV—Effect of Common Active and Inactive Aspirin Product Components on Color Development[a]

Component	Component–Salicylic Acid Ratio	Interference
Lactose	40:1	Nil
Meprobamate	100:1	Nil
Methylcellulose	40:1	Nil
Polyvinylpyrrolidone	20:1	Nil
Phenacetin	60:1	Nil
Calcium stearate	10:1	+20%
Magnesium stearate	10:1	+20%
Stearic acid	10:1	+30%
Alginic acid	10:1	Nil
Ion-exchange resin[b]	10:1	Nil
Caffeine	40:1	Nil
Ethoheptazine citrate	20:1	Nil
	100:1	−15%
Citric acid	15:1	−20%
Dihydrocodeine	20:1	Nil
Codeine phosphate	20:1	Nil
Phenergan HCl	3:1	+100%
Hydrogenated vegetable oil[c]	10:1	Nil
Talc	20:1	Nil
Meperidine HCl	40:1	Nil
Starch	70:1	Nil

[a] Reprinted, with permission, from L. F. Cullen *et al.*, *Ann. N.Y. Acad. Sci.*, **153**, Art. 2, Table 1(1968). [b] Amberlite, Rohm & Haas Co. [c] Sterotex.

It is befitting that the last paper using an iron reagent would be titled "An Automated Colorimetric Method for Determination of Free Salicylic Acid in Aspirin-Containing Products," by Cullen *et al.* (75). The number of salicylic acid determinations required in the development of a stable aspirin product, or in production quality control, can be overburdening. The need to automate the salicylic acid analysis was, and is, great, as the saving in analytical time, effort, manpower, and expense can be tremendous.

The method selected for automation was that of Pankratz and Bandelin (48), as they had reported on the optimum conditions needed in order to obtain the greatest accuracy and reproducibility in the quantitative determination of salicylic acid in pharmaceutical preparations. A flow diagram (Fig. 3) is presented at this time only to show the equipment arrangement utilizing a standard Technicon automated system which was programed at 15 samples/hr. Specificity studies are summarized in Table XV.

Turbidity accounted for the high values given by the presence of calcium and magnesium stearate and stearic acid. The quenching effect (negative interference) was brought about by citric acid which forms a nonionized salt with iron. Phenothiazine derivatives react directly with the ferric iron and so must be removed if present in the aspirin formulation. Linearity of the salicylic acid in the microaperture flow-through cells was excellent. The precision of standard salicylic acid showed a repeatability deviation of 1.5%, while replicate samples of a commercial tablet were within 2%. Good accuracy was demonstrated by recovery of known amounts of standard salicylic acid added to powdered commercial tablets and by comparing this to the manual procedure.

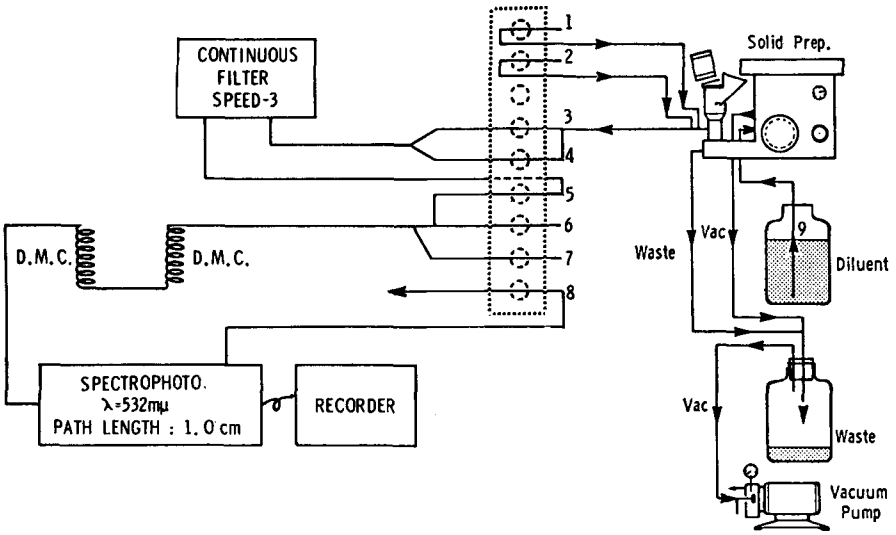


Figure 3—*Flow diagram for the determination of salicylic acid in aspirin-containing products. Key: 1, 0.8 ml./min. air; 2, 4.06 ml./min. SDA No. 30 alcohol; 3, 2.76 ml./min. sample; 4, 2.03 ml./min. sample; 5, 2.76 ml./min.; 6, 2.76 ml./min. $Fe(NO_3)_3$ reagent; 7, 2.00 ml./min. air; 8, 2.50-ml./min. flowcell; and 9, reservoir-SDA No. 30 alcohol. [Reprinted, with permission, from L. F. Cullen* et al., Ann. N. Y. Acad. Sci., *153, Art. 2, Fig. 1 (1968).]*

DETERMINATION OF SALICYLIC ACID IN ASPIRIN AND ASPIRIN PRODUCTS BY UV SPECTROPHOTOMETRY

Until Tinker and McBay (76) introduced their simple and rapid spectrophotometric method of analysis for aspirin and salicylic acid, two separate determinations had to be made for the intact aspirin and hydrolyzed aspirin. In selecting the solvent for the UV studies, they found that in using chloroform A.R. grade, both aspirin and salicylic acid had higher absorbances and stabilities than in either aqueous or alcohol solutions. Also, the greatest difference between maximum and minimum absorbance values for any given concentration was observed in chloroform. The maximum absorbance for aspirin and salicylic acid was found to be 278 and 308 mμ, respectively, as shown in Fig. 4. Beer's law was conformed to in the concentration ranges used. Equations were developed for this two-component mixture, and the application to aspirin, aspirin tablets, or capsules was valid and had an error of less than 0.2%. Samples of aspirin were dissolved in chloroform (filtered if necessary) and read at 308 mμ for salicylic acid content. A portion of the bulk solution was diluted 100 times and then read at 278 mμ for aspirin content. Both readings were against a chloroform blank.

Ebert (77) found that the existing methods for determining amphetamine sulfate, phenacetin, and aspirin in the corresponding tablets involved many laborious time-consuming extractions. After the separations were completed, lengthy titrations or a gravimetric procedure were required. With all this work, very little, if any, reliable information was available in regard to subtle changes such as the presence of salicylic acid which could be formed from the decomposition of the aspirin. Ebert, with meticulous care, designed a three-component spectrophotometric method for the simultaneous determination of aspirin, phenacetin, and salicylic acid. It was not only a more rapid method of analysis, but was found to give increased accuracy and precision. Alcohol was selected as the solvent of choice. In this medium, aspirin was found to have an absorption maximum at 226 mμ, salicylic acid at 235 mμ, and phenacetin at 250 mμ. As there was a great deal of overlapping of the three absorption curves, it was important to prove that the absorbance of this mixture, particularly where the aspirin showed 0, 50, and 100% hydrolysis, represented the sum of the absorbances of the individual compounds comprising the mixture at that wavelength. The validity of this was well proven by Ebert's work. From all these basic data, the appropriate validated equations were derived. The equations were then tried on tablets which had been extracted with ether. The three compounds in alcohol were then determined spectrophotometrically. The estimate of the accuracy and precision compared favorably with those methods used in the past. Though the major emphasis of this work was on the stability of sympathomimetic amine salts when combined with aspirin and phenacetin, interesting facts evolved about the stability of aspirin in these combinations. It might be added that in all the stability studies conducted here, phenacetin did not show any appreciable change.

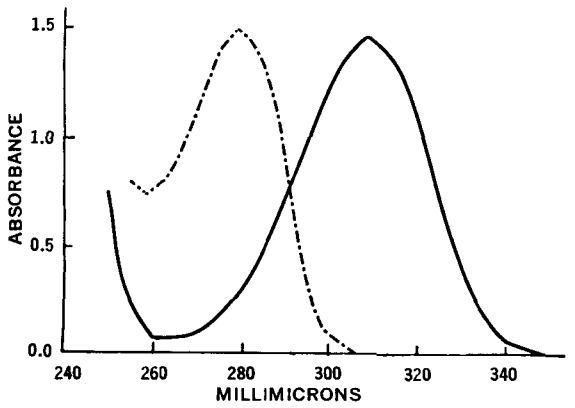


Figure 4—*UV spectrocurves of aspirin (– · –) and of salicylic acid (——) in chloroform.*

Stability studies on tablets containing amphetamine sulfate, aspirin, and phenacetin were conducted in regard to the effects of temperature, moisture, and lubricants on these compounds. All the tablets were packaged in loosely capped amber bottles. These bottles were stored under the following conditions: room temperature, 0% relative humidity; room temperature, 95% relative humidity; 43°, 0% relative humidity, and 43°, 95% relative humidity. After a year's storage and many assays, the important finding was that aspirin decom-

position appeared to be associated with the disappearance of the amphetamine sulfate. The aspirin decomposition appeared to be dependent upon the lubricant present in the following decreasing order: magnesium stearate > talc and magnesium stearate > stearic acid > talc. The increase of temperature accelerated the decomposition of aspirin, but the increase of humidity at the same temperature had a much greater effect. Methamphetamine hydrochloride was substituted for the amphetamine sulfate in a similar stability study, and the conclusions were the same as mentioned for amphetamine sulfate. It is of interest to note that no aspirin decomposition occurred in any of the formulas stored at room temperature, 0% relative humidity, regardless of the ingredients present. In another study, salicylic acid replaced aspirin in the tablet with the result that it had little or no effect on the amphetamine sulfate.

Ebert checked on the possibility that aspirin might be capable of acetylating amphetamine. Working with the pure compounds, he isolated acetylamphetamine which proved this belief. The addition of an equivalent amount of salicylic acid and acetic acid in place of the aspirin in that study did not result in the isolation of any acetylamphetamine. Isolation of any acetylamphetamine from the tablets studied was not successful. Today, with the aid of TLC and GLC, such a decomposition product could readily be detected quantitatively.

Though Leeson's work with aspirin (54) involved chiefly a colorimetric procedure, he used the UV procedure developed by Ebert. This was done to check that no loss of salicylic acid (through volatilization) from the vials on stability had occurred. As the tablets used here did not contain amphetamine sulfate or phenacetin, the interpretation required was simpler. Absolute alcohol was used in place of the 95% alcohol. This would assure better stability of the aspirin during the assay. Readings were taken at 226 mμ (slit width of 0.8 mm.) and at 235 mμ (slit width at 0.6 mm.) against absolute alcohol. On assaying many tablets randomly, the summation (in moles) of the aspirin and salicylic acid found accounted for all the aspirin originally employed. In short, no salicylic acid was being lost through volatilization under the storage conditions.

In the first of a series of many interesting and valuable publications dealing with the determination of the stability of aspirin in many products, Levine (78) introduced a rapid chromatographic assay for APC tablets which included the determination of salicylic acid. Previous papers using this column technique did not discuss the determination of salicylic acid.

The pulverized APC sample was extracted with chloroform containing a small amount of acetic acid (to convert any aspirin salt to the free acid and so be extracted by the chloroform). Without filtering off the insoluble excipient material, an aliquot was diluted with freshly water-washed ether and passed directly to the single duplex column which had just been washed using water-washed ether. The upper segment of the column contained 1 *N* sodium bicarbonate on diatomaceous earth[4] which acted as the supporting phase. The sodium bicarbonate trapped both the aspirin and salicylic acid. The lower segment of the column contained 4 *N* sulfuric acid on diatomaceous earth. The sulfuric acid retained the caffeine while the phenacetin passed through into an evaporating dish. Water-washed ether was passed through the column in small portions to elute quantitatively all the phenacetin. A volumetric flask was placed under the column and sufficient water-washed chloroform was passed through the column to elute quantitatively the caffeine. Immediately, the column was eluted with acetic acid in chloroform (previously water-washed before adding the acetic acid) into another volumetric flask. This last eluate was read immediately in a suitable spectrophotometer at 280 mμ for aspirin and 310 mμ for salicylic acid, as aspirin was not stable in this medium. The caffeine was read at 276 mμ while the phenacetin was evaporating. The residue in the evaporating dish was dissolved in a little chloroform and then diluted with isooctane in a volumetric flask and read at 285 mμ.

This entire procedure, from grinding of the tablets to the final UV readings, took less than 1 hr. Though it was not emphasized, the aspirin should not be left on the column any longer than necessary, as it has been found to hydrolyze readily. From experience it has been found that the phenacetin may be dissolved and diluted with chloroform; thus another solvent (isooctane) is unnecessary.

In calculating, standards of the three drugs and salicylic acid are read in the UV at the stated wavelength in the same medium as the sample. For practical purposes, it was assumed that aspirin's absorbance at 310 mμ was negligible (100 mcg. aspirin/ml. read 0.010). Thus, any reading at 310 mμ was calculated as salicylic acid. From this absorbance value at 310 mμ, the absorbance due to the salicylic acid at 280 mμ can be calculated from the values of the standard salicylic acid read at these two wavelengths and deducted from the total absorbance at 280 mμ; the remainder can be considered to be the absorbance of the intact aspirin.

This partition chromatographic procedure compared excellently with the old and very laborious NF X procedure. The fact of the matter is that Levine's work was so convincing (and deservedly so) that in the next revision of the NF (NF XI) his procedure replaced the old official method except for the FSA limit test. There were also other firsts in this paper; Levine broke tradition with conventional partition chromatography techniques by altering the nature of the immobile phase (neutralizing the sodium bicarbonate phase of the column with acetic acid) as a step in the process. In so doing, the versatility of partition chromatography was thus broadened to permit separations not previously possible. In earlier techniques, the constitution of the immobile phase remains unchanged.

In 1959 and 1960, Smith (79, 80) reported on an AOAC collaborative study of the Levine method for APC. As a result of these studies, the Levine method became official in the Tenth Edition of the Methods of Analysis of the AOAC. These collaborative tests did show a weakness in the procedure, in that the values reported for the salicylic acid content varied appreciably (from 0.15 to 3.17% as hydrolyzed aspirin). As stated before, with care the hydrolysis of the aspirin on the

[4] Celite 545, Johns-Manville Corp., New York, N. Y.

sodium bicarbonate column can be avoided, chiefly by eluting the column as quickly as possible. Smith suggested that the aspirin and salicylic acid content could be calculated by simultaneous equations rather than make the assumptions already discussed in the Levine paper.

Heuermann and Levine (81) and later Heuermann (82) expanded the usefulness of Levine's original work to the analysis of combinations of aspirin, phenacetin, and caffeine with other drugs. The other drugs were pyrilamine maleate, chlorprophenpyridamine maleate, phenindamine tartrate, methapyrilene hydrochloride, doxylamine succinate, thonzylamine hydrochloride, codeine sulfate or phosphate, phenobarbital, or cyclopentylallybarbituric acid. In neither paper were there any salicylic acid values reported, although discussion was made of its determination. An absorbance figure greater than 0.020 at 310 mμ indicated that partial hydrolysis of the aspirin had taken place and must be accounted for in the calculations of the aspirin content of the sample. Simultaneous equations were used in determining the intact aspirin and the FSA content of the sample.

In the application of his partition chromatographic procedure to just aspirin and aspirin tablets, Levine (83) required only the sodium bicarbonate segment of the column. With this paper, he clarified the salicylic acid status in that if 5% or more FSA was found, the aspirin and salicylic acid contents of the given sample were found simultaneously by the described UV procedure. If the FSA was under 5%, another technique was used. (This technique will be described later in this Review Article.)

With the UV procedure, it was noted earlier that aspirin was not stable in chloroform so a little acetic acid was added to acidify the medium. However, in the case of buffered tablets, a stronger acid was needed, not only to stabilize the aspirin but to protect it from the hydrolytic effect of the buffering agents present. This was accomplished by preparing a 0.24 *N* hydrochloric acid solution in methanol and adding a small amount of this solution to the original extraction medium of chloroform. The amount of acid present did not affect the efficiency of the chromatographic column, even if the tablets being assayed were not buffered. An aliquot of the aspirin solution was passed through the column with the aid of more chloroform (no ether was required as with APC tablets). The aspirin was eluted with acetic acid in chloroform and determined at 280 mμ, while the reading at 310 mμ showed the salicylic acid content. If the absorbance at 310 mμ was 0.075 or higher, it represented a concentration of 5% or more FSA. The aspirin reading at 280 mμ was corrected for this FSA absorbance so that the intact aspirin could be reported. If the FSA was below 5%, another method was used in reporting FSA since this spectrophotometric procedure resulted in too low an absorbance reading to be accurate. This procedure was successfully applied to regular aspirin tablets (white), pink aspirin tablets, orange-colored and flavored children's aspirin tablets, enteric-coated tablets, buffered aspirin tablets, and aluminum aspirin tablets.

The stability of aspirin compounded with 10 different kinds of antacids was reported by Kubo *et al.* (84). The samples were stored for a total of 90 days at: 5°, 52% relative humidity; 20°, 75% relative humidity; and 30°, 92% relative humidity. Samples were removed intermittently for assay by a UV spectrophotometric assay. The FSA content was calculated directly from the value at 308 mμ, while the intact aspirin value at 275 mμ had to be corrected for the salicylic acid absorbance value. The antacids studied were aluminum silicate, magnesium carbonate, magnesium trisilicate, calcium gluconate, calcium lactate, sodium phosphate, dried aluminum hydroxide gel, calcium carbonate, magnesium oxide, and sodium bicarbonate. Only the 5° station showed good stability with these antacids. At the 30° station, the last four mentioned antacids were completely incompatible with aspirin, as shown by the high FSA values.

Even though pyrilamine was known to interfere with the salicylic acid reading at 308 mμ, Siegel *et al.* (85) used the Tinker and McBay (76) UV procedure to expedite the analysis of tablets of pyrilamine resin adsorbate with aspirin and ascorbic acid. Tablets were stored at 60° for 1 week and for a total of 12 weeks at 45°. Samples removed at various time intervals showed an unexpected trend because the type of container closure and degree of fill were of prime importance for evaluating these products by accelerated temperature studies. Tablets stored in open bottles, or those with polyethylene snap caps, had greater stability than those with Bakelite screw caps. Filled containers appeared to have greater instability than partially filled ones; bottles that had been opened frequently, compared to those opened only once, appeared more stable. The following explanation was offered by the authors: the passage of air would remove moisture as well as gaseous acidic degradation products which promote instability in these tablets; thus, in tightly closed and well-filled bottles, this would not take place to as large a degree.

Chapman and Harrison (86) determined FSA in soluble aspirin tablets by dissolving the aspirin in glacial acetic acid, filtering, and reading the absorbance at 320 mμ against glacial acetic acid. The salicylic acid content was found from a standard calibration curve of salicylic acid run exactly as the sample. This solvent was selected because aspirin was not stable in chloroform for UV studies, and the results obtained were more reproducible than the BP procedure.

A more thorough study of the Tinker and McBay (76) procedure was reported by Ladomery (87). Chloroform was replaced with absolute spectral alcohol as the solvent for aspirin and salicylic acid. At the wavelength of maximum absorbance for salicylic acid, 300 mμ, Beer's law held through 80 mcg./ml. of absolute spectral alcohol. Similar equations were calculated, only using the absorption data acquired from the alcohol medium. Application of this method was acceptable and accurate.

For APC preparations which contain both barbituric acid derivatives and certain organic bases, it was the objective of Turi (88) to develop a single method rather than using two procedures as described by Heuermann and Levine (81). He was successful with capsules

or tablets containing aspirin, phenacetin, caffeine, itobarbital, and one of four phenothiazine derivatives (chlorpromazine hydrochloride, promethazine hydrochloride, thiethylperazine dimaleate, or thioridazine hydrochloride). No mention, however, was made of determining salicylic acid by this column procedure other than that a prolonged stay (on Column III) would result in a partial *in situ* degradation of the aspirin.

For the determination of aspirin, caffeine, and acetaminophen (APAP), Koshy (89) found that the reversal of the column arrangement described by Heuermann and Levine (81) was all that was required. The three active ingredients could be assayed beside the potential decomposition products, salicylic acid and *p*-aminophenol. After the two columns were prepared in the usual manner and placed in tandem, such that the top column contained the sulfuric acid as the immobile phase and the bottom column contained the sodium bicarbonate as the immobile phase, they were both washed with ether. The powdered sample was dissolved in ethyl acetate, an aliquot of which was then passed through the two columns and collected in a volumetric flask. The columns were further eluted with ether. This fraction contained intact APAP. The columns were then eluted with chloroform. This fraction contained the caffeine. The two columns were then separated, and the bottom column (sodium bicarbonate phase) eluted immediately with acetic acid in chloroform. This fraction contained the aspirin and salicylic acid and was assayed in the usual manner. The top column (sulfuric acid phase), containing *p*-aminophenol, was washed with ether to remove the chloroform. The diatomaceous earth support was then extruded from the column with air under pressure and collected in a beaker. The ether was evaporated from this material, and 0.1 *N* hydrochloric acid was added to dissolve the *p*-aminophenol. This extract was filtered and an aliquot was assayed colorimetrically using 1-naphthol as described by Greenberg and Lester (90).

The effect of water vapor pressure on moisture sorption and the stability of aspirin and ascorbic acid in tablet matrixes reported by Lee *et al.* (91) utilized the Tinker and McBay (76) procedure in evaluating the stability of the aspirin in these studies. Conclusions reached from these studies showed that the moisture adsorptive capacity of each compressed tablet formulation affected the stability of the two drugs to a great extent and were directly related to the moisture sorption and tablet hardness. (The harder the tablet, the less moisture it sorbed and the more stable the drug.) Of the six diluent systems studied, calcium sulfate and cellulose produced the most stable tablets of aspirin and ascorbic acid, while amylose produced the least stable. Under stress-storage conditions, screw-cap glass bottles proved to be a better moistureproof container than snap-top plastic vials. Cellophane and aluminum foil strip packaging materials were about equally effective. Both were more effective than the glass or plastic containers.

Reed and David (92) described a simple direct spectrophotometric determination of salicylic acid in either one complete capsule or one intact tablet of an aspirin containing medicinal, provided no interference by the other components with the salicylic acid absorption at 300 mμ was encountered. The entire dose unit is shaken with alcohol for 1 hr. along with a similar freshly prepared unit dose as a "blank" (as this was generally not available, an equivalent amount of fresh aspirin was weighed and used as the blank). This exposure to an alcoholic medium for 1 hr. could lead to generated hydrolysis in the case of aspirin tablets containing buffers as there is no mention of pH control here or in the aqueous dilution being read at 300 mμ. The sample was read against the blank so that the effect of the intact aspirin would be cancelled out, provided the difference between the two concentrations was not great.

Application of the Tinker and McBay (76) procedure was applied by Day *et al.* (93) in following the stability of two mixtures official in the BPC. They reported the accuracy of the method to be ±2.5%.

In a general paper on the use of UV for analysis of drugs in pharmaceuticals, Sattler (94) applied the method described by Ladomery (87) which was a modification of the Tinker and McBay procedure.

A direct spectrophotometric determination of five compounds, aspirin, salicylamide, caffeine, phenacetin, and salicylic acid, in tablets or powders without any preliminary separation was reported by Clayton and Thiers (95). The powdered sample was extracted with chloroform. From this extract, three aliquots (same volume size) were added to separate volumetric flasks, one to be an acidic medium, another to be a basic medium, and the third a hydrolyzed medium. A mixed solvent consisting of isopropanol, water, and a small amount of hydrochloric acid was added to each flask followed by a solution of 50% sodium hydroxide to the basic and hydrolyzed labeled flasks. After hydrolysis of the aspirin was complete at room temperature (about 15 min.), concentrated hydrochloric acid was added to the hydrolyzed labeled flask, rendering it acid again. On diluting all flasks to volume with the mixed solvent, the solutions were then read at the following wavelengths along with the appropriate reference blank solution. The acidic labeled solution was read at 250, 273, and 310 mμ, the basic labeled solution at 333 mμ, and the hydrolyzed labeled solution at 301 mμ. Using a variety of equations, the content of the individual components was calculated. The comment was made that if salicylic acid were absent, it was possible to omit one step, but the omission is not recommended, since this step provides a measure of any hydrolysis of aspirin which might have occurred during storage or manufacture of the product analyzed.

Use of the isosbestic point as a base line in differential spectrophotometry was applied to aspirin and salicylic acid by Shane and Routh (96). When a series of concentrations of salicylic acid were used to prepare differential absorption spectra, monosodium salicylate (at pH 9) in the reference cell *versus* disodium salicylate (at pH 13.5) in the sample cell, two maxima at 246 and 319 mμ, two minima at 233 and 203 mμ, and two isosbestic points at 268 and 300 mμ were observed. If differential absorption spectra of aspirin solutions were prepared by the same procedure (monosodium acetylsalicylate in the reference cell *versus* disodium salicylate equivalent to the monosodium acetylsalicylate in the sample cell), a

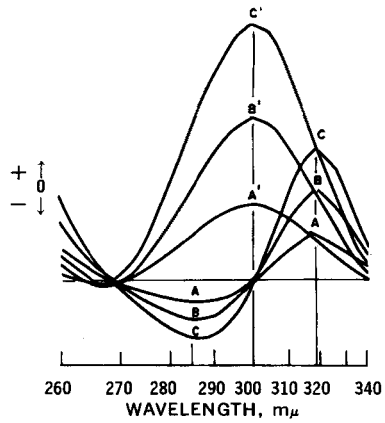


Figure 5—*Differential spectra of salicylic acid (A, B, C) and aspirin (A', B', C'). [Reprinted, with permission, from N. A. Shane and J. I. Routh,* Anal. Chem., **39**, *414, (1967).]*

maximum at 300 mμ, a minimum at 268 mμ, and an isosbestic point at 272 mμ were observed. Figure 5 illustrates the differential absorption spectra of three concentrations of salicylic acid (A, B, and C) compared to the spectra of three concentrations of aspirin (A', B', and C') in the region from 260 to 340 mμ. Each curve is constructed from the two spectra of the same concentration of either salicylic acid or aspirin obtained at pH 9 and 13.5 by subtracting one spectrum from the other spectrum, resulting in the differential absorption spectrum of the given compound. The isosbestic point in differential spectrophotometry would be the wavelength at which each difference curve crosses the zero line. The correlation of the isosbestic point at 300 mμ for salicylic acid, and the maximum at the same wavelength for aspirin, permits the use of the isosbestic point as the zero or base line for the quantitative determination of intact aspirin in the presence of salicylic acid. Thus, one would not need to do a FSA determination as the value reported for aspirin is for the intact aspirin.

A very much needed publication on the significance of salicylic acid sublimation in stability testing of aspirin-containing solids was presented by Gore *et al.* (29). A more than negligible loss of salicylic acid formed from the decomposition of aspirin would preclude the common practice of analytically determining changes in salicylic acid content in solid dosage forms of aspirin as a measure of degrading aspirin. The salicylic acid method could obviously underestimate the extent of decomposition of aspirin and, therefore, provide false confidence in the stability of the tested products. It thus became necessary to develop a method of gauging aspirin stability in solids which would be unaffected by any loss of salicylic acid.

A simultaneous spectrophotometric assay, based chiefly on the work of Edwards (8), was developed for aspirin and salicylic acid in a Clark and Lubs buffer, pH 7.4. It was found that this aqueous medium resulted in improved precision over the chloroform medium used by earlier workers. This slightly alkaline medium afforded a relatively rapid solution of solid aspirin and salicylic acid, and a medium in which slight variation in the pH would not introduce an error into the determination as a consequence of the differential absorption of ionized and unionized aspirin or salicylic acid. The rate of hydrolysis of aspirin has been reported (8) to be independent of pH in the range of 4 through 8. Hydrolysis rate constants were determined at pH 7.4 and 25.5°, and showed a delay of 13 min. between the sample preparation and reading on the spectrophotometer would cause an error of approximately 1%. The actual error was reduced to below 0.1% by maintaining the solutions below 15°, usually at 0°, and reading them within 5–10 min. of their preparation.

Table XVI—Constants for Aspirin and Salicylic Acid UV Assay

Compound	Concentration Range, mcg./ml. of pH 7.4 Buffer	Absorptivity Value at 262 mμ	Absorptivity Value at 296.5 mμ
Aspirin	0–160	3.2	0
Salicylic acid	0–10	3.3	—
Salicylic acid	0–30	—	26.0

Table XVI presents the pertinent data required in the construction of the calibration curves used here.

Before applying this UV procedure, several sublimation studies were conducted in which the need for such an assay was demonstrated quite convincingly. Using an electrobalance, the weights of salicylic acid were monitored continually at the stated temperatures for 12 hr. From this study the following rates of sublimation were calculated (Table XVII).

Table XVII—Sublimation Rates of Salicylic Acid at Various Temperatures

Temperature	Rate of Sublimation, mg./hr.
40 ± 0.05°	0.026
50 ± 0.20°	0.062
70 ± 0.20°	0.372

An Arrhenius-type plot of the apparent zero-order sublimation rates was shown. The slope of the curve was predominately determined by the enthalpy of sublimation of salicylic acid. The observed rates of sublimation may be expected to depend directly upon the area through which the mass transfer occurred. These results, therefore, were not intended to be quantitatively indicative of sublimation loss of salicylic acid during the stability testing, but merely substantiate that such a loss can occur even at moderately elevated temperatures.

A similar sublimation study of purified aspirin revealed no significant loss of weight up to 70°. It was concluded that aspirin does not appreciably sublime under the conditions of the experiment.

With a 9:1 mixture of aspirin–salicylic acid, the results from 12 hr. at 70° indicated that only salicylic acid was lost. This was verified by using the UV procedure discussed.

Aspirin tablets were stored at 50° and 81.2% relative humidity for a period of 98 days. At various time intervals tablets were removed and assayed by first grinding to a fine powder and dissolving in pH 7.4 buffer maintained at 0°. After filtration, an aliquot of the filtrate was further diluted with the cold pH 7.4 buffer and the absorbance measured at 262 and 296.5 mμ. In this application, an error of 0.003 absorbance unit at 262 mμ could contribute an error of approximately 1% in the

Table XVIII—Comparison of Aspirin Tablet Stability Testing Results at 50° and 81.2% Relative Humidity, Based on Determination of Aspirin and Salicylic Acid Content of the Tablets[a]

Time, Days	Aspirin Content Based on Analysis of Aspirin, %	Aspirin Content Based on Analysis of Salicylic Acid, %	Error Due to Sublimation of Salicylic Acid, %
0	100	100	0
15	99.1 ± 0.038	99.1 ± 0.028	0
30	98.4 ± 0.042	98.8 ± 0.215	0.4
45	97.2 ± 0.123	98.7 ± 0.075	1.5
60	97.1 ± 0.178	98.6 ± 0.023	1.5
98	95.0 ± 0.288	97.9 ± 0.311	2.9

[a] Each value is the average of four determinations recorded with ±1 standard deviation.

determination of the aspirin content of a solid consisting of 80% aspirin using a 50-mg. aliquot for analysis. The results of this study are presented in Table XVIII.

The last column does show that salicylic acid is lost from these tablets by sublimation under the stated conditions of the experiment.

By employing the UV procedure presented in this publication, the determination of the residual aspirin, rather than the apparent salicylic acid, in a solid can be used as a valid means of gauging the stability of the formulation. This method of analysis showed an accuracy within at least 1.5%.

DETERMINATION OF SALICYLIC ACID IN ASPIRIN AND ASPIRIN PRODUCTS WITH THE AID OF A FERRIC-ION CHROMATOGRAPHIC COLUMN

Analytically, the need has always been great to separate and isolate the salicylic acid from aspirin as well as other components in the pharmaceutical preparation. The potential susceptibility of aspirin to hydrolysis is constantly prevalent, so the more rapidly the intact aspirin is removed from the medium, once a given procedure is begun, the truer the reported FSA values will be.

Again Levine (83) appeared to be first in breaking away from the traditional methods of determining salicylic acid in aspirin formulations. On using the chromatographic columns he had introduced in 1957 with a diatomaceous earth–2% ferric chloride mix, the passage of a chloroform solution of aspirin and salicylic acid resulted in the trapping of the salicylic acid (shown by a purple zone on the column). The aspirin passed through, and by using several washes with chloroform, the aspirin was completely removed from the column. The bound salicylic acid was then eluted quantitatively with chloroform containing acetic acid. This eluate was read at 310 mμ for FSA. For the procedure to be valid, the purple zone should not reach the bottom of the tube during the washing out of the aspirin. If it does, the procedure must be repeated with another prepared column. As the original procedure was written, this was a major downfall in using this technique, because too many times the purple zone moved partially off the column during the aspirin removal step.

This procedure was applied to pink aspirin tablets, children's flavored and colored aspirin tablets, buffered aspirin tablets, and aluminum aspirin tablets. With the colored tablets, the dyes remained at the top of the column throughout the entire assay. With the buffered tablets, the chloroform-insoluble salts of aspirin or salicylic acid must be transformed to soluble acids in order that the FSA could be eluted in the proper fraction. Acetic acid could not be used since it would dissociate the ferric ion–salicylic acid complex on the column. Boric acid, however, was used for this transformation without affecting the column performance. A solution of boric acid in methanol was added to the sample, followed by chloroform to dissolve the freed aspirin and salicylic acid.

For enteric-coated tablets it was found best to mount in tandem a column containing just diatomaceous earth above the regular ferric ion–diatomaceous earth column. The plain diatomaceous earth column removed the surface-active agents present in these tablets, so the aqueous phase would not be stripped off the regular ferric ion–diatomaceous earth column during both the prewashing step and elution of the salicylic acid. This double column setup could be used also where dyes are present in the original chloroform extract, as well as for large amounts of excipient material in the sample being added to the column.

As will be seen with later papers dealing with this novel approach of Levine's, a delicate balance was being maintained with the ferric chloride content on the diatomaceous earth. A sufficiently low concentration of ferric chloride must be maintained to trap the salicylic acid, but at the same time a sufficiently large quantity of ferric chloride must be present to provide an excess over the amount removed during the washing step. It is thus recommended that the modifications of this procedure, to be discussed in this review, be used rather than the method described in this publication. Just the introduction of this approach to the literature served a very worthwhile purpose as the official compendia now use a procedure based on this original study.

Green (67) applied this described procedure by Levine and as he did not present any comments in his paper, it could be assumed that he did not experience any major difficulty with the columns.

In 1961 Weber and Levine (97) made note that "several investigators have encountered difficulty with the published method" (83). During the elution of the aspirin, the salicylic acid migrated slowly down the column (as evidenced by the position of the purple complex) and spread out into a diffuse band, which sometimes becomes difficult to discern.

In this publication, this was rectified by modifying the ferric chloride reagent. This radical improve-

ment in the chromatographic separation was achieved by having a high concentration of urea in the ferric chloride solution. The resultant effects were tremendous: the band of the ferric–salicylate complex obtained with this new reagent was more deeply colored than that obtained with just the simple ferric chloride reagent. The dense, sharply delineated band migrated only slightly during the elution of the aspirin.

It, therefore, became feasible to use a shorter column which did not require extraordinary care in packing. This column even accommodated larger samples of aspirin.

Optimum conditions were obtained with an immobile phase containing 5% ferric chloride solution which was 10 *M* with respect to urea. The pH must be maintained between 3.1 and 3.3. At lower pH levels the salicylic acid band became diffuse and more loosely retained, while at higher pH levels, recovery of salicylic acid from the column may be incomplete using the specified volume of eluant.

For aspirin or aspirin tablets, the pulverized sample was dissolved with chloroform and passed through the column with the aid of several more portions of chloroform to wash the intact aspirin through the column. A volumetric flask containing some hydrochloric acid in methanol was placed under the column, and the column first eluted with acetic acid in ether followed by chloroform. Concomitantly the absorbance of this solution and of the standard salicylic acid in the same medium was determined at 306 mμ.

For APC tablets and flavored tablets, a column containing a small pad of cotton was placed in tandem over the regular ferric chloride–urea column. The sample in the chloroform was passed first through the cotton column to remove the dyes and any insoluble excipients. Another portion of chloroform was passed through the two columns, and then the top column was discarded. The regular column was then washed with chloroform and the salicylic acid eluted.

As moderate amount of urea was eluted together with the salicylic acid from the column, the hydrochloric acid in the receiving flask was added to maintain acidity of the eluate. The methanol was present to achieve miscibility of the acid with the eluting solvent.

With buffered aspirin tablets, the boric acid was replaced with oxalic acid in methanol, as the oxalic acid was more effective in releasing the bound salicylic acid from the antacid. The oxalic acid solution recovered quantitatively salicylic acid from its calcium salt. However, neither this nor any other reagent thus far tested quantitatively released salicylic acid from aluminum hydroxide gel without causing extensive hydrolysis of aspirin.

Excellent reproducibility on replicate analyses of a wide variety of commercial samples was shown.

Though Weber's publication (98) on the analysis of salicylic acid and benzoic acid does not deal with aspirin, *per se*, it had a valuable innovation regarding the ferric chloride–urea–diatomaceous earth column. When eluting salicylic acid of larger quantities than usual, the occasional leakage of ferric chloride was noted which invalidated the assay. This was seen visually by the presence of a yellow color rather than the usual colorless clear eluate. If any yellow is present in the solution read, the reading at 306 mμ would have to be voided. Weber found that a small layer of diatomaceous earth with 30% phosphoric acid as the immobile phase placed at the bottom of the column on top of the glass wool support, and then followed by the regular ferric chloride–urea–diatomaceous earth mix, resulted in no ferric chloride leakage. The phosphoric acid retained any ferric chloride which might be removed during the elution step of the salicylic acid.

In their study on the formation of acetylcodeine from aspirin and codeine, Jacobs *et al.* (99) used the original Levine procedure (83) in isolating the salicylic acid which resulted from the previously mentioned reaction.

On discussing pharmaceutical heterogeneous systems, Zoglio *et al.* (28) have presented four papers regarding the hydrolysis of aspirin. The salicylic acid resulting from the degradation of aspirin in the various formulations was determined by a modification of Levine's iron–diatomaceous earth procedure (83). The first paper [Kornblum and Zoglio (28)] is discussed in the *Hydrolysis Studies* portion of this review. They demonstrated that calcium or magnesium stearate accelerated the production of salicylic acid from aspirin through the solubilization of aspirin as a calcium or magnesium salt. More aspirin would thus be in solution which in turn would hydrolyze in the existing pH which was conducive to hydrolysis. This effect was not as pronounced when aluminum stearate was used as a lubricant, as the aluminum salt of aspirin has a low solubility in water.

On pursuing this alkali stearate effect, Zoglio *et al.* (28) prepared capsules containing aspirin (20 parts), magnesium stearate (1 part), and 0, 1, 2, 5, 10, and 20 parts of hexamic, maleic, malic, or tartaric acids, or maleic anhydride. These capsules were stored at 22, 40, and 50° for 30 days. A minimum of 20% by weight of hexamic, maleic, or malic acid was required to retard the hydrolysis of aspirin in these capsules. Tartaric acid or maleic anhydride was not effective at the higher temperatures. Besides the lower desired pH contributed by the added acid, the mechanism of inhibiting the degradation of aspirin involved the additive acid and aspirin competing for the magnesium ion.

Maulding *et al.* (28) showed that stearic acid USP, which is nearly a 1:1 mixture of stearic and palmitic acids, promoted the decomposition of aspirin more than either reagent grade stearic or palmitic acid. On preparing various synthetic mixtures of reagent stearic and palmitic acids, the one simulating the amounts of the two acids in stearic acid USP behaved similarly as the stearic acid USP in accelerating the decomposition of aspirin. This ratio of stearic and palmitic acids was also the melting point minimum of the various mixes of these two acids. The possibility exists, therefore, of a liquid or semiliquid being present in formulations containing stearic acid USP which might serve as the medium for aspirin hydrolysis.

The fourth paper dealt with the acceleration of aspirin hydrolysis by various common additives (hexamic acid, aluminum hydroxide calcium stearate, magnesium stearate, or magnesium trisilicate), at a 5 or 10% level of the powder mix or tablet. The formulations con-

taining magnesium trisilicate resulted in the largest amount of FSA (about 13%) after 45 days at 40°. Hexamic acid, under these storage conditions, retarded aspirin hydrolysis. The salicylic acid values resulting from these various formulations were in good agreement with values obtained from extrapolating apparent zero-order rates of aspirin suspensions of the same powder mix as used in the tablet. These studies show that stability prediction for solid dosage forms from apparent zero-order rates of suspensions is feasible and informative.

The determination of the FSA in buffered aspirin tablets by Levine and Weber (100) explored further the usefulness of their ferric chloride–urea–diatomaceous earth column. It was essential that the entire amount of aspirin and salicylic acid be dissolved in the mobile phase before the chromatographic treatment, and that the aspirin was not hydrolyzed during the preparation of that solution. These two requirements are not readily achieved in the case of buffered aspirin tablets. Chloroform solutions of aspirin, in the presence of basic materials such as those which comprise the buffering components of the tablets, undergo hydrolysis together with aspirin anhydride formation. Boric acid stabilizes the solution, at least with respect to the hydrolysis of aspirin, but does not achieve the necessary release of the aspirin and salicylic acid from the buffer components to permit their complete solution in chloroform.

In designing a valid assay procedure, the acid which is used must: (*a*) produce only minimal hydrolysis of aspirin under the conditions of the assay; (*b*) rapidly and completely release aspirin and salicylic acid from the buffer components of the tablet; (*e*) be soluble in chloroform; and (*d*) be readily removed from the chloroform, so that the ferric–salicylic acid complex will not be dissociated during the following step of the analysis.

These requirements were fulfilled by 98–100% formic acid. The distribution of formic acid between chloroform and inorganic acids was greatly in favor of the aqueous phase; therefore, the formic acid was removed from the chloroform solution by passage over dilute hydrochloric acid.

In the analysis of buffered aspirin tablets, two columns are prepared and placed in tandem. The top column consists of diatomaceous earth with 0.05 *N* hydrochloric acid as the immobile phase. It is here that the formic acid in the chloroform is removed. The packing of this column should be such that a flow rate of chloroform of 12 ml./min. is obtained.

The bottom column consists of a layer of diatomaceous earth with 30% phosphoric acid as the immobile phase. The phosphoric acid retains any ferric iron which may be eluted during the procedure. The upper stage of this column consists of the 5% ferric chloride and 10 *M* urea as the immobile phase on diatomaceous earth.

In the procedure, water-saturated solvents were used throughout. To a ground sample of tablets in a volumetric flask, the 98% formic acid was added with swirling to wet the sample completely (not more than 30–45 sec.) followed by chloroform. This mixture was shaken for 10 min. The extent of hydrolysis of aspirin during the period of contact of the sample with the formic acid before dilution lies in the range of 0.01–0.02%/min., so here the extent of hydrolysis will be in the order of 0.01%. After dilution with the chloroform, the hydrolysis of aspirin sharply decreased to an average of only 0.03%/hr. Thus a negligible amount of hydrolysis occurs during the 10-min. shaking period for dissolving the aspirin and salicylic acid. On diluting to volume with chloroform and mixing, the solution was filtered through a loose plug of glass wool. An aliquot of this filtrate was then passed through the double columns which were washed with more chloroform to remove the aspirin. The eluate and the top column were discarded. A receiver containing hydrochloric acid in methanol was placed under the remaining (bottom) column, and ether containing acetic acid was passed through the column followed by chloroform containing acetic acid. This eluate was read at 306 mμ along with a salicylic acid standard in the same medium.

By this method, great strides have been made in regard to freeing salicylic acid from various antacids. It was shown that the formic acid treatment readily recovers salicylic acid from its calcium or magnesium salts but that aluminum salicylate was quite refractory to this treatment. As dried aluminum hydroxide gel is present in several of the commercial buffered aspirin tablets, the need is still acute for a method which will recover salicylic acid from its aluminum salt.

On studying the USP XVII limit test for salicylic acid in aspirin tablets containing buffers, Guttman (101) obtained spurious and nonreproducible results. Low recoveries were explained by an adsorption phenomenon. Significant adsorption of salicylic acid occurred when solutions of chloroform were in contact with chloroform-insoluble agents which are commonly employed as buffers in aspirin tablets. The affinity of salicylic acid for magnesium carbonate and aluminum glycinate was high, but the capacity of these solids for the acid was rather low. These two compounds held tenaciously to the salicylic acid, as it was impossible to elute completely adsorbed material by repeated contacts with fresh solvent.

High recoveries of salicylic acid resulted from a surprisingly rapid transformation of aspirin to a product having the chromatographic characteristics of salicylic acid. This was observed only when solid basic material (here magnesium carbonate) was suspended in the chloroform solution of aspirin. This transformation of aspirin was thus surface catalyzed.

On repeating this experiment with different commercially available buffered aspirin products, the same phenomenon occurred except with one—and it contained citric acid monohydrate besides two antacid compounds. With this sample, the salicylic acid content did not increase appreciably with time of contact with the solid suspension. With all these suspension studies, it was of interest to note that, upon filtration, immediate cessation of salicylic acid production resulted. This was shown by the readings in the UV at 278 and 306 mμ on the chloroform filtrate or when the ferric chloride–urea–diatomaceous earth column procedure (USP XVII) was used.

Table XIX—Results Obtained when Various Methods Were Employed to Estimate Salicylic Acid (SA) Contents of a Number of Commercial Buffered Aspirin Products

Product	Buffers Present	% SA Found Using: USP Procedure	Citric Acid Procedure for FSA	Weber and Levine (97) Procedure	Citric Acid Procedure for Total Nonaspirin Salicylates
1	Aluminum hydroxide Magnesium hydroxide	2.19 2.80 3.03	4.02 4.02 3.89	6.59 6.53	6.64 6.58 6.62
2	Aluminum glycinate Magnesium carbonate	Not detectable	0.211 0.236 0.219	0.64 0.69 0.62 0.67	0.728 0.718 0.739
3	Aluminum hydroxide Glycine Magnesium carbonate	0.455	1.01	3.79 3.91 3.52	3.78 3.58 3.58
4	Calcium phosphate Sodium bicarbonate Citric acid	—	5.22	—	5.92
5	Aluminum hydroxide Glycine Magnesium carbonate	—	0.682	—	2.81
6	Aluminum hydroxide Magnesium hydroxide	—	1.82	—	3.39
7	Calcium carbonate Magnesium carbonate	—	1.96	—	3.37

On further studies with aspirin in chloroform and magnesium carbonate in suspension with and without an equal weight of citric acid monohydrate, it was found that no salicylic acid was produced in 200 min. at 25° with citric acid present. After 60 min. of magnesium carbonate being in contact with the aspirin solution, the addition of citric acid monohydrate resulted in no further salicylic acid production. It was visually observed that the addition of citric acid had a pronounced effect on the nature of the suspension. Marked flocculation of particles was apparent immediately after the addition of citric acid monohydrate. Citric acid which had been previously oven-dried showed a much less marked effect in inhibiting salicylic acid production. This showed that presence of water from the hydrate was essential in this reaction with suspended material. Applying this citric acid monohydrate to the previously studied commercial buffered tablets on an equal weight basis showed that in all systems studied here, the citric acid was effective in markedly reducing the catalytic ability of suspended solids in the production of salicylic acid from the aspirin in chloroform.

Repeating the magnesium carbonate–aspirin adsorption experiment discussed earlier, only this time with and without an equal amount of citric acid monohydrate, it was found that in the presence of citric acid there was no appreciable adsorption of the aspirin from the chloroform. It is also of interest that treatment of antacid compounds in suspension in chloroform with citric acid monohydrate significantly reduced their capacities for adsorbing salicylic acid.

Guttman's study demonstrated very convincingly that finely divided solids of antacids do catalyze a conversion of aspirin to a product which, by the analytical method employed, was determined as salicylic acid. The reaction occurred under essentially anhydrous conditions, and adsorption of the aspirin was apparently a prerequisite for the transformation. The presence of citric acid monohydrate was shown to be effective in inhibiting the formation and adsorption of salicylic acid in the systems reported here. A feasible explanation was given: citric acid monohydrate, which is essentially chloroform insoluble, inhibits the reaction by releasing water of hydration to the surface of the adsorbent. A neutralization reaction takes place in the hydrate layer which modifies the surface characteristics of the adsorbent so as to destroy the adsorption sites. Thus, the catalytic production of salicylic acid from aspirin is never initiated.

Results from studies presented in this paper showed that addition of citric acid monohydrate to the official chromatographic procedure in the original extraction of the sample resulted in almost quantitative recovery of salicylic acid. It was also noted that this citric acid treatment was not effective in displacing salicylic acid from metallic salts which are known to form in buffered aspirin products.

In applying the procedure suggested in the preceding paper, Guttman and Salomon (102) compared its usefulness (and superiority) over the existing USP XVII FSA test for buffered tablets of aspirin. The method consisted of treating, by trituration, a powdered sample with an equal weight of citric acid monohydrate, and dissolving the aspirin and the FSA from the powder mass with chloroform. The remaining residue was treated with an aqueous solution of a strong acid (hydrochloric acid), and this solution was extracted with chloroform. The two chloroform extracts (one containing the FSA and the other containing the nonaspirin salicylates) were combined, and the salicylic acid content determined by the chromatographic method of Weber and Levine (97). With the assumption that the citric acid treatment results only in desorption of salicylic acid and aspirin and does not cause conversion of salicylate salts to free acid, one would have a method available for the estimation of the FSA content

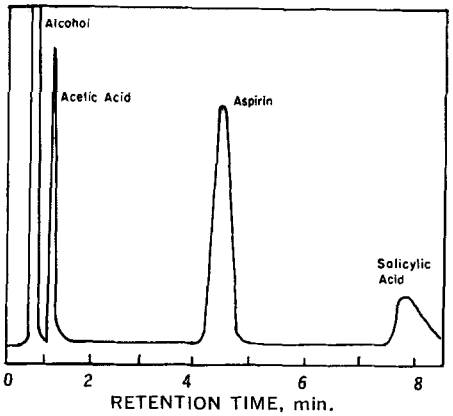


Figure 6—*Gas chromatogram of aspirin in alcohol. [Reprinted, with permission, from J. G. Nikelly,* Anal. Chem., **36**, *2248 (1964).]*

as well as total nonaspirin salicylate contents of buffered tablets of aspirin. Table XIX summarizes the results on applying four different procedures to commercially available buffered aspirin tablets: USP XVII (using boric acid in the initial extraction), Guttman's citric acid procedure, Guttman and Salomon's citric acid procedure for total nonaspirin salicylates, and the Levine and Weber procedure (100) which used formic acid in the initial extraction medium.

Inspection of this table makes obvious that the FSA values, as determined by the USP procedure, were consistently and significantly lower than those determined by the other methods. It is logical to assume that these low values resulted from adsorption of significant amounts of salicylic acid during sample extraction, and to the insensitivity of the procedure to salicylic acid which is present in the sample in the form of salts. The latter two methods appear to be equally precise. It is interesting to note the difference between the citric acid procedure for FSA and the citric acid procedure for total nonaspirin salicylates. The differences reflect the fact that significant amounts of salicylic acid can be present in buffered tablets as chloroform-insoluble salts. It is also apparent that no relationship exists between the FSA content and total nonaspirin salicylate content of these tablets.

DETERMINATION OF SALICYLIC ACID IN ASPIRIN AND ASPIRIN PRODUCTS BY MISCELLANEOUS METHODS

Schulek and Burger (103) stated that on using BrCl as a brominating agent, salicylic acid could be brominated quantitatively in 2 min., even in the presence of aspirin. With the usual amounts of FSA present in aspirin products, it is debatable whether such a titration would be sensitive enough to be of value.

The application of differential thermal analysis (DTA) and thermogravimetric analysis (TGA) to aspirin was published by Wendlandt and Hoiberg (104). No mention was made of the effects of the presence of salicylic acid, but application of this approach would definitely be of interest in evaluating the purity of aspirin.

Gas chromatography has a great potential in the analysis of aspirin products, but only two papers have mentioned the possibilities. Crippen and Freimuth (105) stated that their proposed procedure would differentiate aspirin from salicylic acid only if the temperature of 100–125° and a 1.23–1.83-m. (4–6-ft.) column were used. Nikelly (106) showed (Fig. 6) that under the stated conditions used in the determination of aspirin, the amount of acetic and salicylic acids could be estimated.

In applying an IR spectrophotometric assay of aspirin anhydride, Garrett and Johnson (107) concluded that the insignificance of salicylic acid in these lots of varied purity showed that little definition was lost on ignoring it. Though samples of aspirin were not used here, the usefulness of IR studies on the purity of aspirin in regard to salicylic acid would be of value.

The need for actual physical separation of salicylic acid from aspirin is great. The work by Vietti-Michelina (108) and Wagner (109) on paper chromatographic or paper electrophoretic separation of aspirin and salicylic acid might be applied successfully by using the latest techniques available in this field.

A too often neglected technique in drug studies in the United States is the application of polarography. In 1949 Korshunov *et al.* (110) reported on the reduction of weak acids at a dropping-mercury cathode. The half-wave potentials were given as 1.52 to 1.65 (for 1–7 mmoles/l.) for aspirin and 1.66 to 1.83 (for 1–9 mmoles/l.) for salicylic acid in 0.05 *N* tetramethylammonium iodide. With the more sensitive polarographs of today, such an approach in the analysis of aspirin products could prove to be useful.

Quantitative separation of aspirin and salicylic acid by sephadex G gel filtration was reported by Lee *et al.* (111). No detectable (fluorometrically) hydrolysis of aspirin on the column was noted.

TLC should be an invaluable tool in aspirin stability studies. By using a mixture of hexane, glacial acetic acid, and chloroform (20:5:5), Reimers (112) separated aspirin and salicylic acid. After resolving the mixtures of aspirin–salicylic acid on thin-layer plates [using ascending technique and a hexane, glacial acetic acid, and chloroform mixture (85:15:10), R_f values of 0.2 and 0.35 were obtained for aspirin and salicylic acid, respectively], the spots were removed from the dried plates, packed in an appropriate cell, and determined by UV reflectance spectroscopy. A linear relationship at 302 mμ between absorbance and the square root of the concentration was observed by Frodyma *et al.* (113) with spots containing up to 3.0 μmoles of either compound.

An exhaustive paper on differentiating nonaqueous titration of salicylic acid and aspirin by Lin (114) showed that such a procedure was feasible. Dimethylformamide was the solvent of choice along with the titrant of 0.1 *N* tetrabutylammonium hydroxide in benzene–methanol (10:1) using either a glass–calomel or platinum–calomel electrode pair.

REFERENCES

(1) J. Rath, *Ann.*, **358**, 98(1908); through *Chem. Abstr.*, **2**, 1002(1908).

(2) D. E. Tsakalotos and S. Horsch, *Bull. Soc. Chim.*, **15**, 743(1914); through *Chem. Abstr.*, **9**, 787(1915).

(3) *Ibid.*, **17**, 401(1915); through *Chem. Abstr.*, **10**, 591(1916).

(4) A. Wolf, *Sv. Kem. Tidskr.*, **29**, 109(1917); through *Chem. Abstr.*, **11**, 2634(1917).

(5) C. Morton, *Quart. J. Pharm. Pharmacol.*, **6,** 492(1933).
(6) V. K. La Mer and J. Greenspan, *J. Amer. Chem. Soc.*, **56,** 1492(1934).
(7) J. M. Sturtevant, *ibid.*, **64,** 77(1942).
(8) L. J. Edwards, *Trans. Faraday Soc.*, **46,** 723(1950).
(9) *Ibid.*, **48,** 696(1952).
(10) D. Davidson and L. Auerbach, *J. Amer. Chem. Soc.*, **75,** 5984(1953).
(11) E. Ferroni and R. Baistrocchi, *Sperimentale Sez. Chim. Biol.*, **4,** 7(1953); through *Chem. Abstr.*, **47,** 10974h(1953).
(12) S. Miyamoto, T. Tsuchiya, and T. Miyamoto, *Ann. Rep. Kyoritsu Coll. Pharm.*, **1,** 26(1955); through *Chem. Abstr.*, **50,** 16898(1956).
(13) E. R. Garrett, *J. Amer. Chem. Soc.*, **79,** 3401(1957).
(14) E. R. Garrett, *J. Amer. Pharm. Ass., Sci. Ed.*, **46,** 584(1957).
(15) S. Okano and S. Kojima, *Yakuzaigaku*, **18,** 37(1958); through *Chem. Abstr.*, **53,** 6534f(1959).
(16) M. L. Bender, F. Chloupek, and M. C. Neveu, *J. Amer. Chem. Soc.*, **80,** 5384(1958).
(17) K. C. James, *J. Pharm. Pharmacol.*, **10,** 363(1958).
(18) S. M. Blaug and J. W. Wesolowski, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 691(1959).
(19) E. R. Garrett, *J. Org. Chem.*, **26,** 3660(1961).
(20) M. L. Bender, E. J. Pollock, and M. C. Neveu, *J. Amer. Chem. Soc.*, **84,** 595(1962).
(21) H. Nogami, S. Awazu, and N. Nakajima, *Chem. Pharm. Bull.*, **10,** 503(1962).
(22) L. Nelander, *Acta Chem. Scand.*, **18,** 973(1964).
(23) E. Mario and R. J. Gerraughty, *J. Pharm. Sci.*, **54,** 321 (1965).
(24) T. E. Needham, Jr., and R. J. Gerraughty, *ibid.*, **58,** 62 (1969).
(25) G. Santopadre and S. Bolton, *J. Pharm. Pharmacol.*, **19,** 550(1967).
(26) K. S. Murthy and E. G. Rippie, *J. Pharm. Sci.*, **56,** 1026 (1967).
(27) A. G. Mitchell and J. F. Broadhead, *ibid.*, **56,** 1261(1967).
(28) S. S. Kornblum and M. A. Zoglio, *ibid.*, **56,** 1569(1967); H. V. Maulding, M. A. Zoglio, and E. J. Johnston, *ibid.*, **57,** 1873 (1968); M. A. Zoglio, H. V. Maulding, R. M. Haller, and S. Briggen, *ibid.*, **57,** 1877(1968); H. V. Maulding, M. A. Zoglio, F. E. Pigois, and M. Wagner, *ibid.*, **58,** 1359(1969).
(29) A. Y. Gore, K. B. Naik, D. O. Kildsig, G. E. Peck, V. F. Smolen, and G. S. Banker, *ibid.*, **57,** 1850(1968).
(30) A. R. Fersht and A. J. Kirby, *J. Amer. Chem. Soc.*, **89,** 4853(1967).
(31) *Ibid.*, **89,** 4857(1967).
(32) H. Linke, *Apoth. Ztg.*, **26,** 939, 1083(1911).
(33) H. Melzer, *ibid.*, **26,** 1033(1911).
(34) P. N. Leech, *J. Ind. Eng. Chem.*, **10,** 288(1918).
(35) A. J. Jones, *Chem. Drug.*, **91,** 60(1919).
(36) H. L. Dahm, *J. Ind. Eng. Chem.*, **11,** 29(1919).
(37) A. Nutter-Smith, *Chem. Drug.*, **93,** 89(1920).
(38) A. Nutter-Smith, *Analyst*, **45,** 412(1920).
(39) E. A. Ruddiman, *J. Amer. Pharm. Ass.*, **11,** 796 (1922).
(40) Anon., *Amer. J. Pharm.*, **96,** 592(1924).
(41) H. V. Snidow and H. A. Langenhan, *J. Amer. Pharm. Ass.*, **14,** 694(1925).
(42) H. Valentin and A. Lieber, *Apoth. Ztg.*, **41,** 567(1926).
(43) A. Hoffman, *Dan. Tidsskr. Farm.*, **3,** 82(1929); through *Chem. Abstr.*, **23,** 3050(1929).
(44) R. M. Hitchens, *J. Amer. Pharm. Ass.*, **23,** 1084 (1934).
(45) A. Banchetti, *Ric. Sci.*, **8,** 290(1937).
(46) T. Canback, *Sv. Farm. Tidskr.*, **47,** 621(1943); through *Chem. Abstr.*, **38,** 5644^6(1944).
(47) Y. Tsuzuki and M. Sawada, *Bull. Chem. Soc. Jap.*, **23,** 23(1950).
(48) R. E. Pankratz and F. J. Bandelin, *J. Amer. Pharm. Ass., Sci. Ed.*, **41,** 267(1952).
(49) R. Yamamoto and T. Takahashi, *Ann. Rep. Shionogi Res. Lab.*, **3,** 112(1953).
(50) *Ibid.*, **4,** 79(1954).
(51) D. Ribeiro, D. Stevenson, J. Samyn, G. Milosovich, and A. M. Mattocks, *J. Amer. Pharm. Ass., Sci. Ed.*, **44,** 226(1955).
(52) L. J. Edwards, D. N. Gore, H. D. C. Rapson, and M. P. Taylor, *J. Pharm. Pharmacol.*, **7,** 892(1955).
(53) C. W. Strode, Jr., F. N. Stewart, H. O. Schott, and O. J. Coleman, *Anal. Chem.*, **29,** 1184(1957).
(54) L. J. Leeson, Ph.D. dissertation, University of Michigan, Ann Arbor, Mich., 1957.
(55) L. J. Leeson and A. M. Mattocks, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 329(1958).
(56) F. J. Bandelin and W. Malesh, *J. Amer. Pharm. Ass., Pract. Ed.*, **19,** 152(1958).
(57) C.-M. P. Wirth, *Pharm. Acta Helv.*, **34,** 283(1959).
(58) J. Kral, H. Bieleszova, and Z. Drozdova, *Farm. Obz.*, **29,** 298(1960); through *Chem. Abstr.*, **61,** 2910h(1964).
(59) I. Lippmann, Ph.D. dissertation, University of Michigan, Ann Arbor, Mich., 1960.
(60) M. R. Nazareth and C. L. Huyck, *J. Pharm. Sci.*, **50,** 608 (1961).
(61) *Ibid.*, **50,** 620(1961).
(62) J. D. DeMarco and A. D. Marcus, *ibid.*, **51,** 1010(1962).
(63) H. Okano, H. Kawata, S. Tsutsumi, and Y. Umezaki, *Yakuzaigaku*, **23,** 279(1963); through *Chem. Abstr.*, **61,** 537h(1964).
(64) H. M. Patel and C. L. Huyck, *Mfg. Chem.*, **34,** 100(1963).
(65) I. Grabowska, *Gdanski. Tow. Nauk., Rozpr. Wydz.*, **3,** 193(1964); through *Chem. Abstr.*, **64,** 14029a(1966).
(66) *Ibid.*, **3,** 207(1964); through *Chem. Abstr.*, **64,** 15678b(1966).
(67) A. R. Green, *Australas. J. Pharm.*, **45,** S88(1964).
(68) G. Gold and J. A. Campbell, *J. Pharm. Sci.*, **53,** 52(1964).
(69) A. E. Troup and H. Mitchner, *ibid.*, **53,** 375(1964).
(70) D. Trose and R. Danz, *Pharm. Prax.*, **1966,** 228.
(71) F. Jaminet and G. Evrard, *Pharm. Acta Helv.*, **41,** 601 (1966).
(72) W. Wisniewski and H. Piasecka, *Acta Pol. Pharm.*, **24,** 291 (1967).
(73) K. T. Koshy, A. E. Troup, R. N. Duvall, R. C. Conwell, and L. L. Shankle, *J. Pharm. Sci.*, **56,** 1117(1967).
(74) F. Jaminet and G. Louis, *Pharm. Acta Helv.*, **43,** 153 (1968).
(75) L. F. Cullen, D. L. Packman, and G. J. Papariello, *Ann. N. Y. Acad. Sci.*, **153,** 525(1968).
(76) R. B. Tinker and A. J. McBay, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 315(1954).
(77) W. R. Ebert, Ph.D. dissertation, University of Michigan, Ann Arbor, Mich., 1956.
(78) J. Levine, *J. Amer. Pharm. Ass., Sci. Ed.*, **46,** 687(1957).
(79) G. Smith, *J. Ass. Offic. Agr. Chem.*, **42,** 462(1959).
(80) *Ibid.*, **43,** 241(1960).
(81) R. F. Heuermann and J. Levine, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 276(1958).
(82) R. F. Heuermann, *J. Ass. Offic. Agr. Chem.*, **43,** 243(1960).
(83) J. Levine, *J. Pharm. Sci.*, **50,** 506(1961).
(84) F. Kubo, K. Imaoka, and A. Kaneko, *Ann. Rep. Kyoritsu Coll. Pharm.*, **6/7,** 1(1961–1962); through *Chem. Abstr.*, **60,** 375d (1964).
(85) S. Siegel, R. H. Reiner, J. A. Zelinskie, and E. J. Hanus, *J. Pharm. Sci.*, **51,** 1068(1962).
(86) W. B. Chapman and A. J. Harrison, *J. Ass. Pub. Anal.*, **1,** 64(1963).
(87) L. G. Ladomery, *Australas. J. Pharm.*, **44,** 10(1963).
(88) P. Turi, *J. Pharm. Sci.*, **53,** 369(1964).
(89) K. T. Koshy, *ibid.*, **53,** 1280(1964).
(90) L. A. Greenberg and D. Lester, *J. Pharmacol. Exp. Ther.*, **88,** 87(1946).
(91) S. Lee, H. G. DeKay, and G. S. Banker, *J. Pharm. Sci.*, **54,** 1153(1965).
(92) R. C. Reed and W. W. Davis, *ibid.*, **54,** 1533(1965).
(93) M. D. Day, J. F. Harper, and O. J. Olliff, *Chem. Drug.*, **186,** 127(1966).
(94) H. Sattler, *Pharm. Ztg.*, **111,** 1395(1966).
(95) A. W. Clayton and R. E. Thiers, *J. Pharm. Sci.*, **55,** 404 (1966).
(96) N. A. Shane and J. I. Routh, *Anal. Chem.*, **39,** 414(1967).
(97) J. D. Weber and J. Levine, *J. Pharm. Sci.*, **55,** 78(1966).
(98) J. D. Weber, *J. Ass. Offic. Agr. Chem.*, **48,** 1151(1965).
(99) A. L. Jacobs, A. E. Dilatush, S. Weinstein, and J. J. Windheuser, *J. Pharm. Sci.*, **55,** 893(1966).
(100) J. Levine and J. D. Weber, *ibid.*, **57,** 631(1968).
(101) D. E. Guttman, *ibid.*, **57,** 1685(1968).
(102) D. E. Guttman and G. W. Salomon, *ibid.*, **58,** 120(1969).

(103) E. Schulek and K. Burger, *Magy. Tud. Akad., Kem. Tud. Oszt. Kozlem.*, **12**, 15(1959); through *Chem. Abstr.*, **54**, 12903b (1960).
(104) W. W. Wendlandt and J. A. Hoiberg, *Anal. Chim. Acta*, **29**, 539(1963).
(105) R. C. Crippen and H. C. Freimuth, *Anal. Chem.*, **36**, 273 (1964).
(106) J. G. Nikelly, *ibid.*, **36**, 2248(1964).
(107) E. R. Garrett and J. L. Johnson, *J. Pharm. Sci.*, **51**, 767 (1962).
(108) M. Vietti-Michelina, *Atti Accad. Sci. Torino, Cl. Sci. Fis., Mat. Natur.*, **89**, 383(1954–1955); through *Chem. Abstr.*, **50**, 6255h(1956).
(109) G. Wagner, *Arch. Pharm.*, **289**, 8(1956).
(110) I. A. Korshunov, Z. B. Kuznetsova, and M. K. Shchennikova, *Zhur. Fiz. Khim.*, **23**, 1292(1949); through *Chem. Abstr.*, **44**, 2873d(1950).
(111) K. H. Lee, L. Thompkins, and M. R. Spencer, *J. Pharm. Sci.*, **57**, 1240(1968).
(112) F. Reimers, *Arch. Pharm. Chem*, **74**, 531(1967); through *Chem. Abstr.*, **67**, 67624h(1967).
(113) M. M. Frodyma, V. T. Lieu, and R. W. Frei, *J. Chromatogr.*, **18**, 520(1965).
(114) S. L. Lin, *J. Pharm. Sci.*, **56**, 1130(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received from *Sterling-Winthrop Research Institute, Rensselaer, NY 12144*

The author thanks Agnes F. Cox, Irene D. DeGraff, Laura A. Fantauzzi, Peter Frank, Patricia A. Lanciault, and Kathleen E. Long for making the initial abstract search for papers related to the decomposition of aspirin; Patricia C. Carroll of the library staff for her tenacity in acquiring the original articles discussed in this review; Harold E. Bauer, Frank S. Lukovits, and Walter R. Wiehler for their thoughts and opinions regarding this review; and Ellen V. Miller who so patiently reconstructed the figures presented in this review.

RESEARCH ARTICLES

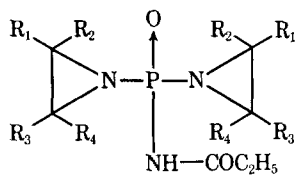
Mechanisms of Reactions of Ring-Substituted Bis(1-aziridinyl)phosphinyl Urethan Antineoplastic Agents

C. K. NAVADA, Z. F. CHMIELEWICZ, and T. J. BARDOS*

Abstract □ Bis(*trans*-2,3-dimethylaziridinyl)phosphinyl urethan (IV) was synthesized and compared with the corresponding *cis*-2,3-dimethyl derivative (III). The comparative alkylating activities and rates of hydrolysis of these two stereoisomeric aziridine derivatives, III and IV, were determined and compared with corresponding data for the monomethyl derivative (V) and two other clinically tested members of this series of antineoplastic agents (dual antagonists), AB-100 (I) and AB-132 (II). The structures of the final hydrolysis products of III, IV, and V were determined and confirmed by direct synthesis. The results indicate that the mechanisms of hydrolysis of III, IV, and V (as that of the unsubstituted aziridine derivative, AB-100) are essentially S_N2, in contrast to the much faster hydrolysis of the 2,2-dimethylaziridine analog, AB-132, which involves a carbonium-ion mechanism. These studies give further support to the hypothesis that the unique pharmacologic properties of AB-132, as compared to other members of this series, may be related to the unique chemical properties of the 2,2-dimethylaziridine moieties.

Keyphrases □ Antineoplastic agents—reaction mechanisms □ Bis(*trans*-2,3-dimethylaziridinyl)phosphinyl urethan—synthesis □ Alkylating activity—*cis*-, *trans*-2,3-dimethylaziridine analogs □ Hydrolysis mechanism—ring-substituted aziridine derivatives □ IR spectrophotometry—identity □ NMR spectroscopy—identity

The synthesis of a series of bis(1-aziridinyl)phosphinyl carbamates, termed "dual antagonists" (I and its analogs containing different carbamate moieties) (1, 2), and their antineoplastic activities in experimental animals (3, 4) and in man (5–9) were previously reported. In an effort to decrease the hematologic toxicity due to the "alkylating" aziridine groups, derivatives were synthesized in which the C-atoms of the aziridine rings were substituted with methyl or ethyl groups (10). One member of this new series, ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinyl carbamate (AB-132, II), has been studied to a considerable extent experimentally (11) as well as clinically (12–17). Its interesting pharmacologic properties [*e.g.*, cholinesterase inhibition (18–20)] and its radiation potentiating effect (21–23) suggested that this compound may act by a different mechanism than the C-unsubstituted aziridine derivatives (24). This conclusion was supported by chemical studies of its hydrolytic and alkylation reactions (11, 25), which indicated that the unique properties of II may be related to the ability of the 2,2-dimethylaziridine group to participate in S_N1 reactions with its substituted carbon (by forming a tertiary carbonium ion) and, alternatively,



I. $R_1 = R_2 = R_3 = R_4 = H$ (AB-100)
II. $R_1 = R_2 = CH_3$; $R_3 = R_4 = H$ (AB-132)
III. $R_1 = R_3 = CH_3$; $R_2 = R_4 = H$ (*cis*) (AB-144)
IV. $R_1 = R_4 = CH_3$; $R_2 = R_3 = H$ (*trans*) (AB-145)
V. $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$ (AB-143)

(103) E. Schulek and K. Burger, *Magy. Tud. Akad., Kem. Tud. Oszt. Kozlem.*, **12**, 15(1959); through *Chem. Abstr.*, **54**, 12903b (1960).
(104) W. W. Wendlandt and J. A. Hoiberg, *Anal. Chim. Acta*, **29**, 539(1963).
(105) R. C. Crippen and H. C. Freimuth, *Anal. Chem.*, **36**, 273 (1964).
(106) J. G. Nikelly, *ibid.*, **36**, 2248(1964).
(107) E. R. Garrett and J. L. Johnson, *J. Pharm. Sci.*, **51**, 767 (1962).
(108) M. Vietti-Michelina, *Atti Accad. Sci. Torino, Cl. Sci. Fis., Mat. Natur.*, **89**, 383(1954–1955); through *Chem. Abstr.*, **50**, 6255h(1956).
(109) G. Wagner, *Arch. Pharm.*, **289**, 8(1956).
(110) I. A. Korshunov, Z. B. Kuznetsova, and M. K. Shchennikova, *Zhur. Fiz. Khim.*, **23**, 1292(1949); through *Chem. Abstr.*, **44**, 2873d(1950).
(111) K. H. Lee, L. Thompkins, and M. R. Spencer, *J. Pharm. Sci.*, **57**, 1240(1968).
(112) F. Reimers, *Arch. Pharm. Chem*, **74**, 531(1967); through *Chem. Abstr.*, **67**, 67624h(1967).
(113) M. M. Frodyma, V. T. Lieu, and R. W. Frei, *J. Chromatogr.*, **18**, 520(1965).
(114) S. L. Lin, *J. Pharm. Sci.*, **56**, 1130(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received from *Sterling-Winthrop Research Institute, Rensselaer, NY 12144*

The author thanks Agnes F. Cox, Irene D. DeGraff, Laura A. Fantauzzi, Peter Frank, Patricia A. Lanciault, and Kathleen E. Long for making the initial abstract search for papers related to the decomposition of aspirin; Patricia C. Carroll of the library staff for her tenacity in acquiring the original articles discussed in this review; Harold E. Bauer, Frank S. Lukovits, and Walter R. Wiehler for their thoughts and opinions regarding this review; and Ellen V. Miller who so patiently reconstructed the figures presented in this review.

RESEARCH ARTICLES

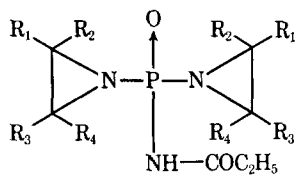
Mechanisms of Reactions of Ring-Substituted Bis(1-aziridinyl)phosphinyl Urethan Antineoplastic Agents

C. K. NAVADA, Z. F. CHMIELEWICZ, and T. J. BARDOS*

Abstract □ Bis(*trans*-2,3-dimethylaziridinyl)phosphinyl urethan (IV) was synthesized and compared with the corresponding *cis*-2,3-dimethyl derivative (III). The comparative alkylating activities and rates of hydrolysis of these two stereoisomeric aziridine derivatives, III and IV, were determined and compared with corresponding data for the monomethyl derivative (V) and two other clinically tested members of this series of antineoplastic agents (dual antagonists), AB-100 (I) and AB-132 (II). The structures of the final hydrolysis products of III, IV, and V were determined and confirmed by direct synthesis. The results indicate that the mechanisms of hydrolysis of III, IV, and V (as that of the unsubstituted aziridine derivative, AB-100) are essentially S_N2, in contrast to the much faster hydrolysis of the 2,2-dimethylaziridine analog, AB-132, which involves a carbonium-ion mechanism. These studies give further support to the hypothesis that the unique pharmacologic properties of AB-132, as compared to other members of this series, may be related to the unique chemical properties of the 2,2-dimethylaziridine moieties.

Keyphrases □ Antineoplastic agents—reaction mechanisms □ Bis(*trans*-2,3-dimethylaziridinyl)phosphinyl urethan—synthesis □ Alkylating activity—*cis*-, *trans*-2,3-dimethylaziridine analogs □ Hydrolysis mechanism—ring-substituted aziridine derivatives □ IR spectrophotometry—identity □ NMR spectroscopy—identity

The synthesis of a series of bis(1-aziridinyl)phosphinyl carbamates, termed "dual antagonists" (I and its analogs containing different carbamate moieties) (1, 2), and their antineoplastic activities in experimental animals (3, 4) and in man (5–9) were previously reported. In an effort to decrease the hematologic toxicity due to the "alkylating" aziridine groups, derivatives were synthesized in which the C-atoms of the aziridine rings were substituted with methyl or ethyl groups (10). One member of this new series, ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinyl carbamate (AB-132, II), has been studied to a considerable extent experimentally (11) as well as clinically (12–17). Its interesting pharmacologic properties [*e.g.*, cholinesterase inhibition (18–20)] and its radiation potentiating effect (21–23) suggested that this compound may act by a different mechanism than the C-unsubstituted aziridine derivatives (24). This conclusion was supported by chemical studies of its hydrolytic and alkylation reactions (11, 25), which indicated that the unique properties of II may be related to the ability of the 2,2-dimethylaziridine group to participate in S_N1 reactions with its substituted carbon (by forming a tertiary carbonium ion) and, alternatively,



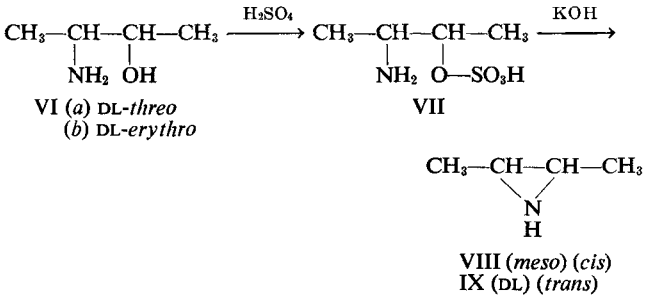
I. $R_1 = R_2 = R_3 = R_4 = H$ (AB-100)
II. $R_1 = R_2 = CH_3$; $R_3 = R_4 = H$ (AB-132)
III. $R_1 = R_3 = CH_3$; $R_2 = R_4 = H$ (*cis*) (AB-144)
IV. $R_1 = R_4 = CH_3$; $R_2 = R_3 = H$ (*trans*) (AB-145)
V. $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$ (AB-143)

in S_N2 reactions with its unsubstituted methylene carbon (25).

In view of the observed profound effect of the 2,2-dimethyl substitution in the aziridine ring on the chemical and pharmacologic properties of II, it was of interest to investigate the chemical reaction mechanisms of the stereoisomeric 2,3-dimethylaziridine derivatives, III and IV (which are position isomers of II), and of the monomethylaziridine derivative, V.

RESULTS AND DISCUSSION

There are three stereoisomeric forms of 2,3-dimethylaziridine: *meso* (*cis*) and L and D (*trans*). The first two of these were synthesized from D- (as well as DL) *threo*-3-amino-2-butanol and L-*erythro*-3-amino-2-butanol, respectively, by Dickey *et al.* (26). By stereospecific syntheses, Dickey *et al.* (26) also established their absolute configurations. In the present work, commercial 3-amino-2-butanol,[1] a mixture of the DL-*threo* and DL-*erythro* isomers (VI), was used as the starting material. Cyclization of the acid sulfates VII gave a mixture of the two optically inactive diastereoisomeric aziridines, VIII and IX. These were separated by fractional distillation, as described in the *Experimental* section, and their NMR spectra were consistent with the configurational assignments of Dickey *et al.* (26) (Scheme I).



Scheme I

The synthesis and physicochemical properties of the ring-substituted bis-aziridinylphosphinyl carbamates III and V were previously reported (10). The *trans*-2,3-dimethyl analog, IV, was synthesized according to the same general procedure, by the reaction of dichlorophosphinyl urethan with DL-*trans*-2,3-dimethylaziridine (IX). Although two diastereoisomers of IV might be expected to arise from this reaction (since two molecules of the racemic aziridine reacted with each molecule of dichlorophosphinyl urethan), only one, sharp melting, crystalline product could be isolated from the reaction mixture by repeated crystallization from *n*-hexane.

The IR spectra of the *cis*- and *trans*-aziridine derivatives, III and IV, are almost superimposable, except in the 700–950 cm.$^{-1}$ region where the differences in the ring-deformation frequencies of the two compounds result in characteristically different absorption patterns. The NMR spectra show the expected differences between the two compounds in the chemical shifts of their ring-CH and -CH_3 protons, corresponding to the differences between the NMR spectra of the aziridines from which they were derived. In the spectra of both III and IV, the multiplets of the ring-CH protons are split by the phosphorus into two symmetrical groups of resonance lines.

The comparative chemical alkylating activities (S_N2-reactivities) of Compounds III, IV, and V were determined according to the previously described method (25, 27) by measuring the rates of alkylation (colored product formation) with γ-(4-nitrobenzyl)-pyridine (NBP) at 80° temperature and standard initial concentrations of the reactants. The initial reaction rates [k'_{80} values (27)] are given in Table I. It is apparent that the *trans*-isomer (IV) is much more reactive than the *cis* (III). This result seems reasonable, considering that in the case of IV the nucleophilic reactant can approach one of the carbon atoms from either side of each aziridine ring; while in the case of III, only one side of each ring is open to nucleophilic attack. Both compounds are considerably less reactive than the "unsubstituted" analog (I), but IV is only slightly less reactive than the monomethyl and gem-dimethyl aziridine derivatives (V and II, respectively) in which one of the aziridine carbons is unsubstituted.

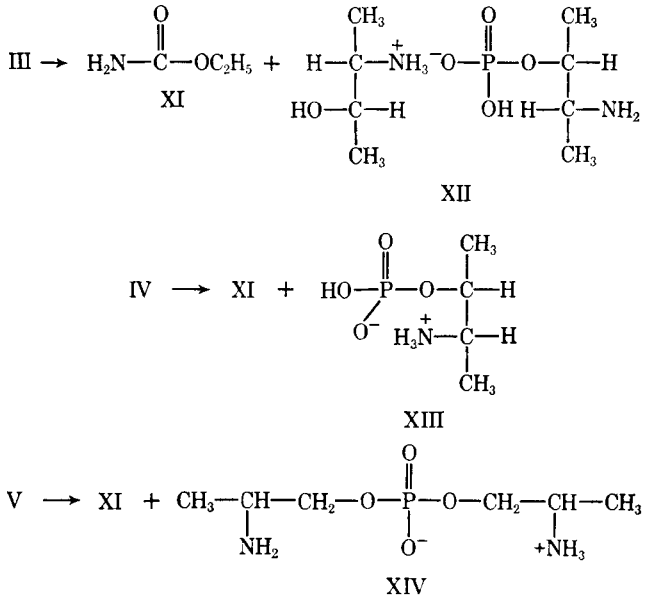
Table I—Comparative Alkylating Activities and Relative Stabilities to Hydrolysis of Ethyl Bis(1-aziridinyl)phosphinyl Carbamates

Compound	Aziridine Ring Substituents	Alkylating Activities,[a] $k'_{80} \times 10^3$ min.$^{-1}$	Half-Lives, hr.,[b] in H_2O at 37°
I	None	3.3[c]	60
II	2,2-CH_3	2.4[c]	2.5
III	*cis*-2,3-CH_3	0.8	125
IV	*trans*-2,3-CH_3	1.9	35
V	2-CH_3	2.0[c]	80

[a] For definition, see *Reference 27.* [b] Based on residual titratable aziridine rings. [c] Data taken from *Reference 25.*

The relative rates of hydrolytic cleavage of the aziridine-rings of Compounds I–V, in distilled water at 37°, can be compared on the basis of the half-life data given in Table I. If the hydrolysis of all compounds was to proceed entirely by an S_N1 mechanism, as was found in the case of the 2,2-dimethyl aziridine analog, AB-132 (II) (11), then the relative rates would depend on the comparative ease with which they are capable of forming a carbonium-ion intermediate. Therefore, the following order of hydrolysis rates would be expected: II > IV ≧ III > V > I. Instead, the rates of hydrolysis show the following sequence: II ≫ IV > I > V > III. Thus, it is evident that the hydrolytic cleavage of the aziridine rings in some of these compounds appears to involve other than S_N1 mechanisms. For example, the 3–4 times faster hydrolysis rate of the *trans*-2,3-dimethylaziridine derivatives IV as compared to the *cis*-isomer III almost parallels their relative S_N2 reactivities in the alkylation of NBP.

Preliminary studies relating to the mechanisms of hydrolysis of I and II were previously reported, indicating that in water solution, I undergoes bimolecular polymerization with its P—N linkages remaining largely intact (25), while the very fast hydrolysis of II proceeds *via* a tertiary carbonium ion and with the cleavage of all three of its P—N bonds (11, 25). To elucidate the mechanisms of hydrolysis of III–V, their final hydrolysis products, obtained after 3 weeks of incubation of each compound in deionized distilled water solution at 37°, were isolated and identified. In all cases, urethan and a phosphate ester were isolated from the hydrolysates as the two major, final hydrolysis products (Scheme II).



Scheme II

[1] Commercial Solvents Corp., New York, N. Y.

The structures of the phosphate esters were identified by elemental analysis, IR and NMR spectra, and alkaline hydrolysis, yielding the known amino alcohols.[2] In addition, most of the phosphate mono- and diesters, salts, and ester-salts that could have resulted from the hydrolysis of Compounds III–V were directly synthesized for comparison with the isolated products (see *Experimental*). The phosphate mono- and diesters were generally prepared by reacting the appropriate aziridine with phosphoric acid. Since the ring-opening reactions of the stereoisomeric 2,3-dimethylaziridines are known to proceed with a single Walden inversion (28), and 2-monosubstituted aziridines are cleaved by strong acids at the unsubstituted 3-position (29), the structures of the phosphate esters were unambiguously defined by their synthesis. However, additional proof of structure was obtained by alkaline hydrolysis to yield the known amino alcohols. The ester-salts were synthesized by reacting the monoesters with one equivalent of the amino alcohol, while the phosphate salts were prepared by combining one equivalent of phosphoric acid with two equivalents of the amino alcohol. If the elemental analysis and melting points did not permit unambiguous identification of the hydrolysis products with the synthetic salts or esters (because of variations in water of crystallization), comparison of the spectra served well for this purpose. In general, the phosphate salts showed much sharper absorption bands in their IR and NMR spectra than the phosphate esters which are zwitterions. Although the positions of the absorption peaks for the phosphate ester and the corresponding salt were similar, it was possible to differentiate between them on the basis of their resolution and splitting patterns. In particular, the splitting by the phosphorus of the CH or CH_2 protons adjacent to the P—O bond in the phosphate esters increased the complexity of the resonance pattern in the 3.5–4.5 p.p.m. region and in the case of XIV gave rise to a well-resolved quartet.

The results show that the *cis*-2,3-dimethylaziridine derivative III yields the phosphate ester-salt of *threo*-3-amino-2-butanol (XII), while the *trans*-2,3-dimethylaziridine analog IV yields the phosphate monoester of *erythro*-3-amino-2-butanol (XIII). If the hydrolysis of the aziridine rings would have proceeded by an S_N1 mechanism, the same carbonium-ion intermediate derived from either III or IV should have given identical mixtures of the phosphates of the two isomeric amino alcohols. Actually, the ring-opening hydrolysis reactions of both III or IV appear to involve in each case a single Walden inversion (*trans*-opening) and, therefore, must be mechanistically S_N2, although they are pseudo-first-order from a kinetic point of view. Furthermore, the structure of the hydrolytic product XIV of the 2-methylaziridine derivative V shows that the ring-opening reaction of this compound occurred at the unsubstituted (less hindered) carbon, thus indicating that the mechanisms of this reaction similarly followed an S_N2 course. The fact that the hydrolysis of all three compounds, III–V, resulted in the formation of a phosphate ester bond, indicates that the ring-opening reaction involves an inter- or intramolecular S_N2 attack of a $P—O^-$ anion on the protonated aziridine ring. This reaction requires either prior or concerted hydrolytic cleavage of a P—N bond, possibly initiated by tautomerization of the O=P—NH— grouping; eventually, all three P—N bonds are cleaved to give the final hydrolysis products.

CONCLUSIONS

On the basis of these results, it is now possible to explain satisfactorily the order of hydrolysis rates for Compounds I–V. Thus, II is the only compound of this series which hydrolyzes by an S_N1 mechanism, and this is consistent with its exceptionally fast rate of hydrolysis. In contrast, the relative rates of hydrolysis of Compounds I, V, and III appear to depend on the comparative steric hindrances of their aziridine moieties to nucleophilic attack. The relative hydrolysis rate of Compound IV is somewhat anomalous and may suggest some smaller contribution of an S_N1 mechanism (which possibility in the case of this compound is not entirely excluded by the structure of the isolated hydrolysis product XIII, since the latter contains only half of the total amount of amino alcohol resulting from the hydrolysis of IV). In any case, the mechanisms of hydrolysis of III–V are largely S_N2 and, therefore, just as the hydrolytic polymerization of the ring-unsubstituted-aziridine derivative I (25), they do not involve carbonium-ion intermediate formation to any substantial degree. The importance of the tertiary carbonium-ion intermediate formed in the hydrolysis of the geminally substituted aziridine derivative II has been previously discussed (11). Other studies on the comparative chemical and biological activities of conventional and "branched-chain" aromatic nitrogen mustards (27) have indicated that the biological activities in a given series of alkylating agents may be related to their different chemical reaction mechanisms (27, 30). On the whole, the results of the present study underline the uniqueness of the 2,2-dimethylaziridine derivative II (AB-132) with respect to its chemical behavior, as compared to other members of this series of alkylating agents.

It should be mentioned that the *in vivo* toxicities and antitumor activities (in several rodent tumor systems)[3] of Compounds I, III–V are qualitatively similar, and that their relative magnitudes closely parallel the relative "S_N2 reactivities" of these compounds with respect to NBP. This is in contrast to the qualitatively and quantitatively different pharmacological activities of AB-132 (II).

EXPERIMENTAL

Comparative alkylating activities and rates of hydrolysis were determined by previously-described procedures (25, 27). The IR spectra were recorded on a Perkin-Elmer model 237 (Infracord), and the NMR spectra were obtained in a Varian Associates model A-60 using deuterium oxide as solvent, except where noted. The melting points were determined on a Mel-Temp melting point apparatus and are uncorrected.

The syntheses of Compounds I–III and V were previously reported (1, 2, 10).

Ethyl [Bis(2,3-*trans*-dimethyl-1-aziridinyl)phosphinyl]carbamate (IV)—Commercially available 3-amino-2-butanol, a mixture of DL-*threo* and DL-*erythro* isomers (VI), was cyclized through its amine sulfate (26) to yield a stereoisomeric mixture of 2,3-dimethylaziridine. The separation of the DL(*trans*)-isomer from the *meso* (*cis*)-isomer, VIII, b.p. 81–83° (771 mm.) [previously employed for the synthesis of III (10)], was accomplished by fractional distillation through a spinning band column, and the fraction, b.p. 75–75.5° (765 mm.) (IX), was collected. NMR spectra (in CCl_4 with tetramethylsilane as internal standard) are as follows: VIII (*cis*): 1.05 (6H, d, J = 6 c.p.s., CH_3), and 2.06 p.p.m. (2H, multiplet, CH); IX (*trans*): 1.12 (6H, d, J = 4 c.p.s., CH_3), and 1.50 p.p.m. (2H, multiplet, CH). The reaction of DL(*trans*)-2,3-dimethylaziridine with dichlorophosphinyl urethan was carried out under nitrogen in the usual manner (10). After filtration, the filtrate was concentrated *in vacuo* at 30° to give an oily residue. Several crystallizations from *n*-hexane gave a pure product, m.p. 103–104°, yield 20%. Ethylenimine assay (31): 102% of the theoretical value (based on two ethylenimine groups and a mol. wt. of 275).

Anal.—Calcd. for $C_{11}H_{22}N_3O_3P$: C, 47.99; H, 8.00; N, 15.26. Found: C, 47.89; H, 7.84; N, 15.42.

IR absorption bands (KBr) ($cm.^{-1}$): 3050, 2960, 1730 (s) (C=O); 1460 (s) sh. 1420, 1440 (m) (C—CH_3, CH_2); 1380 (m) (C—CH_3); 1330 (w) (P=O); 905 (m); 835 (s) (doublet).[4]

NMR (in $CDCl_3$, with tetramethylsilane as internal standard), 1.35 (15H, multiplet, CH_3), 2.42 (4H, multiplet, —CH—N), and 4.15 p.p.m. (2H, quartet, CH_2—O).

Hydrolysis Products—The final hydrolysis products of III–V were obtained by incubating 0.015 mole of each compound with 2.2 moles of deionized distilled water at 37° for 3 weeks. The aqueous suspension was then extracted with ether, and the aqueous layer was lyophilized, washed with ether, and dried. The combined ether extracts, on evaporation of the solvent, yielded in each case a crystalline material which had a melting point and an IR spectrum identical with those of an authentic sample of ethyl

[2] Alkaline hydrolysis of phosphate esters proceeds *via* attack of the hydroxyl ion on the phosphorus (which carries a residual positive charge) and does not involve the asymmetric carbon of the alcohol which is liberated without change in its configuration.

[3] A detailed study on the comparative biological activities of all members of this series of "dual antagonists" in various experimental systems, including a large spectrum of animal tumors, is in preparation and will be published by the cooperating biological investigators.

[4] In the previously reported spectrum of the *cis*-analog III (10), the last three band assignments were erroneously transcribed; this region of the spectrum of III should read: 9.75 μ (1025 $cm.^{-1}$) (s); 9.90 μ (1010 $cm.^{-1}$) (s); 11.00 μ (905 $cm.^{-1}$) (m); and 11.9 μ (835 $cm.^{-1}$) (m).

Table II—Final Hydrolysis Products

	M.p.	Empirical Formula	Analysis, % Calcd.	Found	NMR Spectra δ, p.p.m.
Hydrolysis product of III (XII)	194–195°	$C_8H_{28}N_2O_5P$	C, 37.21 H, 8.92 N, 10.85 P, 12.01	C, 37.08 H, 8.80 N, 11.03 P, 12.17	1.28 (12H, doublet, CH_3) 3.25 (2H, multiplet, CH—N) 3.82 (2H, multiplet, CH—O)
Hydrolysis product of IV (XIII)	275–276°	$C_4H_{12}NO_4P$	C, 28.40 H, 7.10 N, 8.28 P, 18.34	C, 28.53 H, 7.23 N, 8.26 P, 18.12	1.25 (6H, doublet, CH_3) 3.42 (1H, multiplet, CH—N) 4.43 (1H, multiplet, CH—O)
Hydrolysis product of V (XIV)	170–172°	$C_6H_{17}N_2O_4P$	C, 33.96 H, 8.01 N, 13.21 P, 14.62	C, 33.68 H, 7.86 N, 13.13 P, 14.62	1.29 (6H, doublet, CH_3) 2.6–4.2 (6H, multiplet, CH—N, CH_2—O, including overlapping quartet at 3.68)

carbamate. The ether-insoluble products obtained from the aqueous layer were crystallized from methanol–ether. The melting points, elemental analyses, and NMR spectra of these "final hydrolysis products" (XII–XIV) are given in Table II.

Preparation of Phosphate Esters

Phosphate Monoesters—*A. Reaction of Substituted Aziridine with Phosphoric Acid*—To an ice-cooled, stirred solution of the substituted aziridine (0.10 mole) in 10 ml. of water was slowly added an equimolar quantity of 86% phosphoric acid. The resulting solution was stirred at room temperature for 48 hr. and then concentrated to dryness by lyophilization. The products were recrystallized several times from methanol or methanol–ether.

B. Pyrolysis Method—Equimolar quantities of the amino alcohol and phosphoric acid were stirred for 3 hr. at room temperature. The solution was concentrated *in vacuo* at 70°, and the resulting oily residue was heated *in vacuo* (0.8 mm. Hg) for 3 hr. at 160°. The solid residue was recrystallized from methanol or methanol–ether. The product was identical with that obtained by Procedure A, using the corresponding substituted aziridine, by mixed melting point, IR, and NMR.

Phosphate Diesters—The procedure used for the synthesis of the phosphate diesters was essentially similar to that described for the synthesis of the phosphate monoester, Procedure A, except that two equivalents of substituted aziridine (0.2 mole) were reacted with one equivalent (0.1 mole) of phosphoric acid. The products were recrystallized from methanol–ether.

The melting points, elemental analysis, and NMR spectra of the phosphate mono- and diesters are presented in Table III.

Hydrolysis of Phosphate Esters

The phosphate esters were hydrolyzed, to yield the corresponding amino alcohol and sodium phosphate, by refluxing 0.02 mole of the ester for 48 hr. in 100 ml. of 15% sodium hydroxide solution.

Table III—Synthetic Phosphate Esters, Salts, and Half Salts–Half Esters

Compound	M.p.	Empirical Formula	Analysis, % Calcd.	Found	NMR Spectra δ, p.p.m.
(DL-*threo*-Amino-2-butyl) dihydrogen phosphate	86°	$C_4H_{12}NO_4P$	C, 28.40 H, 7.10 N, 8.28	C, 28.25 H, 7.50 N, 7.95	1.26 (6H, doublet, CH_3) 3.24 (1H, multiplet, CH—N) 3.84 (1H, multiplet, CH—O)
Di(DL-*threo*-3-amino-2-butyl) hydrogen phosphate	180–181°	$C_8H_{21}N_2O_4P \cdot H_2O$	C, 37.21 H, 8.92 N, 10.85 P, 12.01	C, 37.26 H, 8.91 N, 11.00 P, 12.15	1.22 (12H, doublet, CH_3) 3.18 (2H, multiplet, CH—N) 3.85 (2H, multiplet, CH—O)
Di-3-(DL-*threo*-2-hydroxy)butylammonium hydrogen phosphate	194–195°	$C_8H_{25}N_2O_6P$	C, 34.78 H, 9.05 N, 10.15	C, 34.79 H, 9.20 N, 10.30	1.36 (12H, doublet, CH_3) 3.23 (2H, multiplet, CH—N) 3.78 (2H, multiplet, CH—O)
3-(DL-*threo*-2-Hydroxy)butylammonium (DL-*threo*-3-amino-2-butyl) hydrogen phosphate	185–187°	$C_8H_{23}N_2O_5P \cdot H_2O$	C, 34.78 H, 9.05 N, 10.15	C, 34.80 H, 9.23 N, 9.99	1.32 (12H, doublet, CH_3) 3.26 (2H, multiplet, CH—N) 3.80 (2H, multiplet, CH—O)
(DL-*erythro*-3-Amino-2-butyl) dihydrogen phosphate	276–278°	$C_4H_{12}NO_4P$	C, 28.40 H, 7.10 N, 8.28 P, 18.34	C, 28.22 H, 7.12 N, 8.26 P, 18.34	1.27 (6H, doublet, CH_3) 3.46 (1H, multiplet, CH—N) 4.42 (1H, multiplet, CH—O)
Di(DL-*erythro*-3-amino-2-butyl) hydrogen phosphate	176–178°	$C_8H_{21}N_2O_4P \cdot 2H_2O$	C, 34.78 H, 9.05 N, 10.15	C, 35.03 H, 9.25 N, 9.88	1.29 (12H, doublet, CH_3) 3.53 (2H, multiplet, CH—N) 4.52 (2H, multiplet, CH—O)
Di(DL-*erythro*-2-hydroxy)butylammonium hydrogen phosphate	199–201°	$C_8H_{25}N_2O_6P$	C, 34.78 H, 9.05 N, 10.15	C, 34.76 H, 9.19 N, 9.89	1.26 and 1.30 (12H, 2 doublets, CH_3) 3.40 (2H, multiplet, CH—N) 4.20 (2H, multiplet, CH—O)
3-(DL-*erythro*-2-Hydroxy)butylammonium (DL-*erythro*-3-amino-2-butyl) hydrogen phosphate	276–277°	$C_8H_{23}N_2O_5P$	C, 37.21 H, 8.91 N, 10.85	C, 36.91 H, 9.08 N, 10.63	1.28 (12H, doublet, CH_3) 3.48 (2H, multiplet, CH—N) 4.25 (2H, multiplet, CH—O)
Di(DL-2-amino-1-propyl) hydrogen phosphate	170–172°	$C_6H_{17}N_2O_4P \cdot CH_3OH$	C, 34.43 H, 8.61 N, 11.48	C, 34.67 H, 8.58 N, 11.58	1.30 (6H, doublet, CH_3) 2.6–4.2 (6H, multiplet, CH—N, CH_2—O)
Di-2-(DL-1-hydroxy)propylammonium hydrogen phosphate	161–163°	$C_6H_{21}N_2O_6P$	C, 29.02 H, 8.46 N, 11.29	C, 28.96 H 8.46 N, 11.38	1.26 (6H, doublet, CH_3) 3.6 (6H, multiplet, CH—N, CH_2O)
Di-1-(DL-2-hydroxy)propylammonium hydrogen phosphate	164–165°	$C_6H_{21}N_2O_6P$	C, 29.02 H, 8.46 N, 11.29	C 28.97 H, 8.58 N, 11.35	1.22 (6H, doublet, CH_3) 3.04 (4H, multiplet, CH_2—N) 4.08 (2H, multiplet, CH—O)

After cooling to room temperature, the solution was saturated with solid sodium hydroxide and extracted with several portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate and, after filtration, the ether was removed *in vacuo*. The oily product was identified by comparison of its IR and NMR spectra with those of an authentic sample of the amino alcohol.

Preparation of Phosphate Salts

To a stirred solution of two equivalents of amino alcohol (0.03 mole) in 20 ml. water was added dropwise one equivalent of phosphoric acid. The reaction mixture was stirred for 1 hr. and then lyophilized. The crude material was recrystallized from methanol. The products were characterized by their melting points, elemental analyses, IR, and NMR spectra (Table III).

Preparation of Phosphate Ester-Salts

To an aqueous solution of the phosphate monoester, prepared as described in Procedure A (0.05 mole in 15 ml. of water), was added 0.05 mole of the corresponding amino alcohol in 10 ml. of water. The mixture was stirred for 24 hr. at room temperature and then lyophilized. The product (95–100% yield) was crystallized from methanol or methanol–ether to yield the phosphate half ester-half salt. Analytical results and spectra are presented in Table III.

REFERENCES

(1) T. J. Bardos, Z. B. Papanastassiou, A. Segaloff, and J. L. Ambrus, *Nature*, **183,** 399(1959).

(2) Z. B. Papanastassiou and T. J. Bardos, *J. Med. Pharm. Chem.*, **5,** 1000(1962).

(3) A. Segaloff, T. J. Bardos, Z. B. Papanastassiou, J. Ambrus, and J. B. Weeth, *Proc. Amer. Ass. Cancer Res.*, **3,** 62(1959).

(4) W. F. Dunning, M. R. Curtis, V. Armaghan, and D. McKenzie, *Cancer Chemother. Rep.*, **12,** 1(1961).

(5) S. McCracken and J. Wolf, *ibid.*, **6,** 52 (1960).

(6) W. G. Thurman, D. J. Fernback, T. M. Holcomb, W. W. Sutow, and J. A. Whitaker, *ibid.*, **12,** 105(1961).

(7) C. L. Brown, D. E. Bergsagel, and W. C. Levin, *ibid.*, **21,** 81(1962).

(8) D. V. Razis, J. L. Ambrus, C. A. Ross, L. Stutzman, J. E. Sokal, A. M. Rejali, and T. J. Bardos, *Cancer*, **14,** 853(1961).

(9) Pacific Veterans Administration Cancer Chemotherapy Group, *Cancer Chemother. Rep.*, **33,** 15(1963).

(10) T. J. Bardos, A. K. Barua, Z. F. Chmielewicz, G. E. Crevar, J. P. Dailey, S. Divald, and Z. B. Papanastassiou, *J. Pharm. Sci.*, **54,** 187(1965).

(11) T. J. Bardos and J. L. Ambrus, *Int. Congr. Chemother., Proc. 3rd*, **2,** 1036(1964).

(12) J. L. Ambrus, J. E. Sokal, C. M. Ambrus, N. Back, and T. J. Bardos, *Proc. Amer. Ass. Cancer Res.*, **3,** 204(1961).

(13) C. A. Ross, J. E. Sokal, J. L. Ambrus, D. V. Razis, and T. J. Bardos, *ibid.*, **3,** 263(1961).

(14) A. L. Watne, G. E. Moore, C. A. Ross, and J. L. Ambrus, *Cancer Chemother. Rep.*, **16,** 421(1962).

(15) C. A. Ross, J. L. Ambrus, J. E. Sokal, H. A. Velasco, L. Stutzman, and D. V. Razis, *ibid.*, **18,** 27(1962).

(16) L. Stutzman, D. V. Razis, J. E. Sokal, J. L. Ambrus, and C. A. Ross, *ibid.*, **18,** 31(1962).

(17) B. G. Delta, C. M. Ambrus, J. L. Ambrus, and L. Stutzman, *ibid.*, **18,** 37(1962).

(18) N. Back, J. L. Ambrus, A. E. Munson, T. J. Bardos, F. F. Foldes, and V. Foldes, *Proc. Amer. Ass. Cancer Res.*, **3,** 301(1962).

(19) F. F. Foldes, J. L. Ambrus, N. Back, T. J. Bardos, and V. Foldes, *Fed. Proc.*, **21,** 335(1962).

(20) N. Back, T. J. Bardos, Z. F. Chmielewicz, E. Achter, and A. E. Munson, *Life Sci.*, **3,** 803(1964).

(21) C. A. Ross, H. A. Velasco, J. E. Sokal, J. L. Ambrus, L. Stutzman, J. Webster, and T. J. Bardos, *Proc. Amer. Ass. Cancer Res.*, **3,** 355(1962).

(22) H. A. Velasco, C. A. Ross, J. H. Webster, J. E. Sokal, L. Stutzman, and J. L. Ambrus, *Cancer*, **17,** 841(1964).

(23) J. H. Webster, J. Sokal, C. A. Ross, H. A. Velasco, J. L. Ambrus, and L. Stutzman, to be published.

(24) T. J. Bardos, *Biochem. Pharmacol.*, **11,** 256(1962).

(25) T. J. Bardos, Z. F. Chmielewicz, and K. Navada, *J. Pharm. Sci.*, **54,** 399(1965).

(26) F. H. Dickey, W. Pickett, and H. J. Lucas, *J. Amer. Chem. Soc.*, **74,** 944(1952).

(27) T. J. Bardos, N. Datta-Gupta, P. Hebborn, and D. J. Triggle, *J. Med. Chem.*, **8,** 167(1965).

(28) R. Chirardell and H. J. Lucas, *J. Amer. Chem. Soc.*, **77,** 106(1955).

(29) S. Gabriel and H. Ohle, *Ber.*, **50,** 804(1917).

(30) T. J. Bardos, Z. F. Chmielewicz, and P. Hebborn, *Ann. N. Y. Acad. Sci.*, **163,** 1006(1969).

(31) E. Allen and W. Seaman, *Anal. Chem.*, **27,** 540(1955).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 8, 1969, from the *Departments of Medicinal Chemistry and Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo, NY 14214*

Accepted for publication February 24, 1970.

Abstracted in part from a thesis submitted by C. K. Navada to the State University of New York at Buffalo in partial fulfillment of the Doctor of Philosophy degree requirements.

This research was supported by U. S. Public Health Service Research Grant No. RO1-CA 06695 from the National Cancer Institute, National Institutes of Health, Bethesda, Md.

* To whom inquiries should be addressed.

Metabolic Fate of Mineral Oil Adjuvants Using ^{14}C-Labeled Tracers I: Mineral Oil

JAMES N. BOLLINGER

Abstract □ This investigation was undertaken to determine the metabolic fate of injected mineral oil using *n*-hexadecane as the tracer. Female white rats and female squirrel monkeys were injected either subcutaneously or intramuscularly with an emulsion containing 1-^{14}C-*n*-hexadecane made with mineral oil and mannide monooleate. The amount of radioactivity remaining at the site of injection and the distribution of radioactivity in the major organs were determined at varying time periods up to 10 months after injection. One week after injection, 85–98% of the 1-^{14}C-*n*-hexadecane-labeled mineral oil remained at the site of injection. After 3 months, 50–60% of the mineral oil remained; and after 10 months, approximately 25–30% of the labeled mineral oil was still at the site of injection. The mineral oil tracer, which migrated from the site of injection, was readily incorporated into lipids. Aside from the site of injection, there did not appear to be any accumulation of the 1-^{14}C-*n*-hexadecane-labeled mineral oil in the major organs.

Keyphrases □ Mineral oil—metabolic fate □ 1-^{14}C-*n*-Hexadecane–mineral oil–mannide monooleate emulsion—distribution □ Radioactivity accumulation—labeled mineral oil injection, monkeys, rats □ Scintillometry—analysis □ TLC—analysis

Mineral oil emulsions are used in many bacterial and viral preparations in present experimental immunization programs. However, little is known concerning the metabolic fate of the mineral oil. Many observations, both present and in the past, show that mineral oil emulsions are not always well tolerated (1). The safety of mineral oil adjuvants has been questioned on the basis of data that indicate mineral oil alone or Freund's (2) complete or incomplete mineral oil adjuvant, given with or without added antigens, may cause or be associated with induction of plasma cell tumors, autoimmune reactions, or excessive formation of focal granulomata (3–6). Some of the questions that have arisen from these observations are whether these adverse effects are a function of an incapability of animals to mobilize and metabolize mineral oil, adverse cellular effects due to emulsifying agents, or the result of bacterial and viral biochemical action on the adjuvant preparation itself or at the site of injection.

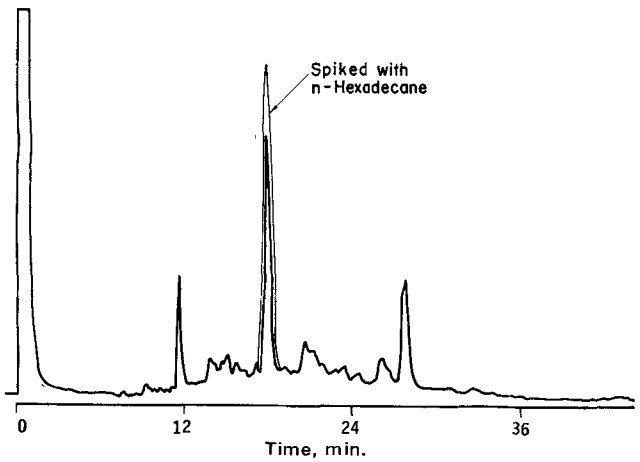


Figure 1—*Gas-liquid chromatograph of mineral oil. Material, mineral oil; column 45.72 m. (150 ft.) capillary coated with Apiezon L; and column temperature, 200°.*

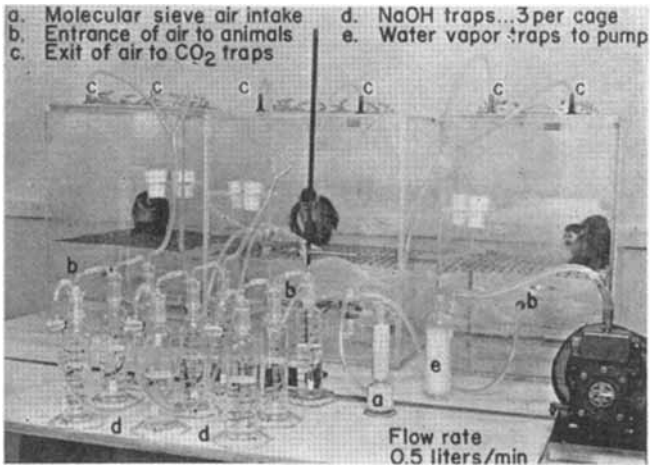


Figure 2—*Plexiglas metabolism cages designed for monkeys.*

Several investigators have attempted to answer some of these questions. Peck *et al.* (7) and Woodhour *et al.* (8) have substituted a vegetable oil for mineral oil in their adjuvant preparation. These investigators reported that the clinical, gross, and histomorphologic reactions were much less than were those obtained in comparable test with Freund's (2) incomplete mineral oil adjuvant. These data, however, could be interpreted to reflect that the ability to metabolize the vegetable adjuvant oils was a factor in lowering toxicity. However, since the extent of metabolism was not measured, no definite conclusion could be drawn. On the other hand, several investigators have shown that straight-chain hydrocarbons (C-16–C-18) are metabolized to fatty acids (9–11).

Investigations by Berlin (12) involving a nonionic emulsifier[1] indicated that the toxic reactions of mineral oil emulsions may be, in part or in whole, related to the level of ester hydrolysis and oxidative changes of the emulsifier. More recently, Hardegree and Pittman (13) have shown that the emulsified tetanus vaccines, which caused sterile abscesses in humans, contained free fatty acids and that a variety of antigens were capable of releasing free fatty acids from a water emulsion of mannide monooleate. At any rate, it would appear that emulsions containing vegetable oil instead of mineral oil would yield as high or higher free fatty acid levels with added antigens. Peck *et al.* (7), however, found less toxicity (guinea pig dermal irritation and mouse peritoneal irritation) with peanut oil than with mineral oil in influenza virus vaccine preparations.

[1] Arlacel A, Atlas Chemical Industries, Inc., Wilmington, DE 19899

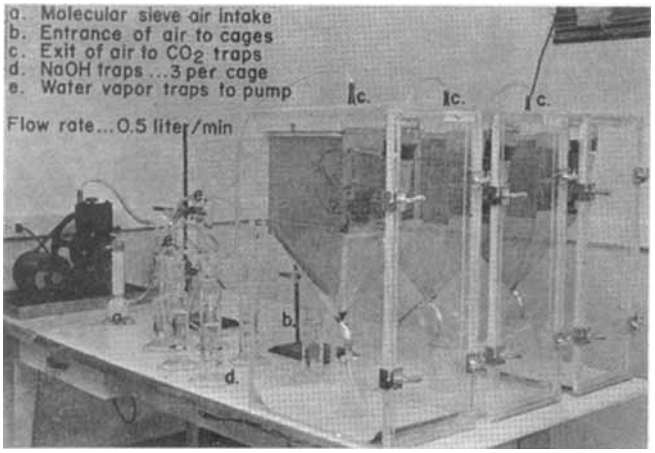


Figure 3—*Plexiglas metabolism cages designed for rats.*

It is the purpose of this study to determine the metabolic fate of the mineral oil in an injected adjuvant emulsion using a major mineral oil component, *n*-hexadecane, as the tracer.

EXPERIMENTAL

Adjuvant Components—A gas-liquid chromatographic analysis of mineral oil[2] is shown in Fig. 1.

TLC analysis of the surfactant, mannide monooleate,[3] shows this material to contain at least 15 components. Identification of the various components has recently been accomplished by O'Neill and Yamauchi (14).

The emulsion used in this study was prepared by using 1 volume surfactant, 9 volumes mineral oil, and 9 volumes water. The resulting emulsions, prepared either by sonic vibration or the double-hubbed needle, double-syringe method, passed the suggested tests for stability (1). However, to eliminate any difficulties with emulsion stability in this investigation, all emulsions were administered within 30 min. after preparation.

^{14}C-Labeled Tracers—A representative ^{14}C-labeled paraffin (*n*-hexadecane) was selected to serve as the ^{14}C-tracer on the basis of its similarity to one of the major components of the mineral oil (Fig. 1), as well as its ability to serve as an effective adjuvant (15). Tritium-labeled materials were considered but are less desirable, since there always exists the possibility of tritium-exchange reactions which invariably complicate interpretation of experimental data (9).

The 1-^{14}C-*n*-hexadecane[4] used in this study had a specific activity of 2.35 mc./mmole. The radiochemical purity was determined with the aid of TLC and a liquid-scintillation spectrometer.[5] Hexane was used to develop and resolve the 1-^{14}C-*n*-hexadecane on thin-layer plates coated with 125 μ of silica gel.[6] Removal of a 3% impurity was accomplished by using a 5 × 0.5-cm. silicic acid column. The impurity remained on the column after elution of the 1-^{14}C-*n*-hexadecane with glass-distilled hexane. Subsequent analysis indicated that there was less than 0.001% radioactive impurity remaining in the 1-^{14}C-*n*-hexadecane tracer.

Emulsions containing the radioactive tracer were prepared by prior mixing of the 1-^{14}C-*n*-hexadecane tracer into the unlabeled mineral oil.

Metabolism Studies—Two types of experimental animals were used in this investigation: albino female rats[7] weighing approximately 250 g. and female squirrel monkeys[8] weighing 500–700 g. A total of 36 rats and 36 monkeys was used.

The dose was administered into the right rear thigh, either subcutaneously or intramuscularly. Prior to injection, several 1-μl. samples were removed from the ^{14}C-labeled emulsion, and a check was made on the distribution uniformity of the tracer within the emulsion. The size of dose administered was 0.1 ml. (approximately 5 μc.) of the emulsion for rats and 0.3 ml. (approximately 15 μc.) for monkeys. The dose was administered with a 1-ml. disposable syringe equipped with a 23-gauge needle. A similar but unlabeled dose of the emulsion was injected into the left thigh. The control legs were examined by a veterinary pathologist during the study and immediately upon death.

After injection of the radioactive emulsions, the animals on 1-, 2-, and 7-day experimental periods were immediately housed in Plexiglas metabolism cages (Figs. 2 and 3). These cages were constructed in such a manner as to allow reasonable freedom of movement and easy access to water and food. The cages were opened for 15–20 min. every 24 hr. for cleaning and to replenish food and water.

In the long-term studies, 1, 2, 3, and 10 months, animals were housed in metal metabolism cages which allowed monitoring of urine and feces but not CO_2.

After the 1-, 2-, or 7-day experimental periods or the 1-, 2-, 3-, or 10-month experimental periods, the animals were killed by exsanguination following anesthetization with sodium pentobarbital. All animals were immediately examined by gross necropsy with particular emphasis placed on the site of injection of an unlabeled emulsion in the left, or control, leg.

To ensure complete removal of the site of injection for radioactivity determinations, entire thighs were excised. The thighs were then placed in a 600-ml. stainless steel beaker, frozen with liquid nitrogen, and shattered with a steel 5.08-cm. (2-in.) engine valve and a hammer. This technique produced a very fine powder out of the muscle, bone, and skin. The crushed legs were then placed into pint jars; 100 ml. of a 2:1 chloroform–methanol solution was added, and the mixture was homogenized with a high-speed blender.[9] After separation into two phases (methanol–water and chloroform), the two phases and the residue were analyzed for radioactivity.

A portion of the major organs of both monkeys and rats was removed for the determination of the radioactivity. Major organs examined were liver, depot fat, spleen, kidney, small intestine, ovary, lung, and, in some cases, the heart, blood, and brain. The inguinal lymph nodes of monkeys were also removed and assayed for radioactivity.

Leg extracts as well as some organ extractions were fractionated by TLC, and attempts were made to ascertain the nature of radioactivity present by the migration on thin-layer plates relative to known standard materials.

Radioactivity Determinations—Respired $^{14}CO_2$ was trapped in NaOH as Na_2CO_3 and then precipitated as $BaCO_3$. The $BaCO_3$ was

Table I—Percentage of ^{14}C-Tracer Remaining in Monkeys and Rats at the Site of Injection after the Administration of Mineral Oil–Mannide Monooleate Emulsion Containing 1-^{14}C-Hexadecane as a Tracer

Route and Time after Injection[a]	Average Percentage Remaining Monkeys, %[b]		Rats, %[b]	
IM, 1 day	3[c]	99.0	3[c]	91.2
Sub Q, 1 day	3	96.8	3	100.0
IM, 2 days	3	98.2	3	86.6
Sub Q, 2 days	2	91.5	3	85.2
IM, 7 days	3	98.8	3	85.2
Sub Q, 7 days	3	96.1	3	89.5
IM, 1 month	2	63.9	2	75.4
Sub Q, 1 month	2	66.9	2	67.3
IM, 2 months	3	77.2	2	77.9
Sub Q, 2 months	2	81.7	2	40.0
IM, 3 months	2	65.9	2	57.9
Sub Q, 3 months	2	61.8	2	49.4
IM, 7 months	1	22.7	—	—
IM, 10 months	4	29.1	6	27.1

[a] IM, intramuscular, and Sub Q, subcutaneous injections. [b] Average percentage based on the total amount recovered and the total amount administered. [c] Number of animals.

[2] Drakeol 6-VR, Pennsylvania Refining Co., Butler, Pa.
[3] Hilltop Laboratories, Inc., Cincinnati, Ohio.
[4] Mallinckrodt Nuclear, St. Louis, MO 63160
[5] Packard Instrument Co, Inc.
[6] Silica gel F254 (Brinkmann), silica gel G (Analabs).
[7] Simonsen Laboratories, Calif.
[8] Charles Chase Corp., Fla.
[9] Omni-Mixer, Ser Vall.

Table II—Thin-Layer Chromatographic Separation and Liquid-Scintillation Counting of the Radioactive Material Remaining at the Site of Injection after 3 and 10 Months

TLC[a] Fraction	TLC Area	3 Months				10 Months,
		IM[b] Rats	IM Monkeys	Sub Q Rats	Sub Q Monkeys	IM Monkeys
		Percentage Values[c]				
1	Phospholipid	1.6	0.2	2.6	0.1	0.4
2	Free sterol	0.4	0.0	0.4	0.0	0.0
3	Free fatty acid	0.6	1.0	1.5	0.2	0.0
4	Triglyceride	3.8	0.6	3.3	0.3	0.0
5	Sterol ester	0.8	0.3	1.0	0.2	4.0
6	Hydrocarbon	92.7	97.6	91.0	98.8	95.7

[a] Pooled leg extracts were applied to silica gel-coated TLC plates and developed first with hexane (to resolve hydrocarbons) and then with 90:10:1 hexane–diethyl ether–acetic acid to resolve lipids. [b] IM, intramuscular injection and Sub Q, subcutaneous injection, of the right thigh. [c] Average percentage of duplicate determinations of the radioactivity recovered from thin-layer chromatographic plates.

Table III—Average Level of Radioactivity in the Major Organs of Monkeys after the Administration of a Mineral Oil–Mannide Monooleate Emulsion Containing 1-^{14}C-*n*-Hexadecane as a Tracer

Time after Injection	Number of Animals	Average Counts/min./100 mg. Wet Tissue							
		Liver	Fat	Spleen	Kidney	Small Intestine	Ovary	Lung	Lymph
1 day	6	142	54	18	11	26	16	21	13
2 days	6	695	472	40	67	88	31	49	27
7 days	6	1232	1701	161	183	130	112	204	46
1 month	4	316	2606	222	397	135	137	324	80
2 months	5	49	1949	59	77	72	71	41	181
3 months	4	144	1596	98	147	68	52	123	20
10 months	4	9	462	15	15	13	16	12	28

Table IV—Average Level of Radioactivity in the Major Organs of Rats after the Administration of a Mineral Oil–Mannide Monooleate Emulsion Containing 1-^{14}C-*n*-Hexadecane as a Tracer

Time after Injection	Number of Animals	Average Counts/min./100 mg. Wet Tissue						
		Liver	Fat	Spleen	Kidney	Small Intestine	Ovary	Lung
1 day	6	34	16	10	14	8	5	14
2 days	6	145	92	56	46	74	46	47
7 days	6	132	365	60	69	53	73	57
1 month	4	87	555	54	114	119	45	24
2 months	4	30	717	25	36	23	109	14
3 months	4	82	2263	40	103	28	82	36
10 months	6	10	647	5	9	12	26	8

removed from the aqueous phase by filtration and dried in an oven at 120° for a minimum of 6 hr. After $BaCO_3$ dried, it was pulverized to a fine powder. Approximately 50-mg. samples were removed and suspended in a scintillation-gel (16) for radioactivity determination. The liquid-scintillation counting efficiency under the study conditions was 86%. Total radioactivity removed *via* respiratory $^{14}CO_2$ was then calculated from the total CO_2 collected over 24-hr. experimental periods.

Urine and feces collected over 24-hr. experimental periods for the 1st week after administration were also assayed for radioactivity. The amount of radioactivity in the feces was determined by a perchloric acid–hydrogen peroxide digestion of 100-mg. samples (17). Radioactive urine was determined by using Bray's method (18). One milliliter of urine was counted, and the total activity eliminated *via* the urine was calculated from total urine volumes.

Radioactivity in the major organs, including blood and feces, was determined by the method of Mahin and Lofberg (17). This method involves perchloric acid digestion of 100-mg. wet tissue samples (0.2 ml. blood). The color is removed from the digested samples with hydrogen peroxide. These samples were then taken up in 20 ml. toluene and ethylene glycol–monoethyl ether (Cellosolve), 2:1, containing the scintillator PPO (6 g./l. counting solution) and counted. Counting efficiency in this system was about 53%.

The fractions obtained from serial scraping of thin-layer chromatographic plates were assayed for radioactivity in the gel-scintillation system as described for $Ba^{14}CO_3$ determinations. Serial scrapings were obtained by the aid of mineral oil and lipid standards[10] and I_2 vapor visualization.

[10] Applied Science Laboratories, State College, PA 16801

RESULTS

The amount of 1-^{14}C-*n*-hexadecane remaining at the site of injection from 1 to 10 months after administration is summarized in Table I. In general, the mineral oil was slowly removed from the site of injection. In monkeys, 96–98% was still present after 1 week, while in rats the amount remaining at the site of injection ranged from 85 to 90% after 1 week. After 1 month, approximately 65–75% remained, and by 3 months both groups of animals had retained 50–60% of the total radioactivity at the site of injection. Ten months after injection, approximately 25–30% was still at the site of injection.

Several mathematical equations (linear and semilogarithmic derived equations) were prepared to predict the length of time required to remove all of the oil from the site of injection. However, since there were too few animals at each time point as well as a considerable amount of scatter of the data, it was found that a confident mathematical prediction could not be made.

Virtually all (95–99%) of the activity remaining at the site of injection in monkeys after 3 and 10 months was present in the hydrocarbon fraction (Table II). On the other hand, 3–4% of the radioactivity found in the legs of rats had been incorporated into triglycerides with 91–93% still associated with the hydrocarbon fraction. A reason for this difference was that the rats had a larger amount of depot fat (triglycerides) in the thigh area than did the monkeys. In both animal groups, a certain amount of radioactivity (1–3%) also was associated with the phospholipids of the leg muscles. McCarthy (11) demonstrated that C-16 and C-18 hydrocarbons were converted *in vivo* into fatty acids of the same chain lengths, suggesting that the incorporation of radioactivity into the various leg lipid fractions was the result of 1-^{14}C-*n*-hexadecane

Table V—Distribution of Radioactivity from 1-^{14}C-*n*-Hexadecane Tracer in Major Lipid Classes of Monkey Tissue 2 Days after Administration

Lipid Classes	Organs					
	Liver, %[a]	Fat, %	Kidney, %	Spleen, %	Ovaries, %	Leg, %
Phospholipids	19.6	0	10.6	0	0	0
Free fatty acids	45.6	0	7.4	18.6	10.0	0
Triglycerides	4.4	57.7	7.4	0	0	0
Hydrocarbons	30.5	42.3	74.6	81.4	90.0	100.0
Radioactivity, average c.p.m./100 mg. tissue	525.0	831.0	58.0	30.0	39.0	

[a] These percents were obtained from serial scraping of TLC and based on the total radioactivity applied to the plates.

Table VI—Distribution of Radioactivity within the Various Lipid Classes of Organs of Monkeys 1 and 3 Months after Administration of a Mineral Oil–Mannide Monooleate Emulsion Containing 1-^{14}C-*n*-Hexadecane as a Tracer

TLC Fraction[b]	Tissues[a]								
	Liver, %	Fat, %	Spleen, %	Kidney, %	Intestine, %	Ovary, %	Lung, %	Brain, %	Lymph, %
1 Month									
Phospholipids	6.0	0.5	14.3	15.1	1.1	0.0	15.0	13.8	0.0
Free sterols	24.1	10.9	42.9	11.5	7.9	16.4	30.7	31.3	0.0
Free fatty acids	7.5	50.0	30.0	20.9	0.0	32.9	17.3	15.6	0.0
Triglycerides	32.3	23.5	5.7	5.0	9.6	27.4	5.5	20.6	18.2
Sterol esters	26.3	11.5	0.0	30.9	72.5	0.0	12.6	13.8	72.8
Hydrocarbon	3.8	3.7	7.2	13.7	9.0	23.3	18.9	5.0	9.1
3 Months									
Phospholipids	59.3	0.9	76.6	70.5	73.0	68.6	88.6	93.2	
Free sterols and free fatty acids	22.0	0.0	16.2	12.9	15.2	17.2	2.5	0.0	
Triglycerides	13.6	96.4	4.6	11.8	6.1	0.0	3.3	0.0	
Sterol esters	3.4	0.0	1.2	2.7	1.5	10.9	4.9	0.0	
Hydrocarbons	1.7	2.7	1.2	2.2	4.6	3.1	1.0	6.7	

[a] Pooled tissue extracts. [b] Serial scraping from TLC. Silica gel plates developed with hexane initially, then with hexane–diethyl ether–acetic acid (90:10:1) system.

Table VII—Elimination of Respiratory $^{14}CO_2$ for 1 Week after the Injection of a Mineral Oil–Mannide Monooleate Emulsion Containing 1-^{14}C-*n*-Hexadecane

Animal	Route of Injection	Days after Injection							
		1	2	3	4	5	6	7	Total
		Percentage Values[a]							
Monkeys	IM	0.01(9)[b]	0.16(6)	0.31(3)	0.33(3)	0.25(3)	0.19(3)	0.28(3)	1.53(3)
	Sub Q	0.01(6)	0.16(5)	0.24(2)	0.28(2)	0.37(2)	0.33(2)	0.25(3)	1.66(3)
Rats	IM	0.05(9)	0.18(6)	0.07(3)	0.10(3)	0.08(3)	0.12(3)	0.10(3)	0.55(3)
	Sub Q	0.12(9)	0.11(6)	0.13(3)	0.32(3)	0.22(3)	0.14(3)	0.17(3)	1.20(3)

[a] Each percentage value represents an average percent of the total radioactive dose eliminated as $^{14}CO_2$ over 24-hr. periods. [b] Numbers in parentheses represent the number of individual determinations.

mobilization to the liver, where it was converted to fatty acids and returned in the form of various lipid classes to leg muscle tissue.

Tables III and IV show the average level of radioactivity found in the major organs of monkeys and rats, respectively, after the administration of a mineral oil mannide monooleate emulsion containing 1-^{14}C-*n*-hexadecane tracer. After the first 24 hr., all organs tested contained radioactivity. The liver had the highest activity of any organ 24 hr. after injection. Two days after injection of the emulsion containing 1-^{14}C-*n*-hexadecane, the level of radioactivity in the liver increased four- to fivefold, as did the level in the depot fat. The radioactivity in all other organs increased to varying degrees. Radioactivity in the depot fat of both animals continued to increase until it became about fourfold over that amount present after 2 days. After 1 month, the amount of radioactivity in the liver of both monkeys and rats decreased; after 2 months, it had returned to those levels recorded for the first 24-hr. period after injection. The specific activity of the depot fat remained high throughout the 3-month period. After 10 months, the radioactivity in the depot fat had decreased to about one-fourth the level measured at 3 months. However, the level of radioactivity in the depot fat was still 50- to 100-fold greater than any other organ. Other than the liver and depot fat, no organ appeared to accumulate large amounts of radioactivity. Most of the detectable radioactivity in the various organs resided in the fat-soluble fraction, with little or none appearing in the water-soluble and residue fractions.

Table V shows the distribution of radioactivity in chloroform–methanol extracts of various organs taken from monkeys 2 days after the administration of an emulsion containing 1-^{14}C-*n*-hexadecane. These data indicate that a substantial portion of the radioactivity in the liver (30%), fat (42%), kidney (74%), spleen (81%), and ovary (90%) was unmetabolized 1-^{14}C-*n*-hexadecane tracer. The remaining radioactivity was located in the various lipid classes such as phospholipids, free fatty acids, and triglycerides, indicating that hexadecane was undergoing metabolism.

By 1 month, however, the amount of radioactivity in the various organs associated with 1-^{14}C-*n*-hexadecane was greatly reduced, and most of the radioactivity (80–95%) was associated with the major lipid classes. After 3 months the major lipid classes contained as much as 95–98% of the radioactivity (Table VI).

Only a small amount of radioactivity from 1-^{14}C-*n*-hexadecane was eliminated the 1st week in the respiratory CO_2 by either monkeys or rats (Table VII). The amount eliminated per day varied considerably among animals, but it was usually less than 0.2% and, in many cases, was as little as 0.01%. As a result, the average total amount

eliminated by respiratory $^{14}CO_2$ the 1st week ranged from 0.5 to 2.0%. The major reason for these low levels was that this material was only slowly mobilized from the site of injection, and its conversion to fatty acids and subsequent CO_2 was limited by the rate of mobilization. There was little or no difference in the rate of mobilization from subcutaneous and intramuscular injections.

Some radioactivity from 1-^{14}C-*n*-hexadecane was eliminated in the urine and feces during the 1st week after administration; however, in both groups of animals, the total amount for the entire week was less than 0.01%.

All animals received an unlabeled dose of the emulsion in the opposite leg. Upon death, each of the control legs was immediately examined by gross necropsy by a veterinary pathologist. At no time was there any indication of any type of infection or a sterile abscess due to the injection.

SUMMARY

This investigation has shown that the *n*-hexadecane component and most likely other straight-chain hydrocarbon components of a mineral oil adjuvant emulsion are very slowly mobilized from the site of injection in monkeys and rats. Results have been presented which show that as much as 25–30% of the ^{14}C-*n*-hexadecane tracer remains at the site of injection after 10 months. The *n*-hexadecane tracer, which is mobilized from the site of injection, was readily metabolized to naturally occurring lipids.

Gross necropsy did not reveal any evidence of any pathological states at the sites of injection.

REFERENCES

(1) Department of Health, Education, and Welfare, Food and Drug Administration, *Ann. Allergy*, **23,** 558(1965).

(2) J. Freund, *J. Advan. Tuberc. Res.*, **7,** 130(1956).

(3) M. Potter and C. L. Robertson, *J. Nat. Cancer Inst.*, **28,** 847(1960).

(4) R. Lieberman, N. Mantel, and W. Humphrey, Jr., *Proc. Soc. Exp. Biol. Med.*, **107,** 163(1961).

(5) S. Levine and E. J. Wenk, *ibid.*, **113,** 898(1963).

(6) J. W. Steiner, B. Langer, and D. L. Schatz, *Arch. Pathol.*, **70,** 424(1960).

(7) H. M. Peck, A. F. Woodhour, T. B. Stein, S. E. McKinney, and M. R. Hilleman, *Proc. Soc. Exp. Biol. Med.*, **116,** 23(1964).

(8) A. F. Woodhour, D. P. Metzgar, T. B. Stein, A. A. Tytell, and M. R. Hilleman, *ibid.*, **116,** 516(1964).

(9) A. G. Ebert, C. R. Schleifer, and S. M. Hess, *J. Pharm. Sci.*, **55,** 923(1966).

(10) M. P. Mitchell and G. Hubscher, *Biochem. J.*, **103,** 23(1967).

(11) R. D. McCarthy, *Biochim. Biophys. Acta*, **84,** 74(1964).

(12) B. S. Berlin, *Ann. Allergy*, **20,** 472(1962).

(13) M. C. Hardegree and M. Pittman, *Proc. Soc. Exp. Biol. Med.*, **123,** 179(1966).

(14) H. J. O'Neill and T. N. Yamauchi, *J. Amer. Oil Chem. Soc.*, **46,** 99A(1969).

(15) A. J. Crowle and C. C. Hu, *Proc. Soc. Exp. Biol. Med.*, **123,** 94(1966).

(16) J. N. Bollinger, W. A. Mallow, J. W. Register, Jr., and D. E. Johnson, *Anal. Chem.*, **19,** 1508(1967).

(17) D. T. Mahin and R. T. Lofberg, *Anal. Biochem.*, **16,** 500 (1966).

(18) G. A. Bray, *ibid.*, **1,** 279(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 15, 1969, from the *Southwest Research Institute, San Antonio, TX 78228*

Accepted for publication February 26, 1970.

This investigation was supported by the Division of Biologics Standards, National Institutes of Health, Contract No. PH 43-67-1104.

The technical assistance throughout the program of Dennis Schmidt was appreciated.

Metabolic Fate of Mineral Oil Adjuvants Using ^{14}C-Labeled Tracers II: Mannide Monooleate

JAMES N. BOLLINGER

Abstract □ This investigation was undertaken to determine the metabolic fate of mannide monooleate when employed in a mineral oil emulsion. Female white rats and female squirrel monkeys were injected subcutaneously or intramuscularly with an emulsion made with mineral oil and surfactant and including either 1-^{14}C-oleate or UL-^{14}C-mannide-labeled mannide monooleate tracer preparations. It was shown that 30–40% of the surfactant mixture is removed from the site of injection after 24 hr. After 1 week, 40–60% of the surfactant is removed from the site of injection; while after 3 months, 10–30% of the surfactant still remains. The 1-^{14}C-oleate-labeled mannide monooleate was largely incorporated into the various lipid classes, while the UL-^{14}C-mannide-labeled mannide monooleate preparation was largely eliminated in the urine. There was some indication that the inguinal lymph nodes of monkeys may have contained unusually large amounts of radioactivity.

Keyphrases □ 1-^{14}C-Oleate-labeled mannide monooleate—metabolic fate □ UL-^{14}C-Mannide-labeled mannide monooleate—metabolic fate □ Mineral oil–mannide monooleate emulsion—subcutaneous, intramuscular injection □ Metabolic fate—mannide monooleate in mineral oil emulsion □ TLC—identity

Freund (1) found that "mannide monooleate"[1] was an effective agent in combining antigens and mineral oils in the form of a water and oil emulsion. However, subsequent use has indicated an infrequent occurrence of cysts following injection of vaccines and allergens (2) made with certain lots of this surfactant. Berlin (3) performed studies which showed that the toxic reactions of a mineral oil–"mannide monooleate" emulsion may be, in part or in whole, related to the level of ester hydrolysis and oxidative changes of this material. Recently, Hardegree and Pittman (4) have shown that the tetanus vaccines which caused sterile abscesses in humans contained free fatty acids and that a variety of these antigens were capable of releasing free fatty acids from a water emulsion of "mannide monooleate."

This study was undertaken to obtain information on the mobilization and metabolism of a mannide monooleate surfactant preparation when it is received as an injected mineral oil emulsion.

[1] Arlacel A, Atlas Chemical Industries, Inc., Wilmington, DE 19899. Quotation marks in text of article indicate the impure mixture; absence of quotation marks in text of article indicates pure mannide monooleate.

eliminated by respiratory $^{14}CO_2$ the 1st week ranged from 0.5 to 2.0%. The major reason for these low levels was that this material was only slowly mobilized from the site of injection, and its conversion to fatty acids and subsequent CO_2 was limited by the rate of mobilization. There was little or no difference in the rate of mobilization from subcutaneous and intramuscular injections.

Some radioactivity from 1-^{14}C-*n*-hexadecane was eliminated in the urine and feces during the 1st week after administration; however, in both groups of animals, the total amount for the entire week was less than 0.01%.

All animals received an unlabeled dose of the emulsion in the opposite leg. Upon death, each of the control legs was immediately examined by gross necropsy by a veterinary pathologist. At no time was there any indication of any type of infection or a sterile abscess due to the injection.

SUMMARY

This investigation has shown that the *n*-hexadecane component and most likely other straight-chain hydrocarbon components of a mineral oil adjuvant emulsion are very slowly mobilized from the site of injection in monkeys and rats. Results have been presented which show that as much as 25–30% of the ^{14}C-*n*-hexadecane tracer remains at the site of injection after 10 months. The *n*-hexadecane tracer, which is mobilized from the site of injection, was readily metabolized to naturally occurring lipids.

Gross necropsy did not reveal any evidence of any pathological states at the sites of injection.

REFERENCES

(1) Department of Health, Education, and Welfare, Food and Drug Administration, *Ann. Allergy*, **23,** 558(1965).

(2) J. Freund, *J. Advan. Tuberc. Res.*, **7,** 130(1956).

(3) M. Potter and C. L. Robertson, *J. Nat. Cancer Inst.*, **28,** 847(1960).

(4) R. Lieberman, N. Mantel, and W. Humphrey, Jr., *Proc. Soc. Exp. Biol. Med.*, **107,** 163(1961).

(5) S. Levine and E. J. Wenk, *ibid.*, **113,** 898(1963).

(6) J. W. Steiner, B. Langer, and D. L. Schatz, *Arch. Pathol.*, **70,** 424(1960).

(7) H. M. Peck, A. F. Woodhour, T. B. Stein, S. E. McKinney, and M. R. Hilleman, *Proc. Soc. Exp. Biol. Med.*, **116,** 23(1964).

(8) A. F. Woodhour, D. P. Metzgar, T. B. Stein, A. A. Tytell, and M. R. Hilleman, *ibid.*, **116,** 516(1964).

(9) A. G. Ebert, C. R. Schleifer, and S. M. Hess, *J. Pharm. Sci.*, **55,** 923(1966).

(10) M. P. Mitchell and G. Hubscher, *Biochem. J.*, **103,** 23(1967).

(11) R. D. McCarthy, *Biochim. Biophys. Acta*, **84,** 74(1964).

(12) B. S. Berlin, *Ann. Allergy*, **20,** 472(1962).

(13) M. C. Hardegree and M. Pittman, *Proc. Soc. Exp. Biol. Med.*, **123,** 179(1966).

(14) H. J. O'Neill and T. N. Yamauchi, *J. Amer. Oil Chem. Soc.*, **46,** 99A(1969).

(15) A. J. Crowle and C. C. Hu, *Proc. Soc. Exp. Biol. Med.*, **123,** 94(1966).

(16) J. N. Bollinger, W. A. Mallow, J. W. Register, Jr., and D. E. Johnson, *Anal. Chem.*, **19,** 1508(1967).

(17) D. T. Mahin and R. T. Lofberg, *Anal. Biochem.*, **16,** 500 (1966).

(18) G. A. Bray, *ibid.*, **1,** 279(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 15, 1969, from the *Southwest Research Institute, San Antonio, TX 78228*

Accepted for publication February 26, 1970.

This investigation was supported by the Division of Biologics Standards, National Institutes of Health, Contract No. PH 43-67-1104.

The technical assistance throughout the program of Dennis Schmidt was appreciated.

Metabolic Fate of Mineral Oil Adjuvants Using ^{14}C-Labeled Tracers II: Mannide Monooleate

JAMES N. BOLLINGER

Abstract □ This investigation was undertaken to determine the metabolic fate of mannide monooleate when employed in a mineral oil emulsion. Female white rats and female squirrel monkeys were injected subcutaneously or intramuscularly with an emulsion made with mineral oil and surfactant and including either 1-^{14}C-oleate or UL-^{14}C-mannide-labeled mannide monooleate tracer preparations. It was shown that 30–40% of the surfactant mixture is removed from the site of injection after 24 hr. After 1 week, 40–60% of the surfactant is removed from the site of injection; while after 3 months, 10–30% of the surfactant still remains. The 1-^{14}C-oleate-labeled mannide monooleate was largely incorporated into the various lipid classes, while the UL-^{14}C-mannide-labeled mannide monooleate preparation was largely eliminated in the urine. There was some indication that the inguinal lymph nodes of monkeys may have contained unusually large amounts of radioactivity.

Keyphrases □ 1-^{14}C-Oleate-labeled mannide monooleate—metabolic fate □ UL-^{14}C-Mannide-labeled mannide monooleate—metabolic fate □ Mineral oil–mannide monooleate emulsion—subcutaneous, intramuscular injection □ Metabolic fate—mannide monooleate in mineral oil emulsion □ TLC—identity

Freund (1) found that "mannide monooleate"[1] was an effective agent in combining antigens and mineral oils in the form of a water and oil emulsion. However, subsequent use has indicated an infrequent occurrence of cysts following injection of vaccines and allergens (2) made with certain lots of this surfactant. Berlin (3) performed studies which showed that the toxic reactions of a mineral oil–"mannide monooleate" emulsion may be, in part or in whole, related to the level of ester hydrolysis and oxidative changes of this material. Recently, Hardegree and Pittman (4) have shown that the tetanus vaccines which caused sterile abscesses in humans contained free fatty acids and that a variety of these antigens were capable of releasing free fatty acids from a water emulsion of "mannide monooleate."

This study was undertaken to obtain information on the mobilization and metabolism of a mannide monooleate surfactant preparation when it is received as an injected mineral oil emulsion.

[1] Arlacel A, Atlas Chemical Industries, Inc., Wilmington, DE 19899. Quotation marks in text of article indicate the impure mixture; absence of quotation marks in text of article indicates pure mannide monooleate.

Table I—Distribution of Radioactivity among the Various Components of UL-^{14}C-Mannide Oleate and 1-^{14}C-Oleate Mannide

Plate Area[c]	Plate I[a] Surfactant[b] Mannide UL-^{14}C, %[d]	Plate I[a] Surfactant[b] Oleate 1-^{14}C, %[d]	Plate II[a] Surfactant[b] Mannide UL-^{14}C, %[d]	Plate II[a] Surfactant[b] Oleate 1-^{14}C, %[d]
a	12.0	6.0	2.9	0.5
b	19.0	2.0	0.8	0.0
c	3.4	7.0	5.7	3.0
d	3.5	8.4	17.9	1.0
e	6.6	7.9	1.2	2.0
f	2.5	8.1	3.8	6.4
g	1.2	2.9	3.9	7.4
h	4.9	3.2	4.1	9.3
i	5.9	9.6	3.1	8.6
j	11.7	24.0	1.3	2.7
k	7.0	7.8	25.2	38.1
l	21.1	13.1	30.1	20.9
m	1.0	0.0	—	—

[a] Refers to systems used in Fig. 1. [b] ^{14}C-Labeled preparations received from Atlas Chemical Industries. [c] Refers to TLC plate areas in Fig. 1. [d] Percentage based on the amount applied to the plates and subsequent serial scrapings of I_2 vapor visualized separation.

Table II—Percentage of ^{14}C-Tracer Remaining in Monkeys and Rats at the Site of Injection after Administration of Mineral Oil–"Mannide Monooleate" Emulsion Containing the 1-^{14}C-Oleate Mannide as a Tracer

Route and Time after Injection	Average Percentage Remaining[a] Monkeys	Rats
IM,[b] 1 day	(3)[c] 78.3	(3)[c] 80.0
Sub Q,[b] 1 day	(3) 82.5	(3) 74.0
IM, 2 days	(3) 77.2	(3) 70.9
Sub Q, 2 days	(3) 77.7	(3) 73.5
IM, 7 days	(3) 41.2	(3) 58.8
Sub Q, 7 days	(3) 41.3	(3) 59.0
IM, 1 month	(2) 49.4	(2) 30.0
Sub Q, 1 month	(2) 37.4	(2) 17.6
IM, 2 months	(2) 46.1	(2) 49.6
Sub Q, 2 months	(2) 30.7	(2) 20.8
IM, 3 months	(2) 35.1	(2) 20.7
Sub Q, 3 months	(2) 22.0	(2) 9.3

[a] Average percentages based on the amount injected. [b] IM, intramuscular; Sub Q, subcutaneous. [c] Numbers in parentheses indicate the number of experimental animals used to determine the percentage ^{14}C-tracer remaining.

EXPERIMENTAL

Adjuvant Components—The adjuvant emulsion used consisted of mineral oil,[2] "mannide monooleate," and water in proportions of 9 parts mineral oil, 1 part surfactant, and 9 parts water.

The surfactant preparation used was obtained from Hilltop Laboratories, Inc., Cincinnati, Ohio. TLC analysis showed the material to contain at least 12 components (Fig. 1). Identification of the various components is tentative in this investigation; however, O'Neill and Yamauchi (5), who recently identified many of the components present in this particular surfactant, indicated that a neutral fraction from the "mannide monooleate" consisted of at least 12 components characteristic of peracylated carbohydrate structures. The intermediate or moderately polar material contained 14 components of the mannide monooleate class. The polar fraction contained predominantly carbohydrate polymers, conjugated dienes, and cyclic fatty acids.

The emulsion used in this study was prepared as described previously (6), using either sonic vibration or a double-hubbed needle, double-syringe method.

^{14}C-Labeled Emulsion—Since the mannide monooleate preparation is not pure but a mixture of monooleate, dioleate, trioleate, *etc.*, and various positional isomers of these esters plus various isomeric forms of dehydrated mannitol and oleic acid, it was decided that the most representative tracer would be ^{14}C-labeled surfactant prepared in the same manner as that used for the preparation of the normally unlabeled material.

Two preparations[3] were used in this study. One was made from 1-^{14}C-oleic acid and unlabeled mannitol, while the other was made from UL-^{14}C-mannitol and unlabeled oleic acid. The specific activity of the 1-^{14}C-oleate mannide was 2.2 μc./mg., and that of the UL-^{14}C-mannide oleate was 2.1 μc./mg. Results of TLC analysis of "mannide monooleate" and radioactivity distribution analysis of the separated components of the two tracers are shown in Fig. 1 and Table I, respectively. The relative amounts of each of the components present in the ^{14}C-labeled material were roughly (visual analysis) the same as those present in the unlabeled material. The data indicate that about 50% of the labeled surfactant is esterified, while the remainder is nonesterified material (chemically altered mannitol and oleic acid).

Dose and Administration—The radioactive tracers were thoroughly mixed with unlabeled surfactant and the mineral oil emulsion prepared as described (6) and used immediately (within 30 min. after preparation).

The size of the dose was 0.1 ml. (approximately 5 μc.) of the emulsion for rats and 0.3 ml. (approximately 15 μc.) for monkeys. The dose was administered with a 1-ml. disposable syringe equipped with a 23-gauge needle. The dose was administered in the right thigh either subcutaneously or intramuscularly, depending upon the phase of the experiment. A total of 60 female white rats and 60 female squirrel monkeys were used in this investigation. Prior to injection, several 1-μl. samples were removed from the ^{14}C-labeled emulsions, and a check was made on the uniformity of distribution of radioactivity within the emulsion. An unlabeled emulsion preparation of the same volume was injected into the left thigh for pathological evaluation by a veterinary pathologist.

After injection of the radioactive emulsions, the animals on the short-term studies (1–7 days) were immediately housed in Plexiglas metabolism cages. In long-term studies (1–3 months), the animals

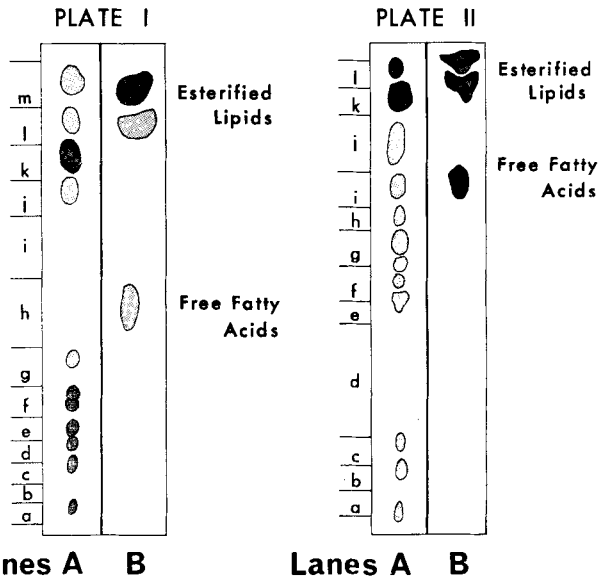


Figure 1—*Plate I shows the distribution of the components of "mannide monooleate" (Lane A) and the relative location of free fatty acids and esterified naturally occurring lipids (Lane B). The letters a–m indicate the components isolated for radioactivity determinations (Table I). The TLC system consisted of 100-μc. silica gel-coated plates developed in a chloroform–diethyl ether–acetic acid (95:5:1) system. Plate II depicts the same materials as Plate I but was developed in a more polar chloroform–diethyl ether–acetic acid (80:20:1) system. The letters depict the areas isolated for radioactivity determinations.*

[2] Drakeol 6-VR, Pennsylvania Refining Co., Butler, Pa.

[3] Both of these tracers were prepared by Atlas Chemical Industries, Inc., Wilmington, DE 19899, with a scale-down of procedures normally employed in the preparation of Arlacel A.

Table III—Percentage of ^{14}C-Tracer Remaining in Monkeys and Rats at the Site of Injection after Administration of Mineral Oil–"Mannide Monooleate" Emulsion Containing the UL-^{14}C-Mannide Oleate as a Tracer

Route and Time after Injection	Average Percentage Remaining[a] Monkeys	Rats
IM, 1 day	(3)[b] 55.0	(3)[b] 67.2
Sub Q, 1 day	(3) 59.5	(3) 68.1
IM, 2 days	(3) 57.3	(3) 56.4
Sub Q, 2 days	(3) 65.7	(3) 58.6
IM, 7 days	(3) 46.1	(3) 54.1
Sub Q, 7 days	(3) 48.5	(3) 52.3
IM, 1 month	(2) 42.1	(2) 30.7
Sub Q, 1 month	(2) 30.7	(2) 19.7
IM, 2 months	(2) 44.4	(2) 39.5
Sub Q, 2 months	(2) 31.9	(2) 17.8
IM, 3 months	(2) 12.3	(2) 13.0
Sub Q, 3 months	(2) 17.1	(2) 8.9

[a] Average percent of the administered radioactivity remaining at the site of injection. [b] Numbers in parentheses indicate the number of experimental animals used to determine the percentage of ^{14}C-tracer remaining.

Table IV—TLC Separation and Liquid-Scintillation Counting of the Radioactivity Remaining at the Site of Injection of Rats and Monkeys 3 Months after Injection of a Mineral Oil–"Mannide Monooleate" Emulsion Containing 1-^{14}C-Oleate Mannide and UL-^{14}C-Mannide Oleate

TLC[a] Fraction	1-^{14}C-Oleate Mannide Rats, %[b]	Monkeys, %	UL-^{14}C-Mannide Oleate Rats, %	Monkeys, %
a–b	4.3	2.6	9.2	3.4
c–g	8.8	5.6	11.1	5.7
h–i	6.3	2.5	6.1	1.2
j–k	21.7	17.7	19.5	12.0
l–m	57.7	71.0	52.1	77.3
Solvent front	1.6	1.2	2.4	0.4

[a] TLC system used consisted of silica gel-coated plates developed in a chloroform–diethyl ether–acetic acid (95:5:1) system. Fractions were scraped from the plates as indicated on Plate I, Fig. 1. [b] Average percentages obtained from the total amount of radioactivity applied to the plates.

were housed in metal metabolism cages which allowed monitoring of urine and feces but not CO_2. Collection of $^{14}CO_2$ was carried out as described in a previous study (6). The determination of mobilization and degree of metabolism were monitored as previously described (6).

RESULTS AND DISCUSSION

Table II shows the average percentage of 1-^{14}C-oleate mannide tracer preparation remaining at the site of injection 1 day to 3 months after administration. Approximately 20–25% of this tracer was removed from the site of injection of rats and monkeys the 1st day after both intramuscular (IM) and subcutaneous injection (Sub Q). Seven days after injection, however, 40% (rats) to 60% (monkeys) had been removed from both sites of injection. After 3 months, 22% (Sub Q) to 35% (IM) still remained at the site of injection in monkeys; in rats the average amount that remained ranged from 9% (Sub Q) to 20% (IM).

The amount of UL-^{14}C-mannide oleate preparation remaining at the site of injection of monkeys and rats for 1 day to 3 months after injection is summarized in Table III. During the first 24 hr. after administration, as much as 35–45% of the radioactivity injected into rats and monkeys had been removed from both IM and Sub Q sites of injection. Two to seven days after injection, the amount remaining in both monkeys and rats changed very slowly, an average of 50% being removed from the site of injection the 1st week. After 1 month, intramuscularly injected animals lost approximately 60%, and subcutaneously injected animals lost 70–80% from the site of injection. Essentially, no change was noted after 2 months. By the 3rd month after injection, both the intramuscularly and subcutaneously injected animals had lost, from both sites of injection, approximately 88% of the radioactivity associated with the UL-^{14}C-mannide oleate preparation.

In general, one-half of the ^{14}C-labeled "mannide monooleate" was removed from the site of injection the 1st week. It took 3 months, however, to remove roughly one-half to three-quarters of the material remaining after 1 week.

In an effort to define the nature of the radioactive material left at the site of injection, the chloroform–methanol leg extracts (6) were applied to TLC plates. The extracts were developed in a chloroform–diethyl ether–acetic acid system (95:5:1), an example of which is shown in Fig. 1. The distribution of radioactivity on the TLC plate (Table IV) indicated that the material remaining at the site of injection of both monkeys and rats was the esterified components of mannide monooleate preparation. The free fatty acids, free sugar, and their altered forms apparently were removed from the site of injection. The nature of the material removed and of that left at the site of injection was not verified chemically.

Distribution of Radioactivity among the Major Organs—The average levels of radioactivity (counts/min./100-mg. wet tissue) of the major organs of monkeys and rats after the administration of 1-^{14}C-oleate and UL-^{14}C-mannide surfactant preparations are presented in Tables V and VI, respectively. All organs of both monkeys and rats incorporated radioactivity within the first 24 hr. However, the level (c.p.m./100-mg. wet tissue) was less with UL-^{14}C-mannide oleate than with the 1-^{14}C-oleate mannide preparation. The depot of fat of those animals receiving 1-^{14}C-oleate-labeled surfactant consistently contained the highest specific activity of any organ except the inguinal lymph nodes. In those animals receiving UL-^{14}C-mannide oleate tracer, the depot fat contained comparatively little radioactivity.

Approximately 75% of the inguinal lymph nodes analyzed had a fairly large amount (200–2000 c.p.m./20-mg. wet tissue) of radioactivity, regardless of the tracers administered. These data seem to indicate that the lymph was a major route in the transport of the surfactant and not necessarily a site of accumulation.

Nature of the Radioactivity in the Major Organs—Attempts were made to evaluate the distribution of radioactivity within the major organs of monkeys 3 months after administration of a mineral

Table V—Average Level of Radioactivity in the Major Organs of Monkeys and Rats after Administration of a Mineral Oil–"Mannide Monooleate" Emulsion Containing 1-^{14}C-Oleate Mannide as the Tracer

Time after Injection	Number[a] of Animals	Liver M	Liver R	Fat M	Fat R	Spleen M	Spleen R	Kidney M	Kidney R	Small Intestine M	Small Intestine R	Ovary M	Ovary R	Lung M	Lung R	Inguinal Lymph Node M
		Average Counts per Minute per 100-mg. Wet Tissue[b]														
1 day	6	153	102	348	405	118	59	149	103	135	59	129	73	65	48	826
2 days	6	104	52	614	316	142	43	149	57	150	36	90	96	103	30	635
7 days	6	399	64	1732	544	199	70	197	74	161	34	92	73	119	54	522
1 month	4	232	49	1977	509	73	29	144	25	55	23	69	38	111	20	469
2 months	4	131	9	1233	612	36	10	46	10	26	11	27	32	20	9	875
3 months	4	331	23	1679	1105	54	28	119	22	38	48	44	80	38	18	253

[a] Number of monkeys (M) and number of rats (R) used in this study. [b] Average counts/min./100-mg. wet tissue except for inguinal lymph nodes, which weighed approximately 20 mg.

Table VI—Average Level of Radioactivity in the Major Organs of Monkeys and Rats after Administration of a Mineral Oil–"Mannide Monooleate" Emulsion Containing UL-^{14}C-Mannide Oleate as the Tracer

Time after Injection	Number[a] of Animals	Liver M	Liver R	Fat M	Fat R	Spleen M	Spleen R	Kidney M	Kidney R	Small Intestine M	Small Intestine R	Ovary M	Ovary R	Lung M	Lung R	Inguinal Lymph Nodes, M
		Average Counts per Minute per 100-mg. Wet Tissue[b]														
1 day	6	33	7	29	8	34	8	46	8	54	9	38	9	20	5	120
2 days	6	25	11	37	12	27	7	39	10	58	12	36	11	27	7	132
7 days	6	43	13	31	13	22	10	30	14	27	10	17	11	55	10	38
1 month	4	44	15	26	18	20	13	70	16	17	10	11	9	20	7	9
2 months	4	12	9	10	8	12	5	38	9	19	6	9	7	6	4	277
3 months	4	31	10	18	13	28	7	161	20	14	10	14	10	12	8	1889

[a] Number of monkeys (M) and number of rats (R) used in the study. [b] Average counts/min./100-mg. wet tissue except for inguinal lymph nodes which weighed approximately 20 mg.

Table VII—Distribution of Radioactivity within Various Organs of Monkeys 3 Months after Administration of a Mineral Oil–"Mannide Monooleate" Emulsion Containing 1-^{14}C-Oleate Mannide as the Tracer

TLC Fraction[b]	Tissues[a]: Liver, %	Fat, %	Spleen, %	Kidney, %	Small Intestine, %	Ovary, %	Lung, %	Brain, %	Inguinal Lymph Nodes, %
a–b	0.0	0.9	60.5	0.0	0.0	35.6	0.0	20.8	10.9
c–e	0.0	0.0	0.0	70.7	0.0	0.5	0.0	14.9	0.0
f–g	2.1	6.5	0.0	29.1	62.9	6.5	44.9	15.8	0.0
h–i	4.2	17.3	16.9	0.0	0.0	35.4	0.0	0.0	7.8
j–k	49.9	11.7	10.8	0.0	37.3	9.5	33.0	19.8	4.2
l–m	31.2	0.0	11.8	0.0	0.0	4.0	22.0	28.7	40.6
Solvent front	12.5	63.6	0.0	0.0	0.0	8.3	0.0	0.0	36.4

[a] Pooled tissue extracts. [b] Probable contents of fractions: (a–b) phospholipids and chemically altered oleic acid; (c–i) chemically altered oleic acid, oleic acid, and monoglycerides; (j–solvent front) esterified surfactant and esterified naturally occurring lipids (triglycerides, sterol esters, and free sterols).

oil–"mannide monooleate" emulsion containing either UL-^{14}C-mannide or 1-^{14}C-oleate-labeled surfactant. The results (Tables VII and VIII) of the serial TLC scrapings and liquid-scintillation counting of the fractions obtained from organ extracts varied considerably. This variation was interpreted as an indication of metabolism once these tracers had reached the various tissues. For example, the depot fat contained some radioactivity associated with free fatty acids and a large amount (64%) associated with newly synthesized triglycerides when the 1-^{14}C-oleate mannide preparation was used. The spleens of the same animals, on the other hand, contained large amounts of radioactivity (60%) at the origin of the TLC plates, indicating that this was probably newly synthesized phospholipids.

The distribution of radioactivity in those monkeys receiving the UL-^{14}C-mannide oleate was completely different from those receiving the 1-^{14}C-oleate mannide preparation. Since no definite chemical studies were performed, these results can best be explained on the basis that the metabolism of the labeled oleate and the labeled mannitol tracers would be expected to be different. The nature of the radioactivity found in the lymph nodes also, unfortunately, remained undetermined. However, it was suspected to be esterified material of the surfactant.

Respiratory $^{14}CO_2$—A substantial amount of radioactivity is eliminated in the respiratory CO_2 of animals given the 1-^{14}C-oleate mannide preparation as the tracer (Table IX). In monkeys, the average daily rate of $^{14}CO_2$ eliminated was approximately 0.9%, yielding a total average of 6% of the administered radioactivity eliminated the 1st week. Similar results were obtained with rats; however, the daily rate was slightly higher, and the total amount eliminated during the 1st week averaged close to 7% of the amount given.

On the other hand, a very small amount (0.1–0.2%) of the UL-^{14}C-mannide oleate tracer preparation was eliminated in the respiratory CO_2 the 1st week after injection. No determinations were made after the 1st week.

Urine and Feces—Table X data show that only a very small amount of radioactivity was eliminated in the urine of monkeys and rats when the 1-^{14}C-oleate-labeled surfactant was administered. The total amount eliminated for 1 week after injection ranged from 0.5 to 1%.

When the UL-^{14}C-mannide surfactant was administered, a large amount of radioactivity, roughly 25% of the dose administered, was eliminated in the urine the first 24 hr. During the 2nd day, the

Table VIII—Distribution of Radioactivity within Various Organs of Monkeys 3 Months after Administration of a Mineral Oil–"Mannide Monooleate" Emulsion Containing UL-^{14}C-Mannide Oleate as the Tracer

TLC Fraction[b]	Tissues[a]: Liver, %	Fat, %	Spleen, %	Kidney, %	Small Intestine, %	Ovary, %	Lung, %	Brain, %	Inguinal[c] Lymph Nodes, %
a–b	64.4	2.4	10.3	22.6	10.3	0.0	17.0	13.4	87.5
c–e	10.7	37.6	4.4	7.5	0.0	25.0	12.6	24.4	10.5
f–g	0.0	12.0	0.0	4.9	0.0	37.5	21.5	0.0	1.8
h–i	4.5	4.8	0.0	7.9	18.2	8.7	0.0	6.3	0.0
j–k	4.1	24.0	38.2	46.0	45.6	28.9	0.0	1.6	0.0
l–m	9.5	0.0	20.6	5.3	12.5	0.0	29.6	54.3	0.0
Solvent front	6.6	20.0	26.5	5.7	13.7	0.0	19.3	0.0	0.0

[a] Pooled tissue extracts. [b] Probable contents of fractions: (a–b) phospholipids and chemically altered oleic acid; (c–i) chemically altered mannitol, mannitol, and monoglycerides; (j–solvent front) esterified surfactant and esterified naturally occurring lipids (triglycerides, sterol esters, and free sterols). [c] The lymph extract was hydrolyzed with perchloric acid prior to TLC separation. TLC separation before hydrolysis yielded results similar to Table VII.

Table IX—Elimination as Respiratory $^{14}CO_2$ of 1-^{14}C-Oleate Mannide and UL-^{14}C-Mannide Oleate from Both Rats and Monkeys for 1 Week after Injection of a Mineral Oil–"Mannide Monooleate" Emulsion

	Days after Injection							
	1, %[b]	2, %	3, %	4, %	5, %	6, %	7, %	Total, %
				1-^{14}C-Oleate Mannide				
Rats (6)[a]	1.96	0.98	0.88	1.12	0.79	0.81	0.70	7.24
Monkeys (6)	0.98	0.83	0.94	0.76	0.88	0.89	0.72	6.00
				UL-^{14}C-Mannide Oleate				
Rats (3)	0.16	0.03	0.01	0.02	0.02	<0.01	<0.01	0.23
Monkeys (3)	0.03	0.03	0.02	0.01	0.02	0.02	0.02	0.15

[a] Number in parentheses is the number of animals analyzed. [b] Average percentage based on the total amount of radioactivity given.

Table X—Elimination of Radioactivity in the Urine of Rats and Monkeys for 1 Week after Administration of a Mineral Oil–Mannide Oleate Emulsion Containing 1-^{14}C-Oleate Mannide or UL-^{14}C-Mannide Oleate as the Tracer

	Days after Injection							
	1, %[a]	2, %	3, %	4, %	5, %	6, %	7, %	Total, %
				1-^{14}C-Oleate Mannide				
Rats (3)[b]	0.27	0.12	0.15	0.15	0.09	0.13	0.09	1.00
Monkeys (3)	0.09	0.12	0.12	0.06	0.07	0.04	0.04	0.54
				UL-^{14}C-Mannide Oleate				
Rats (12)	25.63	2.91	1.98	1.39	1.19	1.15	1.37	35.62
Monkeys (18)	22.87	4.95	3.72	1.90	1.99	1.20	1.73	38.36

[a] Average percentage based on the total amount of radioactivity given. [b] Number in parentheses is the number of animals analyzed.

Table XI—Distribution of Radioactivity in 24-Hr. Urine Samples in Monkeys and Rats 1 Day after Administration of UL-^{14}C-Mannide Oleate as the Tracer

TLC Fraction[a]	Monkeys, %	Rats, %
a–b	46.1	66.2
c–g	53.8	33.4
h–i	0.1	0.2
j–k	0.0	0.2
l–m	0.0	0.0

[a] Probable contents of fractions: (a–g) nonesterified, unaltered, and chemically altered mannitol; (h–m) esterified surfactant.

amount decreased to about 3 and 5% in rats and monkeys, respectively. Three to seven days after injection, the level eliminated in the urine stabilized to about 1% daily. The total amount eliminated the 1st week was about 35–40%. In general, there was no significant difference between monkeys and rats in the total amount eliminated in the urine. The results on the total amount eliminated in the urine of rats agree with those of Porter and Titus (7). However, these investigators studied only the mannide-labeled surfactant. As shown, the 1-^{14}C oleate-labeled material is not eliminated in the urine.

Table XI presents TLC separation and a liquid-scintillation counting analysis of the radioactive components in the urine of those animals receiving the UL-^{14}C-mannide oleate preparation. These data indicate that the material eliminated so rapidly in the urine was the nonesterified sugar components of the surfactant.

No appreciable amount of radioactivity was detected in the feces when either tracer was given.

Pathological Observations—Gross necropsy of all animals indicated that no sterile abscesses, infections, nor other observable pathological conditions were produced at the site of injection. No extensive histological examinations were performed; therefore, it is not possible to state definitely whether any subtle histopathological conditions existed.

SUMMARY

This study has shown that, when using a "mannide monooleate"–mineral oil emulsion, the surfactant was removed from the site of injection faster (40–60% the 1st week) than the mineral oil (1–5% the 1st week) (6). The obvious danger in this phenomenon is that the loss of the emulsifier may allow the mineral oil to coalesce into larger droplet sizes and thereby retard mineral oil mobilization from the site of injection. Those components of the "mannide monooleate" preparation that were retained at the site of injection for longer periods of time (3 months or more) appeared to be the esterified components as opposed to the nonesterified components (free oleic acid, mannitol, and their chemically altered forms), which appeared to be removed within 24 hr. after injection. These data suggest that, had this surfactant been pure mannide monooleate or even its other esterified forms, its longer retention at the site of injection may have also assisted in a more uniform mobilization of mineral oil.

This investigation has yielded additional information on the mobilization and metabolism of the mannide monooleate surfactant preparation when it is received as an injected emulsion. No information was obtained directly which would implicate the surfactant used in this study as a particularly noxious material. Although not tested in this study, this would seem to indicate that the reported toxic effects associated with this material are in some way related to the various antigen preparations.

REFERENCES

(1) J. Freund, *J. Advan. Tuberc. Res.*, **7**, 130(1956).

(2) F. M. Davenport, *Ann. Allergy*, **26**, 288(1968).

(3) B. S. Berlin, *ibid.*, **20**, 472(1962).

(4) M. C. Hardegree and M. Pittman, *Proc. Soc. Exp. Biol. Med.*, **123**, 179(1966).

(5) H. J. O'Neill and T. N. Yamauchi, *J. Amer. Oil Chem. Soc.*, **46**, 99A(1969).

(6) J. N. Bollinger, *J. Pharm. Sci.*, **59**, 1084(1970).

(7) C. C. Porter and D. C. Titus, *Proc. Soc. Exp. Biol. Med.*, **124**, 500(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 15, 1969, from the *Southwest Research Institute, San Antonio, TX 78228*

Accepted for publication March 13, 1970.

This investigation was supported by the Division of Biologics Standards, National Institutes of Health, Contract No. PH 43-67-1104.

The technical assistance throughout the program of Dennis Schmidt was appreciated.

In Vivo Electrometric Study of Carcinogenic Hydrocarbon Interaction with Mouse Epidermis

V. F. SMOLEN, D. E. SNYDER, and R. J. ERB

Abstract □ A bioelectrometric technique was implemented in the study of 3-methylcholanthrene and benzene interaction with the epidermis of hairless mice. In contrast to previous studies of this nature, the experimental results were obtained entirely *in vivo* without causing injury to the mice. The results obtained are analogous to titration curves of amphoteric macromolecules. Inspection of the curves and their temporal variations following the topical application of the hydrocarbons revealed the induction of significant changes in the density of ionogenic groups affixed to the epidermal colloids. The carcinogenic 3-methylcholanthrene was observed to induce consistently a reduction of net cationic fixed-charge density attributable to the loss or discharge of basic nitrogenous groups titratable above, at least, pH 7.4. A discussion of some of the possible mechanisms of this observation is presented. The results of the present study indicate the bioelectrometric method to be complementary to the more direct experimental techniques commonly employed.

Keyphrases □ Carcinogenic hydrocarbon interaction—mouse epidermis □ Epidermis, mouse—3-methylcholanthrene, benzene □ 3-Methylcholanthrene, benzene effects—epidermal net charge density □ Bioelectrometric determination—epidermis–hydrocarbon interaction

The ability of hydrocarbons such as 3-methylcholanthrene (3-MC) to induce malignantization following topical application to skin has been well documented (1). It has been found generally that the greater the extent to which a hydrocarbon possessing a reactive, electron-rich, *K*-region binds to the skin, the greater is its carcinogenic potency (2–4). It has also been demonstrated that binding occurs to protein components of mouse skin (5). The extent of interaction of the hydrocarbons varies with time (2, 4–7), reaching a maximum for 3-MC at approximately 10 hr. (7). The binding of carcinogenic relative to inactive hydrocarbons has been found to be especially high for water-soluble proteins extracted from mouse skin found in a fraction termed "proteins I" (8, 9); the *in vivo* binding of hydrocarbons to nucleic acids appears to remain in some doubt (10). It has been concluded (11) that oxidative biotransformation of the *K*-region of the hydrocarbons yields dicarboxylic acids which bind protein through amide, imide, or ester linkages but most probably through the formation of amides.

The experimental methods employed in these previous investigations concerning the extent and nature of hydrocarbon interaction with mouse skin have been destructive; they necessitated the sacrifice of the animals, disintegration of the skin, and fractionation of its constituents. Such procedures are disadvantaged by the difficulty and necessity of correcting for any *in vitro* binding of the carcinogen which may occur during the extraction process. The observation of significant amounts of *in vitro* binding has led some authors (12) to conclude that carcinogens that were not bound in the intact tissue became bound in the process of disintegrating the tissue. The difficulty of distinguishing between binding sites active *in vivo* and those activated by the *in vitro* treatment of the tissue is readily apparent. Results obtained from the use of destructive methods have, therefore, been open to question with respect to their verity in representing actual *in vivo* conditions.

In the present study, a nondestructive bioelectrometric method, previously described (13, 14), was utilized. It permitted the investigation of hydrocarbon interaction with mouse epidermal colloids to be accomplished entirely *in vivo* under physiological conditions. The completely innocuous nature of the experimental procedure allows each animal to serve as its own control; repeated measurements can be made on the same animal to discern the temporal dependency of the induced effects. The present study was primarily undertaken to examine the ability of the bioelectrometric method to discern the effects of 3-MC on mouse skin and, secondarily, to determine if any responses to the carcinogen previously undetected when employing destructive techniques could be made apparent in an all *in vivo* study.

MATERIALS AND METHODS

Methods—The electrometric method employed in the present study is essentially based upon the Gibbs-Donnan equilibriumt It involves the measurement of electrical potentials developed at the boundary of the tissue and a buffer solution, which may or may not contain the substance whose interaction is to be studied. The results obtained from treated surfaces are compared to untreated controls.

The net density of fixed charge on the tissue surface, arising from the dissociation of ionogenic groups bonded or adsorbed to the surface, can be computed from the measured null point potentials. If these results are obtained under conditions of varying pH and plotted as a function of pH, the curves resemble, and are analogous to, titration curves of amphoteric macromolecules. The shape of these curves is dependent upon the nature of the dissociating groups, the presence of interacting solutes, and the state of aggregation of the colloids composing the tissue surface. The fixed-charge density on tissue surfaces and, therefore, the shape of the titration curves are quite sensitive to interacting substances. Variations in the curves can be interpreted to yield information about the extent and nature of the interaction.

The verity of the measured potentials in representing the fixed-charge density has been established in the principal author's laboratory (unpublished) as well as by others (15). The details of the calculation of the net fixed-charge densities and the elementary interpretation of titration curves have been described (13–15). It has recently been found, in the principal author's laboratory, that for human epidermis the thickness of the colloid phase, whose properties are reflected in the electrometric measurements, is approximately 4 μ. This represents about 40% of the depth of the stratum corneum. Although this same determination has not been performed for mouse skin, which is thinner by several cellular layers, the results of the present study could pertain, at least, to a similar relative depth.

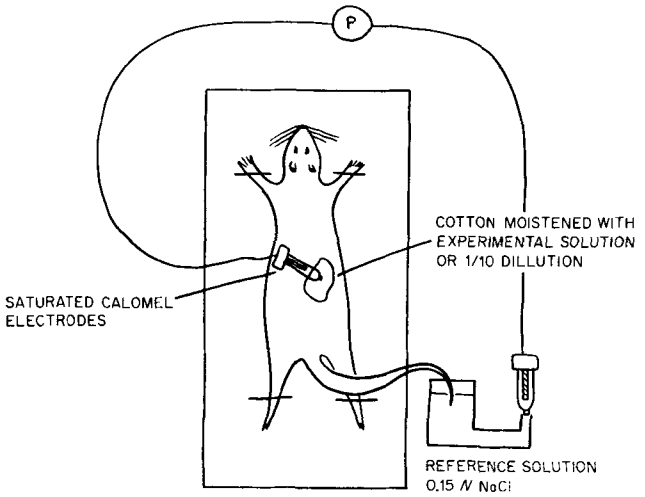


Figure 1—*Experimental arrangement for the determination of fixed-charge density of the epidermal surface of hairless mice.*

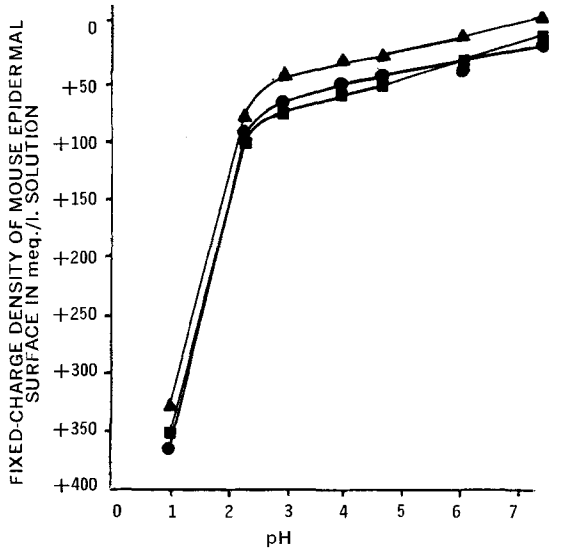


Figure 2—*The average relationship of the fixed-charge density of the epidermal surface colloids of mouse skin to pH. Each point represents the average of 24 determinations on four separate mice studied at each of six different time intervals varying from 1.1 to 50.3 hr. following treatment with benzene (—■—) and 1% 3-MC solution in benzene (—▲—). The remaining curve (—●—) represents results obtained from the untreated control skin areas on each of the mice.*

Figure 1 contains a diagram of the experimental arrangement used in the present investigation with hairless mice. The mice were ether-anesthetized and rendered immobile for the treatments and subsequent measurements by restraining them securely to a specially constructed mouse board prior to their recovery from the anesthetic. The differences of the measured potentials, used for the calculations of the fixed-charge density, are independent of the location of the reference surface on the animal. The tail of each mouse was selected for use in the present case because of its convenience. It was rinsed well with distilled water and 0.15 *N* NaCl and placed into the vessel containing the 0.15 *N* NaCl reference solution. The tail was not disturbed further during the course of the series of measurements corresponding to any given time. The dorsal side of the mouse was cleansed by swabbing with 70% ethanol, followed by a thorough, yet gentle, scrubbing with a solution of soap, and rinsing with distilled water. This cleansing procedure had previously been found to reduce both intrasubject and intersubject variations of the potential differences measured under any given set of conditions (13).

After cleansing, the back of the mouse between the neck and the tail was divided equally into three separate areas by drawing thin lines with an ink marker. The anterior third of the back was treated three times with a liberal application of benzene on a cotton swab and allowed to dry. The posterior third of the back was similarly treated with a 1% w/v solution of 3-MC in benzene. The middle area was left untreated and served as the control. Immediately prior to the measurement of potentials from any of the areas on the mouse, the area was rinsed with distilled water and allowed to hydrate by covering with a pledget of cotton wetted with 0.15 *N* NaCl for approximately 15 min. The electrical potentials were consistently read from the three areas in the order of benzene-treated, control, and 3-MC-treated skin. No significant differences in measurements performed on the three skin areas prior to their treatment were observed.

Titration curves for each treatment area on four separate mice corresponding to approximately 1, 4, 9, 20, 34, and 50 hr. after treatment were constructed over a range of pH from 1.0 to 7.4, using values of fixed-charge density calculated from the observed potential differences.

The data for a titration curve were obtained by swabbing the area with pH 1.0 buffer following the 15-min. soaking with 0.15 *N* NaCl. A 0.5 × 0.5-cm. piece of filter paper saturated with pH 1.0 buffer was placed in the center of the area of interest. Despite any movement by the mouse, the paper was held firmly in place, in part by the weight of the calomel electrode mounted in a movable arm assembly. A potential, E_1, was continuously recorded until observed variations in the potentials were less than approximately 0.2 mv./min.; this stability was usually achieved within 1–2 min. The electrode was then removed, the tip rinsed with saturated KCl solution, and wiped clean. Any remaining excess solution was swabbed from the back of the mouse. A smaller piece of filter paper, wetted with 0.15 *N* NaCl (except at pH 1.0 where a 1:10 dilution of the buffer itself was used), was placed within the area previously covered and was followed immediately by the replacement of the electrode and the recording of a potential, E_2. The electrode and skin surface were treated again as described, and the potential E_1 was redetermined to ensure that conditions had remained constant between the time of E_1 and E_2 measurements. The potential difference, $E_2 - E_1$, termed the dilution potential, E_d, was computed with the average of E_1 values which agreed within 1.0 mv. The E_d values allow the calculation of the fixed-charge density in the manner previously described (13, 14). Following pH 1.0, the fixed-charge densities of the epidermal surface corresponding to pH 2.2, 3.7, 4.6, 6.0, and 7.4 were obtained from dilution potentials measured in the same manner using the isoosmotic buffer solutions of higher pH.

Table I—Significance Levels Obtained by Analysis of Variance

Main Effects and Primary Interactions	*p*
Treatments of skin areas	0.05
Time	0.001
pH	0.001
Time–treatments	0.01
Treatment–pH	N.S.
pH–time	0.001

Materials—The compositions of the isoosmotic buffer solutions have been reported earlier except for the pH 1.0 solution which consisted of 0.10 *M* HCl and 0.05 *M* NaCl. Spectrograde benzene was used. The 3-MC was supplied by Eastman Chemicals.

Hairless, strain HRS/J mice (Jackson Labs, Bar Harbor, Maine) were used in the experiment. The mice were 7-week-old males.

Corning miniature fiber-junction saturated calomel reference electrodes were used in conjunction with a Sargent model SR potentiometric recorder. The potentials were read directly from the recorder chart.

Statistical calculations were conducted with the aid of an IBM 7094 computer.

RESULTS

Gross Graphical and Statistical Results—The experiment can be described as being of three-factor, unsymmetrical design. Four responses, corresponding to the replicate determinations of the dilution potential on four individual mice, were recorded for each factor level. The determinations were made at 1.1, 4.8, 9.4, 20.4, 34.0, and 50.3 hr. and at pH 1.0, 2.2, 2.9, 3.9, 4.6, 6.0, and 7.4 on each of the treated and control areas on the mice. Significance

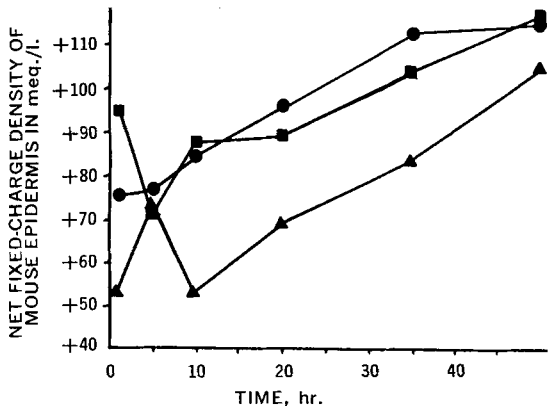


Figure 3—*The relationship of the net fixed-charge density of hairless mouse epidermis, averaged over pH, to the time following treatment of the skin with benzene (—■—), 1% 3-MC in benzene solution (—▲—), as well as for untreated control skin (—●—). Each point is the average of 28 determinations on four different mice.*

levels, resulting from an analysis of variance, for the main effects and primary interactions are shown in Table I; $p > 0.05$ was taken as not significant (N.S.). Some of the effects listed in Table I are graphically illustrated in Figs. 2–4. In Fig. 2, the fixed-charge densities plotted at each pH are averages of the values calculated from observed E_d values at each time and for each mouse. The relatively parallel nature of the titration curves corresponding to each treatment over considerable ranges of pH illustrates the insensitivity of the treatment effects to pH. The relatively small vertical distances between the curves and their crossing can account for the comparatively low order of significance of the treatments. Figure 3 is similar to Fig. 2, except the values of the fixed-charge densities are averaged over pH and plotted as a function of time. The pronounced effect of 3-MC, relative to the benzene control, is clearly evident for the majority of the time course of the study.

It is evident from Fig. 3 that a general trend toward an increased net density of positive fixed charge on the affected colloids underlies the treatment-induced responses. The three curves appear to possess this trend in common, differing only by the superimposition of the treatment-induced effects. Consistent with this trend toward a net increase in cationic fixed-charge density, examination of the titration curves for the untreated control skin areas revealed later curves

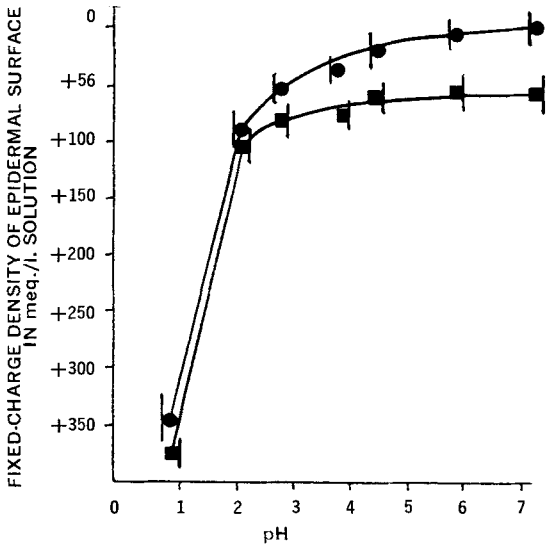


Figure 4—*Comparison of titration curves obtained for untreated mouse epidermis at 1.1 (—●—) and 50.3 (—■—) hr. Each point is the average of four determinations on separate mice plotted ± its standard error. By a paired t test, the curves are significantly different at* $p < 0.01$.

Table II—Statistical Probability Levels (p) Representing the Significance[a] of Observed Differences in Fixed-Charge Density of Benzene-Treated Mouse Epidermis, in Comparison to 1% 3-MC in Benzene Solution-Treated Mouse Skin as Determined from Paired t Tests

Time, hr.	pH 1.0	2.2	2.9	3.9	4.6	6.0	7.4
1.1	—	0.05	0.05	0.02	0.05	—	—
4.8	—	—	0.05	—	—	—	—
9.4	—	0.01	0.001	0.02	0.05	—	—
20.4	—	—	—	—	0.01	—	—
34.0	—	—	—	—	—	—	0.01
50.3	—	—	—	—	—	—	—

[a] Values of $p > 0.05$ were considered as insignificant and correspond to the blanks in the table.

always to lie below earlier curves. The extreme differences in the curves are depicted in Fig. 4 where the 1.1- and 50.3-hr. curves are presented. Each point is plotted with its standard deviation. A paired t test revealed the curves to be significantly different at $p < 0.01$. This agrees with the highly significant pH–time interaction and supports the reality of the underlying general trend toward positivity.

It can be surmised from further inspection of Fig. 3 that, relative to the benzene control, the treatment with 3-MC can be calculated from the vertical differences between the two curves to have induced an overall average reduction of 23% in net cationic fixed-charge density. Exempting the anomaly at 4.8 hr., the reduction appears to decline exponentially with time from 49% at 1.1 hr. to 10% at 50.3 hr. and has an approximate half-life of 20 hr.

To resolve more precisely the influence of pH and time on the effects of the benzene and 3-MC treatments, paired t tests were performed. The significance levels calculated from the tests are presented in Tables II–IV, from which it is evident that the effects are mostly significant at 1.1 and 9.4 hr. and nearly independent of pH. The highly significant differences between the 3-MC (plus benzene as the solvent) and benzene control can partly be attributed to the antagonistic effects of benzene and 3-MC on the net density of fixed charge at these times. This is evident from inspection of the titration curves observed at 1.1 and 9.4 hr. (Figs. 5 and 6) where it can be seen that the benzene and 3-MC displaced curves lie on opposite sides of the untreated control; moreover, in Fig. 3 the benzene curve is observed to lie above the untreated control at 1.1 and 9.4 hr. In each case a reduction of net cationic charge has been elicited by the 3-MC while the benzene has induced an increase at these times; the effect of benzene has subsided, while that of 3-MC has been elevated, at 9.4 hr. relative to 1.1 hr. The opposite effects of benzene and 3-MC are only apparent at 1.1 and 9.4 hr. At all other times the effects of the two hydrocarbons appear additive in reducing the average net density of fixed cationic charge on the involved colloids.

Superficially at least the relatively high significance of the effects of 3-MC at 9.4 and 1.1 hr. may be compared to the maximum in protein binding observed at approximately 10 hr. following treatment of mouse skin (7). That carcinogenic hydrocarbons interact with tissue constituents to alter cellular functions within an hour of their application to mouse skin has also been demonstrated (17, 18).

The appearance and disappearance of statistically significant treatment effects are likely the consequences of the various simul-

Table III—Statistical Levels of Significance[a] Resulting from Paired t Test Comparisons of Fixed-Charge Densities of Benzene-Treated Mouse Epidermis with Untreated Control Skin Areas on the Same Mice

Time, hr.	pH 1.0	2.2	2.9	3.9	4.6	6.0	7.4
1.1	—	0.01	0.05	0.01	0.01	—	—
4.8	—	—	—	—	—	—	0.05[b]
9.4	—	—	0.001	—	—	—	0.01
20.4	—	—	—	—	—	—	—
34.0	—	—	—	—	—	—	—
50.3	0.05[b]	—	—	—	—	—	—

[a] Values of $p > 0.05$ were assumed insignificant. [b] The benzene titration curve was observed to lie above the control.

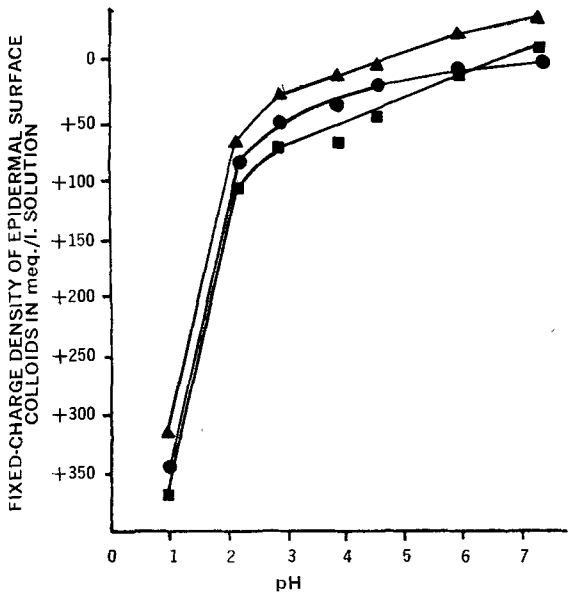


Figure 5—*Titration curves of hairless mouse epidermal surface observed at 1.1 hr. following treatment with benzene (—▲—), 1% 3-MC in benzene solution (—■—), as well as for untreated control skin areas (—●—) on the mice. Each point represents the average of four determinations on individual mice.*

taneously operative rate processes affecting the observed net density of fixed charge in a complex manner. In some instances, it is possible to resolve the influence of some of these processes.

DISCUSSION

Analysis of the Trend toward Positivity and the Time-Dependent Effects of Benzene—Close inspection of the untreated control titration curves in Fig. 4 reveals that the curves begin to diverge appreciably beyond pH 2.2, *i.e.*, in the range of pH where anionogenic groups generally become titratable. On this basis, the observed trend toward positivity can be attributed to a progressively increasing, time-dependent, net loss of anionogenic groups from the epidermal colloids. In contrast, a significant difference between titration curves

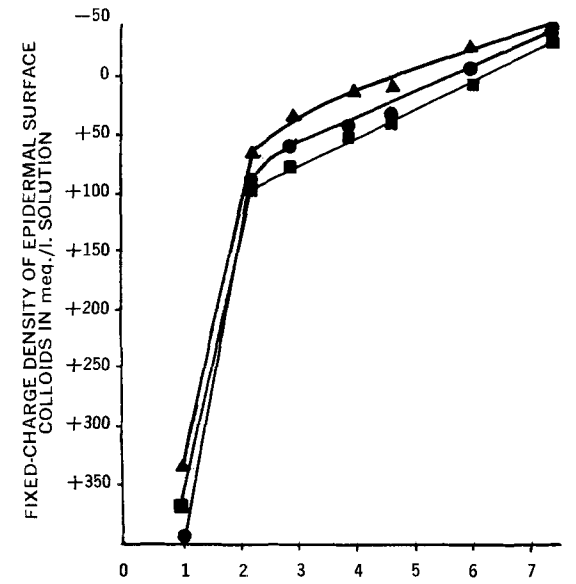


Figure 6—*Titration curves of hairless mouse epidermal surface observed at 9.4 hr. following treatment of the skin with benzene (—■—), 1% 3-MC solution in benzene (—▲—), as well as for untreated control skin (—●—) on the mice. Each point represents the average of four determinations on separate animals.*

Table IV—Statistical Levels of Significance[a] Resulting from Paired *t* Test Comparisons of Fixed-Charge Densities of Mouse Skin Treated with 1% Solution of 3-MC Relative to Untreated Control Skin on the Same Animals

Time, hr.	pH 1.0	2.2	2.9	3.9	4.6	6.0	7.4
1.1	—	—	—	—	—	—	—
4.8	—	—	0.02	—	—	—	—
9.4	—	0.05	0.01	0.01	0.01	0.05	—
20.4	—	—	—	0.05	0.05	0.01	0.02
34.0	—	—	—	—	0.001	—	0.02
50.3	—	—	—	—	—	—	—

[a] Values of $p > 0.05$ were assumed insignificant.

at pH 1.0, coupled with significant vertical differences at higher pH, characteristically accompany a diminution of cationogenic groups titratable in the pH range presently studied. In this case the curves would also be required to approach one another and eventually become coincident once the pH range in which these groups are titratable has been exceeded. Clearly this behavior is not characteristic of the curves in Fig. 4. The loss of anionogenic groups from the epidermal colloids remains the only acceptable explanation of their behavior.

Comparison of the benzene control with the untreated control curves (Figs. 5 and 6) observed at 1.1 and 9.4 hr. reveals a similar loss of anionogenic groups to be responsible for the benzene-induced effects on the fixed-charge densities of the epidermal colloids. An initial acceleration in the loss of anionogenic titratable groups in response to benzene treatment, followed by a slowing in the rate of subsequent loss, could account for the relatively high significant differences between the benzene and control curves at 1.1 hr. and the subsequent loss of significance at later times as the extent of anionogenic group loss from the untreated control skin approaches that for benzene-treated skin. Since carboxyl groups are primarily titratable over the pH range for which the benzene-control differences are significant, it may be speculated that the titratable anionogenic groups were initially diminished by the extraction of fatty acids in the benzene treatment and later lost through further elutriation into the applied 0.15 *N* NaCl and buffered titrating solutions. Joseph *et al.* (19) have made similar observations on toluene-treated skin and derived similar conclusions regarding the effect of the organic solvent.

It can be further hypothesized that the approach of the benzene curve in Fig. 3 to the control curve, in addition to being a consequence of the slowing of the benzene-induced initially accelerated loss of anionogenic materials, is also a result of a slower loss of cationogenic materials from the epidermis which becomes increasingly appreciable with time. Support for this hypothesis is gained from observing that beyond 1.1 hr., the benzene titration curve at pH 1.0 consistently lies above the control, even though the only individual difference that is significant occurs at 50.3 hr. It has been demonstrated (20) that the treatment of skin with organic solvents removes lipoidal substances but does not directly remove hydrophilic materials from skin. However, such treatment predisposes their loss from the skin by leaching when the skin is subsequently exposed to water or aqueous solutions. The loss of these hydrophilic materials is accompanied by a lowering in the water-sorption capacity of the skin. It has been shown that amino groups are predominantly responsible in affecting the water-binding capacity of skin (21). The water-soluble extractives from skin have been identified as nitrogenous and consisting of amino acids and polypeptides (20, 22). The pretreatment of the skin with 70% ethanol and soap may also be expected to have contributed to the elutriative loss of materials from the skin into the applied aqueous solutions. The untreated control skin, however, appears to have been left substantially unaffected with regard to changes in cationic fixed-charge density, as evidenced by the observation of the relatively constant values of positive fixed-charge density at pH 1.1 having a range of 350–380 meq./l. and an overall average of 361 meq./l. The higher values consistently correspond to later times.

In addition to this explication of the observed behavior of the control and benzene-displaced titration curves, the possibility of the changes resulting from the systemic absorption or lateral diffusion of 3-MC into the control areas also exists. To reduce the possibility of

systemic absorption in this study, the mice were isolated between measurements to prevent their licking one another. Iversen and Evensen (16) minimize the importance of systemic absorption of the hydrocarbons in affecting their results. Since each treatment area would be equally affected by systemically absorbed materials, the differences observed between the skin areas could still be attributed to the treatments, even if systemic absorption of the hydrocarbons occurred to an appreciable extent.

Reduction of Net Cationic Charge Density by 3-MC—The approximately constant vertical distance between the 3-MC and other curves in Figs. 2, 5, and 6 demonstrates that the 3-MC-induced diminution in net fixed-cationic charge density does not appreciably rely on pH in the range studied. Such pH independence may be expected to result only if the affected cationic groups are titratable in an alkaline range of pH above the limit of pH 7.4 adopted in the present study. Such basic groups could include the guanidyl group of arginine, the ϵ-amino group of lysine, α-amino groups (23), and some of the nitrogenous groups of purine and pyrimidine bases (24). It is, of course, not possible to discern the individual involvement of each of these groups in the presently observed phenomena. Provided the pK's of the different groups are sufficiently separated, further information might be gained in a future study by extending the alkaline limit of pH and noting the region of pH at which the anticipated coincidence of the 3-MC- and benzene-displaced titration curves is observed. Such observations might conceivably distinguish between the involvement of α-amino, ϵ-amino, and guanidyl groups.

It could be a temptation to conclude that the observed neutralization of charge on the involved base-binding groups, induced by the action of 3-MC, results from the acylation of these groups by carboxylated derivatives of the hydrocarbon. However, the observed up to nearly 50% involvement of the total fixed cationic groups on the colloids is quantitatively grossly inconsistent with the estimated 0.001% of amino acid residues which become conjugated with hydrocarbons (25). The discrepancy would not be appreciably mitigated even if the latter percentage is corrected on the basis of assuming that only strongly basic side-chain amino groups interact with the hydrocarbons. Assuming arginine and lysine to constitute 16.65% of the total amino acids in epidermis (26), a maximum of only 0.006% of their number would be directly bound to the hydrocarbons. Including the possibility of other binding sites as well, *e.g.*, as might occur on nucleic acids, would again diminish this value and add further to the disagreement. A greatly potentiated elutriative loss of water-soluble cationic constituents from the skin, induced by the 3-MC, is not considered a likely mechanism for the unaccounted changes since the observed reduction in net cationic charge, relative to benzene, is maximal at 1.1 hr. As previously discussed, the extent of such losses by leaching increases with time.

Although the results of the present study do not justify the establishment of any firm conclusions regarding the mechanisms by which the 3-MC-induced reduction of fixed cationic charge density is manifested, it is certainly interesting to speculate the possibilities. The simplest possibility to consider is for the interaction of carboxylated derivatives of 3-MC with the tissue colloids to occur through the action of secondary valence forces in addition to the formation of covalent linkages. The severity of the extractive procedures used in direct studies of hydrocarbon–protein interaction only allows the detection of the latter type of combinations. The use of the electrometric method permits changes in cationic fixed-charge density resulting from either type of binding to become demonstrative. The reversible nature of electrostatic and Van der Waals' binding could conceivably allow the resolution of the contribution of each type of interaction to the reduction of net cationic charge density to be elucidated. The removal of the labile interacted hydrocarbon from the skin might be accomplished through extensive washing of the surface. The decline of the 3-MC effect, relative to the benzene control, could have arisen through such a loss of weakly bound anionic derivatives of 3-MC into the applied solutions.

However, if the oxidative carboxylation of the hydrocarbon only occurs concomitantly with its covalent binding to protein (10), it then follows that free anionic forms of the hydrocarbon would not be available for secondary valence force interactions with the proteins. If this is the case, an alternative explanation of the observed diminution in cationic charge density has to be sought.

Because of its very extensive implications, an exceptionally intriguing possibility is the reduction of cationic charge density resulting from the operation of an allosteric mechanism (27). By this means, a cooperative release of protons from the affected macromolecular structures could conceivably result from the interaction of the hydrocarbon at a paucity of cardinal sites; these sites would, however, control the acid–base properties of comparatively large numbers of proton-binding sites. Consideration of Mason's bioelectronic theory (28) of carcinogenesis and Ling's association-induction theory (29, 30) lends some degree of credence to this conjecture. In the framework of Mason's theory, the release of protons from the macromolecular structures could result from the formation of mobile positive holes in the protein molecules following the fusion of conduction bands between the macromolecules and the interacted hydrocarbons. Inductive and electrostatic field effects responsible for the release of protons could be conveyed to the basic side chains in a manner analogous to the mechanisms postulated by Ling (29). A net reduction of cationic charge on macromolecules may be expected to diminish their affinity for forming polysalt complexes with polyanions, such as polynucleotides, or cause the dissociation of such complexes already in existence (31). Such hydrocarbon-induced reductions of cationic charge density on nuclear proteins could conceivably accomplish the derepression of genes central to Pitot and Heidelberger's theory of carcinogenesis (32).

This last hypothesis, in addition to being able to explicate the results observed in the present study, appears to be capable of integrating the various prominent theories of carcinogenesis. Although direct experimental evidence for the hypothesis is at present obviously lacking, its verity could readily be tested on the basis of gaining predictive evidence from both *in vivo* and *in vitro* experimentation. This and the determination of whether the effects of hydrocarbon–tissue interactions involving *K* and *L* regions of the molecules can be distinguished by the bioelectrometric method could provide worthwhile objectives for future experimentation.

CONCLUSIONS

Despite the apparent need for conjecture in some instances to interpret the results, the utility of the bioelectrometric method as a tool complementary to more direct, although destructive, techniques by which tissue–carcinogen interactions can be studied has been demonstrated in the present investigation. It would be of interest in future studies to prolong the experiment to determine whether the method is capable of predicting imminent neoplasia prior to the appearance of morphologic evidence.

REFERENCES

(1) D. B. Clayson, "Chemical Carcinogenesis," Little Brown, Boston, Mass., 1962, pp. 12–19.

(2) C. Heidelberger and M. G. Moldenhauer, *Cancer Res.*, **16,** 442(1956).

(3) V. T. Olivieri and C. Heidelberger, *ibid.*, **18,** 1094(1958).

(4) P. Daudel, N. P. Chenon, H. Boo, P. Jacquignon, A. Lacassagne, G. Prodi, G. Vallie, R. Vasquez, and F. Zajdela, *Bull. Soc. Chem. Biol.*, **42,** 135(1960).

(5) W. G. Wiest and C. Heidelberger, *Cancer Res.*, **13,** 246 (1953).

(6) O. H. Iversen and A. Evensen, "Experimental Skin Carcinogenesis in Mice," Norwegian Universities Press, Boston, Mass., 1962, p. 28.

(7) L. M. Goshman and C. Heidelberger, *Cancer Res.*, **27,** 1678(1967).

(8) G. R. Davenport, C. W. Abel, and C. Heidelberger, *ibid.*, **21,** 591(1961).

(9) C. W. Abel and C. Heidelberger, *ibid.*, **22,** 931(1962).

(10) P. Daudel and R. Daudel, "Chemical Carcinogenesis and Molecular Biology," Interscience, New York, N. Y., 1966, pp. 55–67.

(11) *Ibid.*, pp. 57–58.

(12) H. I. Hadler, V. Darchum, and K. J. Lee, *Natl. Cancer Inst.*, **22,** 661(1959).

(13) V. F. Smolen, *Amer. J. Pharm. Educ.*, **33,** 381(1969).

(14) V. F. Smolen and F. P. Siegel, *J. Pharm. Sci.*, **57,** 378(1968).

(15) N. R. Joseph and M. B. Engel, *Arch. Biochem. Biophys.*, **85,** 209(1959); **84,** 224(1959); and **86,** 117(1960).

(16) O. H. Iversen and A. Evensen, "Experimental Skin Carcinogenesis in Mice," Norwegian Universities Press, Boston, Mass., 1962, p. 24.

(17) *Ibid.*, p. 137.
(18) E. J. Bollum and V. R. Potter, *Cancer Res.*, **19,** 561(1969).
(19) N. R. Joseph, R. Molinard, and F. Bourliere, *Gerontologia*, **1,** 18(1957).
(20) J. H. Blank, *J. Invest. Dermatol.*, **21,** 259(1953).
(21) J. D. Leeder and I. C. Watt, *Phys. Chem.*, **10,** 3289(1960).
(22) M. K. Polano, *J. Soc. Cosmet. Chem.*, **19,** 3(1968).
(23) B. R. Martin, "Introduction to Biophysical Chemistry," McGraw-Hill, New York, N. Y., 1964, p. 79.
(24) *Ibid.*, p. 258.
(25) C. Heidelberger and R. G. Moldenhauer, *Cancer Res.*, **16,** 442(1956).
(26) S. Rothman, "Physiology and Biochemistry of the Skin," University of Chicago Press, Chicago, Ill., 1954, p. 344.
(27) J. Monod, J. P. Chanyeux, and F. Jacob, *J. Mol. Biol.*, **6,** 306(1963).
(28) R. Mason, in "Wave Mechanics and Molecular Biology," L. deBroglie, Ed., Addison-Wesley, Reading, Mass., 1966, pp. 75–83.
(29) G. N. Ling, *Texas Rep. Biol. Med.*, **22,** 244(1964).
(30) G. N. Ling, "A Physical Theory of the Living State," Blaisdell, New York, N. Y., 1962, pp. 518–521.
(31) A. S. Michaels, *Ind. Eng. Chem.*, **10,** 32(1957).
(32) H. Pitot and C. Heidelberger, *Cancer Res.*, **23,** 1694(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 20, 1969, from the *Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication March 3, 1970.

This study was aided partly by a grant from the American Cancer Society and partly by funds provided by the Indiana Elks.

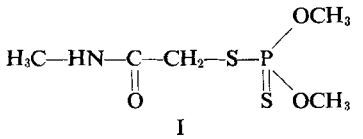
Acute Oral Toxicity of Dimethoate in Albino Rats Fed a Protein-Deficient Diet

ELDON M. BOYD and L. F. MUIS

Abstract □ The oral $LD_{50} \pm SE$ of dimethoate was found to be 147 ± 29 mg./kg., 152 ± 22 mg./kg., and 358 ± 9 mg./kg., respectively, in male albino rats fed for 28 days from weaning on a diet containing: (*a*) 3.5% protein as casein, (*b*) normal amounts of protein as casein, and (*c*) normal amounts of protein as laboratory chow. The toxic syndrome at the range of the LD_{50} was similar in animals of all three dietary groups and consisted of signs of cholinergic stimulation, depression of the central nervous system, anorexia, hypothermia, aciduria, proteinuria, an irritant gastroenteritis, widespread vascular congestion, toxic degenerative changes, dehydration, and loss of weight in body organs.

Keyphrases □ Pesticide toxicity study—rats □ Dimethoate—toxicity study, rats □ Protein-deficiency effect—dimethoate toxicity □ LD_{50}—dimethoate □ Toxic effects, rats—dimethoate

Dimethoate, an organophosphorus pesticide introduced in 1956, is effective as a systemic insecticide against pests such as houseflies, aphids, mites, and grasshoppers (1). It is a white crystalline solid with limited solubility in water but is freely soluble in cottonseed oil. The formula is shown in Structure I. It is



I
dimethoate

absorbed into plants and converted mainly to the toxic oxygen analog (2) which is taken up by insects. Insects are very sensitive to its toxic effects (3), apparently due to less effective detoxifying mechanisms (4), particularly ability to remove the methylamido group which has been termed the "weak link" in detoxification by insects (5). Dimethoate is readily absorbed from the mammalian gastrointestinal tract and biotransformed by conversion of the sulfur group to the oxygen analog, by hydrolysis of the methyl ester groups, and by removal of the methylamido group (2). Pretreatment of mice with SKF 525A lessens (3), while pretreatment with the hepatic enzyme-inducer phenobarbital increases (6), the toxicity of dimethoate. The toxic action of dimethoate is due to inhibition of various body esterases (7). The acute oral LD_{50} of technical dimethoate in male rats has been reported to be between 180 and 325 mg./kg. (2). Tolerance in human foods has been set at 2 p.p.m. (8).

The World Health Organization concluded that further studies were desirable on the worldwide use of dimethoate (2) and requested the authors to investigate its acute oral toxicity in albino rats fed from weaning on diets deficient in protein. The present communication is a report of such studies.

EXPERIMENTAL

The experiments were performed on male albino rats of a Wistar strain.[1] Group I consisted of 137 weanlings, weighing 50–60 g., fed Protein Test Diet—Low[2] which contains 3.5% casein, 81.5% cornstarch, 8% hydrogenated cottonseed oil, 4% salt mix USP XIV, and 3% of an all vitamin mixture (9). A 4-week feeding period was selected from the studies of De Castro and Boyd (10) who found that weanling rats react rather violently to a protein-deficient diet during the first 2 weeks but adjust during the 3rd and 4th weeks. After 28 days of feeding, the animals weighed 50 ± 5 g. (mean ± *SD*).

Group II was a control group of 120 weanlings fed Protein Test Diet—Normal[2] which contains 26% casein, 59% cornstarch, and other ingredients as in Protein Test Diet—Low. At the end of 28 days, their body weight was 199 ± 18 g.

Group III was a protein control group of 128 rats purchased 2 weeks from weaning and fed a standard laboratory chow for 2 weeks.[3] This diet contains 24% protein from various plant and

[1] Obtained from Woodlyn Farms Limited, Guelph, Ontario.
[2] General Biochemicals, Chagrin Falls, Ohio.
[3] The chow was Rockland Rat Diet (Complete), Teklad Inc., Monmouth, Ill.

(17) *Ibid.*, p. 137.
(18) E. J. Bollum and V. R. Potter, *Cancer Res.*, **19,** 561(1969).
(19) N. R. Joseph, R. Molinard, and F. Bourliere, *Gerontologia*, **1,** 18(1957).
(20) J. H. Blank, *J. Invest. Dermatol.*, **21,** 259(1953).
(21) J. D. Leeder and I. C. Watt, *Phys. Chem.*, **10,** 3289(1960).
(22) M. K. Polano, *J. Soc. Cosmet. Chem.*, **19,** 3(1968).
(23) B. R. Martin, "Introduction to Biophysical Chemistry," McGraw-Hill, New York, N. Y., 1964, p. 79.
(24) *Ibid.*, p. 258.
(25) C. Heidelberger and R. G. Moldenhauer, *Cancer Res.*, **16,** 442(1956).
(26) S. Rothman, "Physiology and Biochemistry of the Skin," University of Chicago Press, Chicago, Ill., 1954, p. 344.
(27) J. Monod, J. P. Chanyeux, and F. Jacob, *J. Mol. Biol.*, **6,** 306(1963).
(28) R. Mason, in "Wave Mechanics and Molecular Biology," L. deBroglie, Ed., Addison-Wesley, Reading, Mass., 1966, pp. 75–83.
(29) G. N. Ling, *Texas Rep. Biol. Med.*, **22,** 244(1964).
(30) G. N. Ling, "A Physical Theory of the Living State," Blaisdell, New York, N. Y., 1962, pp. 518–521.
(31) A. S. Michaels, *Ind. Eng. Chem.*, **10,** 32(1957).
(32) H. Pitot and C. Heidelberger, *Cancer Res.*, **23,** 1694(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 20, 1969, from the *Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication March 3, 1970.

This study was aided partly by a grant from the American Cancer Society and partly by funds provided by the Indiana Elks.

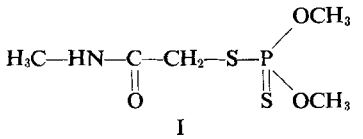
Acute Oral Toxicity of Dimethoate in Albino Rats Fed a Protein-Deficient Diet

ELDON M. BOYD and L. F. MUIS

Abstract □ The oral $LD_{50} \pm SE$ of dimethoate was found to be 147 ± 29 mg./kg., 152 ± 22 mg./kg., and 358 ± 9 mg./kg., respectively, in male albino rats fed for 28 days from weaning on a diet containing: (*a*) 3.5% protein as casein, (*b*) normal amounts of protein as casein, and (*c*) normal amounts of protein as laboratory chow. The toxic syndrome at the range of the LD_{50} was similar in animals of all three dietary groups and consisted of signs of cholinergic stimulation, depression of the central nervous system, anorexia, hypothermia, aciduria, proteinuria, an irritant gastroenteritis, widespread vascular congestion, toxic degenerative changes, dehydration, and loss of weight in body organs.

Keyphrases □ Pesticide toxicity study—rats □ Dimethoate—toxicity study, rats □ Protein-deficiency effect—dimethoate toxicity □ LD_{50}—dimethoate □ Toxic effects, rats—dimethoate

Dimethoate, an organophosphorus pesticide introduced in 1956, is effective as a systemic insecticide against pests such as houseflies, aphids, mites, and grasshoppers (1). It is a white crystalline solid with limited solubility in water but is freely soluble in cottonseed oil. The formula is shown in Structure I. It is



I
dimethoate

absorbed into plants and converted mainly to the toxic oxygen analog (2) which is taken up by insects. Insects are very sensitive to its toxic effects (3), apparently due to less effective detoxifying mechanisms (4), particularly ability to remove the methylamido group which has been termed the "weak link" in detoxification by insects (5). Dimethoate is readily absorbed from the mammalian gastrointestinal tract and biotransformed by conversion of the sulfur group to the oxygen analog, by hydrolysis of the methyl ester groups, and by removal of the methylamido group (2). Pretreatment of mice with SKF 525A lessens (3), while pretreatment with the hepatic enzyme-inducer phenobarbital increases (6), the toxicity of dimethoate. The toxic action of dimethoate is due to inhibition of various body esterases (7). The acute oral LD_{50} of technical dimethoate in male rats has been reported to be between 180 and 325 mg./kg. (2). Tolerance in human foods has been set at 2 p.p.m. (8).

The World Health Organization concluded that further studies were desirable on the worldwide use of dimethoate (2) and requested the authors to investigate its acute oral toxicity in albino rats fed from weaning on diets deficient in protein. The present communication is a report of such studies.

EXPERIMENTAL

The experiments were performed on male albino rats of a Wistar strain.[1] Group I consisted of 137 weanlings, weighing 50–60 g., fed Protein Test Diet—Low[2] which contains 3.5% casein, 81.5% cornstarch, 8% hydrogenated cottonseed oil, 4% salt mix USP XIV, and 3% of an all vitamin mixture (9). A 4-week feeding period was selected from the studies of De Castro and Boyd (10) who found that weanling rats react rather violently to a protein-deficient diet during the first 2 weeks but adjust during the 3rd and 4th weeks. After 28 days of feeding, the animals weighed 50 ± 5 g. (mean ± *SD*).

Group II was a control group of 120 weanlings fed Protein Test Diet—Normal[2] which contains 26% casein, 59% cornstarch, and other ingredients as in Protein Test Diet—Low. At the end of 28 days, their body weight was 199 ± 18 g.

Group III was a protein control group of 128 rats purchased 2 weeks from weaning and fed a standard laboratory chow for 2 weeks.[3] This diet contains 24% protein from various plant and

[1] Obtained from Woodlyn Farms Limited, Guelph, Ontario.
[2] General Biochemicals, Chagrin Falls, Ohio.
[3] The chow was Rockland Rat Diet (Complete), Teklad Inc., Monmouth, Ill.

Table I—Histopathological Observations at Death from Oral Administration of Dimethoate to Albino Rats

Organ	Histopathology
Adrenal glands	Lipoid globules prominent, especially in zona fasciculata
Brain	Meninges and brain hyperemic and congested
Gastrointestinal tract:	
Cardiac stomach	Occasionally submucosa hyperemic
Pyloric stomach	Areas of ulceration of the inner half of the gastric glands
Small bowel	Mild capillary congestion of the lamina propria of the villi
Cecum	Occasionally small infiltrative ulcers of the mucosa and submucosa
Colon	Normal appearance
Heart	Occasionally mild capillary congestion of the myocardium
Kidneys	Congestion of the glomerulus and loop of Henle; venous stasis and thrombosis; occasionally debris beneath Bowman's capsule; cloudy swelling, fine fatty degeneration, and early necrosis of the convoluted tubules
Liver	Sinusoidal congestion and cloudy swelling; fatty degeneration in animals of Group I
Lungs	Usually capillary congestion and venous stasis and thrombosis
Muscle (ventral abdominal wall)	Normal appearance
Pancreas	Deficiency of zymogenic granules in the acinar glands
Salivary (submaxillary) glands	Mucous glands shrunken; serous glands shrunken, granulated, and sometimes vacuolated
Skin	Occasionally ischemic
Spleen	Red pulp contracted
Testes	Deficiency of normal sperm in delayed deaths; inhibition of spermatogenesis in animals of Group I
Thymus gland	Loss of thymocytes, particularly in animals of Group I

animal sources. After 2 weeks of feeding the animals weighed 171 ± 5 g.

At the end of the dieting period, each rat was placed singly in a metabolism cage with water. They received no food for 16 hr. (overnight) to empty the stomach prior to oral administration of dimethoate. Technical dimethoate[4] was freshly dissolved in cottonseed oil USP and given intragastrically in a volume of 20 ml./kg. body weight. Following pilot dose studies in each dietary group, a series of definitive doses, estimated to yield mortality rates from just above 0% to just below 100%, were administered to 10 rats per dose with 15–20 controls given cottonseed oil.

The animal was then returned to its metabolism cage with an excess of diet and drinking water. Clinical signs were measured in units of 1+ to 4+ at hourly intervals during the balance of the 1st day and then at intervals of 24 hr. or as indicated. Body weight gain, food intake, water intake, colonic temperature, urinary volume, urinary blood, urinary glucose and protein output, and urinary pH were measured daily for 5 days.

An autopsy was performed upon all dead animals, the gross pathology was recorded, and a microscopic examination was made upon any organ that appeared abnormal to gross examination. Histopathology was recorded at death upon all organs listed in Table I in representative dead animals of each dietary group.

The wet weight of organs listed in Table II was measured upon animals which could be autopsied within 1 hr. of death to avoid postmortem shifts in organ weights and water levels described by Boyd and Knight (11). Water content was measured upon aliquots of the organs listed in Table III dried to constant weight in a Fisher forced draft isotemp oven at 95° and was calculated as grams water per 100 grams dry weight of tissue.

[4] Cygon, 93.3% dimethoate, Agricultural Division, American Cyanamid Co., Princeton, N. J.

The results were analyzed statistically by the application of *t* tests to the significance of differences between means and by the regression of differences on dose or time. The $LD_{50} \pm SE$ was calculated by linear regression analysis of dose on response. Details of the method, including statistical analysis, have been reviewed by Boyd (12).

RESULTS

Data on calculated lethal doses of dimethoate are assembled in Table IV. The LD_{50} in Group I is identical to that in Group II, and both values are about half that in Group III. The interval to death varies inversely with the dose; *i.e.*, the higher the dose of dimethoate, the more rapid is the occurrence of death. The mean interval to death is identical in all three dietary groups. Pathological lesions seen on gross examination at autopsy are similar in all three dietary groups.

The clinicopathological syndrome of toxicity is essentially the same in animals of all three dietary groups. It will be exemplified by describing changes in animals previously fed laboratory chow (Group III) and by noting significant differences in animals of the other two dietary groups.

The dominant clinical signs of toxicity during the first few hours are tremors, prostration, dacryorrhea, and exophthalmos (Fig. 1). They reach a peak of intensity during the afternoon of the 1st day and begin to disappear at 24 hr. A delayed clinical syndrome, which reaches a peak at 24 hr. (Fig. 2), consists of piloerection, listlessness, sialorrhea, and soiling of the fur. These clinical signs are accompanied by a marked inhibition of food and water intake, hypothermia, and marked loss of body weight (Fig. 3). At 24 and 48 hr., all mean changes shown in Fig. 3 are significant at $p < 0.01$.

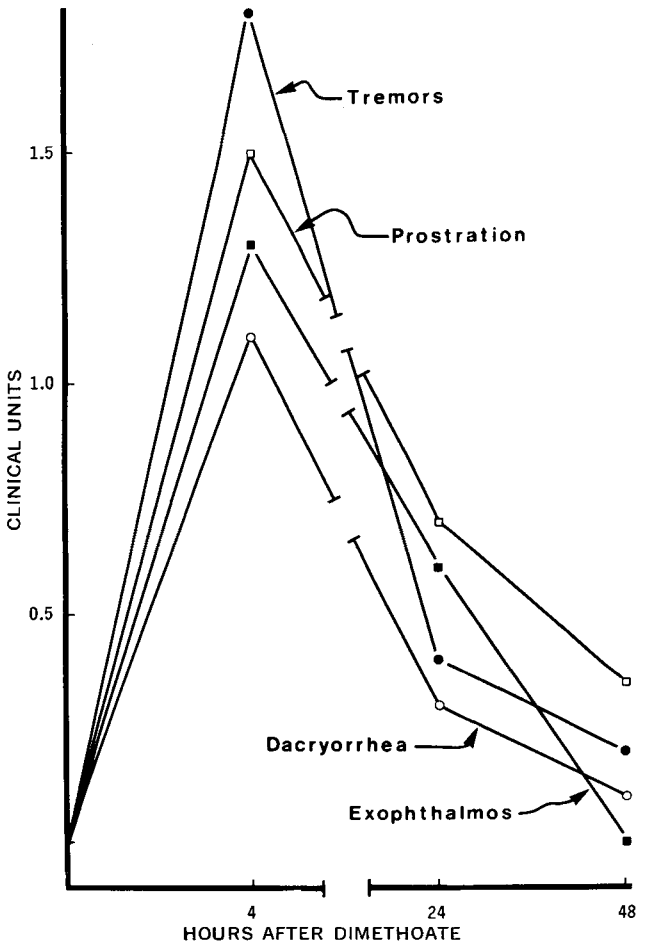


Figure 1—*Mean clinical units of intensity of clinical signs which were dominant during the 1st day of the toxicity syndrome to oral administration of lethal doses of dimethoate in albino rats previously fed laboratory chow.*

Table II—Shifts in the Fresh Wet Weight of Body Organs at Death following Oral Administration of Dimethoate[a]

Organ	Group I (3.5% Casein) $N = 14$ plus 14 Controls	Group II (26% Casein) $N = 15$ plus 14 Controls	Group III (Chow) $N = 15$ plus 15 Controls
Adrenal glands	−1.1	+15.2**[c]	+13.8*
Brain	−5.8*[b]	−0.7	−0.7
Gastrointestinal tract:			
Cardiac stomach	−26.2**	−12.5*	−12.5*
Pyloric stomach	−14.7**	−13.4**	−11.1**
Small bowel	−4.4	−11.1	−18.7**
Cecum	−36.8**	−17.6*	−21.4**
Colon	−18.1**	+2.8	−18.4**
Heart	−3.2	−9.4*	+3.6
Kidneys	+4.3	−11.0*	−8.2**
Liver	−16.6**	−27.8**	−23.8**
Lungs	−17.5*	−26.0**	−0.3
Muscle (ventral abdominal wall)	−13.6*	−3.3	−18.6**
Salivary (submaxillary) glands	−10.3	−23.9**	−28.5**
Skin	−2.3	−13.9*	−10.4*
Spleen	−45.3**	−38.5**	−45.8**
Testes	+18.7*	−13.0*	−1.9
Thymus gland	−44.4**	−33.7**	−14.3*
Residual carcass	−3.6	−17.2**	−7.2*
Autopsy body weight	−7.0*	−13.2**	−14.0**

[a] Weight was measured in grams. The results are expressed as mean percent change from controls fed the same diet but given cottonseed oil with no dimethoate, specifically as $[(\bar{X}d - \bar{X}c)/\bar{X}c] \times 100$, where $\bar{X}d$ is the mean in rats given dimethoate and $\bar{X}c$ in the controls. [b] * indicates that $\bar{X}d - \bar{X}c$ was significant at $p = 0.05$ to 0.02. [c] ** indicates that $\bar{X}d - \bar{X}c$ was significant at $p = 0.01$ or less.

Significant changes in urine are indicated in Fig. 4. At 24 hr., there occurs an oliguria with aciduria; at 72 hr., there is a reactive diuresis and urinary pH returns to normal. Proteinuria, glucosuria,

Table III—Shifts in the Water Levels of Body Organs at Death from Oral Administration of Dimethoate[a]

Organ	Group I (3.5% Casein) $N = 14$ plus 14 Controls	Group II (26% Casein) $N = 15$ plus 14 Controls	Group III (Chow) $N = 15$ plus 15 Controls
Adrenal glands	+3.6	+26.7**[b]	+14.3*[c]
Brain	−1.8	−2.2	−1.6
Gastrointestinal tract:			
Cardiac stomach	−22.6**	−3.3	−16.3**
Pyloric stomach	−6.3**	+4.2	−5.3*
Small bowel	−9.0**	+1.3	−3.5
Cecum	−18.3**	−7.7*	−15.6**
Colon	−10.2*	−3.3	−11.7**
Heart	−10.6**	−6.9**	−6.2**
Kidneys	−4.4*	−5.9*	−9.7**
Liver	−5.3*	−0.9	−3.7*
Lungs	−24.1**	−18.4**	−17.3**
Muscle (ventral abdominal wall)	−13.4**	−16.4**	−9.4*
Salivary (submaxillary) glands	−11.9*	−15.0**	−22.0**
Skin	−14.1*	−15.0*	−11.1*
Spleen	−5.7	−1.9	−3.8
Testes	+4.1	−6.5**	−8.9**
Thymus gland	−23.0**	−11.4**	−15.8**
Residual carcass	−11.1**	−3.6	−8.1**

[a] Water levels were measured as grams water per 100 g. dry weight of tissue. The results are expressed as mean percent change from controls fed the same diet and given cottonseed oil without dimethoate, specifically as $[(\bar{X}d - \bar{X}c)/\bar{X}c] \times 100$, where $\bar{X}d$ is the mean in dimethoate-treated rats and $\bar{X}c$ in controls. [b] ** indicates that $\bar{X}d - \bar{X}c$ was significant at $p = 0.01$ or less. [c] * indicates that $\bar{X}d - \bar{X}c$ was significant at $p = 0.05$ to 0.02.

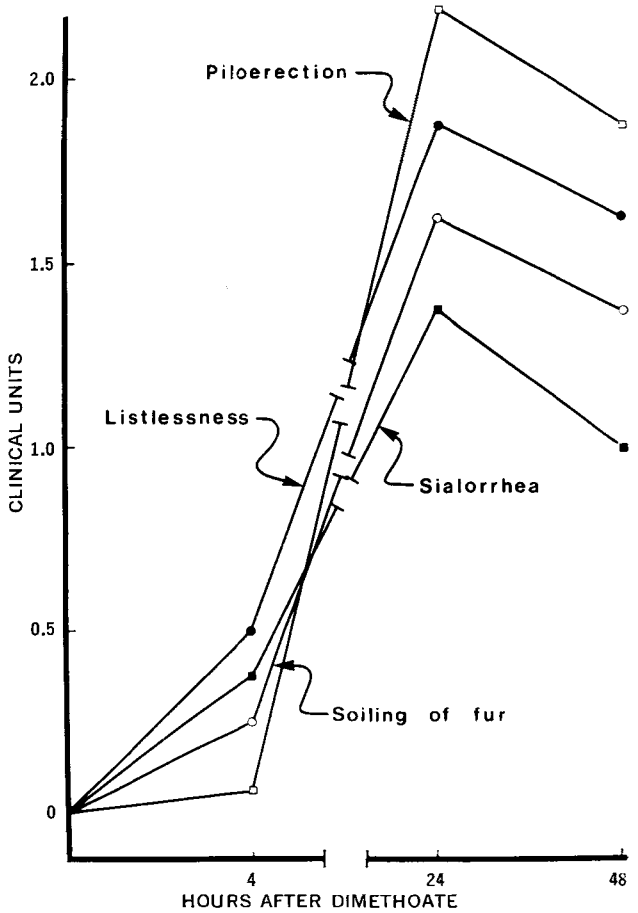


Figure 2—*Mean clinical units of intensity of clinical signs which were dominant at 24–48 hr. after oral administration of lethal doses of dimethoate to albino rats previously fed laboratory chow.*

and hematuria begin to appear during the first 24 hr. and are present in survivors at 48 and 72 hr.

The protein-deficient animals of Group I do not exhibit exophthalmos and have less hypothermia during the first few hours after receiving dimethoate, and piloerection is marked at this time. There is no delayed reaction at 24 hr., and recovery of survivors is rapid in Group I; growth rate, for example, returns to normal during the 2nd day.

In Group II, fed a diet of 26% casein, the initial reaction is similar to that of Group III except that listlessness, piloerection, and sialorrhea are present immediately after dimethoate. Soiling appears at 24 hr., but there is no glycosuria or hematuria. Recovery is delayed to the 3rd day as in Group III.

Microscopically (Table I), there is a moderate, local, irritant gastroenteritis with ulceration in the pyloric or glandular part of the stomach and in the cecum. Cecal ulcers are not particularly common in studies of this nature. After absorption, the most common lesion is vascular congestion. This is accompanied by degenerative changes in the kidneys, liver, pancreas, salivary glands, and testes. There is a stress reaction in the adrenal glands, spleen, and thymus gland and the skin is ischemic. There are signs of kwashiorkor due to protein deficiency in animals of Group I, as indicated by impaired development of several organs.

There is a marked loss of weight in most organs (Table II). Loss of weight is particularly marked in the tissues of the gastrointestinal tract, liver, lungs, muscle, spleen, and thymus gland. Loss of weight is due in part to dehydration as shown by data summarized in Table III. The changes are, in general, similar in animals of all three dietary groups, and the few differences which are found may be seen from values quoted in Tables II and III.

DISCUSSION

The results of this investigation indicated that dimethoate was no more toxic to rats fed a protein-deficient diet than to controls

Table IV—Lethal Doses, Interval to Death, and Gross Pathology following Oral Administration of Lethal Doses of Dimethoate

Measurement	Group I, Protein Test Diet—Low (3.5% Casein)	Group II, Protein Test Diet—Normal (26% Casein)	Group III, Laboratory Chow (24% Protein)
Estimated maximal LD_0, mg./kg.	92†[a]	74†	345
$LD_{50} \pm SE$, mg./kg.	147 ± 29†	152 ± 22†	358 ± 9
Estimated minimal LD_{100}, mg./kg.	202†	230†	371
Hours to death, mean ± SD*[b]	17 ± 4	21 ± 14	22 ± 9
Gross pathology	Congested brain Acute gastritis Renal pallor Hepatitis Pneumonitis	Congested brain Acute gastritis Renal pallor Hepatitis Pneumonitis	Congested brain Acute gastritis Hepatitis Pneumonitis

[a] †, significantly different from results in Group III at $p = 0.02$ or less.
[b] *, see text for variation with dose.

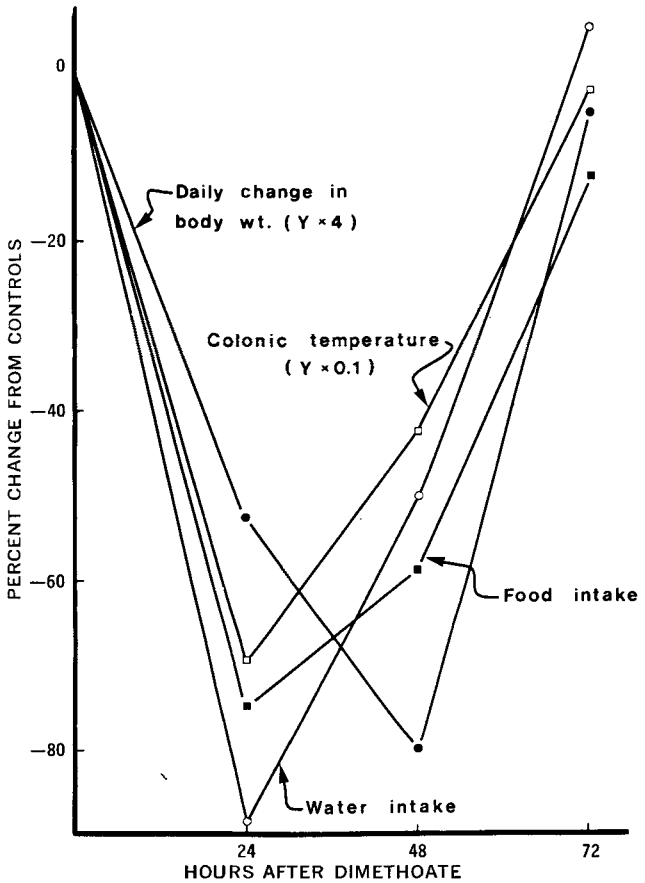


Figure 3—*Significant mean differences in daily body weight gain (Y × 4), colonic temperature (Y × 0.1), food intake (Y × 1), and water intake (Y × 1) following oral administration of lethal doses of dimethoate to albino rats previously fed laboratory chow. Differences were calculated as mean percent change from controls given cottonseed oil, specifically as* $[(\bar{X}_d - \bar{X}_c)/\bar{X}_c] \times 100$, *where* $\bar{X}_d$ *is the mean in dimethoate-treated animals and* $\bar{X}_c$ *the corresponding mean in controls. Note that the ordinate units must be multiplied by the factors indicated in parenthesis to obtain the mean percentage change for daily gain in body weight and for colonic temperature.*

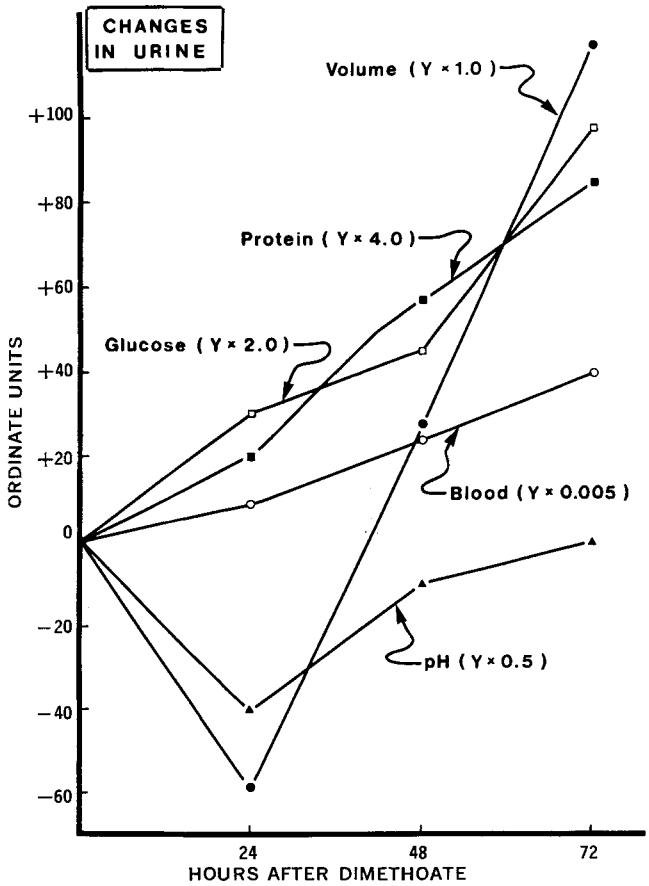


Figure 4—*Significant mean changes in the urine of albino rats previously fed laboratory chow following oral administration of lethal doses of dimethoate. The ordinate units for volume, protein output, and pH represent mean percent change from controls calculated as in Fig. 3. The ordinate units for glucose output indicate milligrams per kilogram body weight per day since there was no glucose in the urine of controls given cottonseed oil. The ordinate for urinary blood indicates units of blood. The ordinate units must be multiplied by the factors indicated in parenthesis to obtain the mean change in each parameter.*

fed adequate amounts of protein as casein. Protein deficiency causes degenerative changes and inhibition of growth in the liver (10) and could be expected to limit production of hepatic detoxifying enzymes. This, in turn, could lessen production of the highly toxic oxygen analog of dimethoate. A decreased production of the oxygen analog could account for no increase in the toxicity of dimethoate in the protein-deficient rat, which is generally more susceptible to pesticide toxicity.

Available data on pesticide toxicity in protein-deficient rats have been summarized in Table V. Dimethoate is the only pesticide studied which is not more toxic to the protein-deficient rat. Five pesticides were twice as toxic to the protein-deficient rat; the five were diazinon and malathion, which are organic phosphorothioates like dimethoate, and chlordane, endrin, and lindane, which are chlorinated organic insecticides. Dicophane or DDT and monuron, an herbicidal chloro compound, were three times as toxic. Four compounds were four times as toxic: chlorpropham, which is an herbicide related to monuron, demeton (an organic phosphorothioate), and two chlorinated insecticides (endosulfan and toxaphene). Carbaryl is a physostigminelike cholinesterase inhibitor of the naphthyl carbamate series and was six times as toxic. Parathion is a *p*-nitrophenyl organic phosphorothioate and was eight times as toxic. Casterline and Williams (29) have reported parathion to be more toxic in young adult rats fed for 30 days on a diet containing no protein than in animals fed a diet containing 15% of protein as casein. Captan is a mercapto-phthalidimide related structurally to thalidomide and was 26 times as toxic.

Table V—Acute Oral LD_{50} of Pesticides in Male Albino Rats Fed for 28 Days from Weaning on a Protein-Deficient Diet Containing 3.5% Casein Compared with Values in Controls Fed a Normal Protein Diet Containing 26% Casein[a]

Pesticide	Protein-Deficient Diet, Group I	Normal Protein Diet, Group II	Quotient: II/I	Reference
Dimethoate	147 ± 29	152 ± 22	1	This paper
Chlordane	137 ± 30	267 ± 44	2	13
Diazinon	215 ± 26	415 ± 39	2	14
Endrin	6.69 ± 0.80	16.6 ± 3.0	2	15
Lindane	95 ± 33	184 ± 16	2	16
Malathion	599 ± 138	1401 ± 99	2	17
Dicophane (DDT)	165 ± 34	481 ± 13	3	18, 19
Monuron	950 ± 240	2880 ± 310	3	20
Chlorpropham	2590 ± 480	10390 ± 1580	4	21
Demeton	2.13 ± 0.37	7.62 ± 0.22	4	22
Endosulfan	24 ± 10	102 ± 16	4	23
Toxaphene	80 ± 19	293 ± 31	4	24
Carbaryl	89 ± 11	575 ± 51	6	25, 26
Parathion	4.86 ± 1.31	37.1 ± 4.9	8	27
Captan	480 ± 110	12600 ± 2100	26	28

[a] The LD_{50} is expressed as mg./kg. ± *SE*.

The results of these investigations indicate that further studies, including clinical toxicology trials, should be done upon pesticides listed toward the bottom of Table V to determine if they present a particular hazard when used in countries where the diet is low in protein. No evidence is present in these investigations to indicate that such a hazard exists for pesticides listed toward the top of Table V.

The type of dietary protein does not appear to be as important a factor contributing to augmented susceptibility to pesticides as is deficiency of protein. Of the pesticides listed in Table V, chlorpropham and monuron were twice as toxic in rats fed laboratory chow as in rats fed normal amounts of protein as casein, while dimethoate was half as toxic. Dietary soy protein in normal amounts has been found, in current studies, to augment the susceptibility of rats to certain drugs such as phenacetin. There may be significant differences depending upon the type of protein in a protein-deficient diet, a problem which has not been studied in this laboratory.

Variation in the concentration of dietary protein involves alteration in the concentration of other dietary ingredients and is usually accomplished by changes in the amount of carbohydrate. The type of carbohydrate selected is of considerable importance. Soluble sugars such as glucose and sucrose should not be used in large amounts, because they can produce dehydration and death (30), and, in sublethal doses, they can add to the toxicity of drugs (31). Starch, on the other hand, produces death only by bowel obstruction from huge doses (32) and is nontoxic in large amounts in the diet. In the present study, therefore, cornstarch was used, rather than a sugar, to replace a lowered concentration of dietary protein.

REFERENCES

(1) E. Y. Spencer, "Guide to Chemicals Used in Crop Protection," 5th ed., Publication 1093, Canada Department of Agriculture, The Queen's Printer, Ottawa, Canada, 1968, p. 186.

(2) FAO/WHO Report, "1967 Evaluation of Some Pesticide Residues in Food. The Monographs," FAO/PL: 1967/M/11/1 and WHO/Food Add./68.30, Food and Agriculture Organization and World Health Organization, United Nations, Geneva, Switzerland, 1968, pp. 117–132.

(3) R. D. O'Brien, *Biochem. J.*, **79**, 229(1961).

(4) S. M. A. D. Zayed, A. Hassan, and I. M. I. Fakhr, *Biochem. Pharmacol.*, **17**, 1339(1968).

(5) R. D. O'Brien, *Can. J. Biochem. Physiol.*, **37**, 1113(1959).

(6) R. E. Menzer and M. H. Best, *Toxicol. Appl. Pharmacol.*, **13**, 37(1968).

(7) D. J. Ecobichon and W. Kalow, *Can. J. Biochem. Pharmacol.*, **41**, 1537(1963).

(8) R. A. Chapman, T.I.L. No. 290, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada, 1967, p. 21.

(9) D. M. Hegsted and Y.-O. Chang, *J. Nutr.*, **85**, 159(1965).

(10) E. S. De Castro and E. M. Boyd, *Bull. W. H. O.*, **38**, 971 (1968).

(11) E. M. Boyd and L. M. Knight, *Toxicol. Appl. Pharmacol.*, **5**, 119(1963).

(12) E. M. Boyd, *Can. Med. Ass. J.*, **98**, 278(1968).

(13) E. M. Boyd and F. I. Taylor, *Ind. Med. Surg.*, **38**, 434 (1969).

(14) E. M. Boyd and E. Carsky, *Acta Pharmacol. Toxicol.*, **27**, 284(1969).

(15) E. M. Boyd and J. Stefac, *Can. Med. Ass. J.*, **101**, 335(1969).

(16) E. M. Boyd and C. P. Chen, *Arch. Environ. Health*, **17**, 156(1968).

(17) E. M. Boyd and T. K. Tanikella, *Arch. Toxikol.*, **24**, 292 (1969).

(18) E. M. Boyd and E. S. De Castro, *Bull. W. H. O.*, **38**, 141 (1968).

(19) E. M. Boyd and C. J. Krijnen, *Bull. Environ. Contam. Toxicol.*, **4**, 256(1969).

(20) E. M. Boyd and I. Dobos, *J. Agr. Food Chem.*, **17**, 1213 (1969).

(21) E. M. Boyd and E. Carsky, *Arch. Environ. Health*, **19**, 621 (1969).

(22) E. M. Boyd and V. Krupa, *Can. J. Pharm. Sci.*, **4**, 35(1969).

(23) E. M. Boyd and I. Dobos, *Arch. Int. Pharmacodyn.*, **178**, 152(1969).

(24) E. M. Boyd and F. I. Taylor, to be published.

(25) E. M. Boyd and M. A. Boulanger, *J. Agr. Food Chem.*, **16**, 834(1968).

(26) E. M. Boyd and C. J. Krijnen, *J. Clin. Pharmacol.*, **9**, 292 (1969).

(27) E. M. Boyd, C. P. Chen, and S. J. Liu, *Arch. Toxikol.*, **25**, 238(1969).

(28) E. M. Boyd and C. J. Krijnen, *J. Clin. Pharmacol.*, **8**, 225 (1968).

(29) J. L. Casterline, Jr., and C. H. Williams, *Toxicol. Appl. Pharmacol.*, **14**, 266(1969).

(30) E. M. Boyd and E. Carsky, *Acta Diabet. Latina*, **4**, 538 (1967).

(31) E. M. Boyd, E. L. Covert, and C. A. Pitman, *Chemotherapy*, **11**, 320(1967).

(32) E. M. Boyd and S. J. Liu, *Can. Med. Ass. J.*, **98**, 492(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 3, 1969, from the *Department of Pharmacology, Queen's University, Kingston, Ontario, Canada.*

Accepted for publication April 10, 1970.

This project was assisted by a grant from the World Health Organization.

Effect of Solvent Composition on Association between Small Organic Species

HARALD KRISTIANSEN, MASAHIRO NAKANO*, NAOMI I. NAKANO, and TAKERU HIGUCHI†

Abstract □ Effects of different organic solvents on the extent of complex formation in binary aqueous organic solvent mixtures were studied by means of spectrophotometry, spectropolarimetry, and solubility technique. In every instance, the stability constants of the complexes decreased as the ratio of organic solvent to water increased. The complexes were much less stable in aqueous dioxane mixtures than in similar mixtures of water and polyhydroxy compounds such as glycerin and sucrose. These studies strongly indicate the significant contribution made by hydrophobic bonding to these interactions and the major role of the water structure.

Keyphrases □ Organic species, small—interaction □ Complex formation—solvent composition effect □ Stability constants, complexes—solvent composition effect □ Solvent effect—small organic species interaction

The uniqueness of water as an environment for biological interactions has been realized in recent years (1). To account for the fact that many solute molecules, notably polymers, associate more strongly in water than in organic solvents, the concept of hydrophobic bonding has been developed (2). The exact role played by water, however, is not yet fully understood. Recent studies of the effect of various solvents on the stability constants of the tetramethylpyrimidopteridinetetrone–*N*,*N*-dimethylcinnamide complex indicated that the complex was less than one-tenth as stable in organic solvents as in water (3). Weber has reported the effect of solvents on quenching of the fluorescence of flavins in the presence of complexing agents (4). The quenching was observed only in water and not in organic solvents, including formamide which is more polar than water.

Sinanoglu and Abdulnur have undertaken theoretical studies on the effect of solvents on the denaturation of deoxyribonucleic acid (5, 6). They indicated that the main source of stability of the helical structure of the nucleic acid in water over organic solvents comes from the large surface enthalpy of water, *i.e.*, large surface tension of water. Subsequently, Moser and Cassidy have examined the extent of quinhydrone formation in various mixtures of water and organic solvents and found a reasonable correlation between surface tension and the extent of complex formation (7).

As a part of the present investigation into the nature of molecular association of small organic species in aqueous solution (3, 8–11), the authors have undertaken a quantitative study of the influence of various organic solvents in mixed solvent systems on the extent of complexation. The main purpose of the present study was to obtain additional information about the factors determining the stability of these complexes in aqueous solution.

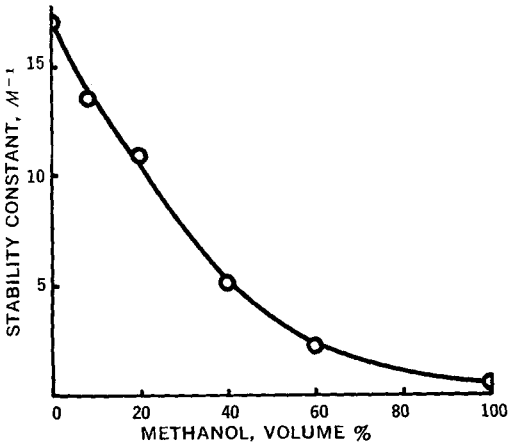


Figure 1—*Stability constant of riboflavin–salicylate-ion complex plotted against solvent composition as measured spectrophotometrically at 25°.*

EXPERIMENTAL

Materials—*trans*-Cinnamic acid, 3,4-dimethoxycinnamic acid, and 1,3,7,9-tetramethylpyrimido(5,4,g)pteridine-2,4,6,8(1H,3H,-7H,9H)-tetrone (abbreviated as TMPPT) were from the same commercial source[1] and were purified and used as previously described (12). Riboflavin, menadione, sodium salicylate, caffeine, sucrose, and glycerin were USP grade chemicals. Caffeine was dried at 105° overnight to obtain an anhydrous compound. Tryptophan[2] was recrystallized from ethanol–water. *N*,*N*-Dimethylcinnamide was synthesized in this laboratory (10). Methanol,[3] acetonitrile,[4] acetone,[3] and dioxane[5] were reagent grade solvents. Water was purified by distillation in an all glass still.

Determination of Stability Constants—The stability constants of 1:1 and 1:2 complexes were defined in the following way:

$$A + B = AB \qquad K_{1:1} = \frac{[AB]}{[A][B]} \qquad \text{(Eq. 1)}$$

$$AB + B = AB_2 \qquad K_{1:2} = \frac{[AB_2]}{[AB][B]} \qquad \text{(Eq. 2)}$$

where brackets refer to equilibrium concentrations. Experimental procedures and calculations of stability constants employed in this study are reported elsewhere; the procedures include spectrophotometric (13), spectropolarimetric (11), and solubility (12, 14) techniques.

RESULTS

Effect of Solvent Composition on Stability Constants of Complexes—*Riboflavin–Salicylate Ion*—Apparent 1:1 stability constants of the riboflavin–salicylate-ion complex in water–methanol mixtures were determined spectrophotometrically; the results are shown in Fig. 1. Displacement of a fraction of water with methanol resulted in a steady decrease in the stability constant of the complex.

Menadione–Caffeine—Effect of solvent composition on the stability constant of the menadione–caffeine complex was also

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Sankyo Kasei Co., Tokyo, Japan.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Chemical Manufacturing Div., Fisher Scientific Co., Fair Lawn, N. J.
[5] J. T. Baker Chemical Co., Phillipsburg, N. J.

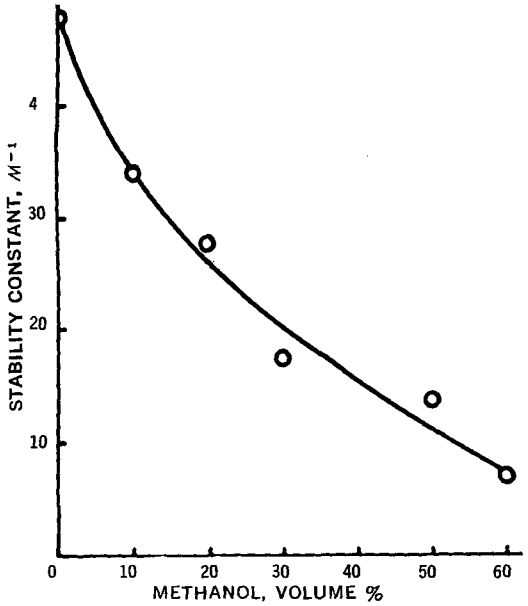


Figure 2—*Stability constant of menadione–caffeine complex plotted against solvent composition as measured spectrophotometrically at 25°.*

investigated spectrophotometrically; the results are shown in Fig. 2. A drastic decrease in stability constants with increasing methanol fraction is also apparent with this complex.

Tryptophan–Caffeine—Effects of environment on the stability constant of this amino acid–alkylxanthine complex were examined spectropolarimetrically. The results presented in Fig. 3 for this complex exhibit the same trend as observed in other systems investigated here.

TMPPT–3,4-Dimethoxycinnamate Ion—The stability constants of 1:1 and 1:2 complexes ($K_{1:1}$ and $K_{1:2}$) formed by the interaction of TMPPT with 3,4-dimethoxycinnamate ion in various compositions of methanol–water mixtures were computed from the phase-solubility diagrams. The stability constants are presented in Fig. 4 as a function of the solvent composition. It is apparent from the figure that both constants decrease with increasing fraction of methanol and the equilibria are highly dependent upon the solvent composition.

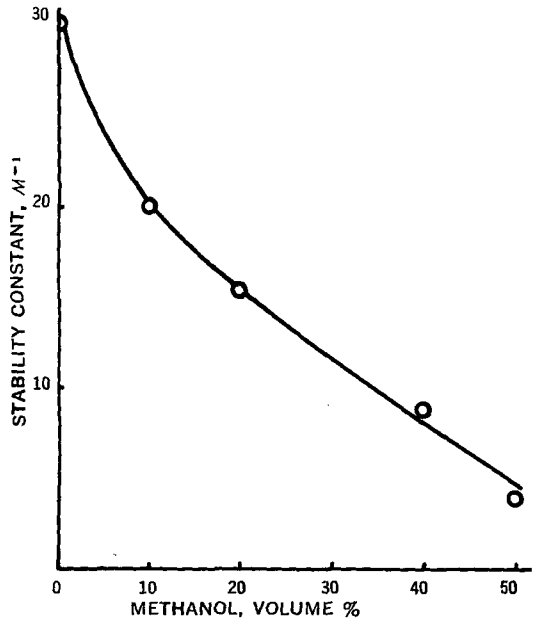


Figure 3—*Stability constant of tryptophan–caffeine complex plotted against solvent composition as measured spectropolarimetrically at 25°.*

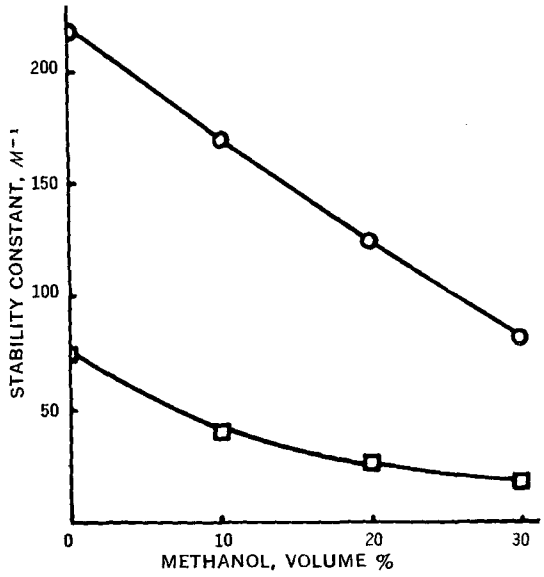


Figure 4—*Stability constant of 1:1 complex (○) and 1:2 complex (□) of TMPPT with 3,4-dimethoxycinnamate ion plotted against solvent composition as measured by solubility technique at 25°.*

TMPPT–Cinnamate Ion—Figure 5 presents the stability constants of the TMPPT–cinnamate-ion complex ($K_{1:1}$) in both water–methanol mixtures and water–dioxane mixtures. These stability constants were determined by the solubility technique and are shown in Fig. 5 as a function of solvent composition. These results again indicate that environment plays a major role in the stability of the complex. Dioxane was more effective in dissociating a complex than methanol. This point was further examined as presented in the next section.

Comparison of Effects of Various Solvents at 10% Level on Stability Constants—Since the stability constants of the complexes tend to be quite low in most organic solvents, the effects of such solvents on stability constants can be more easily evaluated at lower solvent concentrations in water. For this reason, the effects of various organic solvents on the stability constants of complexes were investigated in 10% v/v organic solvent in water. The TMPPT–*N,N*-dimethylcinnamide and menadione–caffeine complexes were chosen as model complexes for this purpose and studied by the solubility method and spectrophotometry, respectively.

The results are shown in Table I together with the values of surface tension of the media. Compounds such as sucrose and glycerin exhibited a much less destabilizing effect on the complexes than the other solvents investigated. Acetonitrile, acetone, and dioxane displayed a larger dissociating effect than the hydroxy compounds, dioxane exhibiting the greatest effect among the solvents investigated.

DISCUSSION

As amply demonstrated in this study, organic solvents have an unfavorable effect on the stacking interactions of organic planar molecules in water. Thus, irrespective of the solutes investigated here and of the methods employed to study these interactions, a steady decrease in the stability of a complex was observed with increasing fractions of organic solvent in aqueous media. Further, the comparison of various organic solvents at the 10% organic solvent–water mixture level indicates that the destabilizing effect of these organic solvents depends upon the nature of solvents and also upon the nature of interacting molecules.

Although interactions in pure organic solvents are greatly reduced in comparison with those in water, there nevertheless exists some associative tendency in pure organic solvents. For instance, the TMPPT–*N,N*-dimethylcinnamide complex has a stability constant of 60.0 M^{-1} at 25° in water, whereas in pure organic solvents such as methanol, acetone, and dioxane, the values range between 2.5 and 4.6 M^{-1} at 25° (3). Yet it is evident that an aqueous environment is necessary for significant interaction to take place between these species. The considerable stability of these complexes

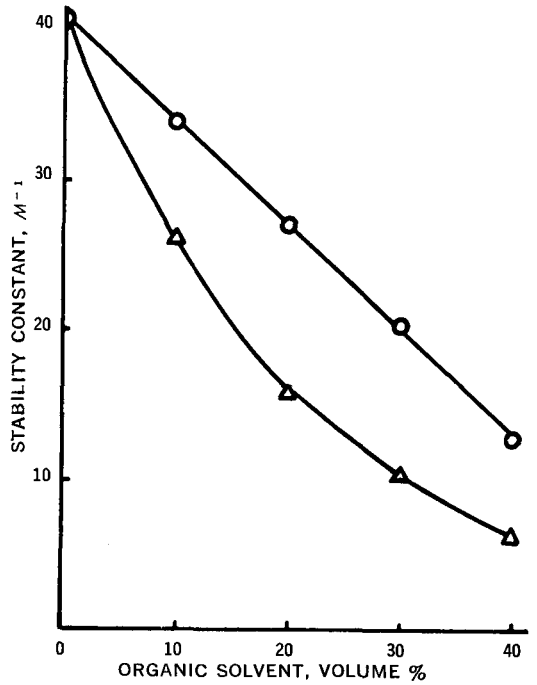


Figure 5—*One-to-one stability constant of TMPPT–cinnamate-ion complex plotted against solvent composition as measured by solubility technique at 25°. Key: O, methanol–water mixture; and Δ, dioxane–water mixture.*

in water may then be regarded as medium-facilitated interactions. Although the exact mechanism by which water uniquely facilitates this type of association is not known, it may be speculated that the structural feature of water plays a major role.

With the exception of the existence of a hydrogen-bonded network, the structure of water still remains controversial. A popular view in recent years is to consider water as a mixture of bulky four-coordinated cluster and dense monomeric water (15, 16). Regarding the influence of solutes upon water structure, Frank outlined the most widely held view (15, 16). Hydrogen-bonding molecules are, in general, expected to alter water structure comparatively little. Those that cannot participate in the four-coordinated clusters, however, tend to break water structure. Sucrose and glycerin are considered to be capable of hydrogen bonding with water because of their polyhydroxyl groups, whereas others such as acetonitrile, acetone, and dioxane can be looked upon as the breakers of the regular structure of water. This may explain the reason why sucrose and glycerin are less effective in dissociating complexes than the nonhydroxyl solvents.

It is interesting to compare the effect of organic solvents on the extent of complexation with that on the micellar properties of surfactants in aqueous solution. It has been observed that critical micelle concentration increases in a monotonic manner with increasing fraction of organic solvent in aqueous media (17, 18). The effect was found to be more pronounced in dioxane–water mixtures than in ethanol–water mixtures. Thus, the relative effects of dioxane and the alcohol on the micellar properties of surfactants are similar to those observed in the present study of complex formation. Since hydrophobic interaction is supposed to be responsible for micelle formation, a similar effect of organic solvents on both micelle and complex formations may be reasonable, and hydrophobic interactions may be considered to be of importance for the association of solute molecules in water.

There are two theories as to the cause of hydrophobic interaction. Hydrophobic bonding is attributed by some workers to the structural restriction of water around nonpolar groups of a solute molecule; *i.e.*, nonpolar groups are surrounded by an "iceberg" zone of water in which the orientation disorder is smaller than bulk water (19, 20). According to this theory, the total number of structurally restricted water molecules will be smaller when two molecules are brought together than when they are separated. This leads to an increase in entropy upon complexation. This positive entropy term

Table I—Stability Constants of Complexes in 10% v/v Organic Solvent in Water at 25°

Organic Solvent	TMPPT–DMCA[a] Complex	Menadione–Caffeine Complex	Surface Tension[b]
None	60.0	48	72.0
Sucrose[c]	56.7	—	72.4
Glycerin	51.0	38	72.8
Methanol	48.7	34	58.2
Acetonitrile	39.9	25	53.8
Acetone	41.5	22	50.9
Dioxane	36.1	—	45.7[d]

[a] *N,N*-Dimethylcinnamide. [b] "Handbook of Chemistry and Physics," 49th ed., R. C. Weast, Ed., The Chemical Rubber Co., Cleveland, Ohio, pp. 28–30. [c] 10% w/v. [d] J. Timmermans, "The Physical-Chemical Constants of Binary Systems in Concentrated Solutions," vol. IV, Interscience, New York, N. Y., 1960, p. 14.

is considered to be the major contribution to the free energy of hydrophobic interaction.

The second school of thought ascribes hydrophobic interaction to the energy gained when two cavities accommodating two single molecules coalesce to one when a complex is formed (5, 6). In this theory the enthalpy term (*i.e.*, negative ΔH) is largely responsible for the favorable interaction in water. This theory thus places a particular emphasis upon the large surface tension of water as being responsible for the unique behavior of water in stabilizing complexes of this nature.

Experimentally, however, negative ΔS for the interactions of simple organic species are more common than positive ΔS as the first theory suggests (11). Fitness of the second theory (enthalpy contribution), on the other hand, gave some suggestive trend. Although the extent of interaction cannot be quantitatively correlated with the surface tension of the media (Table I), surface tension probably is nevertheless related to a common physical property which also influences the intensity of hydrophobic bonding.

Hydrophobic bonding is generally recognized as important for the stability of protein conformations in aqueous solution (2, 20) and is referred to as the intramolecular force operating between the nonpolar side chains of these macromolecules. It is important to emphasize that the systems studied in the present work are interactions between small organic species, and it may be far from secure at this stage to correlate the observed binding in this case with the behavior of large molecules in solution. While the proteins are denatured to varying extent in aqueous organic solvents and in pure organic solvents, the same cannot, of course, take place for the aromatic and heterocyclic species studied here.

As was pointed out in the studies of complex formation between small organic molecules in aqueous solution (8, 9, 12), variation in the ability to bind cannot be certainly explained by hydrophobic forces alone. For example, it has been shown that hydroxy groups on benzoates and cinnamates had a strong enhancing effect on the observed binding in water (8, 9, 12). It is difficult to attribute this effect to a higher hydrophobic character of the interacting species. It has also been demonstrated that the contribution from flexible alkyl side chains was very small (12).

The observation that the binding between organic species dissolved in water apparently takes place most effectively between members of two large, distinct classes of structures (12) has strongly suggested that some selectivity must be operating among the interactants. This again tends to indicate that hydrophobic forces alone cannot be largely responsible, in most instances, for the observed binding, but rather a mechanism of some nonclassical "donor–acceptor" type may be operating. Also the fact that a slight change in molecular structure, notably by introducing substituents having mesomeric effects, results in substantial changes in the observed stability constants (8, 9, 12) appears to support this hypothesis.

Since water may be considered to participate in these interactions, a possible mechanism of the donor–acceptor type apparently should be classified as being not of the classical type but peculiar to water. The authors would expect that the effect of different solvents on the overall stability of a classical charge-transfer complex should reflect competitive solvation effects. Thus, in polar solvents that can act as both donor and acceptor, the medium would be expected to solvate both free solute species and the com-

plex itself. The overall net effect would probably render complex formation less favorable in the presence of such solvents than in an inert solvent.

Such available data concerning the influence of solvents on the stability of charge–transfer complexes suggest that these classical complexes in general are formed in appreciable amount only in nonpolar solvents. For example, the stability constants for the trinitrobenzene–*N*,*N*-dimethylaniline complex decrease with the solvents in the following order (21): cyclohexane > *n*-hexane, *n*-heptane > carbon tetrachloride > chloroform > *s*-tetrachloroethane > 1,4-dioxane. The stability constants in cyclohexane and *n*-hexane were 9.5 and 8.2 M^{-1}, respectively. In chloroform, *s*-tetrachloroethane, and dioxane, they were of much less magnitude, being 1.3, 0.2, and 0.15 M^{-1}, respectively.

The iodine–naphthalene complex decreases in stability with the solvents in the following order (22): *n*-heptane > cyclohexane > carbon tetrachloride > *n*-hexane > chloroform. Similarly, it has been shown that the stability constants for the complex between tetrachlorophthalic anhydride and hexamethylbenzene decrease when the solvents are varied in the following order (23): *n*-hexane > carbon tetrachloride > dibutyl ether > benzotrifluoride > fluorobenzene $\simeq$ benzene $\simeq$ cyclohexanone. The magnitude of the stability constants were reported to be 260 M^{-1} in *n*-hexane and 145 M^{-1} in carbon tetrachloride. For comparison, the constants in dibutyl ether, benzotrifluoride, fluorobenzene, and cyclohexanone were 77, 52, 29, and 23 M^{-1}, respectively.

In all the cited examples it is seen that in going from nonpolar solvents to typical polar solvents such as chloroform, *s*-tetrachloroethane, dibutyl ether, fluorobenzene, benzotrifluoride, cyclohexanone, and dioxane, there is a substantial decrease in the stability of the charge–transfer complexes. If donors and acceptors interact to form complexes that are appreciably ionic in character, these generalizations may no longer apply; in such cases, complex formation may be facilitated in media that promote ionization (24, 25). However, this has by no means been found to be a general rule (25), and the type of complexes which are more stable in polar solvents presumably involve ion radicals and are not simple donor–acceptor complexes. With the exception of some complexes of tetramethylphenylenediamine with various tetrahaloquinones, tetracyanoethylene, and related acceptors, it appears that the known classical charge–transfer complexes are in general formed in appreciable amounts only in nonpolar solvents (25).

Although this discussion suggests that contribution from classical charge–transfer complexes may not be a major factor in aqueous solution because of the competitive participation of water, the authors cannot exclude the possibility that a donor–acceptor mechanism, peculiar to water, may contribute to the stability of the complexes studied. Such a speculation may be justified, since hydrophobic forces alone appear to fail to account for all the observed effects.

CONCLUSIONS

From the preceding discussion, it is apparent that the favorable interaction between small organic species in water may be due to a summation of several factors. The net enhancement in binding by increasing the content of water in the environmental solvent cannot be rationalized on the basis of any single binding mechanism alone. Although hydrophobic bonding and a nonclassical donor–acceptor mechanism may have been the major forces involved, with the contributions from the former possibly of lesser magnitude, further investigations along these lines may shed light on additional factors which have not been evident in this limited study. Since the balance of forces operating between the complex components in pure aqueous solution is not known, it is difficult to separate and interpret the gross effect seen in mixtures of organic solvents with water.

REFERENCES

(1) I. M. Klotz, *Fed. Proc.*, **24**, s-24(1965).

(2) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1(1959).

(3) K. Kakemi, H. Sezaki, T. Mitsunaga, and M. Nakano, to be published; H. Kristiansen and T. Higuchi, to be published.

(4) G. Weber, in "Flavins and Flavoproteins," E. C. Slater, Ed., Elsevier Publishing Co., Amsterdam, The Netherlands, 1966, pp. 15–21.

(5) O. Sinanoglu and S. Abdulnur, *Photochem. Photobiol.*, **3**, 333(1964).

(6) O. Sinanoglu and S. Abdulnur, *Fed. Proc.*, **24**, s-12(1965).

(7) R. E. Moser and H. G. Cassidy, *J. Amer. Chem. Soc.*, **87**, 3463(1965).

(8) T. Higuchi and A. Drubulis, *J. Pharm. Sci.*, **50**, 905(1961).

(9) T. Higuchi and F. D. Pisano, *ibid.*, **53**, 644(1964).

(10) M. Nakano and T. Higuchi, *ibid.*, **57**, 183(1968).

(11) M. Nakano and T. Higuchi, *ibid.*, **57**, 1865(1968).

(12) T. Higuchi and H. Kristiansen, to be published.

(13) M. Nakano and T. Higuchi, to be published.

(14) T. Higuchi and K. A. Connors, *Advan. Anal. Chem. Instr.*, **4**, 117(1965).

(15) H. S. Frank and W. Y. Wen, *Discuss. Faraday Soc.*, **24**, 133(1957).

(16) H. S. Frank, *Fed. Proc.*, **24**, s-1(1965).

(17) P. Becher, *J. Colloid Sci.*, **20**, 728(1965).

(18) M. J. Schick and A. H. Gilbert, *ibid.*, **20**, 464(1965).

(19) G. Némethy and H. A. Scheraga, *J. Chem. Phys.*, **36**, 3401 (1962).

(20) G. Némethy and H. A. Scheraga, *J. Phys. Chem.*, **66**, 1773 (1962).

(21) R. Foster and D. L. Hammick, *J. Chem. Soc.*, **1954**, 2685.

(22) P. A. D. De Maine and J. Peone, Jr., *J. Mol. Spectr.*, **4**, 262(1960).

(23) J. Czekalla and K. O. Meyer, *Z. Phys. Chem. (Frankfurt am Main)*, **27**, 185(1961).

(24) L. J. Andrews and R. M. Keefer, "Molecular Complexes in Organic Chemistry," Holden-Day, San Francisco, Calif., 1964.

(25) G. Briegleb, "Elektronen-Donator-Acceptor-Komplexe," Springer-Verlag, Berlin, Germany, 1961.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 20, 1969, from the *Department of Analytical Pharmaceutical Chemistry and Pharmaceutics, School of Pharmacy' University of Kansas, Lawrence, KS 66044*, and the *School of Pharmacy, University of Wisconsin, Madison, WS 53706*

Accepted for publication February 10, 1970.

This investigation was supported in part by a grant (GM-05830) from the Institute of General Medical Sciences, National Institutes of Health, Bethesda, Md.

H. Kristiansen gratefully acknowledges partial financial support from the Norwegian Research Council for Science and the Humanities.

* Present address: Pharmaceutical Chemistry Division, Food and Drug Directorate, Ottawa 3, Ontario, Canada.

† To whom all correspondence should be addressed.

Acetylcholinesterase Substrates: β-Methylcholine Esters

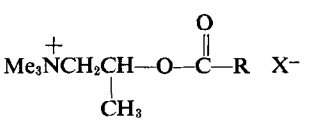
G. M. STEINBERG, M. L. MEDNICK, R. E. RICE, and J. P. MADDOX

Abstract □ *β-Methylcholine propionate* (Compound II) and isobutyrate (Compound III) are "good" substrates for eel acetylcholinesterase. Enzymatic hydrolysis of the corresponding pivalyl ester is barely detectable. For study of acetylcholinesterase under conditions where the "blank" hydrolysis rate of substrate must be reduced to a minimum, Compounds II and III can be used advantageously. Like acetylcholine and β-methylcholine acetate, Compound II shows substrate inhibition. The isobutyryl ester (Compound III) gives no significant substrate inhibition. Kinetic constants for both enzymatic and nonenzymatic hydrolysis are reported for the group of esters. Evidence is presented which suggests that the rate of acylation of the enzyme parallels reactivity with hydroxide ion for acetylcholine and the acetyl and propionyl esters of β-methylcholine. With the isobutyryl ester of β-methylcholine, there is a relative decrease in enzymatic acylation rate.

Keyphrases □ Acetylcholinesterase substrates—β-methylcholine esters □ β-Methylcholine esters—synthesis □ Hydrolysis rates, enzymatic, nonenzymatic—β-methylcholine esters □ Michaelis constants—β-methylcholine esters

For studies (to be published) on oxime reactivation of phosphonylated acetylcholinesterase (AChE) (acetylcholine acetylhydrolase, EC 3.1.1.7), which the authors wished to perform by the continuous monitoring procedure of Kitz *et al.* (1) using an autotitrator, it was important to reduce the "blank" hydrolysis rate of the substrate to a minimum. In the region of pH 9, acetylcholine (ACh), its normal substrate, gives undesirably high "blank" values.

Like most enzymes, AChE has a moderately wide range in its specificity and is capable of hydrolyzing a considerable variety of esters other than ACh (2). It is well known that the rates of aqueous (alkaline) hydrolysis within a family of esters can be decreased by insertion of bulking groups near the ester linkage (3). Recently, Gunter (4) observed that *ortho*-substitution in phenyl acetate, which decreases the alkaline hydrolysis rate, does not interfere with enzymatic hydrolysis by AChE. Hence, it seemed appropriate to examine several homologs of acetyl β-methylcholine with increasing bulk on the acetyl methyl group. The compounds are listed in general structure I. Two of the compounds, Compounds II and III, satisfied the requirements.



Compound	R	X^-
I	$-CH_3$	Br^-
II	$-C_2H_5$	I^-
III	$-CH(CH_3)_2$	I^-
IV	$-C(CH_3)_3$	I^-

β-methylcholine esters

I

Since these compounds may have general utility for studies with AChE, particularly where the "blank" hydrolysis of substrate becomes a problem, the authors explored their properties. In this paper, they report the nonenzymatic and enzymatic hydrolysis rates and Michaelis constants (at pH 7.4).

EXPERIMENTAL

Acetylcholine bromide (ACh) and acetyl β-methylcholine bromide (Compound I) were obtained commercially. The AChE (Worthington Biochemicals) was purified, stable, dry powder from electric eel, Code ECHP, 1000 units/mg. Twenty milligrams of enzyme was dissolved in 1.6 ml. of a previously boiled aqueous solution, pH approximately 7.4, containing KCl (0.225 *M*) and 0.25% gelatin (KCl/gel) to give enzyme concentrate E_w. The concentration of active sites in E_w is estimated to be 2.5×10^{-5} *M*.[1]

Both enzymatic and solvolytic rates were determined from the rate of addition of standard alkali needed to maintain constant pH using a Radiometer TTTl autotitrator, fitted with an ABUl buret, a SBR2 recorder, and a PHA 630 scale expander. The titrator was fitted with the 0.25-ml. buret (0.25 ml. = 100 scale divisions of chart displacement), and the 6-ml. jacketed titration vessel was covered and maintained at 25°. Nitrogen was slowly passed over the reaction solution to minimize CO_2 absorption. The titrant was 0.00353 *M* carbonate-free sodium hydroxide.

Enzymatic Hydrolysis—All measurements were made at pH 7.4, 25°. To 3.0 ml. of 0.225 *M* KCl, there were added 2.0 ml. of aqueous substrate and 15–500 μl. of appropriately diluted (in KCl/gel) enzyme concentrate, E_w. Dilutions ranged from 1/1000 to 1/10,000 (except for Compound IV, in which case undiluted E_w was used). Records were made of initial hydrolysis rates. The enzymatic hydrolysis data are presented in Lineweaver-Burk reciprocal plots (Figs. 1 and 2). For convenience of comparison, the results have been normalized by conversion of the observed rates to those calculated for concentrated enzyme solution, E_w, assuming a direct linear relationship between enzyme concentration and v, the velocity in moles per liter of ester hydrolyzed per minute. The values of $k_{cat.}$ and $K_{m(app.)}$ were computed from the slope and y-intercept using Eqs. 2 and 2a.

Nonenzymatic Hydrolysis—Reactions were run at 25° at the appropriate pH values, which were maintained constant during each run. The 5.0 ml. reaction mixture contained 0.135 *M* KCl and 0.1 *M* ester. Measurements were made of initial hydrolysis rates under conditions where less than 0.1% of the ester was hydrolyzed. The continuous records of alkali delivered (to maintain constant pH) as a function of time were linear over the period of measurement. The values reported have been corrected for the CO_2 blank. At the pH values employed the water component of the observed hydrolytic reaction rate constant, k_{obs}, is negligible (5). Hence the reaction, $RCO_2R' + OH^- \rightarrow RCO_2^- + R'OH$, is first order in ester and in hydroxide ion. Because the autotitrator adds alkali automatically to maintain a constant pH, the rate of addition of alkali is equivalent to the rate of formation of acid.

Synthesis—Compounds II, III, and IV were prepared as follows.

2-Hydroxypropyldimethylamine—To a cooled (8°) stirred solution of dimethylamine (54 g., 1.2 moles) in absolute methanol (300 ml.), 1,2-propylene oxide (58 g., 1.0 mole) was added in small portions over 25 min. while maintaining the temperature below 8°. After this addition, the temperature was permitted to rise to 30° and was held there for 20 min. After removal of the cooling bath, the solution was left to stand 1.5 hr. (maximum temperature 40°). Distillation through a simple head followed by redistillation through a 15 × 1-cm. vacuum-jacketed Vigreux column gave 46 g. (45%) of 2-hydroxypropyldimethylamine, b.p. 126° (uncorrected).

Compounds II, III, and IV—The propionyl and isobutyryl esters were prepared by treating 2-hydroxypropyldimethylamine with a

[1] Calculated from turnover number = 6.7×10^5 min.$^{-1}$, pH 7.4, 0.33 *M* KCl, 0.0073 *M* AChE, 0.25% gelatin, 25°, $K_{m(app.)} = 2.8 \times 10^{-4}$ *M* (H. Michel, private communication).

Table I—Esters II, III, and IV

Compound	Tertiary Aminoester, b.p.[a]	Quaternary Aminoester, m.p.[a]	Formula	Anal., % Calcd.	Found
II	171–171.5°	165–167°	$C_9H_{20}INO_2$	C, 35.89 H, 6.69 N, 4.67 O, 10.62	C, 35.8 H, 6.6 N, 4.7 O, 10.8
III	66.5–67° (10 mm.)	195.5–196.5°	$C_{10}H_{22}INO_2$	C, 38.11 H, 7.04 I, 40.26 N, 4.44 O, 10.15	C, 38.1 H, 6.9 I, 40.2 N, 4.5 O, 10.3
IV	70.5–71.5° (11 mm.)	241–242°	$C_{11}H_{24}INO_2$	C, 40.13 H, 7.35 I, 38.55 N, 4.25 O, 9.72	C, 40.0 H, 7.3 I, 38.4 N, 4.2 O, 10.0

[a] Uncorrected.

small excess of the corresponding acyl anhydride. For the pivalate, the reaction was run in benzene using equivalent quantities of aminoalcohol, pivalyl chloride, and pyridine. The tertiary aminoesters were quaternized by treatment with a small excess of methyl iodide in acetone and recrystallized from acetone or acetone–methanol. The physical properties of the tertiary aminoesters and the quaternary products, together with elemental analyses of the latter, are given in Table I.

RESULTS AND DISCUSSION

Nonenzymatic Hydrolysis—Table II contains the observed rates of reaction with the aqueous solvent. Measurements on each compound were made at two pH values. The essential identity in the pairs of computed k_{OH} values (within the range of experimental error) indicates that for each ester the "water" reaction is negligible. Thus, one can compute the rate of the "blank" hydrolysis reaction, $k_{obs.}$, at any pH above those reported[2] from the relationship $k_{obs.} = k_{OH}[OH^-]$. For convenience in comparison, the actual observed "blank" hydrolysis rates are also given for each of the esters (Table II). It is noteworthy that the effect of increased bulking on going from ACh to Compound III reduces the hydrolysis rate approximately 20×, so that the "blank" for Compound III at pH 9.5 is hardly greater than that of ACh at pH 8.0.

Enzymatic Hydrolysis—In Fig. 1, Lineweaver-Burk reciprocal plots are given for ACh and Compounds I and II. Like ACh, Compounds I and II are good substrates for AChE and each shows marked substrate inhibition at high concentration.[3] Compound III (Fig. 2) does not show substrate inhibition even at the highest concentration of substrate that was used, 0.1 *M*.

Table III contains data on enzymatic hydrolysis at pH 7.4 and substrate concentrations of 5×10^{-3} and 5×10^{-2} *M*, together with the computed ratios of the rates of the enzymatic to nonenzymatic hydrolyses. It also lists the values of $K_{m(app.)}$, computed from the Lineweaver-Burk plots in Figs. 1 and 2. Enzymatic studies were performed at pH 7.4 rather than at another higher pH because it provides data which can be more readily collated with those of other AChE substrate studies, the bulk of which have been performed in the pH 7–7.5 range. It may be assumed that changes in the Michaelis constants due to variation in pH will be substantially the same for the entire group of compounds, so the relative value of v/k_{OH} for the group can be applied at higher pH values.

For the selection of the best substrate for a particular study using an automatic titrating device such as the Radiometer TTTl, one must bear in mind that the conveniently useful range in delivery rate of the buret is perhaps only 20×, *i.e.*, from approximately 1–2 div./min. of chart paper to 20–30 div./min. At pH values above 9, where the "CO_2" blank becomes significant, the range becomes even

Table II—Nonenzymatic Hydrolysis of Substrates

Substrate	pH	Solvolysis: Rate Observed,[a] div./min.	Solvolysis: k_{OH},[b] M^{-1} min.$^{-1}$
Acetylcholine	8.0	5.08	89.6[c]
	8.5	16.56	91.0[c]
β-Methylcholine esters			
(I) Acetyl	9.0	9.35	16.5
	9.5	31.25	17.4
(II) Propionyl	9.0	7.21	12.7
	9.5	22.0	12.3
(III) Isobutyryl	9.0	1.89	3.2
	9.5	7.87	4.4
(IV) Pivalyl	10.0	1.93	0.34
	10.5	7.7	0.43

[a] Div. (on radiometer) = 2.5×10^{-3} ml.; 5.0 ml. of 0.1 *M* ester was present in titration vessel. Titrant, 0.00353 *M* NaOH. [b] $k_{obs.} = k_{OH}[OH^-]$. [c] Reported: 72 and 80.4 (*Reference 5*).

smaller. By increasing the concentration of titrant, the "blank" delivery rate for a given substrate concentration can be reduced. However, by doing so, sensitivity is reduced and, therefore, more of the substrate must be hydrolyzed to obtain measurable hydrolysis records.

Under conditions where the enzyme concentration is the limiting factor, Compound II is best. Its values of v/k_{OH}, the ratio of the enzymatic to the "blank" hydrolysis rates, are equal to or greater than the others. Its lower "blank" value makes it more useful than Compound I. Where the enzyme is nonlimiting, *i.e.*, its concentration can be made high enough to give maximum delivery of titrant, Compound III is preferred because of its lower "blank." The enzymatic hydrolysis of Compound IV is so slow that one may question whether it is hydrolyzed by AChE at all. It is certainly possible that the slight activity is produced by some impurity in the enzyme preparation. However, if it is truly a substrate for AChE, it might be used for monitoring the concentrated enzyme, if and when it becomes available in reasonably large quantities.

β-Methylcholine exists as a DL-pair. It is known that with the acetate ester, only the L(+)-isomer acts as an AChE substrate, while the D(−)-isomer is an inhibitor[4] (10, 11). In these studies, the authors have observed that enzymatic hydrolysis of Compounds II and III give only approximately one-half of the theoretical

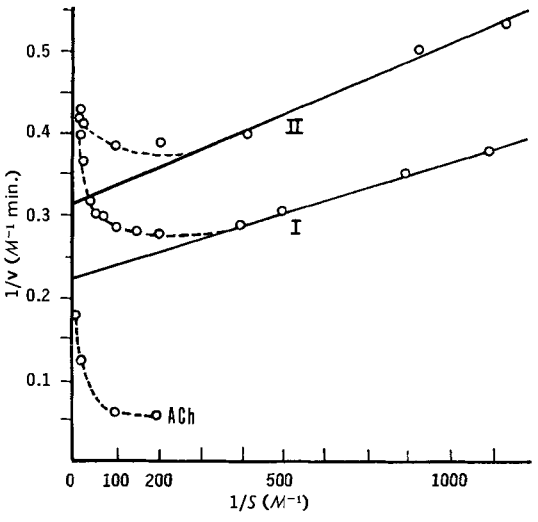


Figure 1—*Lineweaver-Burk reciprocal plots of velocity of substrate hydrolysis as a function of substrate concentration, pH 7.4, 25°. Substrates: ACh and Compounds I and II.* S *represents concentration of racemic mixture.*

[2] Extrapolation probably can be carried to lower pH also. An estimated lower limit for extrapolation is approximately pH 5 (6).

[3] Mounter and Ellin (7) report no substrate inhibition by Compound I. Their studies appear to have been made without addition of supporting electrolyte. If this is so, the ionic strength of the medium changed with each change in concentration of substrate. Since the properties of eel AChE vary significantly with changes in ionic strength (see, for example, *References 8 and 9*), this may be the cause of the difference.

[4] A very rough estimate of the value of K_I for the D(−)-isomer is 10^{-3} *M*, calculated from data in *Reference 11*.

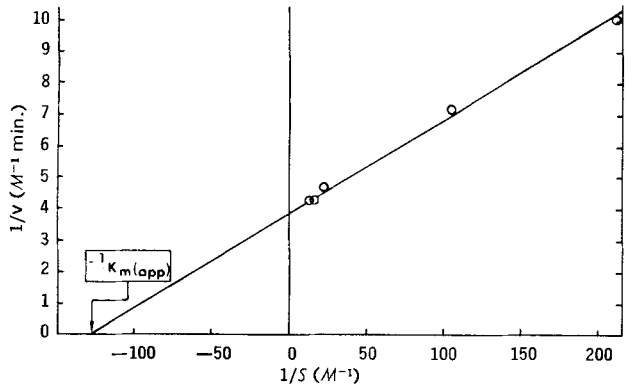


Figure 2—*Lineweaver-Burk plot for Compound III.* S *represents concentration of racemic mixture.*

quantity of acid. Here, too, only one member of each DL-pair acts as substrate. Hence, by using the pure enzymatically active isomers, one should be able to increase the v/OH ratios by at least two times and probably even more, since the inactive isomers may inhibit the enzymatic reaction.

For both the Lineweaver-Burk plots (Figs. 1 and 2) and the computation of $K_{m(app.)}$ (Table III), use was made of total ester concentration and not actual concentration of active isomer.

Observations on Enzyme Kinetics—Since the purposes of this study were pragmatic, the choice of substrates was made entirely on that basis and without regard to theoretical implications of the kinetic results. However, analysis of the data that were obtained points to probable relationships between substrate structure and enzyme kinetic constants which may warrant further investigation.

AChE functions kinetically in a three-step reaction (Eq. 1) (12). Enzymatic hydrolysis follows Michaelis-Menten kinetics (at concentrations below those which cause substrate inhibition) (Eq. 2); however, the kinetic constants are complex functions (Eqs. 3–5) (13).

$$E + \text{ester} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot\text{ester} \xrightarrow{k_2} \text{E-acyl} \xrightarrow{k_3} E + \text{acid} \quad \text{(Eq. 1)}$$

(E-acyl + alcohol)

$$v = \frac{V_{\max.}S}{S + K_{m(app.)}}; \quad \text{where } V_{\max.} = k_{cat.}E_0 \quad \text{(Eq. 2)}$$

$$\frac{1}{v} = \frac{1}{V_{\max.}} + \left(\frac{K_{m(app.)}}{V_{\max.}}\right)\frac{1}{S} \quad \text{(Eq. 2a)}$$

$$k_{cat.} = \frac{k_2k_3}{k_2 + k_3} \quad \text{(Eq. 3)}$$

$$K_{m(app.)} = \frac{K_sk_2}{k_2 + k_3}; \quad K_s = \frac{k_2 + k_{-1}}{k_1} \quad \text{(Eq. 4)}$$

$$\frac{k_{cat.}}{K_{m(app.)}} = \frac{k_2}{K_s} \quad \text{(Eq. 5)}$$

where k_1, k_{-1}, k_2, and k_3 are rate constants; v is the observed rate of product formation; and E_0 and S are, respectively, the concentrations of enzyme and substrate.

Inhibitors can bind to E or E-acyl or to both (Eq. 6).

$$\begin{array}{ccccc} E + \text{ester} & \rightleftharpoons & E\cdot\text{ester} & \rightarrow & \text{E-acyl} \rightarrow E \\ + & & + & & \\ \text{Inh} & & \text{Inh} & & \\ \Big\Updownarrow K_I & & \Big\Updownarrow K_\alpha & & \\ E\text{Inh} & & E\text{Inh-acyl} & \nrightarrow & E \end{array} \quad \text{(Eq. 6)}$$

Binding to E results in competitive inhibition while binding to E-acyl results in noncompetitive inhibition. Inhibition by excess substrate is believed to result from its binding to E-acyl and consequent interference with the deacylation step (14). Binding and interference in this fashion will be observed kinetically only if the relevant kinetic step is rate limiting [with ACh, deacylation is rate limiting; $k_2 = 6$–$10 \times k_3$ (15)]. Thus, the failure of Compound III to give substrate inhibition may be due to its failure to bind to the acyl enzyme, *i.e.*, a marked increase in K_α, or alternatively may be the result of a change in the rate-limiting step, *i.e.*, $k_3 > k_2$.

Table III—Enzymatic Hydrolysis by Eel AChE[a]

Substrate	v, M/min., S_0 = 5×10^{-3} M	v, M/min., S_0 = 5×10^{-2} M	v/k_{OH}, S_0 = 5×10^{-3} M	v/k_{OH}, S_0 = 5×10^{-2} M	$K_{m(app.)}$[b], M
ACh	16.8	7.9	0.185	0.087	2.8×10^{-4}[d]
I	3.5	3.1	0.206	0.182	6.9×10^{-4}[e]
II	2.55	2.4	0.204	0.192	6.9×10^{-4}
III	0.1	0.22	0.0264	0.058	7.6×10^{-3}
IV	—	3×10^{-5}	—	7.7×10^{-5}	—

[a] pH 7.4, 25°; v, moles/l. min. of substrate hydrolyzed, calculated for enzyme concentration E_0. [b] Based upon total ester concentration. [c] S_0, initial substrate concentration. [d] H. Michel (see *Footnote 1*). [e] Reported, 8×10^{-4} M (7).

Table IV—Kinetic Constants for Enzymatic Hydrolysis

Substrate	$k_{cat.}$, min.$^{-1}$	k_2/K_s,[a] M^{-1} min.$^{-1}$	$k_2/K_s\,k_{OH}$
ACh	7×10^5[b]	2.5×10^9[b]	2.8×10^7
I	1.74×10^5	5.0×10^8	3×10^7
II	1.27×10^5	3.7×10^8	3×10^7
III	1.03×10^4	2.7×10^6	7×10^5

[a] Equation 5. Computed from concentration of active ester (one-half total ester concentration) for I, II, and III. [b] See *Footnote 1*.

Table IV contains the values of $k_{cat.}$ computed (Eq. 2a) from the linear portions of the Lineweaver-Burk plots in Figs. 1 and 2 together with the computed values of k_2/K_s (Eq. 5)[5] for each substrate. For the latter calculation, the actual concentration of active isomer was used in computing $K_{m(app.)}$.

It can be seen that for ACh and Compounds I and II, the values of k_2/K_s, the rate constant for acylation of the enzyme divided by the dissociation constant of the enzyme substrate complex, closely parallel the corresponding values of k_{OH}, the rate constant for acylation of the hydroxide ion; *i.e.*, the value of k_2/K_sk_{OH} (Table IV) is constant. If one makes the not unreasonable assumption that the value of K_s does not vary greatly among this group of substrates,[6] then the results suggest that over the range in structural variation represented by ACh and Compounds I and II, the enzyme displays little kinetic selectivity, the differences in reaction rate paralleling the reactivity of the ester toward hydroxide ion.

In the case of Compound III, there is a marked fall in $k_{cat.}$ and in the value of k_2/K_s. Also, as noted earlier, Compound III does not show substrate inhibition. These results suggest that the principal cause of the reduction in the rate of hydrolysis of Compound III by AChE is due to reduction in the rate constant of the acylation step, k_2. Further, the $k_{cat.}$ values for the hydrolysis by eel AChE of the acetyl and isobutyryl esters of phenol are substantially the same.[7] For phenyl acetate, $k_2 \gg k_3$ (17). Therefore, the values of k_3 for the acetate and isobutyrate (and probably the propionate) are closely similar.[8] Hence, the fall in $k_{cat.}$ for Compound III represents a fall in the value of k_2.

The rate of reaction of Compound III with hydroxide is less than that of the esters I and II (Table II). The reduction in its "normalized" reaction rate with enzyme, k_2/K_sk_{OH} (Table IV), is appreciably greater. Thus, with the increase in molecular size or com-

[5] The values of both $k_{cat.}$ and $K_{m(app.)}$ are reduced by the presence of the inhibitory nonsubstrate isomer. However, it can be shown that both are affected to approximately an equal extent, so that the value of k_2/K_s is not appreciably affected.

[6] With alkyl trimethylammonium salts, K_I values vary by no more than 2–3 × over a several carbon atom range in the chain length of the alkyl group. See *Reference 16*.

[7] The $k_{cat.}$ value for phenyl acetate is approximately 3 × greater than that for phenyl isobutyrate (G. M. Steinberg and J. Maddox, to be published).

[8] An inversion in the rate-determining step for the phenyl isobutyrate hydrolysis by AChE is highly unlikely in view of the extremely high rate of the reaction. However, if this was the case, it would mean that k_3 would be greater than $k_{cat.}$ and, hence, would not invalidate the conclusions.

plexity on passing from Compound II to III, one observes the beginning of kinetic selectivity by the enzyme.

It is noteworthy that if one sets $k_2 = k_{OH}[OH^-]$ for ACh, to achieve the enzymatic acylation rate one would have to use a hydroxide-ion concentration of 6×10^4 *M*.

REFERENCES

(1) R. J. Kitz, S. Ginsburg, and I. B. Wilson, *Biochem. Pharmacol.*, **14**, 1471(1965).

(2) "Handbuch Exp. Pharmakol.," vol. 15, G. B. Koelle, Sub. Ed., Springer-Verlag, Berlin, Germany, 1963, p. 308.

(3) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Holt, Rinehart and Winston, New York, N. Y., 1959, p. 318.

(4) C. R. Gunter, unpublished data.

(5) M. R. Wright, *J. Chem. Soc., B*, **1968**, 545.

(6) L. Larsson, *Acta Chem. Scand.*, **8**, 1017(1954).

(7) L. A. Mounter and R. I. Ellin, *Mol. Pharmacol.*, **4**, 452 (1968).

(8) B. D. Roufogalis and J. Thomas, *ibid.*, **4**, 181(1968).

(9) J. P. Changeaux, *ibid.*, **2**, 369(1966).

(10) A. M. Beckett, in "Enzymes and Drug Action," J. L. Mongar and A. V. S. de Reuch, Eds., Little Brown, New York, N. Y., 1962, p. 15.

(11) F. C. G. Hoskin, *Proc. Soc. Exp. Biol. Med.*, **113**, 320(1963).

(12) I. B. Wilson, F. Bergmann, and D. Nachmansohn, *J. Biol. Chem.*, **186**, 781(1950).

(13) B. Zerner and M. L. Bender, *J. Amer. Chem. Soc.*, **86**, 3669(1964).

(14) I. B. Wilson and E. Cabib, *ibid.*, **78**, 202(1956).

(15) J. K. Stoops, Ph.D. thesis, Northwestern University, Evanston, Ill., 1966, p. 35.

(16) J. C. Kellett, Jr., and W. C. Doggett, *J. Pharm. Sci.*, **55**, 414(1966).

(17) R. M. Krupka, *Biochem.*, **3**, 1749(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 11, 1969, from the *Physiology Department, Medical Research Laboratory, Research Laboratories, Edgewood Arsenal, MD 21010*

Accepted for publication March 18, 1970.

Carotid Sinus Pressure Reflex Bioassay for *Veratrum viride*

B. KOROL, A. V. ZUBER*, and L. D. MILLER†

Abstract □ From a total of 238 anesthetized dogs, a series of regression analyses was performed examining the relationship between the variables (sex of the animal, date on which the experiment was performed, effect of the three veratrum alkaloidal drug preparations studied, and the number of days the dogs were stored in the animal house) on the arterial pressure pressor amplitudes of the predrug and postdrug carotid occlusion response and on the blood pressure-lowering effects of the drug treatments. The seasonal date of the experiment and the drug treatments significantly influenced the arterial pressure responses, while sex of the animal or days stored prior to use did not consistently alter the slope constants or arterial pressure responses for the drug subgroups.

Keyphrases □ *Veratrum viride* alkaloids—bioassay □ Carotid sinus pressure reflex—*V. viride* bioassay □ Sex, time of year effects—blood pressure lowering, drugs □ Regression analysis—factors affecting blood pressure lowering

The method presently used for the quantitative assessment of extracts of *Veratrum viride* is based on the progressive loss of the pressor response induced by bilateral occlusion of the common carotid arteries in the anesthetized dogs as reported by O'Dell (1). The potency of the test material is expressed in terms of carotid sinus reflex (CSR) units defined as follows: "One CSR unit represents the amount of intravenously administered hypotensive agent per kilogram of body weight which just abolishes the pressor response to the carotid sinus reflex in dogs" (1).

In a study on anesthetized dogs, Prochnik *et al.* (2) concluded that minimal pressor response to bilateral carotid occlusion is to be expected when the mean arterial pressure is below 88 mm. Hg. Since the regression line approaches zero response at the basal mean arterial pressure level of 60 mm. Hg, it was suggested that minimum variability would be obtained if the carotid occlusion pressor responses were expressed as: (mm. Hg rise due to occlusion × 100)/(mean arterial pressure [mm. Hg] − 60).

Rubin and Burke (3) reported that since the changes in carotid pressor reflex response and in the existent mean arterial pressure are highly correlated, the steeper dose–response curve exhibited by the carotid pressor reflex response would be a more sensitive measure. They also concluded that the *V. viride* hypotensive principles examined did not act *via* adrenergic, sympathetic, or ganglionic blocking actions. However, more recently, Jandhyala and Buckley (4) reported that the effects of cryptenamine, an alkaloidal preparation from *V. viride*, were inhibited by reserpine, α-methyldopa, and adrenalectomy and therefore did act through a mechanism of action involving catecholamines and the adrenal medulla.

The authors have used the method as described by O'Dell (1) for the bioassay of several alkaloidal preparations from *V. viride*. The studies referred to previously alluded to the high correlation between the basal arterial pressure and the pressor response amplitude to bilateral carotid occlusion. The authors have also made unpublished observations on other possible influencing variables, such as time of the year the study was performed and length of time the dog was maintained in the animal quarter. To understand more fully the relationships between these variables, a series of regression analyses was performed on the data obtained over the last few years. This report is concerned with the findings obtained in this study.

plexity on passing from Compound II to III, one observes the beginning of kinetic selectivity by the enzyme.

It is noteworthy that if one sets $k_2 = k_{OH}[OH^-]$ for ACh, to achieve the enzymatic acylation rate one would have to use a hydroxide-ion concentration of 6×10^4 *M*.

REFERENCES

(1) R. J. Kitz, S. Ginsburg, and I. B. Wilson, *Biochem. Pharmacol.*, **14**, 1471(1965).

(2) "Handbuch Exp. Pharmakol.," vol. 15, G. B. Koelle, Sub. Ed., Springer-Verlag, Berlin, Germany, 1963, p. 308.

(3) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Holt, Rinehart and Winston, New York, N. Y., 1959, p. 318.

(4) C. R. Gunter, unpublished data.

(5) M. R. Wright, *J. Chem. Soc., B*, **1968**, 545.

(6) L. Larsson, *Acta Chem. Scand.*, **8**, 1017(1954).

(7) L. A. Mounter and R. I. Ellin, *Mol. Pharmacol.*, **4**, 452 (1968).

(8) B. D. Roufogalis and J. Thomas, *ibid.*, **4**, 181(1968).

(9) J. P. Changeaux, *ibid.*, **2**, 369(1966).

(10) A. M. Beckett, in "Enzymes and Drug Action," J. L. Mongar and A. V. S. de Reuch, Eds., Little Brown, New York, N. Y., 1962, p. 15.

(11) F. C. G. Hoskin, *Proc. Soc. Exp. Biol. Med.*, **113**, 320(1963).

(12) I. B. Wilson, F. Bergmann, and D. Nachmansohn, *J. Biol. Chem.*, **186**, 781(1950).

(13) B. Zerner and M. L. Bender, *J. Amer. Chem. Soc.*, **86**, 3669(1964).

(14) I. B. Wilson and E. Cabib, *ibid.*, **78**, 202(1956).

(15) J. K. Stoops, Ph.D. thesis, Northwestern University, Evanston, Ill., 1966, p. 35.

(16) J. C. Kellett, Jr., and W. C. Doggett, *J. Pharm. Sci.*, **55**, 414(1966).

(17) R. M. Krupka, *Biochem.*, **3**, 1749(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 11, 1969, from the *Physiology Department, Medical Research Laboratory, Research Laboratories, Edgewood Arsenal, MD 21010*

Accepted for publication March 18, 1970.

Carotid Sinus Pressure Reflex Bioassay for *Veratrum viride*

B. KOROL, A. V. ZUBER*, and L. D. MILLER†

Abstract □ From a total of 238 anesthetized dogs, a series of regression analyses was performed examining the relationship between the variables (sex of the animal, date on which the experiment was performed, effect of the three veratrum alkaloidal drug preparations studied, and the number of days the dogs were stored in the animal house) on the arterial pressure pressor amplitudes of the predrug and postdrug carotid occlusion response and on the blood pressure-lowering effects of the drug treatments. The seasonal date of the experiment and the drug treatments significantly influenced the arterial pressure responses, while sex of the animal or days stored prior to use did not consistently alter the slope constants or arterial pressure responses for the drug subgroups.

Keyphrases □ *Veratrum viride* alkaloids—bioassay □ Carotid sinus pressure reflex—*V. viride* bioassay □ Sex, time of year effects—blood pressure lowering, drugs □ Regression analysis—factors affecting blood pressure lowering

The method presently used for the quantitative assessment of extracts of *Veratrum viride* is based on the progressive loss of the pressor response induced by bilateral occlusion of the common carotid arteries in the anesthetized dogs as reported by O'Dell (1). The potency of the test material is expressed in terms of carotid sinus reflex (CSR) units defined as follows: "One CSR unit represents the amount of intravenously administered hypotensive agent per kilogram of body weight which just abolishes the pressor response to the carotid sinus reflex in dogs" (1).

In a study on anesthetized dogs, Prochnik *et al.* (2) concluded that minimal pressor response to bilateral carotid occlusion is to be expected when the mean arterial pressure is below 88 mm. Hg. Since the regression line approaches zero response at the basal mean arterial pressure level of 60 mm. Hg, it was suggested that minimum variability would be obtained if the carotid occlusion pressor responses were expressed as: (mm. Hg rise due to occlusion × 100)/(mean arterial pressure [mm. Hg] − 60).

Rubin and Burke (3) reported that since the changes in carotid pressor reflex response and in the existent mean arterial pressure are highly correlated, the steeper dose–response curve exhibited by the carotid pressor reflex response would be a more sensitive measure. They also concluded that the *V. viride* hypotensive principles examined did not act *via* adrenergic, sympathetic, or ganglionic blocking actions. However, more recently, Jandhyala and Buckley (4) reported that the effects of cryptenamine, an alkaloidal preparation from *V. viride*, were inhibited by reserpine, α-methyldopa, and adrenalectomy and therefore did act through a mechanism of action involving catecholamines and the adrenal medulla.

The authors have used the method as described by O'Dell (1) for the bioassay of several alkaloidal preparations from *V. viride*. The studies referred to previously alluded to the high correlation between the basal arterial pressure and the pressor response amplitude to bilateral carotid occlusion. The authors have also made unpublished observations on other possible influencing variables, such as time of the year the study was performed and length of time the dog was maintained in the animal quarter. To understand more fully the relationships between these variables, a series of regression analyses was performed on the data obtained over the last few years. This report is concerned with the findings obtained in this study.

METHODS

A total of 238 male and female mongrel dogs, weighing between 7.0 and 14.0 kg. each and anesthetized with pentobarbital sodium, 35 mg./kg. i.v., was used throughout.

Following the induction of anesthesia, both common carotid arteries were exposed and isolated by conventional surgical procedures. End arterial pressure was measured from either common carotid artery through a polyethylene tube inserted and fixed in the artery and connected to an E&M Physiograph pressure transducer which was coupled to an E&M Physiograph recorder. The cannula and pressure transducer were filled with a physiological saline solution containing 0.05% heparin sodium. A femoral vein was surgically exposed and used for the administration of all test drug solutions.

The experimental procedure was as follows: after a 5-min. equilibration period, a control pressor response was obtained by the unilateral occlusion of the remaining (either left or right) unused common carotid artery for a period of 45 sec. The test solution was then administered i.v.; 4 min. later, carotid occlusion again was performed. The test drug solution administration and challenging carotid occlusions were made at the time intervals mentioned previously until there was completely diminished carotid occlusion pressor response or until subsequent injections failed to alter the carotid occlusion induced pressor response.

The relationship between the variables, expressed by the regression equation $Y = a + bX$ (5) where the slope constant $b = xy/x^2$ and the intercept a is calculated from this formula by using the mean values of $x(X)$ and $y(Y)$, was examined with the use of a oneway analysis of variance. Using an IBM 360/50 computer, the following regression relationships were made:

A. Influence of sex (male or female) on the relationship between the basal arterial pressure and:

1. The amplitude of the predrug carotid occlusion response.
2. The amplitude of the postdrug carotid occlusion response.
3. The blood pressure-lowering effects by total drug treatment.

B. Influence of date (months October–March—Group 1, and April–September—Group 2) of experiment on the relationships between basal arterial pressure and 1, 2, and 3 of A.

C. Influence of drug: (*a*) cryptenamine alkaloids, (*b*) cryptenamine tannate, and (*c*) crude alkaloids on the relationship between basal arterial pressure and 1, 2, and 3 of A.

D. Influence of date of experiment on the relationship between basal arterial pressure and amplitude of predrug and postdrug carotid occlusion response clustered by drug treatments.

E. Relationship between days stored of dogs in animal house on basal arterial pressure and on amplitude of predrug carotid occlusion response clustered by drugs (*a*), (*b*), and (*c*).

RESULTS

The regression analysis of the relationships between the variables are summarized and presented in Tables I–V.

A. Influence of sex (male or female) on the relationship between the basal arterial pressure and predrug and postdrug carotid occlusion pressor response and on the blood pressure-lowering effects by total drug treatment (Table I).

It can be seen from Table I that the male dog population before drug treatment had a mean basal arterial pressure (BAP), Column Y, of 130.3 mm. Hg; a mean peaked blood pressure response to bilateral carotid artery occlusion (BCO), Column X, of 190.09 mm. Hg; with a regression slope constant, Column b, of 0.7319. The female dogs before drugs did not significantly differ from the male group and had calculated mean values of 132.55 and 195.05 mm. Hg for BAP and BCO response, respectively, and a regression slope of 0.6560.

Table I—Influence of Sex (Male or Female) on the Relationship between Basal Arterial Pressure and Response

Factors[a]	b	Y	X	a
BAP:BCO (B) M	0.7319	130.3	190.09	−8.83
BAP:BCO (B) F	0.6560	132.55	195.05	4.60
BAP:BCO (A) M	0.7424	75.07	101.12	0
BAP:BCO (A) F	0.6961	75.60	104.81	2.64
DD:BP M	−0.0531	10.339	55.091	13.264
DD:BP F	0.0014	9.949	56.780	9.149

[a] BAP = mean basal arterial pressure in mm. Hg; BCO = peak blood pressure (mm. Hg) during carotid occlusion episode; (B) = before drug; (A) = after drug; M = male; F = female; DD = drug dose in mcg./kg.; and BP = blood pressure-lowering response.

After treatment with total drugs, the male and female populations were not significantly different and demonstrated, in the case of the males, BAP, BCO, and slope values of 75.07 mm. Hg, 101.12 mm. Hg, and 0.7424, respectively, while the female population had values of 75.60 mm. Hg, 104.81 mm. Hg, and 0.6961, respectively.

Total drug treatment (DD) in the two sexes was not associated with a difference in: mean blood pressure-lowering activity (BP), Column X; mean dose in mcg./kg., i.v., Column Y; or slope constant, Column b.

B. Influence of time of the year experiment performed on the relationship between the basal arterial pressure and response.

As shown in Table II, the date when the experiment was performed significantly influenced the mean basal arterial pressure levels (Column Y) before as well as after total drug treatment. The experiments performed in the fall–winter (Group 1) showed a lower mean basal arterial pressure value of 129.97 mm. Hg compared to the mean value of 131.90 mm. Hg obtained from the experiments performed in the spring–summer (Group 2). Similarly, after total drugs the mean basal arterial pressure was significantly lower in Group 1 (73.49 mm. Hg) than in Group 2 (75.51 mm. Hg).

Table II—Influence of Date (Fall, Winter—Spring, Summer) of Experiment on the Relationship between Basal Arterial Pressure and Response

Factors[a]	b	Y	X	a
BAP:BCO (B) 1	0.6447	129.97[b]	185.54[c]	10.35
BAP:BCO (B) 2	0.7313	131.90[b]	195.11[c]	−10.78
BAP:BCO (A) 1	0.7437	73.49[d]	95.15[e]	2.73
BAP:BCO (A) 2	0.7162	75.51[d]	105.22[e]	0.15
DD:BP 1	0.158	10.91	56.11	11.80
DD:BP 2	0.0379	9.87	56.32	12.0

[a] BAP = mean basal arterial pressure in mm. Hg; BCO = peak blood pressure (mm. Hg) during carotid occlusion episode; (B) = before drug; (A) = after drug; 1 = experiments performed October through March; 2 = experiments performed April through September; DD = drug dose in mcg./kg.; and BP = blood pressure-lowering response. [b] $p < 0.036$. [c] $p < 0.024$. [d] $p < 0.048$. [e] $p < 0.016$.

The mean peaked blood pressure responses (Column X) also demonstrated significantly different amplitude of response when comparison was made between Group 1 (185.54 mm. Hg) and Group 2 (195.11 mm. Hg) before drug treatment, and Group 1 (95.15 mm. Hg) and Group 2 (105.22 mm. Hg) after total drug treatment.

The date of the experiment did not influence the mean blood pressure-lowering response of total drugs nor the mean total drug dose.

C. Influence of date of experiment performed on the relationship between dose of drug and amplitude of blood pressure-lowering response.

Summarized in Table III are the regression analyses of the relationship between the dose of the drug treatment and the blood pressure-lowering response. The drugs used were: (*a*) cryptenamine alkaloids, (*b*) cryptenamine tannate, and (*c*) an alkaloidal fraction of *V. viride*. It can be seen that the slope constants (Column b) of the relationship between the drug-dose treatment and the blood pressure-lowering response were significantly different, exhibiting negative slopes of −0.0035 and −0.109 for cryptenamine alkaloids and alkaloidal fraction, respectively, while a positive slope constant of 0.214 was obtained for the cryptenamine tannate. In the total population there was no difference in the three drug groups in their mean drug dose or in the mean blood pressure-lowering response.

When the data were analyzed according to the date when the experiment was performed, a similar finding of significantly different slope constants was observed in the October–March (Group 1) experiments.

Here again, cryptenamine alkaloids and alkaloidal fraction demonstrated negative slopes of −0.0404 and −0.622, respectively, and cryptenamine tannate showed a positive slope constant of 0.1638.

Table III—Influence of Date on the Relationship between Blood Pressure-Lowering Response and Dose of Drug Clustered by Drug Groups

Factors[a]	b	Y	X	a
DD:BP *a*	−0.0035[b]	9.27	51.71	9.29
DD:BP *b*	0.214[b]	11.06	52.73	0.22
DD:BP *c*	−0.109[b]	10.40	56.67	16.58
DD:BP *a* 1	−0.0464[c]	9.63[d]	53.25[e]	2.48
DD:BP *b* 1	0.1638[c]	14.0[d,f]	48.95[e,g]	−8.00
DD:BP *c* 1	−0.0622[c]	9.1[d]	63.05[e,g]	3.93
DD:BP *a* 2	0.0091	9.14	59.24	−0.53
DD:BP *b* 2	0.0246	9.80[f]	54.35[g]	1.35
DD:BP *c* 2	−0.1183	10.90	54.35[g]	6.44

[a] DD = drug dose in mcg./kg.; BP = blood pressure-lowering response; *a* = cryptenamine alkaloids; *b* = cryptenamine tannate; *c* = alkaloidal; 1 = experiments performed October through March; and 2 = experiments performed April through September. [b] $p < 0.0567$. [c] $p < 0.06$. [d] $p < 0.03$. [e] $p < 0.045$. [f] $p < 0.031$. [g] $p < 0.024$.

In these experiments there were also significant differences in the mean drug dose employed (Column Y) of 9.63, 14.0, and 9.1 mcg./kg. and between the amplitude of the mean blood pressure-lowering response (Column X) of 53.25, 48.95, and 63.05 mm. Hg for cryptenamine alkaloids, cryptenamine tannate, and alkaloidal fraction, respectively.

When the experiments were performed during April–September, Group 2, there were no significant differences between the slope constants obtained for the three experimental drugs. Also, the mean drug dose as well as the mean arterial pressure-lowering response did not significantly differ between the three subgroups.

When comparison was made of the Date 1 to Date 2 results, it was observed that a cryptenamine tannate dose of 14.0 mcg./kg. (Date 1) was significantly greater than the mean dose of 9.8 mcg./kg. used in the Date 2 experiments. Also, the mean blood pressure-lowering responses of cryptenamine and of alkaloidal fraction of 48.95 and 63.05 mm. Hg, respectively, for Date 1 were significantly different from the Date 2 values of 54.35 mm. Hg for these drug groups.

D. Influence of date experiment performed on the relationship between arterial pressure, drug doses, and the amplitude of the arterial pressure response to bilateral carotid occlusion.

As shown in Table IV, within the Date 1 group there was no significant difference between the three drug subgroups before treatment (B), in slope constant (b), basal mean arterial pressure (Y), or in peaked mean arterial pressure response (X) to carotid occlusion. Similarly, within the Date 2 group there were insignificant differences between the three drug subgroups before treatment (B), as far as slope constant (b) and peaked mean pressor response (X) to carotid occlusion are concerned, although the initial mean arterial pressure (Y) levels between the three subgroups were statistically different. When comparison was made of the regression analysis of each drug subgroup [(*a*), (*b*), or (*c*)] between Dates 1 and 2, before treatment (B), it is observed that there was a statistically significant difference in slope constants of 0.6244 to 0.7256 for cryptenamine alkaloids, 0.6475 to 0.7222 for cryptenamine tannate, and 0.6423 to 0.7182 for alkaloidal fraction. No significant difference in the drug subgroup values was observed between Dates 1 and 2 for the mean basal arterial pressure (Y). There was significant difference in pressor response to bilateral carotid occlusion between Dates 1 and 2 for cryptenamine alkaloids, 185.21 to 193.57 mm. Hg; and for cryptenamine tannate, 180.81 to 199.35 mm. Hg; whereas the difference on the mean response of the alkaloidal fraction was not significantly influenced by the date the experiment was performed.

Table IV—Influence of Date on the Relationship between Basal Arterial Pressure, Drug Treatment, and BCO Response Clustered by Drug Groups

Factors[a]	b	Y	X	a
BAP:BCO (B) *a* 1	0.6244[b]	128.63	185.21[c]	12.98
BAP:BCO (B) *b* 1	0.6475[b]	124.71	180.81[c]	7.64
BAP:BCO (B) *c* 1	0.6423[b]	137.5	191.3	14.6
BAP:BCO (B) *a* 2	0.7256[b]	126.53[d]	193.57[c]	−13.92
BAP:BCO (B) *b* 2	0.7222[b]	139.88[d]	199.35[c]	−4.09
BAP:BCO (B) *c* 2	0.7182[b]	131.62[d]	193.29	−7.20
DD:BCO (A) *a* 1	0.187[e,f]	9.6[g]	17.125[h]	3.193
DD:BCO (A) *b* 1	0.1598[e,f]	14.0[g,i]	23.667[h]	−3.781
DD:BCO (A) *c* 1	0.0361[e,f]	9.368[g]	26.316	−0.941
DD:BCO (A) *a* 2	0.0176[j,f]	9.143	29.6[h]	−0.512
DD:BCO (A) *b* 2	0.0303[j,f]	9.796[i]	31.796[h]	−0.954
DD:BCO (A) *c* 2	0.4620[j,f]	10.873	27.945	−12.90

[a] BAP = mean basal arterial pressure in mm. Hg; BCO = peak blood pressure (mm. Hg) during carotid occlusion episode; (A) = after drug; (B) = before drug; *a* = cryptenamine alkaloids; *b* = cryptenamine tannate; *c* = alkaloidal; 1 = experiments performed October through March; 2 = experiments performed April through September; and DD = drug dose in mcg./kg. [b] $p < 0.03$. [c] $p < 0.05$. [d] $p < 0.005$. [e] $p < 0.05$. [f] $p < 0.01$. [g] $p < 0.03$. [h] $p < 0.03$. [i] $p < 0.05$. [j] $p < 0.001$.

During Date 2, only the slope constants for drug (*a*), 0.0176; drug (*b*), 0.0303; and drug (*c*), 0.4620 were significantly different for the three drug subgroups. The mean drug doses (Y) and the peak mean pressor response to the bilateral carotid occlusion (X) did not vary significantly between the three drug subgroups.

The slope constants for the Date 1 cryptenamine alkaloids, 0.187; cryptenamine tannate, 0.1598; and alkaloidal fraction, 0.0361; significantly differed from their Date 2 values of 0.0176, 0.0303, and 0.4020, respectively. The mean drug dose of cryptenamine tannate of 14.0 mcg./kg. for Date 1 and 9.796 mcg./kg. for the Date 2 experiments were significantly different. The BCO responses (X) also differed significantly for cryptenamine alkaloids and cryptenamine tannate between Date 1 (17.125 and 23.667 mm. Hg) and Date 2 (29.6 and 31.796 mm. Hg), respectively.

E. Relationship of days stored in the animal house of the dogs on the mean basal arterial pressure and on the pressor response to BCO, before drug treatment, separated into drug subgroups.

The data, summarized in Table V, reveal that the duration of days the dogs were stored (dH) in the animal house was not significantly different for the three drug subgroups; however, there was a significant difference for the BAP (Column X) and for the slope constants for the three drug subgroups. The animals which subsequently received cryptenamine alkaloids had a mean BAP of 127.07 mm. Hg and slope constant of 0.0032; for the cryptenamine tannate group, a mean BAP of 135.33 mm. Hg and slope constant of −0.1669; and, in the case of the alkaloid fraction group, a mean BAP of 133.19 mm. Hg with a slope constant of 0.1176.

The influence of the duration of days held on the peaked blood pressure response to BCO did not significantly vary between the three drug subgroups.

DISCUSSION

Examination of the regression analysis presented in the *Results* section leads to the following conclusions:

The sex of the animal does not influence the mean BAP and the magnitude of the pressor response to BCO both before as well as after total drug treatment, nor influence the relationship between total drug-dose treatment and the intensity of the arterial pressure depression.

The date of the experiment, that is, whether the experiment was performed in October through March or in April through September, did have a profound influence on the mean BAP and on the magnitude of the pressor response to BCO both before and after total drug treatment, but did not significantly influence the relationship between total drug-dose treatment and blood pressure-lowering response.

The calculated mean doses of cryptenamine alkaloids, cryptenamine tannate, and the alkaloidal fraction were significantly different.

Table V—Influence of Days Stored in Animal House in the Basal Arterial Pressure and BCO Responses Clustered by Drug Groups

Factors[a]	b	Y	X	a
dH:BAP *a*	0.0032[b]	13.61	127.07[c]	13.20
dH:BAP *b*	−0.1669[b]	14.59	135.33[c]	37.18
dH:BAP *c*	0.1176[b]	13.73	133.19[c]	−1.93
dH:BCO (B) *a*	−0.0569	13.61	191.44	14.706
dH:BCO (B) *b*	−0.1023	14.59	193.79	34.41
dH:BCO (B) *c*	0.0271	13.73	192.76	8.16

[a] dH = days held in storage; BAP = mean basal arterial pressure in mm. Hg; BCO = peak blood pressure (mm. Hg) during carotid occlusion episode; (B) = before drug; *a* = cryptenamine alkaloids, *b* = cryptenamine tannate; and *c* = alkaloidal. [b] $p < 0.0148$. [c] $p < 0.03$.

There was a statistical difference in slope constant, mean drug dose, and in the amplitude of the blood pressure-lowering response for the experiments performed in October through March. When examination was made of the data from experiments performed April through September, there were no calculated significant differences in these relationships. There were no significant differences between the slope constants, mean drug dose, and blood pressure-lowering responses for the three drug subgroups when the data collected from October through March were compared to those obtained in April through September.

There were no differences in the slope constants, mean BAP, and in the amplitude of the pressor response to BCO before drug treatment between the three drug groups for the experiments performed during the October through March period. There was only a significant difference in mean BAP between the three drug groups when the experiments were performed during April through September. However, there were significant differences for the three drug subgroups in slope constant and amplitude of the pressor response to BCO when the two 6-month experimental periods were compared.

After drug treatment, there were significant differences between the three drug groups in slope constant and in mean drug dose for the experiments performed during October–March. Only the slope constants between the three drugs differed significantly when the experiments were performed during April–September. When comparison was made between the two 6-month periods, there was significant difference in slope constants, in mean drug dose (cryptenamine tannate), and in mean peak arterial pressure response to BCO for the cryptenamine alkaloids and cryptenamine groups.

It was observed that although there was no difference between the three drug subgroups in the mean number of days the dogs were stored in the animal house prior to use, there was a significant difference in slope constants and in mean BAP for the three drug subgroups.

Examination of the results reveals that although the mean BAP is markedly lowered by total drug treatment, the slope constants for the relationships between the mean BAP and the pressor response to BCO did not significantly differ. These findings thus support the conclusions of Prochnik *et al.* (2) who suggested that when employing the bilateral carotid occlusion pressor bioassay, correction be made for drug-induced changes in mean BAP and also for the findings that the pressor response was physiologically absent when the arterial pressure reached 60 mm. Hg.

Although there was no difference in mean total drug dose and blood pressure-lowering response, when these factors were separated by drug groups there was significant difference in slope constants. Here, cryptenamine tannate produced the most desirable slope constant indicative of an increase in blood pressure-lowering response with increasing dose. Cryptenamine alkaloids and the alkaloid fraction exhibited negative slopes, thus indicating that increasing drug dose decreases the degree the blood pressure falls. Further separation of these results into time of the year the experiment was performed showed that during October through March, cryptenamine tannate, although administered at a significantly higher dose, again demonstrated a positive slope while the other drugs exhibited negative slope constants in the relationship between drug dose and blood pressure-lowering effects. When the experiments were performed during April through September, cryptenamine alkaloids and cryptenamine tannate showed very low but positive slope constants, while the alkaloid fraction still demonstrated a negative slope constant for the relationship between the drug dose and the intensity of the blood pressure depression. It was of interest to note that for all three drug subgroups there was a significant increase in slope constants, while the peak BCO response (before drugs) only significantly increased for cryptenamine alkaloids and cryptenamine tannate, and no differences were observed in the mean BAP when comparison was made between the October–March and April–September groups. Similar findings were observed when examination of the relationship between drug dose and BCO (after drug) was made.

The days the animals were stored in the animal house prior to use did not consistently affect the slope constants nor the BAP on the BCO response amplitude for the three drug subgroups.

Examination of these results thus indicates that the date at which the experiment was performed did significantly influence most of the examined relationships and if this bioassay procedure continued to be employed, necessary corrections must be applied to adjust for these influences.

These seasonal influences are possibly the result of rhythmic changes in disposition and deposition of adipose tissue and other changes during different seasons as reported by Fisher *et al.* (6) and Hilditch (7). Since in all these experiments pentobarbital was used as the anesthetic, it is quite conceivable that the observed seasonal difference in mean BAP, in pressor response amplitude to BCO, and the blood pressure-lowering effects of the three drugs were the result of either a different level of anesthesia occurring during the different seasons or, more generally, a seasonally induced changed physiological state resulting in differential responding.

It is well known that the physiological state of the preparation can profoundly influence both the nature as well as the intensity of a fixed treatment response. This is particularly in evidence with the alkaloids ibogaine and yohimbine. It was reported (8) that in the anesthetized dog, ibogaine produced a marked lowering of arterial pressure while in the conscious preparation an equal dose of ibogaine appeared to be devoid of hypotensive activity. Likewise, yohimbine is reported (9) to produce a hypertensive response in the conscious animal while evoking an arterial pressure depressor response when the animal is anesthetized with pentobarbital sodium.

In order to examine the possibility that use of the anesthetic is a factor in influencing the responses as described, a series of experiments will be performed in chronically prepared dogs while conscious and in the anesthetized state. With each dog serving as its own control, it will be possible to assess the influence of the anesthetic on many of the relationships examined in this study.

REFERENCES

(1) T. B. O'Dell, *J. Amer. Pharm. Ass., Sci. Ed.*, **41**, 316(1952).

(2) G. Prochnik, G. L. Maison, and J. W. Stutzman, *Amer. J. Physiol.*, **162**, 553(1950).

(3) B. Rubin and J. C. Burke, *J. Pharmacol. Exp. Ther.*, **105**, 409(1952).

(4) B. S. Jandhyala and J. P. Buckley, *J. Pharm. Sci.*, **55**, 888 (1966).

(5) G. W. Snedecor, "Statistical Methods," 4th ed., Iowa State College Press, Ames, Iowa, 1955, p. 543.

(6) H. Fisher, K. G. Hollands, and H. S. Weiss, *Proc. Soc. Exp. Biol. Med.*, **110**, 832(1962).

(7) T. P. Hilditch, "The Chemical Constitution of Natural Fats," Wiley, New York, N. Y., 1947, p. 355.

(8) J. A. Schneider and R. K. Rinehart, *Arch. Int. Pharmacodyn.*, **110**, 92(1957).

(9) S. Gershon and W. J. Lang, *ibid.*, **135**, 31(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 18, 1969, from the *Cochran VA Hospital, St. Louis University School of Medicine, St. Louis, MO 63106 and Neisler Laboratories, Decatur, IL 62526*

Accepted for publication February 24, 1970.

* Present address: Marion Laboratories, Kansas City, Mo.

† Present address: Warren-Teed Co., Columbus, Ohio.

Comparative Distribution, Excretion, and Metabolism of ^{14}C-Labeled Quaternary Ammonium Compounds of Promazine, Chlorpromazine, and Triflupromazine

C. L. HUANG, J. Z. YEH, and I. A. MUNI

Abstract □ The tissue distribution and biotransformation patterns of the quaternary methiodides of promazine, chlorpromazine, and triflupromazine in rats were investigated. After parenteral administration, high concentrations of these compounds were found in the liver, kidneys, and intestines. The majority of these compounds was metabolized by the liver and excreted in the intestines *via* the bile duct. In contrast to the corresponding tertiary amine, the majority of these compounds was excreted unchanged; however, a trace of chlorpromazine sulfoxide methiodide was detected in the urine.

Keyphrases □ Promazine, chlorpromazine, triflupromazine ^{14}C-methiodides—synthesis □ Triflupromazine, chlorpromazine, promazine ^{14}C-methiodides—synthesis □ Biotransformation, distribution—quaternary phenothiazine compounds □ IR spectrophotometry—identity □ UV spectrophotometry—identity □ Scintillometry—analysis □ Radiochromatography—analysis

Molecular modification of phenothiazines with an aim to isolate sedative effect from antihistaminic activity has been contemplated by quaternization of the tertiary amino nitrogen. Albanus *et al.* (1) prepared promethazine hydroxyethyl ammonium chloride[1] and demonstrated that this compound had an antihistaminic activity similar to that of the parent compound but lacked sedative effect. Hanngren (2) reported that this compound caused a decrease in the volume and acidity of the gastric juice in human subjects by inhibiting the gastric basal secretion.

Levine *et al.* (3–5) studied the rate of absorption of monoquaternary ammonium compound of benzomethamine by using *in vivo* intestinal loops in rats. The data indicated that the quaternary ammonium salt was poorly absorbed by the gastrointestinal tract due to its high degree of ionization and the positive charge which promotes the formation of a nonabsorbable complex with the intestinal mucin. Levine stated that more than a passive diffusion was involved in the kinetics of intestinal absorption of benzomethamine and other onium compounds.

The rate of excretion of onium compounds is dependent upon the route of administration and the degree of ionization of the compound. By subcutaneous administration, some 7–37% of radioactivity was recovered in the urine during the same period of time. In a comparative study made by Hansson and Schmitterlöw (6) and Allgen *et al.* (7) on *in vivo* behavior of tertiary and quaternary promethazine, markedly different excretion patterns were found between these compounds. Both compounds were excreted mainly *via* the kidneys, and only 25% was found in the feces after subcutaneous administration. However, with oral administration, tertiary promethazine was excreted mainly in the urine, whereas the majority of the quaternary compound appeared in the feces.

Recently, distribution and excretion patterns of quaternary ammonium salts of mepazine, promethazine, trifluoperazine (8), and perphenazine (9) with one or two moieties of ^{14}C-methyl groups attached to the terminal nitrogen have been studied. The rate of absorption of the i.p. administered ^{14}C-methiodide of these compounds was rapid, as evidenced by the quick disappearance of radioactivity from the injected site and further reflected in fairly high blood levels with peaks, in most cases, at 0.5 hr. after the administration of the compounds. Contrary to the previous speculations of a high blood-brain barrier for these compounds, brain levels of the compounds were low but above the significant level. This finding may provide direct evidence as to why these compounds are active on the CNS.

It appears that the distribution and biotransformation pattern of quaternary phenothiazine compounds are quite different from those of the corresponding tertiary amino derivatives. The majority of the administered quaternary ammonium compounds is found in organs such as liver, intestines, kidneys, gastric mucosa, and pancreas; the majority of the tertiary amine derivatives is found in the lungs and brain. Tertiary phenothiazine derivatives are known to be metabolized *in vivo* largely through four pathways: sulfoxidation, hydroxylation, glucuronidation, and *N*-demethylation; however, quaternary derivatives undergo these metabolic processes to a much lesser extent.

Apparently, two types of major excretion patterns exist, the urinary and fecal types, for these methiodide derivatives. Perphenazine methiodide exhibited the former type of excretion, while methiodides of mepazine, promethazine, and trifluoperazine belonged to the latter category. The difference in the metabolic behavior between these compounds was apparently due to the difference in the affinity of these compounds for the liver cells; perphenazine methiodide could not enter into the active metabolic process of the liver, whereas other compounds actively participated in the excretory mechanism of the liver and thus were excreted in the intestines *via* the bile duct.

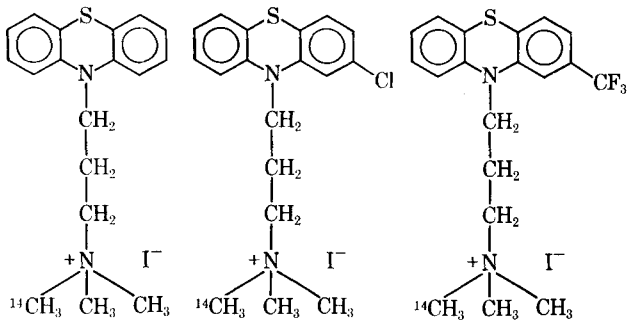
The toxicity of these phenothiazine derivatives did not diminish by quaternization, as evidenced by the fact that the LD_{50} of these compounds administered by the i.p. route was higher than that of the corresponding tertiary amino derivatives. Antimicrobial activities were also retained by these quaternary ammonium salts in that growth of *Staphylococcus aureus* and *Escherichia*

[1] Aprobit, Recit Co., Stockholm, Sweden.

coli was inhibited at the concentrations of 1 and 10 mcg./ml., respectively.

Review of the literature indicated that only a few quaternary ammonium compounds of phenothiazine derivatives were studied extensively in regard to their distribution, excretion, and metabolism in animals. It is the purpose of this study to compare the *in vivo* behavior of three quaternary compounds of promazine, chlorpromazine, and trifluprom azine which are similar in structure except for the functional group attached to the Position 2.

In this report, comparative studies on the tissue distribution and elimination of the methiodide of three structurally related phenothiazine derivatives—promazine, chlorpromazine, and trifluprom azine—are presented. These three compounds differ only by the presence of substituent groups at the Position 2 on the aromatic ring system (Structures I, II, and III).



promazine methiodide-^{14}C I

chlorpromazine methiodide-^{14}C II

trifluprom azine methiodide-^{14}C III

METHODS[2]

Synthesis of Promazine Methiodide (PRZ-MEI) and Promazine Methiodide-^{14}C (PRZ-MEI-^{14}C)—Promazine hydrochloride (0.5 g.) was dissolved in 5 ml. of water, and the solution was adjusted to pH 10 (pHydrion paper) and extracted several times with benzene. The benzene extracts were combined and the solvent removed under reduced pressure to leave an oil (0.42 g.). The oil was dissolved in 5 ml. of acetone, and 0.2 g. of methyl iodide was added. Crystallization commenced within 15 min. at room temperature. The crystals were collected and recrystallized from acetone to yield 0.57 g. (90%) of PRZ-MEI with m.p. 255–257° and R_f 0.71; $\lambda_{max.}$, 210 and 354 mμ.

Anal.—Calcd. for $C_{18}H_{23}IN_2S$: C, 50.70; H, 5.40; N, 6.57. Found: C, 50.80; H, 5.50; N, 6.70.

The free base of promazine (265 mg., 0.93 mmole) obtained was dissolved in 1.5 ml. of acetone and pipeted into the capillary guard of a breakseal tube which contained 85.5 mg. (0.60 mmole) of ^{14}C-methyl iodide (11.7 μc./mg.). The capillary seal was broken to allow 1 ml. (163 mg., 0.60 mmole) of the solution to enter the tube under negative pressure. The tube was stoppered immediately and allowed to stand overnight at room temperature. About 100 mg. of unlabeled methyl iodide was added to ensure a complete quaternization of the promazine base. Then ether was added to the mixture until turbidity occurred. Crystallization commenced within 15 min. The crystals were collected and washed with ether to yield 345 mg. (87%) of PMZ-MEI-^{14}C with m.p. 255–257° and specific activity 3.19 μc./mg. Radiochromatograms showed only one spot with an identical R_f (0.71) with PMZ-MEI, and mixed melting point with PMZ-MEI did not show depression (255–257°).

Synthesis of Chlorpromazine Methiodide (CPZ-MEI) and Chlorpromazine Methiodide-^{14}C (CPZ-MEI-^{14}C)—Chlorpromazine hydrochloride (0.5 g.) in 5 ml. of water was adjusted to pH 10 and extracted several times with benzene. The combined benzene extracts were dehydrated with anhydrous sodium sulfate and evaporated *in vacuo* to leave 0.43 g. of an oil. To the acetone solution (5 ml.) of the oil, 0.2 g. of methyl iodide was added. The mixture was shaken occasionally and left at room temperature overnight. Then ether was added to the mixture to precipitate the product. The precipitate was collected and recrystallized from acetone–ether to yield 0.49 g. (81%) of CPZ-MEI with m.p. 145–147°; $\lambda_{max.}$, 216 and 256 mμ.

Anal.—Calcd. for $C_{18}H_{22}ClIN_2S$: C, 46.91; H, 4.78; N, 6.08. Found: C, 47.23; H, 5.11; N, 5.96.

The oily free base of chlorpromazine (190 mg., 0.6 mmole) in 2 ml. of acetone was reacted with 85.5 mg. (0.6 mmole) of ^{14}C-methyl iodide (11.7 μc./mg.) as described for promazine. The reaction was allowed to proceed for 12 hr. at room temperature; then a suitable amount of ether was added to initiate crystallization. The crystals were collected and recrystallized from acetone–ether to yield 218 mg. (80%) of CPZ-MEI-^{14}C with m.p. 145–147° and specific activity 2.89 μc./mg.

The radiochemical purity and authenticity of this compound were checked by paper chromatography (R_f, 0.79) coupled with a radiochromatogram scanner. The mixed melting point of this compound with CPZ-MEI did not show depression (145–147°).

Synthesis of Trifluprom azine Methiodide (TFP-MEI) and Trifluprom azine Methiodide-^{14}C (TFP-MEI-^{14}C)—Trifluprom azine hydrochloride (0.5 g.) was dissolved in water, and the solution was adjusted to pH 10 by adding sodium hydroxide solution. The oily precipitate appearing in the mixture was extracted several times with ether. The combined ether extracts were dehydrated with anhydrous sodium sulfate and the solvent removed *in vacuo*. The free base of trifluprom azine (0.45 g.) was dissolved in 5 ml. of acetone, and methyl iodide (0.2 g.) was added. The mixture was allowed to stand at room temperature overnight; then a suitable amount of ether was added to initiate crystallization. The crystals were collected and recrystallized from acetone–ether to give 0.38 g. (60%) of TFP-MEI with m.p. 169–170° and R_f 0.81; $\lambda_{max.}$, 216 and 258 mμ.

Anal.—Calcd. for $C_{19}H_{22}F_3IN_2S$: C, 46.16; H, 4.49; N, 5.67. Found: C, 46.33; H, 4.48; N, 5.71.

To the free base of trifluprom azine (224 mg., 0.6 mmole) in 2 ml. of acetone was added ^{14}C-methyl iodide (11.7 μc./mg., 85.5 mg., 0.6 mmole). The reaction was allowed to proceed for 12 hr. at room temperature; then ether was added until crystallization commenced. The crystals were filtered off and recrystallized from acetone–ether to yield 188 mg. (60%) of TFP-MEI-^{14}C with m.p. 169–170° and specific activity 3.11 μc./mg. Radiochemical purity and authenticity of the product were checked by chromatography coupled with radiochromatogram scanner, mixed melting point (169–170°), and cochromatography (R_f 0.81) with an authentic sample of TFP-MEI.

Excretion Studies—In the excretion studies, 1 mg. each of the quaternary ammonium compounds of promazine (3.19 μc./mg.), chlorpromazine (2.89 μc./mg.), and trifluprom azine (3.11 μc./mg.) was injected i.p. to six albino rats weighing 250–280 g. The animals were maintained in metabolic cages with free access to food and water. The urine and feces specimens were collected separately every 8 hr. for a period of 5 days. Urine specimens were diluted five times with water; an aliquot of 0.5 ml. was measured in a planchet, dried, and the activity recorded. It was a thin-layer preparation and no correction for the self-absorption was required. Feces specimens were dried and powdered. An aliquot of 0.1 g. was placed in a planchet and the activity measured. Corrections were made for the self-absorption.

Tissue Distribution Studies—Three groups of albino rats (five rats in each group) weighing 250–280 g. were administered through the intraperitoneal route with 1 mg. each of PRZ-MEI-^{14}C, CPZ-MEI-^{14}C, and TFZ-MEI-^{14}C, respectively. The animals were housed in individual cages and were sacrificed at the following intervals: 0.5, 1, 2, 4, and 8 hr. The following organs and tissues were isolated: liver, lungs, kidneys, spleen, heart, stomach, intestines, blood, brain, muscle (femoral), and bone (femur). The surface of the intestines

[2] Melting points were taken on a Fisher-Johns apparatus and were corrected. UV and IR absorption spectra were recorded on a Perkin-Elmer model 202 and model 137 infracord spectrophotometer, respectively. Paper chromatograms were developed in a solvent system; *n*-butanol–ethanol–water (5:2:2). Albino rats were purchased from Southern Animal Farms, Prattville, Ala. Radioactivity in the tissues was recorded in a G-M counter (Tracerlab, model TGC-2), and radiochromatograms were scanned in a radiochromatogram scanner, Actigraph III (Nuclear-Chicago).

Table I—Urinary and Fecal Excretion of Quaternary ^{14}C-Methiodides of Promazine (PRZ), Chlorpromazine (CPZ), and Triflupromazine (TFZ)[a]

Time Intervals, hr.	Urinary Excretion			Fecal Excretion		
	PRZ	CPZ	TFP	PRZ	CPZ	TFZ
8	12.28	7.23	5.67	0.41	0.40	0.13
16	4.82	1.22	0.97	29.12	19.48	16.96
24	3.98	0.53	0.37	5.20	14.71	13.44
32	2.62	0.44	0.25	3.96	10.28	4.39
40	1.16	0.30	0.17	4.88	5.42	3.00
48	0.85	0.28	0.13	2.61	1.12	0.47
56	0.90	0.22	0.14	1.84	0.52	0.34
64	0.82	0.15	0.07	1.72	1.25	0.87
72	0.86	0.17	0.10	1.10	0.21	0.26
96	0.80	0.42	0.28	1.82	0.82	1.39
120	0.74	0.40	0.28	0.96	0.34	0.52

[a] Expressed in terms of percent of the administered activity.

was rinsed with saline to remove mechanical contamination of the administered material, and the peritoneal cavity was washed several times with detergent to recover the unabsorbed portion of the activity. The whole organs, except for the blood and bone, were homogenized in a homogenizer and diluted with water to an extent that 16 ml. represented 1 g. of the organ. An aliquot of 150 mcg. of the dilution was measured in a planchet, dried, and the activity recorded.

Metabolic Studies—Since the ^{14}C-methyl group is attached to the nitrogen of the 10-substituted side chain of the phenothiazine ring system, it was essential to study its fate *in vivo*. In this study, three rats were injected i.p. with ^{14}C-methiodide (1 mg.) of these compounds. The animals were maintained in a large metabolic jar, and the expired air was passed through a washing bottle containing 40% sodium hydroxide solution for 24 hr. to collect carbon dioxide. The carbon dioxide trapped as sodium carbonate was treated with a calcium chloride solution; the precipitate of the calcium carbonate thus formed was collected and the activity determined.

A portion (usually 0.5 ml.) of the urine collected 24 hr. after the intraperitoneal administration of these compounds was placed linearly on Whatman No. 3 chromatographic paper along with radioactive standards. The chromatogram was developed by an ascending technique in the usual solvent system (10). The chromatogram was dried and scanned in a radiochromatogram scanner to record the activity, and the R_f value of the spot was calculated. Feces collected over a 3-day period were placed in a continuous extraction apparatus and extracted successively with ether for 2 days and then with methanol for 3 days. The methanol extracts were reduced to about 1 ml. *in vacuo*, and an aliquot of 0.1 ml. was placed linearly on Whatman No. 3 chromatographic paper along with the radioactive standard. The chromatogram was developed by an ascending technique in the solvent system described for the urine specimens. After the chromatogram was dried, the activity was recorded in a radiochromatogram scanner and the R_f value was calculated.

Synthesis of Methiodide of Chlorpromazine Sulfoxide—About 0.5 g. of chlorpromazine sulfoxide was dissolved in 5 ml. of acetone and reacted with 0.5 ml. of methyl iodide. The crystal appeared spontaneously. The compound was recrystallized from the acetone–ether mixture to a crystalline powder with m.p. 242–243°. The R_f value was 0.65. This material was used as a reference compound for the identification of metabolites of chlorpromazine methiodide.

Biliary Excretion Studies—Male Holtzman rats weighing about 300 g. were anesthetized by subcutaneous administration of 70 mg./kg. of pentobarbital sodium. Through an abdominal incision, the bile duct was cannulated with a polyethylene tube (PE50, Clay-Adams Inc.). About 1 ml. of the drug solution (1 mg./ml.) was dropped directly into the abdominal cavity before the incision was closed. Bile was collected at the intervals of 0.5, 1, and 2 hr. All animals were sacrificed at the end of 2 hr., and the entire intestines were removed and the radioactivity was recorded as described previously (*Tissue Distribution Studies*). The radioactivity in the urine and bile was determined according to the procedure described under the urinary excretion study.

RESULTS

Excretion of PRZ-MEI, CPZ-MEI, and TFP-MEI—Table I summarizes the urinary and fecal excretion of radioactivity by rats after a single intraperitoneal administration of PRZ-MEI-^{14}C, CPZ-MEI-^{14}C, and TFP-MEI-^{14}C. Total radioactivity recovered in the urine in 5 days was: PRZ-MEI, 29.83%; CPZ-MEI, 11.36%; and TFP-MEI, 8.43% of the administered radioactivity. The majority of the ^{14}C in the urine was recovered within the first 8 hr., and the first 24-hr. excretion accounted for 70–90% of the total urinary radioactivity recovered during the 5-day period. However, fecal excretion was the major route of excretion, which is approximately 2–6 times the urinary excretion for these compounds. The radioactivity in the feces was low in the first 8 hr., but the activity reached its peak in 16 hr. and declined slowly thereafter. Occasional fluctuation of radioactivity was observed which was due to the uneven fecal excretion by these animals. Total radioactivity recovered during the 5-day period was: PRZ-MEI, 53.62%; CPZ-MEI, 54.55%; and TFP-MEI, 51.37% of the administered activity. The ratio between the urinary and fecal excretion for PRZ-MEI, CPZ-MEI, and TFP-MEI was 1:1.8, 1:5, and 1:7.3, respectively. The combined radioactivity of PRZ-MEI recovered in the urine and feces was the highest (83.45% of the administered activity), followed by CPZ-MEI (65.91%) and TFP-MEI (59.80%). The difference in the rate of excretion in these compounds is apparently due to the different substituent groups attached to the Position 2.

Tissue Distribution of PRZ-MEI, CPZ-MEI, and TFP-MEI (Tables II and III)—The blood levels of these ^{14}C-compounds were generally low with peaks at 0.5 hr. The concentrations declined slowly after 1 hr. but remained significant until after 8 hr. Among the three compounds tested, PRZ-MEI showed the highest blood level. The brain level of PRZ-MEI showed an interesting trend of increasing radioactivity in contrast to the decreasing trend of the blood level during the 8-hr. period. However, the brain levels of CPZ-MEI and TFP-MEI were almost parallel to the blood levels. The brain levels declined after 0.5 hr. and were insignificant after 2 hr. In all cases, the intestines showed the highest radioactivity followed by the muscle, liver, and kidneys. Apparently, the liver was

Table II—Distribution of Radioactivity in the Tissues of Rats after Intraperitoneal Administration of Quaternary ^{14}C-Methiodides of Promazine (A), Chlorpromazine (B), and Triflupromazine (C)

Organs	0.5 hr.			1 hr.			2 hr.			4 hr.			8 hr.		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Blood	1.36	0.36	0.33	1.04	0.32	0.26	0.65	0.29	0.20	0.51	0.16	0.09	0.37	0.03	0.07
Brain	0.04	0.01	0.01	0.06	0.01	0.01	0.09	<0.01	<0.01	0.16	<0.01	0.01	0.20	<0.01	<0.01
Heart	0.08	0.05	0.03	0.08	0.05	0.05	0.06	0.03	0.04	0.03	0.02	0.03	0.03	0.01	0.02
Intestines	9.63	18.28	15.76	16.15	25.97	24.58	21.37	26.61	28.66	30.26	33.54	46.27	39.69	50.91	44.02
Kidneys	1.76	1.37	0.95	1.98	0.60	0.69	1.33	0.40	0.87	0.97	0.38	0.35	0.65	0.13	0.28
Liver	6.24	8.38	9.50	10.36	6.43	7.08	7.92	3.20	6.03	6.10	2.50	2.63	4.21	1.12	1.73
Lungs	0.11	0.12	0.04	0.08	0.11	0.14	0.12	0.13	0.07	0.16	0.15	0.02	0.12	0.09	0.02
Muscle	8.38	0.67	3.83	3.47	3.07	1.85	2.78	2.08	1.50	2.40	1.37	1.00	1.89	0.40	0.32
Spleen	0.12	0.66	0.79	0.16	0.38	0.55	0.18	0.13	0.24	0.27	0.27	0.10	0.23	0.08	0.05
Stomach	0.59	0.68	1.71	1.12	0.74	1.13	1.98	1.06	1.37	1.14	0.43	0.88	1.61	0.32	0.68
Urine	0.41	2.13	2.50	0.78	3.07	4.35	1.72	5.67	5.07	4.63	6.98	5.76	11.63	12.04	6.46
Abdominal washings	9.32	4.67	6.54	6.68	1.51	1.96	3.12	0.40	0.60	2.86	0.16	0.10	1.38	0.06	0.05

[a] Expressed in terms of percent of the administered activity.

Table III—Relative Activity[a] of the Intraperitoneally Administered Promazine Methiodide-^{14}C (A), Chlorpromazine Methiodide-^{14}C (B), and Triflupromazine Methiodide-^{14}C (C) in the Tissues of Rats

Organs	0.5 hr.			1 hr.			2 hr.			4 hr.			8 hr.		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Blood	13.6	3.6	3.3	10.2	3.2	2.6	6.5	2.9	2.0	6.0	1.6	0.9	3.7	0.3	0.7
Brain	2.5	1.5	1.6	7.7	1.5	1.0	11.6	0.8	0.7	20.7	0.3	0.2	25.9	0.2	0.1
Heart	21.6	16.3	10.0	21.6	15.2	18.0	16.2	10.0	13.6	8.1	6.9	11.0	8.1	3.2	6.4
Intestines	99.3	282	220	166	389	420	220	436	433	312	446	723	409	699	676
Kidneys	152	176	143	172	86.0	96.9	116	48.0	118	83.8	50.0	43.1	56.6	16.0	38.4
Liver	116	229	276	194	178	219	148	84.0	176	113	58.1	68.7	78.4	30.1	43.1
Lungs	16.2	22.5	6.4	11.9	23.6	24.3	17.7	21.2	5.7	32.6	14.8	5.7	17.7	12.8	4.0
Muscle	38.2	3.0	8.5	15.7	13.9	4.1	12.7	9.5	2.5	10.9	6.3	1.4	8.5	—	1.0
Spleen	43.6	268	326	58.1	163	231	65.3	47.6	75.3	98.1	75.3	33.3	83.6	24.8	19.2
Stomach	73.3	48.8	92.7	140	30.8	107	247	58.6	140	142	22.1	84.9	201	41.1	65.3

[a] A value of 100 represents nonspecific tissue distribution. Relative activity = μc./g. tissue/(total activity/body weight) × 100.

the major organ that metabolized these compounds; it showed a peak level between 0.5 and 1 hr. and maintained a fairly high level throughout the 8-hr. period. The radioactivity accumulated in the intestines reflected the fact that heptobiliary excretion was the major route of elimination for these compounds. The overall radioactivity in the kidneys was lower than that in the liver; however, the specific activity of the kidneys was higher than that of the liver when the radioactivity was expressed in terms of percent specific activity (Table III). The kidney level had its peak at 0.5 to 1 hr. and then steadily declined. The increase of radioactivity in the urine recovered from the urinary bladder by autopsy coincided with the decrease in the kidney level. Abdominal washings were obtained to estimate the rate of absorption of these compounds after the intraperitoneal administration. The recovered radioactivity indicated that the rate of absorption of these compounds by rats was quite rapid; 4.67–9.32% of the administered activity remained in the abdominal cavity at 0.5 hr. but only 0.05–1.38% was recovered after 8 hr.

When the tissue distribution data were expressed in terms of relative activity in respect to the specific activity of the whole body, the distribution pattern of the activity in each organ remained the same. However, the relative specific activity of these organs changed (Table III). The specific activity of PRZ-MEI-^{14}C in the kidneys became the highest, followed by that in the liver at 0.5 hr. The specific activity of TFP-MEI-^{14}C in the spleen was higher than that in the liver and kidneys. A considerably high specific activity was found in the stomach, which had a higher level than that of the heart and lungs. The specific activity of these compounds in the intestines was 1–3 times higher than that of the whole body at 0.5 hr. and eventually it became 4–7 times at 8 hr.

Metabolic Studies—N-*Demethylation* in vivo—No radioactivity was detected in the carbon dioxide collected from animals administered with the ^{14}C-methiodide of promazine, chlorpromazine, and triflupromazine. This finding indicated that *N*-demethylation of the quaternary ammonium compounds did not occur *in vivo*.

Urinary Metabolites—A paper chromatogram of the urine specimens collected from animals given PRZ-MEI-^{14}C and TFP-MEI-^{14}C showed only one spot as indicated on the recording of a radiochromatogram scanner. The R_f values matched those of the corresponding original compounds, indicating that both compounds were excreted in the urine unchanged. The chromatogram of the urine specimens from the CPZ-MEI group of animals showed a trace of the second metabolite with an R_f value of 0.66. It was identified to be its sulfoxide by comparing the R_f value (0.66) and UV absorption spectrum (240 and 310 mμ) with those of the authentic specimen of chlorpromazine sulfoxide methiodide.

Fecal Metabolites—The ether extracts of the feces specimens from animals given the compounds appeared greasy and showed no radioactivity. Most of the radioactivity in the feces was found in methanol extracts; however, about 10–15% of the radioactivity was left in the residue after the methanol extraction. A paper chromatogram of the methanol extract showed only one spot, as indicated on the recording of a radiochromatogram scanner. The R_f values of the spot matched each of the original starting materials (PRZ-MEI, 0.73; CPZ-MEI, 0.78; and TFP-MEI, 0.81).

A study was conducted to establish the rate of fecal excretion of CPZ-MEI-^{14}C after various routes of administration. During the 5-day period, 55% of the administered activity was recovered in the feces after an intraperitoneal administration and about 57% by a subcutaneous route; after an oral route of administration, nearly 98% of the material was recovered within 48 hr. Thus, it was established that fecal excretion was the main route of elimination of CPZ-MEI irrespective of the route of administration. The ratio between the urinary and fecal excretion was as follows: intraperitoneal route, 1 : 5, subcutaneous route, 1 : 2.2; and oral route, 1 : 320. By parenteral routes of administration, usually a slight inflammation was observed at the site of injection.

DISCUSSION

Fecal excretion was the major route of elimination of the *N*-methiodide of promazine, chlorpromazine, and triflupromazine. A substituent group of either Cl or CF_3 at Position 2 on the promazine molecule caused a remarkable change in the rate of elimination of these compounds. The rate of absorption of these compounds after the intraperitoneal route of administration was rapid, as indicated by a rapid disappearance of the compounds from the site of injection. Urinary excretion represented about 8–30% of the administered activity, and 51–55% of the activity was found in the feces. The total excretion in the urine and feces combined was 60–83% of the administered dose in a 5-day period, and a slow excretion continued after 5 days. Apparently, a substituent group of Cl and CF_3 at Position 2 on the promazine molecule remarkably changed the rate of elimination of these compounds, as evidenced by the fact that the extent of excretion for PRZ-MEI (83%) was the highest, followed by CPZ-MEI (66%), with TFP-MEI (60%) the lowest.

By parenteral routes of intraperitoneal and subcutaneous administration of CPZ-MEI, the radioactivity recovered in the feces was 55% and 57%, respectively. However, because of the ionic nature of this compound, it was poorly absorbed by the stomach. Therefore, with oral administration, 98% of the compound was recovered in the feces, and urinary excretion represented only a trace of the compound (9).

Blood level of PRZ-MEI was the highest among the three compounds with a proportionally high brain level. It appeared that the blood-brain barrier is low for PRZ-MEI, which is contrary to the general concept that quaternary ammonium compounds have a high blood-brain barrier. However, the brain level of CPZ-MEI and TFP-MEI was very low and was insignificant against the background after 2 hr.

The majority of the i.p. administered compounds was metabolized by the liver and excreted in the feces. This metabolic route was confirmed by cannulating the common bile duct of animals previously treated with CPZ-MEI. The bile specimen collected in this manner was subjected to paper chromatographic analysis as described previously for urine specimens. A single spot with R_f 0.78 was identified to be the unchanged CPZ-MEI.

Apparently the bile excretion is the major source of radioactivity in the intestines. This is evidenced by the fact that when the common bile dust was ligatured, only a trace of radioactivity was detected in the intestines.

Except for the intestines, the liver and the kidneys are the major organs representing most of the activity. However, the specific activity in the spleen was considerably higher than in other organs.

The methyl groups attached to the terminal nitrogen are quite stable, as evidenced by the negative finding of the radioactivity in the expired air of the animals pretreated with the quaternary compounds. Chromatographic analyses indicated that the majority of the urinary and fecal metabolites was the unchanged drug, except for a metabolite of CPZ-MEI which was identified to be the sulfoxide of CPZ-MEI.

SUMMARY

1. The i.p. administered ^{14}C-methiodides of the three structurally related phenothiazine derivatives—promazine, chlorpromazine, and triflupromazine—were well absorbed by the rats.

2. Promazine methiodide showed higher blood and brain levels than the 2-chloro (chlorpromazine) and 2-trifluoromethyl (triflupromazine) substituted analogs.

3. Liver was the major organ which metabolized the drug and eliminated it to the intestines through biliary excretion.

4. The majority (51–55%) of the administered radioactivity was recovered in the feces, and urinary excretion represented 8–30% of the administered activity.

5. The methyl groups attached to the terminal nitrogen were stable and not demethylated in the metabolic process. No radioactivity was detected in the carbon dioxide collected from the expired air of the animals.

6. Paper chromatography revealed that chlorpromazine methiodide was metabolized to its sulfoxide, while promazine methiodide and triflupromazine methiodide were excreted unchanged.

REFERENCES

(1) L. Albanus, E. Hansson, and C. G. Schmitterlöw, *Acta Pharmacol. Toxicol.*, **18**, 105(1961).

(2) A. Hanngren, *ibid.*, **21**, 116(1964).

(3) R. M. Levine, M. R. Blair, and B. B. Clark, *J. Pharmacol. Exp. Ther.*, **114**, 78(1955).

(4) R. M. Levine and E. M. Pelikan, *ibid.*, **131**, 319(1961).

(5) R. M. Levine, *Pharmacologist*, **3**, 67(1961).

(6) E. Hansson and C. G. Schmitterlöw, *Arch. Int. Pharmacodyn. Ther.*, **131**, 309(1961).

(7) L. G. Allgen, L. Ekman, L. Reio, and S. Ullberg, *ibid.*, **126**, 1(1960).

(8) C. L. Huang and J. Z. Yeh, to be published.

(9) C. L. Huang, G. M. Mir, and J. Z. Yeh, reported at 115th Annual Meeting of American Pharmaceutical Association, Miami meeting, May 1968.

(10) C. L. Huang, *Int. J. Neuropharmacol.*, **6**, 1(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 18, 1969, from the *Department of Pharmacology, School of Pharmacy, University of Mississippi, University, MS 38677*

Accepted for publication February 26, 1970.

This work was supported in part by the Public Health Research Grant MH-11927 from the National Institute of Mental Health, Chevy Chase, Md.

Conformations of *erythro*- and *threo*-Dimethylacetylcholine Iodides in the Solid State

ELI SHEFTER*, PHYLLIS SACKMAN*, WILLIAM F. STEPHEN, Jr.†, and EDWARD E. SMISSMAN†

Abstract □ The structures of *erythro*- and *threo*-α,β-dimethylacetylcholine iodides were determined by X-ray crystallographic procedures. The two molecules have substantially different conformations. The conformation of the *threo*-compound appeared to be dominated by coulombic attraction between the carbonyl oxygen and the quaternary nitrogen group, while in the *erythro*-analog the acyloxy oxygen atom was involved in a similar intramolecular interaction.

Keyphrases □ *erythro*-α,β-Dimethylacetylcholine iodides—structure determinations □ *threo*-α,β-Dimethylacetylcholine iodides—structure determination □ Conformation, structural—*erythro*- and *threo*-α,β-dimethylacetylcholine □ X-ray crystallography—structure determination

Substitution of methyl groups on the α- and/or β-carbons of the acetylcholine molecule (ACh) has dramatic effects on both the muscarinic activity of the analog and the hydrolysis rate of the molecule in the presence of acetylcholinesterase (AChE) (1, 2). Pharmacological studies (2) on the *erythro*(±)- and *threo*(±)-α,β-dimethylacetylcholine compounds indicated that the racemic *erythro*-material is over 300 times more potent as a muscarinic agent than the racemic *threo*-compound. However, *erythro*(±)-dimethylacetylcholine has approximately one-tenth the activity of ACh. When relative rates of hydrolysis by AChE of the two molecules are compared, a reverse situation is found, *i.e.*, the *threo*(±)-material is hydrolyzed at approximately one-tenth the rate of ACh and the *erythro*(±)-analog is negligibly hydrolyzed and possibly acts as an antagonist. In consideration of these results, it was deemed worthwhile to carry out structural studies on these molecules to learn to what extent their electronic and steric features might account for their observed properties. The crystal structures of the *erythro*- and *threo*-compounds are reported in this article.

EXPERIMENTAL

The iodide salts of the racemic mixtures of the two compounds crystallized as prisms from ethanol–ether (for *erythro*-material) and ethanol–benzene (for *threo*-compound) solutions. The individual *threo*-crystals were found to be optically active, and the crystal chosen for the X-ray study was found to contain the α(R)β(R) enantiomer. The crystal data for these compounds are:

erythro-		*threo*-
7.166 (2) Å	*a*	7.592 (3) Å
14.715 (5) Å	*b*	13.229 (4) Å
11.802 (3) Å	*c*	13.322 (3) Å
99.38 (3)°	β	
1.61 g./cm.^{3}	Density meas.	1.54 g./cm.^{3}
1.617 g./cm.^{3}	Density calcd.	1.495 g./cm.^{3}
$P\,2_1/c$	Space group	$P\,2_12_12_1$

The methyl groups attached to the terminal nitrogen are quite stable, as evidenced by the negative finding of the radioactivity in the expired air of the animals pretreated with the quaternary compounds. Chromatographic analyses indicated that the majority of the urinary and fecal metabolites was the unchanged drug, except for a metabolite of CPZ-MEI which was identified to be the sulfoxide of CPZ-MEI.

SUMMARY

1. The i.p. administered ^{14}C-methiodides of the three structurally related phenothiazine derivatives—promazine, chlorpromazine, and triflupromazine—were well absorbed by the rats.

2. Promazine methiodide showed higher blood and brain levels than the 2-chloro (chlorpromazine) and 2-trifluoromethyl (triflupromazine) substituted analogs.

3. Liver was the major organ which metabolized the drug and eliminated it to the intestines through biliary excretion.

4. The majority (51–55%) of the administered radioactivity was recovered in the feces, and urinary excretion represented 8–30% of the administered activity.

5. The methyl groups attached to the terminal nitrogen were stable and not demethylated in the metabolic process. No radioactivity was detected in the carbon dioxide collected from the expired air of the animals.

6. Paper chromatography revealed that chlorpromazine methiodide was metabolized to its sulfoxide, while promazine methiodide and triflupromazine methiodide were excreted unchanged.

REFERENCES

(1) L. Albanus, E. Hansson, and C. G. Schmitterlöw, *Acta Pharmacol. Toxicol.*, **18**, 105(1961).

(2) A. Hanngren, *ibid.*, **21**, 116(1964).

(3) R. M. Levine, M. R. Blair, and B. B. Clark, *J. Pharmacol. Exp. Ther.*, **114**, 78(1955).

(4) R. M. Levine and E. M. Pelikan, *ibid.*, **131**, 319(1961).

(5) R. M. Levine, *Pharmacologist*, **3**, 67(1961).

(6) E. Hansson and C. G. Schmitterlöw, *Arch. Int. Pharmacodyn. Ther.*, **131**, 309(1961).

(7) L. G. Allgen, L. Ekman, L. Reio, and S. Ullberg, *ibid.*, **126**, 1(1960).

(8) C. L. Huang and J. Z. Yeh, to be published.

(9) C. L. Huang, G. M. Mir, and J. Z. Yeh, reported at 115th Annual Meeting of American Pharmaceutical Association, Miami meeting, May 1968.

(10) C. L. Huang, *Int. J. Neuropharmacol.*, **6**, 1(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 18, 1969, from the *Department of Pharmacology, School of Pharmacy, University of Mississippi, University, MS 38677*

Accepted for publication February 26, 1970.

This work was supported in part by the Public Health Research Grant MH-11927 from the National Institute of Mental Health, Chevy Chase, Md.

Conformations of *erythro*- and *threo*-Dimethylacetylcholine Iodides in the Solid State

ELI SHEFTER*, PHYLLIS SACKMAN*, WILLIAM F. STEPHEN, Jr.†, and EDWARD E. SMISSMAN†

Abstract □ The structures of *erythro*- and *threo*-α,β-dimethylacetylcholine iodides were determined by X-ray crystallographic procedures. The two molecules have substantially different conformations. The conformation of the *threo*-compound appeared to be dominated by coulombic attraction between the carbonyl oxygen and the quaternary nitrogen group, while in the *erythro*-analog the acyloxy oxygen atom was involved in a similar intramolecular interaction.

Keyphrases □ *erythro*-α,β-Dimethylacetylcholine iodides—structure determinations □ *threo*-α,β-Dimethylacetylcholine iodides—structure determination □ Conformation, structural—*erythro*- and *threo*-α,β-dimethylacetylcholine □ X-ray crystallography—structure determination

Substitution of methyl groups on the α- and/or β-carbons of the acetylcholine molecule (ACh) has dramatic effects on both the muscarinic activity of the analog and the hydrolysis rate of the molecule in the presence of acetylcholinesterase (AChE) (1, 2). Pharmacological studies (2) on the *erythro*(±)- and *threo*(±)-α,β-dimethylacetylcholine compounds indicated that the racemic *erythro*-material is over 300 times more potent as a muscarinic agent than the racemic *threo*-compound. However, *erythro*(±)-dimethylacetylcholine has approximately one-tenth the activity of ACh. When relative rates of hydrolysis by AChE of the two molecules are compared, a reverse situation is found, *i.e.*, the *threo*(±)-material is hydrolyzed at approximately one-tenth the rate of ACh and the *erythro*(±)-analog is negligibly hydrolyzed and possibly acts as an antagonist. In consideration of these results, it was deemed worthwhile to carry out structural studies on these molecules to learn to what extent their electronic and steric features might account for their observed properties. The crystal structures of the *erythro*- and *threo*-compounds are reported in this article.

EXPERIMENTAL

The iodide salts of the racemic mixtures of the two compounds crystallized as prisms from ethanol–ether (for *erythro*-material) and ethanol–benzene (for *threo*-compound) solutions. The individual *threo*-crystals were found to be optically active, and the crystal chosen for the X-ray study was found to contain the α(R)β(R) enantiomer. The crystal data for these compounds are:

erythro-		*threo*-
7.166 (2) Å	*a*	7.592 (3) Å
14.715 (5) Å	*b*	13.229 (4) Å
11.802 (3) Å	*c*	13.322 (3) Å
99.38 (3)°	β	
1.61 g./cm.^{3}	Density meas.	1.54 g./cm.^{3}
1.617 g./cm.^{3}	Density calcd.	1.495 g./cm.^{3}
$P\,2_1/c$	Space group	$P\,2_12_12_1$

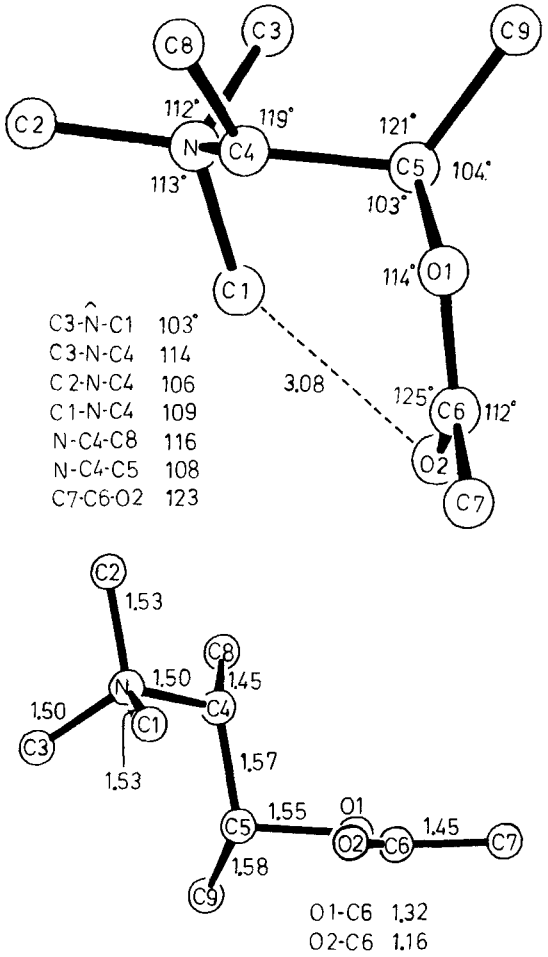


Figure 1—*General views of the* threo-α(*R*)β(*R*)-*dimethylacetylcholine molecule showing intramolecular bond distances and angles.*

Intensity data[1] were collected by the stationary counterstationary crystal technique (3) using balanced filters for Cu Kα radiation (Ni *versus* Co). The intensities of 795 out of 826 unique data for the *threo*-compound and 1140 out of the 1267 independent data for the *erythro*-crystals in the 2 θ-range of 0–100° had intensities significantly greater than their respective background counts. Corrections were applied to the data for α_1-α_2 splitting, Lorentz-polarization factors, and absorption. The absorption correction was an approximate one, based on the anisotropy of transmission of the X-ray beam for a reflection at $\chi = 90°$ for each crystal. The absorption (μ-linear absorption coefficients ~200 cm.$^{-1}$) by these crystals was the greatest source of error in the data.

The structures were obtained by the "heavy atom" technique (4) and refined by least squares, using a block diagonal approximation to the normal equations. During the refinement of the *threo*-structure, the absolute configuration of the enantiomorph in the crystal was derived by considering the agreement between the observed and calculated structure factors (calculations included the anomalous scattering term for iodine) for the α(S)β(S) and α(R)β(R) structures.

The R values for the refined structures are 0.096 and 0.132 for the observed data of the *erythro*- and *threo*-structures, respectively.[2] Weighting schemes were chosen in both structures such that the average weighted difference squared was approximately constant over the whole range of observed structure factors, and the "unobserved" data were given zero weight. The positional and thermal parameters with their respective standard deviations are presented in Table I. No attempt was made to locate the hydrogen positions in these structures.

Table I—Positional and Thermal Parameters and Their Estimated Standard Deviations in Parentheses

Atom	$x/a \cdot 10^4$	$y/b \cdot 10^4$	$x/c \cdot 10^4$	B
		erythro-		
I	−3779(1)	1397(1)	1252(1)	a[a]
C1	1497(21)	2862(11)	1566(15)	2.3(0.3) Å^2
C2	3946(25)	3885(13)	2613(15)	3.0(0.3)
C3	3152(24)	2470(12)	3479(15)	2.8(0.3)
C4	768(20)	3767(10)	3283(13)	1.3(0.2)
C5	−188(18)	4520(9)	2617(11)	1.0(0.2)
C6	−2094(21)	4575(11)	733(13)	2.1(0.3)
C7	−3269(23)	4035(12)	−143(14)	2.8(0.3)
C8	−596(23)	3057(11)	3684(13)	2.5(0.3)
C9	−1188(22)	5139(13)	3382(16)	3.2(0.3)
N	2277(14)	3246(8)	2734(9)	0.9(0.2)
O1	−1535(12)	4105(6)	1707(8)	1.4(0.2)
O2	−1618(15)	5384(8)	625(10)	3.2(0.2)
		threo-		
I	−1144(2)	1559(1)	−1994(1)	b[a]
C1	−3203(35)	1021(16)	895(18)	4.9(0.4) Å^2
C2	−2217(39)	1467(18)	2667(25)	5.9(0.5)
C3	−813(29)	2152(19)	1109(23)	6.3(0.6)
C4	−480(28)	330(16)	1637(18)	3.9(0.4)
C5	−199(27)	5105(12)	4454(16)	3.3(0.4)
C6	−1209(33)	8529(16)	102(23)	5.3(0.5)
C7	−1177(49)	7436(33)	165(37)	8.9(0.9)
C8	−765(39)	5301(24)	2543(28)	7.0(0.6)
C9	−2128(46)	5416(23)	4795(25)	7.3(0.7)
N	−1650(28)	1250(13)	1588(16)	4.8(0.4)
O1	−225(23)	3935(11)	4502(12)	4.7(0.3)
O2	−2552(22)	1019(11)	4862(11)	4.6(0.3)

[a] These atoms (a, b) were refined anisotropically; final values of the coefficients (× 10^4) with their *ESD*'s were:

	B_{11}	B_{12}	B_{33}	B_{12}	B_{13}	B_{23}
a-	124 (4)	21 (1)	22 (1)	21 (2)	−38 (4)	13 (1)
b-	271 (4)	107 (1)	33 (2)	−66 (3)	−7 (3)	−8 (4)

RESULTS AND DISCUSSION

The estimated deviations in the bond distances and angles are on the average 0.04 Å and 2° for the bonding parameters in the two structures. The intramolecular bond lengths and angles are shown in Figs. 1 and 2 for the two compounds. In general, these

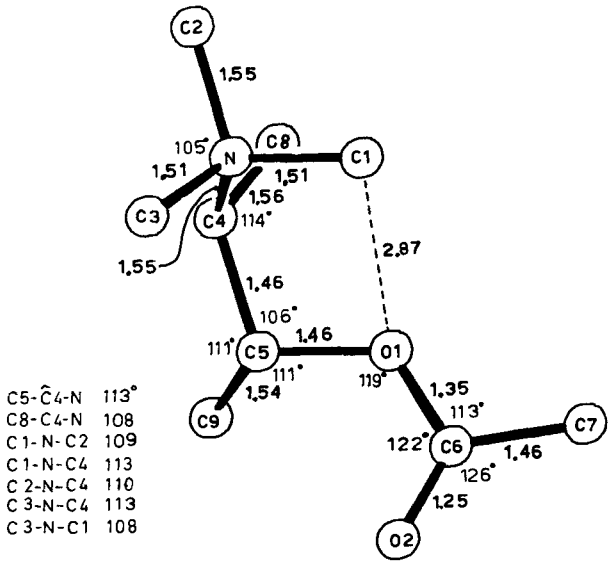


Figure 2—*View of the* erythro-α(*S*)β(*R*)-*dimethylacetylcholine structure with intramolecular bonding parameters noted.*

[1] General Electric XRD-6 diffractometer used for all measurements.
[2] A list of the observed and calculated structure factors is in the Health Sciences Library of the State University of New York at Buffalo, and a copy may be obtained from the librarian.

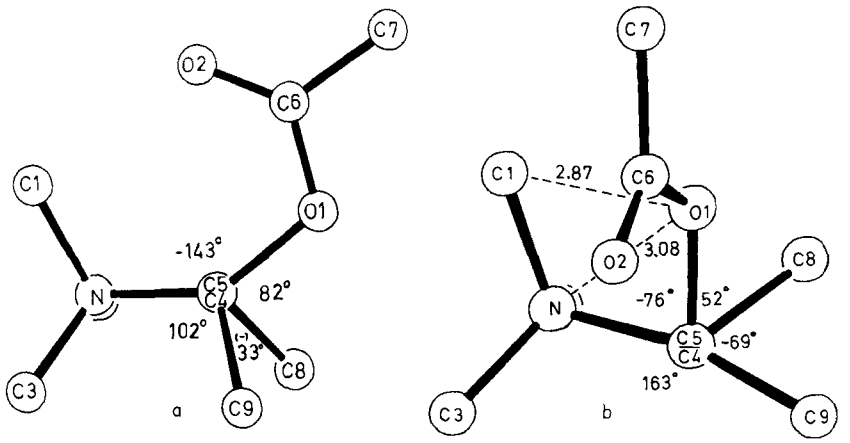


Figure 3—*Conformations of the* threo-$\alpha(R)\beta(R)$ *(a) and* erythro-$\alpha(S)\beta(R)$ *(b) molecules about the C4–C5 bond.*

parameters are similar to one another (*i.e.*, the differences are not *highly* significant) and are in substantially good agreement with comparable bonds in other structures (5–7). There is, however, a substantial distortion of the angles about C4 and C5 of the *threo*-compound away from tetrahedral symmetry. This angular distortion is an inexpensive way of relieving the overcrowding of the C8 and C9 methyl groups in this structure. The distance between the α- and β-methyl groups is quite similar in the two structures (3.18 Å in the *threo*-structure and 3.11 Å in the *erythro*-molecule), even though the C8–C4–C5–C9 torsion angles are markedly different. In both structures, there is also a slight distortion (not *highly* significant) of the angles about C4, possibly resulting from nonbonded repulsions between the quarternary methyl groups and the α-methyl group. This effect can also be seen in Fig. 3.

The spatial disposition of the nonhydrogen atoms of an ACh analog can be described in terms of three torsion angles, herein called ϕ_1, ϕ_2, and ϕ_3 for the N–C4–C5–O1, C4–C5–O1–C6, and C5–O1–C6–C7 groupings of atoms, respectively.[3] These angles are defined in accordance with the Klyne and Prelog (8) rules; for example, the torsion angle for the N–C4–C5–O1 system is the angle between the N–C4 and C5–O1 bonds as viewed down the C4–C5 bond, with a positive value when measured clockwise and a negative value when measured counterclockwise. The values of ϕ_1, ϕ_2, and ϕ_3 of these two molecules are compared with those found for some related molecules in Table II.

From molecular models[4] of the *threo*- and *erythro*-compounds, it is observed that the size of the substituents on the choline residue limits the number of conformations that the two molecules may take up without substantial molecular distortions taking place. Models indicate that the restrictions on ϕ_2 and ϕ_3 are not nearly as great as that on ϕ_1, but the presence of the β-methyl group will sterically prevent free rotation of ϕ_2. The ϕ_3 angles of various analogs of ACh (see Table II) are all relatively close to 180°, this value being the preferred conformation for the concerned atoms in primary esters (10).

A model of the $\alpha(S)\beta(R)$ enantiomer of the *erythro*-compound indicates that ϕ_1 values at approximately $-$*gauche* and $-$*trans*[5] would be sterically favored over other possible rotamers. Repulsions between the β-methyl and the acyloxy oxygen atom and with the cationic methyls would make the *trans*-conformation less favorable. The $\alpha(R)\beta(S)$ minima would be at $+$*gauche* and $+$*trans*. For the *threo*-$\alpha(R)\beta(R)$ stereoisomer, models suggest that minima can be placed at approximately $-$*gauche* and $-$*trans*. The *threo*-$\alpha(S)\beta(S)$ molecule would have similar angular minima but with their signs reversed. A very small amount of angular distortion would result in both compounds when ϕ_1 is 180° due to nonbonded repulsion between the β-methyl and quaternary nitrogen methyls; energy needed to overcome this should be small.

An estimate of the relative rotational barrier for the interconversion of the *gauche-trans*-conformations for the two diastereoisomers is possible from molecular models. This barrier for the *threo*-enantiomers apparently is not very much greater than that for ACh. However, it was only possible to transform the $-$*gauche*-form of the erythro-$\alpha(S)\beta(R)$ enantiomorph to the *trans*-conformation with extensive strains in the bonding parameters, suggesting a large energy barrier due to steric factors. Thus, unless some of the *trans*-rotamer was formed in the synthetic process, a very limited concentration of this conformation would be expected in solution.

In the solid-state structures of these materials, only one conformer is found for each molecule. The value of ϕ_1 found for each molecule suggests that the energy difference between the possible rotamers (based on steric considerations) is influenced greatly by intramolecular attractive forces. These may be described as coulombic attraction between the acidic quaternary nitrogen grouping and the basic ester linkage (11). The *threo*-compound's preference for the $-143°$ conformation over the *gauche* one in this structure may result from crystal forces, such as intermolecular Van der Waals' contacts. A 180° rotamer assumes that no intramolecular attraction exists between the quaternary nitrogen group and the basic oxygens.

Although the hydrogens were not located in these structures, molecular models point to the possibility of further stabilization of the two conformations by N—C—H···O interactions. The latter type of "hydrogen bonds" have been implied as stabilizing factors for ACh molecules in both the solid state and solution (6, 12).

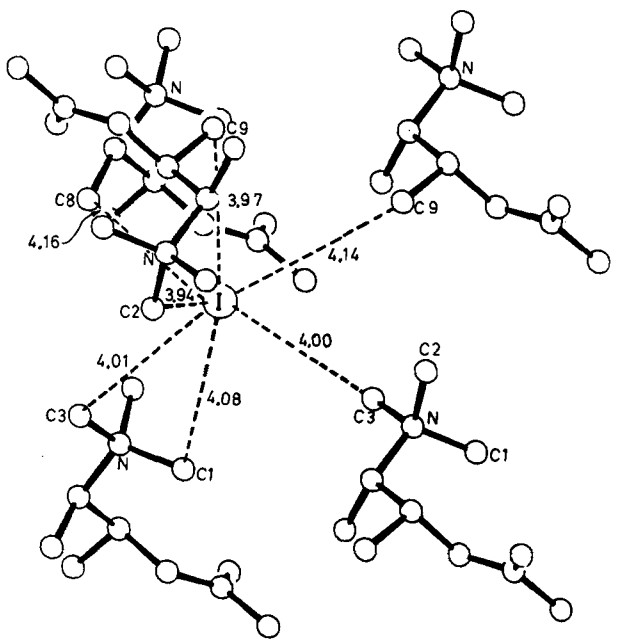


Figure 4—*Packing arrangement about the iodine in the* erythro(±)-*dimethylacetylcholine structure with shortest contacts noted.*

[3] The quaternary nitrogen methyls are in a staggered pattern relative to C4 and C5. This sterically favored arrangement is typical of quaternary nitrogen compounds (6, 7, and 9) and is not felt to be pertinent to the present discussion.

[4] C.P.K. atomic models, Ealing Corp., Cambridge, Mass.

[5] For purposes of simplicity in describing the conformations of ϕ_1, *gauche* and *trans* are used to refer to angles within the range of 60–90° and 140–180°, respectively. The prefixed sign indicates the preference for a particular configuration.

Table II—Conformation Angles and Relative Hydrolytic Rates in the Presence of Acetylcholinesterase of Some ACh Analogs

Compound	ϕ_1	ϕ_2	ϕ_3	Relative Rate[a]	Ref.
ACh Br	77°	79°	167°	100	6
Acetylthiol choline Br	171°	129°	150°	100	11, 14
Acetylselenolcholine I	175°	123°	155°	100[b]	13, 14
erythro-α(R)β(S)-Dimethyl ACh I	76°	−155°	173°	0[c]	2
threo-α(R)β(R)-Dimethyl ACh I	−143°	95°	−175°	10[c]	2
L(+)-β(S)-Methyl ACh I	85°	−147°	175°	54 (46[c])	1, 15
D(+)-α(R)-Methyl ACh I	90°	170°	175°	78 (92[c])	1, 16
	−148°	176°	177°		

[a] Relative to ACh. [b] Rate-determining step different from that in ACh mechanism (14). [c] For racemic mixture.

Further studies are presently being carried out in these laboratories to learn about the conformations of these molecules in solution and the solid state to assess the relative strengths of the intramolecular forces.

No intermolecular contacts in either structure are significantly shorter than the sum of the Van der Waals radii of the atoms or groups involved. The arrangements of molecules about the iodine atoms in these structures are typical of that found in analogous quaternary nitrogen structures (9, 13). The packing arrangement of *erythro*-α,β-dimethyl ACh molecules about iodine is shown in Fig. 4.

Even though definitive evidence is lacking as to the conformation of ACh analogs in biological systems and at the active site of AChE, it is tempting to speculate on the nature of the substrate–enzyme complex from presently available crystallographic, chemical, and biological data. Pauling has made two such attempts, first suggesting (17) that ϕ_1 may be +60° for the ACh molecule bound to the enzyme and more recently that +150° appears to give "optimum" hydrolytic conditions (18). To make these speculations, it was assumed that only one conformation of the substrate is relevant to the esterase for hydrolysis. However, the data in Table II together with experimental data on conformationally constrained ACh analogs (2, 19, 20) suggest that substrates having ϕ_1 values other than +150° are capable of being hydrolyzed by AChE. Along these lines, recent studies on the muscarinic "receptor" (21) and AChE (22) imply a dual mode of substrate binding, a common anionic site which is flanked by two bonding loci for polar and nonpolar side chains of the quaternary trimethylammonium ligands.

The differences in relative rates of the various compounds in Table II suggest that factors such as the electronic feature of the ester linkage and steric repulsion between the methyl groups of the choline moiety and the enzyme surface are probably as important as the ϕ_1 angle in influencing the kinetic processes involved in AChE hydrolysis and cholinergic activity. The importance of the electronic nature of the ester linkage on this rate process has recently been discussed (11). Although it is tempting to speculate on specifics relating to the enzyme–substrate complex and to structural details of each kinetic step for ACh analogs in general, more structural and kinetic data are needed before a highly probable postulate can be put forth which would account for all the factors.

REFERENCES

(1) A. H. Beckett, N. J. Harper, and J. W. Clitherow, *J. Pharm. Pharmacol.*, **15**, 349(1967).

(2) E. E. Smissman, W. L. Nelson, J. B. LaPidus, and J. L. Day, *J. Med. Chem.*, **9**, 458(1966).

(3) T. C. Furnas and D. Harker, *Rev. Sci. Instrum.*, **26**, 449 (1955).

(4) J. M. Robertson and I. Woodward, *J. Chem. Soc.*, **1937**, 219.

(5) E. Shefter, H. G. Mautner, and E. E. Smissman, *Acta Crystallogr., Sect. A*, **25**, S201(1969).

(6) F. G. Canepa, P. Pauling, and H. Sorum, *Nature*, **210**, 907(1966).

(7) C. Chothia and P. Pauling, *ibid.*, **219**, 1156(1968).

(8) W. Klyne and V. Prelog, *Experientia*, **16**, 521(1960).

(9) K. Lonsdale, H. J. Milledge, and L. M. Pant, *Acta Crystallogr.*, **19**, 827(1965).

(10) J. D. Dunitz and P. Strickler, in "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Eds., W. H. Freeman, San Francisco, Calif., 1968, p. 601.

(11) E. Shefter and H. G. Mautner, *Proc. Natl. Acad. Sci.*, **63**, 1253(1969).

(12) M. Martin-Smith, G. A. Smail, and J. B. Stenlake, *J. Pharm. Pharmacol.*, **19**, 649(1967).

(13) E. Shefter and O. Kennard, *Science*, **153**, 1389(1966).

(14) G. R. Hillman and H. G. Mautner, unpublished data.

(15) C. Chothia and P. Pauling, *Chem. Commun.*, **30**, 626(1969).

(16) *Ibid.*, **30**, 746(1969).

(17) P. Pauling, in "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Eds., W. H. Freeman, San Francisco, Calif., 1968, p. 555.

(18) C. Chothia and P. Pauling, *Nature*, **223**, 919(1969).

(19) C. Y. Chiou, J. P. Long, J. G. Cannon, and P. D. Armstrong, *J. Pharmacol. Exp. Ther.*, **166**, 243(1969).

(20) J. B. Robinson, B. Belleau, and B. Cox, *J. Med. Chem.*, **12**, 848(1969).

(21) J. F. Moran and D. J. Triggle, in "Fundamental Concepts in Drug Receptor Interactors," J. Danielli, J. F. Moran, and D. J. Triggle, Eds., Academic, New York, N. Y., 1970, p. 133.

(22) B. Belleau, in "Fundamental Concepts in Drug Receptor Interactions," J. Danielli, J. F. Moran, and D. J. Triggle, Eds., Academic, New York, N. Y., 1970, p. 121.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 31, 1969, from the **School of Pharmacy, Department of Pharmaceutics, State University of New York at Buffalo, Buffalo, NY 14214*, and the *†School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication March 12, 1970.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This work was supported in part by Public Health Service Grants to E.S. (CA-10104) and to W.F.S. and E.E.S. (CA-33233, GM-9254).

The authors thank the Computing Center of the State University of New York at Buffalo for use of their facilities (which are supported in part by Grants NSF-GP-7318 and PHS-P07FR0012605).

Note: A neutron diffraction study was very recently completed by T. F. Brennan, F. Ross, W. C. Hamilton, and E. Shefter (unpublished) on the *erythro*-α,β-dimethylacetylcholine iodide crystals. The results conclusively show that there is no intramolecular C—H . . . O hydrogen interactions.

Interaction of Ascorbic Acid with Silicic Acid

WEN-HUNG WU, TING-FONG CHIN, and JOHN L. LACH

Abstract □ Interaction of ascorbic acid with silicic acid in the solid state was studied using diffuse reflectance spectroscopic techniques. Surface degradation products from ascorbic acid breakdown were effectively observed using diffuse reflectance spectroscopy, whereas the corresponding transmittance spectral method did not give significant information concerning degradation products. Adsorption of ascorbic acid from methanol solution by silicic acid did not occur. However, after evaporation of the methanol, ascorbic acid did undergo strong interaction with silicic acid, probably through hydrogen bonding. Thermal degradation of ascorbic acid in the adsorbed state was found to be different from that in solution. In acid solution, furfural was a major degradation product; whereas in the adsorbed state, furfural could not be detected.

Keyphrases □ Ascorbic acid–silicic acid—solid-state interaction □ Silicic acid–adsorbed ascorbic acid—stability □ Thermal stability—ascorbic acid □ TLC—analysis □ Diffuse reflectance spectroscopy—analysis

Silicic acid, an almost completely inert adsorbent, has found considerable use as a support in chromatography and as a carrier material for some oil-soluble vitamins and other medicinal agents. The surface of silicic acid consists of a plane of exposed silicon atoms to which are attached covalent surface hydroxyl groups, or silanols, the only important adsorption sites. The adsorbed molecules interact with silanols by hydrogen bonding, in which the adsorbed molecule normally acts as an electron donor (1). Gueyne and Duffaut (2) reported complex formation between an alkaline silicate and organic acids, including ascorbic acid. Strohecker and Henning (3) stated that it is not suitable to employ silica gel plates for quantitative assay of ascorbic acid, since too much is lost on the silica gel. Since a loss of ascorbic acid has also been reported to occur when ascorbic acid is present in a vitamin preparation containing oil-soluble vitamins which have been adsorbed onto silicic acid, this study was undertaken to obtain information concerning the nature of this loss. This study also represents a continuing program in the investigation of drug–excipient interactions.

EXPERIMENTAL

Materials—Ascorbic acid (USP reference standard);[1] L-ascorbic acid, reagent grade, m.p. 190–193°;[2] silicic acid, average particle diameter, 3 μ;[3] dehydroascorbic acid;[4] levulinic acid;[5] furfural;[6] 2,6-dichlorophenolindophenol sodium;[7] and 2-diphenylacetyl-1,3-indandione-1-hydrazone, m.p. 241°,[8] were used.

Apparatus and Methods—All diffuse reflectance spectra (DRS) were taken according to the method of Lach and Bornstein (4) by using a spectrophotometer equipped with reflectance attachment.[9]

Determination of Ascorbic Acid—This was performed by the dye method described in USP XVII (5).

TLC for Identification of Degradation Products of Ascorbic Acid—Silica gel G plates (0.25 mm.) and a solvent system of chloroform–*n*-heptane (1:8) were used. Ten grams of darkened sample containing silicic acid, ascorbic acid, and its degradation products was continuously extracted in a continuous extraction apparatus with anhydrous ether for 24 hr. The ethereal extract was concentrated to 1 ml. and then reacted with 2-diphenylacetyl-1,3-indandione-1-hydrazone (DIH) in a mixture of methanol and chloroform using 1 drop hydrochloric acid as a catalyst. The solution was spotted on a plate together with chloroform solutions of a reaction product of furfural and DIH (F-DIH), levulinic acid and DIH (L-DIH), and DIH itself. After development, the plates were examined under UV light. Since DIH shows a bright-yellow fluorescence under UV light,

Table I—Thermal Degradation of Pure Ascorbic Acid at 100°

Time, hr.	Ascorbic Acid Found, mg.	% Degradation	Color Change
0	100	0	White
2	100	0	White
9	99.4	0.6	White
24	99.4	0.6	Slightly yellow
53.5	99.8	0.2	Yellow
140	99.5	0.5	Grey
192	99.4	0.6	Grey

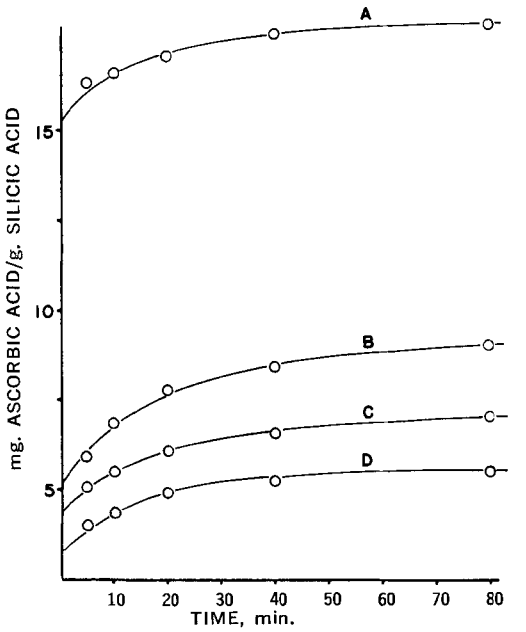


Figure 1—*Desorption and degradation of ascorbic acid (20 mg.) adsorbed on silicic acid (1 g.). Key: A, without heating; B, heated at 100° for 9.5 hr.; C, heated at 100° for 20.5 hr.; and D, heated at 100° for 40.0 hr.*

[1] U. S. Pharmacopeial Convention, Inc.
[2] Fisher Scientific Co.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Pierce Chemical Co., Rockford, Ill.
[5] Eastman Organic Chemicals, Rochester, N. Y.
[6] Fisher Scientific Co.
[7] K & K Laboratory, Inc.
[8] Aldrich Chemical Co., Milwaukee, Wis.

[9] Beckman model DB-G and Beckman reflectance attachment.

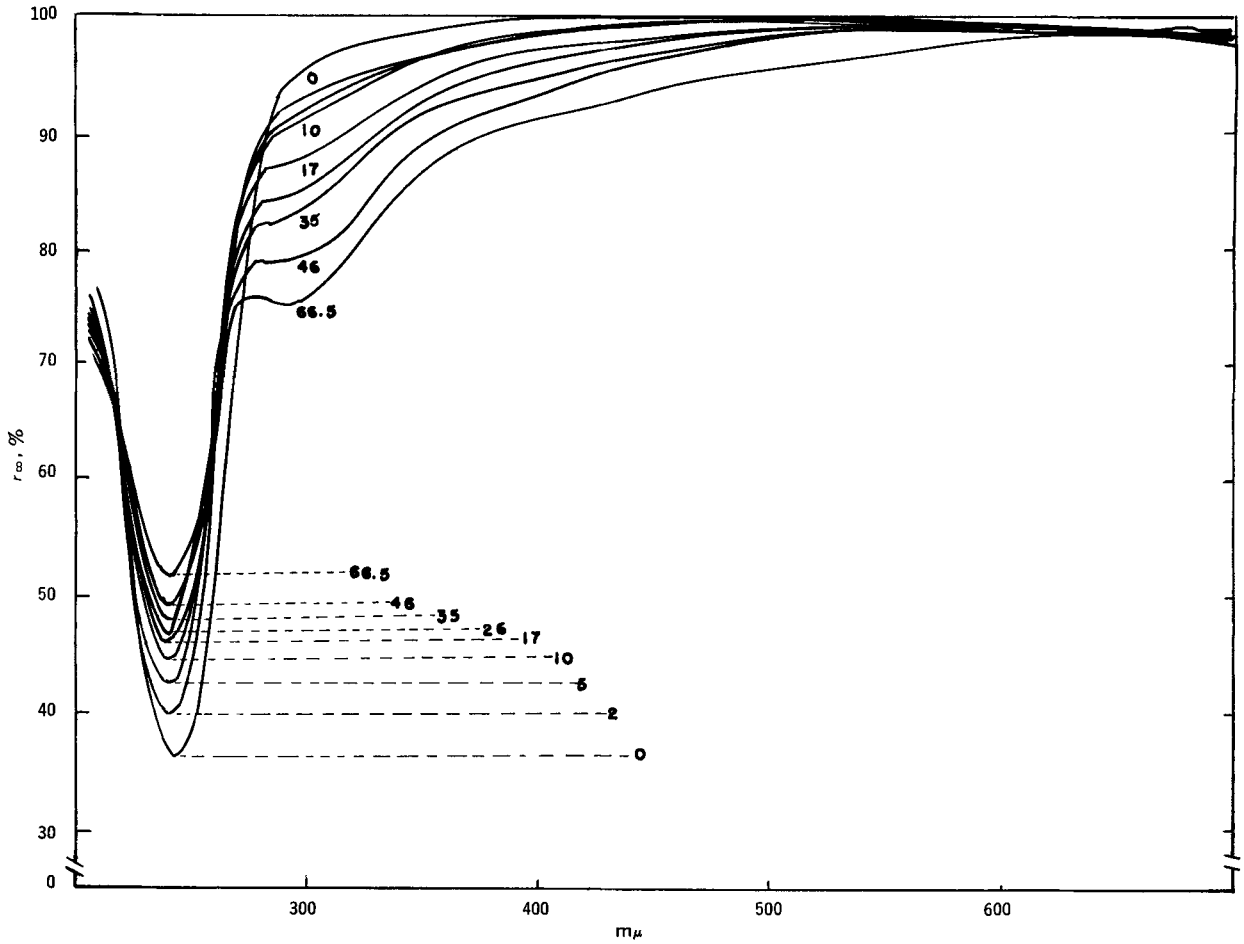


Figure 2—*Diffuse reflectance spectra showing the effects of heating ascorbic acid–silicic acid system at 100°. (The numbers shown are time in hours.)*

all spots containing the DIH moiety appeared as bright spots in the violet background.

RESULTS AND DISCUSSION

Stability of ascorbic acid in the solid state was first studied by heating 100 mg. each of finely powdered ascorbic acid in a number of 50-ml. beakers in a dry oven at 100 ± 0.5°. Quantitative determination of these solid samples was followed at varying time intervals, and the results are shown in Table I. The data in this table indicate that there is no apparent relationship between degradation of ascorbic acid and color change. It is probable that only the surface layer of each ascorbic acid particle was decomposed and that this degradation product(s) could act as a protecting film so that no further decomposition was observed within the time interval studied. Thermal stability of ascorbic acid has been previously studied by several workers (6–8), and their results indicated that pure ascorbic acid is thermally stable at elevated temperatures.

Since silicic acid is known to adsorb various compounds, adsorption of ascorbic acid by silicic acid in methanol solution was undertaken. Ten milligrams of ascorbic acid in 10 ml. methanol was equilibrated with varying quantities of silicic acid for 18 hr. under nitrogen; after centrifugation, the concentration of ascorbic acid in the clear supernatant liquid was determined, and the results are shown in Table II. Since the amount of ascorbic acid that remained in solution phase was approximately constant, it may be concluded that the adsorption of ascorbic acid by silicic acid from the methanol solution was negligible.

However, after complete evaporation of methanol in such equilibrated systems, ascorbic acid was strongly adsorbed to silicic acid since this acid was only very slowly removed from the adsorbent surface by metaphosphoric–acetic acids T.S. This is shown in Fig. 1, Curve A, in which 20 mg. of ascorbic acid/g. of silicic acid was equilibrated in methanol under nitrogen and the solvent evaporated at room temperature under vacuum. Accurately weighed portions of this dried sample were then titrated under nitrogen or after stirring at constant speed for a specified time. As is seen in Fig. 1, approximately 80 min. was required for desorption to occur. These results indicate that ascorbic acid is adsorbed to silicic acid on removal of the methanol and that this adsorption probably occurs through hydrogen bonding between the silanols on the silicic acid and the carbonyl oxygen of ascorbic acid. The lack of quantitative desorption of ascorbic acid as seen in Fig. 1, Curve A, may be attributed to its oxidation in the adsorbed state and will be discussed later.

Stability aspects of ascorbic acid adsorbed on silicic acid were also carried out. Portions of the methanol-equilibrated and vacuum-dried samples were heated at 100 ± 0.5° in a dry oven, and at various time intervals the intact ascorbic acid was determined. The results are shown in Fig. 1, Curves B–D. From this figure and Table

Table II—Adsorption of Ascorbic Acid from Methanol Solution by Silicic Acid

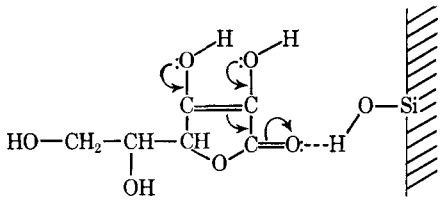
Ascorbic Acid Added, mg.	Silicic Acid Added, g.	Ascorbic Acid Found, mg.[a]
10	0	9.69
10	0.020	9.65
10	0.050	9.65
10	0.100	9.72
10	0.200	9.79
10	0.300	9.79

[a] When 10 mg. of ascorbic acid in 10 ml. methanol was allowed to stand in the dark without tumbling, 9.96 mg. was found after 18 hr.

Table III—Correlation of Reflectance and Degradation of Ascorbic Acid in Adsorbed State

Time for Heating at 100°, hr.	Data from DRS: r_∞ λ245 mμ, %	Data from DRS: $f(r_\infty)$	Ascorbic Acid Found, mg.
0	36.5	0.5524	60
2	40.0	0.4500	56.2
5	42.8	0.3822	—
10	44.9	0.3381	51.9
17	46.2	0.3133	47.8
26	47.0	0.2988	45.0
35	48.1	0.2800	44.1
46	49.2	0.2632	43.9
66.5	51.8	0.2243	42.0

I, it is evident that degradation of ascorbic acid is significantly greater in the adsorbed state than in pure powder form. This accelerated degradation rate may be due simply to a difference in the total surface area, since in this adsorbed state a larger number of ascorbic acid molecules are available for degradation than in the surface of the pure powder. Or it may be due to the fact that in this adsorbed state, as shown in Scheme I, hydrogen bond formation between the carbonyl oxygen of ascorbic acid and the silanol



Scheme I

hydrogen requires a strong bonding of the lone pair electrons on the carbonyl oxygen to the silanol hydrogen. This can induce a general electron shift through the α,β-enone π-orbitals toward this new bond, so that the dissociation of the two hydroxy hydrogens on the

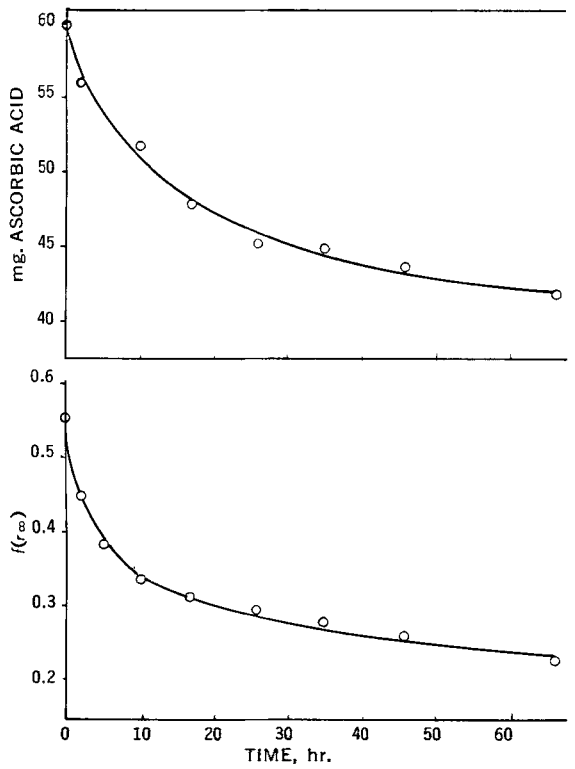


Figure 3—*Correlation of reflectance and degradation of ascorbic acid in adsorbed state.*

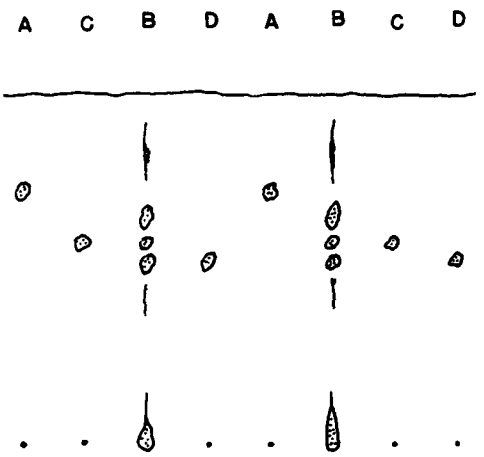


Figure 4—*TLC showing thermal degradation products of ascorbic acid–silicic acid system. Key: A, F-DIH, R_f = 0.71; B, ethereal extract reacted with DIH; C, L-DIH, R_f = 0.58; and D, DIH, R_f = 0.53.*

2- and 3-positions in ascorbic acid is enhanced. This increased dissociation may facilitate the oxidation of ascorbic acid to various carbonyl compounds, presumably through dehydroascorbic acid.

The degradation of pure ascorbic acid in the solid state was also studied using DRS. When a portion of the faintly yellow sample shown in Table I was appropriately diluted with acidified water and the transmittance spectrum taken, the solution showed an absorption maximum at 245 mμ. However, when another portion of the same sample was mixed with silicic acid and the reflectance spectrum taken, two absorption maxima appeared. The one at 245 mμ corresponded to the transmittance spectrum of the acidified ascorbic acid solution. The other, which appeared at 275 mμ, might be a composite maximum due to the various carbonyl compounds produced from the surface degradation of ascorbic acid. Although the quantity of degradation products formed from pure ascorbic acid was small, as shown in Table I, they are mainly concentrated on the surface and are effectively shown in the reflectance spectrum. However, in solution state, they are homogeneously mixed with the intact ascorbic acid molecules, so that the overwhelming amount of ascorbic acid present can sufficiently mask any spectral effects of this small quantity of degradation products. This clearly illustrates the advantage of the DRS method in studying degradation occurring at the surfaces.

When 60 mg. of ascorbic acid was equilibrated with 1 g. of silicic acid in methylene chloride and the dried sample was heated at 100 ± 0.5°, the reflectance spectra taken at varying time intervals showed different intensities; these are given in Fig. 2. As is illustrated in this figure, the intensity of the maximum at 245 mμ gradually decreased with time, whereas a new maximum at about 300 mμ appeared with increasing intensity. This second maximum at 300 mμ is different from that observed in the degradation of pure ascorbic acid, in which case the second maximum appeared at 275 mμ. This suggests that the degradative mechanisms might be different. Along with the spectra, quantitative determinations of ascorbic acid in these equilibrated and heated samples were carried out, and the results are shown in Table III and Fig. 3. For purposes of comparison, reflectance readings, r_∞, at 245 mμ were converted to remission functions, $f(r_\infty)$ since the remission function is directly related to concentration (9). Examination of Fig. 3 does show this remission function concentration dependency which is maintained in the degradated sample, suggesting that quantitized degradation in the solid state can be studied by this technique.

With respect to ascorbic acid it is interesting to note that its degradation products from aqueous solution have been determined by Otani (10) using TLC, and reported to be dehydroascorbic acid, 2,3-diketo-1-gulonic acid, 2-keto-1-gulonic acid, and furfural. Formation of furfural from aqueous solution of ascorbic acid was also discussed by various workers (11–13), the rate of furfural formation being studied in detail by Finholt *et al.* (14).

However, in the present study furfural was not found as part of the degradation products resulting from the breakdown of ascorbic acid in the adsorbed state. Instead, levulinic acid was detected

on thin-layer plates as described in the *Experimental* section. The DIH reagent was used because of its high reactivity toward carbonyl compounds (15, 16). Thermal degradation products of this ascorbic acid–silicic acid system are shown in Fig. 4. Examination of the chromatogram does indicate the absence of furfural, the possible presence of levulinic acid, and, in addition, other unidentified degradation products, and strongly suggests that ascorbic acid in this adsorbed state undergoes a different type of degradation from that in aqueous solution. Although furfural was not detected in this system, it is still possible that any furfural formed could have volatilized from the solid surface or undergone polymerization. It is highly unlikely, however, that levulinic acid results from furfural degradation, since Lamden and Harris (11) pointed out that the formation of furfural in solution degradation does not result from dehydroascorbic acid which, as mentioned before, is probably the first step in the oxidation of ascorbic acid in the adsorbed state. It is, therefore, highly likely that the degradation pathway in the adsorbed state may be quite different from that in solution.

REFERENCES

(1) L. R. Snyder, "Principles of Adsorption Chromatography," Marcel Dekker, New York, N. Y., 1968, p. 160.

(2) J. Gueyne and M. I. Duffaut, Fr. M. 1069, Feb. 19, 1962, Appl. Aug. 31, 1960; 13 pp.; through *Chem. Abstr.*, **59,** 12911c(1963).

(3) R. Strohecker and H. M. Henning, "Vitamin Assay," Verlag Chemil GMBH, Weinheim/Bergstr., 1965, p. 233.

(4) J. L. Lach and M. Bornstein, *J. Pharm. Sci.*, **54,** 730(1965).

(5) "The United States Pharmacopeia," 17th rev., Mack Printing Co., Easton, Pa., 1965.

(6) V. A. Usoltseva, I. G. Chistyakov, and M. D. Nasyrova, *Izv. Vyssh. Ucheb. Zaved., Khim. Khim. Tekhnol.*, **8,** 65(1965); through *Chem. Abstr.*, **63,** 2848f(1965).

(7) C. Duval, *Mikrochim. Ichnoanal. Acta*, **6,** 1073(1964).

(8) J. P. Comer and L. D. Howell, *J. Pharm. Sci.*, **53,** 335(1964).

(9) W. W. Wendlandt and H. G. Hec, "Reflectance Spectroscopy," Interscience, New York, N. Y., 1966, p. 62.

(10) S. Otani, *Yakuzaigaku*, **24,** 293(1964).

(11) M. P. Lamden and R. S. Harris, *Food Res.*, **15,** 79(1950).

(12) I. Tanaka and S. Otani, *Yakuzaigaku*, **22,** 150(1962).

(13) T. Kurata and Y. Sakurai, *Agr. Biol. Chem.*, **31,** 170(1967).

(14) P. Finholt, I. Alsos, and T. Higuchi, *J. Pharm. Sci.*, **54,** 181(1965).

(15) W. A. Mosher, I. S. Bechara, and E. J. Pozomek, *Talanta*, **15,** 482 (1968).

(16) R. A. Braun and W. A. Mosher, *J. Amer. Chem. Soc.*, **80,** 3048(1958).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication March 27, 1970.

This work was supported by Research Grant GM-13178 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Electrostatic Interaction of Oil Droplets with Adsorbed Surface-Active Ions in Dilute Electrolyte Solutions

N. F. H. HO, A. SUZUKI*, and W. I. HIGUCHI

Abstract □ Using the Haydon-Taylor model in which the surfactant head groups are situated at some distance from the oil surface, the interaction energy of electrostatic repulsion (V_R) between oil droplets in dilute electrolyte solutions is derived for the two cases: constant-surface potential and constant-surface charge. For comparison, the repulsive energy is also derived for the constant-surface charge case without the effect of this adsorbed layer model. The Haydon-Taylor model accounts for the penetrability of the electrolyte and dielectric constant in the adsorbed surfactant layer of varying thickness, the degree of surface coverage, particle-size effect, ionic strength, and the dielectric constant in the bulk solution. In the constant-potential case, the resulting V_R equation is identical to the classical Derjaguin, Landau, Verwey, and Overbeek (DLVO) one. Computations show that as flat plates and spheres approach each other, V_R (constant-surface charge with impenetrable adsorbed layer) > V_R (constant-surface charge with penetrable adsorbed layer) > V_R (constant-surface potential). Because of the possible 50- to 100-fold difference in magnitude between them, proper choice of the model is important when considering application of the theory to the rigorous kinetic treatment of the coalescence of o/w emulsions and flocculation of suspensions.

Keyphrases □ Oil droplets, electrostatic interaction—adsorbed surface-active ions □ Electrolyte solutions—oil droplets, adsorbed surface-active ion interaction □ Repulsive interaction—flat plates, spheres □ Emulsions—particle collision probability

In those dispersed systems in which the primary barrier to flocculation (or coalescence) is electrical, the classical theory of the repulsive interaction of overlapping electrical double layers between two particles combined with the attractive interaction due to dispersion forces is used. Moreover, the usual model employed for the repulsive energy requires that the surface potential remains constant during the collision of particles, although the model on constant-surface charge, in which case the surface potential increases during the encounter, is applicable in most dispersed systems (1).

Frens *et al.* (2, 3) showed that the collision of silver iodide colloidal particles in aqueous electrolyte solutions was more appropriately explained by the constant-surface charge condition. They employed the exact solution of the Poisson-Boltzmann equation in the form of elliptical integrals. Recently, while examining the question of the surface potential or charge remaining constant during the mutual approach of particles, Jones and Levine (4) derived approximate expressions in series form, and Muller (5) derived exact equations expressed as elliptical integrals.

Haydon and Taylor (6–8) proposed a model for the adsorption of ionic surface-active agents at the oil/water interface, allowing for the penetration of mobile ions into the adsorbed layer of surfactant molecules situated at some equilibrium distance from the interface. They calculated the potential in the aqueous surface phase and related it to surface-pressure area measurements. The Poisson-Boltzmann equation was

on thin-layer plates as described in the *Experimental* section. The DIH reagent was used because of its high reactivity toward carbonyl compounds (15, 16). Thermal degradation products of this ascorbic acid–silicic acid system are shown in Fig. 4. Examination of the chromatogram does indicate the absence of furfural, the possible presence of levulinic acid, and, in addition, other unidentified degradation products, and strongly suggests that ascorbic acid in this adsorbed state undergoes a different type of degradation from that in aqueous solution. Although furfural was not detected in this system, it is still possible that any furfural formed could have volatilized from the solid surface or undergone polymerization. It is highly unlikely, however, that levulinic acid results from furfural degradation, since Lamden and Harris (11) pointed out that the formation of furfural in solution degradation does not result from dehydroascorbic acid which, as mentioned before, is probably the first step in the oxidation of ascorbic acid in the adsorbed state. It is, therefore, highly likely that the degradation pathway in the adsorbed state may be quite different from that in solution.

REFERENCES

(1) L. R. Snyder, "Principles of Adsorption Chromatography," Marcel Dekker, New York, N. Y., 1968, p. 160.

(2) J. Gueyne and M. I. Duffaut, Fr. M. 1069, Feb. 19, 1962, Appl. Aug. 31, 1960; 13 pp.; through *Chem. Abstr.*, **59,** 12911c(1963).

(3) R. Strohecker and H. M. Henning, "Vitamin Assay," Verlag Chemil GMBH, Weinheim/Bergstr., 1965, p. 233.

(4) J. L. Lach and M. Bornstein, *J. Pharm. Sci.*, **54,** 730(1965).

(5) "The United States Pharmacopeia," 17th rev., Mack Printing Co., Easton, Pa., 1965.

(6) V. A. Usoltseva, I. G. Chistyakov, and M. D. Nasyrova, *Izv. Vyssh. Ucheb. Zaved., Khim. Khim. Tekhnol.*, **8,** 65(1965); through *Chem. Abstr.*, **63,** 2848f(1965).

(7) C. Duval, *Mikrochim. Ichnoanal. Acta*, **6,** 1073(1964).

(8) J. P. Comer and L. D. Howell, *J. Pharm. Sci.*, **53,** 335(1964).

(9) W. W. Wendlandt and H. G. Hec, "Reflectance Spectroscopy," Interscience, New York, N. Y., 1966, p. 62.

(10) S. Otani, *Yakuzaigaku*, **24,** 293(1964).

(11) M. P. Lamden and R. S. Harris, *Food Res.*, **15,** 79(1950).

(12) I. Tanaka and S. Otani, *Yakuzaigaku*, **22,** 150(1962).

(13) T. Kurata and Y. Sakurai, *Agr. Biol. Chem.*, **31,** 170(1967).

(14) P. Finholt, I. Alsos, and T. Higuchi, *J. Pharm. Sci.*, **54,** 181(1965).

(15) W. A. Mosher, I. S. Bechara, and E. J. Pozomek, *Talanta*, **15,** 482 (1968).

(16) R. A. Braun and W. A. Mosher, *J. Amer. Chem. Soc.*, **80,** 3048(1958).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication March 27, 1970.

This work was supported by Research Grant GM-13178 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Electrostatic Interaction of Oil Droplets with Adsorbed Surface-Active Ions in Dilute Electrolyte Solutions

N. F. H. HO, A. SUZUKI*, and W. I. HIGUCHI

Abstract □ Using the Haydon-Taylor model in which the surfactant head groups are situated at some distance from the oil surface, the interaction energy of electrostatic repulsion (V_R) between oil droplets in dilute electrolyte solutions is derived for the two cases: constant-surface potential and constant-surface charge. For comparison, the repulsive energy is also derived for the constant-surface charge case without the effect of this adsorbed layer model. The Haydon-Taylor model accounts for the penetrability of the electrolyte and dielectric constant in the adsorbed surfactant layer of varying thickness, the degree of surface coverage, particle-size effect, ionic strength, and the dielectric constant in the bulk solution. In the constant-potential case, the resulting V_R equation is identical to the classical Derjaguin, Landau, Verwey, and Overbeek (DLVO) one. Computations show that as flat plates and spheres approach each other, V_R (constant-surface charge with impenetrable adsorbed layer) > V_R (constant-surface charge with penetrable adsorbed layer) > V_R (constant-surface potential). Because of the possible 50- to 100-fold difference in magnitude between them, proper choice of the model is important when considering application of the theory to the rigorous kinetic treatment of the coalescence of o/w emulsions and flocculation of suspensions.

Keyphrases □ Oil droplets, electrostatic interaction—adsorbed surface-active ions □ Electrolyte solutions—oil droplets, adsorbed surface-active ion interaction □ Repulsive interaction—flat plates, spheres □ Emulsions—particle collision probability

In those dispersed systems in which the primary barrier to flocculation (or coalescence) is electrical, the classical theory of the repulsive interaction of overlapping electrical double layers between two particles combined with the attractive interaction due to dispersion forces is used. Moreover, the usual model employed for the repulsive energy requires that the surface potential remains constant during the collision of particles, although the model on constant-surface charge, in which case the surface potential increases during the encounter, is applicable in most dispersed systems (1).

Frens *et al.* (2, 3) showed that the collision of silver iodide colloidal particles in aqueous electrolyte solutions was more appropriately explained by the constant-surface charge condition. They employed the exact solution of the Poisson-Boltzmann equation in the form of elliptical integrals. Recently, while examining the question of the surface potential or charge remaining constant during the mutual approach of particles, Jones and Levine (4) derived approximate expressions in series form, and Muller (5) derived exact equations expressed as elliptical integrals.

Haydon and Taylor (6–8) proposed a model for the adsorption of ionic surface-active agents at the oil/water interface, allowing for the penetration of mobile ions into the adsorbed layer of surfactant molecules situated at some equilibrium distance from the interface. They calculated the potential in the aqueous surface phase and related it to surface-pressure area measurements. The Poisson-Boltzmann equation was

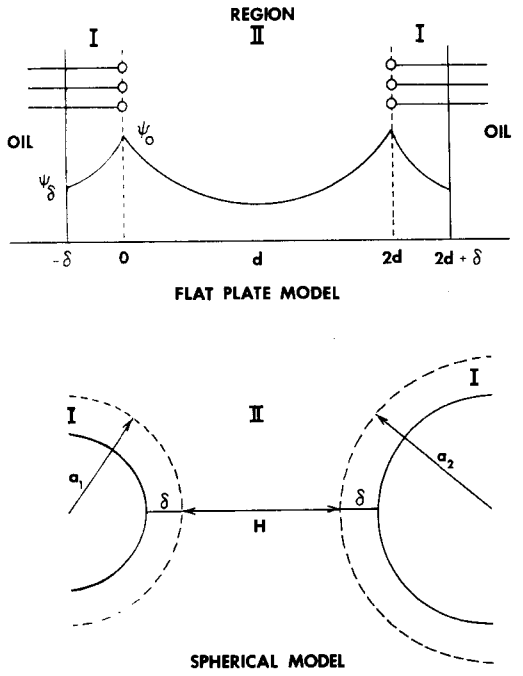


Figure 1—*Distribution of potential between weakly interacting electrical double layers of oil droplets in a dilute electrolyte solution with an ionic surfactant layer extending from the o/w interface. Region I is the surfactant layer penetrable to small ions and Region II is the bulk solution.*

assumed and applied to both sides of the plane of the head groups. Levine *et al.* (9) criticized Haydon and Taylor's mathematical estimation of the extended equilibrium distance of the ionized monolayer and pointed out that the assumption of a Boltzmann distribution of penetrating counterions behind the plane of the ionized monolayer becomes physically somewhat unrealistic in the limit that the available space for ion penetration becomes small as compared to the size of the penetrating ion. Recently, Gingell (10, 11) used the model to calculate the changes in the potential in the surface and aqueous phases between two approaching particles as a possible physical mechanism for intercellular interactions. The purposes of this paper are to utilize the model for interacting particles to derive simple expressions for the repulsive energy of interaction at constant-surface charge and to compare them with the usual energy expressions for constant-surface potential. As will be seen, this becomes necessary for the rigorous study of the kinetics of flocculation (or coalescence) of emulsions whereby one accounts for the probability of random collision of the entire particle-size distribution through Smoluchowski's fundamental equation and Fuchs' stability factor (12, 13). The results of this paper can also apply to suspensions.

THEORY

The Haydon-Taylor model for o/w emulsions containing ionic surfactants in dilute electrolyte solutions is assumed. The adsorbed surfactant molecules at the o/w interface are arranged in such a manner that the ionized head groups extend to some distance from the interface (Fig. 1). To treat the interaction energy of electrostatic repulsion between two oil droplets by way of the energy of interaction between two flat plates, the following system is used.

1. Region I, in which $(-\delta \leq x \leq 0)$ and $(2d \leq x \leq 2d + \delta)$, is the region of the adsorbed surfactant layer and with the dielectric constant ϵ_I. The surface-active ions are mobile, and the ionic head assumes an equilibrium distance δ from the o/w interface. Since solvent and electrolyte are allowed to penetrate this layer, there is an α^2, the volume fraction of this region accessible to small ions, and the characteristic κ_I, the reciprocal Debye-Hückel length. The o/w interface is uncharged, and there is no penetration of ions into the oil phase, thus requiring that $(d\psi/dx)_{-\delta} = 0$ (9).

2. Region II, in which $(0 \leq x \leq 2d)$, is the region of the bulk aqueous phase of dielectric constant ϵ and reciprocal length parameter κ. The size of the surface-active head group is neglected.

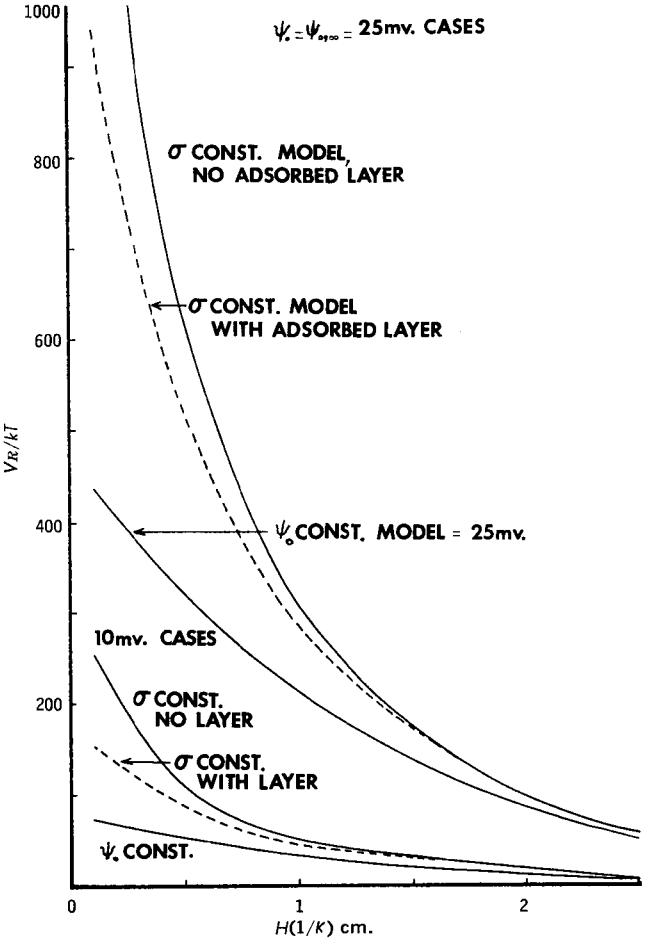


Figure 2—*Comparison of repulsive energy profiles for constant-surface charge, constant-surface charge with a penetrable surfactant layer, and constant charge with an impenetrable surfactant layer models for two equal spheres; $\psi_0 = \psi_{0,\infty} = 10$ and 25 mv., $\kappa = 10^7$ cm.$^{-1}$, $\bullet = 80$, $\epsilon_I = 4$, a = 1.0 μ, $\delta = 1/\kappa$ cm., and $\alpha^2 = 0.75$.*

Repulsive Interaction between Similar Flat Plates—Assuming the modified Gouy-Chapman model of the electrical double layer for a flat plate and the linearized Debye-Hückel approximation for small potentials (<25 mv.), the resulting Poisson equations are:

$$\text{Region I: } \frac{d^2\psi}{dx^2} = \alpha^2\kappa_1{}^2\psi \qquad \text{(Eq. 1)}$$

$$\text{Region II: } \frac{d^2\psi}{dx^2} = \kappa^2\psi \qquad \text{(Eq. 2)}$$

where ψ is the potential and the other terms are previously defined. When the boundary conditions for the left plate are applied, *i.e.*,

$$\text{Region I: } \psi = \psi_0 \text{ at } x = 0$$

$$\frac{d\psi}{dx} = 0 \text{ at } x = -\delta$$

$$\text{Region II: } \psi = \psi_0 \text{ at } x = 0$$

$$\frac{d\psi}{dx} = 0 \text{ and } \psi = \psi_d \text{ at } x = d$$

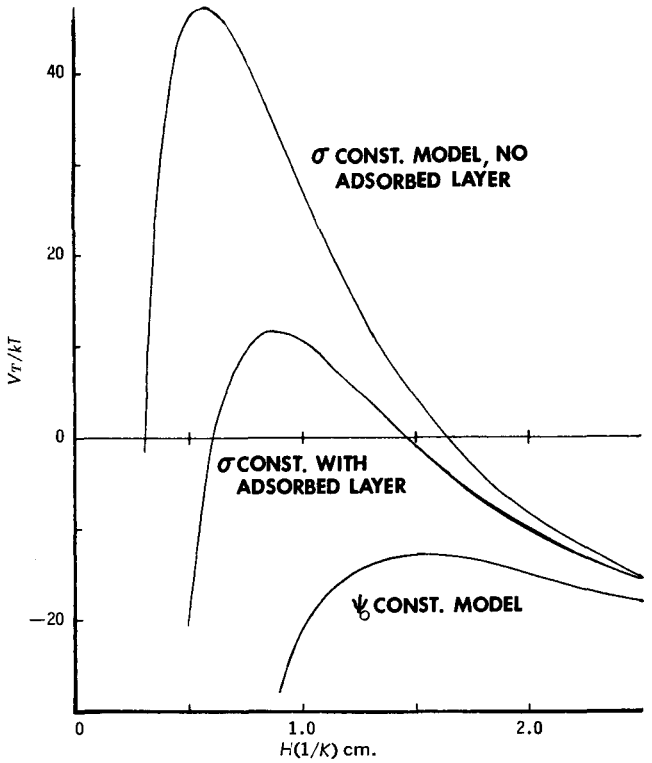


Figure 3—*Comparison of total potential energy profiles between three interacting double-layer models;* $\psi_0 = \psi_{0,\infty} = 25$ *mv.,* $\kappa = 10^7$ *cm.*$^{-1}$, $\epsilon = 80$, $\epsilon_I = 10$, a $= 0.5\ \mu$, $\delta = 1/\kappa$ *cm., and* $\alpha^2 = 0.75$.

the solutions to Eqs. 1 and 2, respectively, are

$$\psi_{I,L} = \frac{\psi_0 \cosh \alpha\kappa_I(\delta + x)}{\cosh \alpha\kappa_I\delta} \qquad (-\delta \leq x \leq 0) \qquad \text{(Eq. 3)}$$

$$\psi_{II,L} = \frac{\psi_0 \cosh \kappa(d - x)}{\cosh \kappa d} \qquad (0 \leq x \leq d) \qquad \text{(Eq. 4)}$$

For the corresponding right plate,

$$\psi_{I,R} = \frac{\psi_0 \cosh \alpha\kappa_I(2d + \delta - x)}{\cosh \alpha\kappa_I\delta} \qquad (2d \leq x \leq 2d + \delta) \qquad \text{(Eq. 5)}$$

$$\psi_{II,R} = \frac{\psi_0 \cosh \kappa(x - d)}{\cosh \kappa d} \qquad (d \leq x \leq 2d) \qquad \text{(Eq. 6)}$$

The surface charge density for the left plate is defined by

$$\sigma_L = \frac{\epsilon_I}{4\pi}\int_{-\delta}^{0}\left(\frac{d^2\psi}{dx^2}\right)_I dx + \frac{\epsilon}{4\pi}\int_{0}^{d}\left(\frac{d^2\psi}{dx^2}\right)_{II} dx \qquad \text{(Eq. 7)}$$

$$\sigma_L = \frac{\psi_0\epsilon\kappa}{4\pi}\left[\frac{\alpha}{b}\tanh \alpha b\kappa\delta + \tanh \kappa d\right] \qquad \text{(Eq. 8)}$$

where $b^2 = \epsilon/\epsilon_I \geq 1$ and, consequently, $\kappa_I = b\kappa$. It can be shown from the symmetry of the system that

$$\sigma_L = \sigma_R \qquad \text{(Eq. 9)}$$

Depending upon the case when the surface potential or charge is constant, Eq. 8 expresses each parameter as a function of plate distances.

When the plates are brought together from infinity, the general expression for the potential energy of repulsion is

$$V_{R(P/P)} = F_{2d} - F_\infty \qquad \text{(Eq. 10)}$$

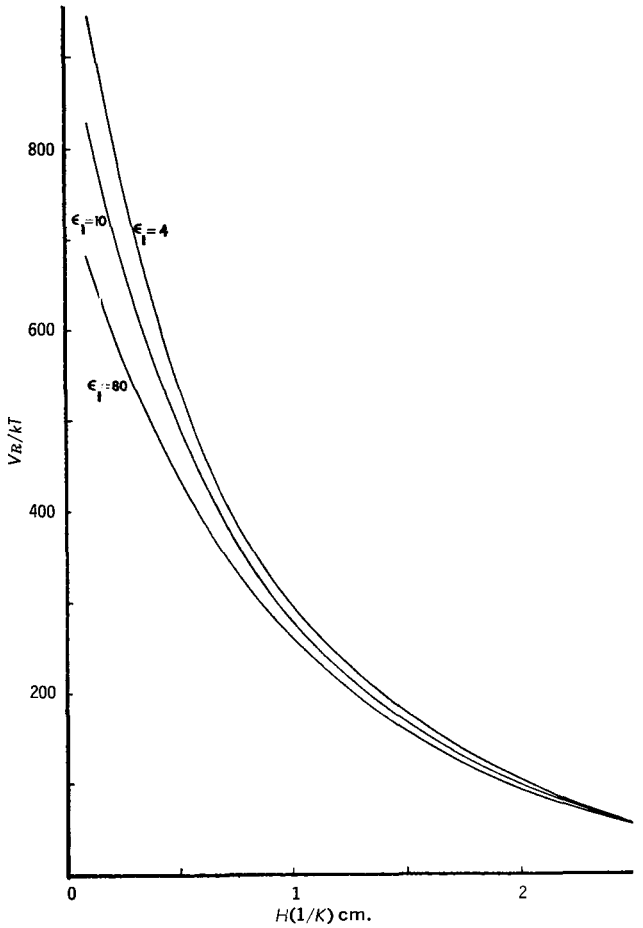


Figure 4—*Effect of the dielectric constant in the penetrable ionic surfactant layer on the repulsive energy of interaction between two equal spheres under the condition of constant charge;* $\psi_{0,\infty} = 25$ *mv.,* $\kappa = 10^7$ *cm.*$^{-1}$, $\epsilon_I = 80$, a $= 1.0\ \mu$, $\delta = 1/\kappa$ *cm., and* $\alpha^2 = 0.75$.

If the surface potential, ψ_0, is constant and small and the potential between the interacting plates is equal to the sum of the potentials of the individual double layers, the free energy of the two similar plates (14) can be approximated by

$$F_{2d} = -\tfrac{1}{2}(\sigma_L\psi_{0L} + \sigma_R\psi_{0R}) = -\sigma\psi_0 \qquad \text{(Eq. 11)}$$

and

$$F_\infty = \lim_{2d\to\infty} F_{2d} \qquad \text{(Eq. 12)}$$

After substituting for σ from Eq. 8 into Eqs. 11 and 12, the potential energy is

$$V_{R(P/P)} = \frac{\epsilon\kappa\psi_0^2}{4\pi}(1 - \tanh \kappa d) \qquad \text{(Eq. 13)}$$

$\psi_0 = \text{const.}$

On the other hand, if the surface charge density, σ, is considered to be constant and small,

$$F_{2d} = \tfrac{1}{2}(\sigma_L\psi_{0L} + \sigma_R\psi_{0R}) = \sigma\psi_0 \qquad \text{(Eq. 14)}$$

Substituting for ψ_0 from Eq. 8 into Eqs. 14 and 12,

$$V_{R(P/P)} = \frac{4\pi\sigma^2}{\epsilon\kappa}\left[\frac{1 - \tanh \kappa d}{(A + 1)(A + \tanh \kappa d)}\right] \qquad \text{(Eq. 15)}$$

$\sigma = \text{const.}$

Table I—Influence of the Constant Potential and Charge Repulsive Energy Models on the Collision Probability of Equal Spherical Particles[a]

ψ_0, mv.	ln W_σ/W_{ψ_0}	W_σ/W_{ψ_0}
5	3.91	5.00×10^1
10	15.65	6.26×10^6
15	35.21	1.95×10^{14}
20	62.60	1.52×10^{25}
25	97.81	$\gg 10^{25}$

[a] $a = 1.0\ \mu$, $\epsilon = 80$, $\kappa H_m \sim 1$.

where $A = \alpha/b \tanh \alpha b \kappa \delta$ $(0 \leq A < 1)$. However, it is recognized from Eq. 8 that because the plates are infinitely apart, the constant-surface charge is

$$\sigma = \frac{\epsilon\kappa}{4\pi} \psi_{0,\infty}(A + 1) \qquad \text{(Eq. 16)}$$

where $\psi_{0,\infty}$ is the surface potential of a single double layer and is comparable in magnitude to the ζ-potential. Thus, Eq. 15 may be expressed again by

$$V_{R(P/P)} = \frac{\epsilon\kappa\psi_{0,\infty}^2(A + 1)}{4\pi} \left(\frac{1 - \tanh \kappa d}{A + \tanh \kappa d}\right) \qquad \text{(Eq. 17)}$$
$$\sigma = \text{const.}$$

If Region I of the adsorbed layer is impenetrable to small ions, *i.e.*, $A = 0$, then Eq. 17 reduces to the interaction energy of repulsion for a classical flat-plate model at constant-surface charge:

$$V_{R(P/P)} = \frac{\epsilon\kappa\psi_{0,\infty}^2}{4\pi} \left(\frac{1 - \tanh \kappa d}{\tanh \kappa d}\right) \qquad \text{(Eq. 18)}$$
$$\sigma = \text{const.}$$

Repulsive Interaction between Spheres—It has already been shown that the approximate interaction energy of repulsion between two spheres can be derived *via* the flat, double-layer approach (15). In general,

$$V_{R(S/S)} = \frac{2\pi a_1 a_2}{a_1 + a_2} \int_H^\infty V_{R(P/P)} dH \qquad \text{(Eq. 19)}$$
$$(a_1, a_2 \gg H)$$
$$(\kappa a_1, \kappa a_2 \gg 1)$$

where a_1 and a_2 are the radii of the corresponding spheres, and H is identical to $2d$ for the plate distances. Analogous to the various cases of $V_{R(P/P)}$ in the previous section, it follows that:

(*a*) If ψ_0 is constant,

$$V_{R(S/S)} = \frac{\epsilon a_1 a_2}{a_1 + a_2} \psi_0^2 \ln (1 + e^{-\kappa H}) \qquad \text{(Eq. 20)}$$

(*b*) If σ is constant and the adsorbed ionic surfactant layer model is assumed,

$$V_{R(S/S)} = \frac{\epsilon a_1 a_2 (A + 1)}{(a_1 + a_2)(A - 1)} \psi_{0,\infty}^2 \ln \left[1 + \frac{(A - 1)}{(A + 1)} e^{-\kappa H}\right] \qquad \text{(Eq. 21)}$$

(*c*) If σ is constant and the adsorbed layer is impenetrable to small ions,

$$V_{R(S/S)} = \frac{\epsilon a_1 a_2}{(a_1 + a_2)} \psi_{0,\infty}^2 \ln \left(\frac{1}{1 - e^{-\kappa H}}\right) \qquad \text{(Eq. 22)}$$

It is noteworthy that, in the constant-surface potential case, the resulting $V_{R(S/S)}$ of Eq. 20 is identical to the classical DLVO one (16). It is implicit in Eqs. 17, 18, 21, and 22 that the potential at the surface (ψ_0) increases as the two particles approach each other. In the limit of $a_1 \gg a_2$, the interaction energy of repulsion for a sphere and a plane can be approximated. Here, the energy is dependent upon the particle with the smallest radius of curvature.

Table II—Correction to the ζ-Potential for the Usual Constant-Surface Potential Model of Repulsion Energy Used when the Constant-Surface Charge Case Is the Physically Applicable Model[a]

	ψ_0, mv.	
ζ, mv.	$\kappa H_m = 0.5$	$\kappa H_m = 1$
10	14.0	12.1
15	21.1	18.2
20	28.1	24.2
25	35.1	30.3
30	42.1	36.3
50	70.2	60.5

[a] Calculations based on Eq. 26 assuming $\zeta \simeq \psi_{0,\infty}$.

It is possible to estimate the volume fraction α^2 in Region I accessible to small ions in terms of the surface charge density:

$$\alpha^2 = 1 - \frac{3r^2\delta\sigma}{a} \qquad \text{(Eq. 23)}$$

where r is the radius of the surfactant chain taken as a cylinder.

RESULTS AND DISCUSSION

Significance of the Repulsive Energy Models on Emulsion Stability—Computation employing Eqs. 20–22 were carried out for a range of conditions. Figure 2 shows that as two particles approach each other, V_R (constant σ without the effect of the adsorbed layer

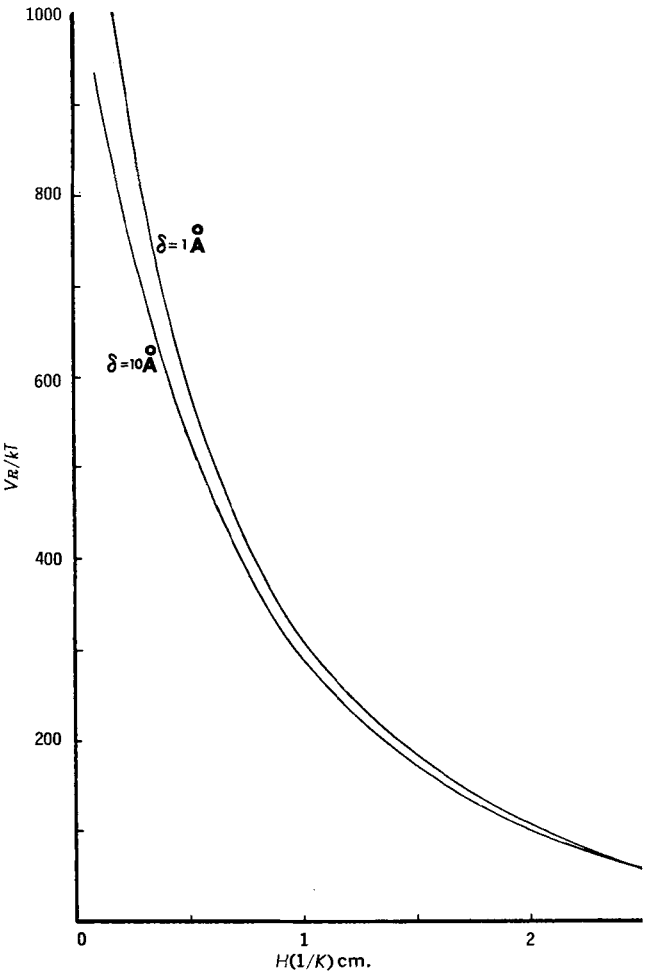


Figure 5—*Effect of the thickness of the adsorbed surfactant layer on the repulsive energy between two equal spheres under the condition of constant charge;* $\psi_{0,\infty} = 25$ *mv.,* $\kappa = 10^7$ *cm.*$^{-1}$, $\epsilon = 80$, $\epsilon_I = 4$, a $= 1.0\ \mu$, *and* $\alpha^2 = 0.75$.

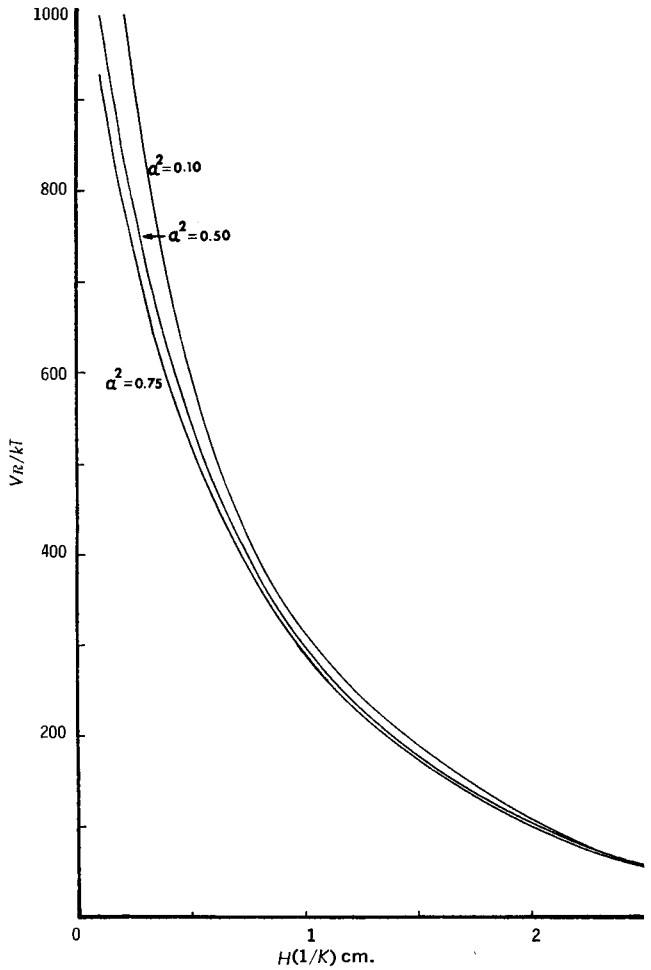


Figure 6—*Effect of the volume in the adsorbed layer available for ion penetration on the repulsive energy curve;* $\psi_{0,\infty} = 25$ *mv.,* $\kappa = 10^7$ *cm.*$^{-1}$, $\epsilon = 80$, $\epsilon_I = 4$, a $= 1.0\ \mu$, *and* $\delta = 1/\kappa$ *cm.*

model) > V_R (constant σ with effect of the model) > V_R (constant ψ_0). Because a 50- to 100-fold difference in magnitude may exist between the three cases at close interparticle distances under certain environmental conditions, *i.e.*, in the order of $0.5/\kappa$ to $1/\kappa$ cm., the proper choice becomes important when one desires to apply it to the rigorous treatment of the coalescence of o/w emulsions and also the flocculation of suspensions. At far distances, the V_R's of the three models approach the same asymptotic values.

To illustrate the importance of the proper choice of the repulsive energy model to predict emulsion stability, consider the two extreme models: V_R (ψ_0 constant) and V_R (σ constant for an ion-impenetrable adsorbed surfactant layer). In general, the probability of collision between two equal spheres is given by the reciprocal of

$$W \sim \frac{1}{2\kappa a} e^{V_{T\max}/kT} \qquad (W \geq 1) \qquad \text{(Eq. 24)}$$

where W is the Fuchs probability factor, and $V_{T\max.}$ is the maximum in a potential energy curve consisting of the sum of the repulsive and attractive energy of interaction ($V_T = V_R + V_A$). Using Eqs. 20, 22, and 24, it can be shown that

$$\ln \frac{W_{\sigma_{\text{const.}}}}{W_{\psi_{0\text{const.}}}} = \left(V_{R\max.}{}_{\sigma_{\text{const.}}} - V_{R\max.}{}_{\psi_{0\text{const.}}}\right)\Big/kT \qquad \text{(Eq. 25)}$$

The results of Eq. 25 for 1.0-μ radius particles in a dilute electrolyte solution, in which the minimum interparticle distance H_m is approximately $1/\kappa$ cm., are found in Table I. Usually, the V_R (ψ_0 constant) model is used to describe the electrostatic barrier, in which case there must be rapid equilibrium of desorption and adsorption of surface charges during collision. However, in most physical systems it is more reasonable that the surface charge remains constant during the encounter, in which case the double-layer overlap leads to an increase in ψ_0 (1). Therefore, depending upon the model chosen, it becomes evident from Table I that one can overestimate or underestimate the probability of collision (or emulsion and suspension stability) by many orders of magnitude. In Fig. 3 the total potential energy curve illustrates another example. There are experimental evidences that may be explained in part by the constant-surface charge model. In the study of the flocculation of latex particles, Higuchi *et al.* (17) required higher concentrations of electrolyte to reproduce the same initial rate, despite repeated efforts to purify the particles. Also, Johnson *et al.* (18) obtained stable arachidic acid sols at zero ζ-potential. However, later they showed some evidence that the structuring of water about the particles might provide an additional repulsive factor (19).

This discussion leads to the questionable use of ζ-potential measurements to predict the stability of dilute aqueous dispersions in which the primary barrier is electrical. Again consider the two extreme cases and equate Eqs. 20 and 22. It follows that

$$\frac{\psi_0{}^2}{\psi_{0,\infty}{}^2} = -\frac{\ln(1 - e^{-\kappa H_m})}{\ln(1 + e^{-\kappa H_m})} \qquad \text{(Eq. 26)}$$

and some results are shown in Table II. If Eq. 20 is used to estimate the repulsive interaction energy and collision probability and if the constant-surface charge model is more correct, then one should use a surface potential higher in magnitude than the observed ζ-potential ($\zeta \simeq \psi_{0,\infty}$). From a practical viewpoint, the low absolute value of the ζ-potential may underestimate the degree of repulsive interaction and stability of a system unless the potential is sufficiently high so that it does not make a difference.

Repulsive Energy According to the Penetrable Adsorbed-Layer Model—To study the behavior of the properties of the adsorbed

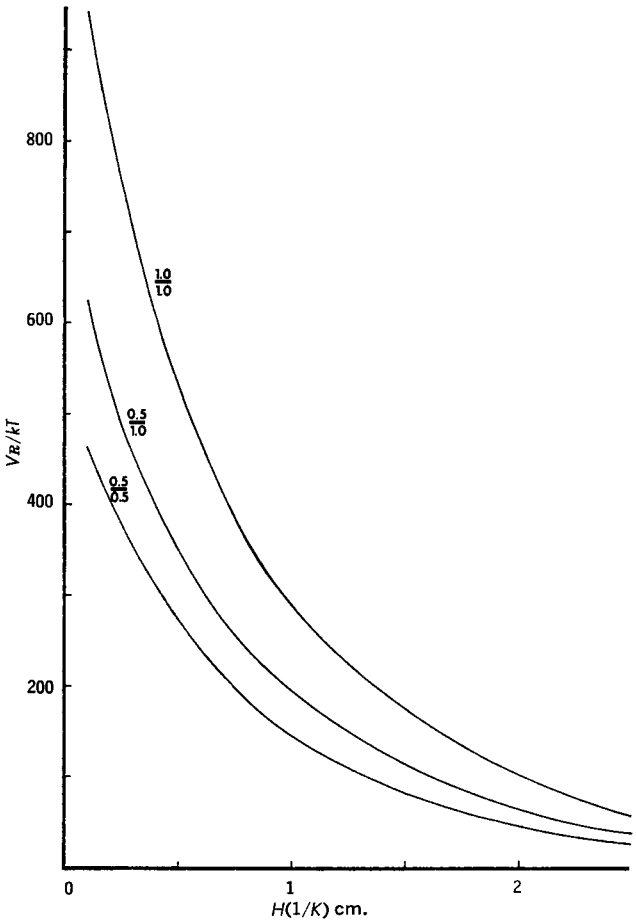


Figure 7—*Effect of the particle size of two spheres on the repulsive energy curve;* $\psi_{0,\infty} = 25$ *mv.,* $\kappa = 10^7$ *cm.*$^{-1}$, $\epsilon = 80$, $\epsilon_I = 4$, $\delta = 1/\kappa$ *cm., and* $\alpha^2 = 0.75$.

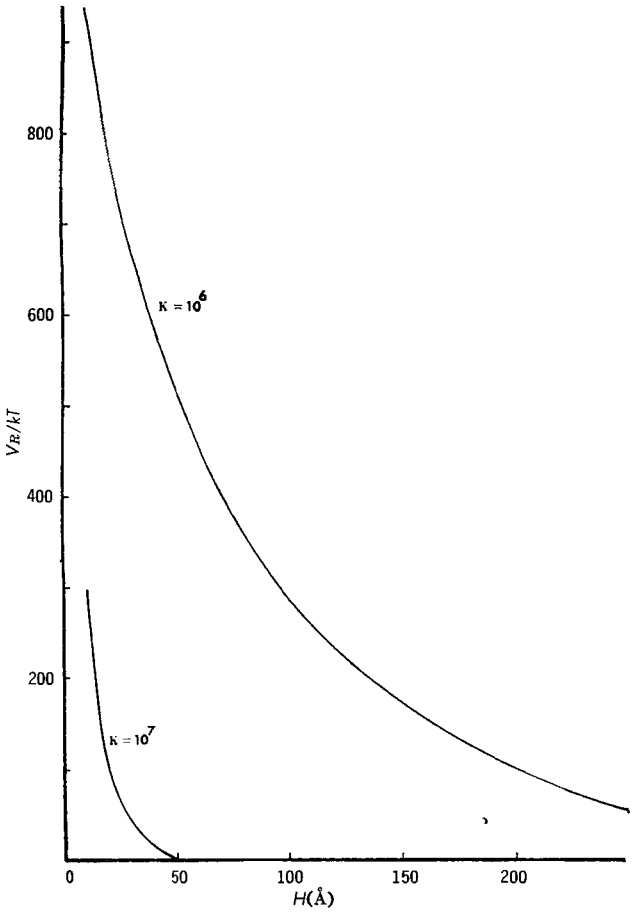


Figure 8—*Effect of the electrolyte concentration on the repulsive energy curve;* $\psi_{0,\infty} = 25$ *mv.*, $\epsilon = 80$, $\epsilon_I = 4$, a $= 1.0\ \mu$, $\delta = 1/\kappa$ *cm., and* $\alpha^2 = 0.75$.

layer of ionic surfactant on the interaction energy of repulsion, Eq. 21 was used. Figures 4–6 show that a decrease in dielectric constant, the thickness of the adsorbed layer, or the volume fraction of the adsorbed layer accessible to small ion penetration leads to a V_R larger in magnitude than that for the opposite situation and, in effect, makes the layer less penetrable to small ions. The thickness of the layer is about 3–10 Å. The low dielectric constant of the solvent in the layer affects the solubility and ionic distribution. Usually, the dielectric constant of the bulk aqueous solution is taken to be 80, while that in the layer is considerably lower, perhaps 4–10.

It is observed that the V_R (constant-surface charge) of the adsorbed-layer model approaches V_R (constant-surface potential) at close particle separations as the values of ϵ_I, δ, and α^2 are increased. The greater penetrability of ions leads to a more effective screening of the surface charges and a decrease in the interaction energy.

In Fig. 7, it is seen how the particle size has a marked effect on the magnitude of the repulsive energy and a consequential increase in the preference for coalescence (or flocculation) of small particles over larger ones. The usual influence of the electrolyte concentration on the V_R *versus* H profiles is shown in Fig. 8.

CONCLUSION

In an o/w emulsion, in which the change in the particle-size distribution with time appears to occur primarily through droplet–droplet coalescence after overcoming an electrostatic barrier to the encounter, the repulsive energy of the constant-surface potential model may prevail in the initial kinetic history of the emulsion. The surface coverage of ionic surfactants is low in a freshly prepared emulsion, say about 5–10% of maximum coverage. Here it is believed that rapid equilibrium of desorption and adsorption of surfactant molecules occurs during the close approach of the droplets. However, at later periods when the total surface area of the system is smaller and the surface coverage higher, the repulsive energy according to the Haydon-Taylor model under the condition of constant-surface charge may then prevail. Intermediate situations between these two periods are also conceivable. Kinetic studies of this nature are being conducted on o/w emulsions with ionic surfactants utilizing Smoluchowski's fundamental flocculation equation treatment reported in an earlier paper (13).

REFERENCES

(1) J. Lyklema, *Advan. Colloid Interface Sci.*, **2,** 65(1968).

(2) G. Frens, D. J. C. Engel, and J. T. G. Overbeek, *Trans. Faraday Soc.*, **63,** 418(1967).

(3) G. Frens, thesis, Utrecht, The Netherlands, 1968.

(4) J. E. Jones and S. Levine, *J. Colloid Interface Sci.*, **30,** 241(1969).

(5) V. M. Muller, *Colloid J. (USSR)*, **31,** 340(1969).

(6) D. A. Haydon and F. H. Taylor, *Phil. Trans. Roy. Soc. (London), Ser. A*, **252,** 225(1960).

(7) *Ibid.*, **252,** 255(1960).

(8) D. A. Haydon, *Proc. Royal Soc. (London), Ser. A*, **258,** 319(1960).

(9) S. Levine, G. M. Bell, and B. A. Pethica, *J. Chem. Phys.*, **40,** 2304(1964).

(10) D. Gingell, *J. Theoret. Biol.*, **17,** 451(1967).

(11) *Ibid.*, **19,** 340(1968).

(12) H. R. Kruyt, "Colloid Science," vol. I, Elsevier, Amsterdam, The Netherlands, 1952, chap. 7.

(13) A. Suzuki, N. F. H. Ho, and W. I. Higuchi, *J. Colloid Interface Sci.*, **29,** 552(1969).

(14) E. J. W. Verwey and J. T. G. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier, Amsterdam, The Netherlands, 1948, chap. 3.

(15) R. Hogg, T. W. Healy, and D. W. Fuerstenau, *Trans. Faraday Soc.*, **62,** 1638(1968).

(16) E. J. W. Verwey and J. T. G. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier, Amsterdam, The Netherlands, 1948, p. 139.

(17) W. I. Higuchi, T. O. Rhee, and D. R. Flanagan, *J. Pharm. Sci.*, **54,** 510(1965).

(18) G. A. Johnson, J. Goldfarb, and B. A. Pethica, *Trans. Faraday Soc.*, **61,** 2321(1965).

(19) G. A. Johnson, S. M. Lecchini, E. G. Smith, J. Clifford, and B. A. Pethica, *Discuss. Faraday Soc.*, **42,** 120(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 11, 1969, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication February 27, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

* Present address: Tanabe Seiyaku, Osaka, Japan.

Molecular-Scale Drug Entrapment as a Precise Method of Controlled Drug Release I: Entrapment of Cationic Drugs by Polymeric Flocculation

HARRIS GOODMAN* and GILBERT S. BANKER

Abstract □ A system of molecular-scale drug entrapment has been developed which provides a physicochemical and highly reproducible method of effecting drug entrapment and subsequent controlled drug release from polymeric matrices. The flocculation of highly concentrated colloidal polymeric dispersions (latices), in the presence of the drug in solution which is to be occluded, provides the entrapment mechanism. A solid-state, highly reproducible molecular entrapment combination of methapyrilene hydrochloride and an acrylic copolymer was prepared and evaluated for sustained-action characteristics. A significant increased duration of action and a reduction of the acute toxicity of methapyrilene in the entrapped form were established by *in vivo* effectiveness studies. The broad application of the entrapment process to acid salts of 11 widely used cationic nitrogen-containing drugs was demonstrated.

Keyphrases □ Polymeric flocculation—cationic drug entrapment □ Flocculation, polymeric—drug-concentration effect □ Drug release, controlled—polymeric matrices □ Release rates—drugs in polymeric matrices □ Dialysis—methyapyrilene–polymer, binding determination

Potentiation and alteration of drug action have been of interest to the medical and pharmaceutical professions for many years. In the last 15 years, prolongation of the therapeutic effects of orally administered medicinals has become of prime interest and concern to the industrial pharmacist.

The methods employed for the production of sustained-action dosage forms can be broadly classified into two categories: phsyicochemical and mechanical. These can be further subdivided into various groups such as gastroresistant coatings, chemical complexes, drug embedments, and ion-exchange techniques.

The majority of the methods employed are based on some type of gross embedment or coating of the medicament to yield the desired effects. The procedures are usually time consuming and laborious, involve mechanical techniques which are difficult to control, and in many cases result in products with excessive variability. Much of this variability can probably be traced to the product design and methods of production.

If optimization of drug action through controlled drug delivery rates from solid oral dosage forms is to become a reality, precise physicochemical methods of controlling drug release must replace earlier empirical and mechanical controlled-release methods.

This investigation was motivated by the need for a simple and highly reproducible procedure for drug entrapment and the development of controlled-release dosage forms employing this principle. The purpose of this study was to investigate the applicability of a molecular entrapment phenomenon as a unique approach to sustained-action dosage forms. The major objectives were:

1. The investigation of the properties of various polymeric materials as they would apply to a sustained-release mechanism based on molecular scale, solid-state entrapment of drugs.
2. The utilization and investigation of a process exhibiting broad application to the molecular entrapment of drugs.
3. The *in vitro* and *in vivo* evaluations of the sustained-action characteristics and activity of a polymer–drug entrapment system.

EXPERIMENTAL

Materials and Equipment—A linear, anionically charged, acrylic copolymer[1] composed of acrylic and methacrylic acids and esters and having a molecular weight exceeding 300,000 was supplied in an emulsion (latex) form, containing 40 ± 0.5% solids. Also used were other polymeric materials evaluated as possible entrapment media.[2,3,4]

The drugs studied were NF or USP grade; where appropriate, the purity was checked by melting point and chloride determinations.

Particle-size reduction and classification of all polymer and polymer–drug systems were accomplished using a comminuting machine (Fitzpatrick model M) and a series of standard sieves. All pH determinations were made on a pH meter (Beckman model N) equipped with a glass electrode (Beckman type E-2). A constant-temperature bath equipped with a mechanism for the rotation of sample bottles, similar to that reported by Souder and Ellenbogen (1), was employed for *in vitro* release-rate studies.

Initial Polymer Screening—Based on a polymer literature screening of toxicity and physical properties, the following polymers were chosen for initial study and aqueous solubility characterization: (*a*) acrylic copolymer emulsion,[1] (*b*) carbohydrate polymers,[2] (*c*) polyethylene glycol,[3] and (*d*) a sodium polyacrylate solution.[4]

Samples evaluated for solubility characteristics were comminuted, and the fraction passing a 60-mesh screen was employed. The two acrylic acid derivatives (the acrylic copolymer emulsion and the sodium polyacrylate solution) were first extracted from their aqueous media to provide the solid polymeric materials for solubility study.

Approximate rates of solubility of the polymeric materials in artificial gastrointestinal fluids were determined employing the rotating-bottle method (1, 2). Seven 1.0-g. samples of each polymer were sprinkled onto the surface of separate 60-ml. portions of artificial gastric fluid USP (without pepsin) in 3-oz. amber glass powder jars. The jars were sealed with a water-resistant electrical tape[5] and were rotated at 41 r.p.m. in a constant-temperature bath at 37 ± 2°. At specified time intervals, a sample bottle was removed and its contents vacuum filtered through a fine sintered-glass filter. The sample was then carefully transferred to a watch glass and dried at 50°. The remaining sample bottles were adjusted in pH as outlined in Table I. The data in Table I illustrate the approximate pH levels for the designated sample time intervals employed and the changes in the test fluids that were made to establish these levels.

[1] Acrysol ASE-75, Rohm & Haas Co., Philadelphia, Pa.
[2] Cerons are water-soluble anionic, cationic, or nonionic etherified carbohydrate polymers available in powder form, Hercules Chemical Co., Wilmington, Del.
[3] Polyglycols, E-4000, 6000, 9000, and 20,000; polyethylene glycol, the Dow Chemical Co., Midland, Mich.
[4] Acrysol G.S. is a sodium polyacrylate polymer supplied as a 12–13% w/v solution, Rohm & Haas Co., Philadelphia, Pa.
[5] Scotch Brand Electrical Tape No. 33, Minnesota Mining and Manufacturing Co., St. Paul, MN 55106

Table I—pH Gradient for Approximate Rate of Solubility Test

Time, hr.	Amount of Fluid Removed, ml.[a]	pH of Test Fluid
1	0.0	1.4
2	20.0	2.1
3	15.0	2.6
4	20.0	5.5
5	30.0	6.9
6	50.0	7.4
8	—	7.4

[a] The clear supernatant fluid removed was replaced by an equal quantity of artificial intestinal fluid USP (prewarmed to 37°).

The dried polymeric material was weighed, and the percentage of polymer dissolved at each time interval was calculated. The acrylic copolymer emulsion showed the most promising rate of solution, and this material was studied by further evaluative methods.

Drug Assay Procedures—Methapyrilene hydrochloride[6] was used because it represents a widely employed compound having characteristics suitable for development as a sustained-action dosage form. The drug is quick acting, having a fairly short duration of action, with single doses exhibiting a therapeutic effect for about 3.5 hr. (3, 4).

A pH-induced differential spectrophotometric method was developed which permitted the analysis of methapyrilene hydrochloride without interference from the polymer (5).

A base-line UV spectrophotometric analysis method, outlined by Reilly and Sawyer (6), was successfully adopted for the quantitative determination of phenylephrine hydrochloride in the presence of the polymeric material. The spectra were all analyzed at pH 12.9 to eliminate any shifts of the absorption maxima of this compound (5). This pH value was found to concur with that reported by Riegelman *et al.* (7).

Entrapment Procedure—Various methods of producing the polymer–drug entrapment systems were attempted. The addition of the acrylic copolymer emulsion to concentrated aqueous solutions of methapyrilene hydrochloride resulted in the flocculation and precipitation of the polymer affording the greatest possibility of drug entrapment since the drug molecules themselves were causing the flocculation. Other methods attempted included the addition of the drug solution to the polymeric system and the acidification of an alkaline aqueous solution of the drug and the polymer.

The following formula and procedure were the basic entrapment method used:

Formula I

Methapyrilene hydrochloride	20.0 g.
Distilled water	100.0 ml.
Acrylic copolymer emulsion	125.0 ml.

The acrylic copolymer emulsion was slowly added to a constantly mixing solution of the methapyrilene in distilled water, resulting in an immediate flocculation and precipitation of the added polymeric material. When the addition of the emulsion was complete, the slurry was allowed to mix for another 5 min. The mixture was then vacuum filtered through a coarse sintered-glass filter, applying vacuum from a water aspirator for about 10 min. The collected material was dried for 4 hr. at 50°. The dried material (a granular white solid) was then comminuted in a comminuting machine, employing a 40-mesh screen and the hammer edge of the blades in the forward position. The comminuted material was then screened through a 60-mesh sieve.

Reproducibility of the entrapment procedure was evaluated by preparing replicate batches and determining the percentage of drug entrapped in each batch.

The effect of drug (solution) concentration on the entrapment product was investigated by utilizing various drug concentrations in aqueous solution and holding constant the amount of polymer emulsion employed.

[6] Methapyrilene hydrochloride (Histadyl HCl), Eli Lilly and Co., Indianapolis, Ind.

Table II—pH Gradient for the *In Vitro* Release-Rate Test

Cumulative Time, hr.	pH of Test Fluid
0.5	1.3
1.5	1.3
2.5	2.3
4.5	6.7
6.5	7.3
8	7.3

Dialysis Studies—A dialysis study was conducted to determine whether methapyrilene hydrochloride was chemically bound to the polymeric material (in solution) in such a manner as to reduce the availability and the therapeutic activity of the drug. A dialysis method similar to that described in the literature (8, 9) was used. Dialysis sacs prepared from a semipermeable cellulosic membrane[7] were used for investigation of free methapyrilene hydrochloride, free acrylic copolymer, and acrylic copolymer–methapyrilene hydrochloride entrapment products. The dialysis samples were rotated for preselected time periods of 48, 72, or 107 hr. at 37°. The dialysis fluid employed was artificial intestinal fluid USP (without pancreatin) adjusted to pH 7.4. This medium caused dissolution of the polymer as well as the drug. The fluids inside the sacs, as well as the surrounding media, were assayed for drug content.

***In Vitro* Release-Rate Procedure**—A rotating-bottle method (1, 2) was used for the evaluation of the powdered and compressed sustained-action dosage forms. The comminuted polymer–drug samples were weighed into six individual dosage units, each to contain 100 mg. of methapyrilene hydrochloride. The samples were carefully transferred to 3-oz. amber glass powder jars and filled with 60 ml. of artificial gastric fluid. Table II illustrates the pH levels for the designated sample time intervals employed. These pH values agree well with those found by Borgstrom (10) in numerous *in vivo* tests. The test fluids were treated with artificial intestinal fluid and 1.0 *N* sodium hydroxide solution in such a manner as to yield the noted pH levels for the designated time intervals.

Suitable aliquots of the filtered test medium were taken (at the specified time periods) and differentially analyzed for methapyrilene hydrochloride.

***In Vivo* Evaluation**—Various methods have been proposed to evaluate the activity and the duration of action of antihistaminic compounds (3, 4, 11, 12). A guinea pig histamine aerosol procedure (4, 12, 13) was employed in this study. Duration of action of an orally administered dose of an acrylic copolymer–methapyrilene hydrochloride entrapment product was compared with that of the free drug.

It has been reported (4, 12) that 1–3 mg./kg. of methapyrilene hydrochloride administered to guinea pigs has shown protective action against histamine vapor for about 3.5 hr. In this study, a dosage level of 2.5 mg./kg. of body weight was employed. The powdered forms of the product were washed through a catheter tube directly into the stomach of the animal. The product and control drug were code labeled so that the investigator performing the actual experiments did not know which system was being given to the individual guinea pigs, thereby reducing prejudiced judgments. A total of 20 experimental runs was made, with 10 test animals receiving the free drug and 10 a polymer–drug entrapment product.

Toxicity Reduction—A reduction in the acute toxicity of a drug should occur with its formulation into a sustained-action dosage product. A preliminary investigation established a dose of 200 mg./kg. as the approximate LD_{83} in rats for orally administered methapyrilene hydrochloride. Six rats were orally dosed with 200 mg./kg. of the free methapyrilene hydrochloride, and another six were given 200 mg./kg. of the drug in a polymer–drug entrapment product. The time of death after oral administration was noted, and a 24-hr. survival time was considered the "cutoff" point for the test procedure.

Flocculation Phenomenon—It was felt that the precipitation of the polymer emulsion in the presence of methapyrilene hydrochloride was due to a flocculation phenomenon. Riddle (14) has stated: "The presence of salts in polymer dispersions usually has

[7] NoJax Casing, Size 30, Visking Co., Chicago, Ill.

the same effect as in other colloidal systems, *i.e.*, the particle size is increased and some of the polymer may coagulate."

It was also hypothesized that as the pH of the acrylic copolymer emulsion (an anionically charged copolymer) was increased, the polymeric compound would become more hydrophilic, resulting in increased solvation and increased stability to electrolytes. Conversely, as the pH decreases, the colloidal system becomes more hydrophobic and more prone to flocculation due to the presence of electrolytic agents.

To test this hypothesis, the acrylic copolymer emulsion was adjusted to pH values of 3.3 and 6.0, maintaining a 20% polymer solids content in both systems. Five-milliliter samples of the system being evaluated were placed in 15.24-cm. (6-in.) (20-ml. capacity) test tubes, and 5 ml. of varying concentrations of sodium chloride or methapyrilene hydrochloride solutions was added. The contents were mixed by inverting the test tubes three or four times. The flocculation value was taken as the minimum concentration of an electrolyte that caused complete flocculation within 2 hr. Complete flocculation was observed as a separation of a voluminous solid phase and a clear aqueous layer.

Flocculation Phenomenon in the Presence of Drugs—Flocculation values for acid salts of 11 widely used cationic nitrogen-containing drug compounds were determined in the copolymer emulsion system. The compounds studied varied in salt form, molecular weight, solubility characteristics, and therapeutic use. Included in this group of drugs were primary, secondary, and tertiary amines and a quaternary ammonium compound.

Preparation of Tablet Samples—Tablets were prepared from acrylic copolymer–methapyrilene hydrochloride entrapment products containing 13.3 and 33.4% active ingredient. In all cases, the tablets contained 100 mg. of drug, and they were compressed (on a Carver laboratory press) directly from the finer than 60-mesh material (hydraulic pressure setting of 8000 lb./in.^{2}). Two sets of tablets were prepared from each of the two employed polymer–drug systems, one set containing 10% starch as a disintegrant while the other contained no disintegrating agent. The tablets containing 13.3% active ingredient were prepared with a 1.27-cm. (0.5-in.) s.c. punch and die set, and those containing 33.4% drug were prepared with a 0.95-cm. (0.375-in.) s.c. punch and die set. Tablet hardness was determined using a hardness tester (Monsanto), and their *in vitro* release rates were determined using the rotating-bottle method.

RESULTS AND DISCUSSION

Polymer Solubility—The powdered polyethylene glycols and the carbohydrate polymers were eliminated from the study since all samples dissolved (all commercial types and molecular weights evaluated) within 1 hr. in artificial gastric fluid.

The polymeric material of the acrylic copolymer emulsion was found to exhibit solubility characteristics desirable for use as a sustained-action matrix. The polymer showed limited solubility in the pH region of 1.4–5.5 (7–11% of the polymer dissolved over a period of 5 hr.). The solubility rate greatly increased as the pH was raised from 5.5 to 6.9 and finally to 7.4. The sodium polyacrylate exhibited similar solubility characteristics in the low pH range but dissolved almost immediately when the pH of the test fluid was raised to 5.5.

The results indicate that the sodium polyacrylate might be suitable as an enterosoluble agent and that the acrylic copolymer has solubility properties more appropriate for a sustained-action preparation. The high molecular weight (over 300,000), available toxicity data (15), and linear nature of this acrylic copolymer (16), coupled with its solubility characteristics, justified further investigation as to its applicability to drug entrapment and sustained-release preparation.

Reproducibility of the Entrapment Procedure—The data in Table III show the assayed amounts of drug found in each of six identically prepared entrapment products. Two analyses were performed on each batch, with the averages being used to calculate the standard deviation of the batches prepared.

Based on the statistical analysis of the experimental data, one can expect that approximately 90% (employing 5 degrees of freedom) of the sample values will fall within ± 5.12 of the sample mean (*i.e.*, 132.87 ± 5.12). The coefficient of variability falls within the 5% limits that are established for most pharmaceutical preparations.

Table III—Reproducibility of the Entrapment Process

Batch No.	mg. of Methapyrilene HCl/g. of Collected Solid	Average of Two Assays
1	135.28 134.59	134.94
2	133.05 139.11	136.08
3	133.94 133.87	133.91
4	130.99 129.77	130.38
5	132.95 131.69	132.32
6	127.22 131.98	129.60

Effect of Drug Concentration on Entrapment Results—Figure 1 depicts the results obtained when various concentrations of methapyrilene hydrochloride solutions were employed with a constant amount of acrylic copolymer emulsion. The same formula (except for the drug concentration) and technique were employed as outlined for the drug entrapment procedure. It appears that a linear relationship exists between the initial drug concentration (in the range of 0.13–2.01 molal) and the amount of drug entrapped in the solid product. The equation for the line covering this portion of the graph was estimated employing the method of least squares.

The equation established was:

$$X_m = 14.22\,(M) + 4.19 \qquad \text{(Eq. 1)}$$

where M represents the molality of the methapyrilene hydrochloride solution and X_m is the amount (g.) of methapyrilene hydrochloride found in 100 g. of the resultant mixture. The sample correlation coefficient (r) found for the points used to calculate the equation was 0.995, indicating that the observed points are all very close to the calculated regression line. It is, therefore, possible to predict accurately the amount of methapyrilene hydrochloride to be found in an entrapment product. This relationship also allows for the reproducible production of products that might exhibit different and desirable effects, which are controllable by the drug–polymer ratio of the system.

No definite explanation can be presented for the break in the curve (Fig. 1) as the molality of the methapyrilene hydrochloride solution exceeds 2.01. The aqueous solubility limit of methapyrilene hydrochloride is being approached at the 3.0 molal concentration level. However, the range of 0.13–2.01 molal solutions presents a very broad and useful area for the production of acrylic copolymer–methapyrilene hydrochloride entrapment compositions.

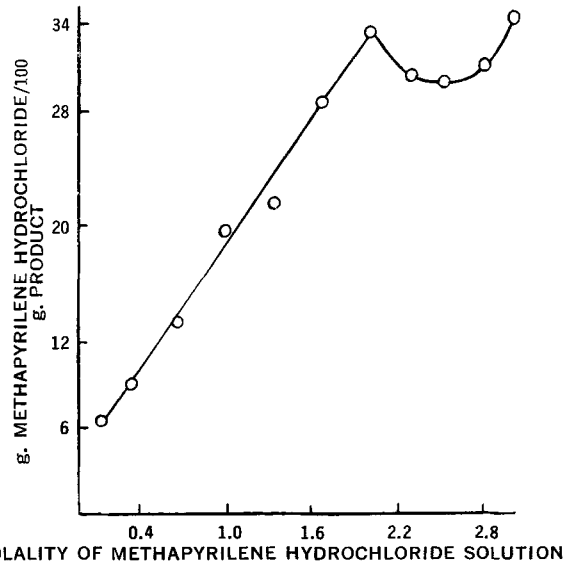


Figure 1—*Effect of drug concentration on the acrylic copolymer–methapyrilene hydrochloride entrapment ratio.*

Table IV—Dialysis of Acrylic Copolymer–Methapyrilene Hydrochloride Entrapment Compositions

Sample No.	Hours Dialyzed	Molarity[a] of System	Molarity Outside the Sac	Molarity Outside / Molarity of System
1[b]	48	7.36	7.24	0.98
2	48	7.68	7.12	0.93
3	48	7.77	7.08	0.91
4[b]	72	7.05	6.97	0.98
5	72	7.39	6.97	0.94
6[b]	107	6.92	6.50	0.94
7	107	7.43	6.50	0.87
8	107	7.42	7.12	0.96
9	107	7.00	6.30	0.90

[a] Molarity of the system was based on the total drug concentration found inside and outside of the dialysis sacs. All molarity concentrations presented are times 10^3. [b] Represents free drug samples, while all others are polymer–drug entrapment compositions.

Drug Availability from Polymer–Drug Solutions—Preliminary dialysis studies showed that the dialysis membrane employed acted as an impermeable barrier towards the polymer molecules (acrylic copolymer) while allowing free passage of the methapyrilene hydrochloride. The pH of the test fluid (range 1.4–7.4) had no apparent effect on the dialysis of the drug across the membrane or upon the impermeability of the membrane towards the polymer molecules. Table IV presents the dialysis data for the drug and polymer–drug entrapment systems.

If complete equilibration occurred, the value obtained by dividing the molarity outside the dialysis sac by the total calculated molarity of the system would be equal to 1; but as can be noted in Table IV, even in the cases of the free drug samples the values attained were not exactly 1. These slight differences of the drug systems from unity may be attributed to errors involved in the dialytic and analytical procedures or to a low order of drug binding to the membrane.

No major differences were noted at the three time intervals employed. It appears that approximately 5–10% of the drug in the polymer–drug product is not passing freely through the membrane and, in some manner, is being bound or retarded by the polymeric system. It was concluded that the major portion (90% or more) of the methapyrilene hydrochloride present in the polymer–drug entrapment products is readily available for passage through a semipermeable membrane. It was felt that this would also be true in a biological system where the membrane of the gastrointestinal tract would act as the semipermeable barrier, excluding the high molecular weight polymer and permitting passage (absorption) of the unbound drug molecules.

***In Vitro* Release-Rate Studies**—Data given in Table V show the release-rate patterns of six individually prepared batches of the acrylic copolymer–methapyrilene hydrochloride entrapment product. In all cases, the samples had been screened through a 60-mesh screen and were weighed to contain 100 mg. of drug (all mixtures contained approximately 13.3% active ingredient) (Table III).

Standard deviations of the release rates for the individual sample periods are shown at the bottom of Table V. Figure 2 represents a graphical presentation of the mean cumulative percent release values plotted against time.

Table V—Release Rates of Acrylic Copolymer–Methapyrilene Hydrochloride Entrapment Compositions

Batch No.	Cumulative Percent Release 0.5 hr.	1.5 hr.	2.5 hr.	4.5 hr.	6.5 hr.	8 hr.
1	54.8	63.2	77.2	87.5	95.9	95.9
2	49.5	71.5	78.3	84.4	101.6	101.6
3	42.3	63.2	70.2	85.2	105.1	—
4	38.1	57.3	66.5	72.9	95.0	95.0
5	45.3	63.2	72.8	76.8	98.7	100.0
6	40.9	57.3	65.4	72.1	98.4	99.3
Mean	45.7	62.6	71.7	79.8	99.1	98.4
SD	6.2	5.2	5.4	6.7	3.7	2.8

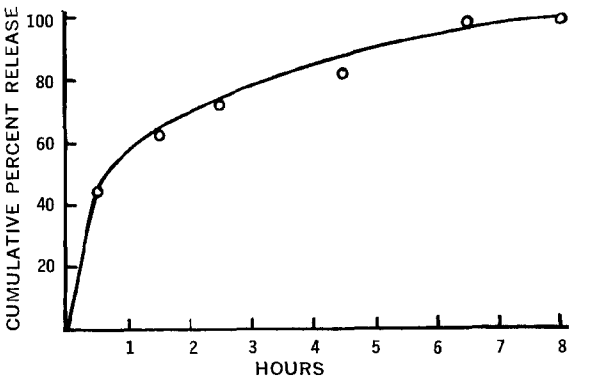


Figure 2—In vitro *release-rate pattern of the acrylic copolymer–methapyrilene hydrochloride entrapment product.*

Several investigators (1, 17–19) have attempted to correlate *in vitro* release-rate patterns with *in vivo* results. Urinary excretion rate data of various sustained-action dosage forms of dextroamphetamine sulfate, phenylpropanolamine hydrochloride, and trimeprazine maleate have been compared with *in vitro* release-rate results obtained with the rotating-bottle method.

The reported *in vitro* release values shown in Table VI were for those products exhibiting suitable sustained-action properties (usually 10–12 hr.) when evaluated *in vivo* by urinary excretion studies. The experimental data (entrapment composition) depicted in Table VI were found to lie within or very close to the values reported for the satisfactory sustained-action products. The entrapment product exhibited *in vitro* sustained-action characteristics in the fine subdivided state without further formulation into granules, pellets, and compression-coated, sugar- or film-coated, or layer tablets.

The results shown in Table VII illustrate the effect of the polymer–drug ratio (percent drug present in the entrapment product) on the release-rate pattern. The polymer–drug ratio may be another means of controlling and changing release-rate patterns.

The 13.3% level of entrapment was used for *in vivo* evaluation because it presented what was believed to be the best *in vitro* characteristics for methapyrilene hydrochloride. The more rapid release patterns may be useful for drugs with a more limited solubility characteristic or when this fine material is formulated into some compacted dosage form.

***In Vitro* Release Rates from Tablet Preparations**—The results in Table VIII show the effect of tableting on the release-rate patterns. The tablets containing 13.3% drug with 10% starch as a disintegrant completely broke apart in about 10 min. The resultant material exhibited almost the identical release-rate pattern as the original 60-mesh screened powder employed for the production of these tablets. This formulation and tableting approach could be used advantageously when the original powdered material exhibits suitable release characteristics.

The tablets (13.3% drug) compressed without any disintegrating agent failed to break apart in the 8-hr. period investigated. In the first 0.5–1.5 hr. of immersion in artificial gastric fluid, the tablets were swollen to about twice their original size. During the initial test period (0–1.5 hr.), 45.6% of the entrapped drug was being released, presumably by a leaching action of the test fluids. This

Table VI—Comparison of Reported Release Values with Experimental Results

Time, hr.	Cumulative Percent Release—Reported Values	Experimental Values
0.5	32–43	45.7
1.5	—	62.6
2	39–69	68.5[a]
2.5	—	71.4
4.5	60–90	79.8
6.5	—	99.1
7	86–98	97.0[a]

[a] These values were graphically determined from Fig. 2.

Table VII—Effect of the Polymer–Drug Ratio on the Release-Rate Pattern

Time, hr.	Percent Drug			
	13.3	19.7	28.5	33.4
	Cumulative Percent Release			
0.5	45.7	60.4	60.4	74.3
1.5	62.6	73.2	71.8	81.3
2.5	71.7	82.8	77.1	83.2
4.5	79.8	87.3	82.3	88.7
6.5	99.1	93.1	92.0	91.7
8	98.4	94.7	94.3	92.6

leaching effect was felt to be the predominant mechanism of release until about the 3rd or 4th hr. when the tablets began to diminish in size due to the slow dissolution of the polymeric material (as the pH of the test fluid increased). The portion of the drug content more tightly entrapped in the polymeric network was then being slowly released as the polymer dissolved.

The tablets containing 33.4% drug prepared with and without the addition of starch had the same general appearance during release-rate evaluation as those containing 13.3% drug without any added disintegrating agent (*i.e.*, initial swelling and then slow dissolution with increasing pH of the test fluids). The total release (in 8 hr.) from these tablets was less than that found for the tablets containing 13.3% drug. This reduction in total release of the two-tablet systems containing 33% drug is undoubtedly due to the increased hardness of these tablets (Table VIII).

The tablets containing 13.3% active ingredient (no starch) and the ones containing 33.4% drug with 10% starch demonstrated very uniform hourly rates of drug release for the test period from 2.5 to 8 hr. (Table IX).

***In Vivo* Studies**—Results shown in Table X were obtained after oral administration of free methapyrilene hydrochloride samples and a polymer–drug entrapment product to guinea pigs, with the animals then being subjected to the histamine aerosol chamber. The 95% confidence interval for the *difference* between the means (pure drug and entrapped drug) was found to be 4.9 ± 1.2 hr., indicating an increased duration of activity of the drug when it is administered in the polymer–drug entrapment system. It was concluded that the drug was slowly being released from the entrapment product in concentrations adequate to maintain a pharmacologic effect over this extended period of time.

Toxicity Reduction—An oral dose of 200 mg./kg. of free methapyrilene hydrochloride was found to kill five out of six rats within 30 min. after administration of the drug. The rats died in an acute convulsive state. One of the six rats survived the preselected 24-hr. cutoff point.

The same dose of methapyrilene hydrochloride administered in a polymer–drug entrapment product had no lethal effect on six rats when observed over a 24-hr. period. The results of this acute toxicity study present further supportive data for the *in vitro* and guinea pig investigations, showing that the polymer–drug entrapment product is preventing the immediate release of all the drug and is protracting its availability over an extended period of time.

Mechanism of Entrapment—Table XI summarizes the results obtained when testing the hypothesis that the acrylic copolymer emulsion system would be more stable to electrolytes as the pH of the system was increased. The results indicate that the original hypothesis was correct and that the presence of methapyrilene hydrochloride was causing flocculation of the polymeric system as exemplified in the drug entrapment procedure.

During the entrapment procedure as the added polymeric system was being flocculated, the drug molecules were entrapped (enclosed) in the formed aggregates, thus retarding their release in subsequent test procedures.

The cause of flocculation can be traced to the added electrolyte (drug molecules), which decreased the thickness of the diffuse ionic layer (of the polymeric material) and thus facilitated the flocculation of the polymeric material. Whether the approaching particles agglomerate or not is determined by the balance of the attractive van der Waals-London forces and the repulsive coulombic forces (20).

Further evidence that the drug was not chemically interacting with the polymer in some salt or direct ionic bond formation was the fact that a chloride analysis showed that approximately 50% of the entrapped drug was still in the chloride salt form. Jirgensons (20) reports that instances in which discharging and resultant flocculation can be explained as an ionic interaction between the ions of the particles and those of the added electrolyte are very rare.

Baron (21) has defined a type of entrapment product, known as inclusion compounds, which does not arise from the linkage of two reactants by means of covalent or coordinate bonds but from the ability of one compound to enclose another spatially. "The enclosed compound (guest) is in a situation whereby it cannot readily

Table IX—Uniformity of Release from Selected Polymer–Drug Tablets

Time Intervals, hr.	Percent Hourly Release	
	13.3% Drug Tablet (No Starch, Hardness 5.0 kg.)	33.4% Drug Tablet (10% Starch, Hardness 9.0 kg.)
2.5–4.5	4.3	4.2
4.5–6.5	4.4	4.2
6.5–8	6.5	4.7

Table X—Measurable Protection Times Afforded to the Guinea Pigs by Free Methapyrilene Hydrochloride and a Polymer–Drug Entrapment Product

Free Methapyrilene HCl		Entrapped Methapyrilene HCl	
Animal No.	Hours Protected	Animal No.	Hours Protected
1	2	10	10
2	6	11	10
3	Sick	12	10
4	2	13	Sick
5	3	8	11
6	1	9	4
7	6	4	9
8	8	5	10
9	6	6	9
5	2	8	10
6	2	9	4
	x_1 = 3.8 hr.		x_2 = 8.7 hr.

Table VIII—Effect of Tableting on the *In Vitro* Release-Rate Pattern

Time, hr.	Cumulative Percent Release					
	13.3% Drug (Fine Powder)	13.3% Drug Tablet (10% Starch)	13.3% Drug Tablet (No Starch)	33.4% Drug (Fine Powder)	33.4% Drug Tablet (10% Starch)	33.4% Drug Tablet (No Starch)
0.5	45.7	45.5	17.2	74.3	16.1	25.3
1.5	62.6	64.9	45.6	81.3	30.0	38.1
2.5	71.7	73.8	59.9	83.2	40.0	51.0
4.5	79.8	81.8	68.1	88.7	48.3	53.8
6.5	99.1	97.0	76.9	91.7	56.7	63.8
8	98.4	100.0	86.7	92.6	63.7	64.4
		Tablet Hardness, 5.0 kg. Tablet Weight, 946–758 mg.			Tablet Hardness, 9.0 kg. Tablet Weight, 299–306 mg.	

Table XI—Flocculation Values of Sodium Chloride and Methapyrilene Hydrochloride for an Acrylic Copolymer Emulsion System

	Flocculation Value[a] pH 3.3	pH 6.0
Sodium chloride	250	1000–1250
Methapyrilene hydrochloride	10	35

[a] Flocculation value is the concentration of the drug in mmoles/l. in the final system required to cause complete flocculation within 2 hr.

leave its position, although it is not actually bonded to the including compound" (21).

The polymer–drug entrapment products formed in this study do not directly fall into this class but may be considered as a type of inclusion system where the host molecules are being held together in a flocculated (aggregated) state entrapping the guest (drug) molecules.

Application of the Process to Entrapment of Other Drugs—Table XII depicts the results obtained employing the flocculation procedure for 11 other cationic drugs. The drugs studied readily caused flocculation of the polymeric material, and the majority of these had flocculation values of about 10–20. The method of drug entrapment described in this study appears applicable to many cationic drugs, without changing the procedure. Possible modifications that could be made in the entrapment process are: (*a*) the use of anionic medicinal agents for the flocculation of cationic polymeric systems; (*b*) the application of nonaqueous solvent systems to cause flocculation; and (*c*) the use of physiologically inert electrolytes to aid in the flocculation and entrapment technique.

Figure 3 illustrates the effect of the phenylephrine concentration employed in the entrapment procedure on the amount of drug found entrapped in the collected solid mixture. The percent of drug concentration of the entrapment product was found to increase sharply as the molality of the solution employed increased from 0.98 to 1.72. Further increases in molality produced only comparatively slight changes in the polymer–drug ratio until the sudden upswing occurring at the molalities of 3.50 and 3.68, similar to the upswing noted with methapyrilene hydrochloride (Fig. 1). The study of acrylic copolymer–phenylephrine hydrochloride entrapment products further exemplifies the applicability of the developed entrapment procedure to other drugs.

SUMMARY AND CONCLUSIONS

The phenomenon of the flocculation of polymeric systems has been evaluated for the molecular entrapment of drugs as a physicochemical approach to the development of oral controlled- or sustained-action dosage forms.

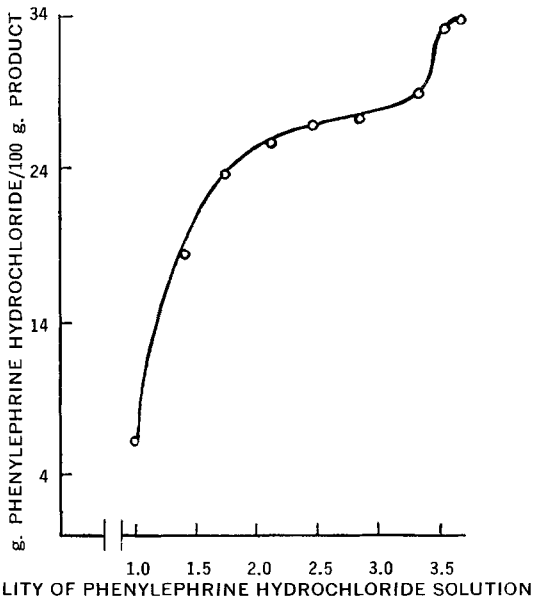


Figure 3—*Effect of drug concentration on the acrylic copolymer–phenylephrine hydrochloride entrapment ratio.*

A solid reproducible molecular entrapment composition of methapyrilene hydrochloride and an acrylic copolymer was prepared and evaluated for sustained-action properties.

In vitro results obtained using the rotating-bottle method indicated satisfactory sustained-release properties of the drug from the entrapment product. The results of *in vivo* studies in guinea pigs were found to correlate with the *in vitro* data. An increase in the continuous duration of action of 4.9 ± 1.2 hr. was shown for the polymer–drug product when compared with a mean duration for the free drug.

A reduction of the acute toxicity of methapyrilene hydrochloride in the entrapped form was established by an *in vivo* investigation in rats.

The polymer–drug entrapment product was found to exhibit characteristic protracted-release patterns in the tableted as well as the fine-powder state.

The broad application of the entrapment process to the acid salts of numerous cationic nitrogen-containing medicinal agents was demonstrated.

Table XII—Flocculation of an Acrylic Copolymer Emulsion System by Acid Salts of Various Cationic Nitrogen-Containing Medicinals

Compound	Molecular Weight	Type of Amine	Category	Flocculation[a] Value
d-Amphetamine sulfate	368.5	Primary	Central stimulant	10
Chlorpromazine hydrochloride	355.3	Tertiary	Tranquilizer	10
Atropine sulfate	694.9	Tertiary	Parasympatholytic	10
Homatropine methylbromide	370.3	Quaternary ammonium	Parasympatholytic	20
Ephedrine hydrochloride	201.7	Secondary	Sympathomimetic	20–25
Phenylephrine hydrochloride	203.7	Secondary	Sympathomimetic	40–50
Morphine sulfate	758.9	Tertiary	Narcotic analgesic	10–15
Dihydrocodeinone bitartrate	494.5	Tertiary	Antitussive	20
Methapyrilene hydrochloride	297.9	Tertiary	Antihistaminic	10
Pyrilamine maleate	401.5	Tertiary	Antihistaminic	10
Chlorpheniramine maleate	390.9	Tertiary	Antihistaminic	10

[a] The flocculation value is the concentration of the drug in mmoles/l. in the final system required to cause complete flocculation within 2 hr.

REFERENCES

(1) J. C. Souder and W. C. Ellenbogen, *Drug Stand.*, **26**, 77 (1958).

(2) E. O. Krueger and E. B. Vliet, *J. Pharm. Sci.*, **51**, 181(1962).

(3) H. M. Lee, R. C. Anderson, and P. N. Harris, *Proc. Soc. Exp. Biol. Med.*, **80**, 458(1952).

(4) S. M. Feinberg, S. Malkiel, T. B. Berstein, and B. J. Hargis, *J. Pharmacol. Exp. Ther.*, **99**, 195(1950).

(5) H. Goodman, C. T. Rhodes, A. M. Knevel, and G. S. Banker, *Can. J. Pharm. Sci.*, **3**, 69(1968).

(6) C. N. Reilly and D. T. Sawyer, "Experiments for Instrumental Methods," McGraw-Hill, New York, N. Y., 1961, pp. 187–188.

(7) S. Riegelman, L. A. Strait, and E. Z. Fischer, *J. Pharm. Sci.*, **51**, 129(1962).

(8) I. M. Klotz, F. M. Walker, and R. B. Proan, *J. Amer. Chem. Soc.*, **68**, 1486(1946).

(9) T. Higuchi and R. Kuramoto, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 393(1954).

(10) B. Borgstrom, A. Dahlqvist, G. Lundh, and J. Sjovall, *J. Clin. Invest.*, **36**, 1521(1957).

(11) H. A. Smith, R. V. Evanson, and G. J. Sperandio, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 94(1960).

(12) H. M. Lee, W. G. Dinwiddle, and K. K. Chen, *J. Pharmacol. Exp. Ther.*, **90**, 83(1947).

(13) H. A. Smith, Ph.D. thesis, Purdue University, Lafayette Ind., 1958.

(14) E. H. Riddle, "Monomeric Acrylic Esters," Reinhold, New York, N. Y., 1954, p. 48.

(15) Rohm & Haas Co., Acrysol ASE-75, Pamphlet AP-9-56, Nov. 1956.

(16) W. A. Kulik, personal communication.

(17) K. G. Shenoy, D. G. Chapman, and J. A. Campbell, *Drug Stand.*, **27**, 77(1959).

(18) K. R. Heimlich, D. R. Macdonnell, A. Polk, and T. L. Flanagan, *J. Pharm. Sci.*, **50**, 213(1961).

(19) K. R. Heimlich, D. R. Macdonnell, T. L. Flanagan, and P. D. O'Brien, *ibid.*, **50**, 231(1961).

(20) B. Jirgensons and M. E. Straumanis, "A Short Textbook of Colloid Chemistry," Wiley, New York, N. Y., 1954, pp. 301–318.

(21) M. Baron, *Org. Chem. Bull.*, **29**, 2(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication March 3, 1970.

* Present address: U. S. Vitamin and Pharmaceutical Corp., New York, N. Y.

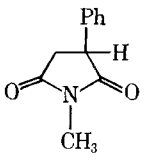
Synthesis and Anticonvulsant Properties of Some Derivatives of *N*-Methyl-2-phenylsuccinimide II

H. C. CLEMSON*, E. O. MAGARIAN†, and J. F. REINHARD*

Abstract □ Several substituted *N*-methyl-2-phenylsuccinimides have been prepared and evaluated for protective activity against convulsions induced by electroshock and pentylenetetrazole.

Keyphrases □ *N*-Methyl-2-phenylsuccinimide derivatives—synthesis □ Anticonvulsant activity—*N*-methyl-2-phenylsuccinimide derivatives □ IR spectrophotometry—structure □ NMR spectroscopy—structure

Phensuximide,[1] *N*-methyl-2-phenylsuccinimide (I), is a well-known anticonvulsant agent which has been employed in the treatment of petit mal epilepsy. The first paper of this series described the synthesis and biological evaluation of several *tert*-aminoalkyl derivatives of Structure I as potential anticonvulsants (1). As part of a continuing study, several additional derivatives of I have been prepared and screened for anticonvulsant properties.

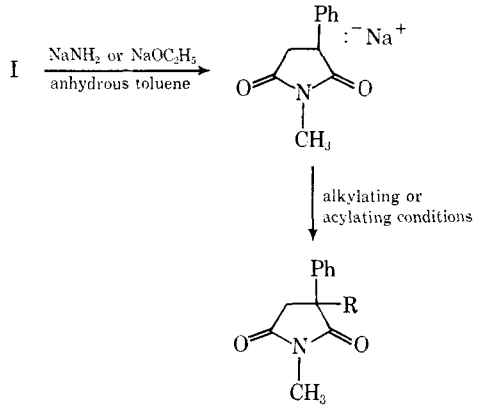


I

[1] Marketed as Milontin by Parke, Davis and Co., Detroit, MI 48232

DISCUSSION

Until recently, the methods used in the preparation of succinimide anticonvulsants have involved the cyclization of appropriate succinonitrile and succinic acid derivatives (2–6). However, the feasibility of direct substitution on the succinimide ring has been demonstrated as a useful synthetic tool (1, 7, 8).



IIa; R = $-CH_2Ph$
IIb; R = $-COCH_3$
IIc; R = $-COPh$

Scheme I

REFERENCES

(1) J. C. Souder and W. C. Ellenbogen, *Drug Stand.*, **26**, 77 (1958).

(2) E. O. Krueger and E. B. Vliet, *J. Pharm. Sci.*, **51**, 181(1962).

(3) H. M. Lee, R. C. Anderson, and P. N. Harris, *Proc. Soc. Exp. Biol. Med.*, **80**, 458(1952).

(4) S. M. Feinberg, S. Malkiel, T. B. Berstein, and B. J. Hargis, *J. Pharmacol. Exp. Ther.*, **99**, 195(1950).

(5) H. Goodman, C. T. Rhodes, A. M. Knevel, and G. S. Banker, *Can. J. Pharm. Sci.*, **3**, 69(1968).

(6) C. N. Reilly and D. T. Sawyer, "Experiments for Instrumental Methods," McGraw-Hill, New York, N. Y., 1961, pp. 187–188.

(7) S. Riegelman, L. A. Strait, and E. Z. Fischer, *J. Pharm. Sci.*, **51**, 129(1962).

(8) I. M. Klotz, F. M. Walker, and R. B. Proan, *J. Amer. Chem. Soc.*, **68**, 1486(1946).

(9) T. Higuchi and R. Kuramoto, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 393(1954).

(10) B. Borgstrom, A. Dahlqvist, G. Lundh, and J. Sjovall, *J. Clin. Invest.*, **36**, 1521(1957).

(11) H. A. Smith, R. V. Evanson, and G. J. Sperandio, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 94(1960).

(12) H. M. Lee, W. G. Dinwiddle, and K. K. Chen, *J. Pharmacol. Exp. Ther.*, **90**, 83(1947).

(13) H. A. Smith, Ph.D. thesis, Purdue University, Lafayette Ind., 1958.

(14) E. H. Riddle, "Monomeric Acrylic Esters," Reinhold, New York, N. Y., 1954, p. 48.

(15) Rohm & Haas Co., Acrysol ASE-75, Pamphlet AP-9-56, Nov. 1956.

(16) W. A. Kulik, personal communication.

(17) K. G. Shenoy, D. G. Chapman, and J. A. Campbell, *Drug Stand.*, **27**, 77(1959).

(18) K. R. Heimlich, D. R. Macdonnell, A. Polk, and T. L. Flanagan, *J. Pharm. Sci.*, **50**, 213(1961).

(19) K. R. Heimlich, D. R. Macdonnell, T. L. Flanagan, and P. D. O'Brien, *ibid.*, **50**, 231(1961).

(20) B. Jirgensons and M. E. Straumanis, "A Short Textbook of Colloid Chemistry," Wiley, New York, N. Y., 1954, pp. 301–318.

(21) M. Baron, *Org. Chem. Bull.*, **29**, 2(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication March 3, 1970.

* Present address: U. S. Vitamin and Pharmaceutical Corp., New York, N. Y.

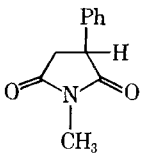
Synthesis and Anticonvulsant Properties of Some Derivatives of *N*-Methyl-2-phenylsuccinimide II

H. C. CLEMSON*, E. O. MAGARIAN†, and J. F. REINHARD*

Abstract □ Several substituted *N*-methyl-2-phenylsuccinimides have been prepared and evaluated for protective activity against convulsions induced by electroshock and pentylenetetrazole.

Keyphrases □ *N*-Methyl-2-phenylsuccinimide derivatives—synthesis □ Anticonvulsant activity—*N*-methyl-2-phenylsuccinimide derivatives □ IR spectrophotometry—structure □ NMR spectroscopy—structure

Phensuximide,[1] *N*-methyl-2-phenylsuccinimide (I), is a well-known anticonvulsant agent which has been employed in the treatment of petit mal epilepsy. The first paper of this series described the synthesis and biological evaluation of several *tert*-aminoalkyl derivatives of Structure I as potential anticonvulsants (1). As part of a continuing study, several additional derivatives of I have been prepared and screened for anticonvulsant properties.

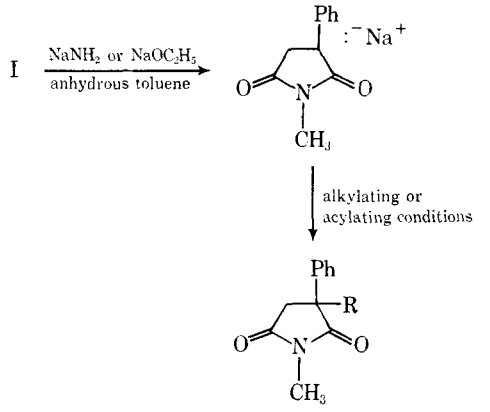


I

[1] Marketed as Milontin by Parke, Davis and Co., Detroit, MI 48232

DISCUSSION

Until recently, the methods used in the preparation of succinimide anticonvulsants have involved the cyclization of appropriate succinonitrile and succinic acid derivatives (2–6). However, the feasibility of direct substitution on the succinimide ring has been demonstrated as a useful synthetic tool (1, 7, 8).



IIa; R = $-CH_2Ph$
IIb; R = $-COCH_3$
IIc; R = $-COPh$

Scheme I

Table I—NMR Spectral Data

No.	Chemical Shift (δ)[a, b]
I	3.00 (2H,M), 3.02 (3H,S), 4.00 (1H,Q), 7.23 (5H,M)
II*a*	2.85 (3H,S), 3.06 (2H,S), 3.10 (1H,D),[c] 3.58 (1H,D),[c] 7.38 (10H,M)
II*b*	2.20 (3H,S), 2.69 (1H,D),[d] 3.03 (3H,S), 4.01 (1H,D),[d] 7.31 (5H,S)
II*c*	2.98 (1H,D),[d] 3.12 (3H,S), 4.07 (1H,D),[d] 7.42 (8H,M), 7.96 (2H,M)
III	3.07 (3H,S), 3.23 (1H,D),[d] 3.88 (1H,D),[d] 5.78 (1H,S), 7.18 (10H,M)
IV	2.60 (3H,S), 3.24 (1H,D),[d] 3.58 (1H,D),[d] 5.49 (1H,S), 7.43 (10H,M)
V	3.15 (3H,S), 3.71 (2H,S), 7.67 (5H,M)

[a] S = singlet, D = doublet, M = multiplet, Q = quartet. [b] All compounds were run as 10% solutions in deuterochloroform, excepting Compound IV whose concentration was 4% in deuterochloroform. [c] J = 13.5 c.p.s. [d] J = 18.0 c.p.s.

The proton in the 2-position of I is relatively acidic, because it is flanked by a benzene ring and a carbonyl group. Consequently, it was readily removed using either sodium amide or sodium ethoxide in anhydrous toluene. The resulting carbanion intermediate was subsequently utilized in alkylating and acylating reactions, as outlined in Scheme I.

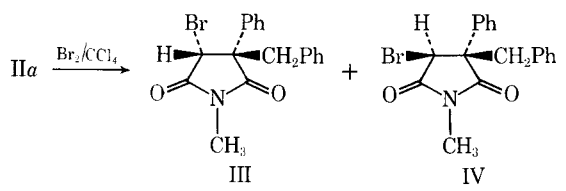
The bromination of II*a* in carbon tetrachloride yielded two compounds which are isomeric (Scheme II). The NMR spectra of the products indicate that they are geometric isomers having Formulas III and IV (Table I). The resonance peak for the methyl protons (2.60δ) in IV appears upfield to the corresponding peak in III (3.07δ).

It would appear from Dreiding stereomodels that Structure IV should possess a strong conformational bias, since the benzyl group is flanked on the one side by the large bromine atom and on the other side by the rather bulky phenyl group. As a result of this strong restriction to free rotation, the benzyl group is so oriented that the methyl group appears in the shielding portion of the induced currents of the aromatic ring. With less restriction to rotation, the benzyl group in III has a decreased influence on the methyl protons; consequently, their resonance peak occurs at a lower field position. The absorption peak for the methyl protons (2.84δ) in II*a*, the parent molecule, is intermediate in position to those in III and IV. Location of the methyl peak in III downfield to that in II*a* may be attributed to the influence of the bromine atom. Compound V, which was prepared by treating I with bromine in carbon tetrachloride (Scheme III), also displayed a similar deshielding effect by its bromine atom (Table I).[2]

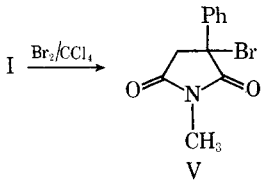
The substitution of the bromine atoms on the benzylic carbons to give diastereomers was also considered. However, the large difference in chemical shifts of the methyl protons cannot be rationalized in terms of these structures. Dreiding stereomodels indicate that the benzyl group should influence the methyl protons to about the same degree in either diastereomer, and that there should be little difference in their chemical shifts.

EXPERIMENTAL

All melting points have been determined on either a Fisher-Johns block or a Kofler micro hot stage and have been corrected.



Scheme II



Scheme III

Pertinent physical data for each of the products are listed in Table II.[3]

The IR spectra were determined on a Beckman IR-8 spectrophotometer using KBr pellets. All succinimide derivatives exhibited the two characteristic frequencies between 1690 and 1775 cm.$^{-1}$ (9–11).

NMR spectra were acquired using a Varian A-60 spectrometer and deuterochloroform solutions with tetramethylsilane as an internal standard. Appropriate data are given in Table I.

***N*-Methyl-2-phenylsuccinimide (I)**—This compound was prepared according to the procedure of Miller and Long (12).

2-Benzyl-*N*-methyl-2-phenylsuccinimide (II*a*)—To 0.10 mole of sodium amide in liquid ammonia (2.3 g. of sodium metal in 150 ml. of liquid ammonia), 18.9 g. (0.10 mole) of *N*-methyl-2-phenylsuccinimide (I) in 150 ml. of anhydrous toluene was added with stirring. After heating at reflux for 3 hr., the mixture was cooled to room temperature and a solution of freshly distilled benzyl chloride (12.7 g., 0.10 mole in 50 ml. of anhydrous toluene) was added with stirring over a period of 15 min. The reaction mixture was heated at reflux for 30 min. and then allowed to stir at room temperature for 12 hr. Finally, it was treated with 20 ml. of water, and the organic layer was separated and dried ($MgSO_4$). The toluene was removed *in vacuo*, yielding an oil which solidified upon standing. The product was recrystallized from 95% ethanol to give 20.2 g. of II*a*.

2-Acetyl-*N*-methyl-2-phenylsuccinimide (II*b*)—A solution of 18.9 g. (0.10 mole) of *N*-methyl-2-phenylsuccinimide (I) and 150 ml. of anhydrous toluene was added to 0.15 mole of sodium ethoxide in 200 ml. of absolute ethanol. The ethanol–toluene azeotropic mixture was distilled, and the volume of the reaction mixture was kept constant by the addition of anhydrous toluene. After the suspension had been cooled to 35°, 15.3 g. (0.15 mole) of acetic anhydride dissolved in 60 ml. of anhydrous toluene was added and the mixture was stirred for 8 hr. at 35°. The mixture was then treated with 100 ml. of a 10% sodium bicarbonate solution. The toluene layer was dried over anhydrous $MgSO_4$ and evaporated *in vacuo* to give a viscous, yellow liquid which solidified when triturated with 95% ethanol. Recrystallization from the same solvent gave 13.6 g. of II*b*.

2-Benzoyl-*N*-methyl-2-phenylsuccinimide (II*c*)—This derivative was prepared in a manner similar to the procedure for II*b* except that 15.5 g. (0.11 mole) of benzoyl chloride was used in place of the anhydride. The product was recrystallized from 95% ethanol, yielding 14.9 g. of II*c*.

Bromination of 2-Benzyl-*N*-methyl-2-phenylsuccinimide (III and IV)—To a mixture of 4.9 g. (0.018 mole) of 2-benzyl-*N*-methyl-2-phenylsuccinimide (II*a*) and 50 ml. of carbon tetrachloride at reflux was added dropwise 2.8 g. (0.018 mole) of bromine over a period of several hours. When the addition was complete, the mixture was heated at reflux for an additional 14 hr., followed by the addition of 50 ml. of water. The organic layer was separated and dried ($MgSO_4$). Evaporation of the carbon tetrachloride solution *in vacuo* gave an oil which solidified upon cooling. Recrystallization from *sec*-butyl alcohol resulted in two fractions. The less soluble fraction gave 1.7 g. of III, and the second fraction yielded 3.8 g. of IV.

2-Bromo-*N*-methyl-2-phenylsuccinimide (V)—A stirred solution of 2.5 g. (0.013 mole) of *N*-methyl-2-phenylsuccinimide (I) in 20 ml. of carbon tetrachloride was treated dropwise with 2.1 g. (0.013 mole) of bromine in 20 ml. of carbon tetrachloride. After the reaction mixture had been heated at reflux for 48 hr., the solvent was removed *in vacuo*. The residue was triturated with 95% ethanol; the solid, thus obtained, was recrystallized from this solvent, affording 2.1 g. of V.

[2] The preparation of V by a different method was previously reported by Izzo (13).

[3] Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and by Micro-Analysis, Inc., Wilmington, Del.

Table II—Derivatives of *N*-Methyl-2-phenylsuccinimide

No.	M.p.	Molecular Formula	Anal., % Calcd.	Found	Yield, %
II*a*	65–67°	$C_{18}H_{17}NO_2$	C, 77.43 H, 6.09 N, 5.02 O, 11.46	C, 77.27 H, 6.15 N, 5.05 O, 11.23	72
II*b*	92–94°	$C_{13}H_{13}NO_3$	C, 67.53 H, 5.63 N, 6.06 O, 20.78	C, 67.38 H, 5.52 N, 6.20 O, 20.64	58
II*c*	147–149°	$C_{18}H_{15}NO_3$	C, 73.72 H, 5.12 N, 4.78 O, 16.38	C, 73.73 H, 5.10 N, 4.78 O, 16.32	51
III	161–164°	$C_{18}H_{16}BrNO_2$	C, 60.37 H, 4.47 Br, 22.32 N, 3.90 O, 8.93	C, 60.21 H, 4.55 Br, 22.35 N, 3.74 O, 9.09	21[a]
IV	117–119°	$C_{18}H_{16}BrNO_2$	C, 60.37 H, 4.47 Br, 22.32 N, 3.90 O, 8.93	C, 60.14 H, 4.36 Br, 22.57 N, 3.76 O, 9.11	48[a]
V	113–115°[b]	$C_{11}H_{10}BrNO_2$	C, 49.29 H, 3.73 Br, 29.82 N, 5.22 O, 11.94	C, 49.50 H, 3.91 Br, 29.62 N, 5.31 O, 12.07	60

[a] Combined yield from the bromination of II*a* = 69%. [b] Lit. m.p. (13) = 110.5–112°.

PHARMACOLOGY

Methods and Materials—The succinimides were tested for anticonvulsant activity by modifications of two methods described by Swinyard *et al.* (14). In the first procedure (maximal electroshock seizures), the compounds were suspended in 10% (aqueous) acacia and administered orally to adult, male, albino mice in groups of 10 at a dosage of 200 mg./kg. At intervals of 0.5, 1, and 2 hr. thereafter, the animals of each group were challenged by delivering 60 ma. (a.c.) through the corneal electrodes for 0.2 sec. The end-point indicating anticonvulsant activity is the abolition of the hindlimb tonic extensor component of the maximal seizure pattern. The time of peak effect was determined by observing which time interval gave optimum protection. Whenever substantial effectiveness was demonstrated in this manner, a second investigation was undertaken to determine potency more precisely. The compound was administered at three or more dosage levels, generally 10 animals per dosage, and the animals were challenged at the predetermined time of peak effect. The dosage of compound required to produce the anticonvulsant end-point in 50% of the animals (ED_{50} with 95% fiducial limits) was computed by the method of Litchfield and Wilcoxon (15).

The second procedure (chemoshock) involved protection against a chemical convulsant, pentylenetetrazol.[4] Again, the compound was administered orally at a dosage of 200 mg./kg. to 10 adult, male, albino mice. At the time of peak effect, a 97% convulsant dosage of pentylenetetrazol (106 mg./kg.) was injected subcutaneously. Anticonvulsant activity was indicated by the failure of clonic convulsive seizures to appear within 1 hr. following pentylenetetrazol. Whenever indicated, ED_{50}'s were determined in the manner previously described.

The mice[5] employed in these investigations were housed 20 per cage and fed Charles River Rat and Mouse Food and tap water *ad libitum*.

[4] Marketed as Metrazol by Knoll Pharmaceutical Co., Orange, NJ 07051

[5] Obtained from the Charles River Breeding Laboratories, N. Wilmington, Mass.

Table III—Anticonvulsant Activity of Succinimide Derivatives in the Mouse

Compound	Fraction of Animals Protected following 200 mg./kg. of Compound: Electroshock	Chemoshock	Time of Peak Activity, hr.
I	9/10[a]	9/10[b]	0.5
II*a*	0/5	0/10	—
II*b*	9/10[c]	9/10[d]	0.5
II*c*	0/10	2/10[e]	—
III	0/10	0/10[f]	—
IV	0/10	0/10	—
V	1/10	2/10[g]	1

[a] ED_{50} = 115 (92–144) mg./kg. [b] ED_{50} = 134 (87–208) mg./kg. [c] ED_{50} = 96 (64–144) mg./kg. [d] ED_{50} = 86 (60–124) mg./kg. [e] ED_{50} = 610 mg./kg. (approx.). [f] 4/10 protected at 400, only 2/10 at 800 mg./kg. [g] 6/10 protected at 400, but only 2/10 at 600 mg./kg.

Results and Discussion—The data summarized in Table III reveal that, in general, anticonvulsant potency was inversely proportional to the size of the side chain in the 2-position. Where this chain contained a benzyl or benzoyl group, anticonvulsant activity was either absent or weak in the sense that total protection could not be achieved at any dosage, owing to the superimposition of the toxicity of the test compound. However, the 2-acetyl derivative (II*b*) was the most active member of the series, equalling the potency of the parent compound, phensuximide (I). A similar relationship between the size of alkyl substituents in some aryldialkylsuccinimides and anticonvulsant potency was reported by Hauck *et al.* (6).

In all of the studies conducted on these succinimides, anticonvulsant activity was in all cases associated with depression of the CNS; yet the hypnotic activity did not appear in response to high dosage, even in the toxic range. Therapeutically, this combination of effectiveness against seizures without risk of undue CNS depression is a distinctly desirable feature of any anticonvulsant, since it must be given daily for prolonged periods.

REFERENCES

(1) H. C. Clemson, E. O. Magarian, G. C. Fuller, and R. O. Langner, *J. Pharm. Sci.*, **57**, 384(1968).

(2) C. A. Miller and L. M. Long, *J. Amer. Chem. Soc.*, **75**, 373 and 6256(1953).

(3) C. A. Miller and R. L. Hull, U. S. pat. 3,183,245 (1965).

(4) J. R. Dice and D. V. Lopiekes, U. S. pat. 2,975,193 (1962).

(5) A. M. Akkerman, G. C. Leeuwen, and J. F. Michels, French pat. 1,404,515 (1965).

(6) F. P. Hauck, Jr., J. Demick, and J. T. Fan. *J. Med. Chem.*, **10**, 611(1967).

(7) D. R. Bryant and C. R. Hauser, *J. Amer. Chem. Soc.*, **83**, 3468(1961).

(8) F. P. Hauck, Jr., and J. T. Fan, *J. Org. Chem.*, **34**, 1703 (1969).

(9) C. Fayat and A. Foucaud, *Compt. Rend.*, **261** (Group 6), 4018(1965).

(10) A. Foucaud, H. Person, and M. Duclos, *Bull. Soc. Chim. Fr.*, **1965**, 2252.

(11) T. Matsuo, *Bull. Chem. Soc. Jap.*, **37**, 1845(1964).

(12) C. A. Miller and L. M. Long, *J. Amer. Chem. Soc.*, **73**, 4895(1951).

(13) P. T. Izzo, *J. Org. Chem.*, **28**, 1713(1963).

(14) E. A. Swinyard, W. C. Brown, and L. S. Goodman, *J. Pharmacol. Exp. Ther.*, **106**, 319(1952).

(15) J. T. Litchfield and R. Wilcoxon, *ibid.*, **96**, 99(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 2, 1970, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, University of Rhode Island, Kingston, RI 02881*

Accepted for publication March 12, 1970.

Abstracted in part from a thesis submitted by H. C. Clemson to the Graduate School, University of Rhode Island, in partial fulfillment of Doctor of Philosophy degree requirements.

This investigation was supported in part by a National Institutes of Health predoctoral research fellowship award, Fellowship No. 5-F1-GM-19,760.

The authors thank Dr. Bruno Vittimberga, Department of Chemistry, University of Rhode Island, for valuable discussions.

* Present address: Graduate School of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115

† Present address: College of Pharmacy, Department of Pharmaceutical Chemistry, University of Kentucky, Lexington, KY 40506

Kinetic Salt Effect in Pharmaceutical Investigations

J. THURØ CARSTENSEN

Abstract □ The use of the kinetic salt effect in kinetic investigations has been widespread since its original derivation by Brønsted and Bjerrum in 1925. Its derivation is tied in with the Debye-Hückel theory, and it would be expected that the concentration range of applicability of the kinetic salt effect would be confined to that of the corresponding Debye-Hückel expression, *i.e.*, less than 0.01 *M*. A review of the pharmaceutical literature shows that applications may be extended to higher concentration ranges. Deviations from the Debye-Hückel expressions by the charged reactants and by the transition complex may be of the same magnitude and sign, and this may be the cause for the concentration range extension.

Keyphrases □ Kinetic salt effect—pharmaceutical solutions □ Ionic strength effect—high ionic concentrations □ Hydrolysis—kinetic salt effect □ Degradation, drug—kinetic salt effect

Properly conducted kinetic studies always, directly or indirectly, take into account the so-called kinetic salt effect. By varying the ionic strength by addition of an inert electrolyte (*e.g.*, NaCl) while keeping other concentrations constant, the rate constants for reacting species will either increase, remain constant, or decrease. Accordingly, the effect is denoted positive, absent, or negative. As shall be seen in the following discussion, the sign or the absence of the kinetic salt effect is a valuable aid in interpretation of mechanisms. Strictly quantitative relations between rate constants and ionic strength are only theoretically valid at exceedingly low concentrations. Since pharmaceutical investigations are most often conducted at ionic strength ranges higher than the theoretical limits, a review of findings from kinetics of pharmaceutical model systems might throw light on the actual range to which the kinetic salt effect can be extended.

THEORY

It can be shown (1) by means of transition-state theory that for a reaction in solution:

$$A + B \rightarrow [AB^{\dagger}] \rightarrow \text{products} \qquad \text{(Eq. 1)}$$

the rate constant, k, is related to the activity coefficients, γ, of the reactants (A and B) and the transition complex ($[AB^{\dagger}]$) by:

$$\log k = \alpha + \log [\gamma_A \gamma_B / \gamma_{[AB^{\dagger}]}] \qquad \text{(Eq. 2)}$$

The charges, z, of the three species are related to one another by $z_A + z_B = z_{[AB^{\dagger}]}$. Applying this and the Debye-Hückel limiting law:

$$\log \gamma = -Q \cdot z^2 \sqrt{\mu} \qquad \text{(Eq. 3)}$$

Table I—Values of $2Q = 3.65 \cdot 10^6 \cdot [\rho/\epsilon^3 T^3]^{0.5}$ at Various Temperatures

Temperature	$2Q^a$
20	1.008
25	1.018
30	1.026
35	1.036
40	1.046
45	1.057
50	1.068
55	1.079
60	1.092
70	1.117
80	1.145
90	1.174
100	1.198

[a] ϵ-values used in the computation are from *Reference 43*, and ρ-values are from *Reference 44*.

to Eq. 2 for a solution of overall ionic strength, μ, leads to the well-known Brønsted-Bjerrum equation (2–5):

$$\log k = \alpha + 2 \cdot Q \cdot z_A \cdot z_B \cdot \sqrt{\mu} \qquad \text{(Eq. 4)}$$

where $2Q = 1.018$ for aqueous solutions at 25°. Since $Q = 1.825 \cdot 10^6 \cdot [\rho/\epsilon^3 T^3]^{0.5}$, where ϵ is the dielectric constant, ρ is density, and T is the absolute temperature, the coefficient to $\sqrt{\mu}$ changes with temperature. A list of values of $2Q$ is given in Table I.

The Debye-Hückel equation is usually only obeyed in ionic strength ranges up to 0.01 (6). Figure 1 is an example of this showing the mean activity coefficients of hydrochloric acid in potassium chloride solutions of varying ionic strength (7). Equation 4 is, therefore, only strictly applicable up to this concentration.

The modified Debye-Hückel equation for higher concentrations is

$$\log \gamma_{\pm} = \frac{z^2 \cdot Q \cdot \sqrt{\mu}}{1 + \beta\sqrt{\mu}} \qquad \text{(Eq. 5)}$$

and holds up to an ionic strength of about $\mu = 0.1$ (8). Using this in the development outlined for Eq. 4 yields

$$\log k = \alpha + 2 \cdot Q \cdot z_A \cdot z_B \cdot \frac{\sqrt{\mu}}{1 + \beta\sqrt{\mu}} \qquad \text{(Eq. 6)}$$

It is recalled that μ is the overall ionic strength, but β depends on the ionic diameter of the reacting species, and this usually is unknown. Linearity and slopes, however, are not very sensitive to the magnitude of β, which is always close to unity; it is the practice of some authors (9) to test kinetic salt effects with this assumption, *i.e.*,

$$\log k = \alpha + 2 \cdot Q \cdot z_A \cdot z_B \cdot \frac{\sqrt{\mu}}{1 + \sqrt{\mu}} \qquad \text{(Eq. 7)}$$

The slope of such a plot should be close to the value of $2 \cdot Q \cdot z_A \cdot z_B$ (which is not necessarily an integer, all depending on tem-

Abstracted in part from a thesis submitted by H. C. Clemson to the Graduate School, University of Rhode Island, in partial fulfillment of Doctor of Philosophy degree requirements.

This investigation was supported in part by a National Institutes of Health predoctoral research fellowship award, Fellowship No. 5-F1-GM-19,760.

The authors thank Dr. Bruno Vittimberga, Department of Chemistry, University of Rhode Island, for valuable discussions.

* Present address: Graduate School of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115

† Present address: College of Pharmacy, Department of Pharmaceutical Chemistry, University of Kentucky, Lexington, KY 40506

Kinetic Salt Effect in Pharmaceutical Investigations

J. THURØ CARSTENSEN

Abstract □ The use of the kinetic salt effect in kinetic investigations has been widespread since its original derivation by Brønsted and Bjerrum in 1925. Its derivation is tied in with the Debye-Hückel theory, and it would be expected that the concentration range of applicability of the kinetic salt effect would be confined to that of the corresponding Debye-Hückel expression, *i.e.*, less than 0.01 *M*. A review of the pharmaceutical literature shows that applications may be extended to higher concentration ranges. Deviations from the Debye-Hückel expressions by the charged reactants and by the transition complex may be of the same magnitude and sign, and this may be the cause for the concentration range extension.

Keyphrases □ Kinetic salt effect—pharmaceutical solutions □ Ionic strength effect—high ionic concentrations □ Hydrolysis—kinetic salt effect □ Degradation, drug—kinetic salt effect

Properly conducted kinetic studies always, directly or indirectly, take into account the so-called kinetic salt effect. By varying the ionic strength by addition of an inert electrolyte (*e.g.*, NaCl) while keeping other concentrations constant, the rate constants for reacting species will either increase, remain constant, or decrease. Accordingly, the effect is denoted positive, absent, or negative. As shall be seen in the following discussion, the sign or the absence of the kinetic salt effect is a valuable aid in interpretation of mechanisms. Strictly quantitative relations between rate constants and ionic strength are only theoretically valid at exceedingly low concentrations. Since pharmaceutical investigations are most often conducted at ionic strength ranges higher than the theoretical limits, a review of findings from kinetics of pharmaceutical model systems might throw light on the actual range to which the kinetic salt effect can be extended.

THEORY

It can be shown (1) by means of transition-state theory that for a reaction in solution:

$$A + B \rightarrow [AB^{\dagger}] \rightarrow \text{products} \qquad \text{(Eq. 1)}$$

the rate constant, k, is related to the activity coefficients, γ, of the reactants (A and B) and the transition complex ($[AB^{\dagger}]$) by:

$$\log k = \alpha + \log [\gamma_A \gamma_B / \gamma_{[AB^{\dagger}]}] \qquad \text{(Eq. 2)}$$

The charges, z, of the three species are related to one another by $z_A + z_B = z_{[AB^{\dagger}]}$. Applying this and the Debye-Hückel limiting law:

$$\log \gamma = -Q \cdot z^2 \sqrt{\mu} \qquad \text{(Eq. 3)}$$

Table I—Values of $2Q = 3.65 \cdot 10^6 \cdot [\rho/\epsilon^3 T^3]^{0.5}$ at Various Temperatures

Temperature	$2Q^a$
20	1.008
25	1.018
30	1.026
35	1.036
40	1.046
45	1.057
50	1.068
55	1.079
60	1.092
70	1.117
80	1.145
90	1.174
100	1.198

[a] ϵ-values used in the computation are from *Reference 43*, and ρ-values are from *Reference 44*.

to Eq. 2 for a solution of overall ionic strength, μ, leads to the well-known Brønsted-Bjerrum equation (2–5):

$$\log k = \alpha + 2 \cdot Q \cdot z_A \cdot z_B \cdot \sqrt{\mu} \qquad \text{(Eq. 4)}$$

where $2Q = 1.018$ for aqueous solutions at 25°. Since $Q = 1.825 \cdot 10^6 \cdot [\rho/\epsilon^3 T^3]^{0.5}$, where ϵ is the dielectric constant, ρ is density, and T is the absolute temperature, the coefficient to $\sqrt{\mu}$ changes with temperature. A list of values of $2Q$ is given in Table I.

The Debye-Hückel equation is usually only obeyed in ionic strength ranges up to 0.01 (6). Figure 1 is an example of this showing the mean activity coefficients of hydrochloric acid in potassium chloride solutions of varying ionic strength (7). Equation 4 is, therefore, only strictly applicable up to this concentration.

The modified Debye-Hückel equation for higher concentrations is

$$\log \gamma_{\pm} = \frac{z^2 \cdot Q \cdot \sqrt{\mu}}{1 + \beta\sqrt{\mu}} \qquad \text{(Eq. 5)}$$

and holds up to an ionic strength of about $\mu = 0.1$ (8). Using this in the development outlined for Eq. 4 yields

$$\log k = \alpha + 2 \cdot Q \cdot z_A \cdot z_B \cdot \frac{\sqrt{\mu}}{1 + \beta\sqrt{\mu}} \qquad \text{(Eq. 6)}$$

It is recalled that μ is the overall ionic strength, but β depends on the ionic diameter of the reacting species, and this usually is unknown. Linearity and slopes, however, are not very sensitive to the magnitude of β, which is always close to unity; it is the practice of some authors (9) to test kinetic salt effects with this assumption, *i.e.*,

$$\log k = \alpha + 2 \cdot Q \cdot z_A \cdot z_B \cdot \frac{\sqrt{\mu}}{1 + \sqrt{\mu}} \qquad \text{(Eq. 7)}$$

The slope of such a plot should be close to the value of $2 \cdot Q \cdot z_A \cdot z_B$ (which is not necessarily an integer, all depending on tem-

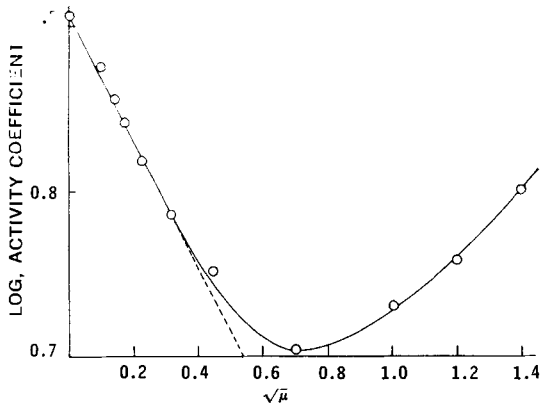


Figure 1—*Logarithm of the activity coefficient of hydrochloric acid as a function of the square root of ionic strength (2).*

perature) and should allow evaluation of the integer value of the product of z_A and z_B.

In the case of higher ionic strengths, an ionic strength effect is possible even if one or both reacting molecules are uncharged. At high ionic strength, if A was charged and B not, then reaction $A + B \rightarrow [AB^{\dagger}]$ would dictate that $z_{\dagger} = z_A$. At high ionic strength, the following relation (10) often holds:

$$-\log \gamma_A = -\log \gamma_{\dagger} = \frac{z^2 \cdot \alpha \cdot \sqrt{\mu}}{1 + \beta\sqrt{\mu}} - b \cdot \mu \qquad \text{(Eq. 8)}$$

At high ionic strength (11), the activity coefficient of the uncharged species adheres to

$$\log \gamma_B = b_B \mu \qquad \text{(Eq. 9)}$$

which, by a treatment similar to the one leading to Eq. 7, yields

$$\log k = \log k_0 + [b_A + b_B - b_{\dagger}] \cdot \mu \qquad \text{(Eq. 10)}$$

i.e., the logarithm of the rate constant should be proportional to the ionic strength. In the case of two uncharged molecules, application of Eq. 9 by itself would lead to an equation of the type of Eq. 10.

Aside from these so-called primary salt effects, a secondary salt effect at times is important. In buffer-catalyzed degradations, an ionized species B^- may be catalytic, whereas the corresponding acid, HB, may not be. The ionization constants of the acid are affected in a manner similar to that described by Eqs. 4 and 10. Increasing ionic strength can affect the concentration of B^- and, in this indirect manner, influence the value of the rate constant.

EXAMPLES

Zero Effect—One of the values of the kinetic salt effect as an experimental tool is that if a decomposition does not exhibit a kinetic salt effect, then reactions of two charged species can be eliminated from consideration, since either z_A or z_B would have to be zero. Examples of this are hydrolysis of the following compounds in aqueous solution: streptozotocin (12), streptovaricin (13), diethylaminoethylsalicylate hydrochloride (14), acetylsalicylic acid anhydride (15), procaine hydrochloride (16, 17), chloramphenicol (18), *N*-acetyl-*p*-aminophenol (19), chlorobutanol (20), ascorbic acid (21, 22), phenobarbital (23), idoxuridine (24), penicillin at low pH (25), morphine (26), cycloserine (27), and hydroxocobalamin (28).

Integer Effects—Where kinetic salt effects do exist, they imply that charged species are of identical or opposite charge. Since even model pharmaceutical systems by necessity are in concentration ranges above 0.01 ionic strength, Eq. 4 cannot be expected to hold. In all of the cases to be cited (29–38), however, linearity prevails, but the slopes differ from $2 \cdot Q \cdot z_A \cdot z_B$, and other evidence has been employed by the respective authors to support proposed mechanisms.

Brooke and Guttmann (29) used kinetic salt effect data in investigating the complex formation between 3-carbomethoxy-1-methylpyridinium cation (NME) and 8-chlorotheophyllinate anion (CT). Figure 2 shows their data plotted both according to Eqs. 4 and 7 and (in spite of the scatter) implies the applicability of Eq. 7

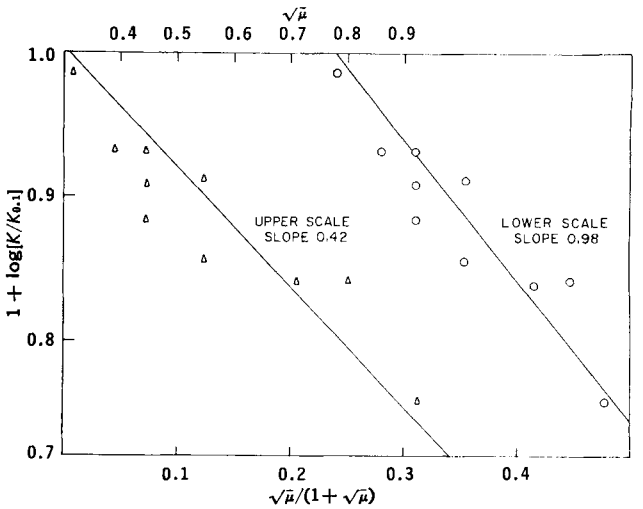


Figure 2—*Kinetic salt effect on degradation of 3-carbomethoxy-1-methylpyridinium cation (29) at 30°, plotted according to Eq. 4 (△, upper scale) and according to Eq. 7 (○, lower scale).*

up to an ionic strength of 1.2. When plotted according to Eq. 7, the slope is close to the theoretical.

Hussain *et al.* (30), in studying the degradation of echothiophate in aqueous solution, supported their proposed mechanism by a negative kinetic salt effect and found linearity up to an ionic strength of 0.02 when plotting was performed according to Eq. 4; the slope (0.9) is close to the theoretical as shown in Fig. 3. It is seen from Fig. 3, however, that use of Eq. 7 extends the useful range to an ionic strength of 0.2 (but not 0.5), still yielding a slope (0.91) close to the theoretical.

Felmeister *et al.* (31) employed the kinetic salt effect to support their contention that dismutation of the semiquinone free radical of chlorpromazine ($C\cdot$) took place *via* a reaction of the type $C\cdot^{++} + H^+ \rightarrow [HC\cdot^{+++}]$. However, they point out that at pH 1.96, the species $H_2C\cdot^{+++}$ might also react with H^+ to form a transition complex. The former type, at 25°, should yield a positive slope of 2.036, as opposed to the value of 1.6 found when plotting was performed according to Eq. 4. As shown in Fig. 4, plotting according to Eq. 7 yields a slope of 2.3, *i.e.*, somewhat closer to the theoretical value, and may be implying that $H_2C\cdot^{+++} + H^+$ plays a part in the overall scheme. The useful range would appear to be up to $\mu = 0.2$, but tabulated data (31) would imply linearity according to Eq. 7 (but not Eq. 4) up to $\mu = 0.5$.

Finholt *et al.* (25) demonstrated a positive kinetic salt effect in penicillin degradation at alkaline pH. Their data are shown in Fig. 5, plotted according to both Eqs. 4 and 7. At pH 6.8, the positive effect is used to postulate penicillin (P) degradation according to the scheme $P^- + HPO_4^= \rightarrow$ products, and the slope of 2 resulting from Eq. 7 would support such a view. The probable mechanism at pH

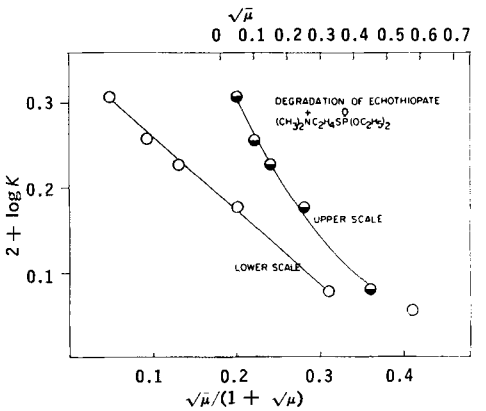


Figure 3—*Kinetic salt effect on degradation of echothiophate at 22° (30), plotted according to Eq. 4 (◒, upper scale) and according to Eq. 7 (○, lower scale).*

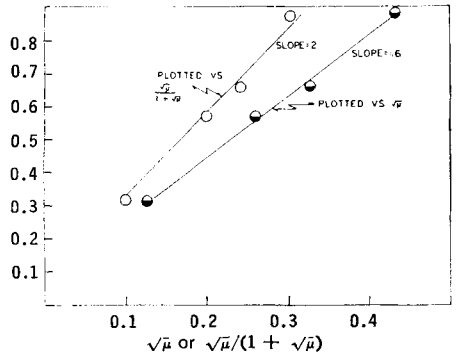


Figure 4—*Kinetic salt effect on degradation of chlorpromazine at 25° (31), plotted according to Eq. 4 (◒) and according to Eq. 7 (○).*

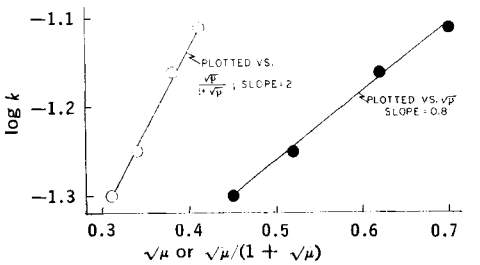


Figure 5—*Kinetic salt effect on degradation of penicillin in phosphate buffer at pH 6.8 and 60° (25), plotted according to Eq. 4 (○) and according to Eq. 7 (●). Plots in borate buffer at pH 8.75 parallel the lines shown in the figure.*

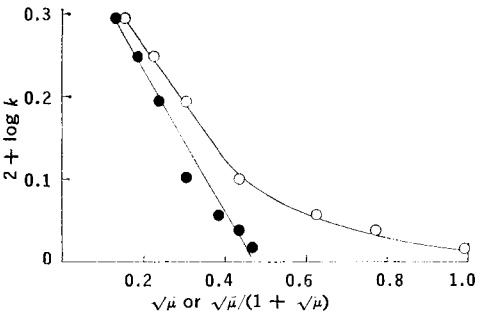


Figure 6—*Kinetic salt effect on degradation of thiamine at pH 6.40 and 96.4° (34), plotted according to Eq. 4 (○) and according to Eq. 7 (●).*

8.75 would be a reaction of the type $P^- + HBO_3^= \rightarrow$ products. This is not in conflict with the reported pH profile since, well below the pK_2 of boric acid, the logarithm of the concentration of $HBO_3^=$ is proportional to pH. In this case, use of Eq. 7 appears valid to an ionic strength of 0.5.

Koshy and Mitchner (32) studied the hydrolysis of 2-(4-phenyl-1-piperazinylmethyl)cyclohexanone (X) at 60°. At pH 5 the kinetic salt effect yields a slope of −0.61 when plotted according to Eq. 4 and a slope of −1.29, *i.e.*, close to the theoretical, when plotted according to Eq. 7. This strongly supports the authors' contention (32) that $X^+ + B^- \rightarrow$ products is the predominant reaction. In this case, both Eqs. 4 and 7 yield linearity up to $\mu = 0.4$.

Fractional Effects—The theoretical slope values, of course, can only be expected when the possibility of parallel reactions can be eliminated (unless both of the same ionic makeup). Szulczewski *et al.* (33) studied the isomerization and hydrolysis of 4,6-diamino-1-(3,5)-dichlorophenyl-1,2-dihydro-2,2-dimethyl-1,3,5-triazine (I) at 65° and a pH of 1.85 and found it to involve both IH^+ and I. When plotted according to Eq. 4, their data yield a slope of 0.5, whereas use of Eq. 7 results in the value 0.86, *i.e.*, still somewhat removed from the theoretical value. Equations 4 and 7 here hold up to $\mu = 0.16$.

A similar situation exists in the degradation of thiamine (34). Here, again, plotting (Fig. 6) according to Eq. 7 extends the useful range of ionic strength to $\mu = 0.22$, but the slope (0.87) is not close to the theoretical. As pointed out by the authors (34), secondary salt effects may be of significance in their system.

The positive kinetic salt effect reported for acid hydrolysis of niacinamide (N) at 89.4° by Finholt and Higuchi (35) yields a slope value of 0.1 if plotted according to Eq. 4 and a slope of 0.6 if plotted according to Eq. 7. Therefore, as pointed out by the authors (35), both $N^+ + H^+$ and $N + H^+$ must be involved, with rate constants of the same order of magnitude.

Other examples of fractional effects are the study on barbital degradation by Goyan *et al.* (36) and the degradations of diethylaminoacetylsalicylate hydrochloride (37) and methylpyrrolidylacetylsalicylate hydrochloride (38) reported by Garrett.

Behavior at High Ionic Strength—At ionic strengths above 1, Eq. 10 may be expected to hold, but the *b*-values are mostly of such low magnitude that absence of ionic strength effect would result in the cases to which it applies. There are three cases in recent pharmaceutical literature where Eq. 10 applies: (*a*) Schroeter's investigation of salicyl alcohol interaction with sulfites (39); (*b*) the investigation of Siegel *et al.* (40) of the hydrolysis of methyl DL-α-phenyl-2-piperidylacetate; and (*c*) Hou and Poole's investigation of degradation of ampicillin at low pH (41).

DISCUSSION

The only case in recent pharmaceutical literature where the extended Debye-Hückel equation has been used is the (nonkinetic) study by Zografi *et al.* (42). The intents of this communication have been to advocate the advantages of using Eq. 7 as a plotting procedure when kinetic salt effects are under study, and to point to the fact that the concentration range in which it may be used may be as high as unity ionic strength, *i.e.*, well beyond the range of both Eqs. 3 and 5.

Table II lists, in tabulated form, the slope values and ionic strength ranges extracted from this survey. It is quite obvious that the range of applicability is considerably higher than what would be expected from the limiting μ-ranges for Eqs. 3 and 5. Only in the case of echothiophate is the maximum allowable ionic strength definitely less than 0.5. Since Eq. 8 holds for ionic strength ranges above $\mu = 0.1$, an equation of type 7 can be derived to yield:

$$\log k = \log k_0 + \frac{2 \cdot z^2 \cdot Q\sqrt{\mu}}{1 + \beta\sqrt{\mu}} - [b_A + b_B - b\dagger] \cdot \mu \quad \text{(Eq. 11)}$$

Table II—Slope Values and Ionic Strength Ranges for the Kinetic Salt Effect in Degradations Reported in Pharmaceutical Literature

Substance	Reference	Slope			Maximum Ionic Strength, μ	Temperature
		Eq. 4	Eq. 7	2Q		
3-Carbomethoxy-1-methylpyridinium cation	29	0.42	1.2	1.03	$\mu_{max.} > 1.2$	30
Echothiophate	30	0.9	0.9	1.01	$0.2 < \mu_{max.} < 0.5$	22
Chlorpromazine	31	1.6	2.3	2.04	$\mu_{max.} > 0.5$	25
Penicillin	25	0.8	2.0	2.18	$\mu_{max.} > 0.5$	60
2-(4-Phenyl-1-piperazinylmethyl)cyclohexanone	32	−0.61	−1.29	−1.09	$\mu_{max.} > 0.4$	60
4,6-Diamino-1-(3,5)-dichlorophenyl-1,2-2,2-dimethyl-1,3,5-triazine	33	0.5	0.86	1.10	$\mu_{max.} > 0.16$	65
Thiamine	34	−0.37	−0.87	−1.19	$\mu_{max.} > 0.22$	96.4
Niacinamide	35	0.1	0.6	1.17	$\mu_{max.} > 0.8$	89.4

An explanation for the extended range could be that, in most cases, the factor $[b_A + b_B - b_\dagger]$ is close to zero. Both b_A and b_B can be determined experimentally by measuring activity coefficients as a function of ionic strength for the reactants A and B. Of course, $b_\dagger$ is not subject to direct experimental determination.

REFERENCES

(1) S. Glasstone, K. Laidler, and H. Eyring, "Theory of Rate Processes," McGraw-Hill, New York, N. Y., 1941.
(2) J. Brønsted, *Z. Physik. Chem.*, **102,** 169(1922).
(3) *Ibid.*, **115,** 337(1925).
(4) N. Bjerrum, *Z. Physik. Chem.*, **108,** 82(1924).
(5) *Ibid.*, **118,** 251(1925).
(6) S. Maron and C. Prutton, "Principles of Physical Chemistry," 4th ed., Macmillan, New York, N. Y., 1965, p. 442.
(7) H. Harned and B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold, New York, N. Y., 1950, p. 575.
(8) K. Denbigh, "Chemical Equilibrium," Cambridge University Press, Cambridge, England, 1961, p. 310.
(9) G. Czapski and H. Schwarz, *J. Phys. Chem.*, **66,** 471(1962).
(10) E. Hückel, *Physik. Z.*, **26,** 93(1925).
(11) P. Debye and J. McAuley, *ibid.*, **26,** 22(1925).
(12) E. R. Garrett, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 767(1959).
(13) *Ibid.*, **48,** 169(1959).
(14) E. R. Garrett, *J. Amer. Chem. Soc.*, **82,** 827(1960).
(15) *Ibid.*, **82,** 711(1960).
(16) A. Marcus and S. Baron, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 85(1959).
(17) T. Higuchi, A. Havinga, and L. Busse, *ibid.*, **39,** 405(1950).
(18) T. Higuchi, A. Marcus, and C. Bias, *ibid.*, **43,** 129(1954).
(19) K. Koshy and J. Lach, *J. Pharm. Sci.*, **50,** 113(1961).
(20) A. Nari and J. Lach, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 390(1959).
(21) P. Finholt, R. Paulssen, and T. Higuchi, *J. Pharm. Sci.*, **52,** 948(1963).
(22) P. Finholt, I. Alsos, and T. Higuchi, *ibid.*, **54,** 181(1965).
(23) F. Tishler, J. Sinsheimer, and J. Goyan, *ibid.*, **51,** 215(1962).
(24) L. Ravin, C. Simpson, A. Zappalla, and J. Gulesich, *ibid.*, **53,** 1064(1964).
(25) P. Finholt, G. Jürgensen, and H. Kristiansen, *ibid.*, **54,** 387(1965).
(26) S. Yeh and J. Lach, *ibid.*, **50,** 35(1961).
(27) L. Malspeis and D. Gold, *ibid.*, **53,** 1173(1964).
(28) A. Marcus and J. Stanley, *ibid.*, **53,** 91(1964).
(29) D. Brooke and D. Guttmann, *ibid.*, **57,** 1677(1968).
(30) A. Hussain, P. Schurman, V. Peter, and G. Milosovich, *ibid.*, **57,** 411(1968).
(31) A. Felmeister, R. Schaubman, and H. Howe, *ibid.*, **54,** 1589(1965).
(32) K. Koshy and H. Mitchner, *ibid.*, **53,** 1381(1964).
(33) D. Szulczewski, C. Shearer, and A. Aguiar, *ibid.*, **53,** 1157 (1964).
(34) J. Windheuser and T. Higuchi, *ibid.*, **51,** 354(1962).
(35) P. Finholt and T. Higuchi, *ibid.*, **51,** 655(1962).
(36) J. Goyan, Z. Sahikh, and J. Autian, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 627(1960).
(37) E. R. Garrett, *J. Amer. Chem. Soc.*, **80,** 4049(1958).
(38) *Ibid.*, **79,** 5206(1957).
(39) L. Schroeter, *J. Pharm. Sci.*, **51,** 258(1962).
(40) S. Siegel, L. Lachman, and L. Malspeis, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 531(1959).
(41) J. Hou and J. Poole, *J. Pharm. Sci.*, **58,** 447(1969).
(42) G. Zografi, P. Patel, and N. Weiner, *ibid.*, **53,** 545(1964).
(43) H. Harned and B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold, New York, N. Y., 1950, p. 118.
(44) "Handbook of Chemistry and Physics," 29th ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1945, p. 1652.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 24, 1969, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*
Accepted for publication February 20, 1970.

In Vitro and *In Vivo* Chlorpromazine Availability from Flocculated Polysalt Complex Systems

P. A. JABLON*, G. S. BANKER, and V. F. SMOLEN

Abstract □ An insoluble drug–polysalt complex of chlorpromazine hydrochloride, sodium carboxymethylcellulose, and protamine sulfate was selected as a model to evaluate the effects of these macromolecular constituents on the *in vitro* and *in vivo* availability of the interacted drug. The *in vitro* liberation of drug from the polysalt complex was studied in simulated gastrointestinal fluids as a function of particle size, pH of formation of the complex flocculate, and presence and absence of enzymes in the medium. The *in vitro* drug-release studies conducted under these varying conditions suggested that the product possessed prolonged-release properties. In contrast, the *in vivo* studies with rats revealed a promoted bioavailability of the drug in the presence of the polysalt complex. Protamine sulfate, a known pinocytotic inducer, was observed to be specifically implicated in this phenomenon.

Keyphrases □ Flocculated complex systems, polysalt—chlorpromazine-^{35}S availability □ Chlorpromazine-^{35}S-polysalt complex systems—*in vivo–in vitro* availability □ Release rates—chlorpromazine-^{35}S-polysalt complex □ pH effect—chlorpromazine-^{35}S-polysalt complex stability □ Scintillometry—analysis

The physical form of a polyelectrolyte salt complex (polysalt) is a function of the charge densities of the interacting species. Macromolecules with high charge densities tend to form precipitates (flocculates), while those with lower charge densities tend to form a gel (coacervate) or quasiliquid product (1). The use of complex coacervates as a means of microencapsulating drugs has been claimed in a number of patents (2–7). Luzzi and Gerraughty (8, 9) have noted that complex coacervates of gelatin and acacia with pentobarbital may be employed to control the liberation of the encapsulated material into gastrointestinal fluids. They studied the release rate of the drug as a function of pH, temperature, ratio of solid to encapsulating material, quantity of hardening agent (formaldehyde), and addition of surfactants. In contrast, polysalt flocculates have received relatively little attention in the pharmaceutical literature.

The present drug–polysalt system was chosen on the basis of preliminary studies. It was found that a polysalt of sodium carboxymethylcellulose (NaCMC) and protamine sulfate interacted with appreciable quantities of

An explanation for the extended range could be that, in most cases, the factor $[b_A + b_B - b_\dagger]$ is close to zero. Both b_A and b_B can be determined experimentally by measuring activity coefficients as a function of ionic strength for the reactants A and B. Of course, $b_\dagger$ is not subject to direct experimental determination.

REFERENCES

(1) S. Glasstone, K. Laidler, and H. Eyring, "Theory of Rate Processes," McGraw-Hill, New York, N. Y., 1941.
(2) J. Brønsted, *Z. Physik. Chem.*, **102,** 169(1922).
(3) *Ibid.*, **115,** 337(1925).
(4) N. Bjerrum, *Z. Physik. Chem.*, **108,** 82(1924).
(5) *Ibid.*, **118,** 251(1925).
(6) S. Maron and C. Prutton, "Principles of Physical Chemistry," 4th ed., Macmillan, New York, N. Y., 1965, p. 442.
(7) H. Harned and B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold, New York, N. Y., 1950, p. 575.
(8) K. Denbigh, "Chemical Equilibrium," Cambridge University Press, Cambridge, England, 1961, p. 310.
(9) G. Czapski and H. Schwarz, *J. Phys. Chem.*, **66,** 471(1962).
(10) E. Hückel, *Physik. Z.*, **26,** 93(1925).
(11) P. Debye and J. McAuley, *ibid.*, **26,** 22(1925).
(12) E. R. Garrett, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 767(1959).
(13) *Ibid.*, **48,** 169(1959).
(14) E. R. Garrett, *J. Amer. Chem. Soc.*, **82,** 827(1960).
(15) *Ibid.*, **82,** 711(1960).
(16) A. Marcus and S. Baron, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 85(1959).
(17) T. Higuchi, A. Havinga, and L. Busse, *ibid.*, **39,** 405(1950).
(18) T. Higuchi, A. Marcus, and C. Bias, *ibid.*, **43,** 129(1954).
(19) K. Koshy and J. Lach, *J. Pharm. Sci.*, **50,** 113(1961).
(20) A. Nari and J. Lach, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 390(1959).
(21) P. Finholt, R. Paulssen, and T. Higuchi, *J. Pharm. Sci.*, **52,** 948(1963).
(22) P. Finholt, I. Alsos, and T. Higuchi, *ibid.*, **54,** 181(1965).
(23) F. Tishler, J. Sinsheimer, and J. Goyan, *ibid.*, **51,** 215(1962).
(24) L. Ravin, C. Simpson, A. Zappalla, and J. Gulesich, *ibid.*, **53,** 1064(1964).
(25) P. Finholt, G. Jürgensen, and H. Kristiansen, *ibid.*, **54,** 387(1965).
(26) S. Yeh and J. Lach, *ibid.*, **50,** 35(1961).
(27) L. Malspeis and D. Gold, *ibid.*, **53,** 1173(1964).
(28) A. Marcus and J. Stanley, *ibid.*, **53,** 91(1964).
(29) D. Brooke and D. Guttmann, *ibid.*, **57,** 1677(1968).
(30) A. Hussain, P. Schurman, V. Peter, and G. Milosovich, *ibid.*, **57,** 411(1968).
(31) A. Felmeister, R. Schaubman, and H. Howe, *ibid.*, **54,** 1589(1965).
(32) K. Koshy and H. Mitchner, *ibid.*, **53,** 1381(1964).
(33) D. Szulczewski, C. Shearer, and A. Aguiar, *ibid.*, **53,** 1157 (1964).
(34) J. Windheuser and T. Higuchi, *ibid.*, **51,** 354(1962).
(35) P. Finholt and T. Higuchi, *ibid.*, **51,** 655(1962).
(36) J. Goyan, Z. Sahikh, and J. Autian, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 627(1960).
(37) E. R. Garrett, *J. Amer. Chem. Soc.*, **80,** 4049(1958).
(38) *Ibid.*, **79,** 5206(1957).
(39) L. Schroeter, *J. Pharm. Sci.*, **51,** 258(1962).
(40) S. Siegel, L. Lachman, and L. Malspeis, *J. Amer. Pharm. Ass., Sci. Ed.*, **48,** 531(1959).
(41) J. Hou and J. Poole, *J. Pharm. Sci.*, **58,** 447(1969).
(42) G. Zografi, P. Patel, and N. Weiner, *ibid.*, **53,** 545(1964).
(43) H. Harned and B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold, New York, N. Y., 1950, p. 118.
(44) "Handbook of Chemistry and Physics," 29th ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1945, p. 1652.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 24, 1969, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*
Accepted for publication February 20, 1970.

In Vitro and *In Vivo* Chlorpromazine Availability from Flocculated Polysalt Complex Systems

P. A. JABLON*, G. S. BANKER, and V. F. SMOLEN

Abstract □ An insoluble drug–polysalt complex of chlorpromazine hydrochloride, sodium carboxymethylcellulose, and protamine sulfate was selected as a model to evaluate the effects of these macromolecular constituents on the *in vitro* and *in vivo* availability of the interacted drug. The *in vitro* liberation of drug from the polysalt complex was studied in simulated gastrointestinal fluids as a function of particle size, pH of formation of the complex flocculate, and presence and absence of enzymes in the medium. The *in vitro* drug-release studies conducted under these varying conditions suggested that the product possessed prolonged-release properties. In contrast, the *in vivo* studies with rats revealed a promoted bioavailability of the drug in the presence of the polysalt complex. Protamine sulfate, a known pinocytotic inducer, was observed to be specifically implicated in this phenomenon.

Keyphrases □ Flocculated complex systems, polysalt—chlorpromazine-^{35}S availability □ Chlorpromazine-^{35}S-polysalt complex systems—*in vivo–in vitro* availability □ Release rates—chlorpromazine-^{35}S-polysalt complex □ pH effect—chlorpromazine-^{35}S-polysalt complex stability □ Scintillometry—analysis

The physical form of a polyelectrolyte salt complex (polysalt) is a function of the charge densities of the interacting species. Macromolecules with high charge densities tend to form precipitates (flocculates), while those with lower charge densities tend to form a gel (coacervate) or quasiliquid product (1). The use of complex coacervates as a means of microencapsulating drugs has been claimed in a number of patents (2–7). Luzzi and Gerraughty (8, 9) have noted that complex coacervates of gelatin and acacia with pentobarbital may be employed to control the liberation of the encapsulated material into gastrointestinal fluids. They studied the release rate of the drug as a function of pH, temperature, ratio of solid to encapsulating material, quantity of hardening agent (formaldehyde), and addition of surfactants. In contrast, polysalt flocculates have received relatively little attention in the pharmaceutical literature.

The present drug–polysalt system was chosen on the basis of preliminary studies. It was found that a polysalt of sodium carboxymethylcellulose (NaCMC) and protamine sulfate interacted with appreciable quantities of

chlorpromazine hydrochloride (CPZ HCl) to form a drug–polysalt complex. This material was insoluble at pH 7.40 and very slowly soluble in 0.1 *N* hydrochloric acid. Another important characteristic was the presence of a proteolyzable polymer. Therefore, it was proposed that a system of this type may be of value in controlling the liberation of the interacted drug in gastrointestinal fluids by utilizing not only its slow dissolution but also the possible destruction of the flocculated drug–polysalt matrix through enzymatic degradation. The present work was directed to elucidate *in vitro* release properties of the drug–polysalt complex in simulated gastrointestinal fluids and to compare these results with the *in vivo* behavior of the system as reflected in its pharmacological response characteristics.

MATERIALS AND METHODS

Materials—All reagents were analytical grade, except barbital NF. The protamine sulfate utilized as the polycation in this work represented a highly purified protein which met all USP requirements.[1] A low-viscosity grade (25–50 cps. in 2% solution) of NaCMC[2] was employed as the polyanion. The specific activity of the ^{35}S-CPZ HCl,[3] initially 3.57 μc./mg., was adjusted to 20,000 d.p.m./mg. with unlabeled drug,[4] and this powder dilution was used in all subsequent experiments. Animals utilized in the *in vivo* portion of this study were 75-day-old albino male rats, Holtzman[5] strain.

Solutions—A pH 7.70 buffer was prepared from 0.05 *M* barbital and sufficient sodium hydroxide. Sodium acetate (0.05 *M*) and hydrochloric acid were employed to prepare a pH 4.75 buffer. Gastrointestinal fluids were prepared according to USP XVII unless otherwise noted. The scintillation cocktail XDC (10) was utilized as a counting solution. When required, approximately 15.0 ml. of the scintillation fluid was added to a low potassium glass counting vial[6] containing 5.0–5.5% colloidal silica pigment.[7] A thick gel promptly formed which was suitable for suspending any materials precipitated from solution by the XDC.

Preparation of Labeled CPZ–Polysalt Complex—Buffered stock solutions of the polymers were prepared. An aliquot of the NaCMC solution was transferred to a beaker containing an accurately weighed quantity of the ^{35}S-CPZ HCl. The mixture was stirred until a precipitate had completely formed. Protamine sulfate solution was then added with constant stirring to flocculate the system. The contents of the beaker were filtered through a medium sintered-glass funnel with the aid of suction. The gummy mass was vacuum dried to constant weight, crushed in a mortar, and assayed for drug content. Drug–polysalts were prepared in this manner from ^{35}S-CPZ HCl, 2 parts protamine sulfate, and 3 parts NaCMC. Drug–polysalt complexes flocculated in buffers at pH values of 4.75 and 7.70 were designated drug–polysalt A and drug–polysalt B, respectively. Tablets of these drug–polysalt materials, weighing approximately 250 mg. and containing approximately 100 mg. of drug, were prepared by direct compression on a Carver laboratory press with a 0.78-cm. (0.31-in.) standard cup punch and die set using an applied load of 500 lb./in.^{2}.

Preliminary work had revealed that this procedure and the order of combination of polymers in the ratio of 2 parts protamine sulfate to 3 parts NaCMC (in the presence of drug) yielded a flocculated product which was optimal with respect to the quantity of drug and colloids cleared from solution. Flocculated drug–polysalts prepared in three separate replications at each pH were found to contain 41.05 ±1.5% and 40.08 ±0.59% w/w of drug for drug–polysalt A and drug–polysalt B, respectively. In the absence of drug, maximum quantities of the polymers were found to be flocculated and cleared from solution when the polymers were combined in electrically equivalent quantities. For example, the apparent equivalent weights of protamine sulfate and NaCMC in the pH 7.70, 0.05 *M* barbital buffer were determined, from the measurement of Donnan membrane potentials, to be 1260 and 615 g. per equivalent of charge, respectively. As anticipated, the most stable flocculates were obtained when the polymers, in the absence of drug, were combined in approximately the ratio of 2 parts protamine to 1 part NaCMC.

Radioassay for ^{35}S-CPZ HCl—Solutions of the labeled drug were counted for 10 min. (or 10,000 counts) in a Packard[6] Tricarb model 574 liquid scintillation spectrometer. The counting efficiency was determined by the internal standard method. Before assay for drug content, samples of the flocculated drug–polysalt were pretreated with hot concentrated sulfuric acid to effect solution.

***In Vitro* CPZ Release Methodology**—A dissolution dialysis technique was selected for the *in vitro* drug-release studies in an attempt to simulate *in vivo* conditions. The polymer impermeable membrane utilized was intended to approximate the behavior of the gastrointestinal mucosal membrane by retaining soluble drug–polymer complexes released from the drug–polysalt system. Only the availability of the free drug, and perhaps drug associated to very small fragments of proteolyzed protamine, would be measured. Therefore, the dissolution dialysis release profile may be expected to be more closely representative of the *in vivo* absorption of CPZ from the polysalt complex than a release profile based upon dissolution alone.

Dissolution dialysis experiments utilized a cell composed of a sac of Nojax[2] cellulose tubing which contained 10 ml. of dialysis fluid and the materials to be dialyzed. Before use, the casing was boiled in 5% acetic acid and flushed well with distilled water. The sac was prepared by cutting the tubing to the required length and tying one end closed. The materials were introduced through the open end of the casing, which was then tied tightly with nylon cord at the upper level of the liquid. Free casing above the closure was knotted around the cord and the excess trimmed off. Sacs prepared in this manner had approximately the same surface area. The sacs were immersed in round, amber, 90-ml. (3-fl. oz.) bottles filled with 50 ml. of the dialysis fluid. The cap of the cell was sealed with tape. The *in vitro* release characteristics of the labeled drug–polysalts were evaluated in simulated gastric fluid, simulated intestinal fluid, and simulated intestinal fluid without enzymes. The drug-release cells were assembled and placed on the reciprocating platform of a shaker-type water bath maintained at 37°. At appropriate intervals during an 8-hr. period, samples of the (outside) fluid were removed from the cells and transferred to counting vials. An equal quantity of the test fluid was then added to the cell to maintain a constant volume. All fluids were freshly prepared and prewarmed to 37° before use. The cells were agitated at a constant rate of approximately 60, 3.8-cm. excursions/min.

***In Vivo* Studies of the Drug–Polysalt System**—The criterion selected for evaluating the *in vivo* characteristics of the flocculated drug–polysalt complex was the depression of spontaneous motor activity in rats. Williamson[8] activity cages (11), which showed negligible differences in sensitivity, were employed to measure this pharmacologic response. The ED_{50} to produce the desired effect in the rat has been reported as 4.4 mg./kg. of CPZ HCl (12). Dragstedt (13) emphasized the importance of administering identical doses of drug in the same fashion when testing products for sustained-release properties. In accordance with this principle, all animals that received a dosage form of the drug as a treatment were given 4.4 mg./kg. of CPZ HCl.

In the first set of *in vivo* experiments, rats were selected at random in groups of three at a time from a colony of 32 previously untreated animals. Each rat in the colony was fasted for 24 hr., orally dosed with polysalt blank (*i.e.*, polymers interacted in the absence of drug), and placed in the Williamson cages for 11.5 hr. The spontaneous activity of these rats was then redetermined in the same manner, except that one of the four treatments was substituted for the polysalt blank. The animals received either a second dose of polysalt blank, a 40% mixture of CPZ HCl in mannitol (drug control), drug–polysalt A, or drug–polysalt B. To optimize sensitivity, the experiments were run from 8:00 p.m. until 7:30 a.m. Rats are nocturnal animals and therefore most active at night. Influences arising from environmental disturbances were minimal

[1] The analytical data and a generous sample were kindly provided by Dr. J. M. McGuire, Eli Lilly and Co., Indianapolis, Ind.
[2] Cellosize CMC, Union Carbide Co., New York, N. Y.
[3] The Radiochemical Centre, Amersham, England.
[4] Thorazine, Smith, Kline & French Laboratories, Philadelphia, Pa.
[5] The Holtzman Co., Madison, Wis.
[6] Packard Instrument Co., Downers Grove, Ill.
[7] Cab-O-Sil, Cabot Corp., Boston, Mass.
[8] Williamson Development Co., West Concord, Mass.

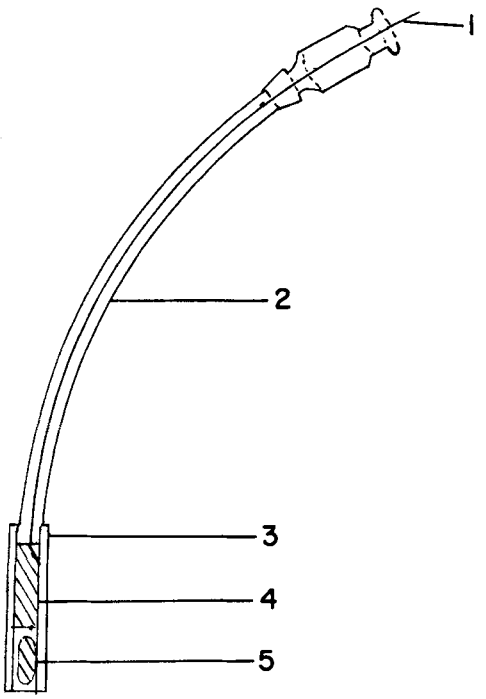


Figure 1—*Device utilized to dose rats orally for* in vivo *activity studies. Key: 1, copper wire; 2, oral dosing needle; 3, capsule holder; 4, epoxy plunger; and 5, capsule section.*

during this time period. Each of the four treatments was replicated on eight different rats.

A dosing device was developed to administer the treatments orally. The apparatus, illustrated in Fig. 1, consisted of an oral dosing needle through which a copper wire bearing a dried epoxy resin plunger was inserted at the far end. A section of catheter tubing was attached to the beveled end of the needle over the plunger. The material that the animal received was weighed into the bottom half of a tared No. 5 gelatin capsule which was subsequently moistened until flexible, folded in half, and placed in the section of tubing. The dosing needle was then inserted into the esophagus of the animal; the copper wire plunger rod was pushed forward to eject the capsule. Since rodents do not have the ability to regurgitate, the capsule and its contents were carried to the stomach by peristalsis. Immediately following dosing with the capsule, the animal received about 0.5 ml. water through the dosing needle.

A second set of *in vivo* experiments was conducted on a new colony of 18 rats according to the general procedure already outlined. The three treatment groups were a drug control of 40% CPZ HCl in mannitol, a physical mixture of 40% CPZ HCl and protamine sulfate, and particles of drug–polysalt which would not pass a 30-mesh screen. Each of the three treatments was replicated on six different rats.

RESULTS AND DISCUSSION

***In Vitro* Release Studies in Simulated Gastric Fluid**—Figure 2 illustrates the dissolution dialysis of various physical forms of the drug in simulated gastric fluid. The uppermost curve in Fig. 2 was obtained with 100 mg. of ^{35}S-CPZ dissolved in 10 ml. of simulated gastric fluid contained within the dialysis sac. This is the same quantity of CPZ as was contained in the drug–polysalts studied in the same manner. However, the dialysis sac served in this case to restrain the ready transfer of CPZ into the outside compartment. It is apparent from the other curves that the presence of a polysalt complex provided a significant additional retardation.

Comparison of the curves for the tablets prepared from drug–polysalt B and small particles of the same material (>30 mesh) reveals the delaying effect of compression on the release of CPZ. This result obviously implicates the surface area of the drug–polysalt as a factor affecting the release of drug from the system.

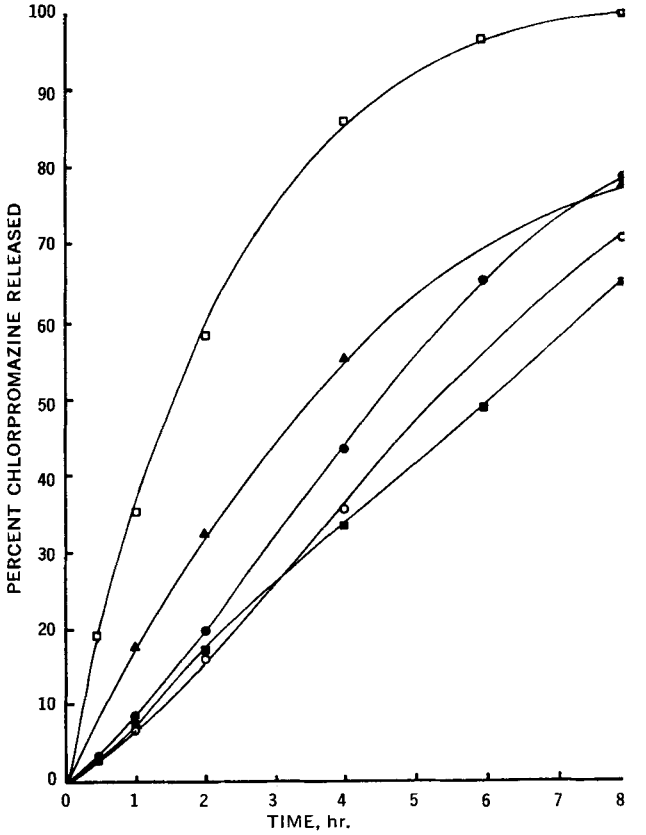


Figure 2—In vitro *release of ^{35}S-labeled chlorpromazine through cellulose sacs into simulated gastric fluid. Key:* □, *dialysis solution control of drug alone;* ●, *tablets drug–polysalt A;* ■, *tablets drug–polysalt B;* ▲, *particles drug–polysalt B (>30 mesh); and* ○, *tablet control (physical mixture of polymer and drug).*

Upon immersion of the drug–polysalt tablets in gastric fluid, the cream-white biconcave disk appeared to swell slightly; a uniform three-dimensional gray band began to form around the outer edge, widen, and move gradually toward the core. After 8–10 hr., the cream-white solid matrix was replaced by a gray, swollen, gel-like structure. From these visual observations, it can be hypothesized that as the simulated gastric fluid penetrated the matrix, it began to solubilize the protamine, forming a network of channels in the

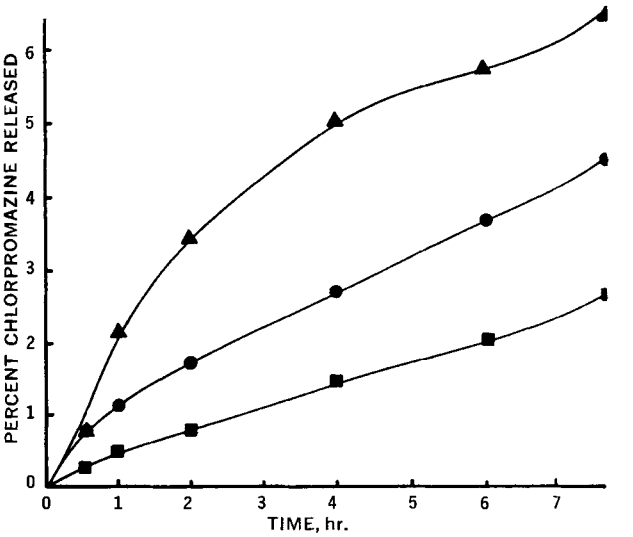


Figure 3—*Release of ^{35}S-labeled chlorpromazine from drug–polysalt tablets into simulated intestinal fluid without enzymes. Key:* ▲, *tablet control (physical mixture of polymers and drugs);* ■, *tablets drug–polysalt A; and* ●, *tablets drug–polysalt B.*

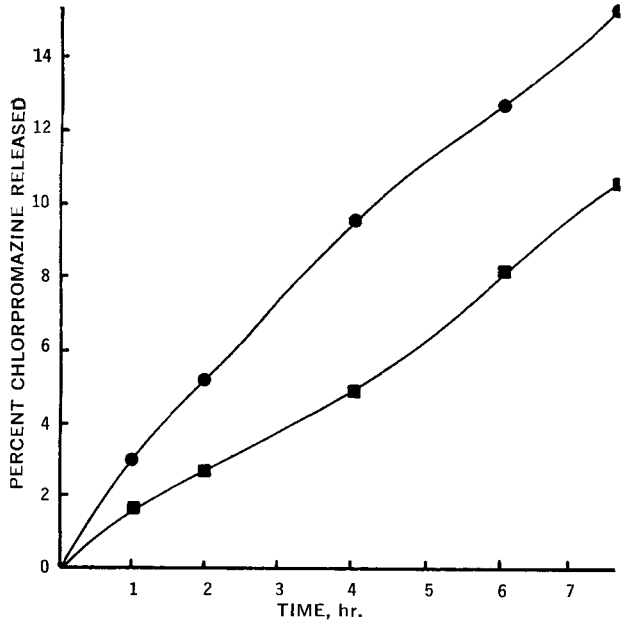


Figure 4—*Release of ^{35}S-labeled drug–polysalt tablets through cellulose membrane into simulated intestinal fluid with enzymes after immersion in simulated gastric fluid for 2 hr. Key: ●, tablets drug–polysalt A; and ■, tablets drug–polysalt B.*

remaining structure of insoluble free acid CMC. The entrapped drug was also solubilized by the gastric fluid; as channels were created, it dissolved and slowly leeched out into the surrounding medium.

CPZ Release in Simulated Intestinal Fluids—At pH 7.4 the tablets swelled rapidly and the matrix soon broke apart into large cream-white particles. The particles, although swollen, appeared to retain their structural integrity in contrast to the behavior of the polysalt complex in gastric fluid. Figure 3 illustrates the slow release of drug from the polysalt system in simulated intestinal fluid without enzymes. Figure 4 presents the dissolution dialysis profile of drug–polysalt A and B tablets in simulated intestinal fluid with enzymes following pretreatment by immersion, for 2 hr., in gastric fluid. This system was intended to simulate *in vivo* conditions where the enzymes can serve as additional means of degrading the complex and liberating the drug. However, in all cases with intestinal fluid, only relatively small amounts of CPZ were released. This can be attributed to the insolubility of the drug and the stability of its polysalt matrix in this medium. It was found that a control study of CPZ dialysis could not be performed for intestinal fluid in the same manner as that done for gastric fluid because of the limited solubility of the drug in this medium.

Influence of pH of Formation on the Stability of the Drug–Polysalt Complex—Since both the drug and polymers are weak electrolytes, the extent of drug–polymer and polymer–polymer electrostatic interactions would be expected to vary with pH. A given drug–polysalt complex should be maximally stable at its pH of formation when combined in electrically equivalent quantities. It is, therefore, also expected that the complex formed at pH 4.75 would be more stable in solutions of lower pH than a corresponding product flocculated at pH 7.70. Conversely, the drug–polysalt complex formed at pH 7.70 should be more stable in solutions at higher pH values relative to the product flocculated at pH 4.75. The release profiles of drug–polysalt complex tablets A and B (shown in Fig. 2) are clearly inconsistent with such expectations. Figure 2 reveals a faster and more complete liberation of CPZ in acid media from the drug–polysalt A tablet than from its B counterpart. Furthermore, visual observations during the study indicated that the tablet matrix of drug–polysalt A was less cohesive than that of the corresponding B product. The observed phenomenon is partly explicable in terms of the greater solubility of CPZ salt relative to free base. In the pH 7.70 formed drug–polysalt complex, the CPZ occurs to a greater extent in the less soluble form of the free base relative to the drug in the pH 4.75 formed complex. Although the relatively slow dissolution of the unionized form of the drug is a factor contributing to the limited release rate of the drug from the tablet matrix, it is not wholly responsible for the slow release. This is indicated in Fig. 3 by the observed faster drug release from a physical mixture of the drug with the polymers relative to the flocculate where, neglecting the occurrence of localized drug–polymer interaction, the rate-limiting factor in the availability of the drug from the tablet may be presumed to be its dissolution in the medium. The similarity in the

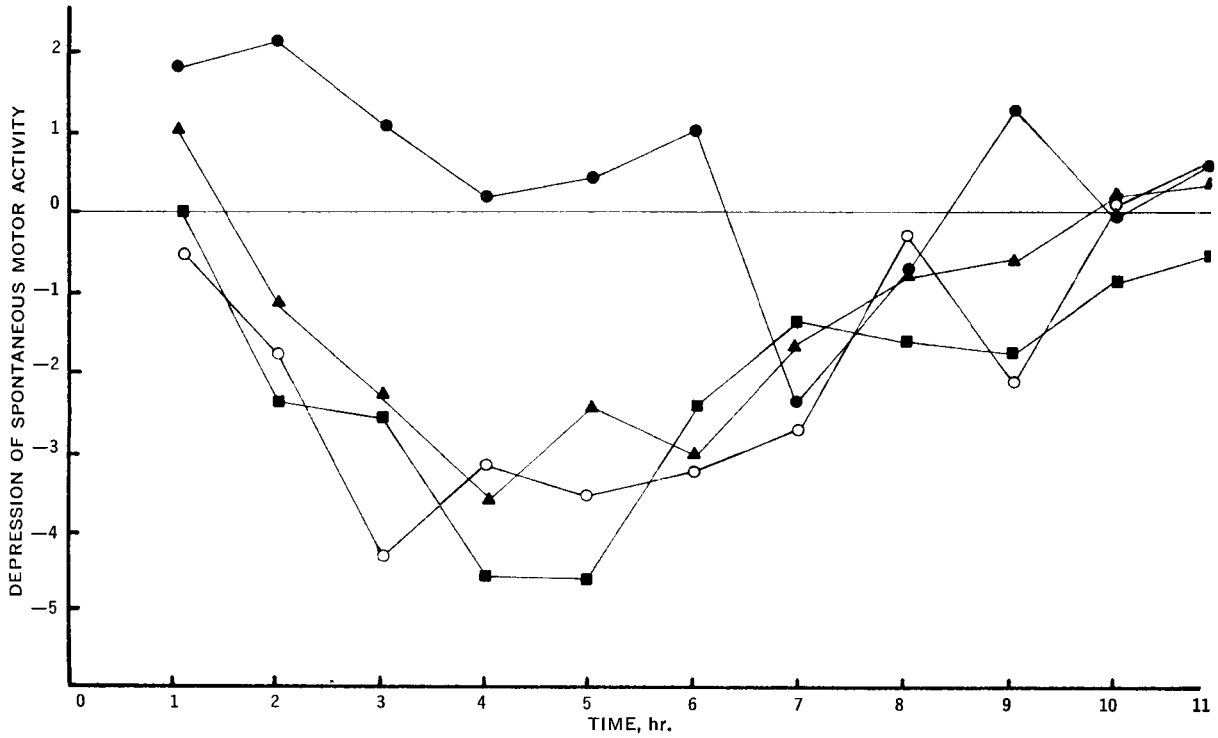


Figure 5—*Chlorpromazine-induced depression of spontaneous motor activity of rats as a function of time following administration of chlorpromazine in different dosage forms. Each point is the average of eight replications on different animals. Key: ●, polysalt blank; ▲, drug control (chlorpromazine HCl and mannitol); ■, drug–polysalt A particles; and ○, drug–polysalt B particles.*

Table I—Summary of Observed Variance Ratio ($F_{obs.}$) Showing Values for Significant Differences among the Four Treatment[a] Means

Time, hr.	Mean Square Treatments	Mean Square Error	$F_{obs.}$ (3, 28)[b]
1	8.948	7.388	1.210
2	31.780	2.000	15.890[c]
3	40.370	7.280	5.540[c]
4	35.783	6.861	5.520[c]
5	38.700	5.250	7.234[c]
6	33.083	9.438	3.505[d]
7	3.533	6.370	0.555
8	7.283	6.191	1.176
9	17.917	4.938	3.628[d]
10	2.083	6.045	0.345
11	2.707	7.063	0.383

[a] The four treatments referred to the polysalt blank, drug control, drug–polysalt A, and drug–polysalt B. [b] $F_{0.95}$ (3, 28) = 2.95; $F_{0.99}$ (3, 28) = 4.57. [c] Significant at $p < 0.01$. [d] Significant at $p < 0.05$.

curves shown in Fig. 2 for drug release from tablets composed of a physical mixture and drug–polysalt B indicates, however, that under acidic conditions in the medium the slow dissolution of the unionized drug may be an important factor in limiting the drug's release.

Another factor may be one similar to the electrolyte plasticization effect observed by Michaels (14); the ions constituting the salt form of the drug can act as a shield to decrease the electrostatic attraction between polycation and polyanion, resulting in a less consolidated system with a greater elasticity and tendency to swell. The looser polymer network formed at pH 4.75 by this mechanism could allow the physically entrapped drug to be more readily released from the matrix.

Proposed Mechanism of Drug–Polysalt Interaction—Based on the preliminary observations and *in vitro* release studies, it can be hypothesized that the mechanism of uptake of the drug by the polysalt involves two consecutive processes. The drug first becomes bound to the NaCMC; upon addition of the protamine polycation, which possesses a greater affinity for the anionic binding sites on the CMC, some bound CPZ is competitively displaced. The released drug may then precipitate from solution due to local saturation and become physically entrapped within the solid polysalt matrix. The drug, therefore, likely exists in both a mechanically entrapped and physically adsorbed form within the polysalt matrix. Its mechanisms of release accordingly include desorption and dissolution.

***In Vivo* Studies of CPZ Availability**—CPZ is cleared very rapidly from the bloodstream (15); the time course of pharmacological response intensity, *i.e.*, magnitude of depression of spontaneous motor activity, was studied in rats in lieu of direct determinations of the drug in body fluids. Male rats were selected because their general level of activity, although lower than the female's, is not subject to the cyclization accompanying the female's estrus cycle. The results of the first study are illustrated in Fig. 5. The "deviation" statistic utilized to describe the treatment-induced effects was obtained as the difference in pretreatment and posttreatment activity readings. The mean of this value for each treatment group was plotted as a function of time. A single factor analysis of variance was employed to indicate the presence of an overall significant difference among the four treatment groups at each hourly interval. At those time periods where the variance ratio, $F_{obs.}$, exceeded the critical value for the 0.05 probability level, comparison tests were employed to determine the exact group(s) responsible. These tests are summarized in Tables I and II. Table I is related to Fig. 5 in that it indicates the times and probability levels at which treatment-induced effects are significant. Table II specifies which treatments are responsible. It is obvious from the results that, contrary to expectation, no prolonged action effect was demonstrated by the drug–polysalt complexes.

However, a closer inspection of the results over a 6-hr. period following the treatments revealed that the depression of activity in one or both of the groups receiving a drug–polysalt was consistently greater than that in the group receiving the drug control. A comparison test, utilizing the average deviation of the two drug–polysalt treatment groups *versus* the drug control group, revealed a statistical difference in activity for the 6-hr. period at approximately the 0.15 probability level.

Since only a heterogeneous mixture of drug–polysalt particles was utilized in the first experiment, it was felt that the use of larger particles (>30 mesh) might provide the desired sustained action. (The small quantity of drug–polysalt complex administered precluded its direct compression into tablets.) Of equal interest was an investigation into the possible cause of the apparently enhanced activity of the drug. The ability of such proteinaceous materials as protamine sulfate to act as inducers of pinocytosis has been well documented in the literature (16–21). Indeed, the striking observations of Whitmore (23), that gelatin microcapsules of streptomycin exhibited enhanced transport into polymorphonuclear leukocytes and macrophages, take on new meaning when he attributes the phenomenon to "phagocytosis" (*sic*) and cites as evidence the high degree of cytoplasmic vacuolation. These facts could conceivably provide a basis on which to explain the enhanced drug activity. Hence, another treatment variable was introduced to determine the effect of protamine sulfate alone on the biological availability of CPZ. Based on these considerations, a second set of *in vivo* experiments was performed. The results are shown in Fig. 6. A single factor analysis of variance (22) for significant differences among the various treatment combinations over an 11.5-hr. period is summarized in Table III. These data clearly demonstrate that the depression of spontaneous activity produced by both treatments was greater than that of the drug control group. Despite the larger particles used, however, no significant sustained action of the drug–polysalt complex was observed. There are several possible explanations for these findings. It is apparent that the presence of protamine alone and with CMC enhances the activity of CPZ. The mechanism by which this occurs is probably not merely Donnan exclusion (24), since drug release *in vitro*, where this nonspecific effect would also be manifested for the interacted system, is delayed rather than facilitated. Although the results of the present study do not provide direct evidence for its occurrence, stimulation of pinocytosis or some other effect of the polymers on the mucosal-absorbing surface in the gastrointestinal tract appears to be a reasonable possibility. If protamine does have the ability to induce cells of the gastrointestinal mucosal-absorbing surface to envacuolate the surrounding medium, it could enhance the absorption of any drug present in that medium. If this was shown to be the case unequivocally, an entirely new concept of drug dosage design could be initiated. However, with the drug–polysalt system, there may be additional components to the mechanism involved in the augmented activity of the drug. Because CPZ is unstable in acidic solution, the polysalt complex possibly also acts to protect the drug from destruction in the stomach and thereby allows a larger fraction of the released medicament to be absorbed intact. Still another

Table II—Comparison Tests at Intervals Showing Significant Differences[a]

Time, hr.	Treatments Compared[b]	$F_{obs.}$ (3, 28)[c]
2	1 × 2	21.13[d]
	1 × 3	38.28[d]
	1 × 4	32.00[d]
3	1 × 2	6.26[d]
	1 × 3	7.22[e]
	1 × 4	15.87[d]
4	1 × 2	8.75[d]
	1 × 3	13.86[d]
	1 × 4	7.66[d]
5	1 × 2	6.73[e]
	1 × 3	19.64[d]
	1 × 4	11.96[d]
6	1 × 2	7.21[e]
	1 × 3	5.19[e]
	1 × 4	8.11[e]
9	1 × 2	2.48
	1 × 3	6.70[e]
	1 × 4	9.23[d]

[a] 2 × 3, 2 × 4, 3 × 4 Comparisons not significant. [b] 1 = polysalt blank, 2 = drug control, 3 = drug–polysalt A, 4 = drug–polysalt B. [c] $F_{0.95}$ (1, 28) = 4.20; $F_{0.99}$ (1, 28) = 7.64. [d] Significant at $p < 0.01$. [e] Significant at $p < 0.05$.

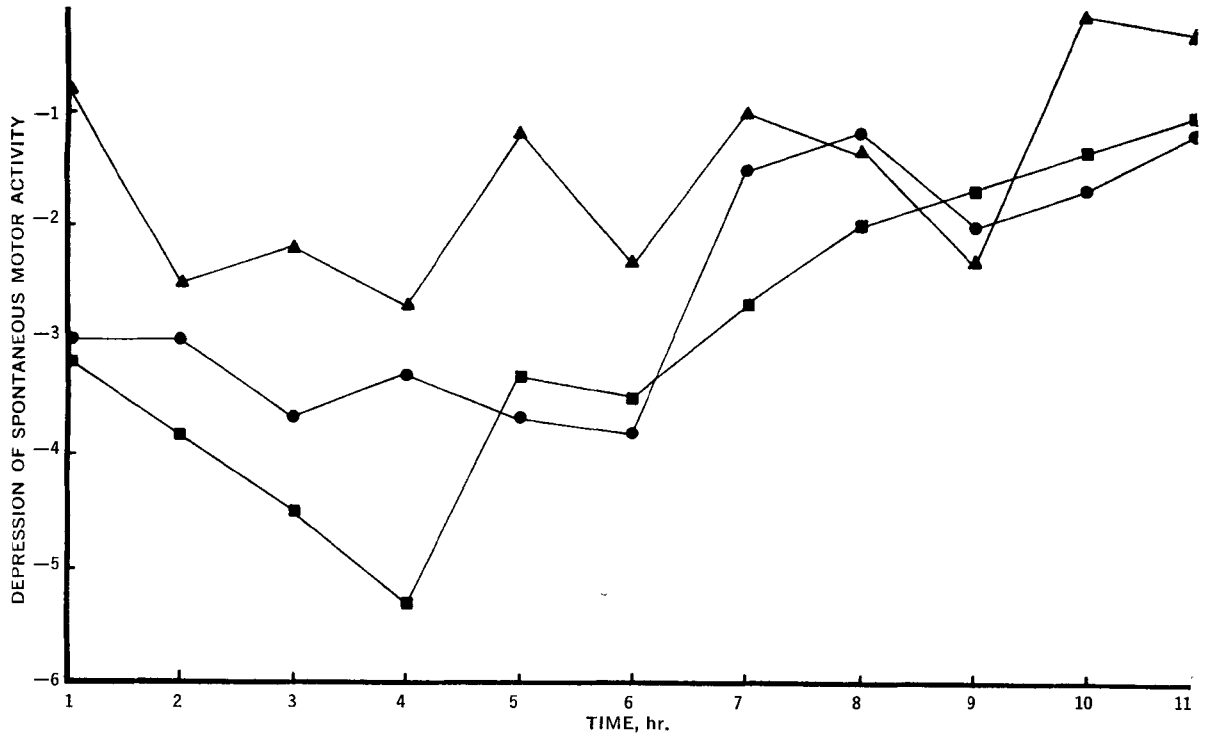


Figure 6—*Chlorpromazine-induced depression of spontaneous motor activity of rats as a function of time following administration of chlorpromazine in different dosage forms. Each point represents the average of six replications on different animals. Key:* ▲, *drug control (chlorpromazine HCl and mannitol);* ●, *chlorpromazine hydrochloride–protamine sulfate physical mixture; and* ■, *chlorpromazine–polysalt particles (>30 mesh).*

alternative possibility in explaining the phenomena is that the drug is released from the system as a protamine complex which is absorbed more rapidly. The CMC may also be implicated in this process, since it can be taken up by mammalian tissue (25); indeed, CPZ itself has been reported to affect absorption through cell membranes (26). Thus, it may even be speculated that the particular combination of materials in the interacted system possesses a maximal ability to affect cellular uptake and enhance the pharmacologic response to CPZ.

The conditions employed in the *in vitro* release studies are obviously only a crude approximation of those actually obtained *in vivo*. Consequently, a diversity of factors may have been operative to produce the clearly evidenced lack of correlation between the *in vitro* and *in vivo* drug availability. Notable among these factors are the small volumes of gastrointestinal fluid available for the dissolution of the drug *in vivo* relative to that used in the *in vitro* study, as well as differences in mechanical agitation of the drug-releasing materials. These differences could contribute significantly to the discrepancy between the *in vitro* and *in vivo* results. Conceivably, *in vivo* the drug-release medium could have become rapidly saturated with drug. This would result in the passage of the drug across the gastrointestinal barrier to become the rate-limiting step in its absorption rather than its release from the dosage form. In this case, the differences in *in vivo* drug availability noted for the different formulations could be attributed to the specific effects of the macromolecular constituents of the dosage form on the barrier properties of the absorbing surfaces and/or their effects on the stability of the drug in the fluids. Because of the demonstrated ability of such materials in altering the permeability properties of cells, the former effect may be considered more likely.

Also, in considering the relatively small volumes of fluid available for action upon the administered materials *in vivo*, it cannot be neglected that gelatin-encapsulated particles were used *in vivo* while compressed tablets were used *in vitro*. The use of gelatin-encapsulated particles was necessitated by the difficulties in compressing the small amounts of drug–polysalt complex comprising the dose of CPZ for the rats. A slow dissolution of the gelatin capsules in the limited fluids available *in vivo* could have slowed the release of the drug in all cases and also masked the differences among the formulations such as were observed *in vitro*.

Table III—Results of Single Factor Analysis of Variance of Treatment Combinations for an 11.5-hr. Period

Treatments[a]	Mean Square Treatments	Mean Square Error	$F_{obs.}$ (1, 10)[b]
1 × 2	330.73	59.43	5.57[c]
1 × 3	705.34	110.03	6.41[c]

[a] 1 = drug control, 2 = protamine sulfate–chlorpromazine hydrochloride physical mixture, 3 = drug–polysalt particles. [b] $F_{0.95}$ (1, 10) = 4.96; $F_{0.99}$ (1, 10) = 10.0. [c] Significant at $p < 0.05$.

CONCLUSIONS

In vitro dissolution dialysis studies clearly indicated a prolongation of drug release from polysalt flocculates. The *in vivo* results with rats did not, however, support this conclusion. Nevertheless, these results do elicit the speculation that, should the observed *in vivo* effects of the polymeric constituents on CPZ availability be a consequence of an active alteration of the permeability properties of the mucosal barrier, *e.g.*, endocytotic induction, it may also be possible, in general, to promote similarly the absorption of irregularly and poorly absorbed orally administered drugs.

REFERENCES

(1) N. Yoshida and C. Thies, *J. Colloid Interface Sci.*, **24,** 29 (1967).

(2) E. Jensen, German pat. 1,185,585 (1965); through *Chem. Abstr.*, **62,** 10632h(1965).

(3) C. Brynko and J. A. Scarpelli, U. S. pat. 3,190,837 (1965); through *Chem. Abstr.*, **63,** P6362h(1965).

(4) E. N. Hiestand, E. Jensen, and P. D. Meister, German pat. 1,249,219 (1967); through *Chem. Abstr.*, **67,** 109438h(1967).

(5) E. Jensen, E. N. Hiestand, and P. D. Meister, German pat. 1,256,196 (1967); through *Chem. Abstr.*, **68,** 6070v(1968).

(6) National Cash Register Co., Brit. pat. 927,157 (1963); through *Chem. Abstr.*, **59,** 6571b(1963).

(7) The Upjohn Co., Brit. pat. 929,401 (1963); through *Chem. Abstr.*, **59,** 14197g(1963).

(8) L. A. Luzzi and R. J. Gerraughty, *J. Pharm. Sci.*, **56,** 634 (1967).
(9) *Ibid.*, **56,** 1174(1967).
(10) G. A. Bruno and J. E. Christian, *Anal. Chem.*, **33,** 1216 (1967).
(11) W. Schallek, A. Kuehn, and D. K. Seppelin, *J. Pharmacol. Exp. Ther.*, **118,** 139(1956).
(12) "An Introduction to Three Phenothiazine Derivatives: Thorazine, Compazine, Stelazine," Smith Kline & French Laboratories, Philadelphia, Pa., p. 14.
(13) C. A. Dragstedt, *J. Amer. Med. Ass.*, **168,** 1652(1958).
(14) A. S. Michaels, *Ind. Eng. Chem.*, **57,** 32(1965).
(15) K. Iwasa, T. Hara, I. Hayashi, and T. Tokui, *Keio J. Med*, **6,** 93(1957); through *Chem. Abstr.*, **52,** 14835h(1958).
(16) J. J-P. Ryser and R. Hancock, *Science*, **150,** 501(1965).
(17) J. J-P. Ryser, *Nature*, **215,** 934(1967).
(18) J. J-P. Ryser, *Science*, **159,** 392(1968).
(19) C. Chapmen-Anressen, *Compt. Rend. Trav. Lab. Carlsberg*, **33,** 73(1962).
(20) V. Schumaker, *Exp. Cell Res.*, **15,** 314(1958).
(21) B. Larsen, *Nature*, **215,** 641(1967).
(22) C. J. Brookes, I. G. Betteley, and S. M. Loxston, "Mathematics and Statistics for Chemists," Wiley, New York, N. Y., 1966, pp. 284–313.
(23) J. Whitmore, *Diss. Abstr. B.*, **28,** 2014(1966).
(24) T. Higuchi, R. Kuramoto, L. Kennon, T. Flanagan, and A. Polk, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 646(1954).
(25) A. L. Burton, *Rev. Can. Biol.*, **20,** 15(1961).
(26) C. DeWaart, T. Brogt, and J. Sietsma, *Acta Physiol. Pharmacol. Neerl.*, **14,** 466(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 13, 1969, from the *Biophysical Pharmaceutics Area of the Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication March 3, 1970.

Abstracted from a thesis submitted by Paul A. Jablon to the Graduate School, Purdue University, Lafayette, IN 47907, in partial fulfillment of the Doctor of Philosophy degree requirements.

This investigation was supported, in part, by a grant from the American Foundation for Pharmaceutical Education and is gratefully acknowledged.

* Present address: Albany College of Pharmacy, Albany, NY 12208

Cytochrome P-450 and Alkaloid Synthesis in *Claviceps purpurea*

S. H. AMBIKE and R. M. BAXTER

Abstract □ Tryptophan, 4-dimethylallyltryptophan, and various analogs of tryptophan were investigated for their effect on binding to cytochrome P-450 and on cytochrome P-450 and total alkaloid levels in *Claviceps purpurea*. These compounds were shown not to affect cytochrome P-450 levels, in contrast to phenobarbital which increased the levels, but rather exhibited stereospecific binding to cytochrome P-450. Those compounds (L-tryptophan and L-4-dimethylallyltryptophan), which showed the highest binding affinity and are known precursors, caused the greatest increase in total alkaloid levels. The significance of these findings is discussed.

Keyphrases □ Cytochrome P-450, alkaloid synthesis—*Claviceps purpurea* □ ^{14}C-L-Tryptophan, tryptophan analogs—cytochrome P-450 □ *Claviceps purpurea* growth—tryptophan analogs □ Colorimetric analysis—spectrophotometer

The occurrence of cytochrome P-450 in a clavine-producing strain of *Claviceps purpurea* has been previously reported (1). Phenobarbital-treated *C. purpurea* exhibited a parallel increase in the cytochrome P-450 and in total alkaloid. Cyanide produced a gradual but marked decrease in both cytochrome P-450 and alkaloid levels. The interconversion of cytochrome P-450 and P-420 has been utilized to provide additional evidence for its presence in *C. purpurea* (2).

Cytochrome P-450 is now recognized as an "oxygen-activating enzyme," participating in the hydroxylation of a wide variety of compounds (3, 4). Substrate interaction with cytochrome P-450 gives rise to two types of difference spectra which have been utilized to measure the nature and degree of interaction of cytochrome P-450 with various compounds. Type I spectral changes are characterized by a difference spectrum, with a trough at 420 mμ and a peak at 385 mμ, and are characteristic of that produced by the interaction of cytochrome P-450 with hexobarbital, aminopyrine, phenobarbital, and chlorpromazine. Type II spectral changes exhibit a difference spectrum, having a peak at 430 mμ and a trough at 390 mμ; the spectrum is produced by the interaction of aniline, nicotine, nicotinamide, *etc.*, with cytochrome P-450. The magnitude of the spectral changes is substrate concentration-dependent (5). It has been suggested (6) that the substrate forms complexes with the oxidized form of cytochrome P-450 and that the rate-limiting step of the reaction is the reduction of the substrate–cytochrome P-450 complex. However, it has also been suggested that the complex is more readily reduced by NADPH cytochrome P-450 reductase than is cytochrome P-450 in the absence of the substrate (7).

The present study is concerned with the possible role of cytochrome P-450 in alkaloid synthesis and extends the previously reported data. The binding of tryptophan, tryptophan analogs, isopentenyl pyrophosphate, and 4-dimethylallyltryptophan to cytochrome P-450 was studied using the difference absorbance. The effect of these compounds on the levels of cytochrome P-450 and total alkaloid and the correlation of these values with effects on binding are also reported.

EXPERIMENTAL

Binding of ^{14}C-L-Tryptophan to Cytochrome P-450—The method utilized was similar to that of Orrenius and Ernster (8) who studied

(8) L. A. Luzzi and R. J. Gerraughty, *J. Pharm. Sci.*, **56,** 634 (1967).
(9) *Ibid.*, **56,** 1174(1967).
(10) G. A. Bruno and J. E. Christian, *Anal. Chem.*, **33,** 1216 (1967).
(11) W. Schallek, A. Kuehn, and D. K. Seppelin, *J. Pharmacol. Exp. Ther.*, **118,** 139(1956).
(12) "An Introduction to Three Phenothiazine Derivatives: Thorazine, Compazine, Stelazine," Smith Kline & French Laboratories, Philadelphia, Pa., p. 14.
(13) C. A. Dragstedt, *J. Amer. Med. Ass.*, **168,** 1652(1958).
(14) A. S. Michaels, *Ind. Eng. Chem.*, **57,** 32(1965).
(15) K. Iwasa, T. Hara, I. Hayashi, and T. Tokui, *Keio J. Med*, **6,** 93(1957); through *Chem. Abstr.*, **52,** 14835h(1958).
(16) J. J-P. Ryser and R. Hancock, *Science*, **150,** 501(1965).
(17) J. J-P. Ryser, *Nature*, **215,** 934(1967).
(18) J. J-P. Ryser, *Science*, **159,** 392(1968).
(19) C. Chapmen-Anressen, *Compt. Rend. Trav. Lab. Carlsberg*, **33,** 73(1962).
(20) V. Schumaker, *Exp. Cell Res.*, **15,** 314(1958).
(21) B. Larsen, *Nature*, **215,** 641(1967).
(22) C. J. Brookes, I. G. Betteley, and S. M. Loxston, "Mathematics and Statistics for Chemists," Wiley, New York, N. Y., 1966, pp. 284–313.
(23) J. Whitmore, *Diss. Abstr. B.*, **28,** 2014(1966).
(24) T. Higuchi, R. Kuramoto, L. Kennon, T. Flanagan, and A. Polk, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 646(1954).
(25) A. L. Burton, *Rev. Can. Biol.*, **20,** 15(1961).
(26) C. DeWaart, T. Brogt, and J. Sietsma, *Acta Physiol. Pharmacol. Neerl.*, **14,** 466(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 13, 1969, from the *Biophysical Pharmaceutics Area of the Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication March 3, 1970.

Abstracted from a thesis submitted by Paul A. Jablon to the Graduate School, Purdue University, Lafayette, IN 47907, in partial fulfillment of the Doctor of Philosophy degree requirements.

This investigation was supported, in part, by a grant from the American Foundation for Pharmaceutical Education and is gratefully acknowledged.

* Present address: Albany College of Pharmacy, Albany, NY 12208

Cytochrome P-450 and Alkaloid Synthesis in *Claviceps purpurea*

S. H. AMBIKE and R. M. BAXTER

Abstract □ Tryptophan, 4-dimethylallyltryptophan, and various analogs of tryptophan were investigated for their effect on binding to cytochrome P-450 and on cytochrome P-450 and total alkaloid levels in *Claviceps purpurea*. These compounds were shown not to affect cytochrome P-450 levels, in contrast to phenobarbital which increased the levels, but rather exhibited stereospecific binding to cytochrome P-450. Those compounds (L-tryptophan and L-4-dimethylallyltryptophan), which showed the highest binding affinity and are known precursors, caused the greatest increase in total alkaloid levels. The significance of these findings is discussed.

Keyphrases □ Cytochrome P-450, alkaloid synthesis—*Claviceps purpurea* □ ^{14}C-L-Tryptophan, tryptophan analogs—cytochrome P-450 □ *Claviceps purpurea* growth—tryptophan analogs □ Colorimetric analysis—spectrophotometer

The occurrence of cytochrome P-450 in a clavine-producing strain of *Claviceps purpurea* has been previously reported (1). Phenobarbital-treated *C. purpurea* exhibited a parallel increase in the cytochrome P-450 and in total alkaloid. Cyanide produced a gradual but marked decrease in both cytochrome P-450 and alkaloid levels. The interconversion of cytochrome P-450 and P-420 has been utilized to provide additional evidence for its presence in *C. purpurea* (2).

Cytochrome P-450 is now recognized as an "oxygen-activating enzyme," participating in the hydroxylation of a wide variety of compounds (3, 4). Substrate interaction with cytochrome P-450 gives rise to two types of difference spectra which have been utilized to measure the nature and degree of interaction of cytochrome P-450 with various compounds. Type I spectral changes are characterized by a difference spectrum, with a trough at 420 mμ and a peak at 385 mμ, and are characteristic of that produced by the interaction of cytochrome P-450 with hexobarbital, aminopyrine, phenobarbital, and chlorpromazine. Type II spectral changes exhibit a difference spectrum, having a peak at 430 mμ and a trough at 390 mμ; the spectrum is produced by the interaction of aniline, nicotine, nicotinamide, *etc.*, with cytochrome P-450. The magnitude of the spectral changes is substrate concentration-dependent (5). It has been suggested (6) that the substrate forms complexes with the oxidized form of cytochrome P-450 and that the rate-limiting step of the reaction is the reduction of the substrate–cytochrome P-450 complex. However, it has also been suggested that the complex is more readily reduced by NADPH cytochrome P-450 reductase than is cytochrome P-450 in the absence of the substrate (7).

The present study is concerned with the possible role of cytochrome P-450 in alkaloid synthesis and extends the previously reported data. The binding of tryptophan, tryptophan analogs, isopentenyl pyrophosphate, and 4-dimethylallyltryptophan to cytochrome P-450 was studied using the difference absorbance. The effect of these compounds on the levels of cytochrome P-450 and total alkaloid and the correlation of these values with effects on binding are also reported.

EXPERIMENTAL

Binding of ^{14}C-L-Tryptophan to Cytochrome P-450—The method utilized was similar to that of Orrenius and Ernster (8) who studied

Table I—Binding Affinity of Cytochrome P-450 of *C. purpurea* and Alkaloid Levels

Compound[a]	Absorbance Difference, $A_{385-420}$	Amount of Cytochrome P-450 in μmoles, $A_{450-490}$	Total Alkaloid in mg./ Flask
Control	0.010	6.10	2.10
L-Tryptophan	0.038	6.51	4.08
D-Tryptophan	0.009	5.86	2.05
DL-Tryptophan	0.018	5.95	2.21
4-Methyl-DL-tryptophan	0.010	6.04	2.14
5-Methyl-DL-tryptophan	0.020	6.05	3.20
6-Methyl-DL-tryptophan	0.010	5.98	2.18
5-Fluoro-DL-tryptophan	0.012	6.20	2.04
4-Fluoro-DL-tryptophan	0.011	5.95	2.15
4-Dimethylallyl-L-tryptophan	0.042	6.09	4.45
Isopentenyl pyrophosphate	0.015	5.95	3.15

[a] Concentration = 2 m*M*.

the binding of ^{14}C-aniline to cytochrome P-450. The cytochrome P-450-containing mycelial pellet preparation (1) was incubated at 37° for 60 min. in 2.5 ml. of a reaction mixture containing 1 ml. of 0.05 *M* tromethamine (pH 7.5). One milliliter of 0.12 *M* KCl, 0.5 ml. of 1.67 *M* $Na_2S_2O_4$, and 1 ml. of 1 m*M* solution ^{14}C-L-tryptophan (sp. act. 9.0 mc./m*M*) were added. After incubation, the sample was diluted to 10 ml. with ice-cold 0.15 *M* KCl and centrifuged at 105,000×*g* for 60 min. The pellet was resuspended in 10 ml. of 0.15 *M* ice-cold KCl and recentrifuged. The washing was repeated three times or until the last washing was devoid of radioactivity. The washed pellet was suspended in 2 ml. of 0.15 *M* KCl and lyophilized. The residue was dissolved in 1 ml. of hyamine hydroxide and 10 ml. of scintillating solution (PPO, 4 g.; POPOP, 100 mg. dissolved in 1 l. of toluene). The radioactivity in counts per minute was determined according to the usual procedure, using a Packard liquid scintillation spectrometer. The results in counts per minute were utilized to determine the percentage of ^{14}C-L-tryptophan bound. The radioactivity in counts per minute was also determined for a 1 m*M* solution of ^{14}C-L-tryptophan and for the combined washings (three).

Effect of Carbon Monoxide on Binding of ^{14}C-L-Tryptophan to Cytochrome P-450—The washed pellet was suspended in 2.5 ml. of the reaction mixture. Carbon monoxide was bubbled into the mycelial pellet suspension for 30 sec. After centrifugation at 105,000 ×*g* for 60 min. and after three washings, the radioactivity was determined as described.

Determination of the Magnitude of Spectral Changes—The mycelial pellet, containing cytochrome P-450, prepared as described (2), was suspended in 2.5 ml. of the reaction mixture. One milliliter of a solution of L-tryptophan (range 0.5–6.0 m*M*/ml.) was added, followed by incubation at 37° for 60 min. The difference in absorbance was measured between the two wavelengths, 385 and 420 mμ. The difference absorbance for the binding of other compounds was determined in a similar manner. The results in Table I

Table II—Binding of ^{14}C-L-Tryptophan to Cytochrome P-450 of Control and Phenobarbital-Treated *C. purpurea*

Series	Source of Cytochrome P-450	Binding of ^{14}C-L-Tryptophan to Cytochrome P-450 in Mycelial Pellet,[a] %	^{14}C-L-Tryptophan in Supernatant,[a] %
1	Control	71.47	27.54
2	Phenobarbital-treated	85.14	14.43
3	Control plus carbon monoxide	11.13	88.41

[a] For details of preparation, see *Experimental.*

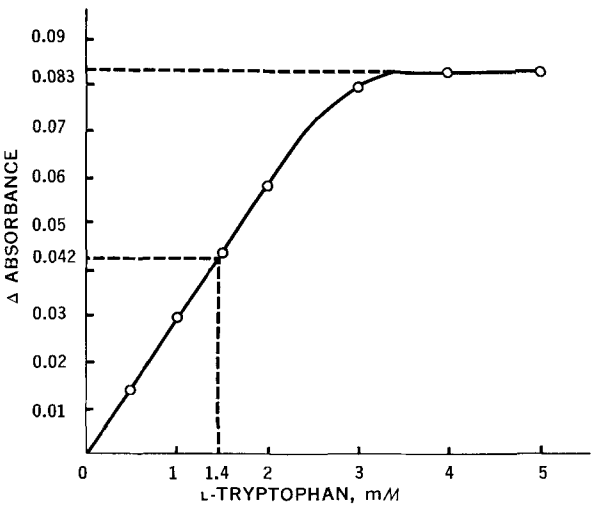


Figure 1—*Binding of* L-*tryptophan to cytochrome P-450 in the mycelial pellet of* C. purpurea.

were obtained using a 1-ml. solution of a concentration sufficient to give a final concentration of 2 m*M*.

Growth of *C. purpurea* in the Presence of Analogs—*C. purpurea* was grown as previously described (2). The analog of tryptophan and other additives were added to a final concentration of 2 m*M* on the 10th day. On the 16th day (6 days after additions), the medium was removed from the mycelial mat and used for estimating total alkaloids by the method of Taber and Vining (9). The preparation of the mycelial pellet was as previously described (2), and the estimation of cytochrome P-450 was by the method of Sato and Omura (10).

RESULTS

A comparison of the binding capacity of cytochrome P-450 in the mycelial pellet preparation from control and phenobarbital-treated *C. purpurea* revealed an increased binding capacity for L-^{14}C-tryptophan for the cytochrome P-450 in the mycelial pellet from phenobarbital-treated *C. purpurea*. Treatment of the mycelial pellet with carbon monoxide in the presence of dithionite resulted in a release of most of the bound L-^{14}C-tryptophan (Table II). Figure 1 illustrates the increase in binding with increasing tryptophan concentration expressed as an absorbance difference ($A_{385-420}$). Maximum binding was observed at a concentration of 5 m*M* of L-tryptophan, with one-half maximum being observed at 1.4 m*M* concentration of L-tryptophan.

Over a pH range from 4.0 to 9.5, binding was maximal at pH 5–5.5 and decreased by approximately 30% at pH 4.0 and 9.5. Table I summarizes the relative binding affinity of cytochrome P-450 as measured by absorbance difference for tryptophan, isopentenyl pyrophosphate, 4-dimethylallyltryptophan, and certain analogs of tryptophan. The effects of these compounds on cytochrome P-450 and total alkaloid levels in *C. purpurea* when added during the alkaloid-producing period are also indicated. It will be observed that the binding affinity is greatest for L-tryptophan and relatively low for D-tryptophan. The analogs of tryptophan used were the DL-isomers; therefore, the values recorded for these should be compared with those recorded for DL-tryptophan. None of the compounds in Table I produced any significant changes in the cytochrome P-450 levels from those of the control. However, L-tryptophan and 4-dimethylallyl-L-tryptophan, which are known precursors of ergot alkaloids, exhibit the highest binding affinities for cytochrome P-450 as well as causing the greatest increases in total alkaloid. The 5-methyl analog of DL-tryptophan exhibited a binding affinity similar to that of DL-tryptophan, but its addition during the alkaloid-producing period of growth of *C. purpurea* resulted in a greater increase in total alkaloid than that produced by DL-tryptophan.

The effects of various analogs of tryptophan when added during the alkaloid-producing period of growth of *C. purpurea* on cytochrome P-450 on total alkaloid levels and the affinity of the cyto-

Table III—Effect of Tryptophan Analogs[a] on Binding Affinity of Cytochrome P-450 for Tryptophan and Alkaloid Levels

Compound[b]	Absorbance Difference, $A_{385-420}$	Amount of Cytochrome P-450 in μmoles, $A_{450-490}$	Total Alkaloid in mg./ Flask
4-Methyl-DL-tryptophan	0.020	6.14	3.12
5-Methyl-DL-tryptophan	0.044	6.10	5.90
6-Methyl-DL-tryptophan	0.030	5.95	3.14
4-Fluoro-DL-tryptophan	0.024	6.10	3.10
5-Fluoro-DL-tryptophan	0.039	5.87	3.87
4-Dimethylallyl-L-tryptophan	0.048	6.14	6.10

[a] Analogs (2 m*M*) and L-tryptophan (2 m*M*) added on the 10th day to the culture of *C. purpurea*. Binding affinity of cytochrome P-450 of mycelial pellet, cytochrome P-450, and alkaloid levels determined on the 16th day (6 days after additions). [b] Concentration = 2 m*M*.

chrome P-450 for L-tryptophan are summarized in Table III. No significant differences were observed in the cytochrome P-450 levels. The cytochrome P-450 of *C. purpurea* grown in the presence of 4-methyl-DL-tryptophan and 4-fluoro-DL-tryptophan exhibited a significantly reduced affinity for L-tryptophan and also a lower total alkaloid. The known precursor 4-dimethylallyl-L-tryptophan, under the same conditions, increased the affinity of the cytochrome P-450 and a significant increase in total alkaloid resulted. The nonprecursor analog, 5-methyl-DL-tryptophan, produced an increase [tryptophan 0.038 (Table I) *versus* 0.044 (Table III)] in binding affinity over that exhibited by the cytochrome P-450 from *C. purpurea* which had not been exposed to any additive and produced an increase in total alkaloid nearly equivalent to that produced by 4-dimethylallyl-L-tryptophan.

The binding affinity of cytochrome P-450 of *C. purpurea* grown in the presence of L-tryptophan exhibited a markedly enhanced affinity for isopentenyl pyrophosphate over that exhibited by cytochrome P-450 from *C. purpurea* which had not been exposed to any additive [0.051 (Table IV) *versus* 0.015 (Table I)]. D-Tryptophan, 5-methyl-DL-tryptophan, and 5-fluoro-DL-tryptophan appeared not to exert a significant effect.

DISCUSSION

The binding of tryptophan to the cytochrome P-450 of the mycelial pellet of *C. purpurea* was both pH and temperature dependent. Maximum binding was observed at a pH of approximately 5.5. Orrenius and Ernster (8) reported that binding to cytochrome P-450 is pH dependent. At 37°, maximum binding was observed after 60 min. The extent of binding observed was 30% of maximum after 15 min., 50% after 30 min., and 80% after 45 min.

Carbon monoxide, which is known to form an enzymically inactive complex with reduced cytochrome P-450 (12), prevents binding of the compound which, once bound, is released in the presence of dithionite and carbon monoxide. Thus, an enzymically functional cytochrome P-450 is necessary for the binding to cytochrome P-450 and for active hydroxylation (8). From Table II, it will be observed that the extent of binding of ^{14}C-L-tryptophan was greatly diminished by carbon monoxide. The addition of dithionite had no effect on the extent of binding, but treatment with carbon monoxide resulted in the release of a large proportion of the already bound ^{14}C-tryptophan.

The binding of tryptophan to the cytochrome P-450 of the mycelial pellet of *C. purpurea* exhibits a high degree of stereospecificity. The data of Table I indicate that the cytochrome P-450 exhibits a relatively high binding affinity for L-tryptophan in contrast to D-tryptophan. The binding affinity exhibited for 4-dimethylallyl-L-tryptophan is at least of the same order as that exhibited for L-tryptophan or greater. These results are particularly significant with respect to the biosynthesis of the ergolene nucleus, since it has been established that ^{14}C-L-tryptophan is more efficiently incorporated into the ergolene nucleus than is ^{14}C-D-tryptophan. In addition, Agurell (13) has reported that 4-dimethylallyl-L-tryptophan is more efficiently utilized than 4-dimethylallyl-D-tryptophan or L-tryptophan. From Table I, it is also apparent that those compounds which exhibited the greatest degree of stereospecific binding also produced the greatest increase in total alkaloid.

Table IV—Effect of Tryptophan Analogs[a] on the Binding Affinity of Cytochrome P-450 for Isopentenyl Pyrophosphate and Alkaloid Levels

Compound[b]	Absorbance Difference, $A_{385-420}$	Amount of Cytochrome P-450 in μmoles, $A_{450-490}$	Total Alkaloid, mg.
L-Tryptophan	0.051	6.10	6.30
D-Tryptophan	0.020	5.85	3.20
DL-Tryptophan	0.030	5.90	4.10
5-Methyl-DL-tryptophan	0.020	5.80	3.38
5-Fluoro-DL-tryptophan	0.025	6.04	4.04

[a] Analogs (2 m*M*) and isopentenyl pyrophosphate (2 m*M*) added on the 10th day to culture of *C. purpurea*. Binding affinity of cytochrome P-450 of mycelial pellet, cytochrome P-450, and alkaloid levels determined on the 16th day (6 days after additions). [b] Concentration = 2 m*M*.

Although various routes have been proposed for the formation of ergot alkaloids, the route proposed by Arigoni (14) would appear of particular interest relative to the results reported here. The pathway proposed by Arigoni is one in which 4-dimethylallyltryptophan is hydroxylated to form an intermediate which is then converted to agroclavine. Thus the results presented in this and earlier reports suggest a role for cytochrome P-450 in the formation of the ergolene nucleus. The most obvious involvement of cytochrome P-450 on the basis of present evidence would be in a reaction in which 4-dimethylallyltryptophan is hydroxylated. However, the high binding affinity exhibited by cytochrome P-450 of the mycelial pellet for L-tryptophan (Table I) and the greatly enhanced affinity for isopentenyl pyrophosphate of the cytochrome P-450 of the mycelial pellet from *C. purpurea* grown in the presence of L-tryptophan (Table IV) suggest the possible involvement of cytochrome P-450 at an earlier stage.

The effect of growing *C. purpurea* in the presence of analogs of tryptophan on the binding affinity of the cytochrome P-450 of the mycelial pellet for L-tryptophan and their effect on cytochrome P-450 and total alkaloid levels are indicated in Table III. No significant changes in cytochrome P-450 levels were produced by any of the compounds. However, those compounds that increased the binding affinity for L-tryptophan (5-methyl-DL-tryptophan and 4-dimethylallyl-L-tryptophan) caused a significant increase in total alkaloids. Those analogs (4-methyl-DL-tryptophan and 4-fluoro-DL-tryptophan) that reduced the binding affinity tended to cause a reduction in total alkaloids. These results offer a possible explanation for earlier observations (15) that the 4-methyl and 4-fluoro analogs inhibited alkaloid synthesis relatively selectively and that such inhibition was only partially reversible by tryptophan. Protein synthesis was not significantly affected by these analogs; thus the effect of 5-methyl-DL-tryptophan in increasing total alkaloid (Table IV) and in increasing the incorporation of ^{14}C-L-tryptophan into agroclavine (15) may be explained in part by its effect on the binding affinity of cytochrome P-450 for L-tryptophan or perhaps 4-dimethylallyl-L-tryptophan.

The binding affinity of cytochrome P-450 of the mycelial pellet of *C. purpurea* for isopentenyl pyrophosphate was altered when *C. purpurea* was grown in the presence of tryptophan and tryptophan analogs. The increase in affinity for isopentenyl pyrophosphate when L-tryptophan was added to the growth medium was the most significant finding observable from the data in Table IV. A stereospecific effect was observable in the alterations in binding affinities (compare L-tryptophan and D-tryptophan).

The results reported here and elsewhere (1) indicate that an increase or decrease in total alkaloid may be paralleled (or the result of): (*a*) an increase or decrease in cytochrome P-450 levels or (*b*) an increase or decrease in binding affinity of cytochrome P-450 for the alkaloid precursors (L-tryptophan and isopentenyl pyrophosphate or 4-dimethylallyl-L-tryptophan). Either or both of these may play a role in the initiation of alkaloid synthesis in *C. purpurea*, which is known to follow the active synthesis of primary metabolites such as protein. Thus, an increase in cytochrome P-450 levels in the presence of the precursors might result in the initiation of alkaloid synthesis. Equally as possible would be the initiation of alkaloid formation as a result of an increase in the binding affinity of existing cytochrome P-450 for alkaloid pre-

cursors. The results in Table IV might allow for the speculation that the increased availability of tryptophan resulting from reduced protein synthesis could sufficiently alter the binding of cytochrome P-450 for isopentenyl pyrophosphate that alkaloid synthesis might proceed, whereas at lower levels of available tryptophan such would not occur because of the low affinity of the cytochrome P-450 for isopentenyl pyrophosphate. Based on the observation that benzyl thiocyanate, which has been shown to enhance tetracycline formation (16), increased total alkaloid in *C. purpurea* to almost the same degree as phenobarbital (15), it is suggestive that cytochrome P-450 may be involved in the formation of certain other secondary cell metabolites in addition to that which has been discussed in this report.

REFERENCES

(1) S. H. Ambike and R. M. Baxter, to be published.

(2) *Ibid.*

(3) J. Schekman, H. Remmer, and R. W. Estabrook, *Mol. Pharmacol.*, **3**, 113(1967).

(4) Y. Imai and R. Sato, *Biochem. Biophys. Res. Commun.*, **22**, 620(1966).

(5) Y. Imai and R. Sato, *J. Biochem.*, **62**, 239(1967).

(6) H. Remmer, J. Schenkman, R. W. Estabrook, J. Gillette, D. Y. Cooper, and O. Rosenthal, *Mol. Pharmacol.*, **2**, 187(1966).

(7) P. L. Gigon, T. E. Gram, and J. R. Gillette, *Biochem. Biophys. Res. Commun.*, **31**, 558(1968).

(8) S. Orrenius and L. Ernster, *Life Sci.*, **6**, 1473(1967).

(9) W. A. Taber and L. C. Vining, *Can. J. Microbiol.*, **4**, 611 (1958).

(10) R. Sato and T. Omura, *J. Biol. Chem.*, **239**, 2370(1964).

(11) D. Y. Cooper, S. Narasimhulu, O. Rosenthal, and R. W. Estabrook, in "Oxidases and Related Redox Systems," T. E. King, H. S. Mason, and M. Morrison, Eds., Wiley, New York, N. Y., 1965, p. 838.

(12) T. Omura and R. Sato, *J. Biol. Chem.*, **239**, 2379(1964).

(13) S. Agurell, *Acta Pharm. Suecica*, **3**, 71(1966).

(14) D. Arigoni, in "Some Aspects of Mevalonoid Biosynthesis," Symposium on "Organic Chemical Approaches to Biosynthesis," London, England, 1965.

(15) N. D. Zahid and R. M. Baxter, presented to the APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

(16) M. H. J. Slezak and M. Vondracek, in "Antibiotics—Advances in Research, Production and Clinical Use," M. Herold and Z. Gabriel, Eds., Butterworths, Toronto, Ontario, Canada, 1966, p. 578.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 22, 1970, from the *Faculty of Pharmacy, University of Toronto, Toronto 5, Ontario, Canada.*

Accepted for publication March 17, 1970.

DRUG STANDARDS

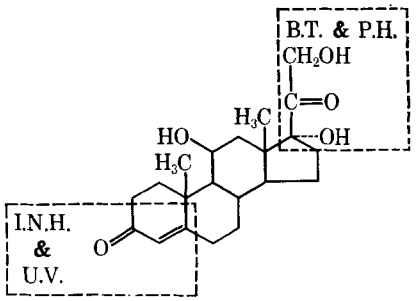
Detection of Decomposition and Analytical Interferences in Pharmaceutical Preparations Containing Corticosteroids

ROBERT E. GRAHAM*, PATRICIA A. WILLIAMS*, and CHARLES T. KENNER†

Abstract □ Since the blue tetrazolium and phenylhydrazine reagents for corticosteroids react with the intact side chain at C_{17}, and the isonicotinic acid hydrazide method and UV spectrophotometry depend upon conjugation in Ring A at the other end of the molecule, the analytical results by the four methods give information concerning decomposition caused by oxidation of the C_{17} side chain and by deconjugation in Ring A. Methods are proposed which allow the detection and determination of both acidic and neutral decomposition products. Measurement of the variation of absorbance with time can be used to detect unidentified interferences in the blue tetrazolium, phenylhydrazine, and isonicotinic acid procedures. The extent of interference of several substances which interfere in at least one of the color reactions is reported. Several examples of the use of the proposed methods to detect and determine decomposition and/or interference are given.

Keyphrases □ Corticosteroids, decomposition determination—methods compared □ Decomposition, corticosteroids—C_{17} side-chain oxidation determination □ Interference—corticosteroid analysis □ Blue tetrazolium, phenylhydrazine, isonicotinic acid hydrazide, UV spectrophotometry methods—analysis

The detection of decomposition and of analytical interferences in pharmaceutical corticosteroid preparations is important in the correct determination of the composition of such preparations. The usual methods of analysis for undecomposed corticoid hormones include the blue tetrazolium reaction (BT) (1–5); the phenylhydrazine–sulfuric acid–alcohol reaction (PH) (6, 7), which is also known as the Porter-Silber reaction; the isonicotinic acid hydrazide reaction (INH) (8), which is also known as the Umberger reaction; and UV spectrophotometry. As is shown in Structure I, the BT and PH reagents react with the C_{17} side chain, while the INH and UV methods depend upon the conjugation of the carbonyl group at C_3 with the double bond between C_4 and C_5 in Ring A of the steroid nucleus. Since



hydrocortisone—portions of molecule measured by the following methods: INH = isonicotinic acid hydrazide, BT = blue tetrazolium, PH = phenylhydrazine H_2SO_4, and UV = ultraviolet

I

cursors. The results in Table IV might allow for the speculation that the increased availability of tryptophan resulting from reduced protein synthesis could sufficiently alter the binding of cytochrome P-450 for isopentenyl pyrophosphate that alkaloid synthesis might proceed, whereas at lower levels of available tryptophan such would not occur because of the low affinity of the cytochrome P-450 for isopentenyl pyrophosphate. Based on the observation that benzyl thiocyanate, which has been shown to enhance tetracycline formation (16), increased total alkaloid in *C. purpurea* to almost the same degree as phenobarbital (15), it is suggestive that cytochrome P-450 may be involved in the formation of certain other secondary cell metabolites in addition to that which has been discussed in this report.

REFERENCES

(1) S. H. Ambike and R. M. Baxter, to be published.

(2) *Ibid.*

(3) J. Schekman, H. Remmer, and R. W. Estabrook, *Mol. Pharmacol.*, **3**, 113(1967).

(4) Y. Imai and R. Sato, *Biochem. Biophys. Res. Commun.*, **22**, 620(1966).

(5) Y. Imai and R. Sato, *J. Biochem.*, **62**, 239(1967).

(6) H. Remmer, J. Schenkman, R. W. Estabrook, J. Gillette, D. Y. Cooper, and O. Rosenthal, *Mol. Pharmacol.*, **2**, 187(1966).

(7) P. L. Gigon, T. E. Gram, and J. R. Gillette, *Biochem. Biophys. Res. Commun.*, **31**, 558(1968).

(8) S. Orrenius and L. Ernster, *Life Sci.*, **6**, 1473(1967).

(9) W. A. Taber and L. C. Vining, *Can. J. Microbiol.*, **4**, 611 (1958).

(10) R. Sato and T. Omura, *J. Biol. Chem.*, **239**, 2370(1964).

(11) D. Y. Cooper, S. Narasimhulu, O. Rosenthal, and R. W. Estabrook, in "Oxidases and Related Redox Systems," T. E. King, H. S. Mason, and M. Morrison, Eds., Wiley, New York, N. Y., 1965, p. 838.

(12) T. Omura and R. Sato, *J. Biol. Chem.*, **239**, 2379(1964).

(13) S. Agurell, *Acta Pharm. Suecica*, **3**, 71(1966).

(14) D. Arigoni, in "Some Aspects of Mevalonoid Biosynthesis," Symposium on "Organic Chemical Approaches to Biosynthesis," London, England, 1965.

(15) N. D. Zahid and R. M. Baxter, presented to the APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

(16) M. H. J. Slezak and M. Vondracek, in "Antibiotics—Advances in Research, Production and Clinical Use," M. Herold and Z. Gabriel, Eds., Butterworths, Toronto, Ontario, Canada, 1966, p. 578.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 22, 1970, from the *Faculty of Pharmacy, University of Toronto, Toronto 5, Ontario, Canada.*

Accepted for publication March 17, 1970.

DRUG STANDARDS

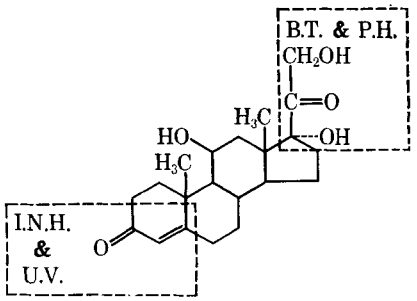
Detection of Decomposition and Analytical Interferences in Pharmaceutical Preparations Containing Corticosteroids

ROBERT E. GRAHAM*, PATRICIA A. WILLIAMS*, and CHARLES T. KENNER†

Abstract □ Since the blue tetrazolium and phenylhydrazine reagents for corticosteroids react with the intact side chain at C_{17}, and the isonicotinic acid hydrazide method and UV spectrophotometry depend upon conjugation in Ring A at the other end of the molecule, the analytical results by the four methods give information concerning decomposition caused by oxidation of the C_{17} side chain and by deconjugation in Ring A. Methods are proposed which allow the detection and determination of both acidic and neutral decomposition products. Measurement of the variation of absorbance with time can be used to detect unidentified interferences in the blue tetrazolium, phenylhydrazine, and isonicotinic acid procedures. The extent of interference of several substances which interfere in at least one of the color reactions is reported. Several examples of the use of the proposed methods to detect and determine decomposition and/or interference are given.

Keyphrases □ Corticosteroids, decomposition determination—methods compared □ Decomposition, corticosteroids—C_{17} side-chain oxidation determination □ Interference—corticosteroid analysis □ Blue tetrazolium, phenylhydrazine, isonicotinic acid hydrazide, UV spectrophotometry methods—analysis

The detection of decomposition and of analytical interferences in pharmaceutical corticosteroid preparations is important in the correct determination of the composition of such preparations. The usual methods of analysis for undecomposed corticoid hormones include the blue tetrazolium reaction (BT) (1–5); the phenylhydrazine–sulfuric acid–alcohol reaction (PH) (6, 7), which is also known as the Porter-Silber reaction; the isonicotinic acid hydrazide reaction (INH) (8), which is also known as the Umberger reaction; and UV spectrophotometry. As is shown in Structure I, the BT and PH reagents react with the C_{17} side chain, while the INH and UV methods depend upon the conjugation of the carbonyl group at C_3 with the double bond between C_4 and C_5 in Ring A of the steroid nucleus. Since



hydrocortisone—portions of molecule measured by the following methods: INH = isonicotinic acid hydrazide, BT = blue tetrazolium, PH = phenylhydrazine H_2SO_4, and UV = ultraviolet

I

Table I—Reported Interferences in Corticosteroid Photometric Methods

Interfering Substance	Reference	BT	PH	INH
Acetone	(36)	+	NR[a]	+
Active hydrogen compounds	(37)	+	NR	NR
Allantoin	(38)	+	NR	NR
Alloxantin dihydrate	(39)	+	NR	NR
Amphotericin B	(36)	+	+	+
Bacitracin	(36)	+	+	None
Benzal acetone	(8)	NR	NR	+
Benzyl styryl ketone	(8)	NR	NR	+
Dyclonine hydrochloride	(36)	+	+	+
Erythromycin	(39)	+	+	+
Erythromycin stearate	(36)	+	−	None
Hydroxy ketones	(39)	+	NR	NR
Iodochlorhydroxyquin	(36)	None	+	+
Lanolin	(36)	+	NR	NR
Nystatin	(36)	+	+	+
Phenothiazine	(34)	NR	−	NR
Polyhydroxyphenols	(37)	+	NR	NR
Polyhydroxythiols	(37)	+	NR	NR
Polymixin B sulfate	(36)	None	+	None
Reducing sugars	(1)	+	NR	NR
Sodium novobiocin	(36)	+	+	+
Stearic acid	(36)	−	Table II	Table II
Tetracyclines	(39)	+	+	+
Xylocaine	(36)	NR	−	NR
Zinc bacitracin	(36)	+	−	None

[a] NR = not stated in reference.

the reactions in these four methods occur with different portions of the molecule, they can be used to detect and distinguish between decomposition which occurs at the C_{17} side chain and decomposition caused by deconjugation in Ring A. Quantitative agreement between all four methods indicates that the corticosteroid is not decomposed and that there is no interference in the determinative steps of any of the methods due to components of the preparation. Differences between the values by the four methods indicate decomposition or interference.

The most common type of corticosteroid decomposition encountered in pharmaceutical preparations is caused by the oxidation of the C_{17} side chain by readily reducible organic and inorganic compounds, by metal ions, and by air in alkaline solutions (9–16). Much less common is the destruction or movement of the double bonds in Ring A caused by absorption of UV light (17–20). The degradation products caused by oxidation of the C_{17} side chain are neutral or acidic in character (9, 11, 21–23) and are a complex mixture of organic acids together with neutral compounds such as aldehydes and ketones. These decomposition products can be separated by extraction into chloroform, since the neutral compounds will be extracted along with the undecomposed steroid while the acidic components will remain in the aqueous phase.

The BT reaction is the official method in USP XVII (24) and NF XII (25) for preparations listed in these compendia. It is more specific than the INH or UV method for corticosteroids since the BT reagent reacts with that portion of the molecule usually involved in decomposition. The BT method is subject to many interferences, as shown in Tables I and II. Table I lists reported interferences with each of the three types of reactions used to determine corticosteroids. Interferences in the UV determination are not included, since a large number of ingredients in corticosteroid preparations absorb in the same region and the interference is relatively easy to detect due to changes in the UV spectra. The PH reaction is specific for the 17,21-dihydroxy-20-keto side chain at C_{17} and is less subject to interference than the BT procedure.

Many substances which interfere in the BT, PH, INH, and UV procedures are usually removed during sample cleanup by extraction, by magnesium silicate[1] column chromatography (26), by TLC (17, 27), by paper chromatography (28–30), and by column-partition chromatography (31, 32). Water-soluble acidic decomposition products and interferences are removed by the use of acidic and basic traps in column procedures and by acidic or basic extractions in separator procedures. Neutral decomposition products extract with the undecomposed corticosteroid.

This paper reports several unreported interferences to the determinative methods (Table II) and suggests methods of evaluation of results by the four methods to detect and determine decomposition in corticosteroid preparations and to detect interferences.

EXPERIMENTAL

Reagents and Equipment—The reagents called for in the references for the individual methods were prepared as directed. All reagents were USP or ACS grade. All corticosteroid standard solutions were prepared from USP, NF, or commercial reference standards.

All measurements were made on a Cary spectrophotometer, model 15.

Methods—In all methods used, the samples were carried through any necessary cleanup and preparative steps. The final solution obtained was evaporated to dryness carefully under air on a steam bath. The residue was dissolved and made to volume with alcohol USP, so that the concentration was approximately 0.010 mg./ml. of the corticosteroid. This solution was used for the determinative steps for all four methods. A standard solution of the corticosteroid of the same concentration was also used in the determinative steps of each method. In all cases, the absorbance spectra of the first standard and the first sample were rescanned after all measurements in the series had been completed.

BT Method—The procedure given in USP XVII (24) and NF XII (25) was followed except that 10.0-ml. aliquots and 1.0 ml. each of the BT reagent and the tetramethylammonium hydroxide reagent were used.

PH Method—The procedure of Silber and Porter (7) was followed without modification.

INH Method—The procedure of Umberger (8) was used, except that the INH reagent was modified by using twice the recommended concentration of hydrochloric acid to increase the sensitivity of the reaction (33).

UV Spectrophotometry—The alcohol USP solution was scanned directly in a 1-cm. cell.

Interference Studies—The interference of several ingredients in corticosteroid preparations, which has not been reported previously, was investigated. The interfering substance was added to a standard solution of hydrocortisone, which was then evaporated to dryness and the residue dissolved in alcohol USP prior to color development. In each case, the absorbance was compared to the absorbance of a standard hydrocortisone solution under the same conditions.

Time Study of Absorbance Variation to Detect Interference—The absorbance of the sample solution and of the standard solution was measured periodically during and after the standard color development period.

Detection of Decomposition or Interference—The absorbance of the sample and standard solution was determined by all four

[1] Florisil, Floridin Co., Pittsburgh, PA 15222

Table II—Interferences in Corticosteroid Photometric Methods

Interfering Substance	Level mg. Interference/ mg. Hydrocortisone	Absorbance: BT Standard	BT Standard + Interference	BT % Difference	PH Standard	PH Standard + Interference	PH % Difference	INH Standard	INH Standard + Interference	INH % Difference
Sorbitan monostearate	250/1	0.585	0.680	+16.2	0.546	T[a]		0.326	0.357	+9.5
Sorbitan monooleate	250/1	0.585	0.748	+27.9	0.546	T		0.326	0.457	+40.2
Ethyl ether peroxides	22/1	0.551	0.618	+12.2	0.379	0.347	−8.4	0.338	0.331	−2.1
Lanolin	600/1	0.585	0.846	+44.6	0.546	T		0.326	0.746	+128.8
Salicylamide	60/1	0.582	0.570	−2.1	0.546	0.549	+0.5	0.326	0.325	−0.3
Sodium lauryl sulfate	20/1	0.564	0.558	−1.1	0.379	T		0.326	0.324	−0.6
Stearic acid	120/1	0.582	0.568	−2.4	0.546	T		0.326	0.325	−0.3
Sulfur	0.05/1	0.588	0.742	+26.2	0.559	0.559	0	0.332	0.332	0.0
Sulfide	0.133/1	0.585	0.709	+21.2	0.546	0.546	0	0.326	0.328	+0.6
Polysorbate 60	90/1	0.570	0.754	+32.3	0.386	T		0.328	0.333	+1.5

[a] T = solution becomes turbid so that absorbance cannot be determined.

methods. Significant differences between the values obtained indicate either decomposition or interference in one or more methods.

Procedure for Determination of Decomposition at C_{17} Side Chain—Prepare an alcohol USP extract of the sample and determine the amount of corticosteroid by either the INH or UV method. This is the total amount of the undecomposed plus any decomposed corticosteroid present.

Dissolve or suspend the sample in an aqueous solution and extract with chloroform. Retain both solutions.

Determine the acidic decomposition products in the aqueous fraction by evaporation of a known aliquot to dryness, followed by determination by the INH or UV method.

Determine the undecomposed corticosteroid in the chloroform fraction by evaporation of a known aliquot to dryness, followed by determination by the BT or PH method.

Determine the neutral decomposition products plus the undecomposed corticosteroid in the chloroform fraction by evaporation of a known aliquot to dryness, followed by determination by the INH or UV method.

RESULTS AND DISCUSSION

The major portion of the determinations in this investigation has been made on hydrocortisone samples and standards, but the results are equally applicable to other corticosteroids since the decomposition pathways and determinative reactions are similar for all corticosteroids.

Several components of corticosteroid preparations were found to cause interference in the determinative steps of at least one of the four corticosteroid methods. The results are summarized in Table II.

Table III—Analysis of Corticosteroid Preparations

Product	Corticosteroid	Concn.	% of Declared Value by BT	PH	INH
Lotion					
1	Hydrocortisone	0.125%	100.0	99.2	99.7
2	Hydrocortisone	0.125%	24.3[a]	2.5	43.8
3	Hydrocortisone	0.25%	97.6[a]	90.6	100.3
4	Hydrocortisone	0.25%	110.8	108.4	109.6
5	Hydrocortisone	0.5%	95.3[a]	90.7	98.7
6	Hydrocortisone	0.5%	99.2[a]	94.4	96.9
7	Hydrocortisone	1%	107.2	107.5	107.4
Cream					
1	Hydrocortisone	0.125%	48.4[a]	30.2	71.1
2	Hydrocortisone	0.125%	82.2[a]	45.0	60.0
3	Hydrocortisone	0.25%	103.6	105.6	101.8
4	Hydrocortisone	0.5%	111.7[a]	102.7	113.2
Ointment					
1	Hydrocortisone acetate	0.5%	104.2[a]	93.4	96.9
Ophthalmic drops					
1	Hydrocortisone acetate	2.5%	78.4	80.6	79.0
2	Prednisolone	0.2%	89.0	...	89.5
Ophthalmic suspension					
1	Dexamethasone	0.1%	106.3	106.8	107.0
2	Prednisolone acetate	0.25%	111.8[a]	99.8	102.1
Tablets					
1	Prednisone	0.75 mg.	90.1		88
2	Prednisone	2.5 mg.	100.0		
3	Prednisone	5 mg.	50.6[a]	45.3	65.1

[a] Interference by variation of absorbance with time.

Table IV—Neutral and Acidic Corticosteroid Decomposition Products Determined in Pharmaceutical Preparations

Pharmaceutical Formulation	Corticosteroid	Corticosteroid Found: % Undecomposed	% Decomposed: Neutral	% Decomposed: Acidic
Capsule	Prednisone (2 mg.)	34	9	57
Tablets (buffered)	Prednisone (5 mg.)	65	15	20
Tablets	Hydrocortisone (4 mg.)	82	5	13
Cream	Hydrocortisone (0.125%)	48	36	16
Cream	Hydrocortisone (0.25%)	53	9	38
Lotion	Hydrocortisone (0.125%)	2	34	64
Lotion	Hydrocortisone (0.125%)	77	10	13
Lotion	Hydrocortisone (0.25%)	84	10	6
Lotion	Hydrocortisone (0.25%)	73	17	10
Lotion	Hydrocortisone (0.25%)	76	12	12

Typical results of determinations by the three methods on samples from different lot or batch numbers of the same preparation are shown in Table III. The difference between the results obtained from samples that have undergone decomposition and the results obtained from undecomposed samples is immediately apparent. The samples of ophthalmic drops are typical of samples in which there is no decomposition but in which the amount of corticosteroid is less than the declared amount. In all cases in which there is a significant discrepancy between the results by the BT and PH methods, a study of the variation of absorbance with time indicated that there was interference in the BT determination. In these cases, the value by the PH method is assumed to be correct.

Decomposition at the C_{17} side chain is indicated whenever the results by BT and PH methods are comparable but are lower than those by the INH or UV method. If no decomposed corticosteroid is found, the difference in values is due to a negative interference in the BT or PH method and/or a positive interference in the INH or UV method. The extent of the decomposition and the amounts of acidic and neutral decomposition products determined for several different corticosteroid preparations are given in Table IV. The amount of neutral decomposition products was determined by subtraction of the values obtained by the BT or PH method from those obtained by the INH or UV method on the chloroform extract of the sample. The value of the total decomposed fraction was calculated by subtraction of the value obtained by the BT or PH method on the chloroform extract from the values obtained by the INH or UV procedure on the alcohol USP extract of the sample.

Decomposition in Ring A is indicated whenever the results by INH and UV methods are comparable and are lower than the values obtained by the BT and PH methods. Decomposition of Ring A is due primarily to absorption of UV light and is not found often in corticosteroid preparations. It is more probable that such a difference in values is due to a positive interference in the BT or PH reaction. Positive interferences in the BT are more probable than negative interferences in the INH or UV procedure, as shown in Tables I and II.

All four methods are subject to interference by components of corticosteroid preparations; detection and elimination of such interferences are important to the correct interpretation of analytical results. Methods of detecting interferences include comparison of the PH and BT results. Differences between these values indicate interference, since the color-development reactions are different even though the reagents react with the same portion of the corticosteroid molecule. The BT reaction is subject to more interferences than the PH reaction, since the BT reagent is less specific for corticosteroids than the PH reagent.

Interference in the BT reaction can be either positive or negative. Positive interferences, such as lanolin, are those that react with the BT reagent to produce the diformazan. Negative interferences are often due to the acidic nature of the interfering substance. The BT reaction is highly pH dependent; substances which lower the pH below the optimum value can cause decrease of color formation or even completely inhibit the color formation. The negative interference of stearic acid and of salicylamide is probably due to pH effect, since increasing amounts of each cause a decrease in the pH of the BT reagent sample solution. As an example, the absorbance of a standard hydrocortisone solution containing 50 mg. of stearic acid was 0.331 compared to an absorbance of 0.608 for the standard alone. The pH of the BT solution containing the stearic acid and standard was 13.15 and that of the BT solution of the standard alone was 13.80.

The PH reaction is also subject to both positive and negative interferences by components of corticosteroid preparations. Interference is caused by some surface-active agents such as polysorbate 60, sorbitan monooleate, and sorbitan monostearate. Interference is also caused by organic acids which are soluble in chloroform but insoluble in the strongly acidic phenylhydrazine–sulfuric acid–alcohol solution used. The insoluble compound raises the base line by light dispersion and causes an increase in the absorbance of the sample which is not always compensated by the blank. Lack of compensation by the blank is probably due to the fact that the particle size of the solid separating from the sample is not always identical to the particle size of the solid separating from the blank. Such differences in size cause differences in the amount of light dispersion and the rate of settling during the actual measurement. The negative interference of phenothiazine (34) in the PH reaction is probably due to a reaction with phenylhydrazine which is not corrected for by the blank used.

Differences between the results by the INH and UV methods also indicate interference in one or both procedures. Positive interference in the INH procedure is caused by substances such as vanillin, which form derivatives with the reagent, or by substances such as oil-soluble vitamins and salicylamide, which absorb in the same region of the spectrum.

Many of the components of corticosteroid preparations interfere with the determination by simple UV spectrophotometry, since they absorb in the same region of the spectrum. Stabilizers such as methylparaben and propylparaben fall into this category. Such interference is usually detected readily by comparison of the absorbance curve of the sample to that of the standard. Interfering substances cause distortions of the spectra which are readily discernible.

Many of the interferences discussed can be detected by time studies of absorbance variation during the color-development period of the samples and standards. This is especially true with the BT procedure, since the absorbance developed by the BT reagent with corticosteroids becomes constant after a certain period of time or continues to increase at a rate dependent upon the individual steroid (35). Remeasurement of the absorbance of

Table V—Detection of Interference in the BT Method by Variation of Absorbance with Time

	Absorbance					Difference
Sample 1. Hydrocortisone Lotion 0.125%						
Minutes		90		126		
Standard hydrocortisone		0.591		0.592		0.001
Sample		0.491		0.528		0.037
Sample 2. Hydrocortisone Lotion 0.125%						
Minutes	30	60	90	150	210	Difference (90–150)
Standard	0.551	0.561	0.568	0.565	0.570	0.003
Sample A (2.1 g.)	0.462	0.490	0.515	0.528	0.540	0.013
Sample B (3.0 g.)	0.668	0.720	0.756	0.784	0.798	0.028

both the standards and samples 20 or 30 min. after the specified time for color development will show any major interference present. Minor interferences usually require an extended time study of absorbance variation during and after the color-development period for both standards and samples. Examples of the detection of interference by the measurement of the variation of absorbance with time are shown in Table V. The increase of 0.037 unit in the absorbance of Sample 1 during the 36 min. after the standard had become constant is an obvious indication that some component in addition to hydrocortisone is reacting with the BT reagent. This particular case of interference was detected by a routine rescan of the absorbance of the first standard and sample of a series of determinations after all samples and standards had been measured. The time lapse of 36 min. was required to measure all samples and standards. Sample 2 is an example of interference that is not immediately apparent by a rescan of the first standard and sample after completion of the series of measurements. In this particular case, a small increase in absorbance was noted for the sample when it was rescanned so that the extended time study was made. This study definitely proves that some interfering substance was present.

Many of these interfering substances may be separated from the corticosteroid in samples by use of a new acetonitrile–diatomaceous earth[2] column procedure (40).

REFERENCES

(1) W. J. Mader and R. R. Buck, *Anal. Chem.*, **24,** 666(1952).

(2) J. J. Callahan, F. Litterio, E. Britt, B. D. Rosen, and J. Owens, *J. Pharm. Sci.*, **51,** 333(1962).

(3) D. E. Guttman, *ibid.*, **55,** 919(1966).

(4) C. A. Johnson, R. King, and C. Vickers, *Analyst*, **85,** 714 (1960).

(5) F. M. Kunze and J. S. Davis, *J. Pharm. Sci.*, **53,** 1259(1964).

(6) C. C. Porter and R. H. Silber, *J. Biol. Chem.*, **185,** 201(1950).

(7) R. H. Silber and C. C. Porter, *ibid.*, **210,** 923(1954).

(8) E. J. Umberger, *Anal. Chem.*, **27,** 768(1955).

(9) T. Chulski and A. A. Forist, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 553(1958).

(10) E. R. Garrett, *J. Pharm. Sci.*, **51,** 445(1962).

(11) D. E. Guttman and P. D. Meister, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 773(1958).

(12) M. L. Leubart and V. R. Mattox, *Nature*, **183,** 820(1959).

(13) A. D. Marcus, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 383 (1960).

(14) T. O. Oesterling and D. E. Guttman, *J. Pharm. Sci.*, **53,** 1189(1964).

(15) S. K. Wahba, S. W. Amin, and N. Rafael, *ibid.*, **57,** 1231 (1968).

(16) J. W. Mauger, A. N. Paruta, and R. J. Gerraughty, *ibid.*, **58,** 574(1969).

(17) E. H. Jensen and D. J. Lamb, *ibid.*, **53,** 402(1964).

(18) K. Savard, H. W. Wotz, P. Marcus, and H. M. Lemon, *J. Amer. Chem. Soc.*, **75,** 6327(1953).

(19) W. E. Hamlin, T. Chulski, R. H. Johnson, and J. G. Wagner, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 253(1960).

(20) D. H. R. Barton and W. C. Taylor, *J. Amer. Chem. Soc.*, **80,** 244(1958).

(21) M. L. Leubart and V. R. Mattox, *Anal. Chem.*, **33,** 559 (1961).

(22) M. L. Leubart and V. R. Mattox, *J. Org. Chem.*, **28,** 1779 (1963).

(23) *Ibid.*, **28,** 1773(1963).

(24) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

(25) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.

(26) I. M. Jakovljevic, P. E. Hartsaw, and G. E. Drummond, *J. Pharm. Sci.*, **54,** 1771(1965).

(27) J. P. Comer and P. E. Hartsaw, *ibid.*, **54,** 524(1965).

(28) J. G. Wagner, J. K. Dale, C. A. Schlagel, P. D. Meister, and R. E. Booth, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 580(1958).

(29) P. D. Meister, C. A. Schlagel, J. E. Stafford, and J. L. Johnson, *ibid.*, **47,** 576(1958).

(30) A. Zaffroni, R. B. Burton, and E. H. Keutman, *Science*, **111,** 6(1950).

(31) A. Bracey, L. Garrett, and P. J. Weiss, *J. Pharm. Sci.*, **55,** 1113(1966).

(32) J. Levine, *ibid.*, **52,** 1015(1963).

(33) F. Kunze, private communication, 1965.

(34) A. Lupovitch, *Clin. Chem.*, **14,** 179(1968).

(35) A. S. Meyer and M. C. Lindberg, *Anal. Chem.*, **27,** 813 (1955).

(36) A. Bracey, L. G. Wayland, N. M. Kantor, and P. J. Weiss, private communication, 1965.

(37) E. F. Salim, P. E. Manni, and J. E. Sinsheimer, *J. Pharm. Sci.*, **53,** 391(1964).

(38) R. J. Kozara, private communication, 1969.

(39) P. E. Manni and J. E. Sinsheimer, *Anal. Chem.*, **33,** 1900 (1961).

(40) R. E. Graham, P. A. Williams, and C. T. Kenner, to be published.

[2] Celite, Johns-Manville Co.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 13, 1970, from the **Dallas District, Food and Drug Administration, Dallas, TX 75204* and † *Southern Methodist University, Dallas, TX 75222*

Accepted for publication March 27, 1970.

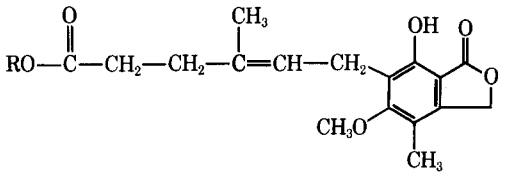
GLC of Mycophenolic Acid and Related Compounds

FRANK E. GAINER and HAROLD J. WESSELMAN

Abstract □ A GLC method is described for the quantitative determination of mycophenolic acid and monosodium mycophenolate. Data are presented to show that both the methyl ester and ethyl ester derivatives of mycophenolic acid can be chromatographed along with a cyclic acid hydrolysis product of mycophenolic acid. In addition, quantitative results are presented for the assay of mycophenolic acid and the monosodium salt in various formulations. Spectral and TLC results are also included.

Keyphrases □ Mycophenolic acid, related compounds—determination □ TLC—separation □ GLC—analysis □ Tetraphenylethylene, chloroform dissolved—internal standard □ IR spectrophotometry—structure □ NMR spectroscopy—structure

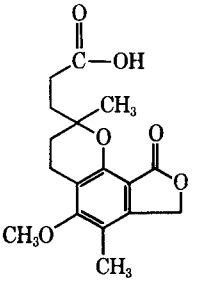
Mycophenolic acid (Structure I) is an antibiotic fermentation product which is isolated from penicillium cultures (1, 2). The antibiotic is known to have



I, R = H
II, R = Na
III, R = CH_3
IV, R = C_2H_5

antibacterial and antifungal properties which have been studied previously (3, 4). Recently, two groups of researchers (5, 6) reported on the antitumor activity of mycophenolic acid, while Williams *et al.* (7) reported that the substance exhibited both antiviral and antitumor properties. Work has been done, both chemical and physical in nature, to elucidate the structure of mycophenolic acid (8, 9) and some related compounds, which were isolated from the culture filtrates of a strain that produces mycophenolic acid (10). Birch and Wright (11) have devised a scheme for the total synthesis of mycophenolic acid. This paper reports the quantitative determination of mycophenolic acid and related compounds by GLC.

Ethyl mycophenolate (IV) may be formed during the isolation of mycophenolic acid from penicillium cultures (12) and recrystallization of the acid by interaction of the acid and ethanol. However, Campbell *et al.* (10) have reported the isolation of ethyl mycophenolate from the culture filtrates of a strain that produces mycophenolic acid. The cyclic compound, 3,4-dihydro-5 - methoxy - 2,6 - dimethyl - 9(7*H*) - oxo - 2*H* - furo-⟨3,4-*h*⟩benzopyran-2-propionic acid (V) was isolated from the reaction vessel after heating the acid at 100° in 10% hydrochloric acid for 18 hr. Methanol leads to partial esterification of mycophenolic



V

acid during extraction processes (12), probably resulting in methyl mycophenolate (III). Monosodium mycophenolate (II) may be formed by adjusting a slurry of mycophenolic acid to pH 7–8 with sodium hydroxide.

No record was found in the literature, either directly or indirectly, reporting the quantitative determination of mycophenolic acid.

EXPERIMENTAL

Equipment—A gas chromatograph (Hewlett-Packard model 402) equipped with a flame-ionization detector was used for the experimental work. The detector signal was fed to a 1-mv. recorder (Honeywell Electronik 16) with a chart speed of 15 in./hr. and a 1-sec. full-scale response. Samples were injected with a 10-μl. syringe (Hamilton No. 701).

Materials—Helium was used as a carrier gas, while electrolytic hydrogen and oxygen were used in the detector. The stationary phase was 3.8% Linde W-98 silicone gum applied by the solution technique to silanized diatomaceous earth (Diatoport S) (80/100 mesh) and packed in a borosilicate glass column, 0.91 m. × 0.64 cm. o.d. Chloroform (Reagent ACS) was used to dissolve the tetraphenylethylene (Reagent ACS) internal standard. The silylating reagent was bis(trimethylsilyl)trifluoroacetamide.[1] Concentrated hydrochloric acid (Reagent ACS) and absolute methanol were employed.

Operating Conditions—The column was operated isothermally at 235°, with the detector block at 280° and the sample injection port at 280°. The helium flow rate was 55 ml./min., with an inlet pressure of 40 psig. Oxygen and hydrogen flow rates were 200 and 50 ml./min., respectively. The electrometer range was 10, with an attenuation of 128. Sample injections of 1.5 μl. were used throughout the study.

Quantitative Analysis—Tetraphenylethylene, 25 mg. in 100 ml. of chloroform, is used as an internal standard.

Mycophenolic Acid—Accurately weigh 50 mg. of mycophenolic acid into a 50-ml. volumetric flask, and dilute to volume with chloroform. Pipet 1.0 ml. of the solution into a 3-ml. butyl-rubber-stoppered ampul, and add 1.0 ml. of the internal standard solution. Carefully evaporate the solution in the ampul to dryness, using a gentle stream of nitrogen and a warm water bath. Add 0.5 ml. of bis(trimethylsilyl)trifluoroacetamide to the ampul, and seal before heating for 1 hr. in a heating block at 80°. Cool to room temperature before chromatographing. Prepare a standard reference solution by accurately weighing 50 mg. of mycophenolic acid reference standard into a 50-ml. volumetric flask and diluting to volume with chloroform. Treat this solution exactly as the sample solution.

[1] Regis, Regisil Chemical Co., Chicago, Ill.

Table I—Mycophenolic Acid Assay Results

Sample	Theory	Found	n	$RSD \pm \%$	$RE\ \%$
Raw material	1000 mg./g.	997.5 mg./g.	12	1.81	−0.25
Raw material	1000	999.3	3		−0.07
Raw material	1000	1002.0	3		+0.20
Capsules with starch	200 mg./capsule	204.4 mg./capsule	5	4.08	+2.18
Capsules with starch	200	198.7	5	1.12	−0.65
Mycophenolic acid + microcrystalline cellulose	570 mg./g.	569.5 mg./g.	3		−0.09
Mycophenolic acid + talc	470	469.3	3		−0.15

Table II—Monosodium Mycophenolate Assay Results

Sample	Theory	Found	n	$RSD \pm \%$	$RE\ \%$
Ampul (10% aqueous)	100 mg./ml.[a]	102.6 mg./ml.[a]	5	1.71	+2.60
Ampul (10% aqueous)	100	103.1	9	2.30	+3.10
Capsules with starch	200 mg./capsule	197.4 mg./capsule	5	4.40	+1.80
Capsules with starch	200	201.0	5	0.69	+0.50
Monosodium mycophenolate + talc (1:1)	500 mg./g.	509.8 mg./g.	3		+1.96

[a] Mycophenolic acid equivalency per unit sample.

Mycophenolic Acid Capsules, 200 mg.—Weigh five filled capsules and empty the contents into a small beaker. Wash the empty capsules with chloroform, discarding the washings. Allow the capsules to dry before weighing. Subtract the weight of the empty capsules from that of the filled capsules to determine the average fill weight. Thoroughly mix the dry powder in the beaker, and accurately weigh a portion of the sample equivalent to 100 mg. of mycophenolic acid. Quantitatively transfer the sample to a 125-ml. separator containing 20 ml. of purified water. Extract the compound with three 20-ml. portions of chloroform, allowing 2 min. for each extraction. Filter the chloroform layer through anhydrous sodium sulfate into a 100-ml. volumetric flask. Wash the sodium sulfate with chloroform, and collect the washings in the flask. Dilute to volume with chloroform and shake well. Pipet 1.0 ml. of this solution into a 3-ml. butyl-rubber-stoppered ampul, and proceed as directed for mycophenolic acid, using the same internal standard and standard reference solution.

Monosodium Mycophenolate—Accurately weigh 100 mg. of sodium mycophenolate into a 100-ml. volumetric flask containing 50 ml. of methanol. Add 0.5 ml. of concentrated hydrochloric acid to the flask and shake well. Dilute to volume with methanol and shake well before transferring 10 ml. of the solution to a butyl-rubber-stoppered ampul. Seal the ampul and place it in a heating block at 80° for 0.5 hr. Cool the contents of the ampul to room temperature. Pipet 1.0 ml. of the solution into a 3-ml. butyl-rubber-stoppered ampul and proceed as with mycophenolic acid. Prepare a standard reference solution by accurately weighing 100 mg. of monosodium mycophenolate standard into a 100-ml. volumetric flask containing 50 ml. of methanol. Treat this solution exactly as the sample solution.

Monosodium Mycophenolate Ampuls, 10%—Pipet 1.0 ml. of the ampul solution into a 100-ml. volumetric flask containing 50 ml. of methanol. Proceed as directed for monosodium mycophenolate, using the same internal standard solution and standard reference solution.

Experimental procedures and details for the GLC of ethyl mycophenolate, methyl mycophenolate, and the cyclic compound are the same as for mycophenolic acid.

Chromatograph the standard and sample solutions, and measure the peak heights of the respective internal standard, standard, and sample peaks.

Calculations—

$$\frac{\text{peak height sample}}{\text{peak height sample internal standard}} = R_1 \qquad \text{(Eq. 1)}$$

$$\frac{\text{peak height standard}}{\text{peak height standard internal standard}} = R_2 \qquad \text{(Eq. 2)}$$

$$\frac{R_1 \times \text{milligram standard}}{R_2 \times \text{milligram sample}} \times \text{standard purity milligram/gram} = \text{milligram active ingredient/gram sample} \qquad \text{(Eq. 3)}$$

Substitute milliliter sample for milligram sample when solutions are assayed.

$$\text{Eq. 3} \times \text{average fill weight of capsule} = \text{milligram active ingredient/capsule} \qquad \text{(Eq. 4)}$$

If the active ingredient is monosodium mycophenolate, the acid equivalency can be obtained by multiplying Eqs. 3 and 4 by the factor 0.935.

RESULTS AND DISCUSSION

Each of the compounds studied, except monosodium mycophenolate, is chloroform soluble; thus, it was convenient to do simple extractions and dilutions with this solvent. Monosodium mycophenolate is very soluble in water and relatively insoluble in most organic solvents except methanol, which was used for this compound.

Since it is more difficult to silylate the sodium salt of a carboxylic acid than the acid itself, initial efforts were made to convert monosodium mycophenolate in methanolic solution to mycophenolic

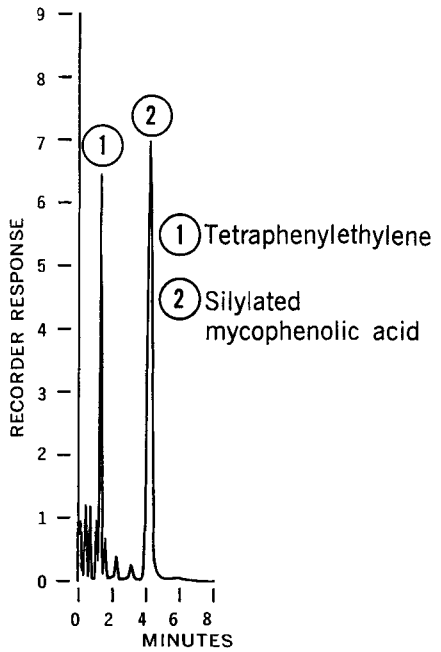


Figure 1—*Typical chromatogram of internal standard and sample.*

Table III—Retention Times of Trimethylsilyl Ethers of Mycophenolic Acid and Related Compounds

Compound	Retention Time,[a] min.
Mycophenolic acid	4.0
Monosodium mycophenolate (as methyl mycophenolate)	3.2
Cyclic compound	4.6
Ethyl mycophenolate	3.6
Methyl mycophenolate	3.2
Tetraphenylethylene[b]	1.2

[a] Using chromatographic parameters described under operating conditions. [b] Internal standard.

acid by the addition of concentrated hydrochloric acid with shaking. This resulted in a solution containing two compounds, as shown by GLC and TLC. The chromatograms suggested that the compounds were methyl mycophenolate and mycophenolic acid. Both IR and NMR confirmed that one component was methyl mycophenolate. Additional checks were not made to determine whether the second component was mycophenolic acid; but the chemistry of the system, along with stability observations, indicated that it was the acid. Since methanol and hydrochloric acid are conducive to ester formation, it was convenient to form methyl mycophenolate from monosodium mycophenolate instead of forming the acid. TLC and GLC of aliquots taken from the reaction mixture at various time intervals indicated that esterification was taking place at a measurable rate even at room temperature. It was possible to obtain complete esterification by heating the reaction mixture at 80° for 0.5 hr. Again, TLC and GLC confirmed that the solution contained only one component, methyl mycophenolate. Chromatography also indicated that no formation and subsequent esterification of the cyclic acid hydrolysis compound (V) were occurring. The methylated cyclic compound has a lower R_f value and a longer retention time than methyl mycophenolate.

To confirm that the sodium salt was being converted to the ester, an alternate method of preparation was employed in which a methanolic solution of mycophenolic acid was treated with gaseous hydrogen chloride. IR and NMR confirmed that the product was methyl mycophenolate. The product, from both methods of preparation, had the same R_f value by TLC.

It was found necessary to heat all compounds studied during the silylating reaction to obtain consistent results. Assay results for mycophenolic acid and monosodium mycophenolate were reproducible, and recoveries were good. Table I shows the results of assays of several lots of mycophenolic acid raw material and formulations. Assay results for monosodium mycophenolate are shown in Table II.

The response of the detector is linear over a range of concentrations from 0.5 to 1.5 mg./ml. for the silylated derivatives of mycophenolic acid and methyl mycophenolate. The continual use of the silylating agent causes a deposit to form on the detector anode, which eventually causes a loss in precision and accuracy. This problem can be eliminated by periodically cleaning the anode in an ultrasonic bath.

Both mycophenolic acid and monosodium mycophenolate are very stable compounds which lend themselves to quantitative assays as silylated derivatives. A typical chromatogram, showing peaks for the internal standard and silylated mycophenolic acid, is shown in Fig. 1. Although no quantitative work was done on ethyl mycophenolate and the cyclic compound, it should be possible to quantitate these compounds by using the same experimental parameters used for mycophenolic acid. Metabolites, other than ethyl mycophenolate, that are related to mycophenolic acid (10) also should be amenable to GLC. It is significant that mycophenolic acid, related metabolites, and other related compounds can be chromatographed by using very similar experimental conditions. Table III presents the retention times for the compounds studied.

REFERENCES

(1) B. Gosio, *Riv. Ig. Sanita Pubb. Ann.*, **7**, 825(1896).

(2) C. L. Alsberg and O. F. Black, U. S. Dept. of Agriculture, Bureau of Plant Industry, *Bull.*, **270**, 7(1913).

(3) H. W. Florey, K. Gilliver, M. A. Jennings, and A. G. Sanders, *Lancet*, **1**, 46(1946).

(4) K. Gilliver, *Ann. Bot. (London)*, **10**, 271(1946).

(5) S. Suzuki, T. Kimura, K. Ando, M. Sawada, and G. Tamura, *J. Antibiot.*, **22**, 297(1969).

(6) S. B. Carter, T. J. Franklin, D. F. Jones, B. J. Leonard, S. D. Mills, R. W. Turner, and W. B. Turner, *Nature*, **223**, 848 (1969).

(7) R. H. Williams, D. H. Lively, D. DeLong, J. C. Cline, M. J. Sweeney, G. A. Poore, and S. H. Larsen, *J. Antibiot.*, **21**, 463(1968).

(8) P. W. Clutterbuck and H. Raistrick, *Biochem. J.*, **27**, 654 (1933).

(9) W. R. Logan and G. T. Newbold, *J. Chem. Soc.*, **1957**, 1946.

(10) I. M. Campbell, C. H. Calzadilla, and N. J. McCorkindale, *Tetrahedron Lett.*, **1966**, 5107.

(11) A. J. Birch and J. J. Wright, *J. Chem. Soc., D*, **1969**, 788.

(12) P. W. Clutterbuck, A. E. Oxford, H. Raistrick, and G. Smith, *Biochem. J.*, **26**, 1441(1932).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 15, 1969, from the *Analytical Development Laboratories, Eli Lilly and Co., Indianapolis, IN 46206*

Accepted for publication March 18, 1970.

The authors thank Dr. E. H. Massey for preparing some of the compounds used in this study and Mr. H. F. Hugar for his assistance in some of the experimental work.

Anhydrotetracycline and 4-Epianhydrotetracycline in Market Tetracyclines and Aged Tetracycline Products

VERNON C. WALTON, MARGARET R. HOWLETT, and GEORGE B. SELZER

Abstract □ A large number of tetracycline samples were tested for the presence of anhydrotetracycline and 4-epianhydrotetracycline when they were fresh and after being stored under normal and adverse conditions. It was found that: (*a*) Newly manufactured tetracycline preparations contain only small amounts of anhydrotetracycline and 4-epianhydrotetracycline. (*b*) Storage under adverse conditions markedly increases the percentage of degradation products, but storage under normal conditions results in only a slow increase in anhydrotetracycline and 4-epianhydrotetracycline. In syrups, this can be correlated with loss in tetracycline potency. (*c*) Citric acid greatly increases the tendency of tetracycline to degrade. (*d*) Tetracycline hydrochloride is more stable than tetracycline phosphate. (*e*) Demethylchlortetracycline is the most stable of the tetracycline derivatives studied. (*f*) Rolitetracycline is extremely unstable.

Keyphrases □ Anhydrotetracycline determination—tetracycline products □ 4-Epianhydrotetracycline determination—tetracycline products □ Column chromatography—separation □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer □ Turbidimetric analysis—biological potency

Degradation products of tetracycline (TC) and 4-epianhydrotetracycline (EATC), in particular, have been implicated in renal dysfunction (1–9). It was, therefore, of interest to determine the amount of degradation in TC products on the market. A large number of market TC products were examined for EATC content. Several lots were tested for stability under normal and adverse storage conditions to determine the amount of EATC that may reasonably be expected in newly manufactured TC products and to reveal what increase in EATC could be expected with time.

Several analytical methods for the determination of 4-epitetracycline (ETC), anhydrotetracycline (ATC), and EATC have been described (10–17). The most convenient are those of Dijkhuis (15), Kelly (16), and Selzer and Wright (17); these have been adopted for use in this study.

EXPERIMENTAL

Methods—*Column Chromatography*—The method of Kelly (16) is a column chromatographic separation of ATC and EATC from each other and from TC and other interfering substances. The column consists of acid-washed diatomaceous earth[1] moistened with 0.1 *M* ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.8; the compounds are eluted with chloroform and determined spectrophotometrically. For this study, the method was modified to ensure that the sample was at pH 7.8 when it was applied to the column. A 5-ml. sample of TC syrup containing 25 mg. of TC/ml. or a 2-ml. sample of pediatric drops containing 100 mg. of TC/ml. was diluted to 10 ml. with 0.1 *M* EDTA buffer, pH 7.8 (prepared by dissolving 0.1 mole of EDTA disodium salt in 800 ml. of water, adjusting the pH to 7.8 with ammonium hydroxide, and diluting to 1 l.). The sample was then brought to pH 7.8 with ammonium hydroxide–water (1:9) and diluted to 25 ml. with the EDTA buffer. One tablet containing 125 mg. of TC or capsule material containing 125 mg. of TC was ground in a mortar and pestle with 10 ml. of 0.1 *M* EDTA buffer, pH 7.8, brought to pH 7.8 with ammonium hydroxide–water (1:9), and diluted to 25 ml. with the EDTA buffer. Each 250-mg. capsule was blended with 25 ml of 0.1 *M* EDTA buffer, pH 7.8, brought to pH 7.8 with ammonium hydroxide–water (1:9), and diluted to 50 ml. with the EDTA buffer. Difficultly soluble samples were dissolved first in 10 ml. of 0.1 *N* HCl, brought to pH 7.8 with ammonium hydroxide–water (1:9), and then diluted to 50 ml. with 0.1 *M* EDTA buffer, pH 7.8.

A 1-ml. aliquot of the diluted sample was then mixed with 1 g. of dry diatomaceous earth, applied to the column, and covered with a 1-cm. layer of diatomaceous earth moistened with 0.1 *M* EDTA buffer, pH 7.8. The method was further modified in that no supporting circle of filter paper was used under the column, no layer of sand was used, and the 0.1 *M* EDTA buffer was not equilibrated with $CHCl_3$ before use.

Spectrophotometric Screening Method—The method of Dijkhuis (15) was used to screen powder, tablet, and capsule samples for total anhydrotetracycline content (ATC + EATC). The sample preparation was modified to ensure the dissolution of samples containing TC base or TC phosphate. A 50-mg. sample of TC powder was weighed into a 50-ml. volumetric flask, and 10 ml. of 0.1 *N* HCl was added. The flask was shaken until the sample dissolved; the contents were then diluted to the mark with water and assayed. Capsule powder or finely ground tablet powder equivalent to 250 mg. of TC hydrochloride was placed in a 250-ml. volumetric flask; 50 ml. of 0.1 *N* HCl was added, and the flask was shaken on a mechanical shaker for 5 min. The sample was then diluted to volume with water and filtered through a fluted-filter paper. The first 20 ml. of filtrate was discarded and the remainder was collected for assay. The calculations were modified in that the formula recommended by Dijkhuis (15) for the calculation of the percent ATC in dosage forms was also used for TC powder.

Paper Chromatography—The method of Selzer and Wright (17) was used. It is an ascending paper chromatographic method for the separation of TC compounds by a resolving solvent of chloroform–nitromethane–pyridine (10:20:3) on 20.3 × 20.3-cm. (8 × 8-in.) Whatman No. 1 paper moistened with McIlvaine's buffer, pH 3.5; the spots are observed by their fluorescence under UV light. To vary the relative positions of the different TC and degradation products on the chromatogram and to facilitate their identification, the chromatographic paper was moistened with any of three buffers: McIlvaine's buffer, pH 3.5; 0.1 *M* EDTA buffer, pH 4.5; or 0.1 *M* EDTA buffer, pH 7.7.

Table I—Total Anhydrotetracyclines Present in Fresh Tetracycline Powder[a]

Sample Number	Manufacturer	ATC + EATC, %
1–4	A	0.17, 0.14, 0.31, 0.17
5–8	B	0.97, 0.86, 0.90, 0.92
9–12	C	0.47, 0.48, 0.57, 0.98
13–18	D	2.06, 1.44, 1.98, 1.59 1.69, 1.27
19–21	E	0.25, 0.71, 0.62
22–25	F	0.24, 0.25, 0.23, 0.21
26–29	G	0.39, 0.46, 0.36, 0.34
30–33	I	0.20, 0.17, 0.66, 0.06
34	J	0.11
35	K	0.25
36–38	L	0.41, 0.09, 0.81

[a] Determined by the method of Dijkhuis (15).

[1] Celite 545, Johns-Manville, New York, N.Y.

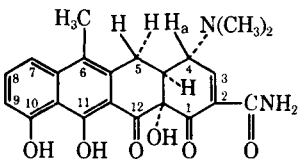
Table II—Total Anhydrotetracyclines Present in Fresh Tetracycline Phosphate Powder[a]

Sample Number	Manufacturer	ATC + EATC, %
1	H	1.44
2	H	1.58
3	H	1.05
4	H	1.07
5	H	1.05

[a] Determined by the method of Dijkhuis (15).

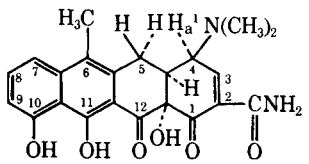
Potency Determination—The biological potencies of the samples of TC used in these studies were determined by the turbidimetric assay method (18) with *Staphylococcus aureus* (ATCC 6538P) as the test organism.

Standards—*ATC–EATC Mixture*—A standard mixture of 50:50 ATC–EATC was prepared as follows: a mixture of 2 g. of TC HCl, 1 ml. of glacial acetic acid, and 20 ml. of water was incubated overnight at 37°. One milliliter of concentrated HCl was added, the mixture was heated on a steam bath for 30 min., and the liquids were removed by lyophilization. The dry powder was dissolved in methanol, and an aliquot was chromatographed on paper as described previously, using each of the three buffers mentioned to moisten the paper. In each case, two spots of equal intensity, corresponding to ATC and EATC, were detected. Analysis of the powder by the column chromatographic method indicated that it contained 48.9% ATC and 50.9% EATC. Comparison of the 100-Mc.p.s. NMR spectrum of a CF_2COOH solution with data in the literature (19) confirmed the identity of the mixture. The bands in a 100-Mc.p.s. spectrum were better resolved, and those bands due to H_a and H_a^1 (Structures I and II) could be integrated. Comparison



anhydrotetracycline

I



4-epianhydrotetracycline

II

of the peak areas indicated that the powder contained 45% ATC and 55% EATC.

Additional quantities of mixed ATC and EATC were prepared by heating TC HCl for 5 min. in 2 *N* HCl (15). The compositions of these materials were determined by the method of Kelly (16) and by comparison of their absorptivities at 356 and 430 mμ.

Anhydrotetracycline HCl Hydrate—Anhydrotetracycline HCl hydrate (Lot No. 63 F 2052, Bristol Laboratories, Syracuse, N.Y.) was used. Paper chromatography in all three buffer systems indicated that the sample contained only a trace of EATC; none could be detected by column chromatography. Integration of the bands corresponding to protons H_a and H_a^1 (Structures I and II) in the 100-Mc.p.s. NMR spectrum of a DMSO-d_6 solution of the sample indicated that the amount of ATC was on the order of 23 times as much as the amount of EATC.

4-Epianhydrotetracycline Sulfate—4-Epianhydrotetracycline sulfate (Lot No. 57 F 536, Bristol Laboratories) was used. Paper chromatography of this compound in all three systems indicated that only a trace of ATC was present; none could be detected by column chromatography. Integration of the bands corresponding to protons H_a and H_a^1 (Structures I and II) in the 100-Mc.p.s. NMR spectrum of a DMSO-d_6 solution of the sample indicated that the amount of EATC was on the order of 10 times as much as the amount of ATC. However, the presence of traces of one or more other compounds was indicated.

Samples—All of the TC samples used in this study had been received as part of the antibiotic certification program.

Table III—Total Anhydrotetracycline and 4-Epianhydrotetracycline Present in Fresh Tetracycline Hydrochloride Tablets[a]

Sample Number	Manufacturer	ATC + EATC, %
1–15	F	0.50, 0.46, 0.53, 0.52, 0.54, 0.46, 0.47, 0.63, 0.59, 0.59, 0.57, 0.58, 0.63, 0.49, 0.54
16–19	B	1.81, 1.62, 1.45, 1.59
20–22	G	0.14, 0.11, 0.69

[a] Determined by the method of Dijkhuis (15).

Table IV—Total Anhydrotetracycline and 4-Epianhydrotetracycline Present in Tetracycline Capsules[a]

Sample Number	Type of Tetracycline	Manufacturer	ATC + EATC, %
1–3	HCl	B	1.38, 1.28, 0.83
4–5	HCl	G	0.29, 0.27
6–15	PO_4	H	1.73, 1.66, 1.62, 1.50 1.76, 1.44, 2.89, 3.05 1.13, 1.14
16–18	PO_4	M	1.58, 0.60, 1.32
19	HCl	M	0.57
20	Base	N	0.65
21–24	HCl	N	0.57, 0.50, 0.46, 0.44
25–28	HCl	O	0.23, 0.32, 0.12, 0.55
29–31	HCl	P	0.21, 0.29, 0.24
32–33	HCl	Q	0.85, 0.68
34	HCl	R	0.33
35–38	HCl	S	0.63, 0.71, 0.71, 0.34
39	HCl	T	0.68
40–49	HCl	U	1.14, 1.09, 1.29, 1.35 1.60, 1.45, 1.41, 1.24 1.31, 1.31

[a] Determined by the method of Dijkhuis (15).

RESULTS AND DISCUSSION

Because pharmaceutical dosage forms of TC contain a number of other ingredients, the column chromatographic procedure and the screening method were tested for interference from such materials. Phenyltoloxamine, aspirin, phenacetin, caffeine, salicylamide, and chlorothen citrate did not interfere with either method. Amphotericin B, neomycin, nystatin, oleandomycin, and triacetyloleandomycin had no detectable effect on the results of the column chromatographic procedure. Although novobiocin caused the EATC band to follow the ATC band quite closely in the column chromatographic procedure, it did not affect the results. However,

Table V—Anhydrotetracycline and 4-Epianhydrotetracycline Found in Fresh Tetracycline Syrups[a]

Sample Number	Manufacturer	ATC, %	EATC, %
1	B	0.27	0.28
2	B	1.81	1.12
3	F	0.06	0.13
4	H	0.13	0.13
5	T	0.13	0.30
6	T	0.09	0.26
7	V	0.39	0.43
8	V	0.72	0.70
9	V	0.11	0.16
10	V	0.11	0.12
11	W	2.93	4.18
12	O	0.19	0.47

[a] Determined by the method of Kelly (16).

Table VI—Comparison of the Loss of Potency of Tetracycline Syrup after Storage[a] with the Amount of Anhydrotetracycline and 4-Epianhydrotetracyclines

Sample Number	Potency Lost, mg./dose	ATC Found, mg./dose	EATC Found, mg./dose
1	0	0.16	0.25
2	0	0.26	0.52
3	0	0.38	0.30
4	0	3.7	4.6
5	2	0.49	0.62
6	5	0.53	0.32
7	5	0.98	1.29
8	9	3.5	3.9
9	10	0.35	0.50
10	11	0.49	0.62
11	12	1.2	0.72
12	13	0.32	0.32
13	13	0.38	0.59
14	16	4.8	4.1
15	17	0.44	0.82
16[b]	17	1.4	1.5
17	23	1.2	0.99
18	25	2.0	2.6
19	28	2.0	2.1
20	61	3.6	4.1
21	32	5.3	5.1

[a] Storage ranged from 12 to 20 months at 25°. [b] An experimental formula.

amphotericin B and nystatin, both of which absorb light at 430 mμ, caused unduly high results in the screening method when they were present in concentrations comparable to those found in commercial products. For this reason, TC products containing amphotericin B or nystatin were tested by the column chromatographic method.

A survey was conducted to find out how much ATC and EATC were present in fresh TC powder. As shown in Table I, the ATC + EATC content of 38 samples of fresh TC HCl bulk material from 11 manufacturers ranged from 0.11 to 2.06%. However, all of the six results over 1% were obtained with material from one manufacturer. Table II shows the total ATC + EATC found in newly manufactured TC PO_4 analyzed by the method of Dijkhuis (15). All of the five samples tested contained between 1 and 2% ATC + EATC.

Another survey was made of the ATC and EATC present in newly manufactured samples of various TC dosage forms. Table III shows that of 22 TC HCl tablets tested, only five contained more than 1% total ATC + EATC. These five samples were produced by one manufacturer and contained less than 2% ATC + EATC. As shown in Table IV, all but two of the 49 TC capsules tested contained less than 2% total ATC + EATC. Table V shows that only two of the 12 fresh TC syrups tested contained more than 1% of either ATC or EATC.

It can thus be seen that the amounts of ATC and EATC found in fresh TC products were quite low. On the basis of these results, limits of 2% EATC in TC powders and 3% EATC in finished TC products have been proposed for inclusion in the *Code of Federal Regulations* (20).

Several studies were conducted to determine the stability of TC products under various conditions. In one study, TC syrups were examined for ATC and EATC after they had been stored for different periods of time. The solid matter in the syrups was either in suspension or easily resuspendable. As shown in Table VI, there is some correlation between loss of biological potency and increase in ATC and EATC content.

In another study, the ability of the TC antibiotics to resist unfavorable storage conditions of elevated temperature and high humidity was investigated. The TC samples were stored at 37° in desiccators containing water (100% relative humidity) or a saturated sodium bromide solution (66% relative humidity) in the desiccant chamber. The samples were tested for potency by the microbiological turbidimetric method (18) and for degradation products by the paper chromatographic method on paper moistened with McIlvaine's buffer, pH 3.5, and by the column chromatographic method.

Several conclusions may be drawn from the data presented in Tables VII and VIII. Demethylchlortetracycline (DMCTC) is the most stable of the TC derivatives when stored under 100% relative humidity at 37°; it showed no loss of potency after storage for 1 month. Chlortetracycline (CTC) is somewhat less stable than DMCTC; after 1 month at 37° and 100% relative humidity, the potency of CTC powder had diminished by 17% and that of capsule material by 14%. Neither CTC nor DMCTC showed evidence of anhydrolike degradation products by the paper chromatographic method. However, TC was less stable; TC products stored 1 month at 37° and 100% relative humidity gave visible evidence of degradation by becoming partially liquid and turning dark brown. Potency losses of from 17 to 79% occurred, and the column chromatographic procedure revealed ATC levels ranging from 2.2 to 7.1% and EATC levels ranging from 3.3 to 14.1%. Paper chromatography indicated that approximately one-half of the TC was converted to the inactive epimer form, ETC. Rolitetracycline (RTC) was even more unstable. Storage at 37° and 100% relative humidity resulted in almost complete destruction of this antibiotic; the only recognizable fragment was a small amount of the epimeric form of RTC. Paper chromatography of degraded oxytetracycline indicated the possible presence of an anhydrolike product.

TC HCl powder and dosage forms were quite stable when stored 2 months at 66% relative humidity and 37°, as shown in Table IX. Only a small fraction of the TC was converted to the anhydro forms. However, TC HCl with added citric acid was almost completely inactivated and formed large amounts of ATC and EATC. Citric acid thus has an adverse effect on TC stability. TC PO_4 is somewhat

Table VII—Results of the Storage of Tetracycline Derivatives for 30 Days at 37° and 100% Relative Humidity

Product	Description after Storage	Potency, mcg./mg. Before Storage	Potency, mcg./mg. After Storage	Paper Chromatography[a]
Pooled demethylchlortetracycline hydrochloride powder	Brown powder	1000	1030	Strong fluorescent spot (DMCTC) at R_f 0.63
Demethylchlortetracycline capsule powder	Light-brown powder	490	480	Two minor spots at R_f 0.19 (Epi-DMCTC) and R_f 0.37
Oxytetracycline hydrochloride powder	Dark-brown	1000	490	Strong fluorescent spot (OTC) at R_f 0.20
Oxytetracycline base powder	Dark-brown liquid with few solid particles		185	Strong blue-green fluorescent spot at origin; R_f 0.39 and 0.96
Rolitetracycline powder	Very dark-brown tar	1000	15	No TC detected. Weak fluorescent spots at R_f 0.38, 0.20, and 0.96. Strong spot at origin.
Chlortetracycline hydrochloride powder	Yellow powder	490	420	Strong fluorescent spot (CTC) at R_f 0.74
Chlortetracycline capsule powder				Weak TC spot at R_f 0.43. No degradation indicated

[a] On paper moistened with McIlvaine's buffer, pH 3.5.

Table VIII—Results of the Storage of Tetracycline Powder and Capsule Materials for 30 Days at 37° and 100% Relative Humidity[a]

Product	pH before Storage	Description after Storage	Potency, mcg./mg. Before Storage	Potency, mcg./mg. After Storage	ATC, %	EATC, %
TC PO_4 powder		Very dark-brown viscous liquid	800	215	2.2	3.3
TC HCl powder	2.38	Dark-brown powder	550	350	2.2	3.7
TC HCl with glucosamine (capsule powder)	2.30	Partially liquid, yellow-brown powder	520	430	7.1	6.5
TC PO_4 capsule powder (Company H)	2.58	Partially liquid, very dark-brown powder	620	130	2.3	5.2
TC PO_4 capsule powder (Company M)	2.46	Very dark-brown liquid	570	160	5.7	14.1

[a] Chromatography was done on paper moistened with McIlvaine's buffer, pH 3.5; strong fluorescent spots for TC at R_f 0.46 and Epi-TC at R_f 0.14; spots for ATC at R_f 0.98 and EATC at R_f 0.60.

Table IX—Results of Storage of Tetracycline Powder and Capsule Materials at 37° and 66% Relative Humidity

Product	Days Stored	Description after Storage	Potency, mcg./mg. Before Storage	Potency, mcg./mg. After Storage	Loss in Potency, %	ATC, %	EATC, %	Paper Chromatography[a]
TC HCl	70	Yellow-brown powder	1000 (est.)	900	10	0.02	0.2	No ATC or EATC detected
TC HCl capsule powder	70	Yellow-brown powder	520	600	0	0.15	None	No ATC or EATC detected
TC HCl with citric acid capsule powder	70	Orange-brown cake	480	35	93	18.5	56.7	Large amount of ATC and EATC. No TC detected
TC PO_4 powder	62	Yellow-brown powder	800	480	40	2.0	3.2	Spots for ATC and EATC visible; large TC and ETC spots
TC PO_4 capsule powder (Company H)	62	Yellow-brown powder	680	450	34	1.3	1.8	Spots for ATC and EATC visible; large TC and ETC spots
TC PO_4 capsule powder (Company M)	62	Yellow-brown powder	600	450	25	1.7	2.3	Spots for ATC and EATC visible; large TC and ETC spots

[a] On paper moistened with McIlvaine's buffer, pH 3.5.

less stable than TC HCl under conditions of 37° and 66% relative humidity; TC PO_4 products stored under these conditions had potency losses ranging from 25 to 40% and small amounts of ATC and EATC were present.

The effects of other active ingredients on the stability of TC PO_4 were also investigated. Samples from two lots of TC PO_4 capsules made by the same manufacturer were stored under adverse and control conditions for 3 years. One lot (A) contained TC PO_4 and no other active ingredients; the other lot (B) contained TC PO_4, phenyltoloxamine citrate, aspirin, phenacetin, and caffeine. Control capsules were stored at room temperature in closed bottles, and the test capsules were stored under adverse conditions in open beakers in a desiccator at 32°. The desiccant chamber was filled with a saturated solution of $Na_2Cr_2O_7$ to ensure a constant relative humidity of 63%. At intervals the capsules were tested for ATC and EATC content. As shown in Table X, the test capsules of both lots at all times contained greater amounts of ATC and EATC than the control capsules; and at all times, Lot B contained greater amounts of ATC and EATC than did Lot A. The final potency of Lot A after being stored under adverse conditions was 24.4% of the labeled potency; after being stored under control conditions, it was 80% of labeled potency. The final potency of Lot B after being stored under adverse conditions was 13.4% of labeled potency; under control conditions, it was 85%. The combination of phenyltoloxamine citrate, aspirin, phenacetin, and caffeine apparently had some additional deteriorating effect on the stability of TC PO_4 capsules stored under adverse conditions.

Table X—Percent of Labeled Potency Present as Anhydrotetracycline and Epianhydrotetracycline in Tetracycline Phosphate Capsule Powder Stored under Normal Conditions and at 32° and 63% Relative Humidity

Months Stored	Normal Conditions ATC, %	Normal Conditions EATC, %	32° and 63% R.H. ATC, %	32° and 63% R.H. EATC, %
		Lot A[a]		
0	0.52	0.27		
4	0.8	0.3	2.0	2.6
9	1.4	0.88	3.3	3.4
32	3.9	2.9	8.6	10.6
		Lot B[b]		
0	1.5	0.44		
4	2.3	0.6	2.7	2.2
9	2.6	1.2	5.7	3.7
	2.8	1.1	3.5	4.0
32	5.1	3.7	13.0	22.1

[a] No other active ingredients present. [b] Contained phenyltoloxamine citrate, aspirin, phenacetin, and caffeine.

REFERENCES

(1) J. M. Gross, *Ann. Intern. Med.*, **58**, 523(1963).

(2) G. W. Frimpter, A. E. Timpanelli, W. J. Eisenmenger, H. S. Stein, and L. I. Erlich, *J. Amer. Med. Ass.*, **184**, 111(1963).

(3) S. R. Sulkowski and J. R. Haserick, *ibid.*, **189**, 152(1964).

(4) W. W. Cleveland, W. C. Adams, and J. B. Man, *J. Pediat.*, **66**, 333(1965).

(5) N. Fulop and A. Drapkin, *New Engl. J. Med.*, **272**, 986(1965).

(6) J. Brodehl, K. Gellissen, W. Hogge, and H. Schumacher, *Helv. Paediat. Acta*, **23**, 373(1968).

(7) K. F. Benitz and H. F. Diermeir, *Proc. Soc. Exp. Biol. Med.*, **115**, 930(1964).

(8) R. R. Lindquist and F. X. Fellers, *Lab. Invest.*, **15,** 864(1966).
(9) M. B. Lowe and E. Tapp, *Arch. Pathol.*, **81,** 362(1966).
(10) L. Rustice and M. Ferappi, *Boll. Chim. Farm.*, **104,** 305 (1967).
(11) D. L. Simmons, C. M. Koorengevel, R. Kubelka, and P. Seers, *J. Pharm. Sci.*, **55,** 219(1966).
(12) D. L. Simmons, H. S. L. Woo, C. M. Koorengeval, and P. Seers, *ibid.*, **55,** 1313(1966).
(13) B. W. Griffiths, *ibid.*, **55,** 353(1966).
(14) A. A. Fernandez, V. T. Noceda, and E. S. Carrera, *ibid.*, **58,** 443(1969).
(15) I. C. Dijkhuis, *Pharm. Weekbl.*, **102,** 1308(1967).
(16) R. G. Kelly, *J. Pharm. Sci.*, **53,** 1551(1964).
(17) G. B. Selzer and W. W. Wright, *Antibiot. Chemother.*, **7,** 292(1957).
(18) "Code of Federal Regulations," Title 21, Part 141c, Section 141c.218(a).
(19) M. Schach von Wittinau and R. K. Blackwood, *J. Org. Chem.*, **31,** 613(1966).
(20) *Fed. Regist.*, **34,** 12286 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 13, 1970, from the *National Center for Antibiotics and Insulin Analysis, Office of Pharmaceutical Sciences, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, DC 20204*

Accepted for publication March 4, 1970.

The NMR work was done by Elizabeth Hansen of the Additives and Instrumentation Branch of the Division of Food Chemistry and Technology, Bureau of Foods, Pesticides, and Product Safety. The potency assays were performed by the Microbiological Assay Branch of the National Center for Antibiotics and Insulin Analysis.

NMR Analysis of Synthetic Corticosteroids of the 1,4-Dien-3-one Type

HAJRO W. AVDOVICH, PAUL HANBURY, and BRUCE A. LODGE

Abstract □ A procedure for the analysis of synthetic corticosteroids of the 1,4-dien-3-one type is described. The method is based upon NMR spectroscopy. Spectra are determined in dimethyl sulfoxide containing an internal reference substance, fumaric acid. Both bulk drugs and formulations can be assayed using this method, and comparison is made with results obtained from official assays on the steroids and their formulations. The average deviation obtained in the NMR method was 0.6%. A procedure for water-soluble 1,4-dien-3-ones is also described. This method uses triethylamine hydrochloride as an internal standard.

Keyphrases □ Corticosteroids, 1,4-dien-3-one type—analysis □ NMR spectroscopy—analysis □ Fumaric acid—internal standard

Synthetic corticosteroids of the 1,4-dien-3-one type are at present assayed (1, 2) by colorimetric methods based on the reduction of certain tetrazolium derivatives by the α-ketol side chain at C-17. Such methods do not distinguish between 1,4-dien-3-ones and related corticosteroids of the 4-en-3-one type, which may be present as impurities and which frequently possess different corticoid activity to that desired in the 1,4-dien-3-one. NMR spectroscopy affords a method of distinguishing easily between the two groups of steroids. 1,4-Dien-3-ones possess three vinylic protons, of which the chemical shift usually differs from that of the single vinylic proton of 4-en-3-ones. It is, therefore, theoretically possible to detect the two groups of compounds in the presence of each other and, hence, to develop a much more specific assay procedure.

Present methods used in the determination of prednisolone sodium phosphate (PSP) (1, 2) are also relatively nonspecific. The BP method (1) for the bulk drug relies upon dissolution in water and measurement of the extinction of the solution at 247 mμ; for

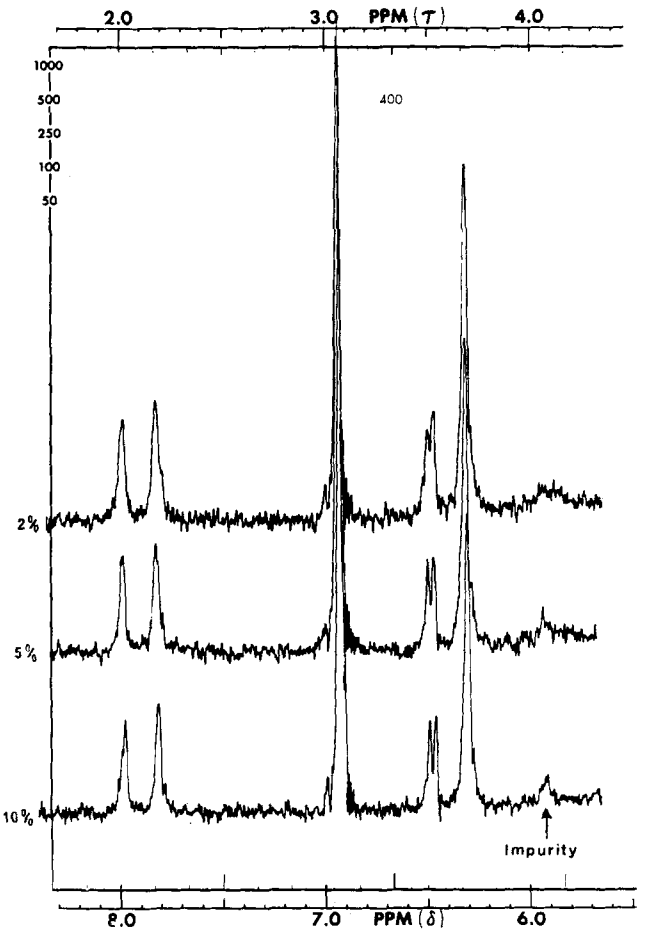


Figure 1—*Partial NMR spectrum of steroidal 1,4-dien-3-one in dimethyl sulfoxide containing fumaric acid and 2, 5, or 10% of added steroidal 4-en-3-one.*

(8) R. R. Lindquist and F. X. Fellers, *Lab. Invest.*, **15,** 864(1966).
(9) M. B. Lowe and E. Tapp, *Arch. Pathol.*, **81,** 362(1966).
(10) L. Rustice and M. Ferappi, *Boll. Chim. Farm.*, **104,** 305 (1967).
(11) D. L. Simmons, C. M. Koorengevel, R. Kubelka, and P. Seers, *J. Pharm. Sci.*, **55,** 219(1966).
(12) D. L. Simmons, H. S. L. Woo, C. M. Koorengeval, and P. Seers, *ibid.*, **55,** 1313(1966).
(13) B. W. Griffiths, *ibid.*, **55,** 353(1966).
(14) A. A. Fernandez, V. T. Noceda, and E. S. Carrera, *ibid.*, **58,** 443(1969).
(15) I. C. Dijkhuis, *Pharm. Weekbl.*, **102,** 1308(1967).
(16) R. G. Kelly, *J. Pharm. Sci.*, **53,** 1551(1964).
(17) G. B. Selzer and W. W. Wright, *Antibiot. Chemother.*, **7,** 292(1957).
(18) "Code of Federal Regulations," Title 21, Part 141c, Section 141c.218(a).
(19) M. Schach von Wittinau and R. K. Blackwood, *J. Org. Chem.*, **31,** 613(1966).
(20) *Fed. Regist.*, **34,** 12286 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 13, 1970, from the *National Center for Antibiotics and Insulin Analysis, Office of Pharmaceutical Sciences, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, DC 20204*

Accepted for publication March 4, 1970.

The NMR work was done by Elizabeth Hansen of the Additives and Instrumentation Branch of the Division of Food Chemistry and Technology, Bureau of Foods, Pesticides, and Product Safety. The potency assays were performed by the Microbiological Assay Branch of the National Center for Antibiotics and Insulin Analysis.

NMR Analysis of Synthetic Corticosteroids of the 1,4-Dien-3-one Type

HAJRO W. AVDOVICH, PAUL HANBURY, and BRUCE A. LODGE

Abstract □ A procedure for the analysis of synthetic corticosteroids of the 1,4-dien-3-one type is described. The method is based upon NMR spectroscopy. Spectra are determined in dimethyl sulfoxide containing an internal reference substance, fumaric acid. Both bulk drugs and formulations can be assayed using this method, and comparison is made with results obtained from official assays on the steroids and their formulations. The average deviation obtained in the NMR method was 0.6%. A procedure for water-soluble 1,4-dien-3-ones is also described. This method uses triethylamine hydrochloride as an internal standard.

Keyphrases □ Corticosteroids, 1,4-dien-3-one type—analysis □ NMR spectroscopy—analysis □ Fumaric acid—internal standard

Synthetic corticosteroids of the 1,4-dien-3-one type are at present assayed (1, 2) by colorimetric methods based on the reduction of certain tetrazolium derivatives by the α-ketol side chain at C-17. Such methods do not distinguish between 1,4-dien-3-ones and related corticosteroids of the 4-en-3-one type, which may be present as impurities and which frequently possess different corticoid activity to that desired in the 1,4-dien-3-one. NMR spectroscopy affords a method of distinguishing easily between the two groups of steroids. 1,4-Dien-3-ones possess three vinylic protons, of which the chemical shift usually differs from that of the single vinylic proton of 4-en-3-ones. It is, therefore, theoretically possible to detect the two groups of compounds in the presence of each other and, hence, to develop a much more specific assay procedure.

Present methods used in the determination of prednisolone sodium phosphate (PSP) (1, 2) are also relatively nonspecific. The BP method (1) for the bulk drug relies upon dissolution in water and measurement of the extinction of the solution at 247 mμ; for

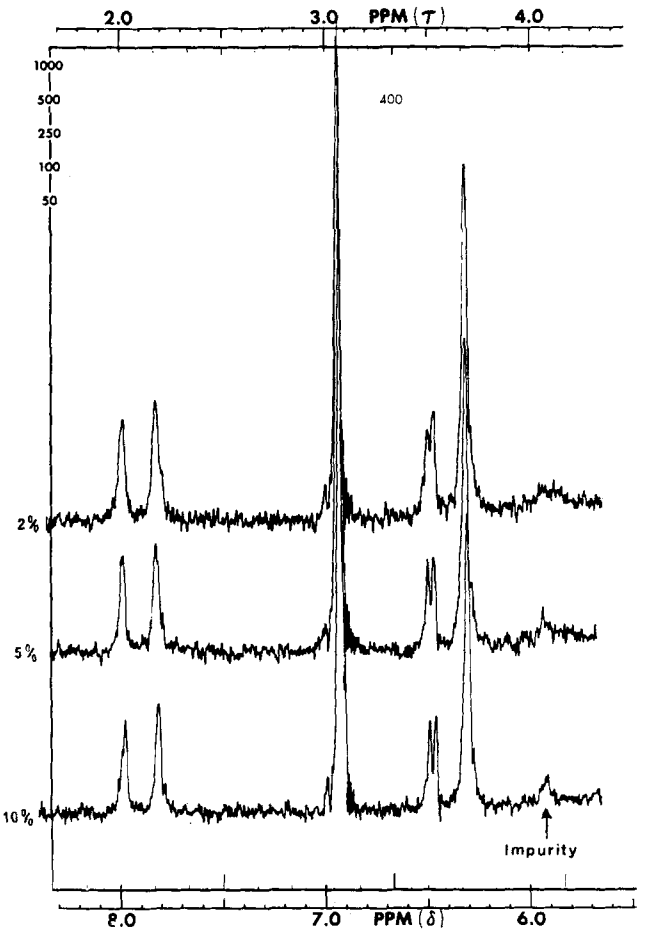


Figure 1—*Partial NMR spectrum of steroidal 1,4-dien-3-one in dimethyl sulfoxide containing fumaric acid and 2, 5, or 10% of added steroidal 4-en-3-one.*

Table I—Analysis of Bulk Water-Insoluble Steroids by NMR

Steroid	Sample No.	Amount Weighed, mg.	Amount Found, mg.	%	Deviation from Mean %
Betamethasone	1	77.9	78.5	100.8	0.5
	2	106.8	107.7	100.8	0.5
Dexamethasone	1	108.0	107.0	99.1	1.2
	2	100.1	100.1	100.0	0.3
Fluocinolone acetonide	1	104.5	105.2	100.7	0.4
Prednisolone	1	82.3	83.3	101.2	0.8
	2	107.3	107.9	100.6	0.3
	3	133.3	134.0	100.5	0.2
	4	140.9	140.0	99.4	0.9
	5	85.9	87.3	101.6	1.3
	6	76.0	77.0	101.3	1.0
	7	75.7	76.3	100.8	0.5
Prednisolone acetate	1	69.4	69.8	100.6	0.3
	2	101.8	102.4	100.6	0.3
	3	95.2	95.3	100.1	0.2
	4	93.2	92.9	99.7	0.6
	5	132.0	133.2	100.9	0.6
	6	96.0	95.8	99.8	0.5
	7	99.3	98.5	99.2	1.1
Prednisone	1	101.0	100.4	99.4	0.9
	2	92.9	93.3	100.4	0.1
	3	89.9	88.8	98.8	1.5
	4	85.8	86.1	100.3	0
	5	106.4	106.0	99.6	0.7
	6	119.4	120.9	101.3	1.0
	7	116.7	117.6	100.8	0.5
Triamcinolone	1	91.7	92.1	100.4	0.1
	2	100.1	99.4	99.3	1.0
	3	104.2	105.1	100.9	0.6
Triamcinolone acetonide	1	83.2	83.0	99.8	0.5

Mean % found = 100.3 SD = 0.7
Average deviation from mean = 0.6%

the formulation, it uses a colorimetric procedure based on a condensation of the steroid with isoniazid. The USP method (2), for both the bulk drug and the formulation, uses alkaline phosphatase to hydrolyze the phosphate ester, followed by extraction of the liberated prednisolone into methylene chloride and measurement of the absorbance of the steroidal solution at 241 mμ.

EXPERIMENTAL

Spectra were obtained at 60 Mc.p.s. using an analytical NMR spectrometer (Varian A-60A). A sweep time of 50 sec. for a chart width of 500 c.p.s. was used for all integrals. A r. f. power of 0.25 milligauss (nominal dial setting) was found to give the maximum integral amplitude (3) and was used for the integrations. Tetramethylsilane in chloroform was used as an external reference to measure chemical shifts.

Procedure (Water-Insoluble Compounds)—*Bulk Drug*—Approximately 100 mg. of steroid was accurately weighed and dissolved in 1 ml. of dimethyl sulfoxide containing fumaric acid (25.40 mg./ml.). The NMR spectrum was integrated 5 times in each direction through the region shown (Fig. 1).

Dosage Forms—Tablets—Twenty 5-mg. or ten 8-mg. tablets were powdered and refluxed for 15 min. with 50 ml. of 95% ethanol. The mixture was filtered, the residue and filter pad were washed with 50 ml. of 95% ethanol, and the combined filtrate was evaporated to dryness by flash evaporation. The residue was dissolved in 1 ml. of dimethyl sulfoxide containing fumaric acid (25.40 mg./ml.). The NMR spectrum was integrated as for the bulk drug.

Injections—Four milliliters (25 mg./ml.) or 2 ml. (40 mg./ml.) of aqueous suspension was carefully measured into a 50-ml. round-bottom flask and evaporated to dryness. One milliliter of dimethyl sulfoxide containing fumaric acid (25.40 mg./ml.) was added to the residue, and the whole was carefully mixed to effect dissolution of the steroid in the solvent. Some of the residue (preservative, suspending agents) was not soluble in the dimethyl sulfoxide and remained adhered to the wall of the flask. A clear solution was obtained, and the NMR spectrum was determined as for the bulk drug.

Procedure (Water-Soluble Compounds)—*Bulk Drug*—Approximately 100 mg. of PSP, previously dried *in vacuo* at 105°, was accurately weighed in a glass-stoppered flask, to which was added about 10 mg. of pure triethylamine hydrochloride, also accurately weighed. The mixture was dissolved in the minimum amount of D_2O (0.5–1.0 ml.). The NMR spectrum was obtained in the usual way (Fig. 2), the signals of interest being integrated 5 times in each direction. The signals at 2.42τ (for PSP) and 6.78τ (for triethylamine hydrochloride) were used as a basis for the assay.

Formulation—Four milliliters of injection of PSP was accurately measured into a 50-ml. round-bottom flask, and the solvent was removed by flash evaporation. Approximately 10 mg. of triethylamine hydrochloride (accurately weighed) was added to the flask, and the mixture was dissolved in the minimum amount of D_2O. The NMR spectrum was obtained and integrated as for the bulk drug.

Calculations—*Bulk Drug*—

amount of steroid (mg.) =

$$\frac{\text{E.W. (steroid)}}{\text{E.W. (standard)}} \times \frac{\text{I (steroid)}}{\text{I (standard)}} \times \text{weight of standard} \quad \text{(Eq. 1)}$$

where E.W. is the molecular weight divided by the number of protons in the signal chosen and I is the integral height.

Tablets—As for the bulk drug, then:

$$\text{amount of steroid/tablet} = \frac{\text{total weight (steroid)}}{\text{no. of tablets in sample}} \quad \text{(Eq. 2)}$$

Table II—Recovery of Steroids from Excipient Mixtures

Mixture	Steroid	Amount Added, mg.	Amount Found, mg.	%
1	Prednisone	97.6	96.3	98.7
2	Prednisone	137.3	135.9	98.9
3	Prednisone	137.0	135.0	98.6
4	Triamcinolone	124.4	125.2	100.6
5	Triamcinolone	106.3	106.9	100.6

Injections—As for bulk drug, then:

$$\text{amount of steroid (mg./ml.)} = \frac{\text{total weight (steroid)}}{\text{no. of ml. in sample}} \quad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

The NMR method, based on the 60 Mc.p.s. spectrum and using an internal standard, has been shown (4) to be suitable as an assay procedure for meprobamate and chemically related substances. The spectrum of a steroidal 1,4-dien-3-one is rather complicated, however, and an internal standard must be chosen with great care, since there are few spaces in the spectrum into which a reference signal might fit. To be of use as a standard, a substance should be an easily purified solid, freely soluble in the solvent of choice. It should not react with the solvent or the substrate and should possess a recognizable, isolated signal in a suitable region of the spectrum. Fumaric acid was chosen as an internal reference standard for use with water-insoluble 1,4-dien-3-ones, because its NMR spectrum in dimethyl sulfoxide shows a single sharp signal at 3.1τ, which fits well into the spectrum of the steroids. Figure 1 shows the part of the spectrum utilized in the analysis; the signals occur at approximately 2.1τ (doublet, C-1 proton), 3.6τ (doublet, C-2 proton), and 3.7τ (singlet, C-4 proton).

Also shown in Fig. 1 is the position of the signal from the vinylic proton in a steroidal 4-en-3-one (4.1τ). Partial spectra were obtained from prepared mixtures containing 2, 5, and 10% of 4-en-3-one in 1,4-dien-3-one to show the intensity of the signal from the impurity. This signal can be seen to be well removed from the primary assay signal (2.1τ) and the standard signal (3.1τ).

The results obtained from the analysis of eight reference steroids are shown in Table I. In two cases, there was very little material available, and only one analysis could be carried out on each. Nevertheless, the results obtained from fluocinolone acetonide and triamcinolone acetonide are both within the average deviation from the mean, which for 30 samples is 0.6%. Eleven of the 30 results are outside the average deviation, which is the equivalent of one standard deviation. This does not appear to be important for practical purposes, however, since the overall range of results is 98.8–101.6%. The NMR method thus appears to be suitable for the assay of bulk steroids of the 1,4-dien-3-one type.

Table II shows that recovery of prednisone and triamcinolone from mixtures with lactose and starch is reproducible and almost complete. The extraction procedure thus appears to be suitable for use in the determination of steroids in dosage forms.

Table III shows the results obtained from some commercial dosage forms of the steroids under consideration. The NMR procedure for tablets is simpler than the colorimetric method because no weighing of the tablets is involved. Recovery of the steroid from the powdered tablets in the colorimetric method involves several extractions of an aqueous suspension of the powder with chloroform, followed by dilution of the chloroform solution. An aliquot is taken, and the chloroform is removed by evaporation on a steam bath; then the residue is dissolved in ethanol. The NMR procedure consists of refluxing with 95% ethanol, filtration, and removal of the solvent by flash evaporation. The residue is dissolved in 1 ml. of the stock solution of fumaric acid in dimethyl sulfoxide. The stock solution was stored in a cool, dark place for several months without deterioration. One problem frequently encountered in extracting drugs from powdered tablets into chloroform is that of emulsion formation. Such emulsions are sometimes very stable, and separation of all the chloroform solution is difficult. This problem does not enter into the NMR procedure at all.

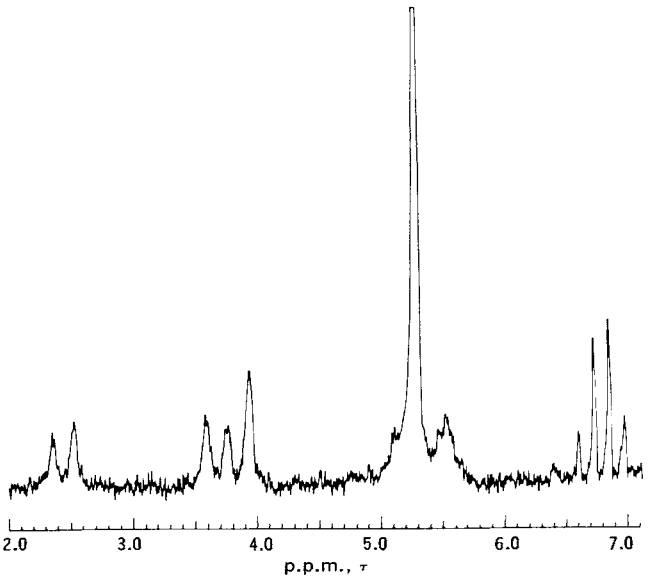


Figure 2—*Partial NMR spectrum of PSP + triethylamine hydrochloride in D_2O.*

The result obtained from Brand C, a prednisone tablet, requires some comment.The colorimetric method was carried out 8 times on this product, using a variety of water–chloroform ratios in the extraction. However, the results showed only 90% of the labeled amount, whereas the NMR procedure showed 98%. The reason for this low result may be that some very bad emulsions were obtained, even when a large excess of chloroform over water was used. The pairs of results obtained from the other five types of tablets compare very well.

Reproducibility of sampling was the chief problem in assaying the injectable preparations, which were both aqueous suspensions; however, the results are all well within the official limits (1, 2). No emulsion problems were encountered with these suspensions, in spite of the presence of suspending agents in the formulations. In the NMR procedure, not all of the dried formulation was soluble in dimethyl sulfoxide, but this apparently did not present any

Table III—Analysis of Dosage Form by NMR and Colorimetric Methods

Sample	Steroid	Labeled Amount, mg.	Found, mg.[a] NMR	Found, mg.[a] Colorimetric
Brand A, tablet	Prednisone	5	5.1	5.0
Brand B, tablet	Prednisone	5	4.9	4.9
Brand C, tablet	Prednisone	5	4.9	4.5[b]
Brand D, tablet	Prednisolone	5	5.1	5.0
Brand E, tablet	Prednisolone	5	4.8	4.9
Brand F, tablet	Triamcinolone	8	8.2	8.2
Brand G, injection	Prednisolone acetate	25/ml.	26.3/ml.	24.9/ml.
Brand H, injection	Triamcinolone acetonide	40/ml.	39.8/ml.	41.5/ml.

[a] Each result is the average of two determinations. [b] This result is the average of eight determinations.

Table IV—Determination of PSP (Bulk Drug) by NMR

Determination No.	Weight $Et_3N \cdot HCl$, mg.	Weight PSP, mg. Added	Found	%
1	10.17	106.2	106.0	99.8
2	12.80	140.8	141.6	100.6
3	13.20	90.6	91.1	100.6
4	9.72	104.1	104.6	100.5
5	10.53	150.3	148.5	98.8
6	9.01	120.2	121.9	101.4
7	11.99	101.5	100.2	98.7
8	12.62	119.2	119.9	100.6
	Mean % = 100.1		SD = 0.95	

problems of recovery based on a comparison of the two pairs of results. It was found, for the formulations studied, that the excipients did not interfere with the NMR method; hence a minimum of cleanup is required.

The NMR procedure, as described, is specific for steroidal 1,4-dien-3-one in the presence of related 4-en-3-ones. This cannot be said of the colorimetric method, which works equally well with other steroids containing the α-ketol side chain. Indeed, it has been shown in this laboratory that such common pharmaceutical aids as ascorbic acid and lactose also produce the color under the reaction conditions used. Since it has been shown (5) that samples of corticosteroids of the 1,4-dien-3-one type sometimes contain significant amounts of related 4-en-3-one precursors, a more specific assay procedure is perhaps desirable.

PSP is a water-soluble corticosteroid and, therefore, the solvent of choice for NMR is D_2O. The almost inevitable presence of a strong signal of 5.3τ (H_2O/HOD) with its attendant spinning side bands presents a considerable problem, since it reduces the space available in which to put a reference signal. Triethylamine hydrochloride was found to be a suitable internal standard, fulfilling all the conditions described for such substances.

The 60-Mc.p.s. spectrum of PSP in D_2O (Fig. 2) shows a broad doublet at 2.42τ (J = 10 c.p.s.), being the signal from the vinylic proton at C-1 in PSP. This signal was compared with that produced by the six equivalent methylene protons of triethylamine hydrochloride at 6.78τ (quartet, J = 7.5 c.p.s.).

In addition to the steroid, the commercial sample of PSP injection contains nicotinamide, phenol, sodium hydroxide, sodium bisulfite, and disodium edetate. Some interference is caused with the signal at 2.42τ (C-1 proton), making accurate integration impossible. However, the signals at 3.66τ (doublet, J = 10 c.p.s., C-2 proton) and 3.93τ (singlet, C-4 proton) are unaffected. The total integral for the two protons was used in the determination, with the necessary correction being made to the calculation.

Results obtained from the analysis of eight samples of PSP are shown in Table IV.

A sample of injection of PSP labeled to contain the equivalent of 20 mg. of prednisolone/ml. was analyzed twice by the NMR method and each time found to contain 18.8 mg./ml., and once by the USP method (2), giving 19.1 mg./ml.

For the injectable sample, the NMR method requires much less manipulation than the official UV method (2) and is also relatively more specific. Comparison of the results obtained for analysis of the commercial preparation showed the two methods to be in good agreement.

CONCLUSION

Synthetic corticosteroids of the 1,4-dien-3-one type can be assayed satisfactorily by means of NMR spectroscopy, even in the presence of related 4-en-3-one precursors. Because of the sensitivity of present NMR spectrometers, the method is chiefly of value for raw material analysis; it is, however, easily adapted for composite tablet assays and for the assay of injectable preparations.

REFERENCES

(1) "British Pharmacopoeia," The Pharmaceutical Press, London, England, 1968.

(2) "The United States Pharmacopeia," 17 rev., Mack Publishing Co., Easton, Pa., 1965.

(3) J. L. Jungnickel and J. W. Forbes, *Anal. Chem.*, **35**, 939 (1965).

(4) J. W. Turczan and T. C. Kram, *J. Pharm. Sci.*, **56**, 1643 (1967).

(5) A. J. Liston, personal communication.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 24, 1969, from the *Research Laboratories, Food & Drug Directorate, Tunney's Pasture, Ottawa, Canada.*

Accepted for publication March 18, 1970.

Quantitative Spectrophotometric Methods for Determination of Sodium Hypochlorite in Aqueous Solutions

A. HUSSAIN*, P. TRUDELL, and A. J. REPTA

Abstract □ Two simple spectrophotometric procedures for the quantitative estimation of hypochlorite in commercially available sodium hypochlorite solutions are presented. One method is based directly on the absorbance of hypochlorite in alkaline aqueous media. The other method takes advantage of the quantitative reaction of hypochlorite and ammonia in alkaline solution to form chloramine, which has a higher molar absorptivity. Results of the spectrophotometric assays are compared with results obtained by the titrimetric procedure of NF XII.

Keyphrases □ Sodium hypochlorite determination—aqueous solutions □ Chloramine determination—Na hypochlorite analysis □ Molar absorptivity—Na hypochlorite, chloramine □ UV spectrophotometry—analysis

Of the various methods available for quantitative determination of sodium hypochlorite in aqueous solution, most are the volumetric or gravimetric type (1–4). All of these methods are relatively laborious, time-consuming, and expensive to do on a routine basis. The method described in the NF XII, while giving good precision and accuracy, requires the preparation of several solutions, some of which must be standardized (1).

The purpose of this work was to develop procedures equally sensitive compared to the NF method, which could be used routinely for the quantitative determination of sodium hypochlorite. The facts that hypochlorite ion has an absorbance maximum at 292.5 mμ in alkaline aqueous solution (5) and that the absorbance shows a direct relationship to concentration of hypochlorite were utilized in one procedure. The other procedure is based on the quantitative reaction of hypochlorite with ammonia to yield chloramine (6), which has an absorbance maximum at 244.3 mμ that is also linearly related to concentration.

EXPERIMENTAL

Reagents—Commercially available sodium hypochlorite solutions were used. Hypochlorous acid solutions were prepared by the method of Higuchi *et al.* (7). Reagent grade ammonium hydroxide (58%) (Mallinckrodt) was diluted with distilled water to yield a 20% ammonium hydroxide solution. All water was distilled from acid permanganate solution in an all-glass still.

Spectrophotometric measurements were carried out using a Cary model 14 spectrophotometer. A Beckman pH meter was used for all pH measurements.

Procedures—*Spectral Changes of Hypochlorous Acid as a Function of pH*—Solutions containing 2.13×10^{-3} *M* hypochlorous acid at various pH values were prepared. The spectral changes were determined by examining the UV spectra from 400 to 250 mμ.

Standardization of Hypochlorous Acid Solutions—A suitable volume (3 ml.) of hypochlorous acid solution at 25° was pipeted into a stoppered conical flask and diluted with about 100 ml. of water. One milliliter of glacial acetic acid and 2 g. of potassium iodide were then added, and the stoppered flask was placed in the dark for 5–10 min. The liberated iodine was then titrated with standard sodium thiosulfate solution, using 1 ml. of starch solution as an indicator.

Iodometric Determinations of Sodium Hypochlorite—These determinations were carried out according to a modification of the procedure described in NF XII. The modification consisted of pipeting 3 ml. of the sample at 25° rather than weighing it.

Spectrophotometric Method I—The molar absorptivity of hypochlorite was determined as follows. Solutions containing concentrations ranging from 8.73×10^{-4} to 34.92×10^{-4} *M* sodium hypochlorite were prepared by pipeting 1, 2, 3, and 4 ml. of a standardized hypochlorous acid solution into 100-ml. volumetric flasks. Two milliliters of 10% sodium hydroxide solution and 75 ml. of distilled water were then added to each flask, and the solutions were allowed to equilibrate at 25°. After equilibrating, the solutions were brought to volume with distilled water at 25°. A plot of the absorbance of each solution in a 1-cm. stoppered cell at 292.5 mμ *versus* concentration was used to calculate the molar absorptivity of hypochlorite ion.

The hypochlorite content of commercial bleaches was determined by pipeting 1 ml. of bleach at 25° into a 200-ml. volumetric flask and adding 175 ml. of water and 1 ml. of 10% sodium hydroxide solution. The solution was then equilibrated at 25° and brought to volume with distilled water of the same temperature. Its absorbance at 292.5 mμ was then determined against a similarly prepared solution containing no bleach. Using the molar absorptivity and a molecular weight at 74.5 g./mole for sodium hypochlorite, the concentration of sodium hypochlorite (in mg./ml.) of bleach solution was calculated.

Spectrophotometric Method II—The molar absorptivity of chloramine was determined by preparing concentrations of chloramine varying from 8.73×10^{-4} to 34.92×10^{-4} *M*. This was done by pipeting 1, 2, 3, and 4 ml. of a standardized hypochlorous acid solution (at 25°) into four 100-ml. volumetric flasks, followed by addition of about 75 ml. of distilled water and 2 ml. of 10% sodium hydroxide solution. The flasks were then equilibrated at 25° in a water bath. To one of the solutions was added 2 ml. of 20% ammonium hydroxide, and the solution was brought to volume with distilled water. The absorbance was measured *immediately* at 244.3 mμ in a 1-cm. stoppered cell using a suitable blank. Each of the remaining solutions was treated in an identical way. A plot of absorbance *versus* concentration was made, and the molar absorptivity was calculated.

Determination of hypochlorite content of commercial bleaches was made by pipeting 1 ml. of bleach at 25° into a 200-ml. volumetric flask. The sample was diluted with 175 ml. of distilled water and 1 ml. of 10% sodium hydroxide solution. The resulting solution was equilibrated at 25°. Two milliliters of 20% ammonium hydroxide solution was then added to the flask, and distilled water was added to bring the solution to a volume of 200 ml. The absorbance was then *immediately* measured at 244.3 mμ against a blank which was prepared in the same way except the bleach was omitted.

DISCUSSION

Spectrophotometric Method I—Since this method was based on the absorbance–concentration relationship of hypochlorite ion, it was necessary to determine the minimum pH required to ensure that all hypochlorous acid was converted to hypochlorite ion. Therefore, several solutions containing identical amounts of hypochlorous acid were adjusted to various pH values, and their

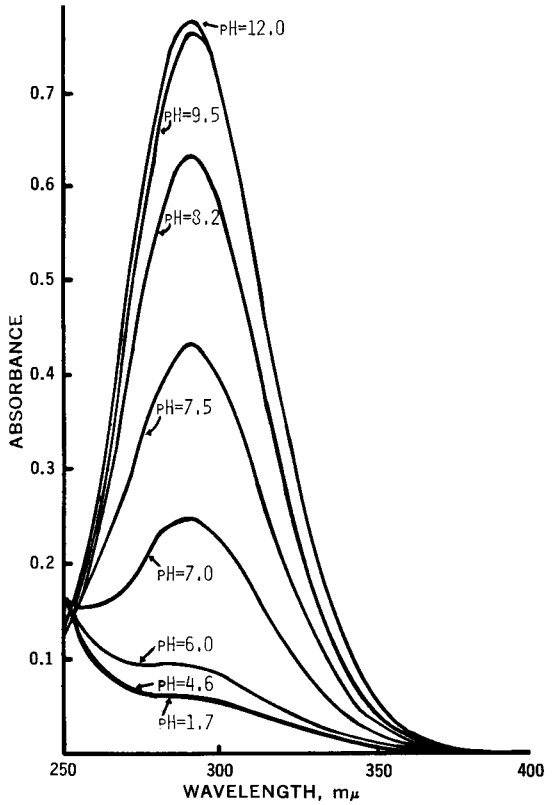


Figure 1—*UV spectral changes of 2.13 × 10^{-3} M hypochlorous acid solutions as a function of pH.* $\lambda_{max.}$ *is 292.5 mμ.*

spectra were obtained by scanning from 400 to 250 mμ. It was found that at pH >10, all hypochlorous acid had been converted to the hypochlorite ion. These data are shown in Fig. 1. All solutions subsequently used in the determination of the absorptivity and in analysis of hypochlorite were made such that pH >10.

From Fig. 1, it was also determined that $\lambda_{max.}$ = 292.5. When absorbance at $\lambda_{max.}$ was plotted against pH (Fig. 2), a typical sigma-shaped curve was obtained. From Fig. 2, the pKa of hypochlorous acid was found to be 7.50, which agrees well with the value of 7.53 previously reported (8).

When the absorbance values at 292.5 mμ of a series of hypochlorite solutions were plotted against their concentrations, a linear relationship was found. From these data the molar absorptivity was calculated (ϵ = 360 ± 2 M^{-1} cm.$^{-1}$).

The quantitative determination of hypochlorite in commercial bleach solution was done by pipeting a 1-ml. sample at 25° into a 200-ml. volumetric flask, adding excess alkali, and bringing to volume with water at 25°. The absorbance of the diluted solution was measured at 292.5 mμ against a blank. The concentration in milligrams of sodium hypochlorite per milliliter of bleach was calculated according to the following equation:

$$\text{mg. of NaOCl/ml. of bleach} = A \times MW \times V_D/\epsilon V_S \quad \text{(Eq. 1)}$$

where: A = absorbance at 292.5 mμ
ϵ = molar absorptivity of hypochlorite (at 292.5 mμ) = 360 M^{-1} cm.$^{-1}$
MW = molecular weight of NaOCl = 74.5
V_D = total volume of diluted solution (in ml.)
V_S = volume of bleach sample solution used (in ml.)

The stability of the solutions was checked, and no change in absorbance at 292.5 mμ was observed over a 2-hr. period.

Spectrophotometric Method II—An earlier report (6) has shown that hypochlorous acid in alkaline solution reacts rapidly in the presence of excess ammonia to form chloramine quantitatively according to the following scheme: $NH_3 + HOCl \rightarrow NH_2Cl + H_2O$.

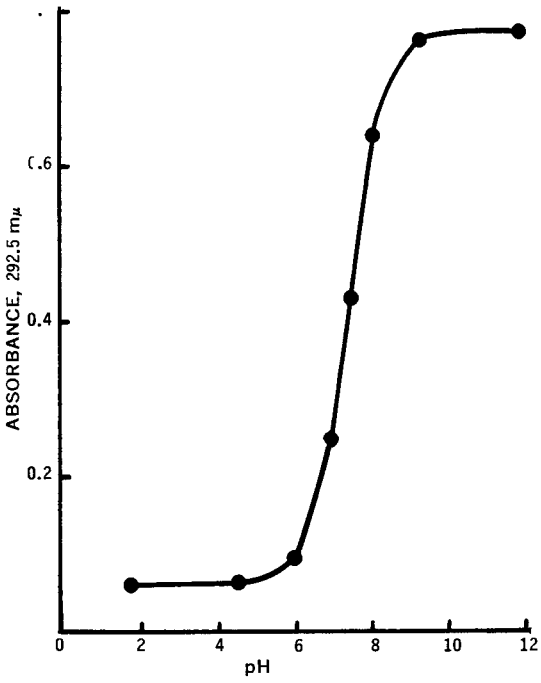


Figure 2—*Absorbance at 292.5 mμ of 2.13 × 10^{-3} M hypochlorous acid solutions as a function of pH.*

This information was used as the basis for the indirect determination of hypochlorite. Figure 3 shows the difference in spectra between hypochlorite solution at pH >10 and a similar solution to which excess ammonia has been added to form chloramine. The $\lambda_{max.}$ shifted from 292.5 mμ for hypochlorite to 244.3 mμ for chloramine. When various concentrations of hypochlorous acid were treated with ammonium hydroxide at pH >10, and the absorbance at 244.3 was plotted against the concentration of chloramine (which is equimolar with hypochlorite), a linear relationship was observed. From the slope of the line, the molar absorptivity was calculated to be 459 ± 3 M^{-1} cm.$^{-1}$. Actual determinations of sodium hypochlorite in commercial bleach samples were similarly

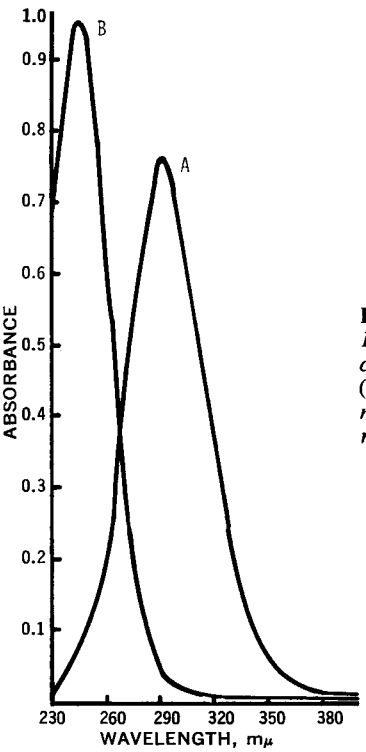


Figure 3—*Spectra of 2.1 × 10^{-3} M solutions of (A) hypochlorite and (B) chloramine (formed by addition of ammonium hydroxide to hypochlorite solutions).*

Table I—Results and Comparisons of Hypochlorite Assay of Three Commercial Bleaches at 25°. Results Expressed as Milligrams of Sodium Hypochlorite per Milliliter of Bleach

Bleach Solution	Assay Method	Number of Determinations	Range of Results, mg./ml.	Mean and Standard Deviation, mg./ml.
1	NF[a]	5	39.5–40.3	39.8 ± 0.3
1	Spectrophotometric method I[b]	5	39.6–39.8	39.6 ± 0.1
1	Spectrophotometric method II[c]	5	39.5–39.8	39.7 ± 0.1
2	NF[a]	7	15.1–15.4	15.2 ± 0.2
2	Spectrophotometric method I[b]	7	15.3–15.4	15.3 ± 0.1
2	Spectrophotometric method II[c]	7	15.0–15.1	15.1 ± 0.1
3	NF[a]	5	59.7–60.2	59.7 ± 0.2
3	Spectrophotometric method I[b]	5	60.0–60.3	60.1 ± 0.2
3	Spectrophotometric method II[c]	5	59.1–59.5	59.3 ± 0.2

[a] Used volume at 25° rather than weighing sample in NF assay. [b] Measured as hypochlorite at 292.5 mμ. [c] Measured as chloramine at 244.3 mμ.

carried out, and the concentration of sodium hypochlorite in milligrams per milliliter of bleach was calculated according to Eq. 1 where now A = absorbance of chloramine at 244.3 mμ, and ϵ = molar absorptivity of chloramine = 459 M^{-1} cm.$^{-1}$.

When the absorbance of chloramine solutions was observed over a period of time, no significant changes were seen in 4–5 min. However, 1–2% decreases in absorbance were observed after 30 min. To minimize the experimental error due to the instability of chloramine, all solutions were prepared up to the point where the ammonium hydroxide was to be added. At this point, one solution at a time was taken and to it were added the ammonium hydroxide and distilled water to bring it to volume. The absorbance was then immediately measured.

RESULTS AND CONCLUSIONS

The hypochlorite content of three different bleach solutions was determined by the NF method and the two spectrophotometric methods; the results are shown in Table I. The results obtained for each sample are in good agreement for all three analytical methods.

The simplicity and ease of the spectrophotometric methods as opposed to the NF method would seem to suggest the former as the methods of choice in the determination of hypochlorite in aqueous solution. In addition, the spectrophotometric methods allow for analysis of much more dilute solutions than the NF method. For example, if a 10-cm. cell and the expanded scale of the Cary 14 spectrophotometer were used, concentrations as low as several micrograms per milliliter could be determined. This could be extremely useful, especially when applied to the determination of active chlorine in water.

REFERENCES

(1) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 365.

(2) A. T. Palin, *J. Inst. Water Eng.*, **3**, 100(1949).

(3) H. C. Marks, D. B. Williams, and G. U. Glasgow, *J. Amer. Water Works Ass.*, **43**, 201(1951).

(4) A. I. Vogel, "A Textbook of Quantitative Inorganic Analysis," 3rd ed., Wiley, New York, N. Y., 1961, pp. 395–396, 571.

(5) R. E. Corbett, W. S. Metcalf, and F. G. Soper, *J. Chem. Soc.*, **1953**, 1927.

(6) I. R. Weil and J. C. Morris, *J. Amer. Chem. Soc.*, **71**, 1664 (1949).

(7) T. Higuchi and J. Hasegawa, *J. Phys. Chem.*, **69**, 796(1965).

(8) "Handbook of Chemistry and Physics," 48th ed., The Chemical Rubber Co., Cleveland, Ohio, 1967, p. D-91.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 12, 1970, from the *Pharmaceutical Development Department, Ayerst Laboratories, Inc., Rouses Point, NY 12979* and the *School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication March 10, 1970.

Partially supported by Grant No. 3123-5038 from the University of Kansas, Lawrence, KS 66044

* Present address: Institute of Pharmaceutical Chemistry, Lawrence, KS 66044

Improved Self-Programming Automated Tablet-Coating System

ALLEN HEYD and JOSEPH L. KANIG

Abstract □ The usefulness of a monitoring system based on moisture sensing is established as a means of automating a tablet-coating process. A radio frequency absorption system was employed to measure continuously the state of dryness of the tablet mass and to indicate when the tablets reached a predetermined satisfactory end-point. The moisture-sensing apparatus was found to monitor the drying rate accurately after the tablets had been sprayed. The analyzer readings were shown to be directly related to conditions of relative humidity existing in the environment surrounding the coating pan. The instrument was extremely sensitive and permitted a constant readout of drying rate and moisture content by means of a specially constructed probe placed within the tablet mass.

Keyphrases □ Tablet-coating system—self-programming, automated □ Automated tablet coating—radio frequency system, moisture measurement □ Diagram—automated tablet-coating system □ Moisture analysis—tablet-coating rate control

One important factor in a tablet-coating process is the drying of the tablet mass after each application of a coating solution. The rate of liquid loss dictates the time period between tablet-coat applications. One does not know if the rate of liquid loss is constant for each application during a coating process, or if the application of successive coats lengthens or shortens the drying time. It becomes a matter of subjective evaluation to determine whether or not a tablet mass is sufficiently dry before the next application. When an automatic timed procedure is used, it is based on the assumption that the drying rate is constant for each cycle of the process. The validity of this assumption has never been clearly established. Drying rates are different for each type of coating solution and are also influenced by tablet shape, tablet size, and batch size. A contention underlying this investigation was that an automatic timed procedure does not take into account either inherent or environmental conditions which may exercise an effect during the coating process and may vary the drying time. In taking these inherent or environmental changes into account, a system must be able to monitor the drying rate continuously and utilize the degree of dryness for controlling an automated process.

Automated tablet-coating systems that have been suggested to date are time controlled (1–6) with the length of each step in the coating cycle determined by a preset timer. Therefore, such systems are insensitive to environmental changes. When an automated system is created around a timed procedure, it must be programmed specifically for all the variables indicative of the properties of a particular batch. Each major variation in size, shape, weight, number, and other features of a tablet batch may require a separate and different program. A system that follows the drying rate, however, may prove to be independent of most of these variables. This report presents a description of an automated tablet-coating system which is entirely programmed and controlled by the moisture content of the tablet mass.

EXPERIMENTAL

Materials—The core tablets used in this investigation consisted of dicalcium phosphate dihydrate[1] lubricated with 1.5% magnesium stearate.[2] Tablets weighing 400 mg. were compressed with 1.01-cm. (0.40-in.) standard concave punches and used without further treatment.

The coating solution used in all the experiments was an 85% (w/v) syrup solution. The solution was colored with 0.1% (w/v) amaranth.[3]

Equipment—The schematic diagram in Fig. 1 illustrates the overall design and arrangement of the equipment which made up the programmed automated tablet-coating system.

The coating solutions were delivered by an airless spray system (A).[4] The pump model used (the "President") had a 28:1 pressure ratio. Two spray guns (B) (Graco automatic Hydra-Spray guns, model 205-163) were suspended inside a 96.52-cm. (38-in.) baffled coating pan (C) in such a position that the spray was directed perpendicular to the surface of the tumbling tablets for maximum, uniform coverage. The guns were suspended from steel tubing which was mounted on the pump chassis. A high-pressure fluid heater (D) (Graco model 206-580, series A), 240 v., was mounted on the rear of the spray system and connected to the fluid lines between the pump and the spray guns. The fluid passing through the guns could then be heated to a desired temperature before it was sprayed. A circulating system (E) (Graco Restrict-A-Flo) was utilized to accomplish continuous fluid circulation for maintaining uniform temperature and homogeneity of the coating liquid. Two timers[5] (F) were mounted on the automatic spray-system control panel. These timers were connected to energize the spray-on and drying cycle. The dry cycle timer was utilized only when the system was manually controlled. The timers were also interconnected with the moisture monitoring–programming device so the entire process could be evaluated for its applicability to automation. The instrument used to measure relative humidity during the coating experiments was a wet-dry bulb psychrometer.[6] Exhaust (G) was supplied with a portable vacuum,[7] and a blower-heater system[8] was used as the source of forced air (H).

[1] Stauffer Chemical Co., Chicago, Ill.
[2] Ruger Chemical Co. Inc., Irvington-on-Hudson, N. Y.
[3] National Aniline Div., Allied Chemical Corp., New York, N. Y.
[4] Graco Hydra-Spray Unit, Gray Co., Inc., Minneapolis, Minn.
[5] Microflex, Eagle Signal Corp., Moline, Ill.
[6] Psychron, model 566-3, Bendix Aviation Corp., Friez Instrument Div., Baltimore, Md.
[7] Craftool model 411, Craftool Inc., New York, N. Y.
[8] Model 7866, Bonat Inc., Norwalk, Conn.

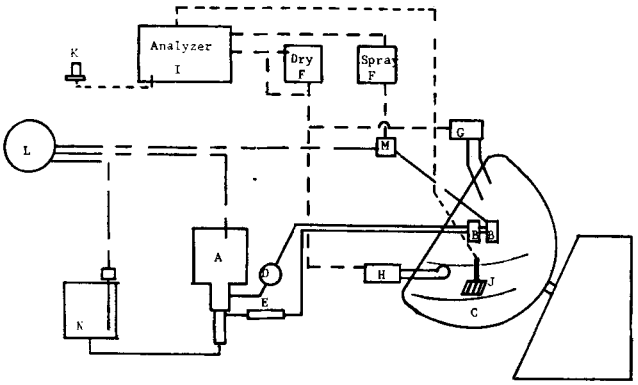


Figure 1—*A schematic diagram of the design and equipment for a self-programming automated tablet-coating system. Key: A, airless spray system; B, spray guns; C, coating pan; D, fluid heater; E, circulating system; F, timers; G, exhaust; H, forced air system; I, Moisture Analyzer; J–K, electrodes, automatic and manual, respectively; L, compressed air; M, solenoid switch; N, mixing tank; ——, coating solution; - - -, electrical line; and — - —, compressed air.*

The instrument selected to measure the state of dryness of the tablet mass was the HFR-6E Moisture Analyzer.[9] The Analyzer (I) operates on the principle of high-frequency conductance caused by power absorption of the material being tested. Power absorption or conductance is the only electrical property which gives accurate indications of moisture content (7). The Analyzer was adapted for in-process control of the various components of the coating system and for continuous readout of tablet moisture content. This was accomplished by replacement of the regular conductance meter with a contact-conductance meter which energized the spray cycle timer. Auxiliary switches were also incorporated into the Analyzer which activated the blower-heater and the exhaust system at the proper coating cycle intervals. A digital contact meter on the Analyzer was set to predetermine the total number of coats applied, after which it deenergized the entire coating apparatus. L-shaped and cup-shaped electrodes (J, K) were used to determine sample moisture content automatically and manually, respectively. Analyzer meter readings did not significantly differ

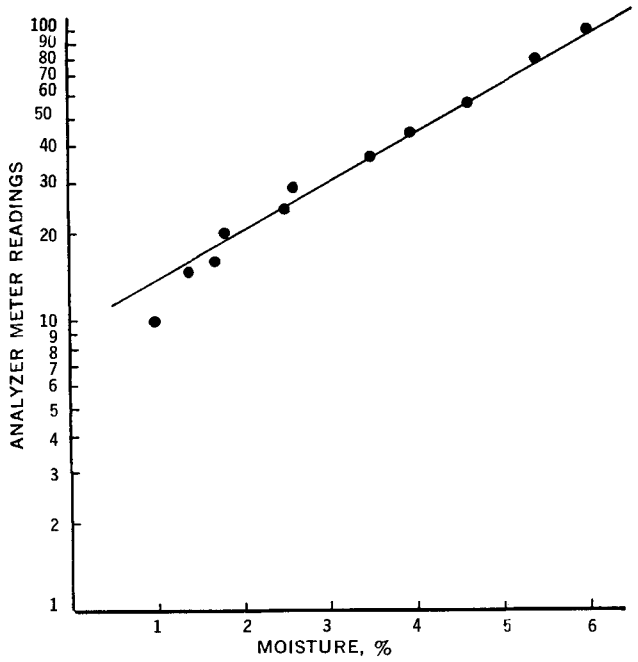


Figure 2—*Moisture calibration curve.*

[9] Boonton Polytechnic Co., Rockaway, N. J.

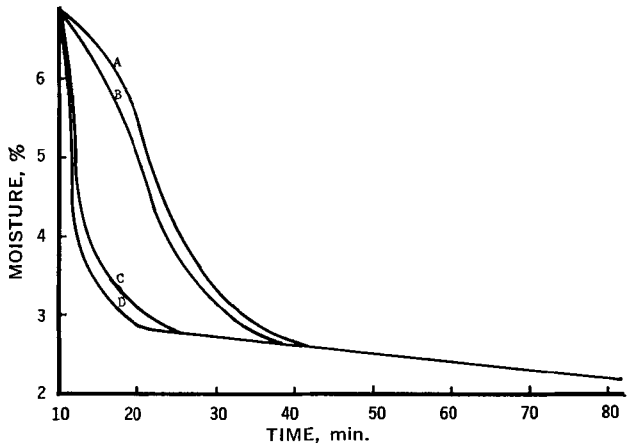


Figure 3—*Analyzer sensitivity to moisture loss. Key: A, at 29°; B, at 29° with exhaust; C, at 29° with forced air and exhaust; and D, with forced air at 50° and exhaust.*

when using either electrode under the same set of conditions. The cup electrode was used only during calibration curve preparation.

Moisture Calibration Curve—A freshly prepared 200-lb. batch of tablets was wetted with 2000 ml. coating solution.

The tablets were allowed to tumble for 20 min. in an open pan without heat, forced air, or exhaust to achieve uniform wetting. Subsequently, three samples were taken at 15-min. intervals from different areas in the pan with the manual Analyzer electrode receptacle. Readings were taken after manipulation of the control switches on the Analyzer panel until a minimum reading on the contact meter (radio frequency conductance) was obtained. The readings in terms of units indicated the dielectric loss and, consequently, the moisture content of the tablet mass. Twenty intact tablets taken from each sample were weighed and placed in 50 ml. of anhydrous methanol, reagent grade, for 12 hr. Five milliliters of each methanol–water sample was then tested for total moisture content using the Karl Fischer method (8).

To establish a calibration curve, readings were plotted against percent moisture on a semilog graph since energy loss due to moisture is a first-order process (7). The resulting curve (Fig. 2), determined by the method of least squares, was then used to interpret readings in terms of actual tablet moisture content. This calibration curve is valid for all volumes of aqueous solution delivered. Therefore, the volumes used in the investigation were appropriate for each experiment.

Automated Coating Procedure—Two hundred pounds of freshly compressed tablets was placed in the coating pan. The automatic electrode was positioned in the tablet mass in such a manner that liquid from the spray nozzle was applied downstream in relation to tablet flow and electrode insertion. Tablet attrition on the electrode walls prevented the buildup of coating solution; therefore, electrode readings were not affected. An equilibrium meter reading of 22, corresponding to 2.2% moisture, was taken and designated as batch dryness for each experiment in the investigation. This reading was arbitrarily chosen to indicate batch dryness because it was extremely difficult to obtain lower readings within an optimum time period. The coating apparatus was put into operation by closing a master switch on the Analyzer. The contact meter indicating dryness activated the spray cycle timer (F). The timer energized a solenoid valve which, in turn, activated the automatic spray guns, allowing the flow and atomization of coating liquid. At the end of the spray cycle time, the solenoid was deenergized and the forced air and exhaust units were energized by the Analyzer. The sequence of events was repeated only when the Analyzer indicated that the predetermined degree of dryness was reached in the tablet mass. On completion of each spray cycle, the digital counter registered one coat. This coating procedure was carried out for repeated coats in each experimental portion of this investigation.

Sensitivity to Moisture Loss—Analyzer sensitivity to moisture loss was determined under four conditions: (*a*) at 29°; (*b*) at 29° with exhaust, (*c*) at 29° with forced air and exhaust; and (*d*) with forced air at 50° and exhaust.

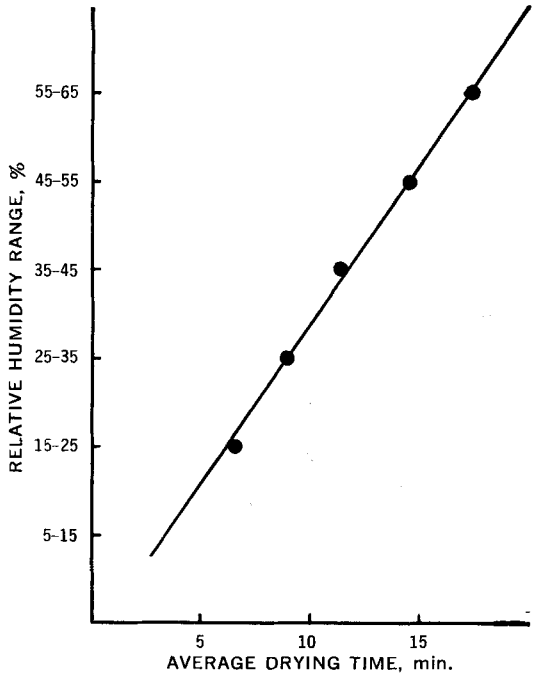


Figure 4—*Relative humidity* versus *drying time.*

The spray cycle timer was set for a 60-sec. interval to deliver 600 ml. of coating liquid and was energized manually. The wetted tablets were tumbled for 10 min. prior to manual sample testing for moisture to assure uniform distribution of coating solution. Sample readings were taken every minute after the equilibration period until a constant moisture content was attained. The coating room temperature was 29° at 60% relative humidity for the series of experiments. Each condition was carried out in duplicate with a fresh batch of tablets.

Sensitivity to Relative Humidity—The automated coating procedure was carried out on freshly prepared batches of tablets for 5 consecutive days. During this time, the ambient relative humidity varied from 15 to 60%. Coating experiments were also carried out at five controlled relative humidity range levels: 15–25 to 55–65%. Each level consisted of 10% units from a low of 15 to a high of 65% relative humidity. These relative humidity levels were attained by atomizing water into the coating-room environment. Spray cycles of 10-sec. duration, delivering 100 ml. of coating solution per cycle, were used. Moisture readings were taken every minute after an initial 3-min. equilibration period until the base reading of 22 was attained.

Sensitivity to Changes in Spray Cycle Time—These experiments were performed at a temperature of 29° and 40% relative humidity to test Analyzer sensitivity to changes in spray cycle time (change in total volume delivered). Spray cycles were begun using a 10-sec. interval and then increased by 5- or 10-sec. increments to a 30-sec. interval. Forced air at 50° was used for drying the tablet mass. Meter readings were taken every 5 min. until a plateau was reached, indicating tablet dryness.

Reproducibility of Coating Cycles—Several coating cycles were run using spray cycles of 30-sec. duration to deliver 300 ml. of coating liquid. The experiments were carried out in triplicate under conditions of 50% relative humidity at a temperature of 29°. Moisture readings were taken every minute after a 5-min. equilibration period until a plateau was reached.

RESULTS AND DISCUSSION

Sensitivity to Moisture Loss—Figure 3 shows that the Analyzer followed moisture loss with time as drying of the tablet mass occurred.[10] The differing drying curve characteristics also indicate

[10] Data points for Figs. 3 and 5 were omitted for the purpose of clarity.

Table I—Effect of Relative Humidity on Drying Time

	Day				
	1	2	3	4	5
Average relative humidity, %	59.3	34.7	27.2	37.5	51.6
Average drying time, min.	18.2	10.0	9.0	11.5	15.0

Table II—Effect of Spraying Cycle Time on Drying Time

Spray Cycle Time, sec.	Drying time, min.
10	10.0
15	13.75
20	19.5
30	28.0

the sensitivity of the instrument to varying drying conditions. It can be seen that forced air significantly increased the drying rate of the tablets. A plateau (batch dryness) was reached in approximately 22 min. as opposed to approximately 40 min. with and without forced air, respectively (Curves C and D *versus* A and B). Exhaust had no significant effect on the rate of moisture loss, as is shown by Curves A and B. The exhaust system could very well have been eliminated from the coating apparatus and would only be of use for collecting dust from an enclosed coating system. There was no significant difference in drying rate when comparing forced air at 29 and 50°, Curves C and D, respectively. Therefore, the major driving force for moisture loss from the surface of the wet tablets was the volume of forced air.

Sensitivity to Relative Humidity—The data in Table I show a decrease in drying time with a decrease in the relative humidity surrounding the coating apparatus. The Moisture Analyzer was quite sensitive to these changes in drying time, and a surprisingly good linear relationship was found between relative humidity and drying time when the data were plotted as a 10-unit range in percent relative humidity *versus* time (Fig. 4).

The Analyzer clearly indicated that there was a change in drying time as the relative humidity surrounding the coating system changed.

Sensitivity to Changes in Spray Cycle Time—Table II shows that the Analyzer was sensitive to a change in moisture when the spray cycle times were varied. It is evident that as the spray cycle time increased, there was a corresponding increase in drying time.

Reproducibility of Coating Cycles—The reproducibility of the coating cycle is quite evident in Fig. 5. In each case, a plateau was reached at approximately 11 min., after which the curves were linear and parallel, indicating approach to dryness.

The importance of using a moisture-monitoring device in tablet coating is illustrated in Figs. 3–5. There was an immediate indication of drying rate and drying time for the tablet mass under specific environmental and tablet mass conditions (Figs. 3 and 4). The state of the drying condition was no longer evaluated subjectively with the attendant, inherent human error; but it was evaluated continuously, quantitatively, and reproducibly as shown in Fig. 5.

In addition, the Analyzer can function as a built-in fail-safe mechanism to correct immediately the drying program of the

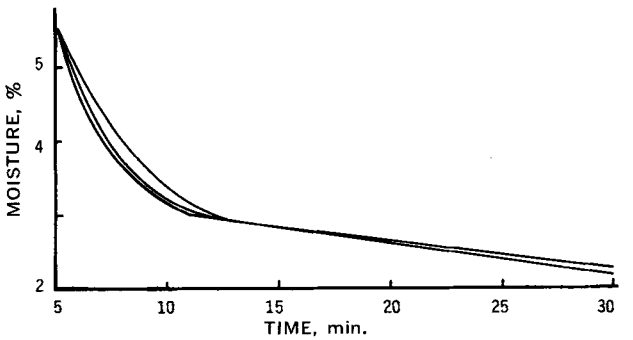


Figure 5—*Reproducibility of coating cycles.*

coating process should there be a failure in one component of the systems such as in the spray delivery timer. Even though timed devices may contain fail-safe mechanisms, such a timed coating process would have to be terminated and reprogrammed manually.

SUMMARY

An automated tablet-coating system was designed which is self-programming and based on the rate of moisture loss. The Moisture Analyzer, which programmed the system, was revealed to be extremely sensitive to the presence of moisture and to moisture loss in the tablet mass. The system quickly adjusts to any change in spray cycle time and also shows a high degree of reproducibility between cycles under similar conditions.

A high degree of sensitivity of the drying cycles to changes in the environmental humidity was also demonstrated by the apparatus. It is, therefore, felt that a moisture-sensing device should be utilized when considering an automated tablet-coating system for production.

REFERENCES

(1) D. E. Wurster, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 451(1959).

(2) R. E. Singiser and W. Lowenthal, *J. Pharm. Sci.*, **50**, 168 (1961).

(3) L. Lachman and J. Cooper, *ibid.*, **52**, 490(1963).

(4) D. S. Mody, M. W. Scott, and H. A. Lieberman, *ibid.*, **53**, 949(1964).

(5) J. Spaulding, *Drug Cosmet. Ind.*, **79**, 766(1956).

(6) G. M. Krause and T. L. Iorio, *J. Pharm. Sci.*, **57**, 1223(1968).

(7) P. S. Miller and J. J. Jones, *Proc. Sci. Sec. Toilet Goods Ass.*, No. 31, May 1959.

(8) "The United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 939.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 30, 1970, from the *Department of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CN 06268,* and *Columbia University College of Pharmaceutical Sciences, New York, NY 10023*

Accepted for publication March 23, 1970.

The experimental portions of this investigation were conducted in the research laboratories of Columbia University College of Pharmaceutical Sciences.

The authors thank Dr. Milo Gibaldi for his suggestions and helpful discussions during this investigation.

Monitoring Volatile Coating Solution Applications in a Coating Pan

RUSSELL J. LANTZ, Jr.*, ALAN BAILEY, and MANFORD J. ROBINSON

Abstract □ Equipment and methods are presented for recording temperature patterns resulting from the evaporation of volatile coating solutions applied to pellets in a rotating pan. These patterns are interpreted in regard to run-to-run replication, effect of application and drying rate, and coating solution distribution. The applicability of this equipment and methodology to control the coating of solid particles is discussed.

Keyphrases □ Coating application, pellets—monitoring method □ Volatile coating solutions—application monitoring □ Diagram—pellet coating equipment with monitor □ Thermal patterns—pellet coating

Sutaria (1) has noted that, until recently, pharmaceutical coating processes were an art because of the apathy shown toward studying the many variables involved in these processes. Sutaria's extensive bibliography indicates that efforts to define and control these variables are now underway and, in some cases, have resulted in the semiautomation of certain aspects of the coating process.

The purpose of this preliminary report is to add to this knowledge by presenting data on methods found useful for monitoring the application of volatile coating solutions to pellets in a rotating pan.

The application and evaporation of coating solutions containing volatile solvents, *e.g.*, acetone, alcohol, and chloroform, produce measurable temperature changes in a bed of pellets in a rotating pan. By placing a thermocouple and thermistors in the pellet bed and recording the temperature changes during coating, it is possible to discern and control certain events and/or trends taking place during the coating process.

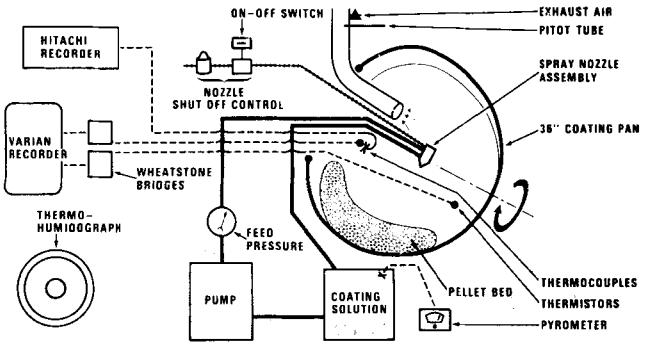


Figure 1—*Equipment diagram.*

EXPERIMENTAL

Equipment and Instrumentation—The coating process was performed in a Stokes 91.4-cm. (36-in.) coating pan.[1] The volume of air moving across and through the pellet bed was monitored at the exhaust with a Dwyer pitot tube and an air velocity meter No. 400.[2] The temperature of the coating solutions was controlled in a 30-l., electrically heated, feed tank. The feed tank temperature was monitored with an Alnor pyrometer[3] coupled to a standard

[1] Model 900-1-8, Pennwalt Corp., Stokes Tableting Equipment Dept., Warminister, Pa.

[2] Dwyer Manufacturing Co., Michigan City, Ind.

[3] Type 1200, Illinois Testing Laboratories, Inc., Chicago, Ill.

coating process should there be a failure in one component of the systems such as in the spray delivery timer. Even though timed devices may contain fail-safe mechanisms, such a timed coating process would have to be terminated and reprogrammed manually.

SUMMARY

An automated tablet-coating system was designed which is self-programming and based on the rate of moisture loss. The Moisture Analyzer, which programmed the system, was revealed to be extremely sensitive to the presence of moisture and to moisture loss in the tablet mass. The system quickly adjusts to any change in spray cycle time and also shows a high degree of reproducibility between cycles under similar conditions.

A high degree of sensitivity of the drying cycles to changes in the environmental humidity was also demonstrated by the apparatus. It is, therefore, felt that a moisture-sensing device should be utilized when considering an automated tablet-coating system for production.

REFERENCES

(1) D. E. Wurster, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 451(1959).

(2) R. E. Singiser and W. Lowenthal, *J. Pharm. Sci.*, **50**, 168 (1961).

(3) L. Lachman and J. Cooper, *ibid.*, **52**, 490(1963).

(4) D. S. Mody, M. W. Scott, and H. A. Lieberman, *ibid.*, **53**, 949(1964).

(5) J. Spaulding, *Drug Cosmet. Ind.*, **79**, 766(1956).

(6) G. M. Krause and T. L. Iorio, *J. Pharm. Sci.*, **57**, 1223(1968).

(7) P. S. Miller and J. J. Jones, *Proc. Sci. Sec. Toilet Goods Ass.*, No. 31, May 1959.

(8) "The United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 939.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 30, 1970, from the *Department of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CN 06268,* and *Columbia University College of Pharmaceutical Sciences, New York, NY 10023*

Accepted for publication March 23, 1970.

The experimental portions of this investigation were conducted in the research laboratories of Columbia University College of Pharmaceutical Sciences.

The authors thank Dr. Milo Gibaldi for his suggestions and helpful discussions during this investigation.

Monitoring Volatile Coating Solution Applications in a Coating Pan

RUSSELL J. LANTZ, Jr.*, ALAN BAILEY, and MANFORD J. ROBINSON

Abstract □ Equipment and methods are presented for recording temperature patterns resulting from the evaporation of volatile coating solutions applied to pellets in a rotating pan. These patterns are interpreted in regard to run-to-run replication, effect of application and drying rate, and coating solution distribution. The applicability of this equipment and methodology to control the coating of solid particles is discussed.

Keyphrases □ Coating application, pellets—monitoring method □ Volatile coating solutions—application monitoring □ Diagram—pellet coating equipment with monitor □ Thermal patterns—pellet coating

Sutaria (1) has noted that, until recently, pharmaceutical coating processes were an art because of the apathy shown toward studying the many variables involved in these processes. Sutaria's extensive bibliography indicates that efforts to define and control these variables are now underway and, in some cases, have resulted in the semiautomation of certain aspects of the coating process.

The purpose of this preliminary report is to add to this knowledge by presenting data on methods found useful for monitoring the application of volatile coating solutions to pellets in a rotating pan.

The application and evaporation of coating solutions containing volatile solvents, *e.g.*, acetone, alcohol, and chloroform, produce measurable temperature changes in a bed of pellets in a rotating pan. By placing a thermocouple and thermistors in the pellet bed and recording the temperature changes during coating, it is possible to discern and control certain events and/or trends taking place during the coating process.

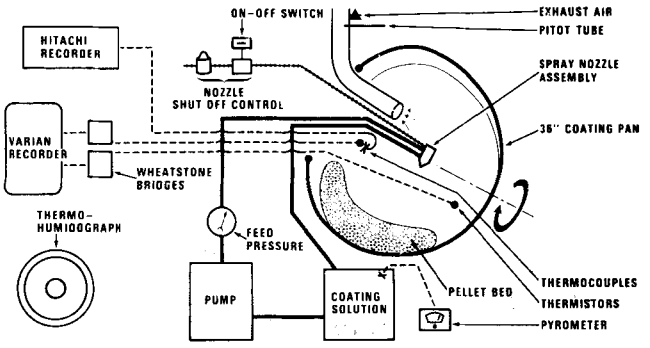


Figure 1—*Equipment diagram.*

EXPERIMENTAL

Equipment and Instrumentation—The coating process was performed in a Stokes 91.4-cm. (36-in.) coating pan.[1] The volume of air moving across and through the pellet bed was monitored at the exhaust with a Dwyer pitot tube and an air velocity meter No. 400.[2] The temperature of the coating solutions was controlled in a 30-l., electrically heated, feed tank. The feed tank temperature was monitored with an Alnor pyrometer[3] coupled to a standard

[1] Model 900-1-8, Pennwalt Corp., Stokes Tableting Equipment Dept., Warminister, Pa.

[2] Dwyer Manufacturing Co., Michigan City, Ind.

[3] Type 1200, Illinois Testing Laboratories, Inc., Chicago, Ill.

Table I—Data Sheet Sample of Conditions Recorded in Addition to Thermal Patterns

Code 20 Time	Pellet-Bed Temp.	Exhaust Air Flow, c.f.m.	Ambient Conditions: Dry Bulb Temp.	Ambient Conditions: % Relative Humidity	Pump Operation and Coating Solution Application: Pump Driving Air, psig.	Pump Operation and Coating Solution Application: Pump Liquid Pressure, psig.	Pump Operation and Coating Solution Application: Coating Solution Applied per Hour, kg.	Pump Operation and Coating Solution Application: Coating Solution Spray Feed Rate, g./min.	Pump Operation and Coating Solution Application: Coating Solution Temp.
10:00 a.m.	15°	200	27.7° (82° F)	31	10	130	4.9	354	67°
11:00 a.m.	15°	211	27.7° (82° F)	31	10	130	4.9	346	68°
12:00 noon	14.5°	211	26.6° (80° F)	33	10	130	4.9	354	64°
1:00 p.m.	14.5°	211	26.6° (80° F)	31	10	130	4.9	344	64°
2:00 p.m.	14.5°	199	26.1° (79° F)	34	10	130	4.9	356	63°
3:00 p.m.	14.5°	200	26.1° (79° F)	34	10	130	4.9	358	65°
4:00 p.m.	15.5°	205	26.1° (79° F)	34	10	125	4.6	355	64°

Table II—Program for Coating Solution Application and Pellet Drying

Code 20 Time	No. of Cycles	Spray Cycle, min.	Dry Cycle, min.	Total Spraying per Hour, min.	Total Amount of Coating Solution Applied per Hour, kg. (Theory)
1st hour	5	2	2	10	3.50
	10	1	3	10	3.50
2nd hour	15	1	3	15	5.25
3rd and 4th hours	15	1	3	15	5.25
5th hour	12	1	4	12	4.2

iron-constantan thermocouple. The coating solutions were pumped by a Grover pump,[4] which delivered 130 psig. pressure to a No. 650067 Spraying Systems[5] T-Jet nozzle mounted on a Spraying Systems 24 AU Auto Jet valve assembly.[5] The spraying and drying cycles were timed with a stopwatch, and the "start" and "stop" of the spray nozzle were controlled by an on-off switch in series with a Skinner electric valve.[6] Ambient conditions were recorded with a Bristol thermo-humidograph.[7]

An Hitachi 165 recorder[8] fitted with a standard iron-constantan thermocouple, placed 3.8 cm. (1.5 in.) deep in the pellet bed and 17.9 cm. (7 in.) in from the pan rim, was used to record pellet bed temperature at this point. The recorder temperature range was from 4 to 21°.

A second recorder,[9] with two type A-21 amplifiers, was used to amplify the signals from two thermistors[10] (nominal resistance: 2000 ohms at 25°). To obtain a range of 13–16° with each amplifier, the thermistors were placed in one arm of a standard Wheatstone bridge, where all arms were at equal resistance at 14°. One thermistor was placed beside the iron-constantan thermocouple, while the second thermistor was moved to different points on the pellet-bed surface. The two separate recording setups were used to give the advantage of the wide temperature range coverage of one recorder and the detailed or expanded scale of a small temperature range recorder, and to provide a reference check of one recorder against the other.

Figure 1 shows the equipment setup.

Materials—Sugar pellets, 16–30 mesh, were used for each coating run. Special denatured No. 30 alcohol was the volatile solvent used in preparing the coating solution.

Procedure—For each coating run, a 50-kg. charge of pellets was placed in the coating pan preset to rotate at 20 r.p.m. The coating solution was prepared, heated, and maintained at 65 ± 3°. A 2-min. spray and 2-min. dry cycle was used until the pellets were wetted and the bed temperature dropped to approximately 15°. The time cycle was then changed to a 1-min. spray and a 3-min. drying period, or a 1-min. spray and a 4-min. drying period, to maintain an equilibrium between the amount of coating solution applied and the rate of evaporation. A sample data sheet and program for coating solution application and pellet drying are seen in Tables I and II, respectively.

Some coating runs were made under near identical conditions to determine if identical thermal patterns could be obtained. In other runs, coating application rates and pellet drying times were varied. The temperature profile across the pellet bed and the circulation of coating solution to the back of the coating pan were also determined. Sixteen coating runs were made to accumulate and recheck the data obtained.

RESULTS AND DISCUSSION

Typical thermal patterns, taken at the same point in the pellet bed with the thermocouple and thermistor setups, are shown in Fig. 2. These identical curves show good uniformity from cycle to cycle in the temperature rise that accompanies the heated coating solution application and the evaporative cooling which occurs during the drying period. The range between the maximum and minimum temperature usually averaged 0.3–0.4° within a normal spray and dry cycle (the lower curve).

Coating runs performed on different days, following nearly identical conditions and procedures, show almost identical thermal traces throughout each run. This is illustrated in Fig. 3, where portions of the trace of each run are shown early and late in the coating process. A comparison of the thermal patterns made early in the run with those made late in the run shows a difference in the heating portion of the curve, which represents the coating solution application. It is possible that the leveling off or temperature decrease at one point in the heating curve may be caused by a partial dissolution of the heavier coating accumulated late in the run as new coating solution is applied. This effect is shown more clearly in an expanded thermal trace of one cycle in Fig. 4.

It was found that a variation in coating solution application rates and/or pellet drying times caused distinguishable differences in the

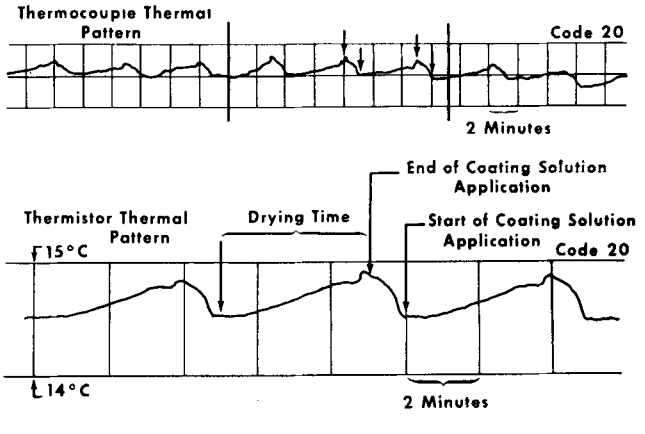


Figure 2—*Typical patterns obtained with thermocouple and thermistor probes.*

[4] Model 2585 TSS, Grover, Montebello, Calif.
[5] Spraying Systems Co., Bellwood, Ill.
[6] Skinner Electric Valve Division, New Britain, Conn.
[7] Bristol Co., Waterbury, Conn.
[8] Hitachi Ltd., Tokyo, Japan.
[9] Model G22, Varian Associates, Palo Alto, Calif.
[10] Fenwal type GB 32 P8, Fenwal Electronics, Inc., Framingham, Mass.

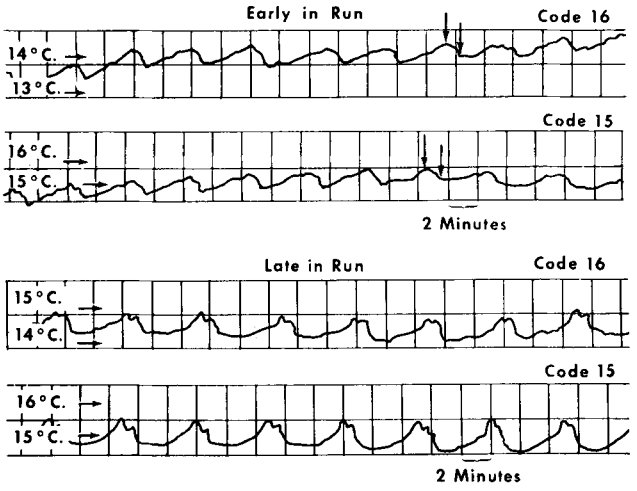


Figure 3—*Run-to-run duplication of thermal patterns.*

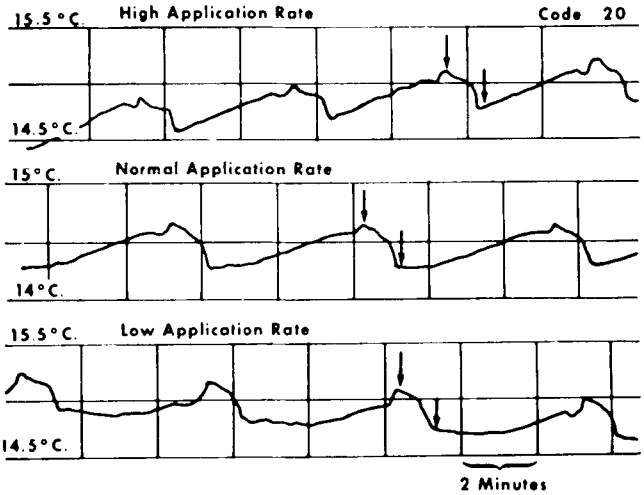


Figure 5—*Effects of coating solution application rate and drying.*

pellet-bed thermal patterns. In Fig. 5 the upper and lower thermal traces show the effect of high and low coating solution application rates, and the middle thermal trace represents a more normal application and drying rate. High application rates of coating solution result in a continuous decrease in temperature during drying, with an overall downward trend in pellet-bed temperature; the low application rate of coating solution shows just the opposite effect. The normal application rate curve illustrates a system in equilibrium, in which the coating solution solvent application rate balances the solvent evaporation rate with no upward or downward trend in pellet-bed temperature.

If the pellets are subjected to an extended period of drying, or if only small amounts of coating solution are applied, two stages of drying, namely the "constant rate period" and the "falling rate period" (2, 3), are revealed by the thermal traces of these pellet beds. In the constant rate period, unbound solvent is removed from a saturated surface at a constant rate under constant conditions. The second stage or falling rate period proceeds after the critical solvent level has been reached,[11] and it is controlled by the internal movement of the solvent to the surface of the pellets. This stage is characterized by a diminishing solvent evaporation rate under constant conditions. In Fig. 6, the constant rate period is shown as a constant decrease in pellet-bed temperature, while the second stage or falling rate period is shown as a gradual increase in pellet-bed temperature.

The distribution of coating solution in a rotating pan is always of concern, because of the poor product circulation patterns set up in some coating pans, including those with improper baffle designs. Improper positioning of the spray nozzle can also result in maldistribution of the coating solution. A means of evaluating coating solution distribution in a rotating pellet bed is illustrated in Figs. 7–9. Figure 8 shows four pellet-bed surface thermal patterns taken at points shown in Fig. 7. Assuming that the magnitude of pellet-bed temperature change is an indication of the amount of coating solution present, it is evident that Position B, just forward of the nozzle, E, receives the most coating solution, followed by Position C just behind the nozzle. Position A receives the third highest quantity of coating solution, while Position D, at the back of the coating pan, receives the least amount. Thermal patterns in Fig. 9, taken at the rear of this particular coating pan in Position D (Fig. 7), indicate that the amount of coating solution reaching this area depends in part on the coating solution application rate.

Measurement of temperature changes should be applicable to any pan coating process (including the coating of pellets and granules and the film coating of tablets) when coatings containing volatile solvents are used. Records of thermal patterns may be particularly useful in determining the day-to-day variability of one coating operator and/or the operator-to-operator variability for a single coating process.

Information which could reduce operator variability can be obtained, in part, from such a series of thermal pattern recordings. This technique may also turn out to be useful in coating process automation. Recording the thermal changes on magnetic tape and running a "playback" of the tape with proper circuitry and control[12] should permit automatic regulation of the pan coating process.

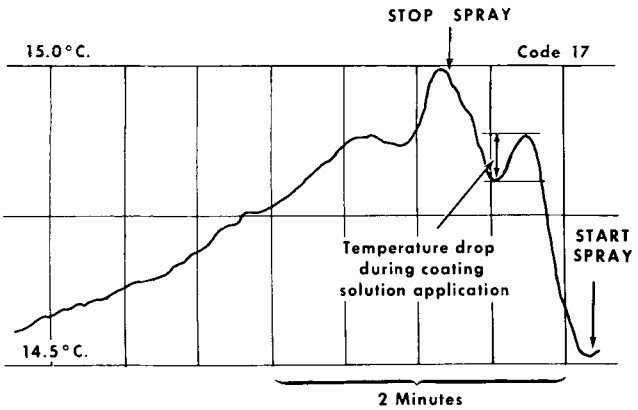


Figure 4—*Temperature drop during coating solution application.*

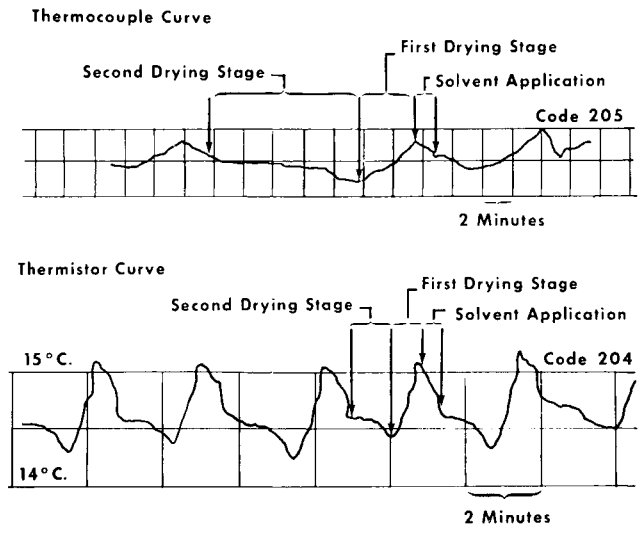


Figure 6—*Thermal patterns indicating two stages of drying.*

[11] The point where the surface no longer remains saturated.

[12] This technique is more feasible if raw materials and ambient conditions can be held constant during the coating of each batch of solid particles.

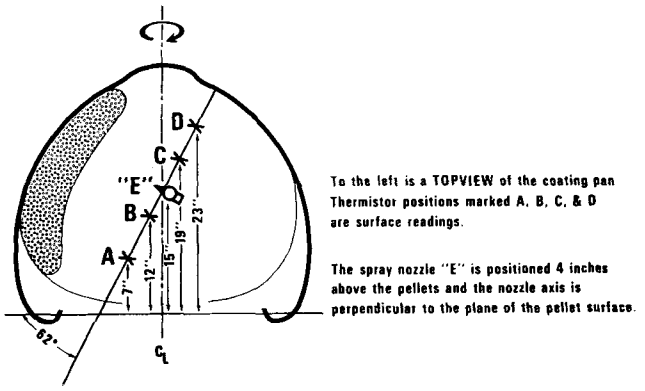


Figure 7—*Nozzle and thermistor positions in the coating pan.*

The recording of temperature profiles across a bed of solid particles during coating may also be correlated with final product results to help in evaluating the design of coating pans and/or baffles for a particular product. This same technique should also be useful in selecting the proper spray nozzle design and determining its position in a coating pan.

SUMMARY

This preliminary report describes a method for measuring temperature changes encountered during the application of volatile coating solutions to pellets in a rotating pan. These temperature changes develop characteristic patterns during the periods of coating solution application and pellet drying, and these patterns are replicable. Distinguishable differences in thermal patterns were found when coating solution application rates and pellet drying times were varied. Thermal traces from extended pellet drying times revealed two stages of drying. Temperature changes recorded at several points on the pellet-bed surface demonstrated differences in coating solution distribution to the pellets.

This method of measuring solid particle-bed temperature changes in a rotating pan should be applicable to the control of day-to-day and operator-to-operator variability; to the automation of the coating process; and to an evaluation of the design of coating pans, baffles, and auxiliary equipment.

Correlations between changes in pan coating conditions and their effects on final product characteristics using this technique will be considered in another paper.

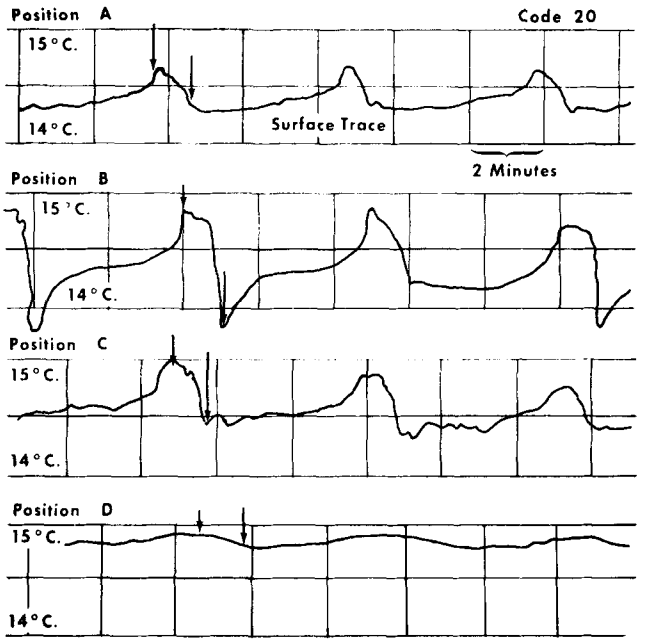


Figure 8—*Distribution of coating solution in the pellet bed.*

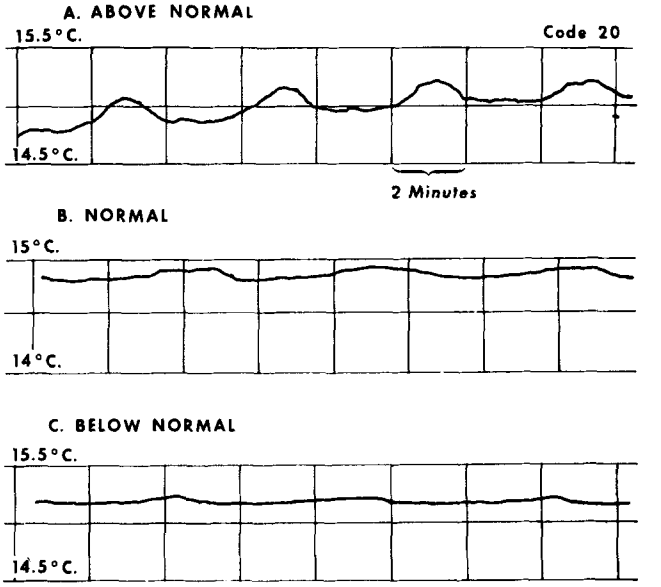


Figure 9—*Coating solution circulation to the back of the coating pan.*

REFERENCES

(1) R. H. Sutaria, *Mfg. Chem. Aerosol News*, **39**, 37(1968).

(2) M. W. Scott, H. A. Lieberman, A. S. Rankell, F. S. Chow, and G. W. Johnston, *J. Pharm. Sci.*, **52**, 284(1963).

(3) R. H. Perry, C. H. Chilton, and S. D. Kirkpatrick, "Perry's Chemical Engineers' Handbook," 4th ed., McGraw-Hill, New York, N. Y., 1963, pp. 15–50.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 11, 1970, from the *Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101*

Accepted for publication April 1, 1970.

Presented in part at the 62nd Annual Meeting of the A.I.Ch.E., Washington, D. C., November 20, 1969.

* Present address: Endo Laboratories, Inc., Garden City, NY 11530

Enhancement of Radioresistance in Mice Treated with Diphenylhydantoin

H. LEVAN*, P. GORDON†, and S. STEFANI*

Abstract □ Diphenylhydantoin, a pharmaceutical compound of the same chemical class as magnesium pemoline, is found to exhibit significant protection against ionizing radiations. It is suggested that the mechanism of the radioprotective action of diphenylhydantoin is related to its influence on nucleic acid metabolism rather than to its anticonvulsant effects on the brain.

Keyphrases □ Diphenylhydantoin—radioresistance enhancement, mice □ Radiation protection—diphenylhydantoin effect, mice □ Irradiation, mice—enhancement of lifespan, diphenylhydantoin effect

Recently, the radioprotective effect of magnesium pemoline, a central nervous system (CNS) stimulant (1–4), has been investigated and reported from these laboratories. These encouraging results led to the studies of other compounds of the same chemical class as magnesium pemoline, among these diphenylhydantoin (DPH). Although pemoline and DPH do not have similar pharmacological actions on the most commonly employed screening system, pemoline is, in fact, 5-phenylpseudohydantoin and has been reported to exert effects similar to DPH and other phenylhydantoins on aspects of learning behavior in the rat and on the enzyme system DNase 1 (5, 6). Because DPH is a relatively nontoxic drug currently in use, while magnesium pemoline still falls in the category of an experimental drug of restricted use, it was felt that the exploration for radioprotective effects exerted by DPH, similar to those of pemoline, might have both theoretical and practical value. In this report, the enhancement of the lifespan of irradiated mice treated with DPH is reported.

METHOD

CF_1 male mice, 50–60 days old, were used. The animals were housed in air-conditioned quarters and had free access to food and water. The experiments started about 10 days after their arrival from the supplier (Carworth Farm). Three hundred mice were divided randomly into two groups. Each group was then divided into 15 cages of 10 animals per cage. A second control group of 60 untreated mice was also used to observe possible pseudomonas infection. The experimental group was treated with 3 mg./kg. of DPH, intraperitoneal route, approximately 15–20 min. before 750 r whole-body X-irradiation; the control group was treated with a 0.3% suspension of tragacanth. Tragacanth was also used to prepare the DPH solution.

Irradiation was carried out with a Maxitron 400 kvp. X-ray machine at 80-cm. target-skin-distance (TSD) and an average exposure rate of 80 r/min. The TSD was determined by measuring the distance between the target and the horizontal level in the cage where the backs of the mice were exposed. The animals were not free to move around in the cage during irradiation. Total exposure was measured by a Victoreen Condenser R-Meter placed in the cage used to irradiate the animals. A turntable rotating at 5 r.p.m. was used to ensure uniform distribution of the radiation field. Less than 3% difference in the total exposure received by the two groups was observed.

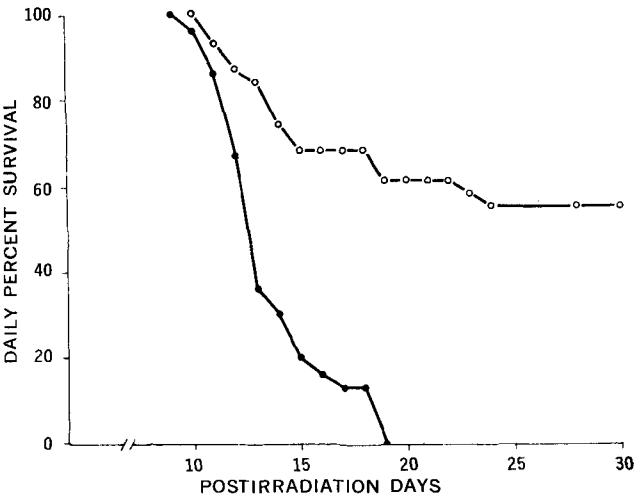


Figure 1—*Survival curves for two groups of CF_1 mice treated with DPH and tragacanth and exposed to 750 r of X-irradiation. Key:* ●—●, *control (tragacanth); and* ○—○, *experimental (3 mg./kg. DPH).*

The animals spent their postirradiation days in the same cage placed at the same position as before irradiation. Cautious measures were taken to assure minimal changes in the environmental factors between preirradiation and postirradiation periods. The experiment was replicated consecutively three times so that a total of 900 mice was used. Mortality was observed up to 30 days for each cage, and the total results were averaged for each of the two groups from all three experimental runs. Statistical analysis of the three combined experimental results was done according to Mewissen (7). The data plotted were thus based on the mortality of 900 animals.

RESULTS AND DISCUSSION

No unusual symptoms or sickness were observed in the 60 untreated, nonirradiated control mice. Figure 1 clearly demonstrates a prolongation of survival of mice treated with DPH. All mortalities in the control group occurred within 10 days. By the 19th day after radiation exposure, all animals in this group had died while over 60% of the animals treated with DPH still survived. Over a period of 2 weeks, the survival in the experimental group decreased from 100 to 55%, and no further mortality was observed after the 24th postirradiation day. Most animals in both groups died between the 11th and 14th day after radiation exposure. The highest mortality of the control group was 30%, 13 days after irradiation, *versus* 13% for the DPH-treated group 12 days following radiation exposure.

Although Laird and Fonner (8) have reported a protective effect of DPH in the hyperacute radiation syndrome wherein death is from convulsion, two aspects of the present experimental design required that the authors postulate a mechanism of action for DPH in this experiment employing X-irradiation at 750 r that is distinct from its classical anticonvulsant effect. The exposure to radiation

employed is one where death is delayed for more than 1 week and is ascribable to hematopoietic depression rather than to CNS effects. Mortality in the experiments of Laird and Fonner occurred following exposures in the 10,000-r range 2–3 days postirradiation and was associated with convulsions, which was not the case in this experiment. Furthermore, DPH was given in this experiment in a single injection at a dose level one-fourth the anticonvulsant dose in mice (9) and would certainly not be expected to exert any anticonvulsant activity 10 days following drug administration.

Potentially important nonanticonvulsant actions of the DPH drug group have been identified by Gordon *et al.* and include antileukemic activity (5) and enhancement of the deteriorated learning and memory characteristic of very old animals (10, 11). Furthermore, both DPH and pemoline have been identified by this group as markedly potentiating the activity of the enzyme DNase 1, while DPH has been found by Shafer (12) to increase markedly the turnover of DNA in normal liver. Thus, DPH may exert the radioprotective action reported here by effects on nucleic acid metabolism, which are in no simple way related to its anticonvulsant action on the brain.

Continuing investigations will include exploring the capacity of DPH to exert radioprotective effects when given after radiation because such an effect for DPH has been observed in these laboratories when it is given within the 1st hour. Further, in a series of preliminary experiments, DPH exerted an effect similar to that of pemoline (5-phenylpseudohydantoin) in altering the growth pattern of Ehrlich carcinoma while prolonging the lifespan of tumor-bearing animals (13–15). The fact that DPH and pemoline have a similar chemical structure and exert similar effects on a nucleic acid-metabolizing enzyme suggests the link between biological effects shared by these two compounds.

REFERENCES

(1) H. LeVan, *Experientia*, **23**, 1058(1967).

(2) H. LeVan and D. L. Hebron, *J. Pharm. Sci.*, **57**, 1033(1968).

(3) H. LeVan, *Experientia*, **24**, 477(1968).

(4) H. LeVan, *Int. J. Clin. Pharm. Ther. Toxicol.*, **6**, 514(1968).

(5) P. Gordon, J. Agoro, and E. R. Brown, to be published.

(6) P. Gordon, O. Callaghan, and B. Doty, *Pharmacologist*, **10**, 169(1968).

(7) D. J. Mewissen, *Int. J. Appl. Radiat. Isotop.*, **4**, 58(1958).

(8) R. D. Laird and R. L. Fonner, *Report No. 262*, Path. Dept. U. S. Army Medical Research Lab., Fort Knox, Ky., 1957.

(9) C. D. Barnes and L. G. Eltherington, "Drug Dosage in Laboratory Animals," University of California Press, Berkeley, Calif., 1965, p. 185.

(10) P. Gordon, "Recent Advances in Biological Psychiatry," vol. 10, Plenum, New York, N. Y., 1968, p. 121.

(11) P. Gordon, S. S. Tobin, B. Doty, and M. Nash, *J. Gerontol.*, **23**, 434(1968).

(12) A Shafer, *J. Oral Ther. Pharm.*, **2**, 319(1966)

(13) H. LeVan and D. L. Hebron, *Experientia*, **24**, 830(1968).

(14) H. LeVan, P. Burlakow, and D. L. Hebron, *Oncology*, **24**, 181(1970).

(15) H. LeVan and P. Burlakow, *Experientia*, **25**, 973(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 13, 1969, from the * *Department of Therapeutic Radiology, V. A. Hospital, Hines, IL 60141, and Department of Radiology, Chicago Medical School, Chicago, IL 60612*, and the † *Departments of Pharmacology and Microbiology, Chicago Medical School, Chicago, IL 60612*

Accepted for publication February 24, 1970.

This work is supported in part by a grant from the Dreyfus Medical Foundation and in part by a grant from the Leukemia Research Foundation.

Erythrina sp. III: Chemical Constituents of *Erythrina suberosa* Roxb. Seeds

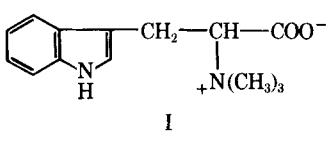
HARKISHAN SINGH and AMRIK SINGH CHAWLA

Abstract □ A phytochemical investigation of *Erythrina suberosa* seeds has resulted in the isolation of erythraline, erysodine, erysotrine, and hypaphorine. This is the first time that erysotrine has been found to occur naturally, although it is well known as a conversion product of other eryso-alkaloids. The alkaloidal constituents were found to vary in different seed collections. The fatty acid composition of the seed oil was examined, and the sterol part from the unsaponifiable matter was found to be composed of sitosterol, stigmasterol, campesterol, and cholesterol.

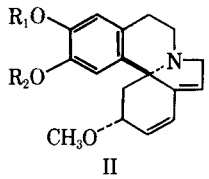
Keyphrases □ *Erythrina suberosa* seeds—phytochemistry, chemical constituents □ Mass spectroscopy—identification □ IR spectrophotometry—identification □ TLC—identification

In India, *Erythrina stricta* Roxb., *E. suberosa* Roxb., and *E. variegata* Linn. var. *orientalis* (Linn.) Merrill (syn. *E. indica* Lam.) have been used in the indigenous system of medicine for various ailments (1).

The authors have started a systematic study of *Erythrina* species growing in India. There have been no earlier reports of chemical investigations of *E. suberosa* and *E. stricta*. Hypaphorine (I) has been isolated from the seeds of *E. variegata* var. *orientalis* (2–6), and Folkers and Koniuszy (6) obtained erythraline



I



II

(II; $R_1,R_2 = -CH_2-$) from the seeds. Subbaratnam (5) isolated a neutral entity, $C_{24}H_{50}O_2$, m.p. 82–84°. The fatty acids present in the seeds have been studied (3, 7). The bark of *E. variegata* var. *orientalis* has been investigated by various workers (3, 8, 9), and preliminary studies on the leaves (3, 10) have been made.

Recently, the present authors fractionated the petroleum ether extract of *E. suberosa* bark into wax esters, alcohols, and acids; alkyl ferulates; and stigmasterol, sitosterol, campesterol, and cholesterol (11). In this paper, results from an investigation on the chem-

employed is one where death is delayed for more than 1 week and is ascribable to hematopoietic depression rather than to CNS effects. Mortality in the experiments of Laird and Fonner occurred following exposures in the 10,000-r range 2–3 days postirradiation and was associated with convulsions, which was not the case in this experiment. Furthermore, DPH was given in this experiment in a single injection at a dose level one-fourth the anticonvulsant dose in mice (9) and would certainly not be expected to exert any anticonvulsant activity 10 days following drug administration.

Potentially important nonanticonvulsant actions of the DPH drug group have been identified by Gordon *et al.* and include antileukemic activity (5) and enhancement of the deteriorated learning and memory characteristic of very old animals (10, 11). Furthermore, both DPH and pemoline have been identified by this group as markedly potentiating the activity of the enzyme DNase 1, while DPH has been found by Shafer (12) to increase markedly the turnover of DNA in normal liver. Thus, DPH may exert the radioprotective action reported here by effects on nucleic acid metabolism, which are in no simple way related to its anticonvulsant action on the brain.

Continuing investigations will include exploring the capacity of DPH to exert radioprotective effects when given after radiation because such an effect for DPH has been observed in these laboratories when it is given within the 1st hour. Further, in a series of preliminary experiments, DPH exerted an effect similar to that of pemoline (5-phenylpseudohydantoin) in altering the growth pattern of Ehrlich carcinoma while prolonging the lifespan of tumor-bearing animals (13–15). The fact that DPH and pemoline have a similar chemical structure and exert similar effects on a nucleic acid-metabolizing enzyme suggests the link between biological effects shared by these two compounds.

REFERENCES

(1) H. LeVan, *Experientia*, **23**, 1058(1967).

(2) H. LeVan and D. L. Hebron, *J. Pharm. Sci.*, **57**, 1033(1968).

(3) H. LeVan, *Experientia*, **24**, 477(1968).

(4) H. LeVan, *Int. J. Clin. Pharm. Ther. Toxicol.*, **6**, 514(1968).

(5) P. Gordon, J. Agoro, and E. R. Brown, to be published.

(6) P. Gordon, O. Callaghan, and B. Doty, *Pharmacologist*, **10**, 169(1968).

(7) D. J. Mewissen, *Int. J. Appl. Radiat. Isotop.*, **4**, 58(1958).

(8) R. D. Laird and R. L. Fonner, *Report No. 262*, Path. Dept. U. S. Army Medical Research Lab., Fort Knox, Ky., 1957.

(9) C. D. Barnes and L. G. Eltherington, "Drug Dosage in Laboratory Animals," University of California Press, Berkeley, Calif., 1965, p. 185.

(10) P. Gordon, "Recent Advances in Biological Psychiatry," vol. 10, Plenum, New York, N. Y., 1968, p. 121.

(11) P. Gordon, S. S. Tobin, B. Doty, and M. Nash, *J. Gerontol.*, **23**, 434(1968).

(12) A Shafer, *J. Oral Ther. Pharm.*, **2**, 319(1966)

(13) H. LeVan and D. L. Hebron, *Experientia*, **24**, 830(1968).

(14) H. LeVan, P. Burlakow, and D. L. Hebron, *Oncology*, **24**, 181(1970).

(15) H. LeVan and P. Burlakow, *Experientia*, **25**, 973(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 13, 1969, from the * *Department of Therapeutic Radiology, V. A. Hospital, Hines, IL 60141, and Department of Radiology, Chicago Medical School, Chicago, IL 60612*, and the † *Departments of Pharmacology and Microbiology, Chicago Medical School, Chicago, IL 60612*

Accepted for publication February 24, 1970.

This work is supported in part by a grant from the Dreyfus Medical Foundation and in part by a grant from the Leukemia Research Foundation.

Erythrina sp. III: Chemical Constituents of *Erythrina suberosa* Roxb. Seeds

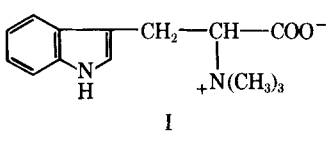
HARKISHAN SINGH and AMRIK SINGH CHAWLA

Abstract □ A phytochemical investigation of *Erythrina suberosa* seeds has resulted in the isolation of erythraline, erysodine, erysotrine, and hypaphorine. This is the first time that erysotrine has been found to occur naturally, although it is well known as a conversion product of other eryso-alkaloids. The alkaloidal constituents were found to vary in different seed collections. The fatty acid composition of the seed oil was examined, and the sterol part from the unsaponifiable matter was found to be composed of sitosterol, stigmasterol, campesterol, and cholesterol.

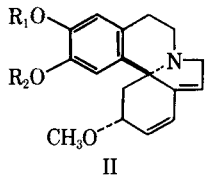
Keyphrases □ *Erythrina suberosa* seeds—phytochemistry, chemical constituents □ Mass spectroscopy—identification □ IR spectrophotometry—identification □ TLC—identification

In India, *Erythrina stricta* Roxb., *E. suberosa* Roxb., and *E. variegata* Linn. var. *orientalis* (Linn.) Merrill (syn. *E. indica* Lam.) have been used in the indigenous system of medicine for various ailments (1).

The authors have started a systematic study of *Erythrina* species growing in India. There have been no earlier reports of chemical investigations of *E. suberosa* and *E. stricta*. Hypaphorine (I) has been isolated from the seeds of *E. variegata* var. *orientalis* (2–6), and Folkers and Koniuszy (6) obtained erythraline



I



II

(II; $R_1,R_2 = -CH_2-$) from the seeds. Subbaratnam (5) isolated a neutral entity, $C_{24}H_{50}O_2$, m.p. 82–84°. The fatty acids present in the seeds have been studied (3, 7). The bark of *E. variegata* var. *orientalis* has been investigated by various workers (3, 8, 9), and preliminary studies on the leaves (3, 10) have been made.

Recently, the present authors fractionated the petroleum ether extract of *E. suberosa* bark into wax esters, alcohols, and acids; alkyl ferulates; and stigmasterol, sitosterol, campesterol, and cholesterol (11). In this paper, results from an investigation on the chem-

Table I—Contents of Fatty Acids Present in *E. suberosa* Seed Oil

Fatty Acid	Percent Weight	Percent Mole
Myristic acid	Traces	
Palmitic acid	12.2	13.4
Stearic acid	2.4	2.4
Oleic acid	38.3	39.2
Linoleic acid	24.7	25.4
Arachidic acid	3.5	3.2
Eicosenoic acid	4.5	4.4
Behenic acid	11.3	9.6
Docosenoic acid	Traces	
Lignoceric acid	3.1	2.4

ical constituents of *E. suberosa* seeds are reported. Three collections, made from Dehradun, Mandi, and Joginder Nagar in India, have been examined; a preliminary communication about the alkaloidal constituents of the seeds has been published (12).

From the powdered seeds, the oil was separated through extraction with petroleum ether. The physical and chemical characteristics of the oil and the chemical characteristics of the separated fatty acids were determined. On spectrophotometric estimation, the oil revealed the presence of 22.9% of diene acids calculated as linoleic acid, 43.7% of mono-ene acids calculated as oleic acid, and 33.4% of saturated fatty acids (found by difference). The fatty acid content in terms of percent weight and percent mole as estimated by GLC of the methyl esters is listed in Table I.

The unsaponifiable matter of the seed oil yielded a sterol material, which the GLC of the trimethylsilyl (TMS) ethers showed to be a mixture of sitosterol (67%), stigmasterol (20%), campesterol (12%), and cholesterol (1%).

The Dehradun seed powder was defatted with petroleum ether and the marc extracted with ethanol. The petroleum ether extract yielded an alkaloidal part (Fraction A). The ethanol extract gave a chloroform-soluble part (Fraction B), which on chromatographic resolution gave a crystalline base identified as erythraline (II; R_1,R_2=—CH_2—). Fraction A, which was resinous, gave erythraline hydriodide. The aqueous alkaline layer remaining after removal of Fraction B from the ethanol extract was acidified, refluxed, and extracted with chloroform to give Fraction C, which did not yield crystalline material. The aqueous layer yielded a water-soluble base identified as hypaphorine (I).

The Mandi seed collection gave Fraction D from the petroleum ether extract, and Fractions E and F from the ethanol extract, representing "free" and "liberated" alkaloidal parts. Fraction D yielded a crystalline hydrochloride which appeared to be erysotrine (II; $R_1 = R_2 = CH_3$) hydrochloride according to elemental and UV data. Fraction F gave crystalline erysodine (II; $R_1 = H$, $R_2 = CH_3$). Contrary to earlier observations (13), it was possible to prepare the hydrochloride of the isolated erysodine. Chromatogaphy of Fraction E led ultimately to crystalline erysotrine hydrochloride and erysodine. A confirmation of the identity of isolated erysotrine hydrochloride came from a mass spectrum of the liberated base from the salt. The base indicated significant peaks at *m/e* 313 (molecular ion M^+; 33% intensity of base peak), 298 (M^+ —CH_3; 35%), and 282 (M^+ —CH_3O; base peak), which compared with the authentic specimen. Hypaphorine was also isolated from the Mandi seeds.

Because of the alkaloidal variations found in the Dehradun and Mandi collections of *E. suberosa* seeds, a third collection, from Joginder Nagar, was investigated. These seeds also yielded the "free" bases erysotrine and erysodine but no erythraline.

The difference in the alkaloidal variations mentioned may be attributed to differences in climatic or soil conditions, and the two plants possibly may belong to different strains. The other aspect of interest is the isolation of erysotrine because never before has erysotrine been found to occur naturally, although it is well known as a conversion product of other erysoalkaloids.

EXPERIMENTAL

Plant Material[1]—The seeds were procured[2] from Dehradun (Uttar Pradesh, India), and also collected from Mandi and Joginder Nagar (Himachal Pradesh, India). The seeds were reduced to moderately coarse powder.

Extractives—The seed powder (Dehradun) (25 g.) was extracted successively in a continuous extraction apparatus, and the percent extractives was determined: petroleum ether (60–80°), 8.10%; benzene, 0.51%; ether, 0.07%; chloroform, 0.64%; acetone, 0.84%; ethanol, 18.20%; and water, 5.30%.

Extraction and Study of Oil—The seed powder (Dehradun) (500 g.) was extracted with petroleum ether (60–80°) for 15 hr. to yield 40 g. of the oil, which had the following characteristics: sp. gr. 25°/25°, 0.9483; n_D^{25} 1.4690; iodine value, 74.6; acid value, 8.6; saponification value, 173; and unsaponifiable matter, 1.6%.

The mixed fatty acid fraction (iodine value, 80.9; saponification equivalent, 281) obtained from the oil was found to have a total diene composition and mono-ene composition of 22.9 and 43.7%, respectively, as determined by the alkali isomerization method of Hilditch *et al.* (14) and using reference values of Hilditch *et al.* (15). By difference the saturated fatty acids come to 33.4%.

The mixed fatty acids were converted to their methyl esters (16). The IR spectrum of the methyl esters showed bands at 2997 cm.$^{-1}$ (olefinic C—H stretching); 2918 and 2846 cm.$^{-1}$ (for CH_2 and CH_3); 1740 cm.$^{-1}$ (C=O stretching); and 1230, 1185, and 1160 cm.$^{-1}$ (C—O stretching for esters).

The methyl esters were analyzed by GLC on a polyester column [20% diethylene glycol succinate on diatomite[3] 2.44 m. (8 ft.) × 0.49 cm. (0.19 in.), 215°] with a thermal conductivity detector. The peak areas were measured by triangulation and the results are given in Table I.

The unsaponifiable part of the seed oil was separated in the usual way. A chromatographic resolution on an alumina column gave a sterol fraction, m.p. 139°. The IR spectrum showed a band at 3440 cm.$^{-1}$ (broad, associated O—H stretching) and a weak but very diagnostic peak at 972 cm.$^{-1}$ for the *trans*-disubstituted double bond of stigmasterol.

The sterol fraction, when resolved as its TMS ether on an SE-30 column [1% on Anakrom SD, 3.04 m. (10 ft.) × 0.32 cm. (0.125 in.), 260°], proved to be a mixture of sitosterol (67%), stigmasterol (20%), campesterol (12%), and cholesterol (1%). A standard mixture of TMS ethers of the corresponding sterols was used for identification of the peaks.[4]

Isolation and Detection of Alkaloids—Dehradun, Mandi, and Joginder Nagar seeds were investigated separately.

[1] The authenticity of the seeds was certified by Dr. T. S. Sareen, Curator, Department of Botany, Panjab University.
[2] Through M/s Pratap Nursery and Seed Stores, Dehradum.
[3] Chromosorb W, Johns-Manville.
[4] These data were obtained through Dr. J. W. Rowe, USDA Forest Products Laboratory, Madison, Wis.

Dehradun Seeds—The seed powder (2.0 kg.) was extracted with petroleum ether (60–80°) in a continuous extraction apparatus. The basic components were removed from the petroleum ether extract by shaking with 2% w/v sulfuric acid. The acid layer (1 l.) was made alkaline with a dilute ammonia solution (150 ml.) and extracted with chloroform. The chloroform extract was worked up to give a residue (0.93 g., Fraction A).

The marc was extracted with ethanol in a continuous extraction apparatus. The residue (300 g.) remaining after evaporation of the solvent was repeatedly treated with 2% w/v sulfuric acid. The acid layer (2 l.) was washed with chloroform (3 × 300 ml.) to remove the nonalkaloidal matter. The acid extract was made alkaline with a dilute ammonia solution (350 ml.) and extracted with chloroform (10 × 300 ml.). The chloroform extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to yield a residue (5.7 g., Fraction B).

Fraction B was chromatographed over alumina (Merck, 170 g.). Elution with benzene gave a sticky solid mass (3.0 g.), which was repeatedly crystallized from absolute ethanol to give a base, m.p. 111–113° [lit. (6) erythraline, m.p. 106–107°].

Anal.—Calcd. for $C_{18}H_{19}NO_3$: N, 4.71. Found: N, 5.11. $[\alpha]_D^{20}$ + 192.5° (c, 0.78, $CHCl_3$) [lit. (6) $[\alpha]_D^{27}$ + 211.8° (c, 0.944, EtOH)]; UV $\lambda_{max.}^{MeOH}$ 292 mμ (log ε 3.6) [lit. (17) 292 mμ (log ε 3.6)].

The base showed no depression on determining mixed melting point with authentic erythraline. The base formed a hydrobromide, m.p. 250–251° dec. [lit. (17) erythraline hydrobromide, m.p. 246°].

Anal.—Calcd. for $C_{18}H_{19}NO_3 \cdot HBr$: C, 57.16; H, 5.33; Br, 21.13; N, 3.70. Found: C, 57.47; H, 5.30; Br, 21.25; N, 3.56. UV $\lambda_{max.}^{MeOH}$ 290 mμ (log ε 3.6). The IR spectra of the base hydrobromide and authentic erythraline hydrobromide were identical.

The hydrochloride of the base was prepared in the usual way and crystallized from dry ether–absolute ethanol to give an entity, m.p. 251–252° dec.

Anal.—Calcd. for $C_{18}H_{19}NO_3 \cdot HCl$: C, 64.79; H, 6.04; Cl, 10.63; N, 4.20. Found: C, 64.66; H, 6.64; Cl, 10.80; N, 4.19.

Fraction A was not resolvable on an alumina column. Its hydriodide, m.p. 247–248° dec., was prepared and showed no depression in melting point on admixture with the hydriodide of erythraline isolated from Fraction B [lit. (6) erythraline hydriodide, m.p. 252–253° dec.]. The IR spectra were also superimposable.

A portion of the aqueous layer left after removal of Fraction B was acidified with dilute sulfuric acid to pH 1.0. It was refluxed for 3 hr., made alkaline with dilute ammonia solution, and extracted with chloroform (5 × 200 ml.). The yield of the residue, after complete solvent removal, was 0.8 g. (Fraction C). It was chromatographed over alumina, but no pure base or salt could be isolated.

The other portion of the aqueous layer was acidified and precipitated with Dragendorff's reagent. A part of the separated precipitate (35 g.) was decomposed with moist silver carbonate (15 g.) and processed to obtain a crystalline material. It was recrystallized from ethanol to give a base, m.p. 250° dec. [lit. (18) hypaphorine, m.p. 253–254°].

Anal.—Calcd. for $C_{14}H_{18}N_2O_2$: C, 68.27; H, 7.37; N, 11.37. Found: C, 67.81; H, 7.11; N, 11.10. The base showed no depression on determining mixed melting point with authentic hypaphorine, and the IR spectra of both were identical.

The base formed a hydrochloride, m.p. 231–232° dec. [lit. (19) hypaphorine hydrochloride, m.p. 234–235°].

Anal.—Calcd. for $C_{14}H_{18}N_2O_2 \cdot HCl$: C, 59.48; H, 6.77; Cl, 12.54; N, 9.91. Found: C, 59.10; H, 6.63; Cl, 12.42; N, 9.80.

The hydrobromide, m.p. 233° dec. [lit. (20) hypaphorine hydrobromide, m.p. 225°], and nitrate, m.p. 221–222° dec. [lit. (18) hypaphorine nitrate, m.p. 223.5–224.5°], of the base were also prepared.

Mandi Seeds—The seed powder (4.8 kg.) was extracted with petroleum ether (60–80°) and the extract was worked as described previously to give an alkaloidal entity (2.5 g., Fraction D). The marc was extracted with ethanol, and the extract was processed to give alkaloidal Fraction E (14 g.).

A benzene-soluble portion (11 g.) of Fraction E was resolved on an alumina column (330 g.). Elution with benzene first gave a syrupy mass (3.0 g.) and then a solid residue (1.5 g.) which crystallized from acetone to give a base, m.p. 208–210° [lit. (13) erysodine, m.p. 204–205°].

Anal.—Calcd. for $C_{18}H_{21}NO_3$: C, 72.22; H, 7.07; N, 4.68. Found: C, 72.34; H, 6.95; N, 4.77. $[\alpha]_D^{20}$ + 220.8° (c, 0.98, $CHCl_3$) [lit. (13) $[\alpha]_D^{27}$ + 248° (c, 0.311, EtOH)], UV $\lambda_{max.}^{MeOH}$ 285 mμ (log ε 3.6) [lit. (17) 285 mμ (log ε 3.6)]. The base showed no depression on determining mixed melting point with authentic erysodine. A mixture with the latter was inseparable on TLC, and the IR spectra were identical.

The hydrochloride was prepared by treating absolute ethanolic solution of the base with ethanolic hydrochloric acid. It was crystallized from dry ether–absolute ethanol to give an entity, m.p. 217–218° dec.

Anal.—Calcd. for $C_{18}H_{21}NO_3 \cdot HCl$: C, 64.40; H, 6.61; Cl, 10.56; N, 4.17. Found: C, 64.66; H, 6.68; Cl, 11.02; N, 4.30.

The syrupy mass was picked up with dry ether and made slightly acidic with ethanolic hydrochloric acid. The separated mass was repeatedly crystallized from dry ether–absolute ethanol to give a product, m.p. 206–208° [lit. (21) erysotrine hydrochloride, m.p. 205–206°].

Anal.—Calcd. for $C_{19}H_{23}NO_3 \cdot HCl$: C, 65.22; H, 6.91; Cl, 10.14; N, 4.01. Found: C, 65.34; H, 7.26; Cl, 10.30; N, 4.02. UV $\lambda_{max.}^{EtOH}$ 232 mμ (ε 19,800) and 284 mμ (ε 3490).

A part of the aqueous layer left after separation of alkaloidal Fraction E was hydrolyzed and worked as for Fraction C to obtain 2.1 g. (Fraction F) of the "liberated" base. The latter was resolved over alumina (53 g.). Elution with chloroform yielded a solid residue (1.1 g.), which crystallized from acetone to give a base, m.p. 208–210°, identified as erysodine.

Anal.—Calcd. for $C_{18}H_{21}NO_3$: C, 72.22; H, 7.07; N, 4.68. Found: C, 72.46; H, 7.04; N, 4.90.

The aqueous layer left after the removal of the "free" (Fraction E) and "liberated" (Fraction F) bases gave hypaphorine when worked in the usual way.

Fraction D (2.5 g.) was chromatographed over alumina (75 g.). Elution with benzene gave a syrupy mass (1.25 g.), which gave an alkaloidal hydrochloride, m.p. 203–204°, identified as erysotrine hydrochloride.

Anal.—Calcd. for $C_{19}H_{23}NO_3 \cdot HCl$: C, 65.22; H, 6.91; N, 4.01. Found: C, 65.24; H, 6.93; N, 3.90.

Joginder Nagar Seeds—This collection, when worked in the same manner as Mandi seeds, led also to the isolation of erysodine and erysotrine, the latter obtained as the hydrochloride.

The isolated bases and salts were identified by comparison with authentic specimens.[5]

REFERENCES

(1) R. N. Chopra, S. L. Nayar, and I. C. Chopra, "Glossary of Indian Medicinal Plants," Council of Scientific & Industrial Research, New Delhi, India, 1956, p. 111.

(2) J. Marañon and J. K. Santos, *Philip. J. Sci.*, **48,** 563(1932); through *Chem. Abstr.*, **26,** 5609(1932).

(3) P. S. Rao, C. V. Rao, and T. R. Seshadri, *Proc. Indian Acad. Sci.*, **7A,** 179(1938).

(4) J. V. Rao, *Curr. Sci.*, **14,** 198(1945).

(5) A. V. Subbaratnam, *J. Sci. Ind. Res., Sect. B,* **15,** 210 (1956).

(6) K. Folkers and F. Koniuszy, *J. Amer. Chem. Soc.*, **62,** 436(1940).

(7) S. P. Pathak and L. M. Dey, *J. Sci. Food Agr.*, **7,** 200(1956).

(8) R. N. Chopra, S. Ghosh, and B. N. Sen, *Indian J. Med. Res.*, **22,** 265(1934).

(9) D. S. Bhakuni and N. M. Khanna, *J. Sci. Ind. Res., Sect. B,* **18,** 494(1959).

(10) S. N. Chakravarti, M. L. Sitaraman, and A. Venkatasubban, *J. Annamalai Univ.*, **2,** 238(1933); through *Chem. Abstr.*, **28,** 1470(1934).

(11) H. Singh, A. S. Chawla, J. W. Rowe, and J. K. Toda, to be published.

(12) H. Singh and A. S. Chawla, *Experientia*, **25,** 785(1969).

(13) K. Folkers and F. Koniuszy, *J. Amer. Chem. Soc.*, **62,** 1677(1940).

(14) T. P. Hilditch, R. A. Morton, and J. P. Riley, *Analyst*, **70,** 68(1945).

(15) T. P. Hilditch, C. B. Patel, and J. P. Riley, *ibid.*, **76,** 81 (1951).

(16) T. P. Hilditch and P. N. Williams, "The Chemical Con-

[5] Through the courtesy of Professor D. H. R. Barton and R. B. Boar, Imperial College of Science and Technology, London, England.

stitution of Natural Fats," 4th ed., Chapman & Hall, London, England, 1964, p. 688.

(17) V. Prelog, K. Wiesner, H. G. Khorana, and G. W. Kenner, *Helv. Chim. Acta*, **32**, 453(1949).

(18) K. Folkers and F. Koniuszy, *J. Amer. Chem. Soc.*, **61**, 1232(1939).

(19) V. Deulofeu, E. Hug, and P. Mazzocco, *J. Chem. Soc.*, **1939**, 1841.

(20) "Dictionary of Organic Compounds," vol. 2, I. Heilbron and H. M. Bunbury, Eds., Eyre & Spottiswoode, London, England, 1953, p. 843.

(21) R. A. Labriola, V. Deulofeu, and B. Berinzaghi, *J. Org. Chem.*, **16**, 90(1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 2, 1970, from the *Department of Pharmaceutical Sciences, Panjab University, Chandigarh-14, India.*

Accepted for publication March 13, 1970.

Abstracted in part from a thesis submitted by Amrik Singh Chawla to the Panjab University in partial fulfilment of Doctor of Philosophy degree requirements.

This work was supported by the University Grants Commission, India.

NMR Analysis of Some Alkyl *p*-Hydroxybenzoates

F. SHIHAB, W. SHEFFIELD, J. SPROWLS, and J. NEMATOLLAHI*

Abstract □ By applying NMR spectrometry, a number of solutions containing unknown quantities of two or three alkyl *p*-hydroxybenzoates (parabens) in 60% polyethylene glycol in water were analyzed for quantification of the individual parabens. The method was found to be accurate, facile, and rapid and seems to possess a promising potential for applicability in related fields of pharmaceutical analysis.

Keyphrases □ Parabens—NMR analysis □ Alkyl *p*-hydroxybenzoates—NMR analysis □ NMR spectroscopy—analysis

The antibacterial and antifungal properties of esters of *p*-hydroxybenzoic acid (parabens) have been of interest to pharmaceutical scientists for the past few decades. Methyl, propyl, butyl, and benzyl parabens have been investigated individually or in combination in pharmaceutical research and development.

A literature survey revealed that UV spectrophotometry is the most widely used method for a quantitative analysis of the parabens (1). This technique has proved quite satisfactory in the quantification of a single paraben, but it is not sufficient for the analysis of a mixture of parabens in solution. This is due to the fact that most parabens possess nearly identical $\lambda_{max.}$ values.

Procedures such as column chromatography (2) and GLC (3) have been reported for the analysis of parabens. The former procedure is time consuming and laborious. If esters are in an aqueous solution, the silylation technique, which should precede injection onto the column, together with other problems inherent to GLC makes the latter procedure less desirable and casts some doubts upon the accuracy of the results.

An exploratory attempt on the applicability of NMR spectrometry for the analysis of parabens revealed the method to be both rapid and accurate and the procedure quite simple.

EXPERIMENTAL

The parabens used in the experiments were all reagent grade. The general procedure for preparing the standard solutions consisted of placing an accurately weighed quantity of the desired ester in a volumetric flask, dissolving, and adjusting the volume with 60% polyethylene glycol (PEG) 400 in water. This vehicle was also used for preparing the solution of the paraben mixtures. Adoption of this solvent was due to its routine use in some other aspects of the research and to illustrate the point that the analysis does not require deuterated solvents.

The NMR spectra were determined using a Varian A-60 NMR spectrophotometer at an ambient temperature.

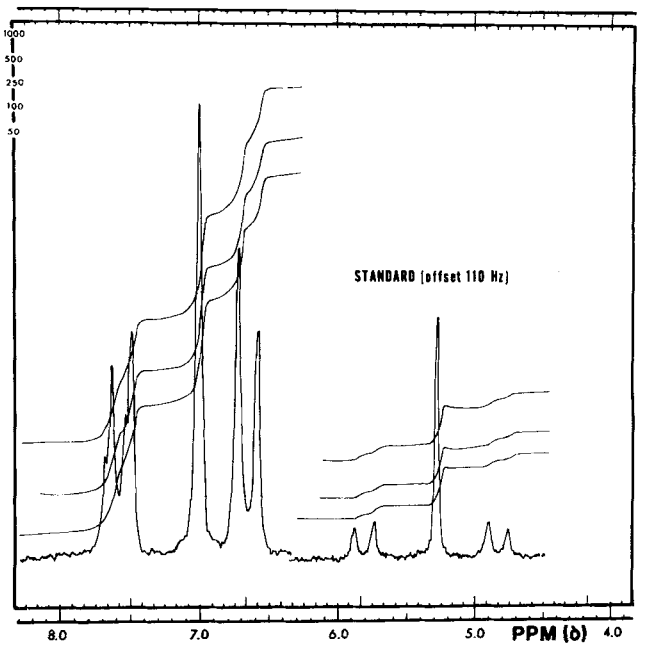


Figure 1—*The NMR spectrum of a mixture of benzyl paraben and methyl paraben in PEG–H_2O. Standard benzyl paraben is depicted at 110 c.p.s. offset.*

RESULTS AND DISCUSSION

Depicted in Fig. 1 is the NMR spectrum of a mixture of benzyl paraben and methyl paraben in 60% PEG in water. Benzyl paraben contains two doublets at δ 6.85 and δ 7.85, which are the results of spin-spin coupling of two pairs of phenyl protons, in two different magnetic environments, in the *p*-hydroxybenzoate moiety of the ester molecule (Structure I). The singlet at δ 7.3 results from the phenyl protons of the benzyl moiety. The two doublets of benzyl paraben superimpose on the doublets of methyl paraben.

stitution of Natural Fats," 4th ed., Chapman & Hall, London, England, 1964, p. 688.

(17) V. Prelog, K. Wiesner, H. G. Khorana, and G. W. Kenner, *Helv. Chim. Acta*, **32**, 453(1949).

(18) K. Folkers and F. Koniuszy, *J. Amer. Chem. Soc.*, **61**, 1232(1939).

(19) V. Deulofeu, E. Hug, and P. Mazzocco, *J. Chem. Soc.*, **1939**, 1841.

(20) "Dictionary of Organic Compounds," vol. 2, I. Heilbron and H. M. Bunbury, Eds., Eyre & Spottiswoode, London, England, 1953, p. 843.

(21) R. A. Labriola, V. Deulofeu, and B. Berinzaghi, *J. Org. Chem.*, **16**, 90(1951).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 2, 1970, from the *Department of Pharmaceutical Sciences, Panjab University, Chandigarh-14, India.*

Accepted for publication March 13, 1970.

Abstracted in part from a thesis submitted by Amrik Singh Chawla to the Panjab University in partial fulfilment of Doctor of Philosophy degree requirements.

This work was supported by the University Grants Commission, India.

NMR Analysis of Some Alkyl *p*-Hydroxybenzoates

F. SHIHAB, W. SHEFFIELD, J. SPROWLS, and J. NEMATOLLAHI*

Abstract □ By applying NMR spectrometry, a number of solutions containing unknown quantities of two or three alkyl *p*-hydroxybenzoates (parabens) in 60% polyethylene glycol in water were analyzed for quantification of the individual parabens. The method was found to be accurate, facile, and rapid and seems to possess a promising potential for applicability in related fields of pharmaceutical analysis.

Keyphrases □ Parabens—NMR analysis □ Alkyl *p*-hydroxybenzoates—NMR analysis □ NMR spectroscopy—analysis

The antibacterial and antifungal properties of esters of *p*-hydroxybenzoic acid (parabens) have been of interest to pharmaceutical scientists for the past few decades. Methyl, propyl, butyl, and benzyl parabens have been investigated individually or in combination in pharmaceutical research and development.

A literature survey revealed that UV spectrophotometry is the most widely used method for a quantitative analysis of the parabens (1). This technique has proved quite satisfactory in the quantification of a single paraben, but it is not sufficient for the analysis of a mixture of parabens in solution. This is due to the fact that most parabens possess nearly identical $\lambda_{max.}$ values.

Procedures such as column chromatography (2) and GLC (3) have been reported for the analysis of parabens. The former procedure is time consuming and laborious. If esters are in an aqueous solution, the silylation technique, which should precede injection onto the column, together with other problems inherent to GLC makes the latter procedure less desirable and casts some doubts upon the accuracy of the results.

An exploratory attempt on the applicability of NMR spectrometry for the analysis of parabens revealed the method to be both rapid and accurate and the procedure quite simple.

EXPERIMENTAL

The parabens used in the experiments were all reagent grade. The general procedure for preparing the standard solutions consisted of placing an accurately weighed quantity of the desired ester in a volumetric flask, dissolving, and adjusting the volume with 60% polyethylene glycol (PEG) 400 in water. This vehicle was also used for preparing the solution of the paraben mixtures. Adoption of this solvent was due to its routine use in some other aspects of the research and to illustrate the point that the analysis does not require deuterated solvents.

The NMR spectra were determined using a Varian A-60 NMR spectrophotometer at an ambient temperature.

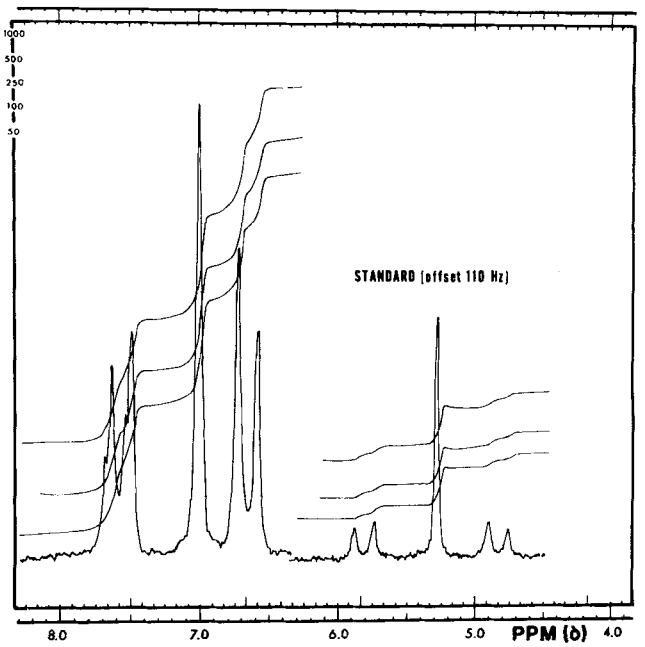
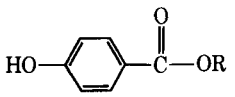


Figure 1—*The NMR spectrum of a mixture of benzyl paraben and methyl paraben in PEG–H_2O. Standard benzyl paraben is depicted at 110 c.p.s. offset.*

RESULTS AND DISCUSSION

Depicted in Fig. 1 is the NMR spectrum of a mixture of benzyl paraben and methyl paraben in 60% PEG in water. Benzyl paraben contains two doublets at δ 6.85 and δ 7.85, which are the results of spin-spin coupling of two pairs of phenyl protons, in two different magnetic environments, in the *p*-hydroxybenzoate moiety of the ester molecule (Structure I). The singlet at δ 7.3 results from the phenyl protons of the benzyl moiety. The two doublets of benzyl paraben superimpose on the doublets of methyl paraben.



$R = CH_3, C_2H_5, C_3H_7, C_4H_9, CH_2Ph$

I

By comparison of the integral values of the peak of the standard to those of the unknown, the concentration of each paraben in the mixture was calculated. The following examples illustrate the calculation of the concentration of individual parabens in mixtures as PEG–water solutions.

The abbreviations used are: standard = st; unknown = u; singlet integral value = SIV; doublet integral value = DIV; triplet integral value = TIV; molarity = *M;* methyl, ethyl, propyl, butyl, and benzyl paraben = MePab, EtPab, PrPab, BuPab, and BzPab, respectively.

Calculation of Concentration of Methyl Paraben and Benzyl Paraben Present in Unknown Quantities as a Mixture in PEG–Water—See Fig. 1. The standard was a known concentration of benzyl paraben solution.

$$\frac{(SIV)_{BzPab}}{(SIV)_{st}} \times (M)_{st} = (M)_{BzPab} \quad \text{(Eq. 1)}$$

$$4/5 \times (SIV)_{BzPab} = (DIV)_{BzPab} \quad \text{(Eq. 2)}$$

$$\text{total } (DIV)_u - (DIV)_{BzPab} = (DIV)_{MePab} \quad \text{(Eq. 3)}$$

$$\frac{(DIV)_{MePab}}{(DIV)_{st}} \times (M)_{st} = (M)_{MePab} \quad \text{(Eq. 4)}$$

Quantification of a mixture of any alkyl paraben mixed with benzyl paraben can be carried out by a similar procedure.

Calculation of Concentration of Methyl Paraben and Propyl Paraben Present in Unknown Quantities as a Mixture in PEG–Water—The standard was a known concentration of methyl paraben solution.

$$4/3 \times (TIV)_{PrPab} = (DIV)_{PrPab} \quad \text{(Eq. 5)}$$

$$\text{total } (DIV)_u - (DIV)_{PrPab} = (DIV)_{MePab} \quad \text{(Eq. 6)}$$

$$\frac{(DIV)_{MePab}}{(DIV)_{st}} \times (M)_{st} = (M)_{MePab} \quad \text{(Eq. 7)}$$

$$\frac{(DIV)_{PrPab}}{(DIV)_{st}} \times (M)_{st} = (M)_{PrPab} \quad \text{(Eq. 8)}$$

Calculation of Concentration of Benzyl Paraben, Methyl Paraben, and Propyl Paraben Present in Unknown Quantities as a Mixture in PEG–Water—The standard was a known concentration of benzyl paraben solution.

$$\frac{(SIV)_{BzPab}}{(SIV)_{st}} \times (M)_{st} = (M)_{BzPab} \quad \text{(Eq. 9)}$$

$$4/5 \times (SIV)_{BzPab} = (DIV)_{BzPab} \quad \text{(Eq. 10)}$$

$$\text{total } (DIV)_u - (DIV)_{BzPab} = (DIV)_{MePab + PrPab} \quad \text{(Eq. 11)}$$

$$4/3\ (TIV)_{PrPab} = (DIV)_{PrPab} \quad \text{(Eq. 12)}$$

$$(DIV)_{MePab + PrPab} - (DIV)_{PrPab} = (DIV)_{MePab} \quad \text{(Eq. 13)}$$

$$\frac{(DIV)_{PrPab}}{(DIV)_{st}} \times (M)_{st} = (M)_{PrPab} \quad \text{(Eq. 14)}$$

$$\frac{(DIV)_{MePab}}{(DIV)_{st}} \times (M)_{st} = (M)_{MePab} \quad \text{(Eq. 15)}$$

Table I—Results of NMR Analyses of Parabens in PEG–H_2O

Alkyl Group	Concn., mole/l., Prepared	Concn., mole/l., Calculated Using NMR Peak Integral Values
	Single	
1. CH_3	0.2500	0.2497
2. C_2H_5	0.3000	0.3000
	Mixture	
3. CH_3 / n-C_3H_7	0.5000 0.3000	0.5006 0.3000
4. CH_3 / n-C_3H_7	0.7500 0.5000	0.7511 0.4989
5. CH_3 / CH_2Ph	0.7500 0.3000	0.7484 0.3000
6. CH_3 / n-C_3H_7 / CH_2Ph	0.5000 0.2500 0.1500	0.5000 0.2516 0.1500

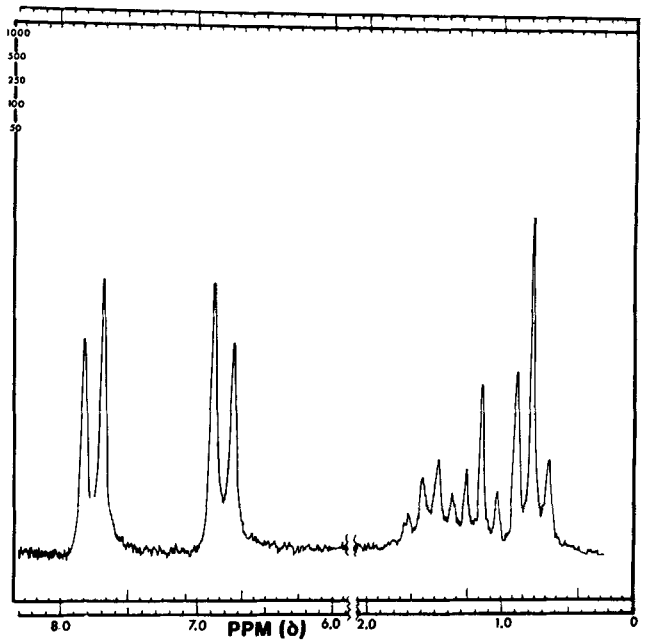


Figure 2—*The NMR spectrum of a mixture of ethyl paraben and propyl paraben in PEG–H_2O.*

A summary of the precision of the experimental procedure is depicted in Table I.

As long as one of the two parabens in a mixture contains a discernible characteristic resonance peak, the individual esters can be determined quantitatively by NMR. Analysis of a mixture of ethyl and propyl paraben, whose triplet peaks are not superimposed, can be cited as an example and is depicted in Fig. 2.

REFERENCES

(1) N. K. Patel and N. E. Foss, *J. Pharm. Sci.*, **53**, 94(1964).

(2) T. Higuchi, K. P. Patel, E. R. Bonow, and J. Landsman, *J. Amer. Pharm. Ass., Sci. Ed.*, **41**, 293(1952).

(3) J. L. Lach and J. S. Sawardeker, *J. Pharm. Sci.*, **54**, 424(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 9, 1970, from the *College of Pharmacy, University of Texas at Austin, Austin, TX 78712*

Accepted for publication March 27, 1970.

This investigation was supported in part by a grant from the Office of the Graduate Dean.

* To whom inquiries and reprint requests should be directed.

Determination of Ethanol in an Elastomeric Matrix by Gas Chromatography

HARRIS I. TARLIN

Abstract □ A gas chromatographic procedure is described for the complete separation and quantitative determination of trace amounts of ethanol in the presence of nine common pharmaceutical compounds. By using a column containing an ethylvinylbenzene polymer, the occluded ethanol content of a single dosage unit can be determined in 8 min.

Keyphrases □ Ethanol, traces—separation, determination, elastomeric matrix, GLC □ Elastomeric matrix—ethanol separation, determination □ GLC—separation, determination

Ethanol has been determined by gas chromatography in liquid cough syrups (1) on a column of styrene-divinylbenzene polymer[1] and in blood (2–5) on a column of ethylvinylbenzene polymer.[2] This polymer has also been used to determine ethanol by gas chromatography in a variety of pharmaceuticals such as paregoric, mercurochrome, elixer phenobarbital, and antihistamine syrups (6). To date, there have been no studies reported in the literature concerning the determination of ethanol in a pharmaceutical dosage form comprising a water-soluble elastomeric material.

This report describes a simple, rapid method for determining ethanol which was occluded in an elastomeric matrix containing lidocaine, propylene glycol, sorbitol, sodium saccharin, polyvinyl alcohol, polyvinylpyrrolidone, gelatin, methylparaben, propylparaben, and common coloring and flavoring agents. The method was developed as part of a quality control program to determine the presence and quantity of ethanol in the finished dosage form prepared from a liquid mixture of the aforementioned components, which was subjected to heat above the boiling point of ethanol during processing.

EXPERIMENTAL

Instruments—A Perkin-Elmer model 811 gas chromatograph with a flame-ionization detector was used. The sensitivity of the detector was 0.005 C./g. The 1.82-m. (6-ft.) glass column, i.d. 0.32 cm. (0.125 in.), o.d. 0.63 cm. (0.25 in.), was packed with ethylvinylbenzene polymer 50/80 mesh, using a vibrator and conditioned for 18 hr. at 200° with helium gas flowing through the column. A small pledget of glass wool was placed in each end of the column. The following gauge pressures were used: helium, 30; hydrogen, 15; and compressed air, 40. The flow rate through the column was 64 ml./min. The column temperature was 122° and the injector temperature was 185°. A Bristol Dynamaster Strip-Chart recorder with a chart speed of 2.54 cm. (1 in.)/min. was used.

Table I—Relationship of Peak Height to Ethanol Concentration

Ethanol, μl./10 ml.	Peak Height	Ethanol, μl./10 ml./ Unit of Peak Height
0.3	3.0	0.10
0.5	5.0	0.10
1.0	10.5	0.095
5.0	51.0	0.098
		Av. = 0.098

Table II—Ethanol Content of Three Batches of Product

	No. of Dose Units	Alcohol Content, μl./unit
Batch 1	4	0.05
	11	0.10
	2	0.15
	6	0.20
	2	0.25
Batch 2	13	0.00
	12	0.05
Batch 3	23	0.00
	1	0.05
	1	0.25

Standard Solution—A stock solution containing 1 μl. of 95% ethanol[3]/ml. was prepared by pipeting 2.0 ml. of alcohol into a 2-l. volumetric flask and diluting to the mark with distilled water.[4] Standard solutions containing 0.3, 0.5, 1.0, and 5.0 μl. of ethanol/10 ml. were prepared by pipeting 0.3, 0.5, 1.0, and 5.0 ml. of stock solution, respectively, into 10-ml. volumetric flasks and diluting to the mark with water. Five microliters of each standard was injected.[5] Table I shows the relationship of peak height[6] to alcohol concentration.

A single dosage unit of 5 cm.[2] of the solid elastomer mixture was cut into two pieces and introduced into a 10-ml. volumetric flask containing 5 ml. of water. The pieces were dissolved with vigorous shaking, and the flask was diluted to the mark with water. Five microliters of the prepared solution was injected. This procedure was carried out for 74 additional dosage units. Ethanol concentrations were calculated as follows: μl. of ethanol/dosage unit = peak height × av. μl. of ethanol/10 ml./unit of peak height.

A standard containing 1 μl. of ethanol/10 ml. of solution was injected throughout this investigation. An evaluation of 10 injections of the standard indicated that steady-state operating conditions were maintained ($p = 0.06$ for $2S$).

Identification of the Ethanol Peak—The ethanol peak was qualitatively identified by the following enrichment method: 8 ml. of a solution consisting of 1 dissolved dosage unit/10 ml. (containing 0.025 μl. of ethanol/ml.) was pipeted into a 10-ml. volumetric flask containing 1 μl. of ethanol. The flask was diluted to the mark with water. The solution thus prepared contained 1.2 μl. ethanol/10 ml. The expected peak height was 13.0, and the expected retention[7] distance was 16.98 ± 0.22 cm. Following are the results of two trials:

	Peak Height	Retention Distance, cm.
Trial 1	13.5	16.9
Trial 2	12.8	16.7

[1] Polypak-2, F. M. Scientific Division, Hewlett Packard.
[2] Porapak Q, Waters Associates, Inc., Framingham, Mass.
[3] Ethyl alcohol USP, 95%, U.S. Industrial Chemical Co. Referred to as ethanol in the following text.
[4] Referred to as water in the following text.
[5] All samples in this study were injected with a 10-μl. Hamilton syringe.
[6] All peak heights in this study were measured on chart paper which was divided into 100 equal units.
[7] The retention distance is the distance in centimeters between the leading slope of the water peak and the maxima of the peak under investigation. The average retention distance for 5 standard ethanol solutions was 16.98 ± 0.22 cm.

This experiment indicated that the observed peak was due to ethanol. A similar procedure was used to identify the first major peak. The peak resulted from the organic impurities in water.

RESULTS AND DISCUSSION

Table II shows a summary of the ethanol content of the three batches studied. The column material used in this investigation has outstanding separation properties for volatile, low-molecular weight compounds and was particularly valuable for this study since no signals were observed from any of the compounds in the dosage form except ethanol.

REFERENCES

(1) H. P. Warrington, Jr., and J. M. Lennon, *J. Pharm. Sci.*, **56**, 404(1967).

(2) J. K. Colehour, *Anal. Chem.*, **39**, 1190(1967).

(3) J. P. Payne, D. V. Foster, D. W. Hill, and D. G. L. Wood, *Brit. Med. J.*, **3**, 819(1967).

(4) G. Freud, *Anal. Chem.*, **39**, 545(1967).

(5) R. N. Baker, A. L. Alenty, and J. F. Zack, Jr., *J. Chromatogr. Sci.*, **7**, 312(1969).

(6) J. T. Piechocki, *J. Pharm. Sci.*, **57**, 134(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 28, 1969, from the *Analytical Laboratory, Astra Pharmaceutical Products, Inc., Worcester, MA 01606*

Accepted for publication February 17, 1970.

The author expresses his appreciation to William Webber and Dr. S. G. Davis for reviewing this manuscript.

Antimicrobial Activity of Some β-Nitrostyrenes

LEONARD R. WORTHEN and HOWARD W. BOND

Abstract □ Nine compounds were tested for antimicrobial activity, and eight of these exhibited varying spectra of inhibition.

Keyphrases □ β-Nitrostyrene derivatives—antimicrobial activity □ Antimicrobial activity, evaluation—β-nitrostyrene derivatives □ Paper disks—microbe-inhibition analysis

In the course of synthesizing some chemical compounds for testing as potential molluscicides, a number of β-nitrostyrene compounds were prepared. This report indicates the preliminary results obtained from testing nine of these compounds, seven of which are new (1), for antibacterial and antifungal activity.

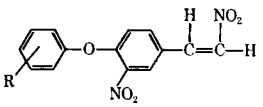
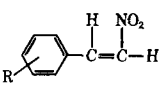
The antimicrobial activity of β-nitrostyrenes has been previously noted by several workers, including Schales and Graefe (2), Bocobo *et al.* (3), and Huitric *et al.* (4).

An impregnated filter paper disk on an inoculated agar plate was selected as the testing method. This technique has the advantage over the hole or cup method because the solvent can be evaporated from the saturated paper disks prior to placing them on the inoculated agar plate. In this manner, any potential antimicrobial effects from the solvent used can be avoided.

EXPERIMENTAL

Materials and Methods—The test organisms used included the following: *Staphylococcus aureus*, strain 209, ATCC 6538; *Escherichia coli*, ATCC 4157; *Pseudomonas aeruginosa*, ATCC 10145; *Bacillus subtilis; Proteus vulgaris; Aspergillus niger*, ATCC 9642; *Trichophyton mentagrophytes*, ATCC 9129; and *Candida albicans*, ATCC 10231. The melted test agar media were inoculated with 0.1 ml. of a 24-hr.-old bacterial culture grown in nutrient broth or a 0.1 ml. saline suspension of a 48-hr.-old fungal culture grown on Sabouraud's agar. Nutrient agar was employed for the bacterial tests

Table I—Antibacterial and Antifungal Activity of Some β-Nitrostyrenes

R	Microbial Spectrum[a] 1	2	3	4	5	6	7	8
								
H	++[b]	++	+	−	−	+	+	+++
p-CH_3O—	++	++	−	−	−	+	++	+++
p-CH_3CONH—	++	++	+	−	−	−	−	+
2,4-Cl_2—	++	+	−	−	−	−	+	+++
Cl_5—	+	−	−	−	−	−	−	−
Br_5—	−	−	−	−	−	−	−	−
								
m-CH_3CO_2—	++	++	++	++	++	+	++	++
p-$CH_3CH_2CO_2$—	++	++	+++	−	++	+	+++	+++
3-CH_3O-4-CH_3CO_2—	++	++	++	−	++	++	+++	+++

[a] Microbial spectrum: Gram-positive: 1, *S. aureus*, strain 209, ATCC 6538; 2, *B. subtilis*. Gram-negative: 3, *E. coli*, ATCC 4157; 4, *P. aeruginosa*, ATCC 10145; 5, *P. vulgaris*. Fungi: 6, *C. albicans*, ATCC 10231; 7, *A. niger*, ATCC 9642; *T. mentagrophytes*, ATCC 9129. [b] Zone of inhibition: − = less than 12.7 mm. (zone includes diameter of disk); + = less than 20 mm., ++ = 21–29 mm., and +++ = more than 30 mm.

This experiment indicated that the observed peak was due to ethanol. A similar procedure was used to identify the first major peak. The peak resulted from the organic impurities in water.

RESULTS AND DISCUSSION

Table II shows a summary of the ethanol content of the three batches studied. The column material used in this investigation has outstanding separation properties for volatile, low-molecular weight compounds and was particularly valuable for this study since no signals were observed from any of the compounds in the dosage form except ethanol.

REFERENCES

(1) H. P. Warrington, Jr., and J. M. Lennon, *J. Pharm. Sci.*, **56**, 404(1967).

(2) J. K. Colehour, *Anal. Chem.*, **39**, 1190(1967).

(3) J. P. Payne, D. V. Foster, D. W. Hill, and D. G. L. Wood, *Brit. Med. J.*, **3**, 819(1967).

(4) G. Freud, *Anal. Chem.*, **39**, 545(1967).

(5) R. N. Baker, A. L. Alenty, and J. F. Zack, Jr., *J. Chromatogr. Sci.*, **7**, 312(1969).

(6) J. T. Piechocki, *J. Pharm. Sci.*, **57**, 134(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 28, 1969, from the *Analytical Laboratory, Astra Pharmaceutical Products, Inc., Worcester, MA 01606*

Accepted for publication February 17, 1970.

The author expresses his appreciation to William Webber and Dr. S. G. Davis for reviewing this manuscript.

Antimicrobial Activity of Some β-Nitrostyrenes

LEONARD R. WORTHEN and HOWARD W. BOND

Abstract □ Nine compounds were tested for antimicrobial activity, and eight of these exhibited varying spectra of inhibition.

Keyphrases □ β-Nitrostyrene derivatives—antimicrobial activity □ Antimicrobial activity, evaluation—β-nitrostyrene derivatives □ Paper disks—microbe-inhibition analysis

In the course of synthesizing some chemical compounds for testing as potential molluscicides, a number of β-nitrostyrene compounds were prepared. This report indicates the preliminary results obtained from testing nine of these compounds, seven of which are new (1), for antibacterial and antifungal activity.

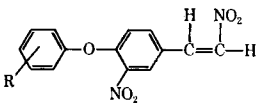
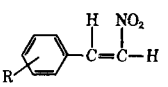
The antimicrobial activity of β-nitrostyrenes has been previously noted by several workers, including Schales and Graefe (2), Bocobo *et al.* (3), and Huitric *et al.* (4).

An impregnated filter paper disk on an inoculated agar plate was selected as the testing method. This technique has the advantage over the hole or cup method because the solvent can be evaporated from the saturated paper disks prior to placing them on the inoculated agar plate. In this manner, any potential antimicrobial effects from the solvent used can be avoided.

EXPERIMENTAL

Materials and Methods—The test organisms used included the following: *Staphylococcus aureus*, strain 209, ATCC 6538; *Escherichia coli*, ATCC 4157; *Pseudomonas aeruginosa*, ATCC 10145; *Bacillus subtilis; Proteus vulgaris; Aspergillus niger*, ATCC 9642; *Trichophyton mentagrophytes*, ATCC 9129; and *Candida albicans*, ATCC 10231. The melted test agar media were inoculated with 0.1 ml. of a 24-hr.-old bacterial culture grown in nutrient broth or a 0.1 ml. saline suspension of a 48-hr.-old fungal culture grown on Sabouraud's agar. Nutrient agar was employed for the bacterial tests

Table I—Antibacterial and Antifungal Activity of Some β-Nitrostyrenes

R	Microbial Spectrum[a] 1	2	3	4	5	6	7	8
								
H	++[b]	++	+	−	−	+	+	+++
p-CH_3O—	++	++	−	−	−	+	++	+++
p-CH_3CONH—	++	++	+	−	−	−	−	+
2,4-Cl_2—	++	+	−	−	−	−	+	+++
Cl_5—	+	−	−	−	−	−	−	−
Br_5—	−	−	−	−	−	−	−	−
								
m-CH_3CO_2—	++	++	++	++	++	+	++	++
p-$CH_3CH_2CO_2$—	++	++	+++	−	++	+	+++	+++
3-CH_3O-4-CH_3CO_2—	++	++	++	−	++	++	+++	+++

[a] Microbial spectrum: Gram-positive: 1, *S. aureus*, strain 209, ATCC 6538; 2, *B. subtilis*. Gram-negative: 3, *E. coli*, ATCC 4157; 4, *P. aeruginosa*, ATCC 10145; 5, *P. vulgaris*. Fungi: 6, *C. albicans*, ATCC 10231; 7, *A. niger*, ATCC 9642; *T. mentagrophytes*, ATCC 9129. [b] Zone of inhibition: − = less than 12.7 mm. (zone includes diameter of disk); + = less than 20 mm., ++ = 21–29 mm., and +++ = more than 30 mm.

and Sabouraud's agar for the fungal tests. All bacterial cultures were incubated at 35° and the fungal cultures at room temperature. The filter paper disks (12.7-mm. diameter) were saturated with 0.1 ml. of a 1:1000 concentration of the compounds to be tested; the solvent was allowed to evaporate before the disks were placed, in duplicate, on the seeded plates. Ethanolic solutions of eight of the compounds were used. The other compound [*m*-(2-nitrovinyl)-phenyl acetate] was employed as a suspension in pyridine. Disks impregnated with an equal amount of the solvent only were dried and used as controls and in no instance exhibited any inhibition. The zones of inhibition were read at the end of 24 hr. of incubation for the bacteria and 48 hr. for the fungi. The results are tabulated in Table I.

DISCUSSION

Nine β-nitrostyrene derivatives were tested by the paper disk method for antimicrobial activity. Of the six 3-nitro-4-substituted phenoxy-β-nitrostyrenes tested, the parent compound had the widest spectrum. The pentachloro derivative was effective against *S. aureus* only, and the pentabromo derivative was inactive. None of these compounds had any activity against *P. vulgaris* or *P. aeruginosa*.

The three ring-substituted β-nitrostyrenes tested all showed good activity against the test organisms. Of particular significance is their activity against the *Proteus* and *Pseudomonas* species tested. One limitation with the most active compound tested, *m*-acetoxy-β-nitrostyrene, was the inability to solubilize it in common organic solvents, even at a 1:1000 concentration. It was placed on the paper disk as a 1:1000 suspension. The results of the tests of the latter series of compounds warrant further study of other derivatives of this group.

REFERENCES

(1) H. W. Bond, J. S. O'Grodnick, and B. H. Pringle, *J. Med. Chem.*, **12**, 1107(1969).

(2) O. Schales and H. A. Graefe, *J. Amer. Chem. Soc.*, **74**, 4486(1952).

(3) F. C. Bocobo, A. C. Curtis, W. D. Block, and E. R. Harrell, *Proc. Soc. Exp. Biol. Med.*, **85**, 220(1954).

(4) A. C. Huitric, R. Pratt, Y. Okano, and W. D. Kumler, *Antibiot. Chemother.*, **6**, 385(1956).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 24, 1969, from the *Departments of Pharmaceutical Chemistry and Pharmacognosy, University of Rhode Island, Kingston, RI 02881*

Accepted for publication February 10, 1970.

This investigation was supported by the Office of Water Resources Research, U. S. Department of Interior, under P.L. 88-379.

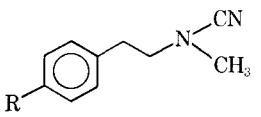
Solvent Effects in the NMR Spectra of Cyano Compounds

EDWARD E. SMISSMAN and ALEXANDROS C. MAKRIYANNIS

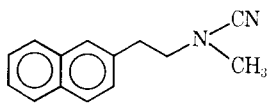
Abstract □ Novel solvent effects in the NMR spectra of certain cyano compounds are reported. A change in the splitting pattern of the methylene groups of *N*-cyano-*N*-methylarylethylamines with a change in solvent was observed in open-chain alkyl cyano compounds containing a terminal aromatic ring.

Keyphrases □ Solvent effects—NMR spectra, cyano compounds □ Cyano compounds, NMR—solvent effects □ NMR spectra—solvent effects, cyano compounds

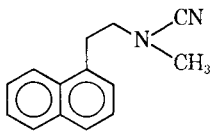
During the synthesis of a number of *N*-cyano-*N*-methylarylethylamines (1), it was observed that the NMR spectra exhibited dramatic changes when the solvent was changed from either carbon tetrachloride or deuterochloroform to benzene. These changes involved: (*a*) a strong upfield shift in the singlet signal due to the *N*-methyl protons, and (*b*) a strong upfield shift of the multiplets due to the four methylene protons, accompanied by a pronounced change in the splitting pattern of the multiplet in all of the compounds where rotation was possible about the C—C bond of the ethylamine. The only compound examined which did not show the change in splitting pattern was *N*-cyanotetrahydroisoquinoline (Compound 5). The observations from the examined spectra are summarized in Table I.



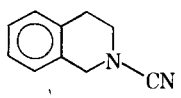
1. R = H
2. R = OCH_3



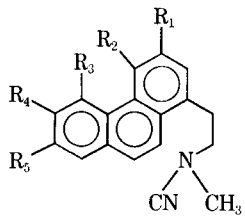
3



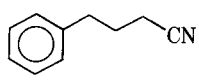
4



5



	R_1	R_2	R_3	R_4	R_5
6.	—OCH_2O—		OH	OMe	H
7.	—OCH_2O—		OAc	OMe	H
8.	H	H	OH	OH	H
9.	OH	OMe	H	OMe	OH



10

$ClCH_2CH_2CN$

11

$BrCH_2CH_2CH_2CN$

12

structures of compounds listed in Table I

and Sabouraud's agar for the fungal tests. All bacterial cultures were incubated at 35° and the fungal cultures at room temperature. The filter paper disks (12.7-mm. diameter) were saturated with 0.1 ml. of a 1:1000 concentration of the compounds to be tested; the solvent was allowed to evaporate before the disks were placed, in duplicate, on the seeded plates. Ethanolic solutions of eight of the compounds were used. The other compound [*m*-(2-nitrovinyl)-phenyl acetate] was employed as a suspension in pyridine. Disks impregnated with an equal amount of the solvent only were dried and used as controls and in no instance exhibited any inhibition. The zones of inhibition were read at the end of 24 hr. of incubation for the bacteria and 48 hr. for the fungi. The results are tabulated in Table I.

DISCUSSION

Nine β-nitrostyrene derivatives were tested by the paper disk method for antimicrobial activity. Of the six 3-nitro-4-substituted phenoxy-β-nitrostyrenes tested, the parent compound had the widest spectrum. The pentachloro derivative was effective against *S. aureus* only, and the pentabromo derivative was inactive. None of these compounds had any activity against *P. vulgaris* or *P. aeruginosa*.

The three ring-substituted β-nitrostyrenes tested all showed good activity against the test organisms. Of particular significance is their activity against the *Proteus* and *Pseudomonas* species tested. One limitation with the most active compound tested, *m*-acetoxy-β-nitrostyrene, was the inability to solubilize it in common organic solvents, even at a 1:1000 concentration. It was placed on the paper disk as a 1:1000 suspension. The results of the tests of the latter series of compounds warrant further study of other derivatives of this group.

REFERENCES

(1) H. W. Bond, J. S. O'Grodnick, and B. H. Pringle, *J. Med. Chem.*, **12**, 1107(1969).

(2) O. Schales and H. A. Graefe, *J. Amer. Chem. Soc.*, **74**, 4486(1952).

(3) F. C. Bocobo, A. C. Curtis, W. D. Block, and E. R. Harrell, *Proc. Soc. Exp. Biol. Med.*, **85**, 220(1954).

(4) A. C. Huitric, R. Pratt, Y. Okano, and W. D. Kumler, *Antibiot. Chemother.*, **6**, 385(1956).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 24, 1969, from the *Departments of Pharmaceutical Chemistry and Pharmacognosy, University of Rhode Island, Kingston, RI 02881*

Accepted for publication February 10, 1970.

This investigation was supported by the Office of Water Resources Research, U. S. Department of Interior, under P.L. 88-379.

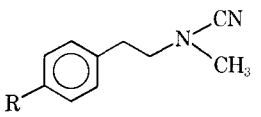
Solvent Effects in the NMR Spectra of Cyano Compounds

EDWARD E. SMISSMAN and ALEXANDROS C. MAKRIYANNIS

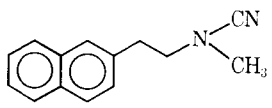
Abstract □ Novel solvent effects in the NMR spectra of certain cyano compounds are reported. A change in the splitting pattern of the methylene groups of *N*-cyano-*N*-methylarylethylamines with a change in solvent was observed in open-chain alkyl cyano compounds containing a terminal aromatic ring.

Keyphrases □ Solvent effects—NMR spectra, cyano compounds □ Cyano compounds, NMR—solvent effects □ NMR spectra—solvent effects, cyano compounds

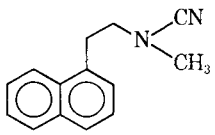
During the synthesis of a number of *N*-cyano-*N*-methylarylethylamines (1), it was observed that the NMR spectra exhibited dramatic changes when the solvent was changed from either carbon tetrachloride or deuterochloroform to benzene. These changes involved: (*a*) a strong upfield shift in the singlet signal due to the *N*-methyl protons, and (*b*) a strong upfield shift of the multiplets due to the four methylene protons, accompanied by a pronounced change in the splitting pattern of the multiplet in all of the compounds where rotation was possible about the C—C bond of the ethylamine. The only compound examined which did not show the change in splitting pattern was *N*-cyanotetrahydroisoquinoline (Compound 5). The observations from the examined spectra are summarized in Table I.



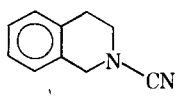
1. R = H
2. R = OCH_3



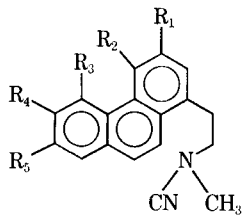
3



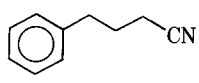
4



5



	R_1	R_2	R_3	R_4	R_5
6.	—OCH_2O—		OH	OMe	H
7.	—OCH_2O—		OAc	OMe	H
8.	H	H	OH	OH	H
9.	OH	OMe	H	OMe	OH



10

$ClCH_2CH_2CN$

11

$BrCH_2CH_2CH_2CN$

12

structures of compounds listed in Table I

Table I—Solvent Effects on Chemical Shifts in the NMR of Cyano Compounds

Compound	Solvent	Chemical Shift (δ) N—CH_3	Chemical Shift (δ) —CH_2—CH_2—
1	CCl_4	2.66	Multiplet; 2.66–3.29
1	C_6H_6	2.00	Multiplet as singlet; 2.47
2	$CDCl_3$	2.87	Multiplet; 2.87–3.51
2	C_6H_6	2.12	Multiplet as singlet; 2.57
3	$CDCl_3$	2.80	Multiplet; 3.07–3.39
3	C_6H_6	2.07	Multiplet as singlet; 2.67
4	$CDCl_3$	2.78	Multiplet as singlet; 3.37
4	C_6H_6	2.05	Multiplet; 2.47–3.26
5	CCl_4	4.30($NCH_2\phi$)	Irregular triplet; 2.88 ($W_{1/2}$ = 13 c.p.s.) Irregular triplet; 3.41 ($W_{1/2}$ = 13 c.p.s.)
5	C_6H_6	3.44($NCH_2\phi$)	Irregular triplet; 1.93 ($W_{1/2}$ = 13 c.p.s.) Irregular triplet; 2.41 ($W_{1/2}$ = 13 c.p.s.)
6	$CDCl_3$	2.80	Multiplets as singlet; 3.27
6	$CDCl_3$–C_6H_6(2:1)	2.38	Multiplet; 2.86–3.17
7	$CDCl_3$	2.81	Multiplet as singlet; 3.31
7	$CDCl_3$–C_6H_6(1:1)	2.12	Multiplet; 2.40–3.18
8	$CDCl_3$	2.83	Multiplet as singlet; 3.42
9	$CDCl_3$	2.90	Multiplet as singlet; 3.41
10	CCl_4		Irregular triplet; 2.77 ($W_{1/2}$ = 14 c.p.s.) Multiplet; 1.33–1.84
10	C_6H_6		Irregular triplet; 3.26 ($W_{1/2}$ = 14 c.p.s.) Multiplet; 2.00–2.92
11	CCl_4		Triplet; 3.73 (J = 6.5 c.p.s.) Triplet; 2.83 (J = 6.5 c.p.s.)
11	C_6H_6		Triplet; 2.88 (J = 6.5 c.p.s.) Triplet; 1.88 (J = 6.5 c.p.s.)
12	CCl_4		Triplet; 3.51 (J = 6 c.p.s.) Multiplet; 1.99–2.70
12	C_6H_6		Triplet; 2.79 (J = 6 c.p.s.) Multiplet; 1.14–1.84

There appears to be no long-range coupling effects between the methylene protons and the protons of the aromatic ring in any of these arylethylamines. Decoupling experiments were performed on Compounds 1, 3, and 4 by irradiating the aromatic region of the spectra. This had no effect on the splitting pattern of the methylene groups when either deuterochloroform or benzene was utilized as the solvent.

The NMR spectrum of phenylbutyronitrile (Compound 10) exhibited equally strong solvent effects, involving upfield shifts accompanied by pronounced changes in the patterns of the methylene signals. The spectra of the alkyl cyano compounds β-chloropropionitrile (Compound 11) and γ-bromobutyronitrile (Compound 12) exhibited upfield shifts of equal magnitude with no change in the splitting pattern, whereas *N,N*-dimethylphenethylamines showed no significant solvent effects in their NMR spectra.

These solvent effects, therefore, appear to be due to the cyano group and can be explained on the basis of a collision complex between the benzene ring and the cyano group. Similar collision complexes have been postulated by Hatton and Richards (2) and have been applied to explain the solvent effects caused by benzene with *N*-methyl formamide (3) and several ketones (4, 5). The general upfield shifts in the case of the nonaromatic protons can be attributed to the anisotropy of the magnetic susceptibility of benzene. The change in the splitting pattern of the methylene peaks, which is observed with all of the open-chain alkyl cyano compounds containing a terminal aromatic ring (Compounds 1–4, 6, 7, 10), can be attributed to: (*a*) a decrease in the shielding effect of benzene when the methylene groups are further removed from the cyano group, and (*b*) conformational changes accompanying the change in solvent.

REFERENCES

(1) E. E. Smissman, A. C. Makriyannis, and E. J. Walaszek, to be published.

(2) J. V. Hatton and R. E. Richards, *Mol. Phys.*, **5**, 139(1962).

(3) L. A. Laplanche and M. T. Rogers, *J. Amer. Chem. Soc.*, **86**, 337(1964).

(4) J. V. Hatton and R. E. Richards, *Mol. Phys.*, **5**, 153(1962).

(5) N. S. Bhacca and D. H. Williams, "Applications of NMR Spectroscopy in Organic Chemistry," Holden-Day, San Francisco, Calif., 1964, p. 66.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 10, 1969, from the *Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication March 27, 1970.

Abstracted in part from a dissertation presented by A. Makriyannis to the Graduate School of the University of Kansas in partial fulfillment of the Doctor of Philosophy degree requirements.

The authors gratefully acknowledge support of this project by McNeil Laboratories, Inc., Fort Washington, Pa.

Monomolecular Film Properties of Some Cellulose Esters

JOEL L. ZATZ and BEVERLY KNOWLES

Abstract □ Cellulose acetate butyrate, cellulose acetate stearate, and cellulose acetate phthalate have been studied as monolayers at the air–water interface. Monolayers of the first two were virtually unaffected by changes in subphase pH, whereas the last one, cellulose acetate phthalate, exhibited large differences under the same conditions. Certain monolayer properties of the cellulose esters have been used to interpret the behavior of these polymers as free films and enteric coatings.

Keyphrases □ Cellulose esters—monomolecular film properties □ Film properties, monomolecular—cellulose acetate butyrate, cellulose acetate stearate, cellulose acetate phthalate □ Polymer monolayers—as model for enteric, film coatings

An approach that has provided information on the properties of film-forming polymers is the study of these materials as monolayers at the air–water interface (1). Such studies give insight into the molecular structure of polymers (1, 2) and may be used to investigate interaction of polymers with other substances, such as plasticizers (3).

Among the polymers used as coatings for pharmaceutical dosage forms, the cellulose esters represent an important group. These materials have previously been studied as free films and as coatings applied to tablets. Antonides and DeKay (4) evaluated various cellulose derivatives for possible use as enteric coatings. Patel *et al.* (5) studied water vapor transmission through free films of cellulose esters as a function of temperature and film thickness. Lachman and Drubulis (6) reported the influence of plasticizers on water vapor transmission through free films of cellulose acetate phthalate.

This study is concerned with the monomolecular film properties of three esters of cellulose, cellulose acetate phthalate (CAP), cellulose acetate butyrate (CAB), and cellulose acetate stearate (CAS). By adjusting the pH of the subphase to values of either 3 or 6.5 before spreading the polymer film, it was possible to assess the effect of a large change in environmental pH on the properties of each polymer studied.

EXPERIMENTAL

Materials and Apparatus—The cellulose derivatives were purchased[1] and purified further to remove residual monomers and other possible contaminants. CAP (32% phthalyl) was purified by precipitation from solution in benzene–ethanol, 1:1, using *n*-hexane as the nonsolvent. The polymer mass was washed with *n*-hexane and dried *in vacuo* at 30° to constant weight. As a result of this procedure, the free phthalic acid content of the polymer was reduced from an initial value of 1.3% w/w to a negligible level. Similarly, CAB (17% butyryl) was precipitated from solution in ethyl acetate by *n*-hexane. The precipitate was washed with *n*-hexane and dried *in vacuo* at 50° to constant weight. CAS was suspended in *n*-hexane, repeatedly washed with that solvent, and dried *in vacuo* at 50°. Water was deionized and then distilled in an all-glass still. The organic liquids employed were of reagent grade and were found to be free of surface-active contaminants (7). The inorganic materials employed were of reagent grade and were not further purified. The Teflon surface balance has already been described (8). Surface pressure was determined by the Wilhelmy plate method.

Procedure—The pH of the subphase was adjusted using HCl to obtain a pH of 3 or a buffer system containing K_2HPO_4 and KH_2PO_4 to obtain a pH of 6.5. The ionic strength of each solution used as subphase was adjusted to 0.1 *M* using KCl. The polymer under investigation was dissolved in a suitable solvent, and the solution was applied to the surface by means of an Agla micrometer syringe. CAB was dissolved in methylene chloride, CAS in benzene, and CAP in a isopropanol–benzene mixture, 1:1. Preliminary experiments showed that essentially the same results were obtained using other spreading solvents (isopropanol–methylene chloride for CAP and methylene chloride–benzene for CAB). After spreading the polymer, 10 or 15 min. was allowed for equilibration. Then the area available to polymer molecules was slowly decreased (at a rate of about 0.02 m.²/mg./min.); surface pressure was determined as a function of available surface area. All studies were carried out at room temperature (25 ± 0.7°).

RESULTS

Plots of surface pressure as a function of available surface area (π–A curves) for the cellulose esters studied are presented in Figs. 1–3. Each plot is based on at least three independent experiments. The reproducibility was such that at a given surface area, the surface pressure found in each experiment was within 0.6 dyne/cm. of the average value plotted in Figs. 1–3.

The π–A curves for CAB are shown in Fig. 1. The results with a subphase pH of 3.2 were nearly identical with those at a pH of 6.5. Monolayers of CAB collapsed to form a stringy, tenacious coagulum.

Some difficulty was encountered in spreading CAS at the surface. Droplets of spreading solution formed from the tip of the Agla syringe appeared to lose their clarity after a few seconds because of polymer adsorption and precipitation at the surface of the drop. Certain proteins are known to behave in a similar manner (9) Since all of the polymer was not in solution when applied to the surface, it is possible that the CAS was not completely spread. The π–A results, given in Fig. 2, show a slight variation in surface pressure as a function of pH. However, at a given surface area, the difference in surface pressure is always less than 2 dynes/cm. and

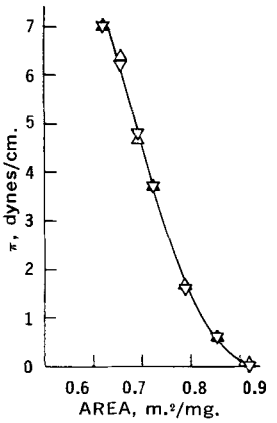


Figure 1—*Surface pressure (π)–area (A) isotherms of CAB at two subphase pH values: ▽, pH = 3.2; and △, pH = 6.5.*

[1] Eastman Organic Chemicals, Rochester, NY 14650

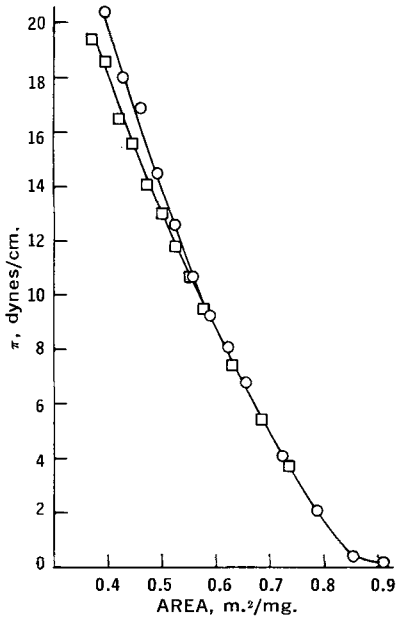


Figure 2—*Surface pressure (π)–area (A) isotherms of CAS at two subphase pH values:* □, *pH = 3.2; and* ○, *pH = 6.5.*

is probably the result of experimental error. The two curves in Fig. 2 are quite similar and indicate that no significant change in the properties of CAS occurs when the subphase pH is changed from 3.2 to 6.5.

Quite another result is obtained for CAP monolayers (Fig. 3). At a pH of 3.1, the π–A curve exhibits a condensed region at areas smaller than about 0.8 m.²/mg. In the same region, the monolayer on a subphase of pH 6.5 appears to be much more expanded. Monolayer collapse pressure at a pH of 3.1 is 16.6 dynes/cm. The corresponding value for a pH of 6.5 is 11.4 dynes/cm. At a pH of 3.1, the extrapolated area at zero pressure of the film is 0.89 m.²/mg., increasing to 1.56 m.²/mg. at a subphase of pH of 6.5.

DISCUSSION

Monolayers of CAB and CAS were virtually unaffected when subphase pH was raised from about 3 to 6.5, whereas large changes occurred in the monolayer properties of CAP. These changes may reflect an alteration in conformation at the interface, which CAB and CAS do not undergo. Therefore, it may be inferred that neither CAB nor CAS could function as an effective enteric coating. Because the properties of these polymers are independent of pH, tablet disintegration could occur in: (*a*) *either* the stomach or small intestine, or (*b*) *neither* the stomach nor small intestine. For both of these polymers, the second alternative was actually found to be the case (4).

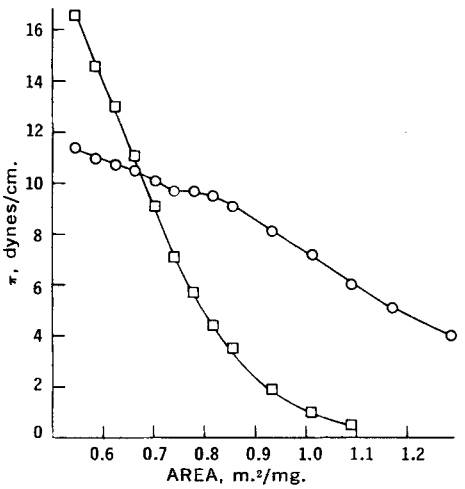


Figure 3—*Surface pressure (π)–area (A) isotherms of CAP at two subphase pH values:* □, *pH = 3.1; and* ○, *pH = 6.5.*

Table I—Some Properties of Unionized Cellulose Esters

Polymer	Collapse Pressure, dynes/cm.	Extrapolated Area at Zero Pressure, m.²/mg.
CAB	7.0	0.82
CAS	19.4	0.74
CAP	16.6	0.89

The change in monolayer properties of CAP with pH is due to the presence of the ionizable carboxyl group in the phthalate moiety, which is presumably uncharged at a pH of 3.1 but negatively charged at a pH of 6.5. The satisfactory performance of CAP as an enteric coating has been ascribed to its insolubility in the strongly acid stomach fluids, where the polymer is uncharged, and its corresponding solubility in the weakly acid small intestine, in which the polymer is charged (10).

A very important property of polymer coatings is permeability to moisture. Moisture permeates polymer films by simple diffusion and by an additional mechanism involving clustering of water molecules about polar centers (11). In a series of related polymers, the most hydrophobic will generally exhibit the lowest moisture permeability.

It is instructive to compare the collapse pressures of the unionized cellulose esters as given in Table I. Collapse pressure, a measure of monolayer stability, is dependent on the balance between adhesional and cohesional forces in the film (12). In a series of compounds, those that exhibit strong intermolecular interactions will have higher collapse pressures. The most important contribution to monolayer stability appears to arise from van der Waals' interactions between hydrophobic groups. Thus, for a group of monolayers of similar constitution, monolayer collapse pressure may be taken as a qualitative measure of "hydrophobicity." Such a relationship has been demonstrated for the half-esters of poly(methyl vinyl ether/maleic anhydride) (3). Based on collapse pressure values, one may conclude that CAS is more hydrophobic than CAB and that films of CAS should be less permeable to moisture than films of CAB. This predicted order is in agreement with experimental results (5). CAP differs from the other cellulose derivatives studied because it contains an additional polar group, the carboxyl group. Its presence would be expected to increase the interaction of CAP with the subphase and to influence the monolayer collapse pressure. For this reason the collapse pressure of monolayers of CAP should, strictly speaking, not be compared with those of CAB and CAS to predict relative moisture permeability of films. Nevertheless, values for both collapse pressure (Table I) and moisture resistance (5) increase in the order CAB, CAP, CAS.

By using polymer monolayers as a model for enteric and film coatings, a great deal of basic information may be obtained within a relatively short time. This new approach may be utilized in the evaluation of new polymers and of polymer mixtures as potential coating materials, and it will hopefully result in the introduction of novel formulations with superior properties.

REFERENCES

(1) J. L. Zatz, N. D. Weiner, and M. Gibaldi, *J. Pharm. Sci.*, **57**, 1440(1968).

(2) J. L. Zatz, N. D. Weiner, and M. Gibaldi, to be published.

(3) J. L. Zatz, N. D. Weiner, and M. Gibaldi, *J. Pharm. Sci.*, **58**, 1493(1969).

(4) H. J. Antonides and H. G. DeKay, *Drug Stand.*, **21**, 205 (1953).

(5) M. Patel, J. M. Patel, and A. P. Lemberger, *J. Pharm. Sci.*, **53**, 286(1964).

(6) L. Lachman and A. Drubulis, *ibid.*, **53**, 639(1964).

(7) J. L. Zatz, *ibid.*, **59**, 117(1970).

(8) A. Felmeister, M. Amanat, and N. Weiner, *Environ. Sci. Technol.*, **2**, 40(1968).

(9) F. MacRitchie and N. F. Owens, *J. Colloid Interface Sci.*, **29**, 66(1969).

(10) L. C. Schroeter, in "Remington's Pharmaceutical Sciences,"

13th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965, p. 595.
(11) V. Stannett and J. L. Williams, *J Polym. Sci., Part C*, **10**, 45(1965).
(12) N. Beredjick, in "Newer Methods of Polymer Characterization," B. Ke, Ed., Interscience, New York, N. Y., 1964, p. 677.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 28, 1969, from the *Department of Pharmacy, College of Pharmacy, Rutgers University, Newark, NJ 07104*
Accepted for publication March 9, 1970.

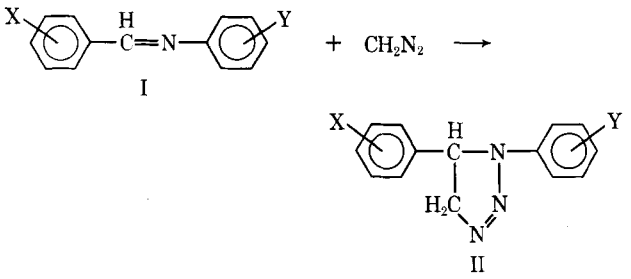
Triazolines VI: Evaluation of 1,5-Diaryl-Δ^2-1,2,3-triazolines and Arylidene Anilines for Herbicidal Activity

PANKAJA K. KADABA

Abstract □ A large number of 1,5-diaryl-Δ^2-1,2,3-triazolines and arylidene anilines (from which the triazolines are derived) have been examined for the first time for herbicidal activity. Although the majority of the compounds as a class showed no activity, those bearing 3 or 4 halogen substituents evinced slight activity in postemergence tests.

Keyphrases □ 1,5-Diaryl-Δ^2-1,2,3-triazolines—herbicidal activity □ Arylidene anilines—herbicidal activity □ Herbicidal activity—1,5-diaryl-Δ^2-1,2,3-triazolines, arylidene anilines

Studies in the author's laboratories on solvation effects and the role of protic–dipolar aprotic solvents in 1,3-cycloaddition reactions (1–5) have helped pave the way to the proper understanding and application of solvation energy to assist bimolecular cycloaddition reactions. Thus, the accelerating effect of water on the 1,3-cycloaddition of diazomethane to Schiff bases (arylidene anilines) (I) has led to a versatile general method for the synthesis of the rarely encountered Δ^2-1,2,3-triazolines (II) (3, 6) (Scheme I). By carrying out



Scheme I

the addition reaction in aqueous dioxane solutions, a variety of previously unknown 1,5-diaryl-1,2,3-triazolines have been obtained in good yields. Earlier methods of syntheses (7, 8) have either failed to give a triazoline adduct or have yielded only insignificant amounts of the products. As a result, there is no reference in the literature to any studies on the biological properties of this group of heterocyclic compounds. The cycloaddition reaction in aqueous dioxane solutions has now made a large number of 1,2,3-triazolines readily available in sufficient quantities to permit, for the first time, a detailed screening of these compounds for biological activity. In this paper, a brief report on the results of screening for herbicidal activity is presented.

About 30 1,5-diaryl-1,2,3-triazolines (II) bearing one or two substituent groups on the *C*-phenyl and/or the *N*-phenyl and the respective arylidene anilines (I), from which the triazolines are derived, were screened for preemergence, postemergence, and defoliant activities. Although the majority of the 1,2,3-triazolines and arylidene anilines showed no activity, compounds bearing 3 or 4 halogen substituents evinced slight activity. The latter compounds caused visible chlorosis, contact and formative effects, and necrosis in the broadleaf species in postemergence applications; the cereals, however, were unaffected. Both cereals and broadleaf species also were not affected in preemergence or defoliant tests.

EXPERIMENTAL

The 1,5-diaryl-1,2,3-triazolines and arylidene anilines containing such substituents as the nitro, chloro, bromo, methyl, methoxy, or carbalkoxy groups on the *C*-phenyl and/or the *N*-phenyl ring, in the *o*-, *m*-, or *p*-positions (with the exception of the carbalkoxy groups, which were present only in the *p*-position of the *N*-phenyl ring) were previously prepared in the author's laboratory (1–6). The *p*-aminobenzoic esters necessary for the preparation of the arylidene *p*-aminobenzoates were synthesized, in a convenient one-step reaction, by refluxing the *p*-aminobenzoic acid with excess alcohol in the presence of commercial boron trifluoride ethyl ether as the catalyst (9).

The test compounds were dissolved in acetone containing 0.5% polysorbate 20[1] and sprayed vertically onto potted seedlings aged 7 days from planting at the time of treatment. The spray volume used was 12 ml. directed evenly over 3 sq. ft. of area, and the spray rates were such that applications equivalent to 0.1 and 1 lb./acre were obtained. Each rate was applied to 12 pots simultaneously (two pots of each of the six species used). Observations were then made from four plants of each broadleaf species (two plants per pot) and 20 plants of each cereal species (10 plants per pot) at intervals of 1–2, 5, and 10–14 days for individual visual effects, the latter comprised of abscission, chlorosis, contact and formative effects, curvature, galling, killing, necrosis, abnormal pigmentation, quilling, adventitious root formation, and stunting.

Compounds bearing one or two halogen atoms, either alone or in conjunction with another group, showed little activity. Those bearing 3 or 4 halogen atoms, either alone or otherwise, produced visual effects, the tetrahalogen compounds being more powerful. Among those tested, 1-(3,4-dichlorophenyl)-5-(2,4-dichlorophenyl)-1,2,3-triazoline and 2,4-dichlorobenzylidene-3-chloroaniline appeared to be the most active and produced a greater variety of visual effects in a greater variety of crops than any of the other compounds.

[1] Tween 20, Atlas Chemical Industries, Wilmington, Del.

13th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965, p. 595.
(11) V. Stannett and J. L. Williams, *J Polym. Sci., Part C*, **10**, 45(1965).
(12) N. Beredjick, in "Newer Methods of Polymer Characterization," B. Ke, Ed., Interscience, New York, N. Y., 1964, p. 677.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 28, 1969, from the *Department of Pharmacy, College of Pharmacy, Rutgers University, Newark, NJ 07104*
Accepted for publication March 9, 1970.

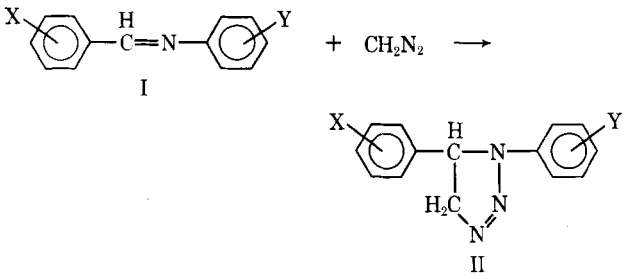
Triazolines VI: Evaluation of 1,5-Diaryl-Δ^2-1,2,3-triazolines and Arylidene Anilines for Herbicidal Activity

PANKAJA K. KADABA

Abstract □ A large number of 1,5-diaryl-Δ^2-1,2,3-triazolines and arylidene anilines (from which the triazolines are derived) have been examined for the first time for herbicidal activity. Although the majority of the compounds as a class showed no activity, those bearing 3 or 4 halogen substituents evinced slight activity in postemergence tests.

Keyphrases □ 1,5-Diaryl-Δ^2-1,2,3-triazolines—herbicidal activity □ Arylidene anilines—herbicidal activity □ Herbicidal activity—1,5-diaryl-Δ^2-1,2,3-triazolines, arylidene anilines

Studies in the author's laboratories on solvation effects and the role of protic–dipolar aprotic solvents in 1,3-cycloaddition reactions (1–5) have helped pave the way to the proper understanding and application of solvation energy to assist bimolecular cycloaddition reactions. Thus, the accelerating effect of water on the 1,3-cycloaddition of diazomethane to Schiff bases (arylidene anilines) (I) has led to a versatile general method for the synthesis of the rarely encountered Δ^2-1,2,3-triazolines (II) (3, 6) (Scheme I). By carrying out



Scheme I

the addition reaction in aqueous dioxane solutions, a variety of previously unknown 1,5-diaryl-1,2,3-triazolines have been obtained in good yields. Earlier methods of syntheses (7, 8) have either failed to give a triazoline adduct or have yielded only insignificant amounts of the products. As a result, there is no reference in the literature to any studies on the biological properties of this group of heterocyclic compounds. The cycloaddition reaction in aqueous dioxane solutions has now made a large number of 1,2,3-triazolines readily available in sufficient quantities to permit, for the first time, a detailed screening of these compounds for biological activity. In this paper, a brief report on the results of screening for herbicidal activity is presented.

About 30 1,5-diaryl-1,2,3-triazolines (II) bearing one or two substituent groups on the *C*-phenyl and/or the *N*-phenyl and the respective arylidene anilines (I), from which the triazolines are derived, were screened for preemergence, postemergence, and defoliant activities. Although the majority of the 1,2,3-triazolines and arylidene anilines showed no activity, compounds bearing 3 or 4 halogen substituents evinced slight activity. The latter compounds caused visible chlorosis, contact and formative effects, and necrosis in the broadleaf species in postemergence applications; the cereals, however, were unaffected. Both cereals and broadleaf species also were not affected in preemergence or defoliant tests.

EXPERIMENTAL

The 1,5-diaryl-1,2,3-triazolines and arylidene anilines containing such substituents as the nitro, chloro, bromo, methyl, methoxy, or carbalkoxy groups on the *C*-phenyl and/or the *N*-phenyl ring, in the *o*-, *m*-, or *p*-positions (with the exception of the carbalkoxy groups, which were present only in the *p*-position of the *N*-phenyl ring) were previously prepared in the author's laboratory (1–6). The *p*-aminobenzoic esters necessary for the preparation of the arylidene *p*-aminobenzoates were synthesized, in a convenient one-step reaction, by refluxing the *p*-aminobenzoic acid with excess alcohol in the presence of commercial boron trifluoride ethyl ether as the catalyst (9).

The test compounds were dissolved in acetone containing 0.5% polysorbate 20[1] and sprayed vertically onto potted seedlings aged 7 days from planting at the time of treatment. The spray volume used was 12 ml. directed evenly over 3 sq. ft. of area, and the spray rates were such that applications equivalent to 0.1 and 1 lb./acre were obtained. Each rate was applied to 12 pots simultaneously (two pots of each of the six species used). Observations were then made from four plants of each broadleaf species (two plants per pot) and 20 plants of each cereal species (10 plants per pot) at intervals of 1–2, 5, and 10–14 days for individual visual effects, the latter comprised of abscission, chlorosis, contact and formative effects, curvature, galling, killing, necrosis, abnormal pigmentation, quilling, adventitious root formation, and stunting.

Compounds bearing one or two halogen atoms, either alone or in conjunction with another group, showed little activity. Those bearing 3 or 4 halogen atoms, either alone or otherwise, produced visual effects, the tetrahalogen compounds being more powerful. Among those tested, 1-(3,4-dichlorophenyl)-5-(2,4-dichlorophenyl)-1,2,3-triazoline and 2,4-dichlorobenzylidene-3-chloroaniline appeared to be the most active and produced a greater variety of visual effects in a greater variety of crops than any of the other compounds.

[1] Tween 20, Atlas Chemical Industries, Wilmington, Del.

REFERENCES

(1) P. K. Kadaba, *J. Heterocycl. Chem.*, **6**, 587(1969).
(2) P. K. Kadaba and J. O. Edwards, *J. Org. Chem.*, **26**, 2331 1961).
(3) P. K. Kadaba, *Tetrahedron*, **22**, 2453(1966).
(4) *Ibid.*, **25**, 3053(1969).
(5) P. K. Kadaba and T. F. Colturi, *J. Heterocycl. Chem.*, **6**, 829(1969).
(6) P. K. Kadaba and N. F. Fannin, *ibid.*, **4**, 301(1967).
(7) A. Mustafa, *J. Chem. Soc.*, **1949**, 234.
(8) G. D. Buckley, *ibid.*, **1954**, 1850.
(9) P. K. Kadaba, M. Tribo, M. Carr, J. Triplett, and A. C. Glasser, *J. Pharm. Sci.*, **58**, 1442(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 26, 1970, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, University of Kentucky, Lexington, KY 40506*

Accepted for publication March 10, 1970.

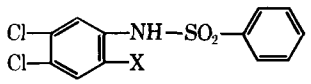
Preparation and Antitumor Activity of Some Schiff Bases of 2′-Amino-4′,5′-dichlorobenzenesulfonanilide and 2′-Amino-*p*-toluenesulfonanilide

JOHN H. BILLMAN and ROBERT L. SCHMIDGALL

Abstract □ Series of variously substituted salicylaldehyde Schiff bases and 2-substituted-*p*-[*N*,*N*-bis(2-chloroethyl)amino]benzaldehyde Schiff bases of 2′-amino-4′,5′-dichlorobenzenesulfonanilide and 2′-amino-*p*-toluenesulfonanilide have been prepared and screened for antitumor activity. None of the compounds showed appreciable activity against L-1210 leukemia.

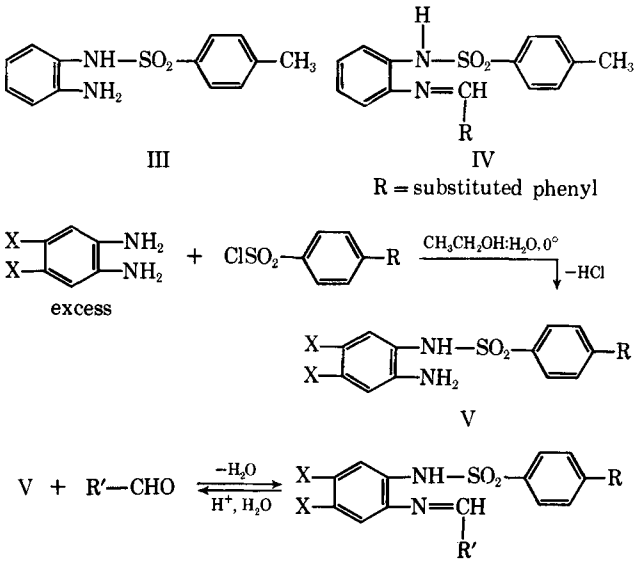
Keyphrases □ 2′-Amino-4′,5′-dichlorobenzenesulfonanilide, Schiff bases—synthesis, antitumor activity evaluation □ 2′-Amino-*p*-toluenesulfonanilide, Schiff bases—synthesis, antitumor activity evaluation □ Antitumor activity evaluation—2′-amino-4′,5′-dichlorobenzenesulfonanilide, 2′-amino-*p*-toluenesulfonanilide □ IR spectrophotometry—structure, analysis

Woolley *et al.* (1–3) have shown that 4′,5′-dichloro-2′-nitrobenzenesulfonanilide (I*a*) is effective in permanently curing some spontaneous mammary cancers of mice. Evidently the not very toxic I*a* functions as an antimetabolite of 1,2-dimethyl-4,5-diaminobenzene and inhibits the biosynthesis of vitamin B_{12}, which is synthesized by the spontaneous cancers but not by the hosts (4). 2′-Amino-4′,5′-dichlorobenzenesulfonanilide (I*b*) was also apparently active but much less potent than I*a*.



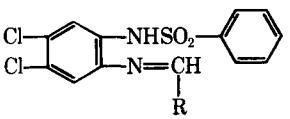
I*a*, X = —NO_2
I*b*, X = —NH_2

In view of the antitumor activity or at least accessibility to the tumor site of I*a* and I*b* plus the convenient handle of the primary amino group of I*b* for further structural modifications, it was decided to prepare some derivatives of Type II. Twelve of these derivatives (Table I) were conveniently synthesized (Scheme I) by condensing the desired aldehyde with I*b*, which was prepared from the corresponding *o*-phenylenediamine and arylsulfonyl chloride. The aldehydes employed were those substituted salicylaldehydes and 2-substituted-*p*-[*N*,*N*-bis(2-chloroethyl)amino]benzaldehydes which have previously shown antitumor activity



Scheme I

either in their own rights or in easily hydrolyzed derivatives (5–15). It was hoped that these new azomethine derivatives (II) would be even more potent antineoplastic drugs than either the active parent amine I*b* or the active aldehyde alone.



R = substituted phenyl
II

It is well known that a majority of tumors contain cells with a lower pH than cells in normal tissues. Fitch and Voegtlin (16) also have shown that the administration of glucose to tumor-bearing animals can produce an even lower pH value for the tumor cells. Since Schiff bases are one class of compounds that hydrolyze readily *in vitro* under mildly acidic conditions,

REFERENCES

(1) P. K. Kadaba, *J. Heterocycl. Chem.*, **6**, 587(1969).
(2) P. K. Kadaba and J. O. Edwards, *J. Org. Chem.*, **26**, 2331 1961).
(3) P. K. Kadaba, *Tetrahedron*, **22**, 2453(1966).
(4) *Ibid.*, **25**, 3053(1969).
(5) P. K. Kadaba and T. F. Colturi, *J. Heterocycl. Chem.*, **6**, 829(1969).
(6) P. K. Kadaba and N. F. Fannin, *ibid.*, **4**, 301(1967).
(7) A. Mustafa, *J. Chem. Soc.*, **1949**, 234.
(8) G. D. Buckley, *ibid.*, **1954**, 1850.
(9) P. K. Kadaba, M. Tribo, M. Carr, J. Triplett, and A. C. Glasser, *J. Pharm. Sci.*, **58**, 1442(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 26, 1970, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, University of Kentucky, Lexington, KY 40506*

Accepted for publication March 10, 1970.

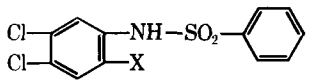
Preparation and Antitumor Activity of Some Schiff Bases of 2′-Amino-4′,5′-dichlorobenzenesulfonanilide and 2′-Amino-*p*-toluenesulfonanilide

JOHN H. BILLMAN and ROBERT L. SCHMIDGALL

Abstract □ Series of variously substituted salicylaldehyde Schiff bases and 2-substituted-*p*-[*N*,*N*-bis(2-chloroethyl)amino]benzaldehyde Schiff bases of 2′-amino-4′,5′-dichlorobenzenesulfonanilide and 2′-amino-*p*-toluenesulfonanilide have been prepared and screened for antitumor activity. None of the compounds showed appreciable activity against L-1210 leukemia.

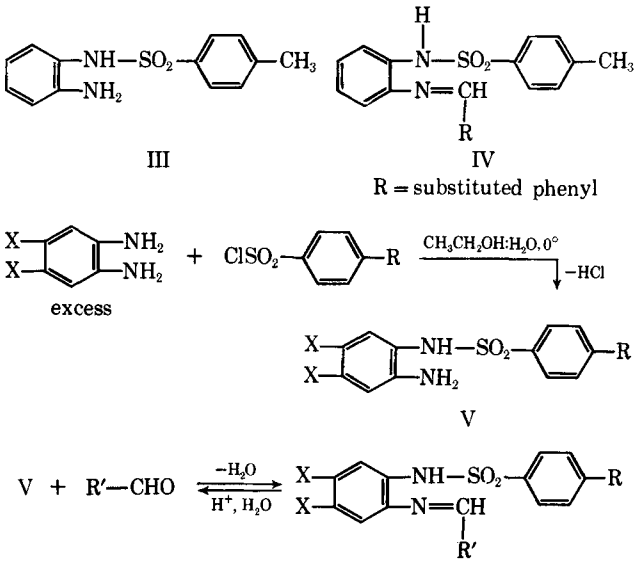
Keyphrases □ 2′-Amino-4′,5′-dichlorobenzenesulfonanilide, Schiff bases—synthesis, antitumor activity evaluation □ 2′-Amino-*p*-toluenesulfonanilide, Schiff bases—synthesis, antitumor activity evaluation □ Antitumor activity evaluation—2′-amino-4′,5′-dichlorobenzenesulfonanilide, 2′-amino-*p*-toluenesulfonanilide □ IR spectrophotometry—structure, analysis

Woolley *et al.* (1–3) have shown that 4′,5′-dichloro-2′-nitrobenzenesulfonanilide (I*a*) is effective in permanently curing some spontaneous mammary cancers of mice. Evidently the not very toxic I*a* functions as an antimetabolite of 1,2-dimethyl-4,5-diaminobenzene and inhibits the biosynthesis of vitamin B_{12}, which is synthesized by the spontaneous cancers but not by the hosts (4). 2′-Amino-4′,5′-dichlorobenzenesulfonanilide (I*b*) was also apparently active but much less potent than I*a*.



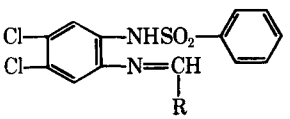
I*a*, X = —NO_2
I*b*, X = —NH_2

In view of the antitumor activity or at least accessibility to the tumor site of I*a* and I*b* plus the convenient handle of the primary amino group of I*b* for further structural modifications, it was decided to prepare some derivatives of Type II. Twelve of these derivatives (Table I) were conveniently synthesized (Scheme I) by condensing the desired aldehyde with I*b*, which was prepared from the corresponding *o*-phenylenediamine and arylsulfonyl chloride. The aldehydes employed were those substituted salicylaldehydes and 2-substituted-*p*-[*N*,*N*-bis(2-chloroethyl)amino]benzaldehydes which have previously shown antitumor activity



Scheme I

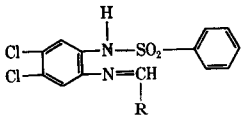
either in their own rights or in easily hydrolyzed derivatives (5–15). It was hoped that these new azomethine derivatives (II) would be even more potent antineoplastic drugs than either the active parent amine I*b* or the active aldehyde alone.

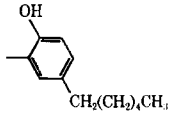
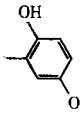
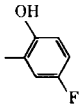
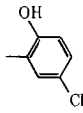
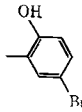
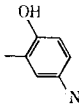
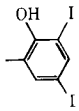
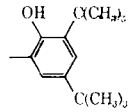
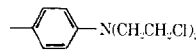
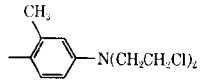
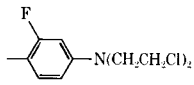


R = substituted phenyl
II

It is well known that a majority of tumors contain cells with a lower pH than cells in normal tissues. Fitch and Voegtlin (16) also have shown that the administration of glucose to tumor-bearing animals can produce an even lower pH value for the tumor cells. Since Schiff bases are one class of compounds that hydrolyze readily *in vitro* under mildly acidic conditions,

Table I—Schiff Base Derivatives of 2′-Amino-4′,5′-dichlorobenzenesulfonanilide



Compd. No.	R	Formula	Yield (pure), %	M.p.	% N Calcd.	% N Found	IR Absorptions, μ —NH- SO_2—	IR Absorptions, μ —C=N—
1		$C_{19}H_{14}Cl_2N_2O_3S$	87.0	166–168°[a]	6.64	6.50	3.14 7.50 8.57	6.20
2		$C_{25}H_{26}Cl_2N_2O_3S$	86.5	135.5–137.5°[a]	5.54	5.53	3.11 7.50 8.60	6.19
3		$C_{20}H_{16}Cl_2N_2O_4S$	92.0	189.0–190.5°[b]	6.21	5.99	3.08 7.54 8.64	6.18
4		$C_{19}H_{13}Cl_2FN_2O_3S$	89.5	179–181°[a]	6.38	6.28	3.08 7.56 8.64	6.16
5		$C_{19}H_{13}Cl_3N_2O_3S$	65.8	181–183°[a]	6.15	6.02	3.16 7.73 8.73	6.20
6		$C_{19}H_{13}BrCl_2N_2O_3S$	70.9	185–186°[a]	5.60	5.66	3.15 7.72 8.72	6.19
7		$C_{19}H_{13}Cl_2N_3O_5S$	76.5	208–210°[e] dec.	9.01	9.09	3.07 7.56 8.60	6.21
8		$C_{19}H_{12}Cl_2I_2N_2O_3S$	91.9	206–208°[d] dec.	4.16	4.46	3.28 7.66 8.63	6.19
9		$C_{27}H_{30}Cl_2N_2O_3S$	82.1	191–193°[a]	5.25	5.28	3.06 7.59 8.59	6.19
10		$C_{23}H_{21}Cl_4N_3O_2S$	89.5	192–194°[b]	7.71	7.66	3.10 7.35 8.59	6.27
11		$C_{24}H_{23}Cl_4N_3O_2S$	94.3	167–169°[b]	7.51	7.36	3.11 7.40 8.57	6.28
12		$C_{23}H_{20}Cl_2FN_3O_2S$	83.7	173–175°[c]	7.46	7.34	3.08 7.41 8.61	6.22

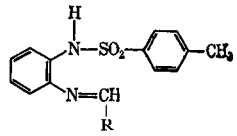
[a] Recrystallized from 2-propanol. [b] Recrystallized from 4:1 2-propanol–acetonitrile. [c] Recrystallized from 1:1 2-propanol–acetonitrile. [d] Recrystallized from 10:1 acetonitrile–*N*,*N*-dimethylformamide.

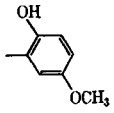
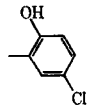
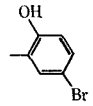
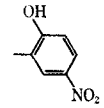
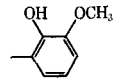
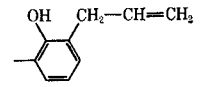
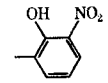
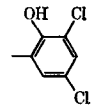
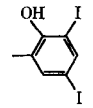
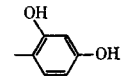
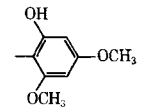
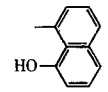
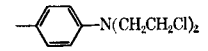
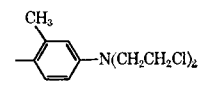
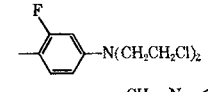
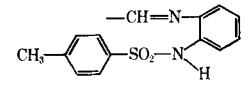
such compounds probably could be hydrolyzed selectively by the tumor cells to liberate the active aldehydes to serve as alkylating agents at the same time as the active amine is freed to act as an antimetabolite.

Even if hydrolysis of the Schiff bases does not occur at the tumor site, the compounds might still be active. Ross *et al.* (10) have pointed out that the azomethine linkage in these compounds can be regarded as an isostere of two cytotoxic agents: the azo compounds and the stilbenes.

Seventeen Schiff bases (IV) (Table II) of 2′-amino-*p*-toluenesulfonanilide (III) were also prepared (Scheme I), using the same or similar aldehydes as were used in the synthesis of Compounds II. Here, if the derivatives IV undergo hydrolysis in the mildly acidic tumor cells, the active aldehydes could still be liberated to serve as

Table II—Schiff Base Derivatives of 2′-Amino-*p*-toluenesulfonanilide



Compd. No.	R	Formula	Yield (pure), %	M.p.	%N Calcd.	%N Found	IR Absorptions, μ —NH— SO_2—	IR Absorptions, μ —C=N—
1		$C_{20}H_{18}N_2O_3S$	98.2	143–144°[a]	7.65	7.82	3.05 7.60 8.73	6.21
2		$C_{21}H_{20}N_2O_4S$	89.5	174–175°[a]	7.07	7.23	3.06 7.51 8.59	6.19
3		$C_{20}H_{17}ClN_2O_3S$	90.2	154–155°[a]	6.99	7.25	3.09 7.55 8.65	6.22
4		$C_{20}H_{17}BrN_2O_3S$	81.9	159.5–160.5°[a]	6.29	6.34	3.08 7.50 8.59	6.29
5		$C_{20}H_{17}N_3O_5S$	96.0	196–197°[b]	10.21	10.44	3.17 7.60 8.72	6.28
6		$C_{21}H_{20}N_2O_4S$	95.1	140.5–142.5°[a]	7.07	6.88	3.11 7.58 8.67	6.23
7		$C_{23}H_{22}N_2O_3S$	82.4	120.0–121.5°[a]	6.89	7.00	3.09 7.55 8.61	6.21
8		$C_{20}H_{17}N_3O_5S$	93.6	161–163°[a]	10.21	10.31	3.10 7.53 8.64	6.19
9		$C_{20}H_{15}Cl_2N_2O_3S$	85.0	171.5–173.5°[a]	6.43	6.20	3.06 7.55 8.58	6.14
10		$C_{20}H_{16}I_2N_2O_3S$	83.9	171–173°[c] dec.	4.53	4.78	3.08 7.60 8.64	6.20
11		$C_{20}H_{16}N_2O_4S$	87.6	188–189°[a] dec.	7.32	7.17	3.11 7.50 8.63	6.10 6.18
12		$C_{22}H_{22}N_2O_5S$	56.3	195–197°[b]	6.57	6.70	3.05 7.50 8.57	6.16
13		$C_{24}H_{20}N_2O_3S$	96.2	186.5–187.5°[d]	6.73	6.52	3.10 7.56 8.60	6.16
14		$C_{24}H_{25}Cl_2N_3O_2S$	94.1	125–126°[b]	8.57	8.50	3.11 7.42 8.64	6.25
15		$C_{25}H_{27}Cl_2N_3O_2S$	98.6	146–147°[b]	8.33	8.42	3.10 7.40 8.62	6.21
16		$C_{24}H_{24}Cl_2FN_3O_2S$	75.5	163–164°[b]	8.26	8.19	3.07 7.39 8.62	6.19
17		$C_{28}H_{26}N_4O_4S_2$	40.4	132–133°[e] dec.	10.25	9.96	2.93 7.40 8.55	6.24

[a] Recrystallized from 2-propanol. [b] Recrystallized from 4:1 2-propanol–acetonitrile. [c] Recrystallized from 1:1 2-propanol–acetonitrile. [d] Recrystallized from acetonitrile. [e] Washed with water, acetone, methanol, and diethyl ether but not recrystallized from any solvent.

alkylating agents but the freed amine (III) would not have any antitumor activity (17). Thus, the primary functions of III would be to serve as a carrier of the active aldehydes to the tumor site and to direct them into the cellular metabolism. III could also serve as a protector of the aldehydes and prevent their destruction through oxidation, reduction, or whatever before they reached the tumor cells where they could be freed to function as antitumor agents.

Antitumor evaluation of the compounds in Tables I and II was carried out[1] using the test system for leukemia L-1210 (intraperitoneal). No appreciable activity was shown by any of the compounds in Tables I and II with this particular test system.

EXPERIMENTAL[2]

2′-Amino-4′,5′-dichlorobenzenesulfonanilide, Ib—Seventy-five grams (0.424 mole) of 4,5-dichloro-*o*-phenylenediamine was suspended in 800 ml. of a 3:2 ethanol–water mixture and cooled to 0–5° in an ice bath. With vigorous stirring and maintenance of the temperature at 0–5°, 61.8 g. (0.350 mole) of benzenesulfonyl chloride was added dropwise over 5 hr. After complete addition, the stirring was continued for 4 hr. with the ice bath removed. Three hundred milliliters of 2.0 *N* hydrochloric acid was then added, and the mixture was stirred another hour. Suction filtration of the reaction mixture provided a wine-red solution and a purple-gray precipitate. The solid was recrystallized from 2-propanol with the hot mother liquor being decolorized with activated charcoal. White crystals of I*b* were obtained after two further recrystallizations from 2-propanol. Yield: 44.8 g., 40.4%. M.p. (lit.) 158° (2); (obs.) 163–165°. IR absorptions, μ: —$NHSO_2$—, 3.10, 7.60, and 8.64; —NH_2, 2.90, 3.00, and 6.20.

Anal.—Calcd. for $C_{12}H_{10}Cl_2N_2O_2S$: N, 8.83. Found: N, 8.80.

Recovery of 22.8 g. of 4,5-dichloro-*o*-phenylenediamine was effected by neutralization of the wine-red solution with 50% aqueous sodium hydroxide.

2′-Amino-4′,5′-dichlorobenzenesulfonanilide Schiff Base Derivatives, II (Table I)—The synthesis of this series of compounds is illustrated by the preparation of 2′-{[5-(*n*-hexyl)salicylidene]amino}-4′,5′-dichlorobenzenesulfonanilide. To a stirring solution of 2.30 g. (0.00730 mole) of 2′-amino-4′,5′-dichlorobenzenesulfonanilide in 50 ml. of absolute ethanol was added dropwise, over 15 min., 1.50 g. (0.00750 mole) of 5-(*n*-hexyl)salicylaldehyde (18) dissolved in 75 ml. of absolute ethanol. The mixture was refluxed for 15 min., chilled, and filtered. The tan solid obtained was recrystallized from 2-propanol to give fluffy, light-yellow crystals. Yield: 3.20 g., 86.5%. M.p. 135.5–137.5°.

Anal.—Calcd. for $C_{25}H_{26}Cl_2N_2O_3S$: N, 5.54. Found: N, 5.53.

2′-Amino-*p*-toluenesulfonanilide, III—A solution of 48.7 g. (0.450 mole) of *o*-phenylenediamine in 300 ml. of a 1:1 aqueous ethanol mixture was cooled to 0–5° in an ice bath. Over a span of 3 hr., 57.2 g. (0.300 mole) of *p*-toluenesulfonyl chloride was added in small portions with rapid stirring at 0–5°. The stirring at 0–5° was continued for another hour after the addition was completed; then the ice bath was removed. Two hundred fifty milliliters of 2.0 *N* hydrochloric acid was added, and stirring was continued for 60 min. at room temperature. After the disubstituted compound had been removed by suction filtration, the clear, dark-red filtrate was neutralized with 50% aqueous sodium hydroxide until a precipitate began to form. To complete the neutralization, solid sodium bicarbonate was slowly added with stirring until the evolution of carbon dioxide ceased. The mixture was chilled thoroughly in an ice bath and then filtered with suction. The resulting off-white solid was recrystallized from 2-propanol to give fluffy, white crystals of III. Yield: 63.8 g., 81.1%. M.p. (lit.) 114° (19), 135–136° (20), 142° (21); (obs.) 139–140°. IR absorptions, μ: —$NHSO_2$—, 3.16, 7.60, and 8.78; —NH_2, 2.91, 3.00, and 6.19.

Anal.—Calcd. for $C_{13}H_{14}N_2O_2S$: N, 10.68. Found: N, 10.65.

2′-Amino-*p*-toluenesulfonanilide Schiff Base Derivatives, IV (Table II)—The following synthesis for 2′-{[2-fluoro-4-(*N*,*N*-bis(2-chloroethyl)amino)benzylidene]amino}-*p*-toluenesulfonanilide is typical of the procedure used for preparing this series of compounds. Five and twenty-five hundredths grams (0.0200 mole) of 2′-amino-*p*-toluenesulfonanilide was dissolved in 50 ml. of dry methanol. To this was added dropwise, with stirring, a second solution of 5.03 g. (0.0220 mole) of 2-fluoro-4-[*N*,*N*-bis(2-chloroethyl)amino]-benzaldehyde (13) contained in 75 ml. of hot methanol. The addition took 15 min. and was followed by 30 min. of refluxing. The reaction mixture was cooled in an ice bath and filtered to obtain a dirty yellow solid. Recrystallization of this solid from a 4:1 2-propanol–acetonitrile mixture afforded bright-yellow crystals which were moderately light sensitive. Yield: 7.70 g., 75.5%. M.p. 163–164°.

Anal.—Calcd. for $C_{24}H_{24}Cl_2FN_3O_2S$: N, 8.26. Found: N, 8.19.

REFERENCES

(1) D. W. Woolley and J. M. Stewart, *Biochem. Pharmacol.*, **11,** 1163(1962).

(2) D. W. Woolley and J. M. Stewart, *J. Med. Chem.*, **6,** 599 (1963).

(3) D. W. Woolley and T. Van Der Hoeven, *Can. J. Chem.*, **43,** 1454(1965).

(4) D. W. Woolley, *J. Exp. Med.*, **93,** 13(1951); *Proc. Soc. Exp. Biol. Med.*, **75,** 745(1950); *J. Amer. Chem. Soc.*, **74,** 5450(1952); *Proc. Nat. Acad. Sci. USA*, **39,** 6(1953); *ibid.*, **41,** 111(1955).

(5) J. H. Billman and J. L. Meisenheimer, *J. Med. Chem.*, **6,** 682(1963).

(6) *Ibid.*, **7,** 115(1964).

(7) *Ibid.*, **8,** 540(1965).

(8) J. H. Billman and M. S. Khan, *J. Med. Chem.*, **11,** 312 (1968).

(9) J. H. Billman and M. S. Khan, *J. Pharm. Sci.*, **57,** 1817 (1968).

(10) W. C. J. Ross, G. P. Warwick, and J. J. Roberts, *J. Chem Soc.*, **1955,** 3110.

(11) F. D. Popp, *ibid.*, **1960,** 5271.

(12) F. D. Popp, *J. Org. Chem.*, **26,** 1566(1961).

(13) F. D. Popp and W. Kirsch, *ibid.*, **26,** 3858(1961).

(14) M. G. Dhapalapur, S. S. Sabnis, and C. V. Deliwala, *J. Med. Chem.*, **11,** 154(1968).

(15) *Ibid.*, **11,** 1014(1968).

(16) R. H. Fitch and G. Voegtlin, *US Pub. Health Serv. Bull.*, **164,** 15(1935).

(17) J. H. Billman, unpublished data.

(18) S. Fujikawa *et al.*, *Yakugaku Zasshi*, **70,** 22(1950).

(19) E. Riesz, R. Pollak, and R. Zifferer, *Monatsh.*, **58,** 147 (1931).

(20) J. H. Billman and R. Chernin, *Anal. Chem.*, **34,** 408(1962).

(21) The Upjohn Company, Netherlands pat. 6,512,029, Mar. 16, 1966.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 6, 1970, from the *Department of Chemistry, Indiana University, Bloomington, IN 47401*

Accepted for publication March 5, 1970.

Abstracted in part from a dissertation submitted by R. L. S. to the Graduate School, Indiana University, in partial fulfillment of Doctor of Philosophy degree requirements.

R. L. Schmidgall acknowledges the financial support of NASA and NSF in the form of predoctoral traineeships.

[1] By the Cancer Chemotherapy National Service Center (CCNSC), Bethesda, Md.

[2] All melting points were taken in open capillaries on a Thomas-Hoover melting point apparatus and are uncorrected. The elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind. The IR spectra were determined in KBr disks on a Perkin-Elmer Infracord spectrophotometer. Aldehydes and other starting materials used were either reagent grade or were purified by distillation or recrystallization from appropriate solvents.

Mechanism of Action of Retinyl Compounds on Wound Healing II: Effect of Active Retinyl Derivatives on Granuloma Formation

K. H. LEE and THEODORE G. TONG

Abstract □ The naturally occurring retinyl derivatives that promote wound healing as measured by their effects on tensile strength of healing wounds also stimulate granuloma formation induced by cotton pellets. The hexosamine and hydroxyproline contents in the granuloma affected by retinyl derivatives are also increased. The mechanisms of action of retinyl derivation on wound healing are discussed.

Keyphrases □ Retinyl compounds—effect on granuloma formation □ Granuloma formation—retinyl derivatives, effects □ Wound healing—mechanism of action, retinyl derivatives

Previous reports have shown that a few retinyl derivatives promote wound healing, and these compounds also reverse the wound-healing retardation action of anti-inflammatory agents (1–3). It is well known that inflammation and mucopolysaccharide synthesis are the two important features which are essential for subsequent healing.

One commonly used method for studying the anti-inflammatory action of drugs is measuring antigranulation effect with the cotton pellet method of Meier *et al.* (4). This method involves subcutaneous implanting of cotton pellets and measuring the size of the granuloma induced after a few days. Anti-inflammatory agents reduce the size or weight of granuloma as compared with that of the control. In the present study, it is found that those retinyl derivatives that promote healing increase the size or weight of the granuloma. These active retinyl derivatives are "inflammatory agents."

The promotion of mucopolysaccharide synthesis action of vitamin A in granulation tissue of an open wound has been demonstrated earlier (1). In the present study, it is found that both hexosamine and hydroxyproline contents in the granulomas of retinoic acid-treated animals are considerably higher than those of the controls. The mechanisms of action of retinyl derivatives on wound healing are discussed.

EXPERIMENTAL

Materials and Special Chemicals—Materials and chemicals used in this study included the following: crystalline hydroxy-L-proline;[1] retinoic acid,[1] all *trans*, Sigma grade, Type XX; synthetic crystalline, β-carotene,[1] Sigma grade, Type I; crystalline lycopene,[1] Blakeslea trispora origin; prednisone;[2] β-ionone[3] (n^{20}_D 1.584); α-ionone[3] (n^{20}_D 1.5030); glucosamine HCl,[4] reagent grade; 2,4-pentanedione[4] (acetylactone), reagent grade; *p*-dimethylaminobenzaldehyde,[4] reagent grade; dental cotton rolls,[5] size 1; polyvinyl sponge;[6] and retinyl palmitate[7] (400,000 units/g.). β-Ionone, as purchased, was a pure preparation, while α-ionone contained 31% of β-ionone. The isomer was satisfactorily separated by using a preparative gas chromatograph as described previously (3). The *p*-dimethylaminobenzaldehyde was twice recrystallized according to the procedure of Adams and Coleman (5).

METHODS

Implantation of Cotton and Polyvinyl Disks—The effect of retinyl compounds on granuloma formation in rats was evaluated with cotton pellet and polyvinyl sponge methods (4, 6). Cotton pellets are disks sliced from dental cotton rolls with a sharp razor blade. Disks weighing 20 ± 0.5 mg. were selected and placed in Petri dishes for sterilization in a steam autoclave for 40 min. The sterilized cotton disks were handled with sterilized instruments and an aseptic technique. Polyvinyl sponges were also prepared in the form of disks. The block of polyvinyl sponge was sliced with a heavy-duty butcher's cold-meat slicer into 3-mm. thick slices. A cork borer, 11 mm. in diameter, was used to cut the slices into uniform disks. The disks were then thoroughly washed with distilled water to remove the preservative and moistening agents. The washed disks were dried at 65° for 48 hr. Disks weighing 22 ± 0.5 mg. were selected and placed in Petri dishes for sterilization in a steam autoclave for 40 min.

Growth of granulation tissue into cotton pellets was induced by subcutaneous implantation at two symmetrical dorsolateral sites on Sprague-Dawley male rats weighing 120 ± 5 g. under ether anesthesia.

The cotton pellet implanted on the right side contained the retinyl compound, and the cotton pellet implanted on the left side served as the control. The compound was introduced to the pellet as its ether solution. The ether was completely evaporated before implantation. On the 7th day after implantation the animals were killed with ether and the body weights were taken. The granulomas were carefully removed and were weighed rapidly on a torsion balance.[8] After drying in an oven at 65° for 48 hr., the dried slices were weighed again.

In other experiments, two cotton disks without drug were implanted dorsolaterally and retinoic acid sodium salt was given orally or intraperitoneally on the day before the operation and on the 2nd day after the operation. On the 7th day after implantation, the granulomas formed were carefully removed, and their wet weights and dry weights were measured as described previously.

For polyvinyl sponge experiments, large Sprague-Dawley male rats weighing 230 ± 5 g. were used. Four sponge disks were implanted subcutaneously at symmetrical dorsolateral sites. Two disks containing drug and two control disks were implanted diagonally 2 cm. from each other. Prednisone was given orally on the day before the operation and also on the 2nd day after the operation. The dosage was 2.5 mg. per rat per day. On the 7th day after implantation, the granulomas were carefully removed and treated as already described.

Chemical Analysis—Individual dry disks were weighed and placed in a Pyrex 13 × 100-mm. culture tube with 1 ml. of 6 *N* HCl. The tube was sealed *in vacuo*, and the content was hydrolyzed at 140° in a constant-temperature heating block for 3 hr.[9] After cooling to room temperature, the seal of the tube was broken and the content was carefully neutralized with 6 *N* NaOH. The content was then filtered with the aid of suction. The original tube and the

[1] Sigma Chemical Co., St. Louis, Mo.
[2] The Upjohn Co., Kalamazoo, Mich.
[3] Aldrich Chemical Co., Inc., Milwaukee, Wis.
[4] Eastman Kodak Co., Rochester, N. Y.
[5] Johnson and Johnson, New Brunswick, N. J.
[6] Unipoint Laboratories, High Point, N. C.
[7] Eastman Kodak Co., N. J.
[8] Roller-Smith precision balance, Bethlehem, Pa.
[9] Hallikainen Instruments, Richmond, Calif.

Table I—Effect of Retinol and Retinoic Acid on Cotton Pellet-Induced Granuloma

	Group I		Group II	
	Left Side	Right Side (Control)	Left Side	Right Side (Control)
No. of animals	8		12	
Drug impregnated:				
Retinyl palmitate, 6000 I.U.	+	−	−	−
Retinoic acid, 2 mg.	−	−	+	−
Pellet wt., mg.	20.0 ± 0.5	20.0 ± 0.5	20.0 ± 0.5	20.0 ± 0.5
Granuloma wet wt., mg.	300.4 ± 7.2	220.3 ± 4.5	332.2 ± 4.9	218.7 ± 2.5
Left/Right	1.4		1.5	
Granuloma dry wt., mg.	54.3 ± 1.6	36.7 ± 2.0	50.9 ± 1.7	35.1 ± 1.9
Left/Right	1.5		1.5	
Change of body wt., g.	+49.5 ± 2.1		+56.1 ± 3.7	

Table II—Effect of Sodium Retinoate[a] on Granuloma Formation

	Group I	Group II	Group III
No. of animals	18	6	6
Drug given	—	RANa, oral	RANa, i.p.
Cotton pellet wt., mg.	20.0 ± 0.5	20.0 ± 0.5	20.0 ± 0.5
Granuloma wet wt., mg.	211.5 ± 4.8	264.9 ± 7.5	274.6 ± 9.9
Granuloma dry wt., mg.	28.6 ± 1.1	41.1 ± 1.6	42.3 ± 1.7

[a] Four milligrams of Na-retinoate was given to each rat on the day of operation and on the 2nd day after operation.

filter were rinsed several times with small portions of distilled water. The filtrate was brought to 10.0 ml. Aliquots were taken for hydroxyproline and glucosamine analyses. Samples containing 5–15 mg. of hydroxyproline were analyzed according to the method described by Neuman and Logan (7). Aliquots of the filtrate containing 0.010–0.030 mg. of glucosamine were analyzed according to the method of Elson and Morgan as modified by Boas (8).

RESULTS

The effects of retinoic acid and vitamin A on cotton pellet-induced granuloma are shown in Table I. Preliminary tests have been done to determine the proper amount of each compound to be used for appreciable, measurable effects. Either 6000 I.U. of retinyl palmitate or 2 mg. of retinoic acid is required to give about 50% increase in granuloma formation as compared with the control.

Both the wet weight and the dry weight of the granuloma induced by cotton pellets are about the same when either drug is applied. The granulomas of the controls are also similar in weight. The ratios of the granuloma induced by cotton pellets impregnated with either drug to the controls are all about 1.5. These differences are highly significant as determined by the Student t test ($p < 0.001$). These results clearly demonstrate that retinyl derivatives increase the size of granuloma induced by the cotton pellet. These results also indicate that retinyl palmitate and retinoic acid are of the same potency. Retinyl palmitate and retinoic acid also had the same activity when tensile strengths of healing wounds were compared (9). In both cases the rats gained body weight at a normal rate, which indicates that the amount of drug used was not showing any toxic effect.

The effects of the sodium salt of retinoic acid, given orally or intraperitoneally, on cotton pellet-induced granuloma are shown in Table II. Group I involves 18 rats receiving no drug. The average wet weight and dry weight of granuloma are about 212 and 29 mg., respectively. These values are about the same as those of the controls shown in Table I. This fact indicates that the implantation of a cotton pellet impregnated with 2 mg. of retinoic acid or 6000 I.U. of retinyl palmitate did not influence the size of their controls. In Group II, six rats were given sodium retinoate orally. The average wet weight and dry weight are significantly larger than those of Group I animals. In Group III, all six rats received sodium retinoate intraperitoneally; their granulomas are about the same as those of Group II animals but are significantly larger than those of the controls.

The effect of prednisone on granulomas induced by retinoic acid-impregnated polyvinyl sponge is shown in Table III. Group I rats received no oral administration of prednisone. The average wet and dry weights of all of the granulomas induced by polyvinyl sponges impregnated with 2 mg. of retinoic acid were 390.9 and 54.8 mg., respectively. The average wet and dry weights of granuloma induced by polyvinyl sponges were 289.5 and 32.9 mg., respectively. The ratio of wet and dry weights of the granulomas induced by retinoic acid-impregnated and control polyvinyl sponges was about 1.5. Group II rats were treated exactly as those of Group I, except prednisone was given to the rats on the day before the opera-

Table III—Effect of Prednisone on Retinoic Acid-Impregnated Polyvinyl Sponge-Induced Granuloma

	Group I (Control)		Group II (Prednisone)	
	With R. A. A	Control B	With R. A. C	Control D
No. of animals	8		6	
Retinoic acid-impregnated, 2 mg.	+	−	+	−
Sponge wt., mg.	22.0 ± 0.5		22.0 ± 0.5	
Granuloma wet wt., mg.	390.9 ± 16.9	289.4 ± 17.5	340.0 ± 15.4	240.5 ± 15.2
A/B	1.4		1.4	
Granuloma dry wt., mg.	54.8 ± 3.4	32.9 ± 3.0	46.3 ± 4.8	27.8 ± 2.3
C/D	1.6		1.6	
Granuloma wet wt., ratio				
A/C		1.15		
B/D		1.20		
Granuloma dry wt., ratio				
A/C		1.18		
B/D		1.18		

Table IV—Effect of a Few Retinol-Related Compounds on Cotton Pellet-Induced Granuloma

Group	No. of Animals	Drug Applied	Body Wt. Change, Av. g.	Granuloma Wt., mg. Wet: Expt.	Wet: Control	Wet: Expt./Control	Wet: p	Dry: Expt.	Dry: Control	Dry: Expt./Control	Dry: p
I	14	β-Carotene, 2 mg.	+53	339.0 ± 12.1	220.5 ± 9.3	1.5	<0.001	38.3 ± 2.0	26.6 ± 1.7	1.4	<0.001
II	6	Lycopene, 2 mg.	+50	244.8 ± 8.7	239.6 ± 6.4	1.0		23.6 ± 1.9	23.0 ± 1.5	1.0	
III	6	β-Ionone, 25 mg.	+47	305.3 ± 10.6	212.7 ± 7.6	1.4	<0.001	37.6 ± 2.6	24.3 ± 2.1	1.5	<0.001
IV	6	α-Ionone, 25 mg.	+49	252.0 ± 6.6	228.8 ± 3.8	1.1		27.5 ± 2.2	28.1 ± 1.7	1.0	

tion and also on the 2nd day after the operation. The average wet and dry weights of the granulomas induced by polyvinyl sponges impregnated with 2 mg. of retinoic acid were 340.0 and 46.3 mg., respectively. These weights are about 20% less than those of the animals receiving no prednisone (Group I). The wet and dry weights of the granulomas induced by polyvinyl sponges without retinoic acid were 240.5 and 27.8 mg., respectively. These weights are also about 20% less than those of the Group I animals. This experiment demonstrated again that retinoic acid enhanced granuloma formation induced by polyvinyl sponges and its activity was inhibited by prednisone. This also confirms the previous findings with another independent method: that prednisone inhibits and retinoic acid promotes healing (2, 3).

The effects of a few retinol-related, naturally occurring compounds in cotton pellet-induced granuloma are shown in Table IV. β-Carotene (Group I) enhances granuloma formation induced by the cotton pellet. It is almost as active as retinoic acid. Lycopene (Group II) has the same structure as β-carotene, except that it has an open trimethyl cyclohexene ring. It is not active. β-Ionone is active while α-ionone is not (Groups III and IV). The activities of these compounds on granuloma formation are essentially the same as their activities on the tensile strength of healing wounds (3).

The total hexosamine contents in the granulomas induced by cotton pellets in the presence and absence of 2 mg. of retinoic acid were 124.0 ± 3.5 and 94.0 ± 2.3 mcg., respectively. The dry weights of the respective granulomas were 55.4 ± 2.1 and 35.5 ± 0.8 mg. The hydroxyproline contents of the experimental and control granulomas were 259.2 ± 7.3 and 200.0 ± 8.5 mcg., respectively.

DISCUSSION

Inflammation and mucopolysaccharide synthesis are the two known important features in wound healing. Anti-inflammatory agents retard healing by their anti-inflammatory and inhibitory actions on mucopolysaccharide synthesis (2, 10–12). Retinol and a few retinyl derivatives can reverse the wound-healing retardation action of a number of anti-inflammatory agents (3, 10). This fact illustrates an important pharmacological principle of using an agent (retinol) to modify the untoward effect of a useful drug (anti-inflammatory agent). Retinol, or either one of the active retinyl derivatives alone, promotes healing (2, 3).

Meier *et al.* (4) introduced a method that makes quantitative studies of the action of cortisone on connective tissue possible. These workers used the cotton pellet as a foreign body to induce granuloma formation. Application of cortisone resulted in a diminution of granuloma size, which can be expressed quantitatively by determining its fresh and dry weights. Cortisone was effective by local as well as by general application, similar concentrations producing the same degree of inhibition of granuloma formed. Since then, this method has been extensively used for assaying steroid and nonsteroid anti-inflammatory agents. Retinol and retinyl derivatives that promote healing cause an increased granuloma mass induced by cotton pellets.

It is interesting to point out that this is the first demonstration of an agent given orally, intraperitoneally, or locally enhancing granuloma formation. This phenomenon definitely shows again the effect of retinol or its active retinyl derivatives and anti-inflammatory agents. It seems justified to call these retinyl compounds "inflammatory agents."

Grindlay and Waugh (6) used essentially the same implantation method to study tissue regeneration, except that they used polyvinyl sponges. The protein, lipid, and mucopolysaccharide contents of the granuloma have been studied.

Jackson *et al.* (13) pointed out that repair of connective tissue is the most basic feature in wound healing, and they used the formation of granuloma induced by polyvinyl sponges to study healing. Sandberg and Zederfeldt (14) found that the rate of gain in tensile strength and hydroxyproline in granuloma was directly related in both rats and rabbits. The results of the present experiment show that only those retinyl derivatives that are active in increasing tensile strength also increase the size and weight of granuloma induced by either the cotton pellet or polyvinyl sponge. Granuloma formation is a useful quantitative method to study wound healing.

The promotion of mucopolysaccharide synthesis action of retinol in granuloma tissue of an open wound has been reported (1). In the present study, both total hexosamine and hydroxyproline contents were increased in the granuloma induced by the cotton pellet impregnated with retinoic acid.

The results obtained in this study suggest that retinol and active retinyl derivatives promote healing by inducing inflammation and increase mucopolysaccharide synthesis mechanisms of action.

REFERENCES

(1) K. H. Lee and T. G. Tong, *J. Pharm. Sci.*, **57**, 1238(1968).
(2) *Ibid.*, **58**, 773(1969).
(3) K. H. Lee and T. G. Tong, to be published.
(4) R. Meier, W. Schuler, and P. Desaulles, *Experientia*, **6**, 469 (1950).
(5) R. A. Adams and G. H. Coleman, *Org. Syn.*, **2**, 17(1922).
(6) J. H. Grindlay and J. M. Waugh, *Arch. Surg.*, **63**, 288(1951).
(7) R. E. Neuman and M. A. Logan, *J. Biol. Chem.*, **184**, 299 (1950).
(8) N. F. Boas, *ibid.*, **204**, 553(1953).
(9) K. H. Lee and T. G. Tong, unpublished data.
(10) K. H. Lee and T. G. Tong, to be published.
(11) K. H. Lee and R. M. Spencer, *J. Pharm. Sci.*, **58**, 464(1969).
(12) *Ibid.*, **58**, 1152(1969).
(13) D. S. Jackson, D. B. Flickinger, and J. E. Dunphy, *Ann. N. Y. Acad. Sci.*, **86**, 943(1960).
(14) N. Sandberg and B. Zederfeldt, *Acta Chir. Scand.*, **126**, 187(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 31, 1969, from the *School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122*

Accepted for publication March 10, 1970.

This work was supported by research funds from the Academic Senate Committee on Research, University of California, San Francisco Medical Center, San Francisco, CA 94122

Presented to the Medicinal Chemistry section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

Action of Antibiotics on Respiratory Tract II: Dicloxacillin

G. BENZI*, F. BERTE, E. BERMUDEZ, and E. ARRIGONI

Abstract □ Dicloxacillin antagonizes *in vitro* the acetylcholine-, histamine-, and barium chloride-induced spasms on the dog bronchial chain. Against the histamine or acetylcholine contraction, dicloxacillin is about 2–3 times less active than ephedrine, about 2000–4000 times less active than isoprenaline, and about 5–6 times more active than ampicillin. *In situ*, the intravenous injection of dicloxacillin induces a decrease of the respiratory frequency with a moderate increase in ventilation.

Keyphrases □ Dicloxacillin—respiratory tract, effects □ Antibiotics, dicloxacillin—effect, respiratory tract □ Bronchospasm—comparison antibiotics, effects

Studies on the ampicillin action on the respiratory tract showed that *in vitro* the antibiotic antagonizes the barium chloride-, histamine-, and acetylcholine-induced spasms on the dog bronchial chains. *In situ*, the antibiotic induces at first a prompt but short increase in ventilation; subsequently, a lesser but persistent activity remains for 2 or 3 hr. (1). The plan of the present research was to study the dicloxacillin action on the airways *in vitro* and *in vivo*. The isoxazolyl group of semisynthetic penicillins has been useful in the therapy of infections caused by penicillase-producing staphylococci (2–7) and, collaterally, possesses a myolytic action on the rabbit-isolated duodenum, jejunum, and ileum; on the guinea-pig-isolated bronchial muscle; and on the rat-isolated intestine (8). Furthermore, dicloxacillin induces a relaxation *in vitro* and *in vivo* of both the terminal bile duct and ureter, normal or hypertonized by various spasmogenic agents (9, 10). The intrabiliary perfusion with dicloxacillin inhibits *in situ* the stimulation of the peripheral end of the right supradiaphragmatic vagus (9), while the perfusion into the ureter with the antibiotic induces no effect on the electrical stimulation of the hypogastric and pelvic nerves (10).

METHODS

The experiments were performed in the dog both *in vitro* and *in situ*. The following were used: dicloxacillin sodium monohydrate, of which amounts are expressed in terms of 3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolyl-penicillanic acid; ampicillin sodium salt, of which amounts are expressed in terms of D(−)-6-(α-amino-α-phenylacetamido)-penicillanic acid; *dl*-isoprenaline hydrochloride; and *l*-ephedrine hydrochloride, the amounts of which are expressed in terms of the base.

Experiments *In Vitro*—Bronchi of 38 mongrel dogs of either sex (weighing 6.6–11.4 kg.) were removed immediately after death. All tissues were carefully dissected and cut into rings, which were tied together in chains with loops of thread. The mucosa was removed to allow for greater freedom of movement of the muscle; the cartilage in each ring was cut so that only the smooth muscle bands were left joining each ring together (11). Four to six rings were suspended in a 50-ml. organ bath containing Tyrode solution gassed with 95% oxygen and 5% carbon dioxide; the temperature was 36.5–37.3°. The tonus level of the preparations was continuously recorded by a strain-gauge lever, giving a magnification of ×15 to 20, tension 200 mg., writing on a kymograph drum. The preparations were left 2 hr. before any drugs were given. Three submaximal doses of acetylcholine were administered, until regular responses were obtained, before any doses of acetylcholine itself or other agonists were tested. The action of dicloxacillin (25–400 mcg./ml.) was evaluated against the stimulation by barium chloride (100–400 mcg./ml.); for the construction of the dose–response curve, dicloxacillin activity was taken as the percent inhibition of the recorded response area by barium chloride stimulation during a 20-min. period of contact.

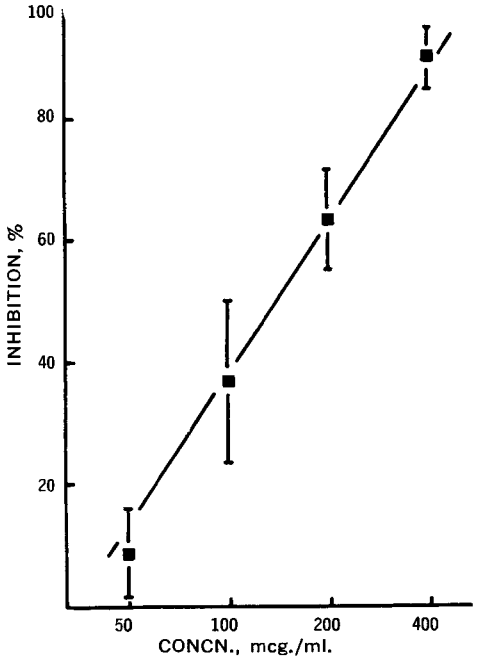


Figure 1—*Log dose–response curve of dicloxacillin action against the stimulation by barium chloride. The ordinate shows the inhibition percent of the contracting action by barium. The concentrations of the antibiotic (mcg./ml.) are plotted on the abscissa in logarithmic scale. The vertical lines indicate standard errors of the means. Six preparations at each dose level.*

For comparative assay with other *in vitro* bronchodilators, the activity of dicloxacillin (200–3200 mcg./ml.), ampicillin (2000–16,000 mcg./ml.), *l*-ephedrine (200–1600 mcg./ml.), and *dl*-isoprenaline (0.125–2.0 mcg./ml.) was evaluated against the stimulation by acetylcholine chloride (0.2–2.0 mcg./ml.) and histamine acid phosphate (1.0–10.0 mcg./ml.). Two dose levels of agonist and antagonist were usually used; the agonist doses were left in contact with the preparation for 0.5–1.0 min. Dicloxacillin and ampicillin were introduced into the bath 10 min., and ephedrine and isoprenaline 30 sec., before the acetylcholine or histamine doses. The ED_{50}, the slope of the curve, the potency ratio, and their 95% confidence limits were calculated (12).

Experiments *In Situ*—The experiments were carried out on 28 mongrel dogs of either sex (weighing 12.4–16.8 kg.) preanesthetized with urethan (0.4 g./kg. i.p.). Anesthesia was induced and maintained by chloralose (80 mg./kg. i.v.); the arterial blood pressure was measured from a cannula inserted into a femoral artery; the intestinal movement and tone were recorded by a rubber balloon inserted into the jejunum. During the succinylcholine chloride (1 mg./kg. i.v.) action, an intratracheal Warne tube was set in place. Through this tube, a little rubber balloon was pushed into a primary or, if possible, secondary bronchus under X-ray examination. Arterial blood pressure and intestinal and respiratory activity were recorded by a Physioscript EE12 Schwarzer polygraph.

Table I—Dog Bronchial Chain *In Vitro:* ED_{50}, Slope Function of Line (S) and Estimated Relative Potency (ERP), with 95% Confidence Limits, of *dl*-Isoprenaline, *l*-Ephedrine, Ampicillin, and Dicloxacillin against Stimulation by Acetylcholine Chloride (0.2–2 mcg./ml.) and Histamine Acid Phosphate (1.0–10.0 mcg./ml.)

Antagonist	Agonist		
	ED_{50}[a]	S[b]	ERP[c]
	Acetylcholine		
Isoprenaline	0.38 (0.20–0.72)	2.51 (1.04–6.02)	26,315 (9500–72,892) D
Ephedrine	520 (247–1902)	2.81 (0.97–8.16)	19 (6.44–56.05) D
Ampicillin	10,000 (4545–22,000)	2.49 (0.69–8.96)	1
Dicloxacillin	1500 (600–3750)	3.58 (0.79–10.13)	6.6 (1.79–20.10) D
	Histamine		
Isoprenaline	0.56 (0.30–1.03)	2.01 (1.12–3.63)	8214 (3042–22,178) D
Ephedrine	450 (214–945)	2.83 (0.97–8.20)	10 (3.4–29.5) D
Ampicillin	4600 (2090–10,120)	2.53 (0.70–9.10)	1
Dicloxacillin	1000 (416–2400)	3.47 (0.77–15.61)	4.6 (1.41–14.95) D

[a] ED_{50} = dose (mcg./ml.) of bronchodilator necessary to reduce by 50% the contraction produced by acetylcholine or histamine. [b] S = fold change in dose required to produce a unit standard deviation change in response along the line; thus S = antilog s = antilog $1/b$, where b and s are, respectively, the slope constant and standard deviation of a line relating log dose of antagonist and probit percent reduction of agonist activity. [c] D = ampicillin and compared drugs differ significantly in potency ($p < 0.05$).

Dicloxacillin (0.25–8.0 mg./kg.) was administered intravenously by a polystan tube inserted into the femoral vein. Two submaximal doses of *dl*-isoprenaline or *l*-adrenaline were given intravenously until regular responses were obtained before any doses of dicloxacillin were tested. To analyze the mechanism of action, dicloxacillin was tested also after: (*a*) cutting the vagi, and (*b*) treatment with atropine sulfate (2–3 mg./kg. s.c.), dibenamine hydrochloride (3–6 mg./kg. i.v.), D(−)INPEA[1] (4–8 mg./kg. i.v.), methysergide maleate (20–40 mcg./kg. s.c. + 10 mcg./kg. i.v.), chlorpheniramine maleate (2–4 mg./kg. s.c.), cyproheptadine hydrochloride (200–400 mcg./kg. i.v.), hexamethonium bromide (200–400 mcg./kg. i.v.), or morphine hydrochloride (4–8 mg./kg. i.v.).

RESULTS AND DISCUSSION

Dicloxacillin (25–400 mcg./ml.) reduces or inhibits the stimulation by barium chloride on the dog bronchial chains *in vitro;* the log dose–response curve is indicated in Fig. 1.

The comparative assay of dicloxacillin, ampicillin, isoprenaline, and ephedrine action against the stimulation by acetylcholine or histamine is indicated in Table I and shows that dicloxacillin is more active than ampicillin in reducing the agonist-induced spasm. It is possible to observe that against histamine- or acetylcholine-induced contraction, dicloxacillin is about 2–3 times less active than ephedrine, about 2000–4000 times less active than isoprenaline, and about 5–6 times more active than ampicillin.

Dicloxacillin, as ampicillin (1) and aminophylline (11), is more effective in preventing a histamine than an acetylcholine bronchospasm. Obviously, it is impossible to correlate directly the present results with clinical conditions, the *in vitro* studies being limited to denervated portions of the airways while many bronchoconstrictive stimuli act, at least in part, *via* reflex mechanisms.

[1] D(−)-1-(4-Nitrophenyl)-2-isopropylaminoethanol HCl.

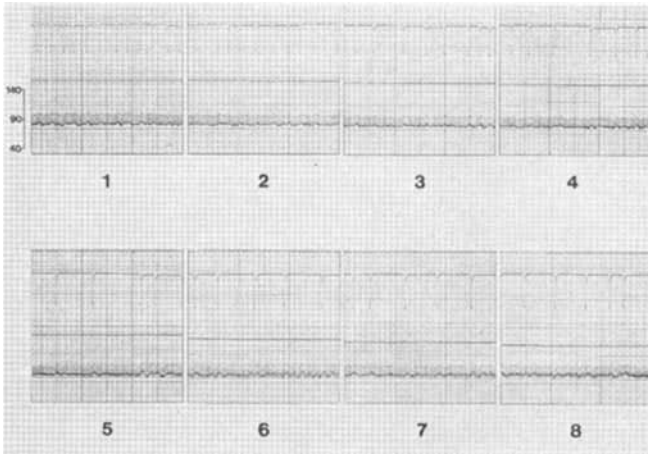


Figure 2—*Action of dicloxacillin intravenously on the ventilation of a dog* in situ. *From top to bottom: tracing recorded by a balloon inserted into the bronchus; blood pressure (mm. Hg). 1 = control condition; 2 = 5 min. after injection intravenously of 2 mg./kg. of dicloxacillin; 3 = 15 min. later; 4 = 30 min. later; 5 = 60 min. later; 6 = 75 min. later; 7 = 90 min. later; and 8 = 120 min. later.*

In situ, the intravenous injection of 0.25–8 mg./kg. of dicloxacillin induces a decrease of the respiratory frequency with a moderate increase in ventilation, as illustrated in Fig. 2. No change in systemic blood pressure occurs during the 2–3 hr. of the antibiotic action on the airways. The comparative evaluation of the action of dicloxacillin and ampicillin upon the dog ventilation is summarized in Fig. 3; ampicillin acts mainly on the depth of respiration, while dicloxacillin acts on the rate.

To analyze the mechanism of action of dicloxacillin *in situ*, it should be noted that: (*a*) the pressure recorded by the bronchial balloon technique reflects merely the effects of lung volume changes

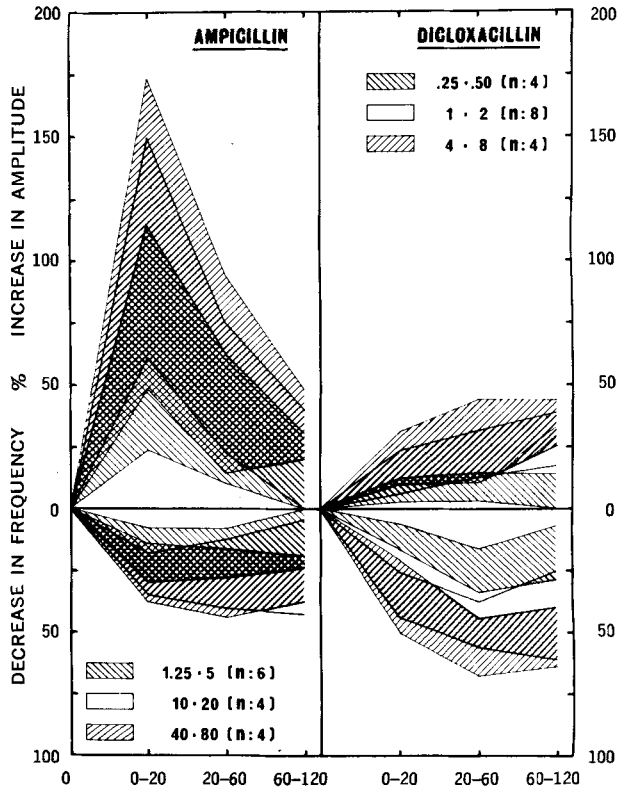


Figure 3—*Dog ventilation* in situ: *range of the percent changes (on ordinate) both in amplitude of the recorded tracing and in frequency of the respiratory rate at various periods of observation (on abscissa, in min.) after the intravenous injection of dicloxacillin (0.25–8 mg./kg.) and ampicillin (1.25–80 mg./kg.).*

on the intrathoracic airways, primarily reflecting changes in tidal volume; (*b*) the pretreatment with morphine antagonizes the action of the antibiotic; (*c*) the dicloxacillin activity is unaffected by the pretreatment with atropine, dibenamine, INPEA, methysergide, chlorpheniramine, cyproheptadine, and hexamethonium; and (*d*) the action of the antibiotic persists after cutting of the vagi.

Consequently, only *in vitro* is it possible to note the myolytic activity of the antibiotic on the bronchial musculature, while *in vivo* it is more likely that dicloxacillin, as ampicillin, induces at least in part an action on the medullary respiratory center.

REFERENCES

(1) G. Benzi, E. Bermudez, and E. Arrigoni, *J. Pharm. Sci.*, **59,** 556(1970).

(2) E. T. Knudsen, D. M. Brown, and G. N. Rolinson, *Lancet*, **2,** 632(1962).

(3) S. Sidell, R. E. Burdick, J. Brodie, R. J. Bulger, and W. M. M. Kirby, *Arch. Intern. Med.*, **112,** 21(1963).

(4) C. F. Gravenkemper, J. V. Bennett, J. L. Brodie, and W. M. M. Kirby, *ibid.*, **116,** 340(1965).

(5) C. M. Kunin, *Clin. Pharmacol. Ther.*, **7,** 166(1966)

(6) C. J. O'Connell and M. B. Lewis, *Amer. J. Clin. Pathol.*, **46,** 55(1966).

(7) G. J. Miraglia and H. I. Basch, *Appl. Microbiol.*, **15,** 566 (1967).

(8) A. Matsuzaki, *Jap. J. Antibiot.*, **21,** 274(1968).

(9) G. Benzi and E. Arrigoni, to be published.

(10) G. Benzi and E. Arrigoni, *Arch. Int. Pharmacodyn.*, **184,** 68 (1970).

(11) M. D. Mc Dougal and G. B. West, *Brit. J. Pharmacol.*, **8,** 26(1953).

(12) J. T. Litchfield, Jr., and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96,** 99(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 5, 1970, from the *Department of Medical Pharmacology, University of Pavia, Pavia, Italy.*

Accepted for publication February 16, 1970.

The authors thank Mr. A. Grandini and Miss C. Provilli for technical assistance.

* To whom correspondence should be addressed.

COMMUNICATIONS

Spectrographic Determination of Traces of Cobalt in Vitamin B_{12} Preparations

Keyphrases □ Cobalt determination—vitamin B_{12} dosage forms □ Emission spectrograph, direct—analysis

Sir:

The assay methods available for determination of vitamin B_{12} fall into four groups: biological, microbiological, chemical, and radioactive (1–3). Most of these methods are time consuming or difficult to perform, and often large samples must be taken for analysis.

A quick and simple analytical assay was needed for evaluation of active ingredients in pharmaceutical preparations containing vitamin B_{12} or its analogs, using only a few centigrams of the test substance.

We have developed a direct emission spectrographic method for rapid analysis of vitamin B_{12} in biological materials, where the amount of nonvitamin B_{12} cobalt is negligible. The method is based upon the cobalt content of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$, 4.35% Co in dry form). Mineralization, fusion, or ashing of organic substances is superfluous in this determination; separation or extraction of traces of Co is similarly unnecessary.

One milliliter of a liquor containing 25–125 mcg. vitamin B_{12}/ml. (corresponding to 1.1–5.4 mcg. Co/ml.) is mixed with 0.1 g. spectroscopically pure graphite powder and evaporated to dryness at 105°. Or, approximately 0.05 g. powdered solid sample is thoroughly mixed with 0.1 g. graphite powder. Then 20 mg. of this mixture or the residue is vaporized in a cupped carbon electrode, using a 14-amp. d.c. arc, and the spectrographic plate is measured as usual (4).

The entire procedure takes about 30 min. for a single determination. In batch analyses the time required is considerably reduced.

The results of this determination are expressed in terms of anhydrous cyanocobalamin. When less stable but more naturally occurring forms of vitamin B_{12}, like hydroxocobalamin and metabolically active coenzyme B_{12}, are also present in the test solution, total vitamin B_{12} activity is directly obtained in this method (calculated as cyanocobalamin equivalents).

Fraudulent addition of inorganic cobalt interferes with this assay.

The most sensitive cobalt line in the UV region, at 3435.5 Å, is chosen as the analytical line. Its intensity is suitable for the range 1–5 mcg. Co/ml. which is usually encountered in pharmaceutical preparations.

There are few ions that interfere with this determination, and it may be termed as specific. However, the intensity of the cobalt line is increased several times in the presence of organic matter existing usually in vitamin preparations. A similar increase of line intensity is observed in flame photometry when using solutions made with combustible organic solvents like methanol or methyl isobutyl ketone (5). For this reason, standard powders are made according to the "addition method," in which increasing amounts of Co are added to the same solution that is being analyzed (4). This "standard addition method" is equivalent, by its nature, to a recov-

on the intrathoracic airways, primarily reflecting changes in tidal volume; (*b*) the pretreatment with morphine antagonizes the action of the antibiotic; (*c*) the dicloxacillin activity is unaffected by the pretreatment with atropine, dibenamine, INPEA, methysergide, chlorpheniramine, cyproheptadine, and hexamethonium; and (*d*) the action of the antibiotic persists after cutting of the vagi.

Consequently, only *in vitro* is it possible to note the myolytic activity of the antibiotic on the bronchial musculature, while *in vivo* it is more likely that dicloxacillin, as ampicillin, induces at least in part an action on the medullary respiratory center.

REFERENCES

(1) G. Benzi, E. Bermudez, and E. Arrigoni, *J. Pharm. Sci.*, **59,** 556(1970).

(2) E. T. Knudsen, D. M. Brown, and G. N. Rolinson, *Lancet*, **2,** 632(1962).

(3) S. Sidell, R. E. Burdick, J. Brodie, R. J. Bulger, and W. M. M. Kirby, *Arch. Intern. Med.*, **112,** 21(1963).

(4) C. F. Gravenkemper, J. V. Bennett, J. L. Brodie, and W. M. M. Kirby, *ibid.*, **116,** 340(1965).

(5) C. M. Kunin, *Clin. Pharmacol. Ther.*, **7,** 166(1966)

(6) C. J. O'Connell and M. B. Lewis, *Amer. J. Clin. Pathol.*, **46,** 55(1966).

(7) G. J. Miraglia and H. I. Basch, *Appl. Microbiol.*, **15,** 566 (1967).

(8) A. Matsuzaki, *Jap. J. Antibiot.*, **21,** 274(1968).

(9) G. Benzi and E. Arrigoni, to be published.

(10) G. Benzi and E. Arrigoni, *Arch. Int. Pharmacodyn.*, **184,** 68 (1970).

(11) M. D. Mc Dougal and G. B. West, *Brit. J. Pharmacol.*, **8,** 26(1953).

(12) J. T. Litchfield, Jr., and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96,** 99(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 5, 1970, from the *Department of Medical Pharmacology, University of Pavia, Pavia, Italy.*

Accepted for publication February 16, 1970.

The authors thank Mr. A. Grandini and Miss C. Provilli for technical assistance.

* To whom correspondence should be addressed.

COMMUNICATIONS

Spectrographic Determination of Traces of Cobalt in Vitamin B_{12} Preparations

Keyphrases □ Cobalt determination—vitamin B_{12} dosage forms □ Emission spectrograph, direct—analysis

Sir:

The assay methods available for determination of vitamin B_{12} fall into four groups: biological, microbiological, chemical, and radioactive (1–3). Most of these methods are time consuming or difficult to perform, and often large samples must be taken for analysis.

A quick and simple analytical assay was needed for evaluation of active ingredients in pharmaceutical preparations containing vitamin B_{12} or its analogs, using only a few centigrams of the test substance.

We have developed a direct emission spectrographic method for rapid analysis of vitamin B_{12} in biological materials, where the amount of nonvitamin B_{12} cobalt is negligible. The method is based upon the cobalt content of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$, 4.35% Co in dry form). Mineralization, fusion, or ashing of organic substances is superfluous in this determination; separation or extraction of traces of Co is similarly unnecessary.

One milliliter of a liquor containing 25–125 mcg. vitamin B_{12}/ml. (corresponding to 1.1–5.4 mcg. Co/ml.) is mixed with 0.1 g. spectroscopically pure graphite powder and evaporated to dryness at 105°. Or, approximately 0.05 g. powdered solid sample is thoroughly mixed with 0.1 g. graphite powder. Then 20 mg. of this mixture or the residue is vaporized in a cupped carbon electrode, using a 14-amp. d.c. arc, and the spectrographic plate is measured as usual (4).

The entire procedure takes about 30 min. for a single determination. In batch analyses the time required is considerably reduced.

The results of this determination are expressed in terms of anhydrous cyanocobalamin. When less stable but more naturally occurring forms of vitamin B_{12}, like hydroxocobalamin and metabolically active coenzyme B_{12}, are also present in the test solution, total vitamin B_{12} activity is directly obtained in this method (calculated as cyanocobalamin equivalents).

Fraudulent addition of inorganic cobalt interferes with this assay.

The most sensitive cobalt line in the UV region, at 3435.5 Å, is chosen as the analytical line. Its intensity is suitable for the range 1–5 mcg. Co/ml. which is usually encountered in pharmaceutical preparations.

There are few ions that interfere with this determination, and it may be termed as specific. However, the intensity of the cobalt line is increased several times in the presence of organic matter existing usually in vitamin preparations. A similar increase of line intensity is observed in flame photometry when using solutions made with combustible organic solvents like methanol or methyl isobutyl ketone (5). For this reason, standard powders are made according to the "addition method," in which increasing amounts of Co are added to the same solution that is being analyzed (4). This "standard addition method" is equivalent, by its nature, to a recov-

ery test. Therefore, experiments for the complete recovery of added vitamin B_{12} are not necessary in this assay.

The cobalt line is measured against the background of the plate, using a densitometer. To prepare standard synthetic powders, 0.1, 0.2, 0.3, and 0.4 ml. of a standard cobalt solution containing 10 mcg. Co/ml. [49.38 mg. cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$, dissolved in 1000 ml. distilled water] are added to four 1-ml. portions of the vitamin solution being analyzed. Each sample is treated as mentioned previously, after mixing with 0.1 g. graphite powder.

An atomic absorption spectrophotometer may be used for this determination. While freedom of interference is better in emission spectrography, because of the higher resolving power of the large spectrographs used, the sensitivities of detection are of the same order of magnitude. Although the atomic absorption method may be superior in rapidity and probably precision, we can mention the following points in favor of emission spectrography:

1. Solid samples can be analyzed directly after drying and grinding with graphite powder.

2. Solvent extraction is not needed for the range of cobalt concentration usually encountered in biological materials.

3. The spectrographic plate is a valuable and permanent record of analytical data, enabling a complete qualitative and quantitative check at any time. This fact is particularly important in legal cases.

In the authors' opinion, emission spectrography and atomic absorption must be used simultaneously and concurrently for the exact determination of traces of metals in biological materials. Their respective results for every metal should doublecheck each other.

In the spectrographic procedure, a "National" carbon cupped electrode, type L 3900, is used as the lower electrode, and a "National" L 3957 is used as the counter electrode. The charge weighed into the cavity is 20 mg. The distance between electrodes, or analytical gap, is 3 mm., which is further increased to 6 mm. during arcing. A d.c. arc, 14-amp., generated from N.S.L. Spec Power,[1] is used as the excitation source. The time of exposure is set to 13 sec., during which up to 3 mcg. of Co in 20 mg. dry residue is completely volatilized. Prearcing is not necessary. A Bausch & Lomb Large Littrow spectrograph is used for this assay, with a fixed slit of 20-μ width and 3-mm. height. The spectrographic plate is Kodak No. 1, size 4 × 10 in., which is developed and dried using a N.S.L. processor. It is measured with a N.S.L. reader (densitometer).

The characteristic curve of the plate is obtained by plotting a preliminary curve, using an iron arc spectrum taken with a two-step filter. This method of plate calibration seems to have a satisfactory precision, because random scatter of experimental points is compensated for in drawing the preliminary curve. The characteristic curve is practically unchanged for a period of laboratory work. Concentrations are plotted against intensities on ordinary graph paper.

[1] National Spectrographic Laboratories Inc., Cleveland, Ohio.

By taking three spectra from each powder, a precision of ±10% was attained.

A large number of vitamin B_{12}-containing preparations such as liver extract and liver injection were analyzed by this method. It was also used to follow separation or purification steps in manufacturing processes (fermentation, extraction, *etc.*). To indicate the precision obtained, we are giving here the results of a routine determination. The analysis of a vitamin B_{12} preparation claimed to contain 40 mcg. vitamin/ml. gave the following results: 1.7, 1.8, 2.0, 2.0, 1.6, and 2.0 mcg. Co/ml. in six different plates. The mean value, 1.85, multiplied by 100/4.35 = 23 gives 42 mcg. cyanocobalamin/ml.

(1) "Methods of Vitamin Assay," 3rd ed., The Association of Vitamin Chemists, Eds., Interscience, New York, N. Y., 1966.
(2) "The Vitamins," vol. II, 2nd ed., W. H. Sebrell, Jr., and R. S. Harris, Eds., Academic, New York, N. Y., 1968.
(3) "The Vitamins," vol. VII, 2nd ed., P. Szent-Györgyi and W. N. Pearson, Eds., Academic, New York, N. Y., 1967.
(4) C. E. Harvey, "Spectrochemical Procedures," A. R. L., California, 1950, pp. 215–254.
(5) H. A. Bryan and J. A. Dean, *Anal. Chem.*, **29**, 1289(1957).

F. M. Farhan
M. Makhani
Laboratory of Spectrography
Faculty of Engineering and Faculty of Pharmacy
University of Tehran
Tehran, Iran

Received November 20, 1969.
Accepted for publication April 6, 1970.
The authors thank Mr. J. Eyvani for his technical assistance.

Synthesis of *O*-Alkyldihydroxyacetone and Derivatives

Keyphrases □ *O*-Alkyldihydroxyacetone, derivatives—synthesis □ IR spectrophotometry—structure □ GLC—identity

Sir:

Recently, Snyder and coworkers (1–4) proposed a biochemical pathway and identified intermediates that demonstrated the biosynthesis of *O*-alkyl ether bonds in glycerolipids from fatty alcohols and dihydroxyacetone-P in cell-free systems. This new metabolic scheme included *O*-alkyldihydroxyacetone and *O*-alkyldihydroxyacetone-P as intermediates. We have now further substantiated the previously reported biochemical reaction sequence (3) by the chemical synthesis of one of the intermediates, namely *O*-alkyldihydroxyacetone (I) (R = octadecyl or hexadecyl) (Scheme I). This preliminary communication describes the synthesis of I which can exist as a keto-enol tautomer; the properties of I are identical with those of the ^{14}C-*O*-alkyldihydroxy-

ery test. Therefore, experiments for the complete recovery of added vitamin B_{12} are not necessary in this assay.

The cobalt line is measured against the background of the plate, using a densitometer. To prepare standard synthetic powders, 0.1, 0.2, 0.3, and 0.4 ml. of a standard cobalt solution containing 10 mcg. Co/ml. [49.38 mg. cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$, dissolved in 1000 ml. distilled water] are added to four 1-ml. portions of the vitamin solution being analyzed. Each sample is treated as mentioned previously, after mixing with 0.1 g. graphite powder.

An atomic absorption spectrophotometer may be used for this determination. While freedom of interference is better in emission spectrography, because of the higher resolving power of the large spectrographs used, the sensitivities of detection are of the same order of magnitude. Although the atomic absorption method may be superior in rapidity and probably precision, we can mention the following points in favor of emission spectrography:

1. Solid samples can be analyzed directly after drying and grinding with graphite powder.

2. Solvent extraction is not needed for the range of cobalt concentration usually encountered in biological materials.

3. The spectrographic plate is a valuable and permanent record of analytical data, enabling a complete qualitative and quantitative check at any time. This fact is particularly important in legal cases.

In the authors' opinion, emission spectrography and atomic absorption must be used simultaneously and concurrently for the exact determination of traces of metals in biological materials. Their respective results for every metal should doublecheck each other.

In the spectrographic procedure, a "National" carbon cupped electrode, type L 3900, is used as the lower electrode, and a "National" L 3957 is used as the counter electrode. The charge weighed into the cavity is 20 mg. The distance between electrodes, or analytical gap, is 3 mm., which is further increased to 6 mm. during arcing. A d.c. arc, 14-amp., generated from N.S.L. Spec Power,[1] is used as the excitation source. The time of exposure is set to 13 sec., during which up to 3 mcg. of Co in 20 mg. dry residue is completely volatilized. Prearcing is not necessary. A Bausch & Lomb Large Littrow spectrograph is used for this assay, with a fixed slit of 20-μ width and 3-mm. height. The spectrographic plate is Kodak No. 1, size 4 × 10 in., which is developed and dried using a N.S.L. processor. It is measured with a N.S.L. reader (densitometer).

The characteristic curve of the plate is obtained by plotting a preliminary curve, using an iron arc spectrum taken with a two-step filter. This method of plate calibration seems to have a satisfactory precision, because random scatter of experimental points is compensated for in drawing the preliminary curve. The characteristic curve is practically unchanged for a period of laboratory work. Concentrations are plotted against intensities on ordinary graph paper.

[1] National Spectrographic Laboratories Inc., Cleveland, Ohio.

By taking three spectra from each powder, a precision of ±10% was attained.

A large number of vitamin B_{12}-containing preparations such as liver extract and liver injection were analyzed by this method. It was also used to follow separation or purification steps in manufacturing processes (fermentation, extraction, *etc.*). To indicate the precision obtained, we are giving here the results of a routine determination. The analysis of a vitamin B_{12} preparation claimed to contain 40 mcg. vitamin/ml. gave the following results: 1.7, 1.8, 2.0, 2.0, 1.6, and 2.0 mcg. Co/ml. in six different plates. The mean value, 1.85, multiplied by 100/4.35 = 23 gives 42 mcg. cyanocobalamin/ml.

(1) "Methods of Vitamin Assay," 3rd ed., The Association of Vitamin Chemists, Eds., Interscience, New York, N. Y., 1966.
(2) "The Vitamins," vol. II, 2nd ed., W. H. Sebrell, Jr., and R. S. Harris, Eds., Academic, New York, N. Y., 1968.
(3) "The Vitamins," vol. VII, 2nd ed., P. Szent-Györgyi and W. N. Pearson, Eds., Academic, New York, N. Y., 1967.
(4) C. E. Harvey, "Spectrochemical Procedures," A. R. L., California, 1950, pp. 215–254.
(5) H. A. Bryan and J. A. Dean, *Anal. Chem.*, **29**, 1289(1957).

F. M. Farhan
M. Makhani
Laboratory of Spectrography
Faculty of Engineering and Faculty of Pharmacy
University of Tehran
Tehran, Iran

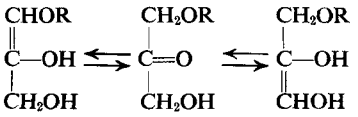
Received November 20, 1969.
Accepted for publication April 6, 1970.
The authors thank Mr. J. Eyvani for his technical assistance.

Synthesis of *O*-Alkyldihydroxyacetone and Derivatives

Keyphrases □ *O*-Alkyldihydroxyacetone, derivatives—synthesis □ IR spectrophotometry—structure □ GLC—identity

Sir:

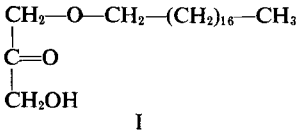
Recently, Snyder and coworkers (1–4) proposed a biochemical pathway and identified intermediates that demonstrated the biosynthesis of *O*-alkyl ether bonds in glycerolipids from fatty alcohols and dihydroxyacetone-P in cell-free systems. This new metabolic scheme included *O*-alkyldihydroxyacetone and *O*-alkyldihydroxyacetone-P as intermediates. We have now further substantiated the previously reported biochemical reaction sequence (3) by the chemical synthesis of one of the intermediates, namely *O*-alkyldihydroxyacetone (I) (R = octadecyl or hexadecyl) (Scheme I). This preliminary communication describes the synthesis of I which can exist as a keto-enol tautomer; the properties of I are identical with those of the ^{14}C-*O*-alkyldihydroxy-



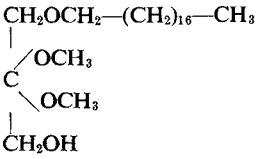
Scheme I

acetone synthesized enzymically by microsomes (4), thereby confirming it as an intermediate in the biosynthesis of these new and metabolically important ether-linked keto lipids.

We have prepared structures with the following general formulas:



I



II

Two synthetic routes were investigated in the synthesis of 1-*O*-octadecyl-2,2-dimethoxy-3-hydroxypropane (II). In the first procedure, acetyl dihydroxyacetone dimethyl ketal was synthesized (5) and then converted to 1-*O*-trityl-2,2-dimethoxy-3-hydroxypropane (III), which had the correct elemental analysis; m.p. 127–128°.

Anal.—Calcd. for $C_{24}H_{26}O_4$: C, 76.16; H, 6.92. Found: C, 76.40; H, 7.01.

IR spectrum: 3500 cm.$^{-1}$ (OH), 2920 cm.$^{-1}$ (C—H), 1600 cm.$^{-1}$ (trityl), 1085–1140 cm.$^{-1}$ (—C—O—C—).

Compound III was reacted with octadecyl bromide in the presence of powdered KOH in benzene (6), resulting in 1-*O*-octadecyl-2,2-dimethoxy-3-*O*-trityl propane (IV). IR spectrum showed loss of OH band absorption, 2920 cm.$^{-1}$ (C—H, substantial increase in aliphatic CH band absorption), 1085–1140 cm.$^{-1}$ (—C—O—C—). Hydrogenolysis of IV with 15% Pd/C in dioxane at 40° and 60 p.s.i. resulted in I.

Anal.—Calcd. for $C_{21}H_{42}O_3$: C, 73.63; H, 12.36. Found: C, 73.59; H, 12.29.

IR spectrum: 3420 cm.$^{-1}$ (OH), 2920 cm.$^{-1}$ (C—H), 1725 cm.$^{-1}$ (—C—C—C—), 1085–1140 cm.$^{-1}$ (—C—O—C—). NMR in $CDCl_3$ showed no methoxy singlet, and the assignments of fragments in the mass spectra were comparable to that of the keto-enol form of I, m.p. 87–89°.

In the second synthetic scheme, batyl alcohol was benzoylated in the presence of pyridine and the crude mono derivative was oxidized with dimethyl sulfoxide and dicyclohexylcarbodiimide in the presence of trifluoroacetic acid (7). This afforded 1-*O*-octadecyl-3-*O*-benzoyl-2-propanone, V.

Anal.—Calcd. for $C_{28}H_{46}O_4$: C, 75.29; H, 10.38. Found: C, 75.26; H, 10.29, m.p. 52–53°.

Compound V was then ketalized to afford 1-*O*-octadecyl-2,2-dimethoxy-3-*O*-benzoylpropane, VI, which was further confirmed with IR. Compound VI was base hydrolyzed to give 1-*O*-octadecyl-2,2-dimethoxy-3-hydroxypropane (II).

Anal.—Calcd. for $C_{23}H_{48}O_4$: C, 71.08; H, 12.45. Found: C, 70.82; H, 12.33. IR spectrum showed 3470 cm.$^{-1}$ (OH), 2920 cm.$^{-1}$ (C—H), 1085–1140 cm.$^{-1}$ (—C—O—C—). NMR δ 3.25 [S, 6. (OCH_3)], m.p. 29–31°.

The two synthetic preparations of *O*-alkyldihydroxyacetone were reduced with $LiAlH_4$ to the corresponding *O*-alkylglycerol, and GLC of the isopropylidene derivatives demonstrated that they had the same retention time as the isopropylidene derivative of authentic batyl alcohol. Acid hydrolysis of the ketone-containing lipids produced octadecanol; periodate oxidation produced *O*-alkylglycolic acids, and they could be reduced with $LiAlH_4$ to *O*-alkylethyleneglycols. These reactions and the details for identifying the products have been described (4). Further work on these ether-linked ketolipids is in progress in our laboratories.

(1) F. Snyder, R. L. Wykle, and B. Malone, *Biochem. Biophys. Res. Commun.*, **34,** 315(1969).

(2) R. L. Wykle and F. Snyder, *ibid.*, **37,** 658(1969).

(3) F. Snyder, B. Malone, and M. L. Blank, *J. Biol. Chem.*, **245,** 1790(1970).

(4) F. Snyder, M. L. Blank, B. Malone, and R. L. Wykle, *ibid.*, **245,** 1800(1970).

(5) C. E. Ballou and H. O. Fischer, *J. Amer. Chem. Soc.*, **78,** 1659(1956).

(6) M. Kates, B. Palameta, and L. S. Yengoyan, *Biochemistry*, **4,** 1595(1964).

(7) K. E. Pfitzner and J. G. Moffatt, *J. Amer. Chem. Soc.*, **87,** 5670(1965).

CLAUDE PIANTADOSI
KHALID S. ISHAQ
Department of Medicinal Chemistry
School of Pharmacy
University of North Carolina
Chapel Hill, NC 27514

FRED SNYDER
Medical Division
Oak Ridge Associated Universities
Oak Ridge, TN 37830

Received April 1, 1970.

Accepted for publication May 5, 1970.

This work has been supported by NIH Research Grant GM12562-06 from the U. S. Public Health Service and the U. S. Atomic Energy Commission.

REVIEWS

Legal Medicine Annual. Edited by CYRIL H. WECHT, M.D., J.D. Appleton-Century-Crofts, 440 Park Ave., New York, NY 10016, 1969. 442 pp. 16.7 × 24.4 cm. Price $14.00.

This book contains 22 monographs by 26 authors covering various aspects of forensic medicine. The largest part of the book is devoted to a discussion of professional liability and forensic toxicology. The remainder of the book is rounded out with a discussion of criminal responsibility, organ transplantation, and abortion laws.

Two chapters that deserve special mention are: *Chapter 8*, dealing with the preparation for trial in drug liability cases. The author gives an excellent discussion of how probing an attorney's inquiry can be into the facts and responsibilities for adverse drug reactions.

Chapter 5 gives a clear overview of the medicolegal problems of hospital liability and the extent to which the courts are forcing hospitals to exercise increasing control over medical staff conduct. While this chapter focuses on potential liability of the medical staff, it is clear that members of the paramedical staff, such as, clinical pharmacists and nurses can face the same types of potential liability.

A book of this nature would not be of interest to the pharmaceutical scientist or pharmacy practitioner unless he has a strong interest in medicolegal problems.

Reviewed by William C. Roemer
Academy of Pharmaceutical Sciences
American Pharmaceutical Association
Washington, DC 20037 ■

Physician's Book Compendium. Edited by MAX CELNIK. Physician's Book Compendium, Inc., 25 West 45th St., New York, NY 10036, 1969. xxvi + 846 pp. 17 × 23.5 cm. Price $29.50.

While the idea behind the "Physician's Book Compendium" of compiling into one source all books from the medical field and giving pertinent bibliographic information is sound and would have useful applications, the product of this idea falls considerably short of its goal, as stated in the Preface, of being "an indispensable reference volume."

Probably the first objectionable feature that the user would notice is the inserts of business reply cards, provided for ordering books. The thickness of these inserts, and their location interspersed throughout the text, makes flipping pages difficult. The next annoyance is the numerous pages of advertisements scattered throughout the volume; they seem to be out of place in a book of this type. The very fact that this book costs nearly thirty dollars and is filled with ads, in itself, might be objectionable to some purchasers of this book.

Had both of these aforementioned materials been placed at the beginning or end of the book, the usefulness of the book would have been somewhat increased, as well as bettering its appearance.

The paragraph summaries, which are not provided for all books, read like the publicity flyers sent out by the publishers. No attempt could be discerned to evaluate or critique any book, an omission which certainly detracts from the value of the compendium. This omission is particularly significant with regard to the older books.

With just a quick check of the section on "Pharmacology," it was noted that nearly half of the books included were published before 1964. One cannot help but wonder if these are all significant, classic books, or if they were included to fill out the space available.

The inclusion of books with a popular approach or nature tends to diminish any remnant image of a scholarly attempt at compiling a technical work.

The use of the same type face, although admittedly a little larger, for both the subheads within sections and the authors' last names adds to the reader's confusion.

While there is apparently a need for this type of book and the editors have made an initial step, it would be difficult, if not impossible, to recommend this book for general use.

Staff Review ■

Contraception: The Chemical Control of Fertility. Edited by DANIEL LEDNICER. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xiv + 269 pp. 15.5 × 24 cm. Price $13.75.

This is an excellent book for researchers who are entering the field of female antifertility. It contains six chapters which deal with biology and chemistry of steroidal and nonsteroidal agents. Each chapter is authored by men who are well versed in their respective fields.

Chapter One introduces the reader to the reproductive cycle of the human female and discusses the interplay between the hypothalamus, pituitary, and ovary.

Chapters Two and Four emphasize the biology of steroidal and nonsteroidal compounds that are either being used as contraceptive agents or have been evaluated in animals. Chapter Two in particular discusses in detail the mode of action and the rationale for selecting the present steroidal contraceptive agents. Chapter Four, on the other hand, primarily emphasizes the biology of nonsteroidal estrogens and antiestrogens. The chemistry is also divided in two chapters. Chapter Three covers the synthesis and the biological activity of various 19-norsteroids and progesteronelike compounds. Other chemical antifertility agents, referred to as nonsteroidals, are discussed in Chapter Five. The free use of flow diagrams and isolated structures brings to light the various routes and rationale that medicinal chemists have explored over the past decade.

Finally, the big plus for the book is the chapter on Assays and Screens. The author has compiled various methods that have been employed by a number of workers in screening for antifertility agents. The use of tissue slides enhances the appreciation of a novice for these assays.

All in all, the book is well written and the chapters are well coordinated. It does have, however, many typographical errors especially in Chapter Five. A chapter on "absorption, excretion, and metabolism" of existing contraceptive agents, in my opinion would have contributed immensely. Also, some mention of male antifertility would have been in order even though the editors did explain its omission in the Preface. The book can certainly be recommended for advanced graduate students and researchers in the field of contraception.

Reviewed by Arvin P. Shroff
Ortho Research Foundation
Raritan, NJ 08869 ■

Isolation and Identification of Drugs (in pharmaceuticals, body fluids and post-mortem material). Edited by E. G. C. CLARKE. The Pharmaceutical Press, 17 Bloomsbury Square, WC-1, London, England. U. S. distributor: Rittenhouse Book Store, 1706 Rittenhouse Square, Philadelphia, PA 19103, 1969. xxii + 870 pp. 14.8 × 22.6 cm. Price $39.00.

Clarke has drawn into monograph form drug identification methods, properties, metabolism, and toxicology. Most of this vast amount of information was available previously in assorted compilations but this book is the first large-scale collation of data of greatest interest to toxicologists, biochemists, pharmacists, pathologists, and forensic and pharmaceutical chemists. There is a clear need for this compilation and one can only regret the rapid obsolescence inherent in these active areas of research. All the more for this admirably printed and bound volume. The book was produced by the Pharmaceutical Society of Great Britain as a companion volume to the *Extra Pharmacopoeia.*

The essence of the work is the Monograph. Most concern commercial drug substances but some pesticides, herbicides, and hallucinogens are included. Unfortunately, the editor does not state criteria for selection or a cutoff date so one wonders at the absence of some recent, noted drugs. As most important compounds are listed, there is little loss in scope of coverage when transplanted to this country. Each monograph ideally contains statements or paragraphs on title, pharmacologic category, synonyms, trade names, structure, physical properties, screening tests, extraction, coded chromatographic systems, UV and IR absorption, references to

REVIEWS

Legal Medicine Annual. Edited by CYRIL H. WECHT, M.D., J.D. Appleton-Century-Crofts, 440 Park Ave., New York, NY 10016, 1969. 442 pp. 16.7 × 24.4 cm. Price $14.00.

This book contains 22 monographs by 26 authors covering various aspects of forensic medicine. The largest part of the book is devoted to a discussion of professional liability and forensic toxicology. The remainder of the book is rounded out with a discussion of criminal responsibility, organ transplantation, and abortion laws.

Two chapters that deserve special mention are: *Chapter 8*, dealing with the preparation for trial in drug liability cases. The author gives an excellent discussion of how probing an attorney's inquiry can be into the facts and responsibilities for adverse drug reactions.

Chapter 5 gives a clear overview of the medicolegal problems of hospital liability and the extent to which the courts are forcing hospitals to exercise increasing control over medical staff conduct. While this chapter focuses on potential liability of the medical staff, it is clear that members of the paramedical staff, such as, clinical pharmacists and nurses can face the same types of potential liability.

A book of this nature would not be of interest to the pharmaceutical scientist or pharmacy practitioner unless he has a strong interest in medicolegal problems.

Reviewed by William C. Roemer
Academy of Pharmaceutical Sciences
American Pharmaceutical Association
Washington, DC 20037 ■

Physician's Book Compendium. Edited by MAX CELNIK. Physician's Book Compendium, Inc., 25 West 45th St., New York, NY 10036, 1969. xxvi + 846 pp. 17 × 23.5 cm. Price $29.50.

While the idea behind the "Physician's Book Compendium" of compiling into one source all books from the medical field and giving pertinent bibliographic information is sound and would have useful applications, the product of this idea falls considerably short of its goal, as stated in the Preface, of being "an indispensable reference volume."

Probably the first objectionable feature that the user would notice is the inserts of business reply cards, provided for ordering books. The thickness of these inserts, and their location interspersed throughout the text, makes flipping pages difficult. The next annoyance is the numerous pages of advertisements scattered throughout the volume; they seem to be out of place in a book of this type. The very fact that this book costs nearly thirty dollars and is filled with ads, in itself, might be objectionable to some purchasers of this book.

Had both of these aforementioned materials been placed at the beginning or end of the book, the usefulness of the book would have been somewhat increased, as well as bettering its appearance.

The paragraph summaries, which are not provided for all books, read like the publicity flyers sent out by the publishers. No attempt could be discerned to evaluate or critique any book, an omission which certainly detracts from the value of the compendium. This omission is particularly significant with regard to the older books.

With just a quick check of the section on "Pharmacology," it was noted that nearly half of the books included were published before 1964. One cannot help but wonder if these are all significant, classic books, or if they were included to fill out the space available.

The inclusion of books with a popular approach or nature tends to diminish any remnant image of a scholarly attempt at compiling a technical work.

The use of the same type face, although admittedly a little larger, for both the subheads within sections and the authors' last names adds to the reader's confusion.

While there is apparently a need for this type of book and the editors have made an initial step, it would be difficult, if not impossible, to recommend this book for general use.

Staff Review ■

Contraception: The Chemical Control of Fertility. Edited by DANIEL LEDNICER. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xiv + 269 pp. 15.5 × 24 cm. Price $13.75.

This is an excellent book for researchers who are entering the field of female antifertility. It contains six chapters which deal with biology and chemistry of steroidal and nonsteroidal agents. Each chapter is authored by men who are well versed in their respective fields.

Chapter One introduces the reader to the reproductive cycle of the human female and discusses the interplay between the hypothalamus, pituitary, and ovary.

Chapters Two and Four emphasize the biology of steroidal and nonsteroidal compounds that are either being used as contraceptive agents or have been evaluated in animals. Chapter Two in particular discusses in detail the mode of action and the rationale for selecting the present steroidal contraceptive agents. Chapter Four, on the other hand, primarily emphasizes the biology of nonsteroidal estrogens and antiestrogens. The chemistry is also divided in two chapters. Chapter Three covers the synthesis and the biological activity of various 19-norsteroids and progesteronelike compounds. Other chemical antifertility agents, referred to as nonsteroidals, are discussed in Chapter Five. The free use of flow diagrams and isolated structures brings to light the various routes and rationale that medicinal chemists have explored over the past decade.

Finally, the big plus for the book is the chapter on Assays and Screens. The author has compiled various methods that have been employed by a number of workers in screening for antifertility agents. The use of tissue slides enhances the appreciation of a novice for these assays.

All in all, the book is well written and the chapters are well coordinated. It does have, however, many typographical errors especially in Chapter Five. A chapter on "absorption, excretion, and metabolism" of existing contraceptive agents, in my opinion would have contributed immensely. Also, some mention of male antifertility would have been in order even though the editors did explain its omission in the Preface. The book can certainly be recommended for advanced graduate students and researchers in the field of contraception.

Reviewed by Arvin P. Shroff
Ortho Research Foundation
Raritan, NJ 08869 ■

Isolation and Identification of Drugs (in pharmaceuticals, body fluids and post-mortem material). Edited by E. G. C. CLARKE. The Pharmaceutical Press, 17 Bloomsbury Square, WC-1, London, England. U. S. distributor: Rittenhouse Book Store, 1706 Rittenhouse Square, Philadelphia, PA 19103, 1969. xxii + 870 pp. 14.8 × 22.6 cm. Price $39.00.

Clarke has drawn into monograph form drug identification methods, properties, metabolism, and toxicology. Most of this vast amount of information was available previously in assorted compilations but this book is the first large-scale collation of data of greatest interest to toxicologists, biochemists, pharmacists, pathologists, and forensic and pharmaceutical chemists. There is a clear need for this compilation and one can only regret the rapid obsolescence inherent in these active areas of research. All the more for this admirably printed and bound volume. The book was produced by the Pharmaceutical Society of Great Britain as a companion volume to the *Extra Pharmacopoeia.*

The essence of the work is the Monograph. Most concern commercial drug substances but some pesticides, herbicides, and hallucinogens are included. Unfortunately, the editor does not state criteria for selection or a cutoff date so one wonders at the absence of some recent, noted drugs. As most important compounds are listed, there is little loss in scope of coverage when transplanted to this country. Each monograph ideally contains statements or paragraphs on title, pharmacologic category, synonyms, trade names, structure, physical properties, screening tests, extraction, coded chromatographic systems, UV and IR absorption, references to

REVIEWS

Legal Medicine Annual. Edited by CYRIL H. WECHT, M.D., J.D. Appleton-Century-Crofts, 440 Park Ave., New York, NY 10016, 1969. 442 pp. 16.7 × 24.4 cm. Price $14.00.

This book contains 22 monographs by 26 authors covering various aspects of forensic medicine. The largest part of the book is devoted to a discussion of professional liability and forensic toxicology. The remainder of the book is rounded out with a discussion of criminal responsibility, organ transplantation, and abortion laws.

Two chapters that deserve special mention are: *Chapter 8*, dealing with the preparation for trial in drug liability cases. The author gives an excellent discussion of how probing an attorney's inquiry can be into the facts and responsibilities for adverse drug reactions.

Chapter 5 gives a clear overview of the medicolegal problems of hospital liability and the extent to which the courts are forcing hospitals to exercise increasing control over medical staff conduct. While this chapter focuses on potential liability of the medical staff, it is clear that members of the paramedical staff, such as, clinical pharmacists and nurses can face the same types of potential liability.

A book of this nature would not be of interest to the pharmaceutical scientist or pharmacy practitioner unless he has a strong interest in medicolegal problems.

Reviewed by William C. Roemer
Academy of Pharmaceutical Sciences
American Pharmaceutical Association
Washington, DC 20037 ■

Physician's Book Compendium. Edited by MAX CELNIK. Physician's Book Compendium, Inc., 25 West 45th St., New York, NY 10036, 1969. xxvi + 846 pp. 17 × 23.5 cm. Price $29.50.

While the idea behind the "Physician's Book Compendium" of compiling into one source all books from the medical field and giving pertinent bibliographic information is sound and would have useful applications, the product of this idea falls considerably short of its goal, as stated in the Preface, of being "an indispensable reference volume."

Probably the first objectionable feature that the user would notice is the inserts of business reply cards, provided for ordering books. The thickness of these inserts, and their location interspersed throughout the text, makes flipping pages difficult. The next annoyance is the numerous pages of advertisements scattered throughout the volume; they seem to be out of place in a book of this type. The very fact that this book costs nearly thirty dollars and is filled with ads, in itself, might be objectionable to some purchasers of this book.

Had both of these aforementioned materials been placed at the beginning or end of the book, the usefulness of the book would have been somewhat increased, as well as bettering its appearance.

The paragraph summaries, which are not provided for all books, read like the publicity flyers sent out by the publishers. No attempt could be discerned to evaluate or critique any book, an omission which certainly detracts from the value of the compendium. This omission is particularly significant with regard to the older books.

With just a quick check of the section on "Pharmacology," it was noted that nearly half of the books included were published before 1964. One cannot help but wonder if these are all significant, classic books, or if they were included to fill out the space available.

The inclusion of books with a popular approach or nature tends to diminish any remnant image of a scholarly attempt at compiling a technical work.

The use of the same type face, although admittedly a little larger, for both the subheads within sections and the authors' last names adds to the reader's confusion.

While there is apparently a need for this type of book and the editors have made an initial step, it would be difficult, if not impossible, to recommend this book for general use.

Staff Review ■

Contraception: The Chemical Control of Fertility. Edited by DANIEL LEDNICER. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xiv + 269 pp. 15.5 × 24 cm. Price $13.75.

This is an excellent book for researchers who are entering the field of female antifertility. It contains six chapters which deal with biology and chemistry of steroidal and nonsteroidal agents. Each chapter is authored by men who are well versed in their respective fields.

Chapter One introduces the reader to the reproductive cycle of the human female and discusses the interplay between the hypothalamus, pituitary, and ovary.

Chapters Two and Four emphasize the biology of steroidal and nonsteroidal compounds that are either being used as contraceptive agents or have been evaluated in animals. Chapter Two in particular discusses in detail the mode of action and the rationale for selecting the present steroidal contraceptive agents. Chapter Four, on the other hand, primarily emphasizes the biology of nonsteroidal estrogens and antiestrogens. The chemistry is also divided in two chapters. Chapter Three covers the synthesis and the biological activity of various 19-norsteroids and progesteronelike compounds. Other chemical antifertility agents, referred to as nonsteroidals, are discussed in Chapter Five. The free use of flow diagrams and isolated structures brings to light the various routes and rationale that medicinal chemists have explored over the past decade.

Finally, the big plus for the book is the chapter on Assays and Screens. The author has compiled various methods that have been employed by a number of workers in screening for antifertility agents. The use of tissue slides enhances the appreciation of a novice for these assays.

All in all, the book is well written and the chapters are well coordinated. It does have, however, many typographical errors especially in Chapter Five. A chapter on "absorption, excretion, and metabolism" of existing contraceptive agents, in my opinion would have contributed immensely. Also, some mention of male antifertility would have been in order even though the editors did explain its omission in the Preface. The book can certainly be recommended for advanced graduate students and researchers in the field of contraception.

Reviewed by Arvin P. Shroff
Ortho Research Foundation
Raritan, NJ 08869 ■

Isolation and Identification of Drugs (in pharmaceuticals, body fluids and post-mortem material). Edited by E. G. C. CLARKE. The Pharmaceutical Press, 17 Bloomsbury Square, WC-1, London, England. U. S. distributor: Rittenhouse Book Store, 1706 Rittenhouse Square, Philadelphia, PA 19103, 1969. xxii + 870 pp. 14.8 × 22.6 cm. Price $39.00.

Clarke has drawn into monograph form drug identification methods, properties, metabolism, and toxicology. Most of this vast amount of information was available previously in assorted compilations but this book is the first large-scale collation of data of greatest interest to toxicologists, biochemists, pharmacists, pathologists, and forensic and pharmaceutical chemists. There is a clear need for this compilation and one can only regret the rapid obsolescence inherent in these active areas of research. All the more for this admirably printed and bound volume. The book was produced by the Pharmaceutical Society of Great Britain as a companion volume to the *Extra Pharmacopoeia.*

The essence of the work is the Monograph. Most concern commercial drug substances but some pesticides, herbicides, and hallucinogens are included. Unfortunately, the editor does not state criteria for selection or a cutoff date so one wonders at the absence of some recent, noted drugs. As most important compounds are listed, there is little loss in scope of coverage when transplanted to this country. Each monograph ideally contains statements or paragraphs on title, pharmacologic category, synonyms, trade names, structure, physical properties, screening tests, extraction, coded chromatographic systems, UV and IR absorption, references to

REVIEWS

Legal Medicine Annual. Edited by CYRIL H. WECHT, M.D., J.D. Appleton-Century-Crofts, 440 Park Ave., New York, NY 10016, 1969. 442 pp. 16.7 × 24.4 cm. Price $14.00.

This book contains 22 monographs by 26 authors covering various aspects of forensic medicine. The largest part of the book is devoted to a discussion of professional liability and forensic toxicology. The remainder of the book is rounded out with a discussion of criminal responsibility, organ transplantation, and abortion laws.

Two chapters that deserve special mention are: *Chapter 8*, dealing with the preparation for trial in drug liability cases. The author gives an excellent discussion of how probing an attorney's inquiry can be into the facts and responsibilities for adverse drug reactions.

Chapter 5 gives a clear overview of the medicolegal problems of hospital liability and the extent to which the courts are forcing hospitals to exercise increasing control over medical staff conduct. While this chapter focuses on potential liability of the medical staff, it is clear that members of the paramedical staff, such as, clinical pharmacists and nurses can face the same types of potential liability.

A book of this nature would not be of interest to the pharmaceutical scientist or pharmacy practitioner unless he has a strong interest in medicolegal problems.

Reviewed by William C. Roemer
Academy of Pharmaceutical Sciences
American Pharmaceutical Association
Washington, DC 20037 ■

Physician's Book Compendium. Edited by MAX CELNIK. Physician's Book Compendium, Inc., 25 West 45th St., New York, NY 10036, 1969. xxvi + 846 pp. 17 × 23.5 cm. Price $29.50.

While the idea behind the "Physician's Book Compendium" of compiling into one source all books from the medical field and giving pertinent bibliographic information is sound and would have useful applications, the product of this idea falls considerably short of its goal, as stated in the Preface, of being "an indispensable reference volume."

Probably the first objectionable feature that the user would notice is the inserts of business reply cards, provided for ordering books. The thickness of these inserts, and their location interspersed throughout the text, makes flipping pages difficult. The next annoyance is the numerous pages of advertisements scattered throughout the volume; they seem to be out of place in a book of this type. The very fact that this book costs nearly thirty dollars and is filled with ads, in itself, might be objectionable to some purchasers of this book.

Had both of these aforementioned materials been placed at the beginning or end of the book, the usefulness of the book would have been somewhat increased, as well as bettering its appearance.

The paragraph summaries, which are not provided for all books, read like the publicity flyers sent out by the publishers. No attempt could be discerned to evaluate or critique any book, an omission which certainly detracts from the value of the compendium. This omission is particularly significant with regard to the older books.

With just a quick check of the section on "Pharmacology," it was noted that nearly half of the books included were published before 1964. One cannot help but wonder if these are all significant, classic books, or if they were included to fill out the space available.

The inclusion of books with a popular approach or nature tends to diminish any remnant image of a scholarly attempt at compiling a technical work.

The use of the same type face, although admittedly a little larger, for both the subheads within sections and the authors' last names adds to the reader's confusion.

While there is apparently a need for this type of book and the editors have made an initial step, it would be difficult, if not impossible, to recommend this book for general use.

Staff Review ■

Contraception: The Chemical Control of Fertility. Edited by DANIEL LEDNICER. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xiv + 269 pp. 15.5 × 24 cm. Price $13.75.

This is an excellent book for researchers who are entering the field of female antifertility. It contains six chapters which deal with biology and chemistry of steroidal and nonsteroidal agents. Each chapter is authored by men who are well versed in their respective fields.

Chapter One introduces the reader to the reproductive cycle of the human female and discusses the interplay between the hypothalamus, pituitary, and ovary.

Chapters Two and Four emphasize the biology of steroidal and nonsteroidal compounds that are either being used as contraceptive agents or have been evaluated in animals. Chapter Two in particular discusses in detail the mode of action and the rationale for selecting the present steroidal contraceptive agents. Chapter Four, on the other hand, primarily emphasizes the biology of nonsteroidal estrogens and antiestrogens. The chemistry is also divided in two chapters. Chapter Three covers the synthesis and the biological activity of various 19-norsteroids and progesteronelike compounds. Other chemical antifertility agents, referred to as nonsteroidals, are discussed in Chapter Five. The free use of flow diagrams and isolated structures brings to light the various routes and rationale that medicinal chemists have explored over the past decade.

Finally, the big plus for the book is the chapter on Assays and Screens. The author has compiled various methods that have been employed by a number of workers in screening for antifertility agents. The use of tissue slides enhances the appreciation of a novice for these assays.

All in all, the book is well written and the chapters are well coordinated. It does have, however, many typographical errors especially in Chapter Five. A chapter on "absorption, excretion, and metabolism" of existing contraceptive agents, in my opinion would have contributed immensely. Also, some mention of male antifertility would have been in order even though the editors did explain its omission in the Preface. The book can certainly be recommended for advanced graduate students and researchers in the field of contraception.

Reviewed by Arvin P. Shroff
Ortho Research Foundation
Raritan, NJ 08869 ■

Isolation and Identification of Drugs (in pharmaceuticals, body fluids and post-mortem material). Edited by E. G. C. CLARKE. The Pharmaceutical Press, 17 Bloomsbury Square, WC-1, London, England. U. S. distributor: Rittenhouse Book Store, 1706 Rittenhouse Square, Philadelphia, PA 19103, 1969. xxii + 870 pp. 14.8 × 22.6 cm. Price $39.00.

Clarke has drawn into monograph form drug identification methods, properties, metabolism, and toxicology. Most of this vast amount of information was available previously in assorted compilations but this book is the first large-scale collation of data of greatest interest to toxicologists, biochemists, pharmacists, pathologists, and forensic and pharmaceutical chemists. There is a clear need for this compilation and one can only regret the rapid obsolescence inherent in these active areas of research. All the more for this admirably printed and bound volume. The book was produced by the Pharmaceutical Society of Great Britain as a companion volume to the *Extra Pharmacopoeia.*

The essence of the work is the Monograph. Most concern commercial drug substances but some pesticides, herbicides, and hallucinogens are included. Unfortunately, the editor does not state criteria for selection or a cutoff date so one wonders at the absence of some recent, noted drugs. As most important compounds are listed, there is little loss in scope of coverage when transplanted to this country. Each monograph ideally contains statements or paragraphs on title, pharmacologic category, synonyms, trade names, structure, physical properties, screening tests, extraction, coded chromatographic systems, UV and IR absorption, references to

quantitative methods, dose, metabolism, and an abstract of the referenced toxicology literature.

Following the monographs are individual collations of each identity test. Drugs are grouped under the standard chromatographic systems. Melting points, UV maxima, and IR bands are listed numerically with the drugs entered opposite. Color test data are compiled by observed reaction. Such devices are familiar and welcome to those experienced in qualitative organic analysis. Hundreds of IR spectra are reproduced, six to a page, and the transparent plastic bookmark carries wavelength and wavenumber grids to be superimposed on the spectra. This is attractive, but of questionable value in view of the difficulty in obtaining sufficiently pure samples and the ready availability of spectra elsewhere. Problems caused by the polymorphism of some categories of drugs also are not signaled. Appendixes include preparation of many reagents, description of color tests, the bibliography, and a good index.

Ten introductory chapters supply general discussion in support of the contents of the monographs. The first chapter contains rapid screening procedures for drug types known to account for most of drug ingestion cases brought to hospitals. Subsequent chapters discuss the three monograph chromatographies, spectrophotometric techniques, the older color and crystal tests, extraction procedures, and drug metabolism. At first notice, Smith's chapter on patterns of drug metabolism may appear unrelated to some readers. Further thought reveals the necessity of knowing excretion and metabolic aspects of drugs in humans. Little value to haphazard searching of the wrong tissues or fluids for evidence of a drug or more commonly, overlooking major metabolites while searching only for unchanged drug. An observation may be made that the monographs and tables are concerned with unchanged drugs and the general omission of metabolite identification is a weakness in this work. This is partly editorial, but also reflects the limited information in this area.

The major weakness of the volume lies in the area of sample isolation. As now offered, the book is largely one of identification methods. Common clean-up techniques aren't presented in a usable form. Isolation methods from pharmaceuticals just aren't treated. It is not a text for isolation and purification of samples.

Clarke's compilation is a practical fusion of qualitative organic analysis and toxicology in monograph form. The reference value of this work is unquestionable. This and the several innovations strongly recommend the volume to laboratories handling compounds of biomedical interest.

Reviewed by Lee T. Grady
Drug Standards Laboratory
American Pharmaceutical Association Foundation
Washington DC 20037 ■

Marine Pharmacology. By MORRIS H. BASLOW. Williams and Wilkins Co., Baltimore, MD 21202, 1969. xiv + 286 pp. 18 × 26 cm. Price $19.75.

The text represents the first comprehensive coverage of the embryonic field of marine pharmacology. It is the outgrowth of the author's interest in this field developed from a graduate course on the subject presented at the University of Hawaii for three years. In its comprehensive overview coverage, one finds chapters ranging from the antibiotic activity of sea water, through the biodynamic principles of various phyla of microorganisms, algae, sponges, jellyfish, marine worms, molluscs, arthropods, echinoderms, and finally culminating in the various higher vertebrates.

Since no other basis for classification is yet reasonable, the taxonomic one used by the author is very effective. Each chapter on a particular phylum is logically arranged and follows a smooth progression of subcategories so designed as to point out bioactive data in an easily readable fashion. References are at the end of each chapter and are numerous and current. For example, the chapter on blue-green algae starts with a brief consideration of the characteristics of the group and the history of reviews on the subject then proceeds through the known chemistry performed on bioactive algal principles, the pharmacology and toxicity of their active substances, and a conclusion based on the potential for the development of useful drugs or other agents from the phylum.

The chapters are profusely illustrated with tables containing pertinent condensed data from numerous investigators. Many chemical structures of known substances are also given as well as reproductions of kymograph and physiograph recordings of effects of the more important marine principles on various test organisms. The final chapter summarizes in table form (10 pages), the pharmacological potential of substances isolated from marine organisms. The index is excellent and complete so that one can start with the name of an organism, the chemical principle, or type of activity and find a summary on these as well as appropriate references in the current scientific literature.

The text is to be highly recommended for use by pharmacologists, pharmaceutical chemists, and pharmacognosists as a prime reference on the subject. The main, and perhaps only, undesirable feature is the high price for this publication. If the publishers wish to make it readily available at all levels of interest, the price should be reduced considerably. In addition, the first eight pages (two of which contain four-color photographs of marine organisms), are printed on glossy paper while the remainder of the text is printed on less expensive yellowish stock paper.

Reviewed by A. H. Der Marderosian
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104 ■

NEW JOURNALS

Comparative and General Pharmacology. Edited by G. A. KERKUT and G. N. WOODRUFF. Scientechnica Ltd., 823–825 Bath Road, Bristol, England BS4 5NU, 1970. i + 128 pp. 18 × 24.5 cm. Price: Annual Subscription $40, Single Copies $11. (*English*)

This quarterly journal will publish original research on all aspects of pharmacology with special emphasis on comparative pharmacology. Occasional review articles and short communications will also be published. The editor, Professor G. A. Kerkut, is in the Department of Physiology and Biochemistry, University of Southampton, Southampton, England. ■

NOTICES

Pharmaceutical Handbook. Edited by R. G. TODD. The Pharmaceutical Press, 17 Bloomsbury Square, W. C. 1 London, England, 1970. xv + 702 pp. 12.5 × 19 cm. Price $9.50.

Chirurgenverzeichnis. By BURKLE DE LA CAMP. Springer-Verlag, Heidelberger Platz 3, 1 Berlin 33, Germany, 1970. viii + 1088 pp. 14.5 × 21 cm. Price $20.90. (*German*)

Gas-Liquid Reactions. By P. V. DANCKWERTS. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. xiii + 276 pp. 15.5 × 23.5 cm. Price $11.50.

Methods of Biochemical Analysis, Vol. 18. Edited by D. GLICK. Wiley, 605 Third Ave., New York, NY 10016, 1970. vi + 421 pp. 15 × 23.5 cm. Price $16.50.

Ingredient X. The Production of Effective Drugs. By LOUIS C. SCHROETER. Pergamon Publishing Co., Maxwell House, Fairview Park, Elmsford, NY 10523, 1969. vii + 157 pp. 13.5 × 20.5 cm. Price $7.75.

Mutation as Cellular Process. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. xi + 244 pp. 15.5 × 23.5 cm.

Homeostatic Regulators. Edited by G. E. W. WOLSTENHOLME and JULIE KNIGHT. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. viii + 327 pp. 15.5 × 23.5 cm.

Foetal Autonomy. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. x + 326 pp. 15.5 × 23.5 cm.

Microbiologie Industrielle et Genie Biochimique. By P. SIMON and R. MEUNIER. Masson et Cie, 120 Boulevard Saint-Germain, Paris, France, 1970. vi + 567 pp. 16.5 × 25 cm. Price 180 fr. (*French*)

quantitative methods, dose, metabolism, and an abstract of the referenced toxicology literature.

Following the monographs are individual collations of each identity test. Drugs are grouped under the standard chromatographic systems. Melting points, UV maxima, and IR bands are listed numerically with the drugs entered opposite. Color test data are compiled by observed reaction. Such devices are familiar and welcome to those experienced in qualitative organic analysis. Hundreds of IR spectra are reproduced, six to a page, and the transparent plastic bookmark carries wavelength and wavenumber grids to be superimposed on the spectra. This is attractive, but of questionable value in view of the difficulty in obtaining sufficiently pure samples and the ready availability of spectra elsewhere. Problems caused by the polymorphism of some categories of drugs also are not signaled. Appendixes include preparation of many reagents, description of color tests, the bibliography, and a good index.

Ten introductory chapters supply general discussion in support of the contents of the monographs. The first chapter contains rapid screening procedures for drug types known to account for most of drug ingestion cases brought to hospitals. Subsequent chapters discuss the three monograph chromatographies, spectrophotometric techniques, the older color and crystal tests, extraction procedures, and drug metabolism. At first notice, Smith's chapter on patterns of drug metabolism may appear unrelated to some readers. Further thought reveals the necessity of knowing excretion and metabolic aspects of drugs in humans. Little value to haphazard searching of the wrong tissues or fluids for evidence of a drug or more commonly, overlooking major metabolites while searching only for unchanged drug. An observation may be made that the monographs and tables are concerned with unchanged drugs and the general omission of metabolite identification is a weakness in this work. This is partly editorial, but also reflects the limited information in this area.

The major weakness of the volume lies in the area of sample isolation. As now offered, the book is largely one of identification methods. Common clean-up techniques aren't presented in a usable form. Isolation methods from pharmaceuticals just aren't treated. It is not a text for isolation and purification of samples.

Clarke's compilation is a practical fusion of qualitative organic analysis and toxicology in monograph form. The reference value of this work is unquestionable. This and the several innovations strongly recommend the volume to laboratories handling compounds of biomedical interest.

Reviewed by Lee T. Grady
Drug Standards Laboratory
American Pharmaceutical Association Foundation
Washington DC 20037 ■

Marine Pharmacology. By MORRIS H. BASLOW. Williams and Wilkins Co., Baltimore, MD 21202, 1969. xiv + 286 pp. 18 × 26 cm. Price $19.75.

The text represents the first comprehensive coverage of the embryonic field of marine pharmacology. It is the outgrowth of the author's interest in this field developed from a graduate course on the subject presented at the University of Hawaii for three years. In its comprehensive overview coverage, one finds chapters ranging from the antibiotic activity of sea water, through the biodynamic principles of various phyla of microorganisms, algae, sponges, jellyfish, marine worms, molluscs, arthropods, echinoderms, and finally culminating in the various higher vertebrates.

Since no other basis for classification is yet reasonable, the taxonomic one used by the author is very effective. Each chapter on a particular phylum is logically arranged and follows a smooth progression of subcategories so designed as to point out bioactive data in an easily readable fashion. References are at the end of each chapter and are numerous and current. For example, the chapter on blue-green algae starts with a brief consideration of the characteristics of the group and the history of reviews on the subject then proceeds through the known chemistry performed on bioactive algal principles, the pharmacology and toxicity of their active substances, and a conclusion based on the potential for the development of useful drugs or other agents from the phylum.

The chapters are profusely illustrated with tables containing pertinent condensed data from numerous investigators. Many chemical structures of known substances are also given as well as reproductions of kymograph and physiograph recordings of effects of the more important marine principles on various test organisms. The final chapter summarizes in table form (10 pages), the pharmacological potential of substances isolated from marine organisms. The index is excellent and complete so that one can start with the name of an organism, the chemical principle, or type of activity and find a summary on these as well as appropriate references in the current scientific literature.

The text is to be highly recommended for use by pharmacologists, pharmaceutical chemists, and pharmacognosists as a prime reference on the subject. The main, and perhaps only, undesirable feature is the high price for this publication. If the publishers wish to make it readily available at all levels of interest, the price should be reduced considerably. In addition, the first eight pages (two of which contain four-color photographs of marine organisms), are printed on glossy paper while the remainder of the text is printed on less expensive yellowish stock paper.

Reviewed by A. H. Der Marderosian
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104 ■

NEW JOURNALS

Comparative and General Pharmacology. Edited by G. A. KERKUT and G. N. WOODRUFF. Scientechnica Ltd., 823–825 Bath Road, Bristol, England BS4 5NU, 1970. i + 128 pp. 18 × 24.5 cm. Price: Annual Subscription $40, Single Copies $11. (*English*)

This quarterly journal will publish original research on all aspects of pharmacology with special emphasis on comparative pharmacology. Occasional review articles and short communications will also be published. The editor, Professor G. A. Kerkut, is in the Department of Physiology and Biochemistry, University of Southampton, Southampton, England. ■

NOTICES

Pharmaceutical Handbook. Edited by R. G. TODD. The Pharmaceutical Press, 17 Bloomsbury Square, W. C. 1 London, England, 1970. xv + 702 pp. 12.5 × 19 cm. Price $9.50.

Chirurgenverzeichnis. By BURKLE DE LA CAMP. Springer-Verlag, Heidelberger Platz 3, 1 Berlin 33, Germany, 1970. viii + 1088 pp. 14.5 × 21 cm. Price $20.90. (*German*)

Gas-Liquid Reactions. By P. V. DANCKWERTS. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. xiii + 276 pp. 15.5 × 23.5 cm. Price $11.50.

Methods of Biochemical Analysis, Vol. 18. Edited by D. GLICK. Wiley, 605 Third Ave., New York, NY 10016, 1970. vi + 421 pp. 15 × 23.5 cm. Price $16.50.

Ingredient X. The Production of Effective Drugs. By LOUIS C. SCHROETER. Pergamon Publishing Co., Maxwell House, Fairview Park, Elmsford, NY 10523, 1969. vii + 157 pp. 13.5 × 20.5 cm. Price $7.75.

Mutation as Cellular Process. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. xi + 244 pp. 15.5 × 23.5 cm.

Homeostatic Regulators. Edited by G. E. W. WOLSTENHOLME and JULIE KNIGHT. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. viii + 327 pp. 15.5 × 23.5 cm.

Foetal Autonomy. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. x + 326 pp. 15.5 × 23.5 cm.

Microbiologie Industrielle et Genie Biochimique. By P. SIMON and R. MEUNIER. Masson et Cie, 120 Boulevard Saint-Germain, Paris, France, 1970. vi + 567 pp. 16.5 × 25 cm. Price 180 fr. (*French*)

quantitative methods, dose, metabolism, and an abstract of the referenced toxicology literature.

Following the monographs are individual collations of each identity test. Drugs are grouped under the standard chromatographic systems. Melting points, UV maxima, and IR bands are listed numerically with the drugs entered opposite. Color test data are compiled by observed reaction. Such devices are familiar and welcome to those experienced in qualitative organic analysis. Hundreds of IR spectra are reproduced, six to a page, and the transparent plastic bookmark carries wavelength and wavenumber grids to be superimposed on the spectra. This is attractive, but of questionable value in view of the difficulty in obtaining sufficiently pure samples and the ready availability of spectra elsewhere. Problems caused by the polymorphism of some categories of drugs also are not signaled. Appendixes include preparation of many reagents, description of color tests, the bibliography, and a good index.

Ten introductory chapters supply general discussion in support of the contents of the monographs. The first chapter contains rapid screening procedures for drug types known to account for most of drug ingestion cases brought to hospitals. Subsequent chapters discuss the three monograph chromatographies, spectrophotometric techniques, the older color and crystal tests, extraction procedures, and drug metabolism. At first notice, Smith's chapter on patterns of drug metabolism may appear unrelated to some readers. Further thought reveals the necessity of knowing excretion and metabolic aspects of drugs in humans. Little value to haphazard searching of the wrong tissues or fluids for evidence of a drug or more commonly, overlooking major metabolites while searching only for unchanged drug. An observation may be made that the monographs and tables are concerned with unchanged drugs and the general omission of metabolite identification is a weakness in this work. This is partly editorial, but also reflects the limited information in this area.

The major weakness of the volume lies in the area of sample isolation. As now offered, the book is largely one of identification methods. Common clean-up techniques aren't presented in a usable form. Isolation methods from pharmaceuticals just aren't treated. It is not a text for isolation and purification of samples.

Clarke's compilation is a practical fusion of qualitative organic analysis and toxicology in monograph form. The reference value of this work is unquestionable. This and the several innovations strongly recommend the volume to laboratories handling compounds of biomedical interest.

Reviewed by Lee T. Grady
Drug Standards Laboratory
American Pharmaceutical Association Foundation
Washington DC 20037 ■

Marine Pharmacology. By MORRIS H. BASLOW. Williams and Wilkins Co., Baltimore, MD 21202, 1969. xiv + 286 pp. 18 × 26 cm. Price $19.75.

The text represents the first comprehensive coverage of the embryonic field of marine pharmacology. It is the outgrowth of the author's interest in this field developed from a graduate course on the subject presented at the University of Hawaii for three years. In its comprehensive overview coverage, one finds chapters ranging from the antibiotic activity of sea water, through the biodynamic principles of various phyla of microorganisms, algae, sponges, jellyfish, marine worms, molluscs, arthropods, echinoderms, and finally culminating in the various higher vertebrates.

Since no other basis for classification is yet reasonable, the taxonomic one used by the author is very effective. Each chapter on a particular phylum is logically arranged and follows a smooth progression of subcategories so designed as to point out bioactive data in an easily readable fashion. References are at the end of each chapter and are numerous and current. For example, the chapter on blue-green algae starts with a brief consideration of the characteristics of the group and the history of reviews on the subject then proceeds through the known chemistry performed on bioactive algal principles, the pharmacology and toxicity of their active substances, and a conclusion based on the potential for the development of useful drugs or other agents from the phylum.

The chapters are profusely illustrated with tables containing pertinent condensed data from numerous investigators. Many chemical structures of known substances are also given as well as reproductions of kymograph and physiograph recordings of effects of the more important marine principles on various test organisms. The final chapter summarizes in table form (10 pages), the pharmacological potential of substances isolated from marine organisms. The index is excellent and complete so that one can start with the name of an organism, the chemical principle, or type of activity and find a summary on these as well as appropriate references in the current scientific literature.

The text is to be highly recommended for use by pharmacologists, pharmaceutical chemists, and pharmacognosists as a prime reference on the subject. The main, and perhaps only, undesirable feature is the high price for this publication. If the publishers wish to make it readily available at all levels of interest, the price should be reduced considerably. In addition, the first eight pages (two of which contain four-color photographs of marine organisms), are printed on glossy paper while the remainder of the text is printed on less expensive yellowish stock paper.

Reviewed by A. H. Der Marderosian
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104 ■

NEW JOURNALS

Comparative and General Pharmacology. Edited by G. A. KERKUT and G. N. WOODRUFF. Scientechnica Ltd., 823–825 Bath Road, Bristol, England BS4 5NU, 1970. i + 128 pp. 18 × 24.5 cm. Price: Annual Subscription $40, Single Copies $11. (*English*)

This quarterly journal will publish original research on all aspects of pharmacology with special emphasis on comparative pharmacology. Occasional review articles and short communications will also be published. The editor, Professor G. A. Kerkut, is in the Department of Physiology and Biochemistry, University of Southampton, Southampton, England. ■

NOTICES

Pharmaceutical Handbook. Edited by R. G. TODD. The Pharmaceutical Press, 17 Bloomsbury Square, W. C. 1 London, England, 1970. xv + 702 pp. 12.5 × 19 cm. Price $9.50.

Chirurgenverzeichnis. By BURKLE DE LA CAMP. Springer-Verlag, Heidelberger Platz 3, 1 Berlin 33, Germany, 1970. viii + 1088 pp. 14.5 × 21 cm. Price $20.90. (*German*)

Gas-Liquid Reactions. By P. V. DANCKWERTS. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. xiii + 276 pp. 15.5 × 23.5 cm. Price $11.50.

Methods of Biochemical Analysis, Vol. 18. Edited by D. GLICK. Wiley, 605 Third Ave., New York, NY 10016, 1970. vi + 421 pp. 15 × 23.5 cm. Price $16.50.

Ingredient X. The Production of Effective Drugs. By LOUIS C. SCHROETER. Pergamon Publishing Co., Maxwell House, Fairview Park, Elmsford, NY 10523, 1969. vii + 157 pp. 13.5 × 20.5 cm. Price $7.75.

Mutation as Cellular Process. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. xi + 244 pp. 15.5 × 23.5 cm.

Homeostatic Regulators. Edited by G. E. W. WOLSTENHOLME and JULIE KNIGHT. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. viii + 327 pp. 15.5 × 23.5 cm.

Foetal Autonomy. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. x + 326 pp. 15.5 × 23.5 cm.

Microbiologie Industrielle et Genie Biochimique. By P. SIMON and R. MEUNIER. Masson et Cie, 120 Boulevard Saint-Germain, Paris, France, 1970. vi + 567 pp. 16.5 × 25 cm. Price 180 fr. (*French*)

quantitative methods, dose, metabolism, and an abstract of the referenced toxicology literature.

Following the monographs are individual collations of each identity test. Drugs are grouped under the standard chromatographic systems. Melting points, UV maxima, and IR bands are listed numerically with the drugs entered opposite. Color test data are compiled by observed reaction. Such devices are familiar and welcome to those experienced in qualitative organic analysis. Hundreds of IR spectra are reproduced, six to a page, and the transparent plastic bookmark carries wavelength and wavenumber grids to be superimposed on the spectra. This is attractive, but of questionable value in view of the difficulty in obtaining sufficiently pure samples and the ready availability of spectra elsewhere. Problems caused by the polymorphism of some categories of drugs also are not signaled. Appendixes include preparation of many reagents, description of color tests, the bibliography, and a good index.

Ten introductory chapters supply general discussion in support of the contents of the monographs. The first chapter contains rapid screening procedures for drug types known to account for most of drug ingestion cases brought to hospitals. Subsequent chapters discuss the three monograph chromatographies, spectrophotometric techniques, the older color and crystal tests, extraction procedures, and drug metabolism. At first notice, Smith's chapter on patterns of drug metabolism may appear unrelated to some readers. Further thought reveals the necessity of knowing excretion and metabolic aspects of drugs in humans. Little value to haphazard searching of the wrong tissues or fluids for evidence of a drug or more commonly, overlooking major metabolites while searching only for unchanged drug. An observation may be made that the monographs and tables are concerned with unchanged drugs and the general omission of metabolite identification is a weakness in this work. This is partly editorial, but also reflects the limited information in this area.

The major weakness of the volume lies in the area of sample isolation. As now offered, the book is largely one of identification methods. Common clean-up techniques aren't presented in a usable form. Isolation methods from pharmaceuticals just aren't treated. It is not a text for isolation and purification of samples.

Clarke's compilation is a practical fusion of qualitative organic analysis and toxicology in monograph form. The reference value of this work is unquestionable. This and the several innovations strongly recommend the volume to laboratories handling compounds of biomedical interest.

Reviewed by Lee T. Grady
Drug Standards Laboratory
American Pharmaceutical Association Foundation
Washington DC 20037 ■

Marine Pharmacology. By MORRIS H. BASLOW. Williams and Wilkins Co., Baltimore, MD 21202, 1969. xiv + 286 pp. 18 × 26 cm. Price $19.75.

The text represents the first comprehensive coverage of the embryonic field of marine pharmacology. It is the outgrowth of the author's interest in this field developed from a graduate course on the subject presented at the University of Hawaii for three years. In its comprehensive overview coverage, one finds chapters ranging from the antibiotic activity of sea water, through the biodynamic principles of various phyla of microorganisms, algae, sponges, jellyfish, marine worms, molluscs, arthropods, echinoderms, and finally culminating in the various higher vertebrates.

Since no other basis for classification is yet reasonable, the taxonomic one used by the author is very effective. Each chapter on a particular phylum is logically arranged and follows a smooth progression of subcategories so designed as to point out bioactive data in an easily readable fashion. References are at the end of each chapter and are numerous and current. For example, the chapter on blue-green algae starts with a brief consideration of the characteristics of the group and the history of reviews on the subject then proceeds through the known chemistry performed on bioactive algal principles, the pharmacology and toxicity of their active substances, and a conclusion based on the potential for the development of useful drugs or other agents from the phylum.

The chapters are profusely illustrated with tables containing pertinent condensed data from numerous investigators. Many chemical structures of known substances are also given as well as reproductions of kymograph and physiograph recordings of effects of the more important marine principles on various test organisms. The final chapter summarizes in table form (10 pages), the pharmacological potential of substances isolated from marine organisms. The index is excellent and complete so that one can start with the name of an organism, the chemical principle, or type of activity and find a summary on these as well as appropriate references in the current scientific literature.

The text is to be highly recommended for use by pharmacologists, pharmaceutical chemists, and pharmacognosists as a prime reference on the subject. The main, and perhaps only, undesirable feature is the high price for this publication. If the publishers wish to make it readily available at all levels of interest, the price should be reduced considerably. In addition, the first eight pages (two of which contain four-color photographs of marine organisms), are printed on glossy paper while the remainder of the text is printed on less expensive yellowish stock paper.

Reviewed by A. H. Der Marderosian
Philadelphia College of Pharmacy and Science
Philadelphia, PA 19104 ■

NEW JOURNALS

Comparative and General Pharmacology. Edited by G. A. KERKUT and G. N. WOODRUFF. Scientechnica Ltd., 823–825 Bath Road, Bristol, England BS4 5NU, 1970. i + 128 pp. 18 × 24.5 cm. Price: Annual Subscription $40, Single Copies $11. (*English*)

This quarterly journal will publish original research on all aspects of pharmacology with special emphasis on comparative pharmacology. Occasional review articles and short communications will also be published. The editor, Professor G. A. Kerkut, is in the Department of Physiology and Biochemistry, University of Southampton, Southampton, England. ■

NOTICES

Pharmaceutical Handbook. Edited by R. G. TODD. The Pharmaceutical Press, 17 Bloomsbury Square, W. C. 1 London, England, 1970. xv + 702 pp. 12.5 × 19 cm. Price $9.50.

Chirurgenverzeichnis. By BURKLE DE LA CAMP. Springer-Verlag, Heidelberger Platz 3, 1 Berlin 33, Germany, 1970. viii + 1088 pp. 14.5 × 21 cm. Price $20.90. (*German*)

Gas-Liquid Reactions. By P. V. DANCKWERTS. McGraw-Hill Book Co., 330 West 42nd St., New York, NY 10036, 1970. xiii + 276 pp. 15.5 × 23.5 cm. Price $11.50.

Methods of Biochemical Analysis, Vol. 18. Edited by D. GLICK. Wiley, 605 Third Ave., New York, NY 10016, 1970. vi + 421 pp. 15 × 23.5 cm. Price $16.50.

Ingredient X. The Production of Effective Drugs. By LOUIS C. SCHROETER. Pergamon Publishing Co., Maxwell House, Fairview Park, Elmsford, NY 10523, 1969. vii + 157 pp. 13.5 × 20.5 cm. Price $7.75.

Mutation as Cellular Process. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. xi + 244 pp. 15.5 × 23.5 cm.

Homeostatic Regulators. Edited by G. E. W. WOLSTENHOLME and JULIE KNIGHT. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. viii + 327 pp. 15.5 × 23.5 cm.

Foetal Autonomy. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. J. & A. Churchill Ltd., 104 Gloucester Place, London, England, 1969. x + 326 pp. 15.5 × 23.5 cm.

Microbiologie Industrielle et Genie Biochimique. By P. SIMON and R. MEUNIER. Masson et Cie, 120 Boulevard Saint-Germain, Paris, France, 1970. vi + 567 pp. 16.5 × 25 cm. Price 180 fr. (*French*)

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

SEPTEMBER 1970
VOLUME 59 NUMBER 9

REVIEW ARTICLE

Steric Aspects of Adrenergic Drugs

P. N. PATIL, J. B. LaPIDUS, and A. TYE

Keyphrases ☐ Adrenergic drugs—steric aspects ☐ Stereoselectivity in biosynthesis—uptake, binding, release ☐ Potentiation, stereoselective—adrenergic drugs ☐ Amines—"indirectly" acting ☐ Drug-receptor theory—adrenergic drugs ☐ Metabolism, central effects—stereoselectivity ☐ α,β-Adrenergic receptors—drug interaction

CONTENTS

... Concepts are extremely useful to the human mind, but concepts may change and change very fast. What remains are the facts, the experimental facts, the discoveries. If a new concept leads to a discovery, you may award a prize without hesitation. But a concept without a discovery would have little chance. Concepts are instruments in scientific research. They help you to make new discoveries. If they don't, they have no justification. And the only way you can prove the justification of a concept—the correctness of a concept—is to see if it not only explains already known facts, but also leads to a new and unexpected discovery. That proves that there is something new in the concept....

Arne Tiselius
(Nobel Laureate)

Adrenergic drugs can almost serve as a prototype for the classical development of a group of medicinally active compounds. The initial lead arises from the observation of physiological activity associated with a natural product. Eventually, the chemical structure of the active substance is revealed, synthetic analogs are prepared, and their pharmacological activities are determined. An empirical body of structure–action relationships develops as a result of these studies, which leads to the synthesis of more compounds and, as knowledge of structural chemistry and pharmacological mechanisms becomes more refined, ideas concerning the relationship of chemical structure and pharmacological action become less empirical.

All these steps have been followed in developing the area of adrenergic drugs to its present state. It is particularly notable that the lead compound, epinephrine, is optically active. The early syntheses leading to racemic material and the pharmacological evaluation of the racemate made it apparent that some stereoselectivity was involved since the racemate was less active than the optically active natural product. Thus, at a very early stage in the development of adrenergic drugs, steric effects were observed. It is surprising that more resolutions were not performed and absolute configurations were not determined for many of the major synthetic analogs until recently. However, some attempts have

been made to review natural asymmetry and pharmacologic action (1, 2). The current revival of interest in this area makes it desirable to review what is known about steric effects and adrenergic agents.

A detailed discussion of adrenergic mechanism would be out of place here, but a brief review of some basic concepts is essential for any meaningful discussion of steric aspects.

Ahlquist (3) suggested the terms α and β for two different types of adrenergic receptors. This classification was based on the comparative effectiveness of the compounds epinephrine, norepinephrine, and isoproterenol on various tissues. In those tissues where the order of activity was epinephrine $\geq$ norepinephrine $\gg$ isoproterenol, the receptors were called α and associated with excitation or contraction. In tissues where the order was isoproterenol $\gg$ epinephrine $\geq$ norepinephrine, the receptors were designated β and associated with inhibition of function or relaxation. The validity of this concept, at least with respect to certain tissues, has been supported by experiments with selective antagonists, some of which block only α-effects and some of which block only β-effects. Other types of adrenergic receptors have been suggested, but most of the experimental work to date has been done with so-called α-agonists and β-agonists and their respective antagonists. A more complete discussion of the concepts of α- and β-receptors may be found in a recent publication (4).

The concept of direct and indirect action has received ample experimental verification. In its simplest sense, it suggests that sympathomimetic agents may act: predominantly directly, that is, at the effector site; predominantly indirectly, by releasing endogenous norepinephrine; or by a combination of these processes. Actually, there is evidence that the mechanism of action of indirect-acting sympathomimetic amines is more complex. Many indirect acting agents appear to block the uptake of norepinephrine as well as cause its release (5).

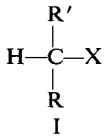
Regardless of the intricacies of the mechanism of action of indirect-acting sympathomimetic agents, it is important to keep in mind that drugs which are closely related structurally, such as the phenethylamines, may produce pharmacologically identical effects by different mechanisms. Analyses of structural or stereochemical requirements for activity are considerably complicated by this fact, and comparative studies of sympathomimetic amines done prior to the establishment of this concept must be reevaluated in modern terms.

Uptake of catecholamines by adrenergic nerves has been shown to proceed against a concentration gradient, and it seems that a saturable active transport system is involved. Iversen (6) has defined two uptake processes: uptake_1 and uptake_2. Uptake_1 is operative at low perfusion concentrations, accumulated norepinephrine is not readily washed out by perfusion with norepinephrine-free medium, and the process appears to be stereoselective for the enantiomers of both norpinephrine and epinephrine. Uptake_2 is operative at higher concentrations. The uptake is characterized by rapid washout of accumulated norepinephrine and complete lack of stereoselectivity. After exogenous catecholamines pass through the neuronal membrane, they may be retained by storage granules inside the neurone. There is considerable evidence for the existence of more than one pool of stored catecholamines within the neurone. Perhaps the most complete information available deals with norepinephrine storage granules isolated from bovine splenic nerves and the adrenal medulla (7). The ability of these isolated storage granules to accumulate the isomers of norepinephrine, epinephrine, and some related compounds has been studied and will be discussed later.

Before leaving the subject of uptake and storage, the effects of cocaine and reserpine must be mentioned. These two drugs have been widely used as pharmacological tools in the study of adrenergic mechanisms. Cocaine apparently acts by inhibiting uptake of catecholamines and related compounds at the adrenergic neurone membrane. On the other hand, reserpine appears to exert its effect on the intraneuronal storage mechanisms and acts to prevent accumulation of norepinephrine or related compounds. Reserpine does not affect neuronal membrane uptake. The recently published monograph by Iversen (6) provides a detailed and extensively documented discussion of various aspects of the uptake and storage processes.

STEREOCHEMICAL NOTATION

Any discussion of stereochemical aspects of drug action must involve the proper use of notation to identify and specify the actual three-dimensional characteristics of the molecules considered. The small capital letters D and L are generally used to denote the configuration of an asymmetric carbon atom (Structure I) in a molecule of the type:

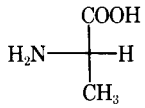


I

The molecule is oriented so that the number 1 carbon of the principal chain is at the top in the usual Fischer projection (8). This is sometimes stated differently, that is, that the carbon in the highest oxidation state is at the top, but since this would be the number 1 carbon, there is no conflict between these statements. If, when the molecule is so oriented, X is on the right, the configuration is said to be D; if X is on the left, the term L is used. These letters should *never* be used to denote sign of rotation. In many cases the lower case letters *d* and *l* (*dextro* and *levo*) have been used to specify sign of rotation, but this can lead to ambiguities. Some authors, particularly in the biological literature, use the upper case and lower case symbols interchangeably. For example, in some studies of sympathomimetic amines, the more active enantiometer of norepinephrine has been referred to as the L-isomer. In these cases, this means levorotatory; but most chemists would interpret this as meaning the "L" configuration, and the L isomer of norepinephrine is dextrorotatory. This example should suffice to show that when either upper case or lower case symbols of this type are used, the reader cannot be sure which enantiomer is being discussed.

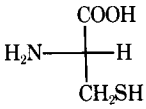
The most reliable notation for specifying absolute configurations is usually referred to as the "sequence

rule." This method has been described in the literature (9) and has been widely accepted. An important distinction between the sequence rule and DL nomenclature is that while the symbols D and L relate the configuration of a molecule to some arbitrary standard, the sequence rule notation is self-consistent for the molecule in question but cannot be used to relate a series of compounds. This can be most clearly seen by considering the following compounds (Structures II and III):



L-alanine

II



L-cysteine

III

These two compounds are obviously related configurationally and have the same absolute configuration. However, due to the difference in atomic number of the substituents, L-alanine has the S-configuration while L-cysteine has the R-configuration.

Medicinal chemists and pharmacologists are often interested in describing structure–action relationships in a series of structurally related drugs. When this is done, it makes little difference which method is used to describe the compounds evaluated as long as the method is unambiguous. For this reason, the symbols D, L, *d*, and *l* should not be used in these cases. Rotations, if desired, should be indicated by either (+) or (−), and absolute configurations by either R or S. Throughout this review, isomers are identified by the signs (+) or (−).

FACTORS COMPLICATING INTERPRETATION OF RESULTS

In every review or symposium, it has become increasingly important to discuss factors that can influence the interpretation of results. This review on optical isomers cannot be an exception. First and most important, when the activity of (−)- and (+)-isomers is compared, it is assumed that both forms are pure. However, this may not be the case. The activity ratios between (−)- and (+)-isomers of isoproterenol were reported as 11.8 for cat blood pressure (10) and 87.45 for dog blood pressure (11). Subsequently, Lands *et al.* (12) obtained higher activity ratios, 1000 for cat blood pressure and 450 for dog blood pressure. They pointed out that constant specific rotation or melting point is not the best criterion for optical purity, but that constant biological activity is.

Occasionally, pharmacologic activity of (−)- and (+)-isomers is expressed in mcg./kg. or mcg./ml. If both isomers have identical salts, it should not influence the calculation of the activity ratio. However, if one isomer is bitartrate salt and the other is hydrochloride salt, it will obviously affect the expression of the activity ratio. Hence, the dose or concentration should be expressed in molar terms, which necessitates knowing the molecular weight of a given drug. Further, it is interesting to know that different physical forms can have different biologic activity. Lands *et al.* (12) found that (+)-isoproterenol as a bitartrate salt is more toxic than when given as a base dissolved with dilute hydrochloric acid. Unfortunately, where the isomers are obtained from outside sources, frequently the physical form of the isomers is not explicitly stated.

Pharmacologic activity of the sympathomimetic agents at adrenergic synapses is complicated by functional integrity of the uptake site (or transfer site) or storage site or by the presence of both α- and β-adrenergic receptors. The enzymes, dopamine-β-hydroxylase, monoamine oxidase (MAO), and catechol-*o*-methyl transferase (COMT), further complicate the situation. Optical isomers of adrenergic drugs are known to interact differently with many of these factors that influence pharmacologic activity. Depending upon the objective of the investigator in a given problem dealing with optical isomers, the following modifications can be made:

1. Reserpine pretreatment can be given to an animal to eliminate stored norepinephrine. Sufficient time must be allowed between reserpine pretreatment and depletion of endogenous norepinephrine. Korol *et al.* (13) studied optical isomers of octopamine on the cardiovascular system of the dog and concluded that both isomers of octopamine are acting directly. However, only 1 hr. was allowed between reserpine treatment and testing of the isomer. It is well known that during the early phase of action of reserpine, the effects of tyramine or phenethylamine are potentiated.
2. Cocaine can be used to inhibit uptake at the adrenergic neuronal membrane.
3. If the pharmacologic effects of closely related agents such as (+)-isomers and corresponding deoxy derivatives[1] are to be studied, it is important that the enzyme, dopamine-β-hydroxylase, be inactivated by a suitable inhibitor. Many deoxy derivatives are good substrates for this enzyme. Shore (14) and Patil *et al.* (15) used a similar approach when activity of deoxy derivatives was compared with corresponding (−)- or (+)-isomers.
4. Comparative pharmacologic effects of α-methylated and non-α-methylated sympathomimetic amines should be studied under the influence of MAO inhibitor because the latter amines are quickly inactivated by enzyme monoamine oxidase. Selection of monoamine oxidase can add one more variable in experimental design. The enzymatic action can be avoided by studying α-methylated amines only.
5. Although in some tissues the role of COMT in termination of pharmacologic effect of (−)-norepinephrine may be negligible, its role for that of (+)-isomer is yet undefined. Recently, fairly stable inhibitors of this enzyme were available. Comparison of activity of both isomers of norepinephrine in the presence of cocaine and under the influence of COMT inhibitor should give a better idea regarding the activity ratio of these antimers. It is difficult to talk about the receptor level when drug effects on a complex parameter such as blood pressure are studied. Studies on isolated tissues are better for controlling the variables.
6. While studying α-adrenergic receptors, β-receptors should be blocked by a suitable β-blocker which has a minimal α-adrenergic blocking property. Rat vas

[1] Amine without β-hydroxyl group. The term deoxy derivative will be used interchangeably with the other name of the amine, for example, deoxymetaraminol or α-methyl-*m*-tyramine.

deferens, seminal vesicles, rabbit aorta, cat spleen, mouse spleen, and cat nictitating membrane are generally used for testing α-adrenergic drug activity. Guinea pig atria, trachea, and rat uterus are generally used for testing β-adrenergic activity. If desired, different tissues from the same species could be used. Every test tissue has its own characteristic. Some sympathomimetic amines produce tachyphylaxis (acute tolerance), and the rate of tachyphylaxis varies with different isomers (16, 17). Two tachyphylactic amines, therefore, cannot be assayed on the same tissue. This introduces some variability in testing procedure and, hence, in the interpretation of results. By taking care of variables in experimental design, facts are clearly exposed. Furchgott (18) has critically evaluated various problems in a study of adrenergic drugs.

Frequently, biological activity of a single isomer is derived from that of the racemic form. However, some wrong assumptions may be made. Various possibilities can occur:

1. Only one isomer is active while the other isomer is practically inactive; for example, (−)-isopropylmethoxamine is a potent β-adrenergic blocker on trachea while (+)-isopropylmethoxamine is practically inactive (15).

2. One isomer is relatively more active than the other isomer. Both the isomers of sotalol are active in blocking β-adrenergic receptors. The pA_2 value for the (−)-isomer is 6.8 and that of the (+)-isomer is 5.15. The effect is stereoselective[2] (19).

3. Both the optical isomers can be equiactive. On the isolated rat vas deferens, isomers of hydroxyamphetamine produce equal effects which do not differ from racemic hydroxyamphetamine (20).

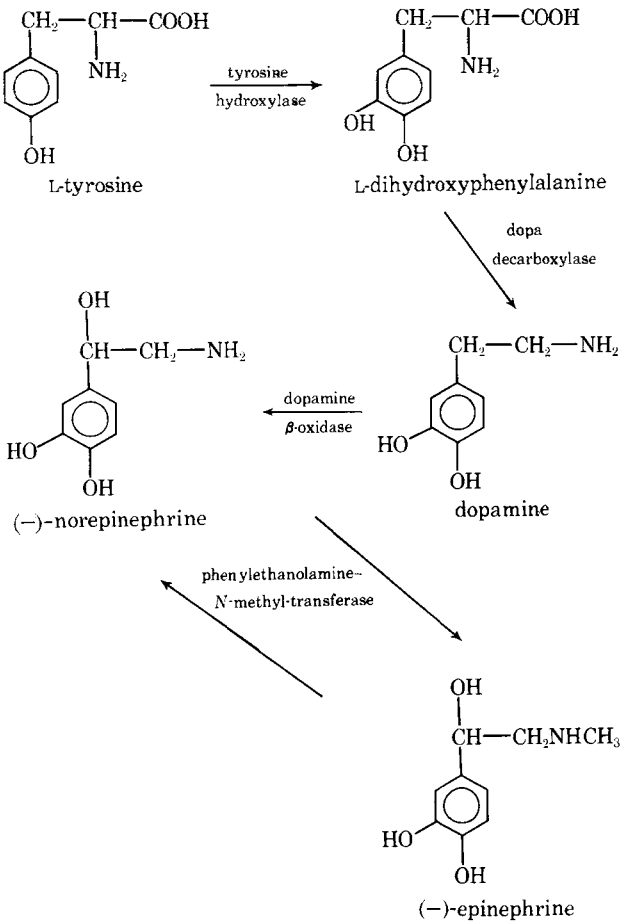
4. A given isomer may not produce any apparent effect, but it may be antagonistic to the pharmacological effects of other isomers. Luduena (21) reported that (+)-isoproterenol antagonizes the effects of (−)-isoproterenol.

5. Similarly, Fielden and Green (22–24) found that (+)-*N*-(1-phenethyl)guanidine has little effect of its own but antagonizes the potent adrenergic neurone-blocking effect of the (−)-form.

6. A very unusual finding reported by Porter *et al.* (25) is that racemic α-methyl-*p*-tyrosine causes greater depletion of norepinephrine from tissues than either isomer alone. In other words, the racemic form can behave as the third molecular species.

Occasionally, the relationship between the activity of (+)- and (−)-isomers may be different when different parameters are used. (+)-β-Hydroxyphenethylguanidine has about one-quarter the norepinephrine-depleting activity of the (−)-isomer. However, as compared to the (−)-form, the (+)-isomer is more effective in preventing ptosis caused by the adrenergic neurone-blocking agent. Such findings might be very useful in analyzing the mechanism of drug action. These observations clearly state that the norepinephrine depletion caused by the drug and the antagonism of the adrenergic neurone blockade are two different mechanisms (26).

Over the years, radiolabeled agents have facilitated research projects on adrenergic drugs, but they also have added another complication. Many labeled sympathomimetic drugs are only available in racemic form. When single desired isomers of high specific activity become available, it will be necessary to reevaluate some previous studies with the racemic form. The role of single molecular species then will be clarified.



Scheme I

BIOSYNTHESIS

The biosynthetic pathway by which norepinephrine and epinephrine are formed has been intensively studied, and a recent review summarizing this work has appeared (27).

All of the work to date has confirmed the original postulation of Blaschko (28) with respect to the major pathway in animals (Scheme I).

The substrates for the first two steps are optically active compounds. Dopamine, while not optically active, yields an optically active product, (−)-norepinephrine, which can then be converted to another optically active compound, (−)-epinephrine. Each of these reactions may, therefore, exhibit some stereochemical features which will be considered at this point.

Tyrosine → Dihydroxyphenylalanine

The action of tyrosine hydroxylase has been studied by several groups, and the properties of the enzyme have been defined (29). D-Tyrosine, tyramine, DL-*m*-tyrosine, and L-tryptophan were found to be inactive as sub-

[2] This term is preferred over stereospecificity.

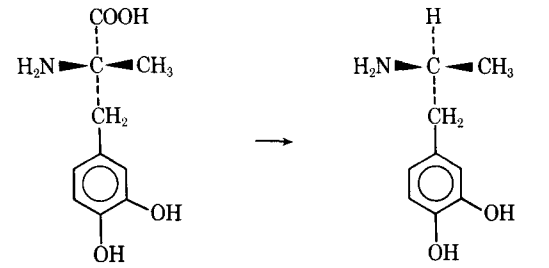
strates, indicating a high degree of structural and stereochemical specificity (30).

Dopa → Dopamine

The enzyme responsible for this conversion is best known as dopadecarboxylase, although the term aromatic L-amino acid decarboxylase is probably more correct (31). As this name suggests, the enzyme is stereospecific because L-aromatic amino acid does not decarboxylate D-dopa (28, 32, 33). It has been shown, however, that D-dopa is converted to dopamine *in vivo*, presumably by conversion to the keto acid followed by transamination to give L-dopa (34).

In addition, it has been demonstrated (35) that the enzymatic decarboxylation of amino acids proceeds with retention of configuration. Obviously, this point is not a major one in the reaction dopa → dopamine, since dopamine contains no asymmetric carbons. It is of major importance, however, in the reaction of α-methylated amino acids, where both the substrate and the product contain asymmetric carbon atoms. The α-methylated analogs of many sympathomimetic amines have been the subject of widespread interest because of their ability to act as false adrenergic transmitters (36).

Biochemically active α-methyldopa has been shown to have the S-configuration (37). This corresponds to the L-configuration of dopa. Decarboxylation of this compound with retention of configuration should yield α-methyldopamine, which also has the S-configuration (Scheme II).



S(−)-α-methyldopa S(+)-α-methyldopamine

Scheme II

The observation that decarboxylation of L-α-amino acids produces dextrorotatory α-methylamines (38), coupled with the proof that the absolute configuration of levorotatory α-methyldopamine (not the natural metabolite) is R (39), establishes the absolute configuration of the natural metabolite as S and serves as proof for the retention of configuration in the α-methyl series.

Dopamine → Norepinephrine

This transformation is catalyzed by the enzyme dopamine-β-oxidase, whose properties have been recently reviewed (40).

The substrate specificity of dopamine-β-oxidase is rather low. In the case of the conversion of dopamine to norepinephrine, substrate stereospecificity is not a factor since the substrate contains no asymmetric carbons. The product, (−)-norepinephrine, has the R-configuration.

In the case of substrate with an α-methyl group, substrate stereospecificity is involved, and several studies have demonstrated that only one enantiomer is β-hydroxylated (41–44).

In the series under discussion, where the sequence rule priorities of functional groups remain essentially the same, those enantiomers with the S-configuration at the β-carbon are active substrates while those with the R-configuration are not. The introduction of the hydroxyl group at the β-carbon is apparently stereospecific, yielding compounds whose absolute configurations at the β-carbon are the same as R(−)-norepinephrine. Thus, S(+)-α-methyldopamine is converted to αS, βR-(−)-α-methylnorepinephrine.

Norepinephrine ⇌ Epinephrine

This interconversion is catalyzed by phenethanolamine-*N*-methyltransferase, which can utilize both enantiomers as substrates, those with the R-configuration showing greater activity (45).

SELECTIVITY OF UPTAKE AND STORAGE MECHANISM FOR OPTICAL ISOMERS

After reviewing different papers on this subject, a need for consistent terminology became evident. For the sake of uniformity, the term accumulation is preferred over deposition, uptake site over transfer site, and release over efflux or chemorelease. In many instances the drug was injected by various routes, and tissue accumulation was observed at a fixed time. This type of tissue accumulation will not distinguish between accumulation by adrenergic nerve endings and extraneuronal uptake. Further, uptake by nerve endings is a two-stage process: (*a*) uptake by neuronal membrane in the cytoplasm, and (*b*) uptake by granular membrane into granules.

In 1963, Kopin and Bridgers (46) investigated the biological differences in (−)- and (+)-isomers of norepinephrine. Rats were subcutaneously injected with equal amounts of either the (±)-norepinephrine-^{14}C and (±)-norepinephrine-^{3}H or the (−)-norepinephrine-^{14}C and (±)-norepinephrine-^{3}H solutions. Animals were killed 1 or 24 hr. later; the ^{3}H–^{14}C ratio in hearts and spleens was determined. From these experiments, the relative role of (−)- and (+)-norepinephrine in tissue uptake and retention was defined. They concluded that both isomers are bound to the tissue to the same extent, and that the disappearance of (+)-norepinephrine-^{3}H is more rapid than that of (−)-isomer. Subsequently, Maickel *et al.* (47) reported that 5 min. after the intravenous injection of (±)-norepinephrine-^{3}H, the ratio of (−)-norepinephrine-^{3}H–(+)-norepinephrine-^{3}H in the rat heart was 11:1. This ratio indicated a higher affinity of uptake mechanism for (−)-norepinephrine. The lack of detection of selectivity of the uptake mechanism by Kopin and Bridgers (46) was then due to methodology. Maickel *et al.* (47) separated the isomers of norepinephrine-^{3}H by an isotope dilution technique. Kopin and Bridgers tested the differences after 1 hr. as compared to 5 min. by former investigators.

More details on the kinetic analysis of uptake of norepinephrine isomers were presented by Iversen (48). Isolated rat heart was perfused for various times with

different concentrations of norepinephrine isomers. The initial rates of uptake by various concentrations obeyed Michaelis-Menton kinetics. On the basis of the Michaelis constant, K_m, (+)-norepinephrine has only one-sixth the affinity for uptake as has (−)-norepinephrine. It is interesting, however, that the $v_{max.}$ for (+)-norepinephrine is higher than that for the (−)-isomer. Under similar experimental conditions, when hearts were perfused for 5 min. at 100 ng./ml. of epinephrine isomers, the tissue accumulation of (+)-epinephrine was only one-third as compared to that of (−)-epinephrine (49). This indicated differences in uptake for isomers of epinephrine. Although accumulation of (−)-norepinephrine in the adrenergic neurone is considered to be an active process (*i.e.*, against a concentration gradient), Born (50) has argued against this concept. The uptake of (−)-norepinephrine in the human erythrocytes occurs by simple diffusion. This process is also stereoselective (51). However, in contrast to the accumulation of (−)-norepinephrine in the adrenergic neurone, the accumulation in the erythrocytes is not inhibited by cocaine. Thus there appears to be some differences between the process of accumulation in these two systems. Selectivity of accumulation of norepinephrine isomers was also studied in the guinea pig heart (52). After intravenous injection of 1 μmole/kg. of the appropriate isomer in the anesthetized guinea pig, the norepinephrine content in the heart and aortic blood was examined. One minute after injection the net uptake of (−)-norepinephrine by the heart was higher than that of (+)-norepinephrine. However, 60 min. afterwards, the net uptake for both isomers appeared to be the same. Possibly because of the small number of observations and the large variability in results, selectivity in uptake might not have been detected.

Westfall (53) used a systematic approach to study the stereoselectivity of epinephrine accumulation in the rat heart. After equal intramuscular doses, 1 mg./kg., both (−)- and (+)-epinephrine caused a greater accumulation of (−)-isomer in the heart, with concomitant greater loss of endogenous norepinephrine. The granular fraction exhibited greater selectivity than other tissue fractions from the heart. Selectivity in accumulation of the physiologically more active isomer of epinephrine was also demonstrated in the mouse heart (54). Approximately 1 hr. after 2-mg./kg. doses, i.p., of epinephrine isomers, the accumulation of (+)-isomer was only one-third of that of (−)-isomer. In mouse femoral muscle, the accumulation difference between (−)- and (+)-isomers was very small.

Work demonstrating the stereoselectivity of norepinephrine isomer uptake and storage has continued to be reported. At relatively high concentrations of norepinephrine isomers, Mueller and Schideman (55) were unable to demonstrate the stereoselectivity in the particular fractions of cat atria. Reinvestigation of the problem with a modified technique eventually did show the differences at low concentrations of these isomers (56). The accumulation of total tritium from (±)-norepinephrine was relatively more inhibited by unlabeled (−)-isomer. Green and Miller (57) reported that *in vitro* labeled epinephrine accumulates in the rat uterus and that (−)-norepinephrine causes greater release of labeled epinephrine while (+)-norepinephrine is significantly less effective. These results indicate a possible stereoselectivity for uptake and/or release in norepinephrine isomers. Tissue perfusion provides a better test system to demonstrate selectivity for uptake than studies in intact animals with various routes of administration. Kirpekar and Wakade (58) used perfused cat spleen to investigate several factors influencing norepinephrine uptake. When (+)-norepinephrine was infused at 0.51 mcg./min. into the arterial supply of the spleen, 55% of the infused amount was recovered in the venous effluent. The recovery for that of (−)-norepinephrine was only 34%. Results indicate a 66% accumulation of (−)-norepinephrine by spleen and only 45% for (+)-norepinephrine.

Greater accumulation of exogenous norepinephrine can be demonstrated in the tissues if endogenous norepinephrine is depleted by a suitable agent which does not drastically block or impair the storage mechanism. Mackenna (59) used prenylamine-treated rabbits to study the uptake of norepinephrine and epinephrine isomers. After injection of 0.41 mg./kg. i.m. of either isomer, as compared to (+)-isomers, the accumulation and retention of (−)-isomers in the heart were higher, with longer duration. Similarly, von Euler and Lishajko (60) investigated the uptake of catecholamines in rabbit hearts depleted by decaborane. When the (−)- and (+)-epinephrines were given with (−)-norepinephrine, simultaneously and in equal amounts, relative to the uptake of (−)-norepinephrine the uptake of (−)-epinephrine was 2–3 times greater than that of (+)-epinephrine. It is suggested that when isomers are taken up in specific stores, the (−)- and (+)-isomers are released at the same rate.

The amine uptake in adrenergic nerve granules from bovine splenic nerves deserves special comment, since the study of specificity of uptake on such a simple system is more meaningful. The spontaneous depletion of endogenous norepinephrine from bovine splenic nerve granules is prevented more by (−)-norepinephrine than by the (+)-isomer. The amine uptake is enhanced by adenosine 5′-triphosphate (ATP). It is concluded that uptake is stereoselective at low concentrations (1 mcg./ml.) for naturally occurring isomers of both norepinephrine and epinephrine (61). The "affinity ratio," (−)-norepinephrine–(+)-norepinephrine, is reported as 5.9:1 (62).

However, reuptake of spontaneously released norepinephrine from the surrounding medium previously distorted the calculations of the affinity ratio of norepinephrine isomers. Von Euler and Lishajko (63) overcame this difficulty by studying the affinity ratio for uptake in the presence of potassium ferricyanide, an agent which continuously removes norepinephrine from the incubation medium of adrenergic nerve granules. The mean value for the affinity ratio found by the new method was 9.4. In the partially purified preparation of norepinephrine storage granules from rat heart, Potter and Axelrod (64) tried to demonstrate the affinity differences between isomers of norepinephrine. However, the differences did not appear to be great or significant.

Under proper conditions, condensation of formaldehyde with tissue catecholamines produces green fluores-

cent adrenergic nerves. Reserpine pretreatment depletes norepinephrine and, hence, fluorescence disappears. It can be reinstituted in the nerves by a suitable catecholamine. In catecholamine-depleted rat iris and salivary glands, after equal doses of either isomers of norepinephrine, an identical fluorescent intensity was observed in the nerves (65–67). Since the method is semiquantitative, small differences in the uptake might not have been detected. The optical isomers may differ in the rate of uptake and show identical accumulation at the end of a fixed time interval. On the other hand, it may be that the uptake mechanism in the rat iris and salivary gland and that in the heart are dissimilar. In his recent study, Iversen (68) was unable to observe differences between (−)- and (+)-isomers of amphetamine in regard to the affinity in the rat iris, but the same isomers showed a 20-fold difference in heart tissue. On this basis, he concluded that the uptake mechanism in two tissues from the same species may be different.

Although (−)- and (+)-isomers of (±)-*erythro*-levonordefrin[3] are resolved, (±)-pseudolevonordefrin or (±)-*threo*-levonordefrin are as yet unresolved. Hence, the question regarding stereospecificity of the levonordefrin molecule for uptake and storage is only partly answered. Muscholl and Lindmar (69) compared the uptake and binding of (−)-levonordefrin and (±)-*threo*-levonordefrin in the perfused rabbit heart. Like (−)-levonordefrin, (±)-*threo*-levonordefrin was readily taken up and retained by the heart with concomitant equivalent loss of norepinephrine. While initial uptake for (−)-levonordefrin, (±)-levonordefrin, and (±)-*threo*-levonordefrin appeared to be the same, their release rates were different. Similarly, in the mouse heart at various times after injection of 20 mcg./kg. i.v. of (±)-*threo*-^{3}H-levonordefrin or either (±)-*erythro*-^{3}H-levonordefrin, there was essentially equal uptake of both *erythro*- and *threo*-levonordefrin (70). But (±)-*threo*-levonordefrin (half-life, 20 hr.) appeared to leave faster than the (±)-*erythro*-form (half-life, 72 hr.). In the catecholamine-depleted rat iris, Patil and Jacobowitz (71) studied reinstitution of fluorescence caused by isomers of levonordefrin. It was observed that (−)- and (+)-levonordefrin produced equal fluorescence intensity, indicating possible equal affinity for uptake and/or storage sites. However, even higher doses of (±)-*threo*-levonordefrin failed to produce fluorescence in the nerves. Thus, histochemical evidence in rat iris is against that found in the heart. As previously stated, it is very likely that uptake characteristics are very different in the two tissues.

Under the influence of the MAO inhibitor, nialamide, the release of labeled (±)-*erythro*-levonordefrin from the mouse heart is not influenced, while (±)-*threo*-levonordefrin is virtually lost after 18 hr. (72). During the first 6 hr. after infusion, (±)-*erythro*-*N*-methyl-levonordefrin[4] retained by the heart was lost with a half-time of 5 to 6 hr. Likewise, (±)-*threo*-*N*-methyl-levonordefrin was taken up by heart and spleen. However, the concentrations did not decrease exponentially with time and the initial rates of loss were greater than those observed with (±)-*erythro*-*N*-methyl-levonordefrin (73, 74). Histochemical work of Patil and Jacobowitz (71) indicates that (±)-*erythro*-*N*-methyl-levonordefrin restores the fluorescence in catecholamine-depleted rat iris, while (±)-*threo*-*N*-methyl-levonordefrin does not. These findings are similar to those for (±)-*erythro*- and (±)-*threo*-levonordefrin.

Shore and Alpers (75) developed a sensitive and specific fluorometric method for the estimation of metaraminol in tissues. Since chemical methods do not distinguish between optical isomers of metaraminol, it has provided a valuable tool for studying stereoselectivity of these molecules. It was observed (76) that after injection of 50 mcg./kg. i.v. of either (−)-metaraminol, (+)-metaraminol, or (±)-deoxymetaraminol, only (−)-metaraminol was retained in the rat heart for up to 24 hr. The tissue concentration of (+)-metaraminol and (±)-deoxymetaraminol fall sharply 10–15 min. after administration. Lack of accumulation of (−)-metaraminol in immunosympathectomized animals indicated that accumulation of (−)-metaraminol in normal animals was in sympathetic nerves only. Intracellular distribution of these agents in different fractions from the heart revealed that (−)-metaraminol displaces heart norepinephrine and is significantly associated with particulate cell fraction, whereas (±)-deoxymetaraminol neither depletes norepinephrine nor is associated significantly with cell particles. Although (+)-metaraminol does not deplete norepinephrine, it shows some association with cell particles (77). *In vitro*, rabbit heart slices also exhibit selective accumulation of (−)-metaraminol over (+)-metaraminol or (±)-deoxymetaraminol. Pretreatment of these slices with imipramine or ouabain markedly inhibited accumulation of both isomers of metaraminol (78). Reserpine pretreatment does not prevent accumulation of (−)- and (+)-metaraminol. But, combined treatment of reserpine and ouabain markedly prevents the accumulation of (−)-metaraminol more than ouabain alone. A similar combination is not synergistic for prevention of the accumulation of (+)-metaraminol. On this basis, it is suggested that reserpine, perhaps by creating local ionic imbalance in the cell, allows ouabain to exert a greater effect on the local Na^+-K^+-ATPase-amine pump-linked mechanism which normally allows the (−)-form of catecholamine to be accumulated more rapidly than the (+)-form (79). Both in the normal and the reserpine-pretreated heart slices, the washout rate for amines is (±)-deoxymetaraminol > (+)-metaraminol > (−)-metaraminol. However, amines are washed out more easily in the reserpine-pretreated animals (80). Using (±)-metaraminol-^{3}H, Lundborg (81) and Lundborg and Stitzel (82) elaborated on the stereoselectivity of (−)- and (+)-metaraminol in the different tissue fractions from mouse heart. The two isomers of metaraminol were able to displace (±)-metaraminol-^{3}H from subcellular fractions of the mouse heart when given 15 min. after administration of the labeled amine. The (−)-form was more effective. If unlabeled isomers were given 24 hr. after (±)-metaraminol-^{3}H, only the (−)-isomer was an effective agent. In the adrenal medullary granules, (−)-metaraminol was more effective in preventing the uptake of (±)-metaraminol-^{3}H than the (+)-form (83).

[3] Cobefrin.
[4] α-Methylepinephrine or dihydroxyephedrine.

Table I—Release of ^{3}H-Norepinephrine (^{3}H-NE) from Mouse Hearts by Optical Isomers and Deoxy Derivatives of Sympathomimetic Agents

Agent	Steric Structure	Dose,[a] mg./kg. s.c.	^{3}H-NE in Heart[b] — % Control	% Depletion
(−)-Norepinephrine bitartrate	1R	2.5	33	67
(+)-Norepinephrine bitartrate	1S	2.5	57	43
Deoxynorepinephrine	—	5	50	50
(−)-Epinephrine bitartrate	1R	2.5	36	64
(+)-Epinephrine bitartrate	1S	2.5	62	38
Deoxyepinephrine	—	5	55	45
(−)-Levonordefrin	1R	2.5	20	80
(±)-Deoxylevonordefrin (methyldopamine)	—	5	39	61
(−)-Metaraminol bitartrate	1R	5	22	78
(±)-Deoxymetaraminol (α-methyl-*m*-tyramine)	—	5	38	62
		10	34	66
(−)-*m*-Octopamine tartrate (*m*-tyramine)	1R	5	38	62
Norephedrine	?	10	68	32
(−)-Deoxynorephedrine	2R	10	86	14
(+)-Deoxynorephedrine	2S	10	58	42
Phentermine	—	10	95	5
Ephedrine HCl	?	10	91	9
(+)-Pseudoephedrine	1S,2R	10	84	16
(+)-Deoxyephedrine	2R	10	62	38
Mephentermine	—	10	100	0

[a] Drugs were administered 60 min. after intravenous ^{3}H-NE. [b] Two hours after drug administration. Data after Daly *et al.* (89).

Histochemically, the accumulation of isomers of dopa has been studied in several tissues. The number of green fluorescent enterochromafinlike cells that could be induced by administration of the (+)-isomer of dopa was usually smaller than that after the (−)-isomer (84). The transport of amino acid into islet cells of the rat pancreas was selective, since accumulation of (+)-dopa could not be demonstrated histochemically even after pretreatment with nialamide. In contrast to this, fluorescence developed in the exocrine cells, both after administration of (−)- and (+)-dopa (85, 86). Similarly, the parafollicular cells of mouse thyroid (87) and the endothelial cells in the capillaries of mouse brain (88) selectively accumulate the (−)-isomer of dopa. When the rat iris is incubated with (−)- or (+)-dopa, a marked diffused fluorescence is seen after (−)-dopa, while (+)-dopa is selectively accumulated in the iris capillaries.[5]

STERIC STRUCTURE–ACTIVITY RELATIONSHIPS FOR RELEASE OR DEPLETION OF ENDOGENOUS AMINES BY SYMPATHOMIMETIC AGENTS

Tissue norepinephrine-depleting effects of certain isomers are described in the previous section. Many studies, which are summarized in this section, were designed for obtaining information regarding a structural requirement for releasing biogenic amines. Daly *et al.* (89) investigated the norepinephrine-releasing potencies of a wide variety of sympathomimetic amines and related compounds. Endogenous cardiac norepinephrine was prelabeled with 5 μc. of (±)-norepinephrine-7-^{3}H with a compound that caused loss of tritiated norepinephrine. Relative activities of some selected agents are presented in Table I. It is clear that monophenolic or diphenolic amines with 1R stereochemistry are better at depleting cardiac norepinephrine than are corresponding isomers with 1S stereochemistry. Nonphenolic amines, in general, were very weak in depleting norepinephrine-7-^{3}H. (+)-Deoxynorephedrine appeared to be a better depleting agent than (−)-deoxynorephedrine. Because enzymes in biotransformation of these agents were not inhibited, it is open to question whether observed differences between (+)- and (−)-deoxynorephedrine are valid. Loss of asymmetry by substitution of one more α-methyl group in deoxynorephedrine and deoxyephedrine made molecules very weak in depleting cardiac ^{3}H-norepinephrine.

Shore (14) compared the norepinephrine-depleting activity of various sympathomimetic amines in which a limited number of optical isomers was included. Drugs were given intraperitoneally to rats; 3 hr. later the change in heart norepinephrine was measured. Drugs used were optical isomers of *p*-hydroxyamphetamine, levonordefrin, and metaraminol. The structure–activity relationships showed again that (−)-metaraminol (1R) in relatively low doses, 1 mg./kg., is the most effective in depleting cardiac catecholamines. Higher doses of deoxymetaraminol (10 mg./kg.) were also effective in lowering the tissue norepinephrine, but this activity was inhibited by benzyloxyamine, an inhibitor of dopamine-β-hydroxylase.

In the isolated perfused rat heart, after labeling endogenous norepinephrine stores, release of tritiated norepinephrine by optical isomers of catecholamines was investigated by Nash *et al.* (90). The release rate for (−)-isomers of norepinephrine, epinephrine, and isoproterenol was higher than for corresponding (+)-isomers. *In vivo*, tyramine is rapidly converted to its β-hydroxylated product, octopamine. Carlsson and Waldeck (91) used this approach to study structure–activity relationships for release of ^{14}C-octopamine that was formed from ^{14}C-tyramine. Mice were injected with 0.2 mg./kg. i.v. of ^{14}C-tyramine. Fifteen minutes was allowed for conversion of ^{14}C-tyramine to ^{14}C-octopamine. Various amines were injected, and after 15 min. the loss of ^{14}C-octopamine from the heart was determined. The steric structure–activity relationship for releasing ^{14}C-octopamine is very similar to that for releasing norepinephrine.

Availability of all four isomers of ephedrine enabled Abdallah *et al.* (17, 92) to investigate the norepinephrine release by these agents. In the perfused rabbit heart, (−)-ephedrine is more effective in both the rate-accelerating effect and releasing the cardiac norepinephrine in the perfusate. However, at equivalent doses, (+)-ephedrine, (−)-pseudoephedrine, and (+)-pseudoephedrine released approximately the same amount of norepinephrine in perfusate, but their heart rate-accelerating effects were not the same.

Recently, synthesis, resolution, and pharmacology of (±)-*threo*-metaraminol and related agents have been carried out by Saari *et al.* (93), Torchiana *et al.* (44), Waldeck (94), and Carlsson *et al.* (95). (−)-α-Methyldopamine (deoxylevonordefrin) is approximately 3 times as potent as its (+)-form in causing norepinephrine depletion from mouse heart, while for similar effects (+)-α-methyl-*m*-tyramine is more active than its (−)-form. A reasonable and logical explanation for the

[5] B. Hamberger and T. Malmfors, personal communication, 1969.

higher norepinephrine-depleting effects of (+)-α-methyl-*m*-tyramine (deoxymetaraminol) is that this agent is converted to (−)-metaraminol, which is a very potent agent in depleting endogenous norepinephrine. The (−)-form may not undergo similar biotransformation; in addition, the (−)-form might be inactive as such. The norepinephrine-depleting effects of the (−)- and (+)-forms of α-methyldopamine are quite opposite to those of isomers of α-methyl-*m*-tyramine. Under *in vitro* conditions when (+)-forms are incubated with semipurified dopamine-β-hydroxylase from bovine adrenal medulla, the corresponding (−)-forms of amine metabolites [namely (−)-levonordefrin from (+)-α-methyldopamine and (−)-metaraminol from (+)-α-methyl-*m*-tyramine] can be obtained. The (−)-forms of both α-methyldopamine and α-methyl-*m*-tyramine failed to yield corresponding β-hydroxylated products. This indicates that marked norepinephrine-depleting effect of (−)-α-methyldopamine might be caused by the parent molecule itself. Furthermore, it is probable that dopamine-β-hydroxylase is also inhibited by this agent. However, exogenous administration of (±)-*threo*-levonordefrin can cause dose-dependent reduction of cardiac norepinephrine, with a concomitant fall of blood pressure in rats (96). As compared to (±)-*threo*-levonordefrin, (±)-*threo*-metaraminol appears to be much less active in depleting cardiac norepinephrine from the heart.

So far, only a limited number of optical isomers have been studied. Validity of any structure–activity relationship can only be studied by investigating structurally similar isomers on one system under well-controlled experiments. Norepinephrine depletion should not only be studied at equimolar doses but at a dose which will cause equivalent depletion or release. The former criterion is easier than the latter one.

INHIBITION OF UPTAKE

Several drugs are known to inhibit the uptake of exogenous norepinephrine into the sympathetic nerve endings. In the isolated perfused rat heart, Burgen and Iversen (97) made a systematic study of chemical structure of sympathomimetic amines and its relationship to inhibition of (±)-^{14}C-norepinephrine uptake. Similarly, in the perfused rabbit heart, Muscholl and Weber (98) studied the inhibition of uptake of levonordefrin by some sympathomimetic amines. The following generalizations can be made regarding steric structure–action relationships: (*a*) In phenolic amines, deoxy derivatives are more potent than their corresponding (−)-isomers which are in turn more potent than (+)-isomers. (*b*) In nonphenolic amines such as ephedrine and norephedrine, deoxy derivatives are also more potent than (−)-isomers. But in the latter case, the situation is complicated by two asymmetric carbons. (*c*) (+)-Deoxynorephedrine is about 20 times more potent in inhibiting norepinephrine uptake than that of (−)-deoxynorephedrine. (*d*) (+)-Norpseudoephedrine is more potent than either (−)-norephedrine or (+)-deoxynorephedrine (Table II). Ross and Renyi (99) investigated effects of a series of amines on accumulation of (±)-norepinephrine-^{3}H in cortex slices from mouse brain. As in the heart, uptake of tritiated norepinephrine resembles that of the enzymatic reaction according to Michaelis-Menton. In a concentration-dependent manner, various sympathomimetic amines prevented accumulation of tritiated norepinephrine in brain slices. However, the slopes of these dose–response curves varied considerably. Isomers, (−) and (+), of metaraminol exhibited distinct differences; the (−)-form was more potent. At the lowest concentration, 0.1 mcg./ml. of (−)-norepinephrine and (±)-norepinephrine, the inhibition of uptake was identical. This possibly indicates that (−)- and (+)-isomers of norepinephrine may not differ in preventing accumulation of tritiated norepinephrine. It is difficult to understand why isomers of metaraminol continue to exhibit stereospecificity at various concentrations while isomers of norepinephrine, amphetamine, and norphenylephrine did not differ in this respect.

Table II—Inhibition of Norepinephrine Uptake by Sympathomimetic Amines in the Rat Isolated Heart

Agent	Steric Configuration	ID_{50},[a] *M*	Relative Inhibition of Uptake[b]
Phenethylamine	—	1.1×10^{-6}	1.00
(−)-Norepinephrine	1R	2.7×10^{-7}	4.07
(+)-Norepinephrine	1S	1.4×10^{-6}	0.78
Deoxynorepinephrine	—	1.7×10^{-7}	6.50
(−)-Epinephrine	1R	1.0×10^{-6}	1.10
(±)-Epinephrine	—	1.4×10^{-6}	0.78
Deoxyepinephrine	—	7.6×10^{-7}	1.45
(−)-Levonordefrin	1R,2S	2.0×10^{-7}	5.50
(±)-Levonordefrin	—	4.2×10^{-7}	2.60
(±)-Deoxylevonordefrin (methyldopamine)	—	1.8×10^{-7}	6.10
(−)-Metaraminol	1R,2S	7.6×10^{-8}	14.40
(−)-Ephedrine	1R,2S	2.2×10^{-6}	0.50
(+)-Deoxyephedrine	2S	6.7×10^{-7}	1.65
Mephentermine	—	1.0×10^{-6}	1.10
(+)-Deoxynorephedrine	2S	1.8×10^{-7}	6.10
(−)-Deoxynorephedrine	2R	3.7×10^{-6}	0.30

[a] Drug concentration producing 50% inhibition of norepinephrine uptake. [b] Relative inhibition = (ID_{50} of phenethylamine)/(ID_{50} of drug). Data in part taken from Burgen and Iversen (97).

Independently and simultaneously, Thoenen *et al.* (100) and Ross *et al.* (101) have observed that tissue accumulation of labeled nonphenolic agents such as amphetamine, norephedrine, and phenylethanolamine are not influenced by cocaine. They have suggested that tissue accumulation of nonphenolic amines is a function of their physical–chemical properties, while that of monophenolic or diphenolic amines is a function of the hydroxyl group. These studies suggest that some precautions are needed in comparing uptake inhibition by phenolic and nonphenolic amines. It is reasonable to compare activity of (−)- and (+)-isomers on any single parameter because their physical–chemical properties are identical. However, to make any generalizations regarding steric structure–activity, a study of a large number of antimeric pairs of both phenolic and nonphenolic amines is required.

IMPLICATIONS OF UPTAKE, STORAGE, INHIBITION OF UPTAKE, AND RELEASE OF ADRENERGIC DRUGS

Endogenously released or exogenously administered (−)-norepinephrine is mainly inactivated by rapid uptake into the adrenergic nerve endings. (+)-Norepineph-

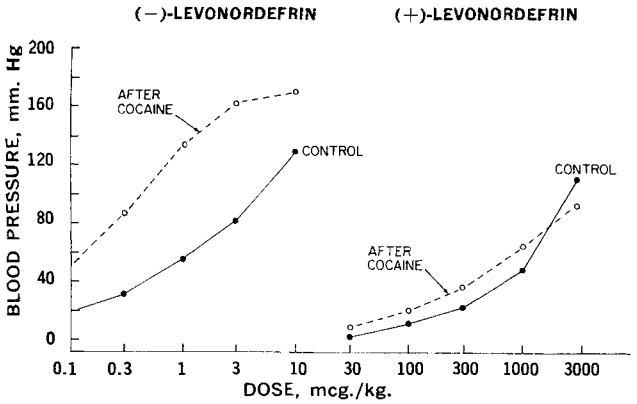


Figure 1—*Stereoselective potentiation of isomers of levonordefrin by cocaine (5 mg./kg. i.v.) in the spinal cat. Data in part obtained from Tye* et al. *(109).*

rine has very little affinity for both uptake and storage site (see *Inhibition of Uptake*). The physiologic implications of these findings are reflected in the transmitter economy. The naturally occurring (−)-norepinephrine and (−)-epinephrine are most efficiently utilized. One important concept that has emerged from the study of uptake and storage mechanisms is the concept of "false neurochemical transmitters." It is possible to substitute a molecule which is weaker than the natural neurohormone in activating pharmacologic receptors. These agents have proved to be very valuable substances in the treatment of hypertension. The classical example is that of α-methyldopa. The biotransformation and the formation of the false neurochemical transmitter are discussed in the previous section.

Because of modern instrumentation and laboratory facilities, many research projects are being carried out at a much faster rate than they were 20–30 years ago. There is also no doubt that many recent studies are better designed than before; however, it is of historical importance to comment on early reports concerning optical isomers of various adrenergic drugs. Tainter (102) and Luduena *et al.* (103) reported that pressor effects of isomers of norepinephrine and epinephrine were potentiated by cocaine. On the other hand, pressor effects of (+)-levonordefrin and (+)-phenylephrine were unaffected or desensitized by cocaine (104, 105). The intestinal smooth muscle of an unanesthetized dog is relaxed by (−)-norepinephrine and (−)-epinephrine. This effect was potentiated by cocaine, while that of (+)-isomers was unaffected or slightly antagonized by cocaine (106).

Trendelenburg (107) and Trendelenburg *et al.* (108) studied sensitization by cocaine for (−)- and (+)-isomers of norepinephrine and epinephrine. The cat nictitating membrane effect caused by (−)-isomers was sensitized more than the corresponding (+)-isomers. Independently, Tye *et al.* (109) also investigated the influence of cocaine on the isomers of norepinephrine, epinephrine, levonordefrin, and phenylephrine. In both normal and catecholamine-depleted cats, the nictitating membrane and blood pressure effects of (−)-isomers were sensitized by cocaine. When studied similarly, the effects of (+)-isomers were less sensitized or desensitized. Unequal potentiation of optical isomers was explained on the basis of previously reported unequal rates of uptake of these agents into the adrenergic neurone. In terms of the structure–activity relationship, it is clear that 1R stereochemistry is necessary for stereoselective or stereospecific sensitization by cocaine. However, the role of the β-hydroxyl group becomes secondary, if the molecule is heavily substituted on nitrogen. (−)-Isoproterenol has 1R stereochemistry at C_1 but is not potentiated by cocaine. Draskóczy and Trendelenburg (110) found that over the wide range of concentrations, (−)- or (+)-norepinephrine is removed from perfusate by the heart at approximately equal rates. In the same tissue, however, cocaine did produce marked stereospecific supersensitivity in favor of the (−)-isomer. Since perfused rabbit heart did not exhibit stereospecific uptake and since cocaine did not potentiate (+)-norepinephrine, they proposed that the lack of potentiation of (+)-norepinephrine by cocaine is caused by low potency of this isomer, which exerts pharmacological effects only in concentrations that saturate the uptake mechanism. The effect of cocaine becomes negligible when the uptake site is saturated (111). It was also demonstrated that in the lower concentration, (+)-norepinephrine, which caused little effect, markedly potentiated the positive inotropic effects of the (−)-isomer. According to these observations, the stereospecificity of the sensitizing effect of cocaine can no longer be ascribed to differences in the rate of uptake of the isomers. Seidehamel *et al.* (112) selected dopamine in an attempt to determine the importance of the positional role of the β-hydroxyl group. This agent is structurally similar to norepinephrine, except it lacks the β-hydroxyl group. The sensitization of the nictitating membrane to these agents produced by cocaine has the following sequence: (−)-norepinephrine > dopamine = (+)-norepinephrine. West *et al.* (113) also attempted to explain the unequal potentiation by cocaine of (−)- and (+)-isomers of norepinephrine, metaraminol, and octopamine in relation to degree of uptake. Cocaine-induced supersensitivity in atria and the rate of uptake occurred in a diminishing fashion: (−)-norepinephrine > (+)-norepinephrine > (−)-metaraminol > (−)-octopamine > (+)-metaraminol > (+)-octopamine. Here, again, saturation of uptake rather than rates of uptake might be an important factor in explaining the rank order. Desipramine, a cocainelike agent, is reported to produce stereoselective sensitization of norepinephrine isomers on rat vas deferens (114). Two other agents, namely prenylamine (115) and α,α′-bis(dimethylammoniumacetaldehyde diethylacetal)-*p,p′*-diacetylbiphenylbromide (DMAE), also behave like cocaine (116). These agents markedly potentiate pressor effects of (−)-norepinephrine and produce little or no potentiation of (+)-norepinephrine. In anesthetized cat nictitating membrane, after the administration of DMAE or cocaine, Wong and Long (116) did not observe a stereospecific sensitization for (−)- and (+)-epinephrine. Thus, it appears that stereospecific or stereoselective sensitization after cocaine or cocainelike agents would be best demonstrated in an antimer which exhibits high isomeric ratio in activation of pharmacologic receptors, as well as high affinity difference for uptake sites [*e.g.*, (−)- a nd (+)-isomers of levonordefrin] (Fig. 1). As compared to other antimers, the isomeric

ratio and difference in uptake for (−)- and (+)-epinephrine are small. Hence, it may be difficult to obtain a clearcut separation by sensitizing effects of one isomer over another.

Before leaving this section, it should be emphasized that stereoselective potentiation of norepinephrine isomers by a given agent should not be taken as a criterion for cocainelike effects. A ganglionic blocker, mecylamine, which does not resemble cocaine in any respect, also causes stereoselective potentiation of norepinephrine isomers (117) (Fig. 2).

Various drug treatments and surgical procedures are known to produce supersensitivity to certain sympathomimetic agents. For further information, readers are referred to an excellent review on this subject (118). Surgical removal of sympathetic ganglia causes degeneration of postganglionic fibers. A few days after the procedure, there is a marked loss of transmitter. The end organ continues to develop a supersensitivity, at least until the 28th postoperative day (119). Denervation supersensitivity has two components: (*a*) cocainelike and (*b*) nonspecific, such as described for decentralization. With any agent that interferes with the release of the neurotransmitter, the end organ develops a supersensitivity which is more like that produced by surgical decentralization. Chronic treatment with reserpine produces a decentralization type of supersensitivity.

The idea of utilizing optical isomers of sympathomimetic amines to study supersensitivity caused by denervation can be traced to as early as 1939 (120). However, it was only after the role of these agents for uptake, binding, and release had been clarified that they became useful tools for analysis of various types of supersensitivities. Trendelenburg (107) reported that 7 days after excision of the superior cervical ganglia of a cat, the denervated nictitating membrane was nearly 100 times more sensitive to (−)-norepinephrine than the corresponding normal side of the same animal. The sensitivity to (+)-norepinephrine, on the other hand, increased only by a factor of 3.5. Soon after this, it was realized that the variable tone of the denervated nictitating membrane of the spinal cat, determined under ether, complicates the accurate estimation of supersensitivities (121). Seidehamel *et al.* (122), therefore, studied the various types of supersensitivities in both normal spinal cat as well as preparations in which tone of the nictitating membrane was reduced to a relatively stable level by acute treatment with reserpine. It was concluded that supersensitivities caused by decentralization and chronic treatment with reserpine cause stereoselective potentiation in favor of (−)-norepinephrine. However, denervation supersensitivity appeared to be nonstereoselective, because both isomers of norepinephrine were equally sensitized by this procedure. The role of COMT becomes important when uptake mechanism is lost. Thus, during the investigation of stereoselectivity and nonstereoselectivity of denervation supersensitivity, the enzyme COMT should be inhibited by a stable enzyme inhibitor like tropolone. Langer *et al.* (119) reported equal sensitizing effects of dopamine and (+)-norepinephrine. Green and Fleming (123) observed that denervated cat spleen *in vitro* produced three- to fourfold potentiation of (−)-norepinephrine, while that

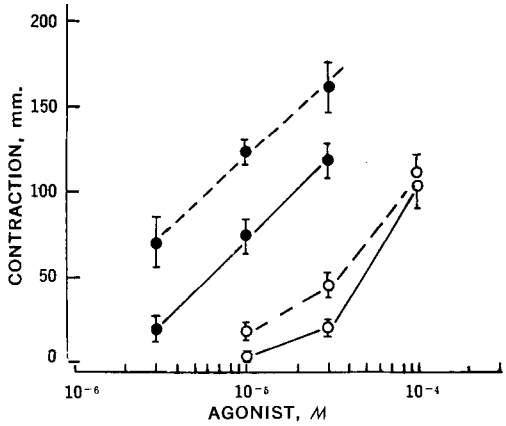


Figure 2—*Stereoselective potentiation of isomers of norepinephrine (NE) by mecamylamine (3-min. incubation). Isolated rat vas deferens was the test organ. Vertical lines are* SEM. *Data from Patil* et al. *(117). Key: ——, control; - - -, with mecamylamine, 3 × 10⁻⁴ M; ●, (−)-NE; and* ○, *(+)-NE.* n = 8.

of (+)-isomer was slightly desensitized. The supersensitivity resulting from chronic reserpine treatment could not be demonstrated in this tissue. Failure to demonstrate such a sensitivity *in vitro* was attributed to the isolational procedural stress. From these reports it appears that the whole problem of change in the activity of optical isomers of norepinephrine in relation to denervation supersensitivity deserves reinvestigation under well-controlled experimental conditions. After denervation, if there is a qualitative change in α-adrenergic receptors (*i.e.*, change in the configuration), it should be reflected in isomeric ratios of norepinephrine. Hence, the isomeric ratio after denervation should be different from that in the normal tissue after administration of cocaine (124).

After a series of well-controlled experiments in reserpine-pretreated spinal cat, Trendelenburg (125) reported an interesting finding with (−)- and (+)-isomers of norepinephrine. During nerve stimulation, after equieffective doses of isomers of norepinephrine, the nictitating membrane effects of (−)-norepinephrine were potentiated more than those of the (+)-isomer. These observations were explained on the basis that during nerve stimulation, injected (−)-norepinephrine is immediately available for release. The (+)-isomer has less affinity for the uptake site and, therefore, will not be stored. Hence, no potentiation will result.

Many sympathomimetic amines and related agents inhibit the uptake of norepinephrine into the sympathetic nerve endings. This implies that relatively more concentration of norepinephrine will then be available for activation of pharmacologic receptors. Since various sympathomimetic amines displayed varying inhibition of uptake of norepinephrine (97), it was anticipated that different sympathomimetic amines should produce varying potentiation of norepinephrine effects. In the isolated rat vas deferens, Swamy *et al.* (126) studied potentiation of (−)-norepinephrine by (+)- and (−)-amphetamine. At all concentrations, 10^{-7}–10^{-5} *M*, of amphetamine, the potentiation of exogenous norepinephrine was identical. In the presence of amphetamine, responses to the effects of endogenously released norepinephrine can be potentiated. Day (127) carried out ex-

periments on the isolated guinea pig vas deferens. The effects of (+)-, (−)-, and (±)-amphetamine were compared on the maximal response to sympathetic nerve stimulation over the frequency range 2–50 pulses/sec., using both pre- and postganglionic stimulation. It was found that both optical isomers and racemic mixture produced identical results. Thus, it appears that on vas deferens, isomers of amphetamine produce identical potentiation of exogenous or endogenous norepinephrine.

Swamy *et al.* (128) used a systematic pharmacologic approach to define some stereochemical characteristics of uptake site. An assumption was made that potentiation of exogenous norepinephrine is a faithful reflection of the ability of molecules to inhibit the uptake of norepinephrine. Only metabolically stable α-methylated amines were selected. Two isolated tissues from rat, vas deferens and right atria, were used to study the potentiating abilities of these agents. On both tissues at relatively low concentrations, 10^{-6} *M*, these drugs produced a potentiation of exogenous norepinephrine. The isomers were ranked according to their abilities to potentiate norepinephrine. In the isolated rat vas deferens, there was not a clearcut separation of steric structure and ability to potentiate norepinephrine. (−)-Metaraminol, which has the highest affinity for uptake site in the heart, was ranked 20th on the vas deferens. (−)-Amphetamine, which has a poor affinity for uptake, was ranked first. Rat vas deferens contains mainly α-adrenergic receptors. A given sympathomimetic amine can compete for both uptake and direct α-adrenergic receptors. If a given agent has higher affinity for both uptake and direct sites, then observed potentiation may not be a true reflection of the amine to inhibit uptake. In other words, norepinephrine, which is spared, will not produce its full effect on α-adrenergic receptors which might be occupied by the amine. In the isolated rat atria, which mainly contains β-adrenergic receptors, the isomers of various amines exhibited distinct stereochemical differences: (*a*) (−)-metaraminol ranked first in 20 agents tested; (*b*) (−)-isomers of norephedrine, ephedrine, and metaraminol ranked higher than their corresponding (+)-isomers; and (*c*) deoxy derivatives, such as α-methyl-*m*-tyramine, α-methyldopamine, amphetamine, and methamphetamine, ranked higher than their corresponding β-hydroxylated (+)-isomers. Thus, if potentiation is the faithful reflection of ability of an amine to inhibit uptake of (−)-norepinephrine, the obvious conclusion is that uptake characteristics differ in rat vas deferens as compared to atria. This view would be consistent with a recent report by Iversen (68).

As early as 1923, Gottlieb (129) studied the optical isomers of cocaine for local anesthetic activity. But a complete steric structure-activity of the cocaine molecule for inhibition of norepinephrine uptake is yet to be defined. Synthesis and/or resolution of these agents appear to be the main task. Schmidt *et al.* (130) investigated central and peripheral effects of (−)-cocaine and (+)-pseudococaine. Only (−)-cocaine was effective in both, producing central sympathetic stimulation and potentiating epinephrine. It can be concluded from these experiments that (+)-pseudococaine might not be an effective inhibitor of epinephrine uptake. However, (−)-cocaine and (+)-pseudococaine have identical local anesthetic properties. This indicates that inhibition of uptake and local anesthetic effects may not be causally related. (−)-Norepinephrine-potentiating activity of close structural analogs of cocaine, tropacocaine, and pseudotropacocaine was investigated on the isolated rat vas deferens (131). Pseudotropacocaine appeared to be slightly more active than tropacocaine. Both of these agents were only 1/30th–1/100th as active as (−)-cocaine. The effects of tyramine were affected differently. A large body of evidence indicates that cocaine, norepinephrine, and tyramine compete for the same site at the adrenergic nerve endings. As expected, effects of norepinephrine were equally shifted by cocaine to the left and right, respectively. However, tropacocaine and pseudotropacocaine did not affect norepinephrine and tyramine response to an equal degree. Tropacocaine caused a slight shift of norepinephrine responses to the left. On the other hand, the same concentration of tropacocaine markedly shifted the effects of tyramine to the right with reduction in maxima. Pseudotropacocaine did not affect tyramine at all, but the effect of norepinephrine was slightly potentiated. These results indicate that a noncompetitive interaction of tropacocaine and pseudotropacocaine with receptors in the adrenergic nerves cannot be excluded.

Because of two asymmetric centers, the antidepressant drug methylphenidate can be exhibited in four stereoisomeric forms. Clinically, only the (±)-*threo*-form is used. The (±)-*erythro*-form is devoid of central effects. Buckner *et al.* (132) evaluated comparative peripheral effects of (±)-*erythro*- and (±)-*threo*-forms of methylphenidate. On the rat vas deferens in potentiating (−)-norepinephrine at equiactive concentrations, (±)-*erythro*-methylphenidate has 1/300th the activity as that of the (±)-*threo*-form. A histochemical technique demonstrated that in rat iris the (±)-*threo*-form markedly inhibited uptake of levonordefrin while the (±)-*erythro*-form was without effect. Implications of these findings are that if CNS stimulant effects are causally related to inhibition of uptake of norepinephrine, a greater potency of (±)-*threo*-methylphenidate over that of the (±)-*erythro*-form explains its higher CNS stimulant activity. The absolute configurations, methylphenidates, pipradrols, and pheniramines, have been determined recently (133).

MECHANISM OF ACTION OF "INDIRECTLY" ACTING β-HYDROXYLATED (+)-ISOMERS AND THEIR CORRESPONDING DEOXY DERIVATIVES

Muscholl (5) has pointed out that "indirectly" acting amines not only cause release of stored norepinephrine but also cause inhibition of reuptake of released norepinephrine. Previously, Patil *et al.* (20) observed that in the normal vas deferens, indirectly acting deoxy derivatives always produced a greater magnitude of pharmacologic effects than their corresponding β-hydroxylated (+)-isomers. For example, deoxylevonordefrin (α-methyldopamine) produced greater effects than (+)-levonordefrin. These results were explained on the basis that deoxy derivatives enter the intraneuronal stores at a faster rate and displace the stored norepinephrine at a faster rate than the corresponding β-hydrox-

ylated (+)-isomer. An incorrectly oriented alcoholic hydroxyl group in (+)-isomer was believed to cause hindrance in uptake of these agents. However, it can be argued that both deoxy derivative and (+)-isomer are taken up at an equal degree and displace equal amounts of norepinephrine. The higher activity of deoxy derivatives then can be explained by greater inhibition of reuptake by the agent. The lower activity of the (+)-isomer is caused by its ability to inhibit the reuptake of released norepinephrine.

A series of experiments was done to test the cocaine-like effect of indirectly acting agents in the reserpine-pretreated rat vas deferens. The concentrations of deoxy derivative and (+)-isomer were the same as those in which they exhibit unequal pharmacological effects on the normal tissue. The deoxy derivative and corresponding (+)-isomer were tested on the contralateral vas deferens of the same reserpine-pretreated rat. It was observed that the deoxy derivative and corresponding (+)-isomer caused equal potentiation of exogenous norepinephrine (134). These observations strengthen the original suggestion that in the rat vas deferens greater indirect effects of the deoxy derivative over the corresponding (+)-isomer is caused by either faster uptake of the agent and/or faster displacement of norepinephrine. The (+)-isomer possibly lacks both effects. This hypothesis is illustrated in Fig. 3.

Since tissue accumulation of nonphenolic amines is not affected by cocaine (100, 101), a very basic question was raised. "Do all indirectly acting agents release the stored norepinephrine by the same mechanism?" Experiments were designed on the rat vas deferens to seek the answer. A group of phenolic and nonphenolic amines was selected. From previous studies (20), it was also known that certain indirectly acting amines produce different maximal effects. To get some clue regarding similarity and dissimilarity of closely related indirectly acting agents, their maximal effects were superimposed. Phenolic amines and nonphenolic amines did show dissimilar behavior. Thus, the results support the hypothesis that all indirectly acting agents do not act by the same mechanism.

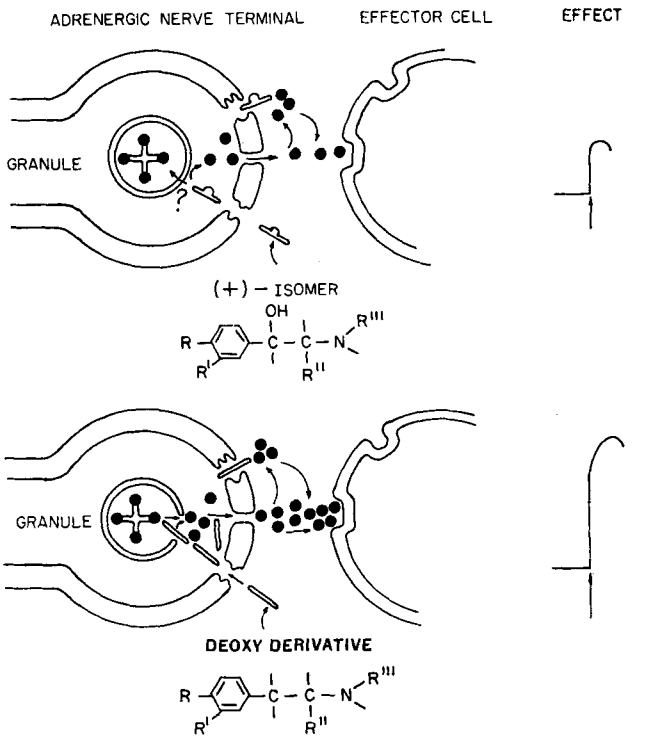


Figure 3—*Illustration of a possible cause of low activity of (+)-isomers when compared with the corresponding deoxy derivatives. Since exogenous norepinephrine (●) is equally potentiated by "indirectly" acting (+)-isomers and their corresponding deoxy derivatives, the inhibition of reuptake of endogenously liberated neurohormone may be the same for both agents. Thus, higher rate of neuronal uptake and/or increase in the basic release mechanism may be the cause for higher amplitude of contraction of deoxy derivative over that of (+)-isomer. In (+)-isomers the OH-group may be incorrectly oriented with respect to transport mechanisms, and the rate of transport, therefore, is slower than that of the corresponding deoxy derivative where such a group is absent. R = H, R′ = OH, R″ = CH_3, R‴ = CH_3. Reproduced, with permission, from Patil* et al., Arch. Int. Pharmacodyn. Ther., *189, 32(1969), and St. Catherine Press, Brugge, Belgium.*

PHARMACOLOGICAL EFFECTS OF OPTICAL ISOMERS OF ADRENERGIC DRUGS— α- AND β-ADRENERGIC AGONISTS

Great credit should go to Cushny (135–137) for his pharmacologic studies on the optical isomers of various substances. He has carefully documented earlier reports concerning optical isomers of many naturally occurring substances (135). Soon after the presence of the epinephrine was demonstrated in adrenal medulla, Cushny (136) examined the pressor effects of naturally occurring (−)-epinephrine and synthetic (±)-epinephrine in the anesthetized dog. The (±)-epinephrine was one-half as active as (−)-epinephrine. From these experiments, it became evident that optical isomers of epinephrine showed selectivity in pharmacologic effects. In the following year, 1909, Cushny (137) compared individual (−)- and (+)-isomers of epinephrine. He found that the (+)-isomer was 1/12th–1/15th as active as (−)-isomer in raising blood pressure.

The single most important contribution in explaining the behavior of optical isomers toward the specific pharmacologic receptors came from the reports of Easson and Stedman (138). The theory was proposed that in an asymmetric molecule like (−)-epinephrine, three of the four groups linked to the asymmetric carbon are concerned in the attachment with the receptor. These groups are: (*a*) the basic nitrogen; (*b*) the aromatic group (with *m*- and *p*-hydroxyl groups which determine the intensity of attachment); and (*c*) the alcoholic hydroxyl group. In the (+)-isomer, since the alcoholic hydroxyl group is oriented in the wrong position, only two-point interaction is expected. This view was strengthened by the fact that deoxyepinephrine, which lacks the alcoholic hydroxyl group, is equiactive with (+)-epinephrine. This theory was elaborated by Blaschko (139) and Beckett (140). It can be illustrated as shown in Fig. 4. On guinea pig ileum, Wilson (141) tested (−)-epinephrine and (+)-epinephrine. According to this theory, the (−)-isomer was more active than the (+)-isomer and epinine was equiactive to (+)-epinephrine. However, Badger (142) collected the results from a number of reports and pointed out that in many cases the activity of deoxy derivatives and (+)-isomers was

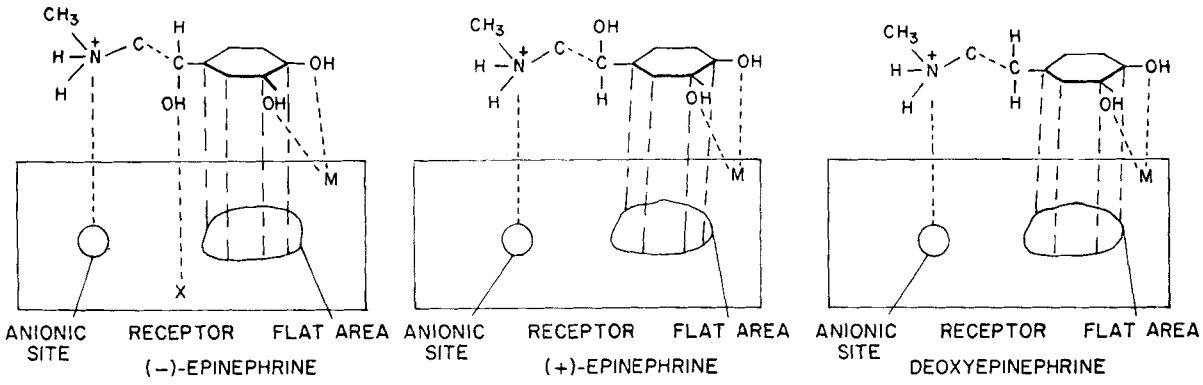


Figure 4—*Suggested interactions of optical isomers of epinephrine and deoxyepinephrine with adrenergic receptors [after Easson and Stedman (138), Belleau (199), and Beckett (140)]. According to Easson and Stedman, the correct orientation of the β-hydroxyl group of (−)-isomer results in higher activity while incorrect orientation of this group in (+)-isomer or the lack of the group in deoxy derivative would result in lesser but equal intensity of effect. In other words, (+)-isomer acts as if the β-hydroxyl group is missing (140).*

not in harmony with the Easson-Stedman hypothesis. For many years, Badger's argument against the theory remained unanswered.

In the meantime, the concept of directly and indirectly acting amines has been widely accepted. Patil *et al.* (20, 143) selected a series of (−)- and (+)-isomers and their deoxy derivatives of sympathomimetic amines in order to test the Easson-Stedman hypothesis. Vas deferens from normal as well as reserpine-pretreated rats were used for the experiments. On the normal vas deferens, the (−)-isomer was always more active than the (+)-isomer, but many corresponding deoxy derivatives were more active than (+)-isomers. When the endogenous norepinephrine in the vas deferens was depleted by reserpine treatment, a very interesting result was obtained. The (−)-isomers retained their higher activity, as in normal tissue, but many (+)-isomers and corresponding deoxy derivatives appeared to have large, unequal, indirect components of action. Both the (+)-isomer and the deoxy derivative were equiactive in the reserpine-pretreated tissues. Results are illustrated in Fig. 5. In other words, in catecholamine-depleted tissues, results were in harmony with the Easson-Stedman hypothesis. These observations suggest that the hypothesis holds true for sites of direct action only (α- or β-adrenergic).

From this study, another question emerged. When catecholamine stores are intact, why are deoxy derivatives more active than the (+)-isomers? A possible explanation is that in (+)-isomers, orientation of the β-hydroxyl group is such that it may cause hindrance of transfer from extraneuronal sites to intraneuronal sites, thereby liberating lesser amounts of neurohormone at slower rates. With the deoxy amines, which lack the β-hydroxyl group, there is no such hindrance to transfer and these amines will be taken up by the storage site more easily and produce greater pharmacologic effects because of a faster rate of release of neurohormone. (This view is discussed and illustrated in a previous section.) Even *in vivo* systems, after depletion of catecholamines, (+)-isomers, and their deoxy derivatives, appear to produce equal pharmacologic effects (16, 144, 145). Dopamine causes biphasic or vasodepressor response in the anesthetized rabbit. Under similar conditions, (+)-norepinephrine produces a pressor effect. It was anticipated that a similar activity difference could be detected on the rabbit aorta. Although the shapes of the dose–response curves of (+)-norepinephrine and dopamine were slightly different, the ED_{50} values were similar (146). (−)-Norepinephrine causes relaxation of the rat fundus strip. Both (+)-norepinephrine and dopamine have some 1/250th the activity of (−)-norepinephrine.[6] The activities of (+)-norepinephrine and deoxynorepinephrine are identical, indicating a possible similar interaction of these drugs as suggested by Easson and Stedman. Furthermore, this theory holds true for the drug effects on skeletal muscle (147).

By use of isomers of norepinephrine, an attempt has been made to characterize the nature of the receptor material. Because cell walls are composed of phospholipids, it is implied that these lipids might be involved in the transport of drug in the adrenergic neurone or in the interaction of drugs at the pharmacologic receptors. For the *in vitro* system, isomers of norepinephrine failed to show differences in their interaction with lecithin (148). This is in contrast to the well-known stereoselectivity of the pharmacologic receptors. Failure of an *in vitro* system to demonstrate a difference should not be taken as evidence against the implication of phospholipids as the receptor material.

On the basis of similar patterns of potency of agonists and dissociation constants of antagonists, Furchgott (18) suggested that α-adrenergic receptors in rabbit aorta, muscle from the corpus of the stomach, and duodenum appear to be of a single type. This suggestion can be reexamined on the basis of activity ratio of optical isomers and the activity ratio of (+)-isomers and the deoxy derivative. If α-adrenergic receptors are identical in different tissues under proper conditions, one should obtain a similar isomeric potency ratio in all these tissues (124). Furthermore, if the Easson-Stedman hypothesis is true for interaction of agonists with α-adrenergic receptors, the activity of (+)-isomers and deoxy derivatives should be identical. Data in Table III are obtained from different reports, because the criteria for obtaining an isomeric ratio for antimers of norepinephrine are relatively constant.

[6] J. R. Vane, personal communication, 1969.

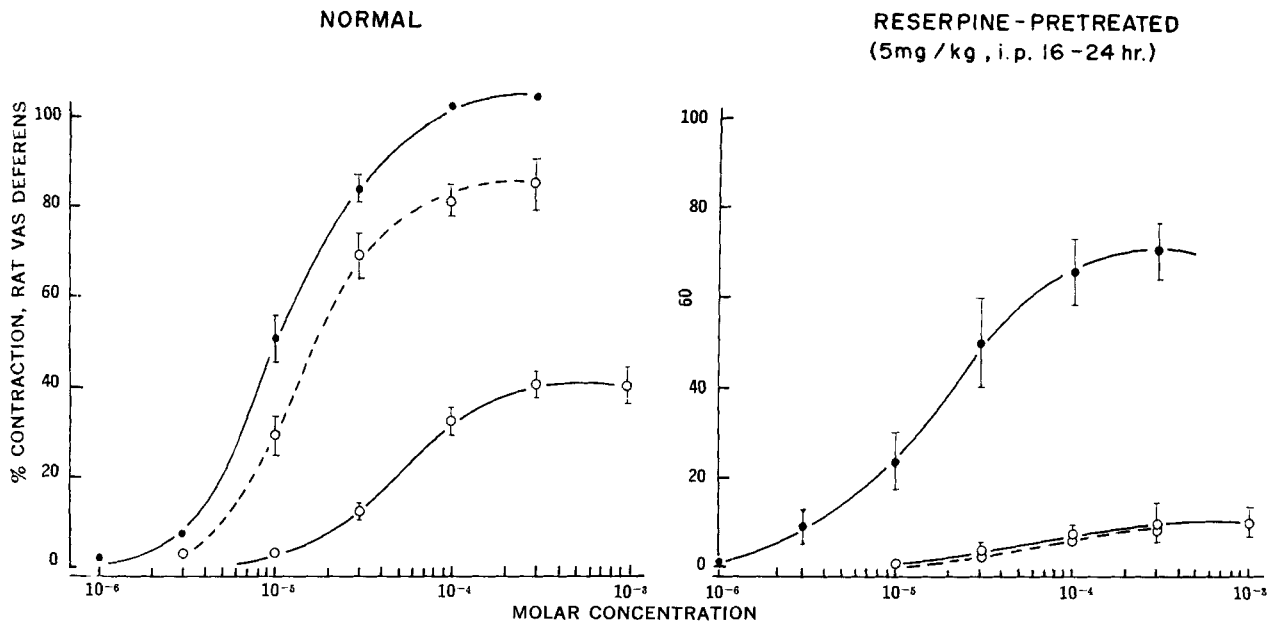


Figure 5—*Dose-response curves of (−)-levonordefrin (—●—), (+)-levonordefrin (—○—), and (+)-deoxylevonordefrin (- -○- -) in normal and reserpine-pretreated rat vas deferens. Note that the Easson and Stedman hypothesis only holds true in catecholamine-depleted tissue. Data after Patil and Jacobowitz (71).*

Fortunately, the isomeric ratios for isomers of norepinephrine from seven different test preparations containing mainly α-adrenergic receptors were readily available. In tissues which contain a high density of adrenergic nerves, the isomeric ratio for norepinephrine should be compared in the presence of cocaine. It can be seen from Table III that within the limits of experimental error, the isomeric ratios for norepinephrine isomers are approximately equal. Furthermore, the activities of (+)-norepinephrine and deoxynorepinephrine were approximately equal. On the basis of this data, new experiments were designed to test the validity of this concept. It was observed that after block of uptake by cocaine, isomeric ratios of norepinephrine on rat vas deferens, seminal vesicles, rabbit aorta, ileum, and spleen are equal (151). Since stereoselectivity is one of the best established properties of receptors, determination of isomeric ratios should provide an excellent criterion to differentiate the receptors. It seems probable, therefore, that the α-adrenergic receptors in all of the mentioned tissues are of a single type. The suggestion by van Rossum (149) regarding different types of α-adrenergic receptors in different tissues appears less tenable.

Conversely, if β-adrenergic receptors are of dissimilar types, the isomeric potency ratio of a given pair of isomers in different tissues containing β-adrenergic receptors should be different (124). The isomeric ratios between (−)- and (+)-norepinephrine for rate-accelerating effects in atria from cat, dog, rabbit, and guinea pig are 60, 10, 10, and 30, respectively.[7] Different isomeric ratios from different species, at least in part, might be a reflection of unequal density of adrenergic enervation. Unfortunately, the effects of isomeric potency ratios are only available from two tissues, heart and bronchioles (Table IV).

Blinks (155) investigated (−)- and (+)-isomers of norepinephrine, epinephrine, and isoproterenol and their deoxy analogs, dopamine, epinine, and deoxyisoproterenol, respectively. Even in the tissues containing β-adrenergic receptors, guinea pig atria, the results were in accordance with the Easson-Stedman hypothesis. Deoxyisoproterenol and (+)-isoproterenol are equipotent weak agonists of β-adrenergic receptors. Both of these agents also have an identical α-adrenergic receptor blocking activity (156, 157). Similarity of interactions of (−)-, (+)-, and their deoxy derivative in tissue containing both α- and β-adrenergic receptors suggests that many molecular features of these sites must be similar. The dissimilarity between α- and β-adrenergic receptors becomes apparent when effects of (−)- and (+)-levonordefrin and (+)-deoxylevonordefrin are examined on both of the adrenergic receptors. (+)-Deoxylevonordefrin and (+)-levonordefrin are equiactive on the reserpine-pretreated rat vas deferens; while on the guinea pig trachea, (+)-deoxylevonordefrin is far more active than (+)-levonordefrin (71).

Apart from these observations, some qualitative differences between deoxy derivatives and (+)-isomers have been reported. Evidence suggests that there may be receptors for initiation of renal vasodilation which are affected by dopamine (deoxynorepinephrine). The renal vasodilatory effects of dopamine are not blocked by any classical antagonist, while those of (+)-norepinephrine are blocked (158). Similarly, it appears from the report of van Rossum (149) that deoxysynephrine causes stimulation of the rabbit jejunum, while (+)-synephrine causes inhibition. On any given tissue, the deoxy derivative and (+)-norepinephrine may interact identically with pharmacologic receptors, as suggested by Easson and Stedman; but if only the deoxy derivative liberates other substances such as histamine or serotonin, then the observed activity difference of (+)-isomer and the deoxy derivative may not appear equal.

[7] J. R. Blinks, personal communication, 1969.

Table III—Relative Activities of Optical Isomers of Norepinephrine (NE) and Deoxynorepinephrine (Dopamine) on Various Tissues which Mainly Contain α-Adrenergic Receptors

Test Parameter	Procedure	Approximate Ratio[a] (+)-NE / (−)-NE	Approximate Ratio[a] (+)-NE / Deoxy NE	Reference No.
Cat blood pressure	Normal	40	1	109, 122,
	Reserpine[b]	47	1	150
	Cocaine	60		
Cat nictitating membrane	Normal	8	1	109, 112,
	Reserpine[b]	8	1	122
	Denervation (R)[b]	128	3	
	Cocaine (R)[b]	80	1	
	Chronic reserpine[c]	58	1	
Cat spleen	Normal	2	—	123
	Cocaine (R)[b]	65	—	
Rabbit aorta	Normal	42	1	146
Rabbit jejunum	Normal	64	10	149
Rat vas deferens	Normal	5	1	20
	Reserpine[b]	5	1	143
	Desipramine	50	—	114

[a] A dose that will cause equivalent effect was selected as a criterion for calculation of dose ratio. [b] Reserpine, 3–5 mg./kg. i.p., was used to deplete catecholamine. [c] Reserpine, 0.1 mg./kg./day for 14 days.

Reuter and Wollert (159) studied the effects of isomers of some sympathomimetic amines on contractility and ^{45}Ca-uptake in isolated guinea pig atria. As compared to (+)-isomers, (−)-isomers of epinephrine and synephrine were more potent in producing contractility and influx of ^{45}Ca. It was concluded that increase of Ca-influx during excitation is responsible for the positive inotropic effects of these agents. If there is a transport system at the site of pharmacologic receptors, it seems likely that calcium is required in transport of these amines at the receptors for the effect; and since (−)-isomers are more effective than (+)-isomers, it seems possible that calcium and a correctly oriented alcoholic hydroxyl group together with basic nitrogen can form a chelate that is to be transported more effectively. The role of divalent metals and their chelates in relation to pharmacologic effects of sympathomimetic amines and their optical isomers should provide an interesting chapter on future developments in understanding the basic mechanisms of drug action. The possible role of metals in the transport of norepinephrine has been discussed by Colburn and Maas (160, 161).

Table IV—Isomeric Ratios of Optical Isomers of Some Sympathomimetic Amines on Tissues Containing Mainly β-Adrenergic Receptors[a]

Isomer	Approx. (+)/(−) Ratio: Rabbit Heart (Rate)	Approx. (+)/(−) Ratio: Perfused Guinea Pig Lung (Bronchodilatation)
(−)-Isoproterenol (−)-Isoproterenol	1500	>800
(−)-Epinephrine (+)-Epinephrine	110	45
(−)-Norepinephrine (+)-Norepinephrine	60	70
(−)-Levonordefrin (+)-Levonordefrin	2600	>3000
(−)-Phenylephrine (+)-Phenylephrine	>10	—

[a] Data condensed from Brown and Lands (152), Luduena and Snyder (153), and Luduena *et al.* (1954).

Table V—Relative Pressor Activity of (−)- and (+)-Isomers of Nonphenolic Sympathomimetic Amines

Isomer	Pressor Effects: Ratio of Activity	Pressor Effects: Isomeric Ratio	Reference No.
(−)-Ephedrine (+)-Ephedrine	1 (pithed cat) 0.3	3	163
(+)-Pseudoephedrine (−)-Pseudoephedrine	1 (pithed cat) <0.1	>10	163
(−)-Norephedrine (+)-Norephedrine	1.0 (pithed dog) 0.68	<2	164
(−)-Norpseudoephedrine (+)-Norpseudoephedrine	1.0 (pithed dog) 0.87	1	164
(−)-Deoxyephedrine (+)-Deoxyephedrine	1.0 (pithed dog) 0.71	1	164
(−)-Deoxynorephedrine (+)-Deoxynorephedrine	1.0 (dog) 0.71	1	164
(−)-Phenylethanolamine (+)-Phenylethanolamine	1.0 (dog) 0.3	3	165
(−)-*N*-Methylphenethanolamine (+)-*N*-Methylphenethanolamine	1 0.25	4	166
(−)-Cyclohexylisopropylamine (+)-Cyclohexylisopropylamine	1.0 (dog) 0.5	2	167
(−)-Cinnamylephedrine (+)-Cinnamylephedrine	1.0[a] (cat) 0.5[a]	2	168

[a] Fall in the blood pressure.

OPTICAL ISOMERS OF NONPHENOLIC AMINES

Since the discovery of ephedrine, many closely related amines have been synthesized and tested for sympathomimetic effects. In early days, the main interest in nonphenolic amines was caused by their central stimulant and prolonged pressor effects. The classical work of Badger and Dale (162) describes pressor activity of many structurally similar amines. Along with these developments, the optical isomers of some nonphenolic amines were also investigated. Many earlier studies were semiquantitative. Hence, an attempt to obtain a coherent summary except for blood pressure effects was a failure. A short report regarding the pressor effects of the isomers is presented in Table V. The pressor effects of nonphenolic amines are now recognized as being mediated through the release of catecholamines from the sympathetic nerve endings. Readers interested in earlier studies regarding optical isomers of nonphenolic amines are urged to read other reports (169–174).

Among nonphenolic amines, the ephedrine isomers present a unique opportunity to study steric structure–activity relationships. Although heart rate and pressor effects of naturally occurring (−)-ephedrine were considered as mixed actions, the investigation of all four isomers of ephedrine revealed that this property is not shared by (+)-ephedrine, and for (+)-pseudoephedrine was mainly "indirect" through the release of endogenous norepinephrine (16). De Meyts and Cession-Fossion (175) extended these observations in rats, that pressor effects of (−)-ephedrine were direct as well as indirect while those

of (+)-pseudoephedrine were mainly indirect. Fifty milligrams per kilogram, i.p., of (−)-ephedrine caused a significant decrease in myocardial catecholamines in rats, while under similar conditions (+)-pseudoephedrine was inactive. Both (−)-ephedrine and (+)-pseudoephedrine did not influence adrenal catecholamines. Light *et al.* (176) examined the vascular effects of ephedrine isomers in dogs. The intraarterial injection of (−)-ephedrine reduced the blood supply to all vascular beds studied, in contrast to the dilation produced by (−)-pseudoephedrine. Renal and vertebral arterial flows were increased and the carotid flow decreased by the (+)-isomers of both ephedrine and pseudoephedrine. Limb flow increased by (+)-ephedrine but decreased by the (+)-pseudoephedrine. These vascular effects probably indicate the indirect or direct activation of either α- or β-adrenergic receptors.

The pattern of the indirect pharmacologic activity of all four ephedrine isomers in the rat vas deferens appears as (−)-ephedrine > (+)-ephedrine ≥ (+)-pseudoephedrine ≫ (−)-pseudoephedrine. The pattern of the potentiation of exogenous norepinephrine by these agents in the reserpine-pretreated tissues also appears to be the same (Table VI). On the isolated rabbit aorta, (−)-ephedrine produces a marked contractile effect while other isomers produce little or no effect (177).

(−)-Ephedrine was introduced in therapeutics as a bronchodilator drug (178) and has been widely used as such. Tye *et al.* (179) investigated the effects of ephedrine isomers on tracheal smooth muscle of guinea pig. All isomers appear to be partial agonists and (−)-ephedrine and (−)-pseudoephedrine were mainly direct acting, the smooth muscle relaxant effects of (+)-ephedrine and (+)-pseudoephedrine were considerably reduced by reserpine-pretreatment. Propranolol, a β-adrenergic blocker, reduced the effects of all isomers. Ephedrines were studied in the presence of tone induced by methacholine. Muscle relaxant effects, therefore, may partly be attributed to competition of ephedrine molecules with that of methacholine.[8]

Pendular movements of rabbit ileum are inhibited only by (−)-ephedrine; the other three isomers produce little or no effect and are antagonistic to the α-adrenergic inhibitory effects of (−)-norepinephrine. The stereospecificity in such antagonism is not very marked. For example, (+)-ephedrine and (−)-pseudoephedrine vary considerably in steric structure, but produce similar antagonistic effects to (−)-norepinephrine (181). (−)-Phenylethanolamine produces inhibition of the rabbit ileum. (+)-Phenylethanolamine and the deoxy derivative, phenethylamine, do not have intrinsic activity, but both agents produce an equal antagonism to norepinephrine effects (182). These observations are consistent with the Easson-Stedman hypothesis (138).

LaPidus *et al.* (183) pointed out the stereochemical similarities between (−)-ephedrine and (−)-pseudoephedrine. In both of these molecules, the functional groups, the phenyl ring, β-hydroxyl group, and the amino group could fit the same three points on a hypothetical receptor. In the anesthetized cat, the pressor and nictitating membrane effects of (−)-ephedrine were promptly terminated by (−)-pseudoephedrine. The pretreatment of an animal with (−)-pseudoephedrine can also prevent the effects of (−)-ephedrine (177). However, the antagonism by (−)-pseudoephedrine can be extended to other indirectly acting amines such as amphetamine and tyramine. The antagonism between (−)-ephedrine and (−)-pseudoephedrine on the cat nictitating membrane or blood pressure may even be physiological.

Table VI—Effects of "Indirectly" Acting Ephedrine Isomers on the Normal Vas Deferens and the Potentiation of Norepinephrine Responses by the Same Agents in the Reserpine-Pretreated Tissues

Isomer	Normal Vas Deferens,[a] % Contraction[b] 10^{-5} *M*	*n*[d]	Reserpine-Pretreated Vas Deferens Response to 3×10^{-7} *M* Norepinephrine in the Presence of 10^{-5} *M* of the Drug[c] % Contraction[e]	*n*[d]
(−)-Ephedrine	25 (±4)	8	49 (± 5)	10
(+)-Ephedrine	11 (±2)	8	20 (±3)	10
(+)-Pseudoephedrine	8 (±2)	8	26 (±4)	10
(−)-Pseudoephedrine	0	8	5 (±1)	10

[a] Data taken from Patil *et al.* (20). [b] With reference to maximal response to norepinephrine = 100. [c] Incubation time 3 min. [d] Number of observations. [e] With reference to maximal response to 3×10^{-4} *M* norepinephrine after the procedure = 100. Data taken from Patil and Patel (180).

Because the pattern of effects of ephedrine isomers varies so greatly from one tissue to another, Kier (184) postulated a pattern of complementary features which represent the α-adrenergic receptor that is activated by ephedrine isomers. Molecular orbital calculations were used to map α-adrenergic receptors. These calculations present an interesting theoretical approach which would gain more recognition if the hypothesis were tested over a wide variety of related molecules and by some experimental means.

Several attempts were made to synthesize the ephedrinelike molecules with a rigid structure. Yelnosky and Katz (185) reported a sympathomimetic action of *cis*-2-amino-4-methyl-5-phenyl-2-oxazoline. Comparison of the structure of this agent with that of ephedrine illustrates a marked similarity in the functional groups. The pharmacological effects are also similar to ephedrine's. Meyer *et al.* (186) synthesized norephedrine homologs, 2-aminotetralol, with a rigid molecular structure. Like norephedrine, this agent could exist in four possible isomers: (+), (−)-*cis*-form and (+), (−)-*trans*-form. Only the *cis*-form was effective as a pressor agent. (+)-*cis*-2-Aminotetralol was about 5 times as active as (−)-*cis*-2-aminotetralol. The arrangement of functional groups in (+)-*cis*-2-aminotetralol was claimed to be like that in the most active form of norephedrine. It should be emphasized that higher pressor potency of these two agents reflects their possible catecholamine-releasing effects. Smissman and Chappell (187) reported on conformationally rigid derivatives of ephedrine. Pharmacological testing on the isolated rat vas deferens revealed that there were no intrinsic effects from all of the agents. However, in 10^{-4} *M* concentrations, effects of exogenous norepinephrine were

[8] R. F. Furchgott, personal communication, 1969.

potentiated. This potentiation indicates that these agents possibly interact with uptake sites. Bulky substituents possibly retard the catecholamine-releasing activity which is seen in the parent ephedrine molecules. It would be interesting to synthesize and test the conformationally rigid analogs of catecholamines such as norepinephrine and epinephrine.

TACHYPHYLAXIS (ACUTE TOLERANCE)

It is outside the scope of this report to review various theories or explanations for tachyphylaxis. Only work pertinent to the optical isomers will be referred to. In the early years, tachyphylaxis to the sympathomimetic amines, such as ephedrine or amphetamine was said to be caused by receptor saturation (188). But when the various sites such as uptake, binding, and direct action were known, it was important to explain the phenomenon of tachyphylaxis in relation to these sites. Abdallah *et al.* (17) studied tachyphylaxis to the ephedrine isomers in the perfused rabbit heart and found that under identical experimental conditions, the rate of development of tachyphylaxis varied with the isomers. (−)-Ephedrine was the most potent tachyphylactic isomer studied. The correlation between norepinephrine recovered in the perfusate and loss of chronotropic effect was not parallel. None of the isomers significantly influenced the total cardiac norepinephrine. *In vivo*, at "equipressor" doses, the tachyphylactic tendencies for ephedrine isomers were (+)-pseudoephedrine > (+)-ephedrine > (−)-ephedrine (16), which is the reverse of the ranking observed *in vitro*. This ranking might be caused by differing disposal rates for the ephedrine isomers *in vivo*; but since cardiovascular reflexes may so greatly influence blood pressure, comparisons between *in vitro* and *in vivo* data are difficult to make. There are some doubts regarding participation of adrenal medulla during ephedrine tachyphylaxis (189). However, in an anesthetized cat, pretreatment with a total of 6 mg./kg. of *N,N*-diisopropyl urea, *N'*-isoamyl urea, and *N'*-diethylaminoethyl urea (P. 286), a specific adrenal medullary blocker, did not influence the pressor effects or the tachyphylactic effects caused by ephedrine isomers (190). If a given indirectly acting amine has a greater affinity for the uptake sites and is not washed off the tissue during perfusion, it may inhibit its own uptake during subsequent injections. This inhibition will result in a reduced displacement of catecholamines from storage sites and a consequent diminishing of tissue response. Similar suggestions have been made earlier by Blaschko (191) and Fawaz and Simaan (192). In other words, different rates of development of tachyphylaxis may be indicative of an amine's ability to prevent its own uptake into the sympathetic nerve endings.

Hanna (193) and Harvey *et al.* (194) found that the tachyphylactic tendencies of amphetamine isomers on dog blood pressure and on rabbit aortic strips were the same for both isomers. The major structural difference between amphetamine and ephedrine is that the latter possesses an alcoholic hydroxyl group. The different rates of development of tachyphylaxis observed with ephedrine isomers in this study may well depend on the orientation of this group. (+)-Pseudoephedrine, a *threo*-isomer, appeared to be less tachyphylactic than (−)-ephedrine and (+)-ephedrine, which are both *erythro*-isomers. Lindmar *et al.* (74) have reported that (±)-pseudo dihydroxyephedrine, a *threo*-form, is washed off the heart more easily than (±)-dihydroxyephedrine, an *erythro*-form. (+)-Pseudoephedrine's lack of tachyphylactic power then may well be caused by its *threo*-conformation which allows it to be more easily washed off. These observations lead to the conclusion that the relative orientation of the β-hydroxyl group and the α-methyl group must be of critical importance in the development of tachyphylaxis in the ephedrine isomers.

Hornykiewicz and Obenaus (195) elaborated on some of the previous observations regarding tachyphylaxis to direct acting amines. In the anesthetized rats, infusion of large amounts of (+)-epinephrine, (+)-norepinephrine, epinine, and (−)- or (+)-phenylephrine induces tachyphylaxis to vasopressor effects of (−)-epinephrine, (−)-norepinephrine, and tetraethylammonium. The tachyphylactic potency of the amines was (−)-phenylephrine > epinine > (+)-norepinephrine = (+)-epinephrine > (+)-phenylephrine. Other peripheral effects of (−)-isoproterenol and (−)-epinephrine were also reduced by infusion of (+)-epinephrine. From these results, it was concluded that tachyphylaxis could result from saturation of α- or β-adrenergic receptors by less active isomers of catecholamines and related agents. In the isolated heart of *venus mercenaria*, (−)-norepinephrine produces negative inotropic effects. The repetition of the same dose produces tachyphylaxis. Optical isomers of amphetamine and ephedrine can prevent the tachyphylactic effects caused by (−)-norepinephrine. (−)-Ephedrine was found to be more potent than (+)-ephedrine, and (+)-amphetamine was found to be more potent than (−)-amphetamine (196, 197).

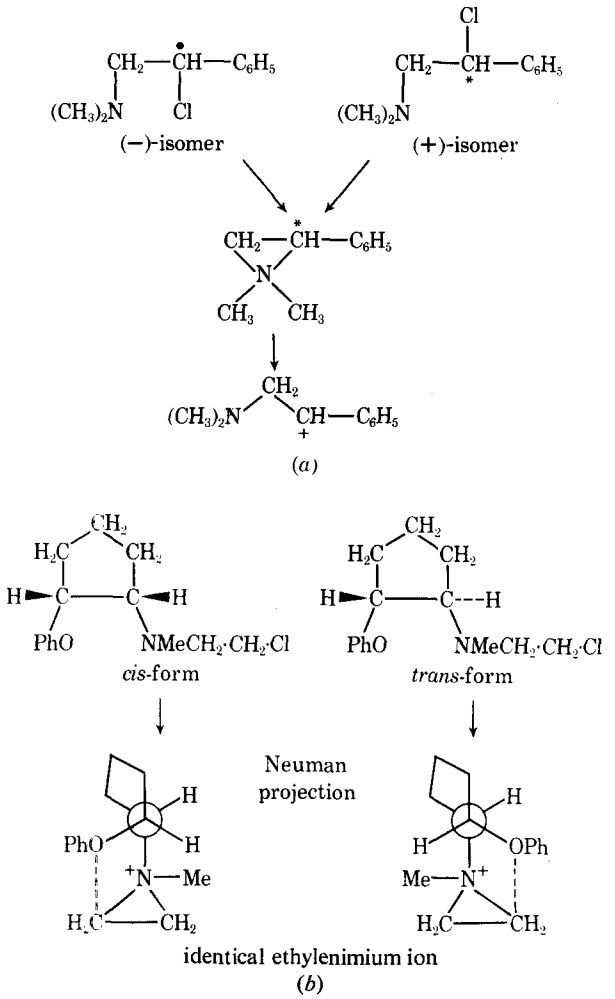
Thus, it appears that sympathomimetic amines can produce tachyphylaxis by the following mechanism or combination of the following mechanisms: (*a*) depletion of small available stores of norepinephrine; (*b*) inhibition of their own uptake by neuronal membrane; (*c*) saturation of α- and/or β-adrenergic receptors; and (*d*) slow excretion or metabolic disposition. The optical isomers of sympathomimetic amines provide a good tool for exploring various mechanisms of sympathomimetic amines.

α-ADRENERGIC BLOCKERS

At present, very little information is available regarding steric aspects of the reversible competitive antagonist of α-adrenergic receptors. Therefore, our knowledge regarding α-adrenergic receptor antagonists is derived solely from irreversible blockers. Nickerson (198) suggested that in the case of β-haloalkylamines, the development of a stable drug receptor bond can be related directly to the chemical reactivity of ethylenimium intermediate formed at physiological pH. Belleau (199) later introduced a concept of isosterism. He suggested a similarity of interaction of (−)-norepinephrine and ethylenimium ion from *N*-(2-chloroethyl)dibenzylamine at the α-adrenergic receptor. It is of interest to note that the suggested interaction of agonist, (−)-noradrenaline, with the α-adrenergic receptor is essentially the same as that postulated by Easson and Stedman (138).

Belleau and Triggle (200) synthesized and resolved two optical isomers of *N,N*-dimethyl-β-chlorophenethylamine.

Equipotent antiadrenaline effects of the two isomeric forms of *N,N*-dimethyl-β-chlorophenethylamine indicate a possible common reactive symmetric molecular species at adrenergic receptors (Scheme III*a*). Similarly, *cis*- and *trans*-isomers of *N*-methyl-*N*-(2′-phenoxycyclopentyl)-2-chloroethylamine yield similar reactive molecular species, and hence they are approximately equipotent with respect to the reactive α-adrenergic site (Scheme III*b*)



Scheme IIIa, b—Common reactive ethylenimium ion from the optical isomers of N,N-*dimethyl-β-chlorophenethylamine and* N-*methyl*-N-(*2′-phenoxycyclopentyl*)-*2-chloroethylamine, respectively.*

(201). There are several thought-provoking reviews available on this subject (202–205).

β-ADRENERGIC BLOCKERS

Since the introduction of dichloroisoproterenol as an antagonist to the inhibitory effects of catecholamines, a number of structurally related chemicals have been synthesized and tested for β-adrenergic blocking properties. All of those agents which produced significant β-adrenergic blockade have asymmetric carbon or carbons. The optical isomers of dichloroisoproterenol (206), pronethalol (206, 207), propranolol (208–211), methoxamine and its derivatives (15, 19, 212), INPEA (213–215), α-methyl-INPEA (216), sotalol (19, 217), butedrine (218–221), and H56/28 (222) have been synthesized, resolved, and tested for pharmacologic activity. The chemical structure and β-adrenergic blocking properties are summarized in Table VII. Examination of data in Table VII reveals that all potent β-blockers markedly resemble the agonists (−)-isoproterenol. The points of similarities are: (*a*) substitution on the phenyl ring; (*b*) alkyl substitution on the nitrogen; and (*c*) alcoholic group in correct stereochemistry with the receptor (1R or D-configuration). The stereochemistry of the alcoholic hydroxyl is the same as that in (−)-isoproterenol. This structural requirement implies that β-adrenergic blockers are relatively more specific in their attachment to the β-receptor than α-adrenergic antagonists to the α-receptors. However, there is a marked similarity between interaction of α- and β-adrenergic agonists with their respective receptors. Substitution of the methyl group adjacent to the carbon-carrying alcoholic hydroxyl group can hinder the effective interaction of agonist or antagonists with the receptor. For example, (−)-pseudobutoxamine fulfills all the structural requirements for a β-adrenergic blocker, but it does not appear to block β-adrenergic receptors. A very interesting study on the conformational aspects of the ephedrine isomers has been carried out by Portoghese (223). He states that: "It appears significant that (−)-ephedrine is the only isomer which possesses both 1R configuration and the *C*-methyl group which projects above the plane of phenethylamine moiety. The (−)-pseudoephedrine also possesses the 1R stereochemistry necessary for direct action, but the *C*-methyl group is oriented below the plane. It is conceivable that the methyl group in the latter isomer hinders effective interaction with the receptor." In terms of this conformational analysis of ephedrine isomers, it becomes apparent why (−)-ephedrine blocks β-adrenergic receptor and (−)-pseudoisomer does not. Similar explanations may hold true for the isomers of methoxamine, isopropylmethoxamine, butoxamine, and α-methyl-INPEA in which there are two asymmetric centers like those in the ephedrine molecule. The actual conformations of these molecules at the receptors are not yet known. Butedrine also has two asymmetric centers, but the methyl substitution is very remote from that of the important functional alcoholic hydroxyl group. As a consequence, it does not appear to influence the interaction of the alcoholic hydroxyl group with the β-adrenergic receptor. Butedrine 1R, 3R and 1R, 3S are almost equiactive.

(−)-Propranolol is more potent in antagonizing isoproterenol-induced tachycardia while its (+)-isomer is much less active. The deoxy analog of propranolol is approximately as active as (+)-propranolol (208). Thus, β-adrenergic blockers are in line with Easson and Stedman's suggestions regarding interactions of asymmetric molecules with the receptors. However, many exceptions appear to have emerged, particularly when a given molecule has more than one asymmetric center in which the nonfunctional group may hinder the attachment of functional groups to receptors. The location of adrenergic receptors in tissue is not known. If β-

Table VII—Isomeric Activity Ratio of β-Adrenergic Blockers

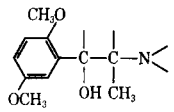
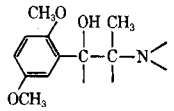
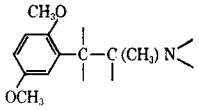
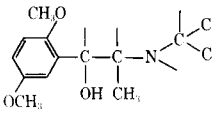
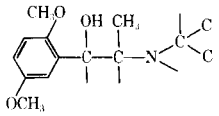
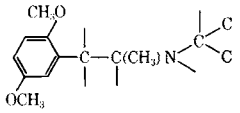
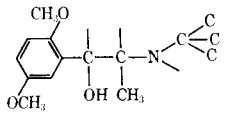
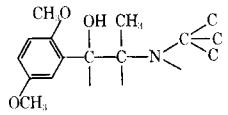
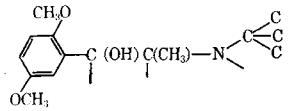
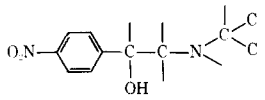
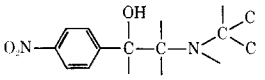
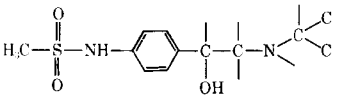
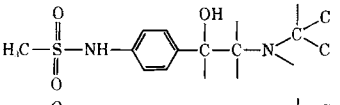
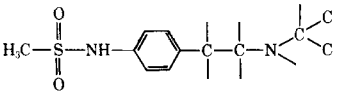
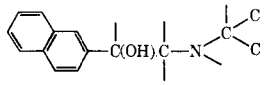
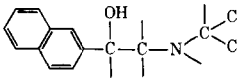
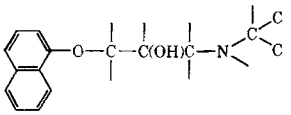
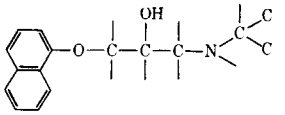
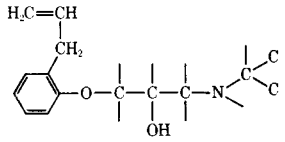
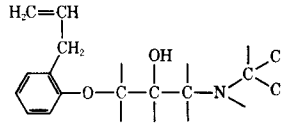
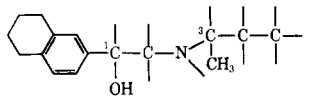
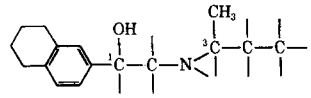
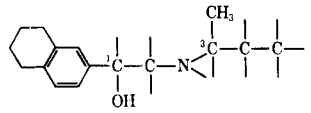
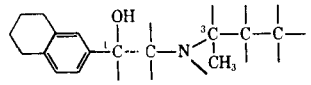
Chemical Structure	Name	pA_2	Isomeric Ratio
	(−)-Methoxamine	6.30	
			77[a]
	(+)-Methoxamine	4.37	
	(±)-Deoxymethoxamine	5.09	
	(−)-Isopropylmethoxamine	6.53	
			>1100[a]
	(+)-Isopropylmethoxamine	<3.50	
	(±)-Deoxyisopropylmethoxamine	4.85	
	(−)-Butoxamine	7.20	
			>1700[a]
	(+)-Butoxamine	<4.0	
	(±)-Pseudobutoxamine	<4.0	
	(−)-INPEA	6.50	
			190[a]
	(+)-INPEA	4.22	
	(−)-Sotalol	6.80	
			44[a]
	(+)-Sotalol	5.15	
	Deoxysotalol	3.88	
	(±)-Pronethalol	7.30	
			>120[a]
	(+)-Pronethalol	5.20	
	(±)-Propranolol	8.50	
			>100[b]

Table VII—*Continued*

Chemical Structure	Name	pA_2	Isomeric Ratio
	(+)-Propranolol	6.50	
	(−)-H56/28	—	
	(+)-H56/28	—	100[c]
	(−)-Butedrine 1R,3R	7.85	
	(+)-Butedrine 1S,3R	<6.00	>70[b]
	(+)-Butedrine 1R,3S	6.70	
	(+)-Butedrine 1S,3S	<5.50[d]	>15[b]

[a] On guinea pig trachea (15, 19). [b] On guinea pig atria for heart rate (222). [c] In anesthetized cat for heart rate (221). [d] Causes marked cardiac depression (220).

receptors are inside the cell and if there is another transport system at the sites of direct action, then it becomes of primary importance to establish a structural requirement for such a system before the Easson-Stedman theory can be rejected.

Different types of β-receptors complicate interpretation of structure–activity study from one tissue to another (224, 225). Substitution of $-CH_3$ group on α-carbon is known to decrease ability of molecules to block β-receptors in the heart. The β-adrenergic receptors in the skeletal muscle are classified as Type β_2. Tremors of skeletal muscles produced by infusion of catecholamines in man are blocked by (±)-propranolol but are unchanged by (+)-propranolol (226). Whether all these β-receptors are different or whether the effects are due merely to physical-chemical properties of β-blockers is not known.

In addition to blocking β-adrenergic receptors, many of these blockers produce direct myocardial depression. Levy and his colleagues (227–230) attempted to correlate the physical-chemical properties with the cardiac effects of various β-blockers and their isomers. However, there appears to be no simple relationship between *in vitro* β-adrenergic blocking action and myocardial depressant effects.

On the other hand, the inhibition of Ca^{++} uptake in cardiac sarcoplasmic reticulum fractions by (−)- and (+)-propranolol correlates with their ability to decrease cardiac contractility (231).

Recently, Serrano and Hardman (232) suggested that a nonionized form of the drug might be essential for the production of β-adrenergic blockade. While this suggestion will require further proof, an interesting experiment could be made by testing (−)- and (+)-isomers of β-adrenergic blockers. Since pKa values of (−)- and (+)-isomers are identical, regardless of change in pH, the relative number of nonionized forms of both drugs should be the same. This would indicate that the difference in pharmacological activity between (−)- and (+)-isomers should remain the same at different pH values.

An attempt has been made to characterize the nature of the β-adrenergic receptor in the guinea pig atria (233). A potent and long-acting β-blocker, propranolol, was used as a tool to tag the receptors. However, uptake of the less active (+)-propranolol was the same as that of the more potent racemate or (−)-isomer. This nonspecific uptake indicates that the antagonist was largely bound to nonspecific sites and that the amount of antagonist bound to the specific sites must be very small. Thus, attempts to identify the pharmacologic receptors have achieved limited success.

Because of their antiarrhythmic effect, there has been considerable clinical interest in β-adrenergic blockers. However, the mechanism of antiarrhythmic action of

these drugs is complicated by their local anesthetic, "quinidinelike," and β-adrenergic blocking effects. Results clearly indicate that adrenergically induced cardiac arrhythmias can be promptly terminated by low doses of (−)-isomers of β-adrenergic blocker. On the other hand, much higher and nearly equivalent doses of both (−)- and (+)-isomers of β-adrenergic blockers are required to prevent or prolong the cardiac arrhythmia induced by ouabain (234–241). Posttetanic potentiation of cat soleus is equally depressed by optical isomers of the β-blocker. On this basis, Standaert *et al.* (242) suggested that termination of digitalis-induced arrhythmia might be a neural phenomenon. In the guinea pig, when arrhythmias were produced by infusion of ouabain, Dohadwalla *et al.* (243) showed that (±)-propranolol is slightly more effective than (+)-propranolol. They attributed this unequal antiarrhythmic effect to the unequal β-adrenergic blocking property and not to the local anesthetic effect. Stickney and Lucchesi (244) elaborated on the ventricular arrhythmias elicited in dogs by central administration of acetylstrophanthidin. Racemic propranolol, but not (+)-propranolol, attenuated the arrhythmias caused by the glycoside.

Some β-adrenergic blockers are known to have a hypotensive effect in man. Kelliher and Buckley (245) studied the possible mechanism of this hypotensive effect. When administered directly into the left lateral ventricle of cat, both (+)- and (±)-propranolol produced nearly equal hypotensive effects. It was concluded that the central hypotensive effect is independent of β-adrenergic blockade. This study should be extended to the other optical isomers which are not local anesthetics. Furthermore, prevention of adrenergically induced arrhythmias may not necessarily be related to local anesthetic effects of β-blockers. For example, (−)-sotalol lacks local anesthetic effect but is a very effective agent in preventing the adrenergically induced arrhythmia. The mechanism of termination of nonadrenergically induced arrhythmia is yet to be clarified. Both the isomers of propranolol cause equal myocardial depression and the local anesthetic effect is also identical. In order to separate local anesthetic effect from quinidine-like effect, the use of deoxypropranolol has been suggested by Ariens (246). Parmley and Brunwald (247) compared myocardial depressant and antiarrhythmic properties of (±)-propranolol, (+)-propranolol, and quinidine. Their study suggests that (+)-propranolol might be a very useful drug in the treatment of certain arrhythmias where β-adrenergic blockade is not desired. Quinidine lowers arterial blood pressure, while (+)-propranolol is without such a clinically undesired effect.

Peripheral vascular effects of propranolol isomers have been studied in the anesthetized dog. Direct intracoronary injections of small doses, in the anesthetized dog, of (+)-propranolol produces transitory reduction in coronary vascular resistance; under similar conditions, (−)-propranolol only increases coronary vascular resistance. This increased resistance may be a reflection of β-blocking effect of (−)-isomer (248). Both isomers of propranolol, when injected directly in the external ilial artery, increased blood flow through the artery to the same extent. This effect is, however, attributed to the local anesthetic effects of the agents (249).

Studies on effects of isomers of adrenergic drugs on metabolic processes are relatively few. Isoproterenol is very potent in releasing free fatty acids *in vitro* from adipose tissue. The (+)/(−) isomeric ratio is approximately 4000 (250). β-Adrenergic blockers competitively inhibit the free fatty acid mobilization stimulated by the adrenergic agents. (−)-Isomers of INPEA, isopropylmethoxamine, and methoxamine are some 100 times more potent than their (+)-isomers (251–255). High concentrations of both (−)- and (+)-INPEA equally reduced the incorporation of labeled glucose in the fat cells. Thus, the phenomenon appears to be nonspecific and unrelated to the β-adrenergic blockade (256).

Any adrenergic drug with a basic chemical structure such as phenethylamine exhibits a variety of characteristic effects at adrenergic synapses. β-Adrenergic blockers are basically derivatives of phenethylamines. It is possible, therefore, that β-receptor antagonists can interact at the same two points (Sites A and B), as suggested for *N*-(2-chloroethyl)dibenzylamine, and can protect α-receptors from the latter (Fig. 6). This two-point interaction would occur equally well with (−)- or (+)-isomers of β-antagonists, and thus the degree of stereoselectivity in protection of α-receptors by β-receptor antagonists would not be marked (257). If β-adrenergic blockers compete with norepinephrine for α-adrenergic receptors, these agents should shift the dose–response curve for norepinephrine to the right in a parallel fashion. Most agents, except isomers of H56/28, produced a potentiation of the effects of exogenous norepinephrine and shifted the curve to the left. This paradoxical observation can be explained by differential interplay between two main factors operative at sympathetic neurones: (*a*) inhibition of uptake, and (*b*) competition at α-adrenergic receptors. Whenever effects of norepinephrine are potentiated by a given blocker, the optical isomers do not differ in this respect. It indicates that inhibition of uptake of norepinephrine by (−)- and (+)-isomers of β-blocker must be similar. Results from biochemical studies tend to support this notion. Either in the adrenergic nerve granule or in the perfused heart, inhibition of uptake of exogenous norepinephrine or epinephrine by (−)- and (+)-isomers of β-blocker is similar (258–261). Since (−)- and (+)-isomers of β-blocker do not differ in either competition at α-receptor or inhibition of uptake and since (−)-isomers are the most potent blockers, the observed effects are not causally related to β-adrenergic blockade. Furthermore, on rabbit aorta, where uptake of agonist is not a critical factor in the determination of competitive antagonism, the pA_2 values of (−)- and (+)-INPEA are equal (262).

All four optical isomers of butedrine were also investigated (263) on the rat vas deferens. Phenylephrine was used as agonist. In the presence of butedrines, the dose–response curve of phenylephrine was depressed. The pD_2' values for noncompetitive antagonism varied from 3.7 to 4.20. This indicates a very small difference in butedrine isomers for the noncompetitive interaction with α-agonists, hence, unrelated to their β-blocking properties.

β-Adrenergic blockers are known to enhance the respiratory difficulty in asthmatics. With the aid of the optical isomers of INPEA, Murmann (264) attempted to analyze the underlying mechanism. He found that except in one species of mice, LD_{50} of histamine was not significantly altered by pretreating mice with either isomer of INPEA. He tentatively, then, suggested that β-adrenergic blockade may not be the cause for the enhancement of respiratory difficulty in asthmatics. However, it appears that in the bronchioles, when the smooth muscle relaxation is blocked by β-adrenergic blockers, there could be bronchoconstriction caused by overpowering of parasympathetics, as well as that of α-adrenergic receptors. Hence, in order to define the role of β-adrenergic blockade in asthmatics, two types of experiments remain to be done: (*a*) to see if (+)-isomers of INPEA will enhance the bronchoconstriction in asthmatics; and (*b*) to compare endotracheal pressure changes after pretreatment of either (−)- or (+)-isomers of β-blockers in experimental animals. The two phases of nicotinic blood pressure effects of acetylcholine in atropine-pretreated animals are well known. The first phase, caused by stimulation of sympathetic ganglia, is selectively antagonized by (−)-INPEA, while (+)-INPEA is ineffective. It is concluded that selective β-adrenergic blockade might be involved at the level of sympathetic ganglia (215).

METABOLIC ASPECTS

Only a limited number of reports concerning this facet are available at present. Epinephrine increases blood sugar; this effect is stereoselective in favor of (−)-isomer. In rabbits, 0.05 mg./kg. of (−)-epinephrine and 1 mg./kg. of (+)-epinephrine produce approximately an equivalent rise in the blood sugar. The effects of the (+)-isomer are transitory, while those of the (−)-isomer last many hours (265). Bowman and Raper (266) studied drugs affecting carbohydrate metabolism on contractions of the rat diaphragm. Potassium-induced depression of the skeletal muscle contraction is readily reversed by sympathomimetic agents. In this respect, (−)-norepinephrine and (−)-epinephrine are 500–1000 times more potent than their respective (+)-isomers. These effects, as they claim, are probably caused by activation of the β-receptor. Anderson and Chen (267) screened the hyperglycemic action of 40 amines. After equimolar doses (0.1 ml. of 0.1 *M* i.v.) in rabbits, the rise in blood sugar was determined. According to this test, the activity of ephedrines was: (−)-pseudoephedrine > (+)-ephedrine = (+)-pseudoephedrine > (−)-ephedrine. The differences between (−)-pseudoephedrine and (−)-ephedrine were striking. The onset of action of (−)-pseudoephedrine was very slow and as much as 40 min. was required for the peak effects. On the other hand, only 10 min. was required for the peak effects of the less active (−)-ephedrine.

A highly attractive hypothesis regarding the role of cyclic 3′,5′-AMP in response to catecholamines has been put forward by Sutherland and his colleagues (268–270). It has been suggested that cyclic 3′,5′-AMP plays an essential role in the hyperglycemic response

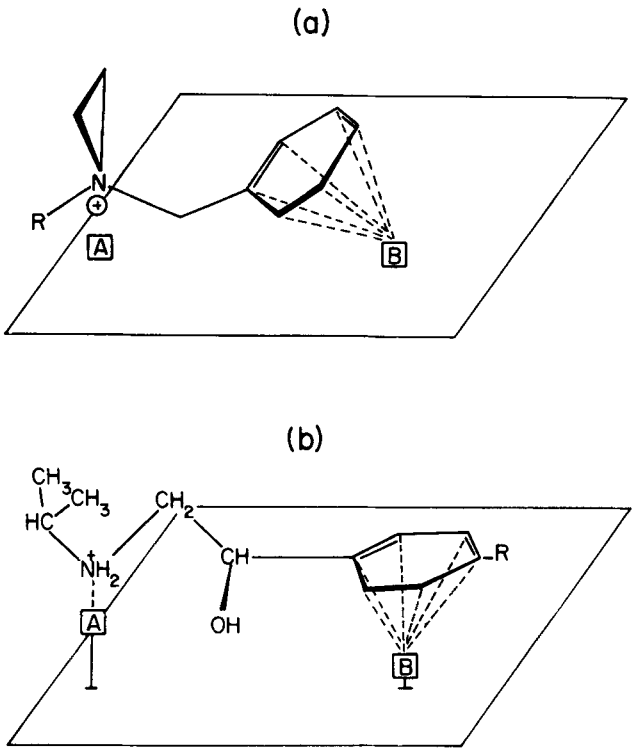


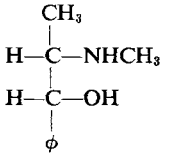
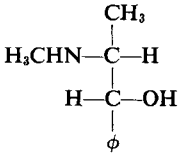
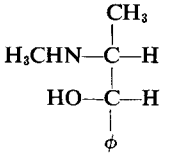
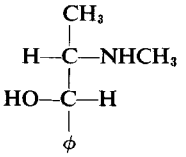
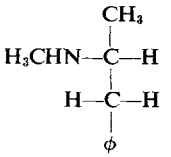
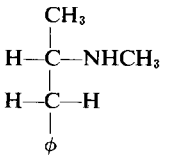
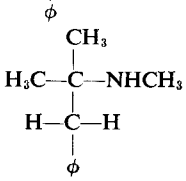
Figure 6—(*a*) *The initial interaction of the ethylenimium ion from* N-(*2-chloroethyl*)*dibenzylamine* ($R = C_6H_5CH_2$) *and the α-adrenergic receptor as suggested by Belleau (199) and Triggle (202).* (*b*) *The possible interaction of β-adrenergic blockers like INPEA* (*where* $R = NO_2$) *and explains the protective action of β-adrenergic blockers against block by* N-(*2-chloroethyl*)*dibenzylamine. Note that the β-hydroxyl group is not involved and thus explains a similar protective effect of* (−)- *and* (+)-*INPEA. Reproduced with permission from Patil* et al., J. Pharmacol. Exp. Ther., *163, 309(1968), and the Williams & Wilkins Co., Baltimore, MD 21202*

to epinephrine, principally through its effect on phosphorylase. Furthermore, it has been been suggested that cyclic 3′,5′-AMP might also be involved in the positive inotropic effects of catecholamines in the heart. The ability of (−)-isoproterenol, (−)-epinephrine, and (−)-norepinephrine to stimulate formation of cyclic 3′,5′-AMP and to produce positive inotropic responses is of a similar nature. (−)-Epinephrine, as compared to its (+)-isomer, is much stronger both in formation of cyclic 3′,5′-AMP and in positive inotropic effects. Recently, Weiss and Costa observed a stereospecific activation of adenylcyclase (271).

McNeill and Brody (272) determined the stereoselectivity of norepinephrine isomers for rat cardiac phosphorylase. Although both forms activated the enzyme to the same extent, (+)-norepinephrine was found to be 1/30th as active as its (−)-form. Thus, adenylcyclase and cyclic 3′,5′-AMP and phosphorylase exhibit stereoselectivity.

A study of the metabolic fate of optical isomers provides an interesting approach to elucidating the nature of various mechanisms that are involved in biotransformation and excretion of the drugs. Considering the specificity of various enzymes, it is unlikely that optical isomers are handled identically by the body. Development of a sensitive and specific analytical method, however, poses a problem. Many methods that are available today do not distinguish between (−)- and

Table VIII—Kinetic Constants for the *In Vitro* *N*-Demethylation of a Series of Sympathomimetic Amines by the 9000×*g* Supernatant Fraction from Rabbit Liver

Drug	Structure	Average K_m (× 10^4 *M*)	Average $\nu_{max.}$[a] (× 10^3)
(−)-Ephedrine (1R,2S)		1.2[b]	1.3
(−)-Pseudoephedrine (1R, 2R)		1.7	1.1
(+)-Ephedrine (1S,2R)		2.7	1.3
(+)-Pseudoephedrine (1S,2S)		2.2	1.8
(−)-Methamphetamine (2R)		3.0	2.6
(+)-Methamphetamine (2S)		4.5	2.6
Mephentermine		5.7	1.7

[a] μmole of HCHO/min./mg. protein. [b] The *Km* and $\nu_{max.}$ values shown are the mean values determined from two or three experimentally independent $1/v$ *versus* $1/s$ plots. The experimental points on each of these plots represented the mean of duplicate rate measurements for each substrate level. The standard error of the mean for the values in the table was about 10%. Data from Dann (282).

(+)-isomers of the drug. Hence, the metabolic fate of (−)- and (+)-isomers from the injected racemate cannot be precisely studied.

The fate and urinary excretion of amphetamine isomers have been studied in various species and in man. Although total urinary excretion of these drugs is pH dependent, the differences in the excretion pattern are not great (273–275). Gunne (276) used the gas chromatographic resolution method (277) for amphetamines and found that after administration of (±)-amphetamine, all subjects excreted approximately equal amounts of both isomers during the first 12 hr. Urine collected after 12 hr. contained a continually decreasing proportion of the (+)-isomer. This slow excretion might be a reflection of higher tissue deposition of (+)-amphetamine (or its metabolites) over that of (−)-amphetamine (42). In rats, 2 days after dosing with equal amounts of (+)- and (−)-amphetamine, the quantity of *p*-hydroxyamphetamine is 48 and 63% of the initial dose, respectively. Relatively more (+)-*p*-hydroxyamphetamine is converted to its β-hydroxylated product, (−)-*p*-hydroxynorephedrine (43) by enzyme dopamine-β-hydroxylase; hence less (+)-*p*-amphetamine will appear in the urine. It is interesting, however, that the ring-hydroxylating enzyme does not appear to show selectivity for amphetamines. This lack of selectivity is in contrast to the fact that (−)-ephedrine is ring hydroxylated while (+)-ephedrine is not (278). There are marked species differences in the metabolism of amphetamine isomers. Axelrod (279) reported that an enzyme system in rabbit liver microsomes catalyzes the deamination of amphetamine to yield phenylacetone and ammonia. This enzyme system prefers (−)-amphetamine as substrate. However, neither (−)- nor (+)-amphetamine is metabolized by a microsomal preparation of rat liver (280). The urinary excretion kinetics of a close structural analog of amphetamine, methamphetamine, was also studied in man (276, 281). The excretion patterns of (+)- and (−)-methamphetamine are similar. Most of the drug is excreted unchanged in 24 hr.; however, a very small amount of the drug is *N*-demethylated. Because more (+)-amphetamine occurs in the urine after (±)-methamphetamine, it is suggested that enzymatic *N*-demethylation may be stereospecific. Similarly, (−)-ephedrine is *N*-demethylated while (+)-ephedrine is not (278). Recently, Dann (282) has investigated demethylation rates of ephedrine isomers. Isolated rabbit liver microsomes were used to determine the enzymatic kinetics. It was found that the $\nu_{max.}$ is the same for all ephedrine isomers, but K_m values for (−)-ephedrine and (−)-pseudoephedrine were approximately twice those of (+)-ephedrine and (+)-pseudoephedrine (Table VIII).

MAO and COMT are the two major enzymes intimately involved in adrenergic drug effects. Although stereochemical substrate specificity was not detected in the early semipurified enzymatic preparations (283–286), it is now demonstrated that indeed these enzymes do show selectivity for the (−)-isomers of norepinephrine, epinephrine, and *m*-octopamine which are better substrates than their corresponding (+)-isomers (287). Kynuramine oxidation by rat liver monoamine oxidase was inhibited more by (+)-isomers (2S) of amphetamine, 2,4-dichloroamphetamine, 4-chloro-*N*-methylamphetamine, than their corresponding (−)-isomers (288). Although available reports indicate that both (−)- and (+)-epinephrine are equally good substrates for COMT (289, 290), a more definite study is needed to establish the stereoselectivity in the highly purified enzyme.

CNS EFFECTS

In 1939, Alles reported that (+)-amphetamine was much superior to its (−)-isomer in antagonizing chloral hydrate hypnosis in rabbits (291). Later, Prinzmetal and Alles (292) confirmed these observations in humans. They concluded that (+)-amphetamine was 2–4 times as

active as (−)-amphetamine in producing CNS stimulation. Tainter *et al.* (293) studied analeptic potency of a series of sympathomimetic amines in rats against hypnotic action of tribromoethanol, chloral hydrate, and pentobarbital. The time required for recovery of the corneal and righting reflexes after a fixed dose of the hypnotic was compared with that observed under the same conditions when various of the supposed analeptics were administered also. Both (−)- and (+)-ephedrine (60 mg./kg.) did not produce any analeptic effects. However, (−)-pseudoephedrine produced a shortening of some of the reflexes under tribromoethanol and chloral hydrate which indicated that with this isomer there was a definite analeptic power. Fairchild and Alles (294) systematically investigated the locomotor activities of all optical isomers of ephedrine, norephedrine, and amphetamine. In mice, the central locomotor activity of the most potent (+)-amphetamine was assigned as 1. The activity ratios of (−)-amphetamine, (+)-norpseudoephedrine, (−)-ephedrine, and (−)-norpseudoephedrine were 4.2, 10, 24.4, and 42.8, respectively. Other isomers, (−)-norephedrine, (+)-norephedrine, (−)-pseudoephedrine, (+)-pseudoephedrine, and (+)-ephedrine, were considered as less effective as central locomotor stimulants and produced measurable activity only at doses approaching lethal amounts. Schulte *et al.* (295) used the jiggle cage to record the central activity of various sympathomimetic amines in rats. When compared at threshold, stimulant doses of (+)-amphetamine were 8 times as potent as its (−)-form. Both forms of pseudoephedrine were much less active than (−)-ephedrine.

The ratio of central stimulant activity using (+)- and (−)-methamphetamine varies from 4 to 8, with the (+)-form more active (296). This activity difference is the same as that exhibited between isomers of amphetamine.

Considering the complexity of the CNS, more than one criterion is needed to obtain a full profile of central stimulant activity. Lanciault and Wolf (297) carefully examined the neuropharmacological properties of the ephedrine isomers. Several standard techniques, including low-frequency electroshock and chemoshock threshold determinations, hexobarbital sleep-time alteration, and a behavioral rating scale, were employed. It was concluded that (−)- and (+)-ephedrines were considerably more potent than (−)- and (+)-pseudoephedrine. None of these agents affected the hexobarbital sleep-time. Interestingly enough, in one test, a given stimulant raises a threshold while the same agent in other tests lowers the seizure threshold. For example, (±)-amphetamine raises the seizure threshold of the low-frequency electroshock test while pentylenetetrazol seizure threshold is lowered by (±)-amphetamine. According to both tests, low-frequency electroshock and chemoshock threshold determinations, (−)- and (+)-ephedrines were more potent than (−)- and (+)-pseudoephedrines. In rabbits, however, (+)-amphetamine was more effective in raising the threshold to electrical convulsions and (−)-amphetamine and (−)- and (+)-ephedrines did not produce any change (298).

Central effects of the optical isomers of phenolic amines such as norepinephrine and epinephrine have been investigated (299). Since these agents produce marked pressor effects, there is the problem of separating peripheral effects from central effects. Moreover, these amines penetrate very poorly into the CNS. To overcome this difficulty, drugs were applied iontophoretically in order to study responses to brainstem neurones in decerebrate cats. The effects of (−)-norepinephrine on the firing rate of spontaneously active neurones have been found to conform to certain well-defined patterns. There are two types of patterns observed with (−)-norepinephrine, excitatory and/or inhibitory. (+)-Norepinephrine inhibited certain neurones which were also inhibited by (−)-norepinephrine; but on neurones excited by (−)-norepinephrine, its effect was weaker or absent. Thus, the excitatory effect shows stereoselectivity whereas inhibitory effect does not. The classical adrenergic blockers did not modify the responses. It is concluded that receptors for norepinephrine on brainstem neurones are of more than one kind and that they do not fit into the α- and β-classification applied to peripheral receptors (300).

An interesting approach to the study of central effects of catecholamines which do not pass the blood–brain barrier is to study them in young chickens where the blood–brain barrier is imperfect or nonexistent. Dewhurst and Marley (301, 302) used this approach to examine central effects of certain phenolic amines. The behavioral, electrocortical, and electromyographic activities were recorded. The phenolic amines produced depressant effects. Levonordefrin was at least 4 times as potent as the (+)-form. (−)-Norepinephrine was twice as potent as (+)-norepinephrine; however, dopamine was more potent than (−)-norepinephrine or (+)-norepinephrine. This pattern differs from that of peripheral sites where (+)-norepinephrine and dopamine are almost equiactive. The nonphenolic amines, such as amphetamine, produced excitatory effects. The (+)-form was more active than the (−)-form of amphetamine.

β-Adrenergic blockers produce variable effects on the CNS. With the aid of optical isomers of INPEA, Murmann *et al.* (303) concluded that, since both isomers were equipotent in producing CNS stimulation and since only one isomer is a potent β-adrenergic blocker, the central effects of β-blocker are unrelated to the blockade of β-adrenergic receptors.

The role of endogenous catecholamines in the peripheral effects of many nonphenolic amines is well defined. However, controversy still exists regarding the role of endogenous catecholamines in central effects of amphetamine and related agents. Wolf *et al.* (304) investigated optical isomers of several nonphenolic amines in the normal, reserpine-pretreated, and α-methyl-*m*-tyrosine-pretreated mice. Ability of the drug to lower chemoconvulsive threshold was used as an index of the central activity. All agents except (−)-pipradrol and (−)-pseudoephedrine demonstrated significant central effects. Reserpine pretreatment lowered the chemoconvulsive threshold to (+)-amphetamine, (−)-amphetamine, (+)-pipradrol, (−)-ephedrine, and (+)-ephedrine, while that after (+)-pseudoephedrine, (−)-norephedrine, and (+)-norpseudoephedrine was not affected. On the other hand, in the α-methyl-*m*-tyrosine-pretreated animals, the chemoconvulsive thresholds

after (+)-amphetamine, (−)-amphetamine, (−)-ephedrine, and (+)-pseudoephedrine were increased. Results from α-methyl-*m*-tyrosine-pretreated animals were assumed to be a better reflection of central catecholamine depletion. It was concluded that central effects of (+)- and (−)-amphetamine, (−)-ephedrine, and (+)-ephedrine possess large indirect components in their activity while those of (+)-pipradrol, (−)-norephedrine, and (+)-norpseudoephedrine are mainly direct. The effects of the optical isomers of amphetamine were also investigated on brain dopamine levels (305, 306), tissue respiration (307), and liver monoamine oxidase inhibition (284). However, both forms of amphetamine isomers produced similar results, indicating that these effects are not causally related to the central effects.

Infusion of (−)-α-methyldopa into the vertebral artery of the cat produces lowering of the blood pressure while (+)-α-methyldopa produces no such effect (308).

No clearcut structure–activity relationship as yet can be formulated for the central effects of sympathomimetic amines. In spite of this, as in the periphery, there are many agents which exhibit stereoselectivity in their central effects.

Amphetamine is well known to produce aggregation toxicity in mice. This effect is more pronounced with (+)-amphetamine. The potency ratio of the isolated/aggregated LD_{50} is 4.9 for (+)-amphetamine and 1.2 for (−)-amphetamine. There was a marked dose-dependent reduction of the brain and heart norepinephrine content after (+)- or (−)-isomers of amphetamine. It was, however, only after the administration of (+)-isomer in aggregated mice that the norepinephrine-depleting effect was enhanced. Hence, it was concluded that the release of endogenous stores of norepinephrine plays a role in the enhanced toxicity of (+)-amphetamine in mice (309). Selectivity of aggregation toxicity was also studied for ephedrine isomers. Except for (+)-pseudoephedrine, all isomers of ephedrine exhibited this phenomenon, but in a magnitude which is much lower than that of (+)-amphetamine (310). The LD_{50} potency ratio (isolated/aggregated) for the most potent ephedrine isomer was only 1.5.

There has been considerable clinical interest in the anorexigenic response of the drugs affecting the CNS. A reliable eating response can be obtained on injection of small quantities of (−)-norepinephrine into the rostral hypothalamus of the rat. (+)-Norepinephrine induces a negligible response, indicating a stereoselective effect. Booth (311) postulated that α-adrenergic modulation of postsynaptic activity by norepinephrine from the nerve ending is involved in the hypothalamic control of feeding in the rat. The stereoselectivity in the feeding behavior for isomers of norepinephrine was also observed by Margules (312). Roszkowski and Kelley (313) developed a screening method for assessing drug inhibition of feeding behavior. (+)-Amphetamine is very effective in producing an inhibition of broth consumption in rats. (−)-Amphetamine was ineffective. Abdallah (314) found (−)-ephedrine to be the most potent of the ephedrine isomers in causing a reduction of food intake in mice. Several other reports indicate that the (+)-form of amphetamine is more of an appetite depressant than the (−)-isomer (315–317). Between the isomers there is crosstolerance to the anorexic effect (318). Thus, it has been difficult to separate the central stimulant effects from the anorexigenic effects of these agents.

Greater loss of sodium from tissue could cause a great loss of body water which in turn reduces the weight of an animal. Because isomers of amphetamine do not significantly differ from one another in causing sodium loss, it cannot be an important factor in the weight-reducing effect of (+)-amphetamine (319).

The potent CNS-stimulating effect of (+)-amphetamine can be demonstrated by increased oxygen consumption in morphine-pretreated dogs or in humans. In this respect, (−)-amphetamine is much less active than the (+)-form (320, 321). *p*-Chloro-substituted amphetamines show some promise as anorexic agents. (+)-*p*-Chloroamphetamine is less of a central stimulant but is a longer-acting anorexigenic agent than (+)-amphetamine (322). (−)-*p*-Chloroamphetamine is more active than (−)-amphetamine; both agents are less active than their respective (+)-forms. The anorexigenic effect of (+)-*p*-chloroamphetamine is unrelated to depletion of brain serotonin because both optical isomers are equally effective in causing depletion of brain serotonin (323). Phenmetrazine and phendimetrazine are known to be effective anorexigenic agents. There is an interesting stereochemical relationship between ephedrine and the phenmetrazine molecule (324, 325). Most observations to date tend to support the view that the anorexigenic effect of (+)-amphetamine may be of central origin.

CONCLUDING REMARKS

An attempt is made to survey a highly scattered and occasionally fragmentary literature on the optical isomers of adrenergic drugs. The facts from the old literature as well as those from the recently published manuscripts are blended in a proper perspective. At adrenergic synapse, stereoselectivity has been observed for: (*a*) all biosynthetic pathways; (*b*) transport in adrenergic neurone; (*c*) prevention of uptake at the neurone; (*d*) binding and retention in the granule; (*e*) enzyme monoamine oxidase; and (*f*) pharmacologic α- and β-adrenergic receptors. Previously, adrenergic blockers were used to classify the pharmacologic receptors. However, blockers don't have to react with the exact configuration of the receptor. In addition, physical–chemical properties of one blocker could differ from those of the other. So far as (−)- and (+)-isomers are concerned, their physical–chemical properties are identical. The evidence obtained from blockers could be regarded as an indirect one. Agonists have to interact with the exact configuration of the receptor to produce the pharmacologic effect. The isomeric activity ratio should serve as a better criterion to differentiate receptors. Even if the adrenergic receptor is isolated, its similarity to that in the tissue will not be proven until isomeric affinity ratios are obtained and compared with the isomeric activity ratio. Thus, optical isomers provide a valuable tool to analyze drug effects at basic levels.

REFERENCES

(1) "Steric Aspects of the Chemistry and Biochemistry of Natural Products," J. K. Grant and W. Klyne, Eds., Cambridge University Press, Cambridge, England, 1960.

(2) C. C. Pfeiffer and E. H. Jenney, *Sci. Ind. Res.*, **26,** 29(1967).
(3) R. P. Ahlquist, *Amer. J. Physiol.*, **153,** 586(1948).
(4) N. C. Moran, *Ann. N. Y. Acad. Sci.*, **139,** 541(1967).
(5) E. Muscholl, *Pharmacol. Rev.*, **18,** 551(1966).
(6) L. L. Iversen, "The Uptake and Storage of Noradrenaline in Sympathetic Nerves," Cambridge University Press, Cambridge, England, 1967.
(7) "Mechanisms of Release of Biogenic Amines," U. S. von Euler, S. Rosell, and B. Uvnäs, Eds., Pergamon, New York, N. Y., 1966.
(8) E. L. Eliel, "Stereochemistry of Carbon Compounds," McGraw-Hill, New York, N. Y., 1962, pp. 87–95.
(9) R. S. Cohn, *J. Chem. Ed.*, **41,** 116(1964).
(10) A. Lindner and C. Stumpf, *Sci. Pharm.*, **21,** 1(1953).
(11) E. Beccari, A. Beretta, and J. Lawendel, *Science*, **118,** 249(1953).
(12) A. M. Lands, F. P. Luduena, and B. F. Tullar, *J. Pharmacol. Exp. Ther.*, **111,** 469(1954).
(13) B. Korol, L. Soffer, and M. L. Brown, *Arch. Int. Pharmacodyn. Ther.*, **171,** 415(1968).
(14) P. A. Shore, *Pharmacol. Rev.*, **18,** 561(1966).
(15) P. N. Patil, A. Tye, and J. B. LaPidus, *J. Pharmacol. Exp. Ther.*, **156,** 445(1967).
(16) *Ibid.*, **148,** 158(1965).
(17) A. H. Abdallah, A. Tye, and P. N. Patil, *Arch. Int. Pharmacodyn. Ther.*, **174,** 454(1968).
(18) R. F. Furchgott, *Ann. N. Y. Acad. Sci.*, **139,** 553(1967).
(19) P. N. Patil, *J. Pharmacol. Exp. Ther.*, **160,** 308(1968).
(20) P. N. Patil, J. B. LaPidus, and A. Tye, *ibid.*, **155,** 1(1967).
(21) F. P. Luduena, *Arch. Int. Pharmacodyn. Ther.*, **137,** 155 (1962).
(22) R. Fielden and A. L. Green, *Brit. J. Pharmacol.*, **24,** 395 (1965).
(23) *Ibid.*, **24,** 408(1965).
(24) *Ibid.*, **26,** 264(1966).
(25) C. C. Porter, J. A. Totaro, A. Burcin, and E. R. Wynosky, *Biochem. Pharmacol.*, **15,** 583(1966).
(26) R. Fielden and A. L. Green, *Brit. J. Pharmacol.*, **32,** 350 (1968).
(27) "Second Symposium on Catecholamines," G. Acheson, Ed., Williams & Wilkins, Baltimore, Md., 1966.
(28) H. Blaschko, *J. Physiol. (London)*, **96,** 50P(1939).
(29) S. Udenfriend, in "Second Symposium on Catecholamines," G. Acheson, Ed., *Pharmacol. Rev.*, **18,** 43(1966).
(30) S. Udenfriend, in "The Harvey Lectures Series 60," Academic, New York, N. Y., 1966, p. 57.
(31) W. Lovenberg, H. Weissbach, and S. Udenfriend, *J. Biol. Chem.*, **237,** 89(1962).
(32) H. Blaschko, *J. Physiol. (London)*, **101,** 337 (1942).
(33) P. Holtz and K. Credner, *Hoppe-Seyler Z., Physiol. Chem.*, **280,** 39(1944).
(34) T. L. Sourkes, M. H. Wiseman-Distler, J. F. Moran, G. F. Murphy, and J. Saint Cyr, *J. Biochem.*, **93,** 469(1964).
(35) B. Belleau and J. Burba, *J. Amer. Chem. Soc.*, **82,** 5751 (1960).
(36) I. J. Kopin, *Ann. Rev. Pharmacol.*, **8,** 377(1968).
(37) S. Yamada, J. Terashima, and K. Achiwa, *Chem. Pharm. Bull.*, **13,** 227(1965).
(38) J. M. van Rossum, *Psychopharmacologia*, **4,** 271(1963).
(39) A. H. Beckett, G. Kirk, and A. J. Sharpen, *Tetrahedron*, **21,** 1489(1965).
(40) S. Kaufman and S. Friedman, *Pharmacol. Rev.*, **17,** 71 (1965).
(41) M. Goldstein, M. R. Mckereghan, and E. Lauber, *Biochem. Biophys. Acta*, **89,** 191(1964).
(42) B. Anagnoste, S. Sirlin, and M. Goldstein, *Fed. Proc.*, **24,** 515(1965).
(43) M. Goldstein, *Int. Congr. Pharmacol. 4th (Abstract)*, **1969,** 92.
(44) M. L. Torchiana, C. C. Porter, and C. A. Stone, *Arch. Int. Pharmacodyn. Ther.*, **174,** 118(1968).
(45) J. Axelrod, in "Second Symposium on Catecholamines," G. Acheson, Ed., *Pharmacol. Rev.*, **18,** 95(1966).
(46) I. J. Kopin and W. Bridgers, *Life Sci.*, **2,** 356(1963).
(47) R. P. Maickel, M. A. Beaven, and B. B. Brodie, *ibid.*, **2,** 953(1963).
(48) L. L. Iversen, *Brit. J. Pharmacol.*, **21,** 523(1963).
(49) *Ibid.*, **24,** 387(1965).
(50) G. V. R. Born, in "Adrenergic Neurotransmission," G. E. W. Wolstenholme and N. O'Connor, Eds., J. & A. Churchill Ltd., London, England, 1968, p. 56.
(51) G. V. R. Born, M. Day, and A. Stockbridge, *J. Physiol. (London)*, **193,** 405(1967).
(52) J. R. Crout, *Arch. Exp. Pathol. Pharmakol.*, **248,** 85(1964).
(53) T. C. Westfall, *Acta Physiol. Scand.*, **63,** 336(1965).
(54) N. E. Andén, *Acta Pharmacol. Toxicol.*, **21,** 59(1964).
(55) R. A. Mueller and F. E. Schideman, *Fed. Proc.*, **23,** 350 (1964).
(56) R. A. Mueller and F. E. Schideman, *J. Pharm. Pharmacol.*, **19,** 398(1967).
(57) R. D. Green, III, and J. W. Miller, *J. Pharmacol. Exp. Ther.*, **152,** 439(1966).
(58) S. M. Kirpekar and A. R. Wakade, *J. Physiol.*, **194,** 609 (1968).
(59) B. R. Mackenna, *Acta. Physiol. Scand.*, **63,** 413(1965).
(60) U. S. von Euler and F. Lishajko, *Int. J. Neuropharmacol.*, **4,** 273(1965).
(61) U. S. von Euler and F. Lishajko, *Acta. Physiol. Scand.*, **60,** 217(1964).
(62) L. Stjärne and U. S. von Euler, *J. Pharmacol. Exp. Ther.*, **150,** 335(1965).
(63) U. S. von Euler and F. Lishajko, *Acta Physiol. Scand.*, **71,** 151(1967).
(64) L. T. Potter and J. Axelrod, *J. Pharmacol. Exp. Ther.*, **142,** 299(1963).
(65) T. Malmfors, *Acta Physiol. Scand., Suppl. 248*, **64,** 1(1965).
(66) H. Corrodi, T. Malmfors, and C. H. Sachs, *ibid.*, **67,** 358 (1966).
(67) B. Hamberger, K. Norberg, and L. Olson, *ibid.*, **69,** 1(1967).
(68) L. L. Iversen, *Arch. Exp. Pathol. Pharmakol.*, **259,** 179 (1968).
(69) E. Muscholl and R. Lindmar, *ibid.*, **257,** 314(1967).
(70) B. Waldeck, *Eur. J. Pharmacol.*, **2,** 208(1967).
(71) P. N. Patil and D. Jacobowitz, *J. Pharmacol. Exp. Ther.*, **161,** 279(1968).
(72) A. Carlsson and B. Waldeck, *Acta Pharmacol. Toxicol.*, **26,** 501(1968).
(73) E. Muscholl and E. Sprenger, *Arch. Exp. Pathol. Pharmakol.*, **254,** 109(1966).
(74) R. Lindmar, E. Muscholl, and E. Sprenger, *ibid.*, **256,** 1(1967).
(75) P. A. Shore and H. S. Alpers, *Life Sci.*, **3,** 551(1964).
(76) P. A. Shore, D. Busfield, and H. S. Alpers, *J. Pharmacol. Exp. Ther.*, **146,** 194(1964).
(77) A. Giachetti and P. A. Shore, *Life Sci.*, **4,** 1455(1965).
(78) A. Giachetti and P. A. Shore, *Biochem. Pharmacol.*, **15,** 607(1966).
(79) F. Berti and P. A. Shore, *ibid.*, **16,** 2271(1967).
(80) A. Giachetti and P. A. Shore, *Pharmacologist*, **10,** 159(1968).
(81) P. Lundborg, *Acta. Physiol. Scand. Suppl. 302*, **64,** 1(1967).
(82) P. Lundborg and R. Stitzel, *Acta. Physiol. Scand.*, **72,** 392 (1968).
(83) P. Lundborg, *ibid.*, **67,** 423(1966).
(84) R. Häkanson, B. Lilja, and C. H. Owman, *Eur. J. Pharmacol.*, **1,** 188(1967).
(85) P. Alm, B. Ehinger, and B. Falck, *Acta Physiol. Scand.*, **76,** 106(1969).
(86) L. Cegrell, B. Falck, and B. Hellman, in "The Structure and Metabolism of Pancreatic Islets," S. E. Brolin, B. Hellman, and H. Knutson, Eds., Pergamon, New York, N. Y., 1964, p. 429.
(87) B. Larson, C. H. Owman, and F. Sundler, *Endocrinology*, **78,** 1109(1966).
(88) A. Bertler, B. Falck, C. H. Owman, and E. Posengren, *Pharmacol. Rev.*, **18,** 369(1966).
(89) J. W. Daly, C. R. Creveling, and B. Witkop, *J. Med. Chem.*, **9,** 273(1966).
(90) C. W. Nash, S. A. Wolfe, and B. A. Ferguson, *Can. J. Physiol. Pharmacol.*, **46,** 35(1968).
(91) A. Carlsson and B. Waldeck, *Acta Pharmacol. Toxicol.*, **24,** 255(1966).
(92) A. H. Abdallah, A. Tye, J. B. LaPidus, and P. N. Patil, *Life Sci.*, **6,** 39(1967).
(93) W. S. Saari, A. W. Raab, and E. L. Englehardt, *J. Med. Chem.*, **11,** 1115(1968).

(94) B. Waldeck, *Eur. J. Pharmacol.*, **5,** 114(1968).
(95) A. Carlsson, J.-J. Meisch, and B. Waldeck, *ibid.*, **5,** 85 (1968).
(96) E.-F. Drews, R. Lindmar, and E. Muscholl, *ibid.*, **3,** 167 (1968).
(97) A. S. V. Burgen and L. L. Iversen, *Brit. J. Pharmacol.*, **25,** 34(1965).
(98) E. Muscholl and E. Weber, *Arch. Exp. Pathol. Pharmakol.*, **252,** 134(1965).
(99) S. B. Ross and A. L. Renyi, *Acta Pharmacol. Toxicol.*, **21,** 226(1964).
(100) H. Thoenen, A. Hürliman, and W. Haefely, *J. Pharm. Pharmacol.*, **20,** 1(1968).
(101) S. B. Ross, A. L. Renyi, and B. Brunfelter, *ibid.*, **20,** 283(1968).
(102) M. L. Tainter, *J. Pharmacol. Exp. Ther.*, **40,** 43(1930).
(103) F. P. Luduena, E. Ananenko, O. H. Siegmund, and L. C. Miller, *ibid.*, **95,** 155(1949).
(104) I. G. Schaumann, *Med. Chem.*, **3,** 383(1936); through "The Chemistry of Organic Medicinal Products," 4th ed., Wiley, New York, N. Y., 1957, p. 468.
(105) M. L. Tainter and A. B. Stockton, *Amer. J. Med. Sci.*, **185,** 832(1933).
(106) C. M. Gruber and R. J. Matthews, Jr., *Arch. Exp. Pathol. Pharmakol.*, **219,** 1(1953).
(107) U. Trendelenburg, *J. Pharmacol. Exp. Ther.*, **148,** 329(1965).
(108) U. Trendelenburg, P. Draskóczy, and S. Pluchino, *ibid.*, **166,** 14(1969).
(109) A. Tye, P. N. Patil, and J. B. LaPidus, *ibid.*, **155,** 24(1967).
(110) P. R. Draskóczy and U. Trendelenburg, *ibid.*, **159,** 66(1968).
(111) S. Z. Langer and U. Trendelenburg, *ibid.*, **167,** 117(1969).
(112) R. J. Seidehamel, P. N. Patil, A. Tye, and J. B. LaPidus, *Life Sci.*, **5,** 2127(1966).
(113) F. R. West, B. Bhagat, N. S. Dhalla, and K. Shein, in "Abstracts of Third International Pharmacological Congress," Sao Paulo, Brazil, 1966. p. 121.
(114) F. Benvenuti, A. Bonaccorsi, and S. Garattini, *J. Pharm. Pharmacol.*, **19,** 477(1967).
(115) H. Obianwu, *Acta Pharmacol. Toxicol.*, **25,** 127(1967).
(116) S. Wong and J. P. Long, *J. Pharmacol. Exp. Ther.*, **156,** 469(1967).
(117) P. N. Patil, C. J. Ross, and A. Tye, unpublished data.
(118) U. Trendelenburg, *Pharmacol. Rev.*, **15,** 225(1963).
(119) S. Z. Langer, P. R. Draskóczy, and U. Trendelenburg, *J. Pharmacol. Exp. Ther.*, **157,** 255(1967).
(120) M. E. Drake, R. John, F. Renshaw, and C. H. Thienes, *Arch. Int. Pharmacodyn. Ther.*, **61,** 494(1939).
(121) S. Z. Langer, *J. Pharmacol. Exp. Ther.*, **154,** 14(1966).
(122) R. J. Seidehamel, P. N. Patil, A. Tye, and J. B. LaPidus, *ibid.*, **153,** 81(1966).
(123) R. D. Green and W. W. Fleming, *ibid.*, **162,** 254(1968).
(124) P. N. Patil, *J. Pharm. Pharmacol.*, **21,** 628(1969).
(125) U. Trendelenburg, *J. Pharmacol. Exp. Ther.*, **151,** 95(1966).
(126) V. C. Swamy, A. Tye, J. B. LaPidus, and P. N. Patil, *Life Sci.*, **6,** 903(1967).
(127) M. D. Day, *J. Pharm. Pharmacol.*, **17,** 619(1965).
(128) V. C. Swamy, A. Tye, and P. N. Patil, *Arch. Int. Pharmacodyn. Ther.*, **182,** 24(1969).
(129) R. Gottlieb, *Arch. Exp. Pathol. Pharmakol.*, **97,** 113(1923).
(130) G. Schmidt, B. Kalischer, and B. Wöckel, *ibid.*, **240,** 523(1961).
(131) J. B. LaPidus, C. J. Ross, D. Canowitz, A. Tye, and P. N. Patil, unpublished data.
(132) C. K. Buckner, P. N. Patil, A. Tye and L. Malspeis, *J. Pharmacol. Exp. Ther.*, **166,** 308(1969).
(133) A. Schafiee, and G. J. Hite, *Med. Chem.*, **12,** 266(1969).
(134) P. N. Patil, D. G. Patel, and A. Tye, *Arch. Int. Pharmacodyn. Ther.*, **182,** 32(1969).
(135) A. R. Cushny, "Biological Relations of Optically Isomeric Substances," Williams & Wilkins, Baltimore, Md., 1926.
(136) A. R. Cushny, *J. Physiol. (London)*, **37,** 130(1908).
(137) *Ibid.*, **38,** 259(1909).
(138) L. H. Easson and E. Stedman, *Biochem. J.*, **27,** 1257(1933).
(139) H. Blaschko, *Proc. Roy. Soc. (London) B*, **137,** 307(1950).
(140) A. H. Beckett, *Fortschr. Arzneimittelforsch.*, **1,** 455(1959).
(141) A. B. Wilson, *J. Physiol. (London)*, **136,** 18P(1957).
(142) G. M. Badger, *Nature (London)*, **159,** 194(1947).
(143) P. N. Patil, J. B. LaPidus, D. Campbell, and A. Tye, *J. Pharmacol. Exp. Ther.*, **155,** 13(1967).
(144) J. H. Burn and M. J. Rand, *J. Physiol. (London)*, **144,** 314(1958).
(145) P. N. Patil, A. Tye, and J. B. LaPidus, *J. Pharmacol. Exp. Ther.*, **149,** 199(1965).
(146) V. C. Swamy, A. Tye, and P. N. Patil, unpublished data.
(147) G. Paterson, *Biochem. Pharmacol. Suppl.*, **12,** 85(1963).
(148) A. W. Cuthbert, B. A. Challingham, S. Warren, and E. Painter, *J. Pharm. Pharmacol.*, **19,** 313(1967).
(149) J. M. van Rossum, *ibid.*, **17,** 202(1965).
(150) J. B. Farmer, *ibid.*, **18,** 261(1966).
(151) P. N. Patil and D. G. Patel, to be published.
(152) T. Brown and A. M. Lands, in "Evaluation of Drug Activities: Pharmacometrics," Academic, New York, N. Y., 1964, p. 353.
(153) F. P. Luduena and A. L. Snyder, *Int. J. Neuropharmacol.*, **3,** 83(1964).
(154) F. P. Luduena, L. von Euler, B. F. Tullar, and A. M. Lands, *Arch. Int. Pharmacodyn. Ther.*, **111,** 392(1957).
(155) J. R. Blinks, *Pharmacologist*, **6,** 176(1964).
(156) F. P. Luduena, in "Abstracts of the 152nd American Chemical Society Meeting," New York, N. Y., 1966.
(157) E. J. Ariens, "Molecular Pharmacology," Academic, New York, N. Y., 1964, p. 243.
(158) L. I. Goldberg, P. Francois, P. F. Sonneville, and J. L. McNay, *J. Pharmacol. Exp. Ther.*, **163,** 188(1968).
(159) H. Reuter and U. Wollert, *Arch. Exp. Pathol. Pharmakol.*, **258,** 288(1967).
(160) R. W. Colburn and J. W. Maas, *Nature*, **208,** 37(1965).
(161) J. W. Maas and R. W. Colburn, *ibid.*, **208,** 41(1965).
(162) G. Badger and H. H. Dale, *J. Physiol. (London)*, **41,** 19(1910).
(163) K. K. Chen, C. K. Wu, and E. Henriksen, *J. Pharmacol. Exp. Ther.*, **36,** 363(1929).
(164) E. E. Swanson, C. C. Scott, H. M. Lee, and K. K. Chen, *ibid.*, **79,** 329(1943).
(165) G. A. Alles and P. K. Knoefel, *Univ. Calif. Pub. Pharmacol.*, **1,** 101(1938).
(166) W. Osten, *Arzneim.-Forsch.*, **5,** 84(1955).
(167) D. F. Marsh and D. A. Herring, *J. Pharmacol. Exp. Ther.*, **97,** 68(1949).
(168) F. H. Schultz, *ibid.*, **70,** 283(1940).
(169) K. K. Chen, *Arch. Int. Med.*, **39,** 404(1927).
(170) P. R. Bromage, *Brit. Med. J.*, **2,** 72(1952).
(171) F. Hauschild, *Arch. Exp. Pathol. Pharmakol.*, **195,** 647 (1940).
(172) K. Schimamoto, S. Uchizumi, and O. Kanauchi, *Jap. J. Pharm. Chem.*, **27,** 460(1955).
(173) D. J. DeJongh, *Acta. Physiol. Pharmacol. Neer.*, **2,** 39(1951).
(174) D. W. Northup and E. J. van Liere, *J. Pharmacol. Exp. Ther.*, **109,** 358(1953).
(175) P. DeMeyts and A. Cession-Fossion, *Arch. Int. Pharmacodyn. Ther.*, **174,** 233(1968).
(176) E. A. Light, L. Wnuck, and E. J. deBeer, *Fed. Proc.*, **19,** 415(1959).
(177) J. B. LaPidus, A. Tye, and P. N. Patil, *J. Pharm. Sci.*, **56,** 1125(1967).
(178) K. K. Chen and C. F. Schmidt, "Ephedrine and Related Substances," Williams & Wilkins, Baltimore, Md., 1930.
(179) A. Tye, R. Baldesberger, J. B. LaPidus, and P. N. Patil, *J. Pharmacol. Exp. Ther.*, **157,** 356(1967).
(180) P. N. Patil and D. G. Patel, unpublished data.
(181) P. N. Patil, J. B. LaPidus, S. Molinari, and A. Tye, *J. Pharm. Sci.*, **56,** 286(1967).
(182) J. M. van Rossum and M. Mujic, *Arch. Int. Pharmacodyn. Ther.*, **155,** 418(1965).
(183) J. B. LaPidus, A. Tye, P. N. Patil, and B. A. Modi, *J. Med. Chem.*, **6,** 76(1963).
(184) M. Kier, *J. Pharmacol. Exp. Ther.*, **164,** 75(1968).
(185) J. Yelnosky and R. Katz, *ibid.*, **141,** 180(1963).
(186) F. Meyer, H.-J. Rimek, and F. Zymalkowski, *Pharmazie*, **20,** 333(1965).
(187) E. E. Smissman and G. S. Chappell, *J. Med. Chem.*, **12,** 429(1969).
(188) C. V. Winder, M. M. Anderson, and H. C. Parke, *J. Pharmacol. Exp. Ther.*, **93,** 63(1948).

(189) R. W. Gardier and A. B. Richards, *Fed. Proc.*, **20,** 111 (1961).
(190) P. N. Patil, D. Campbell, and A. Tye, unpublished data.
(191) H. Blaschko, in "Hypertension, Recent Advances," A. N. Brest and J. H. Moyer, Eds., London Kimpton, 1962, p. 321.
(192) G. Fawaz and J. Simaan, *Brit. J. Pharmacol.*, **24,** 526(1965).
(193) C. Hanna, *Arch. Int. Pharmacodyn. Ther.*, **129,** 191(1960).
(194) H. C. Harvey, T. S. Sulkowski, and D. J. Weeing, *ibid.*, **172,** 301(1968).
(195) O. Hornykiewicz and H. Obenaus, *ibid.*, **173,** 363(1968).
(196) H. P. Ciuchta and D. E. Mann, Jr., *J. Pharm. Sci.*, **50,** 648(1961).
(197) R. F. Orzechowski and D. E. Mann, Jr., *ibid.*, **52,** 337(1963).
(198) M. Nickerson, *Pharmacol. Rev.*, **9,** 246(1957).
(199) B. Belleau, *Can. J. Biochem. Physiol.*, **36,** 731(1958).
(200) B. Belleau and D. J. Triggle, *J. Med. Pharm. Chem.*, **5,** 636(1962).
(201) B. Belleau and P. Cooper, *J. Med. Chem.*, **6,** 579(1963).
(202) D. J. Triggle, in "Advances in Drug Research," vol. 2, Academic, New York, N. Y., 1965, p. 173.
(203) H. J. Smith and H. Williams, *J. Pharm. Pharmacol.*, **17,** 529(1965).
(204) N. B. Chapman and J. D. P. Graham, in "Drugs Affecting Peripheral Nervous System," Marcel Dekker, New York, N. Y., 1967, p. 473.
(205) M. S. K. Ghouri and T. J. Haley, *J. Pharm. Sci.*, **58,** 511(1969).
(206) R. Howe, *Biochem. Pharmacol.*, **12,** 85(1963).
(207) R. Howe and B. S. Rao, *J. Med. Chem.*, **11,** 1118(1968).
(208) R. Howe and R. G. Shanks, *Nature*, **210,** 1336(1966).
(209) L. S. Whitsitt and B. R. Lucchesi, *Life Sci.*, **6,** 939(1967).
(210) D. W. Pollen, A. C. Scott, and W. F. M. Wallace, *Cardiovasc. Res.*, **3,** 7(1969).
(211) A. J. Kaumann and J. R. Blinks, *Fed. Proc.*, **26,** 401(1967).
(212) R. Baltzly and N. B. Mehta, *J. Med. Chem.*, **11,** 833(1968).
(213) L. Almirante and W. Murmann, *ibid.*, **9,** 650(1966).
(214) P. Somani, R. T. Bachand, Jr., W. Murmann, and L. Almirante, *ibid.*, **9,** 823(1966).
(215) Y. Abiko, A. Minamidate, and M. Ito, *Arch. Int. Pharmacodyn. Ther.*, **179,** 130(1969).
(216) P. Somani, *Int. Congr. Pharmacol, 4th (Abstract)*, **1969,** 101.
(217) P. M. Lish, J. H. Weikel, and K. W. Dungan, *J. Pharmacol. Exp. Ther.*, **149,** 161(1965).
(218) C. Casagrande and G. Ferrari, *Farmaco, Ed. Sci.*, **21,** 229(1966).
(219) R. Ferrini, *Arzneim.-Forsch.*, **18,** 48(1968).
(220) R. Ferrini, G. Miragoli, and G. Croce, *ibid.*, **18,** 829(1968).
(221) R. Krell and P. N. Patil, unpublished data.
(222) B. Åblad, M. Brogard, and L. Ek, *Acta Pharmacol. Toxicol. Suppl. 2*, **25,** 9(1967).
(223) P. S. Portoghese, *J. Med. Chem.*, **10,** 1057(1967).
(224) B. Levy, *Arch. Int. Pharmacodyn. Ther.*, **170,** 418(1967).
(225) P. N. Patil, *J. Pharmacol. Exp. Ther.*, **166,** 229(1969).
(226) C. D. Marsden, T. H. Foley, D. A. L. Owen, and R. G. McCallister, *Clin. Sci. (London)*, **33,** 53(1967).
(227) J. V. Levy and V. Richards, *Proc. Soc. Exp. Biol. Med.*, **122,** 373(1966).
(228) J. V. Levy, *Arch. Int. Physiol. Biochem.*, **75,** 381(1967).
(229) J. V. Levy, *Eur. J. Pharmacol.*, **2,** 250(1968).
(230) J. V. Levy. *J. Pharm. Pharmacol.*, **20,** 813(1968).
(231) B. Scales and D. A. D. McIntosh, *J. Pharmacol. Exp. Ther.*, **160,** 261(1968).
(232) J. S. Serrano and H. S. Hardman, *Pharmacologist*, **10,** 299(1968).
(233) L. T. Potter, *J. Pharmacol. Exp. Ther.*, **155,** 91(1967).
(234) B. R. Duce, L. Garberg, and B. Johansson, *Acta Pharmacol. Toxicol., Suppl. 2*, **25,** 41(1967).
(235) B. R. Lucchesi, *J. Pharmacol. Exp. Ther.*, **148,** 94(1965).
(236) R. L. Katz, C. O. Lord, and K. E. Eakins, *ibid.*, **158,** 40(1967).
(237) C. O. Lord, R. L. Katz, and K. E. Eakins, *Anesthesiology*, **29,** 288(1968).
(238) B. R. Lucchesi, L. S. Whitsitt, and J. L. Stickney, *Ann. N. Y. Acad. Sci.*, **139,** 940(1967).
(239) A. M. Barrett and V. A. Cullum, *Brit. J. Pharmacol.*, **34,** 43(1968).
(240) A. M. Barrett, *ibid.*, **21,** 241(1969).
(241) P. Somani and D. L. Watson, *J. Pharmacol. Exp. Ther.*, **164,** 317(1968).
(242) F. G. Standaert, B. Levitt, J. Roberts, and A. Raines, *Eur. J. Pharmacol.*, **6,** 209(1969).
(243) A. N. Dohadwalla, A. S. Freedberg, and E. M. Vaugham-Williams, *Brit. J. Pharmacol.*, **36,** 257(1969).
(244) J. L. Stickney and B. R. Lucchesi, *Eur. J. Pharmacol.*, **6,** 1(1969).
(245) G. J. Kelliher and J. P. Buckley, *Fed. Proc.*, **28,** 482(1969).
(246) E. J. Ariens, *Arch. Pharmakol. Exp. Pathol.*, **257,** 118(1967).
(247) W. W. Parmley and E. Brunwald, *J. Pharmacol. Exp. Ther.*, **158,** 11(1967).
(248) L. S. Whitsitt and B. R. Lucchesi, *Circ. Res.*, **21,** 305(1967).
(249) R. G. Shanks, *Brit. J. Pharmacol.*, **29,** 204(1967).
(250) A. Arnold and J. P. McAuliff, *Biochem. Pharmacol.*, **17,** 475(1968).
(251) J. J. Burns, K. I. Colville, L. A. Lindsay, and R. A. Salvador, *J. Pharmacol. Exp. Ther.*, **144,** 163(1964).
(252) R. A. Salvador, K. I. Colville, S. A. April, and J. J. Burns, *ibid.*, **144,** 172(1964).
(253) W. R. Kukovetz and G. Pöch, *Arch. Exp. Pathol. Pharmakol.*, **256,** 310(1967).
(254) G. Fassina, *J. Pharm. Pharmacol.*, **18,** 399(1966).
(255) K. Stock and E. Westermann, *Life Sci.*, **5,** 1667(1966).
(256) D. O. Allen and J. Ashmore, *Biochem. Pharmacol.*, **18,** 1347(1969).
(257) P. N. Patil, A. Tye, C. May, S. Hetey, and S. Miyagi, *J. Pharmacol. Exp. Ther.*, **163,** 309(1968).
(258) U. S. von Euler, *Circ. Res. Suppl. III*, **20–21,** III-5(1967).
(259) U. S. von Euler and F. Lishakjo, *Acta Physiol. Scand.*, **74,** 501(1968).
(260) J. W. Foo, A. Jowett, and A. Stafford, *Brit. J. Pharmacol.*, **34,** 141(1968).
(261) Y. Cohen, W. Janiec, and J. Bralet, *Therapie*, **23,** 635(1968).
(262) O. D. Gulati, S. D. Gokhale, H. M. Parikh, B. P. Udwadia, and V. S. R. Krishnamurty, *J. Pharmacol. Exp. Ther.*, **166,** 35(1969).
(263) P. N. Patil and D. G. Patel, unpublished data.
(264) W. Murmann, *J. Pharm. Pharmacol.*, **21,** 478(1969).
(265) H. E. Dubin, H. B. Corbit, and L. Freedman, *J. Pharmacol. Exp. Ther.*, **26,** 233(1925).
(266) W. C. Bowman and C. Raper, *Brit. J. Pharmacol.*, **23,** 184(1964).
(267) R. C. Anderson and K. K. Chen, *J. Amer. Pharm. Ass.*, **23,** 290(1934).
(268) T. W. Rall, E. W. Sutherland, and J. Berthet, *J. Biol. Chem.*, **224,** 463(1957).
(269) E. W. Sutherland and G. A. Robison, in "Second Symposium on Catecholamines," *Pharmacol. Rev.*, **18,** 145(1966).
(270) F. Murad, Y. M. Chi, T. W. Rall, and R. W. Sutherland, *J. Biol. Chem.*, **237,** 1233(1962).
(271) B. Weiss and E. Costa, *J. Pharmacol. Exp. Ther.*, **169,** 310(1968).
(272) J. H. McNeill and T. M. Brody, *ibid.*, **165,** 97(1969).
(273) A. H. Beckett and M. Rowland, *J. Pharm. Pharmacol.*, **17,** 628(1965).
(274) L. G. Dring, R. L. Smith, and R. T. Williams, *ibid.*, **18,** 404(1966).
(275) R. W. Fuller and C. W. Hines, *J. Pharm. Sci.*, **56,** 302(1967).
(276) L.-M. Gunne, *Biochem. Pharmacol.*, **16,** 863(1967).
(277) E. Gordis, *ibid.*, **15,** 2124(1966).
(278) Y. Nagase, S. Baba, and A. Matsuda, *J. Pharm. Soc. Japan*, **87,** 123(1967).
(279) J. Axelrod, *J. Biol. Chem.*, **214,** 753(1955).
(280) J. V. Dingell and A. D. Bass, *Biochem. Pharmacol.*, **18,** 1535(1969).
(281) A. H. Beckett and M. Rowland, *J. Pharm. Pharmacol.*, **17,** Suppl. 109S(1965).
(282) R. Dann, M. S. thesis, Ohio State University, Columbus, Ohio, 1968.
(283) H. Blaschko, D. Richter, and H. Schlossmann, *Biochem. J.*, **31,** 2187(1937).
(284) E. Grana and L. Lilla, *Brit. J. Pharmacol.*, **14,** 501(1959).
(285) P. Pratesi and H. Blaschko, *ibid.*, **14,** 261(1959).
(286) H. Blaschko and B. C. Stromblad, *Arzneim.-Forsch.*, **10,** 327(1960).
(287) A. Giachetti and P. A. Shore, *Life Sci.*, **5,** 1373(1966).

(288) R. W. Fuller and C. P. Walters, *Biochem. Pharmacol.*, **16**, 159(1965).
(289) J. Axelrod and R. Tomchick, *J. Biol. Chem.*, **233**, 702(1958).
(290) O. Kraupp, H. Bernheimer, H. Ehringer, and P. Heistracher, *Arch. Exp. Pathol. Pharmakol.*, **238**, 38(1960).
(291) G. A. Alles, *Univ. Calif. Publ. Pharmacol.*, **1**, 129(1939).
(292) M. Prinzmetal and G. A. Alles, *Proc. Soc. Exp. Biol. Med.*, **42**, 206(1939).
(293) M. L. Tainter, L. J. Whitsell, and J. M. Dille, *J. Pharmacol. Exp. Ther.*, **67**, 56(1939).
(294) M. D. Fairchild and G. A. Alles, *ibid.*, **158**, 135(1967).
(295) J. W. Schulte, E. C. Reif, J. A. Bacher, Jr., W. S. Lawrence, and M. L. Tainter, *ibid.*, **71**, 62(1941).
(296) L. W. Roth, R. K. Richards, I. Shemano, and B. B. Morphis, *Arch. Int. Pharmacodyn. Ther.*, **98**, 362(1954).
(297) G. Lanciault and H. H. Wolf, *J. Pharm. Sci.*, **54**, 841(1965).
(298) M. L. Tainter, E. G. Tainter, W. S. Lawrence, E. N. Neuru, R. W. Lackey, F. P. Luduena, H. B. Kirtland, Jr., and R. I. Gonzalez, *J. Pharmacol. Exp. Ther.*, **79**, 42(1943).
(299) J. O. Hoppe, D. K. Seppeling, and A. M. Lands, *ibid.*, **95**, 502(1949).
(300) B. J. Boakes, P. B. Bradley, N. Brookes, and J. H. Wolstencroft, *Brit. J. Pharmacol.*, **32**, 417P(1968).
(301) W. G. Dewhurst and E. Marley, *ibid.*, **25**, 682(1965).
(302) *Ibid.*, **25**, 705(1965).
(303) W. Murmann, L. Almirante, and M. Saccani-Guelfi, *J. Pharm. Pharmacol.*, **18**, 318(1966).
(304) H. H. Wolf, D. E. Rollins, C. R. Rowland, and T. G. Reigle, *Int. J. Neuropharmacol.*, **8**, 319 (1969).
(305) J. R. C. Baird and J. J. Lewis, *Biochem. Pharmacol.*, **13**, 1475(1964).
(306) *Ibid.*, **12**, 577(1963).
(307) J. J. Lewis and D. Pollock, *Life Sci.*, **4**, 21(1965).
(308) M. Henning and P. A. vanZwieten, *J. Pharm. Pharmacol.*, **20**, 409(1968).
(309) K. E. Moore, *J. Pharmacol. Exp. Ther.*, **142**, 6(1963).
(310) H. H. Wolf, M. A. Schuster, and G. Lanciault, *J. Pharm. Sci.*, **53**, 987(1964).
(311) D. A. Booth, *J. Pharmacol. Exp. Ther.*, **160**, 336(1968).
(312) D. L. Margules, *Life Sci.*, **8**, 693(1969).
(313) A. P. Roszkowski and N. M. Kelley, *J. Pharmacol. Exp. Ther.*, **140**, 367(1963).
(314) A. H. Abdallah, *Life Sci.*, **7**, 665(1968).
(315) J. E. Owen, Jr., *J. Exp. Anal. Behav.*, **3**, 293(1968).
(316) J. E. Owen, Jr., *J. Pharm. Sci.*, **52**, 679(1963).
(317) *Ibid.*, **52**, 684(1963).
(318) A. Tagliamonte, P. Tagliamonte, and G. L. Gessa, *Pharmacologist*, **11**, 264(1969).
(319) L. A. Sapirstein, R. Andrews, A. Pultz, and A. Ridolfo, *Proc. Soc. Exp. Biol. Med.*, **82**, 609(1953).
(320) J. L. Morrison and B. E. Abreu, *Fed. Proc.*, **2**, 88(1943).
(321) G. A. Emerson, *ibid.*, **3**, 71(1944).
(322) C. Kaergaard-Nielsen, M. P. Magnussen, E. Kampmann, and H. H. Frey, *Arch. Int. Pharmacodyn. Ther.*, **170**, 428(1967).
(323) R. W. Fuller, C. W. Hines, and J. Mills, *Biochem. Pharmacol.*, **14**, 483(1965).
(324) H. Hoffman, *Arch. Int. Pharmacodyn. Ther.*, **160**, 180(1966).
(325) D. Dvornik and G. Schilling, *J. Med. Chem.*, **8**, 466(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *College of Pharmacy, Ohio State University, Columbus, OH 43210*

Published and unpublished work of the authors of this review cited herein is supported by Grants HE-12215-01, NB-08956-01, GM-17859-04, HE-11556-03, and EY-00500-01, U. S. Public Health Service.

The authors thank Gerald A. Willey of the Research Foundation of Ohio State University for editorial services.

RESEARCH ARTICLES

Interaction of Isoniazid with Magnesium Oxide and Lactose

WEN-HUNG WU, TING-FONG CHIN, and JOHN L. LACH

Abstract □ Interactions of isoniazid with magnesium oxide and with lactose were investigated in the solid state. In the isoniazid–magnesium oxide system, chemisorption as well as physical adsorption of isoniazid molecules onto the magnesium oxide surface was confirmed by diffuse reflectance spectroscopic data. An absorption maximum due to chemisorbed isoniazid molecules was found to occur at 325 mμ, whereas physical adsorption was detected at 268 mμ. The mechanism of surface chemisorption is different from that of the formation of the isoniazid–metal-ion complex in solution. The browning reaction of the isoniazid–lactose system in solid state was also studied using diffuse reflectance spectroscopy. The rates of browning at 95, 100, 105, and 110° were followed by measuring the reflectance at 450 mμ. From the data obtained, the approximate time needed for browning to be perceptible has been predicted. TLC separations of the reaction products confirmed the presence of isonicotinoyl hydrazones of lactose and hydroxymethylfurfural.

Keyphrases □ Isoniazid interaction—magnesium oxide, lactose □ Lactose–isoniazid systems—browning rates □ Magnesium oxide, chemisorption—isoniazid □ Diffuse reflectance spectroscopy—analysis □ IR spectrophotometry—identity

Difficulties in formulating a new pharmaceutical dosage form have often been experienced because of the interactions between the supposed inert adjuvants and the active ingredient itself. Although the nature and intensity of these interactions vary, such interactions may alter the stability, dissolution rate, and, consequently, the absorption of the drug. A literature survey indicates that such interactions involving the formation of complexes have been studied extensively in aqueous solution; relatively few studies have been carried out in the solid state.

Stearic acid and calcium stearate have been shown by Kornblum and Zoglio (1) to catalyze the degradation of aspirin. Ribeiro *et al.* (2) studied the influence of lubri-

(288) R. W. Fuller and C. P. Walters, *Biochem. Pharmacol.*, **16**, 159(1965).
(289) J. Axelrod and R. Tomchick, *J. Biol. Chem.*, **233**, 702(1958).
(290) O. Kraupp, H. Bernheimer, H. Ehringer, and P. Heistracher, *Arch. Exp. Pathol. Pharmakol.*, **238**, 38(1960).
(291) G. A. Alles, *Univ. Calif. Publ. Pharmacol.*, **1**, 129(1939).
(292) M. Prinzmetal and G. A. Alles, *Proc. Soc. Exp. Biol. Med.*, **42**, 206(1939).
(293) M. L. Tainter, L. J. Whitsell, and J. M. Dille, *J. Pharmacol. Exp. Ther.*, **67**, 56(1939).
(294) M. D. Fairchild and G. A. Alles, *ibid.*, **158**, 135(1967).
(295) J. W. Schulte, E. C. Reif, J. A. Bacher, Jr., W. S. Lawrence, and M. L. Tainter, *ibid.*, **71**, 62(1941).
(296) L. W. Roth, R. K. Richards, I. Shemano, and B. B. Morphis, *Arch. Int. Pharmacodyn. Ther.*, **98**, 362(1954).
(297) G. Lanciault and H. H. Wolf, *J. Pharm. Sci.*, **54**, 841(1965).
(298) M. L. Tainter, E. G. Tainter, W. S. Lawrence, E. N. Neuru, R. W. Lackey, F. P. Luduena, H. B. Kirtland, Jr., and R. I. Gonzalez, *J. Pharmacol. Exp. Ther.*, **79**, 42(1943).
(299) J. O. Hoppe, D. K. Seppeling, and A. M. Lands, *ibid.*, **95**, 502(1949).
(300) B. J. Boakes, P. B. Bradley, N. Brookes, and J. H. Wolstencroft, *Brit. J. Pharmacol.*, **32**, 417P(1968).
(301) W. G. Dewhurst and E. Marley, *ibid.*, **25**, 682(1965).
(302) *Ibid.*, **25**, 705(1965).
(303) W. Murmann, L. Almirante, and M. Saccani-Guelfi, *J. Pharm. Pharmacol.*, **18**, 318(1966).
(304) H. H. Wolf, D. E. Rollins, C. R. Rowland, and T. G. Reigle, *Int. J. Neuropharmacol.*, **8**, 319 (1969).
(305) J. R. C. Baird and J. J. Lewis, *Biochem. Pharmacol.*, **13**, 1475(1964).
(306) *Ibid.*, **12**, 577(1963).
(307) J. J. Lewis and D. Pollock, *Life Sci.*, **4**, 21(1965).
(308) M. Henning and P. A. vanZwieten, *J. Pharm. Pharmacol.*, **20**, 409(1968).
(309) K. E. Moore, *J. Pharmacol. Exp. Ther.*, **142**, 6(1963).
(310) H. H. Wolf, M. A. Schuster, and G. Lanciault, *J. Pharm. Sci.*, **53**, 987(1964).
(311) D. A. Booth, *J. Pharmacol. Exp. Ther.*, **160**, 336(1968).
(312) D. L. Margules, *Life Sci.*, **8**, 693(1969).
(313) A. P. Roszkowski and N. M. Kelley, *J. Pharmacol. Exp. Ther.*, **140**, 367(1963).
(314) A. H. Abdallah, *Life Sci.*, **7**, 665(1968).
(315) J. E. Owen, Jr., *J. Exp. Anal. Behav.*, **3**, 293(1968).
(316) J. E. Owen, Jr., *J. Pharm. Sci.*, **52**, 679(1963).
(317) *Ibid.*, **52**, 684(1963).
(318) A. Tagliamonte, P. Tagliamonte, and G. L. Gessa, *Pharmacologist*, **11**, 264(1969).
(319) L. A. Sapirstein, R. Andrews, A. Pultz, and A. Ridolfo, *Proc. Soc. Exp. Biol. Med.*, **82**, 609(1953).
(320) J. L. Morrison and B. E. Abreu, *Fed. Proc.*, **2**, 88(1943).
(321) G. A. Emerson, *ibid.*, **3**, 71(1944).
(322) C. Kaergaard-Nielsen, M. P. Magnussen, E. Kampmann, and H. H. Frey, *Arch. Int. Pharmacodyn. Ther.*, **170**, 428(1967).
(323) R. W. Fuller, C. W. Hines, and J. Mills, *Biochem. Pharmacol.*, **14**, 483(1965).
(324) H. Hoffman, *Arch. Int. Pharmacodyn. Ther.*, **160**, 180(1966).
(325) D. Dvornik and G. Schilling, *J. Med. Chem.*, **8**, 466(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *College of Pharmacy, Ohio State University, Columbus, OH 43210*

Published and unpublished work of the authors of this review cited herein is supported by Grants HE-12215-01, NB-08956-01, GM-17859-04, HE-11556-03, and EY-00500-01, U. S. Public Health Service.

The authors thank Gerald A. Willey of the Research Foundation of Ohio State University for editorial services.

RESEARCH ARTICLES

Interaction of Isoniazid with Magnesium Oxide and Lactose

WEN-HUNG WU, TING-FONG CHIN, and JOHN L. LACH

Abstract □ Interactions of isoniazid with magnesium oxide and with lactose were investigated in the solid state. In the isoniazid–magnesium oxide system, chemisorption as well as physical adsorption of isoniazid molecules onto the magnesium oxide surface was confirmed by diffuse reflectance spectroscopic data. An absorption maximum due to chemisorbed isoniazid molecules was found to occur at 325 mμ, whereas physical adsorption was detected at 268 mμ. The mechanism of surface chemisorption is different from that of the formation of the isoniazid–metal-ion complex in solution. The browning reaction of the isoniazid–lactose system in solid state was also studied using diffuse reflectance spectroscopy. The rates of browning at 95, 100, 105, and 110° were followed by measuring the reflectance at 450 mμ. From the data obtained, the approximate time needed for browning to be perceptible has been predicted. TLC separations of the reaction products confirmed the presence of isonicotinoyl hydrazones of lactose and hydroxymethylfurfural.

Keyphrases □ Isoniazid interaction—magnesium oxide, lactose □ Lactose–isoniazid systems—browning rates □ Magnesium oxide, chemisorption—isoniazid □ Diffuse reflectance spectroscopy—analysis □ IR spectrophotometry—identity

Difficulties in formulating a new pharmaceutical dosage form have often been experienced because of the interactions between the supposed inert adjuvants and the active ingredient itself. Although the nature and intensity of these interactions vary, such interactions may alter the stability, dissolution rate, and, consequently, the absorption of the drug. A literature survey indicates that such interactions involving the formation of complexes have been studied extensively in aqueous solution; relatively few studies have been carried out in the solid state.

Stearic acid and calcium stearate have been shown by Kornblum and Zoglio (1) to catalyze the degradation of aspirin. Ribeiro *et al.* (2) studied the influence of lubri-

cant type and concentration of the lubricant in the decomposition of aspirin in APC tablets. In most cases, an increase in lubricant concentration caused a corresponding increase in decomposition. The most striking effect was observed with calcium stearate, stearic acid, and magnesium stearate. It is conceivable that the metallic salts of these weak organic acids catalyze aspirin degradation: however, the catalytic effect brought about by stearic acid alone suggests that some other mechanism(s) may also be operative. The influence of antacid compounds and tablet diluents on the stability of aspirin has also been investigated by Bandelin and Malesh (3). The authors reported that there is no relationship between the solubility of the adjuvants and the decomposition of aspirin. For example, a highly insoluble magnesium trisilicate produced a high rate of decomposition, while calcium gluconate, a very soluble salt, was less reactive.

Adsorption of antibiotics on antacid materials has been studied by several authors (4, 5). Blaug and Gross (6) showed that the adsorption of some anticholinergic drugs on magnesium trisilicate was so strong that complete desorption was difficult. Chulski and Forist (7), studying the effects of some solid buffering agents on prednisolone, found that the steroid was adsorbed by magnesium trisilicate and that magnesium oxide produced a first-order degradation of the steroid.

Although these studies dealing with drug–adjuvant interactions covered different types of drugs and adjuvants, the nature of the interactions involved and their reaction mechanisms were not fully discussed. This is probably attributable to the fact that reactions in the solid state are usually complex, complicated by numerous parallel and consecutive reactions. However, more important has been the lack of available effective and dependable instrumentation for the study of such interactions in the solid state.

Guillory *et al.* (8) reported on thermal methods using differential thermal analysis (DTA) for the detection of interactions occurring between solid components of pharmaceuticals. Drug–adjuvant interactions in the solid state have been investigated by Lach and Bornstein (9–11), Bighley (12), and McCallister (13), using diffuse reflectance spectroscopy (DRS). The authors covered a wide range of active medicinal agents including antibiotics, analgesics, and anticoagulants. The adjuvants investigated included both metallic and nonmetallic compounds. The degree of strength of the interactions between drugs and adjuvants was interpreted from the spectral shifts observed.

Since the clinical response of any solid dosage form depends not only on the accurate labeled amount of medicament present but also on its actual availability to the patient once administered, a study of these solid-state interactions is of prime importance in an understanding of the formulation of therapeutically effective dosage forms. The purpose of this investigation was to study drug–adjuvant interactions, since drug availability might be significantly altered as a result of these solid–solid interactions. Since isoniazid (INH) does form chelates in solution with various metallic ions (14–16), it is highly probable that similar type interactions would occur on the surface of metallic-containing adjuvants commonly employed in the preparation of solid dosage forms. It is also probable that INH would undergo other types of surface interactions. An investigation dealing with these aspects was therefore undertaken.

EXPERIMENTAL

Reagents—The following were used: isoniazid,[1] recrystallized from 95% ethanol, m.p. 171–172°; MgO USP,[2] heavy, average size 30 μ; KBr,[3] spectroscopic grade; lactose monohydrate,[2] A.R., m.p. 201–202°; silica gel G[4] for TLC; deuterium oxide[4] for spectroscopy, 99.75%; 5-hydroxymethylfurfural,[5] recrystallized from a mixed solvent consisting of equal volumes of ether and petroleum ether, m.p. 31–32°; methanol,[2] A.R.; and methylene chloride,[6] A.R.

Apparatus—All diffuse reflectances were measured using a spectrophotometer[7] equipped with reflectance attachment. The cell consists of a square aluminum block, an aluminum back cover, and two metal bolts. A piece of circular quartz,[8] 3.17 cm. (1.25 in.) in diameter and 0.16 cm. (0.064 in.) in thickness, is fitted into the front side of the block. The detailed dimensions are shown in Fig. 1. The powder compartment is shown as the shaded area. The other apparatus used were: recording spectrophotometer;[9] IR spectrophotometer;[10] vacuum oven;[11] dry oven,[12] analytical, low gradient; heater and circulator,[12] equipped with microset thermoregulator;[13] submersion rotator;[14] and TLC apparatus.[15]

General Procedure for Sample Equilibration—Specified quantities of a drug and an adjuvant were accurately weighed on an analytical balance and transferred into a suitable size amber-colored bottle, which held enough void volume for sample mixing after the powders and solvent (10 ml. solvent/1 g. adjuvant) were added. After the cap was replaced, the bottle was fixed on the rotator and tumbled for 24 hr. at room temperature (25°). The content was poured into a mortar and dried at 25° in a vacuum oven. After the bulk of the solvent was driven off, the powder was thoroughly triturated and then dried again at 35° under vacuum. Finally, the sample was cooled in a vacuum desiccator over calcium sulfate.

Reflectance Measurement—The prepared sample was packed into a cell. Since the process of packing has been reported (17) to cause fluctuation in reflectance reading, it is essential to maintain the same condition for every sample packed. In all cases, pure adjuvant itself was used as a white standard (18).

Preparation of Deuterated INH—One gram of INH was dissolved in 10 ml. of D_2O, and the clear solution was transferred to an ampul which was sealed after the air was replaced by nitrogen. The ampul was allowed to stand in the dark for 24 hr., and the D_2O was evaporated under vacuum until completely dry.

Browning of INH–Lactose Solid System—According to the previously stated procedure, a sample containing 15 mg. INH/1 g. lactose was equilibrated in CH_2Cl_2. For purposes of comparison, lactose containing no INH was treated in the same manner. After the reflectance spectra were taken, both samples were heated at 100 ±0.5° in a dry oven. At various time intervals, the samples were transferred to a desiccator containing calcium sulfate and allowed to cool for 15 min.; the reflectance spectra were then taken. The same experiment was carried out also at 95, 105, and 110°.

Browning of INH–Lactose Mixture in Solution—Three solutions were prepared by dissolving: (*a*) 150 mg. INH, (*b*) 150 mg. INH and 10 g. lactose, and (*c*) 10 g. lactose, respectively, in small amount of water and made to 100-ml. volume. Five milliliters each of the solutions was filled into 10-ml. ampuls and sealed. These ampuls were heated at 100 ± 0.1° in an oil bath; at various time intervals, one ampul containing the respective solution was removed. The reaction was quenched by quick cooling, 1 ml. each of the content was diluted

[1] Conray Products Co., New York, N. Y.
[2] Mallinckrodt Chemical Works, St. Louis, Mo.
[3] Matheson Coleman & Bell.
[4] E. Merck (Germany).
[5] Aldrich Chemical Co., Milwaukee, Wis.
[6] J. T. Baker, Phillipsburg, N. J.
[7] Beckman model DB-G.
[8] Suprasil, Amersil Inc.
[9] Beckman model DK-2.
[10] Beckman IR-10.
[11] Precision Scientific, model 524.
[12] E. H. Sargent & Co.
[13] Precision Scientific.
[14] Scientific Industries, Inc., model SR-250-V.
[15] Desaga/Brinkmann.

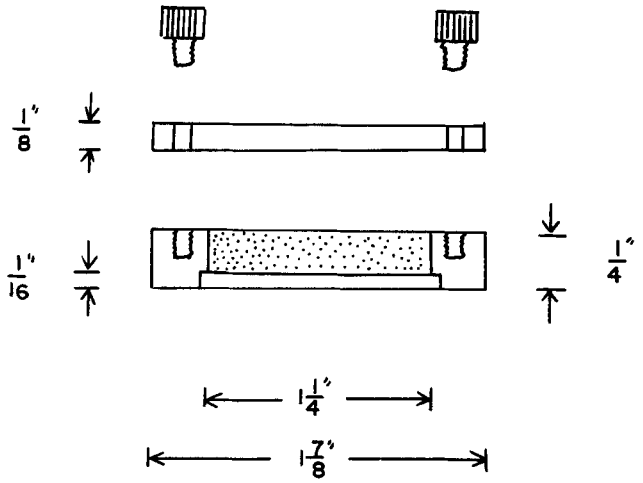


Figure 1—*Cell diagram for diffuse reflectance measurement.*

to 100 ml., and the transmittance spectra were taken from 220–340 mμ.

Preparation of Isonicotinoyl Hydrazone of Hydroxymethylfurfural (INH–HMF)—One gram HMF was dissolved in 8 ml. of methanol and diluted with an equal volume of water. To this solution was added 1 g. of INH and diluted with 5 ml. of water. The clear solution was heated to boiling and then chilled in a refrigerator. The precipitate was filtered and recrystallized from 45 ml. of 50% ethanol, using charcoal as a decolorizing agent. The light-yellow needles obtained were again recrystallized and finally dried at 30° under vacuum, m.p. 216–218° (corrected). $\lambda_{max.}^{H_2O}$ = 320 mμ, $a_{320}^{H_2O}$ = 2697.0. Elemental analysis showed the following:

	C	H	N
Theoretical	58.77	4.52	17.14
Determined	58.86	4.63	17.09

TLC—Silica gel G plates (0.25 mm.) were developed by a solvent mixture made by diluting 60 ml. methanol with $CHCl_3$ to 125 ml. Air-dried plates were then examined under UV light and also exposed to iodine vapor.

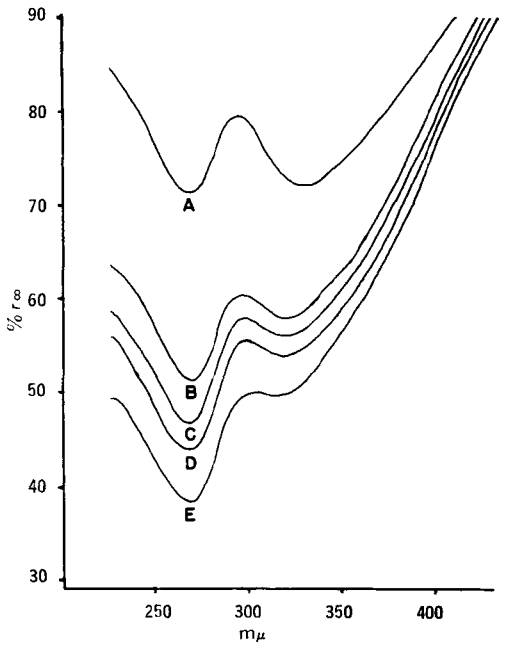


Figure 2—*DRS showing the effects of varying isoniazid concentrations on equilibrated samples using 1.00 g. of magnesium oxide as the adsorbent. Key: A, 3 mg.; B, 7 mg.; C, 10 mg.; D, 13 mg.; and E, 16 mg.*

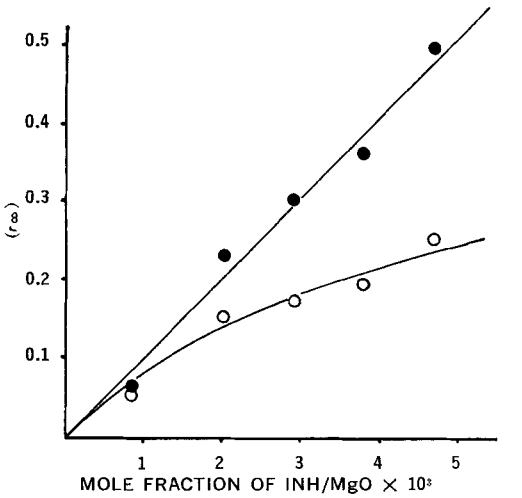


Figure 3—*Relationship between reflectance and concentration of isoniazid. Key: ●, at 268 mμ; and ○, at 325 mμ.*

RESULTS AND DISCUSSION

INH–MgO System—DRS of various samples, containing 3, 7, 10, 13, and 16 mg. INH/1 g. MgO and equilibrated in methanol according to the general procedure previously described, are shown in Fig. 2. In each spectrum, two absorption maxima were observed, one at 268 mμ and the other at 325 mμ. However, the transmittance spectrum of INH in aqueous solution showed only one absorption maximum at 262 mμ. The influence of solvent polarity on this maximum was small, since $\lambda_{max.}$ in $CHCl_3$ was also found at the same wavelength. It was also observed (Fig. 2) that the intensity increase of the maximum at 268 mμ as a function of drug concentration was much greater than that for the maximum at 325 mμ.

These intensity relationships (Table I) are represented by the observed reflectances and expressed as a remission function, which is also referred to as the Kubelka-Munk equation (19):

$$f(r\infty) = \frac{(1 - r\infty)^2}{2r_\infty}$$

where $r\infty$ is a measured reflectance. As seen in the last column of Table I, a comparison of the ratio $f(r\infty)_{268}/(r\infty)_{325}$ indicates that this

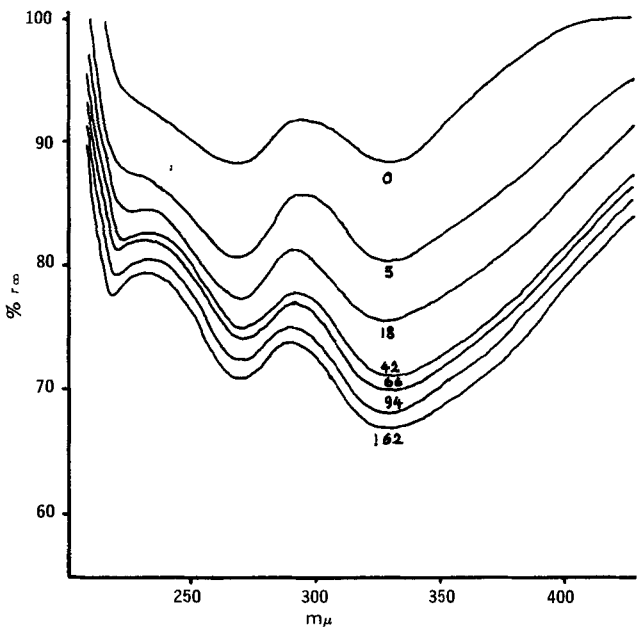


Figure 4—*DRS of a sample prepared by triturating 15 mg. isoniazid with 1 g. magnesium oxide, showing the effects of time on reflectance intensity change. (The numbers shown are time in hours.)*

Table I—Relationship between Concentration of Isoniazid and Reflectances at Two Different Wavelengths

Concentration		Reflectance				
mg. INH/g. MgO	Mole Fraction	$r_{\infty 268}$, %	$f(r_\infty)_{268}$	$r_{\infty 325}$, %	$f(r_\infty)_{325}$	$f(r_\infty)_{268}/f(r_\infty)_{325}$
3	8.82×10^{-4}	71.3	0.05776	72.0	0.05444	1.06
7	2.03×10^{-3}	51.2	9.2326	58.0	0.1521	1.53
10	2.94×10^{-3}	46.8	0.3024	56.0	0.1729	1.75
13	3.82×10^{-3}	43.7	0.3627	54.0	0.1959	1.85
16	4.71×10^{-3}	38.3	0.4970	49.8	0.2530	1.97

concentration effect was considerably more significant for the 268 mμ maximum thanfor the other at 325 mμ. If one assumes that the appearance of these two maxima is due solely to the INH molecules adsorbed onto the MgO surface and that the forces involved in this interaction for these adsorbed molecules are comparable, then this ratio for these two maxima should not change. Theoretically, the ratio of absorbances as well as remission functions at two different wavelengths should be equal to the ratio of the molar absorptivity at the respective wavelengths and should be a constant. Since this is not the case here, it is more likely that the maxima at 268 and 325 mμ are the results of INH molecules being adsorbed by several different mechanisms.

Figure 3 shows a plot of remission function, $f(r_\infty)$, *versus* concentration expressed in mole fraction, *i.e.*, mole INH/mole MgO. Although fluctuation of data was rather significant, it was possible to observe that an approximately linear relationship did exist between the remission function and concentration at 268 mμ, whereas at 325 mμ the remission function approached a plateau. Since in physical adsorption, multiple-layer adsorption may occur, this intensity increase for the 268-mμ maximum with an increase in concentration may be assigned to physical adsorption of INH molecules. This assignment might well be supported by the bathochromic effect observed, which has also been reported by several workers (9, 20) in such adsorption. The second maximum at 325 mμ may be due to chemisorbed INH molecules. Since chemisorption is known to occur only to the extent of formation of a monolayer on the adsorbent surface, the adsorbed molecules in excess of this amount (multilayer) no longer contribute to the intensity of a maximum due to chemisorption, as is seen in the maximum at 325 mμ which gradually shows a plateau at higher concentration and a decrease in the clarity of the peak.

From the molar absorptivity of INH, $a_{262}^{H_2O} = 518.0$, the maximum at 262 mμ can be assigned to a $\pi \rightarrow \pi^*$ transition of the conjugated system in the INH molecule. There is then a possibility of assigning the weaker maximum at 325 mμ in the reflectance spectrum to the $n \rightarrow \pi^*$ transition of the carbonyl group. This is not possible, however, since the intensity of the $n \rightarrow \pi^*$ transition can never exceed that of the $\pi \rightarrow \pi^*$, so the 325-mμ maximum should be always weaker than the 268-mμ maximum. However, this was not observed when the experimental conditions were altered.

To study further the nature of these maxima, 15 mg. of INH/1 g. of MgO was triturated in a mortar for 10 min. and packed into a cell; the reflectance spectrum was then taken at different time intervals. The results are shown in Fig. 4. As is evident from this figure and contrary to Fig. 2, the intensity ratios, $f(r_\infty)_{268}/f(r_\infty)_{325}$, determined within the time interval studied were smaller than 1; as time increased, these ratios gradually decreased and then slowly increased. These relationships between ratios and time calculated from Fig. 4 are shown in Fig. 5, and they may be explained in the following manner. Immediately after the trituration, both chemisorption and physical adsorption have occurred. However, the extent of adsorption in both cases is far from the equilibrium state, as may be observed by a comparison of Fig. 2 and Fig. 4. Consequently, at this stage, except for some of the INH molecules that have been removed from the surface of the INH crystals and chemisorbed onto MgO surface, the rest of the INH molecules remain intact in the crystalline form. However, as the time interval lengthens, some INH molecules become separated from the INH crystalline surface due to particle collision or surface diffusion, and they undergo chemisorption with the large unsaturated MgO surface. This is reflected in the increase in the intensity of the 325-mμ maximum in the diffuse reflectance spectrum. Statistically speaking, it is probable that some of these free INH molecules may also be adsorbed physically to other already chemisorbed molecules, so that the intensity of the maximum at 268 mμ increases but at a much slower rate. This is indicated in Fig. 4, which shows this ratio decrease during the first 66 hr. However, beyond this period the ratio begins to increase. This is probably due to the fact that the unsaturated MgO surface sites are, for the most part, satisfied by the chemisorbed layer of INH molecules and that additional molecules liberated from the INH crystal surface are now physically adsorbed to this layer. If the system just discussed is allowed to attain equilibrium by storage for a sufficient period of time, the reflectance values would be comparable to that of the sample equilibrated in a solvent. Zeitlin *et al.* (18) have confirmed this aspect in their study.

The assignment of the two maxima was further supported by a study that involved equilibration and solvent elution of the drug from the adjuvant surface. Each sample was prepared by equilibrating 5 g. of MgO with 200 mg. of INH dissolved in 50 ml. of methanol and CH_2Cl_2, respectively. The sample was then filtered with gentle

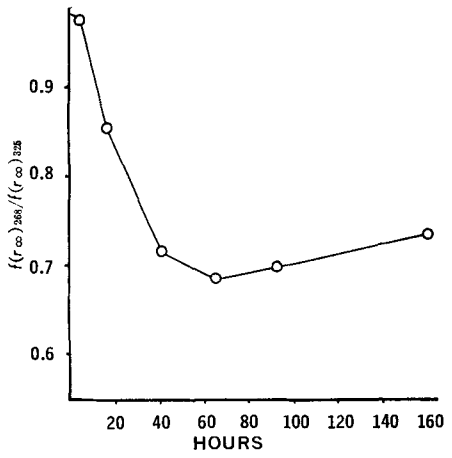


Figure 5—*Relationship between the ratio of two reflectances at different wavelengths and time of a sample prepared by triturating 15 mg. isoniazid with 1 g. magnesium oxide.*

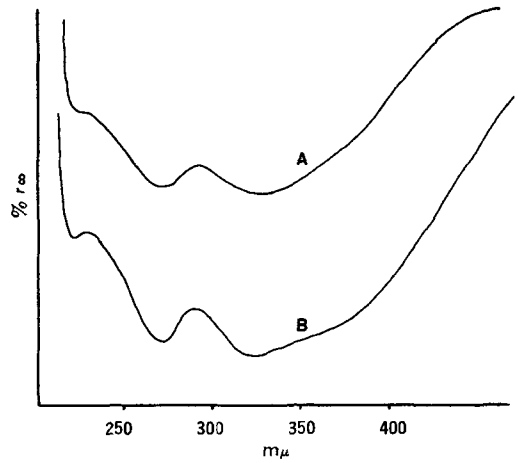


Figure 6—*DRS showing the effects of washing equilibrated dried samples containing 200 mg. isoniazid and 5 g. magnesium oxide. Key: A, equilibrated in methanol and washed with the same solvent; and B, equilibrated in methylene chloride and washed with the same solvent.*

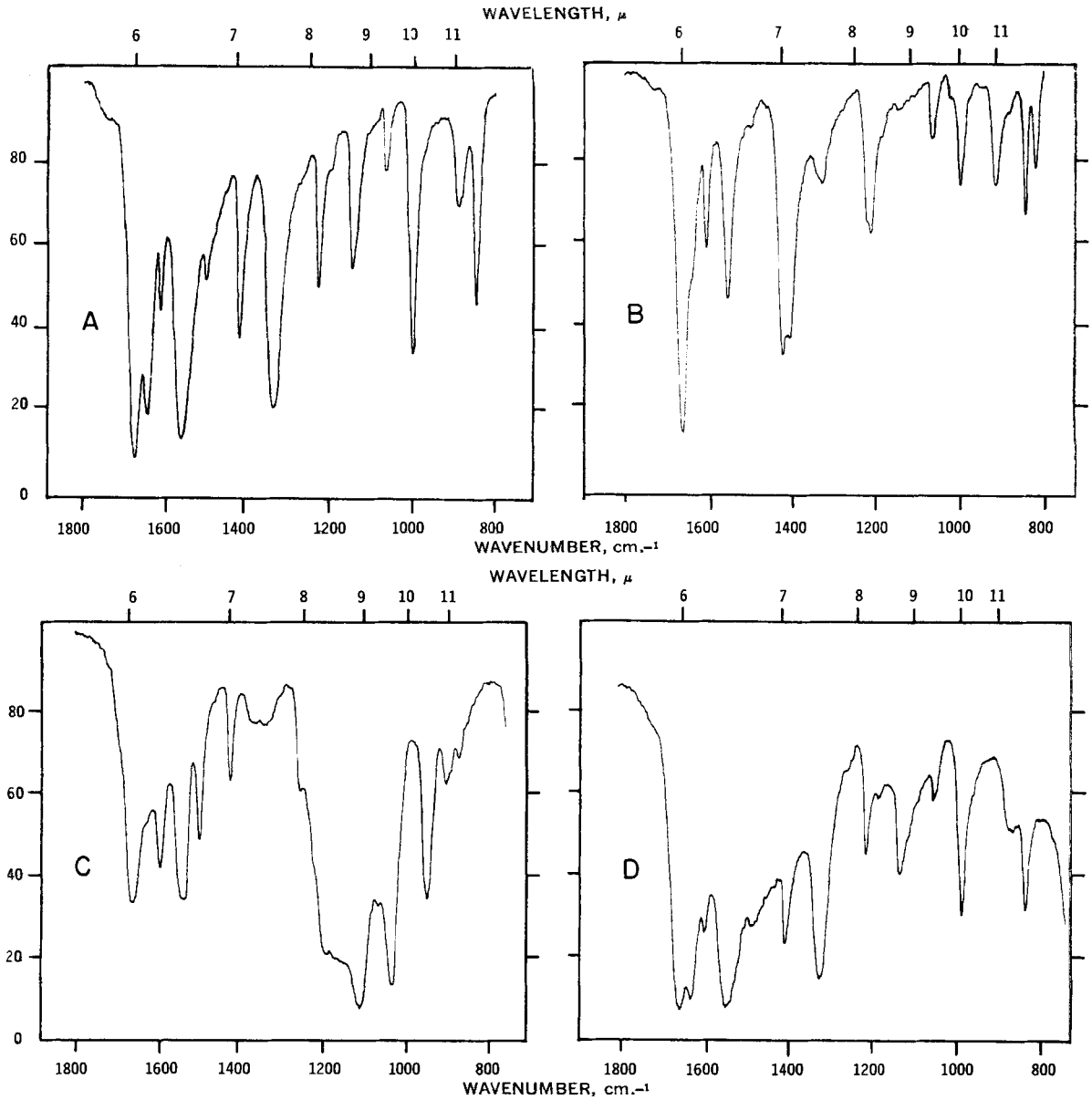
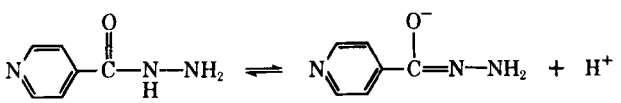


Figure 7—*IR spectra of isoniazid (A), deuterated isoniazid (B), isoniazid–copper complex (C), and isoniazid adsorbed on magnesium oxide (D).*

suction, followed by slow elution with 200 ml. of the respective solvent used in equilibration, and dried at 35° in a vacuum drying oven. Differential thermal spectrograms of these samples indicated that no solvent residue remained. DRS of these samples are shown in Fig. 6. As can be seen, the absorption intensities for the 268-mμ maxima are weaker than for the 325-mμ maxima, and the ratios, $f(r\infty)_{268}/f(r\infty)_{325}$, are less than 1. This is contrary to the ratios calculated from Fig. 2 and listed in Table I. This finding is significant and is due to the fact that in this elution process, physically adsorbed INH molecules are more easily removed than those that are due to chemisorption.

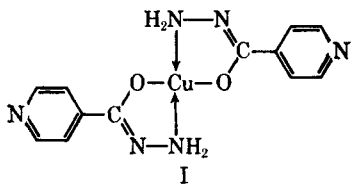
The possibility that this 325-mμ maximum is due to the existence of inter- or intramolecular complexes within the crystal lattice of the INH particle is unlikely in view of the fact that these equilibrated samples were thoroughly eluted with the respective solvents. Under such conditions the presence of crystalline INH particles is very limited.



Scheme I

In their studies dealing with the determination of dissociation or formation constants of INH complexes, various workers (21–24) have reported that spectral shifts of INH solutions do occur with a change in pH. At a pH above 11.6, the 262-mμ maximum is shifted to 300 mμ and is due to ionization of the INH tautomer (Scheme I).

No strong maximum at 325 mμ was observed even at very high pH values. In view of the fact that the pH of a saturated MgO aqueous solution is approximately 10.3, it is highly improbable that the 325-mμ maximum observed in the solid INH–MgO system discussed is due solely to the pH effect. It is interesting to note here that the diffuse reflectance spectrum of a mixture of MgO and INH–Cu complex [the complex prepared according to the procedure of Foye and Duvall (15)] did show a small hump in this 300-mμ region. This would be expected since, in the postulated copper complex structure,



I

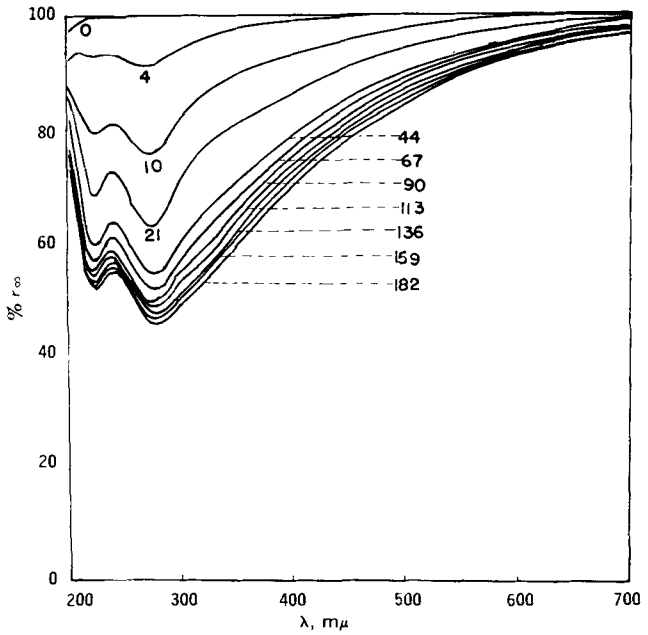


Figure 8—*DRS showing the browning of lactose at 100°. (The numbers shown are time in hours.)*

ionization of the INH tautomer is necessary prior to its formation. The absence of this 300-mμ hump, as seen in Figs. 2, 4, and 6, does suggest that a mechanism or mechanisms other than that involved in the formation of the INH–Cu complex in solution is operative in the INH–MgO equilibrated system. The formation of an INH–Mg complex in solution has not been reported.

These observations are also supported by the IR spectra shown in Fig. 7. Spectra A, B, and C were taken after mixing 1 mg. of INH, deuterated INH, and INH–Cu complex, respectively, with 150 mg. of KBr. Spectrum D was taken of a thoroughly triturated mixture of 1 mg. INH and 3 mg. MgO. This sample was placed in a vacuum desiccator over calcium sulfate for 30 hr. and mixed with 150 mg. of KBr. Spectra A, B, and D portray a strong carbonyl stretching band, νC=O at 1665 cm.$^{-1}$; C exhibits only a weaker band at 1655 cm.$^{-1}$, which is probably due to νC=N rather than the νC$_{-1}$=O. Comparison of A and B at 1636 cm.$^{-1}$ reveals the fact that the 1636-cm.$^{-1}$ band in A is responsible for the bending vibration of an amino group, δ—NH$_2$. Because this amino group bending vibration disappears

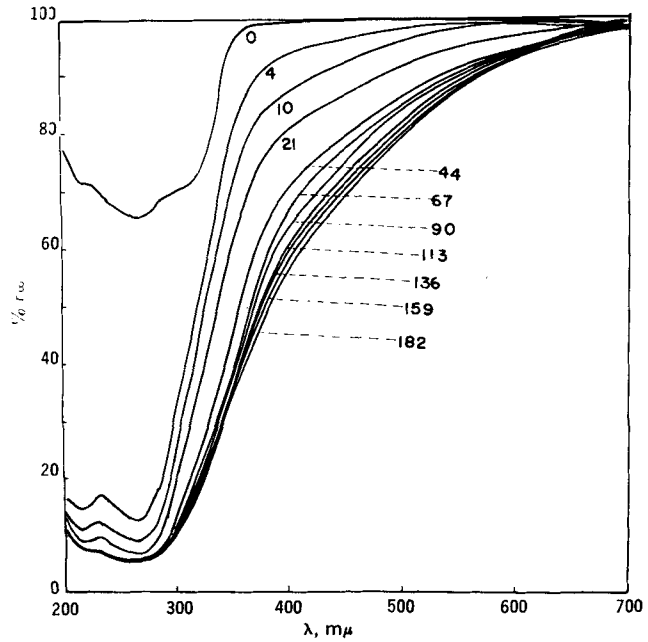


Figure 9—*DRS showing the browning of isoniazid–lactose system at 100°. (The numbers shown are time in hours.)*

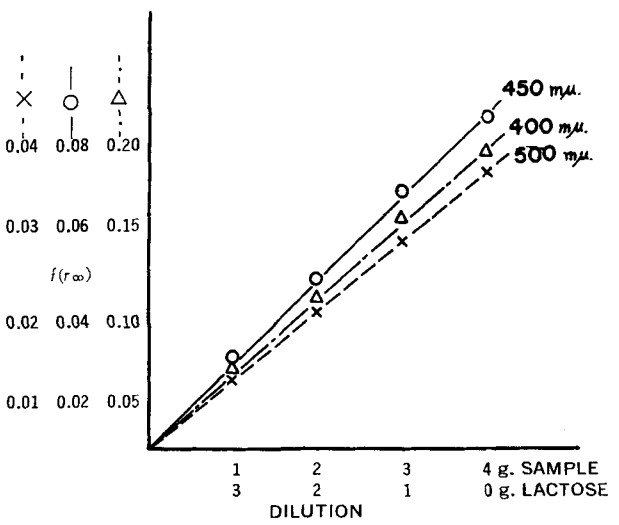


Figure 10—*Relationship between reflectance and dilution of browned substance obtained from heating the isoniazid–lactose system at 100° for 182 hr.*

upon deuteration, it is absent in B. The same band is still present in D but not C. Therefore, it may be assumed that the —NH$_2$ groups in A and D exist in the same free form, while in C it is quite different, probably because of the formation of a coordination bond between cupric ion and the lone pair electrons on the amino nitrogen. However, the most confirmatory evidence is obtained at 1330 and 1110 cm.$^{-1}$, which may be assigned to νC—N and νC—O bands, respectively. Again A and D give the same spectra while C is variant. It is quite natural that Spectrum D shows the 1330-cm.$^{-1}$ band, even if INH has reacted with MgO in a manner similar to that of the INH–Cu complex, because the 1 mg. of INH used is in excess of the amount needed to interact with the surface of the 3 mg. of MgO present. However, the complete absence of a band at 1110 cm.$^{-1}$ indicates that the carbonyl double bond in Spectrum D is still intact. On the other hand, Spectrum C displays a strong νC—O band at 1110 cm.$^{-1}$, which coincides well with the postulated structure of this INH–Cu complex. Hence, it may be concluded that, contrary to that of the INH–Cu complex, the interaction of INH with MgO does not induce significant alteration in the molecular structure of INH. Thus the interaction, as well as the formation of the new UV maximum at 325 mμ in the diffuse reflectance spectrum, is the result of a mechanism different from that involved in this INH–Cu complex.

One possibility is that a donor–acceptor mechanism is operative in this INH–MgO interaction. It is known that the pyridine ring itself is a good donor. This donor capacity, however, might be somewhat reduced by the presence of a carbonyl group that exhibits a negative inductive effect. This effect would not be too significant if one considers the counterbalancing effect of the hydrazide group which may supply enough flow of electrons into the carbonyl group. Electron affinities of many compounds are not well known. However, if the energy level of the antibonding molecular orbital of MgO lies lower

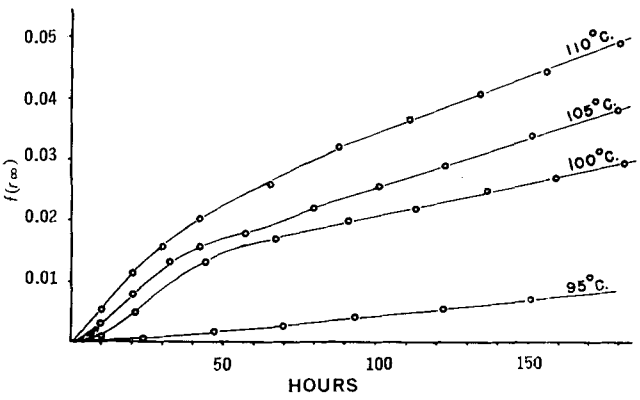


Figure 11—*The rates of browning of lactose at 95, 100, 105, and 110°.*

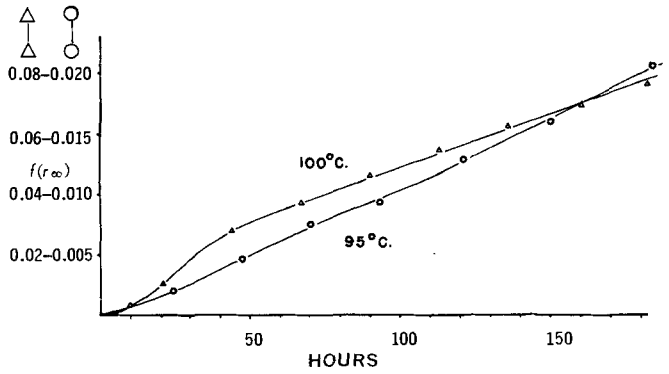


Figure 12—*The rates of browning of the isoniazid–lactose system at 95 and 100°.*

than the antibonding orbital of the conjugated system in the INH molecule, there is a possibility that a π-electron of INH will be transferred to the low-lying antibonding orbital of MgO instead of to the higher energy antibonding orbital of its own. This could happen when the two molecular orbitals approach close proximity to each other so that the overlapping of molecular orbitals actually occurs. Such a situation would require strong adsorption of a substrate onto an adsorbent surface, that is, chemisorption rather than physical adsorption. The immediate consequence of this type of electron transfer is the appearance of a resultant two-charged species which, in turn, would attract each other much more than the orginal two polar molecules. This does indicate the complexity of surface interactions since both chemisorption and physical adsorption are operative.

INH–Lactose System—The reflectance spectra of both lactose and the INH–lactose system are shown in Fig. 8 (Curve O) and Fig. 9 (Curve O). As is evident in Fig. 8 (Curve O), lactose has not been influenced by this equilibration process. Figure 9 (Curve O), which represents the spectrum of the equilibrated INH–lactose sample, shows a broad maximum at 265 mμ and shoulders at both sides of the maximum. Because of the lack of well-separated maxima in the spectrum of the INH–lactose system as compared to that of MgO, a study dealing with concentration effects with respect to intensity changes and spectral shifts was not successful.

Heating of the equilibrated lactose and INH–lactose samples in an oven at 100 ± 0.5° did produce changes in the DRS spectra, as shown in Figs. 8 and 9, as well as browning of the samples. Although no absorption maximum was initially seen in the equilibrated lactose system containing no drug, a maximum did appear at about 275 mμ after heating. In the INH–lactose system, this thermal effect broadened the maximum at 265 mμ. Since no maximum appeared in the visible region of the spectra in Figs. 8 and 9, selection of a proper wavelength at which the extent of browning was to be measured was next undertaken. For this purpose, the darkened sample was subjected to serial dilutions with lactose, and the reflectance spectra were taken. When the remission function, $f(r\infty)$, *versus* extent of dilution was plotted at 400, 450, and 500 mμ, respectively, all

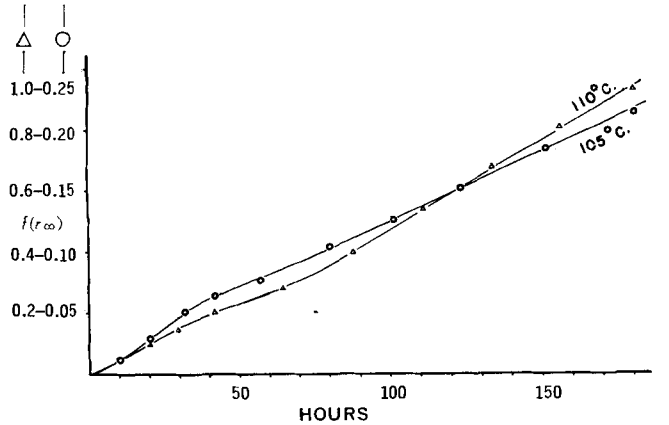


Figure 13—*The rates of browning of the isoniazid–lactose system at 105 and 110°.*

Table II—Apparent Zero-Order Rate Constant, k, of Browning of Lactose and Isoniazid–Lactose Systems

Temperature	Lactose		Isoniazid–Lactose	
	k	log k	k	log k
95°	5.305×10^{-5}	−4.2753	1.238×10^{-4}	−3.9073
100°	1.093×10^{-4}	−3.9614	3.560×10^{-4}	−3.4486
105°	1.574×10^{-4}	−3.8030	1.150×10^{-3}	−2.9393
110°	1.890×10^{-4}	−3.7235	5.860×10^{-3}	−2.2321

showed straight-line relationships as illustrated in Fig. 10, showing the exact conformity with an equation described by Wendlandt and Hecht (25):

$$f(r\infty) = \frac{2.303 \cdot a \cdot C}{S} \qquad \text{(Eq. 2)}$$

where a is the molar absorptivity, C is the molar concentration, and S is the scattering coefficient.

The reflectance at 450 mμ was arbitrarily chosen and employed in the subsequent reflectance measurements for the investigation of the rate of browning in the solid systems. The results are shown in Figs. 11–13. Careful examination of each curve revealed that it consists of two portions. In the first stage of the browning reaction, which in most cases occurred between 0–50 hr., a sigmoidal curve was obtained; in the second stage, an almost straight-line relationship with time was observed. From these two distinct patterns in the reflectance data, it may be assumed that two fundamentally different mechanisms are probably operative. Since each lactose molecule contains 1 molecule of water which may have a direct relationship in the first stage of this browning reaction, a study of the dehydration aspect of lactose was carried out at the same temperature ranges. The results showed that, except at 95°, the complete dehydration of lactose occurred within about 50 hr., which coincided with the time necessary for the completion of the sigmoidal portion of the curve. During this stage of browning, it appears that a true solution phase is formed locally between the water liberated from lactose monohydrate, and that this dehydrated lactose and INH then proceed to react to form: (*a*) a condensation product of lactose and INH, and (*b*) various carbonyl compounds through stepwise degradation of lactose. This initial reaction can be regarded as being in a solution phase and not the so-called solid–solid reaction in the true sense of the meaning.

During the second stage of browning, the reaction may be considered essentially in the solid state. From the straight-line relationship obtained between remission function and time, the browning reaction may be assumed to follow an apparent zero-order rate law; from the slope, the rate constants, k, were calculated and are listed in Table II. However, since the actual reaction is very complicated and many different types of reaction nuclei may be formed during the

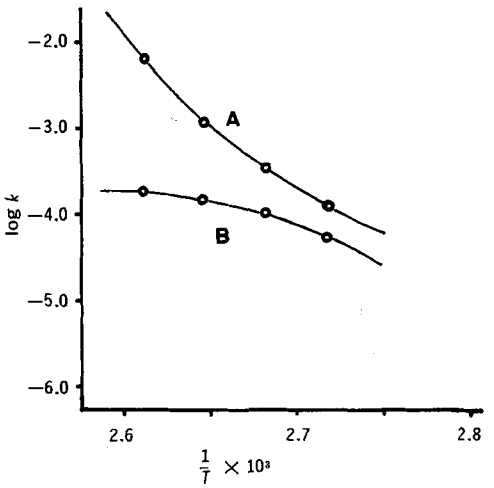


Figure 14—*Temperature dependence of apparent zero-order rate constant for browning reactions of the isoniazid–lactose system (A) and lactose alone (B).*

Table III—Comparison of the Rate of Formation of Hydroxymethylfurfural and Isonicotinoyl Hydrazone of Hydroxymethylfurfural

Time	HMF, *M*	INH–HMF, *M*
0	0	0
5	5.16×10^{-5}	1.63×10^{-4}
10	2.98×10^{-4}	3.15×10^{-4}
16	4.76×10^{-4}	4.63×10^{-4}
21.5	5.67×10^{-4}	5.67×10^{-4}
27	7.16×10^{-4}	7.08×10^{-4}
34	9.74×10^{-4}	7.86×10^{-4}
41	1.03×10^{-3}	1.06×10^{-3}
46	1.22×10^{-3}	1.03×10^{-3}
53	1.21×10^{-3}	1.16×10^{-3}
64	1.34×10^{-3}	1.34×10^{-3}

first stage of the browning reaction, these, along with other nuclei generated in this second stage, may then proceed to form various products. Therefore, the apparent rate constant, k, in this case does not have the usual kinetical significance. It does, however, represent a total overall reaction and does enable the approximate estimation of the rate of reaction at the other temperatures. From the data in Table II, an Arrhenius plot was attempted (Fig. 14). From the curves obtained instead of the usual straight lines, it is obvious that the reaction mechanisms are not consistent throughout the temperature range studied, and this reflects the complexity of the browning reaction. When the curves are extrapolated to room temperature, assuming that the curves do not change their curvature, it is possible to estimate the approximate time required for the extent of browning to be perceptible. Since lactose showed a convex curve that does not intersect with a vertical line down through a point at 25°, it would appear that this browning of lactose would require an infinite time to occur. Brownley and Lachman (26) reported that lactose remained white after storage for 36 months under ambient conditions. On the other hand, the equilibrated mixture of INH and lactose showed a concave curve. From the intersect with the vertical line at 25°, log k was determined and the time required for initial browning to be perceptible, assuming $r\infty = 90\%$, was calculated as approximately 4 years. In this calculation, it is assumed that the straight-line portion of the curve passes through the origin. This 4-year stability of the INH–lactose system will certainly be reduced to some extent when the sample is exposed to excessive moisture. Ritschel and Rahman (27) and Horikoshi and Himuro (28) drew attention to the fact that atmospheric moisture did accelerate the browning of INH tablets.

Since it is well known that browning of many compounds, such as food and food products, is also faster in the presence of moisture, the browning of the INH–lactose system was carried out in solution for purposes of comparison and to isolate reaction products where possible. Prior to heating, Solutions *a* and *b* showed absorption at 262 mμ, which is due to INH as mentioned before (see *Browning of INH–Lactose Mixture in Solution*). Solution *c* did not show any absorbance. After heating, Solution *a* showed a shoulder at about 300 mμ. Solution *b*, which is a mixture of INH and lactose, showed a new strong absorption at 320 mμ. Solution *c* exhibited an intense maximum at 283 mμ, which was identified by TLC as 5-hydroxymethylfurfural (HMF). The new maximum at 320 mμ in Solution *b* was believed to be the result of a reaction between INH and HMF, which was confirmed by TLC using prepared INH–HMF as a standard. The compound INH–HMF has been prepared differently by Knotz (29). Since both HMF and INH–HMF were found to obey Beer's law within 0–0.6 mg. HMF/100 ml. H_2O and 0–0.8 mg. INH–HMF/100 ml. H_2O,[16] the quantities of INH–HMF and HMF produced in Solutions *b* and *c* were determined from the spectra and are listed in Table III. This table indicates that the rates of appearance of both INH–HMF and HMF were approximately equal. From these results, it is assumed that HMF, which is formed from the degradation of lactose in aqueous solution, reacts instantly with INH to form INH–HMF. The high reactivity of both species was substantiated by the fact that swift precipitation of INH–HMF was observable as moderately concentrated solutions of both species were mixed together. Also, an almost theoretical yield was obtained in the preparation of INH–HMF.

[16] With the aid of small amount of methanol.

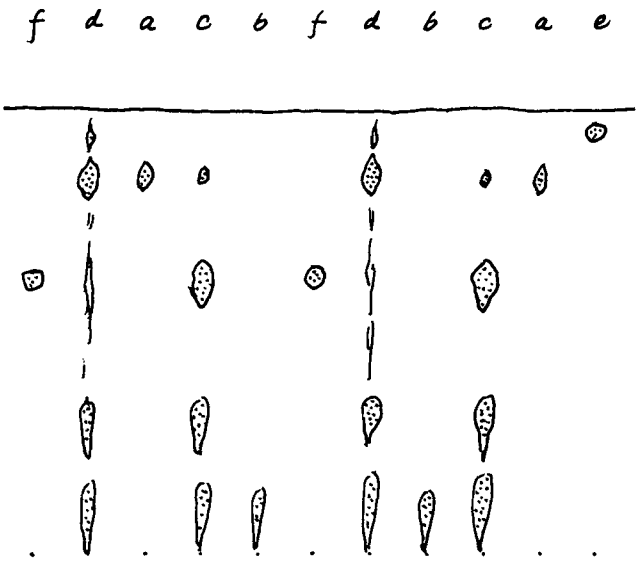
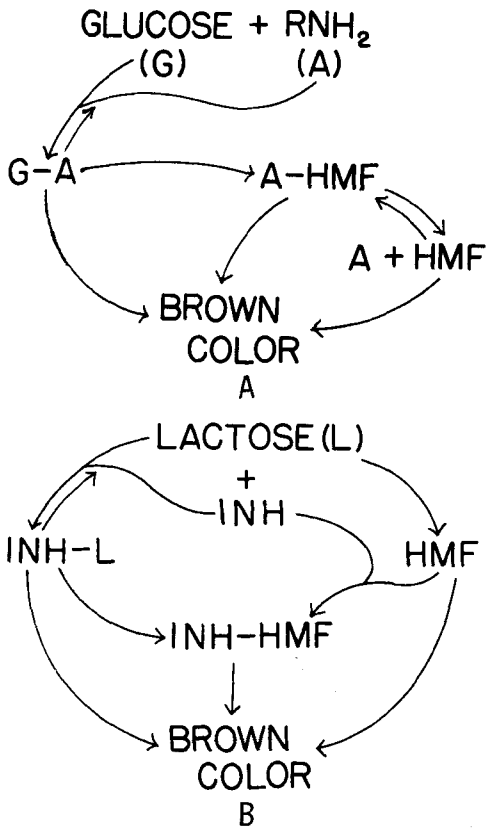


Figure 15—*Thin-layer chromatogram showing thermal degradation products of the isoniazid–lactose system. Key: a, INH–HMF, R_f = 0.85, pink spot under UV light, brown spot by I_2 vapor; b, INH–L, R_f = 0.12, brown spot by I_2 vapor; c, methanol extract of degradation products in solid state; d, methanol extract of degradation products in solution; e, HMF, R_f = 0.96, black spot under UV light, brown spot by I_2 vapor; and f, INH, R_f = 0.62, brown spot by I_2 vapor.*

Since INH–HMF was found in solution degradation, it was interesting to know whether the same compound was also formed in the solid system. Identification of the reaction products in the solid state was carried out using TLC. The highly darkened solid sample was first extracted with anhydrous methanol and then spotted on a plate. Simultaneously, methanol solutions of INH, INH–HMF, HMF, and isonicotinoyl hydrazone of lactose (INH–L) were also spotted. INH-L was tested since Yamamoto and Tanaka (30) reported its formation in INH tablets. The compound was prepared according to a method described by the same authors. The results of TLC are shown in Fig. 15. The presence of INH–HMF and INH–L was evident in this figure. The isolation and confirmation of INH–HMF were carried out first by concentration of a large quantity of methanol extract. This concentrate was then streaked onto a 3.0-mm. thick preparative plate and developed as before. Using UV light as a guide, the pink-color band was scraped off and extracted with anhydrous methanol. The extract was concentrated, diluted with an equal volume of water, and then purified; it showed the same UV and IR spectra when compared with the prepared INH–HMF. Since the quantity of INH–HMF formed in the solid system was very small, many difficulties were encountered in its quantitative determination; consequently, attempts to correlate the rate of browning and the rate of formation of INH–HMF in the solid system were unsuccessful.

The mechanisms of the Maillard-type browning reactions involving glucose and glycine have been discussed in detail by several authors (31, 32); a stepwise reaction scheme was proposed. A simplified reaction scheme is shown in Scheme II A. Although the mechanisms involved in the reactions between sugars and amino acids might resemble the present system, the results from the present studies showed some discrepancies. The degradative mechanisms have been modified and are given in Scheme II B. In the reaction of the INH–lactose system, the formation of INH–HMF may not completely rely on INH–L as a source. Since the formation of INH–L and HMF needs approximately the same conditions, *i.e.*, high temperature and humidity, and since the reactivity of HMF is high, the formation of INH–HMF from INH and HMF is also possible. As shown in Scheme II A, most workers have considered that an equilibrium exists between A − HMF and A + HMF. However, this is not conceivable, except in the presence of catalyst which may promote the hydrolysis of INH–HMF. In fact, the whole process of the browning reaction may be expected to be much more complicated than the proposed scheme, since many parallel pathways may exist in this process. Recently, Mitsuda *et al.* (33) reported that electron spin



Scheme II—Mechanisms af browning reactions. Key: Scheme A, described by Keeney and Bassette (32); and Scheme B, modified

resonance spectroscopy revealed that a free radical reaction was found operative in the aminocarbonyl reaction of food browning.

Although the interaction of INH and lactose does not proceed to an appreciable degree under ambient conditions, the situation may become significant under moderate temperature and moisture conditions as those found in tropical or subtropical climates. It should also be recognized here that during wet granulation procedures, in which both INH and lactose are exposed to higher temperatures and excessive moisture, these degradation aspects cannot be overlooked.

As has been pointed out by Bernstein *et al.* (34), none of the INH derivatives, including hydrazones, is more active on a molar basis than INH itself. It is also interesting to speculate here as to the tuberculostatic activity of darkened tablets of INH–HMF and INH–L itself.

The INH–HMF prepared in this study has been evaluated for its antitubercular activity.[17] Preliminary *in vitro* results using the standard NCBC plate method indicate that INH–HMF and INH have approximately comparable activity. However, due to the fact that their physicochemical properties are sufficiently different (for example, aqueous solubility) and that even if the *in vitro* tests appear to be comparable, it does not necessarily mean that they are clinically equivalent. For example, Kakemi *et al.* (35) studied absorption of INH and its derivatives from the gastrointestinal tract and found that the INH–L is hardly absorbed. A recognition of these INH–lactose reactions and their effects on drug availability and therapeutic response is therefore necessary in the formulation of satisfactory INH dosage forms.

[17] At the State of Iowa Hygienic Laboratory.

REFERENCES

(1) S. S. Kornblum and M. A. Zoglio, *J. Pharm. Sci.*, **56,** 1569 (1967).

(2) D. Ribeiro, D. Stevenson, J. Samyn, G. Milosovich, and A. M. Mattocks, *J. Amer. Pharm. Ass., Sci. Ed.*, **44,** 226(1955).

(3) F. J. Bandelin and W. Malesh, *J. Amer. Pharm. Ass., Pract. Pharm. Ed.*, **19,** 152(1958).

(4) F. E. DiGangi and C. H. Rogers, *J. Amer. Pharm. Ass., Sci. Ed.*, **38,** 646(1949).

(5) H. E. Paul and C. M. Harrington, *ibid.*, **41,** 50(1952).

(6) S. M. Blaug and M. R. Gross, *J. Pharm. Sci.*, **54,** 289(1965).

(7) T. Chulski and A. A. Forist, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 553(1958).

(8) J. K. Guillory, S. C. Hwang, and J. L. Lach, *J. Pharm. Sci.*, **58,** 301(1969).

(9) J. L. Lach and M. Bornstein, *ibid.*, **54,** 1730(1965).

(10) M. Bornstein and J. L. Lach, *ibid.*, **55,** 1033(1966).

(11) J. L. Lach and M. Bornstein, *ibid.*, **55,** 1040(1966).

(12) L. D. Bighley, Ph.D. thesis, University of Iowa, Iowa City, Iowa, 1966.

(13) J. D. McCallister, Ph.D. thesis, University of Iowa, Iowa City, Iowa, 1967.

(14) E. Carl and P. Marquardt, *Z. Naturforsch.*, **7b,** 574(1952).

(15) W. O. Foye and R. N. Duvall, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 285(1958).

(16) H. Wojahn, *Arch. Pharm.*, **287,** 45(1954).

(17) M. M. Frodyma, V. T. Lieu, and R. W. Frei, *J. Chromatogr.*, **18,** 520(1965).

(18) H. Zeitlin, P. Anthony, and W. Jordan, *Science*, **141,** 423 (1963).

(19) P. Kubelka and F. Munk, *Z. Tech. Phys.*, **12,** 593(1931).

(20) G. Kortüm, W. Braun, and G. Herzog, *Angew. Chem. Int. Ed. Engl.*, **2,** 333(1963).

(21) D. S. Goldman, *Science*, **120,** 315(1954).

(22) Q. C. Belles and M. L. Littleman, *Anal. Chem.*, **32,** 720(1960).

(23) K. Nagano, H. Tsukahara, H. Kinoshita, and Z. Tamura, *Chem. Pharm. Bull.*, **11,** 797(1963).

(24) B. Salvesen and S. A. Eikill, *Medd. Nor. Farm. Selsk.*, **27,** 135(1965); through *Chem. Abstr.*, **64,** 9507f(1966).

(25) W. W. Wendlandt and H. G. Hecht, "Reflectance Spectroscopy," Interscience, New York, N. Y., 1966, p. 72.

(26) C. A. Brownley and L. Lachman, *J. Pharm. Sci.*, **53,** 452 (1964).

(27) W. A. Ritschel and M. Rahman, *Sci. Pharm.*, **33,** 105(1965).

(28) I. Horikoshi and I. Himuro, *Yakugaku Zasshi*, **86,** 353(1966).

(29) F. Knotz, *Monatsh. Chem.*, **89,** 718(1958).

(30) R. Yamamoto and H. Tanaka, *Yakuzaigaku*, **17,** 219(1957).

(31) E. L. Richards, *Biochem. J.*, **64,** 639(1956).

(32) M. Keeney and R. Bassette, *J. Dairy Sci.*, **42,** 945(1959).

(33) H. Mitsuda, K. Yasumoto, and K. Yokoyama, *Agr. Biol. Chem.*, **29,** 751(1965).

(34) J. Bernstein, W. P. Jambor, W. A. Lott, F. Pansy, B. A. Steinberg, and H. L. Yale, *Amer. Rev. Tuberc.*, **67,** 354(1953).

(35) K. Kakemi, T. Arita, H. Sezaki, and N. Takasugi, *Chem. Pharm. Bull.*, **13,** 551(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication April 23, 1970.

This work was supported by Research Grant GM-13178 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Abstracted in part from a thesis submitted by Wen-Hung Wu to the University of Iowa, Iowa City, Iowa, in partial fulfillment of the Doctor of Philosophy degree requirements.

Behavior of Erythrocytes in Various Solvent Systems VI: Water–Tetramethylurea

DONALD E. CADWALLADER and JANIS R. PHILLIPS*

Abstract □ Hemolytic behavior of human erythrocytes in water–tetramethylurea (TMU) solutions was investigated. TMU freely permeates human red blood cells; however, hemolysis can be prevented by the inclusion of 0.9% sodium chloride in solutions containing up to 8% TMU. The addition of sodium chloride to solutions containing more than 8% TMU did not prevent hemolysis, discoloration, and precipitation of blood components. Divalent and trivalent anions gave greater protection against hemolysis than did the sodium chloride. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in aqueous solutions.

Keyphrases □ Erythrocytes, behavior—water–tetramethylurea system □ Hemolysis, erythrocytes—water–tetramethylurea system □ Tetramethylurea–sodium chloride system—erythrocyte hemolysis □ Isotonic coefficients—tetramethylurea–water systems

It is well known that to prepare a safe and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous solvent. For this reason, investigations have been made to study the hemolytic effects of aqueous solutions of glycerin, propylene glycol, and liquid polyethylene glycols on rabbit and human erythrocytes, and the hemolytic effects of aqueous dimethylsulfoxide and liquid amides on human erythrocytes (1–5).

This report is concerned with the investigation of water–tetramethylurea (TMU) systems. TMU is a dipolar aprotic solvent and possesses a number of desirable solvent properties. The solvent and reagent properties of TMU have been reviewed by Luttringhaus and Dirksen (6). The solvent has a mild and pleasant odor, a high degree of stability, low reactivity, a high boiling point (177.6°), and a water-white color. It also has the property of being completely miscible with water, alcohols, ether, benzene, chlorinated hydrocarbons, and many other polar and nonpolar solvents.

Due to the possible use of TMU as a drug solvent, Dixon *et al.* carried out toxicity studies on TMU (7). In their studies, TMU was also tested for central nervous system (CNS) pharmacological properties and the ability to increase survival time of tumor-bearing mice due to structural similarities between TMU and drugs capable of producing these effects. It was reported that the acute LD_{50} of TMU for mice was 2230 mg./kg. i.v. and 2920 mg./kg. given orally. Toxic doses were lower with the monkey, the drug being lethal at dosesas low as 750 mg./kg. i.v. TMU was found to possess weak anticonvulsant and tranquilizer activity, and it produced a significant increase in median survival time of mice bearing a plasma cell tumor.

It was the purpose of this investigation to observe the behavior of human erythrocytes in TMU solutions. In each experiment the hemolytic method was utilized. By comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium chloride–water–TMU solutions, it was possible to calculate the *hemolytic isotonic coefficients for sodium chloride in* various water–TMU solutions.

Table I—Values of *i* for Sodium Chloride in Various Water–TMU Solutions, Calculated from Concentrations Causing 25, 50, and 75% Hemolysis of Human Erythrocytes[a]

Tetramethylurea, % v/v	Hemolysis 25%	Hemolysis 50%	Hemolysis 75%	Average
1	1.91	1.94	1.94	1.93
3	1.65	1.72	1.77	1.71
6	1.54	1.62	1.65	1.60
7	1.81	1.88	1.95	1.88

[a] Each value is an average of at least two blood samples.

EXPERIMENTAL

Materials—TMU (Aldrich Chemical Co.) had a specific gravity of 0.972 and was 99% pure. All electrolytes and nonelectrolytes employed in this study were reagent grade.

Collection of Blood—The blood samples used for all experiments were obtained from the forearm veins of a 22-year-old male Caucasian donor. Fresh blood samples were used in all experiments. Approximately 10 ml. of blood was obtained from the donor and placed in a 50-ml. round-bottom flask containing 10–15 glass beads. The flask was rotated gently for about 5 min.; then the blood was decanted into a 50-ml. conical flask and aerated by swirling the flask gently for about 5 min.

Preparation of Solutions—All of the TMU solutions were volume-in-volume percentage preparations. Sodium chloride solutions were prepared on a weight-in-volume basis. Data from a previous paper [*viz.*, Table I, (2)] were used in the preparation of isotonic solutions of calcium chloride, sodium citrate, sodium bromide, sodium sulfate, and sodium tartrate. All pH adjustments were made using Sorensen isotonic buffer systems. Distilled water was used to prepare all solutions.

Quantitative Determination of Percent Hemolysis—In each experiment, the hemolytic method was used to determine the degree of hemolysis of erythrocytes in the TMU solutions. This quantitative method is based on the fact that a hypotonic solution liberates oxyhemoglobin in direct proportion to the number of cells hemolyzed. Into each of two test tubes was transferred 5 ml. of standard sodium chloride solution (0.34%, 0.35% ... 0.45%, 0.46%) and 5 ml. of the mixed solvent system being tested. After the test tubes were brought to a constant temperature by placing in a water bath (37 ± 1°), 0.05 ml. of blood was pipeted into each tube. Each tube was then inverted several times to ensure thorough mixing and allowed to remain 45 min. at 37°. The absorbance of the supernatant liquid after centrifuging was measured using a Klett-Summerson photoelectric colorimeter equipped with a No. 54 filter. To find the percent hemolysis, these absorbance readings were divided by the absorbance readings for 0.05 ml. of blood in 5 ml. of distilled water (standard for 100% hemolysis) and multiplied by 100. A blank, made by placing 0.05 ml. of blood in 5 ml. of 0.9% sodium chloride solution, was used to cancel any light absorbance inherent to the blood sample. Since all solutions of TMU were slightly turbid, TMU solutions corresponding to the concentration range of test solutions were used as blanks to cancel any absorbance that might be caused as a result of this turbidity. Both the standard and the blanks were subjected to the same conditions of standing for 45

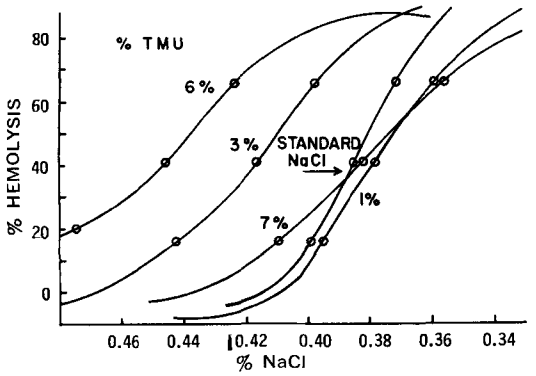


Figure 1—*Hemolysis of human erythrocytes after 45 min. at 37° in various TMU–saline solutions.*

min. at 37° followed by centrifuging. A pH meter (Corning model 7) was used for all pH measurements.

Calculations of *i* Values—Through use of the hemolytic method, concentrations of sodium chloride and the TMU solutions giving the same degree of hemolysis could be determined. Once these concentrations were ascertained, it was possible to calculate isotonic coefficients (*i* values) through use of the following equation:

$$\left(\begin{matrix}i \text{ value for NaCl} \\ \text{in water}\end{matrix}\right)\left(\begin{matrix}\text{g. of NaCl in} \\ \text{100 ml. of water}\end{matrix}\right) = \left(\begin{matrix}i \text{ value for NaCl} \\ \text{in TMU soln.}\end{matrix}\right)\left(\begin{matrix}\text{g. of NaCl in} \\ \text{100 ml. of TMU soln.}\end{matrix}\right) \quad \text{(Eq. 1)}$$

The value of *i* for sodium chloride was taken as 1.86, which is the accepted *i* value for 0.9% sodium chloride in water (8).

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–TMU solutions were plotted on rectangular coordinate graph paper. From these curves, it was possible to determine the concentrations of sodium chloride in g./100 ml. of water and the TMU solvent causing 25, 50, and 75% hemolysis. These values were inserted into Eq. 1, thereby giving the values of *i* for sodium chloride in a particular water–TMU solution at concentrations producing 25, 50, and 75% hemolysis. The various *i* values of TMU solutions are shown in Table I.

Preparation of Hemolysis Curves—Approximately 25 experiments employing human blood were carried out, A standard hemolysis curve (Fig. 1) was constructed from the average readings of these experiments. Hemolysis curves of the various TMU solutions were constructed using the *i* values previously calculated (Table I). Through rearrangement of Eq. 1 to give

$$\left(\begin{matrix}\text{g. of NaCl in 100} \\ \text{ml. of TMU soln.}\end{matrix}\right) = \frac{\left(\begin{matrix}1.86\ i \text{ value for} \\ \text{NaCl in water}\end{matrix}\right)\left(\begin{matrix}\text{g. of NaCl in 100 ml.} \\ \text{of water causing 25\%} \\ \text{hemolysis}\end{matrix}\right)}{\left(\begin{matrix}i \text{ value for NaCl in} \\ \text{TMU solution}\end{matrix}\right)} \quad \text{(Eq. 2)}$$

the grams of sodium chloride per 100 ml. in a TMU solution causing 25% hemolysis were calculated. Similar calculations were carried out at 50 and 75% hemolysis. By plotting these three points, the hemolysis curves for the various TMU solutions were constructed (Fig. 1). From these hemolysis curves (Fig. 1), the amounts of sodium chloride preventing hemolysis of 50% of the erythrocytes (or where 50% hemolysis occurred) in various TMU solutions were determined, and the results are shown in Fig. 2.

RESULTS

In this study it was found that complete hemolysis of human erythrocytes occurred in 0.0–100% TMU solutions after 45 min. at 37°. Upon addition of 0.9% sodium chloride, hemolysis of human erythrocytes was essentially prevented (less than 5%) in solutions containing 0.0–8% TMU. Hemolysis greatly increased in solutions containing 9–12% TMU even with 0.9% sodium chloride. These results are shown in Fig. 3.

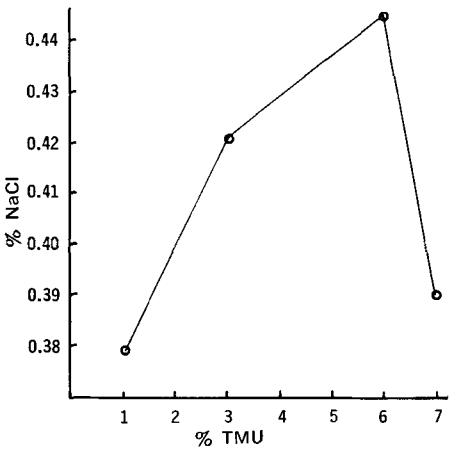


Figure 2—*Amount of sodium chloride preventing hemolysis of 50% of human erythrocytes in various TMU solutions at 37°.*

Reddish-brown solutions resulted when blood was added to 0.9% saline solutions containing 15–16% TMU. More pronounced discoloration and/or precipitation occurred in solutions containing higher concentrations of TMU. Solutions containing 50% TMU revealed a brownish-green color with no precipitation, while those at 75% showed a brown precipitate in addition to the brownish-green color.

It was possible to modify the fragility of human erythrocytes through the addition of hypotonic quantities of sodium chloride (0.34%, 0.35% . . . 0.45%, 0.46%) to various water–TMU solutions. When blood was added to saline solutions containing 0.0–7% TMU, typical sigmoidal hemolysis curves resulted (Fig. 1). These curves were constructed in the manner described in the *Experimental* section of this report utilizing the data presented in Table I.

Calculations of *i* values for sodium chloride in various water–TMU solutions were accomplished through use of Eq. 1 and are shown in Table I.

The pH readings for the TMU solutions were within a range of 5.1–7.0. Addition of isotonic phosphate buffer reduced hemolysis (less than 5%) in solutions containing as much as 10% TMU (Fig. 3).

Aqueous TMU solutions containing isotonic quantities of calcium chloride (1.15%) and sodium bromide (1.51%) gave results similar to those described for the TMU–saline solutions. Addition of sodium citrate (1.84%), sodium sulfate (1.27%), and sodium tartrate (1.61%) reduced hemolysis, producing trace hemolysis in solutions containing as much as 10.5% TMU. The hemolysis curves for TMU solutions containing these divalent and trivalent anion salts were similar to the mentioned curve for phosphate buffer (Fig. 3).

DISCUSSION

From the experimental data gathered in this study, it can be seen that TMU freely permeates human red blood cells and, therefore, offers no protection to these cells from hemolysis. Upon addition of

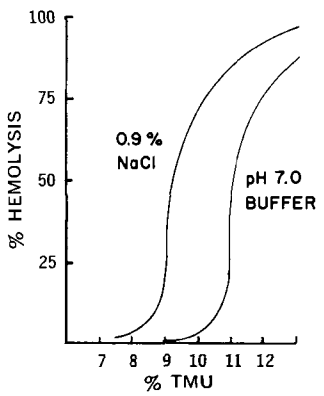


Figure 3—*Hemolysis of human erythrocytes after 45 min. at 37° in various TMU solutions containing 0.9% sodium chloride or pH 7.0 isotonic Sorensen buffer.*

0.9% sodium chloride, complete hemolysis can be prevented in the TMU solutions containing up to 8% TMU. Since addition of 0.9% sodium chloride to these TMU solutions prevents hemolysis and since addition of hypotonic quantities of sodium chloride to these solutions (and TMU solutions of lower concentration) prevents complete hemolysis, the lysis of red cells in solutions containing 7% or less TMU can be attributed to osmotic hemolysis.

There is a critical concentration for TMU at which addition of 0.9% sodium chloride will not prevent hemolysis and/or precipitation of blood components. The transition from nonhemolytic concentrations to destructive concentrations is rather abrupt, as shown by the hemolysis curve in Fig. 3.

Further destruction of red cells occurs as TMU concentrations are increased above the critical concentration. This is apparent as the color changes from reddish-brown to greenish-brown with a brown precipitate.

The van't Hoff factor or isotonic coefficient can be expressed as the ratio of any colligative property of a real solution to that of an ideal solution of a nonelectrolyte (9). The isotonic coefficients (*i* values) for aqueous solutions containing 3 and 6% TMU were found to be less than 1.86. This is evidence that these concentrations offer no protection to human erythrocytes against osmotic hemolysis. However, *i* values calculated for 1 and 7% TMU were slightly higher than 1.86, indicating that these strengths contribute slightly to the tonicity of the extracellular aqueous solutions. This is shown graphically when the concentration of TMU is plotted against the amount of sodium chloride preventing hemolysis of 50% of the erythrocytes (Fig. 2). No *i* values were calculated for 8% TMU solutions since this concentration gave very erratic results and appears to be too critical an area for determination of *i* values.

Upon first observations, pH of the TMU solutions appeared to have some effect upon the solution's tendency to damage human red blood cells, since addition of pH 7.0 Sorensen phosphate buffer considerably reduced hemolysis in solutions containing up to 12% TMU (Fig. 3). However, it did not seem that the reduction of hemolysis was due to the pH effect since the pH range (5.1–7.0) of plain aqueous TMU solutions was near neutral, especially in the higher concentrations. Through further experiments using isotonic quantities of other salts, it was determined that divalent and trivalent anions gave greater protection against hemolysis than did the 0.9% sodium chloride. Therefore, it was surmised that the effect of hemolysis reduction with the phosphate buffer system was due to its phosphate anion properties, not its pH-limiting properties.

In a series of papers (10–15) concerning the hemolysis of human erythrocytes in relation to lattice structure of water, Good established the idea that the effect of a solute upon the structural properties of the water lattice is basic to the mechanism of hemolysis. He found that large simple anions produce simple steric distortion and electrostatic screening which disturb the lattice locally and free water molecules which will tend to associate themselves with a malonamide stabilized zone. Thus the fluidity of the extracellular phase is reduced and hemolysis is slower. Therefore, the large anion will inhibit hemolysis by contributing free water molecules to the malonamide-stabilized lattice. Divalent and trivalent anions used in these studies could possibly act in a similar way whereby they inhibit hemolysis by contributing free water to a TMU-stabilized lattice.

REFERENCES

(1) D. E. Cadwallader, *J. Pharm. Sci.*, **52,** 1175(1963).

(2) D. E. Cadwallader, B. W. Wickliffe, and B. L. Smith, *ibid.* **53,** 927(1964).

(3) B. L. Smith and D. E. Cadwallader, *ibid.*, **56,** 351(1967).

(4) D. E. Cadwallader and J. P. Drinkard, *ibid.*, **56,** 5(1967).

(5) D. E. Cadwallader and J. R. Phillips, *ibid.*, **58,** 1220(1969).

(6) A. Luttringhaus and W. H. Dirksen, *Angew. Chem., Int. Ed. Eng.*, **3,** 260(1964).

(7) R. L. Dixon, R. H. Adamson, M. Ben, and D. P. Rall, *Arch. Int. Pharmacodyn. Ther.*, **160,** 333(1966).

(8) T. S. Grosicki and W. J. Husa, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 632(1954).

(9) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 192.

(10) W. Good, *Biochim. Biophys. Acta*, **44,** 130(1960).

(11) *Ibid.*, **48,** 229(1961).

(12) *Ibid.*, **50,** 485(1961).

(13) *Ibid.*, **52,** 545(1961).

(14) *Ibid.*, **53,** 549(1961).

(15) *Ibid.*, **57,** 104(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 17, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication February 3, 1970.

* Undergraduate Research Participant, NSF/URP Grant GY-4290.

Coumermycin A_1—Biopharmaceutical Studies I

HAROLD L. NEWMARK and JULIUS BERGER

Abstract □ Coumermycin A_1, as the free acid or simple salts, is poorly absorbed from the gastrointestinal tract of animals and humans. It is not appreciably degraded in the fluids of the gastrointestinal tract. Mixture of coumermycin with certain additives, such as sugar amines, can significantly enhance oral absorption in dogs, as measured by the increased blood levels achieved. *N*-Methylglucamine, in a 1:4 ratio of antibiotic to *N*-methylglucamine, enhances blood levels 5–15-fold over the antibiotic alone in both dogs and humans.

Keyphrases □ Coumermycin A_1—absorption, oral administration □ Blood levels, coumermycin A_1—formulation effect □ Stability, coumermycin A_1—gastrointestinal tract □ Microbiological analysis—coumermycin A_1

Coumermycin A_1 is a highly potent antibiotic whose antimicrobial spectrum qualitatively resembles that of novobiocin (1–4). Tests *in vitro* and subcutaneously in mice indicated antibacterial activity, particularly against Gram-positive organisms, at concentrations and doses far lower than for novobiocin (*i.e.*, 1/10 to 1/30). However, early trials of the antibiotic orally in humans gave very poor or nondetectable blood levels (5–7). Blood levels far lower than 1 mcg./ml. were achieved even on doses up to 2 g./day, although several forms of the antibiotic were tested, including the water-soluble disodium salt (7), enteric coating (5, 7), and mixing with polyethylene glycol 300 (Carbowax 300) (7). The total amount of antibiotic accounted for in body fluids, calculated at the maximum blood levels achieved, represented less than 0.1% of the total oral drug intake in these clinical studies.

For mice, the toxicity expressed as LD_{50} in mg./kg. for coumermycin has been reported to be p.o., > 4000 (1); i.p., 183 (1); s.c., 380 (1); and i.m., 500 (8). The median curative dose in experimental *Staphylococcus aureus* (Smith) infection in mice has been reported (1, 3) as 0.13 mg./kg. s.c. and 4.3 mg./kg. p.o. Comparable data were reported by others (10). These large differences in toxicity and activity depending on the route of administration strongly suggest poor absorption. The problem of achieving elevated coumermycin blood levels thus can be posed as one of faulty or poor absorption from the gastrointestinal (GI) tract or of instability of the drug on oral administration. Since the preferred route for antibiotic administration is oral, it was considered essential before embarking on clinical trials to attempt to achieve improved blood levels by this route. The dog was chosen as a test animal since it had been found useful in studies of similar problems with novobiocin (9). Considering the *in vitro* antimicrobial activity of coumermycin in the presence of blood serum, a blood level of at least 1 mcg./ml. was somewhat arbitrarily selected as a desirable goal to allow for a reasonable safety factor, pending eventual clinical verification.

MATERIALS AND METHODS

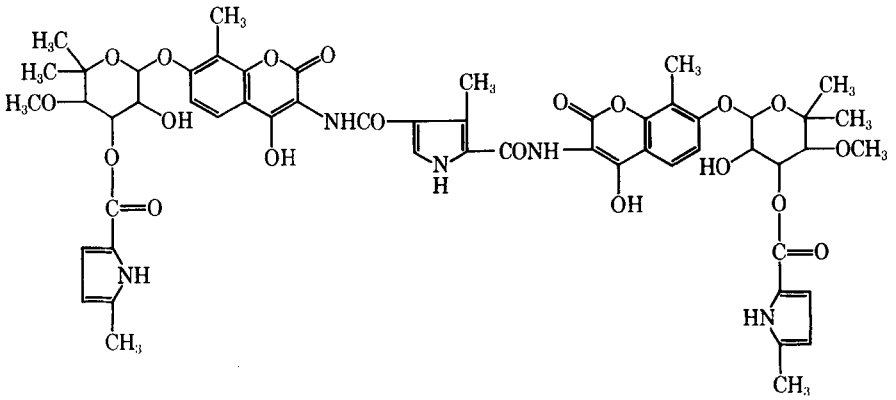
Blood Level Assays—Early studies were performed using an antibiotic bioassay employing serial broth dilutions of 2- or 3-fold, with *Staphylococcus aureus* as the test bacterium. Results of this method had the inherent and substantial potential error of the factor of dilution. During the course of the work, an agar diffusion cup-plate antibiotic bioassay was developed which had a precision of better than ±20% (4). Blood samples (serum) were diluted in 20% dimethylsulfoxide–3% aqueous phosphate buffer solution, pH 6.3, to a concentration which gave inhibition zones against the test organism *S. aureus* within the proper dose–response range (0.5 mcg./ml.). The standard coumermycin monosodium salt was similarly diluted in control blood serum. Quantitative evaluations were obtained as described in (4). Most of the data reported are with the latter method, unless otherwise indicated.

Administration of Drug to Dogs—For oral studies, dogs fasted 12–18 hr. were administered a single dose of about 5 mg./kg. of coumermycin monosodium salt as a fine powder (average diameter 7 μ) in gelatin capsules. Blood samples were taken at various time intervals as indicated later. It was found that administering an oral dose of water to the dog immediately after the drug dose gave higher and more consistent results. Both males and females were used, since no sex-related difference in response was found.

A parenteral aqueous solution of the monosodium salt of coumermycin A_1 was prepared to contain 20 mg./ml. of coumermycin A_1 monosodium salt (Ro 5-4645/10), 10% *N,N*-dimethyl acetamide, 10% ethanol, 40% propylene glycol, and 1.5% benzyl alcohol in water for injection. This solution was injected intravenously at a drug dose of about 10 mg./kg. Blood samples were taken and the sera assayed as previously indicated.

EXPERIMENTAL

Coumermycin A_1 (formerly also known as sugordomycin D-1a or notomycin), mol. wt. 1110, has the chemical structure (2, 4) shown as I.



I

It is a bishydroxycoumarin with two weakly acidic groups, pKa = 5.8–6.2 depending on solvent. [Kawaguchi *et al.* (1, 2) reported the pKa as 7.76 in 75% dioxane–water and 6.35 in 75% dimethyl formamide–water.] The pyrrole groups have very weak acidic properties, pKa above 11. The drug is sensitive to hydrolysis and rearrangement in strongly alkaline solution. A 1% solution of the soluble disodium salt has a pH ~10. This contrasts with novobiocin, pKa about 3.8, which forms a readily soluble monosodium salt which is only slightly alkaline (9).

Coumermycin free acid has no melting point but decomposes at about 245°, as does the crystalline monosodium salt (8). Coumermycin is soluble in aqueous alkali (*i.e.*, above pH 10), dioxane, dimethylformamide, and dimethylacetamide, and moderately soluble in acetone, methyl isobutyl ketone, methyl ethyl ketone, and ethyl acetate. It is poorly soluble in ethanol, methanol, isopropyl alcohol, chloroform, and benzene, and essentially insoluble in carbon tetrachloride, petroleum ether, and neutral and acidic water. It has a slight solubility in aqueous 6 *N* urea. When dissolved in aqueous alkali and neutralized with dilute acid, precipitation is rapid to form a dense particulate solid. Similarly, dilution of organic solvent solutions (*e.g.*, dimethylacetamide) with water results in rapid precipitation. In dilute aqueous alkali solution, coumermycin has strong surfactant properties. These stability, solubility, and physicochemical properties were taken into consideration in the selection of additives to be tested for enhancement of absorption of the drug.

Stability—To determine the importance of the stability of the antibiotic on blood levels, the effect of several experimental conditions was investigated.

Recovery from Blood In Vivo—The parenteral solution of the drug was injected intravenously into dogs at ~10 mg./kg. Blood samples were taken and the sera were bioassayed. The results were as follows (average of two dogs):

Time after Injection, hr.	Blood Levels (Dilution Assay),[1] mcg./ml.
0	0
0.5	160
1.0	160
2.0	70
4.0	70
24.0	3

Assuming a blood volume about 8% of the dog body weight, the peak level of 160 mcg./ml. in blood is about 1.3 times the administered dose. This is essentially 50–200% recovery of the injected drug, considering the errors of the method used. The results thus indicate that the drug was not rapidly destroyed nor eliminated from the blood.

In the Gastrointestinal Tract—In Vitro—An aqueous solution of the freshly prepared disodium salt of coumermycin was added to freshly prepared gastric juice USP to give a concentration of 20 mcg./ml. Full antibiotic activity (dilution bioassay) was maintained when incubated for 4 and 7 hr., respectively, at 37°.

In Vivo—The pyloric sphincter of a dog was tied off surgically. A dose of 500 mg. of coumermycin monosodium salt was administered orally. Blood samples were taken periodically from the gastroepiploic vein leading from the stomach, as well as samples of the gastric contents by aspiration into a syringe with saline. The bioassay results indicated essentially 100% retention of activity in the gastric contents for 4 hr., while the blood samples (1, 2, and 4 hr.) showed <1 mcg./ml.

These tests indicated that coumermycin is relatively stable in the GI tract, certainly in the stomach, and is not destroyed rapidly by the low pH (about 1.5) or enzymes of gastric juice *in vitro* or *in vivo*. It also appears stable to intestinal juice (pH about 8) *in vitro*.

Solubility—It was hypothesized that a system that would solubilize coumermycin at the pH range of the GI tract in aqueous solution would permit more efficient absorption. Several different approaches were made.

[1] It should be pointed out again that the serial broth dilution method, employed in this early exploratory experiment, is subject to a potential twofold error and is probably most likely the reason that the 1- and 4-hr. blood values did not "appear" to decline from the preceding values.

Table I—Coumermycin Blood Levels in Dogs after Single 5-mg./kg. Oral Dose (Antibiotic Content) in Different Formulations

Coumermycin Preparation[a]	Dosage Form	Number of Animals	Average Blood Levels, mcg./ml., Hours after Administration 2	4
Monosodium salt (micronized)	Capsule	6	0.4	0.4
Disodium salt	Capsule	2	1.1	0.8
Dicholine salt	Capsule	2	0.3	0.4
Di NMG salt	Capsule	3	0.3	0.2
Monosodium salt with NMG (1:1)	Capsule	2	1.1	1.3
Monosodium salt with NMG (1:4)	Capsule	3	2.1	3.1
Monosodium salt with NMG (1:10)	Capsule	3	7.5	4.0
Free acid + NMG (1:4)	Capsule	7	1.9	1.2
Monosodium salt with glucosamine (1:4)	Capsule	2	2.9	2.3
Monosodium salt with *N*-acetylglucosamine (1:4)	Capsule	2	1.1	0.8
Monosodium salt with *N*-acetylgalactosamine (1:4)	Capsule	2	2.3	1.6
Monosodium salt, Emulphor, DMA (1:2.5:2.5)[b]	Water-dispersible solution	3	1.5	4
Monosodium salt, poloxalene, DMA (1:2.5:2.5)[b]	Water-dispersible solution[c]	2	2	2
Monosodium salt, 0.5% in 70% DMSO[b]	Solution	2	3.8	6.3
Monosodium salt, urea (1:5)[b]	Capsule	2	1.8	0.8
Monosodium salt, urea, DMA (1:5:4)[b]	Capsule	4	3.8	1.4

[a] Numbers in parentheses are the ratio of the weights of the components used in the test preparation in order of listing. [b] Assays performed by serial dilution method. [c] Administered at 50 mg./kg.

Soluble Salts or Ion-Pairs—A series of disubstituted salts or ion-pairs of coumermycin was prepared and their aqueous solutions neutralized by dilute acid to pH 7. Most of these cases resulted in rapid precipitation of free coumermycin or its monosubstituted salt in the form of dense particles. These included magnesium, sodium, calcium (insoluble), aluminum (insoluble), diethanolamine, triethanolamine, lysine, glycine, trishydroxymethyl aminomethane (THAM), proline, leucine, and choline.

Sugar Amines—When aqueous solutions of coumermycin in dilute alkali are neutralized in the presence of sugar amines, a gel is formed rather than dense particles. This is analogous to the "gel-like" precipitate of amorphous novobiocin formed on careful acidification of its sodium salt solution, as reported previously (8). In particular, *N*-methylglucamine (NMG) aqueous solutions with coumermycin produce aqueous gels on neutralization with acid. A 2.5% solution of coumermycin with 10% NMG (pH about 11) will form an opalescent, solid gel on neutralization by dilute HCl or dilute acetic acid. These gels do not appear to have appreciable crystalline coumermycin. Similar behavior can be observed with glucosamine, *N*-acetylglucosamine, and *N*-acetylgalactosamine. A large molar excess of the sugar amine appears to be required.

Complexing Agents—Coumermycin contains two hydroxycoumarin groups that are potentially capable of forming soluble complexes with polyoxyethylene groups. Indeed, Kawaguchi *et al.* (7) claimed some slight success in using polyethylene glycol 300 (Carbowax 300) to enhance blood levels. It was found that solutions of coumermycin monosodium salt in dimethylacetamide with a series of substances containing polyethylene glycol units, including some nonionic surfactants, did form micellar, slightly opalescent dispersions on dilution with water. These include

Table II—Coumermycin Blood Levels[a] in Humans after Single 250-mg. Oral Dose

Preparation	Blood Levels, mcg./ml., Hours after Dose 0	2	4	8	24
Monosodium salt (micronized)	<0.01	0.01 (0.01–0.1)	0.14 (0.09–0.6)	0.34 (0.15–0.52)	0.16 (0.05–0.44)
Monosodium salt plus NMG (1:4)	<0.01	0.94 (0.25–1.5)	2.4 (1.3–2.8)	2.5 (1.3–4.0)	0.71 (0.4–1.2)

[a] Values are average of six patients in each group; the range is given in parentheses.

polysorbate 80, an emulsifying agent (Emulphor EL-620), a propylene glycol derivative (Pluronic F-68), polyethylene glycol 300, polyethylene glycol 4000, and polyethylene glycol monoricinoleate.

Formulations—A summary is presented in Table I of the data obtained for blood levels in dogs administered different formulations of coumermycin designed to give enhanced levels. A review of these results in dogs resulted in the selection of NMG formulation with the monosodium salt of coumermycin for trial in humans, using a 1:4 weight ratio equivalent to about 1:20 molar ratio. Although higher ratios of NMG to coumermycin increased the blood levels of drug in dogs, practical limitations such as capsule size led to the choice of 1:4 for clinical capsules. NMG has the practical advantages of being commercially available, nonhygroscopic, and chemically stable.

Capsules containing 50 mg. of coumermycin (monosodium salt) with 200 mg. of NMG were tested in humans in comparison to capsules of micronized coumermycin monosodium salt alone. The capsules were administered as a single oral dose of 250 mg. of coumermycin to six humans in each group. The results are presented in Table II.

DISCUSSION

The blood level data presented of coumermycin with NMG in dogs and humans clearly show the enhancement of absorption produced by this additive. At a ratio of 1:4 (drug–NMG), both dogs and humans show a 5–15-fold enhancement of oral absorption of coumermycin as measured by blood levels achieved. This effect is produced by simple physical mixture of the two ingredients in capsules. This represents about a 20 molar ratio of NMG to coumermycin. *In vitro*, this mixture dissolves in water to form a solution at pH 10.5–11.0, which in turn gives a gel-like noncrystalline mass on acidification. Presumably, a similar dissolution and then acidification to form a gel occur in the stomach after oral administration. The resultant gel probably has a greatly enhanced surface area to aid in absorption of coumermycin from the GI tract.

The blood levels achieved with the 1:4 mixture in dogs at 5 mg./kg. p.o. are roughly comparable to those achieved with the same mixture at 250 mg./human p.o. (approximately equivalent to 4 mg./kg.). This substantiates the choice of dogs as a test animal for biopharmaceutical studies of coumermycin.

The NMG mixture with coumermycin rapidly achieves blood levels appreciably higher than the 1 mcg./ml. originally desired on single doses of 250 mg./human, and levels above 1 mcg./ml. are maintained for over 12 hr.

SUMMARY

Coumermycin A_1, as the free acid or simple salts, is poorly absorbed from the GI tract of animals and humans. It is not appreciably degraded in the fluids of the GI tract. Mixtures of coumermycin with certain additivies, such as sugar amines, can significantly enhance oral absorption in dogs, as measured by the increased blood levels achieved. NMG, in a 1:4 ratio of antibiotic to NMG, enhances blood levels 5–15-fold over the antibiotic alone in both dogs and humans.

REFERENCES

(1) N. Kawaguchi, H. Tsukiura, M. Okanishi, T. Miyaki, T. Ohmori, K. Fujisawa, and H. Koskiyama, *J. Antibiot. Ser. A*, **18,** 1(1968).

(2) H. Kawaguchi, T. Naito, and H. Tsukiura, *ibid.*, **18,** 11 (1968).

(3) H. Kawaguchi, T. Miyaki, and H. Tsukiura, *ibid.*, **18,** 220 (1968).

(4) J. Berger, A. J. Schocher, A. D. Batcho, B. Pecherer, O. Keller, J. Maricq, A. E. Karr, B. P. Vaterlaus, A. Furlenmeier, and H. Spiegelberg, *Antimicrob. Ag. Chemother.*, **1965,** 778.

(5) W. Abrams, E. Grunberg, and A. J. Schocher, private communications.

(6) S. Nakazawa, H. Oka, H. Sato, N. Cho, H. Sunada, S. Nohara, K. Kunii, and K. Arai, *Chemotherapy*, **18,** 580(1966).

(7) H. Kawaguchi, H. Yeda, and S. Ishiyama, *Int. Congr. Chemother. 5th*, **1,** 1015(1967).

(8) J. D. Mullins and T. J. Macek, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 245(1960).

(9) H. Kawaguchi, M. Okanishi, and T. Miyaki, U. S. pat. 3,201,386, Aug. 17, 1965.

(10) E. Grunberg and M. Bennett, *Antimicrob. Ag. Chemother.*, **1965,** 786.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 20, 1969, from *Product Development Department and Chemical Research Department, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication February 18, 1970.

The authors thank Drs. J. Gerke, E. Grunberg, A. J. Schocher, and J. Unowsky for various microbiological assays; Dr. E. Whitman for the tests in humans; and Drs. R. Bagdon, R. Banziger, W. Pool, L. Hines, and S. Rubin for assistance in the design and execution of the animal tests.

Coumermycin A_1—Biopharmaceutical Studies II

HAROLD L. NEWMARK, JULIUS BERGER, and J. THURØ CARSTENSEN

Abstract □ Coumermycin monosodium salt in a 1:4 w/w mixture with *N*-methylglucamine is absorbed with an efficiency of about 20–25% in dogs and humans after oral administration. This is 5–15-fold the oral absorption efficiency of the drug alone. On intravenous or oral administration, blood levels decline exponentially after 3–4 hr. in dogs and after 4–6 hr. in humans; the half-lives of these declines are 8–9 hr. in dogs and 8–10 hr. in humans. Blood levels well over 1 mcg./ml. are readily achieved on oral dosage of 4–5 mg./kg. in dogs and humans.

Keyphrases □ Coumermycin A_1—biopharmaceutical characteristics □ Blood levels, coumermycin A_1—administration route effect □ Distribution—coumermycin A_1 □ Microbiological analysis—coumermycin A_1

The antibiotic coumermycin A_1, as the free acid or most simple salts, is poorly absorbed from the gastrointestinal (GI) tract of dogs and humans. In a previous paper, the authors reported that a mixture of the monosodium salt with *N*-methylglucamine (NMG) (1:4 by weight) in capsules enhanced blood levels on oral administration in dogs and humans by 5–15-fold (1). The present studies were made to determine the biopharmaceutical characteristics of this preparation *in vivo*.

EXPERIMENTAL

Materials and Methods—Oral and parenteral preparations were formulated in the following manners. Gelatin capsules of micronized coumermycin A_1 monosodium salt were prepared containing 50 mg. of drug and designated Capsules A. Capsules containing a mixture of 50 mg. of micronized coumermycin A_1 with 200 mg. of NMG were made and designated Capsules B. A fresh parenteral solution of coumermycin A_1, 50 mg./ml., was prepared in a solvent consisting of 30% propylene glycol, 10% ethyl alcohol, 1% benzyl alcohol, 0.5% NMG, and water for injection, with the pH adjusted to 9.4 by sodium hydroxide. In the parenteral solution, there is only a minimal amount of NMG, the ratio of drug to NMG being 1:0.1 or 1/40 of the ratio in the capsules.

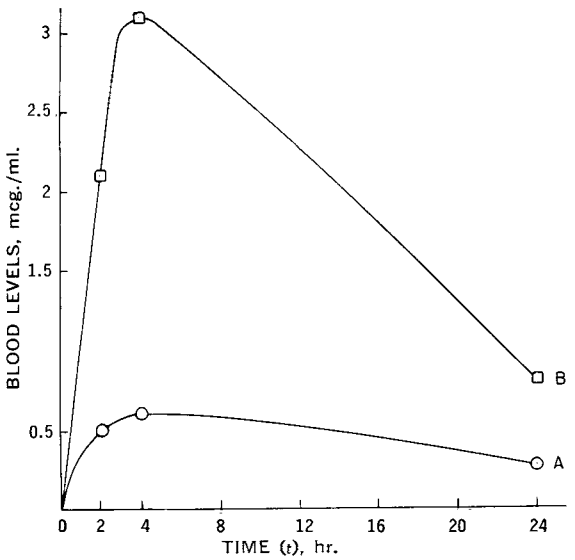


Figure 1—*Coumermycin blood levels in dogs after a single oral dose, 5 mg./kg., of: A, capsules of coumermycin alone; and B, capsules of coumermycin + NMG (1:4). Each group represents the average of six dogs.*

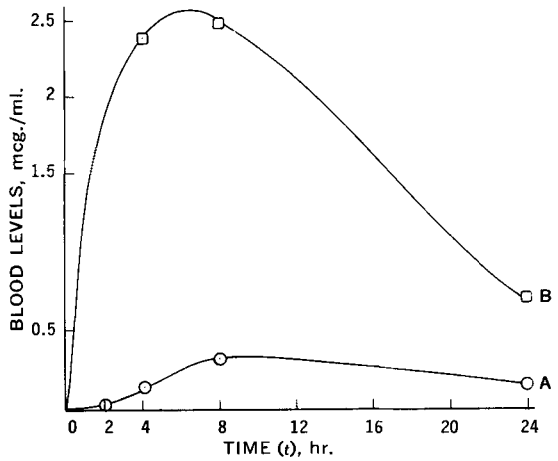


Figure 2—*Coumermycin blood levels in humans after a single oral dose, 4 mg./kg., of: A, capsules of coumermycin alone; and B, capsules of coumermycin + NMG (1:4). Each group represents the average of six subjects.*

Studies in Dogs—Dogs, each weighing about 10 kg., fasted 12–18 hr., were given capsules of drug formulation to provide the equivalent of about 5 mg./kg. Blood samples were taken at intervals and assayed microbiologically by a cup-plate assay for coumermycin concentration (1). Oral absorption efficiency was tested as follows: the parenteral formulation was administered intravenously at 0.5 and 2.5 mg./kg., respectively, to two dogs. Six other dogs were each given orally a single Capsule B. Blood samples were taken at intervals and measured for coumermycin concentration.

The parenteral preparation also was used to permit examination of the difference in blood levels achieved after intramuscular and intravenous administration, measured 1 hr. after injection, in comparison with the oral levels. The effect of repeated daily intravenous injection also was examined after administration at three different dosage levels.

Studies in Humans—Six human subjects were given a single oral dose of five Capsules A, representing a dose of approximately 4 mg./kg. of drug alone. Six other human subjects were given a single oral dose of five Capsules B, similarly representing a dose of approximately 4 mg./kg. of drug content, but in the presence of NMG. Blood samples were taken at intervals and measured microbiologically for coumermycin concentration. For the study of oral absorption efficiency in humans, a single human adult subject (L.R.) was injected intravenously with 50 mg. of coumermycin in parenteral solution; samples of blood were taken periodically for measurement of coumermycin concentration. Subsequently, the same subject was retested with a dose of 100 mg. i.v. In some experiments with dogs and humans, samples of urine and stools were also examined for the presence of excreted, unchanged antibiotic.

RESULTS AND DISCUSSION

The results of a single dose, oral administration, of antibiotic with and without NMG are presented for dogs in Fig. 1 and for humans in Fig. 2. The effect of NMG in enhancing the oral absorption, as indicated by elevation of blood levels, is clearly indicated. From the individual values and comparison of the area under the curves, the enhancement by NMG appears to be in the range of 5–15-fold over the drug alone in both dogs and man. The post-absorptive half-life is calculated to be about 10 hr.

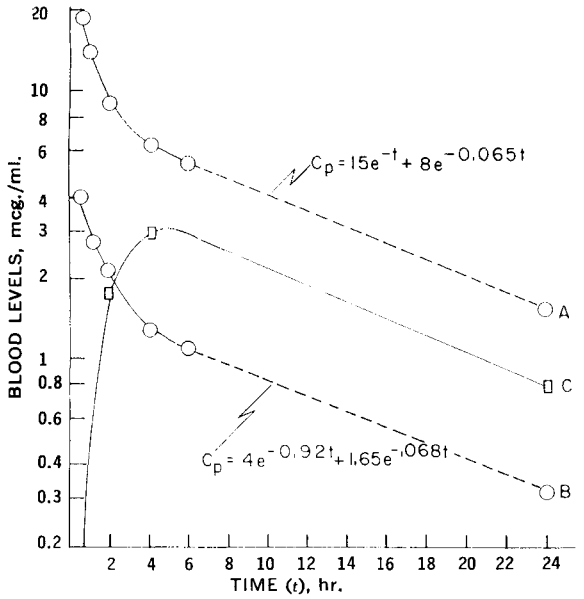


Figure 3—*Blood levels of coumermycin in dogs after 2.5-mg./kg. i.v. dose (A), 0.5-mg./kg. i.v. dose (B), and 2.5 mg./kg. p.o. (C). Data are presented in semilogarithmic form, the linear form of the corresponding equations being indicated in the two intravenous curves. The area under the oral curve is estimated in a linear graph (not shown).*

The efficiency of absorption of coumermycin in the NMG mixture can be calculated from the data of Fig. 3 in dogs and of Fig. 4 in humans. As pointed out by Riegelman *et al.* (2), a peripheral compartment may be detected only in the early phases subsequent to administration of a drug. By considering the present case a two-compartment, open-system model:[1]

$$\text{metabolism, elimination} \xleftarrow{k_{el}} \text{blood} \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} \text{tissue} \quad \text{(Eq. 1)}$$

the equation governing the time-dependent concentration should be: $C_p = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t)$, with a value extrapolated to zero time of $C_p^\circ = A + B$.

The parameters, A, B, α, and β, may now be evaluated from the curves by the feathering technique. The parameter values found are indicated in Figs. 3 and 4.

Once the parameters in the double-exponential decay curve are known, the forward and reverse diffusion constants k_{12} and k_{21} and the disposition constant k_{el} may be determined readily. Values of these for dog (from Fig. 3) and man (from Fig. 4) are listed in Table I.

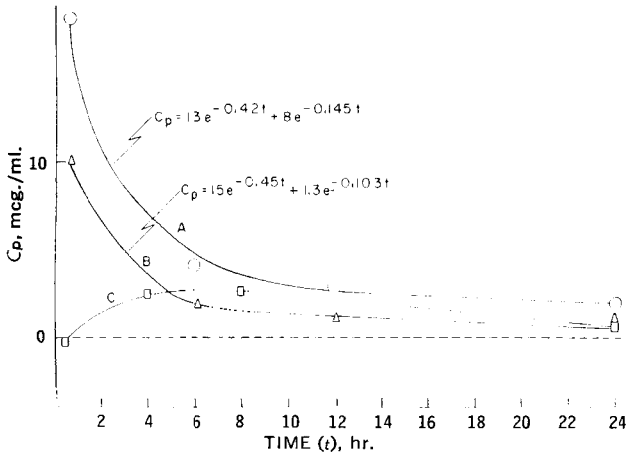


Figure 4—*Blood levels of coumermycin in man after 100-mg. i.v. dose (A), 50-mg. i.v. dose (B), and 250 mg. p.o. (C). The data are presented in linear form, and the appropriate least-squares equations indicated. The area under Curve C can be estimated by graphical means.*

[1] The notations of Riegelman *et al.* (2) are followed here.

The amount of drug in the tissue compartment (T) at time t is governed by the differential equation:

$$\frac{dT}{dt} = k_{12}[A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t)] - k_{21}T \quad \text{(Eq. 2)}$$

This is readily solved, using the integrating factor $\exp(k_{21}t)$ and imposing the initial condition that $T = 0$ at $t = 0$ yields the solution:

$$T = \frac{A \cdot k_{12}}{k_{21} - \alpha}[\exp(-\alpha \cdot t) - \exp(-k_{21}t)] + \frac{B \cdot k_{12}}{k_{21} - \beta}[\exp(-\beta \cdot t) - \exp(-k_{21}t)] \quad \text{(Eq. 3)}$$

This quantity integrated over time is used here as a measure of absorption, *i.e.*,

$$I = \int_0^\infty T dt = \frac{A \cdot k_{12}}{[k_{21} - \alpha]} \cdot \left[\frac{1}{\alpha} - \frac{1}{k_{21}}\right] + \frac{B \cdot k_{12}}{[k_{21} - \beta]} \cdot \left[\frac{1}{\beta} - \frac{1}{k_{21}}\right] \quad \text{(Eq. 4)}$$

Values of this measure of absorption (I) as found in dog and man are listed in Table I.

It is noted that, in the dog, absorption is linearly related to dose (188 = 5.42), whereas proportionality is not quite as apparent in man. In the latter case, however, I depends strongly on the accuracy of $k_{21} - \alpha$ and $k_{21} - \beta$. All dose-independent constants (k_{12}, k_{21}, k_{el}, α, and β) correlate well from low to high dosage. However, only a few points in time are used here, and the values in Table I, at best, should only be used for qualitative comparison. As reported by Wagner and Northam (3), the distribution volumes, when calculated in this fashion, are likely to be overestimates.

Equation 4 cannot be used for estimation of absorption by the oral route, but an insight into the physiological availability of the oral dosage form may be gained by use of the absorption definition of Wagner and Nelson (4), *i.e.*, by determining the area (a) under the curve in Fig. 4 (in linear presentation). In this manner, a value of 19 mcg.-hr./ml. is obtained for the 250-mg. p.o. dose. This is comparable to the 50-mg. i.v. data in Table I, implying a 20% efficiency *via* the oral route.

Similar figures are obtained in the case of the dog, where values are: A_∞ = 138 mcg.-hr./kg.-ml. for 2.5 mg. i.v.; A_∞ = 29 mcg.-hr./kg.-ml. for 0.5 mg./kg. i.v.; and 52 mcg.-hr./kg.-ml. for 5 mg./kg. p.o., so that 5 mg./kg. p.o. is equivalent to about 1 mg./kg. i.v., yielding an efficiency of about 20% *via* the oral route. A more straightforward procedure is to assume that the curve of the 5-mg./kg. p.o. dose, after 4 hr., is approximately equivalent to a hypothetical curve of the 1.25-mg./kg. i.v. dose, obtained by interpolation between 0.5-mg. and 2.5-mg./kg. i.v. doses, *i.e.*, 25% efficiency. Similar treatment of the data in Fig. 4 may be made,

Table I—Biopharmaceutical Parameters of Coumermycin A_1 on Intravenous Injection

Figure	3	3	4	4
Curve	B	A	B	A
B, mcg./ml.	1.65	8.0	8.0	1.3
β, hr.$^{-1}$	0.068	0.065	0.145	0.103
A, mcg./ml.	4	15	13	15
α, hr.$^{-1}$	0.92	1	0.42	0.45
C_p°, mcg./ml.	5.65	23	21	16.3
Dose, D[a]	0.5	2.5	100	50
Distribution volume, $V_p = D/C_p^\circ$, l.	0.89	0.108	4.75	3.3
A/α	4.35	15	55.2	33.3
B/β	24.2	123	31	12.6
C_p°/k_{el}	28.6	138	86	45.9
k_{el}, hr.$^{-1}$	0.20	0.17	0.24	0.36
$\alpha\beta/k_{el} = k_{21}$, hr.$^{-1}$	0.32	0.39	0.25	0.13
$\alpha + \beta - k_{21} - k_{el} = k_{12}$, hr.$^{-1}$	0.47	0.53	0.08	0.06
I, mcg.-hr./ml.	42	188	28	20

[a] Doses in the first two columns refer to mg./kg., in the last two simply to mg. The distribution volume, therefore, refers to l./kg. and liters, respectively.

Table II—Blood Levels in Dogs after Intramuscular and Intravenous Administration

Dose, mg./kg.	Route	Blood Level, 1.0 hr. after Dose, mcg./ml.[a]
10	Intramuscular	26
20	Intramuscular	74
10	Intravenous	85

[a] Each value represents the average of two dogs.

indicating that in humans the oral absorption efficiency of the coumermycin in the mixture with NMG is approximately 20–25%. These figures, of course, are estimates since the two compartments are actually not in steady state of equilibrium throughout the cited period.

The intravenous curves in humans (Fig. 4) show that the early (0.5 hr. after dose) values are essentially dilutions of the drug in the blood volume. Thus, 50 mg. i.v. in a human adult with about 5 l. of blood and fluid in equilibrium with it calculates to 10 mcg./ml. which is what was obtained. A similar calculation for the 100-mg. case yields a value of 20 mcg./ml. as compared to the 18 mcg./ml. actually found. It is noted that it requires 3–4 hr. in dogs and 4–6 hr. in humans before the $A \cdot \exp(-\alpha \cdot t)$-term becomes negligible; after this period the blood level, of course, declines in a conventional first-order fashion. As seen from the limited data here, the overall half-life in humans on 50- and 100-mg. intravenous dose appears somewhat longer than the overall half-life in dogs.

Table II summarizes the data obtained with blood levels in dogs after intramuscular and intravenous administration as measured 1 hr. after the dose. The results suggest some retardation of movement of the drug from the intramuscular site. Thus, it appears that at least twice the dose by the i.m. route is required to achieve the same blood levels as those obtained 1 hr. after i.v. dosing.

The effect of repeated daily i.v. injections of coumermycin A_1 on blood levels in dogs is shown in Table III. Levels were measured 1 hr. after the first, fifth, and tenth daily injection. These data suggest that on i.v. administration, increasing dosage increases blood levels after the first dose but not linearly. After the initial dose, the subsequent increase in blood levels after repeated dosage is greater than the multiple of dose increase. This suggests decreased migration of the drug out of the blood into tissue stores with increased repeated dosage, possibly approaching saturation of these tissues at higher dosage.

Bioassay of urine samples from dogs or humans, even after high blood levels were achieved, indicated the presence of little or no intact coumermycin A_1. On the other hand, substantial amounts of drug were found in the feces of dogs and humans. These data will be reported later.

Table III—Blood Levels in Dogs after Intravenous Administration of 1, 5, and 10 Daily Doses

Dose, mg./kg.	Blood Level, mcg./ml.,[a] 1.0 hr. after Dose Day 1	Day 5	Day 10
1	4.0 ± 0.4	5.4 ± 0.6	6 ± 1.2
3	17.5 ± 0.5	24 ± 6	27 ± 6
9	71 ± 15	135 ± 18	121 ± 14

[a] Each value represents the average of four dogs.

SUMMARY AND CONCLUSIONS

Coumermycin monosodium salt in a 1:4 w/w mixture with NMG is absorbed with an efficiency of about 20–25% in dogs and humans on oral administration, based on the assumption of a two-compartment open model. This is 5–15-fold the oral absorption efficiency of the drug alone. On intravenous or oral administration, blood levels decline exponentially after 3–4 hr. in dogs and 4–6 hr. in humans; the half-lives of these declines are 8–9 hr. in dogs and 8–10 hr. in humans. Blood levels well over 1 mcg./ml. are readily achieved on oral dosage of 4–5 mg./kg. in dogs and humans.

REFERENCES

(1) H. L. Newmark and J. Berger, *J. Pharm. Sci.*, **59**, 1246(1970).
(2) S. Riegelman, J. C. K. Loo, and M. Rowland, *ibid.*, **57**, 117 (1968).
(3) J. G. Wagner and J. I. Northam, *ibid.*, **56**, 529(1967).
(4) J. G. Wagner and E. Nelson, *ibid.*, **53**, 1392(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 20, 1969, from *Product Development Department and Chemical Research Department, Hoffmann-La Roche Inc., Nutley, NJ 07110*, and from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*

Accepted for publication February 18, 1970.

The authors thank Dr. J. Unowsky, Mr. J. Bontempo, and Dr. J. Gerke for microbiological assays; Dr. E. Whitman for the human studies; and Drs. R. Banziger and W. Pool for the animal tests.

Acetylcholine Tachyphylaxis in Isolated Rabbit Atrium and Its Relation to Norepinephrine Stores

M. DAVID MacFARLANE* and THEODORE KOPPANYI

Abstract ☐ Large concentrations of acetylcholine, in the presence of atropine, are known to produce positive inotropic and chronotropic responses in isolated, spontaneously beating rabbit atria. This response is due to release of norepinephrine from atrial tissue. After repeated concentrations of acetylcholine (100 mcg./kg.), in the presence of atropine (3 mcg./kg.), were added to the bath fluid at 10-min. intervals, the usual stimulatory responses to acetylcholine were not detected; *i.e.*, the tissue was tachyphylactic to the actions of acetylcholine. If acetylcholine, in the presence of atropine, was given at intervals of more than 20 min., tachyphylaxis could not be observed. To investigate a possible link between acetylcholine tachyphylaxis and a decrease in the amount of norepinephrine available for release by acetylcholine, the atria were exposed to norepinephrine after acetylcholine tachyphylaxis was produced. Norepinephrine was allowed to remain in contact with tachyphylactic atrial tissues for 5 min. before being washed out. Acetylcholine now elicited its usual stimulatory effect. Epinephrine was also capable of restoring the response of tachyphylactic atria to acetylcholine. Atria excised from rabbits that were pretreated with reserpine did not respond to either acetylcholine or tyramine. When these preparations were incubated with norepinephrine, there was no return of the stimulatory response to acetylcholine, although the response to tyramine was restored.

Keyphrases ☐ Acetylcholine tachyphylaxis—isolated atrium, rabbit ☐ Norepinephrine stores, relationship—acetylcholine tachyphylaxis ☐ Reserpine effect—acetylcholine, tyramine activity ☐ Atropine effect—acetylcholine tachyphylaxis

Tachyphylaxis is the phenomenon in which repeated administration of a drug to a test system at short time intervals leads to progressively smaller and smaller responses induced by the drug (1). Indirectly acting sympathomimetic amines such as amphetamine (2), tyramine (3), and ephedrine (4), as well as cocaine (5), owe their adrenergiclike effects to release of endogenous norepinephrine from the test tissue, and the tissue develops tachyphylaxis due to the gradual depletion of essential norepinephrine stores (6).

It has been demonstrated that large doses of acetylcholine (ACh), when given either in the presence or absence of atropine, released norepinephrine (NE) from isolated cardiac tissue (7, 8). This release has also been shown for indirectly acting sympathomimetic amines (9). Because of this similarity between the indirectly acting sympathomimetic amines and ACh, the authors attempted to produce tachyphylaxis to the positive inotropic and chronotropic actions of ACh on the isolated spontaneously beating rabbit auricles.

It has also been demonstrated by Cowan *et al.* (1, 3, 4) that after production of tachyphylaxis to indirectly acting sympathomimetic amines, an exposure of the tachyphylactic tissue to NE restores, to a lesser or greater degree, the responses to subsequent challenges with the drug. Therefore, the effect of an exposure of ACh–tachyphylactic atrial tissue to NE and epinephrine (E) was tested. Additionally, the absence of responses to ACh in the tachyphylactic preparation was compared to the absence of response to ACh observed in atrial tissues taken from rabbits that were pretreated with reserpine.

It has been proposed (6, 10, 11) that cardiac tissue has a number of storage compartments for NE and that the different compartments may be induced to release this neurohumor by different stimuli. In these studies, the authors also attempted to differentiate between compartments from which ACh and tyramine release NE.

METHODS

All experiments were performed on the isolated, spontaneously beating rabbit atria. Male albino rabbits, weighing 1.2–2.3 kg., were sacrificed by a blow to the nape of the neck. The heart was then rapidly excised and placed in oxygenated Locke solution of the following composition (in g./l.): NaCl, 9.0; KCl, 0.42; $CaCl_2$ (dihydrate), 0.24; $NaHCO_3$, 0.5; and dextrose, 2.0. The right and left atria were separated together from the ventricles and, after washing and removal of excess tissue, were mounted in a 40-ml. organ bath filled with Locke solution at 30 ± 1° through which 100% O_2 was continuously bubbled.

One end of the atrial preparation was attached to a Starling heart lever, loaded to exert a tension of 1 g., which recorded the amplitude of atrial contractions on a smoked paper kymograph. The rate of contraction was recorded using a Thorp impulse counter calibrated so that 1 mm. of deflection was equal to one contraction.

A 1-hr. period was allowed for equilibrium before the experimental protocol was initiated.

The animals used in the studies requiring pretreatment with reserpine were given 5 mg./kg. i.p. 20–24 hr. prior to the experiment.

Statistical analysis of the experimental data was carried out as described by Batson (12). A probability of 5% ($p = 0.05$) was considered maximum for statistical significance.

The following drugs were used in this study: acetylcholine bromide,[1] *l*-adrenaline (*l*-epinephrine) bitartrate,[1] atropine sulfate,[2] levarterenol (*l*-NE) bitartrate monohydrate,[3] tyramine hydrochloride,[4] and reserpine USP[5] (ampuls, 2.5 mg. base/ml.).

The concentrations of drugs, with the exception of reserpine, were expressed in terms of their respective salts. The concentrations of the solutions were such that it was not necessary to add more than 0.3 ml. of the drug solution to the bath fluid.

RESULTS

Dose–response curves to ACh were determined by exposing the atria to progressively increasing concentrations of ACh. ACh was added to the organ bath 3 min. after an addition of atropine (3 mcg./ml.); each addition of ACh was followed by a 30-min. period with numerous washes prior to repetition of the described procedure.

The dose–response curve to ACh (Fig. 1) shows that the force and rate of atrial contraction increased in a linear fashion with increasing concentrations of ACh up to 100 mcg./ml., after which the change in rate decreased with increasing doses of ACh until a negative chronotropic response became apparent.

[1] Eastman Organic Chemicals, Rochester, N. Y.
[2] Merck & Co., Rahway, N. J.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Mann Research Laboratories, New York, N. Y.
[5] Serpasil, CIBA Pharmaceutical Co., Summit, N. J.

Table I—Responses of Isolated Rabbit Atria (n = 8) to ACh (100 mcg./ml.), in the Presence of Atropine (3 mcg./ml.), before and after the Production of Tachyphylaxis

Parameters Measured	Control Values	Change from Control Values: Control Response to ACh ± *SE*	Change from Control Values: Response to ACh ± *SE* after Production of Tachyphylaxis
Force[a]	22 ± 2.1	+9 ± 1.0	−5 ± 1.3[c]
Rate[b]	126 ± 7.8	+36 ± 4.2	−12 ± 2.4[c]

[a] Mean force in mm. [b] Mean rate in beats/min. [c] Comparison of responses to ACh before and after tachyphylaxis; p = 0.05 or less.

The stimulatory response to ACh (100 mcg./ml.), in the presence of atropine (3 mcg./ml.), was characterized by a very short latent period (2–4 sec.) followed by a marked stimulation of both force and rate. After reaching their maximum, the parameters started to return to control levels; when this occurred the bath fluid was exchanged repeatedly. These responses could be reproduced many times when ACh and atropine were given at 30-min. intervals. Results from all subsequent experiments are compared with these described responses.

Production of Tachyphylaxis to ACh—For the demonstration of true tachyphylaxis, certain criteria must be satisfied. The same concentration of ACh must be administered at equal time intervals, and all measured parameters must be allowed to return to predrug levels prior to the next administration of the challenging agent. A concentration of 100 mcg./ml. of ACh was used and was preceded for 3 min. by atropine (3 mcg./ml.). An interval of 10 min. between ACh responses was found to be optimal for the production of tachyphylaxis. When the time interval between responses was prolonged to 20 min. or longer, tachyphylaxis could not be produced. The response to ACh lasted about 1 min.; thus there was a sufficient time interval between drug additions to allow all parameters to return to control levels.

Using these experimental conditions, complete or nearly complete tachyphylaxis was produced following the administration of four doses of ACh. The response of atria to the administration of ACh was considered to be tachyphylactic when the preparation no longer produced an augmentation of atrial contractions. In most experiments, augmentation was replaced by depression. Table I summarizes the measurements obtained in the production of tachyphylaxis.

When the aforementioned schedule of drug additions was continued throughout the duration of the experiment, a spontaneous return of the normal positive inotropic and chronotropic response to ACh could not be observed. However, if after the production of tachyphylaxis the administration of ACh was discontinued for a minimum of 35–45 min., a spontaneous return of the normal response to ACh was observed.

After the establishment of tachyphylaxis to ACh, the indirectly acting sympathomimetic amine, tyramine (5 mcg./ml.), was added to the atrial bath and produced its characteristic augmentation of atrial contractions. Thus, the authors have found no evidence of cross tachyphylaxis between the stimulatory actions of ACh and tyramine.

Reversal of Tachyphylaxis to ACh by Catecholamines—Since the stimulatory response of atria to additions of ACh is the result of a release of NE (7), the development of tachyphylaxis may be attributed to a decrease in the availability of releasable NE and, therefore, should be modified, or perhaps reversed, by exposure to this amine.

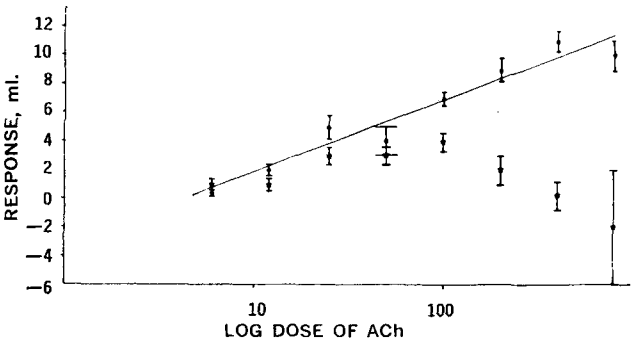


Figure 1—*Dose–response curve to ACh in the presence of atropine (3 mcg./ml.). Key:* ⟊, *force* ± SE; ⟊, *rate* ± SE.

Tachyphylaxis was produced in the manner previously described. Upon production of tachyphylaxis, the atria were incubated with NE (0.025 mcg./ml.) for 5 min. The NE was then washed out and the response to ACh was tested in the presence of atropine. The NE incubation procedure did not interrupt the 10-min. interval between additions of ACh. Table II demonstrates the effect of NE incubation upon the tachyphylaxis to the stimulatory response to ACh. As can be seen, restoration of the normal positive inotropic and chronotropic response was attained. Figure 2 is a tracing of a typical experiment of this type.

Since incubation with NE proved to be effective in restoring the stimulatory response to ACh in tachyphylactic atria, another catecholamine, E (0.025 mcg./ml.), was employed under similar conditions. Table II shows the results of these experiments. The response of the atria to ACh, following E incubation, was restored to control levels as it was following incubation with NE.

Effect of Other Agents upon the Response of Atria to ACh—Atria were excised from rabbits that had received reserpine[6] (5 mg./kg. i.p.) 24 hr. prior to the experimental procedure. In these preparations, the addition of ACh in the presence of atropine failed to produce a positive inotropic and chronotropic response. This was instead supplanted by a negative inotropic and chronotropic response. When reserpine-pretreated atria were incubated with NE (0.025 mcg./ml.) in the manner previously described, there was no restoration of the normal stimulatory response to ACh as was observed in the tachyphylactic atria. Table III summarizes the results of these experiments. However, a restoration of the response to tyramine in reserpine-pretreated atria could be demonstrated following incubation with NE.

DISCUSSION

The positive inotropic and chronotropic effect of ACh on the atropine-pretreated atria is a well-known phenomenon (13–15). Hoffman *et al.* (14) and Middleton *et al.* (16) have proposed that this atrial stimulation is a result of release of an epinephrinelike substance from cardiac tissue, either due to ganglionic stimulation or release from chromaffin tissue. The investigations of Richardson and Woods (7) and of Angelakos and Bloomquist (8) have established that this epinephrinelike substance is, in fact, NE. Thus, one must come to the conclusion that ACh exerts its stimulatory action *via* release of NE in the atropine-pretreated isolated rabbit atria.

Table II—Effect of Incubation with Catecholamines upon Responses of Tachyphylactic Isolated Rabbit Atria to ACh (100 mcg./ml.) in the Presence of Atropine (3 mcg./ml.)

Parameters Measured	Control Values	Catecholamine, CA, 0.025 mcg./ml.	Change from Control Values: Tachyphylactic Response to ACh before CA ± *SE*	Change from Control Values: Control Response to CA ± *SE*	Change from Control Values: Response to ACh after CA ± *SE*
Force[a]	18 ± 1.2	Norepinephrine, n = 6	−7 ± 2.5	+8 ± 1.8	+7 ± 1.4[c]
Rate[b]	138 ± 4.2		−6 ± 1.8	+30 ± 10.8	+18 ± 4.8[c]
Force[a]	20 ± 0.9	Epinephrine, n = 5	−6 ± 2.4	+6 ± 2.1	+8 ± 1.1[c]
Rate[b]	114 ± 8.4		−18 ± 8.4	+30 ± 10.8	+30 ± 9.6[c]

[a] Mean force in mm. [b] Mean rate in beats/min. [c] Comparison of responses to ACh before and after CA; p = 0.05 or less.

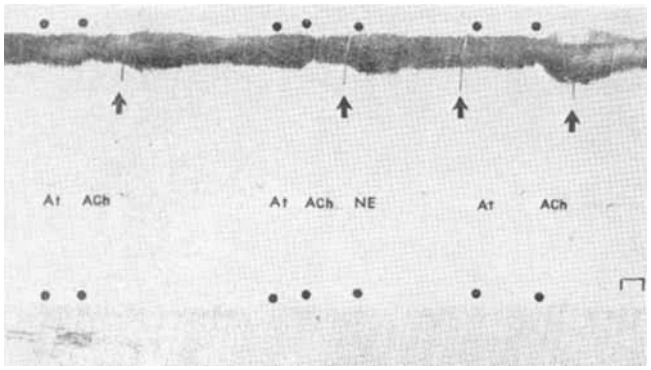


Figure 2—*Effect of NE upon tachyphylaxis to large doses of ACh in isolated rabbit atria.*

The upper tracing measures contractile force and the lower tracing depicts atrial rate. The horizontal bracketed line represents 1 min. The drugs were added at the black dots; ACh, 100 mcg./ml.; At, atropine, 3 mcg./ml.; NE, 0.025 mcg./ml. At the arrows the bath fluid was exchanged three times while the kymograph was arrested. The first two doses of ACh illustrate the response of the tachyphylactic atria to this drug. The third dose of ACh illustrates the response after incubation with NE.

While some experimental evidence (17–19) supports the proposal that ganglionic stimulation is the mechanism by which ACh exerts its effect, results of other studies suggest that this mechanism is not an adequate explanation. Anatomical and histological studies have shown that, although intrinsic ganglia are present in the atria, these structures are all parasympathetic and not sympathetic (20–22). Hirsch *et al.* (23) and Cooper (24), using cardiac transplantation techniques to ensure complete denervation, indicate that ganglionic stimulation by ACh is an unlikely mechanism for the positive inotropic and chronotropic effect in such preparations. Studies using the formaldehyde vapor-condensation technique have shown that there is little or no fluorescence in the atria after sympathetic denervation (25). Lee and Shideman (26), using cat papillary muscle, have demonstrated that, in atropine-pretreated preparations, large doses of ACh produced positive inotropic and chronotropic responses. Careful histological studies of these papillary muscles have shown no ganglia to be present; only sympathetic nerve endings were observed.

It is extremely unlikely, therefore, that ACh produces its stimulatory action by acting through the stimulation of intrinsic sympathetic ganglia in the atria. It is more likely that it is acting by the release of NE from the postganglionic neurons. This may be accomplished in a manner similar to that described by Burn and Rand (27).

In view of this evidence and present experimental data, it would seem more meaningful and correct to characterize the actions of ACh and other nicotinic agents on the atria as indirect actions rather than nicotinic effects. The term nicotinic implies sympathetic ganglionic stimulation, and this is clearly not applicable to atria.

Cowan *et al.* (3, 4), Maengwyn-Davies (28), and Maengwyn-Davies *et al.* (2, 29) have substantiated the general theory of Koppanyi (30) that drugs whose action depends upon the liberation of biogenic substances will show tachyphylaxis due to the gradual depletion of releasable biogenes. Indirectly acting ACh meets these stipulations and is indeed tachyphylactogenic on the rabbit atrium, as are the other indirectly acting atrial stimulants.

Incubation of the atria with NE restores the usual indirect response to ACh, which is lost during tachyphylaxis, presumably by replacement of essential catecholamines. The authors have also shown that E, as well as NE, can reverse the tachyphylaxis to ACh. The restoration of the response to ACh by E is in full agreement with the observations of Potter (31) who showed that E and NE are equally well bound to isolated storage granules. Therefore, in these experiments, E may have acted as a "false transmitter." Another explanation might be that of Raab and Gigee (32) who proposed that E may displace some NE from storage sites and then be demethylated to NE.

There seems to be some question as to whether there is a depletion of NE when the stimulatory response to ACh is absent. Using somewhat different methods, Torchiana and Angelakos (33) have demonstrated that when the stimulatory response to ACh in atropinized preparations was absent, there was no significant depletion of NE from the atrial tissue. Furthermore, they have shown that ACh administered to nonatropinized cardiac tissue causes a liberation of NE, accompanied by an increase rather than a decrease in cardiac catecholamines, without, of course, a positive inotropic and chronotropic response (8). This failure to demonstrate a depletion of NE could be interpreted in two ways. First, these authors administered acetylcholine at 20-min. intervals. At this time interval, there could be a restoration of liberated catecholamines by the atrial tissue since the present authors have shown that ACh administered at 20–30-min. intervals failed to produce tachyphylaxis. Secondly, the ACh releasable store may be small, and depletion of this store would not give a significant reduction in the total catecholamine content of the atria. These proposals may gain support in the observations that the atria is still responsive to tyramine while tachyphylactic to ACh. Moreover, there must be some depletion of NE, as indicated by the observation that tachyphylaxis to ACh is reversed by incubation of the atria with this neurohormone.

In atria excised from reserpine-pretreated rabbits, all agents acting by the release of NE are ineffective. The authors have shown this to be true for ACh, just as it has been shown for nicotine (34), DMPP (35), and tyramine (3). This lack of response in all cases is most probably a result of the depletion of NE by reserpine (36). In the studies reported here, however, atria from reserpine-pretreated rabbits which showed no indirect stimulating response to ACh could not be made to respond upon incubation with catecholamines while the response to tyramine was reinstated. This difference in behavior of indirectly acting ACh and of tyramine and DMPP in atria from reserpine-pretreated animals could be explained by the depressant action of ACh (Table III) and the lack of such depressant action by tyramine. The depressant effect of ACh may be due to stimulation of receptor sites not occupied by atropine, resulting in atrial depression (37).

The lack of cross tachyphylaxis between ACh and tyramine, and the restoration of the response to tyramine but not to ACh in reserpine-pretreated atrium, indicate that these two agents release NE from different stores. When the ACh-releasable store is apparently depleted, the tyramine-releasable store is still unaffected.

The results seem to indicate that there is no uptake of NE by the ACh-releasable compartment in reserpinized atria. If NE was taken up, some would probably be released by ACh, with the result (which is not observed) being a reduction or reversal of the depressant response to ACh. The reserpine-pretreated atria was, however, capable of taking up and releasing NE, as indicated by the restoration of the atrial response to additions of tyramine.

The contrast between the effects of reserpine pretreatment and tachyphylaxis on the atrial response to ACh is quite perplexing. In the case of tachyphylaxis, the negative inotropic and chronotropic effects of large, oft-repeated doses of ACh can be easily converted into atrial stimulation by incubation with catecholamines; thus the tachyphylaxis can be explained by the loss of essential catecholamines. These ACh-releasable catecholamines probably represent only a small fraction of the total catecholamines, because tachyphylaxis is reversed by allowing the atrial preparation to stand without further additions of ACh for 35–45 min.

Table III—Effect of Incubation with NE (0.025 mcg./ml.) on Responses of Reserpine-Pretreated[a] (n = 5) Isolated Rabbit Atria to ACh (100 mcg./ml.) in the Presence of Atropine (3 mcg./ml.)

Parameters Measured	Control Values	Change from Control Values: Tachyphylactic Response to ACh before NE ± *SE*	Change from Control Values: Control Response to NE ± *SE*	Change from Control Values: Response to ACh after NE ± *SE*
Force[b]	20 ± 1.1	−8 ± 2.6	+13 ± 1.1	−8 ± 3.1
Rate[c]	114 ± 10.3	−24 ± 3.0	+42 ± 12.0	−24 ± 5.4

[a] Reserpine, 5 mg./kg. i.p., 24 hr. before sacrifice. [b] Mean force in mm. [c] Mean rate in beats/min.

REFERENCES

(1) F. F. Cowan, *Georgetown Med. Bull.*, **16**, 229(1963).
(2) G. D. Maengwyn-Davies, F. F. Cowan, and T. Koppanyi, *Arch. Int. Pharmacodyn. Ther.*, **160**, 454(1966).
(3) F. F. Cowan, T. Koppanyi, and G. D. Maengwyn-Davies, *ibid.*, **160**, 424(1966).
(4) F. F. Cowan, T. Koppanyi, and G. D. Maengwyn-Davies, *J. Pharm. Sci.*, **52**, 878(1963).
(5) G. D. Maengwyn-Davies and T. Koppanyi, *J. Pharmacol. Exp. Ther.*, **154**, 481(1966).
(6) U. Trendelenburg, "Pharmacology of Cardiac Function," Macmillan, New York, N. Y., 1964, p. 101.
(7) J. A. Richardson and E. F. Woods, *Proc. Soc. Exp. Biol. Med.*, **100**, 149(1959).
(8) E. T. Angelakos and E. Bloomquist, *Arch. Int. Physiol.*, **73**, 397(1966).
(9) R. Lindmar and E. Muscholl, *Arch. Exp. Pathol. Pharmakol.*, **242**, 214(1961).
(10) U. Trendelenburg and J. R. Crout, *J. Pharmacol. Exp. Ther.*, **145**, 151(1964).
(11) U. Trendelenburg, *ibid.*, **147**, 313(1965).
(12) H. D. Batson, "An Introduction to Statistics in the Medical Sciences," Burgess, Minneapolis, Minn., 1956, p. 8.
(13) T. Spadolini and L. J. Domini, *Arch. Physiol.*, **40**, 147(1940).
(14) B. F. Hoffman, H. Hoffman, S. Middleton, and J. Talesnik, *Amer. J. Physiol.*, **144**, 189(1945).
(15) W. C. Lee, L. P. McCarthy, W. W. Zodrow, and F. E. Shideman, *J. Pharmacol. Exp. Ther.*, **130**, 30(1960).
(16) S. M. Middleton, H. H. Middleton, and J. Toha, *Amer. J. Physiol.*, **158**, 31(1949).
(17) C. Heyman and D. Bennati, *Arch. Soc. Biol. Montevideo*, **15**, 56(1952).
(18) S. R. Kottegoda, *Brit. J. Pharmacol.*, **8**, 83(1953).
(19) S. Hukovic, *ibid.*, **14**, 372(1959).
(20) H. H. Woolard, *J. Anat. London*, **40**, 345(1926).
(21) J. F. Nonidez, *Amer. J. Anat.*, **65**, 361(1939).
(22) K. T. Tcheng, *Amer. Heart J.*, **41**, 512(1951).
(23) E. F. Hirsch, V. L. Willman, M. Jellinek, and T. Cooper, *Arch Pathol.*, **76**, 667(1965).
(24) T. Cooper, *Pharm. Rev.*, **18**, 611(1966).
(25) D. Jacobowitz, T. Cooper, and H. B. Barner, *Fed. Proc.*, **25**, 383(1966).
(26) W. C. Lee and F. E. Shideman, *J. Pharmacol. Exp. Ther.*, **126**, 239(1959).
(27) J. H. Burn and M. J. Rand, "Advances in Pharmacology," Academic, New York, N. Y., 1962, p. 2.
(28) G. D. Maengwyn-Davies, *Arch. Int. Pharmacodyn. Ther.*, **156**, 143(1965).
(29) G. D. Maengwyn-Davies, F. F. Cowan, T. Koppanyi, and B. W. Lei, *ibid.*, **160**, 438(1966).
(30) F. F. Cowan, C. Cannon, T. Koppanyi, and G. D. Maengwyn-Davies, *Science*, **134**, 1069(1961).
(31) L. T. Potter, *Pharm. Rev.*, **18**, 439(1966).
(32) W. Raab and A. B. Gigee, *Circ. Res.*, **3**, 553(1955).
(33) M. L. Torchiana and E. T. Angelakos, *Arch. Int. Physiol.*, **71**, 762 (1962).
(34) J. H. Burn and M. J. Rand, *J. Physiol. London*, **144**, 314 (1958).
(35) U. Trendelenburg, *Fed. Proc.*, **21**, 332(1962).
(36) A. A. Bertler, A. Carlsson, and E. Rosenbert, *Naturwissenschaften*, **43**, 521(1956).
(37) T. Koppanyi and M. D. MacFarlane, *Life Sci.*, **3**, 1135 (1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 15, 1969, from the *Department of Pharmacology, Georgetown University, Schools of Medicine and Dentistry, Washington, DC 20007*

Accepted for publication February 17, 1970.

A preliminary report of this work was presented to the Federation of American Societies for Experimental Biology in April 1967.

Supported in part by the Washington Heart Association.

* Present address: University of Southern California, School of Pharmacy, Department of Pharmacology, University Park, Los Angeles, CA 90007

Effect of Sex on Penicillin Blood Levels in Dogs

JOHN W. POOLE*

Abstract ☐ Studies concerned with the oral absorption characteristics of dicloxacillin from various pharmaceutical formulations in beagle dogs suggest that the female of this species shows consistently higher and more prolonged blood serum levels of the antibiotic than the corresponding male. An investigation was initiated to determine the extent of this phenomenon with respect to sodium dicloxacillin monohydrate and other penicillins. The results of these studies suggest that the monobasic penicillin molecules show blood serum level variations after oral administration that are related to the sex of the animal, while a similar correlation does not appear to exist for the amphoteric penicillins. Determination of the biological half-life of the various penicillins in the male and female after intravenous administration indicates that no sexual differences occur with respect to the disappearance of active drug from the blood.

Keyphrases ☐ Penicillin blood levels—dogs ☐ Sex effect, dogs—penicillin blood levels ☐ Blood levels, half-life, corticosteroids—sex effect, dogs ☐ Microbiological test method—analysis

Minor sexual differences in drug response among the animals of a species are frequently encountered in toxicity experiments. For example, L-thyroxin produces a more pronounced depressant effect on weight gain in male than in female rats (1), and the toxicity of hypoglycemic agents is enhanced in female and male rats pretreated with diethylstilbestrol (2). These differences are sometimes quite significant. For example, the antibiotic acetoxycyclohexamide was shown by Pallotta *et al.* (3), in acute and subacute tests, to be about four times as toxic for young female rats as it is for males. In many cases, these differences in response between the sexes can be traced to differences in enzyme activities and rate of metabolism. Male rats metabolize hexobarbital much faster than do females, and the average sleeping time of the male after receiving the drug is only about one-fourth that of the female (4, 5). Recently, in a report by Kernohan and Todd (6), it was suggested that women bleed more readily than men during heparin therapy. A similar conclusion, that women have a higher risk of bleeding with heparin than do men, was made from the studies of Jick *et al.* (7).

The results obtained in a series of investigations on the effect of dosage-form variables on biological avail-

REFERENCES

(1) F. F. Cowan, *Georgetown Med. Bull.*, **16**, 229(1963).
(2) G. D. Maengwyn-Davies, F. F. Cowan, and T. Koppanyi, *Arch. Int. Pharmacodyn. Ther.*, **160**, 454(1966).
(3) F. F. Cowan, T. Koppanyi, and G. D. Maengwyn-Davies, *ibid.*, **160**, 424(1966).
(4) F. F. Cowan, T. Koppanyi, and G. D. Maengwyn-Davies, *J. Pharm. Sci.*, **52**, 878(1963).
(5) G. D. Maengwyn-Davies and T. Koppanyi, *J. Pharmacol. Exp. Ther.*, **154**, 481(1966).
(6) U. Trendelenburg, "Pharmacology of Cardiac Function," Macmillan, New York, N. Y., 1964, p. 101.
(7) J. A. Richardson and E. F. Woods, *Proc. Soc. Exp. Biol. Med.*, **100**, 149(1959).
(8) E. T. Angelakos and E. Bloomquist, *Arch. Int. Physiol.*, **73**, 397(1966).
(9) R. Lindmar and E. Muscholl, *Arch. Exp. Pathol. Pharmakol.*, **242**, 214(1961).
(10) U. Trendelenburg and J. R. Crout, *J. Pharmacol. Exp. Ther.*, **145**, 151(1964).
(11) U. Trendelenburg, *ibid.*, **147**, 313(1965).
(12) H. D. Batson, "An Introduction to Statistics in the Medical Sciences," Burgess, Minneapolis, Minn., 1956, p. 8.
(13) T. Spadolini and L. J. Domini, *Arch. Physiol.*, **40**, 147(1940).
(14) B. F. Hoffman, H. Hoffman, S. Middleton, and J. Talesnik, *Amer. J. Physiol.*, **144**, 189(1945).
(15) W. C. Lee, L. P. McCarthy, W. W. Zodrow, and F. E. Shideman, *J. Pharmacol. Exp. Ther.*, **130**, 30(1960).
(16) S. M. Middleton, H. H. Middleton, and J. Toha, *Amer. J. Physiol.*, **158**, 31(1949).
(17) C. Heyman and D. Bennati, *Arch. Soc. Biol. Montevideo*, **15**, 56(1952).
(18) S. R. Kottegoda, *Brit. J. Pharmacol.*, **8**, 83(1953).
(19) S. Hukovic, *ibid.*, **14**, 372(1959).
(20) H. H. Woolard, *J. Anat. London*, **40**, 345(1926).
(21) J. F. Nonidez, *Amer. J. Anat.*, **65**, 361(1939).
(22) K. T. Tcheng, *Amer. Heart J.*, **41**, 512(1951).
(23) E. F. Hirsch, V. L. Willman, M. Jellinek, and T. Cooper, *Arch Pathol.*, **76**, 667(1965).
(24) T. Cooper, *Pharm. Rev.*, **18**, 611(1966).
(25) D. Jacobowitz, T. Cooper, and H. B. Barner, *Fed. Proc.*, **25**, 383(1966).
(26) W. C. Lee and F. E. Shideman, *J. Pharmacol. Exp. Ther.*, **126**, 239(1959).
(27) J. H. Burn and M. J. Rand, "Advances in Pharmacology," Academic, New York, N. Y., 1962, p. 2.
(28) G. D. Maengwyn-Davies, *Arch. Int. Pharmacodyn. Ther.*, **156**, 143(1965).
(29) G. D. Maengwyn-Davies, F. F. Cowan, T. Koppanyi, and B. W. Lei, *ibid.*, **160**, 438(1966).
(30) F. F. Cowan, C. Cannon, T. Koppanyi, and G. D. Maengwyn-Davies, *Science*, **134**, 1069(1961).
(31) L. T. Potter, *Pharm. Rev.*, **18**, 439(1966).
(32) W. Raab and A. B. Gigee, *Circ. Res.*, **3**, 553(1955).
(33) M. L. Torchiana and E. T. Angelakos, *Arch. Int. Physiol.*, **71**, 762 (1962).
(34) J. H. Burn and M. J. Rand, *J. Physiol. London*, **144**, 314 (1958).
(35) U. Trendelenburg, *Fed. Proc.*, **21**, 332(1962).
(36) A. A. Bertler, A. Carlsson, and E. Rosenbert, *Naturwissenschaften*, **43**, 521(1956).
(37) T. Koppanyi and M. D. MacFarlane, *Life Sci.*, **3**, 1135 (1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 15, 1969, from the *Department of Pharmacology, Georgetown University, Schools of Medicine and Dentistry, Washington, DC 20007*

Accepted for publication February 17, 1970.

A preliminary report of this work was presented to the Federation of American Societies for Experimental Biology in April 1967.

Supported in part by the Washington Heart Association.

* Present address: University of Southern California, School of Pharmacy, Department of Pharmacology, University Park, Los Angeles, CA 90007

Effect of Sex on Penicillin Blood Levels in Dogs

JOHN W. POOLE*

Abstract ☐ Studies concerned with the oral absorption characteristics of dicloxacillin from various pharmaceutical formulations in beagle dogs suggest that the female of this species shows consistently higher and more prolonged blood serum levels of the antibiotic than the corresponding male. An investigation was initiated to determine the extent of this phenomenon with respect to sodium dicloxacillin monohydrate and other penicillins. The results of these studies suggest that the monobasic penicillin molecules show blood serum level variations after oral administration that are related to the sex of the animal, while a similar correlation does not appear to exist for the amphoteric penicillins. Determination of the biological half-life of the various penicillins in the male and female after intravenous administration indicates that no sexual differences occur with respect to the disappearance of active drug from the blood.

Keyphrases ☐ Penicillin blood levels—dogs ☐ Sex effect, dogs—penicillin blood levels ☐ Blood levels, half-life, corticosteroids—sex effect, dogs ☐ Microbiological test method—analysis

Minor sexual differences in drug response among the animals of a species are frequently encountered in toxicity experiments. For example, L-thyroxin produces a more pronounced depressant effect on weight gain in male than in female rats (1), and the toxicity of hypoglycemic agents is enhanced in female and male rats pretreated with diethylstilbestrol (2). These differences are sometimes quite significant. For example, the antibiotic acetoxycyclohexamide was shown by Pallotta *et al.* (3), in acute and subacute tests, to be about four times as toxic for young female rats as it is for males. In many cases, these differences in response between the sexes can be traced to differences in enzyme activities and rate of metabolism. Male rats metabolize hexobarbital much faster than do females, and the average sleeping time of the male after receiving the drug is only about one-fourth that of the female (4, 5). Recently, in a report by Kernohan and Todd (6), it was suggested that women bleed more readily than men during heparin therapy. A similar conclusion, that women have a higher risk of bleeding with heparin than do men, was made from the studies of Jick *et al.* (7).

The results obtained in a series of investigations on the effect of dosage-form variables on biological avail-

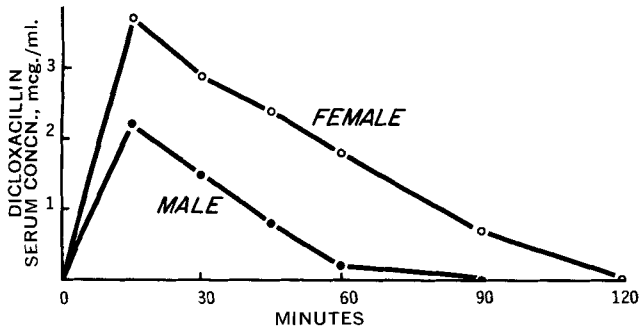


Figure 1—*Mean blood serum concentration of dicloxacillin in male and female beagle dogs after oral administration of 250-mg. doses.*

ability of dicloxacillin in beagle dogs indicated that the female of this species demonstrated consistently better utilization of this drug than the corresponding male. This was shown by higher peak blood serum levels and greater areas under the blood serum level–time curves after oral administration of the antibiotic to female dogs than after similar dosing to the males. A study was initiated to determine the extent of this phenomenon with respect to penicillin compounds as a class.

Specifically, the blood serum levels attained after oral administration of the monobasic penicillins, dicloxacillin and nafcillin, and the amphoteric penicillin, ampicillin, to male and female beagle dogs were determined. The biological half-lives of these drugs in this species were also determined in both sexes after intravenous administration of these compounds. In addition, the results obtained with several other penicillins used in various studies designed to evaluate dosage-form variables have been considered with respect to a difference in biological utilization of these agents between male and female beagle dogs.

EXPERIMENTAL

Compounds—The following were used: ampicillin, anhydrous (Wyeth Laboratories, C-10575); sodium nafcillin monohydrate (Wyeth Laboratories, W-663989); and sodium dicloxacillin monohydrate (Wyeth Laboratories, C-10651).[1]

Procedure—The general procedure used in the blood serum level studies follows. Six dogs, three of each sex, were utilized in each experiment. The test animals were dosed with the appropriate drug in a strict crossover manner. The dogs were fasted overnight; the antibiotic was administered by means of a stomach tube, followed by a standard amount of distilled water. Blood samples were drawn at the appropriate times postadministration and analyzed by a microbiological method utilizing *Sarcina lutea* as the test organism.

In studies in which the drug was administered intravenously, the penicillin was dissolved in an appropriate amount of physiological saline solution just prior to administration.

RESULTS AND DISCUSSION

In a series of investigations concerned with the effect of dosage-form variables on the biological utilization of dicloxacillin, oral administration of the various dosage forms containing this agent to female beagle dogs resulted in significantly higher and greater area under the blood serum level–time curves than after similar administration to male animals of the same species. A typical example of such a study is shown in Fig. 1. In this instance the female animals showed a mean peak serum level of the antibiotic

[1] Dosage forms of the several penicillins employed in these studies were prepared by the Development Section of the Pharmacy Research and Development Division, Wyeth Laboratories, Inc.

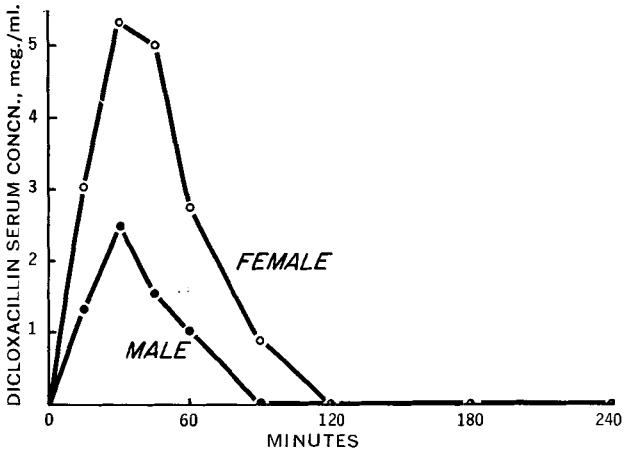


Figure 2—*Mean blood serum concentrations of dicloxacillin in male and female beagle dogs after oral administration of 25-mg./kg. doses.*

of 3.7 mcg./ml., while the male animal showed a mean peak serum level of 2.2 mcg./ml. The areas under the blood serum level–time curves calculated for each sex were 1.20 and 3.34 mcg./ml. × hr. for the male and female dogs, respectively. These results were influenced to some extent by the fact that discrete dosage units containing 250 mg. of the active moiety were administered to each animal in a crossover experiment. Because the females were, generally, slightly smaller than the male animals, this factor may have had a significant effect on the results observed. In addition, the possibility that the females used were "good" absorbers of this agent and the males "poor" absorbers was also considered.

To rule out the significance of these factors, a study was designed in which a 25-mg./kg. dose of penicillin was administered to the two sexes of this species in the form of a powder slurried in water. All the animals used in the initial experiments were omitted from this study. In addition to dicloxacillin, two other penicillins were included in the study, nafcillin and anhydrous ampicillin.

Figure 2 illustrates the difference in blood serum levels attained in this experiment with male and female animals after administration of dicloxacillin at the 25-mg./kg. dose. These results are in agreement with the earlier data obtained utilizing this drug in pharmaceutical dosage forms. The females in this instance showed a mean peak serum level of 5.4 mcg./ml., while the mean level with the males was 2.5 mcg./ml. Similarly, the areas under the blood serum level–time curves were determined to be 4.96 and 1.72 mcg./ml. × hr. for the female and male animals, respectively.

Figure 3 shows the results obtained when nafcillin was administered at the 25-mg./kg. level to beagle dogs of both sexes. In this instance, the females attained a mean peak serum level of 0.68

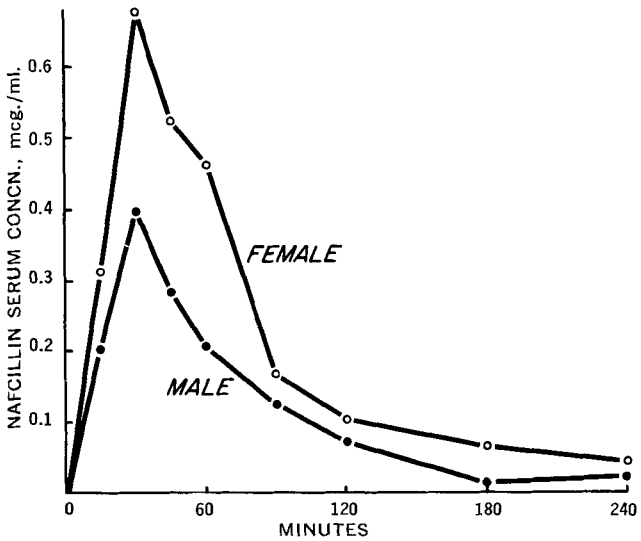


Figure 3—*Mean blood serum concentrations of nafcillin in male and female beagle dogs after oral administration of 25-mg./kg. doses.*

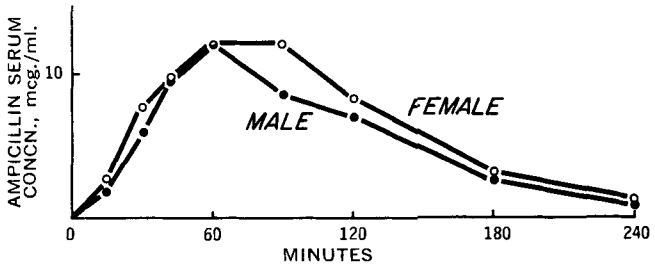


Figure 4—*Mean blood serum concentrations of ampicillin in male and female beagle dogs after oral administration of 25-mg./kg. doses.*

mcg./ml., while the males showed a mean peak serum level of 0.40 mcg./ml. The areas under the blood serum level–time curves for the females and males were calculated to be 0.81 and 0.46 mcg./ml. × hr., respectively.

However, as shown in Fig. 4, after administration of anhydrous ampicillin to animals of both sexes, no significant difference in mean peak serum levels attained or in the area under the blood serum level–time curves between the sexes was observed. The mean peak serum levels attained in this case were 12.1 and 12.2 mcg./ml., and the areas under the blood serum level–time curves were 21.8 and 26.0 mcg./ml. × hr. for the males and females, respectively. Table I summarizes the mean peak serum levels and curve areas obtained in this investigation. The blood serum level data obtained in this study indicate that the monobasic penicillins, such as dicloxacillin and nafcillin, show a significant difference in biological utilization of these agents between male and female beagle dogs. The results obtained with the amphoteric penicillin, ampicillin, did not demonstrate any significant differences in blood serum levels between the sexes.

The results obtained in subsequent experiments utilizing pharmaceutical dosage forms of penicillin G, a monobasic penicillin, and Wy-4508 [6-(1-aminocyclohexanecarboxamido)penicillanic acid], an amphoteric penicillin, are in agreement with the results summarized in Table I.

Figure 5 shows the mean blood serum levels obtained for penicillin G in male and female beagle dogs after oral administration of 500 mg. of the drug as buffered tablets. As was the case with the monobasic penicillins, the female showed a significantly better overall biological utilization of this agent than did the male animal. A mean peak serum level of 58 units/ml. was attained in the females, while a mean peak serum level of 25 units/ml. was noted in the males. Similarly, the areas under the blood serum level–time curves for the female and male animals were determined to be 80 and 48 units/ml. × hr., respectively.

Figure 6 illustrates the results obtained in a study of male and female beagle dogs after oral administration (250 mg.) of a pharmaceutical formulation of the amphoteric penicillin, Wy-4508. The results show that there is no significant difference in the blood serum levels attained between the male and female animals or in the overall biological utilization of this agent after such administration. The peak serum levels obtained were 31.5 mcg./ml. for the male and 27.5 mcg./ml. for the female. The areas under the blood serum level–time curves were 36.0 and 36.6 mcg./ml. × hr. for the males and females, respectively.

One possible mechanism by which the observed sex difference in blood serum levels could be explained is a difference in elimination rate (biological life) for the monobasic penicillins in the males and females. To test this possibility, an intravenous dose of the two

Table I—Peak Blood Serum Level and Areas under Blood Serum Level–Time Curve after Oral Administration of Various Penicillins (25 mg./kg.) to Male and Female Beagle Dogs

Compound	Peak Level, mcg. ml. Male	Peak Level, mcg. ml. Female	Area under Curve, mcg./ml. × hr. Male	Area under Curve, mcg./ml. × hr. Female
Nafcillin[a]	0.40	0.68	0.46	0.81
Dicloxacillin[a]	2.5	5.4	1.72	4.96
Ampicillin	12.1	12.2	21.8	26.0

[a] Administered as the sodium salt of the monohydrate.

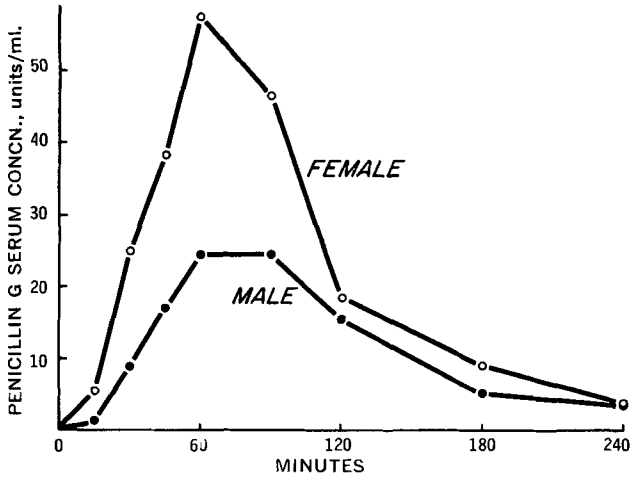


Figure 5—*Mean blood serum concentrations of penicillin G in male and female beagle dogs after oral administration of 500-mg. doses.*

monobasic penicillins, nafcillin and dicloxacillin, along with the amphoteric penicillin, ampicillin, was administered to beagle dogs of each sex and the blood serum level decay followed. The dogs used in this study were the same animals employed in the oral absorption studies with these penicillins. The biological half-life for ampicillin in both sexes was similar, as was expected, on the basis of the oral blood level data. The average half-life values were 35 and 34 min. for the male and female, respectively. However, neither of the monobasic penicillins, dicloxacillin or nafcillin, employed in this study showed a difference in the biological half-life for the male and female animals. The average biological half-life for nafcillin was determined to be 10 min. in each sex, while administration of dicloxacillin resulted in an average biological half-life of 16 min. for the male and 18 min. for the female. The results of these studies are summarized in Table II, where the average biological half-life for the various penicillin products in the male and female animals is listed along with the range of half-lives obtained. These results indicate that the differences in peak serum levels and areas under the blood serum level–time curves noted for the male and female dogs with monobasic penicillins are apparently not related to a difference in elimination rate of these drugs in the two sexes. Other possible mechanisms which may be involved in the differences in serum levels noted are: (*a*) a difference in the metabolism of the monobasic penicillins in the gut wall of the male and female animals or (*b*) a difference in acidity in the gastrointestinal tract of the male and female beagle dogs. This latter hypothesis could account for the lack of any sex difference noted with amphoteric penicillins which would not be as sensitive to the acid environment of the gastrointestinal tract as monobasic penicillins.

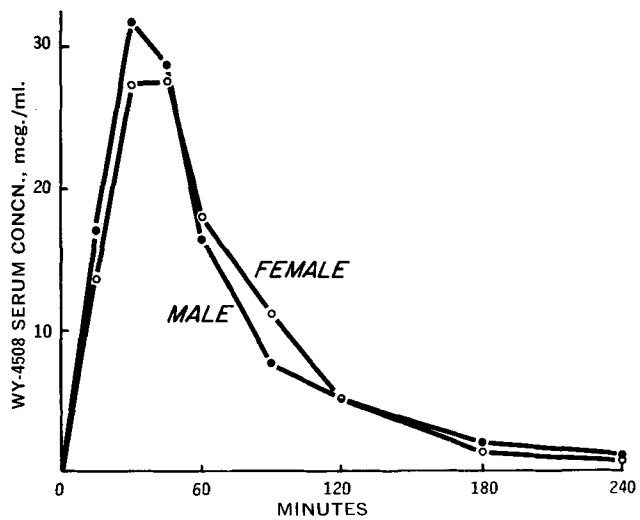


Figure 6—*Mean blood serum concentrations of Wy-4508 in male and female beagle dogs after oral administration of 250-mg. doses.*

Table II—Biological Half-Life of Various Penicillins in Male and Female Beagle Dogs after Intravenous Administration

Sex	Half-Life, min.		
	Nafcillin[a]	Dicloxacillin[a]	Ampicillin
Male	10 (9–11)[b]	16 (12–20)[b]	35 (29–39)[b]
Female	10 (9–11)[b]	18 (15–22)[b]	34 (28–38)[b]

[a] Administered as the sodium salt of the monohydrate. [b] Range of half-life observed.

In any event, the sex of the test animal should be considered in studies concerned with *in vivo* effects of monobasic penicillins in which dogs, particularly beagles, are used. The effect of the animal's sex on the biological utilization of such compounds in other species and in human subjects is not known at this time. Studies in humans are of interest since differences of the magnitude observed in the animal studies may be clinically significant.

SUMMARY

The biological utilization, as demonstrated by blood serum levels and area under the serum level–time curves, for a series of penicillin compounds in male and female beagle dogs has been determined. Results obtained indicate that the female of this species attained a higher mean peak serum level and a greater area under the blood serum level–time curve than did the male after oral administration of the monobasic penicillins studied: dicloxacillin, nafcillin, and penicillin G. The amphoteric penicillins employed, ampicillin and Wy-4508, showed no significant difference in blood serum levels or curve areas after oral administration to male or female beagle dogs. There was no significant difference between the sexes in the elimination rate (half-life) of the monobasic penicillins, sodium dicloxacillin monohydrate and sodium nafcillin monohydrate, or of the amphoteric penicillin, ampicillin, after intravenous administration, which rules out this factor as an explanation for the difference in serum levels noted. A possible reason for the sex difference in serum levels in beagle dogs may be due to a difference in acidity of the gastrointestinal tract and/or to a difference in the gut wall metabolism of the monobasic penicillins in the male and female of this species.

REFERENCES

(1) J. Grossie and C. W. Turner, *Proc. Soc. Exp. Biol. Med.*, **107**, 520(1961).

(2) J. D. McColl and P. Sacra, *Toxicol. Appl. Pharmacol.*, **4**, 631(1962).

(3) A. J. Pallotta, M. G. Kelly, D. P. Rall, and J. W. Ward, *J. Pharmacol. Exp. Ther.*, **136**, 400(1962).

(4) G. P. Quinn, J. Axelrod, and B. B. Brodie, *Biochem. Pharmacol.*, **1**, 152(1958).

(5) B. B. Brodie, *Clin. Pharmacol. Ther.*, **3**, 374(1962).

(6) R. J. Kernohan and C. Todd, *Lancet*, **1**, 621(1966).

(7) H. Jick, D. Slone, I. T. Borda, and S. Shapiro, *New Engl. J. Med.*, **279**, 284(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 11, 1970, from the *Pharmacy Research and Development Division, Wyeth Laboratories, Radnor, PA 19087*

Accepted for publication April 17, 1970.

The author thanks G. Owen for administering the drugs and obtaining blood serum samples, G. Warren and S. Rosenman for the microbiological analysis, and R. Levi for preparation of the various experimental formulations.

Presented to Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

* Fellow, American Foundation for Pharmaceutical Education.

Pharmacokinetic Analysis of Potentiating Effect of Phenylbutazone on Anticoagulant Action of Warfarin in Man

ROBERT A. O'REILLY* and GERHARD LEVY†

Abstract □ Warfarin is eliminated more rapidly but its anticoagulant effect is increased by concomitant administration of phenylbutazone. Pharmacokinetic analysis by recently developed techniques shows that the prewarfarin synthesis rate and the normal degradation of prothrombin complex activity are not affected by phenylbutazone, but that this drug has a pronounced effect on the relationship between synthesis rate of prothrombin complex activity and plasma–warfarin concentration. These observations are consistent with the assumption that phenylbutazone competitively displaces warfarin from nonspecific binding sites in the plasma and tissues (particularly the liver) and thereby increases the interaction of the anticoagulant with its pharmacologic receptor and metabolizing enzyme system.

Keyphrases □ Warfarin activity—phenylbutazone effect □ Phenylbutazone effect, warfarin activity—pharmacokinetics □ Pharmacokinetics—phenylbutazone potentiation, warfarin activity □ Biologic half-life, warfarin—phenylbutazone effect

Drug interactions are usually of three types. A drug may increase or decrease the elimination rate constant of another by stimulating or inhibiting drug metabolizing enzymes, by changing urine pH or the flow rate of urine or bile, and/or by affecting the distribution of the other drug in the body. Another type of drug interaction involves the potentiation or inhibition of the pharmacologic effect of one drug by another without measurably affecting its kinetics of elimination. The third type of drug interaction is one where the gastrointestinal absorption of one drug is increased or decreased by the other. The coumarin anticoagulants exemplify all three of these effects. Induction of microsomal enzymes by heptabarbital increases the rate of elimination of warfarin and bishydroxycoumarin without affecting the relationship between anticoagulant effect and plasma–coumarin concentration in man (1, 2). Heptabarbital apparently also decreases the gastrointestinal absorption of bishydroxycoumarin (3) by mechanisms which are still being studied.

A particularly interesting interaction is that between warfarin and phenylbutazone. The former is more rapidly eliminated in the presence of phenylbutazone, but its anticoagulant effect in man is increased (4).

Table II—Biological Half-Life of Various Penicillins in Male and Female Beagle Dogs after Intravenous Administration

Sex	Half-Life, min.		
	Nafcillin[a]	Dicloxacillin[a]	Ampicillin
Male	10 (9–11)[b]	16 (12–20)[b]	35 (29–39)[b]
Female	10 (9–11)[b]	18 (15–22)[b]	34 (28–38)[b]

[a] Administered as the sodium salt of the monohydrate. [b] Range of half-life observed.

In any event, the sex of the test animal should be considered in studies concerned with *in vivo* effects of monobasic penicillins in which dogs, particularly beagles, are used. The effect of the animal's sex on the biological utilization of such compounds in other species and in human subjects is not known at this time. Studies in humans are of interest since differences of the magnitude observed in the animal studies may be clinically significant.

SUMMARY

The biological utilization, as demonstrated by blood serum levels and area under the serum level–time curves, for a series of penicillin compounds in male and female beagle dogs has been determined. Results obtained indicate that the female of this species attained a higher mean peak serum level and a greater area under the blood serum level–time curve than did the male after oral administration of the monobasic penicillins studied: dicloxacillin, nafcillin, and penicillin G. The amphoteric penicillins employed, ampicillin and Wy-4508, showed no significant difference in blood serum levels or curve areas after oral administration to male or female beagle dogs. There was no significant difference between the sexes in the elimination rate (half-life) of the monobasic penicillins, sodium dicloxacillin monohydrate and sodium nafcillin monohydrate, or of the amphoteric penicillin, ampicillin, after intravenous administration, which rules out this factor as an explanation for the difference in serum levels noted. A possible reason for the sex difference in serum levels in beagle dogs may be due to a difference in acidity of the gastrointestinal tract and/or to a difference in the gut wall metabolism of the monobasic penicillins in the male and female of this species.

REFERENCES

(1) J. Grossie and C. W. Turner, *Proc. Soc. Exp. Biol. Med.*, **107**, 520(1961).

(2) J. D. McColl and P. Sacra, *Toxicol. Appl. Pharmacol.*, **4**, 631(1962).

(3) A. J. Pallotta, M. G. Kelly, D. P. Rall, and J. W. Ward, *J. Pharmacol. Exp. Ther.*, **136**, 400(1962).

(4) G. P. Quinn, J. Axelrod, and B. B. Brodie, *Biochem. Pharmacol.*, **1**, 152(1958).

(5) B. B. Brodie, *Clin. Pharmacol. Ther.*, **3**, 374(1962).

(6) R. J. Kernohan and C. Todd, *Lancet*, **1**, 621(1966).

(7) H. Jick, D. Slone, I. T. Borda, and S. Shapiro, *New Engl. J. Med.*, **279**, 284(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 11, 1970, from the *Pharmacy Research and Development Division, Wyeth Laboratories, Radnor, PA 19087*

Accepted for publication April 17, 1970.

The author thanks G. Owen for administering the drugs and obtaining blood serum samples, G. Warren and S. Rosenman for the microbiological analysis, and R. Levi for preparation of the various experimental formulations.

Presented to Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

* Fellow, American Foundation for Pharmaceutical Education.

Pharmacokinetic Analysis of Potentiating Effect of Phenylbutazone on Anticoagulant Action of Warfarin in Man

ROBERT A. O'REILLY* and GERHARD LEVY†

Abstract □ Warfarin is eliminated more rapidly but its anticoagulant effect is increased by concomitant administration of phenylbutazone. Pharmacokinetic analysis by recently developed techniques shows that the prewarfarin synthesis rate and the normal degradation of prothrombin complex activity are not affected by phenylbutazone, but that this drug has a pronounced effect on the relationship between synthesis rate of prothrombin complex activity and plasma–warfarin concentration. These observations are consistent with the assumption that phenylbutazone competitively displaces warfarin from nonspecific binding sites in the plasma and tissues (particularly the liver) and thereby increases the interaction of the anticoagulant with its pharmacologic receptor and metabolizing enzyme system.

Keyphrases □ Warfarin activity—phenylbutazone effect □ Phenylbutazone effect, warfarin activity—pharmacokinetics □ Pharmacokinetics—phenylbutazone potentiation, warfarin activity □ Biologic half-life, warfarin—phenylbutazone effect

Drug interactions are usually of three types. A drug may increase or decrease the elimination rate constant of another by stimulating or inhibiting drug metabolizing enzymes, by changing urine pH or the flow rate of urine or bile, and/or by affecting the distribution of the other drug in the body. Another type of drug interaction involves the potentiation or inhibition of the pharmacologic effect of one drug by another without measurably affecting its kinetics of elimination. The third type of drug interaction is one where the gastrointestinal absorption of one drug is increased or decreased by the other. The coumarin anticoagulants exemplify all three of these effects. Induction of microsomal enzymes by heptabarbital increases the rate of elimination of warfarin and bishydroxycoumarin without affecting the relationship between anticoagulant effect and plasma–coumarin concentration in man (1, 2). Heptabarbital apparently also decreases the gastrointestinal absorption of bishydroxycoumarin (3) by mechanisms which are still being studied.

A particularly interesting interaction is that between warfarin and phenylbutazone. The former is more rapidly eliminated in the presence of phenylbutazone, but its anticoagulant effect in man is increased (4).

Table I—Effect of Phenylbutazone (PB) on the Elimination of Warfarin[a]

Subject[b]	Age, yr.	Weight, kg.	Date of Experiment Control	Date of Experiment With PB	Biologic Half-Life,[c] hr. Control	Biologic Half-Life,[c] hr. With PB	Extrapolated Initial Plasma-Warfarin Concn.,[c,d] mg./l. Control	Extrapolated Initial Plasma-Warfarin Concn.,[c,d] mg./l. With PB
N-22	22	58	8-7-67	5-21-67	55	37	14.0	17.0
N-27	22	78	6-26-67	7-17-67	59	42	15.7	17.3
N-30	23	70	12-13-67	11-27-67	60	32	16.1	17.0
N-31	25	84	12-17-67	11-27-67	79	51	13.2	13.9
N-32	21	62	8-7-67	8-25-67	59	42	13.6	12.8
N-33	21	85	12-17-67	11-27-67	72	35	13.6	17.2
Mean					64	40	14.3	15.9
SD					9	7	1.2	2.0

[a] Sodium warfarin, 1.5 mg./kg. body weight, alone and on the 4th day of phenylbutazone administration, 200 mg. three times a day. [b] All males. [c] Based on least-squares regression analysis of log C_p values >1 mg./l. [d] Listed only to facilitate description of the data. These values are a complex function of the amount absorbed and of the kinetics of absorption, distribution, and elimination.

This is unusual since more rapid elimination of a drug generally results in a decrease in its total pharmacologic effect (unless the drug is activated by metabolism). For example, the total anticoagulant effect of warfarin is decreased by enzyme inducers such as heptabarbital (1). Recently developed pharmacokinetic techniques (5) now permit a more detailed assessment of the mechanism of potentiation of warfarin by phenylbutazone.

EXPERIMENTAL

A single dose of sodium warfarin, 1.5 mg./kg. body weight, was administered orally to six normal subjects. Plasma-warfarin concentrations and prothrombin complex activity were determined daily for 5 days or more. The same experiment was carried out also during phenylbutazone (PB) administration (200 mg. orally three times a day for at least 8 days). Warfarin was administered on the 4th day of PB administration. Additional details of the study are described elsewhere (4, 6).

THEORETICAL

The theory and methodology for relating the anticoagulant effect of warfarin to its concentration in the plasma have been described in detail in a previous report (5) and will only be summarized here. Coumarin anticoagulants interfere with the synthesis of vitamin K-dependent blood-clotting factors (Factors II, VII, IX, and X) but do not affect their degradation. There is no direct relationship between prothrombin complex activity (P) and warfarin concentration in the plasma (C_p), because the former represents the net effect of the synthesis of various clotting factors and their degradation. Thus,

$$R_{net} = R_{syn.} - R_{deg.} \qquad \text{(Eq. 1)}$$

where R_{net} is the net rate of change in P, $R_{syn.}$ is the rate of synthesis, and $R_{deg.}$ is the rate of degradation of P. Since $R_{deg.} = k_d P$,

$$R_{net} = R_{syn.} - k_d P \quad \text{or} \quad R_{syn.} = R_{net} + k_d P \qquad \text{(Eq. 2)}$$

where k_d is the apparent first-order degradation rate constant for P. Prior to the administration of a coumarin anticoagulant,

$$R_{syn.}^0 = k_d P^0 \qquad \text{(Eq. 3)}$$

In the clinical range of C_p and $R_{syn.}$, there is an essentially linear relationship between $R_{syn.}$ and log C_p which, as has been shown previously (5), is described by the relationship

$$R_{syn.} = R_{syn.}^0 + m \log C_{p\,min.} - m \log C_p \qquad \text{(Eq. 4)}$$

where $C_{p\,min.}$ is the hypothetical minimum effective C_p. A plot of $R_{syn.}$ *versus* log C_p has a slope of $-m$ and extrapolates to $C_{p\,max.}$ at $R_{syn.} = 0$. P and C_p are determined experimentally as a function of time. Administration of a synthesis-blocking dose of warfarin (such that $R_{syn.} = 0$) yields k_d. The change of P with time determines R_{net}. $R_{syn.}$ can then be calculated by means of Eq. 2. Least-squares regression analysis of $R_{syn.}$ as a function of log C_p yields m and $C_{p\,max.}$

RESULTS

PB significantly decreased ($p < 0.01$ by paired t test) the biologic half-life of warfarin from an average of 64 hr. to an average of 40 hr. (Table I). The extrapolated initial C_p was higher during PB administration in five of the six subjects, but the difference was not statistically significant. P^0 was 100% of normal in all subjects in the control period and during PB administration. Also, PB had no effect on k_d and, therefore, did not change $R_{syn.}^0$ (Table II).

The relationship between $R_{syn.}$ and log C_p was profoundly altered by PB. In three of the six subjects the relationship remained approximately linear, but the slope of the regression line ($-m$) decreased appreciably (*e.g.*, Fig. 1). In the other three subjects, data obtained during PB administration show slight curvature (*e.g.*, Fig. 2). Despite

Table II—Effect of Phenylbutazone (PB) on Synthesis Rate ($R_{syn.}^0$) and Degradation Rate Constant (k_d) for Prothrombin Complex Activity

Subject	$R_{syn.}^0$, % of Normal/Day Control	$R_{syn.}^0$, % of Normal/Day With PB	k_d, day^{-1} Control	k_d, day^{-1} With PB
N-22	101	111	1.01	1.11
N-27	95	95	0.95	0.95
N-30	107	107	1.07	1.33[a]
N-31	95	92	0.95	0.92
N-32	92	92	0.92	0.92
N-33	96	96	0.96	0.96
Mean	98	99	0.98(0.98)[b]	1.03(1.01)[b]
SD	5	8	0.05	0.16

[a] Questionable value based on two data points. Control k_d value was used for all calculations. [b] Harmonic mean.

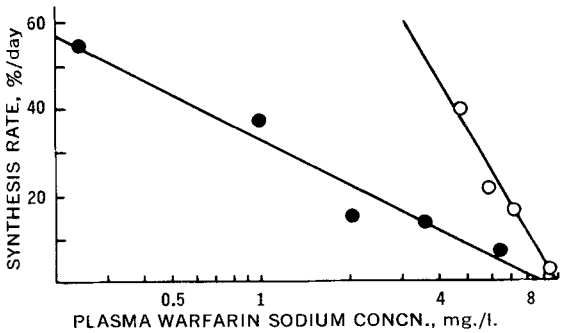


Figure 1—*Effect of phenylbutazone on the relationship between synthesis rate of prothrombin complex activity* ($R_{syn.}$) *and plasma warfarin concentration* (C_p) *in Subject N-33. Key:* ○, *control; and* ●, *with phenylbutazone.*

Table III—Effect of Phenylbutazone (PB) on the Relationship between Prothrombin Complex Activity Synthesis Rate and Plasma Warfarin Concentration

Subject	m, % of Normal/Day		Correlation Coefficient		$C_{p\ max.}$, mg./l.	
	Control	With PB	Control	With PB	Control	With PB
N-22	145	35	0.99	0.96	12.8	11.0
N-27	181	53	0.98	0.95	12.4	8.1
N-30	128	46	0.96	0.92	12.0	8.8
N-31	132	16	0.99	0.85	10.4	20.0
N-32	72	56	0.92	0.96	12.2	6.1
N-33	119	35	0.98	0.98	9.8	8.6
Mean	130	40			11.6	10.4
SD	36	15			1.2	5.0

this, and merely for facilitating a summary of the results, regression lines were fitted also to these data by the method of least squares. This summary is presented in Table III. There was a highly significant ($p < 0.01$) decrease in m during PB administration but no statistically significant change in $C_{p\ max.}$ (by paired t test), although five out of six values were lower during PB administration. Figure 3 shows the results obtained in Subject N-31 in the present study, as well as the results of three additional experiments with warfarin alone. C_p values in the additional experiments were determined spectrophotometrically, while all C_p values in the present study were determined by fluorometry.

DISCUSSION

The biologic half-life of warfarin was significantly decreased during PB administration. PB is a known inducer of drug-metabolizing enzymes (7) but, due to its pronounced affinity to plasma proteins (8), it also displaces warfarin from protein-binding sites (6). There is evidence that decreased plasma protein binding increases the elimination rate constant of coumarin anticoagulants by increasing their concentration at biotransformation sites in the liver (9, 10). On the other hand, PB and bishydroxycoumarin (and, therefore, presumably also warfarin) may be metabolized by the same enzyme system (11); therefore, there may occur mutual inhibition of biotransformation. Thus the observed effect of PB on the elimination of warfarin may be the net result of more than one type of effect, and its direction and magnitude may depend on the dose and duration of PB administration.

The lack of effect of PB on P^0, k_d, and, therefore, on $R_{syn.}{}^0$ shows that PB has no apparent effect on the blood-clotting system. On the other hand, the relationship between $R_{syn.}$ and C_p is considerably changed by PB. Unfortunately, the fluorometric assay for warfarin [which had to be used in this study because PB interferes with the spectrophotometric assay (6)] is not totally specific for unchanged warfarin and is affected also by one or more of its hydroxylated metabolites (12). Particularly the lower C_p values (*i.e.*, the values obtained late in the experiment) are, therefore, somewhat higher than those obtained by spectrophotometry. Consequently, as is exemplified in Fig. 3, the regression lines are steeper (and m values larger) than when C_p is determined spectrophotometrically. However, this has little effect on the *relative* relationships between $R_{syn.}$ and C_p with and without PB.

The effect of PB is unlike that produced by the enzyme inducer heptabarbital (1) and differs also from the pattern observed in a subject who is genetically resistant to warfarin (13). Heptabarbital stimulates the biotransformation of warfarin and, therefore, decreases C_p; but the anticoagulant effect at a given C_p is the same during control periods and during heptabarbital administration, *i.e.*, the "concentration–response" relationship is not altered (1). In the resistant subject, the slope of the $R_{syn.}$ *versus* log C_p plot is normal, but the regression line is displaced laterally to a higher $C_{p\ max.}$ value (13).

PB affects mainly the slope ($-m$) of the $R_{syn.}$–log C_p plot. Qualitatively, this suggests that PB competitively displaces warfarin from nonspecific protein-binding sites in the plasma and tissues[1] and thereby affords more extensive interaction of the anticoagulant with its pharmacologic receptor in the liver. The more rapid elimination of warfarin during PB administration is consistent with this reasoning, since warfarin is eliminated by biotransformation in the liver and the elimination rate of the anticoagulant is a function of its concentration in the liver. The warfarin–PB interaction is an interesting example of the simultaneous occurrence of two separate effects, enhanced elimination (which ordinarily would decrease the total pharmacologic effect) and potentiation of the pharmacologic

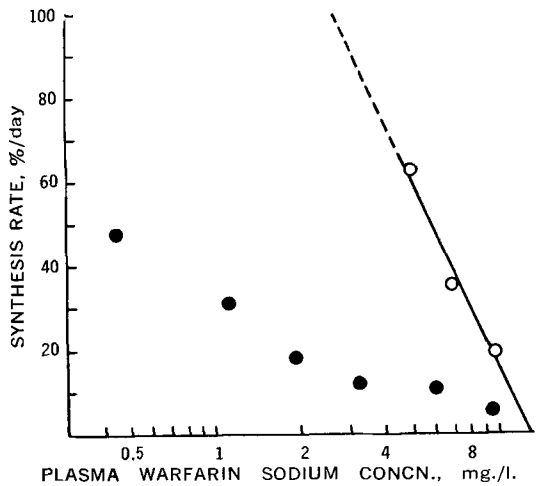


Figure 2—*Effect of phenylbutazone on the relationship between* $R_{syn.}$ *and* C_p *in Subject N-22. Key:* ○, *control; and* ●, *with phenylbutazone.*

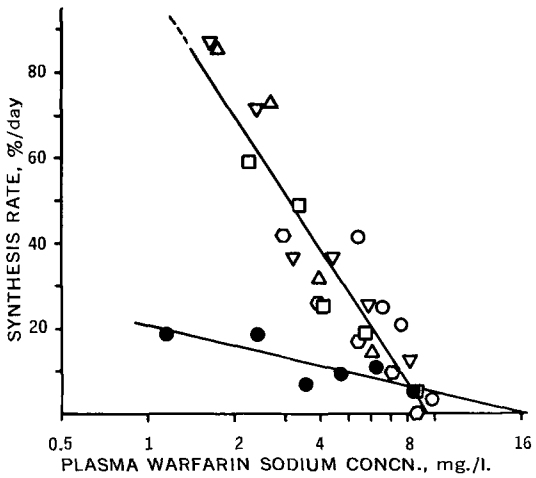


Figure 3—*Effect of phenylbutazone on the relationship between* $R_{syn.}$ *and* C_p *in Subject N-31. Key:* ○, *control; and* ●, *with phenylbutazone. Shown also are data for four other control experiments in which* C_p *was determined spectrophotometrically. The regression line for the control results was fitted to all the experimental data.*

[1] Note, for example, the qualitative similarity between the effect of a constant concentration of displacing agent on the protein binding of a drug at various concentrations (Fig. 3 in *Reference 14*) and the effect of a presumably relatively constant body level of PB on the activity of warfarin as found in the present study.

action of warfarin. Both of these effects seem to be due primarily to a change in the distribution of warfarin in the body.

REFERENCES

(1) G. Levy, R. A. O'Reilly, P. M. Aggeler, and G. M. Keech, *Clin. Pharmacol. Ther.*, **11**, 372(1970).

(2) R. A. O'Reilly, G. Levy, and G. M. Keech, *ibid.*, **11**, 378(1970).

(3) P. M. Aggeler and R. A. O'Reilly, *J. Lab. Clin. Med.*, **74**, 229(1969).

(4) R. A. O'Reilly and P. M. Aggeler, *Proc. Soc. Exp. Biol. Med.*, **128**, 1080(1968).

(5) R. Nagashima, R. A. O'Reilly, and G. Levy, *Clin. Pharmacol. Ther.*, **10**, 22(1969).

(6) P. M. Aggeler, R. A. O'Reilly, L. S. Leong, and P. E. Kowitz, *New Engl. J. Med.*, **276**, 496(1967).

(7) A. H. Conney, *Pharmacol. Rev.*, **19**, 317(1967).

(8) J. J. Burns, R. K. Rose, T. Chenkin, A. Goldman, A. Schulert, and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, **109**, 346 (1953).

(9) G. Levy and R. Nagashima, *J. Pharm. Sci.*, **58**, 1001(1969).

(10) G. Levy, *Proc. Int. Pharmacol. Congr., 4th*, **1970**, Vol. IV, 134.

(11) E. S. Vesell and J. G. Page, *J. Clin. Invest.*, **47**, 2657(1968).

(12) R. A. O'Reilly, *ibid.*, **48**, 193(1969).

(13) R. A. O'Reilly and G. Levy, to be published.

(14) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 28, 1969, from the *Department of Medicine, Santa Clara Valley Medical Center and the Institute for Medical Research of Santa Clara County, and the †Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication April 30, 1970.

Supported in part by Grant HE 8058 from the U. S. Public Health Service.

The technical assistance of Geraldine M. Keech is gratefully acknowledged.

Diffuse Reflectance Studies of Solid–Solid Interactions

JOHN L. LACH and LYLE D. BIGHLEY*

Abstract □ Tetracycline and its derivatives, bishydroxycoumarin, and methantheline bromide have been studied by diffuse reflectance spectroscopy for possible solid–solid interactions with various metallic adjuvants. Examination of the spectra of some of these drug–adjuvant systems show rather small spectral changes, while other spectra show large bathochromic and hyperchromic changes, new band formation, and visual color changes which are indicative of charge–transfer interactions. Although these interactions vary in variety and intensity, they may significantly alter the availability and activity of the medicinal agent in pharmaceutical dosage forms.

Keyphrases □ Solid–solid interaction—determination □ Diffuse reflectance spectroscopy—solid–solid interaction analysis □ Tetracyclines, methantheline bromide, bishydroxycoumarin—metal-ion interactions □ Metal ions–tetracyclines, –methantheline bromide, –bishydroxycoumarin—interactions

Many articles in the pharmaceutical and medical literature deal with the problem of physiologically inactive tablet and capsule formulations. Lach and Bornstein (1), by the use of diffuse reflectance spectroscopy (DRS), have postulated that the apparent inactivity may be due in part to adsorption of the active principle onto an inert adjuvant. They have shown that solid–solid interactions do indeed exist between various chemical entities and a wide variety of adjuvants commonly found in pharmaceutical dosage forms. Although some of the interactions may be of the weak variety, they may, nevertheless, be sufficient to alter the absorption and availability of the medicinal agent.

The study of solid–solid interactions is of interest because of the effects that adjuvants may exhibit when incorporated into pharmaceutical dosage forms. These effects may include: (*a*) changes in the nature and intensity of biological activity caused by complexation with the active ingredient (2); (*b*) modifications of the physical state, particle size, and/or surface area of the drug available to the absorption sites (3); or (*c*) changes in the stability of the active principle (4–7).

The purposes of this study were to continue the investigation of solid–solid interactions of a number of drugs and to gain a better insight into the nature of these interactions.

EXPERIMENTAL

Reagents—Oxytetracycline hydrochlorde,[1] tetracycline hydrochloride,[2] chlortetracycline hydrochloride,[2] demethylchlortetracycline hydrochloride,[2] methantheline bromide,[3] bishydroxycoumarin,[4] magnesium trisilicate, ferric phosphate, aluminum hydroxide, tribasic calcium phosphate, and talc were used.

Apparatus—The following were used: Beckman model DU spectrophotometer with a diffuse reflectance attachment (1), constant-temperature water bath with rotating spindles, 150-ml. amber vials with caps, Parafilm,[5] and a glass desiccator with anhydrous calcium sulfate.[6]

Procedure—*Preparation of the Tetracyclines*—Thirty milligrams of active ingredient (tetracycline HCl, oxytetracycline HCl, chlortetracycline HCl, and demethylchlortetracycline HCl) was weighed for every 2 g. of adsorbent (pharmaceutical adjuvant) used. The powders were placed in 150-ml. amber vials, and 25 ml. of distilled water was added as the dispersion medium. The vial was then covered with Parafilm and capped. Equilibration was allowed to proceed for 2 hr. at 30 ± 0.5° to effect interaction. After equilibration, the suspension was filtered under vacuum and the powder dried

[1] Pfizer and Co.
[2] Lederle and Co.
[3] Searle and Co.
[4] K and K Laboratories.
[5] Marathon Co.
[6] Drierite, Hammond Co.

action of warfarin. Both of these effects seem to be due primarily to a change in the distribution of warfarin in the body.

REFERENCES

(1) G. Levy, R. A. O'Reilly, P. M. Aggeler, and G. M. Keech, *Clin. Pharmacol. Ther.*, **11**, 372(1970).

(2) R. A. O'Reilly, G. Levy, and G. M. Keech, *ibid.*, **11**, 378(1970).

(3) P. M. Aggeler and R. A. O'Reilly, *J. Lab. Clin. Med.*, **74**, 229(1969).

(4) R. A. O'Reilly and P. M. Aggeler, *Proc. Soc. Exp. Biol. Med.*, **128**, 1080(1968).

(5) R. Nagashima, R. A. O'Reilly, and G. Levy, *Clin. Pharmacol. Ther.*, **10**, 22(1969).

(6) P. M. Aggeler, R. A. O'Reilly, L. S. Leong, and P. E. Kowitz, *New Engl. J. Med.*, **276**, 496(1967).

(7) A. H. Conney, *Pharmacol. Rev.*, **19**, 317(1967).

(8) J. J. Burns, R. K. Rose, T. Chenkin, A. Goldman, A. Schulert, and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, **109**, 346 (1953).

(9) G. Levy and R. Nagashima, *J. Pharm. Sci.*, **58**, 1001(1969).

(10) G. Levy, *Proc. Int. Pharmacol. Congr., 4th*, **1970**, Vol. IV, 134.

(11) E. S. Vesell and J. G. Page, *J. Clin. Invest.*, **47**, 2657(1968).

(12) R. A. O'Reilly, *ibid.*, **48**, 193(1969).

(13) R. A. O'Reilly and G. Levy, to be published.

(14) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 28, 1969, from the *Department of Medicine, Santa Clara Valley Medical Center and the Institute for Medical Research of Santa Clara County, and the †Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication April 30, 1970.

Supported in part by Grant HE 8058 from the U. S. Public Health Service.

The technical assistance of Geraldine M. Keech is gratefully acknowledged.

Diffuse Reflectance Studies of Solid–Solid Interactions

JOHN L. LACH and LYLE D. BIGHLEY*

Abstract □ Tetracycline and its derivatives, bishydroxycoumarin, and methantheline bromide have been studied by diffuse reflectance spectroscopy for possible solid–solid interactions with various metallic adjuvants. Examination of the spectra of some of these drug–adjuvant systems show rather small spectral changes, while other spectra show large bathochromic and hyperchromic changes, new band formation, and visual color changes which are indicative of charge–transfer interactions. Although these interactions vary in variety and intensity, they may significantly alter the availability and activity of the medicinal agent in pharmaceutical dosage forms.

Keyphrases □ Solid–solid interaction—determination □ Diffuse reflectance spectroscopy—solid–solid interaction analysis □ Tetracyclines, methantheline bromide, bishydroxycoumarin—metal-ion interactions □ Metal ions–tetracyclines, –methantheline bromide, –bishydroxycoumarin—interactions

Many articles in the pharmaceutical and medical literature deal with the problem of physiologically inactive tablet and capsule formulations. Lach and Bornstein (1), by the use of diffuse reflectance spectroscopy (DRS), have postulated that the apparent inactivity may be due in part to adsorption of the active principle onto an inert adjuvant. They have shown that solid–solid interactions do indeed exist between various chemical entities and a wide variety of adjuvants commonly found in pharmaceutical dosage forms. Although some of the interactions may be of the weak variety, they may, nevertheless, be sufficient to alter the absorption and availability of the medicinal agent.

The study of solid–solid interactions is of interest because of the effects that adjuvants may exhibit when incorporated into pharmaceutical dosage forms. These effects may include: (*a*) changes in the nature and intensity of biological activity caused by complexation with the active ingredient (2); (*b*) modifications of the physical state, particle size, and/or surface area of the drug available to the absorption sites (3); or (*c*) changes in the stability of the active principle (4–7).

The purposes of this study were to continue the investigation of solid–solid interactions of a number of drugs and to gain a better insight into the nature of these interactions.

EXPERIMENTAL

Reagents—Oxytetracycline hydrochlorde,[1] tetracycline hydrochloride,[2] chlortetracycline hydrochloride,[2] demethylchlortetracycline hydrochloride,[2] methantheline bromide,[3] bishydroxycoumarin,[4] magnesium trisilicate, ferric phosphate, aluminum hydroxide, tribasic calcium phosphate, and talc were used.

Apparatus—The following were used: Beckman model DU spectrophotometer with a diffuse reflectance attachment (1), constant-temperature water bath with rotating spindles, 150-ml. amber vials with caps, Parafilm,[5] and a glass desiccator with anhydrous calcium sulfate.[6]

Procedure—*Preparation of the Tetracyclines*—Thirty milligrams of active ingredient (tetracycline HCl, oxytetracycline HCl, chlortetracycline HCl, and demethylchlortetracycline HCl) was weighed for every 2 g. of adsorbent (pharmaceutical adjuvant) used. The powders were placed in 150-ml. amber vials, and 25 ml. of distilled water was added as the dispersion medium. The vial was then covered with Parafilm and capped. Equilibration was allowed to proceed for 2 hr. at 30 ± 0.5° to effect interaction. After equilibration, the suspension was filtered under vacuum and the powder dried

[1] Pfizer and Co.
[2] Lederle and Co.
[3] Searle and Co.
[4] K and K Laboratories.
[5] Marathon Co.
[6] Drierite, Hammond Co.

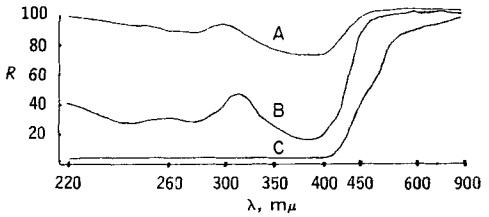


Figure 1—*DRS of chlortetracycline HCl (30 mg.) and magnesium trisilicate (2.0 g.). Key: A, control; B, equilibrated sample; and C, pure chlortetracycline HCl with no adjuvant present.*

in vacuo over anhydrous calcium sulfate. The DRS spectra of the samples were then determined, using magnesium carbonate as the standard.

Preparation of Methantheline Bromide and Bishydroxycoumarin—Sixty milligrams of active ingredient was weighed for every 2 g. of adsorbent. The powders were placed in 150-ml. vials, 10 ml. of distilled water was added to each vial, and equilibration was allowed to proceed as described previously. After equilibration, the dispersion medium was removed by drying *in vacuo* over anhydrous calcium sulfate. The DRS spectra of the samples were determined, using magnesium carbonate as the reference standard.

Preparation of the Control—The control for each experiment was prepared by physically mixing 2 g. of the adsorbent with the indicated amount of dried active ingredient, using a mortar and pestle for the trituration. The DRS of the control was then determined, using magnesium carbonate as the reference standard.

RESULTS AND DISCUSSION

Tetracycline–Adjuvant Interactions—Throughout the following discussion of the different drug–adjuvant systems, a qualitative interpretation of the results will be given because the theory and mechanism of solid–solid interactions are not fully understood at present. However, the authors feel a qualitative interpretation is of value in view of the fact that drug–adjuvant interactions do occur in the solid state. Furthermore, it is important to recognize, prior to dosage formulation, that such surface interactions may occur.

Since Lach and Bornstein (1) showed that oxytetracycline undergoes solid–solid interaction with various adjuvants, since cobalt and nickel complexes of tetracycline and two of its analogs were prepared and their spectral properties studied (8), and because the metal chelation aspects of tetracycline complexes have been investigated extensively in solution (9–13), a DRS study of the various tetracycline–metallic-containing excipients was undertaken. The objective was to determine whether significant spectral differences existed between the different derivatives and whether DRS changes observed in these antibiotic–excipient interactions could be correlated with respect to reported formation constants (9).

Figure 1, which shows the DRS of chlortetracycline hydrochloride and magnesium trisilicate, is an example of the interaction exhibited by tetracycline and its derivatives with this adjuvant. Examination of the figure shows that Spectrum B, which represents the equilibrated sample, has undergone a significant change from that of the physical mixture of the two components (A) and also from the spectrum of the pure drug (C). In addition to these spectral differences, other evidence that an interaction had taken place was noted by a change of color from white or faintly yellow in the physical mixture to a straw yellow in the equilibrated sample. The spectral and color changes occurred with all four tetracycline derivatives. It should be noted here that only in the tetracycline series were the equilibrated samples filtered first and then vacuum dried. Removal of the soluble tetracycline species from solution by the excipient under study further indicates the strength of the interaction.

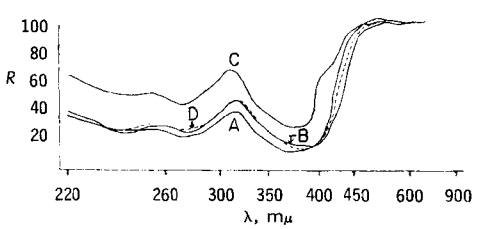


Figure 2—*DRS composite of equilibrated tetracyclines (30 mg.) and magnesium trisilicate (2.0 g.). Key: A, tetracycline HCl; B, chlortetracycline HCl; C, oxytetracycline HCl; and D, demethylchlortetracycline HCl.*

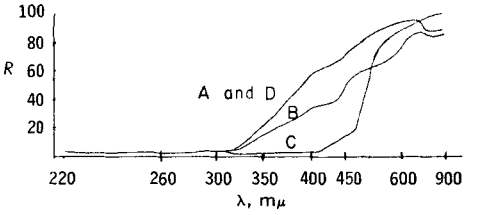


Figure 3—*DRS of tetracycline HCl (30 mg.) and ferric phosphate (2.0 g.). Key: A, control, B, equilibrated sample; C, pure tetracycline HCl with no adjuvant present; and D, pure ferric phosphate with no drug present.*

The equilibrated samples of the tetracycline–magnesium trisilicate series all show a bathochromic shift between 450 and 400 mμ and decreased reflectance throughout the visible and UV region. New band formation is seen at approximately 315 mμ in each case. This band may be considered to be the result of an interaction of the adsorbent with the tetracyclines where the adsorbent facilitates the clarification of an existing peak in this region, or it might represent the reflectance spectrum of a film of drug adsorbed onto the surface of magnesium trisilicate. The suggestion that this peak may be a clarification of an already existing peak can be seen by examining the transmittance spectra of the pure compounds in basic media (1). However, the reflectance spectra of the pure compounds (Spectrum C) do not show a maximum at this wavelength.

It appears that chemical rather than physical interactions have occurred since the spectral changes associated with physical adsorption are usually in the order of 5–10 mμ, while the maximum changes observed here approach 40–45 mμ. This, together with color formation, strongly suggests chemisorption.

Figure 2 shows the composite spectra of the various equilibrated tetracycline–magnesium trisilicate systems. With the exception of small intensity differences and small changes in the bathochromic shifts, the spectra of the equilibrated samples are very similar.

The spectra in Fig. 3 show the tetracycline hydrochloride–ferric phosphate system, which is typical of the spectra of the tetracycline derivatives with this adjuvant. Visual evidence of an interaction was again manifested by color changes. The physical mixtures were faint yellow while the equilibrated samples were tan. An examination of the spectra of the equilibrated samples in the 700–400-mμ region, as compared to the physical mixtures, shows bathochromic shifts of the order of 130 mμ and large intensity changes. Both of these changes are strongly indicative of chemisorption.

Although no differences were observed between the control and equilibrated samples over much of the UV region because of complete absorption by ferric phosphate, it is not unreasonable to assume that spectral changes would also be observed in this region if these samples were diluted with a nonabsorbing material.

Figure 4 shows the spectra of demethylchlortetracycline hydrochloride–aluminum hydroxide. These spectra are again typical of the interaction of the tetracycline series with this adjuvant. The spectra of the equilibrated samples (B) are considerably different from those of the physical mixtures (A). While the physical mixtures showed only limited absorption from approximately 450–350 mμ,

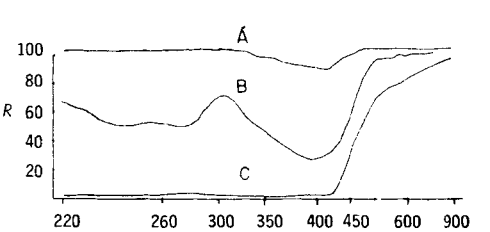


Figure 4—*DRS of demethylchlortetracycline HCl (30 mg.) and aluminum hydroxide (2.0 g.). Key: A, control; B, equilibrated sample; and C, pure demethylchlortetracycline HCl with no adjuvant present.*

Table I—Formation Constants and DRS Bathochromic Shifts of Various Tetracycline–Metal Systems

Tetracycline Derivative	log K' (1:1 Complex)	log K'' (2:1 Complex)	log Ks (Overall)	DRS Batho-chromic Shift, mμ
Aluminum Ion				
Tetracycline	7.4	6.4	19.2	98.0
Chlortetracycline	7.2	ppt.	18.0	87.5
Oxytetracycline	7.0	ppt.	18.0	98.0
Ferric Ion				
Tetracycline	9.9	8.6	25.3	130
Chlortetracycline	9.4	7.2	22.2	125
Oxytetracycline	9.6	7.2	22.5	140
Magnesium Ion				
Tetracycline	—	—	—	57
Chlortetracycline	—	—	—	44
Oxytetracycline	3.8	—	—	45

the equilibrated samples exhibited significant absorption from 500–220 mμ.

The drug concentration of 30 mg. per 2 g. of adjuvant was maintained here, since Lach and Bornstein (1) found that at this concentration the reflectance spectrum presumably represents the chemisorbed single layer. They found that when the drug–adjuvant ratio was increased, the bands in the reflectance spectrum broadened with a limit approaching the spectrum of the pure tetracyclines.

In addition to exhibiting absorption over a wider wavelength range than the physical mixtures, the equilibrated samples showed a bathochromic shift of approximately 80 mμ and a color change from white or faint yellow in the physical mixtures to an intense yellow. These spectral and visual changes are indicative of a chemical interaction of the drug molecules with the available active surface sites.

The large bathochromic shifts observed in these DRS studies of tetracycline–metallic adjuvant systems have also been reported to occur in solution spectra in the presence of metallic ions. Conover (10) has stated that there is a large bathochromic shift of the characteristic long wavelength UV absorption of solutions of tetracycline and its derivatives in an excess of many bivalent and tervalent metallic ions. He reports that these solution band displacements vary quantitatively with various metals, but qualitatively the effects are comparable. It should be pointed out that the observed bathochromic shifts and the nature of the DRS spectra also vary with various metallic adjuvants. However, the spectra of the various tetracycline derivatives for any given metal-containing adjuvant are comparable, which is reasonable and expected.

The bathochromic changes observed in the DRS spectra of these antibiotic–metallic adjuvant interactions were compared with reported formation constants (9) for the metal complexes of the tetracyclines (Table I). Since the present studies deal with surface interactions for which the stoichiometry of the interaction spectrum cannot be determined, a comparison was made between the bathochromic shifts observed in these interactions and log Ks values. Ks has been defined by Albert and Rees (9) as the product of all the partial constants. The bathochromic shifts were determined at a reflectance value between 700 and 380 mμ which exhibited the maximum change.

A comparison of the magnitude of the bathochromic shifts and the changes in the formation constants indicates some correlation,

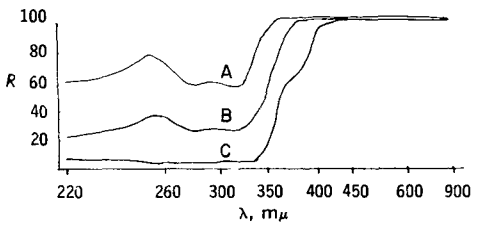


Figure 5—*DRS of bishydroxycoumarin (60 mg.) and magnesium trisilicate (2.0 g.). Key: A, control; B, equilibrated sample; and C, pure bishydroxycoumarin with no adjuvant present.*

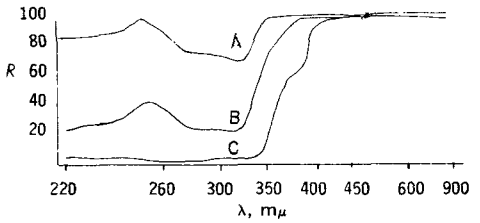


Figure 6—*DRS of bishydroxycoumarin (60 mg.) and aluminum hydroxide (2.0 g.). Key: A, control; B, equilibrated sample; and C, pure bishydroxycoumarin with no adjuvant present.*

because the ferric-containing adjuvant exhibits a larger bathochromic shift than the aluminum-containing adjuvant which, in turn, is larger than the magnesium-containing adjuvant. This is in agreement with the formation constants reported for these ions, since the value for the ferric ion is larger than the aluminum ion, which is larger than the magnesium ion. Although it is interesting that the observed DRS changes do parallel the reported formation constant values for these ions, it is difficult at this time to make a quantitative comparison.

Studies were also conducted to determine whether these drug–adjuvant interactions occurred when physical mixtures were exposed to humid conditions. Dried, physically mixed samples of the tetracyclines and magnesium trisilicate were placed on evaporating dishes in a moisture chamber, which consisted of a wax-sealed desiccator in which the desiccant had been replaced by water. The samples were allowed to stand in this moisture chamber for 4 hr., after which they were removed and dried *in vacuo* over anhydrous calcium sulfate. The spectra of these compounds were determined and found to resemble those shown in Fig. 1. The primary differences observed were that the bathochromic and intensity changes were not as pronounced, nor was the peak at 315 mμ as clearly defined. Possibly the spectral differences between water-equilibrated samples and samples exposed to humid conditions reflect an incomplete interaction such as an interaction of only the surface molecules. It is reasonable to assume that with the equilibration technique, a greater degree of interaction may occur with more pronounced spectral changes, because more surface area is exposed during the procedure.

Interaction of Methantheline Bromide and Bishydroxycoumarin with Various Metallic Adjuvants—The study of bishydroxycoumarin and methantheline bromide for possible drug–adjuvant interactions was prompted by reported therapeutic discrepancies of solid dosage forms of bishydroxycoumarin and certain quaternary compounds (14, 15).

The adjuvants chosen for this study were selected on the basis of the metal fraction which they contain and/or because of their usage in pharmaceutical formulations. Sixty milligrams of active principle was mixed with 2 g. of adjuvant, since studies with 30 mg. of active compound showed no absorbance in either the physical mixtures or the equilibrated samples.

Spectral data presented in Fig. 5 show the bishydroxycoumarin–magnesium trisilicate system. These spectra, as well as the interaction of bishydroxycoumarin with aluminum hydroxide (Fig. 6) and talc (Fig. 7), are representative of a chemisorption interaction. The spectrum of the equilibrated bishydroxycoumarin–tribasic calcium phosphate system, however, is unchanged from the spectrum of the physical mixture.

The equilibrated magnesium trisilicate (Fig. 5), aluminum hydroxide (Fig. 6), and talc (Fig. 7) systems all show a rather large bathochromic shift between 375 and 310 mμ, as well as a hyper-

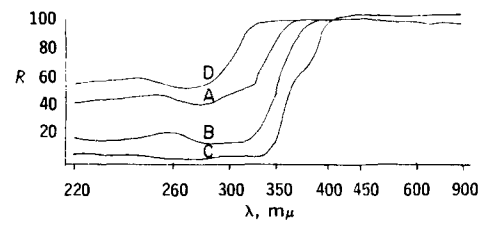


Figure 7—*DRS of bishydroxycoumarin (60 mg.) and talc (2.0 g.). Key: A, control; B, equilibrated sample; C, pure bishydroxycoumarin with no adjuvant present; and D, pure talc with no drug present.*

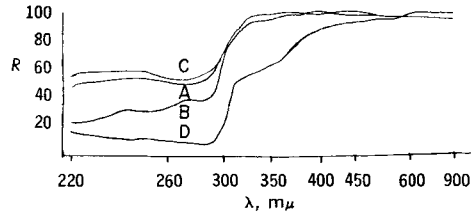


Figure 8—*DRS of methantheline bromide (60 mg.) and talc (2.0 g.). Key: A, control; B, equilibrated sample; C, pure talc with no drug present; and D, pure methantheline bromide with no adjuvant present.*

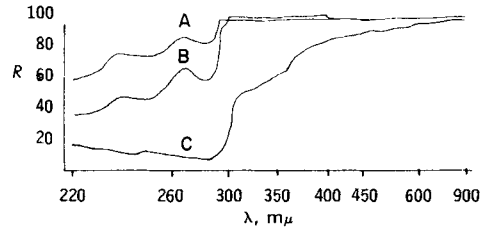


Figure 9—*DRS of methantheline bromide (60 mg.) and tribasic calcium phosphate (2.0 g.). Key: A, control; B, equilibrated sample; and C, pure methantheline bromide with no adjuvant present.*

chromic change throughout the UV region. In the magnesium trisilicate and talc systems, there is a smaller bathochromic change involving a band at 250 mμ, which has shifted to 255 mμ in the equilibrated samples. Since this particular band shift in the bishydroxycoumarin systems occurs only with the magnesium-containing adjuvants, it may indicate chelation of bishydroxycoumarin with magnesium.

Studies involving methantheline bromide and talc (Fig. 8), tribasic calcium phosphate (Fig. 9), and aluminum hydroxide (Fig. 10) showed small bathochromic shifts but rather large intensity changes. However, the interaction with magnesium trisilicate (Fig. 11) appears to be indicative of chemisorption. As in the bishydroxycoumarin–adjuvant systems, no color changes were observed when the samples were equilibrated.

Although it is evident from the spectral data presented that these interactions will vary in intensity, the spectral differences have not been correlated with bioavailability. Desorption and bioavailability experiments of these drug–excipient interactions are currently underway to gain a better understanding of the significance of such interactions.

The chemical properties of the various adjuvants may also influence the spectral changes observed with the methantheline bromide and bishydroxycoumarin systems. The availability of the metal portion in various adjuvants may differ since some surfaces are thought to be more saturated than others. The valency requirements of surface atoms may be more fully satisfied by bonding with nearby atoms, thus making them less available for chemisorption. Nor can hydration effects be overlooked, because hydration may also satisfy the valency requirements of the metallic portion and thereby decrease the number of active sites available for interaction.

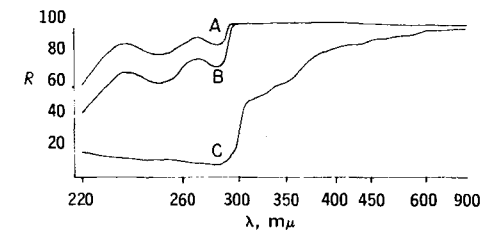


Figure 10—*DRS of methantheline bromide (60 mg.) and aluminum hydroxide (2.0 g.). Key: A, control; B, equilibrated sample; and C, pure methantheline bromide with no adjuvant present.*

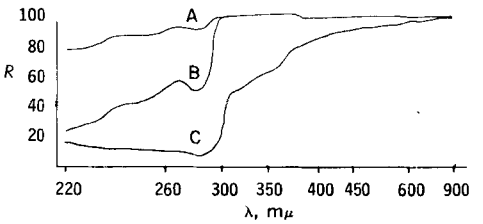


Figure 11—*DRS of methantheline bromide (60 mg.) and magnesium trisilicate (2.0 g.). Key: A, control; B, equilibrated sample; and C, pure methantheline bromide with no adjuvant present.*

A possible explanation for the bishydroxycoumarin–adjuvant interactions may be one of chelation. Further studies are in progress with respect to this hypothesis, and subsequent publications will deal with the mechanism of these interactions.

The therapeutic differences observed in presumably equivalent dosage forms may be due, in part, to surface interactions of the type described in this study. These interactions may exert an effect on the dissolution rate, as preliminary studies currently underway have indicated, and subsequently reduce the gastrointestinal absorption rates.

While it is recognized that drug release from pharmaceutical dosage forms is a complex phenomenon, the fact that surface interactions occur with various inactive adjuvants makes it imperative that the effect of these interactions on drug release be studied. Perhaps a more scientific approach to formulation through a knowledge of drug–adjuvant interactions could prevent many of the discrepancies observed in the therapeutic efficacy of pharmaceutical dosage forms.

REFERENCES

(1) J. L. Lach and M. Bornstein, *J. Pharm. Sci.*, **54,** 1730(1965).

(2) G. Levy and R. H. Reuning, *ibid.*, **53,** 1471(1964).

(3) E. H. Buckwalter and H. L. Dickison, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 661(1958).

(4) E. G. Remmers, W. C. Barringer, G. M. Sieger, and A. P. Doerschuk, *J. Pharm. Sci.*, **53,** 1534(1964).

(5) E. G. Remmers, G. M. Sieger, A. P. Doerschuk, L. Ritter, and J. Weidenheimer, *ibid.*, **51,** 86(1962).

(6) E. G. Remmers, W. C. Barringer, G. M. Sieger, N. Anagnostakos, J. J. Corbett, and A. P. Doerschuk, *ibid.*, **54,** 49(1965).

(7) E. G. Remmers, W. C. Barringer, G. M. Sieger, and A. P. Doerschuk, *ibid.*, **53,** 1452(1964).

(8) W. A. Baker, Jr., and Patrick M. Brown, *J. Amer. Chem. Soc.*, **88,** 1314(1966).

(9) A. Albert and C. W. Rees, *Nature*, **177,** 433(1956).

(10) L. H. Conover, "Symposium on Antibiotics and Mould Metabolites," The Chemical Society, Burlington House, London, England, 1956.

(11) A. Albert, *Nature*, **172,** 201(1953).

(12) J. T. Doluisio and A. N. Martin, *J. Med. Chem.*, **6,** 16 (1963).

(13) *Ibid.*, **6,** 20(1963).

(14) E. Lozinski, *Can. Med. Ass. J.*, **83,** 177(1960).

(15) N. A. Batuyios and E. A. Brecht, *J. Amer. Pharm. Ass., Sci. Ed.*, **46,** 524(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication April 6, 1970.

Abstracted in part from a dissertation presented by Lyle D. Bighley to the Graduate College, University of Iowa, in partial fulfillment of Doctor of Philosophy degree requirements.

* Fellow, American Foundation for Pharmaceutical Education. Henry S. Wellcome Memorial Fellow, 1965–1966.

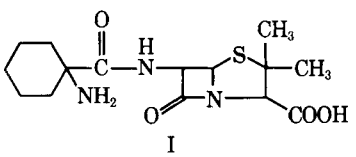
Dissolution Behavior and Solubility of Anhydrous and Dihydrate Forms of Wy-4508, an Aminoalicyclic Penicillin

JOHN W. POOLE* and C. KANTA BAHAL

Abstract □ Anhydrous and dihydrate forms of an aminoalicyclic penicillin (Wy-4508) were compared for solubility in distilled water at temperatures ranging from 7 to 60°. Differences were noted in the physical–chemical properties of these two forms. Below the transition temperature, 61°, the anhydrous form was found to be significantly more soluble than the dihydrate. In addition, the solubility of the anhydrous crystal was shown to be inversely related to temperature. The thermodynamic properties noted for the two forms of the antibiotic have been experimentally evaluated.

Keyphrases □ Penicillin, aminoalicyclic (Wy-4508)—anhydrous, dihydrate forms, solubility □ Temperature effect—aminoalicyclic penicillin solubility □ Dissolution—aminoalicyclic penicillin, anhydrous, dihydrate □ Thermodynamic properties—anhydrous, dihydrate aminoalicyclic penicillin

Many organic and inorganic compounds exist in separate crystalline forms having different physical–chemical properties. Higuchi (1) has pointed out that the resulting variation in thermodynamic properties associated with the differences in crystal forms may be of considerable pharmaceutical importance. The present report is concerned with an investigation of the differences in the physical–chemical properties of two forms of an aminoalicyclic penicillin, Wy-4508 [6-(1-aminocyclohexanecarboxamido)penicillanic acid] (I).



I

Specifically, the solubilities in distilled water of the anhydrous and dihydrate forms of this penicillin were determined, and the thermodynamic properties of these two compounds were experimentally evaluated.

Much of the past work reported on the physical–chemical properties of crystalline hydrates has been concerned with inorganic compounds, as illustrated by the studies of Taylor and Henderson (2) on various hydrates of calcium nitrate and of Hill (3) on calcium sulfate. Recently, several investigations concerned with studies of organic molecules in the anhydrous and hydrated forms have been reported. Two of the hydrated forms of phenobarbital, as well as the anhydrous form, were examined by Eriksson (4) for apparent solubility in water as a function of time. Shefter and Higuchi (5) reported the relative dissolution rates of solvated and nonsolvated crystalline forms of several types of compounds of pharmaceutical interest, including steroids and xanthines. These workers also determined the thermodynamic properties of several crystal systems. In a recent report by the present authors (6), the dissolution and solubility behavior of anhydrous and trihydrate forms of the semisynthetic penicillin, ampicillin, was reported.

EXPERIMENTAL

Apparatus—The following were used: a constant-temperature water bath equipped with a Unitherm Haake constant-temperature circulator[1] and a rotating-bottle apparatus,[2] Swinney hypodermic adapter,[3] Millipore filters (pore size 0.45 μ),[3] and 120-ml. amber bottles with polyseal caps.[4]

Compounds—In all the experiments, anhydrous Wy-4508 (Wyeth Laboratories batch C-10777, m.p. 181–182°) was used. The dihydrate form of Wy-4508 was prepared from the anhydrous material by preparing a saturated solution of the penicillin in 1.0 *N* hydrochloric acid and precipitating the hydrated form at pH 7. IR spectra, differential thermal analysis, and Karl Fischer moisture determinations were obtained for the material and conclusively characterized the anhydrous and dihydrate forms.

Dissolution Procedure—An excess of drug, 4 g., in the appropriate form was added to 50 ml. of distilled water previously equilibrated to the desired temperature. The bottles were rotated at a constant speed in a water bath maintained at the appropriate temperature. Samples withdrawn at definite intervals were filtered through a Millipore filter and diluted immediately to avoid any precipitation of the penicillin in the filtered samples due to supersaturation.

The penicillin content was determined by means of the following iodometric titration procedure. To 2.0-ml. aliquots containing 1–3 mg. of Wy-4508, 2.0 ml. of 1 *N* sodium hydroxide was added, and samples were allowed to stand at room temperature for 15 min. At the end of the time, 2.0 ml. of 1.2 *N* HCl was added, followed by 10 ml. of 0.01 *N* iodine. After 15 min., the excess of iodine was titrated, using 0.01 *N* sodium thiosulfate. For the blank determinations, 10 ml. of 0.01 *N* iodine was added to a 2.0-ml. sample and titrated immediately.

RESULTS AND DISCUSSION

The solubility of the anhydrous form of Wy-4508 at 7, 20, 25, 30, 40, 50, and 60° is shown in Fig. 1. Similar data at 10, 20, 25, 30, 40, 50, and 60° for the dihydrate form of this agent are illustrated

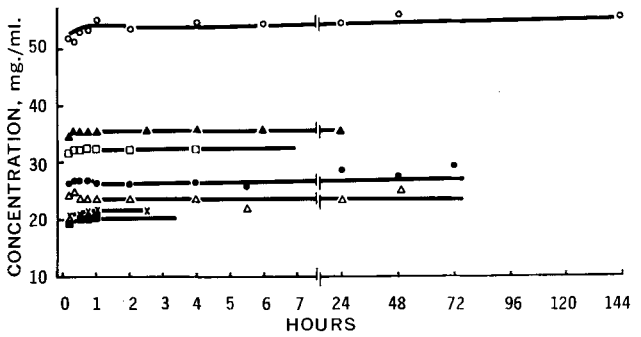


Figure 1—*Solubility of the anhydrous form of Wy-4508 in distilled water at temperatures ranging from 7 to 60°. Key:* ○, *7°;* ▲, *20°;* □, *25°;* ●, *30°;* △, *40°;* ×, *50°; and* ■, *60°.*

[1] Brinkmann Instruments, Westbury, N. Y.
[2] E. D. Menold Sheet Co., Lester, Pa.
[3] Millipore Corp., Bedford, Mass.
[4] Erno Products, Philadelphia, Pa.

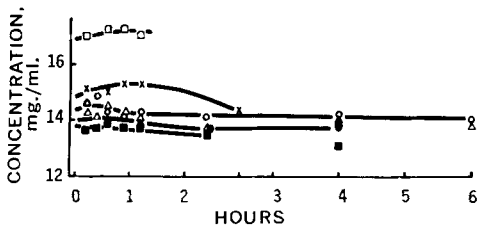


Figure 2—*Solubility of the dihydrate form of Wy-4508 in distilled water at temperatures ranging from 10 to 60°. Key: ○, 10°; ▲, 20°; ■, 25°; ●, 30°; △, 40°; ×, 50°; and □, 60°.*

in Fig. 2. These figures show the concentration of the antibiotic attained in solution as a function of time in the presence of the excess of the solid phase in the appropriate form and under essentially constant agitation. An inverse relationship between temperature and solubility for the anhydrous form of this drug was observed. The dihydrate form of the drug shows no significant change in solubility between 10 and 40°. The negative heat of solution noted for this compound is consistent with the data reported for the anhydrous form of the amphoteric penicillin, ampicillin (6).

An inverse relationship between solubility and temperature is not usually observed, especially with organic compounds. However, from the data presented here and previously (6), it appears that such a relationship (negative heat of solution) is characteristic of the anhydrous form of amphoteric penicillins.

The data shown for the solubility in water for the two forms of Wy-4508 investigated suggest that the equilibrium solubilities observed are good approximations of the true solubility of these crystals. Therefore, the measurements made at the several temperatures permit calculation of the thermodynamic values involved in the transition of the anhydrous form to the dihydrate. The thermodynamic relationship involving polymorphism and solubility is extensively discussed in reports by Shefter and Higuchi (5) and Higuchi *et al.* (7).

A classical van't Hoff-type plot of the apparent equilibrium solubilities observed at the various temperatures of this investigation showed a reasonably good linear relationship for both forms of the antibiotic for temperatures up to 40° (Fig. 3). At the higher temperatures, a deviation from linearity was observed for both forms of Wy-4508. This is probably due to degradation of the penicillin at these higher temperatures.

The transition temperature for the anhydrous–dihydrate crystal system of this compound corresponds to the temperature at which the solubility of the two forms is equal. The transition temperature for this system, obtained by extrapolating the straight-line portions

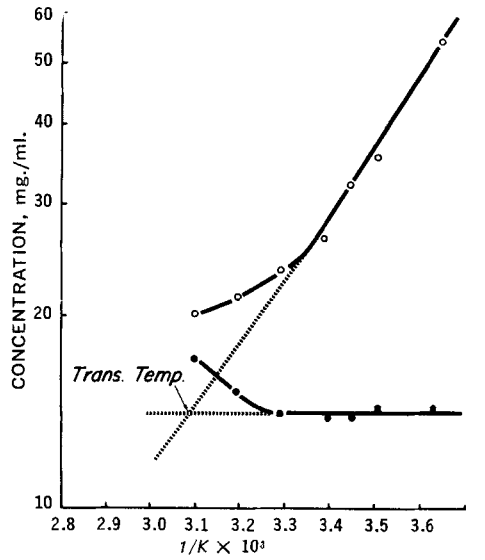


Figure 3—*The van't Hoff-type plot for the anhydrous and dihydrate forms of Wy-4508 in distilled water. Key: ○, anhydrous; and ●, dihydrate.*

of the van't Hoff-type plot, was determined to be 61° (Fig. 3). This plot also points up the inverse relationship between temperature and solubility for the anhydrous form of the drug, whereas the effect of temperatures ranging from 10 to 40° on the solubility of the dihydrate form is insignificant.

The values of the heat of solution for each of the crystal forms were calculated from the slopes of the van't Hoff-type plot and were determined to be −4700 and 0 cal./mole for the anhydrous and dihydrate forms, respectively, The enthalpy of hydration ($\Delta H_{A,H}$) was determined to be −4700 cal./mole. This value is considerably less than that noted for the anhydrous–trihydrate system of ampicillin (6), where the value was determined to be −6400 cal./mole. This is probably due to the fact that only 2 moles of water are involved in the hydration of Wy-4508 as compared to 3 moles of solvent for each mole of ampicillin.

The free energy difference, ΔF_T, between the anhydrous and hydrated forms at constant temperature and pressure is determined by Eq. 1:

$$\Delta F_T = RT \ln \frac{C_s\,(\text{anhydrous})}{C_s\,(\text{dihydrate})} \qquad \text{(Eq. 1)}$$

where C_s is the solubility of the form under consideration at a particular temperature, T, and R is the gas constant. This value, ΔF_T, is a measure of the free energy change involved in a conversion of the anhydrous crystal to the dihydrate crystal. The ΔF_T's at 25 and 37° (corresponding to room and body temperatures) have been determined to be −550 and −390 cal./mole, respectively.

The entropy change, ΔS_T, for the reaction involved in hydrate formation can be calculated by Eq. 2:

$$\Delta S_T = \frac{\Delta H_{A,H} - \Delta F_T}{T} \qquad \text{(Eq. 2)}$$

The values computed for the hydration of the anhydrous to the dihydrate Wy-4508 crystals at 25 and 37° were −13.9 e.u. At the transition temperature of the anhydrous–dihydrate crystal system, ΔF is equal to zero and the entropy change can be calculated by

$$\Delta S\,(\text{trans}) = \frac{\Delta H_{A,H}}{T\,(\text{trans})} \qquad \text{(Eq. 3)}$$

For Wy-4508, ΔS (trans) was determined to be −14.1 e.u. The hydrated species of this system contains 2 molecules of water; the possible intramolecular hydrogen-bond formation between these associated water molecules may account for the relatively large entropy change noted.

The entropy change involved in the fusion of water at 25° is approximately 6 e.u. This decrease in entropy associated with the formation of ice is approximately the same entropy change obtained for the hydration of glutethimide and theophylline (5). Therefore, the energy involved in the transformation of the dehydrated forms of compounds of such types to the hydrate may be related mainly to the decrease in the entropy of water molecules in the hydrate structure. The results obtained for the entropy change in the ampicillin anhydrous–trihydrate system (6), where 3 associated water molecules result in an entropy change of −20 e.u. at 25°, and for the entropy change observed in the present study, where 2 associated water molecules show a change in this value of −13.9 e.u., are consistent with this hypothesis.

The thermodynamic values calculated for the anhydrous–dihydrate Wy-4508 system are summarized in Table I.

The equilibrium solubilities observed in these experiments apparently correspond to the solubilities of anhydrous and dihydrate crystalline phases for the Wy-4508 molecule. At the temperatures

Table I—Thermodynamic Values Calculated for the Anhydrous–Dihydrate Wy-4508 System

Temp.	ΔH, cal./mole Anhydrous	Dihydrate	ΔF_T, cal./mole[a]	ΔS_T, e.u.[a]
	(−4700)	(0)		
25°			−550	−13.9
37°			−390	−13.9
61°			0	−14.1

[a] Calculated for the conversion from the anhydrous to the dihydrate form.

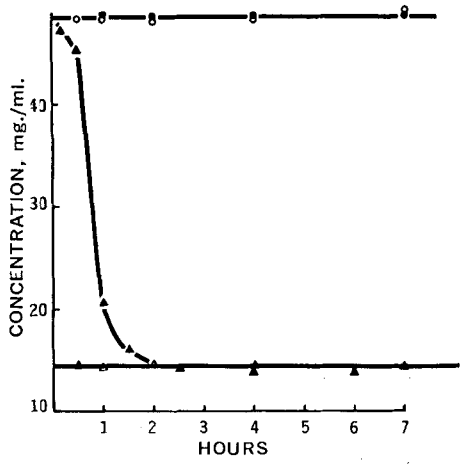


Figure 4—*Influence of seeding anhydrous Wy-4508 with 1 and 10% dihydrate crystals on the solubility in distilled water at 10°. Key:* ○, *anhydrous;* ●, *seeded with 1% dihydrate;* ▲, *seeded with 10% dihydrate; and* △, *dihydrate.*

utilized, there was no evidence of conversion of the more soluble anhydrous form to the less soluble dihydrate species as would be expected strictly from thermodynamic consideration. Undoubtedly, this may be due to steric factors involved in the association of the water molecules in the crystal system and to the relatively high water solubility of this particular amphoteric penicillin. Even at lower temperatures (10°), seeding of the anhydrous form with 1% dihydrate crystals did not result in a rapid conversion of the anhydrous form to the less soluble species. However, when the seed was incorporated at the 10% level, a relatively rapid and complete conversion of the anhydrous to the dihydrate form was observed as shown by the decrease in solubility. These data are summarized in Fig. 4.

SUMMARY

The solubility of the anhydrous and dihydrate forms of Wy-4508 in distilled water has been determined over a temperature range of 7–60°. From the solubility data, the transition temperature for this crystal system was estimated to be 61°. The anhydrous form was found to be significantly more water soluble than the dihydrate at all temperatures below the transition temperature. In addition, the solubility of the anhydrous crystal was shown to be inversely related to temperature (negative heat of solution). The thermodynamic values for the anhydrous–dihydrate Wy-4508 crystal system have been calculated.

REFERENCES

(1) T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 657(1958).
(2) H. S. Taylor and W. N. Henderson, *J. Amer. Chem. Soc.*, **37**, 1688(1915).
(3) A. E. Hill, *ibid.*, **59**, 2243(1937).
(4) S. O. Eriksson, *Svensk. Farm. Tidskr.*, **65**, 353(1961).
(5) E. Shefter and T. Higuchi, *J. Pharm. Sci.*, **52**, 781(1963).
(6) J. W. Poole and C. K. Bahal, *ibid.*, **57**, 1945(1968).
(7) W. I. Higuchi, P. K. Lau, T. Higuchi, and J. W. Shell, *ibid.*, **52**, 150(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 17, 1969, from the *Pharmacy Research and Development Division, Wyeth Laboratories, Radnor, PA 19087*
Accepted for publication April 3, 1970.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.
* Fellow, American Foundation for Pharmaceutical Education.

Effects of Disulfiram on Growth, Longevity, and Reproduction of the Albino Rat

HARALD G. O. HOLCK, PAUL M. LISH*, DAVID W. SJOGREN†, W. W. WESTERFELD‡, and MARVIN H. MALONE§

Abstract □ Chronic oral ingestion of disulfiram at concentrations of 1:1000 and 1:2000 in powdered food retards the growth and reproductive capacity of the albino rat; a concentration of 1:1000 also decreases longevity. Enhancement of diet with ascorbic acid has no antidotal action on the growth and reproductive effects. Chronic disulfiram feeding appears to have no significant effects on whole animal oxygen consumption, blood counts, tissue xanthine oxidase activity, and liver molybdenum content. Calcium deposition in the cerebellum was absent.

Keyphrases □ Disulfiram—chronic feeding studies, rats □ Blood cells, number—disulfiram effect □ Reproduction, longevity, body weight—disulfiram effects □ Oxygen consumption—disulfiram effect □ Ascorbic acid effect—disulfiram activity

While single oral doses and chronic feeding of disulfiram in the absence of ethanol are considered relatively nontoxic (1–3), chronic feeding of rats on diets containing disulfiram (1:400–1:10,000) has been reported to retard growth and increase mortality (4). Muscular incoordination was seen in rats chronically fed the 1:400 and 1:1000 diets by the age of 2 years, and microscopic examination of the cerebellum and basal ganglia revealed calcified masses in rats fed 1:400 disulfiram. In addition to its generally accepted inhibitory effect on liver aldehyde oxidase, disulfiram has been reported to have some antithyroid capacity (5). Disulfiram can inhibit the oxygen uptake of rat liver homogenates by apparently acting as a competitive hydrogen acceptor (6). Ascorbic acid overcomes this inhibition and is reported to be effective intravenously in man (7). The metabolic breakdown of disulfiram into diethyldithiocarbamate, an effective chelator, has also been noted (8).

After preliminary work in this laboratory, it was considered important to document the effects of disulfiram in the young rat during the most rapid phases of growth and to see whether longevity and reproductive capacity would be affected.

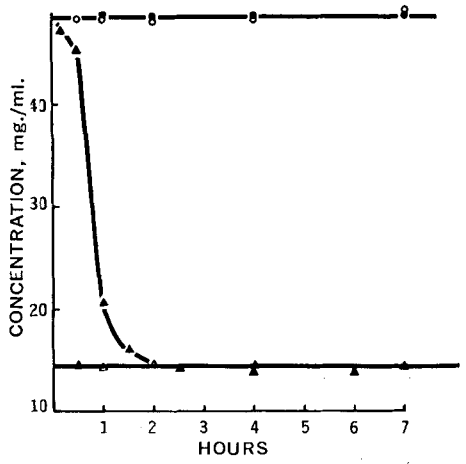


Figure 4—*Influence of seeding anhydrous Wy-4508 with 1 and 10% dihydrate crystals on the solubility in distilled water at 10°. Key:* ○, *anhydrous;* ●, *seeded with 1% dihydrate;* ▲, *seeded with 10% dihydrate; and* △, *dihydrate.*

utilized, there was no evidence of conversion of the more soluble anhydrous form to the less soluble dihydrate species as would be expected strictly from thermodynamic consideration. Undoubtedly, this may be due to steric factors involved in the association of the water molecules in the crystal system and to the relatively high water solubility of this particular amphoteric penicillin. Even at lower temperatures (10°), seeding of the anhydrous form with 1% dihydrate crystals did not result in a rapid conversion of the anhydrous form to the less soluble species. However, when the seed was incorporated at the 10% level, a relatively rapid and complete conversion of the anhydrous to the dihydrate form was observed as shown by the decrease in solubility. These data are summarized in Fig. 4.

SUMMARY

The solubility of the anhydrous and dihydrate forms of Wy-4508 in distilled water has been determined over a temperature range of 7–60°. From the solubility data, the transition temperature for this crystal system was estimated to be 61°. The anhydrous form was found to be significantly more water soluble than the dihydrate at all temperatures below the transition temperature. In addition, the solubility of the anhydrous crystal was shown to be inversely related to temperature (negative heat of solution). The thermodynamic values for the anhydrous–dihydrate Wy-4508 crystal system have been calculated.

REFERENCES

(1) T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 657(1958).
(2) H. S. Taylor and W. N. Henderson, *J. Amer. Chem. Soc.*, **37**, 1688(1915).
(3) A. E. Hill, *ibid.*, **59**, 2243(1937).
(4) S. O. Eriksson, *Svensk. Farm. Tidskr.*, **65**, 353(1961).
(5) E. Shefter and T. Higuchi, *J. Pharm. Sci.*, **52**, 781(1963).
(6) J. W. Poole and C. K. Bahal, *ibid.*, **57**, 1945(1968).
(7) W. I. Higuchi, P. K. Lau, T. Higuchi, and J. W. Shell, *ibid.*, **52**, 150(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 17, 1969, from the *Pharmacy Research and Development Division, Wyeth Laboratories, Radnor, PA 19087*
Accepted for publication April 3, 1970.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.
* Fellow, American Foundation for Pharmaceutical Education.

Effects of Disulfiram on Growth, Longevity, and Reproduction of the Albino Rat

HARALD G. O. HOLCK, PAUL M. LISH*, DAVID W. SJOGREN†, W. W. WESTERFELD‡, and MARVIN H. MALONE§

Abstract □ Chronic oral ingestion of disulfiram at concentrations of 1:1000 and 1:2000 in powdered food retards the growth and reproductive capacity of the albino rat; a concentration of 1:1000 also decreases longevity. Enhancement of diet with ascorbic acid has no antidotal action on the growth and reproductive effects. Chronic disulfiram feeding appears to have no significant effects on whole animal oxygen consumption, blood counts, tissue xanthine oxidase activity, and liver molybdenum content. Calcium deposition in the cerebellum was absent.

Keyphrases □ Disulfiram—chronic feeding studies, rats □ Blood cells, number—disulfiram effect □ Reproduction, longevity, body weight—disulfiram effects □ Oxygen consumption—disulfiram effect □ Ascorbic acid effect—disulfiram activity

While single oral doses and chronic feeding of disulfiram in the absence of ethanol are considered relatively nontoxic (1–3), chronic feeding of rats on diets containing disulfiram (1:400–1:10,000) has been reported to retard growth and increase mortality (4). Muscular incoordination was seen in rats chronically fed the 1:400 and 1:1000 diets by the age of 2 years, and microscopic examination of the cerebellum and basal ganglia revealed calcified masses in rats fed 1:400 disulfiram. In addition to its generally accepted inhibitory effect on liver aldehyde oxidase, disulfiram has been reported to have some antithyroid capacity (5). Disulfiram can inhibit the oxygen uptake of rat liver homogenates by apparently acting as a competitive hydrogen acceptor (6). Ascorbic acid overcomes this inhibition and is reported to be effective intravenously in man (7). The metabolic breakdown of disulfiram into diethyldithiocarbamate, an effective chelator, has also been noted (8).

After preliminary work in this laboratory, it was considered important to document the effects of disulfiram in the young rat during the most rapid phases of growth and to see whether longevity and reproductive capacity would be affected.

Table I—Effects of Disulfiram in the Diet on Body Weight of the Albino Rat[a]

Run	Sex (Number)	Age, Days	Days on Diet	Mean Wt., g. (±*SE*)	Mean Wt., g. (±*SE*)	Significance from Control, *p*	Mean Wt., g. (±*SE*)	Significance from Control, *p*
				Control Diet	**1:2000 Disulfiram**		**1:1000 Disulfiram**	
I	M (20)	30	0	55 (1.3)	56 (2.2)	>0.50	56 (1.7)	>0.50
		60	30	148 (3.3)	129 (4.6)	<0.01	115 (4.0)	<0.001
		90	60	191 (5.7)	169 (6.0)	<0.01	157 (5.0)	<0.001
		120	90	223 (4.9)	195 (4.3)	<0.001	185 (5.4)	<0.001
I	F (22)	30	0	52 (1.8)	51 (2.0)	>0.50	52 (1.9)	>0.50
		60	30	116 (2.6)	103 (4.0)	<0.01	90 (3.1)	<0.001
		90	60	139 (3.1)	127 (3.3)	<0.01	115 (2.9)	<0.001
		120	90	151 (2.8)	141 (3.1)	<0.02	129 (2.6)	<0.001
				Control Diet	**1:8000 Disulfiram**		**1:4000 Disulfiram**	
II	M (10)	30	0	49 (3.6)	50 (3.2)	>0.50	48 (3.2)	>0.50
		60	30	147 (4.2)	158 (5.2)	>0.10	148 (5.7)	>0.50
		90	60	218 (7.9)	225 (5.2)	>0.40	214 (5.0)	>0.50
II	F (10)	30	0	47 (2.8)	45 (2.2)	>0.50	46 (3.0)	>0.50
		60	30	114 (2.4)	116 (3.1)	>0.50	109 (3.3)	>0.20
		90	60	139 (4.7)	140 (3.4)	>0.50	133 (3.8)	>0.30

[a] The disulfiram was mechanically admixed with Purina powdered laboratory chow.

EXPERIMENTAL

All rats were albinos, bred originally from Wistar stock in this laboratory, and were maintained in animal quarters (26°) on unrestricted Purina powdered laboratory chow and tap water. Test diets containing 1:1000, 1:2000, 1:4000, and 1:8000 parts of disulfiram[1] per kg. of Purina powdered laboratory chow were prepared with an electric mixer. When ascorbic acid was added to the drinking water, the solution was prepared daily with tap water. From food consumption data, the daily intake of 1:2000 disulfiram in very young rats was equivalent to about 10 times the dosage of an adult man (0.5 g./day or 7 mg./kg.), while the intake for an adult rat was about 5 times the human dose.

RESULTS AND DISCUSSION

Growth Studies—Young rats were weaned after 30 days, segregated by sex, and randomly divided into three groups. Diets were instituted as shown in the first run of Table I. A significant growth lag was apparent after 30 days of feeding (13 and 22% for males and 11 and 22% for females fed 1:2000 and 1:1000 disulfiram, respectively). This lag was maintained up through 90 days of feeding (13 and 17% for males and 7 and 15% for females consuming 1:2000 and 1:1000, respectively). These results compare favorably with a 10 and 21% reduction, respectively, obtained in a preliminary study with only 10 males per diet. A second run, conducted in the same manner but feeding 1:4000 and 1:8000 disulfiram in the diet (Table I, Run 2), indicated that there was no significant retardation of growth with these concentrations. In another experiment, when feeding of male rats was begun at the age of 85 days and continued for 105 days, there was no significant difference in body weight between the controls and the rats fed 1:6750, 1:4500, 1:3000, and 1:2000 disulfiram (12 rats/test group).

Blood Counts—At the age of 175–185 days, red and white blood cell counts, as well as leucocyte differentials, were determined for another group of male and female rats fed continuously with either control, 1:2000, or 1:1000 disulfiram diets since the age of 30 days (10 males and 10 females/group). Mean red counts for the males were: 10.8, 10.5, and 9.6 million/cu. mm., and for the females: 10.2, 9.5, and 9.8 million/cu. mm., respectively. Mean white cell counts for males were: 13,360, 15,810, and 14,640/cu. mm., and for females: 12,570, 12,030, and 14,870/cu. mm., respectively. These figures, as well as the differential counts, all fell within the normal ranges (9), indicating no important differences between the groups that might be due to a chelation effect. Body weight differences were equivalent to those reported in the previous section.

Oxygen Consumption—At the age of 360–400 days, groups of male rats fed continuously either control, 1:2000, or 1:1000 disulfiram diets since the age of 30 days (9–10 animals/group) were placed in a closed system similar to that described by Maclagan and Sheahan (10) for the estimation of ml. oxygen utilized/kg./hr. at standard conditions. Mean oxygen consumption was not significantly different ($p > 0.50$) for the three populations (815, 793, and 846 ml./kg./hr. S.T.P., respectively). Apparently, these concentrations of disulfiram in the diet had not significantly altered thyroid function.

Table II—Effects of Disulfiram in the Diet on Longevity of the Male Albino Rat[a]

	Number/ Group	Mean Life Span, Days (±*SE*)	Significance from Control, *p*
Control diet	9	944 (33)	—
1:2000 Disulfiram	10	896 (37)	>0.30
1:1000 Disulfiram	10	744 (70)	<0.02

[a] Separated from mothers at the age of 30 days, and test diets begun *ad libitum*.

Effects on Longevity and Body Weight—Utilizing the techniques previously described, three groups of males were maintained on either control, 1:2000, or 1:1000 disulfiram diets until death. As shown in Table II, longevity was significantly reduced only in the animals receiving 1:1000 disulfiram. As in the preceding experiments, no signs of motor incoordination (4) were noted, although a general tendency for poor grooming was seen with the disulfiram-fed animals. The body weight of the rats receiving disulfiram in their food was consistently less than that of the control animals. As in previous experiments, there were no significant differences in the daily food intake between the groups. As with all old rats, a 10–20% incidence of tumors was seen in each of the three groups, but no specific tissue susceptibility was noted. Histologic examinations of the cerebella of two control rats, two on the 1:1000 disulfiram diets, and five on the 1:2000 disulfiram diets showed no calcification.[2] The age at the time of examination ranged from 2.68–2.97, 2.43–2.71, and 2.51–2.71 years, respectively. The age in all instances exceeded the 2-year maximum reported by Fitzhugh *et al.* (4), who found calcium deposition with a diet concentration of 1:400 of disulfiram.

Vitamin Enhancement of Diet—While the rat appears to have some intrinsic capacity to synthesize ascorbic acid (9), an experiment was performed to determine if inclusion of ascorbic acid in the drinking water would reverse or significantly mitigate the disulfiram-induced growth lag. As shown in Table III, a significant

[1] Supplies of disulfiram (Antabuse) were donated by Ayerst, McKenna and Harrison, Ltd.

[2] Histopathologic examinations were performed courtesy of Dr. Charles A. Mebus, D.V.M., Department of Veterinary Science, University of Nebraska.

Table III—Effects of Disulfiram Alone and with Ascorbic Acid on Growth of the Albino Rat

Sex (Number)	Age, Days	Days on Diet	Control Diet, Mean Wt., g. (±*SE*)	1:2000 Disulfiram: Mean Wt., g. (±*SE*)	1:2000 Disulfiram: Significance from Control, *p*	1:1000 Disulfiram, Tap Water: Mean Wt., g. (±*SE*)	1:1000 Disulfiram, Tap Water: Significance from Control, *p*	1:1000 Disulfiram, 0.5% Ascorbic Acid: Mean Wt., g. (±*SE*)	1:1000 Disulfiram, 0.5% Ascorbic Acid: Significance from Control, *p*	Significance between Tests, *p*
M (7)	30	0	56(2.5)	56(3.6)	>0.50	56(3.0)	>0.50	54(5.6)	>0.50	>0.50
	60	30	149(7.4)	131(8.1)	>0.10	122(6.2)	<0.02	85(4.8)	<0.001	<0.001
	90	60	193(12.3)	175(11.5)	>0.30	161(10.1)	>0.05	148(6.7)	<0.01	>0.20
	120	90	222(6.1)	192(4.6)	<0.01	184(9.8)	<0.01	200(7.4)	<0.05	>0.20
F (11)	30	0	48(2.9)	48(3.8)	>0.50	49(2.8)	>0.50	50(3.4)	>0.50	>0.50
	60	30	111(3.2)	97(6.3)	>0.05	82(2.8)	<0.001	76(3.3)	<0.001	>0.10
	90	60	131(3.7)	121(6.2)	>0.10	110(2.7)	<0.001	109(3.4)	<0.001	>0.50
	120	90	146(4.3)	135(5.3)	>0.10	126(2.0)	<0.001	129(2.5)	<0.01	>0.30

Table IV—Effects of Disulfiram Alone and with Ascorbic Acid on First- and Second-Generation Albino Rats

Generation	Sex	Age, Days	Days on Diet	Control Diet: No.	Control Diet: g. (±*SE*)	1:2000 Disulfiram: No.	1:2000 Disulfiram: g. (±*SE*)	1:2000 Disulfiram: Significance, *p*	1:1000 Disulfiram, Tap Water: No.	1:1000 Disulfiram, Tap Water: g. (±*SE*)	1:1000 Disulfiram, Tap Water: Significance, *p*	1:1000 Disulfiram, 0.5% Ascorbic Acid: No.	1:1000 Disulfiram, 0.5% Ascorbic Acid: g. (±*SE*)	1:1000 Disulfiram, 0.5% Ascorbic Acid: Significance, *p*	Significance between Tests, *p*
I	M	30	0	20	44(3.1)	12	41(3.1)	>0.50	9	37(3.0)	>0.10	6	37(1.6)	>0.05	>0.50
		60	30	20	146(5.1)	12	109(5.4)	<0.001	9	100(8.5)	<0.001	6	97(3.1)	<0.001	>0.50
		90	60	20	219(6.0)	12	163(5.6)	<0.001	9	144(7.5)	<0.001	6	133(5.6)	<0.001	>0.20
		120	90	20	260(6.8)	12	201(6.0)	<0.001	9	196(10.3)	<0.001	6	161(4.9)	<0.001	<0.01
II	M	30	0	10[a]	49(3.6)	6	45(3.6)	>0.40	0	0(—)	—	1	65(—)	—	—
I	F	30	0	18	42(0.8)	11	37(2.3)	>0.05	13	38(2.4)	>0.05	4	37(1.9)	>0.05	>0.50
		60	30	18	119(1.9)	11	93(1.7)	<0.001	13	77(4.2)	<0.001	4	92(3.7)	<0.001	<0.02
		90	60	18	153(2.1)	11	135(3.6)	<0.001	13	107(3.7)	<0.001	4	110(3.7)	<0.001	>0.50
II	F	30	0	12[a]	47(2.8)	12	47(2.5)	>0.50	1	40(—)	—	1	52(—)	—	—

[a] Only a representative random sample.

retardation of growth was generally seen after 90 days on the diets (14, 17, and 10% for males, and 8, 14, and 12% for females fed 1:2000 disulfiram, 1:1000 disulfiram, and 1:1000 disulfiram plus ascorbic acid in the drinking water, respectively). With the exception of the males on diet for 30 days, there was no significant difference in weight between animals with and without ascorbic acid on the 1:1000 diet. This exception was unexpected since it appeared to indicate that ascorbic acid might actually enhance disulfiram-induced growth retardation. However, this effect was not seen in the comparable female groups. A separate experiment was then set up to ascertain whether ascorbic acid in the drinking water of rats receiving only the control diet would retard growth or reduce fluid intake. No such effects were seen. In the experiment described in the next section, no consistent ascorbic acid retardation was seen, so this exception in Table III must be discounted as being presently unexplainable.

First- and Second-Generation Studies—In a preliminary study, 1:1000 disulfiram in the diet appeared to have a definite depressant effect on the reproductive capacity of rats, while 1:2000 disulfiram was without effect. At 130–135 days of age, the males and females of the preceding study (Table III) were mated while continuing to receive their assigned diets. The paired rats remained together until the female showed signs of pregnancy or reached the age of 228 days. The young from this mating were separated from their mothers at 30 days of age and placed on the same diet as their parents. These offspring are termed "first generation" in Table IV. First-generation males and females were then mated at the age of 90–95 days, and the young from this union were weighed at 30 days of age and tabulated as "second generation" in Table IV.

There was no significant effect ($p > 0.05$) between treatments as to the body weight of the first generation at the age of 30 days. However, after the diet was in effect for 30 days (60 days of age), the disulfiram-induced retardation of growth was seen (25, 32, and 34% for males, and 22, 35, and 23% for females fed 1:2000 disulfiram, 1:1000 disulfiram, and 1:1000 disulfiram plus ascorbic acid in the drinking water, respectively). There was one isolated instance of apparent ascorbic acid antagonism of disulfiram (females, 30 days of feeding), and one single instance of apparent ascorbic acid

Table V—Xanthine Oxidase Activity Obtained in the Presence (MB) and Absence (NMB) of Methylene Blue

Treatment, No. of Rats[a]	Mean Wt., g. (±*SE*)	Mean Oxygen Consumption, cu. mm./20 min./283 mg. Tissue (±*SE*): Liver NMB	Liver MB	Lung NMB	Lung MB	Intestine NMB	Intestine MB	Spleen NMB	Spleen MB	Kidney NMB	Kidney MB	Mean Liver Molybdenum, μg./g. Dry, Wt.
Control (7)	221(7.0)	18(3.7)	29(4.6)	13(1.2)	19(1.3)	33(3.5)	58(6.0)	17	17	4	8	2.21
1:2000 Disulfiram (8)	172(5.5)	19(3.3)	29(4.0)	8(0.7)	11(0.6)	36(4.2)	50(5.3)	12	15	3	9	2.64
Significance from control, *p*	<0.001	>0.50	>0.50	<0.01	<0.001	>0.50	>0.30					
1:1000 Disulfiram (4)	172(13.4)	21(2.4)	30(3.0)	8(1.0)	12(0.9)	32(3.7)	42(7.5)	10	14	6	12	2.05
Significance from control, *p*	<0.01	>0.50	>0.50	<0.02	<0.01	>0.50	>0.10					

[a] One determination at 37° per animal, except in the cases of kidney and spleen tissue where samples from two animals were pooled per determination.

Table VI—Effects of Disulfiram Alone and with Ascorbic Acid on the Reproduction of the Albino Rat

Treatment	Generation	Litters/ Pairs Mated	Mean Litter Size	Eaten by Mother, %	30-Day Survival, %
Control diet	I	11/12	6.7	2.7	89.2
Control diet	II	11/12	8.2	4.4	72.2
1:2000 Disulfiram	I	9/13	4.9	2.3	81.8
1:2000 Disulfiram	II	9/11	5.8	36.5	34.6
1:1000 Disulfiram	I	8/16	5.5	6.8	54.5
1:1000 Disulfiram	II	5/6	5.6	96.4	3.6
1:1000 Disulfiram[a]	I	5/7	6.4	6.2	31.2
1:1000 Disulfiram[a]	II	2/4	4.5	77.8	22.2

[a] Diet was supplemented with 0.5% ascorbic acid in the drinking water.

synergism with disulfiram (males, 90 days of feeding). Weight comparisons of second-generation animals were hampered by the antiproductive effects of 1:1000 disulfiram; however, there was no significant difference in body weight between second-generation controls and those from parents receiving 1:2000 disulfiram. It would appear in this experiment that disulfiram was not excreted or concentrated to any significant extent in the mother's milk, since offspring reaching 30 days of age did not exhibit significant retardation of growth. Ascorbic acid appears to have no predictable antagonistic effects against disulfiram.

Biochemical Studies—First-generation males (Table IV) were sacrificed and tissue xanthine oxidase activity measured in the presence and absence of methylene blue (11). Methylene blue has the capacity to overcome the disulfiram-induced inhibition of xanthine oxidase almost completely (12). Uric acid–allantoin–creatinine values for urine were determined prior to sacrifice in a manner similar to that of Bass and Place (13). As shown in Table V, lung xanthine oxidase activity was depressed in rats receiving disulfiram; however, this phenomenon is not easily explained since it was also seen with the tissue incubated with methylene blue. With this exception, chronic administration of disulfiram appears to have little to no effect on xanthine oxidase activity. This is in agreement with the finding (14) that xanthine oxidase apparently has little to do with acetaldehyde metabolism *in vivo*. There were no significant differences in the excretory uric acid/creatinine and allantoin/creatinine ratios (15) for the control and test diets. Since both aldehyde oxidase and xanthine oxidase are molybdenum dependent (16), it is of interest to note that the molybdenum content (17) was unaffected by chronic disulfiram feeding.

Effects on Reproduction—Animals were fed control and test diets and mated in a manner similar to that described for the rats in Table IV. The experiment, as summarized in Table VI, indicates that two factors are contributing to the antireproductive effect of disulfiram. By pooling the ratios (litters/pairs mated) for the first and second generations, a 92% incidence of successful conception for rats receiving the control diet is found. This can be contrasted with the 75 and 61% incidence seen for the pooled 1:2000 disulfiram generations and the pooled 1:1000 disulfiram (including those receiving ascorbic acid) generations. However, beyond this anticonceptive effect, one notes that the survival incidence of the young rats born from disulfiram-fed mothers is much less than the controls. Since malformations were not noted at birth and since those surviving 30 days did not have their growth stunted (Table IV), as could be caused by disulfiram excretion in milk, the cause of infant mortality may be due to decreased milk secretion by the mother. This could be a secondary result of chelation. Disturbances of lactation are often interrelated with cannibalism in the albino rat (9).

SUMMARY

Chronic feeding of 1:1000 and 1:2000 disulfiram in the diet retarded the growth of weanling albino rats, while concentrations of 1:4000 and 1:8000 were without effect. Continued feeding of the active concentrations limited the reproductive capacity of young adults by having an anticonceptive effect, as well as by decreasing the survival incidence of the offspring. This latter effect may be due to decreased lactation and secondary cannibalism. Since Lish (18) did not note any significant alterations in the rat estrus cycle it is possible that the mechanism of disulfiram's anticonceptive action involves inhibition of spermatogenesis. In this context, it is interesting to note that the experimental agents of the bis(dichloroacetyl)diamine type, such as *N,N'*-bis(dichloroacetyl)-1,8-octanediamine (WIN-18446) which inhibits spermatogenesis in the rat (19) and man (20), produce pronounced disulfiramlike reactions if the subject also consumes ethanol (21).

Chronic feeding of 1:1000 disulfiram decreases the longevity of rats without any significant accompanying gross symptomatology. This concentration is considered equivalent to about 10 (adult rats) to 20 (young rats) times the average human dose (500 mg./day or 7 mg./kg.). Decreased longevity is probably not correlated with blood dyscrasias.

Effects of disulfiram on growth and reproduction were resistant to supplemental feeding with ascorbic acid and also appeared not to be due to a thiouracil-like effect on the thyroid gland. Tissue xanthine oxidase activity was unaffected as was liver molybdenum by feeding 1:1000 and 1:2000 disulfiram.

REFERENCES

(1) P. J. Hanzlik and A. Irvine, *J. Pharmacol. Exp. Ther.*, **17,** 349(1921).

(2) J. Hald, E. Jacobsen, and V. Larsen, *Acta Pharmacol. Toxicol.*, **4,** 285(1948).

(3) G. P. Child and M. Crump, *ibid.*, **8,** 305(1952).

(4) O. G. Fitzhugh, W. J. Winter, and A. A. Nelson, *Fed. Proc.*, **11,** 345(1952).

(5) J. Christensen and A. Wase, *ibid.*, **13,** 343(1954).

(6) W. W. Nowinski, T. P. Edwards, and J. P. Ellis, *ibid.*, **9,** 95(1950).

(7) J. D. Markham and E. C. Hoff, *J. Amer. Med. Ass.*, **152,** 1597(1953).

(8) F. W. Sunderman, *J. New Drugs*, **4,** 154(1964).

(9) E. J. Farris and J. Q. Griffith, "The Rat in Laboratory Investigation," 2nd ed., J. B. Lippincott Co., Philadelphia, Pa., 1949, pp. 407–412.

(10) N. F. Maclagan and M. M. Sheahan, *J. Endocrinol.*, **6,** 456(1950).

(11) W. W. Westerfeld and D. A. Richert, *J. Biol. Chem.*, **199,** 393(1952).

(12) D. A. Richert, R. Vanderlinde, and W. W. Westerfeld, *ibid.*, **186,** 261(1950).

(13) A. D. Bass and E. F. Place, *ibid.*, **181,** 81(1949).

(14) W. W. Westerfeld and D. A. Richert, *Proc. Soc. Exp. Biol. Med.*, **87,** 524(1954).

(15) A. D. Bass, J. Tepperman, D. A. Richert, and W. W. Westerfeld, *ibid.*, **73,** 687(1950).

(16) B. Mackler, *Fed. Proc.*, **13,** 257(1954).

(17) W. W. Westerfeld and D. A. Richert, *J. Nutr.*, **51,** 85(1953).

(18) P. M. Lish, Master's thesis, University of Nebraska, Lincoln, Nebr., 1951.

(19) F. Coulston, A. L. Beyler, and H. P. Drobeck, *Toxicol. Appl. Pharmacol.*, **2,** 715(1960).

(20) C. G. Heller, D. J. Moore, and C. A. Paulsen, *ibid.*, **3,** 1(1961).

(21) C. G. Heller and D. J. Moore, *Amer. Chem. Soc. Abstr., Div. Med. Chem.*, **April 1963,** 32L.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 20, 1970, from the *Department of Pharmacology, University of Nebraska, Lincoln, NE 68508*

Accepted for publication April 6, 1970.

This research was supported in part by grants from the National Research Council, Medical Division, Problem of Alcohol, and from the Chicago Commission on Alcoholism. The authors wish to thank Loren Braun, Gale E. Demaree, Ira W. Hillyard, Hideko Katayama Kaji, Louise Mues Olson, and R. William Zimmerle for their assistance in executing these and the preliminary experiments.

* Present address: Chromalloy American Corp., Pharmaceutical Group, Oakland, CA 94621

† Present address: 14622 Southern Dr., Dallas, TX 75234

‡ Present address: Department of Biochemistry, State University of New York, Upstate Medical Center, Syracuse, NY 13210

§ Present address: Department of Physiology-Pharmacology, University of the Pacific, Stockton, CA 95204

Stability and Structure of Some Organic Molecular Complexes in Aqueous Solution

JORDAN L. COHEN* and KENNETH A. CONNORS

Abstract □ On the basis of a simple model of 1:1 complex formation between planar molecules, this equation was derived: $\Delta G^0_u = A(G^0_{SL} - G^0_{MS} - G^0_{ML})$, where ΔG^0_u is the standard unitary free energy change for complex formation, A is the area of overlap in the plane-to-plane interaction between the two molecules, and G^0_{IJ} is a free energy of interaction per unit area between I and J. (S, L, and M represent substrate, ligand, and medium.) If the parenthesized term is roughly constant for a series of substrates and ligands in a constant medium, it is suggested that the quantity G^0_{SL} does not play a major role, and that the complex stability is primarily controlled by repulsive solvent interaction terms (G^0_{MS} and G^0_{ML}). It follows that the system will assume an orientation of S and L such as to maximize A. Therefore, maximal overlap areas were estimated for 18 neutral and 32 ionic complexes. A substantial improvement in the area correlation was obtained, and the observation was made that there is no significant difference between the average behavior of neutral and charged complexes. The maximal overlap concept provides a general "first-order" description of molecular complex formation in aqueous solution. Deviations from the line are significant and may be associated with specific structure-stability relationships. Application of the maximal overlap approach leads to average complex structures without introducing arbitrary assumptions about specific group interactions. The structures thus generated are consistent with the chemical reactivity of the complexes.

Keyphrases □ Complexes, organic molecular—aqueous solution □ Stability constants—organic molecular complexes □ Maximal overlap areas, estimated—complexes □ Titration, potentiometric, nonaqueous—analysis □ IR spectrophotometry—structure □ NMR spectroscopy—structure

The earlier suggestion (1) that systematic structural variations in substrates and ligands might be a successful approach to the elucidation of the structures of molecular complexes in solution formed the basis for much of the experimental design, including some of the present work. During the course of these structural investigations, it became increasingly apparent that the authors were studying secondary (specific) effects without having first identified the primary general effect in determining complex stability. This recognition led directly to the conception of the correlation between complex stability and planar area of interactants as described earlier (2). Complexes of neutral interactants gave a rough linear correlation of standard free energy change with planar area of the small interactant; this was interpreted in terms of a simple model of the process. Complexes containing an ionic interactant could not be usefully correlated, and it was suggested that neutral and ionic complexes behave differently in these systems.

The present work was planned to refine the area correlation and to elucidate the differences between neutral and ionic molecular complexes in aqueous solution. An ultimate goal is the ability to determine and to predict complex structures and properties (especially chemical reactivity) in solution, and the area correlation offers a promising point of attack.

EXPERIMENTAL

Materials—Many of the chemicals were prepared or purified as described elsewhere (1, 2). 3-β-Naphthylacrylic acid was prepared from β-naphthaldehyde[1] by the method of Fulton and Robinson (3), and its methyl ester was obtained by treatment with diazomethane. The slightly yellow product was recrystallized twice from ethanol–water, m.p. 88–89° [lit. (4) 93–93.5°]. IR and NMR spectra were consistent with the expected structure (5). Methyl 9-anthroate was prepared by treating 9-anthracene carboxylic acid chloride[1] with methanol in pyridine; it was recrystallized twice from ethanol–water, m.p. 110–111° [lit. (6) 111°]. 2,6-Naphthalenedicarboxylic acid dimethyl ester[2] was used directly, m.p. 185° [lit. (7) 186°]. Naphthalene[3] (resublimed) was used directly, m.p. 79° [lit. (8) 80.3°]. Phenanthrene[1] was recrystallized from ethanol, m.p. 99° [lit. (9) 101°]. The acid chloride of *p*-nitro-*trans*-cinnamic acid was prepared by refluxing the acid (Eastman White Label) with freshly distilled thionyl chloride. The acid chloride was refluxed with methanol to give the methyl ester, which was recrystallized twice from ethanol, m.p. 163° [lit. (10) 161°].

Benzimidazole[1] was recrystallized three times from water, m.p. 172–173° [lit. (11) 171–173°]. Benzimidazole 2-acetonitrile[1] was used directly, m.p. 209–210° [lit. (12) 209.7–210.7°]. 6-Nitrobenzimidazole[1] was recrystallized three times from methanol, m.p. 207–208° [lit. (13) 206°]. Xanthine and hypoxanthine[1] were used directly. Adenine[1] was recrystallized from water and dried at 150° for 8 hr. Nonaqueous titration in glacial acetic acid with acetous perchloric acid (quinaldine red indicator) showed that it was the anhydrous form. 8-Nitrotheophylline[2] was used directly, m.p. 282–283° dec. [lit. (14) 282–283° dec.]. Potentiometric titration with standard base indicated that it was the dihydrate. Theophylline[4] was recrystallized from water, dried at 150° for 4 hr., and then ground to a fine powder and dried for 24 hr., m.p. 270–271°. Nonaqueous titration in *N*,*N*-dimethylformamide with standard sodium methoxide in benzene–methanol solution (thymol blue indicator) showed it to be 100% anhydrous theophylline. Theobromine[4] was recrystallized from water and its purity checked by nonaqueous titration.

3,5-Dinitrobenzoic acid (Eastman White Label) was recrystallized twice from ethanol–water, m.p. 205–207° [lit. (15) 205–207°].

The pKa values were determined spectrophotometrically for many of the ligands; these values were found at 25° in water (ionic strength 0.3 M): 8-nitrotheophylline, 3.55 ± 0.05; hypoxanthine, 8.50 ± 0.06; xanthine, 9.95 ± 0.05 (for conversion to the anion); benzimidazole 2-acetonitrile, 4.20 ± 0.02 and 11.76 ± 0.02; and 6-nitrobenzimidazole, 2.89 ± 0.03 and 10.69 ± 0.05.

Apparatus and Procedures—The equipment and methods employed for stability constant determinations have been described in detail earlier (1, 2, 5, 16). Stability constants were evaluated by the solubility, spectral, and kinetic techniques (17) and are expressed as apparent 1:1 stability constants (K_{11}') in M^{-1}. All constants were determined at 25.0° in aqueous solution of 0.30 M ionic strength; in spectral and kinetic studies the solvent usually contained 0.83% acetonitrile, added as the substrate stock solution. An additional parameter provided by the kinetic method is symbolized q_{11} and is interpreted as the fractional decrease in chemical reactivity of the complexed substrate relative to the uncomplexed substrate (16).

RESULTS

Table I lists the substrate–ligand combinations studied, with their stability constants. Systems were investigated by the solubility

[1] Aldrich Chemical Co., Milwaukee, WI 53210
[2] K and K Laboratories.
[3] J. T. Baker, Phillipsburg, N. J.
[4] NF, Merck & Co., Inc., Rahway, NJ 07065

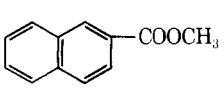
Table I—Apparent Stability Constants at 25° in Aqueous Solution

Substrate	Ligand	K_{11}' (M^{-1}) Solubility	Spectral	Kinetic
Methyl *trans*-cinnamate	Theophylline	25.0	24.5	—
Methyl *trans*-cinnamate	Theophyllinate[a]	—	13.2	13.5
Methyl *trans*-cinnamate	Theobrominate[b]	—	11.6	11.5
Methyl *trans*-cinnamate	8-Bromotheophyllinate	29	28[c]	—
Methyl *trans*-cinnamate	8-Iodotheophyllinate	33	—	—
Methyl *trans*-cinnamate	8-Nitrotheophyllinate	40[d]	—	—
Methyl *trans*-cinnamate	Benzimidazole	—	3.0[e]	—
Methyl *trans*-cinnamate	Benzimidazole cation	5.7	—	—
Methyl *trans*-cinnamate	Benzimidazole 2-acetonitrile cation	5.5	6.0	—
Methyl *trans*-cinnamate	Xanthine anion	—	7.5	—
Methyl *trans*-cinnamate	Hypoxanthine anion	—	2.0	—
Methyl *trans*-cinnamate	Adenine cation	—	2.5	—
Methyl *trans*-cinnamate	3,5-Dinitrobenzoate[f]	9.4	—	—
Methyl 1-naphthoate	Theophyllinate	—	—	25
Methyl 1-naphthoate	Theobrominate	—	—	36
Methyl 1-naphthoate	8-Chlorotheophyllinate	—	—	64
Methyl 2-naphthoate	Theophyllinate	—	—	50
Methyl 2-naphthoate	Theobrominate	—	—	39
Methyl 2-naphthoate	8-Chlorotheophyllinate	120	—	105
Methyl 2-naphthoate	8-Nitrotheophyllinate	230	—	—
Methyl 3-β-naphthylacrylate	Theophyllinate	—	—	41
Methyl 3-β-naphthylacrylate	Theobrominate	—	—	30
Methyl 3-β-naphthylacrylate	8-Chlorotheophyllinate	—	—	58
Methyl *p*-nitrocinnamate	Theophyllinate	—	—	13.5
Methyl 9-anthroate	8-Chlorotheophyllinate	85	—	—
Naphthalene 2,6-dicarboxylic acid, dimethyl ester	8-Chlorotheophyllinate	160	—	—
Cinnamamide	Theophylline	25	—	—
trans-Cinnamic acid anion	Theobrominate	—	5	—
Naphthalene	Theophylline	64	—	—
Naphthalene	Theophyllinate	23	—	
Naphthalene	8-Chlorotheophyllinate	49	—	—
Phenanthrene	Theophyllinate	89	—	—
3,5-Dinitrobenzoate	Theophylline	—	10.3	—
3,5-Dinitrobenzoate	Theophyllinate	—	7.5	—
3,5-Dinitrobenzoate	8-Chlorotheophyllinate	—	13.4	—

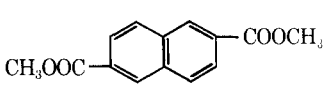
[a] *I.e.*, theophylline anion. [b] Theobromine anion. [c] B. J. Kline, Ph.D. dissertation, Univ. of Wisconsin, Madison, Wis., 1968. [d] 1:1 stoichiometry observed [e] Same value found at two wavelengths. [f] 3,5-Dinitrobenzoic acid anion.

method (*18*) when substrate stability and ligand solubility permitted. Spectral determinations were made on those systems in which a measurable spectral shift occurred upon complexation. The kinetic technique was applied to most of the ester substrates. The reproducibility of most stability constants is about ±10% (the cinnamic acid anion–theobrominate constant is only reliable to ±25% because it was estimated in "infinity" solutions from a kinetic study of methyl cinnamate–theobrominate).

Nearly all of the systems studied include one heterocyclic interactant (methyl cinnamate–3,5-dinitrobenzoate is the only exception), so this limitation must be recognized. As noted earlier, most of the substrates and ligands were selected to provide information on the specific structural requirements for complexing and on the nature of ionic complexes. Another possible feature that was considered was the statistical effect on apparent complex stability (*18*). Thus, methyl 2-naphthoate (I) and 8-chlorotheophyllinate form a complex with $K_{11}' = 120\ M^{-1}$. If 8-chlorotheophyllinate interacts in a specific orientation with methyl 2-naphthoate, and if in a 1:1 complex of 8-chlorotheophyllinate with the dimethyl ester of naphthalene 2,6-dicarboxylic acid (II) this orientation is preserved, then K_{11}' for the complex with II should be twice 120. The observed value is 160 M^{-1}, possibly indicating either that there

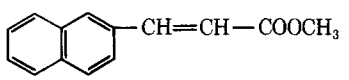


I



II

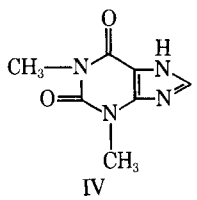
is no specific orientation of the ligand common with I and with II, or that the binding energy of the ligand with II is influenced by the second ester group. Again, comparing the complex naphthalene–theophyllinate ($K_{11}' = 23\ M^{-1}$) with phenanthrene–theophyllinate ($K_{11}' = 89\ M^{-1}$) shows that a simple statistical effect is not the only factor controlling the constant with the larger substrate. (Anthracene could not be studied because of its very low solubility.)



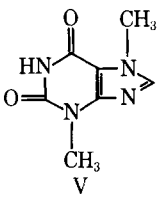
III

Methyl 3-β-naphthylacrylate (III) can be viewed either as a 2-naphthoate with a conjugated side chain or as a methyl cinnamate derivative with a *m,p*-fused aromatic ring. A ligand has a "choice" of positions, one of them characteristic of a naphthoate substrate and one resembling a cinnamate. Comparison of the stability constants for III, for methyl cinnamate, and for methyl 2-naphthoate with common ligands suggests that the ligand preferentially resides at the naphthyl portion of III. This conclusion is strengthened by the q_{11} values, which will be considered later Also consistent with this interpretation is the absence of a useful spectral shift upon complexation, which is typical of benzoate and naphthoate complexes but not of cinnamate complexes.

Some of these data suggest that charge-transfer and simple electrostatic forces are not of major importance in determining complex stability in aqueous solution. The anions of theophylline (IV) and theobromine (V) have very different electronic distributions, but they yield similar K_{11}''s with the common substrate methyl *trans*-cinnamate.



IV



V

Cinnamic acid anion complexes appreciably with the anions theophyllinate (4) and theobrominate. Methyl *p*-nitrocinnamate

Table II—q_{11} Values for Alkaline Hydrolysis of Ester Complexes[a]

Substrate	Ligand	q_{11}
Methyl *trans*-cinnamate	Theophyllinate	0.90
Methyl *trans*-cinnamate	Theobrominate	1.0
Methyl 1-naphthoate	Theophyllinate	0.97
Methyl 1-naphthoate	Theobrominate	0.90
Methyl 1-naphthoate	8-Chlorotheophyllinate	1.0
Methyl 2-naphthoate	Theophyllinate	0.91
Methyl 2-naphthoate	Theobrominate	0.93
Methyl 2-naphthoate	8-Chlorotheophyllinate	0.99
Methyl 3-β-naphthylacrylate	Theophyllinate	0.71
Methyl 3-β-naphthylacrylate	Theobrominate	0.82
Methyl 3-β-naphthylacrylate	8-Chlorotheophyllinate	0.89
Methyl *p*-nitrocinnamate	Theophyllinate	0.82

[a] 25°, 0.3 *M* ionic strength, phosphate or hydroxide buffers, 0.83% acetonitrile.

interacts with theophyllinate to about the same extent as does methyl cinnamate. The anion of 3,5-dinitrobenzoic acid, which Menger and Bender (19) expected to function as an electron acceptor, complexes significantly with several anions (Table I). Thus, with theophylline, a K_{11}' of 10 M^{-1} is observed. Yet a stability constant of 9 M^{-1} is found for the 3,5-dinitrobenzoate–methyl cinnamate complex, and methyl cinnamate complexes with theophylline with K_{11}' equal to 25 M^{-1}. Simple donor–acceptor relationships do not provide a convincing picture of this cycle of numbers.

3,5-Dinitrobenzoate gave $K_{11}' = 13\ M^{-1}$ with 8-chlorotheophyllinate, whereas Guttman and Brooke (20) found a stability constant of 4.7 M^{-1} between 8-chlorotheophyllinate and 3-carbomethoxy-1-methylpyridinium cation; that is, the 8-chlorotheophylline anion interacts more strongly with another anion that it does with a cation.

Earlier results (16) led to an estimate of the uncertainty in q_{11} of about 0.1 unit (q_{11} is constrained, for 1:1 complexes exhibiting inhibition relative to the uncomplexed substrate, to lie in the interval 0–1). In the present study the reactions were simple ester hydrolyses in easily analyzed and controlled systems, and it is estimated that most of the q_{11} values have an uncertainty of about 0.05 unit. These q_{11} values are collected in Table II. Figure 1 shows a plot illustrating the determination of these quantities (16, 17); q_{11} is equal to the reciprocal of the intercept on the ordinate axis.

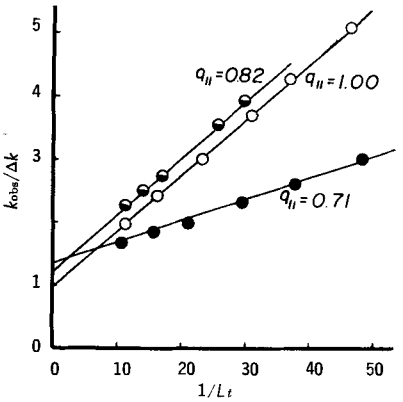


Figure 1—*Kinetic plot showing several complex systems* (L_t *is total ligand concentration*). *Key:* ○, *methyl* trans-*cinnamate–theobrominate;* ◒, *methyl* p-*nitrocinnamate–theophyllinate; and* ●, *methyl 3-β-naphthylacrylate–theophyllinate.*

DISCUSSION

Maximal Overlap Area Correlation—The model of complex formation presented earlier (2) assumes plane-to-plane interaction of substrate *S* and ligand *L* in medium *M*. Equation 1 can be developed from this model:

$$\Delta G_u^0 = A(G_{SL}^0 - G_{MS}^0 - G_{ML}^0) \qquad \text{(Eq. 1)}$$

where ΔG_u^0 is the standard unitary free energy change for complex formation (21), G_{IJ}^0 is a free energy of interaction per unit area between "surfaces" *I* and *J* at the equilibrium separation distance, and *A* is the area of contact between *S* and *L*. The first use of this model took *A* to be the estimated planar area of the smaller of the two interactants (2). A plot of standard unitary free energy change against planar area of the smaller interactant gave a fair linear correlation for complexes in which both *S* and *L* were neutral, but the attempted correlation was not successful when either, or both, of the interactants was an anion. An obvious fault of the treatment is that numerous points corresponded to the same area (because they involved one of the same interactants) yet represented different complex stabilities. This generated vertical lines in the plot. If the model is a good one for complex formation, part of the lack of success apparently was caused by a poor estimate of the overlap area *A*. *A* cannot be greater than the planar area of the smaller interactant, but it may be smaller than this quantity. A refined estimate of the overlap area was made as follows.

A free energy term of the type G_{IJ} assumes an increasingly negative value as attraction between *I* and *J* increases. (When *I* and *J* are separated to infinity, G_{IJ} equals zero.) In a first approximation, the surfaces are considered homogeneous; that is, no preferred specific (group) interactions are considered. Then *A* will assume a value such as to maximize $-\Delta G^0$ subject to these conditions: (*a*) the more negative the value of G_{SL}^0, the greater *A* will be; (*b*) the more positive the value of G_{MS}^0, the greater *A* will be; and (*c*) the more positive the value of G_{ML}^0, the greater *A* will be. In chemical terms, attraction between *S* and *L* will lead to a maximization of overlap area, whereas repulsion would tend to minimize the area. Attraction between *M* and *S* will minimize overlap area, whereas *M–S* repulsion will maximize it, and similarly for *M* and *L*. The final structure is then a compromise position depending upon (possibly) opposing factors.

Since most of these compounds are relatively hydrophobic, it is reasonable to suppose that G_{MS}^0 and G_{ML}^0 are fairly large repulsive quantities. That G_{SL}^0 does not play an overwhelmingly important role is indicated by the marked reduction in K_{11} for the methyl cinnamate–theophylline complex as the water concentration is decreased in water–methanol mixtures (22). It seems possible, therefore, that in many of these complexes a structure is assumed in which the overlap area between substrate and ligand is maximized. Therefore, a plot was made of standard unitary free energy change, ΔG_u^0, against *maximal overlap area.*

Maximal overlap areas were estimated as shown by Fig. 2. The planar area of the smaller molecule is traced from a Corey-Pauling-Koltun molecular model (23) on translucent tracing paper and is darkened. The planar area of the larger molecule is traced on a transparent plastic sheet. These are superimposed and oriented until the area of overlap is visually maximized, with nothing else being taken into consideration. A photocopy of this orientation is made, and the overlap area (hatched area in Fig. 2) is measured by a planimeter. This gives the maximal overlap area for plane-to-plane interaction between the two molecules. Table III gives maximal overlap areas estimated in this way.

Figure 3 shows the correlation of ΔG_u^0 with maximal overlap area for neutral complexes (dark circles). The correlation seems to be slightly better than the original plot against the planar area of the smaller interactant (2). The open circles in Fig. 3 represent complexes in which one or both interactants are ionic. A marked improvement in the correlation of ionic complexes has been achieved with the maximal overlap area concept.

The 50 complexes plotted in Fig. 3 represent about 70 systems, because, for clarity, one point may represent more than one complex. For example, the methyl *trans*-cinnamate–theophylline complex has the same stability constant and overlap area as do several other cinnamate–theophylline complexes, so these additional points were not plotted. A least-squares line calculated for the 50 points gave an ordinate intercept of 0.1 (in units of $-10^{21}\ \Delta G_u^0/N$), indicating that the line passes essentially through the origin, in agreement with the model equation. Separate least-squares lines for the neutral and ionic complexes gave the same slope values, with the standard deviation for neutral complexes being 0.58 and for ionic complexes 0.64, in units of $-\ 10^{21}\ \Delta G_u^0/N$. This treatment has, there-

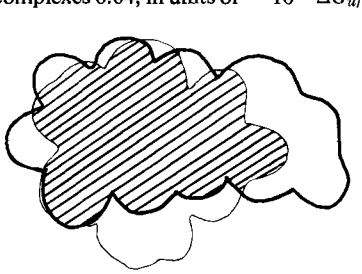


Figure 2—*Maximal overlap area (hatched area) in the methyl* trans-*cinnamate–theophylline complex. Methyl cinnamate is outlined with the heavier line, with the ester group lying to the right.*

Table III—Estimated Maximal Overlap Areas for Complexes

System Number	Substrate	Ligand	Maximal Overlap Area (A^2/molecule)
1	Methyl 2,6-dichloro *trans*-cinnamate	Theophylline	55.6
2	Acetophenone	Theophylline	44.4
3	Methyl *trans*-cinnamate	Benzimidazole	39.5
4	Naphthalene	Benzimidazole	41.2
5	Methyl 1-naphthoate	Theophylline	54.7
6	*trans*-Cinnamaldehyde	Theophylline	49.4
7	Benzalacetone	Theophylline	49.8
8	Methyl *trans*-cinnamate	2-Methyl imidazole	28.4
9	Benzamide	Theophylline	39.5
10	Naphthalene	Theophylline	49.8
11	Methyl crotonate	Theophylline	27.2
12	Methyl benzoate	Theophylline	39.9
13	Methyl hydrocinnamate	Theophylline	34.2
14	*trans*-Cinnamyl acetate	Theophylline	37.9
15	Methyl 2-naphthoate	Theophylline	53.1
16	Methyl *trans*-cinnamate	Theophylline	49.0
17	Methyl *trans*-cinnamate	Caffeine	46.9
18	*trans*-Cinnamamide	Theophylline	45.3
19	Methyl *trans*-cinnamate	Purine anion	33.3
20	Theophylline	Salicylate	39.1
21	Methyl *trans*-cinnamate	Hypoxanthine anion	35.4
22	Methyl *trans*-cinnamate	8-Bromotheophyllinate	48.1
23	Methyl *trans*-cinnamate	3,5-Dinitrobenzoate	39.9
24	Cinnamoylsalicylate	Theophylline	44.9
25	Theophylline	*trans*-Cinnamate	42.4
26	Theophylline	3,5-Dinitrobenzoate	51.9
27	Methyl *cis*-cinnamate	8-Nitrotheophyllinate	44.0
28	Methyl *trans*-cinnamate	8-Nitrotheophyllinate	53.9
29	Methyl 2-naphthoate	8-Nitrotheophyllinate	55.6
30	Methyl *trans*-cinnamate	8-Chlorotheophyllinate	47.8
31	Methyl 2-naphthoate	8-Chlorotheophyllinate	55.6
32	Methyl 1-naphthoate	8-Chlorotheophyllinate	51.0
33	Naphthalene	8-Chlorotheophyllinate	46.5
34	Phenanthrene	8-Chlorotheophyllinate	56.0
35	9-Methyl anthroate	8-Chlorotheophyllinate	56.8
36	Naphthalene 2,6-dicarboxylic acid, dimethyl ester	8-Chlorotheophyllinate	54.3
37	Methyl 3-β-naphthylacrylate	8-Chlorotheophyllinate	54.3
38	Methyl crotonate	Theophyllinate	27.2
39	*p*-Nitrophenyl benzoate	Theophylline	38.7
40	Methyl *cis*-cinnamate	Theophyllinate	42.0
41	Methyl 2-naphthoate	Theophyllinate	53.9
42	Methyl 1-naphthoate	Theophylinate	53.5
43	Methyl *trans*-cinnamate	Theophyllinate	50.2
44	Methyl 3-β-naphthylacrylate	Theophyllinate	51.4
45	Methyl *p*-nitrocinnamate	Theophyllinate	50.2
46	Naphthalene	Theophyllinate	49.0
47	Methyl *trans*-cinnamate	Theobrominate	47.3
48	Methyl 3-β-naphthylacrylate	Theobrominate	49.8
49	Methyl 1-naphthoate	Theobrominate	51.0
50	Methyl 2-naphthoate	Theobrominate	51.0

fore, revealed the result that, in the first-order approximation, all systems behave similarly and, *on the average*, there is no difference between neutral and ionic complexes. This was not obvious on the basis of data for systematically selected substrates and ligands, and it only became clear when so many systems had been studied that the selection was practically random.

The equation of the line in Fig. 3 is

$$-\Delta G_u^0/N = 0.149 \times 10^{-21}\ (\text{cal.}/A^2) \times \text{maximal overlap area } (A^2/\text{molecule}) \quad \text{(Eq. 2)}$$

This slope value is equivalent to 64 dyne/cm. If, as suggested, the *S–L* interaction is of minor importance in determining complex stability, then the average value of the G_{MS}^0 and G_{ML}^0 terms is 32 dyne/cm. This is approximately the value of interfacial tensions between water and some typical hydrophobic organic compounds (24).

The statistical nature of this first-order approximation is apparent from Fig. 3. If another large group of structurally similar substrates and ligands was studied, the points would be expected to define a line close to the one in the figure. However, no single stability constant value could be predicted to within better than a factor of two. The dispersion seen in Fig. 3 may be considered a second-order effect, probably correlatable with specific structural features in the substrate and ligand. (Most of the deviations from the line are too large to be ascribed to misestimates of maximal overlap areas.) In terms of the model and Eq. 1, the quantity ($G_{SL}^0 - G_{MS}^0 - G_{ML}^0$) varies appreciably within the series of complexes represented. This scatter of points does not invalidate the model, which does not include the constraint that the parenthesized quantity should remain constant as *S* and *L* are changed.

Few general inferences can be made at this time concerning the second-order effect (the deviations from the area correlation line) in describing complex stability. One interesting set of data is the stability constants for theophylline and theophyllinate complexes with seven common substrates; the constant for the neutral ligand is nearly always larger than that with the anion, the mean value of the ratio being 2.35 ± 0.73. This is explicable if it is considered that the anion is less hydrophobic than is the neutral ligand, resulting in a more negative G_{ML}^0 term and, hence, decreased complex stability. (Since theophylline was the only ligand that could easily be studied in its neutral and anionic forms, these data earlier led to the too general belief that ionic complexes always behave differently from neutral ones.) That this solvation effect is not the only factor is indicated by stability constants for six common substrates with theophyllinate and 8-chlorotheophyllinate; the ratio of these constants (8-chlorotheophyllinate–theophyllinate) is 2.01 ± 0.33. The larger area of the halogenated xanthine does not account for the effect. These examples indicate a complicated variation in the energy terms even for closely related compounds.

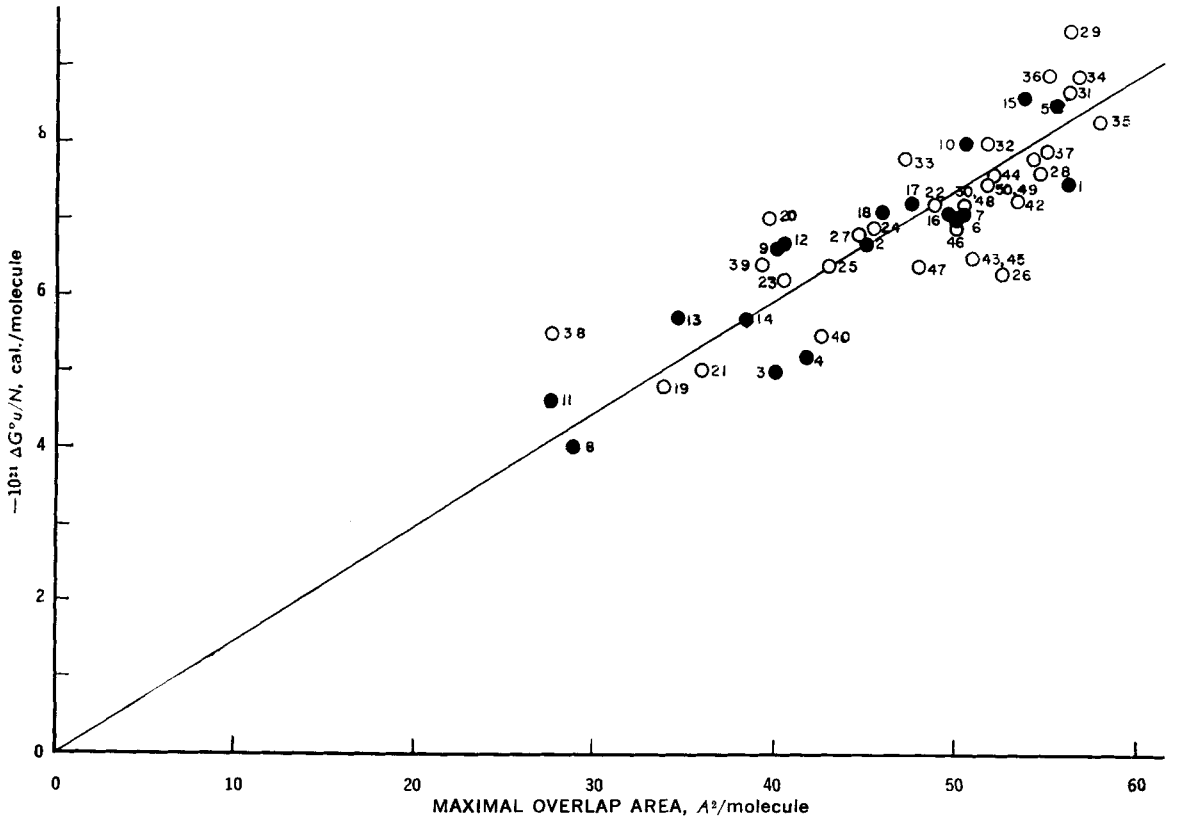


Figure 3—*Plot of standard unitary free energy change for complex formation at 25° in aqueous solution against estimated maximal overlap area. Key: ○, ionic complexes; and ●, neutral complexes. See Table III for complex identities.*

Structure and Reactivity of Complexes—Previous reports have stated q_{11} values for ester hydrolysis to be either about unity (implying direct contact of the ligand with the ester group and, therefore, substantial inhibition of the reaction) (1, 2, 16) or less than 0.5 (with no direct steric interference but a small polar effect on the rate). In this study, ester substrates have been selected to allow a distinction to be made between q_{11} values in the range 0.7–1 (Table II and Fig. 1).

Repetition of the methyl *trans*-cinnamate–theophyllinate study (1) yielded q_{11} = 0.90, which is now thought to be significantly different from unity. The methyl *trans*-cinnamate–theobrominate complex, however, gave a q_{11} value of essentially unity. The stability constants and maximal overlap areas for the two complexes are about the same. The maximal overlap area profiles, developed as described earlier, suggest a possible reversal of the xanthine–cinnamate orientation, with the xanthine occupying a position closer to the ester carbonyl when the ligand is theobrominate; this is consistent with the higher q_{11} value.

Methyl 3-β-naphthylacrylate (III) is a larger molecule than methyl *trans*-cinnamate, and it seemed that a kinetic study with theophyllinate might define the area of interaction between III and this ligand. The q_{11} of 0.71 implies only a small steric involvement with the ester function (a q_{11} of 0.71 corresponds to 0.73 kcal./mole difference in free energy of activation between the complexed and uncomplexed substrate), suggesting that the xanthine lies further away from the ester group than it does in the methyl cinnamate complex. Further, the q_{11} of 0.89 for the 8-chlorotheophyllinate complex of III is consistent with the greater bulk of this xanthine and the possibility that greater steric interference occurs by the ligand in the reaction of the ester function.

It is curious, considering the scatter observed in the area correlation (Fig. 3), that the maximal overlap concept should consistently lead to a picture of the substrate–ligand orientation (that is, the complex structure) that is reasonable in terms of measurable complex reactivity. This leads to the tentative view that the maximal overlap area approach provides a realistic estimate of complex structures in aqueous solution, including an estimate of average complex stability. The actual stability of a particular complex is determined, within this average configuration, by local atomic and group interactions.

REFERENCES

(1) J. A. Mollica, Jr., and K. A. Connors, *J. Amer. Chem. Soc.*, **89**, 308(1967).

(2) K. A. Connors, M. H. Infeld, and B. J. Kline, *ibid.*, **91**, 3597, 5697(1969).

(3) J. D. Fulton and R. Robinson, *J. Chem. Soc.*, **1939**, 200.

(4) V. M. Rodionov and B. J. Kurjev, *Iz. Akad. Nauk, SSSR, Otd. Khim. Nauk*, **1952**, 113; through *Chem. Abstr.*, **47**, 2157h (1953).

(5) J. L. Cohen, Ph.D. dissertation, University of Wisconsin, Madison, Wis., 1969.

(6) R. Karrer, *Helv. Chim. Acta*, **2**, 482(1919).

(7) J. Bradbook and R. M. Linstead, *J. Chem. Soc.*, **1936**, 1739.

(8) R. A. Ward, *J. Phys. Chem.*, **38**, 161(1934).

(9) G. Clav, *Chem. Ber.*, **65**, 846(1932).

(10) R. Pfeiffer and J. Haefelin, *ibid.*, **55**, 1771(1922).

(11) T. C. Bruice and G. L. Schmir, *J. Amer. Chem. Soc.*, **79**, 1663(1957).

(12) R. A. B. Copeland and A. R. Day, *ibid.*, **65**, 1072(1943).

(13) H. L. Bamberger and S. Berle, *Ann. Chem.*, **1893**, 340.

(14) B. F. Deusel, H. Berman, and R. J. Schacter, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 619(1954).

(15) R. Q. Brewster and B. Williams, *Org. Syn.*, **22**, 48(1942).

(16) P. A. Kramer and K. A. Connors, *J. Amer. Chem. Soc.*, **91**, 2600(1969).

(17) K. A. Connors and J. A. Mollica, Jr., *J. Pharm. Sci.*, **55**, 772(1966).

(18) T. Higuchi and K. A. Connors, in "Advances in Analytical Chemistry and Instrumentation," vol. 4, C. N. Reilley, Ed., Interscience, New York, N. Y., 1965, pp. 117–212.

(19) F. M. Menger and M. L. Bender, *J. Amer. Chem. Soc.*, **88**, 131(1966).

(20) D. E. Guttman and D. Brooke, *J. Pharm. Sci.*, **57**, 1677 (1968).

(21) R. W. Gurney, "Ionic Processes in Solution," McGraw-Hill, New York, N. Y., 1953, chap. 6 (Dover Publications edition, 1962).

(22) H. Stelmach, Ph.D. dissertation, University of Wisconsin, Madison, Wis., 1969; P. O. P. Ts'O, N. S. Kondo, R. K. Robins, and A. D. Broom, *J. Amer. Chem. Soc.*, **91**, 5625(1969).
(23) The Ealing Corp., Cambridge, Mass.
(24) A. A. Adamson, "Physical Chemistry of Surfaces," 2nd ed., Interscience, New York, N. Y., 1967, p. 45.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 31, 1969, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*

Accepted for publication March 27, 1970.

The authors thank Dr. Pasupati Mukerjee, who called to their attention the importance of unitary quantities in systems such as these. Computing facilities were provided by the University of Wisconsin Computer Center and the University Research Committee.

Part VI in the series "Modification of Reaction Rates by Complex Formation." For Part V, see H. Stelmach and K. A. Connors, *J. Amer. Chem. Soc.*, **92**, 863(1970).

* Trainee of the National Institute of Dental Research Training Grant, DE-171.

Central Hypotensive Activity of *dl*- and *d*-Propranolol

GERALD J. KELLIHER* and JOSEPH P. BUCKLEY

Abstract □ The intraventricular administration of *dl*-propranolol to α-chloralose-anesthetized cats was followed by a decrease in blood pressure and was devoid of an associated tachycardia. *d*-Propranolol in an equivalent dose produced a hypotensive response which was not statistically different from *dl*-propranolol. These data suggest that there is a central component in the hypotensive response to propranolol and that it is independent of the β-adrenergic blocking activity. The intraventricular administration of 500 mcg. of reserpine base produced greater than a 90% depletion of norepinephrine in all brain regions analyzed within 24 hr. This pretreatment with reserpine also reversed the hypotensive response to both *d*- and *dl*-propranolol as well as converted the decrease in heart rate to an increase in heart rate. Thus, at least one of the amines must be required to produce the hypotensive response. Perfusion of the ventricular system with *dl*-propranolol generally produced an increase in epinephrine along with a decrease in norepinephrine in the brain region analyzed. Under the same conditions, *d*-propranolol also increased epinephrine but led to a decrease in norepinephrine in only 50% of the tissues assayed. These changes in amine levels occurred during the perfusion, which also produced a sustained hypotensive effect lasting the duration of the perfusion. The hypotension associated with propranolol therapy may have a central component that is not dependent on the β-adrenergic blocking property of propranolol, which requires one or more of the brain amines, and leads to an increase in the epinephrine level along with a general decrease in the norepinephrine level of the brain.

Keyphrases □ *dl*-, *d*-Propranolol—central hypotensive activity □ Hypotensive action, *dl*-, *d*-propranolol—intraventricular injection □ β-Adrenergic blocking activity—propranolol □ Catecholamines, brain—propranolol effect

Propranolol antagonizes the cardiovascular effects of β-adrenergic receptor stimulation produced by either stimulation of effector fibers or by sympathomimetic amines. The intravenous administration of propranolol produces a decrease in the sympathetic component to the heart and blocks the chronotropic effects of isoproterenol and epinephrine and the peripheral vasodilatory effects of isoproterenol (1–3). The acute response to propranolol is a decrease in cardiac output, whereas prolonged administration fails to produce this effect (4). Several reports have shown that the intravenous administration of propranolol to normotensive or hypertensive individuals decreased heart rate and cardiac output along with a concomitant decrease in systemic blood pressure but exhibited no significant effects on systemic peripheral resistance (4–6). Epstein *et al.* (5) and Shinebourne *et al.* (6) reported that the increase in arterial pressure that is associated with exercise was abolished by propranolol *via* reduction in cardiac output.

The central nervous system (CNS) manifestations attributed to propranolol classify it as a sedative and general CNS depressant (7–9). Since both propranolol and pronethalol possess these properties while dichloroisoproterenol (DCI) produces CNS stimulation, these authors have concluded that it is the presence of the naphthyl group rather than the β-adrenergic blockade that is responsible for the CNS effects. The depression or tranquilization in humans associated with propranolol administration is invariably apparent only at dosage levels that are many times greater than those necessary to produce β-adrenergic blockade (10–12). Masuoka and Hansson showed that intravenous administration of ^{14}C-labeled propranolol is rapidly taken up by the rat brain and concentrated 50 times greater in the brain than in the blood (13).

The hypotensive effect induced by the administration of β-adrenergic blocking agents in man has been well documented (14–22). This response is not exclusive to humans, because other investigators also have noted depressor effects in animals (23–26). The oral administration of propranolol for several weeks has been reported to result in a gradual and significant decrease in systemic arterial pressure in hypertensive patients (10, 16, 22). In all cases, regardless of the time of onset, the dose of the β-adrenergic blocker was many times that required for the blockade of the β-receptors, thus leading to the hypothesis that this response may not be due to antagonism of these receptors. This hypotensive response was unexpected, since the blocking of a neural mechanism that produces vasodilation in the absence of a significant reduction in cardiac output might be expected to result in a rise in systemic blood pressure

(22) H. Stelmach, Ph.D. dissertation, University of Wisconsin, Madison, Wis., 1969; P. O. P. Ts'O, N. S. Kondo, R. K. Robins, and A. D. Broom, *J. Amer. Chem. Soc.*, **91**, 5625(1969).
(23) The Ealing Corp., Cambridge, Mass.
(24) A. A. Adamson, "Physical Chemistry of Surfaces," 2nd ed., Interscience, New York, N. Y., 1967, p. 45.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 31, 1969, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*

Accepted for publication March 27, 1970.

The authors thank Dr. Pasupati Mukerjee, who called to their attention the importance of unitary quantities in systems such as these. Computing facilities were provided by the University of Wisconsin Computer Center and the University Research Committee.

Part VI in the series "Modification of Reaction Rates by Complex Formation." For Part V, see H. Stelmach and K. A. Connors, *J. Amer. Chem. Soc.*, **92**, 863(1970).

* Trainee of the National Institute of Dental Research Training Grant, DE-171.

Central Hypotensive Activity of *dl*- and *d*-Propranolol

GERALD J. KELLIHER* and JOSEPH P. BUCKLEY

Abstract □ The intraventricular administration of *dl*-propranolol to α-chloralose-anesthetized cats was followed by a decrease in blood pressure and was devoid of an associated tachycardia. *d*-Propranolol in an equivalent dose produced a hypotensive response which was not statistically different from *dl*-propranolol. These data suggest that there is a central component in the hypotensive response to propranolol and that it is independent of the β-adrenergic blocking activity. The intraventricular administration of 500 mcg. of reserpine base produced greater than a 90% depletion of norepinephrine in all brain regions analyzed within 24 hr. This pretreatment with reserpine also reversed the hypotensive response to both *d*- and *dl*-propranolol as well as converted the decrease in heart rate to an increase in heart rate. Thus, at least one of the amines must be required to produce the hypotensive response. Perfusion of the ventricular system with *dl*-propranolol generally produced an increase in epinephrine along with a decrease in norepinephrine in the brain region analyzed. Under the same conditions, *d*-propranolol also increased epinephrine but led to a decrease in norepinephrine in only 50% of the tissues assayed. These changes in amine levels occurred during the perfusion, which also produced a sustained hypotensive effect lasting the duration of the perfusion. The hypotension associated with propranolol therapy may have a central component that is not dependent on the β-adrenergic blocking property of propranolol, which requires one or more of the brain amines, and leads to an increase in the epinephrine level along with a general decrease in the norepinephrine level of the brain.

Keyphrases □ *dl*-, *d*-Propranolol—central hypotensive activity □ Hypotensive action, *dl*-, *d*-propranolol—intraventricular injection □ β-Adrenergic blocking activity—propranolol □ Catecholamines, brain—propranolol effect

Propranolol antagonizes the cardiovascular effects of β-adrenergic receptor stimulation produced by either stimulation of effector fibers or by sympathomimetic amines. The intravenous administration of propranolol produces a decrease in the sympathetic component to the heart and blocks the chronotropic effects of isoproterenol and epinephrine and the peripheral vasodilatory effects of isoproterenol (1–3). The acute response to propranolol is a decrease in cardiac output, whereas prolonged administration fails to produce this effect (4). Several reports have shown that the intravenous administration of propranolol to normotensive or hypertensive individuals decreased heart rate and cardiac output along with a concomitant decrease in systemic blood pressure but exhibited no significant effects on systemic peripheral resistance (4–6). Epstein *et al.* (5) and Shinebourne *et al.* (6) reported that the increase in arterial pressure that is associated with exercise was abolished by propranolol *via* reduction in cardiac output.

The central nervous system (CNS) manifestations attributed to propranolol classify it as a sedative and general CNS depressant (7–9). Since both propranolol and pronethalol possess these properties while dichloroisoproterenol (DCI) produces CNS stimulation, these authors have concluded that it is the presence of the naphthyl group rather than the β-adrenergic blockade that is responsible for the CNS effects. The depression or tranquilization in humans associated with propranolol administration is invariably apparent only at dosage levels that are many times greater than those necessary to produce β-adrenergic blockade (10–12). Masuoka and Hansson showed that intravenous administration of ^{14}C-labeled propranolol is rapidly taken up by the rat brain and concentrated 50 times greater in the brain than in the blood (13).

The hypotensive effect induced by the administration of β-adrenergic blocking agents in man has been well documented (14–22). This response is not exclusive to humans, because other investigators also have noted depressor effects in animals (23–26). The oral administration of propranolol for several weeks has been reported to result in a gradual and significant decrease in systemic arterial pressure in hypertensive patients (10, 16, 22). In all cases, regardless of the time of onset, the dose of the β-adrenergic blocker was many times that required for the blockade of the β-receptors, thus leading to the hypothesis that this response may not be due to antagonism of these receptors. This hypotensive response was unexpected, since the blocking of a neural mechanism that produces vasodilation in the absence of a significant reduction in cardiac output might be expected to result in a rise in systemic blood pressure

(18, 27–29). The mechanism of the hypotensive response to the prolonged oral administration of propranolol has not been elucidated. Several investigators have attempted to explain this hypotensive response on a resetting of the baroreceptor threshold to respond to alterations in systemic blood pressure at a lower level; however, conclusive evidence is currently lacking (30, 31). Frohlich and Page (10) and Waal (21) have suggested that a more fruitful area of research into the mechanism of this hypotensive response would be the elucidation of a possible central component. The purpose of the present study was to investigate the role of the CNS in the hypotensive effects induced by propranolol.

EXPERIMENTAL

Perfusion of Left Lateral Ventricle—Twenty-two adult cats of either sex weighing 2–3 kg. were anesthetized with α-chloralose (65 mg./kg. i.p.) prepared as a 1% solution in warm saline. Systemic blood pressure was recorded from a catheterized femoral artery onto a Grass polygraph, and the ipsilateral femoral vein was catheterized for the administration of drugs. Tracheal intubation was performed, and the animal was ventilated with room air by means of a Harvard respirator. The left lateral ventricle was then prepared for perfusion with artificial cerebrospinal fluid (CSF) (32), following the method described by Bhattacharya and Feldberg (33), by stereotaxically implanting a 22-gauge hypodermic needle which had previously been machined to remove the bevel. The coordinates used were: anterior, 13 mm.; lateral, 2.75 mm.; and horizontal, +7 mm. (34). The successful placement of the cannula was confirmed by the presence of CSF pulsating in the barrel of the cannula. The cannula was then permanently affixed to the skull using dental cement. The perfusate fluid was maintained at 38°, and the lateral ventricle was perfused at a rate of 0.1 ml./min. for 1 hr. prior to drug injection to permit stabilization of the cardiovascular system. After this stabilization period, the perfusion was temporarily interrupted to permit the injection of 0.5 mg. of *d*- or *dl*-propranolol contained in 0.1 ml. of artificial CSF. Perfusion was then resumed.

"Central reserpinization" was performed in 14 cats by implanting a ventricular cannula, following the previously mentioned procedure using an aseptic technique, in cats under pentobarbital anesthesia. Procaine penicillin G (600,000 units i.m.) was administered prior to and on the day following surgery, after which the animal was allowed to recover for 3 days. On the 4th day following surgery, the male cat was removed from the cannula and 500 mcg. of reserpine base[1] in 0.1 ml. of water for injection was then administered into the left lateral ventricle. The cannula then was resealed to effect a functional central sympathectomy (35–38). Twenty-four hours later, the cat was prepared for perfusion of the lateral ventricle as described previously. One-half milligram of *d*- or *dl*-propranolol in 0.1 ml. CSF was administered into the lateral ventricle, and the blood pressure and heart rate were monitored. The brains and hearts from 10 additional adult cats were removed 24 hr. after "central reserpinization" and sectioned into the following regions to delineate the areas adjacent to the ventricular system: medulla, pons, cerebellum, mesencephalon, diencephalon, and telencephalon. These sections were then assayed for norepinephrine content by the method of Brodie *et al.* (39) using a spectrophotofluorometer (Aminco-Bowman).

Eight adult cats weighing 2–3 kg. were prepared for intraventricular perfusion as described and perfused for 1 hr. with either *d*- or *dl*-propranolol (0.5 mg./0.1 ml. CSF, 0.1 ml./min.). The cat then was sacrificed by means of an intravenous injection of air; the brain and heart were rapidly removed, dissected as already described, and frozen. The norepinephrine and epinephrine contents of the various brain sections and myocardia were determined by a modification of the method of Chang (40), in which the fluorescent intensity of the epinephrine in the samples is read within 10 min. following oxidation at an excitation wavelength of 417 mμ (uncorrected) and an emission wavelength of 510 mμ (uncorrected). The samples were then placed in boiling water for 2 min. and cooled to room temperature. The fluorescent intensity of norepinephrine was read at an excitation wavelength of 385 mμ (uncorrected) with an emission wavelength at 485 mμ (uncorrected). The concentrations of epinephrine and norepinephrine were determined using the simultaneous equations described by von Euler and Lishajko (41). All data were analyzed for statistical significance using Student's *t* test unless otherwise indicated.

[1] Serpasil-Ciba.

Table I—Effect of *d*- and *dl*-Propranolol on Blood Pressure of Anesthetized Cats[a]

Compound[b]	Mean Blood Pressure (mm. Hg ± *SE*) Control	After Propranolol	Mean Decrease in Blood Pressure (mm. Hg ± *SE*)	% Decrease
dl-Propranolol	100.4 ± 10.3	70.5 ± 9.9[c]	29.9 ± 6.7[d]	29.8
d-Propranolol	90.3 ± 6.4	72.1 ± 3.9[c]	18.2 ± 3.7	20.2

[a] $N = 11$. [b] 0.5 mg./0.1 ml. CSF, IVT. [c] $p < 0.05$ compared with controls (paired *t* test). [d] Not significant when compared with *d*-propranolol.

RESULTS

Effect of Intraventricular Propranolol on Blood Pressure and Heart Rate of Anesthetized Cats—The intraventricular (IVT) administration of *d*- or *dl*-propranolol produced significant hypotensive responses which are summarized in Table I. Reflexogenic tachycardia in response to the hypotensive effect of propranolol did not occur, but there was a slight but not statistically significant decrease in heart rate (Table II). Although the degree of the hypotensive response to *dl*-propranolol was greater than that produced by the *d*-isomer, there was no statistically reliable difference between the two hypotensive responses. There was, however, a significant difference between *d*- and *dl*-propranolol in the reduction in heart rate produced. The spinal cord of each animal was transected at the C-2 level to ensure that the observed hypotensive response to IVT-administered propranolol was due to a central component; after the blood pressure had stabilized, the experiment was repeated. Neither compound elicited an effect following spinal transection. These data suggest that a portion of the hypotensive response to propranolol is of central origin and may be independent of β-adrenergic receptor blocking activity.

Effect of Central Reserpinization on Central Hypotensive Response to Propranolol—To ascertain the role of endogenous brain amines in the central hypotensive component of propranolol, the compounds were administered IVT to anesthetized cats 24 hr. after administration of reserpine into the lateral ventricle. The data, summarized in Tables III and IV, show that central reserpine pretreatment not only blocked the central hypotensive component previously elicited by both *d*- and *dl*-propranolol but also converted the response to a pressor effect. Whereas propranolol produced a decrease in heart rate in anesthetized nonreserpinized cats, the effect after reserpine was a mild increase in heart rate. These data suggest that the central hypotensive component elicited by both *d*- and *dl*-propranolol is dependent on the presence of central amine stores and a functional sympathetic cardiovascular component. Figure 1 presents a comparison of the effects of IVT propranolol in reserpinized and nonreserpinized anesthetized cats.

Table II—Effect of *d*- and *dl*-Propranolol on Heart Rate of Anesthetized Cats[a]

Compound[b]	Mean Heart Rate/min. ± *SE* Control	After Propranolol	Mean Decrease in Heart Rate/min. ± *SE*	% Decrease
dl-Propranolol	175.5 ± 21.5	150.9 ± 16.2	24.5 ± 7.6[c]	14.0
d-Propranolol	160.5 ± 15.2	158.2 ± 14.7	2.3 ± 1.2	1.4

[a] $N = 11$. [b] 0.5 mg./0.1 ml. CSF, IVT. [c] Significant when compared with *d*-propranolol.

Table III—Effect of Propranolol on Blood Pressure of Anesthetized Cats after "Central Reserpinization"

Compound[a]	Mean Blood Pressure (mm. Hg ± *SE*) Control	After Propranolol	Mean Increase in Blood Pressure (mm. Hg ± *SE*)	% Increase
dl-Propranolol ($N = 6$)	52.2 ± 6.4	65.5 ± 3.0	13.3 ± 9.2	23.8
d-Propranolol ($N = 8$)	47.9 ± 7.2	63.0 ± 7.6	15.1 ± 7.0	24.0

[a] 0.5 mg./0.1 ml. CSF, IVT.

Effect of Central Reserpinization on Brain and Cardiac Norepinephrine Stores—To ascertain whether a functional central sympathectomy, which previous investigators have shown requires a 90% depletion of brain norepinephrine (35–38), had been achieved, 10 adult cats were pretreated IVT with reserpine 24 hr. before sacrifice. The brain was sectioned and analyzed as previously described, and the results of these assays are summarized in Table V. Every section of the brain analyzed showed at least a 90% depletion in the level of norepinephrine; however, cardiac norepinephrine stores were reduced by only 75%. These results confirm those reported by previous investigators for the IVT administration of 500 mcg. of reserpine 24 hr. prior to sacrifice (42–44).

Effect of IVT Perfusion with Propranolol on Regional Distribution of Brain Epinephrine and Norepinephrine—Since *dl*-propranolol has been reported to cause a decrease in brain norepinephrine with a simultaneous increase in cardiac norepinephrine, the effect was investigated in light of the central propranolol component described previously, possibly to correlate it with the hypotensive response. Data on the regional distribution of brain epinephrine and norepinephrine following 1 hr. IVT perfusion with either *d*- or *dl*-propranolol are summarized in Tables VI and VII. These data show that both the *d*- and *dl*-propranolol produced an increase in the level of epinephrine in the various brain regions with one exception. *dl*-Propranolol produced an 11% decrease in the epinephrine level of the diencephalon along with a slight decrease in the level of norepinephrine, although neither amounted to a significant depletion. In all other areas, *dl*-propranolol increased epinephrine content in varying degrees up to 125% in the medulla; however, only in the medulla ($p < 0.0005$), pons ($p < 0.005$), and telencephalon ($p < 0.025$) were these increases statistically significant. The level of norepinephrine was consistently reduced after *dl*-propranolol perfusion, with the effect being greatest in the medulla (32%); however, there was a 22% increase in norepinephrine ($p < 0.01$) in the telencephalon. The reduction in norepinephrine level in the pons ($p < 0.05$) and medulla ($p < 0.05$) proved to be statistically significant.

The epinephrine level was increased in each region of the brain following perfusion with *d*-propranolol, with a maximum of 167% in the medulla. The increase in epinephrine proved to be statistically reliable in the medulla ($p < 0.005$), pons ($p < 0.005$), mesencephalon ($p < 0.05$), and telencephalon ($p < 0.025$). The level of norepinephrine increased in the mesencephalon, diencephalon, and telencephalon but decreased in the medulla, pons, and cerebellum; however, the changes noted in any one region fell short of statistical reliability. The results of perfusing with either *d*- or *dl*-propranolol were parallel in both direction and magnitude of change, with the exception of the mesencephalon and diencephalon.

Table IV—Effect of Propranolol on Heart Rate of Anesthetized Cats after "Central Reserpinization"

Compound[a]	Mean Heart Rate/min. ± *SE* Control	After Propranolol	Mean Increase in Heart Rate/min. ± *SE*	% Increase
dl-Propranolol ($N = 6$)	115.0 ± 6.2	119.2 ± 10.8	4.2 ± 3.0	3.5
d-Propranolol ($N = 8$)	123.7 ± 5.9	132.5 ± 8.3	8.8 ± 2.1	6.7

[a] 0.5 mg./0.1 ml. CSF, IVT.

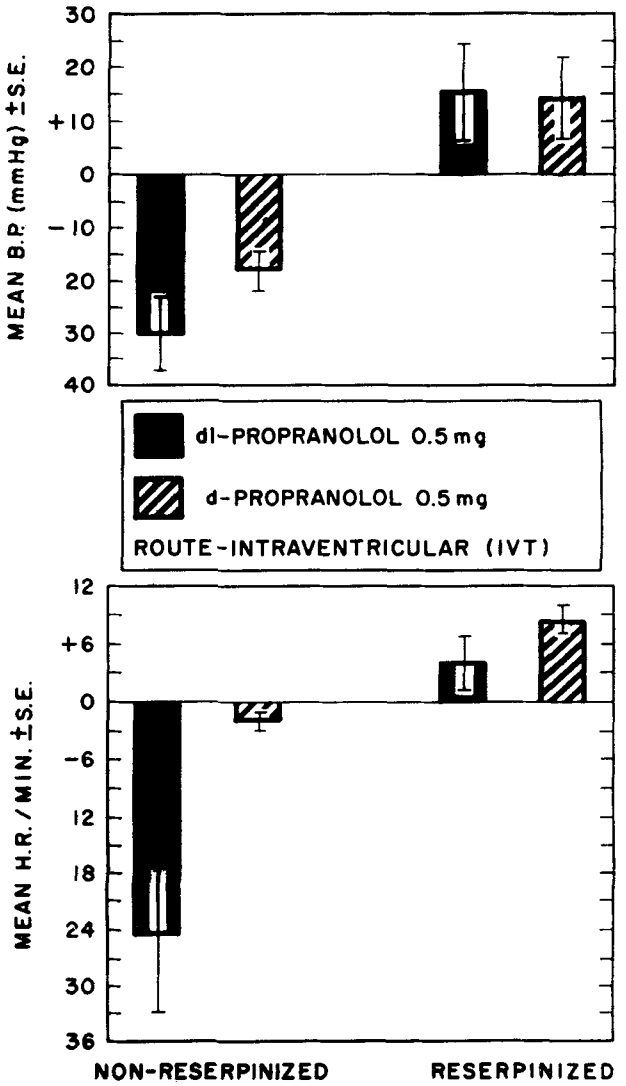


Figure 1—*Effect of "central reserpinization" (500 mcg. reserpine, IVT, 24 hr. prior to experiment) on the blood pressure and heart rate response to propranolol.*

Masuoka and Hansson (13) have studied the CNS distribution of ^{14}C-labeled propranolol in rats and have found that propranolol is highly concentrated in those areas corresponding to the regions where the greatest changes in catecholamine levels have been found: telencephalon, mesencephalon, pons, and medulla. These areas showed the highest and most prolonged concentration of the labeled propranolol. There have been no reports to date on the uptake and distribution of ^{14}C-labeled propranolol in the cat.

The IVT perfusion with either *d*- or *dl*-propranolol resulted in a significant hypotensive response. This response was of a similar

Table V—Effect of "Central Reserpinization" on Regional Distribution of Brain Norepinephrine[a]

Region	Control[b]	Reserpinized[b]	% of Control
Telencephalon	0.248 ± 0.01	0.0240	9.7
Diencephalon	0.416 ± 0.05	0.0217	5.2
Mesencephalon	0.359 ± 0.04	0.0180	5.0
Cerebellum	0.147 ± 0.01	0.0136	9.3
Pons	0.449 ± 0.02	0.0449	10.0
Medulla	0.299 ± 0.03	0.0102	3.4

[a] Expressed as mcg./g. tissue, wet weight; mean ± *SE*. [b] $N = 10$.

Table VI—Effect of IVT Perfusion with *d*-Propranolol[a] on Regional Distribution of Brain Epinephrine[b] and Norepinephrine[b]

Region	Control[c]		Perfused[c]		% Change	
	Epinephrine	Norepinephrine	Epinephrine	Norepinephrine	Epinephrine	Norepinephrine
Medulla	0.012 ± 0.001	0.372 ± 0.051	0.032 ± 0.001	0.280 ± 0.021	+167	−25
Pons	0.009 ± 0.001	0.431 ± 0.023	0.017 ± 0.001	0.394 ± 0.008	+89	−9
Cerebellum	0.024 ± 0.001	0.201 ± 0.011	0.026 ± 0.002	0.189 ± 0.010	+8	−6
Mesencephalon	0.012 ± 0.001	0.308 ± 0.024	0.016 ± 0.001	0.316 ± 0.032	+33	+3
Diencephalon	0.028 ± 0.005	0.358 ± 0.043	0.030 ± 0.001	0.398 ± 0.009	+7	+11
Telencephalon	0.011 ± 0.001	0.162 ± 0.006	0.013 ± 0.001	0.182 ± 0.007	+18	+12

[a] 0.5 mg./0.1 ml. at 0.1 ml./min. for 60 min. [b] Expressed as mcg./g. tissue, wet weight; mean ± *SE*. [c] $N = 4$.

Table VII—Effect of IVT Perfusion with *dl*-Propranolol[a] on Regional Distribution of Brain Epinephrine[b] and Norepinephrine[b]

Region	Control[c]		Perfused[c]		% Change	
	Epinephrine	Norepinephrine	Epinephrine	Norepinephrine	Epinephrine	Norepinephrine
Medulla	0.012 ± 0.001	0.372 ± 0.051	0.027 ± 0.001	0.255 ± 0.011	+125	−32
Pons	0.009 ± 0.001	0.431 ± 0.023	0.016 ± 0.001	0.363 ± 0.014	+78	−16
Cerebellum	0.024 ± 0.001	0.201 ± 0.011	0.025 ± 0.005	0.199 ± 0.010	+4	−1
Mesencephalon	0.012 ± 0.001	0.308 ± 0.024	0.013 ± 0.001	0.268 ± 0.017	+8	−13
Diencephalon	0.028 ± 0.005	0.358 ± 0.043	0.025 ± 0.002	0.356 ± 0.011	−11	−1
Telencephalon	0.011 ± 0.001	0.162 ± 0.006	0.013 ± 0.001	0.198 ± 0.010	+18	+22

[a] 0.5 mg./1.0 ml. CSF at 0.1 ml./min. for 60 min. [b] Expressed as mcg./g. tissue, wet weight; mean ± *SE*. [c] $N = 4$.

magnitude to that seen previously with single IVT injections and persisted through the entire perfusion period. This hypotensive response also was devoid of a concomitant tachycardia. A comparison of the hypotensive response from *d*- and *dl*-propranolol perfusion is summarized in Fig. 2.

DISCUSSION

Many reports have noted a hypotensive effect of propranolol. This response may occur during the acute administration of the compound in which the fall in blood pressure is usually mediated by a decrease in cardiac output (4–6). The acute hemodynamic response to parenterally administered propranolol has been shown to differ from that observed during prolonged administration, because the decrease in cardiac output observed upon acute administration is absent during chronic administration (4, 45). The results of the present studies utilizing the IVT administration of *dl*-propranolol have shown that propranolol can produce a hypotensive response *via* central mechanisms. The hypotensive response produced was statistically significant and was devoid of a concomitant tachycardia, which may suggest the involvement of vasomotor regulatory centers. The results, however, do not rule out the possibility of a vagal action producing the decrease in heart rate and the absence of a concomitant reflexogenic tachycardia.

Several investigators have presented evidence to suggest that many of the effects noted with propranolol, including those on the CNS, are independent of the β-adrenergic blocking activity of the compound (7–9). This also has been shown to be true with the hypotensive response associated with prolonged oral administration of propranolol (4, 31). The present study involved the use of *d*-propranolol, which has been reported to have only 1–2% of the β-blocking activity of the racemic mixture, to differentiate between the hypotensive and β-blocking activity of propranolol (2, 46, 47). The findings suggest that the central hypotensive response induced by *dl*-propranolol is not dependent on β-adrenergic blocking activity, since the *d*-isomer also produces a centrally mediated hypotensive response which was not statistically different from that produced by the racemic mixture. Although Gagnon and Melville (48) have shown that the response to isoproterenol is similar after intravenous or IVT administration (consisting of myocardial augmentation and hypotension), there remains speculation regarding the nature of the adrenergic receptors involved in mediating these responses. Thus, although the *d*-isomer of propranolol has been demonstrated to exhibit only 1–2% of the β-adrenergic receptor blocking activity of *d*-propranolol peripherally, this relationship may not hold true centrally. The β-receptorlike property exhibited centrally may not necessarily demonstrate an equivalent sensitivity to β-adrenergic agonists or antagonists as shown peripherally and thus could give a false indication in the differentiation between central and peripheral β-adrenergic functions. Based on present knowledge, however, the evidence suggests that this central hypotensive response is independent of β-adrenergic blocking activity.

The depletion of brain and cardiac norepinephrine produced by IVT reserpine was similar to that previously reported from this laboratory (44). The depletion of brain amines by reserpine was utilized to study the role played by endogenous brain amines in the centrally mediated hypotensive response demonstrated by both *d*- and *dl*-propranolol. These data suggest that either a level of endogenous brain amine exceeding 10% of normal or a functional central sympathetic component is required for the central hypotensive response to propranolol. These data must be evaluated with the knowledge that reserpine does not selectively deplete norepinephrine but rather produces a gross depletion of other biogenic amines as well.

Propranolol has been reported to produce a decrease in brain catecholamines when 10 mg. i.p./kg./day was administered to mice for 4 days. Other investigators, however, have failed to reproduce these effects with propranolol, using the same dose and time schedule (50, 51). The present studies were also undertaken to determine if perfusion of the brain with propranolol would result in an alteration of the catecholamine content of the brain and to investigate the possibility that there may be a change in the epinephrine–norepinephrine balance. Epinephrine has been reported to repre-

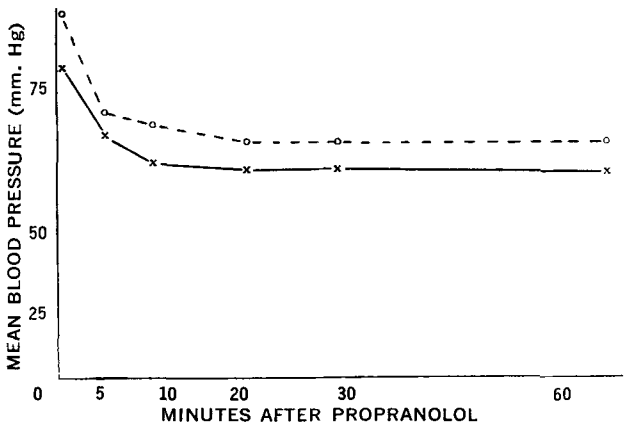


Figure 2—*A comparison of the hypotensive response to IVT perfusion of propranolol, 0.5 mg./0.1 ml. CSF, 0.1 ml./min., in anesthetized cats. Key:* ×—×, dl-*propranolol* (N = 4); *and* o--o, d-*propranolol* (N = 4).

sent 4–17% of the total concentration of catecholamines in the brain (52–54). The rapid metabolism and enzymatic synthesis of epinephrine in the brain suggest that this potent amine may function as a neuroregulatory agent (55–60); some investigators have reported epinephrine to be an inhibitor of synaptic transmission within the CNS (61–63). The data presented here show that there is an alteration in the brain levels of both epinephrine and norepinephrine caused by perfusion with propranolol; however, these alterations are not uniform. The great increase in epinephrine within the pons and medulla strongly suggests that amine alterations associated with vasomotor regulatory centers may play a role in the central hypotensive response of prolonged therapy with propranolol. Furthermore, these data reinforce the idea that determining the whole brain concentration of a particular compound is less desirable than assaying individual brain regions, since an increase in one region may compromise a concurrent decrease in another region when assayed as a whole. This apparently led to the aforementioned reports noting both a decrease or no change in the brain level of norepinephrine following propranolol (45, 50, 51).

REFERENCES

(1) J. W. Black, W. A. M. Duncan, and R. G. Shanks, *Brit. J. Pharmacol.*, **25,** 577(1965).

(2) L. S. Whitsitt and B. R. Lucchesi, *Life Sci.*, **6,** 939(1967).

(3) G. Aberg, T. Dzedin, L. Luncholm, L. Olsson, and N. Svedmyr, *ibid.*, **8,** 353(1969).

(4) M. Ulrych, E. D. Frohlich, H. P. Dustan, and I. H. Page, *Circulation*, **37,** 411(1968).

(5) S. E. Epstein, B. F. Robinson, R. L. Kahler, and E. Braunwald, *J. Clin. Invest.*, **44,** 1745(1965).

(6) E. Shinebourne, J. Fleming, and J. Hamer, *Lancet*, **2,** 1217 (1967).

(7) G. Leszkovszky and L. Tardos, *J. Pharm. Pharmacol.*, **17,** 518(1965).

(8) W. Murmann, L. Almirante, and M. Saccani-Guelfi, *ibid.*, **18,** 317(1966).

(9) E. Grana and D. Sossi, *Farmaco*, **22,** 582(1967).

(10) E. D. Frohlich and I. H. Page, *Physiol. Pharmacol.*, **1,** 1(1966).

(11) J. Fitzgerald, *Brit. Med. J.*, **2,** 372(1967).

(12) H. J. Waal, *ibid.*, **2,** 50(1967).

(13) D. Masuoka and E. Hansson, *Acta Pharmacol. Toxicol.*, **25,** 447(1967).

(14) B. M. C. Prichard, *Brit. Med. J.*, **1,** 1227(1964).

(15) B. M. C. Prichard, *Amer. Heart J.*, **69,** 716(1965).

(16) B. M. C. Prichard and P. M. S. Gillam, *Brit. Med. J.*, **2,** 725(1964).

(17) G. Schroder and L. Werko, *Clin. Pharmacol. Ther.*, **5,** 159 (1964)

(18) G. Schroder and L. Werko, *Amer. J. Cardiol.*, **15,** 58(1965).

(19) R. R. Taylor, E. I. Johnston, and A. D. Jore, *New Engl. J. Med.*, **271,** 877(1964).

(20) B. M. C. Prichard and P. M. S. Gillam, *Amer. J. Cardiol.*, **18,** 387(1966).

(21) H. J. Waal, *Clin. Pharmacol. Ther.*, **7,** 588(1966).

(22) J. W. Paterson and C. T. Dollery, *Lancet*, **2,** 1148(1966).

(23) J. W. Black and J. S. Stephenson, *ibid.*, **2,** 311(1962).

(24) R. S. McNeill, *ibid.*, **2,** 1101(1964).

(25) A. Sekiya and E. M. Vaughan Williams, *Brit. J. Pharmacol.*, **21,** 462(1963).

(26) E. E. Vogin and A. J. Rice, *Arch. Int. Pharmacodyn. Ther.*, **155,** 317(1965).

(27) J. M. Bishop and N. Segal, *J. Physiol.*, **169,** 112P(1963).

(28) D. A. Chamberlain and J. Howard, *Brit. Heart J.*, **26,** 213 (1964).

(29) B. Uvnas, *Physiol. Rev.*, **34,** 608(1954).

(30) B. M. C. Prichard, *Angiologica*, **3,** 318(1966).

(31) D. Dunlop and R. Shanks, *Brit. J. Pharmacol.*, **36,** 132 (1969).

(32) J. K. Merlis, *Amer. J. Physiol.*, **131,** 67(1940).

(33) B. K. Bhattacharya and W. Feldberg, *Brit. J. Pharmacol.*, **13,** 156(1958).

(34) N. Jasper and G. Ajmone-Marsan, "A Stereotaxic Atlas of the Diencephalon of the Cat," National Research Council of Canada, Ottawa, Canada, 1956.

(35) E. Muscholl and M. Vogt, *J. Physiol.*, **141,** 132(1958).

(36) E. Costa, in "Hypertension, Recent Advances," A. N. Brest and J. H. Moyer, Eds., Lea & Febiger, Philadelphia, Pa., 1961, pp. 348–353.

(37) T. E. Gaffney, C. A. Chidsey, and E. Braunwald, *Circ. Res.*, **12,** 264(1963).

(38) G. Sedvall and J. Thorson, *Acta Physiol. Scand.*, **64,** 251 (1965).

(39) B. B. Brodie, M. S. Comer, E. Costa, and A. Dlabac, *J. Pharmacol. Exp. Ther.*, **152,** 340(1966).

(40) C. C. Chang, *Intern. J. Neuropharmacol.*, **3,** 643(1964).

(41) U. S. von Euler and F. Lishajko, *Acta Physiol. Scand.*, **45,** 122(1959).

(42) N. N. Share and K. I. Melville, *J. Pharmacol. Exp. Ther.*, **141,** 15(1963).

(43) N. N. Share and K. I. Melville, *Arch. Int. Pharmacodyn. Ther.*, **153,** 267(1965).

(44) H. H. Smookler, W. B. Severs, W. J. Kinnard, and J. P. Buckley, *J. Pharmacol. Exp. Ther.*, **153,** 485(1966).

(45) A. B. Herring, *Lancet*, **2,** 892(1964).

(46) R. Howe and R. H. Shanks, *Nature*, **210,** 1336(1966).

(47) A. M. Barrett, *J. Pharm. Pharmacol.*, **21,** 241(1969).

(48) D. J. Gagnon and K. I. Melville, *Intern. J. Neuropharmacol.*, **6,** 245(1967).

(49) H. Brunner, P. Hedwall, L. Maitre, and M. Meier, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **245,** 254(1966).

(50) R. Laverty and K. M. Taylor, *J. Pharm. Pharmacol.*, **20,** 605(1968).

(51) T. C. Westfall, *Arch. Int. Pharmacodyn. Ther.*, **167,** 69(1967).

(52) L. M. Gunne, *Acta Physiol. Scand.*, **56,** 324(1962).

(53) A. Bertler and E. Rosengren, *ibid.*, **47,** 350(1959).

(54) M. J. Vogt, *J. Physiol.*, **123,** 451(1954).

(55) A. M. Steinman, S. E. Smerin, and J. D. Barchas, *Science*, **165,** 616(1969).

(56) R. Ciaranello, R. Barchas, G. Byers, D. Stemmle, and J. D. Barchas, *Nature*, **221,** 368(1969).

(57) J. D. Barchas, A. Steinman, S. Smerin, and J. Vernikos-Danellis, *Fed. Proc.*, **27,** 711(1968).

(58) P. L. McGeer and E. G. McGeer, *Biochem. Biophys. Res. Commun.*, **17,** 502(1964).

(59) L. A. Pohorecky, M. J. Zigmond, H. J. Karten, and R. J. Wurtman, *Fed. Proc.*, **27,** 239(1968).

(60) L. A. Pohorecky, M. J. Zigmond, H. J. Karten, and R. J. Wurtman, *J. Pharmacol. Exp. Ther.*, **165,** 190(1969).

(61) D. D. Christ and S. Nishi, *Life Sci.*, **8,** 1235(1969).

(62) E. Bulbring, *J. Physiol.*, **103,** 55(1944).

(63) R. M. Eccles and B. Libet, *ibid.*, **157,** 484(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 4, 1970, from the *Department of Pharmacology, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213*

Accepted for publication March 23, 1970.

Abstracted in part from a thesis submitted by G. J. K. in partial fulfillment of the Doctor of Philosophy degree requirements.

This investigation was supported by Grant GM-1217 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.

The *d*- and *dl*-propranolol used in this study were supplied by Ayerst Laboratories.

* Present address: Department of Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213

Oil–Water Partitioning of Chlorpromazine and Other Phenothiazine Derivatives Using Dodecane and *n*-Octanol

K. S. MURTHY* and GEORGE ZOGRAFI

Abstract □ The apparent partition coefficients of chlorpromazine and some other phenothiazine derivatives in dodecane–water and *n*-octanol–water systems were measured at 30°. Results in the dodecane system at various pH values demonstrated that only the free base form partitions. Intrinsic partition coefficients for all derivatives, except the very polar metabolite chlorpromazine sulfoxide, range from 10^4 to 10^5, indicating the remarkable hydrophobicity of these molecules. Partitioning measurements in *n*-octanol indicate significant extraction of these drugs as ion-pairs, as well as higher intrinsic partition coefficients than in dodecane. Measurement of partitioning at various salt concentrations, utilizing different anions, allowed the calculation of extraction constants. Correlations between intrinsic partition coefficients in dodecane and extraction constants in *n*-octanol are presented. From these studies, it is clear that quantitative studies involving the phenothiazines in heterogeneous systems such as membranes must consider their extreme hydrophobicity and the various factors that influence such behavior.

Keyphrases □ Chlorpromazine, oil–water partitioning—dodecane, *n*-octanol □ Phenothiazine, oil–water partitioning—dodecane, *n*-octanol □ Partition coefficients, apparent—phenothiazines □ Electrolyte, pH effects—apparent partition coefficients, phenothiazines □ Extraction constants—phenothiazines

Previous studies have suggested significant hydrophobic behavior for chlorpromazine and other phenothiazines which appears to be related to their pharmacological activity (1, 2). Such behavior is demonstrated by the very low water solubility of the free base form (3), an apparently high cyclohexane–water partition coefficient (4), significant surface activity at various interfaces (5–7), and accumulation at various biological membranes *in vitro* (8). Measurement of surface activity at charged lipid monolayer surfaces (9, 10), binding to red blood cell ghost membranes at various pH values (11), significant protein binding (12), and accumulation in intestinal membranes during the absorption process (13) all indicate that the ionized form of these drugs participates in hydrophobic interactions when its charge is suitably neutralized by appropriate anions.

In view of these observations, it appeared important to evaluate quantitatively the hydrophobic properties of the phenothiazines so as to characterize the effect of chemical modification and the relative behavior of ionized and free base forms. For this purpose, the influence of variables such as pH, ionic strength, and specific anions on partitioning into dodecane and *n*-octanol was studied.

EXPERIMENTAL

Materials—The chemical structure of each phenothiazine studied is given in Table I. Chlorpromazine (CPZ);[1] chlorpromazine sulfoxide (CPZ-O);[1] 1-chloro, 3-chloro, ethylamino, and butylamino analogs of CPZ;[1] trifluoperazine (TFP);[1] triflupromazine (TPZ);[2] and promazine (PZ)[3] were studied. All buffer ingredients, electrolytes, and urea were of reagent grade. Sodium methane sulfonate and sodium ethane sulfonate were prepared by adding an equivalent amount of sodium hydroxide to the corresponding alkyl sulfonic acid, followed by recrystallization from absolute methanol. The *n*-dodecane and *n*-octanol[4] were a spectrally pure grade.

Determination of Apparent Partition Coefficients—In *n*-octanol–aqueous systems, *n*-octanol saturated with buffer and buffer saturated with *n*-octanol were used to minimize volume changes due to mutual miscibility. Equal volumes of the two solvents with given amounts of solute were placed together and shaken for at least 8 hr., the time required to ensure equilibrium. The two phases were then separated in a separator, and the aqueous phase was analyzed. The difference in amount of solute present before and after equilibration was taken as the amount partitioning into *n*-octanol. Occasional assay of the lipid phase confirmed the validity of this procedure.

For experiments in dodecane–aqueous systems, no presaturation of the two solvents was necessary and solute was analyzed in both phases. Analysis of the phenothiazines was performed spectrophotometrically (Hitachi UV spectrophotometer), employing 95% ethanol as the solvent, following the procedure of Warren *et al.* (14). The wavelengths of maximum absorption are given in Table I. Care was taken to avoid any decomposition of these drugs by eliminating contact with light. Concentrations of solute in the range 10^{-5} to 10^{-4} *M* were chosen to avoid any effects due to micellar aggregation noted earlier for these compounds (15). Temperature was maintained at 30°. In dodecane–water systems, ion strength was always adjusted with KCl; ions used in the *n*-octanol–water system varied and will be specified with the results. When referring to partition coefficients, the ratio of concentration in oil to that in water will always be used.

RESULTS

Partitioning in Dodecane—Dodecane was chosen as a typical hydrocarbon solvent after it had been shown that the oil–water partition coefficients in *n*-octane, *n*-decane, *n*-dodecane, *n*-tetradecane, and *n*-hexadecane did not differ significantly. The choice of dodecane was advantageous since it is readily available in a high quality grade and since mutual miscibility with water is negligible (16). Partition coefficients were found to be independent of: (*a*) the final aqueous concentration of drug up to 10^{-3} *M*; (*b*) the concentration and type of buffer; (*c*) the ionic strength over a range of 0.06 to 0.35; (*d*) different specific counterions used to adjust ionic strength, *e.g.*, Cl^-, Br^-, NO_3^-, and various alkyl sulfonates.

In view of the lack of effect by any of these parameters, particularly ionic strength and counterions, it would appear that only the free base form partitions into dodecane. This agrees with the conclusions of Reese *et al.* (4) concerning CPZ partitioning into cyclohexane and the work of others with many drugs (17–19).

To evaluate this quantitatively, apparent partition coefficients were determined over a pH range of 2.0 to 7.6 (Table II). If the free base is the only form partitioning, then the apparent partition coefficient, P_a, can be expressed in terms of P_t, the true or intrinsic partition coefficient of the free base, and f_B, the fraction of drug present as the free base. Thus,

$$P_a = f_B P_t \qquad \text{(Eq. 1)}$$

[1] Supplied by Smith Kline & French Laboratories.

[2] Supplied by Squibb Co.

[3] Supplied by Wyeth Pharmaceutical Co.

[4] Matheson, Coleman and Bell.

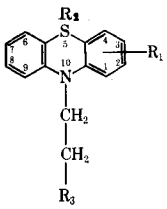


Table I—Chemical Structures of Phenothiazines

Drug	R_1	R_2	R_3	Wavelength of Maximum Absorbance,[a] mμ
Chlorpromazine	2-Cl	—	$-CH_2-CH_2-N(CH_3)_2$	257
Chlorpromazine sulfoxide (CPZ-O)	2-Cl	O	$-CH_2-CH_2-N(CH_3)_2$	239
Promazine (PZ)	H	—	$-CH_2-CH_2-N(CH_3)_2$	254
Triflupromazine (TPZ)	2-CF_3	—	$-CH_2-CH_2-N(CH_3)_2$	259
1-"Chlorpromazine"	1-Cl	—	$-CH_2-CH_2-N(CH_3)_2$	259
3-"Chlorpromazine"	3-Cl	—	$-CH_2-CH_2-N(CH_3)_2$	258
Ethyl "chlorpromazine"	2-Cl	—	$-CH_2-CH_2-N(CH_3)(CH_3)$	255
Butyl "chlorpromazine"	2-Cl	—	$-CH_2-CH_2-CH_2-N(CH_3)_2$	257
Trifluoperazine (TFP)	2-CF_3	—	$-CH_2-CH_2-N\langle\rangle N-CH_3$	260

[a] Solvent is 95% ethanol.

The value of f_B may be determined as

$$f_B = \frac{K_a}{K_a + [H_3O^+]} \qquad \text{(Eq. 2)}$$

where K_a is the dissociation constant of the conjugate acid of these drugs.

From Table II, it is apparent that the change in P_a with each unit of pH is about 10-fold, as predicted by combining Eqs. 1 and 2. Therefore, values of P_t were obtained from the slope of P_a *versus* f_B plots. Values for CPZ and a number of derivatives are listed in Table III, along with calculated values of P_a at pH 7.0. Since TFP has two dissociable groups, the fraction of free base was calculated by taking the two dissociation constants into account (20).

It should be pointed out that there is some uncertainty in the values of pKa for the phenothiazines, except for CPZ-O (Table III), since their significant water insolubility does not allow simple titration in aqueous solution. Thus, techniques such as influence of pH on solubility of the free base (3) and titration in mixed solvent systems (21) must be used. Although both methods agree fairly well, the very high partition coefficients of these compounds dictate that a small error in the choice of pKa will give a significantly different answer for P_t. For example, using a pKa of 9.3 for CPZ gives a P_t of 73,100; at 9.2, one obtains a value of 59,300.[5] Even small differences in pKa due to activity coefficient changes, therefore, will have an effect. For this reason, the authors chose to use values obtained by the same method (3, 23), thus ensuring more consistency when comparing relative hydrophobic properties of the various molecules (Table III). The value of 9.3 for CPZ was confirmed in the laboratory by solubility measurement, and the value for CPZ-O was determined by titration in water since its free base is sufficiently water soluble.

Table II—Effect of pH of the Aqueous Phase on the Apparent Partition Coefficient, P_a, of CPZ between Dodecane and Water at 30°

pH	P_a
2.0	0.003
3.6	0.120
4.0	0.320
5.0	3.80
5.6	13.80
6.6	137.0
7.6	1388

Partitioning in *n*-Octanol—In contrast to the dodecane studies, measurement of P_a in *n*-octanol was significantly influenced by the nature of the aqueous solution. For example, at pH 3.9 in a 0.1 *M* acetate buffer, the addition of 0.125 *M* KCl changed the P_a of CPZ from 4.6 to 32.4, while in 0.125 *M* KBr the value increased to 56.8. Altering the acetate buffer concentration up to 1.0 *M* increased the value to 23.3. These results and comparison of pH dependency with that seen in dodecane suggest that the free base has a higher intrinsic partition coefficient in *n*-octanol and that the ionic species is partitioning as an ion-pair. This can be seen in Table IV; the values of 32.4 and 32.8 mainly represent ionic species partitioning, while values at pH 6.6 and higher represent the free base contribution primarily. Exact analysis of pH data to obtain P_t values is not possible since each value is dependent on the buffer, its concentration, and the concentration of KCl used to adjust ionic strength. It may be assumed, however, that apparent partition coefficients obtained at pH values in excess of 6.6 are essentially due to the free base. Utilizing the value of P_a at pH 6.6 and a pKa of 9.3, a P_t for CPZ of about 250,000 was obtained. This is in good agreement with a value of 220,000 reported by Hansch (24), utilizing a P_a obtained at pH 6.8. Unfortunately, large amounts of drug were needed at such high pH values, and a limited supply of other derivatives did not allow the determination of the exact P_t values in *n*-octanol.

The effect of various electrolytes on the apparent partition coefficient of CPZ at pH 3.9 is presented in Table V by comparing the effect of 0.125 *M* concentrations of electrolyte, all other factors being equal. It may be concluded that inorganic counterions have a significant effect, whereas no differences are noted between the inorganic cations. It is interesting to note a reduced P_a for the methane and ethane sulfonates compared to Cl^- and this will be discussed later. Note also the significant reduction in partitioning when the tetraalkylammonium chlorides are used: the longer the alkyl chain length, the lower the value of P_a. In all cases, except for the tetraalkylammonium ions, increasing the electrolyte concentration increased partitioning; the opposite effect occurred with increasing organic cation concentration. This latter observation suggested a "water-structure" effect, so urea was added to a solution containing KCl. As seen in Table V, urea also decreases the value of P_a significantly.

The effect of counterions on the value of P_a can be measured quantitatively by determining an extraction constant, E_{X^-}, for each anion, X^-. Such a constant may be written for a 1:1 ion-pair

[5] Such large partition coefficients are usually expressed as logarithms (22); in this form, these values are 4.86 and 4.77, respectively.

Table III—Partition Coefficient for Various Phenothiazine Derivatives between Dodecane and Water at 30°

Compound	pKa	P_t	P_a at pH 7.0
CPZ	9.3	73,100	366
CPZ-O	9.0	0.75	0.0075
PZ	9.4	10,400	42
TPZ	9.2	137,000	863
1-CPZ	9.4	61,200	245
3-CPZ	9.2	46,300	292
Ethyl-CPZ	8.7	28,200	553
Butyl-CPZ	9.7	116,000	232
TFP	3.9, 8.1	12,900	97

as follows:

$$E_{X^-} = \frac{(CPZH^+X^-)_{org.}}{(CPZH^+)_{aq.}(X^-)_{aq.}} \quad \text{(Eq. 3)}$$

If one assumes the ion-pair, $CPZH^+X^-$, forms only in the organic phase and, therefore,

$$P_a = \frac{(CPZH^+X^-)_{org.}}{(CPZH^+)_{aq.}} \quad \text{(Eq. 4)}$$

then:

$$P_a = E_{X^-}(X^-)_{aq.} \quad \text{(Eq. 5)}$$

Equations can be written for the possible equilibria involving ion-pairing which occur with divalent anions ($SO_4^=$) or when two positive charges exist on TFP. However, since these constants are dependent on higher powers of drug or anion concentration, no meaningful comparisons independent of drug concentration are possible. Thus, no extraction constants are given for sulfate-ion extraction of CPZ or for chloride-ion extraction of TFP. Figures 1 and 2 show typical plots for 1:1 ion-pair partitioning. Tables VI and VII contain E_{X^-} values obtained from the slopes of these plots as predicted by Eq. 5. This equation predicts a zero intercept. However, in all cases, intercepts due to the acetate ion of the buffer were present. In all cases of 1:1 ion-pairing, no drug concentration dependence was observed for the E_{X^-} values obtained.

DISCUSSION

Partitioning of the Free Base—As was suspected from the earlier evidence of hydrophobic behavior, the intrinsic and apparent partition coefficients of the phenothiazine derivatives in dodecane and *n*-octanol are many orders of magnitude greater than those usually observed with acidic and basic drugs (17–19). The high intrinsic partition coefficient of the free base form demonstrates the need to consider its role when one assesses the effects of these drugs *in vitro* and *in vivo*, even 3–4 pH units below the apparent pKa. Because of these very high partition coefficients, it is important also to recognize the remarkable changes in hydrophobicity that can occur with very small changes in pH. This appears especially true in the pH range of 6–8 where so many *in vitro* and *in vivo* processes are studied. Likewise, at interfaces many steric and electrical factors can produce local pH changes which will alter partitioning without apparently influencing the bulk solution. This suggests, therefore, that any model system, or even a biological system such as a membrane, can produce significant selectivity with these molecules merely on the basis of free base partitioning.

Partitioning of the Ion-Pair—In view of the marked hydrophobic behavior of these molecules in very nonpolar hydrocarbon solvents as the free base, it is not surprising that the ionic species will partition when it can be effectively neutralized by an appropriate anion and solvated by a polar oil phase such as *n*-octanol. The need for solvation of the ion-pair in the oil phase has been studied by Higuchi *et al.* (25), utilizing pharmaceutical amines and mixtures of cyclohexane with chloroform, pentanol, or *p-tert*-butyl phenol. In each case, the proton-donating tendencies of the polar solvent were essential for partitioning of the ion-pair. Enhanced partitioning of tetracycline into cyclohexane when *n*-octanol is added presumably is due also to such a mechanism (26).

Table IV—Effect of pH on CPZ Partitioning in *n*-Octanol

pH	P_a
3.9	32.4
4.2	32.8
5.2	64.0
5.9	135.9
6.6	613.4

Table V—Apparent Partition Coefficient of CPZ into *n*-Octanol in the Presence of Various Salts at 0.125 *M* Concentration

Salt	P_a
Sodium chloride	32.3
Potassium chloride	32.4
Potassium bromide	56.8
Potassium nitrate	50.0
Sodium methane sulfonate	14.8
Sodium ethane sulfonate	25.1
Sodium propane sulfonate	54.5
Ammonium chloride	31.0
Tetramethylammonium chloride	26.1
Tetraethylammonium chloride	18.8
Tetrapropylammonium chloride	16.1
Potassium chloride + 2.0 *M* urea	12.8
Sulfate	15.0

What is most significant in the present study is the very large partition coefficient obtained for these drugs in the presence of very common inorganic ions at commonly encountered ionic strengths (Tables V and VII). These values are much greater than those found in *n*-octanol for most acids and bases in nonionized form (18), zwitterionic form (27), and as ion-pairs (23, 28). The effects of specific ions such as Cl^-, Br^-, NO_3^-, and $SO_4^=$ appear to follow an order expected for counterion binding to amines: the greater the polarizability and hydrophobicity of the anion, the greater this effect. The relatively small value of P_a for the sulfate ion appears to reflect the difficulty of ion-pairing when two CPZ molecules must interact with each sulfate ion to neutralize completely the partitioning species. When this is compared to other amine systems that show no tendency to partition in the presence of sulfate ion (29), the authors again conclude that the phenothiazines indeed are quite hydrophobic.

Comparison of extraction constants for the organic anions, such as acetate, and the alkylsulfonates reveals a significant reduction relative to that due to chloride ion. Likewise, whereas sodium, potassium, and ammonium ion exhibit no influence on partitioning, the tetraalkylammonium ions markedly reduce partitioning. These results may be interpreted by considering the various thermodynamic factors associated with partitioning of hydrophobic ions. In the oil phase, factors tending to promote partitioning will be the

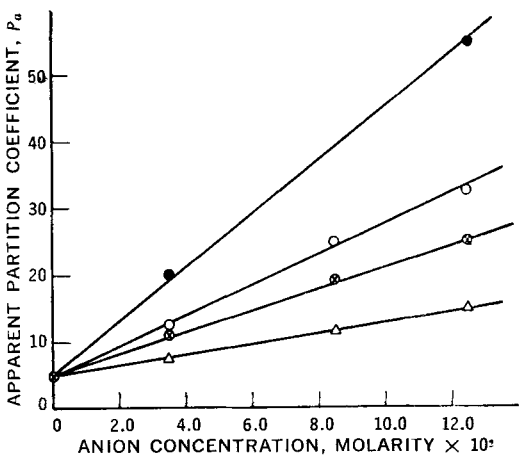


Figure 1—*Apparent partition coefficients for CPZ between* n*-octanol and aqueous buffers, pH 3.9, in the presence of various anions at 30°. Key: ○, chloride; ●, propane sulfonate; ⊗, ethane sulfonate; and △, methane sulfonate.*

Table VI—Extraction Constants for Various Anions with CPZ in *n*-Octanol

Anion	E_{X^-}
Chloride	197
Bromide	383
Nitrate	362
Acetate	213
Methane sulfonate	68
Ethane sulfonate	163
Propane sulfonate	374

electrostatic attraction of the ions (their size, shape, and charge) and the interaction of the ion-pair with the solvent, as already discussed. In the aqueous phase, the hydrophobic drug produces an increase in water structure in its vicinity and hence a decrease in water entropy, the process of transfer, therefore, being favored because of the resultant increase in water entropy after transfer. In the presence of tetraalkylammonium ions, however, partitioning of drug is reduced because a major proportion of water structuring now occurs around the added alkyl groups, reducing the entropy increase ordinarily produced by transfer.[6] This process, a form of salting in, has been noted for micelle formation (30) and surface tension reduction by CPZ (31), and has been discussed theoretically by Diamond (32). It also is presumed that the retarding effect of urea on partitioning into *n*-octanol and dodecane[7] is caused by water-structure effects.

The results obtained with acetate ion and the alkylsulfonates suggest a reduced tendency to partition when compared with chloride ion, which agrees with earlier observations that these substances reduce the surface activity of CPZ (31). It is possible that solvation of the ion-pair in *n*-octanol is not complete, but another factor must be operating since similar effects are seen at the air–solution interface. Again, water-structuring effects should be examined. One possibility is that acetate and the alkylsulfonates work like the tetraalkylammonium ions (32). Another possibility is the formation of an ion-pair in water with a reduced tendency to partition into oil. Diamond (32) has described the tendency of large hydrophobic ions to combine in water as "water-structure-enforced" ion-pairs, which lower the overall free energy of the aqueous phase without the necessity to expel the ion-pair. Whatever these

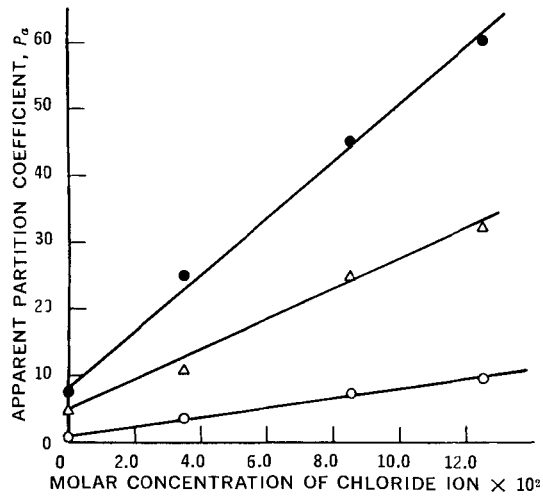


Figure 2—*Apparent partition coefficients for three phenothiazine derivatives between* n-*octanol and aqueous buffer, pH 3.9, in the presence of chloride ion at 30°. Key:* ○, *promazine;* △, *chlorpromazine; and* ●, *triflupromazine.*

[6] The possibility that the tetraalkylammonium ions could partition into octanol, and thus reduce the chloride ion available for CPZ partitioning, exists. However, no detectable partitioning of the tetramethyl- and tetraethylchlorides could be measured, while less than 1% of the tetrapropyl derivative appears to partition.

[7] The value of P_a for CPZ into dodecane at pH 4.0 is reduced in the presence of 2.0 *M* urea from 0.32 to 0.17.

Table VII—Partitioning of Various Derivatives in *n*-Octanol in Presence of KCl

Drug	E_{X^-}	P_a (0.125 *M* KCl)
CPZ	197	32.4
TPZ	384	60.0
PZ	58	8.1
CPZ-O	1.5	0.22
1-CPZ	114	17.0
3-CPZ	430	61.3
TFP	—	49.3

mechanisms are, it is clear that the nature of the aqueous phase must be considered significant in controlling the value of P_a. Furthermore, in the choice of buffers and electrolytes in any study with these drugs, one should keep these phenomena in mind.

Chemical Modification—The influence of chemical modification on the partitioning behavior of the phenothiazine derivatives would, of course, be of great interest when attempting to compare structure to biological activity. The approach used by Hansch *et al.* (22) to correlate *n*-octanol–water partition coefficients with such activity is well known. But, as stated earlier, no attempt was made here to determine values in *n*-octanol because of: (*a*) the limited supply of enough phenothiazines with appropriate substituent group variation, (*b*) the effects of ion-pairing due to buffers and salts present, (*c*) the uncertainty of exact pKa values, and (*d*) the necessity to work at pH values very much lower than the pKa of each drug because of very high intrinsic partition coefficients. However, a number of conclusions should be pointed out concerning structural effects which should be helpful in evaluating phenothiazine structure–activity data obtained in various *in vitro* and *in vivo* studies.

From the data presented in Tables III and VII, it is apparent that comparisons made in dodecane represent the behavior of the free base, while those made in *n*-octanol at low pH represent the ionic species. In both systems the hydrophobic properties of the drug are the major factors contributing to differences between molecules but, in addition, factors controlled by the nature of the ion-pairing mechanism are important in the case of partitioning into octanol. Thus, for example, the P_t values for the 1-, 2-, and 3-Cl derivatives of CPZ in dodecane are close and appear to show a maximum with the 2-Cl compound, whereas the order of extraction constants increases significantly from the 1- to 3-Cl position. The authors suggest that, in the latter case, steric and electronic effects on the ion-binding process are being seen in addition to hydrophobic behavior which occurs in the dodecane system. This latter order of behavior is seen also for adsorption of the ionic species of these derivatives to the air–solution interface (33). Following this reasoning helps to explain the reasonably consistent agreement between the two solvent systems when comparing TPZ and CPZ (about a twofold difference), since now all effects due to position on the ring should be the same. The somewhat poorer quantitative agreement when comparing the relative values of PZ may reflect an additional effect due to differences in size between the hydrogen atom and the other substituents.

Looking at some specific structural effects in both solvents, it is clear that substitution on the phenothiazine ring exerts a significant effect. The marked reduction in hydrophobicity when going from CPZ to CPZ-O clearly indicates why CPZ-O exhibits very little of the physical, chemical, and biological properties of CPZ (1, 9). A relatively large increase in hydrophobicity is also seen when Cl and CF_3 are placed on the aromatic ring, in good agreement with the results of biochemical and pharmacological structure–activity relationships (1, 2). From the results with the ethyl and butyl CPZ analogs and TFP, it is apparent also that the nature of the alkylamino portion is important: the greater the number of alkyl groups, the greater the partitioning tendency. Note, however, in Table III that substitution of this portion of the molecule has the greatest effect on pKa changes. Hence, comparisons near pH 7.0, for example, should be made with great caution so as not to attribute effects to the wrong species.

Finally, the behavior of TFP is interesting because its partitioning is not as great as expected, *i.e.*, the presence of a CF_3 group and more $-CH_2-$ groups than TPZ. The explanation in *n*-octanol centers on the presence of a second dissociated group for a sig-

nificant number of molecules at pH 3.9, which should increase polarity and reduce the ease of ion-pair formation. The presence of the polar nitrogen group also appears to reduce the partitioning tendency of TFP in dodecane, which agrees with the reported reduced water solubility of free base when going from CPZ to TFP (3). It also helps to explain the significantly lower accumulation of TFP relative to CPZ in rat intestinal membrane during drug absorption at pH 6.0 (13).

CONCLUSIONS

Based upon the results of this study, it is clear that studies designed to evaluate the behavior of phenothiazines *in vitro* and *in vivo* must consider the extreme hydrophobicity of these molecules. This study has demonstrated also that great variation in hydrophobicity exists between closely related compounds, as well as between different ion-pairs of the same compound. In biological systems, *in vitro* and *in vivo*, any number of membrane phases and anionic species may exist. Therefore, structure–activity studies must take into account the type of phenomena discussed in this paper when dealing with hydrophobic molecules such as the substituted phenothiazines.

REFERENCES

(1) E. F. Domino, R. A. Hudson, and G. Zografi, "Drugs Affecting the Central Nervous System," vol. 2, A. Burger, Ed., Marcel Dekker, New York, N. Y., 1968, p. 327.

(2) A. T. Florence, *Advan. Colloid Interface Sci.*, **2,** 115(1968).

(3) A. L. Green, *J. Pharm. Pharmacol.*, **19,** 10(1966).

(4) D. R. Reese, G. M. Irwin, L. W. Dittert, C. W. Chong, and J. V. Swintosky, *J. Pharm. Sci.*, **53,** 591(1964).

(5) P. M. Seeman and H. S. Bialy, *Biochem. Pharmacol.*, **12,** 1181(1963).

(6) G. Zografi, D. E. Auslander, and P. L. Lytell, *J. Pharm. Sci.*, **53,** 573(1964).

(7) G. Zografi and I. Zarenda, *Biochem. Pharmacol.*, **15,** 591 (1966).

(8) P. S. Guth and M. A. Spirtes, *Int. Rev. Neurobiol.*, **7,** 231 (1964).

(9) G. Zografi and D. E. Auslander, *J. Pharm. Sci.*, **54,** 1313 (1965).

(10) L. L. M. van Deenen and R. A. Demel, *Biochim. Biophys. Acta*, **94,** 314(1965).

(11) W. O. Kwant and P. Seeman, *ibid.*, **183,** 530(1969).

(12) J. R. Gillette, *Proc. Int. Pharmacol. Meet., 2nd, 1963*, **4,** 9(1965).

(13) J. T. Doluisio, W. G. Crouthamel, G. H. Tan, J. V. Swintosky, and L. W. Dittert, *J. Pharm. Sci.*, **59,** 72(1970).

(14) R. J. Warren, I. B. Eisdorfer, W. E. Thompson, and J. E. Zarembo, *ibid.*, **55,** 144(1966).

(15) W. Scholtan and R. Gonnert, *Med. Chem.*, **5,** 314(1956).

(16) S. G. Frank and G. Zografi, *J. Colloid Interface Sci.*, **29,** 27(1969).

(17) C. A. M. Hogben, D. J. Tocco, B. B. Brodie, and L. S. Schanker, *J. Pharmacol. Exp. Ther.*, **125,** 275(1959).

(18) K. Kakemi, T. Arita, R. Hori, and R. Konishi, *Chem. Pharm. Bull.*, **15,** 1705(1967).

(19) T. B. Vree, A. T. J. M. Muskens, and J. M. van Rossum, *J. Pharm. Pharmacol.*, **21,** 774(1969).

(20) A. N. Martin, J. Swarbrick, and A. Cammarata, "Physical Pharmacy," 2nd ed., Lea & Febiger, Philadelphia, Pa., 1969, p. 202.

(21) L. G. Chatten and L. E. Harris, *Anal. Chem.*, **34,** 1495(1962).

(22) C. Hansch, in "Annual Reports in Medicinal Chemistry," Academic, New York, N. Y., 1967, p. 348.

(23) A. L. Green, *J. Pharm. Pharmacol.*, **19,** 208(1967).

(24) C. Hansch, private communication.

(25) T. Higuchi, A. Michaelis, T. Tan, and A. Hurwitz, *Anal. Chem.*, **39,** 974(1967).

(26) J. T. Dolusio and J. V. Swintosky, *J. Pharm. Sci.*, **53,** 597 (1964).

(27) J. L. Colaizzi and P. R. Klink, *ibid.*, **58,** 1184(1969).

(28) G. M. Irwin, H. B. Kostenbauder, L. W. Dittert, R. Staples, A. Misher, and J. V. Swintosky, *ibid.*, **58,** 301(1969).

(29) G. Schill, R. Modin, and B. Arne Persson, *Acta Pharm. Suecica*, **2,** 119(1965).

(30) J. Steigman, I. Cohen, and F. Spingola, *J. Colloid Sci.*, **20,** 732(1965).

(31) R. M. Patel and G. Zografi, *J. Pharm. Sci.*, **55,** 1345(1966).

(32) R. M. Diamond, *J. Phys. Chem.*, **67,** 2513(1963).

(33) G. Zografi and M. V. Munshi, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 2, 1970, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication April 3, 1970.

This study was supported by Grant NB07134 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Md.

* Present address: Parke-Davis and Co., Detroit, MI 48232

The authors acknowledge the technical assistance of Mr. Nicholas Hotra.

Diffuse Reflectance Studies of Solid–Solid Interactions IV: Interaction of Bishydroxycoumarin, Furosemide, and Other Medicinal Agents with Various Adjuvants

J. DAVID McCALLISTER, TING-FONG CHIN, and JOHN L. LACH

Abstract □ Spectral information comparing the diffuse reflectance of drug–metallic-ion adjuvants with those of the isolated metallic chelates is presented. Various metallic-ion chelates of bishydroxycoumarin and furosemide were prepared and isolated, and their diffuse reflectance spectroscopy spectra were found to be comparable to those of drug–adjuvant equilibrated systems. This information, along with the color changes observed in both the chelate and the interaction product, and the new peaks observed in the UV and visible regions of the spectra suggest that these drug interactions due to chemisorption may proceed by a mechanism similar to that of chelation.

Keyphrases □ Solid–solid interactions—diffuse reflectance spectroscopy □ Drug–metal-ion adjuvants, drug–metal chelates—diffuse reflectance comparison □ Spectral changes, drugs—metal-ion adjuvants □ Diffuse reflectance spectroscopy—analysis □ IR spectrophotometry—identity

A number of articles have recently appeared in the literature which point out the existence of drug–excipient interactions in the solid state (1–5). Although drug–excipient interactions in pharmaceutical dosage forms have been recognized, very little information exists concerning the nature and degree of these physical and chemical surface interactions. Such solid–solid interactions may account, in part, for the discrepancies observed in drug availability of similar dosage forms.

This report represents a part of the continuing study of drug–excipient interaction and deals with mechanistic aspects of these drug–metallic-ion adjuvant interactions.

EXPERIMENTAL

Reagents—The following were used: dioxane-recrystallized bishydroxycoumarin, m.p. 288–289°;[1] alcohol-recrystallized furosemide, m.p. 215° dec.;[2] digoxin, m.p. 266° dec.;[3] indomethacin, m.p. 158°;[4] ergonovine maleate, m.p. 166° dec.;[3] chloramphenicol, m.p. 151°;[5] potassium bromide, spectroscopic grade; activated (basic) alumina (Woelm); magnesium oxide; magnesium trisilicate; calcium sulfate; magnesium carbonate; zinc stearate; zinc oxide; acacia powder; magnesium sulfate, A.R.; ferrous chloride, A.R.; ferrous sulfate, A.R.; aluminum chloride, A.R.; zinc sulfate, A.R.; talc; methanol, A.R.; ethanol USP; and 0.1 *N* NaOH.

Apparatus—The following were used: Beckman DU spectrophotometer with a diffuse reflectance attachment; constant-temperature water bath set at 30 ± 0.5° with rotating spindles; 150-ml. vials with caps sealed with Parafilm;[6] Frease-Precision Scientific vacuum oven; Beckman IR-5A infrared spectrophotometer; Pasadena Hydraulic bench press; and a capillary melting-point apparatus.[7]

[1] K and K Laboratories.
[2] Hoechst Pharmaceuticals.
[3] Burroughs Wellcome & Co.
[4] Merck Sharp and Dohme.
[5] Parke-Davis and Co.
[6] Marathon Co.
[7] Arthur H. Thomas Co.

PROCEDURES

Preparation of Sample—An exact amount of the drug (50 mg. of bishydroxycoumarin, 50 mg. of furosemide, 10 and 20 mg. of digoxin, 10 mg. of ergonovine maleate, and 30 mg. of indomethacin and chloramphenicol) is weighed for every 2 g. of adjuvant weighed. The powders are placed in 150-ml. vials, and 25 ml. of water is added as the dispersion medium. The vial is covered with Parafilm and capped. The sample is equilibrated for 8 hr. at 30 ± 0.5° to effect interaction. After equilibration, the sample is dried under vacuum at 40°. The percent reflectance of this sample is measured, using magnesium carbonate as the reference material.

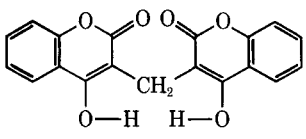
Preparation of Control—The same amounts of drug and of adjuvant are weighed as in the sample preparation and are physically mixed using a mortar and pestle. The percent reflectance of this control is measured, using magnesium carbonate as the reference material.

Preparation of Chelates—Approximately 3 g. of the drug (bishydroxycoumarin or furosemide) is accurately weighed and dissolved, using an equivalent amount of 1.0 *N* NaOH and the volume of the solution adjusted to 50 ml. with distilled water. A molar equivalent quantity of the metal-ion solution is added dropwise to the solution containing the drug while stirring continuously. The addition is continued until the pH drops to approximately 6; then the solution is filtered under vacuum and the insoluble complex is washed well with water to remove impurities and dried in a vacuum oven.

RESULTS AND DISCUSSION

Although no information in the literature describes metallic chelates of bishydroxycoumarin, previous diffuse reflectance spectroscopy (DRS) studies (6) in these laboratories dealing with bishydroxycoumarin–excipient interactions strongly suggested that the mechanism involved could be one of chemisorption due to surface chelation.

An examination of the structure of bishydroxycoumarin (Structure I) suggests that this compound could undergo chelate formation.



I

Attempts to study solution chelate formation of bishydroxycoumarin with metallic ions, based on the DRS information, by the solubility and continuous variation method were unsuccessful.

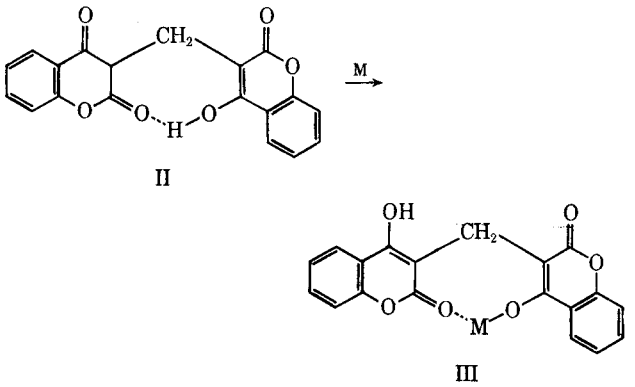
The use of potentiometric titrations, although not totally satisfactory since no significant pH changes were observed, did, however, give evidence of complexation, because visual observation of a precipitate was noted when sodium hydroxide titrant was added to a bishydroxycoumarin solution containing the metal ion under investigation. Since metal hydroxides were also formed in this method, the procedure was modified.

Bishydroxycoumarin was first solubilized by the addition of an equivalent amount of alkali and titrated with an aqueous solution of the metallic ion under investigation, resulting in the formation of highly colored precipitates and suggesting that chelate formation had occurred (Table I).

Table I—Bishydroxycoumarin–Metallic-Ion Chelates

Compound	Color
Bishydroxycoumarin	White
Mg(II) chelate	Light-yellow
Zn(II) chelate	Light-yellow
Fe(II) chelate	Gray
Fe(III) chelate	Brownish-red
Al(III) chelate	Light-yellow

The examination of the IR spectra of these isolated metallic chelates and of the pure bishydroxycoumarin indicated that apparently the accepted conformation (Structure I) of the drug is in disagreement with the present findings. The IR spectrum obtained for bishydroxycoumarin (Fig. 1) does not reveal a free O—H stretching band in the region of 3 μ, as would be expected. Although the spectral data are in agreement with the reported IR spectrum by Hayden *et al.* (7), these authors also fail to account for this in their listed structure. It is interesting to note that free O—H stretching in the 3-μ region is only evident in the chelate, for example, as seen in the zinc chelate (Fig. 2). This suggests that one hydroxyl group of bishydroxycoumarin, in the pure state, is strongly hydrogen bonded to the keto oxygen and that the hydroxyl group on the other coumarin ring would exist primarily in the keto form, which would account for the absence of free O—H stretching in this region. Data based on the IR spectra of the various isolated bishydroxycoumarin chelates indicate that the conformation of this drug and its chelates could be as shown in Scheme I.



Scheme I

Additional evidence supporting this line of reasoning was obtained using Dreiding models. The isolated metallic chelates were very insoluble in all solvent systems tested and, consequently, difficult to purify and characterize; they were highly colored, as listed in Table I.

The adjuvants used in the study of the solid–solid interactions with bishydroxycoumarin were chosen because of their importance in pharmaceutical formulations. In view of the fact that most of the adjuvants used exhibited reflectance values in excess of 100% throughout the wavelengths employed and, therefore, would not contribute to the absorption spectrum of the control or the equilibrated sample, their spectra are not shown.

Figure 3 represents the DRS spectrum of the interaction of magnesium oxide with bishydroxycoumarin. An examination of this figure does indicate a significant difference in the spectrum of the control and that of the equilibrated sample, as evidenced by the

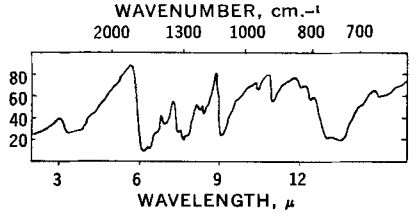


Figure 1—*IR spectrum of bishydroxycoumarin (KBr pellet).*

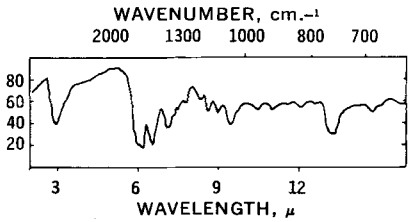


Figure 2—*IR spectrum of the zinc–bishydroxycoumarin chelate (KBr pellet).*

intensified absorption bands in the UV region. There also appear to be two new absorption peaks generated, one between 360 and 390 mμ and the other at approximately 450 mμ.

This band formation in the UV region may be explained as an interaction of the magnesium oxide with bishydroxycoumarin where the adsorption facilitates the clarification of an already existing peak in this region, or it may represent the actual reflectance spectrum of a film of the drug adsorbed onto the surface of the magnesium oxide adsorbent. This is suggested in Fig. 4 where the transmittance curve of bishydroxycoumarin in basic medium shows that the $\lambda_{max.}$ at approximately 320 mμ lies in the same region as that obtained in the DRS spectrum. The appearance of two new bands at approximately 380 and 450 mμ in the visible region of the DRS spectrum in the equilibrated sample and their absence in the transmittance spectrum indicate the presence of a strong chemical surface interaction between bishydroxycoumarin and the magnesium oxide adsorbent. The high degree of interaction is further substantiated by the large intensity change and the significant bathochromic shift observed in the equilibrated sample as compared to that of the control. A comparison of this interaction DRS spectrum to that of the prepared and isolated magnesium chelate shows some interesting similarities. For example, the chelate also shows a new peak at 450 mμ. Both spectra show a hyperchromic effect throughout the UV region. The equilibrated magnesium oxide sample is light yellow in color, quite similar to the isolated magnesium chelate; in addition to the other similarities noted, this suggests that the mechanism involved in these interactions may be similar.

Other magnesium-ion metallic adjuvant–bishydroxycoumarin interactions, although not shown here, are somewhat similar to that of the magnesium oxide systems. In the magnesium carbonate system, there is a hyperchromic effect in the UV region and the possibility of new band formation, broad and distinct, between 375 and 390 mμ, indicative of strong interaction.

There is also a bathochromic shift of 80 mμ between 340 and 420 mμ in the equilibrated *versus* the control spectrum. The interaction of magnesium stearate with bishydroxycoumarin suggests a peak between 375 and 390 mμ, although it is indistinct and quite weak. A bathochromic shift of 40 mμ is observed between 360 and 400 mμ. The new band formation and bathochromic shifts are indicative of strong chemical interactions, and a mechanism similar to that of chelate formation seems reasonable for these interactions.

An examination of the spectrum of the zinc stearate interaction with bishydroxycoumarin (Fig. 5) reveals a slight hyperchromic effect in the UV region with this adjuvant, along with a $\lambda_{max.}$ at approximately 300 mμ.

No new peak formation was observed in the visible region as in the magnesium-containing systems. The DRS spectrum of the isolated zinc–bishydroxycoumarin chelate, however, does show

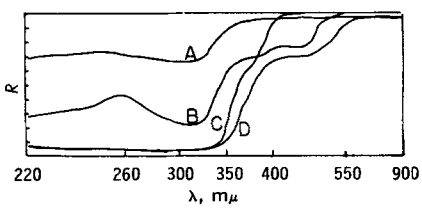


Figure 3—*DRS of bishydroxycoumarin (50 mg.) and magnesium oxide (2.00 g.). Key: A, control (physical mixture); B, equilibrated sample; C, bishydroxycoumarin, 100%; and D, magnesium–bishydroxycoumarin chelate, 100%.*

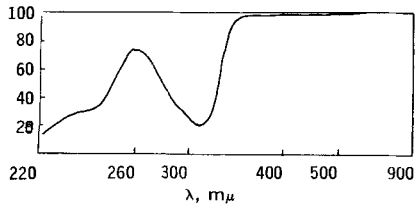


Figure 4—*Transmittance spectra of bishydroxycoumarin (10 mcg./ml.) in 0.1 N NaOH solution.*

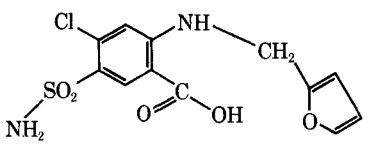
this new band at 450 mμ and a significant bathochromic shift as compared to the spectrum of the equilibrated sample. The absence of new peak generation in the visible region for the zinc stearate–bishydroxycoumarin system as compared to that of the isolated chelate indicates that the zinc in the zinc stearate molecule is less readily available for surface interactions. However, as with the magnesium-containing adjuvant, the degree of surface interactions will vary with other zinc-containing excipients.

Spectral data obtained for the alumina–bishydroxycoumarin interactions were quite similar to those seen with zinc stearate. Again, new peak generation at 450 mμ was observed only in the prepared chelate. It is possible that zinc stearate and alumina have very highly saturated surfaces and, thus, few active sites available for chemisorption.

Although the iron (II) and iron (III) bishydroxycoumarin chelates were prepared, a comparison of their DRS data to that of the equilibrated samples was difficult because of the highly adsorbing nature of the interaction products.

DRS of Furosemide Interactions and Comparison of Isolated Chelates—The interest in studying this drug was due in part to the color changes observed in certain tablet formulations and to the reported discrepancies in absorption when the drug was formulated with certain excipients.[8]

Furosemide (4-chloro-5-sulfamoyl-*N*-furfuryl anthranilic acid), an effective diuretic-saluretic agent (8–10), is similar chemically to the thiazide diuretics. Furosemide is a weak acid with a pKa of approximately 4.7. It forms salts easily with bases such as sodium hydroxide and has the structure shown in Structure IV.



IV

The large spectral and color changes observed in the diffuse reflectance spectrum of the furosemide–metallic-ion adjuvant interactions, as in the bishydroxycoumarin system, again suggest that this compound could undergo chelate formation in aqueous solution, although such chelates have not been previously reported.

Using the previously discussed modified method, the alkali-solubilized furosemide was titrated with a solution of the metallic ion under investigation. The highly colored isolated precipitate did indicate that interaction had occurred (Table II). An examination of the molecular structure using Dreiding models substantiates

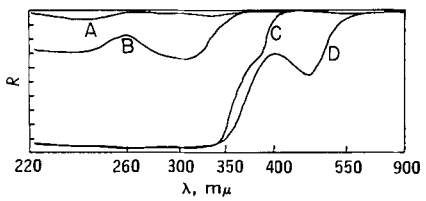


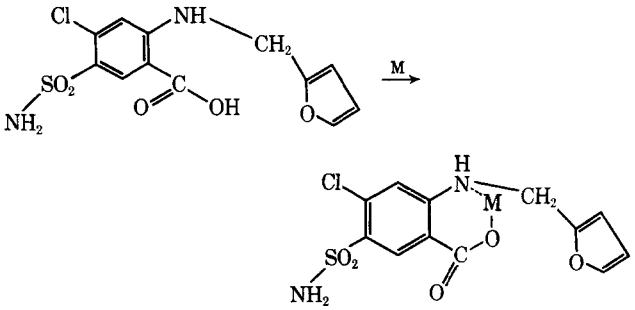
Figure 5—*DRS of bishydroxycoumarin (50 mg.) and zinc stearate (2.00 g.). Key: A, control (physical mixture); B, equilibrated sample; C, bishydroxycoumarin, 100%; and D, zinc–bishydroxycoumarin chelate, 100%.*

[8] Private communication.

Table II—Furosemide and Selected Chelates

Compound	Color	Melting Point
Furosemide	Off-white	215°
Mg(II) chelate	Light-yellow	300°
Zn(II) chelate	Yellow-brown	>360°
Fe(II) chelate	Light-cocoa	200°
Fe(III) chelate	Red-brown	>360°
Al(III) chelate	Yellow-gray	>360°

this line of reasoning, as illustrated in Scheme II.



Scheme II

As in the bishydroxycoumarin system, purification of the isolated chelates was extremely difficult due to their highly insoluble nature. Attempts to characterize these isolated chelates after repeated methanol washings in which the drug was relatively soluble were inconclusive because contamination due to the presence of metallic hydroxides was a problem.

Analysis for furosemide and metallic ion contained in the chelates by spectrophotometric, chelometric, and atomic absorption techniques generally indicated a 1:1 ratio, although a 2:1 ratio for the iron (III) system was suggested. Since the stoichiometric relationship is dependent on an exact metallic-ion determination and since trace amounts of metallic hydroxide contamination can result in a large change in this ratio, further purification studies are in progress to determine this ratio and to characterize the complexes. Although it is recognized that these isolated and methanol-washed chelates are not completely pure, results of an elemental analysis assuming a 1:1 ratio are listed in Table III.

In spite of the difficulties encountered in the purification and stoichiometric determination of these metallic chelates, it is of interest to note that this drug does undergo chelate formation in solution and this is reported here for the first time.

With respect to these solid–solid surface interactions, Fig. 6 represents the DRS of the furosemide–magnesium oxide interaction. Again, as seen in the bishydroxycoumarin–magnesium oxide interaction, equilibration of the furosemide with magnesium oxide results in the clarification of strong absorption bands at 230, 275, and 335 mμ; the bands are comparable to those seen in Fig. 7 which represents the transmittance spectrum of furosemide in methanol.

The clarification of absorption bands after the equilibration procedure indicates that a significant interaction has occurred. Comparable absorption peaks can be seen in the DRS spectrum of the isolated furosemide–magnesium chelate, although of signifi-

Table III—Percent of Various Elements Present in Furosemide Chelates Based on a Probable Stoichiometry of 1:1

Chelate	% Carbon		% Hydrogen		% Nitrogen	
	Theoretical	Actual	Theoretical	Actual	Theoretical	Actual
Mg(II)	40.6	39.53	2.84	3.1	7.92	7.27
Zn(II)	36.4	37.31	2.55	2.91	7.1	7.1
Fe(II)	37.36	41.69	2.61	3.2	7.26	8.03
Fe(III)	37.36	35.90	2.61	2.78	7.26	6.83
Al(III)	40.4	38.39	2.82	2.99	7.85	7.56

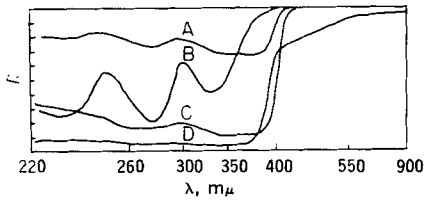


Figure 6—*DRS of furosemide (50 mg.) and magnesium oxide (2.00 g.). Key: A, control (physical mixture); B, equilibrated sample; C, furosemide, 100%; and D, magnesium–furosemide chelate, 100%.*

cantly less clarity, since high absorption results due to use of the pure chelate.

The hypochromic shift noted in the furosemide–magnesium oxide interaction to that of the pure drug and for the isolated chelate is also indicative of a strong surface interaction. This is in contrast to the bathochromic shift observed in the bishydroxycoumarin–magnesium oxide system. The spectral changes observed for furosemide–magnesium trisilicate, magnesium stearate, zinc stearate, and alumina and their isolated chelates are comparable to that of the magnesium oxide system and will not be discussed.

The importance of the strong surface interactions pointed out here cannot be overemphasized with respect to their effect on the therapeutic availability of furosemide. It is possible that these interactions could occur during the wet granulation process of tableting and in the compression of the tablet. The fact that these interactions may occur with different classes of compounds as well as with various adjuvants used in pharmaceutical dosage forms should be recognized, since they may contribute to discrepancies in blood levels and therapeutic efficacy of pharmaceutical dosage forms.

DRS of Interactions of Indomethacin, Digoxin, Chloramphenicol, or Ergonovine Maleate with Excipient—Since these drugs are in widespread use, it was felt that information concerning possible drug–excipient interactions would be of interest. Indomethacin, a recently introduced anti-inflammatory agent, exhibited a strong surface interaction with magnesium oxide and magnesium trisilicate. Figure 8, which illustrates the interaction of indomethacin with magnesium oxide, represents the type of interaction involved. Alumina and acacia exhibited slight interaction with indomethacin, since the DRS spectra of the control and equilibrated sample were comparable.

Due to space limitations, the DRS spectra of the interaction of chloramphenicol, ergonovine maleate, and digoxin with magnesium oxide and magnesium trisilicate are not given; however, a significant interaction was observed in these systems.

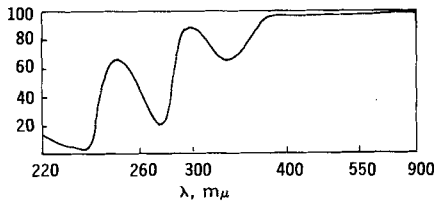


Figure 7—*Transmittance spectrum of furosemide (20 mcg./ml.) in methanol.*

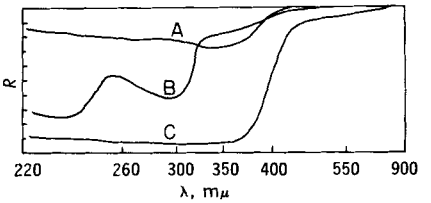


Figure 8—*DRS of indomethacin (30 mg.) and magnesium oxide (2.00 g.). Key: A, control (physical mixture); B, equilibrated sample; and C, indomethacin, 100%.*

SUMMARY

The spectral changes pointed out in this study strongly indicate that solid–solid interactions are not restricted to any particular class of drugs. Although the exact mechanisms of interactions may vary with different drugs, it is significant that they do occur and that they are more widespread than formerly realized.

The nature and strength of these interactions depend on the nature of the drug and of the adjuvant. The nature of adjuvant would include factors such as hydrogen bonding, van der Waals' forces, chemisorption, the amount of adsorbed moisture, and the availability of active sites on the surface of the adjuvant.

The knowledge concerning the availability of the active ingredient cannot be limited to therapeutic aspects but must also encompass the stability of the active ingredient. An awareness of the extent and variety of solid–solid interactions could lead to a more rational and scientific approach to dosage form design.

REFERENCES

(1) F. A. Campagna, G. Curetone, R. A. Mirigian, and E. Nelson, *J. Pharm. Sci.*, **52,** 605(1963).

(2) D. Cook, H. S. Chang, and C. A. Mainville, *Can. J. Pharm. Sci.*, **1,** 69(1966).

(3) J. L. Lach and M. Bornstein, *J. Pharm. Sci.*, **54,** 1730(1965).

(4) R. O. Searl and M. Pernarowski, *Can. Med. Ass. J.*, **96,** 1513(1967).

(5) W. Keller, *Pharmazie*, **15,** 56(1960).

(6) L. Bighley, Ph.D. thesis, University of Iowa, Iowa City, Iowa, 1966.

(7) A. L. Hayden, O. R. Samul, G. B. Selzer, and J. Carol, *J. Ass. Offic. Agr. Chem.*, **45,** 797(1962).

(8) R. J. Timmerman, F. R. Springman, and R. K. Thoms, *Ther. Res.*, **6,** 88(1964).

(9) E. D. Hutchinson, D. Mehta, and G. B. Leonard, *Fed. Proc.*, **23,** 439(1964).

(10) R. T. Manning, *Pharmacologist*, **6,** 190(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication April 10, 1970.

This research was supported in part by Grant GM-13178 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

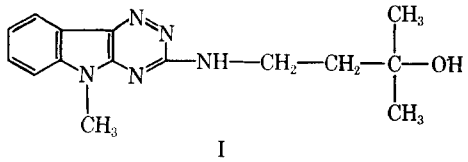
Physical–Chemical Evaluation of 3-(3-Hydroxy-3-methylbutylamino)-5-methyl-*as*. Triazino [5,6-*b*] Indole (SK&F 30097)

LOUIS J. RAVIN, ELIE G. SHAMI*, and ELISABETH RATTIE

Abstract □ X-ray diffraction, IR spectroscopy, and differential scanning calorimetry data are presented for the identification of two polymorphic forms and a hydrate of SK&F 30097. The relative rates of dissolution and the solubilities of the various forms were determined in artificial gastric fluid, water, and 50% ethanol solution. No appreciable difference in the dissolution rate was detected for the respective forms in artificial gastric fluid; however, Form II had a more rapid dissolution rate in the 50% ethanol solution. Dissolution studies in water indicated the formation of a hydrate. Protective colloids were shown to have an effect on the rate of hydrate formation. The change in particle-size distribution of Forms I and II in electrolyte suspension was investigated using the Coulter counter. The relative rate of crystal growth for Form I in the presence of protective colloids was determined. The data presented indicate that methylcellulose slows down the rate of crystal growth of Form I significantly.

Keyphrases □ 3-(3-Hydroxy-3-methylbutylamino)-5-methyl-*as*, triazino [5,6-*b*] indole (SK&F 30097)—identification □ Polymorphic, hydrated forms, SK&F 30097—determination □ Dissolution, solubility rates—SK&F 30097 □ X-ray diffraction—identification □ IR spectrophotometry—structure □ Differential scanning calorimetry—identification

The emphasis on drug availability in pharmaceutical research today requires that the physical–chemical properties of compounds be studied thoroughly prior to preliminary clinical trials. It has been demonstrated that the absorption and therapeutic efficacy of various drugs can be influenced by particle size, solubility, dissolution rate, and wettability (1–4). More recent literature reports stress the importance of the crystal form of a drug and its effect on drug availability (5, 6). The present study deals with the physical–chemical evaluation of a potential antiviral compound, 3-(3-hydroxy-3-methylbutylamino)-5-methyl-*as*, triazino [5, 6-*b*] indole, SK&F 30097 (Structure I).



I

Particular emphasis has been placed on the identification of two crystalline forms and a hydrate and the subsequent evaluation of their physical–chemical properties. Specifically, this study was concerned with an investigation of the relative dissolution behavior of the various forms in several solvent systems and an investigation of the rate of crystal growth in the presence and absence of protective colloids.

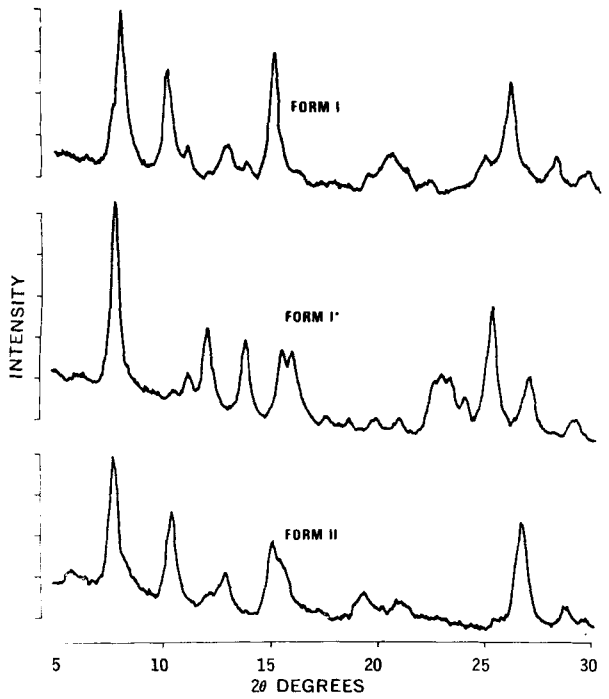


Figure 1—*X-ray diffractograms for Forms I, I*, and II of SK&F 30097.*

EXPERIMENTAL

Materials—A propylene glycol derivative;[1] polyvinylpyrrolidone;[2] carboxymethylcellulose USP; methylcellulose USP; artificial gastric fluid USP; and normal saline solution USP were used. SK&F 30097 Form I was prepared by dissolving SK&F 30097 in HCl solution and subsequently precipitating the compound slowly by adding NaOH solution. The resulting precipitate was filtered and allowed to dry in a vacuum desiccator over phosphorus pentoxide. SK&F 30097 Form I*[3] was prepared by suspending Form I in water and allowing it to be stirred overnight. The suspension was filtered and the filtrate was air-dried at room temperature. SK&F 30097 Form II was prepared by dissolving 1 g. of SK&F 30097 in 20 ml. of hot anhydrous methanol. The resulting solution was filtered and poured into anhydrous ethyl ether which had been previously dried over Linde molecular sieves[4] and cooled in liquid nitrogen. The mixture was shaken and set aside until precipitation was complete. The resulting precipitate was filtered and allowed to dry over phosphorus pentoxide. Extreme care must be taken to maintain anhydrous conditions to prepare Form II.

X-Ray Diffraction Procedure—A diffractometer (General Electric XRD-5) was used. The sample was packed into a planchet having a depression 1.5 mm. deep and 22 mm. in diameter. The flat side of the spatula was used to pack the powder and smooth it so that a uniform level surface was presented to the X-ray beam. Coarse or lumpy powder was first ground in a mortar or mechanical grinder. The instrument variables of the diffractometer were set as follows: (*a*) 1° beam slit and 0.1° detector slit; and (*b*) CuKα radiation, 40 kv., 16 ma., Ni filtered. Intensities were measured by recording the time necessary to count a fixed number of particles.

IR Procedure—The IR spectra for the various forms of SK&F

[1] Pluronic F-68, Wyandotte Chemical Co., Wyandotte, MI 48193
[2] Plasdone C, Antara Chemical Co.
[3] Monohydrate of SK&F 30097 will be designated by Form I*.
[4] Linde Division, Union Carbide Corp., New York, NY 10017

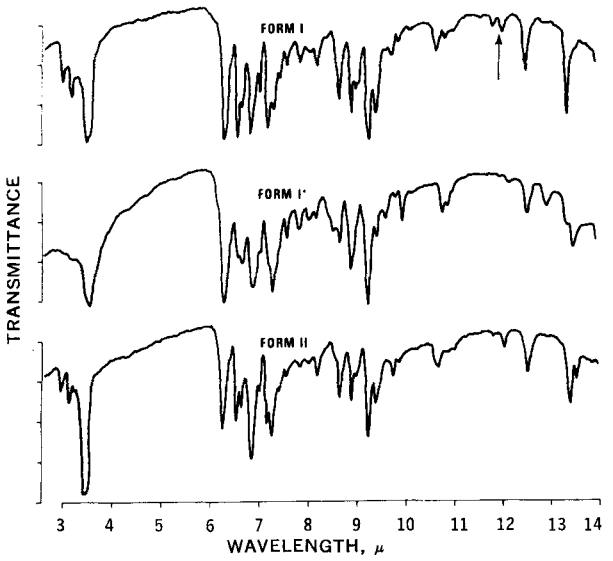


Figure 2—*IR spectra of Forms I, I*, and II of SK&F 30097.*

30097 were determined using a spectrophotometer (Perkin-Elmer model 21). The compounds were run in a mineral oil mull.

Differential Scanning Calorimetry Procedure—A differential scanning calorimeter (Perkin-Elmer DSC-1B) was used. Samples of indium were used to calibrate the instrument, and dry nitrogen at 20 ml./min. was used as the carrier gas. Since the preliminary work was qualitative, the samples were not accurately weighed. The rate of heating used was 20°/min. at a chart speed of 2 in./min.

Dissolution-Rate Studies—The procedure of Milosovich (7) was utilized to run the dissolution experiments under conditions of constant-surface and diffusion-layer thickness. Essentially, 100 mg. of powder was compressed into a disk in a tablet die at a compression force of approximately 5000 p.s.i. The tablet die holding the disk was placed in a plastic holder, and this assembly was placed into 700 ml. of artificial gastric fluid or 50% ethanol solution in a 1-l. glass-jacketed beaker. The solution was maintained at 37° by circulating water from a constant-temperature bath. The stirring rate was maintained at 200 r.p.m. during the experiment. Ten-milliliter samples were removed periodically and replaced by 10 ml. of the appropriate solvent. The samples were analyzed spectrophotometrically using a recording spectrophotometer (Cary model 15).

Dissolution-rate studies were also carried out according to the method of Shefter and Higuchi (8). Five hundred milligrams of the appropriate form was added to 500 ml. of distilled water maintained at 25° in a glass-jacketed 1-l. beaker thermostatically controlled by circulating water from a constant-temperature bath. The suspension was stirred at a rate of 200 r.p.m. Samples were withdrawn periodically and filtered through a syringe fitted with a Swinney filter adapter containing a 0.45-mμ Millipore filter disk. The resulting

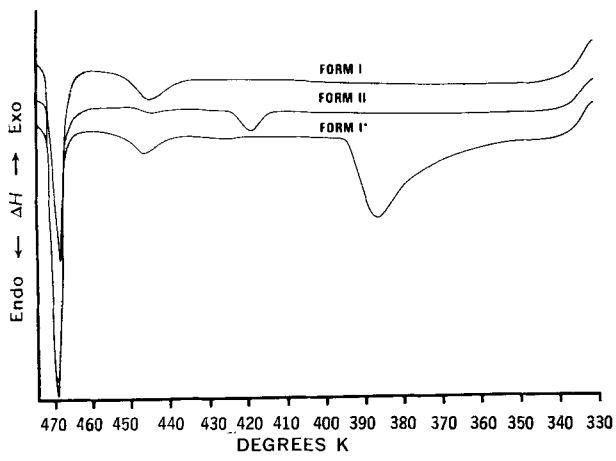


Figure 3—*Thermograms for Forms I, I*, and II of SK&F 30097.*

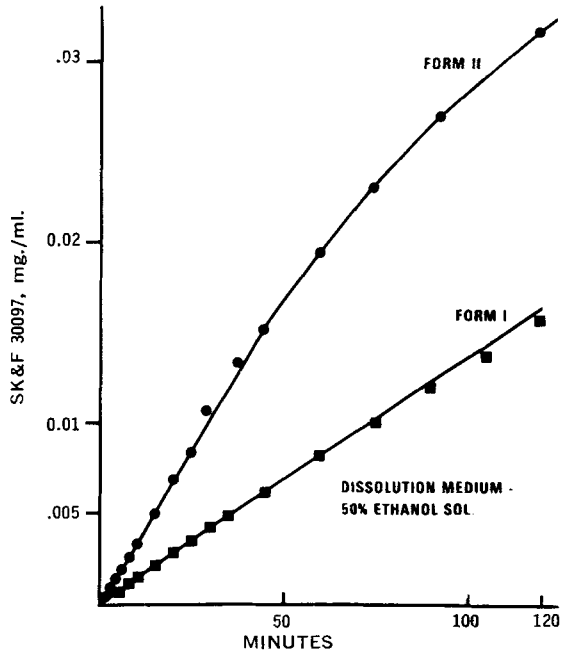


Figure 4—*Dissolution behavior of Forms I and II in 50% ethanol solution.*

clear solutions were analyzed spectrophotometrically (Cary mode 15 recording spectrophotometer). Additional experiments were done in the presence of 0.01% concentration of various protective colloids such as methylcellulose, carboxymethylcellulose, polyvinylpyrrolidone, and propylene glycol derivative.[1] The size of the crystals in the dissolution experiment was not controlled. The same lots of chemical for the various forms of SK&F 30097 were used throughout the study.

Crystal Growth Studies—Crystal growth studies were carried out using a modified procedure of Carless *et al.* (9). A saturated solution of SK&F 30097 was prepared by suspending the compound in normal saline solution containing 0.01% pro-

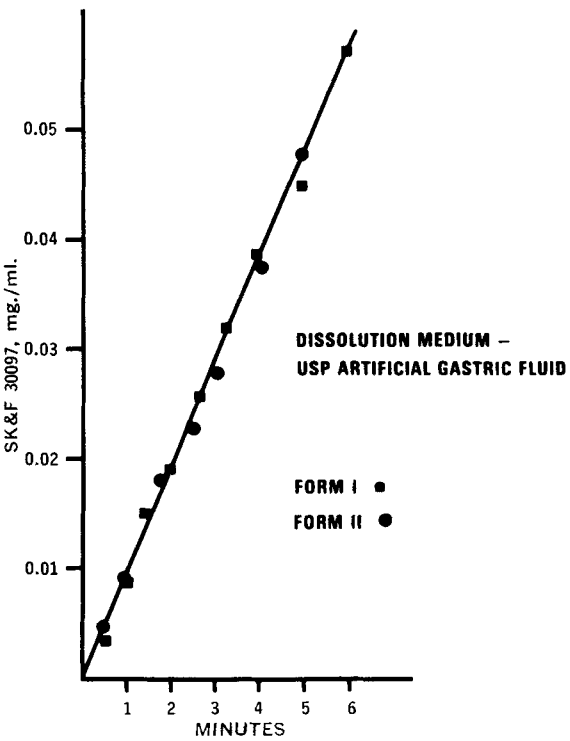


Figure 5—*Dissolution behavior of Forms I and II in artificial gastric fluid.*

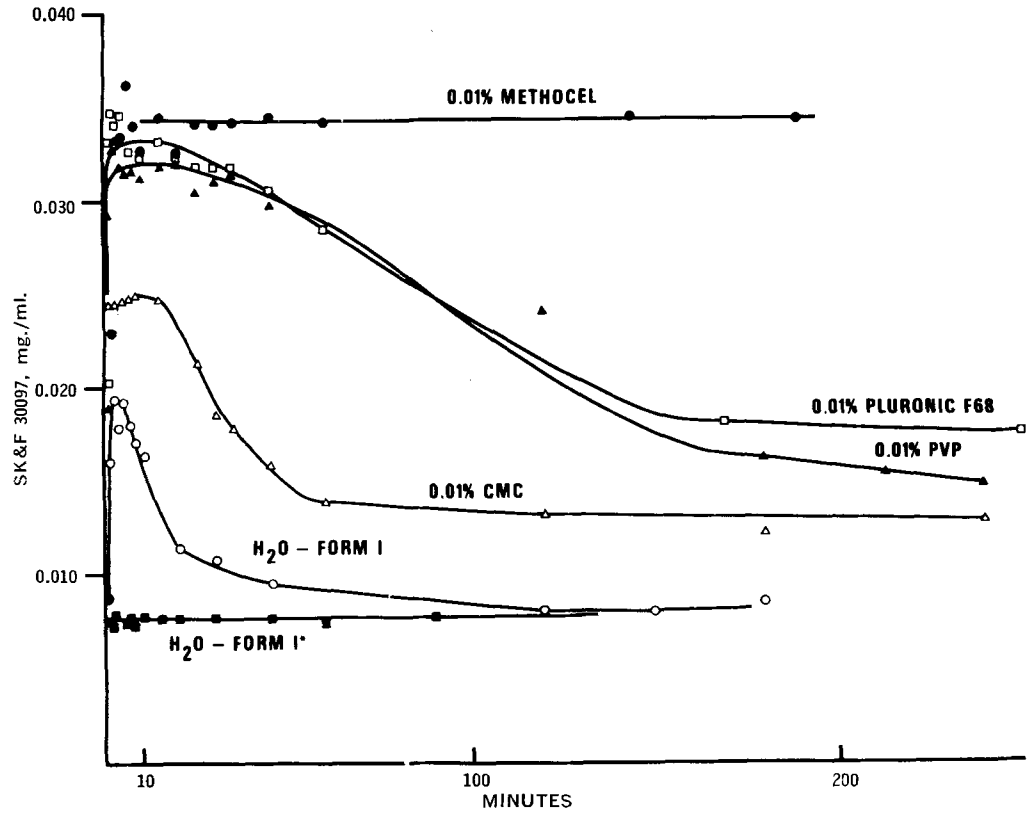


Figure 6—*Dissolution behavior of Forms I and I* in water in the presence of various protective colloids.*

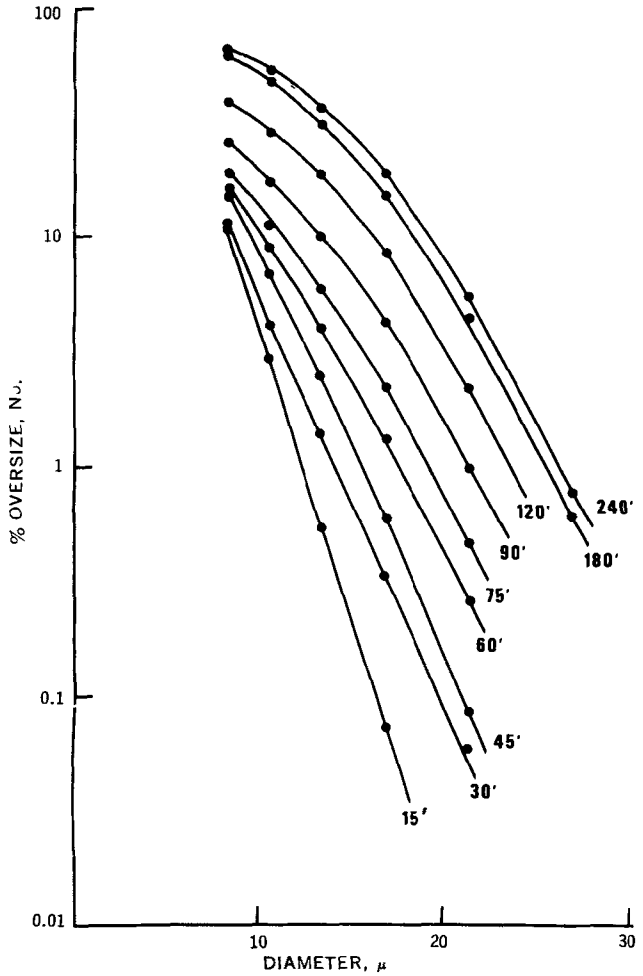


Figure 7—*A plot of change in particle size* versus *diameter at various time intervals for an aqueous suspension of Form I.*

pylene glycol derivative[1] as dispersing agent. The resulting suspension was stirred overnight and subsequently filtered. The filtrate was ready for use in the crystal growth studies. The seed crystals were prepared by suspending 100 mg. of the appropriate form of SK&F 30097 in 50 ml. of saturated solution. The suspension was sonified for 5 min. in an ultrasonic bath[5] to ensure deaggregation of the particles. Four milliliters of the seed suspension was pipeted into 70 ml. of electrolyte solution in a 100-ml. screw-cap bottle. The bottles were placed in a water bath set at 25° and rotated. Samples were removed at fixed intervals, and the contents were transferred to a jacketed glass vessel at 25° for crystal-size counting with the Coulter counter (model B). A 100-μ aperture tube was used during these studies. The tube had previously been calibrated with lycopodium having an average particle size of 27 μ. Experiments were carried out with Form I in electrolyte solution alone and in electrolyte solution containing propylene glycol derivative[1] and 0.01% concentrations of methylcellulose, carboxymethylcellulose, and polyvinylpyrrolidone, respectively. Form I* and Form II were studied in electrolyte solution only.

RESULTS AND DISCUSSION

X-Ray Diffraction and IR Spectroscopy—The X-ray diffraction patterns and the IR spectra for Forms I, I*, and II are shown in Figs. 1 and 2. Distinct differences in the profiles are evident. These distinguishing features can be utilized for the identification and possibly for the analysis of the various forms in mixtures.

Differential Scanning Calorimetry—The thermal behavior of the various forms of SK&F 30097 is illustrated in Fig. 3. The thermogram for Form II shows three endothermic transitions; the first at 143° corresponds to a transformation of Form II to Form I, the second at 165° corresponds to a transformation of Form I to a form that has not been isolated under the conditions of this study, and the third at 183° corresponds to melting. The thermogram for Form I* shows an endotherm at approximately 110° suggesting the presence of water, an endotherm at 165°, and melting at 183°. When Forms I* and II are cooled and reheated, the endotherms at 110 and 143° disappear; the endotherm at 165° is present, along with the endotherm corresponding to melting. In all cases, there

[5] L&R Manufacturing Co., Kearney, N. J.

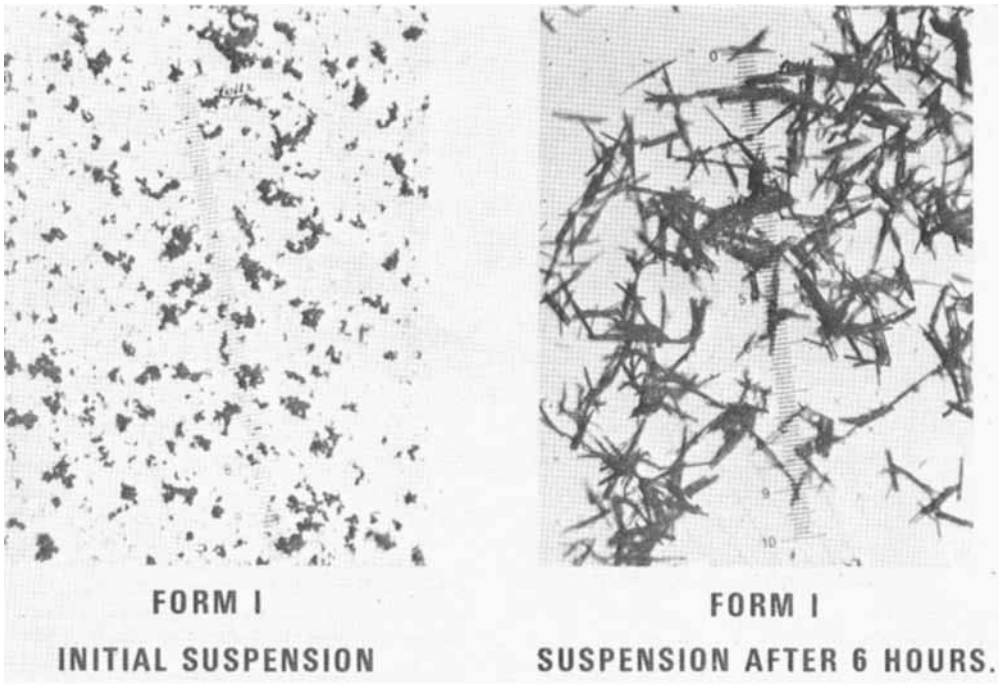


Figure 8—*Photomicrographs showing change in crystal size for a suspension of Form I. Each scale division equivalent to 6 μ.*

appears to be an apparent transformation to another form; however, it has not been isolated or prepared during this study.

Dissolution-Rate Studies—The dissolution behavior of Forms I and II under conditions of constant-surface and diffusion-layer thickness in 50% ethanol solution is illustrated in Fig. 4. As expected, Form II, which is less stable thermodynamically, had a more rapid dissolution rate. After approximately 50 min., the slope of the line for Form II deviates from linearity. This deviation suggests that Form II is being converted to Form I during the dissolution process.

Figure 5 shows the results of a dissolution-rate study for Forms I and II in artificial gastric fluid. There does not appear to be any difference in the dissolution behavior of either form in this medium. On the basis of this information, it was postulated that no appreciable difference in the *in vivo* availability of the various forms would be expected after oral administration. Subsequent studies in beagle dogs indicated that there was no statistical difference in the plasma blood levels obtained after the oral administration of Forms I and II in capsules and in a solution of the hydrochloride salt (10).

The solubility and dissolution-rate data obtained with suspensions of Forms I and I* are shown in Fig. 6. This figure shows the concentration of drug attained in solution as a function of time in the presence of an excess of the solid phase under constant agitation. The anhydrous Form I dissolves much faster than the corresponding hydrated Form I* and yields concentrations supersaturated with respect to the stable anhydrous form.

Since protective colloids are known to retard various nucleation

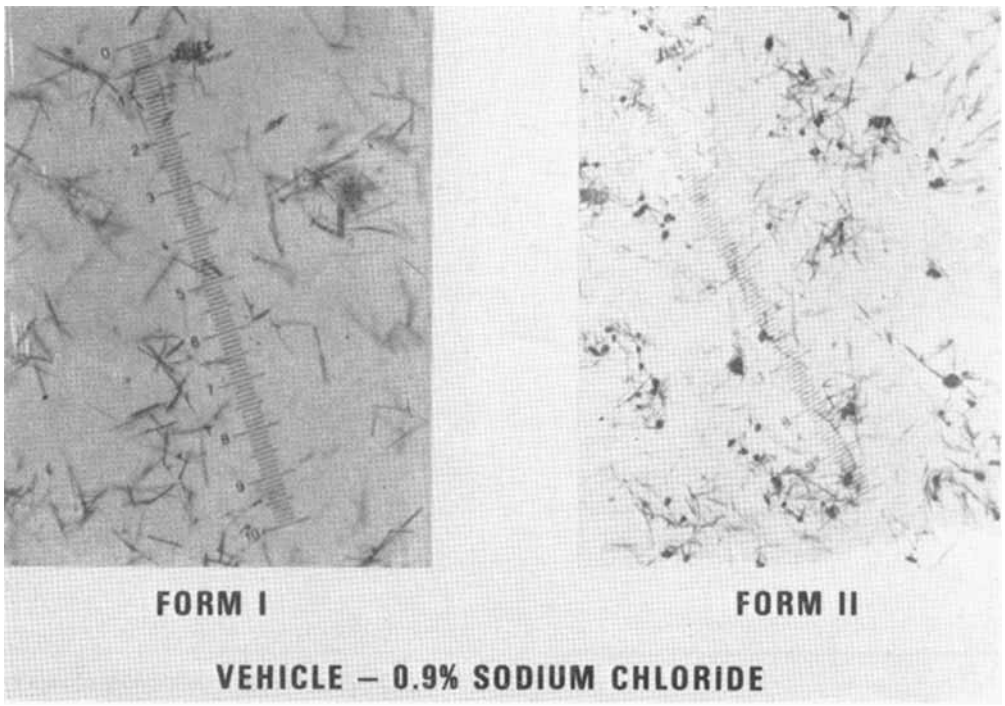


Figure 9—*Photomicrographs depicting crystal growth for Forms I and II after 5 min. in 0.9% sodium chloride solution.*

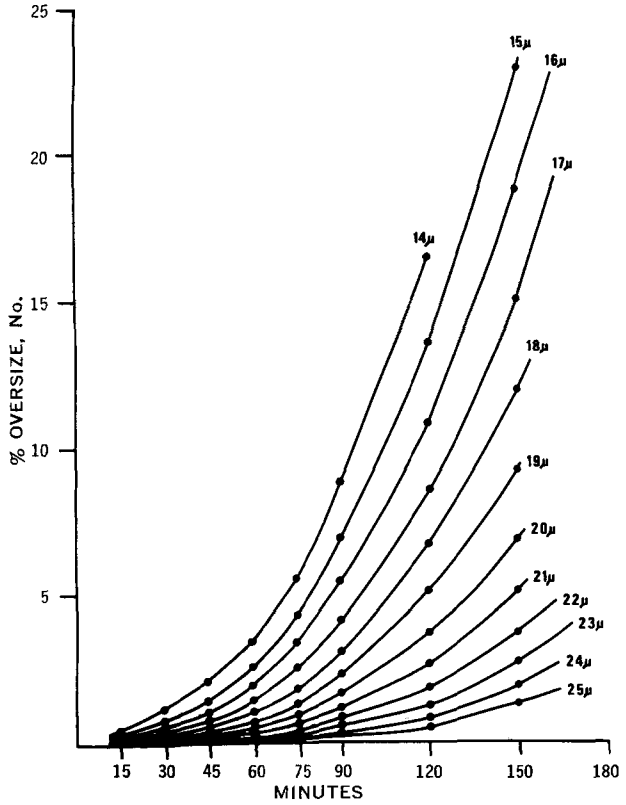


Figure 10—*Change in cumulative count with time for an aqueous suspension of Form I.*

phenomena, their effect on the transformation in this system was investigated. Studies were carried out with propylene glycol derivative,[1] carboxymethylcellulose, polyvinylpyrrolidone, and methylcellulose to determine their influence on the rate of transformation of Form I to Form I*. The dissolution behavior for Form I in 0.01% concentrations of the protective colloids is also shown in Fig. 6. It is apparent that the dissolution process is altered in the presence of the various protective colloids. The slightly higher solubility in the presence of these agents may be attributed to an interaction with the respective colloid. Methylcellulose had a

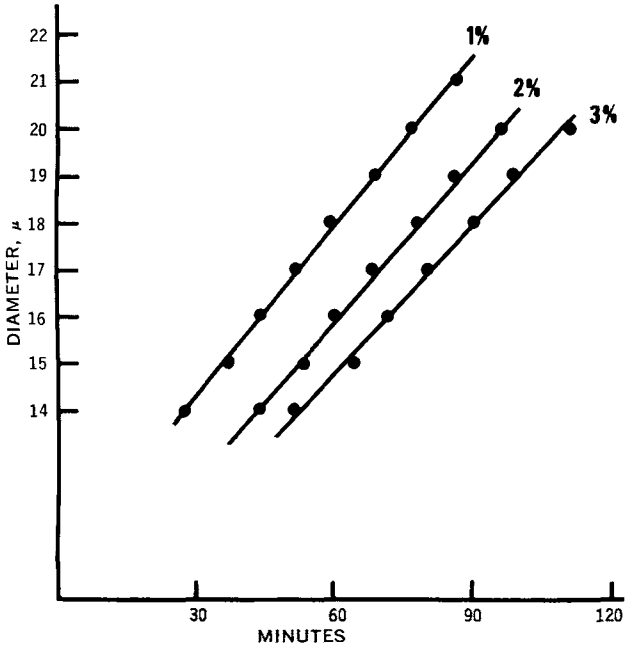


Figure 11—*Rate of growth of Form I in aqueous suspension.*

Table I—Relative Rates of Crystal Growth for Suspensions of SK&F 30097, Form I

Protective Colloid 0.01%	μ/min. × 10^{-4} Above 1%	Above 2%	Above 3%
Propylene glycol derivative	1040	930	880
Propylene glycol derivative plus:			
Carboxymethylcellulose	840	720	640
Polyvinylpyrrolidone	970	870	820
Methylcellulose	3	3.1	3

significant effect on the rate of hydrate formation and, after 48 hr., there was no apparent decrease in solubility similar to that observed with the other additives. Seed crystals of the hydrate form added at this point did not induce the conversion to the hydrate in this system.

Crystal Growth Studies—It became apparent during the preliminary studies that the particle size of Forms I and II increased rapidly when they were placed in aqueous suspension. This change is due to the conversion to Form I*. It was also observed that protective colloids slowed down this process. The dissolution-rate studies reflect these observations. As a result, crystal growth studies were undertaken with the various forms in the presence and absence of protective colloids. Figure 7 illustrates data from a typical crystal growth study with Form I where the change in particle-size distribution is plotted against diameter at various time intervals. There was an increase in particle size for intervals up to 6 hr., and then there was no apparent increase after this time. Photomicrographs of the suspension initially and after 6 hr. are shown in Fig. 8. It is apparent that there is a significant increase in particle size with time. Propylene glycol derivative[1] was used as the dispersing agent during these studies. Since this agent had some effect on the nucleation of Form I, which in turn may affect its crystal growth rate, additional studies were done in its absence. The rate of crystal growth for Forms I and II was extremely rapid.

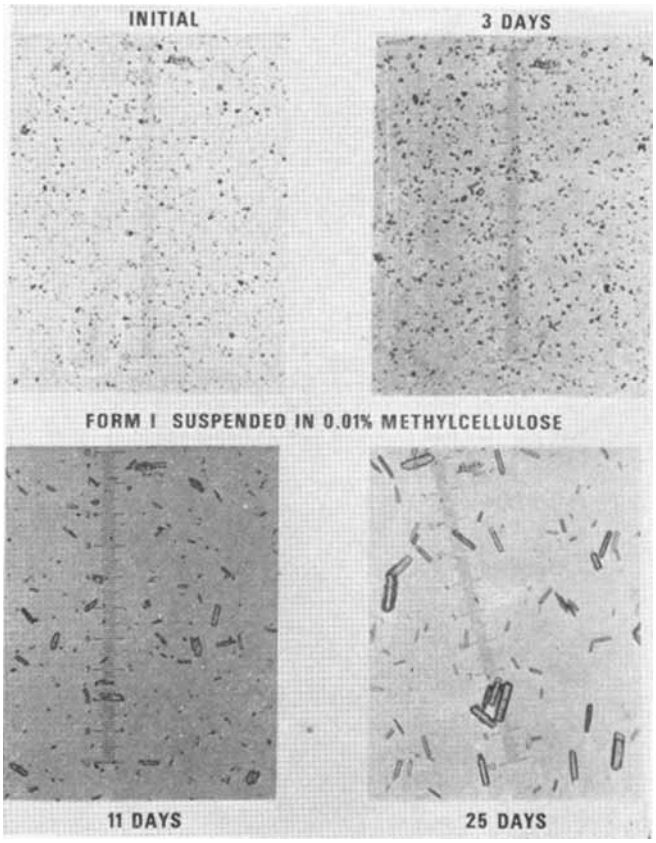


Figure 12—*Photomicrographs showing change in particle size with time for a suspension of Form I in the presence of 0.01% methylcellulose.*

These phenomena are illustrated in Fig. 9, which contains photomicrographs of these forms after 5 min.

The overall rate of crystal growth for Form I of SK&F 30097 was derived according to the method of Edmundson and Lees (11). A plot of percentage number cumulative frequency oversize against time for diameters from 14 to 25 μ is shown in Fig. 10. This size represents the faster growing end of the distribution obtained from Fig. 7. Horizontal lines corresponding to various percentage cumulative counts were made. The intercepts of these lines at the 1, 2, and 3% levels for the various time intervals give the equivalent diameter, which is then plotted against time. Figure 11 shows the rate plot for a typical crystal growth study. The slope of the lines represents the rate of growth of the crystals expressed as an increase in diameter per unit time. Table I contains values showing the relative rates of crystal growth of Form I in the presence and absence of various protective colloids.

The crystal growth in the presence of methylcellulose was approximately 300 times slower than in the presence of the other colloids. Photomicrographs showing the change in particle size with time in the presence of methylcellulose are shown in Fig. 12. There was no apparent increase in crystal size after 3 days; however, after 8 days, there was some evidence of growth. The fact that the Coulter counter measurements indicated a growth rate of 3×10^{-4} μ/min. indicates the extreme sensitivity of this instrument to change in crystal size with time.

SUMMARY

1. The presence of two polymorphic forms and a hydrate of SK&F 30097 has been confirmed by X-ray diffraction, IR spectroscopy, and differential scanning calorimetry.

2. Form II, which was less stable thermodynamically, had a more rapid dissolution rate than Form I in 50% ethanol solution; however, in artificial gastric fluid, there was no apparent difference.

3. Forms I and II readily formed the hydrate in aqueous suspension; protective colloids were shown to affect the rate of hydrate formation.

4. Coulter counter measurements were used to follow the change in particle-size distribution for Form I in electrolyte solution. The relative rates of crystal growth were determined in the presence of various protective colloids. Methylcellulose retarded the rate of crystal growth of Form I significantly.

REFERENCES

(1) J. D. Conklin and F. J. Hailey, *Clin. Pharmacol. Ther.*, **10,** 534(1969).

(2) J. H. Fincher, *J. Pharm. Sci.*, **57,** 1825(1968).

(3) A. J. Aguiar, L. M. Wheeler, S. Fusari, and J. E. Zelmer, *ibid.*, **57,** 1844(1968).

(4) A. J. Aguiar and J. E. Zelmer, *ibid.*, **58,** 983(1969).

(5) J. W. Poole, G. Owen, J. Silverio, J. N. Feyhof, and S. B. Rosenman, *Curr. Ther. Res.*, **10,** 292(1968).

(6) A. J. Aguiar, J. Krc, A. W. Kinkel, and J. C. Samyn, *J. Pharm. Sci.*, **56,** 847(1967).

(7) G. Milosovich, *ibid.*, **53,** 484(1964).

(8) E. Shefter and T. Higuchi, *ibid.*, **52,** 781(1963).

(9) J. E. Carless, M. A. Moustafa, and H. D. C. Rapson, *J. Pharm. Pharmacol.*, **20,** 630(1968).

(10) Smith Kline & French Laboratories, unpublished information.

(11) I. C. Edmundson and K. A. Lees, *J. Pharm. Pharmacol.*, **17,** 193(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 29, 1969, from the *Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 79101*

Accepted for publication March 17, 1970.

The authors are grateful to Dr. D. Zacharias and Mr. R. Warren for the X-ray and IR analyses.

* Present address: Endo Laboratories, Garden City, L. I., N. Y.

Thermodynamics and Kinetics of Covalent Addition of Bisulfite Ion to Pyrimidinium Ions

IAN H. PITMAN* and MARK A. ZISER

Abstract □ Equilibrium and rate constants have been calculated for the reversible covalent addition of bisulfite ion to 2-aminopyrimidinium ion and to its 1-methyl and 4-methyl derivatives. 2-Amino-4,6-dimethylpyrimidinium ion did not appear to add bisulfite ion under the experimental conditions. The 1:1 covalent adducts had very low solubility in aqueous buffers around pH 4. This was consistent with their being zwitterions. Rate-determining steps in adduct formation appeared to involve attack of both bisulfite ion and sulfite ion on the pyrimidinium cation. In the reverse reactions, the zwitterionic adducts appeared to decompose by both nonbase-catalyzed and specific base-catalyzed reactions.

Keyphrases □ Bisulfite ion, kinetics, thermodynamics—covalent addition to pyrimidinium ions □ Covalent addition—bisulfite ion to pyrimidinium ions □ 2-Amino-1,6-dihydropyrimidinium-6-sulfonate—synthesis □ 2-Amino-1,6-dihydro-4-methylpyrimidinium-6-sulfonate—synthesis □ UV spectrophotometry—identification

Many of the known reactions of sodium bisulfite with organic and inorganic molecules have been studied quantitatively, and a considerable amount of thermodynamic and kinetic data is available for use when considering the inclusion of sodium bisulfite as an antioxidant in drug formulations (1). However, very little quantitative data are available on the reversible covalent additions of bisulfite ion to nitrogen-containing heteroaromatics such as pyridines (2), pyrimidines (3, 4), pteridines (5–8), and quinazolines (9, 10), although many drugs belong to these classes of compounds. The occurrence of the addition reaction in a drug formulation would reduce the effective concentration of the bisulfite ion, and the covalent adduct may have different chemical reactivity to that of the augend.

The present study was undertaken to determine the effects of temperature and pH on the kinetics and thermodynamics of addition of bisulfite ion to 2-aminopyrimidinium ion (I; R = R′ = H), 2-amino-1-methylpyrimidinium ion (II), 2-amino-4-methylpyrimidinium ion (I; R = H, R′ = Me), and 2-amino-4,6-dimethylpyrimidinium ion (I; R = R′ = Me). Studies on addi-

These phenomena are illustrated in Fig. 9, which contains photomicrographs of these forms after 5 min.

The overall rate of crystal growth for Form I of SK&F 30097 was derived according to the method of Edmundson and Lees (11). A plot of percentage number cumulative frequency oversize against time for diameters from 14 to 25 μ is shown in Fig. 10. This size represents the faster growing end of the distribution obtained from Fig. 7. Horizontal lines corresponding to various percentage cumulative counts were made. The intercepts of these lines at the 1, 2, and 3% levels for the various time intervals give the equivalent diameter, which is then plotted against time. Figure 11 shows the rate plot for a typical crystal growth study. The slope of the lines represents the rate of growth of the crystals expressed as an increase in diameter per unit time. Table I contains values showing the relative rates of crystal growth of Form I in the presence and absence of various protective colloids.

The crystal growth in the presence of methylcellulose was approximately 300 times slower than in the presence of the other colloids. Photomicrographs showing the change in particle size with time in the presence of methylcellulose are shown in Fig. 12. There was no apparent increase in crystal size after 3 days; however, after 8 days, there was some evidence of growth. The fact that the Coulter counter measurements indicated a growth rate of 3×10^{-4} μ/min. indicates the extreme sensitivity of this instrument to change in crystal size with time.

SUMMARY

1. The presence of two polymorphic forms and a hydrate of SK&F 30097 has been confirmed by X-ray diffraction, IR spectroscopy, and differential scanning calorimetry.

2. Form II, which was less stable thermodynamically, had a more rapid dissolution rate than Form I in 50% ethanol solution; however, in artificial gastric fluid, there was no apparent difference.

3. Forms I and II readily formed the hydrate in aqueous suspension; protective colloids were shown to affect the rate of hydrate formation.

4. Coulter counter measurements were used to follow the change in particle-size distribution for Form I in electrolyte solution. The relative rates of crystal growth were determined in the presence of various protective colloids. Methylcellulose retarded the rate of crystal growth of Form I significantly.

REFERENCES

(1) J. D. Conklin and F. J. Hailey, *Clin. Pharmacol. Ther.*, **10,** 534(1969).

(2) J. H. Fincher, *J. Pharm. Sci.*, **57,** 1825(1968).

(3) A. J. Aguiar, L. M. Wheeler, S. Fusari, and J. E. Zelmer, *ibid.*, **57,** 1844(1968).

(4) A. J. Aguiar and J. E. Zelmer, *ibid.*, **58,** 983(1969).

(5) J. W. Poole, G. Owen, J. Silverio, J. N. Feyhof, and S. B. Rosenman, *Curr. Ther. Res.*, **10,** 292(1968).

(6) A. J. Aguiar, J. Krc, A. W. Kinkel, and J. C. Samyn, *J. Pharm. Sci.*, **56,** 847(1967).

(7) G. Milosovich, *ibid.*, **53,** 484(1964).

(8) E. Shefter and T. Higuchi, *ibid.*, **52,** 781(1963).

(9) J. E. Carless, M. A. Moustafa, and H. D. C. Rapson, *J. Pharm. Pharmacol.*, **20,** 630(1968).

(10) Smith Kline & French Laboratories, unpublished information.

(11) I. C. Edmundson and K. A. Lees, *J. Pharm. Pharmacol.*, **17,** 193(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 29, 1969, from the *Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 79101*

Accepted for publication March 17, 1970.

The authors are grateful to Dr. D. Zacharias and Mr. R. Warren for the X-ray and IR analyses.

* Present address: Endo Laboratories, Garden City, L. I., N. Y.

Thermodynamics and Kinetics of Covalent Addition of Bisulfite Ion to Pyrimidinium Ions

IAN H. PITMAN* and MARK A. ZISER

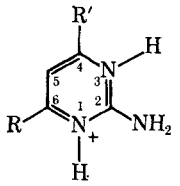
Abstract □ Equilibrium and rate constants have been calculated for the reversible covalent addition of bisulfite ion to 2-aminopyrimidinium ion and to its 1-methyl and 4-methyl derivatives. 2-Amino-4,6-dimethylpyrimidinium ion did not appear to add bisulfite ion under the experimental conditions. The 1:1 covalent adducts had very low solubility in aqueous buffers around pH 4. This was consistent with their being zwitterions. Rate-determining steps in adduct formation appeared to involve attack of both bisulfite ion and sulfite ion on the pyrimidinium cation. In the reverse reactions, the zwitterionic adducts appeared to decompose by both nonbase-catalyzed and specific base-catalyzed reactions.

Keyphrases □ Bisulfite ion, kinetics, thermodynamics—covalent addition to pyrimidinium ions □ Covalent addition—bisulfite ion to pyrimidinium ions □ 2-Amino-1,6-dihydropyrimidinium-6-sulfonate—synthesis □ 2-Amino-1,6-dihydro-4-methylpyrimidinium-6-sulfonate—synthesis □ UV spectrophotometry—identification

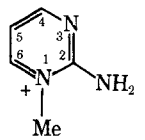
Many of the known reactions of sodium bisulfite with organic and inorganic molecules have been studied quantitatively, and a considerable amount of thermodynamic and kinetic data is available for use when considering the inclusion of sodium bisulfite as an antioxidant in drug formulations (1). However, very little quantitative data are available on the reversible covalent additions of bisulfite ion to nitrogen-containing heteroaromatics such as pyridines (2), pyrimidines (3, 4), pteridines (5–8), and quinazolines (9, 10), although many drugs belong to these classes of compounds. The occurrence of the addition reaction in a drug formulation would reduce the effective concentration of the bisulfite ion, and the covalent adduct may have different chemical reactivity to that of the augend.

The present study was undertaken to determine the effects of temperature and pH on the kinetics and thermodynamics of addition of bisulfite ion to 2-aminopyrimidinium ion (I; R = R′ = H), 2-amino-1-methylpyrimidinium ion (II), 2-amino-4-methylpyrimidinium ion (I; R = H, R′ = Me), and 2-amino-4,6-dimethylpyrimidinium ion (I; R = R′ = Me). Studies on addi-

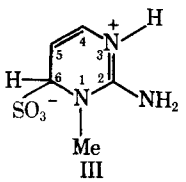
tions to molecules which are widely used as drugs are continuing in the authors' laboratories.



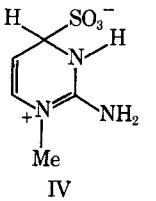
I



II



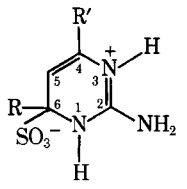
III



IV

RESULTS AND DISCUSSION

The Addition Reaction—Compound II has previously been shown (4) to react with bisulfite ion reversibly to yield, in dilute aqueous solution at 25°, an approximately 0.8:1 mixture of the covalent adducts III and IV. These adducts appear to have similar UV spectra (4), and the spectrum of their equilibrium mixture has the characteristics shown in Table I. Because addition of sodium bisulfite (0.2 *M*) to dilute aqueous solutions of (I; R = R′ = H) and (I; R = H, R′ = Me) produced solutions with similar UV spectra to that of a mixture of III and IV (Table I), it was deduced that covalent addition of bisulfite ion was occurring in these cases also. The addition of sodium bisulfite (1.0 *M*) to saturated aqueous solutions of the pyrimidinium ions (I; R = R′ = H) (II), and (I; R = H, R′ = Me) resulted in the formation of white crystalline products which had the elemental analysis of 1:1 adducts of the respective pyrimidinium ion and bisulfite ion. The reversibility of all of the addition reactions was indicated by the fact that dilution of solutions of the adducts in aqueous sodium bisulfite with water led to reformation of the original pyrimidine. Addition of sodium bisulfite (0.2 *M*) to dilute aqueous solutions of (I; R = R′ = Me) did not lead to any significant changes in its UV spectrum (when the absorbance of the bisulfite ion was taken into account), and it was assumed that covalent addition of bisulfite ion was not occurring to this compound under these conditions.



V

The 1:1 adduct of Compound (I; R = R′ = H) would have the same structure whether addition occurred at C_4 or C_6 and was thus believed to be 2-amino-1,6-dihydropyrimidinium-6-sulfonate (V; R = R′ = H). The strong blocking effects of methyl groups at C_4 and C_6 in Compound (I; R = R′ = Me) to addition at those sites strongly suggest that addition to (I; R = H, R′ = Me) would occur predominantly at C_6 to yield 2-amino-1,6-dihydro-4-methyl-pyrimidinium-6-sulfonate (V; R = H, R′ = Me). Similar blocking effects of *C*-alkyl groups to nucleophilic additions at that site have been observed (11) for nucleophilic additions to other heteroaromatic molecules. More exact structural determinations by NMR spectroscopy of the adducts (V; R = R′ = H) and (V; R = H, R′ = Me) were difficult because of their low solubility in aqueous sodium bisulfite (<1 g./100 ml.). This low solubility is consistent with their being zwitterionic in aqueous buffers between pH 3 and 5.

Because of the acidic or basic nature of the reactants and products in the addition reactions, the reaction mixtures always contained varying concentrations of pyrimidinium ion PyH^+, neutral pyrimidine Py, sulfurous acid H_2SO_3, bisulfite ion HSO_3^-, sulfite ion $SO_3^=$, cation H_2Add^+, zwitterion $HAdd^\pm$, and anion Add^- of the covalent adduct. At any particular pH, the overall addition reaction was

$$Py_T + S_T \underset{k_b}{\overset{k_f}{\rightleftharpoons}} Add_T \qquad \text{(Eq. 1)}$$

where Py_T, S_T, and Add_T were the total pyrimidine, sulfurous acid, and adduct species, respectively, present at any time. The acid-dissociation constants of the various acidic species were measured under experimental conditions and are listed in Table II.

Equilibrium Constants for Addition—Apparent equilibrium constants, $K_{app.}$ values, were calculated from differences in UV absorbance at fixed wavelengths between aqueous buffered solutions of the pyrimidines and similar solutions which had equilibrated after the addition of different amounts of sodium bisulfite. As shown in Fig. 1, the $K_{app.}$ values for addition to the very weakly acidic compound (II) were independent of pH in the pH region investigated, but the values for the more strongly acidic compounds, (I; R = R′ = H) and (I; R = H, R′ = Me), decreased with increasing pH. No evidence could be found for adduct formation by any of the compounds at pH values above 8.5 or below 0.5.

The $K_{app.}$ values were the combined equilibrium constants at each pH for the reactions represented by Eq. 1 and are defined as

$$K_{app.} = \frac{[Add_T]}{[Py_T][S_T]} \qquad \text{(Eq. 2)}$$

If the true addition reactions were

$$PyH^+ + HSO_3^- \overset{K}{\rightleftharpoons} HAdd^\pm \qquad \text{(Eq. 3)}$$

then, in the pH range where $[HSO_3^-] \gg [H_2SO_3]$ or $[SO_3^=]$ and $[HAdd^\pm] \gg [H_2Add^+]$ or $[Add^-]$, values of $K_{app.}$ and K would be related by the identity

$$K_{app.} = \frac{K[H^+]}{(K_{a_p} + [H^+])} \qquad \text{(Eq. 4)}$$

where K_{a_p} is the acid-dissociation constant of the pyrimidinium ion.

Because plots of $K_{app.}$ $([H^+] + K_{a_p})$ against $[H^+]$ were linear between pH 3.3 and 5 for addition to each of the pyrimidinium ions studied, it is believed that these addition reactions are adequately described by Eq. 3 and that the zwitterionic forms of the adducts are the most stable forms in this pH region. Values of the

Table I—UV Spectral Characteristics of 1:1 Bisulfite Adducts

Augend	$\lambda_{max.}$ (log ϵ)
I; R = R′ = H′	255 (3.38)[a]
II	258 (3.36)[a]
I; R = H, R′ = Me	254 (3.17)

[a] These values were corrected for absorbance due to free pyrimidine species at equilibrium and are considered to be more accurate than those in *Reference 3*.

Table II—Acid-Dissociation Constants, at Ionic Strength 10^{-1} *M*, of Pyrimidinium Ions and Sulfurous Acid

Compound	Temperature	pKa (± spread)
I; R = R′ = H	15.5°	3.85 (0.02)
	25.3°	3.77 (0.03)
	36.6°	3.70 (0.03)
II	20.0°	10.75[a] (0.1)
I; R = H, R′ = Me	25.0°	4.41 (0.04)
HSO_3^-	15.1°	6.85 (0.04)
	24.0°	6.94 (0.05)
	37.0°	6.92 (0.03)

[a] At I = 0.01, D. J. Brown, E. Hoerger, and S. F. Mason, *J. Chem. Soc.*, **1955**, 4035.

Table III—Thermodynamic Data for Addition of Bisulfite Ion to Pyrimidinium Ions

Augend	Temperature	K,[a] M^{-1}	K_6,[b] M^{-1}	K_4,[b] M^{-1}	$\Delta H°$, kcal. M^{-1}	$\Delta S°$, cal. M^{-1}
I; R=R′=H	12.45°	4110				
	25.00°	1800			−11.3	−23.1
	36.00°	905				
II	12.45°	1098				
	25.00°	501	222	278	−9.70	−20.1
	36.00°	282				
	36.50°	273				
I; R=H, R′=Me	25.00°	49.0				
I; R=R′=Me	25.00°	<0.1				

[a] K = [HAdd±]/([PyH⁺][HSO_3^-]). [b] K_6 and K_4 are defined in the text.

pH-independent equilibrium constant K were calculated from the slopes of the graphs and are listed in Table III, together with standard enthalpy and entropy changes for addition.

The K values for addition to Compounds (I; R=R′=H) and (I; R=H, R′=Me) are each related to the differences in free energy between a particular adduct and the reactants, because only one covalent adduct is formed in each reaction. On the other hand, an approximately 0.80:1 ratio of III and IV results (4) from addition to II at 25°. Thus, the K value for the addition reaction is really the sum of two microequilibrium constants, $K_6 \simeq K/2.25$ and $K_4 \simeq K/1.8$, where K_6 and K_4 relate to addition at C_6 to yield III and at C_4 to yield IV, respectively. The values of K_6 and K_4 have to be compared with K values for the other compounds in discussion of differences in free energy between a particular adduct and its pyrimidinium ion augend.

It can be seen from Table III that substitution of methyl groups for protons anywhere in the pyrimidinium ring decreases the extent to which covalent addition occurs. The very marked reduction in the value of K produced by methyl groups at the potential sites of addition [comparing additions to (I; R=R′=Me) and (I; R=R′=H)] is probably due to both steric and electronic (+I) destabilization of the adduct and electronic stabilization (+I) of the original pyrimidinium ion. The electronic effects are expected to be the major ones when methyl substitution is at sites distant from the site of addition, *i.e.*, at C_4 and N_1 of the 2-aminopyrimidinium ion. A possible explanation for the lower K value obtained for addition to the compound with the *C*-methyl substituent (I; R=H, R′=Me) than to that with the *N*-methyl substituent (II) would be that the +I effect of the *N*-methyl substituent was partially shielded from the ring by the positive charge on the nitrogen atom.

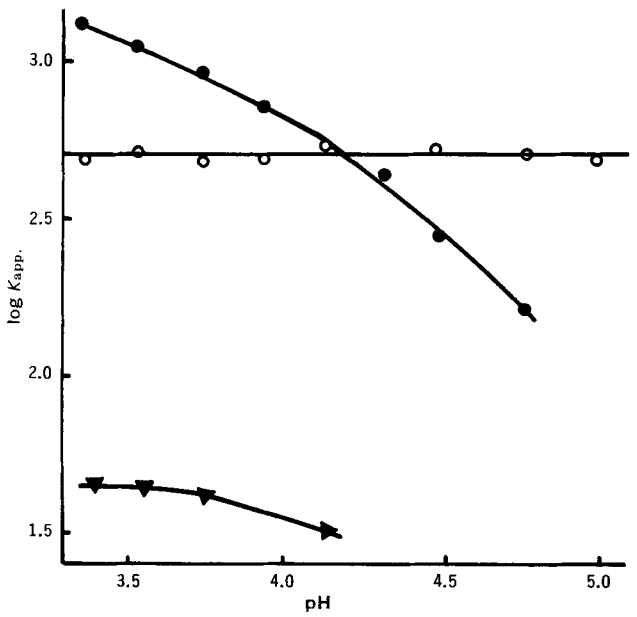


Figure 1—*Plot against pH of log* $K_{app.}$ *for covalent addition of bisulfite ion to:* (●) *(I; R=R′=H),* (○) *(II), and* (▲) *(I; R=H, R′=Me).*

Covalent addition of bisulfite ion to pyrimidinium ions involves saturation of charged imino group >C=$\overset{+}{N}$< and can be compared with the well-known addition to aldehydic or ketonic carbonyl groups >C=O. These reactions have many similarities; they occur readily at room temperature, they are reversible, and they are both inhibited by alkyl substitution at the site of addition. However, as seen by comparison of the thermodynamic data in Table IV with that in Table III, the standard enthalpy change is more favorable and entropy change less favorable for addition to the carbonyl compounds. These differences are most likely due largely to the different "charge types" of the reactions, and they reflect the changes in solvation of reactants and products which accompany the reactions.

Kinetics of Formation and Decomposition of Covalent Adducts (V; R=R′=H) and (V; R=H, R′=Me)—The kinetics of the reversible formation of (V; R=R′=H) and (V; R=H, R′=Me) were studied to determine the effect of total sulfurous acid species concentrations, pH, and buffer-ion concentrations on the covalent addition of bisulfite ion to pyrimidinium ions. These systems were chosen for study because they each involved only one covalent adduct and proceeded cleanly and rapidly at room temperature.

First-order rate constants, $k_{obs.}$ values, for the consumption of the pyrimidinium ion plus the pyrimidine were calculated from the changes in UV absorbance at 302 mμ, which followed mixing of aqueous buffered (succinic acid and borax) solutions of the (I; R=R′=H) or (I; R=H, R′=Me) (6.26×10^{-4} *M*) with equal volumes of solutions containing sodium bisulfite at an initial concentration that was at least 10 times that of the pyrimidine. Values of $k_{obs.}$ increased with increasing pH in the pH range 3.3–4.8 when the initial bisulfite-ion concentration was kept constant, and they increased linearly with increasing initial sodium bisulfite concentrations at constant pH. Examples of this behavior for addition to (I; R=R′=H) are shown as the circles in Fig. 2. Values of the $k_{obs.}$ did not change more than ±2% when the total buffer concentration was varied between 10^{-1} *M* and 10^{-3} *M* at pH 4.20. The ionic strength of all reaction mixtures was maintained at 0.1 *M* with sodium chloride.

When only one covalent adduct is formed in the addition reaction, the rate of consumption of total pyrimidine species according to Eq. 1 would be

$$\text{rate} = k_f[\text{Py}_T][\text{S}_T] - k_b[\text{A}_T] \qquad \text{(Eq. 5)}$$

Table IV—Thermodynamic Data for Addition of Bisulfite Ion to Carbonyl Compounds

Compound	Temperature	K,[a] M^{-1}	$\Delta H°$	$\Delta S°$
CH_3CH_2CHO[b]	20°	12,000		
$CH_3—\overset{\overset{O}{\|}}{C}—CH_3$[b]	20°	290		
C_6H_5CHO[c]	20°	5,000	−17.7	−43

[a] K = [adduct⁻]/([carbonyl compound][HSO_3^-]). [b] M. A. Gubareva, *J. Gen. Chem., USSR*, **17**, 2529(1947). [c] J. A. Sousa and J. D. Margerum, *J. Amer. Chem. Soc.*, **82**, 3013(1960).

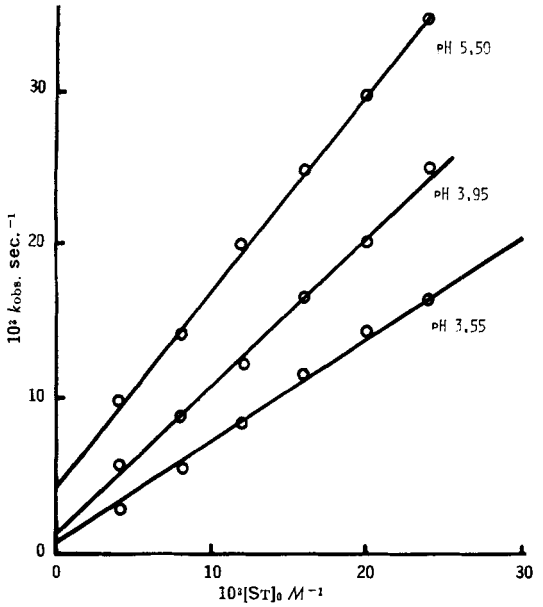


Figure 2—*Plot against* $[S_T]_0$ *of* $k_{obs.}$ *for covalent addition to 2-aminopyrimidinium ion at 25°. Open circles are experimental points and lines were calculated from values of pH-independent rate constants as described in text.*

and the observed first-order rate constant for approach to equilibrium in the presence of a considerable excess of total sulfurous acid species could be related to k_f and k_b values by the identity:

$$k_{obs.} = k_f[S_T]_0 + k_b \qquad \text{(Eq. 6)}$$

where $[S_T]_0$ was the initial sodium bisulfite concentration. Values of k_f were thus calculated from the slope of linear plots of $k_{obs.}$ against $[S_T]_0$ and are listed in Table V.

Although it should be possible to calculate k_b values from the Y-axis intercepts of these plots, this was only done in a few cases because of the extreme sensitivity of these values to small variations in the slopes of the lines. The k_b values were usually calculated from the k_f value and the appropriate $K_{app.}$ value by assuming the principle of microscopic reversibility and using the identity

$$k_b = k_f/K_{app.} \qquad \text{(Eq. 7)}$$

Justification for this assumption came from the fact that at high pH values, the Y-axis intercepts of plots of $k_{obs.}v[S_T]_0$ were relatively large and the graphically determined and calculated k_b values did not vary more than ±3%.

Table V—Rate Constants for Reversible Addition of Bisulfite Ion to Pyrimidinium Ions

Augend	Temperature	pH	$10\,k_f$ M^{-1} sec.$^{-1}$	$10^4\,k_b$ sec.$^{-1}$
I; R = R′ = H	12.00°	3.58	3.50	1.23
		3.98	5.60	4.22
		4.54	7.65	9.99
	25.00°	3.36	5.40	4.16
		3.55	6.90	6.13
		3.76	8.78	9.61
		3.95	9.63	13.4
		4.13	10.8	19.6
		4.32	11.0	27.6
		4.50	12.7	44.7
		4.77	13.5	81.8
	37.00°	3.52	9.85	19.3
		3.93	14.7	47.0
		3.96	15.8	52.8
		4.45	19.8	156.0
I; R = H, R′ = Me	25.00°	3.40	0.703	14.7
		3.56	0.880	21.8
		3.76	1.137	30.2
		4.15	2.24	61.1

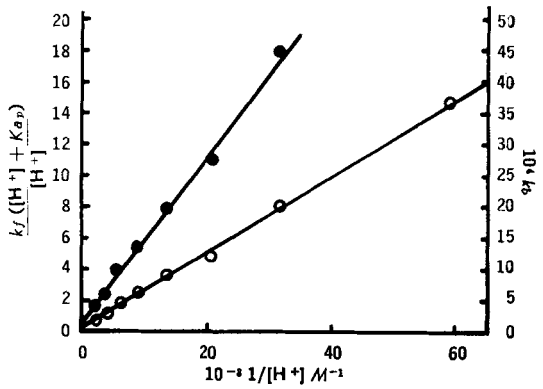


Figure 3—*Plots against* $1/[H^+]$ *of* (○) k_f $([H^+] + K_{a_p})/[H^+]$ and (●) k_b *for addition of bisulfite ion to (I; R = R′ = H) at 25°.*

The pH dependence of k_f and k_b values is consistent with a hypothesis that two independent and competitive reaction paths are followed in the equilibrium reactions. In the forward reaction, both bisulfite ion and sulfite ion react with the pyrimidinium ion; in the reverse reactions, the neutral (zwitterionic) adduct decomposes in both nonbase-catalyzed and specific base-catalyzed reactions. This hypothesis requires that the rate of consumption of pyrimidinium ion would be

$$\text{rate} = k_1[PyH^+][HSO_3^-] + k_2[PyH^+][SO_3^-] - k_{-1}[HAdd^\pm] - k_{-2}[HAdd^\pm][OH^-] \qquad \text{(Eq. 8)}$$

In terms of the total pyrimidine concentration $[Py_T]$ and total sulfurous acid concentrations $[S_T]$, and assuming that the adduct exists predominantly as a zwitterion in the pH region studied, this rate law becomes:[1]

$$\text{rate} = \frac{k_1[H^+]^2 + k_2[H^+]K_{a_{S,2}}}{(K_{a_p} + [H^+])(K_{a_{S,2}} + [H^+])}[Py_T][S_T] - (k_{-1} + k_{-2}[OH^-])[A_T] \qquad \text{(Eq. 9)}$$

Comparison of rate laws 5 and 9 shows that if this hypothesis is correct, plots of $\{k_f(K_{a_p} + [H^+])\}/[H^+]$ against $1/[H^+]$ and of k_b against $1/[H^+]$ should be linear between pH 3 and 5.5 with intercepts on the Y-axis greater than 0.

Plots of this type were obtained for addition to (I; R = R′ = H) and (I; R = H, R′ = Me). An example of results for addition to (I; R = R′ = H) at 25° is shown in Fig. 3. Least-squares treatments of the 8 data points for the forward reaction shown in Fig. 3 showed that they fell on a line with a slope of $2.46 \pm 0.03 \times 10^{-4}$ and with an intercept on the Y-axis (at $1/[H^+] = 0$) of 0.19 ± 0.08. These results indicated that attack of sulfite ion on the pyrimidinium ion ($k_2 = 2103\ M^{-1}$ sec.$^{-1}$) was a much more favorable reaction than attack of bisulfite ion on the pyrimidinium ion ($k_1 \simeq 0.19\ M^{-1}$ sec.$^{-1}$). Similarly, for the decomposition of the covalent adduct (V; R = R′ = H), the 8 data points shown in Fig. 3 lay on a line with slope $1.376 \pm 0.017 \times 10^{-7}$ and an intercept on the Y-axis of $1.03 \pm 0.46 \times 10^{-4}$. These results indicated that the second-order rate constant for the specific base-catalyzed decomposition of the adduct was $1.367 \times 10^7\ M^{-1}$ sec.$^{-1}$, while the first-order rate constant for the nonbase-catalyzed reaction was 10.31×10^{-5} sec.$^{-1}$. The consistency of this data with the proposed mechanisms was demonstrated by the close correspondence between the experimentally determined values of $k_{obs.}$, which are shown as circles in Fig. 2, and the solid lines which were calculated from values of k_1, k_{-1}, k_2, and k_{-2}.

Because of the large standard errors associated with k_1 and k_{-1} values, they could not be used to make meaningful calculations on the enthalpy and entropy of the reactions between bisulfite ion

[1] K_{a_p} and $K_{a_{S,2}}$ are the acid dissociation constants of the pyrimidinium ion and bisulfite ion, respectively. The concentration of H_2SO_3 has been neglected in this equation because it is very much less than $[HSO_3^-]$ between pH 3 and 5.

Table VI—Kinetic Data for Formation of Bisulfite Adducts by Attack of Sulfite Ion on the Pyrimidinium Ion (k_2) and the Base-Catalyzed Decomposition of the Adduct (k_{-2})

Augend	Temperature	k_2, M^{-1} sec.$^{-1}$	ΔH_2^*, kcal. M^{-1}	ΔS_2^*, cal. M^{-1}	$10^{-6}k$, M^{-1} sec.$^{-1}$	ΔH_{-2}^*, kcal. M^{-1}	ΔS_{-2}^*, cal. M^{-1}
I; R = R′ = H	12.0°	754			7.7		
	25.0°	2103	−11.0	−6.2	13.7	−7.7	−2.1
	37.0°	3935			23.2		
I; R = H, R′ = Me	25.0°	170			39.9		

Table VII—Kinetic Data for Formation and Decomposition of Bisulfite Adducts of II at 25°

pH	$10(k_{f_4} + k_{f_6})$, M^{-1} sec.$^{-1}$	$10^4\left(\frac{k_{b_4}K^* + k_{b_6}}{1 + K^*}\right)$ sec.$^{-1}$
3.36	5.00	9.98
3.53	7.60	15.2
3.74	11.4	22.7
3.93	17.6	35.1

and (I; R = R′ = H) or the nonbase-catalyzed decomposition of the adduct. However, the errors associated with k_2 and k_{-2} values were much less, and they were used in Arrhenius plots to calculate enthalpies and entropies of reaction between sulfite ion and (I; R = R′ = H) and for the specific base-catalyzed decomposition of the adduct (V; R = R′ = H). These values are listed in Table VI.

Whereas both bisulfite ion and sulfite ion appeared to be attacking nucleophiles in the addition reaction, it has been reported that the rate-determining step in additions to aldehydes and ketones (12) only involves the attack of sulfite ion on the neutral carbonyl compound. These differences probably arise because of the greater electrophilicity of the charged pyrimidinium ion compared to the uncharged carbonyl compound. This is exemplified by the fact that the second-order rate constant for attack of sulfite ion on neutral benzaldehyde is 240 M^{-1} at 13°, whereas it is 754 M^{-1} for attack on 2-aminopyrimidinium ion at 12°.

Kinetics of Reversible Addition of Bisulfite Ion to II—The changes in UV absorbance which followed mixing of aqueous buffered solutions of II (5.2×10^{-4} M) with solutions of sodium bisulfite ($4.0–20.0 \times 10^{-3}$ M) appeared to be similar to those observed for additions to (I; R = R′ = H) and (I; R = H, R′ = Me). Thus, plots against time of log $(D - D_\infty)$ values which had been calculated from measurements at a wavelength where II was the main absorbing species (303 mμ) were linear through at least two half-lives. Apparent first-order rate constants, $k_{obs.}$ values, were calculated from these types of plots.

The precise interpretation of the meaning of $k_{obs.}$ values for this reaction was complicated by the fact that addition to II yields the isomeric products III and IV, and their relative concentrations (≃1.3:1) at the time when most of the pyrimidine has been consumed is different from that (≃0.8:1) at thermodynamic equilibrium (4). In the extreme, the kinetics for approach to equilibrium of such a system would not be first order. However, because approximate first-order kinetics were observed, it was assumed that the initial reactions between II and bisulfite and sulfite ions led to a "pseudoequilibrium" system and that the subsequent isomerization of the products took place much more slowly.

On this basis, when $[S_T]_0 \gg [Py_T]_0$, the apparent first-order rate constant for approach to the "pseudoequilibrium" condition would be given by Eq. 6:

$$k_{obs.} = (k_{f_4} + k_{f_6})[S_T]_0 + \frac{k_{b_4}K^* + k_{b_6}}{1 + K^*} \qquad \text{(Eq. 10)}$$

where k_{f_4} and k_{f_6} are rate constants for addition at C_4 and C_6, respectively; k_{b_4} and k_{b_6} are the rate constants for decomposition of isomers III and IV, respectively; and $K^* = [III]/[IV]$ at "psuedo-equilibrium." This treatment was consistent with the experimental results, and plots of $k_{obs.}$ against $[S_T]_0$ were linear at several pH values and temperatures. Values of $(k_{f_4} + k_{f_6})$ and $(k_{b_4}K^* + k_{b_6})/(1 + K^*)$ were calculated from such plots and are listed in Table VII.

The pH dependence of $(k_{f_4} + k_{f_6})$ and $(k_{b_4}K^* + k_{b_6})/(1 + K^*)$ values appeared to be similar to that for addition to (I; R = R′ = H), and (I; R = H, R′ = Me) because plots of both $(k_{f_4} + k_{f_6})$ and $(k_{b_4}K^* + k_{b_6})/(1 + K^*)$ against $1/[H^+]$ at constant temperature were linear with intercepts on the Y-axis (when $1/[H^+] = 0$) greater than 0. Thus, it appears that the additions of bisulfite ion to II and decomposition of both III and IV occur by similar mechanisms to those described for addition to (I; R = R′ = H) and (I; R = H, R′ = Me).

The fact that [III]/[IV] ≃1.3 when most of II had been consumed, together with the observation that the pyrimidinium ion was apparently consumed in a first-order reaction, strongly suggests that addition to the carbon atom in II, which is adjacent to the alkylated nitrogen atom, occurs more rapidly than addition to the carbon atom adjacent to the nonalkylated nitrogen atom.

EXPERIMENTAL

Materials and Apparatus—2-Aminopyrimidine and 2-amino-4-methylpyrimidine (K & K Laboratories Inc.) were crystallized from petroleum ether and sublimed before use. 1,2-Dihydro-2-imino-1-methylpyrimidine hydrochloride was prepared as described by Brown and Harper (13), m.p. 277–278°. Sodium bisulfite solutions were prepared immediately before use by dissolving sodium pyrosulfite (Mallinckrodt A.R.) in freshly boiled and cooled distilled water through which oxygen-free nitrogen had been bubbled for 1 hr. One milliliter of methanol was added per 100 ml. of sodium bisulfite solution to stabilize it against oxidation (1). Final bisulfite concentrations were determined by iodometric titration. All solutions were made with freshly boiled and cooled distilled water through which oxygen-free nitrogen had been bubbled for 1 hr.

Spectrophotometric measurements were made on Cary 14 and 16 UV spectrophotometers. Temperatures were controlled to within 0.01° with circulating water from a Lo-temptral 154 water bath (Precision Scientific Co.). The pH values were measured using an Orion model 801/digital pH meter.

Isolation of Bisulfite Adducts—The 1:1 bisulfite adducts of the pyrimidinium ions (I; R = R′ = H) and (I; R = H, R′ = Me) were obtained as white microcrystalline powders following addition of sodium bisulfite (1M) to saturated aqueous solutions of the pyrimidines at 25°. After washing the powders with a little water and drying over calcium chloride at atmospheric pressure, the adducts had the following elemental analysis.

Anal.—Calcd. for 2-amino-1,6-dihydropyrimidinium-6-sulfonate (V; R = R′ = H): C, 27.12; H, 3.98; S, 18.10. Found: C, 27.15; H, 4.12; S, 18.33. Calcd. for 2-amino-1,6-dihydro-4-methylpyrimidinium-6-sulfonate (V; R = H, R′ = Me): C, 31.40; H, 4.73; N, 21.98; S, 16.77. Found: C, 31.18; H, 4.82; N, 21.70; S, 16.88.

Equilibrium Constants—The pKa values were measured by potentiometric titration using the method described by Albert and Serjeant (14). The ionic strength was maintained at 10^{-1} M with sodium chloride. Equilibrium constants for addition of bisulfite ion to the pyrimidinium ions were calculated from the equilibrium concentrations of pyrimidinium ion, bisulfite ion, and covalent adduct which existed following mixing of equal volumes of solutions of the pyrimidine in borax–succinic acid buffers and sodium bisulfite solutions. The ionic strength of all reaction mixtures was maintained at 10^{-1} M with sodium chloride. The equilibrium concentrations were calculated from spectrophotometric measurements at wavelengths where the pyrimidine or pyrimidinium ion was the main absorbing species, and corrections were made for the absorbance of the other species.

Kinetics—Rate constants were calculated from spectrophotometric measurements of concentration changes. Standard kinetic procedures were followed. The ionic strength of all reaction mixtures was maintained at 10^{-1} *M* with sodium chloride.

CONCLUSIONS

1. Covalent addition of bisulfite ion occurs to Position C_4 or C_6 of 2-aminopyrimidinium ion derivatives so long as these positions are not blocked by alkyl substituents and the pH of the system is such that the reactants exist to an appreciable extent as the monoanion (HSO_3^-) and the cation (pyrimidinium ion).
2. Alkyl substituents at any position in the pyrimidinium ring reduce the extent and rate of addition.
3. The covalent adducts are zwitterions, between pH 3 and 5, and have very low water solubility.
4. The mechanism of addition appears to involve attack of both the bisulfite ion and sulfite ion on the pyrimidinium ion. The reverse reaction involves both a nonbase-catalyzed and a base-catalyzed decomposition of the neutral adduct.

REFERENCES

(1) L. C. Schroeter, "Sulfur Dioxide," 1st ed., Pergamon, New York, N. Y., 1966.

(2) N. O. Kaplan, in "The Enzymes," 2nd ed., vol. 3, P. D. Boyer, H. Hardy, and K. Myrbäck, Eds., Academic, New York, N. Y., 1960, pp. 134–135.

(3) D. D. Perrin and I. H. Pitman, *J. Chem. Soc.*, **1965**, 7071.

(4) I. H. Pitman, E. Shefter, and M. Ziser, to be published.

(5) A. Albert and J. J. McCormack, *J. Chem. Soc.*, **1965**, 6930.

(6) M. Viscontini and H. R. Weilenmann, *Helv. Chim. Acta*, **42**, 1854(1959).

(7) C. Van Baalen and H. S. Forrest, *J. Amer. Chem. Soc.*, **81**, 1770(1959).

(8) D. J. Vonderschmitt, K. S. Vitols, F. M. Huennekens, and K. G. Scrimgeour, *Arch. Biochem. Biophys.*, **122**, 488(1967).

(9) T. Higashino, *J. Pharm. Soc. Jap.*, **80**, 245(1960).

(10) W. L. F. Armarego and E. Spinner, *J. Chem. Soc.*, **1961**, 2689.

(11) A. Albert and W. L. F. Armarego, *Advan. Heterocycl. Chem.*, **4**, 1(1965).

(12) C. A. Bishop, R. F. Porter, and K. K. J. Tong, *J. Amer. Chem. Soc.*, **399**, 85(1963).

(13) D. J. Brown and J. S. Harper, *J. Chem. Soc.*, **1963**, 1276.

(14) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen and Co., Ltd., London, England.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 30, 1970, from the *School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication March 18, 1970.

This study was supported in part by a general research grant from the University of Kansas (No. 3475-5038) and NSF Undergraduate Research Participant Grant No. 4210.

* To whom requests for reprints should be directed.

Kinetics of the Hydrolysis of Pilocarpine in Aqueous Solution

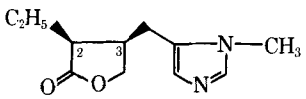
PING-HONG CHUNG, TING-FONG CHIN, and JOHN L. LACH

Abstract □ The kinetics of the hydrolysis of pilocarpine in aqueous solution were investigated utilizing pH-stat titrimetry and polarimetry. A cyclic mechanism was proposed for the hydrolysis, which is catalyzed by both hydrogen ion and hydroxide ion. The appropriate rate constants, equilibrium constants, and the energy of activation for the hydroxide-ion catalyzed hydrolysis were calculated. The hydrolysis in high alkaline pH was found to be accompanied by some epimerization. An optimum condition for the preparation of pilocarpine ophthalmic solution was also suggested.

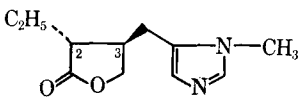
Keyphrases □ Pilocarpine in aqueous solution—hydrolysis □ Kinetics—pilocarpine hydrolysis □ Hydrolysis, pilocarpine—hydrogen-ion catalyzed □ TLC—separation □ UV spectrophotometry—identity □ Titration, pH-stat—pilocarpine degradation determination □ Polarimetry—pilocarpine cyclization

Pilocarpine is used topically as a miotic in the treatment of glaucoma. The isomer, isopilocarpine, although qualitatively similar in its pharmacological effects, is almost completely inactive as a miotic (1). However, little clinical data are available concerning isopilocarpine. Pilocarpine solutions ranging from 0.5 to 10% have been used, although there seems to be no advantage in concentrations above 4%. Pilocarpine is less irritating than physostigmine salicylate and can be employed for long periods without producing undesirable side effects (2).

Although pilocarpine was isolated early in 1875, its structure and that of the isomer were elucidated by Jowett (3, 4) in 1900; total synthesis was achieved by several workers in different ways during the 1930's (5–7). In 1966, the absolute configurations of these compounds were established as (2S:3R) (I) and (2R:3R) (II) for pilocarpine and isopilocarpine, respectively (8).



pilocarpine (2S:3R)
I



isopilocarpine (2R:3R)
II

Pilocarpine possesses a γ-lactone with two asymmetric centers. In the dry state and at high temperature, the two isomers, pilocarpine and isopilocarpine, interconvert, isopilocarpine predominating at equilibrium (8). This property has been used for the preparation of isopilocarpine hydrochloride from pilocarpine hydrochloride (9). It has been shown recently that, in the presence of alkali, isomerization may proceed *via* the enol intermediate (10).

In aqueous solution, pilocarpine presents at least two possible pathways of degradation, including hy-

Kinetics—Rate constants were calculated from spectrophotometric measurements of concentration changes. Standard kinetic procedures were followed. The ionic strength of all reaction mixtures was maintained at 10^{-1} *M* with sodium chloride.

CONCLUSIONS

1. Covalent addition of bisulfite ion occurs to Position C_4 or C_6 of 2-aminopyrimidinium ion derivatives so long as these positions are not blocked by alkyl substituents and the pH of the system is such that the reactants exist to an appreciable extent as the monoanion (HSO_3^-) and the cation (pyrimidinium ion).
2. Alkyl substituents at any position in the pyrimidinium ring reduce the extent and rate of addition.
3. The covalent adducts are zwitterions, between pH 3 and 5, and have very low water solubility.
4. The mechanism of addition appears to involve attack of both the bisulfite ion and sulfite ion on the pyrimidinium ion. The reverse reaction involves both a nonbase-catalyzed and a base-catalyzed decomposition of the neutral adduct.

REFERENCES

(1) L. C. Schroeter, "Sulfur Dioxide," 1st ed., Pergamon, New York, N. Y., 1966.

(2) N. O. Kaplan, in "The Enzymes," 2nd ed., vol. 3, P. D. Boyer, H. Hardy, and K. Myrbäck, Eds., Academic, New York, N. Y., 1960, pp. 134–135.

(3) D. D. Perrin and I. H. Pitman, *J. Chem. Soc.*, **1965**, 7071.

(4) I. H. Pitman, E. Shefter, and M. Ziser, to be published.

(5) A. Albert and J. J. McCormack, *J. Chem. Soc.*, **1965**, 6930.

(6) M. Viscontini and H. R. Weilenmann, *Helv. Chim. Acta*, **42**, 1854(1959).

(7) C. Van Baalen and H. S. Forrest, *J. Amer. Chem. Soc.*, **81**, 1770(1959).

(8) D. J. Vonderschmitt, K. S. Vitols, F. M. Huennekens, and K. G. Scrimgeour, *Arch. Biochem. Biophys.*, **122**, 488(1967).

(9) T. Higashino, *J. Pharm. Soc. Jap.*, **80**, 245(1960).

(10) W. L. F. Armarego and E. Spinner, *J. Chem. Soc.*, **1961**, 2689.

(11) A. Albert and W. L. F. Armarego, *Advan. Heterocycl. Chem.*, **4**, 1(1965).

(12) C. A. Bishop, R. F. Porter, and K. K. J. Tong, *J. Amer. Chem. Soc.*, **399**, 85(1963).

(13) D. J. Brown and J. S. Harper, *J. Chem. Soc.*, **1963**, 1276.

(14) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen and Co., Ltd., London, England.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 30, 1970, from the *School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication March 18, 1970.

This study was supported in part by a general research grant from the University of Kansas (No. 3475-5038) and NSF Undergraduate Research Participant Grant No. 4210.

* To whom requests for reprints should be directed.

Kinetics of the Hydrolysis of Pilocarpine in Aqueous Solution

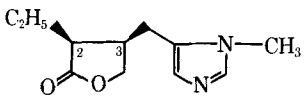
PING-HONG CHUNG, TING-FONG CHIN, and JOHN L. LACH

Abstract □ The kinetics of the hydrolysis of pilocarpine in aqueous solution were investigated utilizing pH-stat titrimetry and polarimetry. A cyclic mechanism was proposed for the hydrolysis, which is catalyzed by both hydrogen ion and hydroxide ion. The appropriate rate constants, equilibrium constants, and the energy of activation for the hydroxide-ion catalyzed hydrolysis were calculated. The hydrolysis in high alkaline pH was found to be accompanied by some epimerization. An optimum condition for the preparation of pilocarpine ophthalmic solution was also suggested.

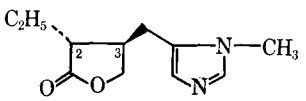
Keyphrases □ Pilocarpine in aqueous solution—hydrolysis □ Kinetics—pilocarpine hydrolysis □ Hydrolysis, pilocarpine—hydrogen-ion catalyzed □ TLC—separation □ UV spectrophotometry—identity □ Titration, pH-stat—pilocarpine degradation determination □ Polarimetry—pilocarpine cyclization

Pilocarpine is used topically as a miotic in the treatment of glaucoma. The isomer, isopilocarpine, although qualitatively similar in its pharmacological effects, is almost completely inactive as a miotic (1). However, little clinical data are available concerning isopilocarpine. Pilocarpine solutions ranging from 0.5 to 10% have been used, although there seems to be no advantage in concentrations above 4%. Pilocarpine is less irritating than physostigmine salicylate and can be employed for long periods without producing undesirable side effects (2).

Although pilocarpine was isolated early in 1875, its structure and that of the isomer were elucidated by Jowett (3, 4) in 1900; total synthesis was achieved by several workers in different ways during the 1930's (5–7). In 1966, the absolute configurations of these compounds were established as (2S:3R) (I) and (2R:3R) (II) for pilocarpine and isopilocarpine, respectively (8).



pilocarpine (2S:3R)
I



isopilocarpine (2R:3R)
II

Pilocarpine possesses a γ-lactone with two asymmetric centers. In the dry state and at high temperature, the two isomers, pilocarpine and isopilocarpine, interconvert, isopilocarpine predominating at equilibrium (8). This property has been used for the preparation of isopilocarpine hydrochloride from pilocarpine hydrochloride (9). It has been shown recently that, in the presence of alkali, isomerization may proceed *via* the enol intermediate (10).

In aqueous solution, pilocarpine presents at least two possible pathways of degradation, including hy-

drolysis to pilocarpic acid and epimerization to isopilocarpine. The most important of these is the opening of the lactone ring. As long as the lactone is intact, the alkaloid maintains its stereochemical configuration (11, 12).

Pilocarpine is relatively stable in solutions of acidic pH (12–14). As the pH increases, pilocarpine progressively becomes unstable (12, 13, 15, 16), especially at elevated temperatures. Its stability has also been shown to be affected by certain buffers. Phosphate and carbonate catalyze the degradation of pilocarpine, whereas borate does not (16, 17). Additions of 0.5% of methylcellulose slightly improve the stability of pilocarpine solutions (18). In all cases, the degradation has been accompanied by a marked drop in pH (14, 16–18), phosphate buffers failing to maintain a constant pH (17).

Since stability studies appearing in the literature were of a preliminary nature, providing limited kinetic information, the purpose of this study was to obtain additional data dealing with the rates of hydrolysis and the mechanisms involved.

EXPERIMENTAL

Reagents—The following were used: pilocarpine nitrate USP; hydrochloric acid, reagent grade; sodium hydroxide, reagent grade; potassium chloride, reagent grade; dibasic potassium phosphate trihydrate, reagent grade; monobasic potassium phosphate, reagent grade; citric acid monohydrate USP; silica gel G, TLC grade; Whatman No. 4 chromatography paper (4 cm. × 25 cm.); methanol, reagent grade; chloroform, reagent grade; and *n*-butanol, reagent grade.

Apparatus—The following were used: radiometer pH-stat (a combination of type TTT1c titrator, SBR2c titrigraph, SBU1a syringe burette, and TTA3 titration assembly), with G2222C glass electrode, K4122 calomel electrode, and B101 0.5-ml. syringe; modified pH-stat titration assembly (19), with G202C glass electrode and K401 calomel electrode; Precision Scientific Co. Temp-Trol circulating system; radiometer PHM4c pH meter; Zeiss polarimeter with sodium lamp, 589-mμ interference filter, and 40-cm. tube; and Beckman DK-2 recording spectrophotometer.

Procedure—(*a*) *Preliminary Test*—A 25-ml. portion of an aqueous 2% pilocarpine nitrate solution was placed in each of three 50-ml. volumetric flasks. One of these was brought to strong alkalinity with sodium hydroxide; another was brought to strong acidity with hydrochloric acid. The flasks were then made up to 50 ml. with water and heated in a boiling water bath for 3 hr. After cooling, the solutions were neutralized to approximately the same pH and were chromatographed using TLC [plates: microscope slides coated with a slurry of 30 g. silica gel G in 65 ml. chloroform–methanol (3:1 v/v) and activated at 100° for 1 hr.; solvent system: chloroform–methanol (1:1 v/v); spots located using iodine vapor] and paper chromatography (paper: 4-cm. × 25-cm. Whatman No. 4 filter paper impregnated with 0.2 *M* pH 6.8 phosphate buffer prepared by mixing approximately equal volumes of solutions of 0.2 *M* KH_2PO_4 and 0.2 *M* K_2HPO_4; mobile phase: water-saturated *n*-butanol; spots located using iodine vapor).

(*b*) *Hydroxide-Ion Catalyzed Hydrolysis*—The pH-stat was utilized to follow hydrolytic degradation. Fifty milliliters of water and an appropriate quantity of potassium chloride (for adjusting ionic strength to 0.1) were placed in the reaction vessel, which was then flushed with nitrogen and sealed from contact with the atmosphere in the manner recommended by Chong *et al.* (19). As the temperature reached the desired value, the pH-stat was turned on to adjust the pH to the preset value. A 0.5-ml. aliquot of stock solution (sample) was introduced into the vessel. When the desired pH equilibrium was achieved, the recording pen was returned to zero to start recording the rate of reaction, and the reaction was allowed to proceed either to completion (for pH 11–12) or until sufficient data were obtained (for pH 8–10). The pseudo-first-order rate constants were then obtained either from a Guggenheim plot or the initial rate method.

(*c*) *Hydrogen-Ion Catalyzed Hydrolysis*—Since the hydrogen-ion catalyzed hydrolysis is negligibly small in comparison to the opposing cyclization reaction, comparable information would be obtained more easily and accurately by following the rate of cyclization. A polarimeter was used to follow the rate of cyclization, because there is a large change in optical activity when pilocarpic acid is cyclized to pilocarpine.

RESULTS AND DISCUSSION

In the preliminary study, as would be expected, all of the heat-treated solutions showed no characteristic absorption in the UV region, except that all exhibited a comparable magnitude of end-absorption. However, it should be noted here that pilocarpine does exhibit a maximum at 215 mμ. The thin-layer chromatograms for the heat-treated acidic and neutral (pilocarpine nitrate solution without addition of acid or alkali) solutions showed a single spot, which had the same R_f value as the reference spot, whereas that for the heat-treated alkaline solution exhibited a much smaller R_f value in spite of producing a single spot. This did indicate that pilocarpine is stable in acidic solution but labile in alkaline solution. By repeating the chromatographic separation in a 0.2 *M* pH 6.8 phosphate buffer-treated paper, using water-saturated *n*-butanol as the mobile phase, the alkaline degraded spot and the reference spot showed significantly different and reproducible R_f values, 0.19 and 0.88, respectively. Repeating the test using weaker alkali, the degraded solution displayed two spots with R_f values of 0.19 and 0.88, respectively, indicating only partial degradation as opposed to one spot in strongly alkaline degraded solution. Authentic pilocarpine and isopilocarpine, when chromatographed by the same techniques, showed, however, almost the same R_f value. Therefore, the described chromatographic techniques could not separate the two isomers and epimerization, if present, would not be detected.

From the titration curves, the heat-treated acidic and neutral solutions showed a single pKa value, 6.85, which is similar to that of pilocarpine nitrate; on the other hand, the alkaline degraded solution, when acidified with hydrochloric acid and titrated immediately with alkali, gave two pKa values, 4.05 and 7.50, respectively. However, if the acidified degraded solution was allowed to stand for several hours, the amount of alkali consumed in the titration for the pKa_1 value, 4.05, was decreased, but that for the pKa_2 value, 7.50, was not affected, except that pKa_2 was shifted toward 6.85. This decrease in alkali consumption probably indicates that the free carboxylic acid group in the degraded solution has undergone cyclization.

When freshly prepared pilocarpine base was dissolved in water and immediately titrated with hydrochloric acid, a pKa value of 6.85 for the conjugate acid was produced. However, upon standing, the same solution of pilocarpine base generated an additional inflection in its titration curve, but the equivalent amount of total acid consumed was unchanged, indicating that an acid created from lactone hydrolysis immediately protonated on the original basic group of the molecule to form a zwitterion.

These observed phenomena are consistent with the fact that the lactone ring in the pilocarpine molecule was hydrolyzed in an alkaline condition and subsequently recyclized when acidified. This has also been confirmed by Baeschlin *et al.* (20). Since the hydrolysis of a lactone produces an acid and since reaction at a constant pH is desirable, a pH-stat that not only keeps the pH constant but also records the rate of reaction was used to follow the hydroxide-ion catalyzed hydrolysis of pilocarpine. The theory behind the use of a pH-stat was discussed by Jacobsen *et al.* (21) and will not be discussed here.

For hydroxide-ion catalyzed hydrolysis, a radiometer pH-stat with a closed-type reaction vessel, as suggested by Chong *et al.* (19), and a Precision Scientific Temp-Trol circulating system were used. In the 11–12 pH range, the entire course of the reaction was recorded; pseudo-first-order rate constants were obtained either from Guggenheim plots (22) or by the differential method (22). Both methods were equally satisfactory as applied to the result in this pH range. At pH 8–10, the reaction at each pH level studied was allowed to proceed until sufficient data were obtained. This procedure was used since the recording of the entire course of the reaction would have taken more than 1 day to complete. The initial rate method was used to obtain the pseudo-first-order rate constants. Since all initial rate *versus* initial concentration plots resulted in straight lines at constant pH, the rate law would have the follow-

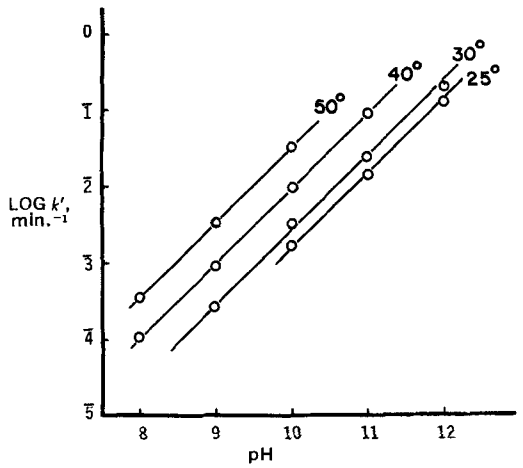


Figure 1—*pH profile of the hydrolysis of pilocarpine in alkaline pH.*

ing form:

$$\frac{-d\,(\text{pilocarpine})}{dt} = k'\,(\text{pilocarpine}) \qquad \text{(Eq. 1)}$$

where k' is the pseudo-first-order rate constant. When log k' was plotted against pH, a straight line was obtained with a slope of 1. Therefore, the rate law for the hydroxide-ion catalyzed hydrolysis of pilocarpine would be:

$$-\frac{d\,(\text{pilocarpine})}{dt} = k_{OH^-}\,(\text{pilocarpine})\,(OH^-) \qquad \text{(Eq. 2)}$$

where k_{OH^-} is the second-order rate constant for the specific hydroxide-ion catalysis.

At pH 7, however, the rate constant was found to be slightly smaller than expected. Intuitively, it could be argued that the *N*-methylimidazole moiety could be acting as a catalyst. Consequently, the possibility of intermolecular catalysis was investigated, using added quantities of *N*-methylimidazole. At pH 10 and up to a concentration of 0.1 *M* of *N*-methylimidazole, no appreciable catalytic effect was observed. (At this pH, *N*-methylimidazole exists entirely in its basic form.)

The effect of ionic strength on alkaline hydrolysis of pilocarpine was also studied at this pH, and no noticeable effect up to the 1.0 *M* potassium chloride level was observed.

This rate constant deviation at pH 7, therefore, probably is due to other facts. For example, the opposing (cyclization) reaction may become significant at this pH, or it may be due to the limitation of pH-stat performance at high buffer capacity and very slow reaction rate. Since recording of sufficient data at this pH would take almost 1 day, the drift in electrode potential could cause a significant error. It should be pointed out that the error recorded by a pH-stat is proportional to the buffer capacity of the system and the magnitude of the potential drift (21).

The temperature effect at different pH's for the hydroxide-ion catalyzed hydrolysis was also studied at 25, 30, 40, and 50°. The pH profiles at these temperatures are shown in Fig. 1 and are consistent with the mentioned rate law. Arrhenius plots for the two pH levels, 9 and 10, are shown in Fig. 2. Both plots show reasonable straight lines of the same slope. The energy of activation was found to be 11.0 kcal./mole after correction for enthalpy of ionization of water. This is in good agreement with the 10.8–12.1 kcal./mole values, as reported by Grace and Symons (23), for the hydroxide-ion catalyzed hydrolysis of substituted and unsubstituted γ-butyrolactones.

In acidic pH, however, pilocarpine is fairly stable. As pointed out by Grace and Symons (23), most γ-lactones are not hydrolyzed appreciably in acid. Therefore, it is difficult to follow the hydrogen-ion catalyzed hydrolysis directly. However, since Long *et al.* (24) reported that the hydrolysis of γ-lactone was catalyzed by hydrogen ion, and since the cleaved acid would recyclize to the lactone in acidic pH, it can be expected that the rate expression would take the form of opposing reactions. Since these reactions would reach equilibrium after a period of time, and since the equilibrium position would lie far toward the lactone in acidic pH, it would be simpler to follow the rate of cyclization than to follow the rate of hydrolysis to obtain this information.

In view of the fact that the opening of the lactone ring in a pilocarpine molecule is accompanied by a large drop in its optical activity (to about 20% of its original value), the rate of cyclization was followed by measuring the optical rotation change with a polarimeter.

To determine the dependence of optical activity on pH, 0.0625 *M* solutions of pilocarpine, which give a reading of about 5° in a 40-cm. polarimeter tube, and similar solutions that had been hydrolyzed completely at pH 12 were adjusted to constant ionic strength (0.2) and to different pH values. Their optical rotations were measured immediately after the adjustment of pH. The optical rotation–pH profiles obtained are shown in Fig. 3. In this figure, it can be clearly seen that the optical rotation–pH profile for the hydrolyzed product, pilocarpic acid, exhibits two inflections which correspond exactly to its two pKa values. This is obvious if the titration curve is superimposed on the optical rotation–pH profile (Fig. 4). In fact, this provides another way for the determination of pKa values of a compound possessing optical activity. This approach is superior to the potentiometric method, since it is not affected by the acidity or alkalinity of the solvent. The presence of optical activity in the completely hydrolyzed product also revealed that the assumption made by Anderson (17), which states that "all products of the degradation other than isopilocarpine have no optical activity," was not valid.

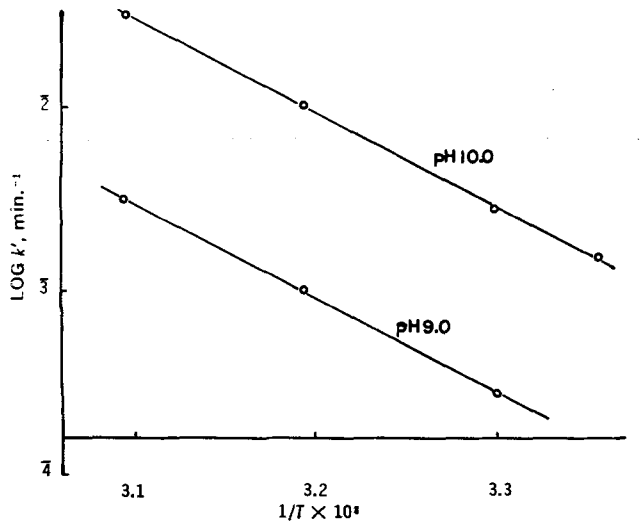


Figure 2—*Arrhenius plot of hydroxide-ion catalyzed hydrolysis of pilocarpine.*

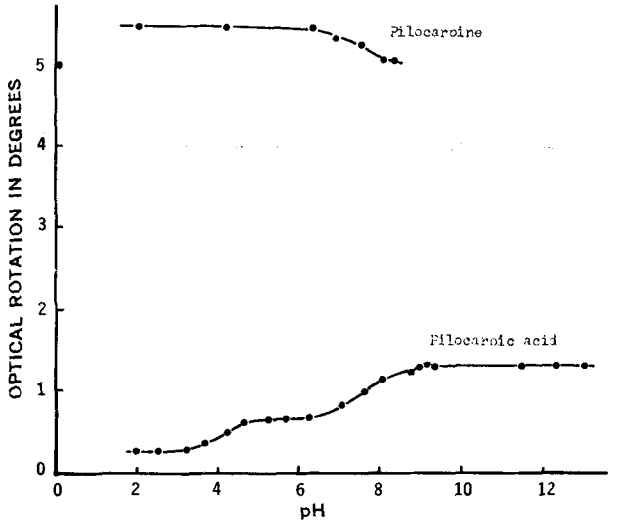
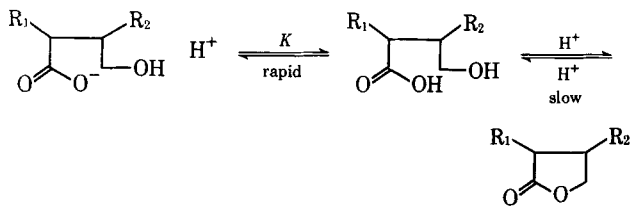


Figure 3—*Optical rotation–pH profile of pilocarpine and pilocarpic acid.*

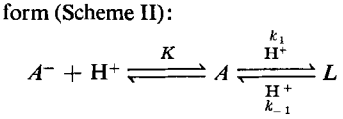
To follow the rate of cyclization, a pilocarpine solution was hydrolyzed at pH 12 for 1 hr. to achieve complete hydrolysis. The hydrolyzed solution was then brought to acidic pH with hydrochloric acid and diluted to a final concentration corresponding to that of a 0.0625 *M* hydrolyzed pilocarpine solution. The pH of the solution was measured, and the cyclization was immediately followed by measuring the optical rotation at different times. These results are shown in Fig. 5. As cyclization proceeds, the optical rotation increases rapidly at first, then gradually slows down, and finally converges to a fixed value which is smaller than the original optical rotation of the corresponding concentration of pilocarpine (Fig. 5). This could indicate that the opposing reactions, namely cyclization and hydrolysis, have attained an equilibrium. Since the rate of the optical rotation change is higher at lower pH, both reactions would be catalyzed by hydrogen ion. If this statement is valid, one should also be able to attain this equilibrium by starting with pilocarpine instead of pilocarpic acid. However, it has been found that the equilibrium optical rotation obtained from pilocarpine is higher than that obtained from pilocarpic acid (Fig. 5). When analyzed for lactone, both equilibrium solutions showed almost equal amounts of lactone remaining, clearly indicating that some epimerization occurred during or after hydrolysis. From the work of Døpke and d'Heureuse (10) and from the fact that the equilibrium optical rotation of the cyclized solution was the same no matter how long the starting compound, pilocarpic acid, remained in an alkaline condition, the former is more likely the case. A study of the extent of epimerization is being conducted in this laboratory and will be reported later.

Assuming that cyclization of pure pilocarpic acid is followed, the simplified mechanism of cyclization may be stated as in Scheme I:



Scheme I

or in symbolic form (Scheme II):



Scheme II

where A^-, A, and L represent pilocarpate, pilocarpic acid, and pilocarpine, respectively; K is the association constant of pilocarpic acid (*i.e.*, $K = 1/K_a$); and k_1 and k_{-1} represent the rate constants of the hydrogen-ion catalyzed cyclization and hydrolysis, respectively (*i.e.*, $k_1 = k_{-H^+}$, and $k_{-1} = k_{H^+}$).

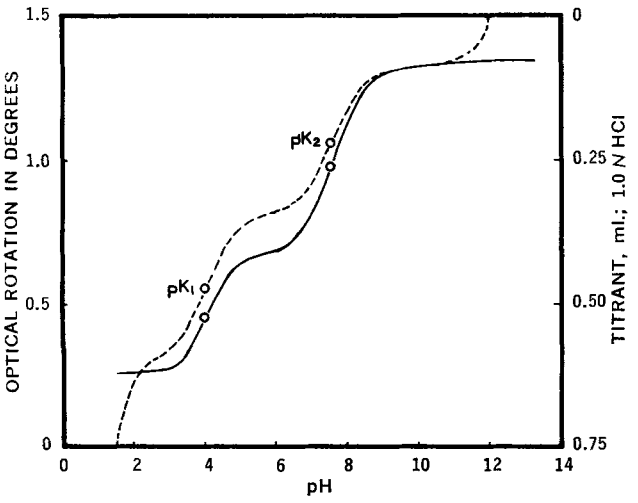


Figure 4—*Optical rotation–pH profile and titration curve of pilocarpic acid. Key: - - - -, titration curve; and —, optical rotation–pH profile.*

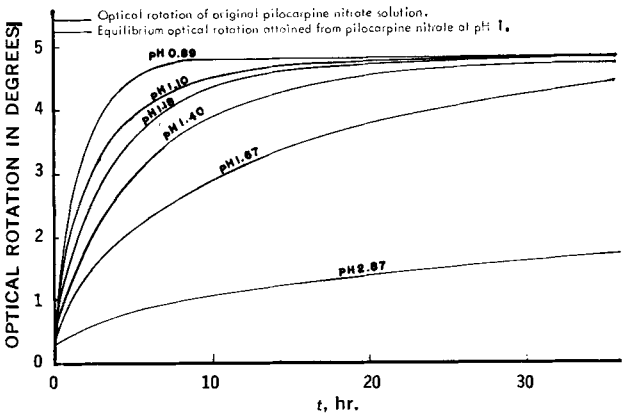


Figure 5—*Optical rotation* versus *time plot for cyclization of pilocarpic acid.*

If one starts with an initial concentration, C_o, then the material balance equation is

$$C_o = (A^-) + (A) + (L) = (A_T) + (L) \qquad \text{(Eq. 3)}$$

where $(A_T) = (A^-) + (A)$. (A) can be expressed in terms of C_o and (L):

$$(A) = \frac{K(H^+)}{K(H^+) + 1}[C_o - (L)] = F[C_o - (L)] \qquad \text{(Eq. 4)}$$

where $F = K(H^+)/[K(H^+) + 1]$.

The rate of formation of pilocarpine is

$$\frac{d(L)}{dt} = k_1(H^+)(A) - k_{-1}(H^+)(L) \qquad \text{(Eq. 5)}$$

Combination of Eqs. 4 and 5 gives

$$\frac{d(L)}{dt} = k_1F(H^+)C_o - (k_1F + k_{-1})(H^+)(L) \qquad \text{(Eq. 6)}$$

which can then be integrated if the pH is kept constant. Upon integration of this equation, using the fact that $(L) = 0$ when $t = 0$, at constant hydrogen-ion concentration, gives

$$\ln \frac{k_1FC_o}{k_1FC_o - (k_1F + k_{-1})(L)_t} = (k_1F + k_{-1})(H^+)t \qquad \text{(Eq. 7)}$$

At equilibrium, $d(L)/dt = 0$, and Eq. 6 gives

$$(L)_{eq} = \frac{k_1F}{k_1F + k_{-1}}C_o \qquad \text{(Eq. 8)}$$

Combination of Eqs. 7 and 8 gives rise to

$$\ln \frac{(L)_{eq}}{(L)_{eq} - (L)_t} = (k_1F + k_{-1})(H^+)t \qquad \text{(Eq. 9)}$$

From the titration curve of pilocarpic acid, K was found to be 1.12×10^4 l./mole.

At pH $\leq$ 2, $F = K(H^+)/[K(H^+) + 1] = 1$, and Eq. 9 can be reduced to

$$\ln \frac{(L)_{eq}}{(L)_{eq} - (L)_t} = (k_1 + k_{-1})(H^+)t \qquad \text{(Eq. 10)}$$

Equation 10 states that a plot of $\log [(L)_{eq}/\{(L)_{eq} - (L)_t\}]$ *versus* t will be a straight line, and the slope is given by

$$\text{slope} = (k_1 + k_{-1})(H^+)/2.303 \qquad \text{(Eq. 11)}$$

It should be noted that this slope is hydrogen-ion concentration dependent.

The quantity "$(L)_{eq}/[(L)_{eq} - (L)_t]$" can be determined from optical rotation measurements, as derived from Scheme III:

$$A^- + H^+ \underset{}{\overset{K}{\rightleftharpoons}} A \underset{H^+}{\overset{H^+}{\rightleftharpoons}} L$$

Scheme III

From this mechanism, the material balance can be written as:

$$C_o = (A^-) + (A) + (L) = (A_T)_t + (L)_t = (A_T)_{eq} + (L)_{eq} \quad \text{(Eq. 12)}$$

for any time t and at equilibrium. For convenience, the following terms are defined:

$[\alpha]_L$ = optical rotation of pilocarpine with concentration C_o
$[\alpha]_{AT}$ = optical rotation of 100% hydrolyzed pilocarpine solution of the same concentration
R_t = optical rotation of sample solution at time t
R_{eq} = optical rotation of sample solution at equilibrium

Since optical rotation is an additive property of a system, the optical rotation of the sample solution can be considered as having contribution from the optically active species present. Thus,

$$R_t = [\alpha]_{AT} \cdot \frac{(A_T)_t}{C_o} + [\alpha]_L \cdot \frac{(L)_t}{C_o} \quad \text{(Eq. 13)}$$

$$R_{eq} = [\alpha]_{AT} \cdot \frac{(A_T)_{eq}}{C_o} + [\alpha]_L \cdot \frac{(L)_{eq}}{C_o} \quad \text{(Eq. 14)}$$

Subtraction of Eq. 13 from Eq. 14 leads to

$$R_{eq} - R_t = \frac{1}{C_o}[[\alpha]_{AT}\{(A_T)_{eq} - (A_T)_t\} + [\alpha]_L\{(L)_{eq} - (L)_t\}] \quad \text{(Eq. 15)}$$

Rearrangement of Eq. 12 produces

$$(A_T)_t - (A_T)_{eq} = (L)_{eq} - (L)_t \quad \text{(Eq. 16)}$$

Combination of Eqs. 15 and 16 renders

$$(L)_{eq} - (L)_t = \frac{C_o}{[\alpha]_L - [\alpha]_{AT}} \cdot (R_{eq} - R_t) \quad \text{(Eq. 17)}$$

$(L) = 0$ when $5 = 0$, and $R_t = R_o$. As a result, Eq. 17 is reduced to

$$(L)_{eq} = \frac{C_o}{[\alpha]_L - [\alpha]_{AT}} \cdot (R_{eq} - R_o) \quad \text{(Eq. 18)}$$

Dividing Eq. 18 by Eq. 17 gives

$$\frac{(L)_{eq}}{(L)_{eq} - (L)_t} = \frac{R_{eq} - R_o}{R_{eq} - R_t} \quad \text{(Eq. 19)}$$

Equation 19 suggests that the quantity "$(L)_{eq}/[(L)_{eq} - (L)_t]$" can be obtained from measurement of the optical rotation of the sample solution at the beginning, at any time t, and at equilibrium, without knowing the concentration.

In the 100% hydrolyzed solution of pilocarpine, isopilocarpic acid is present in much smaller amounts than pilocarpic acid. Since there is a smaller contribution to optical rotation change when isopilocarpic acid is cyclized (about one-half of the value when pilocarpic acid is cyclized), and since isopilocarpic acid cyclizes at a much faster rate ($t_{1/2}$ = 182 sec. at pH 1) (25), to the first approximation, the derived equations can be used to obtain information about cyclization of pilocarpic acid in the hydrolyzed pilocarpine solution.

Thus, the plots of log $[(L)_{eq}/\{(L)_{eq} - (L)_t\}]$ *versus* t for different pH values are illustrated in Fig. 6. Reasonable straight lines are indicated.

Taking the logarithm of Eq. 11 yields

$$\log (\text{slope}) = \log [(k_1 + k_{-1})/2.303] - \text{pH} \quad \text{(Eq. 20)}$$

which implies that a plot of log (slope) against pH will have a straight line with a slope equal to -1, with intercept being log $[(k_1 + k_{-1})/2.303]$. This plot is shown in Fig. 7, and the $(k_1 + k_{-1})$ so obtained is 4.10 l./mole/hr.

At pH greater than 2, Eq. 20 will no longer hold, since $F = K(H^+)/[K(H^+) + 1] \neq 1$, and the slope is now given by

$$\text{slope} = \left[k_1 \frac{K(H^+)}{K(H^+) + 1} + k_{-1}\right] (H^+)/2.303 \quad \text{(Eq. 21)}$$

Upon rearrangement of Eq. 21, Eq. 22 is obtained:

$$\frac{2.303\ (\text{slope})}{(H^+)} = k_1 \frac{K(H^+)}{K(H^+) + 1} + k_{-1} \quad \text{(Eq. 22)}$$

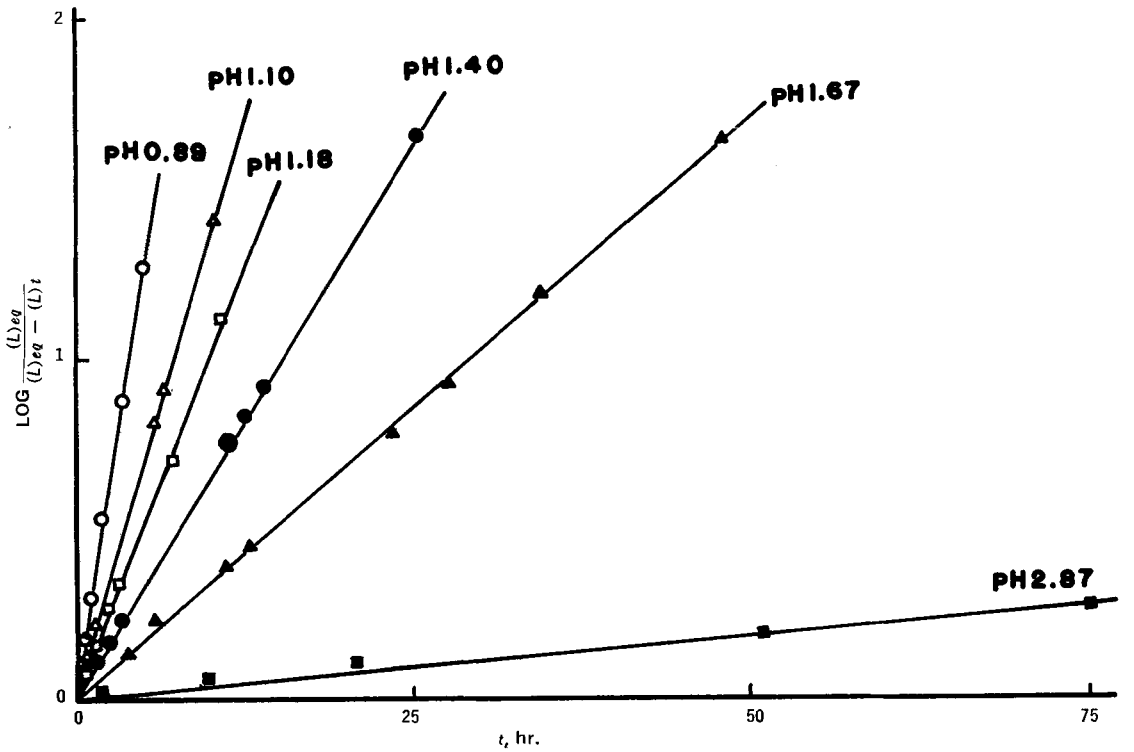


Figure 6—*Cyclization of pilocarpic acid at different pH's.*

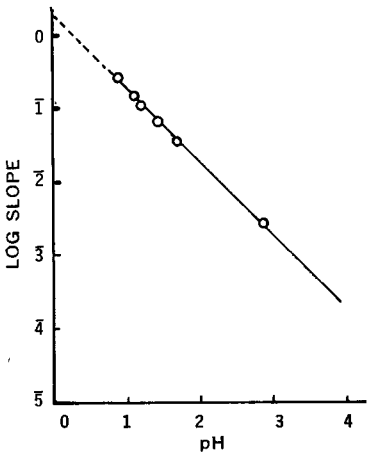


Figure 7—*pH profile of the hydrolysis of pilocarpine in acid pH (log slope* versus *pH plot).*

which denotes that a plot of 2.303 (slope)/(H^+) against $k(H^+)/[K(H^+) + 1]$ will be a straight line with slope equal to k_1 and intercept equal to k_{-1}. However, at a pH higher than 3, the change in pH value becomes significant as the cyclization proceeds. It is, therefore, necessary to use a pH-stat or buffer to maintain the pH constant. The rate of cyclization at a pH higher than 3 was found to be so slow that utilization of a pH-state was impractical. The use of a buffer such as citrate not only failed to maintain the pH constant but also catalyzed the reactions. For this reason, the application of Eq. 22 was unsuccessful.

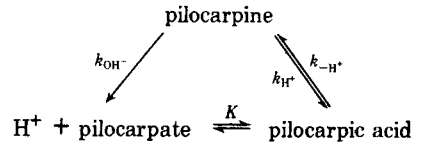
Nevertheless, k_1 and k_{-1} can be calculated from the results obtained in Fig. 7 and the data obtained from the analysis of the cyclized solution for pilocarpine. This is because at low pH (for example, pH = 1), the hydrolyzed product would exist essentially in its acidic form and the ratio k_1/k_{-1} would be equal to $(\text{pilocarpine})_{eq}/[C_o - (\text{pilocarpine})_{eq}]$. Thus, the results from Fig. 7 and from the analysis of cyclized solution give

$$k_1 + k_{-1} = 4.10 \text{ l./mole/hr.} \quad \text{(Eq. 23)}$$

$$k_1/k_{-1} = 29.3 \quad \text{(Eq. 24)}$$

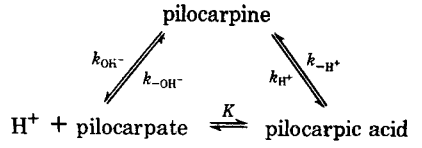
Solution of the simultaneous Eqs. 23 and 24 results in $k_1 = 3.97$ l./mole/hr. and $k_{-1} = 1.35 \times 10^{-1}$ l./mole/hr.

Up to this point, the mechanism for the hydrolysis of pilocarpine can be written as the cyclic equilibrium process shown in Scheme IV:



Scheme IV

where k_{H^+} is equal to k_{-1}, and k_{-H^+} is equal to k_1. By considering "the principle of microscopic reversibility," a reversible reaction for the hydroxide-ion catalyzed pathway can be inferred. Consequently, the complete cyclic mechanism may be stated as in Scheme V:



Scheme V

Here, k_{-OH^-} is defined as the first-order rate constant for the cyclization of pilocarpate to pilocarpine and may be calculated from k_{OH^-}, k_{H^+}, k_{-H^+}, and K. The k_{-OH^-} so calculated is 2.49×10^{-6}/hr.

The hydrolytic information was obtained by following the rate of cyclization to approach equilibrium. (The hydrolysis of isopilocarpine would take the same mechanism but with different values of rate constants.) Since there wasn't noticeable epimerization indicated in acidic pH (14), the hydrolytic information obtained in this study and the postulated degradative pathway do not take into account the epimerization aspect.

The information obtained in this study is different from the recent publications by Baeschlin and Etter (26, 27), in which certain kinetic parameters were not fully considered.

CONCLUSION

1. Hydrolysis of pilocarpine in aqueous solution is a cyclic equilibrium process which is catalyzed by hydrogen ion and hydroxide ion.

2. The equilibrium position depends on pH; it shifts to pilocarpate at high pH and shifts to pilocarpine at low pH. The equilibrium constant and the rate constants at 25°, as well as the energy of activation for the hydroxide-ion catalyzed hydrolysis, were determined and are as follows:

$$K = 1.12 \times 10^4 \text{ l./mole}$$
$$k_{H^+} = 1.35 \times 10^{-1} \text{ l./mole/hr.}$$
$$k_{-H^+} = 3.97 \text{ l./mole/hr.}$$
$$k_{OH^-} = 7.56 \times 10^2 \text{ l./mole/hr.}$$
$$k_{-OH^-} = 2.49 \times 10^{-6}/\text{hr.}$$

$$Ea_{(\text{for } OH^- \text{ catal. hydrolysis})} = 11.0 \text{ kcal./mole}$$

3. It is suggested that pilocarpine solutions be prepared at pH 4–5 for acceptable stability and physiological availability.

4. In alkaline pH, pilocarpine also undergoes some epimerization but at a rate much slower than hydroxide-ion catalyzed hydrolysis.

REFERENCES

(1) R. A. Anderson and F. B. Cowle, *Brit. J. Ophthalmol.*, **52,** 607(1968).

(2) I. H. Leopold and E. Keates, *Clin. Pharmacol. Ther.*, **6,** 262(1965).

(3) H. A. D. Jowett, *J. Chem. Soc.*, **77,** 473(1900).

(4) *Ibid.*, **77,** 851(1900).

(5) W. A. Preobrashenski, N. A. Preobrashenski, and A. F. Wompe, *Ber. Deut. Chem. Ges.*, **66,** 1187(1933).

(6) N. A. Preobrashenski, W. A. Preobrashenski, A. F. Wompe, and M. N. Schtschukina, *ibid.*, **66,** 1536(1933).

(7) A. N. Dey, *J. Chem. Soc.*, **1937,** 1057.

(8) R. K. Hill and S. Barcza, *Tetrahedron*, **22,** 2889(1966).

(9) N. J. Wojciechowski and B. Ecanow, *J. Pharm. Sci.*, **50,** 887(1961).

(10) W. Døpke and G. d'Heureuse, *Tetrahedron Lett.*, **15,** 1807 (1968).

(11) E. Brochmann-Hanssen, P. Schmid, and J. D. Benmaman, *J. Pharm. Sci.*, **54,** 783(1965).

(12) S. Riegelman and D. G. Vaughan, *J. Amer. Pharm. Ass., Pract. Ed.*, **19,** 474(1958).

(13) C. J. Blok, *Pharm. J.*, **155,** 282(1945).

(14) R. Fagerstrøm, *J. Pharm. Pharmacol.*, **15,** 479(1963).

(15) W. H. Morrison and S. M. Truhlsen, *Amer. J. Ophthalmol.*, **33,** 357(1950).

(16) R. A. Anderson and S. D. FitzGerald, *Australas. J. Pharm.*, **48,** s108(1967).

(17) R. A. Anderson, *Can. J. Pharm. Sci.*, **2,** 25(1967).

(18) I. R. Brown, A. E. Dyer, I. N. Elowe, I. E. Stauffer, and G. C. Walker, *ibid.*, **1,** 22(1966).

(19) C. W. Chong, L. W. Dittert, H. B. Kostenbauder, and J. V. Swintosky, *J. Pharm. Sci.*, **56,** 1647(1967).

(20) P. K. Baeschlin, J. C. Etter, and H. Moll, *Pharm. Acta Helv.*, **44,** 301(1969).

(21) C. F. Jacobsen, J. Léonis, K. Linderstrøm-Lang, and M. Ottesen, "Methods of Biochemical Analysis," vol. 4, D. Glick, Ed., Interscience, New York, N. Y., 1957, pp. 171–209.

(22) K. J. Laidler, "Chemical Kinetics," McGraw-Hill, New York, N. Y., 1965, pp. 14–16.

(23) J. A. Grace and M. C. R. Symons, *J. Chem. Soc.*, **1961**, 47.
(24) F. A. Long, W. F. McDevit, and F. B. Dunkle, *J. Phys. Colloid Chem.*, **55**, 813(1951).
(25) A. J. Repta, personal communication.
(26) P. K. Baeschlin and J. C. Etter, *Pharm. Acta Helv.*, **44**, 339 (1969).
(27) *Ibid.*, **44**, 348(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *College of Pharmacy, University of Iowa, Iowa City, IA 52240*
Accepted for publication April 30, 1970.
Abstracted in part from a thesis submitted by Ping-Hong Chung to the University of Iowa in partial fulfillment of Doctor of Philosophy degree requirements.

Solubility Profiles for Several Barbiturates in Hydroalcoholic Mixtures

T. L. BREON and A. N. PARUTA

Abstract □ The solubilities of eight physiologically active barbiturates were determined in binary mixtures of alcohol and water. The solubility curves for these substances varied, showing either solubility maxima or asymptotic solubility isotherms. The dielectric requirement of the barbiturates investigated illustrated an approximate inverse relationship with the number of carbon atoms in the molecule. A similar correlation was found with the solubilities in pure water, with the ratios of the solubilities in ethanol, and at the dielectric requirement to the solubility in water. The therapeutic indexes of duration of action and the period of time involved between administration of the drug and the time when the activity is first manifested increased as the relative polarity of these barbiturates declined. An approximate correlation between activity and solubility ratios is considered.

Keyphrases □ Barbiturates, solubility—ethanol–water systems □ Dielectric requirement—barbiturates □ Polarity, barbiturates—activity correlation □ Solubility ratios—barbiturates, ethanol–water systems

The pharmacological action exerted by a drug molecule in contact with a biological system is the net result of the interactions and extent of interactions with the complex biological environment. The degree as well as the rate of interaction is governed by many parameters, many of which depend on the physical and chemical properties associated with the drug molecule.

To be physiologically active, a drug must be absorbed and distributed throughout the biological fluids. More specifically, it is noted that these actions occur on a molecular level; under these conditions, it would be assumed that solution properties and characteristics are operative. Many biologically active substances are weak electrolytes, and properties such as the pH of the medium, pKa of the drug, concentration gradients, surface tension, and the aqueous and lipid solubilities of the various species contribute to the overall extent of activity.

The biologically active species, to initiate a response, would be presumed to have interacted with cellular constituents; this process is involved with diffusion and permeability as well as those factors previously discussed. Thus, this study is an initial investigation into the possible approximate correlation between solubility characteristics of several barbiturates and therapeutic activity.

The wide variety of available barbiturates certainly attests to the importance of these materials, with a wide spectrum of uses such as sedatives and anticonvulsants. They are derivatives of barbituric acid with a variety of substitutions in the 5-position, and about 20 of these are presently available as therapeutic agents.

Although the general sedative action of all these barbiturates is about the same, they do vary in the duration of depressant action. Since these barbiturates are chemically different, it would be judicious to study them in attempting to relate known duration of action and chemical structure.

Thus, if in a series of barbiturates, a property such as solubility was determined as a function of polarity, there may be an indication of the relative lipoidal nature of these materials. It would be well to consider the solubility of these types of materials in a manner previously described (1). The cosolvent action on these barbiturates by mixtures of decreasing polarity should be instructive.

It might be expected that the position of the dielectric requirement (DR) and the magnitude of solubility at that point would be indicative of the effect of substituents and the relative polarity of the drug molecule. In view of this possibility, some eight barbiturates with a spectrum of values for the onset and duration of action were studied relative to their solubility behavior in hydroalcoholic solutions.

Several long-, intermediate-, and short-acting barbiturates (2) were chosen to test this hypothesis, including barbital as the comparing standard.

The very important work of Hansch and Anderson (3) should be mentioned here since they showed a definitive correlation of the activity of barbiturates with the log of the partition coefficients for various derivatives. This would suggest the importance of the hydrophobic character of substituted barbiturates in a wide variety of biochemical systems.

The model used by Hansch and Anderson (3) is a partitioning between phases to calculate the coefficients or a measure of lipophilicity of these drugs, *i.e.*, the

(23) J. A. Grace and M. C. R. Symons, *J. Chem. Soc.*, **1961**, 47.
(24) F. A. Long, W. F. McDevit, and F. B. Dunkle, *J. Phys. Colloid Chem.*, **55**, 813(1951).
(25) A. J. Repta, personal communication.
(26) P. K. Baeschlin and J. C. Etter, *Pharm. Acta Helv.*, **44**, 339 (1969).
(27) *Ibid.*, **44**, 348(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *College of Pharmacy, University of Iowa, Iowa City, IA 52240*
Accepted for publication April 30, 1970.
Abstracted in part from a thesis submitted by Ping-Hong Chung to the University of Iowa in partial fulfillment of Doctor of Philosophy degree requirements.

Solubility Profiles for Several Barbiturates in Hydroalcoholic Mixtures

T. L. BREON and A. N. PARUTA

Abstract □ The solubilities of eight physiologically active barbiturates were determined in binary mixtures of alcohol and water. The solubility curves for these substances varied, showing either solubility maxima or asymptotic solubility isotherms. The dielectric requirement of the barbiturates investigated illustrated an approximate inverse relationship with the number of carbon atoms in the molecule. A similar correlation was found with the solubilities in pure water, with the ratios of the solubilities in ethanol, and at the dielectric requirement to the solubility in water. The therapeutic indexes of duration of action and the period of time involved between administration of the drug and the time when the activity is first manifested increased as the relative polarity of these barbiturates declined. An approximate correlation between activity and solubility ratios is considered.

Keyphrases □ Barbiturates, solubility—ethanol–water systems □ Dielectric requirement—barbiturates □ Polarity, barbiturates—activity correlation □ Solubility ratios—barbiturates, ethanol–water systems

The pharmacological action exerted by a drug molecule in contact with a biological system is the net result of the interactions and extent of interactions with the complex biological environment. The degree as well as the rate of interaction is governed by many parameters, many of which depend on the physical and chemical properties associated with the drug molecule.

To be physiologically active, a drug must be absorbed and distributed throughout the biological fluids. More specifically, it is noted that these actions occur on a molecular level; under these conditions, it would be assumed that solution properties and characteristics are operative. Many biologically active substances are weak electrolytes, and properties such as the pH of the medium, pKa of the drug, concentration gradients, surface tension, and the aqueous and lipid solubilities of the various species contribute to the overall extent of activity.

The biologically active species, to initiate a response, would be presumed to have interacted with cellular constituents; this process is involved with diffusion and permeability as well as those factors previously discussed. Thus, this study is an initial investigation into the possible approximate correlation between solubility characteristics of several barbiturates and therapeutic activity.

The wide variety of available barbiturates certainly attests to the importance of these materials, with a wide spectrum of uses such as sedatives and anticonvulsants. They are derivatives of barbituric acid with a variety of substitutions in the 5-position, and about 20 of these are presently available as therapeutic agents.

Although the general sedative action of all these barbiturates is about the same, they do vary in the duration of depressant action. Since these barbiturates are chemically different, it would be judicious to study them in attempting to relate known duration of action and chemical structure.

Thus, if in a series of barbiturates, a property such as solubility was determined as a function of polarity, there may be an indication of the relative lipoidal nature of these materials. It would be well to consider the solubility of these types of materials in a manner previously described (1). The cosolvent action on these barbiturates by mixtures of decreasing polarity should be instructive.

It might be expected that the position of the dielectric requirement (DR) and the magnitude of solubility at that point would be indicative of the effect of substituents and the relative polarity of the drug molecule. In view of this possibility, some eight barbiturates with a spectrum of values for the onset and duration of action were studied relative to their solubility behavior in hydroalcoholic solutions.

Several long-, intermediate-, and short-acting barbiturates (2) were chosen to test this hypothesis, including barbital as the comparing standard.

The very important work of Hansch and Anderson (3) should be mentioned here since they showed a definitive correlation of the activity of barbiturates with the log of the partition coefficients for various derivatives. This would suggest the importance of the hydrophobic character of substituted barbiturates in a wide variety of biochemical systems.

The model used by Hansch and Anderson (3) is a partitioning between phases to calculate the coefficients or a measure of lipophilicity of these drugs, *i.e.*, the

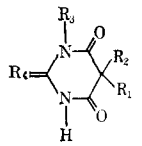


Table I—Summary of the Substituents Found in the Noted Positions for the Barbituric Acid Derivatives Used in this Study

Derivative	R_1	R_2	R_3	R_4
Barbituric acid	—H	—H	H	O
Barbital	$-CH_2CH_3$	$-CH_2CH_3$	H	O
Metharbital	$-CH_2CH_3$	$-CH_2CH_3$	CH_3	O
Butabarbital	$-CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	H	O
Vinbarbital	$-CH_2CH_3$	$-C(CH_3)=CHCH_2CH_3$	H	O
Thiopental	$-CH_2CH_3$	$-CH(CH_2)CH_2CH_2CH_3$	H	S
Thiamylal	$-CH_2CH=CH_2$	$-CH(CH_3)CH_2CH_2CH_3$	H	S
Phenobarbital	$-CH_2CH_3$		H	O
Amobarbital	$-CH_2CH_3$	$-CH_2-CH_2-CH(CH_3)_2$	H	O
Pentobarbital	$-CH_2CH_3$	$-CH(CH_3)-CH_2-CH_2-CH_3$	H	O

greater the lipophilicity, the greater the partition coefficient. It is assumed that the partition coefficients in 1-octanol–water systems are additive in nature.

In the present study, the extent of lipophilicity is measured in a relative fashion, since it is assumed that the molecule possessing the greatest lipophilicity should have the least solubility in the most polar solvent. Further, the ratios of the solubilities, using a standard compound, should indicate the relative polarities of the molecules in a continuously varying spectrum of polarity.

In Table I, the pertinent chemical characteristics are shown for the barbiturates studied. Barbituric acid is also given in Table I and used to illustrate the parent compound.

EXPERIMENTAL

Materials—The materials used in this study were as follows: barbituric acid,[1] m.p. 252–255°; metharbital,[2] m.p. 151–155°; butabarbital,[3] m.p. 166–168°; thiamylal,[4] m.p. 133–135°; barbital,[5] m.p. 189°; pentobarbital,[6] m.p. 131°; amobarbital,[7] m.p. 153°; and phenobarbital USP, m.p. 176°. Thiopental was prepared from the sodium salt.[8] The sodium salt was dissolved in a quantity of distilled water, and the free acid precipitated by the addition of 1.0 *M* hydrochloric acid[9] solution. The slurry was filtered and washed with three portions of distilled water. The melting point range of the dried precipitate was 156–158°. Melting points of pooled and dried samples from the gravimetric procedure were also made and found not to vary more than ± 1–2° outside the range of the original material. This was done to ascertain if any aberrant behavior such as hydrate formation or crystalline modification (polymorphism) occurred in these binary solvent mixtures.

Hydroalcoholic solvents were prepared volumetrically by the use of burets, previously determined densities for absolute ethyl alcohol,[10] and distilled water at ambient room temperature. These mixtures ranged from 0.0 to 100.0% w/w distilled water in 2.5% increments and represent a polarity range in terms of dielectric constant values of 24.3–78.5.

A pH 10.7 buffer was prepared with 71 g. of anhydrous sodium dibasic phosphate[11] (reagent grade) dissolved in 1000 ml. of distilled water and adjusted to pH 10.7 with 1.0 *M* sodium hydroxide[12] solution.

Equipment—A rotating apparatus was constructed which held 48 screw-capped glass vials of 21-ml. volume and revolved at 32 r.p.m. The vials were rotated in such a way that the solute traversed the full length of the vial twice per revolution, thus causing sufficient agitation of the contents. No caking was observed in any of the samples. This apparatus was immersed in a 10-gal. water bath maintained at 25.0 ± 0.3° by a Tecan Tempunit.[13]

A Cary model 16 spectrophotometer,[14] a Mettler type H6T[15] analytical balance, a Leeds Northup model 7401 pH meter,[16] and a Sorvall model GLC-1[17] centrifuge were utilized in the assay procedure. Computational treatment of the data was aided through utilization of an IBM System 360 model 50 digital computer.[18]

Procedure—The procedures used in this study have been previously given (4) and consist essentially of a gravimetric analytical technique with a spectrophotometric check run. Each solubility curve represents the average values from at least three runs of the 41 samples comprising the total variation in solvent composition.

[1] Aldrich Chemical Co., Milwaukee, Wis., lot 072281.
[2] Gemonil, Abbott Laboratories, North Chicago, Ill., lot 685-7608.
[3] McNeil Laboratories, Fort Washington, Pa., lot 5086.
[4] Surital, Parke, Davis and Co., Detroit, Mich., lot 405838.
[5] Merck & Co., Rahway, N. J., lot 51115.
[6] Abbott Laboratories, North Chicago, Ill., lot 12130.
[7] Ruger Chemical Corp., N. J., lot 105 3180.
[8] Abbott Laboratories, North Chicago, Ill., lot 780-7657.
[9] Mallinckrodt Chemical Works, New York, N. Y.
[10] U.S. Industrial Chemicals Co., New York, N. Y.
[11] Fisher Scientific Co., Fair Lawn, N. J.
[12] Mallinckrodt Chemical Works, New York, N. Y.
[13] Techne (Cambridge) Limited, Cambridge, England.
[14] Cary Instruments, Belmont, Mass.
[15] Mettler Instrument Corp., Hightstown, N. J.
[16] Leeds and Northup Co., Philadelphia, Pa.
[17] Sorvall, Newtown, Conn.
[18] International Business Machines, Armonk, N. Y.

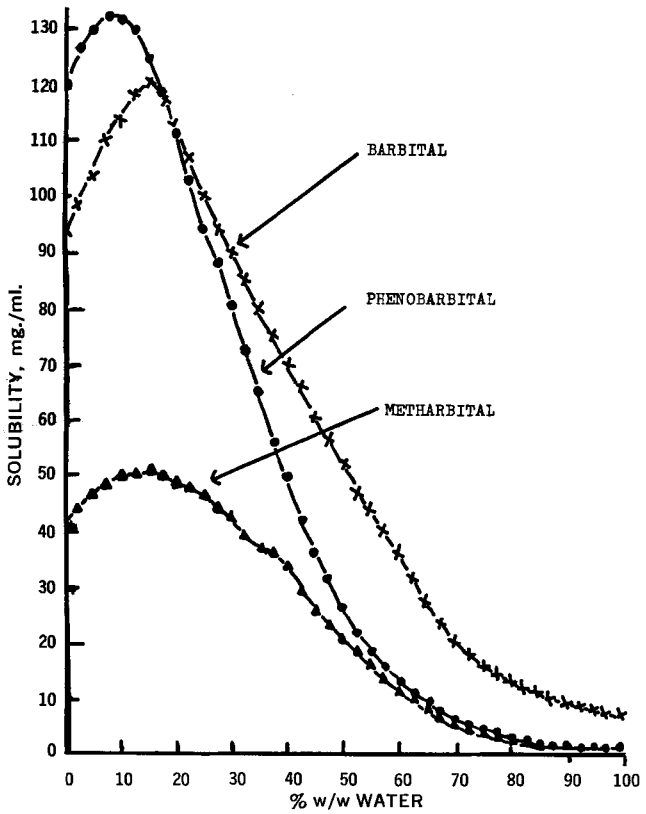


Figure 1—*Solubilities of the barbiturates noted are plotted in milligrams per milliliter at 25° as a function of the weight percent water.*

RESULTS AND DISCUSSION

In Fig. 1, the solubilities of barbital, phenobarbital, and metharbital in milligrams per milliliter as a function of the percent w/w water in binary mixtures are presented. The solubility curves show maxima at 10% w/w water for phenobarbital and 15% w/w water for barbital. Metharbital, on the other hand, shows a maximum at 15% w/w water and a shoulder at 35% w/w water. These values lead to DR (dielectric requirement) values of 29.0 for phenobarbital, 30.6 for barbital, and 30.6 and 41.3 for metharbital, respectively. The values for barbital and phenobarbital agree with those previously given (5–7).

The basic correlation of the interpretation of solubility phenomena in terms of dielectric constants is substantiated by considering Khalil and Martin's (5) value for barbital. The value of the solubility parameter is given as approximately 13.5 in their excellent work on model membranes. The DR of barbital in this present study was found to have a value of 31, which gives a solubility parameter value of 13.7 from a previously presented relationship (6), which has been modified slightly and presented in another communication (8).

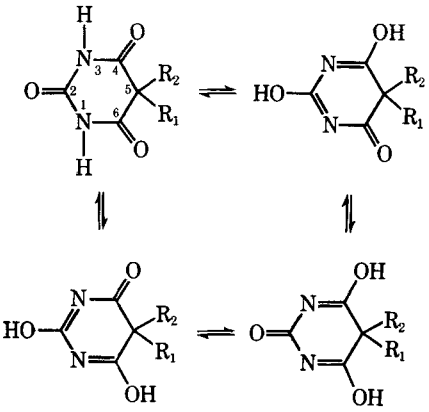
Further, Khalil and Martin (5) obtained a value for the solubility parameter of salicylic acid as 10.8, which coincides well with the value of 10.5 derived on a dielectric constant basis (8).

It is interesting to note the nature of these curves, since a crossover occurs in the magnitude of solubility. Barbital is seen to have greater solubility than phenobarbital over a wide range of polarity, *i.e.*, dielectric constant values of 33 to 78, whereas phenobarbital has greater solubility in the range of 24 to 33. These three barbiturates have been grouped together for convenience, since these materials possess a DR and approximately the same duration of action. The solubility of these substances varies about 5–13% w/v in pure alcohol to the maxima. The solubility of these substances are summarized in Table II.

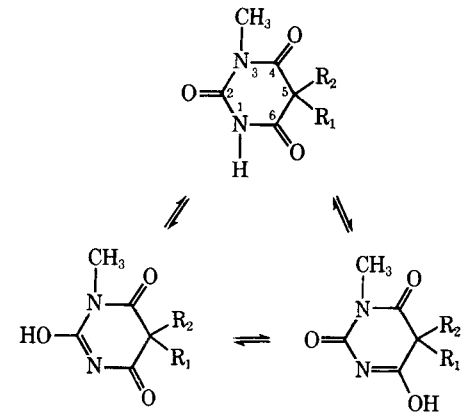
In the case of metharbital, it is noted from Fig. 1 that the addition of a methyl group to the R_3 position of the molecule dramatically reduces the maximum solubility as compared to barbital with a hydrogen at this position. A DR is observed at 30.6 and a solubility at this point of 51.2 mg./ml. It is also seen that a shouldering effect, unique to this derivative, is observed in the range of 40–44 in terms of dielectric constants. The pharmacological activity of this derivative is also unusual in that it not only produces sedation but possesses anticonvulsant properties.

A brief consideration of the chemical structure of the metharbital molecule will also yield some unusual characteristics relative to the nonmethylated analogs. It has been reported (9) that the barbituric acid derivatives undergo enol–keto tautomerism. Molecules devoid of alkyl substituents on the nitrogen atoms can provide a maximum of two (N—H) hydrogen atoms which would be available for contribution to monoenolized and dienolized structures with the three neighboring carbonyl groups.

Scheme I*a* illustrates the three possible dienolized structures as



a—derivatives devoid of *N*-alkyl substituents showing only the dienol combinations



b—*N*-alkyl derivatives showing all possible combinations of enol species

Scheme Ia,b—Enol–keto tautomerism of the barbituric acid derivatives

well as the keto form. It is noted from this illustration that the carbonyl group at carbon-2 has twice the number of chances of becoming enolized as those at carbon-4 or 6, due to its vicinal position to both nitrogen atoms. Metharbital, on the other hand, has only one (N—H) hydrogen available. With the methyl group on the nitrogen at position-3, only two possible monoenolized species can form, as shown in Scheme I*b*. Other effects being equal, the chances of the carbonyl group at position-2 or 6 being converted to the enol form are equal.

Tautomerism is not a static situation where tautomeric species of molecules exist in only keto or enol forms, but rather it is a dynamic equilibrium where active hydrogen atoms are rapidly interchanging between the various species.

It may be possible that the limitations imposed on the tautomerism of the metharbital molecule by the *N*-methyl group could cause the shouldering effect on the solubility profile. A second possibility for this unusual behavior for a barbiturate derivative is that the polarity of the various tautomeric forms is different. The assay procedure, not being specific for any particular species of this molecule, would detect the cumulative solubility of all the various species present.

Table II—Summary of the Solubility of the Barbiturate Noted in Ethanol–Water Mixtures in Milligrams per Milliliter at 25° as a Function of w/w Percent Water and Dielectric Constant

w/w % Water	Dielectric Constant, ε	Solubility, mg./ml. Barbital	Phenobarbital	Methabarbital
0.0	24.3	92.3	118.4	41.9
2.5	25.5	98.6	122.6	43.7
5.0	26.5	103.1	127.8	46.1
7.5	27.6	110.0	131.1	47.9
10.0	29.0	113.3	132.3	50.0
12.5	29.7	118.3	130.6	50.7
15.0	30.6	120.7	126.4	51.2
17.5	31.5	117.2	120.6	50.9
20.0	32.7	112.5	112.3	50.3
22.5	33.8	107.7	104.0	49.3
25.0	34.7	100.1	97.7	48.0
27.5	36.4	94.3	90.2	46.0
30.0	37.5	90.2	82.4	44.7
32.5	38.6	85.1	78.2	43.4
35.0	39.8	80.8	70.3	39.0
37.5	41.3	75.6	62.1	36.6
40.0	42.8	70.0	52.0	36.2
42.5	44.2	66.3	46.6	33.4
45.0	45.7	60.2	41.1	28.9
47.5	47.5	56.5	36.3	26.2
50.0	49.0	51.6	30.6	23.5
52.5	50.5	47.7	25.4	21.2
55.0	52.0	43.1	21.2	18.7
57.5	53.6	39.2	18.0	16.2
60.0	55.4	34.1	15.0	14.0
62.5	57.0	30.6	11.5	12.1
65.0	58.4	28.3	10.0	10.2
67.5	60.0	24.1	7.9	8.6
70.0	61.7	20.9	6.2	7.4
72.5	63.3	17.1	5.1	6.3
75.0	64.5	15.6	4.5	5.3
77.5	66.1	14.2	4.0	4.5
80.0	67.5	13.3	3.0	4.0
82.5	68.9	12.5	2.7	3.5
85.0	70.2	11.1	2.5	3.2
87.5	71.7	10.1	2.3	2.9
90.0	73.2	9.0	1.9	2.7
92.5	74.5	8.0	1.7	2.5
95.0	75.7	7.5	1.5	2.3
97.5	77.1	7.4	1.3	2.2
100.0	78.5	7.3	1.2	2.0

The solubility data for butabarbital are found in Table III. Figure 2 represents the isothermal data graphically as solubility in milligrams per milliliter as a function of solvent composition. This molecule is identical to barbital with the exception of the ethyl group on the R_2 position, which is replaced with a *sec*-butyl group. The addition of these two carbon atoms decreased the solubility over the entire range of the solvent composition.

Reber and Pathamanon (10) have determined the solubility of vinbarbital in ethanol–water mixtures. A tabulation of values derived from smoothing their data is found in Table III and plotted in Fig. 2, as described previously. This particular derivative has a fifth carbon atom and an olefinic bond added to the substituent at the R_2 position of the butabarbital molecule.

For the purpose of obtaining the solubility profiles for vinbarbital, special treatment of the data presented by Reber and Pathamanon (10) was necessary. Their profile consisted of 11 pieces of data, only one of which corresponded to an exact solvent composition used in this study. It was necessary, therefore, to analyze their data and determine the apparent solubility of vinbarbital in each of the 41 solvent systems employed for the remaining compounds. Rather than arbitrarily picking points off a plot of their data, the data were subjected to a polynomial regression. A previously compiled and published digital computer program (11) was employed in double precision. This program is based on a mathematical method presented by Ostle (12). The coefficients of an eighth-degree polynomial, representing the best fit of a curve to the data of Reber and Pathamanon (10), were computed. From this equation, the apparent solubilities of vinbarbital in hydroalcoholic solvents of identical composition to those used in this study were calculated. It is suggested by Ostle (12) that this particular method is not valid for data presented in uneven increments of the independent variable. However, it is felt that the published solubility data for vinbarbital closely approximate even increments of solvent composition and, therefore, a close approximation of the true equation describing the data should be rendered by this method. A comparison of the original data in Table IV may be made with the values computed by this method which are found in Table III.

Amobarbital, the third of the intermediate-acting barbiturates of this group, is also illustrated in Fig. 2. However, in this case, the solubility curve rises asymptotically toward a maximum value in pure ethanol. The DR would presumably have a value of about or less than the dielectric constant of pure ethanol, *i.e.*, 24.3. The solubility data for these compounds are given in Table III.

Three barbiturates of short to ultrashort duration were also studied. Pentobarbital, the 5-ethyl-5-(1-methyl butyl) derivative of barbituric acid, showed a solubility curve also running asymptotically toward pure alcohol. Again, it would be assumed that the DR would be about 24.3, that being the value for pure ethanol. Although there is a discrimination between amobarbital, the 5-ethyl-5-isopentyl derivative, and pentobarbital based on duration of action, both possess similar solubility curves and magnitudes of solubility. Certainly, there is considerable overlap in the duration

Table III—Summary of the Solubility of the Barbiturate Noted in Ethanol–Water Mixtures in Milligrams per Milliliter at 25° as a Function of w/w Percent Water and Dielectric Constant

w/w % Water	Dielectric Constant, ε	Solubility, mg./ml. Amobarbital	Butabarbital	Vinbarbital
0.0	24.3	219.6	84.0	62.3
2.5	25.5	217.1	85.9	62.7
5.0	26.5	213.4	87.9	63.1
7.5	27.6	210.3	89.3	63.3
10.0	29.0	205.6	90.1	63.0
12.5	29.7	196.6	90.6	62.2
15.0	30.6	191.9	89.6	61.0
17.5	31.5	182.2	88.5	59.2
20.0	32.7	172.1	85.9	56.9
22.5	33.8	160.1	82.6	54.2
25.0	34.7	145.8	79.2	51.3
27.5	36.4	137.5	73.4	48.1
30.0	37.5	123.6	68.6	44.8
32.5	38.6	110.9	63.6	41.3
35.0	39.8	104.2	58.6	37.9
37.5	41.3	88.2	53.7	34.4
40.0	42.8	81.0	48.2	31.1
42.5	44.2	67.0	43.0	27.8
45.0	45.7	62.3	38.4	24.7
47.5	47.4	51.8	33.4	21.8
50.0	49.0	40.0	29.4	19.0
52.5	50.5	33.4	24.8	16.4
55.0	52.0	25.5	21.1	14.0
57.5	53.6	19.5	17.7	11.8
60.0	55.4	16.4	14.5	9.8
62.5	57.0	11.2	11.9	8.1
65.0	58.4	9.6	9.6	6.6
67.5	60.0	7.8	7.5	5.3
70.0	61.7	7.4	6.4	4.2
72.5	63.3	5.3	4.8	3.3
75.0	64.5	2.6	3.7	2.6
77.5	66.1	2.2	2.9	2.1
80.0	67.5	1.7	2.4	1.7
82.5	68.9	1.2	2.0	1.4
85.0	70.2	1.1	1.7	1.2
87.5	71.7	0.96	1.5	1.1
90.0	73.2	0.84	1.4	1.0
92.5	74.5	0.70	1.2	0.9
95.0	75.7	0.68	1.1	0.8
97.5	77.1	0.64	1.0	0.7
100.0	78.5	0.56	0.9	0.7

Table IV—Summary of the Solubility of Vinbarbital in Ethanol–Water Mixtures in Milligrams per Milliliter at 25° as a Function of w/w Percent Water (10)

w/w % Water	Solubility, mg./ml.
0.16	62.3
7.56	63.3
20.96	55.8
28.77	46.6
38.62	32.8
48.85	20.20
58.26	11.38
67.62	5.03
79.87	1.76
90.20	0.96
100.00	0.71

of sedative effect for these materials; however, although amobarbital has an intermediate duration, it is closer to short-acting pentobarbital (especially chemically) than it is to intermediate-acting vinbarbital and butabarbital.

The following two compounds to be discussed differ from the previously mentioned derivatives, because the oxygen at the R_4 position has been replaced with a sulfur atom in the ultrashort-acting thiamylal and thiopental. The effect of this substitution may decrease the polarity of the molecule from its oxy-analog. On the electronegativity scale, the value for the oxygen atom is 1 unit higher than those for the sulfur and carbon atoms which are approximately equal. Thus, the chemical bond between the oxygen at the R_4 position and the adjacent carbon atom may be more polar in character than the similar situation with the sulfur atom.

Aside from this substitution, the chemical structure of thiopental varies from barbital by a 1-methylbutenyl group replacing an ethyl substituent on the R_2 position.

Table V and Fig. 3 tabulate and graphically illustrate the solubility data for thiopental, in the manner previously described.

The ultrashort-acting barbiturate thiamylal is similar in chemical structure to thiopental, with the exception of an allyl substituent replacing an ethyl group in the R_1 position. Data for this derivative are found in Table V. A plot of these data, in the usual fashion, is represented in Fig. 3. This curve is unique in that no maximum is observed, but the solubility profile rises sharply toward pure

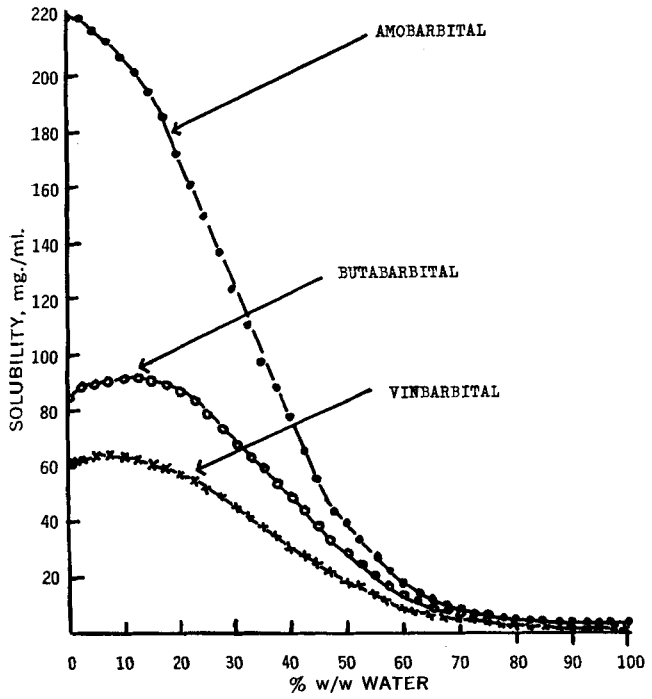


Figure 2—*This plot parallels that for Fig. 1 for the barbiturates noted.*

Table V—Summary of the Solubility of the Barbiturate Noted in Ethanol–Water Mixtures in Milligrams per Milliliter at 25° as a Function of w/w Percent Water and Dielectric Constant

w/w % Water	Dielectric Constant, ϵ	Solubility, mg./ml. Pentobarbital	Thiopental	Thiamylal
0.0	24.3	250.4	56.3	160.8
2.5	25.5	250.4	62.3	149.9
5.0	26.5	246.2	74.2	135.4
7.5	27.6	243.3	97.1	124.6
10.0	29.0	236.1	94.9	112.7
12.5	29.7	226.4	86.6	102.0
15.0	30.6	218.6	79.9	93.2
17.5	31.5	208.8	71.6	82.3
20.0	32.7	193.1	63.7	71.8
22.5	33.8	180.4	55.4	61.9
25.0	34.7	169.3	50.4	54.9
27.5	36.4	157.1	41.1	43.3
30.0	37.5	137.1	36.3	37.7
32.5	38.6	123.4	31.0	32.3
35.0	39.8	110.6	38.0	28.7
37.5	41.3	98.3	23.5	23.1
40.0	42.8	85.0	18.8	18.1
42.5	44.2	72.0	16.3	15.4
45.0	45.7	62.5	14.0	13.0
47.5	47.4	51.0	11.2	10.3
50.0	49.0	40.0	9.1	8.2
52.5	50.5	34.5	7.3	6.5
55.0	52.0	31.3	5.4	4.7
57.5	53.6	24.3	4.5	3.4
60.0	55.4	20.6	3.2	2.5
62.5	57.0	15.5	2.4	1.96
65.0	58.4	13.2	2.0	1.41
70.0	61.7	10.3	0.9	0.73
72.5	63.3	7.8	0.7	0.51
75.0	64.5	5.5	0.5	0.35
77.5	66.1	4.0	0.30	0.23
80.0	67.5	3.1	0.28	0.19
82.5	68.9	2.7	0.23	0.15
85.0	70.2	2.1	0.19	0.12
87.5	71.7	1.7	0.17	0.10
90.0	73.2	1.4	0.15	0.09
92.5	74.5	0.9	0.12	0.07
95.0	75.7	0.6	0.11	0.06
97.5	77.1	0.5	0.09	0.06
100.0	78.5	0.5	0.08	0.05

ethanol, having a dielectric constant of about 24.3. This would indicate that a DR of less than 24.3 would exist for this compound.

It was noted in the case of all the barbituric acid derivatives that some portion of the solubility isotherm possessed a fair degree of linearity relative to the solvent composition. The rates calculated as the slopes of the straight line best representing these approximately linear sections are summarized in Table VI, along with the ranges in solvent composition in which this relationship is valid.

The limits of solvent composition, within which these rates are operative, lie well within the range of pharmaceutical interest. It might be assumed then that there would be some pharmaceutical formulation advantages in this information.

It would be pertinent then to consider the changes in solubility produced by altering the substituent groups on the barbital molecule in a relative manner where barbital is used as the standard of comparison.

The ratios computed in this manner for metharbital are shown and the data plotted as a function of w/w percent water in Fig. 4. It can be seen in this illustration that the magnitude of solubility of metharbital is substantially lower than that of barbital over the entire range of solvent composition. The peak observed between 30 and 40% water by weight corresponds to the shoulder on the solubility profile. An inflection on this curve may be observed at 15% w/w water, which corresponds to the maximum solubility of both metharbital and barbital.

Phenobarbital, on the other hand, produces an interesting spectrum of values, wherein at low polarities the ratio is greater than

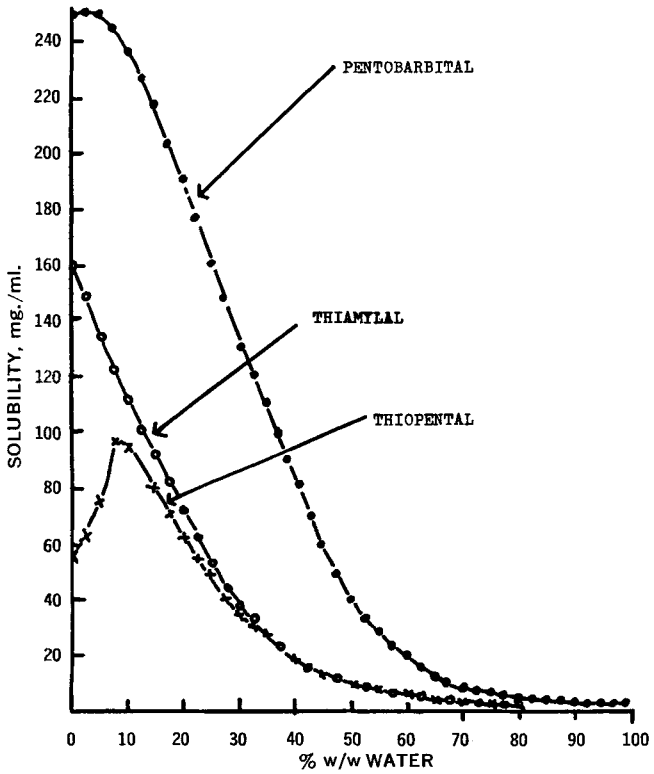


Figure 3—*Solubilities of the noted barbiturates are illustrated as described in Fig. 1.*

unity and is isodielectric at 20% w/w water. The fair degree of linearity for the ratio curve up to about 70% w/w water implies a unique discriminatory effect due to the phenyl substituent in the incremental polarity shifting due to the varying aqueous content. This would seem to support the concept of a continuous polarity spectrum in ethanol–water mixtures possessing constant forces of interaction or dissolution. The approximate slope for this line is

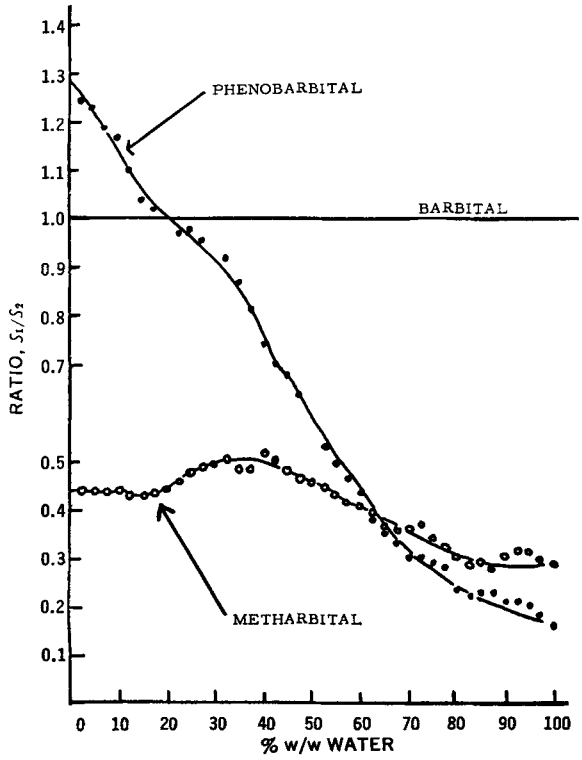


Figure 4—*Ratio of the solubility of phenobarbital and metharbital relative to barbital (defined as unity) is given as a function of the weight percent water.*

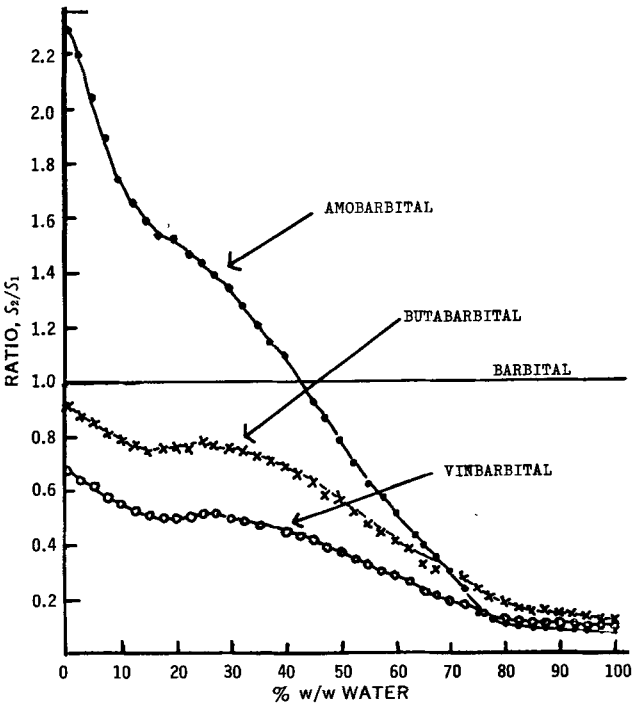


Figure 5—*This plot parallels that of Fig. 4 for amobarbital, butabarbital, and vinbarbital.*

1.4×10^{-2}/% change in water by weight. Thus a 10% change in water content from 10 to 20% would decrease the ratio by 0.14.

In Fig. 5, the ratio curves for amobarbital, butabarbital, and vinbarbital are shown. These three curves are similar in that there is an inflection point at about 15% w/w water and again, as expected, the curves converge from about 70–100% w/w water. Amobarbital also possesses an isodielectric point at 42.5% w/w water. In Fig. 6, the ratio curves are given for pentobarbital, thiamylal, and thiopental. Pentobarbital shows an inflection point at about 20% w/w water and an isodielectric point at 45% w/w water. A very large change in the ratio is seen, from a 270% increase in pure ethanol due to the 1-methyl butyl group to about a 300% reduction in pure water. This ninefold change in solubility ratio varies nonlinearly throughout the intermediate range.

The ratio curves for thiamylal and thiopental are also interesting, the "spike" for thiopental due to the sharp maximum in the solubility profile. Further, the convergence of these plots extends over a very wide spectrum of concentration values, *i.e.*, 30–100% w/w water.

To attempt to collate this information in a coherent fashion, the solubility values in pure water, pure ethanol, and at the DR (where applicable) were considered.

In Table VII, a summary of the duration and onset of action (13, 14) is shown for the compounds in this study, as well as the pertinent characteristics of the solubility profiles.

Table VI—Summary of Rates of Change in Solubility in Milligrams/Percent w/w Water, Calculated from the Linear Portion of the Solubility Profiles

Derivative	Range in % w/w Water	Rate in mg./% w/w Water
Barbital	15.0–55.0	−1.8
Phenobarbital	15.0–50.0	−2.9
Metharbital	20.0–60.0	−1.0
Butabarbital	20.0–55.0	−1.9
Vinbarbital	20.0–60.0	−1.2
Amobarbital	15.0–50.0	−4.4
Pentobarbital	10.0–50.0	−5.0
Thiopental	10.0–30.0	−3.0
Thiamylal	0.0–30.0	−4.0

Table VII—Summary of the Dielectric Requirement (DR), the Solubilities in Absolute Ethanol, in Water, and at the Dielectric Requirement in Milligrams per Milliliter, as a Function of the Duration and Onset of Action

Derivative	Duration of Action		Onset of Action	DR	Solubility in Ethanol, mg./ml.	Solubility at DR, mg./ml.	Solubility in Water, mg./ml.
	Reference 13	*Reference 14*	*Reference 13*				
Barbital	Long	Long	30–60 min.	30.6	92	121	7.3
Metharbital	Long	Long	30–60 min.	30.6	42	51	2.00
Phenobarbital	Long	Long	20–40 min.	29.7	118	132	1.20
Butabarbital	Intermediate	Short to intermediate	20–30 min.	29.7	84	91	0.86
Vinbarbital	Intermediate	Short to intermediate	20–30 min.	27.6	62	63	0.70
Amobarbital	Intermediate	Short to intermediate	20–30 min.	24.3	219	219	0.56
Pentobarbital	Short	Short to intermediate	20–30 min.	24.3	250	250	0.50
Thiopental	Ultrashort	Ultrashort	30 sec.	27.6	56	97	0.08
Thiamylal	Ultrashort	Ultrashort	20–60 sec.	24.3	161	161	0.05

Table VIII—Summary of the Therapeutic Action, and the Ratios of the Solubility in Ethanol and at the Dielectric Requirement (DR) to the Solubility in Water

Derivative	Duration (*Reference 13*)	Onset (*Reference 14*)	Sol. in Ethanol / Sol. in Water	Sol. at DR / Sol. in Water	Group
Barbital	Long	30–60 min.	12	16	I
Metharbital	Long	30–60 min.	21	25	I
Phenobarbital	Long	20–40 min.	98	110	II
Butabarbital	Intermediate	20–30 min.	98	104	II
Vinbarbital	Intermediate	20–30 min.	88	91	II
Amobarbital	Intermediate	20–30 min.	390	390	III
Thiopental	Ultrashort	30 sec.	670	1200	III
Thiamylal	Ultrashort	20–60 sec.	2300	2300	III
Pentobarbital	Short	20–30 min.	500	500	III

The onset of action for all the compounds listed is after the oral dose except with thiamylal and thiopental which are given intravenously. Intravenous administration of barbiturates other than thiamylal and thiopental would have an onset of action about 3–5 min., with barbital and phenobarbital at the upper value and the others at the lower. The DR's for these compounds shift to lower values as the duration of action decreases, indicating greater lipophilicity for the ultrashort barbiturates. The solubilities of these compounds in pure ethanol, pure water, or at the DR show no discernible pattern relative to direction and magnitude.

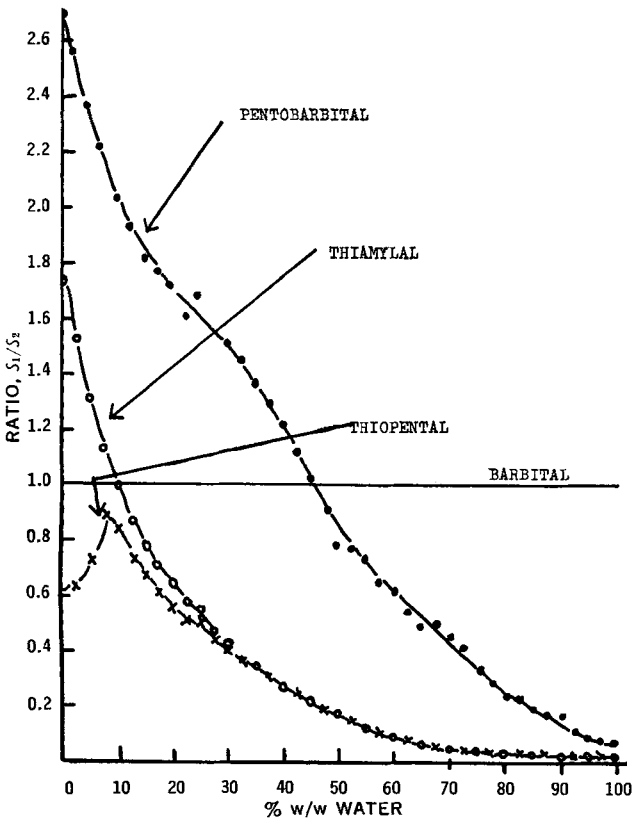


Figure 6—*This plot parallels that of Fig. 4 for pentobarbital, thiopental, and thiamylal.*

It was thought, however, that some sort of approximate correlation should exist relative to the pharmacological parameters and physical properties, although this relationship is undoubtedly complex (13). In Table VIII, the ratios of the solubilities of these compounds in pure ethanol and at the DR relative to water are shown. For compounds with a long duration of action, these ratios have values of about 10 to 100. However, if phenobarbital is omitted, the ratios vary from about 10 to 25. For the compounds listed as intermediate in action, these ratios vary from about 100 to 400 for butabarbital through amobarbital. Again, overlapping is suggested by these values since amobarbital is substantially greater than either butabarbital or vinbarbital. For those barbiturates possessing short or ultrashort action, the ratios are substantially larger, ranging in value from 500 to 2300, which indicates relatively greater lipophilicity for these substances. Since amobarbital is closer to pentobarbital in the properties of the solubility curves, they might also be considered in a given group.

Thus, a classification scheme for the barbiturates might be considered from both points of view, *i.e.*, pharmacological values and physical measurements. The last column of Table VIII breaks down the nine barbiturates in this study into three groups denoted by a Roman numeral. Although this type of classification would be very limited, it would provide a means by which exact and simple physical measurements could place a compound in the appropriate pharmacological range.

This correlation is rather good in view of the nature of the solvents. Such a relationship might be expected with pure water and ethanol which anchor the ends of the spectrum of solvent composition. Between these end-points, however, nonideal solvents are involved; this cosolvency phenomenon produces solubilities that deviate from those which may be expected of ideal solutions.

It may be conjectured that the magnitude of these ratios are an indication of the extent to which these compounds become concentrated in the less polar biological fluids. Thus, the derivatives

possessing a higher ratio, *i.e.*, thiopental and thiamylal, become concentrated to a higher degree in the body lipids than do barbital or metharbital and might be expected to be ultrashort acting.

In considering these solubility data in total, an approximate correlation has been observed between the lipophilic nature of the various barbiturate analogs and their therapeutic action. One must view this study with proper perspective in relation to the numerous other physical and chemical properties as well as the various biopharmaceutical parameters which all contribute to the variation in the final therapeutic activity possessed by the members of this series. The net result of the complex interaction of these and other factors determines the type and degree of the pharmacological activity involved.

The underlying concept of the pH-partition hypothesis as an approximate model would seem to be confirmed. However, such phenomena as binding, detoxification, active or passive diffusion, and complexation would play important roles in biological readouts which were not patternized or aberrant in behavior.

It is anticipated that several studies on the barbiturates and sulfonamides relative to solubility and partitioning will be undertaken in these laboratories and will be the subject of future communications.

REFERENCES

(1) A. N. Paruta, *J. Pharm. Sci.*, **58**, 204(1969).

(2) "Remington's Pharmaceutical Sciences," part V, No. 67, Mack Publishing Co., Easton, Pa., 1965.

(3) C. Hansch and S. M. Anderson, *J. Med. Chem.*, **10**, 745 (1967).

(4) A. N. Paruta and S. A. Irani, *J. Pharm. Sci.*, **55**, 1060 (1966).

(5) S. A. Khalil and A. N. Martin, *ibid.*, **56**, 1225(1967).

(6) A. N. Paruta, B. J. Sciarrone, and N. G. Lordi, *ibid.*, **51**, 704(1962).

(7) N. G. Lordi, B. J. Sciarrone, T. J. Ambrosio, and A. N. Paruta, *ibid.*, **53**, 463(1964).

(8) *Ibid.*, **53**, 1349(1964).

(9) R. E. Stuckey, *J. Pharm. Pharmacol.*, **14**, 217(1941).

(10) L. Reber and C. Pathamanon, *Amer. J. Pharm.*, **139**, 234 (1967).

(11) System/360 Scientific Subroutine Package (360A-CM-03X), Version III, Programmers Manual, International Business Machines Corp., White Plains, N. Y., 1968, p. 408.

(12) B. Ostle, "Statistics in Research," Iowa State College Press, Ames, Iowa, 1954, chap. 6.

(13) T. C. Daniels and E. C. Jorgensen, in "Textbook of Organic Medicinal and Pharmaceutical Chemistry," 4th ed., C. O. Wilson and O. Gisvold, Eds., J. P. Lippincott, Philadelphia, Pa., 1962, pp. 334, 336–340.

(14) S. K. Sharpless, in "The Pharmacological Basis of Therapeutics," 3rd ed., L. S. Goodman and A. Gilman, Eds., Macmillan, New York, N. Y., 1968, p. 107.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 2, 1969, from the *College of Pharmacy, University of Rhode Island, Kingston, RI 02881*

Accepted for publication May 11, 1970.

Abstracted in part from a dissertation presented by Thomas L. Breon to the Graduate School, University of Rhode Island, in partial fulfillment of the Master of Science degree requirements.

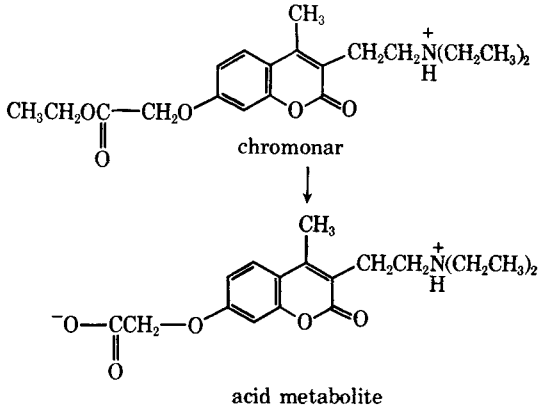
Metabolism and Excretion of Chromonar and Its Metabolite in Dog and Man

YVONNE C. MARTIN and RONALD G. WIEGAND

Abstract □ A simple method for the detection of chromonar and its acid metabolite by fluorescence techniques is described. Following oral or intravenous administration, chromonar is rapidly hydrolyzed to its metabolite, the corresponding acid. No further metabolism is observed. The metabolite distributes according to a single-compartment model. The plasma half-life is 1 hr. in man and dog. Excretion of the metabolite into the bile accounts for approximately 25% of the dose, and excretion into the urine accounts for the remainder.

Keyphrases □ Chromonar—metabolism, excretion □ Metabolite, chromonar—*in vitro, in vivo* determination □ Blood levels, chromonar and acid metabolite—human, dog □ Excretion—chromonar acid metabolite □ TLC—separation, identification □ Fluorometry—analysis

The coumarin compound chromonar[1] is used for the treatment of angina pectoris in Germany and Japan. The early report by Klarwein and Nitz (1) demonstrated that when the drug comes in contact with biological tissues, it is rapidly hydrolyzed to the corresponding acid (Scheme I), which exists as a zwitterion at the pH of the blood and urine. The present authors, therefore expected that the tissue distribution and further metabolism of the compound would be minimal and that the study of plasma and urine concentrations of the acid metabolite at various times after dosage would provide the information necessary to assess the way that chromonar is handled by the body.



Scheme I—Metabolism of Chromonar

[1] 3-(β-Diethylamino-ethyl)-4-methyl-7-carbethoxy-methoxy-2-oxo-(1,-2-chromene). Also known as Cassella 4489, Abbott-27053, and Intensain hydrochloride. Chromonar is marketed by the Cassella Co. of Germany.

possessing a higher ratio, *i.e.*, thiopental and thiamylal, become concentrated to a higher degree in the body lipids than do barbital or metharbital and might be expected to be ultrashort acting.

In considering these solubility data in total, an approximate correlation has been observed between the lipophilic nature of the various barbiturate analogs and their therapeutic action. One must view this study with proper perspective in relation to the numerous other physical and chemical properties as well as the various biopharmaceutical parameters which all contribute to the variation in the final therapeutic activity possessed by the members of this series. The net result of the complex interaction of these and other factors determines the type and degree of the pharmacological activity involved.

The underlying concept of the pH-partition hypothesis as an approximate model would seem to be confirmed. However, such phenomena as binding, detoxification, active or passive diffusion, and complexation would play important roles in biological readouts which were not patternized or aberrant in behavior.

It is anticipated that several studies on the barbiturates and sulfonamides relative to solubility and partitioning will be undertaken in these laboratories and will be the subject of future communications.

REFERENCES

(1) A. N. Paruta, *J. Pharm. Sci.*, **58**, 204(1969).

(2) "Remington's Pharmaceutical Sciences," part V, No. 67, Mack Publishing Co., Easton, Pa., 1965.

(3) C. Hansch and S. M. Anderson, *J. Med. Chem.*, **10**, 745 (1967).

(4) A. N. Paruta and S. A. Irani, *J. Pharm. Sci.*, **55**, 1060 (1966).

(5) S. A. Khalil and A. N. Martin, *ibid.*, **56**, 1225(1967).

(6) A. N. Paruta, B. J. Sciarrone, and N. G. Lordi, *ibid.*, **51**, 704(1962).

(7) N. G. Lordi, B. J. Sciarrone, T. J. Ambrosio, and A. N. Paruta, *ibid.*, **53**, 463(1964).

(8) *Ibid.*, **53**, 1349(1964).

(9) R. E. Stuckey, *J. Pharm. Pharmacol.*, **14**, 217(1941).

(10) L. Reber and C. Pathamanon, *Amer. J. Pharm.*, **139**, 234 (1967).

(11) System/360 Scientific Subroutine Package (360A-CM-03X), Version III, Programmers Manual, International Business Machines Corp., White Plains, N. Y., 1968, p. 408.

(12) B. Ostle, "Statistics in Research," Iowa State College Press, Ames, Iowa, 1954, chap. 6.

(13) T. C. Daniels and E. C. Jorgensen, in "Textbook of Organic Medicinal and Pharmaceutical Chemistry," 4th ed., C. O. Wilson and O. Gisvold, Eds., J. P. Lippincott, Philadelphia, Pa., 1962, pp. 334, 336–340.

(14) S. K. Sharpless, in "The Pharmacological Basis of Therapeutics," 3rd ed., L. S. Goodman and A. Gilman, Eds., Macmillan, New York, N. Y., 1968, p. 107.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 2, 1969, from the *College of Pharmacy, University of Rhode Island, Kingston, RI 02881*

Accepted for publication May 11, 1970.

Abstracted in part from a dissertation presented by Thomas L. Breon to the Graduate School, University of Rhode Island, in partial fulfillment of the Master of Science degree requirements.

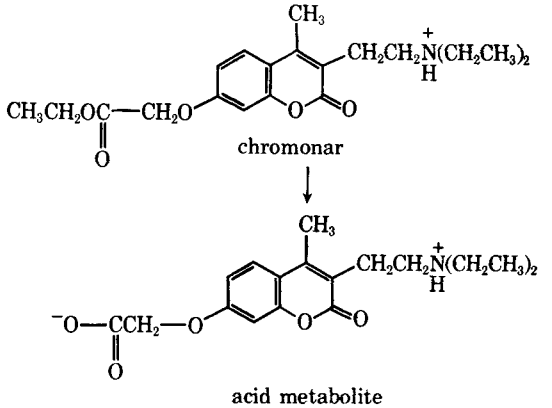
Metabolism and Excretion of Chromonar and Its Metabolite in Dog and Man

YVONNE C. MARTIN and RONALD G. WIEGAND

Abstract □ A simple method for the detection of chromonar and its acid metabolite by fluorescence techniques is described. Following oral or intravenous administration, chromonar is rapidly hydrolyzed to its metabolite, the corresponding acid. No further metabolism is observed. The metabolite distributes according to a single-compartment model. The plasma half-life is 1 hr. in man and dog. Excretion of the metabolite into the bile accounts for approximately 25% of the dose, and excretion into the urine accounts for the remainder.

Keyphrases □ Chromonar—metabolism, excretion □ Metabolite, chromonar—*in vitro, in vivo* determination □ Blood levels, chromonar and acid metabolite—human, dog □ Excretion—chromonar acid metabolite □ TLC—separation, identification □ Fluorometry—analysis

The coumarin compound chromonar[1] is used for the treatment of angina pectoris in Germany and Japan. The early report by Klarwein and Nitz (1) demonstrated that when the drug comes in contact with biological tissues, it is rapidly hydrolyzed to the corresponding acid (Scheme I), which exists as a zwitterion at the pH of the blood and urine. The present authors, therefore expected that the tissue distribution and further metabolism of the compound would be minimal and that the study of plasma and urine concentrations of the acid metabolite at various times after dosage would provide the information necessary to assess the way that chromonar is handled by the body.



Scheme I—Metabolism of Chromonar

[1] 3-(β-Diethylamino-ethyl)-4-methyl-7-carbethoxy-methoxy-2-oxo-(1,-2-chromene). Also known as Cassella 4489, Abbott-27053, and Intensain hydrochloride. Chromonar is marketed by the Cassella Co. of Germany.

Table I—Kinetic Parameters ($\pm SD$) of the Appearance and Disappearance of the Acid Metabolite Following Intravenous Administration of Chromonar to Dogs[a]

Dog	Dose, mg./kg.	Approximate Half-Life, hr., Chromonar	Conversion Constant, Chromonar to the Acid Metabolite, $hr.^{-1}$	Metabolite Disappearance Constant, $hr.^{-1}$	Metabolite Half-Life, hr.	Metabolite Apparent Relative Volume of Distribution, V_d', l./kg.
1	2	0.04	18.6 ± 3.6	1.11 ± 0.02	0.62	0.356 ± 0.022
	5	0.02	85.3 ± 13.1	0.76 ± 0.10	0.92	0.482 ± 0.018
	9	0.01	167 ± 44	0.94 ± 0.20	0.74	0.541 ± 0.036
2	2	0.04	23.2 ± 3.8	0.89 ± 0.12	0.78	0.500 ± 0.033
	5	0.04	20.6 ± 3.7	0.85 ± 0.14	0.81	0.429 ± 0.029
	10	0.04	25.7 ± 5.1	0.36 ± 0.11	1.94	0.563 ± 0.034
3[b]	10	0.02	7.1 ± 1.5	1.09 ± 0.21	0.64	0.408 ± 0.050
4[b]	10	0.02	6.5 ± 1.0	0.95 ± 0.13	0.73	0.458 ± 0.039
5	5			0.87	0.80	
6	5			0.91	0.76	
Mean		0.03		0.87	0.87	0.467

[a] Calculated from the plasma levels of the metabolite and whole blood levels of chromonar. [b] These dogs received 10 mg./kg. chromonar intravenously 6 days/week for 25 months prior to this study.

Table II—Kinetic Parameters ($\pm SD$)[a] of Disappearance of the Metabolite from Plasma Following Oral Administration of Chromonar to Dogs

Dog	Dose, mg./kg.	Lag, hr.	Metabolite Disappearance Constant, $hr.^{-1}$	Metabolite Half-Life, hr.	Metabolite Apparent Relative Volume of Distribution, l./kg.
7	50	0.25	0.71 ± 0.04	0.97	5.78 ± 0.19
8	150	0.25	0.48 ± 0.05	1.44	7.35 ± 0.07
9	150	0.25	0.65 ± 0.04	1.06	2.80 ± 0.11
Mean			0.616	1.16	5.31

[a] Estimated by the method of Wiegand and Sanders (2), the variance was minimized by manipulation of the time lag.

Since both compounds are highly fluorescent, it was possible to modify the original assay method of Klarwein and Nitz (1) to make it more sensitive. To determine the amount of chromonar in a sample, it was extracted immediately after collection into ether from basic solution; then it was reextracted into acid, and the fluorescence of the acid phase was determined. The amount of the acid metabolite in a sample was determined by the fluorescence of the supernatant solution after trichloroacetic acid precipitation.

EXPERIMENTAL

Reagents—Chromonar and the acid metabolite[1] were used. The standards used in the analytical procedures were homogeneous on TLC in Solvent Systems I and II. The chromonar hydrochloride, which was administered to animals and to human subjects, contained 0.6% of 3-(β-diethylamino-ethyl)-4-methyl-7-hydroxy-2-oxo-(1,2-chromene), which is the immediate chemical precursor of chromonar. The fluorescence spectrum of this compound did not interfere with the determination of chromonar or the acid metabolite.

Diethyl-*p*-nitrophenyl monothiophosphate[2] was the commercial product. All other reagents were commercial products of reagent grade.

Fluorescence Analysis of Chromonar—Within 5 sec. after withdrawal from the subject, the blood sample (usually 1 ml.) was shaken vigorously by hand for 1 min. in a 50-ml. ground-glass-stoppered centrifuge tube with 1 ml. 1 *M* tris chloride buffer, pH 8.6, and 25 ml. diethyl ether. These ether extracts are stable for several hours. When a convenient number of samples had been extracted, within 1–3 hr. of the original extraction, the tubes were centrifuged at low speed and 20 ml. of the ether phase was transferred to a second tube which contained 3 ml. 0.1 *N* HCl. The samples were then mechanically shaken for 10 min. and centrifuged. The ether layer was discarded, and the fluorescent intensity of the aqueous layer was determined in an Aminco-Bowman spectrophotofluorometer. The excitation wavelength was 334 mμ and the emission wavelength was 400 mμ. A tissue blank, standard chromonar solution, and tissue blank plus standard were analyzed with each set of samples.

Fluorescence Analysis of Chromonar plus Its Acid Metabolite—When the total of chromonar plus the acid metabolite is to be analyzed, there is no need for the pH 8.6 extraction. Consequently, the sample is treated with trichloroacetic acid to precipitate proteins, and the fluorescence is read on the supernatant solution.

Plasma or urine samples were used as such or diluted with isotonic saline. Feces were homogenized with five volumes of isotonic saline and then further diluted with saline if necessary. Samples could be stored at −15° for several weeks with no apparent degradation of the acid metabolite.

For the analysis, 2 ml. of 5% trichloroacetic acid was added with vigorous shaking to 0.1 ml. of the sample. The fluorescence of the clear supernatant layer was determined after the samples were centrifuged. A tissue blank, standard, and tissue blank plus standard were included with each set of samples.

TLC of Urine, Bile, and Fecal Samples—The methods and solvent systems were similar to those used by Klarwein and Nitz (1). Following sample applications the TLC plates (Analtech silica gel G, 200 μ thick) were first developed with water, then dried for 1 hr. at 100°, and then redeveloped with one of the following systems: I, ethanol–chloroform–water in a 10:2:1 volume ratio; II, ethylacetate–isopropyl alcohol–ammonium hydroxide in a 9:7:4 volume ratio; or III, chloroform–isopropyl alcohol–acetic acid–water in a 4:2:1:1 volume ratio. The location of fluorescent spots was detected under long wavelength UV light by comparison with authentic compounds; chromonar and the acid metabolite emit a characteristic yellow-green color.

To quantitate the acid metabolite after chromatography of a known volume of sample, the spots with the same R_f as the standard were scraped off and extracted with several portions of 95% ethanol. The samples were diluted with water, and the metabolite

[2] Parathion, K&K Laboratories, Inc., Plainview, N. Y.

content was then determined in 5% trichloroacetic acid solution as previously described.

***In Vitro* Conversion of Chromonar to Its Metabolite by Human Plasma**—Freshly collected human plasma from three subjects was incubated with chromonar. At 1, 2, 3, 4, and 5 min., samples were removed and analyzed for the concentration of unchanged drug.

Dog Blood Level Studies—The plasma concentration of chromonar and its acid metabolite was determined after intravenous administration of chromonar in isotonic saline to six dogs. Dogs 1 and 2 were 10-kg. female mongrels which had been anesthetized by 3 mg./kg. s.c. morphine followed in 15 min. by 250 mg./kg. sodium barbital. Three doses were given to each animal, with intervals of 2 hr. Blood samples were taken from the jugular vein. Dogs 3 (female, 9.5 kg.) and 4 (male, 9.6 kg.) were on a chronic toxicity study for 25 months at the same dose. They were not anesthetized. Blood samples from these dogs were taken from the femoral vein. Female mongrels, Dogs 5 (5.5 kg.) and 6 (14 kg.), were anesthetized as described. The ureters and common bile ducts were cannulated, and the total urinary and biliary output was collected for 4 hr.

The plasma concentration of the acid metabolite was also determined after oral administration of chromonar in a capsule. Dog 7 was a 14.5-kg. male. Dogs 8 (female, 7.1 kg.) and 9 (male, 13.8 kg.) had been on a chronic toxicity study for 25 months at the same dose. None of these dogs was anesthetized. Blood samples from these dogs were taken from the femoral or jugular vein.

Human Blood Level Studies—In a multiple-dose study, 12 normal volunteers were divided into two groups of six. The first group, Subjects 1–6, took 150 mg. of chromonar orally on Day 1 and, for the following 6 days (2–7 inclusively), 150 mg. orally, t.i.d. On the 8th day of the experiment, they were given a final oral dose of 150 mg. In addition, Subjects 1 and 2 received an intraduodenal dose of 150 mg. prior to Day 1. The second group was given 40 mg. of chromonar intravenously on Day 1; for the subsequent 6 days, they received 150 mg. of the drug orally, t.i.d. On the 8th day, they were given a second 40 mg. of chromonar intravenously. Plasma samples were collected from blood taken from the femoral vein on Days 1 and 8 at appropriate times. Urine samples were collected prior to and 0–4, 4–8, and 8–24 hr. following drug administration.

Six additional normal adult subjects were given three 150-mg. oral doses of chromonar tablets with 6 hr. between doses. Plasma was obtained from blood drawn at 0, 1, 2, 4, 6, 8, 10, 12, 14, and 24 hr.

Excretion of the Acid Metabolite by Humans—To quantitate the amount of acid metabolite recovered in urine and feces, four subjects (19–22) were given a single 150-mg. tablet of chromonar. Urine and feces were collected for 4 days after drug administration.

Calculation of Kinetic Constants from Blood Level Studies—For the calculation of the disappearance constant and the apparent relative volume of distribution of the metabolite, the computer program of Wiegand and Sanders (2) was used. The curve of the plasma levels of the metabolite, which was determined following the oral administration of chromonar to dogs, often indicated an apparent delay in absorption of 10–15 min. In these cases the kinetic constants were recalculated using several time lags. The lag which resulted in the best fit (lowest sum of squares of deviations of experimental points from the calculated curve) was chosen, and these values are reported in the tables. For the case of blood levels of the metabolite following oral administration of chromonar to humans, data were not sufficient for use of this program. In the case of intravenous administration of chromonar to dogs, the first-order constant that describes the increase in plasma concentration of the metabolite relates to the conversion of chromonar to its metabolite rather than to absorption of drug, as in the original formulation of the program (3).

For intravenous administration of chromonar to humans, plasma samples were not taken until 0.5 hr. after the dosing. Thus, the conversion to the acid metabolite was complete. Since the disappearance curve suggested a single-compartment model, the plasma half-life of the metabolite was calculated for these subjects, assuming an exponential decline of plasma levels with time. The slope of the least-squares line of the logarithm of concentration against time gives the first-order disappearance constant, k_d. The plasma half-life, $t_{1/2}$, is related to k_d by the expression $t_{1/2} = \ln 2/k_d$. The intercept of this plot is c_o, the theoretical initial plasma concentration. However, this theoretical initial concentration is also equal to the dose of the drug, a_o, divided by the apparent relative volume of distribution of the drug V_d'. Thus, V_d' was evaluated as $V_d' = a_o/c_o$.

Table III—Recovery of the Acid Metabolite 4 hr. after the Intravenous Administration of 5 mg./kg. Chromonar to Dogs

Dose, %	Dog 5	Dog 6
Urine	53.2	75.3
Bile	27.4	16.6
Total recovery, %	80.6	91.9

Calculation of Area under the Blood Level Curves—Areas under the acid metabolite plasma level curves for the subjects who had received chromonar orally were calculated by the addition of the area of each triangular or trapezoidal segment of the actual curve. The areas on Day 8 were corrected for the area due to the initial plasma concentration of the metabolite.

Areas under the metabolite blood level curve following intravenous administration were calculated from the extrapolated theoretical initial plasma concentration of the metabolite. The relationship is: area $= c_o/k_d$.

RESULTS

Analytical Methods—Klarwein and Nitz (1) reported that diethyl-*p*-nitrophenyl monothiophosphate could be used to prevent the hydrolysis of chromonar. However, in preliminary investigations, it was observed that when diethyl-*p*-nitrophenyl monothiophosphate was used, a slight excess of this inhibitor (twice the concentration necessary for inhibition of the serum esterase) quenched the fluorescence of the chromonar in the final solution. Thus, diethyl-*p*-nitrophenyl monothiophosphate was not used in the present method; instead, all samples analyzed for the concentration of chromonar were extracted immediately (within 5 sec.) from the basic solution with ether. With this procedure, 95–100% of the standard chromonar which had been added to a blood or tissue homogenate sample was recovered. A typical blank blood sample corresponded to 0.02 mcg. chromonar/ml.

In the assay method for the total of chromonar and its acid metabolite, the simple trichloroacetic acid precipitation to remove most of the interfering substances from plasma, urine, bile, and feces was practical because of the intense fluorescence of the drugs. A relatively selective and sensitive spectrophotofluorometer is also necessary. In this procedure, a typical plasma blank in the determination of either chromonar or its acid metabolite corresponded to 0.01 mcg./ml.

For both methods the range of concentrations over which the fluorescence is linear with respect to concentration was 0.04–10.0 mcg./ml. sample. Above this range the fluorescence decreased with concentration. Since the concentration of drug in certain urine and bile samples exceeded 10 mcg./ml., appropriate dilutions were necessary.

The intensity of fluorescence of chromonar and its metabolite depends on the pH of the sample (1). It was, therefore, essential that the samples, standards, and blanks were prepared in the same way. If a sample exhibited too high a fluorescence, it was not further diluted with water but rather with the same concentration of acid that had been used in the original determination.

***In Vitro* Conversion of Chromonar to Its Metabolite by Human Plasma**—The half-life ($\pm SD$) was calculated to be 2.68 ± 0.96 min.; thus, chromonar is extremely short-lived in human plasma.

Blood Levels in Dogs—Calculations derived from the plasma concentration of chromonar and its metabolite at various times after an intravenous dose of chromonar are summarized in Table I. The half-life of chromonar is very short in the dog; essentially none remained in the blood after 10 min. (0.17 hr.). The plasma half-life of the metabolite averaged 0.87 hr. This relatively short half-life represents either efficient excretion or rapid metabolism of the metabolite. The decline in plasma levels of the metabolite parallels the decline of coronary vasodilation.[3] The half-life of the metabolite was not significantly shorter in Dogs 3 and 4, which had been on a chronic study for 25 months prior to this study.

[3] T. D. Darby and Y. C. Martin, unpublished observations.

Table IV—Calculations from Plasma Levels of the Metabolite in Six Subjects Given 40 mg. Chromonar Intravenously

Subject	Sex	Dose, mg./kg.	Day	Metabolite Disappearance Constant, hr.,$^{-1}$ $\pm SD$	p^a	Metabolite Half-Life, hr.	Intercept C_o, mcg./ml.	Area, hr. mcg./ml.	Metabolite Apparent Relative Volume of Distribution, l./kg.
7	M	0.48	1	0.670 ± 0.071	0.50	1.03	1.20	1.79	0.40
			8	0.735 ± 0.052		0.94	1.36	1.85	0.35
8	F	0.70	1	0.858 ± 0.102	0.26	0.81	0.89	1.04	0.79
			8	0.716 ± 0.043		0.96	1.33	1.86	0.53
9	M	0.52	1	0.903 ± 0.106	0.63	0.76	1.51	1.67	0.34
			8	0.804 ± 0.019		0.86	1.20	1.49	0.43
10	M	0.52	1	0.843 ± 0.026	0.79	0.82	1.46	1.73	0.36
			8	0.856 ± 0.038		0.81	1.33	1.55	0.39
11	F	0.77	1	0.855 ± 0.033	0.31	0.81	1.75	2.05	0.44
			8	0.905 ± 0.032		0.77	1.92	2.12	0.40
12	F	0.66	1	0.957 ± 0.053	0.50	0.72	1.53	1.60	0.43
			8	0.882 ± 0.088		0.79	1.81	2.05	0.36
Mean			1	0.861 ± 0.063	0.68	0.80	1.39	1.65	0.46
Mean			8	0.830 ± 0.043		0.84	1.49	1.82	0.41
Mean			1 and 8			0.82	1.44	1.74	0.44

[a] Probability value, which should be less than 0.05 if slopes are significantly different on Days 1 and 8 in same subject.

With oral administration of chromonar, a 0.25-hr. time lag in absorption was observed (Table II); this is approximately the disintegration time of the gelatin capsules used to administer the drug. The mean half-life of the metabolite in blood was 1.16 hr.; this is somewhat longer than that determined after intravenous administration.

In the case of oral dosage, the V_d' term as calculated also includes the fraction of the drug that is not absorbed. The ratio of the intravenous to oral volumes of distribution, 0.088, indicates that in the dog 8.8% of an oral dose of chromonar is absorbed.

Excretion of the Metabolite by Dogs—Within 4 hr. after a single intravenous dose of 5 mg./kg. of chromonar, an average of 86% of the dose was recovered in the urine and bile as the metabolite (Table III). The identity of the fluorescent material was confirmed by quantitative TLC. At least 90% of the fluorescence was cochromatographic with the authentic sample of the acid metabolite. Since the plasma half-life of the metabolite is the same in these dogs (Dogs 5 and 6) as in the others studied, one may conclude that the major route of disappearance of the metabolite from blood is excretion into the urine and bile. There is no evidence for further metabolism or tissue accumulation of the drug. In these cases the bile was collected as it was excreted. Since the plasma half-life of the acid metabolite was not shortened by this procedure, enterohepatic circulation apparently does not occur.

Human Blood Level Studies—The mean half-life of the acid metabolite following the intravenous administration of chromonar was 0.80 hr. (Table IV). There were no apparent differences between males and females. When these six subjects (7–12) were given 150 mg. chromonar, t.i.d., orally for 6 days and the intravenous study was repeated, the half-life was not significantly altered. There was also no significant change in the intercept, the area under the plasma curve, or the apparent relative volume of distribution. Thus, as in the case of the dog, there is apparently no metabolic adaptation to chromonar after continued administration.

The actual values for the plasma concentration of the metabolite following intravenous administration of chromonar to Subject 11 are plotted in Fig. 1. This is graphic illustration of the lack of metabolic adaptation by repeated doses of chromonar. The plot of the logarithm of concentration against time is linear, which confirms the appropriateness of using the single-compartment model for distribution of the metabolite.

The standard deviation of the values calculated for the first-order disappearance constants of the plasma levels in each subject is usually approximately 10% of the value of the constant. This also indicates that the data are fit very well by a single-term equation or a single-compartment model. There was no indication of a deviation from simple first-order disappearance in any of the data.

The six subjects (1–6) who were given oral doses of chromonar separated by 6 days of 150-mg. t.i.d. dosage showed no consistent change in the area under the metabolite plasma curve (Table V). The area under the curve is a measure of availability of drug by the oral route. The two subjects (1 and 2) who received an intraduodenal dose showed essentially the same area under the plasma curve after this dose as after oral drug, indicating that chromonar may be absorbed from the intestine.

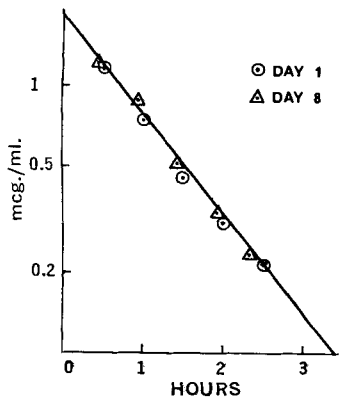


Figure 1—*Plasma levels of the acid metabolite after single 40-mg. i. v. doses of chromonar to Subject 11 (Table IV). On Days 2–7, 150 mg. chromonar t.i.d. was given orally.*

Table V—Calculated Areas under the Metabolite Plasma Level Curves in Six Subjects Given 150 mg. Chromonar Orally

Subject	Sex	Dose, mg./kg.	Day	Area, hr. mcg./ml.
1	M	2.23	0(duodenal)	1.48
			1	2.33
			8	1.58
2	M	2.13	0(duodenal)	1.01
			1	1.33
			8	1.73
3	M	2.06	1	2.52
			8	1.10
4	F	3.00	1	2.46
			8	1.52
5	F	1.99	1	1.50
			8	1.63
6	F	2.28	1	2.44
			8	2.11
Mean ($n=6$)			1	2.10
			8	1.61

Table VI—Human Urine Recoveries of the Metabolite Following Administration of Chromonar

Subject	mg. Acid Metabolite Recovered in Urine: Day 1 0–4 hr.	Day 1 4–8 hr.	Day 1 8–24 hr.	Day 1 %	Day 8 0–4 hr.	Day 8 4–8 hr.	Day 8 8–24 hr.	Day 8 %
				Oral				
1 duod.	18.0	1.3	1.5	20.5				
oral	33.8	5.0	0.2	26.0	18.6	9.7	0.5	19.2
2 duod.	25.4	2.7	0.4	19.0				
oral	25.4	5.9	1.1	21.6	43.3[a]	3.6[b]	0.6	31.7
3	9.4	4.2	1.3	9.4	31.8	3.2	1.4	24.3
4	21.2	12.5	0.0	22.5	25.2	11.5	1.6	32.2
5	27.4	9.4	0.2	25.7	2.07	8.97	0.74	7.87
6	47.9	8.9	0.0	37.9	36.0	4.7	0.0	27.2
			Mean	23.8			Mean	23.8
				Intravenous				
7	27.0	2.9	0.3[c]	78.0	18.6[d]	2.5[e]	0.9	55.0
8	19.5	6.2	1.7[e]	68.5	33.0	2.6	0.8[c]	91.0
9	11.8	2.2	4.1[c]	45.3	22.2	1.5		59.3
10	19.0	1.5[f]	0.2	54.3	24.3[g]	1.0	1.6	67.3
11	14.8	0.5	0.3	39.0[h]	18.3[c]	1.3	0	49.0
12	24.5	6.4	1.9	82.0	33.9	2.9	2.7	98.8
			Mean ($n=5$)	65.6			Mean ($n=6$)	70.1

[a] 0–6 hr. [b] 6–13 hr. [c] Might be overlap with next dose or previous. [d] 0–2 hr. [e] 2–6 hr. [f] 4–10.5 hr. [g] 0–5 hr. [h] Refrigerated, but not frozen, for 4 days; not included in mean.

Table VII—Recovery of the Metabolite in Urine and Feces Following an Oral Dose of 150 mg. Chromonar

Recovery of the Metabolite, hr.	Subject 19	20	21	22
		Urine, mg. Recovered		
0–24 hr.	25.3	0.2	43.4	58.2
24–48 hr.	5.4	40.9	1.2	0.0
48–72 hr.	1.2	0.2	0.0	0.0
72–96 hr.	0.0	0.2	0.2	0.2
Total	32.8	41.5	44.8	58.2
		Feces, mg. Recovered		
0–24 hr.	0.5	0.0	23.6	0.0[a]
24–48 hr.	44.0	39.6	21.0	7.6[a]
48–72 hr.	24.9	31.4	25.4	7.6[a]
72–96 hr.	12.9	10.5	2.1	3.2[a]
Total	82.3	81.5	72.1	18.4
Total g. samples	327	253	276	94
Total mg. excreted	115.5	123.0	116.9	76.6
% of dose recovered as the metabolite[b]	83	89	84	55

[a] Low weights of fecal samples in this subject (see text). [b] Corrected for difference in molecular weights and purity of administered drug.

The actual serum levels of the metabolite following oral administration of chromonar to Subject 2 are plotted in Fig. 2. Note that the rate of decline of serum levels of the metabolite is approximately the same as that of Subject 11 to whom the dose was administered intravenously. The variation in the amount of chromonar absorbed by the same subject on different days is often seen.

A comparison of the areas under the plasma curves after intravenous and oral dosage allows estimation of the fraction of the dose absorbed orally. Correction for the dose by each route must be made. Calculated on this basis from the data in Tables IV and V, 32% of the drug was absorbed on the 1st day of oral administration; on the 8th day, 25% was absorbed. Thus, the overall average absorption was 28%.

After a 6-day (t.i.d.) regimen of chromonar orally, the mean plasma concentration of the metabolite was 0.06 mcg./ml. 10 hr. following the last dose. (This compares with a mean of 0.9 mcg./ml. at the peak following an oral dose.) Thus, there was no appreciable accumulation of the metabolite.

The plasma concentrations of the metabolite when the drug was given at 6-hr. intervals (Subjects 13–18) are summarized in Fig. 3. The pattern of absorption does not appear to vary from one dose to another. There was also no accumulation during the day, and by 24 hr. the plasma levels of the metabolite were essentially zero. The plot of the mean plasma concentration against time was fit very well by a calculated curve in which first-order absorption and elimination were assumed. A value of 1.60 hr.$^{-1}$ was used for the

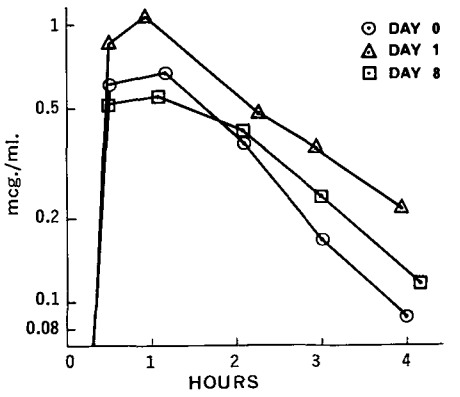


Figure 2—*Plasma levels of the acid metabolite after single duodenal (Day 0) or oral (Days 1 and 8) doses of 150 mg. chromonar to Subject 1 (Table V). On Days 2–7, 150 mg. chromonar t.i.d. was given orally.*

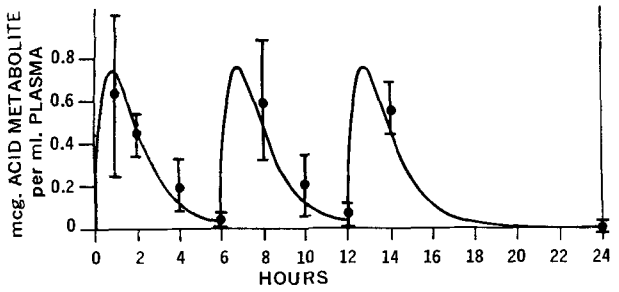


Figure 3—*Plasma levels of the acid metabolite. Three 150-mg. tablets were given with 6 hr. between doses.*

absorption constant, 0.820 hr.$^{-1}$ for the disappearance constant, 1.53 l./kg. for the apparent volume of distribution, and 2.28 mg./kg. for the dose.

From the intravenous studies the apparent volume of distribution of the acid metabolite in man was 0.44 l./kg. (Table IV).

Human Excretion Studies—The urinary excretion data from Subjects 1–12 are summarized in Table VI. Excretion of the metabolite occurred primarily in the first 4 hr. after administration. In the second 4-hr. period, relatively less drug was excreted after intravenous dosage than after oral dosage, which is consistent with the plasma curves and with the expected delay after oral administration due to the absorption phase. When the drug was administered intravenously, an average of 68% of the dose of chromonar was excreted into the urine as the metabolite, with the remainder probably excreted into the bile. From the urinary excretion data for these subjects, it is also possible to calculate the percent of an oral dose which was absorbed. Calculated from the average percent of the doses excreted in the urine, absorption was 36% on the 1st day and 34% on the 8th day, for an overall average of 35%. This agrees very well with the figure of 28% absorption determined from the plasma levels of the same subjects.

Following a single oral dose of chromonar, an average of 77% of the dose is recovered as the acid metabolite in urine plus feces (Table VII). The fecal weights from Subject 22 were very low; excluding this subject, the recoveries averaged 85%. The identity of the fluorescent material in the samples was confirmed by TLC in Solvent Systems II and III.

Bishydroxycoumarin, ethyl biscoumacetate, and warfarin are well-known drugs which also contain the coumarin ring system. All three of these drugs are completely metabolized, although the coumarin portion of the molecules is usually not degraded (4–6). The apparent multicompartment distribution reported for bishydroxycoumarin (7) is not seen with the acid metabolite of chromonar.

SUMMARY

Following the oral or intravenous administration of chromonar, the drug is rapidly hydrolyzed to the corresponding acid. It is largely in this form that the drug circulates in the blood and is excreted into the bile and urine. In dogs as well as humans, the plasma half-life of the acid metabolite is approximately 1 hr. The decline in plasma levels conform to a single-compartment model. Excretion, not further metabolism, accounts for the decline in blood levels of this metabolite. Biliary excretion is an important factor; in the dog an average of 22% of an intravenous dose was recovered in the bile and 64% in the urine. There is no evidence of accumulation of the metabolite when chromonar is administered on a chronic basis.

REFERENCES

(1) M. Klarwein and R. E. Nitz, *Arzneim.-Forsch.*, **15**, 555(1965).

(2) R. G. Wiegand and P. G. Sanders, *J. Pharm. Exp. Ther.*, **146**, 271(1964).

(3) A. Goldstein, L. Aronow, and S. Kalman, "Principles of Drug Action," Harper & Row, New York, N. Y., 1968, p. 322.

(4) R. Nagashima, G. Levy, and E. J. Sarcione, *J. Pharm. Sci.*, **57**, 1881(1968).

(5) R. T. Williams, "Detoxication Methods," Wiley, New York, N. Y., 1959, p. 631.

(6) R. A. O'Reilly, P. M. Aggeler, M. S. Hoag, and L. Leong, *Thromb. Diath. Haemorrh.*, **8**, 82(1962).

(7) R. Nagashima, G. Levy, and R. A. O'Reilly, *J. Pharm. Sci.*, **57**, 1888(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 11, 1969, from *Chemical Pharmacology Department, Abbott Laboratories, North Chicago, IL 60064*

Accepted for publication April 23, 1970.

The technical assistance of Erna Scherfling and Harold Brondyk is gratefully acknowledged.

Content Uniformity in Rectal Suppositories

IVO SETNIKAR and FEBO FONTANI

Abstract □ The content variability in five types of suppositories was evaluated in terms of coefficient of variation. The observed content variability had a coefficient of variation in the range of 1.2–4.5; the method variability had a coefficient of variation in the range of 0.3–4.3. Method variability interferes with the assessment of the actual content variability and, according to the official content uniformity specifications, may lead to the rejection of complying samples or to the acceptance of noncomplying samples. The rationale and the structure of the official content uniformity specifications were studied. The performance of official content uniformity specifications declines as method variability increases. An alternative approach for the evaluation and the restriction of content variability, based on the coefficient of variation, may be advantageous in some instances.

Keyphrases □ Suppositories, rectal—content uniformity □ Content uniformity, suppositories—official specifications □ Variability, drug content—suppositories

In discrete dosage forms, the content accuracy and the content unit-to-unit uniformity of the active ingredient are basic and obvious requirements for assuring reliable and constant therapeutic effects. Several investigators (1–5) have studied the manufacturing, analytical, and statistical problems involved in the control of content uniformity of tablets and capsules. In fact, content uniformity specifications are already given for some tablets in the USP XVII (6) and in the NF XII (7).

But content nonuniformity may affect other dosage forms besides tablets and capsules. For instance, sedimentation, heterogeneous repartition during the melting-casting process, or weight variations (8) may result in an excessive unit-to-unit content variation in rectal suppositories, which are dosage forms designed either to develop a local therapeutic effect or to serve as the vehicle for a drug with a general action. Especially in the second instance, the content uniformity of the active ingredient is as important as for oral dosage forms. Nevertheless, little attention has been given to this subject, apart from Elste *et al.* (9) who investigated some assay methods of content in individual suppositories.

The present investigation was undertaken to study the analytical and statistical aspects of the content uniformity control in rectal suppositories and to give a further contribution to this subject.

EXPERIMENTAL

Materials—Five types of suppositories, containing from 0.19 to 30% active principle, were investigated:

1. DIN, *i.e.*, 2.3-g. suppositories containing 15 mg. of dehydroepiandrosterone sodium sulfate in a water-soluble base of polyethylene glycol 1450 and 6000,[1] glyceryl monostearate, propylene glycol, and succinonitrile.
2. VAL, *i.e.*, 2.0-g. suppositories containing 100 mg. dimenhydrinate in a water-insoluble base of theobroma oil.
3. GAM, *i.e.*, 2.7-g. suppositories containing 800 mg. phenprobamate in a water-insoluble base of triglycerides of natural fatty acids.[2]
4. MAL, *i.e.*, 2.1-g. suppositories containing 4 mg. dimefline hydrochloride (3-methyl-7-methoxy-8-dimethylaminomethylflavone hydrochloride) and 500 mg. aminopyrine in a water-insoluble base of triglycerides of natural fatty acids.[3]
5. TEF, *i.e.*, 2.1-g. suppositories containing 276 mg. of theophylline and 46 mg. of ethylenediamine (to form aminophylline) in a water-insoluble base of theobroma oil.

The suppositories were manufactured by the melting-casting process previously outlined (8). Before casting, the active ingredients were in suspension in the melted base and the homogeneity was maintained by continuous mechanical stirring.

Methods—Ten replicates of the following assays were done: (*a*) bulk drug used for the preparation of suppositories; (*b*) drug content in samples equivalent in weight to one suppository (samples were taken from a mass obtained by homogenizing, without melting, 30 suppositories of each type) and (*c*) drug content in individual suppositories after weighing each suppository to within a precision of 0.1 mg.

The drug of replicates (*a*) and the samples of replicates (*b*) and (*c*) belonged to the same production batch.

The assays were done manually by the following methods.

Bulk Drugs—Dehydroepiandrosterone Sodium Sulfate—The bromometric method for Δ^5-steroids, according to Gorac (10), was used.

Dimenhydrinate—Assay was done with perchloric acid in glacial acetic acid as proposed by Meulenhoff and Van Sonsbeek (11).

Phenprobamate—Kjeldahl's method of nitrogen determination, using the accelerator "selenium mixture Merck" proposed by Wieninger (12), was used.

Dimefline Hydrochloride—This method involved titration in glacial acetic acid after adding mercuric chloride solution, using perchloric acid and methyl violet as indicator. One milliliter of 0.1 *N* perchloric acid is equivalent to 35.98 mg. of dimefline hydrochloride.

Aminopyrine—Perchloric acid method in anhydrous medium (13) was used.

Ethylenediamine—The method of De Lorenzi (14), based on the addition of formaldehyde to neutralized solutions of ethylenediamine hydrochloride and titration of the developed acidity with 0.1 *N* sodium hydroxide, was followed.

Theophylline—About 250 mg., exactly weighed, was dissolved in 30 ml. of anhydrous pyridine previously neutralized to thymolphthalein. The solution was then titrated with 0.1 *N* alcoholic potassium hydroxide to a deep-blue color. One milliliter of 0.1 *N* potassium hydroxide is equivalent to 18.02 mg. of anhydrous theophylline.

Drugs Vehicled in the Suppository Bases—DIN—The procedure described by Clark and Thompson (15) for the pure ingredient was adapted to suppositories. One suppository was dissolved in 100

[1] Carbowax 1450 and 6000, Union Carbide Corp., New York, N. Y.
[2] Imhausen H, Imhausen Werke, Witten-Ruhr, Germany.
[3] Imhausen W, Imhausen Werke, Witten-Ruhr, Germany.

Table I—Analytical Statistics Obtained on Bulk Drug, on Homogenized Suppositories, and on Individual Suppositories

Suppositories and Entries[a]	Content of Active Ingredient— Bulk Drug, %	Homogenized Suppository, mg./g.	Individual Suppository, mg./Suppository	Suppository Weight, mg.
DIN				
NC	98.27[b]		15	
ACF	98.35	6.06	14.7	2308
CV	0.29	4.33	2.44	1.62
R%	−0.6–0.3	−5.1–6.7	−5.1–2.4	−1.9–3.1
NR			−7.2–0.0	
FLM_{95}	98.15–98.55	5.87–6.25	14.4–14.9	2281–2335
VAL				
NC	100		100	
ACF	100.15	51.44	102.1	1977
CV	0.14	0.57	3.45	1.57
R%	−0.1–0.3	−1.1–0.9	−4.2–7.4	−2.3–2.5
NR			−2.1–10.3	
FLM_{95}	100.05–100.25	51.23–51.65	99.6–104.6	1956–1998
GAM				
NC	100		800	
ACF	99.86	300	784	2675
CV	0.36	1.13	1.84	1.48
R%	−0.6–0.7	−1.4–1.7	−2.0–2.8	−1.7–2.7
NR			−4.0–0.6	
FLM_{95}	99.60–100.12	297–302	773–795	2647–2703
MAL–DIM				
NC	100		4.00	
ACF	100.04	1.93	3.97	2060
CV	0.14	0.66	1.20	1.06
R%	−0.2–0.3	−0.6–1.1	−2.3–2.0	−1.4–2.2
NR			−3.0–1.3	
FLM_{95}	99.93–100.15	1.92–1.94	3.94–4.00	2046–2074
MAL–AMI				
NC	100		500	
ACF	100.03	242.5	499	
CV	0.15	0.30	1.74	
R%	−0.2–0.2	−0.5–0.4	−2.0–3.3	
NR			−2.2–3.2	
FLM_{95}	99.92–100.14	242.0–243.0	493–505	
TEF–ETHYL				
NC	100		46	
ACF	99.57	20.7	43.5	2088
CV	0.15	3.56	3.45	1.27
R%	−0.2–0.1	−6.2–3.2	−4.8–6.2	−2.0–2.0
NR			−10.0–0.4	
FLM_{95}	99.46–99.67	20.1–21.2	42.4–44.6	2069–2107
TEF–THEO				
NC	90.85[c]		276	
ACF	90.42	134	280	
CV	0.26	3.19	4.47	
R%	−0.5–0.4	−2.5–6.1	−6.6–5.4	
NR			−5.1–7.0	
FLM_{95}	90.25–90.59	131–137	271–289	

[a] NC = nominal content; ACF = average content found; *CV* = coefficient of variation; R% = range found, calculated in percentage of the ACF; NR = range in percentage of the NC; FLM_{95} = 0.05 *p* fiducial limits of the ACF. [b] Water content 1.73%. [c] Water content 9.15%.

ml. of methanol by refluxing for 15 min. and, after cooling, was diluted to 200.0 ml. with methanol. The mixture was left to rest, and 2.0 ml. of the decanted limpid liquid was evaporated to dryness in a test tube. One milliliter of antimony trichloride reagent (150 g. $SbCl_3$ in 50 ml. of acetic anhydride) was added, and the test tube was kept for 5 min. in a water bath at 50 ± 2°. After cooling, 2.0 ml. of acetic anhydride and 3.0 ml. of acetic acid were added. After 40 min., the developed color was measured and compared with a standard solution of dehydroepiandrosterone sodium sulfate submitted to the same procedure.

VAL—One suppository was added to 100.0 ml. of water in a stoppered flask and heated to 45° until the base melted. The flask was shaken and cooled to room temperature. The content was filtered, and 5.0 ml. of the filtrate was diluted to 100.0 ml. with 0.01 *N* sodium hydroxide. The absorbance was determined at 276 mμ using 0.01 *N* sodium hydroxide as blank. As the standard, a solution of dimenhydrinate in 0.01 *N* sodium hydroxide was used. Dimenhydrinate was taken from the same batch used for manufacturing the suppositories.

GAM—One suppository was stirred in 50 ml. of petroleum ether (40–70°) until the base dissolved. The insoluble drug was quantitatively filtered on a tared sintered-glass funnel and washed with three 20-ml. portions of petroleum ether. The funnel was dried to constant weight in a vacuum over P_2O_5. The weight of the residue (melting at 100–105°) was taken as the phenprobamate in the sample.

MAL: Assay of Dimefline Hydrochloride (MAL–DIM)—One suppository was dissolved in 50.0 ml. of chloroform. A portion of 20.0 ml. of this solution was evaporated to dryness on a water bath. To the residue, 50 ml. of 0.1 *N* hydrochloric acid was added. The mixture was heated and stirred on a water bath at 45° for 5 min., cooled, diluted to 100.0 ml. with 0.1 *N* hydrochloric acid, and filtered; 25.0 ml. of the filtrate was diluted to 50.0 ml. with 0.1 *N* hydrochloric acid. On this solution the absorbance was determined at 309 mμ using 0.1 *N* hydrochloric acid as blank. The *a* for dimefline hydrochloride is 61.9.

MAL: Assay of Aminopyrine (MAL–AMI)—To another portion of 20.0 ml. of the chloroformic solution, prepared for the MAL–DIM assay, 20 ml. of glacial acetic acid was added. The titration was done with 0.1 *N* perchloric acid, using methyl violet as indicator. One milliliter of 0.1 *N* perchloric acid corresponds to 23.13 mg. of aminopyrine.

TEF: Assay of Ethylenediamine (TEF–ETHYL)—One suppository was stirred in 50 ml. ethyl ether until the base dissolved. After extraction with four 20-ml. portions of water, the aqueous extracts

were filtered through a filter paper and diluted to 100.0 ml. with water (Solution A). To this solution, 0.1 *N* hydrochloric acid was added until the solution was neutral to methyl orange indicator. Five milliliters of 40% aqueous formaldehyde, previously neutralized to the same indicator, was added; the developed acidity was titrated with 0.1 *N* sodium hydroxide. One milliliter of 0.1 *N* sodium hydroxide corresponds to 6.0 mg. of anhydrous ethylenediamine.

TEF: Assay of Theophylline (TEF–THEO)—Exactly 1 ml. of Solution A, prepared as already described, was diluted to 250.0 ml. with 0.01 *N* sodium hydroxide, and the absorbance was measured at the maximum (about 275 mμ). The amount of drug in the sample was calculated according to the British Pharmacopoeia (16) taking 65.0 as the value of *a* for anhydrous theophylline.

RESULTS

Compliance with the Official Content Uniformity Specifications (OCUS)—The content ranges of the active ingredient (Table I, Entry NR) in the investigated suppositories were within the limits prescribed in the first step of the OCUS given by USP XVII (6) for some oral dosage forms. In fact, in the 10 specimens investigated, the active ingredient found was always within the $M \pm 0.15\ M$ limits (M = nominal content). The OCUS may, therefore, be extended to rectal suppositories, where a heterogeneous distribution of discrete particles of the active ingredients between the individual dosage units is likely, as in oral dosage forms with a relatively small quantity of active ingredient compared to the inert ingredient (3, 4).

Assay Method for Content Uniformity Tests—The large amount of interfering vehicle and the small quantities of active ingredient present in the individual suppositories necessitated different assay methods for the bulk drug from those for the content of each suppository. Table I shows the performance of these assay methods in the different conditions, namely: (*a*) on the bulk drug, (*b*) on the homogenized suppository mass, and (*c*) on the individual suppositories. The analytical intrinsic variability, expressed by the coefficient of variation (*CV*), was usually smaller in the assays of the bulk drug than in the content assays on suppositories. In several instances, the difference between the *CV* of the two types of assay was significant to a $p < 0.05$ level. [The critical value for the ratio between the larger and the smaller *CV* is 1.56 (17).]

Since the assay method on the bulk drug must usually be changed or modified for assaying the content in individual suppositories, its performance cannot be taken as a standard for content assay. The most representative standard is the assay with the elimination of the unit-to-unit content variability, *i.e.*, the assay on samples, of the size of one dosage unit, taken from homogenized suppositories.

Relationship between Content Variability and Observed Unit-to-Unit Variability—The unit-to-unit variability found in the content assays of the individual suppositories is composite and depends on the variability inherent in the analytical method as well as on the actual content variability.

It has been assumed that the different variables follow a Gaussian distribution (1, 18), so that they may be related by the following equation:

$$CV^2_{obs.} = CV^2_{ana.} + CV^2_{con.} \qquad \text{(Eq. 1)}$$

where $CV_{obs.}$ is the *CV* observed, $CV_{ana.}$ is the *CV* inherent in the analytical method, and $CV_{con.}$ is the *CV* depending on the actual content variability. Equation 1 shows that $CV_{con.}$ cannot be estimated directly from $CV_{obs.}$, unless $CV_{ana.}$ is negligible compared to $CV_{obs.}$. Some $CV_{ana.}$'s found, represented by the *CV* obtained on the homogenized suppository mass, were rather large, namely in the same range as those found by other authors in assays of the content in individual tablets or capsules with drug combinations or with small quantities of active ingredient compared to the inert ingredients (3, 18–21). The $CV_{ana.}$, therefore, interfered with the estimation of the $CV_{con.}$. As a matter of fact, the $CV_{obs.}/CV_{ana.}$ ratio was not significantly greater ($p < 0.05$) than 1 in the DIN, TEF–ETHYL, and TEF–THEO content assays (Table II). Thus, in these suppositories, there is no proof of an actual content variability, since this is concealed by the assay method variability. Conversely, the presence of an actual content variability is demonstrable at a $p < 0.05$ level in the VAL, GAM, MAL–DIM, and MAL–AMI suppositories (Table II).

Table II—Analysis of the Variabilities Observed in the Assays

Suppository Type	Active Ingredient, %	$\frac{CV_{obs.}{}^a}{CV_{ana.}}$	$\frac{CV_{obs.}{}^a}{\sqrt{CV^2_{ana.} + CV_W{}^2}}$
DIN	0.65	0.56	0.53
VAL	5.0	6.03	1.99
GAM	29.3	1.63	0.99
MAL–DIM	0.19	1.82	0.96
MAL–AMI	24.3	5.80	1.58
TEF–ETHYL	2.06	0.97	1.00
TEF–THEO	13.4	1.40	1.31

[a] The critical value for the ratio is 1.56 ($\alpha = 0.05$, one-tailed).

Table III—Regressions of Content over the Weight of Different Types of Suppositories

Suppository Type	Regression[a]	t^b of Difference ($a = 0$; $b = 1$)
DIN	$C = (0.68 \pm 0.52) + (0.32 \pm 0.52)\ W$	1.31
VAL	$C = (0.64 \pm 0.77) + (0.36 \pm 0.77)\ W$	0.83
GAM	$C = (-0.09 \pm 0.21) + (1.09 \pm 0.21)\ W$	0.42
MAL–DIM	$C = (0.76 \pm 0.40) + (0.24 \pm 0.40)\ W$	1.92
MAL–AMI	$C = (-0.24 \pm 0.56) + (1.24 \pm 0.56)\ W$	0.42
TEF–ETHYL	$C = (-0.05 \pm 0.89) + (1.05 \pm 0.89)\ W$	0.06
TEF–THEO	$C = (-1.16 \pm 0.99) + (2.16 \pm 0.99)\ W$	1.18

[a] The regressions are standardized on average content and on average weight units. [b] $t = 2.31$ for $\alpha = 0.05$.

The actual content variability may depend on a heterogeneous dispersion of the active ingredient in the suppository mass, or on the suppository-to-suppository weight variability, or on both. The relationship between the different coefficients of variation is

$$CV^2_{con.} = CV_W{}^2 + CV^2_{het.} \qquad \text{(Eq. 2)}$$

where CV_W represents the unit-to-unit weight variability, and $CV_{het.}$ is the coefficient of variation due to heterogeneous dispersion of the active ingredient in the vehicle. From Eqs. 1 and 2, a heterogenous distribution of the active ingredient is demonstrable when $CV_{obs.} > \sqrt{CV^2_{ana.} + CV_W{}^2}$. This seems to be the case of VAL and of MAL–AMI suppositories (Table II). Table II shows that it is not possible to relate heterogeneous distribution to the percentage of active ingredient in the dosage form, as is often the case in solid oral dosage forms (22).

Correlation between Content and Weight—The correlation between content and weight may be studied using the linear regression (23) of content (C) over the suppository weight (W) in the equation

$$C = a + bW \qquad \text{(Eq. 3)}$$

Comparison becomes easier by standardizing the regression, *i.e.*, by adopting as units average content and average weight. By this procedure, Eq. 3 shows a strict weight dependence of content when $a = 0$ and $b = 1$.

The regressions calculated by the least-squares method are given in Table III. Consistency with the hypothesis that $a = 0$ and $b = 1$ was tested with the Student's t test. Even though the regressions obtained apparently differ greatly from the theoretical one in which weight depends on content (expressed by $C = W$), the obtained results do not disprove such dependence. Data scatter, however, prevents any valid conclusion.

Correlation between Drugs in Combination—The TEF suppositories contain a combination of ETHYL and of THEO; the MAL suppositories contain a combination of DIM and of AMI. Since in both suppository types, the two ingredients were assayed simulta-

Table IV—Linear Regressions between Active Ingredients in MAL and TEF Suppositories[a]

$$\text{DIM} = (0.52 \pm 0.18) + (0.48 \pm 0.18)\,\text{AMI}$$
$$t_a = 2.95;\ t_b = 2.70$$
$$\text{ETHYL} = (0.94 \pm 0.27) + (0.07 \pm 0.27)\,\text{THEO}$$
$$t_a = 3.48;\ t_b = 0.26$$

[a] The critical t value is 2.26 ($\alpha = 0.05$).

neously in each individual suppository, the possible correlation between the two ingredients may be investigated by the linear regression. The regressions found, standardized into units of average content, are given in Table IV.

No statistically significant correlation was found between ETHYL and THEO, and a very small one ($0.05 > p > 0.02$) was found between DIM and AMI. For these suppositories the content uniformity found on one of the two combined drugs is not transferable to the other one, since each active ingredient varies independently.

Content Accuracy—Content accuracy, *i.e.*, correspondence between the actual average content and the labeled content, may either be investigated on a composite sample or evaluated from the average of the contents of the individual dosage units. In the latter case, the coefficient of variation of the mean (*CVM*) indicates the precision by which content accuracy is evaluated. The $p = 0.05$ confidence limits are the product of *CVM* multiplied by the appropriate t value (2.26 for 9 degrees of freedom).

Table I shows that the $p = 0.05$ confidence limits of the average content covered or were very close to the labeled content in most instances. The largest difference between the actual average content and the labeled content was found for TEF–ETHYL suppositories (−5.4%). Nevertheless, the TEF–ETHYL suppositories may still conform to the specification that the content must be between 95 and 105% of the nominal content, since the upper confidence limits are only 3% lower than the nominal content. Therefore, there is no proof to a $p = 0.05$ level that the content found is lower than 97%.

It should be emphasized that the content accuracy found is not the true one, since it depends also on the accuracy of the assay method. The performance of the method in terms of accuracy cannot be investigated with statistical tools, as in the case of uniformity, but must be evaluated by comparing different methods, preparing samples with known quantities of the substance under investigation, *etc.* (24). While theoretically it is impossible to be sure that an assay method is 100% accurate, in practice some methods, especially when a comparison with a proper standard is involved, yield an acceptable accuracy.

In the case of TEF–ETHYL suppositories, it was found that the method used was inaccurate because of suppository base interference. The alternative accurate method, which is now under investigation, has a wide intrinsic variability and, therefore, is not suitable for evaluating content uniformity. The results of the study on content accuracy of TEF–ETHYL suppositories will be the subject of another paper.

DISCUSSION

The samples of the different types of rectal suppositories conformed to USP XVII OCUS (16) for tablets. It may be shown, however, that this does not imply that every sample taken from the investigated lots of suppositories complies with the OCUS or that the OCUS are appropriate for restricting content variability in rectal suppositories. In fact, the OCUS call for comment before evaluating their adaptability to more general conditions.

Structure of the OCUS—The OCUS are based on sampling plans devised for "attributes" and for the restriction of "defective" specimens in a lot. The content is a continuous variable, which is transformed into an attribute by confronting the content found to the $M \pm 0.15\,M$ limits (M is the labeled content), a transformation which implies by itself a loss of information. Nevertheless, the OCUS "attributes" plan was adopted by USP XVII after a multilaboratory study (2), mainly because it was shown that some variables, *e.g.*, the weights of sterile solids, occasionally may not follow a Gaussian distribution (25). In these cases, the "attributes" plan may be advantageous because it is more "robust," *i.e.*, less susceptible to the biasing effects of non-Gaussian distributions on the evaluation of variability.

The "attributes" plan of the OCUS, however, is not flexible; even a minor change in sample size, in acceptance number, or in critical limits radically changes the performance of the plan. Furthermore, the OCUS plan does not take intrinsic analytical variability into account and so sometimes leads to wrong conclusions.

The TEF–ETHYL suppositories, for instance, would not comply with the OCUS in approximately 1.7% of the samplings. More generally, an ideal lot with a perfect content uniformity, investigated for compliance with the OCUS with an assay procedure having an intrinsic variability expressed by a $CV_{\text{ana.}} = 5$, would not comply in approximately 1% of the samplings. Conversely, the same analytical variability might result in acceptance of a bad lot.

One reason for the choice of the "attributes" plan for the OCUS was the extensive experience already acquired with similar plans for the control and restriction of weight variability in different types of dosage forms. It should be remembered, however, that weight uniformity is investigated with a very precise and accurate method, namely the analytical balance, with an inherent $CV_{\text{ana.}}$ usually smaller than 0.1. When introduced into the equation

$$CV^2_{\text{obs.}} = CV^2_{\text{ana.}} + CV_W{}^2 \qquad \text{(Eq. 4)}$$

the $CV_{\text{ana.}}$ is negligible, so CV_W is directly evaluatable from the $CV_{\text{obs.}}$. Moreover, for measuring weight, there is only one well-known and well-defined analytical method independent of a particular drug or formulation.

On the other hand, content uniformity is tested by methods which may have large intrinsic variabilities, interfering with the evaluation of the true content variability. Further, each drug and often each formulation call for a particular assay method, with a different intrinsic variability and, therefore, also a different consequence on the evaluation of true content variability.

For these reasons, the experience acquired with the weight uniformity specifications cannot be transferred to the control of content uniformity, unless the assay method is extremely precise and accurate.

Another point which should be emphasized is that the results obtained with the OCUS depend on the precision *and* on the accuracy of content of active ingredient and of its assay method. As a matter of fact, the name "test for content uniformity" is misleading, since actually the allowed variability decreases with the increase of inaccuracy, as shown in Fig. 1. Since the inaccuracy actually found depends also on the random composition of the sample and is biased by the imprecision and the inaccuracy of the assay method, a single specimen may sometimes be classified as within the $M \pm 0.15\,M$ limits and sometimes as outside. The OCUS do not provide for a clear discrimination between good and defective specimens or for an explicit and fixed margin for content variability, so that among other drawbacks the OCUS cannot be transferred to the production control charts or to the inspection of portions of the lot which are larger than the sample sizes required by the OCUS.

When discussing weight variability, it was shown that the confidence in the results increases with the precision of the assay method. Since compliance with the OCUS depends both on precision and on accuracy, the assay method used for the OCUS must be accurate, a requirement which does not always go hand in hand with precision, economy, and sometimes even feasibility. This, for instance, is the case with biological assay methods, where the investigation of content uniformity can be performed only with inaccurate and unspecific methods (26). The need for different methods of testing accuracy and uniformity of content is becoming more and more obvious with the introduction of automated methods of analysis and is considered in the new editions of the USP and of the NF (21, 27, 28). But different methods, one for accuracy and the other for uniformity of content, are incompatible with the very structure of the OCUS.

In conclusion, the OCUS have several disadvantages. These are: (*a*) lack of flexibility so that it is very difficult to adapt the OCUS to different sample sizes, to the control of larger portions of a lot, or to control during production; (*b*) misleading results due to the neglect of the intrinsic analytical variability of the assay method; (*c*) dependence of content variability on content accuracy, which in

some instances prevents the use of precise methods for investigating content uniformity; (*d*) indefinite allowance for variability, impeding generalization, and the transfer of the OCUS to production control charts; (*e*) "yes or no" type of results, whereas often the knowledge of the degree of compliance or noncompliance of the sample is desirable to alert the producer or consumer to the presence of a borderline condition; (*f*) difficulty of gaining better knowledge of the OCUS through the experience acquired by content variability investigations, since most practical and theoretical studies evaluate the content as a continuous variable and express content variability in terms of standard deviation; and (*g*) loss of information due to the transformation of a continuous variable into an attribute.

These disadvantages are of secondary importance when the intrinsic variability of the assay method is small. But when the analytical variability is large enough to interfere with the evaluation of the actual content variability, or when a precise but inaccurate method is appropriate for checking content uniformity, an alternative approach to the control and the restriction of content variability seems desirable, *e.g.*, one based on a "variables" sampling plan, as already advanced by Breuning and King (29), or based on the *CV* since many published investigations evaluate content uniformity through the *CV* method.

Content Uniformity Specifications Based on the Restriction of the Coefficient of Variation (CUS-*CV*)—According to Eq. 1 the basic acceptance condition of a CUS-*CV*, which takes into account the analytical variability, is

$$CV^2_{con.} = CV^2_{obs.} - CV^2_{ana.} \leqslant AQL^2 \qquad \text{(Eq. 5)}$$

where *AQL* is the acceptance quality level for content uniformity. Since the $CV_{ana.}$ depends on the assay method used for the content determination of the particular drug in the individual dosage form, the content uniformity specification must state for each drug and for each dosage form: (*a*) the official method for the content assay in the individual dosage units, and (*b*) the maximum method variability allowed for accepting the results (as for biological assay methods).

It has been shown (30) that the $CV_{con.}$ consistent with a 95% acceptance probability of USP XVII OCUS is 5.4 in the first sample of 10 and 6.2 in the composite sample of 30 dosage units. The *AQL*

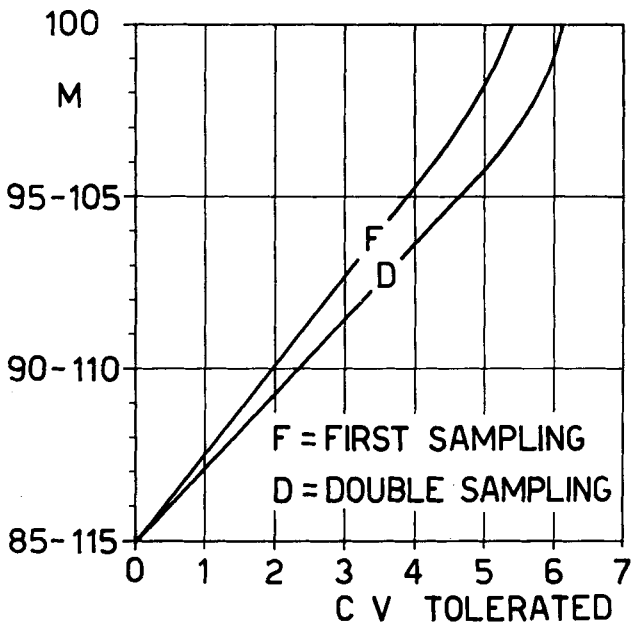


Figure 1—*Relationship between the content accuracy and the* CV *tolerated for the producer, according to the content uniformity specification of USP XVII. Ordinate: average content, as a percentage of nominal content; abscissa:* CV *tolerated for the producer. The tolerated* CV *is maximum for an average content equal to the nominal value and vanishes when the actual content approaches 85 or 115% of the labeled content. The "first" and the "double" sampling curves reflect the implications of the first and second sampling steps described in the USP specification.*

Table V—Limits for $CV_{con.}$ at Different Sample Sizes

Sample Size, units	$CV_{con.}$[a] *AQL'*	$CV_{con.}$[a] *UQL*
10	3.7	6.9
15	4.1	6.6
20	4.3	6.4
30	4.5	6.3
60	4.7	6.0
100	4.9	5.9

[a] Calculated according to Eq. 5.

for content uniformity may, therefore, be located between 5.4 and 6.2, *e.g.*, restrictively at 5.4.

The *AQL'* of a sample should give a 95% confidence that the *CV* in the lot is equal to or lower than 5.4. Therefore, the *AQL'*s were calculated from the one-tailed 95% fiducial limits of Table H of Davies (17) for different sample sizes and are given in Table V. Also, the unacceptable quality level (*UQL*), *i.e.*, the quality found which gives 95% confidence that the $CV_{con.}$ in the lot is higher than 5.4, depends on the sample size and is given in Table V.

In an actual inspection for uniformity, one may, for instance, start with a sample of 10 units and then take any further decision, *i.e.*, upon acceptance, rejection, or expansion of the sample, on comparing the $CV_{con.}$ found with the values of Table V. In this way, the sample size is open and flexible.

As already pointed out, the OCUS link together two types of quality levels: one for uniformity and one for accuracy.

The requirements implied in the OCUS must, therefore, be translated into terms of *AQL* for accuracy consistent with the *AQL* for uniformity. According to Breuning and King (29), this condition is satisfied by expressing accuracy as the *CVM*. In a sample of 10 and for a $CV_{con.} = 5.4$, the $CVM = 5.4/\sqrt{10} = 1.71$. This *CVM* means that about 99% of the averages of the assays on 10 units, or of the assays of composite samples of 10 units, should be within 95 and 105% of the nominal content, a condition which in fact is similar to the requirements of several monographs of USP XVII.

In sum, the CUS-*CV* may be outlined as the *AQL'* of the $CV_{con.}$ found on the basis of Eq. 5 should be within the values given in Table V for the different sample sizes. Since the OCUS implies also a specification for content accuracy, it must be added that the average content, assayed with a given accurate method on a composite sample of 10 homogenized dosage units, or calculated from 10 assays on individual dosage units, should be within 95 and 105% of the labeled content. The analytical variability must be considered in terms of $CV_{ana.}$ or of $CVM_{ana.}$ in order to rid the found average content of the bias due to analytical variability.

These specifications are both flexible and transferable to production control charts.

The main argument against a CUS-*CV* plan is that it is less "robust" than the "attributes" plan when the distribution of the investigated variable is non-Gaussian (25). A non-Gaussian distribution, however, seems an exception rather than the rule for the content of active ingredients (19). On the other hand, the advantages of the greater "robustness" of the OCUS are diminished by the greater room for uncertainty inherent in an "attributes" plan. In fact, according to Pietra and Setnikar (30), the *AQL* implied by the OCUS attribute plan for a *lot* is equal to 5.4 (or to 6.2 after the second sampling) and the *UQL* is equal to 12. This difference for lot *CV* is greater than the differences for sample *CV* listed in Table V. Although lot conditions are not comparable to sample conditions, the latter usually imply a larger margin of uncertainty but are, nevertheless, the actual conditions for inspecting uniformity.

Given the advantages and disadvantages both of the "attributes" plan and of the *CV* plan, reconsideration of the general approach to content uniformity control would seem to be indicated. The present investigation aims to be a contribution to this subject.

REFERENCES

(1) E. Brochmann-Hanssen and J. C. Medina, *J. Pharm. Sci.*, **52,** 630(1963).

(2) T. N. T. Olson and I. Lee, *ibid.*, **55,** 1(1966).

(3) L. Lachman and H. D. Silvestrowicz, *ibid.*, **53,** 1234(1964).

(4) K. E. Schulte, G. Henke, and D. Herpel, *Arch. Pharm.*, **300**, 899(1967).
(5) C. Roberts, *Technometrics*, **11**, 161(1969).
(6) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 905.
(7) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 449.
(8) I. Setnikar and V. Pietra, *J. Pharm. Sci.*, **58**, 112(1969).
(9) U. Elste, U. Winkhaus, and H. Duda, *Deut. Apoth. Ztg.*, **106**, 568(1966).
(10) S. Gorac, *Magy. Kem. Foly.*, **70**, 161(1964); through *Chem. Abstr.*, **61**, 1711(1964).
(11) J. Meulenhoff and J. J. M. Van Sonsbeek, *Pharm. Weekbl.*, **91**, 453(1956).
(12) F. M. Wieninger, *Wochschr. Brau.*, **53**, 251(1936).
(13) "Pharmacopée Francaise," 8th ed., Impr. Maisonneuve, St. Ruffine, France, 1965, p. 93.
(14) F. De Lorenzi, *Boll. Chim. Farm.*, **94**, 343(1955).
(15) L. D. Clark and H. Thompson, *Science*, **107**, 429(1948).
(16) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1968, p. 36.
(17) O. L. Davies, "Statistical Methods in Research and Production," Oliver and Boyd, London, England, 1958, p. 382.
(18) R. Tawashi and P. Speiser, *Pharm. Acta Helv.*, **39**, 734(1964).
(19) C. D. Smith, T. P. Michaels, M. J. Chertkoff, and L. P. Sinotte, *J. Pharm. Sci.*, **52**, 1183(1963).
(20) J. P. Comer, P. Hartsan, and C. E. Stevenson, *ibid.*, **57**, 147(1968).
(21) M. Geller, O. W. A. Weber, and B. Z. Senkowski, *ibid.*, **58**, 477(1969).
(22) W. N. French, F. Matsui, D. Cook, and L. Levi, *ibid.*, **56**, 1622(1967).
(23) J. M. Airth, D. F. Bray, and C. Radecka, *ibid.*, **56**, 233 (1967).
(24) W. J. Youden, *J. Ass. Off. Agr. Chem.*, **45**, 169(1962).
(25) J. D. Haynes and M. Schnall, "Drug Sterile Solids Weight Variation and Recommendation for Specification," unpublished report to P.M.A., March 27, 1963.
(26) E. R. Garrett, *J. Pharm. Sci.*, **51**, 672(1962).
(27) N. R. Kuzel, H. E. Roudebush, and C. E. Stevenson, *ibid.*, **58**, 381(1969).
(28) J. W. Myrich, *ibid.*, **58**, 1018(1969).
(29) H. L. Breuning and E. P. King, *ibid.*, **51**, 1187(1962).
(30) V. Pietra and I. Setnikar, *ibid.*, **59**, 530(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 22, 1970, from the *Research Division of Recordati s.a.s., Milano, Italy.*

Accepted for publication March 27, 1970.

USP Collaborative Study of the Assay of Atropine and Scopolamine Dosage Forms

LEE T. GRADY and RUPERT O. ZIMMERER, Jr.

Abstract □ Several tablet and injection dosage forms of atropine and scopolamine are covered by USP XVIII monographs. Gas chromatographic unit dose assay and content uniformity procedures were developed for these monographs. Interlaboratory reliability was evaluated by means of a collaborative study. The methods afford substantial improvements in sensitivity, specificity, and speed over previous official methods.

Keyphrases □ Atropine, scopolamine dosage forms—analysis □ Scopolamine, atropine dosage forms, analysis—collaborative study □ Content uniformity method—atropine, scopolamine dosage forms □ GLC—analysis

Previous official methods of assaying dosage forms for belladonna alkaloids have relied largely on titrimetry. These methods lacked sensitivity and specificity. Unit doses could not be assayed and decomposition products were not excluded. Such problems aroused some criticism. A notable exception was an IR method (1) which, although failing in sensitivity, did offer specificity with some control over decomposition. Assay methods for USP XVIII were desired which would be accurate, reliable, and highly specific and yet be sufficiently sensitive, precise, and rapid to allow content uniformity determinations on unit doses.

Various other approaches to belladonna alkaloid analysis may be noted. A colorimetric method (2, 3) was applied to preparations containing phenobarbital along with the alkaloids, and a dye–complex method was applied to atropine tablets and elixir (4). Neither of these approaches distinguishes one belladonna alkaloid from another. A fluorometric method has been reported for atropine (5). Paper chromatography (6, 7), partition-column chromatography (2, 3, 8), countercurrent distribution (9), TLC (10), and TLC with densitometry (11) have all achieved separation of scopolamine from atropine–hyoscyamine.

Initial efforts in the gas chromatography of belladonna alkaloids were reported by Kazyak and Knoblock (12), Brochmann-Hanssen and Fontan (13), Jain and Kirk (14), and Solomon *et al.* (15). Penner (16) studied atropine assay by GLC, both as a silyl derivative and later untreated, using tetraphenylethylene as the internal standard. Alber (17) recently reported a broad study of the gas chromatography of drugs and alkaloids using the methylphenylpolysiloxane liquid phase which was used in this collaboration.

The procedures developed for this collaborative study are related to a method previously reported (18) for dose forms of belladonna alkaloids containing phenobarbital. Other official methods[1] were developed earlier for belladonna alkaloids using anthracene as an internal standard for control of injection volume alone.

MATERIALS

Methylene Chloride—Gas chromatography or 99 mole % grade was used.

[1] Hyoscyamine sulfate tablets NF and morphine and atropine sulfates tablets NF.

(4) K. E. Schulte, G. Henke, and D. Herpel, *Arch. Pharm.*, **300**, 899(1967).
(5) C. Roberts, *Technometrics*, **11**, 161(1969).
(6) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 905.
(7) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 449.
(8) I. Setnikar and V. Pietra, *J. Pharm. Sci.*, **58**, 112(1969).
(9) U. Elste, U. Winkhaus, and H. Duda, *Deut. Apoth. Ztg.*, **106**, 568(1966).
(10) S. Gorac, *Magy. Kem. Foly.*, **70**, 161(1964); through *Chem. Abstr.*, **61**, 1711(1964).
(11) J. Meulenhoff and J. J. M. Van Sonsbeek, *Pharm. Weekbl.*, **91**, 453(1956).
(12) F. M. Wieninger, *Wochschr. Brau.*, **53**, 251(1936).
(13) "Pharmacopée Francaise," 8th ed., Impr. Maisonneuve, St. Ruffine, France, 1965, p. 93.
(14) F. De Lorenzi, *Boll. Chim. Farm.*, **94**, 343(1955).
(15) L. D. Clark and H. Thompson, *Science*, **107**, 429(1948).
(16) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1968, p. 36.
(17) O. L. Davies, "Statistical Methods in Research and Production," Oliver and Boyd, London, England, 1958, p. 382.
(18) R. Tawashi and P. Speiser, *Pharm. Acta Helv.*, **39**, 734(1964).
(19) C. D. Smith, T. P. Michaels, M. J. Chertkoff, and L. P. Sinotte, *J. Pharm. Sci.*, **52**, 1183(1963).
(20) J. P. Comer, P. Hartsan, and C. E. Stevenson, *ibid.*, **57**, 147(1968).
(21) M. Geller, O. W. A. Weber, and B. Z. Senkowski, *ibid.*, **58**, 477(1969).
(22) W. N. French, F. Matsui, D. Cook, and L. Levi, *ibid.*, **56**, 1622(1967).
(23) J. M. Airth, D. F. Bray, and C. Radecka, *ibid.*, **56**, 233 (1967).
(24) W. J. Youden, *J. Ass. Off. Agr. Chem.*, **45**, 169(1962).
(25) J. D. Haynes and M. Schnall, "Drug Sterile Solids Weight Variation and Recommendation for Specification," unpublished report to P.M.A., March 27, 1963.
(26) E. R. Garrett, *J. Pharm. Sci.*, **51**, 672(1962).
(27) N. R. Kuzel, H. E. Roudebush, and C. E. Stevenson, *ibid.*, **58**, 381(1969).
(28) J. W. Myrich, *ibid.*, **58**, 1018(1969).
(29) H. L. Breuning and E. P. King, *ibid.*, **51**, 1187(1962).
(30) V. Pietra and I. Setnikar, *ibid.*, **59**, 530(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 22, 1970, from the *Research Division of Recordati s.a.s., Milano, Italy.*

Accepted for publication March 27, 1970.

USP Collaborative Study of the Assay of Atropine and Scopolamine Dosage Forms

LEE T. GRADY and RUPERT O. ZIMMERER, Jr.

Abstract □ Several tablet and injection dosage forms of atropine and scopolamine are covered by USP XVIII monographs. Gas chromatographic unit dose assay and content uniformity procedures were developed for these monographs. Interlaboratory reliability was evaluated by means of a collaborative study. The methods afford substantial improvements in sensitivity, specificity, and speed over previous official methods.

Keyphrases □ Atropine, scopolamine dosage forms—analysis □ Scopolamine, atropine dosage forms, analysis—collaborative study □ Content uniformity method—atropine, scopolamine dosage forms □ GLC—analysis

Previous official methods of assaying dosage forms for belladonna alkaloids have relied largely on titrimetry. These methods lacked sensitivity and specificity. Unit doses could not be assayed and decomposition products were not excluded. Such problems aroused some criticism. A notable exception was an IR method (1) which, although failing in sensitivity, did offer specificity with some control over decomposition. Assay methods for USP XVIII were desired which would be accurate, reliable, and highly specific and yet be sufficiently sensitive, precise, and rapid to allow content uniformity determinations on unit doses.

Various other approaches to belladonna alkaloid analysis may be noted. A colorimetric method (2, 3) was applied to preparations containing phenobarbital along with the alkaloids, and a dye–complex method was applied to atropine tablets and elixir (4). Neither of these approaches distinguishes one belladonna alkaloid from another. A fluorometric method has been reported for atropine (5). Paper chromatography (6, 7), partition-column chromatography (2, 3, 8), countercurrent distribution (9), TLC (10), and TLC with densitometry (11) have all achieved separation of scopolamine from atropine–hyoscyamine.

Initial efforts in the gas chromatography of belladonna alkaloids were reported by Kazyak and Knoblock (12), Brochmann-Hanssen and Fontan (13), Jain and Kirk (14), and Solomon *et al.* (15). Penner (16) studied atropine assay by GLC, both as a silyl derivative and later untreated, using tetraphenylethylene as the internal standard. Alber (17) recently reported a broad study of the gas chromatography of drugs and alkaloids using the methylphenylpolysiloxane liquid phase which was used in this collaboration.

The procedures developed for this collaborative study are related to a method previously reported (18) for dose forms of belladonna alkaloids containing phenobarbital. Other official methods[1] were developed earlier for belladonna alkaloids using anthracene as an internal standard for control of injection volume alone.

MATERIALS

Methylene Chloride—Gas chromatography or 99 mole % grade was used.

[1] Hyoscyamine sulfate tablets NF and morphine and atropine sulfates tablets NF.

Buffer—Prepare 0.2 *M*, pH 9.0 buffer, standardized against the glass electrode, by dissolving 34.8 g. dibasic potassium phosphate in 900 ml. water. Adjust to pH 9.0 and make to 1 l. with water.

Atropine Sulfate Standard Solution—Dissolve 30.0 mg. atropine sulfate in distilled water in a 100-ml. volumetric flask to obtain a solution of 0.30 mg./ml. Prepare fresh daily.

Homatropine Hydrobromide Standard Solution—Similarly, prepare a final concentration of 0.04 mg./ml. by weighing 40.0 mg. homatropine hydrobromide.

Scopolamine Hydrobromide Standard Solution—Similarly, prepare a final concentration of 0.25 mg./ml. by weighing 25.0 mg. scopolamine hydrobromide.

PROCEDURES

Atropine Sulfate Tablets—Place 1 tablet, or its equivalent from a composite of 20 tablets, in 5 ml. buffer in a 30-ml. separator and add exactly 1.0 ml. homatropine standard solution. Extract with 10 ml. methylene chloride, passing the separated organic layer through 2 g. anhydrous sodium sulfate supported by a small pledget of glass wool in a funnel. Evaporate the methylene chloride at reduced pressure to about 0.3 ml. Inject an appropriate volume, about 1 μl., into the chromatographic system. Perform the assay in duplicate. Repeat the procedure, pipeting duplicate 1.0-, 2.0-, 3.0-, and 4.0-ml. aliquots of atropine standard solution in place of the tablets.

Measure the height, *H*, of the atropine and homatropine peaks in each chromatogram and calculate $R = H$ atropine/H homatropine. Plot the value of *R* obtained from the standards *versus* amount of standard atropine sulfate added. Determine the amount of atropine sulfate in the sample preparation directly from the graph.

Atropine Sulfate Injection—Proceed as directed under tablets, substituting 1.0 ml. or the measured contents of a single-dose container of the injection for the tablets.

Atropine Sulfate Ophthalmic Solution—Pipet 1.0- or 2.0-ml. aliquots of the preparation into duplicate 50-ml. volumetric flasks and make to volume with distilled water so that the final concentration of atropine sulfate is 200–800 mcg./ml. Pipet 1.0 ml. of the sample preparation in place of the tablet, and proceed as directed in the assay for atropine sulfate tablets. Multiply the amount of atropine sulfate in the sample preparation by the dilution factor.

Scopolamine Hydrobromide Tablets—Place 1 tablet, or its equivalent from a 20-tablet composite, in 5 ml. buffer in a 30-ml. separator and add exactly 1.0 ml. atropine sulfate standard solution. Extract with 10 ml. methylene chloride, filtering the organic layer through 2 g. anhydrous sodium sulfate supported by a small pledget of glass wool in a funnel. Evaporate the solution under reduced pressure to about 0.3 ml. Inject an appropriate quantity, about 1 μl., into the chromatographic system. Perform the assay in duplicate. Repeat the procedure, substituting duplicate 1.0-, 2.0-, and 3.0-ml. aliquots of scopolamine hydrobromide standard solution for the tablet.

Measure the peak heights, *H*, of atropine and scopolamine in each chromatogram, and calculate the ratio $R = H$ scopolamine/H atropine. Plot the ratios of the standards *versus* the amount of scopolamine hydrobromide added. Read the amount of scopolamine hydrobromide in the tablet or sample preparation from the calibration graph.

Scopolamine Hydrobromide Injection—Proceed as directed in the assay for the tablets, substituting 1.0 ml. or the measured contents of a single-dose container of the injection for the tablet.

GAS CHROMATOGRAPHY

Analyses should be performed using 0.6-1.2-m. glass columns, 4 mm. i.d., packed with 3% w/w methylphenylsilicone oil[2] on 80/100- or 100/120-mesh silanized, acid-washed, flux-calcined diatomite. Flame-ionization detectors are used. Helium carrier gas is used at a flow of about 60 ml./min. The temperature of the injection port is not more than 25° above that of the column; on-column injection is preferred. Column temperature and flow may be adjusted to permit rapid (5–10 min.) and optimum analysis, about 210° for an 0.6-m. column or 225° for the 1.2-m. column.

Low-polarity methylphenylsilicone[2] is coated on silanized, acid-washed, flux-calcined diatomite. A special curing sequence has been found to increase inertness and efficiency: maintain the column at 250° for 1 hr. with helium flowing to remove oxygen and solvents, stop the flow of helium and heat at about 340° for 4 hr., lower temperature to 250°, and condition with the helium flowing until stable. A suitable initial test for support inertness, which is valuable with any low-polarity liquid phase, is the delivery of a single symmetric peak for injected cholesterol with no evidence of decomposition. The alkaloid peaks should be symmetric with little tailing.

PROTOCOL

The previously discussed details of methods and materials were supplied[3] to the collaborators along with samples of commercial dosage forms. The stated objective of the study was to evaluate these gas chromatographic methods for use in USP XVIII monographs on atropine sulfate tablets, injection, and ophthalmic solution, and on scopolamine hydrobromide tablets and injection.

Separate report sheets for each alkaloid were supplied. These requested, in addition to assay results, the identities of the instruments, support material, and supplier. Chromatographic data requested were column parameters, retention times, occurrence of peaks in reagent blanks, resolution factors (19), and efficiencies (20). The variance of an individual drug–standard ratio was to be reported for eight injections of a single sample. Procedural variance was to be estimated by 6–8-fold assay of a single bottle of an injection or of a tablet composite.

The following paragraphs were included in the protocol by way of explanation to the collaborators.

Both atropine and scopolamine are available in multiple strengths for each of the official items (tablets, injection, and solution). These procedures were prepared with this in mind and feature the use of a standard curve hinged on a single amount of internal standard. This course was chosen rather than manipulating all sample preparations to a single specified concentration,[4] because a calibration curve must be prepared in the process of approving a column for use in a single-point assay. Such a single-point approach requires a linear standard curve passing exactly through zero, an unnecessarily strict and often unattainable requirement for some drugs.

The isolation scheme is the simplest possible. Basic phosphate buffer is added so that the pH of the aqueous phase is 9 and is used instead of alkali to minimize ester cleavage. Homatropine was chosen as the "extracted" internal standard for atropine preparations for several reasons. It differs from atropine only in a methylene group and the nature of substitution of the carbinol; thus, chromatographic and chemical characteristics are similar. Multiple extractions or complete recoveries along the way are rendered unnecessary, since the molecular ratio of standard to analyte is controlled from the first step of the assay. Because of the close chemical similarities, minor alkaline ester cleavage or amine degradation also is controlled. Similarly, atropine is chosen as the standard for scopolamine. Both standards are or will be readily available. Heat and air during evaporation should be avoided.

Belladonna alkaloids are polar compounds, and the particular difficulties associated with the GLC of amines are well known. Improperly or partially cured and conditioned columns often cause extensive tailing of such compounds. An additional problem can be partial, on-column dehydration (13, 15) of atropine and scopolamine. Although the preparation of less polar derivatives may allow successful chromatography in poorer systems, the additional steps and problems are appreciable. Modern phases, supports, and column treatments have extended greatly the range of molecules that can be chromatographed directly, without prior formation of less polar derivatives. The authors had successfully determined belladonna alkaloids previously and found no reason for including derivatization in the assays. The key to this assay is in the selection of the column.

[2] OV-17. This oil contains approximately equal proportions of methyl and phenyl radicals. Other proportions are, or are becoming, available. Substitution is permitted but only if the chromatographic parameters discussed herein are met.

[3] During the summer of 1969 with all responses completed by midfall.

[4] USP XVIII since has specified a single-sample concentration but retains the calibration curve.

Table I—Assay Results: Individual Laboratory Average Assay Values for Samples Supplied

Collaborator	Replicates, n	Atropine SO_4 0.4-mg. Tab.	Atropine SO_4 0.3-mg. Tab.	Atropine SO_4 0.6-mg. Tab.	Atropine SO_4 0.4 mg./0.5 ml.	Scopolamine HBr 0.43 mg./ml.	Scopolamine HBr 0.32 mg./ml.
A	2	0.398	0.275	0.552	0.395	0.397	0.313
B	2	0.383	0.280	0.592	0.390	0.425	[0.365]
C	4–9	0.420	0.304	0.633	0.390	0.430	0.312
D	3–10	0.390	0.295	0.605	0.405	0.438	0.315
E[a]	2	0.388	0.293	0.614	0.389	0.418	0.312
F	2	0.390	0.300	0.600	0.403	0.433	0.310
G	2	0.412	0.295	0.602	0.405	0.400	0.315
H	2–4	0.407	0.265	0.566	0.370	—	—
I[b]	1	0.415	0.320	[0.510]	0.420	0.430	0.315
$\bar{x}$, mg.		0.401	0.295	0.595	0.396	0.422	0.313
$\bar{s}$, mg.		0.013	0.014	0.027	0.014	0.015	0.002
$\bar{cv}$ %		3.3	4.8	4.5	3.5	3.6	<1

[a] Diatoport S support; the others used OV-17 on Gas-Chrom Q. [b] OV-1 on Chromosorb WHP.

It was recognized that some collaborators would prefer purchasing a prepared packing. The Drug Standards Laboratory (DSL) arranged for a lot,[5] tested by DSL, to be reserved by Applied Science Laboratories, State College, Pa. The collaborators were advised that after packing the column, the described curing and conditioning were necessary.

RESULTS AND DISCUSSION

Each assay value in Table I is the average of that collaborator's individual values (as given in the second column), whether he reported duplicate or 10-fold determinations. In this way, each collaborator's result is not allowed to obscure interlaboratory variation.

Accuracy—The average assay values given in Table I are compared in Table II to the manufacturer's in-house extraction–titrimetry values determined at time of pass. The assay results by the GLC method exhibited less deviation from declared contents than the in-house extraction–titrimetry methods. On the average, the GLC values appeared to run about 0.5% lower. However, this GLC method does not measure decomposition products and should yield more realistic values.

Reference standards for each drug will now be available. What effect this standardization would have on the interlaboratory variation or on the actual assay values cannot be predicted, although some reduction in interlaboratory variation may be anticipated. With regard to assay values, results from Laboratory A, for example, averaged 3.2% below results from other laboratories, suggesting some bias in procedure, very likely related to the standards used. Laboratory H, which used a programmed temperature run, reported assay values approximately 5% lower than other laboratories. No other systematic error was apparent.

Overall, the GLC methods given here must be judged as accurate and suitable for the official USP assay methods in light of the following features.

Reliability—The data were evaluated grossly in the following manner. A mean, standard deviation, and coefficient of variation were generated for each dosage form. In four of six cases, the magnitude of the standard deviation was similar; since chromatographic properties for the two drug standard situations were essentially similar, it appears that all the data in Table I may be pooled. An average, $\bar{cv}$, of the nine coefficients of variation was obtained. The deviation of the individual assay values from the mean, $\bar{x}$, for that dosage form was considered; if $x - \bar{x} \geq 3\,(\bar{cv})\,(\bar{x})$, with $\bar{x}$ calculated without rejection, then the assay value was rejected, with the additional limitation that only one value from any column could be rejected. Only the two values in brackets out of the 52 values in Table I were rejected. This course was chosen to allow conservative estimation of reliability.

A new average of the nine recalculated coefficients of variation was then calculated to be 3.4%. This value is one estimate of the overall reliability of the methods and is composed of both intralaboratory and interlaboratory variations, as well as of significant deviations from the protocol. True reliability, of course, is a composite of this variation with accuracy, specificity, sensitivity, and precision. The contribution of each factor to the overall reliability will be discussed.

Table II—Comparison of Assay Values

Dosage Form	Manufacturer	USP Collaborative
Atropine Sulfate Tablets		
0.4 mg.	94.0%	100.0%
0.3 mg.	95.0	98.4
0.6 mg.	97.3	99.2
Atropine Sulfate Injection		
0.4 mg./ml.	103.6	99.0
Scopolamine HBr Injection		
0.43 mg./ml.	101.0	98.4
0.32 mg./ml.	100.4	98.0
Average	99.7	99.2

Specificity and Sensitivity—The inherent specificity and sensitivity of gas chromatography, combined here with an extracted internal standard, are utilized by these methods. In addition to serving as the monograph assay, the comparison of the sample chromatogram to that of the reference standard serves also as a strong identity test. Degradation products do not interfere. Tropic acid is not extracted in the first step, and tropine and scopine elute prior to the standards and drugs. No collaborator reported evidence of decomposition products.

The methods are directly applicable to available strengths of dosage forms covered by the monographs identified in the object of the collaborative study. Indeed, only a fraction of a percent of the available sample is actually analyzed. Use of this sensitivity in proof of content uniformity of tablets is obvious; however, this application depends also on intralaboratory chromatographic precision.

Precision—Collaborators supplied data for both the reproducibility of the ratio obtained upon multiple injection of a single drug-

Table III—Precision Data Presented as Coefficients of Variation

Laboratory	Atropine R	Atropine R, %	Atropine Assay	Scopolamine R	Scopolamine R, %	Scopolamine Assay
A[a]	1.58	0.47	0.87	1.37	1.31	1.48
B	0.49	2.81	5.56	0.32	2.93	—
C	0.97	0.57	—	0.43	0.62	—
D	0.52	0.6	0.6	0.45	1.0	2.2
E[b]	0.89	0.65	0.79	0.80	0.68	0.99
F	1.07	0.65	2.92	1.06	1.04	1.24
G	1.10	0.73	—	1.01	0.82	0.65
H	1.11	2.30	3.43	—	—	—
I[c]	1.19	3.2	2.7	1.19	3.2	2.7

[a] Electronic integrator. [b] Diatoport S support. [c] Disk integrator, also used OV-1/Chromosorb WHP instead of recommended packing; all others used OV-17 on Gas-Chrom Q.

[5] OV-17 on Gas-Chrom Q.

Table IV—Chromatographic Data

Laboratory	Retention Time (Atropine), min.	Resolution Factor: Homatropine/ Atropine	Resolution Factor: Atropine/ Scopolamine	Plates: Homatropine	Plates: Atropine	Plates: Scopolamine	Column, m.
A	5, 4	—	—	1450	1270	1420	1.2
B	11	3.0	4.3	470	600	760	0.9
C	4.9, 4.6	—	>20	(2680)	1550, 1200	1390	(0.6)
D	3.7	3.6	4.8	960	1080	1140	0.6
E	5.5, 3.5	3.8	3.7	620	690, 430	570	0.55
F	4.9, 4.3	—	—	950	1360	1580	1.2
G	4.3	3.5	4.9	1010	1480	1520	1.2
H	4.5[a]	6.0	—	—	—	—	1.2
I	5, 4	1.7	1.8	1080	1420	1530	2.0

[a] Programmed run, 135–210° at 10°/min.

standard mixture and of the ratio obtained from multiple assay of a single commercial item. Their results are found in Table III. The directions are for peak height ratios; however, two laboratories used area-measuring devices.

Laboratory H erroneously did a programmed 10° per minute run, and the degraded precision is, therefore, understandable. Laboratories B and F generated a variable in obtaining sample preparations which F did not carry over from atropine to scopolamine. Otherwise, the assay manipulations appear to contribute little to (im)precision which, therefore, must be largely dependent on chromatographic precision. An approximation of the difference in the two sets of precision data is about 0.5% in coefficient of variation. Indeed, any significant departure from this level should signal a possible procedural error to the analyst.

Without rejecting any value, the average of coefficients of variation in R is 1.3% for atropine and 1.4% for scopolamine, with a further estimate of about 1.9% for dosage form assay, which is moderately good for a GLC method. Several values can be accounted for by graphical error alone. This is completely satisfactory (21) for use in content uniformity testing.

The collaborative procedures construct calibration curves for the alkaloids with four points for atropine and three points for scopolamine, based on the range of anticipated concentration of sample preparations from the range of dose strengths. Nonlinear absorption, tailing, and relative graphical error could be expected to cause somewhat greater imprecision in the lower values on the calibration curve than in the higher values. These factors would similarly affect precisions for the absolute sizes of samples injected. A plot of the percent coefficient of variation against the value of R for each alkaloid, as given in Table III, failed to support any such correlation between precision and magnitude of R. Therefore, the precision discussions in this report must pool all values irrespective of magnitude or the identity of the drugs. Variations in sample size injected and magnitude of R conversely cannot be cited as causes of interlaboratory variations. This also is consistent with the judgment that reference standards are the only further vehicle needed to control interlaboratory variation in these assays.

Dosage Forms—The 0.4-mg. atropine sulfate tablets were compressed tablets; the other two were hypodermic tablets. The results show the method to be equally applicable. Although the protocol gave a separate procedure, scopolamine hydrobromide tablets were not issued to collaborators. The atropine sulfate ophthalmic solutions available are sufficiently concentrated to require an initial 25- or 50-fold dilution to enter the assay concentration range. Thus, samples were not issued to collaborators. Results in this laboratory have shown that the procedure works as well for the nonofficial ophthalmic solutions containing cellulose derivatives.

General Observations—Intralaboratory precision was found to be primarily a matter of chromatographic reproducibility. It would appear worthwhile to prepare a packed column giving good performance, particularly where use is anticipated in content uniformity testing. The collaborators' data are of some guidance here.

The chromatographic parameters reported by the collaborators are given in Table IV. A minimum resolution of R, not less than 3.0, is one reasonable standard which an analyst might apply to a column. Laboratory I reported much lower resolution (Table III) and experienced poor precision; both results may reflect the use of a different (OV-1) phase. The authors' initial studies (18) leading to the choice of packing would support this conclusion. Similarly, efficiencies of the order of 1000 plates for any peak appear suitable, but much less efficiency can be tolerated, as shown by Laboratory E using a 0.6-m. column. Some of the reported efficiencies appear in error for the claimed column lengths. Most collaborators reported linear standard curves with the intercept near or at the origin.

However, perhaps the parameter most predictive of precision would be tailing. The authors found that tailing factors[6] larger than 1.6 correlated with diminished precision and, for this reason, the protocol sent to collaborators stated that peaks should be symmetric with little tailing.

Laboratory H erroneously used a programmed temperature run. This commonly degrades both precision and accuracy,[7] and the authors are strongly against this unnecessary operation. Speed also is lost.

The collaborators offered several comments on procedure. Laboratory H found the concentrates following evaporation were stable for 1 or 2 days. The authors made similar observations but do not recommend unnecessary sample storage. Laboratory H also reported that initial experiments indicated that evaporation at 60° in an airstream was satisfactory.

Laboratory A reported an extraneous peak in the homatropine and scopolamine standards; the homatropine appeared to contain 1.5% atropine. Such problems are best avoided by reference standards.

No collaborator reported evidence of decomposition, probably because the alkaloids were injected as free bases at moderate temperatures, with injection block temperatures only slightly greater than on-column temperatures. There have been reports (15, 22) of additional injection-site decomposition, possibly related to the glass wool at the top of the column or the injection of salts. The authors used commercial silanized glass wool and did not observe decomposition.

SUMMARY

The gas chromatographic assay methods for atropine sulfate tablets, injection, and ophthalmic solution and for scopolamine hydrobromide tablets and injection, now covered by USP XVIII monographs, have been studied in a nine-laboratory, collaborative study. The drug is extracted once from alkaline buffer along with added internal standard, homatropine for atropine and atropine for scopolamine. The extract is concentrated and injected into a defined system without further treatment. The methods are accurate, reliable, sensitive, highly specific, rapid, and reasonably precise. The methods are suited to content uniformity testing and serve as strong identity tests for all these monographs. Some interlaboratory variations suggest the need for alkaloid reference standards keyed to these methods to control systematic error. No changes in the procedures were found necessary.

REFERENCES

(1) "Official Methods of Analysis of the Association of Official Agricultural Chemists," 10th ed., AOAC, Philadelphia, Pa., 1965, p. 540 (32.035–32.036).

[6] TF = $(a + b)/2a$, measured at 5% of peak height.

[7] The protocol uses peak heights; automatic peak area methods would be expected to maintain quantitative value.

(2) S. A. Koch, J. Levine, and N. Zenker, *J. Pharm. Sci.*, **54,** 1046(1965).
(3) A. Bracey and G. Selzer, *ibid.*, **57,** 464(1968).
(4) V. Das Gupta and N. M. Ferguson, *Amer. J. Hosp. Pharm.*, **26,** 168(1969).
(5) L. A. Roberts, *J. Pharm. Sci.*, **58,** 1015(1969).
(6) M. Sterescŭ and N. Popovici, *Pharmazie*, **14,** 313(1959).
(7) J. Reichelt, *ibid.*, **9,** 968(1954).
(8) G. Schill and A. Agren, *Svensk Farm. Tidskr.*, **56,** 55(1952); through *Chem. Abstr.*, **46,** 6324b (1952).
(9) H. Brauniger and G. Borgwordt, *Pharmazie*, **10,** 591(1955).
(10) F. Wartman-Hafner, *Pharm. Acta Helv.*, **41,** 406(1966); through *Chem. Abstr.*, **65,** 19925g (1966).
(11) B. L. Wu Chu, E. S. Mika, M. J. Solomon, and F. A. Crane, *J. Pharm. Sci.*, **58,** 1073(1969).
(12) L. Kazyak and E. C. Knoblock, *Anal. Chem.*, **35,** 1448(1963).
(13) E. Brochmann-Hanssen and C. R. Fontan, *J. Chromatogr.*, **19,** 296(1965).
(14) N. C. Jain and P. L. Kirk, *Microchem. J.*, **12,** 229(1967).
(15) M. J. Solomon, F. A. Crane, B. L. Wu Chu, and E. S. Mika, *J. Pharm. Sci.*, **58,** 264(1969).
(16) P. S. Penner, 1968, private communication.
(17) L. L. Alber, *J. Ass. Offic. Anal. Chem.*, **52,** 1295(1969).
(18) R. O. Zimmerer, Jr., and L. T. Grady, *J. Pharm. Sci.*, **59,** 87(1970).
(19) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 773.
(20) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, p. 789.
(21) W. N. French, F. Matsui, D. Cook, and L. Levi, *J. Pharm. Sci.*, **56,** 1622(1967).
(22) E. Brochmann-Hanssen and A. B. Svendsen, *ibid.*, **51,** 1095 (1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 24, 1970, from the *Drug Standards Laboratory, Washington, DC 20037*

Accepted for publication June 3, 1970.

The authors are grateful to these cocollaborators: R. D. Poe, Alcon Laboratories, Inc.; J. B. Kottemann and B. Ross, Food and Drug Administration; H. J. Wesselman and J. M. Woodside, Eli Lilly and Co.; L. N. Mattson, Scientific Associates, Inc.; E. E. Thiemer and J. W. Kouten, Smith, Miller and Patch, Inc.; P. W. Dame, D. C. Davis, and C. L. Graham, The Upjohn Co.; and N. K. Scott, Vitarine Co., Inc. They also thank J. M. Woodside, Eli Lilly and Co., and C. W. Ferry, Burroughs Wellcome and Co., Inc., who supplied dosage forms for this study and their in-house assay values.

The Drug Standards Laboratory is jointly sponsored by the American Medical Association, the American Pharmaceutical Association Foundation, and the United States Pharmacopeial Convention, Inc.

Determination of Total Iron in Hematinics by Atomic Absorption Spectrophotometry

HARRIS I. TARLIN and MARTIN BATCHELDER

Abstract □ The total iron content of six hematinic preparations was determined rapidly, precisely, and accurately by atomic absorption spectrophotometry. Hematinics comprising iron–carbohydrate complexes required ashing prior to assaying by atomic absorption spectrophotometry while those with an iron chelate or simple salt structure may be determined directly by atomic absorption spectrophotometry. A statistical evaluation of the data indicated that the atomic absorption spectrophotometry method was equivalent to the official colorimetric and volumetric methods and to a classical gravimetric procedure.

Keyphrases □ Iron in dosage forms—analysis □ Atomic absorption spectroscopy—analysis □ Colorimetric analysis—spectrophotometer □ Titration—iron analysis □ Gravimetric analysis—iron

Hematinic preparations generally fall into three structural categories: iron–carbohydrate complexes, iron chelates, and iron salts. Preparations consisting of iron–carbohydrate complexes are usually assayed for total iron by a lengthy colorimetric (1) or gravimetric procedure.[1] The usual USP (2) or NF (3) procedure for determining the total iron content of iron chelates and iron salts involves a sodium thiosulfate or ceric sulfate titration. Extensive studies (4, 5) indicate that atomic absorption spectrophotometry (AAS) offers a technique for assaying iron which is relatively free from interfering ions. To date, no studies have been reported in the literature concerning the assay of total iron in hematinics by AAS.

[1] The procedure used in this study was a slight modification of the gravimetric iron assay procedure described in most quantitative analysis textbooks. (See H. H. Willard, N. H. Furman, and C. E. Bricker, "Elements of Quantitative Analysis," 4th ed., D. Van Nostrand, Princeton, N. J., 1956, pp. 335, 336.)

EXPERIMENTAL

Instruments—A Perkin-Elmer model 303 double-beam spectrophotometer, equipped with an iron hollow cathode lamp and single-slot burner head, was used for all atomic absorption measurements. The instrument was optimized with a 10-p.p.m. standard iron solution. A sensitivity of 0.18 mcg./ml. for 1% absorption was achieved. Instrument parameters appear in Table I. All colorimetric measurements were carried out on a Perkin-Elmer model 202

Table I—Instrument Parameters

Wavelength	248.3 mμ
Hollow cathode lamp current	30 ma.
Fuel	Acetylene (flow meter at 9)[a]
Oxidizer	Air (flow meter at 9)[a]
Aspiration rate	1.8 ml./min.
Slit	No. 3
Meter response	No. 2
Recorder	Perkin-Elmer model No. 165

[a] Perkin-Elmer Burner Control Box No. 303-0240.

(2) S. A. Koch, J. Levine, and N. Zenker, *J. Pharm. Sci.*, **54,** 1046(1965).
(3) A. Bracey and G. Selzer, *ibid.*, **57,** 464(1968).
(4) V. Das Gupta and N. M. Ferguson, *Amer. J. Hosp. Pharm.*, **26,** 168(1969).
(5) L. A. Roberts, *J. Pharm. Sci.*, **58,** 1015(1969).
(6) M. Sterescŭ and N. Popovici, *Pharmazie*, **14,** 313(1959).
(7) J. Reichelt, *ibid.*, **9,** 968(1954).
(8) G. Schill and A. Agren, *Svensk Farm. Tidskr.*, **56,** 55(1952); through *Chem. Abstr.*, **46,** 6324b (1952).
(9) H. Brauniger and G. Borgwordt, *Pharmazie*, **10,** 591(1955).
(10) F. Wartman-Hafner, *Pharm. Acta Helv.*, **41,** 406(1966); through *Chem. Abstr.*, **65,** 19925g (1966).
(11) B. L. Wu Chu, E. S. Mika, M. J. Solomon, and F. A. Crane, *J. Pharm. Sci.*, **58,** 1073(1969).
(12) L. Kazyak and E. C. Knoblock, *Anal. Chem.*, **35,** 1448(1963).
(13) E. Brochmann-Hanssen and C. R. Fontan, *J. Chromatogr.*, **19,** 296(1965).
(14) N. C. Jain and P. L. Kirk, *Microchem. J.*, **12,** 229(1967).
(15) M. J. Solomon, F. A. Crane, B. L. Wu Chu, and E. S. Mika, *J. Pharm. Sci.*, **58,** 264(1969).
(16) P. S. Penner, 1968, private communication.
(17) L. L. Alber, *J. Ass. Offic. Anal. Chem.*, **52,** 1295(1969).
(18) R. O. Zimmerer, Jr., and L. T. Grady, *J. Pharm. Sci.*, **59,** 87(1970).
(19) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 773.
(20) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, p. 789.
(21) W. N. French, F. Matsui, D. Cook, and L. Levi, *J. Pharm. Sci.*, **56,** 1622(1967).
(22) E. Brochmann-Hanssen and A. B. Svendsen, *ibid.*, **51,** 1095 (1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 24, 1970, from the *Drug Standards Laboratory, Washington, DC 20037*

Accepted for publication June 3, 1970.

The authors are grateful to these cocollaborators: R. D. Poe, Alcon Laboratories, Inc.; J. B. Kottemann and B. Ross, Food and Drug Administration; H. J. Wesselman and J. M. Woodside, Eli Lilly and Co.; L. N. Mattson, Scientific Associates, Inc.; E. E. Thiemer and J. W. Kouten, Smith, Miller and Patch, Inc.; P. W. Dame, D. C. Davis, and C. L. Graham, The Upjohn Co.; and N. K. Scott, Vitarine Co., Inc. They also thank J. M. Woodside, Eli Lilly and Co., and C. W. Ferry, Burroughs Wellcome and Co., Inc., who supplied dosage forms for this study and their in-house assay values.

The Drug Standards Laboratory is jointly sponsored by the American Medical Association, the American Pharmaceutical Association Foundation, and the United States Pharmacopeial Convention, Inc.

Determination of Total Iron in Hematinics by Atomic Absorption Spectrophotometry

HARRIS I. TARLIN and MARTIN BATCHELDER

Abstract □ The total iron content of six hematinic preparations was determined rapidly, precisely, and accurately by atomic absorption spectrophotometry. Hematinics comprising iron–carbohydrate complexes required ashing prior to assaying by atomic absorption spectrophotometry while those with an iron chelate or simple salt structure may be determined directly by atomic absorption spectrophotometry. A statistical evaluation of the data indicated that the atomic absorption spectrophotometry method was equivalent to the official colorimetric and volumetric methods and to a classical gravimetric procedure.

Keyphrases □ Iron in dosage forms—analysis □ Atomic absorption spectroscopy—analysis □ Colorimetric analysis—spectrophotometer □ Titration—iron analysis □ Gravimetric analysis—iron

Hematinic preparations generally fall into three structural categories: iron–carbohydrate complexes, iron chelates, and iron salts. Preparations consisting of iron–carbohydrate complexes are usually assayed for total iron by a lengthy colorimetric (1) or gravimetric procedure.[1] The usual USP (2) or NF (3) procedure for determining the total iron content of iron chelates and iron salts involves a sodium thiosulfate or ceric sulfate titration. Extensive studies (4, 5) indicate that atomic absorption spectrophotometry (AAS) offers a technique for assaying iron which is relatively free from interfering ions. To date, no studies have been reported in the literature concerning the assay of total iron in hematinics by AAS.

[1] The procedure used in this study was a slight modification of the gravimetric iron assay procedure described in most quantitative analysis textbooks. (See H. H. Willard, N. H. Furman, and C. E. Bricker, "Elements of Quantitative Analysis," 4th ed., D. Van Nostrand, Princeton, N. J., 1956, pp. 335, 336.)

EXPERIMENTAL

Instruments—A Perkin-Elmer model 303 double-beam spectrophotometer, equipped with an iron hollow cathode lamp and single-slot burner head, was used for all atomic absorption measurements. The instrument was optimized with a 10-p.p.m. standard iron solution. A sensitivity of 0.18 mcg./ml. for 1% absorption was achieved. Instrument parameters appear in Table I. All colorimetric measurements were carried out on a Perkin-Elmer model 202

Table I—Instrument Parameters

Wavelength	248.3 mμ
Hollow cathode lamp current	30 ma.
Fuel	Acetylene (flow meter at 9)[a]
Oxidizer	Air (flow meter at 9)[a]
Aspiration rate	1.8 ml./min.
Slit	No. 3
Meter response	No. 2
Recorder	Perkin-Elmer model No. 165

[a] Perkin-Elmer Burner Control Box No. 303-0240.

Table II—Analysis of Iron–Carbohydrate Complexes

Type of Sample	% of Labeled Amount[a]			
	Gravimetric	Colorimetric	Atomic Absorption—Direct	Atomic Absorption—Ashed
Iron–carbohydrate complex 1				
Batch 1	101.96	101.96	90.54	98.76
Batch 2	98.86	98.86	88.84	101.66
Batch 3	100.28	100.20	90.34	100.24
Batch 4	102.42	102.40	94.38	98.72
Batch 5	99.44	NA[b]	NA[b]	98.58
Batch 6	101.32	NA[b]	93.12	98.54
Batch 7	99.98	NA[b]	NA[b]	98.14
Iron–carbohydrate complex 2				
Batch 1	100.70	97.95	84.70	96.80
Batch 2	96.60	95.50	85.35	96.55
Batch 3	99.05	97.63	85.55	97.05
Iron–carbohydrate complex 3				
Batch 1	100.01	100.78	93.12	99.84
SD	±0.129[c]	±0.135[d]		±0.334[e]

[a] Based on a minimum of two assays. [b] NA = not analyzed. [c] Based on three determinations of iron–carbohydrate complex 1. [d] Based on six determinations of iron–carbohydrate complex 2. [e] Based on eight determinations of iron–carbohydrate complex 2.

double-beam UV-visible spectrophotometer using a 1-cm. Corex cell and a slit width of 25.

Reagents—Iron standard solution for atomic absorption spectroscopy was used.[2] All other reagents were ACS, USP, or NF grade. Distilled water was used for all solutions. Iron standard solutions used for calibrations were 7, 10, and 12 mcg. Fe/ml., respectively.

PROCEDURES

Colorimetric Method—The total iron content of the three iron–carbohydrate preparations was determined colorimetrically by the 2,2′-bipyridine method described in USP XVII (1). Calculation of the mg. Fe/ml. solution was according to the following equation:

$$100 \times \frac{A_u}{A_s} \times \frac{d}{W} = \text{mg. Fe/ml. solution} \qquad \text{(Eq. 1)}$$

where A is the absorbance of the sample, A_s is the absorbance of the standard; d is the density of the sample measured with a pycnometer at 20°, and W is the weight of the sample in grams.

Titration Method—Tablet and sustained-release capsule preparations were assayed for iron following the USP XVII (2) sodium thiosulfate titration procedure. For the hard gelatin capsule, the NF XII (3) ceric sulfate titration procedure was followed.

Gravimetric Method—Accurately weigh a portion of iron–carbohydrate solution equivalent to about 90 mg. of iron into a platinum crucible. Ash the sample with a Meker burner for 30 min., and dissolve the ash in a 400-ml. beaker by boiling with 75 ml. of 6 *N* HCl. Dilute with water to 150–200 ml. and make alkaline with NH_4OH. Acidify with nitric acid and again make alkaline with an excess of NH_4OH. Heat to 70–80° for approximately 1 min. and allow to cool and stand for 2 hr. Filter through Whatman No. 41-H filter paper and wash the precipitate thoroughly with 1% NH_4OH. Ash the filtered precipitate in a tared platinum dish and determine the weight of the ash. Calculate the mg. total Fe/ml. solution as follows:

$$WA \times \frac{699.4}{W} \times d = \text{mg. total Fe/ml. solution} \qquad \text{(Eq. 2)}$$

where WA is the weight of the ash in grams, d is the density of the sample measured with a pycnometer at 20°, and W is the weight of the sample in grams.

ATOMIC ABSORPTION WITH ASHING[3]

Iron–Carbohydrate Preparations—Accurately weigh a portion of solution equivalent to about 90 mg. of Fe into a platinum crucible and ash for 30 min. over a Meker burner. Dissolve the ash in a 400-ml. beaker by boiling with 75 ml. of 6 *N* HCl. After the solution is cool, transfer to a 1-l. volumetric flask and dilute to the mark with water. Pipet a 6.0-ml. aliquot from the 1-l. volumetric flask into a 50-ml. volumetric flask and dilute to the mark with water. Aspirate the sample against a blank consisting of 100 ml. of 6 *N* HCl prepared according to the same dilution sequence as the sample. Calculate the mg. Fe/ml. solution as follows:

$$\text{mg. Fe/ml. solution} = \frac{(F_{AV})\,(A)\,(0.001)}{W/d \times 1/1000 \times 6/50} \qquad \text{(Eq. 3)}$$

where F_{AV} is the average mcg. Fe/ml./absorbance unit (determined from standards). F_{AV} was calculated as follows:

$$F \text{ (for a given standard)} = \frac{\text{concentration of standard, mcg. Fe/ml.}}{\text{absorbance}}$$

$$F_{AV} = \frac{\Sigma F}{\text{no. of standards measured}}$$

A = absorbance of the sample

d = density of the sample measured with pycnometer at 20°

Tablet—Weigh and finely powder 20 tablets. Weigh a portion of powder equivalent to about 90 mg. of Fe into a platinum crucible. Follow the procedure used for iron–carbohydrate preparations in the *Atomic Absorption with Ashing* section starting with "and ash for 30 min." up to "transfer to a 1-l. volumetric flask and dilute to the mark with water." Filter a portion of the solution through Whatman No. 2 filter paper. Pipet 6.0 ml. of the filtrate into a 50-ml. volumetric flask, and dilute to the mark with water. After aspirating the sample, calculate the mg. Fe/tablet as follows:

$$\text{mg. Fe/tablet} = F_{AV} \times A \times \frac{50}{6} \times \frac{1000}{1000} \times \frac{\text{average tablet weight, g.}}{\text{sample weight, g.}} \qquad \text{(Eq. 4)}$$

Table III—Ratio of Variance

	Critical, 5%	Calculated Value
Colorimetric *vs.* gravimetric *vs.* AAS with ashing[a]	3.88	1.88
Colorimetric *vs.* gravimetric *vs.* AAS with ashing[b]	6.94	3.63
AAS direct *vs.* AAS with ashing[c]	7.71	27.07

[a] Based on seven batches of iron–carbohydrate complex 1. [b] Based on three batches of iron–carbohydrate complex 2. [c] Based on five batches of iron–carbohydrate complex 1. These results showed significant difference at the 0.01 level.

[2] Fisher Scientific.

[3] In all the following AAS assays, the sample was aspirated against a blank consisting of the appropriate volume of 6 *N* HCl which was subjected to the same dilution sequence as the sample.

Table IV—Analysis of Tablet and Capsules

Type of Sample	Amount of Fe Declared	mg. Fe/Tablet or Capsule[a]		
		Titration	Atomic Absorption—Direct	Atomic Absorption—Ashed
Tablet	40 mg./tablet	39.25	40.48	38.95
Capsule (hard gelatin)	37.52 mg./capsule	39.48	40.54	38.95
Capsule (sustained release)	50 mg./capsule	50.11	52.23	51.71
SD		±2.10[b]	±1.79[b]	

[a] Based on a minimum of two assays. [b] Based on four determination of the sustained-release capsule.

where F_{AV} is the average mcg. Fe/ml./absorbance unit, and A is the absorbance of the sample.

Hard Gelatin Capsule and Sustained-Release Capsule—Weigh the contents of 20 capsules, mix thoroughly, and weigh a portion equivalent to about 90 mg. of Fe into a platinum crucible. Follow the procedure used for iron–carbohydrate preparations in the *Atomic Absorption with Ashing* section starting with "and ash for 30 min." up to "transfer to a 1-l. volumetric flask and dilute to the mark with water." Filter a portion of the solution through Whatman No. 2 filter paper, pipet 6.0 ml. of the filtrate into a 50-ml. volumetric flask, dilute to the mark with water, and aspirate. Calculate the mg. Fe/capsule as follows:

$$\text{mg. Fe/capsule} = F_{AV} \times A \times \frac{50}{6} \times \frac{1000}{1000} \times \frac{\text{average net fill weight, g.}}{\text{sample weight, g.}} \quad \text{(Eq. 5)}$$

where F_{AV} is the average mcg. Fe/ml./absorbance unit, and A is the absorbance of the sample.

ATOMIC ABSORPTION BY DIRECT DILUTION

Iron–Carbohydrate Preparations—Accurately weigh a portion of solution equivalent to about 90 mg. of Fe into a tared beaker. Quantitatively transfer the solution to a 1-l. volumetric flask with 30 ml. of water, add 100 ml. of 6 *N* HCl, and dilute to the mark with water. Pipet a 6.0-ml. aliquot into a 50-ml. volumetric flask, dilute to the mark with water, and aspirate. Calculate the mg. Fe/ml. as described in the section entitled *Atomic Absorption with Ashing: Iron–Carbohydrate Preparation.*

Tablet, Hard Capsule, and Sustained-Release Capsule—Weigh 20 tablets or the contents of 20 capsules. Weigh a portion of tablet powder or capsule content equivalent to about 90 mg. of Fe and transfer to a 1-l. volumetric flask with 30 ml. of water. Add 100 ml. of 6 *N* HCl,[4] and dilute to the mark with water. Filter a portion of the solution through Whatman No. 2 filter paper, pipet 6.0 ml. of filtrate into a 50-ml. volumetric flask, and dilute to the mark with water. Aspirate the sample against a blank. Calculate the mg. Fe/tablet or mg. Fe/capsule as described in the section entitled *Atomic Absorption with Ashing: Tablet* or *Atomic Absorption with Ashing: Hard Gelatin Capsule and Sustained-Release Capsule.*

In the case of individual sustained-release capsules, the following procedure is used. Add the contents of one capsule to a 500-ml. volumetric flask. Add 50 ml. of 6 *N* HCl and 100 ml. of water, heat until solution is effected, and dilute to the mark with water. Filter a portion of the solution through Whatman No. 2 filter paper, pipet 6.0 ml. of the filtrate into a 50-ml. volumetric flask, and dilute to the mark with water. Aspirate the sample against a blank and calculate the mg. Fe/capsule as follows:

$$\text{mg. Fe/capsule} = F_{AV} \times A \times \frac{500}{1000} \times \frac{50}{6} \quad \text{(Eq. 6)}$$

[4] It was necessary to heat the contents of the sustained-release capsule to effect solution after adding 6 *N* HCl. The insoluble material observed was no doubt due to the coating excipients commonly used in sustained-release preparations.

Table V—Recovery Studies

Type of Sample	Amount of Fe[a] Added, mcg.	% Fe Recovered[b] by Atomic Absorption
Iron–carbohydrate complex	100	98[c]
Capsule	100	101.6[d]
Tablet	100	100.4[d]

[a] In all cases, the final concentration of the added iron was 2 mcg./ml. [b] Average recovery for two trials. [c] Ashed before analysis. [d] Assayed directly without ashing.

where F_{AV} is the average mcg. Fe/ml./absorbance unit, and A is the absorbance of the sample.

Recovery Study—Known amounts of iron were added to each of the three dosage forms studied, and the iron content was determined by AAS.

RESULTS AND DISCUSSION

The assay results for the three iron–carbohydrate preparations are summarized in Table II. These results show that there is a significant difference between iron–carbohydrate preparations assayed directly by AAS and those ashed prior to assay by AAS. Although the standard deviation of the AAS ashed method is greater than the colorimetric or gravimetric method, the analysis of variance shown in Table III indicates that there is no significant difference between the colorimetric, gravimetric, and AAS with ashing method. The assay results summarized in Table IV indicate that the tablet and capsules studied may be analyzed directly for iron by AAS. Table V summarizes data from the recovery study.

It is recommended that an initial study of a hematinic preparation should consist of an ashed and a direct AAS assay procedure. This generally will give a measure of interferences caused by organic molecules that complex or chelate the iron or excipients which may refract or absorb light and thus interfere with the assay.

REFERENCES

(1) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 326.

(2) "The United States Pharmacopeia," 17th rev., 1st supplement, Mack Publishing Co., Easton, Pa., 1967, p. 12.

(3) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 169.

(4) D. J. David, *At. Absorption Newslett.*, **1**, 9(1962).

(5) J. E. Allan, *Spectrochim. Acta*, **10**, 800(1959).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 16, 1970, from the *Analytical Laboratory, Astra Pharmaceutical Products, Inc., Worcester, MA 10606*

Accepted for publication April 1, 1970.

The authors express their appreciation to William Webber, Dr. S. G. Davis, and Sheila Tarlin for reviewing this manuscript and to Frances Edgeworth and John Andronic for assisting with the colorimetric and gravimetric assay procedures.

Assay of Chloramphenicol and Its Esters in Formulations

M. S. KARAWYA* and M. G. GHOURAB

Abstract □ A method for the estimation of chloramphenicol and its esters in some pharmaceutical preparations is described. The method is based on measuring the violet color produced by the interaction of chloramphenicol with hydroxylamine and ferric chloride in an alcoholic medium.

Keyphrases □ Chloramphenicol, esters in dosage forms—analysis □ Colorimetric analysis—spectrophotometer □ Hydroxylamine HCl, ferric chloride—color formation, chloramphenicol

The estimation of chloramphenicol and its esters has been, and is still, the subject of much investigation. The need for further investigation is evidenced by the fact that most of the methods reported for the estimation of chloramphenicol are subject to difficulties during their application. These difficulties may be attributed to the unfavorable solubility of chloramphenicol or its derivatives in the solvent of the experiment, as in case of titrimetric (1) and polarographic methods (2). Most color reagents used in colorimetric assays (3–7) may interact also with the decomposition products of chloramphenicol, thus giving erroneous results. These decomposition products may also interfere in spectrophotometric methods (8). Aihara *et al.* (4) proposed a method based on measuring the color developed from the action of ferric chloride on the hydroxamic acid which is produced by the interaction of chloramphenicol and hydroxylamine in aqueous alkaline medium. The method, however, is applicable only to free chloramphenicol and not to its water-insoluble esters.

In the present investigation, the authors used the hydroxylamine reaction in establishing another colorimetric method suitable for estimating chloramphenicol and its esters in various pharmaceutical forms.

EXPERIMENTAL

Reagents—The following were used: (*a*) authentic samples of chloramphenicol, chloramphenicol palmitate, chloramphenicol stearate, and chloramphenicol succinate; (*b*) standard test solution of chloramphenicol or its esters: a 0.2% solution of chloramphenicol or any of the mentioned derivatives was prepared by dissolving 100 mg. of the sample in absolute ethanol and adjusting to 50 ml. with the same solvent; (*c*) ethanolic hydroxylamine hydrochloride reagent: a saturated solution of hydroxylamine hydrochloride in absolute ethanol; (*d*) ethanolic sodium hydroxide solution: a saturated solution of sodium hydroxide in absolute ethanol; (*e*) ethanolic hydrochloric acid: 20 ml. of hydrochloric acid (35% w/v) completed to 100 ml. with absolute ethanol; (*f*) ferric chloride solution: 1% w/v in distilled water; and (*g*) ethanolic succinic acid solution: 91.3 mg. of succinic acid dissolved in absolute ethanol and adjusted to 50 ml. with the same solvent.

Analytical grade reagents were used whenever possible.

In a spectrophotometric study of the violet color resulting from the action of hydroxylamine hydrochloride and ferric chloride on chloramphenicol, a Prolabo spectrophotometer was used. The optimum wavelength for the measurement was found to be 505 mμ.

Procedure—Pipet a volume of the ethanolic solution of chloramphenicol or its esters, equivalent to 1–5 mg., into a small conical flask. (In the case of free chloramphenicol, add 1 ml. of succinic acid solution.) Add 1 ml. of hydroxylamine hydrochloride solution to the solution in the flask, and then add 1 ml. of sodium hydroxide solution. Complete to 5 ml. with absolute ethanol and heat on a water bath at 85–95° for 5 min. Transfer the flask to a refrigerator freezer and allow to stand for 15 min. Transfer the contents of the flask quantitatively to a 10-ml. measuring tube with the aid of absolute ethanol (previously cooled to the same temperature as the mixture) and adjust the volume to approximately 7.5 ml. Add to the mixture, in the following succession, 1 ml. of distilled water (cooled as described), 1 ml. of 20% hydrochloric acid in absolute ethanol, and 0.5 ml. of ferric chloride solution; mix well. Measure the violet color after 2–5 min. in a 1-cm. cell at 505 mμ against a blank made simultaneously, omitting the addition of hydroxylamine reagent. The results are deduced from absorbance–concentration curves of chloramphenicol esters according to the sample analyzed. In the case of the chloramphenicol base, the results are deduced by comparing with standard preparations containing the same amount of succinic acid treated simultaneously.

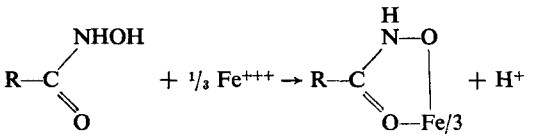
Precision of the Method—Absorbance *versus* concentration of chloramphenicol palmitate was measured and the deviation was 1.5%.

Mechanism of Reaction—It has been suggested (4) that hydroxylamine reacts with the amide group of chloramphenicol to give a hydroxamic acid. The addition of ferric ion to the hydroxamic acid solution produces a color (9–11) which could be utilized in the quantitative measurement. Feigl *et al.* (12) suggested that a carboxylic acid cannot be converted into a hydroxamic acid by the action of hydroxylamine and sodium hydroxide, while its ester can be converted under the same conditions (Scheme I).

$$\mathrm{RCOOR' + NH_2OH + NaOH \rightarrow RCO(NHONa) + R'OH + H_2O}$$
$$\mathrm{RCO(NHONa) + HCl \rightarrow RCONHOH + NaCl}$$

Scheme I

The hydroxamic acid formed gives a violet color with ferric chloride due to the formation of a water-soluble inner complex with ferric ion (Scheme II).



Scheme II

Accordingly, the esters of chloramphenicol would behave similarly.

Range—The color produced with chloramphenicol esters obeys Beer's law in the range of 300–500 mcg./ml. for the palmitate, 300–575 mcg./ml. for the stearate, and 300–485 mcg./ml. for the succinate.

Reproducibility—The reproducibility of the results is measured by applying the proposed method on solutions of known concentrations of chloramphenicol and its esters. The results are shown in Table I.

Application of the Method on Pharmaceutical Preparations—*Extraction of Chloramphenicol and Its Derivatives*—With a formulated preparation, it is necessary first to isolate chloramphenicol from the other accompanying ingredients. This can be accomplished by extracting the chloramphenicol or its derivatives by means of a suitable organic solvent as follows.

Chloramphenicol eye drops contain chloramphenicol, boric acid, borax, and phenyl mercuric nitrate. A volume equivalent to 50 mg. of chloramphenicol is mixed with an equal volume of phosphate buffer at pH 6.5 in a separating funnel and shaken with successive portions of chloroform (4 × 20 ml.) until complete

Table I—Assay of Solutions of Known Concentration of Chloramphenicol or Its Derivatives

Chloramphenicol			Chloramphenicol Palmitate			Chloramphenicol Succinate		
Added Amount, mcg.	Found Amount, mcg.	Error, %	Added Amount, mcg.	Found Amount, mcg.	Error, %	Added Amount, mcg.	Found Amount, mcg.	Error, %
250	255.7	2.3	300	305.7	1.9	300	308.1	2.7
275	277.0	0.70	350	353.5	1.0	325	325.0	0.0
300	301.0	0.30	375	375.0	0.0	380	351.0	0.3
350	351.0	0.30	400	401.0	0.25	375	374.0	0.3
400	401.5	0.40	425	424.0	−0.2	400	398.0	−0.5
425	424.0	−0.2	475	471.0	−0.8	425	419.0	−1.4
450	440.5	−2.1	500	491.5	−1.7	450	436.5	−3.0

extraction is effected. The chloroform is evaporated, and the residue is dissolved in 50 ml. of ethanol.

Chloramphenicol ear drops contain chloramphenicol and propylene glycol. A volume equivalent to 50 mg. chloramphenicol is directly extracted with chloroform (4 × 15 ml.), and the procedure described for eye drops is followed.

Chloramphenicol suppositories contain chloramphenicol in a suppository base. A weight of the suppositories equivalent to 50 mg. chloramphenicol is shaken with hot water and allowed to cool; the base is extracted with petroleum ether (3 × 10 ml.). The chloramphenicol remaining in the liquid is then extracted with chloroform (4 × 15 ml.) and treated as previously described.

Chloramphenicol palmitate suspension contains chloramphenicol palmitate and suspending, coloring, and flavoring agents.

Chloramphenicol palmitate and streptomycin suspension contains chloramphenicol palmitate, streptomycin sulfate, and suspending, coloring, and flavoring agents.

Chloramphenicol stearate suspension contains chloramphenicol stearate and suspending, coloring, and flavoring agents.

A volume of each of these three suspensions equivalent to 50 mg. of chloramphenicol is treated as described for eye drops.

Chloramphenicol succinate injection contains chloramphenicol succinate with a diluent in a powder form. An equivalent to 50 mg. of chloramphenicol is dissolved in 50 ml. ethanol.

All of the formulations were supplied from freshly prepared batches. The resulting ethanolic solutions of chloramphenicol or its esters were then assayed as described under *Procedure*. The results obtained were compared with those of the Aihara *et al.* (4) method, the spectrophotometric method (1), and the α-naphthol method (6). They are compiled in Table II.

Estimation of Chloramphenicol in Degraded Samples—The estimation of chloramphenicol in degraded samples of palmitate suspension, eye drops, and ear drops was carried out to examine the potentialities of the proposed method.

Degradation of chloramphenicol was achieved by incubating the preparation at different temperatures for 2 months. The suspension darkened in color and acquired a caramel-like odor. The eye drops and ear drops acquired a yellow color. The results were also compared with those of the spectrophotometric (1) and α-naphthol methods (6) and are represented in Table III.

Table II—Percentage of Chloramphenicol and Its Esters with Respect to the Labeled Amounts in Different Formulations

Samples	Proposed Method	Aihara *et al.* Method	Spectrophotometric Method	α-Naphthol Method
Chloramphenicol	99	99	98	99.9
Ear drops	97.5	98	98.2	97
Eye drops	96	97	96.2	96
Capsules	99	100	99.5	101
Suppositories	101	100	103	102
Chloramphenicol palmitate	105	Negative	102	97
Suspension	107	Negative	105	102
Suspension and streptomycin	103	Negative	107	101
Chloramphenicol stearate	98	Negative	100	98
Suspension	102	Negative	105	101
Chloramphenicol succinate	99.8	Negative	101	97
Injection	101	Negative	102	98

RESULTS AND DISCUSSION

The ethanol used in the experiment was found advantageous due to its solubilizing effect and for increasing the sensitivity of the reaction. Minimum sensitivity is given by 70% ethanol and maximum sensitivity by 90% ethanol. The performance of the interaction in absolute ethanol is 5 times as sensitive as in ethanol 70%. However, the presence of about 15% water is necessary to dissolve the resulting sodium chloride and ferric hydroxamate. To choose the optimum pH for the hydrolysis step, many alkalies and buffer solutions were tried. When using boric acid buffer (pH 10), a faint green, fluorescent, unstable color was produced. The addition of 1 ml. of phosphate buffer (pH 8) together with 1 ml. of sodium hydroxide solution stabilized the final violet color, but the sensitivity of the reaction decreased. The use of higher concentrations of phosphate buffer inhibited the resulting color. Higher and lower concentrations of sodium hydroxide solution and hydroxylamine reagent were tried, and 1 ml. of saturated ethanolic solutions of each was found most convenient for the color formation. At concentrations below 300 mcg./ml. of chloramphenicol, another grade of color is formed and this does not obey Beer's law.

The interaction was favored by heating. The time and temperature of heating affected the sensitivity of the reaction; heating for 5 min. at a temperature of 85–95° was found most suitable for the completion of the reaction. In the acidification step, the optimum pH was found in the range of 2.1–2.3; this was obtained by employing 1 ml. of ethanolic 20% hydrochloric acid. Hydrochloric acid buffer and organic acids were found to lessen the sensitivity of the color.

The intensity of the color increases greatly by cooling and reaches its maximum at 0° and falls to a minimum at 15°. The time of cooling affects the intensity of the produced color; the maximum color is obtained after 15 min. of cooling.

It is of great importance to adhere to the specified amount and concentration of ferric chloride (0.5 ml. of 1% solution); lower

Table III—Percentage of Chloramphenicol with Respect to Labeled Amounts in Degraded Samples

	Proposed Method	Naphthol Method	UV Method	Proposed Method	Naphthol Method	UV Method
	37°			45°		
Palmitate suspension	102	101	102	90	104	110
Eye drops	97	96	98	91	96.9	101
Ear drops	100	99	99.5	90	99.5	107
	55°			60°		
Palmitate suspension	82	110	140	76	130	[a]
Eye drops	80	101	110	50	[a]	[a]
Ear drops	64	104	130	35	[a]	[a]

[a] Discordant results.

concentrations gave incomplete reactions, and higher concentrations vitiated the violet color and changed it to yellow. When impure ferric chloride or an unfresh solution of it was employed, the addition of 2 drops of hydrogen peroxide (10 vol.) restored and potentiated the violet color. However, excess hydrogen peroxide was found unfavorable as the color changed to orange. The violet color, produced by the action of ferric chloride, develops within 2 min. and should be measured within 2–5 min. The color is not affected by light but is very sensitive to temperature.

Free chloramphenicol gave the same reaction as the salts, but the sensitivity was 5 times lower ($\leq$15 mg.) and the results were not reproducible. The addition of a carboxylic acid, however, was found to increase the sensitivity of the reaction. Many acids were tried, and succinic acid gave the highest sensitivity ($\leq$3 mg.). Although succinic acid alone gave negative reactions with the reagent, its presence affected the sensitivity of the reaction. Therefore, equal amounts of succinic acid were added to both test and standard preparations.

Due to the slight yellowish color produced by the action of sodium hydroxide on chloramphenicol or its esters, the blank experiment was done exactly like the experiment, omitting the addition of hydroxylamine hydrochloride reagent.

Data presented in Table I indicate the accuracy of the proposed method and the reproducibility of the results. The percentage of error with respect to the added and found amounts (Table I) ranges from $\pm$2.1 to 2.3 in chloramphenicol, from $\pm$1.7 to 1.9 in chloramphenicol palmitate, and from $\pm$2.7 to 3 in chloramphenicol succinate. The method was found applicable to different freshly formulated preparations, and the results (Table II) are comparable with those of the Aihara *et al.* method (4), the spectrophotometric method, and the α-naphthol method (6). The method of Aihara *et al.* (4), however, was not applicable to chloramphenicol esters. The comparison of the proposed method to the α-naphthol method and the spectrophotometric method on degraded samples of chloramphenicol and its palmitate produced varying results (Table III). The chloramphenicol analysis of the heated preparation decreased with an increase in temperature in the case of the proposed method and increased with the other two methods. This fact indicates that by means of the hydroxylamine method, chloramphenicol and its esters can be determined, while some of the degradation products interfere in the results of the other two methods. In addition, the color produced by the α-naphthol method was not the same in all cases; it ranged from greenish to bluish.

REFERENCES

(1) D. S. Masterson, Jr., *J. Pharm. Sci.*, **57**, 305(1968).

(2) A. J. Glazko, L. M. Wolf, and W. A. Dill, *Arch. Biochim.*, **23**, 411(1949).

(3) J. Levine and H. Fischbach, *Antibiot. Chemother.*, **1**, 59 (1951).

(4) T. H. Aihara, H. Machida, and Y. Yoneda, *J. Pharm. Soc. Jap.*, **77**, 1318(1957).

(5) E. Hennig and H. Heypoth, *Pharm. Zentralh.*, **103**, 810 (1964).

(6) D. S. Masterson, Jr., *J. Pharm. Sci.*, **57**, 305(1968).

(7) A. Spitzer, A. Schweizer, and V. Popvici, *Farmacia (Bucharest)*, **12**, 503(1964).

(8) R. B. Mukherjee and B. N. Durra, *J. Proc. Inst. Chem. India*, **37**, 17(1965).

(9) T. Higuchi, A. D. Marcus, and C. D. Bois, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 135(1954).

(10) J. H. Ford, *Anal. Chem.*, **19**, 1004(1947).

(11) N. Bohnos, A. C. Dornbush, L. I. Feldman, J. H. Martin, E. Pelcak, and T. H. Williams, *Antibiot. Ann.*, **54**, 49(1953).

(12) F. Feigl, "Spot Tests in Organic Analysis," 6th ed., Elsevier, Amsterdam, The Netherlands, 1960, p. 250.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 27, 1969, from the *Pharmacognosy Department, Faculty of Pharmacy, Cairo University*, and the *Memphis Chemical Co., Zeitoun, Cairo.*

Accepted for publication April 3, 1970.

* Present address: Faculty of Pharmacy, Kasr-el-Aini, Cairo, Egypt, U.A.R.

Application of Absorbance Ratios to Analysis of Pharmaceuticals VI: Analysis of Binary Mixture Using a Reference Spectrum

M. J. CHO and M. PERNAROWSKI

Abstract □ Binary mixtures may be resolved by using absorbance ratio values and a reference spectrum for one component in the mixture. The method is based on the differences between the absorbance values for the mixture at any two wavelengths and the values for a solution containing only one component in the mixture. Three constants are required to resolve the mixture, but only one of these is an absorptivity value. Unlike the absorbance ratio method described in the literature, this method does not depend on the use of a wavelength at which the two components in the mixture have identical absorptivity values.

Keyphrases □ Binary mixture analysis—reference spectrum □ Caffeine–Na benzoate—spectral characteristics □ Absorbance ratios—analysis □ UV spectrophotometry—analysis, absorbance ratios

The ratio of two absorbance values determined on the same solution at two different wavelengths is a constant. These ratios may be used to determine both the relative and absolute concentrations of the components in a binary mixture (1). However, absolute concentrations (w/v) cannot be determined unless one of the two wavelengths chosen for the analysis represents an isoabsorptive point.

Isoabsorptive points (those wavelengths at which the two components in a mixture have identical absorptivity values) are difficult to isolate. The mathematical derivations in the next section show that absolute concentration values can be obtained by analyzing the mixture at wavelengths that do not represent isoabsorptive points, and that the number and nature of the constants in the derived equations are the same as those associated with the absorbance ratio method of analysis (1). This method of analysis is based, therefore, on the use of two absorbance ratio values (Q values), one absorptivity value, and the differences, at two wave-

concentrations gave incomplete reactions, and higher concentrations vitiated the violet color and changed it to yellow. When impure ferric chloride or an unfresh solution of it was employed, the addition of 2 drops of hydrogen peroxide (10 vol.) restored and potentiated the violet color. However, excess hydrogen peroxide was found unfavorable as the color changed to orange. The violet color, produced by the action of ferric chloride, develops within 2 min. and should be measured within 2–5 min. The color is not affected by light but is very sensitive to temperature.

Free chloramphenicol gave the same reaction as the salts, but the sensitivity was 5 times lower ($\leq$15 mg.) and the results were not reproducible. The addition of a carboxylic acid, however, was found to increase the sensitivity of the reaction. Many acids were tried, and succinic acid gave the highest sensitivity ($\leq$3 mg.). Although succinic acid alone gave negative reactions with the reagent, its presence affected the sensitivity of the reaction. Therefore, equal amounts of succinic acid were added to both test and standard preparations.

Due to the slight yellowish color produced by the action of sodium hydroxide on chloramphenicol or its esters, the blank experiment was done exactly like the experiment, omitting the addition of hydroxylamine hydrochloride reagent.

Data presented in Table I indicate the accuracy of the proposed method and the reproducibility of the results. The percentage of error with respect to the added and found amounts (Table I) ranges from $\pm$2.1 to 2.3 in chloramphenicol, from $\pm$1.7 to 1.9 in chloramphenicol palmitate, and from $\pm$2.7 to 3 in chloramphenicol succinate. The method was found applicable to different freshly formulated preparations, and the results (Table II) are comparable with those of the Aihara *et al.* method (4), the spectrophotometric method, and the α-naphthol method (6). The method of Aihara *et al.* (4), however, was not applicable to chloramphenicol esters. The comparison of the proposed method to the α-naphthol method and the spectrophotometric method on degraded samples of chloramphenicol and its palmitate produced varying results (Table III). The chloramphenicol analysis of the heated preparation decreased with an increase in temperature in the case of the proposed method and increased with the other two methods. This fact indicates that by means of the hydroxylamine method, chloramphenicol and its esters can be determined, while some of the degradation products interfere in the results of the other two methods. In addition, the color produced by the α-naphthol method was not the same in all cases; it ranged from greenish to bluish.

REFERENCES

(1) D. S. Masterson, Jr., *J. Pharm. Sci.*, **57**, 305(1968).

(2) A. J. Glazko, L. M. Wolf, and W. A. Dill, *Arch. Biochim.*, **23**, 411(1949).

(3) J. Levine and H. Fischbach, *Antibiot. Chemother.*, **1**, 59 (1951).

(4) T. H. Aihara, H. Machida, and Y. Yoneda, *J. Pharm. Soc. Jap.*, **77**, 1318(1957).

(5) E. Hennig and H. Heypoth, *Pharm. Zentralh.*, **103**, 810 (1964).

(6) D. S. Masterson, Jr., *J. Pharm. Sci.*, **57**, 305(1968).

(7) A. Spitzer, A. Schweizer, and V. Popvici, *Farmacia (Bucharest)*, **12**, 503(1964).

(8) R. B. Mukherjee and B. N. Durra, *J. Proc. Inst. Chem. India*, **37**, 17(1965).

(9) T. Higuchi, A. D. Marcus, and C. D. Bois, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 135(1954).

(10) J. H. Ford, *Anal. Chem.*, **19**, 1004(1947).

(11) N. Bohnos, A. C. Dornbush, L. I. Feldman, J. H. Martin, E. Pelcak, and T. H. Williams, *Antibiot. Ann.*, **54**, 49(1953).

(12) F. Feigl, "Spot Tests in Organic Analysis," 6th ed., Elsevier, Amsterdam, The Netherlands, 1960, p. 250.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 27, 1969, from the *Pharmacognosy Department, Faculty of Pharmacy, Cairo University*, and the *Memphis Chemical Co., Zeitoun, Cairo.*

Accepted for publication April 3, 1970.

* Present address: Faculty of Pharmacy, Kasr-el-Aini, Cairo, Egypt, U.A.R.

Application of Absorbance Ratios to Analysis of Pharmaceuticals VI: Analysis of Binary Mixture Using a Reference Spectrum

M. J. CHO and M. PERNAROWSKI

Abstract □ Binary mixtures may be resolved by using absorbance ratio values and a reference spectrum for one component in the mixture. The method is based on the differences between the absorbance values for the mixture at any two wavelengths and the values for a solution containing only one component in the mixture. Three constants are required to resolve the mixture, but only one of these is an absorptivity value. Unlike the absorbance ratio method described in the literature, this method does not depend on the use of a wavelength at which the two components in the mixture have identical absorptivity values.

Keyphrases □ Binary mixture analysis—reference spectrum □ Caffeine–Na benzoate—spectral characteristics □ Absorbance ratios—analysis □ UV spectrophotometry—analysis, absorbance ratios

The ratio of two absorbance values determined on the same solution at two different wavelengths is a constant. These ratios may be used to determine both the relative and absolute concentrations of the components in a binary mixture (1). However, absolute concentrations (w/v) cannot be determined unless one of the two wavelengths chosen for the analysis represents an isoabsorptive point.

Isoabsorptive points (those wavelengths at which the two components in a mixture have identical absorptivity values) are difficult to isolate. The mathematical derivations in the next section show that absolute concentration values can be obtained by analyzing the mixture at wavelengths that do not represent isoabsorptive points, and that the number and nature of the constants in the derived equations are the same as those associated with the absorbance ratio method of analysis (1). This method of analysis is based, therefore, on the use of two absorbance ratio values (Q values), one absorptivity value, and the differences, at two wave-

lengths, between the absorbance values for a solution and the values for a reference solution containing one of the two components in the mixture.

THEORY

Hypothetical spectrophotometric curves for X and Y are shown in Fig. 1 and will be used to clarify the derivations. It is assumed that Beer's law is obeyed at all wavelengths and at all spectrophotometrically significant concentrations.

The total absorbance of a mixture containing X and $Y(A_M)$ is equal to the sum of the absorbances due to $X(A_X)$ and $Y(A_Y)$. Therefore, at λ_4,

$$A_{M4} = a_6C_X + a_1C_Y \qquad \text{(Eq. 1)}$$

C_X and C_Y are the concentrations of X and Y in the mixture. Similarly, at λ_2,

$$A_{M2} = a_2C_X + a_5C_Y \qquad \text{(Eq. 2)}$$

The absorbance values, at λ_4 and λ_2, for a reference solution containing only X are:

$$A_{X4} = a_6C_{XR} \qquad \text{(Eq. 3)}$$

$$A_{X2} = a_2C_{XR} \qquad \text{(Eq. 4)}$$

C_{XR} is the concentration of X in the reference solution.

Subtracting Eq. 3 from Eq. 1 and rearranging yield:

$$a_1C_Y = D_4 - a_6C_X + a_6C_{XR} \qquad \text{(Eq. 5)}$$

D_4 is defined as $A_{M4} - A_{X4}$. Similarly, at λ_2,

$$a_5C_Y = D_2 - a_2C_X + a_2C_{XR} \qquad \text{(Eq. 6)}$$

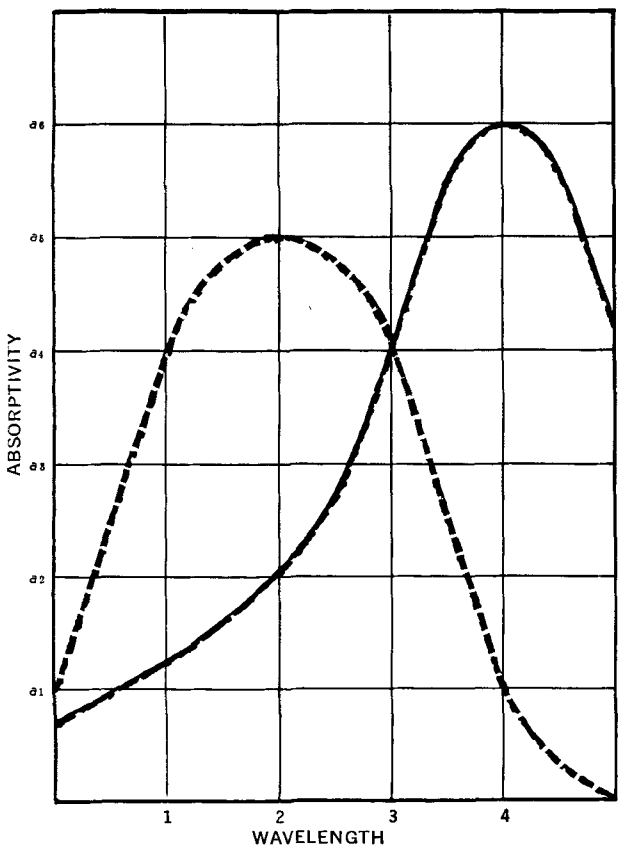


Figure 1—*Hypothetical spectrophotometric curves for* X (——) *and* Y (- - -).

Dividing Eq. 6 by Eq. 5 yields:

$$\frac{a_5C_Y}{a_1C_Y} = \frac{D_2 - a_2C_X + a_2C_{XR}}{D_4 - a_6C_X + a_6C_{XR}} \qquad \text{(Eq. 7)}$$

However, a_5/a_1 is equal to Q_Y, the absorbance ratio value for pure Y. Therefore:

$$Q_YD_4 - a_6Q_YC_X + a_6Q_YC_{XR} = D_2 - a_2C_X + a_2C_{XR} \qquad \text{(Eq. 8)}$$

Dividing both sides of Eq. 8 by a_6 and substituting Q_X, the absorbance ratio value for pure X, for a_2/a_6 yield:

$$\frac{Q_YD_4}{a_6} - Q_YC_X + Q_YC_{XR} = \frac{D_2}{a_6} - Q_XC_X + Q_XC_{XR} \qquad \text{(Eq. 9)}$$

After rearranging:

$$C_X = C_{XR} + \frac{D_2 - Q_YD_4}{a_6(Q_X - Q_Y)} \qquad \text{(Eq. 10)}$$

Therefore, the absolute concentration of $X(C_X)$ in a solution containing X and Y can be determined by measuring the differences, at two wavelengths, between the absorbance values for the mixture and the absorbance values for a reference solution containing only X. The constants, Q_X and Q_Y, are concentration independent and are easily determined by measuring absorbance values of solutions containing only X or Y.

The absorptivity value, a_6, is determined by measuring absorbance values, at the specified wavelength, of accurately prepared solutions containing only X.

The method described herein was checked experimentally by determining the absolute concentrations of sodium benzoate in solutions containing sodium benzoate and caffeine.

EXPERIMENTAL

Apparatus—A UV spectrophotometer[1,2] was used.

Reagents—Sodium benzoate USP and caffeine USP were used.

Spectral Characteristics of Sodium Benzoate and Caffeine—Caffeine and sodium benzoate (in water) absorb a maximum of radiant energy at 273 and 225 mμ, respectively. An examination of the sodium benzoate spectrum indicated that the analysis of this substance in the presence of caffeine could be carried out by determining absorbance values at 225 mμ (the absorption maximum for sodium benzoate) and at 235 mμ (a region in which the spectra have slight inflection points).

Two series of solutions were prepared, one containing caffeine and the other containing sodium benzoate. The absorbance value of each solution was determined at 225 and 235 mμ. The Q:235:225 values for caffeine and sodium benzoate were then calculated by dividing the absorbance at the first wavelength (235 mμ) by that at the second wavelength (225 mμ). Based on seven such solutions, the Q:235:225 value for caffeine was 0.721 ± 0.002; for sodium benzoate, it was 0.619 ± 0.003.

A series of solutions was prepared to contain from 5.0 to 14.0 mg. of sodium benzoate per liter of solution. The absorptivity value for sodium benzoate at 225 mμ was found to be 59.2 ± 0.3.

Procedure—Accurately dilute the solution containing caffeine and sodium benzoate. Determine absorbance values at 225 and 235 mμ.

Prepare a reference solution, by dilution, to contain a known quantity of sodium benzoate. (The reference solution used in these analyses contained 14 mg. of sodium benzoate per liter.) Determine absorbance values at 225 and 235 mμ.

Subtract the values for the reference solution from the values for the mixture. Substitute these D_2 and D_4 values into the numerical form of Eq. 10:

$$C_X \text{ (g./l.)} = 0.014 + \frac{D_2 - 0.721D_4}{59.2\,(0.619 - 0.721)} \qquad \text{(Eq. 11)}$$

Calculate the concentration of sodium benzoate in the solution.

[1] Beckman model DU-2.
[2] Bausch & Lomb Spectronic 505.

Table I—Analysis of Sodium Benzoate in Sodium Benzoate–Caffeine Mixtures

mg. Drug/l.		mg. Sodium Benzoate/l.
Sodium Benzoate	Caffeine	Recovered
4.5	7.5	4.54
5.0	7.0	5.06
5.0	5.0	5.02
6.0	6.0	6.05
6.5	5.5	6.32
7.0	5.0	7.11

Sixteen solutions were prepared to contain from 4.5 to 7.5 mg. of sodium benzoate per liter. Caffeine and sodium benzoate injection USP usually contains equal quantities of caffeine and sodium benzoate. However, to test the accuracy of the method, the solutions contained from three parts of sodium benzoate for every five parts of caffeine to five parts of sodium benzoate for every three parts of caffeine. To check the accuracy of the method further, a second set of six solutions was prepared and analyzed. The results for this set are shown in Table I. The percent recovery for the 22 solutions was 100.1%; the standard deviation value was ± 2.9%.

DISCUSSION

The caffeine–sodium benzoate mixture can be resolved by using simultaneous equations (2) or by the absorbance ratio method of analysis (1). If the former method is used, four absorptivity values must be determined. If the latter method is used, one absorptivity value and two absorbance ratio values must be determined. Absorbance ratio values are concentration independent; for this reason, the absorbance ratio method of analysis yields accurate results more quickly than does the method utilizing simultaneous equations. However, the absorbance ratio method of analysis yields absolute concentrations only if one of the two wavelengths chosen for the analysis is an isoabsorptive point. Such points are difficult to isolate, and the absorptivity values at such wavelengths cannot be determined with accuracy.

The method of analysis described here utilizes three constants. The absorbance ratio values are the same as those used in the absorbance ratio method of analysis. The absorptivity value, on the other hand, is determined at a wavelength at which one of the components absorbs a maximum of radiant energy. It is, therefore, not necessary to isolate an isoabsorptive point to calculate the absolute concentration of a drug in a mixture.

The method does require the use of a reference solution. However solutions must be prepared to determine constants, and one of these can be designated as the reference solution. This solution must, however, contain a quality of drug which will yield absorbance values somewhat greater than those observed for the mixture. If the difference between the absorbance values at the two specified wavelengths is small, the error in the analysis will be greater than necessary.

The results reported here indicate that the method is capable of yielding reasonably accurate results. The maximum percent recovery observed for the 22 solutions was 105.8%. The minimum percent recovery was 95.6%. A reexamination of these two solutions indicated that slight changes in absorbance readings can result in fairly substantial changes in percent recovery. This implies that each absorbance value should be determined twice (and the mean value used in the calculation) and that a stable, single-beam spectrophotometer should be used to measure absorbance values. Since the solutions were read in sequence, the values could not be rejected. Even so, the overall percent recovery for the 22 solutions was 100.1%. A standard deviation value of ±2.9% is acceptable if the speed and ease of analysis are taken into consideration.

The caffeine concentration in the solutions can be determined by using an equation similar to that given for sodium benzoate. Both equations, therefore, are based on absorbance differences. However, the equations may be derived by summing absorbance values. If this is done, the form of Eq. 10 is the same except that the C_{XR} term becomes negative.

Absorbance ratio values are used in both this method of analysis and that previously published (1). The appropriate references (1, 3) should, therefore, be consulted for a full discussion of the advantages and limitations of the use of such ratios in analysis.

REFERENCES

(1) M. Pernarowski, A. M. Knevel, and J. E. Christian, *J. Pharm. Sci.*, **50**, 943(1961).

(2) M. Pernarowski, in "Pharmaceutical Chemistry," vol. 2, L. G. Chatten, Ed., Marcel Dekker, New York, N. Y., 1969, p. 25.

(3) M. Pernarowski, A. M. Knevel, and J. E. Christian, *J. Pharm. Sci.*, **50**, 946(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 11, 1969, from the *Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver 8, B. C., Canada.*

Accepted for publication April 2, 1970.

Lactose USP (Beadlets) and Dextrose (PAF 2011): Two New Agents for Direct Compression

N. L. HENDERSON and A. J. BRUNO

Abstract □ Lactose USP (beadlets) and dextrose (PAF 2011) have been evaluated and compared with two commonly used agents for direct compression—spray-dried lactose USP and anhydrous lactose USP—as to their relative physical properties and stability for tableting by direct compression. The compression characteristics of all four materials were defined with the aid of a rotary tablet machine instrumented with strain gauges. Compression and ejection forces were monitored continuously and correlated with tablet hardness, friability, weight variation, and disintegration times. It was concluded that the two new agents are generally superior to the other materials tested for use as fillers for direct compression. Both exhibited excellent flow and compressional characteristics. Neither agent represents an improvement over the older materials with regard to physical stability.

Keyphrases □ Lactose (beadlets), dextrose, evaluation—direct compression □ Dextrose, lactose beadlets—spray-dried, anhydrous lactose comparison □ Tablets, direct compression—lactose beadlets, dextrose, anhydrous, spray-dried lactose □ Physical properties, tablets—lactose beadlets, spray-dried, anhydrous lactose, dextrose

Most therapeutic agents lack the compression and flow characteristics required for tableting on a rotary tablet press and must be processed prior to the tableting operation. Classically, the techniques of granulation, either the wet method or the dry granulation method, have been used for this purpose. Each of these time-consuming procedures often necessitates the use of specialized equipment. Wet granulation is unsuitable for materials that are heat or solvent labile. In addition, the incorporation of active ingredients into granules often leads to decreased drug availability.

At present, one of the most promising alternatives to granulation techniques is direct compression. It is for this reason that the concept of compressing pharmaceuticals directly into tablets has received much attention in the past decade (1–8).

The process of direct compression, by definition, involves the blending of the active ingredient with a compressible, free-flowing agent for direct compression, along with required lubricants and disintegrating agents. Direct compression allows optimum utilization of production time. Since the active ingredient is not incorporated into a granule, tablet dissolution becomes more dependent on compression force and less subject to other, less controllable parameters.

The ideal agent for direct compression should be compressible, free-flowing, inert with respect to chemical and physical reactivity, and relatively inexpensive. To date, no single agent has been found that is suitable for all direct compression formulas. If such an agent was available, it would surely gain rapid and widespread acceptance throughout the pharmaceutical industry.

Samples of two new agents for direct compression, *i.e.*, lactose USP (beadlets)[1] and dextrose–corn syrup solids,[2] hereafter termed dextrose (PAF 2011), were obtained for study. The first of these materials is a form of lactose USP monohydrate, spray-dried, which has been specially processed to form white, free-flowing "beads" having a very faint caramel-like odor similar to that of spray-dried lactose. Dextrose (PAF 2011) is produced by the controlled hydrolysis of starch and is composed of 95–96% dextrose combined with 4–5% higher saccharides. The result is a white, free-flowing, odorless material composed of aggregated, porous, crystalline beads. The two materials have been evaluated and compared with two agents commonly used for direct compression, namely, spray-dried lactose USP[1] and anhydrous lactose USP,[3] as to their relative physical properties and stability with respect to tableting by direct compression.

Microcrystalline cellulose, a widely used agent for direct compression, was not included in this study for comparative purposes since it has been reviewed thoroughly in previous publications (3, 9).

EXPERIMENTAL

Materials—Lactose USP was employed as a control in physical and color stability tests. Lubrication of tablets was accomplished by the addition of 1% of either magnesium stearate USP or stearic acid USP. Starch USP was used as the disintegrating agent in samples used to determine disintegration times. Where the effect of amines on the tablets was to be determined, *d*-amphetamine sulfate USP or phenylephrine hydrochloride USP was incorporated in the formulations at a concentration of 10% by weight.

Test Methods and Equipment—*Tablet Machines*—The compression characteristics of all four materials for direct compression were defined with the aid of a Stokes RBB2 tablet machine instrumented to permit the simultaneous monitoring of tablet compression and ejection forces (10). The selection of this machine was based on the fact that it is routinely used in tablet production and the die-filling operation is dependent on the force of gravity and the flow properties of the material to be tableted. The press was fitted with standard concave punches 0.87 cm. ($^{11}/_{32}$ in.) in diameter and was set to operate at 1200 tablets/min.

[1] Foremost Dairies, Inc., San Francisco, Calif. In the case of the lactose USP (beadlets), only one lot of material was available for testing.

[2] Available as Celutab (Dextrose PAF 2011) from Penick and Ford, Ltd., Cedar Rapids, Iowa.

[3] Sheffield Chemical Co., Norwich, N. Y.

Table I—Physical Properties of Sugars for Direct Tablet Compression

Material	Tap Density, g./ml.	—Angle of Repose— Control[a]	After Exposure[b]	Particle-Size Range, μ
Lactose USP (beadlets)	0.63	39–41	46–48	15–300
Spray-dried lactose USP	0.64	44–46	48–50	15–300
Anhydrous lactose USP	0.60	53–55	58–60	15–450
Dextrose (PAF 2011)	0.67	39–41	54–56	75–350
Lactose USP	0.70	62–64	70–72	5–175

[a] Control angle of repose. [b] Angle of repose after exposure to 75% R.H. in open containers for 5 days.

The many samples of tablets required for evaluation of the physical and color stability of the various sugars were prepared with the aid of a Stokes Model F tablet machine using 1.27-cm. (0.5-in.) standard concave tooling.

Hardness—Tablet hardness was determined on a hardness tester[4] which was modified to operate from a compressed air line. All values were expressed as the average of the values obtained for 10 individual tablets.

Friability—Friability was determined in a Roche Friabilator (11). Samples of 10 tablets were weighed, subjected to rotation for 20 min. at 25 r.p.m., and then reweighed after careful dusting. The percentage of tablet weight lost was then calculated. Tablets that capped were taken as 100% loss, and the number capped was recorded.

Disintegration Time—Disintegration times were determined using the standard USP apparatus both with and without disks (12). These data were reported as the average time required for 12 tablets to disintegrate.

Bulk Density—The bulk density of each sugar was determined according to the tap method of Butler and Ramsey (13).

Particle Size—Particle size was determined by sieve analysis. Sizing was accomplished in an End Shake[5] sieve shaker using a series of 20- to 200-mesh stainless steel U. S. Standard sieves. The unit was operated for 15 min. The sample for each test was 100 g. The finite particle shape was determined by examination of each material under low power (100×) magnification.

Angle of Repose—The angle of repose for each substance was measured by a tilting box technique as previously reviewed by Train (14).

Color Stability Testing—Tests were carried out by exposing samples of 10 tablets, in open glass Petri dishes, to one of the following conditions: (*a*) heat storage at 56° for 10 days; (*b*) moisture storage at 75% R.H. for 30 days; (*c*) heat/moisture storage at 37°/75% R.H. for 10 days; or (*d*) light exposure to 600 foot candles (fc.) fluorescent illumination for 12 weeks (15).

Initial and Equilibrium Moisture Content—The initial moisture content of each material was determined by loss on drying in a vacuum oven after exposure for 16 hr. at 60° in open containers.

To determine the hygroscopicity of these agents for direct compression, tared samples of each were stored at various relative humidities in open containers in desiccators at 25° for a 7-day period. Samples were then weighed to determine the percentage of moisture picked up.

RESULTS AND DISCUSSION

Some of the physical properties of four sugars for direct compression are summarized in Table I. Data for lactose USP were also included for comparison.

The angle of repose of a powder is often regarded as providing a measure of the internal friction of the material. The determination of this angle, therefore, serves to quantify the relative degree of flowability of materials (16, 17). All of the agents for direct compression tested were found to have a low angle of repose, indicating excellent flow properties. It should be noted that, after exposure to 75% R.H. for 5 days in open containers, an increase in angle of repose resulted. Dextrose (PAF 2011) exhibited the greatest decrease in flowability. This material was found to be considerably more hygroscopic than the lactose sugars (Table II).

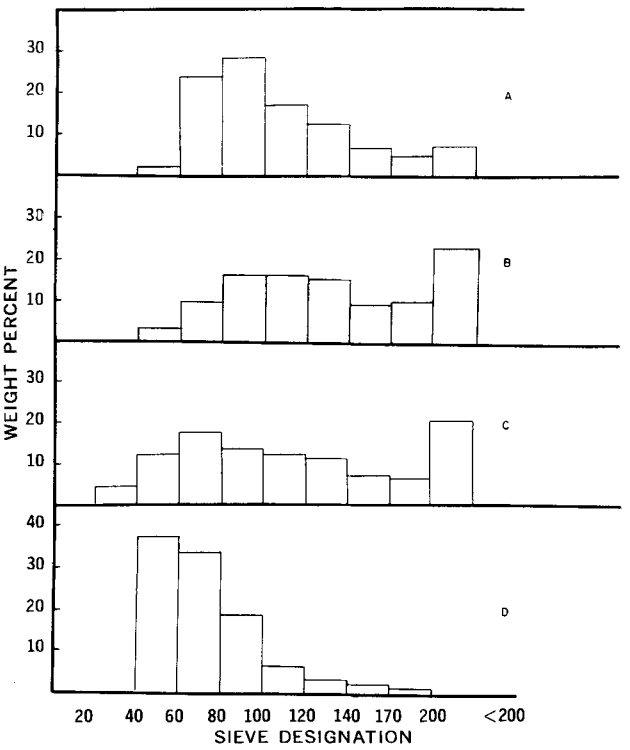


Figure 1—*Histograms displaying particle-size distribution of four materials for direct compression. Key: A, lactose USP (beadlets); B, spray-dried lactose USP; C, anhydrous lactose USP; and D, dextrose (PAF 2011).*

Sieve analysis indicated a wide range of particle size for each sugar tested. In general, when a material has a narrow particle-size distribution, it will exhibit less tablet-to-tablet weight variation than a material with a wider distribution of particle sizes. Dextrose (PAF 2011) was found to have not only the most narrow particle-size distribution, but it also contained the smallest percentage of "fines" (Fig. 1).

Color Stability—Several references indicate that tablets prepared from lactose tend to discolor upon storage (18, 19). This phenomenon is accelerated by the presence of amines and/or certain lubricants, and it is dependent upon the temperature, humidity, and illumination under which the tablet is stored. The mechanism governing this process has been previously elucidated (18, 20).

A study was undertaken to determine the degree of discoloration produced in the five sugars (four agents for direct compression plus a lactose USP control) when stored in open containers under four testing conditions—heat, moisture, heat/moisture, and light—in the presence of lubricants and selected amines. Results of visual observation are summarized in Table III. Samples stored at 37° at 75% R.H. for 10 days exhibited no more color change than those stored at either 56° or 75% R.H.

There is no evidence that stearic acid USP produces any less discoloration than magnesium stearate USP under these conditions. Control samples, which had been stored in a cool, dark environment (in closed containers) for a 12-week period, showed the same degree of discoloration as the test samples. Unlubricated control samples showed no color change after a 12-week storage period in closed containers. This indicates that the lubricants alone are capable of causing discoloration.

It was found that the incorporation of amines, such as *d*-amphetamine sulfate USP or phenylephrine hydrochloride USP, in the formulations both accentuated and accelerated the degree of darkening produced under all test conditions. In the presence of amines, magnesium stearate USP showed a greater tendency than stearic acid USP toward producing discolored sugar tablets.

Of the four materials tested for direct compression, only anhydrous lactose USP was able to withstand adequately the effects

[4] Strong-Cobb Arner Co.
[5] Newark Wire Cloth Co., Newark, N. J.

Table II—Sorption of Water Vapor in Humidity Chambers

Sample	Initial Moisture, %	% Moisture Present[a] 11	31	51	75	84	93	100
Lactose USP (beadlets)	0.22	0.22	0.22	0.22	0.22	0.22	1.0	17.0
Spray-dried lactose USP	0.20	0.50	0.50	1.0	1.0	1.0	1.5	21.5
Anhydrous lactose USP	0.24	0.24	0.24	0.24	1.0	1.5	3.0	27.0
Dextrose (PAF 2011)	8.50	9.0	9.0	9.5	10.5	27.0	60.0	76.0
Lactose USP	0.16	0.16	0.16	0.16	0.16	0.16	0.16	17.5

[a] After exposure to specified humidity for 7 days at 25° in open containers.

Table III—Color Stability of Sugar Tablets after Exposure to Different Storage Conditions in Open Containers

Sugar	Lubricant	Degree of Discoloration (Visual Observation) 56° for 10 Days	75% R.H. R.T. for 7 Days	600 fc. for 12 Weeks
Lactose USP (beadlets)	Mg stearate	Slight	Slight	Slight
	Stearic acid	Slight	Slight	Slight
Spray-dried lactose USP	Mg stearate	Slight	Slight	Slight
	Stearic acid	Slight	Slight	Slight
Anhydrous lactose USP	Mg stearate	None	None	None
	Stearic acid	None	None	None
Dextrose (PAF 2011)	Mg stearate	Slight	Slight	None
	Stearic acid	Slight	Slight	None
Lactose USP	Mg stearate	None	Slight	None
	Stearic acid	None	Slight	None

of high temperature, humidity, and exposure to light. These findings agree with those of Batuyios (21). Neither lactose USP (beadlets) nor dextrose (PAF 2011) represents a substantial improvement over spray-dried lactose USP with regard to overall physical and color stability.

Compression Characteristics—The compression characteristics of the four sugars for direct compression were defined with the aid of a rotary tablet machine instrumented with strain gauges. Compression and ejection forces were monitored continuously and correlated with the tablet parameters of hardness, friability, tablet-to-tablet weight variation, and disintegration time.

Figure 2, illustrating waveforms resulting from compression and ejection events, is included for comparative purposes. The top tracing is a series of typical compression responses, the mean value for which was about 1200 lb. force (each major division is equivalent to 786 lb.). The lower tracing represents the response obtained from ejection-force measurements using an ejection cam instrumented with metal foil strain gauges (10). A mean value of about 22 lb. force was obtained in this instance (each major division is equivalent to 12.5 lb.).

Tablets were prepared from each of the four materials for direct compression at two levels of pressure: a "normal" pressure level (approximately 2200 lb.) to simulate typical production conditions and a "high" pressure level (approximately 4100 lb.) to encourage compressional difficulties. The physical specifications of the tablets prepared are listed in Table IV.

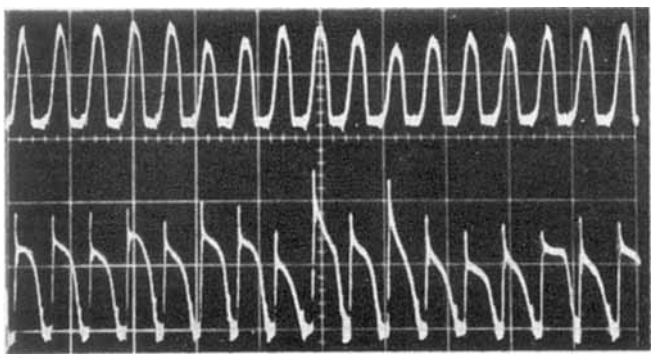


Figure 2—*Oscilloscopic tracing of force waveforms obtained from Stokes RBB2 tablet press. See text for explanation.*

After careful examination of the compression curves and evaluation of the physical properties of the tablets, those prepared from dextrose (PAF 2011) were found to be the hardest and least friable of the four agents tested. At "normal" pressure and speed, tablets prepared from spray-dried lactose USP began capping after 6 hr. This was not the case with the other three materials. Figure 3A shows a waveform resulting from the ejection of spray-dried lactose USP tablets 15 min. after commencement of operation. Figure 3B is a similar photograph taken 6 hr. later. The twin peak effect is indicative of capping insofar as the materials tested in this study are concerned. The initial peak is representative of the force required to overcome die wall–tablet adhesion. Under ordinary conditions, this force is rapidly dissipated as the tablet emerges from the die. However, when capping and/or lamination occur, the rapid expansion of the tablet results in an increase in force which manifests itself as the second peak in the ejection waveform. The possibility that these second peaks were the result of buildup of material in the dies or on the lower punch tips was minimized because both these sites were checked for cleanliness at regular intervals throughout the run.

At the higher pressure, tablets prepared from anhydrous lactose USP began capping as depicted by the ejection waveforms shown in Fig. 4. The secondary peaks in this figure are not as pronounced as those in Fig. 3B. However, when one compares the waveforms in Fig. 4 with those seen in a typical noncapping situation (Fig. 2), the secondary peak becomes more apparent. A secondary peak of this type indicates a latent tendency toward capping; the tablets do not cap as they come off the machine but cap during handling

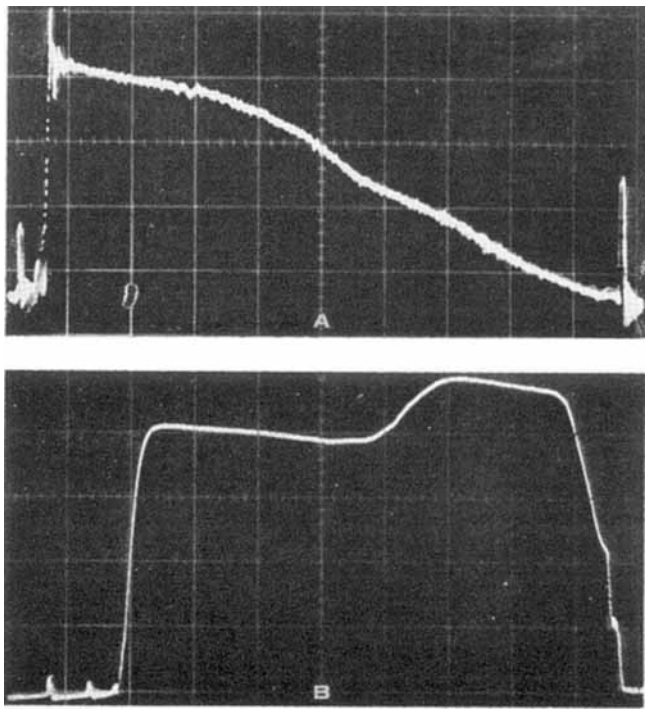


Figure 3—*Oscilloscopic tracing resulting from the ejection of spray-dried lactose USP tablets; each large division represents 6.27 lb. Key: A, no capping; and B, capping.*

Table IV—Compression Specifications of Four Sugars for Direct Tablet Compression[a]

Materials	Compression Force, lb.[b]	Weight, g.[c]	Thickness, cm. (in.)[c]	Hardness[c]	Friability (% Loss)[d]	No. of Capped Tablets
Lactose USP (beadlets)	2230	0.200	0.337 (0.133)	11.3	1.2	0
	4108	0.198	0.314 (0.124)	15.8	11.8	1
Spray-dried lactose USP	2219	0.201	0.337 (0.133)	7.0	1.8	0
	4083	0.201	0.314 (0.124)	14.0	60.9	5
Anhydrous lactose USP	2231	0.204	0.337 (0.133)	8.1	1.6	0
	3974	0.203	0.314 (0.124)	16.2	21.9	2
Dextrose (PAF 2011)	2153	0.201	0.337 (0.133)	12.3	0.7	0
	4120	0.201	0.314 (0.124)	19.8	1.0	0

[a] All tablets lubricated with 0.5% each magnesium stearate USP and stearic acid USP. [b] Average of 20 consecutive events. [c] Average of 10 readings. [d] Capped tablets taken as 100% loss.

and packaging. Tablets prepared from lactose USP (beadlets) also exhibited, to a lesser degree, a latent tendency toward capping at high pressure when run with standard concave punches.

It was decided to subject the two best performers [lactose USP (beadlets) and dextrose (PAF 2011)] to a more severe test. Each material was run on the same tablet machine at 1200 tablets/min., using extra deep concave (modified ball) tooling at the high pressure level. Under these conditions, pronounced capping was evident in the case of the lactose USP (beadlets). In contrast, tablets prepared from dextrose (PAF 2011) did not cap under these same test conditions (Fig. 5).

Weight Variation—Extremely close weight tolerances were obtained with all agents except anhydrous lactose USP. To determine the magnitude of tablet-to-tablet weight variation, each material was run for 1 hr. on the instrumented RBB2 tablet press. The presence of a blank station in the die table served to index the compression forces at each individual station. It was, therefore, possible to determine if fluctuations in compression force levels were due to tablet-to-tablet weight variation or to tooling variation (*i.e.*, the combined length of the upper and lower punches was either more or less than the norm for the particular set of tooling). Figure 6 shows a series of waveforms resulting from the compression of dextrose (PAF 2011) in which a pattern of variable peak heights is evident. Since this pattern recurs in successive revolutions, one may assume that it is due to tooling variation. Hence, the dextrose (PAF 2011) exhibited remarkably close weight tolerances. In comparison, the waveforms for anhydrous lactose USP depicted in Fig. 7 showed a considerable degree of fluctuating peak heights. Since the pattern obtained in this case does not recur at regular intervals, the force variations cannot be attributed to tooling variations.

Samples were taken from the machine at 10-min. intervals. Twenty tablets were selected, at random, from these samples and weighed individually on a semimicro balance. The statistical data obtained are tabulated in Table V.

Table V—Weight Variation of Four Sugar Tablets Prepared by Direct Compression

Sample	Mean Weight[a]	Standard Deviation
Lactose USP (beadlets)	201.94	±0.7466
Spray-dried lactose USP	197.77	±0.9177
Anhydrous lactose USP	199.41	±1.4949[b]
Dextrose (PAF 2011)	195.63	±0.9652

[a] Average of 20 tablets and expressed in mg. [b] $p = 0.0165$. Significant at 95% confidence limits.

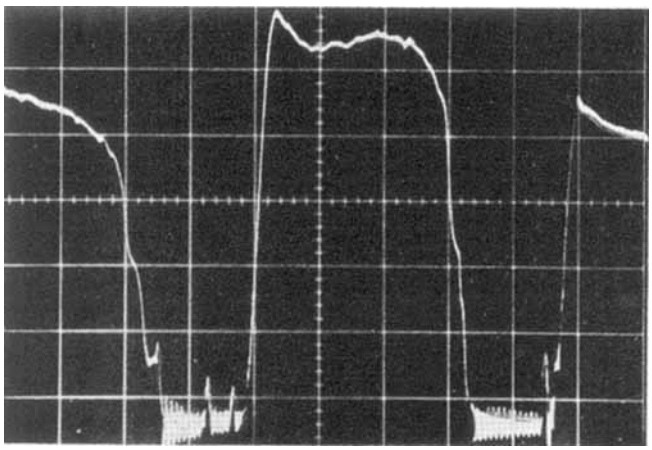


Figure 4—*Oscilloscopic tracing resulting from the ejection of anhydrous lactose USP tablets; each large division represents 12.5 lb.*

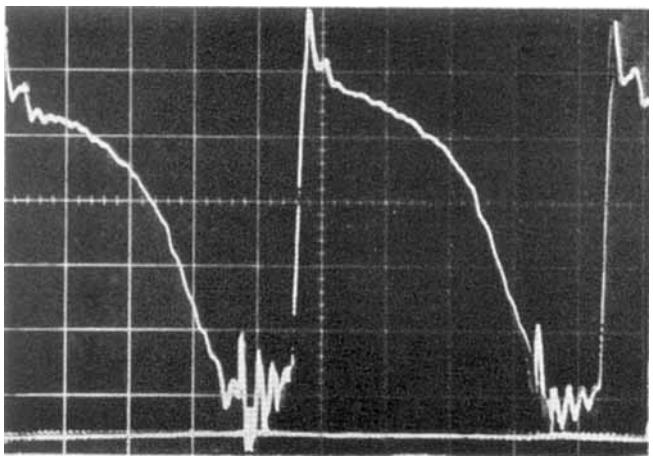


Figure 5—*Oscilloscopic tracing resulting from the ejection of dextrose (PAF 2011) tablets; each large division represents 6.27 lb.*

Disintegration—To obtain disintegration information, samples were prepared from each of the four agents for direct compression according to the following formula:

Ingredients	g./Tablet
Material for direct compression	0.190
Starch USP	0.010
Magnesium stearate USP	0.001
Stearic acid USP	0.001
	0.202

Tablets were compressed on the instrumented RBB2 tablet

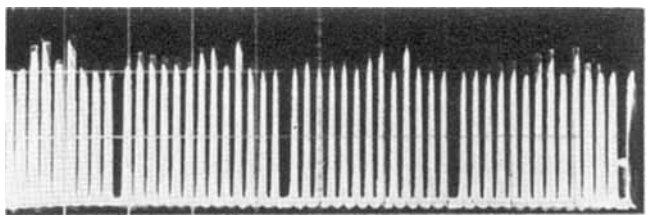


Figure 6—*Oscilloscopic tracing resulting from the compression of dextrose (PAF 2011); each large division represents 786 lb.*

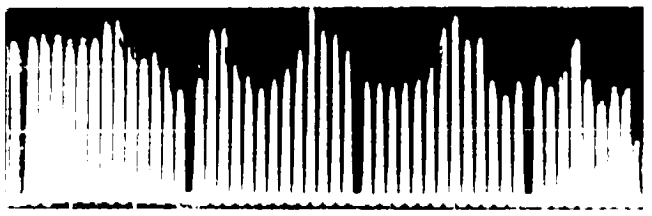


Figure 7—*Oscilloscopic tracing resulting from the compression of anhydrous lactose USP; each large division represents 786 lb.*

machine. The uniformity of the compression force, weight, and tablet thickness was maintained within narrow limits. Disintegration times, as determined by the official USP method (12), are recorded in Table VI.

Although all samples had disintegration times of less than 9 min., those prepared from dextrose (PAF 2011) exhibited the shortest and most uniform times. This observation could well have been predicted in view of the difference in solubility between dextrose and lactose.

SUMMARY AND CONCLUSIONS

Lactose USP (beadlets) and dextrose (PAF 2011) have been evaluated and compared with two commonly used agents for direct compression, namely, spray-dried lactose USP and anhydrous lactose USP, as to their relative physical properties and stability for tableting by direct compression.

The physical properties, *i.e.*, bulk density, angle of repose, and particle-size distribution, of all four agents were determined. The effects of heat, moisture, and light, as well as the presence of selected amines and lubricants, on the physical properties and color stability of tablets prepared from the materials were compared with a lactose USP control. The compression characteristics of all four materials were defined with the aid of an instrumented rotary tablet machine. Compression and ejection forces were monitored continuously and correlated with tablet hardness, friability, weight variation, and disintegration time.

Both dextrose (PAF 2011) and lactose USP (beadlets) exhibited excellent flow characteristics, as evidenced by minimal tablet-to-tablet weight variation. The hygroscopic nature of the dextrose (PAF 2011) detracts considerably from its overall physical stability. Neither new agent represented a significant improvement over the older materials with regard to color stability. Of the four agents tested, only anhydrous lactose USP was able to withstand adequately the exposure to heat, light, and moisture.

The compressional characteristics of dextrose (PAF 2011) were superior to those of the lactose sugars. When identical formulations were tested, dextrose (PAF 2011) produced the hardest and least friable tablets. The compression characteristics of lactose USP (beadlets) were superior to those of spray-dried lactose USP and anhydrous lactose USP.

It is felt that both dextrose (PAF 2011) and lactose USP (beadlets) warrant serious consideration for use as agents for direct compression in the pharmaceutical industry.

Table VI—Disintegration Time for Four Sugar Tablets Prepared by Direct Compression

Sample	Weight, g.[a]	Thickness, cm. (in.)[a]	Compression Force, lb.[b]	Disintegration Time, sec. With Disks[c]	Disintegration Time, sec. Without Disks[c]
Lactose USP (beadlets)	0.202	0.337 (0.133)	2257	310	440
Spray-dried lactose USP	0.198	0.235 (0.132)	2201	190	320
Anhydrous lactose USP	0.199	0.337 (0.133)	2237	400	510
Dextrose (PAF 2011)	0.196	0.235 (0.132)	2187	130	160

[a] Average of 10 tablets. [b] Average of 10 consecutive events. [c] Average of 12 tablets.

REFERENCES

(1) G. Milosovich, *Drug Cosmet. Ind.*, **92**, 557(1963).

(2) M. W. Scott, H. A. Lieberman, D. Maher, and F. Chow, paper presented to the Eastern Regional Meeting, Industrial Pharmacy Section, APhA, 1961.

(3) C. D. Fox, M. D. Richman, G. E. Reier, and R. Shangraw, *Drug Cosmet. Ind.*, **92**, 161(1963).

(4) J. Jaffe and N. E. Foss, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 26(1959).

(5) A. M. Raff, M. J. Robinson, and E. V. Svedres, *J. Pharm. Sci.*, **50**, 76(1961).

(6) K. P. Perlman, *Diss. Abstr.*, **23**, 603(1962).

(7) R. N. Duvall, K. T. Koshy, and R. E. Dashiell, *J. Pharm. Sci.*, **54**, 1196(1965).

(8) G. M. Kovac, paper presented to the Industrial Pharmacy Section, APhA, Las Vegas, Nev., 1962.

(9) G. E. Reier, Ph.D. thesis, University of Maryland, College Park, Md., 1964.

(10) P. E. Wray, J. G. Vincent, F. W. Moller, and G. J. Jackson, paper presented at annual meeting, APhA, Dallas, Texas, 1966.

(11) E. G. E. Shafer, E. G. Wollish, and C. E. Engel, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 114(1956).

(12) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 919–920.

(13) A. Q. Butler and J. C. Ramsey, *Drug. Stand.*, **20**, 222(1952).

(14) D. Train, *J. Pharm. Pharmacol.*, **10**, 127T(1958).

(15) L. Lachman, C. J. Swartz, and J. Cooper, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 213(1960).

(16) A. C. Todd, paper presented at Particle Characteristics Conference, Union, N. J., 1968.

(17) B. S. Neuman, in "Flow Properties of Disperse Systems," J. J. Hermans, Ed., Interscience, New York, N. Y., 1953, chap. 10.

(18) R. A. Castello and A. M. Mattocks, *J. Pharm. Sci.*, **51**, 106(1962).

(19) W. C. Gunsel and L. Lachman, *ibid.*, **52**, 178(1963).

(20) C. A. Brownley and L. Lachman, *ibid.*, **53**, 452(1964).

(21) N. H. Batuyios, *ibid.*, **55**, 727(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 12, 1969, from the *Pharmaceutical Product Development Department, Lederle Laboratories, Pearl River, NY 10965*

Accepted for publication April 10, 1970.

The authors thank J. Callahan and R. A. Nash for their valuable contributions to this work.

Permeability of Double-Layer Films I

TSUNETO KURIYAMA, MICHIHARU NOBUTOKI, and MICHIO NAKANISHI

Abstract □ To make a pharmaceutical preparation that contains a water-unstable ingredient, it is preferable that the preparation be coated with a less permeable film. When rapid disintegration is required, however, the coating film should be made as thin as possible. The present study was carried out on the permeability of the double-layer film in order to create a thin film with sufficient protectivity from moisture. It was found that the permeability of most double-layer films had a directional property and could be made thinner than single films. This finding suggests the potential usefulness in the manufacturing process of pharmaceutical preparations.

Keyphrases □ Films, double layer—permeability □ Permeation, water vapor—double-layer films □ Solvent evaporation rate—effect on film

Coating is an important and basic technique which is widely used in the field of pharmaceutics. Film coating of tablets is a typical example. One important purpose of coating is to prevent moisture from permeating into preparations, because decomposition of the ingredient is often attributable to hydrolysis (and oxidation). For protection from moisture, it is of primary importance to select a proper coating agent. Several kinds of coating agents are available, but the selection is not always easy because a coating agent should fulfill various incidental requirements. A coating agent should be soluble in the gastric or intestinal fluids as desired, while its solvents should not react with the ingredients and its water vapor permeability should be as low as possible. The water vapor permeability of a film may be reduced by making the film thicker, but such films may prolong the time of tablet disintegration in the digestive fluids. Therefore, the thickness of the coating film is limited. It may be possible to create a more useful coating film by combining currently available agents. For this purpose, two methods may be feasible: making a film from a mixed solution or combining single films to make a multilayer film. The authors studied the latter and found that the multilayer film had very interesting and useful properties which were worthy of reporting.

To study quantitatively the water vapor permeability of a coating film, it may be advisable to perform an experiment on a simple system such as the free film rather than on a complicated system such as the film-coated tablet. Many reports are available concerning the permeability of a single film (1–5). In this report, the water vapor permeability was measured on the double-layer film as well as its constituent single films in a manner similar to that described by the former reporters.

EXPERIMENTAL

Cell for Measuring Water Vapor Permeability—A cell was assembled according to the method of Patel *et al.* (1). The cell was filled with distilled water and horizontally sealed with a sample film. The initial weight of the cell was accurately measured after 1 hr. of thermal equilibration at a given temperature and humidity environment (generated by the Tabai Lucifer model TL-21P). The cell was again weighed after 20 hr. at the same conditions and the decrease of the weight was calculated. In this experiment, it was necessary to maintain a constant distance between the sample film and the surface of water in the cell to avoid inconsistencies in the data obtained. The shortest possible distance was advisable (6), but for practical reasons the authors used a distance of 1.5 cm.

Film Preparation—(*a*) *Single Films*—The coating agents were dissolved in suitable solvents, and the film solutions were then poured on horizontal glass plates equipped with a holding frame around the perimeter. The solvent was allowed to evaporate gradually. In this process, the rate of evaporation was controlled by setting a cover which limited the diffusion of solvent vapor. The thickness of the film was adjusted by changing the concentration (5–15%) and the quantity of the solution poured on the glass plate. Free films were peeled from the plates. Each free film was cut into circles 4 cm. in diameter to make the sample films. These samples were dried in a vacuum for 12 hr. to remove residual solvent.

(*b*) *Double-Layer Films*—It was necessary to use a double-layer free film which contained no air layer between, because an actual multilayer-coating film on the tablet contains no air layer. Two single films, which had the same thickness but were of different types, were selected from the prepared films and combined to form a double-layer film by using a solvent which dissolved only a surface of either film.

Measurement of Thickness of Film—The thickness of a single film produced in the manner described was measured with a dial gauge (precision: 0.001 mm.). The thickness of a double-layer film was taken as a total of the thickness of each single film.

Materials—Coating agents used are shown in Table I.

Measurement of Water Vapor Permeability—Several films were selected to distribute properly the thickness of each film in the range of 0.05–0.4 mm., and their water vapor permeability was measured. Permeability is generally described as follows (8):

$$q = PAt(p_1 - p_2)/l \qquad \text{(Eq. 1)}$$

Quantity (q) of water vapor, which passes through a given film at a given temperature, depends upon area (A), thickness (l), vapor pressure difference ($\Delta p = p_1 - p_2$), and time (t). The proportional coefficient (P) was termed the permeability coefficient. Permeability (Q), which is defined in Eq. 2, was obtained at a given condition:

$$Q = q/At \quad \text{(g./m.}^2\text{ 24 hr.)} \qquad \text{(Eq. 2)}$$

Comparison was made on Q-values for various samples in terms of 0.3-mm. film thicknesses. It was, of course, very hard to produce a sample film of the exact thickness intended, and Q-value depended upon the thickness (l) of a film. But because the relationship between $1/Q$ and l was linear, Q for exactly 0.3-mm. thickness was obtained graphically.

RESULTS AND DISCUSSION

In the case of preparing sample films, the rate of evaporation of the solvent was a problem. Too rapid evaporation produced turbidity in the film, and the water vapor permeability of the film depended upon such turbidity, producing inconsistencies in data. The rate of evaporation was controlled to make the films transparent. All results reported were obtained from transparent sample films.

The relationship between $1/Q$ and l should be a straight line passing through the origin according to Eq. 1. But this experiment revealed that the line did not pass through the origin but intersected

Table I—Abbreviations and Solvents of Coating Agents Used

Abbreviation	Chemical Name	Manufacturer (Specification)	Solvent Used for Making Film
EC	Ethylcellulose	Dow Corning (50 cps.)	Ethyl acetate
MC	Methylcellulose	Shin-etsu Chemical Co. (25 cps.)	Methylene chloride + methyl alcohol (1:1)
AC	Cellulose acetate	Wako Pure Chemical Co.	Acetone
CAB	Cellulose acetate butylate	Eastman Organic Chemicals Co.	Acetone
SH	Shellac	Gifu Shellac Manuf.	Methyl alcohol
PVA	Polyvinyl alcohol	Hayashi Pure Chemicals (500)	Water
HPC	Hydroxypropylcellulose	Freunt Ind.	Methyl alcohol + chloroform (1:1)
AEA	Polyvinyl acetate diethylamino acetal	Sankyo Co.	Acetone
HECAP	Hydroxyethylcellulose acetate phthalate	Yoshitomi Pharm. Ind. (7)	Acetone
CC	Cellulose acetate phthalate-2-diethylaminoethylester	Wako Pure Chemicals	Methyl alcohol
CAP	Cellulose acetate phthalate	Wako Pure Chemicals	Acetone

the ordinate ($1/Q$-axis), indicating that the permeability of coating film does not perfectly agree with the classical theory (Eq. 1).

This fact has been reported also by Patel *et al.* (1), who pointed out that the linear plots resulted for various samples extrapolated to the same point on the abscissa (l-axis). However, the latter was not always supported experimentally, as shown in Fig. 1, as Patel himself suspected contingency. In the case of double-layer films also, the relationships between $1/Q$ and l were linear, just as in Fig. 1.

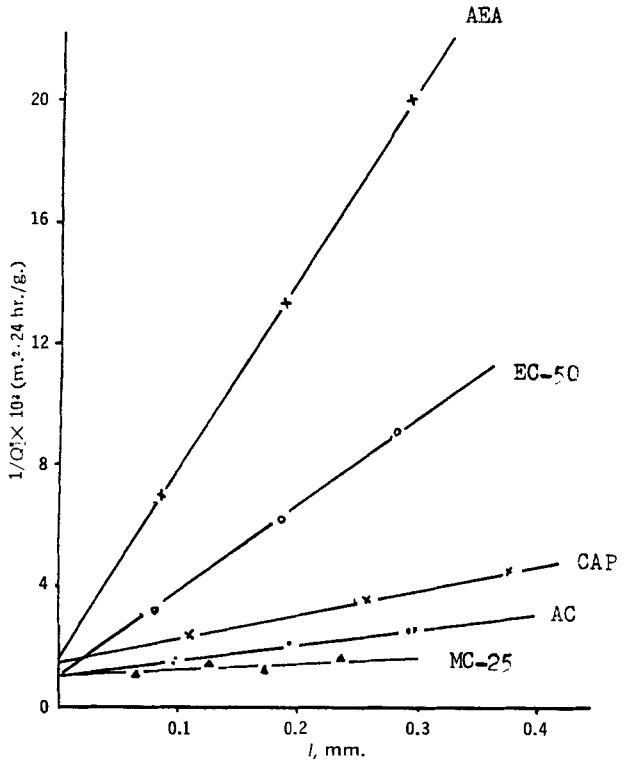


Figure 1—*The relationship between the resistance for permeation* (1/Q) *and the film thickness* (l). *Temperature, 40°; water vapor pressure of higher pressure side* p_1, *5.53 cm. Hg (100% R.H.); water vapor pressure of lower pressure side* p_2, *4.20 cm. Hg (76% R.H.).*

Table II shows the results obtained. "Types" in the table are defined in Table III.

Table II shows the permeability for double-layer films as well as that for single-layer films, which are the components of the double-layer films. Differences in permeability were noted even in the same double-layer film, depending upon which layer was set on the side of higher humidity or depending upon the direction of permeation. This property is called "two-sidedness" by Rogers *et al.* (9, 10). In the present report, the double-layer film is expressed as $X + Y$ (or $Y + X$), which means that the film is made from material X and material Y, and X-layer (or Y-layer)—written before the + mark—is set on the side of higher humidity, and Y-layer (or X-layer)—written after the + mark—is set on the side of lower humidity. In Table III, A, B, C, and D are defined as follows: when X is more permeable than Y, A refers to the permeability of X and B refers to that of Y; C refers to the permeability observed for $Y + X$, and D to the permeability for $X + Y$. Therefore, A is always larger than B, and C or D is usually between A and B.

It was found, however, that D can be greater than A or that C can be smaller than B in some cases. In Table III, all the possible orders of A, B, C, and D are listed; they are classified into four groups. Twelve orders of A, B, C, and D are called by "types" in this report. Table III also shows the number of cases falling under each "type." Rogers *et al.* (9) and Ninneman and Simerl (10) measured the permeability of double-layer films used for packaging and observed the "two-sidedness." "Two-sidedness" is expressed as C $\neq$ D, according to the definition already mentioned. This property, which was confirmed in the present experiment on coating films, may be useful in pharmaceutics. More concretely, moisture should be minimized in a tablet which contains a water-unstable ingredient; in this sense, it will be practically useful to utilize the "two-sidedness" of the double-layer film for protecting the tablet from moisture. If coating layers are properly combined, the film-coated tablet can be dried easily even at a mild condition and, at the same time, the tablet will absorb little moisture. In addition, Table III indicates a much greater merit of double-layer film coating. Permeability of the double-layer film and that of each layer were compared on the same unit thickness basis in the present experiment. It revealed the

Table II—Water Vapor Permeability[a] of Double-Layer Film and Its Elemental Single Films

Double-Layer Film Combinations	Q^b	Single Film Name	Q^b	Type[c]	Double-Layer Film Combinations	Q^b	Single Film Name	Q^b	Type[c]
EC-50 + HECAP[d]	102	HECAP	123	2	CAB + MC-25	194	MC-25	625	8
HECAP + EC-50[e]	150	EC-50	104		MC-25 + CAB	262	CAB	109	
CAB + HECAP	92	HECAP	123	2	CC + PVA	526	PVA	667	2
HECAP + CAB	164	CAB	109		PVA + CC	714	CC	625	
AEA + HECAP	74	HECAP	123	8	AC + HECAP	168	HECAP	123	8
HECAP + AEA	87	AEA	49		HECAP + AC	161	AC	385	
CAP + AEA	132	CAP	263	8	CAP + MC-25	260	MC-25	625	10
AEA + CAP	80	AEA	49		MC-25 + CAP	340	CAP	263	
CAB + AEA	62	CAB	109	7	HECAP + CAP	136	CAP	263	8
AEA + CAB	97	AEA	49		CAP + HECAP	139	HECAP	123	
EC-50 + PVA	168	EC-50	104	8	AEA + MC-25	87	MC-25	625	8
PVA + EC-50	225	PVA	667		MC-25 + AEA	92	AEA	49	
AEA + PVA	69	PVA	667	8	AEA + AC	122	AC	385	8
PVA + AEA	142	AEA	49		AC + AEA	125	AEA	49	
CAB + PVA	190	PVA	667	8	EC-50 + CAB	112	CAB	109	11
PVA + CAB	255	CAB	109		CAB + EC-50	106	EC-50	104	
CAB + AC	204	AC	385	8	CC + MC-25	676	MC-25	625	11
AC + CAB	236	CAB	109		MC-25 + CC	625	CC	625	
CAP + PVA	296	PVA	667	8	CC + HPC	526	HPC	556	9
PVA + CAP	350	CAP	263		HPC + CC	625	CC	625	
EC-50 + AEA	75	AEA	49	7	CAB + CAP	141	CAP	263	8
AEA + EC-50	90	EC-50	104		CAP + CAB	167	CAB	109	
AC + MC-25	427	AC	385	8	AC + EC-50	213	EC-50	104	8
MC-25 + AC	556	MC-25	625		EC-50 + AC	191	AC	385	
MC-25 + PVA	459	PVA	667	10	SH + EC-50	15	EC-50	104	8
PVA + MC-25	625	MC-25	625		EC-50 + SH	19	SH	8	
HECAP + PVA	212	PVA	667	8	MC-25 + EC-50	244	EC-50	104	8
PVA + HECAP	238	HECAP	123		EC-50 + MC-25	192	MC-25	625	
AC + PVA	485	PVA	667	8	CAP + EC-50	179	EC-50	104	8
PVA + AC	649	AC	385		EC-50 + CAP	143	CAP	263	

[a] Measured under the following conditions: temperature, 40°; vapor pressure at higher pressure side p_1, 55.3 mm. Hg (100% R.H. at 40°); vapor pressure at lower pressure side p_2, 42.0 mm. Hg (76% R.H. at 40°). Every double-layer film is composed of two kinds of single films which have the same thickness. [b] Dimension of permeability Q; g./m.^{2} 24 hr., value obtained for 0.3-mm. thickness both of double-layer and single films. [c] Defined in Table III. [d] HECAP was set at the lower pressure side. [e] EC-50 was set at the lower pressure side.

presence of a particularly interesting effect of the "two-sidedness," as shown in Table III. For example (HECAP + EC-50, HECAP + CAB, CC + PVA), some double-layer films made from the materials X and Y showed greater permeability than that of either X or Y film when water vapor permeated from one direction and, on the contrary, their permeability was smaller than that of either X or Y film when water vapor permeated from the other direction (Group α). This means that some double-layer films can accomplish effective protection from moisture when the combined thickness of the layers is less than that of a single film. A coating film on a tablet should be less permeable as well as thinner, because it should effectively protect the ingredients from moisture and be disintegrated quickly in the digestive fluid. These two requirements generally disagree with each other, but the effect of the "two-sidedness" (Group α) will be a great help to the reconciliation of these antinomous requirements. The incidence of the Group γ is naturally the highest. No case classifiable into Group β was found, as was assumed theoretically. Examples of the Groups α and δ are actually observed.

Table III—Classification of Permeability of Double-Layer Films

Type	Permeability[a] Larger	← →		Smaller	Cases	Group
1	C[a]	A[a]	B[a]	D[a]	0	
2	D	A	B	C	3	α
3	A	B	C	D	0	
4	A	B	D	C	0	
5	C	D	A	B	0	β
6	D	C	A	B	0	
7	A	C	D	B	2	
8	A	D	C	B	20	γ
9	A	C	B	D	1	
10	A	D	B	C	2	
11	C	A	D	B	2	δ
12	D	A	C	B	0	

[a] A, permeability of more permeable single film; B, permeability of less permeable single film; C, permeability of double-layer film when its more permeable layer was set on the lower vapor pressure side; D, permeability of double-layer film when its more permeable layer was set on the higher vapor pressure side.

SUMMARY

Water vapor permeability of the coating film, single- and double-layer films, was measured at a certain condition. It was revealed that plotting the relationship between the resistance for permeation ($1/Q$) and the film thickness (l) gave a straight line, but the line did not intersect the $1/Q$-axis at the origin, indicating that the permeability of coating film does not perfectly agree with the classical theory. The permeability of the double-layer film varies, depending upon the direction of permeation (two-sidedness). The "two-sidedness" was classified into some groups according to the relationship of permeability of each single film and that of the composite film. The most remarkable characteristic was observed in the Group α, which was interpreted to mean that the permeability of the double-layer film from one side and that from the other side are larger or/and smaller than that of any of its elemental single films of the same thickness. This fact is very important and may be useful from the viewpoint of pharmaceutical technology.

REFERENCES

(1) M. Patel, J. M. Patel, and A. P. Lemberger, *J. Pharm. Sci.*, **53**, 286(1964).

(2) T. Higuchi and A. Aguiar, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 574(1959).
(3) J. Kanig and H. Goodman, *J. Pharm. Sci.*, **51**, 77(1962).
(4) B. J. Munden, H. G. DeKay, and G. S. Banker, *ibid.*, **53**, 395(1964).
(5) L. Lachman and A. Drubulis, *ibid.*, **53**, 639(1964).
(6) B. Takeda, *Nippon Kagaku Zasshi*, **74**, 207(1953).
(7) I. Sako, Jap. pat. 438595.
(8) A.S.T.M. D-697-42-T, R. M. Barrer, *Trans. Faraday Soc.*, **35**, 628(1939).
(9) C. E. Rogers, V. Stannet, and N. Szwarc, *Ind. Eng. Chem.*, **49**, 1933(1957).
(10) K. W. Ninneman and L. E. Simerl, *Pulp Pap. Mag. Can.*, **63**, T68(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 31, 1969, from the *Research Laboratories of Yoshitomi Pharmaceutical Industries, Ltd., Yoshitomi-Chō, Chikujo-Gun, Fukuoka-Prefecture, Japan.*

Accepted for publication December 29, 1969.

Abstracted from a thesis submitted by T. Kuriyama to the Graduate School, Kyushu University, in partial fulfillment of Doctor of Philosophy degree requirements.

Thanks are expressed to Professor S. Iguchi for his interest in the work and useful discussions. The authors also wish to thank Mr. M. Tasaka, Executive Director, Yoshitomi Pharmaceutical Industries, Ltd., for his encouragement and his kind permission to publish this paper.

NOTES

Permeability of Double-Layer Films II

TSUNETO KURIYAMA, MICHIHARU NOBUTOKI, and MICHIO NAKANISHI

Abstract □ Most of the combinations of coating films showed "two-sided" permeability, while this property has not been reported for films composed of hydrophobic layers only. Any coating film is more or less hydrophilic, and its water vapor permeability varies according to the mean humidity condition in which the film is placed. It was found that double-layer films with "two-sidedness" have characteristic relations (as classified into Groups α, β, γ, and δ) between specificity of permeability and humidity range.

Keyphrases □ Permeability, two-sided—double-layer films □ Double-layer films—permeability, relationship of water vapor, humidity

In the first part of this study, the authors reported the so-called "two-sided" directional property of the water vapor permeability of double-layer films, and classified this property into four groups: α, β, γ, and δ (1). In double-layer films with two-sided water vapor permeability of the Group α, the permeability is either smaller or greater than that of either component layer, depending on the direction of permeation. The latter feature is a phenomenon that cannot be elucidated in a simple manner. Ninneman and Simerl (2) studied the permeability of multilayer films and introduced the following equation:

$$\Delta p = Q \sum_{i=1}^{n} \frac{l_i}{P_i} \qquad \text{(Eq. 1)}$$

where Δp stands for the difference in water vapor pressure between the higher humidity atmosphere and lower humidity atmosphere (*i.e.*, $p_1 - p_2$), Q is the permeability of an n-layer film, l_i is the thickness of the ith layer in the n-layer film, and P_i is the permeability coefficient of the ith layer. This equation may estimate the permeability of the multilayer film at a certain condition, where the material and the thickness of each component layer are given. It cannot explain the two-sided property of the multilayer film, however, because the permeability thus calculated is independent of the permutation of component layers of a film. In other words, Eq. 1 is practically applicable to the hydrophobic film only but not to the coating film, which is more or less of a hydrophilic nature.

Equation 3 is derived from Eq. 2 (1), which is known to be applicable to the hydrophobic film.

$$q = P \frac{p_1 - p_2}{l} At \qquad \text{(Eq. 2)}$$

$$\frac{1}{Q} = \frac{At}{q} = \frac{l}{P \Delta p} \qquad \text{(Eq. 3)}$$

where A is the area of film through which water vapor is permeated, t is the time of permeation, and q is the quantity of moisture permeated. But Eq. 3 does not always apply to the coating (hydrophilic) film. While $1/Q$ should be proportional to l according to Eq. 3, previously reported experiments (1) revealed that the value of $1/Q$ for $l=0$ did not come to zero in spite of the linearity of the relationship between $1/Q$ and l. This finding suggests that the permeability of the coating film is different from that of the usual hydrophobic film and its behavior is rather complex. The two-sided property of the coating film may be attributed to these very facts.

Takeda (3), studying the permeability of cellulose films, introduced the following equation:

$$\frac{1}{Q} = \frac{l}{P \Delta p} + \frac{2}{k \Delta p} \qquad \text{(Eq. 4)}$$

where k is a coefficient which indicates how easily water molecules drive into the film from the higher humidity atmosphere. This equation explains that the relationship between $1/Q$ and l is not proportional;

(2) T. Higuchi and A. Aguiar, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 574(1959).
(3) J. Kanig and H. Goodman, *J. Pharm. Sci.*, **51**, 77(1962).
(4) B. J. Munden, H. G. DeKay, and G. S. Banker, *ibid.*, **53**, 395(1964).
(5) L. Lachman and A. Drubulis, *ibid.*, **53**, 639(1964).
(6) B. Takeda, *Nippon Kagaku Zasshi*, **74**, 207(1953).
(7) I. Sako, Jap. pat. 438595.
(8) A.S.T.M. D-697-42-T, R. M. Barrer, *Trans. Faraday Soc.*, **35**, 628(1939).
(9) C. E. Rogers, V. Stannet, and N. Szwarc, *Ind. Eng. Chem.*, **49**, 1933(1957).
(10) K. W. Ninneman and L. E. Simerl, *Pulp Pap. Mag. Can.*, **63**, T68(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 31, 1969, from the *Research Laboratories of Yoshitomi Pharmaceutical Industries, Ltd., Yoshitomi-Chō, Chikujo-Gun, Fukuoka-Prefecture, Japan.*

Accepted for publication December 29, 1969.

Abstracted from a thesis submitted by T. Kuriyama to the Graduate School, Kyushu University, in partial fulfillment of Doctor of Philosophy degree requirements.

Thanks are expressed to Professor S. Iguchi for his interest in the work and useful discussions. The authors also wish to thank Mr. M. Tasaka, Executive Director, Yoshitomi Pharmaceutical Industries, Ltd., for his encouragement and his kind permission to publish this paper.

NOTES

Permeability of Double-Layer Films II

TSUNETO KURIYAMA, MICHIHARU NOBUTOKI, and MICHIO NAKANISHI

Abstract □ Most of the combinations of coating films showed "two-sided" permeability, while this property has not been reported for films composed of hydrophobic layers only. Any coating film is more or less hydrophilic, and its water vapor permeability varies according to the mean humidity condition in which the film is placed. It was found that double-layer films with "two-sidedness" have characteristic relations (as classified into Groups α, β, γ, and δ) between specificity of permeability and humidity range.

Keyphrases □ Permeability, two-sided—double-layer films □ Double-layer films—permeability, relationship of water vapor, humidity

In the first part of this study, the authors reported the so-called "two-sided" directional property of the water vapor permeability of double-layer films, and classified this property into four groups: α, β, γ, and δ (1). In double-layer films with two-sided water vapor permeability of the Group α, the permeability is either smaller or greater than that of either component layer, depending on the direction of permeation. The latter feature is a phenomenon that cannot be elucidated in a simple manner. Ninneman and Simerl (2) studied the permeability of multilayer films and introduced the following equation:

$$\Delta p = Q \sum_{i=1}^{n} \frac{l_i}{P_i} \qquad \text{(Eq. 1)}$$

where Δp stands for the difference in water vapor pressure between the higher humidity atmosphere and lower humidity atmosphere (*i.e.*, $p_1 - p_2$), Q is the permeability of an n-layer film, l_i is the thickness of the ith layer in the n-layer film, and P_i is the permeability coefficient of the ith layer. This equation may estimate the permeability of the multilayer film at a certain condition, where the material and the thickness of each component layer are given. It cannot explain the two-sided property of the multilayer film, however, because the permeability thus calculated is independent of the permutation of component layers of a film. In other words, Eq. 1 is practically applicable to the hydrophobic film only but not to the coating film, which is more or less of a hydrophilic nature.

Equation 3 is derived from Eq. 2 (1), which is known to be applicable to the hydrophobic film.

$$q = P \frac{p_1 - p_2}{l} At \qquad \text{(Eq. 2)}$$

$$\frac{1}{Q} = \frac{At}{q} = \frac{l}{P \Delta p} \qquad \text{(Eq. 3)}$$

where A is the area of film through which water vapor is permeated, t is the time of permeation, and q is the quantity of moisture permeated. But Eq. 3 does not always apply to the coating (hydrophilic) film. While $1/Q$ should be proportional to l according to Eq. 3, previously reported experiments (1) revealed that the value of $1/Q$ for $l=0$ did not come to zero in spite of the linearity of the relationship between $1/Q$ and l. This finding suggests that the permeability of the coating film is different from that of the usual hydrophobic film and its behavior is rather complex. The two-sided property of the coating film may be attributed to these very facts.

Takeda (3), studying the permeability of cellulose films, introduced the following equation:

$$\frac{1}{Q} = \frac{l}{P \Delta p} + \frac{2}{k \Delta p} \qquad \text{(Eq. 4)}$$

where k is a coefficient which indicates how easily water molecules drive into the film from the higher humidity atmosphere. This equation explains that the relationship between $1/Q$ and l is not proportional;

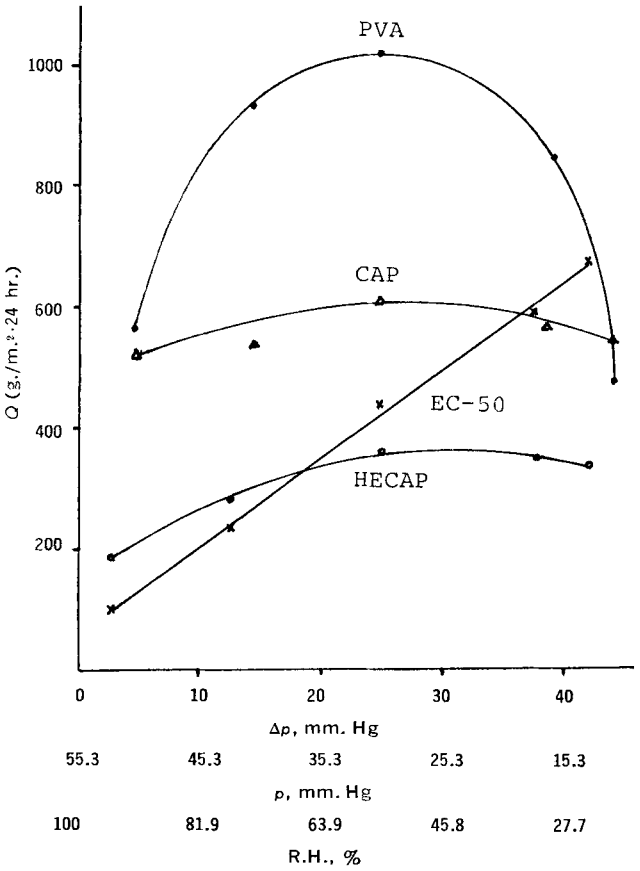


Figure 1—*Relationship between* Q *and* Δp. *Temperature, 40°; thickness of film, 0.1 mm.;* p_1 = *100% R.H* = *55.3 mm. Hg; and* $\Delta p = p_1 - p$.

i.e., the value of $1/Q$ is not zero even when the value of l equals zero. The authors attempted to elucidate various features of two-sidedness by using Eq. 4, but the results did not clarify every finding of a series of experiments.

The present report describes the results of a study of the two-sided permeability of double-layer films and the permeability of the component single-layer films according to the changes of p_1 and p_2. The symbol p_1 means a certain fixed water vapor pressure at the higher humidity side, and p_2 means that at the lower humidity side. The symbol p is used as the variable of either p_1 or p_2.

EXPERIMENTAL

Method—Experiments were performed in the same manner as reported in the preceding part of this study (1). Various p_2 (water vapor pressure of lower humidity atmosphere) conditions and temperatures were generated by a Tabai Lucifer model TL-21P. The permeation cell containing a solution to generate various p_1 (water vapor pressure of higher humidity atmosphere) conditions in the cell was placed in the cited apparatus. The weight decrease of the cell after permeation was measured.

Materials—The materials used are the same as in the previous report (1); they are designated by abbreviations defined in that paper.

RESULTS AND DISCUSSION

The permeability (Q) of the single films was determined at 40°, where water vapor pressure at the higher humidity side p_1 was 55.3 mm. Hg (relative humidity, 100%), and water vapor pressure at the lower humidity side p_2 was varied over a wide range. Some of the results thus obtained are illustrated in Fig. 1.

The figure shows the results with 0.1-mm.-thick films only; similar results were obtained with films of various thicknesses.

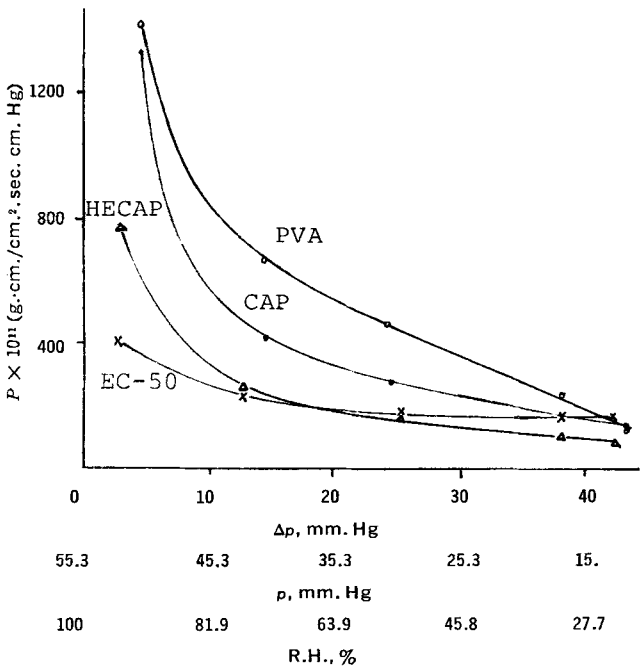


Figure 2—*Relationship between* P *and* Δp. *Temperature, 40°; thickness of film, 0.1 mm.;* p_1 = *100% R.H.* = *55.3 mm. Hg; and* $\Delta p = p_1 - p$.

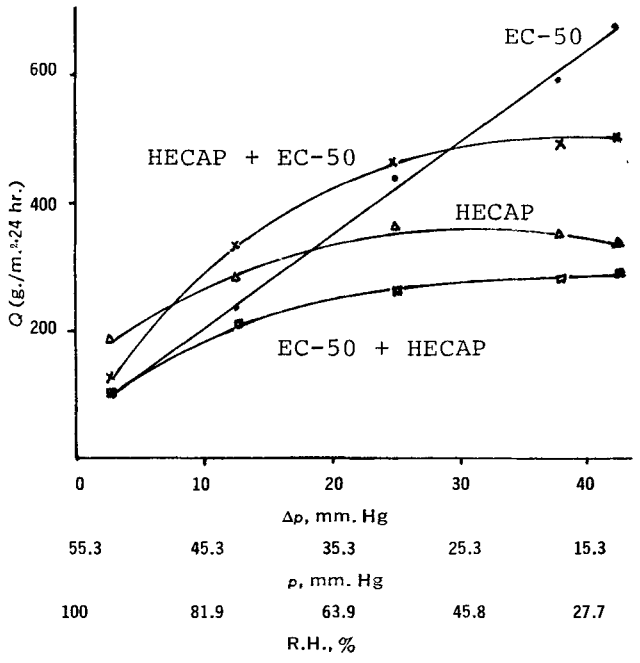


Figure 3—*Relationship between* Q *and* Δp. *Temperature, 40°; composition of double-layer films:*

(Higher humidity side)		*(Lower humidity side)*
HECAP	+	*EC-50*
EC-50	+	*HECAP*

Thickness of film: double-layer film, 0.05 mm. + 0.05 mm.; single film, 0.1 mm.; p_1 = *100% R.H.* = *55.3 mm. Hg; and* $\Delta p = p_1 - p$.

Since it was difficult to obtain directly in a single experiment films of exactly 0.1-mm. thickness, several films were selected so that the thickness of each film might be distributed in a range from 0.05 to 0.4 mm. and the respective water vapor permeability was measured at various humidity conditions. Plotting the relationship between permeation resistance ($1/Q$) and film thickness (l) gave a straight line. Thus, the permeability (Q) of the film of a given thickness (l) in various conditions could be obtained.

Figure 1 suggests some noteworthy points. According to Eq. 2, the relationship between Q ($=q/A$) and Δp ($=p_1 - p_2$) should be linear and proportional. As shown in Fig. 1, however, most of the obtained curves do not agree with Eq. 2. In particular, the value of Q for PVA does not increase after the maximum point, even when the value of Δp increases. The cause of this apparently controversial phenomenon may be that the permeability, Q, depends not only on the pressure difference between the higher vapor pressure side and the lower side, but also on the mean humidity condition to which the test film is subjected. This feature may be called the water vapor pressure dependency of permeability.

The permeability coefficient (P) for a hydrophobic film is constant, because water vapor does not interact with the film, and the experimental data correspond to Eq. 1 or 2. On the other hand, for hydrophilic films such as coating films, the value of P changes according to the humidity condition, because water vapor interacts with the film; thus Eq. 1 or 2 does not apply to the hydrophilic film. The relationship between P and Δp was obtained from Eq. 2 by using the data of Fig. 1. Figure 2 shows the results obtained. The value of P changed sharply, especially for the PVA film.

Figure 3 illustrates the relationship between Q and Δp for the double-layer film, which has the remarkable two-sided feature of permeation. For comparison, this figure also shows the curves obtained with each single-component film. The permeability Q of the single films and of the double-layer film is expressed on the basis of 0.1-mm. thickness. It was found (Fig. 3) that two-sided features, classified as Group α and Group δ (1), occurred over different humidity ranges. These features will, of course, change according to the sort of double-layer film or the combination of single films used therein.

SUMMARY

With both single-component films and a double-layer film, the relationship between water vapor permeability (Q) and humidity condition was studied. It was found that the permeability of the films depended not only upon the difference in vapor pressure (Δp) between the higher and lower humidity sides, but also upon the mean humidity condition to which the test film was subjected. It was also found that a double-layer film with two-sided permeability displayed the feature of Group α in a certain humidity range and the feature of Group δ in another humidity range.

REFERENCES

(1) T. Kuriyama, M. Nobutoki, and M. Nakanishi, *J. Pharm Sci.*, **59**, 1341 (1970).

(2) K. W. Ninneman and L. E. Simerl, *Pulp Pap. Mag. Can.*, **63**, T68(1962).

(3) B. Takeda, *Nippon Kagaku Zasshi*, **74**, 207(1953).

(4) C. E. Rogers, V. Stannet, and N. Szwarc, *Ind. Eng. Chem.*, **49**, 1933(1957).

(5) M. Patel, J. M. Patel, and A. P. Lemberger, *J. Pharm. Sci.*, **53**, 286(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 12, 1969, from the *Research Laboratories of Yoshitomi Pharmaceutical Industries, Ltd., Yoshitomi-Cho, Chikujo-Gun, Fukuoka-Prefecture, Japan.*

Accepted for publication March 10, 1970.

Abstracted in part from a thesis submitted by T. Kuriyama to the Graduate School, Kyushu University, in partial fulfillment of Doctor of Philosophy degree requirements.

Thanks are expressed to Professor S. Iguchi for his interest in the work and useful discussions. The authors also thank Mr. M. Tasaka, Executive Director, Yoshitomi Pharmaceutical Industries, Ltd., for his encouragement and his kind permission to publish this paper.

GLC Determination of Guaiacol Glyceryl Ether in Blood

WILLIAM R. MAYNARD, Jr., and ROBERT B. BRUCE

Abstract □ A method has been developed for the determination of guaiacol glyceryl ether in blood by extraction with methylene chloride followed by conversion to the heptafluorobutyrate ester and quantitation using an electron-capture detector. Blood levels following oral administration of the drug indicate a rapid absorption and elimination with a half-life of 1 hr.

Keyphrases □ Guaiacol glyceryl ether—GLC determination, in blood □ NMR—identification □ GLC—determination, guaiacol glyceryl ether, in blood

Guaiacol glyceryl ether (GGE) has been used for many years as an expectorant and, more recently, it has been claimed to have activity as a muscle relaxant (1) and as an hypocholesteremic (2–5), and to reduce platelet adhesiveness (6). In spite of its extensive use and study, no methods appear to be available for its determination in man following usual dosages. Morgan *et al.* (7) studied blood levels in dogs, and Mizutani and Naito (8) have determined blood levels in rabbits. However, relatively large doses were administered in each case, and these methods do not appear adaptable to present needs. Therefore, a method has been developed using GLC which appears to be satisfactory.

EXPERIMENTAL

The method is based on extraction of GGE from blood with methylene chloride and conversion to the heptafluorobutyrate ester and quantitation by GLC using an electron-capture detector. Mephenesin [3-(*o*-toloxy)-1,2-propanediol] is used as an internal standard.

The procedure is carried out as follows. To 5.0 ml. of blood, add 1.0 mcg. of mephenesin, 3 ml. of distilled water, and 0.5 ml. of 2 *N* H_2SO_4. Then extract with 20 ml. of redistilled methylene chloride by shaking for 10 min. Separate the phases by centrifuging, and dry the methylene chloride extract by passing it through a layer of anhydrous sodium sulfate in a funnel. Repeat the extraction with an

Since it was difficult to obtain directly in a single experiment films of exactly 0.1-mm. thickness, several films were selected so that the thickness of each film might be distributed in a range from 0.05 to 0.4 mm. and the respective water vapor permeability was measured at various humidity conditions. Plotting the relationship between permeation resistance ($1/Q$) and film thickness (l) gave a straight line. Thus, the permeability (Q) of the film of a given thickness (l) in various conditions could be obtained.

Figure 1 suggests some noteworthy points. According to Eq. 2, the relationship between Q ($=q/A$) and Δp ($=p_1 - p_2$) should be linear and proportional. As shown in Fig. 1, however, most of the obtained curves do not agree with Eq. 2. In particular, the value of Q for PVA does not increase after the maximum point, even when the value of Δp increases. The cause of this apparently controversial phenomenon may be that the permeability, Q, depends not only on the pressure difference between the higher vapor pressure side and the lower side, but also on the mean humidity condition to which the test film is subjected. This feature may be called the water vapor pressure dependency of permeability.

The permeability coefficient (P) for a hydrophobic film is constant, because water vapor does not interact with the film, and the experimental data correspond to Eq. 1 or 2. On the other hand, for hydrophilic films such as coating films, the value of P changes according to the humidity condition, because water vapor interacts with the film; thus Eq. 1 or 2 does not apply to the hydrophilic film. The relationship between P and Δp was obtained from Eq. 2 by using the data of Fig. 1. Figure 2 shows the results obtained. The value of P changed sharply, especially for the PVA film.

Figure 3 illustrates the relationship between Q and Δp for the double-layer film, which has the remarkable two-sided feature of permeation. For comparison, this figure also shows the curves obtained with each single-component film. The permeability Q of the single films and of the double-layer film is expressed on the basis of 0.1-mm. thickness. It was found (Fig. 3) that two-sided features, classified as Group α and Group δ (1), occurred over different humidity ranges. These features will, of course, change according to the sort of double-layer film or the combination of single films used therein.

SUMMARY

With both single-component films and a double-layer film, the relationship between water vapor permeability (Q) and humidity condition was studied. It was found that the permeability of the films depended not only upon the difference in vapor pressure (Δp) between the higher and lower humidity sides, but also upon the mean humidity condition to which the test film was subjected. It was also found that a double-layer film with two-sided permeability displayed the feature of Group α in a certain humidity range and the feature of Group δ in another humidity range.

REFERENCES

(1) T. Kuriyama, M. Nobutoki, and M. Nakanishi, *J. Pharm Sci.*, **59**, 1341 (1970).

(2) K. W. Ninneman and L. E. Simerl, *Pulp Pap. Mag. Can.*, **63**, T68(1962).

(3) B. Takeda, *Nippon Kagaku Zasshi*, **74**, 207(1953).

(4) C. E. Rogers, V. Stannet, and N. Szwarc, *Ind. Eng. Chem.*, **49**, 1933(1957).

(5) M. Patel, J. M. Patel, and A. P. Lemberger, *J. Pharm. Sci.*, **53**, 286(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 12, 1969, from the *Research Laboratories of Yoshitomi Pharmaceutical Industries, Ltd., Yoshitomi-Cho, Chikujo-Gun, Fukuoka-Prefecture, Japan.*

Accepted for publication March 10, 1970.

Abstracted in part from a thesis submitted by T. Kuriyama to the Graduate School, Kyushu University, in partial fulfillment of Doctor of Philosophy degree requirements.

Thanks are expressed to Professor S. Iguchi for his interest in the work and useful discussions. The authors also thank Mr. M. Tasaka, Executive Director, Yoshitomi Pharmaceutical Industries, Ltd., for his encouragement and his kind permission to publish this paper.

GLC Determination of Guaiacol Glyceryl Ether in Blood

WILLIAM R. MAYNARD, Jr., and ROBERT B. BRUCE

Abstract □ A method has been developed for the determination of guaiacol glyceryl ether in blood by extraction with methylene chloride followed by conversion to the heptafluorobutyrate ester and quantitation using an electron-capture detector. Blood levels following oral administration of the drug indicate a rapid absorption and elimination with a half-life of 1 hr.

Keyphrases □ Guaiacol glyceryl ether—GLC determination, in blood □ NMR—identification □ GLC—determination, guaiacol glyceryl ether, in blood

Guaiacol glyceryl ether (GGE) has been used for many years as an expectorant and, more recently, it has been claimed to have activity as a muscle relaxant (1) and as an hypocholesteremic (2–5), and to reduce platelet adhesiveness (6). In spite of its extensive use and study, no methods appear to be available for its determination in man following usual dosages. Morgan *et al.* (7) studied blood levels in dogs, and Mizutani and Naito (8) have determined blood levels in rabbits. However, relatively large doses were administered in each case, and these methods do not appear adaptable to present needs. Therefore, a method has been developed using GLC which appears to be satisfactory.

EXPERIMENTAL

The method is based on extraction of GGE from blood with methylene chloride and conversion to the heptafluorobutyrate ester and quantitation by GLC using an electron-capture detector. Mephenesin [3-(*o*-toloxy)-1,2-propanediol] is used as an internal standard.

The procedure is carried out as follows. To 5.0 ml. of blood, add 1.0 mcg. of mephenesin, 3 ml. of distilled water, and 0.5 ml. of 2 *N* H_2SO_4. Then extract with 20 ml. of redistilled methylene chloride by shaking for 10 min. Separate the phases by centrifuging, and dry the methylene chloride extract by passing it through a layer of anhydrous sodium sulfate in a funnel. Repeat the extraction with an

Table I—Recovery of Known Amounts of GGE Added to Control Blood

GGE—mcg./ml. Blood— Added	Found	Recovery, %
0.080	0.078	97.5
0.16	0.15	92.5
0.20	0.19	97.0
1.00	1.02	102.0
2.00	1.89	94.4
3.00	3.07	101.0

additional 10 ml. of methylene chloride, and combine the extracts. Wash sodium sulfate with an additional 3 ml. of methylene chloride which is added to the combined extract. Evaporate the combined extracts under a stream of nitrogen to 100 μl. Add 300 μl. of a solution of heptafluorobutyric anhydride in methylene chloride (1 ml./100 ml.), mix, heat to 60° for 30 sec., and allow to stand for 10 min. with occasional mixing. Evaporate to complete dryness with nitrogen, add 0.50–3.0 ml. of redistilled ether, mix, and inject 1–4 μl. into the gas chromatograph.

The gas chromatograph used in this study was a Barber-Coleman, Series 5000, with a ^{63}Ni-detector. The column was 1.21 m. (4 ft.) long, stainless steel, and contained 3% XE-60 on diatomaceous earth.[1] Temperatures were: column, 144°; detector, 280°; and injection port, 260°. The flow rate was 40 ml./min. of nitrogen. The retention time of mephenesin heptafluorobutyrate was 4 min. 50 sec. and that of GGE heptafluorobutyrate was 6 min. 10 sec. The column was preconditioned by four rapid injections of 4 μl. of a mixture of heptafluorobutyric anhydride in ether (1:4) and

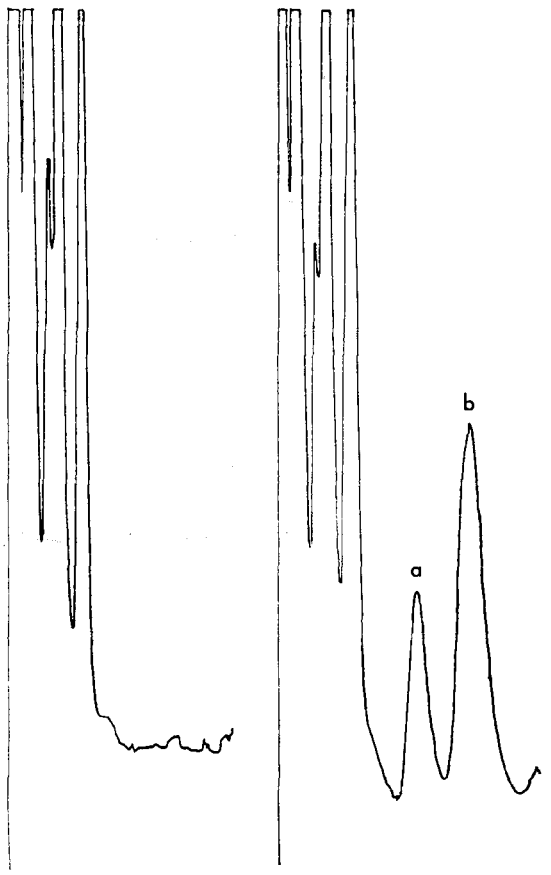


Figure 1—*Chromatograms showing results of the analysis of control blood (left) and blood sample from subject receiving GGE (right): a, mephenesin heptafluorobutyrate; and b, GGE heptafluorobutyrate.*

[1] Gas-Chrom Q, Applied Science Laboratories, Inc., State College, PA 16801

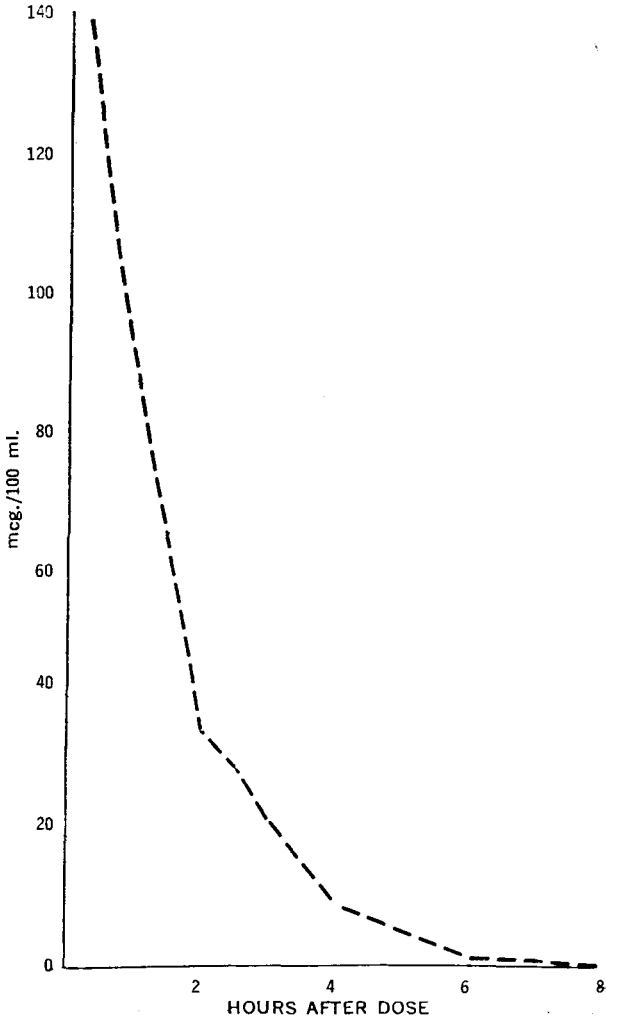


Figure 2—*Average blood levels following the oral administration of 600 mg. of GGE to three human subjects.*

allowing the column to remain at 144° for 30 min. The column temperature was raised to 200° for 2 hr. The column exit was disconnected during this preconditioning.

Three normal male subjects were administered an oral liquid dose of 600 mg. of GGE. The subjects received no solid food from the midnight before drug administration until 2 hr. after drug administration. Blood samples were drawn at frequent intervals for 8 hr. after the dose.

RESULTS AND DISCUSSION

GGE has been determined (9, 10) by GLC in pharmaceutical preparations. These methods did not give the sensitivity needed for blood level determination. Amides and esters prepared from halogenated acid chlorides or anhydrides give a high response with the electron-capture detector. Heptafluorobutyric anhydride was used because it reacts easily and quickly with GGE at room temperature and can be readily separated from the ester by simple evaporation. The procedure is straightforward and simple to carry out. The only precaution is to remove completely the solvent—methylene chloride—before the sample is injected into the gas chromatograph, since the detector is very sensitivie to halogenated compounds.

The results found from the analysis of blood samples to which known amounts of GGE had been added are shown in Table I. The standard deviation at the 1.00-mcg./ml. level was 0.046. These results appear to be satisfactory for the analysis of blood. Chromatograms from actual analysis of blood samples are shown in Fig. 1. A comparison of the chromatogram of the control blood with that of the sample containing GGE shows that nothing present in

the control interferes with the determination. Some uninvestigated materials are eluted earlier than the esters of GGE and mephenesin. Quantitation is made comparing peak heights of the internal standard with that of the GGE ester.

A large amount of the ester was prepared by the procedure described to determine whether reaction had occurred with both or only one of the hydroxyl groups. NMR spectra showed that only the primary hydroxyl had reacted.

The results from the blood level determinations following the oral dose are shown in Fig. 2. GGE is readily absorbed, with the maximum amount determined occurring in the 0.25-hr. sample. The half-life was 1.00 hr. Detectable amounts of the drug were no longer present in the 8-hr. samples of any of the subjects, indicating rapid metabolism and excretion.

REFERENCES

(1) H. Hafner, *Wien. Med. Wochenschr.*, **114**, 523(1964).

(2) R. D. Eastham and E. P. Griffiths, *Lancet*, **7441**, 795(1966).

(3) J. L. Silverman and H. A. Wurzel, *Amer. J. Med. Sci.*, **254**, 491(1967).

(4) H. R. Baumgartner, M. Cronquist, P. Wobman, and F. Streuli, *Schweiz. Med. Swchr.*, **97**, 1674(1967).

(5) J. Franco and L. R. Grams, *Clin. Res.*, **16**, 41(1968).

(6) S. Naito and M. Mizutani, *Chem. Pharm. Bull.*, **17**, 822 (1969).

(7) A. M. Morgan, E. B. Truitt, Jr., and J. M. Little, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 374(1957).

(8) M. Mizutani and S. Naito, *Chem. Pharm. Bull.*, **15**, 1422 (1967).

(9) B. R. Rades and E. S. Aranda, *J. Pharm. Sci.*, **57**, 847(1968).

(10) M. Koibuchi, T. Shibazaki, T. Minamakawa, and Y. Nishimura, *J. Pharm. Soc. Jap.*, **88**, 877(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 10, 1970, from the *Research Laboratories, A. H. Robins Company, Inc., Richmond, VA 23220*

Accepted for publication March 18, 1970.

The authors thank Dr. Russell C. Seckendorf, Clinical Pharmacology, A. H. Robins Company, for the administration of the drug and collection of blood samples.

Antiradiation Compounds XIV: Dithiocarbamates of Aminothiophenes

WILLIAM O. FOYE, JAMES MICKLES, and GERARD M. BOYCE

Abstract □ Dithiocarbamates of 2-amino-3-cyano(or carbethoxy)-4,5-dialkylthiophenes and a corresponding furan have been obtained. Dithiocarbamate formation of 2-amino-3-cyano-4,5-diphenylfuran resulted in a conversion to the corresponding thiophene dithiocarbamate. A dithiocarbamate trithiocarbonate of 2-amino-3-carbethoxy-4-mercaptomethylthiophene was also synthesized, and ring closure of the 2-amino-3-cyanothiophenes to thiopheno[2,3-*d*]pyrimidines was observed. None of the compounds tested showed radiation-protective or antimalarial properties.

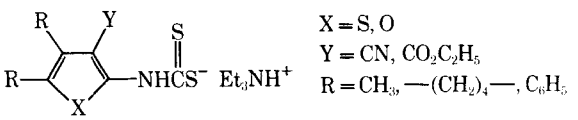
Keyphrases □ Antiradiation compounds—dithiocarbamates of aminothiophenes □ Aminothiophenes, dithiocarbamate derivatives—radiation-protective capacity, antimalarial properties □ IR spectrophotometry—structure

Although thiophene derivatives have not appeared frequently with radiation-protective properties, a basic derivative, *N*-phenyl-2-thiophenecarboxamidine, has been reported to have appreciable protection in rats (1). Since several heterocyclic dithiocarbamates in the pyridine, pyrimidine, and acridine series (2) are radiation protective, dithiocarbamates of thiophenes and furans having basic functions appeared to be logical candidates as radiation-protective compounds. Methods for obtaining thiophenes and furans having primary amino substituents in the ring have recently been announced (3), and the conversion of compounds of this type to dithiocarbamates has been attempted. Inclusion of this sulfur-containing function provides a thiol anion capable of undergoing rapid hydrogen-atom exchange reactions (4), which could account for radiation protection.

PROCEDURE

Preparation of 2-aminothiophenes was carried out by the method of Gewald *et al.* (5) with modifications. This procedure involved the base-catalyzed condensation of a carbonyl compound with an active methylene nitrile and sulfur. Using methyl ethyl ketone and malononitrile, the reaction was found to be best catalyzed with morpholine, with excess ketone as the solvent. Using methyl ethyl ketone and malononitrile, the product was 2-amino-3-cyano-4,5-dimethylthiophene; with ethanol as the solvent, the product was 2-butylidenemalononitrile. By the same procedure, but with ethanol as the solvent, the following were obtained: 2-amino-3-carbethoxy-4,5-dimethylthiophene, 2-amino-3-cyano-4,5-tetramethylenothiophene, and 2-amino-3-carbethoxy-4,5-tetramethylenothiophene. Also obtained by the same general procedure, without sulfur, were 2-amino-3-cyano-4,5-dimethylfuran and the corresponding 4,5-diphenyl compound.

Attempts to form the dithiocarbamates of the 2-aminothiophenes previously mentioned, using carbon disulfide and ethanol as the solvent, gave only small yields over a period of 24–72 hr. In the case of 2-amino-3-cyano-4,5-dimethylthiophene, the thiourea was formed instead. By using the procedure of Fairfull and Peak (6), however, triethylammonium salts of the dithiocarbamates (I) of the aminothiophenes and one of the aminofurans were obtained in good yield and sufficiently pure for analysis.



I

The attempted conversion of 2-amino-3-cyano-4,5-diphenylfuran to the dithiocarbamate gave a product having a poor analysis for the ethyl ester of the dithiocarbamate. By allowing the reaction to take place during a much longer time (2 weeks), a product was obtained for which the analysis indicated formation of the dithiocarbamate salt of 2-amino-3-cyano-4,5-diphenylthio-

the control interferes with the determination. Some uninvestigated materials are eluted earlier than the esters of GGE and mephenesin. Quantitation is made comparing peak heights of the internal standard with that of the GGE ester.

A large amount of the ester was prepared by the procedure described to determine whether reaction had occurred with both or only one of the hydroxyl groups. NMR spectra showed that only the primary hydroxyl had reacted.

The results from the blood level determinations following the oral dose are shown in Fig. 2. GGE is readily absorbed, with the maximum amount determined occurring in the 0.25-hr. sample. The half-life was 1.00 hr. Detectable amounts of the drug were no longer present in the 8-hr. samples of any of the subjects, indicating rapid metabolism and excretion.

REFERENCES

(1) H. Hafner, *Wien. Med. Wochenschr.*, **114**, 523(1964).

(2) R. D. Eastham and E. P. Griffiths, *Lancet*, **7441**, 795(1966).

(3) J. L. Silverman and H. A. Wurzel, *Amer. J. Med. Sci.*, **254**, 491(1967).

(4) H. R. Baumgartner, M. Cronquist, P. Wobman, and F. Streuli, *Schweiz. Med. Swchr.*, **97**, 1674(1967).

(5) J. Franco and L. R. Grams, *Clin. Res.*, **16**, 41(1968).

(6) S. Naito and M. Mizutani, *Chem. Pharm. Bull.*, **17**, 822 (1969).

(7) A. M. Morgan, E. B. Truitt, Jr., and J. M. Little, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 374(1957).

(8) M. Mizutani and S. Naito, *Chem. Pharm. Bull.*, **15**, 1422 (1967).

(9) B. R. Rades and E. S. Aranda, *J. Pharm. Sci.*, **57**, 847(1968).

(10) M. Koibuchi, T. Shibazaki, T. Minamakawa, and Y. Nishimura, *J. Pharm. Soc. Jap.*, **88**, 877(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 10, 1970, from the *Research Laboratories, A. H. Robins Company, Inc., Richmond, VA 23220*

Accepted for publication March 18, 1970.

The authors thank Dr. Russell C. Seckendorf, Clinical Pharmacology, A. H. Robins Company, for the administration of the drug and collection of blood samples.

Antiradiation Compounds XIV: Dithiocarbamates of Aminothiophenes

WILLIAM O. FOYE, JAMES MICKLES, and GERARD M. BOYCE

Abstract □ Dithiocarbamates of 2-amino-3-cyano(or carbethoxy)-4,5-dialkylthiophenes and a corresponding furan have been obtained. Dithiocarbamate formation of 2-amino-3-cyano-4,5-diphenylfuran resulted in a conversion to the corresponding thiophene dithiocarbamate. A dithiocarbamate trithiocarbonate of 2-amino-3-carbethoxy-4-mercaptomethylthiophene was also synthesized, and ring closure of the 2-amino-3-cyanothiophenes to thiopheno[2,3-*d*]pyrimidines was observed. None of the compounds tested showed radiation-protective or antimalarial properties.

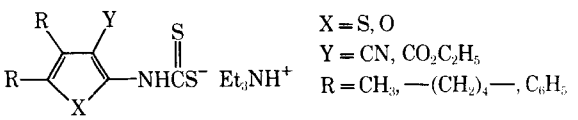
Keyphrases □ Antiradiation compounds—dithiocarbamates of aminothiophenes □ Aminothiophenes, dithiocarbamate derivatives—radiation-protective capacity, antimalarial properties □ IR spectrophotometry—structure

Although thiophene derivatives have not appeared frequently with radiation-protective properties, a basic derivative, *N*-phenyl-2-thiophenecarboxamidine, has been reported to have appreciable protection in rats (1). Since several heterocyclic dithiocarbamates in the pyridine, pyrimidine, and acridine series (2) are radiation protective, dithiocarbamates of thiophenes and furans having basic functions appeared to be logical candidates as radiation-protective compounds. Methods for obtaining thiophenes and furans having primary amino substituents in the ring have recently been announced (3), and the conversion of compounds of this type to dithiocarbamates has been attempted. Inclusion of this sulfur-containing function provides a thiol anion capable of undergoing rapid hydrogen-atom exchange reactions (4), which could account for radiation protection.

PROCEDURE

Preparation of 2-aminothiophenes was carried out by the method of Gewald *et al.* (5) with modifications. This procedure involved the base-catalyzed condensation of a carbonyl compound with an active methylene nitrile and sulfur. Using methyl ethyl ketone and malononitrile, the reaction was found to be best catalyzed with morpholine, with excess ketone as the solvent. Using methyl ethyl ketone and malononitrile, the product was 2-amino-3-cyano-4,5-dimethylthiophene; with ethanol as the solvent, the product was 2-butylidenemalononitrile. By the same procedure, but with ethanol as the solvent, the following were obtained: 2-amino-3-carbethoxy-4,5-dimethylthiophene, 2-amino-3-cyano-4,5-tetramethylenothiophene, and 2-amino-3-carbethoxy-4,5-tetramethylenothiophene. Also obtained by the same general procedure, without sulfur, were 2-amino-3-cyano-4,5-dimethylfuran and the corresponding 4,5-diphenyl compound.

Attempts to form the dithiocarbamates of the 2-aminothiophenes previously mentioned, using carbon disulfide and ethanol as the solvent, gave only small yields over a period of 24–72 hr. In the case of 2-amino-3-cyano-4,5-dimethylthiophene, the thiourea was formed instead. By using the procedure of Fairfull and Peak (6), however, triethylammonium salts of the dithiocarbamates (I) of the aminothiophenes and one of the aminofurans were obtained in good yield and sufficiently pure for analysis.



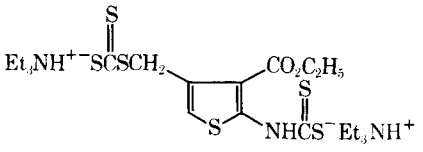
I

The attempted conversion of 2-amino-3-cyano-4,5-diphenylfuran to the dithiocarbamate gave a product having a poor analysis for the ethyl ester of the dithiocarbamate. By allowing the reaction to take place during a much longer time (2 weeks), a product was obtained for which the analysis indicated formation of the dithiocarbamate salt of 2-amino-3-cyano-4,5-diphenylthio-

phene (I, R = C_6H_5, Y = CN). During this prolonged reaction, the furan ring apparently opened and reacted with carbon disulfide to give the corresponding thiophene.

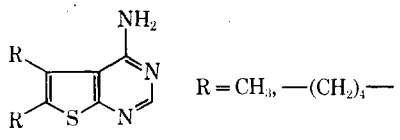
IR absorption of the dithiocarbamates containing cyano groups showed either very weak or no peaks for cyano absorption. This may be attributed to interaction with the adjacent, negatively charged dithiocarbamate group. In addition, absorption due to C=S occurred at lower wavenumbers than in the case of the 3-carbethoxy compounds, again indicating interaction with cyano groups.

2-Amino-3-ethoxycarbonyl-4-mercaptomethylthiophene was obtained by a modification of the method of Gewald and Schinke (7) for the disulfide. The mercaptan was not isolated but was treated with carbon disulfide and triethylamine to give the dithiocarbamate trithiocarbonate (II) from condensation at both the amino and thiol functions. A previous dithiocarbamate trithiocarbonate, prepared from cysteine, was found to be strongly radiation protective in mice (8). A similar attempt to obtain 2-amino-3-cyano-4-methyl-5-mercaptothiophene gave the sulfide.



II

Thiopheno[2,3-*d*]pyrimidines (III) were also obtained using the method of Gewald (9) for synthesis of furo[2,3-*d*]pyrimidines. These compounds resulted on refluxing the appropriate 3-cyanothiophene with formamide and acetic anhydride.



III

BIOLOGICAL TESTING RESULTS

Antiradiation screening of several of the compounds was carried out.[1] Tests were carried out in mice *versus* 825 r (X-rays) or 950 r (γ-rays) with an observation period of 30 days. The dithiocarbamate of 2-amino-3-carbethoxy-4,5-dimethylthiophene (I) was inactive against 950 r (γ-rays), and the dithiocarbamate trithiocarbonate (II) and the two thiopheno[2,3-*d*]-pyrimidines (III) were inactive against 825 r (X-rays).

The three compounds (II and III) were also screened for antimalarial activity in mice infected with *Plasmodium berghei* and in *Aedes aegypti* infected with *Plasmodium gallinaceum;* they were inactive in both tests. Also, the dithiocarbamates of the 2-amino-3-cyano(or carbethoxy)-4,5-dialkylthiophenes (I) were inactive in the mouse test.

It may be concluded from the lack of radiation-protective activity for the dithiocarbamate trithiocarbonate (II) reported here and the powerful protective effect of the corresponding cysteine derivative (8) that a rigid structure connecting these functions is detrimental to radiation protection.

EXPERIMENTAL

Analyses for carbon, hydrogen, and nitrogen were performed.[2] Sulfur analyses were done by Parr bomb peroxide fusion. Melting points were taken on a Mel-Temp apparatus and are corrected. IR absorption spectra were obtained with a Perkin-Elmer model 137B spectrometer.

Triethylammonium 3-Cyano-4,5-dimethylthiophene-2-dithiocarbamate—A mixture of 5.3 g. (0.035 mole) of 2-amino-3-cyano-4,5-dimethylthiophene (5), 42 ml. (0.600 mole) of carbon disulfide, 76.5 ml. (0.550 mole) of triethylamine, and 2.5 ml. of absolute ethanol formed a red-orange solution. The solution was stirred at room temperature for 48 hr. and yielded 3.3 g. (28%) of orange-yellow product; m.p. 241–242°; IR (KBr) 880 (β-ring), 1135 (C=S), 2200 (C≡N, weak) $cm.^{-1}$

Anal.—Calcd. for $C_{14}H_{23}N_3S_3$: C, 51.02; H, 7.03; N, 12.75; S, 29.19. Found: C, 51.37; H, 6.97; N, 12.29; S, 29.63.

Triethylammonium 3-Carbethoxy-4,5-dimethylthiophene-2-dithiocarbamate—A mixture of 7.0 g. (0.035 mole) of 2-amino-3-carbethoxy-4,5-dimethylthiophene (5), 42 ml. (0.600 mole) of carbon disulfide, 76.5 ml. (0.550 mole) of triethylamine, and 2.5 ml. of absolute ethanol formed a red-orange solution, which was allowed to stand at room temperature for 2 days, giving 7.5 g. (56%) of bright-yellow crystals; m.p. 105–107°; IR (KBr) 990 (C=S), 1650 (C=O) $cm.^{-1}$.

Anal.—Calcd. for $C_{16}H_{28}N_2O_2S_3$: C, 51.02; H, 7.49; N, 7.44; S, 25.54. Found: C, 50.86; H, 7.11; N, 7.15; S, 25.10.

Triethylammonium 3-Cyano-4,5-tetramethylenothiophene-2-dithiocarbamate—A mixture of 8.9 g. (0.05 mole) of 2-amino-3-cyano-4,5-tetramethylenothiophene (5), 60 ml. (0.86 mole) of carbon disulfide, 109 ml. (0.78 mole) of triethylamine, and 3.5 ml. of absolute ethanol formed a yellow solution, which was allowed to stand for 3 days at room temperature, giving 6.5 g. (36%) of rust-colored product; m.p. 210–212°; IR (KBr) 875 (β-ring), 1115 (C=S) $cm.^{-1}$.

Anal.—Calcd. for $C_{16}H_{25}N_3S_3$: C, 54.04; H, 7.09; N, 11.82; S, 27.05. Found: C, 54.11; H, 6.82; N, 11.70; S, 27.50.

Triethylammonium 3-Carbethoxy-4,5-tetramethylenothiophene-2-dithiocarbamate—A mixture of 11.25 g. (0.05 mole) of 2-amino-3-carbethoxy-4,5-tetramethylenothiophene (5), 60 ml. (0.86 mole) of carbon disulfide, 109 ml. (0.78 mole) of triethylamine, and 3.5 ml. of absolute ethanol formed a yellow solution, which was allowed to stand at room temperature for 3 days, giving 11.3 g. (56%) of bright-yellow product; m.p. 101–103°; IR (KBr) 965 (C=S), 1660 (C=O) $cm.^{-1}$.

Anal.—Calcd. for $C_{18}H_{30}N_2O_2S_3$: C, 53.69; H, 7.51; N, 6.96; S, 24.89. Found: C, 53.97; H, 7.36; N, 6.75; S, 24.76.

***N,N'*-Bis(3-cyano-4,5-dimethyl-2-thienyl)thiourea**—A solution of 2-amino-3-cyano-4,5-dimethylthiophene (5) (3.04 g., 0.02 mole) in 50 ml. of absolute ethanol was treated with carbon disulfide (42 ml., 0.06 mole). The yellow solution was stirred at room temperature for 2 days, and 0.95 g. (27%) of yellow compound was obtained which did not melt below 300°; IR (KBr) 1290 (C=S, thioamide), 2200 (C≡N) $cm.^{-1}$.

Anal.—Calcd. for $C_{15}H_{14}N_4S_3$: C, 52.0; H, 4.1; S, 27.7. Found: C, 51.7; H, 4.5; S, 27.5.

Triethylammonium 3-Cyano-4,5-dimethylfuran-2-dithiocarbamate—A solution of 3.1 g. (0.02 mole) of 2-amino-3-cyano-4,5-dimethylfuran (9), 60 ml. (0.44 mole) of triethylamine, and 32.5 ml. (0.44 mole) of carbon disulfide was stirred at room temperature for 2 weeks. The orange solid was triturated with absolute ethanol, giving 0.64 g. (9%) of compound which slowly decomposed on standing; m.p. 195–200° dec.; IR (KBr) 880 (β-ring), 1145 (C=S) $cm.^{-1}$.

Anal.—Calcd. for $C_{14}H_{23}N_3OS_2$: C, 53.7; H, 7.3; N, 13.3; S, 20.4. Found: C, 53.3; H, 7.4; N, 12.7; S, 20.5.

Triethylammonium 3-Cyano-4,5-diphenylthiophene-2-dithiocarbamate—A solution of 2.28 g. (0.009 mole) of 2-amino-3-cyano-4,5-diphenylfuran (9), 10.5 ml. (0.150 mole) of carbon disulfide, 19.1 ml. (0.138 mole) of triethylamine, and 6 ml. of absolute ethanol was allowed to stand at room temperature for 2 weeks. A yield of 0.61 g. (15%) of orange crystals was obtained; m.p. 256–258°; IR (KBr) 987 (C=S), 2050 (C≡N) $cm.^{-1}$.

Anal.—Calcd. for $C_{24}H_{27}N_3S_3$: C, 63.5; H, 6.0; N, 9.2; S, 21.2. Found: C, 63.4; H, 5.8; N, 9.0; S, 20.7.

Bis(triethylammonium)-3-carbethoxy-2-dithiocarbamate-4-thienyltrithiocarbonate—A solution of 7.3 g. (0.06 mole) of 1,3-dimercaptoacetone and 6.8 g. (0.06 mole) of ethyl cyanoacetate in 30 ml. of absolute ethanol was treated with 3 ml. of triethylamine. After 15 min., 35 ml. (0.47 mole) of carbon disulfide and 45 ml. (0.30 mole) of triethylamine were added; the solution was stirred for 1 hr. and allowed to stand overnight at room temperature. A red oil was separated and crystallized by the addition of 200 ml. of absolute ethanol. After being stirred for 2 hr., the yellow product (10.8 g., 31%) was isolated; m.p. 106–108°; IR (KBr) 1010 (C=S), 1125 (C=S), 1670 (C=O) $cm.^{-1}$.

[1] At the Walter Reed Army Institute of Research; results reported through the courtesy of Dr. D. P. Jacobus.

[2] By Weiler and Strauss, Oxford, England, or by Carol Fitz, Needham, Mass.

Anal.—Calcd. for $C_{22}H_{41}N_3O_3S_6$: C, 46.20; H, 7.23; N, 7.35; S, 33.64. Found: C, 45.97; H, 7.05; N, 7.05; S, 33.62.

Bis(2-amino-3-cyano-4-methyl-5-thienyl)sulfide—A mixture of 58 g. (1 mole) of acetone, 13.2 g. (0.2 mole) of malononitrile, and 6.4 g. (0.2 g. atom) of sulfur was treated dropwise with 20 ml. of triethylamine. The mixture was stirred at 30–35° for 7 hr. and allowed to stand at room temperature for 36 hr. After addition of 400 ml. of aqueous ethanol (1:1) and vigorous stirring, a tan, crystalline product was obtained which was extracted with boiling ethanol, giving 1.85 g. (3%) of product; m.p. 255–257°.

Anal.—Calcd. for $C_{12}H_{10}N_4S_3$: C, 47.03; H, 3.29; N, 18.28; S, 31.39. Found: C, 47.14; H, 3.38; N, 18.15; S, 31.28.

4-Amino-5,6-dimethylthiopheno[2,3-*d*]pyrimidine—A mixture of 3.04 g. (0.02 mole) of 2-amino-3-cyano-4,5-dimethylthiophene (5), 30 ml. of formamide, and two drops of acetic anhydride was refluxed at 160–165° for 2 hr. After being cooled, a solid product was isolated and recrystallized from dioxane, giving 1.48 g. (39%) of white crystals; m.p. 261–263°; IR (KBr) 1650 (NH_2), 3400 (NH_2) $cm.^{-1}$.

Anal.—Calcd. for $C_8H_9N_3S$: C, 53.47; H, 5.21; N, 23.22; S, 18.25. Found: C, 53.61; H, 5.01; N, 23.44; S, 17.94.

4-Amino-5,6-tetramethylenothiopheno[2,3-*d*]pyrimidine—A mixture of 3.56 g. (0.02 mole) of 2-amino-3-cyano-4,5-tetramethylenothiophene (5), 30 ml. of formamide, and two drops of acetic anhydride was refluxed at 165–170° for 2 hr. After being cooled, a solid product was isolated and recrystallized from dioxane, giving 0.7 g. (17%) of white product; m.p. 261–263°; IR (KBr) 1635 (NH_2), 3350 (NH_2) $cm.^{-1}$.

Anal.—Calcd. for $C_{10}H_{11}N_3S$: C, 58.23; H, 6.32; N, 21.15; S, 15.62. Found: C, 58.51; H, 5.94; N, 20.67; S, 16.01.

REFERENCES

(1) I. Baev and S. Robev, *C. R. Acad. Bulg. Sci.*, **13**, 733(1960).

(2) W. O. Foye and D. H. Kay, *J. Pharm. Sci.*, **57**, 345(1968); W. O. Foye, D. H. Kay, and P. R. Amin, *ibid.*, **57**, 1793(1968).

(3) K. Gewald, *Chem. Ber.*, **98**, 3571(1965); V. I. Shvedov, V. K. Ryzhkova, and A. N. Grimev, *Khim. Getero. Soedin.*, **1967**, 239.

(4) S. G. Cohen, in "Organosulfur Chemistry," M. J. Janssen, Ed., Interscience, New York, N. Y., 1967, chap. 3.

(5) K. Gewald, E. Schinke, and H. Bottcher, *Chem. Ber.*, **99**, 94 (1966).

(6) A. E. S. Fairfull and D. A. Peak, *J. Chem. Soc.*, **1955**, 796.

(7) K. Gewald and E. Schinke, *Chem. Ber.*, **99**, 2712(1966).

(8) R. I. H. Wang, W. Dooley, Jr., W. O. Foye, and J. Mickles, *J. Med. Chem.*, **9**, 394(1966).

(9) K. Gewald, *Chem. Ber.*, **99**, 1002(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 1, 1970, from the *Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115*

Accepted for publication March 18, 1970.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This investigation was supported by Research Grant RH00297 from the National Center for Radiological Health, U. S. Public Health Service, Bethesda, Md.

N-Substituted Aminoethanethiols and *N*-Substituted Aminoethanethiol *S*-Sulfonic Acids as Radioprotective Agents

F. I. CARROLL and MONROE E. WALL

Abstract □ Several *N*-substituted aminoethanethiols and *N*-substituted aminoethanethiol *S*-sulfonic acids were tested as potential radioprotective agents. 2-(2′-Carbamidoethylamino)-ethanethiol (I*a*), 2-(2′-carboxyethylamino)-ethanethiol (I*b*), 2-(2′-cyanoethylamino)-ethanethiol (I*c*), and 2-(2′-carbamidoethylamino)-ethanethiol *S*-sulfonic acid (II*a*) exhibited significant protective effects against ionizing radiation. Compound I*a* showed the highest activity and was selected for further radiation-protection test studies. The structure–activity relationships of this class of compounds are discussed.

Keyphrases □ Aminoethanethiols, *N*-substituted—radioprotective capacity, structure–activity relationships □ Aminoethanethiol *S*-sulfonic acids, *N*-substituted—radioprotective capacity, structure–activity relationships □ Radioprotective agents—aminoethanethiols, aminoethanethiol *S*-sulfonic acids, *N*-substituted

A recent list of the various types of compounds that show radioprotective properties has appeared and their structure–activity relationships have been discussed (1). Aminoalkylthiols constitute the most effective class of radioprotective agents. The initial discovery that 2-mercaptoethylamine (MEA) offered protection to mice against ionizing radiation (2) led to the synthesis of several hundred derivatives of this compound. Structural requirements necessary for radioprotective activity have evolved from the test results on these compounds and have been summarized (1). This effect was not observable when one or two alkyl substituents were placed on the carbon containing the thiol function of MEA (3, 4).[1] Subsequently, it was found that some *N*-substituted aminoethanethiols and *N*-substituted aminoethanethiol *S*-sulfonic acids, prepared in this laboratory, showed significant protection against ionizing radiation. In this report the radioprotection test results on these compounds are presented, and their structure–activity relationships are discussed.

[1] Subsequent antiradiation test results have shown that 2-mercapto-2-methylaminopropane hydrochloride, when administered at 90 mg./kg. i.p. using CMCTW as vehicle, gave 67% survival to mice irradiated with 825 r. (See footnotes to Table II for explanation of test data.)

Anal.—Calcd. for $C_{22}H_{41}N_3O_3S_6$: C, 46.20; H, 7.23; N, 7.35; S, 33.64. Found: C, 45.97; H, 7.05; N, 7.05; S, 33.62.

Bis(2-amino-3-cyano-4-methyl-5-thienyl)sulfide—A mixture of 58 g. (1 mole) of acetone, 13.2 g. (0.2 mole) of malononitrile, and 6.4 g. (0.2 g. atom) of sulfur was treated dropwise with 20 ml. of triethylamine. The mixture was stirred at 30–35° for 7 hr. and allowed to stand at room temperature for 36 hr. After addition of 400 ml. of aqueous ethanol (1:1) and vigorous stirring, a tan, crystalline product was obtained which was extracted with boiling ethanol, giving 1.85 g. (3%) of product; m.p. 255–257°.

Anal.—Calcd. for $C_{12}H_{10}N_4S_3$: C, 47.03; H, 3.29; N, 18.28; S, 31.39. Found: C, 47.14; H, 3.38; N, 18.15; S, 31.28.

4-Amino-5,6-dimethylthiopheno[2,3-*d*]pyrimidine—A mixture of 3.04 g. (0.02 mole) of 2-amino-3-cyano-4,5-dimethylthiophene (5), 30 ml. of formamide, and two drops of acetic anhydride was refluxed at 160–165° for 2 hr. After being cooled, a solid product was isolated and recrystallized from dioxane, giving 1.48 g. (39%) of white crystals; m.p. 261–263°; IR (KBr) 1650 (NH_2), 3400 (NH_2) $cm.^{-1}$.

Anal.—Calcd. for $C_8H_9N_3S$: C, 53.47; H, 5.21; N, 23.22; S, 18.25. Found: C, 53.61; H, 5.01; N, 23.44; S, 17.94.

4-Amino-5,6-tetramethylenothiopheno[2,3-*d*]pyrimidine—A mixture of 3.56 g. (0.02 mole) of 2-amino-3-cyano-4,5-tetramethylenothiophene (5), 30 ml. of formamide, and two drops of acetic anhydride was refluxed at 165–170° for 2 hr. After being cooled, a solid product was isolated and recrystallized from dioxane, giving 0.7 g. (17%) of white product; m.p. 261–263°; IR (KBr) 1635 (NH_2), 3350 (NH_2) $cm.^{-1}$.

Anal.—Calcd. for $C_{10}H_{11}N_3S$: C, 58.23; H, 6.32; N, 21.15; S, 15.62. Found: C, 58.51; H, 5.94; N, 20.67; S, 16.01.

REFERENCES

(1) I. Baev and S. Robev, *C. R. Acad. Bulg. Sci.*, **13**, 733(1960).

(2) W. O. Foye and D. H. Kay, *J. Pharm. Sci.*, **57**, 345(1968); W. O. Foye, D. H. Kay, and P. R. Amin, *ibid.*, **57**, 1793(1968).

(3) K. Gewald, *Chem. Ber.*, **98**, 3571(1965); V. I. Shvedov, V. K. Ryzhkova, and A. N. Grimev, *Khim. Getero. Soedin.*, **1967**, 239.

(4) S. G. Cohen, in "Organosulfur Chemistry," M. J. Janssen, Ed., Interscience, New York, N. Y., 1967, chap. 3.

(5) K. Gewald, E. Schinke, and H. Bottcher, *Chem. Ber.*, **99**, 94 (1966).

(6) A. E. S. Fairfull and D. A. Peak, *J. Chem. Soc.*, **1955**, 796.

(7) K. Gewald and E. Schinke, *Chem. Ber.*, **99**, 2712(1966).

(8) R. I. H. Wang, W. Dooley, Jr., W. O. Foye, and J. Mickles, *J. Med. Chem.*, **9**, 394(1966).

(9) K. Gewald, *Chem. Ber.*, **99**, 1002(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 1, 1970, from the *Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115*

Accepted for publication March 18, 1970.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This investigation was supported by Research Grant RH00297 from the National Center for Radiological Health, U. S. Public Health Service, Bethesda, Md.

N-Substituted Aminoethanethiols and *N*-Substituted Aminoethanethiol *S*-Sulfonic Acids as Radioprotective Agents

F. I. CARROLL and MONROE E. WALL

Abstract □ Several *N*-substituted aminoethanethiols and *N*-substituted aminoethanethiol *S*-sulfonic acids were tested as potential radioprotective agents. 2-(2′-Carbamidoethylamino)-ethanethiol (I*a*), 2-(2′-carboxyethylamino)-ethanethiol (I*b*), 2-(2′-cyanoethylamino)-ethanethiol (I*c*), and 2-(2′-carbamidoethylamino)-ethanethiol *S*-sulfonic acid (II*a*) exhibited significant protective effects against ionizing radiation. Compound I*a* showed the highest activity and was selected for further radiation-protection test studies. The structure–activity relationships of this class of compounds are discussed.

Keyphrases □ Aminoethanethiols, *N*-substituted—radioprotective capacity, structure–activity relationships □ Aminoethanethiol *S*-sulfonic acids, *N*-substituted—radioprotective capacity, structure–activity relationships □ Radioprotective agents—aminoethanethiols, aminoethanethiol *S*-sulfonic acids, *N*-substituted

A recent list of the various types of compounds that show radioprotective properties has appeared and their structure–activity relationships have been discussed (1). Aminoalkylthiols constitute the most effective class of radioprotective agents. The initial discovery that 2-mercaptoethylamine (MEA) offered protection to mice against ionizing radiation (2) led to the synthesis of several hundred derivatives of this compound. Structural requirements necessary for radioprotective activity have evolved from the test results on these compounds and have been summarized (1). This effect was not observable when one or two alkyl substituents were placed on the carbon containing the thiol function of MEA (3, 4).[1] Subsequently, it was found that some *N*-substituted aminoethanethiols and *N*-substituted aminoethanethiol *S*-sulfonic acids, prepared in this laboratory, showed significant protection against ionizing radiation. In this report the radioprotection test results on these compounds are presented, and their structure–activity relationships are discussed.

[1] Subsequent antiradiation test results have shown that 2-mercapto-2-methylaminopropane hydrochloride, when administered at 90 mg./kg. i.p. using CMCTW as vehicle, gave 67% survival to mice irradiated with 825 r. (See footnotes to Table II for explanation of test data.)

Table I—Radiation-Protective Activities of *N*-Substituted Aminoethanethiols

$HSCH_2CH_2\overset{+}{N}H_2RA^-$

Compound I	R	A	Vehicle of Administration	pH of Preparation	Approx. LD_{50}, mg./kg.	Drug[a] Dose, mg./kg.	Radiation[b] Dose, r	No. Mice	Mortality by Days[c]	30-Day Survival, %[d]
a	$CH_2CH_2CONH_2$	Tos	Saline[e]	5.5	> 900	500	1100	15	000000/00000/00003/10000/00000/00000	73
			Saline	5.5		250	1100	15	000000/00000/00122/00000/00000/00000	67
			Saline	5.7		900	1000	10	000000/00000/00000/00000/00000/00000	100
			Saline	5.6		450	1000	10	000000/00000/00100/00000/00000/00000	90
			Saline	5.7		175	1000	15	000000/00023/24100/3	0
b	$CH_2CH_2CO_2H$	Tos	Water	6.9	>1200	1200	1000	15	000000/00000/00000/10010/00000/00000	87
			Water	6.9		600	1000	15	000000/00002/03411/00000/00000/00000	27
			Saline	7.8		300	800	19	000000/00012/55311/00000/00000/00000	5
			PEG[f]	5.5		593	800	6	000000/01100/02100/01	0
c	CH_2CH_2CN	Tos	Saline	5.2	500	300	825	15	000001/00001/11001/01100/10000/00000	47
			Saline	5.2		150	825	15	000000/00022/4223	0
d	$CH_2CH_2CO_2C_2H_5$	HSO_4	Water	6.8	1700	600	800	15	000000/00003/23213/01	0
e	$CH_2CH_2CO_2C_2H_5$	Tos	Water	6.2	1700	1000	825	15	001000/00010/30120/01010/11000/00000	20
			Water	6.2		500	825	15	000000/00002/54103	0
f	$CH_2CH(CH_3)CO_2(CH_2)_2\overset{+}{N}H(CH_3)_2$	2Cl	Water	6.7	200	100	825	15	000000/00022/42201/11	0
g	$CH_2CH(CH_3)CO_2(CH_2)_2\overset{+}{N}HC(CH_3)_3$	2Cl	Water	5.9	700	400	825	13	100000/00003/5211	0
h	$CH(CH_3)CH_2CO_2C_2H_5$	Tos	Water	5.5	2000	1300	825	15	001000/00011/41320/01000/00000/00000	7
			Water	5.5		650	825	15	000000/00011/54020/101	0
i	$CH_2CH(CH_3)CO_2CH_3$	Tos	Water	6.0	2200	1000	825	13	000001/00004/21311	0
j	$CH_2CH(CH_3)CO_2CH_2CH(CH_3)_2$	Tos	Water	6.5	1000	500	825	15	000000/00002/27201/1	0
k	$CH(CH_3)CH_2CO_2CH_3$	Tos	Water	6.3	1500	1000	825	15	000000/00000/03523/00010/00000/00000	7
			Water	6.3		500	825	15	000000/00011/35310/1	0

[a] Compound administered intraperitoneally as 0.5–10% solution 15 min. before irradiation. [b] 800–825 r (X-rays); 1000–1100 r (γ-rays). [c] The number of animals dying on Days 0 through 30. [d] Control mice did not survive 30 days. [e] Physiological saline solution. [f] Polyethylene glycol.

Table II—Radiation-Protective Activities of *N*-Substituted Aminoethanethiol *S*-Sulfonic Acids

$^-O_3S_2CH_2CH_2\overset{+}{N}H_2R$

Compound II	R	Vehicle of Administration	pH of Preparation	Approx. LD_{50}, mg./kg.	Drug[a] Dose, mg./kg.	Radiation[b] Dose, r	No. Mice	Mortality by Days[c]	30-Day Survival, %[d]
a	$CH_2CH_2CONH_2$	Water	5.5	1200	800	1000	15	000000/00001/02000/00000/01101/00100	53
		Water	5.5		400	1000	15	000000/00210/46001/00000/00000/00000	7
b	$CH_2CH_2CONHCH(CH_3)_2$	Saline[e]	5.6	400	250	1000	15	000000/00012/04321/2	0
c	$CH_2CH_2CONHC(CH_3)_2$	Water	6.0	350	200	1000	15	000000/00012/35012/01	0
d	$CH_2CH(CH_3)CONH_2$	CMCTW[f]	5.7	750	600	1000	15	100000/00002/04011/00000/00000/00000	40
		CMCTW	5.7		300	1000	15	000000/10012/07021/1	0
e	$CH(CH_3)CH_2CN$	CMCTW	5.6	850	600	1000	15	000000/00001/12211/11100/02000/00000	13
		CMCTW	5.6		300	1000	15	000000/00001/03533	0
f	$CH(C_6H_5)CH_2CN$	CMCTW	5.5	> 800	200	1000	15	000000/00000/375	0
g	$CH_2CH(CH_3)CO_2CH_3$	Water	5.9	>1000	800	1000	15	000001/00011/5601	0
h	$CH_2CO_2C_2H_5$	Water	5.5	>2000	2000	1000	15	000000/00001/63300/11	0

[a] Compound administered intraperitoneally as 0.5–10% solution 15 min. before irradiation. [b] 800–825 r (X-rays); 1000–1100 r (γ-rays). [c] The number of animals dying on Days 0 through 30. [d] Control mice did not survive 30 days. [e] Physiological saline solution. [f] 0.3% methylcellulose and 0.1% polysorbate 80.

METHOD

The synthesis of all the compounds listed in Tables I and II has been reported previously (5, 6). The irradiation was performed utilizing either a 300-kvp. GE Maxitron Unit, dose rate in air 45 r/min., or a ^{60}Co irradiator, which contained a 1200-c. source, with dose rate between 100–50 r/min. (7). Female mice of the Walter Reed Bagg Swiss or Inbred Charles River (ICR) strain, 5–6 weeks old and weighing 21–25 g., were used. Forty mice were exposed to whole body lethal irradiation. Equal numbers of control mice injected with only the vehicle used for the particular drug evaluation were irradiated simultaneously. The mice were exposed in a perforated Lucite dish which rotated continuously during exposure. A 30-day period for survival was observed. All control animals died before the 21st day following exposure. Survival of treated mice was interpreted as good (>45% survival), fair (25–44% survival), slight (1–24% survival), and none (0% survival).[2]

RESULTS AND DISCUSSION

Structure–activity relationship studies on MEA and its derivatives have established that the presence of a basic function and a free thiol group or function readily convertible to a free thiol *in vivo* is necessary for high radioprotective effect. Alkylation of the amino group of MEA gives compounds with varied activities. Simple *N,N*-dialkyl derivatives of MEA exhibited little or no effect, whereas significant effects were observed in *N*-(2′-phenethylamino)-ethanethiol and *N*-(2′-thienylethylamino)-ethanethiol (8). Good radioprotection was also found in several other *N*-substituted aminoethanethiols. The results are summarized in Table I. Significant activity was observed in the case of the 2-carbamidoethyl derivative (I*a*), 2-carboxyethyl derivative (I*b*), or 2-cyanoethyl derivative (I*c*), and the activity was considerably reduced with the 2-carbethoxyethyl derivatives (I*d* and I*e*). This reduction of activity could possibly be connected with the ester function, since several other ester derivatives (I*f–k*) offered either slight activity or no effect at all.

A similar structure–activity relationship was also observed in the *N*-substituted aminoethanethiol *S*-sulfonic acids: good activity when the *N*-alkyl group was 2-carbamidoethyl (II*a*) and reduced activity when a methyl group was placed alpha to the amide function, *N*-alkyl equal 2-carbamidopropyl (II*d*). Placement of alkyl substituents on the amide nitrogen, Compounds II*b* and II*c*, resulted in a complete loss of activity. With the exception of Compound II*e*, which showed only slight activity, the remaining compounds were inactive.

The good radioprotective property of Compounds I*a–c* and II*a*, with their relatively low toxicity, has created additional interest in these agents. In particular, the amide (I*a*) that showed the highest activity was selected for further radioprotection studies.

The *S*-triphenylmethyl derivative (III) of I*a* was tested for potential latent antiradiation activity but proved to be completely inactive.

$$(C_6H_5)_3CSCH_2CH_2NHCH_2CH_2CONH_2$$

III

[2] These antiradiation screening tests were performed at the Walter Reed Army Institute of Research, Washington, D.C., under the direction of J. P. Jacobus.

REFERENCES

(1) W. O. Foye, *J. Pharm. Sci.*, **58,** 283(1969), and references cited.

(2) Z. M. Bacq, A. Herve, J. Lecomte, P. Fisher, J. Blavier, G. Dechamps, H. LeBihan, and P. Rayet, *Arch. Int. Physiol.*, **59,** 442 (1951).

(3) F. I. Carroll, J. D. White, and M. E. Wall, *J. Org. Chem.*, **28,** 1236(1963).

(4) *Ibid.*, **28,** 1240(1963).

(5) F. I. Carroll, H. M. Dickson, and M. E. Wall, *ibid.*, **30,** 33 (1965).

(6) D. Rosenthal, G. Brandrup, K. H. Davis, Jr., and M. E. Wall, *ibid.*, **30,** 3689(1965).

(7) D. L. Klayman, M. M. Grenan, and D. P. Jacobus, *J. Med. Chem.*, **12,** 510(1969).

(8) A. F. Ferris, O. L. Salerni, and B. A. Schutz, *ibid.*, **9,** 391 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 14, 1969, from the *Chemistry and Life Sciences Laboratory, Research Triangle Institute, Research Triangle Park, NC 27709*

Accepted for publication March 18, 1970.

This investigation was supported by the Department of the Army and the U. S. Army Medical Research and Development Command, Contract No. DA-49-193-MD-2164.

The authors are indebted to T. R. Sweeney and J. P. Jacobus of the W.R.A.I.R. for the antiradiation test data, as well as many helpful discussions during the course of this investigation.

Drug Absorption from the Rectum III: Aspirin and Some Aspirin Derivatives

WERNER LOWENTHAL, JOSEPH F. BORZELLECA, and CHARLES D. CORDER, Jr.

Abstract ☐ The rectal absorption of aspirin, aluminum aspirin, and calcium carbaspirin was studied in dogs. Cocoa butter, polysorbate 61, polyethylene glycol mixture, and a mixture of natural saturated vegetable fatty acid glycerides were the bases used. The areas under the plasma concentration–time curves, peak salicylate levels, and the time the peak levels occurred were used as the criteria for comparison The absorption of aluminum aspirin from cocoa butter and the polyethylene glycol mixture was poor. Plasma salicylate levels from aspirin and calcium carbaspirin in the polysorbate 61 base were minimal. The highest peak and largest area under the curve were seen with calcium carbaspirin in vegetable fatty acid glycerides base The commercial aspirin product had the lowest peak and smallest area under the curve but the earliest peak. The two latest peaks in salicylate plasma levels were observed following the use of cocoa butter base suppositories. No conclusions can be reached concerning any differences in absorption of aspirin or calcium carbaspirin in the various bases or the commercial aspirin product.

Keyphrases ☐ Drug absorption—aspirin, derivatives, rectum, dog ☐ Aspirin, derivatives—drug absorption, rectum, dog ☐ Rectal absorption, dog—aspirin, derivatives ☐ Suppositories, absorption—aspirin, derivatives, dog

Suppositories resemble a type of sustained-release tablet where it is desired that the matrix rapidly disintegrates. The drug is incorporated into a wax-type matrix which can be either hydrophilic, such as polyethylene glycol waxes, or hydrophobic, such as cocoa butter. With this in mind, one would expect the ordinary suppository to release the drug more slowly than a rapidly disintegrating tablet. This should result in slower absorption and possibly a lower peak. In addition, there is relatively little fluid present in the rectum compared to the stomach and intestine. As a result, as the suppository matrix liquifies, the drug is diffused through a viscous medium to get to the absorbing membrane. This also decreases the rate of absorption and may explain the erratic and incomplete absorption that occurs in the rectum.

Aspirin suppositories became official in the USP XVII and are widely used. Coldwell and Boyd (1) reported that LD_{50} of rectally administered suppositories to male albino rats was significantly less than the LD_{50} of orally administered aspirin.

Only a few *in vivo* studies have been reported (2). Thomsen (3) indicated that polysorbate 60 at 20% concentration modified aspirin absorption from a mixture of natural saturated vegetable fatty acid glycerides base.[1] The surfactant caused an earlier peak level. Coldwell *et al.* (4) studied the effect of dosage form and route of administration on the absorption and excretion of aspirin in 10 human volunteers. Results indicated that oral absorption from tablets (640 mg.) proceeded uniformly with a peak in 2 hr. Absorption from suppositories (640 mg.) was more variable. Aspirin tablets given rectally had lower levels and gave erratic blood level patterns. The rate of disappearance of salicylate from plasma was slower for the rectal route than the oral route. No salicylate could be detected in the plasma following administration of an aspirin suspension rectally. Recovery of salicylate from urine was less for the tablets and the suspension given rectally than for tablets given orally or for aspirin rectal suppositories. Neuwald and Kunze (5) found that *in vitro* dissolution tests with suppositories were misleading and did not predict *in vivo* absorption.

Since there is a potential bioavailability problem with rectal suppositories and due to the potential toxic hazard, especially in children, it was decided to investigate the effects of various bases on the absorption of aspirin, aluminum aspirin,[2] and calcium carbaspirin (calcium aspirin carbamide).[3]

Aspirin is soluble to the extent of 1 g. in 100 ml. water at 37° (6); aluminum aspirin NF is "insoluble"; and 1 g. calcium carbaspirin dissolves in 4.33 ml. of water at 20° (7). These were used to represent aspirin compounds with three different solubilities. The rate of absorption and the amount absorbed are dependent upon the rate of release of the drug from the dosage form matrix and the rate of solution of the drug in the rectum. To evaluate these factors, the three forms of aspirin were incorporated in four different suppository bases. The bases were: (*a*) cocoa butter USP;[4] (*b*) a mixture containing partial glycerides or triglycerides of natural saturated vegetable fatty acids of C_{12-18} chain length with m.p. 33.5–35.5°, saponification value of 220–230, iodine value (Kaufman) of >7, and a hydroxyl value of 50–56[1] (S-55); (*c*) polysorbate 61 [polyoxyethylene (4) sorbitan monostearate];[5] and (*d*) a mixture of polyethylene glycols (PEG).

Cocoa butter and S-55 are hydrophobic bases melting below the normal body temperature of humans and dogs. Cocoa butter is widely used and may be considered a "standard" against which other bases are compared. S-55 represents the new synthetic bases which are being used as cocoa butter substitutes. Polysorbate 61 is a tan waxy solid, dispersible in water, with a pour-point of approximately 38° and a hydrophile–lipophile balance of 9.6, and represents the nonionic surfactant type of base. The polyethylene glycol mixture is a water-soluble base but does not melt at normal body temperature.

[1] Witepsol S-55, Chemische Werke Witten, G.m.b.H., Riches-Nelson, Inc.

[2] Abbott Laboratories, North Chicago, IL 60064
[3] Calurin, Dorsey Laboratories, a Division of the Wander Co.
[4] Charles Huisking & Co., Inc.
[5] Tween 61, Atlas Chemical Industries, Inc., Wilmington, DE 19899

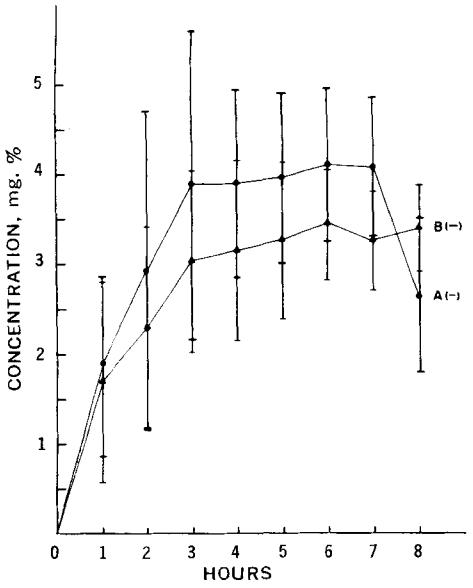


Figure 1—*Plasma salicylate levels for cocoa butter base suppositories. A, aspirin, standard deviation (—); B, calcium carbaspirin, standard deviation (—).*

As a result, three different drugs were to be studied in four different bases, resulting in 12 different products. A commercial aspirin product in a water-soluble base was included for comparative purposes.

EXPERIMENTAL

Procedures and Methods—These were the same as those previously reported (2). The dogs used weighed between 11.3 and 14.5 kg. Dogs were used in this study because of their larger size and because the rectal physiology of the dog and the human are similar. Four or five dogs were used for each preparation.

Analytical Procedure—These were the same as those previously reported (2).

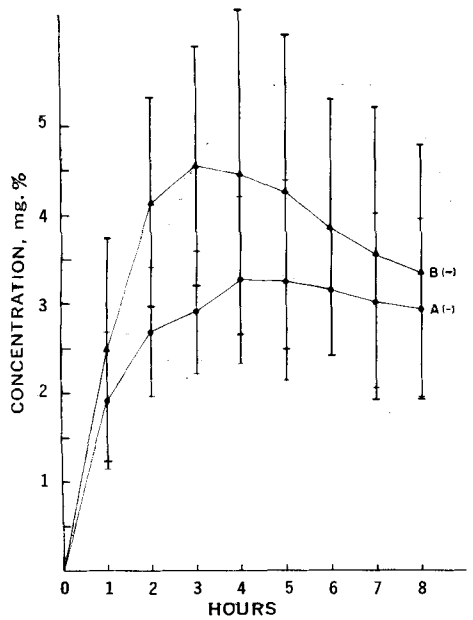


Figure 2—*Plasma salicylate levels for S-55 base suppositories. A, aspirin, standard deviation (—); and B, calcium carbaspirin, standard deviation (—).*

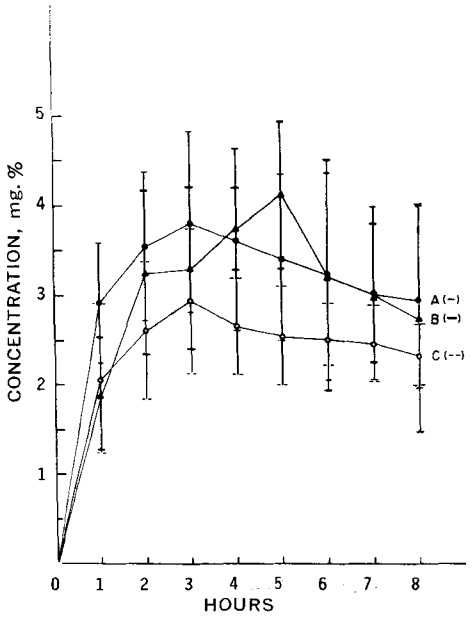


Figure 3—*Plasma salicylate levels for PEG mixture suppository base and the commercial aspirin product. A, aspirin, standard deviation (—); B, calcium carbaspirin, standard deviation (—); and C, commercial aspirin product, standard deviation (— —).*

Suppository Formulation—Aspirin[6] USP comminuted through an 80-mesh screen, aluminum aspirin[2] NF, and calcium carbaspirin[3] were used. The suppositories were made by the hot-melt method using metal molds. Drug displacement in the four bases was first determined, and the amount of base required was calculated (8). The drugs were mixed with the melted base and poured into molds; the molten mass was allowed to solidify in a refrigerator. The suppositories were removed from the molds and stored in a refrigerator in a well-closed container until used. The same molds were always used. The PEG base was made from 6 parts polyethylene glycol 1540[7] and 4 parts polyethylene glycol 6000[7]

A 330-mg. dose of aspirin base or equivalent amounts of aluminum aspirin and calcium carbaspirin were used. This dose was lower than the one used in the previous study. This was deemed necessary to reduce the interference by the biotransformation systems (9).

RESULTS AND DISCUSSION

Aluminum aspirin was poorly absorbed from cocoa butter and PEG bases, and in two dogs no salicylate could be detected. When salicylate was detected in the plasma, its appearance was later then either the aspirin or calcium carbaspirin. Due to the insignificance of the low levels obtained, the data were not tabulated. The peak was about 1.21 mg.% in 4–7 hr. in cocoa butter and about 0.81 mg.% in 3–7 hr. in PEG base.

Calcium carbaspirin in polysorbate 61 base caused defecation and expulsion of the suppositories in 3 out of 4 dogs. Although there was no visual evidence of damage to the mucosa nor was any bleeding evident, irritation may have occurred and could have caused expulsion. Aspirin in polysorbate 61 base did not cause defecation, but the suppository or parts of it was removed in 3 out of 4 dogs. Again there was no visual evidence of mucosal damage or blood. The salicylate levels for these two products were very low and were not tabulated because they would be of doubtful value. The peak level for calcium carbaspirin was 1.84 mg.% in 8 hr., for aspirin it was 1.74 mg.% in 4–8 hr.

Individual dogs were fairly consistent in the salicylate levels they exhibited after administration of the various products; *e.g.*, dogs that showed high salicylate levels generally did so for all products.

[6] Aspirin, Merck & Co., Inc., Rahway, NJ 07065
[7] Carbowax 1540 and Carbowax 6000, Union Carbide Chemicals Co., New York, NY 10017

Table I—Data from Graphical Concentration of Salicylate in Plasma–Time Plots

Preparation	Area under Curve, mg./hr.	Peak Level	
		Height,[a] mg. %	Time,[a] hr.
Aspirin–cocoa butter	26.1	4.10 (2.85–6.27)	6(3–7)
Calcium carbaspirin–cocoa butter	21.9	3.46 (2.85–4.40)	6(4–6)
Aspirin–commercial product	18.9	2.94 (1.86–3.70)	3(2–4)
Aspirin–PEG	24.9	3.80 (2.77–4.95)	3(3–4)
Calcium carbaspirin–PEG	23.8	4.12 (3.28–4.91)	5(4–6)
Aspirin–S-55	21.7	3.27 (2.48–4.30)	4(2–5)
Calcium carbaspirin–S-55	29.0	4.56 (2.95–6.39)	3(3–4)

[a] Obtained by averaging data for individual dogs. Numbers in parenthesis are the ranges.

The average salicylate levels at the various sampling intervals and the standard deviations are shown in Figs. 1–3. As can be seen, there are no trends or consistencies among the drugs or bases.

Areas under the curves were determined with a planimeter.[8] These findings, together with the height of the peaks and time the peak levels occurred, are given in Table I. Using this information the products were ranked in the following ways: (*a*) order of decreasing area under the curves; (*b*) order of decreasing peak height; and (*c*) order of increasing length of time for peak blood level to occur.

Decreasing Area under the Curve:

1. Calcium carbaspirin—S-55
2. Aspirin—cocoa butter
3. Aspirin—PEG
4. Calcium carbaspirin—PEG
5. Calcium carbaspirin—cocoa butter
6. Aspirin—S-55
7. Commercial aspirin product

Decreasing Height of Peak:

1. Calcium carbaspirin—S-55
2. Calcium carbaspirin—PEG
3. Aspirin—cocoa butter
4. Aspirin—PEG
5. Calcium carbaspirin—cocoa butter
6. Aspirin—S-55
7. Commercial aspirin product

Time Peak Occurs:

1. Commercial aspirin product
2. Calcium carbaspirin—S-55
3. Aspirin—PEG
4. Aspirin—S-55
5. Calcium carbaspirin—PEG
6. Calcium carbaspirin—cocoa butter
7. Aspirin—cocoa butter

From these rankings the following conclusions can be made:

1. Calcium carbaspirin in S-55 base has the highest peak and the largest area under the curve but only the second earliest peak.
2. The commercial aspirin suppositories in a water-soluble base had the smallest area under the curve and the lowest peak height, but the peak occurred earliest.
3. Calcium carbaspirin in cocoa butter base ranked fifth in area under the curve and peak height and sixth in time of occurrence of the peak.
4. Aspirin in S-55 base ranked sixth in area under the curve and height of peak level but was fourth in time that the peak level occurred.
5. The two latest peaks in salicylate blood levels were observed following the use of cocoa butter base suppositories.

No conclusions can be made concerning any difference in absorption of aspirin or calcium carbaspirin in cocoa butter, PEG, or the synthetic fatty base. Aluminum aspirin appears to be only poorly absorbed from the rectum in 8 hr. The reasons may be the insolubility of the salt and the lack of fluid to dissolve it. Also the reason postulated by Levy and Procknal (10) that a protective gel forms around the drug to reduce its bioavailability may also occur in the rectum. Polysorbate 61 appears to cause irritations resulting in expulsion of the suppositories, although absorption from this base does occur. There was no visual evidence of tissue damage or bleeding due to polysorbate 61. The results here do confirm those of Neuwald and Kunze (5) who reported that aspirin and calcium salicylate absorption was identical. The peak blood levels were of similar height. Cummings *et al.* (11) reported that a polymeric condensation product of Al_2O_3 and aspirin from the interaction of aluminum isopropoxide and aspirin was absorbed equally well as was aspirin after oral administration. Absorption of the aluminum aspirin was delayed about 0.5 to 1 hr, but the total salicylate excreted in the urine was the same for the two products. This aluminum aspirin compound should also be tested for rectal absorption.

[8] K and E Compensating Polar Planimeter, model 4236M.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 3, 1970, from the *Departments of Pharmacy and Pharmacology, Health Sciences Division, Virginia Commonwealth University, Richmond, VA 23219*

Accepted for publication March 27, 1970.

This investigation was supported in part by a grant from the U. S. Office of Naval Research, Number N-00014-68-A-0510.

The assistance of Charles O'Rear in the assay of the suppositories is gratefully acknowledged. The cooperation of Abbott Laboratories, Dorsey Laboratories, Riches-Nelson, Inc., Atlas Chemical Industries, Inc., and Union Carbide Chemicals Co. for supplying the drugs and the chemicals is also gratefully acknowledged.

REFERENCES

(1) B. B. Coldwell and E. M. Boyd, *Can. J. Physiol. Pharmacol.*, **44**, 909(1966).

(2) W. Lowenthal and J. F. Borzelleca, *J. Pharm. Sci.*, **54**, 1790(1965).

(3) H. W. L. Thomsen, *An. Fac. Quim. Farm. Univ. Chile*, **18**, 158(1966); through *Chem. Abstr.*, **68**, 103679m(1968).

(4) B. B. Coldwell, G. Solomonraj, E. M. Boyd, J. Jantz, and A. B. Morrison, *Clin. Toxicol.*, **2**, 111(1969).

(5) F. Neuwald and F. Kunze, *Arzneim.-Forsch.*, **14**, 1162 (1964).

(6) "The Merck Index," 8th ed., Merck & Co., Inc., Rahway, N. J., 1968, p. 13.

(7) Dorsey Laboratory, Calurin Specifications.

(8) "Remington's Pharmaceutical Sciences," 13th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965, pp. 551–552.

(9) A. J. Cummings, B. K. Martin, and R. Renton, *Brit. J. Pharmacol.*, **26**, 461(1966).

(10) G. Levy and J. A. Procknal, *J. Pharm. Sci.*, **51**, 294(1962).

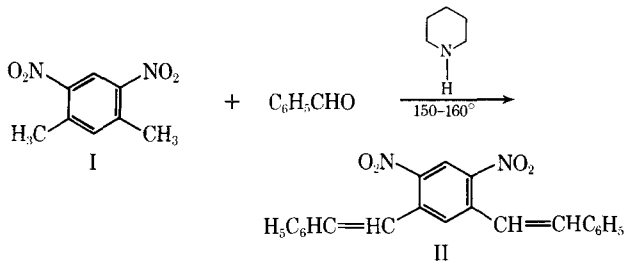
(11) A. J. Cummings, B. K. Martin, and L. F. Wiggins, *J. Pharm. Pharmacol.*, **15**, 56(1963).

Preparation and Biological Activity of Substituted 1,3-Distyryl-4,6-dinitrobenzenes

Keyphrases □ 1,3-Distyryl-4,6-dinitrobenzenes—synthesis □ Cytotoxic, antimicrobial activity—1,3-distyryl-4,6-dinitrobenzenes

Sir:

In 1931, it was shown by Ruggil *et al.* (1) that one molecule of 4,6-dinitro-1,3-xylene (I) condensed with two molecules of benzaldehyde to give 1,3-distyryl-4,6-dinitrobenzene (II) (Scheme I). As an extension of this approach, we studied the condensation of a variety of



Scheme I

substituted aromatic aldehydes with 4,6-dinitro-1,3-xylene. The condensations were conducted by heating under reflux a solution of the appropriate aromatic aldehyde (0.2 mole), Compound I (0.1 mole), and piperidine (10 ml.) for 20–90 min. at 150–160°. The reaction mixture was diluted with a large volume of benzene or 75% ethanol, and the crystalline products (III) (Table I) were isolated by filtration and drying. The yields of 1,3-distyryl-4,6-dinitrobenzenes ranged between 40 and 60% and furnished satisfactory elemental (C, H, and N) analyses.

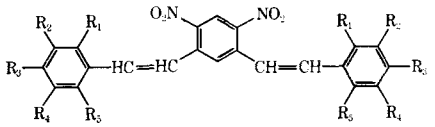
These compounds were examined for their antimicrobial activities (2). In these tests, the microorganisms were grown in agar media. The compounds to be tested were dissolved in acetone at concentrations of 1 mg./ml. and applied to paper disks of 13-mm. diameter. After incubation, the diameters of the zones of growth inhibition were measured. The compounds which showed significant antimicrobial activity are given in Table I.

Some of these compounds were also subjected to L-1210 *in vitro* assay for cytotoxic activity (3). In these screening experiments, the samples were weighed (about 5–10 mg.) into glass homogenizers (12-ml. size) sterilized with 0.1 ml. of 70% ethanol and about 0.1 ml. dimethylsulfoxide (DMSO) was added to help solubilize. The sample was ground with sterile water to make a suspension containing L-1210 leukemic cells. The tubes were stoppered and incubated at 37° for 3 days; then cell counts were made on each tube by a Coulter counter. The percent inhibition and the ID_{50} values were calculated. Values of 1 or less for ID_{50} were considered potentially

Table II—Cytotoxic Activity of Compounds III*f* and III*g*[a]

Compound		ID_{50}
III*f*	R_2 = OCH_3, R_3 = OH	0.188
III*g*	R_2 = OH, R_3 = OCH_3	0.648

[a] See Table I for Structure III.



III

Table I—Chemical and Biological Activity Data of Compounds III[a]

Test Organism	Compounds				
	III*a* R_1 = OH, R_5 = NO_2	III*b* R_1 = OCH_3	III*c* R_2 = NO_2	III*d* R_1 = OC_2H_5, R_2 = OCH_3	III*e* R_1 = Cl
	Zones of Inhibition, mm.				
Bacillus subtilis	29	25	25	17	29
Bacillus cereus	19	18	17		18
Staphylococcus aureus	25	22	23	17	25
Mycobacterium phlei	25	25	25	16	24
Bacillus subtilis (synthetic agar)	42	40	37	30	34
Escherichia coli (synthetic agar)	25	25	24		25
Propionibacterium thonii	37	32	33	16	30
Trigonopsis variabilis	21	20	21	17	22
Glomerella cingulata			20		
Chlorella vulgaris	30	22	25		20
Melting point	207–208°	218–219°	300°	161–162°	228–229°

[a] All R groups are H unless otherwise specified.

active. Only the compounds given in Table II showed significant cytotoxic activity.

(1) P. Ruggil, A. Zimmerman, and R. Thouvay, *Helv. Chim. Acta*, **24**, 1250(1931).
(2) L. J. Hanka, *Abstracts, Int. Congr. Chemother., Proc., 5th*, **B 912**, 351(1967).
(3) H. H. Buskirk, *Proc. Tissue Culture Ass.*, **20**, 23(1969).

ROBERT E. HARMON
S. K. GUPTA
J. L. HANSEN
Western Michigan University
Kalamazoo, MI 49001

L. J. HANKA
Research Laboratories
The Upjohn Company
Kalamazoo, MI 49001

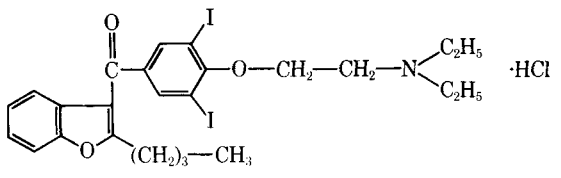
Received March 23, 1970.
Accepted for publication May 15, 1970.
The work at Western Michigan University was supported by Grant CA-6140 from the National Institutes of Health. The assistance of Mr. H. H. Buskirk of The Upjohn Company in testing these compounds for cytotoxic activity is gratefully acknowledged.

Observations on the Micelle Formation of 2-Butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl Ketone Hydrochloride (SK&F 33134-A) by NMR Spectroscopy

Keyphrases □ 2-Butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride (SK&F 33134-A)—micelle formation □ Critical micelle concentration—SK&F 33134-A □ NMR spectroscopy—micelle formation determination

Sir:

In recent years, several papers (1–3) have dealt with the use of high-resolution NMR for the determination of CMC. In our laboratories, we have used NMR to show the existence of micelles in a 5% aqueous solution of SK&F 33134-A(I).[1] Micelle formation has not previously been reported for this system.



I

All measurements were carried out with a Jealco C60H NMR spectrometer equipped with a variable temperature probe. Spectra were recorded at temperatures ranging from 25 to 95°. Figure 1 illustrates the NMR spectrum for a 5% solution of SK&F 33134-A in D_2O at 25°. There are no sharp resonance lines as one would expect under normal conditions. Instead, there is a large broadening effect of all resonance lines. We attribute this broadening to dipolar interaction in the micellar structure where molecular motion is se-

[1] Marketed as Cordarone by Labaz Laboratories in several European countries.

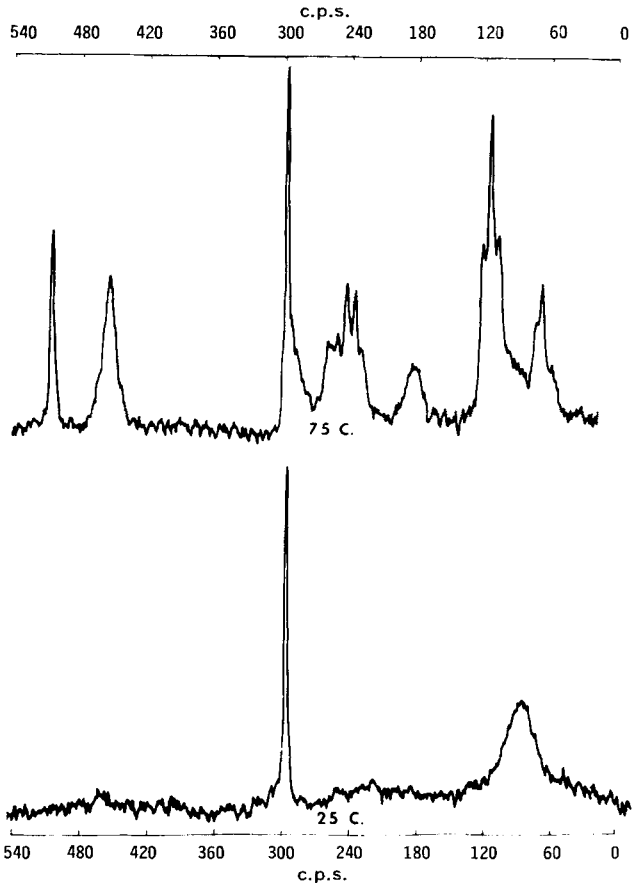


Figure 1—*NMR spectra for a 5% solution of SK&F 33134-A in D_2O at 25 and 75°, respectively.*

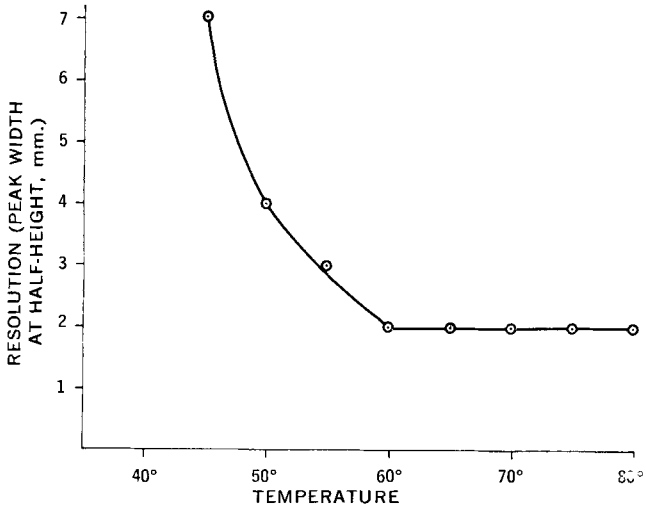


Figure 2—*A plot showing the effect of temperature on the resolution (peak width at half-height) at 504 c.p.s. for a 5% solution of SK&F 33134-A in D_2O.*

active. Only the compounds given in Table II showed significant cytotoxic activity.

(1) P. Ruggil, A. Zimmerman, and R. Thouvay, *Helv. Chim. Acta*, **24**, 1250(1931).
(2) L. J. Hanka, *Abstracts, Int. Congr. Chemother., Proc., 5th*, **B 912**, 351(1967).
(3) H. H. Buskirk, *Proc. Tissue Culture Ass.*, **20**, 23(1969).

ROBERT E. HARMON
S. K. GUPTA
J. L. HANSEN
Western Michigan University
Kalamazoo, MI 49001

L. J. HANKA
Research Laboratories
The Upjohn Company
Kalamazoo, MI 49001

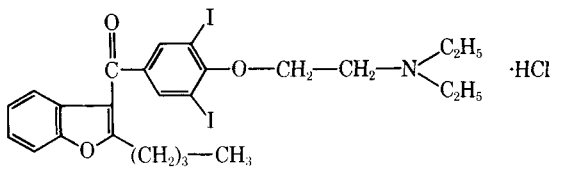
Received March 23, 1970.
Accepted for publication May 15, 1970.
The work at Western Michigan University was supported by Grant CA-6140 from the National Institutes of Health. The assistance of Mr. H. H. Buskirk of The Upjohn Company in testing these compounds for cytotoxic activity is gratefully acknowledged.

Observations on the Micelle Formation of 2-Butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl Ketone Hydrochloride (SK&F 33134-A) by NMR Spectroscopy

Keyphrases □ 2-Butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride (SK&F 33134-A)—micelle formation □ Critical micelle concentration—SK&F 33134-A □ NMR spectroscopy—micelle formation determination

Sir:

In recent years, several papers (1–3) have dealt with the use of high-resolution NMR for the determination of CMC. In our laboratories, we have used NMR to show the existence of micelles in a 5% aqueous solution of SK&F 33134-A(I).[1] Micelle formation has not previously been reported for this system.



I

All measurements were carried out with a Jealco C60H NMR spectrometer equipped with a variable temperature probe. Spectra were recorded at temperatures ranging from 25 to 95°. Figure 1 illustrates the NMR spectrum for a 5% solution of SK&F 33134-A in D_2O at 25°. There are no sharp resonance lines as one would expect under normal conditions. Instead, there is a large broadening effect of all resonance lines. We attribute this broadening to dipolar interaction in the micellar structure where molecular motion is se-

[1] Marketed as Cordarone by Labaz Laboratories in several European countries.

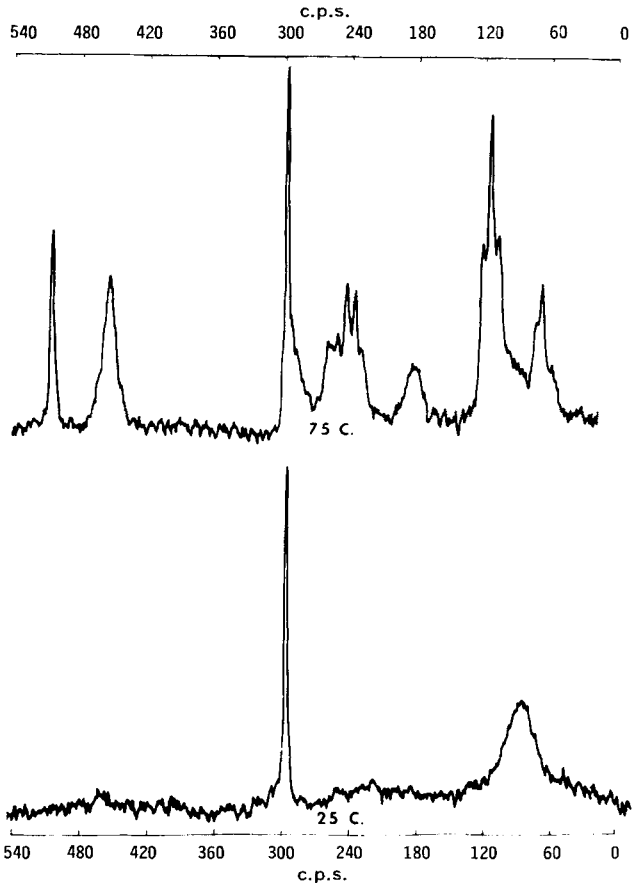


Figure 1—*NMR spectra for a 5% solution of SK&F 33134-A in D_2O at 25 and 75°, respectively.*

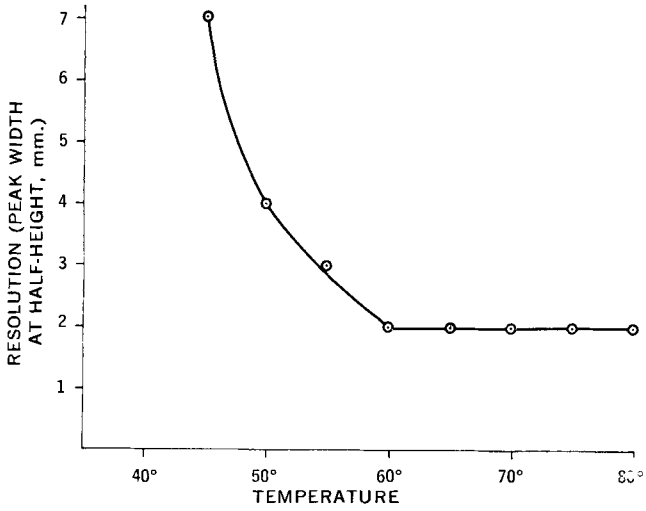


Figure 2—*A plot showing the effect of temperature on the resolution (peak width at half-height) at 504 c.p.s. for a 5% solution of SK&F 33134-A in D_2O.*

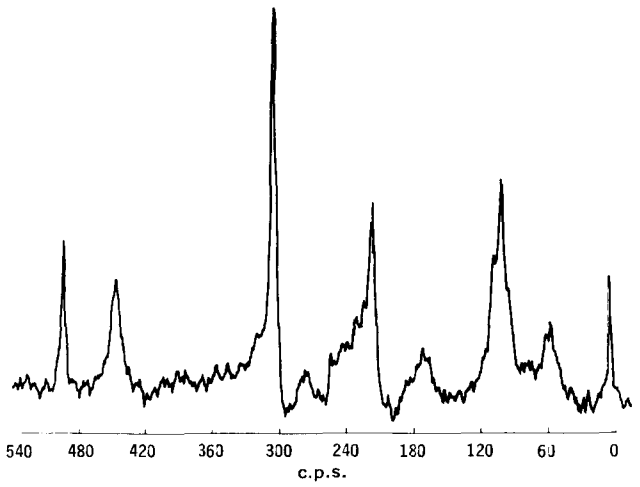


Figure 3—*NMR spectrum for SK&F 33134-A solution diluted with methanol-d_4.*

verely inhibited. Raising the temperature of this solution in small increments and recording the NMR spectra at selected temperature intervals resulted in spectra having increasing resolutions, with corresponding increases in signal intensities. The spectrum for SK&F 33134-A at 75° is also shown in Fig. 1. The sharpening of the spectrum with an increase in signal intensity suggests the gradual breakdown of the micellar structure as the temperature increases. The resolution reaches its maximum at approximately 60°. We assume that the micellar structure is completely disrupted at this temperature. The effect is further illustrated in Fig. 2, which contains a plot of band width at half-height of the aromatic protons on the iodinated benzene ring at 504 c.p.s. (resolution) *versus* temperature. The fact that band width at half-height reaches a minimum constant value at approximately 60° again illustrates the apparent breakdown of the micellar structure at this temperature.

Water-miscible organic solvents have been shown to have some effect on micelle formation (4). Figure 3 illustrates the effect of the addition of methanol-d_4 to the micellar solution of SK&F 33134-A at 25°. The NMR spectrum becomes highly resolved, indicating that the micelle has been disrupted.

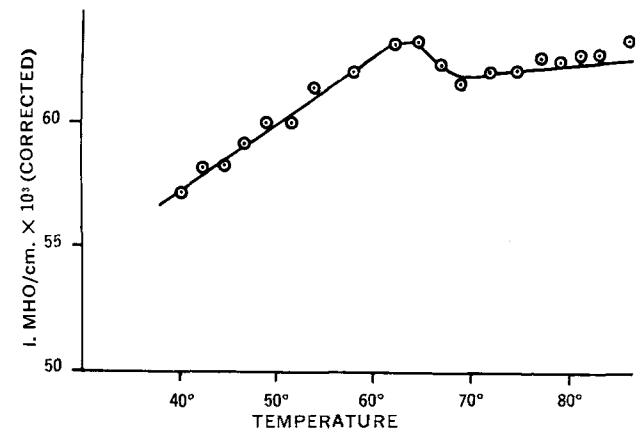


Figure 4—*Plot showing the effect of temperature on the specific conductance of a 5% aqueous solution of SK&F 33134-A.*

To substantiate the effect of temperature on the micellar state of SK&F 33134-A, conductivity studies were done over the same temperature range. A Serfass conductivity bridge, model RCM15B1, and a Beckman K 1.00/cm. conductivity cell were used. An aqueous solution of SK&F 33134-A was poured into two small jacketed glass vessels connected to each other and to a constant-temperature bath. The conductivity cell was immersed into one vessel and a thermometer into the other vessel. The temperature of the water bath was increased slowly. The temperature and the conductivity of the test solution were recorded. The results are shown in Fig. 4. The conductivity increases gradually to 60° and then becomes relatively constant. These conductivity data are in good agreement with the NMR data, indicating the presence of micelles at 25° and the subsequent complete destruction of the micelles as the temperature is increased. In both cases the temperature effect is reversible.

(1) J. Clifford and B. A. Pethica, *Trans. Faraday Soc.*, **61**, 182 (1965).

(2) J. F. Yan and M. B. Palmer, *J. Colloid. Interface Sci.*, **30**, 177 (1969).

(3) J. C. Eriksson, *Acta Chem. Scand.*, **17**, 1478(1963).

(4) J. L. Moilliet and B. Collie, "Surface Activity," D. Van Nostrand, New York, N. Y., 1951, p. 52.

RICHARD J. WARREN
R. JOHN STEDMAN*
ELIE G. SHAMI†
ELISABETH S. RATTIE
LOUIS J. RAVIN

Research and Development Division
Smith Kline & French Laboratories
Philadelphia, PA 19101

Received January 30, 1970.
Accepted for publication June 3, 1970.

* Present address: Temple University School of Pharmacy, Philadelphia, Pa.

† Present address: Endo Laboratories, Garden City, L.I., N.Y.

Imidazole and Pyrazole Bis(2-fluoroethyl)triazenes

Keyphrases □ Bis(2-fluoroethyl)triazene derivatives—synthesis □ Antileukemic activity—triazenoimidazoles

Sir:

Among a considerable number of 5-(disubstituted-triazeno) and 5-(monosubstituted-triazeno) derivatives of imidazole-4-carboxamide and of imidazole-4-carboxylic acid esters tested against lymphoid leukemia L-1210, 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (I, NSC-82196) has proved to be the most effective. In certain of the standard L-1210 tests, a majority of the afflicted mice treated with NSC-82196

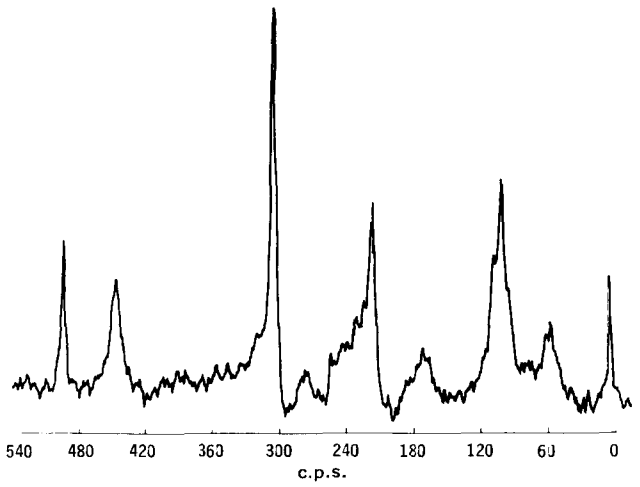


Figure 3—*NMR spectrum for SK&F 33134-A solution diluted with methanol-d_4.*

verely inhibited. Raising the temperature of this solution in small increments and recording the NMR spectra at selected temperature intervals resulted in spectra having increasing resolutions, with corresponding increases in signal intensities. The spectrum for SK&F 33134-A at 75° is also shown in Fig. 1. The sharpening of the spectrum with an increase in signal intensity suggests the gradual breakdown of the micellar structure as the temperature increases. The resolution reaches its maximum at approximately 60°. We assume that the micellar structure is completely disrupted at this temperature. The effect is further illustrated in Fig. 2, which contains a plot of band width at half-height of the aromatic protons on the iodinated benzene ring at 504 c.p.s. (resolution) *versus* temperature. The fact that band width at half-height reaches a minimum constant value at approximately 60° again illustrates the apparent breakdown of the micellar structure at this temperature.

Water-miscible organic solvents have been shown to have some effect on micelle formation (4). Figure 3 illustrates the effect of the addition of methanol-d_4 to the micellar solution of SK&F 33134-A at 25°. The NMR spectrum becomes highly resolved, indicating that the micelle has been disrupted.

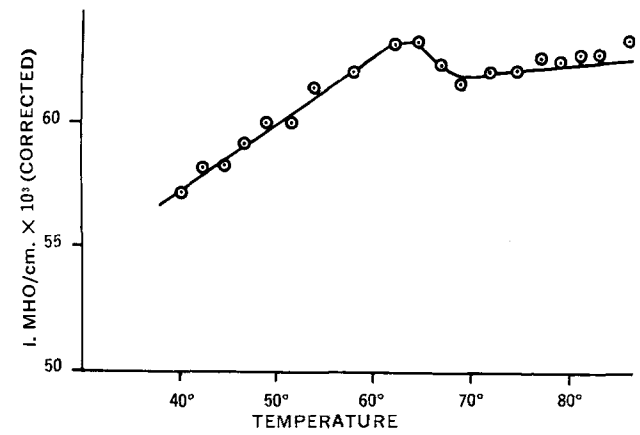


Figure 4—*Plot showing the effect of temperature on the specific conductance of a 5% aqueous solution of SK&F 33134-A.*

To substantiate the effect of temperature on the micellar state of SK&F 33134-A, conductivity studies were done over the same temperature range. A Serfass conductivity bridge, model RCM15B1, and a Beckman K 1.00/cm. conductivity cell were used. An aqueous solution of SK&F 33134-A was poured into two small jacketed glass vessels connected to each other and to a constant-temperature bath. The conductivity cell was immersed into one vessel and a thermometer into the other vessel. The temperature of the water bath was increased slowly. The temperature and the conductivity of the test solution were recorded. The results are shown in Fig. 4. The conductivity increases gradually to 60° and then becomes relatively constant. These conductivity data are in good agreement with the NMR data, indicating the presence of micelles at 25° and the subsequent complete destruction of the micelles as the temperature is increased. In both cases the temperature effect is reversible.

(1) J. Clifford and B. A. Pethica, *Trans. Faraday Soc.*, **61**, 182 (1965).

(2) J. F. Yan and M. B. Palmer, *J. Colloid. Interface Sci.*, **30**, 177 (1969).

(3) J. C. Eriksson, *Acta Chem. Scand.*, **17**, 1478(1963).

(4) J. L. Moilliet and B. Collie, "Surface Activity," D. Van Nostrand, New York, N. Y., 1951, p. 52.

RICHARD J. WARREN
R. JOHN STEDMAN*
ELIE G. SHAMI†
ELISABETH S. RATTIE
LOUIS J. RAVIN

Research and Development Division
Smith Kline & French Laboratories
Philadelphia, PA 19101

Received January 30, 1970.
Accepted for publication June 3, 1970.

* Present address: Temple University School of Pharmacy, Philadelphia, Pa.

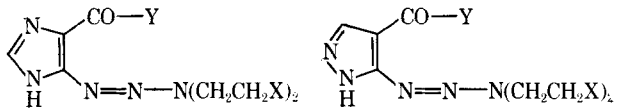
† Present address: Endo Laboratories, Garden City, L.I., N.Y.

Imidazole and Pyrazole Bis(2-fluoroethyl)triazenes

Keyphrases □ Bis(2-fluoroethyl)triazene derivatives—synthesis □ Antileukemic activity—triazenoimidazoles

Sir:

Among a considerable number of 5-(disubstituted-triazeno) and 5-(monosubstituted-triazeno) derivatives of imidazole-4-carboxamide and of imidazole-4-carboxylic acid esters tested against lymphoid leukemia L-1210, 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (I, NSC-82196) has proved to be the most effective. In certain of the standard L-1210 tests, a majority of the afflicted mice treated with NSC-82196



I: Y = NH_2; X = Cl
II: Y = NH_2; X = F
III: Y = OCH_3; X = F
IV: Y = NH_2; X = F
V: Y = OC_2H_5; X = F

The data in Table I show that the two bis(2-fluoroethyl)triazenoimidazoles (II and III) increased the lifespan of leukemic mice by 50–70% at tolerated doses. By way of comparison, the doses of NSC-82196 reported (1, 2) to be most effective are 300–625 mg./kg. for single-dose treatment and 50–100 mg./kg./day for daily treatment. The data appear to justify the following conclusions: (*a*) the bis(2-fluoroethyl)triazenes are more toxic than is NSC-82196; (*b*) at doses tolerated by the host animals, II and III are less effective than NSC-82196; and (*c*) in the standard L-1210 test system, the increases in lifespan caused by II and III are comparable to those produced by the corresponding dimethyltriazenes of the amide (NSC-45388) (8) and methyl ester (NSC-87982) (5) series.

Both the v-triazole (9) and the pyrazole (10) analogs of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388, DIC) cause significant increases in the lifespan of mice bearing L-1210, and other triazenopyrazole amides and esters have likewise demonstrated activity against L-1210 (7). However, in tests conducted in accordance with the protocols of the Cancer Chemotherapy National Service Center, amide IV displayed minimal activity, whereas the ethyl ester did not significantly increase survival time as a result of either the single-dose or the daily therapeutic regimens. Again, both are more toxic than NSC-82196.

Amides II and IV, like NSC-82196, undergo a change in aqueous solutions to ionic transformation products (11, 12), but the bis(2-fluoroethyl)triazeno derivatives are considerably more stable than NSC-82196. Esters III and V likewise undergo a change, presumed to be the same type. Obviously, the greater toxicity of II–V, in comparison with NSC-82196, may be due to replacement of chloro groups by fluoro groups. It is also conceivable that the lower toxicity of NSC-82196 results in part from its instability. If this is true, the instability may be advantageous.

(1) Y. F. Shealy and C. A. Krauth, *Nature*, **210**, 208(1966).
(2) G. Hoffman, I. Kline, M. Gang, D. D. Tyrer, J. M. Venditti, and A. Goldin, *Cancer Chemother. Rep. (Part I)*, **52**, 715(1968).
(3) I. Wodinsky, J. Swiniarski, and C. J. Kensler, *ibid.*, **52**, 393 (1968).
(4) G. R. Pettit and R. L. Smith, *Can. J. Chem.*, **42**, 572(1964).
(5) Y. F. Shealy, C. A. Krauth, R. F. Pittillo, and D. E. Hunt, *J. Pharm. Sci.*, **56**, 147(1967).
(6) C. C. Cheng, R. K. Robins, K. C. Cheng, and D. C. Lin, *ibid.*, **57**, 1044(1968).
(7) Y. F. Shealy and C. A. O'Dell, unpublished data.
(8) Y. F. Shealy, J. A. Montgomery, and W. R. Laster, Jr., *Biochem. Pharmacol.*, **11**, 674(1962).
(9) Y. F. Shealy and C. A. O'Dell, *J. Med. Chem.*, **9**, 733(1966).
(10) C. N. Noell and C. C. Cheng, *ibid.*, **12**, 545(1969).
(11) D. J. Abraham, J. S. Rutherford, and R. D. Rosenstein, *ibid.*, **12**, 189(1969).
(12) Y. F. Shealy, C. A. Krauth, L. B. Holum, and W. E. Fitzgibbon, *J. Pharm. Sci.*, **57**, 83(1968).

Y. Fulmer Shealy
C. Allen O'Dell
Kettering-Meyer Laboratories
Southern Research Institute
Birmingham, AL 35205

Received March 12, 1970.
Accepted for publication May 22, 1970.
The work described was supported by Contract No. PH43-64-51 from Chemotherapy, National Service Center, National Institutes of Health, U. S. Public Health Service. Elemental analyses and spectrophotometric determinations were performed under the supervision of Dr. W. C. Coburn, Jr., by members of the Molecular Spectroscopy Section of this Institute. Screening of Compounds II–V was performed under the auspices of Chemotherapy, National Service Center, Contract No. PH43-65-594, and under the supervision of Dr. F. M. Schabel, Jr., and Dr. W. R. Laster, Jr., of the Chemotherapy Department of this Institute.

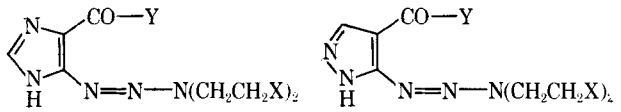
Aggregation Mechanisms in Pharmaceutical Suspensions

Keyphrases ☐ Suspensions, pharmaceutical—aggregation mechanism ☐ Flocculation, coagulation—suspensions

Sir:

The method of prevention of impaction and caking in pharmaceutical suspensions by controlled flocculation is usually credited to Haines and Martin (1). The work of these authors is, however, sometimes quoted in review articles (2, 3) without reference to the important criticisms subsequently published by Wilson and Ecanow (4) and Ecanow *et al.* (5). We have endorsed (6) some of these criticisms, but have suggested that several generalizations proposed by Ecanow and his coworkers were based on inadequately controlled experiments. The purpose of this communication is to clarify some aspects of suspension theory recently commented upon by Ecanow *et al.* (7), since this area is of considerable importance to the pharmaceutical formulator.

We are grateful to Ecanow *et al.* (7) for amplifying some points in their earlier paper (5), since we had previously found that the almost complete absence of experimental data, such as particle size of the drug and concentration of electrolyte, made an adequate appraisal impossible. Despite the recent criticisms of these authors, we see no reason to retract from our claim that Figure 1 in *Reference 6* demonstrates differences between coagulation and flocculation. Ecanow and coworkers appear to have forgotten that suspensions of drugs in anionic surfactants (1, 8) and cationic and nonionic surfactants (9) in the absence of electrolyte cake on storage. The control suspension described in the uppermost curve of Figure 1 in *Reference 6* caked after ultimate sedimentation. However, we do not consider it semantically or scientifically helpful to refer to this process as coagulation for the following reason. If the particles were slightly smaller, they would remain in permanent colloidal suspension due to Brownian motion



I: Y = NH_2; X = Cl
II: Y = NH_2; X = F
III: Y = OCH_3; X = F
IV: Y = NH_2; X = F
V: Y = OC_2H_5; X = F

The data in Table I show that the two bis(2-fluoroethyl)triazenoimidazoles (II and III) increased the lifespan of leukemic mice by 50–70% at tolerated doses. By way of comparison, the doses of NSC-82196 reported (1, 2) to be most effective are 300–625 mg./kg. for single-dose treatment and 50–100 mg./kg./day for daily treatment. The data appear to justify the following conclusions: (*a*) the bis(2-fluoroethyl)triazenes are more toxic than is NSC-82196; (*b*) at doses tolerated by the host animals, II and III are less effective than NSC-82196; and (*c*) in the standard L-1210 test system, the increases in lifespan caused by II and III are comparable to those produced by the corresponding dimethyltriazenes of the amide (NSC-45388) (8) and methyl ester (NSC-87982) (5) series.

Both the v-triazole (9) and the pyrazole (10) analogs of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388, DIC) cause significant increases in the lifespan of mice bearing L-1210, and other triazenopyrazole amides and esters have likewise demonstrated activity against L-1210 (7). However, in tests conducted in accordance with the protocols of the Cancer Chemotherapy National Service Center, amide IV displayed minimal activity, whereas the ethyl ester did not significantly increase survival time as a result of either the single-dose or the daily therapeutic regimens. Again, both are more toxic than NSC-82196.

Amides II and IV, like NSC-82196, undergo a change in aqueous solutions to ionic transformation products (11, 12), but the bis(2-fluoroethyl)triazeno derivatives are considerably more stable than NSC-82196. Esters III and V likewise undergo a change, presumed to be the same type. Obviously, the greater toxicity of II–V, in comparison with NSC-82196, may be due to replacement of chloro groups by fluoro groups. It is also conceivable that the lower toxicity of NSC-82196 results in part from its instability. If this is true, the instability may be advantageous.

(1) Y. F. Shealy and C. A. Krauth, *Nature*, **210**, 208(1966).
(2) G. Hoffman, I. Kline, M. Gang, D. D. Tyrer, J. M. Venditti, and A. Goldin, *Cancer Chemother. Rep. (Part I)*, **52**, 715(1968).
(3) I. Wodinsky, J. Swiniarski, and C. J. Kensler, *ibid.*, **52**, 393 (1968).
(4) G. R. Pettit and R. L. Smith, *Can. J. Chem.*, **42**, 572(1964).
(5) Y. F. Shealy, C. A. Krauth, R. F. Pittillo, and D. E. Hunt, *J. Pharm. Sci.*, **56**, 147(1967).
(6) C. C. Cheng, R. K. Robins, K. C. Cheng, and D. C. Lin, *ibid.*, **57**, 1044(1968).
(7) Y. F. Shealy and C. A. O'Dell, unpublished data.
(8) Y. F. Shealy, J. A. Montgomery, and W. R. Laster, Jr., *Biochem. Pharmacol.*, **11**, 674(1962).
(9) Y. F. Shealy and C. A. O'Dell, *J. Med. Chem.*, **9**, 733(1966).
(10) C. N. Noell and C. C. Cheng, *ibid.*, **12**, 545(1969).
(11) D. J. Abraham, J. S. Rutherford, and R. D. Rosenstein, *ibid.*, **12**, 189(1969).
(12) Y. F. Shealy, C. A. Krauth, L. B. Holum, and W. E. Fitzgibbon, *J. Pharm. Sci.*, **57**, 83(1968).

Y. Fulmer Shealy
C. Allen O'Dell
Kettering-Meyer Laboratories
Southern Research Institute
Birmingham, AL 35205

Received March 12, 1970.
Accepted for publication May 22, 1970.
The work described was supported by Contract No. PH43-64-51 from Chemotherapy, National Service Center, National Institutes of Health, U. S. Public Health Service. Elemental analyses and spectrophotometric determinations were performed under the supervision of Dr. W. C. Coburn, Jr., by members of the Molecular Spectroscopy Section of this Institute. Screening of Compounds II–V was performed under the auspices of Chemotherapy, National Service Center, Contract No. PH43-65-594, and under the supervision of Dr. F. M. Schabel, Jr., and Dr. W. R. Laster, Jr., of the Chemotherapy Department of this Institute.

Aggregation Mechanisms in Pharmaceutical Suspensions

Keyphrases ☐ Suspensions, pharmaceutical—aggregation mechanism ☐ Flocculation, coagulation—suspensions

Sir:

The method of prevention of impaction and caking in pharmaceutical suspensions by controlled flocculation is usually credited to Haines and Martin (1). The work of these authors is, however, sometimes quoted in review articles (2, 3) without reference to the important criticisms subsequently published by Wilson and Ecanow (4) and Ecanow *et al.* (5). We have endorsed (6) some of these criticisms, but have suggested that several generalizations proposed by Ecanow and his coworkers were based on inadequately controlled experiments. The purpose of this communication is to clarify some aspects of suspension theory recently commented upon by Ecanow *et al.* (7), since this area is of considerable importance to the pharmaceutical formulator.

We are grateful to Ecanow *et al.* (7) for amplifying some points in their earlier paper (5), since we had previously found that the almost complete absence of experimental data, such as particle size of the drug and concentration of electrolyte, made an adequate appraisal impossible. Despite the recent criticisms of these authors, we see no reason to retract from our claim that Figure 1 in *Reference 6* demonstrates differences between coagulation and flocculation. Ecanow and coworkers appear to have forgotten that suspensions of drugs in anionic surfactants (1, 8) and cationic and nonionic surfactants (9) in the absence of electrolyte cake on storage. The control suspension described in the uppermost curve of Figure 1 in *Reference 6* caked after ultimate sedimentation. However, we do not consider it semantically or scientifically helpful to refer to this process as coagulation for the following reason. If the particles were slightly smaller, they would remain in permanent colloidal suspension due to Brownian motion

because of their high mutual repulsion. This is the *exact opposite* of coagulation in true colloids. The reason why the particles come together at the base of the container is that they sediment individually and roll over one another until they can go no farther. The repulsion only extends a short distance from the particle surface and is not sufficient to keep them far apart. We prefer to call this process "impaction," thus preserving what Chwala (10) called the "sedimentation paradox." The control suspension of Ecanow *et al.* (7) showed no sedimentation after 7 days due to the presence of glycerol. We would suggest, however, that if it were allowed to sediment, it would impact as did similar suspensions recently described (11). This is another reason why we feel that the inclusion of thickening agents in this type of study may mask important effects.

We naturally accept the statement by these authors that interactions between electrolytes and surfactants are concentration dependent and, if reference is made to our earlier paper (8), it will be found that the interaction of the ammonium salt of the same surfactant was tested under a wide range of electrolyte concentrations. Similar tests were also performed with the purer sodium salt used subsequently (6, 12). Only in much higher concentrations of electrolyte than were used did opalescence occur. We accept, of course, that there is bound to be interaction between ions of opposing charge in solution, whether they are surfactants or simple inorganic ions. Such interactions within the double layer are responsible for the reduction of ζ-potential and subsequent coagulation. Yet, we maintain that this type of interaction should not be called flocculation unless a definite precipitation of the surfactant–metal complex occurs, giving a continuous physical bridge between the particles. This would be analogous to the flocculation of colloids by starch polymer molecules described by La Mer (13). We would remind Ecanow *et al.* (5) that they used the precipitation effect as the decisive criterion for coagulation or flocculation.

We would also like to answer an earlier criticism, by Wilson and Ecanow (4), of the validity of the concept of flocculation (or rather coagulation) of large particles by ζ-potential reduction and van der Waals' attraction. They refer to the statement by Kruyt (14) of the possibility of long-range London–van der Waals' forces between particles of 2–5 μm. in diameter and the statement that such flocculation has not been experimentally verified.[1] Kruyt was referring to *secondary minimum* flocculation. This is only possible with intermediate concentrations of electrolyte, and we have shown (12, 15) that aggregation in the secondary minimum is unlikely to be the explanation of our results. We have produced calculated energy-of-interaction curves to show that coagulation is more likely in the primary minimum. The depth of this minimum is restricted by the film of surfactant at the solid–liquid interface and makes the suspension still readily redispersible. This is because particle–particle attraction is strong enough to maintain the open structure of the coagulum, although, as Ecanow *et al.* (7) pointed out, such structures can be broken down by centrifuging.

The results of the work by Schenkel and Kitchener (16) on the coagulation of 10-μm. polymer particles show that, at the highest concentrations of electrolyte, rapid coagulation occurred, forming an open structure. In this case the particles seized on contact, since there was no film of surfactant at the interface.

We suggest that secondary minimum coagulation may be the cause of the effect noted by Ecanow *et al.* with the monovalent ions. Such coagulation would produce very loosely bound aggregates which would settle out more rapidly, but interparticulate forces might not be strong enough to maintain the open structure under the accumulating weight of the growing sediment. The wetting action of the surfactant would still be maintained and would assist the particles to slide over one another.

Calculations and carefully controlled measurements, similar to those previously published (12), may substantiate this point.

We would also like to question the distinction which Wilson and Ecanow (4) and Ecanow *et al.* (7) have made between hydrophobic and hydrophilic particles. The drug particles under consideration are fundamentally hydrophobic and, for this reason, need the presence of an adsorbed layer of amphophilic surfactant to form suspensions. It is important to realize that the stabilizing effect of surfactant films only extends a comparatively short distance from the particle surface (17). For this reason, the diagrams published by Ecanow *et al.* (18) are so far from true scale as to be misleading.

It is accepted that, in suspensions of pharmaceuticals, Brownian motion is less important, but it is not, as Ecanow *et al.* (5) state, negligible. We have calculated (6, 11) that for an aqueous suspension of drug particles, Brownian motion was a greater source of displacement than sedimentation up to a diameter of 2 μm. For sizes above this value, differential sedimentation rates in polydisperse systems will also cause particle collisions and coagulation. Since, however, glycerol will slow down both Brownian motion and sedimentation, we still consider its use likely to complicate experiments designed to differentiate between aggregation mechanisms. We have also shown (9) that, in some systems, glycerol itself can cause aggregation. We have studied aggregation phenomena in model paracolloidal systems, using a Coulter counter and a digital computer (19–21). It is apparent that much of the theory developed for colloidal systems may, with certain modifications, be applied to dispersions of particles in the 2–10-μm. regions, this being a size range of peculiar importance to the pharmaceutical formulator. Our results indicate that there is no abrupt particle-size boundary between colloidal and paracolloidal systems but rather a gradual change of properties exists. With regard to the question of period of induction, it is important to stress that coagulation rate is number concentration dependent. Although a suspension may have a small proportion by weight of particles in the region where Brownian motion is important, this can be a significant proportion by number.

We have provided further evidence (12) that coagulation in our suspensions is qualitatively analogous to

[1] Experimental verification of secondary minimum flocculation has been published by Schenkel and Kitchener (16).

that in a lyophobic colloid.

We found that similar coagulation curves could be produced with sodium, calcium, and aluminum salts and that the only real difference was in the position of the vertical region of the sigmoid curve (12). The effect of valency was approximately as would be predicted by the Schulze-Hardy rule. The suspensions coagulated with the calcium chloride were particularly interesting since, with the highest concentration of electrolyte used, a further step in the sedimentation height curve was observed. Compatibility tests between the electrolyte and surfactant showed that, at this concentration, slight precipitation occurred. We interpret this as being perhaps the point of transition between coagulation and flocculation.

Ecanow *et al.* (5, 7, 18) appear to argue that since coagulation and flocculation must produce qualitatively different results and that we merely found quantitative differences, we could not be studying two different phenomena. We find it difficult to answer such circular reasoning. We would still maintain, on the basis of carefully controlled experiments whose results are compared with fundamental theory, that both coagulation and flocculation can produce suspensions that remain free from caking on storage.

(1) B. A. Haines, Jr., and A. N. Martin, *J. Pharm. Sci.*, **50**, 228 (1961).

(2) R. A. Nash, *Drug Cosmet. Ind.*, **97**, 843(1965).

(3) P. H. Elworthy, *Pharm. J.*, **199**, 107(1967).

(4) R. G. Wilson and B. Ecanow, *J. Pharm. Sci.*, **52**, 757(1963).

(5) B. Ecanow, R. Grundman, and R. Wilson, *Amer. J. Hosp. Pharm.*, **23**, 404(1966).

(6) B. A. Matthews and C. T. Rhodes, *J. Pharm. Pharmacol., Suppl.*, **20**, 204S(1968).

(7) B. Ecanow, B. Gold, R. Levinson, H. Takruri, and W. Stansazek, *Amer. Perfum. Cosmet.*, **84**, 30(1969).

(8) B. A. Matthews and C. T. Rhodes, *J. Pharm. Sci.*, **57**, 569 (1968).

(9) R. D. C. Jones, B. A. Matthews, and C. T. Rhodes, *ibid.*, **59**, 518(1970).

(10) A. Chwala, *Kolloidchem. Beih.*, **31**, 222(1930).

(11) B. A. Matthews and C. T. Rhodes, *Pharm. Acta Helv.*, **45**, 52(1970).

(12) B. A. Matthews and C. T. Rhodes, *J. Pharm. Sci.*, **59**, 521 (1970).

(13) V. K. La Mer, *J. Colloid Sci.*, **19**, 291(1964).

(14) H. R. Kruyt, "Colloid Science," Elsevier, Amsterdam, The Netherlands, 1952, p. 324.

(15) B. A. Matthews, Ph.D. thesis, University of London, 1969.

(16) J. H. Schenkel and J. A. Kitchener, *Trans. Faraday Soc.*, **56**, 161(1960).

(17) R. H. Ottewill and T. Walker, *Kolloid Z.*, **227**, 108(1968).

(18) B. Ecanow, B. Gold, and C. Ecanow, *Amer. Perfum. Cosmet.*, **84**, 27(1969).

(19) B. A. Matthews and C. T. Rhodes, *J. Colloid Interface Sci.*, **28**, 71(1968).

(20) *Ibid.*, **32**, 332(1970).

(21) *Ibid.*, **32**, 339(1970).

B. A. Matthews
J. R. Geigy A. G.
4000 Basle 21
Switzerland

C. T. Rhodes
Faculty of Pharmaceutical Sciences
University of British Columbia
Vancouver 8
Canada

Received December 24, 1969.
Accepted for publication June 4, 1970.

Effect of Macromolecules on Aqueous Solubility of Cholesterol

Keyphrases ☐ Cholesterol, cholesterol-26-^{14}C solubility—macromolecule effects ☐ Macromolecular substances—cholesterol solubility effect ☐ Pectin, acacia, dextrans effect—cholesterol solubility ☐ Scintillometry—analysis

Sir:

Intravenous administration of dextran solution has been suggested for treatment of hypercholesterolemia and atherosclerosis (1), and numerous investigations have been carried out to determine the effect of dextran on experimental hypercholesterolemia (2–6). Oral administrations of pectin and acacia have been studied for their hypocholesterolemic effects (7–14). The cited reports contain various and conflicting statements concerning the *in vivo* effect, value, and mechanisms of action of macromolecular substances for lowering serum cholesterol. However, there have been no reports of studies conducted to determine the *in vitro* effects of these carbohydrate macromolecules on the solubilization of cholesterol. It was, therefore, considered desirable to study the effect of these macromolecules on the aqueous solubility of cholesterol.

Cholesterol concentrations were determined using a radioactive technique suitable for very low amounts of cholesterol in water. A 10.0-ml. volume of a benzene stock solution, containing 10 mcg. of cholesterol and 0.1 μc. of cholesterol-26-^{14}C/ml., was transferred to a 125-ml. iodine flask, and the solvent was evaporated under a mild stream of nitrogen with constant shaking. A 50-ml. volume of the aqueous solution of the particular macromolecular substance was added to the flask. The dissolution studies were carried out at 30 ± 0.5°, and the test solutions were agitated using magnetic stirrers. At predetermined intervals of time, samples were withdrawn from the system and filtered immediately through a Millipore filtration assembly containing 0.45-μ filter paper. An accurately measured 0.20-ml. volume of the particle-free filtrate was pipeted into a liquid-scintillation vial. To this was added 15 ml. of dioxane–naphthalene phosphor (15), and the vials were shaken for 30 sec. to ensure thorough mixing. Duplicate samples, along with appropriate standards and blanks, were counted directly using a liquid-scintillation system.[1] The counts per minute involved after 12 hr. were between 150 and 415.

The data from the solubility studies are illustrated in Fig. 1. Each point on the solubility curves represents an average of at least eight determinations. Pectin (0.5%) and acacia (0.5%) solutions significantly increased the aqueous solubility of cholesterol, while slight increases were observed for the solubility of cholesterol in dextran solutions. The results for both high and low molecular weight dextrans[2] were similar.

[1] Unilux II, Nuclear Chicago.
[2] Dextran T70 and T40, Pharmacia Laboratories.

that in a lyophobic colloid.

We found that similar coagulation curves could be produced with sodium, calcium, and aluminum salts and that the only real difference was in the position of the vertical region of the sigmoid curve (12). The effect of valency was approximately as would be predicted by the Schulze-Hardy rule. The suspensions coagulated with the calcium chloride were particularly interesting since, with the highest concentration of electrolyte used, a further step in the sedimentation height curve was observed. Compatibility tests between the electrolyte and surfactant showed that, at this concentration, slight precipitation occurred. We interpret this as being perhaps the point of transition between coagulation and flocculation.

Ecanow *et al.* (5, 7, 18) appear to argue that since coagulation and flocculation must produce qualitatively different results and that we merely found quantitative differences, we could not be studying two different phenomena. We find it difficult to answer such circular reasoning. We would still maintain, on the basis of carefully controlled experiments whose results are compared with fundamental theory, that both coagulation and flocculation can produce suspensions that remain free from caking on storage.

(1) B. A. Haines, Jr., and A. N. Martin, *J. Pharm. Sci.*, **50**, 228 (1961).

(2) R. A. Nash, *Drug Cosmet. Ind.*, **97**, 843(1965).

(3) P. H. Elworthy, *Pharm. J.*, **199**, 107(1967).

(4) R. G. Wilson and B. Ecanow, *J. Pharm. Sci.*, **52**, 757(1963).

(5) B. Ecanow, R. Grundman, and R. Wilson, *Amer. J. Hosp. Pharm.*, **23**, 404(1966).

(6) B. A. Matthews and C. T. Rhodes, *J. Pharm. Pharmacol., Suppl.*, **20**, 204S(1968).

(7) B. Ecanow, B. Gold, R. Levinson, H. Takruri, and W. Stansazek, *Amer. Perfum. Cosmet.*, **84**, 30(1969).

(8) B. A. Matthews and C. T. Rhodes, *J. Pharm. Sci.*, **57**, 569 (1968).

(9) R. D. C. Jones, B. A. Matthews, and C. T. Rhodes, *ibid.*, **59**, 518(1970).

(10) A. Chwala, *Kolloidchem. Beih.*, **31**, 222(1930).

(11) B. A. Matthews and C. T. Rhodes, *Pharm. Acta Helv.*, **45**, 52(1970).

(12) B. A. Matthews and C. T. Rhodes, *J. Pharm. Sci.*, **59**, 521 (1970).

(13) V. K. La Mer, *J. Colloid Sci.*, **19**, 291(1964).

(14) H. R. Kruyt, "Colloid Science," Elsevier, Amsterdam, The Netherlands, 1952, p. 324.

(15) B. A. Matthews, Ph.D. thesis, University of London, 1969.

(16) J. H. Schenkel and J. A. Kitchener, *Trans. Faraday Soc.*, **56**, 161(1960).

(17) R. H. Ottewill and T. Walker, *Kolloid Z.*, **227**, 108(1968).

(18) B. Ecanow, B. Gold, and C. Ecanow, *Amer. Perfum. Cosmet.*, **84**, 27(1969).

(19) B. A. Matthews and C. T. Rhodes, *J. Colloid Interface Sci.*, **28**, 71(1968).

(20) *Ibid.*, **32**, 332(1970).

(21) *Ibid.*, **32**, 339(1970).

B. A. Matthews
J. R. Geigy A. G.
4000 Basle 21
Switzerland

C. T. Rhodes
Faculty of Pharmaceutical Sciences
University of British Columbia
Vancouver 8
Canada

Received December 24, 1969.
Accepted for publication June 4, 1970.

Effect of Macromolecules on Aqueous Solubility of Cholesterol

Keyphrases ☐ Cholesterol, cholesterol-26-^{14}C solubility—macromolecule effects ☐ Macromolecular substances—cholesterol solubility effect ☐ Pectin, acacia, dextrans effect—cholesterol solubility ☐ Scintillometry—analysis

Sir:

Intravenous administration of dextran solution has been suggested for treatment of hypercholesterolemia and atherosclerosis (1), and numerous investigations have been carried out to determine the effect of dextran on experimental hypercholesterolemia (2–6). Oral administrations of pectin and acacia have been studied for their hypocholesterolemic effects (7–14). The cited reports contain various and conflicting statements concerning the *in vivo* effect, value, and mechanisms of action of macromolecular substances for lowering serum cholesterol. However, there have been no reports of studies conducted to determine the *in vitro* effects of these carbohydrate macromolecules on the solubilization of cholesterol. It was, therefore, considered desirable to study the effect of these macromolecules on the aqueous solubility of cholesterol.

Cholesterol concentrations were determined using a radioactive technique suitable for very low amounts of cholesterol in water. A 10.0-ml. volume of a benzene stock solution, containing 10 mcg. of cholesterol and 0.1 μc. of cholesterol-26-^{14}C/ml., was transferred to a 125-ml. iodine flask, and the solvent was evaporated under a mild stream of nitrogen with constant shaking. A 50-ml. volume of the aqueous solution of the particular macromolecular substance was added to the flask. The dissolution studies were carried out at 30 ± 0.5°, and the test solutions were agitated using magnetic stirrers. At predetermined intervals of time, samples were withdrawn from the system and filtered immediately through a Millipore filtration assembly containing 0.45-μ filter paper. An accurately measured 0.20-ml. volume of the particle-free filtrate was pipeted into a liquid-scintillation vial. To this was added 15 ml. of dioxane–naphthalene phosphor (15), and the vials were shaken for 30 sec. to ensure thorough mixing. Duplicate samples, along with appropriate standards and blanks, were counted directly using a liquid-scintillation system.[1] The counts per minute involved after 12 hr. were between 150 and 415.

The data from the solubility studies are illustrated in Fig. 1. Each point on the solubility curves represents an average of at least eight determinations. Pectin (0.5%) and acacia (0.5%) solutions significantly increased the aqueous solubility of cholesterol, while slight increases were observed for the solubility of cholesterol in dextran solutions. The results for both high and low molecular weight dextrans[2] were similar.

[1] Unilux II, Nuclear Chicago.
[2] Dextran T70 and T40, Pharmacia Laboratories.

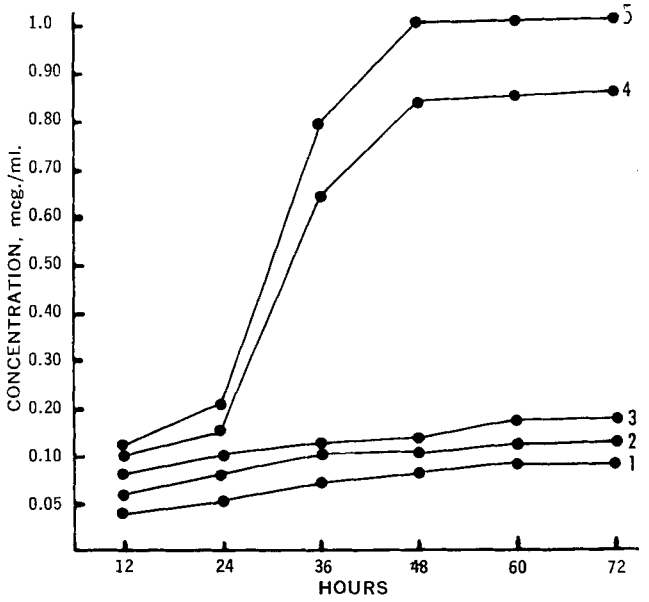


Figure 1—*Effect of various macromolecules on the aqueous solubility of cholesterol at 30°. Key: 1, water; 2, 6% dextran; 3, 10% dextran; 4, 0.5% acacia; and 5, 0.5% pectin.*

Statistical analyses of the data by means of t and F tests at 95% confidence levels indicate the differences in solubilities between various test solutions to be significant.

It is well known that dextran, pectin, and acacia form colloidal solutions. It is possible that cholesterol might be suspended, dispersed, or adsorbed on these colloidal particles. Another possibility is that these macromolecules might entrap the relatively smaller molecule of cholesterol. Brintzinger and Beier (16) suggested a number of mechanisms for the ability of gum acacia to increase the solubility of substances such as benzoic acid, anisic acid, sulfanilic acid, strychnine, and nitroaniline. The suggested mechanisms included adsorption on the acacia micelle, solution in the micelle, adsorption exchange between the added substance and water of hydration, and formation of chemical bonds.

The large increase in the apparent solubility of cholesterol in 0.5% pectin solution is most interesting when related to the findings of Wells and Ershoff (8). They found that pectin inhibited the increase in liver cholesterol following cholesterol feeding of rats, even when the rats were fed on alternate days with cholesterol. These authors suggested that more is involved than the simple tying up of cholesterol (possibly by the formation of a nonabsorbable pectin–cholesterol complex). Another possibility they suggested was that pectin induces changes in the intestinal flora which result in greater degradation of cholesterol, thereby leaving less of this material available for absorption. The present finding that pectin increases cholesterol solubility suggests the possibility that cholesterol in a solubilized form might be more susceptible to degradation in the intestine. Other possibilities are that cholesterol is adsorbed or dispersed in the colloidal particles formed by pectin or that a water-soluble complex is formed which is nonabsorbable. The fact that significant increases in the solubility of cholesterol in acacia and pectin solutions were observed after 24 hr. suggests that there may be more than one mechanism.

Since in the current studies the solubility of cholesterol was greatly increased in acacia and pectin solutions, it would appear that acacia might have the same *in vivo* cholesterol-lowering effect as pectin. However, Lin *et al.* (7) reported that addition of pectin to a basal diet containing cholesterol increased the excretion of exogenous cholesterol, while the addition of acacia or arabinose to this ration produced practically no change in the amount of fecal lipids and in the recovery of exogenous cholesterol in the feces.

Flotte and Buxton (2, 3) and Ditzel and Dyerberg (6) observed that administration of dextran has a profound effect in lowering serum cholesterol. Ditzel and Dyerberg discussed the possible modes of action of dextran on plasma lipids and cholesterol. They postulated that a dextran–lipid complex might influence cholesterol synthesis or breakdown or facilitate cholesterol transport through the cell membranes by altering its solubility characteristics or charge. Rothschild *et al.* (17) postulated that the mode of action of dextran might be explained by a stimulating effect on the reticuloendothelial system. The current findings suggest that dextran might facilitate at least part of the transport redistribution of cholesterol by an increase in the apparent solubility of cholesterol.

Further investigations are being conducted to determine the effect of a variety of macromolecules on the aqueous solubility of cholesterol and related hormone drugs and to elucidate the mechanism(s) of action of solubilization phenomena.

(1) G. Lusztig, L. Zozsa, M. Perneczky, and L. Sajtos, *Z. Ges. Inn. Med. Ihre Grenzgeb.*, **17**, 152(1962).
(2) C. T. Flotte and R. W. Buxton, *Circulation (Suppl.)*, **28**, 721 (1963).
(3) *Ibid.*, **32**, 85(1965).
(4) D. M. Brahmankar and W. E. Connor, *Circ. Res.*, **21**, 817 (1967).
(5) H. Losel and W. Appel, *Fortschr. Med.*, **85**, 517(1967).
(6) J. Ditzel and J. Dyerberg, *J. Atheroscler. Res.*, **10**, 5(1969).
(7) T. M. Lin, K. S. Kim, E. Karvinen, and A. C. Ivy, *Amer. J. Physiol.*, **188**, 66(1957).
(8) A. F. Wells and B. H. Ershoff, *J. Nutr.*, **74**, 87(1961).
(9) H. Fisher, P. Griminger, H. S. Weiss, and W. G. Siller, *Science*, **146**, 1063(1964).
(10) H. D. Fausch and T. A. Anderson, *J. Nutr.*, **85**, 145(1965).
(11) H. Fisher, G. W. Van der Noot, W. S. McGrath, and P. Griminger, *J. Atheroscler. Res.*, **6**, 190(1966).
(12) H. Fisher, W. G. Siller, and P. Griminger, *ibid.*, **6**, 292(1966).
(13) H. Fisher, P. Griminger, and W. G. Siller, *ibid.*, **7**, 381 (1967).
(14) T. A. Anderson and R. D. Bowman, *Proc. Soc. Exp. Biol. Med.*, **130**, 665(1969).
(15) G. A. Bray, *Anal. Biochem.*, **1**, 279(1960).
(16) H. Brintzinger and H. G. Beier, *Kolloid-Z.*, **64**, 300(1933).
(17) M. A. Rothschild, M. Oratz, E. Wimer, and S. S. Schreiber, *Proc. Soc. Exp. Biol. Med.*, **104**, 478(1960).

DEVENDRA K. MADAN
DONALD E. CADWALLADER
Department of Pharmacy
School of Pharmacy
University of Georgia
Athens, GA 30601

Received March 16, 1970.
Accepted for publication June 4, 1970.

Objective Visual Evaluation of the Relative Content of Major and Minor Defects in Tablets and Capsules

Keyphrases ☐ Tablets, capsules—relative defect determination ☐ Defects, tablets and capsules—visual determination method

Sir:

Recently, it became desirable for our Quality Control Department to develop and subsequently implement a reasonably objective analysis for the evaluation of major and minor physical defects in tablets and capsules. The results would then be used to determine if additional processing was required prior to packaging.

Even though major and minor physical defects are routinely monitored by most pharmaceutical manufacturers, we were unable to find a reference regarding a test method in the literature. Consequently, a visual method has been developed which allows for the monitoring of major and minor physical defects in tablets and capsules.

Basically, the method consists of filling a rectangular plastic (Plexiglas) tray, a monolayer in depth, with the test tablets or capsules. The tray is placed on a workbench and positioned under a lamp containing a 100-watt incandescent lightbulb. The tray's width is placed parallel to the front bench edge about 7.62–15.24 cm. (3–6 in.) from the edge. The lamp is connected to a timer[1] that activates illumination of the lightbulb for a required amount of time.

Table I—Definition of Major and Minor Defects

Major Defects	Minor Defects
Surface spots	Polishing not uniform
Breaks or cracks	Feathered edges
Coated base tablet exposed	Chips
Foreign particulate matter[a]	Pitting or pimples
	Atypical mottling
	Nonuniform color
	Nonuniform size or shape
	Not smooth
	Smeared printing
	Thin-coated edges

[a] Detection of this major defect results in rejection of the lot until it is freed of any health hazard resulting from this defect.

Subsequently, the start button of the timer is depressed and the tablets are scanned for both major and minor defects (Table I); the defective tablets are marked, using a felt-tipped pen (or equivalent). Automatically, the light is turned off, signifying the end of the analysis. The number and types of defects are sorted, and the results are recorded on an assay report form. The report is designed so that the number and types of defects are identified.

An empty tray is superimposed over the tablet-containing tray, and the two trays are rotated 180° around the x- or y-axis so that the reverse side of the tablets are exposed. The original tray is removed, and the assay is performed on the new exposed side of the samples. The procedure is repeated until the total number of tablets or capsules required in the evaluation is assayed, and the results are recorded accumulatively.

The total number of units assayed is related to batch size and based on Military Standard 105D. Using the average tablet or capsule weight, the number of units to be tested are weighed into a beaker for subsequent transfer to the testing tray. A double sampling plan is used in which the acceptable quality level (A. Q. L.) is 0.65 and 4.0%, respectively, for major and minor defects.

Two sets of trays (two identical trays per set) are required to accommodate capsules, capsule-shaped tablets, and the conventional standard-shaped tablets. All the trays are 22.86 × 15.24 cm. (9 × 6 in.); however, the trays used for capsules and capsule-shaped tablets have an inner depth of 0.32 cm. (0.125 in.), and the trays used for the standard-shaped tablets have an inner depth of 0.16 cm. (0.062 in.).

Since the size of the tablet or capsule being assayed dictates the number of units in each tray load, the time interval (length of time light is illuminated) is correspondingly adjusted. In effect, the illumination duration is longest for the smallest tablet assayed. After a year of using this technique, we have developed the following formula for determining the time interval for a given sample:

$$P \text{ seconds/tablet} \times \frac{\text{total number of tablets to be assayed}}{\text{number of filled trays}} = \text{seconds/tray} \quad \text{(Eq. 1)}$$

The value of P chosen should reflect the desired rigidity of the inspection. In our laboratory, P has an average value of 0.035 sec./tablet. If 1000 tablets were to be tested and five filled trays were involved, the time interval would be 7 sec./tray. If the last tray is less than half a tray, half the time interval is used. Otherwise, the tray is considered a full tray.

We found that the number of tablets scanned per tray varies between analysts, and the formula attempts to compromise these differences. Otherwise, the natural tendency of the analyst is to become overcritical of the sample appearance.

J. C. Stone
Quality Control Laboratories
Warren-Teed Pharmaceuticals Inc.
Columbus, OH 43215

Received April 13, 1970.
Accepted for publication May 27, 1970.
The author thanks Mr. Frank Blackett for his advice and counsel during the assemblement of the testing equipment, and the invaluable assistance provided by the Quality Control Department.

[1] Model M-1M, Industrial Timer Corporation, Parsippany, N. J.

REVIEWS

Thin-Layer Chromatography. Edited by EGON STAHL. Springer-Verlag New York, Inc. 175 5th Ave. New York, NY 10010, 1969. xxiv + 1041 pp.

Egon Stahl's *Thin-Layer Chromatography* (second edition) is the most complete and current compilation of TLC theory and practice available today. The book, published in 1967 in German, is an expansion of his first work, organized in much the same manner. There is the same review of nomenclature before each compound class, the same history, and a much expanded theory section covering new apparatus and additional techniques.

The book has a much more complete section on heterocyclic nitrogen compounds than Kirchner's *Thin-Layer Chromatography*. Phenothiazines, the diazepines, carbamate esters, compounds of antihistamine activity, and the narcotics are all more thoroughly covered by Stahl than in the other available books on the market. Moreover, most charts list the structural formula where feasible or the chemical name of the compound in addition to its common name. The amino acid section is also well done. There is an extensive listing of general test conditions and solvent systems for these compounds.

The volume, however, is not specific in its treatment of the steroids. For the most part, it lists general systems for the various groupings of steroids and does not treat each drug individually. An incomplete and inconsistent index is also a hindrance, varying between listing drugs as to their activity, group name, or the name of one compound in the group. However, no existing TLC handbook approaches the amount of data contained in this volume and, as such, this work is a definitive addition to any TLC library.

Reviewed by Dorothy K. Wolf
Drug Standards Laboratory
American Pharmaceutical Association Foundation
Washington, DC 20037 ■

Cationic Surfactants. Vol. 4. Edited by ERIC JUNGERMANN. Marcel Dekker, New York, NY 10016, 1970. xiv + 652 pp. 16 × 23.5 cm. Price $37.50.

This, the fourth volume in Dekker's Surfactant Science Series, is concerned with the organic, physical, and analytical chemistry of cationic surfactants. A fourth section deals with the biological aspects of this class of surfactants.

The eighteen authors are drawn from both industry and the universities. Following an introductory chapter, the next six chapters deal with the synthesis of straight chain and cyclical alkylammonium compounds, petroleum-derived and polymeric forms, and nonnitrogen-containing surfactants. Brief references to the applications, physical properties, and toxicities of the compounds under discussion are made. As would be expected, major emphasis is placed on commercial products. Considering the potentially unexciting subject matter, the presentation is clear and readable.

There then follows a group of six chapters concerned with the physical chemistry of cationic surfactants. Specific topics covered are micelle formation in aqueous and nonaqueous media, adsorption by a variety of substrates, and coacervation. A brief section on adsorption on biological substrates has obvious pharmaceutical implications. In most cases the treatment is sufficiently basic; inevitably, much of the discussion is common to all classes of surfactants.

A single chapter comprises the section on the analytical chemistry of cationic surfactants. The approach taken is a practical one, with emphasis placed on methods that have been developed for carefully purified surfactants. The chapter considers both qualitative and quantitative aspects of analysis. Most discussion of the latter centers around absorptiometric, volumetric, and gravimetric analysis.

The final section, on the biology of these compounds, contains two chapters that review the germicidal properties and toxicology, respectively, of cationic surfactants. In the chapter on germicidal properties, little consideration is given to a discussion of the mechanisms of action involved. However, the descriptive literature in this area is covered satisfactorily. Cationic surfactant toxicology is well summarized in the final chapter, which also contains a comprehensive listing of reported toxicities.

While this text undoubtedly represents a comprehensive presentation on cationic surfactants, its major appeal to the pharmaceutical scientist will probably be limited to the last two sections which comprise approximately one-third of the total material.

Reviewed by James Swarbrick
School of Pharmacy
University of Connecticut
Storrs, CT 06268 ■

Pharmaceutical Chemistry, Volume 2: Instrumental Techniques. Edited by L. G. CHATTEN. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xiv + 773 pp. 17 × 24 cm. Price $17.75.

Eighteen authors and the editor have collaborated to present 19 chapters in this second volume of a two-volume set on the analytical aspects of pharmaceutical chemistry. When the objectives stated in the preface are compared with the final written work, this reviewer finds that, in general, the mission has been accomplished. In an attempt to evaluate this book, each chapter has been examined and the following comments are respectfully set down.

The chapters may be conveniently divided into three groups: (1) topics involving electromagnetic radiation; (2) topics dealing with electrochemistry; and (3) subjects not classified as either (1) or (2). In many cases, questions, problems, and answers as well as experiments have been provided.

The first group of items is concerned with analytical techniques utilizing electromagnetic radiation in its various forms. The first two chapters concerning absorption spectrophotometry in the visible and ultraviolet region and in the infrared are well written and employ pretty much the standard approach to the subjects. In the first case, spectrophotometric titrations and a welcome treatment of the chemistry of chromogenesis in some colorimetric analyses are included. The presentation of structure–spectra correlations is done well; unknown spectra are provided for practice and study.

In both chapters, the experiments are appropriate but the instrumental features receive only brief attention. Raman spectroscopy, becoming more useful as instrument prices diminish, is discussed in a separate chapter. The theory is examined carefully and requires an appreciation of group theory and fundamental spectroscopy for adequate understanding. At present, no good examples of the application of this technique to pharmaceuticals are evident.

In the division of fluorometry, the explanations of theory, derivations of equations, and the examination of pharmaceutical examples are excellent. Particularly noteworthy is the portion discussing spectra–structural correlations. The choice of experiments includes the study of the physiological availability of riboflavin. The 16 pages of the atomic absorption chapter are used with efficient economy, the various points being well presented. This simple technique is certain to be used to a much greater extent than it is today. The qualitative elucidation of the nuclear magnetic resonance phenomena is clearly written as is the section of interpretation of spectra, so very useful in organic compound identification. There is little quantitative work in this area, however. Turbidimetry and

REVIEWS

Thin-Layer Chromatography. Edited by EGON STAHL. Springer-Verlag New York, Inc. 175 5th Ave. New York, NY 10010, 1969. xxiv + 1041 pp.

Egon Stahl's *Thin-Layer Chromatography* (second edition) is the most complete and current compilation of TLC theory and practice available today. The book, published in 1967 in German, is an expansion of his first work, organized in much the same manner. There is the same review of nomenclature before each compound class, the same history, and a much expanded theory section covering new apparatus and additional techniques.

The book has a much more complete section on heterocyclic nitrogen compounds than Kirchner's *Thin-Layer Chromatography*. Phenothiazines, the diazepines, carbamate esters, compounds of antihistamine activity, and the narcotics are all more thoroughly covered by Stahl than in the other available books on the market. Moreover, most charts list the structural formula where feasible or the chemical name of the compound in addition to its common name. The amino acid section is also well done. There is an extensive listing of general test conditions and solvent systems for these compounds.

The volume, however, is not specific in its treatment of the steroids. For the most part, it lists general systems for the various groupings of steroids and does not treat each drug individually. An incomplete and inconsistent index is also a hindrance, varying between listing drugs as to their activity, group name, or the name of one compound in the group. However, no existing TLC handbook approaches the amount of data contained in this volume and, as such, this work is a definitive addition to any TLC library.

Reviewed by Dorothy K. Wolf
Drug Standards Laboratory
American Pharmaceutical Association Foundation
Washington, DC 20037 ■

Cationic Surfactants. Vol. 4. Edited by ERIC JUNGERMANN. Marcel Dekker, New York, NY 10016, 1970. xiv + 652 pp. 16 × 23.5 cm. Price $37.50.

This, the fourth volume in Dekker's Surfactant Science Series, is concerned with the organic, physical, and analytical chemistry of cationic surfactants. A fourth section deals with the biological aspects of this class of surfactants.

The eighteen authors are drawn from both industry and the universities. Following an introductory chapter, the next six chapters deal with the synthesis of straight chain and cyclical alkylammonium compounds, petroleum-derived and polymeric forms, and nonnitrogen-containing surfactants. Brief references to the applications, physical properties, and toxicities of the compounds under discussion are made. As would be expected, major emphasis is placed on commercial products. Considering the potentially unexciting subject matter, the presentation is clear and readable.

There then follows a group of six chapters concerned with the physical chemistry of cationic surfactants. Specific topics covered are micelle formation in aqueous and nonaqueous media, adsorption by a variety of substrates, and coacervation. A brief section on adsorption on biological substrates has obvious pharmaceutical implications. In most cases the treatment is sufficiently basic; inevitably, much of the discussion is common to all classes of surfactants.

A single chapter comprises the section on the analytical chemistry of cationic surfactants. The approach taken is a practical one, with emphasis placed on methods that have been developed for carefully purified surfactants. The chapter considers both qualitative and quantitative aspects of analysis. Most discussion of the latter centers around absorptiometric, volumetric, and gravimetric analysis.

The final section, on the biology of these compounds, contains two chapters that review the germicidal properties and toxicology, respectively, of cationic surfactants. In the chapter on germicidal properties, little consideration is given to a discussion of the mechanisms of action involved. However, the descriptive literature in this area is covered satisfactorily. Cationic surfactant toxicology is well summarized in the final chapter, which also contains a comprehensive listing of reported toxicities.

While this text undoubtedly represents a comprehensive presentation on cationic surfactants, its major appeal to the pharmaceutical scientist will probably be limited to the last two sections which comprise approximately one-third of the total material.

Reviewed by James Swarbrick
School of Pharmacy
University of Connecticut
Storrs, CT 06268 ■

Pharmaceutical Chemistry, Volume 2: Instrumental Techniques. Edited by L. G. CHATTEN. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xiv + 773 pp. 17 × 24 cm. Price $17.75.

Eighteen authors and the editor have collaborated to present 19 chapters in this second volume of a two-volume set on the analytical aspects of pharmaceutical chemistry. When the objectives stated in the preface are compared with the final written work, this reviewer finds that, in general, the mission has been accomplished. In an attempt to evaluate this book, each chapter has been examined and the following comments are respectfully set down.

The chapters may be conveniently divided into three groups: (1) topics involving electromagnetic radiation; (2) topics dealing with electrochemistry; and (3) subjects not classified as either (1) or (2). In many cases, questions, problems, and answers as well as experiments have been provided.

The first group of items is concerned with analytical techniques utilizing electromagnetic radiation in its various forms. The first two chapters concerning absorption spectrophotometry in the visible and ultraviolet region and in the infrared are well written and employ pretty much the standard approach to the subjects. In the first case, spectrophotometric titrations and a welcome treatment of the chemistry of chromogenesis in some colorimetric analyses are included. The presentation of structure–spectra correlations is done well; unknown spectra are provided for practice and study.

In both chapters, the experiments are appropriate but the instrumental features receive only brief attention. Raman spectroscopy, becoming more useful as instrument prices diminish, is discussed in a separate chapter. The theory is examined carefully and requires an appreciation of group theory and fundamental spectroscopy for adequate understanding. At present, no good examples of the application of this technique to pharmaceuticals are evident.

In the division of fluorometry, the explanations of theory, derivations of equations, and the examination of pharmaceutical examples are excellent. Particularly noteworthy is the portion discussing spectra–structural correlations. The choice of experiments includes the study of the physiological availability of riboflavin. The 16 pages of the atomic absorption chapter are used with efficient economy, the various points being well presented. This simple technique is certain to be used to a much greater extent than it is today. The qualitative elucidation of the nuclear magnetic resonance phenomena is clearly written as is the section of interpretation of spectra, so very useful in organic compound identification. There is little quantitative work in this area, however. Turbidimetry and

REVIEWS

Thin-Layer Chromatography. Edited by EGON STAHL. Springer-Verlag New York, Inc. 175 5th Ave. New York, NY 10010, 1969. xxiv + 1041 pp.

Egon Stahl's *Thin-Layer Chromatography* (second edition) is the most complete and current compilation of TLC theory and practice available today. The book, published in 1967 in German, is an expansion of his first work, organized in much the same manner. There is the same review of nomenclature before each compound class, the same history, and a much expanded theory section covering new apparatus and additional techniques.

The book has a much more complete section on heterocyclic nitrogen compounds than Kirchner's *Thin-Layer Chromatography*. Phenothiazines, the diazepines, carbamate esters, compounds of antihistamine activity, and the narcotics are all more thoroughly covered by Stahl than in the other available books on the market. Moreover, most charts list the structural formula where feasible or the chemical name of the compound in addition to its common name. The amino acid section is also well done. There is an extensive listing of general test conditions and solvent systems for these compounds.

The volume, however, is not specific in its treatment of the steroids. For the most part, it lists general systems for the various groupings of steroids and does not treat each drug individually. An incomplete and inconsistent index is also a hindrance, varying between listing drugs as to their activity, group name, or the name of one compound in the group. However, no existing TLC handbook approaches the amount of data contained in this volume and, as such, this work is a definitive addition to any TLC library.

Reviewed by Dorothy K. Wolf
Drug Standards Laboratory
American Pharmaceutical Association Foundation
Washington, DC 20037 ■

Cationic Surfactants. Vol. 4. Edited by ERIC JUNGERMANN. Marcel Dekker, New York, NY 10016, 1970. xiv + 652 pp. 16 × 23.5 cm. Price $37.50.

This, the fourth volume in Dekker's Surfactant Science Series, is concerned with the organic, physical, and analytical chemistry of cationic surfactants. A fourth section deals with the biological aspects of this class of surfactants.

The eighteen authors are drawn from both industry and the universities. Following an introductory chapter, the next six chapters deal with the synthesis of straight chain and cyclical alkylammonium compounds, petroleum-derived and polymeric forms, and nonnitrogen-containing surfactants. Brief references to the applications, physical properties, and toxicities of the compounds under discussion are made. As would be expected, major emphasis is placed on commercial products. Considering the potentially unexciting subject matter, the presentation is clear and readable.

There then follows a group of six chapters concerned with the physical chemistry of cationic surfactants. Specific topics covered are micelle formation in aqueous and nonaqueous media, adsorption by a variety of substrates, and coacervation. A brief section on adsorption on biological substrates has obvious pharmaceutical implications. In most cases the treatment is sufficiently basic; inevitably, much of the discussion is common to all classes of surfactants.

A single chapter comprises the section on the analytical chemistry of cationic surfactants. The approach taken is a practical one, with emphasis placed on methods that have been developed for carefully purified surfactants. The chapter considers both qualitative and quantitative aspects of analysis. Most discussion of the latter centers around absorptiometric, volumetric, and gravimetric analysis.

The final section, on the biology of these compounds, contains two chapters that review the germicidal properties and toxicology, respectively, of cationic surfactants. In the chapter on germicidal properties, little consideration is given to a discussion of the mechanisms of action involved. However, the descriptive literature in this area is covered satisfactorily. Cationic surfactant toxicology is well summarized in the final chapter, which also contains a comprehensive listing of reported toxicities.

While this text undoubtedly represents a comprehensive presentation on cationic surfactants, its major appeal to the pharmaceutical scientist will probably be limited to the last two sections which comprise approximately one-third of the total material.

Reviewed by James Swarbrick
School of Pharmacy
University of Connecticut
Storrs, CT 06268 ■

Pharmaceutical Chemistry, Volume 2: Instrumental Techniques. Edited by L. G. CHATTEN. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1969. xiv + 773 pp. 17 × 24 cm. Price $17.75.

Eighteen authors and the editor have collaborated to present 19 chapters in this second volume of a two-volume set on the analytical aspects of pharmaceutical chemistry. When the objectives stated in the preface are compared with the final written work, this reviewer finds that, in general, the mission has been accomplished. In an attempt to evaluate this book, each chapter has been examined and the following comments are respectfully set down.

The chapters may be conveniently divided into three groups: (1) topics involving electromagnetic radiation; (2) topics dealing with electrochemistry; and (3) subjects not classified as either (1) or (2). In many cases, questions, problems, and answers as well as experiments have been provided.

The first group of items is concerned with analytical techniques utilizing electromagnetic radiation in its various forms. The first two chapters concerning absorption spectrophotometry in the visible and ultraviolet region and in the infrared are well written and employ pretty much the standard approach to the subjects. In the first case, spectrophotometric titrations and a welcome treatment of the chemistry of chromogenesis in some colorimetric analyses are included. The presentation of structure–spectra correlations is done well; unknown spectra are provided for practice and study.

In both chapters, the experiments are appropriate but the instrumental features receive only brief attention. Raman spectroscopy, becoming more useful as instrument prices diminish, is discussed in a separate chapter. The theory is examined carefully and requires an appreciation of group theory and fundamental spectroscopy for adequate understanding. At present, no good examples of the application of this technique to pharmaceuticals are evident.

In the division of fluorometry, the explanations of theory, derivations of equations, and the examination of pharmaceutical examples are excellent. Particularly noteworthy is the portion discussing spectra–structural correlations. The choice of experiments includes the study of the physiological availability of riboflavin. The 16 pages of the atomic absorption chapter are used with efficient economy, the various points being well presented. This simple technique is certain to be used to a much greater extent than it is today. The qualitative elucidation of the nuclear magnetic resonance phenomena is clearly written as is the section of interpretation of spectra, so very useful in organic compound identification. There is little quantitative work in this area, however. Turbidimetry and

related methods are presented together, but the discussion is somewhat difficult to read. Many equations are introduced and the treatment includes light-scattering measurements. Numerous references are listed and classified, but the single experiment chosen is disappointing. It was hoped that some work in light scattering might be suggested.

Refractometry and polarimetry are discussed in two relatively short sections. The theoretical discussion of refractometry is good and the use of this property in monitoring chromatographic eluants and in structural studies is presented together with its well-known application in liquid identification. The theory involving polarimetry and related properties, such as optical rotatory dispersion and circular dichroism, is most complex and cannot be handled in a few pages as is evident from this chapter. The discussion of circularly and plane polarized light and the resolution of the former into the latter is not quite clear. Optical rotatory dispersion is treated in terms of the octant rule. The proposed experiments deal with pharmaceutical compounds or preparations and are good. Unfortunately, quantitative analysis by polarimetry is limited by lack of instrumental sensitivity.

Crystallography is introduced in a chapter which describes properties that are measurable optically. Although most interesting, the work proved very hard to read. Concomitant performance of the suggested experiments seems necessary. Possibly the use of photomicrographs, colored if colors are to be seen, together with diagrams using various colors to emphasize coordinate axes, angles, and lines would have improved this important section. The techniques of X-ray analysis in terms of topics such as powder diffraction, single crystal studies, and X-ray fluorescence are part of a chapter that reads well and cites good pharmaceutical examples, both in cases of identity study and quantitative determinations. Considering the analyses possible with the powder technique and the trace level detection possible when fluorescence is measured, it is disappointing that some experiments are not proposed for those fortunate enough to have an X-ray spectrometer.

Electrochemical topics are covered in five chapters. The presentation on potentiometry is rather standard and includes a discussion of electron tubes and some simple circuits. However, the use of 12 photographs of commercial instruments and injudiciously plotted curves is not making the most of space. The omission of specific ion electrodes is surprising. The conductimetric and high-frequency methods are discussed in a chapter entitled *Current Flow Methods*. The discussions are done well and include measurement circuits and Wheatstone bridge concepts with a description of critical micelle measurements. Coulometry and chronopotentiometry are written in a direct, crisp, clear, readable style. The pertinent equations and the salient differences between the techniques are made plain. At the end of each subsection, the pharmaceutical applications are listed and an experiment using an appropriate pharmaceutical is supplied for each technique.

The polarography and amperometry chapters are well done. The various equations necessary for polarography are derived, and concepts are lucidly introduced and developed. In addition to classical polarography, solid microelectrodes and measurements in nonaqueous media are discussed. The commentary on instrumental aspects is pertinent, and the experiments and summary of pharmaceutical applications are good. It was disappointing not to find any mention of AC polarography. Both one- and two-polarized electrode systems used in amperometric titrations are considered and the applications to pharmaceuticals are summarized.

The final group of topics includes chapters on mass spectrometry, gas chromatography, and radiochemistry. The survey of mass spectrometry begins with a good introduction to theory, but the study of fragmentation patterns clearly requires more intense work than could be presented in the available space. Although good use has been made of this technique, together with pyrolysis and gas chromatography with simplified interpretation by means of computer techniques, the utility in quantitative analysis, discussed in this section, has not been established. Gas chromatography is developed in the usual manner and seems generally satisfactory. However, the discussion of the detectors and their mode of operation is disappointing, as is the omission of pyrolysis techniques. Likewise, the choice of experiments is not imaginative with work such as the separation of steroids and the monitoring of barbiturates in the urine published in the literature. The closing chapter on radiochemistry presents a logical development of the theory and measurements appropriate to this area. However, the very important aspect of radiation safety procedures, decontamination, and waste disposal, as well as radioaseptic techniques, has not been included. The experiments are disappointing; it was hoped that procedures such as neutron-activation analysis (if a neutron source is available), scintillation spectrometry, or nuclide standardization and calibration would be included.

This reviewer recommends that this book be considered as a text for a course in instrumental analysis. It may be necessary to supplement this work from many available sources if some aspects of electronics or instrumental function and design are to be taught. But this is not a serious drawback since this text presents the analysis of pharmaceuticals, information not available in a text at this level before.

Reviewed by Thomas Medwick
College of Pharmacy
Rutgers University
Newark, NJ 07104 ■

Consulting. Establishing and Maintaining An Independent Practice By RICHARD A. STEMM. SiSi: Stemm's Information Systems & Indexes, P. O. Box 42576, Los Angeles, CA 90050, 1970. iii + 29 pp. 13.5 21 cm. Price $2.50.

Despite its brevity, this booklet, written by Richard Stemm, contains a vast amount of information for the practicing or aspiring consultant. The author states in the Preface that the primary value of the publication is helping an individual objectively plan and prepare a course of action to establish himself as an independent consultant.

The material appears to be applicable to individuals in most fields and specialties. Among the topics covered are proposing, quoting, negotiating, contracts, fees, and costs. The booklet also contains forms for the National Consulting Register, a division of the author's organization.

Staff Review ■

Codeine and Its Alternates for Pain and Cough Relief. By NATHAN B. EDDY, HANS FRIEBEL, KLAUS-JURGEN HAHN, and HANS HALBACH. World Health Organization, Geneva, Switzerland, 1970. i + 253 pp. 17.5 × 24 cm. Price $6.00.

This review of the analgesic and antitussive effects of codeine and its alternates was originally published, in five installments, in the *Bulletin of the World Health Organization* during 1968 and 1969.

The five chapters included are codeine, exclusive of its antitussive action; alternates for pain relief; the antitussive action of codeine—mechanism, methodology, and evaluation; potential alternates for cough relief; and discussion and summary.

Codeine, which has been used increasingly for the relief of pain and cough since it was first isolated in 1832 from morphine, is an effective analgesic and antitussive either alone or in combination with other compounds. This publication reviews the literature concerning the experimental and clinical effectiveness of codeine and a wide range of alternative substances.

Effectiveness and lack of undesirable side-effects, including tolerance, dependence, and liability to abuse, are among the leading characteristics to be considered in the evaluation of codeine and its potential alternates. These aspects are taken up in this review.

Staff Review ■

related methods are presented together, but the discussion is somewhat difficult to read. Many equations are introduced and the treatment includes light-scattering measurements. Numerous references are listed and classified, but the single experiment chosen is disappointing. It was hoped that some work in light scattering might be suggested.

Refractometry and polarimetry are discussed in two relatively short sections. The theoretical discussion of refractometry is good and the use of this property in monitoring chromatographic eluants and in structural studies is presented together with its well-known application in liquid identification. The theory involving polarimetry and related properties, such as optical rotatory dispersion and circular dichroism, is most complex and cannot be handled in a few pages as is evident from this chapter. The discussion of circularly and plane polarized light and the resolution of the former into the latter is not quite clear. Optical rotatory dispersion is treated in terms of the octant rule. The proposed experiments deal with pharmaceutical compounds or preparations and are good. Unfortunately, quantitative analysis by polarimetry is limited by lack of instrumental sensitivity.

Crystallography is introduced in a chapter which describes properties that are measurable optically. Although most interesting, the work proved very hard to read. Concomitant performance of the suggested experiments seems necessary. Possibly the use of photomicrographs, colored if colors are to be seen, together with diagrams using various colors to emphasize coordinate axes, angles, and lines would have improved this important section. The techniques of X-ray analysis in terms of topics such as powder diffraction, single crystal studies, and X-ray fluorescence are part of a chapter that reads well and cites good pharmaceutical examples, both in cases of identity study and quantitative determinations. Considering the analyses possible with the powder technique and the trace level detection possible when fluorescence is measured, it is disappointing that some experiments are not proposed for those fortunate enough to have an X-ray spectrometer.

Electrochemical topics are covered in five chapters. The presentation on potentiometry is rather standard and includes a discussion of electron tubes and some simple circuits. However, the use of 12 photographs of commercial instruments and injudiciously plotted curves is not making the most of space. The omission of specific ion electrodes is surprising. The conductimetric and high-frequency methods are discussed in a chapter entitled *Current Flow Methods*. The discussions are done well and include measurement circuits and Wheatstone bridge concepts with a description of critical micelle measurements. Coulometry and chronopotentiometry are written in a direct, crisp, clear, readable style. The pertinent equations and the salient differences between the techniques are made plain. At the end of each subsection, the pharmaceutical applications are listed and an experiment using an appropriate pharmaceutical is supplied for each technique.

The polarography and amperometry chapters are well done. The various equations necessary for polarography are derived, and concepts are lucidly introduced and developed. In addition to classical polarography, solid microelectrodes and measurements in nonaqueous media are discussed. The commentary on instrumental aspects is pertinent, and the experiments and summary of pharmaceutical applications are good. It was disappointing not to find any mention of AC polarography. Both one- and two-polarized electrode systems used in amperometric titrations are considered and the applications to pharmaceuticals are summarized.

The final group of topics includes chapters on mass spectrometry, gas chromatography, and radiochemistry. The survey of mass spectrometry begins with a good introduction to theory, but the study of fragmentation patterns clearly requires more intense work than could be presented in the available space. Although good use has been made of this technique, together with pyrolysis and gas chromatography with simplified interpretation by means of computer techniques, the utility in quantitative analysis, discussed in this section, has not been established. Gas chromatography is developed in the usual manner and seems generally satisfactory. However, the discussion of the detectors and their mode of operation is disappointing, as is the omission of pyrolysis techniques. Likewise, the choice of experiments is not imaginative with work such as the separation of steroids and the monitoring of barbiturates in the urine published in the literature. The closing chapter on radiochemistry presents a logical development of the theory and measurements appropriate to this area. However, the very important aspect of radiation safety procedures, decontamination, and waste disposal, as well as radioaseptic techniques, has not been included. The experiments are disappointing; it was hoped that procedures such as neutron-activation analysis (if a neutron source is available), scintillation spectrometry, or nuclide standardization and calibration would be included.

This reviewer recommends that this book be considered as a text for a course in instrumental analysis. It may be necessary to supplement this work from many available sources if some aspects of electronics or instrumental function and design are to be taught. But this is not a serious drawback since this text presents the analysis of pharmaceuticals, information not available in a text at this level before.

Reviewed by Thomas Medwick
College of Pharmacy
Rutgers University
Newark, NJ 07104 ■

Consulting. Establishing and Maintaining An Independent Practice By RICHARD A. STEMM. SiSi: Stemm's Information Systems & Indexes, P. O. Box 42576, Los Angeles, CA 90050, 1970. iii + 29 pp. 13.5 21 cm. Price $2.50.

Despite its brevity, this booklet, written by Richard Stemm, contains a vast amount of information for the practicing or aspiring consultant. The author states in the Preface that the primary value of the publication is helping an individual objectively plan and prepare a course of action to establish himself as an independent consultant.

The material appears to be applicable to individuals in most fields and specialties. Among the topics covered are proposing, quoting, negotiating, contracts, fees, and costs. The booklet also contains forms for the National Consulting Register, a division of the author's organization.

Staff Review ■

Codeine and Its Alternates for Pain and Cough Relief. By NATHAN B. EDDY, HANS FRIEBEL, KLAUS-JURGEN HAHN, and HANS HALBACH. World Health Organization, Geneva, Switzerland, 1970. i + 253 pp. 17.5 × 24 cm. Price $6.00.

This review of the analgesic and antitussive effects of codeine and its alternates was originally published, in five installments, in the *Bulletin of the World Health Organization* during 1968 and 1969.

The five chapters included are codeine, exclusive of its antitussive action; alternates for pain relief; the antitussive action of codeine—mechanism, methodology, and evaluation; potential alternates for cough relief; and discussion and summary.

Codeine, which has been used increasingly for the relief of pain and cough since it was first isolated in 1832 from morphine, is an effective analgesic and antitussive either alone or in combination with other compounds. This publication reviews the literature concerning the experimental and clinical effectiveness of codeine and a wide range of alternative substances.

Effectiveness and lack of undesirable side-effects, including tolerance, dependence, and liability to abuse, are among the leading characteristics to be considered in the evaluation of codeine and its potential alternates. These aspects are taken up in this review.

Staff Review ■

related methods are presented together, but the discussion is somewhat difficult to read. Many equations are introduced and the treatment includes light-scattering measurements. Numerous references are listed and classified, but the single experiment chosen is disappointing. It was hoped that some work in light scattering might be suggested.

Refractometry and polarimetry are discussed in two relatively short sections. The theoretical discussion of refractometry is good and the use of this property in monitoring chromatographic eluants and in structural studies is presented together with its well-known application in liquid identification. The theory involving polarimetry and related properties, such as optical rotatory dispersion and circular dichroism, is most complex and cannot be handled in a few pages as is evident from this chapter. The discussion of circularly and plane polarized light and the resolution of the former into the latter is not quite clear. Optical rotatory dispersion is treated in terms of the octant rule. The proposed experiments deal with pharmaceutical compounds or preparations and are good. Unfortunately, quantitative analysis by polarimetry is limited by lack of instrumental sensitivity.

Crystallography is introduced in a chapter which describes properties that are measurable optically. Although most interesting, the work proved very hard to read. Concomitant performance of the suggested experiments seems necessary. Possibly the use of photomicrographs, colored if colors are to be seen, together with diagrams using various colors to emphasize coordinate axes, angles, and lines would have improved this important section. The techniques of X-ray analysis in terms of topics such as powder diffraction, single crystal studies, and X-ray fluorescence are part of a chapter that reads well and cites good pharmaceutical examples, both in cases of identity study and quantitative determinations. Considering the analyses possible with the powder technique and the trace level detection possible when fluorescence is measured, it is disappointing that some experiments are not proposed for those fortunate enough to have an X-ray spectrometer.

Electrochemical topics are covered in five chapters. The presentation on potentiometry is rather standard and includes a discussion of electron tubes and some simple circuits. However, the use of 12 photographs of commercial instruments and injudiciously plotted curves is not making the most of space. The omission of specific ion electrodes is surprising. The conductimetric and high-frequency methods are discussed in a chapter entitled *Current Flow Methods*. The discussions are done well and include measurement circuits and Wheatstone bridge concepts with a description of critical micelle measurements. Coulometry and chronopotentiometry are written in a direct, crisp, clear, readable style. The pertinent equations and the salient differences between the techniques are made plain. At the end of each subsection, the pharmaceutical applications are listed and an experiment using an appropriate pharmaceutical is supplied for each technique.

The polarography and amperometry chapters are well done. The various equations necessary for polarography are derived, and concepts are lucidly introduced and developed. In addition to classical polarography, solid microelectrodes and measurements in nonaqueous media are discussed. The commentary on instrumental aspects is pertinent, and the experiments and summary of pharmaceutical applications are good. It was disappointing not to find any mention of AC polarography. Both one- and two-polarized electrode systems used in amperometric titrations are considered and the applications to pharmaceuticals are summarized.

The final group of topics includes chapters on mass spectrometry, gas chromatography, and radiochemistry. The survey of mass spectrometry begins with a good introduction to theory, but the study of fragmentation patterns clearly requires more intense work than could be presented in the available space. Although good use has been made of this technique, together with pyrolysis and gas chromatography with simplified interpretation by means of computer techniques, the utility in quantitative analysis, discussed in this section, has not been established. Gas chromatography is developed in the usual manner and seems generally satisfactory. However, the discussion of the detectors and their mode of operation is disappointing, as is the omission of pyrolysis techniques. Likewise, the choice of experiments is not imaginative with work such as the separation of steroids and the monitoring of barbiturates in the urine published in the literature. The closing chapter on radiochemistry presents a logical development of the theory and measurements appropriate to this area. However, the very important aspect of radiation safety procedures, decontamination, and waste disposal, as well as radioaseptic techniques, has not been included. The experiments are disappointing; it was hoped that procedures such as neutron-activation analysis (if a neutron source is available), scintillation spectrometry, or nuclide standardization and calibration would be included.

This reviewer recommends that this book be considered as a text for a course in instrumental analysis. It may be necessary to supplement this work from many available sources if some aspects of electronics or instrumental function and design are to be taught. But this is not a serious drawback since this text presents the analysis of pharmaceuticals, information not available in a text at this level before.

Reviewed by Thomas Medwick
College of Pharmacy
Rutgers University
Newark, NJ 07104 ■

Consulting. Establishing and Maintaining An Independent Practice By RICHARD A. STEMM. SiSi: Stemm's Information Systems & Indexes, P. O. Box 42576, Los Angeles, CA 90050, 1970. iii + 29 pp. 13.5 21 cm. Price $2.50.

Despite its brevity, this booklet, written by Richard Stemm, contains a vast amount of information for the practicing or aspiring consultant. The author states in the Preface that the primary value of the publication is helping an individual objectively plan and prepare a course of action to establish himself as an independent consultant.

The material appears to be applicable to individuals in most fields and specialties. Among the topics covered are proposing, quoting, negotiating, contracts, fees, and costs. The booklet also contains forms for the National Consulting Register, a division of the author's organization.

Staff Review ■

Codeine and Its Alternates for Pain and Cough Relief. By NATHAN B. EDDY, HANS FRIEBEL, KLAUS-JURGEN HAHN, and HANS HALBACH. World Health Organization, Geneva, Switzerland, 1970. i + 253 pp. 17.5 × 24 cm. Price $6.00.

This review of the analgesic and antitussive effects of codeine and its alternates was originally published, in five installments, in the *Bulletin of the World Health Organization* during 1968 and 1969.

The five chapters included are codeine, exclusive of its antitussive action; alternates for pain relief; the antitussive action of codeine—mechanism, methodology, and evaluation; potential alternates for cough relief; and discussion and summary.

Codeine, which has been used increasingly for the relief of pain and cough since it was first isolated in 1832 from morphine, is an effective analgesic and antitussive either alone or in combination with other compounds. This publication reviews the literature concerning the experimental and clinical effectiveness of codeine and a wide range of alternative substances.

Effectiveness and lack of undesirable side-effects, including tolerance, dependence, and liability to abuse, are among the leading characteristics to be considered in the evaluation of codeine and its potential alternates. These aspects are taken up in this review.

Staff Review ■

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

OCTOBER 1970
VOLUME 59 NUMBER 10

REVIEW ARTICLE

Microencapsulation

LOUIS A. LUZZI

Keyphrases ☐ Microencapsulation—review ☐ Coacervation simple, complex—microencapsulation ☐ Phase separation—microencapsulation ☐ Interfacial polymerization—microencapsulation ☐ Electrostatic methods—microencapsulation of aerosols ☐ Pharmaceutical applications—microcapsules

Microencapsulation may be thought of as a method of wrapping small entities in individual, protective coatings. These coatings may be designed to protect, separate, or aid in storage and handling. Alternatively, the coatings may be constructed so that the encapsulated material is released under prescribed conditions to control or prolong the action from the microscopic-size capsules. The conditions for release may be made dependent upon moisture, pH, physical force, or combinations thereof. The mechanism for release may be associated with leaching, erosion, rupture, or other such actions, depending upon wall construction.

Microencapsulation encompasses both science and technology. A great deal has been written concerning current applications and future possibilities for encapsulating those materials whose original activities, actions, or stabilities may be altered or controlled by packaging in microcapsules. The technology of microencapsulation, involving several varied disciplines, is advancing rapidly, but the application of the principles involved has not yet approached its full potential. Knowledge from several disciplines is often essential to the technology of microencapsulation. For example, it may be necessary for pharmacology and therapeutics to join with colloidal and polymer chemistry for the encapsulation of medicinals.

Much of the available information pertaining to microcapsules and microencapsulation is found in the patent literature. Many applications of microencapsulation, employing varied and ingenious techniques, are patented. However, as is typical with patent literature, control and testing data as well as completeness of details essential for reproducibility are either lacking or alluded to in ambiguous terms. Definitive literature in these areas is limited and will probably remain so for some time, due to security measures on the part of those companies holding patent rights.

In this review of microencapsulation, an attempt will be made to bring together the published information from the scientific, patent, and company-prepared literature. In cases in which the scientific or patent literature is accompanied by company brochures, only the former literature will be discussed and the latter will be referenced.

Microcapsules are measured in microns and usually fall into the range of from several to approximately 200 μ (1). Some of the literature (2) alludes to microcapsules as a form produced by a specific mechanism which yields discretely packaged materials regardless of size, as opposed to discrete, microscopic packages of material. For this paper, microcapsules are defined as being discrete packages of material in the size range of 0.5–200 μ (3), and the term applies to all small, discrete packages produced by any number of techniques. Figures 1–3 illustrate microcapsules in different stages of preparation.

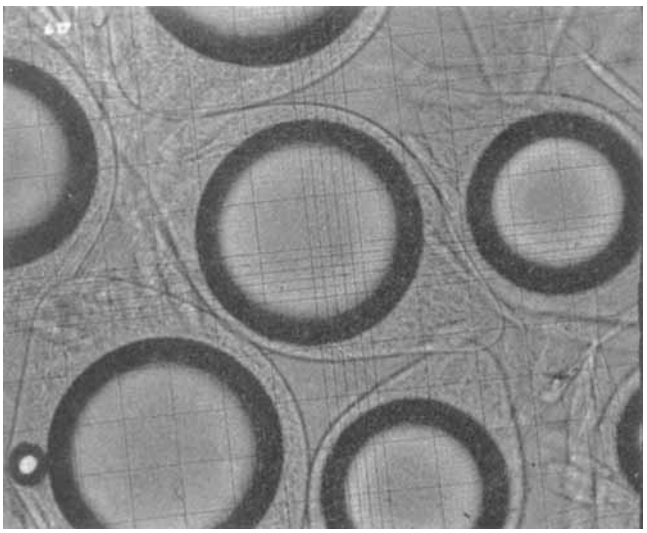


Figure 1—*Microcapsules of individual oil droplets before drying. (Courtesy of The National Cash Register Co.)*

SURVEY OF MICROENCAPSULATION METHODS

The processes of microencapsulation have been used to encase particles of liquids, solids, or gases. These particles must be immersible in the liquid phase containing the material that will ultimately form the capsule wall. The first practical use of microencapsulation appears in United States patents issued to Green and Schleicher (4–6). The principles of microencapsulation enumerated in these patents involve both simple and complex coacervation. Coacervation, as a method of microencapsulation, was also claimed in two other patents by Green and Schleicher (7) and by Green (8); these patents were filed at the same time but issued several years later.

Coacervation—The term "coacervation" has been used by chemists to describe the salting out of or phase separation of lyophilic solids into liquid droplets rather than into solid aggregates (9). The term was introduced into colloidal chemistry by Bungenberg de Jong and Kruyt (10) to describe the flocculation or separation of liquids from solution where at least one of the liquids contained a colloidal solute.

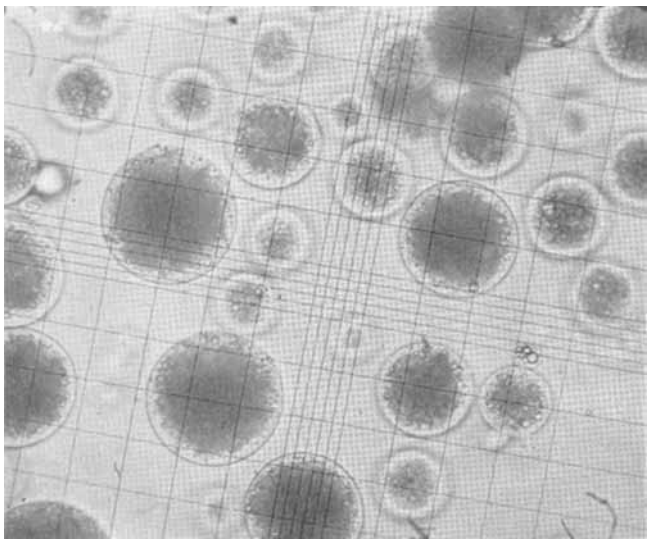


Figure 2—*Capsules within a capsule or aggregate microcapsules containing a liquid, before drying. (Courtesy of The National Cash Register Co.)*

Coacervation has been subdivided into two categories: simple coacervation and complex coacervation. Briefly, simple coacervation usually deals with systems containing only one colloidal solute, while complex coacervation usually deals with systems containing more than one colloid (11).

Simple coacervation is a process involving the addition of a strongly hydrophilic substance to a solution of a colloid. This added substance causes two phases to be formed, one phase rich in colloidal droplets and the other poor in such droplets. This process depends primarily on the degree of hydration produced. For example, addition of alcohol or sodium sulfate, as typical hydrophilic substances, to an aqueous solution of gelatin can lead to phase formation. When suitable conditions, including the presence of suitable nuclei, are prevalent, microcapsules may result.

Complex coacervation (Fig. 4) is primarily dependent on pH. It has been reported that in gum arabic–gelatin systems, complex coacervation occurred and microcapsules formed at pH values below the isoelectric point (IEP) of the gelatin but would not occur above this pH. At pH values below the IEP of gelatin, it becomes positively charged while acacia particles retain their negative charge regardless of pH. The same was found to be true of other systems containing two dispersed colloids, one of which was ampholytic (12).

Two mechanisms have been suggested for the formation of microcapsules by these methods (13): (*a*) individual coacervate droplets may be drawn to and coalesce about particles immiscible in the system, or (*b*) a single coacervate droplet may encompass one or a group of immiscible nuclei. Either or both mechanisms may be operative in a given system. However, unpublished data seem to indicate that, in certain cases, complex coacervate systems may be controlled to yield reproducible "compound capsules." That is, capsules may be formed which contain smaller, previously formed capsules from the same mother liquid, without first removing the smaller capsules or rejuvenating the liquid.

Phase Separation—This technique involves the dispersion of a polymer or copolymer in a solvent system in which the nucleus material is not soluble. Another liquid, which is miscible with the solvent system but which must be a nonsolvent for the polymer and nuclei, may be added. The rate of stirring, subsequent dispersion of the polymer-rich phase, and the rate of addition of the nonsolvent may be controlled to regulate the porosity of the capsule wall.

Several other methods of microencapsulation have been reported. These include interfacial polymerization, electrostatic methods, mechanical processes, and vacuum metalization.

Interfacial Polymerization—Microencapsulation by this method is a process whereby a monomer is made to polymerize at the interface of two immiscible substances. If the internal phase is a liquid, it is possible to disperse or solubilize the monomer in this phase and to

emulsify the mixture in the external phase until the desired particle size is reached. At this point, a crosslinking agent may be added to the external phase. Since there is usually some migration of the monomer from the internal to the external phase, and since it is preferred that the crosslinking agent not transfer to the internal phase, the bulk of any polymerization will take place at the interface (15).

An interesting dual-walled capsule formulation embodying a combination of coacervation and interfacial polymerization was disclosed by Brynko and Scarpelli (16). They prepared dual-walled oil-containing capsules whose inner wall consisted of polymerized styrene–divinylbenzene monomer with an outer wall of a gelatin–acacia coacervate shell. The monomer was dissolved in the internal phase, and polymerization took place during and after coacervation. The individual processes were controlled by temperature adjustment.

Electrostatic Methods—Preparation of microcapsules by these methods (17, 18) involve a bringing together of the wall material and the material to be encapsulated when both are aerosols. The material must be liquid during the encapsulation stage and must be capable of wetting the core material. Should the internal phase be liquid during the process, then it must have the higher interfacial tension to ensure encapsulation. The two aerosols must be oppositely charged; this can be accomplished prior to the mixing of the two. Three chambers are used in this process, with two of the chambers being atomization chambers and the third a mixing chamber. Oppositely charged ions are generated and directed into the two atomization chambers and are deposited on the liquid drops while they are being atomized. The capsules are allowed to cool in order to solidify the coating and then are collected by an appropriate aerosol collection system.

Mechanical Methods—One mechanical method (19) for producing microcapsules involves the use of counter-rotating disks. The inner rotating disk produces small particles of the core material. These particles are projected toward a point on the outer counter-rotating disk, which is lined with liquid coating material and has a row of orifices. When a sufficient weight of core material has collected at a particular orifice, centrifugal force separates the core and wall-forming material from the rotating cylinder, thus completing capsule formation. The amount of wall material applied is related to the initial concentration of the wall solution and the rate at which the solution flows onto the outer disk.

Another method (20) involves the dripping of the material to be encapsulated onto a continuous film of the liquified wall material and subsequent collection of the capsules. Here, again, the accumulated weight of core material causes the film to separate, with subsequent capsule formation (Fig. 5).

Still another method for the mechanical coating of small particles is the Wurster fluidized-bed coating technique (21–23). Figure 6 shows that the apparatus may consist of a vertical, somewhat conical column. A gas, carrying the coating material, is introduced at the base or constricted part of the column at a velocity high enough to suspend the particles. The gas velocity

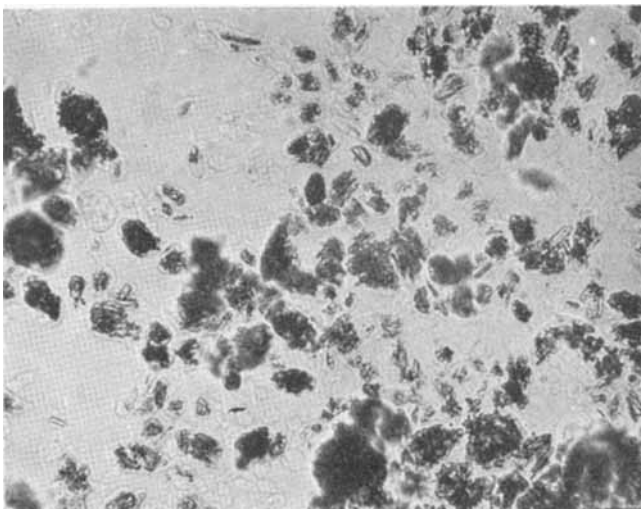


Figure 3—*Microencapsulated solid before drying.*

in the flared part of the column is greatly decreased, so the particles cannot be supported in this region and they fall outward and downward into the constricted region where they are again lifted by the gas flow. The wall material is dissolved in a solvent (usually volatile) and is sprayed onto the supported particles, in a fine mist, from a nozzle located near the bottom of the column. The solution coats the suspended particles, and heated air drives off the solvent. When the particles are sufficiently dry, the air flow is cut off and the coated product falls to the bottom of the apparatus for collection. The amount of wall material applied is generally proportional to the atomizing time, since the coating material is sprayed at a uniform rate and the particles

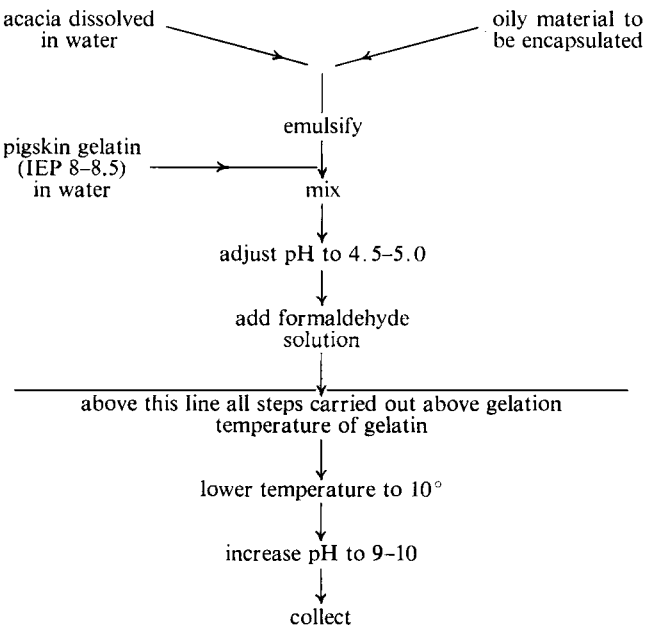


Figure 4—*Flow diagram of a typical method of manufacture of microcapsules* via *complex coacervation. Emulsification may be carried out with or without gelatin present. The particle size of the emulsified oil may be controlled, thus affecting the size of the ultimate capsule. Adjustment of pH to 4.5 causes coacervate droplets to form and encapsulate the emulsified oil. Formaldehyde addition, lowering of temperature, and increase in pH all fix the coacervate droplets in place about nuclei.*

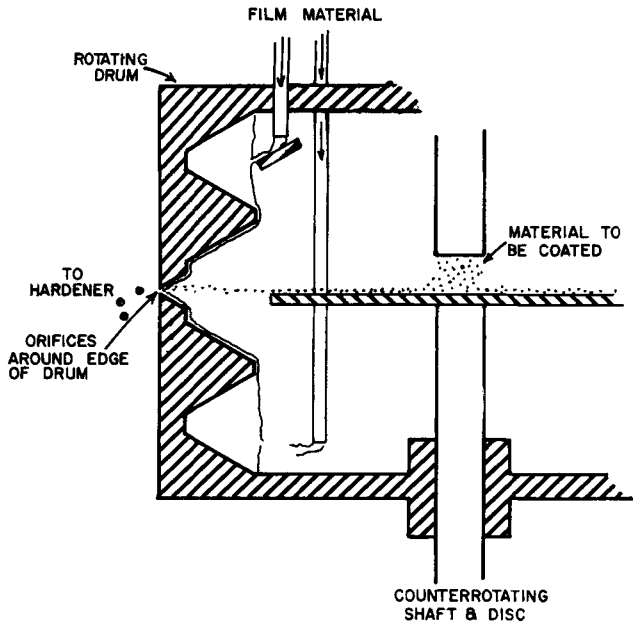


Figure 5—*Schematic of a counter-rotating, multiorifice apparatus for the coating of particles.*

are uniformly exposed to the spray. The time and air velocity required to coat the particles depend upon: (*a*) the starting surface area of the particles to be coated (the smaller the particle, the greater the surface area per pound of material to be coated and, therefore, the longer the time needed to coat); (*b*) the desired thickness of the coating; (*c*) the weight of particles coated per batch; and (*d*) the rate of flow of the coating liquid.

Vacuum Metalization—Although not directly applicable to pharmaceuticals when using metallic substances as the coating material, this process presents a very interesting methodology (24, 25). The apparatus (Fig. 7) consists of a container in which a vacuum can be pulled and which is equipped with a heated metal vaporization mechanism, a refrigerated hopper, and an inclined refrigerated vibrating tray. The particles to be coated are fed onto the tray and, in their cooled state, condense the metal vapors emerging from the heated crucible. The thickness of the coating may be regulated by controlling the rate at which the particles descend the inclined tray. Other factors which may be controlled to regulate wall thickness are the rate of metal vaporization and its consequent condensation.

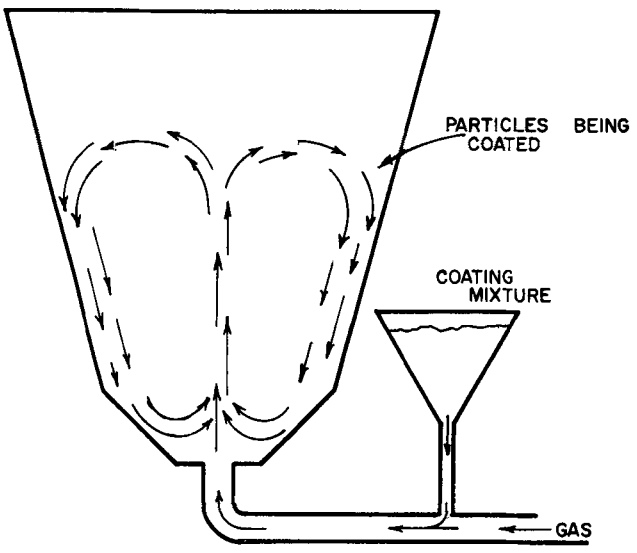


Figure 6—*Schematic of the Wurster coating apparatus.*

Many more devices have been reported. However, most of the principles have been discussed here. The specific mechanisms not mentioned are usually part of, or a combination of, several of those discussed.

PREPARATION OF MICROCAPSULES

Bungenberg de Jong (26) described many coacervate systems along with the behavior of coacervate droplets under a variety of conditions. Although he did not attempt the technique of microencapsulation, his descriptions and observations are the bases for the current technology of microencapsulation *via* coacervation. The first practical use of the process of coacervation was made by Wagner (3), Green and Schleicher (4), and Green (27, 28). Two of the early patents (3, 4) indicated that various oils, some of which contained dissolved dyes, had been encapsulated in gelatin–acacia microcapsules.

One procedure (4) consisted of dissolving 20 g. of acacia in 160 g. of water and emulsifying into this 80 g. of trichlorodiphenyl containing 3–6%, by weight, of color reactant materials. Emulsification was continued until the oil droplet size was from 2 to 5 μ. Next, 20 g. of gelatin was dissolved in 160 g. of water and mixed with the emulsion. This mixture of colloids and oil was then diluted by adding approximately 525 g. of water, at which time (the patent states) coacervation occurred and the gelatin–acacia complex formed about the oil droplets; all ingredients were kept at 50°. The mass was then cooled by pouring it into a quantity of cold (0°) water. The mixture was agitated and allowed to stand for 1 hr. at not more than 25°. The pH was adjusted to between 7 and 9 with sodium hydroxide, and the material was left in this state for at least 30 min. The capsules were hardened by adding 20 g. of formaldehyde solution USP and mixed at 3° or lower for about 10 min. Since the ultimate use of this material was for the preparation of "carbonless carbon paper,"[1] the capsules were not dried but the water content of the mixture was adjusted to suit this application.

In another patent (5), Green and Schleicher, again using similar materials, related that encapsulation may be brought about by adjusting pH and by controlling the degree of hydration of pigskin gelatin through salt addition. Using the pH adjustment method, the oily material was emulsified into an acacia solution; a solution of pigskin gelatin, with an IEP of pH 8, was added to the emulsion. Then, the pH of the mixture was brought to 6.5 with a 20% w/w solution of sodium hydroxide. Three hundred grams of this mixture was diluted with 700 g. of water at 50°. The pH of the diluted mixture was then lowered to approximately 4.5 with diluted acetic acid. It was during this step that the coacervate droplets formed and were deposited about

[1] The National Cash Register Co., Dayton, Ohio.

the oil droplets. While still at 50°, 2.19 g. of formaldehyde solution was added to help fix in place the coacervate droplets. The mixture was cooled to 10° and, subsequently, the pH was brought to 9. The cooling and pH adjustment further immobilized the formed shell.

Another method of microencapsulation is given in the same patent and describes coacervation by salt addition. In this case, the emulsion, containing 100 parts of 10% w/w gelatin and 20 parts of oil but no acacia, is treated in a similar manner, except that coacervation is induced by slowly adding, with stirring, a strongly hydrophilic salt solution. In this instance, 1.51 l. of a 20% solution of sodium sulfate was added at 50°. The mixture was then placed in 37.85 l. of 7% sodium sulfate solution at about 20°. The material was filtered and washed to remove the salt and was later treated with formaldehyde solution.

In later patents, Green and Schleicher (7) and Green (8) presented schematic diagrams for the preparation of microcapsules by both simple and complex coacervate systems. They also offered further explanation of these reactions and included an enumeration of other core materials consisting of naturally occurring and synthetic oils. A list of anions and cations arranged according to their relative hydrophilic nature was given to aid in determining the efficiency of various salts which may be used for simple coacervate systems.

In other patents involving coacervation of phase separation, several inventors added to the technology of microencapsulation. Materials other than, and in addition to, gelatin, albumin, casein, pectin, acacia, *etc.*, have been used. Solids as well as oils have been encapsulated, wall materials have been altered, sealing coats have been applied, and various combinations of natural and synthetic materials have been used as coating agents. The following is a sampling of the many variations perpetrated to attain microcapsules having various "improved" characteristics. An attempt will be made, in this section, to discuss patent information in a chronological manner, using filing dates as a guide.

Brynko and Scarpelli (16), in their patented process of making dual-walled capsules, dissolved two monomers, dichlorodiphenyl monomer and styrene monomer, in an internal oil phase. The oil phase was emulsified into an aqueous phase containing acacia, gelatin, and (to act as the catalyst for the monomers) potassium persulfate. The initial pH of the emulsion was 6.5 and the temperature was 55°. The pH was subsequently lowered and the temperature brought to 15°. Polymerization of the monomers began with the initial mixing of the phases and was substantially completed at the interface by the time coacervation had been accomplished. To harden the capsule walls, formaldehyde solution was added and the pH was raised to 10. Agglomeration of capsules was controlled by the addition of a maleic anhydride copolymer such as the polyvinylmethyl ether–maleic anhydride copolymer.

Brynko and Scarpelli (29) also reported that dual-walled capsules have been formed by causing deposition, *via* coacervation, of a lower molecular weight copolymer followed by an addition of a higher molecular weight copolymer. They reported a mixture of polyethylene–maleic anhydride copolymer, of approximate 1000–2000 molecular weight, in an aqueous solution containing gelatin and acacia at 35° and at pH 9. The material to be encapsulated was dispersed in the mixture and the pH was lowered to 4.8. A wall thickness of about 5 μ was found when coacervation was completed using the prescribed quantities of materials. The pH was then raised to about 6.8 to allow for the addition of an aqueous medium containing polyethylene–maleic anhydride copolymer having a molecular weight of 60,000–70,000. At pH 6.8, decoacervation took place and, consequently, it was important that the time during which the mixture was at this pH was at a minimum. Upon addition of the higher molecular weight copolymer, the pH was lowered to approximately 5. The resultant deposition yielded capsules with a total wall thickness of about 100 μ. When the mixture was cooled to 13°, the walls became rigid and solid but were still in a reversible state; formaldehyde, glutaraldehyde, or a similar crosslinking material was used to fix the wall material.

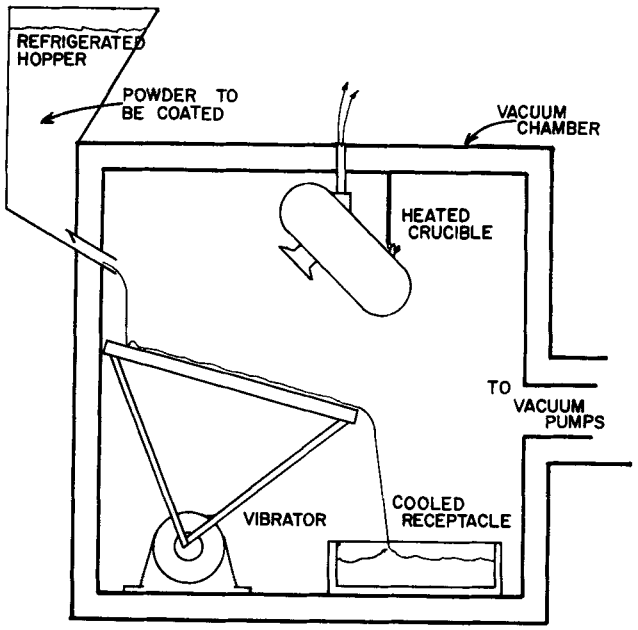


Figure 7—*Vacuum metalization apparatus for encapsulation.*

In another patent (30), fractionated gelatin along with acacia was used as the wall-forming material. Fractionation was accomplished by heating a solution of pigskin gelatin (IEP 8) at pH 7 and treating this with ethyl alcohol. Two distinct layers formed, one rising to the top and the other sinking. The lower layer was collected, dried, and comminuted. The powdered gelatin fraction, along with acacia, was dissolved in water, and capsules were formed by complex coacervation. Discrete capsules were collected and were found to be more pervious to light than capsules formed using nonfractionated gelatin.

Jensen (31) reported a method for encapsulation of water-soluble solid materials. The solid material was first coated with a liquefied lipid material by one of several mechanical methods. The coated solid was then encapsulated, using either simple or complex coacer-

Table I—Effect of pH on Diffusion Rate of Dye

pH of Material prior to Spray Drying	Diffusion Rate of Dye, % dye released/min.
4.00	0.125
5.00	0.125
6.00	0.128
7.25	0.180
8.50	0.302

vation or a partially hydrolyzed maleic anhydride copolymer. The solubility of the partially hydrolyzed copolymer was found to vary in different hydroalcoholic systems; the solubility was also found to decrease with the addition of selected salts. For example, completely hydrolyzed styrene–maleic anhydride copolymer was found to be about 2% soluble in water but at least 20% soluble in a 50–50 mixture of methanol and water. When the partially hydrolyzed copolymer was used, phase separation and consequent encapsulation of suspended material were brought about by the addition of an appropriate solvent or a salt such as magnesium sulfate. The sulfate salt was found to be effective over a wide concentration range. An adjustment of pH, either up or down depending on the polymer, was necessary to reduce the solubility of the wall material, thereby ensuring irreversibility.

Another method for the encapsulation of hydrophilic materials that involves precoating was disclosed by Heistand *et al.* (32). In this case, the precoating procedure was carried out from nonaqueous systems. For example, 50% hydrolyzed styrene–maleic anhydride copolymer was dissolved in a nonaqueous liquid. Into this an aqueous solution of the desired core material was emulsified. A third liquid, soluble in the organic liquid but not a solvent for the polymer, was added to induce phase separation and encapsulation of the suspended aqueous phase. The precoat was hardened, separated, and dried. The resultant precoated material was then suspended in an aqueous system from which encapsulation was brought about by any of several previously mentioned methods.

In a patent granted to Jensen (33), styrene–maleic anhydride copolymers and the hydrolyzed species of these copolymers were used as part of the negatively charged species to effect encapsulation *via* complex coacervation. It is stated that encapsulation by this method produces a shell which is less permeable than that obtained using, for example, gelatin and acacia. The gelation or cooling step may be eliminated when using this combination of materials.

Another method of preparing less permeable microcapsules was patented by Jensen (34) and involves controlling pH during the drying procedure. This included a spray-drying process which was used to obtain a free-flowing powder form of microcapsules and which was carried out at pH values between 4 and 6. It is stated in the patent that although the reason for the decrease in permeability was unknown and unexpected, nevertheless there existed an optimum between pH 4 and 6. Table I is a presentation of some of the data generated for this patent. The data were collected from 1 g. of dry product having a gelatin–acacia shell and suspended in 250 ml. of chloroform and stirred at a constant rate. The rate of diffusion of an encapsulated dye was measured spectrophotometrically using filtered aliquots of the chloroform suspension (Table I).

Jensen and Wagner (35) also were granted a patent in which they described the preparation of microcapsules using a partially hydrolyzed styrene–maleic acid copolymer. The method for the preparation of the acid copolymer was disclosed in this case.

In a patent entitled "Encapsulation in Natural Products" (36), the lipophilic material to be encapsulated was dispersed in a solution of egg albumin and, in order to bring about encapsulation, was heated to between 70 and 90°. In examples given in this patent, other techniques that aid coagulation were cited. These included addition of formaldehyde solution, alternations of ratios, variations of temperature and time, and use of additional polymers. The inventor stated that one advantage of this process was that it was particularly suitable for volatile substances since only moderately high temperatures were necessary.

Capsules with a wall material consisting of acacia and low viscosity ethylcellulose (ratio not more than 2:1) and gelatin were disclosed in a patent granted to Maierson (37). The process of encapsulation follows that of Green and Schleicher (5) and Green (6), differing only in the inclusion of ethylcellulose in the lipid phase. It was claimed that this modification of the process causes ethylcellulose to be deposited at the inside of the capsule walls, thereby plugging pores which may be present in the walls. Deposition of the ethylcellulose was said to be brought about by the presence of an aqueous phase. The film of ethylcellulose continues to deposit during coacervation and acts as host for acacia–gelatin droplets.

A cyclic process (38) for the manufacture of microcapsules that may contain acetylsalicylic acid and which employs nonaqueous dispersion media was disclosed in a patent issued to Miller and Anderson. Given as "Example I" of encapsulation in this patent is the following:

"This example utilizes (a) cyclohexane as the solvent vehicle, (b) butyl rubber . . . to maintain the wall material solution as a separate phase, (c) aspirin of a particle size . . . , and (d) ethylcellulose . . . solution in a 20% alcohol/toluene solvent as a wall material.

"Into a 600 ml. vessel, there are introduced, with agitation sufficient to produce liquid entities of ethylcellulose–cyclohexane solution of several microns average drop size at 80°, 200 grams of a 3% by weight, solution of the specified butyl rubber in cyclohexane, 4 grams of the specified ethylcellulose, and 48 grams of the specified particulate size acetylsalicylic acid, to form a system which is heated to 80°." (38)

When the desired dispersion had been reached, the system was cooled and the wall began to form at about 70°. The cooling was carried to room temperature. The capsules were removed and the remaining liquid reconstituted to its original proportions of starting ingredients. The capsules were recovered, in an appropriate manner, and dried.

Anderson *et al.* (39) also patented a method of encapsulation for aspirin using ethylcellulose. In this system, the aspirin particles were first wetted with an aqueous buffering solution of monobasic potassium phosphate previously adjusted to pH 2.3 with phosphoric acid. The aspirin particles were dried and the encapsulation procedure begun.

Ethylcellulose, acetic anhydride, and polyethylene were dispersed in cyclohexane at room temperature and solubilized by raising the temperature to 80°. At this point, the buffered aspirin particles were dispersed with continued agitation, and the temperature was lowered to approximately 25°. The capsules thus formed were separated from the insoluble polyethylene particles by washing through a screen. Polyethylene was used in this system as a phase separation inducing material, which caused no hydrolysis of aspirin as may have been the case with water-soluble materials.

When the phase separation of a polymer is used as the method of coating small particles, difficulties are sometimes encountered in recovering discretely coated nuclei. To overcome this, Rowe (40) incorporated mineral silicates into the system and thus minimized adhesion and coalescence of capsules. Generally speaking, when phase separation of polymers is to be the method of encapsulation, an organic solvent solution of the polymer is prepared, the particles to be coated are dispersed in it, and an organic nonsolvent for both the polymer and particles is added to the dispersion. A polymer-rich dispersed phase separates during the addition of the nonsolvent; this phase, while dispersed, may migrate to and coat suspended particles. It was found that incorporation of the mineral silicate (40) (talc was preferred) into the system during the addition of the nonsolvent minimized coalescence.

Another method of preventing capsules from aggregating during the isolation or drying step was patented by Maierson (41). In this method, cationic surfactants were added to redispersed capsules prior to drying. More than one surfactant was added, in varied sequences, to achieve optimum particle dispersion.

Ranney (42) compiled techniques from the U. S. patent literature pertaining to microencapsulated products. The techniques were abstracted from 81 U. S. patents, some of which have been presented here and some of which are yet to be discussed. He also included numerous flow charts and schematics obtained from the literature.

STUDIES OF MICROENCAPSULATION SYSTEMS

The most widely known application of microcapsules to date is, of course, manifest in the copying material produced by The National Cash Register Co. Their major product, in this area, is record paper commonly known as "carbonless carbon paper." Several methods of microencapsulation are utilized to encapsulate the system of leuco dyes used in this process. In all cases, however, the dyes are dissolved in an oily base prior to encapsulation. The formed capsules are coated or spread on paper along with a material which will cause color formation of the leuco dyes when the capsules are ruptured and contact between reactants is possible. This is not only the major application but, as mentioned earlier, is the first to become widely used in a commercial product. As noted, the technology has been widely patented and there has been a rapid diversification of application. Materials ranging from volatile oils to solids and semisolids are now being encapsulated. The materials used as encapsulating agents are just as varied, ranging from the gelatin–acacia complexes to ethylcellulose, hexamethylenediamine, and combinations of two or more of these or similar materials. In spite of the wealth of technology, and with one or two exceptions, microcapsules and the microencapsulation processes remain a curiosity. Figure 8 illustrates some of the characteristics of microcapsules. Numerous potential applications have been advanced in various patents and elsewhere (1, 43, 44) and have served to stimulate exploration of the area.

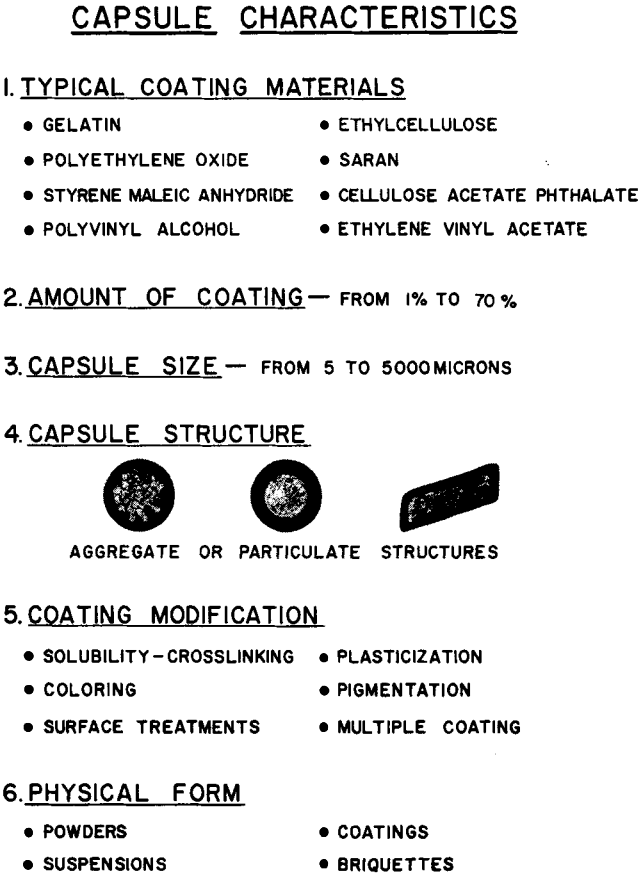


Figure 8—*Some capsule characteristics. (Courtesy of The National Cash Register Co.)*

The discussion to this point has primarily involved the patent literature. As such, it was descriptive in areas of technology but lacked depth of detail due to the nature of the literature covered. Several definitive treatments of restricted areas of microencapsulation have appeared in the literature. The object of an important, continuing, in-depth study (45–52) has been to encapsulate enzymes in semipermeable microcapsules for use in enzyme-replacement therapy. Thus far, these investigators have reported several successes using both shunt systems and, in certain cases, interperitoneal injection. They have encapsulated catalase,

Table II—Effect of Acid Value of Encapsulated Oil on Permeability of Microcapsule Wall[a]

Acid in Oil, %	Acid Value of Oil	Oil Extracted in 60 Min., ml.	Oil Extracted as Total Weight of Sample, %
0	0	0.012	1.02
1	2.15	0.012	1.02
2	4.22	0.061	5.18
3	6.08	0.091	7.70
4	8.24	0.248	21.08
5	10.47	0.320	27.20

[a] Reprinted, with permission, from L. A. Luzzi and R. J. Gerraughty, *J. Pharm. Sci.*, **53**, 431(1964).

urease, and erythrocyte hemolysates as well as detoxicants useful in conjunction with the experimental enzyme therapy.

Various encapsulation methods have been used by these investigators in attempts to achieve workable systems. Microcapsules have been prepared with walls of nylon, collodion, and heparin-complexed collodion. The nylon microcapsules are of special interest, since neither the methods by which they may be prepared nor the products themselves have, as yet, been discussed in this presentation. The nylon monomer, 1,6-hexanediamine, was dissolved in water along with a solution of the material to be encapsulated. This was emulsified into a mixture of chloroform and cyclohexane until the desired particle size was reached. At this point, a suitable polymerizing agent, such as sebacyl chloride, which had been dissolved in some of the external phase of the emulsion, was added. As there was migration of the nylon monomer across the interface, and since a polymerizing agent was present in the external phase, polymerization of the nylon took place in the immediate vicinity of the aqueous droplet. Improved capsules, in the sense that they existed individually by virtue of strong negative charges, were achieved when 4,4′-diamino-2,2′-diphenyldisulfonic acid was substituted for 50% of the nylon monomer.

Luzzi *et al.* (53) modified this technique to produce a sustained-release pharmaceutical dosage form. In this work, nylon was used to encapsulate a barbiturate. Particle-size range of the resultant free-flowing powder was noted, and kinetic data were given for the release of medication from tablets formulated in different manners.

Table III—Effect of Final pH on Permeability of Capsule Walls when a Solid Barbiturate Has Been Encapsulated *via* Complex Coacervation[a]

Final pH	After 0.5 Hr. in Gastric Fluid[b]	After 1 Hr. in Intestinal Fluid	After 2 Hr. in Intestinal Fluid	Total in Gastric and Intestinal Fluids
6.5	27.0	14.7	23.5	50.5
7.0	23.7	28.4	29.3	53.0
7.5	29.4	13.1	22.4	51.8
8.0	28.6	17.7	27.2	55.8
8.8	36.7	33.7	36.0	72.7

[a] Reprinted, with permission, from L. A. Luzzi and R. J. Gerraughty, *J. Pharm. Sci.*, **56**, 634(1967). [b] All values expressed as percent of encapsulated phase.

In other work by Luzzi and Gerraughty (54), some selected oils used in pharmacy were encapsulated and evaluated for their effect on capsule permeability (Table II). The capsules were prepared by complex coacervation, using gelatin and acacia, and contained oils with a variety of acid and saponification values and surfactant properties. In additional publications by the same authors (55, 56), a similar encapsulating system, modified for the encapsulation of solids, was evaluated. Manufacturing variables such as starting pH, starting temperature, ratio of encapsulated material to wall material, quantity of denaturant, and final pH (Table III) were examined to determine the effect on capsule formation. Techniques of altering and controlling the release of drugs from microcapsules were also studied.

Phares and Sperandio (57) used a simple coacervate encapsulating system to investigate the possibility of coating pharmaceuticals. They investigated the encapsulation of five solids and two liquids, with emphasis on regulation of coating thickness and on controlling the volume of coacervate. These authors (58) also investigated the preparation of phase diagrams for coacervate systems, using parameters of physical measurement rather than chemcial analysis methods which had previously been used.

Very basic work has been and is being carried out in the area of phase separation by Veis and coworkers (59–63). Although microencapsulation was not cited as the objective in these references, the information presented is important for an understanding of the area. These authors have explored phase separation in complex and simple coacervate systems, both with and without fractionated gelatin. In some cases, mathematical models were prepared; in others, the degree of separation of polymeric material was examined.

ADDITIONAL PRESENT AND FUTURE APPLICATIONS

Although wide ranging applications have been found for microcapsules, their potential use in pharmaceuticals has not yet been realized. The following brief overview of a few current and projected applications may suffice to stimulate further the reader's imagination.

The first application was, as mentioned, the preparation of "carbonless carbon paper." Many other uses for these capsules have since followed. The encapsulation of magnetizable materials was disclosed by Schleicher and Boughman (64). The encapsulated material may be permanently magnetized or may be magnetized by magnetic fields applied to the capsules. When the encapsulated and magnetizable materials are light-opaque platelets, light transmission through the area covered by these capsules may be controlled by the direction of the magnetic field. This type of application has potential in fields such as computerology, printing, data processing, copying, and light shuttering (Fig. 9).

Another application for microcapsules may be found when it is desired to apply a thin film of a volatile or partially volatile mixture on surfaces. One such application (65, 66) involves the encapsulation of ad-

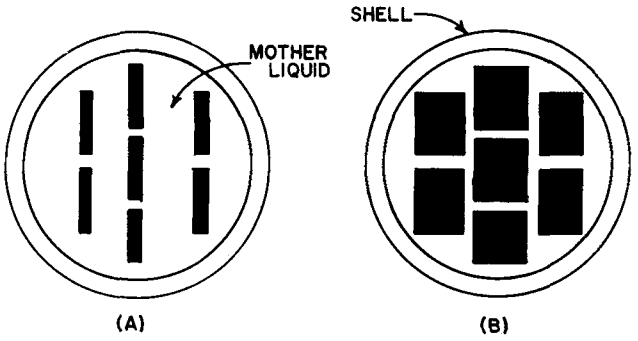


Figure 9—*Schematic of encapsulated magnetic pigments in different magnetic fields.*

hesives and subsequent distribution of the encapsulated adhesive on a surface. In the encapsulated form, the adhesive is nontacky and remains so until the capsules are ruptured. Various adhesive systems have been advanced and include heat reactivatable, resin-catalyst, solvent reactivatable, and totally encapsulated systems.

Another application (67) using films of microcapsules involves absorbent material which may be used for cleaning and wiping. In this case, the pressure applied during use releases an encapsulated soil-removing liquid. Similar products are becoming increasingly popular in the advertising media for fragrances and foods.

Other microencapsulated products, such as aspirin, have been marketed, and the potential for products seems to be unlimited. This is due to the variety of techniques that exists for encapsulating particles as a means of packaging, separating, and/or storing materials on a microscopic level for later release under controlled conditions. Pharmaceutical applications such as taste-masking of bitter drugs, formulation of prolonged-action medicinals, separation of incompatible materials, protection of moisture- and light-sensitive drugs, and formulations for enzyme-replacement therapy are areas currently receiving considerable attention.

Other areas, allied to pharmaceuticals, which are being investigated include agricultural chemicals and foods. In agriculture, microcapsules of insecticides, fungicides, and various microorganisms, for example, have been made in attempts to increase the longevity of action of the encapsulated material.

The food industry is using microcapsules as containers for maintaining the quality of fats, oils, and flavors. The encapsulated materials are released during the preparation of the meal or during ingestion.

Perhaps the most imaginative area for the application of encapsulated materials is in aerospace studies. Cosmic dosimeters utilizing encapsulated live bacteria are already in use. Measurement of survival of bacteria during a space trip can indicate how much cosmic radiation was encountered. Another possibility in the area of space travel may be in the construction of urethan-type shelters. This application depends on the encapsulation of a polymer which foams when the capsules are broken. This and other applications may be found in many of the references cited to this point; however, no attempt has been made to exhaust this type of reference. References 68–81 have been included in the list so that the interested reader may pursue the topic further.

REFERENCES

(1) Harvard Business School, "Report on Microencapsulation," Management Reports, Boston, Mass., 1963.
(2) "Microcapsulation, Big Rewards from Thinking Small," Business Management Magazine, January, 1966.
(3) J. G. Wagner, U. S. pat. 3,069,370(1962).
(4) B. K. Green and L. Schleicher, U. S. pat. 2,730,456(1956).
(5) B. K. Green and L. Schleicher, U. S. pat. 2,730,457(1956).
(6) B. K. Green, U. S. pat. 2,712,507(1955).
(7) B. K. Green and L. Schleicher, U. S. pat. 2,800,457(1957).
(8) B. K. Green, U. S. pat. 2,800,458(1957).
(9) "The Condensed Chemical Dictionary," 4th ed., Reinhold, New York, N. Y., 1950, p. 178.
(10) H. G. Bungenberg de Jong and H. R. Kruyt, *Proc. Kon. Ned. Akad. Wetensch.*, **32**, 849(1929).
(11) H. G. Bungenberg de Jong, in "Colloid Science," vol. II, H. R. Kruyt, Ed., Elsevier Publishing Co., Amsterdam, The Netherlands, 1949, p. 339.
(12) *Ibid.*, p. 341.
(13) L. A. Luzzi, Ph.D. dissertation, University of Rhode Island, Kingston, R. I., 1966.
(14) Unpublished data from these laboratories.
(15) T. M. S. Chang, *Science*, **146**, 524(1964).
(16) C. Brynko and J. A. Scarpelli, U. S. pat. 2,969,331(1961).
(17) G. Langer and G. Yamote, U. S. pat. 3,159,874(1964).
(18) J. A. Herbig, "Encyclopedia of Chemical Technology," 2nd ed., vol. 13, 1967, p. 446.
(19) G. R. Somerville, Jr., U. S. pat. 3,015,128(1962).
(20) C. F. Raley, Jr., W. J. Burkett, and J. S. Swearingen, U. S. pat. 2,766,478(1956).
(21) D. E. Wurster, U. S. pat. 2,648,609(1953).
(22) D. E. Wurster, U. S. pat. 2,799,241(1957).
(23) J. A. Herbig, "Encylcopedia of Chemical Technology," 2nd ed., vol. 13, 1967, p. 450.
(24) C. W. Baier and R. W. Steeves, U. S. pat. 2,846,971(1958).
(25) *Chem. Eng.*, **68**, 52(1961).
(26) H. G. Bungenberg de Jong, in "Colloid Science," vol. 2, H. R. Kruyt, Ed., Elsevier Publishing Co., Amsterdam, The Netherlands, 1949, pp. 243–487.
(27) B. K. Green, U. S. pat. 1,722,507(1955).
(28) B. K. Green, U. S. pat. 2,800,451(1957).
(29) C. Brynko and J. A. Scarpelli, U. S. pat. 3,190,837(1965).
(30) W. H. Anthony, U. S. pat. 3,041,288(1962).
(31) E. H. Jensen, U. S. pat. 3,265,629(1966).
(32) E. N. Heistand, J. G. Wagner, and E. L. Knoechel, U. S. pat. 3,242,051(1966).
(33) E. H. Jensen, U. S. pat. 3,265,630(1966).
(34) E. H. Jensen, U. S. pat. 3,043,782(1962).
(35) E. H. Jensen and J. G. Wagner, U. S. pat. 3,069,370(1962).
(36) S. Soloway, U. S. pat. 3,137,631(1964).
(37) T. Maierson, U. S. pat. 3,205,175(1965).
(38) R. E. Miller and J. L. Anderson, U. S. pat. 3,155,590(1964).
(39) J. L. Anderson, G. L. Gardner, and N. H. Yoshida, U. S. pat. 3,341,416(1967).
(40) E. L. Rowe, U. S. pat. 3,336,155(1967).
(41) T. Maierson, U. S. pat. 3,436,452(1969).
(42) M. W. Ranney, "Microencapsulation Technology," Noyer Development Corp., 1969.
(43) "NCR, Encapsulation—Pharmaceuticals," The National Cash Register Co., Dayton, Ohio.
(44) "NCR, Encapsulation—Applications," The National Cash Register Co., Dayton, Ohio.
(45) T. M. S. Chang, *Science*, **146**, 524(1964).
(46) T. M. S Chang, F. C. MacIntosh, and S. G. Mason, *Can. J. Physiol. Pharmacol.*, **44**, 115(1966).
(47) T. M. S. Chang and F. C. MacIntosh, *Pharmacologist*, **6**, 198(1964).
(48) T. M. S. Chang, *Trans. Amer. Soc. Artif. Intern. Organs*, **12**, 13(1966).
(49) T. M. S. Chang, *Sci. J.*, **3**, 62(1967).
(50) T. M. S. Chang, L. F. Johnson, and O. J. Ransome, *Can. J. Physiol. Pharmacol.*, **45**, 706(1967).

(51) T. M. S. Chang and M. J. Poznansky, *J. Biomed. Mater. Res.*, **2**, 187(1968).
(52) T. M. S. Chang and M. J. Poznansky, *Nature*, **218**, 243 (1968).
(53) L. A. Luzzi, M. A. Zoglio, and H. V. Maulding, to be published.
(54) L. A. Luzzi and R. J. Gerraughty, *J. Pharm. Sci.*, **53**, 429 (1964).
(55) *Ibid.*, **56**, 634(1967).
(56) *Ibid.*, **56**, 1174(1967).
(57) R. E. Phares, Jr., and G. J. Sperandio, *J. Pharm. Sci.*, **53**, 515(1964).
(58) *Ibid.*, **53**, 518(1964).
(59) A. Veis and C. Arenyi, *J. Phys. Chem.*, **64**, 1203(1960).
(60) *Ibid.*, **65**, 1798(1961).
(61) *Ibid.*, **67**, 1960(1963).
(62) A. Veis, J. Anesey, and J. Cohen, *Arch. Biochem. Biophys.*, **98**, 104(1962).
(63) A. Veis, E. Bodor, and S. Mussell, *Biopolymers*, **5**, 37(1967).
(64) L. Schleicher and C. S. Boughman, U. S. pat. 2,971,916 (1961).
(65) H. J. Eichel, U. S. pat. 2,1988,461(1958).
(66) H. J. Eichel, U. S. pat. 3,317,433(1967).
(67) R. E. Miller, U. S. pat. 2,980,941(1961).
(68) H. F. Huber and H. J. Stroble, *Tappi*, **49**, 41A(1966).
(69) J. A. Bakan, "Microencapsulation Via Coacervation—Phase Separation," presented at the National Industrial Research Conference, Land O'Lakes, Wis., June 1966.
(70) C. Thies, *J. Phys. Chem.*, **70**, 3783(1966).
(71) N. Yoshida and C. Thies, *J. Colloid Interface Sci.*, **24**, 29 (1968).
(72) R. P. Arens and N. P. Sweeny, U. S. pat. 3,423,489(1966).
(73) S. A. Bell, M. Berdick, and W. M. Holliday, *J. New Drugs*, **6**, 284(1966).
(74) J. Rotstein, E. I. Estrin, C. Cunningham, M. Gilbert, A. Jordan, J. Lamstein, M. Safrin, E. Wimer, and J. Silson, *J. Clin. P'Col.*, March-April 1967.
(75) M. J. Thomas, "Some NCR Encapsulation Techniques and Applications," 53rd Mid-Year Meeting Proceedings of the Chemical Specialties Manufacturers Association, Inc., New York, N. Y.
(76) *Chem. Week*, December 21, 1963.
(77) *Ibid.*, February 27, 1965.
(78) J. A. Bakan, "Microencapsulation as Applied to Pharmaceutical Products," presented at the Eastern Regional IPT Section, Academy of Pharmaceutical Sciences, Philadelphia, Pa., Oct. 1968.
(79) "Encapsulated Liquid Crystals," The National Cash Register Co., Dayton, Ohio.
(80) C. Thies, *J. Phys. Chem.*, **70**, 3783(1966).
(81) E. S. Raun and R. D. Jackson, *J. Econ. Entomol.*, **59**, 620 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Pharmacy, University of Georgia, Athens, GA 30601*

RESEARCH ARTICLES

Thermodynamic Analysis of Structure–Activity Relationships of Drugs: Prediction of Optimal Structure

TAKERU HIGUCHI and STANLEY S. DAVIS*

Abstract □ A new quantitative and comprehensive approach relating structures of congeneric drugs to their relative biological activities is presented. The analysis is derived on the basis that structure–activity relationships represent a family, a different situation applying to each phenomenon such as drug absorption, drug transport, drug transformation, and drug excretion. The present treatment considers the relationship under equilibrium or quasiequilibrium conditions, thus permitting rigorous thermodynamic treatment. On the basis of the effect of structural changes on the distributive tendencies of the drug in various body tissues, including the receptor site, relationships have been derived which are surprisingly in good agreement with available experimental data. The approach suggests a rational way to predict the degree of lipophilicity which would result in maximal activity.

Keyphrases □ Structure–activity relationships, drugs—optimal structure prediction □ Thermodynamic analysis—structure–activity relationships □ Equilibrium conditions, model compartments—thermodynamic activity, drugs □ Energy change—aqueous–lipid partitioning

Persistent efforts have been made over many decades to bring some satisfactory order to the correlation of the relative activities of drugs with their molecular structure. The last few years have seen a great upsurge in interest in this direction. In this publication, the authors: (*a*) review many of the earlier hypotheses and theories dealing with structure–activity relationships, and (*b*) present a new formulation of the problem based on thermodynamics.

The proposed approach, which will be treated in depth later in this paper, assumes that any observed biological activity in the animal or any test system usually involves one or more time-independent situations and a large number of time-dependent processes such as drug absorption, drug transport, drug transformation, and drug excretion. Since structural alterations affect each of these differently, it would appear highly unlikely that any single relationship can account for the observed situation. The present treatment has been largely limited to analysis of the effects of structural changes on the time-invariant activity of drugs.

As a general approximation, overall interaction of a drug molecule with its receptor site appears to be resolvable into two parts. The first is highly specific in nature and is presumably responsible for the "lock and

(51) T. M. S. Chang and M. J. Poznansky, *J. Biomed. Mater. Res.*, **2**, 187(1968).
(52) T. M. S. Chang and M. J. Poznansky, *Nature*, **218**, 243 (1968).
(53) L. A. Luzzi, M. A. Zoglio, and H. V. Maulding, to be published.
(54) L. A. Luzzi and R. J. Gerraughty, *J. Pharm. Sci.*, **53**, 429 (1964).
(55) *Ibid.*, **56**, 634(1967).
(56) *Ibid.*, **56**, 1174(1967).
(57) R. E. Phares, Jr., and G. J. Sperandio, *J. Pharm. Sci.*, **53**, 515(1964).
(58) *Ibid.*, **53**, 518(1964).
(59) A. Veis and C. Arenyi, *J. Phys. Chem.*, **64**, 1203(1960).
(60) *Ibid.*, **65**, 1798(1961).
(61) *Ibid.*, **67**, 1960(1963).
(62) A. Veis, J. Anesey, and J. Cohen, *Arch. Biochem. Biophys.*, **98**, 104(1962).
(63) A. Veis, E. Bodor, and S. Mussell, *Biopolymers*, **5**, 37(1967).
(64) L. Schleicher and C. S. Boughman, U. S. pat. 2,971,916 (1961).
(65) H. J. Eichel, U. S. pat. 2,1988,461(1958).
(66) H. J. Eichel, U. S. pat. 3,317,433(1967).
(67) R. E. Miller, U. S. pat. 2,980,941(1961).
(68) H. F. Huber and H. J. Stroble, *Tappi*, **49**, 41A(1966).
(69) J. A. Bakan, "Microencapsulation Via Coacervation—Phase Separation," presented at the National Industrial Research Conference, Land O'Lakes, Wis., June 1966.
(70) C. Thies, *J. Phys. Chem.*, **70**, 3783(1966).
(71) N. Yoshida and C. Thies, *J. Colloid Interface Sci.*, **24**, 29 (1968).
(72) R. P. Arens and N. P. Sweeny, U. S. pat. 3,423,489(1966).
(73) S. A. Bell, M. Berdick, and W. M. Holliday, *J. New Drugs*, **6**, 284(1966).
(74) J. Rotstein, E. I. Estrin, C. Cunningham, M. Gilbert, A. Jordan, J. Lamstein, M. Safrin, E. Wimer, and J. Silson, *J. Clin. P'Col.*, March-April 1967.
(75) M. J. Thomas, "Some NCR Encapsulation Techniques and Applications," 53rd Mid-Year Meeting Proceedings of the Chemical Specialties Manufacturers Association, Inc., New York, N. Y.
(76) *Chem. Week*, December 21, 1963.
(77) *Ibid.*, February 27, 1965.
(78) J. A. Bakan, "Microencapsulation as Applied to Pharmaceutical Products," presented at the Eastern Regional IPT Section, Academy of Pharmaceutical Sciences, Philadelphia, Pa., Oct. 1968.
(79) "Encapsulated Liquid Crystals," The National Cash Register Co., Dayton, Ohio.
(80) C. Thies, *J. Phys. Chem.*, **70**, 3783(1966).
(81) E. S. Raun and R. D. Jackson, *J. Econ. Entomol.*, **59**, 620 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Pharmacy, University of Georgia, Athens, GA 30601*

RESEARCH ARTICLES

Thermodynamic Analysis of Structure–Activity Relationships of Drugs: Prediction of Optimal Structure

TAKERU HIGUCHI and STANLEY S. DAVIS*

Abstract □ A new quantitative and comprehensive approach relating structures of congeneric drugs to their relative biological activities is presented. The analysis is derived on the basis that structure–activity relationships represent a family, a different situation applying to each phenomenon such as drug absorption, drug transport, drug transformation, and drug excretion. The present treatment considers the relationship under equilibrium or quasiequilibrium conditions, thus permitting rigorous thermodynamic treatment. On the basis of the effect of structural changes on the distributive tendencies of the drug in various body tissues, including the receptor site, relationships have been derived which are surprisingly in good agreement with available experimental data. The approach suggests a rational way to predict the degree of lipophilicity which would result in maximal activity.

Keyphrases □ Structure–activity relationships, drugs—optimal structure prediction □ Thermodynamic analysis—structure–activity relationships □ Equilibrium conditions, model compartments—thermodynamic activity, drugs □ Energy change—aqueous–lipid partitioning

Persistent efforts have been made over many decades to bring some satisfactory order to the correlation of the relative activities of drugs with their molecular structure. The last few years have seen a great upsurge in interest in this direction. In this publication, the authors: (*a*) review many of the earlier hypotheses and theories dealing with structure–activity relationships, and (*b*) present a new formulation of the problem based on thermodynamics.

The proposed approach, which will be treated in depth later in this paper, assumes that any observed biological activity in the animal or any test system usually involves one or more time-independent situations and a large number of time-dependent processes such as drug absorption, drug transport, drug transformation, and drug excretion. Since structural alterations affect each of these differently, it would appear highly unlikely that any single relationship can account for the observed situation. The present treatment has been largely limited to analysis of the effects of structural changes on the time-invariant activity of drugs.

As a general approximation, overall interaction of a drug molecule with its receptor site appears to be resolvable into two parts. The first is highly specific in nature and is presumably responsible for the "lock and

key" relationship between the two interactants (1). It is suggested that this part of the interaction involves those portions of the drug and receptor species that are in intimate fixed contact with each other. The second, which is largely unspecific in nature, is generally considered to arise simply from hydrophobic interaction between the lipoidal parts of the drug molecule and various lipophilic portions of the receptor. The present treatment is limited essentially to the influence on drug activity arising from the effects of changes in its molecular structure on the latter contribution to drug–receptor binding.

It is recognized, however, that the effect on activity of any structural changes in the drug molecule cannot always be totally ascribed to one or the other of the two interaction categories. Introduction of a hydrophobic grouping in the near vicinity of the specific site, for example, may increase the nonspecific interactions while interfering with the "lock and key" relationship. However, if the specific-type binding is assumed to be limited to a fractionally small part of the molecular surface of the drug, it would appear that most structural changes would not affect the specific interaction but would manifest their effect essentially through their influence on hydrophobic binding. Most serious approaches relating group contribution to drug activity have been limited primarily to effects arising from the same unspecific part of the overall interaction.

EARLIER APPROACHES

Some of the more interesting earlier studies on structure–activity relationships were those made by Overton (2), Meyer (3), and Meyer and Hemmi (4), who related narcotic activity to partition coefficients and suggested that narcosis occurred when a definite molar concentration was reached in the receptive lipid biophase. In a normal homologous series, for example, the increase in activity upon the addition of a methylene group was shown to be in the narrow range of 2.5–3.3 times, depending on the nature of the drug series and the test organism. Many physicochemical properties of aliphatic compounds, which depend on an equilibrium between two phases, increase or decrease with change in chain length in a similar manner. The increase in biological response with chain length does not continue indefinitely as predicted by this relationship; instead, a cutoff point is reached where higher homologs have little or no activity. Hansch (5) rightly commented that such effects are of extreme importance and should be explained by any proposed model dealing with structure–activity relationships.

Ferguson (6) rationalized the picture of narcotic action using thermodynamics and suggested that an equilibrium exists between the extracellular phase and the phase at the site of action (receptor site) such that substances present at the same proportional saturation in a given medium have the same degree of biological action. Or, as stated by Brink and Posternak (7), "equal degrees of narcosis are produced at about equal thermodynamic activities."[1] This generalization, unlike the earlier theories of Overton (2) and Meyer (3), has the great advantage of not requiring any specific mechanism of action. Ferguson (6) also considered that physical toxicity involved no chemical reaction and that the narcotic substance left the body unchanged (8). For example, the substitution of a halogen in a hydrocarbon leaves the potency practically unchanged, and no specific narcotic character can be ascribed to the chlorine atoms in chloroform, their presence merely lowers the vapor pressure of methane to a level convenient for administration.

McGowan (9) related the bioactivity of organic compounds to their size (parachor), while Mullins (10) examined the problem with the help of the solubility parameter concept (11). The latter concluded that narcosis, by chemically inert molecules, took place when constant fraction of the total volume of some nonaqueous phase in the cell was occupied by narcotic molecules. If the narcotic behaved ideally in the biophase, the thermodynamic activity multiplied by the volume fraction of narcotic was a constant. Higher values of thermodynamic activity, which occurred when a homologous series was ascended, were attributed to an increase in the activity coefficient of the narcotic in the biophase.

Crisp and Marr (12) examined the action of a range of narcotic substances from a more strictly thermodynamic standpoint and concluded that the mechanism of narcotic action in small organisms is only consistent with an equilibrium condition between the narcotic in the biophase and in the external medium. They felt that no theory that relied solely on a rate process, such as diffusion through a lipoid layer, could account adequately for the facts. However, Hansch and Fujita (13) recently concluded that, in the great majority of cases, a true equilibrium condition is rarely achieved and that a probabilistic approach may be far more realistic. These aspects will be discussed at greater length with relation to the proposed model.

By far the most widely known and employed linear free energy approach to stucture–activity correlation is that due to Hansch (5, 14–16) and Hansch and Fujita (13). Originally, a four-parameter approach was suggested:

$$\log BR = k\pi^2 + k'\pi + \rho\sigma + k'' \qquad \text{(Eq. 1)}$$

where π is a constant derived from partition studies between water and 1-octanol, σ is the Hammett constant, and ρ is a reaction constant derived from regression analysis. Correlation between biological response (BR) and chemical structure was achieved with a great many diverse systems. When only the π-term was necessary for good correlation, the response was considered to be controlled by a physical process (*e.g.*, partitioning of the drug), whereas a chemical interaction was thought to be responsible for correlations dominated by the σ-term. However, this type of one-parameter approach was limited, no doubt, to the complex nature of the biological test systems which would include problems such as drug penetration, possible differential rates of metabolism and excretion, and steric, electronic, and hydrophobic interactions with critical sites in the biophase. Also of equal importance is the very limited accuracy of much of the biological data.

In free energy terms the response can be considered as being governed by one rate-limiting process for which K_{BR} is the equilibrium constant (14). Then:

$$\Delta F_{BR}^0 = \Delta F_{L/H}^0 + \Delta F_{\text{electronic}}^0 + \Delta F_{\text{steric}}^0 \propto \log K_{BR} \qquad \text{(Eq. 2)}$$

where L/H = hydrophobic and BR = biological response.

In partial terms for substituent effects and for a true equilibrium condition:

$$\delta_x F_{BR}^0 = \delta_x F_{L/H}^0 + \delta_x F_{\text{electronic}}^0 + \delta_x F_{\text{steric}}^0 \propto \delta_x \log K_{BR} \qquad \text{(Eq. 3)}$$

Attempts can then be made to associate the various free energy terms with definite physicochemical constants (Table I). For example,

$$\delta_x F_{L/H}^0 = f(\log P,\ \pi,\ R_M,\ \Delta R_M,\ \beta, \text{ and parachor}) \qquad \text{(Eq. 4)}$$

$$\delta_x F_{\text{electronic}}^0 = f[\sigma,\ \textit{etc.},\ \text{quantum mechanically calculated electron densities or chemical shifts (NMR)}] \qquad \text{(Eq. 5)}$$

The $\delta_x F_{\text{steric}}^0$ terms are somewhat difficult to ascribe to definite parameters, although E_S and E_S^C have been used. Often, a two-parameter approach seems to be successful.

Detailed examples of the use of the various parameters can be found in one of the many recent reviews of Hansch's work (13–15). In some cases, this has indicated that, while steric interactions are extremely important [for example, see Portoghese (17)], the concept of "lock and key" fit of drug and substrate has been overemphasized at the expense of hydrophobic bonding (14).

[1] The use of the term "thermodynamic activity" in this sense is questionable. As defined by G. N. Lewis (*Proc. Amer. Acad. Arts Sci.*, **37**, 49(1901), activity of Component A can only be compared to some other state of A. The authors will treat this in greater detail in a subsequent paper.

Hansch's approach is not without its shortcomings (18) and by and large it cannot cope with steric factors or with metabolic inactivation processes too successfully. Also, some surprising results appear in regression analysis. For example, in one case the derived equations would indicate that the mode of action of thiobarbiturates differs from that of the analogous barbiturates (19).

The linear free energy relations of Zahradnik (20, 21) are also of the basic Hammett type but are restricted in their application to aliphatic compounds of the type R—X. They can be written as:

$$\log(BR_i/BR_0) = \alpha\beta \qquad \text{(Eq. 6)}$$

where BR terms refer to the response of the species (i) and the reference species (0); β is a constant characterizing the alkyl substituent R and its value is independent of the nature of the functional group X; and α characterizes the susceptibility of the biological system to the influence of the substituents R. The two constants are mutually independent. The β is linearly related to the logarithm of the activity coefficient of the drug and Hansch's π (22). Success in correlation has been demonstrated for situations where specific electronic and steric effects are not crucial, and the approach has also been extended (21) using Hammett and Taft substituent constants.

In continuing investigations designed to elucidate structure–activity relationships, Purcell *et al.* (23) attempted correlation with a wide range of parameters, including dielectric and surface-active properties, dipole moments, and electronic structure. A number of attempts have also been made to apply molecular orbital methods, but progress has been slow due to the lack of a reasonable theoretical framework within which to work (24). Hansch (5) has not attempted to factor out hydrogen bonding, although Purcell *et al.* (23) have suggested ways of dealing with such a term.

Table I lists some of the parameters that have been used in linear free energy correlations of the Hansch type. In multiparameter correlations, the mathematical significance of the various terms, π, σ, *etc.*, as well as π^2, σ^2, and $\sigma\pi$, are determined using regression analysis. The physicochemical significance of the various squared and product terms is not clear.

Table I—Linear Free Energy Correlation Parameters (log BR = $a(1) + b(2) + \cdots + k$)

	Parameter
1. π	Hydrophobic bonding constant from partition coefficients[a–e]
2. π^2	
3. σ	Hammett linear free energy constant[a–g]
4. σ^2	
5. σ^*	Taft aliphatic constant[h, i]
6. σ_I	Inductive parameter[j]
7. σ_+	Electrophilic radical constant[j]
8. σ_p	Electronic effect of substituent attached to side chain and positions *ortho* to it[k]
9. σ_m	
10. $\sigma^\cdot$	Radical constant[j]
11. $\pi\sigma$, *etc.*	?[l]
12. E_s	Taft steric parameter[m]
13. E_s^c	Hancock's corrected steric parameter[g]
14. N	Number of carbon atoms in substituent[n, o]
15. nH	Number of hydrogens attached to protonated nitrogen[g]
16. Log P	Partition coefficient[p]
17. Log VP	Vapor pressure (from GLC)[q]
18. P_E	Molar electronic polarizability[o]
19. γ	Arbitrary steric constant[r]
20. μ^2	Dipole moment[s]
21. $t_{1/2}$	Reaction parameter[q]
22. $S_o(Z)$	Superdelocalizability[t, u]
23. $f_{oxy.}(E)$	Frontier electron density (ether oxygen)[t, u]
24. ϵ	Electron density on nitrogen[v]
25. pKa	(*Reference 1*)
26. ΔpKa	Dissociation constant difference between parent and derivative[w]
27. E_R	Constant obtained from hydrogen abstraction reaction of nuclear-substituted cumenes[j]
28. Δk	Hydrogen bonding parameter[x]
29. P	Orientation polarization of amide groups[z]
30. A	Wheland's atomic localization energy[h]
31. [P]	Parachor[y]
32. E_A	Interaction parameter (hydrogen bonding)[z, aa]
33. F	Molar attraction constant[bb]
34. πF	?[bb]
35. R_M	Chromatography constant derived from R_f[cc]
36. *HOMO*	Energy of highest occupied molecular orbital[dd]
37. [P^*]	Adjusted parachor[ee, ff]
38. δ_c	Occupation number (extended Huckel theory)[gg]
39. e_c	Electron density by extended Huckel theory[gg]
40. ϵ_c	As 39, but by complete neglect of differential overlaps[gg]
41. θ	Total interaction energy hydride ion[gg]
42. Δ_E	Eigen value differences[gg]
43. δ_E	Incipient transition state energy differences[gg]
44. M	Molecular weight[ff]

[a] C. Hansch and T. Fujita, *J. Amer. Chem. Soc.*, **86**, 1616(1964). [b] C. Hansch, *Ann. Rept. Med. Chem.*, **1966**, 347. [c] C. Hansch, *ibid.*, **1967**, 348. [d] C. Hansch, *Farmaco, Sci. Ed.*, **23**, 293(1968). [e] C. Hansch, in *Proc. Int. Pharmacol Meet., 3rd*, 141 (1967). [f] K. Kakemi, T. Arita, R. Hori, and R. Konishi, *Chem. Pharm. Bull.*, **15**, 1705(1967). [g] C. Hansch, A. R. Steward, and J. Iwasa, *J. Med. Chem.*, **8**, 868(1965). [h] R. Zahradnik, *Experientia*, **18**, 534(1962). [i] C. Hansch and E. J. Lien, *Biochem. Pharmacol.*, **17**, 709(1968). [j] C. Hansch, *J. Med. Chem.*, **11**, 920(1968). [k] C. Hansch, R. M. Muir, T. Fujita, P. P. Maloney, F. Geiger, and M. Streich, *J. Amer. Chem. Soc.*, **85**, 2817(1963). [l] C. Hansch, K. Kiehs, and G. L. Lawrence, *ibid.*, **87**, 5770(1965). [m] C. Hansch and E. W. Deutsch, *Biochim. Biophys. Acta*, **126**, 117(1966). [n] W. R. Smithfield and W. P. Purcell, *J. Pharm. Sci.*, **56**, 577(1967). [o] A. Cammarata, *J. Med. Chem.*, **12**, 314(1969). [p] C. Hansch and S. M. Anderson, *ibid.*, **10**, 745(1967). [q] N. J. Turner and R. D. Battershell, "Abstracts, 155th National Meeting, American Chemical Society," No. A56, San Francisco, Calif., April 1968. [r] R. W. Fuller, M. N. Marsh, and J. Mills, *J. Med. Chem.*, **11**, 397(1968). [s] J. W. McFarland, L. H. Conover, A. L. Howes, J. E. Lynch, O. R. Chisholm, W. C. Austin, R. L. Cornwell, J. C. Danilewicz, W. Courtney, and D. H. Morgan, "Abstracts, 155th National Meeting, American Chemical Society," San Francisco, Calif., April 1968. [t] K. Fukui, K. Morokume, C. Nagata, and A. Imamura, *Bull. Chem. Soc. Japan*, **34**, 1224(1961). [u] K. Fukui, C. Nagata, and A. Imamura, *Science*, **132**, 87(1960). [v] C. Hansch, E. W. Deutsch, and R. N. Smith, *J. Amer. Chem. Soc.*, **87**, 2738(1965). [w] T. Fujita, *J. Med. Chem.*, **9**, 797(1966). [x] W. P. Purcell, J. G. Beasley, R. P. Quintana, and J. A. Singer, *J. Med. Chem.*, **9**, 297(1966). [y] J. C. McGowan, P. N. Atkinson, and L. H. Ruddle, *J. Appl. Chem.*, **16**, 99(1966). [z] J. C. McGowan, *ibid.*, **2**, 323(1952). [aa] N. C. Deno and H. E. Berkheimer, *J. Chem. Eng. Data*, **5**, 1(1960). [bb] J. A. Ostrenga, *J. Med. Chem.*, **12**, 349(1969). [cc] C. B. C. Boyce and B. V. Milborrow, *Nature*, **208**, 537(1965). [dd] W. B. Neely, H. C. White, and A. Rudzik, *J. Pharm. Sci.*, **57**, 1176(1968). [ee] J. C. McGowan, *Rec. Trav. Chim. Pays-Bas*, **75**, 193(1965). [ff] A. Leo, C. Hansch, and C. Church, *J. Med. Chem.*, **12**, 766(1969). [gg] R. B. Hermann, H. W. Culp, R. E. McMahon, and M. M. Marsh, *ibid.*, **12**, 749(1969).

The choice of terms is often bewildering; and in many cases, little can be said about a particular parameter except that it "works" and gives improved correlation. The complexity of some of the more recent equations is also disturbing. A recent illustrative example cited by Hansch (25) but not recommended contains seven terms to correlate 16 data points:

$$\log BR = -0.123\,\pi^2 + 0.633\,\pi - 1.823\,\sigma^{\cdot 2} + 3.162\,\sigma^\cdot - 0.796(\pi\sigma^\cdot) + 0.639\,E_s + 1.450 \qquad \text{(Eq. 7)}$$

Leo *et al.* (26) recently examined some of the parameters currently used in structure–activity relationships and their improvement, if any, over simple correlations using molecular weight. In general, the octanol–water partition coefficient was more suitable for satisfactory correlation than polarizability, molar attraction constant, parachor, or adjusted parachor. A great number of linear free energy constants (27) have yet to be tested but this would appear to be only a matter of time.

Free and Wilson (28) developed a purely mathematical approach to structure–activity relationships from an original proposal by Bruice *et al.* (29). Here it is assumed that the contribution due to each substituent is additive and constant, regardless of substituent variation in the remainder of the molecule. Although being restricted to a series of chemically related species, it has the great apparent advantage over linear free energy methods in that no physicochemical data are required. Smithfield and Purcell (30) discussed the application and requirements of the method. These are: (*a*) closely re-

lated analogs that provide a gradual change in biological response; (*b*) accurate biological data; and (*c*) additive activity parameters. Although somewhat limited for these reasons, it has been used with success by Purcell and Clayton (31), Ban and Fujita (32), and many others. A similar additive method is that of Kopecký *et al.* (33) in which constants are fitted to a semiempirical equation using regression analysis. This too is limited, at present, to specialized classes of compounds.

Singer and Purcell (34) compared the linear free energy and Free and Wilson (28) types of correlation technique. Each has its own merits and disadvantages. In particular, the Free and Wilson additive constant group contribution concept is not suitable for cases where there is a parabolic relation between the partition coefficient and biological response.

Interrelationships between the various methods have also been discussed by Cammarata (24) who showed that many of the physicochemical approaches to the study of drug action can be related, in a quantitative manner, to the principle of hard and soft acids. Here the drug–receptor interaction was discussed in terms of each pair of interacting atoms making an independent contribution to the electronic, steric, and desolvation free energies of interaction.

A NEW PROPOSAL BASED ON THE EQUILIBRIUM MODEL

It is apparent from the preceding analysis of the various approaches to structure–activity relationships that no single proposed system has been widely accepted and, at the same time, been based on rational grounds. To a certain degree, this has been due apparently to the fact that a single, simple correlation was sought when none was possible for the multifaceted situation. The authors wish to suggest a more limited approach which seems to be rational, relatively simple, and perhaps more useful. It is designed for any test system that rapidly achieves distributive equilibrium or quasi-equilibrium with respect to the added drug. It assumes, in brief, that: (*a*) any test system, whether a culture of microorganisms, a mouse, or a man, consists of a number of widely differing physical regions having widely differing affinities for the added drug species, and (*b*) biological activity is determined by the relative amount distributed to the receptor from the total system.

For the purpose of analysis, the authors make the following conditions and fundamental assumptions.

1. A biological test system can be represented by t number of accessible compartments, w, 1, 2, 3 . . . $t + r$, where compartment w is the aqueous phase; 1, 2, 3, *etc.*, are tissue, lipoidal, protein, *etc.*, phases; and r is the receptor. The receptor can either be some definite site (*e.g.*, an enzyme surface) or some unspecialized region in some cells. The effective volume of each compartment is V_w, V_1, V_2, *etc.*

2. Thermodynamic equilibrium or quasiequilibrium is reached in all accessible phases, and the thermodynamic activity of the drug in the rth compartment is the same as that in the aqueous, first, second, *etc.*, all with reference to a common standard state. If a drug is added to the aqueous compartment, it will be distributed to all the other available compartments according to Nernst's distribution law.

3. For a series of drugs of closely related structures, biological activity is proportional to the fraction of the active sites occupied. If the fractions occupied are made the same, then equal biological response will be elicited.

4. Essentially all of the administered drug will be distributed to the various accessible body compartments, and only an insignificant amount will actually be attached to the receptor site.

Apparent observed overall activities of a series of drugs in any animal test system will obviously be dependent on effects of structural changes on, for example, the process of absorption, the process of transport to the area of the receptor site, the process of excretion, the process of chemical transformation into metabolites, *etc.* As is apparent, the authors have in the present analysis restricted the definition of drug activity to equilibrium situations, the term drug activity being related to the intensity of biological response observed when the test drug is assumed to be completely distributed over all of the readily accessible tissue and fluid space. The equilibrium, or more correctly, the pseudoequilibrium definition of drug activity can be, to a certain extent, considered as the intrinsic (time-independent) activity, and other (time-dependent) effects can be considered modifications of it. The pseudoequilibrium situation commonly prevails, because a significant number of the body compartments of the test systems are not accessible to many drugs.

Table II—Affinity Constants for the CH_2 Group

Solvent	F
Carbon tetrachloride	4.6
Cyclohexane	4.5
Chloroform	4.4
Hexane	4.2
Benzene	4.2
Ethyl ether	3.6
Octanol	3.2
Oleic acid	3.1
Ethyl acetate	3.0
n-Butanol	2.6
2-Butanol	2.2
3-Pentanone	2.1

The influence of the distributive effects on drug activity can be formalized as follows. Consider the situation when an amount of drug, S, is administered to the test system. It is evident that

$$S = C_wV_w + C_1V_1 + C_2V_2 + \cdots C_iV_i$$
$$= C_wV_w + \sum_{i=1}^{i=t} C_iV_i \qquad \text{(Eq. 8)}$$

where C's refer to the effective concentrations in each accessible biophase compartment and V's to their volumes. As previously stated, the number of accessible compartments is taken as t, and the amount of drug incorporated into the receptor phase is normally negligible.

If the drug distribution between the aqueous phase and each biophase is assumed to follow a linear partition isotherm, a distribution constant can be defined:

$$K_i = \frac{C_i}{C_w} \qquad \text{(Eq. 9)}$$

and

$$S = C_w\left(V_w + \sum_{i=1}^{i=t} K_iV_i\right) \qquad \text{(Eq. 10)}$$

The effective concentration of the drug on the receptor can then be formulated by solving for C_w in Eq. 10:

$$C_r = K_rC_w = \frac{SK_r}{V_w + \sum_{i=1}^{i=t} K_iV_i} \qquad \text{(Eq. 11)}$$

or

$$E = \frac{C_r}{S} = \frac{K_r}{V_w + \sum_{i=1}^{i=t} K_iV_i} \qquad \text{(Eq. 12)}$$

E being the concentration of drug produced on the receptor per unit amount of drug administered. It relates directly to the expected relative activity of the drug.

What change in activity can be expected on this basis when, for example, a hydrogen in the reference (parent) drug is replaced by a methyl group? It is apparent from Eq. 12 that the activity of the new derivative will differ from that of the parent compound as the chemical change influences the various K, the partition coefficient, values in the equation. For a system where $V_w \gg \sum_{i=1}^{i=t} K_iV_i$ (that is, for a system where the bulk of the drug is in the aqueous phase), it is evident that the substitution of a methyl for a hydrogen will produce a marked increase in lipophilicity and activity corresponding to a similar increase in K_r (usually of the order of 2–3×), the partition coefficient of the receptor site. This relates as a first approximation to the amount of free energy necessary to bring a methylene group from aqueous to the receptor bond state. It is evident that if two methylene groups are introduced (*e.g.*, by substituting with C_2H_5 rather than CH_3), the increase in activity will correspond to the

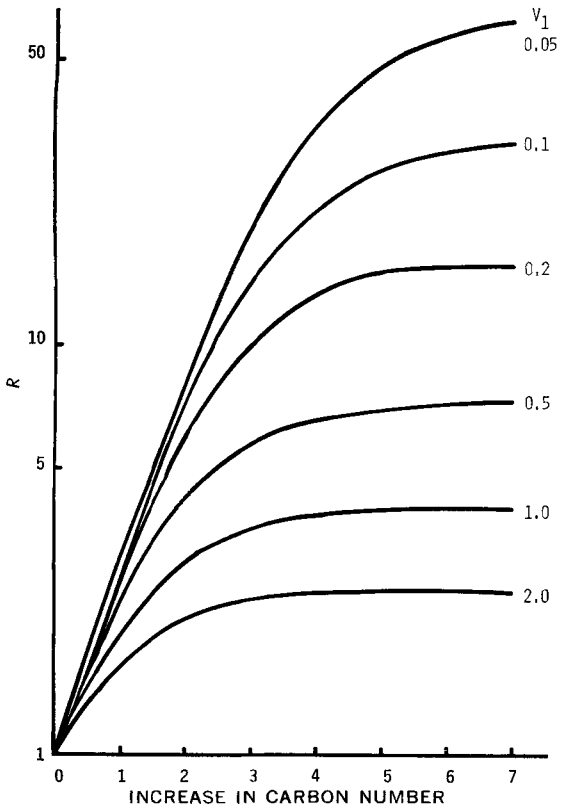


Figure 1—*Two-compartment model analysis of drug distribution:* $F_{(CH_2)r} = 3$ *and* $F_{(CH_2)l} = 3$.

square of the first; *i.e.*, if the first increase is by a factor of 2.5, the second will be approximately $(2.5)^2$ or 6.25. This follows essentially the group contribution approach developed by Hansch and others (5).

For the situation, $V_w < \sum_{i=1}^{i=t} K_i V_i$, *i.e.*, for systems in which the drug has been largely distributed into the tissue phases leaving only a small fraction in the aqueous, substitution of a methyl for a hydrogen may be expected to lead to a decrease in activity in instances where the receptor may be intermediate in polarity. This can be seen by using a highly simplified test system consisting only of an aqueous phase and a single lipoidal phase in addition to the receptor. For such a case:

$$E = \frac{K_r}{V_w + K_l V_l} \cong \frac{K_r}{K_l V_l} \quad \text{since } K_l V_l \gg V_w \quad \text{(Eq. 13)}$$

where subscript l refers to the lipoidal phase. It is evident that K_r may still increase by a factor of 2.5, but K_l may increase by a factor of 4.5, the expected decrease in activity in this example being 2.5/4.5 or by a factor of $^5/_9$. For the same system, introduction of C_2H_5 for CH_3 will be expected to produce a $(^5/_9)^2$ decrease in activity.

These concepts, as they apply to Eq. 12, can be generalized. Thus, when substituents α, β, *etc.*, are introduced, the effective drug concentration on the receptor is

$$E = \frac{K_r^*(F_\alpha)_r(F_\beta)_r \cdots}{V_w + \sum_{i=1}^{i=t} [K_i^*(F_\alpha)_i(F_\beta)_i \cdots]V_i} \quad \text{(Eq. 14)}$$

where the asterisk (*) refers to the partitioning properties of the parent reference drug into compartment i, and the F's are the factorial group contribution in modifying them with respect to each biophase. An F value, as used here, is the ratio of the partition coefficient of a substituted substance to the partition coefficient of the parent compound. For the example of the methyl substitution given in this illustration, $F_{(CH_2)r} = 2.5$ and $F_{(CH_2)l} = 4.5$. It is evident that for systems obeying equilibrium or pseudoequilibrium conditions, Eq. 14 will permit prediction of the effects of such substituents whose F values are known.

For comparison of relative activities of derived compounds with those of their parents, it is convenient to define another function:

$$R = E/E^* = \frac{K_r\left(V_w + \sum_{i=1}^{i=t} K_i^* V_i\right)}{K_r^*\left(V_w + \sum_{i=1}^{i=t} K_i V_i\right)} \quad \text{(Eq. 15)}$$

It is apparent that if $R > 1$, a derived compound is more active than its parent; conversely, if $R < 1$, it will be less active.

The relationships discussed can perhaps be seen more effectively graphically. For the two-compartment model discussed, select the situation such that $V_w = 1$ and $K_l^* = 0.33$. The resulting activity values expressed as R as functions of the number of added methylene groups are shown in Figs. 1–3. In all cases, R initially increases in magnitude with increase in chain length. When the volume of the lipoidal phase is somewhat less than that of the aqueous phase, this increase is more or less geometric in nature. However, the linear (geometric) region does not continue indefinitely, and a maximum or limiting value of R is obtained. If $F_{(CH_2)l} = F_{(CH_2)r}$, a plateau region is reached where further increase in chain length has little effect on R. If $F_{(CH_2)l}$ is greater than $F_{(CH_2)r}$, a maximum value for R is obtained. The exact shapes of the various hypothetical curves are dependent on the values of V_l and $F_{(CH_2)l}$, $F_{(CH_2)r}$. If $F_{(CH_2)l} = 2F_{(CH_2)r}$, a parabolic relationship results (Fig. 3). The position in the alkyl chain for maximum activity depends on the difference between the affinity factors (F values) of the receptor and lipoidal phase and the volume of the lipoidal phase. As the difference between $F_{(CH_2)r}$ and $F_{(CH_2)l}$ becomes greater, maximal R occurs at lower chain lengths. A similar result is obtained by increasing the volume of the lipoidal phase.

It is evident that for any real multicompartment system, the fractional distribution of drug into various compartments will be such that it will move from the aqueous phase gradually toward the most lipophilic compartment as the drug itself is made more lipoidal. In the extreme cases, the drug will be found largely in the aqueous or in the most lipoidal compartment (adipose tissues), which correspond roughly to the simplified example. The receptor sites, because of their locations, may be expected to offer intermediate environments

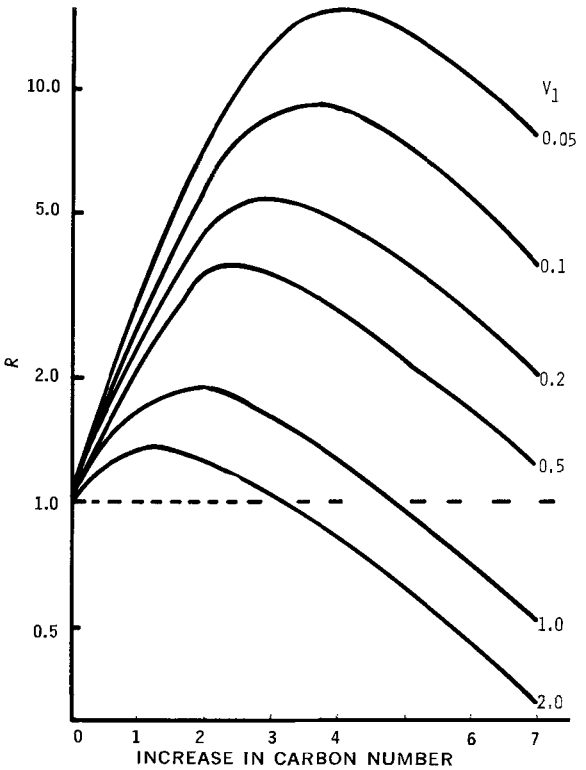


Figure 2—*Two-compartment model analysis of drug distribution:* $F_{(CH_2)r} = 3$ *and* $F_{(CH_2)l} = 4$.

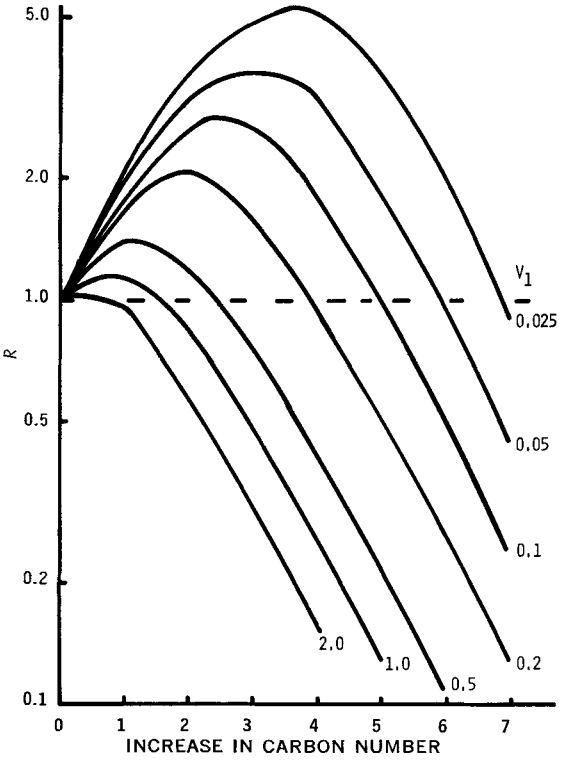


Figure 3—*Two-compartment model analysis of drug distribution:* $F_{(CH_2)_r} = 2$ *and* $F_{(CH_2)_l} = 4$.

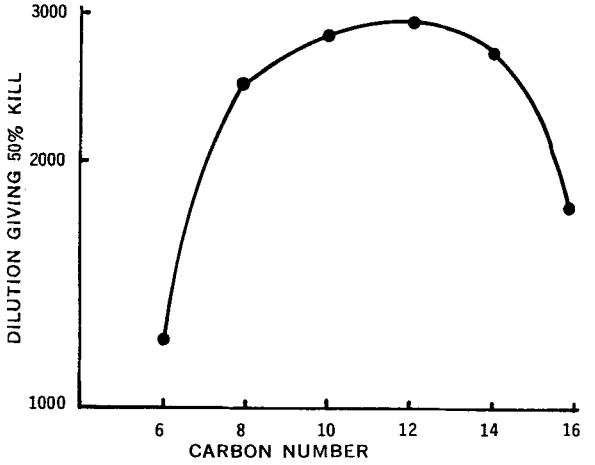


Figure 4—*Dilution of alkyl rhodanates required for 50% kill of green chrysanthemum aphid. [Plotted from the data of E. E. Bousquet, P. O. Salzberg, and H. F. Dietz,* Ind. Eng. Chem., *27, 1342(1935).]*

The authors found that for nearly all nonpolar lipoidal solvents such as benzene, cyclohexane, carbon tetrachloride, chloroform, and hexane, the free energy change in transferring a methylene function from water to the lipoidal environment is such that an addition of a CH_2 produces a partition coefficient increase by a factor close to 4.5 at 25° (Table II). Similarly, a CF_2 produces an increase by a factor of 5.5. It would appear, therefore, that if the effect on K_r is by a factor of 2–3, any significant increase in lipophilicity can be expected to lead to decreased activity at longer chain lengths. It is apparent that at some point of balanced lipophilicity, the optimal concentration of the drug on the receptor surface will be obtained. The so-called "parabolic" relationship between drug activity and lipophilicity, therefore, is a thermodynamically predictable situation, as shown in Figs. 1–3. Figure 4 shows an example of the "parabolic" relationship in biological response data between drug activity and lipophilicity.

Affinity constants, the F values, corresponding to effects of individual groupings on distributions of the drug between water and various lipoidal media (solvents) can most conveniently be estimated by partitioning experiments. For many systems, this would involve simply measuring the partition coefficient of a selected compound and that of the same compound containing in addition the grouping under study. The affinity constant for a methylene group, for example, can be evaluated by measuring the factorial increase in the partition coefficient of, for example, *p*-propylphenol as compared to that of *p*-butylphenol, the difference corresponding to the free energy of transfer of a methylene from water to the selected solvent. Some of these constants have been evaluated in the authors' laboratory in this manner, including the use of the ion-pair extraction procedure described earlier (35). It is evident that if the F values for all common substituent groupings were available, it would permit much greater insight into the potential distributive behavior of new drugs.

The $F_{(CH_2)}$ for inert hydrocarbon solvents, determined as described, is in the region of 4.2–4.5, while for polar liquids this value appears to be considerably lower and is dependent on the nature of the polar liquid in question. Some representative values calculated from partition data are shown in Table II. Values for other isolated groupings in various solvents can be also obtained from published data or from partition experiments. For example, $F_{(C_6H_6)}$ is 1000 in cyclohexane and 60 in octanol. The addition of a halogen atom brings about an increase in the partition coefficient in the majority of cases; for aromatic species, this increase can be correlated quite well with the size of the added groupings. Although different F values for the halogens are evident for different solvents, detailed accurate partition experiments will be necessary before a table of values similar to Table II can be derived for them.

Polar groupings, of course, present a very diverse picture, and a great variety of F values will be possible, depending on the nature of the lipid phase. To a first approximation, the hydroxyl group, for example, has an F value of 2×10^{-2} in octanol and 4×10^{-4} in cyclohexane. In general, polar groupings will have a solubilizing effect in water and will increase the hydrophilic nature of a drug species. Some insight into the possible variation in values of F can be gained by calculating activity coefficient values for various functional groups in different organic solvents (Table III). The magnitudes of the various contributions can be rationalized largely on the basis of hydrogen bonding interactions. When both hydrogen-donating and hydrogen-accepting groupings are present in a single drug molecule, there is always the possibility of intramolecular bonding resulting in marked departure from additivity situations for these groupings.

A semiquantitative concept of the effect of adding various functional groups to a hypothetical drug molecule can be obtained by using the F values given, employing arbitrary values for K_l, V_l, and V_w, and considering that in the simple two-compartment model the lipoidal phase is similar to an inert hydrocarbon (cyclohexane) and the receptor biophase is similar to a long-chain alcohol (octanol). The calculated R values (Table IV) show that the quantity of substituted drug reaching the receptor site, as compared to the parent drug, depends on the partition coefficient of the parent drug and the volume

Table III—Activity Coefficients of Aliphatic Functional Groups in Different Organic Solvents

Group	Solvent					
	Benzene	Chloroform	Carbon Tetrachloride	Octanol	Ether	Cyclohexane–Hexane
CH_2	1.00	0.96	0.92	1.32	1.15	1.00
COOH	64.5	45.7	74.2	0.58	1.41	50.0
C=O	2.6	0.56	6.1	2.6	2.0	(7)
OH	(1)	5.5	35	0.5	7	65
NH_2	1.5	0.25	2		0.75	5

Table IV—Substitution of a Hypothetical Drug; Effect of Partition Coefficient and Lipid Phase Volume on the Ratio R

Partition Coefficient of Parent Drug	Volume of Lipid Phase	R —CH_2	R —C_6H_5	R —OH
0.01	0.1	3.2	30.0	0.020
	1.0	3.1	5.5	0.0202
	10.0	2.5	0.65	0.022
0.1	0.1	3.1	5.5	0.0202
	1.0	2.5	0.65	0.022
	10.0	1.13	0.12	0.04
1.0	0.1	2.5	0.65	0.022
	1.0	1.13	0.12	0.04
	10.0	0.76	0.066	0.22
10.0	0.1	1.13	0.12	0.04
	1.0	0.76	0.066	0.22
	10.0	0.72	0.060	1.96
100.0	0.1	0.76	0.066	0.22
	1.0	0.72	0.060	1.96
	10.0	0.71	0.060	14.4

of the lipoidal compartment, all other factors being equal. The usual generalization that the addition of a hydrophobic grouping results in a greater quantity of drug at the receptor site and the converse for a hydrophilic grouping are of limited value. The CH_2 and C_6H_5 groups will give increased drug concentration at the receptor site and, hence, biological activity when K_l and V_l are small. However, when K_l and V_l become larger, the drug will be contained almost exclusively in the lipoidal phase. This is especially true for the C_6H_5 grouping in the present example, where R becomes very much less than unity, indicating a greatly reduced activity for the substituted form. At the other extreme, a hydroxyl group brings about reduced activity at low K_l and V_l but has the opposite effect at higher values.

One major point of interest resulting from this approach is that it suggests that within the stated limitations it is possible to predict the degree of lipophilicity required to elicit the optimal activity for a given drug series. As pointed out earlier, whether a given substituent will effect an improvement in the drug activity will depend on whether R as defined in Eq. 15 is greater than 1 or not, the optimal lipophilicity for the drug usually corresponding to $R = 1$ when a single CH_2 grouping is added. It is evident that if, for example, a methylene group is added to a parent drug molecule and the resulting derivative drug possesses essentially the same biological activity, R would be close to unity and these structures would represent nearly the peak in the parabolic relationship between activity and the number of, for example, methylene carbons. For the simple two-compartment model systems consisting only of the aqueous and lipoidal phases, R can be readily estimated by using Eq. 15 in terms of the relative amounts of the drug found in the aqueous phase (V_w) and in the lipid phase (V_l). In Fig. 5, R corresponding to addition of a single methylene is shown for $F_{(CH_2)l} = 4.5$ as a function of the logarithm of $K_l^* V_l/V_w$, the ratio of amount of drug concentrated in the lipid phase to that in the aqueous for several values of $F_{(CH_2)r}$, the affinity constant for the receptor site. Although the selection of the affinity constant for the receptor affects the ratio at which point $R = 1$, the maximum point in the parabolic relationship, the effect is relatively small, the ratio of the amounts of the drug in the two phases for $R = 1$ being 0.40 for $F_{(CH_2)r} = 2.00$, 0.56 for $F_{(CH_2)r} = 2.25$, and 1.25 for $F_{(CH_2)r} = 3.0$. Since the $F_{(CH_2)}$ values for most receptors would usually fall in the range of 2.25–2.50, assuming that the receptor site has an intermediate polar nature, the ratio would normally be expected to be within the range of 0.40–1.00 for maximum drug activity. Since this variance is well within the range produced by a single methylene group, it would appear that normally a rather sharp maximum in activity could be expected when approximately one-half to an equivalent amount of drug is concentrated in the adipose and other lipoidal tissues, as compared to that found free in the aqueous phase of the test system. This postulate is readily amenable to experimental test.

Although this approach was based on the simplified two-phase model, it is more widely applicable to real animal systems than it may first appear. The adipose tissue in man is normally the largest and the most important lipophilic depot, along with fat deposits in the circulatory and other systems. It is evident that it is largely to these accessible, similar, essentially nonpolar lipid deposits that lipophilic drugs tend to accumulate as they are made increasingly hydrophobic.

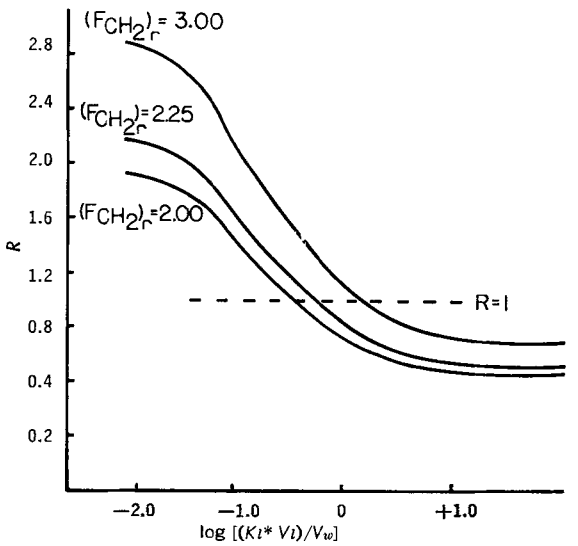


Figure 5—*Influence of distribution ratio and affinity constant for receptor site on the relative activity of drug containing CH_2 grouping over that of the parent compound.*

The present general approach has been described in terms of equilibrium interaction with a definite, although usually unidentified, receptor site. It is evident that essentially the same development and conclusions would apply to systems in which the drug activities are governed by rates of transport across membrane barriers. Since in such situations the rate is directly influenced by the equilibrium concentration on the surface of the barrier, all of the relationships derived apply with equal validity.

The authors recognize the fact that the present treatment attempts to treat an extremely complex problem in a simplistic way. Situations have been ignored that involve irreversible interaction with the receptor, induced conformational changes in the receptor, nonlinear distribution function (for the receptor and any of the remaining biophases), and any other complicating factor. The authors have not considered the specific interactions between the receptor and the drug nor between the competing sites in other biophases. The analysis treats the test system as being in equilibrium, a state that is not altogether realistic for any large living organism. Yet the present approach points to the start that must be taken in recognizing the influence of substituents on distributive tendencies to compartments other than the receptor in affecting the observed apparent activity.

REFERENCES

(1) "Molecular Pharmacology," E. J. Ariens, Ed., Academic, New York, N. Y., 1964, vol. I, p. 119; vol. II, p. 201.

(2) E. Overton, "Studien über die Narkose," G. Fischer, Jena, East Germany, 1901.

(3) H. Meyer, *Arch. Exp. Pathol. Pharmacol.*, **42**, 109(1899).

(4) K. H. Meyer and H. Hemmi, *Biochem. Z.*, **277**, 39(1935).

(5) C. Hansch, *Proc. Int. Pharmacol. Meet., 3rd, 1966*, **7**, 141 (1967).

(6) J. Ferguson, *Proc. Roy. Soc., Ser. B*, **127**, 387(1939).

(7) F. Brink and J. M. Posternak, *J. Cell. Comp. Physiol.*, **32**, 211(1948).

(8) J. Ferguson, *Chem. Ind. London*, **1964**, 818.

(9) J. C. McGowan, *J. Appl. Chem.*, **2**, 323(1952).

(10) L. J. Mullins, *Chem. Rev.*, **54**, 289(1954).

(11) J. H. Hildebrand and R. L. Scott, "Regular Solutions," Prentice Hall, Englewood Cliffs, N. J., 1962.

(12) D. J. Crisp and D. H. A. Marr, *Proc. Int. Congr. Surface Activ., 2nd*, **4**, 310(1957).

(13) C. Hansch and T. Fujita, *J. Amer. Chem. Soc.*, **86**, 1616 (1964).

(14) C. Hansch, *Ann. Rept. Med. Chem.*, **1966**, 347.

(15) *Ibid.*, **1967**, 348.
(16) C. Hansch, *Farmaco, Sci. Ed.*, **23**, 293(1968).
(17) P. S. Portoghese, *J. Pharm. Sci.*, **54**, 1077(1965).
(18) B. M. Bloom, *Ann. Rept. Med. Chem.*, **1965**, 236.
(19) C. Hansch, A. R. Steward, S. M. Anderson, and D. Bentley, *J. Med. Chem.*, **11**, 1(1968).
(20) R. Zahradnik, *Experientia*, **18**, 534(1962).
(21) R. Zahradnik, *Arch. Int. Pharmacodyn. Ther.*, **135**, 311 (1962).
(22) J. Kopecký and K. Bocek, *Experientia*, **23**, 125(1967).
(23) W. P. Purcell, J. G. Beasley, R. P. Quintana, and J. A. Singer, *J. Med. Chem.*, **9**, 297(1966).
(24) A. Cammarata, *ibid.*, **12**, 314(1969).
(25) C. Hansch, *ibid.*, **11**, 920(1968).
(26) A. Leo, C. Hansch, and C. Church, *ibid.*, **12**, 766(1969).
(27) P. R. Wells, "Linear Free Energy Relationships," Academic, New York, N. Y., 1968.
(28) S. M. Free and J. W. Wilson, *J. Med. Chem.*, **7**, 395(1964).
(29) T. C. Bruice, N. Kharasch, and R. J. Winzler, *Arch. Biochem. Biophys.*, **62**, 305(1956).
(30) W. R. Smithfield and W. P. Purcell, *J. Pharm. Sci.*, **56**, 577 (1967).
(31) W. P. Purcell and J. M. Clayton, *J. Med. Chem.*, **11**, 199 (1968).
(32) T. Ban and T. Fujita, *ibid.*, **12**, 353(1969).
(33) J. Kopecký, K. Bocek, and D. Vlachová, *Nature*, **207**, 981 (1965).
(34) J. A. Singer and W. P. Purcell, *J. Med. Chem.*, **10**, 1000 (1967).
(35) A. F. Michaelis and T. Higuchi, *J. Pharm. Sci.*, **58**, 201 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 30, 1970, from the *Department of Analytical Pharmaceutical Chemistry and Pharmaceutics, School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication May 19, 1970.

* Fulbright postdoctoral research fellow, on leave from School of Pharmacy, University of London, England.

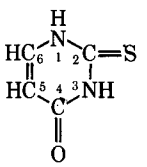
Metal Complexes of Thiouracils I: Stability Constants by Potentiometric Titration Studies and Structures of Complexes

EDWARD R. GARRETT and DENNIS J. WEBER

Abstract □ The divalent metals, Cu^{++}, Pb^{++}, Cd^{++}, Ni^{++}, and Zn^{++}, complex with the 5- and/or 6-alkyl-substituted thiouracils, HU. Significant concentrations of MU^+ and MU_2 complexes in homogeneous solution for all but Cu^{++} permitted estimation of the respective K_1 and K_2 stability constants by potentiometric titrations, where the log K_1 values were directly proportional to the pKa values of the parent thiouracils. Thus, the complex with 5,6-dimethyl-2-thiouracil (pKa′ 8.08) was the most stable, and the complex with 5-carboethoxy-2-thiouracil (pKa′ 6.43) was the least stable of those studied. The initial MU^+ complex is formed by the covalent bonding of the divalent cation at the anionic sulfur. When sulfhydryl formation in thiouracil is blocked by prohibiting tautomerization, as with 6-methyl-*N,N′*-diethyl-2-thiouracil, or by alkylation of the sulfur, as with 2-ethylmercapto-4-hydroxypyrimidine, no complexation with metal ions was observed. Pb^{++} and Cd^{++} ions have stability constants, K_1, for MU^+ formation with thiouracils that are 100 times greater than with Ni^{++} or Zn^{++}. No complexation of thiouracils with Fe^{+++}, Fe^{++}, Co^{++}, Ca^{++}, or Mn^{++} was observed. The MU_2 complex is formed by the covalent bonding of the divalent metal to two sulfur anions; this bis(6-*n*-propyl-2-thiouracil)-cadmium (II) is the first complex to precipitate from solution on the titration of cadmium ion and 6-*n*-propyl-2-thiouracil at 25 and 35°. The structure was confirmed by elemental analysis and IR spectra of synthesized compounds. In all other cases of studied complexation of Cd^{++} and Pb^{++} with 2-thiouracil, 6-methyl-2-thiouracil, 5-methyl-2-thiouracil, 5,6-dimethyl-2-thiouracil, 5-carboethoxy-2-thiouracil, and 6-*n*-propyl-2-thiouracil, the first complex that precipitated on potentiometric titration had a 1:1 stoichiometry and was most probably the cyclic dimer, M_2U_2, bis(thiouracil-metal) (II), although the polynuclear polymer, M_nU_n, was possible. The heightened acidity of the 4-OH of the initial MU^+ complex promoted dissociation at low pH values and subsequent covalent bonding of the divalent cation to the sulfur of one thiouracil and the oxygen of another. The resultant M_2U_2 or M_nU_n structure was confirmed by elemental analysis and IR spectra of synthesized complexes. The formed and precipitated complexes of Pb^{++} and Cd^{++} as MU^+, MU_2, and M_2U_2 were stable, at least in mildly alkaline solutions, whereas those of Ni^{++} and Zn^{++} were destroyed in mild alkali with the final precipitation of the metal hydroxides.

Keyphrases □ Thiouracils–metals—complexation □ Complexes, thiouracil–metal—stability constants □ Metal–thiouracil complexes —structure □ Solubility, aqueous—thiouracils □ Potentiometric titration—analysis □ IR spectrophotometry—structure □ UV spectrophotometry—structure

The antithyroid activities of 5-methyl-, 6-methyl-, and 5,6-dimethyl-substituted thiouracils have been claimed to be 0.7, 1.0, and 1.2 relative to 2-thiouracil (Structure I*a*) (1–3).



HU I*a*

The present antithyroid derivative of choice, because of its claimed maximal activity and low toxicity in the intact animal, is 6-*n*-propyl-2-thiouracil (1–5). Alkylation of thiouracil at the N-1, N-3, or sulfur positions greatly reduced (2), and substitution by electronegative groups at the 5- or 6-position practically eliminated, any antithyroid activity (1–3).

Cupric ion has been implicated in thyroid function (6). The copper content of the normal and pathologic thyroid has been determined (7) and verified by Kasanen and Viitanen (8) who found elevated copper levels in toxic and nontoxic goiters. The formation of diiodoty-

(15) *Ibid.*, **1967**, 348.
(16) C. Hansch, *Farmaco, Sci. Ed.*, **23**, 293(1968).
(17) P. S. Portoghese, *J. Pharm. Sci.*, **54**, 1077(1965).
(18) B. M. Bloom, *Ann. Rept. Med. Chem.*, **1965**, 236.
(19) C. Hansch, A. R. Steward, S. M. Anderson, and D. Bentley, *J. Med. Chem.*, **11**, 1(1968).
(20) R. Zahradnik, *Experientia*, **18**, 534(1962).
(21) R. Zahradnik, *Arch. Int. Pharmacodyn. Ther.*, **135**, 311 (1962).
(22) J. Kopecký and K. Bocek, *Experientia*, **23**, 125(1967).
(23) W. P. Purcell, J. G. Beasley, R. P. Quintana, and J. A. Singer, *J. Med. Chem.*, **9**, 297(1966).
(24) A. Cammarata, *ibid.*, **12**, 314(1969).
(25) C. Hansch, *ibid.*, **11**, 920(1968).
(26) A. Leo, C. Hansch, and C. Church, *ibid.*, **12**, 766(1969).
(27) P. R. Wells, "Linear Free Energy Relationships," Academic, New York, N. Y., 1968.
(28) S. M. Free and J. W. Wilson, *J. Med. Chem.*, **7**, 395(1964).
(29) T. C. Bruice, N. Kharasch, and R. J. Winzler, *Arch. Biochem. Biophys.*, **62**, 305(1956).
(30) W. R. Smithfield and W. P. Purcell, *J. Pharm. Sci.*, **56**, 577 (1967).
(31) W. P. Purcell and J. M. Clayton, *J. Med. Chem.*, **11**, 199 (1968).
(32) T. Ban and T. Fujita, *ibid.*, **12**, 353(1969).
(33) J. Kopecký, K. Bocek, and D. Vlachová, *Nature*, **207**, 981 (1965).
(34) J. A. Singer and W. P. Purcell, *J. Med. Chem.*, **10**, 1000 (1967).
(35) A. F. Michaelis and T. Higuchi, *J. Pharm. Sci.*, **58**, 201 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 30, 1970, from the *Department of Analytical Pharmaceutical Chemistry and Pharmaceutics, School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication May 19, 1970.

* Fulbright postdoctoral research fellow, on leave from School of Pharmacy, University of London, England.

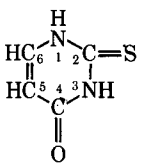
Metal Complexes of Thiouracils I: Stability Constants by Potentiometric Titration Studies and Structures of Complexes

EDWARD R. GARRETT and DENNIS J. WEBER

Abstract □ The divalent metals, Cu^{++}, Pb^{++}, Cd^{++}, Ni^{++}, and Zn^{++}, complex with the 5- and/or 6-alkyl-substituted thiouracils, HU. Significant concentrations of MU^+ and MU_2 complexes in homogeneous solution for all but Cu^{++} permitted estimation of the respective K_1 and K_2 stability constants by potentiometric titrations, where the log K_1 values were directly proportional to the pKa values of the parent thiouracils. Thus, the complex with 5,6-dimethyl-2-thiouracil (pKa′ 8.08) was the most stable, and the complex with 5-carboethoxy-2-thiouracil (pKa′ 6.43) was the least stable of those studied. The initial MU^+ complex is formed by the covalent bonding of the divalent cation at the anionic sulfur. When sulfhydryl formation in thiouracil is blocked by prohibiting tautomerization, as with 6-methyl-*N,N′*-diethyl-2-thiouracil, or by alkylation of the sulfur, as with 2-ethylmercapto-4-hydroxypyrimidine, no complexation with metal ions was observed. Pb^{++} and Cd^{++} ions have stability constants, K_1, for MU^+ formation with thiouracils that are 100 times greater than with Ni^{++} or Zn^{++}. No complexation of thiouracils with Fe^{+++}, Fe^{++}, Co^{++}, Ca^{++}, or Mn^{++} was observed. The MU_2 complex is formed by the covalent bonding of the divalent metal to two sulfur anions; this bis(6-*n*-propyl-2-thiouracil)-cadmium (II) is the first complex to precipitate from solution on the titration of cadmium ion and 6-*n*-propyl-2-thiouracil at 25 and 35°. The structure was confirmed by elemental analysis and IR spectra of synthesized compounds. In all other cases of studied complexation of Cd^{++} and Pb^{++} with 2-thiouracil, 6-methyl-2-thiouracil, 5-methyl-2-thiouracil, 5,6-dimethyl-2-thiouracil, 5-carboethoxy-2-thiouracil, and 6-*n*-propyl-2-thiouracil, the first complex that precipitated on potentiometric titration had a 1:1 stoichiometry and was most probably the cyclic dimer, M_2U_2, bis(thiouracil-metal) (II), although the polynuclear polymer, M_nU_n, was possible. The heightened acidity of the 4-OH of the initial MU^+ complex promoted dissociation at low pH values and subsequent covalent bonding of the divalent cation to the sulfur of one thiouracil and the oxygen of another. The resultant M_2U_2 or M_nU_n structure was confirmed by elemental analysis and IR spectra of synthesized complexes. The formed and precipitated complexes of Pb^{++} and Cd^{++} as MU^+, MU_2, and M_2U_2 were stable, at least in mildly alkaline solutions, whereas those of Ni^{++} and Zn^{++} were destroyed in mild alkali with the final precipitation of the metal hydroxides.

Keyphrases □ Thiouracils–metals—complexation □ Complexes, thiouracil–metal—stability constants □ Metal–thiouracil complexes —structure □ Solubility, aqueous—thiouracils □ Potentiometric titration—analysis □ IR spectrophotometry—structure □ UV spectrophotometry—structure

The antithyroid activities of 5-methyl-, 6-methyl-, and 5,6-dimethyl-substituted thiouracils have been claimed to be 0.7, 1.0, and 1.2 relative to 2-thiouracil (Structure I*a*) (1–3).



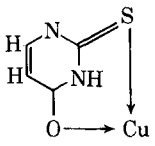
HU I*a*

The present antithyroid derivative of choice, because of its claimed maximal activity and low toxicity in the intact animal, is 6-*n*-propyl-2-thiouracil (1–5). Alkylation of thiouracil at the N-1, N-3, or sulfur positions greatly reduced (2), and substitution by electronegative groups at the 5- or 6-position practically eliminated, any antithyroid activity (1–3).

Cupric ion has been implicated in thyroid function (6). The copper content of the normal and pathologic thyroid has been determined (7) and verified by Kasanen and Viitanen (8) who found elevated copper levels in toxic and nontoxic goiters. The formation of diiodoty-

rosine and thyroxine is increased when cupric ion is added to homogenates of thyroid gland (9). Other evidence that cupric ion aids in the formation of thyroxine, by formation of iodine from iodide, has been presented (10–12).

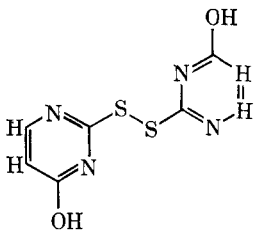
Since cupric ion and other heavy metals precipitate thiouracil and its derivatives from aqueous solution, Libermann (13, 14) conjectured that complexing ability and antithyroid activity may be correlated. He assumed that completeness of precipitation could be taken as a measure of the stability of the complex. However, this assumption is not necessarily valid. Some ethylenediaminetetraacetic acid (EDTA) complexes have high stability *and* high water solubility. Libermann (14) suggested the structure for the cupric–thiouracil complex (Structure I*b*), which assumed a 1:1 stoichiometry of



I*b*

metal to ligand and chelate binding of the cupric ion by the sulfur at the 2-position and the oxygen at the 4-position. Consideration of the stereochemistry of 2-thiouracil and the square-planar nature of cupric ion shows that the proposed structure is impossible because the phenolic oxygen and thionyl sulfur are coplanar and physically distant.

The oxidation of thiouracil by iodine has been shown to occur with ease at physiological pH values. The product is the disulfide (Structure I*c*) (15):



I*c*

The disodium salt of this disulfide is stable, but the free acid readily disproportionates to thiouracil and higher oxidation products (15).

This ease of the oxidation of 2-thiouracil by iodine suggests that thiouracil's antigoitrogenic mechanism of action may be the reduction of iodine to the ineffective iodide. However, since cupric ion has been implicated in thyroid function at the level of iodine production, the removal of cupric or cuprous ion by complexation with thiouracil could be an alternate explanation for its mode of action. If the complexation of cupric or cuprous copper is important in the mechanism of action of the thiouracils, then the stability constant of the complex may be larger for copper than with other physiological metal ions. Furthermore, it may be possible to correlate the stability constant of the copper complex or complexes of thiouracils and other metals with the biological activity of the particular thiouracil derivative. The correlation can only be expected under the conditions of equal concentrations at the site of action. Any differences in the *in vivo* solubility or stability of the thiouracil derivative should be considered.

The principal purpose of these studies was to provide quantitative information on the complexation of metal ions with thiouracils. The types of metal ions which complex, the effect of thiouracil substituents on the stability constants, and the structure of the complexes were to be determined. This first paper in the series applies the method of potentiometric titrations to the study of aqueous solutions of metal complexes of thiouracil that maintain homogeneous solutions for portions of the titrations. This excludes the study of solutions of mixtures of copper ions and thiouracils that give immediate precipitation by this method. The complexes of lead, cadmium, nickel, zinc, ferric, ferrous, manganese, calcium, and cobaltous ions with 2-thiouracil (TU). 6-*n*-Propyl-2-thiouracil (PTU), 6-methyl-2-thiouracil (6MTU), 5-methyl-2-thiouracil (5MTU), 5,6-dimethyl-2-thiouracil (5,6DMTU), 5 carboethoxy-2-thiouracil (5CETU), *N*,*N'*-diethyl-6-methyl-2-thiouracil, and 2-ethylmercapto-4-hydroxypyrimidine (2EM4HP) are to be considered, however.

EXPERIMENTAL

Purification of 2-Thiouracil (TU)—2-Thiouracil[1] was recrystallized from hot water. The product was washed with water and acetone and dried in a vacuum oven at 80°, m.p. 322–323° dec. (all melting points are uncorrected); literature value 310–312° dec. (16), about 340° (17). Equivalent weight 130.3; calculated for $C_4H_4N_2OS$ 128.1. IR spectrum (18), $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 3020 (NH); 1680 (C=O); 1280, 1240, 1177. UV spectrum (16), (0.1 *M* $HClO_4$), $\lambda_{max.}$ 273 (ϵ 13,700), $\lambda_{max.}$ 212 (ϵ 16,600).

Purification of 6-*n*-Propyl-2-thiouracil (PTU)—The compound[2] was recrystallized from hot water and dried at 80°, m.p. 219–221°; literature value 219–221° (19). Equivalent weight 170.0; calculated for $C_7H_{10}N_2OS$ 170.2. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 3100 (NH); 1650 (C=O); 1550, 1240, 1190. UV spectrum (0.1 *M* $HClO_4$), $\lambda_{max.}$ 272 (ϵ 15,840), $\lambda_{max.}$ 214 (ϵ 15,840).

Purification of 6-Methyl-2-thiouracil (6MTU)—The compound[3] was recrystallized from hot water and dried in a vacuum oven at 50°, m.p. 331–332° dec.; literature value >300° (1). Equivalent weight 142.1; calculated for $C_5H_6N_2OS$ 142.1. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 3100 (NH); 1640 (C=O); 1195, 1165. UV spectrum (0.1 *M* $HClO_4$), $\lambda_{max.}$ 274 (ϵ 15,460), $\lambda_{max.}$ 213 (ϵ 15,760).

Purification of 5,6-Dimethyl-2-thiouracil (5,6DMTU)—This material[4] was recrystallized from hot water and dried at 50° in a vacuum oven, m.p. 286–287° dec.; literature value 283–285° (1). Equivalent weight 156.5; calculated for $C_6H_8N_2OS$ 156.2. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 3210, 3110 (NH); 1660 (C=O); 1600, 1210, 1130. UV spectrum (0.1 *M* $HClO_4$), $\lambda_{max.}$ 276 (ϵ 17,340), $\lambda_{max.}$ 215 (ϵ 14,020).

Purification of 5-Methyl-2-thiouracil (5MTU)—5-Methyl-2-thiouracil[5] was recrystallized from hot water, washed with water, and dried at 50° in a vacuum oven, m.p. 334° dec; literature value not available (17). Equivalent weight 141.2; calculated for $C_5H_6N_2OS$ 142.1. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 3090 (NH); 1640 (C=O); 1240, 1200, 1165. UV spectrum (0.1 *M* $HClO_4$), $\lambda_{max.}$ 274 (ϵ 15,450), $\lambda_{max.}$ 213 (ϵ 15,730).

Purification of 5-Carboethoxy-2-thiouracil (5CETU)—5-Carboethoxy-2-thiouracil[6] was used as received, m.p. 245–246°; literature value 245° (17). Equivalent weight 197.5; calculated for $C_7H_8N_2O_3S$

[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Nutritional Biochemical Corp., and K & K Laboratories, Plainview, N. Y.
[3] Nutritional Biochemical Corp. and K & K Laboratories.
[4] K & K Laboratories.
[5] Sigma Chemical Co., St. Louis, Mo.
[6] Cyclo Chemical Corp., Los Angeles, Calif.

200.2. UV spectrum (0.1 M $HClO_4$), $\lambda_{max.}$ 310 (ϵ 15,121), $\lambda_{max.}$ 269 (ϵ 18,991), $\lambda_{max.}$ 213 (ϵ 10,762).

Purification of 2-Thio-6-aminouracil—2-Thio-6-aminouracil was used as received, m.p. 330°; literature value 295° (17). UV spectrum (0.1 M $HClO_4$), $\lambda_{max.}$ 275 (ϵ 18,413), $\lambda_{max.}$ 202 (ϵ 5856).

Synthesis and Purification of 6-Methyl-*N*,*N*'-diethyl-2-thiouracil (X)—This material was synthesized by the procedure of Lacey (20). *N*,*N*'-Diethylthiourea[7] (3.2 g.) was added to 20 ml. of glacial acetic acid and brought to a boil in a round-bottom flask fitted with a reflux condenser and a dropping funnel containing 8.6 g. of diketene.[8] The diketene was added over a 0.5-hr. period, and the reaction was allowed to cool overnight. The reaction was further heated for 0.5 hr. and then cooled; the acetic acid was removed by vacuum evaporation. Twenty milliliters of water was added to the residue; the mixture was shaken to emulsify and put into a refrigerator overnight. The precipitated contents were recrystallized from hot water and dried in a vacuum oven at 50°, m.p. 97–98°; literature value 97–98° (20). IR spectrum, $\bar{\nu}$ in $cm.^{-1}$ (Nujol mull): 1680 (C=O); 1250, 1105. UV spectrum (H_2O), $\lambda_{max.}$ 278 (ϵ 13,100), $\lambda_{max.}$ 222 (ϵ 15,250). Potentiometric titration with 0.1 N NaOH indicated that no titratable acid function was present. The yield was 73%.

Synthesis and Purification of 2-Ethylmercapto-4-hydroxypyrimidine (2EM4HP) (IX)—Eight grams of 2-thiouracil (0.062 mole) was added to 2.49 g. NaOH (0.062 mole) in 100 ml. of water, and acetone was added; the solution was cooled overnight in a refrigerator. A precipitate of the sodium salt of 2-thiouracil formed (8.3 g., 0.055 mole), which was filtered and collected.

The sodium salt of 2-thiouracil (0.055 mole) and ethyl iodide (0.06 mole, 9.35 g.) were added to 120 ml. of 95% ethanol in a round-bottom flask and refluxed until the sodium thiouracil had gone into solution. It was necessary to add an additional 4 g. (0.026 mole) of ethyl iodide during the reaction to put the sodium thiouracil into solution. The reaction mixture was cooled and the ethanol removed by vacuum evaporation. A white residue was left, which was recrystallized from ethanol once and then finally purified by sublimation, m.p. 152–153°; literature value 152° (17). IR spectrum, $\bar{\nu}$ in $cm.^{-1}$ (Nujol mull): 1660 (C=O), 1270, 1170, 1540. UV spectrum (H_2O), $\lambda_{max.}$ 280 (ϵ 5500), $\lambda_{max.}$ 230 (ϵ 11,750). Equivalent weight 156.4; calculated for $C_6H_8N_2OS$ 156.2.

Anal.—Calcd. for $C_6H_8N_2OS$: C, 46.13; H, 5.16; N, 17.94; S, 20.52. Found: C, 46.40; H, 5.31; N, 18.04; S, 20.37.[9]

Potentiometric Titrations—All potentiometric titrations were performed using a Sargent model D automatic titrator equipped with a 2.5-ml. capacity syringe buret. The sample solutions were titrated under nitrogen in water-jacketed titration cells at constant temperature. A Beckman combination electrode with Ag–AgCl reference was used. The pH standardization at 4 and 7 was checked before and after each titration. In no case was the pH drift larger than 0.05 pH unit and usually was less than 0.02 unit.

The 0.1 N sodium hydroxide titrant was prepared and maintained carbonate-free. Sample solutions for titration were prepared with constant concentrations of thiouracil (2×10^{-3} M) and varying concentrations of metal ion, as listed in Table I. The ionic strength was maintained constant at 0.006 M by substituting 0.03 M sodium perchlorate for equal volumes of 0.01 M divalent metal nitrate, since the ratio of the molar concentrations of sodium perchlorate to divalent metal nitrate is 1:3 for equal ionic strengths.

The pKa' values of the various thiouracils (Table II) were determined by potentiometric titration with standard alkali, with an initial ionic strength of 0.006 M and estimated from the pH of half-neutralization. The pKa' values at 25.0° were the averages of at least three separate determinations and had standard deviations of less than 0.05. The pKa' values at 35.0 and 45.0° were usually single determinations made at the same time and under the same conditions as the titrations of the metal–thiouracil mixtures. The equivalent weights of the thiouracils have been given under the characterization of the individual compounds.

Standardization of Metal-Ion Solutions—Analytical grade nitrate salts of cupric, cadmium, lead, nickel, ferric, cobalt, calcium, zinc, and manganese were used to prepare stock solutions in distilled water. The stock solutions were standardized using the mercury, mercury-EDTA electrode[10] (21, 22). The general procedure was to titrate potentiometrically solutions containing 2-ml. aliquots of 0.01–0.04 M metal nitrate, 25.0 ml. of buffer (22), and 1 drop of 1.0×10^{-3} M Hg–EDTA solution (22) with 0.1000 M EDTA. The reference electrode was a saturated calomel electrode. In the case of cobalt, an excess of standard calcium was added, and the excess was determined by titration with EDTA according to the general procedure outlined. Since the procedure of Reilley *et al.* (22) cannot be used to standardize ferric ion, the procedure of Pribil *et al.* (23) was used. The procedure was to titrate a solution containing 5 ml. of 0.01 M ferric ion, 25.0 ml. of a pH 3 chloroacetic acid buffer (0.2 M), and 1 drop of a 0.01 M ferrous perchlorate solution. The titrant was 0.1000 M EDTA, and the electrode system consisted of a platinum indicator electrode and a calomel reference. Since ferrous ion is easily oxidized, fresh ferrous-ion stock solutions were prepared as needed from analytical grade $FeSO_4 \cdot 7H_2O$[11] and boiled, nitrogen-purged water.

Aqueous Solubility of Thiouracils—Saturated aqueous solutions (50 ml. of 0.1 M $HClO_4$ in 100-ml. sealed flasks) of 2-thiouracil, 6-*n*-propyl-2-thiouracil, 6-methyl-2-thiouracil, 5-methyl-2-thiouracil, 5,6-dimethyl-2-thiouracil, 5-carboethoxy-2-thiouracil, and 6-amino-2-thiouracil were equilibrated at 25.0° in a controlled temperature shaker bath. Filter sticks (Sargent No. S-30417) were used to remove samples from the equilibrated solutions. The first filtrate was discarded; 2.0-ml. aliquots of the filtered, equilibrated solution were taken, appropriately diluted with 0.1 M $HClO_4$, and read on the Cary spectrophotometer or the Beckman DU against a 0.1 M $HClO_4$ blank solution. Repetitive samples were taken with time to assure complete equilibration. Possible oxidation (15) of the equilibrating solutions of TU, PTU, 5MTU, and 6MTU were denied by the fact that the absorbances at several wavelengths were of the same ratio to similar absorbances for fresh solutions of these thiouracils. The calculated solubilities are listed in Table II.

Spectrophotometric Titrations of Thiouracils—Thirty milliliters of solutions, 0.921×10^{-4} and 1.00×10^{-4} M in 2-thiouracil and 8.00×10^{-3} M in sodium perchlorate, contained in a water-jacketed titration cell (25.0°), were titrated with 1.00 N NaOH and 0.0499 N NaOH, respectively. Nitrogen gas, free of carbon dioxide (passed through an Ascarite tube) and saturated with water (passed through a sparger tube immersed in water) at 25.0°, was passed into the cell for each run. The cell solution was stirred with a magnetic stirrer. A constant-rate buret (Sargent) equipped with a 2.5-ml. syringe buret was used to deliver the titrant to the cell. The volume delivered could be read to the nearest 0.5 μl. A microaperture flow cell (Beckman catalog No. 97290) was connected by polyethylene tubing (Clay-Adams Co., No. PE 200) to the titration cell and to a 5.0-ml. gastight syringe (Hamilton). After each addition of standard alkali, the gastight syringe was actuated by hand to draw the titration cell solution into the flow cell and the spectrum recorded *versus* a water blank. The gastight syringe was actuated several times before each spectrum was recorded to ensure thorough mixing of any solution that may have remained in the flow cell or tubing. The pH of the solution was read after mixing using a glass-calomel electrode system and a Radiometer pH meter (No. TTT 1). The total volume change during the titration of the sample solution was less than 2% and was considered negligible.

The absorbances at the $\lambda_{max.}$ and other wavelengths were plotted against pH. The pKa' values were determined from the intercept of plots of $\log [(A - A_{H^+})/(A_{OH^-} - A)]$ *versus* pH, where A is the absorbance at a given pH value, A_{H^+} is the absorbance in 0.10 M $HClO_4$, and A_{OH^-} is the absorbance at pH 9 in accordance with the expression (24):

$$\log \left(\frac{A - A_{H^+}}{A_{OH^-} - A} \right) = pKa' - pH \qquad \text{(Eq. 1)}$$

The pKa' values determined by spectrophotometric titrations are given in Table II.

Synthesis of Bis(2-thiouracil)cadmium (II) or $Cd(TU)_2$ (IV)—A solution containing 0.06 mole of 2-thiouracil in 2 l. of hot water was prepared. To the 2-thiouracil solution was added slowly, with stirring, a solution containing 0.03 mole of cadmium nitrate in about 200 ml. of water. The resulting mixture was allowed to stand 0.5 hr. on low heat (about 70°). It was then allowed to cool to room temperature, and the pH was adjusted to 6.5 with concentrated NaOH.

[7] Eastman Organic Chemical Co., Rochester, N. Y.
[8] K & K Laboratories, Plainview, N. Y.
[9] Huffman Laboratories Inc., Wheatridge, Colo.
[10] Sargent Co., Chicago, Ill.
[11] Matheson Coleman & Bell, Cincinnati, Ohio.

Table I—Composition of Solutions and Estimated Logarithmic Stability Constants of 1:1 (MU^+) and 1:2 (MU_2) Metal Complexes of Substituted Thiouracils from Potentiometric Titrations

Ligand[a]	Metal	$10^3[M^{++}]_0$	Temperature	Slope[b]	log K_1[c]	log K_2[c]
2TU	Pb^{++}	2.00	25.0°	1.00	4.68(4.68)[b]	3.07
		1.80	25.2°	1.15	4.69	3.69
		1.60	25.2°	1.15	4.70	3.54
		1.40	25.1°	1.11	4.72	3.47
		1.20	25.0°	1.11	4.73	3.42
		1.00	24.9°	1.08	4.76	3.37
		0.800	24.9°	1.12	4.76	3.49
		0.600	24.9°	1.10	4.84	3.47
		0.400	24.9°	1.08	4.83	3.41
		0.200	24.9°	1.07	4.75	3.49
					4.74 ± 0.05	3.44 ± 0.15
		2.00	34.95°	1.10	4.52(4.58)[b]	3.24
		0.800	34.85°	1.10	4.62	3.40
					4.67 ± 0.05	3.32 ± 0.08
		2.00	44.85°	1.00	4.52(4.54)[b]	2.62
		0.800	45.15°	1.16	4.49	3.37
					4.50 ± 0.01	2.99 ± 0.37
	Cd^{++}	2.00	25.1°	1.00	4.21(4.21)[b]	3.12
		1.80	25.3°	1.36	3.88	4.00
		1.60	24.9°	1.12	4.10	3.57
		1.40	25.3°	1.23	4.03	3.55
		1.20	25.1°	1.36	3.85	3.94
		1.00	25.1°	1.22	4.02	3.46
		0.800	25.1°	1.59	4.13	3.58
		0.600	25.1°	1.33	3.98	3.31
		0.400	25.0°	1.60	4.07	3.36
		0.200	25.0°	1.60	3.96	3.03
					4.02 ± 0.10	3.49 ± 0.29
		2.00	34.7°	1.31	4.07	3.68
		0.80	34.7°	1.26	4.10	3.86
					4.08 ± 0.01	3.77 ± 0.09
		2.00	45.0°	1.10	4.05	3.20
		0.800	45.0°	1.25	3.93	3.70
					3.99 ± 0.06	3.45 ± 0.25
	Ni^{++}	60.00	25.0°	1.13	2.46	3.78
		6.00	25.0°	1.11	2.59	3.57
		4.00	25.0°	1.01	2.38(2.43)[b]	1.69
		1.60	25.0°	1.08	2.60	2.12
		1.00	25.0°	1.20	2.40	2.84
					2.49 ± 0.09	2.66 ± 0.71
PTU	Pb^{++}	2.00	25.8°	1.00	4.79(4.82)[b]	3.34
		1.60	25.8°	1.00	4.76(4.77)[b]	—
		1.00	25.8°	1.12	4.84	3.48
		0.400	25.8°	1.24	4.67[d]	3.26
		0.200	25.8°	1.13	4.50[d]	—
					4.79 ± 0.03	3.36 ± 0.09
		2.00	34.80°	1.08	4.58(4.65)[b]	3.50
		0.800	34.80°	1.15	4.69	3.32
					4.63 ± 0.05	3.41 ± 0.09
		2.00	44.85°	1.00	4.43(4.47)[b]	3.32
		0.800	44.58°	1.05	4.45	3.32
					4.44 ± 0.01	3.27 ± 0.04
	Cd^{++}	2.00	25.0°	—	4.16	4.22
		1.60	25.3°	1.43	—	4.73
		1.00	25.3°	1.66	4.17	4.81
		0.400	25.3°	1.42	4.31[d]	4.20
		0.200	25.3°	2.06	—	—
					4.16 ± 0.005	4.49 ± 0.28
		2.00	35.1°	1.49	3.95	4.64
		0.800	34.9°	1.48	3.78	4.97
					3.86 ± 0.08	4.80 ± 0.17
		2.00	44.9°	1.58	4.01	4.35
		1.20	44.9°	1.42	3.65	4.19
					3.83 ± 0.18	4.27 ± 0.08
	Ni^{++}	2.00	25.9°	1.91	1.34	3.49
		1.60	25.9°	—	0.30[d]	4.46[d]
		1.00	25.9°	—	1.36	3.53
					1.35 ± 0.01	3.51 ± 0.02
	Zn^{++}	2.00	25.9°	—	2.16	3.62
6MTU	Pb^{++}	2.00	26.0°	1.00	4.63(4.66)[b]	3.29
		1.60	25.5°	1.00	4.73(4.80)[b]	3.33
		1.00	25.5°	1.22	4.68	3.45

(*Continued*)

Table I—(*Continued*)

Ligand[a]	Metal	$10^3[M^{++}]_0$	Temperature	Slope[b]	log K_1[c]	log K_2[c]
		0.400	25.0°	1.22	4.73	3.00
					4.69 ± 0.04	3.26 ± 0.16
		2.00	35.1°	1.00	4.70(4.74)[b]	3.51
		0.800	35.1°	1.10	4.80	3.59
					4.75 ± 0.05	3.55 ± 0.04
		2.00	44.9°	1.02	4.54	3.28
		0.80	44.95°	1.19	4.52	3.48
					4.53 ± 0.01	3.38 ± 0.10
	Cd^{++}	2.00	26.0°	1.25	4.15	3.43
		1.60	25.5°	1.33	4.16	3.59
		1.00	25.5°	1.32	4.09	3.70
		0.40	26.0°	1.96	3.67[d]	4.15[d]
					4.13 ± 0.03	3.57 ± 0.11
		2.00	35.2°	1.15	4.31	3.89
		0.800	35.2°	1.47	4.35	3.96
					4.33 ± 0.02	3.92 ± 0.03
		2.00	44.85°	1.00	4.26(4.33)[b]	3.82
		0.800	35.0°	1.46	4.18	3.80
					4.22 ± 0.0	3.81 ± 0.01
	Ni^{++}	2.00	26.0°	1.53	4.13	3.18
5MTU	Pb^{++}	2.00	25.0°	1.00	4.75(4.75)[b]	3.14
		1.60	25.3°	1.06	4.74	3.34
		1.00	24.5°	1.20	4.85	3.24
		0.400	24.5°	1.20	4.89	3.57
					4.80 ± 0.06	3.32 ± 0.16
		2.00	35.05°	1.00	4.65(4.66)[b]	3.25
		0.800	34.9°	1.08	4.73	3.12
					4.69 ± 0.04	3.18 ± 0.06
		2.00	44.85°	1.02	4.51(4.55)[b]	3.30
		0.800	44.85°	1.14	4.59	3.13
					4.55 ± 0.04	3.21 ± 0.08
	Cd^{++}	2.00	25.0°	1.07	4.28(4.36)[b]	3.47
		1.60	25.0°	1.32	4.27	3.75
		1.00	25.3°	1.42	4.22	3.92
		0.400	25.0°	1.93	3.79[b]	4.48[b]
					4.25 ± 0.03	3.71 ± 0.18
		2.00	34.85°	1.16	4.14	3.82
		0.800	34.9°	1.28	4.25	3.93
					4.19 ± 0.05	3.87 ± 0.07
		2.00	44.75°	1.14	3.97	3.23
		0.800	44.95°	1.44	4.11	3.85
					4.04 ± 0.07	3.54 ± 0.31
	Ni^{++}	2.00	26.0°	1.49	2.30[d]	2.84
		1.60	25.3°	1.21	2.72	2.74
		1.00	25.0°	1.31	2.61	1.63[d]
					2.66 ± 0.05	2.79 ± 0.05
5,6DMTU	Pb^{++}	2.00	25.8°	1.00	5.01(5.04)[b]	4.04
		1.60	25.0°	1.00	4.98(5.04)[b]	3.86
		1.00	25.3°	1.00	5.03(5.04)[b]	3.83
		0.400	25.3°	1.37	4.79[d]	3.92
					5.01 ± 0.02	3.91 ± 0.08
		2.00	34.85°	1.00	5.04(5.02)[b]	4.37
		0.800	34.85°	1.06	4.99	4.72
					5.01 ± 0.02	4.54 ± 0.17
		2.00	45.35°	1.00	4.81(4.86)[b]	4.23
		0.800	44.9°	1.32	4.82	4.13
					4.81 ± 0.00	4.18 ± 0.05
	Cd^{++}	2.00	25.5°	1.00	4.52[c](4.53)[b]	—
		1.60	25.5°	1.14	4.36	3.99
		1.00	25.3°	1.32	4.37	4.24
		0.400	25.5°	1.31	4.37	4.11
					4.37 ± 0.004	4.11 ± 0.10
		2.00	34.85°	1.11	4.50	4.23
		0.800	34.95°	1.33	4.40	4.40
					4.45 ± 0.05	4.31 ± 0.08
		2.00	44.85°	1.23	4.28	4.21
		0.800	45.0°	1.67	4.19	4.45
					4.23 ± 0.04	4.33 ± 0.12
	Ni^{++}	1.00	25.5°	1.38	3.03	2.99
5CETU	Pb^{++}[e]	2.00	25.5°	—	ppt.	ppt.

(*Continued*)

Table I—(*Continued*)

Ligand[a]	Metal	$10^3[M^{++}]_0$	Temperature	Slope[b]	log K_1[c]	log K_2[c]
		0.800	25.5°	—	ppt.	ppt.
		2.00	35.0°	—	ppt.	ppt.
		0.800	35.0°	—	ppt.	ppt.
		2.00	45.0°	—	ppt.	ppt.
		0.800	45.0°	—	ppt.	ppt.
	Cd^{++}	2.00	24.8°	1.25	3.57	3.74
		0.800	25.0°	1.75	3.48	3.56
					3.52 ± 0.04	3.65 ± 0.09
		2.00	35.4°	1.54	3.50	3.72
		0.800	34.5°	2.28	—	—
		2.00	44.85°	1.73	3.79	3.98
		0.800	44.85°	2.03	3.55	3.94
					3.67 ± 0.12	3.96 ± 0.02

[a] The ligand concentration was 0.002 *M*, ionic strength was 0.006 *M*, and initial volume of the titrating solution was 25.00 ml. The ligand abbreviations represent the following compounds: (TU) 2-thiouracil; (PTU) 6-*n*-propyl-2-thiouracil; (6MTU) 6-methyl-2-thiouracil; (5MTU) 5-methyl-2-thiouracil; (5,6DMTU) 5,6-dimethyl-2-thiouracil; and (5CETU) 5-carboethoxy-2-thiouracil. [b] These are the slopes of plots of log $(1 - \bar{n})/\bar{n}$ *versus* $p[U^-]$, where $\bar{n}$ is the degree of formation of a postulated 1:1 complex (MU^+) in accordance with $\log (1 - \bar{n})/\bar{n} = pK_1 + p[U^-]$. For those cases where the slope was consistent with the theoretical expectation of unity, it can be postulated that the only complex present in significant concentration is the 1:1 complex, MU^+. The values of log K_1 estimated from the intercept of these plots for unit slopes are given in parentheses. The estimations of pK_1 and slope values were limited to those portions of the titrations where homogeneous solutions were maintained. [c] Log K_1 and log K_2 values were derived from the slope and intercept values of plots of $\bar{n}/(1 - \bar{n})[U^-]$ *versus* $[(\bar{n} - 2)/(\bar{n} - 1)][U^-]$ in accordance with $\bar{n}/(1 - \bar{n})[U^-] = K_1 + [(\bar{n} - 2)/(\bar{n} - 1)][U^-] K_1K_2$, where K_1 is the stability constant for the formation of the 1:1 complex, MU^+, and K_2 is the stability constant for the formation of MU_2 from MU^+. The mean values of the log K values are given for each temperature with the estimated standard deviations. [d] This value was considered anomalous and was not included in the calculation of the mean values. [e] There was immediate precipitation on addition of $Pb(NO_3)_2$ to the 5CETU solution.

The resulting suspension was warmed again, cooled to room temperature, and filtered through a medium fritted-glass funnel. The product was washed with cold water and acetone and dried in a vacuum oven at 60°. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 1620, 1550, 1530, 1300, 1210, 1180, 827.

Anal.—Calcd. for $C_8H_6CdN_4O_2S_2$: Cd, 30.65. Found: Cd, 31.54, 30.96.

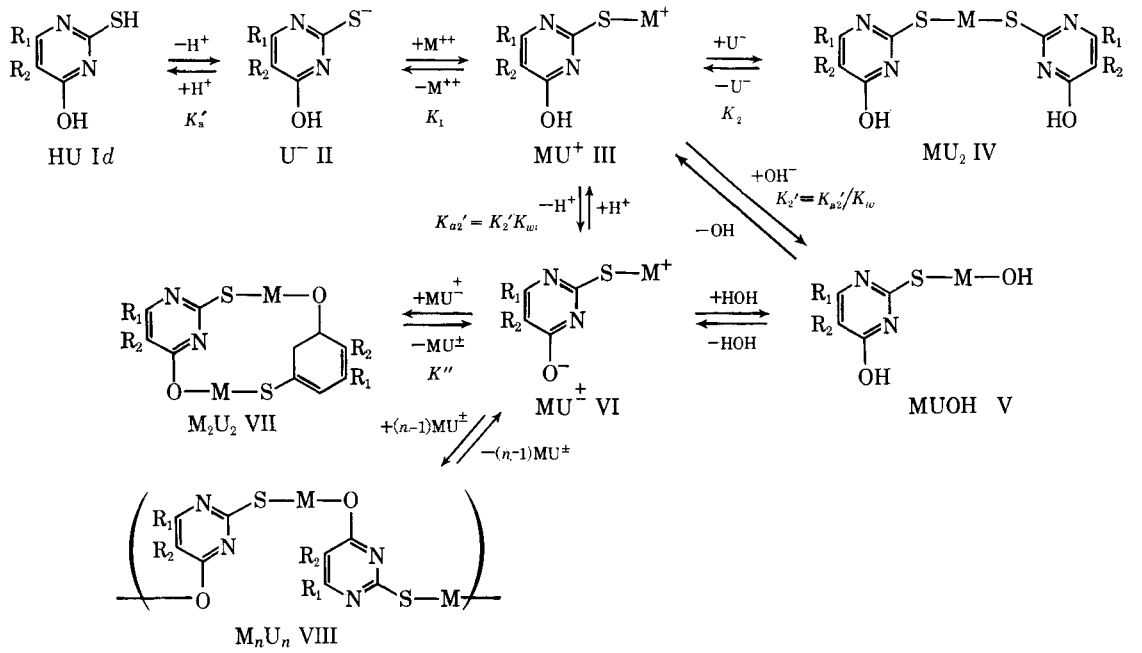
Synthesis of Bis(2-thiouracil)lead (II) or $Pb(TU)_2$ (IV)—The procedure for the synthesis of bis(2-thiouracil)lead (II) was exactly the same as in the case of bis(2-thiouracil)cadium (II) except that lead nitrate was used in place of cadmium nitrate. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 1660, 1630, 1560, 1500, 1280, 1000, 815.

Anal.—Calcd. for $C_8H_6N_4O_2PbS_2$: Pb, 44.90. Found: Pb, 44.51.

Synthesis of Bis(2-thiouracil-cadmium) (II) or $Cd_2(TU)_2$ (VII)—A solution containing 0.06 mole of 2-thiouracil in a minimum of hot water was added slowly, with stirring, to a 1-l. solution containing 0.06 mole of cadmium nitrate heated to the same temperature (about 80°) as the hot thiouracil solution. The resulting mixture was maintained at the initial temperature for 0.5 hr. and then allowed to cool to room temperature. Concentrated sodium hydroxide was added to pH 6.8, and the resulting suspension was reheated. After 30 min. the suspension was cooled to room temperature and the product filtered through a medium fritted-glass funnel. The resulting product was washed with water and then acetone and dried in the vacuum oven at 60°. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 3400, 1570, 1510, 1335, 1020.

Anal.—Calcd. for $C_8H_4Cd_2N_4O_2S_2$: Cd, 47.2. Found: Cd, 45.8.

Synthesis of Bis(2-thiouracil-lead) (II) or $Pb_2(TU)_2$ (VII)—The procedure for bis(2-thiouracil-lead) (II) was exactly the same as in the case of bis(2-thiouracil-cadmium) (II) with lead nitrate substituted for cadmium nitrate. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 1560, 1520, 1430, 1330, 1000, 820.



Scheme I—Relations among the divalent metal-ion complexes of thiouracils except that no experimental evidence exists in support of MUOH (V). MU^+ (III) and MU_2 (IV) exist in significant concentrations in solution, and characterized MU_2 (IV), M_2U_2 (VII), and/or M_nU_n (VIII) are precipitated from solutions of metal-ion and thiouracil mixtures.

Table II—Intrinsic Solubilities[a] at 25.0° and pKa′ Values of Various Thiouracils

Ligand	Solubility, moles/l.	pKa′[b] 25.0°	35.0°	45.0°
2-Thiouracil	5.53×10^{-3}	7.46(7.52)	7.22	7.09
6-*n*-Propyl-2-thiouracil	7.07×10^{-3}	7.76(7.80)	7.48	7.17
6-Methyl-2-thiouracil	3.75×10^{-3}	7.73(7.94)	7.65	7.41
5-Methyl-2-thiouracil	3.58×10^{-3}	7.71(7.80)	7.57	7.35
5,6-Dimethyl-2-thiouracil	8.79×10^{-3}	8.08	8.06	7.76
5-Carboethoxy-2-thiouracil	7.97×10^{-3}	6.43	6.40	6.27
2-Ethylmercapto-4-hydroxypyrimidine	—	7.01	—	—
6-Amino-2-thiouracil	1.79×10^{-3}	—	—	—

[a] Determined from the absorbances of saturated solutions in 0.1 *M* $HClO_4$ where the absorptivity values for wavelengths of maximum absorbance have been given in the *Experimental* section. [b] Determined by potentiometric titration with standard alkali with an initial ionic strength of 0.006 *M*, where the pKa′ was estimated from the pH of half-neutralization. The pKa′ values at 25.0° were the averages of at least three separate determinations and had standard deviations less than 0.05. The parenthetical pKa′ values at 25.0° were determined by spectrophotometric titration of 10^{-4} *M* thiouracil from the intercept values of plots of log $[(A - A_{H+})/(A_{OH-} - A)]$ *versus* pH, where A is the absorbance at a given wavelength at a given pH value, A_{H+} is the absorbance in 0.10 *M* $HClO_4$, and A_{OH-} is the absorbance at pH 9 in accordance with the expression log $[(A - A_{H+})/(A_{OH-} - A)]$ = pKa′ − pH.

Anal.—Calcd. for $C_8H_4N_4O_2PbS_2$: Pb, 62.15. Found: Pb, 62.54.

Synthesis of Bis(6-*n*-propyl-2-thiouracil)cadmium (II) or Cd(PTU)$_2$ (IV)—The preparation of this complex was performed in the same manner as for bis(2-thiouracil)cadmium (II). The molar amounts of 6-*n*-propyl-2-thiouracil and cadmium nitrate were 0.06 and 0.03, respectively. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 3100, 1630, 1500, 1270, 1220, 1175, 1015, 970, 830.

Anal.—Calcd. for $C_{14}H_{18}CdN_4O_2S_2$: C, 37.29; H, 4.02; Cd, 24.9; N, 12.43; S, 14.22.[12] Found: C, 37.94; H, 4.15; Cd, 24.7; N, 11.95; S, 13.77.

Synthesis of Bis(6-*n*-propyl-2-thiouracil-lead) (II) or Pb$_2$(PTU)$_2$ (VII)—This complex was prepared by the same procedure as for bis(2-thiouracil-cadmium) (*II*). The molar amounts of 6-*n*-propyl-2-thiouracil and lead nitrate used were 0.06. IR spectrum, $\bar{\nu}$ in cm.$^{-1}$ (Nujol mull): 1550, 1420, 1280, 1165, 1020, 825.

Anal.—Calcd. for $C_{14}H_{18}N_4O_2PbS_2$: Pb, 55.2. Found: Pb, 55.4.

Analysis of Cadmium Content of Complexes—Accurately weighed (200-mg.) samples of the dried cadmium complex were dissolved in 150 ml. of 0.01 *M* H_2SO_4 and heated (70°) until all of the sample had dissolved. Approximately 150 mg. of sodium hydrogen sulfide in 15 ml. of water was added, and the resulting cadmium sulfide precipitate was digested for about 2 hr. until the crystals were large. The precipitate was filtered onto a tared, fritted-glass funnel, washed with warm water, and dried in a vacuum oven at 50° overnight. The dried precipitate was weighed in the funnel, and the cadmium was calculated by multiplying the weight of cadmium sulfide by the gravimetric factor 0.7780 (*25a*).

Analysis of Lead Content of Complexes—Accurately weighed samples (300 mg.) of the lead complex were digested in 100 ml. of 1.0 *M* nitric acid until everything had dissolved. The volume was reduced to 25 ml. by evaporation; 100 ml. of a solution 2.0 *M* in sulfuric acid and 1.0 *M* in sodium sulfate was added. The precipitate was digested to give large crystals, and the volume was reduced by evaporation to about 50 ml. The precipitate was collected on a tared, fritted-glass funnel, washed with water, dried at 120° overnight, and weighed. The gravimetric factor is 0.6832 (*25b*).

THEORY AND METHODS OF CALCULATION

The derivation of the general equation for the calculation of stability constants from potentiometric titration data was first given by Bjerrum (26). An excellent summary of computational techniques is also available (27). The following derivations are made for the specific circumstances in this study. They do not assume the formation or precipitation of significant amounts of the hydroxides of the free metal ion, *i.e.*, MOH^+ or $M(OH)_2$, and require all species (ligands, metal ions, and complexes) to be in solution and in instantaneous equilibrium. They can be and were applied only under such valid circumstances. All possible species and complexes and the rational equilibria among them are shown in Scheme I. The discussion of the following section is in respect to these relationships.

[12] Elemental analysis by Huffman Laboratories Inc., Wheatridge, Colo.

Equations for Stability Constants of Complexes of Thiouracil (HU) and Doubly Charged Metal Cations (M^{++}) where the Significant Complexes in Solution Have Only the Forms MU^+ and MU_2—The formation of 1:1 (MU^+) and 2:1 (MU_2) complexes of thiouracil anion (U^-) and metal ion (M^{++}) in homogeneous solution can be formulated as:

$$\underset{\text{I}}{HU} \underset{+H^+}{\overset{-H^+}{\rightleftarrows}} \underset{K_a'}{U^-} \underset{-M^{++}}{\overset{+M^{++}}{\rightleftarrows}} \underset{\text{III}}{MU^+} \underset{-U^-}{\overset{+U^-}{\rightleftarrows}} \underset{K_2\ \text{IV}}{MU_2} \qquad \text{(Eq. 2)}$$

The apparent acid-dissociation constant of the thiouracils is:

$$K_a' = \frac{[U^-][H^+]\gamma_\pm}{[HU]} \qquad \text{(Eq. 3)}$$

where $[U^-]$ and $[HU]$ are the molar concentrations of thiouracil anion, II, and undissociated thiouracil acid, I, respectively; and $[H^+]\gamma_\pm = a_{H^+} = 10^{-pH}$ is the hydrogen-ion activity, where $\gamma_\pm$ is the mean activity coefficient of the hydrogen-ion concentration, $[H^+]$. The stability constant for the first complex of metal ion with thiouracil is:

$$K_1 = \frac{[MU^+]}{[M^{++}][U^-]} \qquad \text{(Eq. 4)}$$

where $[MU^+]$ is the concentration of the first complex, III; and $[M^{++}]$ is the concentration of free metal ion. The step stability constant for the formation of the 1:2 complex is:

$$K_2 = \frac{[MU_2]}{[MU^+][U^-]} \qquad \text{(Eq. 5)}$$

where $[MU_2]$ is the concentration of the second complex, IV. The overall stability constant, the product of K_1 and K_2, is:

$$\beta_2 = K_1K_2 = \frac{[MU_2]}{[M^{++}][U^-]^2} \qquad \text{(Eq. 6)}$$

The mass balance equations for thiouracil, metal-ion, and sodium hydroxide titrant are:

$$[HU]_0 = [U^-] + [HU] + [MU^+] + 2[MU_2] \qquad \text{(Eq. 7)}$$

$$[M^{++}]_0 = [M^{++}] + [MU^+ + [MU_2] \qquad \text{(Eq. 8)}$$

$$[NaOH] = [U^-] + [MU^+ + 2[MU + [OH^-] - [H^+] \qquad \text{(Eq. 9)}$$

The initial stoichiometric concentrations of thiouracil and metal ion in the solution to be titrated are given by $[HU]_0$ and $[M^{++}]_0$. The stoichiometric concentration of alkali, calculated on the basis that of the amount added at any point in the titration none had yet been consumed, is given by [NaOH]. The concentration of hydroxyl ion in Eq. 9 is the sum of the hydroxyl ion from the titrant and from the dissociation of water. Since a hydrogen ion is produced when a water molecule dissociates, the hydrogen-ion concentration corrects the hydroxyl-ion concentration for this phenomenon, so the resultant equation only accounts for the hydroxyl ion due to the titrant.

The degree of formation, $\bar{n}$, is defined as the average number of ligands bound to a metal ion:

$$\bar{n} = \frac{[MU^+] + 2[MU_2]}{[M^{++}]_0} \qquad \text{(Eq. 10)}$$

When the appropriately rearranged Eqs. 3 and 7 are substituted into Eq. 10,

$$\bar{n} = \frac{[HU]_0 - [U^-]\left(1 + \frac{[H^+]\gamma_\pm}{K_a'}\right)}{[M^{++}]_0} \qquad \text{(Eq. 11)}$$

When Eq. 9 is subtracted from Eq. 7,

$$[HU_0] - [NaOH] = [HU] - [OH^-] + [H^+] \qquad \text{(Eq. 12}a\text{)}$$

If the potentiometric titrations are performed near neutrality and/or significant thiouracil is present during the titrations, the differences between the relatively small quantities of $[OH^-]$ and $[H^+]$ can be ignored and

$$[HU]_0 - [NaOH] = [HU] \qquad \text{(Eq. 12}b\text{)}$$

This was true for all cases. The value for [HU] in Eq. 12*b* may be obtained from a rearrangement of Eq. 3. After substitution of this value for [HU] into Eq. 12*b*, the following equation is obtained on rearrangement:

$$[U^-] = \frac{[HU]_0 - [NaOH]}{[H^+]\gamma_\pm/K_a'} \qquad \text{(Eq. 13)}$$

The right-hand side of Eq. 13 contains only experimentally obtainable quantities; therefore, $[U^-]$ can be calculated. Substitution of $[U^-]$ into Eq. 11 allows the calculation of $\bar{n}$.

Substitution of the rearranged equilibrium expressions for $[MU^+]$, $[MU_2]$, (Eqs. 4 and 6) and the mass balance (Eq. 8) for $[M^{++}]_0$ into Eq. 10 gives, on simplification and rearrangement, a relation between $\bar{n}$, K_1, and β_2:

$$\frac{\bar{n}}{(1 - \bar{n})[U^-]} = \left(\frac{\bar{n} - 2}{\bar{n} - 1}\right)\beta_2[U^-] + K_1 \qquad \text{(Eq. 14)}$$

Equation 14 is linear when $\bar{n}/(1 - \bar{n})\,[U^-]$ is plotted against $[(\bar{n} - 2)/(\bar{n} - 1)]\,[U^-]$. The slope is β_2 and the intercept is K_1. If β_2 is assumed to be zero, Eq. 14 reduces to an equation whose logarithmic transformation is:

$$\log \frac{1 - \bar{n}}{\bar{n}} = pK_1 + p[U^-] \qquad \text{(Eq. 15)}$$

where pK_1 and $p[U^-]$ represent the negative logarithm of K_1 and $[U^-]$, respectively. Equation 15 is linear when $\log (1 - \bar{n})/\bar{n}$ is plotted against $p\,[U^-]$ and has a slope of 1 and an intercept of pK_1.

Equations for Stability Constants of Complexes of Thiouracil (HU) and Doubly Charged Metal Cations (M^{++}) where Significant Complexes in Solution Have Only the Forms MU^+, MU_2, and MUOH (or $MU^\pm$)—The formation of MUOH (V), the mixed ligand complex (or its reaction equivalent zwitterion $MU^\pm$, VI) can conceivably occur by reaction of III, Scheme I, or Eq. 2, with OH^- (or by its equivalent, the removal of a H^+):

$$\begin{array}{ccccc} MU^\pm & \underset{+H^+}{\overset{-H^+}{\rightleftarrows}} & MU^+ & \underset{-OH^-}{\overset{+OH^-}{\rightleftarrows}} & MUOH \\ \text{VI}\ K_{a_2}'/K_2'K_w & & \text{III}\ K_2' = K_{a_2}'/K_w & & \text{V} \end{array} \qquad \text{(Eq. 16)}$$

This derivation of the equations for the calculation of the stability constants of MU^+ (III) and MUOH (V) assumes that no significant amounts of MOH^+ and/or $M(OH)_2$ are formed and all species are in solution and at equilibrium. The use of the symbol MUOH in the following equations may stand for either MUOH (V) or $MU^\pm$ (VI) or the sum of both. The fact that both are products of hydroxyl-ion reaction with MU^+ (III) makes them mathematically equivalent in all calculations.

The expressions for the apparent acid-dissociation constants, K_a', and the equilibrium constant, K_1, for the first complex, MU^+ (III), have already been given (Eqs. 3 and 4). The step stability constants, K_2', of the mixed ligand complex is:

$$K_2' = \frac{[MUOH]}{[MU^+][OH^-]\gamma_\pm'} \qquad \text{(Eq. 17)}$$

where [MUOH] is the molar concentration of the mixed ligand complex (V), and $[OH^-]\gamma_\pm' = a_{OH^-} = 10^{-pOH} = 10^{-(pK_w - pH)}$ is the activity of hydroxyl ions. The mean activity coefficient is given by $\gamma_\pm'$. The expression for the autoprotolytic constant of water is:

$$K_w = [H^+]\,\gamma_\pm\,[OH^-]\,\gamma_\pm' \qquad \text{(Eq. 18)}$$

The overall stability constant, the product of K_w, K_1, and K_2', for the mixed ligand complex is:

$$\beta_{11} = K_1K_2'K_w = \frac{[MUOH][H^+]\gamma_\pm}{[M^{++}][U^-]} \qquad \text{(Eq. 19)}$$

The value of $[OH^-]\,\gamma_\pm'$ has been substituted by $K_w/[H^+]\,\gamma_\pm$ from Eq. 18. The mass balance equations for thiouracil, metal-ion, and sodium hydroxide titrant are:

$$[HU]_0 = [U^-] + [HU] + [MU^+] + [MUOH] + 2[MU_2] \qquad \text{(Eq. 20)}$$

$$[M^{++}]_0 = [M^{++}] + [MU^+] + [MUOH] + [MU_2] \qquad \text{(Eq. 21)}$$

$$[NaOH] = [U^-] + [MU^+] + 2[MUOH] + 2[MU_2] + [OH^-] - [H^+] \qquad \text{(Eq. 22)}$$

When the degree of formation for the complexes MU^+, MUOH, and MU_2 is considered

$$\bar{n} = \frac{[MU^+] + [MUOH] + 2[MU_2]}{[M^{++}]_0} \qquad \text{(Eq. 23)}$$

Substitution of rearranged Eqs. 3 and 20 into Eq. 23 results in:

$$\bar{n} = \frac{[HU]_0 - [U^-]\left(1 + \frac{[H^+]\gamma_\pm}{K_a'}\right)}{[M^{++}]_0} \qquad \text{(Eq. 11)}$$

Subtracting Eq. 22 from Eq. 20, substituting [HU] from Eq. 3 and [MUOH] from Eq. 19, and dropping the relatively small quantities $[OH^-]$ and $[H^+]$ give:

$$\frac{[HU]_0 - [NaOH]}{[H^+]\gamma_\pm/K_a' - \beta_{11}[M^{++}]/[H^+]\gamma_\pm} = [U^-] \qquad \text{(Eq. 24)}$$

The left-hand side of Eq. 24 contains the free metal-ion concentration; therefore, the value of $[U^-]$ cannot be accurately calculated unless the free or uncomplexed metal-ion concentration is determinable. If the assumption is made that MUOH is not present in any significant amount during some interval in the titration, then Eq. 24 reduces to Eq. 13. This assumption would permit the observed data to conform to Eq. 14. Equation 24 is valid when MUOH is present, whether or not significant amounts of MU_2 are formed.

The relation of $\bar{n}$ to K_1, β_2, and β_{11} for mixed ligand complexes is derived by substitution of the equilibrium expressions for $[MU^+]$ (Eq. 4), [MUOH] (Eq. 19), $[MU_2]$ (Eq. 6), and $[M^{++}]$ (Eq. 21) into Eq. 23 to give:

$$\bar{n} = \frac{K_1[U^-] + \beta_{11}\frac{[U^-]}{[H^+]\gamma_\pm} + 2\beta_2[U^-]^2}{1 + K_1[U^-] + \beta_{11}[U^-]/[H^+]\gamma_\pm + \beta_2[U^-]^2} \qquad \text{(Eq. 25)}$$

Rearrangement of Eq. 25 by multiplication of both sides by the denominator of the right-hand expression and collection of similar terms gives:

$$\frac{\bar{n}}{(1 - \bar{n})[U^-]} = K_1 + \frac{\beta_{11}}{[H^+]\gamma_\pm} + \frac{\bar{n} - 2}{\bar{n} - 1}\beta_2[U^-] \qquad \text{(Eq. 26)}$$

which is not linear when $\bar{n}/(1 - \bar{n})\,[U^-]$ is plotted against $1/[H^+]\gamma_\pm$ or $[(\bar{n} - 2)/(\bar{n} - 1)]\,[U^-]$ when $\bar{n}$ is calculated from Eq. 11 for $[U^-]$ calculated from Eq. 24. When it is assumed that the concentration of MUOH is not significant, β_{11} approaches zero, Eq. 26 reduces to Eq. 14, $[U^-]$ may be calculated from Eq. 13, and only potentiometric titration data are needed. When it is assumed that the concentration

of MU_2 is not significant and that only MU^+ and MUOH complexes may exist, β_2 approaches zero and Eq. 26 becomes

$$\frac{\bar{n}}{1 - \bar{n}\,[U^-]} = K + \frac{\beta_{11}}{[H^+]\gamma_\pm} \qquad \text{(Eq. 27)}$$

which is linear when $\bar{n}/(1 - \bar{n})\,[U^-]$ is plotted against $1/[H^+]\gamma_\pm$ with a slope of β_{11} and an intercept of K_1 when $\bar{n}$ is calculated from Eq. 11 for $[U^-]$ calculated from Eq. 24, which demands an independent estimate of metal-ion concentration in addition to the potentiometric titration data.

Equations for Stability Constants of Thiouracil (HU) and Doubly Charged Metal Cations (M^{++}) in the General Case where Complexes Have Only the Possible Forms MU^+, MU_2, $MU^\pm$ (or MUOH), and M_2U_2 or n Complexes as M_nU_n—The formation of positively charged MU^+ (III) and neutral $MU^\pm$ (VI) [or its equivalent MUOH (V)] 1:1 complexes, as well as the polynuclear complexes, M_2U_2 (VII), can be formulated as an extension of Eq. 16:

$$\underset{\text{VII}}{M_2U_2} \underset{-MU^\pm}{\overset{+MU^\pm}{\rightleftharpoons}} \underset{K_2''}{\underset{\text{VI}}{MU^\pm}} \underset{-(n-1)MU^\pm}{\overset{+(n-1)MU^\pm}{\rightleftharpoons}} \underset{\text{VIII}}{M_nU_n} \qquad \text{(Eq. 16}a\text{)}$$

Molecular models of M_2U_2 (VII) in Scheme I are easily formed and exhibit no strain. It is also possible to write polynuclear complexes, M_nU_n, of variable n in the linear form of VIII in Scheme I. This derivation of the equations to relate stability constants of MU^+, $MU^\pm$, M_2U_2, and polynuclear complexes, M_nU_n, assumes that no significant amounts of MOH^+ are formed and all species are in solution and in equilibrium.

The expressions for the apparent acid-dissociation constant of the ligand and the stability constant of the first complex (MU^+, III) have been given (Eqs. 3 and 4). The expression for the acid-dissociation constant of MU^+ (III) to $MU^\pm$ (VI) is:

$$K_{a_2}' = \frac{[MU^\pm][H^+]\gamma_\pm}{[MU^+]} \qquad \text{(Eq. 28)}$$

The equation for the step stability constant of M_2U_2 (VII) is:

$$K_2'' = \frac{[M_2U_2]}{[MU^\pm]^2} \qquad \text{(Eq. 29)}$$

Substitution of the equilibrium expression for $[MU^+]$ (Eq. 4) into Eq. 17 and collection of constants give:

$$\beta_{a2} = K_{a_2}'K_1 = \frac{[MU^\pm][H^+]\gamma_\pm}{[M^{++}]\,[U^-]} \qquad \text{(Eq. 30)}$$

where β_{a2} has the same value as β_{11} in Eq. 19. Substitution of the expression for $[MU^\pm]$ from Eq. 30 into Eq. 29 and collection of constants give:

$$\beta_{22} = K_2''\beta_{a2} = \frac{[M_2U_2][H^+]^2\,\gamma_\pm{}^2}{[M^{++}]\,[U^-]^2} \qquad \text{(Eq. 31)}$$

The mass balance equations for thiouracil, metal-ion, and sodium hydroxide titrant are:

$$[HU]_0 = [U^-] + [HU] + [MU^+] + [MU^\pm] + 2[M_2U_2] + 2[MU_2] \qquad \text{(Eq. 32)}$$

$$[M^{++}]_0 = [M^{++}] + [MU^+] + [MU^\pm] + 2[M_2U_2] + [MU_2] \qquad \text{(Eq. 33)}$$

$$[NaOH] = [U^-] + [MU^+] + 2[MU^\pm] + 4[M_2U_2] + 2[MU_2] + [OH^-] - [H^+] \qquad \text{(Eq. 34)}$$

The degree of formation when the complexes MU^+, MU_2, $MU^\pm$, and M_2U_2 are considered is:

$$\bar{n} = \frac{[MU^+] + [MU^\pm] + 2[M_2U_2] + 2[MU_2]}{[M^{++}]_0} \qquad \text{(Eq. 35)}$$

Equation 35, when substituted by rearranged Eqs. 3 and 32, is

$$\bar{n} = \frac{[HU]_0 - [U^-]\left(1 + \dfrac{[H^+]\gamma_\pm}{K_a'}\right)}{[M^{++}]_0} \qquad \text{(Eq. 11)}$$

Subtracting Eq. 34 from Eq. 32; substituting Eqs. 3, 30, and 31 for [HU], $[MU^\pm]$, and $[M_2U_2]$, respectively; and dropping the relatively small quantities $[OH^-]$ and $[H^+]$ give:

$$[U^-] = \frac{[HU]_0 - [NaOH]}{[H^+]\gamma_\pm/K_a' - \beta_{a2}[M^{++}]/[H^+]\gamma_\pm - 2\beta_{22}[M^{++}]^2[U]/[H^+]^2\gamma_\pm{}^2} \qquad \text{(Eq. 36)}$$

where β_{a2} (Eq. 30) has the same value as β_{11} (Eq. 19).

The general equation for the ligand-anion concentration, where n polynuclear complexes M_nU_n (VIII) exist for all n values in addition to the complex MU^+ (III) and independent of whether an MU_2 (IV) complex is present or not, is:

$$[U^-] = \frac{[HU]_0 - [NaOH]}{[H^+]\gamma_\pm/K_a' - \sum_0^n \left(\dfrac{n\beta_{nn}[M^{++}]^n[U^-]^{n-1}}{[H^+]\,\gamma_\pm{}^n}\right)} \qquad \text{(Eq. 37)}$$

where $MU^\pm$ (or its equivalent, MUOH) is considered to be a polynuclear complex of the form M_1U_1. When $n = 1$, the only complexes in solution can be MU^+, $MU^\pm$, and possibly MU_2, and Eq. 37 reduces to Eq. 24. When $n = 0$ or if it is assured that M_nU_n complexes including $MU^\pm$ (or MUOH) are not significant during the titration, the only complexes in solution are MU^+ and possibly MU_2, and Eq. 37 reduces to Eq. 13 which can be calculated from the homogeneous potentiometric titrations. The values of $[U^-]$ in Eqs. 25 and 37 are functions of the free or uncomplexed metal-ion concentration and cannot be calculated accurately unless this concentration is obtained in addition to the potentiometric titration data.

The relation of $\bar{n}$ to K_1, β_2, β_{a2}, and β_{22} is derived by substitution of the equilibrium expressions for $[MU^+]$ (Eq. 4), $[MU_2]$ (Eq. 6), $[MU^\pm]$ (Eq. 30), $[M_2U_2]$ (Eq. 31), and $[M^{++}]_0$ (Eq. 33) into Eq. 35 to give:

$$\bar{n} = \frac{K_1[U^-] + 2\beta_2[U^-]^2 + \beta_{a2}[U^-][H^+]^{-1}\gamma_\pm{}^{-1} + 2\beta_{22}[M^{++}][U^-]^2[H^+]^{-2}\gamma_\pm{}^{-2}}{1 + K_1[U^-] + \beta_2[U^-]^2 + \beta_{a2}[U^-][H^+]^{-1}\gamma_\pm{}^{-1} + 2\beta_{22}[M^{++}][U^-]^2[H^+]^{-2}\gamma_\pm{}^{-2}} \qquad \text{(Eq. 38)}$$

which can be rearranged to:

$$\frac{\bar{n}}{(1 - \bar{n})[U^-]} = K_1 + \frac{\beta_{a2}}{[H^+]\gamma_\pm} + \left(\frac{\bar{n} - 2}{\bar{n} - 1}\right)\beta_2[U^-] + \frac{2\beta_{22}[M^{++}][U^-]}{[H^+]^2\gamma_\pm{}^2} \qquad \text{(Eq. 39)}$$

The general equation to relate $\bar{n}$ and the stability constants for all possible complexes in solution, MU^+, MU_2, and n complexes of the form M_nU_n (which includes $MU^\pm$ or MUOH as M_1U_1) is:

$$\bar{n} = \frac{K_1[U^-] + 2\beta_2[U^-]^2 + \sum_0^n n\beta_{nn}[M^{++}]^{n-1}[U^-]^n[H^+]^{-n}\gamma_\pm{}^{-n}}{1 + K_1[U^-] + \beta_2[U^-]^2 + \sum_0^n n\beta_{nn}[M^{++}]^{n-1}[U^-]^n[H^+]^{-n}\gamma_\pm{}^{-n}} \qquad \text{(Eq. 40)}$$

which, when $n = 0$, 1, and 2, reduces to Eqs. 14, 25 or 26, and 38 or 39, respectively. Only in the case where $n = 0$ and the significant complexes in solution can be only MU^+ and MU_2 will $\bar{n}$ be independent of the free metal-ion concentration and can it be calculated from potentiometric titration data alone.

RESULTS

Potentiometric Titrations of Various Thiouracil–Metal-Ion Mixtures—The addition of an amount of cupric nitrate as low as 2×10^{-4} M to a 2×10^{-3} M aqueous solution of a 2-thiouracil (2-thiouracil, 6-*n*-propyl-2-thiouracil, 6-methyl-2-thiouracil, 5-methyl-2-thiouracil, and 5,6-dimethyl-2-thiouracil) caused an immediate drop in pH from about 5.5 to about 3.0 and the immediate formation of a precipitate, the amount of which increased with time. Attempts at potentiometric titration of such mixtures with smaller concentrations of thiouracils in the presence of minimal amounts of cupric nitrate were unsuccessful in maintaining a homogeneous solution. Since the complexes of cupric ion and the thiouracils had such low

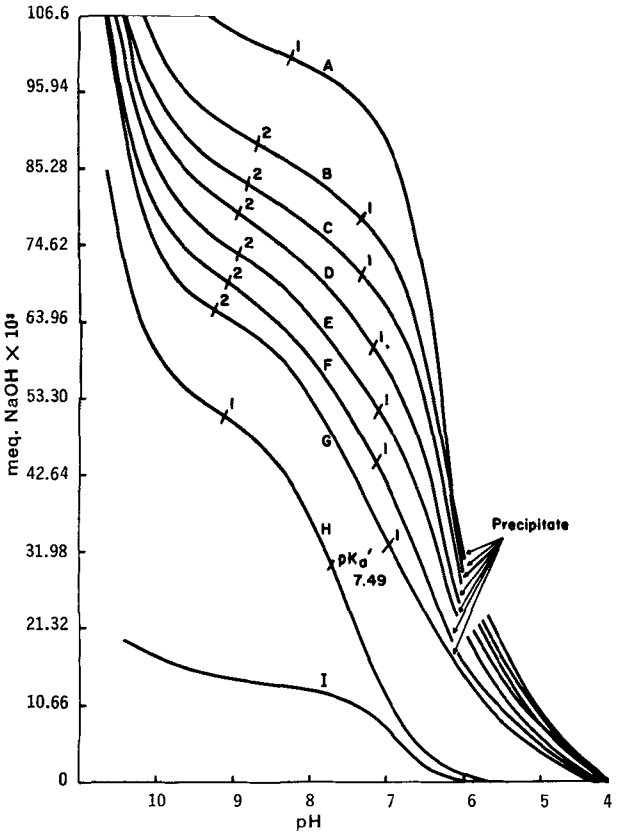


Figure 1—*Potentiometric titration curves of aqueous solutions of lead nitrate and 2-thiouracil with μ = 0.006 at 25.0°. Twenty-five-milliliter solutions were 2.00 × 10^{-3} M in 2-thiouracil and: (A), 2.00 × 10^{-3} M; (B), 1.60 × 10^{-3} M; (C), 1.40 × 10^{-3} M; (D), 1.20 × 10^{-3} M; (E), 1.00 × 10^{-3} M; (F), 8.00 × 10^{-4} M; (G), 6.00 × 10^{-4} M; and (H), 0 M in lead nitrate. Curve I is the titration of 25.0 ml. of 4.00 × 10^{-4} M lead nitrate. The titer of alkali between inflections 1 and 2 are: (B), 6.50 × 10^{-3}; (C), 1.14 × 10^{-2}; (D), 1.76 × 10^{-2}; (E), 2.10 × 10^{-2}; (F), 2.30 × 10^{-2}; and (G), 3.03 × 10^{-2} meq.*

solubilities, it was technologically infeasible to use the potentiometric titration method which demands instantaneous equilibration in homogeneous solution for the estimation of complexation constants of copper-ion–thiouracil complexes.

Fortunately this was not the general case. Homogeneous solutions were maintained for significant titer additions to mixtures of other metal-ion solutions and various 2-thiouracils.

Typical Potentiometric Titration Curves of Metal-Ion–Thiouracil Mixtures which Initially Form Homogeneous Solutions; Thiouracil–Lead-Ion Mixtures—A typical set of potentiometric titration curves is given in Fig. 1 for solutions of 25.00 ml. of 2.00 × 10^{-3} *M* 2-thiouracil (25.0°, μ = 0.006). Various amounts of lead nitrate have been added to each of these solutions so that the original concentration ranged from 2.00 × 10^{-3} *M* to 0.00 *M* $Pb(NO_3)_2$ for Curves A to H, Fig. 1. The plots are given in terms of the milliequivalent of NaOH necessary to achieve an observed pH value for each of these solutions. Homogeneous solutions were maintained for a portion of the titrations, and the points of precipitation are clearly indicated in Fig. 1. They occurred in excess of pH 6, except for the particular case (Curve H) of the thiouracil in the absence of lead ion where no precipitation was observed throughout the titration. The titration curve for 25.00 ml. of 4.00 × 10^{-4} lead nitrate is given in Curve I.

When the titrations of the precipitating solutions were continued past the points of precipitation, two apparent inflections, labeled as 1 and 2 in Fig. 1, were observed in all cases, except for the equimolar solution (Curve A) of Pb^{++} and 2-thiouracil when only one inflection was observed. The total milliequivalents of NaOH consumed by the equimolar solution (Curve A, Fig. 1) to the first observed inflection at pH 8.3 closely corresponded to twice the milliequivalents of NaOH necessary to neutralize the same concentration of 2-thiouracil alone (Curve H, Fig. 1). This strongly indicates that the *precipitated* lead–thiouracil complex is of 1:1 stoichiometry and that the precipitate is the net result of equal numbers of lead ions displacing two protons per molecule from equal numbers of thiouracil molecules. If the added milliequivalents of NaOH necessary to reach the pH of this inflection 1 (Curve A, Fig. 1) had been greatly in excess of twice the milliequivalents necessary to neutralize the ligand, it could be proposed that additional NaOH reacted with the precipitated complex and destroyed it or that the metal hydroxide $Pb(OH)_2$ was precipitated simultaneously. If all the metal had been reacted to form hydroxide, the milliequivalents of NaOH to this inflection 1 would have been three times the milliequivalents of NaOH necessary to neutralize the ligand alone. IR analysis of the precipitate, isolated at pH 7.5, gave a curve identical to the IR curve of bis(2-thiouracil-cadmium) (II), M_2U_2 (VII).

Further confirmation is obtained from the titrations (Curves B–G, Fig. 1) of solutions where the 2-thiouracil concentrations are in molar excess of $Pb(NO_3)_2$ concentrations. The titrations of the excess 2-thiouracil in these curves appear to occur after the completion of the precipitation of the Pb^{++}–thiouracil complex of apparent 1:1 stoichiometry at inflection 1. The milliequivalents of titer between this inflection 1 and inflection 2 may represent the neutralization of the excess 2-thiouracil. It is apparent that this titer between the inflections (Curves B–G, Fig. 1) is for a compound of pKa 7.5, a fact consistent with the pKa of 2-thiouracil (Curve H, Fig. 1).

The 10^2 meq. of NaOH consumed between inflections 1 and 2 for the various titrations of Fig. 1 were: B, 0.7 (1.0); C, 1.2 (1.5); D, 1.8 (2.0); E, 2.1 (2.5); F, 2.3 (3.0); and G, 3.0 (3.5). The parenthetical values are the molar excess of 10^2 meq. of 2-thiouracil over Pb^{++} ion; the close correspondence with the titration values is apparent. Exact

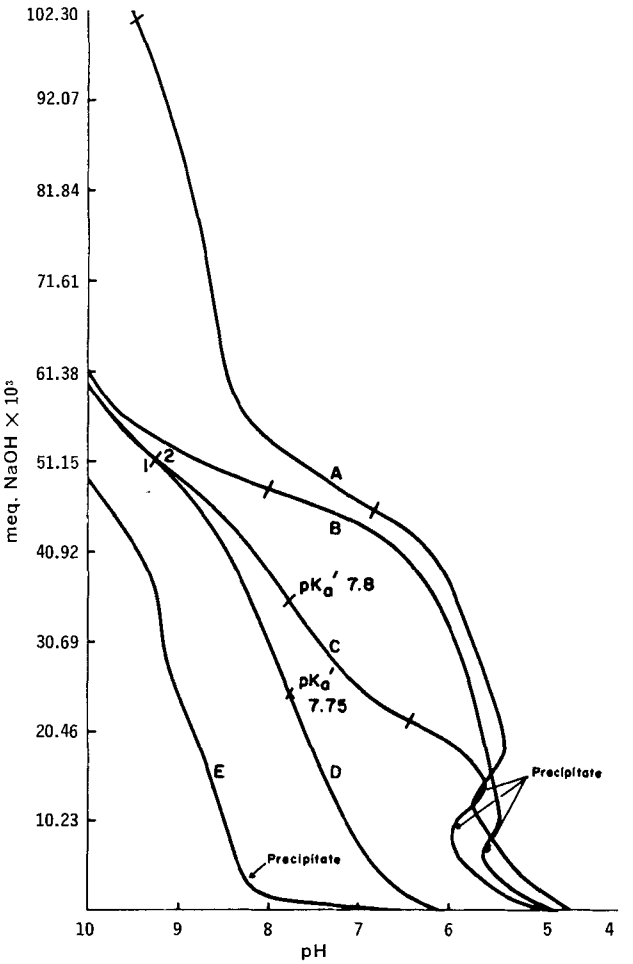


Figure 2—*Potentiometric titration curves of aqueous mixtures of cadmium nitrate and 6-n-propyl-2-thiouracil (PTU) with μ = 0.006 at 25.0°. Twenty-five-milliliter solutions were 2.00 × 10^{-3} M in PTU and: (A), 2.00 × 10^{-3} M; (B), 1.00 × 10^{-3} M; (C), 4.00 × 10^{-4} M; and (D), 0 M in Cd^{++}. Curve E is the titration of 25 ml. of 8.00 × 10^{-4} M cadmium nitrate.*

coincidence of these values is not to be expected since it is based on the titration of a heterogeneous and precipitating system which is not in instantaneous equilibria. If the values are taken as absolute, the precipitates may be a mixture of 1:1 stoichiometry and a compound of higher ratio of thiouracil to metal with the former predominating.

Similar titration curves with Pb^{++} ion were observed for the variously 5- and 6-alkyl-substituted 2-thiouracils listed in Table I. Only in the case of the ligand 5-carboethoxy-2-thiouracil was there immediate precipitation on the addition of lead ion.

Titration of Thiouracil–Cadmium-Ion Mixtures—Potentiometric titrations of mixtures of cadmium ion and the variously 5- and 6-alkyl-substituted 2-thiouracils listed in Table I, except 6-*n*-propyl-2-thiouracil, gave sets of curves very similar to those obtained on titration of thiouracil mixtures with lead ion (Fig. 1). Precipitation occurred during the titration at about pH 6.5, so the calculation of stability constants could be based on a region of homogeneous solution. The milliequivalents of NaOH consumed to inflection 1 for the equimolar mixtures of Cd^{++} and substituted 2-thiouracils (Curve A, Fig. 1) corresponded to twice the milliequivalents of NaOH consumed by the same concentration of ligand alone (Curve H, Fig. 1) to indicate strongly the precipitation of a complex of metal ion–thiouracil of 1:1 stoichiometry, which was not destroyed by the addition of further alkali to result in $Cd(OH)_2$. As stated previously, the formation or precipitation of $Cd(OH)_2$ would have consumed milliequivalents NaOH greatly in excess of twice the available milliequivalents of ligand. If the cadmium–thiouracil complex had been completely hydrolyzed, or if the cadmium had been completely precipitated as the hydroxide, three times the milliequivalents of ligand would have been consumed as NaOH.

The IR curve of the precipitate from such titrations was isolated at pH 10.5 and was identical to the IR curve of synthesized bis(2-thiouracil-cadmium) (II), M_2U_2 (VII).

Further confirmation was obtained from the titrations of solutions where the thiouracil concentrations were in molar excess of the $Cd(NO_3)_2$ concentrations. These titrations were similar to Curves B–G of Fig. 1 for the lead-ion studies in that the titration of excess thiouracil assigned to the milliequivalents NaOH between inflections 1 and 2 (about pH 7 and 9) were consistent with the thiouracil added to the original solutions in excess of 1:1 molar stoichiometry with the cadmium ion.

Anomalous Titration Curves of 6-*n*-Propyl-2-thiouracil–Cadmium-Ion Mixtures—The titration curves of cadmium-6-*n*-propyl-2-thiouracil solutions at 25 and 35° (Fig. 2) differed from the curves obtained from mixtures of cadmium ion and the other studied 5- and/or 6-substituted 2-thiouracils or obtained from thiouracil–lead-ion mixtures (Fig. 1). The titration curves for mixtures of cadmium ion and 0.002 *M* 6-*n*-propyl-2-thiouracil (Fig. 2) showed precipitation near pH 6 as before. However, the first inflection, near pH 8.0, for all concentrations of cadmium ion equal or greater than 0.001 *M* (Curves A and B, Fig. 2) occurred when milliequivalents of NaOH were consumed that were equal to the milliequivalents of NaOH consumed by the ligand alone. Only when the total cadmium concentration was less than 0.001 *M* (Curve C, Fig. 2), *i.e.*, when it was less than half of the ligand concentration, did the titration curve give any indication of any excess or uncomplexed ligand. Such excess 6-*n*-propyl-2-thiouracil is postulated to be titrated between the designated inflections of Curve C, Fig. 2, and characterized by the 7.8 pKa′ which is essentially the titration curve of 6-*n*-propyl-2-thiouracil alone (Curve D, Fig. 2) in the absence of cadmium ion. Such a behavior on titration, where the synonymous inflections of Curves A and B (Fig. 2) at the same milliequivalents of NaOH are considered to represent the complete precipitation of all available complex, must demand that the metal to thiouracil precipitate have a 1:2 stoichiometry. The fact that when the added Cd^{++} was in excess of this 1:2 stoichiometry (Curve A, Fig. 2) indicated hydroxide-ion consumption by the excess cadmium ion is confirmatory. This is readily seen from the similarities of the titration curves above pH values of 8 for Curve A and Curve E, where Curve E is for the alkaline potentiometric titration of cadmium ion alone.

The above analysis was performed on titration curves obtained at 25 and 35°. However, at 45° the potentiometric titrations of cadmium–6-*n*-propyl-2-thiouracil solutions gave curves which were similar to Fig. 1. This indicates that at elevated temperatures the precipitation of cadmium complexes of 6-*n*-propyl-2-thiouracil of 1:1 stoichiometry is preferred over the precipitation of cadmium–6-*n*-propyl-2-thiouracil complexes of 1:2 stoichiometry that occurs at lower temperatures.

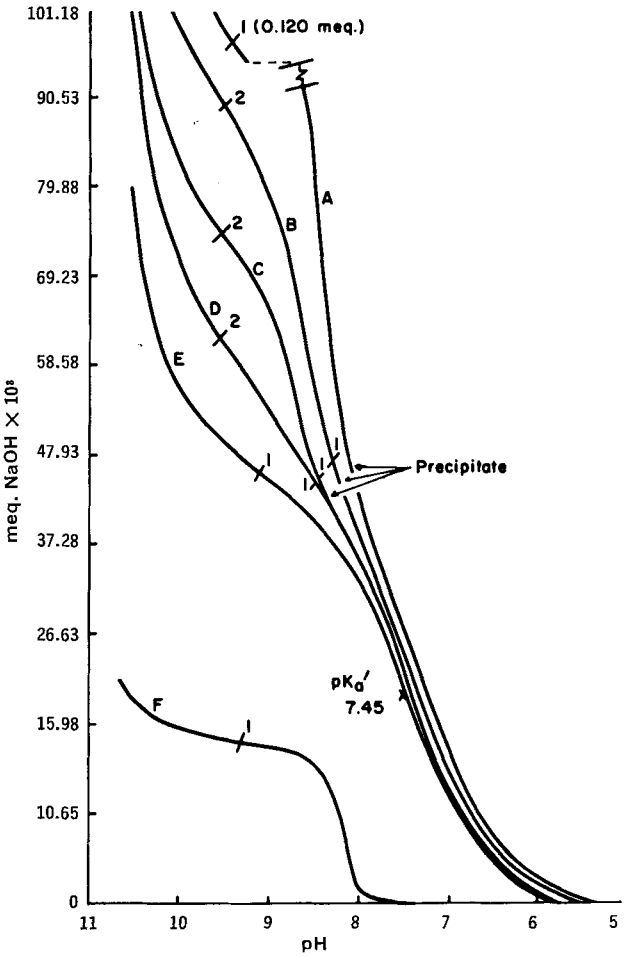


Figure 3—*Potentiometric titration curves of aqueous mixtures of nickel nitrate and 2-thiouracil (2TU) with μ = 0.006 at 25.0°. Twenty-five-milliliter solutions were 2.00 × 10⁻³ M in 2TU and: (A), 2.00 × 10⁻³ M; (B), 1.20 × 10⁻³ M; (C), 8.00 × 10⁻⁴ M; (D), 4.00 × 10⁻⁴ M; and (E), 0 M in nickel nitrate. Curve F is the titration of 25 ml. of 4.00 × 10⁻⁴ M nickel nitrate.*

Titration of Nickel–Thiouracil and Zinc–Thiouracil Mixtures—Titrations of 2-thiouracil, 6-*n*-propyl-2-thiouracil, 6-methyl-2-thiouracil, 5-methyl-2-thiouracil, and 5,6-dimethyl-2-thiouracil in the presence of nickel gave curves that showed an initial pH drop from 6.0 to 5.5 and precipitation during the titration near pH 8 (Fig. 3 Curves A–D).

Titration of 6-*n*-propyl-2-thiouracil in the presence of zinc gave an initial pH drop from 6.5 to 6.0 and precipitation occurred near pH 7.3. The titration curves for the zinc complexes were very similar to those found for nickel (Fig. 3).

The potentiometric titrations of equimolar solutions of Zn^{++} or Ni^{++} and thiouracil (Curve A, Fig. 3) when continued past the points of precipitation did not give evidence of clear-cut stoichiometry of the precipitating complexes as had been so evident in the lead and cadmium cases. The total milliequivalents of NaOH consumed to the observed inflection in such a titration was greatly in excess of twice the milliequivalents of NaOH which would have been necessary to neutralize the thiouracil ligand alone (Curve E, Fig. 3). This is definite indication that metal hydroxide precipitation, $M(OH)_2$, must also occur concomitant with the possible precipitation of a metal–thiouracil complex. The fact that the milliequivalents of NaOH consumed between inflections 1 and 2, in Curves B–D, Fig. 3, presumably assignable to the excess thiouracil if the metal–thiouracil complex was at least 1:1, had no relation to milliequivalents of such excess thiouracil, was further evidence that the added hydroxide ion was consumed by the metal and that any metal–thiouracil complexes were probably disrupted by the addition of this excess hydroxide ion.

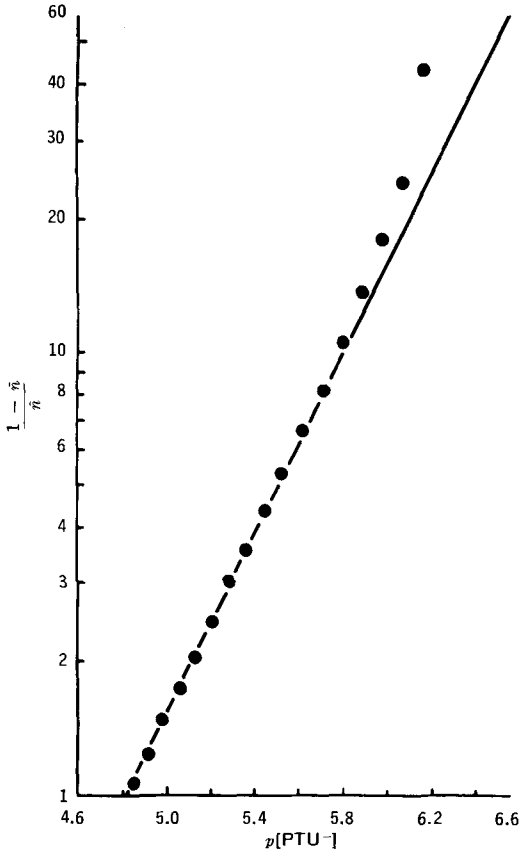


Figure 4—*Plot of log* $(1-\bar{n})/\bar{n}$ *against the negative logarithm of 6-n-propyl-2-thiouracil-anion concentration* (p[*PTU*$^-$]) *obtained from a lead nitrate* (*2.00* $\times$ 10^{-3} M)–*PTU* (*2.00* $\times$ 10^{-3} M) *mixture in water with* μ = *0.006 at 25.8°. Slope of plot is 1.00 and log* K_1 *is 4.82 from the* pK_1 *intercept.*

Titration of 2-Thiouracil and 6-*n*-Propyl-2-thiouracil in the Presence of Other Metal Ions—Potentiometric titrations of 2-thiouracil and 6-*n*-propyl-2-thiouracil in the presence of ferric, ferrous, manganese, calcium, and cobaltous ions gave no indication of any complex formation. The titration curves of the mixtures could be assigned to uncomplexed ligand in the case of calcium and manganese and to hydrolysis of the metal ion in the case of ferric, ferrous, and cobaltous ions.

Titrations of Sterically Blocked Thiouracil in the Presence of Cu (II), Cd (II), and Pb (II)—Solutions of 2-ethylmercapto-4-hydroxypyrimidine (2EM4HP) and *N,N'*-diethyl-6-methyl-2-thiouracil in the presence of cadmium, lead, and cupric ions were titrated with standard alkali. The titration curve of 2EM4HP in the presence of cadmium ion was the same as the titration of the ligand alone. The titration curves of 2EM4HP in the presence of cupric and lead ion could be assigned to a simple summation of the metal hydrolysis and ligand titration curves and indicated no apparent complexation. The titration of solutions of *N,N'*-diethyl-6-methyl-2-thiouracil alone showed no titratable group, and the titrations of metal ion mixtures gave curves showing only metal hydrolysis. In the case of 2EM4HP, the pKa′ of the 4-hydroxy group was 7.01 at 25°. The fact that no complexation occurred at the 4-hydroxy position, even when it could form an anion more easily than 2-thiouracil itself (pKa′ 7.49), argues for complexation at the sulfur position in the sterically unblocked compounds.

Calculation of Complexation Constants from Potentiometric Titrations—Those portions of the potentiometric titration curves where homogeneous solutions are maintained prior to the points of precipitation (Figs. 1–3) permit the estimation of complexation constants. The highest pH values at which usable data could be obtained in these studies were about 6.5 since the precipitation which occurred destroyed the equilibrium conditions.

Such feasible titration data were analyzed on the basis of the derived equations. First, the assumption was made that the only significant concentration of complex in solution derived from the doubly charged metal cation M^{++}, and the thiouracil was the 1:1 complex, MU^+, with the anion of the latter. If this were true, derived Eq. 15:

$$\log \frac{1-\bar{n}}{\bar{n}} = pK_1 + p\,[U^-] \qquad \text{(Eq. 15)}$$

would be applicable and the plot of log $(1-n)/n$ against $p\,[U^-] = -\log[U^-]$ should have a slope of one and an intercept of pK_1, a measure of the complexation constant of MU^+. The values of $\bar{n}$ were calculated from:

$$\bar{n} = \frac{[HU]_0 - [U^-]\left(1 + \frac{[H^+]\,\gamma_\pm}{K_a'}\right)}{[M^{++}]_0} \qquad \text{(Eq. 11)}$$

where $[HU]_0$ and $[M^{+2}]_0$ are the molar concentrations of the added thiouracil and metal ion, respectively, where K_a' is the dissociation constant of the thiouracil acid, where $[H^+]\,\gamma_\pm$ is the activity of the hydrogen ion, a_{H^+}, and is calculated from $a_{H^+} = 10^{-pH}$ where the pH is experimentally observed, and where the concentration of thiouracil anion $[U^-]$ is calculated at the same pH value from:

$$[U^-] = \frac{[HU]_0 - [NaOH]}{[H^+]\gamma_\pm/K_a'} \qquad \text{(Eq. 13)}$$

where [NaOH] is calculated on the premise that it would have been the concentration of the added milliequivalent of NaOH at the point in the titration if no NaOH had been reacted or consumed. Typical plots in accordance with Eq. 15 are given in Figs. 4 and 5 and the tabulated slopes of such plots are listed for all available titration studies in Table I. In those cases where the solutions were prepared so that the metal-ion and the thiouracil concentration were equimolar, the slopes of such plots were unity and it was reasonable that the intercept represented the pK_1 of the MU^+ complex (Table I). However, when the total metal-ion concentration $[M^{++}]_0$ was made up to be less than the total thiouracil concentration $[HU]_0$, the estimated slopes of such plots were in excess of unity (Table I). At very high ligand–metal ratios, these slopes approached two in some cases. This would be expected if appreciable amounts of MU_2 were formed which would be favored under conditions of high lig-

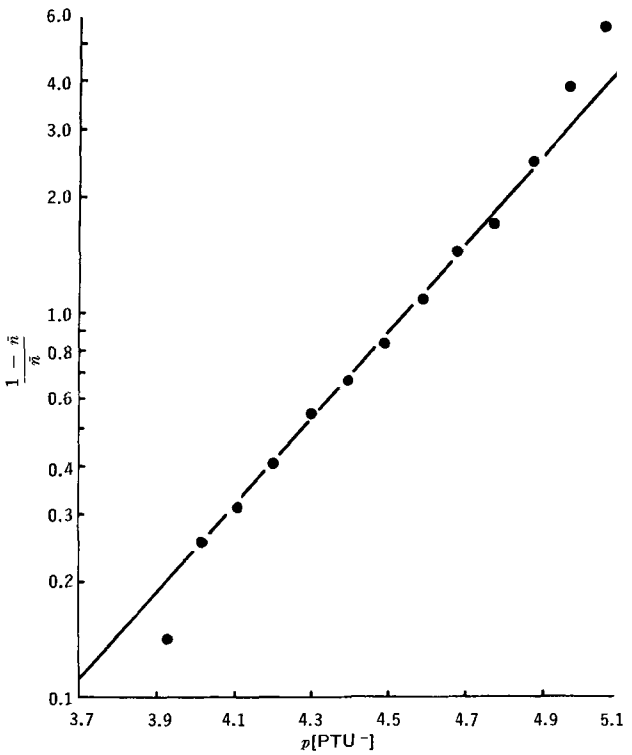


Figure 5—*Plot of log* $(1-\bar{n})/\bar{n}$ *against the negative logarithm of 6-n-propyl-2-thiouracil anion concentration* (p[*PTU*$^-$]) *obtained from a lead nitrate* (*2.00* $\times$ 10^{-4} M)–*PTU* (*2.00* $\times$ 10^{-3} M) *mixture in water with* μ = *0.006 at 25.8°. Slope of plot is 1.13.*

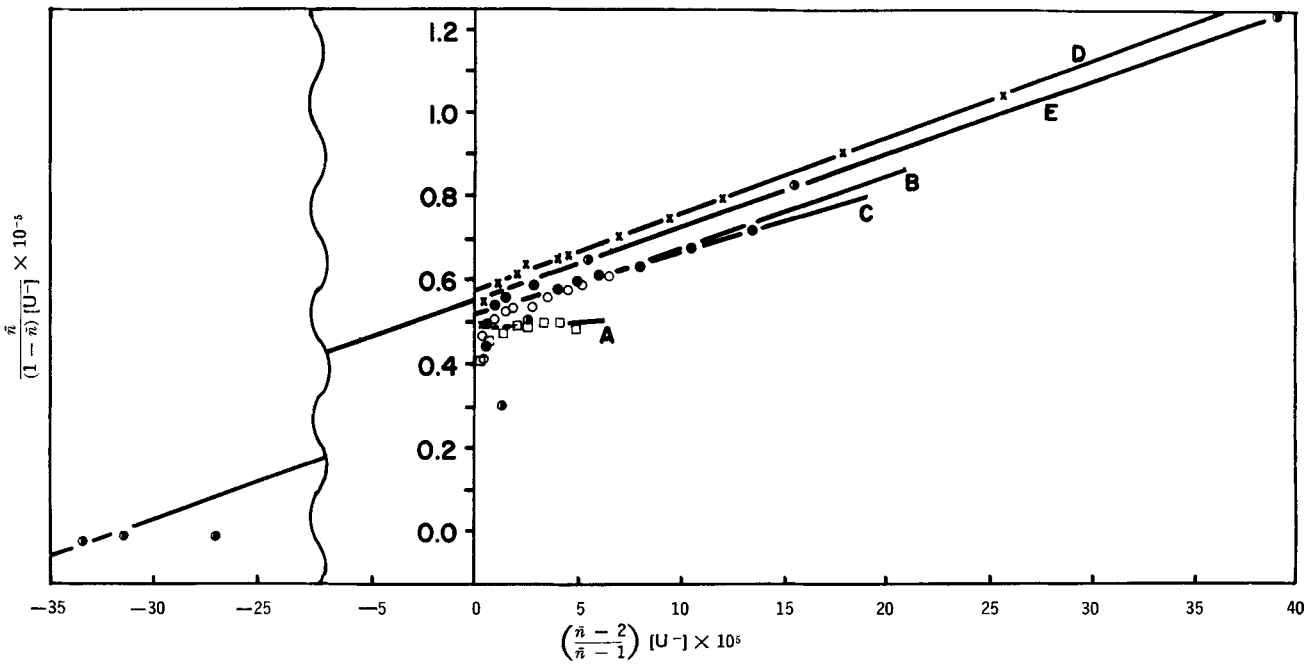


Figure 6—*Plots of* $\bar{n}/(1-\bar{n})[2TU^-]$ *against* $[(\bar{n}-2)/(\bar{n}-1)]$ $[2TU^-]$ *from aqueous mixtures of 2-thiouracil* ($[2TU] = 2.00 \times 10^{-3}$ M) *and lead nitrate with* $\mu = 0.006$ *at 25.0°. Key: (A), 2.00* $\times 10^{-3}$ M Pb^{++}; *(B), 1.80* $\times 10^{-3}$ M Pb^{++}; *(C), 1.40* $\times 10^{-3}$ M Pb^{++}; *(D), 8.00* $\times 10^{-4}$ M Pb^{++}; *and (E), 2.00* $\times 10^{-4}$ M Pb^{++}. *Intercept on ordinate is* K_1 *and slope is* K_1K_2.

and concentrations and as the titrations proceeded. At these higher slopes the premises for the use of such plots derived from Eq. 15 become invalid, and equations based on the postulates of additional complexes in solution must be used.

Thus, the assumption was made that there were two significant complexes in solution, the 1:1 complex MU^+ and the 1:2 complex MU_2. If this were true, the derived:

$$\frac{\bar{n}}{(1-\bar{n})[U^-]} = \left(\frac{\bar{n}-2}{\bar{n}-1}\right)\beta_2[U] + K_1 \qquad \text{(Eq. 14)}$$

would be applicable and the plot of $\bar{n}/\{(1-\bar{n})[U^-]\}$ against $(\bar{n}-2/\bar{n}-1)[U^-]$ would be linear for all thiouracil–metal-ion mixtures and the statistically significant estimated slopes β_2 and intercepts K_1 would be the same for all such mixtures. The $\bar{n}$ and $[U^-]$ data are calculated from the experimental values and Eqs. 11 and 13, as has been explained previously, and typical plots are given in Fig. 6.

The derived values of log K_1 and log K_2 (where $K_2 = \beta_2/K_1$) are related to the complexation constants of MU^+ and MU_2 (from MU^+), respectively, and are listed in Table I. The facts that the calculated values of log K_1 and log K_2 did not vary with the total metal-ion concentration (Table I) were consistent with the premise that only MU^+ and MU_2 complexes are in significant concentrations in the homogeneous solutions of mixtures of metal ions and thiouracils maintained prior to the observance of precipitation on the further addition of alkali. It has been shown in the *Theory and Methods of Calculation* section that the presence of significant amounts of other complexes in solution, such as $MU^{\pm}$ (VI) or its equivalent MUOH (V), M_2U_2 (VII) or M_nU_n (VIII) would make $[U^-]$ a function of the metal-ion concentration as denoted in Eqs. 24, 36, or 37. If this were true, plots (Fig. 5) made on the assumption of only significant concentrations of MU^+ (III) and MU_2 (IV) in the homogeneous solutions in accordance with Eq. 14 and on the premise of $[U^-]$ being independent of M^{++} concentration (Eq. 13) would not be expected to be linear. This can be readily seen from the forms of Eqs. 26 and 39 where it is apparent that log K_1 and log K_2 values estimated from plots in accordance with Eq. 14 would vary widely as a function of the metal-ion concentration, $[M^{++}]_0$. Since such plots were linear (Fig. 6) and there was no significant variation of the derived log K_1 and log K_2 values with M^{++} concentration (Table I), it is reasonable to accept the premise that only the MU^+ (III) and MU_2 (IV) complexes are in significant concentrations in the titrated homogeneous solutions prior to the observed precipitation on further addition of alkali.

DISCUSSION

Stability Constants of the Significantly Soluble MU^+ and MU_2 Complexes—Analyses of the homogeneous portions of the alkaline titration curves of solutions of mixtures of lead, cadmium, nickel, or zinc nitrate and various thiouracils [2-thiouracil (TU), 6-*n*-propyl-2-thiouracil (PTU), 6-methyl-2-thiouracil (6MTU), 5-methyl-2-thiouracil (5MTU), 5,6-dimethyl-2-thiouracil (5,6DMTU), and 5-carboethoxy-2-thiouracil (5CETU)] to the pH values of precipitation were consistent with the facts that the species present in significant concentrations in the homogeneous solutions of the titration must be the MU^+ (III) and MU_2 (IV) complexes of Scheme I. Plots (Fig. 5) in accordance with Eq. 14 where $[U^-]$ was calculated from Eq. 13 demonstrated linearity for all the metal ions and thiouracils studied that showed significant complexation. The values of log K_1 and log K_2 that were calculated from such plots (Eq. 14 and Fig. 5) did not show significant variation with various metal-ion concentrations $[M^{++}]_0$ (Table I). These facts are indicative that complexes such as MUOH (V), $MU^{\pm}$ (VI), M_2U_2 (VII), or M_nU_n (VIII) of Scheme I were not of significant concentrations in the alkaline titrated homogeneous solutions prior to precipitation.

The stability constant K_1 (Table I) is the largest for the formation of the MU^+ complex from Pb^{++} and Cd^{++} with 5,6DMTU and smallest from Cd^{++} with 5CETU. The log K_1 values were available for CdU^+ complexes with the substituted thiouracils that had the highest range in pKa′ values (Table II). A plot of such log K_1 values against these pKa′ values at 25.0° showed definite linearity and conformed to the expression:

$$\log K_1 = 0.50\ \text{pKa}' + 0.32 \qquad \text{(Eq. 41)}$$

Single alkyl substitution at the 5 or 6 position of 2-thiouracil slightly increased the pKa′ values to about 7.7 from 7.5 and similarly increased the log K_1 value of the CdU^+ complex. Simultaneous alkyl substitution at the 5 or 6 position significantly elevated the pKa′ to 8.1 and the log K_1.

Substitution of the electronegative carboethoxy group at the 5 position significantly reduced both the pKa′ to 6.43 and log K_1 value. This is readily understandable in that the electronegative 5-carboethoxy group (R_2 in Id, Scheme I) would be expected to reduce the electron availability of the *para* sulfur atom. Thus the sulfur's electronic charge would be reduced to result in lessened ability to bind both hydrogen ions and metal cations with the concomitant

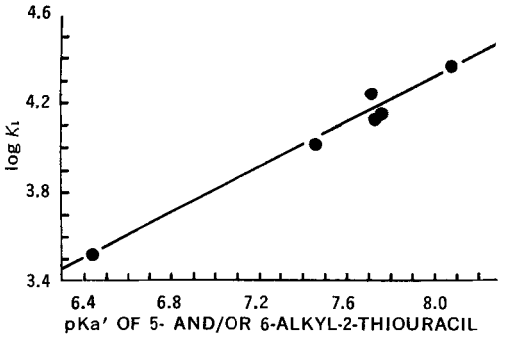


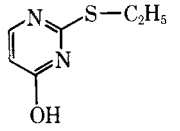
Figure 7—*Apparent linear relation between the logarithm of the stability constant* K_1 *for the formation of the* CdU^+ *complex in solution,* $Cd^{++} + U^- \overset{K_1}{\rightleftarrows} CdU^+$ *(where* U^- *is the 5- and/or 6-alkyl 2-thiouracil anion) and the pKa′ for the dissociation of the respective thiouracil acid,* $HU \overset{Ka'}{\rightleftarrows} H^+ + U^-$.

results of lowered pKa′ (or higher acidity) and diminished affinity, K_1, of thiouracils for metal ions.

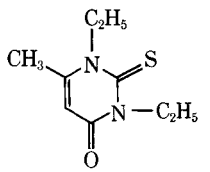
No obvious relations were observed as to the significant effects of 5 and/or 6 substituents of 2-thiouracil and their related pKa′ values on the formation constants, K_2, of the MU_2 complexes (Table I). No significant temperature effects could be concluded within the narrow temperature range studied.

The potentiometric titration studies demonstrated that the order of decreasing stability of complexes of divalent metal cations with thiouracils (Table I) to form MU^+ is $Pb^{++} > Cd^{++} \gg Ni^{++} \sim Zn^{++}$. Metal ions which do not complex thiouracils are Fe^{+++}, Fe^{++}, Co^{++}, Ca^{++}, and Mn^{++}.

Evidence for the Necessity of a Potential Anionic Sulfur in Thiouracils for Divalent Metal-Ion Complexation—The increase in pKa′ of the ionizable sulfhydryl group of the thiouracil is related to the stability of the metal complex (Fig. 7 and Table I) and implicates the need for an anionic sulfur in thiouracil for metal complex formation. In addition, when the dissociable proton is removed by alkylation as in 2-ethylmercapto-4-hydroxypyrimidine (IX), no complexation with metal ions was observed. Since the acidic hydroxyl group, pKa′ 7.01, still exists at the 4 position in IX, it can be stated that metal ions do not readily bind to the oxygen at the 4 position in the *parent* thiouracils. Alkylation of the N-1 and N-3 nitrogens of thiouracil, as in *N,N′*-diethyl-6-methyl-2-thiouracil (X) also destroyed the ability to complex metal ions. These substitutions on both the ring nitrogens prohibit the tautomeric formation of the dissociable sulfhydryl group of the complexing thiouracils, HU (I) in Scheme I. This is further evidence that metal-ion complexation occurs at the sulfur atom and is dependent on the formation of the sulfur anion.



IX



X

Possible Structures of Precipitates from Alkaline Titrated Solutions of Metal–Thiouracil Complexes—Although only concentrations of MU^+ and MU_2 complexes appear to be significantly present in the homogeneous solutions of mixtures of metal ions and thiouracils on alkaline potentiometric titration, this does not exclude the probability that the MUOH (V), $MU^{\pm}$ (VI), M_2U_2 (VII), and M_nU_n (VIII) complexes given in Scheme I may also be present.

It is apparent that if the concentration of any one of these complexes is limited by its solubility, precipitation will occur as the titrations proceed. It is also apparent from Scheme I that concentrations of all complexes must increase with the addition of hydroxyl ions. It may be presumed *a priori* that the charged complexes MU^+ and $MU^{\pm}$ would not readily or preferentially precipitate as such, or as salts with appropriate counterions.

Alkaline titrations of precipitating equimolar thiouracil–metal-ion solutions of Pb^{++} and Cd^{++} with all thiouracils (Fig. 1, Curve A), except for the Cd^{++}–PTU mixture (Fig. 2, Curve A), consumed titer up to the inflection pH of complete precipitation that was twice the alkaline titer expected for the titration of the thiouracil alone. Solutions (except for Cd^{++} and PTU mixtures) that contained less than equimolar amounts of metal to ligand showed that uncomplexed free ligand consumed equivalent moles of alkali but that the formed precipitate consumed twice the milliequivalents of alkali expected for the titration of that amount of thiouracil that was equimolar with the added Pb^{++} or Cd^{++} ions (Fig. 1, Curves B–G). These facts indicated that the complex precipitated must have a stoichiometry of 1:1, metal to ligand, or some multiple of 1:1. This behavior required that the species MU_2 present in homogeneous solutions for all Pb^{++} or Cd^{++} with all thiouracils (Table I), except for Cd^{++} and PTU mixtures, was not the precipitating species.

These phenomena may be summarized in the following expressions given in reference to Scheme I. The expected stoichiometry for neutralization of thiouracil is:

$$\underset{\text{I}}{HU} + OH^- \rightarrow \underset{\text{II}}{U^-} + HOH \qquad \text{(Eq. 42)}$$

If no more than the milliequivalent of NaOH necessary to neutralize the total amount of available thiouracil is necessary to complete the precipitation of adducts of divalent metal ion and thiouracil (Fig. 2), then:

$$M^{++} + \underset{\text{I}}{HU} + OH^- \rightleftarrows M^{++} + \underset{\text{II}}{U^-} + HOH \rightleftarrows \underset{\text{III}}{MU^+} + HOH \qquad \text{(Eq. 43)}$$

and

$$\underset{\text{III}}{MU^+} + \underset{\text{I}}{HU} + OH^- \rightleftarrows \underset{\text{III}}{MU^+} + \underset{\text{II}}{U^-} + HOH \rightarrow \underset{\text{IV}}{MU_2}\downarrow + HOH \qquad \text{(Eq. 44)}$$

and, as in the special case of 6-*n*-propyl-2-thiouracil and cadmium ion, the precipitated complex may have the stoichiometry, metal to ligand, of 1:2. Further confirmation was obtainable from the facts that when cadmium ion was equal to, or in excess of, one-half the available moles of PTU (Fig. 2, Curves A and B), the precipitation of complex (as monitored by the alkaline titration to the appropriate pH inflection where the precipitation of $Cd(OH)_2$ could not possibly interfere) was completed when the milliequivalents of added NaOH were just equivalent to the available PTU in accordance with Eqs. 43 and 44. Furthermore, $Cd(OH)_2$ precipitation, indicative of uncomplexed cadmium ion, was observed only when the cadmium-ion concentration was in excess of half the molar concentration of PTU (Fig. 2, Curve A). Free uncomplexed PTU was only observed (Fig. 2, Curve C) when the cadmium-ion concentration was less than half the molar concentration of PTU.

If twice the milliequivalents of NaOH necessary to neutralize the thiouracil completes the precipitation of equimolar amounts of divalent metal ion and thiouracil (Fig. 1), then in reference to Scheme I,

$$\underset{\text{I}}{HU} + M^{++} + 2OH^- \rightleftarrows \underset{\text{II}}{MU^+} + OH^- + H_2O \rightarrow \underset{\text{V}}{MUOH}\downarrow + H_2O \qquad \text{(Eq. 45)}$$

or

$$\underset{\text{I}}{HU} + M^{++} + 2OH^- \rightleftarrows \underset{\text{VI}}{MU^{\pm}} + 2H_2O \qquad \text{(Eq. 46)}$$

where

$$\underset{\text{VI}}{2MU^{\pm}} \rightarrow \underset{\text{VII}}{M_2U_2}\downarrow \qquad \text{(Eq. 47)}$$

or

$$\underset{\text{VI}}{nMU^{\pm}} \rightarrow \underset{\text{VIII}}{M_nU_n}\downarrow \qquad \text{(Eq. 48)}$$

There was no tendency for such precipitated complexes of lead and cadmium ion with the various thiouracils to be disrupted at relatively high concentrations of hydroxyl ions, at least to a pH of 9.0. This is indicative of the fact that the complexes of lead and cadmium of Scheme I have relatively high stabilities.

Consumption of more than twice the milliequivalents of NaOH necessary to neutralize the available thiouracil to the inflection corresponding to complete precipitation (Fig. 3) would be indicative of ready disruption and lessened stability of complexes such as those of Scheme I. It would imply that the metal hydroxides would be more readily formed on the further addition of hydroxide ion.

Consider the example when 3 times the milliequivalents of NaOH necessary to neutralize the total amount of available thiouracil complete the precipitation of an equimolar solution of a divalent metal ion and a thiouracil (Fig. 3, Curve A). Then,

$$\underset{\text{I}}{HU} + M^{++} + 3OH^- \rightarrow M(OH)_2 \downarrow + \underset{\text{II}}{U^-} + H_2O \quad \text{(Eq. 49)}$$

Alternatively the possible precipitated complexes of MU_2, MUOH, M_2U_2, or M_nU_n in equilibrium with their relatively unstable counterparts in solution can be disrupted by additional alkali as

$$\underset{\text{IV}}{MU_2} + 2(OH^-) \rightleftarrows \underset{\text{III}}{MU^+} + \underset{\text{II}}{U^-} + 2(OH^-) \rightarrow M(OH)_2 \downarrow + \underset{\text{II}}{2U^-} \quad \text{(Eq. 50)}$$

and

$$\begin{array}{ccc} & \underset{\text{V}}{MUOH} + OH^- \rightarrow M(OH)_2 \downarrow + \underset{\text{II}}{U^-} \\ & \uparrow\downarrow & \\ \frac{1}{n}\underset{\text{VII or VIII}}{M_nU_n} + HOH + OH^- & \uparrow\downarrow & \uparrow \\ & \uparrow\downarrow & \\ & \underset{\text{VI}}{MU^{\pm}} + OH^- + HOH \rightarrow \underset{\text{III}}{MU^+} + 2OH^- & \end{array} \quad \text{(Eq. 51)}$$

In the cases of the complete titrations after precipitation of the equimolar mixtures of Ni^{++} or Zn^{++} with thiouracils the alkaline titers to the completion of precipitation were greatly in excess of twice the titer necessary to neutralize the available thiouracil, HU, alone (Fig. 3, Curve A). This definitely indicates that the potential complexes of Scheme I for Ni^{++} and Zn^{++} with thiouracils were readily disrupted in solution by hydroxide ion in accordance with Eqs. 49–51. This is not unexpected since the stability constant of the MU^+ complexes for nickel and zinc are about a hundred times smaller than those for lead and cadmium (Table I).

Confirmation of Structures of Precipitates and Complexes of Cadmium and Lead Complexes of Thiouracils—The equilibria of Scheme I are consistent with the potentiometric titration studies. The precipitation of a relatively stable MU_2 (IV) complex from Cd^{++} and 6-*n*-propyl-2-thiouracil and of relatively stable either MUOH, M_2U_2, or M_nU_n complexes from Cd^{++} and Pb^{++} with the other thiouracils (Table I) is highly probable and consistent with the data.

These high probabilities were put to the test by the deliberate synthesis, isolation, and characterization of MU_2 (IV) and M_2U_2 (VII) or M_nU_n (VIII) complexes. The MU_2 complexes were prepared by the use of forcing conditions where a solution of the metal ion was added to the thiouracil solution in molar excess. The complexes of 1:1 stoichiometry were prepared from equimolar concentrations of thiouracil and metal ion where the thiouracil solution was added to that of the metal ion. The precipitation was effected by adjustment of the pH of the solutions to 6.5 (see *Experimental*).

The synthesized MU_2 (IV) complexes of bis(2-thiouracil)-cadmium (II), $Cd(TU)_2$; bis(2-thiouracil)-lead (II), $Pb(TU)_2$; and bis-(6-*n*-propyl-2-thiouracil)-cadmium (II), $Cd(PTU)_2$, and the synthesized M_2U_2 (VII) (or M_nU_n, VIII) complexes of bis(2-thiouracil-cadmium) (II), $Cd_2(TU)_2$; bis(2-thiouracil-lead) (II), $Pb_2(TU)_2$; and bis(6-*n*-propyl-2-thiouracil-lead) (II), $Pb_2(PTU)_2$, had the proper metal analyses and the proper complete elemental analysis [as in the Cd $(PTU)_2$ case] to confirm fully these assigned structures.

Further confirmation of the validity of the assignment of the MU_2 structure, IV, of Scheme I was obtained from the IR spectra. The synthesized $Cd(TU)_2$, $Pb(TU)_2$, and $Cd(PTU)_2$ had a strong absorption band at 1630 $cm.^{-1}$ assignable to the absorption of the tautomerizable carbonyl group at the 4 position of thiouracils (28). Strong absorption bands between 1440 and 1660 $cm.^{-1}$ indicated the presence of C=N bonds (29) that were not bound to metal ion. In contrast, the IR spectrum of the synthesized 6-methyl-*N,N'*-diethyl-2-thiouracil (X), a compound incapable of tautomeric formation o C=N bonds, did not demonstrate such C=N absorption bands.

Further confirmation of the validity of the assignment of an M_2U_2 (or M_nU_n) structure, VII (or VIII) of Scheme I, was obtained also from the IR spectra. The synthesized $Cd_2(TU)_2$, $Pb_2(TU)_2$, and $Pb_2(PTU)_2$ (or their M_nU_n equivalents) showed no significant band above 1570 $cm.^{-1}$ that could be assigned to a potential carbonyl group at the 4 position of the thiouracil ring (28) but did show bands assignable to the C=N group (29). The validity of secondary metal binding to the 4-oxygen is well indicated.

Further evidence for such an assignment as in VII or VIII of Scheme I is that the pKa′ of 2-ethylmercapto-4-hydroxypyrimidine (IX) is 7.01 compared to 7.46 for 2-thiouracil. Since alkyl substitution at the sulfur atom increased the acidity of the potential hydroxyl group at the 4 position, it is expected that substitution by a cation on the sulfur with retention of positive charge, as with MU^+ (III), would further increase the acidity. This facilitated dissociation of the 4-hydroxy group of the MU^+ (III) complex to $MU^{\pm}$ (VI) should be highly favored at lowered pH values and promote ready reaction with other MU^+ or $MU^{\pm}$ molecules to form the M_2U_2 (VII) or M_nU_n (VIII) polynuclear complexes of Scheme I early in the alkaline potentiometric titrations. Such neutral cyclic or polynuclear complexes with lessened hydrophilic groups must, in general, have lower solubilities and precipitate more readily than the MU_2 (IV) complexes which have been shown to exist readily at lower pH values (Fig. 5 and Table I).

The one observed exception was the cadmium and 6-*n*-propyl-2-thiouracil mixture where the neutral 1:2 complex $Cd(PTU)_2$ preferentially precipitated at the lower temperatures of 25 and 35°. Equations 43, 44, and 46–48 are valid explanations of the multiple equilibria and precipitation that may occur with some mixtures of metal ion and a thiouracil. It can be argued that the larger alkyl group significantly decreased the solubility of the MU_2 complex, significantly increased the pKa_2' of the 4-OH group of the $MU^{\pm}$ complex, or significantly increased the stability constant, K_2, of the MU_2 complex for PTU. Of these, the first and third arguments are most probable. The latter is confirmed by the data of Fig. 1, where K_2 for MU_2 formation exceeded K_1 for MU^+ formation by more than two-fold for the cadmium complex of PTU; whereas for the cadmium complexes of CETU and 5,6MTU, they were approximately equal and for lead and cadmium complexes of the other thiouracils studied, K_1 was 5–20-fold greater than K_2.

The precipitates produced by the potentiometric titrations of solutions containing 2-thiouracil in the presence of lead and cadmium ions were isolated, at pH 7.5 and 10.5, respectively, washed, and the IR spectra recorded. The IR spectra of these precipitates were identical with the spectra of the synthesized M_2U_2 complexes $Pb_2(TU)_2$ and $Cd_2(TU)_2$, respectively. Thus, it can be concluded that complexes of the form MUOH (V) are neither of significant concentration in homogeneous solutions of Cd^{++} or Pb^{++} ions with thiouracil, nor are they readily precipitated from such solutions on the addition of alkali. Equations 44, 47, and 48 from Scheme I are valid explanations of the multiple equilibria and precipitation that occur with most mixtures of Pb^{++} or Cd^{++} and thiouracils.

The elemental and IR analyses of the synthesized $Pb_2(TU)_2$, $Cd_2(TU)_2$ and $Pb_2(PTU)_2$ complexes do not permit rigorous discrimination between the preferred assignments as the cyclic dimers M_2U_2 (VII) or as the polynuclear polymers M_nU_n (VIII). When the synthesized $Cd_2(TU)_2$ was recrystallized from an ammoniacal solution and dried, its IR spectrum was almost exactly the same as the original $Cd_2(TU)_2$. However the physical form was noncrystalline in nature and appeared as "paper pulp." It is possible that the ammonia complexed with the cadmium and opened the ring of $Cd_2(TU)_2$, VII, and, on removal of the ammonia, the polymeric structure $Cd_n(TU)_n$ (VIII) was formed.

REFERENCES

(1) G. W. Anderson, I. F. Halverstadt, W. H. Miller, and R. O. Roblin, *J. Amer. Chem. Soc.*, **67**, 2197(1945).

(2) E. B. Astwood, A. Bissell, and A. M. Hughes, *Endocrinology*, **37**, 456(1945).
(3) R. H. Williams and G. A. Kay, *J. Med. Sci.*, **213**, 198(1947).
(4) "New and Nonofficial Drugs," 1st ed., J. B. Lippincott, Philadelphia, Pa., 1963, p. 759.
(5) L. S. Goodman and A. Gilman, "The Pharmacological Basis of Therapeutics," 3rd ed., Macmillan, New York, N. Y., 1965, p. 1489.
(6) M. J. Seven and L. A. Johnson, "Metal-Binding in Medicine," 1st ed., J. B. Lippincott, Philadelphia, Pa., 1960, pp. 321–324.
(7) H. J. Koch, Jr., and E. R. Smith, *J. Clin. Endocrinol.*, **16**, 123(1956).
(8) A. Kasanen and I. Viitanen, *Acta Med. Scand.*, **153**, 467 (1956).
(9) B. Weiss, *J. Biol. Chem.*, **201**, 31(1953).
(10) D. M. Fawcett and S. Kirkwood, *ibid.*, **205**, 795(1953).
(11) A. Taurog, G. D. Potter, and I. L. Chaikoff, *ibid.*, **213**, 119 (1955).
(12) G. S. Serif and S. Kirkwood, *ibid.*, **233**, 109(1958).
(13) D. Libermann, *Nature*, **164**, 142(1949).
(14) D. Libermann, *Bull. Soc. Chim. Biol.*, **31**, 1325(1949).
(15) W. H. Miller, R. O. Roblin, and E. B. Astwood, *J. Amer. Chem. Soc.*, **67**, 2201(1945).
(16) H. C. Koppel, R. H. Springer, R. K. Robins, and C. C. Cheng, *J. Org. Chem.*, **26**, 792(1961).
(17) D. J. Brown, "The Pyrimidines," 1st ed., Interscience, New York, N. Y., 1962, pp. 586–619.
(18) L. N. Short and H. W. Thompson, *J. Chem. Soc.*, **1952**, 168.
(19) "The Merck Index," 7th ed., Merck and Co., Rahway, N. J., 1960, p. 865.
(20) R. N. Lacey, *J. Chem. Soc.*, **1954**, 839.
(21) C. N. Reilley and R. W. Schmid, *Anal. Chem.*, **30**, 947 (1958).
(22) C. N. Reilley, R. W. Schmid, and D. W. Lamson, *ibid.*, **30**, 953(1958).
(23) R. Pribil, Z. Kondela, and B. Matzska, *Coll. Czech. Chem. Commun.*, **16**, 80(1951).
(24) A. E. Gillam and E. S. Stern, "Electron Absorption Spectroscopy," 2nd ed., Edward Arnold Publishers, Ltd., London, England, 1960, p. 289.
(25) N. H. Furman, "Standard Methods of Chemical Analysis," 6th ed., D. Van Nostrand, Princeton, N. J., 1962, (*a*) p. 251 and (*b*) p. 560.
(26) S. Bjerrum, "Metal Amine Formation in Aqueous Solution," P. Haase and Sons, Copenhagen, Denmark, 1941.
(27) F. J. C. Rossotti and H. Rossotti, "The Determination of Stability Constants," 1st ed., McGraw-Hill, New York, N. Y., 1961, p. 176.
(28) W. C. Schneider and I. F. Halverstady, *J. Amer. Chem. Soc.*, **70**, 2626(1948).
(29) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," 2nd ed., Wiley, New York, N. Y., 1962, p. 283.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 3, 1970, from the *College of Pharmacy, University of Florida, Gainesville, FL 32601*

Accepted for publication May 28, 1970.

This investigation was supported in part by Public Health Service Research Grants Number 5 R01 GMO9864-01 through 05 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and Public Health Service Research Grant Number 9 R01 CA 10738-06 from the National Cancer Institute.

D. J. Weber acknowledges support by an NIH predoctoral fellowship, GPM-19085. A grant-in-aid to D. J. Weber by The Upjohn Company, Kalamazoo, Mich., is also gratefully acknowledged.

Abstracted in part from a thesis submitted by D. J. Weber to the University of Florida in partial fulfillment of the Doctor of Philosophy degree requirements.

In Vitro Release of Chloramphenicol from Polymer Beads of α-Methacrylic Acid and Methylmethacrylate

S. C. KHANNA* and P. SPEISER

Abstract □ The *in vitro* release behavior of chloramphenicol from four different bead polymers containing methylmethacrylate and α-methacrylic acid in various buffer solutions has been studied. The concentration of α-methacrylic acid in the copolymer beads and the pH and ionic strength of the buffer solutions were observed to influence the release rate of the chloramphenicol from these beads. The beads containing no α-methacrylic acid did not release the drug in any buffer solution, and the beads containing only α-methacrylic acid released the drug at almost the same rate in all buffer solutions. The smaller beads released the drug more quickly than the larger ones.

Keyphrases □ Polymer beads—chloramphenicol release □ Chloramphenicol release—α-methacrylic acid, methylmethacrylate beads □ α-Methacrylic acid, concentration effect—release rates, polymer beads □ pH, ionic strength effects—chloramphenicol release, polymer beads

In an earlier publication (1), the possibility of utilizing the bead polymerization method for the preparation of a sustained-release dosage form was discussed. Physical barriers are used in the majority of the prolonged-release dosage forms to decrease the rate of drug release to the absorption site. The swelling or dissolution property of the polymer materials in which the drug is embedded is the major contributing factor in the release of drug from such dosage forms. Nelson (2) reported that the dissolution or release rate of a drug from a dosage form is the rate-determining factor in the absorption and physiological availability of the drug. Hence, an *in vitro* release procedure may be used to screen the materials worthy of inclusion as a potential physical barrier for sustained-release products. Furthermore, it may show the direction in which the right copolymers or polymers for the purpose may be found. The final required sustained-release dosage form containing these beads may consist of a single specimen of the polymer beads or a mixture of many different polymer and copolymer beads.

As the drug is incorporated in a large number of small individual beads, the chances of consistent availability of the drug at the intended site of the gastrointestinal tract increase considerably. In the present work, the

(2) E. B. Astwood, A. Bissell, and A. M. Hughes, *Endocrinology*, **37**, 456(1945).
(3) R. H. Williams and G. A. Kay, *J. Med. Sci.*, **213**, 198(1947).
(4) "New and Nonofficial Drugs," 1st ed., J. B. Lippincott, Philadelphia, Pa., 1963, p. 759.
(5) L. S. Goodman and A. Gilman, "The Pharmacological Basis of Therapeutics," 3rd ed., Macmillan, New York, N. Y., 1965, p. 1489.
(6) M. J. Seven and L. A. Johnson, "Metal-Binding in Medicine," 1st ed., J. B. Lippincott, Philadelphia, Pa., 1960, pp. 321–324.
(7) H. J. Koch, Jr., and E. R. Smith, *J. Clin. Endocrinol.*, **16**, 123(1956).
(8) A. Kasanen and I. Viitanen, *Acta Med. Scand.*, **153**, 467 (1956).
(9) B. Weiss, *J. Biol. Chem.*, **201**, 31(1953).
(10) D. M. Fawcett and S. Kirkwood, *ibid.*, **205**, 795(1953).
(11) A. Taurog, G. D. Potter, and I. L. Chaikoff, *ibid.*, **213**, 119 (1955).
(12) G. S. Serif and S. Kirkwood, *ibid.*, **233**, 109(1958).
(13) D. Libermann, *Nature*, **164**, 142(1949).
(14) D. Libermann, *Bull. Soc. Chim. Biol.*, **31**, 1325(1949).
(15) W. H. Miller, R. O. Roblin, and E. B. Astwood, *J. Amer. Chem. Soc.*, **67**, 2201(1945).
(16) H. C. Koppel, R. H. Springer, R. K. Robins, and C. C. Cheng, *J. Org. Chem.*, **26**, 792(1961).
(17) D. J. Brown, "The Pyrimidines," 1st ed., Interscience, New York, N. Y., 1962, pp. 586–619.
(18) L. N. Short and H. W. Thompson, *J. Chem. Soc.*, **1952**, 168.
(19) "The Merck Index," 7th ed., Merck and Co., Rahway, N. J., 1960, p. 865.
(20) R. N. Lacey, *J. Chem. Soc.*, **1954**, 839.
(21) C. N. Reilley and R. W. Schmid, *Anal. Chem.*, **30**, 947 (1958).
(22) C. N. Reilley, R. W. Schmid, and D. W. Lamson, *ibid.*, **30**, 953(1958).
(23) R. Pribil, Z. Kondela, and B. Matzska, *Coll. Czech. Chem. Commun.*, **16**, 80(1951).
(24) A. E. Gillam and E. S. Stern, "Electron Absorption Spectroscopy," 2nd ed., Edward Arnold Publishers, Ltd., London, England, 1960, p. 289.
(25) N. H. Furman, "Standard Methods of Chemical Analysis," 6th ed., D. Van Nostrand, Princeton, N. J., 1962, (*a*) p. 251 and (*b*) p. 560.
(26) S. Bjerrum, "Metal Amine Formation in Aqueous Solution," P. Haase and Sons, Copenhagen, Denmark, 1941.
(27) F. J. C. Rossotti and H. Rossotti, "The Determination of Stability Constants," 1st ed., McGraw-Hill, New York, N. Y., 1961, p. 176.
(28) W. C. Schneider and I. F. Halverstady, *J. Amer. Chem. Soc.*, **70**, 2626(1948).
(29) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," 2nd ed., Wiley, New York, N. Y., 1962, p. 283.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 3, 1970, from the *College of Pharmacy, University of Florida, Gainesville, FL 32601*

Accepted for publication May 28, 1970.

This investigation was supported in part by Public Health Service Research Grants Number 5 R01 GMO9864-01 through 05 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and Public Health Service Research Grant Number 9 R01 CA 10738-06 from the National Cancer Institute.

D. J. Weber acknowledges support by an NIH predoctoral fellowship, GPM-19085. A grant-in-aid to D. J. Weber by The Upjohn Company, Kalamazoo, Mich., is also gratefully acknowledged.

Abstracted in part from a thesis submitted by D. J. Weber to the University of Florida in partial fulfillment of the Doctor of Philosophy degree requirements.

In Vitro Release of Chloramphenicol from Polymer Beads of α-Methacrylic Acid and Methylmethacrylate

S. C. KHANNA* and P. SPEISER

Abstract □ The *in vitro* release behavior of chloramphenicol from four different bead polymers containing methylmethacrylate and α-methacrylic acid in various buffer solutions has been studied. The concentration of α-methacrylic acid in the copolymer beads and the pH and ionic strength of the buffer solutions were observed to influence the release rate of the chloramphenicol from these beads. The beads containing no α-methacrylic acid did not release the drug in any buffer solution, and the beads containing only α-methacrylic acid released the drug at almost the same rate in all buffer solutions. The smaller beads released the drug more quickly than the larger ones.

Keyphrases □ Polymer beads—chloramphenicol release □ Chloramphenicol release—α-methacrylic acid, methylmethacrylate beads □ α-Methacrylic acid, concentration effect—release rates, polymer beads □ pH, ionic strength effects—chloramphenicol release, polymer beads

In an earlier publication (1), the possibility of utilizing the bead polymerization method for the preparation of a sustained-release dosage form was discussed. Physical barriers are used in the majority of the prolonged-release dosage forms to decrease the rate of drug release to the absorption site. The swelling or dissolution property of the polymer materials in which the drug is embedded is the major contributing factor in the release of drug from such dosage forms. Nelson (2) reported that the dissolution or release rate of a drug from a dosage form is the rate-determining factor in the absorption and physiological availability of the drug. Hence, an *in vitro* release procedure may be used to screen the materials worthy of inclusion as a potential physical barrier for sustained-release products. Furthermore, it may show the direction in which the right copolymers or polymers for the purpose may be found. The final required sustained-release dosage form containing these beads may consist of a single specimen of the polymer beads or a mixture of many different polymer and copolymer beads.

As the drug is incorporated in a large number of small individual beads, the chances of consistent availability of the drug at the intended site of the gastrointestinal tract increase considerably. In the present work, the

Table I—Polymeric Beads of α-Methacrylic Acid and Methylmethacrylate with Chloramphenicol

Preparation No.	Monomeric Mixture Polymerized, % α-Methacrylic Acid	Methylmethacrylate	Chloramphenicol in Beads, %
1	100	–	9.5
2	66.6	33.3	15.0
3	33.3	66.6	19.5
4	–	100.0	7.0

influence of the following factors on the *in vitro* release of chloramphenicol USP embedded in beads of α-methacrylic acid, methylmethacrylate, or mixtures thereof has been studied: (*a*) content of α-methacrylic acid in the polymer beads, (*b*) pH and ionic strengths of the buffer solutions, and (*c*) size of the bead.

EXPERIMENTAL

***In Vitro* Release Test**—A modified USP tablet disintegration test apparatus was used under the same conditions as previously described (3). In each glass tube, a fine nylon filter was fitted so that the beads could be separated from the buffer solution from time to time. Approximately 100–150-mg. beads from an 800–1000-μ diameter sieve fraction were accurately weighed and eluted with 50 ml. of buffer solution. At a fixed time interval, selected according to the polymer and the buffer solution used, the buffer solution was filtered through the fine nylon filter fitted in the tube and was replaced with the same amount of the fresh buffer solution. The amount of chloramphenicol released was determined spectrophotometrically at the 278-mμ wavelength. This procedure was repeated until either the whole of the embedded drug was released or a maximum period of 14 hr. was reached. Triplicate experiments were performed with each buffer solution, and the results are the average of these.

Buffer Solution Used—Various buffer solutions in the range of pH of gastrointestinal fluids have been used as eluting liquids for the *in vitro* release of chloramphenicol from the polymer beads. Buffer solutions of pH 1.2 (HCl–NaCl), 3.2, 5.2, 6.2, 7.2, and 8.2 (all phosphates), each with ionic strengths of 0.1, 0.2, and 0.3, were prepared. Buffer solutions of either pH 8.2 or of ionic strength 0.3 are less common in the human gastrointestinal tract, but they were selected in this study to check the effect of these factors at this high limit on the release behavior of chloramphenicol from the polymer beads.

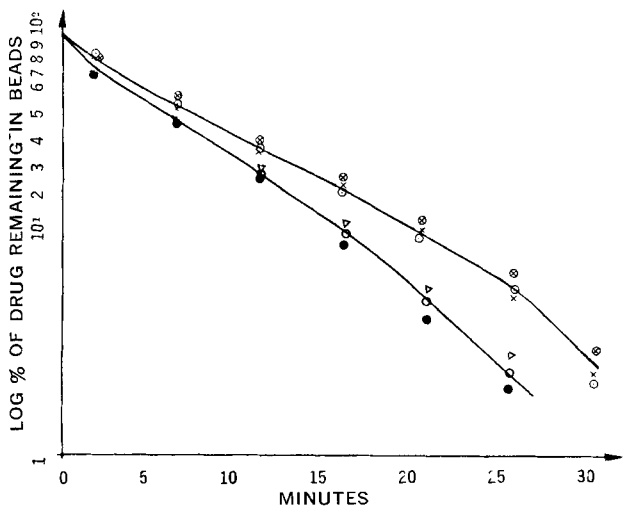


Figure 1—*Release of chloramphenicol from Preparation 1 in different buffer solutions (μ = ionic strength). Key: ○, pH 1.2, μ 0.1; ▽, pH 1.2, μ 0.2; ●, pH 1.2, μ 0.3; ⊗, pH 5.2, μ 0.1; ⊙, pH 5.2, μ 0.2; and ×, pH 5.2, μ 0.3.*

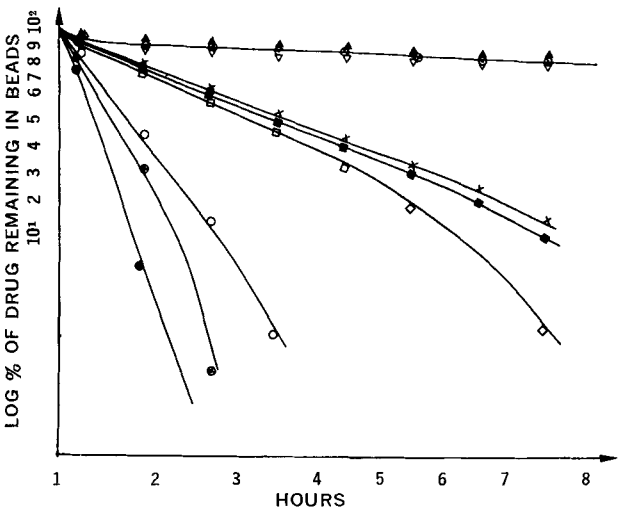


Figure 2—*Release of chloramphenicol from Preparation 2 in different buffer solutions (μ = ionic strength). Key: ▲, pH 3.2, μ 0.1; ⊙, pH 3.2, μ 0.2; ▽, pH 3.2, μ 0.3; ×, pH 5.2, μ 0.1; ■, pH 5.2, μ 0.2; □, pH 5.2, μ 0.3; ○, pH 7.2, μ 0.1; ⊗, pH 7.2, μ 0.2; and ●, pH 7.2, μ 0.3.*

To calculate the amount of the substances for the preparation of the buffer solutions, the following equations were used (4):

$$\text{pH} = \text{pK}_n + \log \frac{(\text{salt})}{(\text{acid})} - \frac{A\,(2n-1)\,\sqrt{\mu}}{1+\sqrt{\mu}} \qquad \text{(Eq. 1)}$$

$$\mu = 1/2 \sum C_i Z_i^2 \qquad \text{(Eq. 2)}$$

All symbols have the same meaning as in the literature (4).

The pH's of the buffer solutions were determined using a Metrohm pH meter, type E 396, and the buffers were adjusted to the correct value if required. The ionic strength, if required, was adjusted by the addition of sodium chloride. All substances used were of analytical quality.

Beads Used—The materials used and the method of preparation of these polymeric beads in the presence of chloramphenicol USP have already been reported (1). The composition of the beads used in the present study is shown in Table I.

Bead Size Used—Sieve analysis of the polymeric beads was carried out (1). The fraction left on each sieve (400–500 μ, 500–630 μ, 630–800 μ, and 800–1000 μ) was used to study the effect of bead size on the release rate of chloramphenicol from the polymeric beads.

RESULTS AND DISCUSSION

To select the appropriate polymer or copolymer of α-methacrylic acid and methylmethacrylate for incorporation into the sustained-release dosage form, the influence of different ionic strengths and the pH's of the various buffer solutions on the release behaviors of chloramphenicol embedded in them has been studied. Although gastric juice varies individually in composition (5), the acidity ranges generally in terms of pH values from 1.2 to 2.5. In some healthy persons, it may even exhibit higher pH values. The pH values of the fluids from the duodenum to the large intestine may vary from 5 to 8 (6). Therefore, the release studies of the drug from the polymeric beads were carried out in buffer solutions from pH 1.2 to 8.2. Although the ionic strengths of the gastrointestinal fluids are constant under normal conditions, they may change due to uptake of ionic substances during meals, *etc.* Therefore, the influence of the different ionic strengths (0.1, 0.2, and 0.3) at all the pH levels of the buffer solutions on the release of drug from beads also has been studied. As the enzymatic activity of gastrointestinal fluids is of minor importance in the release behaviors of the drug from the polymers, pure buffer solutions of either HCl–NaCl or phosphates having the given pH's and ionic strengths have been used.

In the course of the experiments, it became evident that in most cases the release of chloramphenicol from the various polymer and

Table II—Average $t_{20}\%$, $t_{50}\%$, and $t_{80}\%$[a] of Chloramphenicol Released from Different Bead Formulations in Various Buffer Solutions

Buffer Solutions		Preparation No. 1 Time, min.			Preparation No. 2 Time, hr.			Preparation No. 3 Time, hr.		
pH	Ionic Strength	t_{20}	t_{50}	t_{80}	t_{20}	t_{50}	t_{80}	t_{20}	t_{50}	t_{80}
1.2	0.1	1.2	5.0	13.0						
	0.2	1.2	5.4	13.5	—	—	—	—	—	—
	0.3	1.2	5.0	13.0						
3.2	0.1	1.4	6.2	16.4	6.5	—	—			
	0.2	1.4	5.8	16.0	4.75	—	—	—	—	—
	0.3	1.5	6.5	18.5	4.5	—	—			
5.2	0.1	1.4	6.6	17.5	0.8	2.6	5.9			
	0.2	1.3	6.0	16.8	0.65	2.4	5.5	—	—	—
	0.3	1.3	6.5	18.2	0.55	2.1	4.75			
6.2	0.1							25.0[b]	—	—
	0.2	—	—	—	—	—	—	17.0[b]	—	—
	0.3							15.0[b]	—	—
7.2	0.1				0.25	0.8	1.8	1.6	6.5	15.5[b]
	0.2	—	—	—	0.20	0.6	1.35	1.0	2.9	6.4
	0.3				0.15	0.5	0.95	0.45	1.5	3.4
8.2	0.1							1.25	5.5	14.0[b]
	0.2	—	—	—	—	—	—	0.60	2.0	4.5
	0.3	—	—	—	—	—	—	0.35	1.2	2.6

[a] The values for each were taken from Figs. 1–3. [b] The values obtained by extrapolation.

copolymer beads of α-methacrylic acid and methylmethacrylate gave curves of a higher order when plotted as percent cumulative release against time, or as logarithms of the amount of drug remaining in the beads (as a percentage) against time, or as the amount of drug released against the square root of time. However, since most of the drug release data from the various bead formulations showed that up to 80% of the release of the chloramphenicol apparently followed a first-order rate, except the release during the first interval, it was decided to represent the results as logarithms of the amount of chloramphenicol remaining in the beads as a percentage against time (Figs. 1–3). The faster release rate in the beginning may be due to the presence of the drug on the surfaces of the beads. The orthogonal polynomials were used where adequate data were available to calculate the best fitting equations for the regression of the complete curve of log percent of drug remaining in beads against time. In extreme cases, polynomials up to the third degree were required to provide an adequate equation. The comparison of the functions of these equations was rather complex; therefore, the times for release of 20, 50, and 80% of chloramphenicol (represented as t_{20}, t_{50}, and t_{80}) from beads were used as the comparative measures to prove the influence of the various factors in the study on the release behavior of the drug from polymeric beads (Table II). In a few cases, where even 20% release over a study period could not be obtained from the experimental values, it was calculated from the respective regression equations.

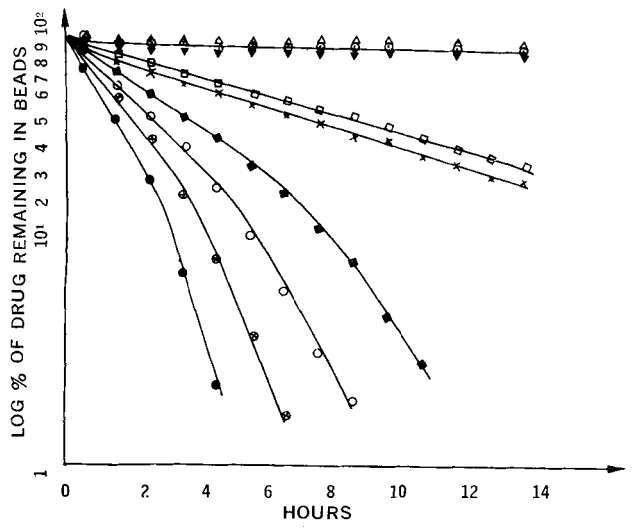


Figure 3—*Release of chloramphenicol from Preparation 3 in different buffer solutions (μ = ionic strength). Key: △, pH 6.2, μ 0.1; ⊙, pH 6.2, μ 0.2; ▼, pH 6.2, μ 0.3; □, pH 7.2, μ 0.1; ×, pH 7.2, μ 0.2; ■, pH 7.2, μ 0.3; ○, pH 8.2, μ 0.1; ⊗, pH 8.2, μ 0.2; and ●, pH 8.2, μ 0.3.*

Influence of α-Methacrylic Acid Content in Bead Polymers on Release of Chloramphenicol—In the absence of α-methacrylic acid in the polymeric beads, Preparation 4, the release of the drug did not take place in buffer solutions of pH 8.2 and below. The release from these beads in buffer solution of higher pH's than this was not carried out. In copolymers, Preparations 3 and 2, incorporating 33.3 and 66.6% α-methacrylic acid, respectively, the release of chloramphenicol started in the buffer solutions of pH 6.2 and 3.2, respectively. The release of chloramphenicol from these copolymer beads in the buffer solutions of pH 5.2 and 2.2, respectively, was almost negligible. The beads containing only α-methacrylic acid, Preparation 1, released the drug quite rapidly in buffer solutions of pH 1.2 and above.

Thus, the content of α-methacrylic acid in the polymer and copolymers influences the onset of the release of chloramphenicol in the buffer solutions. The higher the acidic content in the polymeric beads, the lower is the pH at which the release of drug starts. The results are given in Table III.

There is a rather complicated relationship between the α-methacrylic acid content of the polymer and the properties (pH and ionic strength) of the buffer solutions. This is observed from the irregular release behavior of chloramphenicol from these polymeric beads in the different buffer solutions. For this reason, a quantitative comparison between α-methacrylic acid content in the polymers and the release rates of drug from them in buffer solutions seems difficult to establish. However, it may be observed (Table II) that the higher the α-methacrylic acid content in the polymeric beads, the quicker the release of the drug from them into the buffer solutions.

Influence of pH's and Ionic Strengths of Buffer Solutions on Release Rate of Chloramphenicol from Beads—The polymeric beads containing only α-methacrylic acid showed no significant change in the release rate of chloramphenicol embedded in these beads with variation of either pH or ionic strength of the buffer solution (Table II). It is possible that, due to the large number of acidic groups in the polymer beads, identical solubility and swelling of the beads occurred in the acidic to neutral buffer solutions.

Table III—Influence of Acidic Content in Beads on the Onset of Drug Release

	Preparation No. 1	2	3	4
pH of buffer solution (ionic strength = 0.1)	≥1.2	≥3.2	≥6.2	≤8.2

Table IV—Average Release of Chloramphenicol from Polymeric Beads of Different Diameter

Bead Diameter, μ	Preparation No. 1, Buffer Solution pH 5.2, Ionic Strength = 0.1, Time, min. t_{50}	Preparation No. 1, Buffer Solution pH 5.2, Ionic Strength = 0.1, Time, min. t_{80}	Preparation No. 2, Buffer Solution pH 5.2, Ionic Strength = 0.1, Time, hr. t_{50}	Preparation No. 2, Buffer Solution pH 5.2, Ionic Strength = 0.1, Time, hr. t_{80}	Preparation No. 3, Buffer Solution pH 7.2, Ionic Strength = 0.1, Time, hr. t_{50}	Preparation No. 3, Buffer Solution pH 7.2, Ionic Strength = 0.1, Time, hr. t_{80}
400– 500	1.8	3.5	1.0	1.9	3.5	8.4
500– 630	3.0	7.8	1.3	3.9	4.3	9.2
630– 800	4.0	9.7	2.0	4.0	6.4	15.1
800–1000	7.0	17.4	2.5	5.9	6.5	15.5

However, with the increase of either pH or ionic strength of the buffer solutions, the rate of release of chloramphenicol from the polymer beads containing either 66.6 or 33.3% of α-methacrylic acid was enhanced (Table II, Figs. 2 and 3). In the case of a preparation containing 66.6% α-methacrylic acid in the polymer, the most pronounced increase in the release of chloramphenicol took place in buffer solutions having pH's between 3.2 and 5.2; for a preparation containing 33.3% α-methacrylic acid, the same effect was observed in buffer solutions of pH's between 6.2 and 7.2.

The ionic strength had a more pronounced effect in the case of the bead formulation containing 33.3% α-methacrylic acid than in the case of one containing 66.6% α-methacrylic acid. This change in the release rate of the drug may be due to different swelling and solubility properties of the polymer beads in various buffer solutions.

Influence of the Bead Diameter on the Release Rate—As the release of chloramphenicol from the copolymer beads was found to be dependent on the ionic strengths and pH's of the eluting buffer solutions, it was considered that these beads could be ion-exchange-type resins. The release rate of the drug from such resins is inversely proportional to the radius of the spherical particles. Thus, the release behavior from different bead sizes of the first three preparations was studied. Preparation 4 could not be considered for the study because no release took place from this preparation in any buffer solution below pH 8.2 (Table III). However, no such relationship between t_{50} release and 1/radius could be observed. It may be that the fraction of the sieve-analyzed beads taken in these release studies is not representative of the uniform size distribution of the beads in that fraction. Moreover, the geometry of these beads is altered by swelling and dissolution; hence, the mean radius taken will not be correct for such correlation. However, the results (Table IV) clearly show that the release rate of drug increases with decreasing bead size. Therefore, the uniform release from the sustained-release dosage form may be obtained by varying the particle size in the formulation.

SUMMARY

The release behavior of the drug from these beads in the buffer solutions depends mainly on the amount of α-methacrylic acid content in the polymer. The polymer beads containing 100% α-methacrylic acid released the drug easily in strong acidic buffer solutions of pH 1.2 and above. Hence, these beads may be used to form the initial dose portion in a sustained-release dosage form. The copolymer beads containing 66.6 and 33.3% α-methacrylic acid released the drug in buffer solutions of pH 3.2 and 6.2, respectively, and over. These may be incorporated as a sustained-release portion in such dosage forms. The combination of these beads in the appropriate proportion will show a right release pattern for a sustained-release dosage form.

On the basis of this study, copolymer beads of α-methacrylic acid and methylmethacrylate having the property of predetermined release of drug in specific buffer solution may possibly be prepared. The release rate of the sustained-release portion may also be controlled by varying the bead sizes.

REFERENCES

(1) S. C. Khanna, T. Jecklin, and P. Speiser, to be published.

(2) E. Nelson, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 607(1957).

(3) S. C. Khanna, M. Soliva, and P. Speiser, *J. Pharm. Sci.*, **58**, 1385(1969).

(4) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, pp. 202, 264.

(5) G. Dotevall, *Acta Med. Scand.*, **170**, 59(1961).

(6) Documenta Geigy, Wissenschaftliche Tabellen, 7. Aufgabe, J. R. Geigy A. G., Pharma, Basel, Switzerland, pp. 647–654.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 15, 1969, from the *School of Pharmacy, Federal Institute of Technology, Zürich, Switzerland.*

Accepted for publication April 17, 1970.

Abstracted from the dissertation submitted by S. C. Khanna to the Swiss Federal Institute of Technology in partial fulfillment of Doctor of Natural Sciences degree requirements.

The authors thank Dr. M. Soliva for his active participation in the discussion.

* Present address: J. R. Geigy S. A., Galenic Research Section, Basel, Switzerland.

Micellar Solubilization of Barbiturates II: Solubilities of Certain Barbiturates in Polyoxyethylene Stearates of Varying Hydrophilic Chain Length

M. WAFIK GOUDA*, A. A. ISMAIL†, and M. M. MOTAWI†

Abstract □ A study has been made of the solubilities of a series of 5,5-disubstituted barbituric acid derivatives in aqueous solutions of polyoxyethylene stearates of varying polyoxyethylene chain length. Except for phenobarbital, which forms an insoluble precipitate complex with the solubilizers, all other barbiturates studied were micellarly solubilized. On a molar basis, solubility increases with an increase in hydrophilic chain length but decreases if solubility is expressed in terms of the amount solubilized per ethylene oxide unit. A possible explanation for such a pattern was given. The partition coefficient, K, of the drug molecules between a micellar pseudophase and an aqueous phase was found to be dependent on both the polar effect and the number of carbon atoms of the substituents on the 5-position. The formation of an insoluble precipitate complex by phenobarbital was attributed to the presence of the aromatic phenyl group in the molecule.

Keyphrases □ Barbiturates, 5,5-disubstituted—solubility □ Micellar solubilization—barbiturates □ Solubilization, barbiturates—polyoxyethylene stearates □ Hydrophilic chain length, polyoxyethylene stearates—barbiturates solubilization

The micellar solubilization of certain barbiturate drugs in polysorbates 20, 40, 60, and 80[1] was previously reported (1). These polysorbates have the same hydrophilic portion in their molecule but differ in the length of the carbon atom chain of their lipophilic portion. This investigation was undertaken to study the effect of the hydrophilic group of nonionic solubilizers and the chemical structure of solubilizates on the degree of solubilization. Polyoxyethylene stearates are nonionic surfactants used as solubilizing agents in the same manner as polysorbates (2). Polyoxyl 40 stearate was official in USP XVII as a pharmaceutic aid. A group of four polyoxyethylene stearates[2] was chosen as the solubilizing agents for this study. Since these solubilizers are all of one chemical type, differing only in ethylene oxide content, they serve as a good model to compare solubilization of the barbiturates as a function of hydrophilic chain length.

EXPERIMENTAL

Materials—The following surfactants[2] were used as received: polyoxyethylene 30 monostearate, polyoxyethylene 40 monostearate, polyoxyethylene 50 monostearate, and polyoxyethylene 100 monostearate.

The barbiturates used and their melting points[3] were: phenobarbital, 5-ethyl-5-phenylbarbituric acid, m.p. 173–174°; barbital, 5,5-diethylbarbituric acid, m.p. 185–187°; amobarbital, 5-ethyl-5-isoamylbarbituric acid, m.p. 153–155°; diallylbarbituric acid, 5,5-diallylbarbituric acid, m.p. 169–170°; and cyclobarbital, 5-(1-cyclohexenyl)-5-ethylbarbituric acid, m.p. 89–91°.

[1] Tween 20, 40, 60, and 80, Atlas Chemical Industries, Inc., Wilmington, DE 19899

[2] Myrj 51, 52, 53, and 59, Atlas Chemical Industries, Inc., Wilmington, DE 19899

[3] Uncorrected melting points determined with a Thomas-Hoover Unimelt.

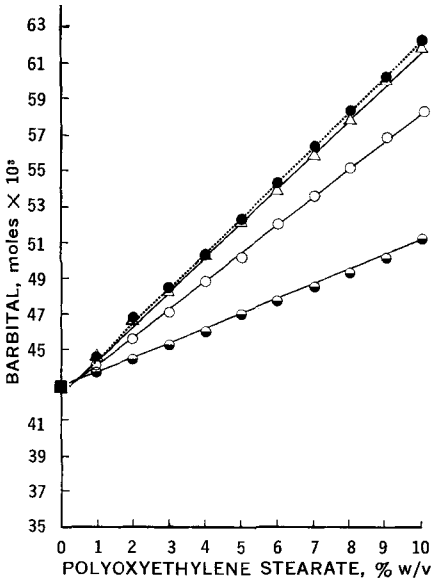


Figure 1—*Solubility of barbital in polyoxyethylene stearate solutions at 30°. Key: ■, solubility in water; ●, polyoxyethylene 30 stearate; △, polyoxyethylene 40 stearate; ○, polyoxyethylene 50 stearate; and ◒, polyoxyethylene 100 stearate.*

Assay Procedure—The differential UV procedure of Walker *et al.* (3), using a Unicam SP 500 spectrophotometer, was used to assay for the barbiturates.

Solubility Determinations—The solubilities of the barbiturates in solutions of polyoxyethylene stearates in 0.003 N sulfuric acid at 30° were determined by the procedure described earlier (1). Equilibrium solubility was attained after 24 hr.

RESULTS AND DISCUSSION

The solubilization of barbital, diallylbarbituric acid, cyclobarbital, amobarbital, and secobarbital in the polyoxyethylene stearate surfactants is shown in Figs. 1–5. The concentrations of the polyoxyethylene stearates used were well beyond their CMC (4). All of these barbiturate drugs showed a linear increase in solubilities in the presence of the solubilizers characteristic of micellar solubilization of such polar solubilizates.

To compare the solubilizing power of the different homologs of solubilizer, the slopes of the solubilization isotherms were calculated using the method of least squares. Table I shows the solubilization capacities expressed as moles drug per gram solubilizer, moles drug per mole solubilizer, and moles drug per equivalent of ethylene oxide in the surfactant. The latter values are the slopes of the solubilization isotherms if the moles of solubilizate were to be plotted against the surfactant concentration in equivalents of ethylene oxide per liter. The solubilizing power of the solubilizers, expressed on a molar basis, is found to increase slightly but gradually as the polyoxyethylene chain increases. However, when the solubilizing power of the surfactants is expressed in terms of moles drug per gram surfactant or moles drug per ethylene oxide equivalent, the

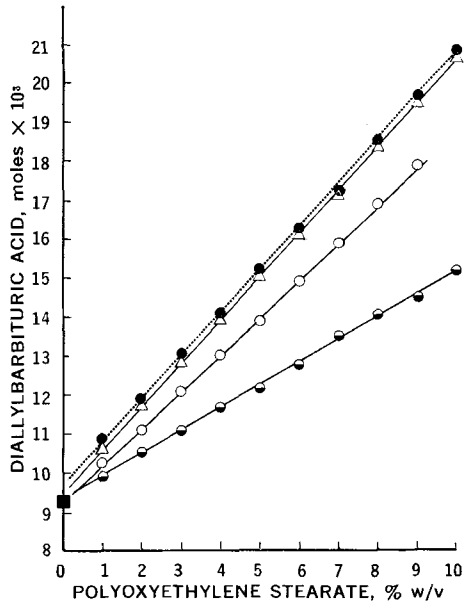


Figure 2—*Solubility of diallylbarbituric acid in polyoxyethylene stearate solutions at 30°. Key: ■, solubility in water; ●, polyoxyethylene 30 stearate; △, polyoxyethylene 40 stearate; ○, polyoxyethylene 50 stearate; and ◒, polyoxyethylene 100 stearate.*

efficiency of solubilization decreases with the increasing length of the polyoxyethylene chain.

Other authors have reported a similar pattern for the solubilization of benzoic acid derivatives in the same polyoxyethylene stearate surfactants (5) and for the solubilization of benzaldehyde and *p*-methyl benzaldehyde in polyoxyethylene ethers of varying hydrophilic chain length (6). No explanation for such a pattern was given. A possible explanation could be forwarded, however, on the basis of the study of Schick *et al.* (7) of the effect of the polyoxyethylene chain length of polyoxyethylene ethers on the micellar weight and the number of surfactant molecules per micelle. From light-scattering measurements, these authors found that, for polyoxyethylene ethers of branched nonylphenol and *n*-decanol having a hydrophobic chain of 10.5 and 12 carbon atoms, respectively, both the aggregate number (number of molecules per micelle) and the aggregate molecular weight of the micelles decrease as the polyoxyethylene chain increases from 10 to 50 ethylene oxide units. However, for *n*-octadecanol ethers with a hydrophobic chain of 18 carbon atoms, the increase in ethylene oxide units from 14 to 100 resulted in a decrease of the aggregate number but an increase of the aggregate molecular weight of the micelle. This increase in micellar weight was also found to be associated with increase in the micellar size. Such an increase in both micellar weight and micellar size was not linearly parallel to the increase in ethylene oxide units. For example, the aggregate molecular weight for the *n*-octadecanol ether surfactants with 14 ethylene oxide units was found to be 330,000; while for the surfactant with 100 ethylene oxide units, it was found to be 465,000. This means that the micellar size per ethylene oxide equivalent will actually be seen to decrease with the increasing length of the polyoxyethylene chain. Since the authors here are dealing with surfactants having the same 18 carbon atom hydrophobic chain length, it seems reasonable to expect that if the solubilization efficiency is expressed on a molar basis, it will increase with the hydrophilic chain length due to formation of larger micelles which could accommodate more solubilizate. On the other hand, if the solubilization efficiency is expressed in terms of number of moles solubilized per ethylene oxide equivalent, then it is expected to decrease as the hydrophilic chain increases.

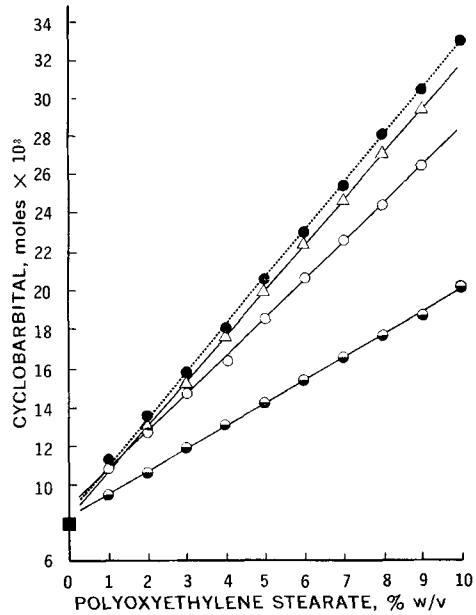


Figure 3—*Solubility of cyclobarbital in polyoxyethylene stearate solutions at 30.° Key: ■, solubility in water; ●, polyoxyethylene 30 stearate; △, polyoxyethylene 40 stearate; ○, polyoxyethylene 50 stearate, and ◒, polyoxyethylene 100 stearate.*

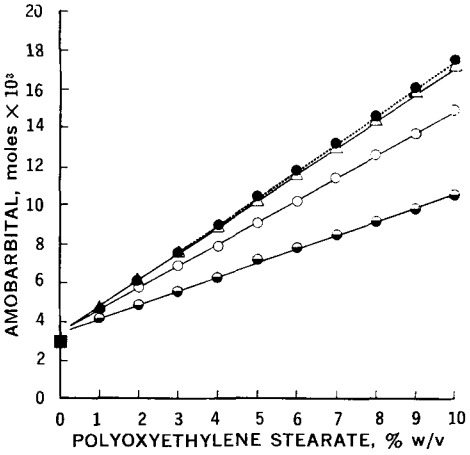


Figure 4—*Solubility of amobarbital in polyoxyethylene stearate solutions at 30°. Key: ■, solubility in water; ●, polyoxyethylene 30 stearate; △, polyoxyethylene 40 stearate; ○, polyoxyethylene 50 stearate; and ◒, polyoxyethylene 100 stearate.*

The effect of the chemical structure of the solubilizate on the degree of solubilization was determined by regarding the solubilization

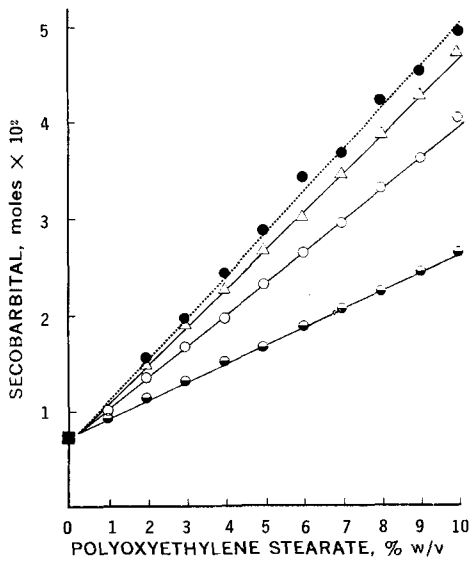


Figure 5—*Solubility of secobarbital in polyoxyethylene stearate solutions at 30°. Key: ■, solubility in water; ●, polyoxyethylene 30 stearate; △, polyoxyethylene 40 stearate; ○, polyoxyethylene 50 stearate; and ◒, polyoxyethylene 100 stearate.*

Table I—Solubilities and Partition Coefficients of Barbiturates in Polyoxyethylene Stearate Solutions at 30°

Drug	Surfactant	Solubility: mole Drug/ g. Surfactant × 10^4	Solubility: mole Drug/ Ethylene Oxide equiv.[a] × 10^2	Solubility: mole Drug/ mole Surfactant[b] × 10^2	Partition Coefficient K [micelles]/[water] × 10^{-2}
Barbital	Polyoxyethylene 30 stearate	1.95	1.07	31.2	4.04
	Polyoxyethylene 40 stearate	1.93	1.00	39.4	5.09
	Polyoxyethylene 50 stearate	1.60	0.79	39.8	5.15
	Polyoxyethylene 100 stearate	0.85	0.40	39.8	5.15
	Polysorbate 60[c]	—	—	25.1	3.17
Diallylbarbituric acid	Polyoxyethylene 30 stearate	1.13	0.62	18.2	10.89
	Polyoxyethylene 40 stearate	1.12	0.58	22.9	13.72
	Polyoxyethylene 50 stearate	0.96	0.47	23.9	14.32
	Polyoxyethylene 100 stearate	0.59	0.28	27.8	16.62
	Polysorbate 60[c]	—	—	17.3	10.48
Cyclobarbital	Polyoxyethylene 30 stearate	2.42	1.33	38.8	27.11
	Polyoxyethylene 40 stearate	2.24	1.17	45.7	31.99
	Polyoxyethylene 50 stearate	1.97	0.97	49.0	34.26
	Polyoxyethylene 100 stearate	1.18	0.55	55.1	38.53
	Polysorbate 60[c]	—	—	33.8	23.97
Amobarbital	Polyoxyethylene 30 stearate	1.42	0.78	22.8	43.29
	Polyoxyethylene 40 stearate	1.38	0.72	28.2	53.76
	Polyoxyethylene 50 stearate	1.15	0.56	28.5	54.36
	Polyoxyethylene 100 stearate	0.72	0.34	33.6	64.07
	Polysorbate 60[c]	—	—	—	—
Secobarbital	Polyoxyethylene 30 stearate	4.33	2.37	69.2	54.88
	Polyoxyethylene 40 stearate	4.01	2.09	81.9	65.03
	Polyoxyethylene 50 stearate	3.31	1.63	82.2	65.27
	Polyoxyethylene 100 stearate	1.88	0.88	87.8	69.71
	Polysorbate 60[c]	—	—	—	—

[a] Percentages of ethylene oxide per surfactant molecules are: polyoxyethylene 30 stearate, 80.0% w/w; polyoxyethylene 40 stearate, 84.5% w/w; polyoxyethylene 50 stearate, 89.5% w/w; and polyoxyethylene 100 stearate, 94.0% w/w (5). [b] Calculations are based on the following molecular weights: polyoxyethylene 30 stearate, 1604; polyoxyethylene 40 stearate, 2044; polyoxyethylene 50 stearate, 2484; and polyoxyethylene 100 stearate, 4684. [c] The molar solubilities and partition coefficients of the drugs in polysorbate 60 were obtained from *Reference 1*.

as a partition between a micellar phase and a water phase. The partition coefficient, K, associated with this process was calculated using the equation reported previously (1). Table I lists the values of K for the various barbiturates in the different polyoxyethylene stearates. The values of K for the same drugs in polysorbate 60, polyoxyethylene 20 sorbitan monostearate, is also included for comparison. The order (decreasing) of the partition coefficients for the mentioned barbiturates between the micellar pseudophase and the aqueous phase was: secobarbital > amobarbital > cyclobarbital > diallylbarbituric acid > barbital. This same order was found for the partition coefficients of the same barbiturates between the polysorbate micellar phase and the aqueous phase (1). It is also the order of their distribution coefficients between 1-octanol and water (8). As expected, the value of K is dependent on the lipophilic character, as well as the inductive effect of the substituents on the 5-position of the barbituric acid molecule. The extent of solubilization, as expressed by K, was found to be higher for the polyoxyethylene stearate solubilizers than for polysorbate 60. Both types of solubilizers have a stearate hydrophobic chain length but the former has more ethylene oxide units. The higher solubilizing power of the polyoxyethylene stearates is considered to be due to their larger size micelles.

The results obtained for these barbiturates indicate that their solubilization in the polyoxyethylene stearate solutions is essentially a micellar solubilization and similar in mechanism to their solubilization in polysorbate solutions. The solubilization mechanism appears to be essentially an inclusion within the micelles. For nonionic surfactants containing polyoxyethylene chains, the micelle may be considered to consist of two parts, an inner core of hydrocarbon tails and an outer shell of hydrated polyoxyethylene (9). The position of the solubilizate molecule inside the micelle cannot be determined with certainty from these results. However, it seems reasonable to expect that the lipophilic character of the substituents on the 5-position of the barbiturate molecule, as well as their influence on the rest of the molecule, will determine whether the solubilizate molecule is predominantly within the hydrocarbon core or the polyoxyethylene shell of the micelles.

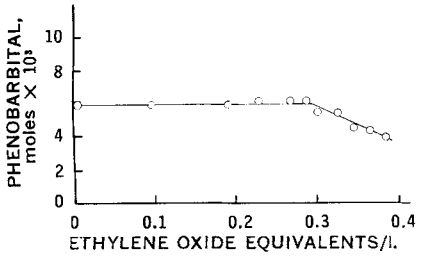


Figure 6—*Interaction of phenobarbital with polyoxyethylene 40 stearate at 30.°*

The interaction of phenobarbital with the polyoxyethylene stearate surfactants resulted in the formation of an insoluble precipitate. The effect of varying polyoxyethylene 40 stearate concentrations, expressed as ethylene oxide equivalents per liter, on the solubility of phenobarbital is shown in Fig. 6. The results are typical of the solubilization curves obtained with all the solubilizers studied. This phase diagram shows a plateau region, indicating that phenobarbital interacts strongly with the surfactant and forms an insoluble precipitate complex. The stoichiometric ratios of the phenobarbital–surfactant complex formed in the plateau region could be calculated from the phase diagrams (10). Analysis of this region shows that 1 molecule of phenobarbital reacts with 3 equivalents of ethylene oxide. This stoichiometric ratio of 1:3 was obtained for all the surfactants studied. Because of the heterogenicity of the surfactants, this value is regarded as an approximation.

Higuchi and Lach (10) found that while pentobarbital and barbital do not interact with polyethylene glycols, phenobarbital forms an insoluble complex with a stoichiometric ratio of 1:2 phenobarbital-PEG. Chakravarty *et al.* (11) showed that polyoxyethylene 40 stearate interacted with phenol and resorcinol to form insoluble complexes. Saito and Shinoda (12) reported that benzene mixes with the polyoxyethylene shell of polyoxyethylene nonylphenyl ethers and depresses the cloud points of their solution to below 0°. Such a depression results in turbidity and separation into two phases at any temperature above the cloud point. The strong interaction of phenobarbital with the surfactants is attributed to the presence of the aromatic ring in the molecule, the phenyl group causing an increased interaction between the barbiturate molecule and the polyoxyethylene chain of the surfactant resulting in the formation of an insoluble precipitate complex.

REFERENCES

(1) A. A. Ismail, M. Wafik Gouda, and M. M. Motawi, *J. Pharm. Sci.*, **59**, 220(1970).

(2) Atlas Powder Co. Catalog, ILD-102-3M-6-1965.
(3) J. J. Walker, R. S. Fischer, and J. J. McHugh, *Amer. J. Clin. Pathol.*, **18**, 451(1948).
(4) Paul Becher, in "Nonionic Surfactants," M. J. Schick, Ed., Marcel Dekker, New York, N. Y., 1967, chap. 15.
(5) F. W. Goodhart and A. N. Martin, *J. Pharm. Sci.*, **51**, 52 (1962).
(6) A. G. Mitchell and L. S. C. Wan, *ibid.*, **53**, 1467(1964).
(7) M. J. Schick, S. M. Atlas, and F. R. Eirich, *J. Phys. Chem.*, **66**, 1326(1962).
(8) C. Hansch and S. M. Anderson, *J. Med. Chem.*, **10**, 745 (1967).
(9) T. Nakagawa, in "Nonionic Surfactants," M. J. Schick, Ed., Marcel Dekker, New York, N. Y., 1967, chap. 17.
(10) T. Higuchi and J. L. Lach, *J. Amer. Pharm. Ass., Sci. Ed* **43**, 465(1954).
(11) D. Chakravarty, J. L. Lach, and S. M. Blaug, *Drug Stand.*, **25**, 137(1957).
(12) H. Saito and K. Shinoda, *J. Colloid Interface Sci.*, **24**, 10 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 23, 1970, from the **School of Pharmacy, University of Montana, Missoula, MT 59801*, and the *†College of Pharmacy, University of Alexandria, Alexandria, U.A.R.*

Accepted for publication May 22, 1970.

* To whom all inquiries should be directed.

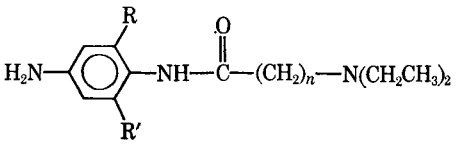
Potential Antiarrhythmic Agents II: Effects of Amide Reversal and *ortho*-Methylation on Activity of Procaine Amide

D. K. YUNG, M. M. VOHRA, and I. CHU

Abstract □ Substitution of a methyl group at one or both of the *ortho*-positions of the benzene ring in procaine amide and procaine provides analogs that are more active in prolonging the refractory period of isolated rabbit atria than procaine amide itself. These analogs, however, fail to abolish ouabain-induced ventricular and aconitine-induced atrial arrhythmias in cats. On the other hand, analogs like 2-diethylamino-4′-amino-2′,6′-dimethylacetanilide dihydrochloride monohydrate, 4-amino-*N*-(2-diethylaminoethyl)-2′,6′-dimethylbenzamide, 2-diethylaminoethyl 4-amino-2-methylbenzoate, and 2-diethylaminoethyl 4-amino-2,6-dimethylbenzoate produce a significant increase in the amount of ouabain required to elicit ectopic rhythm in cats when administered before the infusion of the glycoside. Of these four compounds, the last three also show local anesthetic activity in the corneal reflex test in rabbits. Reversal of the amide group in procaine amide significantly reduces the activity in prolonging the refractory period of cardiac tissue and does not seem to improve the antiarrhythmic activity of the parent compounds.

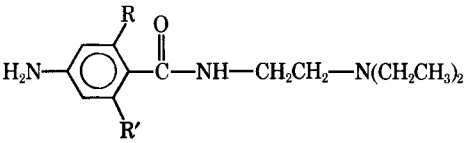
Keyphrases □ Antiarrhythmic agents, potential—synthesis □ Procaine amide activity—amide reversal, *ortho*-methylation, effects □ Structure-activity relationships—procaine amide derivatives □ IR spectrophotometry—identity

It is frequently noted that several pairs of compounds with analogous pharmacological activities can be obtained by reversing the position of the functional group. For example, a large increase in analgesic activity is reportedly caused by this type of reversal in the ester functional group of meperidine (1). As part of a continuing investigation on the structure–activity relationships of procaine amide (2), it was, therefore, considered of interest to determine whether this type of isosterism is possible in procaine amide. Accordingly, 3-diethylamino-4′-aminopropionanilide (I*a*) was synthesized. For comparative purposes, 2-diethylamino-4′-aminoacetanilide (I*b*), 3-diethylamino-4′-amino-2′,6′-dimethylpropionanilide (I*c*), and 2-diethylamino-4′-amino-2′,6′-dimethylacetanilide (I*d*) were also prepared. Compounds I*c* and I*d* can be regarded as analogs of lidocaine, which is useful clinically in the prevention and treatment of cardiac arrhythmias.



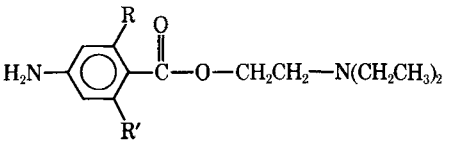
I*a*, $n = 2$; R and R′ = H
I*b*, $n = 1$; R and R′ = H
I*c*, $n = 2$; R and R′ = CH_3
I*d*, $n = 1$; R and R′ = CH_3

The fact that 4-amino-*N*-(2-diethylaminoethyl)-2-chlorobenzamide was 4 times as active as procaine amide in blocking atrial fibrillation in dogs (3) prompted the preparation of 4-amino-*N*-(2-diethylaminoethyl)-2-methylbenzamide (II*a*) and 4-amino-*N*-(2-diethylaminoethyl)-2,6-dimethylbenzamide (II*b*) in an attempt to study the effect on activity of substitution of one or two methyl groups on the benzene ring *ortho* to the amide linkage in procaine amide.



II*a*, R = CH_3; R′ = H
II*b*, R and R′ = CH_3

Two additional compounds, 2-diethylaminoethyl 4-amino-2-methylbenzoate (III*a*) and 2-diethylaminoethyl 4-amino-2,6-dimethylbenzoate (III*b*), were included in



III*a*, R = CH_3; R′ = H
III*b*, R and R′ = CH_3

(2) Atlas Powder Co. Catalog, ILD-102-3M-6-1965.
(3) J. J. Walker, R. S. Fischer, and J. J. McHugh, *Amer. J. Clin. Pathol.*, **18**, 451(1948).
(4) Paul Becher, in "Nonionic Surfactants," M. J. Schick, Ed., Marcel Dekker, New York, N. Y., 1967, chap. 15.
(5) F. W. Goodhart and A. N. Martin, *J. Pharm. Sci.*, **51**, 52 (1962).
(6) A. G. Mitchell and L. S. C. Wan, *ibid.*, **53**, 1467(1964).
(7) M. J. Schick, S. M. Atlas, and F. R. Eirich, *J. Phys. Chem.*, **66**, 1326(1962).
(8) C. Hansch and S. M. Anderson, *J. Med. Chem.*, **10**, 745 (1967).
(9) T. Nakagawa, in "Nonionic Surfactants," M. J. Schick, Ed., Marcel Dekker, New York, N. Y., 1967, chap. 17.
(10) T. Higuchi and J. L. Lach, *J. Amer. Pharm. Ass., Sci. Ed* **43**, 465(1954).
(11) D. Chakravarty, J. L. Lach, and S. M. Blaug, *Drug Stand.*, **25**, 137(1957).
(12) H. Saito and K. Shinoda, *J. Colloid Interface Sci.*, **24**, 10 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 23, 1970, from the **School of Pharmacy, University of Montana, Missoula, MT 59801*, and the *†College of Pharmacy, University of Alexandria, Alexandria, U.A.R.*

Accepted for publication May 22, 1970.

* To whom all inquiries should be directed.

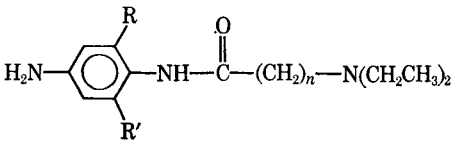
Potential Antiarrhythmic Agents II: Effects of Amide Reversal and *ortho*-Methylation on Activity of Procaine Amide

D. K. YUNG, M. M. VOHRA, and I. CHU

Abstract □ Substitution of a methyl group at one or both of the *ortho*-positions of the benzene ring in procaine amide and procaine provides analogs that are more active in prolonging the refractory period of isolated rabbit atria than procaine amide itself. These analogs, however, fail to abolish ouabain-induced ventricular and aconitine-induced atrial arrhythmias in cats. On the other hand, analogs like 2-diethylamino-4′-amino-2′,6′-dimethylacetanilide dihydrochloride monohydrate, 4-amino-*N*-(2-diethylaminoethyl)-2′,6′-dimethylbenzamide, 2-diethylaminoethyl 4-amino-2-methylbenzoate, and 2-diethylaminoethyl 4-amino-2,6-dimethylbenzoate produce a significant increase in the amount of ouabain required to elicit ectopic rhythm in cats when administered before the infusion of the glycoside. Of these four compounds, the last three also show local anesthetic activity in the corneal reflex test in rabbits. Reversal of the amide group in procaine amide significantly reduces the activity in prolonging the refractory period of cardiac tissue and does not seem to improve the antiarrhythmic activity of the parent compounds.

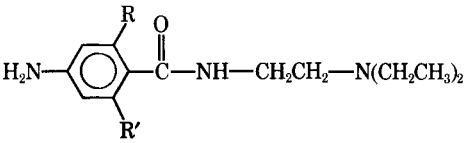
Keyphrases □ Antiarrhythmic agents, potential—synthesis □ Procaine amide activity—amide reversal, *ortho*-methylation, effects □ Structure-activity relationships—procaine amide derivatives □ IR spectrophotometry—identity

It is frequently noted that several pairs of compounds with analogous pharmacological activities can be obtained by reversing the position of the functional group. For example, a large increase in analgesic activity is reportedly caused by this type of reversal in the ester functional group of meperidine (1). As part of a continuing investigation on the structure–activity relationships of procaine amide (2), it was, therefore, considered of interest to determine whether this type of isosterism is possible in procaine amide. Accordingly, 3-diethylamino-4′-aminopropionanilide (I*a*) was synthesized. For comparative purposes, 2-diethylamino-4′-aminoacetanilide (I*b*), 3-diethylamino-4′-amino-2′,6′-dimethylpropionanilide (I*c*), and 2-diethylamino-4′-amino-2′,6′-dimethylacetanilide (I*d*) were also prepared. Compounds I*c* and I*d* can be regarded as analogs of lidocaine, which is useful clinically in the prevention and treatment of cardiac arrhythmias.



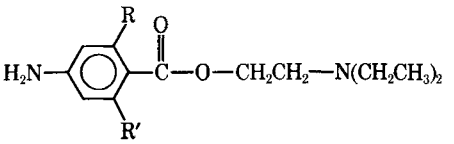
I*a*, $n = 2$; R and R′ = H
I*b*, $n = 1$; R and R′ = H
I*c*, $n = 2$; R and R′ = CH_3
I*d*, $n = 1$; R and R′ = CH_3

The fact that 4-amino-*N*-(2-diethylaminoethyl)-2-chlorobenzamide was 4 times as active as procaine amide in blocking atrial fibrillation in dogs (3) prompted the preparation of 4-amino-*N*-(2-diethylaminoethyl)-2-methylbenzamide (II*a*) and 4-amino-*N*-(2-diethylaminoethyl)-2,6-dimethylbenzamide (II*b*) in an attempt to study the effect on activity of substitution of one or two methyl groups on the benzene ring *ortho* to the amide linkage in procaine amide.



II*a*, R = CH_3; R′ = H
II*b*, R and R′ = CH_3

Two additional compounds, 2-diethylaminoethyl 4-amino-2-methylbenzoate (III*a*) and 2-diethylaminoethyl 4-amino-2,6-dimethylbenzoate (III*b*), were included in



III*a*, R = CH_3; R′ = H
III*b*, R and R′ = CH_3

Table I—3-Diethylamino-4′-amino-2′,6′-dimethylpropionanilide 2HCl (Ic) and Intermediates

Compound	Yield, %	M.p.	Solvent of Recrystn.	Formula	Anal., % Calcd.	Found
4-Nitro-2,6-dimethylaniline	90	161.5–162°[a]	Ethanol (50%)	$C_8H_{10}N_2O_2$	—	—
3-Chloro-4′-nitro-2′,6′-dimethylpropionanilide	69	218.5–220°	Ethanol	$C_{11}H_{13}ClN_2O_3$	C, 51.47 H, 5.10 N, 10.92	C, 51.55 H, 5.13 N, 10.74
3-Diethylamino-4′-nitro-2′,6′-dimethylpropionanilide	80	80–82°	*n*-Hexane–acetone	$C_{15}H_{23}N_3O_3$	C, 61.41 H, 7.90 N, 14.32	C, 61.40 H, 7.84 N, 14.30
3-Diethylamino-4′-nitro-2′,6′-dimethylpropionanilide picrate	72	174–176°	Methanol–acetone	$C_{21}H_{26}N_6O_{10}$	C, 48.27 H, 5.02	C, 48.30 H, 5.38
3-Diethylamino-4′-amino-2′,6′-dimethylpropionanilide 2HCl (Ic)	60	237–238°	Absolute ethanol	$C_{15}H_{27}Cl_2N_3O$	C, 53.57 H, 8.09	C, 53.34 H, 8.23
3-Diethylamino-4′-amino-2′,6′-dimethylpropionanilide dipicrate	68	207–209°	Methanol–acetone	$C_{27}H_{31}N_9O_{15}$	C, 44.93 H, 4.33	C, 45.00 H, 4.41

[a] Reported m.p., 163.5–164.5° (8).

Table II—4-Amino-*N*-(2-diethylaminoethyl)-2-methylbenzamide (IIa) and Intermediates

Compound	Yield, %	B.p.	Formula	Anal., % Calcd.	Found
4-Nitro-2-methylbenzoic acid	91	–[a]	—	—	—
4-Nitro-2-methylbenzoyl chloride	90	164–168°/18 mm.[b]	$C_8H_6ClNO_3$	C, 48.12 H, 3.03 N, 7.02	C, 48.40 H, 3.27 N, 6.86
4-Nitro-*N*-(2-diethylaminoethyl)-2-methylbenzamide	82	170–174°/0.1 mm.[c]	$C_{14}H_{21}N_3O_3$	C, 60.19 H, 7.58	C, 60.20 H, 7.67
4-Nitro-*N*-(2-diethylaminoethyl)-2-methylbenzamide picrate	84	–[d]	$C_{20}H_{24}N_6O_{10}$	C, 47.24 H, 4.76	C, 47.24 H, 4.86
4-Amino-*N*-(2-diethylaminoethyl)-2-methylbenzamide (IIa)	87	182°/0.25 mm.	$C_{14}H_{23}N_3O$	C, 67.45 H, 9.30 N, 16.86	C, 66.87 H, 9.05 N, 17.11

[a] M.p. 150–151°, reported m.p. 153° (9). [b] M.p. 33–34°. [c] M.p. 63–64°. [d] M.p. 162–164°, after recrystallization from methanol and acetone.

this study to determine whether or not steric protection of the ester group in procaine can produce active antiarrhythmic substances. The observation of activity in 2-diethylaminoethyl 2,3,5,6-tetramethylbenzoate (4) made the proposed structural modification appealing.

EXPERIMENTAL

Chemical Synthesis[1]

Compounds Ia (3-diethylamino-4′-aminopropionanilide HCl) and Ib (2-diethylamino-4′-aminoacetanilide HCl) were prepared according to the procedure reported by DiGangi (5) and Lofgren and Lundquist (6), respectively. The procedure of Dahlbom *et al.* (7) was followed in preparing Compound Ic (3-diethylamino-4′-amino-2′,6′-dimethylpropionanilide 2HCl) and 2-diethylamino-4′-amino-2′,6′-dimethylacetanilide. Compound Ic and its intermediates are new compounds whose physical data and yields are listed in Table I. The dihydrochloride salt of 2-diethylamino-4′-amino-2′,6′-dimethylacetanilide (Id) has not been reported previously.

2-Diethylamino-4′-amino-2′,6′-dimethylacetanilide 2HCl—Yield was 95%, m.p. 250–252°, after recrystallization from absolute ethanol.

Anal.—Calcd. for $C_{14}H_{21}N_3O \cdot 2HCl \cdot H_2O$: C, 49.41; H, 8.00; N, 12.35. Found: C, 49.41; H, 7.86; N, 12.28.

Compound IIa [4-amino-*N*-(2-diethylaminoethyl)-2-methylbenzamide] was synthesized by the reaction of 4-nitro-2-methylbenzoic acid, which was prepared according to the method of Peltier (9), with thionyl chloride, followed by condensation of the acid chloride with *N*,*N*-diethylethylenediamine and reduction of the resultant 4-nitro-*N*-(2-diethylaminoethyl)-2-methylbenzamide with iron-hydrochloric acid. Attempts to make the hydrochloride salt of Compound IIa failed because of the hygroscopic nature of the salt. The physical data and yields of Compound IIa and its intermediates are summarized in Table II.

The preparation of Compound IIb [4-amino-*N*-(2-diethylaminoethyl)-2,6-dimethylbenzamide] was analogous to that of Compound IIa. 4-Nitro-2,6-dimethylbenzoic acid was obtained by converting 4-nitro-2,6-dimethylaniline to 4-nitro-2,6-dimethylbenzonitrile *via* the Sandmeyer reaction, followed by acidic hydrolysis of the nitrile. Attempts to prepare the hydrochloride salt of Compound IIb were unsuccessful because of the hygroscopic nature of the salt. Compound IIb and its intermediates have been reported in the literature (10). The intermediate 4-nitro-*N*-(2-diethylaminoethyl)-2,6-dimethylbenzamide and Compound IIb are liquids; for purpose of identification, picrate salts were prepared.

4-Nitro-*N*-(2-diethylaminoethyl)-2,6-dimethylbenzamide Picrate—Yield was 85%, m.p. 189–191°, after recrystallization from acetone and methanol.

Anal.—Calcd. for $C_{21}H_{26}N_6O_{10}$: C, 48.27; H, 5.02. Found: C, 48.15, H, 5.43.

4-Amino-*N*-(2-diethylaminoethyl)-2,6-dimethylbenzamide Dipicrate—Yield was 90%, m.p. 200–203°, after recrystallization from acetone and methanol.

Anal.—Calcd. for $C_{27}H_{31}N_9O_{15}$: C, 44.93; H, 4.33. Found: C, 44.80; H, 4.51.

Compounds IIIa (2-diethylaminoethyl 4-amino-2-methylbenzoate) and IIIb (2-diethylaminoethyl 4-amino-2,6-dimethylbenzoate) were synthesized by a previously reported procedure (10). While Compound IIIb and its intermediates have appeared in the literature (10), Compound IIIa and its intermediates are new compounds. The physical data and yields of Compound IIIa and its intermediates are listed in Table III. Compounds IIIa and IIIb failed to form stable hydrochloride salts. Since 2-diethylaminoethyl 4-nitro-2,6-dimethylbenzoate and Compound IIIb are liquids, picrate salts were prepared.

[1] Melting points were taken with a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Elemental analyses were performed by Weiler and Strauss, Oxford, England. The absorption peaks of IR spectra of all compounds synthesized were as expected. IR spectra were recorded on a Perkin-Elmer model 237B spectrophotometer.

Table III—2-Diethylaminoethyl 4-Amino-2-methylbenzoate (III*a*) and Intermediates

Compound	Yield, %	B.p.	Formula	Anal., % Calcd.	Anal., % Found
4-Nitro-2-methylbenzoyl chloride			See Table II		
2-Diethylaminoethyl 4-nitro-2-methylbenzoate	82	122–124°/0.05 mm.	$C_{14}H_{20}N_2O_4$	C, 59.98 H, 7.19 N, 10.00	C, 60.21 H, 7.45 N, 9.64
2-Diethylaminoethyl 4-nitro-2-methylbenzoate picrate	62	—[a]	$C_{20}H_{23}N_5O_{11}$	C, 47.17 H, 4.55	C, 47.28 H, 4.75
2-Diethylaminoethyl 4-amino-2-methylbenzoate (III*a*)	98	146–150°/0.1 mm.	$C_{14}H_{22}N_2O_2$	C, 67.17 H, 8.86 N, 11.19	C, 67.15 H, 9.10 N, 11.26
2-Diethylaminoethyl 4-amino-2-methylbenzoate picrate	56	—[b]	$C_{20}H_{25}N_5O_9$	C, 50.10 H, 5.26	C, 49.66 H, 5.40

[a] M.p. 173.5–175°, after recrystallization from methanol and acetone. [b] M.p. 155–157°, after recrystallization from methanol and acetone.

Table IV—Effect of Compounds on the Maximum Stimulation Rates (MSR) of Isolated Rabbit Atria

Compound	Concn., mcg./ml.	Average Percent Depression, MSR	Activity
Procaine amide	10	10.4 ± 1.5	1
	20	15.1 ± 2.2	1
	30	19.8 ± 4.7	1
3-Diethylamino-4′-amino-propionanilide HCl (I*a*)	30	5.1 ± 1.0	0.3
2-Diethylamino-4′-amino-acetanilide HCl (I*b*)	30	5.4 ± 1.2	0.3
3-Diethylamino-4′-amino-2′,6′-dimethylpropion-anilide 2HCl (I*c*)	10	18.2 ± 4.4	1.8
	20	24.9 ± 2.5	1.7
	30	34.4 ± 4.7	1.7
2-Diethylamino-4′-amino-2′,6′-dimethylacetani-lide 2HCl H_2O (I*d*)	10	18.5 ± 2.8	1.8
	20	26.0 ± 3.6	1.7
	30	33.5 ± 5.3	1.7
4-Amino-*N*-(2-diethylamino-ethyl)-2-methylbenzamide (II*a*)	10	14.6 ± 1.1	1.4
	20	21.9 ± 3.4	1.5
	30	28.0 ± 2.6	1.4
4-Amino-*N*-(2-diethylamino-ethyl)-2,6-dimethyl-benzamide (II*b*)	10	17.1 ± 1.7	1.6
	20	30.7 ± 1.4	2.0
	30	36.3 ± 2.2	1.8
2-Diethylaminoethyl 4-amino-2-methylbenzoate (III*a*)	5	26.7 ± 2.0	—
	10	42.4 ± 6.8	4.1
2-Diethylaminoethyl 4-amino-2,6-dimethylbenzoate (III*b*)	5	23.4 ± 2.2	—
	10	48.0 ± 7.6	4.6

2-Diethylaminoethyl 4-Nitro-2,6-dimethylbenzoate Picrate—Yield was 96%, m.p. 169–171°, after recrystallization from acetone and methanol.

Anal.—Calcd. for $C_{21}H_{25}N_5O_{11}$: C, 48.18; H, 4.81; N, 13.37. Found: C, 48.17; H, 4.86; N, 13.70.

2-Diethylaminoethyl 4-Amino-2,6-dimethylbenzoate Dipicrate—Yield was 83%, m.p. 107–111°, after recrystallization from acetone and methanol.

Anal.—Calcd. for $C_{27}H_{30}N_8O_{16}$: C, 44.88; H, 4.18. Found: C, 45.14; H, 4.50.

Determination of pKa Values—An accurately weighed amount of the compound (about 120 mg.) was dissolved in a stoichiometric quantity of 0.1 *N* HCl or in water, and the solution was diluted to 200 ml. with freshly boiled distilled water. To the solution was added a known amount of 0.1 *N* NaOH. The pH of the solution was then determined by means of a pH meter. The pKa value of the compound was calculated, using the formula $pKa = pH - \log [B]/[BH^+]$.

Pharmacological Testing

For the biological testing, compounds that formed hydrochloride salts were dissolved in distilled water. The others were dissolved in a stoichiometric amount of 0.4 *N* HCl. All solutions were then buffered with potassium phosphate solution of pH 7.4, followed by dilution with normal saline to give the desired concentrations. Procaine amide and procaine were used in the form of hydrochloride salts.

Determination of Maximum Stimulation Rate (MSR)—The method of determination was that reported by Dawes (11). The rate and amplitude of contraction of the rabbit atria were recorded by a force displacement transducer on an E & M model DMP-4A physiograph. Four or more determinations were carried out, using at least two isolated atria for each compound. The average percent depression on the MSR for procaine amide was arbitrarily set to have an activity of 1 unit.

General Methods for Experiments on Cats—Cats of either sex, weighing from 2 to 4 kg., were anesthetized with pentobarbital[2] (35 mg./kg. i.p.). Artificial respiration was maintained throughout the experiment. Mean atrial blood pressure was recorded from the femoral artery with a Statham P-23C pressure transducer. Electrocardiograms were recorded on a standard Lead II. All recordings were made on a direct ink-writing Beckman type R dynograph. The solutions of the test compounds were injected intravenously through indwelling polyethylene catheters in the femoral vein, followed by a 2-ml. saline flush.

Prior to the testing for antiarrhythmic activity, the cardiotoxicity of Compounds I*c*, I*d*, II*a*, II*b*, III*a*, and III*b* was studied by injecting the compound intravenously at increasing doses of 1, 2.5, 5, and 10 mg./kg. at 10-min. intervals until ectopic rhythm appeared in the electrocardiograms. The total dose was taken as the maximum tolerated dose.

Determination of Activity in Termination of Ouabain-Induced Arrhythmias—The method used to induce arrhythmias with ouabain was that described by Raper and Wale (12). An initial loading dose of ouabain (40 mcg./kg.) was injected intravenously into cats; starting 30 min. later, doses of 10 mcg./kg. were repeated at 15-min. intervals until a persistent ventricular tachycardia was produced. When arrhythmias had persisted for 10 min., increasing doses of the compound to be tested were given every 10 min. until the maxi-

[2] Nembutal.

Table V—Arrhythmic and Lethal Doses of Ouabain[a] in Cats Pretreated with Compounds

Compound[b]	Approx. LD_{50}, mg./kg.	Dose of Compd,[c] mg./kg.	No. of Cats	Average Dose to Ectopic Rhythm, mcg./kg.	p[d]	Average Lethal Dose, mcg./kg.	p[d]
Control	—	—	3	104 ± 6	—	158 ± 10	—
Procaine amide	290	18.5	2	143 ± 15	<0.01	228 ± 1	<0.01
Ic	194	8.5	1	110	—	162	—
Id	192	18.5	3	131 ± 4	<0.01	212 ± 23	<0.05
IIa	186	18.5	1	118	—	168	—
IIb	209	8.5	3	129 ± 11	<0.05	215 ± 20	>0.01
IIIa	86	18.5	3	143 ± 5	<0.01	210 ± 27	<0.05
IIIb	67	8.5	3	128 ± 5	<0.01	193 ± 31	>0.05

[a] Ouabain administered by intravenous infusion at a rate of 5 mcg./kg./min. [b] Compounds administered by slow intravenous injection before ouabain infusion. [c] Maximum tolerated dose of the compounds. [d] p values compared with control.

mum tolerated dose was reached. The criterion used to define antiarrhythmic activity was the reversion to sinus rhythm for a period of not less than 30 min.

Determination of Activity in Prevention of Ouabain-Induced Arrhythmias—In this series of experiments the compound to be tested was injected intravenously in cats in one single dose. Ouabain was administered 10 min. later by intravenous infusion at the rate of 5 mcg./kg./min. The amount of ouabain required to produce ectopic rhythm and death was determined.

Determination of Activity in Termination of Aconitine-Induced Arrhythmias—Atrial arrhythmias were produced in cats according to the procedure described by Schmid and Hanna (13). A 0.05% solution of aconitine nitrate in normal saline was used. Compounds Id and IIIa were administered intravenously at the rate of 1 mg./kg./min., and Compounds IIb and IIIb were administered at the rate of 0.5 mg./kg./min.

Determination of Surface Anesthesia in Rabbits—Albino rabbits of either sex, weighing 2 to 3 kg., were used. The eyelashes of the rabbit were clipped off; into each conjunctival sac was instilled 0.5 ml. of a 2% solution of the test compound. Normal saline solution was used as a control. The corneal reflex was elicited by touching the cornea with a feather five times at 2, 5, 10, 15, 20, 30, and 35 min. after the compound was applied. The degree of anesthetic activity was expressed in terms of percent anesthesia. For example, a score of 50 failures of corneal reflex out of a possible maximum of 120 gave 41.7% anesthesia.

Acute Toxicity Studies in Mice—Male albino mice, weighing 20 to 35 g., were used in the tests. Compounds were injected intraperitoneally. The median lethal dose (LD_{50}) for each compound was calculated by the method of Litchfield and Wilcoxon (14) and was based on 1 day's observations.

RESULTS AND DISCUSSION

Results in Table IV indicate that although Compound Ia showed some activity in prolonging the refractory period of isolated rabbit atria at a concentration of 30 mcg./ml., the activity, being only one-third of the activity of procaine amide, was hardly significant. Shortening the hydrocarbon chain length between the carbonyl carbon and the terminal amino nitrogen atoms in Compound Ia failed to improve the activity of the parent compound. This suggests that reversing the amide functional group in procaine amide does not improve the antiarrhythmic activity of the compound.

The activity of procaine amide to depress the MSR of isolated rabbit atria was increased significantly ($p < 0.05$) when one or two methyl groups were substituted at the *ortho*-positions of the benzene ring. This information led to the synthesis of the two lidocaine analogs, Compounds Ic and Id. These two compounds had an activity of about 1.7 times that of procaine amide in depressing the MSR. Compared with the corresponding nonmethylated compounds, Ia and Ib, the methylated ones were at least 5 times more active.

In view of the favorable effect of the methyl substituents, it was considered of interest to modify the structure of procaine in the same fashion. Accordingly, Compounds IIIa and IIIb were prepared. When tested on isolated rabbit atria, both compounds demonstrated marked depressant action on the force and rate of contraction of the atria at a concentration of 30 mcg./ml. Determinations of MSR were, therefore, carried out at lower concentrations, 5 and 10 mcg./ml. Results showed that Compounds IIIa and IIIb were about 4 times more active than procaine amide.

Since Compounds Ic, Id, IIa, IIb, IIIa, and IIIb showed significant prolongation of the refractory period of isolated rabbit atria, they were tested for their ability to antagonize or prevent ouabain-induced arrhythmias. Prior to these experiments, the maximum tolerated dose of the compounds was determined. For Compounds Ic, IIb, and IIIb, the maximum tolerated dose was found to be 8.5 mg./kg.; for Compounds IIa and IIIa, the maximum was 18.5 mg./kg. It appeared that compounds with a methyl substituent at both *ortho*-positions of the benzene ring had a higher cardiotoxicity, as indicated by the lower maximum tolerated dose value, than those that were only mono-*ortho* substituted.

None of the compounds tested appeared to show any activity in terminating arrhythmias induced by ouabain. To make certain that the animals were not refractory, procaine amide was given after each experiment in doses of 28.5 to 38.5 mg./kg., and this resulted in the successful conversion to sinus rhythm. It should be noted, however, that procaine amide was also ineffective at doses of 8.5 and 18.5 mg./kg.

The effectiveness of the compounds in preventing ouabain-induced arrhythmias was determined by the increase in the amount of the glycoside needed to evoke ectopic rhythm, as indicated in the electrocardiograms by three successive QRS complexes not clearly related to a preceding P wave (15), and to cause the death of the animal. Results of the determinations are listed in Table V. On a weight basis, the di-*ortho*-methyl-substituted procaine amide analog (IIb) showed a higher prophylactic activity against arrhythmias than procaine amide, whereas the monosubstituted analog (IIa) did not appear to have any activity at all. The corresponding analogs of procaine, Compounds IIIa and IIIb, also showed protective action against ouabain-induced arrhythmias. Again, on a weight basis, the di-*ortho*-methyl-substituted compound (IIIb) was more active than the monosubstituted analog (IIIa).

After Compounds Id, IIb, IIIa, and IIIb were found to be active prophylactically against ventricular arrhythmias caused by ouabain, it was decided to test them for activity in abolishing established atrial arrhythmias induced by aconitine. None of the compounds showed any activity.

The effects of Compounds Ic, Id, IIa, IIb, IIIa, and IIIb on blood pressure were studied. The compounds did not demonstrate any significant effect on the blood pressure, although a transient lowering of blood pressure was observed with Compounds IIb and IIIa at cumulative doses of 6 mg./kg.

Table VI—Local Anesthetic Activity of Compounds and Their Ionization Constant (H_2O, 25°)

Compound	No. of Tests	Average Percent Anesthesia	pKa
Saline (control)	–	—	—
Procaine	6	34.6	8.86[a]
Id	3	Inactive	8.24
IIb	3	54.1	8.68
IIIa	2	41.3	8.80
IIIb	3	41.7	8.64

[a] Reported pKb value, 5.2 (16).

Since many antiarrhythmic agents seem to possess some degree of local anesthetic action, those compounds, namely, Id, IIb, IIIa, and IIIb, were tested for local anesthetic activity. Table VI is the summary of the results of the testing. Due to the limited number of tests, the data in this table were not analyzed statistically to determine if the average percent anesthesia of the compounds was significantly higher than that of procaine. Therefore, the results should be treated qualitatively rather than quantitatively. It is not surprising that Compounds IIb, IIIa, and IIIb showed local anesthetic activity, because in addition to structural similarity these compounds had a pKa value very close to that of procaine.

REFERENCES

(1) R. H. K. Foster and A. J. Carman, *J. Pharmacol. Exp. Ther.*, **91**, 195(1947).

(2) D. K. Yung, L. G. Chatten, and D. P. MacLeod, *J. Pharm. Sci.*, **57**, 2073(1968).

(3) D. B. Reisner and M. D. Cordasco, *J. Org. Chem.*, **23**, 1403 (1958).

(4) R. B. Arora, V. N. Sharma, and B. R. Madan, *J. Pharm. Pharmacol.*, **8**, 323(1956).

(5) F. E. DiGangi, *J. Amer. Pharm. Ass., Sci. Ed.*, **44**, 135(1955).

(6) N. Lofgren and B. Lundquist, *Svensk Kem. Tid.*, **58**, 206 (1946); through *Chem. Abstr.*, **43**, 10246(1949).

(7) R. Dahlbom, C. Tegner, and N. Willman, *Acta Chem. Scand.*, **13**, 1145(1959).

(8) B. M. Wepster, *Recueil*, **73**, 809(1954).

(9) D. Peltier, *C. R.*, **235**, 1660(1952); through *Chem. Abstr.*, **48**, 1311d(1954).

(10) British pat., Laaketehdas Orion Oy., 896,720; through *Chem. Abstr.*, **57**, 11113g(1962).

(11) G. S. Dawes, *Brit. J. Pharmacol.*, **1**, 90(1946).

(12) C. Raper and J. Wale, *Eur. J. Pharmacol.*, **4**, 1(1968).

(13) J. R. Schmid and C. Hanna, *J. Pharmacol. Exp. Ther.*, **156**, 331(1967).

(14) T. J. Litchfield, Jr., and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96**, 99(1949).

(15) A. K. Reynolds and M. L. Horne, *Can. J. Physiol. Pharmacol.*, **47**, 165(1969).

(16) N. A. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 219.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 18, 1970, from the *College of Pharmacy, Faculty of Health Professions*, and the *Department of Pharmacology, Faculty of Medicine, Dalhousie University, Halifax, N. S., Canada.*

Accepted for publication April 30, 1970.

Abstracted from a thesis submitted by I. Chu in partial fulfillment of the Master of Science degree requirements.

This work was supported by the Medical Research Council of Canada *via* Grant MA-3081, and by Smith Kline & French Inter-American Corporation (Canada).

In Vivo Evaluation of Absorption and Excretion of Pentylenetetrazol-10-^{14}C from Sustained-Release and Nonsustained-Release Tablets

WILLIAM R. EBERT*, ROBERT W. MORRIS*, SUSAN G. ROWLES†, HENRY T. RUSSELL‡, GORDON S. BORN§, and JOHN E. CHRISTIAN§

Abstract □ Sustained-release and nonsustained-release tablets containing pentylenetetrazol-10-^{14}C were administered orally to human volunteers. The levels of the drug and/or its labeled metabolites in the plasma and urine were determined by liquid scintillation counting. These data showed that the sustained-release tablets provided a consistent plasma level of ^{14}C for about 12 hr. and that the drug and/or its labeled metabolites were excreted in the urine at a fairly constant rate during this period. The nonsustained-release tablets given in divided doses resulted in three separate peak plasma-^{14}C levels and a urinary excretion pattern similar to that of the sustained-release tablet. A single dose of the nonsustained-release tablet was followed by a peak plasma-^{14}C level, which decreased during the 12 hr. after administration, and by a fairly constant rate of urinary excretion of ^{14}C during this period.

Keyphrases □ Pentylenetetrazol-10-^{14}C—absorption, excretion □ Tablets, sustained-, nonsustained-release—pentylenetetrazol-10-^{14}C □ Absorption, excretion—pentylenetetrazol-10-^{14}C from sustained-, nonsustained-release tablets □ Scintillometry—analysis

Sustained-release[1] and nonsustained-release tablets containing pentylenetetrazol and niacin in common therapeutic dosages have been evaluated for their *in vivo* performance in humans by following niacin–plasma levels (1). This evaluation was accomplished by using ^{14}C-labeled niacin in the tablets and determining the plasma and urine levels of niacin-^{14}C and/or its labeled metabolites subsequent to oral administration of the tablets. The results of this study showed that after ingestion of sustained-release tablets, the plasma level of niacin-^{14}C and/or its labeled metabolites was sustained for a 12-hr. period. In contrast, three doses of nonsustained-release tablets, administered at 4-hr. intervals, resulted in three peak plasma levels. The drug excretion patterns observed after both dosage regimens were similar.

Because of the various chemical, pharmacological, and metabolic differences between niacin and pentylenetetrazol, different absorption and excretion patterns for the two drugs were expected. The present paper reports a study of the absorption and excretion patterns characteristic of pentylenetetrazol administered orally, combined with niacin, in both sustained-release and nonsustained-release dosage forms. Pentylenetetrazol-10-^{14}C was used in this study to permit determination of these patterns by radiotracer techniques similar to those originally reported by Rosen and Swintosky (2) for following the appearance of a drug in human plasma and urine.

[1] Geroniazol TT, Philips Roxane Laboratories, Columbus, Ohio.

Since many antiarrhythmic agents seem to possess some degree of local anesthetic action, those compounds, namely, Id, IIb, IIIa, and IIIb, were tested for local anesthetic activity. Table VI is the summary of the results of the testing. Due to the limited number of tests, the data in this table were not analyzed statistically to determine if the average percent anesthesia of the compounds was significantly higher than that of procaine. Therefore, the results should be treated qualitatively rather than quantitatively. It is not surprising that Compounds IIb, IIIa, and IIIb showed local anesthetic activity, because in addition to structural similarity these compounds had a pKa value very close to that of procaine.

REFERENCES

(1) R. H. K. Foster and A. J. Carman, *J. Pharmacol. Exp. Ther.*, **91**, 195(1947).

(2) D. K. Yung, L. G. Chatten, and D. P. MacLeod, *J. Pharm. Sci.*, **57**, 2073(1968).

(3) D. B. Reisner and M. D. Cordasco, *J. Org. Chem.*, **23**, 1403 (1958).

(4) R. B. Arora, V. N. Sharma, and B. R. Madan, *J. Pharm. Pharmacol.*, **8**, 323(1956).

(5) F. E. DiGangi, *J. Amer. Pharm. Ass., Sci. Ed.*, **44**, 135(1955).

(6) N. Lofgren and B. Lundquist, *Svensk Kem. Tid.*, **58**, 206 (1946); through *Chem. Abstr.*, **43**, 10246(1949).

(7) R. Dahlbom, C. Tegner, and N. Willman, *Acta Chem. Scand.*, **13**, 1145(1959).

(8) B. M. Wepster, *Recueil*, **73**, 809(1954).

(9) D. Peltier, *C. R.*, **235**, 1660(1952); through *Chem. Abstr.*, **48**, 1311d(1954).

(10) British pat., Laaketehdas Orion Oy., 896,720; through *Chem. Abstr.*, **57**, 11113g(1962).

(11) G. S. Dawes, *Brit. J. Pharmacol.*, **1**, 90(1946).

(12) C. Raper and J. Wale, *Eur. J. Pharmacol.*, **4**, 1(1968).

(13) J. R. Schmid and C. Hanna, *J. Pharmacol. Exp. Ther.*, **156**, 331(1967).

(14) T. J. Litchfield, Jr., and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96**, 99(1949).

(15) A. K. Reynolds and M. L. Horne, *Can. J. Physiol. Pharmacol.*, **47**, 165(1969).

(16) N. A. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 219.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 18, 1970, from the *College of Pharmacy, Faculty of Health Professions*, and the *Department of Pharmacology, Faculty of Medicine, Dalhousie University, Halifax, N. S., Canada.*

Accepted for publication April 30, 1970.

Abstracted from a thesis submitted by I. Chu in partial fulfillment of the Master of Science degree requirements.

This work was supported by the Medical Research Council of Canada *via* Grant MA-3081, and by Smith Kline & French Inter-American Corporation (Canada).

In Vivo Evaluation of Absorption and Excretion of Pentylenetetrazol-10-^{14}C from Sustained-Release and Nonsustained-Release Tablets

WILLIAM R. EBERT*, ROBERT W. MORRIS*, SUSAN G. ROWLES†, HENRY T. RUSSELL‡, GORDON S. BORN§, and JOHN E. CHRISTIAN§

Abstract □ Sustained-release and nonsustained-release tablets containing pentylenetetrazol-10-^{14}C were administered orally to human volunteers. The levels of the drug and/or its labeled metabolites in the plasma and urine were determined by liquid scintillation counting. These data showed that the sustained-release tablets provided a consistent plasma level of ^{14}C for about 12 hr. and that the drug and/or its labeled metabolites were excreted in the urine at a fairly constant rate during this period. The nonsustained-release tablets given in divided doses resulted in three separate peak plasma-^{14}C levels and a urinary excretion pattern similar to that of the sustained-release tablet. A single dose of the nonsustained-release tablet was followed by a peak plasma-^{14}C level, which decreased during the 12 hr. after administration, and by a fairly constant rate of urinary excretion of ^{14}C during this period.

Keyphrases □ Pentylenetetrazol-10-^{14}C—absorption, excretion □ Tablets, sustained-, nonsustained-release—pentylenetetrazol-10-^{14}C □ Absorption, excretion—pentylenetetrazol-10-^{14}C from sustained-, nonsustained-release tablets □ Scintillometry—analysis

Sustained-release[1] and nonsustained-release tablets containing pentylenetetrazol and niacin in common therapeutic dosages have been evaluated for their *in vivo* performance in humans by following niacin–plasma levels (1). This evaluation was accomplished by using ^{14}C-labeled niacin in the tablets and determining the plasma and urine levels of niacin-^{14}C and/or its labeled metabolites subsequent to oral administration of the tablets. The results of this study showed that after ingestion of sustained-release tablets, the plasma level of niacin-^{14}C and/or its labeled metabolites was sustained for a 12-hr. period. In contrast, three doses of nonsustained-release tablets, administered at 4-hr. intervals, resulted in three peak plasma levels. The drug excretion patterns observed after both dosage regimens were similar.

Because of the various chemical, pharmacological, and metabolic differences between niacin and pentylenetetrazol, different absorption and excretion patterns for the two drugs were expected. The present paper reports a study of the absorption and excretion patterns characteristic of pentylenetetrazol administered orally, combined with niacin, in both sustained-release and nonsustained-release dosage forms. Pentylenetetrazol-10-^{14}C was used in this study to permit determination of these patterns by radiotracer techniques similar to those originally reported by Rosen and Swintosky (2) for following the appearance of a drug in human plasma and urine.

[1] Geroniazol TT, Philips Roxane Laboratories, Columbus, Ohio.

Table I—*In Vitro* Determination of Cumulative Pentylenetetrazol and Niacin Release from Sustained-Release Tablets

Time, hr.	Mean % Pentylenetetrazol Released ± *SD* — Chemical Assay	Mean % Pentylenetetrazol Released ± *SD* — ^{14}C Assay	Mean % Niacin Released ± *SD*
1[a]	40.5 ± 0.9[b]	41.8 ± 0.6[b]	30.8 ± 0.2[c]
4[d]	69.9 ± 1.9[e]	73.8 ± 1.0[e]	58.0 ± 2.0[f]
8[d]	89.8 ± 0.3[g]	92.9 ± 0.7[g]	77.8 ± 1.0[h]

[a] Mean of six tablets; simulated gastric solution test fluid. [b] Established limit for commercial forms is 40.0 ± 3.0%. [c] Established limit for commercial forms is 30.0 ± 5.0%. [d] Mean of three tablets; simulated intestinal solution test fluid. [e] Established limit for commercial forms is 73.0 ± 5.0%. [f] Established limit for commercial forms is 57.5 ± 7.5%. [g] Established limit for commercial forms is 92.5 ± 7.5%. [h] Established limit for commercial forms is 82.5 ± 7.5%.

EXPERIMENTAL

Radioactive Pentylenetetrazol—Pentylenetetrazol-10-^{14}C with a specific activity of 17.8 μc./mg., as synthesized[2] by Stiver, and with radiochemical purity established by TLC (3) was diluted (4) with nonradioactive pentylenetetrazol NF XII. Three separate dilutions of pentylenetetrazol-^{14}C were prepared, and the resulting specific activities were 23.4 μc./300 mg., 18.5 μc./100 mg., and 9.3 μc./100 mg. Each dilution was prepared by dissolving pentylenetetrazol-10-^{14}C and pentylenetetrazol NF XII in methanol (99.85%) with stirring. The methanol was evaporated over a steam bath. The pentylenetetrazol-^{14}C thus obtained was then cooled to room temperature and dried in a vacuum desiccator at 0.05 mm. at room temperature for 6 hr.

Radioactive Tablets—Three different compressed tablet formulations were manufactured from the pentylenetetrazol-^{14}C dilutions as follows.

Formula A—Sustained-release tablets, of the insoluble matrix type, from which the active ingredients are slowly leached; identical to commercial forms,[1] with the exception of the use of pentylenetetrazol-^{14}C. These tablets contained pentylenetetrazol-^{14}C, 300 mg. (equivalent to 23.4 μc. of ^{14}C); niacin, 150 mg.; fatty substances; and inert excipients.

Formula B—Nonsustained-release tablets containing pentylenetetrazol-^{14}C, 100 mg. (equivalent to 18.5 μc. of ^{14}C); niacin, 50 mg.; and inert excipients.

Formula C—Nonsustained-release tablets containing pentylenetetrazol-^{14}C, 100 mg. (equivalent to 9.3 μc. of ^{14}C); niacin, 50 mg.; and inert excipients.

Quality Control—Tablets were selected at random from all three tablet formulations; they were subjected to routine quality control tests to establish the conformance of the pentylenetetrazol-^{14}C-containing sustained-release tablets to the commercial forms and to determine that the physical and chemical characteristics of the nonsustained-release tablets were satisfactory with reference to commercially available tablets of similar composition. Tablets were tested for pentylenetetrazol content, niacin content, weight, thickness, and hardness. The disintegration time of the nonsustained-release tablets was determined by the USP Tablet Disintegration Test, and the *in vitro* release rate was determined for the sustained-release tablets.

***In Vitro* Release Rate Tests**—Before the sustained-release tablets were administered to human subjects, the *in vitro* release rates of pentylenetetrazol and niacin were determined to establish conformance of the radioactive tablets to commercial forms. The USP Tablet Disintegration Test Apparatus (5) was used to determine the *in vitro* release rates as follows. Six tablets were chosen at random and tested individually by placing one tablet in one of the six tubes of the basket-assembly, which was then immersed in 600 ml. of simulated gastric fluid T.S. (6) at 37 ± 1°. After operation of the apparatus for 1 hr., the basket was removed, rinsed, and transferred immediately to 600 ml. of simulated intestinal fluid T.S. (7) at 37 ± 1° in which the apparatus was operated for 7 hr.

At the end of the 1st hour, samples of the gastric test fluid were assayed for pentylenetetrazol by both the liquid scintillation counting technique and by gravimetric analysis. In the latter procedure, an aliquot of the test fluid was saturated with ammonium sulfate and the pentylenetetrazol quantitatively extracted with carbon tetrachloride. After evaporation of the solvent, the residue was dissolved in a small volume of ether and evaporated to dryness; the process then was repeated. The weight of the residue was determined after drying in a vacuum at room temperature to constant weight. The niacin was quantitated by UV spectrophotometry with a correction for the background absorbance due to the gastric test fluid. Samples of the intestinal test fluid were withdrawn at 4 and 8 hr. after the start of the test and were assayed similarly after acidification of the release solutions.

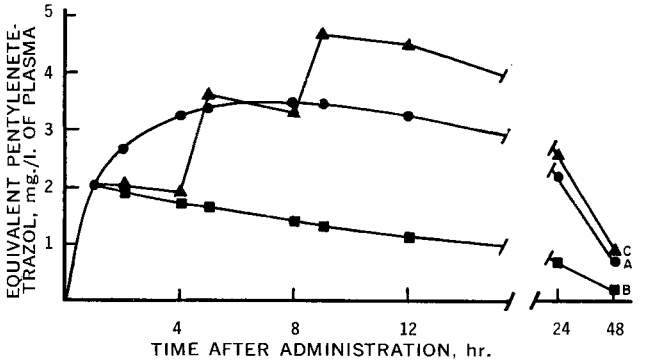


Figure 1—*Average plasma levels of equivalent pentylenetetrazol following oral administration of pentylenetetrazol-^{14}C tablets. Group A: One sustained-release tablet. Group B: One nonsustained-release tablet. Group C: One nonsustained-release tablet at 0, 4, and 8 hr. For clarity, standard deviations are not shown on the curves but are given in Table II.*

***In Vivo* Protocol**—Twelve healthy adult human volunteers, determined by medical history and physical examinations to be free of any disorders associated with abnormal absorption or excretion patterns, were used in this study. The subjects were all Caucasian males, ranging in age from 25 to 34 yr. and weighing between 72.3 and 102.8 kg. Plasma and urine samples were obtained from all subjects before the experiment began for later use in background correction of sample assays. All subjects fasted for 12 hr. prior to the experiment and for 5 hr. after it began.

The 12 volunteers were assigned experimental subject numbers at random. Each of four subjects (Group A: Subjects 1, 2, 3, and 4) was given one tablet of Formula A, the sustained-release dosage form. Each of four other subjects (Group B: Subjects 5, 6, 7, and 8) was given one tablet of Formula B, a nonsustained-release tablet. Each of four additional subjects (Group C: Subjects 9, 10, 11, and 12) was given one tablet of Formula C, a nonsustained-release tablet, at time zero and again at 4 and 8 hr. after time zero. All tablets were ingested with water.

Ten-milliliter blood samples were withdrawn from all subjects at 1, 2, 4, 5, 8, 9, 12, 24, and 48 hr. after time zero. All blood samples were treated with 20 mg. of disodium EDTA, and the plasma was immediately separated by centrifugation and frozen until assayed. Total urinary collections were made for all subjects during the following intervals after time zero: 0–3, 3–6, 6–9, 9–12, 12–24, 24–36, 36–48, and 48–72 hr. The urine was frozen until analyzed.

Assay of ^{14}C—Liquid scintillation counting techniques were employed to determine the quantity of ^{14}C in each plasma and urine sample. In a preliminary experiment, replicate plasma samples were prepared for counting by three different methods, using Insta-Gel emulsifier,[3] hyamine hydroxide[4] solution, and perchloric acid. All three methods gave satisfactory results, but the Insta-Gel emulsifier system was determined most suitable on the basis of its simplicity and low quenching characteristics (counting efficiencies of approximately 80% were obtained).

Remaining plasma samples were prepared for counting as follows. Exactly 1 ml. of plasma was slowly added to a counting vial containing 10 ml. of emulsifier (Insta-Gel). The vial was tightly capped and allowed to remain at room temperature for 20 hr., with

[2] Synthesis conducted at the Bionucleonics and Medicinal Chemistry Departments, Purdue University, Lafayette, Ind.

[3] Packard Instrument Co., Inc., Downers Grove, Ill.

[4] *p*-(Diisobutyl-cresoxyethoxy ethyl)dimethylbenzylammonium hydroxide.

Table II—Plasma Levels of Equivalent Pentylenetetrazol following Oral Administration of Pentylenetetrazol-^{14}C Tablets

Time after Administration, hr.	Mean[a] Equivalent Plasma Pentylenetetrazol,[b] mg./l. ± *SD*		
	Group A[c]	Group B[d]	Group C[e]
1	2.1 ± 0.3	2.1 ± 0.3	2.1 ± 0.2
2	2.7 ± 0.3	2.0 ± 0.2	2.0 ± 0.2
4	3.2 ± 0.3	1.7 ± 0.1	1.8 ± 0.2
5	3.4 ± 0.3	1.7 ± 0.1	3.6 ± 0.9
8	3.5 ± 0.2	1.4 ± 0.0	3.3 ± 0.4
9	3.5 ± 0.2	1.3 ± 0.1	4.7 ± 0.5
12	3.2 ± 0.3	1.1 ± 0.1	4.5 ± 0.5
24	2.2 ± 0.4	0.7 ± 0.1	2.7 ± 0.6
48	0.7 ± 0.1	0.2 ± 0.1	0.9 ± 0.4

[a] Mean of four subjects. [b] Any ^{14}C-labeled metabolites have been equated to the administered pentylenetetrazol-^{14}C. [c] Group A: One sustained-release tablet containing 300 mg. of pentylenetetrazol-^{14}C. [d] Group B: One nonsustained-release tablet containing 100 mg. of pentylenetetrazol-^{14}C. [e] Group C: One nonsustained-release tablet containing 100 mg. of pentylenetetrazol-^{14}C at 0, 4, and 8 hr.

occasional shaking. Upon cooling to 4°, a viscous one-phase system was obtained.

Urine samples were prepared for counting by adding 1.0-ml. aliquots to 15 ml. of a scintillation solution containing 2,5-diphenyloxazole (PPO), 10.0 g.; naphthalene, 80.0 g.; *p*-xylene, 143 ml.; *p*-dioxane, 429 ml., and a sufficient quantity of 2-ethoxyethanol to make 1 l.

All samples were cooled to 4° and were counted at that temperature in a Packard Tri-Carb liquid scintillation spectrometer equipped with bialkali photomultiplier tubes. The discriminators were set at 50 and 900, and the gain was adjusted to optimize the counting rate. All samples were counted for a length of time sufficient to assure counting errors less than 5% (at the 95% confidence level) in all cases and less than 1% for the majority of the samples.

All sample count rates were corrected for background and converted to absolute count rates by the internal standardization method of quench correction. The results of the sample assays are expressed in terms of "equivalent pentylenetetrazol," thus equating any ^{14}C-labeled metabolites to the administered pentylenetetrazol-^{14}C, and assuming that the specific activities of all labeled compounds present in the urine and plasma are identical to the specific activity of the administered pentylenetetrazol-^{14}C.

RESULTS AND DISCUSSION

Tablet Assays—The *in vitro* release rate data for the sustained-release tablets (Formula A) are given in Table I. These data indicate that the radioactive sustained-release tablets met the specifications of the corresponding commercial form.

The data for quality control tests for total pentylenetetrazol, total niacin, weight, thickness, hardness, and disintegration time are not summarized in this paper but showed that all tablets tested conformed to corresponding commercially available forms.

Plasma Data—The average plasma levels of equivalent pentylenetetrazol are plotted for each test group in Fig. 1. This graph shows that the plasma levels of the parent compound and/or its labeled metabolites were identical (±0.01 mg./l.) for all three groups 1 hr. after drug administration. Plasma concentrations of equivalent pentylenetetrazol gradually rose during the 1–4-hr. period after administration of the sustained-release tablet and then remained constant (3.2–3.5 mg./l.) until about the 12th hour. However, during the 1–4-hr. period following administration of the nonsustained-release tablet, plasma-^{14}C levels gradually decreased. Typical rises and falls in plasma levels of equivalent pentylenetetrazol were observed following the repeated administration of the nonsustained-release tablet. The plasma level of equivalent pentylenetetrazol decreased continually after the peak seen 1 hr. after single-dose administration of the nonsustained-release tablet.

Table II gives the mean plasma levels of equivalent pentylenetetrazol and the standard deviations for all three groups at the various times studied. Student *t* values (8) were determined to compare the mean plasma levels of ^{14}C of the different groups at various times. At the 99% confidence level (p = 0.01), there were no statistically significant differences between the mean plasma levels of any

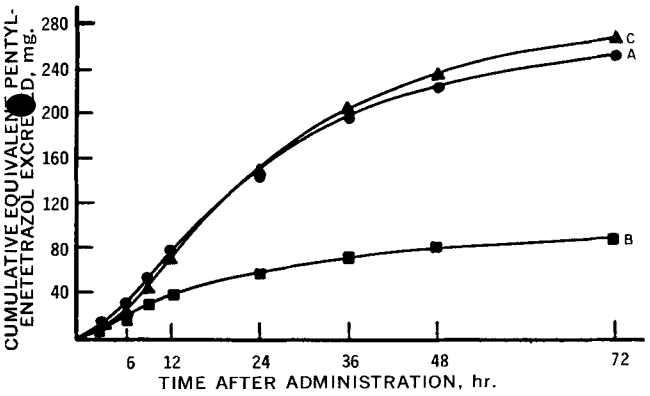


Figure 2—*Cumulative average urinary excretion of equivalent pentylenetetrazol following oral administration of pentylenetetrazol-^{14}C tablets. Group A: One sustained-release tablet. Group B: One nonsustained-release tablet. Group C: One nonsustained-release tablet at 0, 4, and 8 hr. For clarity, standard deviations are not shown on the curves but are given in Table III for the noncumulative excretion data. (The value of Group C, 24–36-hr. collection, includes an estimate of the amount of pentylenetetrazol present in a quantity of urine which was voided but not collected for analysis. (One subject forgot to collect one urine sample voided during this interval.)*

two groups 1 hr. after drug administration. At all times after 1 hr., the mean plasma levels of Groups A and B (the sustained-release tablet group and the single-dose, nonsustained-release tablet group) were significantly different. This indicates that the sustained-release tablet was still releasing pentylenetetrazol after the 1st hour, whereas the peak plasma level resulting from dosage with one nonsustained-release tablet occurred prior to or approximately at 1 hr.

Statistically significant differences were found between Groups A and C (the sustained-release tablet group and the group that received three divided doses of a nonsustained-release tablet) only at 2, 4, 9, and 12 hr. These results suggest that the sustained-release tablet released about one-third of the pentylenetetrazol rapidly and that the remainder was smoothly and continuously released.

As expected, there was no statistically significant difference between Groups B and C (the groups receiving nonsustained-release tablets in single doses and in three divided doses) at 1, 2, or 4 hr. From 5 hr. on, differences between these two groups were significant.

Urine Data—Urinary excretion of equivalent pentylenetetrazol proceeded at a nearly constant rate for approximately 36 hr. after administration of the sustained-release tablet and then gradually decreased (Fig. 2). A similar excretion pattern was shown by those subjects receiving three nonsustained-release tablets at 4-hr. intervals. A constant rate of urinary excretion of equivalent pentylenetetrazol was evident for approximately 24 hr. following single oral

Table III—Urinary Excretion of Equivalent Pentylenetetrazol following Oral Administration of Pentylenetetrazol-^{14}C Tablets

Time after Administration, hr.	Mean[a] Equivalent Pentylenetetrazol[b] Excreted, mg. ± *SD*		
	Group A[c]	Group B[d]	Group C[e]
0–3	12.3 ± 4.4	9.3 ± 3.9	8.8 ± 3.0
3–6	19.5 ± 2.7	9.3 ± 3.4	13.9 ± 2.8
6–9	23.0 ± 4.9	10.8 ± 1.5	19.7 ± 7.2
9–12	24.7 ± 4.1	8.2 ± 1.3	26.4 ± 7.7
12–24	65.9 ± 5.2	21.5 ± 0.4	79.7 ± 10.6
24–36	51.8 ± 11.9	12.8[f] ± 4.9	55.3 ± 11.5
36–48	27.2 ± 5.5	7.9 ± 2.0	31.2 ± 7.0
48–72	23.5 ± 4.8	7.6 ± 1.6	30.6 ± 10.6

[a] Mean of four subjects. [b] Any ^{14}C-labeled metabolites have been equated to the administered pentylenetetrazol-^{14}C. [c] Group A: One sustained-release tablet containing 300 mg. of pentylenetetrazol-^{14}C. [d] Group B: One nonsustained-release tablet containing 100 mg. of pentylenetetrazol-^{14}C. [e] Group C: One nonsustained-release tablet containing 100 mg. of pentylenetetrazol-^{14}C at 0, 4, and 8 hr. [f] This value includes an estimate of the amount of pentylenetetrazol present in a quantity of urine which was voided but not collected for analysis. (Subject forgot to collect one urine sample voided during this interval.)

administration of the nonsustained-release tablet, with the rate decreasing after the 24-hr. point.

The mean equivalent pentylenetetrazol excretion values and the standard deviations for the three groups are given in Table III. Student t values (8) were calculated to compare the mean ^{14}C excretion values of the different groups at various times. Statistically significant ($p = 0.01$) differences were seen between Groups A and B at all collection intervals after the first (0–3 hr.).

No significant differences between Groups A and C were present at any of the collection intervals. These results indicate that urinary excretion of 300 mg. of pentylenetetrazol proceeds at approximately the same rate whether the dose is administered in one sustained-release tablet or divided and administered in three doses at 4-hr. intervals. These results also indicate that the same fraction of pentylenetetrazol was absorbed from the sustained-release tablets as from the nonsustained-release tablets.

There were no significant differences between Groups B and C until the fourth collection (9–12 hr.). Mean excretion values for these two groups were significantly different in all of the last five urine collections.

SUMMARY

Sustained-release and nonsustained-release tablets containing pentylenetetrazol-10-^{14}C were administered to human subjects. The resulting plasma and urine concentrations of equivalent pentylenetetrazol were determined by liquid scintillation counting techniques. Subjects receiving the sustained-release tablets exhibited smoothly sustained plasma levels of equivalent pentylenetetrazol for a period of about 12 hr. and a nearly linear urinary excretion rate of ^{14}C during a period of 36 hr. Subjects receiving three doses of nonsustained-release tablets at 4-hr. intervals exhibited typical rises and falls in plasma ^{14}C levels and an excretion pattern similar to that of the subjects receiving the sustained-release tablets. Subjects receiving a single dose of a nonsustained-release tablet showed one peak plasma ^{14}C level which then decreased continuously. A fairly constant rate of urinary ^{14}C excretion was evident for 24 hr. The results of this study showed that the sustained-release tablet produced absorption and excretion patterns similar to those obtained following three doses of the drug administered in nonsustained-release form at 4-hr. intervals.

REFERENCES

(1) W. R. Ebert, R. W. Morris, and R. L. Bogner, *Del. Med. J.*, **39**, 152(1967).

(2) E. Rosen and J. V. Swintosky, *J. Pharm. Pharmacol.*, **12**, 237T(1960).

(3) J. F. Stiver, J. B. Data, and J. E. Christian, to be published.

(4) J. F. Stiver, Ph.D. thesis, Purdue University, Lafayette, Ind., 1970.

(5) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 919.

(6) *Ibid.*, p. 1075.

(7) *Ibid.*, p. 1076.

(8) N. M. Downie and R. W. Heath, "Basic Statistical Methods," 2nd ed., Harper and Row, New York, N. Y., 1965, pp. 138–143, 298.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 31, 1969, from *Philips Roxane Laboratories, Inc., Columbus, OH 43216* and the *Bionucleonics Department, Institute for Environmental Health, Purdue University, Lafayette, IN 47907*

Accepted for publication April 22, 1970.

The authors gratefully acknowledge the assistance of R. C. Anderson, Research and Development Department, Philips Roxane Laboratories, Inc.

* Present address: Research and Development Department, Philips Roxane Laboratories, Inc., Columbus, OH 43216

† Present address: School of Pharmacy, Ferris State College, Big Rapids, MI 49307

‡ Present address: Pathology Department, St. Elizabeth Hospital, Lafayette, IN 47907

§ Present address: Bionucleonics Department, Purdue University, Lafayette, IN 47907

Permeability of Double-Layer Films III

TSUNETO KURIYAMA, MICHIHARU NOBUTOKI, and MICHIO NAKANISHI

Abstract □ Moisture permeability of most double-layer films has a directional property. This "two-sidedness" may be brought about mainly by a change in the permeability coefficient as a result of the change in vapor pressure. To utilize this characteristic, it should be clarified as to how the permeability coefficient varies. For this purpose the differential permeability coefficient was calculated, making it easy to estimate the permeability of moisture under various conditions and making it possible to obtain the distribution of both vapor pressure and the water concentration in double-layer films. When the permeability on single films under various moisture conditions is given, the "two-sidedness" feature of double-layer films made from them will be grasped.

Keyphrases □ Double-layer films—theoretical considerations □ Films, double layer—moisture permeability □ Differential permeability coefficients—double-layer films □ Water concentration, vapor pressure—films

Previous reports (1, 2) dealt with variations of "two-sidedness" in the moisture permeability of double-layer films with changing conditions. It is very important to investigate the cause (or principle) of these phenomena. Rogers *et al.* (3) explained the two-sidedness skillfully, even though they did not classify such characteristics as were reported in a previous report (1). Their theory can be regarded as applicable to understand various types of two-sidedness and their behavior under changing moisture conditions. As stated in a previous report (2), the permeability coefficient, P, is not constant but varies with the moisture changes. Rogers *et al.* (3) introduced the concept of the differential permeability coefficient to solve this problem. The following theoretical considerations are mainly based on these ideas.

EXPERIMENTAL

The experimental method and the abbreviation for each film are the same as those in previous reports (1, 2).

Cell for Measuring Water Vapor Permeability—The cell and measuring method are modifications of those of Patel *et al.* (4). Permeation through a sample film was determined by measuring weight change of the cell at a certain condition.

administration of the nonsustained-release tablet, with the rate decreasing after the 24-hr. point.

The mean equivalent pentylenetetrazol excretion values and the standard deviations for the three groups are given in Table III. Student t values (8) were calculated to compare the mean ^{14}C excretion values of the different groups at various times. Statistically significant ($p = 0.01$) differences were seen between Groups A and B at all collection intervals after the first (0–3 hr.).

No significant differences between Groups A and C were present at any of the collection intervals. These results indicate that urinary excretion of 300 mg. of pentylenetetrazol proceeds at approximately the same rate whether the dose is administered in one sustained-release tablet or divided and administered in three doses at 4-hr. intervals. These results also indicate that the same fraction of pentylenetetrazol was absorbed from the sustained-release tablets as from the nonsustained-release tablets.

There were no significant differences between Groups B and C until the fourth collection (9–12 hr.). Mean excretion values for these two groups were significantly different in all of the last five urine collections.

SUMMARY

Sustained-release and nonsustained-release tablets containing pentylenetetrazol-10-^{14}C were administered to human subjects. The resulting plasma and urine concentrations of equivalent pentylenetetrazol were determined by liquid scintillation counting techniques. Subjects receiving the sustained-release tablets exhibited smoothly sustained plasma levels of equivalent pentylenetetrazol for a period of about 12 hr. and a nearly linear urinary excretion rate of ^{14}C during a period of 36 hr. Subjects receiving three doses of nonsustained-release tablets at 4-hr. intervals exhibited typical rises and falls in plasma ^{14}C levels and an excretion pattern similar to that of the subjects receiving the sustained-release tablets. Subjects receiving a single dose of a nonsustained-release tablet showed one peak plasma ^{14}C level which then decreased continuously. A fairly constant rate of urinary ^{14}C excretion was evident for 24 hr. The results of this study showed that the sustained-release tablet produced absorption and excretion patterns similar to those obtained following three doses of the drug administered in nonsustained-release form at 4-hr. intervals.

REFERENCES

(1) W. R. Ebert, R. W. Morris, and R. L. Bogner, *Del. Med. J.*, **39**, 152(1967).

(2) E. Rosen and J. V. Swintosky, *J. Pharm. Pharmacol.*, **12**, 237T(1960).

(3) J. F. Stiver, J. B. Data, and J. E. Christian, to be published.

(4) J. F. Stiver, Ph.D. thesis, Purdue University, Lafayette, Ind., 1970.

(5) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 919.

(6) *Ibid.*, p. 1075.

(7) *Ibid.*, p. 1076.

(8) N. M. Downie and R. W. Heath, "Basic Statistical Methods," 2nd ed., Harper and Row, New York, N. Y., 1965, pp. 138–143, 298.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 31, 1969, from *Philips Roxane Laboratories, Inc., Columbus, OH 43216* and the *Bionucleonics Department, Institute for Environmental Health, Purdue University, Lafayette, IN 47907*

Accepted for publication April 22, 1970.

The authors gratefully acknowledge the assistance of R. C. Anderson, Research and Development Department, Philips Roxane Laboratories, Inc.

* Present address: Research and Development Department, Philips Roxane Laboratories, Inc., Columbus, OH 43216

† Present address: School of Pharmacy, Ferris State College, Big Rapids, MI 49307

‡ Present address: Pathology Department, St. Elizabeth Hospital, Lafayette, IN 47907

§ Present address: Bionucleonics Department, Purdue University, Lafayette, IN 47907

Permeability of Double-Layer Films III

TSUNETO KURIYAMA, MICHIHARU NOBUTOKI, and MICHIO NAKANISHI

Abstract □ Moisture permeability of most double-layer films has a directional property. This "two-sidedness" may be brought about mainly by a change in the permeability coefficient as a result of the change in vapor pressure. To utilize this characteristic, it should be clarified as to how the permeability coefficient varies. For this purpose the differential permeability coefficient was calculated, making it easy to estimate the permeability of moisture under various conditions and making it possible to obtain the distribution of both vapor pressure and the water concentration in double-layer films. When the permeability on single films under various moisture conditions is given, the "two-sidedness" feature of double-layer films made from them will be grasped.

Keyphrases □ Double-layer films—theoretical considerations □ Films, double layer—moisture permeability □ Differential permeability coefficients—double-layer films □ Water concentration, vapor pressure—films

Previous reports (1, 2) dealt with variations of "two-sidedness" in the moisture permeability of double-layer films with changing conditions. It is very important to investigate the cause (or principle) of these phenomena. Rogers *et al.* (3) explained the two-sidedness skillfully, even though they did not classify such characteristics as were reported in a previous report (1). Their theory can be regarded as applicable to understand various types of two-sidedness and their behavior under changing moisture conditions. As stated in a previous report (2), the permeability coefficient, P, is not constant but varies with the moisture changes. Rogers *et al.* (3) introduced the concept of the differential permeability coefficient to solve this problem. The following theoretical considerations are mainly based on these ideas.

EXPERIMENTAL

The experimental method and the abbreviation for each film are the same as those in previous reports (1, 2).

Cell for Measuring Water Vapor Permeability—The cell and measuring method are modifications of those of Patel *et al.* (4). Permeation through a sample film was determined by measuring weight change of the cell at a certain condition.

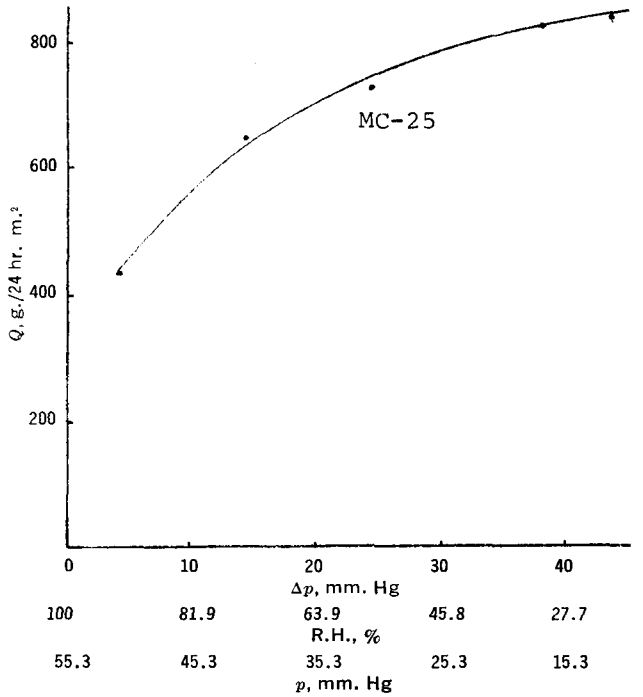


Figure 1—*Relationship between* Q *and* Δp. *Temperature, 40°; thickness of film, 0.2 mm.;* p_1 = *100% R.H. = 55.3 mm. Hg; and* $\Delta p = p_1 - p$.

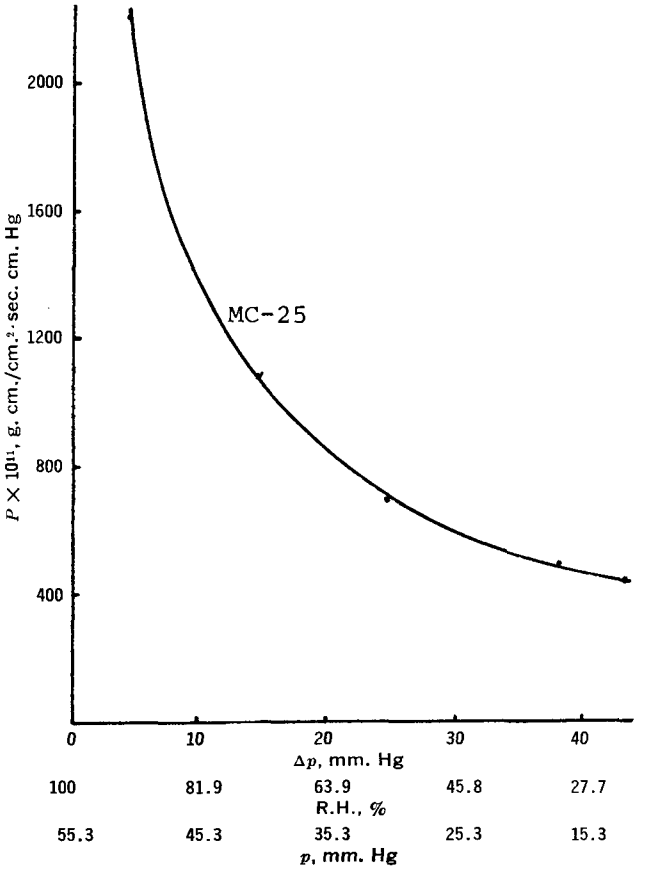


Figure 2—*Relationship between* P *and* Δp. *Temperature, 40°; thickness of film, 0.2 mm.;* p_1 = *100% R.H. = 55.3 mm. Hg; and* $\Delta p = p_1 - p$.

Film Preparation—*Single Film*—Free film was prepared by casting a solution of coating agent on a glass plate.

Double-Layer Film—Two single films were combined to form a double-layer film by using a solvent that dissolved only a surface of either film.

Measurement of Film Thickness—The thickness of a single film is expressed as the mean value of 12 different points measured with a dial gauge (precision 0.001 mm.) in a sample film (4 cm. in diameter). Standard deviation of the thickness in a sample film was less than 0.0006 mm. at 0.05-mm. thickness, 0.0008 mm. at 0.15-mm. thickness, and 0.001 mm. at 0.25-mm. thickness.

Materials—The coating agents used are as follows:

Abbreviation	Chemical Name	Manufacturer (Specification)
EC	Ethylcellulose	Dow Corning (50 cps.)
MC	Methylcellulose	Shin-etsu Chemical Co. (25 cps.)
HECAP	Hydroxyethylcellulose acetate phthalate	Yoshitomi Pharmaceutical Industries (5)

All permeability data were determined as the mean of six observations at the same condition.

RESULTS AND DISCUSSION

Figure 1 shows the relationship between the pressure difference, Δp, and the moisture permeability, Q, across methylcellulose (MC-25) film of 0.2-mm. thickness.

Application of Eq. 1 to this relationship gives the permeability coefficient P. The result is shown in Fig. 2.

$$Q = \frac{q}{At} = P\frac{p_1 - p_2}{l} = P\frac{\Delta p}{l} \qquad \text{(Eq. 1)}$$

where l = thickness of a film, A = permeation area of the film, t = time of permeation, p_1 = water vapor pressure at the side of higher humidity, p_2 = water vapor pressure at the side of lower humidity, P = permeability coefficient of moisture, Q = moisture permeability, and q = quantity of permeated moisture.

Figure 2 shows that the permeability coefficient is not a constant but a function of the humidity condition in which the film is placed. Each section that is rectangular to the direction of permeation across a film may have a certain differential permeability coefficient, depending upon the humidity condition in which the section is located. What is obtained in an experiment is permeability coefficient P, which corresponds to the integrated value of all differential permeability constants involved. This idea is expressed as follows:

$$P = \frac{1}{\Delta p}\int_{p_2}^{p_1} P'\,dp \qquad \text{(Eq. 2)}$$

Table I—Calculation of Differential Permeability Coefficient, p_1 = 5.53 cm. Hg

p, cm. Hg	$\Delta p = p_1 - p$, cm. Hg	Qp_1p, g./24 hr. m.²	$Ql = p\Delta p$, g./sec. cm.	P_{p_1p}, g./sec. cm. cm. Hg	P', g./sec. cm. cm. Hg
5.03	0.5	460	1067×10^{-11}	2134×10^{-11}	
4.93	0.6	485	1125×10^{-11}	1875×10^{-11}	580×10^{-11}
4.83	0.7	506	1174×10^{-11}	1677×10^{-11}	490×10^{-11}
4.73	0.8	528	1225×10^{-11}	1531×10^{-11}	510×10^{-11}
4.63	0.9	550	1276×10^{-11}	1418×10^{-11}	510×10^{-11}

Table II—Calculation of P_{pp_2} (p_2 = 1.03 cm. Hg)

P, cm. Hg	$\Delta p = p - p_2$, cm. Hg	P', g./sec. cm. cm. Hg	$\int_{p_2}^{p} P'\,dp$, g./sec. cm.	P_{pp_2}, g./sec. cm. cm. Hg	Q_{pp_2}, g./24 hr. m.²
1.03	0.0	90×10^{-11}	9×10^{-11}	90×10^{-11}	
1.13	0.1	70×10^{-11}	16×10^{-11}	80×10^{-11}	3.5
1.23	0.2	90×10^{-11}	25×10^{-11}	83×10^{-11}	7.2
1.33	0.3	50×10^{-11}	30×10^{-11}	75×10^{-11}	9.7
1.43	0.4	90×10^{-11}	39×10^{-11}	78×10^{-11}	13.5

where P' is the differential permeability coefficient.

Differentiating Eq. 2 gives Eq. 3:

$$P' = \frac{d(P\,\Delta p)}{dp} \qquad \text{(Eq. 3)}$$

Figure 2 gives the relationship between P and Δp (or p), which may produce P' with the help of Eq. 3. The process of this calculation is summarized in Table I. In this table, each P and Q has a suffix consisting of two letters. The first letter refers to the water-vapor pressure of the side of higher humidity; the second letter refers to the water vapor pressure of the side of lower humidity. For instance, Q_{p_1p} is the Q which is obtained by fixing the water vapor pressure on p_1 at the side of higher humidity and by varying the vapor pressure, p, at the side of lower humidity. In a like manner, the correspondent permeability coefficient of moisture is expressed as P_{p_1p}.

The results shown in Table I were calculated from the data of Fig. 1, not from data of Fig. 2. Although the four values observed in Fig. 2 are converted from Fig. 1, the curve in Fig. 2 is not as accurate as that in Fig. 1, because the former is more sharply curved than the latter. As is understood from Eq. 3, even a small error in P will have a strong influence on the resulting P'. The results obtained in Table I show the relationship that corresponds to the function $P' = f(p)$ under a certain condition. Figure 2 shows the observed values corresponding to Eq. 4. Here, using the relation of $P' = f(p)$ of Table I, P_{pp_2} can be obtained (Eq. 5). P_{pp_2} is the permeability coefficient for varying vapor pressure at the side of higher humidity under constant vapor pressure at the lower side. Moreover, P_{pp_2} gives Q_{pp_2} according to Eq. 1.

$$P_{p_1p} = \frac{1}{p_1 - p}\int_{p}^{p_1} P'\,dp \qquad \text{(Eq. 4)}$$

$$P_{pp_2} = \frac{1}{p - p_2}\int_{p_2}^{p} P'\,dp \qquad \text{(Eq. 5)}$$

These calculations were made in accordance with Table II, and the results are shown in Fig. 3.

P_{p_1p} is considerably different from P_{pp_2}. This means that the moisture permeability will be different when the mean value of vapor pressure is not the same, even if the difference of pressure across the film is constant. The calculated P' values were considerably divergent as plotted in Fig. 3. This divergence was caused, as mentioned previously, by the fact that the values of P' were strongly influenced by the slightest errors of P (errors of observation and plotting). P' values used in Table II are the raw results from Table I, so the P_{pp_2} values obtained are divergent too. However, if the compensated values of P' obtained from the regression curve in Fig. 3 are used, a better curve can be obtained around P_{pp_2}. Rogers *et al.* (3) explained the two-sidedness of the permeability without calculating P'. Their idea shows that it is possible to obtain Q_{pp_2}

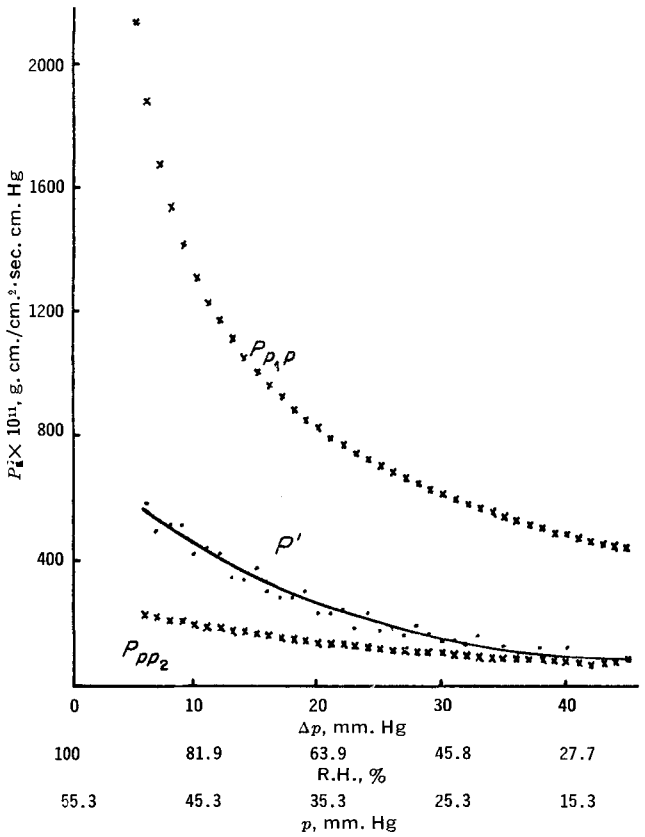


Figure 3—*Δp-profile of* P', P_{p_1p}, *and* P_{pp_2}. *Material, MC-25; thickness of film, 0.2 mm.; temperature, 40°;* p_1 = *100% R.H.* = *55.3 mm. Hg; and* $\Delta p = p_1 - p$. P_{p_1p} = *permeability coefficient observed by fixing the water vapor pressure on* p_1 *at the side of higher humidity and by varying the vapor pressure* p *at the side of lower humidity.* P_{pp_2} = *permeability coefficient calculated fixing the water vapor pressure on* p_2 *at the side of lower humidity and varying the vapor pressure* p *at the side of higher humidity.* P' = *differential permeability coefficient calculated.*

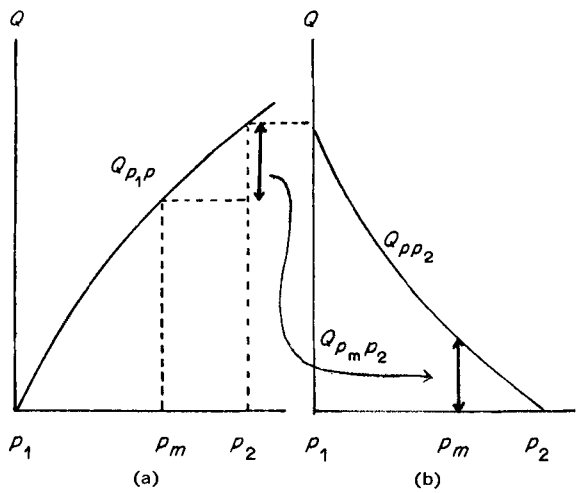


Figure 4—*Rogers' graphical method to obtain* Q_{pp_2} *from* Q_{p_1p}

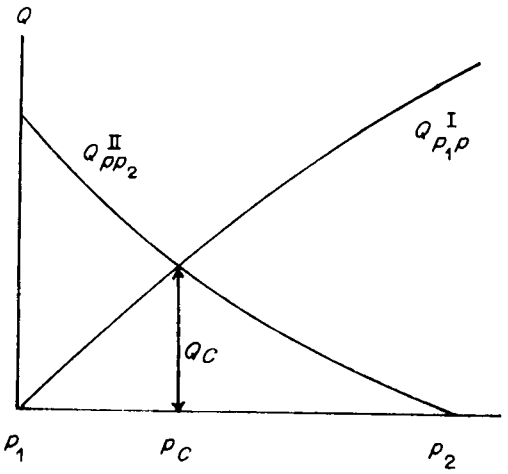


Figure 5—*Graphical estimation of permeability* (Q_c) *of double-layer film composed of Material I (higher humidity side) and Material II (lower humidity side).*

graphically, as shown in Fig. 4, if Q_{p_1p} is given. (This is, in principle, the same method as that used in Table I and/or II.)

Figure 5 gives the combination of $Q^{I}_{p_1p}$ and $Q^{II}_{pp_2}$, where $Q^{I}_{p_1p}$ means the moisture permeability across Film I when the vapor pressure p_1, at the side of higher humidity, is constant with varying vapor pressure p at the lower side. $Q^{II}_{pp_2}$ is Q_{pp_2} of Film II, which is obtained by the method shown in Fig. 4 using the data $Q^{II}_{p_1p}$. Rogers *et al.* (3) indicated that Q_c in Fig. 5 corresponds to the moisture permeability of double-layer film made from I and II, where Film I faces the p_1 side and Film II faces the p_2 side. As a model, Fig. 6 (a and b) illustrates different combinations of films with different moisture permeability characteristics.

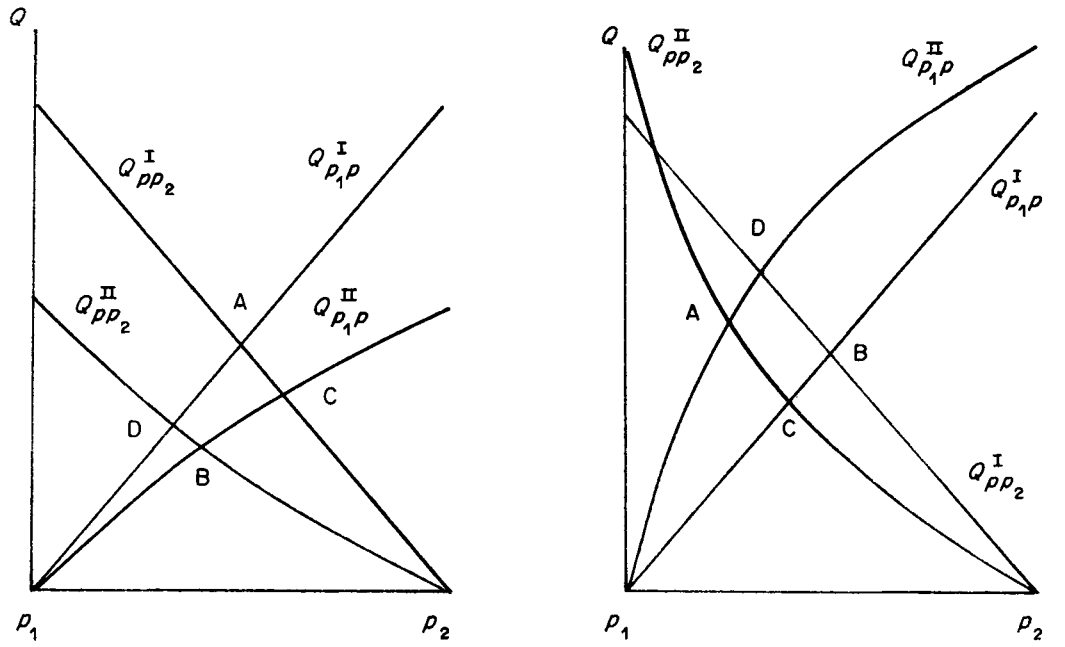
Rogers *et al.* (3) explained that the two-sided characteristic of the double-layer film results from the different Q around the intersecting points C and D. When the thicknesses of Films I and II are the same, A, B, C, and D all correspond to the permeability across the films of the same thickness. The characteristics of these double-layer films are classified according to the order of magnitude of Q's around A, B, C, and D as mentioned in an earlier report (1). Figure 6a belongs to group γ, and Figure 6b belongs to group α. Only if the actual data of $Q^{I}_{p_1p}$ and $Q^{II}_{p_1p}$ are given can $Q^{I}_{pp_2}$ and $Q^{II}_{pp_2}$ be determined graphically, where p_2 can be determined to be any value within a range of the mentioned data. For instance, the observed Q_{p_1p} for both ethylcellulose EC-50 of 0.1-mm. thickness and hydroxyethylcellulose acetate phthalate (HECAP) of 0.1-mm. thickness can produce Fig. 7.

Each intersecting point, A, B, C, or D, in Fig. 7 is plotted against humidity conditions to obtain Fig. 8. Figure 8 is utilized to compare the permeation property of the double-layer film with that of each of its single films, where the double-layer film is made from EC-50 and HECAP of 0.1-mm. thickness and single films are EC-50 and HECAP of 0.2 mm. The observed values corresponding to Fig. 8 were plotted in Fig. 9.

When the two graphs are compared, the shapes are seen to be similar, but the predicted graph (Fig. 8) seems to be more compressed in the direction of the ordinate than the observed ones (Fig. 9). These facts may be explained as follows. According to the method of Rogers *et al.* (3) shown in Fig. 4, the moisture permeability is halved when two films of the same kind with the same thickness are piled; that is, the thickness of the film is doubled. In other words, the moisture permeability is inversely proportional to the thickness of the film (or $1/Q$ is in direct proportion to l). Thus, Fig. 4b is obtained when Fig. 4a is turned over symmetrically around the line (parallel to the p ordinate) that passes through the point $^1/_2 \cdot Q$. However, as mentioned in an earlier report (1), the moisture permeability is not exactly in inverse proportion to the thickness but is slightly more than one-half, even if the thickness of the film is doubled. The main reason Fig. 8 differs from Fig. 9 may be attributed to the assumed inversely proportional relation.

Although the following processing method involves some theoretical inadequacies, it may be practically useful to estimate the two-sided property of the double-layer film.

1. The moisture permeability is measured at several Δp levels about single Films I and II, which may be combined to form a



	Film Material	*Water Vapor Pressure (Fixed)*	*Water Vapor Pressure (Varied)*
$Q^{I}_{p_1p}$	*I*	p_1 (*higher pressure side*)	p (*lower pressure side*)
$Q^{I}_{pp_2}$	*I*	p_2 (*lower pressure side*)	p (*higher pressure side*)
$Q^{II}_{p_1p}$	*II*	p_1 (*higher pressure side*)	p (*lower pressure side*)
$Q^{II}_{pp_2}$	*II*	p_2 (*lower pressure side*)	p (*higher pressure side*)

Figure 6—*Two-sidedness of double-layer films (Rogers' method).*

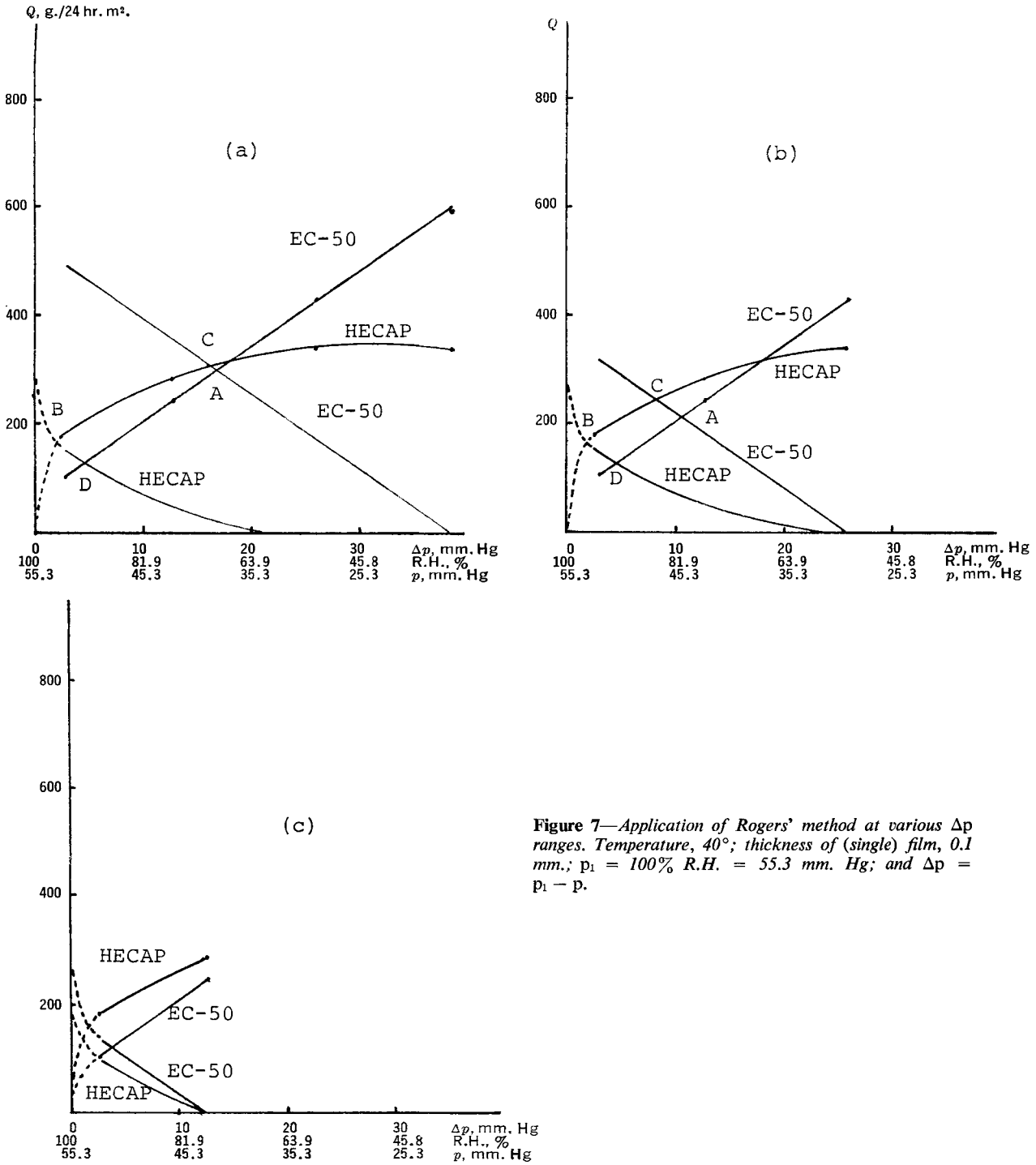


Figure 7—*Application of Rogers' method at various* Δp *ranges. Temperature, 40°; thickness of (single) film, 0.1 mm.;* p_1 = *100% R.H.* = *55.3 mm. Hg; and* Δp = $p_1 - p$.

double-layer film with several levels of thickness. (Although Δp can be set as either $p_1 - p$ or $p - p_2$, the following explanation is carried out using $\Delta p = p_1 - p$.)

2. The data are arranged in terms of: abscissa = l, ordinate = $1/Q$, and parameter = Δp, which afford a linear relationship. The graphs are obtained for I and II.

3. From the graphs obtained in Step 2, the figure is depicted for I and II in terms of: abscissa = Δp, ordinate = Q, and parameter = l. To evaluate the permeability of the double-layer film which consists of I and II when each elemental layer has thickness l_1, the parameter is enough to be l_1 and $2l_1$. In other words, the Q–Δp (or Qp_1p) curve is obtained for both I and II with the thickness l_1 and $2l_1$, respectively.

4. The Q_{pp_2} curve for a given p_2 is obtained by turning the Q_{p_1p} curve of a film with thickness l_1 symmetrically around the line that passes through the point $Q_{p_1p_2}$ of the film with thickness $2l_1$ and is parallel to the Δp axis. The four curves obtained, $Q^{\mathrm{I}}_{p_1p}$, $Q^{\mathrm{I}}_{pp_2}$, $Q^{\mathrm{II}}_{p_1p}$, and $Q^{\mathrm{II}}_{pp_2}$, are drawn in a graph as in Fig. 6.

In this method, different from that of Rogers *et al.* (3), the value of Q along the line of symmetry is not $1/2 \cdot Q_{p_1p_2}$ for l_1 but $Q_{p_1p_2}$ for $2l_1$. From the intersect points in the graph thus obtained, the two-sided characteristic of the double-layer film is evaluated. According to this method, the permeability of any combination of films can be determined only if the data for each single film are given, and the permeabilities of single films can be compared with each other under the same conditions on the any-accurate-thickness (l_1) basis (although it is very difficult to make a film with an exact l_1 thickness). Figure 10 is an example of the result thus obtained with a double-layer film made from EC-50 and HECAP.

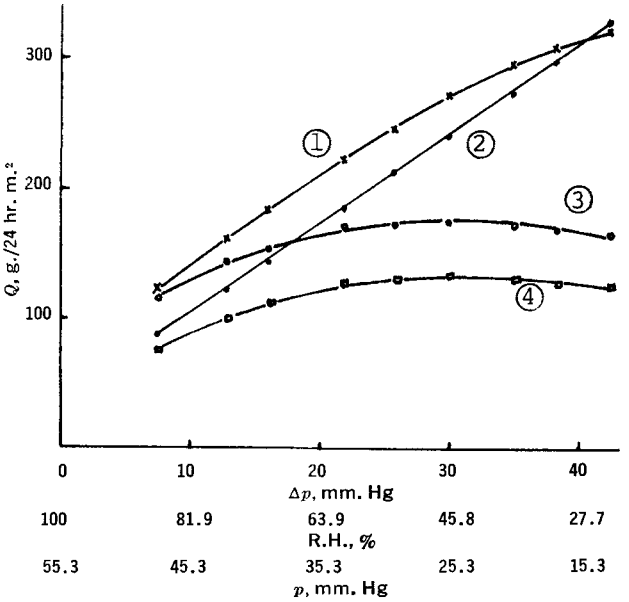


Figure 8—*Estimation of two-sidedness of moisture permeability. Temperature, 40°;* p_1 *= 100% R.H. = 55.3 mm. Hg; and* $\Delta p = p_1 - p$. *Materials:* ①, *HECAP (0.1 mm.) + EC-50 (0.1 mm.);* ②, *EC-50 (0.2 mm.);* ③, *HECAP (0.2 mm.); and* ④, *EC-50 (0.1 mm.) + HECAP (0.1 mm.).*

Figure 10 closely resembles Fig. 9, indicating the usefulness of the method to predict the two-sided property. This graphical method does not necessarily match with the detailed facts. (For instance, $Q^{I}_{p_2p_2}$ does not come to zero.) However, the Q values obtained from the intersecting points make it possible to determine the two-sided property of permeability of the double-layer film with tolerable accuracy.

The differential permeability coefficient can be utilized for another purpose. The $\Delta p/l$ in Eq. 1 is the gradient of vapor pressure across

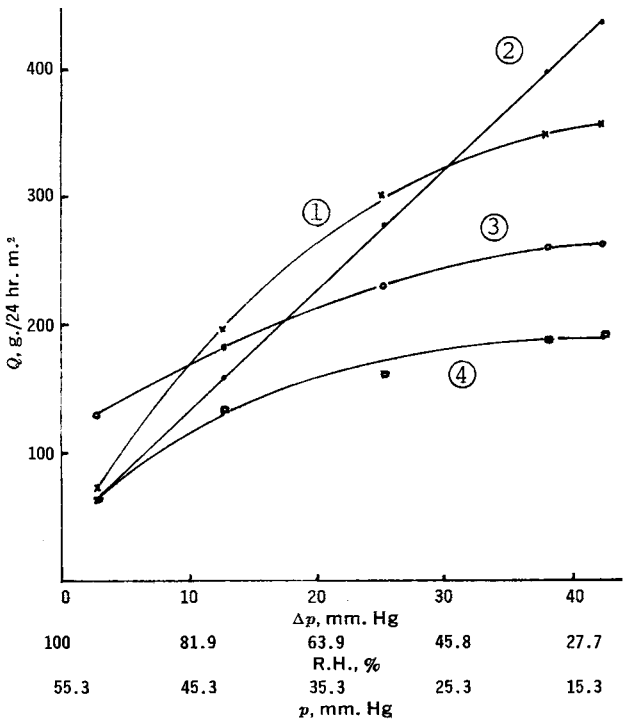


Figure 9—*Two-sidedness of moisture permeability observed. Temperature, 40°;* p_1 *= 100% R.H. = 55.3 mm. Hg; and* $\Delta p = p_1 - p$. *Materials:* ①, *HECAP (0.1 mm.) + EC-50 (0.1 mm.);* ②, *EC-50 (0.2 mm.);* ③, *HECAP (0.2 mm.); and* ④, *EC-50 (0.1 mm.) + HECAP (0.1 mm.).*

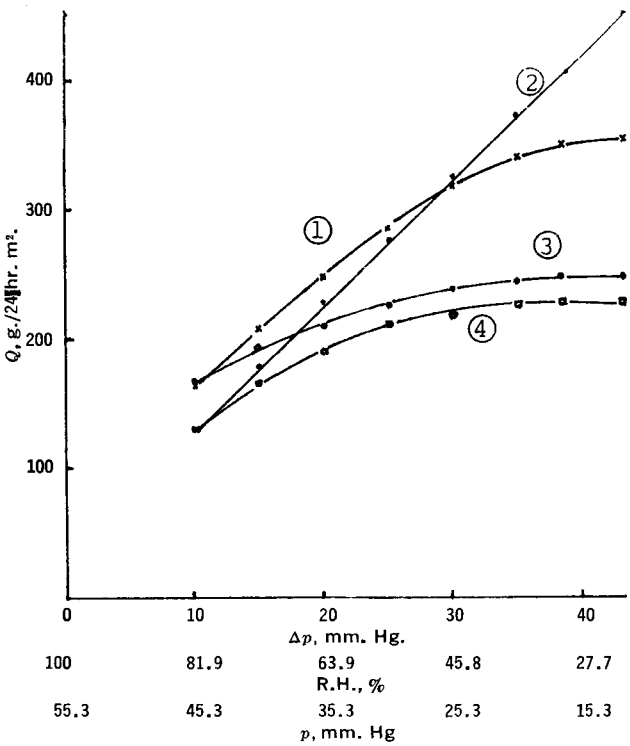


Figure 10—*Estimation of two-sidedness of moisture permeability. Temperature, 40°;* p_1 *= 100% R.H. = 55.3 mm. Hg; and* $\Delta p = p_1 - p$. *Materials:* ①, *HECAP (0.1 mm.) + EC-50 (0.1 mm.);* ②, *EC-50 (0.2 mm.);* ③, *HECAP (0.2 mm.); and* ④, *EC-50 (0.1 mm.) + HECAP (0.1 mm.).*

the film. After permeation arrives at a steady state of condition, the moisture transmitting the cross section of the direction of permeation is of the same quantity as Q, passing through the film itself. Considering the cross section, Eq. 1 then can be written as Eq. 6:

$$Q = -P' \frac{dp}{dx} \qquad \text{(Eq. 6)}$$

where x is the distance from the surface of higher humidity to the aimed cross section of the direction of permeation. This equation may then be correlated to the equation of Fick's law (Eq. 7) on diffusion:

$$Q = -D \frac{\partial c}{\partial x} \qquad \text{(Eq. 7)}$$

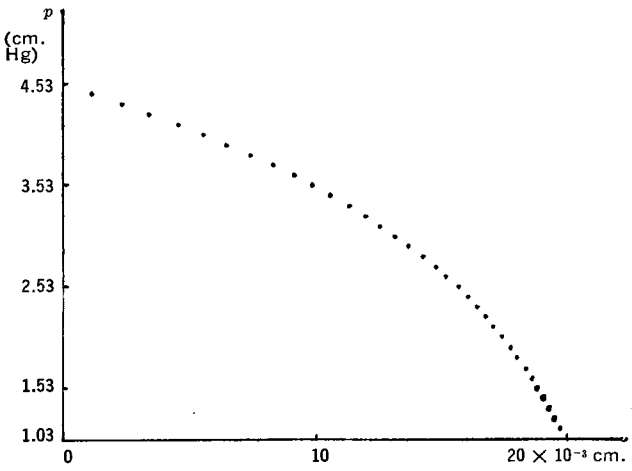


Figure 11—*Water vapor pressure distribution in film (calculated). Film material, MC-25; temperature, 40°;* p_1 *= 4.53 cm. Hg; and* p_2 *= 1.03 cm. Hg.*

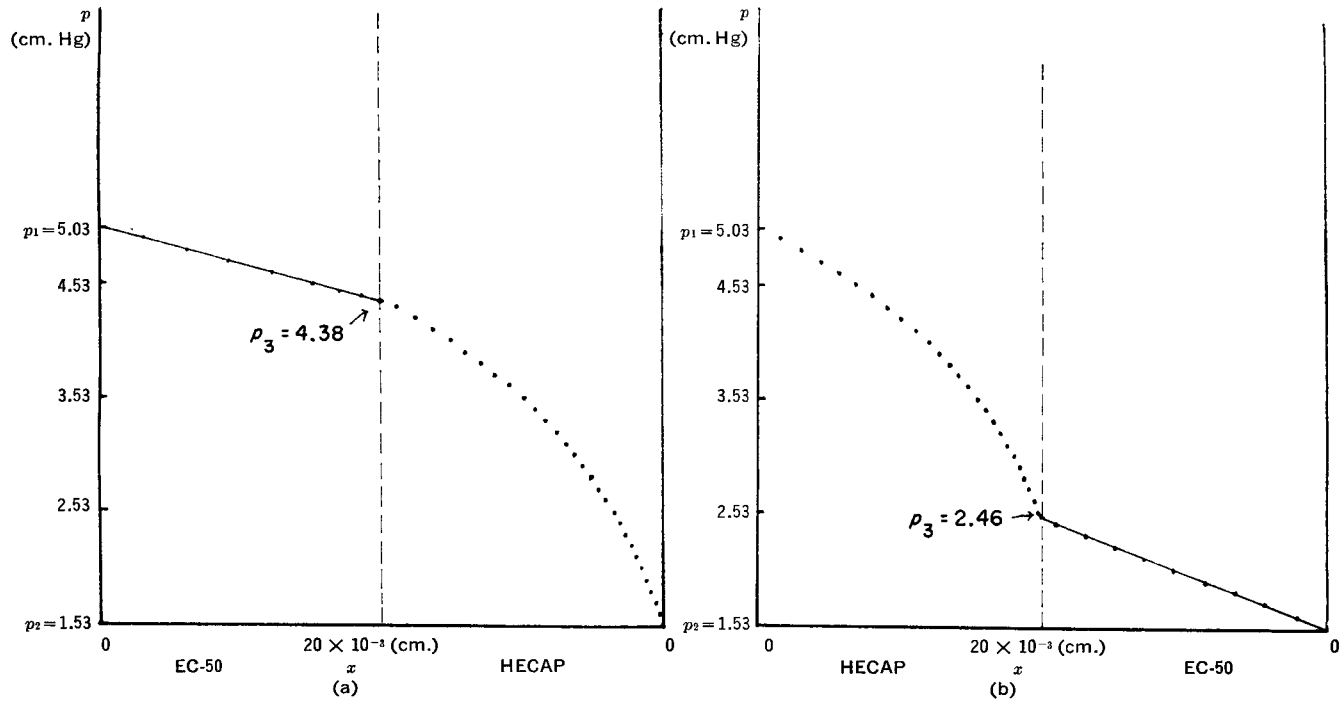


Figure 12—*Water vapor pressure distribution (calculated) of double-layer film. Temperature, 40°;* p_1 = *5.03 cm. Hg; and* p_2 = *1.53 cm. Hg.*

While the diffusion coefficient, D, in Eq. 7 is constant, the differential permeability coefficient, P', in Eq. 6 is a function of the water-vapor pressure. Equation 8 is obtained when Eq. 6 is integrated in the range between $x = 0$ and $x = x$, *i.e.*, between p_1 and p where p_1 = the water vapor pressure at the side of higher humidity, and p = the vapor pressure in equilibrium to cross section across the film ($p_1 > p > p_2$).

$$x = \frac{1}{Q_{p_1p}} \int_{p}^{p_1} P'dp \qquad \text{(Eq. 8)}$$

The distribution of the vapor pressure across the film can be obtained from Fig. 10. Table III shows the calculation process which gives the relation of p to x. This calculation was carried out using the P' value which was calculated in Table I and revised in Fig. 3. These results produce Fig. 11.

The curve of the vapor pressure distribution in MC-25 (hydrophylic film) was concluded not to be linear, although it was linear in the case of a hydrophobic film. Gillespie (6) investigated the water concentration of each layer in some multilayer films and obtained similar results: the curve of water concentration distribution was linear on polystyrene (hydrophobic) and sigmoid on cellophane (hydrophilic). Figure 11 supports this result, although it was not sigmoid. However, Fig. 11 shows the distribution of the vapor pressure; if the vapor pressure distribution is converted into the distribution of water concentration, it will give a sigmoid curve. The reason for this follows. Although the relation of the water vapor pressure, p, to the water concentration, c, which is in equilibrium with the pressure, follows Henry's law (Eq. 9) in general,

$$c = Sp \qquad \text{(Eq. 9)}$$

the relation given by Eq. 10 is valid, as shown by Henley (7) with the propane–polyethylene system, when p varies over a wide range:

$$S = ap + b \qquad \text{(Eq. 10)}$$

where both a and b are constants.

Substituting Eq. 10 into Eq. 9 gives Eq. 11:

$$c = ap^2 + bp \qquad \text{(Eq. 11)}$$

This indicates that c is a function of the second order of p. This has been also supported experimentally by Gillespie (6). The ordinate of Fig. 11 can be converted into the water concentration distribution by using Eq. 11, and then the obtained curve will be more similar to the sigmoid.

Table III—Calculation of Water Vapor Pressure Distribution in Film[a]

p, cm. Hg	P', g./sec. cm. cm. Hg	$\int_{p}^{p_1} P'\,dp$, g./sec. cm.	x, cm.
4.53	445×10^{-11}	0×10^{-11}	0×10^{-3}
4.43	420×10^{-11}	44.5×10^{-11}	1.25×10^{-3}
4.33	400×10^{-11}	86.5×10^{-11}	2.43×10^{-3}
4.23	380×10^{-11}	126.5×10^{-11}	3.57×10^{-3}
4.13	360×10^{-11}	164.5×10^{-11}	4.64×10^{-3}
...	...	...	...
...	...	...	...
...	...	...	...
1.23	76×10^{-11}	694.3×10^{-11}	19.58×10^{-3}
1.13	74×10^{-11}	701.7×10^{-11}	19.79×10^{-3}
1.03	72×10^{-11}	709.1×10^{-11}	20.00×10^{-3}

[a] $p_1 = 4.53$ cm. Hg, $p_2 = 1.03$ cm. Hg, $l = 0.02$ cm., $Q_{p_1p_2} = 1/l \int_{p_2}^{p_1} P'dp = \frac{709.1 \times 10^{-11}}{0.02} = 35.46 \times 10^{-3}$ (g. /sec. cm.^{2}) = 306 (g./24 hr. m.^{2}).

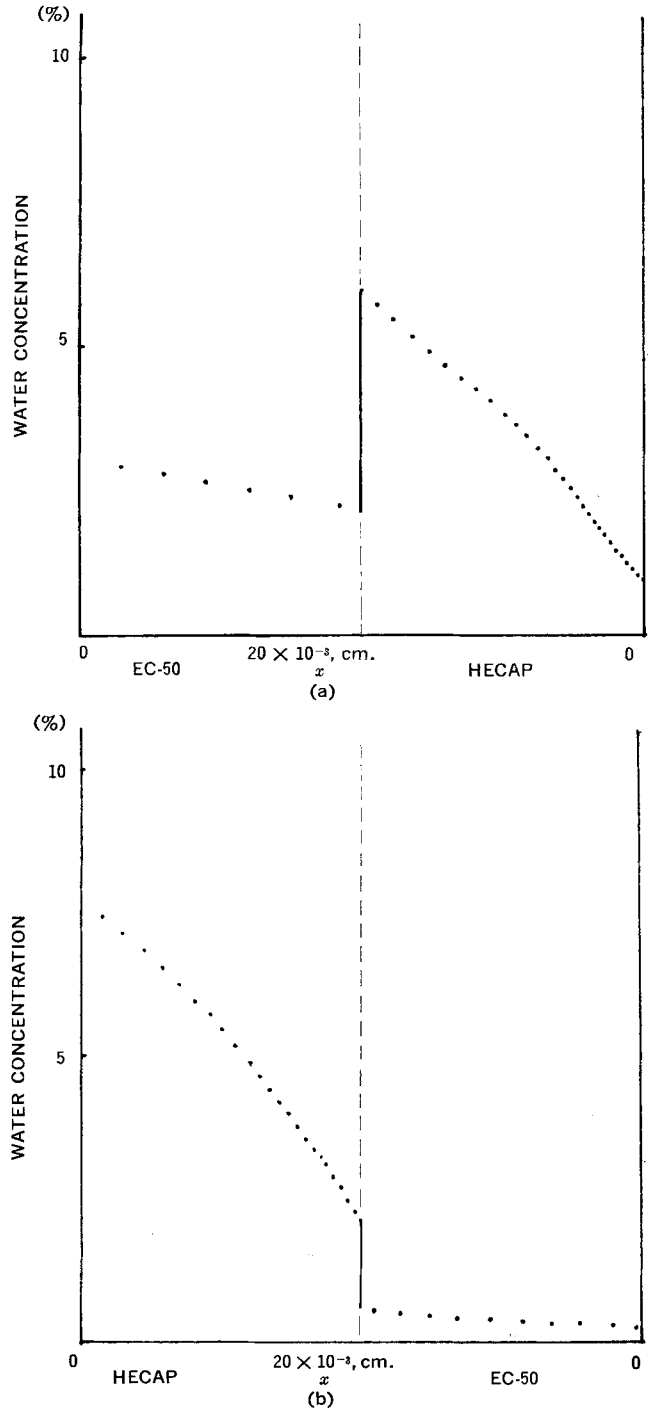


Figure 13—*Water concentration distribution in double-layer film. x = film thickness.*

For a double-layer film of EC-50 and HECAP, the distribution of the water vapor pressure can be shown as in Fig. 12 and the distribution of water concentration can be shown as in Fig. 13. These distributions change, depending upon which side faces the higher humidity atmosphere. In addition, Fig. 13a shows that moisture permeation can occur even against the gradient of water concentration in some cases, although it does not occur against that of the vapor pressure. The curve of the water concentration distribution is not so sharply S-shaped as observed by Gillespie. The difference may be caused mainly by the different experimental conditions between Gillespie's and the present study. Gillespie dealt with multilayer film having air layers between each elemental layer and, of course, the material of the film was different.

SUMMARY

The differential moisture permeability coefficient of a film was actually obtained by calculation. Using this coefficient, the permeability for any humidity condition, not only the condition in which the experiment was actually carried out, can be estimated. The authors introduced a graphical method to estimate the two-sided characteristic of moisture permeability for the double-layer film under changing humidity conditions. In addition, the distributions of both water vapor pressure and water concentration in a film were found to be determined by calculation, even in the case of very thin films for which other determination methods cannot be applied. In hydrophilic films, the curve of the water vapor pressure distribution was not linear. The distribution of the water vapor pressure within the double-layer film with a particularly remarkable two-sidedness was expressed by a specific figure, and the figure changed depending upon which side faced the higher humidity atmosphere. Across the interface of films, moisture permeation was found to occur possibly against the water concentration.

REFERENCES

(1) T. Kuriyama, M. Nobutoki, and M. Nakanishi, *J. Pharm. Sci.*, **59,** 1341(1970).
(2) *Ibid.*, **59,** 1344(1970).
(3) C. E. Rogers, V. Stannet, and M. Szwarc, *Ind. Eng. Chem.*, **49,** 1933(1957).
(4) M. Patel, J. M. Patel, and A. P. Lemberger, *J. Pharm. Sci.*, **53,** 286(1964).
(5) I. Sako, Japanese pat. 438,595.
(6) T. Gillespie, *J. Polym. Sci., Part A-1*, **4,** 933(1966).
(7) E. J. Henley, *AIChE. J.*, **12,** 1030(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 12, 1970, from the *Research Laboratories of Yoshitomi Pharmaceutical Industries, Ltd., Yoshitomi-Cho, Chikujo-Gun, Fukuoka-Prefecture, Japan.*

Accepted for publication April 22, 1970.

Abstracted from a thesis submitted by T. Kuriyama to the Graduate School, Kyushu University, in partial fulfillment of Doctor of Philosophy degree requirements.

Thanks are expressed to Prof. S. Iguchi for his interest in the work and useful discussions; also to Mr. M. Tasaka, Executive Director of Yoshitomi Pharmaceutical Industries, Ltd., for his encouragement and his kind permission to publish this paper.

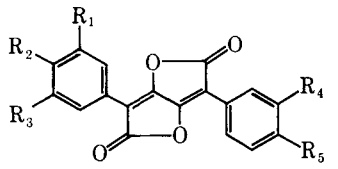
Isolation of Diphenyl-Substituted Tetronic Acids from Cultures of *Paxillus atrotomentosus*

M. C. GAYLORD, R. G. BENEDICT, G. M. HATFIELD, and L. R. BRADY

Abstract □ Pigments which accumulated in surface cultures of *Paxillus atrotomentosus* (Batsch) Fr. were studied. The nutrient broth and mycelium of 2-month-old cultures were blended, and the pigments were partitioned into ether. Two pigments were isolated by dry-column chromatography using a silica gel adsorbent and ether saturated with concentrated HCl as a chromatographic solvent. The major pigment was identified as xerocomic acid, and the minor pigment was shown to be atromentic acid. Identification of these acids was based on UV and IR spectra, mass spectral fragmentation patterns, high-resolution mass spectroscopy, and properties of their acetyl derivatives. Examination of fresh carpophores of *P. atrotomentosus* failed to demonstrate detectable amounts of either of these tetronic acids. These pigments have been reported to occur in fresh carpophores of some species in the Boletaceae and Gomphidiaceae, but this is the first reported presence of any diphenyl-substituted tetronic acid in the saprophytic culture of a fungus.

Keyphrases □ Tetronic acids, diphenyl substituted—*Paxillus atrotomentosus* cultures □ Pigment formation—*P. atrotomentosus* cultures □ Column chromatography—separation □ Paper chromatography—identification □ Electrophoresis—separation monitoring

Diphenyl-substituted tetronic acids (pulvinic acid derivatives) have long been known to be characteristic constituents of certain lichens (1, 2). Experimental evidence (3) suggests that polyporic acid, a terphenylquinone present in basidiomycetes as well as in lichens, is a precursor of pulvinic acid lactone (I) and calycin in the thalli of the lichen *Pseudocyphellaria crocata*. A number of terphenylquinones are known to occur in basidiomycetes (4), especially in the Hydnaceae, but information on the occurrence of pulvinic acid derivatives in basidiomycetes is restricted essentially to recent reports on the presence of atromentic acid (II), gomphidic acid (III), variegatic acid (IV), and xerocomic acid (V) in carpophores of some species in the Boletaceae and Gomphidiaceae (Table I).



	R_1	R_2	R_3	R_4	R_5
I, pulvinic acid lactone	H	H	H	H	H
II, atromentic acid lactone	H	OH	H	H	OH
III, gomphidic acid lactone	OH	OH	OH	H	OH
IV, variegatic acid lactone	OH	OH	H	OH	OH
V, xerocomic acid lactone	OH	OH	H	H	OH

Pigmentation was noted in surface cultures of *Paxillus atrotomentosus* (Batsch) Fr., and studies were initiated to determine the identity of the pigments produced by vegetative mycelia of this agaric. Carpophores of *P. atrotomentosus* are known to contain the terphenylquinone, atromentin (9), but no information is available in the literature on the pigments formed by vegetative growth of this species or on the occurrence of tetronic acids in carpophores of the fungus.

Table I—Occurrence of Diphenyl-Substituted Tetronic Acids in Carpophores of Basidiomycetes

Species	Reported Tetronic Acid
Boletaceae	
Boletus appendiculatus Fr.	Variegatic acid (5)
B. calopus Fr.	Variegatic acid (6)
B. erythropus (Fr.) Secr.	Variegatic acid (5, 6)
Suillus bovinus (Fr.) Kuntze	Variegatic acid (5)
S. variegatus (Fr.) Kuntze	Variegatic acid (5, 7)
Xerocomus chrysenteron (St. Amans) Quél.	Atromentic acid (6), variegatic acid (6), xerocomic acid (6)
Gomphidiaceae	
Gomphidius glutinosus (Fr.) Fr.	Atromentic acid (8), gomphidic acid (8), xerocomic acid (8)

EXPERIMENTAL

Origin of Culture and Carpophores—A culture of *P. atrotomentosus* was used in this study.[1] Stock cultures were maintained on a recommended cherry agar and were transferred monthly.

Carpophores of this mushroom were obtained from the Quinault River area of western Washington during September 1969.[2]

Vegetative Growth of *P. atrotomentosus*—Liquid nutrient medium for surface cultivation of the fungus was prepared by steaming 1 kg. of pitted fresh, or unsweetened frozen, sour cherries in 1 l. of distilled water for 2 hr. Twenty milliliters of strained cherry decoction and 30 ml. of distilled water were added to each 500-ml. Roux bottle, and the nutrient solutions were autoclaved for 15 min. at 15 lb. pressure. Sterile nutrient solutions were inoculated immediately after preparation, using a homogenate obtained by removing the mycelium of *P. atrotomentosus* from 10–14-day-old agar-slant cultures and blending for 5 sec. with a small volume of sterile water.

Preliminary observations suggested that the best results were obtained when the cultures were incubated at 20°, that a pigment with chromatographic properties of atromentic acid was present in the cultures after 35 days, and that maximum pigment accumulation occurred between 50 and 60 days. Shortly after this optimal period, the cultures tended to undergo degradative changes, and pigments were no longer extractable with ether. Similar deteriorations occurred very rapidly in cultures incubated at 30° or exposed to intense light for a significant period. Thus, the cultures employed in this study were incubated routinely in the dark at 20° for 50–60 days.

Very little growth was evident in the cultures at the end of the 1st week. Fine vegetative mycelia gradually radiated from the submerged fragments of inoculum. Brown spherical aggregations began to appear in zonations around each growing point; after 21 days, scattered buff-colored tufts of aerial mycelium were developing from the dark-brown aggregations. In 38 days a yellowish-beige mycelial growth covered approximately one-half of the yellow-colored nutrient broth in the Roux bottles. Between 50 and 60 days, aerial mycelia became yellow-orange at the edges of the mycelial mats and fluoresced a bright orange under UV light. Microscopic examination

[1] Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

[2] Identification was provided by Dr. D. E. Stuntz, Department of Botany, University of Washington, Seattle, Wash.

Table II—Chromatographic Separation of Selected Terphenylquinones and Pulvinic Acid Derivatives

Compound	Chromatographic System[a] and R_f Values					
	A	B	C	D	E	F
Aurantiacin	0.85	0.64	0.99	0.99	—	—
Thelephoric acid	0.63	0	0.05	0	—	—
Polyporic acid	0.15	0.98	0.70	0.72	—	—
Pulvinic acid lactone	0.84	0.94	0.25	0.95	—	—
Pulvinic acid	0.63	0.90	0.54	0.92	—	—
Atromentin	0.21	0.74	0.52	0.35	0.71	0.93
Atromentic acid lactone	0.83	0.81	0	0.99	0.97	0.87
Atromentic acid	0.53	0.64	0.07–0.17	0.72	0.90	0.82
Xerocomic acid	0.50	0.58	0.04–0.12	0.50	0.85	0.93

[a] System A: silica gel G thin layer, methyl ethyl ketone–H_2O–formic acid (250:25:1); System B: silica gel G thin layer, ether saturated with concentrated HCl; System C: polyamide thin layer, methyl ethyl ketone–H_2O–formic acid (250:25:1); System D: kieselguhr G thin layer, benzene–methyl formate–formic acid (13:5:4); System E: Whatman No. 1 paper, *n*-butanol–acetone–H_2O (2:5:2); and System F: Whatman No. 1 paper, H_2O–methyl ethyl ketone–diethylamine (921:77:2).

of the aerial mycelia at this stage revealed the presence of specialized strand formations; these strands resembled the dark velvety hyphal structures found with numerous brown incrustations on the stipe of the mature carpophore of *P. atrotomentosus*.

Degradative changes in the cultures appeared to commence shortly after a complete mycelial mat was developed. These changes were associated with a rapid transition of mycelial color from yellow-orange to brown. The color of the nutrient broth also changed from yellow to brown.

Chromatographic Examination—Various chromatographic procedures were examined for their utility in detecting pigments in the cultures and in monitoring the isolation and purification manipulations. The four TLC and two paper chromatographic systems which offered the greatest value are listed in Table II. The silica gel G[3] and kieselguhr G[3] TLC plates were activated at 110° for 30 min. The polyamide[4] system had been developed to examine fungal terphenylquinones (10), and the kieselguhr system had been used successfully with certain diphenyl-substituted tetronic acids (6, 8). The paper chromatographic systems were particularly useful for distinguishing atromentic and xerocomic acids.

The various pigments could be detected on the chromatograms without the use of any visualization reactions. However, observations of the chromatograms under longwave UV light provided some information which aided in distinguishing the various pigments. Atromentin, aurantiacin, polyporic acid, and thelephoric acid absorb UV light without fluorescing. The fluorescent colors noted with the other compounds were: atromentic acid, dull yellow; atromentic acid lactone and pulvinic acid, orange; pulvinic acid lactone, bright yellow; and xerocomic acid, dull orange.

Electrophoretic Examination—A paper electrophoretic procedure was also found to be useful in monitoring the isolation and purification manipulations. Samples were applied 20 cm. from the positive edge of 46 × 57-cm. sheets of Whatman No. 3 paper. The paper was then carefully dampened with a pH 2 buffer (formic acid–acetic acid–H_2O, 1:4:28). The dampened sheet was placed on a rack, covered, and run at 2500 v. and 100 ma. for 2 hr.; the sheet was covered with a crude fraction of nonane (b.p. approx. 100°), since the high voltage required the use of a cooling system.

Atromentic and xerocomic acids moved distances of 6.5 and 5.8 cm., respectively, toward the positive pole under the experimental conditions. Atromentic acid lactone and atromentin showed no migration in this electrophoretic system, and polyporic acid and pulvinic acid apparently were either eluted or ran off the sheet in the 2-hr. time interval.

Isolation and Purification of Pigments from *P. atrotomentosus* Cultures—Stability problems with some terphenylquinones and hydroxylated diphenyl-substituted tetronic acids and the suspected distribution of pigments in both the mycelium and culture broth suggested the desirability of extracting the total culture mixture without drying. The pooled mycelium and nutrient broth from 56-day-old cultures were blended in a large blender, and the mixture was extracted by shaking repeatedly with ether. Approximately 50 l. of ether was required to extract exhaustively the pigments from 50 Roux bottle cultures. The initial ether extract was dried over anhydrous Na_2SO_4. The ether solution was separated, and the solvent was removed under reduced pressure at 32°. Most of the pigmented material in the resulting red residue was soluble in water; the pigments were dissolved in approximately 60 ml. of cold water and separated from less polar impurities by filtration. Pigments in the aqueous filtrate were partitioned into ether, and the ether was removed to give a crude pigment fraction. Precautions were taken during all of the purification manipulations to minimize exposure of the pigments to light.

The chromatographic and electrophoretic monitoring systems indicated the presence of one major pigment and one minor component in the crude pigment fraction. Dry-column chromatography (11) was selected for the separation of these substances. Silica gel was washed successively with water, methanol, and ether. The washed silica gel was dried at 140° for 24 hr. and then was equilibrated with 10% w/v water for 3 hr. in a ball mill. The crude pigment fraction was adsorbed on five times its weight of silica gel, and aliquots of this material were added to the top of approximately 1.8 × 30-cm. silica gel columns. Ether saturated with concentrated HCl was used as the chromatographic solvent system. The major and minor pigments, which were subsequently established to be xerocomic and atromentic acids, respectively, migrated at approximately R_f 0.51 and 0.60. These bands were removed from the columns, the pigments were eluted from the adsorbent with the HCl-saturated ether, and the pigment materials were rechromatographed individually on a second series of columns for ultimate separation. Each 100 mg. of the crude pigment fraction yielded 75 mg. of xerocomic acid and 2 mg. of atromentic acid. Some pigmented material could not be eluted from the adsorbent and remained at the top of the columns. No evidence of a pigment band was noted at R_f 0.80, the approximate migration of atromentin in the dry-column system.

Table III shows the relative weights of the various components found in 50 Roux-bottle cultures which were harvested at 56 days. After the blended cultures were extracted with ether, the mycelium and nutrient broth were separated and freeze-dried.

Identification of Xerocomic Acid[5]—The UV spectra of the major pigment, $\lambda_{max.}^{methanol}$ 408 and 260 mμ, $\lambda_{max.}^{water}$ 378 and 256 mμ, and $\lambda_{max.}^{0.01\ M\ NaHCO_3}$ 620, 362, 320 (sh), and 230 mμ, agreed with the published absorption properties of xerocomic acid (6) and a related tetronic acid (5). The IR spectrum (KBr pellet), with absorption peaks at 3190, 2882, 2558, 1739, 1675, 1600, and 1513 $cm.^{-1}$, was also in general agreement with the reported spectrum for xerocomic acid (6).

The mass spectral fragmentation of diphenyl-substituted tetronic acids has been studied (12), and the fragmentation patterns of some hydroxylated pulvinic acid derivatives have been confirmed (6, 8). Water is readily lost from these tetronic acids, and the molecular ions in the mass spectra correspond to the respective lactones. The tetronic acid lactones exhibit two basic fragmentation series. The first series involves successive elimination of small fragments; this established fragmentation series for xerocomic acid lactone is *m/e* 338 → 310 → 282 →226. The second series involves cleaving the lactone into two equal or approximately equal ions and subsequent elimination of small fragments. Xerocomic acid lactone, an unsym-

[3] Obtained from Brinkmann Instruments Inc., Westbury, N. Y.
[4] Obtained from Alupharm Chemicals, New Orleans, La.

[5] UV and IR spectra were obtained with a Beckman UV spectrophotometer, model DB, and a Beckman IR spectrophotometer, model IR5A, Beckman Instruments, Inc., Fullerton, Calif. Mass spectra were determined with a Picker-AEI MS9 mass spectrometer, Picker Nuclear Division, White Plains, N. Y.

Table III—Weight of Culture Components and Pigment Fractions from 50 Roux Bottle Cultures of *P. atrotomentosus* Harvested at 56 Days

	Dry Weight, g.	Yield Based on Total Solids, %
Extracted mycelia	20.03	43.7
Solids from extracted nutrient broth	19.55	42.7
Initial ether extract	6.17	13.5
Partitioned crude pigment fraction	2.76	6.0
Xerocomic acid	2.07	4.5
Atromentic acid	0.055	0.001

metrical molecule, fragments initially into ions *m/e* 177 and 161, and these undergo the following fragmentations: *m/e* 177 → 149 → 121 → 75 and *m/e* 161 → 133 → 105 → 77.

The mass spectrum of the isolated pigment revealed ions at *m/e* 338, 310, 282, 226, 177, 161, 149, 133, 121, 105, 77, and 75. These observations agree completely with the known fragmentation patterns of xerocomic acid lactone. The high-resolution parent ion peak at *m/e* 338.0426, both observed and calculated for $C_{18}H_{10}O_7$, and other ionic fragments in the high-resolution mass spectrum further supported the identity of xerocomic acid lactone.

Confirmation of the identity of the major pigment as xerocomic acid was obtained by preparing an acetyl derivative, using an established procedure (6). The tetronic acid (50 mg.) was refluxed for 5 min. with 1 ml. of acetic anhydride and a trace of concentrated H_2SO_4. The yellow needles of triacetylxerocomic acid lactone, which separated upon cooling, were removed by filtration and recrystallized from glacial acetic acid. The properties of this acetyl derivative, m.p. 225°[6] (lit. 221–223°), UV spectrum $\lambda_{max.}^{acetone}$ 379 mμ and IR spectrum (KBr pellet) 1821, 1764, 1658, 1499, 1364, and 1205 cm.$^{-1}$, were generally consistent with those reported previously (6). The mass spectrum of the acetyl derivative revealed a parent ion of *m/e* 464, an initial loss in mass equivalent to three acetyl groups to give fragments *m/e* 338 and 43, and fragmentation patterns of *m/e* 338 which were identical to those observed for xerocomic acid lactone.

Identification of Atromentic Acid—The UV spectra $\lambda_{max.}^{ethanol}$ 391 and 257 mμ, $\lambda_{max.}^{water}$ 375 and 258 mμ, and $\lambda_{max.}^{0.01\ M\ NaHCO_3}$ 360 (sh), 320, and 238 mμ agreed with those of a reference sample of atromentic acid, the identity suggested for the minor pigment by the chromatographic and electrophoretic studies.

Atromentic acid lactone is symmetrical, and the mass spectral fragmentation patterns would be represented by *m/e* 322 → 294 → 266 → 238 → 210 and *m/e* 322→ 161 → 133 → 105 → 77. The mass spectrum of the isolated material was in complete agreement with these fragmentation patterns with ion peaks at *m/e* 322, 294, 266, 238, 210, 161, 133, 105, and 77. The high-resolution parent ion peak at *m/e* 322.1474, both observed and calculated for $C_{18}H_{10}O_6$, and other ionic fragments in the high-resolution mass spectrum provided additional evidence for the identity of atromentic acid lactone.

A crystalline acetyl derivative was not obtained due to the limited availability of the minor pigment, but a small quantity of the acetylated product was prepared and purified on a silica gel column using the ether–HCl solvent. The acetyl derivative was eluted from the chromatographic adsorbent, and this solution was used to obtain a mass spectrum. A parent ion was observed at *m/e* 406, as anticipated for diacetylatromentic acid lactone. Fragments equivalent to two acetyl substituents were lost to give an ion peak at *m/e* 322, and the fragmentation patterns of this product were identical to those observed for atromentic acid lactone.

Examination of Carpophores of *P. atrotomentosus* for Tetronic Acids—Fresh carpophores (5 kg.) of the mushroom were placed in a blender and reduced to a pulpy slurry. Sufficient water was added to maintain the fluidity of the slurry. The blended mixture was extracted exhaustively by shaking with several portions of ether and then with cold methanol. The ether and methanol extracts were studied using the various chromatographic and electrophoretic procedures previously described. Atromentin was readily detected in the ether extract and was observed to be present in the methanol extract. Using these experimental procedures, no evidence for the presence of pulvinic acid derivatives was noted in the limited quantity of mushrooms available.

[6] Determined with a Kofler micro hot-stage apparatus.

RESULTS AND DISCUSSION

A temperature of 20° and an incubation period of approximately 2 months were selected as standard conditions for growing the vegetative mycelium of *P. atrotomentosus* in surface cultures. The cultures were grown in the dark to prevent any light-induced destruction or transformation of the pigments, and precautions were employed during all experimental manipulations to minimize exposure to light. Concern for stability also prompted extraction of the pigments from a blended mixture of the fresh mycelium and nutrient broth. A number of fungal terphenylquinones (10) and pulvinic acid derivatives (5–7) are known to present stability problems under certain conditions, and avoidance of a drying process would lessen the probability of artifact formation of the type that has recently been suggested for amitenone in carpophores of *Suillus bovinus* (13). Bovinone was obtained as the major pigment when fresh carpophores of *S. bovinus* were extracted without the application of heat, and the amitenone present in the dried mushroom may be of chemical rather than biologic origin.

No atromentin was detected in the cultures of *P. atrotomentosus*. The water-insoluble portion of the initial ether extract and the ether-exhausted slurry were treated with a $NaHCO_3$ solution and reextracted to exclude the possibility that atromentin was present as its leuco form, which is known to account for a significant percentage of this terphenylquinone in carpophores of this species (9).

Chromatographic, electrophoretic, and UV and IR spectral data all suggested and supported the conclusion that the pigments isolated from the cultures were diphenyl-substituted tetronic acids. The mass spectra and mass spectral fragmentation patterns of the acids and their acetyl derivatives established the number and distribution of hydroxyl substituents. The major pigment in the cultures was xerocomic acid, and atromentic acid was isolated in a considerably lower concentration. These observations provide the first established formation of pulvinic acid derivatives by the vegetative growth phase of a basidiomycete.

Several hydroxylated pulvinic acid derivatives are known to occur in the fresh carpophores of some mushrooms (5–8), especially members of the Boletaceae. The Boletaceae and the Paxillaceae are recognized as being closely related (14), and the possibility that pigments represent chemotaxonomic links between the two families has been noted (5). The recent report of atromentin in carpophores of *S. bovinus* (13) and the discovery of pulvinic acid derivatives in cultures of *P. atrotomentosus* provide further experimental support for such chemotaxonomic relationships and for the close relationship between terphenylquinones and pulvinic acid derivatives.

The distinctive metabolic capabilities of carpophores and vegetative growth of *P. atrotomentosus* raise a very interesting question of comparative biochemistry. The extremely limited information on the biosynthetic relationships of terphenylquinones and tetronic acids (3) suggests that atromentin is a precursor of atromentic acid; this sequence would imply that the vegetative phase of this species has a more complete biosynthetic capability than the specialized fruiting body. Experimentally controlled carpophore development of this species in the laboratory has never been achieved, but vegetative cultures may offer a tool for clarification of biosynthetic relationships and some biologic control mechanisms.

REFERENCES

(1) M. E. Hale, Jr., "The Biology of Lichens," Edward Arnold Ltd., London, England, 1967, pp. 105–107.

(2) W. Karrer, "Konstitution und Vorkommen der organischen Pflanzenstoffe," Birkhäuser Verlag, Basel, Switzerland, 1958, pp. 444–448.

(3) W. S. G. Maass and A. C. Neish, *Can. J. Bot.*, **45,** 59(1967).

(4) R. G. Benedict and L. R. Brady, in "Fermentation Advances," Academic, New York, N. Y., 1969, pp. 63–88.

(5) P. C. Beaumont, R. L. Edwards, and G. C. Elsworthy, *J. Chem. Soc., C,* **1968,** 2968.

(6) W. Steglich, W. Furtner, and A. Prox, *Z. Naturforsch.*, **23B,** 1044(1968).

(7) R. L. Edwards and G. C. Elsworthy, *Chem. Commun.*, **1967,** 373.

(8) W. Steglich, W. Furtner, and A. Prox, *Z. Naturforsch.*, **24B**, 941(1969).

(9) F. Kögl, H. Becker, A. Detzel, and G. de Voss, *Ann. Chem.*, **465**, 211(1928).

(10) M. C. Gaylord, J. R. DeBoer, and L. R. Brady, *J. Pharm. Sci.*, **56**, 1069(1967).

(11) B. Loev and M. M. Goodman, *Chem. Ind. (London)*, **1967**, 2026.

(12) R. M. Letcher, *Org. Mass Spectrom.*, **1**, 805(1968).

(13) P. C. Beaumont and R. L. Edwards, *J. Chem. Soc., C*, **1969**, 2398.

(14) R. Singer, "The Agaricales in Modern Taxonomy," 2nd ed., J. Cramer, Weinheim, West Germany, 1962, p. 694.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle, WA 98105*

Accepted for publication May 26, 1970.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This investigation was supported in part by National Institutes of Health Research Grant GM 07515-10.

The authors wish to thank Dr. William S. Chilton, Department of Chemistry, University of Washington, for consultation on the electrophoretic studies and for access to specially designed electrophoretic equipment.

Application of Clearance and Volume of Distribution to the Plateau Principle of Drugs

SAMUEL A. CUCINELL* and WILLIAM PERL†

Abstract □ The concentration of drug in plasma after continuous administration (plateau concentration, C_∞) has been defined as the relationship of the dosage per unit time and the half-life of elimination ($t_{1/2}$) to the volume of distribution (V_d) of a drug. The C_∞ determinants, V_d and $t_{1/2}$, have been determined in single-dose and continuous-infusion experiments. The data derived have been used to predict C_∞ for a series of substances when administered by continuous intravenous infusion. Alterations in V_d and $t_{1/2}$ of a drug may occur under clinical situations. This is reflected in changes in the plateau concentration, despite a constant dosage per unit time. An experimental example of deoxycholic acid decreasing the volume of distribution of bromsulfophthalein is given.

Keyphrases □ Plateau principle—plasma drug concentration □ Deoxycholic acid effect—volume of distribution □ Volume of distribution—plasma concentration plateau □ Drug administration rate—plasma concentration plateau

Drugs are often administered by continuous or repeated administration over long enough periods so that a relatively constant, or plateau concentration, of drug in the plasma is achieved and maintained. This plateau concentration will, in large measure, determine the effectiveness or toxicity of a drug. Bishydroxycoumarin,[1] quinidine, the anesthetics, antibiotics, and digitalis depend on a constant drug level in plasma to maintain their desired clinical response. In many cases, the drug may be regarded as distributed in a so-called volume of distribution into which the drug is administered at a constant rate and out of which the drug is removed by first-order kinetics. This model leads to a relationship of the plateau concentration of the drug to its rate of administration, its volume of distribution, and its first-order rate constant of removal. This relationship has been called the plateau principle (1). The purpose of this paper is to report on various types of kinetic experiments which demonstrate the self-consistency and utility of the plateau principle.

[1] Dicumarol.

THEORY

The basic formulas of the one-compartment model are summarized for convenience.

Drug is infused into the body at a constant rate, I (mg./min.). Upon entering the body, the drug is assumed to equilibrate "instantaneously" among the various body tissues, so the quantity $Q(t)$ (mg.) of drug in the body at time t (min.) is expressed as

$$Q(t) = V_d C_p(t) \qquad \text{(Eq. 1)}$$

where V_d (ml.) is by definition the constant volume of distribution and $C_p(t)$ (mg./ml.) is the concentration of drug in plasma at time t. The rate of removal $L(t)$ (mg./min.) of drug from the body, either as separation from the body or loss of identity within the body, is assumed expressible by the first-order expression

$$L(t) = GC_p(t) \qquad \text{(Eq. 2)}$$

where G (ml./min.) is by definition a constant clearance. The mass balance equation for the drug is, by Eqs. 1 and 2,

$$V_d(dC_p/dt) = I - GC_p(t) \qquad \text{(Eq. 3)}$$

which has the solution starting from zero drug plasma concentration (1)

$$C_p(t) = C_{p\infty}(1 - e^{-kt}) \qquad \text{(Eq. 4)}$$

In Eq. 4, the plateau concentration $C_{p\infty}$ (mg./ml.) is given by

$$C_{p\infty} = I/G \qquad \text{(Eq. 5)}$$

and the rate constant k(min.$^{-1}$) is given by

$$k = G/V_d \qquad \text{(Eq. 6)}$$

Equations 5 and 6 give

$$C_{p\infty} = I/kV_d \qquad \text{(Eq. 7)}$$

Substitution of the half-life $t_{1/2} = 0.693/k$ into Eq. 7 gives

$$C_{p\infty} = It_{1/2}/0.693V_d \qquad \text{(Eq. 8)}$$

If, after the plateau concentration is reached (to a given accuracy), the infusion is suddenly stopped, the decay of drug plasma concentration is described by Eq. 3, with $I = 0$, as

$$-V_d(dC_p/dt) = GC_p(t) \qquad \text{(Eq. 9)}$$

with solution

$$C_p(t) = C_{p\infty}e^{-kt} \qquad \text{(Eq. 10)}$$

(8) W. Steglich, W. Furtner, and A. Prox, *Z. Naturforsch.*, **24B**, 941(1969).

(9) F. Kögl, H. Becker, A. Detzel, and G. de Voss, *Ann. Chem.*, **465**, 211(1928).

(10) M. C. Gaylord, J. R. DeBoer, and L. R. Brady, *J. Pharm. Sci.*, **56**, 1069(1967).

(11) B. Loev and M. M. Goodman, *Chem. Ind. (London)*, **1967**, 2026.

(12) R. M. Letcher, *Org. Mass Spectrom.*, **1**, 805(1968).

(13) P. C. Beaumont and R. L. Edwards, *J. Chem. Soc., C*, **1969**, 2398.

(14) R. Singer, "The Agaricales in Modern Taxonomy," 2nd ed., J. Cramer, Weinheim, West Germany, 1962, p. 694.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle, WA 98105*

Accepted for publication May 26, 1970.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This investigation was supported in part by National Institutes of Health Research Grant GM 07515-10.

The authors wish to thank Dr. William S. Chilton, Department of Chemistry, University of Washington, for consultation on the electrophoretic studies and for access to specially designed electrophoretic equipment.

Application of Clearance and Volume of Distribution to the Plateau Principle of Drugs

SAMUEL A. CUCINELL* and WILLIAM PERL†

Abstract □ The concentration of drug in plasma after continuous administration (plateau concentration, C_∞) has been defined as the relationship of the dosage per unit time and the half-life of elimination ($t_{1/2}$) to the volume of distribution (V_d) of a drug. The C_∞ determinants, V_d and $t_{1/2}$, have been determined in single-dose and continuous-infusion experiments. The data derived have been used to predict C_∞ for a series of substances when administered by continuous intravenous infusion. Alterations in V_d and $t_{1/2}$ of a drug may occur under clinical situations. This is reflected in changes in the plateau concentration, despite a constant dosage per unit time. An experimental example of deoxycholic acid decreasing the volume of distribution of bromsulfophthalein is given.

Keyphrases □ Plateau principle—plasma drug concentration □ Deoxycholic acid effect—volume of distribution □ Volume of distribution—plasma concentration plateau □ Drug administration rate—plasma concentration plateau

Drugs are often administered by continuous or repeated administration over long enough periods so that a relatively constant, or plateau concentration, of drug in the plasma is achieved and maintained. This plateau concentration will, in large measure, determine the effectiveness or toxicity of a drug. Bishydroxycoumarin,[1] quinidine, the anesthetics, antibiotics, and digitalis depend on a constant drug level in plasma to maintain their desired clinical response. In many cases, the drug may be regarded as distributed in a so-called volume of distribution into which the drug is administered at a constant rate and out of which the drug is removed by first-order kinetics. This model leads to a relationship of the plateau concentration of the drug to its rate of administration, its volume of distribution, and its first-order rate constant of removal. This relationship has been called the plateau principle (1). The purpose of this paper is to report on various types of kinetic experiments which demonstrate the self-consistency and utility of the plateau principle.

[1] Dicumarol.

THEORY

The basic formulas of the one-compartment model are summarized for convenience.

Drug is infused into the body at a constant rate, I (mg./min.). Upon entering the body, the drug is assumed to equilibrate "instantaneously" among the various body tissues, so the quantity $Q(t)$ (mg.) of drug in the body at time t (min.) is expressed as

$$Q(t) = V_d C_p(t) \qquad \text{(Eq. 1)}$$

where V_d (ml.) is by definition the constant volume of distribution and $C_p(t)$ (mg./ml.) is the concentration of drug in plasma at time t. The rate of removal $L(t)$ (mg./min.) of drug from the body, either as separation from the body or loss of identity within the body, is assumed expressible by the first-order expression

$$L(t) = GC_p(t) \qquad \text{(Eq. 2)}$$

where G (ml./min.) is by definition a constant clearance. The mass balance equation for the drug is, by Eqs. 1 and 2,

$$V_d(dC_p/dt) = I - GC_p(t) \qquad \text{(Eq. 3)}$$

which has the solution starting from zero drug plasma concentration (1)

$$C_p(t) = C_{p\infty}(1 - e^{-kt}) \qquad \text{(Eq. 4)}$$

In Eq. 4, the plateau concentration $C_{p\infty}$ (mg./ml.) is given by

$$C_{p\infty} = I/G \qquad \text{(Eq. 5)}$$

and the rate constant k(min.$^{-1}$) is given by

$$k = G/V_d \qquad \text{(Eq. 6)}$$

Equations 5 and 6 give

$$C_{p\infty} = I/kV_d \qquad \text{(Eq. 7)}$$

Substitution of the half-life $t_{1/2} = 0.693/k$ into Eq. 7 gives

$$C_{p\infty} = It_{1/2}/0.693V_d \qquad \text{(Eq. 8)}$$

If, after the plateau concentration is reached (to a given accuracy), the infusion is suddenly stopped, the decay of drug plasma concentration is described by Eq. 3, with $I = 0$, as

$$-V_d(dC_p/dt) = GC_p(t) \qquad \text{(Eq. 9)}$$

with solution

$$C_p(t) = C_{p\infty}e^{-kt} \qquad \text{(Eq. 10)}$$

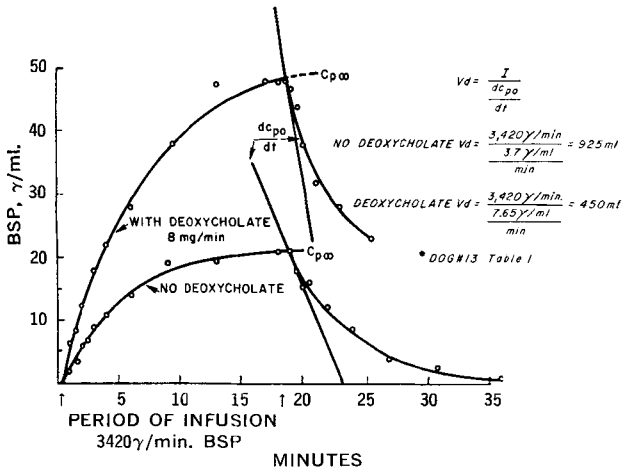


Figure 1—*Plasma BSP concentration with constant infusion.*

Just after stoppage of the infusion, the time rate of change of $C_p(t)$ changes discontinuously from the value zero just before stoppage to the value given by Eq. 9 as

$$-V_d(dC_p/dt)_0 = GC_p(0) = GC_{p\infty} \qquad \text{(Eq. 11)}$$

Substitution of G from Eq. 5 and solving for V_d give

$$V_d = -I/(dC_p/dt)_0 \qquad \text{(Eq. 12)}$$

Equations 12 and 8 yield

$$C_{p\infty} = -(dC_p/dt)_0 t_{1/2}/0.693 \qquad \text{(Eq. 13)}$$

The value of V_d from Eq. 12 applies only to a single-compartment model with monoexponential elimination. Although in practice this may be true for some drugs, additional compartments (multiexponential) may be observed, yielding a low value of V_d from Eq. 12 compared to Eq. 8 (2).

In the present constant-infusion experiments, the experimental data were I (exp.), $C_{p\infty}$ (exp.), $(dC_p/dt)_0$ (exp.), and $t_{1/2}$ (exp.) from the slope of the semilogarithmic plot of the decay curve after stoppage of the infusion (Fig. 1). $C_{p\infty}$ (exp.) was compared with the theoretical $C_{p\infty}$ obtained by Eq. 13 from $(dC_p/dt)_0$ (exp.) and $t_{1/2}$ (exp.) (Table I). Equivalently, the theoretical V_d obtained by Eq. 12 from I (exp.) and $(dC_p/dt)_0$ (exp.) was compared with the theoretical V_d obtained by Eq. 8 from I (exp.), $C_{p\infty}$ (exp.), and $t_{1/2}$ (exp.).

A series of sudden-injection experiments was also performed. Theoretical first-order kinetics (3) predicts that the response to sudden injection is essentially the time derivative of the response to constant infusion. Hence, in the present model, if the amount Q_0 of drug is suddenly injected, the plasma concentration will be

$$C_p(t) = C_{p0}e^{-kt} \qquad \text{(Eq. 14)}$$

where the zero-time intercept C_{p0} yields the volume of distribution V_d by

$$V_d = Q_0/C_{p0} \qquad \text{(Eq. 15)}$$

and the rate constant k is given by Eq. 6. The experimental data in these experiments were the amount injected Q_0 (exp.), the zero-time intercept C_{p0} of the straight-line fit of the semilogarithmically plotted plasma concentration against time, and the half-life $t_{1/2}$ (exp.) $= 0.693/k$ (exp.) from the slope of the semilogarithmic plot. Constant-infusion experiments on the same animals yielded the constant-infusion rate I (exp.) and the plateau concentration $C_{p\infty}$ (exp.). A comparison of theory and experiment was made by first calculating V_d by Eq. 15 from Q_0 (exp.) and C_{p0} (exp.) and then substituting this V_d and $t_{1/2}$ (exp.), I (exp.) into Eq. 8 to yield a $C_{p\infty}$ for comparison with $C_{p\infty}$ (exp.).

In some sudden-injection experiments, the plasma concentration decayed multiexponentially at first before becoming monoexponential (Fig. 2). In these cases the relation

$$V_d = Q_0/C_{p0}(1 + B) \qquad \text{(Eq. 16)}$$

was used instead of Eq. 15, where B is a correction derived in the *Appendix*.

In all experiments the clearance G was calculated by Eq. 6 (Table II).

If the drug is removed from the body primarily from one organ, then the rate of removal of drug can be expressed by the Fick principle as

$$L(t) = F[C_a(t) - C_v(t)] \qquad \text{(Eq. 17)}$$

where F (ml./min.) is the rate of blood flow through the organ, and $C_a(t)$ and $C_v(t)$ are the concentrations of drug, respectively, in the arterial blood inflow to and in the venous blood outflow from the organ. Equations 2 and 17 yield

$$G = \lambda EF \qquad \text{(Eq. 18)}$$

where

$$E = [C_a(t) - C_v(t)]/C_a(t) \qquad \text{(Eq. 19)}$$

is the so-called extraction ratio from whole blood and

$$\lambda = C_a(t)/C_p(t) \qquad \text{(Eq. 20)}$$

is the constant-partition coefficient of drug between whole blood and plasma.

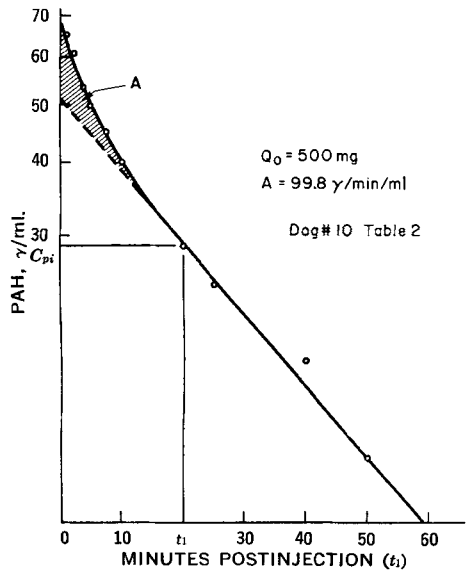


Figure 2—*Plasma decay of PAH after single dose.*

EXPERIMENTAL

Mongrel dogs of both sexes were anesthetized intravenously with pentobarbital, 50 mg./kg. Polyethylene catheters were placed in the forepaw and in the femoral veins. Isotonic solutions of bromsulfophthalein (BSP), antipyrine, *p*-aminohippuric (PAH) acid, and phenolsulfonphthalein (PSP) (all in saline) were used. In a constant-infusion experiment, the solution was infused into the paw vein at a constant rate by means of a Cambridge syringe pump. Blood was sampled from the catheter in the femoral vein into a heparinized syringe at intervals of 30 sec. for eight samples, every minute for the next four samples, and every 2 min. for the final four samples. The same schedule was followed for the single-injection experiments. There was never more than a 2% change in hematocrit throughout the experiment.

In those experiments in which two different infusions were made, the start of the second infusion was delayed until the plasma level of drug from the previous experiment had been zero for at least 2 hr. If this precaution was not taken, the BSP clearance often did not follow a first-order decay; instead the second infusion caused an almost linear rise in dye concentration throughout the 16-min. period of infusion. This suggests that the Tm for BSP had been exceeded and the rate of removal of BSP from plasma was no longer a first-order function but rather depended on liver capacity (4).

Values of V_d and k were obtained in each of six dogs by the single-injection technique. BSP (25 mg.), PSP (4.0 mg.), and PAH (500 mg.) were given intravenously as a single bolus in 5 ml. saline. Two

Table I—Continuous Infusion of BSP

Dog	Experimental			Calculated				
	I,[a] γ/min.	$t_{1/2}$,[b] min.	$C_{p\infty}$,[c] γ/ml.	$C_{p\infty}$,[d] γ/ml.	G,[e] ml./min.	V_d,[f] ml.	V_d,[g] ml.	Percent of Error of $C_{p\infty}$
4	5400	2.0	26	35	207	438	599	+25
7	9850	3.3	64	74	154	625	733	+6
9	9850	1.9	70	61	141	455	345	−8
8	9850	2.3	70	65	141	505	467	−7
10	3180	2.6	7.8	8.6	508	1370	1520	+10
	9600	2.9	27	31	355	1298	1480	+15
13	3420	3.9	21	21.5	163	925	930	−2
	3420[h]	4.0	48	45.5	71	450	440	−5
15	3820	4.0	58	57	66	382	380	−2
	3820[h]	4.2	65	77	59	300	356	+17
11	3420	4.0	26	39	131	513	759	+30
	3420[h]	3.9	42	62	81	316	458	+50

[a] I = rate of administration of BSP. [b] $t_{1/2}$ = half-life of plasma decay determined (from semilogarithmic plot) upon discontinuance of infusion. [c] $C_{p\infty}$ (experimental) = steady-state concentration (Fig. 2). [d] $C_{p\infty}$ (calculated) = obtained from $(dC_p/dt)_0$ and $t_{1/2}$ by Eq. 13. [e] G = determined from experimental values by Eq. 5. [f] V_d = volume of distribution; determined from the initial slope upon discontinuance of infusion, by Eq. 12. [g] V_d = determined from experimental values by Eq. 8. [h] Deoxycholic acid, 8 mg./min., infused with BSP.

hours after the plasma level of the test drug had reached zero, a constant infusion of the same drug was started.

In the case of antipyrine, the constant-infusion study was done 1 week after the single injection of 500 mg. The blood in the case of antipyrine was drawn every minute for the first four samples, every 5 min. for the next four samples, and every 15 min. for the next six samples.

The BSP and PSP were analyzed by diluting serum (1:3) with 0.001 *N* NaOH and reading the absorbance in a DU spectrophotometer at 560 mμ. The PAH and antipyrine were analyzed by the methods of Smith (5) and Brodie and Axelrod (6), respectively.

RESULTS

The V_d and $t_{1/2}$ were determined for a series of representative substances by both the single-injection and the continuous-infusion techniques. Data from the continuous infusion of BSP are shown in Table I. The $C_{p\infty}$ calculated from these data are in fair approximation with the experimentally observed $C_{p\infty}$. The clearance G, or volume freed of drug per unit time, was determined by Eq. 5. The volume of distribution, V_d, determined from Eq. 12 was in fair agreement with that determined from Eq. 8.

To demonstrate the consistency of these equations, deoxycholic acid was added to the infusion of BSP (Fig. 1). This caused an increase in $C_{p\infty}$ for the same dosage of BSP in the same dog which had received BSP alone 2 hr. previously. This change in $C_{p\infty}$ could have been due, by Eq. 8, to an increase in $t_{1/2}$ or a decrease in V_d. It is seen from Table I that V_d, determined from the initial slope, decreased and $t_{1/2}$ remained the same. The G must decrease by Eq. 6. Now G is also equal to $F(C_a - C_v)/C_a$, Eq. 18, with $\lambda = 1$. Either the blood flow or the fraction of BSP extracted from plasma must, therefore, have been depressed by deoxycholic acid to correspond with the depression of G. Since Demling (7), using a thermoelectric technique, was able to demonstrate that liver blood flow was not depressed by deoxycholate administration, the fraction of BSP removed from the plasma was probably decreased.

There is a tendency for the estimated $C_{p\infty}$ to be in excess of the experimental value (Table I). It may be that BSP has depots other than the plasma and liver which are more slowly filled. In this case the initial slope method, Eq. 12, would yield low values of V_d and, hence, by Eq. 8, too high values for $C_{p\infty}$.

In a similar manner, V_d and $t_{1/2}$ may be determined from Eqs. 14 and 15 by means of a single intravenous injection. Values of $C_{p\infty}$ predicted from Eq. 8, making use of V_d and $t_{1/2}$ obtained in this fashion, were in fair agreement with the $C_{p\infty}$ experimentally determined for BSP, PAH, PSP, and antipyrine by continuous infusion (Table II). The V_d and $t_{1/2}$ obtained from the single intravenous injection may be used to estimate the $C_{p\infty}$ of these substances during continuous administration. There is no consistent trend in the error of estimating $C_{p\infty}$ from the single-dose value of V_d and $t_{1/2}$.

Only in single-dose experiments with PAH was it necessary to correct for multiexponential kinetics by Eq. 16.

DISCUSSION

The clearance G determines, or is defined by, the plateau plasma concentration $C_{p\infty}$ in response to long-time, constant infusion of the drug at rate I (Eq. 5). It is clear that $C_{p\infty}$ can change, despite a constant I, if G changes. In the following, various pharmacological situations in which $C_{p\infty}$ changes are discussed in terms of G, V_d, and $t_{1/2}$. For convenience in visualization, the pair of fundamental parameters, G and V_d, may be replaced by the equivalent pair, $t_{1/2}$

Table II—Calculation of $C_{p\infty}$ from Data Obtained from Single Intravenous Injection

	V_d,[a] ml.	$t_{1/2}$,[b] min.	G,[c] ml./min.	$C_{p\infty}$, Predicted,[d] γ/ml.	I, mg./min.	$C_{p\infty}$, Experimental,[e] γ/ml.	$C_{p\infty}$, % Error
Dog 10 PAH	9400	25	250	43	10.6	53	−20
Dog 70 PAH	4600	12	264	62	16.5	66	−7
Dog 70 PSP	600	2.5	165	6.1	1.02	7.4	−19
Dog 205 BSP	300	4.5	46	72	3.30	63	+12
Dog 18 BSP	482	3.75	89	44	3.82	55	−20
Dog 14 Antipyrine	8500	100	59	58	3.4	65	−10

[a] V_d, determined from zero-time intercept after single injection by Eq. 15 or 16. [b] $t_{1/2}$, half-time of semilogarithmic plot of decay curve. [c] G, determined from V_d and $t_{1/2}$ by Eq. 6 with $k = 0.693/t_{1/2}$. [d] $C_{p\infty}$ (predicted), calculated from Eq. 8. [e] $C_{p\infty}$ (experimental), determined by constant infusion in each dog 2 hr. after completion of single dose.

Table III—Change in Values of G with Dose before and after Induction of Drug-Metabolizing Enzymes[a]

Dog	Wt, kg.	V_d, l. 50 mg./kg.	V_d, l. 20 mg./kg.	$t_{1/2}$, hr. 50 mg./kg.	$t_{1/2}$, hr. 20 mg./kg.	G, l./hr. 50	G, l./hr. 20
6	14	12	10.5	5.5	2.8	1.5	2.6 prestimulation
		15	14	2.0	1.8	5.3	5.5 poststimulation
1	10.2	10.2	5.7	7.7	1.5	1.0	2.6 prestimulation
		13.5	7.4	2.0	1.3	4.7	4.1 poststimulation
2	11.5	8.6	7.7	6.0	2.2	1.0	2.4 prestimulation
		9.6	8.0	2.5	1.8	2.7	3.0 poststimulation

[a] All data obtained from single injection.

and V_d, where by Eq. 6

$$t_{1/2} = 0.693\ V_d/G \qquad \text{(Eq. 21)}$$

For convenience in interpretation, the pair $t_{1/2}$ and V_d are assumed to be independent variables experimentally as well as theoretically. This is probably rarely if ever qualitatively correct, but one or the other predominates quantitatively.

To illustrate the utility of the concept of volume of distribution, the displacement of drugs from plasma binding sites by competing substances in the plasma may be interpreted as an increase in volume. Sulfa drugs displacing bilirubin from plasma binding sites may precipitate kernicteris in the newborn at relatively low plasma concentrations of bilirubin (8). Phenylbutazone increases the antibacterial activity of the sulfa drugs (9). In these cases, the pharmacological activity or toxicity of the drug is increased while the plasma concentration is decreased. In terms of Eq. 15, V_d has increased, causing C_{p0} to decrease at constant Q_0.

The volume of distribution may also be made to decrease. In this case, another drug may compete for binding sites in an organ other than the plasma. Thus, in Table I, deoxycholic acid when infused with BSP increased $C_{p\infty}$ and decreased V_d while $t_{1/2}$ remained unchanged. This implies (Eq. 21) a decrease in G which means that the drug is not being removed from the circulation as fast as the control, despite a constant $t_{1/2}$. Weiner *et al.* (10) have shown that methandrostenolone in man is capable of increasing the $C_{p\infty}$ of oxyphenbutazone while the $t_{1/2}$ remains unchanged. The implication that V_d is thereby decreased (Eq. 21) would indicate that methandrostenolone is capable of displacing oxyphenbutazone from extravascular binding sites. Norethandrolone and iopanoic acid appear capable of inhibiting liver storage and excretion of BSP in man (11). It is possible that this is a reflection of competition for liver binding sites. This is a case of V_d decreasing at constant I and $t_{1/2}$. Hence G is decreased (Eq. 21), which causes $C_{p\infty}$ to increase (Eq. 5).

Whereas V_d may be altered while the $t_{1/2}$ remains unchanged, the opposite may also occur. $C_{p\infty}$ may change with $t_{1/2}$ while V_d remains constant. The liver enzyme systems that metabolize a large number of drugs may be stimulated by phenobarbital or a number of other compounds (12). These enzymes will break down diphenylhydantoin, bishydroxycoumarin, and antipyrine at an increased rate after phenobarbital pretreatment. For example, an individual receiving an intravenous injection of a drug has a much longer $t_{1/2}$ before receiving phenobarbital than after receiving phenobarbital for a sufficient time. In terms of Eq. 21, G has increased.

It has been noticed that this effect is more readily demonstrated with a high dose of drug than with a low dose (13). The data in Table III demonstrate the differences observed in V_d and $t_{1/2}$ when a dog is given a 50-mg./kg. and a 20-mg./kg. dose of diphenylhydantoin intravenously. G at 20 mg./kg. was higher (2.6 l./hr.) than at 50 mg./kg. (1.5 l./hr.). Assuming that liver blood flow was not affected by the diphenylhydantoin, the fraction E of drug cleared from the blood was higher at the lower dose than at the higher dose (Eq. 18). After pretreatment of the dogs with barbiturates, the G for both dosage levels had increased (about 4 l./hr.), although the $t_{1/2}$ at the low dose was unchanged. This suggests that fraction E of the drug removed by the liver was no longer dose dependent. Also, it is clear that the induction of drug-metabolizing enzymes is prominent at low as well as high dosage levels in terms of an increase in G. The induction at the lower dose level manifests itself more as an increase in both V_d and G than as a decrease in $t_{1/2}$.

The $t_{1/2}$ may also be increased, forcing an elevation of $C_{p\infty}$, without an increase in I. Bishydroxycoumarin is capable of inhibiting the metabolism of diphenylhydantoin, thereby prolonging the $t_{1/2}$ and potentiating the effect of the anticonvulsant (14). V_d tends to remain unchanged. Thus, there is a decrease in G (Eq. 21), which in turn would cause an increase in the level of $C_{p\infty}$ (Eq. 5).

SUMMARY

The plateau principle is experimentally described for BSP, PAH, PSP, and antipyrine in terms of the volume of distribution (V_d), the half-life of plasma decay ($t_{1/2}$), and the rate of administration (I).

The plasma concentration of a drug may be altered by changes in V_d or $t_{1/2}$ despite a constant I. Deoxycholic acid infusion increased $C_{p\infty}$ for BSP by decreasing the V_d, while $t_{1/2}$ remained constant. $C_{p\infty}$ may be predicted by prior determination of V_d and $t_{1/2}$ for drugs with approximately monoexponential decay kinetics.

APPENDIX

For a single-injection experiment, a better approximation of the volume of distribution than Eq. 15 is given by

$$V_d = \left\{Q_0 - G_0 \int_0^{t_1} C_p(t)\, dt\right\} / C_{pi} \qquad \text{(Eq. A1)}$$

where C_{pi} is the drug plasma concentration at time t_1 on the final monoexponential portion of the plasma decay curve (Fig. 2). The numerator in Eq. A1 takes account of the drug which is lost from the body, by Eq. 2, at the actual (elevated) plasma concentration before the distribution of drug corresponding to the final monoexponential behavior has occurred. If the final monoexponential $C_{p0}\, e^{-kt}$ is added and subtracted to $C_p(t)$ in Eq. A1 and the integration

$$\int_0^{t_1} C_{p0}\, e^{-kt}\, dt = (C_{p0} - C_{pi})/k \qquad \text{(Eq. A2)}$$

is carried out, the result is

$$V_d C_{pi} = Q_0 - GA - G(C_{p0} - C_{pi})/k \qquad \text{(Eq. A3)}$$

where A is the area between the actual plasma decay curve and the monoexponential curve back-extrapolated to time zero (cross-hatched area in Fig. 2 measured on a linear plot). Substitution of Eq. 6 for G in Eq. A3 and solving for V_d yield

$$V_d = \frac{Q_0}{C_{p0} + kA} = \frac{Q_0}{C_{p0}(1 + B)} \qquad \text{(Eq. A4)}$$

where

$$B = kA/C_{p0} = 0.693A/t_{1/2}C_{p0} \qquad \text{(Eq. A5)}$$

REFERENCES

(1) A. Goldstein, L. Aronow, and S. M. Kalman, "Principles of Drug Action," Harper and Row, New York, N. Y., 1968.

(2) S. Riegelman, J. Loo, and M. Rowland, *J. Pharm. Sci.*, **57**, 128(1968).

(3) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell, Waltham, Mass., 1966.

(4) H. O. Wheeler, J. I. Meltzer, and S. E. Bradley, *J. Clin. Invest.*, **39**, 236(1960).

(5) H. Smith, "Principles of Renal Physiology," Oxford University Press, Oxford, England, 1956.

(6) B. B. Brodie and J. Axelrod, *J. Biol. Chem.*, **179**, 25(1949).
(7) L. Demling, "Thermoelektrische Durschblutringsmissumgen in der menschlichen Leber. Vortr. geh. Internat. Kongr. Gastrointerologie, Leindein, 1960," as quoted by H. Henning and L. Demling, in "Progress in Liver Diseases," vol. 1, Grune and Stratton, 1961.
(8) G. B. O'Dell, *J. Clin. Invest.*, **38**, 823(1959).
(9) A. H. Anton, *J. Pharm. Exp. Ther.*, **134**, 291(1961).
(10) W. Weiner, A. A. Siddiqui, R. T. Shahani, and P. G. Dayton, *Proc. Soc. Exp. Biol. Med.*, **124**, 1170(1967).
(11) L. J. Schoenfield and W. Foulk, *J. Clin. Invest.*, **43**, 1419 (1964).
(12) A. K. Conney, *Pharm. Rev.*, **19**, 317(1967).
(13) P. G. Dayton, S. A. Cucinell, M. Weiss, and J. M. Perel, *J. Pharm. Exp. Ther.*, **158**, 305(1967).
(14) J. M. Hansen, M. Kristensen, L. Shovsted, and L. K. Christensen, *Lancet*, 265 (July 30, 1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 3, 1969, from the *New York University Research Service, Goldwater Memorial Hospital, Welfare Island, N. Y.*

Accepted for publication May 26, 1970.

Supported in part by U. S. Public Health Service Research Grant HE 07482 from the National Heart Institute and by Grants U 1579 and U 1761 of the Health Research Council of New York and Grant GM 14186-01.

*Present address: Department of Medicine, Division of Clinical Pharmacology, Emory University School of Medicine, Atlanta, GA 30303

†Present address: Department of Medicine, New Jersey College of Medicine and Dentistry, Newark, NJ 07103

Application of Molecular Sieve Technique in Solubilization Studies of Benzoic Acid in Solutions of Cetomacrogol 1000

M. DONBROW, E. AZAZ, and R. HAMBURGER

Abstract □ The applicability of a new technique in solubilization studies, using a molecular sieve, was tested on a system consisting of benzoic acid and cetomacrogol 1000 (cetostearyl ether of polyoxyethylene) in aqueous solutions. The data obtained are in good agreement with those found by other methods. Some advantages of the method are outlined.

Keyphrases □ Benzoic acid—solubilization study □ Cetomacrogol 1000 solution—benzoic acid solubility □ Micellar solubilization, benzoic acid in cetomacrogol 1000—quantitative determination □ Molecular sieve technique—solubilization study □ Spectrophotometry, UV, visual—analysis

The importance of theoretical and pharmaceutical aspects of micellar solubilization of drugs has been well recognized in recent years (1–4). Nevertheless, only three methods are available in routine practice for quantitative investigation of the phenomenon.

The conventional solubility method has been used by almost all investigators either as a basic tool or comparatively. Its main disadvantage is that it is limited to saturated systems; hence the dependence of solubilization on the concentration of unbound solubilizate cannot be studied by this method. Another major disadvantage lies in the fact that many additives decrease the cloud point (4, 5). This means that although turbidity is often used as a criterion for saturation with liquid solubilizates, it is not necessarily an indication of maximum solubility (6–10). Furthermore, the determination of the turbidimetric end-point is subject to error.

Equilibrium dialysis was first introduced into solubilization studies by Patel and Kostenbauder (11). This method solved the problems encountered in the solubility method. Although widely accepted, it is time consuming and requires preliminary work on the selection of a proper membrane for each system. Nylon membranes used by Patel and Foss (12) are stated to swell and bind phenolic compounds; rubber membranes tried by Matsumoto *et al.* (13) varied in thickness; and methylcellulose membranes are attacked by certain surfactants (14).

Potentiometric titration was first used for solubilization studies of organic acids and bases by Donbrow and Rhodes (15–17). Although rapid and elegant enough to have been adopted for routine use (15–23), it is restricted to ionizing solubilizates in which only the unionized form undergoes micellar solubilization. It is thus unsuitable for studies on the solubilization of acids and bases of pronounced amphiphilic properties such as local anesthetics or acid derivatives of steroidal hormones, their ionized form, as well as the unionized, being solubilized (24).

In view of the increasing importance of studies on nonsaturated systems, additional methods of a more general nature and greater scope than potentiometric titration and quicker than equilibrium dialysis would be advantageous. Such methods would also be valuable for crosschecking.

A molecular sieve technique which promised to meet these needs, has in fact been applied to methyl *p*-hydroxybenzoate by Ashworth and Heard (25). Dextran gel, with a suitable degree of crosslinking, was used in a static way similar to a semipermeable membrane in dialysis. The small molecules (the solute) are distributed between the swollen gel and the external liquid, while the surfactant is unable to penetrate the internal gel phase and remains in the external liquid. The solute distribution normally follows a linear relation. (See also Eqs. 5 and 6 in *Results and Discussion*, and Eq. 1 in *Experimental*.)

The object of the present work is to broaden the applicability of this method by testing it on another

(6) B. B. Brodie and J. Axelrod, *J. Biol. Chem.*, **179**, 25(1949).
(7) L. Demling, "Thermoelektrische Durschblutringsmissumgen in der menschlichen Leber. Vortr. geh. Internat. Kongr. Gastrointerologie, Leindein, 1960," as quoted by H. Henning and L. Demling, in "Progress in Liver Diseases," vol. 1, Grune and Stratton, 1961.
(8) G. B. O'Dell, *J. Clin. Invest.*, **38**, 823(1959).
(9) A. H. Anton, *J. Pharm. Exp. Ther.*, **134**, 291(1961).
(10) W. Weiner, A. A. Siddiqui, R. T. Shahani, and P. G. Dayton, *Proc. Soc. Exp. Biol. Med.*, **124**, 1170(1967).
(11) L. J. Schoenfield and W. Foulk, *J. Clin. Invest.*, **43**, 1419 (1964).
(12) A. K. Conney, *Pharm. Rev.*, **19**, 317(1967).
(13) P. G. Dayton, S. A. Cucinell, M. Weiss, and J. M. Perel, *J. Pharm. Exp. Ther.*, **158**, 305(1967).
(14) J. M. Hansen, M. Kristensen, L. Shovsted, and L. K. Christensen, *Lancet*, 265 (July 30, 1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 3, 1969, from the *New York University Research Service, Goldwater Memorial Hospital, Welfare Island, N. Y.*

Accepted for publication May 26, 1970.

Supported in part by U. S. Public Health Service Research Grant HE 07482 from the National Heart Institute and by Grants U 1579 and U 1761 of the Health Research Council of New York and Grant GM 14186-01.

*Present address: Department of Medicine, Division of Clinical Pharmacology, Emory University School of Medicine, Atlanta, GA 30303

†Present address: Department of Medicine, New Jersey College of Medicine and Dentistry, Newark, NJ 07103

Application of Molecular Sieve Technique in Solubilization Studies of Benzoic Acid in Solutions of Cetomacrogol 1000

M. DONBROW, E. AZAZ, and R. HAMBURGER

Abstract □ The applicability of a new technique in solubilization studies, using a molecular sieve, was tested on a system consisting of benzoic acid and cetomacrogol 1000 (cetostearyl ether of polyoxyethylene) in aqueous solutions. The data obtained are in good agreement with those found by other methods. Some advantages of the method are outlined.

Keyphrases □ Benzoic acid—solubilization study □ Cetomacrogol 1000 solution—benzoic acid solubility □ Micellar solubilization, benzoic acid in cetomacrogol 1000—quantitative determination □ Molecular sieve technique—solubilization study □ Spectrophotometry, UV, visual—analysis

The importance of theoretical and pharmaceutical aspects of micellar solubilization of drugs has been well recognized in recent years (1–4). Nevertheless, only three methods are available in routine practice for quantitative investigation of the phenomenon.

The conventional solubility method has been used by almost all investigators either as a basic tool or comparatively. Its main disadvantage is that it is limited to saturated systems; hence the dependence of solubilization on the concentration of unbound solubilizate cannot be studied by this method. Another major disadvantage lies in the fact that many additives decrease the cloud point (4, 5). This means that although turbidity is often used as a criterion for saturation with liquid solubilizates, it is not necessarily an indication of maximum solubility (6–10). Furthermore, the determination of the turbidimetric end-point is subject to error.

Equilibrium dialysis was first introduced into solubilization studies by Patel and Kostenbauder (11). This method solved the problems encountered in the solubility method. Although widely accepted, it is time consuming and requires preliminary work on the selection of a proper membrane for each system. Nylon membranes used by Patel and Foss (12) are stated to swell and bind phenolic compounds; rubber membranes tried by Matsumoto *et al.* (13) varied in thickness; and methylcellulose membranes are attacked by certain surfactants (14).

Potentiometric titration was first used for solubilization studies of organic acids and bases by Donbrow and Rhodes (15–17). Although rapid and elegant enough to have been adopted for routine use (15–23), it is restricted to ionizing solubilizates in which only the unionized form undergoes micellar solubilization. It is thus unsuitable for studies on the solubilization of acids and bases of pronounced amphiphilic properties such as local anesthetics or acid derivatives of steroidal hormones, their ionized form, as well as the unionized, being solubilized (24).

In view of the increasing importance of studies on nonsaturated systems, additional methods of a more general nature and greater scope than potentiometric titration and quicker than equilibrium dialysis would be advantageous. Such methods would also be valuable for crosschecking.

A molecular sieve technique which promised to meet these needs, has in fact been applied to methyl *p*-hydroxybenzoate by Ashworth and Heard (25). Dextran gel, with a suitable degree of crosslinking, was used in a static way similar to a semipermeable membrane in dialysis. The small molecules (the solute) are distributed between the swollen gel and the external liquid, while the surfactant is unable to penetrate the internal gel phase and remains in the external liquid. The solute distribution normally follows a linear relation. (See also Eqs. 5 and 6 in *Results and Discussion*, and Eq. 1 in *Experimental*.)

The object of the present work is to broaden the applicability of this method by testing it on another

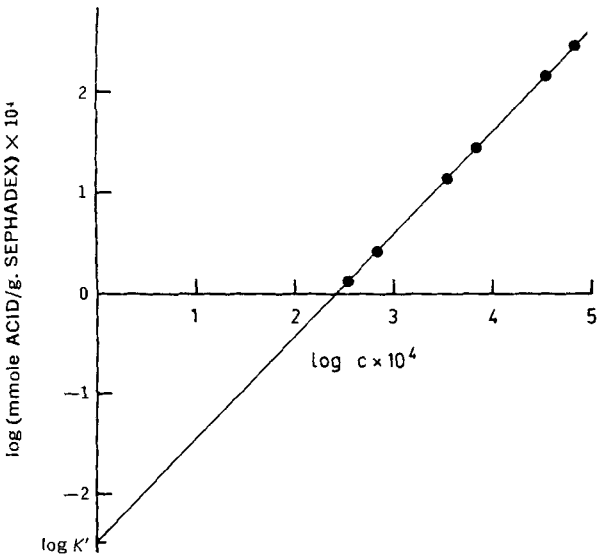


Figure 1—*Log–log plot of distribution of benzoic acid between Sephadex G25-fine and aqueous 0.005* N *HCl at 25°. (See Eq. 1 in* Experimental.)

system, benzoic acid in cetomacrogol solutions, for which data for comparison with other methods are available (18, 19, 21, 22, 26).

EXPERIMENTAL

Materials—Cetomacrogol 1000 BPC[1] was used (n_D^{60}, 1.451; n_D^{25} for 20% w/v solution, 1.360). The material, dried at 60° in vacuum, gave by combustion analysis the following results: C, 58.66; H, 9.62; O, 31.72. Assuming a molecular weight of 1300, this ratio fits the formula: $CH_3(CH_2)_{15}(OCH_2CH_2)_{24}OH$. Benzoic acid, analytical grade; dextran gel;[2] and Sephadex G25-fine[2] were also used. Water regain equalled 2.5 ± 0.2 g./g. dry gel. Particle size, 20–80 μ, was confirmed microscopically.

Procedure—*Determination of Internal Solvent Volume*—Ten milliliters of 0.1% solution of dextran blue 2,000,000[2] was added to about 3.5-g. samples of the gel swollen in 15 ml. 0.05 *N* NaCl. After the samples were shaken for 1 hr. at 25°, they were decanted and centrifuged. The concentration of the dye was determined by measuring the absorbance at 620 mμ directly, without further dilution. The water regain was measured on three samples of the gel. The mean value of a given batch was found to be 2.33 g./g. dry gel (± 0.05 g./g. dry gel).

Determination of K' *(Distribution Coefficient of Benzoic Acid between Aqueous 0.005* N *HCl and Sephadex G25-Fine)*—Assuming a linear distribution of benzoic acid between the gel and the external phase (Eq. 1) and aiming at equal amounts of benzoic acid outside and inside the gel (Eq. 2), the optimal weight of dry gel for a given external volume was calculated from Eq. 3:

$$M = CK' \qquad \text{(Eq. 1)}$$

$$MW = CK'W = CV \qquad \text{(Eq. 2)}$$

or

$$W = \frac{V}{K'} \qquad \text{(Eq. 3)}$$

where M = mmoles of benzoic acid bound to 1 g. gel; C = concentration of the free acid in the external phase in mmoles; W = weight of the dry gel in grams; K' = the distribution coefficient in (mmoles acid/g. gel)/(mmoles acid/l.); and V = aqueous external volume in liters = $V_t - 2.33\ W$ (where V_t is total aqueous volume and 2.33 g./g. dry gel is water regain of the gel).

Substituting K' of 0.0045 l./g., as approximated from the preliminary experiment, and external volume (V) of 0.002 l., suitable quantities of Sephadex (W) were about 0.6 g. The samples were weighed accurately from weighing bottles into known volumes of aqueous 0.005 *N* HCl (thus minimizing moisture uptake from the air). This concentration of HCl was maintained in all subsequent stages of the work to suppress the ionization of benzoic acid. The gel was allowed to swell at room temperature for 3 hr. Solutions of benzoic acid of varying concentration were added and made up to constant volume of 0.004 l.(V_t). The samples were shaken at 25° for 1 hr.

After equilibration, aliquot portions of the external phase were decanted, diluted suitably with 0.005 *N* HCl, and centrifuged to remove any fine particles present. The absorbance was read spectrophotometrically at the 230 or 273-mμ maximum. Blanks were prepared using the same procedure but omitting the benzoic acid (Sephadex was found to release traces of UV absorbing materials.)

Distribution of Benzoic Acid between Cetomacrogol 1000 and Aqueous Solutions of 0.005 N *HCl*—The procedure and amounts were identical with those used in the previous stage, except that cetomacrogol 1000 as well as benzoic acid was added from stock solutions to the conditioned gel. The concentration of cetomacrogol in the samples was 2.3–2.6% (varying with the weight of dry gel). Equilibration and analysis were carried out as previously. At the dilutions of surfactant studied, Beer's law was observed. The $\lambda_{max.}$ and $\epsilon_{max.}$ were as in water.

The optimum amount of dry gel (W) for a given external volume (V') in this case may be calculated from Eq. 4, aiming to equalize the amount of benzoic acid outside and inside the gel:

$$CWK' = CV' + CGK'' \qquad \text{(Eq. 4a)}$$

or

$$W = \frac{1}{K'}(V' + GK'') \qquad \text{(Eq. 4b)}$$

where C, K', V, and W are as defined for Eq. 1; V' = external volume corrected for micelle partial volume (27) = $V - 0.84\ G$; G = weight of micelles in sample in grams; and $K'' = K_1K_2$ = apparent distribution coefficient of benzoic acid between cetomacrogol and

Table I—Change in Concentration of Benzoic Acid Solution Shaken with Sephadex G25-fine at 25° with Time[a]

Time, hr.	Benzoic Acid Concentration, mmoles
0	0.145[b]
0.25	0.115
1	0.115
24	0.115
96	0.115
120	0.101
144	0.068
168	0.064
192	0.050
264	0.000

[a] Total aqueous volume 50 ml., Sephadex about 3.5 g. [b] Hypothetical, calculated from total acid added.

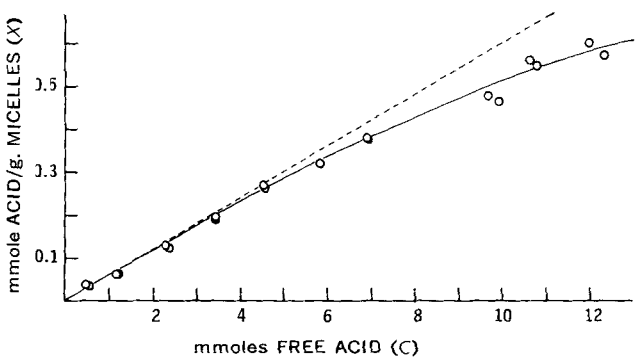


Figure 2—*Adsorption isotherm of benzoic acid in 2% w/v cetomacrogol and 0.005* N *HCl at 25°. (See text for description.) Key:* ——, *experimental isotherm,* X = $(K_1K_2C)/(1 + K_1C)$; *and* - - -, *slope of the initial portion of the isotherm* X = K_1K_2C.

[1] Marketed as "Texofor AIP," by Glovers Chemicals Ltd., Wortley Low Mills, Leeds, England.
[2] Pharmacia, Uppsala, Sweden.

water in l./g., neglecting its decreasing value with rising concentration (see *Results and Discussion*).

RESULTS AND DISCUSSION

From Fig. 1, plotted in log–log form for convenience, it is evident that the uptake of benzoic acid by Sephadex follows a linear relation over the range studied, and that the assumption made in Eq. 1 is valid.

The value of 4.46×10^{-3} l./g. found for K', the distribution coefficient, was reproducible over a wide concentration range ($1 \times 10^{-2} - 1M$), provided that the equilibration time was limited. If the period of contact was extended above 96 hr. (Table I), benzoic acid reacted further with the gel. Such reactions have been observed for other aromatic and heterocyclic compounds (28).

With regard to the solubilization of benzoic acid in cetomacrogol 1000, some typical results are plotted in Fig. 2. These results clearly demonstrate that, as already reported (19, 22), there is a deviation from linear distribution.

The values of C and X (Fig. 2) were calculated from Eqs. 5 and 6, respectively:

$$C_0V = C_fV + K'CW \qquad \text{(Eq. 5}a\text{)}$$

$$C = \frac{(C_0 - C_f)V}{K'W} \qquad \text{(Eq. 5}b\text{)}$$

$$C_fV = CV' + GX \qquad \text{(Eq. 6}a\text{)}$$

$$X = \frac{C_fV - CV'}{G} \qquad \text{(Eq. 6}b\text{)}$$

where C, G, K', V, V', and W are as defined earlier (Eqs. 1–4*b*); C_0 = initial concentration of benzoic acid in mM; C_f = final total concentration of benzoic acid outside the gel in mM; and X = mmoles of benzoic acid bound/1 g. micelles.

The results plotted in Fig. 2 may be represented by use of Langmuir's equation (Eq. 7):

$$X = \frac{K_1K_2C}{1 + K_1C} \qquad \text{(Eq. 7)}$$

where X = mmoles acid bound to 1 g. surfactant; C = concentration of free acid in mM; and K_1 and K_2 = adsorption parameters in l./mmoles and mmoles/g., respectively.

By means of the reciprocal form of Langmuir's equation (Eq. 8):

$$\frac{1}{X} = \frac{1}{K_2} + \frac{1}{K_1K_2C} \qquad \text{(Eq. 8)}$$

linearity was obtained over the full range (Fig. 3).

Unfortunately, the individual values of K_1 and K_2 could not be obtained by graphical methods. As can be seen from Fig. 3, the intercept ($1/K_2$) in the plot is very small and K_2 is subject to a very large error. This shortcoming has been pointed out previously (22).

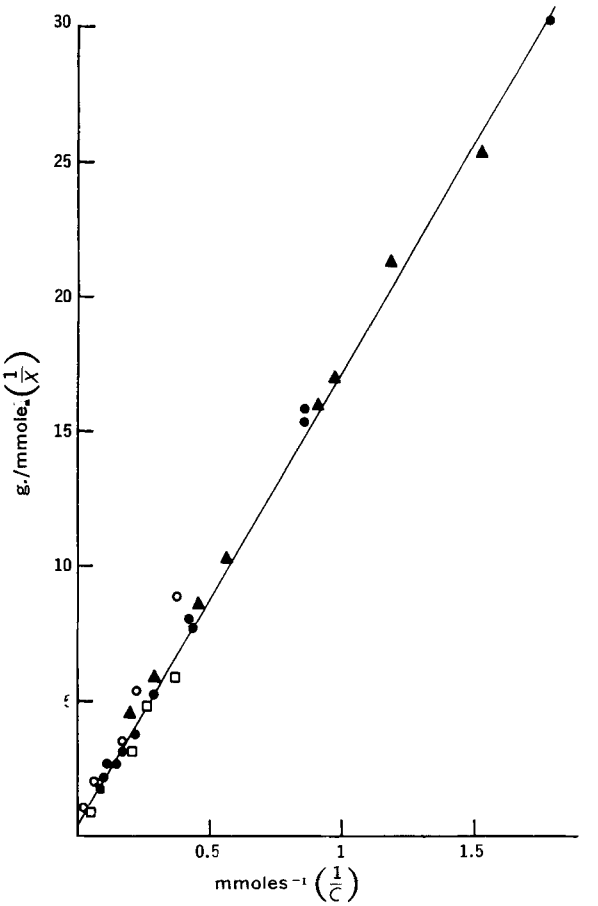


Figure 3—*Adsorption isotherm of benzoic acid in cetomacrogol at 25° from data obtained by different methods:*

$$\frac{1}{X} = \frac{1}{K_2} + \frac{1}{K_1K_2C}$$

Key: ●, *molecular sieve, present work;* ▲, *Donbrow and Azaz (21);* □, *Mitchell and Brown (18); and* ○, *Donbrow* et al. *(19, 22).*

To overcome this difficulty, a X/C *versus* X plot was tried using the rearranged form of Langmuir's equation (Eq. 9):

$$X/C = K_1K_2 - K_1X \qquad \text{(Eq. 9)}$$

The slope was, however, very small, giving a large error in K_1.

Values of K_1 and K_2 were, in fact, calculated statistically from a

Table II—Comparison of Adsorption Parameters Obtained by Molecular Sieve Method with Those Obtained by Other Methods

K_1K_2, l./g.						
Statistical Mean,[a] $K_1K_2 \times 10^2$	Standard Deviation, $S \times 10^2$	Range of Individual Runs, $K_1K_2 \times 10^2$	Maximal Concn. of Free Acid, mmoles	Cetomacrogol Concn., % w/v	Methods Used	References
4.4[b]	0.046	4.2–4.8	29.2	4–12	Potentiometric titrations and solubility	19, 22
5.9[c]	0.32	4.8–6.5	20.2	1.3–13	Potentiometric titrations	18[c]
5.6[d]	0.055	4.4–6.9	10.73	19	Potentiometric titrations	21
6.05[e]	0.17	—[e]	12.5	2.2–2.6	Molecular sieve	Present work (Fig. 3)

[a] Data were treated by the least-squares method using a Control Data Corp. computer to obtain the range of the individual runs. The means reported were combined parameters obtained by using all the individual results in the least-squares calculations. [b] The published data (19) were corrected for surfactant partial volume (27), replotted, and treated sta tistically. [c] These values were calculated from the potentiometric titration data of Mitchell and Brown (18) by changing the units and substituting into Langmuir's equation (Eq. 8). A plot of $1/x$ *versus* $1/c$ gave a straight line, from the slope of which the value of K_1K_2 was found to be 5.9×10^{-2} l./g. In the original publication, results were reported as distribution coefficients. The solubility method gave the lowest distribution coefficient (4.8×10^{-2} l./g.) as would be expected. Results within the same range were obtained by these authors by pH measurements (one point titration) and the equilibrium dialysis method. [d] The experimental procedure was similar to the one used previously (19, 22). [e] All repeated experiments were included.

very large number of results and will be reported (21). For the objective of this present work, the product of the two parameters $K_1K_2 = K''$, which can be determined accurately from either type of plot (Eq. 8 or 9), is sufficient to characterize the system. This combined parameter can be regarded as a linear distribution coefficient for a limited range of benzoic acid concentration in cetomacrogol 1000 solutions (Fig. 2, straight-line extrapolation from initial points). The concept of K'' as a distribution coefficient is derived from Eq. 7 which, at low values of the binding constant K_1 or low concentrations of adsorbate ($K_1C \ll 1$), approaches $X = K_1K_2C = K''C$.

Table II shows that the value of 6.05×10^{-2} for K'' by the molecular sieve method is of the same order as values obtained by other methods; the standard deviation, which falls within the range previously obtained, is acceptable. Since the method was found to be rapid and reproducible, it should prove to be suitable for routine solubilization studies, not only for benzoic acid in nonionic surfactants such as cetomacrogol 1000 but also for other unsaturated solubilized systems.

REFERENCES

(1) B. A. Mulley, in "Advances in Pharmaceutical Sciences," vol. I, A. H. Beckett and J. E. Carless, Eds., Academic, London, England, and New York, N. Y., 1964.

(2) J. Swarbrick, *J. Pharm. Sci.*, **54,** 1229(1965).

(3) L. Sjoblom, in "Solvent Properties of Surfactant Solutions," K. Shinoda, Ed., Marcel Dekker, New York, N. Y., 1967.

(4) T. Nakagawa, in "Nonionic Surfactants," N. J. Schick, Ed., Marcel Dekker, New York, N. Y., 1967.

(5) N. H. J. Weiden and L. B. Norton, *J. Colloid Sci.*, **8,** 606 (1953).

(6) T. Higuchi and J. L. Lach, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 465(1954).

(7) D. Guttman and T. Higuchi, *ibid.*, **45,** 659(1956).

(8) B. A. Mulley and A. D. Metcalf, *J. Pharm. Pharmacol.*, **8,** 774(1956).

(9) A. G. Mitchell and L. S. C. Wan, *J. Pharm. Sci.*, **53,** 1467(1964).

(10) A. G. Mitchell, *J. Pharm. Pharmacol.*, **15,** 761(1963).

(11) N. K. Patel and H. B. Kostenbauder, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 289(1958).

(12) N. K. Patel and N. E. Foss, *J. Pharm. Sci.*, **53,** 94(1964).

(13) H. Matsumoto, H. Matsumura, and S. Iguchi, *Chem. Pharm. Bull.*, **14,** 385(1966).

(14) M. Donbrow, unpublished data.

(15) M. Donbrow and C. T. Rhodes, *J. Pharm. Pharmacol.*, **15,** 233(1963).

(16) *Ibid.*, **17,** 258(1965).

(17) M. Donbrow and C. T. Rhodes, *Int. Kongr. Pharm. Wiss. Vortr. Originalmitt. M.*, **1963,** 397(1964).

(18) A. G. Mitchell and K. F. Brown, *J. Pharm. Pharmacol.*, **18,** 115(1966).

(19) M. Donbrow and C. T. Rhodes, *J. Chem. Soc., Suppl.* 2, **1964,** 6166.

(20) M. Donbrow and J. Jacobs, *J. Pharm. Pharmacol.*, **18,** 92S (1966).

(21) M. Donbrow and E. Azaz, to be published.

(22) M. Donbrow, P. Molyneux, and C. T. Rhodes, *J. Chem. Soc., A.*, **1967,** 561.

(23) W. P. Evans, *J. Pharm. Pharmacol.*, **16,** 323(1964).

(24) E. G. Rippie, D. J. Lamb, and P. W. Romig, *J. Pharm. Sci.*, **53,** 1346(1964).

(25) R. W. Ashworth and D. D. Heard, *J. Pharm. Pharmacol.*, **18,** 98S(1966).

(26) N. K. Patel and N. E. Foss, *J. Pharm. Sci.*, **54,** 1495(1965).

(27) A. T. Florence, *J. Pharm. Pharmacol.*, **18,** 384(1966).

(28) B. J. Gellote, *J. Chromatogr.*, **3,** 330(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 24, 1969, from the *Pharmacy Department, Hebrew University School of Pharmacy, Jerusalem, Israel.*

Accepted for publication June 3, 1970.

Some Factors Affecting Release and Availability of Drugs from Hard Gelatin Capsules

B. J. McGEE, D. R. KENNEDY, and G. C. WALKER

Abstract □ Acetylsalicylic acid, acetylsalicylic acid and lactose, and acetylsalicylic acid and dibasic calcium phosphate were administered orally in No. 3 and No. 4 hard gelatin capsules to rabbits, using a Latin square design. Plasma levels were determined at 1-, 2-, and 3-hr. intervals; an analysis of variance showed that at hour 3, there was a difference between the average for the No. 3 and No. 4 capsules, irrespective of excipient. The significantly better plasma levels obtained with the more tightly compacted No. 4 capsules may be due to the diffusion of gastric juice through the gelatin which created higher pressure within the capsules. Dissolution determinations were made using the Levy beaker method and the oscillating tube method. The mean plasma levels at the end of 1, 2, and 3 hr. were lower than the concentrations obtained at the corresponding hours from the *in vitro* study, and direct correlation of the two sets of data could not be made. Hard gelatin capsules containing acetylsalicylic acid alone broke down slowly *in vitro*.

Keyphrases □ Capsules, hard gelatin—drug release, availability □ *In vivo–in vitro* release rates—drug from capsules □ Aspirin release, capsules—excipient effect □ Gelatin capsule size effect—aspirin release rates

Encapsulation remains a popular method for administering medication because of the general view that capsules readily break down upon ingestion to release the enclosed medicament (1). It was thought desirable to investigate the effects of two different excipients and the effects of two different pressures of fill on the rate of absorption of acetylsalicylic acid (ASA) from hard gelatin capsules in the rabbit. ASA is reported to be absorbed rapidly from all parts of the gastrointestinal tract and may serve as a "marker" to assess the effect of formulation and dosage form characteristics on absorption rate (1). *In vitro* dissolution

very large number of results and will be reported (21). For the objective of this present work, the product of the two parameters $K_1K_2 = K''$, which can be determined accurately from either type of plot (Eq. 8 or 9), is sufficient to characterize the system. This combined parameter can be regarded as a linear distribution coefficient for a limited range of benzoic acid concentration in cetomacrogol 1000 solutions (Fig. 2, straight-line extrapolation from initial points). The concept of K'' as a distribution coefficient is derived from Eq. 7 which, at low values of the binding constant K_1 or low concentrations of adsorbate ($K_1C \ll 1$), approaches $X = K_1K_2C = K''C$.

Table II shows that the value of 6.05×10^{-2} for K'' by the molecular sieve method is of the same order as values obtained by other methods; the standard deviation, which falls within the range previously obtained, is acceptable. Since the method was found to be rapid and reproducible, it should prove to be suitable for routine solubilization studies, not only for benzoic acid in nonionic surfactants such as cetomacrogol 1000 but also for other unsaturated solubilized systems.

REFERENCES

(1) B. A. Mulley, in "Advances in Pharmaceutical Sciences," vol. I, A. H. Beckett and J. E. Carless, Eds., Academic, London, England, and New York, N. Y., 1964.

(2) J. Swarbrick, *J. Pharm. Sci.*, **54,** 1229(1965).

(3) L. Sjoblom, in "Solvent Properties of Surfactant Solutions," K. Shinoda, Ed., Marcel Dekker, New York, N. Y., 1967.

(4) T. Nakagawa, in "Nonionic Surfactants," N. J. Schick, Ed., Marcel Dekker, New York, N. Y., 1967.

(5) N. H. J. Weiden and L. B. Norton, *J. Colloid Sci.*, **8,** 606 (1953).

(6) T. Higuchi and J. L. Lach, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 465(1954).

(7) D. Guttman and T. Higuchi, *ibid.*, **45,** 659(1956).

(8) B. A. Mulley and A. D. Metcalf, *J. Pharm. Pharmacol.*, **8,** 774(1956).

(9) A. G. Mitchell and L. S. C. Wan, *J. Pharm. Sci.*, **53,** 1467(1964).

(10) A. G. Mitchell, *J. Pharm. Pharmacol.*, **15,** 761(1963).

(11) N. K. Patel and H. B. Kostenbauder, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 289(1958).

(12) N. K. Patel and N. E. Foss, *J. Pharm. Sci.*, **53,** 94(1964).

(13) H. Matsumoto, H. Matsumura, and S. Iguchi, *Chem. Pharm. Bull.*, **14,** 385(1966).

(14) M. Donbrow, unpublished data.

(15) M. Donbrow and C. T. Rhodes, *J. Pharm. Pharmacol.*, **15,** 233(1963).

(16) *Ibid.*, **17,** 258(1965).

(17) M. Donbrow and C. T. Rhodes, *Int. Kongr. Pharm. Wiss. Vortr. Originalmitt. M.*, **1963,** 397(1964).

(18) A. G. Mitchell and K. F. Brown, *J. Pharm. Pharmacol.*, **18,** 115(1966).

(19) M. Donbrow and C. T. Rhodes, *J. Chem. Soc., Suppl.* 2, **1964,** 6166.

(20) M. Donbrow and J. Jacobs, *J. Pharm. Pharmacol.*, **18,** 92S (1966).

(21) M. Donbrow and E. Azaz, to be published.

(22) M. Donbrow, P. Molyneux, and C. T. Rhodes, *J. Chem. Soc., A.*, **1967,** 561.

(23) W. P. Evans, *J. Pharm. Pharmacol.*, **16,** 323(1964).

(24) E. G. Rippie, D. J. Lamb, and P. W. Romig, *J. Pharm. Sci.*, **53,** 1346(1964).

(25) R. W. Ashworth and D. D. Heard, *J. Pharm. Pharmacol.*, **18,** 98S(1966).

(26) N. K. Patel and N. E. Foss, *J. Pharm. Sci.*, **54,** 1495(1965).

(27) A. T. Florence, *J. Pharm. Pharmacol.*, **18,** 384(1966).

(28) B. J. Gellote, *J. Chromatogr.*, **3,** 330(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 24, 1969, from the *Pharmacy Department, Hebrew University School of Pharmacy, Jerusalem, Israel.*

Accepted for publication June 3, 1970.

Some Factors Affecting Release and Availability of Drugs from Hard Gelatin Capsules

B. J. McGEE, D. R. KENNEDY, and G. C. WALKER

Abstract □ Acetylsalicylic acid, acetylsalicylic acid and lactose, and acetylsalicylic acid and dibasic calcium phosphate were administered orally in No. 3 and No. 4 hard gelatin capsules to rabbits, using a Latin square design. Plasma levels were determined at 1-, 2-, and 3-hr. intervals; an analysis of variance showed that at hour 3, there was a difference between the average for the No. 3 and No. 4 capsules, irrespective of excipient. The significantly better plasma levels obtained with the more tightly compacted No. 4 capsules may be due to the diffusion of gastric juice through the gelatin which created higher pressure within the capsules. Dissolution determinations were made using the Levy beaker method and the oscillating tube method. The mean plasma levels at the end of 1, 2, and 3 hr. were lower than the concentrations obtained at the corresponding hours from the *in vitro* study, and direct correlation of the two sets of data could not be made. Hard gelatin capsules containing acetylsalicylic acid alone broke down slowly *in vitro*.

Keyphrases □ Capsules, hard gelatin—drug release, availability □ *In vivo–in vitro* release rates—drug from capsules □ Aspirin release, capsules—excipient effect □ Gelatin capsule size effect—aspirin release rates

Encapsulation remains a popular method for administering medication because of the general view that capsules readily break down upon ingestion to release the enclosed medicament (1). It was thought desirable to investigate the effects of two different excipients and the effects of two different pressures of fill on the rate of absorption of acetylsalicylic acid (ASA) from hard gelatin capsules in the rabbit. ASA is reported to be absorbed rapidly from all parts of the gastrointestinal tract and may serve as a "marker" to assess the effect of formulation and dosage form characteristics on absorption rate (1). *In vitro* dissolution

Table I—Average Concentrations Obtained in Dissolution Determinations of No. 3 Capsules Containing ASA[a]

Time, min.	Average Concentration,[b] mg./100 ml. Hydrochloric Acid–Water, pH 1.2	Simulated Gastric Fluid	Simulated Intestinal Fluid
10	0	1.0	0
20	0.8	3.3	2.5
30	3.5	10.0	7.8
45	6.8	13.3	13.0
60	10.0	15.3	15.0
90	13.8	16.5	17.3
120	16.0	17.3	18.3
180	18.0	20.0	20.0
240	19.8		

[a] Oscillating tube method, 100-mesh screen. [b] Average of three determinations.

tests were carried out to complement the *in vivo* work to determine if any correlation existed between the two sets of data.

METHODS AND PROCEDURES

ASA BP was used in all cases (British Drug Houses, 20-mesh crystals). The excipients were water-soluble α-lactose and the relatively water-insoluble dibasic calcium phosphate, both of which are used as fillers and diluents in capsules as well as tablets.

The rabbit was used as the test animal. Each rabbit received all four of the following combinations, as well as the control ASA at weekly intervals: ASA alone in a No. 3 capsule, ASA and lactose in No. 3 and No. 4 capsules, ASA and dibasic calcium phosphate in No. 3 and No. 4 capsules. Ten rabbits were used in two 5 × 5 Latin squares, and one rabbit died during the treatment schedule. An analysis of variance was carried out, ignoring the "week" as if the data were from a randomized block experiment and omitting Rabbit 5 from which two observations were missing.

It was determined that 200 mg. of ASA would produce measurable plasma salicylate levels and that 200 mg. of ASA and 66.6 mg. of excipient could be contained in both No. 3 and No. 4 empty gelatin capsules (Parke-Davis), thus affording a relatively greater compaction pressure in the No. 4 capsule. No. 3 capsules containing 200 mg. of ASA alone were also used, because it was felt that the same crystals without excipient or compaction would serve to point out any differences between excipients or compaction pressures. Ten capsules of each combination and size were selected at random and assayed individually according to the method of Routh and Dryer (2). The largest difference between the mean mixture weights of the combination variables was found to be 0.0003 g. For practical purposes the weights may be considered identical.

***In Vitro* Tests**—Dissolution-rate studies were begun using the Levy and Hayes (3) "beaker method," so that the low agitation intensity which Levy regards as important for *in vitro* dissolution-rate measurements to reflect *in vivo* conditions might be achieved (4). This method was found to be unsatisfactory for use with capsules; it was necessary to use the "oscillating tube" method which involves a relatively high agitation intensity as the dissolution fluid is forced through a 100-mesh screen on the bottom of a Plexiglas cylinder (4). Dissolution rates were determined for the No. 3 capsules containing ASA alone using the Levy and Hayes beaker method and the oscillating tube method with distilled water adjusted to pH 1.2 with hydrochloric acid as the dissolution medium. Dissolution rates for each combination variable were determined using the oscillating tube method and either gastric fluid, simulated test solution USP, or intestinal fluid, simulated test solution USP (5) as the dissolution medium. The intrinsic dissolution of the ASA crystals was determined in each simulated test solution using the oscillating tube method. The samples at the end of each time period were assayed by the method of Routh and Dryer (2) (Tables I–IV).

Table II—Average Concentrations Obtained in Dissolution Determinations of No. 3 and No. 4 Capsules Containing ASA and Lactose[a]

Time, min.	Average Concentration,[b] mg./100 ml. No. 3 Capsule SGF	No. 3 Capsule SIF	No. 4 Capsule SGF	No. 4 Capsule SIF
10	5.3	1.0	15.3	8.5
20	10.5	10.0	16.1	16.3
30	13.3	12.0	17.5	19.9
45	14.3	15.5	18.1	
60	16.3	19.8	18.9	
90	18.5		19.1	
120	19.5		19.4	
180	19.8		19.5	

[a] Oscillating tube method, 100-mesh screen. [b] Average of three determinations: SGF, simulated gastric fluid; and SIF, simulated intestinal fluid.

Table III—Average Concentrations Obtained in Dissolution Determinations of No. 3 and No. 4 Capsules Containing ASA and Dibasic Calcium Phosphate[a]

Time, min.	Average Concentration,[b] mg./100 ml. No. 3 Capsule SGF	No. 3 Capsule SIF	No. 4 Capsule SGF	No. 4 Capsule SIF
10	6.8	3.5	7.8	8.8
20	16.5	14.8	19.8	19.6
30	19.0	19.8		
45	19.8			

[a] Oscillating tube method, 100-mesh screen. [b] Average of three determinations: SGF, simulated gastric fluid; and SIF, simulated intestinal fluid.

***In Vivo* Tests**—The rabbit was mildly sedated using halothane. A gag was placed between the teeth, and the capsule was placed well back in the throat and washed down with a little water. Blood samples were withdrawn from the marginal ear vein at 1-, 2-, and 3-hr. intervals after administration of the capsule. The samples were collected in calibrated centrifuge tubes and centrifuged, and the liquid was used for analysis. Plasma blank values were subtracted from the sample values. The plasma was analyzed according to the method of Routh and Dryer (2).

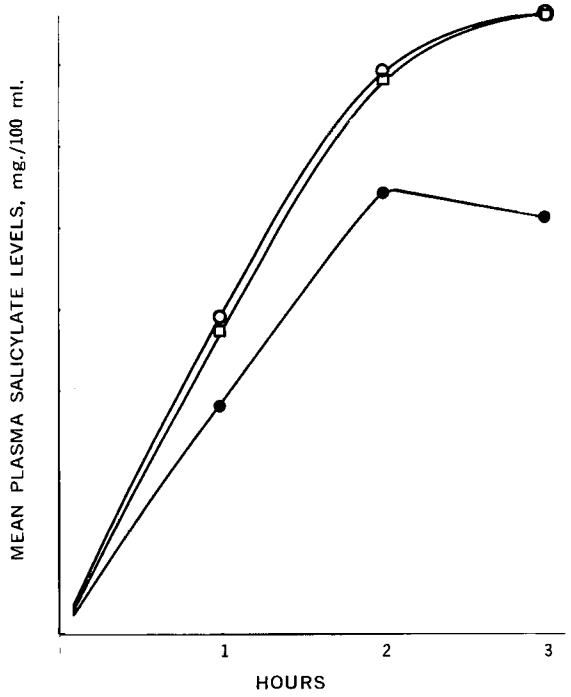


Figure 1—*Mean plasma salicylate levels after administration of ASA in No. 3 and No. 4 hard gelatin capsules. Key:* ○, *ASA + lactose (No. 4);* □, *ASA (No. 3); and* ●, *ASA + lactose (No. 3).*

Table IV—Dissolution of 20-Mesh ASA Crystals in Both Gastric Fluid, Simulated Test Solution and Intestinal Fluid, Simulated Test Solution[a]

Time, min.	Average Concentration,[b] mg./100 ml. SGF	SIF
10	11.5	19.3
20	12.5	20.3
30	13.0	
45	16.3	
60	18.8	
90	19.5	
120	19.8	
180	19.4	

[a] Oscillating tube method, 100-mesh screen. [b] Average of three determinations: SGF, simulated gastric fluid; and SIF, simulated intestinal fluid.

RESULTS AND DISCUSSION

Table V and Figs. 1 and 2 show the mean plasma salicylate levels in mg./100 ml. obtained at 1, 2, and 3 hr. after administration of the capsule. The mean plasma salicylate values were low. A multiple regression analysis[1] (6), in which there was no detailed examination of differences among the five treatment means, showed no suggestion of week differences. An analysis of variance (Table VI) showed that by the 3rd hour, the highly compacted combinations in No. 4 capsules were producing better plasma levels than the No. 3 capsules, with or without excipients.

The better plasma salicylate levels obtained with the tightly compacted No. 4 capsules may be due to the diffusion of gastric juice through the gelatin, which created a higher osmotic pressure within the capsules, thereby leading to more rapid breakdown and absorption. It is difficult to explain the excellent *in vivo* performance of the ASA in a No. 3 capsule.

Under the low agitation conditions of the Levy beaker method, a No. 3 capsule containing ASA alone floated in the hydrochloric acid–water solution for 2 hr. before settling to the bottom of the beaker. Figure 3 presents sketches of the appearance of the capsule at various times. The concentration of ASA was found to be 5.5 mg./100 ml. at the end of 4 hr. A small amount of pepsin was added to the beaker, and the thin gelatin bag broke within 1 min. The beaker method using distilled water, adjusted to pH 1.2 with hydrochloric acid, was felt to be unsatisfactory for dissolution determinations with capsules. A modified Levy–Hayes method, using weighted capsules to prevent floating, has been suggested for overcoming the results experienced in this study (7).

Using the oscillating tube method, the hard gelatin capsules showed distortion and did not appear to break down readily even in the presence of the digestive enzyme used and the relatively high agitation intensities of the oscillating tube method. The average concentration (three determinations) of ASA at the end of 4 hr. was 19.8 mg./100 ml. using hydrochloric acid–water (pH 1.2) as the dissolution medium and 20.0 mg./100 ml. after 3 hr. using gastric fluid, simulated test solution. Intrinsic dissolution determinations showed that ASA dissolves well when unencumbered by gelatin.

The mean plasma salicylate levels at the end of 1, 2, and 3 hr. were far lower than the concentrations obtained at the corresponding hours in the *in vitro* study, so that any attempt to correlate the

Table V—Mean Plasma Salicylate Levels from the Determination of Plasma Salicylate Concentration in Rabbits at 1, 2, and 3 hr. following Administration of ASA Combinations in Hard Gelatin Capsules

Dosage Form	Mean Plasma Salicylate Levels, mg./100 ml. Hour 1	Hour 2	Hour 3
ASA in No. 3 capsules	3.7	6.8	7.6
ASA plus lactose in No. 3 capsules	2.8	5.4	5.1
ASA plus lactose in No. 4 capsules	3.9	6.9	7.6
ASA plus dibasic calcium phosphate in No. 3 capsules	3.9	6.6	6.9
ASA plus dibasic calcium phosphate in No. 4 capsules	4.9	7.0	8.3

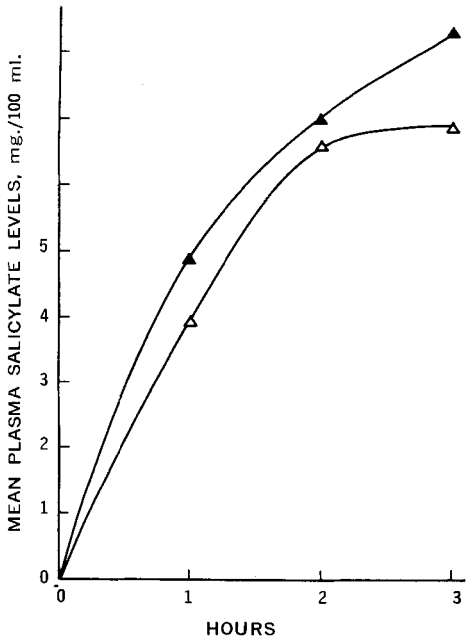


Figure 2—*Mean plasma salicylate levels after administration of ASA in No. 3 and No. 4 hard gelatin capsules. Key: △, ASA + dibasic calcium phosphate (No. 3); and ▲, ASA + dibasic calcium phosphate (No. 4).*

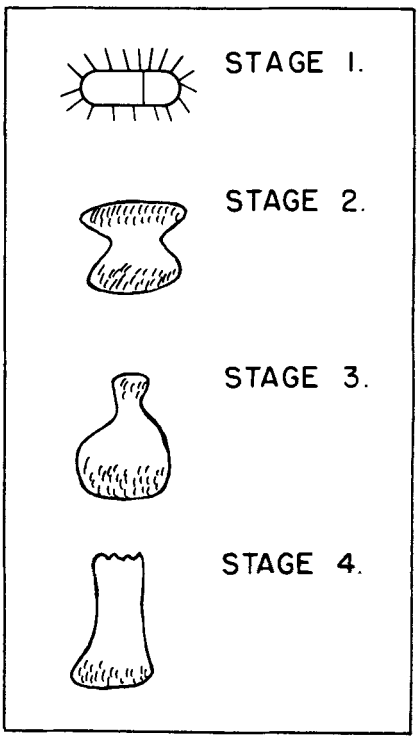


Figure 3—*Appearance of capsule at various stages using the Levy and Hayes beaker method. Stage 1, whiskers or needles, 3 min.; Stage 2, mushroom, 8–10 min.; Stage 3, pycnidium, 25–120 min.; and Stage 4, cylinder, over 120 min.*

[1] Thanks are extended to Professor D. W. Reid and Mrs. Bazoki of the Department of Epidemiology and Biometrics, School of Hygiene, University of Toronto, for their suggestions regarding the experimental design and analysis of the data obtained.

Table VI—Analysis of Data from Plasma Level Determinations

	Hour 1		Hour 2		Hour 3	
	df	*MS*	*df*	*MS*	*df*	*MS*
Control *vs.* average of others	1	0.31	1	0.63	1	2.89
Capsules	1	8.70	1	8.12	1	34.41[a]
Fillers	1	9.71	1	3.93	1	14.95
Capsules + fillers	1	0.01	1	2.94	1	3.00
Rabbits	8	6.70	8	22.18[b]	8	16.03[a]
Error	32	3.86	32	5.83	32	5.50

[a] Significant at the 5% level. [b] Significant at the 1% level.

two sets of data was considered of little value. A rank order correlation may, however, be observed in that the mean plasma salicylate values, as well as dissolution values, are higher for the more highly compacted No. 4 capsules and in the case of dibasic calcium phosphate. The low plasma salicylate levels may be due to a failure to break down *in vivo*. The *in vitro* agitation intensities resulting from the dissolution medium being forced through the 100-mesh screen were reported by Levy (4) to be far greater than the agitation encountered in the stomach, a fact that could account for the widespread difference in concentration levels between the two studies.

In vitro observations showed that the ASA became moist; over a period of time the gelatin stretched and, together with the mechanical action of striking against the 100-mesh screen, assisted in the breakdown. The stretching effect was not as noticeable in the case of capsules containing excipients.

The low plasma salicylate levels obtained *in vivo* together with the *in vitro* behavior of the capsules would seem to indicate that the gelatin in the hard gelatin capsules had been modified in some manner, either by the acidity of the gastric juice, or by the acidity of the ASA (pKa 3.5) within the microenvironment of the capsule, or by a combination of the two. If due to the weakly acidic drug alone or to a combination of drug and gastric juice, this could be of importance for other drugs of similar properties when encapsulated. If due to the acidity of the gastric juice alone, it would appear that the release of any drug enclosed in hard gelatin capsules could be affected adversely. Gelatin is obtained by heat denaturation of collagen and is built of three strands, which are joined primarily by hydrogen bonding between the strands (8). It is possible that the stretching of these strands accounted for the appearance of the hard gelatin capsules in the *in vitro* tests and perhaps for the low plasma salicylate levels.

REFERENCES

(1) G. Levy, *J. Pharm. Sci.*, **50**, 388(1961).

(2) J. I. Routh and R. L. Dryer, "Standard Methods of Clinical Chemistry," vol. 3, Academic, New York, N. Y., 1959, pp. 194–199.

(3) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262**, 1053(1960).

(4) G. Levy, *J. Pharm. Sci.*, **52**, 1039(1963).

(5) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 1072.

(6) G. W. Snedecor, "Statistical Methods," 5th ed., Iowa State University Press, Ames, Iowa, 1956, p. 237.

(7) J. W. Poole, "Some Experiences in the Evaluation of Formulation Variables on Drug Availability," Proceedings of the DIA Symposium on Formulation Factors Affecting Therapeutic Performance of Drug Products, **3**, 8(1969).

(8) A. White, P. Handler, and E. Smith, "Principles of Biochemistry," 3rd ed., The Blakiston Division, McGraw-Hill, New York, N. Y., 1964, pp. 768–769.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 20, 1969, from the *Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada.*

Accepted for publication May 5, 1970.

The authors express their thanks to Smith Kline & French Inter-American Corp. for financial support during this investigation.

Factors Influencing Solvolysis of Corticosteroid-21-phosphate Esters

G. L. FLYNN and D. J. LAMB

Abstract □ The solvolysis of methylprednisolone-21-phosphate in dilute aqueous solution (<0.005 *M*) is qualitatively similar to that observed for methylphosphate and other simple monoalkyl phosphates, particularly in the pH range 3–8. In more concentrated solutions (>0.02 *M*), however, there is an acceleration of reaction velocities and marked deviation from the expected pH dependency. This change in chemical behavior is attributed to association colloid formation. Support for this mechanism is drawn from hydrolysis-rate data obtained as a function of concentration and independently determined critical micelle concentration values.

Keyphrases □ Corticosteroid-21-phosphate esters—solvolysis, micelle formation □ Solvolysis, corticosteroid-21-PO_4 esters—pH profile, activation energy, aggregation effect □ Critical micelle concentration determination—conductivity, surface-tension methods □ Phosphate, inorganic—analysis

The term phosphate ester is extremely ambiguous because it applies to several distinctly different classes of compounds, each characterized by unique chemical behavior. Furthermore, chemistries in each phosphate ester class can be appreciably different both qualitatively and quantitatively, depending on the types and proximities of neighboring atoms within the molecule. Additional complexity arises from the biformity of the carbon–oxygen–phosphorus linkage. Solvolysis, for instance, may entail carbon–oxygen cleavage, phosphorus–oxygen splitting, or both, depending on the reaction conditions (1).

The hydrolyses of monoalkyl phosphates are characterized by these diversities. The prototype of this phosphate ester class is methylphosphate. It is a relatively simple molecule whose aqueous stability has been extensively studied (2). At pH values below zero, the conjugate acid species predominates, and the solvolysis takes place with both carbon–oxygen and phosphorus–oxygen splitting. The neutral molecule is the principal species in pH range 0–2, and it cleaves exclusively at the carbon–oxygen bond. Above pH 3, the monoanion,

Table VI—Analysis of Data from Plasma Level Determinations

	Hour 1		Hour 2		Hour 3	
	df	*MS*	*df*	*MS*	*df*	*MS*
Control *vs.* average of others	1	0.31	1	0.63	1	2.89
Capsules	1	8.70	1	8.12	1	34.41[a]
Fillers	1	9.71	1	3.93	1	14.95
Capsules + fillers	1	0.01	1	2.94	1	3.00
Rabbits	8	6.70	8	22.18[b]	8	16.03[a]
Error	32	3.86	32	5.83	32	5.50

[a] Significant at the 5% level. [b] Significant at the 1% level.

two sets of data was considered of little value. A rank order correlation may, however, be observed in that the mean plasma salicylate values, as well as dissolution values, are higher for the more highly compacted No. 4 capsules and in the case of dibasic calcium phosphate. The low plasma salicylate levels may be due to a failure to break down *in vivo*. The *in vitro* agitation intensities resulting from the dissolution medium being forced through the 100-mesh screen were reported by Levy (4) to be far greater than the agitation encountered in the stomach, a fact that could account for the widespread difference in concentration levels between the two studies.

In vitro observations showed that the ASA became moist; over a period of time the gelatin stretched and, together with the mechanical action of striking against the 100-mesh screen, assisted in the breakdown. The stretching effect was not as noticeable in the case of capsules containing excipients.

The low plasma salicylate levels obtained *in vivo* together with the *in vitro* behavior of the capsules would seem to indicate that the gelatin in the hard gelatin capsules had been modified in some manner, either by the acidity of the gastric juice, or by the acidity of the ASA (pKa 3.5) within the microenvironment of the capsule, or by a combination of the two. If due to the weakly acidic drug alone or to a combination of drug and gastric juice, this could be of importance for other drugs of similar properties when encapsulated. If due to the acidity of the gastric juice alone, it would appear that the release of any drug enclosed in hard gelatin capsules could be affected adversely. Gelatin is obtained by heat denaturation of collagen and is built of three strands, which are joined primarily by hydrogen bonding between the strands (8). It is possible that the stretching of these strands accounted for the appearance of the hard gelatin capsules in the *in vitro* tests and perhaps for the low plasma salicylate levels.

REFERENCES

(1) G. Levy, *J. Pharm. Sci.*, **50**, 388(1961).

(2) J. I. Routh and R. L. Dryer, "Standard Methods of Clinical Chemistry," vol. 3, Academic, New York, N. Y., 1959, pp. 194–199.

(3) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262**, 1053(1960).

(4) G. Levy, *J. Pharm. Sci.*, **52**, 1039(1963).

(5) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 1072.

(6) G. W. Snedecor, "Statistical Methods," 5th ed., Iowa State University Press, Ames, Iowa, 1956, p. 237.

(7) J. W. Poole, "Some Experiences in the Evaluation of Formulation Variables on Drug Availability," Proceedings of the DIA Symposium on Formulation Factors Affecting Therapeutic Performance of Drug Products, **3**, 8(1969).

(8) A. White, P. Handler, and E. Smith, "Principles of Biochemistry," 3rd ed., The Blakiston Division, McGraw-Hill, New York, N. Y., 1964, pp. 768–769.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 20, 1969, from the *Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada.*

Accepted for publication May 5, 1970.

The authors express their thanks to Smith Kline & French Inter-American Corp. for financial support during this investigation.

Factors Influencing Solvolysis of Corticosteroid-21-phosphate Esters

G. L. FLYNN and D. J. LAMB

Abstract □ The solvolysis of methylprednisolone-21-phosphate in dilute aqueous solution (<0.005 *M*) is qualitatively similar to that observed for methylphosphate and other simple monoalkyl phosphates, particularly in the pH range 3–8. In more concentrated solutions (>0.02 *M*), however, there is an acceleration of reaction velocities and marked deviation from the expected pH dependency. This change in chemical behavior is attributed to association colloid formation. Support for this mechanism is drawn from hydrolysis-rate data obtained as a function of concentration and independently determined critical micelle concentration values.

Keyphrases □ Corticosteroid-21-phosphate esters—solvolysis, micelle formation □ Solvolysis, corticosteroid-21-PO_4 esters—pH profile, activation energy, aggregation effect □ Critical micelle concentration determination—conductivity, surface-tension methods □ Phosphate, inorganic—analysis

The term phosphate ester is extremely ambiguous because it applies to several distinctly different classes of compounds, each characterized by unique chemical behavior. Furthermore, chemistries in each phosphate ester class can be appreciably different both qualitatively and quantitatively, depending on the types and proximities of neighboring atoms within the molecule. Additional complexity arises from the biformity of the carbon–oxygen–phosphorus linkage. Solvolysis, for instance, may entail carbon–oxygen cleavage, phosphorus–oxygen splitting, or both, depending on the reaction conditions (1).

The hydrolyses of monoalkyl phosphates are characterized by these diversities. The prototype of this phosphate ester class is methylphosphate. It is a relatively simple molecule whose aqueous stability has been extensively studied (2). At pH values below zero, the conjugate acid species predominates, and the solvolysis takes place with both carbon–oxygen and phosphorus–oxygen splitting. The neutral molecule is the principal species in pH range 0–2, and it cleaves exclusively at the carbon–oxygen bond. Above pH 3, the monoanion,

which cleaves exclusively at the phosphorus–oxygen bond, is formed and its concentration determines the reaction velocity well into the basic pH range.

Phosphates are of interest pharmaceutically because they provide a means of making soluble derivatives out of highly insoluble compounds. Additionally, some types of phosphate esters are sufficiently stable to allow the formulation of solutions with practical shelflives. These characteristics translated into biopharmaceutical terms mean instantaneous blood levels and facile parenteral administration. Most important or widely used of the pharmaceutical phosphate esters are the corticosteroid-21-phosphates. This report deals with their chemical behavior in aqueous solution as a function of concentration, pH, and other reaction conditions.

EXPERIMENTAL

Sample Preparation—In the kinetic studies, solutions were prepared at predetermined concentrations using distilled water and appropriate amounts of buffering agents and organic phosphate. In the pH 3–6 region, these were adjusted to pH at room temperature using either a concentrated solution of sodium hydroxide or concentrated hydrochloric acid. In the very low and high pH ranges, the samples were adjusted to pH at the temperature of the run. Initial concentrations were then determined by the Porter–Silber procedure (3, 4). After pH adjustment, the samples were filled in appropriately sized glass vials (usually 5 ml.) and placed in constant-temperature oil baths maintained within ±0.1° of the indicated temperatures. These were removed and analyzed on a suitable, predetermined schedule.

In the surface-tension determinations of critical micelle concentrations (CMC), a concentrated solution of the drug in distilled water was prepared and then diluted by 50 or 60% successively to levels far below the expected CMC. These were analyzed for surface tension as a function of concentration. Conductimetric studies were carried out on solutions prepared in a tight range around the expected CMC values (1.5×10^{-3} *M* to 3×10^{-2} *M*).

Equipment—Spectrophotometric determinations were performed on a Cary 11 recording spectrophotometer. The pH adjustments were monitored on either a Beckman model GS or a Corning expanded scale meter. The surface-tension measurements were done on a Cenco DuNoüy 70545 tensiometer and conductivity measurements on a Serfass conductivity bridge model RCM 15B1.

Inorganic Phosphate Procedure—*Chemicals*—All of the following chemicals were used without further purification: methylene chloride (distilled in glass); hydrochloric acid, reagent grade; potassium phosphate, monobasic, crystals A.R.; *N,N*-dimethylformamide (distilled in glass); ammonium molybdate A.R.; glacial acetic acid, reagent grade; cupric sulfate (anhydrous) A.R.; ascorbic acid USP; sodium acetate A.R., granular; stannous chloride, crystals A.R.; and sodium phosphate, dibasic (anhydrous) A.R.

Hydrocortisone-21-phosphate (disodium salt), prednisolone-21-phosphate (disodium salt), and dexamethasone-21-phosphate (disodium salt) were used as received.[1] Methylprednisolone-21-phosphate (M-21-P) (disodium salt) (Upjohn) was purified *via* continuous methylene chloride extraction for micellar kinetic studies. Otherwise, it was used as received.

Reagents—Originally the Mokrasch (5) procedure was employed. On later runs, a modified procedure with the following reagents was used:

1. Ammonium molybdate—2.0% $(NH_4)_6Mo_7 \cdot 4H_2O$ in double-distilled water.
2. Acetate buffer—310 ml. of glacial acetic acid, 49 g. of potassium acetate, and 48 mg. of cupric sulfate dissolved in double-distilled water and diluted to 1000 ml.
3. Ascorbic acid—2% ascorbic acid in distilled water.
4. Stannous chloride—20% stannous chloride in hydrochloric acid; 1 ml. diluted to 100 ml. with distilled water prior to use.
5. Standard inorganic phosphate solution—300 mg. (accurately weighed) of Na_2HPO_4 (dried 2 hr. at 110°) in 100 ml. of distilled water; 10 ml. diluted to 200 ml. with distilled water (approximately 2×10^{-4} *M*). When buffer systems were used, the standard was prepared in them.

Procedure—Total sample was removed from the ampuls, placed in a 60-ml. separator, and extracted with two 25-ml. portions of methylene chloride to remove formed sterol. An aliquot of the extracted sample was then appropriately diluted. To separate 100-ml. volumetric flasks, the following were added: (*a*) 2 ml. of standard, q.s. to 10 ml.; (*b*) 2 ml. of standard and 2–5 ml. of sample q.s. to 10 ml.; (*c*) 2–5 ml. of sample [appropriate size varied from concentration to concentration but same amount of sample used in (*b*) and (*c*) in any case for given run]; and (*d*) 10 ml. distilled water (blank).

This was followed immediately for each solution with 50 ml. of acetate buffer, 5 ml. of ammonium molybdate solution, 5 ml. of ascorbic acid solution, and 5 ml. of stannous chloride solution, mixing well after each addition. These were then diluted to volume with acetate buffer and allowed to develop for 30 min. The absorbance of the samples at 740 mμ against the blank was determined within 3 hr. using 1-cm. cells.

Calculations—

$$[\text{M-21-P}]_t = [\text{M-21-P}]^0 - [IP]_t \quad \text{(Eq. 1)}$$

$$= [\text{M-21-P}]^0 - [S] \times \frac{A_{mp}}{A_s} \times \text{D.F.} \times \frac{\Delta A_{mp}{}^t}{\Delta A_{mp}{}^0} \quad \text{(Eq. 2)}$$

where:

$[\text{M-21-P}]_t$ = molar concentration of methylprednisolone-21-phosphate with respect to time
$[\text{M-21-P}]^0$ = initial molar concentration of methylprednisolone-21-phosphate (independently determined); alternately, the inorganic phosphate infinity value was used as material balance was good
$[IP]_t$ = inorganic phosphate molar concentration with respect to time
$[S]$ = molar concentration of phosphate standard
A_{mp} = absorbance of sample
A_s = absorbance of standard
D.F. = dilution factor
$\Delta A_{mp}{}^t$ = absolute difference in the spiked and nonspiked sample at time, *t*
$\Delta A_{mp}{}^0$ = absolute difference in the spiked and nonspiked sample initially; equal to A_s

CMC Determinations—*1. Surface-Tension Measurements*—These were made on the tensiometer at room temperature. Each dilution was read three times and the values were averaged. It was found that pH was relatively invariant at pH = 7.5, and no adjustments were made. Experiments run at lower pH were done on solutions that had been accurately pH adjusted.

2. Conductimetric and Spectrophotometric Determinations—These were run on solutions diluted to between 0.75 and 15 mg./ml. corresponding to 1.5×10^{-3} *M* and 3×10^{-2} *M*, respectively. The conductimetric measurements were carried out in a jacketed-glass beaker in which the temperature was maintained within 0.1° of that indicated. The cell constant of the conductance probe was 1.0.

pKa Determinations—Solutions of M-21-P were prepared to contain 1.33 mg./ml. NaCl was added to give an ionic strength of 0.15. Titration curves were run on a radiometer titrator at the indicated temperature using 0.09823 *N* HCl as the titrant. The pKa_2 values were read directly from the graph at the half-neutralization point. The pKa_1 values were estimated from the one and one-half neutralization point and were corrected for water content of the phosphate sample. Equation 3 was used:

$$pKa_1 = pH_1 - \log \frac{[A^- + H^+]}{[HA - H^+]} \quad \text{(Eq. 3)}$$

THEORETICAL CONSIDERATIONS

From the beginning of these studies, it was realized that spurious, unquenchable reactions would be occurring simultaneously with the solvolytic reaction in the pH range of interest. To avoid the potentially serious complications resulting from these secondary decompositions, a method of monitoring the reaction, which was either sensitive to the presence of intact organic phosphate or capable of assessing formed inorganic phosphate, was sought. Two established analytical procedures, the Porter–Silber (3, 4) procedure

[1] Supplied by Merck & Co., Rahway, N. J.

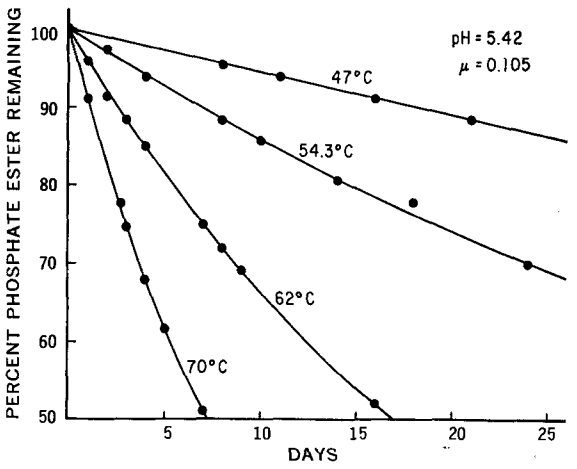


Figure 1—*Typical curves for the disappearance of M-21-P from aqueous media as a function of temperature at pH 5.42 and an ionic strength of 0.105.*

and the Mokrasch (5) inorganic phosphate procedure, were deemed suitable. After some experimentation with each, the inorganic phosphate procedure was chosen. This procedure is based on the reduction of phosphomolybdate to form a colored product. It was developed expressly for the determination of inorganic phosphate in the presence of labile organic phosphate and thus was particularly suited to the investigation.

The removal of formed neutral species was found to be a necessary condition to the successful application of this technique. Otherwise, color development was erratic. In addition, each sample was assayed with a counterpart spiked with a known amount of inorganic phosphate. The procedure is similar to that used in scintillation counting where counting efficiency is determined by adding an exact quantity of unstable isotope (^3H, ^{14}C, *etc.*) to the sample after it has been counted for its unknown concentration. In both cases the known concentration corrects the value of the unknown for variances in the sensitivity of the assay. Thus, any tendency for color development to drift was offset by including the drift factor, $\Delta A_{mp}{}^t/\Delta A_{mp}{}^0$, in the calculations (Eq. 2). With these precautions the infinity time values by the inorganic phosphate method were within experimental error of the independently determined initial concentrations.

The solvolysis of M-21-P between pH 0 and 8 was expected to be pseudo-first-order based on literature reports (6, 7) for similar compounds:

$$\frac{d[\text{M-21-P}]}{dt} = -k_{obs.}\,[\text{M-21-P}] \qquad \text{(Eq. 4)}$$

where [M-21-P] stands for the instantaneous concentration of M-21-P and $k_{obs.}$ is the observed pseudo-first-order rate constant at a given pH, temperature, ionic strength, *etc.* The integrated form of this equation becomes the familiar

$$[\text{M-21-P}]_t = [\text{M-21-P}]^0\, e^{-k_{obs.}t} \qquad \text{(Eq. 5)}$$

Since $[\text{M-21-P}]_t = [\text{M-21-P}]^0 - [\text{inorganic phosphate}]$, then

$$[\text{M-21-P}]^0 - [\text{inorganic phosphate}] = [\text{M-21-P}]^0\, e^{-k_{obs.}t} \qquad \text{(Eq. 6)}$$

or, on a percentage basis:

$$\frac{[\text{M-21-P}]^0 - [\text{inorganic phosphate}]}{[\text{M-21-P}]^0} \times 100 = 100\, e^{-k_{obs.}t} \qquad \text{(Eq. 7)}$$

Semilogarithmic plots of either intact M-21-P concentration or percent M-21-P remaining *versus* time should yield straight lines intercepting at log $[\text{M-21-P}]^0$ (Eq. 6) or log 100 (Eq. 7), respectively, with slopes of $k_{obs.}/2.303$.

RESULTS

The disappearance of M-21-P in reaction systems typical of those considered here is shown as a function of time and pH in Fig. 1.

Table I—Buffers Used in M-21-P Kinetic Studies

pH	Buffer
0.02	1.0 *M* $HClO_4$
0.72	0.2 *M* $HClO_4$
1.24	0.0645 *M* HCl, 0.05 *M* KCl
1.61	0.0263 *M* HCl, 0.05 *M* KCl
2.50	0.05 *M* Phthalate, 0.396 *M* HCl
3–8	Either 0.02 *M* citrate or 0.02 *M* bisulfite, 0.02 *M* citrate

Table II—Rate Constants at pH = 4.06 at 70° for Solvolysis of Several Corticosteroid-21-phosphate Esters

	$k^{70°}$ (days^{-1})
Methylprednisolone	0.151
Dexamethasone	0.148
Prednisolone	0.156
Hydrocortisone	0.148

This figure shows the residual percentage of the initial M-21-P in solutions consisting initially of 2×10^{-3} *M* M-21-P at several temperatures and pH = 5.42. The buffers used in the studies are from a paper by Bunton *et al.* (2) and are outlined in Table I.

As would be expected on the basis of Eq. 7, semilogarithmic plots of the residual percentage of M-21-P against time are linear. Typical pseudo-first-order curves are shown in Fig. 2 for the reaction at several temperatures and pH = 5.32. The solvolyses of prednisolone-21-phosphate, hydrocortisone-21-phosphate, and dexamethasone-21-phosphate proceeded in a similar fashion as shown by a single pH and temperature study summarized in Table II. These rates are indistinguishable within experimental error from one another.

The pH dependency of the reaction velocity is shown in Fig. 3. These data are for the reaction of solutions initially of 2×10^{-3} *M* concentration at 70°. Included with these data for later consideration are the 70° literature data of Marcus (8) for the solvolysis of hydrocortisone-21-phosphate and 101° data extrapolated to 70° for methylphosphate (2) using an activation energy of 30.6 kcal./mole.

The temperature dependency of the reaction was determined at multiple points above pH = 3. As predicted by the Arrhenius equation, plots of the log of the observed rate constant against the reciprocal of the absolute temperature yielded straight lines (Fig. 4). The lines representing different values of pH were also parallel. The activation energy values calculated from the slopes of these lines are tabulated in Table III. The average of these values, 27.5 kcal./mole, is assumed to be the best estimate of the activation energy.

Because of the striking diversity in these results and previous results on hydrocortisone-21-phosphate and the obvious structural

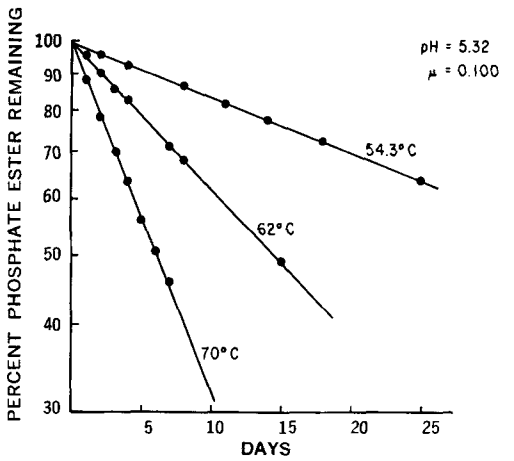


Figure 2—*Semilogarithmic plots showing the disappearance of M-21-P as a function of temperature at pH 5.32 and an ionic strength of 0.100.*

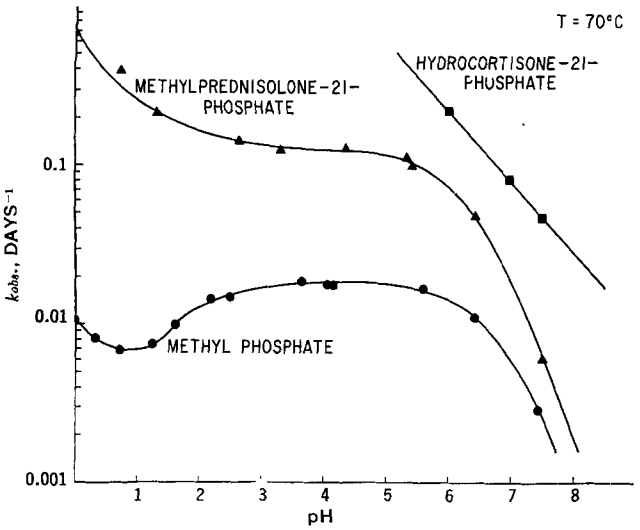
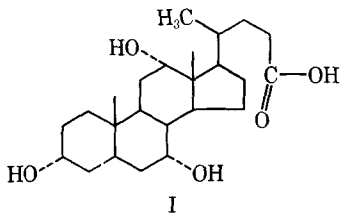


Figure 3—*The 70° pH profiles for methylphosphate, M-21-P, and hydrocortisone-21-phosphate.*

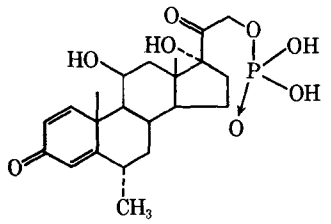
semblance between the corticosteroid phosphates and the surface-active bile acids (Structures I and II), studies attempting to relate the

mol. wt. = 409



I

mol. wt. = 454



II

I—cholic acid II—methylprednisolone-21-phosphate

observed differences to molecular aggregation effects were initiated. Typical results of surface-tension measurements of serially diluted solutions of M-21-P at room temperature and pH = 7.5 are shown in Fig. 5. These are plotted in the usual fashion as the surface tension *versus* the log of the molar concentration. Distinct breaks in the curves at approximately 0.02 *M* were observed. Because of the tedium of the procedure and interest in expanding the data to include surface-tension measurements as a function of pH and temperature, conductimetric studies supplanted surface-tension experiments. The data from a representative temperature experiment at pH = 7.5 are graphically presented in Fig. 6. It appears from this plot that there is a slight increase in the CMC with increasing temperature. CMC values fall between 0.01 and 0.02 *M*, which is in good agreement with the surface-tension studies. Neither method yielded sharply defined CMC's. Conductimetric studies were also performed at 25° and several pH values. At low pH, where the free acid predominates, the solubility of the compound was too low to make measurements. CMC's measured above pH = 3 were not distinguishable from one another.

Table III—Apparent Activation Energy as a Function of pH for the Solvolysis of M-21-P

pH	E_a
7.52	27.0
6.45	27.3
5.42	26.3
5.32	28.9
4.35	29.4
3.32	27.6

With the amphiphilic character of the compound definitely established, studies were conducted to assess the effect of association colloid formation on the reaction. Unfortunately, in the pH range of maximum interest, the data gathered were only crudely quantitative due to an inability to control pH in bufferless systems. Therefore, the results will only be reported here in qualitative terms. The reaction rate was found to be invariant with concentration at pH = 4 in solutions ranging from 0.005 to 0.16 *M* (0.005, 0.01, 0.02, 0.04, 0.08, and 0.16 *M*). In these studies, pH drifts were found to be no more than several hundredths of a unit throughout the concentration range. However, on reactions run at pH = 7 and 7.5, a different behavior was observed for series of solutions of the same concentrations. For the dilute solutions, pH shifts to more acidic conditions exceeded a full pH unit. As the concentration increased, the pH drift narrowed to slightly over half a unit. The downward shift in pH would tend to accelerate the reaction. Despite this, the reaction in 0.16 *M* solutions was consistently observed to be 2–3 times more rapid than in 0.08 *M* solutions. There appeared to be little difference between 0.08 and 0.04 *M* solutions. Below 0.04 *M*, the pH shifts became exaggerated and there was an apparent increase in reaction rate.

DISCUSSION

Analysis of the pH–Rate Profile—The pH profile for all alkyl phosphates is a composite of the individual species profiles, and its shape is determined by their relative concentrations and rates (1). Four species are possible: the conjugate acid, the free acid, the monoanion, and the dianion. The conjugate acid is formed in highly acidic solution (pH $\ll$ 1.0) and thus is only of marginal concern for the steroid-21-phosphates. In pH range 1–8, the instability of these compounds can be explained on the basis of the independent reactivities of the free acid and monoanion. An M-21-P species profile as a function of pH is given in Table IV. These data are calculated using averaged, independently determined pK_{a_1} and pK_{a_2} values of 2.55 and 6.04, respectively, and Eqs. 8–10:

$$C_N = \frac{[H^+]^2}{K_{a_1}K_{a_2} + K_{a_1}[H^+] + [H^+]^2} \quad \text{(Eq. 8)}$$

$$C_M = \frac{K_{a_1}[H^+]}{K_{a_1}K_{a_2} + K_{a_1}[H^+] + [H^+]^2} \quad \text{(Eq. 9)}$$

$$C_D = 1 - [C_N + C_M] = \frac{K_{a_1}K_{a_2}}{K_{a_1}K_{a_2} + K_{a_1}[H^+] + [H^+]^2} \quad \text{(Eq. 10)}$$

where C is the fractional concentration and subscripts N, M, and D refer to the neutral species, monoanion, and dianion, respectively.

Comparison of the pH profiles of methylphosphate and M-21-P between pH 0 and 2, where the neutral species prevails, indicates that the trough found for methylphosphate is leveled out for its corticosteroid counterpart (Fig. 3). Reaction rates at pH = 1.0 differ by approximately two orders of magnitude. Similar plateauing is observed in the profiles of α-D-glucose-1-phosphate (9) and monobenzyl phosphate (10). Unlike these two cases, the increased reactivity of the neutral species cannot be attributed to a change from an S_N2 solvolytic displacement to an S_N1 carbonium-ion mechanism. The carbonium ion of M-21-P would be even less energetically favored than that for methylphosphate due to the electron-withdrawing α-carbonyl. A probable explanation for the increased reactivity is stabilization of the transition stated (S_N2 mechanism with sp^2 hybridization) due to π-orbital overlap with the electron-rich neighboring oxygen atom (11). A similar effect of similar or greater magnitude for α-carbonyls has been noted in other solvolytic displacements (12). The plateau in the M-21-P profile suggests that the specific second-order rate constants for the cleavage of the neutral species and monoanion are of the same order of magnitude.

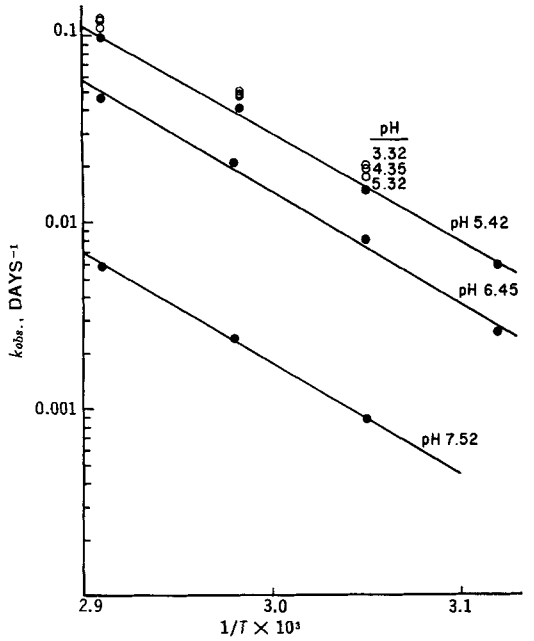


Figure 4—*Arrhenius plots for the hydrolysis of M-21-P at several values of pH.*

In the pH region of pharmaceutical interest, the principal degradative route for phosphate monoesters is the cleavage of the monoanion. The qualitative similarity of the reaction for M-21-P and methylphosphate above pH = 3 is readily apparent from Fig. 3. The reaction proceeds with P—O splitting. The specific requirements for the hydrolysis of the monoanion led Butcher and Westheimer (13) to postulate a mechanism in which an unstable monomeric metaphosphate-ion intermediate is formed. This mechanism is consistent with the relative rapidity of the hydrolysis, the requirement of protonation of one of the phosphate oxygens, P—O splitting, and the marked insensitivity of the hydrolytic velocity to the nature of the leaving group. At 70° the monoanion rate constant for M-21-P is just over seven times that found for methylphosphate. Because of an approximately 3-kcal./mole difference in observed activation energies, this factor shrinks as the temperature is lowered. Regardless, this difference is significant and likely attributable to electronic effects of the α-carbonyl on the strength of the P—O bond. An interesting fact discovered in these studies is that, in dilute solution, the nature of the steroid nucleus has little effect on the specific second-order monoanion instability constant for the corticosteroid-21-phosphates, at least insofar as hydrocortisone, methylprednisolone, dexamethasone, and prednisolone are different. This suggests that the overriding influence on the observed rate is the adjacent carbonyl and that the M-21-P data can be considered representative of the stability of related steroid phosphates.

Throughout the course of the kinetic studies at premicellar concentrations, there was no noticeable effect of buffers on the reaction rate. Buffers could be interchanged or changed in concentration without a measurable reaction velocity change. This is not too surprising because at low pH, the buffer species present are extremely poor nucleophiles. At high pH the mechanism precludes buffer involvement but, neglecting this, the reaction requirements are such that two negatively charged species must be brought together and this is energetically unfeasible.

Because of doubts regarding the relative validity of the available methods for following the progress of the reaction, the disappearance of M-21-P was also followed directly using the Porter–Silber procedure (3). Results by this procedure were comparable with those obtained following the appearance of inorganic phosphate. For instance, at 54.3° and pH = 5.4, the inorganic phosphate procedure yielded a value for $k_{obs.}$ of 1.45×10^{-2} day^{-1} while the value by the Porter–Silber procedure was 1.83×10^{-2} day^{-1}. These results were obtained on ampuls from the same set. It was implicit in these studies that the formation of spurious products would have negligible effect on the principal reaction. In other words, it was assumed that changes in the molecule prior to solvolysis, if any, would not appreciably affect processes at the phosphate moiety. Products were assumed to be inert. The dominance of the solvolysis as the major instability, the linearity of plots, and the reproducibility of data by both of these methods attest to the credibility of these assumptions.

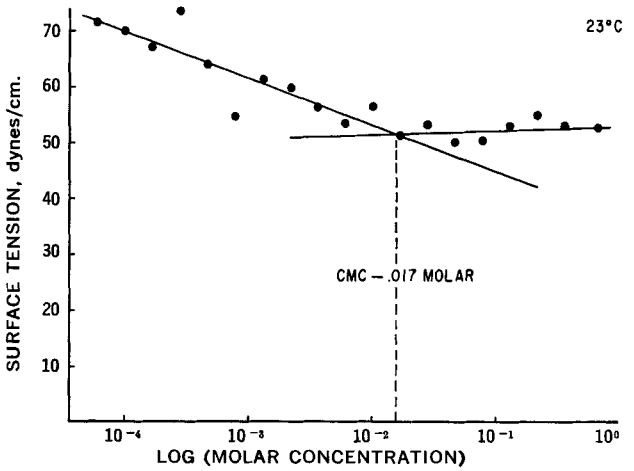


Figure 5—*Determination of the CMC of M-21-P by surface-tension measurement. Each point is an average of three determinations.*

The dianion of M-21-P is virtually unreactive. Negative linear dependency on the pH is observed far into the high pH range, and this dependency parallels the concentration of the monoanion. Presumably, the concentration of the monoanion should eventually become negligible, resulting in a plateau in the pH profile. This point, if it is reached at all, is well above pH = 8 based on the observations in this study.

Micellization and Its Probable Influence on Reaction Rate—Strong evidence has already been presented for the formation of association colloids in these systems. In addition, slight but unmistakable deviations from Beer's law were observed above the CMC. Similarly, the solubility of methylprednisolone in solutions of M-21-P increases sharply as the CMC is exceeded.

The enthalpy for the formation of the micelles, ΔH_m, is calculable from the available data and Eq. 11:

$$\Delta H_m = -RT^2 \left(\frac{d \ln \text{CMC}}{dT}\right) \qquad \text{(Eq. 11)}$$

and was found to be approximately −1.0 kcal./mole at 40°. This is entirely consistent with the behavior of other systems (14). The significance of this value is that it indicates the marked temperature insensitivity of micellization. Over a narrow temperature range,

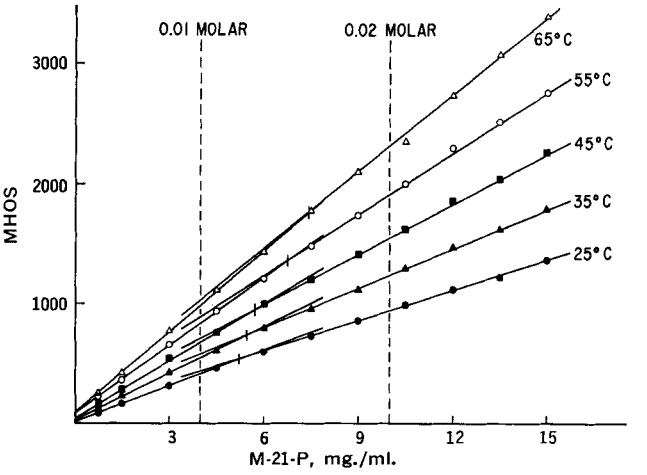


Figure 6—*Determination of the CMC of M-21-P by conductance measurement. Each line represents a different temperature. In all cases, the apparent CMC fell between 0.01 and 0.02* M.

Table IV—M-21-P Species Concentrations of $f(pH)^a$

pH	Free Acid (N)	Monoanion (M)	Dianion (D)
0	0.997	0.003	Negligible
1	0.973	0.027	Negligible
2	0.78	0.12	Negligible
3	0.262	0.738	Negligible
4	0.034	0.956	0.008
5	0.003	0.914	0.083
6	Negligible	0.523	0.477
7	Negligible	0.099	0.901
8	Negligible	0.011	0.989
9	Negligible	Negligible	0.998
10	Negligible	Negligible	1.000

[a] M-21-P total = 1.0.

the fraction of molecules participating in micelles to total surfactant molecules remains relatively constant. In other words, temperature effects parallel those of chemical equilibria rather than chemical reactions.

Another factor to be kept in mind is the salt effect or, more properly, the gegenion effect on micellization. Many investigators working with diverse systems have observed that the logarithm of the CMC changes linearly with the logarithm of the concentration of gegenion (14):

$$\ln \text{CMC} = -K \ln Cg + \text{constant} \qquad \text{(Eq. 12)}$$

In practical terms, this contraindicates the use of buffers in well-designed studies on micellar kinetics. It also suggests that the hydrocortisone-21-phosphate reaction systems of Marcus (8) were of significant micellar character. His working phosphate concentration was 0.02 M, slightly above the CMC, and his studies were performed in 0.20 M phosphate buffer providing as little as 0.2 M and up to 0.4 M concentration of positively charged ions (gegenions), depending on pH. The activation energy reported by Marcus for the solvolysis of hydrocortisone-21-phosphate is 17.0 kcal./mole, fully 10 kcal./mole less than that found for M-21-P and 13 kcal./mole less than the average for this entire class of compounds (1). A shifting micellar fraction with temperature can account for much of these substantial differences.

Controlling variables in micellar kinetic studies can be a difficult job. Not only do the usual parameters such as temperature and pH have to be controlled, but also gegenion concentration and impurities. Buffers can have a multiplicity of effects. Therefore, several studies at pH $\simeq$ 4 and pH > 7.0 were initiated in bufferless systems. There was definitely no micellar facilitation of the reaction at low pH. At high pH, it appeared as if the reactions were accelerated above the CMC, particularly when changing pH effects, which ran counter to the micellar effects, were taken into account. The insensitivity to micellization at pH $\simeq$ 4 is explicable when the peculiar requirements and mechanism of the phosphate monoanion decomposition are considered. The rate-determining step, the production of a metaphosphate anion, is a unimolecular reaction reflecting the intrinsic instability of a given molecule. With the exception of factors influencing protonation, the presence of neighboring molecules would not play a major role in the reaction. It is implicit here that the pH at the micellar surface is not appreciably different from the bulk pH. This is not necessarily true at higher pH. The surface potential, ψ, is becoming increasingly negative. If the hydrogen ions are present in a Boltzman distribution represented by Eq. 13:

$$[H^+]_s = [H^+]_b \exp. - (|e|\psi/kt) \qquad \text{(Eq. 13)}$$

where $[H^+]_s$ and $[H^+]_b$ are the surface and bulk hydrogen-ion concentrations, respectively, then the pH at the micellar surface is expected to lag behind that of the bulk solution. This will change the ratio of phosphate monoanions to dianions relative to the bulk and, therefore, produce an apparent increase in rate. This factor and the gegenion effect together could help produce the rather unusual pH and temperature dependency observed by Marcus (8) in the pH range 5–8.

REFERENCES

(1) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," vol. II, W. A. Benjamin, New York, N. Y., 1966, pp. 1–15.

(2) C. A. Bunton, D. R. Llewellyn, and K. G. Oldham, *J. Chem. Soc.*, **1958,** 3574.

(3) R. H. Silber and C. C. Porter, *J. Biol. Chem.*, **210,** 923(1954).

(4) R. E. Peterson, A. Karrer, and S. L. Guerra, *Anal. Chem.*, **29,** 144(1957).

(5) L. C. Mokrasch, *ibid.*, **33,** 432(1961).

(6) M. C. Bally, *Bull. Soc. Chim. France*, **9,** 340, 405(1942).

(7) A. Desjobert, *ibid.*, **14,** 809(1947).

(8) A. D. Marcus, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 383(1960).

(9) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, **1958,** 3588.

(10) J. Kumamoto and F. H. Westheimer, *J. Amer. Chem. Soc.*, **77,** 2515(1955).

(11) J. March, "Advanced Organic Chemistry: Reactions, Mechanism, and Structure," McGraw-Hill, New York, N. Y., 1968, pp. 284–285.

(12) A. Streitwieser, Jr., "Solvolytic Displacement Reactions," McGraw-Hill, New York, N. Y., 1962, pp. 28–29.

(13) W. Butcher and F. H. Westheimer, *J. Amer. Chem. Soc.*, **77,** 2420(1955).

(14) K. Shinoda, T. Nakagawa, B. Tamamushi, and T. Isemura, "Colloidal Surfactants," Academic, New York, N. Y., 1963, pp. 31–33.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication June 3, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

The authors express their appreciation to Mr. Russell Smith for his assistance in the work and to Mr. William Beyer, Upjohn Control Division, who improved the Mokrasch inorganic phosphate procedure. The improvements yield a procedure which is not encumbered with cloudiness and in which labile phosphates are more stable. Thanks are also extended to Drs. William Higuchi, Harry Kostenbauder, and Pasupati Mukerjee for helpful discussions.

Solute Fluxes in Fat Absorption

R. L. S. WILLIX

Abstract □ Diffusion rates of fatty acid (oleic and palmitic acids) in bile salt solution and in polyoxyethylene–polyoxypropylene copolymer solution depend on the amount of fatty acid solubilized and also on any physical restrictions to flow. Micelles of the two types of surfactant are significantly different in size. Assuming a passive process for the uptake of fatty acid, diffusion rates correlate with the observed uptake of fatty acid into everted intestinal sacs of the rat.

Keyphrases □ Fat absorption—solute fluxes, *in vitro* □ Diffusion cells—fat transfer □ Radioactive solute—fat transfer, diffusion cells □ Refractometry, differential—diffusion rates □ Scintillometry—analysis

The surface-active property of the natural surfactants, bile salts, in mediating lipolysis (1) and absorption by the small intestine of hydrophobic material, otherwise poorly soluble in an aqueous medium (2–4), is well accepted.

Synthetic nonionic surfactants, such as polyoxyethylene–polyoxypropylene copolymer,[1] also promote absorption of free fatty acids by everted intestinal sacs of the rat but less effectively than bile salts (5). They exert an effect *in vivo* from which conclusions are obscured due to the absence of a clear distinction between uptake from micellar solutions compared with uptake from emulsions (6).

Phase separation by ultracentrifugation has established that, for any one concentration of either surfactant and lipid composition, a fixed amount of fatty acid partitions between the micellar and emulsion phases (6); and for absorption *in vitro*, there is a clear relationship between the amount of fatty acid (oleic acid) solubilized and uptake into the everted intestinal sac (5).

If it is generally agreed that lipid is absorbed almost exclusively from the isotropic phase of intestinal content, micellar solubilization could promote uptake by increasing the chemical potential or effective concentration of lipid in the systems considered. An understanding of the mechanism, however, requires a knowledge of the dynamic nature of the physicochemical events in the absorptive process. Diffusion coefficients in the aqueous phase and the interfacial resistances to fatty acid movement between emulsion globules, micelles, and the surrounding aqueous medium are the kinetic quantities that provide the link between solubilization and uptake in this luminal stage of fat absorption.

The method involves the measurement of the fatty acid flux across a confined volume of solution, in practice a solution that is limited to the interior of a diaphragm and made less sensitive to gradients that lead to bulk flow in free solution. In addition, the use of diaphragms of different porosities provides data on solute fluxes when the movement of fatty acid in one or more of its states of aggregation, monomers, micelles, or emulsion globules is restricted.

The method has been applied to lipids in a qualitative manner (7) and follows from studies (8, 9) on the effect of solubilizing agents in increasing the driving force for diffusion of water-insoluble dyes. The capacity of Millipore filters, under conditions of free filtration, in separating emulsion and micellar phases of lipid dispersed by bile salts has been previously demonstrated (10). Using Millipore and sintered-glass diaphragms, the present study provides quantitative data on the diffusive movement of fatty acid, specifically oleic acid, in lipid systems of bile salts and of polyoxyethylene–polyoxypropylene copolymer.

The validity of a diffusion-limited model for the luminal stage of fatty acid absorption implies the presence in the gut of a still layer near the absorptive cell. While this is not proven, the glycocalyx coat could well produce an unstirred layer of fluid of greater thickness than at a clean cell surface; the presence of microvilli with some of the characteristics of physical pores (11) improves the analogy with the physicochemical model, making the data on fatty acid movement in its different states of aggregation perhaps directly applicable to the biological situation.

An understanding of the luminal stage of the absorptive process through the comparison of different surfactants may then provide insight into the much more complicated *in vivo* problem where, as yet, few clearcut physicochemical parameters have emerged.

EXPERIMENTAL

Materials—Sodium taurocholate (NaTC) moved as a single spot on activated silica gel at room temperature with ethyl acetate–methanol–glacial acetic acid (70:20:10). It was used as supplied.[2]

Sodium taurodeoxycholate (NaTDC) was synthesized from taurine and deoxycholic acid by the method of Norman (12) as modified by Hofmann (13). Bile salts were used in a 4:1 ratio with NaTC–NaTDC in all experiments.

Polyoxyethylene–polyoxypropylene copolymer (nominal molecular weight 8000) had a specific refractive index increment of 0.139 ml. $g.^{-1}$ and was used as supplied.

Glycerol 1-monooleate (monoolein) was shown by TLC to contain small amounts (~5%) of diglyceride and free fatty acid. By GLC the fatty acid composition was $C_{12:0}$ 1, $C_{16:1}$ 5, $C_{18:1}$ 85, $C_{18:2}$ 5.

Oleic acid and palmitic acid were ~98% pure by GLC. Radiochemical purity of oleic acid-1-^{14}C was certified[3] as 95–98% pure $C_{18:1}$ of which 7% was elaidic acid; 97% of the material was free fatty acid by TLC. Palmitic acid-9,10-^{3}H was certified 96% pure; 94% of the activity was in the fatty acid band.

Sodium dihydrogen phosphate and monohydrogen disodium phosphate were used as supplied.[4]

Methods—Two types of diaphragm cells are described. One uses a sintered-glass disk of ~30-μ average pore diameter, 4.9 cm.[2]

[1] Pluronic F68, Wyandotte Chemicals Corp., Wyandotte, MI 48193

[2] Koch-Light Laboratories, Colnbrook, England.
[3] Radiochemical Centre, Amersham, England.
[4] Ajax Chemicals, Sydney, Australia.

Table I—Cell Constants

Diaphragm	Cell Constant β (cm.$^{-2}$)
Sintered glass (SG), 30 μ	0.25 ± 0.02
Millipore (MP), 1.2 μ	9.54 ± 1.1
Millipore, 0.010 μ	4.37 ± 0.06

in area and 2 mm. thick, in the Stokes modification (14) of the Northrop–Anson (15) diffusion cell, in which stirring is effected by two polyethylene-encased[5] metal bars mounted one on each side close to, but not in contact with, the diaphragm. The cell was filled by suction and the lower chamber was closed with a tightly fitting ground-glass stopper. A film of silicone grease was placed on the external joint, and the entire lower chamber port was enclosed in a small polyethylene bag. The cell was immersed vertically in the thermostat and held in a rigid clamp to minimize vibration.

When steady-state conditions prevailed in the diaphragm, the solution in the upper chamber was removed and replaced with the solution of interest, either a nonradioactive solution of lower concentration if a chemical concentration gradient was to be maintained across the diaphragm or a nonradioactive solution of the same lipid concentration if the system was to be at chemical (but not isotopic) equilibrium. The upper chamber was closed with a ground-glass stopper with a perforation to the atmosphere, and the run was commenced by activating the stirrer with rotating magnets. At the end of the diffusion period, the entire solution (24.2 ml.) from each chamber was removed for analysis. Radioactivity measurements were made on triplicate samples (refractive index measurements also were made if surfactant diffusion in concentration gradient experiments were being followed).

The other diaphragm cell utilized Millipore cellulose acetate filters (25 mm. in diameter, 0.15 mm. thick) of different porosities, mounted vertically to expose 2.90 cm.^{2} to the solutions in each chamber (initially always 20 ml. in volume) and stirred by centrally located metal bars encased in Perspex and driven by rotating magnets mounted on the cell support platform. Solutions in both chambers were always of the same composition and concentration in lipid, *i.e.*, at chemical but not isotopic equilibrium.

At timed intervals over the period of one experiment, 0.5-ml. samples (usually six in all) were removed from each chamber for radioactivity analysis, but the liquid level at the end of the diffusion period never fell below the top of the diaphragm. Before use the cell was tested for leaks with an impermeable barrier.

Cells were calibrated using KCl as the standard diffusing solute. Chloride ion in stock solutions and in each chamber at the experiment end was determined by AgCl turbidity in 50% ethanol–water (after dilution to reduce Cl^- concentration to a convenient level) according to the method of Luce *et al.* (16). The calibration curve at 350 mμ was linear up to 14 mcg./ml. Cl.

Where surfactant diffusion rates were monitored, differential refractometry (in 10-cm. cells) with a Hilger–Rayleigh interferometer was used. The instrument was calibrated with sucrose solutions of known refractive index to give drum readings with respect to the refractive index: $\Delta l/\Delta n = 4.81 \times 10^{-6}$. However, since the solute concentration term in the diffusion equation (discussed later) is independent of the unit of measurement, drum readings were later taken directly as measures of concentration. When such diffusion rates were followed in the presence of lipid, lipid concentrations were such that the refractive index measurement of $\Delta n/\Delta c = 0.140$ ml. g.$^{-1}$ polyoxyethylene–polyoxypropylene copolymer compared with 0.139 ml. g.$^{-1}$ polyoxyethylene–polyoxypropylene copolymer in the absence of lipid. From this it was concluded that, at these lipid concentrations, lipid contributed little to the refractive index and that refractive index changes were due mainly to the movement of surfactant.

For radiochemical assay, fatty acids were extracted with a 3:1 v/v quantity of a 1:1:1 v/v/v mixture of ethanol–heptane–diethyl ether. Two further extractions with the upper phase of ethanol–water–heptane–diethyl ether ensured that > 99% of the fatty acid was in the organic extract, the solvent was driven off under dry N_2, and the residue was counted in a liquid scintillation counter[6] after dissolution in toluene containing 2,5-diphenyloxazole (4 g./l.) and 1,4-bis-2-(5-phenyloxazolyl)benzene (0.05 g./l.) as scintillation solutes. Count times were such as to achieve 1% standard deviation. Corrections for quenching were made by the channels ratio method.

Solutions taken to be nonradioactive at the start of a diffusion period were tested for adventitious amounts of radioactivity.

Emulsions and micellar solutions of lipid were prepared by 5-min. ultrasonic irradiation (40 w. at 20 kc.) at constant anode current on a Branson sonifier.

Solubilization was determined by separation of emulsion and micellar phases by ultracentrifugation at 10^7 *g* min. (~36°), at the end of which the lower aqueous micellar phase was sampled and analyzed for radioactivity.

All solutions were 0.15 *M* NaH_2PO_4/0.07 *M* Na_2HPO_4 in phosphate buffer at pH 6.4 ± 0.1, and all experiments were performed at 37 ± 1°.

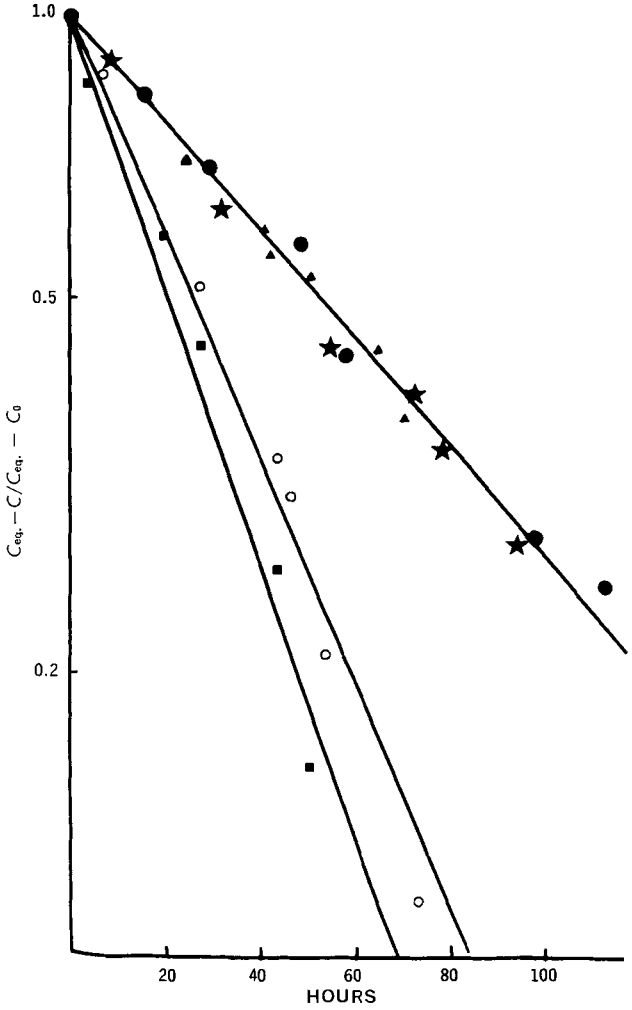


Figure 1—*Semilogarithmic plots of* ^{14}C *and* ^3H *isotopic concentration function* $(C_{eq.} - C)/(C_{eq.} - C_0)$ versus *time for 4:4:2 mM monoolein–oleic acid-*^{14}C*–palmitic acid-*^3H *equilibrating through 10-mμ MP. Key: mM 4:1 sodium taurocholate–sodium taurodeoxycholate: ●, 2.5; ▲, 5.0; ★, 7.5; ○, 10.0; and ■, 15.0.*

RESULTS AND DISCUSSION

Diffusion Equation—The usual application of Fick's law of diffusion as applied to diaphragms (17) is followed:

$$\log \frac{C_{eq.} - C}{C_{eq.} - C_0} = -\bar{D}_T \left[\frac{A}{2.3l} \frac{V_P + V_Q}{V_P V_Q}\right] t \qquad \text{(Eq. 1)}$$

where A is the total area of the diaphragm pores exposed at right angles to the direction of flow, l the thickness of the diaphragm (and associated unstirred layers of solution), V_P and V_Q the volumes of

[5] Polythene.
[6] Nuclear Chicago.

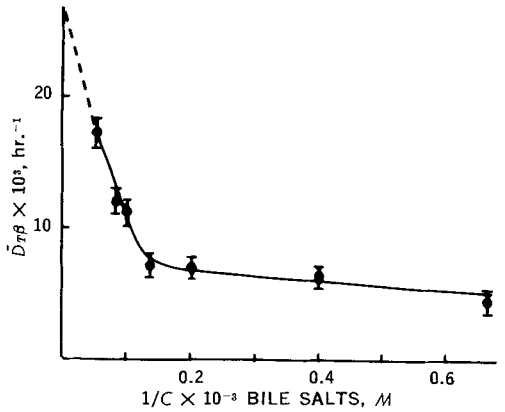


Figure 2—*Fatty acid equilibration rate* $\overline{D}_T\beta$ *as a function of the reciprocal of the bile salt concentration, 4:4:2 m*M *monoolein–oleic acid–palmitic acid, through 10-m*μ *MP.*

each compartment P and Q, $C_{eq.}$ the concentration or specific activity of the diffusing solute at equilibrium, C_0 initially, and C at time t. $\bar{D}$ is the average value of the diffusion coefficient for the concentration range of the experiment and is expressed as $\bar{D}_T$ to allow for all states of aggregation of fatty acid.

The expression in square brackets is the cell constant β and is determined with KCl, given that $D_{KCl} = 2.45 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ in 0.5 M solution at 37° (Table I). Three measurements with separate Millipore (MP) filters of each stated pore size were made for β, whereas β for the sintered-glass (SG) disk was obtained from nine separate determinations over the period of use (several months) to detect significant erosion. The 8% variability in β for the SG disk is higher than can be achieved with such diaphragms, but was considered satisfactory for the experiments described here. With SG diaphragms, diffusion coefficients were calculated from one experimental point. Since kinetic curves were not obtained, the results were more liable to error than in the MP procedure. This was partly offset by the duplication of experiments with the SG diaphragms.

Tortuosity and blocked capillaries in MP filters are serious limitations if such structural characteristics are a feature of many pores in the one filter and are not even approximately reproduced from filter to filter. However, the range in β for any one porosity suggested no gross irregularities.

Preliminary experiments established a rate of stirring above which the results were insensitive to stirring rate, *i.e.*, the "still" layers associated with the diaphragm were at their minimum thickness. In this respect, one limitation of the method is whether the dimensions of the unstirred layer are comparable for KCl and lipid solutes. When considered against the overall thickness of the diaphragm, the effect is likely to be small.

With MP filters, semilogarithmic plots of the concentration term–time give acceptable straight lines (Fig. 1). Correlation coefficients were never less than −0.95. The gradients $\bar{D}_T\beta$, obtained by regression analysis, were within 8% for both oleic acid and palmitic acid loss from one compartment and gain in the other compartment in any one experiment. Such parallelism in the rates of loss and gain suggest steady-state conditions in the diaphragm and the reasonable balance of activity-limited retention by the diaphragm.

Bile Salts—*Effect of Solubilization*—Figure 1 shows fatty acid, equilibration rates across a 0.010-μ MP where, in each experiment both chambers P and Q have the same lipid composition (4 mM oleic acid, 2 mM palmitic acid, and 4 mM monoolein), with one chamber containing ^{14}C- and ^{3}H-labeled fatty acids; bile salt concentrations alter from experiment to experiment. The effect of surfactant concentration on equilibration rate ($\bar{D}_T\beta$) is shown in Fig. 2.

Fatty acid solubilization in this system (Fig. 3*a*) suggests a CMC in the region 2.0–2.5 mM bile salts with total solubilization above 10.0 mM. When the gradient $\bar{D}_T\beta$ is expressed as a function of the reciprocal of the bile salt concentration (Fig. 2), no change in $\bar{D}_T\beta$ is apparent until the bile salt concentration reaches ~7.0 mM. This may be a result of pore blockage by fine globules, so appreciable transport rates are achieved only when there is near total solubilization. On the other hand, the solubilization curve reveals that only 30% of the fatty acid is solubilized at this bile salt concentration and that an increase in the solubilizing power, apparent from the change in the gradient of the solubilization curve, occurs in this region. A significant change in the flux is then to be expected near this surfactant concentration.

Furthermore, although all the lipid was solubilized near 10 mM bile salts, the diffusion function continued to increase with increasing surfactant concentration. At 10 mM bile salts and the stated lipid concentration, about 40 molecules of lipid are associated with each micelle on the basis of an even distribution of solubilizates and an aggregation number of ~40 for the bile salt micelle (discussed later). Given the tendency of lipid to swell micelles (18, 19), increasing the concentration of surfactant with the same overall lipid concentration would suggest the formation of more, and smaller, micelles. An increase in the rate of equilibration $\bar{D}_T\beta$ with increasing surfactant concentration is then understandable on the basis of micelle size. The reciprocal concentration plot is made to allow extrapolation to such high bile salt concentrations ($1/C = 0$), *i.e.*, so large a number of micelles that on the average the amount of lipid per micelle (at this fixed total lipid concentration) scarcely alters the dimension of the micelle. The extrapolated $\bar{D}_T$ so determined is 1.7×10^{-6} cm.^{2} sec.$^{-1}$.

Effect of Pore Size—The diffusion coefficient of oleic acid solubilized in bile salts and equilibrating through the SG diaphragm is 3.9×10^{-6} cm.^{2} sec.$^{-1}$ (Table II). The difference between this figure and 1.7×10^{-6} cm.^{2} sec.$^{-1}$ is probably due to the factor ~3 between micellar diameter (~30 Å) and average pore size not being adequate for the process to approach that of diffusion in free solution, *i.e.*, with no geometrical limitations on movement. This fits with the observation that for the same lipid mixture in 10 mM bile salts equilibrating through a 1.2-μ MP, $\bar{D}_T$ is 4.4×10^{-6} cm.^{2} sec.$^{-1}$ (Table II).

The 20% lower $\bar{D}_T$ when there is a chemical concentration dif-

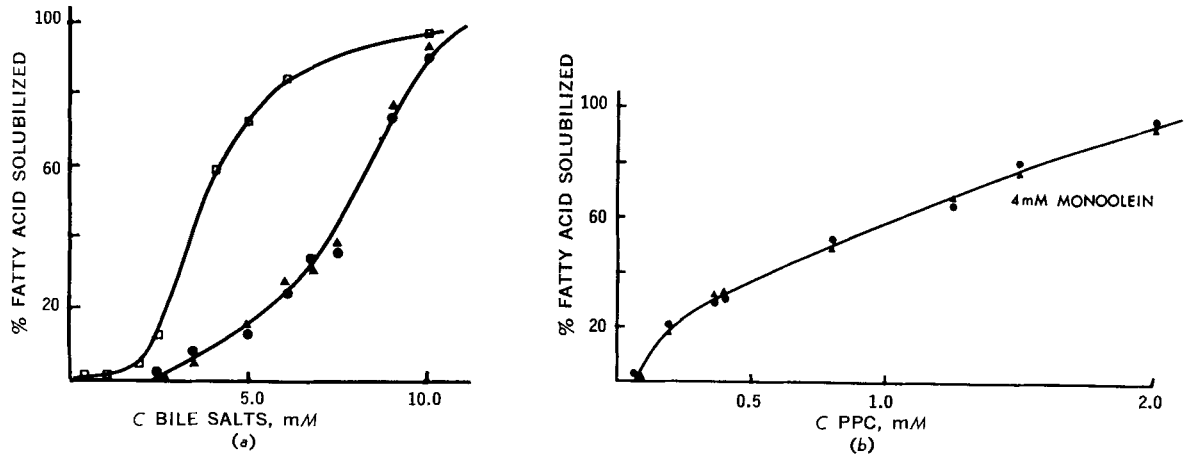


Figure 3—(*a*) *Fatty acid solubilization in bile salt solution. Total lipid concentrations: oleic acid* mM: □, *1.0, and* ●, *4.0; palmitic acid* mM: ▲, *2.0; and monoolein* mM: *upper curve, 1.0, and lower curve, 4.0.* (*b*) *Fatty acid solubilization in polyoxyethylene–polyoxypropylene copolymer (PPC) solution. Total lipid concentrations: oleic acid* mM, ●, *4.0; palmitic acid* mM, ▲, *2.0; and monoolein* mM, *4.0.*

Table II—Diffusion Coefficients $\bar{D}_T$ in Bile Salt Solutions

Chamber P		Chamber Q			$\bar{D}_T \times 10^6$ (cm.^{2} sec.$^{-1}$)	
Bile Salt, mM	Lipid, mM[a]	Bile Salt, mM	Lipid, mM	Diaphragm	Bile Salts	Oleic Acid
10.0	—	5.0	—	30-μ SG	5.28	
10.0	1.0 OA, 1.0 MO	5.0	—	30-μ SG	5.43	3.25
					4.75	3.40
					4.63	3.10
					5.0 ± 0.4	3.2 ± 0.2
10.0	1.0 OA, 1.0 MO	10.0	1.0 OA, 1.0 MO	30-μ SG		3.47
						4.47
						3.9 ± 0.5
10.0	4.0 OA, 4.0 MO	10.0	4.0 OA, 4.0 MO	1.2-μ MP		4.4 ± 0.5
2.0	1.0 OA, 1.0 MO	2.0	—	30-μ SG		0.59
2.0	1.0 OA, 1.0 MO	2.0	1.0 OA, 1.0 MO	30-μ SG		0.41
						0.33
						0.37 ± 0.04
2.0	4.0 OA, 4.0 MO (2.0 PA)	2.0	4.0 OA, 4.0 MO (2.0 PA)	0.010-μ MP		0.25 ± 0.04

[a] MO = monoolein, OA = oleic acid, and PA = palmitic acid.

ference (rather than just an isotopic concentration gradient) is outside the error limits for the experiments described.

For bile salt concentrations below the CMC, that is, lipid exists mainly in the emulsion phase, the kinetic constant is effectively the same when a 0.010-μ MP rather than a 30-μ SG diaphragm is interposed between the equilibrating systems; this figure is an order of magnitude lower than when the lipid is totally solubilized.

Polyoxyethylene–Polyoxypropylene Copolymer—*Effect of Pore Size*—When each chamber contains lipid of composition 4:4:2 mM in monoolein–oleic acid–palmitic acid and 2 mM polyoxyethylene–polyoxypropylene copolymer, equilibration rates over the MP porosity range from 0.010 to 1.2 μ are as shown in Fig. 4. The CMC for this nonionic surfactant is near 0.10 mM, based on solubilization data in Fig. 3*b*, and 2.0 mM polyoxyethylene–polyoxypropylene copolymer ensures better than 95% solubilization of fatty acid in the lipid system used. Hence, the interpretation is not complicated by the presence of an emulsion phase. Nevertheless, the equilibration rate is roughly constant over the MP porosity range from 0.010 to 0.200 μ and increases with larger pore sizes, even when variation in the cell constant β is allowed for (lower curve of Fig. 4).

In the lower porosity range, $\bar{D}_T$ is 3.2 × 10^{-7} cm.^{2} sec.$^{-1}$ and increases to 9.7 × 10^{-7} cm.^{2} sec.$^{-1}$ for the 1.20-μ MP; from this rate/reciprocal pore dimension display, ~3 μ is the MP dimension at which the diffusion coefficient of fatty acid in the 2.0 mM polyoxyethylene–polyoxypropylene copolymer system approaches that obtained with the SG diaphragm corresponding to free diffusion (Table III). Due to the steep nature of the plot in this region, this can only be an estimate; but the point emphasized is that although the fatty acid is solubilized, the flux is much reduced below 0.200 μ.

Effect of Solubilization—A detailed study of the effect of solubilization on equilibration rate was not carried out in the polyoxyethylene–polyoxypropylene copolymer system, since preliminary experiments suggested that similar effects to the bile salt system were being observed; that is, when micelles are freely transported and emulsion globules restricted in their movement, increased solubilization due to an increasing concentration of surfactant, at a fixed lipid composition, results in an increased flux. The difference is that these effects are observable in the 1-μ pore range compared with 100 times lower for the bile salt micelle.

This behavior is expected from the data in Fig. 5, which summarizes the throughput of fatty acid in ultrafiltration experiments, under a differential pressure of 60 cm. Hg. While only approximate at best, ultrafiltration does show that with increasing polyoxyethylene–polyoxypropylene copolymer concentration the proportion

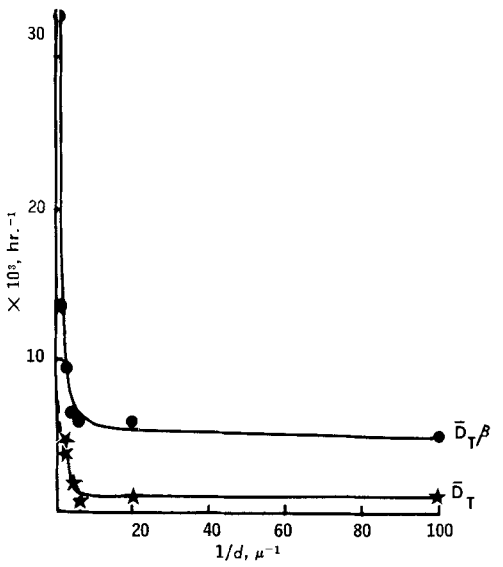


Figure 4—*Fatty acid equilibration rate* $\bar{D}_T\beta$, *upper curve,* $\bar{D}_T$, *lower curve,* versus *reciprocal of average pore dimension (MP): 4:4:2* m*M monoolein–oleic acid–palmitic acid in 2.0* m*M polyoxyethylene–polyoxypropylene copolymer.*

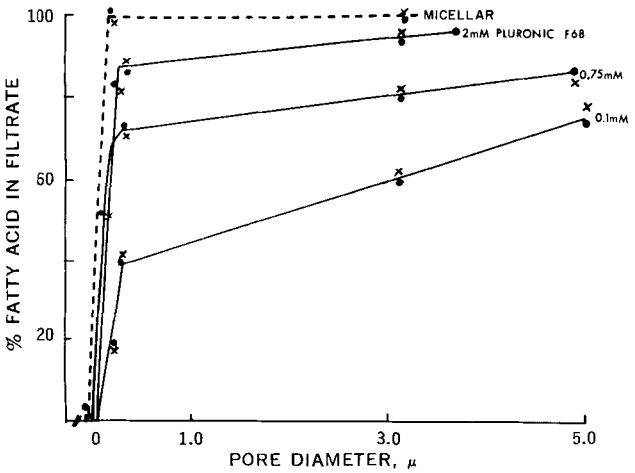


Figure 5—*Ratio of fatty acid, filtrate/unfiltered dispersion as a function of average pore dimension (MP): 4:4:2* m*M monoolein–oleic acid–palmitic acid in polyoxyethylene–polyoxypropylene copolymer. Key: oleic acid* m*M,* ●, *4.0; palmitic acid* m*M,* ×, *2.0;* ——, *filtration of emulsion–micellar dispersion; and* - - -, *filtration of micellar solution obtained by ultracentrifugation of 2* m*M polyoxyethylene–polyoxypropylene copolymer–lipid dispersion.*

Table III—Diffusion Coefficients $\bar{D}_T$ in Polyoxyethylene–Polyoxypropylene Copolymer (PPC) Solutions

Chamber P		Chamber Q			$\bar{D}_T \times 10^6$ (cm.^{2} sec.$^{-1}$)	
PPC, mM	Lipid, mM	PPC, mM	Lipid, mM	Diaphragm	PPC	Oleic Acid
2.0 (16 mg./ml.)	—	0.1	—	30-μ SG	2.68	
2.0	1.0 OA, 1.0 MO	1.0	—	30-μ SG	1.83	2.20
					1.89	2.05
					2.63	2.01
					2.49	1.13
					2.2 ± 0.4	1.8 ± 0.4
2.0	1.00 OA, 1.0 MO	2.0	1.0 OA, 1.0 MO	30-μ SG		2.0
2.0	4.0 OA, 4.0 MO	2.0	4.0 OA, 4.0 MO	0.010-μ MP		0.32 ± 0.04
2.0	4.0 OA, 4.0 MO	2.0	4.0 OA, 4.0 MO	1.20-μ MP		0.97 ± 0.11

of fatty acid in the filtrate increases appreciably only with filters above 0.100-μ pore size. Moreover, while the bulk of fatty acid in polyoxyethylene–polyoxypropylene copolymer micellar solution is transmissible through 0.300-μ pores (Fig. 5), pore sizes in excess of 5.0 μ are necessary to recover all the fatty acid from a filtered polyoxyethylene–polyoxypropylene copolymer stabilized emulsion.

By contrast, a 0.010-μ MP under the same hydrostatic pressure retains no fatty acid from an emulsion stabilized with 2.0 mM bile salts.

The diffusion data in the polyoxyethylene–polyoxypropylene copolymer system are summarized in Table III.

Comparison of Micelles of the Two Surfactants—At 37°, pH 6.4, $Na^+ = 0.15$ M, and in the absence of lipid, the micellar molecular weights (MMWs) of 4:1 NaTC–NaTDC is 2.0×10^4 (*i.e.*, aggregation number 40) and 5×10^5 for polyoxyethylene–polyoxypropylene copolymer from a light-scattering study made in conjunction with the present investigation. The former value is in fair agreement with 1.2–2.3×10^4 (depending on the sodium-ion concentration) for the taurodeoxycholate micelle at 25° (20). MMWs of the magnitude found for polyoxyethylene–polyoxypropylene copolymer are not unusual for nonionic surfactants (21).

From the Stokes relation, the diffusion coefficient D is inversely proportional to the radius. If the diffusing entities are roughly spherical and their densities about the same, D is inversely proportional to $\sqrt[3]{\text{MMW}}$. Also, if the two media have about the same viscosity,

$$\frac{D^{BS}}{D^{PPC}} \sim \frac{\sqrt[3]{5 \times 10^5}}{\sqrt[3]{2 \times 10^4}} = 2.9 \qquad \text{(Eq. 2)}$$

Above the CMC the apparent diffusion coefficient $\bar{D}_T$ is the weighted mean of the coefficient for monomolecular and micellar species (22, 23):

$$\bar{D}_T C_T = D_{\text{mono.}} C_{\text{mono.}} + D_{\text{mic.}} C_{\text{mic.}} = L_1 X_1 + L_2 X_2 \qquad \text{(Eq. 3)}$$

where C's are the concentrations (see *Appendix*).

If solubilization does not greatly alter the CMC, *i.e.*, the CMC of each surfactant in the absence of lipid solubilizate is close to that in the presence of lipid, then from the observed values of the CMC by the solubilization method and the measured diffusion coefficients of monomeric and micellar surfactant:

$$\frac{\bar{D}^{BS}}{\bar{D}^{PPC}} = \frac{4.9 \times 10^{-6}}{1.6 \times 10^{-6}} \sim 3.0 \qquad \text{(Eq. 4)}$$

in fair agreement with the expected ratio.

Because of the necessarily lower concentrations of surfactants and the interferometric method of estimation, $D_{\text{mono.}}$ has low accuracy. However, the monomeric contribution to the total flux is relatively small, and relatively large errors in the detemination of the diffusion coefficient of monomer result in relatively small changes in $D_{\text{mic.}}$

Correspondingly, using 6×10^{-6} cm.^{2} sec.$^{-1}$ for the diffusion coefficient of monomeric oleic acid (24) and 4×10^{-5} M for its solubility (6), the diffusion coefficients of the lipid-containing (1.0 mM solutions) bile salt and polyoxyethylene–polyoxypropylene copolymer micelles are 3.9×10^{-6} cm.^{2} sec.$^{-1}$ and 1.8×10^{-6} cm.^{2} sec.$^{-1}$, respectively, based on the movement of radioactively labelled fatty acid.

CONCLUSIONS

An increase in the driving force for diffusion of lipid with an increase in solubilization is clearly apparent and, provided that events in the luminal stage determine the kinetics of absorption of fatty acid, this would account for the improved fatty acid uptake observed in everted sacs from micellar systems compared with emulsions (5).

Another factor that emerges from this study is the necessity for unrestricted movement of micelles if their maximum contribution to the diffusive flux is to be achieved. With bile salt micelles, the transport function approaches the diffusion coefficient in free solution for pore sizes somewhat in excess of 10 mμ; whereas with polyoxyethylene–polyoxypropylene copolymer micelles, dimensions greater than 200 mμ are necessary for unhampered micellar movement.

The significance of these findings to fat absorption *in vivo* is not clearcut, but a reasonable expectation is that incomplete mixing in the gut preserves a layer of fluid near the absorptive cell in which diffusion-controlled processes dominate. Physical restrictions on the movement of micelles due to the microvilli and the associated glycocalyx coat are possible, but further experiments are required to assess their importance.

However, even neglecting the possible existence of a greater macroscopic barrier to the movement of the polyoxyethylene–polyoxypropylene copolymer micelle, the factor of 2 between the diffusion coefficients of the lipid-containing bile salt micelle compared with the lipid-containing polyoxyethylene–polyoxypropylene copolymer micelle would largely account for the difference of absorption rates in everted intestinal sacs observed for the two types of micellar systems.

APPENDIX

The usual application of irreversible thermodynamics is followed.

The flux of fatty acid (*e.g.*, oleic acid) in the systems described may be written

$$J_T = J_1 + J_2 + J_3 \qquad \text{(Eq. A1)}$$

where the subscripts 1, 2, and 3 refer to the contributions of monomers, micelles, and emulsion globules ("monomer" including all associated forms of fatty acid that exist outside the micelles and emulsions).

Flows and forces are related by

$$J_i = \sum_k L_{ik} X_k \qquad \text{where } i = 1, 2, 3 \ldots n \qquad \text{(Eq. A2)}$$

and L_{ik} is the coefficient that expresses the effect of the force X_k on the flow J_i. This set of n equations includes all possible interactions between flows and forces in the system.

In the absence of driving forces such as temperature and pressure gradients, the driving forces are equatable to chemical potential gradients or concentration gradients if the usual equivalence between thermodynamic activity and concentration, at low solute concentrations, is assumed.

Since the flux of surfactant can also be described as the sum of monomer J_I and micellar J_{II} fluxes, the fatty acid flux can be wholly described by

$$J_1 = L_1 X_1 + L_{12} X_2 + L_{13} X_3 + L_{1I} X_1 + L_{1II} X_{II} \qquad \text{(Eq. A3)}$$

$$J_2 = L_2X_2 + L_{21}X_1 + L_{23}X_3 + L_{2I}X_I + L_{2II}X_{II} \quad \text{(Eq. A4)}$$

$$J_3 = L_3X_3 + L_{31}X_1 + L_{32}X_2 + L_{3I}X_I + L_{3II}X_{II} \quad \text{(Eq. A5)}$$

The first term in each equation is, as expected, an expression of Fick's diffusion law, $J_i = L_iX_i = -D(dc_i)/(dx)$. For example, in Eq. A3 the flux of monomer J_1 is related to the concentration of monomer X_1 through the diffusion coefficient L_1, the second term takes into account "coupling" between monomer fatty acid and micellar fatty acid, and the third term takes into account coupling between monomer oleic acid and emulsified oleic acid. The last two terms in Eq. A3 allow for association between monomer oleic acid and monomer and micellar surfactant, respectively, and so on for J_2 and J_3. These equations attempt to account for all associations between fatty acid and surfactant in their different aggregated forms (with the proviso that the presence of monoolein alters the description little).

From the reciprocal relation postulate of Onsager, $L_{12} = L_{21}$, $L_{13} = L_{31}$, *etc.* The near similarity of $\bar{D}_T$ in an emulsified system when the movement of emulsion globules is, or is not, restricted by the diaphragm, *e.g.*, 0.010-μ MP compared with SG, suggests that the flux of emulsion is small, *i.e.*, $J_3 \sim 0$. Correspondingly and considering the relatively small interface of the emulsion, that is, molecules aggregated in the emulsion must interact less with fatty acid in free solution compared with the extent of interaction if the same amount of material was all dispersed in solution, coupling between emulsion and monomeric and emulsion and micellar fatty acid may be neglected. Then

$$J_1 = L_1X_1 + L_{12}X_2 + L_{1I}X_I + L_{1II}X_{II} \quad \text{(Eq. A6)}$$

and

$$J_2 = L_2X_2 + L_{21}X_1 + L_{2I}X_I + L_{2II}X_{II} \quad \text{(Eq. A7)}$$

are the only quantities of importance.

Coupling between surfactant and fatty acid movement should be apparent in the comparison of two systems, one where there is a net concentration gradient of surfactant and the other where chemical concentrations are the same in both chambers, but a difference exists only in the concentration of isotopic label. The identity (within the limits stated) of $\bar{D}_T$ for oleic acid in polyoxyethylene–polyoxypropylene copolymer diffusing in sintered glass under the two conditions described suggests that

$$L_{1I} \sim L_{1II} \sim L_{2I} \sim L_{2II} = 0 \quad \text{(Eq. A8)}$$

Considering the weights of monomers and micelles of bile salts compared with polyoxyethylene–polyoxypropylene copolymer, one may be led to expect that if interaction between the flows of fatty acid and surfactant were significant, a lesser effect would be observed with bile salts than with the nonionic surfactant. The reverse is the case, which is the justification for the application of Eq. A8 to the bile salt system.

The argument is somewhat complicated in a system with a solute concentration gradient by the movement of solvent (*i.e.*, water) in the opposite direction to the flow of solute, which may reduce the observed (*i.e.*, mutual) solute diffusion coefficient from the isotopic (~differential or thermodynamically ideal) value. However, this solvent flow effect is likely to be small when the chemical potential differences are as small as in the systems described here. The magnitude of solute concentrations in the present work also justifies comparisons between 1 mM and 4 mM lipid systems. Then,

$$J_T = L_1X_1 + L_2X_2 + L_{12}(X_1 + X_2) \quad \text{(Eq. A9)}$$

from the mentioned equivalence of L_{12} and L_{21}. L_{12} is likely to be small. But since the extent of this coupling between monomer and micellar fatty acid is uncertain, Eq. A9 is best written

$$J_T = (L_1 + L_{12})X_1 + (L_2 + L_{12})X_2 = L_1'X_1 + L_2'X_2 \quad \text{(Eq. A10)}$$

The measured diffusion coefficients L_1' and L_2' equal L_1 and L_2, respectively, if L_{12} is negligibly small.

REFERENCES

(1) G. Benzonana and P. Desnuelle, *Biochim. Biophys. Acta*, **105**, 121(1965).

(2) A. F. Hofmann and B. Borgstrom, *Fed. Proc.*, **21**, 43(1962).

(3) J. M. Johnston and B. Borgstrom, *Biochim. Biophys. Acta*, **84**, 412(1964).

(4) W. J. Simmonds, *Amer. J. Clin. Nutr.*, **22**, 266(1969).

(5) N. E. Hoffman, *Biochim. Biophys. Acta*, **196**, 193(1970).

(6) W. J. Simmonds, T. G. Redgrave, and R. L. S. Willix, *J. Clin. Invest.*, **47**, 1015(1968).

(7) C. W. Carr, *J. Colloid Sci.*, **20**, 923(1965).

(8) R. B. Dean and J. R. Vinograd, *J. Phys. Chem.*, **46**, 1091 (1942).

(9) K. J. Mysels, in "Pesticidal Formulations Research," J. W. Valkenburgh, Ed., Advances in Chemistry Series 86, American Chemical Society, 1969, chap. 4.

(10) B. Borgstrom, *J. Lipid Res.*, **8**, 598(1967).

(11) A. M. Ugolev, "Physiology and Pathology of Membrane Digestion," Plenum, New York, N. Y., 1968.

(12) A. Norman, *Arkiv Kemi*, **8**, 331(1955).

(13) A. F. Hofmann, *Acta Chem. Scand.*, **17**, 173(1963).

(14) R. H. Stokes, *J. Amer. Chem. Soc.*, **72**, 763(1950).

(15) J. H. Northrop and M. L. Anson, *J. Gen. Physiol.*, **12**, 543 (1929).

(16) E. N. Luce, E. C. Denice, and F. E. Akerlund, *Ind. Eng. Chem.*, **15**, 365(1943).

(17) R. H. Stokes, "Electrolyte Solutions," Butterworth's, London, England, 1955.

(18) E. B. Feldman and B. Borgstrom, *Lipids*, **1**, 430(1966).

(19) F. P. Woodford, *J. Lipid Res.*, **10**, 539(1969).

(20) J. P. Kratohvil and H. T. DelliColli, *Can. J. Biochem.*, **46**, 946(1968).

(21) R. R. Balmbra, J. S. Clunie, J. M. Corkill, and J. F. Goodman, *Trans. Faraday Soc.*, **60**, 979(1964).

(22) D. G. Kolp, R. G. Laughlin, F. P. Krause, and R. E. Zimmerer, *J. Phys. Chem.*, **67**, 51(1963).

(23) W. L. Courchene, *J. Phys. Chem.*, **68**, 1870(1964).

(24) L. G. Longworth, *J. Amer. Chem. Soc.*, **75**, 5705(1953).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 4, 1970, from the *Department of Physiology, University of Western Australia, Nedlands, Western Australia.*

Accepted for publication May 11, 1970.

This work was supported by the Australian Research Grants Committee.

The author is indebted to Professor W. J. Simmonds for many helpful discussions and to Professor J. P. Quirk for use of the interferometer and light-scattering photometer.

Present address: Department of Chemistry, Western Australian Institute of Technology, Hayman Road, South Bentley 6102, Australia.

Inhibitors of Monoamine Oxidase VI: Effects of Substitution on Inhibitory Activity of 6(or 8)-Substituted β-Carbolines

BENG T. HO, KO-CHIN LI, K. E. WALKER, L. WAYNE TANSEY, PATRICIA M. KRALIK, and WILLIAM M. McISAAC

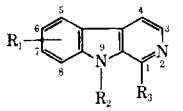
Abstract □ A number of 6(or 8)-substituted aromatic β-carbolines were synthesized, and their inhibitory activities toward monoamine oxidase were compared with their tetrahydro congeners. A considerable difference in the effects of 6(or 8)-substitution on the inhibitory activities existed between these aromatic and tetrahydro-β-carbolines. Influence of 9-methyl substitution on activities was greater with the tetrahydro than the aromatic series; as a result, 9-methyltetrahydro-β-carbolines were generally much better inhibitors of the enzyme than the corresponding 9-hydrogen-tetrahydro-β-carbolines. An amino group at C_1 of β-carboline caused a fivefold decrease in inhibitory activity. This decrease was likely due to the steric hindrance by the bulk of the amino group. Aromatic β-carbolines were prepared by the palladium-on-charcoal catalyzed dehydrogenation of the corresponding tetrahydro-β-carbolines. Methylation of the N_9 of aromatic β-carboline was carried out with methyl iodide in the presence of sodium hydride. Nitration of β-carboline gave a mixture of two isomeric products, 6- and 8-nitro-β-carbolines, which were separated with hot chloroform. Catalytic reduction converted the nitro compounds to 6- and 8-amino-β-carbolines, respectively. The positions of the amino group was confirmed by NMR spectrometry.

Keyphrases □ Monoamine oxidase inhibitors—synthesis □ β-Carbolines, 6(or 8)-substituted—synthesis □ Structure–activity relationship—monoamine oxidase inhibitors □ NMR spectroscopy—structure □ UV spectrophotometry—structure

In the previous papers (1, 2) the monoamine oxidase inhibitory activity of several 6(or 8)-substituted tetrahydro-β-carbolines and their 9-methyl analogs has been reported. It was found that, in general, replacement of hydrogen on the C_6 position of tetrahydro-β-carboline (I*a*) caused a slight reduction in inhibitory activity. An even greater decrease of activity resulted when the same position of 9-methyltetrahydro-β-carboline (I*b*) was substituted (1). Introduction of a methyl group on C_8 of I*a* and I*b*, however, did not affect the activity of these two compounds. In the present study, a number of 6(or 8)-substituted aromatic β-carbolines (V–XV) were synthesized, and their inhibitory activities were compared with those of the tetrahydro series.

A considerable difference in the effects of 6(or 8)-substitution on the inhibitory activities existed between these aromatic β-carbolines and their tetrahydro congeners. Replacement of the C_6-hydrogen of II by a methoxy group resulted in nearly a 1.5 time decrease in activity (Table I, Compound VI), whereas a greater loss (fourfold) of activity was observed by a similar substitution in the tetrahydro series (Table II Compounds I*a* and I*c*). When the 9-methyl compounds of each series were compared, 6-methoxy substitution on the 9-methyltetrahydro-β-carboline (I*b*) gave Compound I*d*, which was 10 times less active than I*b* as an inhibitor; however, only less than a threefold decrease in activity of 9-methyl-β-carboline (III) resulted from

Table I—Inhibition of Monoamine Oxidase



Compound	R_1	R_2	R_3	I_{50},[a] m*M*
II	H	H	H	0.029[b]
III	H	CH_3	H	0.010[b]
IV	H	H	CH_3	0.14[b]
V	H	H	NH_2	0.15
VI	6-OCH_3	H	H	0.043
VII[c]	6-OCH_3	CH_3	H	0.028
VIII	6-CH_3	H	H	0.12
IX	6-CH_3	CH_3	H	0.070
X	6-Cl	H	H	0.024
XI	6-NH_2	H	H	0.63
XII	8-OCH_3	H	H	0.12
XIII	8-CH_3	H	H	0.072
XIV[c]	8-CH_3	CH_3	H	0.020
XV	8-NH_2	H	H	0.58

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Data from *Reference 2*. [c] Hydrochloride salt.

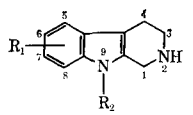
the conversion of III to 6-methoxy-9-methyl-β-carboline (VII). Substitution of chlorine on the C_6 position had less effect on both series, because X was equally as active as II, and I*e* was only slightly less active than I*a*. The greater (fourfold) loss of activity resulting from the methyl substitution of C_6 of II was unexpected in view of the similarity in sizes among CH_3, Cl, and OCH_3 and the weaker electron-donating nature of CH_3 than OCH_3.

It was reported earlier (1) that a methyl group on C_8 of I*a* and I*b* exerted little effect on the inhibitory activity (Table II, Compounds I*f* and I*g*). The same substitution on C_8 of the aromatic series, on the other hand, caused a reduction in the inhibition of the enzyme; XIII and XIV were, respectively, 2.5 and 2.0 times weaker inhibitors than II and III.

Effects of 9-methyl substitution were greater in the tetrahydro than in the aromatic series. For instance, a 34-fold increase in activity was achieved by the methylation of I*a* to I*b*, a 13-fold increase from I*c* to I*d*,[1] and nearly a 24-fold increase from I*f* to I*g* (Table II). Introduction of a methyl group to N_9 of II gave only a threefold increase in inhibition; a 1.5 time increase was observed as a result of conversion of VI to VII, a 1.7 time increase from VIII to IX, and a 3.6 time increase from XIII to XIV (Table I). These data further indicated that if aromatic β-carbolines and their tetrahydro congeners were bound at the same site on monoamine

[1] The previously reported value of 3.6 m*M* (1) should be the I_{50} for 2-methyl-6-methoxy-1,2,3,4-tetrahydro-β-carboline.

Table II—Inhibition of Monoamine Oxidase



Compound	R_1	R_2	I_{50},[a] m*M*
I*a*	H	H	0.34
I*b*	H	CH_3	0.010
I*c*	6-OCH_3	H	1.30
I*d*	6-OCH_3	CH_3	0.10
I*e*	6-Cl	H	0.42
I*f*	8-CH_3	H	0.38
I*g*	8-CH_3	CH_3	0.016

[a] All data from *References 1* and *2*.

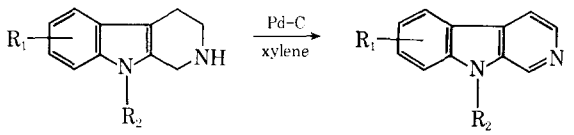
oxidase, it would appear to have different binding conformations for each series (3).

Decreases of 22- and 20-fold, respectively, in activity were found when an amino group was placed on either C_6 or C_8 of II (Table I, Compounds XI and XV). Such magnitude of reduction in activity, however, was not observed with the 6-methoxy compound (VI). Since the amino group is smaller in size than the methoxy group, the greater loss of activity found with XI and XV was, therefore, not due to the steric hindrance caused by the substituent on C_6 or C_8.

Replacement of C_1-hydrogen of II with an amino group resulted in about a fivefold decrease in activity. In view of the finding that V was an inhibitor of equal activity as 1-methyl-β-carboline (IV), this decrease was most likely attributed to the steric hindrance by the bulk of the amino or alkyl group (2).

CHEMISTRY

Aromatic β-carbolines, such as 6-methoxy-(VI), 6-methyl-(VIII), 6-chloro-(X), 8-methoxy-(XII), and 8-methyl-(XIII), were prepared by the palladium-on-charcoal (Pd-C) catalyzed dehydrogenation of the corresponding 1,2,3,4-tetrahydro-β-carbolines (I) in boiling xylene (Scheme I). The reaction would appear to require a

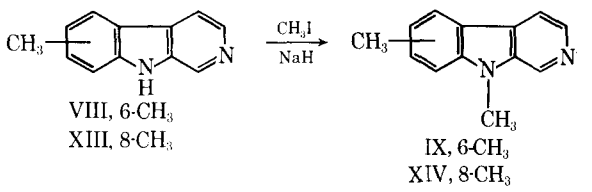


VI, R_1 = 6-OCH_3; R_2 = H
VII, R_1 = 6-OCH_3; R_2 = CH_3
VIII, R_1 = 6-CH_3; R_2 = H
X, R_1 = 6-Cl; R_2 = H
XII, R_1 = 8-OCH_3; R_2 = H
XIII, R_1 = 8-CH_3; R_2 = H

Scheme I

fresh and active catalyst; in a few instances when an old catalyst was used in the preparation of methoxylated β-carbolines, only the starting material was recovered. Due to the availability of 8-methyl-1-oxotetrahydro-β-carboline commercially, the preparation of XIII by the present method was simpler and more economical than by the method of Cook *et al.* (4) who utilized the condensation of 7-methyltryptophan with formaldehyde, followed by $K_2Cr_2O_7$ oxidation. The synthesis of 8-methyltetrahydro-β-carboline (I*f*) has previously been reported (1).

The very low yield of Compound X was apparently attributed to the dehalogenation of the 6-chloro atom by Pd-C during the dehydrogenation; unsubstituted β-carboline (XVI) was solated as a by-product. Attempts to prepare X by other methods were unsuccessful. Substitution of Raney nickel for Pd-C as the

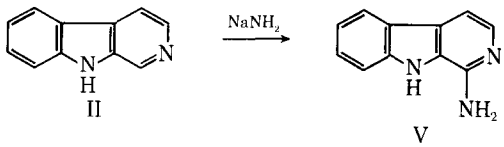


VIII, 6-CH_3
XIII, 8-CH_3
IX, 6-CH_3
XIV, 8-CH_3

Scheme II

catalyst resulted in the isolation of an equal amount of the unchanged 6-chlorotetrahydro-β-carboline (I*e*) and the dehalogenated product II. No reaction took place when 1-oxo-6-chlorotetrahydro-β-carboline was refluxed with phosphorus oxychloride. This reagent has been reported to give 9-alkyl-β-carbolines from 1-oxo-9-alkyl-1,2,3,4-tetrahydro-β-carbolines (5). Chloranil (tetrachloro-*p*-benzoquinone) has been shown to be an excellent dehydrogenating agent for the preparation of carbazoles from tetrahydrocarbazoles (6). However, the use of *p*-benzoquinone in the dehydrogenation of 6-chlorotetrahydro-β-carboline failed to give X.

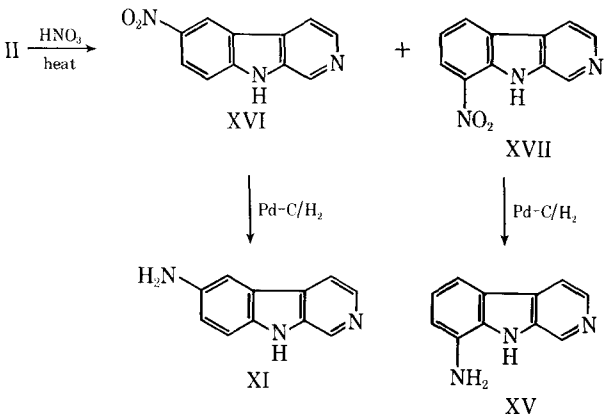
Methylation of VIII and XIII with methyl iodide in the presence of sodium hydride gave the dimethyl-β-carbolines IX and XIV, respectively (Scheme II). 1-Amino-β-carboline (V) was obtained from the treatment of β-carboline (II) with sodium amide according to the procedure reported in the literature (9) (Scheme III).



Scheme III

Nitration of β-carboline (II) and the reduction of the nitro-β-carboline to amino-β-carboline have been reported by Saxena (7). In his preparation, only one isomer was isolated. The position of the nitro group was not established other than to assume that it was the 6-nitro-β-carboline (XVI). No indication was given by Saxena on whether the 6-nitro-β-carboline prepared by him was free of contamination of other isomers. However, in carrying out the nitration of 1-methyl-β-carboline, Synder *et al.* (8) reported the isolation of two products. The higher-melting nitro compound (67% yield) was then reduced, diazotized, and converted to a bromo compound whose physical properties were identical with those of 6-bromo-1-methyl-β-carboline. The position of the nitro group in the lower-melting compound (20% yield) was assumed by them to be the isomer 8-nitro-1-methyl-β-carboline.

In the present work, two isomeric nitro-β-carbolines from the nitration of II (Scheme IV) were found to vary with their R_f values on TLC, retention times on gas chromatograms, as well as IR and UV spectra. Separation of these two isomers was achieved by the difference in their solubilities in hot chloroform. Reduction of the nitro group by Pd-C catalyst gave 6-amino-β-carboline (XI) and 8-amino-β-carboline (XV), respectively (Scheme IV). The authors chose to differentiate the positions of the amino group in the two products, XI and XV, by their NMR spectra; XI (60 Mc.p.s.): τ (D_2O-DCl),



Scheme IV

1.31 (singlet, H-1), 1.80 (doublet, J = 6.0 c.p.s., H-3), 1.89 (doublet, J = 6.0 c.p.s., H-4), 1.94 (doublet, J = 2.0 c.p.s., H-5), 2.27 (doublet of doublets, J = 2.0 and 8.7 c.p.s., H-7), 2.57 (doublet, J = 8.7 c.p.s., H-8). Due to the limited solubility of XV in D_2O-DCl, its NMR spectrum was run with a 100 Mc.p.s. spectrometer: τ (D_2O-DCl), 0.82 (singlet, H-1), 1.44 (doublet, J = 6.3 c.p.s., H-3), 1.55 (doublet, J = 6.3 c.p.s., H-4), 1.67 (doublet, J = 7.9 c.p.s., H-5), 2.07 (doublet, J = 7.9 c.p.s., H-7), 2.48 (triplet, J = 7.9 c.p.s., H-6). An earlier attempt to convert 6-nitrotetrahydro-β-carboline into XVI, which could then be used for further characterization of the 6-nitro isomer, was unsuccessful.

EXPERIMENTAL[2]

Preparation of Substituted Aromatic β-Carbolines from Tetrahydro-β-carbolines—A mixture of 20 mmoles of the corresponding substituted 1,2,3,4-tetrahydro-β-carbolines (1, 2) in 150 ml. of xylene and 500 mg. of 5 or 10% Pd-C catalyst was refluxed for 6 hr. or overnight and then filtered hot. The filtrate was cooled in ice; the deposited product was collected on a filter and recrystallized from a suitable solvent. Accordingly, the following compounds were prepared: 6-methoxy-β-carboline (VI), 45%, m.p. 206–207° (toluene); $\lambda_{max.}$ (EtOH) 217, 234, 249, 259 (s), 292, 299, 361, 371 mμ.

Anal.—Calcd. for $C_{12}H_{10}N_2O$: C, 72.71; H, 5.09; N, 14.14. Found: C, 73.01; H, 4.77; N, 14.09.

6-Methyl-β-carboline (*VIII*)—27%, m.p. 190–191° (benzene); $\lambda_{max.}$ (EtOH) 220, 240, 255 (s), 265 (s), 285, 297, 357, 372 mμ.

Anal.—Calcd. for $C_{12}H_{10}N_2$: C, 79.09; H, 5.53; N, 15.37. Found: C, 79.19; H, 5.55; N, 15.11.

6-Chloro-β-carboline (*X*)—0.9%, m.p. 270–271° (chloroform); $\lambda_{max.}$ (EtOH) 238, 249, 331, 345 mμ.

Anal.—Calcd. for $C_{11}H_7ClN_2$: C, 65.20; H, 3.48; N, 13.82. Found: C, 65.49; H, 3.38; N, 13.96.

8-Methoxy-β-carboline (*XII*)—62%, m.p. 204–205° (xylene); $\lambda_{max.}$ (EtOH) 216, 243, 268, 278, 287, 343, 354 (s) mμ.

Anal.—Calcd. for $C_{12}H_{10}N_2O$: C, 72.71; H, 5.09; N, 14.14. Found: C, 72.68; H, 5.11; N, 14.04.

8-Methyl-β-carboline (*XIII*)—90%, m.p. 233–234.5° (benzene); $\lambda_{max.}$ (EtOH) 217, 237, 338, 352 mμ. A 229–230° m.p. has been reported for this compound, which was prepared by the treatment of 7-methyltryptophan with formaldehyde followed by potassium dichromate oxidation (4).

6-Methoxy-9-methyl-β-carboline (*VII*)—Ether–HCl was added to the xylene filtrate and the hydrochloride salt of VII was collected on a filter; yield, 83%, m.p. 254–257°. Recrystallization from EtOH gave 50%, m.p. 258–259°; $\lambda_{max.}$ (EtOH) 220, 235, 248, 270, 286 (s), 292, 299, 370, 380 mμ. A 262–263° m.p. has been recorded for the hydrochloride salt of VII prepared by the treatment of the 1-oxo-6-methoxy-9-methyltetrahydro-β-carboline with $POCl_3$ (5).

Alkylation of Substituted β-Carbolines–*8,9-Dimethyl-β-carboline* (*XIV*)—To a stirred suspension of 0.2 g. (12 mmoles) of 8-methyl-β-carboline (XIII) in 20 ml. of dimethylformamide was added slowly 0.6 g. (17 mmoles) of sodium hydride (50% suspension in mineral oil). After stirring at room temperature for 4 hr., the mixture was cooled in ice, and 1.8 g. (13 mmoles) of methyl iodide was added. Stirring was continued overnight, and 150 ml. of chloroform was then added. The chloroform layer was washed with water (ten 100-ml. portions), dried (anhydrous sodium sulfate), and evaporated *in vacuo*, leaving an oil. When a solution of this oil in 150 ml. of dry ether was mixed with an excess of ether–HCl, a hydrochloride salt of the product precipitated. Recrystallization from ethanol gave 1.8 g. (65%), m.p. 298–300°.

Anal.—Calcd. for $C_{13}H_{12}N_2 \cdot HCl$: C, 67.10; H, 5.63; N, 12.04. Found: C, 66.75; H, 5.86; N, 11.98.

A small protion of this hydrochloride salt was neutralized with 10% sodium hydroxide to yield 8,9-dimethyl-β-carboline (XIV), m.p. 69–71°; $\lambda_{max.}$ (EtOH) 247, 292, 350, 364 mμ. A 68–70° m.p. has been recorded for the compound prepared from 1,7-dimethyltryptophan and formaldehyde followed by oxidation of the condensation product with potassium dichromate (4).

6,9-Dimethyl-β-carboline (*IX*)—In a similar manner as the preparation of XIV, a mixture of 1.6 mmoles of 6-methyl-β-carboline (VIII) in 10 ml. of dimethylformamide and 2.8 mmoles of sodium hydride was stirred for 30 min. Methyl iodide (2.8 mmoles) was added, and the stirring was continued for 1 hr. The product, m.p. 121–122°, was obtained in a 38% yield. Recrystallization from benzene–hexane did not raise the melting point; $\lambda_{max.}$ (EtOH) 220, 238, 255 (s), 265, 285, 297, 357, 367 mμ.

Anal.—Calcd. for $C_{13}H_{12}N_2$: C, 79.56; H, 6.16; N, 14.27. Found: C, 79.73; H, 6.24; N, 14.13.

1-Amino-β-carboline (V)—This was prepared from β-carboline (II) and $NaNH_2$ according to the procedure of Snyder *et al.* (9), m.p. 200–201° (lit. m.p. 198–200°); $\lambda_{max.}$ (EtOH) 225, 243, 256 (s), 273 (s), 280, 290, 339, 353 mμ.

Nitration of β-Carboline—A mixture of 4.96 g. (29 mmoles) of -carboline (II) and 60 ml. of concentrated nitric acid was stirred at ice temperature for 2 hr. and then heated on a steam bath until the solid dissolved (approximately 15 min.). The hot solution was poured onto 30 g. of crushed ice, and the bright-yellow solid that precipitated was collected on a filter and washed several times with water. This nitrate salt was dissolved in a minimum amount of water (about 900 ml.). Upon neutralization with 10% sodium hydroxide, the free amine precipitated; yield 4.88 g. (78%). Both silica gel TLC [$CHCl_3$–MeOH (9:1)] and gas chromatography (3% SE 30 on Varaport 30) showed that it was a mixture of two products.

The mixture was treated with about 4 l. of hot chloroform and then immediately filtered. The insoluble solid, 2.64 g. (42%) and with R_f = 5.8, was recrystallized 3 times from acetone to give 850 mg., m.p. 340–342°; $\lambda_{max.}$ (EtOH) 237, 267, 295, 350 mμ.

Anal.—Calcd. for $C_{11}H_7N_3O_2$: C, 61.97; H, 3.31; N, 19.71. Found: C, 61.83; H, 3.39; N, 19.54. The assignment of 6-nitro-β-carboline (XVI) to this compound was based on the NMR spectrum of its reduction product, 6-amino-β-carboline (XI).

When this chlorofom filtrate was evaporated *in vacuo*, 1.65 g. (26%) of bright-yellow solid was obtained. Two recrystallizations from a minimum amount of dimethylformamide gave 0.5 g., m.p. 320° dec.; R_f = 7.3. $\lambda_{max.}$ (EtOH) 228, 263, 292, 302 mμ.

Anal.—Calcd. for $C_{11}H_7N_3O_2$: C, 61.97; H, 3.31; N, 19.71. Found: C, 62.01; H, 3.45; N, 19.68. On the basis of the NMR spectrum of its reduction product (XV), the compound was believed to be the 8-nitro-β-carboline (XVII).

6-Amino-β-carboline (XI)—A mixture of 0.85 g. (4 mmoles) of 6-nitro-β-carboline (XVI), 200 ml. of ethanol, and 0.5 g. of 5% Pd-C catalyst was shaken with hydrogen at an initial pressure of 3 atm. until the consumption of hydrogen ceased. After filtration, the solution was evaporated *in vacuo*, and the residue was twice recrystallized from ethanol yielding 0.33 g. (45%) of product, m.p. 302–303° [lit. (7) m.p. 268°]; $\lambda_{max.}$ (EtOH) 242, 296, 304, 381 mμ. Silica gel TLC [butanol–acetic acid–water (4:1:1)]: R_f = 4.5.

Anal.—Calcd. for $C_{11}H_9N_3$: C, 72.11; H, 4.95; N, 22.94. Found: C, 71.81; H, 5.50; N, 22.69.

8-Amino-β-carboline (XV)—In a similar manner as described in the preparation of X, the 8-nitro-β-carboline (XVII) was catalytically reduced. The product, 8-amino-β-carboline was recrystallized from ethanol–ether to yield 62%, m.p. 242° dec.; $\lambda_{max.}$ (EtOH) 232, 252, 292, 360 mμ. Silica gel TLC [*n*-butanol–acetic acid–water (4:1:1)]: R_f = 6.4.

Anal.—Calcd. for $C_{11}H_9N_3$: C, 72.11; H, 4.95; N, 22.94. Found: C, 71.90; H, 5.11; N, 22.69.

Assay—Mitochondrial monoamine oxidase from beef liver was isolated and purified as previously described (2). All the stock solutions of inhibitors, except VIII and IX, were prepared in 0.01 *N* HCl. Compounds VIII and IX were dissolved in 0.02 *N* HCl. Incubation was carried out with tryptamine-2-^{14}C hydrochloride according to the previously described procedure (2).

[2] Melting points are corrected and were taken on a Mel-Temp apparatus. Qualitative UV spectra were obtained with a Beckman spectrophotometer model DB-G.

REFERENCES

(1) B. T. Ho, W. M. McIsaac, and L. W. Tansey, *J. Pharm. Sci.*, **58,** 998(1969).

(2) B. T. Ho, W. M. McIsaac, K. E. Walker, and V. Estevez, *ibid.*, **57,** 269(1968).

(3) B. T. Ho, W. M. McIsaac, L. W. Tansey, and K. E. Walker, *ibid.*, **58,** 219(1969).

(4) J. W. Cook, K. M. Gailey, and J. D. London, *J. Chem. Soc.*, **1954,** 568.

(5) Farbenfabriken Bayer Akt.-Ges., Ger. pat. 1,044,821, Nov. 27, 1958; through *Chem. Abstr.*, **55,** 3642(1961).

(6) B. M. Barclay and N. Campbell, *J. Chem. Soc.*, **1945**, 530.

(7) J. P. Saxena, *Indian J. Chem.*, **4**, 148(1965).

(8) H. R. Snyder, S. M. Parmeter, and L. Katz, *J. Amer. Chem. Soc.*, **70**, 222(1948).

(9) H. R. Snyder, H. G. Walker, and F. X. Werber, *ibid.*, **71**, 527(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 5, 1970, from *Texas Research Institute of Mental Sciences, Houston, TX 77025*

Accepted for publication June 3, 1970.

This work was supported in part by Grant MH-11168, U. Public Health Service, Bethesda, Md.

The authors thank Mrs. Geraldine Tasby for her assistance.

Kinetics and Mechanisms of Action of Drugs on Microorganisms XI: Effect of Erythromycin and Its Supposed Antagonism with Lincomycin on the Microbial Growth of *Escherichia coli*

EDWARD R. GARRETT, SAMUEL M. HEMAN-ACKAH, and GEORGE L. PERRY

Abstract □ The steady-state growth of *Escherichia coli* in broth cultures is inhibited by erythromycin with a new steady-state growth-rate constant ($k_{app.}$), which is linearly related to drug concentrations in the range 0–10.0 mcg. ml.$^{-1}$ as $k_{app.} = k_0 - k_E E$, where k_E is the inhibitory-rate constant for drug concentration E of drug-free rate constant k_0. The $k_{app.}$ at $E > 10.0$ mcg. ml.$^{-1}$ adheres to a kinetic model, which implies the saturation of a limited number of receptor sites in accordance with the equation $k_{app.} = k_0 - k_a E/(1 + k_b E)$, where k_a and k_b are constants of proportionality related to drug concentration partitioned into the biophase and its affinity for available receptor sites. The dependence of *E. coli* growth rate on drug concentrations is invariant with the organism population or broth concentrations. However, values for k_a increase 10-fold as the pH of broth is increased from 6.80 to 7.80 while k_0 remains constant. This indicates that the unprotonated fraction of the drug concentration contributes to the activity. Lincomycin in Phase I-affected growth has the same formal dependency on concentration as does erythromycin with a potency ratio of 6.68:1, erythromycin base to lincomycin base, on a weight basis. The combined effects of erythromycin and lincomycin in Phase I of its effect are not antagonistic on the growth rate of *E. coli* in the subinhibitory range and can be predicted on the basis of adding equivalent amounts in accordance with the coincident response–dose curves of erythromycin and lincomycin (Phase I).

Keyphrases □ Erythromycin effect—*Escherichia coli* steady-state growth □ Lincomycin growth, Phase I—erythromycin effect □ Reversibility—erythromycin antimicrobial activity □ Microbiological analysis—erythromycin action on *E. coli* □ Kinetics—erythromycin action on *E. coli*.

The mode of action of the macrolide erythromycin is generally ascribed to inhibition of protein synthesis (1–9). Erythromycin binds exclusively to the 50 S subunit of ribosomes from *Escherichia coli* (1, 4, 10), *Staphylococcus aureus* (11), *Bacillus subtilis* (6, 8, 12), *Bacillus megaterium* (2, 13), and *Bacillus stearothermophilus* (2) in reconstituted cell-free systems to inhibit polypeptide synthesis which has been stated to be a rapid and reversible process (8). Tanaka *et al.* (4) observed maximum binding of the 50 S ribosomal subunit of *E. coli* Q. 13 at very low concentrations of erythromycin (~0.6 mcg. ml.$^{-1}$). Only about one-tenth of that amount was found to bind to the ribosomes of resistant mutants.

It has been speculated (8) that erythromycin competes with the transport RNA carrying the peptidyl radical involved in amino acid polymerization for a common binding site on the 50 S subunit of ribosomes. An alternative proposed model (12) for erythromycin's protein inhibition is that it inhibits a "translocase" factor which is necessary for the transfer of the peptidyl t-RNA elongated by a single aminoacyl residue from a ribosomal acceptor site to a donor site.

Conflicting views have been expressed about the modes of action of those antibiotics (*e.g.*, erythromycin, lincomycin, and chloramphenicol), which are supposed to have the common 50 S ribosomal binding site (7, 8, 10, 12). In fact, combinations of erythromycin and lincomycin have been claimed to be antagonistic (2, 3, 8, 10, 12), which would not necessarily follow from an assumption of similar modes of action.

The application of microbial kinetics to the quantification and prediction of antimicrobial action has been demonstrated (14–22). The effects of subinhibitory concentrations of drugs on the growth of bacteria have been studied in simple reproducible systems with *E. coli* strain B/r as the test organism to derive kinetic parameters that may characterize antibacterial action and drug–receptor interaction and to provide insight into the possible mechanisms of drug action.

This paper presents the results of such studies on the action of erythromycin on the growth of *E. coli*. It considers the formal dependence of the kinetic constants of growth inhibition on antibiotic concentration as affected by inoculum size, composition and pH of the media, and reversibility of the erythromycin concentrations. In addition, the actions of combinations of erythromycin and lincomycin on microbial growth, as compared with their *a priori* expectation, are considered.

(6) B. M. Barclay and N. Campbell, *J. Chem. Soc.*, **1945**, 530.

(7) J. P. Saxena, *Indian J. Chem.*, **4**, 148(1965).

(8) H. R. Snyder, S. M. Parmeter, and L. Katz, *J. Amer. Chem. Soc.*, **70**, 222(1948).

(9) H. R. Snyder, H. G. Walker, and F. X. Werber, *ibid.*, **71**, 527(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 5, 1970, from *Texas Research Institute of Mental Sciences, Houston, TX 77025*

Accepted for publication June 3, 1970.

This work was supported in part by Grant MH-11168, U. Public Health Service, Bethesda, Md.

The authors thank Mrs. Geraldine Tasby for her assistance.

Kinetics and Mechanisms of Action of Drugs on Microorganisms XI: Effect of Erythromycin and Its Supposed Antagonism with Lincomycin on the Microbial Growth of *Escherichia coli*

EDWARD R. GARRETT, SAMUEL M. HEMAN-ACKAH, and GEORGE L. PERRY

Abstract □ The steady-state growth of *Escherichia coli* in broth cultures is inhibited by erythromycin with a new steady-state growth-rate constant ($k_{app.}$), which is linearly related to drug concentrations in the range 0–10.0 mcg. ml.$^{-1}$ as $k_{app.} = k_0 - k_E E$, where k_E is the inhibitory-rate constant for drug concentration E of drug-free rate constant k_0. The $k_{app.}$ at $E > 10.0$ mcg. ml.$^{-1}$ adheres to a kinetic model, which implies the saturation of a limited number of receptor sites in accordance with the equation $k_{app.} = k_0 - k_a E/(1 + k_b E)$, where k_a and k_b are constants of proportionality related to drug concentration partitioned into the biophase and its affinity for available receptor sites. The dependence of *E. coli* growth rate on drug concentrations is invariant with the organism population or broth concentrations. However, values for k_a increase 10-fold as the pH of broth is increased from 6.80 to 7.80 while k_0 remains constant. This indicates that the unprotonated fraction of the drug concentration contributes to the activity. Lincomycin in Phase I-affected growth has the same formal dependency on concentration as does erythromycin with a potency ratio of 6.68:1, erythromycin base to lincomycin base, on a weight basis. The combined effects of erythromycin and lincomycin in Phase I of its effect are not antagonistic on the growth rate of *E. coli* in the subinhibitory range and can be predicted on the basis of adding equivalent amounts in accordance with the coincident response–dose curves of erythromycin and lincomycin (Phase I).

Keyphrases □ Erythromycin effect—*Escherichia coli* steady-state growth □ Lincomycin growth, Phase I—erythromycin effect □ Reversibility—erythromycin antimicrobial activity □ Microbiological analysis—erythromycin action on *E. coli* □ Kinetics—erythromycin action on *E. coli*.

The mode of action of the macrolide erythromycin is generally ascribed to inhibition of protein synthesis (1–9). Erythromycin binds exclusively to the 50 S subunit of ribosomes from *Escherichia coli* (1, 4, 10), *Staphylococcus aureus* (11), *Bacillus subtilis* (6, 8, 12), *Bacillus megaterium* (2, 13), and *Bacillus stearothermophilus* (2) in reconstituted cell-free systems to inhibit polypeptide synthesis which has been stated to be a rapid and reversible process (8). Tanaka *et al.* (4) observed maximum binding of the 50 S ribosomal subunit of *E. coli* Q. 13 at very low concentrations of erythromycin (~0.6 mcg. ml.$^{-1}$). Only about one-tenth of that amount was found to bind to the ribosomes of resistant mutants.

It has been speculated (8) that erythromycin competes with the transport RNA carrying the peptidyl radical involved in amino acid polymerization for a common binding site on the 50 S subunit of ribosomes. An alternative proposed model (12) for erythromycin's protein inhibition is that it inhibits a "translocase" factor which is necessary for the transfer of the peptidyl t-RNA elongated by a single aminoacyl residue from a ribosomal acceptor site to a donor site.

Conflicting views have been expressed about the modes of action of those antibiotics (*e.g.*, erythromycin, lincomycin, and chloramphenicol), which are supposed to have the common 50 S ribosomal binding site (7, 8, 10, 12). In fact, combinations of erythromycin and lincomycin have been claimed to be antagonistic (2, 3, 8, 10, 12), which would not necessarily follow from an assumption of similar modes of action.

The application of microbial kinetics to the quantification and prediction of antimicrobial action has been demonstrated (14–22). The effects of subinhibitory concentrations of drugs on the growth of bacteria have been studied in simple reproducible systems with *E. coli* strain B/r as the test organism to derive kinetic parameters that may characterize antibacterial action and drug–receptor interaction and to provide insight into the possible mechanisms of drug action.

This paper presents the results of such studies on the action of erythromycin on the growth of *E. coli*. It considers the formal dependence of the kinetic constants of growth inhibition on antibiotic concentration as affected by inoculum size, composition and pH of the media, and reversibility of the erythromycin concentrations. In addition, the actions of combinations of erythromycin and lincomycin on microbial growth, as compared with their *a priori* expectation, are considered.

EXPERIMENTAL

Organism—Replicate slants of *E. coli* strain B/r were used in all experiments. The slants had been prepared from a single colony and were stored in a refrigerator at 4°.[1]

Culture Media—Bacto Antibiotic Medium 3[2] was rehydrated according to the specifications of the manufacturer to peptone broth USP. The media were filtered twice through Millipore 0.45-μ HA filters and autoclaved at 120° for 15 min. The pH of the media was 7.05 ± 0.05, with the exception of those that were used to study the antibacterial activity as a function of pH. To obtain media with a pH in the range of 6.8–7.8, various amounts of Millipore-filtered 1.7 *N* HCl and 2 *N* NaOH, respectively, were added to the culture media aseptically after the sterilization.

Antibiotic—An assayed sample of erythromycin lactobionate[3] (670 mcg. base eq. mg.$^{-1}$) was used and will be referred to here as erythromycin I. An assayed sample of lincomycin hydrochloride[4] (895 mcg. base eq. mg.$^{-1}$) was also used. The references to concentrations of drugs throughout this paper refer to these samples of antibiotics.

Bacterial Cultures—An aliquot (5 ml.) of culture medium was inoculated from a fresh slant, and the culture was allowed to grow for 12 hr. at 37.5° in an incubator. A sample of 0.5 ml. was then diluted 100-fold into fresh medium. The growth of the culture was followed up to 2 × 10^7 *E. coli* ml.$^{-1}$. Samples of 0.5 ml. of this culture (undiluted or suitably diluted in broth) were finally added to replicate volumes of 49.0 ml. broth in loosely capped 125-ml. conical flasks. The growth of culture was followed to a desired inoculum size, and 0.5 ml. of drug was added. The cultures were maintained at 37.5 ± 0.1° in a 50-gal. constant-temperature water bath equipped with a shaker. All pipets and media used for the dilutions of the cultures were prewarmed to protect the organisms from temperature shocks.

Total Count Method—The method has been previously described (16). Samples of 1.00 ml. were withdrawn at 20-min. intervals from the cultures. They were diluted to obtain counts within a range of 10,000–30,000 counts/50 μl. on the Coulter counter, model B.[5] The diluent used was a Millipore 0.45-μ HA-filtered aqueous solution of 0.85% NaCl and 1% formaldehyde. The instrument was equipped with a 30-μ orifice. The settings were: aperture current, 5; amplification, 8; gain, 10; lower threshold, 13; and upper threshold, maximum. The total counts were corrected for the background count of the particular batch of medium used and diluted in the same way as the sample. The background counts in general did not exceed 1000 counts/50 μl.

Viable Count Method—Samples of 0.50 ml. were withdrawn from the cultures and appropriately diluted into sterilized 0.85% saline solution so that 50–150 colonies per plate would result. From these dilutions, aliquots of 1.00 ml. were pipeted onto each of three replicate agar plates. The plates were incubated for 48 hr. at 37.5°, and the colonies were counted on a Colony counter, model C-110.[6]

Effect of Antibiotic Concentration on Growth Rates—Fresh solutions of the respective antibiotics were aseptically prepared for each experiment. They were sufficiently diluted so that aliquots of 0.5 ml. added to 49.5 ml. culture volumes yielded the desired drug concentrations (Table I). The solutions were added to the cultures growing at 37.5° in the logarithmic phase at an organism population of 1.3 × 10^6 *E. coli* ml.$^{-1}$. Samples were withdrawn every 20 min. and counted by both the viable and total cell count methods. One culture without drug was studied in each experiment as the control to obtain the growth-rate constant in absence of the drug. The growth curves for 0–250 mcg. ml.$^{-1}$ at pH 7.05 were obtained (Fig. 1). A similar experiment was performed for 0–50 mcg. ml.$^{-1}$ erythromycin I, but counts were obtained by only the total cell count method (Fig. 2).

[1] The strain B/r of *E. coli* is an "in house" strain originally obtained from the Bacteriology Department of the University of Maine (Dr. D. B. Pratt) and has been used in both the Departments of Bacteriology and Pharmaceutics of this university for the last 10 years. In previous publications on "Kinetics and Mechanisms of Action of Drugs on Microorganisms I–X" (14–22), the authors have referred to this *E. coli* as strain B/r. A sample of this strain is being sent to the American Type Culture Collection for appropriate "official" numbering.

[2] Difco Laboratories, Detroit, Mich.

[3] Supplied by Abbott Laboratories, North Chicago, Ill.

[4] Supplied by The Upjohn Co., Kalamazoo, Mich.

[5] Coulter Electronics Co., Hialeah, Fla.

[6] New Brunswick Scientific Co., New Brunswick, N. J.

Table I—Growth-Rate Constants ($k_{app.}$ in sec.$^{-1}$) and Calculated Parameters for Erythromycin-Affected and Lincomycin-Affected *E. coli* in Broth at 37.5° and pH 7.05

Drug Concentrations, mcg. ml.$^{-1}$ × n^a	10^5 $k_{app.}$ Erythromycin I	10^5 $k_{app.}$ Lincomycin HCl, Phase I	10^5 $k_{app.}$ Lincomycin HCl, Phase II
0	60.1	60.1	60.1
2.5	56.9	56.9	—
5.0	54.6	53.6	—
7.5	50.6	50.0	41.7
10.0	45.6	47.5	35.1
20.0	36.9	34.8	26.2
30.0	25.5	24.8	14.5
40.0	18.0	18.6	8.4
50.0	13.4	14.3	5.7
10^5 k_E (ml. mcg.$^{-1}$ sec.$^{-1}$)[b]	1.50	0.27	—
10^5 k_a (ml. mcg.$^{-1}$ sec.$^{-1}$)[c]	1.64	0.35	0.63
10^5 k_a/k_b (sec.$^{-1}$)	117.65	117.65	66.7
10^2 k_b (ml. mcg.$^{-1}$)	1.39	0.28	0.93

[a] n = 1 for erythromycin; n = 0.2 for lincomycin, *i.e.*, actual concentration of lincomycin is five times that stated. [b] Calculated from the slope of the plot of $k_{app.}$ *versus* concentration from 0 to 10 mcg. ml.$^{-1}$ erythromycin I or 0 to 100 mcg. ml.$^{-1}$ lincomycin HCl. [c] k_a, k_b, and k_a/k_b are estimated from the slopes and intercepts of $D/(k_0 - k_{app.})$ *versus* D, where D is concentration of drug and k_0 is $k_{app.}$ at D = 0 for the expression $D/(k_0 - k_{app.}) = D(k_b/k_a) + 1/k_a$ for erythromycin I > 10 mcg. ml.$^{-1}$ and lincomycin HCl in the steady-state Phase I > 100 mcg. ml. and for all studied lincomycin concentrations in the second steady-state Phase II that occurs after several generations of lincomycin-affected growth (22).

Effect of Broth Concentration on Drug-Affected Growth Rates—Various strengths of peptone broth USP, buffered at pH 7, were prepared so that the concentrations of broth ingredients were

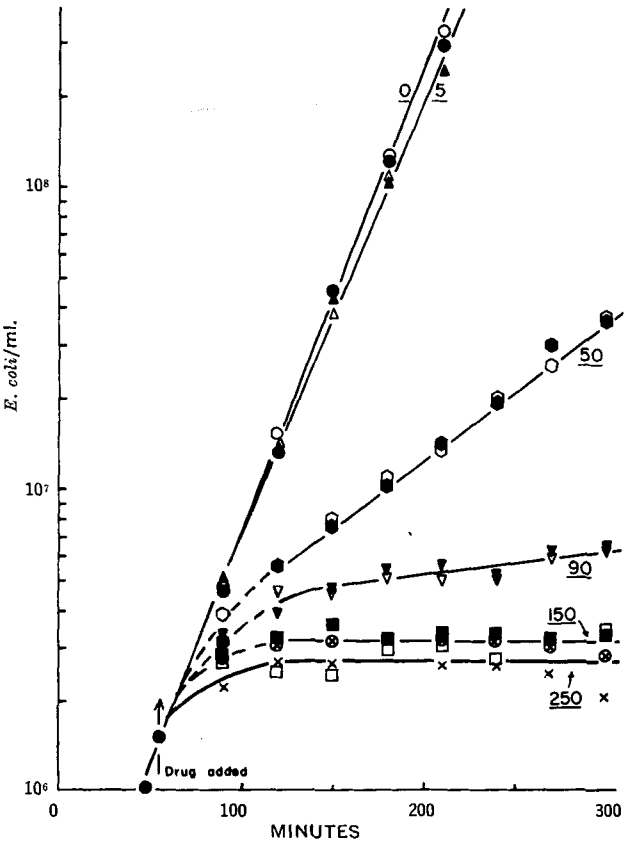


Figure 1—*Semilogarithmic plot of* E. coli/*ml. against time after broth inoculation in the presence of erythromycin I. Each curve is labeled in terms of micrograms per milliliter of erythromycin I. The open symbols are total counts obtained by the Coulter counter. The solid symbols are viable counts obtained by colony counts.*

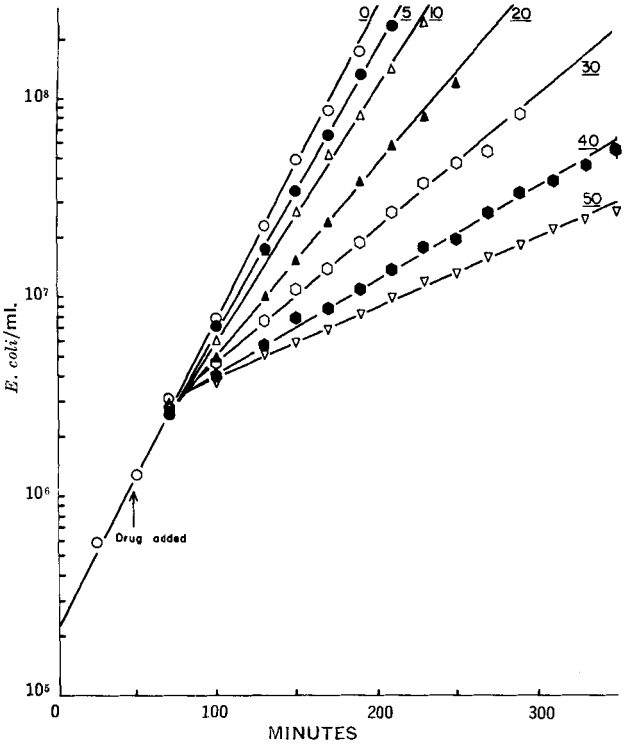


Figure 2—*Typical semilogarithmic plots of* E. coli *growth by total counts (Coulter) in the presence of the labeled concentrations of erythromycin I in micrograms per milliliter at 37.5° and pH 7.05.*

normal, halved, and doubled. Six replicate 49.5-ml. volumes of each kind were inoculated with 0.5 ml. culture containing 2×10^7 *E. coli* ml.$^{-1}$ in the logarithmic growth. They were maintained at 37.5°; when the cell concentration reached 10^6 ml.$^{-1}$, 0.5 ml. of drug solutions was added to achieve erythromycin I concentrations ranging from 5–80 mcg. ml.$^{-1}$ (Table II). The sixth replicate contained no drug. Coulter counts were obtained from samples withdrawn every 20 min.

Effect of Organism Population on Drug-Affected Growth Rates—Each flask of three sets of six replicate 49.5-ml. volumes of culture medium was inoculated with 0.5 ml. of appropriately diluted culture growing in the logarithmic phase. The three sets were allowed to grow at 37.5° to cell concentrations of 1.6×10^4, 1.3×10^5, and 1.1×10^6 *E. coli* ml.$^{-1}$, respectively. Aliquots (0.5 ml.) of drug solutions were added to achieve erythromycin I concentrations from 2.5–12.5 mcg. ml.$^{-1}$ (Table III). One culture in each set contained no drug. Coulter counts were obtained from samples withdrawn every 20 min.

Reversibility of Drug Action—A 49.5-ml. volume of broth was inoculated at zero time with *E. coli* to a cell concentration of 2×10^5 ml.$^{-1}$ (Figs. 3 and 4). The culture was allowed to grow at 37.5°

Table II—Growth-Rate Constants ($k_{app.}$) for Erythromycin-Affected *E. coli* in Various Broth[a] Concentrations at pH 7.05 and 37.5°

Erythromycin I Concentration, mcg./ml.$^{-1}$	10^5 $k_{app.}$, sec.$^{-1}$ Peptone (USP) Broth[a]	Doubled Nutrients	One-Half Nutrients
0	61.1	57.2	58.6
5	54.3	52.3	52.4
10	45.7	45.8	45.4
20	37.5	38.9	34.0
40	22.7	24.8	26.4
80	5.9	7.5	5.4

[a] The normal composition in grams per liter of broth was: beef extract, 1.5; yeast extract, 1.5; peptone, 5.0; and dextrose, 1.0. The K_2HPO_4 of 3.68 g./l. and KH_2PO_4 of 1.32 g./l. were maintained for all three broths. However the NaCl was 3.5 g./l. for the normal, 0.37 for the doubled, and 4.40 for the halved nutrients prepared from Bacto Antibiotic Medium 3 (Difco Laboratories, Detroit, Mich.).

Table III—Growth-Rate Constants ($k_{app.}$) for Erythromycin-Affected *E. coli* of Various Organism Populations at the Time of Drug Addition

Erythomycin I Concentration, mcg. ml.$^{-1}$	10^5 $k_{app.}$ sec.$^{-1}$ —*E. coli*/ml. at Time of Drug Addition— 9.0×10^3	1.0×10^6	1.10×10^6
0	61.5	61.0	59.5
2.5	59.5	55.8	56.0
5.0	51.8	52.1	49.9
7.5	46.2	45.4	46.8
10.0	42.0	41.2	41.6
12.5	34.6	35.0	37.9

(Curves A). At 50 min., when the growth was in the logarithmic phase and the cell concentration was 10^6 *E. coli* ml.$^{-1}$, 0.5 ml. of a sufficiently diluted erythromycin I solution was added to achieve a final concentration of 50 mcg. ml.$^{-1}$ (Curves B). At 125 min., after the culture of Curve B had settled into a new steady-phase growth, aliquots of 5 and 0.5 ml. were added to 45 and 49.5 ml. fresh broths, respectively, so that both organisms and drug concentrations were diluted 10-fold (Curve C, Fig. 3) and 100-fold (Curve C, Fig. 4). The drug-free culture of Curve A was likewise diluted 10-fold (Curve D, Fig. 3) and 100-fold (Curve D, Fig. 4). At the same time, aliquots of the cultures of Curve B (Figs. 3 and 4) were diluted 10-fold (Curve E, Fig. 3) and 100-fold (Curve E, Fig. 4), respectively, in broths containing enough erythromycin I so that the drug concentration was restored to 50 mcg. ml.$^{-1}$. Dilutions of the cultures of Curve B were also made 10-fold (Fig. 3) and 100-fold (Fig. 4) in broths (Curves F) containing enough erythromycin I to increase the drug concentration about 2-fold (*i.e.*, 100 mcg. ml.$^{-1}$).

At 200 min., when the cultures of Curves C containing 5 mcg. ml.$^{-1}$ (Fig. 3) and 0.5 mcg. ml.$^{-1}$ (Fig. 4) of erythromycin I had emerged from the lag phase, 0.5-ml. aliquots of solutions containing enough erythromycin I were added to reestablish both drug concentrations to 50 mcg. ml.$^{-1}$ (Curves G, Figs. 3 and 4).

Whenever any variation was effected, samples of the broth culture were taken and counted on the Coulter every 10 min. for the first 30 min. and thereafter at intervals of 30 min.

Effect of pH on Drug-Affected Growth Rates—Sufficient amounts of 1 *N* HCl and 2 *N* NaOH were added to the broth to obtain pH values of 6.80, 7.05, 7.20, 7.60, 8.20, and 8.40 (Fig. 5). Six replicate 49.5-ml. volumes of each broth were inoculated with 0.5 ml. of appropriately diluted *E. coli* culture in the logarithmic phase of growth. They were maintained at 37.5° until the organism population reached 10^6 *E. coli* ml.$^{-1}$. Drug solutions (0.5 ml.) were added to five replicates to achieve the desired concentrations of antibiotic shown in Fig. 4. The sixth replicate in each set contained no drug. Coulter counts were obtained from samples withdrawn every 20 min.

Comparison of Growth Rates of Erythromycin-Affected Organisms with Lincomycin-Affected Organisms—Solutions (0.5 ml.) of erythromycin I were added to five replicate 49.5-ml. volumes of broth inoculated with *E. coli* in the logarithmic phase of growth and containing an organism population of 10^6 ml.$^{-1}$ at 37.5°. The final concentration of the drug achieved in the broths ranged from 2.5 to 50 mcg. ml.$^{-1}$ erythromycin I. This was repeated for concentrations of lincomycin HCl, from 12.5 to 250 mcg. ml.$^{-1}$ (Table I). Counts were obtained from samples withdrawn every 20 min. The relationships between the apparent growth-rate constants, $k_{app.}$, and drug concentrations are shown in Fig. 6.

Action of Erythromycin and Lincomycin Combinations—Replicate 49-ml. samples of cultures in the steady-state growth (Curve A, Fig. 7) with 10^6 ml.$^{-1}$ *E. coli* were treated with antibiotic solutions. Aliquots (0.5 ml.) of appropriately diluted erythromycin I or lincomycin HCl solutions were added to separate cultures of Curve A 50 min. after inoculation. The resultant plots are given as Curve B in Fig. 7 for final concentrations of 15.8 mcg. ml.$^{-1}$ of erythromycin I or 60 mcg. ml.$^{-1}$ of lincomycin HCl or as Curve D for final concentrations of 31.6 mcg. ml^{-1} of erythromycin I or 120 mcg. ml.$^{-1}$ of lincomycin HCl. Seventy-five minutes after the addition of the drug, when the culture had settled to a new steady-state growth, aliquots (0.5 ml.) of appropriately diluted lincomycin HCl or erythromycin I solutions were added to the replicate solution that had been treated previously with the alternate drug. The resultant curve

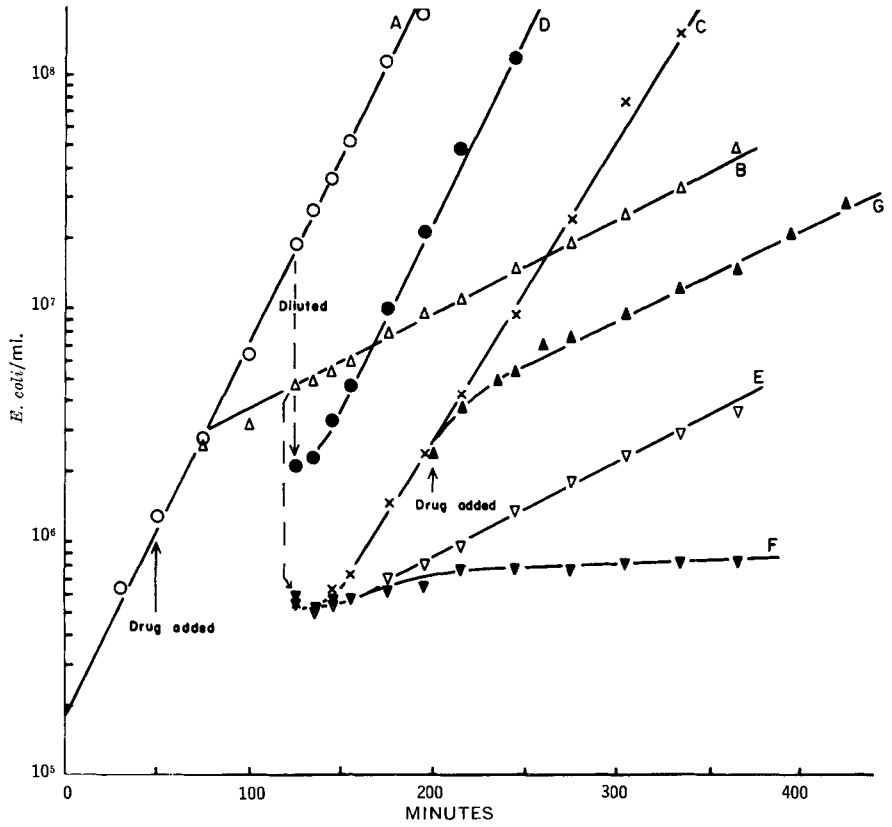


Figure 3—*Semilogarithmic plots of reversibility studies of* E. coli *growth with time on addition of erythromycin I and 1:10 dilution of culture with broth. Curve A is without drug. Curve B is after addition of 50 mcg./ml. Curves C, E, F, and G are after 1:10 dilution of the Curve B culture and have final drug concentrations of 5, 50, 100, and 50 mcg./ml., respectively. The dilution broth contained the erythromycin I in the cases of Curves E and F. There was no drug in the diluent in the case of Curve C, and drug was added at 200 min. for Curve G. Curve D is after 1:10 dilution of the culture of Curve A with no drug added.*

for both replicates, independent of the order of addition, was Curve C, Fig. 7. The final concentrations of the drugs in the cultures, represented by Curve C, were 15.8 mcg. ml.$^{-1}$ erythromycin I and 60.0 mcg. ml.$^{-1}$ lincomycin HCl. Coulter counts were obtained on samples that were withdrawn from the treated cultures every 20 min.

Effect of Mixtures of Graded Equipotent Concentrations of Erythromycin and Lincomycin—Equipotent solutions of 42.55 mcg. ml.$^{-1}$ erythromycin I and 200 mcg. ml.$^{-1}$ lincomycin HCl were prepared. They were then mixed in graded proportions using 100–0% of the lincomycin solution and the residual percentage of the erythromycin solution (Fig. 8). This was repeated to give nine graded equipotent mixtures for each of three antibiotic equivalent concentrations. Replicate 49.5-ml. cultures in steady-state growth that contained 10^6 *E. coli* ml.$^{-1}$ were each inoculated with an aliquot (0.5 ml.) of one of the antibiotic mixtures. Coulter counts were obtained for samples withdrawn every 20 min.

RESULTS

The addition of graded concentrations of erythromycin to growing balanced cultures of *E. coli* strain B/r demonstrated a linear semilogarithmic plot (Figs. 1–4) shortly after the addition or dilution of the antibiotic concentration in accordance with Eq 1:

$$\ln N = k_{app.}t + \text{intercept} \qquad \text{(Eq. 1)}$$

where N is the number of organisms, t is time, and $k_{app.}$ is obtained from the slope of the appropriate plots in the steady-state growth. In the absence of antibiotic, $k_{app.}$ is k_0 and the intercept is the natural logarithm of the inoculum size, ln N_0, at time zero.

Coincidence of Total and Viable Counts of Organisms—The coincidence of total (Coulter count) and viable (colony count) numbers of *E. coli* ml.$^{-1}$ in drug-free and in erythromycin-treated growing balanced cultures could not be denied for all erythromycin concentrations up to 150 mcg. ml.$^{-1}$ (Fig. 1). There is no significant evidence of kill in the time intervals studied at doses above the inhibitory concentrations. This evidence of the bacteriostatic action of erythromycin permits the use of total counts to determine the growth rates of organisms affected by subinhibitory concentrations of erythromycin.

Effect of Drug Concentrations on Growth Rates—The derived apparent growth-rate constants, $k_{app.}$ (Eq. 1), for various concentrations of erythromycin I were obtained from the slopes of the plots of Fig. 2 and are listed in Table I.

The extent of inhibition is directly proportional to the erythromycin I concentration, E, in the range 0–10 mcg. ml.$^{-1}$ in accordance with the expression:

$$k_{app.} = k_0 - k_E E \qquad \text{(Eq. 2)}$$

where k_E is defined as the specific inhibitory-rate constant. The $k_{app.}$ is not a linear function of increasing drug concentration when $E > 10$ mcg. ml.$^{-1}$, but it asymptotically approaches zero (Figs. 5 and 6). A plot of $E/(k_0 - k_{app.})$ *versus* E is reasonably linear for those concentrations >10 mcg. ml.$^{-1}$ but shows deviations at concentrations of 0–10 mcg. ml.$^{-1}$. This implies quantitative adherence of the action of erythromycin I on *E. coli* to a previously derived kinetic model (20, 22):

$$\frac{E}{(k_0 - k_{app.})} = E\left(\frac{k_b}{k_a}\right) + \frac{1}{k_a} \qquad \text{(Eq. 3)}$$

at erythromycin I concentrations, $E > 10$ mcg. ml.$^{-1}$. The k_a and k_b are constants of proportionality, which may be related to drug availability in the biophase and drug affinity to receptor or binding sites. The calculated values for the constants k_a and k_b were obtained from the slope and intercept of the linear portion of such a plot in accordance with Eq. 3. Typical plots are given for the data of Fig. 5 in Fig. 9.

Analysis of Culture Broth Variations—The apparent growth-rate constants, $k_{app.}$, obtained at different concentrations of erythromycin I at different concentrations of nutrients in broth are given in Table II. Variation of these growth-rate constants among the different broths is not significantly different from that observed in daily variation in organism growth rates. There is no significant inactivation or binding of the erythromycin by broth constituents at the nutrient concentrations studied.

Effect of Organism on Drug-Affected Growth Rates—The apparent growth-rate constants, $k_{app.}$, for *E. coli* obtained at various concentrations of erythromycin I for three different organism concentrations at the time of drug addition are given in Table III. There are no significant differences among the growth rates for the different organism concentrations at any drug concentration in the range studied. In the absence of any evidence for systematic influence of organism population on growth-rate constants, it is

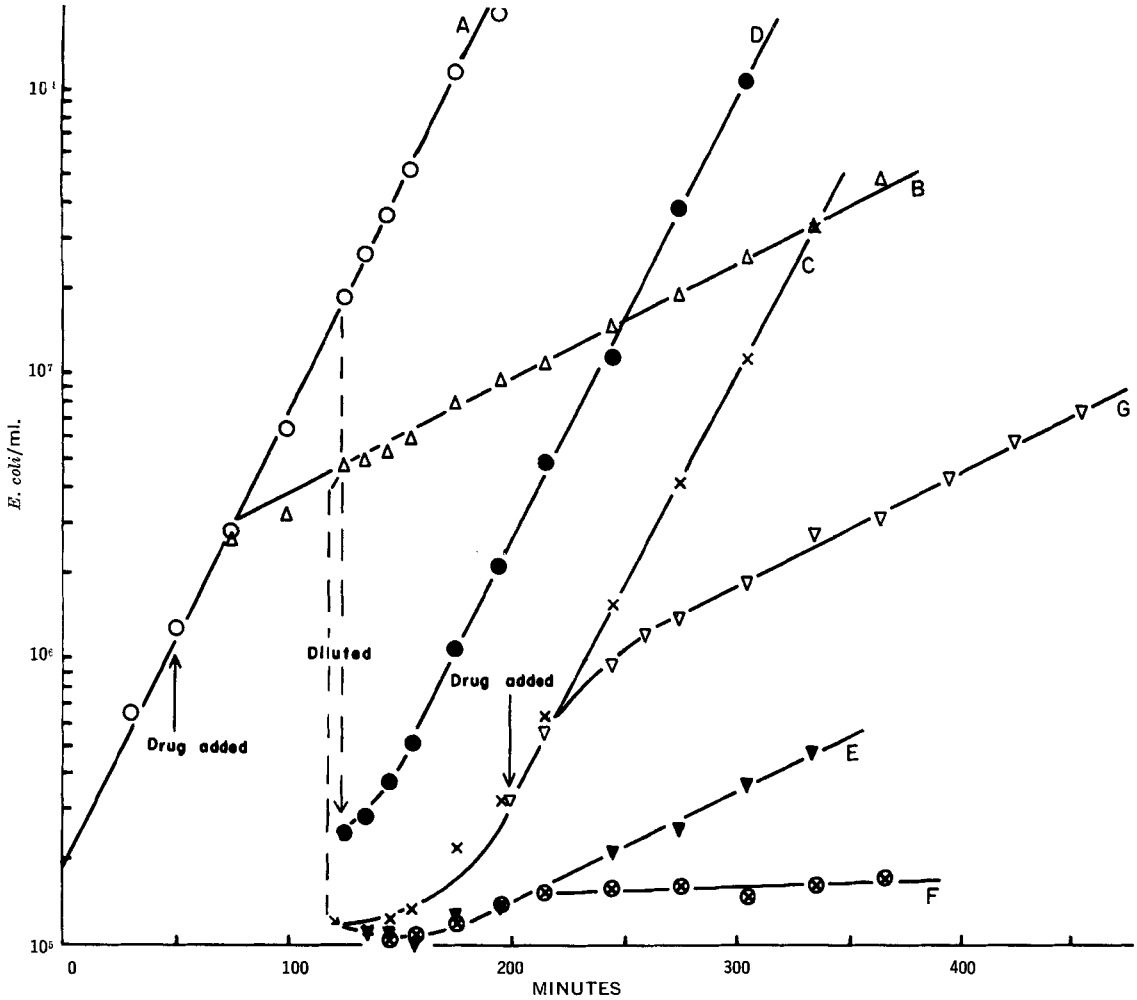


Figure 4—*Semilogarithmic plots of reversibility studies of* E. coli *growth with time on addition of erythromycin l and 1:100 dilution of culture with broth. Curve A is without drug. Curve B is after addition of 50 mcg./ml. Curves C, E, F, and G are after 1:100 dilution of the Curve B culture and have final concentrations of 0.5, 50, 100, and 50 mcg./ml., respectively. The dilution broth contained the erythromycin l in the cases of Curves E and F. There was no drug in the diluent in the case of Curve C, and drug was added at 200 min. for Curve G. Curve D is after 1:100 dilution of the culture of Curve A with no drug added.*

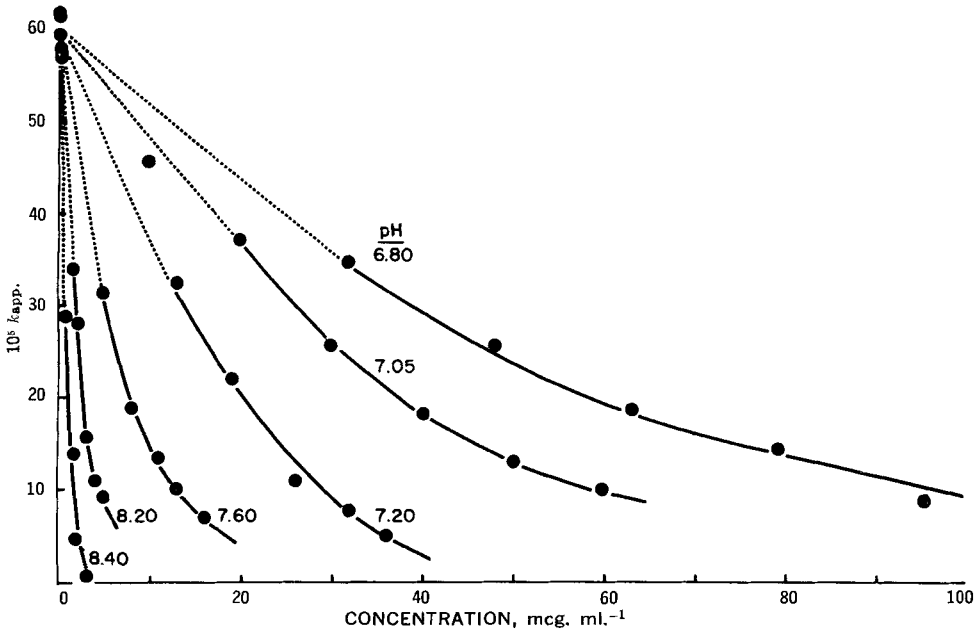


Figure 5—*Dependence of the apparent growth-rate constant,* $k_{app.}$ *in sec.*$^{-1}$, *for* E. coli *on erythromycin concentration at various pH values and at 37.5°.*

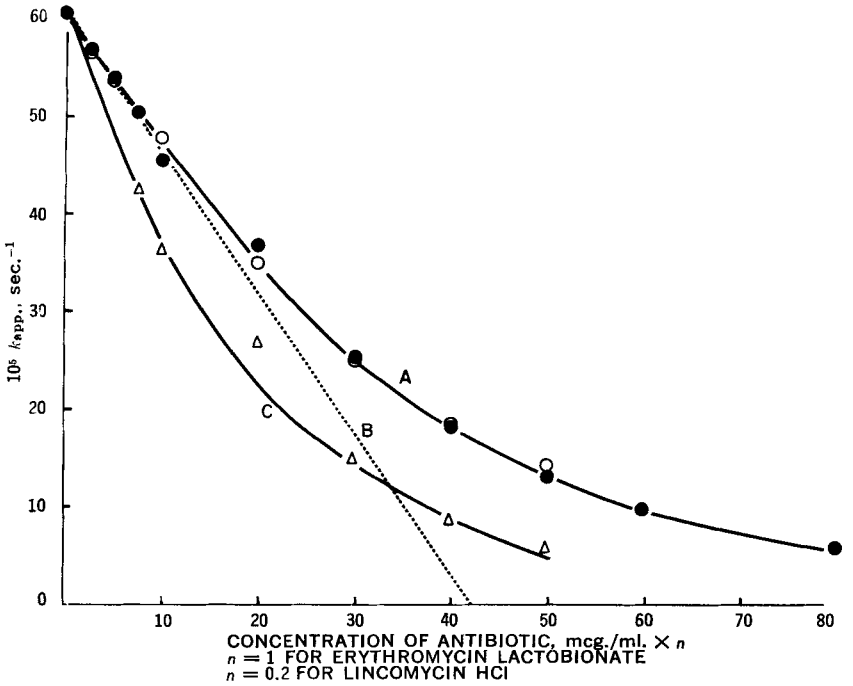


Figure 6—*Demonstration of the coincidence of the dependencies of the apparent growth-rate constants,* $k_{app.}$ *in sec.*$^{-1}$, *for* E. coli *at 37.5° and pH 7.05 on equipotent concentrations of erythromycin l and lincomycin HCl. Curve A represents this dependence, where the closed symbols are for erythromycin l and the open symbols for lincomycin HCl in its Phase 1 growth and where the actual concentrations of lincomycin HCl are five times the stated values. The dashed line, Curve B, demonstrates the linear dependency of* $k_{app.}$ *on drug concentration at low drug concentrations. Curve C represents the* $k_{app.}$ *dependence for Phase II steady state of lincomycin-affected microbial growth.*

concluded that the drug is neither metabolized by the organisms nor depleted in the medium as a result of adsorption to cellular components, inactivation by excretory products of metabolism, and other interactions which may be functions of organism numbers. The effective drug concentrations may, therefore, be assumed to remain constant over the period of study.

Reversibility of Drug Action—The equilibrium of drug between the nutrient medium and the biophase in the microorganism is readily achieved. It took about 30–50 min. after drug addition for the culture to attain a new steady-state phase of growth (Figs. 1–3). The steady-state growth of *E. coli* inhibited by 50 mcg. ml.$^{-1}$ erythromycin l (Curves B in Figs. 3 and 4) reverted to a new steady state when diluted 10-fold and 100-fold, respectively (Curves C in Figs. 3 and 4). The new growth-rate constants were coincident with those found in the presence of the resultant concentrations of the drug, *i.e.*, 5 and 0.5 mcg. ml.$^{-1}$, respectively. The new steady state was attained after 20–30 min. of an apparent initial lag phase. Both drug-free (Curves D in Figs. 3 and 4) and drug-affected cultures (Curves C in Figs. 3 and 4) showed this initial lag period on dilution into fresh broth, so that this may be attributed to a possible need for cell rejuvenation before reestablishment of the new steady state or to a consequence of the shock of dilution.

Cultures inhibited by low concentrations of the drug, *i.e.*, 5 and 0.5 mcg. ml.$^{-1}$ erythromycin l, were further inhibited by addition of more drug to a final concentration of 50 mcg. ml.$^{-1}$, with predictable growth-rate constants (Curves G in Figs. 3 and 4). The time for equilibration of drug between the medium and biophase was also 20–30 min. Similarly, 10-fold and 100-fold dilutions of the culture of Curve B by fresh medium with the same concentration of drug (Curves E of Figs. 3 and 4) achieved similar steady-state growth rates after the same initial lag phase periods of 20–30 min. There were no significant changes in growth-rate constants. A similar dilution of the culture of Curve B into fresh medium containing high concentrations of the drug to increase the final concentration twofold (Curves F of Figs. 3 and 4) produced further inhibition and new steady-state growth rates were attained after 20–30 min. with predictable rate constants.

Thus it was concluded that the action of subinhibitory concentrations of erythromycin on intact *E. coli* cells is readily reversed in finite time intervals. Time lags introduced by dilution effects were the same in the presence or absence of erythromycin.

Effect of pH on Drug-Affected Growth Rates—The apparent growth-rate constants, $k_{app.}$, obtained for *E. coli* in broth at pH values 6.80–8.40 in the absence and presence of graded erythromycin l concentrations are plotted in Fig. 5. Growth-rate constants for drug-free cultures are not significantly affected by pH; the values for drug-affected cultures are significantly decreased at higher pH values for the same erythromycin concentrations (Fig. 5). Since it has been shown (22) that numbers of microorganisms in the

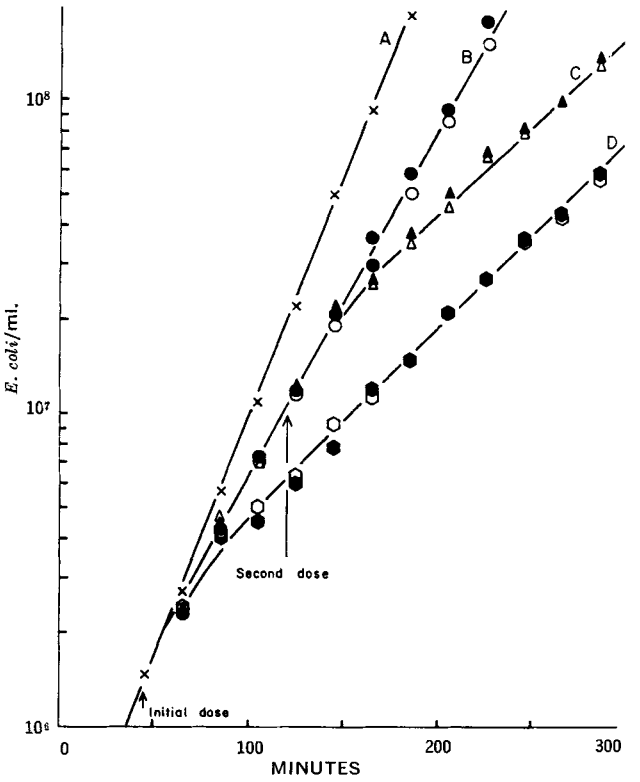


Figure 7—*Nonsignificant effects of order of addition of equipotent erythromycin l and lincomycin HCl on growth rates of* E. coli. *Curve A is for growth of* E. coli *in the absence of drug. Curve B is for growth of* E. coli *in the presence of 15.8 mcg./ml. erythromycin l (closed symbols) or equipotent 60 mcg./ml. lincomycin HCl (open symbols). Curve C is when equipotent lincomycin HCl (60 mcg./ml.) is added to the erythromycin-affected culture of Curve B (closed symbols), or when equipotent erythromycin l (15.8 mcg./ml.) is added to the lincomycin-affected culture of Curve B (open symbols). Curve D is when 200 mcg./ml. lincomycin HCl (open symbols) and 31.6 mcg./ml. erythromycin l (closed symbols) are added to the culture of Curve A.*

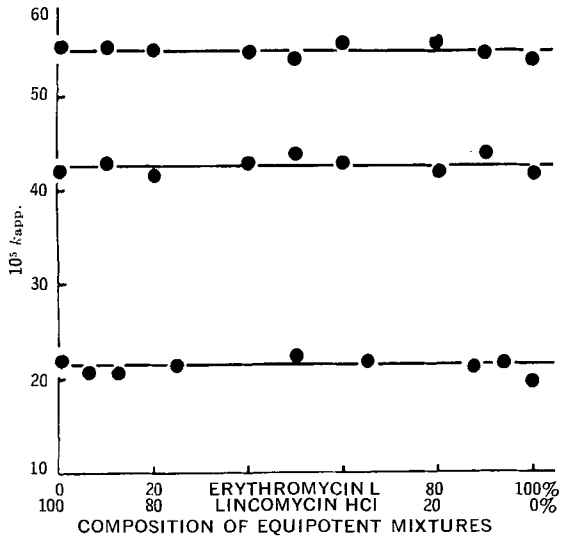


Figure 8—*Effect of continuously varied erythromycin l and lincomycin HCl fractions in equipotent mixtures at three different potency levels on the apparent growth-rate constants,* $k_{app.}$ *in sec.*$^{-1}$, *of* E. coli *at pH 7.05 and 37.5°. Erythromycin l is presumed to be five times as potent as lincomycin HCl on a weight-to-weight basis.*

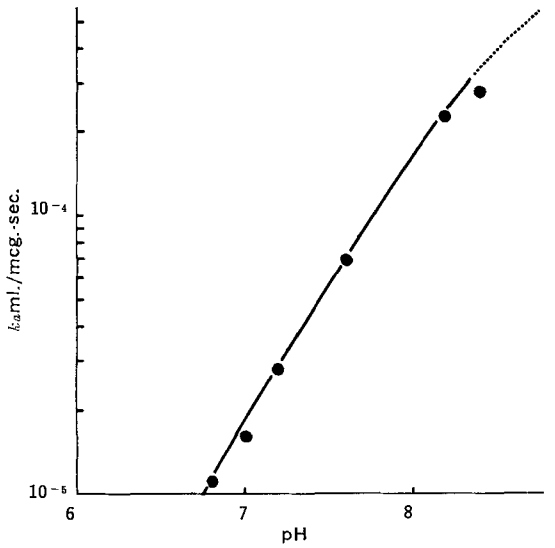


Figure 10—*Semilogarithmic plot of the apparent inhibitory-rate constant,* k_a, *for the effect of erythromycin l on the growth of* E. coli *at 37.5°. The drawn line is consistent with the expression* $k_a = k_a^*(K_s/(K_a + [H^+])$, *where* $k_a^* = 1.11 \times 10^{-3}$ *ml./mcg.-sec. is the intrinsic inhibitory-rate constant for the nonprotonated erythromycin l of pKa' 8.8, and* k_a *is obtained from* $k_{app.} = k_0 - k_aE/(1 + k_bE)$.

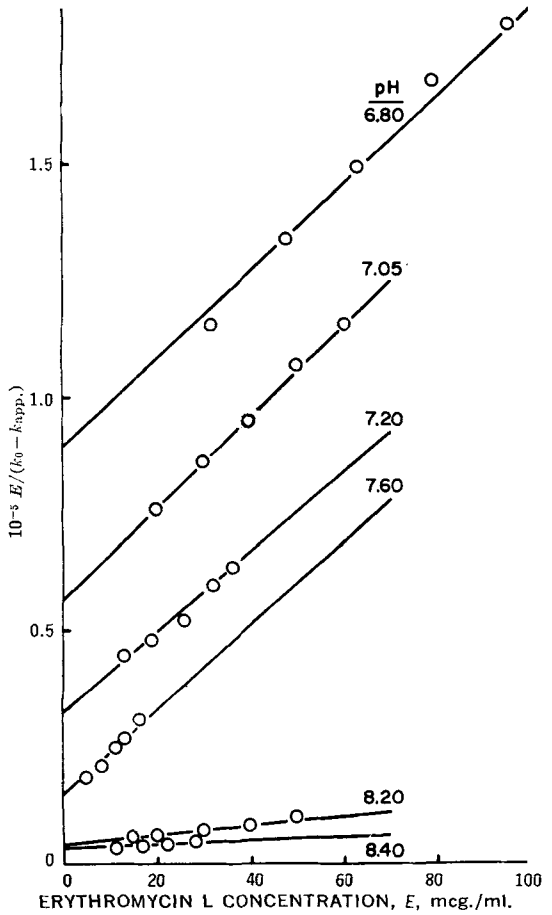


Figure 9—*Demonstration of saturation kinetics for the action of erythromycin l at higher concentrations,* E, *on the apparent growth-rate constants,* $k_{app.}$, *of* E. coli *at 37.5°. The curves are labeled as to the pH values of the media and are plotted in accordance with the expression* $E/(k_0 - k_{app.}) = (k_b/k_a)E + 1/k_a$ *where* k_b/k_a *and* $1/k_a$ *are the slopes and intercepts, respectively, and* k_0 *is the rate constant in the absence of drug.*

growing culture have no significant effect on the pH of the medium up to 10^8 ml.$^{-1}$ organisms, the variations in growth-rate constants are attributed to variations in the initial pH values of the broths. The calculated values of k_a obtained from the intercepts of the plots of Fig. 9 in accordance with Eq. 3 are plotted as a function of pH in Fig. 10. The values of k_a increased about 10-fold for a unit increase in the pH over the range 6.80–7.60. The slope of the log k_a *versus* pH plot tended to lessen above that pH value.

Effect of Order of Addition of Lincomycin and Erythromycin on Microbial Growth—The dependence of the growth-rate constants of erythromycin-affected cultures on drug concentration is similar to that of lincomycin-affected cultures in Phase I of growth (22). The plot of $k_{app.}$ *versus* concentration (Fig. 6) for erythromycin l is coincident with that for lincomycin HCl when the actual lincomycin concentration is reduced by an average factor of 5.0. This implies that 5.0 weight units of lincomycin HCl are equipotent to 1.0 weight unit of erythromycin l, *i.e.*, there is a molar potency ratio of 11.9. Aqueous solutions of erythromycin l lose potency during storage at refrigerator or room temperature. Consequently, the potency ratio between erythromycin l and lincomycin HCl may be far less than the figure quoted, unless freshly prepared aqueous solutions are compared.

There were no significant differences among the growth-rate inhibitions produced by 31.6 mcg. ml.$^{-1}$ of erythromycin l (Curve D, Fig. 7), equipotent 120 mcg. ml.$^{-1}$ lincomycin HCl (Curve D, Fig. 7), and equipotent combinations of 15.8 mcg. ml.$^{-1}$ erythromycin l and 60.0 mcg. ml.$^{-1}$ lincomycin HCl (Curve C, Fig. 7) where Curves C and D had the same slopes. Also there was no significant difference in the effective inhibition by the combinations (Curve C, Fig. 7) when the equipotent amount of erythromycin l was added after 75 min. of lincomycin-affected growth (Curve B, Fig. 7) or when the equipotent lincomycin HCl was added after 75 min. of erythromcyin-affected growth (also Curve B, Fig. 7). Thus, the order of addition of the antibiotics showed no significant difference in ultimate growth inhibition.

Effect of Mixtures of Graded Equipotent Concentrations of Erythromycin and Lincomyin—The growth-rate constants of cultures affected by mixtures of erythromycin and lincomycin are plotted in Fig. 8. The mixtures contained 100–0% erythromycin l and the residual percentage of equipotent lincomycin HCl solution. The mixtures were prepared so as to be *a priori* equipotent in their combined action on *E. coli* growth, in accordance with Fig. 6, at three different levels of action. The null slopes of the plots of the $k_{app.}$ for all the *a priori* equipotent mixtures demonstrate the lack of any significant bacteriostatic antagonism or synergism (23) in the subinhibitory range.

DISCUSSION

The steady growth rates of *E. coli*, as affected by concentrations of erythromycin, are unaffected by significant changes in the contituents of the nutritive media (Table II) and by organism population (Table III). The extent of inhibition of growth ($k_0 - k_{app.}$) is not directly proportional to drug concentration over the complete range (Fig. 6) in accordance with Eq. 2 and suggests that some saturable process, such as the binding of the drug to a limited number of receptor sites, may become rate determining at higher drug concentrations.

An operative kinetic model, similar to that which defined the kinetics of lincomycin action (22) on *E. coli*, may be applied:

$$E \overset{K_1}{\rightleftharpoons} E_1 + R \overset{K_2}{\rightleftharpoons} E_1R \qquad \text{(Eq. 4)}$$

where E, the erythromycin concentration in the culture medium, is in equilibrium with E_1, the concentration within the cell or biophase, which reversibly binds to a free receptor site R to form a drug–receptor complex E_1R. On the basis of the assumption that the rate of increase in microbial numbers in a balanced culture is proportional to a net rate of protein synthesis above a minimum rate required for life-sustaining processes and is proportional to the number of organisms initially present in the culture, the following expression has been derived (18) to quantify the extent of inhibition of growth produced by the action of the drug:

$$k_0 - k_{app.} = \frac{k_aE}{1 + k_bE} \qquad \text{(Eq. 5)}$$

where k_0 is the growth-rate constant of the drug-free culture; $k_{app.}$ is the growth-rate constant of culture affected by the concentration, E, of erythromycin; $k_b = K_1K_2$, the product of the partition coefficient, K_1, and the affinity constant, K_2, of Eq. 4; and $k_a = qK_1K_2$, where q is a proportionality constant. It may be that at low drug concentrations (0–10 mcg. ml.$^{-1}$), the inhibition of microbial growth is affected by reaction of the drug with only a small fraction of the available receptor sites. Thus K_2, k_a, and k_b are small with $k_bE << 1$; Eq. 5 simplifies to Eq. 2, which is an expression for the observed linear dependence of the growth-rate constant on the drug concentration in this concentration range (Fig. 6 and Table I).

At higher drug concentrations, >10 mcg. ml.$^{-1}$, it is possible that the already complexed receptor sites either reduce the availability of remaining sites by steric effects, configurational changes, protective colloid action, *etc.*, or transform them to another type of reduced but constant affinity. Whatever the mechanism, it takes progressively greater concentrations of the drug to bind the remaining sites. Thus, the $k_{app.}$ does not remain linearly dependent on erythromycin concentrations. The arithmetic transformation of Eq. 5 is Eq. 3, so that the linear plots of $E/(k_0 - k_{app.})$ *versus* E for concentrations >10 mcg. ml.$^{-1}$ (Fig. 9) demonstrate adherence of the data to Eq. 5. The calculated value of $k_a/k_b = 117.25 \times 10^{-6}$ from such a plot (Table I) is approximately 2 k_0, and it is the same with lincomycin (22).

The addition of the drug to a balanced culture results in inhibition of growth, with a new steady state of growth attained after 30–50 min. (Figs. 1–4). Dilution of drug-affected organisms into drug-free or drug-containing media established a new steady-state growth of the culture within similar time intervals. The rate transitions were no slower than those occurring on dilution of the drug-free culture into fresh media. Cultures inhibited by erythromycin reverted to growth rates coincident with those found in the presence of very small concentrations of the antibiotic when they were diluted into fresh broths. Conversely, cultures inhibited by low concentrations of the antibiotic were further and quickly inhibited by addition of more antibiotics to predictable growth-rate constants (Figs. 3–6). It is concluded that the action of erythromycin on bacterial culture is readily reversible.

Progressively larger amounts of erythromycin are needed to produce the same degree of inhibition of growth of *E. coli* as the pH is increased (Fig. 5). This confirms the literature (24, 25) that the minimum inhibitory concentration (M.I.C.) for erythromycin action on Gram-positive and Gram-negative organisms decreases with an increase in pH. Erythromycin base has a pKa = 8.8 (25), and the enhanced antibacterial action observed at higher pH values may be assigned to the unprotonated fraction, f, of the total drug which contributes to the activity. If it is postulated that only the unprotonated species partitions into the microorganism (26), the inhibitory-rate constant, k_a, of Eq. 5 should be a function of this unprotonated fraction, f, and then

$$k_a = k_a^*f = k_a^* \left(\frac{K_a}{K_a + [H^+]}\right) \qquad \text{(Eq. 6)}$$

where the intrinsic inhibitory-rate constant of the unprotonated species is $k_a^* = qK_1^*K_2$, K_1^* is the intrinsic partition coefficient between medium and microbial biophase for the uncharged species, qK_2 is invariant in that the biophase pH is considered constant, and f can be defined by the parenthetical expression of Eq. 6 (22).

A plot of log k_a *versus* pH must, therefore, approach a slope of unity when $[H^+] > K_a$, and the slope should tend to decrease as K_a becomes less than $[H^+]$, as in Fig. 10. The calculated value for k_a^* is $11.08 \pm 0.70 \times 10^{-4}$ (ml./mcg.-sec.), from which it is estimated that at pH 8.8 the activity of erythromycin will be 30-fold that at pH 7.0.

The coincidence of the plot of $k_{app.}$ against concentration for erythromycin I and lincomycin HCl in Phase I (22) (Fig. 6), when the latter is presumed to have one-fifth the potency of erythromycin on a weight basis, is not inconsistent with the same mechanisms of action for the two antibiotics. Antagonism would not be predicted for the action of combinations of these two antibiotics (23) on the premise of this same peculiar dependency of growth inhibition on dose (Fig. 6). This *a priori* prediction for *E. coli* is well verified by the fact that equipotent mixtures of lincomycin and erythromycin demonstrate the same inhibitory effect on the growth of *E. coli* at any magnitude of inhibition in the subinhibitory range (Fig. 8). Also, the sequence of addition of erythromycin and lincomycin produced no differences in growth-rate inhibition when the resultant drug combinations were equipotent (Fig. 7). For freshly prepared solutions, when the potencies of the antibiotic samples are taken into account, erythromycin base is 6.68 times as potent as the lincomycin base at 37.5° and pH 7.05 in the system against *E. coli*.

These observations are consistent with the facts that each of these compounds binds to the 50 S ribosomal subunit (2, 3, 7, 9, 10) in cell-free extracts and thus may inhibit polypeptide synthesis similarly in the intact living cell by interfering with the function of transfer RNA. If there is no peculiar or physicochemical interaction, the combined action of the two drugs should be predictable on the basis of the potencies determined from their individual dose–response relations (23) and assigned to their relative ability to compete for binding sites, where the relative potencies are functions of the products of their relative affinity for the receptor site, K_2, and their partition into the biophase, K_1, as per Eq. 4.

Since the *a priori* prediction and the kinetic results do not demonstrate antagonism, the problem is to reconcile this condition with the statements of supposed antagonism that exist in the literature (2, 3, 8, 10, 12). Most of these statements of antagonism are based on studies of protein synthesis in cell-free extracts, where the criteria of effect are degrees of amino acid incorporation (3, 8) where added erythromycin is supposed to reverse the inhibition of amino acid incorporation by lincomycin. It is apparent, however, that in balanced growth cultures of intact cells such as *E. coli*, no such antagonistic effects appear.

The present studies on such combinations were based on the Phase I lincomycin-affected steady state of *E. coli* growth (22). However, lincomycin has a second mode of action on a growing culture after prolonged contact with the cells. The $k_{app.}$ dependency on lincomycin concentration during this Phase II steady state of lincomycin-affected growth is different from that of the Phase I dependency and that of erythromycin-affected growth (Fig. 6). Thus, Phase II lincomycin effects may be expected to be different than erythromycin, and the observed cell-free antagonisms may be possible. The action of combinations of erythromycin and lincomycin in Phase II will be investigated further to test this hypothesis.

REFERENCES

(1) D. Apirion, *J. Mol. Biol.*, **30,** 255(1967).

(2) F. N. Chang, quoted by B. Weisblum and J. Davies, *Bacteriol. Rev.*, **32,** 439(1968).

(3) F. N. Chang and B. Weisblum, in "Antibiotics 1—Mechanism of Action," D. Gotlieb and P. D. Show, Eds., Springer-Verlag, New York, N. Y., 1967, p. 440.

(4) K. Tanaka, H. Teraoka, M. Tamari, E. Otaka, and S. Osawa, *Science*, **12,** 576(1968).

(5) S. B. Taubman, A. G. So, F. E. Young, E. W. Davie, and J. W. Corcoran, *Amer. Soc. Microbiol.*, **1968,** 395.
(6) S. B. Taubman, N. R. Jones, F. E. Young, and J. W. Corcoran, *Biochem. Biophys. Acta*, **123,** 438(1966).
(7) J. M. Wilhem and J. W. Corcoran, *Biochemistry*, **6,** 2578 (1967).
(8) J. M. Wilhem, N. L. Oleinick, and J. W. Corcoran, *Amer. Soc. Microbiol.*, **1967,** 236.
(9) R. Wolfe and F. E. Hahn, quoted by F. E. Hahn, in "Antibiotics 1—Mechanism of Action," D. Gotlieb and P. D. Show, Eds., Springer-Verlag, New York, N. Y., 1967, pp. 379–385.
(10) K. Igarashi, H. Ishitsuka, and A. Kaji, *Biochem. Biophys. Res. Commun.*, **37,** 499(1969).
(11) J. C. H. Mao, *Biochem. Pharmacol.*, **16,** 2441(1967).
(12) B. Weisblum and J. Davies, *Bacteriol. Rev.*, **32,** 493(1968).
(13) E. Cudliffe and K. McQuillen, *J. Mol. Biol.*, **30,** 137(1967).
(14) E. R. Garrett and M. R. W. Brown, *J. Pharm. Pharmacol.*, **Suppl. 15,** 185T(1963).
(15) M. R. W. Brown and E. R. Garrett, *ibid.*, **52,** 179(1964).
(16) E. R. Garrett and G. H. Miller, *ibid.*, **54,** 427(1964).
(17) E. R. Garrett, O. K. Wright, G. H. Miller, and K. L. Smith, *J. Med. Chem.*, **9,** 203(1966).
(18) E. R. Garrett, G. H. Miller, and M. R. W. Brown, *J. Pharm. Sci.*, **55,** 593(1966).
(19) E. R. Garrett, *Arzeim.-Forsch.*, **16,** 1364(1966).
(20) E. R. Garrett and O. K. Wright, *J. Pharm. Sci.*, **56,** 1576 (1967).
(21) E. R. Garrett, J. B. Mielck, J. K. Seydel, and H. J. Kessler, *J. Med. Chem.*, **12,** 740(1969).
(22) J. B. Mielck and E. R. Garrett, *Chemotherapy*, **14,** 337(1969).
(23) E. R. Garrett, *Antibiot. Chemother.*, **8,** 8(1958).
(24) Abbott Records, "Erythromycin—A Review of Its Properties and Clinical Status," Abbott Laboratories, Scientific Division, North Chicago, Ill. 1966.
(25) V. Lorian, *Int. Congr. Chemother., Proc. 6th*, abstract A4-38 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 16, 1970, from the *College of Pharmacy, University of Florida, Gainesville, FL 32601*

Accepted for publication June 3, 1970.

Presented at the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

Biosynthesis of Deuterated Benzylpenicillins I: Solvent Deuterium Oxide Participation

BRUCE C. CARLSTEDT*, HENRY L. CRESPI, MARTIN I. BLAKE†, and JOSEPH J. KATZ

Abstract □ The 53–414 strain of *Penicillium chrysogenum* was cultured in a defined medium containing glucose, acetate, lactate, and phenylacetic acid as carbon sources and 99.8% deuterium oxide as solvent. Partially deuterated benzylpenicillin was isolated from the culture. The extent of solvent participation in the biosynthesis of the penicillin molecule was determined by analysis of the proton magnetic resonance spectra. Incorporation of deuterium appears almost complete at two positions: the C-3 position of the thiazolidine ring and the C-6 position of the β-lactam ring. A partial incorporation of deuterium at the C-5 position is also observed. The deuterium atoms in the C-5 and C-6 positions apparently arise during biosynthesis of the amino acid cysteine. The deuterium atom at the C-3 position apparently arises either in the biosynthesis of the amino acid valine or in the closing of the thiazolidine ring.

Keyphrases □ Deuterated benzylpenicillin—biosynthesis □ *Penicillium chrysogenum* cultures—benzylpenicillin deuteration □ Biosynthesis, deuterated benzylpenicillin—*P. chrysogenum* cultures □ Proton magnetic resonance—analysis, structure □ IR spectrophotometry—structure

A variety of organisms has been successfully cultured in pure deuterium oxide (D_2O) (1); in several instances, pharmacologically active principles containing deuterium have been isolated and studied. Nona *et al.* (2–4) studied the effects of D_2O on the growth of *Penicillium janczewskii*. Deuterated griseofulvin was isolated and its antifungal activity evaluated. Mrtek *et al.* (5, 6) cultured a strain of *Claviceps purpurea* in D_2O and examined the biosynthesis of deuterated clavine alkaloids isolated from the culture. Katz and Crespi (7) reviewed the literature on isotope effects in biological systems.

Shaffer *et al.* (8) observed the effects of D_2O on the growth rate and morphology of *Penicillium notatum*, but penicillin production was not investigated. Mohammed *et al.* (9, 10) reported the effects of D_2O on the growth and antibiotic production of 13 low-producing strains of two species of *Penicillium* and three high-producing strains of *Penicillium chrysogenum*. Here again, deuterated antibiotic was not isolated. Behrens *et al.* (11) synthesized deuterophenylacetyl-^{15}N-DL-valine and found the deuterophenylacetyl moiety was incorporated into the benzylpenicillin molecule. Demain (12) used partially deuterated benzylpenicillin, which contained five deuterium atoms in the benzene ring, to study the stability of penicillin during fermentation. Laskar and Mrtek (13) synthesized and studied the activity of a benzylpenicillin which was fully deuterated in the benzyl moiety. A reduction in antibiotic activity was reported.

In the present study, *P. chrysogenum* 53-414, a mutant of the Wisconsin Q-176 strain, was cultured in a defined medium containing D_2O as the solvent. Partially deuterated benzylpenicillin was isolated, and the degree of solvent participation in its biosynthesis was determined by proton magnetic resonance (PMR) analysis of this isotope hybrid compound.[1]

EXPERIMENTAL

Preparation of Slants—*P. chrysogenum*, Wisconsin strain 53–414, was obtained from the American Type Culture Collection (number 12690) in the lyophilized form. Agar slants were prepared as described by Mohammed *et al.* (9). The lyophilized material was suspended in Difco nutrient broth, streaked on the surface of the agar

[1] "Isotope hybrid compound" refers to a compound that contains one type of atom but also more than trace amounts of one of its isotopes (14).

(5) S. B. Taubman, A. G. So, F. E. Young, E. W. Davie, and J. W. Corcoran, *Amer. Soc. Microbiol.*, **1968,** 395.
(6) S. B. Taubman, N. R. Jones, F. E. Young, and J. W. Corcoran, *Biochem. Biophys. Acta*, **123,** 438(1966).
(7) J. M. Wilhem and J. W. Corcoran, *Biochemistry*, **6,** 2578 (1967).
(8) J. M. Wilhem, N. L. Oleinick, and J. W. Corcoran, *Amer. Soc. Microbiol.*, **1967,** 236.
(9) R. Wolfe and F. E. Hahn, quoted by F. E. Hahn, in "Antibiotics 1—Mechanism of Action," D. Gotlieb and P. D. Show, Eds., Springer-Verlag, New York, N. Y., 1967, pp. 379–385.
(10) K. Igarashi, H. Ishitsuka, and A. Kaji, *Biochem. Biophys. Res. Commun.*, **37,** 499(1969).
(11) J. C. H. Mao, *Biochem. Pharmacol.*, **16,** 2441(1967).
(12) B. Weisblum and J. Davies, *Bacteriol. Rev.*, **32,** 493(1968).
(13) E. Cudliffe and K. McQuillen, *J. Mol. Biol.*, **30,** 137(1967).
(14) E. R. Garrett and M. R. W. Brown, *J. Pharm. Pharmacol.*, **Suppl. 15,** 185T(1963).
(15) M. R. W. Brown and E. R. Garrett, *ibid.*, **52,** 179(1964).
(16) E. R. Garrett and G. H. Miller, *ibid.*, **54,** 427(1964).
(17) E. R. Garrett, O. K. Wright, G. H. Miller, and K. L. Smith, *J. Med. Chem.*, **9,** 203(1966).
(18) E. R. Garrett, G. H. Miller, and M. R. W. Brown, *J. Pharm. Sci.*, **55,** 593(1966).
(19) E. R. Garrett, *Arzeim.-Forsch.*, **16,** 1364(1966).
(20) E. R. Garrett and O. K. Wright, *J. Pharm. Sci.*, **56,** 1576 (1967).
(21) E. R. Garrett, J. B. Mielck, J. K. Seydel, and H. J. Kessler, *J. Med. Chem.*, **12,** 740(1969).
(22) J. B. Mielck and E. R. Garrett, *Chemotherapy*, **14,** 337(1969).
(23) E. R. Garrett, *Antibiot. Chemother.*, **8,** 8(1958).
(24) Abbott Records, "Erythromycin—A Review of Its Properties and Clinical Status," Abbott Laboratories, Scientific Division, North Chicago, Ill. 1966.
(25) V. Lorian, *Int. Congr. Chemother., Proc. 6th*, abstract A4-38 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 16, 1970, from the *College of Pharmacy, University of Florida, Gainesville, FL 32601*

Accepted for publication June 3, 1970.

Presented at the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

Biosynthesis of Deuterated Benzylpenicillins I: Solvent Deuterium Oxide Participation

BRUCE C. CARLSTEDT*, HENRY L. CRESPI, MARTIN I. BLAKE†, and JOSEPH J. KATZ

Abstract □ The 53–414 strain of *Penicillium chrysogenum* was cultured in a defined medium containing glucose, acetate, lactate, and phenylacetic acid as carbon sources and 99.8% deuterium oxide as solvent. Partially deuterated benzylpenicillin was isolated from the culture. The extent of solvent participation in the biosynthesis of the penicillin molecule was determined by analysis of the proton magnetic resonance spectra. Incorporation of deuterium appears almost complete at two positions: the C-3 position of the thiazolidine ring and the C-6 position of the β-lactam ring. A partial incorporation of deuterium at the C-5 position is also observed. The deuterium atoms in the C-5 and C-6 positions apparently arise during biosynthesis of the amino acid cysteine. The deuterium atom at the C-3 position apparently arises either in the biosynthesis of the amino acid valine or in the closing of the thiazolidine ring.

Keyphrases □ Deuterated benzylpenicillin—biosynthesis □ *Penicillium chrysogenum* cultures—benzylpenicillin deuteration □ Biosynthesis, deuterated benzylpenicillin—*P. chrysogenum* cultures □ Proton magnetic resonance—analysis, structure □ IR spectrophotometry—structure

A variety of organisms has been successfully cultured in pure deuterium oxide (D_2O) (1); in several instances, pharmacologically active principles containing deuterium have been isolated and studied. Nona *et al.* (2–4) studied the effects of D_2O on the growth of *Penicillium janczewskii*. Deuterated griseofulvin was isolated and its antifungal activity evaluated. Mrtek *et al.* (5, 6) cultured a strain of *Claviceps purpurea* in D_2O and examined the biosynthesis of deuterated clavine alkaloids isolated from the culture. Katz and Crespi (7) reviewed the literature on isotope effects in biological systems.

Shaffer *et al.* (8) observed the effects of D_2O on the growth rate and morphology of *Penicillium notatum*, but penicillin production was not investigated. Mohammed *et al.* (9, 10) reported the effects of D_2O on the growth and antibiotic production of 13 low-producing strains of two species of *Penicillium* and three high-producing strains of *Penicillium chrysogenum*. Here again, deuterated antibiotic was not isolated. Behrens *et al.* (11) synthesized deuterophenylacetyl-^{15}N-DL-valine and found the deuterophenylacetyl moiety was incorporated into the benzylpenicillin molecule. Demain (12) used partially deuterated benzylpenicillin, which contained five deuterium atoms in the benzene ring, to study the stability of penicillin during fermentation. Laskar and Mrtek (13) synthesized and studied the activity of a benzylpenicillin which was fully deuterated in the benzyl moiety. A reduction in antibiotic activity was reported.

In the present study, *P. chrysogenum* 53-414, a mutant of the Wisconsin Q-176 strain, was cultured in a defined medium containing D_2O as the solvent. Partially deuterated benzylpenicillin was isolated, and the degree of solvent participation in its biosynthesis was determined by proton magnetic resonance (PMR) analysis of this isotope hybrid compound.[1]

EXPERIMENTAL

Preparation of Slants—*P. chrysogenum*, Wisconsin strain 53–414, was obtained from the American Type Culture Collection (number 12690) in the lyophilized form. Agar slants were prepared as described by Mohammed *et al.* (9). The lyophilized material was suspended in Difco nutrient broth, streaked on the surface of the agar

[1] "Isotope hybrid compound" refers to a compound that contains one type of atom but also more than trace amounts of one of its isotopes (14).

Table I—Composition of Protio Culture Media

Seed Culture Medium		Protio Culture Medium	
Dextrose	6.00 g.	Lactose	3.00 g.
Lactose	1.00 g.	Dextrose	0.50 g.
Standard salt mixture	10.00 ml.	Standard salt mixture	10.00 ml.
NH_4 Acetate	0.35 g.	NH_4OH	0.559 g.
NH_4 Lactate (80%, syrupy)	0.693 g.	Lactic acid	0.520 g.
		Acetic acid	0.273 g.
Water to make	100.0 ml.	Water to make	100.00 ml.
$CaCO_3$	1.50 g.		

Standard Salt Mixture

KH_2PO_4	3.00 g.
$MgSO_4 \cdot 7H_2O$	0.25 g.
$FeSO_4 \cdot 7H_2O$	0.10 g.
$CuSO_4$	0.005 g.
$ZnSO_4 \cdot 7H_2O$	0.02 g.
$MnSO_4 \cdot H_2O$	0.02 g.
$CaCl_2$	0.05 g.
Water to make	100.00 ml.

slants, and allowed to grow at room temperature for 5–7 days. The agar slants were stored at 5°; fresh slants were prepared every 4–6 months from lyophilized spores.

Preparation of Seed Culture—The seed culture medium was prepared according to the formula shown in Table I. The pH of the medium was adjusted to 6.9 with 25% KOH prior to the addition of $CaCO_3$. Fifty milliliters of medium was transferred into a series of 250-ml. conical flasks, and the flasks were autoclaved. Each flask was inoculated with a loopful of the aerial mycelium from an agar slant and placed on an Eberbach rotary shaker at 185–190 r.p.m. for 96 hr. The resulting inoculum, consisting of pellets ranging from 1 to 3 mm. in diameter, was recovered aseptically by collection on filter paper in a Büchner funnel. For H_2O cultures, the mycelial pad was washed with distilled water and stored in a sterile container. For D_2O cultures, the mycelial pad was rinsed once with distilled water, rinsed twice with D_2O, and stored in a sterile container.

Water Culture Techniques—The protio medium was prepared according to the formula shown in Table I. The pH was adjusted to 6.85 with 25% KOH (aqueous) prior to autoclaving. Three hundred and fifty milliliters of the medium was added to a series of 2-l. conical flasks and autoclaved. Twenty-eight grams (wet weight) of the seed culture mycelium was added to each culture flask by aseptic technique. All flasks were placed on an Eberbach rotary shaker at 185–190 r.p.m. for approximately 160 hr. at which time the cultures were harvested. Phenylacetic acid (PAA), as the potassium salt, was added as a sterile solution at the rate of 29.2 mg. PAA/350 ml. culture medium/day. Protio cultures were used as controls and for penicillin isolation studies.

Deuterated Culture Methods—A modified replacement technique was used for culturing *P. chrysogenum* in D_2O. The D_2O nutrient medium (Table II) differed from that of the protio cultures in the following ways: D_2O replaced water as the solvent, a vitamin mixture (Table II) was added, and a daily addition of carbohydrate was required. The D_2O nutrient medium was adjusted to apparent[2] pH (15) 6.9 with 25% KOH in D_2O; 50 ml. was transferred into 250-ml. conical flasks and autoclaved. One milliliter of the vitamin mixture was added to each flask. Each flask was inoculated with 4 g. (wet weight) of mycelial pad which had been previously rinsed with D_2O. The flasks were allowed to ferment on an Eberbach rotary shaker at 185–190 r.p.m. for approximately 160 hr. Anhydrous D-glucose, exchanged once with D_2O, was dissolved in sufficient D_2O to give a final concentration of 0.125 g./1.5 ml., transferred to a multiple-dose vial, and autoclaved.

PAA in D_2O was neutralized with 2.5% KOH in D_2O. The solution was diluted with D_2O to give a final concentration of 4.17 mg./0.5 ml., added to a multiple-dose vial, and autoclaved.

Daily addition of the D-glucose and PAA was accomplished with a sterile syringe and needle by injection through the cotton plug.

Bioassay Procedures—A 1-ml. aliquot of the culture medium was removed each day by means of a sterile disposable pipet and was placed in a 3-ml. test tube which was stoppered to reduce dilution of the deuterium by atmospheric moisture. The aliquot was used for three analyses: pH or apparent pH, penicillin titer, and D_2O content where applicable. The USP XVII (16) bioassay procedure was employed, except that the test organism was *Sarcina lutea* (17), obtained from the American Type Culture Collection (number 9341). The organism was maintained on an agar slant of Difco Bacto Antibiotic Medium 1 and was reslanted every 7–10 days. A 10-ml. base layer of Difco Bacto Antibiotic Medium 2 and a 6-ml. seed layer of Difco Bacto Antibiotic Medium 1 were utilized. Sodium penicillin G[3] was used as the reference standard. Microliter (10–100) portions of the aliquot were diluted to 10 ml. with 1% phosphate buffer, pH 6.0. Reference standard penicillin was included with each cup plate assay. The plates were incubated at 30° for 18–24 hr. A graph of inhibition zone diameter *versus* penicillin concentration was plotted. Titer values were read from the graph, multiplied by the dilution factor, and expressed as units per milliliter of culture medium. No correction factor was applied for the deuterated penicillin. All values of penicillin are in terms of sodium penicillin G reference.

Table II—Composition of D_2O Nutrient Culture Medium and Vitamin Mixture

D_2O Nutrient Culture Medium		Vitamin Mixture[a]	
Dextrose	1.00 g.	Biotin	0.005 g.
Standard salt mixture[b]	10.00 ml.	Inositol	0.005 g.
		Ca pantothenate	0.005 g.
NH_4OH	0.55 g.	Pyridoxine HCl	0.005 g.
Acetic acid	0.273 g.	Thiamine HCl	0.005 g.
Lactic acid	0.52 g.	D_2O to make	100.00 ml.
D_2O to make	100.00 ml.		

[a] Filtered through Millipore filter for sterilization. [b] Prepared as in Table I but with D_2O as the solvent.

Determination of pH—The pH of the protio cultures and the apparent pH of the D_2O nutrient cultures were determined daily on the aliquot removed from the culture medium.

Deuterium Analysis—D_2O content was determined daily by the spectrophotometric method of Crespi and Katz (18). Aliquots for analysis were centrifuged to yield a clear sample prior to determination.

Extraction Procedures—Centrifugation of the broth and mycelium and vacuum filtration on a Büchner funnel through double filter paper yielded a culture broth which contained essentially all of the penicillin produced in the fermentation. The extraction procedure was modified from a general solvent transfer method described by Chain (19). The method utilized amyl acetate, buffer, chloroform, and water as the four separate extraction steps. The method of Seifter and Richardson (20) involved amyl acetate, bicarbonate buffer, ether, and silica gel column with buffer elution as the four extraction steps. In the present study, butyl and/or amyl acetate were used as the nonpolar solvents. The broth was cooled to 3–5° in an ice-salt bath, acidified to pH 2.0 with phosphoric acid, and then shaken with cold (0–5°) 20% butyl acetate in amyl acetate saturated with water. Emulsions were broken by centrifugation. The butyl acetate–amyl acetate fraction was extracted with cold (0–5°) 3% pH 7 phosphate buffer. The buffer fraction was acidified to pH 2.0 and extracted with cold (0–5°) amyl acetate saturated with water. The amyl acetate fraction was extracted into distilled water with 2.5% KOH until the pH approached 7 as recorded on the pH meter. The water fraction was lyophilized, and the crude extract was stored in a vacuum desiccator.

Isolation and Identification of Penicillin—Initial purification of the crude extract was accomplished by recrystallization. The crude extract was dissolved in 85% acetone in water and kept cold in an ice-salt bath. Small flakes of penicillin formed upon addition of cold acetone. These flakes were isolated by centrifugation, dissolved in water, lyophilized, and the residue was stored in a vacuum desiccator. This proved to be sufficient purification for the protio penicillin, but further purification was required for the partially deuterated penicillin. The *N*-ethyl piperidine salt of the partially deuterated penicillin was prepared (21) and recrystallized, first from chloroform by additions of cold acetone and then from chloroform by additions of cold amyl acetate. The *N*-ethyl piperidine salt of a reference

[2] The apparent pH of a D_2O solution is that pH observed with a pH meter. The pH of a solution is the apparent pH plus 0.4 unit.

[3] Nutritional Biochemicals Corp.

Table III—Chemical Shifts (δ, p.p.m.) of Protio and Partially Deuterated Benzylpenicillins[a]

Compound	Side-Chain Phenyl	β-Lactam CH—CH (*J*, c.p.s.)	Thiazolidine —CH—	Side-Chain —CH_2—	Thiazolidine $(CH_3)_2$
I. K benzylpenicillin[b,c,d]	7.38	5.54d(4 c.p.s.), 5.43d(4 c.p.s.)	4.26	3.68	1.58, 1.51
II. K benzylpenicillin[e,c,f]	7.38	5.53d(4 c.p.s.), 5.43d(4 c.p.s.)	4.23	3.68	1.56, 1.49
III. K benzylpenicillin[g,c,f]	7.37	5.53d(4 c.p.s.), 5.43d(4 c.p.s.)	4.22	3.68	1.56, 1.49
IV. K partially deuterated benzylpenicillin[h,c,f]	7.36	5.50	4.22[i]	3.68	1.54, 1.46

[a] d denotes doublet; *J* values in c.p.s. [b] Green *et al.* (28). [c] Referred to DSS. [d] Solvent—D_2O. [e] Purchased from Nutritional Biochemicals Corp. [f] Solvent—apparent pH 7.4 0.01 *M* phosphate buffer D_2O. [g] Protio benzylpenicillin prepared in this laboratory. [h] Partially deuterated benzylpenicillin prepared in this laboratory. [i] Very small peak.

penicillin G was also prepared for comparative purposes. The IR spectrum of isolated material was obtained with a Beckman IR 7 spectrophotometer using a Nujol mull on Irtran II plates. The PMR spectra were obtained on a Varian HA 100 spectrometer (probe temperature at 31°) after dissolving the sample in 0.01 *M* phosphate D_2O buffer (apparent pH 7.4) or in deuteriochloroform. The lock material was hexamethyldisiloxane (HMS), internally for deuteriochloroform solutions and as an external capillary for D_2O solutions. The chemical shifts for D_2O solutions (Table III) were referred to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS). The chemical shifts for the $CDCl_3$ solutions (Table IV) were referred to tetramethylsilane (TMS). Bioassays were performed on the partially deuterated penicillin as described under *Bioassay Procedures.*

RESULTS AND DISCUSSION

It has been demonstrated on numerous occasions that organisms cultured in media containing high concentrations of heavy water experience a severe inhibition in the biosynthesis of metabolic products. Experiences of the authors with ergot alkaloids (6), belladonna alkaloids (22), penicillin (9, 10), and griseofulvin (2) have been reported. Thus, poor yields of penicillin obtained in this study (Fig. 1) were not unexpected, nor does this represent a serious deterrent in studying the biosynthesis of this antibiotic.

Isolation of penicillin on a laboratory scale has been described (11) but does present certain difficulties, principally because of the extreme instability of penicillin at pH values below and above neutral. This is compounded in the present studies by low titer values imposed by the inhibitory effects of heavy water and by the necessity for developing a defined medium for biosynthetic studies.

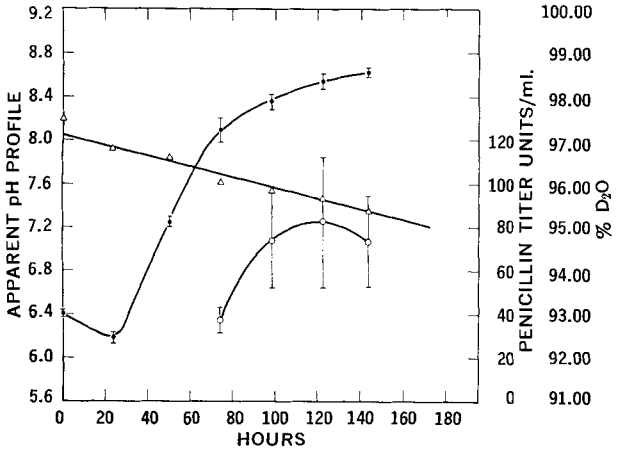


Figure 1—*Typical growth curves for* P. chrysogenum *53-414 in D_2O nutrient medium. Key:* ●, *apparent pH profile;* ○, *penicillin titer; and* △, *D_2O dilution.*

Knowledge of the composition of the medium and the availability of nutrient components and precursors in deuterated form is essential if the biosynthetic pathways are to be charted. Although optimal growth and penicillin production are achieved when corn-steep liquor is incorporated into the nutrient medium, its use here was precluded because of its unspecified composition. Further, the introduction of hydrogen atoms in this way would interfere with biosynthetic studies involving fully deuterated nutrient media needed for the production of fully deuterated penicillin.

The objectives of this study were: (*a*) to select a suitable strain of *Penicillium*, which would produce adequate amounts of penicillin under the constraints of a deuterated environment; (*b*) to develop an appropriate, defined, nutrient medium yielding maximum penicillin titers; (*c*) to isolate the antibiotic in a sufficiently pure state to permit identification, characterization, and analysis of deuterium distribution within the penicillin molecule; and (*d*) to determine the extent of participation of solvent D_2O in the biosynthesis of penicillin.

Five strains of *P. chrysogenum*, Q-176, 49-133, 51-20, 51-20 F3-64, and 53-414, were examined in preliminary studies for their ability to grow and produce antibiotic on a defined medium containing heavy water as solvent. The last two strains listed demonstrated the best performance in a series of growth and production studies. After further experimentation, the 53-414 strain was selected for the present work on the basis of higher penicillin titers (about 80 units per ml.).

For the protio cultures, the nutrient medium described by Singh and Johnson (23) was utilized. Cultures of the test organisms in this medium were used as controls, as a reference for establishing the appropriate medium and culture conditions for the D_2O nutrient cultures, and to provide a source material for the development of suitable extraction, isolation, and purification procedures. The composition of the protio culture media is shown in Table I. Since only one form of penicillin, benzylpenicillin, was desired in the fermentation broth, phenylacetic acid was added as a precursor to the nutrient.

In the development of an appropriate medium for the deuterio cultures, an attempt was made to include only those components that were also available in fully deuterated form. This was considered at this time since in subsequent studies the production of a fully deuterated penicillin was planned. The basic D_2O nutrient culture medium is listed in Table II and is an adaptation of the protio medium of Singh and Johnson (23). Lactose is a component of the protio medium, since it is slowly metabolized and represents a continuous source of glucose. Soltero and Johnson (24) demonstrated that increased penicillin titers resulted when cultures were fed glucose or sucrose on a continuous basis rather than only initially as a single addition of lactose which presumably has a similar effect as continuous or repeated additions of glucose. However, since deuterated lactose is not available at this time, the authors resorted to daily addition of glucose. Deuterioglucose is readily available from algae grown in heavy water.

The culturing procedure employed in this study is a modification of the replacement medium technique suggested by Halliday and Arnstein (25) for studying the metabolites in penicillin biosynthesis.

Table IV—Chemical Shifts (δ, p.p.m.) of Protio and Partially Deuterated *N*-Ethyl Piperidyl Benzylpenicillins in $CDCl_3$[a]

Compound	Side-Chain Phenyl	Amide —NH—	β-Lactam CH—CH (*J*, c.p.s.)	Thiazolidine —CH—	Side-Chain $—CH_2—$	Thiazolidine $(CH_3)_2$
V. Benzylpenicillinic acid[b,c]	7.34	6.35d(8.5 c.p.s.)	5.52, 5.70d(4 c.p.s.) 5.49d(4 c.p.s.)	4.38	3.67	1.52, 1.45
VI. NEP-Benzylpenicillin[c,d]	7.37	6.22d(7.5 c.p.s.)	5.62, 5.69d(4 c.p.s.), 5.63d(4 c.p.s.)	4.35	3.70	1.63, 1.51
VII. NEP-Partially deuterated benzylpenicillin[c,e]	7.37	6.22	5.62	—	3.70	1.62, 1.52

[a] d denotes doublet; *J* values in c.p.s. [b] Green *et al.* (28). [c] Referred to TMS. [d] Prepared as in *Experimental* section from reference benzylpenicillin. [e] Prepared from IV in Table III.

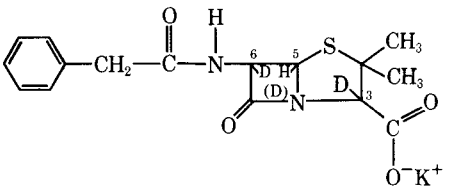
Nona *et al.* (3) and Mrtek *et al.* (6) used a replacement technique in culturing *P. janczewskii* and *C. purpurea*, respectively, in deuterated culture media. In their experiments the organism was allowed to mature in a protio medium and was then introduced into a D_2O medium containing only deuterated substrates. Hopefully the protio organism would synthesize deuterio metabolic products from the deuterated substrates. The technique was applied successfully for the production of deuterated griseofulvin and certain deuterated ergot alkaloids. It has not yet been successful for deuterated belladonna alkaloids when the method (26) is applied to a higher plant. The modification of replacement technique as applied in the present study involved the preparation of the seed culture in a protio medium. The mycelial pad from the protio seed culture was rinsed with D_2O and used as the inoculum for the deuterated culture.

The beneficial effect of a vitamin mixture addition on the culturing of organisms in a deuterated medium has been demonstrated by Mohan *et al.* (27) with *Torulopsis utilis*. Nona *et al.* (2) and Mrtek *et al.* (5) also reported the effects of adding selected vitamins to the culture medium. After preliminary experimentation the vitamin combination shown in Table II was found to enhance penicillin production.

Figure 1 shows the change in apparent pH, the dilution of deuterium by exchange with atmospheric moisture, and penicillin titer values for a typical D_2O nutrient culture over a 160-hr. study period. The apparent pH profile shows an expected immediate drop to an apparent pH 6.2 followed by a rise to a plateau value of apparent pH 8.6. If the fermentation was permitted to continue beyond that indicated in Fig. 1, the apparent pH would eventually drop. The data for the curves in Fig. 1 are based on the observations from thirty 50-ml. cultures. Maximum penicillin titers (70–80 units per ml.) are obtained after about 120 hr. of fermentation, at which point the apparent pH begins to plateau. At this apparent pH, autolysis begins and penicillin breakdown is evident. Over the course of study the D_2O content of the medium diminished at the rate of about 0.26% per day.

Because of the extreme lability of the penicillin molecule in solutions of low pH, caution was exercised during the processing of the fermentation broth. The primary problem in the extraction procedure was the formation of emulsions in the first step, which was overcome by centrifugation. After recovery of the crude extract in lyophilized form, the penicillin was recrystallized from 85% acetone in water by additions of cold acetone. The partially deuterated benzylpenicillin was found to be 60% pure by bioassay. The IR spectrum showed characteristic carbonyl absorptions at 1770, 1665, and 1608 $cm.^{-1}$, which confirmed the presence of penicillin but also showed a broad shoulder between 1550 and 1575 $cm.^{-1}$ that indicated the possible presence of carbonyl groups other than those of the penicillin. The PMR spectrum showed additional peaks in the upfield region (0.70–2.30 p.p.m.) which were not due to the benzylpenicillin.

Chemical shifts for ordinary and partially deuterated benzylpenicillins are given in Tables III and IV. PMR spectrum for potassium benzylpenicillin in D_2O (II) shows a resonance at 7.38 p.p.m. for the five protons of the phenyl ring and two doublets at 5.53 and 5.43 p.p.m. due to the C-5 and C-6 protons (Structure I) of the β-lactam ring, respectively, in the downfield region. The amide proton is exchanged with the deuterium of the solvent and is not observed.



partially deuterated benzylpenicillin

I

In the upfield region of the spectrum, the C-3 proton of the thiazolidine ring is observed at 4.23 p.p.m. and the methylene protons of the benzyl side chain at 3.68 p.p.m. The two methyl groups on the thiazolidine ring appear as singlets at 1.58 and 1.51 p.p.m. The PMR spectrum for the isotope hybrid compound isolated in this study (IV) shows the same resonances for the phenyl, side-chain methylene, and thiazolidine methyl groups. The resonances at 4.22 and 5.43 p.p.m. are almost completely absent and a singlet is observed at 5.50 p.p.m. Interpretation of these spectra indicates the possibility of two points of full deuteration, which are surmised to be the C-3 position of the thiazolidine ring and the C-6 position of the β-lactam ring. Also, the C-5 position of the β-lactam ring appears to be partially deuterated.

C-3 Position—In accord with the assignments made by Green *et al.* (28), the resonance at 4.22 p.p.m. (Table III) is almost completely absent (5–10% normal value) and indicates extensive deuteration at the C-3 position.[4] It has been shown that penicillin is biosynthesized from L-cysteine (29) and valine (30). The C-3 position of the thiazolidine ring arises from the α-carbon of valine, and an examination of the pathway for biosynthesis of valine (31) reveals that a deuterium atom from the solvent may be incorporated at the α-carbon by transamination. Arnstein and Crawhill (32) suggested a mechanism for the formation of the thiazolidine-β-lactam ring. In the final step for the thiazolidine ring closure, the suggested pathway allows for incorporation of a proton, or a deuterium atom, at the C-3 position, so that the deuterium atom evidenced here may be due either to previous incorporation into the valine or this last ring closure step.

C-5 and C-6 Positions—The assignments made by Green *et al.* (28) for potassium benzylpenicillin in D_2O (I) are in agreement with this work, except for the β-lactam ring protons at C-5 and C-6. They assigned the resonance at 5.43 p.p.m. to the C-5 proton and the resonance at 5.54 p.p.m. to the C-6 proton (Table III). In the partially deuterated benzylpenicillin isolated in this study, the doublet at 5.43 p.p.m. is essentially absent and the doublet at 5.53 p.p.m. is replaced by a singlet at 5.50 p.p.m. (Table III). The C-5 and C-6 positions of the β-lactam ring arise from the β- and α-carbon atoms, respectively, of cysteine (29). From an examination of the biosynthetic pathways for formation of cysteine (33), it is observed that a deuterium atom from the solvent may be incorporated at the α-carbon of cysteine (*i.e.*, the C-6 position of penicillin) by trans-

[4] The presence of protons at these points might be due to the 3–5% water in the medium or a preferential isotope incorporation of the protons over the deuterium atoms during biosynthesis.

amination. This position should then be essentially fully deuterated. Thus, the observation of deuteration at the position which normally resonates at 5.43 p.p.m. indicates that this resonance represents the C-6 proton rather than the C-5 proton. The authors conclude, therefore, that the assignments by Green *et al.* (28) for the β-lactam protons are inverted. This observation is further confirmed by the data from Table IV in which chemical shifts for penicillin in organic solvents are listed.

The assignments of Green *et al.* (28) (V) are for benzylpenicillinic acid in $CDCl_3$. The *N*-ethyl piperidyl salts of protio (VI) and partially deuterated (VII) benzylpenicillins were used in this study. Green *et al.* (28) assigned the resonance at 5.52 p.p.m. to the C-5 proton, the two doublets centered at 5.70 and 5.49 p.p.m. to the C-6 proton (which is coupled to the C-5 proton and the amide proton), and the doublet at 6.35 p.p.m. to the amide proton. Resonances observed for *N*-ethyl piperidyl protiobenzylpenicillin (VI) differ slightly but are in good agreement with these assignments. The spectrum observed for the *N*-ethyl piperidyl partially deuterated benzylpenicillin (VII) shows singlets at 6.22 and 5.62 p.p.m. The singlet observed at 5.62 p.p.m. is the same resonance as that assigned to the C-5 proton and indicates its presence in the partially deuterated benzylpenicillin. Agreement with the conclusion that the resonance observed in D_2O solutions at 5.50 p.p.m. (Table III) is due to the C-5 proton is thus retained. Also, if the protons had been present at the C-6 position, splitting of the amide resonance would have been observed. The change in assignments to 5.54 p.p.m. for the C-5 proton and 5.43 p.p.m. for the C-6 proton indicated by this study would involve only those assignments for potassium benzylpenicillin in D_2O solution.

The assignments made by Green *et al.* (28) for other penicillins or benzylpenicillins in other solvents or under different conditions than those examined in this study are assumed correct, since factors such as solvent or conformational effects could be influencing the chemical shifts. Incorporation of deuterium at the C-6 position is probably greater than 90%,[4] since there is no observable splitting of the singlet peak at 5.50 p.p.m. (Table III). Partial deuteration at the C-5 position is estimated to be 50–75%. The amide proton in the $CDCl_3$ spectrum for VII (Table IV) was used as an internal reference. Saur *et al.* (34) observed isotopic exchange of deuterium in the β-carbon protons of phosphoenolpyruvate when examining deuterium isotope effects on fermentation of hexoses. Thus, deuterium may have been incorporated at the β-carbon of 3-phosphohydroxypyruvate during the biosynthesis of the cysteine (33). Arnstein and Crawhill (32) suggested that one of the β-protons of cysteine is retained during ring closure of penicillin biosynthesis and thus deuteration at the C-5 position would be observed.

Further studies will attempt to biosynthesize a fully deuterated benzylpenicillin, to examine its potency in relation to protiopenicillin, and to examine the fate of d_3-acetate in the fermentation.

REFERENCES

(1) J. J. Katz and H. L. Crespi, *Science*, **151,** 1187(1966).

(2) D. A. Nona, M. I. Blake, and J. J. Katz, *J. Pharm. Sci.*, **56,** 1063(1967).

(3) D. A. Nona, M. I. Blake, H. L. Crespi, and J. J. Katz, *ibid.*, **57,** 975(1968).

(4) *Ibid.*, **57,** 1993(1968).

(5) R. G. Mrtek, H. L. Crespi, M. I. Blake, and J. J. Katz, *J. Pharm. Sci.*, **54,** 1450(1965).

(6) *Ibid.*, **56,** 1234(1967).

(7) J. J. Katz and H. L. Crespi, in "Isotope Effects on Reaction Rates," C. J. Collins and N. S. Bowman, Eds., to be published.

(8) R. L. Shaffer, H. L. Crespi, and J. J. Katz, *Botan. Gazz.*, **119,** 24(1957).

(9) K. Mohammed, D. A. Nona, and M. I. Blake, *J. Pharm. Sci.*, **57,** 2049(1968).

(10) *Ibid.*, **59,** 419(1970).

(11) O. K. Behrens, J. Corse, R. G. Jones, E. C. Kleiderer, Q. F. Soper, F. R. Van Abeele, L. M. Larson, J. C. Sylvester, W. J. Haines, and H. E. Carter, *J. Biol. Chem.*, **175,** 765(1948).

(12) A. L. Demain, *Antibiot. Chemother.*, **VII,** 361(1957).

(13) P. A. Laskar and R. G. Mrtek, personal communication.

(14) H. L. Crespi, R. M. Rosenberg, and J. J. Katz, *Science*, **161,** 795(1968).

(15) A. K. Covington, M. Paabo, R. A. Robinson, and R. G. Bates, *Anal. Chem.*, **40,** 700(1968).

(16) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 832.

(17) F. Kavanagh and L. J. Dennin, in "Analytical Microbiology," F. Kavanagh, Ed., Academic, New York, N. Y., 1963, pp. 327–339.

(18) H. L. Crespi and J. J. Katz, *Anal. Biochem.*, **2,** 274(1961).

(19) E. Chain, "Antibiotics," vol. II, Oxford University Press, London, England, 1949, pp. 722–732.

(20) E. Seifter and G. A. Richardson, U. S. pat. 2,870,064, Jan. 20, 1959; through *Chem. Abstr.*, **53,** 7519f(1959).

(21) H. T. Clarke, J. R. Johnson, and R. Robinson, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 90.

(22) R. A. Uphaus, F. A. Crane, M. I. Blake, and J. J. Katz, *J. Pharm. Sci.*, **54,** 202(1965).

(23) K. Singh and M. J. Johnson, *J. Bacteriol.*, **56,** 339(1948).

(24) F. V. Soltero and M. J. Johnson, *Appl. Microbiol.*, **2,** 41 (1954).

(25) W. J. Halliday and H. R. V. Arnstein, *Biochem. J.*, **64,** 380 (1956).

(26) F. A. Crane, M. I. Blake, R. A. Uphaus, and J. J. Katz, to be published.

(27) V. S. Mohan, H. L. Crespi, and J. J. Katz, *Nature*, **193,** 189 (1962).

(28) G. F. H. Green, J. E. Page, and S. E. Stanforth, *J. Chem. Soc.*, **1965,** 1595.

(29) H. R. V. Arnstein and P. T. Grant, *Biochem. J.*, **57,** 360 (1954).

(30) *Ibid.*, **57,** 353(1954).

(31) A. White, P. Handler, and E. Smith, "Principles of Biochemistry," 3rd ed., McGraw-Hill, New York, N. Y., 1964, p. 520.

(32) H. R. V. Arnstein and J. C. Crawhill, *Biochem. J.*, **67,** 180 (1957).

(33) G. N. Cohen, "Biosynthesis of Small Molecules," Harper and Row, New York, N. Y., 1967, pp. 22–23.

(34) W. K. Saur, H. L. Crespi, E. A. Halevi, and J. J. Katz, *Biochemistry*, **7,** 3529(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Chemistry Division, Argonne National Laboratory, Argonne, IL 60439*

Accepted for publication June 3, 1970.

Presented at the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This study was performed under the auspices of the U. S. Atomic Energy Commission, Washington, D. C.

The authors acknowledge the assistance of Mrs. G. D. Ryan and Mrs. G. N. McDonald in obtaining the PMR spectra.

* Argonne Universities Association–Argonne National Laboratory Fellowship appointee.

† Present address: Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

Interaction of Hexachlorophene with Human Epidermis I: *In Vivo* Bioelectrometric Study of pH Influence

ROLLAND I. POUST and VICTOR F. SMOLEN

Abstract □ The effects of pH and hexachlorophene on the net fixed-charge density of the colloids composing the epidermal surface were studied *in vivo* employing human subjects. The results are analogous to titration curves of amphoteric macromolecules. The study was performed over a pH range of 1.1–12.3 in the presence and absence of saturated concentrations of hexachlorophene. Both the control and hexachlorophene titration curves were nonhysteretic when initiated at either extreme of the pH range; this finding indicates reversibility of the observed pH and hexachlorophene-induced changes. The presence of hexachlorophene decreased the net fixed-charge density of the tissue surface below pH 5.6. Above pH 5.6, a net increase was consistently observed. Mechanistically, the hexachlorophene-induced alterations in fixed-charge density are hypothesized as resulting from allosterically effected changes in the dissociation constants of ionogenic protein side groups located vicinally to hexachlorophene interaction sites. The bioelectrometric method allowed the study to be performed without causing any injury or discomfort to the subjects. The results of the present study suggest a further utility for the bioelectrometic method in studies of the substantive antibacterial behavior of hexachlorophene on human skin.

Keyphrases □ Hexachlorophene interaction—human epidermis □ Epidermal surface colloids—acid-base binding properties □ Charge density, fixed—human epidermis □ Bioelectrometric study —pH effect, hexachlorophene–human epidermis interaction

Following transient exposure, the substantive effectiveness of a skin antibacterial such as hexachlorophene largely depends upon the extent of its deposition onto the colloids composing the stratum corneum as well as the manner in which it is subsequently available to affect microorganisms. Commonly employed experimental techniques do not always permit the substantive activity of skin antibacterials to be readily evidenced, particularly under conditions of actual product usage. The recovery and quantification of very small amounts of the antibacterial agents are often necessary. Some techniques require the excision of the treated skin and are, therefore, inapplicable for extended and routine use with human subjects. Techniques that involve the disintegration of the skin and fractionation of its constituents are further disadvantaged by the obvious difficulty of distinguishing and correcting for antibacterial bound to the colloid *in vivo* and that which becomes interacted *in vitro* through the activation of binding sites by the *in vitro* treatment of the tissue.

A bioelectrometric method, which has been successfully applied in studies of the interaction of other substances (1–5)—some of which have been phenolic (6)—could conceivably be applied to allow the detection of residual, bound quantities of hexachlorophene on human skin *in vivo* under realistic conditions of product usage, without any chance of injury resulting from the measurements.

The purpose of the present study was to determine the sensitivity of the bioelectrometric method in detecting changes in the net fixed-charge density of the epidermal surface occurring in response to the interaction of hexachlorophene. The interpretation of the results of the investigation was anticipated to provide some mechanistic insight into the nature of observed phenomena.

METHOD

The application of the bioelectrometric method to the study of solute interaction with tissue surfaces is dependent on the detection of solute-induced changes in the net fixed-charge density of the surface colloids relative to suitable controls. The fixed charge originates from the dissociation of counterions from immobile ionogenic groups covalently bonded or otherwise sorbed onto the surface. The magnitude of the fixed-charge density, at any given composition of applied solution, determines the magnitude of the electrical potential difference developing across the colloid–aqueous boundary. The measurement of a potential, E_1, includes this equilibrium phase boundary potential as well as all other potentials developed in the circuit. These extraneous potentials have been found to remain constant for the short period of time required to replace the initial solution in contact with the tissue surface with a dilution of this same solution, followed by the immediate recording of a second potential, E_2.[1] The difference of these two measured potentials ($E_2 - E_1$), termed the dilution potential, E_d, is devoid of extraneous potentials and includes only those potential differences developed between the aqueous medium at the tissue surface and the bulk of the applied solution. This consideration allows the interpretation of E_d in terms of the familiar Donnan equilibrium (7) and its treatment as a diffusion potential (8). Under the conditions of its measurement, E_d is characteristic only of the tissue surface rather than reflecting the properties of the bulk or interior of the tissue phase.

General equations relating the dilution potential and fixed-charge density (f) have been presented in earlier reports (1, 2). Determinations of the fixed-charge density of tissue surfaces under conditions of varying pH resemble and are analogous to pH titration curves of amphoteric macromolecules (9) or insoluble colloids (10, 11) when plotted as a function of pH. The form of these curves is dependent upon the nature of the ionizing groups, the presence of interacting solutes, and the state of aggregation of the colloids composing the tissue surface. The fixed-charge density on tissue surfaces is often quite sensitive to interacting substances, as reflected in alterations in the shapes of titration curves. Such changes can often be interpreted to yield information concerning the extent and nature of the interaction.

Details of the experimental arrangement, preparation of the tissue surface, and precautions concerning how the measurements are to be performed have been reported earlier for human finger epidermis (2). Briefly, the experimental procedure consists of standardizing the condition of the skin by thorough cleansing and allowing the skin to become prehydrated by soaking in 0.15 *N* NaCl for approximately 15 min. This treatment also removes labile, water-soluble, ionogenic materials (12), which would otherwise be elutriated during the measurements. Such losses contribute to uncontrolled variations in the measured potentials. As shown in Fig. 1, the experimental finger and a saturated calomel electrode are placed together into a buffered isoosmotic solution, which may or may not contain the solute whose interaction with the surface is of interest. The reference finger is placed into another beaker containing 0.15 *N* NaCl solution along with another saturated calomel electrode. Neither the reference finger nor the electrode is further

[1] The solutions used for the measurment of E_2 were 10-fold dilutions with regard to all components except the hexachlorophene.

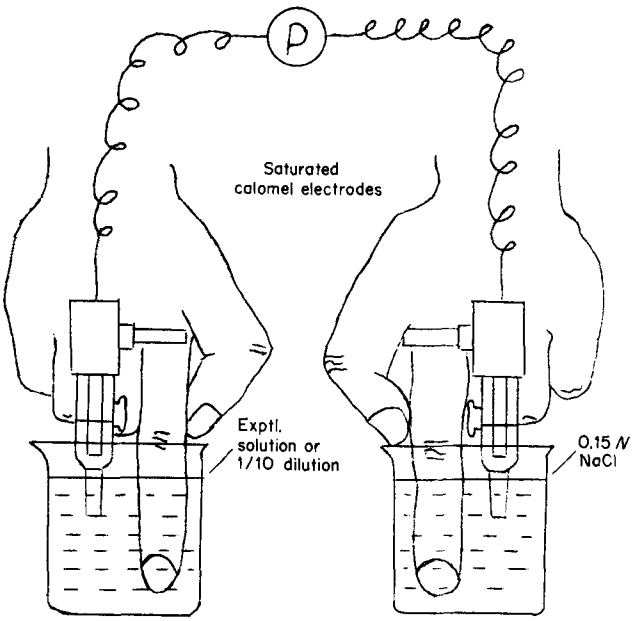


Figure 1—*Diagram of experimental arrangement for the electrometric study of hexachlorophene interaction with human finger epidermis. P = potentiometric device.*

disturbed during the experiment.

The internal circuit between the experimental and reference fingers is completed by the body of the subject. The electrodes in the external circuit are connected to a potentiometric device used for the null point reading of the developed potentials. The potential, E_1, is recorded until its observed variations become less than approximately 0.2 mv./min. When the epidermis is pretreated as previously described, this stability is usually achieved within 1 min. following immersion. Following the measurement of E_1, the finger is removed and immersed in a 1/10 dilution of the same solution; the potential, E_2, is immediately recorded. The finger is then removed from the 10-fold dilution and reimmersed in the previous solution. The potential, E_1, is again measured and compared to the previously determined value to ensure that the extraneous potentials in the circuit have remained constant. Agreement within 1.0 mv. was taken as acceptable. The mean of the two measured values was used to obtain the dilution potential, E_d.

MATERIALS

Corning, miniature, fiber-junction, calomel reference electrodes were used in conjuction with a Heath pH electrometer-recorder. The potentials were read directly from the recorder chart. The potential measurements were performed on three male, human volunteers ranging from 20 to 25 years of age. All reagents were of analytical grade, except for hexachlorophene.[2]

The buffered solutions used for the determination of E_1 were each prepared to an ionic strength of 0.15 and a buffer capacity of 0.0288. This buffer capacity is approximately equivalent to that of a 0.05 *M* phosphate buffer at pH 7.21. The ionic strength is the same as that of isotonic sodium chloride. The compositions of these solutions are listed in Table I. Tenfold dilutions of these solutions, as well as of each of these solutions saturated with hexachlorophene, were also prepared and used in the experiment.

Calculations were performed with the aid of a CDC 6500 digital computer. Calibration curves, relating measured values of the dilution potential to corresponding theoretical values of the fixed-charge density, were constructed with a model 563 Calcomp digital incremental plotter.[3] Representative curves, corresponding to several buffer solutions, are shown in Fig. 2. Figure 3 contains a corresponding nomograph. The verity of the calibration curves was tested experimentally through the *in vitro* measurement of E_d values corresponding to zero net fixed-charge density, *i.e.*, E_d^0 values.

In the absence of fixed charge, the E_d^0 values are tantamount to liquid junction potentials across a free diffusion-type boundary (8). Such boundaries may be conveniently formed using a filter paper bridge between each solution and its 10-fold dilution. However, an error can result in such measurements if a fixed charge resides on the filter paper. To determine whether an error is introduced from this source, diffusion potential measurements were performed with the formation of a liquid junction, between 0.1 and 0.01 *N* KCl solutions, within the filter paper. The measured potential had a mean value of 0.8 mv., indicating that the filter paper was effectively neutral and suitable as a matrix for the formation of free diffusion boundaries. Diffusion potentials measured in this manner have previously been found to agree generally within 1 mv. with values determined using free diffusion boundaries formed within glass capillaries (1).

The averages of six determinations of E_d^0 values are listed in Table II with their corresponding standard deviations. The agreement between experimental and calculated values is most often within 1 mv. and is considered acceptable. The presence of saturated concentrations of hexachlorophene in both the buffers and their 10-fold dilutions does not appear to influence the magnitude of the measured potentials.

To determine the influence of pH on the interaction of hexachlorophene with the epidermal colloids, the fixed-charge density of the tissue surface was determined at each of the 12 pH values de-

Table I—Composition of Buffer Solutions Employed in the Determination of Titration Curves of Tissue Surfaces (Concentrations in Moles/Liter)

pH	Components	Sodium Chloride
1.0	Hydrochloric acid, 0.1	0.05
2.2	Hydrochloric acid, 0.0061	0.1439
2.9	Hydrochloric acid, 0.0013	0.1487
3.7	Sodium acetate, 0.0136; acetic acid, 0.155	0.1364
4.6	Sodium acetate, 0.0211; acetic acid, 0.0304	0.1289
5.5	Sodium acetate, 0.0821; acetic acid, 0.0055	0.0679
6.5	Sodium phosphate, dibasic, 0.0148; sodium phosphate, monobasic, 0.0762	0.0344
7.4	Sodium phosphate, dibasic, 0.0319; sodium phosphate, monobasic, 0.0206	0.0337
8.5	Ammonium hydroxide, 0.015; ammonium chloride, 0.0816	0.0684
10.0	Ammonium hydroxide, 0.0613; ammonium chloride, 0.0104	0.1396
11.5	Sodium hydroxide, 0.00316	0.1438
13.0	Sodium hydroxide, 0.1	0.05

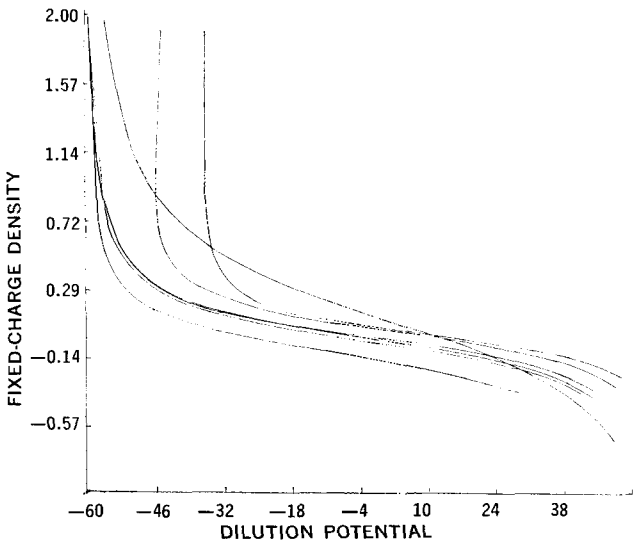


Figure 2—*Plot of calibration curves for several buffered measuring solutions using the digital incremental plotter.*

[2] Supplied by Armour-Dial, Inc., Chicago, Ill.
[3] California Computer Products, Inc., Anaheim, Calif.

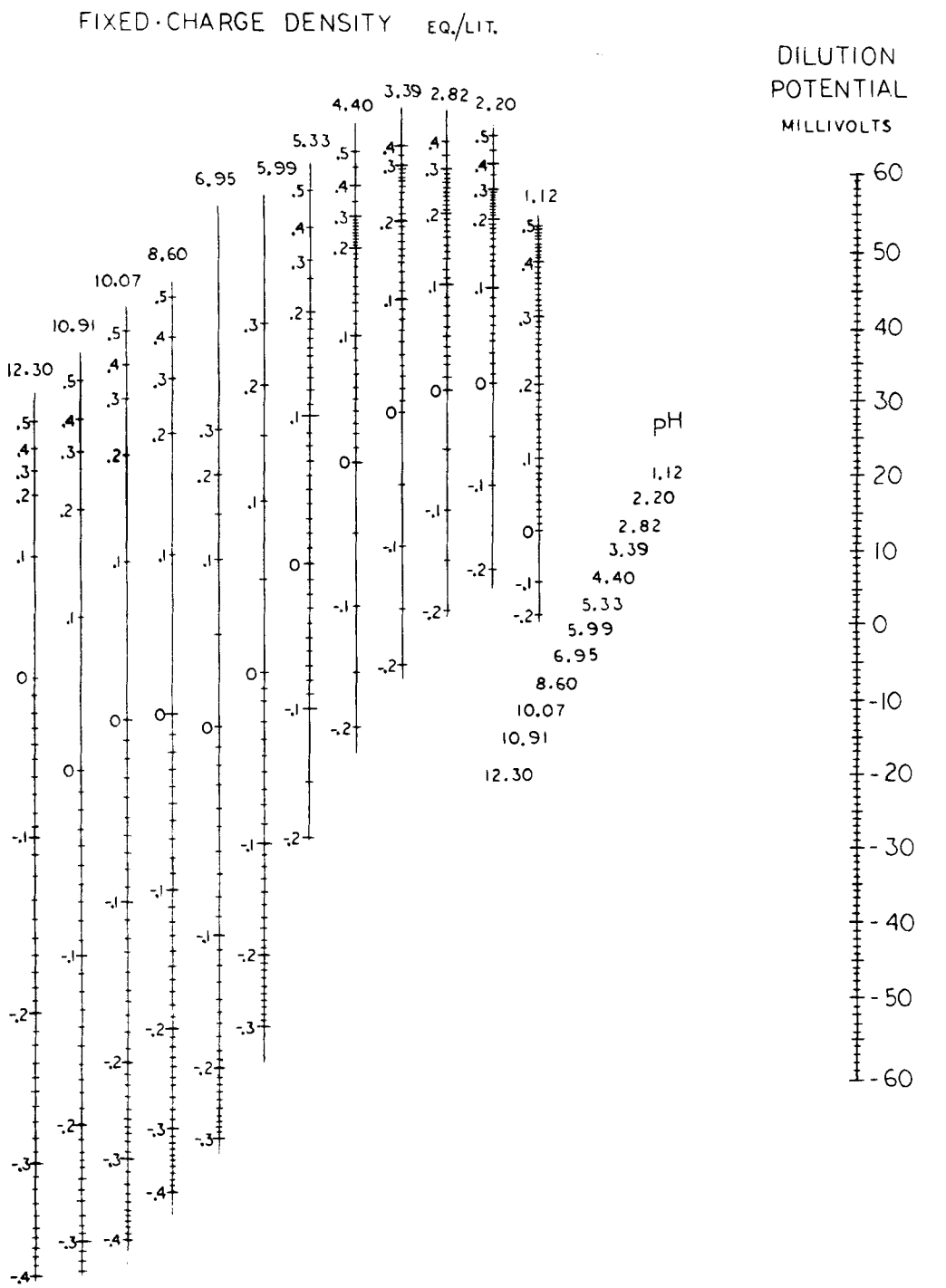


Figure 3—*Alignment Chart relating experimentally observed values of the dilution potential to the corresponding density of fixed charge. To read the fixed-charge density, a straight line is passed to the left from the dilution potential, through the decimal point of the pH of the solution, and intersected with the fixed-charge density scale corresponding to the same solution pH.*

scribed, both in the absence and presence of hexachlorophene. The use of the controls permitted the changes in fixed-charge density due to hexachlorophene interaction to be evidenced. Measurement of E_d values for the control points were made following the attainment of an apparent equilibrium of the solutions with the surface; usually only a few minutes were required. However, the measurements with hexachlorophene-containing solutions were made after 46 min. to allow time for complete interaction with the epidermal surface.

RESULTS AND DISCUSSION

Normal and Hexachlorophene-Displaced Titration Curves—Figure 4 demonstrates the influence of pH on the net fixed-charge density of normal and hexachlorophene-treated human finger epidermis. The results resemble and are analogous to hydrogen-ion titration curves of proteins. A reversibility in the effects of pH and hexachlorophene on the fixed-charge density was indicated by a lack of any systematic differences between titration curves, differing only in that the measurements were initiated at low and high pH. This conclusion was found to be further supported statistically by the results of a paired t test comparison performed between the up-curves and the down-curves; such curves were statistically the same in the case of both the control and hexachlorophene-treated skin. It was, therefore, concluded that the interactions of the solution components with the colloids constituting the epidermal surface are reversible.

Inspection of the curves reveals that, relative to the control curve below approximately pH 5, hexachlorophene induces a net decrease in the density of fixed positive charge on the epidermal surface and causes a small shift in the isoelectric point in the direction of a more acidic pH. In contrast, above the isoelectric point, near pH 5, a hexachlorophene-induced reduction in the fixed anionic charge density of the epidermal surface is observed. It is pertinent to note that, in excess of this pH, the proportion of hexachlorophene [pKa =

Table II—Comparison of Calculated and Experimental Dilution Potentials for Control and Hexachlorophene-Containing Buffer Solutions

pH of Buffer	Calculated	Observed Potentials	
		Plain Buffer	HCP in Buffer
1.12	31.87	31.58 ± 1.63	33.65 ± 1.36
2.20	−5.89	−3.56 ± 0.24	−3.30 ± 0.65
2.82	−12.04	−11.50 ± 1.64	−8.72 ± 0.93
3.39	−11.14	−12.80 ± 2.96	−9.50 ± 1.30
4.40	−10.42	−8.25 ± 0.48	−9.57 ± 1.12
5.33	−3.83	−2.43 ± 0.29	−4.17 ± 0.87
5.99	4.65	6.83 ± 0.23	6.83 ± 0.90
6.95	4.72	6.10 ± 0.34	6.60 ± 0.60
8.60	−5.81	−4.95 ± 0.19	−5.20 ± 1.19
10.07	−11.68	−9.70 ± 0.18	−8.23 ± 1.41
10.91	−13.36	−10.90 ± 0.32	−10.78 ± 0.98
12.30	−31.57	−27.15 ± 0.35	−31.47 ± 1.50

5.4 (12)] existing in solution as anions relative to unionized hexachlorophene increases very rapidly with increased alkalinity. It, therefore, appears that the reduction in net positive surface charge observed at low pH may be reasonably attributed to the results of the interaction of unionized hexachlorophene, while hexachlorophene anions are apparently responsible for the reduction of net negative charge in the less acidic and alkaline pH range. Although an interaction of anions with a negatively charged surface, which results in a reduction of negative charge density, may initially appear to be a rather unique phenomenon, it has previously been observed to occur in similar studies involving phenolic (6) and other anions (2, 4). The interaction of anions with proteins in solution above their isoelectric point has also been commonly reported (13).

Mechanism of Hexachlorophene Effects—Solute-induced changes in fixed-charge density, which result in the displacement of titration curves such as observed for the effects of hexachlorophene, can result from an irreversible loss of titrable groups from the surface or through the masking of their charge. Changes in the fixed-charge density, which might occur from the elutriative loss of charged materials from the skin (14, 15), are minimized by the treatment of the skin performed prior to the measurements. Any further elutriative losses, which could conceivably occur during the measurements, are apparently minimal as indicated by the previously described reversible nature of the effects. Since irreversible chemisorption is obviously also not a plausible mechanism, the hexachlorophene effects must be attributed to a masking of fixed surface charge.

Since at low pH the hexachlorophene is unionized and at high pH the interaction of anions lowers the anionic fixed-charge density, the observed phenomena must occur by a means other than a direct neutralization such as could result from salt binding of the hexachlorophene to the ionogenic fixed surface groups. Since hexachlorophene is a dihydric phenol, its mode of interaction with proteins may be expected to resemble that attributed to phenols in general. Küntzel (16), in describing the action of phenols on collagen, suggested that their interaction with proteins occurs through hydrogen bonding of the phenols with the peptide linkages constituting the protein backbone. The affinity of the interacting molecules for the protein is postulated to result from a polarity induced into their aromatic nuclei by the phenolic hydroxyl group; there results a development of activated CH groups with alternative net positive and negative partial charges. The CH group adjacent to the hydroxyl is postulated as the primary binding site within the molecule.

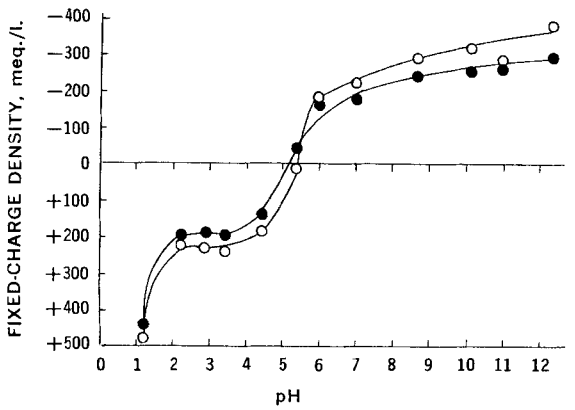


Figure 4—*Control* (○) *and hexachlorophene-displaced* (●) *titration curves of human finger epidermis. Each point is the average of six replicates on one subject.*

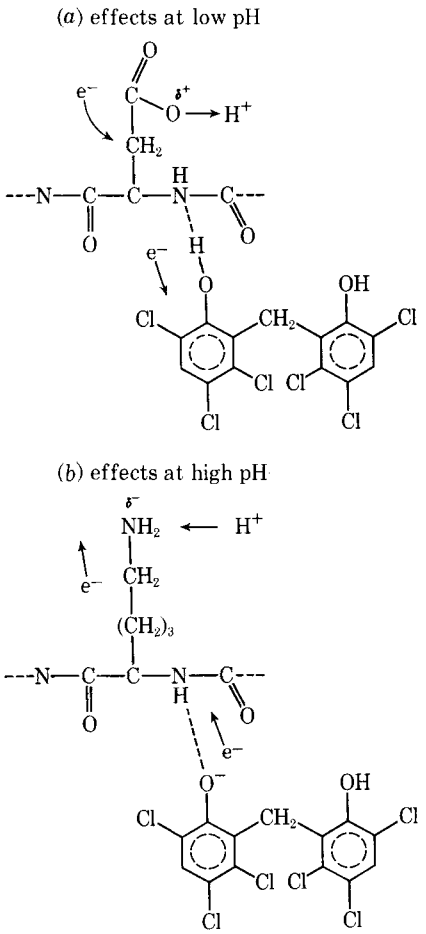
Pankhurst (17), on the other hand, suggested that the binding of phenolic molecules occurs directly by the formation of hydrogen bonds between the phenolic hydroxyl and the peptide nitrogen. Conceivably both sites may be operative and serve to complement one another; however, the direct hydrogen bonding of the hydroxyl appears to be more reasonable in providing the primary mode of interaction (18). In any event, only peptide linkages could occur in sufficient number to provide the binding sites for the extraordinarily large quantity of phenols commonly observed to be sorbed by proteins. That the sorption does not directly involve ionogenic groups is indicated by the mineral acid-binding capacity of collagen, for example, remaining unchanged (18). The degree of dissociation of ionogenic groups located vicinally to hexachlorophene-interacted peptide sites may, however, be influenced indirectly by hexachlorophene binding. Such influences can be propagated through space by direct electrostatic field effects or through the atoms constituting the protein itself through an inductive delocalization of electrons (19). Both mechanisms can operate to alter the acidic pK's of ionizable vicinal groups. The observed changes in fixed-charge density of the epidermal surface, therefore, can be postulated to occur as a consequence of an uptake or release of protons occurring concomitantly with the binding of hexachlorophene with peptide linkages vicinal to the affected ionogenic groups. Electron inductive effects have been shown to be effectively transmitted through as many as five saturated carbon atoms (20). Pauling (21) described the transmission of inductive effects through peptide linkages to be facilitated by the high polarizability, due to the partial resonance, of the peptide amide bond, which has been discerned to possess a transmissivity for inductive effects approximately tantamount to two methylene groups (22). Ling (22) and Szent-Györgi (23) convincingly postulated that such effects can be transmitted through hydrogen bonds to alter the reactivity of groups located in positions neighboring the binding sites of hydrogen bond-forming interactants.

Based on these considerations, the electronic mechanisms operative in the manifest effects of hexachlorophene on the epidermal fixed-charge density can be postulated to occur as diagrammatically described in Scheme I.

The influence of hexachlorophene at low pH must primarily be directed toward the fixed carboxyl groups, which are titrable within the pH range of 2–6 (9). Carboxyl groups are plentiful in the stratum corneum. It has been found (24) that aspartyl and glutamyl residues comprise approximately 21.7% of the weight of keratin, the major proteinaceous constituent of stratum corneum.

At low pH the hydrogen bonding of the electrophilic phenolic hydroxyl with the peptide linkage may be envisaged to induce a withdrawal of delocalizeable electronic charge from the carboxyl group of a vicinal aspartyl or glutamyl side group, resulting in a lowering of the acidic pK of the groups. The accompanying release of protons into the milieu manifests as a reduction in the net positive fixed-charge density of the surface. The high density of carboxyl groups and their separation from the polypeptide backbone by only one or two methylene groups contribute to an expected high probability that such groups may be located within the inductive influence of bound hexachlorophene. The presence of the three electronegative chlorine atoms on the aromatic nuclei of the hexachlorophene molecule may be expected to contribute substantially to its electrophilic inductive efficacy.

At high pH the electrophilic character of the hexachlorophene can be considered as abrogated by the excess electrons resulting from the dissociation of protons from the phenolic groups. Scheme I also illustrates the electronic mechanism postulated as responsible for the observed lowering of fixed negative charge density of the epidermal surface at high pH. The groups primarily affected in the upper pH range are likely constituted by the ϵ-amino group of lysine, which together with the guanidyl group of arginine and the phenolic group of tyrosine comprises approximately 13% of the amino acid content of keratin (24). Delocalization of electronic charge originating in the bound hexachlorophene to groups normally dissociating their protons in the upper pH range is postulated to increase their acidic pK's and manifest in a lower net density of fixed charge on the surface. A similar increase in the pK's of titrable groups is apparently responsible for the reported anion-induced



Scheme I—Simplified diagrammatic representation of the postulated electronic inductive mechanisms of hexachlorophene-induced changes in the fixed-charge density of human epidermis

shifts in the position of hydrogen-ion titration curves of wool and soluble proteins in the direction of increased alkalinity along the pH axis (9, 10, 25). The proteins were reported to appear to combine stoichiometrically with both hydrogen ions and the anions inducing the shifts (26, 27).

The possibility of direct electrostatic salt binding between fixed cationic groups on the colloids and hexachlorophene anions cannot be entirely excluded. Such interactions would depend upon the availability of both fixed cationic groups and hexachlorophene anions. The interactions would be expected to manifest in a net reduction of fixed positive charge density and could possibly be contributing to the effects of hexachlorophene observed in the vicinity of pH below 5.7 in which imidazolyl and α-amino groups (9) can be expected to be involved. However, if salt binding of hexachlorophene is contributing to its observed effects, it cannot be resolved from the operation of the inductive mechanisms which have been postulated as primarily responsible.

SUMMARY AND CONCLUSIONS

The results of the present study clearly indicate that the bioelectrometric method is capable of detecting the influence of hexachlorophene on the acid–base binding properties of the colloids constituting the epidermal surface. The application of the method has revealed aspects of the interaction of hexachlorophene, which are undetectable by other more conventional experimental techniques. The mechanistic interpretation of the observed hexachlorophene-induced displacement of the titration curve of the epidermal surface, although speculative, is in general agreement with related results previously reported and the postulations of other investigators.

The further utility of the electrometric method in the investigation of the kinetic and equilibrium interaction behavior of hexachlorophene with human skin *in vivo* and the manner in which the results relate to its substantive antibacterial properties are topics of subsequent reports in the present series.

REFERENCES

(1) V. F. Smolen and F. P. Siegel, *J. Pharm. Sci.*, **57,** 378(1968).
(2) V. F. Smolen, *Amer. J. Pharm. Educ.*, **33,** 381(1969).
(3) V. F. Smolen, D. E. Snyder, and R. J. Erb, to be published.
(4) N. R. Joseph and M. B. Engel, *Arch. Biochem. Biophys.*, **85,** 209(1959).
(5) N. R. Joseph, H. P. Catchpole, D. M. Laskin, and M. B. Engel, *ibid.*, **84,** 224(1959).
(6) M. B. Engel, N. R. Joseph, D. M. Laskin, and H. R. Catchpole, *Amer. J. Physiol.*, **201,** 621(1961).
(7) E. A. Guggenheim, *J. Phys. Chem.*, **33,** 842(1929).
(8) G. Milazzo, "Electrochemistry," Elsevier, Amsterdam, The Netherlands, 1963, pp. 65–69, 138–142.
(9) C. Tanford, *Advan. Protein Chem.*, **17,** 69(1962).
(10) P. Alexander and R. J. Hudson, "Wool—Its Chemistry and Physics," Reinhold, New York, N. Y., 1954, pp. 181–244.
(11) M. B. Engel and N. R. Joseph, *Arch. Biochem. Biophys.*, **86** 117(1960).
(12) W. Mahler, *J. Amer. Chem. Soc.*, **76,** 3920(1954).
(13) F. W. Putnam, *Advan. Protein Chem.*, **4,** 79(1948).
(14) M. K. Polano, *J. Soc. Cosmet. Chem.*, **19,** 3(1968).
(15) I. H. Blank, E. Gould, and A. Theobald, *J. Invest. Dermatol.*, **42,** 364(1963).
(16) A. Küntzel, in "Handbuch Der Gerbere Icheme," vol. I, part 1, W. Grassmann, Ed., Springer, Vienna, Austria, 1944, p. 519; through K. H. Gustavson, "The Chemistry and Reactivity of Collagen," Academic, New York, N. Y., 1956, pp. 192–195.
(17) K. G. A. Pankhurst, "Surface Phenomena in Chemistry and Biology," Pergamon, New York, N. Y., 1958, p. 115.
(18) K. H. Gustavson, "The Chemistry and Reactivity of Collagen," Academic, New York, N. Y., 1956, pp. 190–195.
(19) G. N. Ling, *Texas Rep. Biol. Med.*, **22,** 244(1964).
(20) V. Schwarz, S. Hermanek, and J. Trotanek, *Chem. Ind., London*, **1960,** 1212.
(21) L. Pauling, "The Nature of the Chemical Bond," Cornell University Press, Ithaca, N. Y., 1948, pp. 276–281.
(22) G. N. Ling, *Biopolymers, Symp.*, **1,** 91(1964).
(23) A. Szent-Györgi, "Bioelectronics," Academic, New York, N. Y., 1968, pp. 35–50.
(24) R. G. Crounse, *Nature*, **200,** 539(1963).
(25) J. Steinhardt and G. M. Zaiser, *Advan. Protein Chem.*, **10,** 160(1955).
(26) J. Steinhardt, *J. Res. Nat. Bur. Stand.*, **28,** 191(1942).
(27) J. Steinhardt, C. H. Fugitt, and M. Harris, *ibid.*, **28,** 29 (1942).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Biophysical Pharmaceutics Area of the Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication June 9, 1970.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

Abstracted in part from a thesis by R. I. Poust in partial fulfillment of the Doctor of Philosophy degree requirements.

This study was supported by a grant from Armour-Dial, Inc., Chicago, Ill.

The technical assistance of Mr. Michael Crosby is acknowledged.

Adsorption of Methylene Blue by Potato Starch: Effect of Methanol, Dioxane, Sucrose, and Urea in Aqueous Systems

ARVIND L. THAKKAR, WILLIAM L. WILHAM, and GEORGE ZOGRAFI*

Abstract □ Methylene blue is adsorbed by potato starch from aqueous solution as a monomer or dimer, depending upon its concentration. The adsorption phenomenon can be described by the Langmuir equation. The effect of methanol, dioxane, sucrose, and urea on methylene blue adsorption has been examined. These water-miscible additives shift the monomer–dimer equilibrium of the dye in the direction of the monomer and reduce the extent of methylene blue adsorption without significantly affecting the maximum possible adsorption. This appears to indicate that their effect is primarily on the effective concentration of the dye. At high concentrations, methanol and dioxane apparently further reduce adsorption by affecting the surface of potato starch.

Keyphrases □ Methylene blue—potato starch adsorption □ Starch, potato—methylene blue adsorption □ Methanol, dioxane, sucrose, urea effects—methylene blue adsorption □ Sucrose effect—methylene blue visible spectrum □ Colorimetric analysis—spectrophotometer

Enhanced adsorption of water-soluble certified dyes to various solid dosage form ingredients has been suggested as a means of overcoming the problems of dye migration during the drying of tablet granulations (1). During a study of anionic dye adsorption on various starches, significant adsorption was noted for all types of starch except potato starch (1, 2). Schoch and Maywald (3) observed also that Congo Red, a negatively charged dye, is not adsorbed by potato starch, presumably because of the presence of phosphate groups not ordinarily found in the other starches (4). In view of this negative charge, potato starch would be expected to adsorb cationic substances significantly. Schoch and Maywald (3) found that the cationic dye, methylene blue, strongly stains potato starch granules, but no quantitative studies on the adsorption of cationic substances by potato starch have appeared.

In the present study, methylene blue was chosen as a model dye in view of the information available concerning its state in solution (5–8). In dilute aqueous solutions, the dye exists principally in its monomeric form, while in concentrated solution the dimeric and multimeric forms predominate as a result of hydrophobic self-association. Since the monomer–multimer equilibria are sensitive to various polar additives (5), the effect of such substances on methylene blue adsorption was also investigated. The additives examined were dioxane, methanol, sucrose, and urea.

EXPERIMENTAL

Materials—Methylene blue USP and potato starch (purified powder)[1] were used as received. Water contents of the trihydrate dye and the starch were determined and were taken into consideration when recording their weights. Reagent grade dioxane, methanol, and sucrose and urea USP were used without further purification. Dye solutions for adsorption studies, with or without additives, were always freshly prepared using double-distilled water.

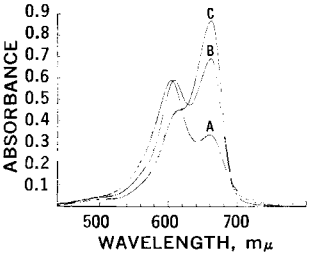


Figure 1—*Effect of concentration upon the visible spectrum of methylene blue at room temperature. Key: A, 1.2 × 10⁻³ M, 0.1-mm. cells; B, 1.2 × 10⁻⁴ M, 1-mm. cells; and C, 1.2 × 10⁻⁵ M, 10-mm. cells.*

Spectral Studies—To illustrate the effect of concentration upon the visible spectrum of methylene blue, the following concentrations were employed: 1.2×10^{-3} *M*, 1.2×10^{-4} *M*, and 1.2×10^{-5} *M*; the cells used were of 0.1-, 1-, and 10-mm. light pathlengths, respectively. The effect of additives upon the spectrum of the dye was examined at constant dye concentration (1.2×10^{-4} *M*) and various additive concentrations, using 1-mm. cells. Spectra were recorded at room temperature on a spectrophotometer (Cary 15).

Adsorption Studies—All adsorption experiments were done at a controlled room temperature of 26 ± 1°. Approximately 0.5 g. of potato starch was accurately weighed into each of a series of vials. Ten milliliters of a dye solution was added to each vial, and the vials were stoppered with butyl rubber closures. It was initially ascertained that these stoppers adsorbed no methylene blue. The adsorption vials were shaken occasionally over 4 hr. Preliminary experiments indicated that this time was more than sufficient for the various systems to attain equilibrium.

Upon equilibration, 1 ml. of clear, granule-free, supernatant dye solution was withdrawn from each vial. It was then suitably diluted with water, and its concentration was determined spectrophotometrically at 660 mμ. A spectrophotometer (Cary 15) and 1-cm. glass cells were used. When the systems contained additives, it was necessary to make sure that the dye solutions were diluted to a point where the additive concentrations were too low to interfere with the spectral procedure. This precaution precluded adsorption studies at relatively low equilibrium dye concentrations in the presence of additives. From the concentration of dye solutions before and after contact with starch, the amount of dye adsorbed was determined.

RESULTS

Spectral Properties of Methylene Blue—The changes in the visible spectrum of methylene blue, reflecting changes in the state of aggregation with increasing dye concentration, are shown in Fig. 1. The peak at ~660 mμ has been assigned to the monomeric form, which predominates in dilute aqueous solutions. The dimeric form absorbs at ~590 mμ; the population of this form increases as the dye concentration is increased. Possibility of higher multimers has been proposed for some time; recently, existence of a trimer was conclusively proven and a rather broad peak at ~580 mμ (not shown in Fig. 1) was ascribed to the trimer (8). Because of these spectral properties, the Beer's law relationship is applicable only in a limited concentration range, up to ~1×10^{-5} *M*, when absorbance cells of 1-cm. light pathlength are used.

Figure 2 illustrates the effect of increasing additive concentrations upon the spectrum of the dye. Relative effectiveness of these

[1] Fisher Scientific Co., Pittsburgh, Pa.

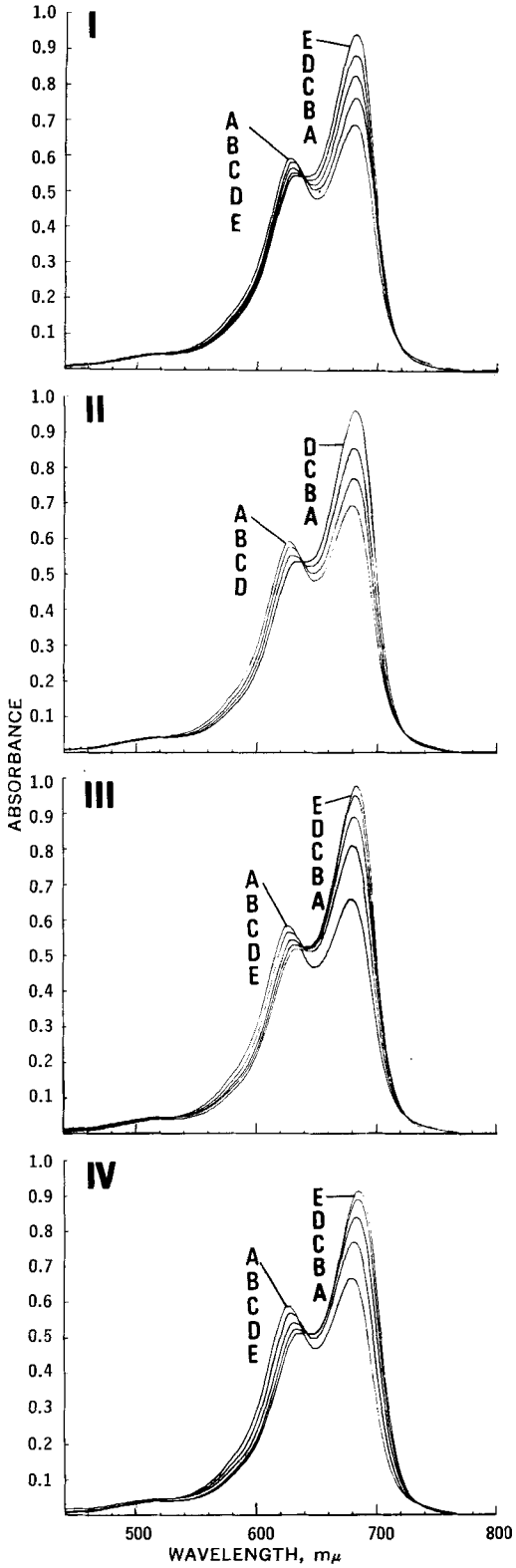


Figure 2—*Effect of additive concentration upon the visible spectrum of methylene blue at room temperature. I: Methanol, A, 0%; B, 5%; C, 10%; D, 15%; and E, 20% v/v. II: Dioxane, A, 0%; B, 2.5%; C, 5%; and D, 10% v/v. III: Sucrose, A, 0 M; B, 0.5 M; C, 1 M; D, 1.5 M; and E, 2.0 M. IV: Urea, A, 0 M; B, 1 M; C, 2 M; D, 3 M; and E, 4 M. Dye concentration: 1.2 × 10⁻⁴ M; 1-mm. cells.*

additives in diminishing the self-association of methylene blue may be examined by considering the ratio of the absorbance of the monomer peak to that of the dimer peak. Plots of monomer/dimer ratio (M/D ratio) *versus* the concentration of additives are shown in

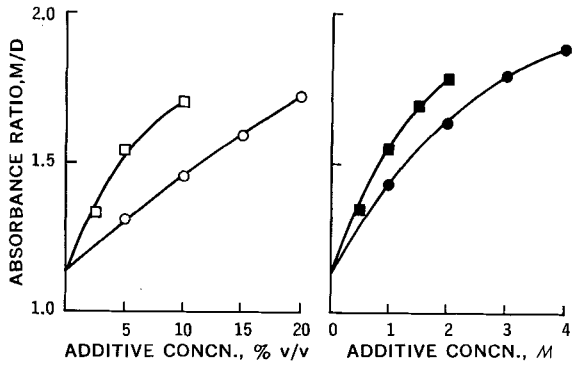


Figure 3—*Relationship between additive concentration and absorbance ratio of the monomer and dimer peaks. Key: ○, methanol; □, dioxane; ■, sucrose; and ●, urea.*

Fig. 3. Sucrose is more effective than urea, on a molar basis, in reducing the self-association of the dye. This observation has an interesting parallel in the work of Emerson and Holtzer (9) who found that at equimolar concentrations, sucrose is more effective than urea in raising the CMC of dodecyltrimethylammonium bromide. Of the two organic solvents employed, dioxane is more effective than methanol at equal volume concentration.

Adsorption Studies—The Langmuir equation for adsorption from solution may be written in its linear form as follows:

$$\frac{C}{x/M} = \frac{C}{k_2} + \frac{1}{k_1 k_2} \qquad \text{(Eq. 1)}$$

where C is the concentration of the solute at equilibrium, x/M is the amount of solute adsorbed per unit weight of the adsorbent, and k_1 and k_2 are constants. The constant k_1 represents the tendency for adsorption to occur and may be considered as an equilibrium constant for the adsorption process; k_2 represents the maximum value of x/M at a given temperature determined by the number of available sites or the surface area of the adsorbent. If a system follows the Langmuir adsorption equation, then plots of $C/(x/M)$ *versus* C should be linear. The constants k_1 and k_2 may be calculated from the values of the slope and intercept of such Langmuir plots.

Figure 4 is a Langmuir plot for the adsorption of methylene blue on potato starch from water. The plot shows two linear regions of significantly different slopes. The lower region corresponds to dilute solutions in which methylene blue exists predominantly as the monomer; the higher region corresponds to solutions in which the dimers predominate. These regions are referred to hereafter as the monomer

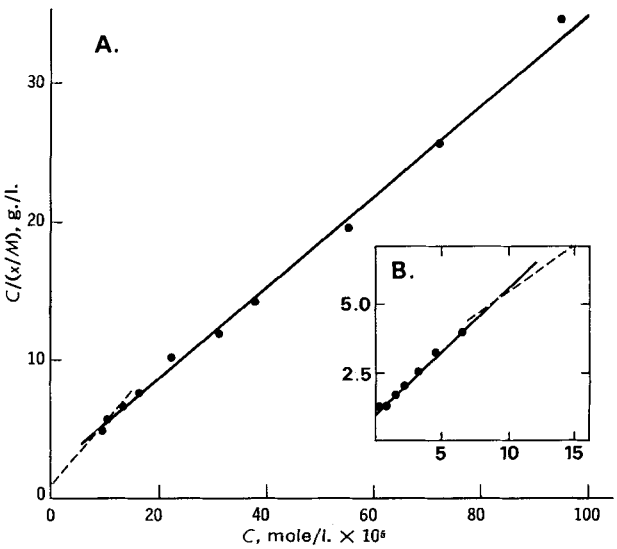


Figure 4—*Langmuir plots for adsorption of methylene blue on potato starch from water. A: Solid line and data points are for dimer range; dashed line is for monomer range. B: Solid line and data points are for monomer range; dashed line is for dimer range. The lines drawn are calculated least-squares lines.*

Table I—Langmuir Constants for Adsorption of Methylene Blue on Potato Starch from Aqueous Solutions at 26°

Dye Concentration Range	Langmuir Constants k_1, l./mole	Langmuir Constants k_2, moles/g.	Correlation Coefficient of Linear Fit
Low, predominantly monomer	0.466×10^4	2.18×10^{-5}	0.996
High, predominantly dimer	1.54×10^4	3.04×10^{-5}	0.998

and dimer regions, respectively. Since the trimer is formed at relatively high concentrations, it was assumed that within the dye concentration range employed in this study the fraction of trimer present was negligible.

The Langmuir constants calculated from the linear plot of Fig. 4, along with the correlation coefficients of the linear fits, are listed in Table I.

Effect of Additives—Figures 5–8 show, respectively, the effect of methanol, dioxane, sucrose, and urea upon the adsorption of methylene blue by potato starch. Over the dye concentration range employed, adsorption decreases with an increase in the additive content of the system. This finding is in agreement with previous observations made with dye–starch systems containing water-miscible organic solvents (2, 10, 11). In the experiments reported here, it was not possible to obtain valid results at low dye concentrations because the presence of additives gave rise to difficulties in the analytical procedure, as indicated in the *Experimental* section. The data obtained in the presence of additives, therefore, refer to relatively high dye concentrations (dimer range).

All the systems examined followed the Langmuir equation. Representative Langmuir plots, for the systems containing sucrose, are shown in Fig. 9. The Langmuir constants and the correlation coefficients for all the systems examined are listed in Table II. For comparison, the values of M/D ratio for these systems are also listed. The values listed in Table II make the following points apparent: (*a*) k_1 decreases with an increase in the additive content of the system; (*b*) k_1 decreases as the M/D ratio increases (or as the relative amount of dimer decreases); and (*c*) k_2 remains relatively unchanged (except at 10% volume concentration of methanol or dioxane).

DISCUSSION

Effect of Additives upon Self-Association of Methylene Blue—All four additives employed in this study shift the monomer/dimer equilibrium of methylene blue in aqueous solution toward the direction of the monomer. The organic solvents, methanol and dioxane, probably act this way by interacting with water and by lowering the dielectric constant of the system (12). However, it seems unlikely that the same mechanism is operative with urea and sucrose; urea increases the dielectric constant of water (13), whereas sucrose decreases it (14–16). Mukerjee and Ghosh (5) suggested that urea affects the monomer/multimer equilibrium of methylene blue by disrupting the structure of water. Uedeira and Uedeira (17) suggested that sucrose also diminishes self-association in some aromatic azo dyes by a similar mechanism.

Additionally, urea and sucrose are said to have opposite effects upon water structure (18); urea is believed to be a "structure breaker" and sucrose a "structure maker." The terms structure maker and structure breaker, as applied to the action of urea on water, have been the subjects of recent controversies (19, 20) of both a mechanistic as well as a semantic nature. In view of such controversies, it is difficult to say what specific mechanisms are responsible for the actions of the four additives used in this study. However, these additives all have the potential for interacting with water and they all diminish self-association of methylene blue in aqueous solution.

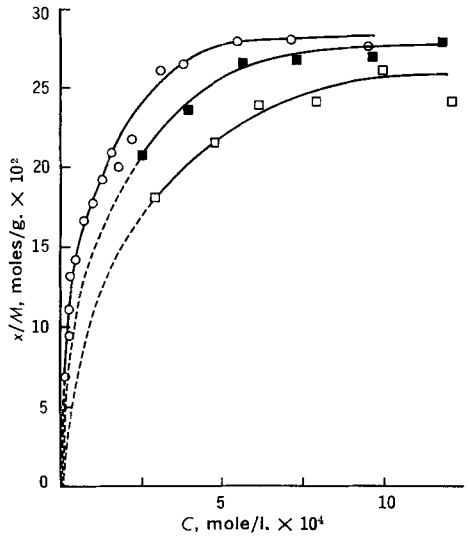


Figure 5—*Isotherms for adsorption of methylene blue on potato starch. Key:* O, *water;* ■, *5% methanol; and* □, *10% methanol.*

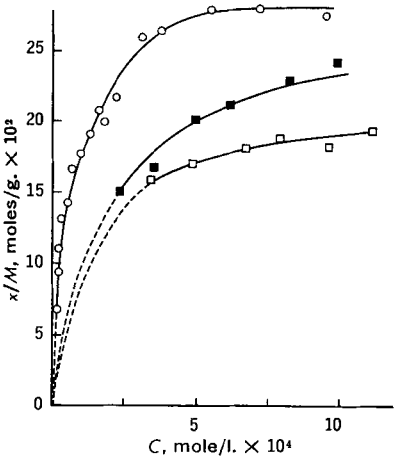


Figure 6—*Isotherms for adsorption of methylene blue on potato starch. Key:* O, *water;* ■, *5% dioxane; and* □, *10% dioxane.*

Adsorption of Methylene Blue from Water—Giles *et al.* (21) and Giles (22) suggested that methylene blue is adsorbed as the dimer[2] on several nonporous adsorbents. The results of the present study show that, although at relatively high concentrations the dimer may be preferentially adsorbed, at very low concentrations, in which the monomer predominates, an appreciable amount of the dye is adsorbed on potato starch. It would appear that if adsorption experiments were performed with only relatively high concentrations of methylene blue, the monomer region might remain undetected. Additional evidence for the possibility of monomer adsorption comes from the

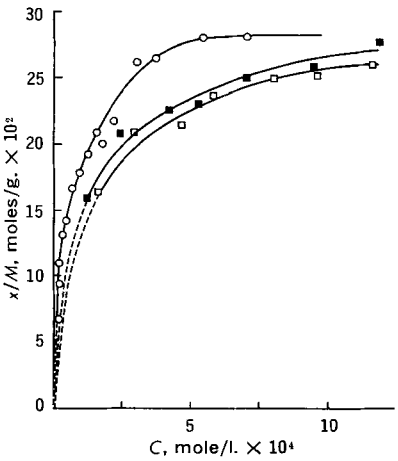


Figure 7—*Isotherms for adsorption of methylene blue on potato starch. Key:* O, *water;* ■, *0.5* M *sucrose; and* □, *1.0* M *sucrose.*

[2] The work of Giles *et al.* (21) and Giles (22) suggests that dimer adsorption of methylene blue involves adsorption of one molecule upon the adsorbent with the second molecule stacked lengthwise on top of the first. Dimer adsorption is not believed to involve side-by-side arrangement of two molecules on the surface of the adsorbent.

Table II—Langmuir Constants for Adsorption of Methylene Blue on Potato Starch from Aqueous Systems Containing Additives and M/D Ratios for These Systems

System	Langmuir Constants[a] k_1, l./mole $\times 10^{-4}$	k_2, moles/g. $\times 10^5$	Correlation Coefficient of Linear Fit	M/D Absorbance Ratio[b]
Water (dimer range)	1.54	3.04	0.998	1.129
5% (v/v) Methanol	0.76	3.11	0.980	1.316
10% (v/v) Methanol	0.67	2.87	0.997	1.458
5% (v/v) Dioxane	0.40	3.07	0.997	1.545
10% (v/v) Dioxane	0.39	2.47	0.983	1.796
0.5 *M* Sucrose	0.74	3.07	0.996	1.350
1.0 *M* Sucrose	0.36	3.08	0.994	1.545
0.5 *M* Urea	0.77	2.97	0.995	1.279
1.0 *M* Urea	0.47	3.08	0.997	1.429

[a] Determined at experimental temperature of 26 ± 1°. [b] At methylene blue concentration = 1.2×10^{-4} *M*.

work of Iimura (23), who found that more methylene blue was adsorbed by some clays when the initial dye concentration in the system was high than when methylene blue was added in small successive amounts until the same total concentration was reached in the system. This observation may be interpreted as follows: When the initial concentration is high, methylene blue exists principally as the dimer, and it is adsorbed as a monolayer predominantly of dimers; when small successive amounts of methylene blue are added, it exists in the unaggregated (monomer) form which is adsorbed.

If the two regions of the Langmuir plot (Fig. 4) corresponded strictly to monomers and dimers, then one would expect that k_2 (dimer) should be twice as large as k_2 (monomer). Experimentally, such is not the case; k_2 (dimer) is only 1.39 times k_2 (monomer). This finding suggests that in neither the "monomer" nor the "dimer" region is there adsorption of only one dye species. The two regions of the Langmuir plot represent the predominant species.

Additionally, the specific surface area calculated from both of the k_2 values and a molecular area of 120 Å^2 for methylene blue (22) do not agree with the specific surface area determined by porosity measurements (24). These values (in m.^{2}/g.) are: 15.6 [from k_2 (monomer)], 11.0 [from k_2 (dimer)], and 0.2 (from porosity measurements).[3] These much higher values from methylene blue adsorption measurements may be due to penetration and diffusion of the dye solution into the starch grain. Thus the actual area available for adsorption would be much greater than the outer grain surface. A similar lack of correlation between the specific surface area values obtained by different methods was found in a study of the adsorption of various anionic certified dyes by several starches (2). The structure and porosity of the starch grain are important factors to be considered in the study of adsorption by the grain; both the external and the internal surfaces of the grain probably participate when dissolved substances are adsorbed.

Adsorption in the Presence of Additives—The equilibria between various forms of methylene blue can be represented as shown in Scheme I:

$$\begin{array}{ccc} \text{monomer}_{\text{solution}} & \overset{1}{\rightleftharpoons} & \text{dimer}_{\text{solution}} \\ {\scriptstyle 3}\,\updownarrow & & {\scriptstyle 2}\,\updownarrow \\ \text{monomer}_{\text{adsorbed}} & & \text{dimer}_{\text{adsorbed}} \end{array}$$

Scheme I

Equilibrium 3 comes into the picture, apparently, only at very low dye concentration. Under the conditions employed in this study, dimer adsorption is predominant. The Langmuir constants listed in Table II refer to adsorption characteristics of methylene blue dimer.

The decreasing value of k_1 when the systems contain increasing amounts of additives indicates essentially what Figs. 5–8 show,

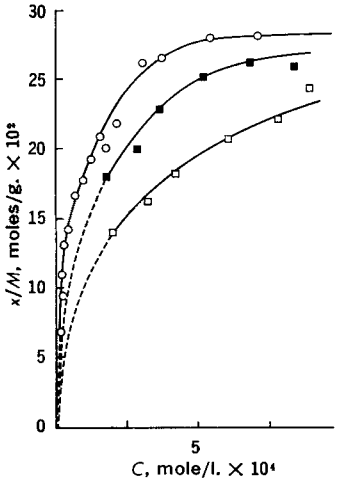


Figure 8—*Isotherms for adsorption of methylene blue on potato starch. Key:* ○, *water;* ■, *0.5* M *urea; and* □, *1.0* M *urea.*

i.e., the extent of adsorption decreases with an increase in the amount of additive. All the additives employed in this study shift Equilibrium 1 in the direction of the monomer, resulting in a decreased effective concentration of the dimer. The relative amount of dimer is still much greater than that of monomer and, since dimer is the predominant adsorbing species, decreased adsorption is reflected in lower k_1 values.

The relatively unchanged values of k_2 (or maximum possible amount of dye adsorbed) with increasing additive contents of the system indicate that the number of sites available for adsorption remains essentially unaltered. This observation indicates that the additives primarily affect the dye rather than the starch. However, at organic solvent concentrations of 10% (v/v) or higher, the starch is apparently affected. This is reflected in diminished values of both k_1 and k_2. When the systems contained 50% or higher volumes of methanol or dioxane, dye adsorption was virtually eliminated. Starch is known to contain water of hydration as part of its grain structure. It is possible that organic solvents in excess shrink or dehydrate this hydrophilic polymer.

Apparent adsorption, as noted in this study for methylene blue, can also occur with a variety of cationic substances including drugs.

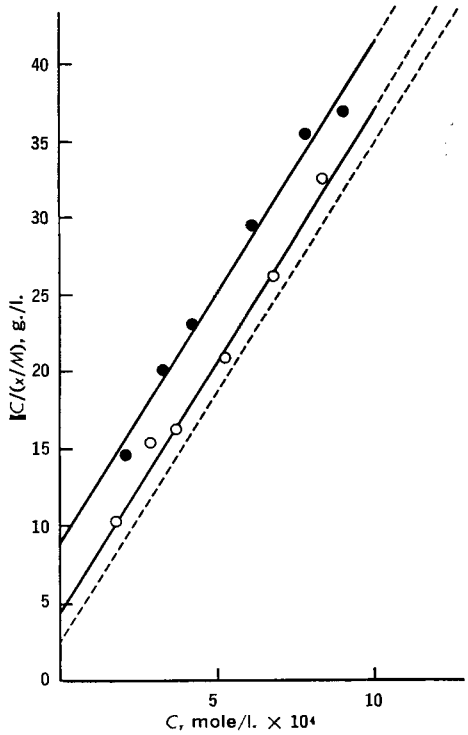


Figure 9—*Langmuir plot for adsorption of methylene blue on potato starch from aqueous sucrose solutions. Key:* - - -, *water (dimer range);* ○, *0.5* M *sucrose; and* ●, *1.0* M *sucrose.*

[3] The cooperation of H. M. Rootare of the Department of Dental Materials, University of Michigan, in specific surface area determination is gratefully acknowledged.

In the case of dyes, enhanced adsorption is desirable for uniform coloring of solid dosage forms. However, in the case of drugs, adsorption to a seemingly inert excipient such as starch may affect their release and availability. Problems due to drug adsorption by excipients may also arise during *in vitro* evaluation of the solid dosage forms and during quantitative analysis for the active ingredient. This would be particularly true for potent drugs that have low effective doses. Adsorption studies during the preformulation stages in the development of a drug product can provide clues to such problems.

REFERENCES

(1) G. Zografi and A. M. Mattocks, *J. Pharm. Sci.*, **52,** 1103 (1963).

(2) G. Zografi, Ph.D. thesis, University of Michigan, 1961.

(3) T. J. Schoch and E. C. Maywald, *Anal. Chem.*, **28,** 382(1956).

(4) T. Posternak, *Helv. Chim. Acta*, **18,** 1351(1935).

(5) P. Mukerjee and A. K. Ghosh, *J. Phys. Chem.*, **67,** 193(1963).

(6) M. J. Blandamer, J. A. Brivati, M. F. Fox, M. C. R. Symons, and G. S. P. Verma, *Trans. Faraday Soc.*, **63,** 1850(1967).

(7) E. Rabinowitch and L. F. Epstein, *J. Amer. Chem. Soc.*, **63,** 69(1941).

(8) E. Braswell, *J. Phys. Chem.*, **72,** 2477(1968).

(9) M. F. Emerson and A. Holtzer, *ibid.*, **71,** 3320(1967).

(10) A. L. Thakkar and G. Zografi, unpublished data; A. L. Thakkar, M.S. thesis, Columbia University, 1964.

(11) J. Huebner and K. Venkataraman, *J. Soc. Dyers Colour.*, **42,** 110(1926).

(12) G. N. Lewis, T. T. Megel, O. Goldschmid, and J. Bigeleisen, *J. Amer. Chem. Soc.*, **65,** 1150(1943).

(13) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic, New York, N. Y., 1958, p. 323.

(14) A. A. Maryott and C. G. Malmberg, *J. Res. Nat. Bur. Stand.*, **45,** 299(1950).

(15) G. Akerlof, *J. Amer. Chem. Soc.*, **54,** 4125(1932).

(16) A. N. Paruta, *J. Pharm. Sci.*, **53,** 1252(1964).

(17) M. Uedeira and H. Uedeira, *Kolloid Z. Z. Polym.*, **194,** 148(1964).

(18) G. E. Walrafen, *J. Chem. Phys.*, **44,** 3726(1965).

(19) M. Abu-Hamidayyah, *J. Phys. Chem.*, **69,** 2720(1965).

(20) S. Subramanian, D. Balasubramanian, and J. C. Ahluwalia, *ibid.*, **73,** 266(1969).

(21) C. H. Giles, I. A. Easton, R. B. McKay, C. C. Patel, N. B. Shah, and D. Smith, *Trans. Faraday Soc.*, **62,** 1963(1966).

(22) C. H. Giles, *J. Amer. Chem. Soc.*, **91,** 759(1969).

(23) K. Iimura, *Nendo Kagaku*, **6,** 14(1966); through *Chem. Abstr.*, **69,** 90008k(1968).

(24) H. M. Rootare and C. F. Prenzlow, *J. Phys. Chem.*, **71,** 2733(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 13, 1970, from the *Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46206*

Accepted for publication June 3, 1970.

* College of Pharmacy, University of Michigan, Ann Arbor, MI 48101

DRUG STANDARDS

Spectrophotometric Determination of Diphenhydramine Hydrochloride in an Antiallergic Cream

FABRIZIO De FABRIZIO

Abstract □ A specific method for the quantitative determination of diphenhydramine hydrochloride in a cream formulation has been developed. The method entails the extraction of diphenhydramine by chloroform, further purification of the extracted base by column chromatography using alginic acid, and its spectrophotometric determination in the eluate at 258 mμ.

Keyphrases □ Diphenhydramine cream—analysis □ Column chromatography—separation □ UV spectrophotometry—analysis

The isolation of a pharmacologically active ingredient from a pharmaceutical formulation containing surfactants is often difficult. Various authors have followed different procedures for the removal of unwanted ingredients. Jones (1) described a method for the determination of diethylstilbestrol in a water-dispersible suppository using column chromatography followed by TLC. Gottlieb (2) used refluxing with an organic solvent to break down the emulsion and subsequently recovered the active drug (which was also diethylstilbestrol) using an aluminum column. More recently, Forman (3) developed an assay for dienestrol in a cream[1] using urea-inclusion chemistry to remove the excess of monostearin. The information obtained from any of these studies is valuable and may indicate a general approach to analysis employing the two basic steps of extraction and cleanup. However, each of them is a specific case which depends upon the physical and chemical properties of the active component.

Therefore, it is reasonable to assume that different methods of separation may be required for differing formulations containing the same active ingredients and for differing active ingredients contained in similar formulations. Diphenhydramine hydrochloride is found in various combinations in commercially available pharmaceutical preparations, and various assays for its determination have been reviewed (4–6). No procedure, however, has been reported involving the quantification of diphenhydramine hydrochloride in an

[1] The analysis was applied to a product containing 2% of diphenhydramine hydrochloride and marketed as "Allergin Cream."

In the case of dyes, enhanced adsorption is desirable for uniform coloring of solid dosage forms. However, in the case of drugs, adsorption to a seemingly inert excipient such as starch may affect their release and availability. Problems due to drug adsorption by excipients may also arise during *in vitro* evaluation of the solid dosage forms and during quantitative analysis for the active ingredient. This would be particularly true for potent drugs that have low effective doses. Adsorption studies during the preformulation stages in the development of a drug product can provide clues to such problems.

REFERENCES

(1) G. Zografi and A. M. Mattocks, *J. Pharm. Sci.*, **52,** 1103 (1963).

(2) G. Zografi, Ph.D. thesis, University of Michigan, 1961.

(3) T. J. Schoch and E. C. Maywald, *Anal. Chem.*, **28,** 382(1956).

(4) T. Posternak, *Helv. Chim. Acta*, **18,** 1351(1935).

(5) P. Mukerjee and A. K. Ghosh, *J. Phys. Chem.*, **67,** 193(1963).

(6) M. J. Blandamer, J. A. Brivati, M. F. Fox, M. C. R. Symons, and G. S. P. Verma, *Trans. Faraday Soc.*, **63,** 1850(1967).

(7) E. Rabinowitch and L. F. Epstein, *J. Amer. Chem. Soc.*, **63,** 69(1941).

(8) E. Braswell, *J. Phys. Chem.*, **72,** 2477(1968).

(9) M. F. Emerson and A. Holtzer, *ibid.*, **71,** 3320(1967).

(10) A. L. Thakkar and G. Zografi, unpublished data; A. L. Thakkar, M.S. thesis, Columbia University, 1964.

(11) J. Huebner and K. Venkataraman, *J. Soc. Dyers Colour.*, **42,** 110(1926).

(12) G. N. Lewis, T. T. Megel, O. Goldschmid, and J. Bigeleisen, *J. Amer. Chem. Soc.*, **65,** 1150(1943).

(13) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic, New York, N. Y., 1958, p. 323.

(14) A. A. Maryott and C. G. Malmberg, *J. Res. Nat. Bur. Stand.*, **45,** 299(1950).

(15) G. Akerlof, *J. Amer. Chem. Soc.*, **54,** 4125(1932).

(16) A. N. Paruta, *J. Pharm. Sci.*, **53,** 1252(1964).

(17) M. Uedeira and H. Uedeira, *Kolloid Z. Z. Polym.*, **194,** 148(1964).

(18) G. E. Walrafen, *J. Chem. Phys.*, **44,** 3726(1965).

(19) M. Abu-Hamidayyah, *J. Phys. Chem.*, **69,** 2720(1965).

(20) S. Subramanian, D. Balasubramanian, and J. C. Ahluwalia, *ibid.*, **73,** 266(1969).

(21) C. H. Giles, I. A. Easton, R. B. McKay, C. C. Patel, N. B. Shah, and D. Smith, *Trans. Faraday Soc.*, **62,** 1963(1966).

(22) C. H. Giles, *J. Amer. Chem. Soc.*, **91,** 759(1969).

(23) K. Iimura, *Nendo Kagaku*, **6,** 14(1966); through *Chem. Abstr.*, **69,** 90008k(1968).

(24) H. M. Rootare and C. F. Prenzlow, *J. Phys. Chem.*, **71,** 2733(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 13, 1970, from the *Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46206*

Accepted for publication June 3, 1970.

* College of Pharmacy, University of Michigan, Ann Arbor, MI 48101

DRUG STANDARDS

Spectrophotometric Determination of Diphenhydramine Hydrochloride in an Antiallergic Cream

FABRIZIO De FABRIZIO

Abstract □ A specific method for the quantitative determination of diphenhydramine hydrochloride in a cream formulation has been developed. The method entails the extraction of diphenhydramine by chloroform, further purification of the extracted base by column chromatography using alginic acid, and its spectrophotometric determination in the eluate at 258 mμ.

Keyphrases □ Diphenhydramine cream—analysis □ Column chromatography—separation □ UV spectrophotometry—analysis

The isolation of a pharmacologically active ingredient from a pharmaceutical formulation containing surfactants is often difficult. Various authors have followed different procedures for the removal of unwanted ingredients. Jones (1) described a method for the determination of diethylstilbestrol in a water-dispersible suppository using column chromatography followed by TLC. Gottlieb (2) used refluxing with an organic solvent to break down the emulsion and subsequently recovered the active drug (which was also diethylstilbestrol) using an aluminum column. More recently, Forman (3) developed an assay for dienestrol in a cream[1] using urea-inclusion chemistry to remove the excess of monostearin. The information obtained from any of these studies is valuable and may indicate a general approach to analysis employing the two basic steps of extraction and cleanup. However, each of them is a specific case which depends upon the physical and chemical properties of the active component.

Therefore, it is reasonable to assume that different methods of separation may be required for differing formulations containing the same active ingredients and for differing active ingredients contained in similar formulations. Diphenhydramine hydrochloride is found in various combinations in commercially available pharmaceutical preparations, and various assays for its determination have been reviewed (4–6). No procedure, however, has been reported involving the quantification of diphenhydramine hydrochloride in an

[1] The analysis was applied to a product containing 2% of diphenhydramine hydrochloride and marketed as "Allergin Cream."

Table I—Results of Determination of Diphenhydramine Hydrochloride in Eight Synthetic Mixtures[a]

Mixture Containing	Taken, g.	Found for Eight Determinations, g.[b]
Diphenhydramine hydrochloride	2.00	1.97, 2.01, 2.10, 1.98, 2.04, 1.96, 2.01, 2.10

[a] The preparation also contains emulsifying wax, liquid paraffin, parabens, glycerin, water, color, and menthol. [b] Mean percentage recovery of diphenhydramine hydrochloride = 100.5%.

emulsified cream base. The procedure described in this paper is a modification of an analysis method for the determination of diphenhydramine hydrochloride in a cough mixture (6). The method entails the recovery of the diphenhydramine by chloroform extraction and further purification through an alginic acid column, followed by quantitative spectrophotometry at 258 mμ.

EXPERIMENTAL

Apparatus—A Beckman DB spectrophotometer and 4-cm. square, fused silica cells were used. A glass column, 30 × 1.8 cm., with a stem, 5 cm., was fitted with a buret key.

Reagents—The following were used: cation-exchange resin, alginic acid, 40–100 mesh (available from British Drug Houses); 2 *N* hydrochloric acid in water; 0.1 *N* hydrochloric acid in water; 5% hydrochloric acid in water; 95% ethanol in water; and 80% ethanol in water. Except where otherwise specified, all reagents were of British Drug Houses' Analar quality.

Standard Solutions—The following solutions were prepared with suitable reference standards: (*a*) diphenhydramine hydrochloride, 2 g./100 ml. in 5% hydrochloric acid; and (*b*) diphenhydramine hydrochloride, 3.2 mg./100 ml. in 0.1 *N* hydrochloric acid.

Column Preparation—Alginic acid, about 4 g., was slurried in water and allowed to soak for 4 hr. The slurry was poured into a glass column which had been fitted with a cotton wool plug and allowed to settle. The column was washed with 2 *N* hydrochloric acid, until the absorbance of the eluate (pathlength 4 cm.) was less than 0.005 at 258 mμ, and then with distilled water until the eluate was neutral to litmus. Finally, 25 ml. of 80% ethanol was passed through the column.

Sample Treatment—An amount corresponding to approximately 2 g. of cream (about 40 mg. diphenhydramine hydrochloride) was accurately weighed into a 150-ml. separator. The cream was suspended in 6 ml. of 5% hydrochloric acid. The suspension was extracted with four successive portions of 15 ml. each of chloroform; each extraction was filtered through a pledget of cotton wool into a 100-ml. volumetric flask, and the solution was brought to volume. An aliquot of 20 ml. was pipeted into a 25-ml. volumetric flask. The chloroform was evaporated to dryness on a water bath with the aid of a current of air. The residue was dissolved in 95% ethanol, and the solution was brought to volume. An aliquot, 10 ml., was pipeted onto the prepared column, and the solution was allowed to pass through the column at a rate of 1 ml./min. The column was then washed with 50 ml. of 80% ethanol divided into two portions, also at a rate of 1 ml./min., and finally with 200 ml. of water at a rate of 4 ml./min. Diphenhydramine was subsequently eluted with 0.1 *N* hydrochloric acid at a rate of 1 ml./min. The first 5 ml. of eluate was discarded and the balance collected into a 100-ml. volumetric flask until 100 ml. was collected. The absorbance of the solution was then determined at 258 mμ, using 4-cm. cells and 0.1 *N* hydrochloric acid as blank.

Table II—Analysis of Diphenhydramine Hydrochloride in Eight Commercial Formulations

Preparation	Claim for Diphenhydramine Hydrochloride, g. %	Found, % of Claim
A	2	105.30
B	2	98.73
C	2	100.23
D	2	99.45
E	2	102.70
F	2	100.68
G	2	98.10
H	2	99.79

RESULTS AND DISCUSSION

The cream examined was of the oil–water type. The oil phase contained menthol as a coolant. A nonionic emulsifying wax was used as the emulgent, as well as providing a cream of the required consistency. The aqueous phase contained diphenhydramine hydrochloride, parabens, red color, and glycerin as a humectant. In a preliminary study, attention was focused on the possibility of finding a combination of solvents which would help liquify the cream and at the same time facilitate the diphenhydramine extraction. The most suitable system found was chloroform–water. By initially acidifying the cream, the difficulty, which is normally encountered in alkaloidal extractions using chloroform–water systems, of extraction of the active ingredient favoring the aqueous phase instead of the chloroform phase is overcome. In addition to diphenhydramine, the chloroform extract contains menthol, liquid paraffin, parabens, and the emulsifying wax. For this reason, the residue after the evaporation of chloroform was dissolved in 95% ethanol instead of water which would have given a turbid solution. Although the alcoholic solution used does not dissolve the oily particles in the residue, these are easily eliminated by filtration of the solution through a pledget of cotton wool. When the procedure was followed to analyze eight synthetic mixtures of the cream (prepared in a manner similar to commercial formulation), the results in Table I were obtained.

When the method was applied to eight commercial products, the results in Table II were obtained.

The reasonable results obtained with commercial and empirical products establish the validity of this procedure.

REFERENCES

(1) L. N. Jones, M. Seidman, and B. C. Southworth, *J. Pharm. Sci.*, **57**, 646(1968).

(2) S. Gottlieb, *J. Amer. Pharm. Ass., Sci. Ed.*, **37**, 147(1948).

(3) B. J. Forman and L. T. Grady, *J. Pharm. Sci.*, **58**, 1262(1969).

(4) "The United States Pharmacopeia" 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 207.

(5) J. Levine, *J. Pharm. Sci.*, **54**, 485(1965).

(6) F. De Fabrizio, *J. South African Chem. Inst.*, **XX**, 194(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 16, 1970, from the *Control Pharmaceutical Laboratory of Biopharm (Pty.) Limited, Johannesburg, Republic of South Africa.*

Accepted for publication April 3, 1970.

Acetonitrile–Diatomaceous Earth Column for Corticosteroids

ROBERT E. GRAHAM*, PATRICIA A. WILLIAMS*, and CHARLES T. KENNER†

Abstract □ A column of acetonitrile on diatomaceous earth is used to hold the corticosteroid on the column during the wash of the column with *n*-heptane to remove decomposition products and interfering substances, after which the corticosteroid and acetonitrile are eluted from the column with chloroform. The recommended procedure significantly reduces or completely eliminates the interference of various substances and certain decomposition products in the blue tetrazolium, phenylhydrazine, isonicotinic acid hydrazide, and UV methods of determination. The column can be readily modified to include acidic, basic, or neutral aqueous-trap layers when necessary. There is no significant difference in precision between the proposed procedure and the normal precision of the determinative methods. Results on typical pharmaceutical preparations, some of which show evidence of extensive decomposition, are given.

Keyphrases □ Corticosteriod dosage forms—analysis □ Acetonitrile–diatomaceous earth column—corticosteroid separation □ Column chromatography—separation □ Blue tetrazolium, phenylhydrazine, isonicotinic acid hydrazide methods—corticosteroid analysis □ UV spectrophotometry-analysis

The determination of corticosteroids in many pharmaceutical preparations, other than those official in USP (1) and NF (2), can be difficult due to the interference of certain ingredients and because official methods do not always detect the presence of decomposition products of corticosteroids in the preparation. As a consequence, many "cleanup" procedures have been proposed to remove interferences and/or decomposition products before the actual final determinative step in the analysis of corticosteroids.

Levine (3) has reviewed the column partition chromatography of steroids and listed solka floc (4), silica (5, 6), siliconized diatomaceous earth (7), and diatomaceous earth (8) as the solid supports used. Jakovljevic *et al.* (9) used magnesium–silica gel[1] column chromatography with various solvent systems to remove the more polar interferences. Bracey *et al.* (10) utilized a column of methanol and water on acid-washed diatomaceous earth to remove interfering antibiotics from corticosteroid preparations.

This paper reports a new column partitioning chromatographic procedure which effectively traps the corticosteroid in an acetonitrile layer on a diatomaceous earth column while interferences are removed by washing with *n*-heptane. The acetonitrile and corticosteroid are then removed from the column with chloroform. The method may be modified readily for the removal of acidic, basic, and/or other water-soluble interferences.

EXPERIMENTAL

Apparatus—The following were used: Cary model 15 recording spectrophotometer; glass chromatographic columns, 2.2 × 25 cm. constricted at one end to 0.4 × 5 cm.; and aluminum tamping tool to fit chromatographic column.

Reagents—*Solvents*—All solvents were spectro, certified, analytical reagent, USP, or distilled-in-glass grade.

Acetonitrile, *n*-heptane, chloroform, methanol, and alcohol USP were used.

Acetonitrile–*n*-heptane (mutually saturated): mix 30 ml. of acetonitrile and 400 ml. of *n*-heptane in a separator. Agitate vigorously for at least 2 min. and separate when both layers are completely clear. These saturated solutions are to be used whenever *n*-heptane or acetonitrile is called for in these directions.

Chloroform (water saturated): add 50 ml. of water to 400 ml. of chloroform in a separator. Agitate vigorously for 2 min. and separate only when both layers are clear.

Diatomaceous earth,[2] acid washed, was used.

Standards—Hydrocortisone, hydrocortisone acetate, prednisolone, prednisolone acetate, prednisone (all USP reference standards); dexamethasone (NF reference standard); betamethasone (Schering Corp.); and flurandrenolone (Eli Lilly) were used.

All standard solutions were prepared as 1.00 mg./100 ml. in alcohol USP or as 100 mg./100 ml. in alcohol USP.

Reagents used in the determinative steps were prepared as specified in the method reference.

Proposed Column Procedure—*Preparation of Column*—Acetonitrile layer: insert a glass wool plug in the bottom of the chromatographic column. Thoroughly mix 4.0 g. of diatomaceous earth with 4.0 ml. of acetonitrile, transfer to the column, and pack firmly.

Aqueous trap layer: when it is necessary to remove water-soluble neutral impurities, an aqueous trap layer is used above the acetonitrile layer. Mix 2.0 g. of diatomaceous earth with 2.0 ml. of water, transfer to the column above the acetonitrile layer, and tamp firmly. If this type of trap is used, the chloroform must be saturated with water.

Aqueous basic trap layer: when it is necessary to remove water-soluble acidic interferences, a basic trap layer is used in the column above the acetonitrile layer. Mix 2.0 g. of diatomaceous earth with 2.0 ml. of either 8% (w/v) $NaHCO_3$ or 10% (w/v) Na_2CO_3, transfer to the column, and pack firmly above the acetonitrile layer. When this type of trap is used, the chloroform must be water saturated.

Aqueous acidic trap layer: when it is necessary to remove water-soluble basic interferences, both an aqueous layer and an acidic layer are used above the acetonitrile layer. Mix 1.0 g. of diatomaceous earth with 1.0 ml. of water and transfer to the column above the acetonitrile layer. Mix 2.0 g. of diatomaceous earth with 2.0 ml. of 1 *N* HCl, transfer to the column above the aqueous layer, and tamp firmly. Water-saturated chloroform is required in this case.

Sample layer: dissolve the sample residue, prepared as directed under *Sample Preparation*, in 1.0 ml. of acetonitrile and 2.0 ml. of *n*-heptane. Mix the solution thoroughly with 3.0 g. of diatomaceous earth, transfer to the column above the acetonitrile or other trap layer, and pack firmly. Dry wash the sample beaker with 1 g. of diatomaceous earth and with glass wool, both of which are transferred to the top of the column. Retain the sample beaker for washing with *n*-heptane and chloroform.

Elution of Column—Wash the sample beaker successively with six 25-ml. portions of *n*-heptane, which are transferred to the column. The liquid head should be maintained between 8 and 12 cm. above the column bed until the last wash, which is allowed to drain completely from the column. Wash the tip of the column with alcohol and discard the *n*-heptane and alcohol wash. Rinse the sample beaker with five 25-ml. portions of chloroform and pour each through the column, maintaining a liquid head of 8–12 cm. above the column bed until the last portion, which is allowed to drain completely from the column. Rinse the tip of the column with chloroform and add to the eluate. Evaporate the eluate carefully to dryness under air in the hood to ensure that the acetonitrile is evaporated completely and that fumes of acetonitrile are not generated in the open laboratory since acetonitrile vapors are toxic.

[1] Florisil, Floridin Co., Pittsburgh, PA 15222

[2] Celite 545, Johns-Manville Products Corp., New York, NY 10016

Table I—Precision of Column Procedure

Sample Number	Taken, mg.	Recovery[a] of Hydrocortisone Standards, mg. BT Method	PH Method	INH Method	UV Method
1	2.01	2.01	1.98	2.00	2.04
2	2.01	2.02	1.98	2.01	2.03
3	2.01	2.01	2.01	2.01	2.03
4	2.01	2.01	2.01	2.02	2.03
5	2.01	1.99	2.00	2.03	2.03
6	2.01	2.02	2.01	2.03	2.04
Av.	2.01	2.01	2.00	2.02	2.03
SD^b (%)		0.60	0.60	0.60	0.20

[a] Compared to aliquots of the same sample determined identically but without going through the column procedure. [b] Calculated from the range by the method of Dean and Dixon (14).

Preparation of Sample Solution for Determinative Procedures—Dissolve the residue from the chloroform eluate in alcohol USP and dilute accurately to a volume that will contain approximately 1 mg. of corticosteroid/100 ml. Use proper size aliquots of this solution for determination by one or more of the determinative procedures.

Determinative Procedures—Blue tetrazolium (BT) method: the procedure given in USP XVII (1) and NF XII (2) was followed, except that 10.0-ml. aliquots and 1.0 ml. each of the BT reagent and of the tetramethylammonium hydroxide reagent were used.

Phenylhydrazine (PH) method: the procedure of Silber and Porter (11) was followed without modification.

Isonicotinic acid hydrazide (INH) method: the procedure of Umberger (12) was used, except that the INH reagent was modified by using twice the recommended concentration of hydrochloric acid to increase the sensitivity of the reaction (13).

UV spectrophotometry: The alcohol USP solution was scanned directly in a 1-cm. cell.

Sample Preparation—*Lotions, Creams, and Ointments*—The sample size should be sufficient to contain approximately 5 mg. of the corticosteroid. For preparations declared on a weight–volume basis, transfer the selected volume to a beaker, using a pipet calibrated "to contain," and wash the contents of the pipet into the beaker with warm alcohol USP. For preparations declared on a weight–weight basis, weigh an accurate sample as rapidly as possible into a beaker. In either case, add warm alcohol USP up to a total volume of 30 ml. and heat on the steam bath with periodic agitation to incipient boiling. Cool in an ice bath until the residue solidifies and then decant the liquid into a 100-ml. volumetric flask. Repeat the extraction with three 20-ml. portions of warm alcohol, decanting each into the flask after cooling. Adjust to room temperature and dilute to volume with alcohol USP. Filter if necessary. Carefully evaporate a 20.00-ml. aliquot to dryness on the steam bath and continue as directed under "Sample Layer."

Drops, Injectables, and Suspensions—Accurately measure a volume of the sample containing approximately 1 mg. of corticosteroid into a beaker. Carefully evaporate to dryness under air on a steam bath and continue as directed under "Sample Layer."

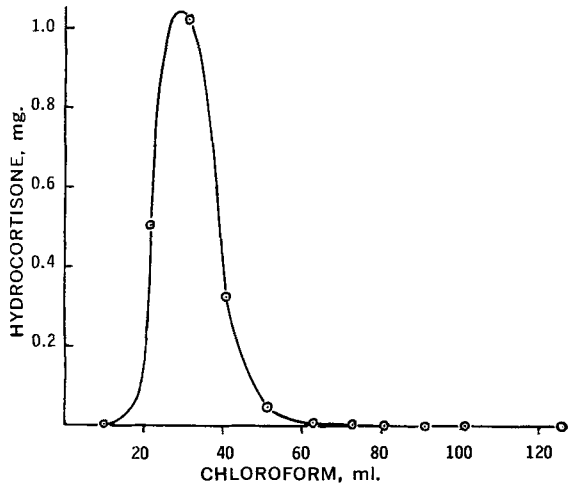


Figure 1—*Elution curve for removal of corticosteroid and acetonitrile from column with chloroform.*

Table II—Precision of Determinative Methods

Sample[a] No.	Absorbance BT Method	PH Method	INH Method	UV Method
1	0.548	0.387	0.340	0.451
2	0.551	0.394	0.338	0.451
3	0.551	0.387	0.340	0.450
4	0.551	0.391	0.340	0.450
5	0.550	0.386	0.347	0.447
6	0.551	0.386	0.339	0.451
Av.	0.550	0.388	0.341	0.450
SD^b	0.0012	0.0032	0.0036	0.0016
SD (%)	0.22	0.82	1.05	0.35

[a] Samples of hydrocortisone standard (1.00 mg.) were dissolved in chloroform, evaporated to dryness, dissolved, and diluted to volume in alcohol USP; aliquots were determined by each method. [b] Calculated from the range by method of Dean and Dixon (14).

Tablets—Weigh 20 tablets to obtain the average tablet weight. Grind the 20 tablets to pass a 60-mesh screen, mix thoroughly, and accurately weigh a sample containing approximately 1 mg. of corticosteroid into a beaker. Cover the sample with 1.0 ml. of methanol, swirl periodically during a 10-min. period, and add 1.0 ml. of water. Mix the solution thoroughly with 3.0 g. of diatomaceous earth; continue as directed under "Sample Layer" beginning with "...transfer to the column above the acetonitrile...." Use water-saturated chloroform in the elution step.

Buffered tablets that contain alumina or magnesium carbonate will not disperse properly under the conditions listed and must be dissolved by the following procedure. Prepare the column with the acetonitrile layer and an aqueous trap layer as directed previously. Add 1.0 ml. of dilute HCl to the accurately weighed sample and allow to stand for 5–10 min. to ensure complete solution of the oxide and/or carbonate. Add 2.0 ml. of water and 3.0 g. of diatomaceous earth and mix thoroughly. Transfer to the column; continue as directed under the "Sample Layer" beginning with "Dry wash the beaker...." Use chloroform saturated with water in the elution step.

Capsules—Weigh the net contents of 20 capsules to determine the average capsule content. Mix thoroughly and accurately weigh a sample containing approximately 1 mg. of the corticosteroid into a beaker. Proceed as directed under *Tablets* beginning with "Cover the sample with 1.0 ml. of methanol...."

METHOD DEVELOPMENT

Elution Curve—The column was prepared as directed under *Column Preparation*, including a sample of approximately 2 mg. of reference standard hydrocortisone. To ensure constant flow conditions, a 12-cm. liquid head was maintained in the column during elution by use of a separator. The 150-ml. *n*-heptane wash was evaporated to dryness, dissolved in alcohol USP, and scanned by UV. The spectrum showed no evidence of the presence of hydrocortisone. The chloroform eluate was caught in ten 10-ml. fractions and two 25-ml. fractions. Each fraction was taken to dryness,

Table III—Capacity of Column

Hydrocortisone Taken, mg.	Hydrocortisone Recovered, mg.	Recovery, %
1.00	1.02	102
2.00	2.02	101
4.00	4.00	100
6.00	5.92	99
8.00	7.89	99
10.0	9.84	98
15.0	15.1	101
20.0	19.8	99
	Av.	99.9%
	SD^a	1.4%

[a] Calculated from the range by method of Dean and Dixon (14).

Table IV—Applicability of Column Procedure to Various Corticosteroids

Corticosteroid	Taken, mg.	Recovered, mg.	Recovered, %
Betamethasone	2.120	2.155	101.7
Cortisone acetate	2.036	2.054	100.9
Deoxycorticosterone acetate	2.015	2.026	100.5
Dexamethasone	2.080	2.104	101.2
Flurandrenolone	2.003	1.994	99.6
Hydrocortisone acetate	2.000	1.952	97.6
Prednisolone	2.000	2.000	100.0
Prednisolone acetate	2.180	2.176	99.8
Prednisone	2.080	2.105	101.5
		Av.	100.3
		SD[a]	1.39%

[a] Calculated from the range by the method of Dean and Dixon (14).

Table V—Removal of Interferences in Products by Proposed Procedures

Method of Analysis	Declared Value, %[a] BT	PH	INH	UV
Hydrocortisone Cream, 0.125%[b]				
Direct	101.4[c]	95.0	113.8[c]	ND[d]
Recommended procedure	99.2[c]	94.6	96.9	ND[d]
Buffered Prednisolone Tablets, 5 mg.				
Direct	65.8[c]	44.8	84.6	ND[d]
Recommended procedure	50.5[c]	45.3	64.8	65.1

[a] Average of duplicates. [b] Interferences present according to manufacturer's declaration were lanolin, stearic acid, and parabens. [c] Variation of absorbance with time indicates unidentified interference is present. [d] ND—not determined by this method.

dissolved in alcohol USP, and determined by UV spectrophotometry. The elution curve, shown in Fig. 1, indicates that the corticosteroid was completely eluted in the first 100 ml. of eluate. Since over 99.5% was eluted in the first 80 ml. of eluate, the use of 125 ml. of chloroform in the recommended procedure includes a definite safety factor.

Replication Studies—Six 2.00-ml. aliquots of a standard hydrocortisone solution containing 1.00 mg./ml. were determined by the suggested column procedure. Aliquots of the final alcohol solution were analyzed by all four determinative steps and compared to values obtained from aliquots of the same standard solution determined directly without being put through the column. The values are shown in Table I. The standard deviation, as calculated from the range by the method of Dean and Dixon (14), varies from 0.20% for the UV method to 0.60% for the PH method, with an average of 0.50% for all four determinative procedures. This is approximately the same as the average standard deviation for six replicate 1.00-mg. samples of standard hydrocortisone which were dissolved in 100 ml. of chloroform, evaporated carefully to dryness, made to volume with alcohol USP, and determined by all four determinative procedures. The results, shown in Table II, have an average standard deviation of 0.61%.

Column Capacity Study—Samples of from 1.00 to 20.0 mg. of hydrocortisone were placed on and eluted from the column by the suggested procedure. The eluates were evaporated to dryness, dissolved in alcohol, and analyzed by UV spectrophotometry. The results were compared to identical samples which were not placed on and eluted from the column and are reported as percent recovery in Table III. Samples containing at least 20 mg. of corticosteroid can be determined safely by the suggested procedure.

Corticosteroid Applicability Studies—Since all of the method-development work utilized hydrocortisone, other corticosteroid standards were analyzed by the proposed column procedure utilizing the INH procedure and compared to identical standards which were not put on and eluted from the column. The results are shown in Table IV. The recoveries ranged from 97.6 to 101.7%, with an average recovery of 100.3% and a standard deviation calculated from the range by the method of Dean and Dixon (14) of 1.39%.

Interference Studies—The efficiency of the suggested procedure for the complete removal or reduction of interferences in the various determinative methods was investigated in sample preparations and by the addition of the interfering substances to hydrocortisone standards. In each case, the measurement was made on aliquots of the alcohol extract of the sample or of the extract of the standard plus the interfering substance. One aliquot was measured directly and the second was put through the column separation step before measurement. In the investigation involving the standard plus added interference, a standard without the interfering substance was also determined directly.

The results for two typical samples are shown in Table V. The values for the hydrocortisone cream show interference in bot the BT and INH procedures, but none in the PH method when run directly. The interference in both BT and INH measurements was detected using the variation of absorbance with time method (15). The column separation procedure reduces the amount of interference in the BT method and completely removes the substances that interfere in the INH measurement.

Results for the buffered prednisolone tablets show the versatility of the suggested procedure in the detection and estimation of decomposition products in corticosteroid preparations. When run directly, interference was indicated only in the BT method, and this interference was not completely removed by the column procedure. Since the INH procedure depends upon conjugation in Ring A of the corticosteroid, and the BT and PH reagents react with the side chain at C_{17} (15), the difference between the values by INH and PH when run directly indicates that the C_{17} side chain in approx-

Table VI—Removal of Certain Interferences by Recommended Procedure

Interfering Substance	Interfering Substance, mg./mg. Hydrocortisone	Absorbance Method of Measurement: Blue Tetrazolium — Standard Only, Direct	Blue Tetrazolium — Standard + Interference, Direct	Blue Tetrazolium — Standard + Interference, Procedure	Phenylhydrazine — Standard Only, Direct	Phenylhydrazine — Standard + Interference, Direct	Phenylhydrazine — Standard + Interference, Procedure	Isonicotinic Acid Hydrazide — Standard Only, Direct	Isonicotinic Acid Hydrazide — Standard + Interference, Direct	Isonicotinic Acid Hydrazide — Standard + Interference, Procedure
Lanolin	600/1	0.580	0.839	0.600	0.540	T[a]	0.538	0.334	0.764	0.348
Polysorbate 60	25/1	0.580	0.611	0.573	0.540	T[a]	0.521	0.334	0.332	0.338
Sodium lauryl sulfate	20/1	0.580	0.574	0.573	0.540	T[a]	0.537	0.334	0.332	0.328
Sorbitan monooleate	250/1	0.580	0.742	0.637	0.540	T[a]	0.552	0.334	0.468	0.362
Sorbitan monostearate	250/1	0.580	0.674	0.607	0.540	T[a]	0.539	0.334	0.366	0.335
Sulfide[b]	0.133/1	0.580	0.703	0.580	0.540	0.540	0.540	0.334	0.336	0.333
Sulfur	0.05/1	0.580	0.732	0.580	0.540	0.540	0.538	0.334	0.334	0.332

[a] T means solution becomes turbid so that absorbance cannot be determined. [b] Acetonitrile column modified with aqueous trap layer.

Table VII—Typical Samples Analyzed by the Proposed Procedure

Product	Corticosteroid	Interfering Substances Present[b]	Declared Value, %[a] Method of Analysis BT	PH	INH
A. Samples Showing Little or No Decomposition					
Cream 0.25%	Hydrocortisone	a, c	103.6[c]	105.6[c]	101.8[c]
Lotion 0.125%	Hydrocortisone	c, e	100.0[c]	99.2[c]	99.7[c]
Lotion 0.25%	Hydrocortisone	c	110.8	108.4	109.6
Lotion 0.50%	Hydrocortisone	a, c, g	99.2[d]	94.4	96.9
Lotion 1%	Hydrocortisone	a	107.2[c]	107.5[c]	107.4[c]
Drops 25 mg./ml.	Hydrocortisone acetate	—	78.4	80.6	79.0
Drops 0.2%	Prednisolone	—	89.0	ND[e]	89.5
Suspension 0.1%	Dexamethasone	—	106.3	106.8	107.0
Suspension 0.25%	Prednisolone acetate	c	111.8[c,d]	99.8[c]	102.1[c]
Tablets 0.75 mg	Prednisone	d	90.1[c,f]	ND[e]	88.0[c,f]
B. Samples Showing Decomposition					
Cream 0.125%	Hydrocortisone	a, c, g	48.4[d]	30.2	71.1
Cream 0.125%	Hydrocortisone	a, c, g	82.2[d]	45.0	60.0
Cream 0.50%	Hydrocortisone	a, c, g	111.7[d]	102.7	113.2
Lotion 0.125%	Hydrocortisone	a, c, g	24.3[d]	2.5	43.8
Lotion 0.25%	Hydrocortisone	c, e, f	97.6[c,d]	90.6[c]	100.3[c]
Lotion 0.50%	Hydrocortisone	a, c, g	95.3[d]	90.7	98.7
Ointment 0.50%	Hydrocortisone acetate	h	104.2[d]	93.4	96.9
Suspension 0.1%	Dexamethasone	—	98.5[c]	ND[e]	103.7[c]

[a] Average of duplicates. [b] Interfering substances present according to manufacturer's declared content: a, lanolin; b, magnesium stearate; c, parabens; d, salicylamide; e, sodium lauryl sulfate; f, sorbitan monostearate; g, stearic acid; and h, selenium sulfide. [c] Single determination. [d] Variation of absorbance with time indicates presence of unidentified interference. [e] ND, not determined by this method. [f] Salicylamide was removed by extraction of $CHCl_3$ eluate with 0.25 *N* NaOH before evaporation to dryness.

imately 40% of the declared amount of the corticosteroid had been oxidized to neutral or acidic products. The column procedure for buffered tablets of this type requires an aqueous trap layer to remove HCl from the chloroform, and this aqueous layer also removes any acidic decomposition products. The difference between the INH values before and after being put through the column indicates that approximately half of the decomposition products were acidic. The difference between the INH and PH results after being put through the column indicates that the other half of the decomposition products were neutral. These results also indicate that only 45% of the declared amount was actually present in the sample at the time of the analysis.

The results of the investigation using standards with added interfering substances are summarized in Table VI. The substances added have been reported to interfere in at least one of the determinative methods (15). The proposed procedure either eliminates the interference completely or greatly reduces it at the level shown. The first column of figures under each method is the absorbance for the standard alone run directly, the second column is the standard plus interference run directly, and the third column is the absorbance of standard plus interference after being separated by the proposed procedure. The differences in the values in the three columns indicate the extent of interference and the efficiency of removal.

RESULTS AND DISCUSSION

The results on typical undecomposed and partially decomposed corticosteroid preparations are summarized in Table VII. In each case in which there is significant disagreement between the BT and PH values, a study of the variation of absorbance with time indicates that some unidentified interfering substance is present. It is also apparent that such discrepancies are found more often in samples that have undergone decomposition, as indicated by significant differences between values obtained by the INH procedure and values obtained by the PH or BT method.

Diethyl ether was used instead of chloroform during part of this investigation for the removal of the corticosteroid from the column. It was found, however, that some bottles of ether contain ether peroxides, which cause decomposition of the corticosteroid during the evaporation step and also interfere with all four determinative methods. When ether is used, the column must be kept completely filled during the elution step to ensure that the corticosteroid is completely eluted from the column. Since chloroform has none of these disadvantages, it is the solvent of choice for the elution step. Isooctane (2,2,4-trimethylpentane) was substituted for the *n*-heptane in several analyses but did not improve the results obtained.

The few minor limitations of the proposed procedure include the care that must be used in the evaporation of acetonitrile, because it is toxic and because any acetonitrile left unevaporated will cause gross interference in the BT procedure. Also, if the liquid head in the column is maintained above 12 cm. during the *n*-heptane wash, some acetonitrile will be stripped from the column. This could lead to some of the corticosteroid also being removed during the wash step.

Some substances, which interfere in one or more of the determinative steps, are sufficiently soluble in both water and chloroform to be removed from an aqueous trap layer in the elution step. If this occurs, the eluate is evaporated, redissolved in acetonitrile–*n*-heptane, and transferred to a new column without an aqueous trap layer. One example of an interfering substance of this type is a decomposition product of corticosteroids thought to be a glyoxal.

Preliminary work with the column indicates that it can be used to improve the analysis of other types of steroids and may be used to separate with ease certain important steroids occurring in pharmaceutical preparations. Investigative work is continuing on the use of the column for such separations.

REFERENCES

(1) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

(2) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965.

(3) J. Levine, *J. Pharm. Sci.*, **52,** 1015(1963).

(4) P. B. Baker, F. Dobson, and S. W. Stroud, *Nature*, **168,** 114(1951).

(5) W. J. Haines, "Recent Progress in Hormone Research," vol. 7, Academic, New York, N. Y., 1952, p. 255.

(6) E. Heftmann and D. F. Johnson, *Anal. Chem.*, **26,** 519 (1954).

(7) C. J. O. R. Morris and D. C. Williams, *Biochem. J.*, **54,** 470 (1953).

(8) D. Banes, *J. Amer. Pharm. Ass., Sci. Ed.*, **42,** 669(1953).

(9) I. M. Jakovljevic, P. E. Hartsaw, and G. E. Drummond, *J. Pharm. Sci.*, **54**, 1771(1965).
(10) A. Bracey, L. Garrett, and P. J. Weiss, *ibid.*, **55**, 1113(1966).
(11) R. H. Silber and C. C. Porter, *J. Biol. Chem.*, **210**, 923(1954).
(12) E. J. Umberger, *Anal. Chem.*, **27**, 768(1955).
(13) F. Kunze, private communication, Food and Drug Administration, U.S. Dept. of H.E.W., 1965.
(14) R. B. Dean and W. J. Dixon, *Anal. Chem.*, **23**, 636(1951).
(15) R. E. Graham, P. A. Williams, and C. T. Kenner, *J. Pharm. Sci.*, **59**, 1152(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 13, 1970, from the *Dallas District, Food and Drug Administration, Dallas, TX 75204, and †Southern Methodist University, Dallas, TX 75222

Accepted for publication April 17, 1970.

Quantitative Determination of Butaperazine by TLC

A. J. KAPADIA, M. A. BARBER, and A. E. MARTIN

Abstract □ A method for the separation and determination of butaperazine in the presence of its degradation products is described. A sample is streaked onto a thin layer of silica gel G under a stream of nitrogen. The chromatogram is developed with isopropyl alcohol–ammonia (1 *N*) (4:1). The separated butaperazine is removed from the silica gel by elution with methanol and is determined quantitatively by UV spectroscopy. Details of the elution technique are described. Using the proposed method, quantitative recoveries are obtained from tablets and syrups.

Keyphrases □ Butaperazine in dosage forms—analysis □ TLC—separation □ UV spectrophotometry—analysis

The use of psychotropic drugs for the treatment of patients with emotional or mental disorders has led to widespread use of phenothiazine derivatives. One such derivative is butaperazine,[1] 2-(*n*-butyryl)-10-[3-(4-methyl-1-piperazinyl)-propyl]-phenothiazine.

The literature provides ample indication that several types of decomposition take place in these compounds. One type involves oxidation at the sulfur atom, leading to sulfoxide and eventually to sulfone (1). The quinonoid-type oxidation products of phenothiazine have been described (2–4). Huang and Sands (5, 6) studied the effect of UV irradiation on chlorpromazine solution under aerobic and anaerobic conditions. They found that under the former condition, oxidation prevails and the sulfoxide and *N*-oxide are formed; however, under the latter condition, the polymerization processes predominate.

The degree of deterioration and the type and amount of decomposition product pose difficult problems for the analyst. Consequently, for the purpose of establishing stability, it is necessary to devise a relatively simple but versatile separation, one that would be applicable to the quantitative determination of the phenothiazine derivative in experimental formulations.

Recently, Blazek (7) reviewed the procedures available for quantitative determination of phenothiazine derivatives. Included among the methods for these compounds are colorimetric (8), titrimetric (9), UV absorption spectrophotometric, and chromatographic procedures. One might choose any one of these except for the following considerations. The first two procedures are not selective for the undegraded compound. The UV method (10, 11) is an accurate and convenient means of assaying formulations containing phenothiazine derivatives, but it is unsuitable in badly degraded formulations because of the presence of other UV absorbing species. A paper chromatography technique (12, 13) was not selected because of degradation and tailing occurring during analysis (14, 15). Gas chromatographic procedures have been used for phenothiazines, but the present authors observed that butaperazine, because of its high boiling point and low thermal stability, was too low in volatility to be eluted quantitatively without extensive thermal decomposition from the several columns that were tried. Thus, they eliminated GLC from further consideration.

Since its introduction by Stahl (16), TLC has assumed a position of analytical importance for both the separation and analysis of complex inorganic, organic, and biological mixtures. Several papers have been published which describe quantitative thin-layer techniques (16–19). These methods can be classified as direct or indirect.

In the direct method, the developed chromatogram is quantitatively evaluated by measuring spot size or area or by densitometry. Thus, measurement is accomplished without removing the sought-for substance from the support. An indirect method implies removal of the separated substance from the plate. This may be followed by elution of the sample from the adsorbent and analysis of the eluant, usually by spectrophotometry or colorimetry. Indirect methods have the advantage in that spectra of the samples are readily obtained as part of the analysis. These may provide important additional information concerning identity and purity (20). Spencer and Beggs (21) have pointed out certain precautions which must be taken if an indirect method is to give precise, accurate results.

The authors report an application of the indirect method, utilizing TLC for the physical separation of butaperazine from its degradation products and, subsequently, quantitative determination of butaperazine using UV spectroscopy. Possible sources of error in the method are examined. The initial steps in the oxidative decomposition of butaperazine are shown.

[1] Marketed as Repoise by A. H. Robins Co., Richmond, VA 23220

(9) I. M. Jakovljevic, P. E. Hartsaw, and G. E. Drummond, *J. Pharm. Sci.*, **54**, 1771(1965).
(10) A. Bracey, L. Garrett, and P. J. Weiss, *ibid.*, **55**, 1113(1966).
(11) R. H. Silber and C. C. Porter, *J. Biol. Chem.*, **210**, 923(1954).
(12) E. J. Umberger, *Anal. Chem.*, **27**, 768(1955).
(13) F. Kunze, private communication, Food and Drug Administration, U.S. Dept. of H.E.W., 1965.
(14) R. B. Dean and W. J. Dixon, *Anal. Chem.*, **23**, 636(1951).
(15) R. E. Graham, P. A. Williams, and C. T. Kenner, *J. Pharm. Sci.*, **59**, 1152(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 13, 1970, from the *Dallas District, Food and Drug Administration, Dallas, TX 75204, and †Southern Methodist University, Dallas, TX 75222

Accepted for publication April 17, 1970.

Quantitative Determination of Butaperazine by TLC

A. J. KAPADIA, M. A. BARBER, and A. E. MARTIN

Abstract □ A method for the separation and determination of butaperazine in the presence of its degradation products is described. A sample is streaked onto a thin layer of silica gel G under a stream of nitrogen. The chromatogram is developed with isopropyl alcohol–ammonia (1 *N*) (4:1). The separated butaperazine is removed from the silica gel by elution with methanol and is determined quantitatively by UV spectroscopy. Details of the elution technique are described. Using the proposed method, quantitative recoveries are obtained from tablets and syrups.

Keyphrases □ Butaperazine in dosage forms—analysis □ TLC—separation □ UV spectrophotometry—analysis

The use of psychotropic drugs for the treatment of patients with emotional or mental disorders has led to widespread use of phenothiazine derivatives. One such derivative is butaperazine,[1] 2-(*n*-butyryl)-10-[3-(4-methyl-1-piperazinyl)-propyl]-phenothiazine.

The literature provides ample indication that several types of decomposition take place in these compounds. One type involves oxidation at the sulfur atom, leading to sulfoxide and eventually to sulfone (1). The quinonoid-type oxidation products of phenothiazine have been described (2–4). Huang and Sands (5, 6) studied the effect of UV irradiation on chlorpromazine solution under aerobic and anaerobic conditions. They found that under the former condition, oxidation prevails and the sulfoxide and *N*-oxide are formed; however, under the latter condition, the polymerization processes predominate.

The degree of deterioration and the type and amount of decomposition product pose difficult problems for the analyst. Consequently, for the purpose of establishing stability, it is necessary to devise a relatively simple but versatile separation, one that would be applicable to the quantitative determination of the phenothiazine derivative in experimental formulations.

Recently, Blazek (7) reviewed the procedures available for quantitative determination of phenothiazine derivatives. Included among the methods for these compounds are colorimetric (8), titrimetric (9), UV absorption spectrophotometric, and chromatographic procedures. One might choose any one of these except for the following considerations. The first two procedures are not selective for the undegraded compound. The UV method (10, 11) is an accurate and convenient means of assaying formulations containing phenothiazine derivatives, but it is unsuitable in badly degraded formulations because of the presence of other UV absorbing species. A paper chromatography technique (12, 13) was not selected because of degradation and tailing occurring during analysis (14, 15). Gas chromatographic procedures have been used for phenothiazines, but the present authors observed that butaperazine, because of its high boiling point and low thermal stability, was too low in volatility to be eluted quantitatively without extensive thermal decomposition from the several columns that were tried. Thus, they eliminated GLC from further consideration.

Since its introduction by Stahl (16), TLC has assumed a position of analytical importance for both the separation and analysis of complex inorganic, organic, and biological mixtures. Several papers have been published which describe quantitative thin-layer techniques (16–19). These methods can be classified as direct or indirect.

In the direct method, the developed chromatogram is quantitatively evaluated by measuring spot size or area or by densitometry. Thus, measurement is accomplished without removing the sought-for substance from the support. An indirect method implies removal of the separated substance from the plate. This may be followed by elution of the sample from the adsorbent and analysis of the eluant, usually by spectrophotometry or colorimetry. Indirect methods have the advantage in that spectra of the samples are readily obtained as part of the analysis. These may provide important additional information concerning identity and purity (20). Spencer and Beggs (21) have pointed out certain precautions which must be taken if an indirect method is to give precise, accurate results.

The authors report an application of the indirect method, utilizing TLC for the physical separation of butaperazine from its degradation products and, subsequently, quantitative determination of butaperazine using UV spectroscopy. Possible sources of error in the method are examined. The initial steps in the oxidative decomposition of butaperazine are shown.

[1] Marketed as Repoise by A. H. Robins Co., Richmond, VA 23220

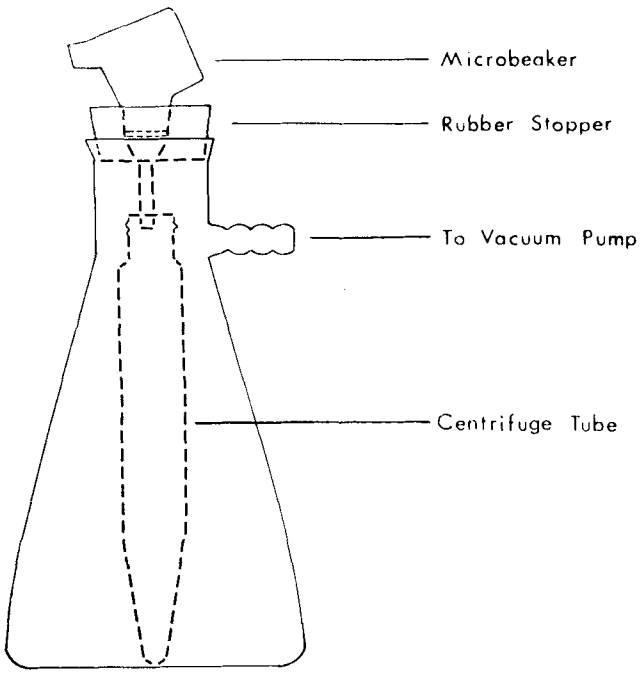


Figure 1—*Elution assembly for quantitative recovery of sample.*

EXPERIMENTAL

Apparatus—The following were used: Hamilton syringe, fixed needle, blunt tip, 100 and 250 μl.; microbeaker, Pyrex, filter type with fine-porosity fritted disk; glass plates (20 × 20 cm.); and Shandon Chromatank (Cat. No. SAB-2843) lined with solvent-saturated filter paper.

Chemicals—All chemicals and reagents used were analytical reagent grade. The chemical purity of standard butaperazine was checked by melting point and TLC. Solubility analysis gave further indication of the purity of the standard.

Phosphate Buffer Solution, pH 5.3—Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), 38.0 g., and 2.0 g. disodium hydrogen phosphate (Na_2HPO_4) were dissolved and diluted to 1 l. with water.

Preparation of Thin-Layer Plates—A slurry of silica gel G was prepared from 30 g. of Merck silica gel G, grain size 10–40 μ, and 60 ml. water. This was applied to glass plates with an appropriate spreader set at 250 μ. The plates were air dried for 15 min. and then activated at 105° and stored over anhydrous silica gel. Subsequent work has shown that the air-dried plates without activation yield the same type of separation.

Developing Solvent—The developing system consisted of 4 parts isopropyl alcohol and 1 part 1 *N* ammonia. The system was prepared just prior to use and used only once.

Preparation of the Standard—The standard solution of butaperazine phosphate or maleate was prepared in phosphate buffer solution (pH 5.3) such that it contained a concentration of the salt equivalent to 1.91 mg./ml. of the base. The solution was thoroughly mixed, and 10-ml. aliquots were transferred to a separator. The aqueous solution was extracted, using successively one 10-ml. and three 5-ml. portions of chloroform. The standard solution was prepared just prior to use.

Application of Drug and Development of Chromatogram—Using a precision syringe, a predetermined amount of chloroform extract was applied to a plate. The organic extract was transferred as a narrow streak 4 cm. long. An appropriate streak was achieved by applying successive 10-μl. portions of the solution, evaporating each portion with a slow stream of nitrogen. This application was repeated until a total of about 0.1 mg. of butaperazine base was applied on the same streak. One hundred microliters of the standard solution previously described was applied as a single streak.

Another streak using the sample solution was applied in the same manner on the other side of the plate. The exact quantity applied depended, of course, on the expected sample concentration.

The prepared plates (two or more) were placed into separate chromatographic chambers. Each chamber was equilibrated for 2 hr.

Table I—Precision of the Whole Procedure

Amount Butaperazine Taken, mcg.	Absorbance at 278 mμ after TLC (Uncorrected)	Absorptivity
76.58	0.392	51.2
76.58	0.398	52.0
77.09	0.396	51.4
96.37	0.484	50.2
96.37	0.489	50.7
96.37	0.495	51.4
96.37	0.498	51.7
76.58	0.391	51.1
76.58	0.393	51.3
76.58	0.404	52.8
$\bar{x}$		51.3
SD		0.56

and contained 150 ml. developing solvent. The chromatogram was allowed to develop in the dark and terminated when the solvent front ascended to a height of 15–16 cm. The developed plate was dried in a vacuum desiccator and was subsequently examined briefly under an UV lamp (254 mμ) to locate the separated butaperazine. The butaperazine appeared as a bright-orange to pink fluorescent streak at R_f approximately 0.5. This area was carefully marked, taking care not to overlap any other fluorescent areas.

Quantitative Determination of Butaperazine—Quantitative determination was accomplished by carefully removing the portion of silica gel adsorbent containing the butaperazine. The powder was removed by scraping onto nonabsorbent paper and was then transferred to a microbeaker with the aid of a small funnel. Methanol (3 ml.) was added and, after mixing gently, the mixture was allowed to stand for 15 min. The methanolic extract was collected by filtration, using the filtration assembly shown in Fig. 1. Elution of butaperazine was continued, using two additional 3-ml. portions of methanol. The combined extracts were finally diluted to 10 ml. with

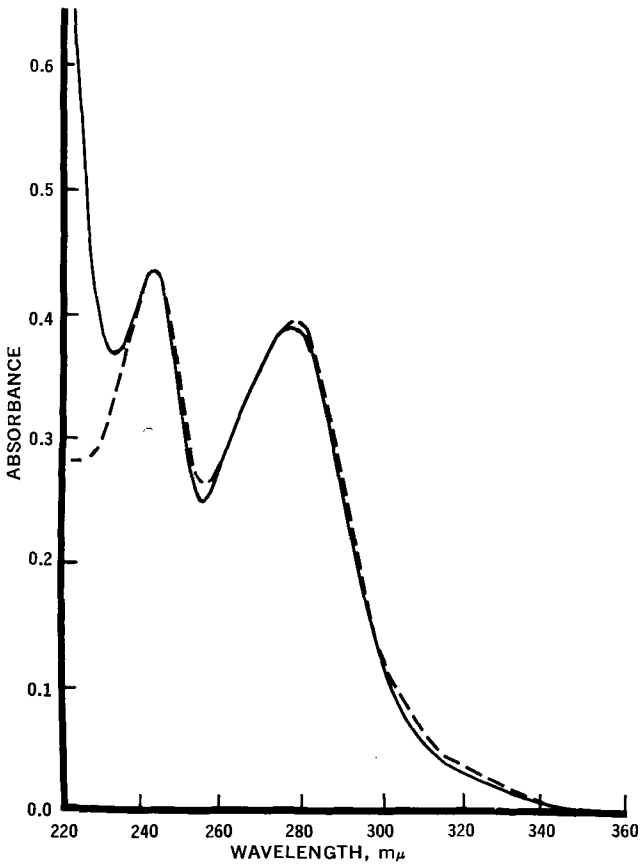


Figure 2—*UV absorption spectra of butaperazine in methanol. Key: ——, before TLC; and - - -, after TLC.*

Table II—Total Recovery: Butaperazine following TLC

Absorbance before TLC, 278 mμ	Absorbance after TLC,[a] 278 mμ	Recovery, %
0.396	0.384	97.0
0.394	0.390	99.0
0.391	0.388	99.2
0.495	0.476	96.2
0.495	0.481	97.2
0.495	0.487	98.4
0.497	0.490	98.6
0.391	0.383	98.0
0.395	0.385	97.5
0.389	0.395	101.8
$\bar{x}$		98.3

[a] Corrected for analytical blank.

methanol and mixed. The UV spectrum of the methanolic solution was then recorded in 1.0-cm. cells, using methanol as the reference.

Assay of Experimental Preparations—Each formulation was treated individually because of differences in the concentration of butaperazine. The general procedure for film-coated tablets involved reducing several weighed tablets in a mortar to a fine powder and passing the grind through a No. 50 sieve. A weighed portion of the sieved grind was then transferred to a separator using phosphate buffer solution. After mixing thoroughly, the mixture was extracted completely with chloroform. Each portion of the chloroform extract was transferred through a pledget of chloroform-washed cotton into a flask. During the transfer, the withdrawal of any insoluble matter was avoided. The chloroform extract was either concentrated or diluted to volume, depending upon the concentration of butaperazine. It was then chromatogrammed and assayed. Usually the standard and sample were run concurrently; therefore, absorbance values of the butaperazine eluates were not corrected for adsorbent blank.

RESULTS AND DISCUSSION

To compare UV spectra, a standard butaperazine maleate solution was prepared and extracted with chloroform as described. One aliquot was diluted directly with methanol. An equal aliquot was chromatographed, the butaperazine was eluted, and the solution was finally diluted to volume with methanol. As seen in Fig. 2, absorbance spectra before and after TLC were essentially identical above 240 mμ. Below this wavelength, the spectrum of an unchromatographed sample exhibits a higher absorbance. This observed spectral difference reflects the presence of chloroform in the unchromatographed sample. Although 242 mμ could not be utilized as an analytical wavelength because of interferences, use of the absorbance maximum at 278 mμ provided a direct measure of intact butaperazine. At this wavelength, there is little interference in the analytical blank or from formulation excipients. The position of maximum absorbance and absorbancy coefficient did not change significantly above 240 mμ during chromatography. This observation indicates that the responsible chromophore is not modified during chromatography.

Two-dimensional TLC, using the same solvent system for development in both directions, confirmed the stability of butaperazine during chromatography. No evidence of decomposition or alteration of the butaperazine spot was noted.

The spectral absorbance of the silica gel was obtained by collecting portions of the surface layer in a region near the butaperazine spot and processing them in exactly the same manner as the sample. Absorbance of the filtrate was found to be 0.008 at 278 mμ, using a 1.0-cm. pathlength. This value is on the order of a few percent of the absorbance for the chromatographed samples.

Treatment of the eluant to remove most of the finely divided, insoluble particles of the adsorbent is an important step in improving the precision of the method. The 0.008 figure cited, which was repeatedly obtained, agreed closely with the published value of Spencer and Beggs (21).

A standard curve was prepared from aqueous solutions of butaperazine carried through the procedure described. At 278 mμ, a linear relationship exists between the absorbance and the amount of butaperazine in the range 45–123 mcg.

The precision of the method was established by measuring the absorbance at 278 mμ and calculating the absorptivity for known samples. Table I summarizes the precision data obtained for the whole procedure (including sample preparation, extraction, TLC, and elution). The experimentally determined mean absorptivity of a set of 10 assay runs on known samples was 51.3. The standard deviation (single measurement) was 0.56.

Experiments were conducted to determine the total recovery of butaperazine after TLC and elution (Table II). The average recovery (10 trials) was 98.3% (range 96–102%).

These high recoveries may be attributed partly to the method of elution chosen. Thus, butaperazine on the silica gel was exposed repeatedly to fresh solvent. Minimizing the volume of eluting solvent and filtering concurrently decreased losses from filtration and transfer.

To assess application of the method to degraded samples, mixtures of butaperazine, butaperazine sulfoxide, and sulfone were prepared so as to simulate various degrees of oxidation. These mixtures were assayed for intact butaperazine (Table III). As expected, recoveries were uniformly high, averaging better than 97%.

The assay method was further tested for its applicability in the presence of oxidative degradation products of butaperazine. Buffered aqueous solutions of butaperazine maleate were treated with 0.3 ml. and 0.6 ml. hydrogen peroxide (30%) for 30, 60, and 120 min. After each interval, the solutions were extracted with chloroform. The amount of butaperazine remaining was determined, using the procedures described (Table IV). In summary, hydrogen peroxide treatment causes a loss of butaperazine, which increases with time of exposure. Table III reveals that, as the amount of peroxide is increased, decomposition of butaperazine increases. Examination of TLC plates confirmed that the butaperazine spot became smaller and fainter with time and/or increasing peroxide concentration. The other change noted was in the sulfoxide spot, which increased in size and intensity with time. Thus, the sulfoxide appeared to be the major product for this oxidative degradation.

Table III—Recovery of Butaperazine in the Presence of Its Oxidation Products

mg. Taken			Intact Butaperazine	
Butaperazine	Butaperazine Sulfoxide	Butaperazine Sulfone	mg. Found	Recovery, %
19.15	1.7	1.7	18.58	97.0
19.87	3.4	3.4	19.35	97.4
19.87	5.1	5.1	19.35	97.4

Table IV—Degradation of Butaperazine with 30% Hydrogen Peroxide

Interval after Addition of H_2O_2, min.	Absorbance at 278 mμ —30% H_2O_2 Added, ml.— 0.3	0.6
0	0.496	0.475
30	0.459	0.432
60	0.441	0.395
120	0.389	0.290

Table V—Assay of Butaperazine in Experimental Formulations: Application of TLC Method

Formulation	Storage Conditions	Labeled Amount of Butaperazine	Label Claim Found, %
Tablets	12 months at RT	51.3 mg./tablet	97.2
	12 months at 37°		99.4
	12 months at 50°		97.8
Syrup	Initial	5 mg./ml.	98.2
	12 months at RT		98.3
Injectables	3 months at RT	5 mg./ml.	96.8
	3 months at 37°		98.5
	3 months at 50°		99.0

Unbuffered aqueous solutions of butaperazine phosphate were placed in a commercial light-stability testing unit. After 7 and 15 days of exposure, the solutions were assayed. As expected, the butaperazine content had decreased by 16 and 22%, respectively.

Aqueous solutions of butaperazine buffered at pH 5.3 were sealed in glass ampuls and autoclaved at 115° for 30, 60, and 120 min. The ampuls were cooled, opened, and assayed for butaperazine. An initial 10% loss was noted, which did not increase with time. Atmospheric oxygen initially present in the vial and test solution was suspected. When consumed, no further oxidation occurred. This suspicion was confirmed by autoclaving ampuls in which oxygen was replaced with nitrogen. As predicted, these solutions did not show a significant loss of butaperazine.

Tablets, syrups, and injectables were assayed by the method described. These results are shown in Table V. The excipients, coloring agents, diluents, and fillers employed in the various formulations did not interfere.

It is evident that the formulations tested exhibited no significant decomposition. Storage at elevated temperatures (37 and 50°) for extended periods did not change this overall picture.

Several experimental factors were found to be critical in obtaining precise, accurate results. The area of the sample streak increases during development; therefore, care must be taken to keep the initial application narrow and uniform. Evaporation of the organic solvent must be accomplished quickly with a nonoxidizing drying agent. This precaution minimizes the size of the sample streak and prevents oxidative decomposition.

Use of the solvent system for more than one separation is not permissible, because decreased separation of butaperazine and decomposition products occurs and the R_f values change unpredictably.

Investigation was begun early to elucidate the major decomposition products of butaperazine and to compare them to those of other phenothiazines.

Chloroform solutions of butaperazine, its sulfoxide, and sulfone[2] were compared by TLC to the chloroform extract of degraded aqueous samples of butaperazine. These results showed that the sulfoxide and sulfone migrate slower than butaperazine in an isopropanol–NH_3 system. In addition, all were well resolved from butaperazine. In a degraded sample, one of the bands corresponded to the position of the sulfoxide streak. This band was eluted with methanol. The UV spectrum of the eluate was identical to that of the synthetic sulfoxide. The band corresponding to the synthetic sulfone could not be isolated by the techniques employed.

When a developed TLC plate was exposed for a few days to normal atmosphere, it was noted that the fluorescence of the butaperazine band changed with time. The resulting fluorescence was similar to that of a degraded sample.

TLC of grossly degraded samples often revealed the presence of products other than those described. Oxidative decomposition apparently is not the only degradation pathway, although, undoubtedly, the predominant one. Degradation apparently proceeds as follows: [butaperazine]...[sulfoxide]...[sulfone]...[other oxides].

[2] The identity of these synthesized compounds was established by CHN analysis and by UV, IR, and NMR spectroscopy.

REFERENCES

(1) S. P. Massie, *Chem. Rev.*, **54,** 797(1954).

(2) S. Granick, L. Michaelis, and M. P. Schubert, *J. Amer. Chem. Soc.*, **62,** 1802(1940).

(3) L. Michaelis, S. Granick, and M. P. Schubert, *ibid.*, **63,** 351 (1941).

(4) L. Michaelis, M. P. Schubert, and S. Granick, *ibid.*, **62,** 204(1940).

(5) C. L. Huang and F. L. Sands, *J. Pharm. Sci.*, **56,** 259(1967).

(6) C. L. Huang and F. L. Sands, *J. Chromatogr.*, **13,** 246(1964).

(7) J. Blazek, *Pharmazie*, **22,** 129(1967).

(8) F. M. Forrest, I. S. Forrest, and A. S. Mason, *Amer. J. Psychiat.*, **118,** 300(1961).

(9) G. Dusinsky, *Pharmazie*, **13,** 478(1958).

(10) T. L. Flanagan, T. H. Lin, W. J. Novick, I. M. Ronolish, C. A. Bocker, and E. J. Van Loom, *J. Med. Pharm. Chem.*, **1,** 263(1959).

(11) A. Felmeister, Ph.D. thesis, Rutgers—The State University, New Brunswick, N. J., 1963.

(12) G. Nadian and G. Sobolewski, *J. Chromatogr.*, **2,** 544(1959).

(13) I. B. Eisdorfer and W. C. Ellenbogen, *ibid.*, **4,** 329(1960).

(14) B. J. Cudzinowicz, *J. Gas Chromatogr.*, **4,** 110(1966).

(15) J. G. Kirchner, J. M. Miller, and R. G. Rice, *J. Agr. Food Chem.*, **2,** 1031(1954).

(16) E. Stahl, *Pharmazie*, **11,** 633(1956).

(17) M. S. J. Dallas, *J. Chromatogr.*, **33,** 193(1968).

(18) E. J. Shellard and M. Z. Alan, *ibid.*, **33,** 347(1968).

(19) E. J. Shellard, "Quantitative Paper and Thin-Layer Chromatography," Academic, New York, N. Y., 1968.

(20) W. L. Stanley and S. H. Vennier, *J. Ass. Offic. Agr. Chem.*, **40,** 582(1957).

(21) R. D. Spencer and B. H. Beggs, *J. Chromatogr.*, **21,** 52 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 29, 1969, from the *Analytical Research Laboratories, A. H. Robins Co., Inc., Richmond, VA 23220*

Accepted for publication April 20, 1970.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

The authors wish to express their sincere appreciation to Farbenfabriken Bayer AG, Germany, for the generous supply of butaperazine sulfoxide and sulfone.

Separation and Determination of Anhydrotetracycline, 4-Epianhydrotetracycline, Tetracycline, and 4-Epitetracycline in a Tetracycline Mixture

PETER P. ASCIONE and GEORGE P. CHREKIAN

Abstract □ A column chromatographic method for the determination of anhydrotetracycline, 4-epianhydrotetracycline, tetracycline, and 4-epitetracycline has been developed. The chromatogram involves the separation of these tetracyclines on a column of acid-washed diatomaceous earth treated with buffer consisting of 0.1 *M* ethylenediaminetetraacetic acid disodium salt at pH 7.0, glycerin, and polyethylene glycol 400, followed by a modified spectral determination of column eluates.

Keyphrases □ Tetracycline mixture—anhydrotetracycline, 4-epianhydrotetracycline, tetracycline, 4-epitetracycline determination □ Column chromatography—separation □ Colorimetric analysis—spectrophotometry

The method described in this paper was modified and adapted from previous studies (1, 2). Known tetracycline mixtures were determined with recoveries of 98.0 to 102.0% for anhydrotetracycline (ATC), 4-epianhydrotetracycline (EATC), tetracycline (TC), and 4-epitetracycline (ETC).

In the course of work in these laboratories on the stability of TC, it was necessary to find a specific and accurate method for the determination of degraded and nondegraded TC in crystalline and pharmaceutical preparations. The methods described by various workers (3–7) for separation of degraded TC from TC have been found workable and reproducible here, but they had the disadvantage of not determining all the components of a TC mixture on a single chromatogram.

TLC and column chromatographic methods were developed in these laboratories for the separation, examination, and determination of TC (1, 2). With modification of both the column chromatographic and spectrophotometric method, a specific and accurate method of assay for ATC, EATC, TC, and ETC, in TC mixtures, has been developed. This method allows a separate analysis for all components, thus providing a scheme for determining the column recovery quantitatively.

EXPERIMENTAL

Materials and Methods—Reagents, the preparation of diatomaceous earth[1] and chromatographic columns, and the apparatus have been described previously (2). A pH of 7.0 was maintained.

Determination of ATC, EATC, TC, and ETC—The general procedure, column preparation, and sample preparation for crystalline TC HCl or capsules and tablets were as described for demethylchlortetracycline HCl (2).

Sample Preparation—*TC Neutral and Syrups*—This was the same as described for demethylchlortetracycline neutral (2), except that 20 mg. of TC neutral or 1.0 ml. of syrup equivalent to 20–25 mg. of TC was used.

[1] Celite 545, Johns-Manville Corp., New York, NY 10016

Table I—Analysis of Synthetic Mixtures of ATC, EATC, TC, and ETC

No.	Standard Mixtures	Amount Present, mg.	Amount Found, mg.	Recovered, %
1	ATC HCl	2.58	2.55	98.8
	EATC HCl	2.32	2.31	99.6
	TC HCl	19.01	18.98	99.8
	ETC HCl	0.51	0.50	98.0
2	ATC HCl	2.32	2.27	97.8
	EATC HCl	3.29	3.30	100.3
	TC HCl	18.91	18.98	100.4
	ETC HCl	0.50	0.51	102.0

Development of Column—Use a 25-ml. glass-stoppered graduate as the primary receiver under the column. Add 20 ml. of benzene to the column. When the solvent level reaches the top of the column packing, add 60 ml. of chloroform. After collecting 10 ml. (Cut 1), remove the primary receiver and replace with a 10-ml. graduate. Collect 5 ml. in this graduate and discard. Replace the 10-ml. graduate with another 25-ml. glass-stoppered graduate. After collecting 15 ml. (Cut 2), remove the second graduate and replace with a 50-ml. volumetric flask. Collect the eluate in this flask until the level of the solvent in the column again reaches the top of the packing. Replace the 50-ml. volumetric flask (Cut 3) with a 10-ml. graduate, and add 40 ml. of 50% *n*-butanol in chloroform to the column. When the volume of eluate in the graduate reaches 8 ml., replace the 10-ml. graduate (Cut 4) with a 50-ml. glass-stoppered graduate and collect all of the last solvent added to the column (Cut 5).

Assay of Column Cuts—Determine the absorbance of Cut 1 (ATC) and Cut 2 (EATC) in a 1-cm. cell against chloroform at a wavelength of 438 mμ on a suitable spectrophotometer.

To Cut 3 (TC), add Cut 4 and mix well. Add 2.0 ml. of alkaline methanol solution and dilute to volume with chloroform. Determine the absorbance of this solution in a 1-cm. cell against chloroform, at a wavelength of 366 mμ, on a suitable spectrophotometer within 10 min. after making the solution alkaline. To Cut 5 (ETC), add 2.0 ml. of alkaline methanol; adjust the volume to the nearest milliliter graduation with chloroform, mix, and record the volume. Determine the absorbance of this solution in a 1-cm. cell against chloroform, at a wavelength of 366 mμ, within 10 min. after making the solution alkaline.

RESULTS AND DISCUSSION

In earlier work (1, 2), the separation and determination of TC by TLC and column chromatography suggested the development of this column chromatographic technique. The effectiveness of the column for the separation of ATC, EATC, TC, and ETC was demonstrated by the use of TLC (1). The column eluates were collected in fractions of 5 ml. and examined by TLC. Figure 1 shows the chromatography of column eluates. According to the results of TLC, the TC was separated on a column of acid-washed diatomaceous earth treated with EDTA, polyethylene glycol 400 (PEG 400), and glycerin at pH 7.0, using benzene, chloroform, and butanol as the developing solvents. The ATC and EATC are visible yellow bands which can be seen during chromatography. The TLC shows the distribution of the TC components isolated on

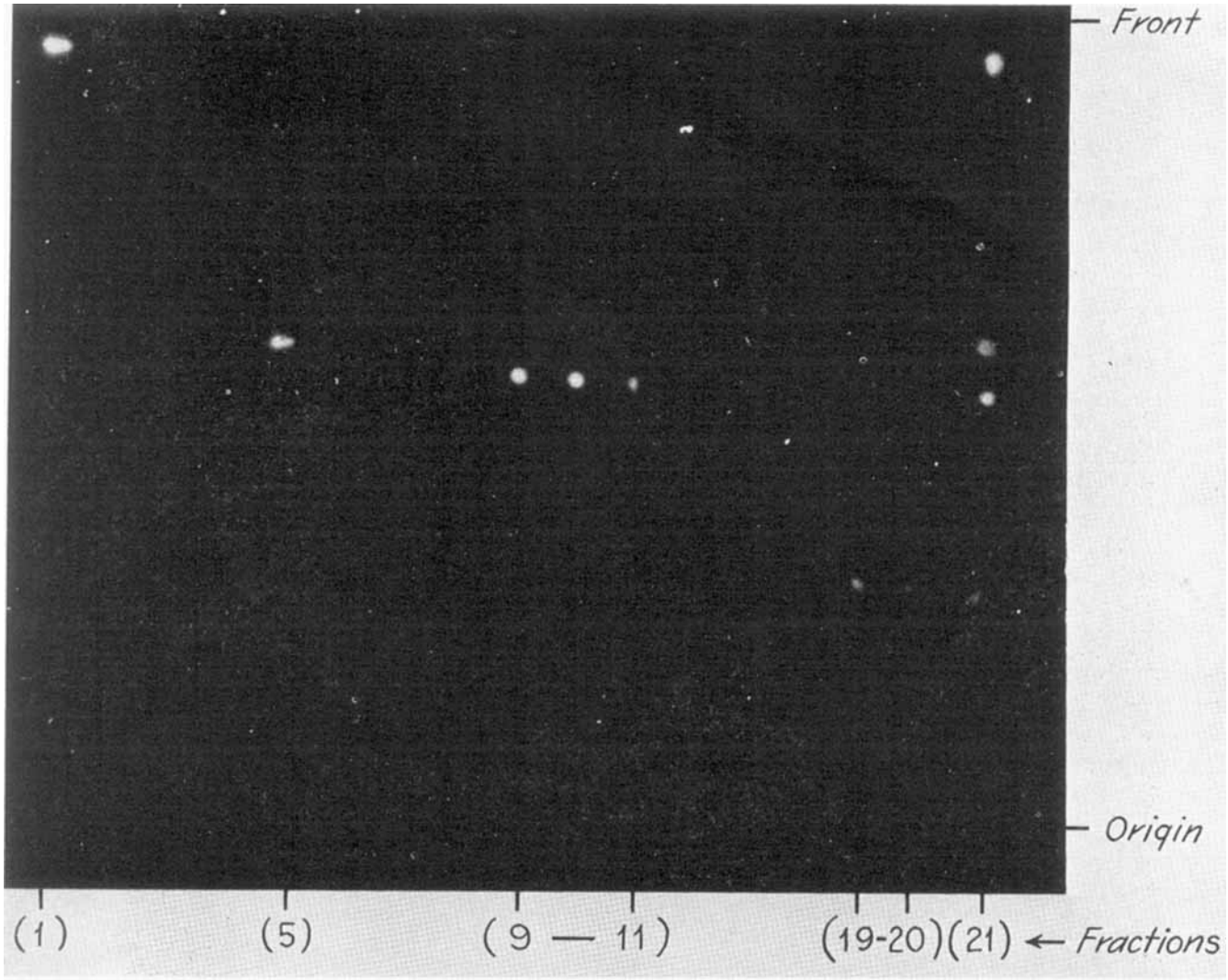


Figure 1—*TLC of TC mixture column fractions: (1) ATC; (5) EATC; (9-11) TC; (19-20) ETC; and (21) reference standard mixture.*

the column and having R_f values similar to those of standard test references.

Absorption Spectra—Absorption spectra in the region of 350–550 mμ were determined on a Cary model II recording spectrophotometer. Standard solutions of ATC and EATC, containing 0.25 mg./ml., were prepared by dissolving 25 mg. of standard in 10 ml. of methanol and diluting to 100 ml. with chloroform. Six milliliters of the chloroform solution was transferred to a 25-ml. volumetric flask and then diluted to 25 ml. with chloroform. Figures 2 and 3 show the spectra of ATC and EATC with both maxima at 438 mμ.

Through examination of the anhydro standards in these laboratories, the absorptivity for both ATC and EATC at 438 mμ was found to be 1.85×10^{-2}. The absorptivity is defined as the optical

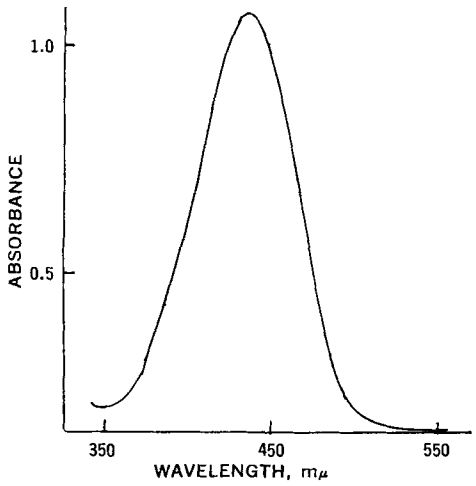


Figure 2—*Absorption spectrum of ATC.*

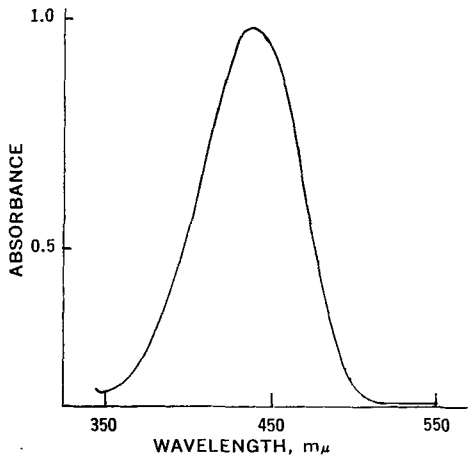


Figure 3—*Absorption spectrum of EATC.*

density at the wavelength of each particular TC for a solution containing 1 mcg. of TC per milliliter, using a 1-cm. cell.

A standard calibration curve of ATC HCl was prepared in chloroform solutions. In the analysis of ATC HCl, a linear relationship over the range of 0.025–1.5 mg./50 ml. is obtained. The absorptivity for TC and ETC was determined in alkaline methanol at 366 mμ and found to be 3.29×10^{-2} as previously described (2). The determination of absorbances of the column chromatography eluates provided a quantitative spectrophotometric method for assaying ATC, EATC, TC, and ETC in solvent solutions when compared with standards treated in a like manner.

To test the accuracy of this column chromatographic procedure for TC mixtures, a known mixture was made of standards of ATC, EATC, TC, and ETC, which was analyzed by the column procedure with the recoveries shown in Table I.

In the two experiments performed, recoveries of 98.0–102.0% were obtained on TC mixtures. Quantitative analysis of synthetic mixtures of TC was achieved with high degree of accuracy (Table I) on the column of acid-washed diatomaceous earth treated with buffer consisting of 0.1 *M* EDTA, glycerin, and PEG 400 at pH 7.0. Therefore, this method has the advantage of determining the entire content of a TC mixture on a single chromatogram.

REFERENCES

(1) P. P. Ascione, J. B. Zagar, and G. P. Chrekian, *J. Pharm. Sci.*, **56,** 1393(1967).

(2) *Ibid.*, **56,** 1396(1967).

(3) R. G. Kelly, *J. Pharm. Sci.*, **53,** 353(1964).

(4) B. W. Griffiths, *ibid.*, **55,** 353(1966).

(5) D. L. Simmons, C. K. Koorengevel, R. Kubelka, and P. Seers, *ibid.*, **55,** 219(1966).

(6) A. A. Fernandez, V. T. Noceda, and E. S. Carrerd, *ibid.*, **58,** 443(1969).

(7) D. L. Simmons, R. J. Ranz, H. S. L. Woo, and P. Picotte, *J. Chromatogr.*, **43,** 141(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 20, 1970, from the *Department of Analytical Development, Quality Control Section, Lederle Laboratories, Pearl River, NY 10965*

Accepted for publication April 30, 1970.

The authors thank John B. Zager of these laboratories and Mrs. D. Budd of the Literature Service Department for their valuable assistance with this report.

Spectrophotometric Assay of Potassium Permanganate Tablets (USP XVII)

A. MANCOTT and J. TIETJEN

Abstract □ Assay of potassium permanganate tablets (USP XVII) was accomplished by a spectrophotometric procedure. This method is comparable in accuracy to the USP XVII titrimetric procedure.

Keyphrases □ Potassium permanganate tablets—analysis □ Colorimetric analysis—spectrophotometer

The quantitative determination of potassium permanganate by a spectrophotometric method has been reported by Bastian *et al.* (1). The application of this method to the assay of USP XVII potassium permanganate tablets, as compared to the standard USP XVII titrimetric assay for potassium permanganate tablets (2), is reported in this article.

The spectrophotometric method reported here is comparable in accuracy to the USP XVII assay, but it is considerably simpler to do and results in an appreciable saving of time, labor, and materials.

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with a spectrophotometer[1] (slit width 5 Å). Matched cells with a 1-cm. optical path were used.

Reagents and Chemicals—Potassium permanganate solution (0.1 *N*)[2] was standardized against 0.1 *N* oxalic acid solution.[3] Potassium permanganate tablets USP XVII (300 mg.)[4] were assayed. All other reagents used where of the highest commercial grade available.

[1] Bausch and Lomb, model 505.
[2] Fisher Certified reagent.
[3] Fisher Certified reagent.
[4] Eli Lilly and Co., Indianapolis, Ind.

Table I—Absorbance of Known Potassium Permanganate Solutions

Concentration of $KMnO_4$, mg./l.	Absorbance
44.354	0.693
47.515	0.743
50.676	0.792
53.737	0.841
56.898	0.891
60.059	0.939
63.220	0.988

Procedure—Standardized solutions of potassium permanganate were prepared and their absorbances measured. Twenty 300-mg. potassium permanganate tablets USP XVII were weighed and finely powdered. An accurately weighed portion of the powder, 50–55 mg., was dissolved in water and diluted to 1 l. The absorbance of the solution was measured at 526 mμ and compared with the standards to determine its concentration. The same samples were also assayed according to the USP XVII titrimetric procedure.

RESULTS AND DISCUSSION

Absorbance readings for the standardized potassium permanganate solutions in the concentration range of 44–64 mg./l. were obtained (Table I). A graph of absorbance *versus* concentration was linear with a slope of 0.0156.

The percent potassium permanganate in the sample used is found from:

$$\% KMnO_4 = \frac{A}{0.0156 \times W} \times 100 \qquad \text{(Eq. 1)}$$

where A = absorbance, and W = weight in milligrams of the $KMnO_4$ sample.

USP XVII standards for potassium permanganate tablets contain not less than 95% and not more than 105% of the labeled amount of $KMnO_4$ for tablets of 300 mg. or more, and not less

density at the wavelength of each particular TC for a solution containing 1 mcg. of TC per milliliter, using a 1-cm. cell.

A standard calibration curve of ATC HCl was prepared in chloroform solutions. In the analysis of ATC HCl, a linear relationship over the range of 0.025–1.5 mg./50 ml. is obtained. The absorptivity for TC and ETC was determined in alkaline methanol at 366 mμ and found to be 3.29×10^{-2} as previously described (2). The determination of absorbances of the column chromatography eluates provided a quantitative spectrophotometric method for assaying ATC, EATC, TC, and ETC in solvent solutions when compared with standards treated in a like manner.

To test the accuracy of this column chromatographic procedure for TC mixtures, a known mixture was made of standards of ATC, EATC, TC, and ETC, which was analyzed by the column procedure with the recoveries shown in Table I.

In the two experiments performed, recoveries of 98.0–102.0% were obtained on TC mixtures. Quantitative analysis of synthetic mixtures of TC was achieved with high degree of accuracy (Table I) on the column of acid-washed diatomaceous earth treated with buffer consisting of 0.1 *M* EDTA, glycerin, and PEG 400 at pH 7.0. Therefore, this method has the advantage of determining the entire content of a TC mixture on a single chromatogram.

REFERENCES

(1) P. P. Ascione, J. B. Zagar, and G. P. Chrekian, *J. Pharm. Sci.*, **56,** 1393(1967).

(2) *Ibid.*, **56,** 1396(1967).

(3) R. G. Kelly, *J. Pharm. Sci.*, **53,** 353(1964).

(4) B. W. Griffiths, *ibid.*, **55,** 353(1966).

(5) D. L. Simmons, C. K. Koorengevel, R. Kubelka, and P. Seers, *ibid.*, **55,** 219(1966).

(6) A. A. Fernandez, V. T. Noceda, and E. S. Carrerd, *ibid.*, **58,** 443(1969).

(7) D. L. Simmons, R. J. Ranz, H. S. L. Woo, and P. Picotte, *J. Chromatogr.*, **43,** 141(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 20, 1970, from the *Department of Analytical Development, Quality Control Section, Lederle Laboratories, Pearl River, NY 10965*

Accepted for publication April 30, 1970.

The authors thank John B. Zager of these laboratories and Mrs. D. Budd of the Literature Service Department for their valuable assistance with this report.

Spectrophotometric Assay of Potassium Permanganate Tablets (USP XVII)

A. MANCOTT and J. TIETJEN

Abstract □ Assay of potassium permanganate tablets (USP XVII) was accomplished by a spectrophotometric procedure. This method is comparable in accuracy to the USP XVII titrimetric procedure.

Keyphrases □ Potassium permanganate tablets—analysis □ Colorimetric analysis—spectrophotometer

The quantitative determination of potassium permanganate by a spectrophotometric method has been reported by Bastian *et al.* (1). The application of this method to the assay of USP XVII potassium permanganate tablets, as compared to the standard USP XVII titrimetric assay for potassium permanganate tablets (2), is reported in this article.

The spectrophotometric method reported here is comparable in accuracy to the USP XVII assay, but it is considerably simpler to do and results in an appreciable saving of time, labor, and materials.

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with a spectrophotometer[1] (slit width 5 Å). Matched cells with a 1-cm. optical path were used.

Reagents and Chemicals—Potassium permanganate solution (0.1 *N*)[2] was standardized against 0.1 *N* oxalic acid solution.[3] Potassium permanganate tablets USP XVII (300 mg.)[4] were assayed. All other reagents used where of the highest commercial grade available.

[1] Bausch and Lomb, model 505.
[2] Fisher Certified reagent.
[3] Fisher Certified reagent.
[4] Eli Lilly and Co., Indianapolis, Ind.

Table I—Absorbance of Known Potassium Permanganate Solutions

Concentration of $KMnO_4$, mg./l.	Absorbance
44.354	0.693
47.515	0.743
50.676	0.792
53.737	0.841
56.898	0.891
60.059	0.939
63.220	0.988

Procedure—Standardized solutions of potassium permanganate were prepared and their absorbances measured. Twenty 300-mg. potassium permanganate tablets USP XVII were weighed and finely powdered. An accurately weighed portion of the powder, 50–55 mg., was dissolved in water and diluted to 1 l. The absorbance of the solution was measured at 526 mμ and compared with the standards to determine its concentration. The same samples were also assayed according to the USP XVII titrimetric procedure.

RESULTS AND DISCUSSION

Absorbance readings for the standardized potassium permanganate solutions in the concentration range of 44–64 mg./l. were obtained (Table I). A graph of absorbance *versus* concentration was linear with a slope of 0.0156.

The percent potassium permanganate in the sample used is found from:

$$\% KMnO_4 = \frac{A}{0.0156 \times W} \times 100 \qquad \text{(Eq. 1)}$$

where A = absorbance, and W = weight in milligrams of the $KMnO_4$ sample.

USP XVII standards for potassium permanganate tablets contain not less than 95% and not more than 105% of the labeled amount of $KMnO_4$ for tablets of 300 mg. or more, and not less

than 93% and not more than 107% for tablets of less than 300 mg.

Determinations of five potassium permanganate 300-mg. tablet samples were performed. The mean percent of the labeled amount was 99.65 with a *SD* of 0.43 when assayed spectrophotometrically and 99.89 with a *SD* of 0.59 when assayed titrimetrically.

REFERENCES

(1) R. Bastian, R. Weberling, and F. Palilla, *Anal. Chem.*, **22**, 160(1950).

(2) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 566, 567.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 29, 1969, from the *Chemistry Department, Queensborough Community College, City University of New York, Bayside, NY 11364*

Accepted for publication April 6, 1970.

Quantitative Separation of Free Estrogens by Liquid Partition Chromatography

G. J. KROL, R. P. MASSERANO, J. F. CARNEY, and B. T. KHO

Abstract □ A mixture of structurally related estrogens containing estrone, equilin, equilenin, 17α-estradiol, 17α-dihydroequilin, and 17α-dihydroequilenin was separated by a partition column chromatographic system based on a lipophilic polydextran stationary support and a composite organic solvent. The chromatographic column yielded 25 theoretical plates per centimeter of column height at 0.6 ml./min. flow rate; column efficiency was studied as a function of solvent flow rate, and an inverse relationship between the two parameters was observed. Since the complete separation of the six estrogens required 10 hr., the column was shortened to yield a 3-hr. elution, which separated completely four of the estrogens with only partial overlap between estrone and equilin. The overlap between estrone and equilin was resolved quantitatively by specific colorimetric and fluorometric determinations. The method may be scaled up for preparative purposes, it is applicable to other steroids that are too labile for gas chromatography, and the same chromatographic column can be used repeatedly.

Keyphrases □ Estrogens, free—quantitative separation □ Chromatography, liquid partition—separation □ Fluorometry—analysis □ Colorimetric analysis—spectrophotometer □ UV spectrophotometry—analysis

The separation and quantitative determination of estrogens were studied by a number of investigators. The problem was already approached by gas chromatography (1, 2), TLC (3), and liquid chromatography (4–6). However, no quantitative chromatographic method applicable to an estrogen mixture containing closely related structures such as estrone, equilin, equilenin, estradiol, dihydroequilin, and dihydroequilenin was reported in the literature. Although this study is based only on the analysis of an arbitrary mixture of these synthetic free estrogens, the sulfate esters of these free estrogens are the principal ingredients of the naturally occurring estrogenic hormones (7, 8).

Another consideration was the need for a chromatographic procedure that would be applicable to the separation of structurally related free estrogens for preparative purposes. Liquid column chromatography is ideally suited for this purpose. Furthermore, such a system may also be applicable to the analysis of other steroid structures that are too labile for gas chromatography. For example, Vandenheuvel and Horning (9) observed that gas chromatography of C-21 steroids containing an α-ketol side chain led to side-chain cleavage. Other internal rearrangements of C-21 steroids were observed by Brooks (10). Such rearrangements could lead to complications in the quantitation and the

Table I—Elution Volumes of Free Estrogens as a Function of the Solvent System

Solvent System[a]	Estrone N[e]	Estrone El. Vol.[f]	Estrone Fr. Vol.[g]	Estrone Ovl. Vol.[h]	Equilin El. Vol.	Equilin Rel. Vol.[i]	Equilin Fr. Vol.	Equilenin El. Vol.	Equilenin Rel. Vol.	Equilenin Fr. Vol.	Estradiol[b] El. Vol.	Estradiol[b] Fr. Vol.	DHEQ[c] El. Vol.	DHEQ[c] Fr. Vol.	DHEQN[d] El. Vol.	DHEQN[d] Fr. Vol.
A	1770	88	12	4.5	97	1.10	14	116	1.32	24						
B	1090	80	13	5.5	88	1.10	14	104	1.30	22						
C	910	75	17	8.5	83	1.11	16	96	1.28	20	161	24	185	26	230	32
D	1500	112	18	3.0	127	1.12	18	164	1.45	28	226	32	269	36		
E	700	71	18	9.0	80	1.13	18	99	1.40	19	138	31	159	33	211	46
F	950	62	16	6.0	70	1.13	12	84	1.36	20	111	22	132	26	160	40

[a] Solvent system: A = cyclohexane–xylene–methanol–triethylamine (400:400:75:5); B = cyclohexane–toluene–methanol–trimethylamine (400:400:75:5); C = cyclohexane–benzene–methanol–trimethylamine (400:400:75:5); D = cyclohexane–benzene–methanol (500:150:75); E = cyclohexane–benzene–methanol (500:150:75); and F = cyclohexane–benzene–methanol–trimethylamine (500:150:75:5). Column height for Systems A, B, C, and D = 65 ± 5 cm.; and column height for Systems E and F = 45 ± 2 cm. All elutions were carried out at 0.6 ± 0.1 ml./min. flow rate. All volumes except the relative volume are expressed in milliliters. [b] 17α- or 17β-Estradiol. [c] 17α- or 17β-Dihydroequilin. [d] 17α- or 17β-Dihydroequilenin. [e] Theoretical plate number; based on elution volume and peak width at half peak height. [f] The elution volume of a given estrogen at its maximum concentration. [g] The total volume of eluent containing a given estrogen fraction. [h] The overlap volume between estrone and equilin. [i] The elution volume of a given estrogen relative to the elution volume of estrone (estrone relative volume = 1).

than 93% and not more than 107% for tablets of less than 300 mg.

Determinations of five potassium permanganate 300-mg. tablet samples were performed. The mean percent of the labeled amount was 99.65 with a *SD* of 0.43 when assayed spectrophotometrically and 99.89 with a *SD* of 0.59 when assayed titrimetrically.

REFERENCES

(1) R. Bastian, R. Weberling, and F. Palilla, *Anal. Chem.*, **22**, 160(1950).

(2) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 566, 567.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 29, 1969, from the *Chemistry Department, Queensborough Community College, City University of New York, Bayside, NY 11364*

Accepted for publication April 6, 1970.

Quantitative Separation of Free Estrogens by Liquid Partition Chromatography

G. J. KROL, R. P. MASSERANO, J. F. CARNEY, and B. T. KHO

Abstract □ A mixture of structurally related estrogens containing estrone, equilin, equilenin, 17α-estradiol, 17α-dihydroequilin, and 17α-dihydroequilenin was separated by a partition column chromatographic system based on a lipophilic polydextran stationary support and a composite organic solvent. The chromatographic column yielded 25 theoretical plates per centimeter of column height at 0.6 ml./min. flow rate; column efficiency was studied as a function of solvent flow rate, and an inverse relationship between the two parameters was observed. Since the complete separation of the six estrogens required 10 hr., the column was shortened to yield a 3-hr. elution, which separated completely four of the estrogens with only partial overlap between estrone and equilin. The overlap between estrone and equilin was resolved quantitatively by specific colorimetric and fluorometric determinations. The method may be scaled up for preparative purposes, it is applicable to other steroids that are too labile for gas chromatography, and the same chromatographic column can be used repeatedly.

Keyphrases □ Estrogens, free—quantitative separation □ Chromatography, liquid partition—separation □ Fluorometry—analysis □ Colorimetric analysis—spectrophotometer □ UV spectrophotometry—analysis

The separation and quantitative determination of estrogens were studied by a number of investigators. The problem was already approached by gas chromatography (1, 2), TLC (3), and liquid chromatography (4–6). However, no quantitative chromatographic method applicable to an estrogen mixture containing closely related structures such as estrone, equilin, equilenin, estradiol, dihydroequilin, and dihydroequilenin was reported in the literature. Although this study is based only on the analysis of an arbitrary mixture of these synthetic free estrogens, the sulfate esters of these free estrogens are the principal ingredients of the naturally occurring estrogenic hormones (7, 8).

Another consideration was the need for a chromatographic procedure that would be applicable to the separation of structurally related free estrogens for preparative purposes. Liquid column chromatography is ideally suited for this purpose. Furthermore, such a system may also be applicable to the analysis of other steroid structures that are too labile for gas chromatography. For example, Vandenheuvel and Horning (9) observed that gas chromatography of C-21 steroids containing an α-ketol side chain led to side-chain cleavage. Other internal rearrangements of C-21 steroids were observed by Brooks (10). Such rearrangements could lead to complications in the quantitation and the

Table I—Elution Volumes of Free Estrogens as a Function of the Solvent System

Solvent System[a]	Estrone N[e]	Estrone El. Vol.[f]	Estrone Fr. Vol.[g]	Estrone Ovl. Vol.[h]	Equilin El. Vol.	Equilin Rel. Vol.[i]	Equilin Fr. Vol.	Equilenin El. Vol.	Equilenin Rel. Vol.	Equilenin Fr. Vol.	Estradiol[b] El. Vol.	Estradiol[b] Fr. Vol.	DHEQ[c] El. Vol.	DHEQ[c] Fr. Vol.	DHEQN[d] El. Vol.	DHEQN[d] Fr. Vol.
A	1770	88	12	4.5	97	1.10	14	116	1.32	24						
B	1090	80	13	5.5	88	1.10	14	104	1.30	22						
C	910	75	17	8.5	83	1.11	16	96	1.28	20	161	24	185	26	230	32
D	1500	112	18	3.0	127	1.12	18	164	1.45	28	226	32	269	36		
E	700	71	18	9.0	80	1.13	18	99	1.40	19	138	31	159	33	211	46
F	950	62	16	6.0	70	1.13	12	84	1.36	20	111	22	132	26	160	40

[a] Solvent system: A = cyclohexane–xylene–methanol–triethylamine (400:400:75:5); B = cyclohexane–toluene–methanol–trimethylamine (400:400:75:5); C = cyclohexane–benzene–methanol–trimethylamine (400:400:75:5); D = cyclohexane–benzene–methanol (500:150:75); E = cyclohexane–benzene–methanol (500:150:75); and F = cyclohexane–benzene–methanol–trimethylamine (500:150:75:5). Column height for Systems A, B, C, and D = 65 ± 5 cm.; and column height for Systems E and F = 45 ± 2 cm. All elutions were carried out at 0.6 ± 0.1 ml./min. flow rate. All volumes except the relative volume are expressed in milliliters. [b] 17α- or 17β-Estradiol. [c] 17α- or 17β-Dihydroequilin. [d] 17α- or 17β-Dihydroequilenin. [e] Theoretical plate number; based on elution volume and peak width at half peak height. [f] The elution volume of a given estrogen at its maximum concentration. [g] The total volume of eluent containing a given estrogen fraction. [h] The overlap volume between estrone and equilin. [i] The elution volume of a given estrogen relative to the elution volume of estrone (estrone relative volume = 1).

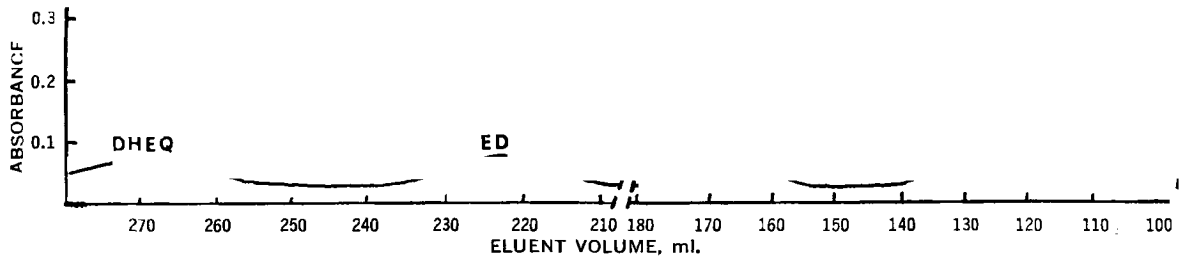


Figure 1—*Separation of estrone (ES), equilin (EQ), equilenin (EQN), estradiol (ED), and dihydroequilin (DHEQ) on Sephadex LH-20 (0.9 × 68-cm. column) with cyclohexane–benzene–methanol (500:150:75) solvent. Flow rate: 0.6 ± 0.1 ml./min.*

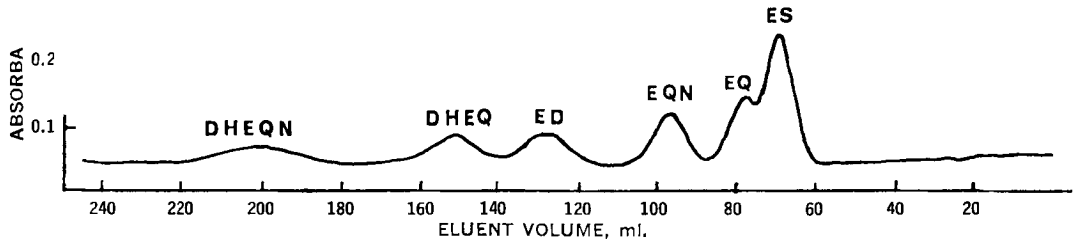


Figure 2—*Separation of estrone (ES), equilin (EQ), equilenin (EQN), estradiol (ED), dihydroequilin (DHEQ), and dihydroequilenin (DHEQN) on Sephadex LH-20 (0.9 × 45-cm. column) with cyclohexane–benzene–methanol (500:150:75) solvent. Flow rate: 1.6 ± 0.1 ml./min.*

degree of specificity of the gas chromatographic separation.

The chromatographic approach selected in this study was based on the need for a selective separation system which would be applicable to both analytical and preparative purposes. The choice of a partition chromatographic system involving a lipophilic polydextran gel support and a nonaqueous solvent system was suggested by the work of Nystrom and Sjovall (11) and Seki (12) with other steroids. However, the previous work (11, 12) was not quantitative and not directly applicable to the present requirements.

The chromatographic system reported in this paper yielded 3020 theoretical plates and separated the six estrogens. However, the complete separation required 10 hr. Since the rate-determining step is the separation of estrone and equilin, the authors decided, for practical considerations, to base the quantitative analytical method on a less time-consuming chromatographic system which separates all of the estrogens except estrone and equilin. The short column separation required 3 hr. Furthermore, the partial overlap between estrone and equilin was resolved quantitatively by three independent nonchromatographic procedures. One procedure depended on a colorimetric reaction which was specific for equilin (13). The other two depended on a relatively higher specific fluorometric (14) and colorimetric response of estrone as compared to equilin after heating in a sulfuric acid–methanol–water solution. The latter procedures were modified to enhance the selectivity for estrone.

Since the chromatographic solvent selected for the complete separation of the estrogen mixture was relatively volatile and inert, the separation may be adapted to preparative purposes. An analogous chromatographic system was also found applicable to C-21 steroid structures containing labile side chains and to steroids that are prone to dehydration under gas chromatographic conditions (15).

EXPERIMENTAL

Apparatus—All chromatographic separations were carried out in 0.9-cm. (i.d.) and 50–125-cm. columns.[1] Chromatograms were obtained with a flow-cell[2] and recording spectrophotometer[3] system. A pulseless pump[4] was used to vary the elution flow rate. A spectrofluorometer[5] was used for the fluorometric analyses. UV and colorimetric determinations were carried out with a recording spectrophotometer.[6]

Reagents and Materials—The following solvents were used: reagent grade glacial acetic acid, benzene, methanol, concentrated sulfuric acid, toluene, triethylamine, trimethylamine, xylene, and spectroquality cyclohexane;[7] reagent grade benzene was redistilled prior to use. Free estrogen samples were of reference standard purity.[8] The commercially available lipophilic gel[9] was used without pretreatment.

Preparation of Column—The gel was suspended in the chromatographic solvent (200 ml. of solvent/10 g. of gel), and the resulting slurry was equilibrated by shaking for at least 2 hr. The gel was allowed to settle, excess solvent was decanted, and a fresh portion of solvent was added. This procedure was repeated three times. The final slurry was transferred to the column by gravity feed and packed at elution pressure to the desired length.

Chromatography—An aliquot (0.5–1 ml.) containing a mixture of free estrogens (50–200-mcg. amounts) in a given chromatographic solvent was applied quantitatively to the column. The column was eluted with the aid of a pulseless pump at flow rates ranging from 0.4–2 ml./min. The elution patterns were recorded by the flow-cell spectrophotometer system, and fractions were collected at appropri-

[1] Obtained from Fisher and Porter Co., Warminster, Pa.
[2] Obtained from Arthur H. Thomas Co., Philadelphia, Pa.
[3] Beckman DB.
[4] Waters Associates, Inc., Framingham, Mass.
[5] Perkin-Elmer model 203.
[6] Cary model 14.
[7] Matheson, Coleman & Bell.
[8] Prepared by Ayerst Research Laboratories, Montreal, P.Q., Canada.
[9] Sephadex LH-20, Pharmacia Fine Chemicals Inc., New Market, N. J.

Table II—Elution Volumes of Estrogens as a Function of Column Height[a]

Col. Hgt., cm.	Estrone N[e]	Estrone El. Vol.[f]	Estrone Fr. Vol.[g]	Estrone Ovl. Vol.[h]	Equilin El. Vol.	Equilin Rel. Vol.[i]	Equilin Fr. Vol.	Equilenin El. Vol.	Equilenin Rel. Vol.	Equilenin Fr. Vol.	Estradiol[b] El. Vol.	Estradiol[b] Fr. Vol.	DHEQ[c] El. Vol.	DHEQ[c] Fr. Vol.	DHEQN[d] El. Vol.	DHEQN[d] Fr. Vol.
44	820	71	18	9.0	80	1.13	18	99	1.40	19	138	31	164	33	215	46
68	1500	112	18	3.0	127	1.12	18	164	1.45	28	223	32	269	36		
120	3020	196	22	0.0	222	1.13	24	280	1.43	30						

[a] Solvent system: cyclohexane–benzene–methanol (500:150:75); flow rate: 0.6 ± 0.1 ml./min. All volumes except the relative volume are expressed in milliliters. [b] 17α- or 17β-Estradiol. [c] 17α- or 17β-Dihydroequilin. [d] 17α- or 17β-Dihydroequilenin. [e] Theoretical plate number; based on elution volume and peak width at half peak height. [f] The elution volume of a given estrogen at its maximum concentration. [g] The total volume of eluent containing a given estrogen fraction. [h] The overlap volume between estrone and equilin. [i] The elution volume of a given estrogen at its maximum concentration relative to the elution volume of estrone (estrone relative volume = 1).

Table III—Elution Volumes of Free Estrogens as a Function of Flow Rate[a]

Flow Rate, ml./min.	Estrone N[e]	Estrone El. Vol.[f]	Estrone Fr. Vol.[g]	Estrone Ovl. Vol.[h]	Equilin El. Vol.	Equilin Rel. Vol.[i]	Equilin Fr. Vol.	Equilenin El. Vol.	Equilenin Rel. Vol.	Equilenin Fr. Vol.	Estradiol[b] El. Vol.	Estradiol[b] Fr. Vol.	DHEQ[c] El. Vol.	DHEQ[c] Fr. Vol.	DHEQN[d] El. Vol.	DHEQN[d] Fr. Vol.
0.3	1130	69	14	3.0	79	1.14	12	120	1.74	22						
0.6	800	70	16	6.0	78	1.13	16	97	1.40	19	132	26	161	33		
0.8	700	71	18	9.0	80	1.13	18	99	1.40	19	137	31	159	33	211	46
1.6	420	70	20	10.0	79	1.13	18	97	1.40	22	130	28	155	33	209	42

[a] Solvent system: cyclohexane–benzene–methanol (500:150:75). All elutions were carried out on the same (45-cm.) column. [b] 17α- or 17β-Estradiol. [c] 17α- or 17β-Dihydroequilin. [d] 17α- or 17β-Dihydroequilenin. [e] Theoretical plate number; based on peak width at half peak height. [f] The elution volume of a given estrogen at its maximum concentration. [g] The total volume of eluent containing a given estrogen fraction. [h] The overlap volume between estrone and equilin. [i] The elution volume of a given estrogen at its maximum concentration relative to the elution volume of estrone (estrone relative volume = 1).

ate elution volumes. A 100-ml. fraction free of any estrogens was collected for a blank in UV determinations.

Quantitation—Each fraction was evaporated to dryness at 50° under nitrogen. The estrogen fractions were reconstituted quantitatively with methanol in a 5-ml. volumetric flask. The 100-ml. blank fraction was reconstituted in a 10-ml. volumetric flask. Each fraction was scanned by a recording spectrophotometer against methanol and an appropriate blank, which was prepared from the 100-ml. fraction. Aliquots of each fraction were then withdrawn for the fluorometric and colorimetric analyses.

The fluorometric analysis was applied only to the fraction containing estrone and equilin. The determination required approximately 2–4 mcg. of estrone, which was contained in 50–100 μl. of methanol. The solution was pipeted into a 10-ml. volumetric flask, which was made up to volume with concentrated sulfuric acid, water, and methanol (8:1:1 parts by volume). The volumetric flasks were heated at 80° for 15 min. After cooling to room temperature, the fractions and standard reference solutions were analyzed spectrofluorometrically by activating at 273 mμ and reading the emission at 470 mμ.

By increasing the concentration range of the estrogens by a factor of 10–20, the fluorometric procedure was adapted to colorimetric analysis of estrone, equilin, equilenin, estradiol, 17α-dihydroequilin, and 17α-dihydroequilenin. After heating and cooling, the sulfuric acid solutions were scanned by a recording spectrophotometer from 700 to 420 mμ.

The chromatographic fractions containing estrone and equilin were also analyzed by a modified Kober test which is specific for equilin (13).

RESULTS AND DISCUSSION

Chromatography—The efficiency of the chromatographic system was studied as a function of solvent composition, column height, and flow rate. Table I presents the column plate numbers, the peak elution volumes, and the volumes of estrogen fractions as a function of the solvent system. Figures 1 and 2 illustrate representative elution patterns obtained with the selected solvent system.

These data and preliminary data indicated that a combination of a short-chain alcohol, cyclohexane, and an aromatic solvent yielded the most effective separation. Such a combination not only facilitated the miscibility of the first two components but also introduced selective interactions between the steroid molecules and the mobile phase. The selectivity of this system was apparently enhanced by the favorable partition coefficients resulting from marked contrast between the polarity of the methanol-solvated stationary phase and the relatively low polarity of the mobile phase. The structure and the relative ratio of the aromatic component to cyclohexane have been found to be rather critical. A somewhat favorable effect was also observed on addition of tertiary amines. This effect could be attributed to "masking" of the common phenolic group, which has a predominant effect on the polarity and solubility of estrogens, and subsequent enhancement of less significant structural variations between different estrogen structures. However, the somewhat favorable effect of tertiary amines was offset by the odor of the resulting solvent mixtures and complications in the quantitation procedure; thus, for quantitative analytical purposes, the amines were omitted from the solvent mixture.

Although the chromatographic system studied separated estrogens that differed only by one double bond, it did not separate the α- and β-isomers of estradiol, dihydroequilin, and dihydroequilenin. Table II reflects the results of the column height study. As anticipated, the degree of separation and the column plate number increased with increasing column height. However, due to practical considerations such as flow rate, there was a natural limitation to the height of the column. Increasing the pressure to increase the flow rate tended to compress the gel phase and thus increase the resistance to solvent flow.

Increasing the flow rate also led to decreasing column efficiency (increasing height of the theoretical plate). At higher flow rates, the operation is at nonequilibrium, and the diffusion-dependent mass transfer Cv term of the Van Deemter equation (16) is directly proportional to the square of the thickness of liquid film (d_f) on the support and inversely proportional to the diffusion coefficient of a solute in the solvent phase (D_e). Since in the chromatography system investigated, d_f is rather large while D_e is rather small, the Cv term made a relatively large contribution to the Van Deemter equation and thus led to the inverse relationship between column efficiency and solvent flow rate. These effects are apparent in Table III.

Quantitation—Since the system selected for quantitation did not yield complete separation between estrone and equilin, additional specificity was built into the quantitation procedure. The UV determination is suitable for quantitation of equilenin, estradiol, dihydroequilin, and dihydroequilenin which are separated from each other. However, the UV spectra of estrone and equilin are identical; thus the UV determination of the combined estrone and equilin fraction gives only the sum of their concentrations. Combining the result of the UV determination with a fluorometric and a colorimetric procedure, which is relatively selective for estrone,

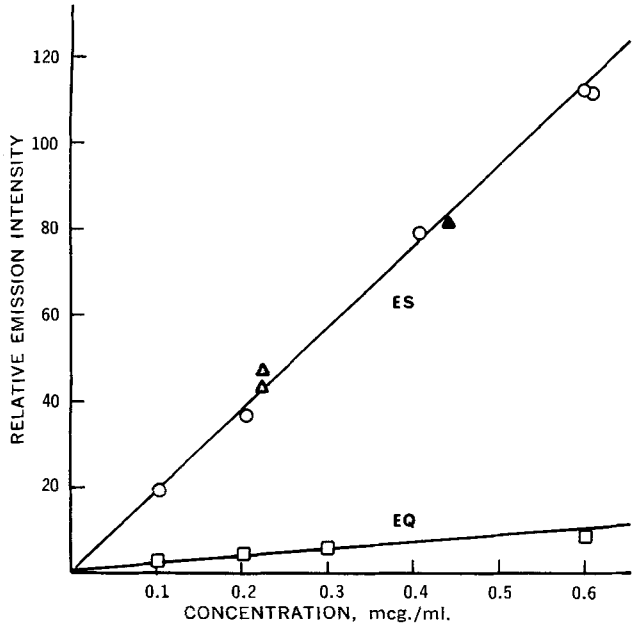


Figure 3—*Relative emission intensity of estrone (ES) and equilin (EQ) at 273-mμ activation wavelength and 470-mμ emission wavelength. Key: □, equilin (EQ); ○, estrone (ES); △, 0.2 mcg. of ES + 0.2 mcg. of EQ; and ▲, 0.4 mcg. of ES + 0.2 mcg. of EQ.*

and a modified Kober procedure, which is selective for equilin, gives three independent methods for individual determination of the estrone and equilin mixture.

The fluorometric procedure adopted in this study is based on a method developed by Bates and Cohen (14). However, the authors have observed that a mixture of sulfuric acid, methanol, and water yields a greater response than the sulfuric acid and water solution recommended by Bates and Cohen (14). By utilizing a spectrofluorometer rather than a fluorometer, a significant gain was also obtained in the specificity of estrone determination. The relative response ratio of estrone to equilin, observed by Bates and Cohen (14) with a filter fluorometer, was 4:1, while the ratio observed in the present study with the aid of a spectrofluorometer was 10.8:1 (Fig. 3). Given this relative response ratio and the total concentration of the two estrogens in an unknown mixture, one can postulate the following equations, which resolve the composition of the unknown mixture:

$$R = R'Es + R''Eq \qquad \text{(Eq. 1)}$$

$$R'' = \frac{R'}{10.8} \qquad \text{(Eq. 2)}$$

where R = observed fluorescence response of the estrone and equilin fraction; Es = mcg. of estrone in the sample; Eq = mcg. of equilin in the sample; R' = the fluorescence response for estrone (in response units per mcg. of estrone); and R'' = the fluorescence response for equilin (in response units per mcg. of equilin).

Note that concentrations are not involved since all samples are in the same volume.

Table IV—Observed Maximum Absorption Wavelength and Absorptivity in the Acid-Induced Colorimetric Determination

Estrogen	Maximum Absorption Wavelength, mμ	Absorptivity ($a \times 10^{-2}$)
Estrone	453	14.5
Equilin	453	7.0
Equilenin	480	11.6
Estradiol	455	4.0
17α-Dihydroequilin	490	19.0
17α-Dihydroequilenin	455	5.1

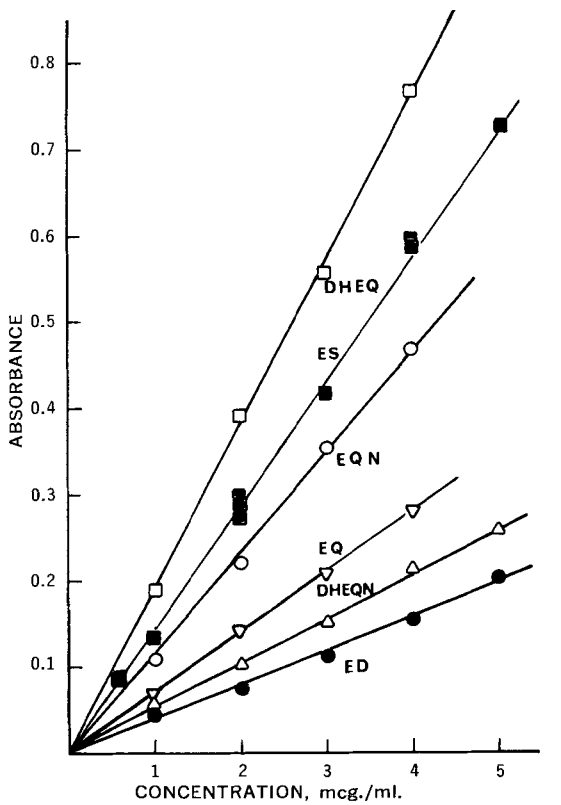


Figure 4—*Colorimetric absorbance of estrone (ES, at 453 mμ), equilin (EQ, at 453 mμ), equilenin (EQN, at 480 mμ), estradiol (ED, at 455 mμ), 17α-dihydroequilin (DHEQ, at 490 mμ), and 17α-dihydroequilenin (DHEQN, at 455 mμ).*

From UV determination:

$$Eq = C_T - Es \qquad \text{(Eq. 3)}$$

where C_T = total mcg. of estrone and equilin in the mixed fraction.

By substitution:

$$Es = 1.10\,\frac{R}{R'} - 0.102\,C_T \qquad \text{(Eq. 4)}$$

Equation 4 was applied to the analysis of several fractions containing estrone and equilin, and quantitative results were obtained. However, some chromatographic fractions, which apparently contained an unknown impurity that had a significant quenching effect, yielded values considerably lower than expected. These fractions were analyzed by the colorimetric procedure which, although less sensitive than the fluorometric analysis, was considerably more reliable. A set of equations analogous to those already listed was also applied to the colorimetric analysis of the fraction containing both estrone and equilin. The relative colorimetric response ratio of estrone to equilin was observed to be 2:1. As an independent check of the determination of equilin in the combined estrone and equilin fraction, a modified Kober test was utilized (13).

The colorimetric analysis was also applied to the determination of the remaining estrogen fractions. In this application, no blank correction was necessary; since there was no overlap between the remaining estrogens, the directly observed absorbances were used in the determination. Table IV summarizes the absorptivities and the wavelengths of maximum absorbance observed in the colorimetric determination. Figure 4 illustrates the relative colorimetric responses of the six estrogens. It is of interest to note the differences in the relative response of each of the estrogens. Jones and Hähnel (17) postulated a carbonium-ion mechanism for the formation of an acid-induced steroid chromophore. Since the double bond in Ring B of the equilin structure could affect adversely the carbonium-ion formation, this mechanism could explain the observed decrease in the absorptivity of equilin as compared to estrone. However, the

Table V—Comparative Recovery Data

Chromatographic Fraction	Theo. Input, mcg.	% Recovery Obtained by Different Methods: UV[a] A	UV[a] B	Fluorometry[a] A	Fluorometry[a] B	Colorimetry[a] A	Colorimetry[a] B	Modified Kober[a] A	Modified Kober[a] B
Estrone	210	97	100	94	103	96	95	100	104
Equilin	109			105	95	100	109	104	103
Equilenin	50.5	97	97			90	93		
Estradiol	99.3	100	106			102	110		
Dihydroequilin	104.0	98	110			97	99		
Dihydroequilenin	49.8	94	90			105	95		

[a] A and B values correspond to different elutions.

observed relative magnitude of absorptivities of other estrogens is not as readily explained and thus merits further investigation.

Table V summarizes the quantitative results observed in this study.

SUMMARY AND CONCLUSIONS

The partition chromatographic system investigated was found to be sufficiently selective to separate completely six estrogens which are characterized by only minor differences in the molecular structure. The chromatographic column used for this purpose yielded 3020 theoretical plates, 25 theoretical plates per centimeter of column height at 0.6 ml./min. flow rate. However, this separation required 10 hr. and, due to a relatively large contribution of the mass transfer factor to column efficiency, it was not possible to increase the efficiency of the system by increasing the solvent flow rate. This limitation was compensated by selecting a shorter column (800 theoretical plates) and resolving the partial overlap between estrone and equilin by specific fluorometric and colorimetric reactions. Since the elution volumes were reproducible, columns could be monitored manually without elaborate instrumentation. However, the separation procedure requires 3 hr. and thus is still relatively impractical. To reduce the elution time, the authors are now investigating the possibility of further optimization of their chromatographic system.

REFERENCES

(1) T. Lukkainen, W. J. A. Vandenheuvel, and E. C. Horning, *Biochem. Biophys. Acta*, **62,** 153(1962).

(2) G. Schilling, Ayerst Research Laboratories, Montreal, P. Q., Canada, unpublished data.

(3) J. C. Touchstone, A. K. Balin, and P. Knapstein, *Steroids*, **13,** 199(1969).

(4) H. VanBaelen, W. Heyns, and P. DeMoor, *J. Chromatogr.*, **30,** 226(1967).

(5) A. R. Hurwitz, H. J. Burke, and R. A. Marra, *J. Pharm. Sci.*, **56,** 1509(1967).

(6) P. J. Stanchak and H. S. Strickler, *Clin. Chem.*, **14,** 838 (1968).

(7) G. A. Grant and D. Beall, in "Recent Progress in Hormone Research," vol. 5, G. Pincus, Ed., Academic, New York, N. Y., 1950, pp. 307–320.

(8) L. F. Fieser and M. Fieser, "Steroids," Reinhold, New York, N. Y., 1959, pp. 477, 478.

(9) W. J. A. Vandenheuvel and E. C. Horning, *Biochem. Biophys. Res. Commun.*, **3,** 356(1960).

(10) C. J. A. Brooks, *Anal. Chem.*, **37,** 636(1965).

(11) E. Nystrom and J. Sjovall, *Anal. Biochem.*, **12,** 235(1965).

(12) T. Seki, *J. Chromatogr.*, **29,** 246(1967).

(13) J. H. Graham, *J. Pharm. Sci.*, **54,** 1665(1965).

(14) R. W. Bates and H. Cohen, *Endocrinology*, **47,** 166(1950).

(15) G. J. Krol and N. G. Nash, unpublished data.

(16) L. R. Snyder, "Principles of Adsorption Chromatography," Marcel Dekker, New York, N. Y., 1968, pp. 100–102.

(17) H. A. Jones and R. Hähnel, *Steroids*, **13,** 693(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 16, 1969, from the *Analytical Development Department, Ayerst Laboratories Inc., Rouses Point, NY 12979*

Accepted for publication May 11, 1970.

Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

The authors wish to thank Dr. D. Beall, Dr. W. Hausmann and Mr. P. Neil for their interest, helpful discussions, and assistance in the preparation of this manuscript. They also thank Miss H. Archibald for the colorimetric analyses and Dr. G. Schilling for supplying the estrogen reference compounds.

Factors Affecting Dose Variation in Meter Valves

AVINASH M. CONTRACTOR, M. DAVID RICHMAN*, and RALPH F. SHANGRAW

Abstract □ Dose variation in commercial meter valves appears to be within acceptable pharmaceutical limits. Changes in delivered dose weights were found to occur during container emptying due to formulation fractionation. A more serious problem results from failure of the meter chamber to fill uniformly when the container is almost empty (~15% remaining). Three valves were found to be superior to others in overcoming this problem. Storage position influenced drainback in meter valves, particularly at later stages of container emptying.

Keyphrases □ Aerosol meter valves—dosage weight variation □ Propellent, formulation effects—aerosol dosage weight □ Fractionation—aerosols □ GLC—analysis

The need for official standards for weight variation of doses obtained from aerosol meter valves has long been recognized. The Chemical Specialties Manufacturers Association is attempting to draw up safe and reasonable specifications. Although much data on meter valves have been generated by the pharmaceutical industry, little has been published. Notable exceptions to this lack of published data are found in articles by Porush *et al.* (1), Young *et al.* (2), Grim *et al.* (3), and Contractor *et al.* (4). These authors have emphasized the importance of uniformity in delivery from meter valves as indispensable in dependable pharmaceutical preparations. Limits of ±15% of the calculated dose have been suggested. However, no articles have addressed themselves to dose variations arising from such critical factors as formulation pressure, container emptying, long standing of containers between actuations, and valve design. Comparison between commercially available meter valves, using identical formulations and evaluation methods, was undertaken with the hope that data would be generated upon which reasonable standards for meter valve performance and dose variation could be based.

EXPERIMENTAL

Valves—Meter valves for use with pharmaceuticals are available in sizes of 200, 100, and 50 μl., the last being the most common. The meter valves employed in this study are listed in Table I. All valves studied, except valve R 50I, contained acetal resin[1] stems with rubber stem seals supported by stainless steel gaskets. The R 50I valve was similar but the stem was made of stainless steel. The resin has replaced stainless steel in many valve stems due to the lower cost and a greater flexibility in design. Valves R 50I-EC and S 50I-EC have an emptying cup to assist in complete product removal from the container. Valve V 50I-DA has such a design that the last traces of product drain into the valve.

Formulations—The same four formulations used in the previous study and listed in Table II, representing various types of products, were employed (4). Since the purpose of the study was to investigate factors affecting dose variation under various conditions, the formulations tested were kept as simple as possible, with no active ingredient in them.

Testing Procedures—Ten replicates of each valve and each formulation were used to provide data in the following areas:

1. Dose-to-dose variation at four levels of container emptying: initial, 10, 50, and 80%.
2. Maximum amount removable before dose became substandard and erratic.
3. Effect of storage position and time on dose.
4. Effect of formulation on dose uniformity.
5. Effect of container emptying on the ratio of formulation components.

The functionality of valves covering dose variation within and between valves has been presented in an earlier paper (4). The testing procedures and parameters measured were the same, except for the quantitative measurement of the ratio of propellent blends before and during container use. Gas chromatography utilizing new sampling procedures was used for the quantitative determination of propellent blends.

The instrument employed was an Aerograph (model A-700 Atuoprep) gas chromatograph with thermal conductivity detection. The column was of stainless steel, 6.09 m. × 0.95 cm. (20 ft. × 0.375 in.), containing 30% SE 30, 60–80-mesh diatomaceous earth.[2] The columns were conditioned at 150° for 24 hr. with a helium flow of 6 ml./min. Analysis was performed isothermally with column temperature at 55°, injector block temperature at 130°, and detector temperature at 190°. The carrier gas, helium, was at an inlet pressure of 50 p.s.i. and a flow rate of 200 ml./min., and the filament current was maintained at 150 ma. A Honeywell recorder (Electronic 15) was employed at a speed of 1.01 cm./min. (0.4 in./min.).

Sampling Technique—A 3.81-cm. (1.5-in.) 25G regular point hypodermic needle[3] was attached to the top of the stem of a 50-μl. valve, using a piece of polyethylene tubing as packing. The needle was carefully introduced into the injector block, and the valve was actuated by pressing the container against the injector block. A sample of about 50 μl. was thus injected through the needle. The pressurized container acted as a pressure syringe and delivered about a 50-μl. sample every time the valve was actuated. By comparing the ratio of the peak heights, proportions of components were calculated, because the volumes of samples were about and not exactly 50 μl. Initial sample composition served as a standard control for comparing subsequent sample compositions at various levels of container emptying.

RESULTS AND DISCUSSION

The mean dose delivered from each valve for each formulation at various levels of container emptying can be seen in Tables III and IV. Since the doses were measured in milligrams, there is a significant difference in dose between formulations due to the differences in densities of the various propellent and propellent–alcohol blends. Although the differences between the same size valves of different types and manufacturers were considerable, these differences are not of importance in determining the precision of

[1] Delrin, E. I. du Pont de Nemours, Wilmington, Del.

[2] Gas-Chrom P, Applied Science Laboratories, State College, Pa.

[3] Becton, Dickinson and Co., Rutherford, N. J.

Table I—Meter Valve Specifications[a]

Experimental Identification Number	Chamber Size, μl.	Actuation Position	Type of Stem	Container	Manufacturer
E 100U	100	Upright	Acetal resin	Glass vial[b]	Emson Res., Inc., Bridgeport, Conn.
E 50U	50	Upright	Acetal resin	Glass vial	Emson Res., Inc., Bridgeport, Conn.
E 50I	50	Inverted	Acetal resin	Glass vial	Emson Res., Inc., Bridgeport, Conn.
V 100U	100	Upright	Acetal resin	Glass vial	Valve Corp. of Am., Bridgeport, Conn.
V 50U	50	Upright	Acetal resin	Glass vial	Valve Corp. of Am., Bridgeport, Conn.
V 50I	50	Inverted	Acetal resin	Glass vial	Valve Corp. of Am., Bridgeport, Conn.
V 50I-DA	50	Inverted	Acetal resin	Glass vial	Valve Corp. of Am., Bridgeport, Conn.
R 50I	50	Inverted	Stainless stl.	Aluminum tb.[c]	Riker Labs., Northridge, Calif.
R 50I-EC	50	Inverted	Stainless stl.	Glass vial	Riker Labs., Northridge, Calif.
S 50I-EC	50	Inverted	Acetal resin	Glass vial	(Experimental Valve, English)

[a] Actuator buttons: Emson S-1, orifice 0.020 in Gasket: Buna rubber. [b] Plastic-coated round glass vial, model S-1409F1 (20 ml.), Wheaton Plasticote Corp., Mays Landing, N. J. [c] Aluminum tube, 2.2 × 5.79 cm. ($^7/_8$ × $2^9/_{32}$ in.), Emson Research, Inc., Bridgeport, Conn.

Table II—Composition of Test Formulations

Ingredients	% w/w I	II	III	IV
Ethanol (absolute)	0	0	50	0
Propellent 12[a]	50	75	25	0
Propellent 114[b]	50	25	25	0
Propellent C-318[c]	0	0	0	100
~ Pressure at 25° (77°F.), p.s.i.g.	53	67	13	29

[a] Dichlorodifluoromethane. [b] Dichlorotetrafluoroethane. [c] Octafluorocyclobutane.

each type of valve. They are, however, of great importance in terms of product development departments, because each formulation must be tailored to each specific valve, even though the valves are labeled to contain equal volumes (*i.e.*, 50 or 100 μl.).

The precision of meter valves is extremely good and would certainly compare favorably to other dosage forms when limited to a single formulation and a single level of container emptying. The precision exhibited by inverted valves is generally better than that of the upright valves.

It would appear from Tables III and IV that the precision was influenced by formulation. Formulation IV gave an unusually high level of precision, while Formulation III generally exhibited low precision. These results indicate possible container-emptying effects due to fractionation. Formulation IV, containing only propellent C 318, could not fractionate. On the other hand, Formulation III, containing propellents and alcohol with significantly different densities and vapor pressures, would be most prone to show fractionation effect. The result of this fractionation is proportionally higher vaporization of component with higher vapor pressure, thereby leaving the liquid phase gradually more and more concentrated in the component with lower vapor pressure as the level of container emptying increases. To confirm this container-emptying effect due to fractionation, Formulations I, II, and III were subjected to quantitative analysis, using gas chromatography. Figures 1 and 2 are the plots of data obtained by quantitative analysis of the liquid phase at various levels of container emptying. These figures confirm the fractionation effect.

Although container-emptying effects are not large, they are real and should not be ignored. Formulations that minimize these effects would appear to be preferable. Fractionation effects in themselves are not critical in regard to dose of active ingredient as the formulation is measured by volume in the metering chamber. Vaporization of the propellent into the headspace would tend to concentrate any active ingredient during container use. However, persons evaluating aerosols should be aware that weights will change in most formulations with container emptying. Even when

Table III—Mean Dose[a] Delivered, mg., from Meter Valves at Various Levels of Container Emptying

Level of Emptying	Type of Meter Valve E 100U	V 100U	V 50U	E 50U	E 50I	V 50I	R 50I	V 50I-DA	R 50I-EC	S 50I-EC
Formulation I										
Initial	165.4	134.0	69.5	74.5	68.0	69.0	71.0	73.0	68.3	87.6
10%	163.5	130.2	68.7	74.6	68.1	69.2	71.7	75.2	68.7	88.7
50%	163.0	130.2	68.9	73.2	68.5	69.7	72.5	76.2	69.6	91.3
80%	159.2	131.0	68.3	72.6	68.9	69.8	72.2	77.4	70.5	92.9
Total mean	162.8	131.3	68.9	73.3	68.3	69.4	71.8	76.1	69.3	90.1
Coeff. of variation, %	4.24	5.18	3.63	6.21	2.08	4.90	3.39	5.73	2.22	3.80
Formulation II										
Initial	168.6	123.1	65.9	75.5	65.4	65.8	70.9	75.4	69.7	89.4
10%	161.9	123.0	64.9	75.1	66.0	65.6	72.2	76.2	70.6	90.8
50%	156.0	122.1	64.2	74.0	68.0	67.4	73.0	77.3	71.6	92.9
80%	146.2	121.8	64.2	72.0	68.5	67.2	73.4	78.3	72.3	94.6
Total mean	158.2	122.5	64.8	74.2	66.9	66.5	71.6	76.7	71.0	91.9
Coeff. of variation, %	7.96	3.43	2.64	4.31	2.48	5.26	3.38	1.51	2.43	2.87
Formulation III										
Initial	89.1	74.4	39.8	38.2	38.4	41.0	46.4	43.4	40.1	43.3
10%	88.7	71.7	38.5	37.8	37.4	38.7	44.4	43.0	39.3	47.8
50%	79.1	67.7	37.8	35.9	37.3	37.9	43.9	41.8	37.8	46.9
80%	70.5	67.6	36.0	33.7	36.8	36.9	42.8	40.0	36.8	45.5
Total mean	81.8	70.4	38.0	36.4	37.5	38.9	44.1	42.0	38.5	47.1
Coeff. of variation, %	12.35	4.69	5.26	6.87	5.01	3.78	2.15	3.33	3.94	5.41

[a] Average of 40 values.

Table IV—Mean Dose[a] Delivered, mg., from Meter Valves at Various Levels of Container Emptying

Level of Emptying	Type of Meter Valve: E 50I	V 50I	V 50I-DA	R 50I-EC	S 50I-EC
	Formulation IV				
Initial	64.2	71.1	79.6	72.7	96.7
10%	64.1	70.7	79.2	73.0	97.7
50%	64.0	70.2	79.0	72.8	97.1
80%	63.9	70.1	79.4	72.8	97.6
Total mean	64.1	70.5	79.3	72.8	97.0
Coeff. of variation, %	1.5	1.73	1.28	1.61	1.42

[a] Average of 40 values.

Table V—Percent Remaining in Package after Dose Fell below Acceptable Limits[a]

Formulation	Type of Valve: E 100V	V 100U	E 50U	V 50U	E 50I	V 50I	R 50I	V 50I-DA	R 50I-EC	S 50I-EC
I	18	14	19	16	11	15	16	0.9	2.3	3.7
II	16	13	12	11	12	12	15	0.6	1.8	3.2
III	7	10	8	9	7	8	11	0.2	1.1	1.3
IV					8	11		0.7	2.3	3.9
Average	13.7	12.3	13.0	12.0	9.5	11.5	14.0	0.6	1.9	3.0

[a] Sudden decrease (>10%) in the weight of individual doses for two successive actuations.

Table VI—Comparison of Initial Dose after 16-hr. Storage with Doses Obtained at 45-min. Intervals[a]

Formulation	Valve Type: E 100U	V 100U	E 50U	V 40U
I Initial	164[b]/165[c]	129/134	75.4/74.5	70.1/69.5
80% *CE*	164/159	126/131	70.1/74.6	68.3/68.3
II Initial	173/169	125/123	79.4/75.5	66.3/65.9
80% *CE*	157/146	123/122	73.0/72.0	64.6/64.2
III Initial	91.0/89.1	74.1/74.4	42.9/38.2	40.3/39.8
80% *CE*	72.2/70.5	68.4/67.6	34.2/33.7	35.2/36.0

[a] Upright valves stored in upright position. [b] Initial dose after 16-hr. storage (average of 10 values). [c] Doses obtained at 45-min. intervals between actuations (average of 40 values).

Table VII—Comparison of Initial Dose after 16-hr. Storage with Doses Obtained at 45-min. Intervals (Inverted Valves)

Formulation	Valve Type: E 50I (Stored Upright)	V 50I (Stored Upright)	R 50I (Stored Inverted)	V 50I-DA (Stored Inverted)	R 50I-EC Upright	R 50I-EC Inverted	S 50I-EC Upright	S 50I-EC Inverted
I Initial	58.9[a]/68.0[b]	68.3/69.8	73.0/72.0	75.6/73.9	67.0/68.3	68.1/68.3	85.7/87.6	88.0/87.6
80% *CE*	27.8/68.7	34.7/69.0	73.3/71.2	78.3/79.4	69.9/70.5	70.5/70.5	70.0/92.9	91.3/92.9
II Initial	35.7/65.4	53.9/65.8	72.2/70.9	76.9/75.4	66.4/69.7	69.9/69.7	88.7/89.4	89.8/89.4
80% *CE*	18.0/68.5	19.1/67.2	72.8/70.4	79.9/78.2	70.9/72.3	72.6/72.3	89.3/94.6	94.1/94.6
III Initial	37.8/37.5	40.5/41.0	46.9/46.4	44.4/43.4	38.6/40.1	39.9/40.1	46.1/48.3	48.7/48.3
80% *CE*	35.7/36.8	35.1/36.9	43.3/41.8	40.9/40.0	35.2/36.8	38.0/36.8	42.0/45.5	45.1/45.5

[a] Initial dose after 16-hr. storage (average of 10 values). [b] Doses obtained at 45-min. intervals (average of 40 values).

Table VIII—Comparison of Initial Dose after 16-hr. Storage with Doses Obtained at 45-min. Intervals (Inverted Valves), Formulation IV

	Valve Type: E 50I		V 50I		V 50I-DA		R 50I-EC		S 50I-EC	
	Inverted	Upright	Inverted	Upright	Inverted	Upright	Inverted	Upright	Inverted	Upright
Initial	67.0[a]/64.2[b]	30.3/64.2	74.4/71.1	24.9/71.1	82.6/80.0	54.3/80.0	73.8/72.7	71.5/72.7	97.2/96.7	94.9/96.7
80% *CE*	65.2/63.9	23.9/63.9	71.5/70.0	14.4/70.0	79.5/79.0	10.9/79.0	73.7/72.8	71.7/72.8	98.3/97.6	94.0/97.6

[a] Initial dose after 16-hr. storage (average of 10 values). [b] Doses obtained at 45-min. intervals (average of 40 values).

these effects are included, individual doses fall well within ±15% of mean for most of the valves and formulations.

Although the weight variation of doses delivered from meter valves would appear in most cases to meet USP and NF weight variation standards for capsules and small weight tablets, other areas of concern do exist. One major problem with most meter valves is the determination of the point at which the dose falls below acceptable limits. Using the arbitrary end-point of two successive individual doses being at least 10% less than the previous dose, the percent remaining in the aerosol package with each type of valve was determined. These values are shown in Table V. The results, to say the least, are disturbing, with the exception of three valves (V 50I-DA, R 50I-EC, and S 50I-EC). A significant quantity remains in the container beyond the point where doses fall below acceptable limits. In most cases the patient is not able to determine either visually or audibly that any change in dose has occurred. Such a phenomenon is not or would not be tolerated in any other dosage form. Its acceptance in aerosols becomes particularly questionable when it is obvious that valves do exist that effectively eliminate the problem. The apparent formulation effect which indicates that Formulation III is better than I or II is only a result of product densities. Approximately the same number of doses remains in the container for all formulations tested. The end-point for the upright valves is a function of the length of the dip

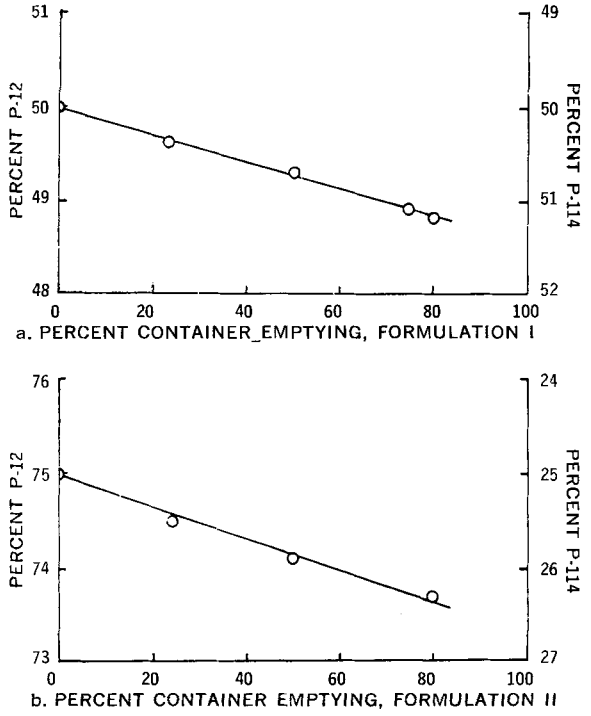


Figure 1—*Effect of container emptying on ratio of propellents 12/114.*

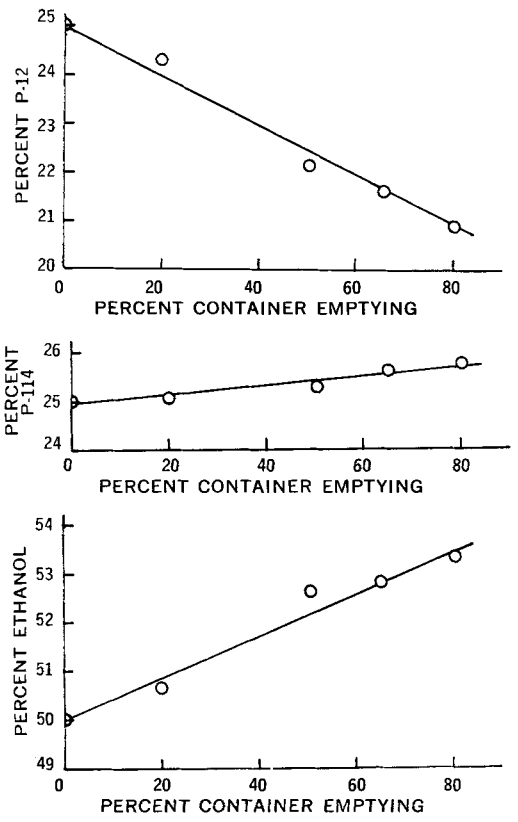


Figure 2—*Effect of container emptying on proportion of P-12/P-114/ethanol.*

tube and is generally more variable than that found in inverted valves.

The fact that initial doses from meter valves after a period of nonuse are often low has long been recognized. In many cases it was believed that the time necessary to cause a significant decrease in the weight delivered was longer than would ordinarily be encountered in practice. The data presented in Tables VI–VIII indicate that a problem does exist under ordinary use conditions for some valves. As can be seen in Table VI, no significant difference appears to exist with upright valves between the dose obtained after a 16-hr. storage and those obtained at 45-min. intervals. These data were collected from containers stored in the upright position. Observation of the bottles and dip tubes indicates that the liquid remains in the dip tube in contact with the bottom of the valve stem at all times, preventing drainback.

Inverted valves appear to exhibit a problem as can be seen in Table VII. Doses obtained from inverted valves after 16-hr. storage in the upright position showed a significant loss in weight, with the exception of only one valve (R 50I-EC). In addition, this loss of weight is even larger at later stages of container emptying, amounting to from one-third to one-half of the dose obtained from 45-min. actuations. Of equal importance is the fact that when inverted valves are stored in the inverted position, no significant differences occur, as can be seen from the data for valves R 50I, V 50I-DA, R 50I-EC, and S 50I-EC.

To verify the importance of storage position and eliminate any variations due to fractionation, valves E 50I, V 50I, V 50I-DA, R 50-I-EC, and S 50I-EC were studied in both upright and inverted storage conditions, using Formulation IV. The results of the study are shown in Table VIII. These data show conclusively that drainback does occur in inverted valves without efficient emptying cups and that this drainback is a function of storage position. Although the cause is not obvious, container emptying accentuates the drainback problem. Unfortunately, data were not collected at intermediate levels of container emptying in such a form to indicate when this effect first begins to occur. At least one manufacturer has designed an inverted valve, which effectively decreases drainback, by adding a chamber around the lower tank opening. At least one commercial product avoids the problem because packaging and labeling are designed so that containers are customarily stored in the inverted position. It would appear that all producers using inverted valves might well adopt this policy.

SUMMARY AND CONCLUSIONS

1. Container-emptying effects resulting from propellent fractionation and causing changes in the weights of doses delivered do occur in inhalation formulations.

2. A new simple technique is described to measure the extent of fractionation, using a gas chromatograph.

3. A significant decrease in dose weights at the latter stages of container emptying is a serious problem in metered aerosols. Three valves appear to be significantly superior to all others in minimizing variation of dose through the latter stages of container emptying.

4. Doses delivered from inverted meter valves after standing all night in an upright position are significantly lower than those delivered at 45-min. intervals. These effects are magnified at latter stages of container emptying. The problems can be solved by designing packaging and labeling so that containers with inverted valves are stored in an inverted position.

REFERENCES

(1) I. Porush, C. G. Thiel, and J. G. Young, *J. Amer. Pharml Ass., Sci. Ed.*, **49**, 70(1960).

(2) J. G. Young, I. Porush, C. G. Thiel, S. Cohen, and C. H. Stimmel, *ibid.*, **49**, 72(1960).

(3) W. M. Grim, J. B. Portnoff, F. A. Testaino, and R. O. Toberman, *Aerosol Age*, **13**, 22(1968).

(4) A. M. Contractor, R. F. Shangraw, and M. D. Richman, *Drug Cosmet. Ind.*, **105**, 44(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Department of Pharmacy, Schoo. of Pharmacy, University of Maryland, Baltimore, MD 21201*

Accepted for publication May 6, 1970.

Presented at the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This work was supported by research funds from Barr-Stalfort, Inc., Baltimore, Md.

* Present address: Pharmaceutical Aerosol Division, Barr-Stalfort, Inc., Baltimore, Md.

Bentonite–Cellulose Systems: Flow Behavior of Mixed Dispersions and Mechanical Properties of Composite Films

HANS SCHOTT

Abstract □ Bentonite and microcrystalline cellulose are used as thickening agents and binders; their interaction was studied in suspension and in the solid state. Mixtures of the two solids covering the whole range of compositions were prepared by blending their aqueous suspensions under high shear. The rheological behavior of the mixed suspensions and the mechanical properties of composite films prepared by drying thin layers of these suspensions were investigated as a function of composition. Based on the volume-percentage of the dry components, bentonite was about five times more effective in increasing the viscosity of aqueous suspensions than cellulose. Cellulose tended to produce yield values. Bentonite formed strong films when its aqueous suspensions were dried; neither pure cellulose nor a mixture containing 88 vol. % cellulose formed coherent films. In the range of 0–77 vol. % cellulose, the in-plane and perpendicular tensile strengths of the composite films decreased only moderately with increasing cellulose content.

Keyphrases □ Bentonite–cellulose systems—flow behavior, mechanical properties □ Cellulose–bentonite systems—flow behavior, mechanical properties □ Films, bentonite–cellulose—relative composition effect □ Rheological behavior, bentonite–cellulose suspensions—relative composition effect

Bentonite and finely dispersed cellulose are used as thickening agents and binders in suspensions, ointments, and tablets (1, 2). The rheology of aqueous bentonite dispersions (3–5) and cellulose suspensions (6, 7), as well as the film-forming ability of sodium bentonite (8), has been reported. The present work deals with the flow behavior of mixed dispersions, containing varying proportions of cellulose and clay, and with the mechanical properties of films prepared therefrom. The purpose was to determine whether there is any synergism in thickening and binding; for instance, whether partial replacement of bentonite by cellulose boosts the viscosity of bentonite suspensions, or whether the presence of cellulose increases the strength of solid bentonite. The interaction between clays and cellulose is described in previous work (9, 10).

EXPERIMENTAL

Materials—Microcrystalline cellulose[1] is manufactured by acid hydrolysis of wood pulp with a high α-cellulose content to the "level-off degree of polymerization," followed by mechanical disintegration (11, 12). Suspensions were made by dispersing the powder in water with a Waring blender. They were permitted to hydrate 24 hr. before admixing clay dispersions.

Sodium bentonite[2] was slurried in a Waring blender with water for 10 min. at top speed and was decanted from settled out grit and heavy particles. This reduced the clay concentration from 28 to 21 g./l. The supernatant dispersion was passed through a column of a commercial cation-exchange resin[3] in the sodium form. The pH of a dispersion containing 21 g./l. was 9.8. Since the microcrystalline cellulose is partly soluble in alkaline media, the pH of the clay dispersion was adjusted to 5–7 as follows: a small amount of the sodium bentonite dispersion was passed through a column of the cation-exchange resin in the hydrogen form, which reduced its pH to 2.2. It was then mixed immediately with enough of the sodium form to obtain the desired pH.

The water was twice distilled. Neither bentonite nor cellulose contained water-soluble impurities. Mixed suspensions were prepared by combining the required amounts of clay dispersions and cellulose suspensions of known concentrations and stirring together in a Waring blender, followed by two passes through a hand homogenizer. Lower viscosities and yield values were found if homogenization was omitted. The total solids content of dispersions was sometimes determined by drying at 125° to ensure against concentration changes due to evaporation of water. The nominal and actual compositions always agreed within ±0.01%, expressed as solids content.

After viscosity measurements, the suspensions were concentrated, spread into thin layers on metal or glass trays, and dried in a forced-circulation oven. As a routine precaution, the composition of a few films was determined from the ash content at 600°. The difference between nominal and actual composition was always below 0.1% in terms of percentage composition values. This shows that preferential settling out of bentonite or of cellulose from the suspensions during handling did not occur.

Methods—Viscosity measurements were made with a rotational Synchro-Lectric viscometer,[4] using chiefly the No. 1 and occasionally the No. 2 spindle. The instrument was used at 2, 4, 10, and 20 r.p.m. with the guard in place. The 650-ml. samples were measured in 800-ml. beakers. All measurements were made at 22–23.5°.

Because the viscosity of suspensions containing cellulose rose very rapidly even after short rest periods, the flow measurements had to be restricted to those observable after elimination of thixotropic thickening. Reproducible results were obtained when the suspensions were stirred for 10 min. with a magnetic stirrer at top speed immediately before measuring viscosities. Sometimes, thixotropic increases in viscosity occurred even during viscosity measurements. In those cases, the suspensions were stirred between readings as often as was necessary to obtain reproducible values.

Flow curves were drawn as revolutions per minute (rate of shear) *versus* scale divisions of torque (shear stress); the full scale was 500 divisions. Yield values, Y, are reported as scale divisions for No. 1 spindle at the point where the extrapolated flow curve intersected the shear stress axis. They are only approximate values, particularly for those systems where rapid thixotropic buildup of viscosity introduced some uncertainty in the readings at 2 r.p.m.

There are two convenient and representative ways of defining the viscosity of shear-thinning liquids, some of which have yield values. The first, "differential viscosity," is defined here as the reciprocal slope of the secant to the flow curve at the points for 10 and 20 r.p.m. Since the instrument had only four speeds, this was more reproducible than drawing the tangent to the flow curve at a given r.p.m. and using its reciprocal slope (13, 14). The differential viscosities, $\eta^*_{\text{dif.}}$, in centipoises (cps.), were calculated as

$$\eta^*_{\text{dif.}} = K(\Delta \text{ div.}/\Delta \text{ r.p.m.}) \qquad \text{(Eq. 1)}$$

which became

$$\eta^*_{\text{dif.}} = 1.96\left[\frac{\text{div. (20 r.p.m.)}}{1.06} - \text{div. (10 r.p.m.)}\right] \qquad \text{(Eq. 2)}$$

[1] Avicel-PH, FMC Corp., Marcus Hook, Pa.
[2] Volclay bentonite, 325 mesh, American Colloid Co., Skokie, Ill.
[3] Amberlite IR-120, Rohm & Haas Co., Philadelphia, Pa.
[4] Model RVF, Brookfield Engineering Laboratories, Inc., Stoughton, Mass.

for the No. 1 spindle, and

$$\eta^*_{dif.} = 8.2\left[\frac{\text{div. (20 r.p.m.)}}{1.17} - \text{div. (10 r.p.m.)}\right] \quad \text{(Eq. 3)}$$

for the No. 2 spindle; div. (20 r.p.m.) is the number of scale divisions on the torque scale ranging from 0–500 at the speed of 20 r.p.m. The numerical constants were determined by calibrating the instrument with a 60% sucrose solution (48 cps. at 23.2°).

A second way of describing the flow behavior is as "apparent plastic viscosity" (15), by analogy to plastic and pseudoplastic liquids (16). Apparent plastic viscosities, $\eta^*_{pl.}$, in cps., for the No. 1 spindle were calculated as

$$\eta^*_{pl.} = \frac{\text{div. (20 r.p.m.)}}{1.06} - Y \quad \text{(Eq. 4)}$$

This is the reciprocal slope of the secant to the flow curve at the point of 20 r.p.m. and the yield value or, if $Y = 0$, at the origin. Such computations are necessary because, while the suspensions containing only cellulose gave nearly linear flow curves above the yield value between 4 and 20 r.p.m., many of the suspensions containing clay gave flow curves that were convex toward the shear stress axis, and the two kinds of curves must be compared. For Newtonian liquids, differential viscosity and apparent plastic viscosity are identical with the Newtonian viscosity.

The mechanical properties of the films were measured after conditioning at 23° and 50% relative humidity, using a recording stress-strain tester equipped with strain gauges.[5] Tensile strength in the plane of the film (x–y direction) was determined on strips 1.27 cm. (0.5 in.) wide and with a 2.54-cm. (1.0-in.) distance between the clamps, which were elongated at the rate of 2%/min. To compensate for differences in density, the tensile strength is expressed as breaking length, *i.e.*, the smallest length of film sufficient to cause it to break under the load of its own weight. The tensile strength perpendicular to the plane of the film (z-direction) was determined by gluing a 2.54 × 2.54-cm. (1 × 1-in.) film specimen with epoxy cement between two cubic aluminum blocks of 2.54-cm. (1-in.) side length, attaching one to the upper jaw of the tensile testing machine and the other to the crosshead, and lowering the latter at the rate of 0.025 cm./min. (0.01 in./min.). The z-direction tensile strength represents resistance to delamination. The epoxy cement did not appreciably penetrate the clay film because, except for the layer immediately adjoining the aluminum surface, the ruptured films swelled and dispersed freely in water.

RESULTS

Flow Properties—Typical flow curves are shown in Fig. 1. Differential and apparent plastic viscosities and approximate yield values are listed in Table I. Compositions are expressed as volume percent of dry material, with a density of 1.5 g./cm.^{3} for cellulose, 2.8 g./cm.^{3} for clay, and 1.0 g./cm.^{3} for water. Yield values are expressed as scale divisions for the No. 1 spindle, full scale being 500 divisions. As indicated by Table I, clay tends to build up viscosity faster than cellulose, and cellulose tends to produce yield values faster than clay with increasing concentration.

The effect on the differential viscosity of gradually replacing clay with microcrystalline cellulose while maintaining the total concentration of suspended solids constant at 1.64 vol. % of dry material is shown in Fig. 2. Also shown is the effect of concentration on the differential viscosity of dispersions of pure clay. The abscissa scale representing clay concentration is the same for both curves. Therefore, the upper, broken curve refers to compositions containing a concentration of cellulose equal to 1.64 vol. % minus the indicated clay concentration.

If the two curves coincided, microcrystalline cellulose would have the same thickening effect on clay dispersions as water, *i.e.*, none. If the broken curve representing the viscosity of mixed suspensions at constant total concentration was a straight horizontal line located at the differential viscosity of a pure clay dispersion containing 1.64 vol. %, namely 135 cps., cellulose would have the same thickening effect on clay dispersions as an equal volume of additional clay. The broken curve was only slightly above the solid curve, indicating that microcrystalline cellulose exerts only a modest thickening effect on clay dispersions.

[5] Instron Corp., Canton, Mass.

Table I—Differential Viscosities, Apparent Plastic Viscosities, and Yield Values for Suspensions Containing Na^+/H^+ Bentonite and/or Microcrystalline Cellulose

Clay, vol. %	Cellulose, vol. %	Differential Viscosity, cps.	Apparent Plastic Viscosity, cps.	Yield Value, Five-Hundredths of Full Scale
0.730	0	13	11.5	0
0.864	0	16	15	0
1.024	0	19	18.5	0
1.118	0	22	23	0
1.199	0	26	30	0
1.213	0	28	32	0
1.317	0	33	41.5	1
1.430	0	47	64	2
1.552	0	78	108	9
1.650	0	135	194	22
0	1.616	7	7	0
0	3.149	9	11	9
0	3.252	10	13	7
0	3.355	11	12	8
0	3.430	10	11	9
0	3.459	9	12	10
0	3.562	13	14.5	9
0	3.666	12	18	16
0	3.770	11	19	17
0	3.874	12	21	20
0	3.947	14	18	25
0	3.978	18	28	26
0	4.082	18	23	27
0	4.186	16	25	32
0.182	1.441	11	8.5	0
0.365	1.265	12	10.5	0
0.552	1.087	15	12.5	0
0.736	0.907	18	14	1
0.921	0.727	19	21	1
1.108	0.547	24	28	2
1.299	0.360	41	49	3
1.486	0.183	72	97	9
0.344	3.085	71	—	—
0.257	3.172	44	—	—
0.129	3.294	29	20	56
0.150	3.840	39	40	152
0.300	3.700	107	120[a]	610[a]
0.514	2.915	123	140[a]	550[a]

[a] Divisions measured with No. 2 spindle × 4.

The reverse situation is shown in Fig. 3. Starting with a cellulose suspension of 3.430 vol. % and replacing small but increasing amounts with clay at a constant volume fraction of dry solids caused

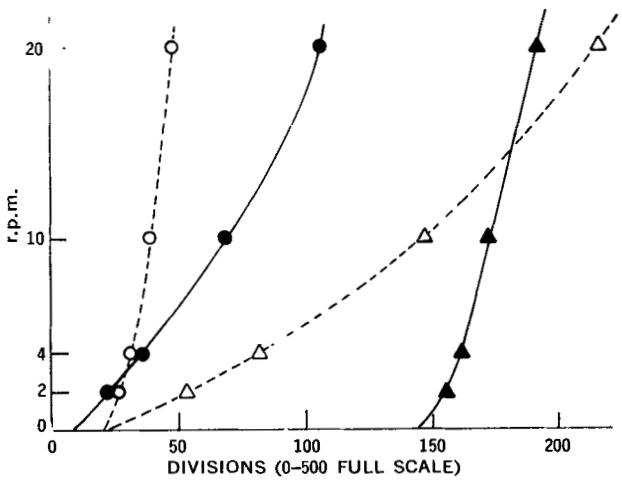


Figure 1—*Typical flow curves for suspensions of bentonite, microcrystalline cellulose, and their mixtures. Key:* ○, *3.874 vol. % cellulose;* ●, *1.486 vol. % clay + 0.183 vol. % cellulose;* △, *1.650 vol. % clay; and* ▲, *3.840 vol. % cellulose + 0.150 vol. % clay.*

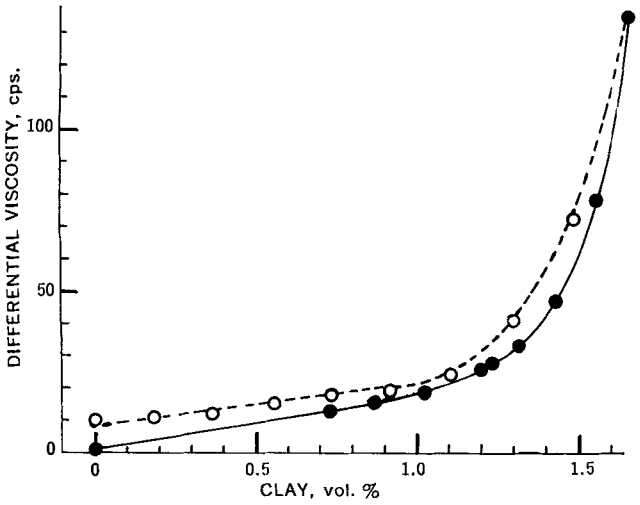


Figure 2—*Differential viscosity of clay dispersions as a function of clay content, and the effect of replacing clay with cellulose. Key:* ●—●, *clay only; and* O- - -O, *gradual replacement of clay with cellulose at a constant solids content of 1.64 ± 0.02 vol. %.*

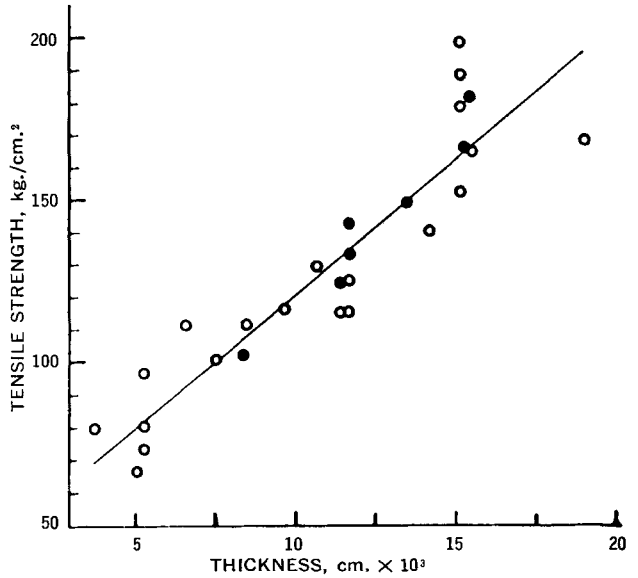


Figure 4—*Effect of thickness on the tensile strength of Na^+ bentonite films. Rate of elongation:* O, *2%/min.; and* ●, *10%/min.*

a rapid increase in differential viscosity. In the range of compositions studied, this increase amounted to about 170 cps./vol. % replaced. If the increase in viscosity per volume percent was the same for cellulose as for clay, the broken curve would be a straight horizontal line located at the differential viscosity of the 3.430 vol. % cellulose suspension, namely 10 cps. The rapid divergence of the two curves shows that for suspensions of microcrystalline cellulose, bentonite is a far more effective thickening agent than are additional amounts of cellulose. Similar plots and the same conclusions can be obtained with apparent plastic viscosities.

The following numerical examples show how the relative thickening efficiency of clay and that of cellulose were compared. From Fig. 2, it is seen that a suspension containing 0.47 vol. % clay and 1.64 − 0.47 = 1.17 vol. % cellulose has the same differential viscosity as a 0.80 vol. % clay dispersion. Therefore, 0.80 − 0.47 = 0.33 vol. % clay is approximately equivalent in thickening power to 1.17 vol. % cellulose, or 1 vol. % clay is equivalent to 3.55 vol. % cellulose. From Table I, a 4.0-vol. % cellulose suspension is seen to have the same differential viscosity as the interpolated value for a 0.98-vol. % clay dispersion, and a 3.8-vol. % cellulose suspension the same as a dispersion containing 0.73 vol. % clay. The equivalent of 1 vol. % clay is, therefore, 4.08 and 5.20 vol. % cellulose, respectively. The values obtained by this kind of calculation, based on differential viscosities, ranged from 1:3.0 to 1:9.0, with a mean equivalent thickening ratio of 1:4.5 vol. % dry clay–vol. % dry cellulose. Similar calculations for apparent plastic viscosities gave a range of 1:3.2–1:10.8 for equivalent thickening ratios of clay–cellulose and a mean of 1:4.9. The large spread in the values may be due to the fact that the structural mechanism by which bentonite thickens a cellulose suspension is different from the one operative in a dispersion of pure bentonite. The 4.5 and 4.9 ratios are only meaningful as an order of magnitude.

According to Einstein's law, the viscosity of dispersions is determined only by the total volume fraction of the dispersed phase and not by its particle size. The present systems are strongly non-Newtonian; even the viscosity of very dilute bentonite dispersions was found to be 70 times larger than that predicted by Einstein's law, owing to the interaction between particles (5). This interaction is responsible for the pronounced thickening observed. Thickening, *i.e.*, high viscosities and yield values, results from three-dimensional networks formed by particle–particle association. Small particle sizes, as shown by high specific surface areas, and highly asymmetric particle shapes are conducive to thickening (*Reference 13*, p. 338; *Reference 14*, p. 180). The increase in viscosity with de-

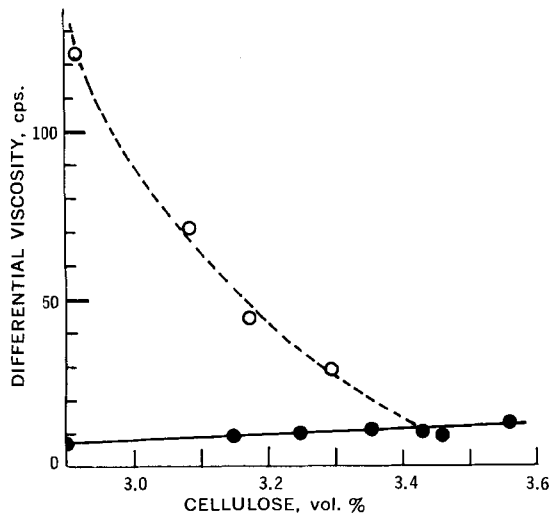


Figure 3—*Differential viscosity of cellulose suspensions as a function of cellulose content, and the effect of replacing cellulose with clay. Key:* ●—●, *cellulose only; and* O- - -O, *gradual replacement of cellulose by clay at a constant solids content of 3.430 ± 0.004 vol. %.*

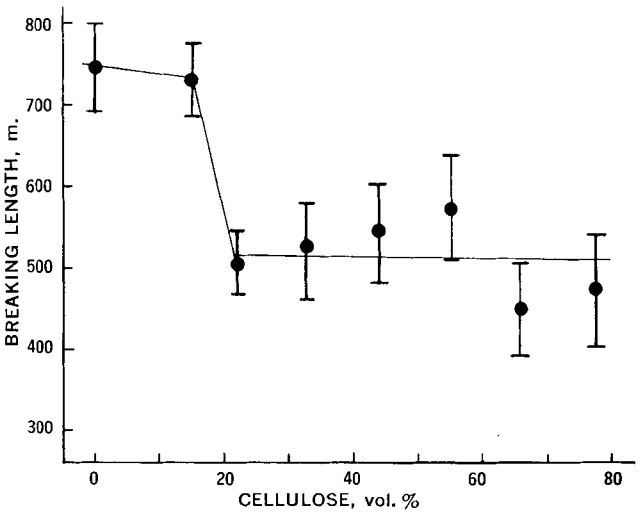


Figure 5—*Effect of microcrystalline cellulose on the breaking length of Na^+/H^+ bentonite films.*

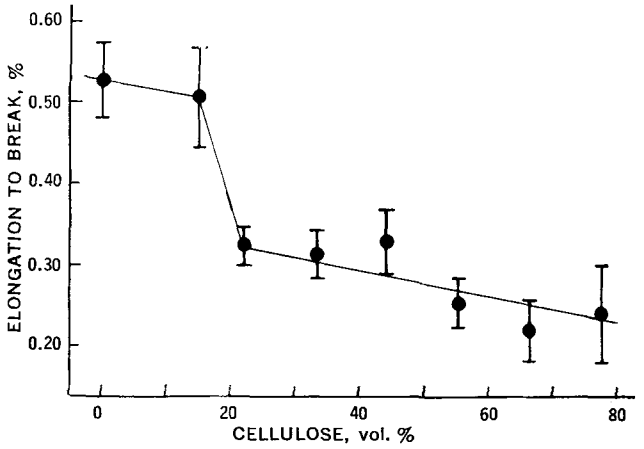


Figure 6—*Effect of microcrystalline cellulose on the elongation to break of Na^+/H^+ bentonite films.*

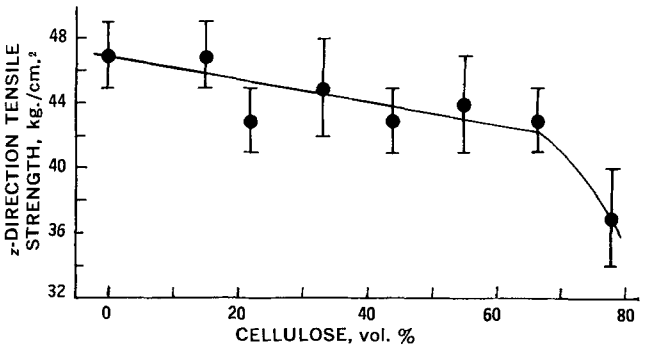


Figure 7—*Effect of microcrystalline cellulose on the z-direction tensile strength of Na^+/H^+ bentonite films.*

creasing particle size has also been attributed to a solvation shell surrounding the particles, which increases their effective volume (17).

The kind of bentonite used here has stacks of 3–4-lattice layers as primary particles. These swell in water by inserting three layers of water, each 3 Å thick, between adjacent clay layers (5, 18). Neglecting the lateral area, the specific surface area corresponding to the basal planes is 240 m.²/g. The primary particles in microcrystalline cellulose, which had been stirred for 10 min. at top speed in a Waring blender and given two passes through a hand homogenizer, were rods or laths with a median length between 4.5 and 6.5 μ and a median diameter or width between 1.0 and 1.6 μ when wet. According to these microscopic measurements, the specific surface area of the water-swollen particles was about 3 m.²/g.

The bentonite lamellas are far more asymmetrical than the rods of microcrystalline cellulose. The higher specific surface area of the clay particles compared to the cellulose particles, and their greater asymmetry, should produce an equivalent thickening ratio far greater than 1:4.5 or 1:4.9. This may indicate the existence of some specific effects which enhance the thickening power of the microcrystalline cellulose or reduce that of bentonite. Hydrogen-bond formation resulting in more and stronger particle-to-particle attachments for cellulose is unlikely to be responsible for the smaller than expected difference in thickening, because bentonite particles can adhere to cellulose surfaces by hydrogen bonds (as proton acceptors) as readily as two cellulose surfaces adhere to each other (9). There seems to be no reason why the hydration shell surrounding a cellulose particle should be substantially deeper than that around a clay particle of the same size. Perhaps electrostatic effects, specifically the repulsive component of the interaction between the more highly charged clay particles, are involved. Another possible explanation is that the mechanical shearing to which the microcrystalline cellulose was subjected, like the beating of paper-making pulp, frazzled the rods, rendering their surface fuzzy. Microfibrils emanating from the rods need not be visible at the magnification of 500× used in these microscopic observations, yet they could increase the surface area of these rods and considerably enhance their ability to form three-dimensional networks in suspension. This would also explain why the rods seemed to occur in clumps.

Film Properties—Neither pure microcrystalline cellulose nor a mixture containing 88 vol. % cellulose formed coherent films when their aqueous suspensions were dried without external pressure. For the remaining range of compositions, the apparent densities of the conditioned films, *d*, *i.e.*, the weight divided by length × width × thickness, increased with increasing weight % clay (% CL) according to the equation

$$d = 0.544 + 0.0134\%\ \text{CL} \qquad \text{(Eq. 5)}$$

Using 1.5 and 2.8 g./cm.³ as bulk densities of cellulose and clay, the equation indicates that the void volume of the films increased with increasing cellulose content, from 30% for pure clay films to 44% for films containing 77.6 vol. % cellulose. The high void volume associated with microcrystalline cellulose is useful for the absorption of oils and pharmaceuticals in tablets or gels (2).

The mechanical properties of films of pure clay were found to improve with increasing thickness (Fig. 4). This runs counter to the usual observation of increasing strength of films and fibers with decreasing cross-sectional area owing to the reduced probability of finding flaws. For this reason, only films of thickness between 0.010 and 0.015 cm. were tested in later work. As can be seen from Fig. 4, the proportionality between tensile strength and thickness cannot extend to films much thinner than those studied in the present work. Otherwise, extrapolation of the least-squares line based on films in the thickness range of 50–150 μ would result in a tensile strength of nearly 30 kg./cm.² for a film of zero thickness. The correlation coefficient between tensile strength (dependent variable) and film thickness for the data of Fig. 4 is 0.909. At 26 degrees of freedom, this corresponds to a level of significance better than 0.1%.

In Figs. 5–7, each point is the average of at least eight measurements, and the bars represent ± the standard deviation of the averages. The initial 15 vol. % cellulose had no noticeable effects on breaking length and elongation. Higher loadings of cellulose reduced the former by approximately 30% to a nearly constant level independent of cellulose loading. Except for the film containing 77.6 vol. % cellulose, the *z*-direction tensile strength decreased only very slightly with increasing cellulose content (note the expanded ordinate scale of Fig. 7).

The fact that finely dispersed cellulose lowered the strength of sodium bentonite or hydrogen bentonite films only moderately indicates that the adhesion of cellulose to clay is of the same order of magnitude as the adhesion of clay to itself. The strength of the pure bentonite films indicates that the latter is considerable. Mixed cellulose–bentonite films swelled freely and disintegrated readily in water over the entire range of compositions.

REFERENCES

(1) J. B. Sprowls and H. M. Beal, "American Pharmacy," 6th ed., J. B. Lippincott, Philadelphia, Pa., 1960, pp. 290, 291.

(2) O. A. Battista and P. A. Smith, *Ind. Eng. Chem.*, **54(9)**, 20(1962).

(3) H. Van Olphen, "An Introduction to Clay Colloid Chemistry," Interscience, New York, N. Y., 1963, pp. 135–146.

(4) A. Kahn, *Proc. Nat. Conf. Clays Clay Miner., 6th*, **1957**, 220.

(5) H. Schott, *J. Colloid Interface Sci.*, **26**, 133(1968).

(6) J. Hermans, Jr., *J. Polym. Sci., Part C*, **2**, 129(1963).

(7) N. Z. Erdi, M. M. Cruz, and O. A. Battista, *J. Colloid Interface Sci.*, **28**, 36(1968).

(8) E. A. Hauser and D. S. LeBeau, *J. Phys. Chem.*, **42**, 961 (1938).

(9) H. Schott, *Text. Res. J.*, **35**, 612(1965).

(10) H. Schott, *J. Amer. Oil Chem. Soc.*, **45**, 414(1968).

(11) O. A. Battista, S. Coppick, J. A. Howsmon, F. F. Morehead, and W. A. Sisson, *Ind. Eng. Chem.*, **48**, 333(1956).

(12) O. A. Battista, *J. Polym. Sci., Part C*, **9**, 135(1965).

(13) W. Philippoff, "Viskositaet der Kolloide," Theodor Steinkopff, Dresden, East Germany, 1942, p. 3.

(14) E. K. Fischer, "Colloidal Dispersions," Wiley, New York, N. Y., 1950, p. 15.

(15) E. N. da C. Andrade, "Viscosity and Plasticity," Heffer & Sons, Cambridge, England, 1947, Lecture I.
(16) H. Green, "Industrial Rheology and Rheological Structures," Wiley, New York, N. Y., 1949, pp. 19, 20.
(17) A. C. Zettlemoyer and G. W. Lower, *J. Colloid Sci.*, **10**, 29(1955).
(18) H. Schott, *Kolloid-Z. Z. Polym.*, **199**, 158(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 29, 1969, from *Temple University School of Pharmacy, Philadelphia, PA 19140*

Accepted for publication April 30, 1970.

The author is indebted to Mr. J. F. Wichmann of the U. S. Forest Products Laboratory, Madison, Wis., for measuring the mechanical properties of the films.

NOTES

Cautions Regarding the Physical Interpretation of Statistically Based Structure–Activity Relationships

ARTHUR CAMMARATA*, RICHARD C. ALLEN*, J. K. SEYDEL†, and E. WEMPE†

Abstract □ The distinction between the use of multiple regression analysis as a predictive tool and as a means of investigating controlling physical characteristics in structure–activity studies often is unrecognized. Three examples of complications that can arise with either of these goals in mind are discussed. The first is an illustration of a "false" parabolic dependence of activity on lipophilicity; the second deals with unrecognized interrelationships between certain physical parameters; and the third is a situation where a number of statistically significant correlations can be presented, each of which may be given a differing physical interpretation.

Keyphrases □ Structure–activity relationships—precautions concerning interpretation □ Multiple regression analysis—use, misuse

Within the past few years, multiple regression analysis has been exploited as a statistical tool for the evaluation of structure–activity data. One goal of these analyses is the derivation of a regression equation which will provide estimates of the biological potencies for additional structural entities within a series. A second goal is the determination of the physical and chemical properties of a given series of compounds which are most influential in affecting the observed biological potencies. It is often not recognized that each of these goals represents a separate problem, because the multiple regression approach usually makes use of physically meaningful parameters. As a consequence, any regression equation that correlates structure–activity data can be given a physical interpretation. At times, however, a quirk within a set of structure–activity data can lead to a statistically significant regression equation which provides a poor, if not erroneous, reflection of the physical factors affecting biological potency. At other times, a physical interpretation becomes difficult because a number of correlations can be presented for the same data, each of which involves one or more parameters that could be given a differing physical interpretation. Three examples to illustrate these complications are discussed in this report.

EXAMPLE 1

The minimum inhibitory concentrations against *Escherichia coli* for a variety of congeneric sulfanilamides have been correlated linearly with the Hammett σ-value or with the pKa for the compounds (1, 2). Other congeneric sulfanilamide series have been found (3) which require the addition of π or of π and π^2 terms in a multiple regression model in order to gain a correlation with their bacteriostatic activities. A π term in combination with σ or pKa in a regression equation indicates that lipophilic and electronic factors, respectively, are controlling biological potency. When both π and π^2 appear in a regression equation, the biological activities are parabolically related to the lipophilicities of the compounds; *i.e.*, there is an optimal lipophilicity to observe a maximum biological response within the series.

A reasonable approach to follow if regression equations are to be used as a guide to further syntheses is first to synthesize and test a relatively few compounds which vary over a wide range with respect to their expected electronic (σ) or lipophilic (π) characteristics. If the subsequent regression analysis requires the addition of a physical parameter other than that chosen as an initial criterion to correlate the activity data, it could be said that the additional parameter (or parameters) reflects a real physical requirement for the system under study. Following this approach, N^1-benzoylsulfanilamides having substituents covering extremes in Hammett σ-values (Compounds 1–7; Table I) are found to have their bacteriostatic potencies correlated by the equation

$$\log(1/C) = \underset{(-4.99)}{-0.81}\ (\pm 0.16)\sigma + \underset{(3.74)}{1.18}\ (\pm 0.31)\pi \quad \text{(Eq. 1)}$$
$$\underset{(-3.38)}{-1.19}\ (\pm 0.35)\pi^2 + 5.10$$

N	s	R	$F(3,3)$
7	0.12	0.98	34.67

In Eq. 1, the standard error for the estimate of a coefficient appears in parentheses after the coefficient; in parentheses below the coefficient is the t test. The statistics for the equation are the standard error of the estimate s, the multiple correlation coefficient R, and the F-ratio.

Based on Eq. 1, it may be concluded that electronic and lipophilic factors control the bacteriostatic activities for this series of sulfanilamides and that there is an optimum lipophilicity for the series. If the latter conclusion is true, a regression equation based on the activities for a larger number of N^1-benzoylsulfanilamides should retain the π and π^2 terms as found in Eq. 1, since the lipophilicities for the additional compounds should lie on the same parabola as is found for the smaller series. In this particular instance, an extension

(15) E. N. da C. Andrade, "Viscosity and Plasticity," Heffer & Sons, Cambridge, England, 1947, Lecture I.
(16) H. Green, "Industrial Rheology and Rheological Structures," Wiley, New York, N. Y., 1949, pp. 19, 20.
(17) A. C. Zettlemoyer and G. W. Lower, *J. Colloid Sci.*, **10**, 29(1955).
(18) H. Schott, *Kolloid-Z. Z. Polym.*, **199**, 158(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 29, 1969, from *Temple University School of Pharmacy, Philadelphia, PA 19140*

Accepted for publication April 30, 1970.

The author is indebted to Mr. J. F. Wichmann of the U. S. Forest Products Laboratory, Madison, Wis., for measuring the mechanical properties of the films.

NOTES

Cautions Regarding the Physical Interpretation of Statistically Based Structure–Activity Relationships

ARTHUR CAMMARATA*, RICHARD C. ALLEN*, J. K. SEYDEL†, and E. WEMPE†

Abstract □ The distinction between the use of multiple regression analysis as a predictive tool and as a means of investigating controlling physical characteristics in structure–activity studies often is unrecognized. Three examples of complications that can arise with either of these goals in mind are discussed. The first is an illustration of a "false" parabolic dependence of activity on lipophilicity; the second deals with unrecognized interrelationships between certain physical parameters; and the third is a situation where a number of statistically significant correlations can be presented, each of which may be given a differing physical interpretation.

Keyphrases □ Structure–activity relationships—precautions concerning interpretation □ Multiple regression analysis—use, misuse

Within the past few years, multiple regression analysis has been exploited as a statistical tool for the evaluation of structure–activity data. One goal of these analyses is the derivation of a regression equation which will provide estimates of the biological potencies for additional structural entities within a series. A second goal is the determination of the physical and chemical properties of a given series of compounds which are most influential in affecting the observed biological potencies. It is often not recognized that each of these goals represents a separate problem, because the multiple regression approach usually makes use of physically meaningful parameters. As a consequence, any regression equation that correlates structure–activity data can be given a physical interpretation. At times, however, a quirk within a set of structure–activity data can lead to a statistically significant regression equation which provides a poor, if not erroneous, reflection of the physical factors affecting biological potency. At other times, a physical interpretation becomes difficult because a number of correlations can be presented for the same data, each of which involves one or more parameters that could be given a differing physical interpretation. Three examples to illustrate these complications are discussed in this report.

EXAMPLE 1

The minimum inhibitory concentrations against *Escherichia coli* for a variety of congeneric sulfanilamides have been correlated linearly with the Hammett σ-value or with the pKa for the compounds (1, 2). Other congeneric sulfanilamide series have been found (3) which require the addition of π or of π and π^2 terms in a multiple regression model in order to gain a correlation with their bacteriostatic activities. A π term in combination with σ or pKa in a regression equation indicates that lipophilic and electronic factors, respectively, are controlling biological potency. When both π and π^2 appear in a regression equation, the biological activities are parabolically related to the lipophilicities of the compounds; *i.e.*, there is an optimal lipophilicity to observe a maximum biological response within the series.

A reasonable approach to follow if regression equations are to be used as a guide to further syntheses is first to synthesize and test a relatively few compounds which vary over a wide range with respect to their expected electronic (σ) or lipophilic (π) characteristics. If the subsequent regression analysis requires the addition of a physical parameter other than that chosen as an initial criterion to correlate the activity data, it could be said that the additional parameter (or parameters) reflects a real physical requirement for the system under study. Following this approach, N^1-benzoylsulfanilamides having substituents covering extremes in Hammett σ-values (Compounds 1–7; Table I) are found to have their bacteriostatic potencies correlated by the equation

$$\log(1/C) = \underset{(-4.99)}{-0.81}\ (\pm 0.16)\sigma + \underset{(3.74)}{1.18}\ (\pm 0.31)\pi \quad \text{(Eq. 1)}$$
$$\underset{(-3.38)}{-1.19}\ (\pm 0.35)\pi^2 + 5.10$$

N	s	R	$F(3,3)$
7	0.12	0.98	34.67

In Eq. 1, the standard error for the estimate of a coefficient appears in parentheses after the coefficient; in parentheses below the coefficient is the t test. The statistics for the equation are the standard error of the estimate s, the multiple correlation coefficient R, and the F-ratio.

Based on Eq. 1, it may be concluded that electronic and lipophilic factors control the bacteriostatic activities for this series of sulfanilamides and that there is an optimum lipophilicity for the series. If the latter conclusion is true, a regression equation based on the activities for a larger number of N^1-benzoylsulfanilamides should retain the π and π^2 terms as found in Eq. 1, since the lipophilicities for the additional compounds should lie on the same parabola as is found for the smaller series. In this particular instance, an extension

Table I—*In Vitro* Activities against *Escherichia coli* of N^1-Benzoylsulfanilamides

Compound Number	Benzoyl Substituent	σ	π	Activities,[a] log (1/C)
1	4-OMe	−0.27	0.08	5.40
2	4-Me	0.17	0.42	5.40
3	H	0.0	0.0	5.25
4	4-Cl	0.23	0.87	5.10
5	3-CF_3	0.42	1.07	4.65
6	4-NO_2	0.78	0.02	4.50
7	4-CN	0.63	−0.31	4.05
8	3-Me	−0.07	0.52	5.40
9	4-iso-Pr	−0.15	1.40	5.40
10	4-Et	−0.15	0.92	5.62
11	4-*n*-Pr	−0.15	1.42	5.18
12	3,4-Me	−0.24	0.94	5.40
13	3-Me, 4-MeO	−0.44	0.60	5.25

[a] These activities were determined in the laboratories of J. K. S. and have been reported in previous discussions by J. K. Seydel and E. Wempe, *Arzeim.-Forsch.*, **14**, 705(1964), and by A. Cammarata, *J. Med. Chem.*, **11**, 1111(1968).

of the series (Compounds 1–13, Table I) eradicates the π, π^2 dependence:

$$\log(1/C) = \underset{(-4.51)}{-0.88\ (\pm 0.19)\sigma} \underset{(1.81)}{+0.59\ (\pm 0.32)\pi} \underset{(-1.62)}{-0.39\ (\pm 0.24)\pi^2} + 5.04 \qquad \text{(Eq. 2)}$$

N	s	R	$F(3,9)$
13	0.21	0.91	14.78

The equation correlating the total set of data is, therefore,

$$\log(1/C) = \underset{(-6.05)}{-1.06\ (\pm 0.17)\sigma} + 5.15 \qquad \text{(Eq. 3)}$$

N	s	R	$F(1,11)$
13	0.22	0.87	36.10

which indicates that only electronic factors influence the activities of these compounds.

With Eq. 3 as a guide, it is readily found that Compound 7 alone led to the π, π^2 dependence found in Eq. 1. This compound represents a terminal point displaced relative to the overall linear trend of activity with σ which holds for the other compounds (Compounds 1–6). When Compound 7 is deleted from the set used to derive Eq. 1, the resultant regression equation becomes

$$\log(1/C) = \underset{(-5.73)}{-0.84\ (\pm 0.14)\sigma} + \underset{(1.07)}{0.57\ (\pm 0.53)\pi} \underset{(-1.21)}{-0.63\ (\pm 0.52)\pi^2} + 5.17 \qquad \text{(Eq. 4)}$$

N	s	R	$F(3,2)$
6	0.11	0.98	20.11

The statistics for the coefficients in Eq. 4 clearly indicate that the π and π^2 terms should be deleted.

For the 13 N^1-benzoylsulfanilamides considered, a linear correlation between their bacteriostatic potency and their Hammett σ-values is suitable for directing later syntheses. An eventual π, π^2 dependence will most probably be found as a wider variety of multiple substitutions are made on the N^1-benzoyl moiety. In this event, it is likely that the optimum lipophilicity found will differ substantially from the optimum lipophilicity calculated on the basis of Eq. 1.

EXAMPLE 2

In testing alternative physical indexes in attempted correlations of structure–activity data, the rationale commonly used is to attribute physical significance only to those terms that appear as statistically significant in the derived regression equation. For example, if the use of the Hammett σ-constant does not lead to a regression equation in which this index is shown to be statistically significant, the inference that may be drawn is that an alternative electronic index, such as group polarizability P_E or group dipole moment μ, might be more suitable. These alternative indexes are often considered as measures of electronic properties not encompassed by the Hammett σ-value. Seldom, however, are these more specific electronic indexes investigated with respect to the general electronic index they are intended to displace. The result of this type of an investigation can considerably complicate the physical interpretation of a regression equation for a given body of structure–activity data.

Table II—Group Dipole Moments and Hammett σ-Values

Group	σ_p[a]	μ, obs.[b]	μ, estd.	$\lvert d \rvert$
SO_2[c]	0.728	−5.14	−4.00	1.14
SO[c]	0.567	−4.08	−3.33	0.75
CN	0.628	−4.05	−3.58	0.46
NO_2	0.778	−4.01	−4.20	0.19
COMe	0.516	−2.96	−3.12	0.16
CHO	0.216	−2.96	−1.86	1.09
CF_3	0.551	−2.60	−3.26	0.66
CCl_3	0.42[d]	−2.07	−2.72	0.65
$CHCl_2$	0.34[d]	−2.03	−2.38	0.35
CH_2Cl	0.184	−1.82	−1.73	0.08
Cl	0.226	−1.60	−1.91	0.31
OH	−0.357	−1.60	−0.51	2.11
Br	0.232	−1.57	−1.93	0.36
F	0.062	−1.48	−1.23	0.25
I	0.276	−1.42	−2.12	0.70
SMe	−0.047	−1.18	−0.77	0.40
Me	−0.170	0.35	−0.26	0.61
$SiMe_3$	−0.01	0.42	−0.93	1.35
OMe	−0.268	1.28	0.15	1.12
NH_2	−0.660	1.52	1.77	0.25
NMe_2	−0.600	1.61	1.53	0.08

[a] Hammett σ-values for *para*-substituents as given by K. B. Wiberg, "Physical Organic Chemistry," Wiley, New York, N. Y., 1964, p. 410. [b] L. E. Sutton, in "Determination of Organic Structures by Physical Methods," E. A. Braude and F. C. Nachod, Eds., Academic, New York, N. Y., 1955. [c] Assumed to have a Me group substituted on the S atom. The slight contribution made by the dipole moment of the Me group is neglected. [d] Calculated based on a quantum perturbation theory approach, F. L. J. Sixma, *Rec. Trav. Chim.*, **72**, 673(1953).

Certain pharmacological agents have been indicated as having their effect on a biological system more adequately interpreted in terms of the group dipole moments, μ, than of the Hammett σ-values for the substituents (4–6). An investigation of the relation between the group dipole moments and the Hammett σ-values for the substituents found in Table II, however, reveals a significant correlation between the two indexes:

$$\mu = \underset{(-9.16)}{-4.162\ (\pm 0.454)\sigma} - 0.969 \qquad \text{(Eq. 5)}$$

N	s	R	$F(1,19)$
21	0.83	0.90	83.88

Thus, a correlation of biological potency which involves μ or σ should be similarly interpreted. If, in two respective regression

Table III—Neuraminidase Inhibition by 1-Phenoxymethyl-3,4-dihydroisoquinolines

Phenoxy Substituent	σ	π	μ_v	log (1/C)
4-NO_2	0.78	0.50	−4.01	2.903
4-Br	0.27	1.13	−1.57	2.767
4-CN	0.66	0.14	−4.05	2.839
4-Cl	0.23	0.93	−1.60	2.807
4-F	0.06	0.31	−1.48	2.634
H	0.0	0.0	0.0	2.577
4-Me	−0.17	0.48	0.35	2.682
4-OMe	−0.27	−0.12	0.31	2.620
4-OH	−0.37	−0.87	0.00	2.244
4-OEt	−0.24	0.38	0.31	2.650
4-OPr	−0.25	0.88	0.31	2.790
4-OBu	−0.32	1.38	0.31	2.785
4-*t*-Bu	−0.20	1.68	0.35	3.149
3-Me	−0.07	0.56	0.18	2.782
3-F	0.34	0.47	−0.74	2.665
3-Cl	0.37	1.04	−0.80	2.818

Table IV—Acetyl Transferase-Catalyzed Acylation of Substituted Anilines by Acetylaminophenylazobenzenesulfonic Acid

Substituted Aniline	Charge Density on N of Aniline[a]	σ^-	π	Acylation Rate, log A[b]
4-Br	1.849	0.23	1.13	0.049
4-Cl	1.849	0.23	0.93	0.037
4-Me	1.853	−0.17	0.48	0.0
H	1.851	0.0	0.0	−0.155
4-NO_2	1.827	1.27	0.50	−0.468
4-SO_2NH_2	1.841	0.91	−1.16	−0.745

[a] From the results of Hückel molecular orbital calculations reported by A. Perault and B. Pullman, *Biochim. Biophys. Acta*, **66**, 86(1963). [b] From the data reported by K. B. Jacobson, *J. Biol. Chem.*, **236**, 343 (1961).

analyses, it is found that μ contributes significantly, whereas σ does not, or the converse, this finding alone is not sufficient to warrant an alternative interpretation. The compounds considered may have biological potencies that more closely parallel the order of one index than they do the other, but the indexes are not sufficiently independent, on the basis of Eq. 5, to make a distinction between the two possible physical interpretations.

Recently, viral neuraminidase inhibition potencies have been reported (6) for the compounds shown in Table III. The regression equation correlating these data was given (6) by

$$\log(1/C) = \underset{(8.78)}{0.271\ (\pm 0.031)\pi} + \underset{(1.68)}{0.061\ (\pm 0.036)\mu_v} + \underset{(2.95)}{0.029\ (\pm 0.010)\mu_v^2} + 2.551 \qquad \text{(Eq. 6)}$$

N	s	R	$F(3,12)$
16	0.079	0.927	28.96

A more appropriate representation of the correlation, based on the statistics for the coefficients of Eq. 6, is expressed as

$$\log(1/C) = \underset{(8.11)}{0.265\ (\pm 0.032)\pi} + \underset{(3.67)}{0.014\ (\pm 0.003)\mu_v^2} + 2.548 \qquad \text{(Eq. 7)}$$

N	s	R	$F(2,13)$
16	0.081	0.916	36.89

Equation 7 indicates that these compounds have their potencies determined by lipophilic factors and by the component of the group dipole moment, μ_v, which is directed along the 1,4-axis of the substituted moiety.

Upon comparing the electronic index μ_v^2 to its analog σ^2, as is suggested by Eq. 5, it is found that these indexes are related:

$$\mu_v^2 = 30.235\,\sigma^2 - 1.048 \qquad \text{(Eq. 8)}$$

N	s	R	$F(1,14)$
16	2.115	0.925	83.89

A much improved correlation can be obtained by using the unresolved group dipole moments:

$$\mu^2 = 29.659\,\sigma^2 - 0.205 \qquad \text{(Eq. 9)}$$

N	s	R	$F(1,14)$
16	1.483	0.960	164.16

Since the quantity σ^2 has been indicated as having a variety of possible origins (7) and has been shown to correlate with a free-radical index E_r (8) and a charge-transfer index E_{LEMO} (9), it is found that at least three differing physical interpretations can be presented for the electronic term found in Eq. 7. The resolution of the possible alternatives is, therefore, seen as a problem separate from the use of Eq. 7, or related forms, as a predictor of new agents. The most convenient equation to use for guiding the synthesis of new compounds may contain physical parameters which only obliquely reflect the actual factors controlling the observed response.

EXAMPLE 3

Another illustration of the potential complications attending the physical interpretation of derived regression equations is provided by the data found in Table IV. A more limited statistical analysis of the enzymatic acylation rates for this system has been reported (10). Here the authors would like to point out that at least five different statistically significant regression equations will correlate the data:

$$\log A = -0.028\ (\pm 0.004)\,\mu^2 + 0.012 \qquad \text{(Eq. 10)}$$

N	s	R	$F(1,4)$
6	0.111	0.952	38.58

$$\log A = \underset{(3.54)}{0.085\ (\pm 0.024)\,\mu} + \underset{(3.40)}{0.216\ (\pm 0.063)\pi} - 0.112 \qquad \text{(Eq. 11)}$$

N	s	R	$F(2,3)$
6	0.112	0.963	24.88

$$\log A = \underset{(-15.27)}{-0.335\ (\pm 0.022)\,\sigma^-} + \underset{(16.94)}{0.252\ (\pm 0.015)\pi} - 0.155 \qquad \text{(Eq. 12)}$$

N	s	R	$F(2,3)$
6	0.028	0.997	399.2

$$\log A = \underset{(8.99)}{18.169\ (\pm 2.021)\,q_N} + \underset{(12.16)}{0.290\ (\pm 0.024)\pi} - 33.82 \qquad \text{(Eq. 13)}$$

N	s	R	$F(2,3)$
6	0.048	0.993	140.7

$$\log A = \underset{(-6.96)}{-0.272\ (\pm 0.039)\sigma^2} + \underset{(8.41)}{0.264\ (\pm 0.031)\pi} - 0.179 \qquad \text{(Eq. 14)}$$

N	s	R	$F(2,3)$
6	0.061	0.989	85.96

There is little doubt that a charge-related property is affecting the enzymatic acylation rates, according to these correlations, and it is also likely that the lipophilicity of the compounds contributes an effect. An explicit description of the electronic interaction mechanism is difficult, however, because of the various physical significances that can be attributed to the different electronic indexes. In this particular case, Eq. 13 may be preferred as a basis for interpretation, since the charge density on the aniline nitrogen is the most fundamental of the electronic indexes that can be used.

CONCLUSIONS

From the examples presented, it is clear that a physical interpretation for a correlation of biological activities with some combination of physical parameters should be made with caution. It is also evident that the ability of a regression equation to act as a predictor for the biological activities of compounds within a series cannot be presented in full support of the interpretation lent to the terms appearing in the regression equation.

At present, a safe approach to follow if the intent is to gain insight into physical factors influencing the action of drug agents is: (*a*) investigate a correlation for internal consistency, as may be illustrated by *Example 1*; (*b*) establish whether the use of fundamental linear free energy (σ, π) and molecular orbital (q, S^E, S^N) indexes lead to essentially equivalent conclusions, *e.g.*, Eqs. 12 and 13 complement one another; and (*c*) when indexes having little precedent in correlating the rates and equilibria of simple chemical systems become involved in a correlation—*viz.*, μ, μ^2, and σ^2, investigate compounds designed specifically to distinguish between alternative physical interpretations. In the latter instance, since Hammett σ-values are simply additive, whereas group dipole moments are vectorially additive, it might be expected that multisubstituted compounds should provide the more appropriate test of dipole control of a biological response.

REFERENCES

(1) J. K. Seydel, *Mol. Pharmacol.*, **2**, 259(1966).

(2) A. Cammarata and R. C. Allen, *J. Pharm. Sci.*, **56**, 640(1967).

(3) T. Fujita and C. Hansch, *J. Med. Chem.*, **10**, 991(1967).

(4) J. W. Mc Farland, *Nat. Med. Chem. Symp. Amer. Chem. Soc. Proc., 11th*, **1968**.

(5) J. W. McFarland, L. H. Conover, H. L. Howes, Jr., J. E. Lynch, D. R. Chisholm, W. C. Austin, R. L. Cornwell, J. C. Danilewicz, W. Courtney, and D. H. Morgan, *J. Med. Chem.*, **12,** 1066(1969).
(6) M. S. Tute, *ibid.*, **13,** 48(1970).
(7) A. Cammarata and S. J. Yau, *ibid.*, **13,** 94(1970).
(8) A. Cammarata, S. J. Yau, J. H. Collett, and A. N. Martin, *Mol. Pharmacol.*, **6,** 65(1970).
(9) R. C. Allen and C. Cavallito, to be published.
(10) C. Hansch, E. W. Deutsch, and R. N. Smith, *J. Amer. Chem. Soc.*, **87,** 2738(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 11, 1970, from **Temple University, School of Pharmacy, Laboratory of Physical Medicinal Chemistry, Philadelphia, PA 19140,* and the *†Chemistry Department, Borstel Research Institute for Experimental Biology and Medicine, 2061 Borstel, Germany.*

Accepted for publication April 1, 1970.

Abstracted in part from a thesis presented by R. C. Allen to the Medical College of Virginia, Richmond, Va., in partial fulfillment of the Doctor of Philosophy degree requirements.

Solubilization of Some Steroid Hormones in Aqueous Solutions of Bile Salts

ARVIND L. THAKKAR

Abstract □ Solubilities of testosterone propionate, methyltestosterone, and 19-nortestosterone in aqueous sodium cholate and deoxycholate were determined. Solubilizing capacity values show that deoxycholate is a better solubilizer than cholate and that both bile salts solubilize more 19-nortestosterone than other testosterone derivatives. A possible mode of solubilization is discussed.

Keyphrases □ Steroid hormones—solubilization, aqueous solutions, bile salts □ Testosterone derivatives—solubilizing effect of sodium cholate, deoxycholate □ UV spectrophotometry—analysis

The ability of bile salts to enhance the water solubility of steroid hormones was noted as early as 1944 by Cantarow *et al.* (1). Since that time, micellar solubilization of steroids has been studied extensively by Ekwall (2) and Sjöblom (3). However, bile salt solubilization of hormonal steroids appears not to have been examined in detail. This study was undertaken to examine the solubilization of testosterone propionate, methyltestosterone, and 19-nortestosterone by the anionic surfactants, sodium cholate and sodium deoxycholate, and is part of a larger study of the solubilization of steroidal hormones by steroidal surfactants. A recent report from this laboratory (4) dealt with the solubilization of some androgenic steroids by ethoxylated cholesterol, a nonionic surfactant.

EXPERIMENTAL

Materials—Sodium cholate,[1] sodium deoxycholate,[1] methyltestosterone NF, testosterone propionate USP, and 19-nortestosterone[2] were used as received. Moisture contents of the bile salts, determined by drying overnight *in vacuo* at 110°, were taken into consideration when recording their weights. Bile salt solutions, prepared with distilled water, were not buffered; their pH ranged from 7.0 to 7.8 for cholate and from 7.2 to 8.6 for deoxycholate.

Solubility Determinations—Solubilities were determined by equilibration of several concentrations of bile salt solutions with the steroids, followed by spectrophotometric analyses of suitably diluted aliquots, as described previously (4). For solutions in which enhancement of steroid solubility was minimal, dilution with 50% (v/v) methanol was still necessary to lower the bile salt concentration to a point where it would not interfere with the UV spectrophotometric analytical procedure. In such cases, cells of 5-cm. pathlength were used. 19-Nortestosterone, which was not included in the previous study, has maximum absorbance in 50% (v/v) aqueous methanol at 244 mμ, with a molar absorption coefficient of 17.3×10^3.

[1] Special enzyme grade, Mann Research Laboratories, Inc., New York, N. Y.
[2] Purchased from Organon, Inc., West Orange, N. J.

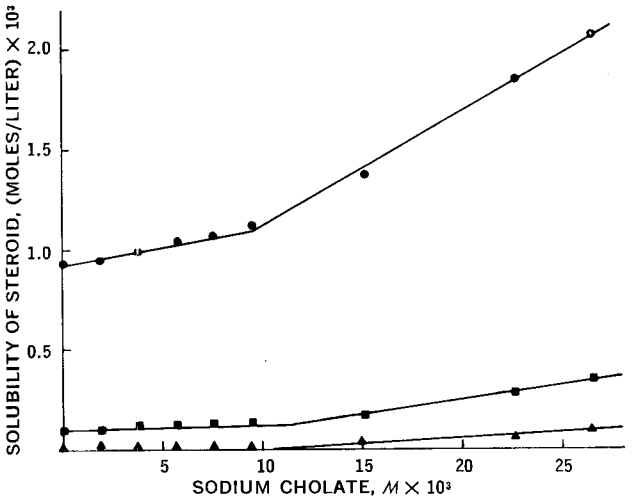


Figure 1—*Solubility of steroids in aqueous solutions of sodium cholate at 30°. Key:* ●, *19-nortestosterone;* ■, *methyltestosterone; and* ▲, *testosterone propionate.*

RESULTS AND DISCUSSION

Figures 1 and 2 show the relationship between solubility of the steroid solubilizates and the concentration of sodium cholate and sodium deoxycholate, respectively. At low concentrations of bile salts, only a marginal change in steroid solubility is observed. After these initial stages, up to $\sim 1.0 \times 10^{-2}$ *M* for sodium cholate and $\sim 6.0 \times 10^{-3}$ *M* for sodium deoxycholate, steroid solubility increases linearly with bile salt concentration. This behavior conforms well to the general features of micellar solubilization, but it is at variance with the report of Lach and Pauli (5) who found that the solubility of testosterone increased at a higher rate below the apparent critical micelle concentration (CMC) of deoxycholate than above it. In a comprehensive paper dealing with the solubiliza-

(5) J. W. McFarland, L. H. Conover, H. L. Howes, Jr., J. E. Lynch, D. R. Chisholm, W. C. Austin, R. L. Cornwell, J. C. Danilewicz, W. Courtney, and D. H. Morgan, *J. Med. Chem.*, **12,** 1066(1969).
(6) M. S. Tute, *ibid.*, **13,** 48(1970).
(7) A. Cammarata and S. J. Yau, *ibid.*, **13,** 94(1970).
(8) A. Cammarata, S. J. Yau, J. H. Collett, and A. N. Martin, *Mol. Pharmacol.*, **6,** 65(1970).
(9) R. C. Allen and C. Cavallito, to be published.
(10) C. Hansch, E. W. Deutsch, and R. N. Smith, *J. Amer. Chem. Soc.*, **87,** 2738(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 11, 1970, from **Temple University, School of Pharmacy, Laboratory of Physical Medicinal Chemistry, Philadelphia, PA 19140,* and the *†Chemistry Department, Borstel Research Institute for Experimental Biology and Medicine, 2061 Borstel, Germany.*

Accepted for publication April 1, 1970.

Abstracted in part from a thesis presented by R. C. Allen to the Medical College of Virginia, Richmond, Va., in partial fulfillment of the Doctor of Philosophy degree requirements.

Solubilization of Some Steroid Hormones in Aqueous Solutions of Bile Salts

ARVIND L. THAKKAR

Abstract □ Solubilities of testosterone propionate, methyltestosterone, and 19-nortestosterone in aqueous sodium cholate and deoxycholate were determined. Solubilizing capacity values show that deoxycholate is a better solubilizer than cholate and that both bile salts solubilize more 19-nortestosterone than other testosterone derivatives. A possible mode of solubilization is discussed.

Keyphrases □ Steroid hormones—solubilization, aqueous solutions, bile salts □ Testosterone derivatives—solubilizing effect of sodium cholate, deoxycholate □ UV spectrophotometry—analysis

The ability of bile salts to enhance the water solubility of steroid hormones was noted as early as 1944 by Cantarow *et al.* (1). Since that time, micellar solubilization of steroids has been studied extensively by Ekwall (2) and Sjöblom (3). However, bile salt solubilization of hormonal steroids appears not to have been examined in detail. This study was undertaken to examine the solubilization of testosterone propionate, methyltestosterone, and 19-nortestosterone by the anionic surfactants, sodium cholate and sodium deoxycholate, and is part of a larger study of the solubilization of steroidal hormones by steroidal surfactants. A recent report from this laboratory (4) dealt with the solubilization of some androgenic steroids by ethoxylated cholesterol, a nonionic surfactant.

EXPERIMENTAL

Materials—Sodium cholate,[1] sodium deoxycholate,[1] methyltestosterone NF, testosterone propionate USP, and 19-nortestosterone[2] were used as received. Moisture contents of the bile salts, determined by drying overnight *in vacuo* at 110°, were taken into consideration when recording their weights. Bile salt solutions, prepared with distilled water, were not buffered; their pH ranged from 7.0 to 7.8 for cholate and from 7.2 to 8.6 for deoxycholate.

Solubility Determinations—Solubilities were determined by equilibration of several concentrations of bile salt solutions with the steroids, followed by spectrophotometric analyses of suitably diluted aliquots, as described previously (4). For solutions in which enhancement of steroid solubility was minimal, dilution with 50% (v/v) methanol was still necessary to lower the bile salt concentration to a point where it would not interfere with the UV spectrophotometric analytical procedure. In such cases, cells of 5-cm. pathlength were used. 19-Nortestosterone, which was not included in the previous study, has maximum absorbance in 50% (v/v) aqueous methanol at 244 mμ, with a molar absorption coefficient of 17.3×10^3.

[1] Special enzyme grade, Mann Research Laboratories, Inc., New York, N. Y.
[2] Purchased from Organon, Inc., West Orange, N. J.

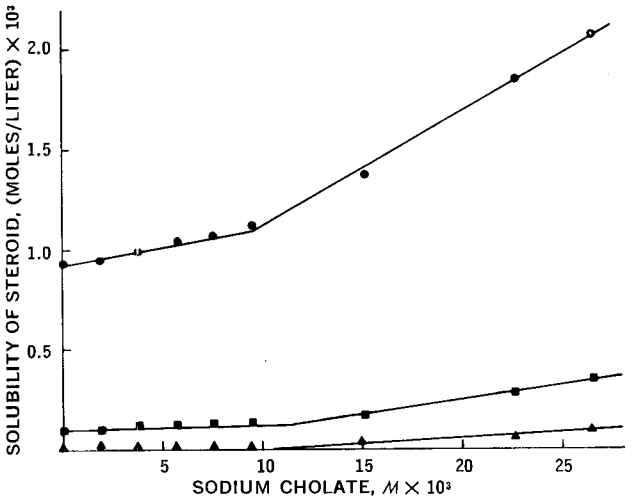


Figure 1—*Solubility of steroids in aqueous solutions of sodium cholate at 30°. Key:* ●, *19-nortestosterone;* ■, *methyltestosterone; and* ▲, *testosterone propionate.*

RESULTS AND DISCUSSION

Figures 1 and 2 show the relationship between solubility of the steroid solubilizates and the concentration of sodium cholate and sodium deoxycholate, respectively. At low concentrations of bile salts, only a marginal change in steroid solubility is observed. After these initial stages, up to $\sim 1.0 \times 10^{-2}$ *M* for sodium cholate and $\sim 6.0 \times 10^{-3}$ *M* for sodium deoxycholate, steroid solubility increases linearly with bile salt concentration. This behavior conforms well to the general features of micellar solubilization, but it is at variance with the report of Lach and Pauli (5) who found that the solubility of testosterone increased at a higher rate below the apparent critical micelle concentration (CMC) of deoxycholate than above it. In a comprehensive paper dealing with the solubiliza-

Table I—Solubilizing Capacity of Bile Salts for Steroids at 30°

Steroids	Solubilizing Capacity,[a] —(Mole Steroid/Mole Bile Salt) × 10^3— Sodium Cholate	Sodium Deoxycholate	Reciprocal of Solubilizing Capacity, —Mole Bile Salt/Mole Steroid— Sodium Cholate	Sodium Deoxycholate
Testosterone propionate	6.61	34.19	151	29
Methyltestosterone	16.20	30.49	62	32
19-Nortestosterone	62.11	163.2	16	6

[a] These values are calculated by the method of least squares from the linear plots, above the apparent CMC, in Figs. 1 and 2. Correlation coefficients of the linear relationships were higher than 0.90 in all the cases.

tion of bile acids, cholesterol, and several other lipoidal substances by bile salts, Ekwall (2) has shown that micelle formation in aqueous bile salt solutions takes place in a number of discrete stages. In the present work, the bile salt concentration ranges in which the rather sharp increases in steroid solubility commence are in reasonably good agreement with the "first concentration limits," 1.3–1.8 × 10^{-2} *M* for cholate and 5–6 × 10^{-3} *M* for deoxycholate, reported by Ekwall (2), and also with the CMC values reported by Bates *et al.* (6), using other solubilizates.

The solubilizing capacities of the bile salts for the steroids examined are listed in Table I. It is evident from Table I that deoxycholate is a better solubilizer than cholate. This is in agreement with previous findings (2, 6) with other solubilizates. Table I also shows that both bile salts solubilize 19-nortestosterone to the greatest extent. Water solubilities (in moles/liter at 30°) of the steroids examined are in the following order: 19-nortestosterone (9.59 × 10^{-4}) > methyltestosterone (1.02 × 10^{-4}) > testosterone propionate (0.06 × 10^{-4}). The ability of 19-nortestosterone to become solubilized to the greatest extent may be due to its inherently high affinity for water. Sjöblom (3) has also reported similar data for solubilization of 19-nortestosterone in aqueous solutions of polysorbate 20. In the concentration range examined, both of the bile salts solubilized more methyltestosterone than testosterone propionate. However, as Table I shows, the solubilizing capacity of deoxycholate for testosterone propionate is greater than that for methyltestosterone. These results and those of a previous study (4) thus show that there is no apparent relationship between the water solubility of a steroid and its property of becoming micellarly solubilized.

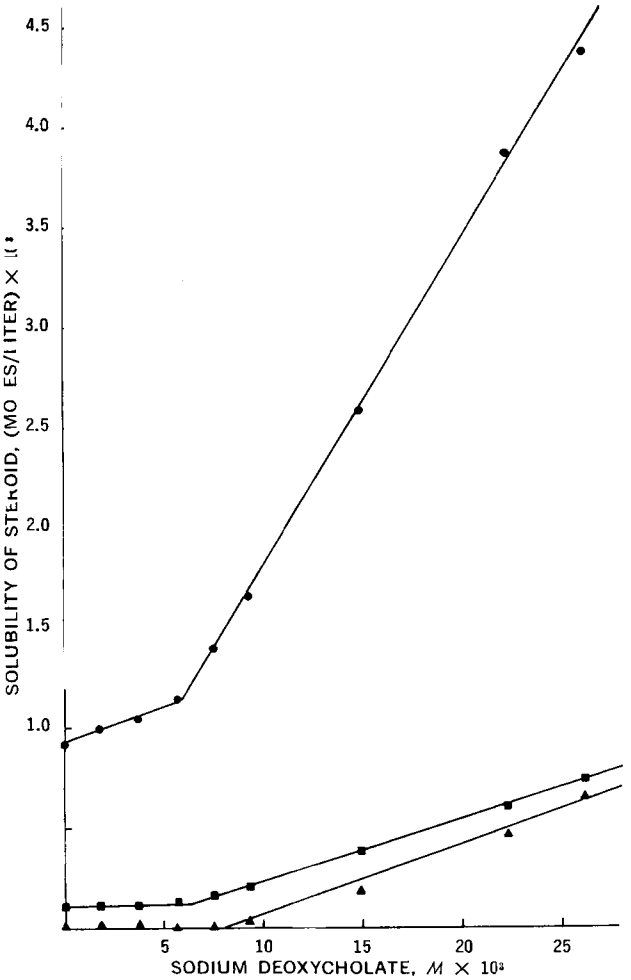


Figure 2—*Solubility of steroids in aqueous solutions of sodium deoxycholate at 30°. Key:* ●, *19-nortestosterone;* ■, *methyltestosterone; and* ▲, *testosterone propionate.*

A closer examination of Figs. 1 and 2 shows that significant increases in the solubility of 19-nortestosterone take place before the apparent CMC's of the bile salts are exceeded. This may be due to formation of mixed micelles of the bile salt and 19-nortestosterone, or it may be that the bile salts increase the solubility of 19-nortestosterone by cosolvation effects. It is interesting that 19-nortestosterone, a steroid molecule without one angular methyl group, is considerably more hydrophilic than other testosterone derivatives.

Recently, Small *et al.* (7, 8) proposed that cholate and deoxycholate micelles are formed by hydrophobic association of the hydrocarbon backs of the rigid steroid nuclei in such a way that the hydrophilic sides, containing the hydroxyl groups and the negatively charged ionic groups, are exposed to water. In the absence of excess counterion concentration, as in this study, these micelles remain small in comparison with classical detergent micelles, their aggregation number ranging from 2 to 9 (7). Upon examining space-filling (Stuart–Breiglib) molecular models, Small has suggested that up to 9 or 10 cholate molecules may associate hydrophobically.

Small and Admirand (9) have shown that at 30° about 6–9 moles of bile salt will solubilize 1 mole of lithocholate. The reciprocal solubilizing capacity values for 19-nortestosterone determined in this study are similar to those for lithocholate. There is a structural similarity between lithocholate and 19-nortestosterone; both have polar groups at each end of their molecules. These observations suggest that the mechanism responsible for the solubilization of these two steroids may be similar. Working with space-filling molecular models (Corey–Pauling–Koltun), it is possible to surround one molecule of either 19-nortestosterone or lithocholate by bile salt molecules in such a way that the hydrophobic parts of the molecules are in contact and the polar functions are exposed to the surroundings. The number of bile salt molecules required for this purpose is consistent with the reciprocal solubilizing capacity values.

For methyltestosterone and testosterone propionate, the solubility data do not permit postulation of the solubilization mechanism. It seems reasonable, however, to expect that the effective average micelle size and volume would change when these solubilizates are incorporated, regardless of the mechanism, in micellar solutions of bile salts. Light scattering, viscosity, and ultracentrifugation studies would reflect such changes and might, therefore, provide useful clues for understanding these solubilized systems.

REFERENCES

(1) A. Cantarow, K. E. Paschkis, A. E. Rakoff, and L. Hansen, *Endocrinology*, **35**, 129(1944).

(2) P. Ekwall, "International Conference on the Biochemical Problems of Lipids," Butterworth Scientific Publishers, London, England, 1953, p. 103; P. Ekwall, K. Fontell, and A. Sten, *Int.*

Congr. Surface Activity, Proc. 2nd, **I**, 353(1957) and references cited therein.

(3) L. Sjöblom, in "Solvent Properties of Surfactant Solutions," K. Shinoda, Ed., Marcel Dekker, New York, N. Y., 1967, p. 221, and references cited therein.

(4) A. L. Thakkar and P. B. Kuehn, *J. Pharm. Sci.*, **58**, 850 (1969).

(5) J. L. Lach and W. A. Pauli, *ibid.*, **55**, 32(1966).

(6) T. R. Bates, M. Gibaldi, and J. L. Kanig, *ibid.*, **55**, 191(1966).

(7) D. M. Small, in "Molecular Association in Biological and Related Systems," R. F. Gould, Ed., Advances in Chemistry Series, Monograph No. 84, Amer. Chem. Soc., Washington, D. C., 1968, p. 31.

(8) D. M. Small, S. A. Penkett, and D. Chapman, *Biochim. Biophys. Acta*, **176**, 178(1969).

(9) D. M. Small and W. Admirand, *Nature*, **221**, 265(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 17, 1969, from the *Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46206*

Accepted for publication March 17, 1970.

The author acknowledges the technical assistance of W. L. Wilham and P. B. Kuehn in carrying out portions of the laboratory work.

Alkaloids of Tylophora I: Isolation of Six New Alkaloids

KOPPAKA V. RAO*, RICHARD WILSON, and BERNICE CUMMINGS

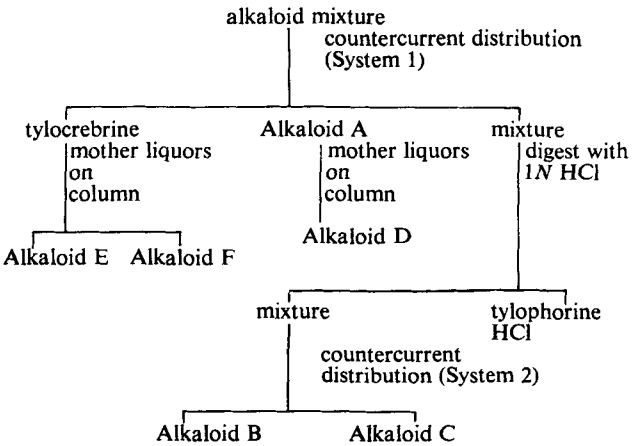
Abstract □ *Tylophora crebriflora* (N. O. Asclepiadaceae) is a slender vine found chiefly in northeastern Australia. In a detailed examination of the plant, six alkaloids have been isolated which have not previously been shown to be present in this genus. The methods for their isolation and their physical characteristics are described.

Keyphrases □ *Tylophora crebriflora*—separation, isolation, physical properties, six alkaloids □ IR spectrophotometry—structure, identification □ UV spectrophotometry—structure, identification

In a series of papers during the 1950's, Govindachari *et al.* (1–5) described the isolation, structure, and synthesis of two alkaloids, tylophorine and tylophorinine, present in the Indian plant *Tylophora indica*. These alkaloids are built up of a dibenzo[*f,h*]-pyrrolo[1,2*b*]-isoquinoline skeleton. From a related Australian plant, *Tylophora crebriflora*, Gellert *et al.* (6) described the isolation of a third member named tylocrebrine, together with a minor amount of tylophorine. The two were shown to be isomeric, differing in the arrangement of the methoxyl groups. During routine screening by the Cancer Chemotherapy National Service Center (CCNSC), it was observed that tylocrebrine showed significant antileukemic activity. At the request of CCNSC to provide tylocrebrine for possible clinical trials, these studies were initiated.

EXPERIMENTAL

The dried plant, *Tylophora crebriflora*, was obtained from Australia.[1] The total alkaloid fraction could be readily isolated by the following steps: (*a*) extraction with 1% methanolic acetic acid; (*b*) concentration; (*c*) partition between ethyl acetate and 0.2 *N* HCl (aq.); and (*d*) extraction of the aqueous layer at pH 9–10 with chloroform. The crude mixture of alkaloids represented a yield of approximately 0.15%.



Scheme I—Separation of the alkaloids of Tylophora crebriflora. *System 1: 3% aqueous acetic acid–chloroform–ethyl acetate (10:7:3) System 2: 3% aqueous acetic acid–chloroform–*n*-butanol (5:4:1)*

The mixture was separated into its components by the use of countercurrent distribution and chromatography on a commercial adsorbent,[2] as indicated in Scheme I. In addition to the two known members, tylocrebrine and tylophorine, the extracts yielded six new alkaloids.

Tylocrebrine and Alkaloid A are the major components, each being present to the extent of about 40% of the total. Next in abundance are tylophorine and Alkaloids B and C, which account for approximately 4–5% each. The rest is made up of the other three members, Alkaloids D, E, and F.

The analytical data and physical properties of the new members are shown in Tables I and II. In general, Alkaloids A–E show

[1] The plant material used in this study was collected, identified, and supplied by the Department of Forestry of Queensland, Brisbane, Queensland, Australia, in 1964. (A voucher specimen was preserved at Chas. Pfizer & Co., Inc., Maywood, N. J.)

[2] Florisil, Floridin Co., Pittsburgh, Pa.

Congr. Surface Activity, Proc. 2nd, **I**, 353(1957) and references cited therein.

(3) L. Sjöblom, in "Solvent Properties of Surfactant Solutions," K. Shinoda, Ed., Marcel Dekker, New York, N. Y., 1967, p. 221, and references cited therein.

(4) A. L. Thakkar and P. B. Kuehn, *J. Pharm. Sci.*, **58**, 850 (1969).

(5) J. L. Lach and W. A. Pauli, *ibid.*, **55**, 32(1966).

(6) T. R. Bates, M. Gibaldi, and J. L. Kanig, *ibid.*, **55**, 191(1966).

(7) D. M. Small, in "Molecular Association in Biological and Related Systems," R. F. Gould, Ed., Advances in Chemistry Series, Monograph No. 84, Amer. Chem. Soc., Washington, D. C., 1968, p. 31.

(8) D. M. Small, S. A. Penkett, and D. Chapman, *Biochim. Biophys. Acta*, **176**, 178(1969).

(9) D. M. Small and W. Admirand, *Nature*, **221**, 265(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 17, 1969, from the *Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46206*

Accepted for publication March 17, 1970.

The author acknowledges the technical assistance of W. L. Wilham and P. B. Kuehn in carrying out portions of the laboratory work.

Alkaloids of Tylophora I: Isolation of Six New Alkaloids

KOPPAKA V. RAO*, RICHARD WILSON, and BERNICE CUMMINGS

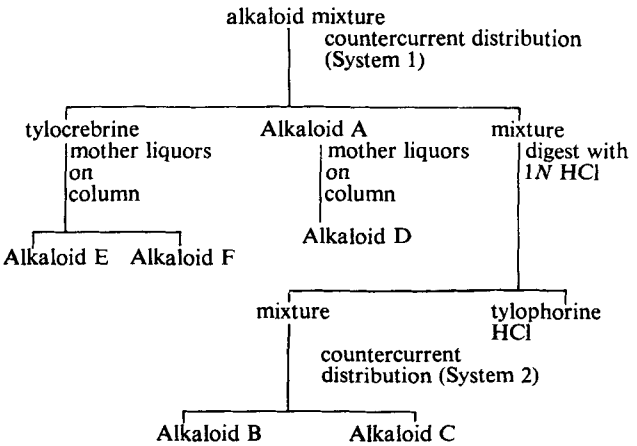
Abstract □ *Tylophora crebriflora* (N. O. Asclepiadaceae) is a slender vine found chiefly in northeastern Australia. In a detailed examination of the plant, six alkaloids have been isolated which have not previously been shown to be present in this genus. The methods for their isolation and their physical characteristics are described.

Keyphrases □ *Tylophora crebriflora*—separation, isolation, physical properties, six alkaloids □ IR spectrophotometry—structure, identification □ UV spectrophotometry—structure, identification

In a series of papers during the 1950's, Govindachari *et al.* (1–5) described the isolation, structure, and synthesis of two alkaloids, tylophorine and tylophorinine, present in the Indian plant *Tylophora indica*. These alkaloids are built up of a dibenzo[*f,h*]-pyrrolo[1,2*b*]-isoquinoline skeleton. From a related Australian plant, *Tylophora crebriflora*, Gellert *et al.* (6) described the isolation of a third member named tylocrebrine, together with a minor amount of tylophorine. The two were shown to be isomeric, differing in the arrangement of the methoxyl groups. During routine screening by the Cancer Chemotherapy National Service Center (CCNSC), it was observed that tylocrebrine showed significant antileukemic activity. At the request of CCNSC to provide tylocrebrine for possible clinical trials, these studies were initiated.

EXPERIMENTAL

The dried plant, *Tylophora crebriflora*, was obtained from Australia.[1] The total alkaloid fraction could be readily isolated by the following steps: (*a*) extraction with 1% methanolic acetic acid; (*b*) concentration; (*c*) partition between ethyl acetate and 0.2 *N* HCl (aq.); and (*d*) extraction of the aqueous layer at pH 9–10 with chloroform. The crude mixture of alkaloids represented a yield of approximately 0.15%.



Scheme I—Separation of the alkaloids of Tylophora crebriflora. *System 1: 3% aqueous acetic acid–chloroform–ethyl acetate (10:7:3) System 2: 3% aqueous acetic acid–chloroform–*n*-butanol (5:4:1)*

The mixture was separated into its components by the use of countercurrent distribution and chromatography on a commercial adsorbent,[2] as indicated in Scheme I. In addition to the two known members, tylocrebrine and tylophorine, the extracts yielded six new alkaloids.

Tylocrebrine and Alkaloid A are the major components, each being present to the extent of about 40% of the total. Next in abundance are tylophorine and Alkaloids B and C, which account for approximately 4–5% each. The rest is made up of the other three members, Alkaloids D, E, and F.

The analytical data and physical properties of the new members are shown in Tables I and II. In general, Alkaloids A–E show

[1] The plant material used in this study was collected, identified, and supplied by the Department of Forestry of Queensland, Brisbane, Queensland, Australia, in 1964. (A voucher specimen was preserved at Chas. Pfizer & Co., Inc., Maywood, N. J.)

[2] Florisil, Floridin Co., Pittsburgh, Pa.

Table I—Characteristics of the Tylophora Alkaloids

Property	Alkaloid A	Alkaloid B	Alkaloid C
1. Melting point	212–214°	222–224°	223–225°
2. Formula	$C_{24}H_{27}NO_5$	$C_{23}H_{25}NO_4$	$C_{23}H_{25}NO_5$
3. Analysis	Calcd. / Found	Calcd. / Found	Calcd. / Found
	C, 70.40 / C, 70.26	C, 72.80 / C, 72.41	C, 69.85 / C, 69.42
	H, 6.65 / H, 6.62	H, 6.64 / H, 6.69	H, 6.37 / H, 6.44
	N, 3.42 / N, 3.43	N, 3.69 / N, 3.61	N, 3.54 / N, 3.55
	OMe, 30.31 / OMe, 30.16	OMe, 24.53 / OMe, 24.49	OMe, 23.07 / OMe, 23.54
4. UV spectrum	$\lambda_{max.}$ / log ϵ	$\lambda_{max.}$ / log ϵ	$\lambda_{max.}$ / log ϵ
	262 / 4.837	262 / 4.818	262 / 4.777
	285(sh) / 4.425	284(sh) / 4.386	265(sh) / 4.359
	305(sh) / 4.028	302(sh) / 4.057	303(sh) / 4.143
5. Rotation $[\alpha]_D^{25}$ (C, 1 in chloroform)	−32	−63	34
6. R_f: Citric acid–formamide–chloroform[a]	0.2–0.3	0–0.1	0–0.1
Formamide–chloroform	0.65–0.75	0.5–0.6	0.3–0.4

[a] Whatman No. 1 sheets immersed in 5% citric acid, dried, moistened before use with 30% formamide in methanol, and developed with chloroform saturated with formamide.

Table II—Characteristics of the Tylophora Alkaloids

Property	Alkaloid D	Alkaloid E	Alkaloid F
1. Melting point	186–188°	198–200°	137–138°
2. Formula	$C_{25}H_{29}NO_6$	$C_{25}H_{29}NO_5$	$C_{24}H_{29}NO_4$
3. Analysis	Calcd. / Found	Calcd. / Found	Calcd. / Found
	C, 68.32 / C, 68.36	C, 70.90 / C, 70.67	C, 72.88 / C, 72.74
	H, 6.65 / H, 6.80	H, 6.80 / H, 6.80	H, 7.39 / H, 7.37
	N, 3.19 / N, 3.19	N, 3.31 / N, 3.88	N, 3.54 / N, 3.52
	OMe, 35.30 / OMe, 34.95	OMe, 36.64 / OMe, 36.21	OMe, 31.39 / OMe, 31.40
4. UV spectrum	$\lambda_{max.}$ / log ϵ	$\lambda_{max.}$ / log ϵ	$\lambda_{max.}$ / log ϵ
	262 / 4.843	263 / 4.878	240(sh) / 4.198
	282(sh) / 4.410		288 / 4.001
5. Rotation $[\alpha]_D^{25}$	−16.5	−69	−42.5
6. R_f: Citric acid–formamide–chloroform	0.4–0.5	0.7–0.8	0.7–0.8

characteristics very similar to those of tylocrebrine, thus showing similarity in chemical structure. Alkaloid F has somewhat different spectral properties, and the exact significance of these results will be discussed in a subsequent paper.

REFERENCES

(1) T. R. Govindachari, B. R. Pai, and K. Nagarajan, *J. Chem. Soc.*, **1954**, 2801.

(2) T. R. Govindachari, M. V. Lakshmikantham, K. Nagarajan, and B. R. Pai, *Tetrahedron*, **4**, 311(1958).

(3) T. R. Govindachari, M. V. Lakshmikantham, K. Nagarajan, and B. R. Pai, *Chem. Ind.*, **1957**, 1484.

(4) T. R. Govindachari, M. V. Lakshmikantham, B. R. Pai, and S. Rajappa, *Tetrahedron*, **9**, 53(1960).

(5) T. R. Govindachari, B. R. Pai, S. Rajappa, and N. Viswanathan, *Chem. Ind.*, **1959**, 950.

(6) E. Gellert, T. R. Govindachari, M. V. Lakshmikantham, I. S. Ragade, R. Rudzats, and N. Viswanathan, *J. Chem. Soc.*, **1962**, 1008.

(7) E. Gellert and R. Rudzats, *J. Med. Chem.*, **7**, 361(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 8, 1969, from *The John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., Maywood, NJ 07607*

Accepted for publication May 11, 1970.

This investigation was supported by Contract PH43-68-45 from the Cancer Chemotherapy National Service Center, National Institutes of Health, U. S. Public Health Service.

* To whom correspondence should be addressed. Present address: College of Pharmacy, University of Florida, Gainesville, FL 32601

Dermophilic Insect Repellents with Perdurable Efficacy

RONALD P. QUINTANA*, ANDREW LASSLO*, LORRIN R. GARSON*, CARROLL N. SMITH†, and IRWIN H. GILBERT†

Abstract □ The synthesis of the monohexanoate, monopropionate, and monobenzoate esters of dihydroxyacetone is reported; the compounds were designed to provide long-lasting insect-repellent efficacy. The insectifugal properties of these compounds and those of the corresponding monoundecanoate ester are also described. While several of the compounds exerted significant repellency, the perdurable effect of dihydroxyacetone monohexanoate (II) was particularly noteworthy.

Keyphrases □ Dihydroxyacetone—synthesis of monohexanoate, -propionate, -benzoate esters □ Insect repellents, dermophilic, long-lasting—dihydroxyacetone monohexanoate, -propionate, -benzoate, -undecanoate esters, evaluation □ Synthesis—dihydroxyacetone monohexanoate, -propionate, -benzoate esters, insect-repellent activity

This approach to the development of dermophilic[1] insect repellents with prolonged activity involves the synthesis of compounds, designed for topical application, incorporating an insect-repellent component along with a component capable of anchoring to the skin; the respective entities are linked together by chemical bonds, constituting the so-called "precursor molecule" (1–4). The specifics of the rationale have been discussed in detail relatively recently (3). While the precursor molecule may be an effective repellent *per se*, it is the gradual breakdown of the precursor molecule, anchored to the epidermis, that is expected to provide long-lasting protection by means of the sustained release of the repellent component.

In two preceding communications (3, 4), the authors reported the preparation of dihydroxyacetone monoundecanoate (I). Based upon the effects elicited by I and upon the observations of Drake and Melamed (5), it was felt that structural designs inherent in the monohexanoate, monopropionate, and monobenzoate esters of dihydroxyacetone (II, III, and IV, respectively) should contribute substantially to the elucidation of relationships between chemical constitution and insect repellency (6). These compounds, like I, derive their dermophilic properties from the α-hydroxymethyl ketone moiety (7), retaining the hydroxyacetone ester function known to enhance susceptibility substantially to the hydrolytic process (8). In addition to the synthesis and the insectifugal efficacies of Compounds II, III, and IV, the insect repellency of I is reported here for the first time.

Compounds II–IV were prepared by procedures patterned after the direct acylation employed in the synthesis of I (4). Their properties are summarized in Table I. Compound II was also prepared by the method utilizing the diazoketone intermediate (3); however, the former method of preparation turned out to be a much more feasible one. The conditions associated with monomer and dimer formation in a monoester of dihydroxyacetone were discussed earlier (4); the physicochemical characteristics of Compounds II–IV parallel those observed in the referenced communication. Since the formulation used in the insect-repellent tests assured conversion to the sought monomeric state in each instance (4), circumstances did not warrant characterization of the monomers and dimers of the three new compounds.

The repellency of Compounds I–IV against *Aedes aegypti* mosquitoes is summarized in Table II. Compound II completely prevented biting at the 4- and 8-hr. test intervals and effected a dramatic reduction in biting at the 26-hr. test interval compared to the corresponding control. It is important to note the similarity in effects produced by Compounds I and II at the 22- and 26-hr. intervals; in both cases there was a resurgence of repellent effects from the 22-hr. test to that at 26 hr.

The test 22 hr. after application (after the overnight period) tends to coincide with the point following a period of limited exertion and limited perspiration, while that at 26 hr. appears to coincide with a 4-hr. active period following the overnight hours. If these premises are accepted, the results may be interpreted in terms of enhanced hydrolytic release of the repellent component prior to the 4-, 8-, and 26-hr. testing points. The repellency level of Compounds I and II with respect to the USDA standard deet, at the 26-hr. interval, is also noteworthy, particularly that of Compound II.

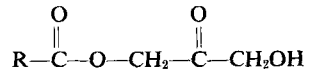
In addition to the tests summarized in Table II, Compound II was subsequently evaluated at additional time intervals; the treated subjects remained in a warm, humid room for a 3-hr. period immediately prior to the evaluation. Under these conditions, Compound II provided essentially complete protection 16 and 20 hr. after application to the skin [biting at 16 hr., 1.9% (control 64%, $LSD_{0.05}$ 10.8); biting at 20 hr., 9.7% (control 64%, $LSD_{0.05}$ 10.8)].

The repellent efficacy of Compound III is also apparent; the latter provided almost complete protection 4 and 8 hr. after application. The fact that it exerted no repellency at the subsequent test intervals can be interpreted in terms of a comparatively increased rate of hydrolysis normally associated with propionic acid esters with respect to those of substantially larger aliphatic acids. The relatively higher volatility of the *released* propionic acid with respect to the larger homologs also could be a contributing factor.

The specific contributions of the precursor molecules' intrinsic repellency and of the released acid-components have not been ascertained. It is apparent, however, that hydrolytic release of the latter is associated with maximum protection.

[1] Dermophilic properties include all those chemical and physical characteristics of an organic molecule that contribute to its affinity for the skin.

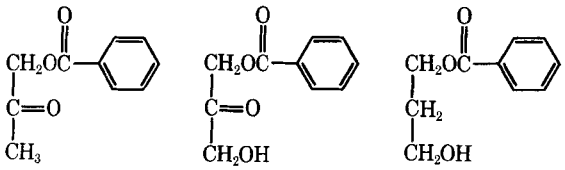
Table I—Esters of Dihydroxyacetone



No.	R	B.p. (mm.)	M.p.	Yield, %	Formula	Anal., % Calcd.	Found
II	n-C_5H_{11}	114–116°(0.25)	...	52.0	$C_9H_{16}O_4$	C, 57.43 H, 8.57	C, 57.57 H, 8.43
III	C_2H_5	80–82°(0.10)	...	21.2	$C_6H_{10}O_4$	C, 49.31 H, 6.90	C, 49.52 H, 6.92
IV	C_6H_5	...	86.7–89.2°[a]	25.4	$C_{10}H_{10}O_4$	C, 61.85 H, 5.19	C, 61.86 H, 5.18

[a] The analytical sample was obtained by recrystallization from ethanol after the more insoluble dihydroxyacetone dibenzoate (V) had been removed by crystallization from this solvent. The latter was obtained in 6% (crude) yield; the analytical sample (*Anal.*—Calcd. for $C_{17}H_{14}O_5$: C, 68.45; H, 4.73. Found: C, 68.34; H, 4.68.) melted at 123.6–124.1°; Romo (9) reports m.p. 118–119°.

The lack of insectifugal activity observed with Compound IV is somewhat surprising when one considers that the two closely related analogs, 1-hydroxy-2-propanone benzoate (VI) and 1,3-propanediol monobenzoate (VII), are effective repellents (10).



VI IV VII

These findings constitute the results of exploratory work involving structural designs, with gradual changes in chemical constitution or physical properties or both, which enabled correlations between molecular configuration and biological response in terms of concepts reasonably well established in contemporary theoretical chemistry. The work currently in progress leads to the anticipation of even more encouraging results; some of these are expected to be reported in the authors' next communication.

EXPERIMENTAL

Synthetic Work[2]—The esters of dihydroxyacetone, listed in Table I, were prepared by the procedure reported in the authors' preceding communication (4). The hexanoyloxyacetic acid (VIII) used in the earlier cited alternate method (3) for the preparation of dihydroxyacetone monohexanoate (II) distilled at 130–132° (1.8 mm.), m.p. 30.7–33.9°, $\nu_{max.}^{CHCl_3}$ 1745 cm.$^{-1}$ (carbonyl), and was obtained in a 46.0% (59.6 g.) yield.

Anal.—Calcd. for $C_8H_{14}O_4$: C, 55.16; H, 8.10. Found: C, 55.37; H, 8.04.

The latter (VIII) was converted through 1-hexanoyloxy-3-diazoacetone (IX) to Compound II, b.p. 100–102° (0.1 mm.).

Anal.—Calcd. for $C_9H_{16}O_4$: C, 57.43; H, 8.57. Found: C, 57.52; H, 8.69.

Evaluation of Insect-Repellent Activity—Female *Aedes aegypti* mosquitoes, 7–8 days old, were confined in small cylindrical cages (4 × 12 cm.). The sides of the cages were clear plastic; one end was covered with gauze and the other end was fitted with a plastic slide closure. Mosquitoes in stock cages were immobilized by exposure to a low temperature, and six females were placed in each small cage. The cages were then held in a warm room for at least 1 hr. to permit the mosquitoes to recover before tests were begun. Three squares, each 25 cm.^{2}, were outlined on the skin of each forearm of three subjects, and each square was treated with 20 mg./cm.^{2} of the repellent. The latter was applied as an ethanol solution heated on a steam bath for 30 min. prior to application. Tests were made by placing the end of the cage equipped with the slide in contact with a treated area on a human arm and by opening the slide to give the mosquitoes direct access to the treated skin for a period of 1 min. In each test period, cages of mosquitoes were exposed to untreated areas of the skin to provide checks on the percentage biting. Three tests on each of three subjects were employed in determining average values.

In the tests reported in Table II, the treated subjects remained in a room maintained at 27.7° (82°F.) and 78–80% relative humidity during the 8-hr. workday. In the subsequent evaluations conducted on Compound II, the subjects remained in the temperature- and humidity-controlled room only for 3 hr. immediately prior to the repellency test.

Table II—Percent of Mosquitoes (*Aedes aegypti*) Biting Forearms of Human Volunteers at Various Intervals after Topical Application

Repellent[a]	*LSD*[b]	Percent Biting at Hours Indicated[c] 4	8	22	26	30
Compd. I	14.4	24.2	22.2	52.4	13.1	28.3
Control	(14.4)	(69.7)	(59.2)	(40.8)	(40.8)	(59.2)
Compd. II	9.4	0[d]	0	61.2	8.2	53.8
Control	(9.4)	(79.7)	(68.4)	(57.4)	(57.4)	(76.8)
Compd. III	11.6	5.5	2.8	73.4	81.6	77.3
Control	(11.6)	(62.9)	(62.9)	(75.1)	(75.1)	(62.9)
Compd. IV	9.4	57.3	53.2	63.1	68.2	83.3
Control	(9.4)	(79.7)	(68.4)	(57.4)	(57.4)	(76.8)
Deet[e]	12.1		0		55.7	
Control	(12.1)		(69.5)		(68.7)	

[a] Application rate 20 mg./cm.^{2}, applied in ethanol solution. [b] Least significant difference at the 0.05 level. [c] Average of three tests on each of three subjects with six mosquitoes per test. [d] In addition, one subject received no bites when exposed to a stock cage (containing 1000–1500 mosquitoes) for 3 min. [e] *N,N*-Diethyl-*m*-toluamide; application rate 3 mg./cm.^{2}.

REFERENCES

(1) R. P. Quintana, L. R. Garson, and A. Lasslo, *Can. J. Chem.*, **46**, 2835(1968).

(2) L. R. Garson and R. P. Quintana, *J. Med. Chem.*, **12**, 538 (1969).

(3) R. P. Quintana, L. R. Garson, and A. Lasslo, *Can. J. Chem.*, **47**, 853(1969).

(4) L. R. Garson, R. P. Quintana, and A. Lasslo, *ibid.*, **47**, 1249(1969).

(5) N. L. Drake and S. Melamed, U.S. pat. 2,627,489(1953).

(6) L. R. Garson and M. E. Winnike, *J. Med. Entomol.*, **5**, 339 (1968).

(7) K. Laden and R. Zielinski, *J. Soc. Cosmet. Chem.*, **16**, 777 (1965); K. Heyns, U. Sage, and H. Paulsen, *Carbohyd. Res.*, **2**, 328(1966).

(8) E. Schätzle, H. Urheim, M. Thürkauf, and M. Rottenberg, *Helv. Chim. Acta*, **46**, 2418(1963).

(9) J. Romo, *J. Org. Chem.*, **21**, 1038(1956).

[2] Boiling points are uncorrected. Melting points are corrected; they were determined with a Büchi melting-point apparatus. IR spectra were obtained with a Perkin-Elmer model 137B spectrophotometer. Analyses were performed by Dr. G. Weiler and Dr. F. B. Strauss, Oxford, England, and by Galbraith Laboratories, Inc., Knoxville, Tenn.

(10) "Chemicals Evaluated as Insecticides and Repellents at Orlando, Fla.," W. V. King, Compiler, Agricultural Handbook No. 69, Entomology Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C., 1954, pp. 279, 284.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 15, 1969, from the *Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee Medical Units, Memphis, TN 38103 and the †Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture, Gainesville, FL 32601

Accepted for publication April 6, 1970.

This work was supported by the U. S. Army Medical Research and Development Command, Washington, D. C., through Research Contract No. DA-49-193-MD-2636.

The authors thank Miss B. D. Bramham for assistance in the syntheses. They also acknowledge the assistance of C. E. Schreck, H. K. Gouck, R. D. Godwin, K. H. Posey, and J. Jackson in the evaluation of mosquito repellency.

Interactions of Drugs with Proteins I: Binding of Tricyclic Thymoleptics to Human and Bovine Plasma Proteins

H. J. WEDER* and M. H. BICKEL

Abstract □ Data on the binding of imipramine, desipramine, and 3-chlorodesmethylimipramine to plasma proteins have been obtained over a wide range of ligand concentration using a modified equilibrium dialysis technique. Plasma proteins other than albumin do not appreciably contribute to complex formation with the drugs studied. Fifty-nine percent imipramine is bound to albumin in the plasma level range reached under therapeutic conditions. However, the association constants of the complex is low. Species differences in the binding capacity of albumin were observed. Apparently, atypical binding behavior was disclosed for desipramine and 3-chlorodesmethylimipramine. A binding model is discussed, and values of binding parameters are given.

Keyphrases □ Thymoleptics, tricyclic—binding to human, bovine plasma proteins □ Proteins, human, bovine plasma—imipramine, desipramine, 3-chlorodesmethylimipramine, interaction, binding, model

These studies on the binding of drugs and other compounds to plasma proteins are aimed at gaining insight into the following problems:

1. Possible influence on the pharmacokinetics of a drug by its interactions with proteins.
2. Mechanism of interactions between drugs or model compounds and proteins and physicochemical interpretation.
3. Critique of methods used in the study of drug–protein interactions.

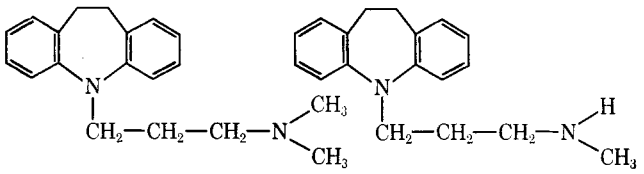
Part I contains data on the complex formation of imipramine, its active metabolite desipramine (desmethylimipramine), and 3-chlorodesmethylimipramine with bovine albumin and human albumin, γ-globulin, plasma, and serum in concentration ranges including the one met with under therapeutic conditions. The first two drugs are used clinically; the latter is a metabolite of the clinically used 3-chloroimipramine accumulating in rats (1) and human (2).

Imipramine (IP) is one of the few drugs about which much of the metabolic and pharmacokinetic data is known (3–8). In addition, physicochemical data of all its major metabolites have been reported (9). Sensitive methods exist for determining imipramine, desipramine, and 3-chlorodesmethylimipramine (5, 6, 10, 11).

Data on IP binding to plasma proteins were published by Tinao and Gomez-Guillen (12) in 1963. Data and species differences in the binding of desipramine to plasma proteins have recently been reported by Borgå *et al.* (13). Earlier, Gillette (14, 15) reported on the binding of imipramine to liver microsomes, which presumably is the reason for the high concentrations of imipramine and related drugs in lung, liver, or kidney tissue in the rat *in vivo* (5, 8). Beside many reports on interactions between thymoleptics and membranes [reviewed by Glowinski and Baldessarini (16)], an interaction between imipramine and the outer membrane of blood platelets has also been reported (17).

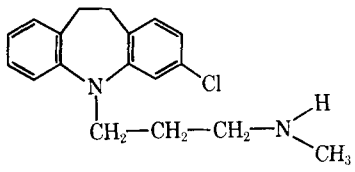
EXPERIMENTAL

Materials—The hydrochlorides of imipramine,[1] desipramine[1] (DMI), and 3-chlorodesmethylimipramine[1] (CDMI) were used.



imipramine
I

desipramine
II



3-chlorodesmethylimipramine
III

Also used were 10,11-^{14}C-imipramine hydrochloride[2] (8.05 mc./mmole) and ^{3}H-acetic anhydride[2] (in benzene, 500 mc./mmole). Gas chromatographic (11) and spectrophotometric tests showed

[1] Supplied by Geigy Ltd., Basel, Switzerland.
[2] Acquired from Radiochemical Centre, Amersham, England.

(10) "Chemicals Evaluated as Insecticides and Repellents at Orlando, Fla.," W. V. King, Compiler, Agricultural Handbook No. 69, Entomology Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C., 1954, pp. 279, 284.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 15, 1969, from the *Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee Medical Units, Memphis, TN 38103 and the †Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture, Gainesville, FL 32601

Accepted for publication April 6, 1970.

This work was supported by the U. S. Army Medical Research and Development Command, Washington, D. C., through Research Contract No. DA-49-193-MD-2636.

The authors thank Miss B. D. Bramham for assistance in the syntheses. They also acknowledge the assistance of C. E. Schreck, H. K. Gouck, R. D. Godwin, K. H. Posey, and J. Jackson in the evaluation of mosquito repellency.

Interactions of Drugs with Proteins I: Binding of Tricyclic Thymoleptics to Human and Bovine Plasma Proteins

H. J. WEDER* and M. H. BICKEL

Abstract □ Data on the binding of imipramine, desipramine, and 3-chlorodesmethylimipramine to plasma proteins have been obtained over a wide range of ligand concentration using a modified equilibrium dialysis technique. Plasma proteins other than albumin do not appreciably contribute to complex formation with the drugs studied. Fifty-nine percent imipramine is bound to albumin in the plasma level range reached under therapeutic conditions. However, the association constants of the complex is low. Species differences in the binding capacity of albumin were observed. Apparently, atypical binding behavior was disclosed for desipramine and 3-chlorodesmethylimipramine. A binding model is discussed, and values of binding parameters are given.

Keyphrases □ Thymoleptics, tricyclic—binding to human, bovine plasma proteins □ Proteins, human, bovine plasma—imipramine, desipramine, 3-chlorodesmethylimipramine, interaction, binding, model

These studies on the binding of drugs and other compounds to plasma proteins are aimed at gaining insight into the following problems:

1. Possible influence on the pharmacokinetics of a drug by its interactions with proteins.
2. Mechanism of interactions between drugs or model compounds and proteins and physicochemical interpretation.
3. Critique of methods used in the study of drug–protein interactions.

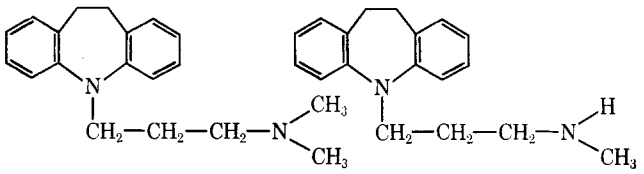
Part I contains data on the complex formation of imipramine, its active metabolite desipramine (desmethylimipramine), and 3-chlorodesmethylimipramine with bovine albumin and human albumin, γ-globulin, plasma, and serum in concentration ranges including the one met with under therapeutic conditions. The first two drugs are used clinically; the latter is a metabolite of the clinically used 3-chloroimipramine accumulating in rats (1) and human (2).

Imipramine (IP) is one of the few drugs about which much of the metabolic and pharmacokinetic data is known (3–8). In addition, physicochemical data of all its major metabolites have been reported (9). Sensitive methods exist for determining imipramine, desipramine, and 3-chlorodesmethylimipramine (5, 6, 10, 11).

Data on IP binding to plasma proteins were published by Tinao and Gomez-Guillen (12) in 1963. Data and species differences in the binding of desipramine to plasma proteins have recently been reported by Borgå *et al.* (13). Earlier, Gillette (14, 15) reported on the binding of imipramine to liver microsomes, which presumably is the reason for the high concentrations of imipramine and related drugs in lung, liver, or kidney tissue in the rat *in vivo* (5, 8). Beside many reports on interactions between thymoleptics and membranes [reviewed by Glowinski and Baldessarini (16)], an interaction between imipramine and the outer membrane of blood platelets has also been reported (17).

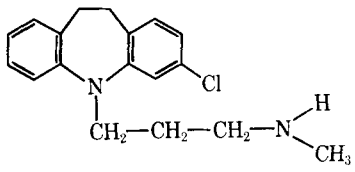
EXPERIMENTAL

Materials—The hydrochlorides of imipramine,[1] desipramine[1] (DMI), and 3-chlorodesmethylimipramine[1] (CDMI) were used.



imipramine
I

desipramine
II



3-chlorodesmethylimipramine
III

Also used were 10,11-^{14}C-imipramine hydrochloride[2] (8.05 mc./mmole) and ^{3}H-acetic anhydride[2] (in benzene, 500 mc./mmole). Gas chromatographic (11) and spectrophotometric tests showed

[1] Supplied by Geigy Ltd., Basel, Switzerland.
[2] Acquired from Radiochemical Centre, Amersham, England.

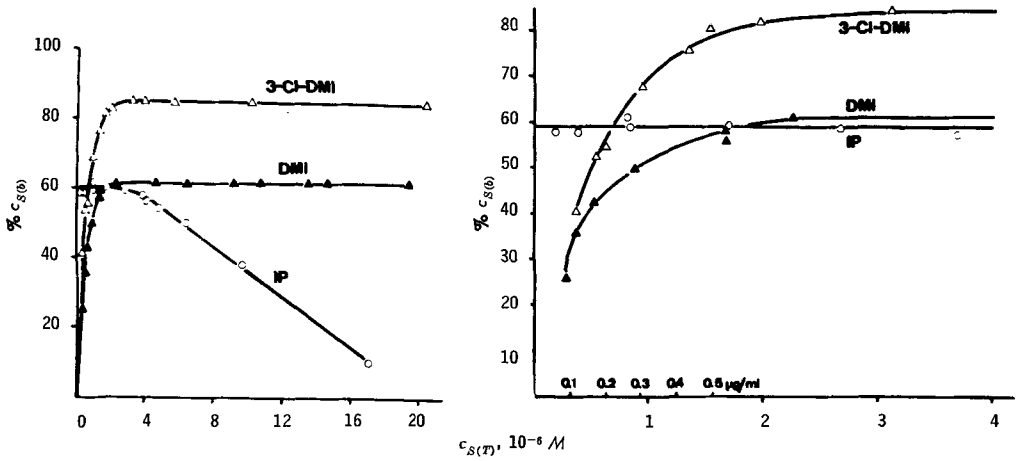


Figure 1—*Fraction of IP, DMI, and CDMI bound to HA, pH 7.4, 20°. HA 4% = $6.15 \cdot 10^{-4}$ M. % $c_{S(b)}$ = fraction of ligand bound. $c_{S(T)}$ = total molar ligand concentration.*

purities of >99% for all compounds. The radiochemical purities were >98%.

Demineralized crystallized bovine albumin[3] (BA), electrophoretically pure human albumin[4] (HA), and γ-globulin[4] (HGLO) were used. All reagents used were of analytical purity.

Normal human plasma (NHP) and serum (NHS) were pooled and stored at −15° for no longer than 1 week.

The ligands (IP, DMI, and CDMI) were dissolved in 0.01 *M* phosphate buffer containing 0.9% NaCl, at pH 7.4 ± 0.05 and ionic strength of 0.19. The proteins (BA, HA, and HGLO) were dissolved in the same medium by gentle agitation.

Methods—Unbound and total ligand concentrations were determined after equilibrium dialysis. The dialysis chamber was developed in this laboratory and will be described in a subsequent paper (18).

Analytical Methods—^{14}C-IP in aqueous solutions was measured by direct liquid scintillation counting. Protein quenching was corrected for by the channel ratio method. DMI and CDMI were determined by the isotope derivative method described by Hammer and Brodie (10). In all cases, a Packard Tri-Carb 314 E scintillation spectrometer and the following scintillation system were used: 4 g./l. 2,5-bis-[5′-*tert*-butylbenzoxazolyl(2′)]-thiophene (BBOT) and 40 g./l. naphthalene in dioxane–xylene–ethyleneglycol monomethyl ether (3:1:3, v/v).

RESULTS

Table I shows the fraction of the total concentrations of IP, DMI, and CDMI bound to HA, HGLO, BA, serum, and plasma. The values for two total concentrations are given for each ligand, the first concentration representing the range of plasma levels observed in therapy (2, 19, 20). The albumin concentrations of the sera showed a mean value of 4.1%.

The fractions of IP, DMI, and CDMI bound in a 4% HA solution as a function of total ligand concentration in the range of 0.1–20 µ*M* are depicted in Fig. 1. Whereas a typical curve results with IP, the curves obtained with DMI and CDMI represent apparently atypical cases.

IP in the concentration range of 0.2 µ*M* (0.06 mcg./ml.)–3 µ*M* (0.95 mcg./ml.), *i.e.*, in the therapeutic range, is bound at a fraction of 58.9 ± 2.8%. A further increase of the total ligand concentration up to 17.2 µ*M* (54.5 mcg./ml.) leads to a gradual decrease to a bound fraction of 10.5 ± 1.9%.

The DMI fraction bound shows a sharp increase from 23.5 ± 2.1% to 61.2 ± 2.4% in the (therapeutic) concentration range 0.27 µ*M* (0.08 mcg./ml.)–3 µ*M* (0.91 mcg./ml.). With a further increase to 19.3 µ*M* (5.85 mcg./ml.), the fraction bound remains constant.

An analogous atypical curve is obtained with CDMI; from 35.2 ± 1.8% to 84.2 ± 3.1% are bound in the range of 0.3 µ*M* (0.1 mcg./ml.)–3 µ*M* (1.0 mcg./ml.), the fraction bound again remaining constant at higher concentrations. Thus, 3 µ*M* represents a critical concentration in the atypical cases of DMI and CDMI.

DISCUSSION

An interaction between albumin and the ligands IP, DMI, and CDMI is demonstrated by the results. The experiments did not reveal an influence on the binding affinity of HA by other plasma proteins. It must be concluded that albumin is the only complexing partner for these ligands in plasma. Species differences are observed for all three ligands, the binding affinity being significantly higher with bovine than with HA.

The therapeutic treatment of patients with IP or DMI leads to plasma levels of 0.005–0.3 mcg./ml. (2, 19, 20). Similar concentrations are observed with other tricyclic drugs, although the absolute values are very low compared with many other classes of drugs. According to the results, up to 70% of IP, DMI, and CDMI in plasma are bound to albumin in the therapeutic concentration range, leaving a free concentration of 30% or more. These data are of little help for the estimation of a possible influence on the pharmacokinetics and pharmacodynamics of the drugs by their interactions with plasma albumin, In this respect, a far better key is provided by the absolute values of the binding affinity, *i.e.*, the association constant, because only the stability of these complexes can possibly be related with pharmacological phenomena. Therefore, information should be obtained on the main forces responsible for drug–protein complexes and not merely on their stabilizing factors.

The following model allows the estimation of the association constant of the IP–HA complex under the experimental conditions used. The c_p ($6.15 \cdot 10^{-4}$ *M*) is the concentration of albumin *P*, where each molecule *P* can bind *n* ligand molecules *S*. Thus *P* has *n* binding sites. If all the *n* binding sites are occupied with ligands, the total binding capacity per liter solution is $c_p \cdot n$. This capacity, however, can only be reached if the concentration of the free ligands, $c_{S(f)}$, is infinite. With relatively low ligand concentrations, the binding sites are only partially occupied in a statistical manner. A correlation between the number of occupied and unoccupied binding sites can be derived under the assumption that the reactivity of all binding sites is equal and that no interactions occur between binding sites. If *A* is a binding site of albumin, *S* a ligand molecule, and *AS* an occupied binding site, then the association or affinity constant *k* for the binding reaction $A + S \rightleftharpoons AS$ is

$$k = \frac{c_{AS}}{c_A \cdot c_{S(f)}} \qquad \text{(Eq. 1)}$$

If, under the experimental conditions, *r* out of *n* binding sites are occupied, then

$$c_{AS} = c_p \cdot r \qquad \text{(Eq. 2)}$$

and

$$c_A = c_p \cdot n - c_p \cdot r \qquad \text{(Eq. 3)}$$

Thus, Eq. 1 becomes

$$k = \frac{c_p \cdot r}{(c_p \cdot n - c_p \cdot r) c_{S(f)}} \qquad \text{(Eq. 4)}$$

[3] Acquired from Poviet Producten N.V., Amsterdam, The Netherlands.

[4] Supplied by the Central Laboratories, Swiss Red Cross, Berne, Switzerland.

Table I—Percent of Total Concentration IP, DMI, and CDMI Bound to Plasma Proteins[a]

Macromolecular System Protein Concn.		Total Concentration IP 100% = 0.2 μM = 0.063 mcg./ml.	IP 100% = 10 μM = 3.16 mcg./ml.	DMI 100% = 0.3 μM = 0.091 mcg./ml.	DMI 100% = 10 μM = 3.02 mcg./ml.	DCMI 100% = 0.3 μM = 0.101 mcg./ml.	DCMI 100% = 10 μM = 3.37 mcg./ml.
BA	4%	72.3 ± 3.6	45.9 ± 2.0	35.5 ± 1.8	75.9 ± 3.4	46.1 ± 1.8	90.2 ± 4.2
HA	4%	60.0 ± 1.2	36.4 ± 1.3	28.3 ± 2.0	62.3 ± 2.9	37.5 ± 1.9	83.3 ± 3.8
HGLO	0.7%	0	<1	0	<1	0	<1
HA + HGLO	4% + 0.7%	58.9 ± 2.1	38.2 ± 1.4	30.3 ± 2.4	65.1 ± 3.2	40.1 ± 2.3	82.7 ± 3.6
NHS		64.7 ± 2.8	40.0 ± 1.2	32.7 ± 2.1	68.4 ± 3.4	35.3 ± 2.0	85.2 ± 4.0
NHP		59.1 ± 2.6	37.7 ± 1.8	30.8 ± 2.5	61.2 ± 3.1	38.6 ± 1.8	81.8 ± 4.1

[a] pH 7.4, 20°, ± *SD* (6–10 experiments). For abbreviations, see *Experimental.*

or

$$c_p \cdot r = \frac{c_p \cdot n}{1 + [1/(k \cdot c_{S(f)})]} \qquad \text{(Eq. 5)}$$

Figure 2 shows the plot of the free ligand concentration $c_{S(f)}$ against the total ligand concentration $c_{S(T)} = c_p \cdot r + c_{S(f)}$. A curve is thereby obtained for each albumin complex with the three ligands used. Curves 4 ($k = 0$) and 5 ($k = \infty$) represent the cases of total lack and of infinitely high binding affinity, respectively. Thus, the sequence of the association constants for the complexes in the concentration range 1–11 μM is $k_{CDMI} > k_{DMI} > k_{IP}$. Only IP fits the binding model introduced, whereas the atypically behaving DMI and CDMI do not. However, with all three ligands, no deviation from the ideal binding curve can be observed in the lowest concentration range (0 – 1 μM). In this range the sequence of binding affinities is $k_{IP} > k_{CDMI} > k_{DMI}$ (Table I).

Graphical determination of the binding capacity of HA for IP leads to a value of about 5.1 μM (Fig. 2). By calculation, an association constant of $k_{IP} = 0.5 \cdot 10^4\ M^{-1}$ is obtained. According to Fig. 2 the binding capacities of albumin for DMI and CDMI must be much higher than for IP. A hypothetical explanation of the atypical curves of DMI and CDMI could be a conformational change of the protein by a primary interaction, which then results in an increase of available binding sites, possibly of higher reactivity.

It is unlikely that at therapeutic concentrations the binding of IP to plasma albumin exerts an appreciable influence on pharmacokinetics and thus pharmacodynamics, since the association constant of the IP–HA complex is less than $10^4\ M^{-1}$ (21, 22).

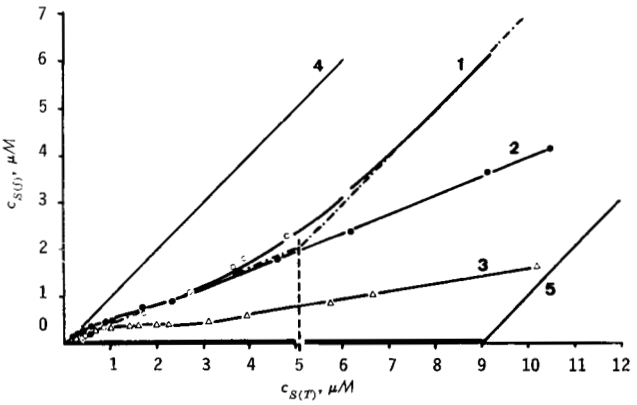


Figure 2—*Free ligand concentration* $c_{S(f)}$ *as a function of total concentration* $c_{S(T)}$. *For conditions, see Fig. 1. Key: 1 = IP, 2 = DMI, 3 = CDMI, and 4 and 5 = theoretical binding curves for* k = 0 *and* k = ∞, *respectively.*

Further results on the binding mechanism as well as thermodynamic data will be presented and discussed in a subsequent report (18).

REFERENCES

(1) M. H. Bickel, M. Flückiger, and M. Baggiolini, *Arch. Pharmakol. Exp. Pathol.*, **256,** 360(1967).

(2) M. H. Bickel and H. J. Weder, unpublished results.

(3) B. Herrmann and R. Pulver, *Arch. Int. Pharmacodyn. Ther.*, **126,** 454(1960).

(4) B. Herrmann, W. Schindler, and R. Pulver, *Med. Exp.*, **1,** 381(1960).

(5) J. V. Dingell, F. Sulser, and J. R. Gillette, *J. Pharmacol.*, **143,** 14(1964).

(6) M. H. Bickel and M. Baggiolini, *Biochem. Pharmacol.*, **15,** 1155(1966).

(7) J. L. Crammer and B. Scott, *Psychopharmacologia*, **8,** 461(1966).

(8) M. H. Bickel and H. J. Weder, *Arch. Int. Pharmacodyn. Ther.*, **173,** 433(1968).

(9) M. H. Bickel and H. J. Weder, *J. Pharm. Pharmacol.*, **21,** 160(1969).

(10) W. Hammer and B. B. Brodie, *J. Pharmacol.*, **157,** 503(1967).

(11) H. J. Weder and M. H. Bickel, *J. Chromatogr.*, **37,** 181 (1968).

(12) M. M. Tinao and F. Gomez-Guillen, *Arch. Inst. Farmacol. Exp. (Madrid)*, **15,** 169(1963).

(13) O. Borgå, D. L. Azarnoff, and F. Sjöqvist, *J. Pharm. Pharmacol.*, **20,** 571(1968).

(14) J. R. Gillette, *Biochem. Pharmacol.*, **12S,** 106(1963).

(15) J. R. Gillette, *Proc. Int. Pharmacol. Meet., 2nd*, **4,** 9(1965).

(16) J. Glowinski and R. J. Baldessarini, *Pharmacol. Rev.*, **18,** 1201(1966).

(17) D. J. Boullin and R. A. O'Brien, *J. Pharm. Pharmacol.*, **20,** 583(1968).

(18) H. J. Weder and M. H. Bickel, to be published.

(19) J. P. Moody, A. C. Tait, and A. Todrick, *Brit. J. Psychiat.*, **113,** 183(1967).

(20) W. Hammer and F. Sjöqvist, *Life Sci.*, **6,** 1895(1967).

(21) B. K. Martin, *Nature*, **207,** 274(1965).

(22) *Ibid.*, **207,** 959(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 21, 1969, from the *Medizinisch-Chemisches Institut, University of Berne, Berne, Switzerland.*

Accepted for publication April 14, 1970.

* Present address: Institute of Molecular Biology and Biophysics, Federal Institute of Technology, Zurich, Switzerland.

Use of Tetraphenylethylene in Quantitative GLC of Hyoscyamine and Scopolamine

BANG-LING WU CHU and EDWARD S. MIKA

Abstract □ Hyoscyamine and scopolamine as free bases were simultaneously quantitated by GLC, using tetraphenylethylene as an internal standard. A linear relationship was found between the ratios of the integrated peak areas of alkaloid to the internal standard and the actual weight ratios of alkaloid to the internal standard for both hyoscyamine and scopolamine. Pure compounds and extracts from *Hyoscyamus niger* Linne powder were analyzed. The combined precision of the extraction procedure of plant materials with the GLC procedure was determined.

Keyphrases □ Tetraphenylethylene, internal standard—quantitative GLC of hyoscyamine, scopolamine □ GLC, hyoscyamine, scopolamine, quantitative—tetraphenylethylene, internal standard □ Hyoscyamine—simultaneous GLC quantification with scopolamine using tetraphenylethylene as internal standard □ Scopolamine—simultaneous GLC quantification with hyoscyamine using tetraphenylethylene as internal standard

Brochmann-Hanssen and Svendsen (1) successfully separated a large number of alkaloid mixtures from various plant sources by GLC. Solomon *et al.* (2) reported quantitative determination of atropine and scopolamine by GLC. No information was available pertaining to the use of a proper compound as an internal standard for the quantitation of the two major tropane alkaloids. Furthermore, no estimate of the combined precision of the alkaloid extraction procedure involving plant materials with the GLC procedure has been reported. Recently, Zimmerer and Grady (3) described an assay procedure of hyoscyamine, atropine, scopolamine, and phenobarbital in unit doses of tablets and elixirs, using homatropine as an internal standard.

The importance of using an internal standard to obtain reproducible quantitative results in any assay procedures by GLC has been emphasized by various investigators (4–9). This paper reports the quantitative determination of hyoscyamine and scopolamine simultaneously as pure compounds and from plant extracts, using tetraphenylethylene (TPE) as an internal standard by employing both isothermal and programmed temperature GLC.

EXPERIMENTAL

Equipment—A linear programmed temperature gas chromatograph (Perkin-Elmer model 881), equipped with a hydrogen flame-ionization detector and a 1-mv. recorder (Sargent model SR, S-72180-20) with a chart speed of 1 in./min. and 1-sec. full-scale response, was used. A pH meter (Beckman zeromatic), centrifuge, and flash evaporator were used for the extraction of plant powders.

Materials—The carrier gas was helium. Hydrogen and air were used in the flame-ionization detector. Dual borosilicate glass columns, 1.83-m. (6-ft.) × 0.19-cm. (0.075-in.) inside diameter, were packed with 2.5% of methyl silicone gum rubber[1] on diatomite aggregate,[2] DMCS 80/100 mesh. The prepared packing material (Perkin-Elmer Co.) was introduced into the columns under reduced pressure with uniform vibration. A minimum of Pyrex glass wool was used to hold the packing material in place in the column. The columns were conditioned and maintained by the injection of Silyl-8[3] into the chromatograph once every 2 weeks before any sample analysis was made. Seeds of *Hyoscyamus niger* Linne (Solanaceae), annual variety, were used.[4] Macroscopic and microscopic examination of aerial organs of flowering plants in this laboratory confirmed identity as hyoscyamus NF XI (henbane). Plants which were field grown, oven-dried at 50°, and ground to 40 mesh served as the standard plant powder. Hyoscyamine[5] and scopolamine[6] free bases, as well as their hydrobromide salts,[7] were used. TPE was also used.[6] All other chemicals used were analytical reagent grade.

Operating Conditions—The sample was chromatographed isothermally at 200° for 6 min., followed by programmed temperatures from 200 to 290° at the rate of 6°/min. The injector temperature was maintained at 300°. The helium flow rate was 100 ml./min., with an inlet pressure of 24 psig. Air and hydrogen inlet pressures were 48 and 24 psig., respectively. Attenuations of ×100, ×50, ×20, and ×10 were used.

Standard Curves—Nine separate chloroform solutions were prepared by weighing out known amounts of hyoscyamine, scopolamine, and the internal standard, TPE, into 10-ml. volumetric flasks. The solutions were chromatographed, and the integrated peak areas of hyoscyamine, scopolamine, and TPE were obtained. A standard curve was then established by plotting the ratio of peak areas of hyoscyamine to TPE *versus* the weight ratio of the two compounds. A similar curve was obtained for scopolamine. On alternate days during this study, fresh solutions of the two alkaloids and the internal standard were used to determine the reproducibility of the standard curves.

Quantitation of the Alkaloids of Hyoscyamus Powder—*Extraction Procedure*—Hyoscyamine and scopolamine were extracted from standard hyoscyamus powder as total alkaloids by the procedure previously described (10).

Preparation of Sample Solution—A stock solution was prepared by dissolving a known amount of TPE in chloroform. A definite volume of this standard TPE solution was added to an aliquot of the plant extract by means of a lambda pipet and thoroughly mixed. The mixed solution was then chromatographed. The usual sample size injected was approximately 2 μl. Pure hyoscyamine and scopolamine as free bases were used to check the reproducibility of the procedure each day that plant extracts were analyzed.

Calculations—The amounts of hyoscyamine and scopolamine in hyoscyamus powder were determined by computing the alkaloid to TPE peak area ratio from the chromatogram, obtaining the cor-

Table I—Gas Chromatographic Data

Parameter	Hyoscyamine	Scopolamine	Tetraphenylethylene
Retention[a]	0.87	(1.00)	1.13
Asymmetry, A_s (11)	1.10	1.16	1.00
Theoretical plates (11)	17,000	19,000	42,000
Response[b]	1.10	(1.00)	2.47

[a] Actual retention time = × 15.1 min.; see Fig. 1. [b] Equimolar amounts, ratio of integrated peak areas.

[1] SE 30/S.
[2] Chromosorb G, acid-washed.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Obtained from Dr. Lynn Brady, University of Washington, Seattle, Wash.
[5] New York Quinine and Chemical Works, Inc.
[6] Aldrich Chemical Co. Inc.
[7] S. B. Penick & Co.

Table II—Simultaneous Determination of Hyoscyamine and Scopolamine in Standard Hyoscyamus Powder

Weight of Plant Powder, g.	Alkaloids Found, mcg.		Alkaloid Concentration, mg./100 g. dry wt.	
	Hyoscyamine	Scopolamine	Hyoscyamine	Scopolamine
4.35	244	544	5.6	12.5
3.54	202	439	5.7	12.4
4.46	236	540	5.3	12.1
4.55	264	573	5.8	12.6
Mean			5.6	12.4
95% Confidence limits (12) of mean			5.3 and 5.9	12.1 and 12.7

Table III—Recovery Data

Weight of Plant Powder, g.	Theoretical Amount,[a] mg.		Amount Recovered, mg.		Percent Recovery	
	Hyoscyamine	Scopolamine	Hyoscyamine	Scopolamine	Hyoscyamine	Scopolamine
4.60	3.27	5.66	3.18	5.39	96.9	94.6
4.36	3.25	5.63	3.30	5.50	101.7	97.5
4.56	3.27	5.65	3.05	5.67	92.9	100.4
4.77	3.28	5.68	3.25	5.86	99.0	103.5
Mean					97.6	99.0
95% Confidence limits (12) of mean					91.7 and 103.5	93.0 and 105.1

[a] Consists of 3.01 mg. hyoscyamine and 5.09 mg. scopolamine which was added volumetrically to each plant sample, alkaloid content of which was calculated from data in Table II.

responding weight ratio of alkaloid to TPE from the standard curve, and multiplying by the weight of TPE in the sample. The value so obtained was then converted to alkaloid concentration in mg./100 g. dry weight of powdered plant specimen.

Recovery Studies—A solution of hyoscyamine hydrobromide and scopolamine hydrobromide as a mixture was prepared by dissolving known amounts of the two alkaloid salts in 10 ml. distilled water. An aliquot of this solution was added to a weighed hyoscyamus powder sample and extracted and analyzed by this method to estimate the recovery of the added pure alkaloids.

RESULTS AND DISCUSSION

For accurate quantitative analysis, the internal standardization technique was used. The virtues of using an internal standard have been well established (3–9).

Figure 1 is a typical gas chromatogram of a mixture of pure hyoscyamine and scopolamine, with TPE added as an internal standard. Extracts of *Hyoscyamus niger* L. with TPE added showed similar chromatographic characteristics. The three compounds were well separated from one another with no overlapping, and TPE was found in the proximity of hyoscyamine and scopolamine. Therefore, in these studies, TPE can be used as an internal standard for each pure alkaloid individually, as a mixture, or in plant extracts. Moreover, TPE is readily available commercially, does not exist in plant extracts, remains stable in chloroform solution for relatively long periods of time, and does not decompose within this operating temperature range of GLC. Various other compounds such as acetanilid, pilocarpine, and pilocarpine hydrochloride were investigated in this study for their ability to serve as an internal standard; however, TPE proved to be the most satisfactory.

Brochmann-Hanssen and Svendsen (1) separated hyoscyamine and scopolamine from hyoscyamus leaf extract by GLC at 200° isothermally. Solomon *et al.* (2) reported a GLC procedure involving temperature programming only from 150 to 275° at the rate of 6°/min. for the separation of these two alkaloids from plant extracts. During initial experimentation, both procedures were compared using certain root extracts of *H. niger* as the sample. Data obtained showed the presence of a few compounds eluted between the solvent

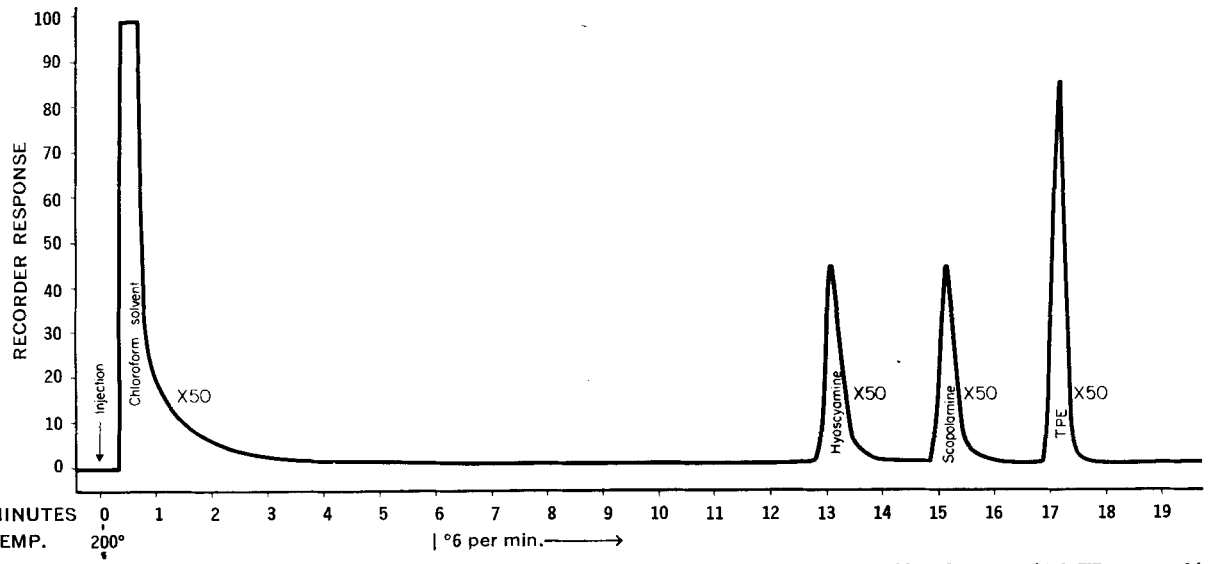


Figure 1—*Typical gas chromatogram of a mixture of pure hyoscyamine and scopolamine bases in chloroform to which TPE was added as the internal standard. Extracts of* Hyoscyamus niger *L. with TPE added showed similar chromatographic characteristics.*

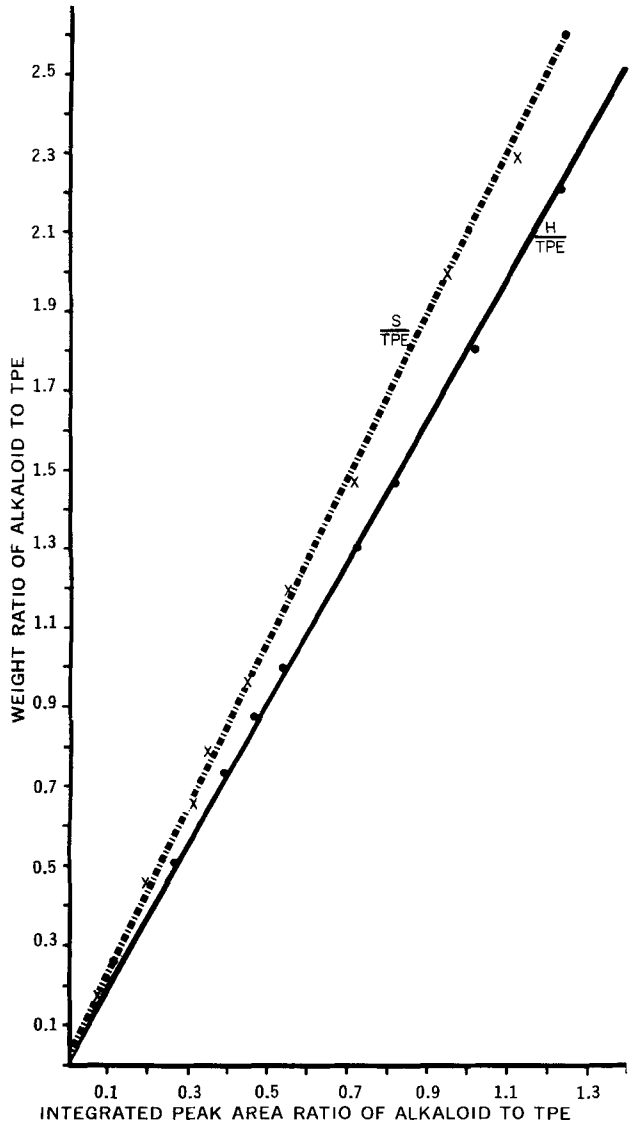


Figure 2—*Standard curves for hyoscyamine and scopolamine with TPE as the internal standard.*

peak and the hyoscyamine peak, resulting in chromatograms that did not afford the desired resolution. A technique was desired where the two alkaloids in question would have greater retention times and also greater differences in retention time so as to minimize or eliminate any possible interfering effect of the solvent and/or any other substances on the quantitation of hyoscyamine and scopolamine. A combination of both isothermal and programmed temperature GLC yielded well-separated symmetrical peaks (Table I). The number of theoretical plates suggested high column efficiency. The resolution factors (8) between hyoscyamine and scopolamine and between scopolamine and TPE were 2.2 and 2.4, respectively, indicating complete resolution.

A linear relationship was established between the weight ratios of the alkaloid to the internal standard and the integrated area ratios of the two. Figure 2 shows the standard curves for hyoscyamine and scopolamine with TPE as the internal standard. Maximum precision and accuracy were obtained when the integrated peak area ratio of alkaloid to TPE was near unity. Each value shown in Fig. 2 is the average of three independent determinations. The standard curves thus obtained were checked and redetermined with fresh standard solutions. The use of an internal standard greatly simplified the analytical procedure and calculations.

Samples of hyoscyamus powder were analyzed by this method, and the results are shown in Table II. The combined precision of the liquid–liquid extraction technique with the GLC procedure was examined by adding known amounts of the two alkaloid salts to hyoscyamus powder. The results obtained from the recovery studies are presented in Table III. In all cases, quantitative recoveries of the added alkaloids were obtained.

Brochmann-Hanssen and Svendsen (1) pointed out that the amount of glass wool placed on top of the column packing was closely associated with the degree of decomposition or dehydration of the alkaloids. Special care was taken in using only the minimum amount of glass wool practicable during column packing. As shown in Fig. 1, no additional peaks apparently due to decomposition were present when pure alkaloids or henbane extracts to which TPE was added were chromatographed. The treatment of columns by injection of Silyl-8, which is a mixture of three trimethylsilyl donors, also aids in enhancing the column efficiency and preventing breakdown of sensitive compounds.

It should be emphasized that under the conditions employed, GLC did not differentiate between hyoscyamine and atropine. Zimmerer and Grady (3) arrived at the same conclusion and thus used the term "hyoscyamine-atropine" in their discussion.

The results obtained in this study clearly demonstrate that TPE is an excellent internal standard in the quantitative GLC determination of hyoscyamine and scopolamine, irrespective whether present as authentic free bases, mixtures, or in plant extracts.

REFERENCES

(1) E. Brochmann-Hanssen and A. B. Svendsen, *J. Pharm. Sci.*, **51,** 1095(1962).

(2) M. J. Solomon, F. A. Crane, B. L. Wu Chu, and E. S. Mika, *ibid.*, **58,** 264(1969).

(3) R. O. Zimmerer and L. T. Grady, *ibid.*, **59,** 87(1970).

(4) S. D. Nogare and R. S. Juvet, Jr., "Gas-Liquid Chromatography," Interscience, New York, N. Y., 1962, pp. 256, 257.

(5) E. Brochmann-Hanssen, *J. Pharm. Sci.*, **51,** 1017(1962).

(6) E. Brochmann-Hanssen and A. B. Svendsen, *ibid.*, **52,** 1134 (1963).

(7) G. E. Martin and J. S. Swinehart, *Anal. Chem.*, **38,** 1789 (1966).

(8) L. S. Ettre and A. Zlatkis, "The Practice of Gas Chromatography," Interscience, New York, N. Y., 1967, pp. 157, 401–403.

(9) N. Y. Mary, *J. Pharm. Sci.*, **58,** 1089(1969).

(10) B. L. Wu Chu, E. S. Mika, M. J. Solomon, and F. A. Crane, *ibid.*, **58,** 1073(1969).

(11) O. E. Schupp, "Technique of Organic Chemistry, Gas Chromatography," vol. 13, Interscience, New York, N. Y., 1968, pp. 22, 43.

(12) N. T. J. Bailey, "Statistical Methods in Biology," Wiley, New York, N. Y., 1959, p. 49.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 28, 1970, from the *Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680*

Accepted for publication April 6, 1970.

Aspirin-Induced Occult Gastrointestinal Blood Loss: Local *versus* Systemic Effects

JACK R. LEONARDS* and GERHARD LEVY†

Abstract □ The purpose of this study was to determine if occult gastrointestinal blood loss produced by therapeutic doses of aspirin in man is a local or systemic effect. The daily oral administration of from 3.9 to 5.2 g. aspirin in tablets for 8 days increased the average daily blood loss from 0.3 to 6.4 ml. in nine normal subjects. Intravenous administration of from 2.7 to 3.4 g. aspirin as the sodium salt per day for 3 days to the same subjects caused no measurable gastrointestinal blood loss above control values. The average bleeding time increased from a control value of 2.6 to 4.5 min. during oral aspirin administration and to 4.1 min. during intravenous administration of the drug. The absence of gastrointestinal bleeding due to intravenous aspirin and the similarity in the degree of prolongation of bleeding time by both oral and intravenous aspirin indicate that gastrointestinal bleeding is a local effect of the drug and is not related to changes in the bleeding time.

Keyphrases □ Aspirin—induced gastrointestinal blood loss, local *versus* systemic effects □ Gastrointestinal bleeding—aspirin-induced, local, systemic effects

The mechanism of aspirin-induced gastrointestinal blood loss has been the subject of considerable discussion. Some investigators believe that it is a local[1] effect (1, 2), while others consider it due to a systemic action of aspirin (3, 4). It has been suggested that the prolongation of bleeding time produced by aspirin may possibly play a role in the causation of gastrointestinal blood loss (5). The study described here was initiated for the purpose of assessing the degree of gastrointestinal blood loss caused by the relatively large oral doses of aspirin used for the relief of rheumatism and arthritis, and to compare this with that produced by intravenous aspirin. Bleeding times were determined during a control period and during oral and intravenous aspirin administration to establish the possible relationship between this effect and gastrointestinal blood loss.

METHODS

Nine healthy, ambulatory subjects (sex, age, and weight listed in Table I) had their red blood cells labeled with ^{51}Cr as described in a previous paper (6). They collected their stools over a 35-day period for the determination of fecal blood loss (6). The first 8 days served as a control period. From 3.9 to 5.2 g. aspirin as 0.3-g. tablets (Bayer) were taken daily, in four equal doses, for the next 8 days. After a rest period of 11 days, 2.7–3.4 g. aspirin as sodium acetylsalicylate dissolved in 250 ml. of normal saline was injected intravenously at a constant rate over a 1–2-hr. period each day for 3 days.

Bleeding times were obtained on the last 2 days of the control and oral aspirin periods and on each day of the intravenous aspirin period at the end of the injection. At least six stab wounds (three on each arm) were made on each day, and the reported bleeding times are averages of 12 to 36 individual determinations. The stab wounds were made with a disposable lancet (Dade Hemolet) with a blade 4 mm. long and 1.4 mm. wide at the base, which was attached to a specially modified spring-type automatic lancet holder.

[1] In this discussion, "local effect" refers to the topical exposure of the gastrointestinal mucosa to ingested aspirin.

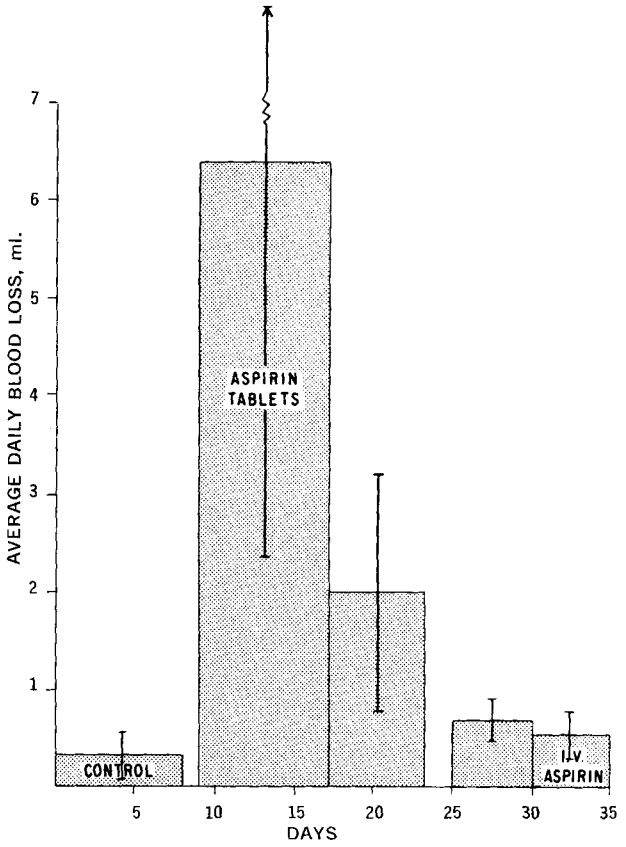


Figure 1—*Average daily occult gastrointestinal blood loss in nine normal subjects during a control period, during oral administration of 3.9–5.2 g. aspirin in tablet form per day, during postdrug periods, and while receiving 2.7–3.4 g. aspirin intravenously for 3 days. Vertical bars are standard deviations.*

RESULTS

The results of the study are summarized in Table I. Oral administration of aspirin caused a pronounced and statistically significant increase in average daily blood loss (ADBL), which persisted for at least 6 days after the last day of drug ingestion. The total average blood loss due to aspirin was 59 ml. above control values, based on the bleeding observed from the 10th to the 23rd day of the study. One individual lost 129 ml. of blood during this period, or 117 ml. in excess of the control value. Intravenous administration of aspirin did not cause any measurable bleeding under the experimental conditions. There was no statistically significant difference (by paired *t* test) in the ADBL during the intravenous aspirin period and the initial control period. The time course of ADBL in the various control, treatment, and rest periods is shown in Fig. 1.

The average bleeding time was 2.6 min. in the control period. There was a statistically significant ($p < 0.01$) increase in bleeding time to 4.5 min. during oral aspirin administration and to 4.1 min. in the period when aspirin was given intravenously (Table I).

Table I—Effect of Aspirin Tablets and Intravenous Aspirin on Gastrointestinal Blood Loss and Bleeding Time in Man

	Subject									Mean	
	G.P.	R.F.	A.T.	R.N.	C.D.	J.E.	M.G.	M.B.	T.F.	Value	*SD*
Sex	M	M	M	M	F	F	M	M	F		
Weight, kg.	66	71	73	77	65	52	77	66	57		
Age, yr.	26	24	24	19	19	20	22	24	24		
Daily dose, oral aspirin, g.	4.5	5.2	3.9	5.2	4.5	3.9	5.2	4.5	3.9		
Daily dose, i.v. aspirin, g.	2.7	2.7	2.7	2.7	3.0	2.7	3.4	3.4	3.0		
ADBL,[a] ml.											
1st–8th day (control)	0.2	0.3	0.3	0.35	0.3	0.3	0.85	0.2	0.3	0.3	0.2
10th–17th day (aspirin tablets)	9.2	8.2	3.7	8.1	3.4	3.2	14.6	5.4	1.7	6.4	4.0
18th–23rd day	1.4	2.2	2.0	2.3	0.9	3.2	2.0	3.7	0.25	2.0	1.1
26th–30th day	1.1	1.0	0.5	0.6	0.5	0.5	0.9	0.5	0.8	0.7	0.2
31st–35th day (i.v. aspirin)	0.7	0.6	0.7	0.5	0.3	0.5	0.4	0.9	0.4	0.6	0.2
				Average Bleeding Time, min.							
Control	1.9	2.3	3.1	2.9	3.3	2.9	2.4	2.0	2.8	2.6	0.5
Aspirin tablets	2.7	4.5	5.7	4.6	5.3	5.5	3.1	4.9	4.4	4.5	1.0
i.v. Aspirin	3.2	3.3	5.0	4.0	6.0	4.5	4.0	3.6	3.6	4.1	0.9

[a] Average daily blood loss.

DISCUSSION

The relatively large oral doses of aspirin tablets employed in this study caused appreciable gastrointestinal blood loss. The ADBL of 6.4 ml. observed in this study during daily administration of 4.5 g. aspirin on the average may be compared to an average of 2.3 ml. in 15 subjects who received daily doses of only 2.6 g. of the same aspirin tablets in a previous study (7). Gastrointestinal bleeding due to aspirin is, therefore, of increasing significance when relatively high doses of the drug are used.

No bleeding was observed during intravenous administration of aspirin to the subjects in this study, although the same or larger doses caused significant gastrointestinal blood loss when given orally (7). The period of oral administration of aspirin (8 days) was considerably longer than the period of intravenous administration (3 days). However, comparison of the ADBL of the nine subjects during the first 3 days of oral administration of aspirin (5.7 ml.) with that during the 3 days of intravenous administration (0.45 ml.) yields essentially the same results as those listed in Table I for the longer time periods. Both sets of data show statistically significant ($p < 0.01$ by paired t test) blood loss during oral administration of aspirin and no evidence of aspirin-induced bleeding when the drug was given intravenously. Due to technical problems, intravenous aspirin was always given after a period of oral aspirin administration rather than in a crossover fashion, but it has already been shown (6, 7) that the bleeding response to aspirin is not affected by previous aspirin administration when followed by an 8-day rest period.

The results of this study are consistent with those of Anderson (1) and Davison *et al.* (2) who did not detect any gastric erosions or bleeding after parenteral administration of aspirin to guinea pigs, rabbits, and dogs in doses that caused significant gastric damage when given by the oral route. Simultaneously with the preliminary report of this study (8), Cooke and Goulston (9) recently reported that intravenous infusion of 1 g. aspirin twice daily for 3 days did not increase the fecal blood loss in 15 healthy human subjects above control levels. However, they did not challenge their subjects with oral aspirin.

The paper by Grossman *et al.* (3) has been cited frequently in support of the contention that aspirin-induced gastrointestinal blood loss in man is a systemic effect. These investigators reported a barely statistically significant increase in ADBL from intravenous aspirin administration, but it should be noted that most of the subjects had peptic ulcer; some had bled within 2 weeks of the study, and much more bleeding was observed when the same dose of aspirin was administered orally (2.1 ml. *versus* 0.8 ml./day in excess of control values). There has been a number of reports of gastric hemorrhage following parenteral administration of aspirin to rats (10, 11) and cats (12), but the doses used were massive (300 to 1300 mg./kg.) and often lethal. It is unrealistic to extrapolate the results obtained under these extreme conditions to the therapeutic doses used in man. Brodie and Chase (13) recently reported a statistically significant increase in the incidence of gastric hemorrhage in rats given 64 mg. aspirin/kg., either orally or intraperitoneally. They noted, however, that hemorrhage was much less severe and the lesions were smaller when the drug was given intraperitoneally. Since the serosal side of the stomach is exposed directly to aspirin particles when the drug suspension is injected intraperitoneally, this effect could well have been a local one.

The prolongation of bleeding time during aspirin administration observed in this study is in agreement with the reports of other investigators (5). The prolonged bleeding time during intravenous aspirin administration was not a carryover from the oral aspirin period, since the bleeding times determined in several of the subjects just prior to the first intravenous dose of aspirin was similar to their control times. The rigid standardization of the stab wound (due to the use of an automatic lancet) and the large number of determinations accounted for the very small variability of the bleeding times.

The results of this study show that gastrointestinal blood loss in normal human subjects receiving therapeutic doses of aspirin is a local effect and that it is not related to the prolongation of bleeding time produced by aspirin. It is, therefore, reasonable that the gastrointestinal bleeding liability of aspirin preparations can be reduced or even eliminated (7, 14, 15) by appropriate pharmaceutical formulation designs. Such preparations would be particularly important for the treatment of rheumatoid arthritis, which usually requires relatively high doses of aspirin. Unfortunately, none of the presently available preparations is ideally suitable for this purpose, mainly because of the high sodium content or limited buffer capacity. The feasibility of developing more suitable preparations for this purpose is now being explored.

REFERENCES

(1) K. W. Anderson, *Arch. Int. Pharmacodyn. Ther.*, **152**, 379 (1964).

(2) C. Davison, D. H. Hertig, and R. DeVine, *Clin. Pharmacol. Ther.*, **7**, 239(1966).

(3) M. I. Grossman, K. K. Matsumoto, and R. J. Lichter, *Gastroenterology*, **40**, 383(1961).

(4) R. Menguy, *ibid.*, **51**, 430(1966).

(5) L. F. Gast, *Ann. Rheum Dis.*, **23**, 500(1964).

(6) J. R. Leonards and G. Levy, *Clin. Pharmacol. Ther.*, **8**, 400 (1967).

(7) J. R. Leonards, *Gastroenterology*, **44**, 617(1963).

(8) J. R. Leonards and G. Levy, *Pharmacologist*, **11**, 269(1969).
(9) A. R. Cooke and K. Goulston, *Brit. Med. J.*, **3**, 330(1969).
(10) H. G. Barbour and V. C. Dickerson, *Arch. Int. Pharmacodyn. Ther.*, **58**, 78(1938).
(11) M. Levrat and R. Lambert, *Gastroenterologia*, **94**, 273(1960).
(12) A. Lynch, H. Shaw, and G. W. Milton, *Gut*, **5**, 230(1964).
(13) D. A. Brodie and B. J. Chase, *Gastroenterology*, **53**, 604 (1967).
(14) J. R. Leonards and G. Levy, *Clin. Pharmacol. Ther.*, **10**, 571 (1969).
(15) J. R. Leonards and G. Levy, *J. Pharm. Sci.*, **58**, 1277(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 10, 1970, from the **Department of Biochemistry, School of Medicine, Case-Western Reserve University, Cleveland, OH 44106* and the *† Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication April 6, 1970.

Effect of Micelle Formation on Optical Rotatory Dispersion of β-D-Octyl Glucoside

P. MUKERJEE, J. PERRIN, and E. WITZKE

Abstract □ The optical rotatory dispersion of a nonionic surfactant, β-D-octyl glucoside, has been investigated in aqueous solutions in the UV region. The rotatory dispersion curves at any concentration can be represented by a one-term Drude equation. The specific rotation at any wavelength shows an increase at the CMC, which can be determined reliably from this change in specific rotation. The rotatory dispersion curve for the surfactant in micellized form has been derived and compared with that of the nonmicellized surfactant below the CMC. The change is small and can be ascribed to a "medium" effect, arising from the difference in the local refractive index at the micelle surface, as compared to the bulk solvent. This interpretation is compatible with the currently accepted ideas on the fluid nature of the micelle core and suggests a lack of any conformational restraint at the micellar interface.

Keyphrases □ Optical rotatory dispersion, β-D-octyl glucoside—micelle formation effect □ β-D-Octyl glucoside—optical rotatory dispersion, micelle formation effect □ Micelle formation, effect—optical rotatory dispersion, β-D-octyl glucoside

Many chemical and biochemical reactions and interactions of interest occur at interfaces, *e.g.*, monolayers, micelles, enzymes, or membranes. The local molecular environment at an interface and the presence of an asymmetry and other peculiarities in dielectric properties (1) are factors of considerable importance in understanding the properties of molecules at interfaces. It has been pointed out recently that for studying many such interactions, the interface between a micelle and the solution provides a convenient locus whose composition is capable of a considerable controlled variation (2, 3). The use of optically active surfactant monomers or solubilized molecules offers the possibility of using optical activity as a probe for studying properties of interfaces and of understanding the effect of the interface composition on the optical activity itself. The present paper reports what appears to be the first such study on a simple model surfactant system, β-D-octyl glucoside.

β-D-Octyl glucoside has a CMC in aqueous solution of 0.024–0.025 *M* at 25° (4, 5). The micelles probably contain about 30 monomers (6), the hydrocarbon chains forming a spheroidal core. The glucoside head groups presumably remain exposed to water both in the monomeric and micellar forms. This, by itself, would suggest that there should be no change in their optical activity. In fact, however, the packing of the chains produces a high effective concentration of the head groups in the interfacial layer (2), which interact strongly enough with each other to counter the micelle-forming tendency of the aliphatic chain rather substantially. This is apparent from the following comparison.

Recently the CMC of a hypothetical octyl chain, unencumbered by any head group, was estimated to be about 3×10^{-3} *M* (3). The CMC of octyl glucoside is higher by a factor of about 8. The head group self-interaction thus makes the standard free energy of micelle formation for octyl glucoside more positive by $kT \ln 8$ or about $2kT$'s per monomer, where k is the Boltzmann constant and T the absolute temperature (3). The glucoside groups at the micelle surface are thus in a considerably different local environment when compared to the free monomers.

EXPERIMENTAL

Materials—The octanol used was a Baker analyzed reagent, which was purified further by vacuum distillation, the middle one-third portion being collected.

β-D-Octyl Glucoside—Glucose, on acetylation followed by bromination (7), yielded acetobromoglucose, m.p. 89°. The bromo compound was reacted with octanol in dry absolute ether in the presence of silver oxide to give β-tetraacetyl octyl glucoside, m.p. 63–64°. After deacetylation in sodium methylate solutions, β-D-octyl glucoside was obtained. It was recrystallized twice from ethyl acetate, washed with Skelly-A, and dried under vacuum. The compound melts over a wide range, 65–99° (8). The intermediate compounds were purified by recrystallization before proceeding to the next step of the preparation.

Anal.—Calcd. for C, 57.5; H, 9.7. Found: C, 57.8; H, 9.5.

Apparatus and Experimental Procedure—The optical rotatory dispersion (ORD) measurements were carried out in a Cary model 60 spectropolarimeter. The cell compartment was thermostated at 25 ± 0.2°. Five-centimeter cells were used. All solutions were optically clear, and double-distilled water was used. The ORD measurements were made in the 250–370-mμ region.

RESULTS

Figure 1 shows the variation of the observed rotation at 320 mμ as a function of concentration. To magnify the small differences observed, a deviation plot is presented. The data show the usual curvature near the CMC. If the CMC region is excluded, the data

(8) J. R. Leonards and G. Levy, *Pharmacologist*, **11**, 269(1969).
(9) A. R. Cooke and K. Goulston, *Brit. Med. J.*, **3**, 330(1969).
(10) H. G. Barbour and V. C. Dickerson, *Arch. Int. Pharmacodyn. Ther.*, **58**, 78(1938).
(11) M. Levrat and R. Lambert, *Gastroenterologia*, **94**, 273(1960).
(12) A. Lynch, H. Shaw, and G. W. Milton, *Gut*, **5**, 230(1964).
(13) D. A. Brodie and B. J. Chase, *Gastroenterology*, **53**, 604 (1967).
(14) J. R. Leonards and G. Levy, *Clin. Pharmacol. Ther.*, **10**, 571 (1969).
(15) J. R. Leonards and G. Levy, *J. Pharm. Sci.*, **58**, 1277(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 10, 1970, from the **Department of Biochemistry, School of Medicine, Case-Western Reserve University, Cleveland, OH 44106* and the *† Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication April 6, 1970.

Effect of Micelle Formation on Optical Rotatory Dispersion of β-D-Octyl Glucoside

P. MUKERJEE, J. PERRIN, and E. WITZKE

Abstract □ The optical rotatory dispersion of a nonionic surfactant, β-D-octyl glucoside, has been investigated in aqueous solutions in the UV region. The rotatory dispersion curves at any concentration can be represented by a one-term Drude equation. The specific rotation at any wavelength shows an increase at the CMC, which can be determined reliably from this change in specific rotation. The rotatory dispersion curve for the surfactant in micellized form has been derived and compared with that of the nonmicellized surfactant below the CMC. The change is small and can be ascribed to a "medium" effect, arising from the difference in the local refractive index at the micelle surface, as compared to the bulk solvent. This interpretation is compatible with the currently accepted ideas on the fluid nature of the micelle core and suggests a lack of any conformational restraint at the micellar interface.

Keyphrases □ Optical rotatory dispersion, β-D-octyl glucoside—micelle formation effect □ β-D-Octyl glucoside—optical rotatory dispersion, micelle formation effect □ Micelle formation, effect—optical rotatory dispersion, β-D-octyl glucoside

Many chemical and biochemical reactions and interactions of interest occur at interfaces, *e.g.*, monolayers, micelles, enzymes, or membranes. The local molecular environment at an interface and the presence of an asymmetry and other peculiarities in dielectric properties (1) are factors of considerable importance in understanding the properties of molecules at interfaces. It has been pointed out recently that for studying many such interactions, the interface between a micelle and the solution provides a convenient locus whose composition is capable of a considerable controlled variation (2, 3). The use of optically active surfactant monomers or solubilized molecules offers the possibility of using optical activity as a probe for studying properties of interfaces and of understanding the effect of the interface composition on the optical activity itself. The present paper reports what appears to be the first such study on a simple model surfactant system, β-D-octyl glucoside.

β-D-Octyl glucoside has a CMC in aqueous solution of 0.024–0.025 *M* at 25° (4, 5). The micelles probably contain about 30 monomers (6), the hydrocarbon chains forming a spheroidal core. The glucoside head groups presumably remain exposed to water both in the monomeric and micellar forms. This, by itself, would suggest that there should be no change in their optical activity. In fact, however, the packing of the chains produces a high effective concentration of the head groups in the interfacial layer (2), which interact strongly enough with each other to counter the micelle-forming tendency of the aliphatic chain rather substantially. This is apparent from the following comparison.

Recently the CMC of a hypothetical octyl chain, unencumbered by any head group, was estimated to be about 3×10^{-3} *M* (3). The CMC of octyl glucoside is higher by a factor of about 8. The head group self-interaction thus makes the standard free energy of micelle formation for octyl glucoside more positive by $kT \ln 8$ or about $2kT$'s per monomer, where k is the Boltzmann constant and T the absolute temperature (3). The glucoside groups at the micelle surface are thus in a considerably different local environment when compared to the free monomers.

EXPERIMENTAL

Materials—The octanol used was a Baker analyzed reagent, which was purified further by vacuum distillation, the middle one-third portion being collected.

β-D-Octyl Glucoside—Glucose, on acetylation followed by bromination (7), yielded acetobromoglucose, m.p. 89°. The bromo compound was reacted with octanol in dry absolute ether in the presence of silver oxide to give β-tetraacetyl octyl glucoside, m.p. 63–64°. After deacetylation in sodium methylate solutions, β-D-octyl glucoside was obtained. It was recrystallized twice from ethyl acetate, washed with Skelly-A, and dried under vacuum. The compound melts over a wide range, 65–99° (8). The intermediate compounds were purified by recrystallization before proceeding to the next step of the preparation.

Anal.—Calcd. for C, 57.5; H, 9.7. Found: C, 57.8; H, 9.5.

Apparatus and Experimental Procedure—The optical rotatory dispersion (ORD) measurements were carried out in a Cary model 60 spectropolarimeter. The cell compartment was thermostated at 25 ± 0.2°. Five-centimeter cells were used. All solutions were optically clear, and double-distilled water was used. The ORD measurements were made in the 250–370-mμ region.

RESULTS

Figure 1 shows the variation of the observed rotation at 320 mμ as a function of concentration. To magnify the small differences observed, a deviation plot is presented. The data show the usual curvature near the CMC. If the CMC region is excluded, the data

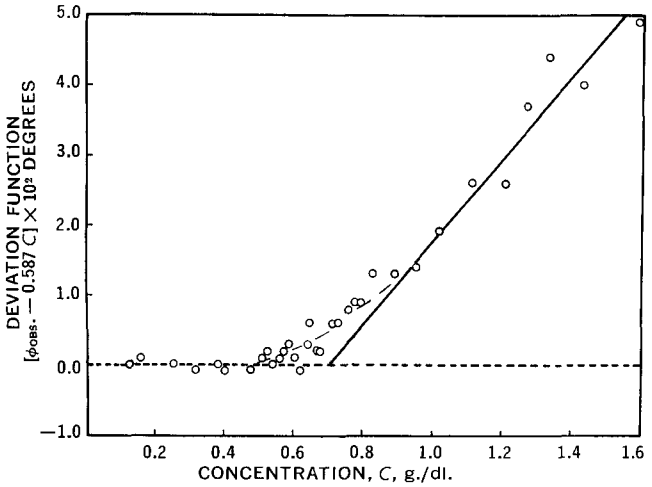


Figure 1—*Variation in optical rotation at 320 mμ with concentration of octyl glucoside at 25°. The ordinate records the difference between the observed rotation, ϕ, and that calculated as 0.587 C, C being the concentration of octyl glucoside in grams per 100 ml.*

can be represented by two straight lines, one below and one above the CMC, whose point of intersection corresponds to the CMC. The value observed, 0.70% or 0.024 mole/l., is in good agreement with the value of 0.024–0.025 *M* obtained from surface-tension measurements (4, 5). ORD measurements are thus capable of determining CMC's for some optically active surfactants. The technique should be of some value for naturally occurring optically active surfactants such as bile salts.

To determine the nature and extent of the change in the ORD curves, the rotation data were analyzed by using the simple Drude equation:

$$[\alpha]_\lambda = A/(\lambda^2 - \lambda_0^2) \qquad \text{(Eq. 1)}$$

n which $[\alpha]_\lambda$ is the specific rotation measured at the wavelength λ. A and λ_0 are constants (9). For exhibiting the changes observed on micelle formation on a magnified scale, Eq. 2, which is a rearranged form of Eq. 1, was used, as suggested by Heller (10):

$$\frac{1}{[\alpha]_\lambda \lambda^2} = \frac{1}{A} - \frac{\lambda_0^2}{A\lambda^2} \qquad \text{(Eq. 2)}$$

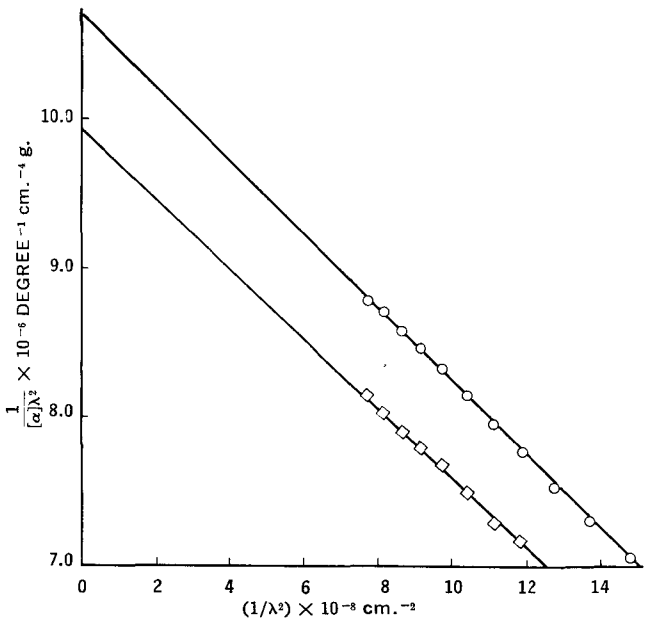


Figure 2—*Plots of specific rotation–wavelength data for octyl glucoside at 25° according to Eq. 2. Key: ○, below the CMC (monomeric); and □, micellar (see text).*

A plot of $1/[\alpha]_\lambda\lambda^2$ *versus* $1/\lambda^2$ should yield a straight line if Drude's equation is obeyed. From the intercept and the slope, A and λ_0 can be determined.

The upper curve in Fig. 2 shows the data below the CMC. Each point is a mean of three measurements at three different concentrations. This curve represents the monomer. To obtain the curve representing the optical rotatory power of octyl glucoside in the micellar form, the change in the observed rotation, $\Delta\phi$, at a particular wavelength was determined from the difference in ϕ between two concentrations, one considerably above and one slightly above the CMC. From this $\Delta\phi$ and the corresponding difference in concentration, ΔC, after a suitable correction for the pathlength of the cell, $[\alpha]_\lambda^m$, the specific rotation of the surfactant in the micellar form was obtained. The rationale behind this method of calculation is the well-founded assumption that above the CMC the monomer concentration changes very little with the total concentration. Here, also, the plotted points are the means of three sets of calculations of $[\alpha]_\lambda^m$, using three different combinations of concentrations. The average variation in the specific rotations estimated at different concentrations was 0.2–0.3%.

Figure 2 shows that the data are linear within experimental error; thus the simple Drude equation is obeyed. The values of A obtained from the least-squares fitted straight lines are 9.32×10^6 and 10.08×10^6 (degree^{-1} cm.$^{-4}$ g.) for the monomeric and micellar forms, respectively, while the corresponding values of λ_0 are 1525 and 1531 Å. Thus, the change in the ORD on micelle formation arises mainly from a change of about 8% in A, the change in λ_0 being barely significant.

DISCUSSION

Although this analysis shows that the simple Drude equation is obeyed and that the primary change on micelle formation is in A rather than λ_0, an unequivocal interpretation is difficult; the apparent simplicity of the Drude equation may be misleading.

For regions far from optically active absorption bands, the specific rotation can be written as (11)

$$[\alpha]_\lambda = \left(\frac{n^2+2}{3}\right)\left(\frac{9600\pi cN}{Mh\lambda^2}\right)\sum_i \frac{R_i}{\nu_i^2 - \nu^2} \qquad \text{(Eq. 3)}$$

where n is the refractive index of the medium, c is the velocity of light, N is Avogadro's number, M is the molecular weight of the substance, h is Planck's constant, ν is the frequency of incident light, ν_i is the frequency characterizing the electronic transition to an excited state, and R_i is the "rotatory strength" of the transition. Replacing the frequencies by the corresponding wavelengths, and condensing several quantities into the new variable A_i, yield

$$[\alpha]_\lambda = \left(\frac{n^2+2}{3}\right)\sum_i \frac{A_i\lambda_i^2}{\lambda^2 - \lambda_i^2} \qquad \text{(Eq. 4)}$$

Although Eq. 4 has the form of the Drude Eq. 1, it clearly shows that λ and A in Eq. 1, where A includes the refractive index factor, are complicated averages.

By considering the physical realities of the situation at a micellar interface, however, it is possible, qualitatively, to ascribe at least a part of the difference in A between free and micellized monomers to the Lorentz correction factor, $(n^2 + 2)/3$. At the micellar interface, the glucose moieties are present at a high concentration, roughly 3 molal or 35% by weight (3), and close to the hydrocarbon core of the micelle. The refractive index, at the sodium D line (5893 Å), of a 35% by weight solution of glucose is estimated to be 1.390 (12),[1] as compared to 1.3330, the value of water. This, by itself, can increase the Lorentz factor by 4%. The actual effect may be substantially higher because of two additional factors. The frequency dependence of the refractive index (dispersion) is expected to be substantially higher for glucose than for water and, therefore, the refractive increment for glucose solutions should be higher at the lower wavelengths at which the ORD measurements were made,

[1] Experimental values of the refractive index of D-glucose solutions at such high concentrations were not available. The refractive increments of D-glucose and sucrose up to 10% concentrations are very similar, however. Therefore, the authors used the value for a 35% solution of sucrose, 1.3902. D-Fructose has a very similar value, also.

2500–3700 Å. The proximity of the hydrocarbon core of the micelle may also be a contributory factor, because of the higher refractive index of octane (n_D = 1.3975) as compared to water. Thus, although no firm conclusion is possible, the change in optical rotation on micelle formation is not incompatible with a "medium" effect, operating through the Lorentz factor, and no conformational restraints at the micelle surface need be invoked. This tentative conclusion is in accord with the accepted fluid nature of the micelle core (3). It should clearly be of some interest to study monomers or solubilized species in micelles containing optically active absorption bands in experimentally accessible wavelength regions.

Finally, note the observed curvature near the CMC in Fig. 1. This is another piece of evidence against the phase-separation model for micellization, the arguments against which have been summarized recently (3).

REFERENCES

(1) P. Mukerjee and A. Ray, *J. Phys. Chem.*, **70,** 2144(1966).
(2) P. Mukerjee and K. Banerjee, *ibid.*, **68,** 3567(1964).
(3) P. Mukerjee, *Advan. Colloid Interface Sci.*, **1,** 241(1967).
(4) C. R. Bury and J. Browning, *Trans. Faraday Soc.*, **49,** 209 (1953).
(5) K. Shinoda, T. Yamanaka, and K. Kinoshita, *J. Phys. Chem.*, **63,** 648(1959).
(6) R. R. Balmbra, J. S. Clunie, J. M. Corkill, and J. F. Goodman, *Trans. Faraday Soc.*, **60,** 979(1964).
(7) R. Whistler and M. Wolfrom, Eds., "Methods in Carbohydrate Chemistry," vol. II, Academic, New York, N. Y., 1963, p. 221.
(8) C. R. Noller and W. C. Rockwell, *J. Amer. Chem. Soc.*, **60,** 2076(1938).
(9) C. Djerassi, "Optical Rotatory Dispersion," McGraw-Hill, New York, N. Y., 1960, p. 5.
(10) W. Heller, *J. Phys. Chem.*, **62,** 1569(1958).
(11) P. Urnes and P. Doty, *Advan. Protein Chem.*, **16,** 401(1961).
(12) "Handbook of Chemistry and Physics," The Chemical Rubber Co., 46th ed., pp. 1065, 1066.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 30, 1970, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*

Accepted for publication April 23, 1970.

2-Amino-2-oxazoline Formation by Cyclization of 1-(2-Hydroxyethyl)-2-methyl-2-thiopseudoureas

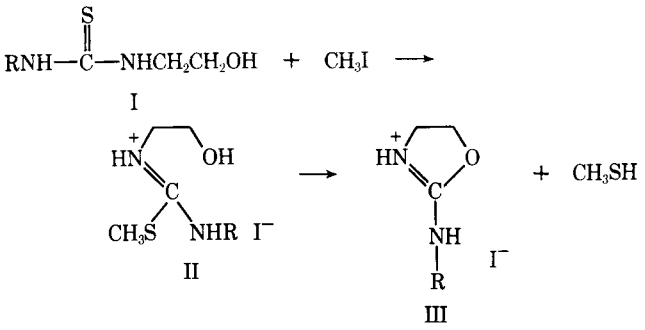
DANIEL L. KLAYMAN, ROBERT J. SHINE*, and ARLESS E. MURRAY, Jr.

Abstract □ 1-(2-Hydroxyethyl)-3-substituted-2-methyl-2-thiopseudourea hydriodides, when heated in polar solvents, were found in many instances to result in the formation of 2-amino-2-oxazoline derivatives with the simultaneous evolution of methyl mercaptan. The rate of the reaction is apparently influenced by the group substituted in the 3-position of the thiopseudourea. Similarly, the 5,6-dihydro-4*H*-1,3-oxazine ring system could be prepared by starting with a 1-(3-hydroxypropyl)-2-thiourea.

Keyphrases □ 2-Amino-2-oxazoline formation—using thiopseudoureas, cyclization □ Thiopseudoureas—in formation of 2-amino-2-oxazoline □ IR spectrophotometry—identity

An investigation of the influence of the degree of *N*-substitution of *S*-methylthiopseudoureas, which are subjected to alkaline hydrolysis, on the rate of methyl mercaptan evolution was reported earlier (1). In the course of that study the preparation of the *S*-methyl derivative of 1-(2-hydroxyethyl)-3-benzoyl-2-thiourea (I*f*), a potential antiradiation agent, was attempted. It was found that methyl mercaptan was readily evolved when the *S*-methyl derivative (II*f*) was heated in polar organic solvents such as acetonitrile, alcohols, and acetone, even in the absence of base. Methyl mercaptan was formed as a consequence of the intramolecular displacement of the methylthio group by the hydroxyl group to give 2-benzamido-2-oxazoline hydriodide (III*f*) in 73% yield.

To examine further this interesting reaction, a number of other 1-(2-hydroxyalkyl)-2-thioureas were prepared and subsequently treated with methyl iodide (Scheme I).



Scheme I

Simply heating the 2-methyl-2-thiopseudourea hydriodides in polar solvents in several cases led to methyl mercaptan evolution. The rate of this evolution, which reflects the extent of the cyclization reaction, was found to be strongly influenced by the R group of the 1-(2-hydroxyalkyl)-2-thiourea (I). Oxazoline formation proceeds smoothly when there is a benzoyl group in the 3-position of a 1-(2-hydroxyalkyl)-2-thiourea. 1-(2-Hydroxypropyl)-3-benzoyl-2-thiourea (I*l*), on heating with methyl iodide in ethanol, gave 2-benzamido-5-methyl-2-oxazoline hydriodide (III*l*). When 1-(3-hydroxypropyl)-3-benzoyl-2-thiourea was used (I*k*), the cyclization reaction gave the six-membered heterocycle—*viz.*, 2-benzamido-5,6-dihydro-4*H*-1,3-oxazine hydriodide (III*k*), in good yield.

The evolution of methyl mercaptan was slower when the *S*-methyl derivatives of 1-(2-hydroxyethyl)-3-phenyl-2-thiourea (I*d*) and 1-(2-hydroxypropyl)-3-phenyl-2-

2500–3700 Å. The proximity of the hydrocarbon core of the micelle may also be a contributory factor, because of the higher refractive index of octane (n_D = 1.3975) as compared to water. Thus, although no firm conclusion is possible, the change in optical rotation on micelle formation is not incompatible with a "medium" effect, operating through the Lorentz factor, and no conformational restraints at the micelle surface need be invoked. This tentative conclusion is in accord with the accepted fluid nature of the micelle core (3). It should clearly be of some interest to study monomers or solubilized species in micelles containing optically active absorption bands in experimentally accessible wavelength regions.

Finally, note the observed curvature near the CMC in Fig. 1. This is another piece of evidence against the phase-separation model for micellization, the arguments against which have been summarized recently (3).

REFERENCES

(1) P. Mukerjee and A. Ray, *J. Phys. Chem.*, **70,** 2144(1966).
(2) P. Mukerjee and K. Banerjee, *ibid.*, **68,** 3567(1964).
(3) P. Mukerjee, *Advan. Colloid Interface Sci.*, **1,** 241(1967).
(4) C. R. Bury and J. Browning, *Trans. Faraday Soc.*, **49,** 209 (1953).
(5) K. Shinoda, T. Yamanaka, and K. Kinoshita, *J. Phys. Chem.*, **63,** 648(1959).
(6) R. R. Balmbra, J. S. Clunie, J. M. Corkill, and J. F. Goodman, *Trans. Faraday Soc.*, **60,** 979(1964).
(7) R. Whistler and M. Wolfrom, Eds., "Methods in Carbohydrate Chemistry," vol. II, Academic, New York, N. Y., 1963, p. 221.
(8) C. R. Noller and W. C. Rockwell, *J. Amer. Chem. Soc.*, **60,** 2076(1938).
(9) C. Djerassi, "Optical Rotatory Dispersion," McGraw-Hill, New York, N. Y., 1960, p. 5.
(10) W. Heller, *J. Phys. Chem.*, **62,** 1569(1958).
(11) P. Urnes and P. Doty, *Advan. Protein Chem.*, **16,** 401(1961).
(12) "Handbook of Chemistry and Physics," The Chemical Rubber Co., 46th ed., pp. 1065, 1066.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 30, 1970, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*

Accepted for publication April 23, 1970.

2-Amino-2-oxazoline Formation by Cyclization of 1-(2-Hydroxyethyl)-2-methyl-2-thiopseudoureas

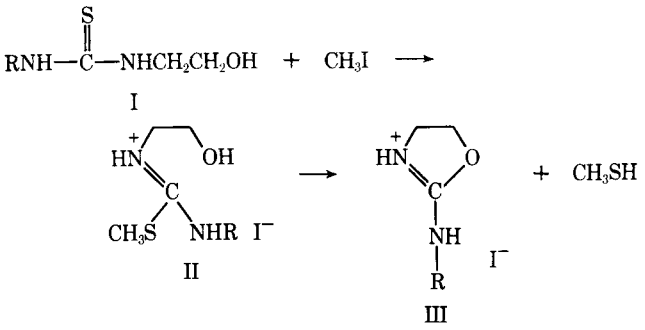
DANIEL L. KLAYMAN, ROBERT J. SHINE*, and ARLESS E. MURRAY, Jr.

Abstract □ 1-(2-Hydroxyethyl)-3-substituted-2-methyl-2-thiopseudourea hydriodides, when heated in polar solvents, were found in many instances to result in the formation of 2-amino-2-oxazoline derivatives with the simultaneous evolution of methyl mercaptan. The rate of the reaction is apparently influenced by the group substituted in the 3-position of the thiopseudourea. Similarly, the 5,6-dihydro-4*H*-1,3-oxazine ring system could be prepared by starting with a 1-(3-hydroxypropyl)-2-thiourea.

Keyphrases □ 2-Amino-2-oxazoline formation—using thiopseudoureas, cyclization □ Thiopseudoureas—in formation of 2-amino-2-oxazoline □ IR spectrophotometry—identity

An investigation of the influence of the degree of *N*-substitution of *S*-methylthiopseudoureas, which are subjected to alkaline hydrolysis, on the rate of methyl mercaptan evolution was reported earlier (1). In the course of that study the preparation of the *S*-methyl derivative of 1-(2-hydroxyethyl)-3-benzoyl-2-thiourea (I*f*), a potential antiradiation agent, was attempted. It was found that methyl mercaptan was readily evolved when the *S*-methyl derivative (II*f*) was heated in polar organic solvents such as acetonitrile, alcohols, and acetone, even in the absence of base. Methyl mercaptan was formed as a consequence of the intramolecular displacement of the methylthio group by the hydroxyl group to give 2-benzamido-2-oxazoline hydriodide (III*f*) in 73% yield.

To examine further this interesting reaction, a number of other 1-(2-hydroxyalkyl)-2-thioureas were prepared and subsequently treated with methyl iodide (Scheme I).

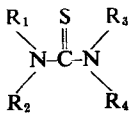


Scheme I

Simply heating the 2-methyl-2-thiopseudourea hydriodides in polar solvents in several cases led to methyl mercaptan evolution. The rate of this evolution, which reflects the extent of the cyclization reaction, was found to be strongly influenced by the R group of the 1-(2-hydroxyalkyl)-2-thiourea (I). Oxazoline formation proceeds smoothly when there is a benzoyl group in the 3-position of a 1-(2-hydroxyalkyl)-2-thiourea. 1-(2-Hydroxypropyl)-3-benzoyl-2-thiourea (I*l*), on heating with methyl iodide in ethanol, gave 2-benzamido-5-methyl-2-oxazoline hydriodide (III*l*). When 1-(3-hydroxypropyl)-3-benzoyl-2-thiourea was used (I*k*), the cyclization reaction gave the six-membered heterocycle—*viz.*, 2-benzamido-5,6-dihydro-4*H*-1,3-oxazine hydriodide (III*k*), in good yield.

The evolution of methyl mercaptan was slower when the *S*-methyl derivatives of 1-(2-hydroxyethyl)-3-phenyl-2-thiourea (I*d*) and 1-(2-hydroxypropyl)-3-phenyl-2-

Table I—Hydroxyalkyl-2-thioureas (I)

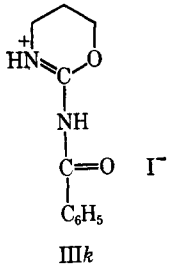


	R_1	R_2	R_3	R_4	M.p.	Yield, %	Recrystn. Solvent	Anal., % Calcd.	Found
a	H	H	H	CH_2CH_2OH	82–84°	79	MeOH–Et_2O	C, 29.49 H, 6.71 N, 23.31 S, 26.68	C, 29.49 H, 6.71 N, 23.51 S, 26.68
b	CH_3	H	H	CH_2CH_2OH	73°[a]	92	EtOH		
c	*tert*-C_4H_9	H	H	CH_2CH_2OH	147°	87	EtOH	C, 47.69 H, 9.15 N, 15.89 S, 18.19	C, 47.89 H, 9.09 N, 15.92 S, 18.06
d	C_6H_5	H	H	CH_2CH_2OH	140°[b]	97	EtOH		
e	$C_6H_5CH_2CH_2$	H	H	CH_2CH_2OH	92–94°	93	$CHCl_3$	C, 58.90 H, 7.19 N, 12.49 S, 14.29	C, 59.11 H, 7.36 N, 12.42 S, 14.27
f	C_6H_5CO	H	H	CH_2CH_2OH	128°[c]	51	MeOH		
g	C_6H_5	CH_3	H	CH_2CH_2OH	104–105°	93	EtOH	C, 57.11 H, 6.71 N, 13.32 S, 15.25	C, 57.39 H, 6.56 N, 13.37 S, 15.10
h	C_6H_5	H	CH_2CH_2OH	CH_2CH_2OH	98°	91	$CHCl_3$	C, 54.97 H, 6.71 N, 11.66 S, 13.35	C, 54.69 H, 6.72 N, 11.72 S, 13.28
i	C_6H_5	H	H	$CH_2CH_2CH_2OH$	82–84°	87	$CHCl_3$	C, 57.11 H, 6.71 N, 13.32 S, 15.25	C, 56.95 H, 6.52 N, 13.11 S, 15.20
j	C_6H_5	H	H	$CH_2CH(OH)CH_3$	108–109°	92	CH_3CN	C, 57.11 H, 6.71 N, 13.32 S, 15.25	C, 56.76 H, 6.82 N, 13.22 S, 15.14
k	C_6H_5CO	H	H	$CH_2CH_2CH_2OH$	110°	30	CH_3CN	C, 55.44 H, 5.92 N, 11.76 S, 13.46	C, 55.67 H, 6.22 N, 12.11 S, 13.37
l	C_6H_5CO	H	H	$CH_2CH(OH)CH_3$	115–116°	34	EtOH	C, 55.44 H, 5.92 N, 11.76 S, 13.46	C, 55.07 H, 5.79 N, 11.72 S, 13.41

[a] *Reference 2*, m.p. 73°. [b] L. Knorr and P. Rössler, *Ber.*, **36**, 1278(1903), m.p. 138°; *Reference 4*, m.p. 139°. [c] *Reference 4*, m.p. 128°.

thiourea (I*j*) were used than with the comparable benzoyl derivatives described previously. 2-Anilino-2-oxazoline (III*d*) and 2-anilino-5-methyl-2-oxazoline (III*j*) hydriodides were obtained in good yield after several hours of refluxing in ethanol. The *S*-methyl derivative of 1-(3-hydroxypropyl)-3-phenyl-2-thiourea (I*i*), in contrast to its benzoyl analog, failed to cyclize to any appreciable extent, as was further indicated by only a meager evolution of methyl mercaptan.

S-Methyl derivatives of 1-(2-hydroxyethyl)-2-thioureas, which were substituted in the 3-position by methyl (I*b*), *tert*-butyl (I*c*), and phenethyl (I*e*), on refluxing in acetonitrile or ethanol gave only trace quantities of methyl mercaptan, indicating failure of the cyclization reaction to occur. The thiopseudoureas could be isolated from the reaction mixture. When the *S*-methyl derivatives of 1,1-di(2-hydroxyethyl)-3-phenyl-2-thiourea (I*h*) and 1-(2-hydroxyethyl)-3-methyl-3-phenyl-2-thiourea (I*g*) were refluxed in the usual polar solvents, considerably less than one equivalent of methyl mercaptan was evolved, resulting in their limited conversion to oxazolines. Compound III*g* could be isolated in poor yield, however, when the reaction was performed in water.



III*k*

In an attempt to make the difficultly accessible 2-amino-2-oxazoline hydriodide (III*a*), the previously unknown 1-(2-hydroxyethyl)-2-thiourea (I*a*) was prepared by the reaction of benzoylisothiocyanate with 2-aminoethanol. The resultant compound, I*f*, was hydrolyzed with dilute sodium hydroxide, giving I*a* in good yield. The *S*-methyl derivative of this thiourea failed to cyclize to any appreciable extent. A successful synthesis of 2-amino-2-oxazoline (isolated as the picrate salt) was achieved, however, by removing the benzoyl group of III*f* by hydrolysis with 1:1 hydrochloric acid for about 2 hr. Its homolog, 2-amino-5,6-dihydro-4*H*-1,3-oxazine picrate, was prepared similarly from its benzamido derivative (III*k*).

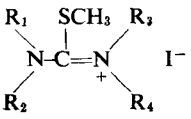


Table II—*S*-Methyl Thiopseudourea Hydriodides (II)

Compound[a]	M.p.	Yield, %	Recrystn. Solvent	Molecular Formula	Anal., % Calcd.	Found
a	93°	61	CH_3CN	$C_4H_{11}IN_2OS$	C, 18.33 H, 4.23 N, 10.67 S, 12.23	C, 18.55 H, 4.20 N, 10.72 S, 12.04
b	89–90°	86	CH_3CN	$C_5H_{13}IN_2OS$	C, 21.75 H, 4.75 N, 10.14 S, 11.61	C, 21.97 H, 4.85 N, 10.34 S, 11.75
c	158–159°	96	EtOH	$C_8H_{19}IN_2OS$	C, 30.19 H, 6.02 N, 8.81 S, 10.08	C, 29.92 H, 5.85 N, 8.75 S, 10.23
d	110–111°	96	CH_3CN	$C_{12}H_{19}IN_2OS$	C, 39.35 H, 5.23 N, 7.65 S, 8.75	C, 39.41 H, 5.34 N, 7.76 S, 8.69
h	95–97°	64	CH_3CN	$C_{12}H_{19}IN_2O_2S$	C, 37.70 H, 5.01 N, 7.33 S, 8.39	C, 37.59 H, 4.87 N, 7.24 S, 8.53
i	89.5°	70	CH_3CN	$C_{11}H_{17}IN_2OS$	C, 37.51 H, 4.87 N, 7.95 S, 9.10	C, 37.53 H, 5.04 N, 8.09 S, 9.27
j	107–108°	60	EtOH	$C_{11}H_{17}IN_2OS$	C, 37.51 H, 4.87 N, 7.95 S, 9.10	C, 37.74 H, 4.81 N, 7.91 S, 9.27

[a] Derived from the thiourea indicated by the same letter in Table I.

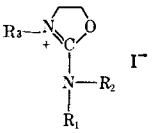


Table III—2-Amino-2-oxazoline Hydriodides (III)

Compound[a]	M.p.	Yield, %	Recrystn. Solvent	Molecular Formula	Anal., % Calcd.	Found
d	158°	30	2-PrOH	$C_9H_{11}IN_2O$	C, 37.25 H, 3.82 N, 9.69	C, 37.52 H, 4.03 N, 9.54
f	194–196°	87	EtOH	$C_{10}H_{11}IN_2O_2$	C, 37.76 H, 3.49 N, 8.81	C, 37.84 H, 3.50 N, 8.83
g	128° dec.[b]	18	EtOH	$C_{10}H_{13}IN_2O$	C, 39.49 H, 4.31 N, 9.21	C, 39.13 H, 4.32 N, 8.91
k	132°[c]	72	CH_3CN	$C_{11}H_{13}IN_2O_2$	C, 39.78 H, 3.94 N, 8.43	C, 39.74 H, 4.18 N, 8.38
l	157°	47	CH_3CN	$C_{11}H_{13}IN_2O_2$	C, 39.78 H, 3.94 N, 8.43	C, 40.09 H, 3.85 N, 8.38

[a] Derived from the thiourea indicated by the same letter in Table I. [b] Reaction was run in water to give a complex mixture of products which included the amino-oxazoline. [c] 2-Benzamido-5,6-dihydro-4*H*-1,3-oxazine hydriodide.

Adcock *et al.* (2) reported the use of 2.5 equivalents of sodium ethoxide in boiling ethanol to effect the cyclization of certain 1-(2-hydroxyethyl)-2-methyl-2-thiopseudoureas. However, it appears that base is not necessary if the R groups of II are sufficiently activating. The resultant increase in the net positive charge on the carbon atom bearing the methylthio group facilitates the attack by the hydroxyl oxygen atom.

While this investigation was in progress, Budde and Salerni (3) in a related reaction reported the cyclization of the *S*-ethyl derivative of 1-(2-hydroxyethyl)-1-(2-acetamidoethyl)-3-phenyl-2-thiourea to give a 2,3-disubstituted oxazoline.

EXPERIMENTAL[1]

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. IR spectra were determined on a

[1] Microanalyses were performed by Mr. Joseph F. Alicino, Metuchen, NJ 08840

Beckman IR-5 or Perkin-Elmer 221 spectrophotometer as KBr pellets.

Hydroxyalkyl-2-thioureas—To 0.2 mole of an alkyl or aryliso-thiocyanate in 100 ml. of ethanol was added portionwise 0.2 mole of an amino-alcohol, and the resultant solution was heated for 0.5 hr. on a steam bath. In some cases, the hydroxyalkyl-2-thioureas crystallized from the cooled solution; in other instances, the solutions were concentrated or evaporated to dryness to induce crystallization.

1-(Hydroxyalkyl)-3-benzoyl-2-thioureas—These compounds were prepared essentially by the method of Douglass and Dains (4). To 44.6 g. (0.55 mole) of sodium thiocyanate dissolved in 500 ml. of acetonitrile or acetone was added slowly 70.3 g. (0.50 mole) of benzoyl chloride, and the resulting mixture was heated on a steam bath for 10 min. The mixture was then cooled, and the amino-alcohol (0.50 mole) was added with constant stirring. The resultant mixture was heated on a steam bath for 15 min. and poured into a large volume of water. The oily layer generally crystallized on cooling to give the 1-(hydroxyalkyl)-3-benzoyl-2-thiourea (Table I).

1-(2-Hydroxyethyl)-2-thiourea (Ia)—To 22.4 g. (0.1 mole) of 1-(2-hydroxyethyl)-3-benzoyl-2-thiourea was added 100 ml. of 6% NaOH solution, and the resulting solution was heated on a steam bath for 10 min. The cooled solution was then made slightly acidic with 6 *N* H_2SO_4; the benzoic acid, which separated, was removed by filtration. The filtrate was adjusted to neutrality with dilute NaOH solution. The solvent was removed under reduced pressure, giving a residue which was extracted with three 100-ml. portions of hot CH_3CN. The CH_3CN extracts were evaporated to dryness, and the Ia was recrystallized (Table I).

Reaction of Hydroxyalkyl-2-thioureas with Methyl Iodide—To 1-(hydroxyalkyl)-2-thiourea (0.05 mole) dissolved in 40 ml. of acetonitrile or ethanol was added methyl iodide (0.06 mole), and the solution was heated under reflux. When methyl mercaptan evolution was very faint or not detected within 0.5 hr., the solution was refluxed for 2 hr. and cooled or concentrated under reduced pressure to give the *S*-methylthiopseudourea hydriodide. The latter gave a positive nitroprusside test (1) when heated with base (Table II).

When a copious evolution of methyl mercaptan was detected within the first 0.5 hr. of refluxing, heating was continued until methyl mercaptan evolution virtually ceased, generally within 6–12 hr. The solution was cooled or concentrated, causing separation of the 2-amino-2-oxazoline derivative (or 2-amino-5,6-dihydro-4*H*-1,3-oxazine derivative) as the hydriodide salt. The IR spectra of these compounds showed a characteristic peak at 5.9–6.1 μ (C=N) (Table III).

2-Amino-2-oxazoline (IIIa)—A mixture of 1.0 g. (0.0031 mole) III*f* in 10 ml. of 1:1 HCl was agitated and heated under reflux for about 2 hr. until solution was complete. The benzoic acid, which separated on cooling, was removed and the filtrate was concentrated several times with water to remove the excess acid. The syrup was treated with a saturated ethanolic solution of picric acid to give 0.45 g. (46%) of 2-amino-2-oxazoline picrate as fine needles (from water), m.p. 192–195° [lit. (5) m.p. 186–188°].

2-Amino-5,6-dihydro-4*H*-1,3-oxazine—2-Benzamido-5,6-dihydro-4*H*-1,3-oxazine hydriodide (III*k*, 1.0 g., 0.0030 mole) was treated as described previously to give 0.37 g. (37%) of 2-amino-5,6-dihydro-4*H*-1,3-oxazine picrate as fine needles (from water), m.p. 207–208° [lit. (6) m.p. 200°].

REFERENCES

(1) D. L. Klayman and R. J. Shine, *Anal. Chim. Acta*, **41**, 408 (1968).

(2) B. Adcock, A. Lawson, and D. H. Miles, *J. Chem. Soc.*, **1961**, 5120.

(3) W. L. Budde and O. L. Salerni, *J. Heterocycl. Chem.*, **6**, 419 (1969).

(4) I. B. Douglass and F. B. Dains, *J. Amer. Chem. Soc.*, **56**, 719(1934).

(5) S. Gabriel, *Ber.*, **22**, 1139(1889).

(6) S. Gabriel and W. E. Lauer, *ibid.*, **23**, 87(1890).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 22, 1969, from the *Division of Medicinal Chemistry, Walter Reed Army Institute of Research, Washington, DC 20012*

Accepted for publication April 3, 1970.

The authors thank Mr. David Kenny for technical assistance.

* Present address: Department of Chemistry, Trinity College, Washington, DC 20017

Comparison of Effects of 1,8-Dihydroxyanthraquinone and 1,5-Dihydroxyanthraquinone on Different Segments of Rabbit Gastrointestinal Tract

THEODORE H. EICKHOLT, LAWRENCE J. DANNA*, LYNN J. VERZWYVELT*, GERALD P. WAGUESPACK*, JOHNNY L. BUNCH*, and DANIEL D. WINSLOW*

Abstract □ Using an isolated muscle bath technique, five different segments of the rabbit gastrointestinal tract (duodenum, jejunum, ileum, and ascending and descending colon) were used to record the effects of two doses of 1,5-dihydroxyanthraquinone. Transducer-recorded tracings were made of contractions per minute, interval between contractions, and amplitude of contractions. The results were evaluated; statistical tests among comparisons of the three parameters between the two drug doses, control values, and previous values for 1,8-dihydroxyanthraquinone again indicated the parameter of interval between contractions to be a statistically significant measurement of activity. No statistically significant differences in effects were shown between the two drugs.

Keyphrases □ 1,8- *versus* 1,5-Dihydroxyanthraquinone—effect on gastrointestinal tract, rabbit □ Gastrointestinal tract, rabbit—effects, 1,8- *versus* 1,5-dihydroxyanthraquinone

The normal activity of different segments of the rabbit gastrointestinal tract has been previously delineated, using an isolated muscle bath technique (1).

The effects of 15- and 30-mg. doses of 1,8-dihydroxyanthraquinone on these different segments of rabbit intestinal tract have been studied with this technique and statistically compared with the standards (2). The results of the statistical analyses of the transducer-

Beckman IR-5 or Perkin-Elmer 221 spectrophotometer as KBr pellets.

Hydroxyalkyl-2-thioureas—To 0.2 mole of an alkyl or aryliso-thiocyanate in 100 ml. of ethanol was added portionwise 0.2 mole of an amino-alcohol, and the resultant solution was heated for 0.5 hr. on a steam bath. In some cases, the hydroxyalkyl-2-thioureas crystallized from the cooled solution; in other instances, the solutions were concentrated or evaporated to dryness to induce crystallization.

1-(Hydroxyalkyl)-3-benzoyl-2-thioureas—These compounds were prepared essentially by the method of Douglass and Dains (4). To 44.6 g. (0.55 mole) of sodium thiocyanate dissolved in 500 ml. of acetonitrile or acetone was added slowly 70.3 g. (0.50 mole) of benzoyl chloride, and the resulting mixture was heated on a steam bath for 10 min. The mixture was then cooled, and the amino-alcohol (0.50 mole) was added with constant stirring. The resultant mixture was heated on a steam bath for 15 min. and poured into a large volume of water. The oily layer generally crystallized on cooling to give the 1-(hydroxyalkyl)-3-benzoyl-2-thiourea (Table I).

1-(2-Hydroxyethyl)-2-thiourea (Ia)—To 22.4 g. (0.1 mole) of 1-(2-hydroxyethyl)-3-benzoyl-2-thiourea was added 100 ml. of 6% NaOH solution, and the resulting solution was heated on a steam bath for 10 min. The cooled solution was then made slightly acidic with 6 *N* H_2SO_4; the benzoic acid, which separated, was removed by filtration. The filtrate was adjusted to neutrality with dilute NaOH solution. The solvent was removed under reduced pressure, giving a residue which was extracted with three 100-ml. portions of hot CH_3CN. The CH_3CN extracts were evaporated to dryness, and the Ia was recrystallized (Table I).

Reaction of Hydroxyalkyl-2-thioureas with Methyl Iodide—To 1-(hydroxyalkyl)-2-thiourea (0.05 mole) dissolved in 40 ml. of acetonitrile or ethanol was added methyl iodide (0.06 mole), and the solution was heated under reflux. When methyl mercaptan evolution was very faint or not detected within 0.5 hr., the solution was refluxed for 2 hr. and cooled or concentrated under reduced pressure to give the *S*-methylthiopseudourea hydriodide. The latter gave a positive nitroprusside test (1) when heated with base (Table II).

When a copious evolution of methyl mercaptan was detected within the first 0.5 hr. of refluxing, heating was continued until methyl mercaptan evolution virtually ceased, generally within 6–12 hr. The solution was cooled or concentrated, causing separation of the 2-amino-2-oxazoline derivative (or 2-amino-5,6-dihydro-4*H*-1,3-oxazine derivative) as the hydriodide salt. The IR spectra of these compounds showed a characteristic peak at 5.9–6.1 μ (C=N) (Table III).

2-Amino-2-oxazoline (IIIa)—A mixture of 1.0 g. (0.0031 mole) III*f* in 10 ml. of 1:1 HCl was agitated and heated under reflux for about 2 hr. until solution was complete. The benzoic acid, which separated on cooling, was removed and the filtrate was concentrated several times with water to remove the excess acid. The syrup was treated with a saturated ethanolic solution of picric acid to give 0.45 g. (46%) of 2-amino-2-oxazoline picrate as fine needles (from water), m.p. 192–195° [lit. (5) m.p. 186–188°].

2-Amino-5,6-dihydro-4*H*-1,3-oxazine—2-Benzamido-5,6-dihydro-4*H*-1,3-oxazine hydriodide (III*k*, 1.0 g., 0.0030 mole) was treated as described previously to give 0.37 g. (37%) of 2-amino-5,6-dihydro-4*H*-1,3-oxazine picrate as fine needles (from water), m.p. 207–208° [lit. (6) m.p. 200°].

REFERENCES

(1) D. L. Klayman and R. J. Shine, *Anal. Chim. Acta*, **41**, 408 (1968).

(2) B. Adcock, A. Lawson, and D. H. Miles, *J. Chem. Soc.*, **1961**, 5120.

(3) W. L. Budde and O. L. Salerni, *J. Heterocycl. Chem.*, **6**, 419 (1969).

(4) I. B. Douglass and F. B. Dains, *J. Amer. Chem. Soc.*, **56**, 719(1934).

(5) S. Gabriel, *Ber.*, **22**, 1139(1889).

(6) S. Gabriel and W. E. Lauer, *ibid.*, **23**, 87(1890).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 22, 1969, from the *Division of Medicinal Chemistry, Walter Reed Army Institute of Research, Washington, DC 20012*

Accepted for publication April 3, 1970.

The authors thank Mr. David Kenny for technical assistance.

* Present address: Department of Chemistry, Trinity College, Washington, DC 20017

Comparison of Effects of 1,8-Dihydroxyanthraquinone and 1,5-Dihydroxyanthraquinone on Different Segments of Rabbit Gastrointestinal Tract

THEODORE H. EICKHOLT, LAWRENCE J. DANNA*, LYNN J. VERZWYVELT*, GERALD P. WAGUESPACK*, JOHNNY L. BUNCH*, and DANIEL D. WINSLOW*

Abstract □ Using an isolated muscle bath technique, five different segments of the rabbit gastrointestinal tract (duodenum, jejunum, ileum, and ascending and descending colon) were used to record the effects of two doses of 1,5-dihydroxyanthraquinone. Transducer-recorded tracings were made of contractions per minute, interval between contractions, and amplitude of contractions. The results were evaluated; statistical tests among comparisons of the three parameters between the two drug doses, control values, and previous values for 1,8-dihydroxyanthraquinone again indicated the parameter of interval between contractions to be a statistically significant measurement of activity. No statistically significant differences in effects were shown between the two drugs.

Keyphrases □ 1,8- *versus* 1,5-Dihydroxyanthraquinone—effect on gastrointestinal tract, rabbit □ Gastrointestinal tract, rabbit—effects, 1,8- *versus* 1,5-dihydroxyanthraquinone

The normal activity of different segments of the rabbit gastrointestinal tract has been previously delineated, using an isolated muscle bath technique (1).

The effects of 15- and 30-mg. doses of 1,8-dihydroxyanthraquinone on these different segments of rabbit intestinal tract have been studied with this technique and statistically compared with the standards (2). The results of the statistical analyses of the transducer-

Table I—Summary of Averages and Standard Deviations of Measurements of Rabbit Intestinal Activity Obtained with 15 mg. 1,5-Dihydroxyanthraquinone

Segment	No. of Measurements	Contractions/min.	Interval between Contractions, sec.	Amplitude of Contractions, mm.
Duodenum	18	16.0 ± 0.89	1.8 ± 0.1[a]	0.93 ± 0.63
Jejunum	18	13.8 ± 1.68	2.2 ± 0.3[a]	2.22 ± 1.87
Ileum	18	11.1 ± 1.42	2.7 ± 0.1[a]	4.26 ± 4.01[a]
Ascending colon	18	7.9 ± 1.34[a]	3.7 ± 1.1[a]	1.03 ± 1.10
Descending colon	18	5.9 ± 0.90	5.2 ± 1.2[a]	1.62 ± 0.43

[a] Significantly different from normal averages at $p = 0.05$.

Table II—Summary of Averages and Standard Deviations of Measurements of Rabbit Intestinal Activity Obtained with 30 mg. 1,5-Dihydroxyanthraquinone

Segment	No. of Measurements	Contractions/min.	Interval between Contractions, sec.	Amplitude of Contractions, mm.
Duodenum	18	15.6 ± 1.26	1.9 ± 0.1[a]	1.88 ± 1.72
Jejunum	18	12.4 ± 0.49	2.4 ± 0.2[a]	1.69 ± 1.21[a]
Ileum	18	10.1 ± 1.18	2.9 ± 0.2[a]	3.65 ± 2.31[a]
Ascending colon	18	6.2 ± 0.69[a]	4.8 ± 0.7[a]	0.65 ± 0.33
Descending colon	18	8.6 ± 1.97[a]	3.7 ± 0.9[a]	1.06 ± 1.78

[a] Significantly different from normal averages at $p = 0.05$.

recorded tracings indicated that the parameter of interval between contractions was a statistically significant measurement of activity.

Therefore, the purposes of this study were to observe the effects of 1,5-dihydroxyanthraquinone on the different segments of the rabbit gastrointestinal tract and to compare these statistically with the previous normal and 1,8-dihydroxyanthraquinone figures to determine the value of the parameter of interval between contractions as a significant measurement of activity for drug-effect studies.

EXPERIMENTAL

Adult albino rabbits were anesthetized with ether, and 2–3-cm. segments of duodenum, jejunum, ileum, and ascending and descending colon were expediently removed and maintained in oxygenated Tyrode's solution in the same manner as in the previously delineated standards and 1,8-dihydroxyanthraquinone studies (1, 2).

As in the previous studies, the Tyrode's solution in glass distilled water again contained sodium chloride, 0.8%; potassium chloride, 0.02%; calcium chloride, 0.02%; magnesium chloride, 0.01%; sodium bicarbonate, 0.1%; sodium diphosphate, 0.005%; and glucose, 0.1%; 15 ml. was used to bathe the intestinal segment in the isolated muscle bath chamber (1, 2).

Similar to the standard and 1,8-dihydroxyanthraquinone studies, the temperature was maintained at 38°, and the preparation was oxygenated using an air flow slowly (2–3 bubbles/sec.) bubbled through the solution (1, 2). The segment was attached to the muscle hooks, and the intestinal activity was again recorded *via* transducer (E & M isotonic myograph) and physiograph recorder (E & M) (1, 2).

The segments were allowed to acclimate to the muscle bath environment, following which normal intestinal activity for the different segments was recorded. Six different rabbits were used to provide six different samples of duodenum, jejunum, ileum, and ascending and descending colon for each of the two doses of the 1,5-dihydroxyanthraquinone in the same manner as in the 1,8-dihydroxyanthraquinone studies (2).

The anthraquinone was 1,5-dihydroxyanthraquinone.[1] The drug was placed in Tyrode's solution so as to provide 0.1 and 0.2% concentrations, providing 15 and 30 mg./15 ml. isolated muscle bath chamber, respectively, for the low and high doses.

The effects of each dosage were recorded for a minimum of 15 min. for each of the six different samples of the five different segments and were completely repeated at the second dosage for six different samples of the five different segments, identical to the design of the 1,8-dihydroxyanthraquinone studies (2).

From each recording of each individual gut segment, amplitude of contraction, interval between contractions, and contractions per minute were measured and averaged as in the two previous studies (1, 2).

Standard deviations were calculated; analyses of variance with F test and Scheffe's S method tests were made between combinations of treated and control intestinal segments (3).

[1] Eastman Organic Chemicals, P 2246.

RESULTS

Table I summarizes the averages and standard deviations of the amplitude of contraction, interval between contractions, and contractions per minute obtained with the low dose (15 mg.) of 1,5-dihydroxyanthraquinone.

Table II summarizes the averages and standard deviations of the amplitude of contraction, interval between contractions, and contractions per minute obtained with the high dose (30 mg.) of 1,5-dihydroxyanthraquinone.

Table III summarizes the similar parameters obtained with the low dose (15 mg.) of 1,8-dihydroxyanthraquinone, and Table IV summarizes those obtained with the high dose (30 mg.) of 1,8-dihydroxyanthraquinone.

Table V summarizes the averages and standard deviations of the amplitude of contraction, interval between contractions, and contractions per minute obtained with the normal untreated intestinal segments.

Of the 150 possible comparisons of the three parameters between drug-treated and normal averages, 31 were significantly different statistically at $p = 0.05$, including the 15 from the 1,8-dihydroxyanthraquinone (2).

All 10 comparisons of the interval between contractions for both the 15 and 30 mg. 1,5-dihydroxyanthraquinone and normal averages were significantly different statistically at $p = 0.05$. There were no significant differences between any of the comparisons between the 15- and 30-mg. doses of 1,5-dihydroxyanthraquinone; there was one significant with the 1,8-dihydroxyanthraquinone, the ileum comparisons of amplitude of contractions (2).

The remaining six significant comparisons were jejunum *versus* jejunum amplitude of contractions between 30-mg. dose and normal averages (this is compared to the 15-mg. dose of the 1,8-dihydroxyanthraquinone which was significant); ileum *versus* ileum amplitude of contractions between both treatments and normal averages; ascending colon *versus* ascending colon contractions per minute between both treatments and normal averages; and the descending colon *versus* descending colon contractions per minute between 30-mg. dose and normal averages.

Table III—Summary of Averages and Standard Deviations of Measurements of Rabbit Intestinal Activity Obtained with 15 mg. 1,8-Dihydroxyanthraquinone

Segment	No. of Measurements	Contractions/min.	Interval between Contractions, sec.	Amplitude of Contraction, mm.
Duodenum	18	14.4 ± 1.05	1.9 ± 0.3[a]	0.73 ± 0.36
Jejunum	18	12.4 ± 1.65	2.5 ± 0.4[a]	1.05 ± 0.46[a]
Ileum	18	9.8 ± 2.04	3.2 ± 0.6[a]	1.52 ± 1.35[a,b]
Ascending colon	18	8.1 ± 2.75[a]	4.5 ± 2.5[a]	4.62 ± 5.79
Descending colon	18	7.2 ± 1.54	4.4 ± 0.9[a]	1.51 ± 1.63

[a] Significantly different from normal averages at $p = 0.05$. [b] Significantly different from 30-mg. dose average at $p = 0.05$.

Table IV—Summary of Averages and Standard Deviations of Measurements of Rabbit Intestinal Activity Obtained with 30 mg. 1,8-Dihydroxyanthraquinone

Segment	No. of Measurements	Contractions/min.	Interval between Contractions, sec.	Amplitude of Contraction, mm.
Duodenum	18	15.2 ± 1.41	1.9 ± 0.2[a]	2.84 ± 1.92
Jejunum	18	13.4 ± 0.90	2.3 ± 0.2[a]	2.87 ± 2.44
Ileum	18	10.3 ± 1.10	2.7 ± 1.0[a]	7.04 ± 2.06[b]
Ascending colon	18	7.7 ± 1.55[a]	3.5 ± 0.9[a]	0.18 ± 0.13
Descending colon	18	6.4 ± 1.69	4.3 ± 1.2[a]	0.35 ± 0.22

[a] Significantly different from normal averages at $p = 0.05$. [b] Significantly different from 15-mg. dose average at $p = 0.05$.

Table V—Summary of Averages and Standard Deviations of Measurements of Normal, Untreated Rabbit Intestinal Activity

Segment	No. of Measurements	Contractions/min.	Interval between Contractions, sec.	Amplitude of Contraction, mm.
Duodenum	21	16.7 ± 1.68	3.9 ± 0.1	2.56 ± 2.50
Jejunum	21	12.4 ± 2.31	5.0 ± 1.5	6.53 ± 3.46
Ileum	21	10.5 ± 1.27	6.2 ± 1.1	9.17 ± 0.68
Ascending colon	15	2.1 ± 0.83	15.0 ± 8.2	1.68 ± 0.57
Descending colon	15	4.3 ± 1.68	13.4 ± 5.0	3.91 ± 2.38

SUMMARY AND CONCLUSIONS

Five different segments of the rabbit intestinal tract—duodenum, jejunum, ileum, and ascending and descending colon—were utilized in recording the activity of these segments in response to 15- and 30-mg. doses of 1,5-dihydroxyanthraquinone, using an *in vitro* isolated muscle bath technique.

Contractions per minute, interval between contractions, and amplitude of contraction were recorded, determined, and averaged; standard deviations were calculated; and analyses of variance with *F* test and Scheffe's *S* method tests were performed on segment combinations between the 1,5-dihydroxyanthraquinone-treated and normal segments and the 1,8-dihydroxyanthraquinone-treated segments.

Of the 150 comparisons of the three parameters between drug-treated and normal averages, 31 were significantly different statistically at $p = 0.05$ and included all comparisons of interval between contractions for both drug treatments and normal averages.

Comparing the actual figures (averages) of the three parameters for the five segments of the high dose of the 1,5-dihydroxyanthraquinone and the normal averages, 5/5 values for interval between contractions for the high dose were less than the normal figures and all showed statistically significant differences.

Comparing the actual values of the three parameters for the five segments of the low dose of the 1,5-dihydroxyanthraquinone and the normal averages, 5/5 values for interval between contractions for the low dose were less than the normal figures and all again showed statistically significant differences.

Comparisons of effects between the high and low doses of 1,5-dihydroxyanthraquinone on the three parameters showed no statistically significant differences. Comparisons of the activity between the high doses of both drugs on the three parameters and comparisons between the low doses of the two drugs showed no statistically significant differences.

The statistical tests between figures of both drugs *versus* normal show considerable agreement in the numbers of statistically significant differences and agree much of the time with specific segments.

The results from this *in vitro* study and their statistical comparisons show again the parameter of interval between contractions to be a statistically significant measurement of activity.

REFERENCES

(1) T. H. Eickholt, R. H. Box, and N. D. Courville, *J. Pharm. Sci.*, **56**, 1328(1967).

(2) T. H. Eickholt, N. D. Courville, L. J. Danna, L. J. Verzwyvelt, and G. P. Waguespack, *ibid.*, **58**, 1289(1969).

(3) R. E. Kirk, "Experimental Design: Procedures for the Behavioral Sciences," Brooks/Cole, Belmont, Calif., 1968, pp. 90, 91.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 2, 1969, from the *Department of Pharmacology and Toxicology, School of Pharmacy, Northeast Louisiana State College, Monroe, LA 71201*

Accepted for publication April 1, 1970.

The authors wish to acknowledge H. L. Dockery, Director, Academic Computer Center, for assistance with the statistical analyses.

* Special problems undergraduate student in pharmacology.

New Compounds: Some 3,4,5-Trimethoxyphenyl Analogs of Analgesics

VASANT G. TELANG* and CHARLES I. SMITH†

Abstract □ Starting from 3,4,5-trimethoxybenzoic acid, the syntheses of the 3,4,5-trimethoxyphenyl analogs of meperidine hydrochloride and ketobemidone hydrochloride are described.

Keyphrases □ Meperidine HCl, 3,4,5-trimethoxyphenyl analogs—synthesis □ Ketobemidone HCl, 3,4,5-trimethoxyphenyl analogs—synthesis □ NMR spectrometry—structure □ IR spectrophotometry—structure

A number of naturally occurring compounds having action on the CNS possess a 3,4,5-trimethoxyphenyl group. For the past 15 years, a considerable amount of work on 3,4,5-trimethoxyphenyl compounds of possible psychotropic activity has been done (1). Many of these compounds have little chemical relationship except the possession of 3,4,5-trimethoxyphenyl groups. One laboratory reported previously on the synthesis of some 3,4,5-trimethoxyphenyl analogs of antihistamines (2) and anticonvulsants (3). Since analgesics are known to have a distinct effect upon the CNS, the drugs selected for the present studies were the important synthetic substitutes for the narcotic analgesic morphine. They are meperidine hydrochloride[1] and ketobemidone hydrochloride.[2]

The synthetic routes are shown in Scheme I. The 3,4,5-trimethoxybenzoic acid (I) was reduced to the corresponding alcohol according to the procedure reported by DiFazio (4). This alcohol was converted to 3,4,5-trimethoxybenzyl chloride (II) following the procedure of Drake and Tuemmler (5). It remained stable without any change in color in an evacuated desiccator over sodium hydroxide pellets for more than 6 months at room temperature, in contrast to a previous report by Tsao (6) that this product slowly darkens even when stored in the refrigerator. Acetone has been reported (4, 7) to be the best of several solvents tried for the conversion of II to 3,4,5-trimethoxyphenylacetonitrile (III). By reaction in dimethyl sulfoxide (DMSO) at 55–60°, the desired nitrile was obtained in approximately the same yield but the reaction time was decreased from 10 days (acetone) to 3 hr. Alkylation of III followed by treatment of the resulting product with hydrogen chloride gave α,α-bis(2-dimethylaminoethyl)-(3,4,5-trimethoxyphenyl)acetonitrile dihydrochloride (IV). Pyrolysis of IV at its melting point to cause evolution of trimethylamine and give the cyclized product V failed. Only a black residue which did not melt when tested up to 350° was obtained. Therefore, III was alkylated with 2,2′-dichloro-*N*-methyldiethylamine to obtain the cyclized product V. Sodium amide or *n*-butyllithium worked equally well as the base for this condensation reaction.

Hydrolysis of V according to the method reported by Eisleb (8) failed. Several attempts, using either acid or alkali at elevated temperatures under pressure, did not yield the desired product. Finally, refluxing V with an excess of 2.2 *M* ethanolic sulfuric acid for 4 days and then hydrolyzing the imino ester gave the desired ethyl ester VIII. It is known that the carboxyl group on a quaternary carbon atom reacts sluggishly, probably because the alkyl groups dominate so much space in the neighborhood of the carboxyl group that they tend to block the intermediate ionic addition complex (9). Similar steric hindrance of the 4-cyano group in V is the probable reason for the failure in facile hydrolysis of the nitrile. Compound VIII was also prepared by condensation of 2,2′-dichloro-*N*-methyldiethylamine with ethyl 3,4,5-trimethoxyphenylacetate (VII) in the presence of sodium ethoxide.

Compound V reacted with ethylmagnesium bromide only at low temperatures. Addition of ethylmagnesium bromide at 0° and then stirring the reaction mixture at 20° for 24 hr. gave the intermediate ketimine, which finally was hydrolyzed to the corresponding ketone by warming with dilute acid. Similar trials at higher temperatures did not yield any product. It appears, therefore, that the intermediate magnesium adduct of the ketimine is unstable above 20°. A similar observation was reported recently by Muren (10).

EXPERIMENTAL

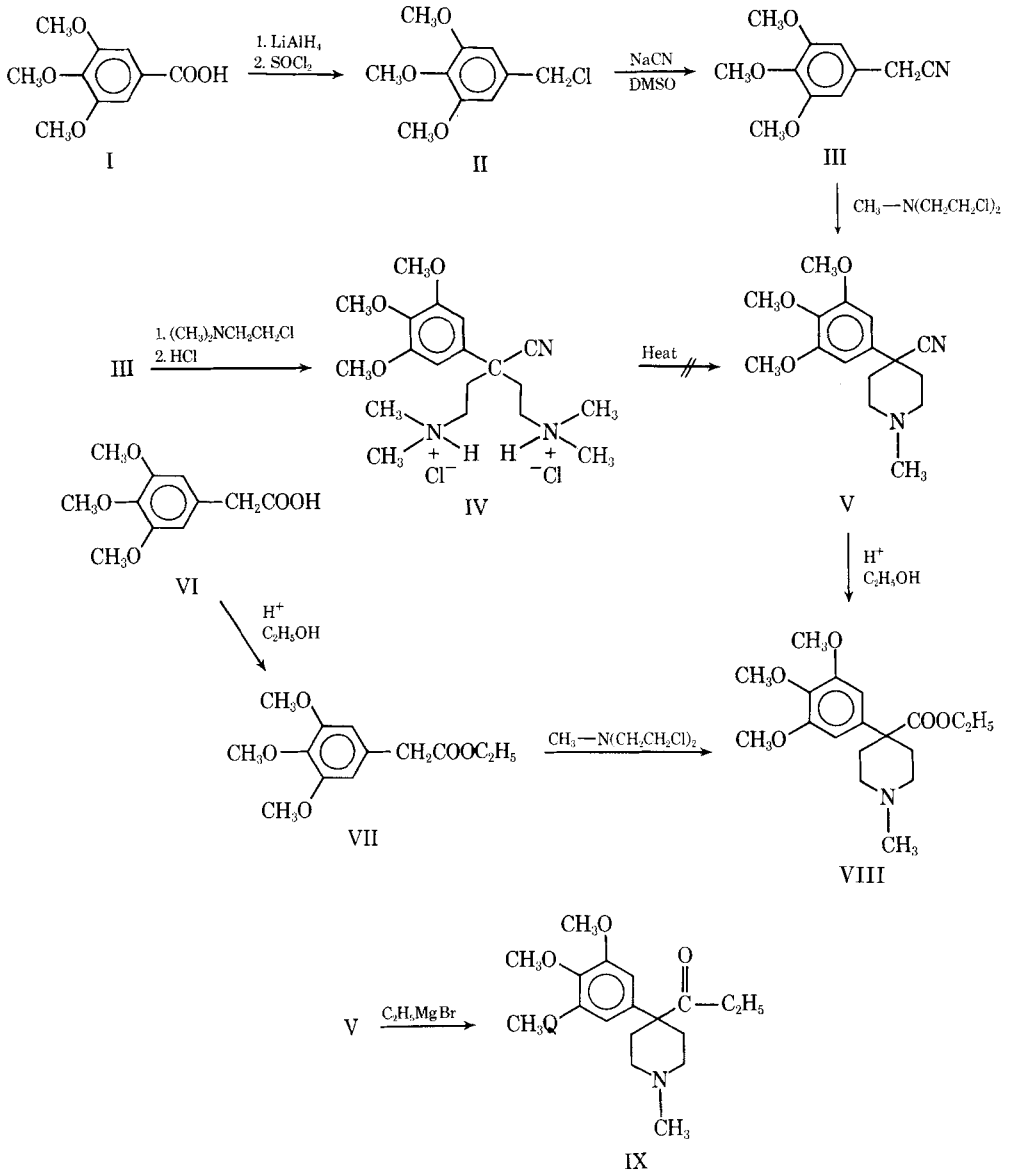
All melting points were determined with a Thomas-Hoover melting-point apparatus and are uncorrected. IR spectra were recorded with Beckman model 8 or Perkin-Elmer 237B grating IR spectrophotometers. NMR spectra were determined with Varian Associates A-60 and A-60D instruments, using tetramethylsilane or 3-(trimethylsilyl)propanesulfonic acid sodium salt as the internal standard. Unless otherwise specified, all NMR spectra were obtained with approximately 15% concentrations; s refers to singlet, d to doublet, t to triplet, q to quadruplet, and m to multiplet. Microanalyses were performed by Micro-Analysis, Inc., Wilmington, Del., and M-H-W Laboratories, Garden City, Mich.

3,4,5-Trimethoxyphenylacetonitrile (III)—3,4,5-Trimethoxybenzyl chloride (II) was prepared according to the method reported by Drake and Tuemmler (5). A mixture of 2.18 g. (0.0101 mole) of II, 0.49 g. (0.010 mole) of sodium cyanide, and 190 ml. of DMSO was heated with magnetic stirring for 3 hr. at 55–60°. Subsequently, the mixture was cooled, diluted with four times its volume of water, and then extracted with three 100-ml. portions of ether. The combined pale-yellow ethereal extracts were washed with 6 *N* hydrochloric acid and with water, and then they were dried over anhydrous magnesium sulfate. Removal of the ether under vacuum yielded a pale-yellow oil, which crystallized upon cooling to provide 1.2 g. (58%) of yellow needles, m.p. 75–76°.

α,α - Bis(2 - dimethylaminoethyl) - (3,4,5 - trimethoxyphenyl)acetonitrile dihydrochloride (IV)—Sodium amide was freshly prepared, starting with 7.5 g. (0.33 g. atom) of sodium and about 400

[1] Marketed as Demerol hydrochloride by Winthrop Laboratories, N. Y.; 4-carbethoxy-1-methyl-4-phenylpiperidine hydrochloride.

[2] Named Cliradon hydrochloride and used experimentally as an analgesic; ethyl 4-(*m*-hydroxyphenyl)-1-methyl-4-piperidyl ketone hydrochloride.



Scheme I

ml. of anhydrous liquid ammonia, according to the procedure of Hancock and Cope (11). The ammonia was allowed to evaporate as the reaction flask achieved room temperature. Then 150 ml. of dry benzene and 15 g. (0.072 mole) of III were added. The mixture was stirred with heating for 1 hr. and then was cooled. Next, 21 g. (0.14 mole) of 2-chloro-*N*,*N*-dimethylethylamine hydrochloride was added with stirring, and the reaction mixture was stirred and heated at reflux for 24 hr. It was cooled, 100 ml. of water was added, and the two layers were separated. The aqueous layer was extracted with benzene. The combined benzene extracts were dried, and the solvent was removed under vacuum to yield a red residue, which was dissolved in anhydrous ether. Dry HCl gas was bubbled into the ethereal solution, whereupon a copious pale-yellow solid precipitated; yield 18.4 g. (60.6%). Decolorization with activated carbon[3] and four recrystallizations from hot absolute ethanol gave a white solid, the dihydrochloride, m.p. 270°.

Anal.—Calcd. for $C_{19}H_{31}N_3O_3 \cdot 2HCl$: C, 54.03; H, 7.87; Cl, 16.79; N, 9.95; O, 11.36. Found: C, 54.27; H, 7.79; Cl, 16.47; N, 9.83; O, 11.51.

4-Cyano-4-(3,4,5-trimethoxyphenyl)-1-methylpiperidine (V)—A mixture of 2.07 g. (0.0100 mole) of III, 1.07 g. (0.0274 mole) of sodium amide,[4] and 1.92 g. (0.0100 mole) of 2,2′-dichloro-*N*-methyldiethylamine hydrochloride (12) in 50 ml. of toluene was refluxed for 12 hr. with stirring. It was cooled and decomposed with water. The organic layer was separated and extracted with three 20-ml. portions of 10% hydrochloric acid. The acidic extracts were combined, made alkaline with 40% aqueous sodium hydroxide solution, and then extracted with ether. After the ethereal layer was dried over magnesium sulfate, the ether was removed under vacuum to obtain 1.5 g. (52%) of V as an oily residue. This was redissolved in ether, and dry hydrogen bromide gas was bubbled in to precipitate a dark solid. This solid was separated and recrystallized four times from hot absolute ethanol with decolorization (Norite) to yield a white granular powder, the hydrobromide of V, m.p. 244.5°. The IR spectrum showed a broad ammonium band (2700–2250 cm.$^{-1}$) and a nitrile peak (2242 cm.$^{-1}$). The NMR spectrum (D_2O) showed peaks of δ values: 2.45 (t, 4βH, piperidine ring); 3.18 (s, 3H, N—CH_3); 3.60 (t, 4αH, piperidine ring); 4.0 (d, 9H, methoxys); and 7.05 (s, 2H, aromatic).

Anal.—Calcd. for $C_{16}H_{22}N_2O_3 \cdot HBr$: C, 51.76; H, 6.24; Br, 21.52; N, 7.55; O, 12.93. Found: C, 52.42; H, 6.43; Br, 21.36; N, 7.48; O, 12.85.

4 - Carbethoxy - 4 - (3,4,5 - trimethoxyphenyl) - 1 - methylpiperidine (VIII)—*Method A*—To an ice-cold solution of 2.9 g. (0.010 mole) of V in 210 ml. of absolute ethanol, previously dried according to the procedure of Vogel (13), was added dropwise 30 ml. of concentrated sulfuric acid. This reaction mixture was stirred mag-

[3] Norite, American Norit Co., Jacksonville, Fla.
[4] Gray powder, Fisher Scientific Co., Fairland, N. J.

netically and heated at reflux for 4 days. After being cooled, it was poured upon 300 g. of ice and the resulting aqueous solution was boiled for 5 hr. and cooled. The solution was made alkaline with 40% aqueous sodium hydroxide solution, and the resulting turbid solution was extracted with ether. The ethereal extract was dried over magnesium sulfate and the ether was removed under vacuum to obtain 1.7 g. (50%) of an oily residue (VIII). The IR spectrum (film) of this oil showed a strong peak for ester carbonyl (1718 $cm.^{-1}$), ester C—O—C stretch (1316 and 1066 $cm.^{-1}$), and complete absence of any nitrile peak. The NMR spectrum (CCl_4) showed peaks of δ values: 1.18 (t, 3H, C—CH_3); 2.34 (m, 11H, 8 piperidine ring and 3 N—CH_3); 3.75 (d, 9H, methoxys); 4.12 (q, 2H, CH_2 of ethyl); and 6.64 (s, 2H, aromatic). This oily free base (VIII) was dissolved in dry ether, and dry HCl gas was bubbled in to produce a white granular precipitate. This hydrochloride was separated, decolorized (Norite), and recrystallized twice from absolute ethanol and ether, m.p. 198–199°.

Anal.—Calcd. for $C_{18}H_{27}NO_5 \cdot HCl$: C, 57.82; H, 7.55; Cl, 9.48; N, 3.75; O, 21.40. Found: C, 57.83; H, 7.47; Cl, 9.58; N, 3.91; O, 21.28

Method B—Sodium ethoxide was prepared by reacting 1.5 g. (0.065 g. atom) of sodium with 100 ml. of absolute ethanol. Most of the alcohol was distilled and 100 ml. of dry toluene was added, of which 50 ml. was distilled to ensure complete removal of ethanol. Then 7.6 g. (0.030 mole) of ethyl 3,4,5-trimethoxyphenylacetate (VII) was added, and the reaction mixture was warmed for 1 hr. and cooled. Next 4.7 g. (0.030 mole) of 2,2′-dichloro-*N*-methyldiethylamine was added, and the mixture was stirred and heated for 24 hr. under an atmosphere of dry nitrogen. The contents were cooled and diluted with water. The organic layer was separated and extracted with dilute hydrochloric acid. The acidic aqueous layer was made alkaline with 40% sodium hydroxide solution and extracted with ether. The ethereal extract was dried over sodium sulfate, and the ether was evaporated to obtain 3.5 g. (35%) of oily residue (VIII). The IR spectrum of this oil was identical with that of the oily free base obtained by Method A.

Ethyl 4-(3,4,5-Trimethoxyphenyl)-1-methyl-4-piperidyl Ketone (IX)—A solution of 0.58 g. (0.0020 mole) of V in 20 ml. of dry benzene was added to a solution of ethylmagnesium bromide prepared by reacting 0.44 g. (0.0040 mole) of ethyl bromide with 0.096 g. (0.0040 g. atom) of magnesium in 10 ml. of dry ether under an atmosphere of dry nitrogen. During the addition, the reaction was maintained at 0° and, after the addition, was stirred for 24 hr. at 20°. The reaction mixture was decomposed with 10 ml. of 3 *N* hydrochloric acid and heated for 1 hr. on a steam bath while the organic layer was allowed to evaporate. The mixture was cooled and made alkaline with 3 *N* sodium hydroxide solution. The resulting turbid solution was extracted with chloroform. The combined extracts were dried and the chloroform was evaporated. The residual oil, on TLC examination (basic alumina; ethyl acetate–benzene, 7:3, as the developing solvent), showed two distinct spots. The spot with the lower R_f value proved to be the desired ketone (IX), which was isolated by chromatography using ethyl acetate–benzene (7:3) on a basic alumina[5] column. The eluting solvents were evaporated under reduced pressure; the residual oil, 0.27 g. (42%), was dissolved in minimal ether and treated with ethereal HCl. The precipitate obtained was decolorized (Norite) once and recrystallized twice from chloroform–hexane so as to yield a white fluffy powder, m.p. 226–228° (dec.). The IR spectrum of this hydrochloride showed a broad ammonium band, complete absence of a nitrile band, and the presence of a carbonyl band (1699 $cm.^{-1}$). The NMR spectrum of the hydrochloride salt ($CDCl_3$) showed peaks of δ values: 0.93 (t, 3H, CH_3); 2.55 (m, 13H, 8 piperidine ring, 3 N—CH_3 and 2 CH_2 of ethyl); 3.86 (d, 9H, methoxys); and 6.44 (s, 2H, aromatic).

Anal.—Calcd. for $C_{18}H_{27}NO_4 \cdot HCl$: C, 60.41; H, 7.89; N, 3.91. Found: C, 60.56; H, 7.88; N, 4.01.

REFERENCES

(1) L. H. Schlager, *Arzeim.-Forsch.*, **13,** 226(1963).
(2) L. T. DiFazio and P. F. Smith, *J. Med. Chem.*, **9,** 631(1966).
(3) V. S. Palkar and P. F. Smith, *ibid.*, **8,** 883(1965).
(4) L. T. DiFazio, Ph.D. thesis, University of Rhode Island, 1964.
(5) N. L. Drake and W. B. Tuemmler, *J. Amer. Chem. Soc.*, **77,** 1206(1955).
(6) M. U. Tsao, *ibid.*, **73,** 5495(1951).
(7) B. Silverman, Ph.D. thesis, University of Florida, 1961.
(8) O. Eisleb, *Ber.*, **74,** 1433(1941).
(9) L. F. Fieser and M. Fieser, "Advanced Organic Chemistry," Reinhold, New York, N. Y., 1962, p. 811.
(10) J. F. Muren, *J. Med. Chem.*, **13,** 140(1970).
(11) E. M. Hancock and A. C. Cope, in "Organic Syntheses," coll. vol. III, E. C. Horning, Ed., Wiley, New York, N. Y., 1965, p. 219.
(12) W. E. Hanby and H. N. Rydon, *J. Chem. Soc.*, **1947,** 513.
(13) A. I. Vogel, "Elementary Practical Organic Chemistry, Part I," Wiley, New York, N. Y., 1957, p. 146.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 24, 1970, from the **College of Pharmacy, University of Minnesota, Minneapolis, MN 55455,* and the *†College of Pharmacy, University of Rhode Island, Kingston, RI 02881*

Accepted for publication May 11, 1970.

Abstracted in part from a thesis submitted by V. G. T. to the University of Rhode Island in partial fulfillment of the Doctor of Philosophy degree requirements.

[5] Basic Alumina AG10, 100-200 mesh, Bio-Rad Laboratories, Richmond, Calif.

Lidocaine: Evidence for the *trans*-Configuration

Keyphrases ☐ Lidocaine—*trans*-configuration ☐ NMR spectroscopy—structure ☐ IR spectrophotometry—structure

Sir:

A recent communication (1) gave IR data which purported to show that lidocaine was an unusual example of an acyclic secondary amide possessing the *cis*-configuration. The bases for this spatial assignment were: (*a*) the amide II band situated at a lower frequency (1494 cm.$^{-1}$) than that usually associated with *trans*-amides (>1500 cm.$^{-1}$), and (*b*) an invariance with dilution of the single N—H stretching band at 3312 cm.$^{-1}$, presumably arising from *cis*-dimeric association.

Entirely different conclusions were reached in recently published studies regarding spatial properties of some closely related acetanilides (2) and α-haloacetanilides (3, 4), including the immediate precursor of lidocaine (5), 2-chloro-2′,6′-acetoxylidide (I).

The strong dependence of both the NMR spectra and chemical reactivity of the α-halogen on the spatial configuration of these anilides was amply demonstrated. It was shown that *ortho*-substituted tertiary α-haloacetanilides could exist in two rotomeric forms, the predominant isomer, A, being spatially constituted with the α-methylene group *cis* and orthogonal to the anilide ring. In this configuration, the α-halogen was quite unreactive and its chemical properties contrasted sharply with the other rotomer, B, possessing the α-methylene group in a much less constricted environment, *i.e.*, *trans* and away from the anilide ring.

Moreover, secondary (*N*-hydrogen) α-haloacetanilides were shown to be entirely constituted with the α-methylene *trans* and away from the anilide ring; their NMR spectral properties and chemical reactivity thus closely resembled the B rotomers of the tertiary anilides. This was consistent with the usual *trans*-assignment (amidic proton *trans* to carbonyl oxygen) for most secondary amides, although contrasting slightly with findings for similar, hindered, secondary α-unsubstituted acetanilides (2), where, for instance, 2′,6′-acetoxylidide (II) is only 74% rather than all *trans* (B configuration).

Relevant to this communication was the finding, substantiated in other systems (2, 6, 7), that the *cis*- or *trans*-spatial configuration strongly and consistently influenced the NMR resonance of selected groupings in the amide. Thus, the α-methyl or α-methylene and nucleophilic groups attached thereto, which were constituted *trans* and away from the aromatic ring, were deshielded and absorbed at a lower field than rotomers that possessed the α-methylene *cis* and orthogonal to the ring. Likewise, in tertiary amides the N—CH_3 *cis* to the carbonyl oxygen was upfield from the *trans*-N—CH_3 group.

Moreover, the ability to separate tertiary α-haloacetanilide rotomers A and B into pure form given sufficient *ortho*-bulk (*i.e.*, *ortho-tert*-butyl) provides a convenient means, by simple nucleophilic displacement, for preparing new compounds of known spatial configurations. Under controlled conditions, reactive rotomer B can alkylate a nucleophile many times faster than its equilibration to A. Thus, a new compound with configuration B may be prepared, and its equilibration to an equilibrium mixture of A and B may be observed and measured.

Therefore, confirmation of the *cis*- or *trans*-configuration of lidocaine can be ascertained from a comparison of its spectra with selected materials, including rotomers of known configurations. Accordingly, Table I compiles pertinent NMR and IR absorptions for lidocaine; I; II; the tertiary anilide homolog of lidocaine, *N*-methyl-2-diethylamino-2′,6′-acetoxylidide (III); 2-chloro-2′-*tert*-butyl-6′-ethylacetanilide (IV); 2-diethylamino-2′-*tert*-butyl-6′-ethylacetanilide (V); and the A and B forms of its *N*-methyl homolog, 2-diethylamino-*N*-methyl-2′-*tert*-butyl-6′-ethylacetanilide (VI).

Table I—Spectral Data for Lidocaine and Related Materials

	Pertinent NMR Shift δ CCl_4[a]				Pertinent IR, cm.$^{-1}$ CCl_4[b]	
Material	N—CH_3 Singlet	$XCH_2C(=O)$ Singlet	N—$(CH_2CH_3)_2$ Quartet	N—$(CH_2CH_3)_2$ Triplet	νN—H	Amide II[c]
Lidocaine	—	3.01	2.62	1.10	3328	1497
I	—	4.15	—	—	3410	1507
II A[d]	—	1.71	—	—	3238[e]	1543[e]
II B[d]	—	2.05	—	—	—	—
III A[d]	3.05	2.74	2.52	0.80	—	—
III B[d]	3.30	3.30	[f]	1.10	—	—
IV	—	4.05	—	—	3424	1508
V	—	3.06	2.69	1.12	3338	1495
VI A[g]	3.05	2.73	2.53	0.81	—	—
VI B	3.29	3.29	2.69	1.10	—	—

[a] For NMR shifts of respective tertiary α-haloamides, see *Reference 3*. [b] Beckman IR 12 in dry CCl_4 at 0.033–0.046 *M*. [c] As verified by sensitivity to deuteration. [d] Spectra of both rotomers from equilibrium mixture, 74% IIB, $CDCl_3$; >90% IIIA. [e] KBr pellet. [f] Obscure. [g] VIB was prepared from IIE (*Reference 3*) with excess diethylamine, then equilibrated in CCl_4 over several days to about 90% VIA.

Examination of the tertiary anilides, III and VI, shows that the B isomer is present at equilibrium, as in the α-halo series, only in minor amounts; hence, substitution of halogen by diethylamino does not produce any unusual change in rotomer distribution. It could be reasonably questioned then why such an unusual departure from normal *trans*, as recently claimed (1), would take place with secondary α-amino-acetanilides, such as lidocaine.

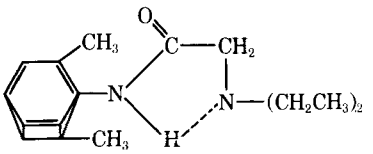
The *trans*-tertiary amides (carbonyl oxygen *cis* to anilide ring), VIB and IIIB, as predicted, have resonance absorptions for N—CH_3 downfield from those for VIA and IIIA. Moreover, the α-methylene and N—$(CH_2CH_3)_2$ groups also are downfield from these more highly shielded groups in VIA and IIIA. The resonance absorptions for these latter two moieties in lidocaine, positioned between IIIA, VIA and IIB, VIB, are of little use for structural determinations. Compelling, however, is the consistent position of the N—$(CH_2\underline{CH_3})_2$ triplet. Materials possessing the α-methylene and, hence, diethylamino *cis* and over the anilide ring have this moiety well within the aromatic shielding zone (6) and therefore at a higher field (δ 0.8 p.p.m.) than the δ 1.1 observed for materials constituted with the α-methylene *trans* to the anilide ring.

In contrast, the position of the amide II band does not appear to be a reliable criterion for spatial assignments, a conclusion previously reached (8). As was found for similar compounds (9, 10), *trans*-α-chloroacetanilides I and IV absorb very close to 1500 $cm.^{-1}$, at considerably lower frequencies than the amide II band normally found in *trans*-α-unsubstituted acetamides. (Note that II, predominately *trans*, has a prominent amide II band at about 1540 $cm.^{-1}$.)

Assignment of the *trans*-configuration to lidocaine can be reconciled with the observed single, solvent invariant N—H stretch and lower than usual amide II band by reference to the internally bonded structure shown for *trans*-lidocaine. Ample precedent for this type of hydrogen bonding has been noted in *trans*-α-halo- and α-alkoxy acetamides (9, 10). Such internal bonding could produce a single solvent invariant νN—H; the νN—H found for lidocaine at even 100 $cm.^{-1}$ lower frequency than the same function in α-halo-substituted amides is a tribute to the strong amido–amino (N—H- - -N) association in this amide (11).

As suggested for similar association in α-haloacetanilides (9), such bonding could force an equilibrium shift to a wholly *trans*-configuration (as contrasted with some minor *cis* in materials such as II). Finally, the amide II band near 1500 $cm.^{-1}$ found for lidocaine (and related *trans*-materials, I, IV, V) could arise from a lesser amount of intermolecular association. As shown earlier (10), α-substitution and steric hindrance to intermolecular association cause a frequency drop in this band. Protonation of the amino group would lessen the internal bonding, and the spectral characteristics presumably found for lidocaine hydrochloride (1) could revert more closely to those of "normal" *trans*-amides.

The assignment of the *trans*-associated structure for lidocaine is further strengthened by its behavior to vapor phase osmometric measurements in benzene. In this nonbonding solvent, as well as carbon tetrachloride, lidocaine over a 0.1–0.005 *M* concentration range shows no evidence of intermolecular association and is strictly monomeric. In contrast, certain authentic *cis* and dimerically bonded amides such as 2-pyridone have been shown to give a corresponding multiple of its molecular weight under these conditions (12).



trans-lidocaine

(1) G. A. Neville and D. Cook, *J. Pharm. Sci.*, **58**, 636(1969).
(2) H. Kessler and A. Rieker, *Ann.*, **70**, 857(1967).
(3) J. P. Chupp and J. F. Olin, *J. Org. Chem.*, **32**, 2297(1967).
(4) J. P. Chupp, J. F. Olin, and H. K. Landwehr, *ibid.*, **34**, 1192 (1969).
(5) "The Merck Index," 6th ed., Merck & Co., Inc., Rahway, N. J., 1952, p. 335.
(6) C. E. Johnson and F. A. Bovey, *J. Chem. Phys.*, **29**, 1012 (1958).
(7) L. A. LaPlanche and M. T. Rogers, *J. Amer. Chem. Soc.*, **85**, 3728(1963); *ibid.*, **86**, 337(1964).
(8) L. J. Bellamy, "Advances in Infrared Group Frequencies," Methuen and Co. Ltd., Great Britain, 1968, chap. 8.
(9) R. A. Nyguist, *Spectrochim. Acta*, **19**, 509(1963).
(10) R. D. McLachlan and R. A. Nyguist, *ibid.*, **20**, 1397(1964).
(11) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond," W. H. Freeman, San Francisco, Calif., 1960, chaps. 5 and 6.
(12) M. H. Krackov, C. M. Lee, and H. G. Mautner, *J. Amer. Chem. Soc.*, **87**, 892(1965).

J. P. CHUPP
Research Department
Agricultural Division
Monsanto Company
St. Louis, MO 63166

Received January 12, 1970.
Accepted for publication May 27, 1970.
The author is indebted to Dr. J. Freeman for the IR spectra and to C. Scott for vapor phase osmometric measurements.

Extrinsic Optical Activity from a Micellar Solution

Keyphrases ☐ Micellar solutions—extrinsic optical activity ☐ Sulfaethidole—betaine-induced optical activity ☐ Polarimetry—analysis

Sir:

Extrinsic optical activities have been observed following the interaction of macromolecules with suitable small molecules (1–3) and following the interaction of optically active solvents with solutes (4, 5). We now report optical activity induced into a symmetrical molecule by an optically active surfactant in the micellar form. L- and D-*N*-decyl-*N*,*N*-dimethylalanine hydrobromides (betaines) were used as the surfactants; their

Examination of the tertiary anilides, III and VI, shows that the B isomer is present at equilibrium, as in the α-halo series, only in minor amounts; hence, substitution of halogen by diethylamino does not produce any unusual change in rotomer distribution. It could be reasonably questioned then why such an unusual departure from normal *trans*, as recently claimed (1), would take place with secondary α-amino-acetanilides, such as lidocaine.

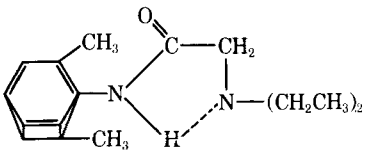
The *trans*-tertiary amides (carbonyl oxygen *cis* to anilide ring), VIB and IIIB, as predicted, have resonance absorptions for N—CH_3 downfield from those for VIA and IIIA. Moreover, the α-methylene and N—$(CH_2CH_3)_2$ groups also are downfield from these more highly shielded groups in VIA and IIIA. The resonance absorptions for these latter two moieties in lidocaine, positioned between IIIA, VIA and IIB, VIB, are of little use for structural determinations. Compelling, however, is the consistent position of the N—$(CH_2\underline{CH_3})_2$ triplet. Materials possessing the α-methylene and, hence, diethylamino *cis* and over the anilide ring have this moiety well within the aromatic shielding zone (6) and therefore at a higher field (δ 0.8 p.p.m.) than the δ 1.1 observed for materials constituted with the α-methylene *trans* to the anilide ring.

In contrast, the position of the amide II band does not appear to be a reliable criterion for spatial assignments, a conclusion previously reached (8). As was found for similar compounds (9, 10), *trans*-α-chloroacetanilides I and IV absorb very close to 1500 $cm.^{-1}$, at considerably lower frequencies than the amide II band normally found in *trans*-α-unsubstituted acetamides. (Note that II, predominately *trans*, has a prominent amide II band at about 1540 $cm.^{-1}$.)

Assignment of the *trans*-configuration to lidocaine can be reconciled with the observed single, solvent invariant N—H stretch and lower than usual amide II band by reference to the internally bonded structure shown for *trans*-lidocaine. Ample precedent for this type of hydrogen bonding has been noted in *trans*-α-halo- and α-alkoxy acetamides (9, 10). Such internal bonding could produce a single solvent invariant νN—H; the νN—H found for lidocaine at even 100 $cm.^{-1}$ lower frequency than the same function in α-halo-substituted amides is a tribute to the strong amido–amino (N—H- - -N) association in this amide (11).

As suggested for similar association in α-haloacetanilides (9), such bonding could force an equilibrium shift to a wholly *trans*-configuration (as contrasted with some minor *cis* in materials such as II). Finally, the amide II band near 1500 $cm.^{-1}$ found for lidocaine (and related *trans*-materials, I, IV, V) could arise from a lesser amount of intermolecular association. As shown earlier (10), α-substitution and steric hindrance to intermolecular association cause a frequency drop in this band. Protonation of the amino group would lessen the internal bonding, and the spectral characteristics presumably found for lidocaine hydrochloride (1) could revert more closely to those of "normal" *trans*-amides.

The assignment of the *trans*-associated structure for lidocaine is further strengthened by its behavior to vapor phase osmometric measurements in benzene. In this nonbonding solvent, as well as carbon tetrachloride, lidocaine over a 0.1–0.005 *M* concentration range shows no evidence of intermolecular association and is strictly monomeric. In contrast, certain authentic *cis* and dimerically bonded amides such as 2-pyridone have been shown to give a corresponding multiple of its molecular weight under these conditions (12).



trans-lidocaine

(1) G. A. Neville and D. Cook, *J. Pharm. Sci.*, **58**, 636(1969).
(2) H. Kessler and A. Rieker, *Ann.*, **70**, 857(1967).
(3) J. P. Chupp and J. F. Olin, *J. Org. Chem.*, **32**, 2297(1967).
(4) J. P. Chupp, J. F. Olin, and H. K. Landwehr, *ibid.*, **34**, 1192 (1969).
(5) "The Merck Index," 6th ed., Merck & Co., Inc., Rahway, N. J., 1952, p. 335.
(6) C. E. Johnson and F. A. Bovey, *J. Chem. Phys.*, **29**, 1012 (1958).
(7) L. A. LaPlanche and M. T. Rogers, *J. Amer. Chem. Soc.*, **85**, 3728(1963); *ibid.*, **86**, 337(1964).
(8) L. J. Bellamy, "Advances in Infrared Group Frequencies," Methuen and Co. Ltd., Great Britain, 1968, chap. 8.
(9) R. A. Nyguist, *Spectrochim. Acta*, **19**, 509(1963).
(10) R. D. McLachlan and R. A. Nyguist, *ibid.*, **20**, 1397(1964).
(11) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond," W. H. Freeman, San Francisco, Calif., 1960, chaps. 5 and 6.
(12) M. H. Krackov, C. M. Lee, and H. G. Mautner, *J. Amer. Chem. Soc.*, **87**, 892(1965).

J. P. CHUPP
Research Department
Agricultural Division
Monsanto Company
St. Louis, MO 63166

Received January 12, 1970.
Accepted for publication May 27, 1970.
The author is indebted to Dr. J. Freeman for the IR spectra and to C. Scott for vapor phase osmometric measurements.

Extrinsic Optical Activity from a Micellar Solution

Keyphrases ☐ Micellar solutions—extrinsic optical activity ☐ Sulfaethidole—betaine-induced optical activity ☐ Polarimetry—analysis

Sir:

Extrinsic optical activities have been observed following the interaction of macromolecules with suitable small molecules (1–3) and following the interaction of optically active solvents with solutes (4, 5). We now report optical activity induced into a symmetrical molecule by an optically active surfactant in the micellar form. L- and D-*N*-decyl-*N*,*N*-dimethylalanine hydrobromides (betaines) were used as the surfactants; their

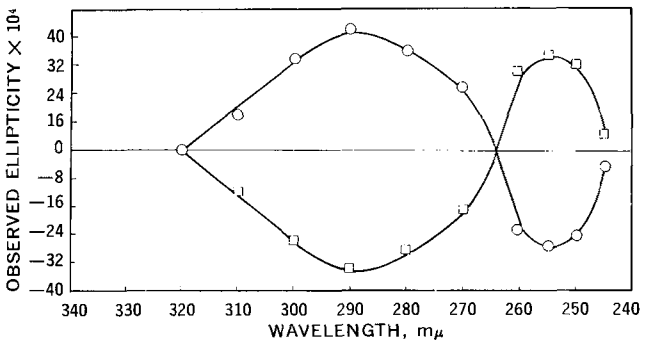


Figure 1—*Extrinsic circular dichroism curves for 7.0 × 10⁻⁴ M SETD in an 8.9 × 10⁻² M aqueous betaine solution. Key:* O, D-*betaine;* □, L-*betaine; and S/N ratio = 20:1. All measurements were made in a 0.1-cm. cell.*

CMC's, measured by optical rotatory dispersion and circular dichroism, have been found to be approximately 1×10^{-2} *M* at 25° (6, 7). Sulfaethidole (SETD) was used as the optically inactive molecule; this drug has been found to become optically active when bound to bovine serum albumin (8), giving peaks in ellipticity at 280 (negative) and 257 mμ (positive) which are consistent with the UV spectra.

All measurements were made in a 6002 attachment to a Cary 60 spectropolarimeter[1] at 25°. Under the experimental conditions, the betaine showed no optical activity above 240 mμ (7), and the SETD alone showed no activity at any wavelengths. Figure 1 shows the optical activity induced in the SETD molecules by the L- and D-betaines at concentrations considerably above their CMC. Peaks of opposite sign are seen at wavelengths of 288 and 255 mμ. We have not observed any induced optical activities at concentrations of betaine below the CMC.

Figure 2 shows the effect of betaine concentration on the ellipticity at 285 mμ with the SETD concentration constant. These ellipticities are small, as is the signal–noise (S/N) ratio, but it appears that the plot cuts the betaine concentration axis at approximately the CMC. At a concentration of 6.0×10^{-2} *M*, the ellipticity seems to have reached a plateau. This is probably the result of all the SETD being solubilized by the micellar betaine, so that subsequent additions of betaine can cause no further interaction. These extrinsic effects probably are due to the interaction of the hydrophobic core of the micelle with the hydrophobic portion of the drug molecule. It is possible that the extrinsic effects observed in aqueous macromolecular solutions are also predominantly hydrophobic in origin.

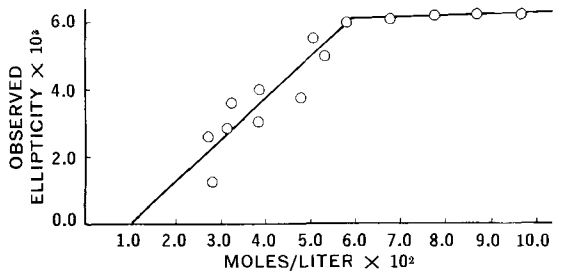


Figure 2—*Plot of observed ellipticity at 285 mμ against* D-*betaine concentration for a constant SETD concentration of 7.0 × 10⁻⁴ M. S/N ratio = 20:1 at largest ellipticities and 4:1 at the lowest. All measurements were made in a 0.2-cm. cell.*

[1] Cary Instruments, Monrovia, Calif.

(1) E. R. Blout, *Biopolym. Symp.*, **1**, 397(1964).
(2) C. F. Chignell, *Life Sci.*, **7**, 1181(1968).
(3) J. H. Perrin and P. A. Hart, *J. Pharm. Sci.*, **59**, 431(1970).
(4) E. Axelrod, G. Barth, and E. Bunnenberg, *Tetrahedron Lett.*, **57**, 5031(1969).
(5) B. Bosnich, *J. Amer. Chem. Soc.*, **88**, 2606(1966).
(6) S. Bonkoski and J. H. Perrin, *J. Pharm. Pharmacol.*, **20**, 934 (1968).
(7) S. Bonkoski and J. H. Perrin, *J. Pharm. Sci.*, **58**, 1428(1969).
(8) J. H. Perrin, V. Averhart, and H. Kostenbauder, unpublished observations.

J. H. Perrin
P. Idsvoog
School of Pharmacy
University of Wisconsin
Madison, WI 53706

Received May 13, 1970.
Accepted for publication June 29, 1970.

Macromolecular Dissolution: Temperature Effects on Polymer–Drug Preparation

Keyphrases □ Macromolecular dissolution—temperature of product formation effect □ Polyethylene maleic anhydride–phenylpropanolamine interaction temperature—effect on dissolution rate □ Temperature of preparation, polymer–drug system—physicochemical properties

Sir:

Polymer–drug interaction systems have been a source of study for application toward the design of prolonged-release dosage forms (1–3). The general method of preparing these systems has differed from investigator to investigator and between the types of polymers and drugs. However, at no time has the effect of preparation temperature on dissolution and/or drug release from the polymer been investigated.

This report concerns the effects of preparation tem-

Table I—Dissolution Rates as Effected by Preparation Temperature

Temperature	Dissolution Rate, × 10^{-1}, Δ Refractometer Scale Units/min.
27°	4.14
40°	3.36
45°	3.82
50°	3.83
55°	2.89
60°	0.24
80°	0.26
100°	0.09

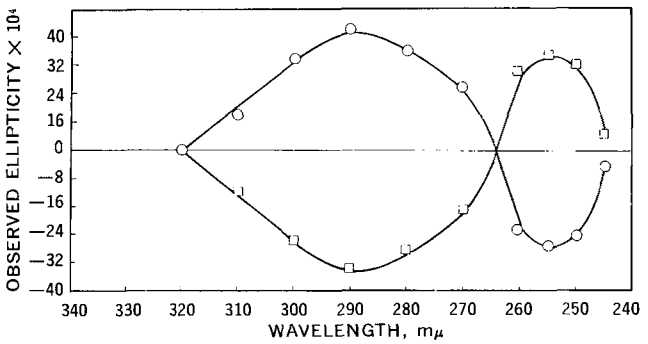


Figure 1—*Extrinsic circular dichroism curves for 7.0 × 10⁻⁴ M SETD in an 8.9 × 10⁻² M aqueous betaine solution. Key:* O, D-*betaine;* □, L-*betaine; and S/N ratio = 20:1. All measurements were made in a 0.1-cm. cell.*

CMC's, measured by optical rotatory dispersion and circular dichroism, have been found to be approximately 1×10^{-2} *M* at 25° (6, 7). Sulfaethidole (SETD) was used as the optically inactive molecule; this drug has been found to become optically active when bound to bovine serum albumin (8), giving peaks in ellipticity at 280 (negative) and 257 mμ (positive) which are consistent with the UV spectra.

All measurements were made in a 6002 attachment to a Cary 60 spectropolarimeter[1] at 25°. Under the experimental conditions, the betaine showed no optical activity above 240 mμ (7), and the SETD alone showed no activity at any wavelengths. Figure 1 shows the optical activity induced in the SETD molecules by the L- and D-betaines at concentrations considerably above their CMC. Peaks of opposite sign are seen at wavelengths of 288 and 255 mμ. We have not observed any induced optical activities at concentrations of betaine below the CMC.

Figure 2 shows the effect of betaine concentration on the ellipticity at 285 mμ with the SETD concentration constant. These ellipticities are small, as is the signal–noise (S/N) ratio, but it appears that the plot cuts the betaine concentration axis at approximately the CMC. At a concentration of 6.0×10^{-2} *M*, the ellipticity seems to have reached a plateau. This is probably the result of all the SETD being solubilized by the micellar betaine, so that subsequent additions of betaine can cause no further interaction. These extrinsic effects probably are due to the interaction of the hydrophobic core of the micelle with the hydrophobic portion of the drug molecule. It is possible that the extrinsic effects observed in aqueous macromolecular solutions are also predominantly hydrophobic in origin.

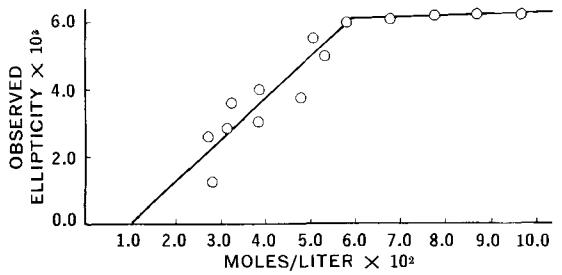


Figure 2—*Plot of observed ellipticity at 285 mμ against* D-*betaine concentration for a constant SETD concentration of 7.0 × 10⁻⁴ M. S/N ratio = 20:1 at largest ellipticities and 4:1 at the lowest. All measurements were made in a 0.2-cm. cell.*

[1] Cary Instruments, Monrovia, Calif.

(1) E. R. Blout, *Biopolym. Symp.*, **1**, 397(1964).
(2) C. F. Chignell, *Life Sci.*, **7**, 1181(1968).
(3) J. H. Perrin and P. A. Hart, *J. Pharm. Sci.*, **59**, 431(1970).
(4) E. Axelrod, G. Barth, and E. Bunnenberg, *Tetrahedron Lett.*, **57**, 5031(1969).
(5) B. Bosnich, *J. Amer. Chem. Soc.*, **88**, 2606(1966).
(6) S. Bonkoski and J. H. Perrin, *J. Pharm. Pharmacol.*, **20**, 934 (1968).
(7) S. Bonkoski and J. H. Perrin, *J. Pharm. Sci.*, **58**, 1428(1969).
(8) J. H. Perrin, V. Averhart, and H. Kostenbauder, unpublished observations.

J. H. Perrin
P. Idsvoog
School of Pharmacy
University of Wisconsin
Madison, WI 53706

Received May 13, 1970.
Accepted for publication June 29, 1970.

Macromolecular Dissolution: Temperature Effects on Polymer–Drug Preparation

Keyphrases □ Macromolecular dissolution—temperature of product formation effect □ Polyethylene maleic anhydride–phenylpropanolamine interaction temperature—effect on dissolution rate □ Temperature of preparation, polymer–drug system—physicochemical properties

Sir:

Polymer–drug interaction systems have been a source of study for application toward the design of prolonged-release dosage forms (1–3). The general method of preparing these systems has differed from investigator to investigator and between the types of polymers and drugs. However, at no time has the effect of preparation temperature on dissolution and/or drug release from the polymer been investigated.

This report concerns the effects of preparation tem-

Table I—Dissolution Rates as Effected by Preparation Temperature

Temperature	Dissolution Rate, × 10^{-1}, Δ Refractometer Scale Units/min.
27°	4.14
40°	3.36
45°	3.82
50°	3.83
55°	2.89
60°	0.24
80°	0.26
100°	0.09

Table II—Bound Moisture as a Function of Reaction and Drying Temperature

Temperature	Dried Polymer–Drug, mg.	Bound Moisture, mg.	Bound Moisture, %
40°	182.6	20.3	12.52
45°	179.7	17.4	10.72
50°	178.6	16.3	10.04
55°	174.6	12.3	7.58
60°	173.2	10.9	6.72
80°	173.0	10.7	6.59
100°	162.0[a]	—	—

[a] Polymer–drug sample used as reference containing essentially no moisture.

perature on the dissolution of a polyethylene maleic anhydride–phenylpropanolamine interaction system that was recently devised (4). Samples of the interaction system were prepared at 27, 40, 45, 50, 55, 60, 80, and 100°; aqueous solutions of the polymer and drug were mixed in a 1:1 stoichiometric ratio at each indicated temperature. The solutions were evaporated to dryness, and the powder was compressed into 1-g. nondisintegrating disks on a laboratory press using a 1.6-cm. (0.63-in.) punch and die set. Dissolution studies were then performed using a refractometric method previously described (5). In addition, 10-ml. samples from a stock solution containing 16.40 mg./ml. of polymer–drug in a 1:1 ratio were oven dried to constant weight at the stated temperatures to determine bound moisture.

The results of these experiments are summarized in Tables I and II. It is apparent from an examination of Table I that the dissolution rates of the polymer–drug are essentially the same between the temperature range of 27–55°, with the higher rate at 27°. However, an abrupt, approximately 10-fold, decrease in rate is shown at 60 and 80°, with another significant drop in rate at 100°. These results clearly indicate that temperature has a pronounced effect on some physicochemical property of the polymer–drug system during preparation.

The data in Table II summarize the temperature effect on bound moisture in the polymer–drug system. There is a continuous decrease in bound moisture with an increase in drying temperature, the percent being approximately equal from 40 to 50° and from 55 to 80°. This table shows a rank order correlation with the data in Table I, because both dissolution rate and bound moisture decrease with an increase in temperature. In both studies, 55° appears to be the inflection point at which temperature has the most pronounced effect on dissolution and bound moisture.

Moisture, it appears, is a factor contributing to this dissolution phenomenon. Therefore, water associated with the molecules of the polymer–drug system influences the dissolution mechanism of this polymeric material. Prior to polymer dissolution, solvent imbibition and hydration must occur to form a swollen gel; subsequently, gel disintegration must occur for dissolution to proceed. This situation suggests a dynamic system which encompasses several rate phenomena such as solvent penetration, hydration, swelling, and gel disintegration, all of which determine the dissolution rate of the polymer. The state of hydration, therefore, has an apparent influence on the dynamics of polymer–drug dissolution as suggested by this report.

Other factors, which may contribute to a change in dissolution characteristics when a polymer–drug system is prepared at different temperatures, are stability of the individual components comprising the system and the type of interaction system formed.

The effect of preparation temperature on dissolution could not be attributed to instability of the components of the system studied. The integrity of the pure polymer was confirmed, since no change in dissolution rate was found when a solution of pure polymer was dried at 27 and 100° and then tested. In addition, polyethylene maleic anhydride was reported by the supplier to be stable to any rupture of molecules and does not break down into smaller units at temperatures below 200° (6). The spectra for phenylpropanolamine did not change when a solution of the drug at the concentration used for interaction system preparation was heated at 100° for 2 days, the time required for preparation of the polymer–drug systems.

Ethylene maleic anhydride hydrolyzes in water to form the free acid, each monomer containing two carboxyl groups. Dicarboxylic acids form salts or amides with amines depending upon the reaction temperature (7). It is, therefore, proposed that the polymer and drug may interact at the lower and intermediate temperatures to form a salt. As the temperature increases, a greater proportion of the interaction system formed may be the amide form. The salt form, being more hydrophilic, would have a greater affinity than the amide for bound water. The amide, being more hydrophobic, would show a lesser degree of attraction for moisture. One would, therefore, have a lower rate of hydration and subsequent dissolution, which are consistent with the experimental data.

I am presently engaged in studies to elucidate further the mechanism of this dissolution phenomenon and to determine the effect of preparation temperature on drug release from the polymer.

(1) C. R. Willis, Jr., and G. S. Banker, *J. Pharm. Sci.*, **57,** 1598 (1968).

(2) S. Srinivas and G. S. Banker, Ph.D. thesis, Purdue University, Lafayette, Ind., 1966.

(3) T. H. Simon and G. S. Banker, Ph.D. thesis, Purdue University, Lafayette, Ind., 1963.

(4) A. Heyd, D. O. Kildsig, and G. S. Banker, to be published.

(5) A. Heyd, D. O. Kildsig, and G. S. Banker, *J. Pharm. Sci.*, **59,** 947(1970).

(6) R. T. Haynes, private communication.

(7) R. T. Morrison and R. N. Boyd, "Organic Chemistry," Allyn and Bacon, Boston, Mass., 1959, p. 679.

ALLEN HEYD

Division of Pharmaceutics
School of Pharmacy
University of Connecticut
Storrs, CT 06268

Received May 13, 1970.

Accepted for publication June 25, 1970.

Present address: Vick Divisions, Research and Development, Mount Vernon, NY 10553

The author thanks Paul Michelson for his assistance in the laboratory as an undergraduate research technician.

Identification of *p*-Nitrophenyl Glucoside as a Urinary Metabolite

Keyphrases ☐ *p*-Nitrophenol-2,6-^{14}C urinary metabolites—determination ☐ Paper chromatography—separation, identification ☐ Rechromatography procedure—separation, identification

Sir:

In 1969, Gessner and Vollmer (1) reported that mouse liver microsomes fortified with uridine diphosphoglucose (UDPG) can effect glucosylation of *p*-nitrophenol (PNP) to *p*-nitrophenyl glucoside (PNPG). The pathway appeared to be about 5 times less active than glucuronidation of PNP when carried out by the same microsomal preparations fortified with uridine diphosphoglucuronic acid (UDPGA). In view of the fact that glucosides of phenols have not been previously detected among mammalian metabolites, it was of interest to test whether the pathway manifests itself at all *in vivo*. We found PNPG in the urine of PNP-treated mice. The metabolite was characterized chromatographically by employing a novel technique, which permits a chromatographic sample to be subjected to consecutive chromatography in several solvent systems without elution of the material. The method is described below.

Male, Albany Swiss mice, weighing 23–30 g., were injected intraperitoneally with a 50 mg./kg., 0.145 mc./kg., dose of radioactive PNP[1] in a quantity of isotonic saline which was equivalent to 25 ml./kg. per dose. Urine from six mice was pooled and subjected to lyophilization. The residue of lyophilization was dissolved in distilled water; the concentrate thus obtained was used for chromatography. Descending chromatography was carried out on Whatman No. 1 paper strips in the solvents listed in Table I. Authentic reference compounds, PNP, PNPG, *p*-nitrophenylglucuronic acid (PNPGA), and *p*-nitrophenylsulfate (PNPS) were chromatographed contemporaneously for comparison of the R_f values of metabolites with those of authentic compounds. The compounds were detected on chromatograms with the aid of UV light; they appeared as quenching spots. Radioactive metabolites were located by scanning chromatographic strips with the aid of the Nuclear Chicago 4 pi chromatogram scanner, model 1002. In this manner, R_f values of the metabolites were located and compared to the R_f values of the authentic compounds. The presence of a radioactive peak at the R_f of PNPG on the initial chromatogram was interpreted as an indication of the possible presence of PNPG. The hypothesis was further tested by subjecting the chromatographically separated radioactive metabolite to chromatography in other solvents.

A novel method of rechromatography was developed, one that does not require elution of the substance under investigation. Verbal report of the method was made in 1969 in conjunction with the identification of PNPG as an *in vitro* metabolite (1). The method is suitable for consecutive chromatography in several solvents and permits purification and characterization of an unknown substance by its chromatographic mobility. The paper chromatogram is developed in the usual manner, and the region with the R_f range under investigation is cut out as a strip. The cutout strip is then pinned at two opposite ends (using rustless pins) to two strips of chromatographic paper; a short leader-strip is for dipping into the solvent, and a long strip is for the development of the chromatogram. Thus assembled, the chromatogram is developed in the usual manner. Again the region with the R_f value of interest can be cut out from the chromatogram and subjected to chromatography in another solvent. The process can be repeated several times with little loss of the original material, and data from several solvents can be obtained from experiments on a single chromatographic sample of a substance.

Table I—Comparison of the Chromatographic Mobility of a Urinary Metabolite with that of Authentic *p*-Nitrophenylglucoside

Solvent System	R_f[a] of PNPG	R_f[a] of Metabolite
I *n*-Butanol–pyridine–water (14:3:3)	0.63	0.63
II Benzene–acetic acid–water (125:72:3)	0.48	0.44
III *n*-Propanol–ethyl acetate–water (7:1:2)	0.73	0.74
IV Methanol–formic acid–water (16:3:1)	0.68	0.66
V 1 *N* Sodium formate–formic acid (200:1)	0.77	0.77

[a] Chromatography was carried out on Whatman No. 1 paper strips in a consecutive manner as described in the text.

The method of consecutive chromatography was used for the detection of PNPG in the urine of PNP-treated mice as follows. The concentrate described previously was chromatographed in Solvent I, *n*-butanol–pyridine–water (14:3:3), and the radioactive peak with the R_f of PNPG was cut out and subjected to chromatography in Solvent II, benzene–acetic acid–water (125:72:3). Again the chromatogram that was developed in Solvent II was cut up, and the radioactive peak with R_f of PNPG was subjected to chromatography in Solvent III, *n*-propanol–ethyl acetate–water (7:1:2). The process was repeated with the chromatogram from each solvent in turn. Chromatography in Solvent III was followed by chromatography in Solvent IV, methanol–formic acid–water (16:3:1), and then by chromatography in Solvent V, 1 *N* sodium formate–formic acid (200:1). The R_f values of the reference PNPG and radioactive metabolite were noted in each solvent, and the results are summarized in Table I.

Thus, the presence of PNPG in the urine of PNP-treated mice was established by consecutive chromatography in five solvent systems. Data in the table show that in each solvent system the radioactive peak of metabolite behaved like PNPG. Our experiments showed that in the first solvent system, PNPG overlapped with PNPS. However, the two components separated out in the second solvent system and, thereafter, a single radioactive peak with the mobility of PNPG was detectable on chromatograms. The chroma-

[1] *p*-Nitrophenol-2,6-^{14}C from Tracerlabs, Waltham, Mass.

tographically pure PNPG was present in a small quantity and accounted for about 1–2% of the urinary metabolites. We also noted from our experiments that 24–50% of the administered PNP was excreted in urine in the form of metabolites of PNP in the first 5 hr., and that PNPGA and PNPS were the major products detectable on chromatograms at that time. The two metabolites accounted for about 95% of the urinary metabolites. This observation is in accord with an earlier work of Robinson *et al.* (2). In the 5-hr. urine, PNPGA and PNPS appeared to be present in equal amounts.

The fact that PNPG was present only in small amounts in the urine explains why the metabolite eluded detection in the past. However, low urinary excretion need not necessarily imply low production of this metabolite at all times in the body, but may be related to the fact that PNPG is not sufficiently acidic for extensive renal excretion. *In vitro*, the glucosylation pathway appears quite active (1). In this context perhaps it is significant that *N*-acetylglucosamine conjugation of steroids is also a pathway that appears to be very active *in vitro* (3), but such metabolites are detected with difficulty in mammalian urine (4).

(1) T. Gessner and C. A. Vollmer, *Fed. Proc.*, **28**, 545(1969).
(2) D. Robinson, J. N. Smith, and R. T. Williams, *Biochem. J.*, **50**, 221(1951).
(3) D. C. Collins, H. Jirku, and D. S. Layne, *J. Biol. Chem.*, **243**, 2928(1968).
(4) M. Arcos and S. Lieberman, *Biochem. J.*, **66**, 2032(1967).

T. Gessner
N. Hamada
Department of Biochemical Pharmacology
School of Pharmacy
State University of New York at Buffalo
Buffalo, NY 14214

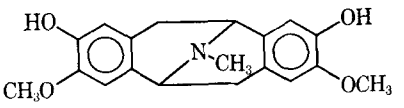
Received March 12, 1970.
Accepted for publication June 10, 1970.
This investigation was partially supported by Grant ES-00013 from the U. S. Public Health Service. Some technical assistance from Mr. Dennis Bogyo is gratefully acknowledged.

Total Synthesis of (±)-Bisnorargemonine

Keyphrases ☐ (±)-Bisnorargemonine—synthesis ☐ IR spectrophotometry—identification ☐ UV spectrophotometry—identification ☐ NMR spectroscopy—identification ☐ Mass spectroscopy—identification

Sir:

We had previously (1) postulated Structure I for bisnorargemonine based on its unambiguous NMR spectrum, in which the chemical shifts of the aromatic and methoxyl protons appeared to be unequivocal for the structural assignment. Nevertheless, since a structure based on spectral evidence alone should be substantiated by synthesis, we have completed the necessary synthesis



I

by a 14-step procedure and an alternate 16-step procedure.

In the shorter synthesis, we started with the preparation of *O*-benzylvanillin, which was condensed with hippuric acid to yield 5-keto-2-phenyl-4-(4′-benzyloxy-3′-methoxybenzylidene)-4,5-dihydroöxazole. The latter then was hydrolyzed with barium hydroxide to give 3-methoxy-4-benzyloxyphenyl-pyruvic acid, which was readily oxidized with hydrogen peroxide to provide the desired 3-methoxy-4-benzyloxyphenylacetic acid, m.p. 114–116° [lit. (2) m.p. 116°]. This acid was converted to its acid chloride with thionyl chloride prior to reaction with β-methoxy-β-(3-benzyloxy-4-methoxyphenyl)ethylamine, which was obtained by treating the nitromethane adduct of *O*-benzylisovanillin with sodium methoxide, according to Rosenmund *et al.* (3), to give 1-methoxy-1-(3-benzyloxy-4-methoxyphenyl)-2-nitroethane,[1] m.p. 100–102°, which was then reduced with lithium aluminum hydride. The resulting amide, m.p. 96.5–98.5°, was then submitted to Bischler–Napieralski cyclization (4) with phosphorus oxychloride to give 1-(3′-methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxyisoquinoline, m.p. 146–147°.

Another route to this isoquinoline was achieved by dehydrogenation of the known 1-(3′-methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline (5) with 10% palladium-on-carbon and rebenzylation of the resulting phenolic isoquinoline with benzyl chloride according to Lee and Soine (6). This route also served to confirm the identity of the isoquinoline from the first method. The tetrahydroisoquinoline was prepared, according to Tomita and Kunimoto (5), by condensation of 3-methoxy-4-benzyloxyphenylacetyl chloride with β-(3-benzyloxy-4-methoxyphenyl)-ethylamine. Subsequent Bischler–Napieralski cyclization with phosphorus pentachloride gave 1-(3′-methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxy-3,4-dihydroisoquinoline hydrochloride. The latter, on reduction with sodium borohydride in aqueous methanol, provided the desired tetrahydroisoquinoline.

The isoquinoline obtained by either of the two methods then was quaternized with methyl iodide and reduced with sodium borohydride in pyridine, according to Barton *et al.* (7), to yield 1-(3′-methoxy-4′-benzyloxybenzyl)-2-methyl-6-benzyloxy-7-methoxy-1,2-dihydroisoquinoline, m.p. 61–64°. Acid cyclization of this product, by the method of Battersby and Binks (8), gave (±)-bisnorargemonine, m.p. 231.5–232.5°. Except for melting point and rotational differences, the natural and synthetic compounds were shown to be identical. The identity of natural and synthetic materials was established by comparative UV, IR, NMR, and mass

[1] This compound has been prepared but not described by E. Brochmann-Hanssen and K. Hirai, *J. Pharm. Sci.*, **57**, 940(1968). Private communication with the authors indicates that the compound was obtained as a pale-yellow oil with suitable chromatographic and NMR characteristics. Our solid product meets similar criteria and is assumed to be identical to their product.

tographically pure PNPG was present in a small quantity and accounted for about 1–2% of the urinary metabolites. We also noted from our experiments that 24–50% of the administered PNP was excreted in urine in the form of metabolites of PNP in the first 5 hr., and that PNPGA and PNPS were the major products detectable on chromatograms at that time. The two metabolites accounted for about 95% of the urinary metabolites. This observation is in accord with an earlier work of Robinson *et al.* (2). In the 5-hr. urine, PNPGA and PNPS appeared to be present in equal amounts.

The fact that PNPG was present only in small amounts in the urine explains why the metabolite eluded detection in the past. However, low urinary excretion need not necessarily imply low production of this metabolite at all times in the body, but may be related to the fact that PNPG is not sufficiently acidic for extensive renal excretion. *In vitro*, the glucosylation pathway appears quite active (1). In this context perhaps it is significant that *N*-acetylglucosamine conjugation of steroids is also a pathway that appears to be very active *in vitro* (3), but such metabolites are detected with difficulty in mammalian urine (4).

(1) T. Gessner and C. A. Vollmer, *Fed. Proc.*, **28**, 545(1969).
(2) D. Robinson, J. N. Smith, and R. T. Williams, *Biochem. J.*, **50**, 221(1951).
(3) D. C. Collins, H. Jirku, and D. S. Layne, *J. Biol. Chem.*, **243**, 2928(1968).
(4) M. Arcos and S. Lieberman, *Biochem. J.*, **66**, 2032(1967).

T. Gessner
N. Hamada
Department of Biochemical Pharmacology
School of Pharmacy
State University of New York at Buffalo
Buffalo, NY 14214

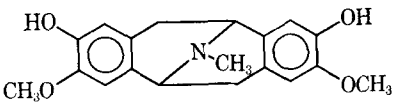
Received March 12, 1970.
Accepted for publication June 10, 1970.
This investigation was partially supported by Grant ES-00013 from the U. S. Public Health Service. Some technical assistance from Mr. Dennis Bogyo is gratefully acknowledged.

Total Synthesis of (±)-Bisnorargemonine

Keyphrases ☐ (±)-Bisnorargemonine—synthesis ☐ IR spectrophotometry—identification ☐ UV spectrophotometry—identification ☐ NMR spectroscopy—identification ☐ Mass spectroscopy—identification

Sir:

We had previously (1) postulated Structure I for bisnorargemonine based on its unambiguous NMR spectrum, in which the chemical shifts of the aromatic and methoxyl protons appeared to be unequivocal for the structural assignment. Nevertheless, since a structure based on spectral evidence alone should be substantiated by synthesis, we have completed the necessary synthesis



I

by a 14-step procedure and an alternate 16-step procedure.

In the shorter synthesis, we started with the preparation of *O*-benzylvanillin, which was condensed with hippuric acid to yield 5-keto-2-phenyl-4-(4′-benzyloxy-3′-methoxybenzylidene)-4,5-dihydroöxazole. The latter then was hydrolyzed with barium hydroxide to give 3-methoxy-4-benzyloxyphenyl-pyruvic acid, which was readily oxidized with hydrogen peroxide to provide the desired 3-methoxy-4-benzyloxyphenylacetic acid, m.p. 114–116° [lit. (2) m.p. 116°]. This acid was converted to its acid chloride with thionyl chloride prior to reaction with β-methoxy-β-(3-benzyloxy-4-methoxyphenyl)ethylamine, which was obtained by treating the nitromethane adduct of *O*-benzylisovanillin with sodium methoxide, according to Rosenmund *et al.* (3), to give 1-methoxy-1-(3-benzyloxy-4-methoxyphenyl)-2-nitroethane,[1] m.p. 100–102°, which was then reduced with lithium aluminum hydride. The resulting amide, m.p. 96.5–98.5°, was then submitted to Bischler–Napieralski cyclization (4) with phosphorus oxychloride to give 1-(3′-methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxyisoquinoline, m.p. 146–147°.

Another route to this isoquinoline was achieved by dehydrogenation of the known 1-(3′-methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline (5) with 10% palladium-on-carbon and rebenzylation of the resulting phenolic isoquinoline with benzyl chloride according to Lee and Soine (6). This route also served to confirm the identity of the isoquinoline from the first method. The tetrahydroisoquinoline was prepared, according to Tomita and Kunimoto (5), by condensation of 3-methoxy-4-benzyloxyphenylacetyl chloride with β-(3-benzyloxy-4-methoxyphenyl)-ethylamine. Subsequent Bischler–Napieralski cyclization with phosphorus pentachloride gave 1-(3′-methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxy-3,4-dihydroisoquinoline hydrochloride. The latter, on reduction with sodium borohydride in aqueous methanol, provided the desired tetrahydroisoquinoline.

The isoquinoline obtained by either of the two methods then was quaternized with methyl iodide and reduced with sodium borohydride in pyridine, according to Barton *et al.* (7), to yield 1-(3′-methoxy-4′-benzyloxybenzyl)-2-methyl-6-benzyloxy-7-methoxy-1,2-dihydroisoquinoline, m.p. 61–64°. Acid cyclization of this product, by the method of Battersby and Binks (8), gave (±)-bisnorargemonine, m.p. 231.5–232.5°. Except for melting point and rotational differences, the natural and synthetic compounds were shown to be identical. The identity of natural and synthetic materials was established by comparative UV, IR, NMR, and mass

[1] This compound has been prepared but not described by E. Brochmann-Hanssen and K. Hirai, *J. Pharm. Sci.*, **57**, 940(1968). Private communication with the authors indicates that the compound was obtained as a pale-yellow oil with suitable chromatographic and NMR characteristics. Our solid product meets similar criteria and is assumed to be identical to their product.

spectral analyses. Such criteria left no doubt that the original structural assignment (1) was correct.

(1) T. O. Soine and L. B. Kier, *J. Pharm. Sci.*, **52,** 1013(1963).
(2) R. L. Douglas and J. M. Gulland, *J. Chem. Soc.*, **1931,** 2893.
(3) K. W. Rosenmund, M. Nothnagel, and H. Riesenfeldt, *Ber.*, **60,** 392(1927).
(4) W. M. Whaley and T. R. Govindachari, in "Organic Reactions," vol. 6, Wiley, New York, N. Y., 1951, p. 74.
(5) M. Tomita and J. Kunimoto, *J. Pharm. Soc. Japan*, **80,** 1245 (1960).
(6) K. H. Lee and T. O. Soine, *J. Pharm. Sci.*, **57,** 1922(1968).
(7) D. H. R. Barton, R. H. Hesse, and G. W. Kirby, *J. Chem. Soc.*, **1965,** 6379.
(8) A. R. Battersby and R. Binks, *J. Chem. Soc.*, **1955,** 2888.

CHUNG-HSIUNG CHEN
TAITO O. SOINE*
Department of Medicinal Chemistry
College of Pharmacy
University of Minnesota
Minneapolis, MN 55455

KUO-HSIUNG LEE
Department of Medicinal Chemistry
School of Pharmacy
University of North Carolina
Chapel Hill, NC 27514

Received April 16, 1970.
Accepted for publication June 17, 1970.
This investigation was supported by Grant No. NB 08427 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
* To whom requests for reprints should be directed.

Improved Method for Measuring Output Potential of Specific Ion Electrodes

Keyphrases □ Ion electrodes, specific—output potential measurement □ pH meter, expanded scale—ion electrode potential output determination

Sir:

Since Frant and Ross (1) announced the invention of a single crystal lanthanum fluoride electrode for the determination of fluoride-ion activity, numerous articles have appeared in the literature describing applications of the device. It has been used to determine fluoride in bone (2), urine (3), chromium plating baths (4), tungsten (5), toothpaste (6), and numerous other samples.

In these methods, either an expanded scale analog or digital pH meter was used to measure fluoride-ion activity electrode potential. When using an expanded scale pH meter, it is possible to read electrode potentials to about ±1 mv. According to the report of Lingane (7), this would represent an error of ±0.017 pF unit, or a relative accuracy of ±1.7%. With a digital pH meter, it is possible to measure potentials to ±0.1 mv., with a correspondingly greater relative accuracy.

When determining the fluoride content of some pharmaceutical preparations without a digital pH meter, it was necessary in the authors' laboratory to devise a method of measuring the electrode potential more accurately than can be done directly with an expanded scale analog instrument. This was accomplished by using an expanded scale pH meter as an electrometer coupling between the electrode and a variable range strip-chart recorder, which involves simply connecting the recorder into the appropriate electrical output jacks of the meter. By so doing, and by choosing the proper input range of the recorder, it is possible to expand almost any portion of a standard curve [*i.e.*, almost any millivolt range of the plot: log (concentration F^-) *versus* millivolts] to full-scale deflection on the chart.

The most convenient way is to operate the pH meter in the pH (expanded scale) mode. This is done to keep the calibration circuit of the meter activated.

For the present work, a Corning model 10 expanded scale pH meter was used. While some of its characteristics (*i.e.*, deactivation of the calibration circuit when operated in the millivolt mode) may not be common, simple modifications of this procedure should make it applicable to individual needs.

After choosing the desired concentration range for the standard curve, the fluoride and reference electrodes are placed in the least concentrated standard sample. The calibration knob of the pH meter is then adjusted to bring the recorder pen to zero. Thus, at this concentration, the recorder will sense a zero potential from the electrode. The electrodes are then placed in the most concentrated standard, and the span control of the recorder is adjusted to bring the pen to fullscale deflection. To do this, the span control of the recorder must be infinitely variable between any two coarse span settings. One or two standards of intermediate concentrations are then used, and the pen deflection is noted.

When this is done, the numbers read from the chart become arbitrary units, not millivolts. However, the relationship, log (concentration F^-) *versus* recorder reading, is still linear. The concentration range is chosen on the basis of a compromise between a range narrow enough to allow sufficient accuracy and one wide enough to suppress electronic noise from the electrode. In the authors' laboratory, a three or fourfold concentration range was found reasonable. This range requires the recorder to have a fullscale deflection of about 1.5 mv.

In addition to a high degree of accuracy, several advantages are realized with the use of the recorder:

1. It provides a superb method of determining when the electrodes reach equilibrium; one need only note the point at which the needle ceases to drift. In extremely dilute solutions, equilibration time may be 30–45 min.

2. As reported by Strinivasan and Rechnitz (8), the electrode was found to drift, necessitating frequent recalibration. Because the slope of the calibration curve did not change, recalibration could be accomplished in a few minutes by adjusting the reading of one of the standard solutions to its original value on the recorder with the calibration knob of the pH meter.

3. The recorder gives a graphic presentation of the electronic noise produced by the electrode. This can be significant when the recorder is operated in the 1–2 mv. range. With the recorder, one can read potentials in spite of noise.

While work using this procedure in the authors' laboratory was conducted with a fluoride electrode, the

spectral analyses. Such criteria left no doubt that the original structural assignment (1) was correct.

(1) T. O. Soine and L. B. Kier, *J. Pharm. Sci.*, **52,** 1013(1963).
(2) R. L. Douglas and J. M. Gulland, *J. Chem. Soc.*, **1931,** 2893.
(3) K. W. Rosenmund, M. Nothnagel, and H. Riesenfeldt, *Ber.*, **60,** 392(1927).
(4) W. M. Whaley and T. R. Govindachari, in "Organic Reactions," vol. 6, Wiley, New York, N. Y., 1951, p. 74.
(5) M. Tomita and J. Kunimoto, *J. Pharm. Soc. Japan*, **80,** 1245 (1960).
(6) K. H. Lee and T. O. Soine, *J. Pharm. Sci.*, **57,** 1922(1968).
(7) D. H. R. Barton, R. H. Hesse, and G. W. Kirby, *J. Chem. Soc.*, **1965,** 6379.
(8) A. R. Battersby and R. Binks, *J. Chem. Soc.*, **1955,** 2888.

CHUNG-HSIUNG CHEN
TAITO O. SOINE*
Department of Medicinal Chemistry
College of Pharmacy
University of Minnesota
Minneapolis, MN 55455

KUO-HSIUNG LEE
Department of Medicinal Chemistry
School of Pharmacy
University of North Carolina
Chapel Hill, NC 27514

Received April 16, 1970.
Accepted for publication June 17, 1970.
This investigation was supported by Grant No. NB 08427 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
* To whom requests for reprints should be directed.

Improved Method for Measuring Output Potential of Specific Ion Electrodes

Keyphrases □ Ion electrodes, specific—output potential measurement □ pH meter, expanded scale—ion electrode potential output determination

Sir:

Since Frant and Ross (1) announced the invention of a single crystal lanthanum fluoride electrode for the determination of fluoride-ion activity, numerous articles have appeared in the literature describing applications of the device. It has been used to determine fluoride in bone (2), urine (3), chromium plating baths (4), tungsten (5), toothpaste (6), and numerous other samples.

In these methods, either an expanded scale analog or digital pH meter was used to measure fluoride-ion activity electrode potential. When using an expanded scale pH meter, it is possible to read electrode potentials to about ±1 mv. According to the report of Lingane (7), this would represent an error of ±0.017 pF unit, or a relative accuracy of ±1.7%. With a digital pH meter, it is possible to measure potentials to ±0.1 mv., with a correspondingly greater relative accuracy.

When determining the fluoride content of some pharmaceutical preparations without a digital pH meter, it was necessary in the authors' laboratory to devise a method of measuring the electrode potential more accurately than can be done directly with an expanded scale analog instrument. This was accomplished by using an expanded scale pH meter as an electrometer coupling between the electrode and a variable range strip-chart recorder, which involves simply connecting the recorder into the appropriate electrical output jacks of the meter. By so doing, and by choosing the proper input range of the recorder, it is possible to expand almost any portion of a standard curve [*i.e.*, almost any millivolt range of the plot: log (concentration F^-) *versus* millivolts] to full-scale deflection on the chart.

The most convenient way is to operate the pH meter in the pH (expanded scale) mode. This is done to keep the calibration circuit of the meter activated.

For the present work, a Corning model 10 expanded scale pH meter was used. While some of its characteristics (*i.e.*, deactivation of the calibration circuit when operated in the millivolt mode) may not be common, simple modifications of this procedure should make it applicable to individual needs.

After choosing the desired concentration range for the standard curve, the fluoride and reference electrodes are placed in the least concentrated standard sample. The calibration knob of the pH meter is then adjusted to bring the recorder pen to zero. Thus, at this concentration, the recorder will sense a zero potential from the electrode. The electrodes are then placed in the most concentrated standard, and the span control of the recorder is adjusted to bring the pen to fullscale deflection. To do this, the span control of the recorder must be infinitely variable between any two coarse span settings. One or two standards of intermediate concentrations are then used, and the pen deflection is noted.

When this is done, the numbers read from the chart become arbitrary units, not millivolts. However, the relationship, log (concentration F^-) *versus* recorder reading, is still linear. The concentration range is chosen on the basis of a compromise between a range narrow enough to allow sufficient accuracy and one wide enough to suppress electronic noise from the electrode. In the authors' laboratory, a three or fourfold concentration range was found reasonable. This range requires the recorder to have a fullscale deflection of about 1.5 mv.

In addition to a high degree of accuracy, several advantages are realized with the use of the recorder:

1. It provides a superb method of determining when the electrodes reach equilibrium; one need only note the point at which the needle ceases to drift. In extremely dilute solutions, equilibration time may be 30–45 min.

2. As reported by Strinivasan and Rechnitz (8), the electrode was found to drift, necessitating frequent recalibration. Because the slope of the calibration curve did not change, recalibration could be accomplished in a few minutes by adjusting the reading of one of the standard solutions to its original value on the recorder with the calibration knob of the pH meter.

3. The recorder gives a graphic presentation of the electronic noise produced by the electrode. This can be significant when the recorder is operated in the 1–2 mv. range. With the recorder, one can read potentials in spite of noise.

While work using this procedure in the authors' laboratory was conducted with a fluoride electrode, the

method should be equally applicable to all other specific ion electrodes.

(1) M. S. Frant and J. W. Ross, Jr., *Science*, **154,** 1553(1966).
(2) L. Singer and W. D. Armstrong, *Anal. Chem.*, **40,** 613(1968).
(3) M. W. Sun, *J. Amer. Ind. Hyg. Ass.*, **30,** 133(1969).
(4) M. S. Frant, *Plating*, **54,** 702(1967).
(5) B. A. Raby and W. E. Sunderland, *Anal. Chem.*, **39,** 1304 (1967).
(6) N. Shane and D. Miele, *J. Pharm. Sci.*, **57,** 1260(1968).
(7) J. J. Lingane, *Anal. Chem.*, **39,** 881(1967).
(8) K. Strinivasan and G. A. Rechnitz, *ibid.*, **40,** 509(1968).

LAWRENCE T. SENNELLO
RONALD J. GORSKI
Analytical Research Department
Abbott Laboratories
North Chicago, IL 60064

Received May 8, 1970.
Accepted for publication June 25, 1970.

BOOKS

REVIEWS

Editor's Note: *While it is not usual Journal practice to run more than one review on a particular book, "The Theory and Practice of Industrial Pharmacy" appeared to warrant a broader approach as it is an initial attempt to produce a textbook in the area of industrial pharmacy. For this reason, we are providing the opinions of two reviewers in the book reviews which follow.*

The Theory and Practice of Industrial Pharmacy. Vol. 1. Edited by LEON LACHMAN, HERBERT A. LIEBERMAN, and JOSEPH L. KANIG. Lea & Febiger, Philadelphia, PA 19106, 1970. xii + 811 pp. 15.5 × 23 cm. Price $24.50.

In their preface, the editors state that a good textbook on industrial pharmacy has been sorely needed. This is indeed the case and this book represents the first modern comprehensive treatment of industrial pharmacy to be published in the English language.

The book has three coeditors and thirty-eight contributors. As a result some chapters are truly outstanding and others are distinctly disappointing.

The book is written in four sections. These sections deal with the principles of pharmaceutical processing, dosage forms, quality control, and industrial pharmaceutical law and structures of pharmaceutical companies.

The first third of the book deals with principles of pharmaceutical processing and is written from a unit operations standpoint. Unit operations which are of particular importance in pharmaceutical processing are covered in separate chapters and include drying, mixing, milling, dispersion, clarification and filtration, compaction and compression, heat transfer, and fluid flow. Other specialized pharmaceutical processes treated are sterilization and tablet coating. One of the most outstanding chapters in the entire book appears to be very much out of place in the pharmaceutical processing or unit operations section and that is the chapter on biopharmaceutics. This chapter which was contributed by Professor Gibaldi is perhaps the most outstanding single chapter on the subject of biopharmaceutics to have been written to this date.

Section two treats on an individual chapter basis all of the various major classes of pharmaceutical dosage forms, including tablets, capsules, liquids, emulsions, semisolids, suspensions, suppositories, sterile products, aerosols, and sustained-release products. This section is entitled in the table of contents, "Dosage Forms: Design and Evaluation." Unfortunately dosage form design principles and drug product evaluation are either inadequately treated or are virtually missing from many of the chapters in this section. For example, in the chapter on capsules, no mention is made of the importance of availability studies or methods of characterizing *in vitro* availability. In most of the chapters in this section mechanical methods of manufacture are stressed. In the approximate 40% of the book dealing with dosage forms the chapter on sustained-action dosage forms by Eriksen, the chapter on sterile products by Avis, and the chapter on aerosols by Sciarra are outstanding in organization and content. The chapter on pilot plant scale-up techniques by Michelson is also well done, but seems strangely out of place, not to have been located in part one of the book dealing with pharmaceutical processing.

The nearly 100 pages of the book dealing with quality control contain two chapters coauthored by Dr. Lachman, one dealing with kinetic principles of stability testing and the other with quality assurance. These are very well written and represent a significant textbook compilation on these subjects to the literature.

In the design, organization, and writing of their book the editors have overlooked one important aspect of industrial pharmacy today. That aspect is the role of preformulation research and the application of physical-chemical principles in a systematic manner for the rational design of pharmaceutical drug products and dosage forms. It is obvious from the examination of the table of contents that the authors were at some loss as to the location of the section on biopharmaceutics. This excellent chapter together with a section on preformulation, its implications in drug product design, and the components of a total preformulation system would have provided a very good foundation for the dosage form section of the book. Unfortunately the preformulation concept is not treated by the book, making the foundation for the following dosage form section inadequate, and accentuating the lack of total information on basic principles of dosage form design and evaluation.

Even though this book contains some obvious omissions, quite often is incomplete on the subjects of dosage form design principles and evaluation, and is questionably organized in two instances, the book does represent a creditable and laudable effort to cover a broad and very difficult area. This book is recommended as a textbook for any advanced pharmaceutics or manufacturing graduate course. The book warrants consideration for elective undergraduate manufacturing pharmacy courses. It is doubtful that the book will find wide-scale acceptance as an undergraduate text based on the specialized subjects treated and the nature of the material being covered. The book should, however, have a prominent place on the shelf of every pharmacy school and pharmaceutical laboratory library. It will also undoubtedly be a valuable contribution to the working bookshelf of many industrial and development pharmacists.

Reviewed by Gilbert S. Banker
School of Pharmacy and Pharmacal Sciences
Purdue University
Lafayette, IN 47907 ■

The Theory and Practice of Industrial Pharmacy. Vol. 1. Edited by LEON LACHMAN, HERBERT A. LIEBERMAN, and JOSEPH L. KANIG. Lea & Febiger, Philadelphia, PA 19106, 1970. xii + 811 pp. 15.5 × 23 cm. Price $24.50.

The preparation of this first attempt at a text on industrial pharmacy was undoubtedly difficult. The very scope of the subject must have given rise to many hard decisions finally arrived at with much hesitation, breadth *versus* depth alone being one of the most weighty of these.

method should be equally applicable to all other specific ion electrodes.

(1) M. S. Frant and J. W. Ross, Jr., *Science*, **154,** 1553(1966).
(2) L. Singer and W. D. Armstrong, *Anal. Chem.*, **40,** 613(1968).
(3) M. W. Sun, *J. Amer. Ind. Hyg. Ass.*, **30,** 133(1969).
(4) M. S. Frant, *Plating*, **54,** 702(1967).
(5) B. A. Raby and W. E. Sunderland, *Anal. Chem.*, **39,** 1304 (1967).
(6) N. Shane and D. Miele, *J. Pharm. Sci.*, **57,** 1260(1968).
(7) J. J. Lingane, *Anal. Chem.*, **39,** 881(1967).
(8) K. Strinivasan and G. A. Rechnitz, *ibid.*, **40,** 509(1968).

LAWRENCE T. SENNELLO
RONALD J. GORSKI
Analytical Research Department
Abbott Laboratories
North Chicago, IL 60064

Received May 8, 1970.
Accepted for publication June 25, 1970.

BOOKS

REVIEWS

Editor's Note: *While it is not usual Journal practice to run more than one review on a particular book, "The Theory and Practice of Industrial Pharmacy" appeared to warrant a broader approach as it is an initial attempt to produce a textbook in the area of industrial pharmacy. For this reason, we are providing the opinions of two reviewers in the book reviews which follow.*

The Theory and Practice of Industrial Pharmacy. Vol. 1. Edited by LEON LACHMAN, HERBERT A. LIEBERMAN, and JOSEPH L. KANIG. Lea & Febiger, Philadelphia, PA 19106, 1970. xii + 811 pp. 15.5 × 23 cm. Price $24.50.

In their preface, the editors state that a good textbook on industrial pharmacy has been sorely needed. This is indeed the case and this book represents the first modern comprehensive treatment of industrial pharmacy to be published in the English language.

The book has three coeditors and thirty-eight contributors. As a result some chapters are truly outstanding and others are distinctly disappointing.

The book is written in four sections. These sections deal with the principles of pharmaceutical processing, dosage forms, quality control, and industrial pharmaceutical law and structures of pharmaceutical companies.

The first third of the book deals with principles of pharmaceutical processing and is written from a unit operations standpoint. Unit operations which are of particular importance in pharmaceutical processing are covered in separate chapters and include drying, mixing, milling, dispersion, clarification and filtration, compaction and compression, heat transfer, and fluid flow. Other specialized pharmaceutical processes treated are sterilization and tablet coating. One of the most outstanding chapters in the entire book appears to be very much out of place in the pharmaceutical processing or unit operations section and that is the chapter on biopharmaceutics. This chapter which was contributed by Professor Gibaldi is perhaps the most outstanding single chapter on the subject of biopharmaceutics to have been written to this date.

Section two treats on an individual chapter basis all of the various major classes of pharmaceutical dosage forms, including tablets, capsules, liquids, emulsions, semisolids, suspensions, suppositories, sterile products, aerosols, and sustained-release products. This section is entitled in the table of contents, "Dosage Forms: Design and Evaluation." Unfortunately dosage form design principles and drug product evaluation are either inadequately treated or are virtually missing from many of the chapters in this section. For example, in the chapter on capsules, no mention is made of the importance of availability studies or methods of characterizing *in vitro* availability. In most of the chapters in this section mechanical methods of manufacture are stressed. In the approximate 40% of the book dealing with dosage forms the chapter on sustained-action dosage forms by Eriksen, the chapter on sterile products by Avis, and the chapter on aerosols by Sciarra are outstanding in organization and content. The chapter on pilot plant scale-up techniques by Michelson is also well done, but seems strangely out of place, not to have been located in part one of the book dealing with pharmaceutical processing.

The nearly 100 pages of the book dealing with quality control contain two chapters coauthored by Dr. Lachman, one dealing with kinetic principles of stability testing and the other with quality assurance. These are very well written and represent a significant textbook compilation on these subjects to the literature.

In the design, organization, and writing of their book the editors have overlooked one important aspect of industrial pharmacy today. That aspect is the role of preformulation research and the application of physical-chemical principles in a systematic manner for the rational design of pharmaceutical drug products and dosage forms. It is obvious from the examination of the table of contents that the authors were at some loss as to the location of the section on biopharmaceutics. This excellent chapter together with a section on preformulation, its implications in drug product design, and the components of a total preformulation system would have provided a very good foundation for the dosage form section of the book. Unfortunately the preformulation concept is not treated by the book, making the foundation for the following dosage form section inadequate, and accentuating the lack of total information on basic principles of dosage form design and evaluation.

Even though this book contains some obvious omissions, quite often is incomplete on the subjects of dosage form design principles and evaluation, and is questionably organized in two instances, the book does represent a creditable and laudable effort to cover a broad and very difficult area. This book is recommended as a textbook for any advanced pharmaceutics or manufacturing graduate course. The book warrants consideration for elective undergraduate manufacturing pharmacy courses. It is doubtful that the book will find wide-scale acceptance as an undergraduate text based on the specialized subjects treated and the nature of the material being covered. The book should, however, have a prominent place on the shelf of every pharmacy school and pharmaceutical laboratory library. It will also undoubtedly be a valuable contribution to the working bookshelf of many industrial and development pharmacists.

Reviewed by Gilbert S. Banker
School of Pharmacy and Pharmacal Sciences
Purdue University
Lafayette, IN 47907 ■

The Theory and Practice of Industrial Pharmacy. Vol. 1. Edited by LEON LACHMAN, HERBERT A. LIEBERMAN, and JOSEPH L. KANIG. Lea & Febiger, Philadelphia, PA 19106, 1970. xii + 811 pp. 15.5 × 23 cm. Price $24.50.

The preparation of this first attempt at a text on industrial pharmacy was undoubtedly difficult. The very scope of the subject must have given rise to many hard decisions finally arrived at with much hesitation, breadth *versus* depth alone being one of the most weighty of these.

method should be equally applicable to all other specific ion electrodes.

(1) M. S. Frant and J. W. Ross, Jr., *Science*, **154,** 1553(1966).
(2) L. Singer and W. D. Armstrong, *Anal. Chem.*, **40,** 613(1968).
(3) M. W. Sun, *J. Amer. Ind. Hyg. Ass.*, **30,** 133(1969).
(4) M. S. Frant, *Plating*, **54,** 702(1967).
(5) B. A. Raby and W. E. Sunderland, *Anal. Chem.*, **39,** 1304 (1967).
(6) N. Shane and D. Miele, *J. Pharm. Sci.*, **57,** 1260(1968).
(7) J. J. Lingane, *Anal. Chem.*, **39,** 881(1967).
(8) K. Strinivasan and G. A. Rechnitz, *ibid.*, **40,** 509(1968).

LAWRENCE T. SENNELLO
RONALD J. GORSKI
Analytical Research Department
Abbott Laboratories
North Chicago, IL 60064

Received May 8, 1970.
Accepted for publication June 25, 1970.

BOOKS

REVIEWS

Editor's Note: *While it is not usual Journal practice to run more than one review on a particular book, "The Theory and Practice of Industrial Pharmacy" appeared to warrant a broader approach as it is an initial attempt to produce a textbook in the area of industrial pharmacy. For this reason, we are providing the opinions of two reviewers in the book reviews which follow.*

The Theory and Practice of Industrial Pharmacy. Vol. 1. Edited by LEON LACHMAN, HERBERT A. LIEBERMAN, and JOSEPH L. KANIG. Lea & Febiger, Philadelphia, PA 19106, 1970. xii + 811 pp. 15.5 × 23 cm. Price $24.50.

In their preface, the editors state that a good textbook on industrial pharmacy has been sorely needed. This is indeed the case and this book represents the first modern comprehensive treatment of industrial pharmacy to be published in the English language.

The book has three coeditors and thirty-eight contributors. As a result some chapters are truly outstanding and others are distinctly disappointing.

The book is written in four sections. These sections deal with the principles of pharmaceutical processing, dosage forms, quality control, and industrial pharmaceutical law and structures of pharmaceutical companies.

The first third of the book deals with principles of pharmaceutical processing and is written from a unit operations standpoint. Unit operations which are of particular importance in pharmaceutical processing are covered in separate chapters and include drying, mixing, milling, dispersion, clarification and filtration, compaction and compression, heat transfer, and fluid flow. Other specialized pharmaceutical processes treated are sterilization and tablet coating. One of the most outstanding chapters in the entire book appears to be very much out of place in the pharmaceutical processing or unit operations section and that is the chapter on biopharmaceutics. This chapter which was contributed by Professor Gibaldi is perhaps the most outstanding single chapter on the subject of biopharmaceutics to have been written to this date.

Section two treats on an individual chapter basis all of the various major classes of pharmaceutical dosage forms, including tablets, capsules, liquids, emulsions, semisolids, suspensions, suppositories, sterile products, aerosols, and sustained-release products. This section is entitled in the table of contents, "Dosage Forms: Design and Evaluation." Unfortunately dosage form design principles and drug product evaluation are either inadequately treated or are virtually missing from many of the chapters in this section. For example, in the chapter on capsules, no mention is made of the importance of availability studies or methods of characterizing *in vitro* availability. In most of the chapters in this section mechanical methods of manufacture are stressed. In the approximate 40% of the book dealing with dosage forms the chapter on sustained-action dosage forms by Eriksen, the chapter on sterile products by Avis, and the chapter on aerosols by Sciarra are outstanding in organization and content. The chapter on pilot plant scale-up techniques by Michelson is also well done, but seems strangely out of place, not to have been located in part one of the book dealing with pharmaceutical processing.

The nearly 100 pages of the book dealing with quality control contain two chapters coauthored by Dr. Lachman, one dealing with kinetic principles of stability testing and the other with quality assurance. These are very well written and represent a significant textbook compilation on these subjects to the literature.

In the design, organization, and writing of their book the editors have overlooked one important aspect of industrial pharmacy today. That aspect is the role of preformulation research and the application of physical-chemical principles in a systematic manner for the rational design of pharmaceutical drug products and dosage forms. It is obvious from the examination of the table of contents that the authors were at some loss as to the location of the section on biopharmaceutics. This excellent chapter together with a section on preformulation, its implications in drug product design, and the components of a total preformulation system would have provided a very good foundation for the dosage form section of the book. Unfortunately the preformulation concept is not treated by the book, making the foundation for the following dosage form section inadequate, and accentuating the lack of total information on basic principles of dosage form design and evaluation.

Even though this book contains some obvious omissions, quite often is incomplete on the subjects of dosage form design principles and evaluation, and is questionably organized in two instances, the book does represent a creditable and laudable effort to cover a broad and very difficult area. This book is recommended as a textbook for any advanced pharmaceutics or manufacturing graduate course. The book warrants consideration for elective undergraduate manufacturing pharmacy courses. It is doubtful that the book will find wide-scale acceptance as an undergraduate text based on the specialized subjects treated and the nature of the material being covered. The book should, however, have a prominent place on the shelf of every pharmacy school and pharmaceutical laboratory library. It will also undoubtedly be a valuable contribution to the working bookshelf of many industrial and development pharmacists.

Reviewed by Gilbert S. Banker
School of Pharmacy and Pharmacal Sciences
Purdue University
Lafayette, IN 47907 ■

The Theory and Practice of Industrial Pharmacy. Vol. 1. Edited by LEON LACHMAN, HERBERT A. LIEBERMAN, and JOSEPH L. KANIG. Lea & Febiger, Philadelphia, PA 19106, 1970. xii + 811 pp. 15.5 × 23 cm. Price $24.50.

The preparation of this first attempt at a text on industrial pharmacy was undoubtedly difficult. The very scope of the subject must have given rise to many hard decisions finally arrived at with much hesitation, breadth *versus* depth alone being one of the most weighty of these.

All in all, the book is an excellent beginning and the editors are congratulated in obtaining the consistently good help of so many competent contributors.

But there are serious omissions in a text that by its title claims exposition of both theory and practice, and there are inclusions that seem a bit unnecessary here, however necessary and important as ancillary materials that have been very properly treated in other works.

As to the latter, it seems that for the most part the chapters on heat transfer, momentum transfer, and fluid flow have been well treated elsewhere and need not have been included here, however well done. In actual practice an engineering department would or should function competently in these areas.

It is interesting to note that the technological details of dosage form development in the various areas are never tied in with control and production in the sense that in a well-run outfit development should issue detailed specifications for the finished job and assume the responsibility for the accuracy of these regardless of the degree of control and production involvement during the planning stages of new drug design. The chapter on pilot plant scale-up techniques is well done technically, but needs the finishing touch of stress on the responsibilities to be assumed and by whom. This is the important connecting link between development and production and is an extremely important administrative factor in the pursuit of industrial pharmacy. Interestingly, it is often disregarded in practice as it is disregarded in this text.

Little if any reference is made to the construction and control of labeling information, to include both label and package circular, yet these are grave responsibilities that come much earlier in the scheme of things than the mechanical control of finished labeling.

In the chapter on quality assurance, reference is made to a zero-defect concept. It is unfortunate that this widely used term is repeated here since it is a physical and psychologic anomaly. Yet the notion, as developed by Sinotte and others, is a most interesting approach to quality control because it deals with the fallibilities of the human which, after all is said and done, constitute the fundamental need for control. But the term "zero defect" should be replaced with one of greater semantic digestibility. Personnel may and should be qualified not only by training, experience, and the like, but also by their emotional attitudes toward the job after training and motivational incentives have been applied. The applicable paragraph on page 715 could and should be greatly expanded by competent psychological treatment. It is not too difficult to calculate a standard deviation, but a human can ruin its importance.

The control of bulk raw material is well spiced with general statements such as "adequate systems," "appropriate records," and our old friend "careful handling." A few pages later these are followed by a quite satisfactory handling of statistical quality control for the purposes of this text, but in neither case nor in any other area of this volume is much attention given to how samples are taken, and where and when, and by whom. It is one thing to give a blessing to Military Standards sampling plans, but quite another to show how these are not complete and practical answers to the requirements of practice. The reader should like to know who trains the sampler or his supervisor and how this is done and what responsibilities reside in these operators. Often no one else sees what they do, or how they do it.

Automated sampling and assay procedures have already solved many problems in quality control, as this reviewer prophesized many years ago, and as this volume gives adequate description, but we need more definitive treatment of hand sampling and bench assay operation in a text on industrial pharmacy too, it would seem. The day is somewhat in the future when relatively small pharmaceutical operations will have disappeared. These need specific and truly applicable help in these areas of quality control far more than their more sophisticated counterparts.

This reviewer is impelled once again to point out that it seems almost axiomatic that authors in this field must use terms such as, "carefully," "adequate," "absolutely correct," "proper," "suitable," "reasonable," and the like. This volume has far too many such terms. As a text it needs none of them, or at the most, few. But it does need definitive statements in these applicable areas.

In no place was there found any statement as to the organizational responsibilities and authorities of the director of quality control. This matter has been so avoided or mishandled in the industry generally that a careful discussion of it would seem to be in order. The statement in the last paragraph on page 748 is good, but it is altogether too general for a text without more detailed support.

The matter of patents is well discussed and is quite informative to those of little experience in the field, which is as it should be. It would have been most helpful however, if some discussion of the manner in which ideas are developed in the group research that pertains today, and the difficulty of assigning the proper credit to individuals who participate in invention. These are the very facts of life to directors of development, or should be. Patents protect and provide incentive, but they are not the research "thing" itself. The "thing" itself is the heart and soul of industrial pharmacy.

But this is an excellent "first try" in the elaboration of a text that has been long sorely needed. Careful revision will make it into a most valuable resource. Such revision will surely follow its use as a text, a use which is recommended.

Reviewed by Sereck H. Fox
Professor of Industrial Pharmacy
Wayne State University
Detroit, MI 48202 ■

European Pharmacopoeia. Vol. 1. Published under the direction of the Council of Europe (Partial Agreement) in accordance with the Convention on the Elaboration of a European Pharmacopoeia. Maisonneuve S. A., 57-Sainte-Ruffine, France, 1969. i + 401 pp. 15.5 × 24.5 cm. (*English and French versions available*)

The publication of the *European Pharmacopoeia* represents a significant step toward greater cooperation across national boundaries in the field of public health. The European Pharmacopoeia Commission was established by a Convention signed in September 1964 by the following countries: Belgium, France, Federal Republic of Germany, Italy, Luxembourg, the Netherlands, Switzerland, and the United Kingdom.

For the past five years, some hundred scientists, representing these eight countries, have been preparing this compilation. Application of the standards established in this volume must be made before January 1, 1972, with this volume gradually replacing the traditional national pharmacopoeias.

This volume contains general methods of analysis as well as 51 monographs on specific basic medicinal substances. A second volume of this compendium containing biological substances such as hormones, vaccine serum, and antibiotics is scheduled for publication next year.

Staff Review ■

Pharmacology and Patient Care. By Solomon Garb, Betty Jean Crim, and Garf Thomas. Springer, 200 Park Ave. So., New York, NY 10003, 1970. x + 597 pp. 14 × 21.5 cm. Price $8.95 (hard cover), $6.75 (soft).

This, the third edition of *Pharmacology and Patient Care*, is somewhat broader than the first two editions. Garf Thomas, Chief Hospital Pharmacist, University of Missouri Medicial Center, has been added as a coauthor, in an attempt to give this book, originally published as a textbook for nurses, a more interdisciplinary, paramedical approach.

This edition contains several new chapters, with all the chapters from the preceding editions being updated. Of particular interest are the chapters on "Drug Interaction and Incompatibilities" and "Pharmaceuticals and Society."

Staff Review ■

All in all, the book is an excellent beginning and the editors are congratulated in obtaining the consistently good help of so many competent contributors.

But there are serious omissions in a text that by its title claims exposition of both theory and practice, and there are inclusions that seem a bit unnecessary here, however necessary and important as ancillary materials that have been very properly treated in other works.

As to the latter, it seems that for the most part the chapters on heat transfer, momentum transfer, and fluid flow have been well treated elsewhere and need not have been included here, however well done. In actual practice an engineering department would or should function competently in these areas.

It is interesting to note that the technological details of dosage form development in the various areas are never tied in with control and production in the sense that in a well-run outfit development should issue detailed specifications for the finished job and assume the responsibility for the accuracy of these regardless of the degree of control and production involvement during the planning stages of new drug design. The chapter on pilot plant scale-up techniques is well done technically, but needs the finishing touch of stress on the responsibilities to be assumed and by whom. This is the important connecting link between development and production and is an extremely important administrative factor in the pursuit of industrial pharmacy. Interestingly, it is often disregarded in practice as it is disregarded in this text.

Little if any reference is made to the construction and control of labeling information, to include both label and package circular, yet these are grave responsibilities that come much earlier in the scheme of things than the mechanical control of finished labeling.

In the chapter on quality assurance, reference is made to a zero-defect concept. It is unfortunate that this widely used term is repeated here since it is a physical and psychologic anomaly. Yet the notion, as developed by Sinotte and others, is a most interesting approach to quality control because it deals with the fallibilities of the human which, after all is said and done, constitute the fundamental need for control. But the term "zero defect" should be replaced with one of greater semantic digestibility. Personnel may and should be qualified not only by training, experience, and the like, but also by their emotional attitudes toward the job after training and motivational incentives have been applied. The applicable paragraph on page 715 could and should be greatly expanded by competent psychological treatment. It is not too difficult to calculate a standard deviation, but a human can ruin its importance.

The control of bulk raw material is well spiced with general statements such as "adequate systems," "appropriate records," and our old friend "careful handling." A few pages later these are followed by a quite satisfactory handling of statistical quality control for the purposes of this text, but in neither case nor in any other area of this volume is much attention given to how samples are taken, and where and when, and by whom. It is one thing to give a blessing to Military Standards sampling plans, but quite another to show how these are not complete and practical answers to the requirements of practice. The reader should like to know who trains the sampler or his supervisor and how this is done and what responsibilities reside in these operators. Often no one else sees what they do, or how they do it.

Automated sampling and assay procedures have already solved many problems in quality control, as this reviewer prophesized many years ago, and as this volume gives adequate description, but we need more definitive treatment of hand sampling and bench assay operation in a text on industrial pharmacy too, it would seem. The day is somewhat in the future when relatively small pharmaceutical operations will have disappeared. These need specific and truly applicable help in these areas of quality control far more than their more sophisticated counterparts.

This reviewer is impelled once again to point out that it seems almost axiomatic that authors in this field must use terms such as, "carefully," "adequate," "absolutely correct," "proper," "suitable," "reasonable," and the like. This volume has far too many such terms. As a text it needs none of them, or at the most, few. But it does need definitive statements in these applicable areas.

In no place was there found any statement as to the organizational responsibilities and authorities of the director of quality control. This matter has been so avoided or mishandled in the industry generally that a careful discussion of it would seem to be in order. The statement in the last paragraph on page 748 is good, but it is altogether too general for a text without more detailed support.

The matter of patents is well discussed and is quite informative to those of little experience in the field, which is as it should be. It would have been most helpful however, if some discussion of the manner in which ideas are developed in the group research that pertains today, and the difficulty of assigning the proper credit to individuals who participate in invention. These are the very facts of life to directors of development, or should be. Patents protect and provide incentive, but they are not the research "thing" itself. The "thing" itself is the heart and soul of industrial pharmacy.

But this is an excellent "first try" in the elaboration of a text that has been long sorely needed. Careful revision will make it into a most valuable resource. Such revision will surely follow its use as a text, a use which is recommended.

Reviewed by Sereck H. Fox
Professor of Industrial Pharmacy
Wayne State University
Detroit, MI 48202 ■

European Pharmacopoeia. Vol. 1. Published under the direction of the Council of Europe (Partial Agreement) in accordance with the Convention on the Elaboration of a European Pharmacopoeia. Maisonneuve S. A., 57-Sainte-Ruffine, France, 1969. i + 401 pp. 15.5 × 24.5 cm. (*English and French versions available*)

The publication of the *European Pharmacopoeia* represents a significant step toward greater cooperation across national boundaries in the field of public health. The European Pharmacopoeia Commission was established by a Convention signed in September 1964 by the following countries: Belgium, France, Federal Republic of Germany, Italy, Luxembourg, the Netherlands, Switzerland, and the United Kingdom.

For the past five years, some hundred scientists, representing these eight countries, have been preparing this compilation. Application of the standards established in this volume must be made before January 1, 1972, with this volume gradually replacing the traditional national pharmacopoeias.

This volume contains general methods of analysis as well as 51 monographs on specific basic medicinal substances. A second volume of this compendium containing biological substances such as hormones, vaccine serum, and antibiotics is scheduled for publication next year.

Staff Review ■

Pharmacology and Patient Care. By Solomon Garb, Betty Jean Crim, and Garf Thomas. Springer, 200 Park Ave. So., New York, NY 10003, 1970. x + 597 pp. 14 × 21.5 cm. Price $8.95 (hard cover), $6.75 (soft).

This, the third edition of *Pharmacology and Patient Care*, is somewhat broader than the first two editions. Garf Thomas, Chief Hospital Pharmacist, University of Missouri Medicial Center, has been added as a coauthor, in an attempt to give this book, originally published as a textbook for nurses, a more interdisciplinary, paramedical approach.

This edition contains several new chapters, with all the chapters from the preceding editions being updated. Of particular interest are the chapters on "Drug Interaction and Incompatibilities" and "Pharmaceuticals and Society."

Staff Review ■

All in all, the book is an excellent beginning and the editors are congratulated in obtaining the consistently good help of so many competent contributors.

But there are serious omissions in a text that by its title claims exposition of both theory and practice, and there are inclusions that seem a bit unnecessary here, however necessary and important as ancillary materials that have been very properly treated in other works.

As to the latter, it seems that for the most part the chapters on heat transfer, momentum transfer, and fluid flow have been well treated elsewhere and need not have been included here, however well done. In actual practice an engineering department would or should function competently in these areas.

It is interesting to note that the technological details of dosage form development in the various areas are never tied in with control and production in the sense that in a well-run outfit development should issue detailed specifications for the finished job and assume the responsibility for the accuracy of these regardless of the degree of control and production involvement during the planning stages of new drug design. The chapter on pilot plant scale-up techniques is well done technically, but needs the finishing touch of stress on the responsibilities to be assumed and by whom. This is the important connecting link between development and production and is an extremely important administrative factor in the pursuit of industrial pharmacy. Interestingly, it is often disregarded in practice as it is disregarded in this text.

Little if any reference is made to the construction and control of labeling information, to include both label and package circular, yet these are grave responsibilities that come much earlier in the scheme of things than the mechanical control of finished labeling.

In the chapter on quality assurance, reference is made to a zero-defect concept. It is unfortunate that this widely used term is repeated here since it is a physical and psychologic anomaly. Yet the notion, as developed by Sinotte and others, is a most interesting approach to quality control because it deals with the fallibilities of the human which, after all is said and done, constitute the fundamental need for control. But the term "zero defect" should be replaced with one of greater semantic digestibility. Personnel may and should be qualified not only by training, experience, and the like, but also by their emotional attitudes toward the job after training and motivational incentives have been applied. The applicable paragraph on page 715 could and should be greatly expanded by competent psychological treatment. It is not too difficult to calculate a standard deviation, but a human can ruin its importance.

The control of bulk raw material is well spiced with general statements such as "adequate systems," "appropriate records," and our old friend "careful handling." A few pages later these are followed by a quite satisfactory handling of statistical quality control for the purposes of this text, but in neither case nor in any other area of this volume is much attention given to how samples are taken, and where and when, and by whom. It is one thing to give a blessing to Military Standards sampling plans, but quite another to show how these are not complete and practical answers to the requirements of practice. The reader should like to know who trains the sampler or his supervisor and how this is done and what responsibilities reside in these operators. Often no one else sees what they do, or how they do it.

Automated sampling and assay procedures have already solved many problems in quality control, as this reviewer prophesized many years ago, and as this volume gives adequate description, but we need more definitive treatment of hand sampling and bench assay operation in a text on industrial pharmacy too, it would seem. The day is somewhat in the future when relatively small pharmaceutical operations will have disappeared. These need specific and truly applicable help in these areas of quality control far more than their more sophisticated counterparts.

This reviewer is impelled once again to point out that it seems almost axiomatic that authors in this field must use terms such as, "carefully," "adequate," "absolutely correct," "proper," "suitable," "reasonable," and the like. This volume has far too many such terms. As a text it needs none of them, or at the most, few. But it does need definitive statements in these applicable areas.

In no place was there found any statement as to the organizational responsibilities and authorities of the director of quality control. This matter has been so avoided or mishandled in the industry generally that a careful discussion of it would seem to be in order. The statement in the last paragraph on page 748 is good, but it is altogether too general for a text without more detailed support.

The matter of patents is well discussed and is quite informative to those of little experience in the field, which is as it should be. It would have been most helpful however, if some discussion of the manner in which ideas are developed in the group research that pertains today, and the difficulty of assigning the proper credit to individuals who participate in invention. These are the very facts of life to directors of development, or should be. Patents protect and provide incentive, but they are not the research "thing" itself. The "thing" itself is the heart and soul of industrial pharmacy.

But this is an excellent "first try" in the elaboration of a text that has been long sorely needed. Careful revision will make it into a most valuable resource. Such revision will surely follow its use as a text, a use which is recommended.

Reviewed by Sereck H. Fox
Professor of Industrial Pharmacy
Wayne State University
Detroit, MI 48202 ■

European Pharmacopoeia. Vol. 1. Published under the direction of the Council of Europe (Partial Agreement) in accordance with the Convention on the Elaboration of a European Pharmacopoeia. Maisonneuve S. A., 57-Sainte-Ruffine, France, 1969. i + 401 pp. 15.5 × 24.5 cm. (*English and French versions available*)

The publication of the *European Pharmacopoeia* represents a significant step toward greater cooperation across national boundaries in the field of public health. The European Pharmacopoeia Commission was established by a Convention signed in September 1964 by the following countries: Belgium, France, Federal Republic of Germany, Italy, Luxembourg, the Netherlands, Switzerland, and the United Kingdom.

For the past five years, some hundred scientists, representing these eight countries, have been preparing this compilation. Application of the standards established in this volume must be made before January 1, 1972, with this volume gradually replacing the traditional national pharmacopoeias.

This volume contains general methods of analysis as well as 51 monographs on specific basic medicinal substances. A second volume of this compendium containing biological substances such as hormones, vaccine serum, and antibiotics is scheduled for publication next year.

Staff Review ■

Pharmacology and Patient Care. By Solomon Garb, Betty Jean Crim, and Garf Thomas. Springer, 200 Park Ave. So., New York, NY 10003, 1970. x + 597 pp. 14 × 21.5 cm. Price $8.95 (hard cover), $6.75 (soft).

This, the third edition of *Pharmacology and Patient Care*, is somewhat broader than the first two editions. Garf Thomas, Chief Hospital Pharmacist, University of Missouri Medicial Center, has been added as a coauthor, in an attempt to give this book, originally published as a textbook for nurses, a more interdisciplinary, paramedical approach.

This edition contains several new chapters, with all the chapters from the preceding editions being updated. Of particular interest are the chapters on "Drug Interaction and Incompatibilities" and "Pharmaceuticals and Society."

Staff Review ■

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

NOVEMBER 1970
VOLUME 59 NUMBER 11

REVIEW ARTICLE

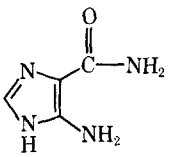
Syntheses and Biological Activity of 5-Aminoimidazoles and 5-Triazenoimidazoles

Y. FULMER SHEALY

Keyphrases ☐ 5-Aminoimidazoles—methods of synthesis ☐ Triazenoimidazoles—synthesis, stability ☐ Antineoplastic activity—triazenoimidazoles ☐ Microbiological activity—triazenoimidazoles ☐ Clinical studies—5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide

I. INTRODUCTION

In 1945, Stetten and Fox (1) isolated a heterocyclic amine that accumulated (1, 2) during sulfonamide bacteriostasis of *Escherichia coli* and also detected this compound in sulfonamide-inhibited cultures of other bacteria. Shive *et al.* (3), proceeding from the hypothesis that purine biosynthesis had been affected, showed that the heterocyclic amine was 5-aminoimidazole-4-carboxamide[1] (I) (AIC[2]), an imidazole that had been synthesized more than 20 years earlier by Windaus and Langenbeck (4) as a potential intermediate for the chemical synthesis of purines (5). Shive *et al.* (3) correctly postulated that AIC "functions as a precursor of purine bases or is formed from a precursor of purines" and that a *p*-aminobenzoic acid "coenzyme [formyltetrahydrofolic acid] functions in combining a single carbon unit into the pyrimidine ring." Subsequent studies of purine biosynthesis revealed that the 1-(β-D-ribofuranosyl) 5′-phosphate derivatives of AIC and of four other imidazoles constitute the middle group of steps in the biosynthetic pathway to purine ribonucleotides. All imidazole moieties of these compounds have an amino group (or its formyl derivative) at the 5-position of the imidazole ring; all, save the first, have a carboxyl or a carboxamide group at the 4-position.



I
AIC

Other imidazole derivatives possess various types and varying degrees of biological significance. The physiological and pharmacological importance of histidine and histamine is well known, and the literature relating to these compounds, their derivatives, and their analogs is extensive. Certain imidazole structures are reported to display antiprotozoal, antifungal, antibacterial, monoamine oxidase-inhibitory, her-

[1] When a nitrogen atom of the imidazole ring has an exocyclic substituent, that nitrogen atom is assigned position 1. Prior to 1967, the subject indexes of *Chemical Abstracts* listed Compound I as 5(or 4)-aminoimidazole-4(or 5)-carboxamide, because the position (which should be number 1) of the labile ring proton cannot be fixed. This nomenclature convention, in which the number of the alternative numbering sequence is included in parentheses, had been employed for similar imidazoles that have a proton on a ring nitrogen and substituents at positions 4 and 5. In recent subject indexes, Compound I is named 5-aminoimidazole-4-carboxamide, and this simplified nomenclature is also employed for similar imidazoles. In agreement with later *Chemical Abstracts* usage and for the sake of simplicity, a single number, without the parenthetical alternative numbering system, will be used thoughout this review to designate positions 4 and 5. In the structures of compounds, such as AIC, having the labile proton instead of another substituent (alkyl, ribofuranosyl, *etc.*) on a nitrogen atom, the proton is generally placed on the nitrogen atom that corresponds to the point of attachment of the ribofuranosyl group of the biosynthetic imidazoles.

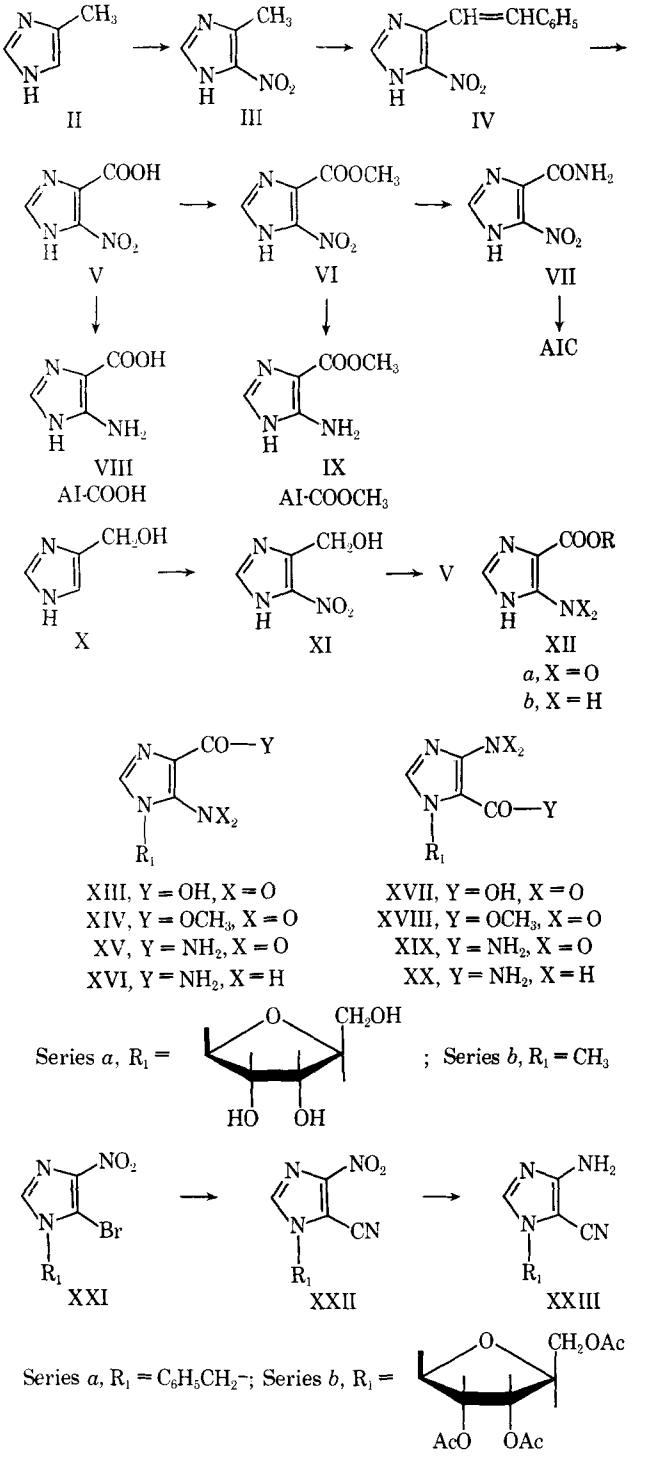
[2] The letter designations AIC and AICA are both employed in the literature for Compound I. Since either abbreviation could also be logically used for 5-aminoimidazole-4-carboxylic acid, the ribonucleotide of which is a precursor of the ribonucleotide of I, neither is specific. The shorter abbreviation will, therefore, be used in this article.

bicidal, and other types of activity. The following examples are illustrative: the activity of imidazole-4,5-dicarboxamide and some of its derivatives against coccidiosis in poultry (6, 7); the activity of 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole (metronidazole) (8–10) and other nitroimidazoles (*e.g.*, 10–15) against *Trichomonas vaginalis*, other *Trichomonas* species, *Entamoeba histolytica*, and other protozoa; and the broad-spectrum antibacterial and antiprotozoal activity of 2-amino-5-(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole and related structures (16). The antiprotozoal activity of metronidazole is clinically important in the oral treatment of trichomoniasis (14, 17–19) and amoebiasis (14, 20, 21).

However, to limit the scope of this review, it deals, first, with chemical syntheses and biological properties of 5-aminoimidazoles related to those in the biosynthetic pathway to purine ribonucleotides, the principal emphasis being on imidazoles bearing a member of the carboxylic acid family of groups (carboxyl, ester, amide, thioamide, amidine, nitrile, *etc.*) *ortho* to the amino group. Secondly, it summarizes the chemical and biological properties of triazenoimidazoles related to the imidazoles mentioned.[3] An excellent review of the entire imidazole field covers the period from 1919 to 1950 (22). Recent reviews (23, 24) differ from the present review in that: (*a*) they were limited to nucleosides and nucleotides of imidazoles, and (*b*) consideration of these types of derivatives was not confined to 5-aminoimidazoles.

II. SYNTHESES OF 5-AMINOIMIDAZOLES[4, 5]

Chemical syntheses of 5-aminoimidazole derivatives are presented by describing the basic methods for forming the imidazole ring with an amino group (or a precursor group) at position 5 rather than by structural type (amide, ester, nitrile, *etc.*). To preserve the continuity of certain synthetic sequences, further transformations of the structures obtained from a basic route are also frequently included in the section in which the basic method is described.



Scheme I

The synthetic methods of Sections B, C, and D are related and, in some of their variations, may include similar or common intermediates. However, they were developed at different times by different investigators; to simplify the presentation, they are treated separately.

A. Reduction of the Nitro Group (Windaus–Langenbeck and Sarasin–Wegmann Routes)—Because of its importance in aromatic chemistry, reduction of a nitro group (or an arylazo group) naturally received initial attention as a method for preparing 5-aminoimidazoles. These early efforts to synthesize 5-aminoimidazole (AI) and its methyl derivatives met with difficulties

[3] Triazenoimidazoles and aminoimidazoles are included in the same review because of the derivation, structures, and biological activity of the former. There is no intention to imply that inhibition of biochemical reactions involving aminoimidazoles is the primary mechanism of action of the triazeno derivatives (Part IV).

[4] To minimize both the number of letter designations and the number of long systematic names, three abbreviations will be employed in Parts II and III, with some exceptions in Part III where commonly accepted letter designations are shown with the structures. The three are AI for 5-aminoimidazole, AIC for Compound I, and AICAR for the ribonucleotide of Compound I. Admittedly, the latter two are not consistent; but there is no reason to change the generally accepted AICAR, and the reasons for choosing AIC are explained in *Footnote 2*. These three letter designations are employed in combination with the formulas of functional or protecting groups and in combination with names of groups or substituents to designate certain derivatives. For brevity, the terms ribofuranoside or ribonucleoside and ribonucleotide will be used for imidazoles bearing the β-D-ribofuranosyl and the β-D-ribofuranosyl 5′-phosphate groups at the position of these groups in the biosynthetic imidazoles (*i.e.*, adjacent to the amino group). This shorthand nomenclature method is illustrated by the following examples: 2-methyl-AIC ribonucleotide for 5-amino-2-methyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide 5′-(dihydrogen phosphate) (LX*d*); 1-cyclohexyl-AI-COOH for 5-amino-1-cyclohexylimidazole-4-carboxylic acid (XXXVII; R′ = R₂ = H, R₁ = cyclohexyl); and AI-COOCH₃ for 5-aminoimidazole-4-carboxylic acid methyl ester (IX).

[5] For convenience, carboxyl, amino, and phosphoric acid groups are depicted in their unionized forms throughout this review. However, ionic species are present in the biochemical environments in which imidazole derivatives having these groups are biosynthesized and transformed, and they are usually the forms isolated from chemical reactions.

owing to the instability of the products under the conditions of the chemical reductions (25–27, *cf.*, 28). Yields were low or nil because of degradation by ring-opening, but both AI (29) and its 4-methyl derivative (30) were subsequently obtained (as dihydrochlorides) in moderate yields by chemical reduction of the corresponding nitro compounds. The examples of prime interest in the present context have a carboxamide, a carboxyl, or similar group at position 4. During the period that difficulties were being experienced in preparing the simple aminoimidazoles, Windaus and Langenbeck (4) succeeded in synthesizing AIC and AI-$COOCH_3$[4] (IX) by a route that began with 4-methylimidazole (II) and terminated in the reduction of a nitro group (Scheme I). A nitration (25), an aldol-type condensation, and oxidation of the resulting styryl derivative (IV) yielded the key intermediate, 5-nitroimidazole-4-carboxylic acid (V), which had been obtained earlier by a less productive method (31). The nitro amide (VII) (4, 32) was prepared from the ester (VI) and reduced catalytically to AIC (4). Further studies and modifications of the route to V and IX were made by Allsebrook *et al.* (33), who also used the method to prepare the two nitro acids (XIII*b* and XVII*b*) having a methyl group on a ring nitrogen atom. The route was employed by Shive *et al.* (3) to prove the structure of AIC isolated from bacterial cultures; by Rabinowitz (34) to prepare (in weakly basic solution) 5-aminoimidazole-4-carboxylate (VIII), a catabolite of xanthine formed by extracts of *Clostridium cylindrosporum;* by Allsebrook *et al.* (33) to prepare 5-ureidoimidazole-4-carboxylic acid and its methyl ester for purine synthesis; by Taylor *et al.* (35) to prepare 5-aminoimidazole-4-carbohydroxamic acid; and by Robinson and Shepherd (36) to prepare 5-nitroimidazole-4-carboxylic acid hydrazide.

Kulev and Gireva (37) and Gireva and Dobychina (38) described an improved synthesis of the nitro acid (V); 4-(hydroxymethyl)imidazole (X), prepared from invert sugar, was nitrated and 4-(hydroxymethyl)-5-nitroimidazole (XI) was oxidized in high yield to V in the nitration mixture. These authors and others have reported the preparation of several esters (XII*a*) of V (38–42), the reduction of some of the nitro esters to esters (XII*b*) of 5-aminoimidazole-4-carboxylic acid (38, 40, 41), the preparation of acyl and sulfonyl [including bis(2-chloroethyl)aminobenzoyl and bis-(2-chloroethyl)aminobenzenesulfonyl] derivatives of the amino group of XII*b* ($R = C_2H_5$) (43), and the synthesis of *N*-aryl carboxamide analogs of VII (44, 45).

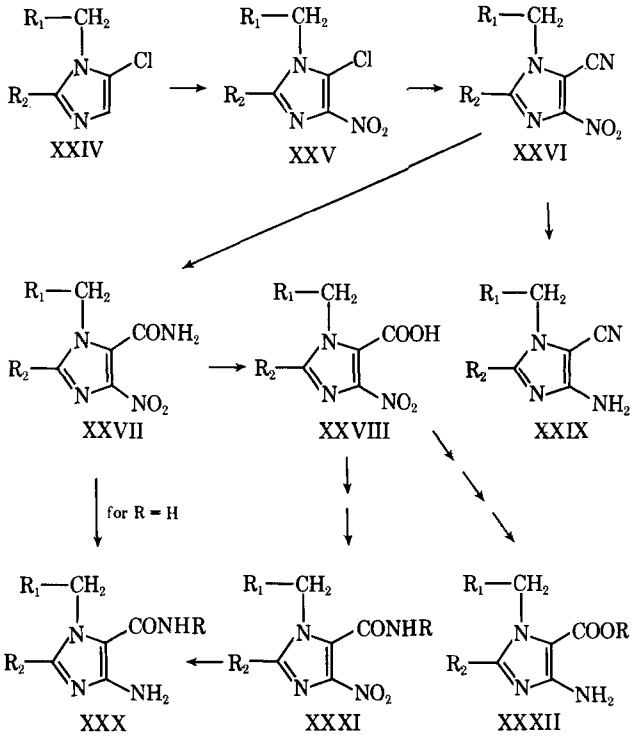
A synthesis (*cf.*, Parts IIB and IIE) of AIC ribonucleoside (XVI*a*) based on the nitro acid (V) was accomplished by Baddiley *et al.* (32, 46). Glycosidation of the silver or chloromercury salt of methyl 5-nitroimidazole-4-carboxylate (VI) with 2,3,5-tri-*O*-benzoylribofuranosyl chloride evidently gave a mixture of ribofuranosides (XIV*a* and XVIII*a* tribenzoates) that was transformed by ammonia to a mixture of the nitro amides (XV*a*, XIX*a*), which were separated. Catalytic reduction of each isomer gave AIC ribonucleoside (XVI*a*) and its isomer, 4-amino-1-(β-D-ribofuranosyl)imidazole-5-carboxamide (XX*a*). Methylation of the silver salt of the nitro amide (VII) gave the 1-methyl derivative (XV*b*) (32, 33) and its isomer (XIX*b*), which was also prepared from XVIII*b* (32); catalytic reduction of XV*b* afforded 1-methyl-AIC (XVI*b*).

Several findings on alkylation of these nitroimidazoles are germane to aminoimidazoles because of their potential or actual precursor role. Methyl 1-methyl-4-nitroimidazole-5-carboxylate (XVIII*b*) was the only reported product of methylation of the silver salt of VI (32, 33); the nitro styryl derivative (IV), like VII, gave a mixture of *N*-methyl isomers (32), and alkylation of the sodium salts of IV and its 2-methyl derivative gave mixtures of isomers (15). Ikehara *et al.* (47) isolated XVIII*a* tribenzoate as the main product of glycosidation of the chloromercury derivative of VI with 2,3,5-tri-*O*-benzoylribofuranosyl chloride and, in contrast, 4-methyl-5-nitro-1-β-D-ribofuranosylimidazole tribenzoate as the main product (44%) of glycosidation of 4-methyl-5-nitroimidazole (III). These results suggest either that the position of attack varies with the type of nitroimidazole substrate, the alkylating agent, and the conditions or that the methods of isolation or detection sometimes failed to reveal a second isomer.

In another synthesis of aminoimidazole nucleosides *via* glycosidation of nitro derivatives, more conclusive evidence of the position of predominant attack was obtained. Rousseau *et al.* (48, 49) employed an acid-catalyzed fusion procedure to glycosidate VI and 4-bromo-5-nitroimidazole with tetra-*O*-acetyl-β-D-ribofuranose. The *O*-triacetyl derivative of XVIII*a* was obtained in 83% yield, and the isomer could not be detected chromatographically. Although 4-bromo-5-nitroimidazole is not a product of the Windaus–Langenbeck route, it may be appropriate at this point to note that benzylation of this compound in solution (DMF) afforded a 78% yield of the derivative (XXI*a*) with the alkyl group adjacent to the bromo group[6] (50) and that 5-bromo-4-nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole (XXI*b*) was isolated in 72% yield from the acid-catalyzed fusion procedure (49), whereas less than a 5% yield of the isomeric ribofuranoside could be isolated. The amino derivatives, XXIII*a* (50) and XXIII*b* (48, 49), were obtained in yields of 99 and 87%, respectively, by catalytic hydrogenation of the nitro derivatives (XXII); similarly, XX*a* was obtained in high yield from XVIII*a* triacetate by the sequence consisting of amination of the ester group, deacetylation, and catalytic hydrogenation (49).

Another synthesis of aminoimidazolecarboxylic acid derivatives originated soon after the Windaus–Langenbeck route and produces structures related to those just discussed. In a sequence (Scheme II) of high-yielding steps (XXIV–XXVII, XXX), Sarasin and Wegmann (51) converted 5-chloro-1-methylimidazole (XXIV, $R_1 = R_2 = H$) to 4-amino-1-methylimidazole-5-carboxamide (XXX, $R = R_1 = R_2 = H$). The total synthetic route begins with the Wallach imidazole-ring formation (52, 53) from an *N,N'*-dialkyloxamide (XXXIII) and results in the introduction of a substituent on the imidazole-ring nitrogen adjacent to the car-

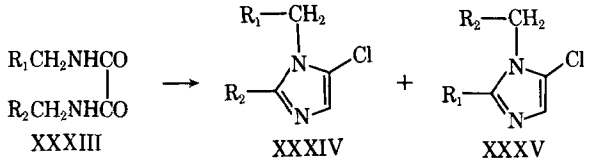
[6] Benzylation of the sodium salt gave a mixture of approximately equal amounts of the isomers.



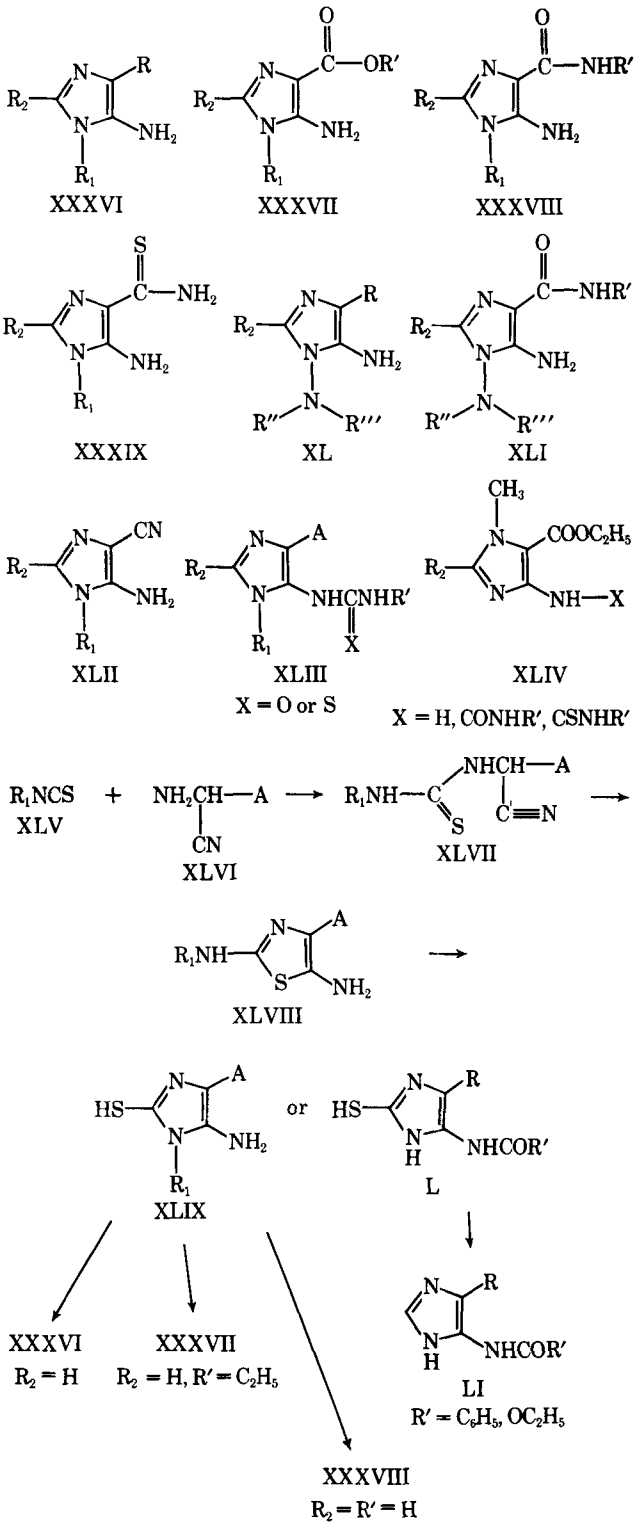
Scheme II

boxyl-type group (*cf.*, XVII–XX, XXII, XXIII) rather than at the position of the ribofuranosyl group in the biosynthetic imidazoles. In its most general form, the route is represented by XXIV–XXXII (Scheme II). Although hydrolysis of the amides (XXVII) to acids (XXVIII) is difficult, Mann and Porter (54) introduced a modification that gave high yields of the carboxylic acids (XXVIII) (*cf.*, 55). From the acids, *N*-substituted amides (XXXI, R_1 and R_2 = H or CH_3) (54–56), nitro esters (54), and a hydroxamic acid (57) have been prepared *via* acid chlorides. Originally, the nitro group of XXVI and XXVII was reduced chemically (tin and hydrochloric acid) (51, 55, 58, 59), but catalytic reduction to amino amides (XXX) (54, 56, 60–62), esters (XXXII, $R_1 = R_2 = H$) (54), and carbonitriles (XXIX) (60) is superior. However, incomplete reduction of the nitro group of 1-methyl-4-nitroimidazole-5-carbonitrile (XXVI, $R_1 = R_2 = H$) to an hydroxylamino group has been observed (63). The aminoimidazole derivatives resulting from most of these investigations (51, 54, 56, 58–64) were employed as intermediates for the synthesis of *N*-alkylpurines.

Recent investigations (61, 62, 65, 66) have extended the scope of and have clarified the reaction of oxamides with phosphorus pentachloride, the initial step of the overall synthesis of the amino amides (XXX). The isomers isolated have always been the 5-chloroimidazoles (51, 58, 61, 62, 67). In the recent study of several products by Trout and Levy (62), vapor-phase chroma-



Scheme III



In XLV–XLIX, A = H, CH_3, C_6H_5, $—COOC_2H_5$, or $—CONH_2$ and R_1 = H, CH_3, C_6H_5, $—COC_6H_5$, $—COOC_2H_5$, but not in all possible combinations.

Scheme IV

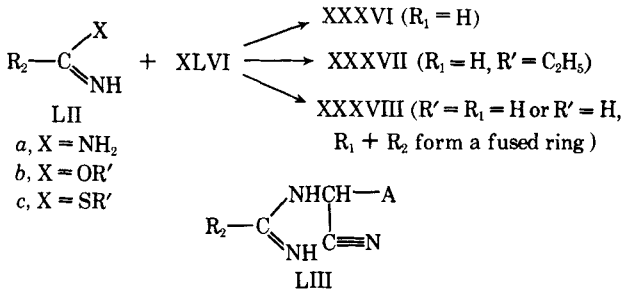
tography and TLC failed to reveal any of the 4-chloro derivatives. An unsymmetrical oxamide (XXXIII) (Scheme III) gave two separable 5-chloro-1,2-dialkylimidazoles (XXXIV and XXXV) (61). Several 4-amino-1,2-dialkylimidazole-5-carboxamides (XXX) were prepared by the Sarasin–Wegmann route (XXIV–XXVII, XXX)

in the course of these studies (61, 62) and used for the synthesis of purines (64).

5-Chloro-1-methyl-4-nitroimidazole (XXV, R_1 = R_2 = H) (51, 67) has also served as a starting point for the synthesis of some 4-amino-1-methylimidazole-5-sulfonamides (68, 69).

B. From α-Aminonitriles (Cook–Heilbron and G. Shaw Methods)—Synthetic routes that begin with α-aminonitriles were pioneered by Cook, Heilbron, G. Shaw, and coworkers and have proved to be exceptionally important in syntheses of 5-aminoimidazoles (XXXVI–XLII), including the nucleotides of the biosynthetic pathway (Part III). In the first route (Scheme IV) (70–75), reactions of isothiocyanates (XLV) with α-aminonitriles (XLVI) produce 2,5-diaminothiazoles (XLVIII) *via* thioureas (XLVII), which may or may not be isolated. Under the influence of mild aqueous base, these 5-aminothiazoles rearrange to 5-amino-2-mercaptoimidazoles (XLIX, or the tautomeric 5-amino-2-thioxoimidazolines). The group (R_1) at position 1 is derived from the isothiocyanate, whereas the substituent (A) at position 4 is that present on the α-carbon atom of the aminonitrile. When an acylisothiocyanate is one of the reactants, the acyl group appears on the 5-amino group of the imidazole derivative (L). Presumably, the 1-acylimidazole is first formed, and then acyl migration occurs under the influence of the basic conditions of the thiazole → imidazole rearrangement (70). The mercapto (or thioxo) substituent at position 2 may be removed by Raney nickel desulfurization (71–73), or it may be alkylated to afford compounds such as 2-(methylthio) and 2-(benzylthio) derivatives (75, 76). This synthetic route has produced 5-aminoimidazoles devoid of a functional substituent at position 4 (71–73), 5-aminoimidazole-4-carboxylic acid esters (73), or 5-aminoimidazole-4-carboxamides (73–75) with (XLIX, L) or without (LI, XXXVI–XXXVIII with R_2 = H) a sulfur function at position 2. Treatment of a thiazole ester (XLVIII; A = $COOC_2H_5$, R_1 = CH_3) with ammonia and subsequent desulfurization also gave amides, 1-methyl-2-mercapto-AIC and 1-methyl-AIC (XVI*b* or XXXVIII; R_1 = CH_3, R_2 = R′ = H) (73). Recently, 2-mercapto-AIC was prepared by the route XLV–XLIX (77) (see last paragraph of this section).

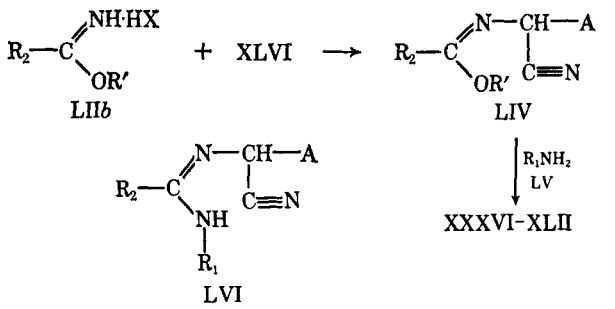
In a second route (Scheme V) (70, 74, 78, 79) based on α-



Scheme V

aminonitriles, the imidazole ring is formed by interaction of an amidine (LII*a*), an imidic acid ester (imidate) (LII*b*), or an imidic acid thioester (thioimidate) (LII*c*) with an appropriate α-aminonitrile (XLVI). This type of reaction produces 2-substituted derivatives and may be visualized as proceeding through an intermediate amidine (LIII). Again, the 5-amino group and the 4-substituent are furnished by the α-aminonitrile; the substituent at the 2-position is determined by the structure (LII) of the amidine or imidic acid derivative. This method was responsible for an early synthesis (74) of AIC from formamidine (LII*a*, R_2 = H). Soon after its role in purine biosynthesis had been discovered, AIC labeled at the 5-position with ^{14}C was prepared (80, 81) (*cf.*, Part IIC) from ethyl formimidate (LII*b*, R′ = C_2H_5) and labeled 2-aminocyanoacetamide (XLVI, A = $CONH_2$). Thioimidate (LII*c*) hydrochlorides were considered (79) to give more satisfactory results in this type of synthesis, and they were utilized in the preparation of a considerable number of 5-aminoimidazoles (XXXVI; R_1 = H, R = hydrogen, alkyl, or aryl) (79, 82, 83), 5-aminoimidazolecarboxylates (XXXVII; R_1 = H, R′ = C_2H_5) (79, 82, 83), and 5-aminoimidazole-4-carboxamides (XXXVIII; R_1 = R′ = H) (74, 84) with several different substituents at position 2. Several reported observations are of interest in the light of subsequent syntheses of this type: (*a*) the imidates (LII*b*) were used as free bases (79–81); (*b*) the thioimidate (LII*c*) hydrochloride sometimes failed to give imidazoles (or gave low yields) owing, apparently, to replacement by the α-aminonitrile of the imino group rather than the thioether group (82, 83); and (*c*) an intermediate imidate (LIV; R_2 = $C_6H_5CH_2$—, A = —$COOC_2H_5$), rather than an imidazole, was obtained when an equivalent of acid was present during the reaction of ethyl aminocyanoacetate with a starting imidate (LII*b;* R_2 = $C_6H_5CH_2$—, R′ = C_2H_5).[7] More recently, bicyclic compounds that are 1,2-disubstituted AIC derivatives by virtue of ring fusion at positions 1 and 2 of the imidazole ring have been synthesized from cyclic imidates and aminocyanoacetamide (85–90). These reactions of *N*-substituted imidates were usually performed in the presence of a catalytic amount of acid.

By preparing intermediate linear imidates (LIV), either in the isolated form or in solution, G. Shaw and coworkers (91–95) added an extra dimension of versatility to this method of synthesis. The general route (Scheme VI) is shown by Structures LII*b*, XLVI,



Scheme VI

LIV, and XXXVI–XLII. As before, the starting imidate supplies the carbon atom at position 2 with its sub-

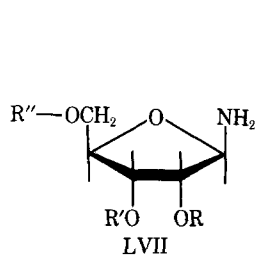
[7] Observations by Abraham, Baker, Barltrop, Chain, Waley, and Robinson cited in *Reference 79*.

stituent, but a primary amine (LV), or ammonia, then completes the imidazole ring by furnishing the nitrogen atom at position 1 with its substituent. The amidine (LVI) may be assumed to be an intermediate in this process (Scheme VI). Thus, appropriate choices of α-aminonitriles, imidates, and primary amines permit, in the absence of complications in specific cases consequent to the use of such sensitive intermediates, the introduction of various substituents at positions 1, 2, and 4 of 5-aminoimidazoles. From such linear imidates (LIV) and primary amines or ammonia, 1-alkyl and 1,2-dialkyl 5-aminoimidazoles (XXXVI, R = H) (92), 1-substituted and 1,2-disubstituted derivatives of AIC (XXXVIII, R′ = H) and of its *N*-methylamide analog (XXXVIII, R′ = CH_3) (91, 92, 96, 97), and 1-substituted 5-aminoimidazole-4-carboxylic acid esters (XXXVII, R_2 = H) (95) have been synthesized. Included among the AIC derivatives prepared by the Shaw method were 1,1′-bis derivatives (97) joined by an alkylene chain. When the primary amines were replaced in the synthetic sequence by hydrazine, a substituted hydrazine (LV, R_1 = R″R‴N—), or a semicarbazide, then 1,5-diaminoimidazoles (XL, XLI) were obtained (94, 97). Moreover, beginning the sequence with α-aminocyanothioacetamide (XLVI, A = —$CSNH_2$) produced thioamide analogs of AIC (XXXIX) (93), which have also been prepared by thiation of amides or addition of hydrogen sulfide to nitriles (Part IIF). The synthesis of the thioamides was sometimes frustrated by thiazole formation and other complications, but these difficulties could be circumvented by choosing specific esters of imidic acid hydrochlorides as reactants with aminocyanothioacetamide. Thus, benzyl acetimidate hydrochloride produced 2-methyl derivatives (XXXIX, R_2 = CH_3), whereas isopentyl formimidate (but not certain other formimidic acid esters) gave thioamides unsubstituted (R_2 = H) at position 2. (However, in a nonaqueous solvent the isopentyl ester gave a thiazole.) The formation of 5-amino-2-methylimidazole-4-thiocarboxamide (XXXIX; R_1 = H, R_2 = CH_3) from ethyl acetimidate free base is consistent with earlier observations (79) that imidate free bases and α-aminonitriles form 5-aminoimidazoles without a requirement for added ammonia. Only recently, after aminomalononitrile (XLVI, A = CN) became available as a result of studies related to prebiological syntheses (Part IID), has a carbonitrile (XLII) been synthesized by this route (96).

Representatives and derivatives of the general structures XXXVI–XLIV were synthesized during the course of these studies from aminoimidazoles obtained from the basic routes. Thus, carbonitriles (XLII) were also prepared by dehydration of some 1-alkyl- and 1,2-dialkyl-AIC derivatives with pyrophosphoryl chloride (96). Earlier, representatives of XLII had been obtained by treating thioamides (XXXIX) with mercuric chloride and methylamine (93). 1-Cyclohexyl derivatives of XXXVIII (1-cyclohexyl-AIC and some of its *N*-alkyl derivatives), as well as an analogous *N,N*-disubstituted amide, were synthesized from 1-cyclohexyl-AI-COOH by the active ester, mixed anhydride, or dicyclohexylcarbodiimide methods of peptide synthesis (98, 99). Since the ultimate aim of much of the earlier work on the routes from α-aminonitriles was the synthesis of purines, further transformations of the initially obtained aminoimidazole derivatives were directed toward that end. Conversion of the amino group to ureido and to thioureido groups with isocyanates and isothiocyanates, respectively, afforded urea and thiourea derivatives (XLIII, XLIV) (70, 73, 75, 78, 79, 82, 83, 100–102) for cyclization to purines. Alkylation of the imidazole ring with diazomethane, methyl iodide, or methyl sulfate gave some 1-methyl-4-aminoimidazoles (XLIV, X = H) (101, 102) for the preparation of *N*-alkylpurines *via* urea or thiourea derivatives.

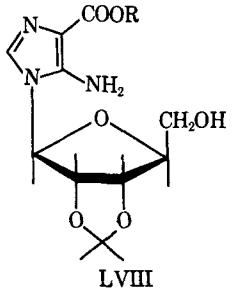
The linear imidates (LIV, A = $COOCH_3$ or $CONH_2$) derived from methyl α-aminocyanoacetate or α-aminocyanoacetamide served not only as sources of alkyl and aryl derivatives but also as precursors of the biosynthetic ribonucleotides (Part III) and the corresponding ribonucleosides. Interaction of the appropriate linear imidate and 2,3,5-tri-*O*-benzoylribofuranosylamine (LVII*a*) (103) gave, after removal of protecting groups, AIC ribonucleoside (XVI*a*) (92) and the methyl ester of AI-COOH ribonucleoside (95, 104). Although 5-aminoimidazole-4-carboxylic acids readily undergo decarboxylation in the acid form, AI-COOH ribonucleoside could be obtained as its calcium salt (95) or the pyridine salt of its isopropylidene derivative (LVIII*a*) (98) after alkaline hydrolysis of derivatives of the ethyl or methyl esters. AIC ribonucleoside was also prepared (105) by amidation of the isopropylidene derivative (LVIII*b*) (95, 104, 105) of AI-$COOCH_3$. The ribofuranoside analogs, 1-galactosyl-2-methyl-AI and 2-methyl-1-xylopyranosyl-AIC, were synthesized from glycosylamines and linear imidates (LIV) (92).

The ribonucleotides were first synthesized *via* the linear imidate method (*cf.*, Part IIE) by phosphorylating the 5′-hydroxyl group of isopropylidene derivatives of the nucleosides with 2-cyanoethylphosphate and dicyclohexylcarbodiimide (106) or with pyrophosphoryl chloride (107). Thus, phosphorylation of the isopropylidene derivative (LVIII*b*) of AI-$COOCH_3$ ribonucleoside and subsequent removal of protecting groups led to the isolation of AI-COOH ribonucleotide (Part III) (95, 104) and its methyl ester (105) in the form of their barium salts. Succino-AICAR (Part III) was then synthesized in 15% yield from LVIII*b* by a sequence of operations consisting of coupling the pyridine salt of LVIII*a* with dimethyl aspartate in the presence of dicyclohexylcarbodiimide, phosphorylation, removal of protecting groups, and purification by ion-exchange methods (98, 108). Subsequently, this sequence of steps was employed for the preparation of analogs of succino-AICAR in which the aspartyl moiety is replaced by other amino acid residues (LIX) (109); a similar sequence of steps that began with the pyridine salt of LVIII*a* and proceeded *via* active esters—the 2,4-dinitrophenyl and the pentachlorophenyl esters (LVIII*c*, LVIII*d*)—also produced succino-AICAR (99). From nucleosides obtained by the linear imidate method, AICAR (Part III) was prepared by the following methods (99, 105) (including the usual deblocking and purification operations): (*a*) phosphorylation of



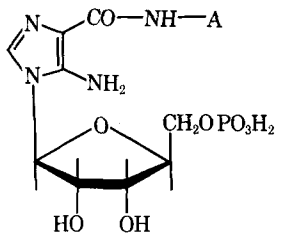
LVII

a, R = R′ = R″ = C_6H_5CO—
b, R + R′ = isopropylidene, R″ = H_2O_3P—



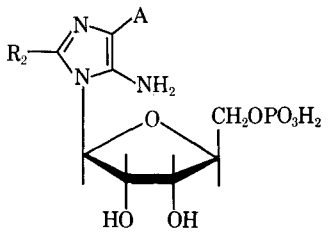
LVIII

a, R = H
b, R = CH_3
c, R = 2,4-dinitrophenyl
d, R = pentachlorophenyl



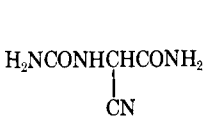
LIX

—NH—A derived from the following amino acids: D-aspartic; L-, D-, and DL-*threo*-β-methylaspartic; DL-*erythro*-β-methylaspartic; DL-β,β-dimethylaspartic; L-glutamic; and glycine.

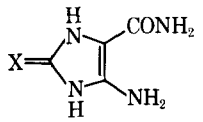


LX

a, R_2 = H, A = CN
b, R_2 = H, A = $CONHCH_3$
c, R_2 = H, A = $CON(C_2H_5)_2$
d, R_2 = CH_3, A = $CONH_2$



LXI



LXII

a, X = O
b, X = S

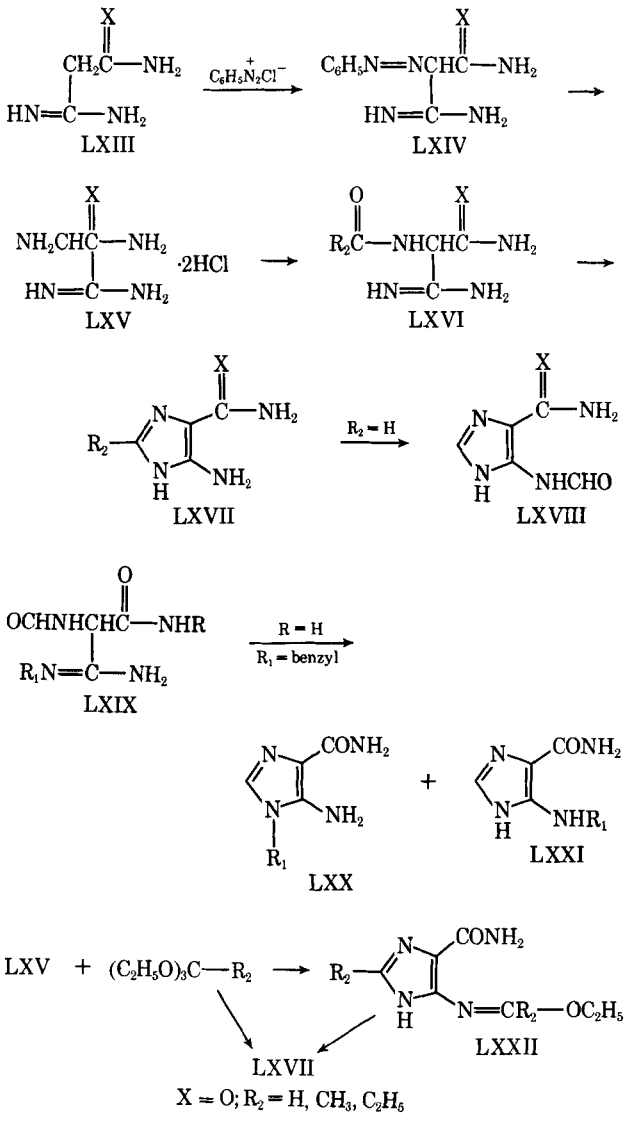
LVIII*b* followed by amidation (40–50% yield) of the ester group with ammonia; (*b*) phosphorylation of the 2′,3′-*O*-isopropylidene derivative of AIC ribonucleoside; (*c*) amidation of the pyridine salt of LVIII*a* with ammonia in the presence of dicyclohexylcarbodiimide; and (*d*) phosphorylation of the active ester LVIII*c* followed by amidation. Method *a* is apparently superior to the other three methods. Subsequently, it was found (96) that the major product of phosphorylation of isopropylidene AIC ribonucleoside with pyrophosphoryl chloride is, after deblocking, AI-CN ribonucleotide (LX*a*) formed by concomitant dehydration of the amide group. As mentioned, alkyl derivatives of AIC were similarly dehydrated to nitriles. The last method (*d*) applied in the reverse order, *i.e.*, amidation followed by phosphorylation, also yielded the *N*-methyl (LX*b*) and *N*,*N*-diethyl (LX*c*) analogs of AICAR (99).

After some of these syntheses of ribonucleotides from ribofuranosides had been performed, 2′,3′-*O*-isopropylideneribosylamine 5′-phosphate (LVII*b*) was synthesized (110, 111) and used for syntheses of imidazole ribonucleotides directly from the linear imidate intermediates. The ribonucleotides of AIC and AI-$COOCH_3$ were obtained from reactions of LVII*b* with LIV (A = —$CONH_2$ or —$COOCH_3$) (110, 111), and 2-methyl-AIC ribonucleotide (LX*d*) was similarly formed in very low yield (111).

A synthesis of 2-hydroxy-AIC (or the tautomeric 5-amino-2-oxoimidazoline-4-carboxamide, LXII*a*) and of ^{13}C-labeled LXII*a* is similar to the thiourea–thiazole method of Cook, Heilbron, and coworkers. The urea (LXI) obtained from aminocyanoacetamide (XLVI, A = —$CONH_2$) cyclized in aqueous base to LXII*a* (112). The analogous 2-mercapto-AIC (LXII*b*), mentioned previously, was formed from XLVI and potassium thiocyanate in acidic solution without identification of intermediates (77).

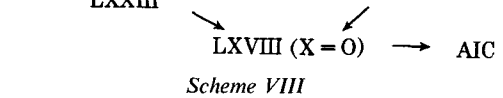
C. From Aminomalonic Acid Derivatives (E. Shaw Method)—Soon after AIC had been identified as a biologically significant compound, E. Shaw and Woolley (113) devised a new synthesis (Scheme VII) that is also applicable to similar aminoimidazoles. The basic intermediate is a three-carbon unit, typified by malonamamidine (LXIII, X = O), obtainable from nitriles such as ethyl cyanoacetate and malononitrile. The 2-amino group is introduced by coupling an aryldiazonium salt at the active methylene group of LXIII and reducing the resulting azo derivative (LXIV). Reduction with zinc in formic acid affords a formamide derivative (LXVI, R_2 = H) that can be thermally cyclized to the imidazole (LXVII). In this way, AIC was synthesized (Scheme VII) from malonamamidine (LXIII, X = O) (113), and the analogous carboxamidine (LXVII; R_2 = H, X = NH) was prepared from malonodiamidine (LXIII, X = NH) (114). Both gave formyl derivatives (LXVIII) on treatment with formic acid and acetic anhydride (114). Similarly, from ethyl chloroformate and aminomalonodiamidine (LXV, X = NH), which was isolated by substituting hydrochloric acid for formic acid in the reduction of the phenylazo derivative (LXIV), 5-amino-2-hydroxyimidazole-4-carboxamidine (LXVII; R_2 = OH, X = NH) was obtained (Scheme VII) (115), and radioactive AIC labeled with ^{14}C at position 5 was synthesized (116) by the E. Shaw route (see Part IIB for labeled AIC). The phenylazo derivative (LXIV) can also be reduced catalytically (117), and the cyclization of LXVI (R_2 = H, X = O) is more conveniently effected in refluxing formic acid (117) or with triethyl orthoformate (118, 119). 5-Formamidoimidazole-4-carboxamide (LXVIII, X = O) obtained by the first modification of the procedure is easily hydrolyzed to AIC.

The adaptability of the method for the introduction of other substituents was demonstrated with syntheses of *N*-benzyl-AIC from *N*-benzyl cyanoacetamide *via* LXIX with R_1 = H and R = benzyl (120); 2-methyl-AIC *via* LXVI (R_2 = CH_3) by substituting acetic acid for formic acid in the reduction (121); and the



Scheme VII

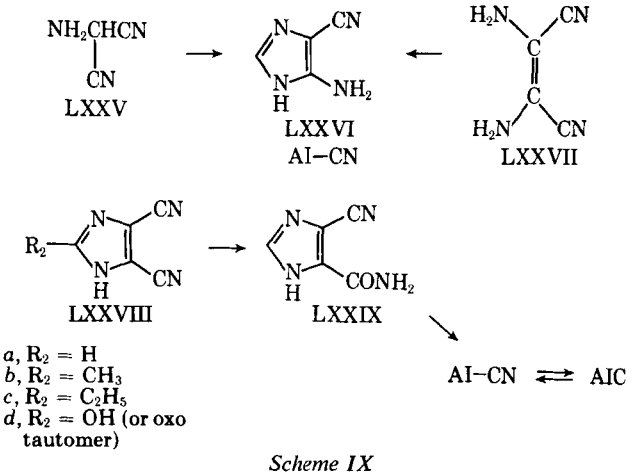
N-benzylamidine (LXIX; R = H, R_1 = benzyl) analog of LXVI. Intermediates of the latter type can cyclize to either a 1-substituted AIC (LXX) or to an AIC substituted on the amino group (LXXI). Either of the two benzyl AIC derivatives could be obtained as the major product by properly choosing the reaction conditions (122). A further modification by Richter *et al.* (118) yields 2-substituted AIC derivatives. The reaction of aminomalonamamidine dihydrochloride (LXV, X = O) (112, 113) with a molar equivalent of an orthoester gives a 2-substituted AIC derivative (LXVII); an excess of the orthoester produces an alkoxy alkyl (or aryl) methyleneamino derivative (LXXII), which can be cyclized to a purine or hydrolyzed to the AIC derivative (LXVII).



Scheme VIII

Another route (Scheme VIII) (123, 124) to AIC that begins with malononitrile and proceeds through oximinomalonodiamidoxime (LXXIII) (125) is essentially a variation of the E. Shaw method. Nitrosation, rather than azo coupling, provides the precursor group at the 2-position, and reaction of hydroxylamine at the nitrile groups then gives the three-carbon unit (LXXIII) similar to LXIV. Reduction of LXXIII with zinc and formic acid gives formyl-AIC (LXVIII). Alternatively, the furazan (1,2,5-oxadiazole) (LXXIV) (125) may be prepared from LXXIII and aqueous base (or from malononitrile without isolating LXXIII) and converted to formyl-AIC by treatment with formic acid and reduction with zinc–formic acid (123). Reduction of other furazans or furazanopyrimidines obtained from LXXIV also gave 5-formamidoimidazole-4-carboxamide (LXVIII) (123).

D. From Hydrogen Cyanide—Interest in prebiotic synthesis of purines stimulated investigations of simple precursor molecules that give rise to adenine (126, 127). Oró and Kimball (128) proved that AIC and 5-aminoimidazole-4-carboxamidine (LXVII; X = NH, R_2 = H) (AI-amidine), as well as formamidine, were among products formed from hydrogen cyanide and aqueous ammonia. They proposed (128, 129) a multistep route to these compounds which included formamidine and 2-aminomalononitrile, a hydrogen cyanide trimer. Ferris and Orgel (130, 131) succeeded in preparing the long-sought (*e.g.*, 75, 132) aminomalononitrile (LXXV) and showed that interaction of this compound with formamidine in ethanol did, indeed, give an imidazole, 5-aminoimidazole-4-carbonitrile (LXXVI, AI-CN), which was isolated in 35% yield[8] (Scheme IX). This imidazole can be hydrolyzed to AIC



Scheme IX

(131, 133, 134) or prepared from it by dehydration with thionyl chloride in pyridine (130, 131) or with phosphorus oxychloride (135).

However, aminomalononitrile also reacted with cyanide in aqueous solution (130, 131) to give the more stable hydrogen cyanide tetramer, diaminomaleonitrile

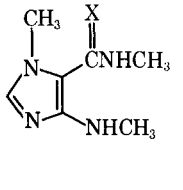
[8] This reaction is the same type as the Cook–Heilbron synthesis of AIC (74) (Part IIB).

(LXXVII) (136–138 and references cited), and both the tetramer and AI-CN were stated to be among the products of Oró's hydrogen cyanide–aqueous ammonia reactions (130, 131). Further studies (133, 139) showed that the yields of AI-CN were 5–50%, depending on reaction conditions, from formamidine and aminomalononitrile in *aqueous* solutions; that reaction of the latter compound with cyanide to the tetramer (LXXVII), however, was faster and proceeded in yields of 60–80%; that the tetramer also produced very low yields of AI-CN, AIC, and AI-amidine by reaction with formamidine; and that formation of the tetramer and its subsequent reaction with formamidine could be responsible for AI-CN, AIC, and the amidine formed in solutions of HCN and aqueous ammonia. In dilute aqueous solutions, the tetramer was converted photochemically *via* its *trans*-isomer (139, 140) to AI-CN in yields as high as 80% (139). (However, AI-CN is subject to photochemical degradation, and the yields quoted were determined by analytical methods.) As a result of these studies, the proposed steps in the pathway from hydrogen cyanide and ammonia to imidazoles and purines were revised to place greater emphasis on the role of the tetramer (LXXVII) (139, 141, 142).

Since the aim of these studies was to demonstrate the formation of purines under postulated primitive earth conditions, much of the work was performed with dilute aqueous solutions; yields were only of secondary importance. Wakamatsu *et al.* (143) and Yamada *et al.* (144, 145) then found, however, that anhydrous solutions of ammonia and hydrogen cyanide (or sodium cyanide and ammonium chloride) yield adenine and imidazole-4,5-dicarbonitrile (LXXVIII*a*) in addition to the tetramer (144) and a pyrimidopyrimidine (146). Yields of LXXVIII*a*, determined analytically, under optimal conditions were only about 20% (144), but the starting materials are simple. In anhydrous ammonia, the formation of LXXVIII*a* from the tetramer was attributed to reversion of the tetramer to aminomalononitrile (146).

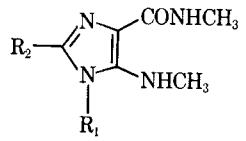
Prior to these studies of either the aqueous or the anhydrous ammonia–hydrogen cyanide systems, the tetramer (LXXVII) had been converted to imidazole-4,5-dicarbonitriles (LXXVIII*a–c*) with triethyl orthoesters (147), to the 2-oxoimidazoline (LXXVII*d*, OH-tautomer) with phosgene (148), and to LXXVIII*b* with ethyl acetimidate (149). The relevance of the formation of dinitriles from hydrogen cyanide or its oligomers to aminoimidazoles is that AI-CN and AIC were prepared from LXXVIII*a* in good yields by the following sequence of steps: partial hydrolysis to the monoamide (LXXIX) (150, 151), a Hofmann hypobromite reaction to AI-CN (150, 152), and alkaline hydrolysis to AIC (150 and references already cited). AI-CN, LXXVIII*a* and its 1-substituted derivatives, and other dicarbonitriles derivable from hydrogen cyanide or its oligomers are potential precursors of other aminoimidazole derivatives (153–155).

E. By Ring Cleavage of Purines—Much of the early work on 5-amino-4-substituted imidazoles was motivated by plans to form a pyrimidine ring from the substituents and, thereby, to produce purines. The reverse process, cleavage of the pyrimidine ring of purines, has



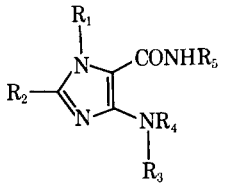
LXXX

a, X = O
b, X = S



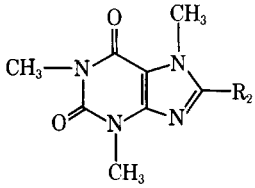
LXXXI

a, $R_1 = R_2 = H$
b, $R_1 = CH_3$; R_2 = H, CH_3, $NH_2CH_2CH_2$—
c, R_1 = H; $R_2 = NO_2$
d, R_1 = H; $R_2 = NH_2$, Cl, Br

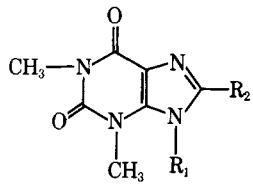


LXXXII

a, R_3 = COOH; $R_1 = R_4 = CH_3$;
R_2 = H or COOH; $R_5 = CH_3$ or C_2H_5
b, $R_2 = R_3$ = H; $R_4 = R_5 = CH_3$;
R_1 = OH or 2,3-dihydroxypropyl
c, $R_1 = R_4 = R_5 = CH_3$; R_2 = H; $R_3 = CH_3$ or C_2H_5
d, $R_1 = R_5 = C_6H_5CH_2$—; $R_2 = R_3 = R_4$ = H
e, R_1 = glucopyranosyl; $R_2 = R_3 = R_4$ = H;
R_5 = H or glucopyranosyl
f, $R_1 = R_2 = R_3 = R_5$ = H; $R_4 = CH_3$
g, $R_1 = R_3 = R_5 = C_6H_5CH_2$—; R_2 = H; R_4 = CHO



LXXXIII

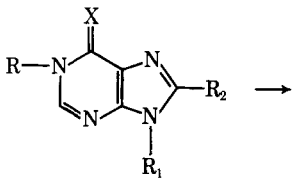


LXXXIV

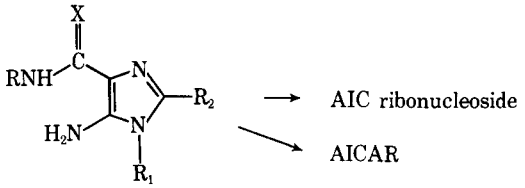
furnished 5-aminoimidazole-4-carboxylic acid derivatives and has become increasingly important as a route to these compounds. Imidazoles obtained by this method have sometimes been formed inadvertently during studies of purines, sometimes as a means of establishing the structure of certain purines, and sometimes by design. The formation of imidazoles from purines is part of the larger area of pyrimidine-ring cleavage and rearrangement reactions of fused pyrimidine heterocycles such as pteridines, quinazolines, 8-azapurines, and thiadiazolopyrimidines. The products of cleavage of these ring systems are usually similar in structure to the aminoimidazolecarboxylic acid derivatives under consideration here. Correlation of findings from these areas is obviously beyond the scope of this review. The following discussion simply narrates the historical highlights and attempts to record most of the known examples of formation of derivatives of the aminoimidazolecarboxylic acid-type from purines.[9]

The action of barium, sodium, or potassium hydroxide solutions on the methylated purines (caffeine, theophylline, and theobromine) provided the first

[9] Demonstrations of the formation of imidazoles by ring opening of purines have not always included isolation, nor have the yields always been of practical significance.



LXXXV



LXXXVI

LXXXVI*a–k*

a, R = $C_6H_5CH_2$—; *b*, R = *p*-$CH_3C_6H_4SO_2$—; *c*, R = NH_2—; *d*, R = CH_3OCH_2—
for LXXXVI*a–d*: X = O, R_2 = H, R_1 = β-D-ribofuranosyl
e, R = CH_3OCH_2—; *f*, R = $HOOCCH_2CH_2$—
for LXXXVI*e–f*: X = O, R_2 = H, R_1 = β-D-ribofuranosyl 5′-(dihydrogen phosphate)
g, X = O, R = R_1 = $C_6H_5CH_2$—, R_2 = H
h, X = O, R = CH_3, R_2 = H, R_1 = 2′,3′-*O*-ethoxymethylene-β-D-ribofuranosyl
i, X = O, R = glucopyranosyl, R_1 = H or glucopyranosyl, R_2 = H
j, X = S, R = benzyl, R_1 = β-D-ribofuranosyl, R_2 = H
k, formyl derivative of LXXXVI with X = S, R = R_2 = H, R_1 = β-D-ribofuranosyl 5′-(dihydrogen phosphate)

Scheme X

examples of this method of synthesis. Caffeidine [1, *N* - dimethyl - 4 - (methylamino)imidazole - 5 - carboxamide] (LXXX*a*) was obtained from caffeine (LXXXIII, R_2 = H) (156–158 and earlier references cited in these publications) and from 8-carbamoylcaffeine (LXXXIII, R_2 = $CONH_2$) (159). The initial products resulting from alkaline degradation of caffeine, 8-carbamoylcaffeine, and 1-ethyltheobromine (160) were believed to be the carbamic acids (LXXXII*a*) (158, 161). Later, thiocaffeine and theophylline (LXXXIV, R_1 = R_2 = H) were degraded by alkaline conditions to thiocaffeidine (LXXX*b*) (162) and to theophyllidine (LXXXI*a*) (163), respectively. More recently, some isocaffeines (LXXXIV, R_1 = CH_3) were similarly cleaved to 1,*N*-dimethyl-5-(methylamino)imidazole-4-carboxamides (LXXXI*b*) (164), and some 7- and 8-substituted theophyllines produced (165) 1-substituted *N*-methyl-4 - (methylamino)imidazole - 5 - carboxamides (LXXXII*b*) and a 2-substituted theophyllidine (LXXXI*c*), respectively (165). Certain 2-substituted theophyllidines (LXXXI*d*) (165) were also obtained from 2-nitrotheophyllidine. Methylation (55, 158, 166) and ethylation (156) of caffeidine gave dialkylamino derivatives (LXXXII*c*), and the methylamino group of LXXX*a* was converted to benzoyl, urea, and nitroso derivatives (158). Caffeidine has also been transformed to its methyl ester analog *via* the formyl derivative of its methylamino group and the nitroso derivative of its amide group (167).

Purines not substituted on the ring-nitrogen atoms are ionizable in alkaline media. The anion formed is then stabilized to attack by nucleophiles. In the methylated derivatives just discussed, the two carbonyl groups in the pyrimidine ring, as well as the *N*-alkyl substituents, contribute to labilization of the purine system. During the year that the degradation of theophylline was recorded, the initial reports (120, 168) of the extensive investigations of purine *N*-oxides by Brown (169) and coworkers and of the purposeful labilization of the purine ring by E. Shaw appeared. These series of publications served to emphasize the potential utility of purines substituted on ring-nitrogen atoms for imidazole formation. Prior evidence, to be presented, by Baker and Joseph (170) of the increased susceptibility of nitrogen-substituted purines to ring opening may have gone unrecognized because of the specialized (cyclonucleoside) structure.

E. Shaw synthesized (Scheme X) AIC ribonucleoside (*cf.*, Parts IIA and IIB) from inosine (LXXXV; R = R_2 = H, R_1 = β-D-ribofuranosyl) or its triacetate by first labilizing the pyrimidine ring by placing a substituent (R) at position 1. The first method (120) consisted of alkaline ring cleavage of 1-benzylinosine and removal of the benzyl group of *N*-benzyl-AIC ribonucleoside (LXXXVI*a*) with sodium and liquid ammonia. The overall yield suffered because of the difficulty of the debenzylation step. The second method (171) consisted of tosylation of inosine triacetate, alkaline ring opening of 1-(*p*-toluenesulfonyl)inosine triacetate, hydrazinolysis of the resulting *N*-(*p*-toluenesulfonyl)-AIC ribonucleoside (LXXXVI*b*), and reductive fission of the acid hydrazide (LXXXVI*c*) to the amide (AIC ribonucleoside). A third method (172) gave AIC ribonucleoside from inosine triacetate and AICAR from the di(*p*-nitrophenyl) ester of the 2′,3′-*O*-isopropylidene derivative of inosinic acid [inosine 5′-(dihydrogen phosphate) or IMP, Part III] as follows: the methoxymethyl group was introduced at position 1, the pyrimidine ring was again opened under alkaline conditions, the labilizing methoxymethyl group was simultaneously removed hydrolytically from the carboxamide group of LXXXVI*d* and of the dinitrophenyl isopropylidene derivative of LXXXVI*e*, and the protecting groups were removed. This method constituted the first chemical synthesis of AICAR. The β-alanine analog (LXXXVI*f*) of succino-AIC ribonucleotide was similarly prepared by employing the principle of ring labilization (173). Inosinic acid was alkylated with propiolactone at position 1 by careful control of the pH; alkaline ring opening of 1-(2-carboxyethyl)inosinic acid then gave LXXXVI*f*.

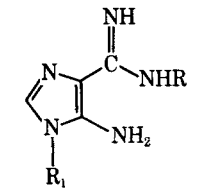
Other aminoimidazolecarboxamides have been formed by alkaline cleavage of substituted hypoxanthines—mostly 1-substituted derivatives—as follows: 1,*N*-dibenzyl-AIC (LXXXVI*g*) (174) from 1,9-dibenzylhypoxanthine (LXXXV; X = O, R = R_1 = benzyl, R_2 = H); 4-amino-1,*N*-dibenzylimidazole-5-carboxamide (LXXXII*d*) from 1,7-dibenzylhypoxanthine (175); the ethoxymethylene derivative of *N*-methyl-AIC ribonucleoside (LXXXVI*h*) (176) from the corresponding 1-methylinosine; several glucopyranosyl-AIC derivatives (LXXXVI*i*, LXXXII*e*) (175) from various glucopyranosylhypoxanthines; and 5-(methylamino)imidazole - 4 - carboxamide (LXXXII*f*) (177) from 3-methylhypoxanthine, 3-methyladenine, or 6-(dimethylamino)-3-methylpurine. The last two purines may have been converted to 3-methylhypoxan-

thine prior to ring opening. Cleavage of hypoxanthinium salts is outlined near the end of this section (IIE).

In addition, ring opening of certain purine-6(1*H*)-thiones (LXXXV, X = S) (6-mercaptopurines) has resulted in imidazole formation. 1-Benzyl-9-β-D-ribofuranosylpurine-6(1*H*)-thione, the sulfur analog of 1-benzylinosine, was cleaved to AI-CSNHCH$_2$C$_6$H$_5$ ribonucleoside (LXXXVI*j*) (178); 1-methylpurine-6-(1*H*)-thione (LXXXV; R = CH$_3$, R$_1$ = R$_2$ = H) gave AIC on treatment with aqueous ammonia (179); and 5-formamido-1-β-D-ribofuranosylimidazole-4-thiocarboxamide 5′-(dihydrogen phosphate) (formyl-thio-AIC ribonucleotide, LXXXVI*k*) was reported (180) to be formed from 6-mercaptopurine ribonucleotide pyridinium salt.

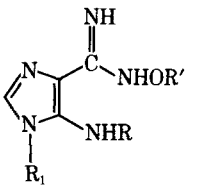
As mentioned, initial investigations of purine *N*-oxides and 1-substituted hypoxanthines were reported almost simultaneously. Although the isolation of 4-guanidinoimidazole after vigorous acidic hydrolysis of guanine (181) and of AIC after treatment of hypoxanthine with zinc and sulfuric acid (182) has been reported, acidic ring cleavage of the common purines unsubstituted on ring-nitrogen atoms usually results in extensive degradation (183, 184 and references cited). However, under milder, but still strenuous, conditions, it was possible to isolate 5-aminoimidazole-4-carboxamidine (LXXXVII*a*) in 10% yield by acidic hydrolysis of adenine (183). In contrast, the oxide of adenine prepared by Stevens and Brown (168) was cleaved under much less vigorous conditions to 5-aminoimidazole-4-carboxamidoxime (LXXXVIII*a*) in yields in excess of 75% (168, 185). This reaction established the structure of the oxide as adenine 1-*N*-oxide (LXXXIX*a*); catalytic reduction of the carboxamidoxime produced the amidine (LXXXVII*a*) (*cf.*, Parts IIC and IID), and hydrolysis gave AIC. Formation of the amidoxime by acidic ring opening of 2-methyladenine 1-*N*-oxide (LXXXIX*b*) (186) and of the 2′-, 3′-, and 5′-phosphates of adenosine 1-*N*-oxide (LXXXIX*c*) (with the expected concomitant loss of the ribofuranosyl group) was also demonstrated (187). Ring cleavage of adenosine 1-*N*-oxide and its 2′-, 3′-, and 5′-phosphates by aqueous sodium hydroxide produced, respectively, the ribonucleoside (LXXXVIII*b*) and the 2′-, 3′-, and 5′-phosphates of LXXXVIII*b* (187).

Cleavage of 1-*N*-oxides by acetic anhydride apparently proceeds through the initial acetylation of the *N*-oxide group (186). Adenine 1-*N*-oxide and 2,6-diaminopurine 1-*N*-oxide were cleaved by refluxing acetic anhydride, and an oxadiazole ring was formed by the reagent; the product was a 3-(5-acetamidoimidazol-4-yl)oxadiazole (XC*a*), which was obtained also from LXXXVIII*a* and acetic anhydride. The 2-acetoxy derivative (XC*b*) was likewise formed from 8-hydroxyadenine 1-*N*-oxide. Under milder conditions the product obtained from adenine 1-*N*-oxide was the formamide (XC*c*), and XC*a* was evidently formed from it by an acyl-exchange reaction. Both XC*a* and XC*b* were hydrolyzed under acidic conditions to the 5-aminoimidazole derivatives (XC*d* and XC*e*) (186). In other studies of purine oxides, 4-acetyl-5-aminoimidazole was obtained by acid hydrolysis of 6-methylpurine



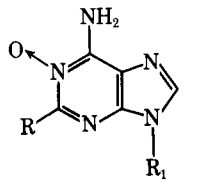
LXXXVII

a, R$_1$ = R = H
b, R = H, R$_1$ = β-D-ribofuranosyl
c, R = CH$_3$, R$_1$ = H
d, R = benzyl, R$_1$ = H
e, R = —CH$_2$COOH, R$_1$ = H



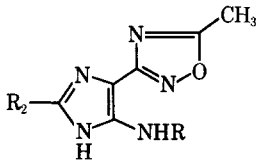
LXXXVIII

a, R$_1$ = R = R′ = H
b, R = R′ = H, R$_1$ = β-D-ribofuranosyl
c, R = CHO, R$_1$ = R′ = C$_2$H$_5$
d, R = H, R′ = benzyl, R$_1$ = β-D-ribofuranosyl



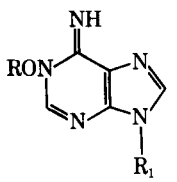
LXXXIX

a, R = R$_1$ = H
b, R = CH$_3$, R$_1$ = H
c, R = H, R$_1$ = β-D-ribofuranosyl



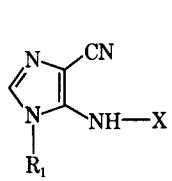
XC

a, R = —COCH$_3$, R$_2$ = H
b, R = —COCH$_3$, R$_2$ = —OCOCH$_3$
c, R = —CHO, R$_2$ = H
d, R = R$_2$ = H
e, R = H, R$_2$ = OH



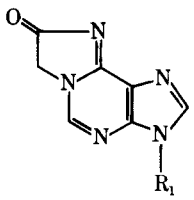
XCI

a, R = R$_1$ = C$_2$H$_5$
b, R = benzyl, R$_1$ = β-D-ribofuranosyl



XCII

a, X = —CONH$_2$, R$_1$ = β-D-ribofuranosyl
b, R$_1$ = X = CH$_3$

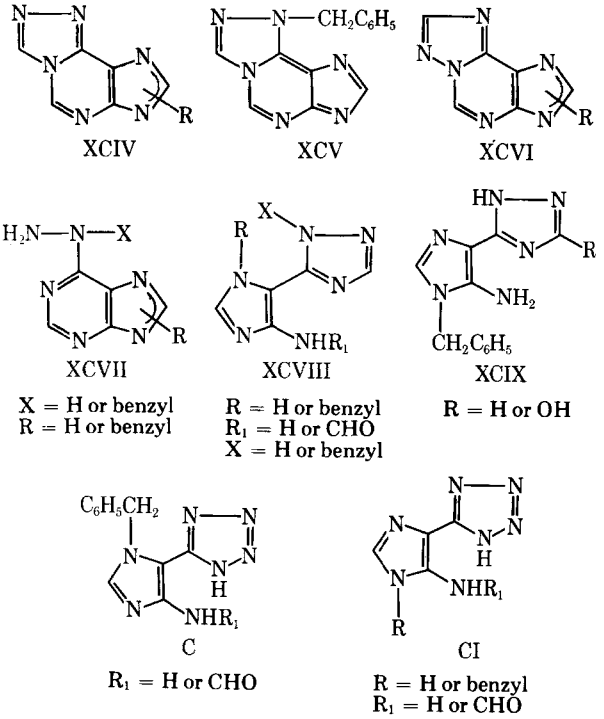


XCIII

a, R$_1$ = H
b, R$_1$ = CH$_3$

1-*N*-oxide (188); the amidoxime (LXXXVIII*a*) and AIC were minor components of the acid hydrolysate of 1-hydroxyisoguanine (189); 5-chloro-AIC was one of several products of the action of hydrochloric acid on hypoxanthine 3-*N*-oxide (190); and 1-β-D-ribofuranosyl - 5 - ureidoimidazole - 4 - carbonitrile (XCII*a*) was one of the products isolated, in low yield, after photolysis of adenine 1-*N*-oxide (191).

Syntheses of imidazoles from purine *N*-oxides and from purines having an alkyl group on a ring-nitrogen atom converged when the *N*-oxide group was alkylated to 1-alkoxy derivatives (XCI) (192, 193). As 1-substituted adenine derivatives, such compounds are highly susceptible to the well-known rearrangement, generally believed to involve ring opening and reclosure, that results in placing the 1-substituent on the 6-amino group. This reaction was, indeed, observed to occur with 1-alkoxyadenines (192). However, the substituted imidazolecarboxamidoxime (LXXXVIII*c*) could be isolated in high yield by cleavage of XC*a* in neutral solution at low temperature (192). Likewise, 1-benzyloxyadenosine (XCI*b*) was cleaved in methanolic ammonia to a substituted imidazolecarboxamidoxime (LXXXVIII*d*) (193). Reductive removal of the benzyloxy group gave the amidine analog (LXXXVII*b*) of AIC ribonucleoside, and diazotization of LXXXVII*b*

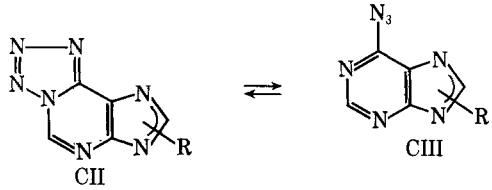


provided a convenient synthesis of 2-azaadenosine. [2-Azaadenine 1-*N*-oxide and 2-azaadenosine 1-*N*-oxide had also been prepared (194) by diazotizing the amidoximes LXXXVIII*a*, LXXXVIII*b*.] At this point it may be recalled that ring fission of adenine 1-*N*-oxide by acetic anhydride, discussed previously, evidently proceeds *via* a 1-acyloxy derivative (186) analogous to the 1-alkoxyadenines.

Although 1-alkyladenines readily rearrange in basic media to 6-(alkylamino)purines, they may undergo cleavage to carboxamidines in acidic solutions. Brookes and Lawley showed that 5-amino-*N*-methylimidazole-4-carboxamidine (LXXXVII*c*) (195) and the *N*-benzyl derivative (LXXXVII*d*) (196) are formed by acidic cleavage of 1-methyladenine and 1-benzyladenine, respectively. Acidic degradation of 1-methyladenosine and 1-benzyladenosine removed the ribofuranosyl group and gave the same carboxamidines (LXXXVII*c*, LXXXVII*d*) (196).

Cleavage of the pyrimidine ring of a cyclic adenine derivative (XCIII*a*), formed from N^6-glycyladenine, also occurs under acidic conditions (197). The ring-cleavage products isolated—and probably formed *via* 1-carboxymethyladenine (197, 198)—were the *N*-substituted amidine LXXXVII*e* and AIC. Acidic hydrolysis of the related cyclic intermediate (XCIII*b*) from N^6-glycyl-9-methyladenine produced, among other products, a small amount of 1-methyl-AIC (XVI*b*) (199). An earlier acidic cleavage of *N*-(purin-6-yl)aspartic acid, the aglycone of adenylosuccinic acid (Part III), resulted in the formation of AIC and was postulated to involve a cyclic intermediate (200).

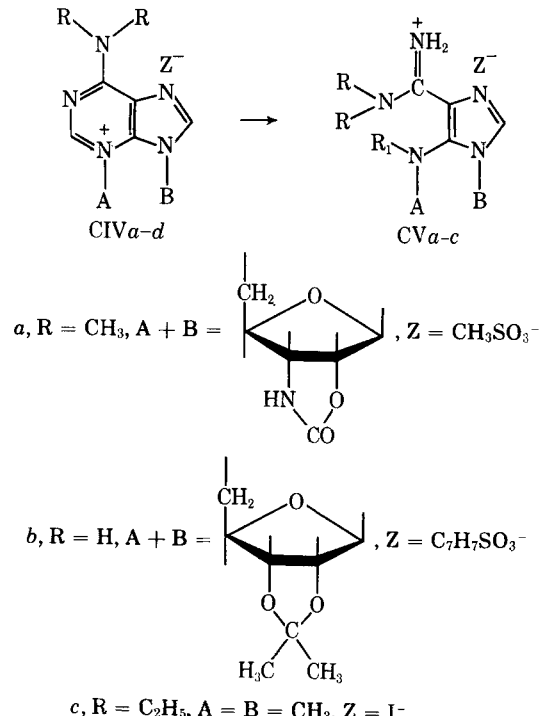
5-Aminoimidazol-4-yl heterocycles that are structurally similar to the imidazolyloxadiazoles (XC) already described have been obtained from purines to which a heterocyclic ring is fused at positions 1 and 6 (tricyclic heterocycles) (XCIV–XCVI, CII) or from



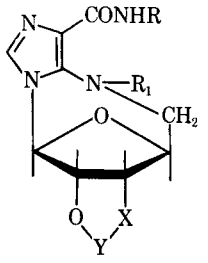
Scheme XI

purines that can form such tricyclic compounds (XCVII, CIII). Temple *et al.* (201–203), in a series of publications, described the synthesis of 4-aminoimidazol-5-yl-*s*-triazoles (XCVIII), 5-aminoimidazol-4-yl-*s*-triazoles (XCIX), and aminoimidazolyltetrazoles (C and CI) by acid- or base-catalyzed ring cleavage. The presence of a benzyl group on one of the imidazole ring-nitrogen atoms may aid, but is not necessary for, opening of the pyrimidine ring. The aminoimidazoles represented by XCVIII and XCIX (201, 203) were obtained from appropriately substituted *s*-triazolo[3,4-*i*]purines (XCIV, XCV) or from the isomeric *s*-triazolo[5,1-*i*]purines (XCVI). Furthermore, the 6-hydrazinopurines from which the *s*-triazolo[3,4-*i*]purines were prepared also underwent ring cleavage to derivatives of type XCVIII or XCIX. When formic acid, diethoxymethyl acetate, or phosgene was the reactant, cyclization to an *s*-triazolo[3,4-*i*]purine may have preceded ring opening. But, when 7-benzyl-6-hydrazinopurine was converted to XCVIII (R = benzyl, R_1 = X = H) with mineral acid, prior formation of a tricyclic system was not possible. Similarly, the 5-[amino (or formamido)imidazolyl]tetrazoles (C and CI) (202) were isolated in good yields by acidic or basic cleavage of the tetrazolopurines (CII), which are in tautomeric equilibrium with the 6-azidopurines (CIII) (Scheme XI).

Quaternized purines, positively charged in the purine ring, may be expected to be even more susceptible

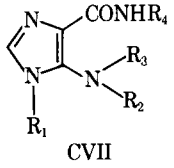


Scheme XII



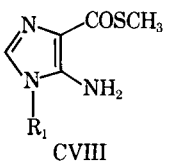
CVI

a, R = R_1 = H, X = NH, Y = CO
b, R = $C_6H_5CH_2$—, R_1 = CHO, X = O, Y = $C(CH_3)_2$
c, R = R_1 = H, X = O, Y = $C(CH_3)_2$



CVII

a, R_1 = H, R_2 = R_4 = benzyl, R_3 = CHO
b, R_1 = R_3 = H, R_2 = R_4 = benzyl
c, R_1 = R_2 = benzyl, R_3 = R_4 = H
d, R_1 = R_2 = benzyl, R_3 = CHO, R_4 = H
e, R_1 = R_2 = CH_3, R_3 = R_4 = H



CVIII

R_1 = H or CH_3

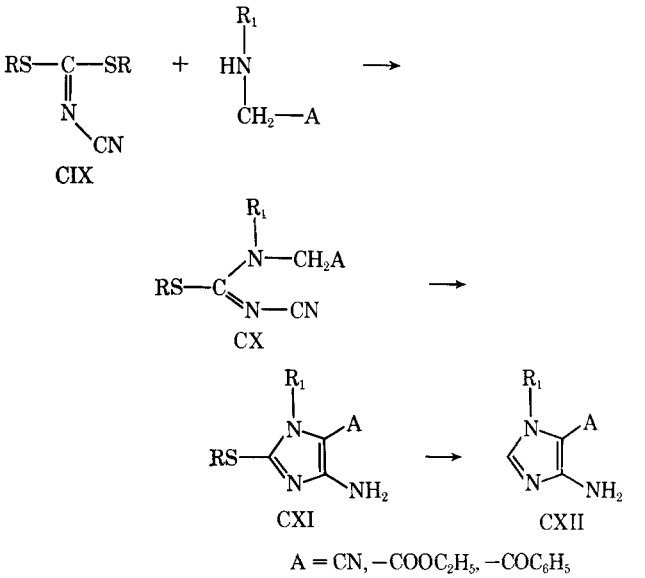
to nucleophilic opening of either the pyrimidine or the imidazole ring than are uncharged species. The studies of puromycin provided an example of pyrimidine-ring opening of a quaternary derivative. As mentioned, Baker and Joseph (170) found that the cyclonucleoside (CIV*a*) (Scheme XII) was easily cleaved by dilute base to CV*a* (R_1 = CHO). Further, mild basic treatment hydrolyzed the amidine and the formamide groups, yielding an AIC ribonucleoside analog (CVI*a*). Michelson (204) mentioned the formation of a similar amidine (CV*b*, R_1 = H) from the cyclonucleoside of 2′,3′-isopropylideneadenosine (CIV*b*); Montgomery *et al.* (174) observed the formation of an *N*-benzyl-AIC ribonucleoside analog (CVI*b*) by facile ring cleavage of the isopropylidene-protected cyclonucleoside of 1-benzylinosine, a hypoxanthinium salt. Kusashio and Yoshikawa (205) isolated small amounts of CVI*c* and carbonitrile analogs of CVI*c* (R_1 = CHO and H) during studies of the phosphorylation of 2′,3′-*O*-isopropylideneinosine with phosphorus oxychloride containing a small quantity of water or with pyrophosphoryl chloride. These imidazoles were presumably formed from the cyclonucleoside of inosine. Montgomery and his coworkers (174, 206) also found that derivatives of aminoimidazolecarboxamides were formed from quaternary salts under mild conditions as follows: CVII*a* and LXXXII*g* from 1,3-dibenzyl- and 1,3,7-tribenzylhypoxanthinium bromides, respectively; and CVII*c* from 3,9-dibenzyl-6-(dimethylamino)-purinium bromide (CIV*d*). Compounds CVII*b* and CVII*d* were prepared from CVII*a* and CVII*c*, respectively. Cleavage of the dimethylaminopurinium derivative (CIV*d*) might have proceeded by initial hydrolysis at position 6 to 3,9-dibenzylhypoxanthine; however, its similarity to cyclonucleosides (CIV*a*, CIV*b*) (Scheme XII), which first yielded the amidines (CV*a*, CV*b*), is evident, and ring opening could have preceded hydrolytic removal of the dimethylamino group. Furthermore, Marsico and Goldman (207) found that alkaline degradation of 6-(diethylamino)-3,9-dimethylpurinium iodide (CIV*c*) produced the amidine CV*c* (R_1 = H); further degradation yielded the nitrile (XCII*b*) and finally 1-methyl-5-(methylamino)imidazole-4-carboxamide (CVII*e*), which was independently synthesized by lithium aluminum hydride reduction of formyl 1-methyl-AIC. Although the foregoing studies were not designed to determine relative facility of ring opening, it appears that, in general, the quaternized derivatives yielded imidazoles under milder conditions than uncharged, ring-nitrogen-substituted derivatives.

Other reported examples of imidazole formation by ring opening include the interception of 1-methyl-4-ureidoimidazole-5-carbonitrile during the alkaline hydrolysis of 2-chloro-7-methyladenine (208); isolation of 2-(methylsulfonyl)AIC ribonucleoside (or a derivative) after treatment of 8-(methylsulfonyl)guanosine with *tert*-butoxide ion in dimethyl sulfoxide (209); and the nonenzymatic formation of AI ribonucleoside from adenosine, a ketopentose, cupric ions, and pyrophosphate (210).

Finally, an interesting acid-catalyzed ring opening of 6-(methylthio)purines was recently reported by Albert (211) and afforded the first imidazole thiol esters (CVIII), one of which was converted by ammonia to AIC.

At the beginning of this section, it was stated that ring-opening and rearrangement reactions of purines are related, but the transitory formation of imidazole derivatives during rearrangement reactions has not been treated here. During such reactions, facile reclosure of a pyrimidine ring to a more stable purine may preclude ring opening as a source of imidazoles. Likewise, the fact that the imidazole, rather than the pyrimidine, ring of purines is sometimes cleaved has not been discussed, but this fact is relevant to ring opening as a method of imidazole synthesis.

F. Miscellaneous Methods—1-Substituted-4-aminoimidazoles, which belong to the structural type furnished by the Sarasin–Wegmann method, are formed by a route (Scheme XIII) that begins with *N*-cyanoiminodithiocarbonic acid esters (CIX) (212). The imidazole



A = CN, $-COOC_2H_5$, $-COC_6H_5$

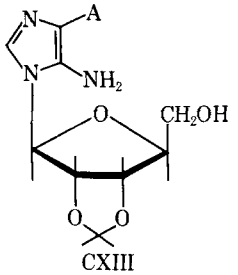
Scheme XIII

ring is formed (CXI) by base-catalyzed cyclization of the intermediate isothioureas (CX). The thioether group at position 2 may be removed with Raney nickel (CXII).

Approaches to 1-substituted derivatives of AI and AI-COOH (VIII) similar to the steps in the biosynthesis of inosinic acid have been studied by Shaw and coworkers (*cf.*, Part III). The 1-cyclohexyl and ribonucleotide derivatives of AI, the latter in admixture with formylglycinamidine ribonucleotide (FGAM), were obtained by decarboxylation of the amino acids under careful control to minimize ring opening (213). The influence of metal ions, pH, and other factors on the decarboxylation has been studied (214). Conversely, carboxylation of these two AI derivatives with bicarbonate gave the amino acids (215), and cyclization of the *N*-cyclohexyl derivatives of glycinamidine (with ethyl formimidate) and formylglycinamidine gave small amounts of 1-cyclohexyl-AI (213, 215).

Interconversions of carboxamide and carbonitrile and of thiocarboxamide and carbonitrile groups are potential sources of derivatives of all three types. Dehydration of AIC to AI-CN (LXXVI) and hydrolysis of the latter compound to AIC were described in Part IID; hydrolysis of nitrile products of the Sarasin–Wegmann method to amides was depicted in Part IIA; and conversion of some 1-alkyl-5-aminoimidazole-4-carboxamides and -thiocarboxamides to the corresponding nitriles was mentioned in Part IIB. The thiocarboxamide analog (AI-$CSNH_2$) (XXXIX, R_1 = R_2 = H) of AIC was first prepared by thiation of AIC with phosphorus pentasulfide (47, 216) and by the linear imidate method of Shaw and Butler (93) (Part IIB). Recently, thiation of AIC has been improved (217, 218), but conversion of the nitrile to AI-$CSNH_2$ with a methanolic alkaline solution of hydrogen sulfide proved to be superior (217, 219). Treatment of AI-$CSNH_2$ with formic acid and sodium formate yielded the 5-formamidoimidazole-4-thiocarboxamide (LXVIII, X = S).

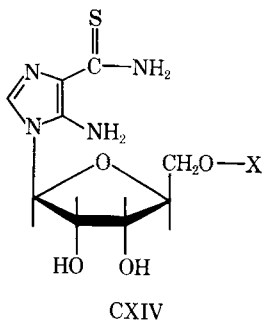
These reactions have been extended to include the syntheses of the thioamide analogs of AIC ribonucleoside and ribonucleotide (217, 219). 5-Amino-1-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carbonitrile (CXIII*a*), obtained by dehydrating AIC



CXIII

a, A = CN

b, A = $-CSNH_2$



CXIV

a, X = H

b, X = PO_3H_2

ribonucleoside with *p*-toluenesulfonylchloride in pyridine (220), was transformed by the hydrogen sulfide procedure to isopropylidene AI-$CSNH_2$ ribonucleoside (CXIII*b*). The usual mildly acidic removal of the isopropylidene group furnished the thioamide analog of AIC ribonucleoside (CXIV*a*), which reverted to the nitrile upon treatment with methyl iodide and base. Phosphorylation of CXIII*b* by the phosphorus oxychloride–trimethyl phosphate method, removal of the isopropylidene group, and application of the usual ion-exchange purification procedures yielded the thiocarboxamide analog (CXIV*b*) of AICAR.

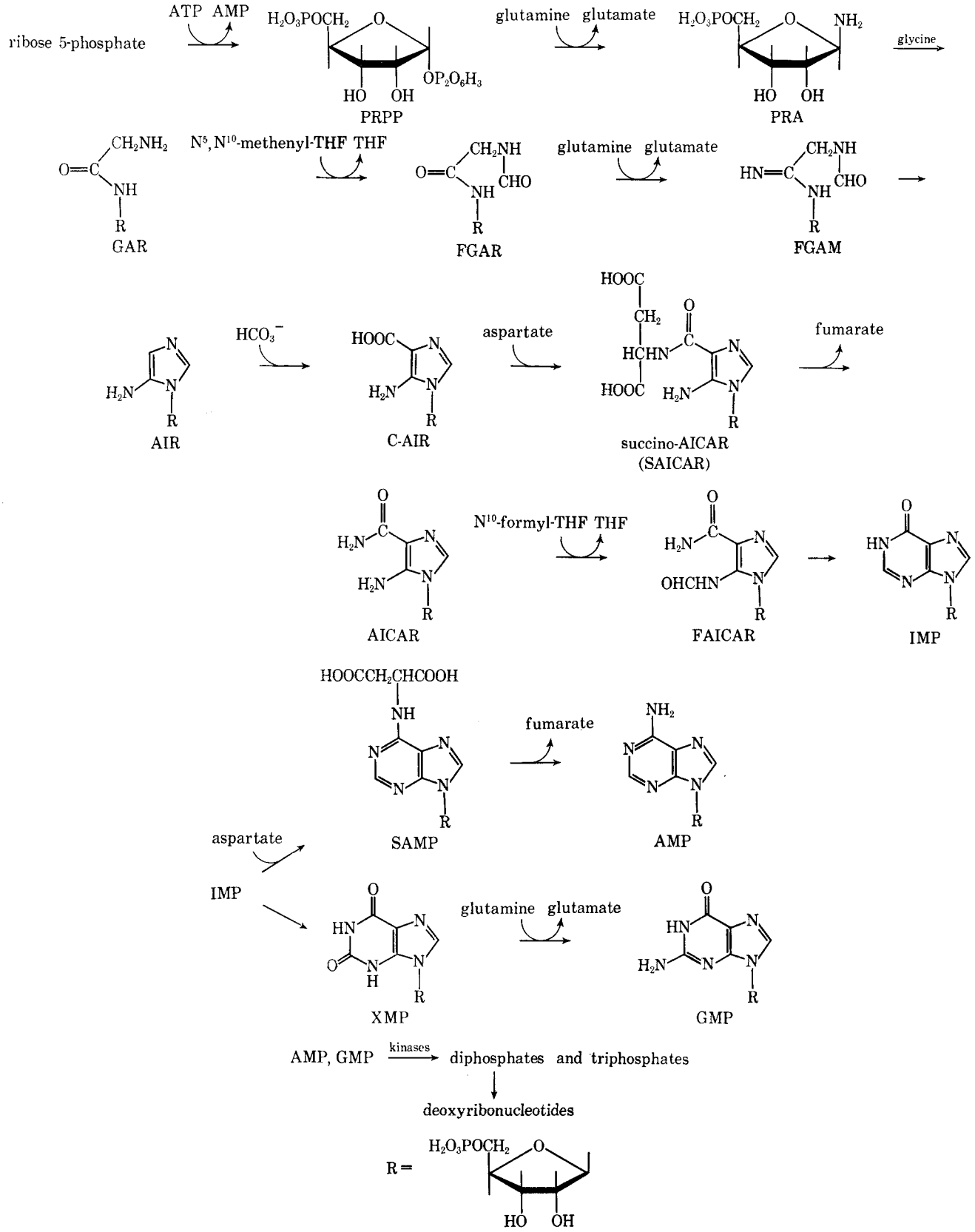
Other interconversions and further transformations of functional groups, many of which have been mentioned in the preceding sections, provide access to desired derivatives. Some examples involving the amino group are its conversion by orthoesters to alkoxyanils (50, 93, 94, 221, 222) exemplified by LXXII; the preparation of 5-formamido derivatives (93–95, 114, 117, 217), which are of potential interest because a derivative of this type is the immediate biosynthetic precursor of inosinic acid; and the formation of ureido, thioureido, and guanidino derivatives (167, 212, 223, 224, and references cited in Parts IIA and IIB).

Investigations of the biosynthesis of aminoimidazole ribonucleotides by bacteria were an integral part of fundamental studies of purine biosynthesis. Microbiological synthesis has become an important source of AIC ribonucleoside (225–232), AIC ribonucleotide (227, 233–235), and succino-AIC ribonucleotide or the analogous ribonucleoside (227, 236) as a result of the use of bacterial species, mutant strains, or yeasts that accumulate these compounds. Microbiological synthesis has provided these compounds in quantities sufficient for the employment of AIC ribonucleoside as a starting material for the synthesis of purine ribonucleosides (224, 237) and for consideration of AICAR and succino-AICAR as food-seasoning agents (Part III).

III. BIOCHEMISTRY AND BIOLOGICAL ACTIVITY OF 5-AMINOIMIDAZOLES[4]

AIC and its ribofuranosyl derivatives, alone or in combination with other metabolites or antimetabolites, have been the subjects of many biochemical, microbiological, pharmacological, and other biological investigations. These studies are too numerous to be recounted here; rather, the purpose of items 1–7 of Part III is to point out several known biological roles of aminoimidazoles in order to emphasize their importance in metabolic processes.

1. The prominent role of ribonucleotides of aminoimidazoles in the biosynthesis of purines is now well known. The postulated (3) association of AIC, or a derivative, with purine biosynthesis came during a period when studies designed to identify the ultimate sources (*e.g.*, glycine and formate) of the individual atoms of the purine ring and when investigations probing the role of folic acid in one-carbon metabolism were being intensively pursued. The identification of AICAR, rather than AIC itself, as a true precursor of purines resulted from the discoveries, along with other findings, of the precursor role of inosinic acid (IMP) for hypoxanthine (238) and of the accumulation of AIC ribonucleoside (XVI*a*) (239–243) and AICAR (240, 244) during sulfonamide bacteriostasis. The convergence and integration of these studies of simple



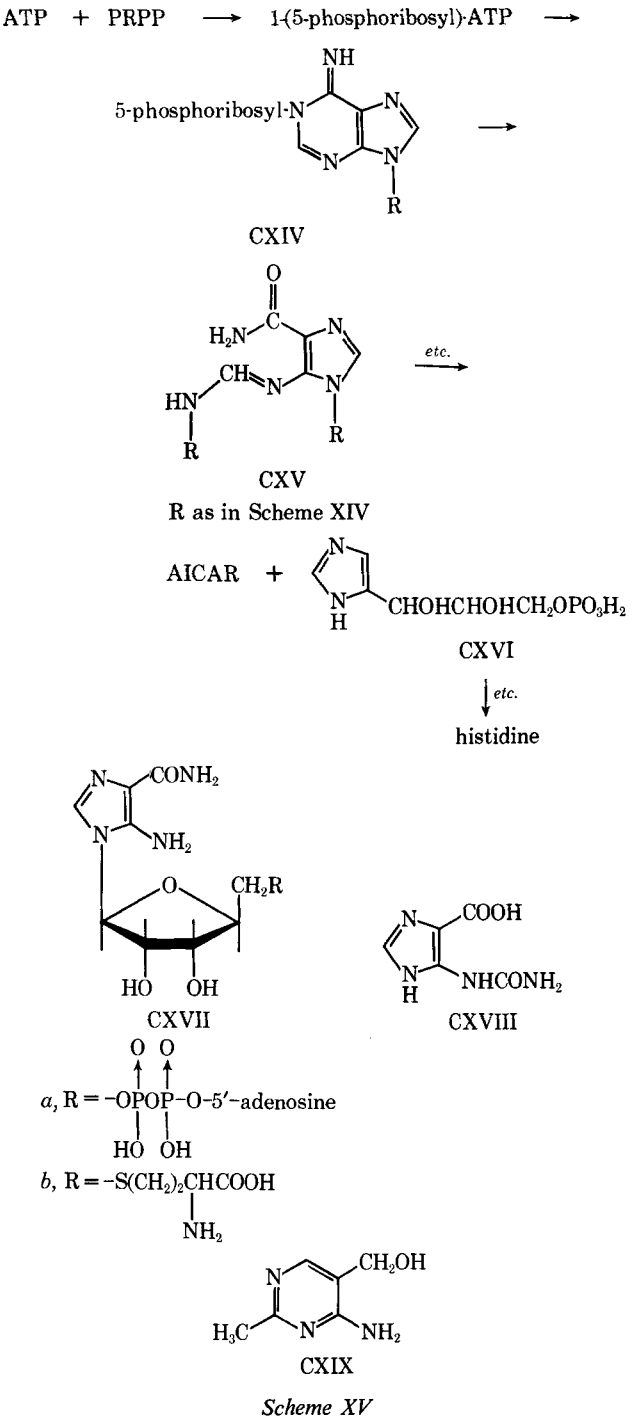
Scheme XIV—Biosynthesis of purine ribonucleotides

purine precursors, aminoimidazole derivatives, and folic acid coenzymes led to the delineation of the complete sequence of steps in the synthesis *de novo* of inosinic acid (Scheme XIV). The chronology of these events and the detailed biochemical processes of the individual steps have been reviewed (245-248) and will not be reexamined here. More recent reviews (249–252) have dealt with the effects of inhibitors of some of the

reactions of Scheme XIV,[5, 10, 11] the principal emphasis being, of necessity, on the actions of purines and purine analogs on the initial and the late steps.

The prominent role of aminoimidazole ribonucleotides in purine synthesis *de novo* is evident from Scheme XIV. Only certain salient aspects (arbitrarily selected) of the synthesis *de novo* of inosinic acid will be mentioned. (*a*) Glutamine plays a donor role in two steps (PRPP → PRA and FGAR → FGAM), and tetrahydrofolate derivatives insert a one-carbon unit in two of the steps (GAR → FGAR and AICAR → FAICAR). (Glutamine is not the only nitrogen source for PRPP → PRA. Interference with N^{10}-formyltetrahydrofolate metabolism was responsible for blocking the formylation of AIC ribonucleotide and its resulting accumulation during sulfonamide bacteriostasis.) (*b*) The formation of PRA is subject to feedback inhibition by purine derivatives and purine analogs. (*c*) Salvage pathways for the utilization of preformed purines, AIC, AIC ribonucleoside, and purine ribonucleosides are available to certain cells and organisms. (*d*) The transformations of succino-AICAR to AICAR and of adenylosuccinic acid to AMP are apparently effected by the same enzyme, adenylosuccinase (253, 254). (*e*) Biotin apparently does not participate directly in the carboxylation of AIR; impairment of aspartate synthesis was probably responsible for evidence of biotin-deficiency inhibition of this step (255).

In addition to these selected fragments of biochemical information, the following chemical findings are relevant to the role of aminoimidazoles in purine biosynthesis. (*a*) Evidence for the nonenzymatic formation of PRA from ribose 5-phosphate and aqueous ammonia has been presented (256), and derivatives of PRA have been employed in chemical syntheses of imidazole nucleotides (Part IIB). (*b*) 5-Aminoimidazole-4-carboxylic acids are easily decarboxylated to the corresponding aminoimidazoles, which are likewise unstable. Decarboxylations of AI-COOH (VIII) (34), its ribonucleoside (214), its ribonucleotide (C-AIR) (213,



Scheme XV

214, 257), and its 1-cyclohexyl derivative (XXXVII; R_1 = C_6H_{11}, R_2 = R′ = H) (95, 213, 214) occur in mildly acidic or neutral media; certain metal ions have a stabilizing influence (214). (*c*) Using AI-COOH ribonucleotide and 1-cyclohexyl AI-COOH (as a model compound), Franks *et al.* (213) have further shown that the steps from GAR to C-AIR may be caused to proceed nonenzymatically in the reverse direction simply by varying the pH and by heating. (*d*) By these degradative methods and by synthetic procedures, the open-chain precursors (GAR, FGAR, and FGAM) and the analogous cyclohexyl derivatives have been formed by purely chemical means (110, 111, 213, 258). (*e*) Nonenzymatic carboxylation of AIR with bicar-

[10] Abbreviations used in Scheme XIV are: ATP = adenosine triphosphate = adenosine 5′-(tetrahydrogen triphosphate)*; AMP = adenosine monophosphate = 5′-adenylic acid*; THF = tetrahydrofolate; PRPP = 5-phosphoribosylpyrophosphate = ribofuranose 5-(dihydrogen phosphate) 1-(trihydrogen pyrophosphate)*; PRA = 5-phosphoribosylamine = 2-amino-2-deoxyribose 5-(dihydrogen phosphate)*; GAR = glycinamide ribonucleotide = 2-amino-*N*-β-D-ribofuranosylacetamide 5′-(dihydrogen phosphate)*; FGAR = formylglycinamide ribonucleotide = 2-formamido-*N*-β-D-ribofuranosylacetamide 5′-(dihydrogen phosphate)*; FGAM = formylglycinamidine ribonucleotide = 2-formamido-*N*-β-D-ribofuranosylacetamidine 5′-(dihydrogen phosphate)*; AIR = 5-aminoimidazole ribonucleotide = 5-amino-1-β-D-ribofuranosylimidazole 5′-(dihydrogen phosphate)*; C-AIR = 5-amino-4-carboxyimidazole ribonucleotide = 5-amino-1-β-D-ribofuranosylimidazole-4-carboxylic acid 5′-(dihydrogen phosphate)*; succino-AICAR or SAICAR = 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide = *N*-[(5-amino-1-β-D-ribofuranosylimidazol-4-yl)carbonyl]aspartic acid 5′-(dihydrogen phosphate)*; AICAR = 5-aminoimidazole-4-carboxamide ribonucleotide = 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide 5′-(dihydrogen phosphate)*; FAICAR = 5-formamidoimidazole-4-carboxamide ribonucleotide = 5-formamido-1-β-D-ribofuranosylimidazole-4-carboxamide 5′-(dihydrogen phosphate)*; IMP = inosine monophosphate = 5′-inosinic acid*; SAMP = adenylosuccinic acid = *N*-(9-β-D-ribofuranosyl-9*H*-purin-6-yl)aspartic acid 5′-(dihydrogen phosphate); XMP = 5′-xanthylic acid*; GMP = 5′-guanylic acid*. (**Chemical Abstracts* names.)

[11] See *References 247–249* for the enzyme, metal-ion, and ATP or GTP requirements for these steps and for other species (phosphate, ADP, and water) either required for or produced in the various steps. Systematic enzyme names are given in *Report of the Commission on Enzymes of the International Union of Biochemistry*, Pergamon Press, New York, N. Y., 1961.

bonate at 85° has been demonstrated (215). (*f*) Cusack *et al.* (215) have pointed out that most of the forward reactions to inosinic acid and most of those that are enzymatically reversible have been performed chemically under relatively mild conditions, and they have suggested a possible relevance of these findings to primeval synthesis.

2. AICAR is a coproduct of an intermediate in histidine biosynthesis (*e.g.*, 259, 260 and references cited). Adenosine triphosphate is glycosidated at position 1 by PRPP; a ring opening, analogous to one of the chemical methods of synthesis previously described, then produces an AIC ribonucleotide derivative (CXV) (Scheme XV). In several steps, AICAR and 4-imidazolylglycerol phosphate (CXVI) are formed, the latter arising from the ribofuranosyl group of PRPP, the nitrogen atom at position 1 of ATP, and nitrogen donated by glutamine. Histidine is formed from CXVI in several additional steps. It is interesting to note certain biochemical interrelationships: (*a*) AICAR may be recycled to ATP *via* IMP and AMP, and (*b*) biodegradation of histidine in several steps *via* urocanic acid yields formiminoglutamic acid, a one-carbon donor for tetrahydrofolate (261).

3. The pyrophosphate (CXVII*a*) of AICAR and AMP is formed from AIC and nicotinamide adenine dinucleotide (NAD) in the presence of NAD glycohydrolase from beef spleen (262–265). Enzymatic conversion of the dinucleotide (CXVII*a*) to the pyrophosphate of IMP and AMP and incorporation of the imidazole moiety into nucleic acids have been demonstrated. Evidence has also been presented for the transfer of a deoxyribofuranosyl group from pyrimidine deoxyribofuranosides to AIC by bacterial enzymes (266, 267).

4. Xanthine is degraded by extracts of certain species of *Clostridia* to 5-aminoimidazole-4-carboxylic acid (VIII) (34). The amino acid is formed *via* 4-ureidoimidazole-5-carboxylic acid (CXVIII) (268) and undergoes further degradation to 5-aminoimidazole (34). Again, it is interesting for correlative purposes to note that the latter compound is further degraded to formiminoglycine, which is a one-carbon source for tetrahydrofolic acid (THF). The sequential formation of 5-formimino-THF, 5,10-methenyl-THF, and 10-formyl-THF is effected by enzymes from *C. cylindrosporum* extracts (269). Thus, interrelationships among purines, aminoimidazole derivatives, and folic acid coenzymes may be found in catabolic, as well as anabolic (Scheme XIV), processes.

5. AIC has been identified as a urinary excretion product of healthy human beings and of animals (270–275). It is normally excreted by adult human beings in quantities of about 1 mg. per day (271, 273, 276, 277), and for most individuals it is comparable to creatinine in the constancy of its excretion (273, 276). The excretion levels may be elevated in abnormal circumstances such as during acute leukemia (276, 278); after oral administration of adenine, glycine, or AIC (279); or during folic acid deficiency (280). More recently, AIC ribonucleoside has been identified as one of several ribonucleosides excreted by normal subjects and by patients with leukemia or gout (281).

6. A compound believed to be 5-aminoimidazole-4-carboxamide 5′-*S*-homocysteinylriboside {5-amino-1-[5-*S*-(3-amino-3-carboxypropyl)-5-thio-β-D-ribofuranosyl]imidazole-4-carboxamide} (CXVII*b*) has been isolated from the urine of patients with homocystinuria (282, 283).

7. In studies with mutants of *Salmonella typhimurium*, Newell and Tucker (284, 285) showed that purines and the pyrimidine moiety (CXIX) of thiamine share the early parts of their biosynthetic pathways. The evidence indicates that this pyrimidine is formed from AIR. The transformation of AIR to the pyrimidine requires the loss of the ribofuranosyl group and the addition of one carbon atom in the ring and the two carbon atoms in the exocyclic groups.

In addition to their involvement in these metabolic processes, aminoimidazolecarboxylic acid derivatives have displayed certain types of biological activity. 5-Amino-2-thioxoimidazoline-4-carboxamide (LXII*b* or, in the thiol tautomeric form, 2-mercapto-AIC) was reported to be moderately inhibitory to Ehrlich ascites carcinoma in mice (77). However, imidazoline-2-thione (2-mercaptoimidazole) and several of its derivatives that do not have the functional groups of the biosynthetic imidazoles appear to be more active against this neoplasm; some of these same compounds—LXII*b* was not included—are also effective in inhibiting solid rodent neoplasms (77, 286). Imidazoline-2-thione and some related compounds inhibited thymidine uptake by ascites cells (77). The activity of LXII*b* in the Ehrlich ascites test may, therefore, be unrelated to its formal resemblance to AIC.

Among 15 analogs or derivatives of AIC and of AIC ribonucleoside tested against Nakahara-Fukuoka sarcoma (a tumor especially sensitive to 6-mercaptopurine and related compounds), only 5-formamidoimidazole-4-thiocarboxamide (LXVIII, X = S) and 5-amino-1-β-D-ribofuranosylimidazole-4-thiocarboxamide (CXIV*a*) were active (287). Further evaluation (288) of these two compounds against seven mouse tumors and leukemias showed that they are inhibitory to adenocarcinoma 755, Ehrlich carcinoma (ascites), sarcoma 180 (ascites), leukemia L-1210, C1498 leukemia, SR-61 leukemia, and Nakahara-Fukuoka sarcoma. 6-Mercaptopurine and its ribonucleoside, employed as reference compounds in these tests, were also active against the same mouse neoplasms. A subline of SR-61 leukemia resistant to these two purine anticancer agents was also resistant to the two imidazole derivatives. These results, the fact that the structures of the two imidazoles suggest that they are potential precursors of 6-mercaptopurine, and the observation (289) that 6-mercaptopurine is excreted after administration of the two compounds to mice suggest that the observed activity may be due to 6-mercaptopurine formed *in vivo* rather than to the two imidazoles *per se*. The thiocarboxamide analog (XXXIX, $R_1 = R_2 = H$) of AIC was reported to be weakly active against the Ehrlich ascites carcinoma and adenocarcinoma 755 (288); however, in an earlier test, it did not significantly increase the lifespan of animals with Ehrlich ascites carcinoma (290).

Inhibition of the growth of a rat sarcoma by ethyl 5-aminoimidazole-4-carboxylate (XII*b*, R = C_2H_5) and its *p*-[bis(2-chloroethyl)amino]phenylsulfonyl derivative has also been reported (43).

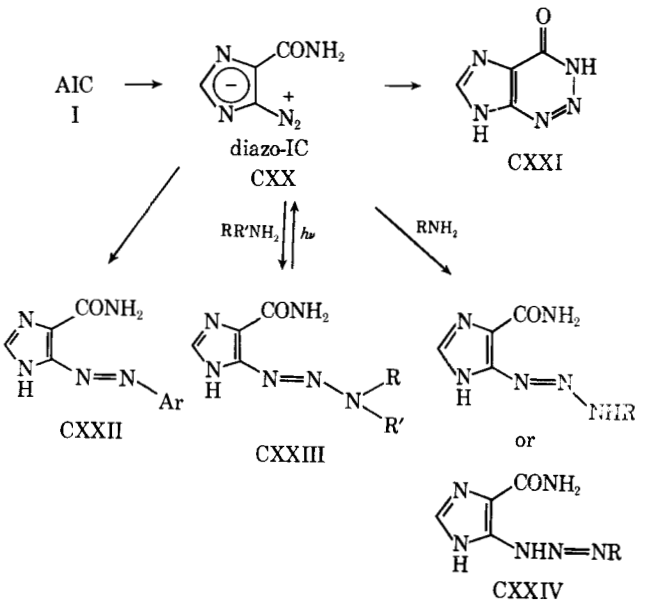
The activity of 8-azaguanine against mouse leukemia L-1210 was found to be potentiated by AIC (291). This effect resulted from inhibition by AIC of the enzyme guanine deaminase (292). Inhibition of adenylosuccinase by an analog of succino-AICAR also represents activity at the enzyme level and deserves mention because of its potential importance for further investigation. Burrows *et al.* (109) found that the L-*threo*-β-methylaspartic acid analog of succino-AICAR is a competitive inhibitor of the enzyme.

AICAR is reported to possess potent activity in enhancing the flavor of a wide variety of food products (227, 233, 293). It is also claimed that succino-AICAR has flavor-enhancing activity (227).

IV. TRIAZENOIMIDAZOLES AND RELATED COMPOUNDS

Interest in triazenoimidazoles began with the prototype, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388, DIC), which was synthesized by Shealy *et al.* (294) for evaluation as a potential anticancer agent. Initial biological evaluation revealed activity in prolonging the survival time of mice with lymphoid leukemia L-1210 and in inhibiting the growth of the solid tumors adenocarcinoma 755 (Ca755) and sarcoma 180 (S180) in mice (295). This evidence of activity against more than one type of neoplasm led to pharmacological studies and clinical trials under the auspices of the Cancer Chemotherapy National Service Center (CCNSC). The chemistry and the biological properties of this compound and of related triazenoimidazoles are discussed in this section. There is no intention to imply that biological activities of the types to be mentioned are confined to imidazole triazenes.

Diazotization of 5-aminoimidazoles, followed by the addition of a coupling agent, such as 1- or 2-naphthol or the Bratton–Marshall reagent [*N*-(1-naphthyl)ethylenediamine] (296), usually produces intensely colored solutions. This property has frequently been exploited in characterizing 5-aminoimidazoles (*e.g.*, 26, 27, 30, 73, 82, 92) and is the basis for an assay procedure for excreted AIC (273). Diazotization of AIC (Scheme XVI) initially gave 2-azahypoxanthine[12] (CXXI) (1, 297), the product of intramolecular coupling of the diazo group with the adjacent amide nitrogen atom. Later, an intermediate in the formation of 2-azahypoxanthine was isolated and characterized as 5-diazoimidxazole-4-carboxamide (CXX) (298). [The substance that initially precipitated from one of the earlier diazotizations (297) might have been CXX.] This compound readily cyclizes to 2-azahypoxanthine over a wide range of pH values, particularly at pH 7 and in basic solutions. Despite this propensity for intramolecular coupling, which is amply precedented in the formation of benzo-*v*-triazines (299), intermolecular



Scheme XVI

coupling with aromatic compounds and with amines was sufficiently competitive to permit isolation (usually in good yields) of the resulting arylazo (CXXII) (294), dialkyl- or arylalkyl-triazeno (CXXIII) (294, 300), and monoalkyl- or monoaryl-triazeno (CXXIV) (301, 302) derivatives.

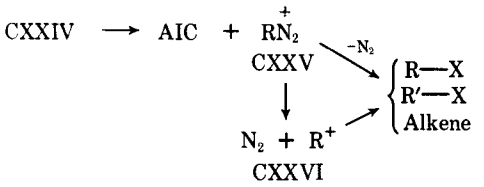
In the initial studies (294), representative disubstituted-triazeno derivatives (CXXIII) (including DIC) in phosphate buffer (pH 7), as well as DIC in 0.1 *N* hydrochloric acid, were shown by UV spectroscopy to be essentially unchanged in the dark during the periods of observation (limited to 1–2 days). Exposure of these solutions to ambient light resulted in the formation of 2-azahypoxanthine (CXXI). The disubstituted-triazeno derivatives (CXXIII) were evidently dissociated by certain wavelengths of light to CXX and the secondary amine (RR'NH), and CXX then cyclized to CXXI. Further observations (300) by TLC of the dimethyltriazeno (DIC) and the butylmethyltriazeno (CXXIII; R = CH_3, R' = C_4H_9) derivatives in 50% aqueous methanol or ethanol showed that the formation of 2-azahypoxanthine is rapid in sunlight or in 365-mμ light (*cf.*, 303), slow under fluorescent lighting, and very slow or negligible under incandescent lighting.[13]

[12] *Chemical Abstracts* names: 4*H*-imidazo[4,5-*d*]-*v*-triazin-4-one, 3,7-dihydro-; 7*H*-imidazo[4,5-*d*]-*v*-triazin-4-ol.

[13] These *qualitative* observations simply indicate a trend and should not be interpreted to be applicable to all lighting situations involving sunlight and artificial lighting. They indicate that suitable precautions should be exercised in handling solutions of triazenoimidazoles. It cannot be emphasized too strongly that the behavior of solutions of these and similar compounds will depend on the wavelengths of light to which they are exposed and on the intensity of the sensitizing light that reaches these solutions. Since the intensity of light decreases rapidly with distance at rates that depend on the geometrical arrangement of the solution and the light source, relatively small differences in the distance of a solution from a light source (*e.g.*, a 365-mμ lamp) catalyzing decomposition can make considerable differences in the apparent rate of decomposition. [The intensity of light from a germicidal lamp was found to be inversely proportional to distance at short distances and roughly inversely proportional to the *square* of the distance at greater distances (304). The Inverse Square Law applies to a point source of light.] For these reasons, exact comparisons of the rates of change of a compound in different solutions or of the rates of change of different compounds cannot be made unless the solutions are exposed under comparable conditions to light of the same wavelength and intensity. Comparisons of stability of different compounds might also be made at the light wavelengths that are optimal for the decomposition of each compound. Obviously, much effort would be needed to acquire the data necessary for precise comparisons.

Loo and Stasswender (303, *cf.*, 305, 306) developed a colorimetric method for determining DIC in biological material, based on the deliberate dissociation of DIC, by exposing solutions containing both this compound and the Bratton–Marshall reagent to UV light in the 365-mμ range. Taken together, these studies indicated that DIC is stable in the dark for at least 24 hr. in phosphate buffer (294), for at least 4–7 days in 50% alcohol (300), and for at least 1 month in refrigerated 0.1 *N* hydrochloric acid solutions (303). Considerable (and various degrees of) dissociation of several disubstituted-triazeno derivatives (CXXIII) in Krebs–Ringer phosphate buffer (pH 7.4) within 20 hr. has been reported (307) but protection from light was not mentioned. In agreement with the observations at pH 7, Skibba *et al.* (306) recently reported DIC to be stable for at least 24 hr. in the dark in lactated Ringer's solution (pH 6.5).[14]

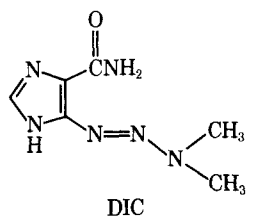
In contrast, it was found (Scheme XVII) that simple



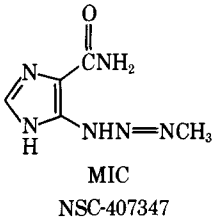
Scheme XVII

monoalkyltriazeno derivatives (CXXIV) are unstable in aqueous and alcoholic solutions even in the dark, that AIC is formed during the decomposition of these derivatives, and that the decomposition follows first-order kinetics (302). Within the series consisting of CXXIV with R = methyl, ethyl, *n*-butyl, cyclohexyl, and *tert*-butyl, stability appeared to decrease in the order in which these compounds are listed. This decomposition was assumed to be similar to deamination of primary amines by nitrosation. Deamination *via* decomposition of triazenes of the benzenoid series is known to occur in organic solvents (308, 309) or in aqueous acids (310–312). Although the mechanistic details of deamination reactions are complex (313–315), they suggested that the simple monoalkyltriazenoimidazoles dissociate, in essence, in accordance with the equations shown (302), producing the unstable alkyldiazonium ion (CXXV) in addition to AIC. The alkyldiazonium ion, in turn, evolves nitrogen and forms a carbonium ion (CXXVI). Some form[15] of the carbonium ion or the alkyldiazonium ion may rearrange (R′), form an alkene, or react with nucleophiles available *in vitro* or *in vivo* (water; hydroxyl, amino, or thiol groups; electron-rich centers of heterocycles; *etc.*). Certain monosubstituted-triazeno derivatives (CXXIV), in which the substituent is an aryl group or a more complex alkyl moiety, are more stable (302); the mode of decomposition may be different or may yield a more stable diazo derivative.

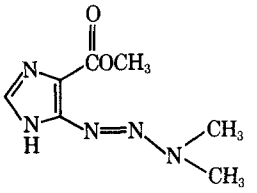
In the initial synthesis (294) of 5-(3,3-disubstituted-1-triazeno)imidazole-4-carboxamides (CXXIII), the substituents on the triazeno group included straight-chain alkyl, cyclic alkyl, aralkyl, and aryl groups, and the number of carbon atoms in the straight-chain groups was doubled [from the dimethyltriazeno (DIC) to the



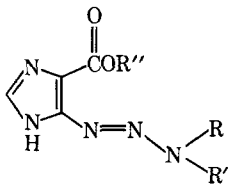
DIC
NSC-45388



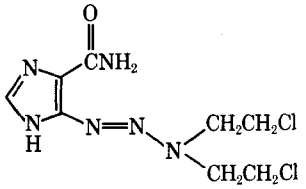
MIC
NSC-407347



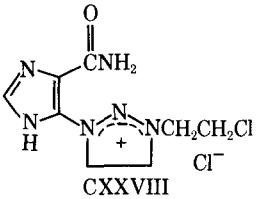
NSC-87982



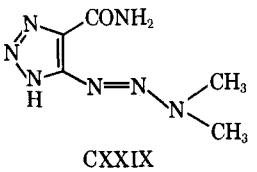
CXXVII



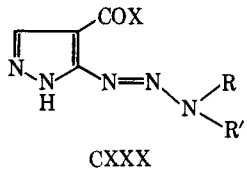
NSC-82196



CXXVIII



CXXIX



CXXX

a, X = OCH_3 or OC_2H_5
b, X = NH_2

dioctyltriazeno derivative]. Because of the antileukemic (L-1210) activity of DIC, its *v*-triazole analog (316), and some methyltriazenes of the benzenoid series (317), one of the alkyl substituents in a later group of dialkyltriazenoimidazolecarboxamides was a methyl group (300). Routine screening of the dialkyltriazeno derivatives against mouse neoplasms indicated that those derivatives in which at least one of the alkyl substituents is a methyl group are the most effective against L-1210.[16] Thus, in addition to DIC, the methyl propyl (NSC-76418), methyl butyl (NSC-70874), methyl isobutyl (NSC-83113), methyl pentyl (NSC-87981), methyl cyclohexyl (NSC-83111), and methyl 2-hydroxyethyl (NSC-83112) derivatives all caused an increase in lifespan (ILS) of treated leukemic mice, as did 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MIC) (302). The maximum values of the ILS observed in preliminary tests of these compounds fall within the range 40–70% (Reference 300 and additional tests os NSC-83112). By way of comparison, preliminary testt of DIC against L-1210 indicated the ILS to be abouf

[14] In the author's laboratories, there was no significant decrease in the absorbance of DIC in Krebs-Ringer buffer during 24 hr. in the dark and only a small decrease during 4 days.

[15] Summaries of theories on the nature of the carbonium ion (hot, nonsolvated, vibrationally excited, solvent-caged) from deamination reactions and the roles of the alkyldiazonium ion and the counterion may be found in *References 309* and *313–315*.

[16] Since doses in some of the L-1210 tests were determined by prior results from S180 and Ca755 tests, more detailed testing might reveal some activity among derivatives that failed the preliminary L-1210 tests.

50–60% (295); further, extensive evaluation has given values of the ILS of 60–90%, depending on the dose and on the route and frequency of administration (318). Activity by methyltriazeno derivatives was also found among the analogous triazenoimidazolecarboxylic acid esters; methyl 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxylate (NSC-87982) increased the lifespan of leukemic mice by 60% (319). At dose levels determined by the L-1210 primary screening protocols, other (but not all) methylalkyltriazenoimidazole esters (CXXVII) have likewise caused some increase in the lifespan of treated leukemic mice (320).

Antineoplastic activity of triazenoimidazoles is not confined to leukemia L-1210. DIC is active against other leukemias and inhibits the growth of solid rodent tumors (295, 318), and most of the aforementioned methylalkyl derivatives also inhibit Ca755 and S180 (300). Derivatives of this group selected for tests against intramuscular Walker carcinosarcoma 256 (IMW256) in rats inhibited tumor growth (300), and an initial test of MIC against the subcutaneous form of this tumor suggested modest inhibition (302). Inhibition of solid tumor growth was frequently accompanied by loss of weight by the host animals. The apparent association of L-1210 activity with at least one methyl group may not apply to other tumors. Modest inhibition of S180 by triazenoimidazolecarboxamides lacking a methyl group was observed (300). Futhermore, certain monoaryltriazeno derivatives of the imidazolecarboxylate variety (CXXVII) that did not inhibit L-1210 in primary screening did display modest inhibition of IMW256 (320). Such derivatives, of course, cannot form a methyl or alkyl carbonium ion; rather, nonenzymic dissociation should produce a diazoimidazole, an aryldiazonium ion, or both. (Aryldiazonium ions are more stable than the alkyl variety, and their coupling properties are well known.) In addition, Hano *et al.* (307) described some dialkyl- and monoalkyltriazenoimidazolecarboxamides (CXXIII and CXXIV) in addition to those mentioned and evaluated the new and some of the old derivatives against the Ehrlich carcinoma. Within the series of derivatives of CXXIII in which R and R′ were identical and comprised of one to five carbon atoms, the dipropyltriazene and DIC were considered to be the most active against the solid form of the tumor (321). The butyl methyl and the methyl propyl derivatives were subsequently shown to display similar activity (307). None of the dialkyltriazeno derivatives inhibited proliferation of the ascites form of the Ehrlich carcinoma, but two monosubstituted-triazeno derivatives (CXXIV, R = ethyl or hydroxyethyl) were reported to increase lifespan. The potentiating effect (322) of MIC on the activity of 8-azaguanine against Ehrlich ascites is probably due to AIC, which is known (301, 302, 307) to be formed, as previously explained, from the triazene and which is known to potentiate the activity of 8-azaguanine by inhibiting guanine deaminase (see Part III).

The uncertainties of the association of structural elements with activity are emphasized by the fact that the most effective derivative in the L-1210 test system is one that does not bear a methyl group; namely, 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (NSC-82196) (323, 324). At *certain* dose levels and dosage schedules, a majority of leukemic mice treated with this compound survived until tests were discontinued after long periods (2–8 months) (323, 325). In addition to these encouraging effects in the standard L-1210 test, Hoffman *et al.* (325) demonstrated the effectiveness of NSC-82196 in advanced L-1210 leukemia. Intraperitoneal administration produced a high proportion of 60-day survivors on certain treatment schedules; oral administration was less effective. Knowledge of the L-1210 test system gained from correlations of inoculum size to average lifespan and from cell-kill kinetic studies (326) indicates that leukemic cells had been completely eradicated from the long-term survivors of the L-1210 tests. Using spleen-colony and host-survival criteria, Wodinsky *et al.* (327) showed that the activity of both NSC-82196 and DIC against L-1210 extends also to an L-1210 line resistant to 6-mercaptopurine. This study indicated a low level of crossresistance of the 6-MP-resistant line to NSC-82196 but not to DIC. Additionally, Tyrer *et al.* (328) developed a strain of L-1210 resistant to NSC-82196 and showed that the resistant variant is crossresistant to DIC and to nitrosoureas; but, interestingly, it is sensitive to certain nitrogen mustard derivatives such as cyclophosphamide and melphalan.

Unfortunately for purposes of handling and administration, this compound is beset by problems of instability. In addition to its susceptibility to light-catalyzed dissociation, it undergoes an internal alkylation to an ionic transformation product in solution and even, slowly, in the solid state (323, 324), but it can be preserved in the solid form at low temperatures. The *v*-triazolinium salt structure (CXXVIII), the most likely among several candidate structures, was identified as the structure of the ionic isomer by an X-ray crystal structure analysis (329). The quality of specimens of NSC-82196 was originally estimated from distinctive differences in the IR spectra of the two compounds. Determinations of NSC-82196 based on a microbiological assay (330), light-catalyzed dissociation to 5-diazoimidazole-4-carboxamide (303, 305), a UV absorption method (331), and titrimetric determination of the ionic isomer (332) have been described. The latter method also provided information on the rate of change of NSC-82196 in solution. A nonaqueous titration procedure developed by Sternglanz (333) provides a direct determination of NSC-82196 and other triazenoimidazole amides and esters.

Bis[3,3-(2-fluoroethyl)-1-triazeno] derivatives of the imidazole (CXXIII and CXXVII) and pyrazole (CXXX) types have likewise demonstrated activity against L-1210, but they are, thus far, more toxic and less active than NSC-82196 (334*a*). The association of L-1210 activity with methyltriazenes also applies, as mentioned previously, to the *v*-triazole analog (CXXIX) (316) of DIC and to certain triazenopyrazolecarboxylic acid esters (CXXX*a*) (334*b*) and triazenopyrazolecarboxamides (CXXX*b*) (334*b*, 335). The triazeno-*v*-triazoles (316) and the triazenopyrazoles (334*b*, 335) appear[13] to be more stable to light than the triazenoimidazoles.

In addition to the antineoplastic effects produced by certain triazenoimidazoles, antimicrobial activity has also been demonstrated. Methyl 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxylate (NSC-87982) (319) is strongly inhibitory *in vitro* to a broad spectrum of Gram-positive and Gram-negative bacteria, mycobacteria, yeasts, filamentous fungi, and algae (319, 336, 337). Strains of *Escherichia coli* and *Streptococcus faecalis* resistant to various antibiotics and antimetabolites were not crossresistant to NSC-87982 (338). Activity *in vivo* was demonstrated against *Staphylococcus aureus* in mice (336), and significantly high blood levels and detectable concentrations in several mouse tissues were found after administration by various routes (337). It has also been shown that a number of related 5-(disubstituted-triazeno)imidazole-4-carboxylic acid esters have broad-spectrum antimicrobial activity *in vitro* (320). Additionally, from among a number of bacteria sensitive to DIC, a strain of *E. coli* resistant to glutamic acid γ-hydrazide (and sensitive to DIC at a concentration of 5 mcg./ml. in the disk-plate method) was selected for a microbiological method of assay of DIC in blood and tissues (339). Recently, inhibition of *E. coli* B (340) by DIC and several of its homologs (at concentrations of about 10 mcg./ml.) and inhibition of *Bacillus subtilis* (341) by DIC were reported. Although the antimicrobial activity of the 3,3-bis(2-chloroethyl)-1-triazeno derivative (NSC-82196) is minimal, inhibition of two species of *Mycobacteria* and of drug-resistant *E. coli* strains at high concentrations was observed (330).

Pharmacological studies of DIC have been conducted in the dog (342–344), rat (305, 345), mouse (339), monkey (305), and man (306, 342, 343, 345, 346); DIC and some of its homologs have also been studied in rabbits and cats (347). NSC-82196 has been subjected to pharmacological and clinical evaluation (305, 348). In clinical trials, DIC (NSC-45388) has brought about objective remissions of malignant melanoma in some of the treated patients (306, 349). Objective responses by several other cancers have also been observed in patients treated with DIC (349).

The discussion, thus far, has dealt with triazenoimidazoles. Although 5-diazoimidazole-4-carboxamide (CXX, diazo-IC, NSC-22420), the precursor of the carboxamides, is an unstable compound in solution, there are reports that it also has various types of biological activity; these findings have possible relevance to the question of the mechanism of action of triazenoimidazoles prepared from it. Reports of antineoplastic activity include inhibition of human epidermoid carcinoma (H.Ep.-2) cells in culture (298), subcutaneous Walker carcinoma 256 (298), and Ehrlich ascites carcinoma (298, 321). Antimicrobial activity by diazo-IC (or, because of the instability of CXX, a mixture of diazo-IC and 2-azahypoxanthine) in the form of inhibition of *Mycobacterium tuberculosis* has been reported (350). Furthermore, diazo-IC completely inhibits *E. coli in vitro* (290, 340) at low concentrations and is strongly inhibitory to *B. subtilis* (341). A third type of activity is represented by the potent inhibition of xanthine oxidase by the diazo compound and by two thioazo derivatives prepared from it (351).

At this time, there are unanswered questions concerning both the relationship of structure to activity and the mechanism of action of triazenoimidazoles and related compounds. Several potential mechanisms of action may be considered, and these are not necessarily mutually exclusive. The structural relationship to, and chemical derivation from, the imidazole moieties of the imidazole ribonucleotides suggested *a priori* that the triazenoimidazoles or diazo-IC might interfere in some way with imidazole and purine metabolism (295, 298). A second possibility that was also considered (295) is that the triazenes may be latent forms of the active agent—the corresponding diazo compound for disubstituted-triazeno derivatives or, in the case of NSC-82196, the diazo compound and bis(2-chloroethyl)amine. A third possibility is that the *monosubstituted*-triazeno derivatives are carrier forms (302) of a reactive species (an alkyldiazonium ion or a carbonium ion) and, on the basis of recent findings (345), that the disubstituted-triazeno derivatives may act similarly after *N*-dealkylation.

In what is, of necessity, a speculative vein, a number of findings may be brought to bear on the question of mechanism of action without, at this time, providing a definitive answer. Some observations tend to place the triazenoimidazoles in the class of chemically reactive anticancer drugs ("biological alkylating agents") such as alkylating agents and diazo compounds. Studies by Wilkoff *et al.* (352) of the cell-kill kinetics of DIC and NSC-82196 in cultures of leukemia L1210 cells indicate that these drugs are probably not cell-cycle-stage specific agents. Their behavior in this system, therefore, is similar to that of certain chemically reactive drugs. The crossresistance to nitrosoureas of the L-1210 variant that is resistant to NSC-82196 (328) is consistent with the reactive species idea, but the lack of crossresistance to other drugs having a bis(2-chloroethyl)amino group is not. The earlier findings of Pittillo and Hunt (338) that sulfur-containing amino acids reversed the inhibition of *E. coli* ATCC9637 and of *Saccharomyces cerevisiae* by NSC-87982 and the inhibition of another *E. coli* strain by NSC-82196 suggested (along with observations of crossresistance) involvement of a chemically reactive species as one, but not the sole, mechanism of action (330). Subsequently, Yamamoto (340) reported that cysteine abolished the inhibitory action of diazo-IC on *E. coli* B and suggested that this compound might be the active form for inhibition of this organism by dialkyltriazenoimidazolecarboxamides. More direct evidence in this connection is the discovery by Saunders and Saunders (341) that the inhibitory action of DIC on *B. subtilis* is markedly increased by exposing cultures growing in the presence of the compound to light and that a strain resistant to DIC is also resistant to the diazo compound. These studies in bacteria also indicated that RNA and protein synthetic processes are inhibited by NSC-87982 in *E. coli* (338), that DNA synthesis is inhibited in *E. coli* B by diazo-IC (340), and that DNA synthesis in *B. subtilis* is inhibited by DIC (341).

In the studies of Pittillo and Hunt (330), the partial protection to NSC-82196 inhibition of an *E. coli* strain

afforded by AIC suggested the involvement of purine metabolism. The observations in these studies of NSC-82196 and of NSC-87982 of collateral sensitivity of bacteria resistant to certain purine analogs (330, 337, 338) and of partial crossresistance to 8-azaguanine and to 2,6-diaminopurine of an *E. coli* strain resistant to NSC-82196 (330) also tended to implicate purine metabolism. In this connection, the low level of crossresistance to NSC-82196 by 6-mercaptopurine-resistant L-1210 cells (327) should be recalled. The statement made at the outset that potential mechanisms of action should not be considered mutually exclusive is illustrated by the report of Peters and McGeer (290) that diazo-IC, as well as some related compounds, showed inhibitory effects on the incorporation of glycine into hypoxanthine by pigeon liver homogenates.

A mechanism involving some form of alkyldiazonium ion or carbonium ion must be considered for the monoalkyltriazenoimidazoles because of their demonstrated behavior *in vitro*, as explained previously. Housholder and Loo (353) and Skibba *et al.* (345) reported that patients given DIC excrete large amounts of AIC; the latter investigators found that DIC is *N*-demethylated by rat liver microsomes to formate and AIC. It is appropriate to recall at this point that MIC not only forms AIC in solution but also has antineoplastic activity. If metabolic *N*-demethylation results in the formation of MIC, then a mechanism involving some form of an alkyldiazonium ion or a carbonium ion must also be considered for DIC and other disubstituted triazenoimidazoles. By analogy with organic deamination reactions, some form of these highly reactive species should be generated and should react with nucleophilic centers such as those listed earlier in this section. Recently, Preussmann *et al.* (354), in a comprehensive paper on the mechanism of carcinogenesis of phenyltriazenes, reported that formaldehyde and aniline are formed by incubation of 3,3-dimethyl-1-phenyltriazene with rat liver or lung microsomal fractions in the presence of oxygen and an NADPH-generating system, that formaldehyde or acetaldehyde are formed similarly from certain other carcinogenic dimethyl- or diethyltriazenes of the phenyl and pyridyl series, and that 3-methyl-1-phenyltriazene is a potent carcinogen. Their proposed mechanism of carcinogenesis consists of oxidative dealkylation of a 3,3-dialkyl-1-aryltriazene *in vivo* to an aryl monoalkyltriazene, decomposition of this intermediate to a carbonium ion, and alkylation by this species of "biopolymers, probably nucleic acids." (The authors mention certain, as yet, unexplained phenomena such as the failure to observe tumors in the liver or lungs.) The later stages of their mechanism are essentially those proposed (302) for the dissociation of 5-(3-alkyl-1-triazeno)imidazole-4-carboxamides (CXXIV), and their findings are similar to those of Skibba *et al.* (345), except that formate was identified in the latter study. Whether *N*-dealkylation is the basis of a mechanism of action or simply a catabolic process remains to be seen. One may speculate that the particular mechanism by which a triazene acts (such as reversion to a diazo derivative, interference in purine metabolism, oxidative *N*-dealkylation, or combinations of these processes) may depend on the biochemical environment in which it is placed or to which it is transported.[17]

REFERENCES

(1) M. R. Stetten and C. L. Fox, Jr., *J. Biol. Chem.*, **161,** 333(1945).

(2) C. L. Fox, Jr., *Proc. Exp. Biol. Med.*, **51,** 102(1942).

(3) W. Shive, W. W. Ackermann, M. Gordon, M. E. Getzendaner, and R. E. Eakin, *J. Amer. Chem. Soc.*, **69,** 725(1947).

(4) A. Windaus and W. Langenbeck, *Ber.*, **56,** 683(1923).

(5) W. Langenbeck, *Angew. Chem.*, **60,** 297(1948).

(6) A. C. Cuckler, L. R. Chapin, C. M. Malanga, E. F. Rogers, H. J. Becker, R. L. Clark, W. J. Leanza, A. A. Pessolano, T. Y. Shen, and L. H. Sarett, *Proc. Soc. Exp. Biol. Med.*, **98,** 167(1958).

(7) D. K. McLoughlin and D. K. Chester, *Poultry Sci.*, **38,** 353(1959); through *Chem. Abstr.*, **53,** 19137c(1959).

(8) C. Cosar and L. Julou, *Ann. Inst. Pasteur, Paris*, **96,** 238 (1959).

(9) M. Bock, *Arzneim.-Forsch.*, **11,** 587(1961).

(10) C. Cosar, C. Crisan, R. Horclois, R. M. Jacob, J. Robert, S. Tchelitcheff, and R. Vaupré, *ibid.*, **16,** 23(1966).

(11) G. P. Ellis, C. Epstein, C. Fitzmaurice, L. Goldberg, and G. H. Lord, *J. Pharm. Pharmacol.*, **16,** 801(1964).

(12) *Ibid.*, **19,** 102(1967).

(13) E. Grunberg, G. Beskid, R. Cleeland, W. F. DeLorenzo, E. Titsworth, H. J. Scholer, R. Richle, and Z. Brener, *Antimicrob. Ag. Chemother.*, **1968,** 513.

(14) I. de Carneri, *Arzneim.-Forsch.*, **19,** 382(1969).

(15) P. N. Giraldi, V. Mariotti, and I. de Carneri, *J. Med. Chem.*, **11,** 66(1968).

(16) G. Berkelhammer and G. Asato, *Science*, **162,** 1146(1968).

(17) P. Durel, V. Roiron, A. Siboulet, and L. J. Borel, *Brit. J. Vener. Dis.*, **36,** 21(1960).

(18) H. C. Hesseltine and Y. Lefebvre, *J. Amer. Med. Ass.*, **184,** 1011(1963).

(19) Editorial, *ibid.*, **184,** 1036(1963).

(20) S. J. Powell, A. J. Wilmot, and R. Elsdon-Dew, *Ann. Trop. Med. Parasitol.*, **61,** 511(1967).

(21) P. J. Taylor, *Ariz. Med.*, **25,** 641(1968).

(22) K. Hofmann, "Imidazole and Its Derivatives," part 1, Interscience, New York, N. Y., 1953.

(23) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic, New York, N. Y., 1963.

(24) L. B. Townsend, *Chem. Rev.*, **67,** 533(1967).

(25) R. G. Fargher and F. L. Pyman, *J. Chem. Soc.*, **115,** 217 (1919).

(26) R. G. Fargher, *ibid.*, **117,** 668(1920).

(27) F. L. Pyman, *ibid.*, **121,** 2616(1922).

(28) I. E. Balaban, *ibid.*, **1930,** 268.

(29) G. Hunter and J. A. Nelson, *Can. J. Res.*, **19** (Sec. B), 296 (1941).

(30) R. Weidenhagen and R. Herrmann, *Ber.*, **68,** 2205(1935).

(31) A. Windaus and H. Opitz, *ibid.*, **44,** 1721(1911).

(32) J. Baddiley, J. G. Buchanan, F. E. Hardy, and J. Stewart, *J. Chem. Soc.*, **1959,** 2893.

(33) W. E. Allsebrook, J. M. Gulland, and L. F. Storey, *ibid.*, **1942,** 232.

(34) J. C. Rabinowitz, *J. Biol. Chem.*, **218,** 175(1956).

(35) E. C. Taylor, C. C. Cheng, and O. Vogl, *J. Org. Chem.*, **24,** 2019(1959).

[17] *Addendum added in proof*—The following publications dealing with the mechanism of action, metabolism, and biological effects of DIC and NSC-82196 appeared since this manuscript was completed:

(1) C. L. Vogel, V. T. De Vita, R. P. Lisak, and M. W. Kies, *Cancer Res.*, **29,** 2249(1969).

(2) J. L. Skibba, D. D. Beal, G. Ramirez, and G. T. Bryan, *ibid.*, **30,** 147(1970).

(3) C. L. Vogel, C. Denham, T. P. Waalkes, and V. T. De Vita, *ibid.*, **30,** 1651(1970).

(4) P. P. Saunders and G. A. Schultz, *Biochem. Pharmacol.*, **19,** 911 (1970).

(5) J. L. Skibba, G. Ramirez, D. D. Beal, and G. T. Bryan, *ibid.*, **19,** 2043(1970).

(36) B. Robinson and D. M. Shepherd, *J. Pharm. Pharmacol.*, **14,** 9(1962).
(37) L. P. Kulev and R. N. Gireva, *Zh. Prikl. Khim.*, **30,** 811 (1957); through *Chem. Abstr.*, **51,** 17897a(1957) [*J. Appl. Chem. USSR*, **30,** 858(1957)].
(38) R. N. Gireva and N. S. Dobychina, *Izv. Tomsk. Politekhn. Inst.*, **102,** 108(1959); through *Chem. Abstr.*, **59,** 1622d(1963).
(39) L. P. Kulev and A. M. Rozhkov, *Zh. Obshch. Khim.*, **27,** 1389(1957) [*J. Gen. Chem. USSR*, **27,** 1470(1957)].
(40) L. P. Kulev and V. R. Koroleva, *ibid.*, **29,** 2401(1959) [*J. Gen. Chem. USSR*, **29,** 2366(1959)].
(41) V. R. Koroleva, *Izv. Tomsk. Politekhn. Inst.*, **111,** 23(1961); through *Chem. Abstr.*, **57,** 14594b(1962).
(42) R. N. Gireva, V. E. Golubev, T. A. Boldyreva, and V. E. Chistyakov, *Sin. Prir. Soedin., Ikh Analogov Fragmentov, Akad. Nauk SSSR, Otd. Obshch. Tekhn. Khim.*, **1965,** 54; through *Chem. Abstr.*, **65,** 2158d(1966).
(43) R. N. Gireva, G. A. Aleshina, L. F. Mal'tseva, T. V. Mikhailova, and O. I. Petrova, *Khim.-Farm. Zh.*, **2,** 39(1968).
(44) R. N. Gireva, N. N. Gavrilov, and V. M. Mar'yanovskii, *Khim. Geterotsikl. Soedin., Sb. 1: Azotsoderzhashchie Geterotsikly*, **1967,** 119; through *Chem. Abstr.*, **70,** 96709g(1969).
(45) R. N. Gireva, N. N. Gavrilov, G. A. Aleshina, A. V. Evseeva, and N. V. Nikitina, *ibid.*, **1967,** 123; through *Chem. Abstr.*, **70,** 96710a(1969).
(46) J. Baddiley, J. G. Buchanan, and J. Stewart, *Proc. Chem. Soc.*, **1957,** 149.
(47) M. Ikehara, N. Nakazawa, and H. Nakayama, *Chem. Pharm. Bull.*, **10,** 660(1962).
(48) R. J. Rousseau, L. B. Townsend, and R. K. Robins, *Chem. Commun.*, **1966,** 265.
(49) R. J. Rousseau, R. K. Robins, and L. B. Townsend, *J. Amer. Chem. Soc.*, **90,** 2661(1968).
(50) N. J. Leonard, K. L. Carraway, and J. P. Helgeson, *J. Heterocycl. Chem.*, **2,** 291(1965).
(51) J. Sarasin and E. Wegmann, *Helv. Chim. Acta*, **7,** 713(1924).
(52) O. Wallach, *Ann. Chem.*, **214,** 278(1882).
(53) O. Wallach, *Ber.*, **16,** 534(1883).
(54) F. G. Mann and J. W. G. Porter, *J. Chem. Soc.*, **1945,** 751.
(55) A. Heymons, *Ber.*, **66,** 1017(1933).
(56) F. F. Blicke and H. C. Godt, *J. Amer. Chem. Soc.*, **76,** 3653(1954).
(57) M. H. Fisher, W. H. Nicholson, and R. S. Stuart, *Can. J. Chem.*, **39,** 1336(1961).
(58) F. Montequi, *An. Soc. Espan. Fís. Quím.*, **24,** 731(1926); through *Chem. Abstr.*, **21,** 1448(1927).
(59) *Ibid.*, **25,** 182(1927); through *Chem. Abstr.*, **21,** 3353(1927).
(60) R. N. Prasad and R. K. Robins, *J. Amer. Chem. Soc.*, **79,** 6401(1957).
(61) G. E. Trout and P. R. Levy, *Rec. Trav. Chim.*, **84,** 1257 (1965).
(62) *Ibid.*, **85,** 765(1966).
(63) E. C. Taylor and P. K. Loeffler, *J. Org. Chem.*, **24,** 2035 (1959).
(64) G. E. Trout and P R. Levy, *Rec. Trav. Chim.*, **85,** 1254 (1966).
(65) P. M. Kochergin, *Zh. Obshch. Khim.*, **34,** 3402(1964) [*J. Gen. Chem. USSR*, **34,** 3444(1964)].
(66) P. M. Kochergin, *ibid.*, **34,** 2735(1964) [*J. Gen. Chem. USSR*, **34,** 2758(1964)].
(67) I. E. Balaban and F. L. Pyman, *J. Chem. Soc.*, **125,** 1564 (1924).
(68) F. F. Blicke and C.-M. Lee, *J. Org. Chem.*, **26,** 1861(1961).
(69) M. H. Fisher, W. H. Nicholson, and R. S. Stuart, *Can. J Chem.*, **39,** 501(1961).
(70) I. Heilbron, *J. Chem. Soc.*, **1949,** 2099.
(71) A. H. Cook, J. D. Downer, and I. Heilbron, *ibid.*, **1948,** 1262.
(72) C. W. Capp, A. H. Cook, J. D. Downer, and I. Heilbron, *ibid.*, **1948,** 1340.
(73) A. H. Cook, J. D. Downer, and I. Heilbron, *ibid.*, **1948,** 2028.
(74) A. H. Cook, I. Heilbron, and E. Smith, *ibid.*, **1949,** 1440.
(75) A. H. Cook and E. Smith, *ibid.*, **1949,** 3001.
(76) *Ibid.*, **1949,** 2329.
(77) G. Weitzel, F. Schneider, H. Guglielmi, J. Sander, J. Durst, and W.-D. Hirschmann, *Z. Physiol. Chem.*, **346,** 208(1966).
(78) I. M. Heilbron and A. H. Cook, British patents 683523, 683593, and 683594 (1952); through *Chem. Abstr.*, **48,** 2093a–e (1954).
(79) A. H. Cook, A. C. Davis, I. Heilbron, and G. H. Thomas, *J. Chem. Soc.*, **1949,** 1071.
(80) C. S. Miller, S. Gurin, and D. W. Wilson, *Science*, **112,** 654(1950).
(81) C. S. Miller, S. Gurin, and D. W. Wilson, *J. Amer. Chem. Soc.*, **74,** 2892(1952).
(82) H. Bader, J. D. Downer, and P. Driver, *J. Chem. Soc.*, **1950,** 2775.
(83) H. Bader and J. D. Downer, *ibid.*, **1953,** 1636.
(84) I. Nakata, *Meiji Yakka Daigaku Kenkyu Kiyo*, **2,** 66(1963); through *Chem. Abstr.*, **61,** 1864b(1964).
(85) R. G. Glushkov and O. Yu. Magidson, *Zh. Obshch. Khim.*, **31,** 1173(1961) [*J. Gen. Chem. USSR*, **31,** 1085(1961)].
(86) *Ibid.*, **31,** 1906(1961) [*J. Gen. Chem. USSR*, **31,** 1783 (1961)].
(87) R. G. Glushkov and O. Yu. Magidson, *Khim. Geterotsikl. Soedin.*, **2,** 192(1966) [*Chem. Heterocycl. Compd.*, **2,** 134(1966)].
(88) R. G. Glushkov and O. Yu. Magidson, *Khim. Geterotsikl. Soedin., Akad. Nauk Latv. SSR*, **1965,** 85; through *Chem. Abstr.*, **63,** 13258f(1965).
(89) V. G. Granik and R. G. Glushkov, *Khim.-Farm. Zh.*, **1,** 16(1967); through *Chem. Abstr.*, **68,** 68955z(1968).
(90) R. G. Glushkov and A. R. Todd, *Khim. Geterotsikl. Soedin.*, **1968,** 433; through *Chem. Abstr.*, **69,** 106668p(1968).
(91) G. Shaw and R. N. Warrener, *Proc. Chem. Soc.*, **1958,** 193.
(92) G. Shaw, R. N. Warrener, D. N. Butler, and R. K. Ralph, *J. Chem. Soc.*, **1959,** 1648.
(93) G. Shaw and D. N. Butler, *ibid.*, **1959,** 4040.
(94) R. N. Naylor, G. Shaw, D. V. Wilson, and D. N. Butler, *ibid.*, **1961,** 4845.
(95) G. Shaw and D. V. Wilson, *ibid.*, **1962,** 2937.
(96) M. Greenhalgh, G. Shaw, D. V. Wilson, and N. J. Cusack, *J. Chem. Soc., C*, **1969,** 2198.
(97) C. L. Leese and G. M. Timmis, *J. Chem. Soc.*, **1961,** 3816.
(98) G. Shaw and D. V. Wilson, *ibid.*, **1963,** 1077.
(99) I. E. Burrows and G. Shaw, *J. Chem. Soc., C*, **1967,** 1088.
(100) A. H. Cook, J. D. Downer, and I. Heilbron, *J. Chem. Soc.*, **1949,** 1069.
(101) A. H. Cook and G. H. Thomas, *ibid.*, **1950,** 1884.
(102) H. Bader and J. D. Downer, *ibid.*, **1953,** 1641.
(103) J. Baddiley, J. G. Buchanan, R. Hodges, and J. F. Prescott, *ibid.*, **1957,** 4769.
(104) G. Shaw and D. V. Wilson, *Proc. Chem. Soc.*, **1961,** 381.
(105) G. Shaw, D. V. Wilson, and C. P. Green, *J. Chem. Soc.*, **1964,** 2650.
(106) G. M. Tener, *J. Amer. Chem. Soc.*, **83,** 159(1961).
(107) H. Grunze and W. Koransky, *Angew. Chem.*, **71,** 407(1959).
(108) G. Shaw and D. V. Wilson, *Proc. Chem. Soc.*, **1962,** 115.
(109) I. E. Burrows, G. Shaw, and D. V. Wilson, *J. Chem. Soc., C*, **1968,** 40.
(110) R. Carrington, G. Shaw, and D. V. Wilson, *Tetrahedron Lett.*, **1964,** 2861.
(111) R. Carrington, G. Shaw, and D. V. Wilson, *J. Chem. Soc.*, **1965,** 6864.
(112) L. H. Smith, Jr., and P. Yates, *J. Amer. Chem. Soc.*, **76,** 6080(1954).
(113) E. Shaw and D. W. Woolley, *J. Biol. Chem.*, **181,** 89(1949).
(114) E. Shaw, *ibid.*, **185,** 439(1950).
(115) E. Shaw and D. W. Woolley, *ibid.*, **194,** 641(1952).
(116) T. Noguchi, *Pharm. Bull. (Japan)*, **4,** 130(1956).
(117) J. A. Montgomery, K. Hewson, R. F. Struck, and Y. F. Shealy, *J. Org. Chem.*, **24,** 256(1959).
(118) E. Richter, J. E. Loeffler, and E. C. Taylor, *J. Amer. Chem. Soc.*, **82,** 3144(1960).
(119) T. Shirodokoro, M. Fukuzawa, N. Ito, M. Yamada, and M. Kakehi, Japanese patent 20550 (1965); through *Chem. Abstr.*, **64,** 2096f(1966).
(120) E. Shaw, *J. Amer. Chem. Soc.*, **80,** 3899(1958).
(121) Y. Hirata, I. Teshima, and K. Iwashita, *Res. Rept. Nagoya Ind. Sci. Res. Inst.*, No. **8,** 70(1955); through *Chem. Abstr.*, **51,** 5758i(1957).

(122) E. Shaw, *J. Org. Chem.*, **30,** 3371(1965).
(123) T. Ichikawa, T. Kato, and T. Takenishi, *J. Heterocycl. Chem.*, **2,** 253(1965).
(124) T. Takenishi, T. Kato, and T. Ichikawa, Japanese patent 15577 (1966); through *Chem. Abstr.*, **66,** 10958c(1967).
(125) G. Longo, *Gazz. Chim. Ital.*, **61,** 575(1931); through *Chem. Abstr.*, **26,** 710(1932).
(126) J. Oró, *Biochem. Biophys. Res. Commun.*, **2,** 407(1960).
(127) J. Oró and A. P. Kimball, *Arch. Biochem. Biophys.*, **94,** 217(1961).
(128) *Ibid.*, **96,** 293(1962).
(129) J. Oró, *Nature*, **191,** 1193(1961).
(130) J. P. Ferris and L. E. Orgel, *J. Amer. Chem. Soc.*, **87,** 4976 (1965).
(131) *Ibid.*, **88,** 3829(1966).
(132) W. Ruske and E. Ruske, *Chem. Ber.*, **91,** 2505(1958).
(133) J. P. Ferris and L. E. Orgel, *J. Amer. Chem. Soc.*, **88,** 1074(1966).
(134) Ajinomoto Co., Inc., French patent 1437213 (1966); through *Chem. Abstr.*, **65,** 20136g(1966).
(135) K. Suzuki, T. Saito, T. Meguro, I. Kumashiro, and T. Takenishi, Japanese patent 6910 (1967); through *Chem. Abstr.*, **68,** 68995n(1968).
(136) R. L. Webb, S. Frank, and W. C. Schneider, *J. Amer. Chem. Soc.*, **77,** 3491(1955).
(137) H. Bredereck, G. Schmötzer, and H.-J. Becher, *Ann. Chem.*, **600,** 87(1956).
(138) T. Völker, *Angew. Chem.*, **72,** 379(1960).
(139) R. A. Sanchez, J. P. Ferris, and L. E. Orgel, *J. Mol. Biol.*, **30,** 223(1967).
(140) Y. Yamada, N. Nagashima, Y. Iwashita, A. Nakamura, and I. Kumashiro, *Tetrahedron Lett.*, **1968,** 4529.
(141) R. A. Sanchez, J. P. Ferris, and L. E. Orgel, *J. Mol. Biol.*, **38,** 121(1968).
(142) R. Sanchez, J. Ferris, and L. E. Orgel, *Science*, **153,** 72 (1966).
(143) H. Wakamatsu, Y. Yamada, T. Saito, I. Kumashiro, and T. Takenishi, *J. Org. Chem.*, **31,** 2035(1966).
(144) Y. Yamada, I. Kumashiro, and T. Takenishi, *ibid.*, **33,** 642(1968).
(145) Ajinomoto Co., Inc., Netherlands application 6403034 (1964); through *Chem. Abstr.*, **62,** 6496b(1965).
(146) Y. Yamada, I. Noda, I. Kumashiro, and T. Takenishi, *Bull. Chem. Soc. Jap.*, **42,** 1454(1969).
(147) D. W. Woodward, U. S. patent 2534331 (1950); through *Chem. Abstr.*, **45,** 5191d(1951).
(148) *Ibid.*, U. S. patent 2534332 (1950); through *Chem. Abstr.*, **45,** 5191i(1951).
(149) H. Bredereck and G. Schmötzer, *Ann. Chem.*, **600,** 95 (1956).
(150) Y. Yamada, I. Kumashiro, and T. Takenishi, *Bull. Chem. Soc. Jap.*, **41,** 241(1968).
(151) Ajinomoto Co., Inc., Netherlands application 6507892 (1965); through *Chem. Abstr.*, **64,** 15893f(1966).
(152) *Ibid.*, French patent 1437212 (1966); through *Chem. Abstr.*, **66,** 2563q(1967).
(153) Y. Yamada, I. Kumashiro, and T. Takenishi, *Bull. Chem. Soc. Jap.*, **41,** 1237(1968).
(154) Ajinomoto Co., Inc., French patent 1537384 (1968); through *Chem. Abstr.*, **71,** 81425h(1969).
(155) Y. Yamada and I. Kumashiro, British patent 1158965 (1969); through *Chem. Abstr.*, **71,** 101889t(1969).
(156) E. Schmidt, *Ber.*, **14,** 813(1881).
(157) E. Fischer and O. Bromberg, *ibid.*, **30,** 219(1897).
(158) H. Biltz and H. Rakett, *ibid.*, **61,** 1409(1928), and earlier references cited therein.
(159) A. Einhorn and E. Baumeister, *ibid.*, **31,** 1138(1898).
(160) M. W. Van Der Slooten, *Rec. Trav. Chim.*, **15,** 188(1896).
(161) "Beilstein's Handbuch der Organischen Chemie," 4th ed., **25,** 511, 512 (1936); 2nd suppl., **25,** 402(1954).
(162) A. M. Khaletskii and M. S. Eshman, *Zh. Obshch. Khim.*, **18,** 2116(1948); through *Chem. Abstr.*, **43,** 3794d(1949).
(163) H. Auterhoff and M. F. Hebler, *Arzneim.-Forsch.*, **19,** 621 (1959).
(164) E. S. Golovchinskaya, O. A. Kolganova, L. A. Nikolaeva, and E. S. Chaman, *Zh. Obshch. Khim.*, **33,** 1650(1963) [*J. Gen. Chem. USSR*, **33,** 1608(1963)].
(165) A. M. Bellini and A. Fabian, *Ann. Chim. (Rome)*, **54,** 621 (1964); through *Chem. Abstr.*, **61,** 14661d(1964).
(166) H. Biltz and H. Rakett, *Ber.*, **64,** 1970(1931).
(167) R. M. Hoskinson, *Aust. J. Chem.*, **21,** 1913(1968).
(168) M. A. Stevens and G. B. Brown, *J. Amer. Chem. Soc.*, **80,** 2759(1958).
(169) G. B. Brown, in "Progress in Nucleic Acid Research and Molecular Biology," vol. 8, J. N. Davidson and W. E. Cohn, Eds., Academic, New York, N. Y., 1968, pp. 209–255.
(170) B. R. Baker and J. P. Joseph, *J. Amer. Chem. Soc.*, **77,** 15(1955).
(171) E. Shaw, *ibid.*, **81,** 6021(1959).
(172) *Ibid.*, **83,** 4770(1961).
(173) C. M. Baugh and E. N. Shaw, *Biochim. Biophys. Acta*, **114,** 213(1966).
(174) J. A. Montgomery, K. Hewson, S. J. Clayton, and H. J. Thomas, *J. Org. Chem.*, **31,** 2202(1966).
(175) G. T. Rogers and T. L. V. Ulbricht, *J. Chem. Soc., C*, **1968,** 1929.
(176) A. Holy and K. H. Scheit, *Chem. Ber.*, **99,** 3778(1966).
(177) B. C. Pal and C. A. Horton, *J. Chem. Soc.*, **1964,** 400.
(178) J. A. Montgomery and H. J. Thomas, *J. Org. Chem.*, **28,** 2304(1963).
(179) G. B. Elion, *ibid.*, **27,** 2478(1962).
(180) M. R. Atkinson, J. F. Jackson, R. K. Morton, and A. W. Murray, *Nature*, **196,** 35(1962).
(181) G. Hunter, *Biochem. J.*, **30,** 1183, 1189(1936).
(182) S. Friedman and J. S. Gots, *Arch. Biochem. Biophys.*, **39,** 254(1952).
(183) L. F. Cavalieri, J. F. Tinker, and G. B. Brown, *J. Amer. Chem. Soc.*, **71,** 3973(1949).
(184) R. H. Lindsay, W. K. Paik, and P. P. Cohen, *Biochim. Biophys. Acta*, **58,** 585(1962).
(185) R M. Cresswell and G. B. Brown, *J. Org. Chem.*, **28,** 2560 (1963).
(186) M. A. Stevens, H. W. Smith, and G. B. Brown, *J. Amer. Chem. Soc.*, **82,** 1148(1960).
(187) *Ibid.*, **81,** 1734(1959).
(188) M. A. Stevens, A. Giner-Sorolla, H. W. Smith, and G. B. Brown, *J. Org. Chem.*, **27,** 567(1962).
(189) J. C. Parham, J. Fissekis, and G. B. Brown, *ibid.*, **32,** 1151 (1967).
(190) H. Kawashima and I. Kumashiro, *Bull. Chem. Soc. Jap.*, **42,** 750(1969).
(191) F. Cramer and G. Schlingloff, *Tetrahedron Lett.*, **1964,** 3201.
(192) T. Fujii, T. Itaya, C. C. Wu, and S. Yamada, *Chem. Ind. (London)*, **1966,** 1967.
(193) J. A. Montgomery and H. J. Thomas, *Chem. Commun.*, **1969,** 458.
(194) M. A. Stevens, H. W. Smith, and G. B. Brown, *J. Amer. Chem. Soc.*, **82,** 3189(1960).
(195) P. Brookes and P. D. Lawley, *J. Chem. Soc.*, **1960,** 539.
(196) P. Brookes, A. Dipple, and P. D. Lawley, *J. Chem. Soc., C*, **1968,** 2026.
(197) G. B. Chheda and R. H. Hall, *Biochemistry*, **5,** 2082(1966).
(198) G. B. Chheda, R. H. Hall, and P. M. Tanna, *J. Org. Chem.*, **34,** 3498(1969).
(199) G. B. Chheda and R. H. Hall, *ibid.*, **34,** 3492(1969).
(200) C. E. Carter, *J. Biol. Chem.*, **223,** 139(1956).
(201) C. Temple, Jr., C. L. Kussner, and J. A. Montgomery, *J. Org. Chem.*, **30,** 3601(1965).
(202) *Ibid.*, **31,** 2210(1966).
(203) *Ibid.*, **32,** 2241(1967).
(204) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic, New York, N. Y., 1963, p. 20.
(205) K. Kusashio and M. Yoshikawa, *Bull. Chem. Soc. Jap.*, **41,** 142(1968).
(206) J. A. Montgomery, H. J. Thomas, and K. Hewson, *Chem. Ind. (London)*, **1965,** 1596.
(207) J. W. Marsico and L. Goldman, *J. Org. Chem.*, **30,** 3597 (1965).
(208) E. Shaw, *ibid.*, **27,** 883(1962).
(209) M. Ikehara and K. Muneyama, *Chem. Pharm. Bull.*, **14,** 46(1966).
(210) M. E. King and C. E. Carter, *Biochim. Biophys. Acta*, **44,** 232(1960).

(211) A. Albert, *Chem. Commun.*, **1969,** 500; *J. Chem. Soc.*, **1969,** 2379.
(212) R. Gompper, M. Gäng, and F. Saygin, *Tetrahedron Lett.*, **1966,** 1885.
(213) M. Franks, C. P. Green, G. Shaw, and G. J. Litchfield, *J. Chem. Soc., C*, **1966,** 2270.
(214) G. J. Litchfield and G. Shaw, *Chem. Commun.*, **1965,** 563.
(215) N. J. Cusack, G. J. Litchfield, and G. Shaw, *ibid.*, **1967,** 799.
(216) G. H. Hitchings and G. B. Elion, U. S. patent 2756228 (1956).
(217) A. Yamazaki, I. Kumashiro, T. Takenishi, and M. Ikehara, *Chem. Pharm. Bull.*, **16,** 2172(1968).
(218) S. Yamazaki, T. Meguro, and I. Kumashiro, Japanese patent 23428 (1967); through *Chem. Abstr.*, **69,** 36126j(1968).
(219) *Ibid.*, Japanese patent 00705 (1968); through *Chem. Abstr.*, **69,** 52137u(1968).
(220) T. Meguro, T. Saito, I. Kumashiro, and T. Takenishi, Japanese patent 1534 (1966); through *Chem. Abstr.*, **64,** 12781e (1966).
(221) E. C. Taylor and P. K. Loeffler, *J. Amer. Chem. Soc.*, **82,** 3147(1960).
(222) A. Yamazaki, I. Kumashiro, and T. Takenishi, *Chem. Pharm. Bull.*, **16,** 1561(1968).
(223) N. P. Sen and P. L. McGeer, *J. Chromatogr.*, **20,** 147(1965).
(224) A. Yamazaki, I. Kumashiro, and T. Takenishi, *J. Org. Chem.*, **32,** 1825(1967).
(225) T. Shiro, A. Yamanoi, S. Konishi, S. Okumura, and M. Takahashi, *Agr. Biol. Chem. (Tokyo)*, **26,** 785(1962); through *Chem. Abstr.*, **58,** 7121b(1963).
(226) J. R. De Zeeuw, U. S. patent 3173848 (1965); through *Chem. Abstr.*, **63,** 2359d(1965).
(227) H. T. Huang, *Biochemistry*, **4,** 58(1965).
(228) H. T. Huang, U. S. patent 3271386 (1966); through *Chem. Abstr.*, **66,** 36593x(1967).
(229) H. Hama, H. Okada, I. Kinoshita, T. Shiro, and T. Sumita, Japanese patent 13795 (1966); through *Chem. Abstr.*, **65,** 19275c (1966).
(230) T. Hirahara, Y. Suzuki, and T. Nakamura, Japanese patent 27660 (1967); through *Chem. Abstr.*, **69,** 18036a(1968).
(231) S. Konishi, T. Shiro, and N. Katsuya, Japanese patent 19833 (1968); through *Chem. Abstr.*, **70,** 18925k(1969).
(232) O. Kanemitsu, T. Akimoto, M. Ito, and S. Mimura, Japanese patent 20717 (1968); through *Chem. Abstr.*, **70,** 27669g (1969).
(233) Chas. Pfizer and Co., British patent 1011346 (1965); through *Chem. Abstr.*, **64,** 7330c(1966).
(234) K. Mitsugi, T. Shiro, S. Okumura, and M. Takahashi, Japanese patent 20551 (1965); through *Chem. Abstr.*, **64,** 4231c (1966).
(235) Kyowa Fermentation Industry Co., Ltd., French patent 1425028 (1966); through *Chem. Abstr.*, **65,** 9700d(1966).
(236) S. Knoishi, I. Maeyashiki, and T. Shiro, Japanese patent 19832 (1968); through *Chem. Abstr.*, **70,** 27662z(1969).
(237) A. Yamazaki, I. Kumashiro, and T. Takenishi, *J. Org. Chem.*, **32,** 3032, 3258(1967).
(238) G. R. Greenberg, *J. Biol. Chem.*, **190,** 611(1951).
(239) G. R. Greenberg, *J. Amer. Chem. Soc.*, **74,** 6307(1952).
(240) G. R. Greenberg, *Fed. Proc.*, **12,** 651(1953).
(241) G. R. Greenberg and E. L. Spilman, *J. Biol. Chem.*, **219,** 411(1956).
(242) J. M. Weaver and W. Shive, *J. Amer. Chem. Soc.*, **75,** 4628 (1953).
(243) J. S. Gots, *Nature*, **172,** 256(1953).
(244) G. R. Greenberg, *J. Biol. Chem.*, **219,** 423(1956).
(245) G. R. Greenberg and L. Jaenicke, in "Chemistry and Biology of Purines," Ciba Foundation Symposium, G. E. W. Wolstenholme and C. M. O'Connor, Eds., J. & A. Churchill Ltd., London, England, 1957, pp. 204–232.
(246) J. M. Buchanan, J. G. Flaks, S. C. Hartman, B. Levenberg, L. N. Lukens, and L. Warren, *ibid.*, pp. 233–252.
(247) J. M. Buchanan and S. C. Hartman, in "Advances in Enzymology," vol. 21, F. F. Nord, Ed., Interscience, New York, N. Y., 1959, pp. 199–261.
(248) J. M. Buchanan, in "The Nucleic Acids," vol. 3, E. Chargaff and J. N. Davidson, Eds., Academic, New York, N. Y., 1960, pp. 303–322.
(249) G. B. Elion and G. H. Hitchings, in "Advances in Chemotherapy," vol. 2, A. Goldin, F. Hawking, and R. J. Schnitzer, Eds., Academic, New York, N. Y., 1965, pp. 91–177.
(250) M. E. Balis, in "Frontiers of Biology," vol. 10, A. Neuberger and E. L. Tatum, Eds., North-Holland Publishing Co., Amsterdam, The Netherlands, 1968, chaps. 1 and 2.
(251) L. L. Bennett, Jr., and J. A. Montgomery, in "Methods in Cancer Research," vol. 3, H. Busch, Ed., Academic, New York, N. Y., 1967, pp. 549–631.
(252) D. D. Schroeder, A. J. Allison, and J. M. Buchanan, *J. Biol. Chem.*, **244,** 5856(1969).
(253) J. S. Gots and E. G. Gollub, *Proc. Nat. Acad. Sci. USA*, **43,** 826(1957).
(254) R. W. Miller and J. M. Buchanan, *J. Biol. Chem.*, **237,** 491(1962).
(255) F. Ahmad, P. Missimer, and A. G. Moat, *Can. J. Biochem.*, **43,** 1723(1965).
(256) D. P. Nierlich and B. Magasanik, *J. Biol. Chem.*, **240,** 366(1965).
(257) L. N. Lukens and J. M. Buchanan, *ibid.*, **234,** 1799(1959).
(258) R. Carrington and G. Shaw, *J. Chem. Soc., C*, **1968,** 1957.
(259) D. W. E. Smith and B. N. Ames, *J. Biol. Chem.*, **240,** 3056 (1965).
(260) A. E. Shedlovsky and B. Magasanik, *ibid.*, **237,** 3725(1962).
(261) D. M. Greenberg, *Ann. Rev. Biochem.*, **33,** 633(1964).
(262) S. G. A. Alivisatos and D. W. Woolley, *J. Amer. Chem. Soc.*, **77,** 1065(1955).
(263) S. G. A. Alivisatos and D. W. Woolley, *J. Biol. Chem.*, **221,** 651(1956).
(264) S. G. A. Alivisatos, L. La Mantia, F. Ungar, and B. L. Matijevitch, *ibid.*, **237,** 1212(1962).
(265) A. Liakopoulou and S. G. A. Alivisatos, *ibid.*, **240,** 3110 (1965).
(266) W. S. MacNutt, *Nature*, **166,** 444(1950).
(267) W. S. MacNutt, *Biochem. J.*, **50,** 384(1952).
(268) J. C. Rabinowitz and W. E. Pricer, Jr., *J. Biol. Chem.*, **218,** 189(1956).
(269) J. C. Rabinowitz and W. E. Pricer, Jr., *J. Amer. Chem. Soc.*, **78,** 5702(1956).
(270) G. Ya. Vilenkina, *Vopr. Med. Khim.*, **2,** 450(1956); through *Chem. Abstr.*, **52,** 20491g(1958).
(271) A. E. Braunshtein and G. Ya. Vilenkina, *Biokhimiya*, **23,** 887(1958) [*Biochemistry USSR*, **23,** 839(1958)].
(272) D. M. Smith, R. M. Paul, E. G. McGeer, and P. L. McGeer, *Can. J. Biochem. Physiol.*, **37,** 1493(1959).
(273) P. L. McGeer, E. G. McGeer, and M. C. Griffin, *ibid.*, **39,** 591(1961).
(274) P. L. McGeer, E. G. McGeer, and A. J. Wood, *Can. J. Comp. Med. Vet. Sci.*, **25,** 211(1961).
(275) N. F. Tolkachevskaya and G. Ya. Vilenkina, *Vopr. Med. Khim.*, **11,** 14(1965); through *Chem. Abstr.*, **64,** 8720f(1966).
(276) P. L. McGeer, E. G. McGeer, and R. Hasselback, *Can. Med. Ass. J.*, **85,** 437(1961).
(277) R. F. Coward and P. Smith, *Clin. Chim. Acta*, **12,** 206 (1965).
(278) G. Ya. Vilenkina and F. E. Fainshtein, *Vopr. Med. Khim.*, **7,** 301(1961); through *Chem. Abstr.*, **55,** 27624b(1961).
(279) M. Nishida, Y. Matsui, and Y. Nobuoka, *Yakugaku Kenkyu*, **35,** 128(1963); through *Chem. Abstr.*, **61,** 16549h(1964).
(280) V. Herbert, *Amer. J. Clin. Nutr.*, **20,** 562(1967).
(281) K. Fink and W. S. Adams, *Arch. Biochem. Biophys.*, **126,** 27(1968).
(282) T. L. Perry, S. Hansen, H.-P. Bar, and L. MacDougall, *Science*, **152,** 776(1966).
(283) T. L. Perry, S. Hansen, L. MacDougall, and P. D. Warrington, *Clin. Chim. Acta*, **15,** 409(1967).
(284) P. C. Newell and R. G. Tucker, *Nature*, **215,** 1384(1967).
(285) P. C. Newell and R. G. Tucker, *Biochem. J.*, **106,** 279 (1968).
(286) G. Weitzel, F. Schneider, H. Guglielmi, F. Seif, W.-D. Hirschmann, and J. Durst, *Hoppe-Seyler's Z. Physiol. Chem.*, **348,** 1277(1967).
(287) A. Hoshi, K. Kumagai, and K. Kuretani, *Chem. Pharm. Bull.*, **16,** 2080(1968).

(288) A. Hoshi, F. Kanzawa, K. Kumagai, and K. Kuretani, *Cancer Chemother. Rep.* (Part 1), **53,** 165(1969).
(289) A. Hoshi, Y. Ohsaka, and K. Kuretani, *Chem. Pharm. Bull.*, **17,** 1720(1969).
(290) D. A. Peters and P. L. McGeer, *Can. J. Physiol. Pharmacol.*, **46,** 195(1968).
(291) H. G. Mandel and L. W. Law, *Cancer Res.*, **14,** 808(1954).
(292) H. G. Mandel, J. L. Way, and P. K. Smith, *Biochim. Biophys. Acta*, **23,** 402(1957).
(293) H. T. Huang and G. M. Shull, U. S. patent 3355301 (1967); through *Chem. Abstr.*, **68,** 28636q(1968).
(294) Y. F. Shealy, C. A. Krauth, and J. A. Montgomery, *J. Org. Chem.*, **27,** 2150(1962).
(295) Y. F. Shealy, J. A. Montgomery, and W. R. Laster, Jr., *Biochem. Pharmacol.*, **11,** 674(1962).
(296) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128,** 537(1939).
(297) D. W. Woolley and E. Shaw, *ibid.*, **189,** 401(1951).
(298) Y. F. Shealy, R. F. Struck, L. B. Holum, and J. A. Montgomery, *J. Org. Chem.*, **26,** 2396(1961).
(299) J. G. Erickson, P. F. Wiley, and V. P. Wystrach, in "The Chemistry of Heterocyclic Compounds," vol. 10, A. Weissberger, Ed., Interscience, New York, N. Y., 1956, chap. 1.
(300) Y. F. Shealy, C. A. Krauth, S. J. Clayton, A. T. Shortnacy, and W. R. Laster, Jr., *J. Pharm. Sci.*, **57,** 1562(1968).
(301) C. A. Krauth, Y. F. Shealy, and J. A. Montgomery, *J. Ala. Acad. Sci.*, **33,** 37(1962).
(302) Y. F. Shealy and C. A. Krauth, *J. Med. Chem.*, **9,** 34(1966).
(303) T. L. Loo and E. A. Stasswender, *J. Pharm. Sci.*, **56,** 1016 (1967).
(304) A. D. McLaren and D. Shugar, "Photochemistry of Proteins and Nucleic Acids," Macmillan, New York, N. Y., 1964, pp. 361, 362.
(305) R. B. Field, G. Hoffman, V. S. Waravdekar, and I. Kline, *Cancer Chemother. Rep.* (Part 1), **53,** 199(1969).
(306) J. L. Skibba, G. Ramirez, D. D. Beal, and G. T. Bryan, *Cancer Res.*, **29,** 1944(1969).
(307) K. Hano, A. Akashi, I. Yamamoto, S. Narumi, and H. Iwata, *Gann*, **59,** 207(1968).
(308) E. H. White and H. Scherrer, *Tetrahedron Lett.*, **1961,** 758.
(309) E. H. White, H. Maskill, D. J. Woodcock, and M. A. Schroeder, *ibid.*, **1969,** 1713.
(310) O. Dimroth, *Ber.*, **38,** 670(1905).
(311) L. V. Alferova, B. A. Dolgoplosk, and V. A. Kropachev, *Vysokomolekul. Soedin.*, **2,** 3(1960); through *Chem. Abstr.*, **54,** 23650f(1960).
(312) A. N. Lovtsova, T. N. Shatkina, and O. A. Reutov, *Dokl. Akad. Nauk SSSR*, **183,** 1091(1968) (English translation, p. 1072).
(313) A. Streitwieser, Jr., *J. Org. Chem.*, **22,** 861(1957).
(314) H. Zollinger, "Azo and Diazo Chemistry," Interscience, New York, N. Y., 1961, pp. 120–136.
(315) E. H. White and D. J. Woodcock, in "The Chemistry of the Amino Group," S. Patai, Ed., Interscience, London, England, 1968, chap. 8.
(316) Y. F. Shealy and C. A. O'Dell, *J. Med. Chem.*, **9,** 733(1966).
(317) Y. F. Shealy and C. A. Krauth, unpublished data.
(318) W. R. Laster, Jr., and F. M. Schabel, Jr., unpublished data.
(319) Y. F. Shealy, C. A. Krauth, R. F. Pittillo, and D. E. Hunt, *J. Pharm. Sci.*, **56,** 147(1967).
(320) Y. F. Shealy, C. A. O'Dell, and C. A. Krauth, unpublished data.
(321) K. Hano, A. Akashi, I. Yamamoto, S. Narumi, Z. Horii, and I. Ninomiya, *Gann*, **56,** 417(1965).
(322) H. Nishio, I. Yamamoto, K. Kariya, and K. Hano, *Chem. Pharm. Bull.*, **17,** 539(1969).
(323) Y. F. Shealy and C. A. Krauth, *Nature*, **210,** 208(1966).
(324) Y. F. Shealy, C. A. Krauth, L. B. Holum, and W. E. Fitzgibbon, *J. Pharm. Sci.*, **57,** 83(1968).
(325) G. Hoffman, I. Kline, M. Gang, D. D. Tyrer, J. M. Venditti, and A. Goldin, *Cancer Chemother. Rep.* (Part 1), **52,** 715 (1968).
(326) H. E. Skipper, F. M. Schabel, Jr., and W. S. Wilcox, *Cancer Chemother. Rep.*, **35,** 1(1964).
(327) I. Wodinsky, J. Swiniarski, and C. J. Kensler, *ibid.*, **52,** 393(1968).
(328) D. D. Tyrer, I. Kline, M. Gang, A. Goldin, and J. M. Venditti, *Cancer Chemother. Rep.* (Part 1), **53,** 229(1969).
(329) D. J. Abraham, J. S. Rutherford, and R. D. Rosenstein, *J. Med. Chem.*, **12,** 189(1969).
(330) R. F. Pittillo and D. E. Hunt, *Cancer Chemother. Rep.*, **51,** 213(1967).
(331) A. P. Simonelli, S. C. Mehta, and W. I. Higuchi, 116th Annual Meeting, APhA, Montreal, Canada, May 1969.
(332) R. H. James, P. D. Sternglanz, and Y. F. Shealy, *J. Pharm. Sci.*, **58,** 1193(1969).
(333) P. D. Sternglanz, *ibid.*, **59,** 708(1970).
(334) Y. F. Shealy and C. A. O'Dell, (*a*) *ibid.*, **59,** 1358(1970); and (*b*) to be published.
(335) C. W. Noell and C. C. Cheng, *J. Med. Chem.*, **12,** 545 (1969).
(336) R. F. Pittillo and D. E. Hunt, *Appl. Microbiol.*, **15,** 531 (1967).
(337) D. E. Hunt and R. F. Pittillo, *Proc. Soc. Exp. Biol. Med.*, **125,** 919(1967).
(338) R. F. Pittillo and D. E. Hunt, *ibid.*, **126,** 555(1967).
(339) D. E. Hunt and R. F. Pittillo, *Cancer Chemother. Rep.*, **52,** 553(1968).
(340) I. Yamamoto, *Biochem. Pharmacol.*, **18,** 1463(1969).
(341) P. P. Saunders and G. F. Saunders, Abstracts, 158th National Meeting, ACS, Sept. 1969, No. BIOL 333.
(342) T. L. Loo, E. A. Stasswender, J. H. Jardine, and E. Frei, III, *Proc. Amer. Ass. Cancer Res.*, **8,** 42(1967), abstract 163.
(343) T. L. Loo, J. K. Luce, J. H. Jardine, and E. Frei, III, *Cancer Res.*, **28,** 2448(1968).
(344) T. L. Loo, B. B. Tanner, G. E. Housholder, and B. J. Shepard, *J. Pharm. Sci.*, **57,** 2126(1968).
(345) J. L. Skibba, G. Ramirez, D. D. Beal, R. O. Johnson, and G. T. Bryan, *Proc. Amer. Ass. Cancer Res.*, **10,** 83(1969), abstract 328.
(346) C. MacDonald, N. Wollner, F. Ghavimi, and J. Zweig, *ibid.*, **8,** 43(1967), abstract 167.
(347) K. Hano, A. Akashi, Y. Suzuki, I. Yamamoto, S. Narumi, and H. Iwata, *Jap. J. Pharmacol.*, **17,** 668(1967).
(348) S. K. Carter and J. W. Newman, *Cancer Chemother. Rep.* (Part 3), **1,** 63(1968).
(349) J. K. Luce and W. G. Thurman, *Proc. Amer. Ass. Cancer Res.*, **10,** 53(1969), abstract 209.
(350) G. N. Pershin and L. I. Shcherbakova, *Farmakol. Toksikol.*, **26,** 712(1963).
(351) H. Iwata, I. Yamamoto, and K. Muraki, *Biochem. Pharmacol.*, **18,** 955(1969).
(352) L. J. Wilkoff, E. A. Dulmadge, and G. J. Dixon, *Cancer Chemother. Rep.* (Part 1), **52,** 725(1968).
(353) G. E. Housholder and T. L. Loo, *Life Sci.*, **8** (Part 1), 533 (1969).
(354) R. Preussmann, A. von Hodenberg, and H. Hengy, *Biochem. Pharmacol.*, **18,** 1(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Kettering-Meyer Laboratories, Southern Research Institute, Birmingham, AL 35205*

The author is grateful to Mrs. Joan K. Whitworth for preparing the manuscript and for assistance with source materials; to Mr. Vladimir Minic for assistance with literature compilations; and to Chemotherapy, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Contracts PH43-64-51 and SA-43-ph-1740, and to the C. F. Kettering Foundation for support of investigations cited from these laboratories.

Corneal Absorption Reinforcement of Certain Mydriatics

EUNICE S. N. WANG and E. ROY HAMMARLUND*

Abstract □ By using a prebuffer technique or by adding buffer and/or viscolizer to certain dilute mydriatic solutions, similar physiological effects were obtained as were given by unbuffered solutions that were 10 times more concentrated. Isotonic, sterile, 2.6% sodium borate solution was found to be an effective buffer for the following solutions: 0.1% homatropine HBr, and 1% phenylephrine HCl plus 0.1% cyclopentolate HCl. These concentrations provided adequate dilation for routine eye examinations. The addition of 0.5% hydroxypropyl methylcellulose, 4000 cps., to buffered mydriatic solutions did not show any further increase in pupil size of Caucasians compared to the same buffered solutions without viscolizer. However, when used similarly in the eyes of Orientals, the viscous solutions produced a more marked dilating effect. No eye irritation was reported by any of the human test subjects for any of the dilute experimental eye drops tested. The results of the animal experimentation indicated that the several biological buffers tested [tromethamine, 2-(*N*-morpholino)ethanesulfonic acid, and *N*,*N*-bis(2-hydroxyethyl)glycine] were not as effective as sodium borate for increasing the corneal absorption of the medicinal agents used. The pKa value of cyclopentolate HCl, determined by titration, was found to be 7.93.

Keyphrases □ Mydriatics—corneal absorption reinforcement □ Corneal absorption, mydriatics—reinforcement □ Buffer effect—corneal absorption □ pH effect—mydriatic activity □ Cyclopentolate HCl—pKa determination

Good therapy involves the efficient use of a drug so that the body encounters minimal chemical challenge to achieve the desired effect. In the case of ophthalmic solutions, modification of the vehicle must be continuously studied since it has the potential for improving therapy.

Because the cornea of the eye is more permeable to lipid-soluble than to water-soluble substances, the nonionized form of a drug, which is more lipid soluble, will be absorbed through the cornea more rapidly than the lipid-insoluble ionized form. The degree of dissociation of a drug in tears (normal pH 7.4), predictable from its pKa, and the capacity of tear buffers to overcome the buffer effect of the drug play vital roles in corneal drug absorption. The short-term alteration of pH also may affect the rate of corneal uptake.

Since the physiological effect of alkaloidal eye drops results from an absorption of the free base portion of the alkaloid, greater response should be obtained if the drugs are administered in alkaline media. However, due to the instability of many alkaloids in alkaline solution, this procedure is not usually possible. Previous studies by Boberg-Ans *et al.* (1) have shown that pretreatment of the eye with a drop of sterile, isotonic, 2.6% sodium borate solution (pH 9.2) can markedly reduce the dose required for an alkaloidal drug to produce a mydriatic or miotic response in the eye.

In 1966, Good *et al.* (2) described a series of organic buffers, called "biological buffers," for use in biological systems. No report has appeared on the effect of these biological buffers on the corneal absorption of ophthalmic drugs.

The objective of this study was to investigate some related aspects of prebuffering the eye or of buffering certain mydriatic eye drops with various alkaline buffers along with the utilization of some viscolizers in order to obtain increased physiological response in both rabbit and human eyes.

Since Howard and Lee (3) and others have shown that there is a difference in mydriatic response between persons of different races, both Oriental and Caucasian subjects were included in this study. In addition, the previously unreported dissociation constant for cyclopentolate HCl was determined experimentally.

EXPERIMENTAL AND RESULTS

Subjects and Materials—The experimental subjects employed were Caucasian and Oriental humans, both male and female, and New Zealand white rabbits, female, weight 2–2.5 kg. The mydriatics used were cyclopentolate HCl,[1] homatropine HBr USP,[2] and phenylephrine HCl USP.[3] The buffers utilized were sodium borate decahydrate,[4] *N*,*N*-bis(2-hydroxyethyl)glycine,[5] 2-(*N*-morpholino)ethanesulfonic acid,[6] and tromethamine.[7] The viscolizers employed were hydroxypropyl methylcellulose 4000 cps.[8] and polyvinyl alcohol.[9]

Preparation of Aqueous Solutions of Mydriatics and Buffers—All aqueous drug and buffer solutions (without viscolizer) were prepared and sterilized by filtration through sterile membrane filters[10] (0.45 μ) into sterile dropper bottles; subsequent transfers were made with sterile pipets. Adjustment of the tonicity of the test solutions was not considered. All eye drops prepared were stored in 0.5-oz., light-resistant, sterile, all-glass dropper bottles. To standardize the size of the drops instilled, only droppers that delivered 71–74 drops of water to give exactly 4 ml. when dropped vertically into a small cylindrical graduate were used.

Preparation of Ophthalmic Vehicles Containing Viscolizers—A double concentrated solution of each viscolizer, *i.e.*, 1% hydroxypropyl methylcellulose and 2.8% polyvinyl alcohol, was prepared

[1] Cyclogyl, Schieffelin and Co., New York, N. Y.
[2] Merck Co., Inc., Rahway, N. J.
[3] Neo-Synephrine HCl, Winthrop Laboratories, New York, N. Y.
[4] Reagent grade, J. T. Baker Chemical Co., Phillipsburg, N. J.
[5] Bicine, A grade, Calbiochem, Los Angeles, Calif.
[6] MES, A grade, Calbiochem, Los Angeles, Calif.
[7] Tris, buffer grade, Nutritional Biochemicals Corp., Cleveland, Ohio.
[8] Methocel, 90 HG, premium, Dow Chemical Co., Midland, Mich.
[9] 99% hydrolyzed; Matheson, Coleman and Bell, Cincinnati, Ohio.
[10] Swinnex, Millipore Filter Corp., Bedford, Mass.

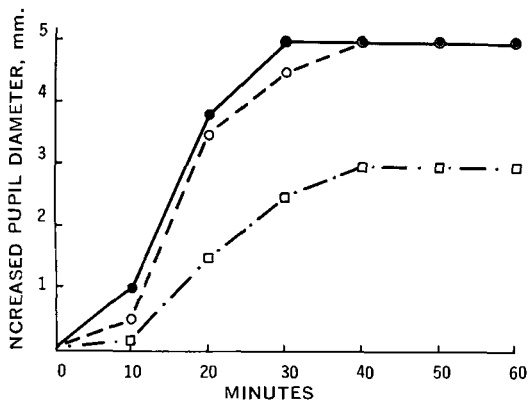


Figure 1—*Mydriatic effect of dilating solutions on humans (Caucasian), both with and without borate prebuffer. Key: ●, unbuffered concentrated dilating solution; ○, dilute dilating solution with borate prebuffer; and □, unbuffered dilute dilating solution.*

and autoclaved at 15 p.s.i. for 30 min. After cooling, a clear dispersion resulted. Equal volumes of the doubly concentrated sterile stock viscolizer solution and the doubly concentrated sterile aqueous mydriatic solution were mixed together before instillation, thereby giving the desired final experimental concentration. The final viscolizer concentrations of 0.5% hydroxypropyl methylcellulose and 1.4% polyvinyl alcohol were selected because those concentrations are often employed commercially.

Measurement of Pupil Size—Rabbits were placed on top of empty rabbit cages, the height of which subdued them sufficiently so that no further restraint was necessary; humans were seated in an evenly lighted room. A ruler with a millimeter scale was held firmly against the face, and the horizontal diameter of the pupil was estimated to the nearest 0.5 mm. in a similar manner in all cases. The pupil diameters were measured before the initial instillation of drops and at various time intervals thereafter. The results are presented as the average millimeter of increased diameter of at least two human or three rabbit subjects in each case.

Although statistical analysis could have been used in this study, it would contribute little. The range of values from which the averages were derived for the controls did not overlap those for the test subjects in any instance.

Determination of Threshold for Mydriatics in Rabbits—Before undertaking the mydriatic study in rabbits, it was necessary to ascertain the proper concentration of drug to give the desired response in rabbit eyes. The threshold concentration sought was the minimum concentration that would give a measurable mydriatic response but which was still small enough so that any increase in response brought about by any adjuvant would be apparent. The rabbit threshold concentrations were found to be 0.0075% for

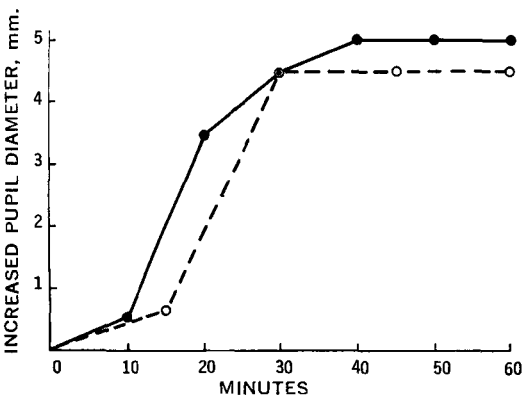


Figure 2—*Mydriatic effect of dilute dilating solution on humans (Caucasian) with borate prebuffer and in freshly prepared, 8-, and 24-hr.-old borate buffers. Key: ●, identical results from prebuffered eye, freshly prepared buffered dilute dilating solution, and 8-hr.-old buffered dilute dilating solution; and ○, 24-hr.-old buffered dilute dilating solution.*

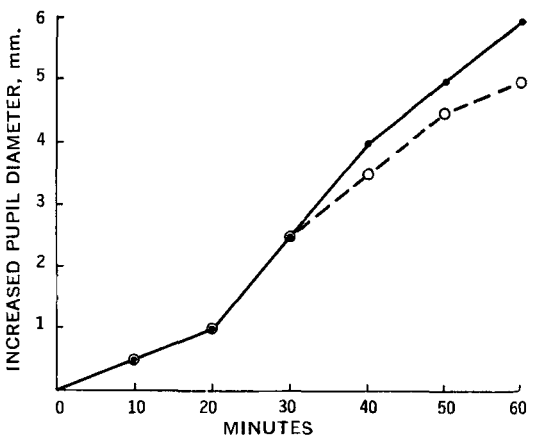


Figure 3—*Mydriatic effect on Orientals of buffered dilute dilating solution with and without viscolizer. Key: ●, borate-buffered dilute dilating solution with hydroxypropyl methylcellulose viscolizer; and ○, same solution without viscolizer.*

homatropine HBr and 0.02 and 0.002%, respectively, for the combination phenylephrine HCl and cyclopentolate HCl solution.

Comparison of Effects of Combination Solution of Phenylephrine HCl and Cyclopentolate HCl in Different Concentrations, Both with and without Sodium Borate Prebuffering—The combination solution of 10% phenylephrine HCl and 1% cyclopentolate HCl, which is used routinely in many eye clinics, is designated as "concentrated dilating solution," and a combination solution of exactly one-tenth this concentration is designated as "dilute dilating solution" throughout this study. The mydriatic effect and relative amount of eye irritation of both solutions were tested on human subjects. One drop of the concentrated dilating solution was instilled in one eye of the test subject, and one drop of the dilute dilating solution was instilled immediately in the other eye. Measurements of the pupil size were taken immediately before and every 10 min. after instillation for 1 hr. Figure 1 shows that the dilute dilating solution gave a weaker response than did the concentrated solution. The test subjects reported that the concentrated dilating solution was quite painful to the eye but that the dilute dilating solution was not.

In another series of experiments, one drop of sterile 2.6% sodium borate solution (pH 9.2) was first instilled in one eye of the test subject. After a few seconds, any excess buffer solution on the eye lid was wiped off with soft tissue, and this prebuffered eye received one drop of the dilute dilating solution while the unbuffered eye received one drop of the concentrated dilating solution. Figure 1 shows that there was a similar mydriatic response in both eyes, with both pupils reaching the same maximum diameter at about the same time. The degree of dilation of both eyes was confirmed by an ophthalmologist at the eye clinic as being adequate for any routine eye examination; the borate prebuffer solution gave no eye irritation.

The foregoing experiments were first carried out on rabbit eyes using the previously mentioned threshold concentration of the combination dilating solution. The results on rabbits corresponded to those reported in Fig. 1 for humans.

Use of Combination 1% Phenylephrine HCl and 0.1% Cyclopentolate HCl Solution in a Sodium Borate Buffered Vehicle, Both with and without Viscolizer—To lessen the number of drops that must be given a patient, an attempt was made to combine the borate buffer solution (both with and without viscolizer) and the dilute dilating solution so that the technique of prebuffering the eye was unnecessary. The buffered drops had a pH of 9.2. A brief study of the stability of the agents in aqueous, viscous and nonviscous, alkaline solutions was in order.

Comparison tests of the degree of mydriasis obtained were conducted first with rabbit eyes and then with a series of humans, using freshly prepared, dilute dilating solution in sodium borate buffer *versus* similar solutions which were 8 and 24 hr. old. All tests were made by instilling in a like manner one solution in one eye while the solution to which it was being compared was instilled in the other. The results for the average of three humans for each solution are presented in Fig. 2. The rabbit tests gave

similar responses. Clinically satisfactory and practically identical mydriatic and cycloplegic effects were obtained from the prebuffered solution and the freshly prepared and the 8-hr. old alkaline solution, but the 24-hr.-old alkaline solution produced slightly less mydriatic effect than did the others. No irritation to the eye was felt by any of the subjects receiving any of these alkaline, dilute dilating solutions. There was no change in physical appearance of the 24-hr.-old alkaline solution. However, after standing at room temperature for 3 days, the solution turned slightly yellow.

The addition of 0.5% hydroxypropyl methylcellulose to the freshly prepared sodium borate buffered, dilute dilating solution did not produce any increased mydriasis in the eyes of Caucasians because they were already at maximum dilation. However, when this viscous, dilute dilating solution was compared in Oriental eyes to the same solution without viscolizer, an increase in the degree of mydriasis in Oriental eyes was produced by the more viscous solution (Fig. 3). Since Oriental eyes are known to be more difficult to dilate than those of Caucasians, it was not surprising that one drop of the buffered, viscous, dilute dilating solution did not produce a completely satisfactory dilation, as shown in Fig. 3. It was necessary to instill a second drop 15 min. later into the eyes of Orientals to effect comparable dilation; these results are shown in Fig. 4 along with a one-drop dose of concentrated dilating solution. Comparison with Fig. 1 shows how much less effective one drop of concentrated dilating solution is for Orientals (Fig. 4) than for Caucasians (Fig. 1).

Effect of Various Prebuffers on Rabbit Corneal Absorption of Certain Mydriatics—In this study on rabbits, the enhanced mydriatic effect produced by using 2.6% sodium borate solution as a prebuffer was compared to the effect given by use of the following three biological buffers: 0.2 *M* *N,N*-bis(2-hydroxyethyl)glycine, 0.2 *M* 2-(*N*-morpholino)ethanesulfonic acid, and 0.2 *M* tromethamine for aqueous homatropine HBr solutions. The tests were all made in a similar manner, with sodium borate prebuffer solution being instilled in one eye of each rabbit and one of the biological buffer solutions dropped in the other eye prior to the instillation of the mydriatic solution. The diameter of the pupils was measured every 15 min. thereafter for 2 hr. and represented the average given by three or more rabbits in all cases. The results, shown in Fig. 5, indicate that the largest increase in mydriatic effect was given by the buffer with the higher pH and the smallest increase was given by the buffer with the lowest pH. A similar test on rabbits was made using sodium borate prebuffer solution in one eye and no buffer in the other eye to find out how much the mydriatic effect of a combination solution of phenylephrine HCl and cyclopentolate HCl is increased in rabbit eyes by prebuffering with sodium borate. The results, shown in Fig. 6, indicate that prebuffering markedly reinforces the mydriatic effect of phenylephrine and cyclopentolate solution in rabbit eyes.

Effect of Viscolizers on Rabbit Corneal Absorption of Homatropine HBr Solution—A series of rabbits received one drop of aqueous 0.0075% homatropine HBr solution in one eye and one drop of the same homatropine solution prepared in either 1.4% polyvinyl alcohol or 0.5% hydroxypropyl methylcellulose in the other eye. Single-drop doses rather than measured volumes were used, because when the droppers were calibrated, the sizes of the drops were found to vary less than 6% whether or not they contained a viscolizer. The procedure was carried out in the same manner as described previously; the measurement of the pupil diameters was taken every 15 min. for 2 hr. The results are shown in Fig. 7 and indicate that both viscolizers reinforce the mydriatic effect of homatropine HBr solution but that the increase given by hydroxypropyl methylcellulose was much greater than that given by polyvinyl alcohol. This increased mydriatic effect produced by the drops containing 0.5% hydroxypropyl methylcellulose paralleled the somewhat greater viscosity that it had, *i.e.*, 13.2 cps. *versus* 2.3 cps. for the drops containing 1.4% polyvinyl alcohol.

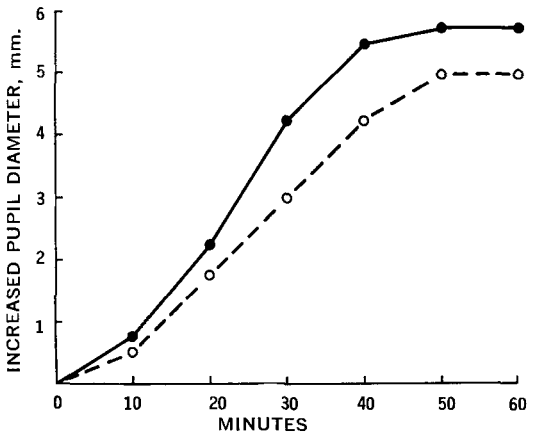


Figure 4—*Mydriatic effect on Orientals of concentrated dilating solution and two doses of buffered, viscous, dilute dilating solutions. Key: ●, borate-buffered, viscous, dilute dilating solution (two doses instilled); and ○, unbuffered concentrated dilating solution (one dose instilled).*

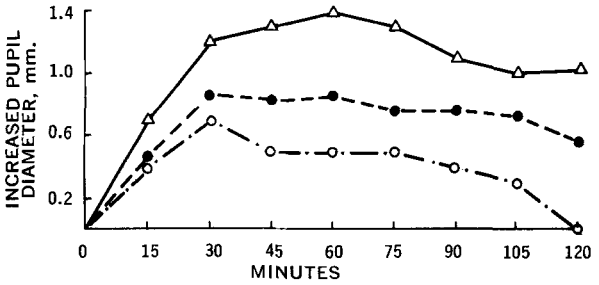


Figure 5—*Effect of various prebuffer solutions on rabbit corneal absorption of 0.0075% homatropine HBr solution. Key: △, with sodium borate prebuffer (pH 9.2); ●, with no buffer or with N,N-bis(2-hydroxyethyl)glycine prebuffer (pH 8.4) or with tromethamine prebuffer (pH 8.3); and ○, with 2-(N-morpholino)ethanesulfonic acid prebuffer (pH 6.2).*

Determination of pKa of Cyclopentolate HCl—An aqueous solution of known concentration of cyclopentolate HCl was neutralized with a stoichiometric quantity of standardized NaOH solution to liberate free cyclopentolate. A titration curve of the free cyclopentolate was obtained by titrating the neutralized solution with standardized dilute HCl, using a Beckman Zeromatic pH meter. The midpoint of the titration curve was ascertained, and the pKa value of cyclopentolate HCl was determined by finding the pH value that corresponded to this midpoint. In this way the pKa of cyclopentolate HCl was found to be 7.93, as shown in Fig. 8.

DISCUSSION AND CONCLUSIONS

The experimental data obtained from this investigation indicate that the physiological response of several mydriatic eye drops can be enhanced by proper utilization of certain buffers and viscolizers, with 2.6% sodium borate solution being the most effective buffer and 0.5% hydroxypropyl methylcellulose being the most effective viscolizer of those tested.

A comparison of the amount of the free base portion of an amine ophthalmic drug present at pH 9.2 (the pH of 2.6% sodium borate

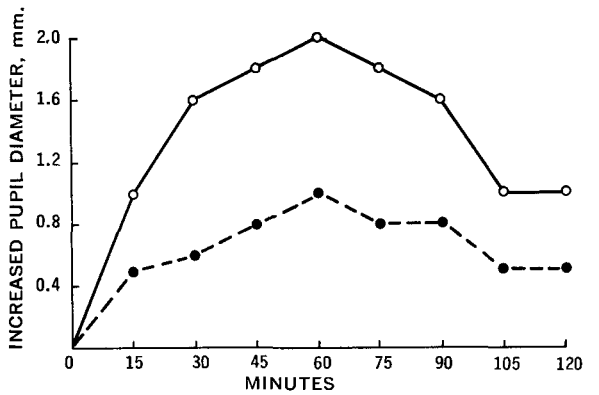


Figure 6—*Mydriatic effect on rabbits of combination solution of 0.02% phenylephrine HCl and 0.002% cyclopentolate HCl, both with and without borate prebuffer. Key: ○, with sodium borate prebuffer; and ●, without sodium borate prebuffer.*

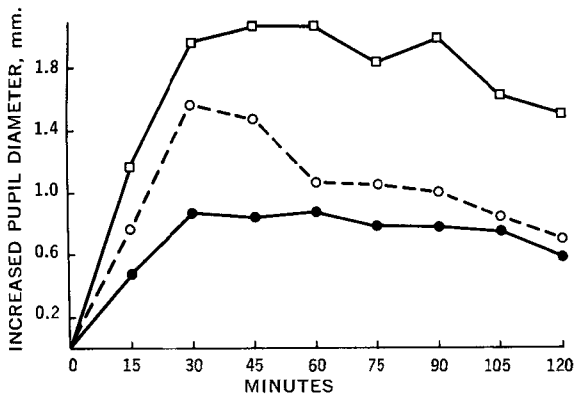


Figure 7—*Effect of viscolizers on rabbit corneal absorption of 0.0075% homatropine HBr solution. Key: ●, 0.0075% homatropine HBr solution; ○, same plus 1.4% polyvinyl alcohol; and □, same plus 0.5% hydroxypropyl methylcellulose.*

solution) and at pH 7.4 (the pH of normal tear fluid) by using the pKa of the particular drug can be used to predict whether or not buffering the eye to pH 9.2 would enhance the corneal absorption of this drug. For example, with phenylephrine HCl, which has a pKa of 8.86, the amount of free base present at pH 9.2 would at maximum be about 22 times greater than that present at pH 7.4; in a similar manner for homatropine HBr (pKa 9.7), the free base form would be about 48 times greater at pH 9.2. In the case of pilocarpine HCl with a pKa of only 6.85, the concentration of the free base portion at pH 9.2 is only about 1.3 times greater than that present at pH 7.4. This increase in quantity of free base is too small an amount to show any measurable increase in miotic effect. A study of pilocarpine HCl showed no measurable decrease in pupil diameter due to the prebuffer treatment with sodium borate.

Since the pH on the surface of the eye may fluctuate due to the loss of carbon dioxide and sometimes become as high as 8 (rather than 7.4), and since the pH of the mixed sodium borate solution and tear fluid which is continuously being diluted with more tears will decrease and be lower than 9.2 for much of the time after its initial instillation, the actual ratio of the free base molecules of instilled amine drops on buffered and unbuffered eyes will be considerably smaller than the theoretical calculated value. This study showed that the mydriatic effect of the combination solution of phenylephrine HCl and cyclopentolate HCl and of homatropine HBr each was increased about 10-fold when the sodium borate prebuffer method or borate-buffered drops were used. If the theoretical optimum buffering conditions could have prevailed, the mydriatic solutions should have given physiological responses of 2–4 times the experimental findings.

Although the most appropriate pH for eye drops is usually reported as being 7.4 for maximum eye comfort, Trolle-Lassen (4) and others have found that the eye can tolerate an isotonic solution up to about pH 9.7 as long as it has no other irritating properties. Therefore, the sterile, isotonic, 2.6% sodium borate solution is a suitable nonirritating buffer for ophthalmic use whenever the therapeutic drug has a sufficiently high pKa value for its effect to be appreciably enhanced.

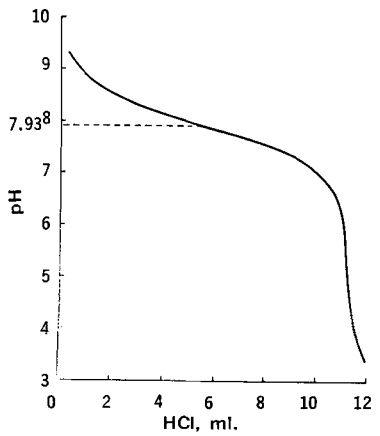


Figure 8—*Titration curve of cyclopentolate.*

Recommended Dilating Solutions for Routine Eye Examination—The following two rather dilute mydriatic preparations will give a physiological response equal to that of the frequently used more concentrated dilating solution composed of 10% phenylephrine HCl and 1% cyclopentolate HCl:

1. If the administration of a drop dose of each of two separate solutions presents no problem, it is recommended that one drop of sterile, 2.6% aqueous sodium borate solution be first instilled in the eye, followed immediately by one drop of a sterile, aqueous, combination solution of 1% phenylephrine HCl and 0.1% cyclopentolate HCl. This is the so-called prebuffering technique.

2. If it is desired to have the total medication in a single drop, as it might be for children, one drop of a special solution should be instilled which is prepared by mixing together equal volumes of the aqueous mydriatic and viscous buffer solutions not more than 8 hr. prior to their use. The buffer solution is composed of double concentrations of sodium borate, 5.2%, and hydroxypropyl methylcellulose, 1.0%, and has been sterilized in an autoclave. The mydriatic solution consists of double concentrations of phenylephrine HCl, 2%, and cyclopentolate HCl, 0.2%, and has been sterilized by filtration. Equal volumes of these two solutions, each in a sterile bottle, are mixed by pouring the viscolizer and buffer solution into the dropper bottle containing the mydriatic solution and shaking the contents well for a few seconds to effect complete mixing. The resulting solution will have the recommended concentration of 1.0% phenylephrine HCl, 0.1% cyclopentolate HCl, 0.5% hydroxypropyl methylcellulose, and 2.6% sodium borate, and it should not be used more than 8 hr. after being mixed.

While the use of the buffered solution has the advantage of requiring the instillation of only a single drop, it also has the obvious disadvantage of requiring that the two separate solutions must be mixed together daily since the mixture is only stable for 1 day. Also, when using the prebuffer technique, if there is any excess buffer solution on the cornea or lids following its instillation, it should be wiped off gently with tissue before instillation of the mydriatic drops. Otherwise, due to the occasional limiting capacity of the cul-de-sac of the eye, a portion of the medicinal drop will flow out from the eye and down the cheek. The partial loss of the drop will cause a deficient response. Use of the preparation with both medicament and buffer combined in one solution will avoid this disadvantage.

For use on races other than Caucasian, it is recommended for either buffering method that a repeat instillation be made about 15 min. after the first one to ensure adequate dilation.

Duration of Activity and Decreased Side Effects—All of the dilute mydriatic solutions used with prebuffer or with both buffer and viscolizer studied in this investigation gave mydriatic effects which were of considerably shorter duration than those given by the more concentrated dilating solutions used without buffer, although the duration of action of the various solutions is not being specifically reported in these results. Since it has been found that angle closure glaucoma can be precipitated by dilating the pupil (5), the shorter duration of mydriasis should be a decided advantage for using a dilute mydriatic solution with a buffer rather than a nonbuffered, more concentrated one.

Carpenter (6) states that cyclopentolate in its usual and more concentrated dosage has been found to produce disorientation, hallucinations, apprehension, and cerebral disfunction in some cases, lasting for as long as 11 months in one instance. Another report (7) concludes that cyclopentolate is a strong enough hallucinogen to produce symptoms similar to those of LSD in some persons. Since an appreciable amount of any instilled eye drops descends through the nasolacrimal duct into the nasopharynx and is eventually swallowed or absorbed through the pharyngeal mucosa, a 10-fold less concentration of any therapeutic agent instilled into the eye—if it produces an acceptable therapeutic response—should have the added advantage of producing considerably less side effects.

REFERENCES

(1) J. Boberg-Ans, K. F. Grove-Rasmussen, and E. R. Hammarlund, *Brit. J. Ophthalmol.*, **43**, 670(1959).

(2) N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M Singh, *J. Biochem.*, **5**, 467(1966).

(3) H. J. Howard and T. R. Lee, *Proc. Soc. Exp. Biol. Med.*, **24**, 700(1927).
(4) C. Trolle-Lassen, *Pharm. Weekbl.*, **93**, 148(1958).
(5) E. Friedman, *J. Amer. Med. Ass.*, **205**, 928(1968).
(6) W. T. Carpenter, *Arch. Ophthalmol.*, **78**, 445(1967).
(7) H. H. Mark, *J. Amer. Med. Ass.*, **186**, 430(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 23, 1970, from the *College of Pharmacy, University of Washington, Seattle, WA 98105*

Accepted for publication May 27, 1970.

This research was supported in part by Research Grant EY 00482 from the National Eye Institute, National Institutes of Health, Bethesda, Md.

Abstracted in part from a thesis submitted by Eunice S. N. Wang to the Graduate School, University of Washington, Seattle, Wash., in partial fulfillment of Master of Science degree requirements.

The authors wish to thank Dr. Carl Kupfer, Director, National Eye Institute, National Institutes of Health, formerly Chairman, Ophthalmology Department, University of Washington School of Medicine, for his help with the clinical portion of this investigation.

*To whom inquiries should be addressed.

Interactions of Drugs with Proteins II: Experimental Methods, Treatment of Experimental Data, and Thermodynamics of Binding Reactions of Thymoleptic Drugs and Model Dyes

H. J. WEDER* and M. H. BICKEL

Abstract □ The binding to bovine albumin of the model dyes and drugs—bromocresol green, eosin, imipramine, and desipramine—has been studied using equilibrium dialysis, ultracentrifuge sedimentation, and difference spectrophotometry. An improved apparatus for equilibrium dialysis has been developed. Bromocresol green interacts with two types of binding sites: four ligands are bound by H-bonds, electron-donator-acceptor (and possibly hydrophobic) forces stabilized by electrostatic forces, and three to four ligands are bound by electrostatic forces only. Eosin is bound by van der Waals' forces and electron-donator-acceptor forces to three binding sites and by electrostatic forces to six binding sites. Imipramine interacts with only one type of binding site by van der Waals' and possibly hydrophobic forces, stabilized by dipole-dipole forces and involving tyrosyl residues. There are $n = 6$ binding sites, and the intrinsic association constant $k = 5 \times 10^3$ M^{-1}. Desipramine binding exhibits a more complicated mechanism, probably involving exposure of additional binding sites upon a drug-induced conformational change. Given concentrations of drug and protein yield the free-drug concentration and degree of binding as experimental values. From these the following parameters for each type of binding site have been determined by computer: n, k, free-enthalpy change, enthalpy change, and entropy change. From these parameters, as well as from spectral shifts and dependence on pH, ionic strength, and temperature, the modes and forces of interaction have been deduced according to previously discussed binding models and methods. Identical results are obtained by dialysis and ultracentrifugation if pH, ionic strength, and temperature are kept constant.

Keyphrases □ Plasma protein-drugs—interactions □ Thymoleptics, dyes—bovine albumin binding □ Dyes, thymoleptics—thermodynamics, bovine serum binding □ Thermodynamics—drugs-albumin binding □ Dialysis, equilibrium—albumin binding □ UV spectrophotometry—analysis

It is generally assumed that a small molecule interacting with a biopolymer induces a conformational change which is responsible for the action of the small molecule (drug). Similar interactions or binding of drugs also occur with biopolymers not involved in pharmacological action, *i.e.*, with unspecific receptors such as plasma proteins.

The binding of a small molecule can influence the chemical reactivity at different sites of the macromolecule. These phenomena are mainly due to long-range electrostatic forces, to shorter range specific interactions such as hydrogen and hydrophobic bonds (1), and finally to proton dispersion forces; the latter obey the same laws as London dispersion forces (2). In addition, primary drug–protein complexes are often stabilized by charge transfer forces. These forces, however, should not be used to estimate the overall complex stability, since the interactions are mainly due to van der Waals–London forces. The stability of a drug–protein complex is expressed by its association constant, which is also important for the pharmacokinetic behavior of the drug.

Numerous methods are currently used for the study of drug–protein interactions (3). Thermodynamic methods and optical rotatory dispersion (ORD) or circular dichroism measurements are tools for the detection of drug-induced conformational changes of a biopolymer. Equilibrium dialysis and ultracentrifugation, as well as spectroscopy in the visible and UV range, allow the determination of association constants. The resulting energy and entropy effects are useful parameters for the interpretation of the mechanism of interaction. Difference spectra in the 220–310-mμ range yield information on conformational changes in the environment of phenylalanyl, tyrosyl, and tryptophyl residues. High resolution NMR spectroscopy is a powerful tool for the study of primary binding sites and of drug atoms interacting with the macromolecule. Information on the mobility of the interacting groups can, in this way, be obtained by the determination of relaxation times.

In this paper, the authors present the results of experiments with triphenylmethane dyes and bovine albumin designed to test methods used in the study of drug–protein interactions. Some of these methods were used in the earlier study of tricyclic thymoleptic drugs (4). This paper also contains additional data on the thermodynamics and binding mechanism of the drugs mentioned.

(3) H. J. Howard and T. R. Lee, *Proc. Soc. Exp. Biol. Med.*, **24**, 700(1927).
(4) C. Trolle-Lassen, *Pharm. Weekbl.*, **93**, 148(1958).
(5) E. Friedman, *J. Amer. Med. Ass.*, **205**, 928(1968).
(6) W. T. Carpenter, *Arch. Ophthalmol.*, **78**, 445(1967).
(7) H. H. Mark, *J. Amer. Med. Ass.*, **186**, 430(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 23, 1970, from the *College of Pharmacy, University of Washington, Seattle, WA 98105*

Accepted for publication May 27, 1970.

This research was supported in part by Research Grant EY 00482 from the National Eye Institute, National Institutes of Health, Bethesda, Md.

Abstracted in part from a thesis submitted by Eunice S. N. Wang to the Graduate School, University of Washington, Seattle, Wash., in partial fulfillment of Master of Science degree requirements.

The authors wish to thank Dr. Carl Kupfer, Director, National Eye Institute, National Institutes of Health, formerly Chairman, Ophthalmology Department, University of Washington School of Medicine, for his help with the clinical portion of this investigation.

*To whom inquiries should be addressed.

Interactions of Drugs with Proteins II: Experimental Methods, Treatment of Experimental Data, and Thermodynamics of Binding Reactions of Thymoleptic Drugs and Model Dyes

H. J. WEDER* and M. H. BICKEL

Abstract □ The binding to bovine albumin of the model dyes and drugs—bromocresol green, eosin, imipramine, and desipramine—has been studied using equilibrium dialysis, ultracentrifuge sedimentation, and difference spectrophotometry. An improved apparatus for equilibrium dialysis has been developed. Bromocresol green interacts with two types of binding sites: four ligands are bound by H-bonds, electron-donator-acceptor (and possibly hydrophobic) forces stabilized by electrostatic forces, and three to four ligands are bound by electrostatic forces only. Eosin is bound by van der Waals' forces and electron-donator-acceptor forces to three binding sites and by electrostatic forces to six binding sites. Imipramine interacts with only one type of binding site by van der Waals' and possibly hydrophobic forces, stabilized by dipole-dipole forces and involving tyrosyl residues. There are $n = 6$ binding sites, and the intrinsic association constant $k = 5 \times 10^3$ M^{-1}. Desipramine binding exhibits a more complicated mechanism, probably involving exposure of additional binding sites upon a drug-induced conformational change. Given concentrations of drug and protein yield the free-drug concentration and degree of binding as experimental values. From these the following parameters for each type of binding site have been determined by computer: n, k, free-enthalpy change, enthalpy change, and entropy change. From these parameters, as well as from spectral shifts and dependence on pH, ionic strength, and temperature, the modes and forces of interaction have been deduced according to previously discussed binding models and methods. Identical results are obtained by dialysis and ultracentrifugation if pH, ionic strength, and temperature are kept constant.

Keyphrases □ Plasma protein-drugs—interactions □ Thymoleptics, dyes—bovine albumin binding □ Dyes, thymoleptics—thermodynamics, bovine serum binding □ Thermodynamics—drugs-albumin binding □ Dialysis, equilibrium—albumin binding □ UV spectrophotometry—analysis

It is generally assumed that a small molecule interacting with a biopolymer induces a conformational change which is responsible for the action of the small molecule (drug). Similar interactions or binding of drugs also occur with biopolymers not involved in pharmacological action, *i.e.*, with unspecific receptors such as plasma proteins.

The binding of a small molecule can influence the chemical reactivity at different sites of the macromolecule. These phenomena are mainly due to long-range electrostatic forces, to shorter range specific interactions such as hydrogen and hydrophobic bonds (1), and finally to proton dispersion forces; the latter obey the same laws as London dispersion forces (2). In addition, primary drug–protein complexes are often stabilized by charge transfer forces. These forces, however, should not be used to estimate the overall complex stability, since the interactions are mainly due to van der Waals–London forces. The stability of a drug–protein complex is expressed by its association constant, which is also important for the pharmacokinetic behavior of the drug.

Numerous methods are currently used for the study of drug–protein interactions (3). Thermodynamic methods and optical rotatory dispersion (ORD) or circular dichroism measurements are tools for the detection of drug-induced conformational changes of a biopolymer. Equilibrium dialysis and ultracentrifugation, as well as spectroscopy in the visible and UV range, allow the determination of association constants. The resulting energy and entropy effects are useful parameters for the interpretation of the mechanism of interaction. Difference spectra in the 220–310-mμ range yield information on conformational changes in the environment of phenylalanyl, tyrosyl, and tryptophyl residues. High resolution NMR spectroscopy is a powerful tool for the study of primary binding sites and of drug atoms interacting with the macromolecule. Information on the mobility of the interacting groups can, in this way, be obtained by the determination of relaxation times.

In this paper, the authors present the results of experiments with triphenylmethane dyes and bovine albumin designed to test methods used in the study of drug–protein interactions. Some of these methods were used in the earlier study of tricyclic thymoleptic drugs (4). This paper also contains additional data on the thermodynamics and binding mechanism of the drugs mentioned.

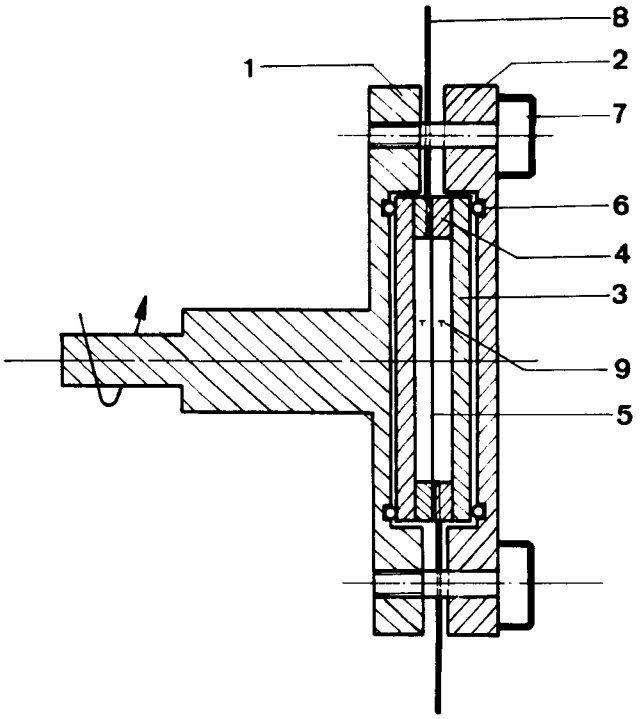


Figure 1—*Apparatus for equilibrium dialysis. Key: 1, Plexiglas core; 2, Plexiglas lid; 3, glass disk; 4, Teflon ring; 5, membrane; 6, rubber ring; 7, binding screw; 8, Cr–Ni tubes, 1.4-mm. diameter; and 9, surface of liquid in dialysis cells. For further details, see* Experimental.

EXPERIMENTAL

Materials—The purity of the dyes, bromocresol green (BKG) and eosin (EOS),[1] was checked by TLC. All other compounds used were described previously (4). The buffer solutions are indicated in the respective tables and figures.

Spectrophotometry—All measurements were carried out at 20 ± 2° with a Unicam UV spectrophotometer SP 800 and a 1-cm. cell. The following molar extinction coefficients have been determined for BKG (ϵ^{20}_{616}) and EOS (ϵ^{20}_{515}), respectively: 2.93×10^4 and 5.94×10^4 (pH 7.0 phosphate buffer, 0.01 *M*), 2.04×10^4 and 5.50×10^4 (pH 5.0 disodiumcitrate buffer, 0.05 *M*), and 1.32×10^4 and 3.26×10^4 (pH 3.5 citric acid–phosphate buffer 0.05 *M*). The relative errors were <3%.

To use a spectral change for the determination of the free and protein-bound dye, the following conditions must be fulfilled:

1. There is no absorbance of the protein in the spectral range used.
2. Free and bound ligands obey Beer's law in the concentration range used.
3. Extinction coefficient (ϵ) of bound ligand is independent of degree of binding (r).
4. There is the appearance of an isosbestic point ($\epsilon_{free} = \epsilon_{bound}$).

Calculations of association constants can be carried out according to Benesi and Hildebrand (6) or Hammes and Schimmel (7). The molar ratio of an interaction can be determined by extrapolation of the difference spectrophotometric titration curve at saturation ($r = n$) obtained by varying the concentration of either ligand or protein.

Equilibrium Dialysis—The fraction of a ligand bound to a protein can be determined by measuring the free ligand concentration in the protein-free compartment. Membrane effects can be controlled if the ligand concentration can be determined in both compartments. Under certain conditions (8), the Donnan effect must be corrected for (9, 10). The devices used for equilibrium dialysis range from simple cellophane bags (11–13) to specially designed apparatus (14, 15). The one developed by the authors contains two cells (3.0 ml., 0.27-cm. width), separated by a regenerated cellulose membrane of a medium pore size of 3–5 mμ and a weight of 12.8 g./m.^{2}. The vertically positioned wheel-shaped double cell is rotated at 100 r.p.m. Each cell contains two metal tubes in opposite position. They are used for filling and emptying the cells and are connected by a plastic tube during dialysis. Further details are shown in Fig. 1. The main advantages (14) of this device, as compared to others, are a constant solution volume (pressure compensation), minimal dilution effects, short equilibration times, and low risk of denaturation.

To suppress Donnan effects, the buffer solutions contained 0.9% NaCl. The dialyses were carried out at 20 ± 1 and 4 ± 1°, respectively. The experimentally determined equilibration times were in the 2–3 hr. range for all ligands used [imipramine (IP), desipramine (DMI), BKG, and EOS]. Addition of these ligands to the buffer cell or to the protein cell led to identical results. In the latter case, an incubation time of 30 min. was allowed before dialysis. The binding reactions have been found to be reversible. The weak adsorption of IP and DMI to the membrane has been controlled by measuring the equilibration concentrations in both cells. The concentrations were determined either by measurement of radioactivity (4) or spectrophotometry.

Ultracentrifugation—In a strong gravitational field, sedimentation of proteins and protein–ligand complexes can be obtained whereas the free ligand concentration remains unchanged. The method described by Steinberg and Schachman (16, 17) has been used.

Ultracentrifugation was carried out at 200,000 × *g* for 16 hr. at 21 ± 1°.

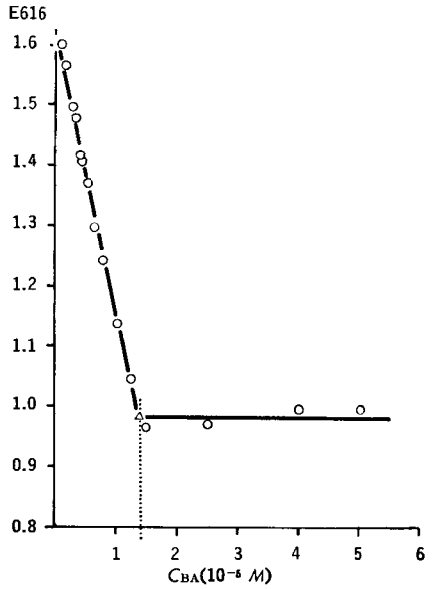


Figure 2—*BKG–BA interaction (pH 7.00). Saturation curve obtained by spectrophotometric titration. For further details, see* Results.

CALCULATION OF BINDING PARAMETERS

The basic theory of the calculation of ligand–protein equilibria has been described in detail (11, 13, 18, 19). In many cases, a clear interpretation of experimental data is hampered by the occurrence of more than one type of binding site, one or more interacting types of binding sites, or by cooperative binding phenomena. In certain cases, particularly when important chemical and physical characteristics of the macromolecule are known, the correction function, $f(r)$, can lead to the desired result (20).

Various curve-fitting techniques can be employed for the determination of the number of individual binding sites and association constants by solving a set of i simultaneous linear equations for a system of i binding types (21).

A graphic method, employed when the characteristics and concentration of the protein are unknown, has been described by Rosenthal (22), making it possible to determine the physicochemical parameters of binding systems which consist of one ligand and one or more proteins containing one or more noninteracting binding

[1] Purchased from Fluka Ltd., Buchs, Switzerland.

Table I—BKG–BA Interaction Showing Binding Parameters and Comparison of Methods at 21°

Method	pH	Ionic Strength	n_1[a]	n_2[a]	k_1,[b] 10^5 l./mole	k_2, 10^3 l./mole	$\Delta F_1°$,[c] cal./mole	$\Delta F_2°$, cal./mole
DIA[d]	7.0	0.19[e]	4.07 ± 0.18	3–4[f]	5.48 ± 0.25	—	−7718	—[f]
UCS[g]	7.0	0.19[e]	4.11 ± 0.21	3–4[f]	5.62 ± 0.34	—	−7732	—[f]
UCS	7.0	0.04[h]	4.19 ± 0.12	3–4[f]	13.2 ± 0.4	—	−8231	—[f]
TIT[i]	7.0	0.04[h]	3.6	—	—	—	—	—
UCS	5.0	0.24[j]	58 ± 2	44 ± 4	0.06 ± 0.03	1.31 ± 0.10	−4942	−4192
UCS	3.5	0.25[k]	108 ± 8	0	0.47 ± 0.04	0	−5113	0

[a] n = number of (primary and secondary) binding sites. [b] k = intrinsic association constant. [c] $\Delta F°$ = free-enthalpy change. [d] DIA = equilibrium dialysis. [e] Phosphate buffer, 0.01 *M* + NaCl, 0.9%. [f] See *Discussion*. [g] UCS = ultracentrifuge sedimentation. [h] Phosphate buffer, 0.01 *M*. [i] TIT = spectrophotometric titration. [j] Disodium citrate buffer, 0.05 *M*. [k] Citric acid–phosphate buffer, 0.05 *M*.

sites. This method also yields information about the distribution of the ligands on the different types of binding sites.

Equations for the characterization of pure hydrophobic interaction mechanisms between phenols (23) or polycyclic hydrocarbons (24) and proteins have been developed from the protein association constants and partition coefficients of the ligand in question. Crothers (25) developed a computational method for the calculation of binding isotherms for heterogeneous polymer systems.

Theoretical studies toward determination of the binding functions of ligands that interact with a polymerizing protein system have been carried out by Nichol *et al.* (26). According to these authors, pronounced sigmoidal binding curves, as in the case of allosteric systems, are to be expected.

RESULTS

BKG–Bovine Albumin (BA)—The binding equilibrium of BKG and BA was investigated using three methods: difference spectrophotometry, equilibrium dialysis, and sedimentation in the ultracentrifuge.

The spectrophotometric titration of 5×10^{-5} *M* BKG solutions in 0.01 *M* phosphate buffer, pH 7.0, with corresponding BA solutions (molar excess range of BKG 1 to 100) yielded at saturation a binding stoichiometry of BA–BKG = 1:3.6 (Fig. 2). Difference spectra, involving constant BA concentrations and variable BKG concentrations with corresponding BKG solutions as reference, show under like conditions absorption maxima at 413 and 632 mμ, the longwave red-shift maximum yielding the same stoichiometry (1:3.8). Since the absorption maxima of free BKG are 400 and 616 mμ, the charge transfer band of the albumin-bound BKG exhibits a shift of 13 and 16 mμ, respectively. At 616 mμ, a simultaneously appearing hypochromic effect of 32 ± 4% can be measured.

The evaluation of the experimental binding curves, as obtained by means of dialysis and ultracentrifugation, is based on the following model, which has also been successfully employed in the case of EOS–albumin interaction. This model assumes that the use of dilute solutions allows the activities of the components to be equated to their concentrations and, furthermore, that interactions between individual binding sites as well as cooperative binding phenomena are absent or negligible. If r is the mean degree of binding, *i.e.*, number of moles of bound ligand per mole of protein, and c_f the concentration in moles/liter of the free ligand at equilibrium, then

$$r = \sum_{i=1}^{i=q} \frac{n_i \cdot k_i \cdot c_f}{1 + k_i \cdot c_f} \qquad \text{(Eq. 1)}$$

where n_i represents the maximal number of the equivalent binding sites of q different types, and k_i is the corresponding intrinsic association constants. In the case of two types of binding sites, Eq. 1 becomes

$$r = \frac{n_1 \cdot k_1 \cdot c_f}{1 + k_1 \cdot c_f} + \frac{n_2 \cdot k_2 \cdot c_f}{1 + k_2 \cdot c_f} \qquad \text{(Eq. 2)}$$

or for one type of binding site,

$$r = \frac{n_1 \cdot k_1 \cdot c_f}{1 + k_1 \cdot c_f} \qquad \text{(Eq. 3)}$$

or

$$\frac{r}{c_f} = k_1 \cdot n_1 - k_1 \cdot r \qquad \text{(Eq. 4)}$$

Thus, r/c_f as a function of r (Scatchard plot) yields the number of binding sites, n_1, when $r/c_f \to 0$ and the negative slope of the straight line corresponds to the intrinsic association constant k_1. Assuming that $r \neq f(\Delta F_e)$, where ΔF_e is the electrostatic free-energy change, the Scatchard plot yields a curve resulting from interference of the individual curves representing the two types of binding sites. Hence, Eq. 4 can be written

$$\frac{r}{c_f} = \frac{r_1 + r_2}{c_f} = k_1 \cdot n_1 - k_1 \cdot r_1 + k_2 \cdot n_2 - k_2 \cdot r_2 =$$
$$k_1 \cdot n_1 + k_2 \cdot n_2 - [(k_1 - k_2)r_1 + k_2 \cdot r] \qquad \text{(Eq. 5)}$$

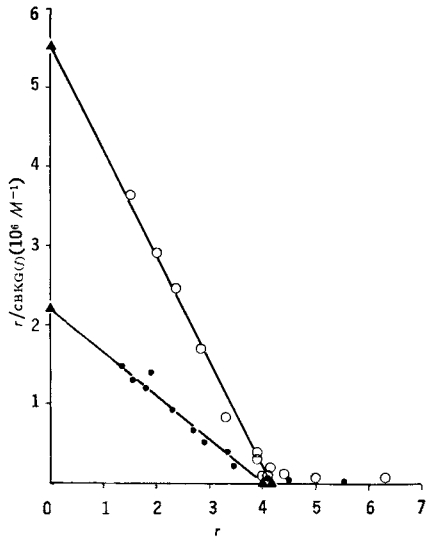


Figure 3—*BKG–BA interaction (pH 7.00). Scatchard plots for primary binding sites. Key: ●, equilibrium dialysis, ionic strength 0.19; and ○, ultracentrifuge sedimentation, ionic strength 0.04. For further details, see* Results.

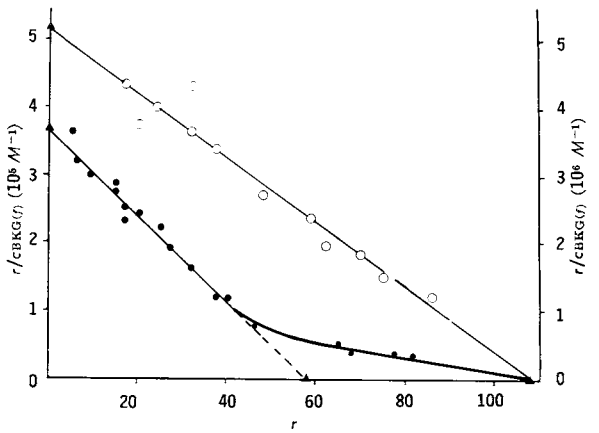


Figure 4—*BKG–BA interaction. Scatchard plots for binding at pH 5.0* (○) *(left scale) and pH 3.5* (●) *(right scale). Ultracentrifuge sedimentation. For further details, see* Results.

Table II—EOS–BA Interaction Showing Binding Parameters by Ultracentrifuge Sedimentation at 21°[a]

pH	Ionic Strength	n_1	n_2	k_1, 10^6 l./mole	k_2, 10^5 l./mole	$\Delta F_1°$, cal./mole	$\Delta F_2°$, cal./mole
7.0	0.04[h]	3.9 ± 0.1	5.8 ± 0.4	5.62 ± 0.22	2.31 ± 0.17	−9077	−7213
7.0	0.19[e]	3.8 ± 0.1	5.6 ± 0.5	9.70 ± 0.51	1.09 ± 0.08	−9324	−6773
5.0	0.24[i]	7.6 ± 0.4	10.6 ± 0.4	0.80 ± 0.04	1.28 ± 0.03	−7938	−6868
3.5	0.24[i]	65 ± 5	0	4.09 ± 0.41	0	−8891	0
3.5	0.25[k]	61 ± 6	0	1.34 ± 0.12	0	−8240	0

[a] For identification of [e], [h], [i], and [k], see Table I.

and in the limit, when r_1, r_2, and $r \rightarrow$ zero,

$$\frac{r}{c_f} \rightarrow k_1 \cdot n_1 + k_2 \cdot n_2 \qquad \text{(Eq. 6)}$$

For $r/c_f \rightarrow 0$,

$$r \rightarrow n_1 + n_2 \qquad \text{(Eq. 7)}$$

From Eq. 5, one obtains the negative slope:

$$-\frac{d(r/c_f)}{dr} = k_2 + (k_1 - k_2)\frac{dr_1}{dr} \qquad \text{(Eq. 8)}$$

For $k_1 \gg k_2$ and low values of r, $dr_1/dr = 1$ as a first approximation; hence the negative slope is $-k_1$, valid for the experimental range $dr_1/dr \simeq 0$, where $d(r/c_f)/dr$ approaches $-k_2$ and r reaches its limiting value of $n_1 + n_2$.

For the determination of the slopes $-k_1$ and $-k_2$, n_1 and n_2, as well as of the y-intercept $k_1n_1 + k_2n_2$ for $r \rightarrow 0$, the authors developed a best-fit computer program which also permitted the calculation of the standard free-energy (free-enthalpy) changes, $\Delta F_1°$ and $\Delta F_2°$, from the obtained intrinsic association constants.

The binding parameters obtained for the BKG–BA interaction at pH 7.0, 5.0, and 3.5 are listed in Table I; the corresponding binding curves are shown in Figs. 3 and 4. In all dialysis and ultracentrifuge experiments, three BKG concentrations were used (4×10^{-5}, 10^{-4}, and 5×10^{-4} M), and the BA concentration was varied within the range 5×10^{-6} to 1.25×10^{-4} M.

EOS–BA—For the characterization of this interaction, spectrophotometry and ultracentrifugation were employed.

Spectrophotometric titrations at 23° of 2.5×10^{-5} M EOS solutions at pH 7.0, 5.0, and 3.5 with 2.5×10^{-5} to 3.1×10^{-7} M BA solutions of corresponding pH values yielded at saturation BA–EOS stoichiometries of 1:3.2, 1:2.1, and 1:0, respectively. Normal and difference spectra demonstrate a red-shift maximum between 532 and 534 mμ and a clear isobestic point at 520–522 mμ (Fig. 5).

The binding parameters, as obtained from ultracentrifuge experiments, are listed in Table II. In these experiments, the EOS concentration was held constant at 2.5×10^{-5} M, whereas the BA concentration was varied in the range 6×10^{-8} to 1.25×10^{-5} M.

IP–BA—Free-enthalpy, enthalpy, and entropy changes of the IP–BA interaction were determined by means of equilibrium dialysis in an IP concentration range (2×10^{-5} to 2×10^{-4} M) which produces 1:1 complex formation; the BA concentration was 2×10^{-5} M. When $r = 1$ (1:1 complexing), Eq. 3 becomes

$$r = \frac{k_1 \cdot c_f}{1 + k_1 \cdot c_f} \qquad \text{(Eq. 9)}$$

or

$$\frac{1}{r} = \frac{1}{c_f \cdot k_1} + 1 \qquad \text{(Eq. 10)}$$

The $1/r$ plotted as a function of $1/c_f$ yields, at 20 and 4°, intercepts of 1.04 ± 0.05 and 0.98 ± 0.07, respectively. The binding parameters and thermodynamic functions are listed in Table III. Analogous experiments with human albumin (HA) yielded an approximately 35% lower association constant.

Binding studies performed in the gravitational field of the ultracentrifuge, with a 13.7 to 360 molar excess of IP and BA concentrations of 10^{-5} and 0.5×10^{-5} M, yield the straight-line binding curve depicted in Fig. 6. The binding parameters calculated according to Eq. 4 are listed in Table IV.

DMI–BA—The interaction of DMI and BA, as studied under the same experimental conditions (21°, pH 7.0, ionic strength I = 0.04), is of a more complex nature. The Scatchard plot yields, for values of r in the range 0–8, a curve with an initially positive slope $d(r/c_f)/dr$ and a maximum at $r \simeq 6$; therefore, the model introduced previously cannot be employed for the DMI–BA interaction. For the interpretation of this mechanism of interaction, an r *versus* c_f plot (Fig. 7) seems to be useful. In the concentration range leading to 1:1 complex formation, the DMI interaction is of the type described for IP.

DISCUSSION

BKG–BA Interactions—Within the limits of experimental error, various methods [equilibrium dialysis, ultracentrifuge sedimentation, and ultrafiltration (27, 28)] yield under identical experimental conditions the same binding parameters such as association constants and number of binding sites (compare Table I). If the intrinsic association constant determined by Rodkey (27) by means of ultrafiltration and that determined in this paper are averaged, the result is $k_1 = 5.45 \times 10^5$ l./mole, with a relative error of 3.5%. In this context, the number of primary binding sites as a function

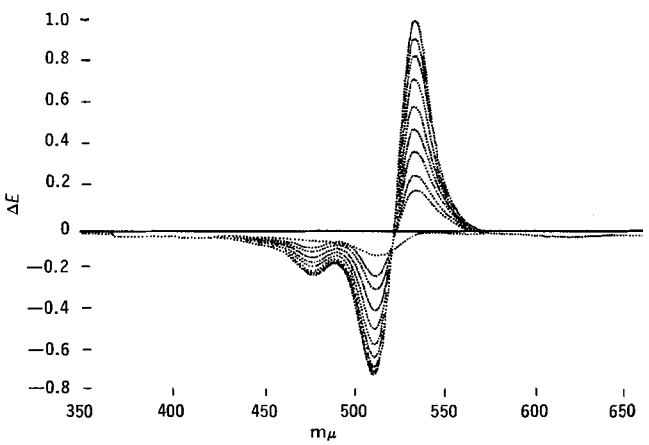


Figure 5—*EOS–BA interaction. Difference spectra by spectrophotometric titrations of EOS with albumin solutions. For further details, see* Results.

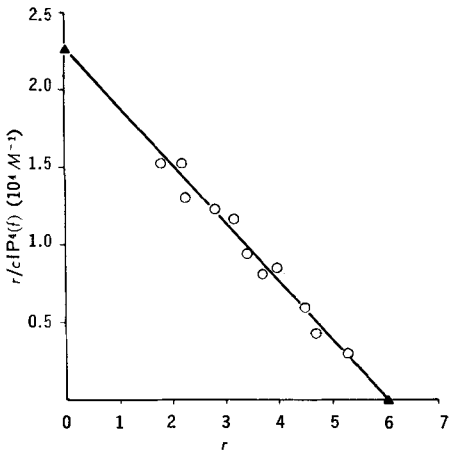


Figure 6—*IP–BA interaction (pH 7.00). Scatchard plot. Ultracentrifuge sedimentation, ionic strength 0.04. For further details, see* Results.

Table III—IP–Albumin Interaction Showing Binding Parameters and Thermodynamic Functions (pH 7.4) by Equilibrium Dialysis[a]

Albumin	Temperature	k_1, 10^3 l./mole	$\Delta F_1°$, cal./mole	$\Delta H_1°$,[b] cal./mole	$\Delta S_1°$,[c] cal. × degree^{-1} × mole^{-1}
Bovine	20°	6.00 ± 0.25	−5063	−5720	−2.2
Bovine	4°	10.60 ± 0.49	−5100		
Human	20°	3.82 ± 0.25	−4800		

[a] For other symbols and the buffer used, see Table I. Ionic strength 0.19. [b] $\Delta H_1°$ = enthalpy change. [c] $\Delta S_1°$ = entropy change.

of pH is of interest. Spectrophotometric titration yielded $n_1 = 3.6$ at pH 7.0. At pH 5.0 and 3.5, the red shift of the difference spectrum has disappeared, but ultracentrifuge experiments yield n_1 values of 58 ± 2 and 108 ± 8. This allows the conclusion that the additional binding sites are chemically different from the ones exhibited at pH 7.0. It can be assumed that the four binding sites observed in the native state of the albumin are lost by unfolding of the protein at low pH. It must be recalled that at pH 7.0, secondary binding sites ($n_2 = 3$ to 4) are also detectable. However, because $k_2 \ll k_1$, n_2 cannot be determined accurately from the experimental data; k_2 is about 5×10^6 l./mole and is thus comparable with the observed intrinsic association constant at pH 3.5, $k_1 = 4.7 \times 10^6$ l./mole.

The albumin molecule is known to undergo a reversible conformation change at pH ≃ 4 (29). Electrostatic interaction forces within the albumin molecule cause an expansion of the molecule to take place, leading to the formation of a species of the so-called *F*-form having greater electrophoretic mobility and higher viscosity (30). In contrast, the *N*-form (native state) has significantly more compact packing. The binding forces for hydrophobic molecules are strongly diminished by an *N–F* transition (30, 31).

At pH 3.5, the binding mechanism is relatively simple: the albumin molecule carries a positive excess charge, and the *N–F* transition has occurred. BKG is present as a univalent anion, so the obtained free-enthalpy change of $\Delta F_1° \simeq -5100$ cal./mole can be attributed to electrostatic interactions. The number of binding sites, $n_1 = 108 \pm 8$, may be accounted for by the basic (cationic) amino acid residues. This binding model (Eq. 3) is confirmed by the straight line in Fig. 4 and by the agreement of the experimentally determined n_1k_1 value (5.15×10^6 l./mole) and the calculated one (5.11×10^6 l./mole).

At pH 5.0, two different binding curves are obtained. Since $k_1 > k_2$, the latter value and thus $\Delta F_2°$ may have been somewhat overestimated (Table I). A more realistic value for $\Delta F_2°$ would be around −3800 cal./mole. It is likely that $\Delta F_1°$ (−4950 cal./mole) represents electrostatic interactions involving the monovalent BKG anion and $\Delta F_2°$ (−3800 cal./mole) those with the bivalent BKG anion. In the BKG–HA system, Rodkey (28) observed a 20% decrease in the association constant at pH 4.8 as compared to pH 3.4. A weak conformational change of the albumin molecule at pH 5.0 appears sufficient for the exposure of the same number of binding sites as is observed in the case of the *N–F* transition. Conformational changes induced by ligand–protein interaction or protein unfolding also should not be excluded.

Table IV—IP–BA Interaction Showing Binding Parameters by Ultracentrifuge Sedimentation at 21°[a]

pH	Ionic Strength	n_1	k_1, 10^3 l./mole	$\Delta F_1°$, cal./mole
7.4	0.04[h]	6.31 ± 0.24	3.59 ± 0.12	−4781
7.0	0.04[h]	6.05 ± 0.19	3.76 ± 0.13	−4808
7.0	0.19[e]	6.12 ± 0.16	6.36 ± 0.41	−5114

[a] For identification of [h] and [e], see Table I.

At pH 7.0, the binding mechanism appears not to be of a solely electrostatic nature. This is not surprising since the albumin molecule, which is in its native state, has a negative excess charge, causing electrostatic repulsion forces with respect to the BKG which exists as the bivalent anion only at pH 7.0. At an ionic strength of 0.19, the negative free-enthalpy change for the primary reaction ($n_1 \simeq 4$) is approximately 4000 cal./mole higher than the $\Delta F_1°$ assumed for the bivalent anion at pH 5.0. However, $-\Delta F_1°$ is increased by about 500 cal./mole through the decrease of the ionic strength by a factor of 5. This confirms the participation of stabilizing electrostatic interaction forces.

In conclusion, the following interpretation of the BKG–BA interaction emerges: (*a*) four primary binding sites predominantly interacting by means of proton donator–acceptor forces (hydrogen bonds), electron donator–acceptor forces, and possibly hydrophobic forces (total $-\Delta F_1° = 4000$ cal./mole), and stabilized further by electrostatic forces ($-\Delta F_1° \simeq 3800$ cal./mole, dependent on ionic strength); and (*b*) three to four secondary binding sites apparently interacting by means of electrostatic forces only ($-\Delta F_2° \simeq 5000$ cal./mole, $k_2 \simeq 5 \times 10^4$ l./mole).

Hydrophobic binding of straight-chain fatty acids by BA, involving stabilizing electrostatic forces, were also observed by Ray *et al.* (32).

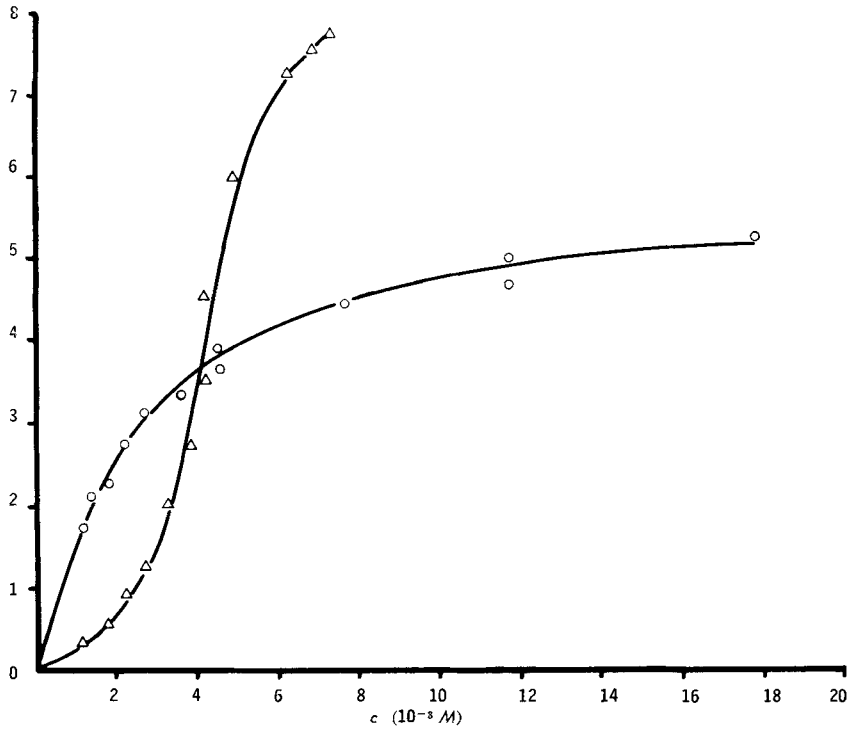


Figure 7—*IP–BA and DMI–BA interactions. Degree of binding* (r) versus *free concentration* (c_f). *Key:* ○, *imipramine*; *and* △, *desipramine. Ultracentrifuge sedimentation, pH 7.0, ionic strength 0.04. For further details, see* Results.

EOS–BA Interactions—At pH 3.5, the simple binding model with one type of binding site is again confirmed. It also can be assumed that electrostatic forces predominate because of the unfolding of the albumin molecule, its positive excess charge, and the charge distribution of the ligand. An increase of the ionic strength by a factor of about 3 produces a decrease of the free-enthalpy change by about 700 cal./mole (Table II), but the number of binding sites remains unchanged ($n_1 \simeq 60$) and is considerably smaller in comparison to BKG.

At pH 7.0, the ionic strength dependence of the free-enthalpy change is opposite for the two types of binding sites. The primary binding sites ($n_1 \simeq 4$) are likely to interact by means of van der Waals' forces, since an increased electrolyte concentration reduces the dielectric constant of water and thus increases the association constant (by a factor of about 2). Although in a strictly physical sense the van der Waals forces apply only to dispersion forces, the authors employ the term in a broader sense according to Martin (33), whereby it should be noted that dipole–dipole interactions are stabilized by energy effects and true dispersion forces by entropy effects. The free-enthalpy change involving the secondary binding sites ($n_2 \simeq 6$) is approximately 2000 cal./mole smaller than $-\Delta F_1°$. However, in this case, electrostatic forces appear to predominate, since an increase of the ionic strength by a factor of 5 reduces the $-\Delta F_2°$ by about 450 cal./mole (Table II).

Spectrophotometric titrations at pH 7.0 and 5.0 yielded binding stoichiometries of BA–EOS = 1:3.2 and 1:2.1, respectively.

The binding mechanism at pH 5.0 appears to be more specific than for BKG, since $n_1 + n_2$ is smaller by about 80 binding sites. An accurate interpretation of the binding mechanism at this pH, which is the approximate isoelectric point of albumin, is again difficult. Despite comparable ionic strengths, $-\Delta F_1°$ is about 1400 cal./mole lower than at pH 7.0.

Finally, from the interaction data at hand, no structural relationships between ligand and protein can be derived. A possible participation of ϵ-amino groups of lysine is indicated by preliminary experiments showing that the majority of these groups are blocked for dinitrofluorobenzene after binding of BKG or EOS.

IP–BA and DMI–BA Interactions—From the temperature dependence, the resulting thermodynamic functions, and the ionic strength dependence of the IP–BA binding reaction in the 1:1 complexing range, it can be concluded that participation of van der Waals' forces is predominant. From the negative enthalpy change of 5700 cal./mole, it can be concluded that the forces under consideration are energetically stabilized, probably by dipole–dipole interactions. Hydrophobic forces cannot be excluded, since the entropy function ($\Delta S_i° = -2.2$ cal. $\times$ degree^{-1} $\times$ mole^{-1}) is very small. However, it is certain that they do not constitute the main interaction forces in contrast with sulfonamide–albumin binding, where Clausen (34) measured positive ΔS values of 20–30 cal. $\times$ degree^{-1} $\times$ mole^{-1}. In the case of IP, a decrease of the ionic strength by a factor of 5 produces at constant temperature a reduction of the intrinsic association constant by almost 50% and a corresponding reduction of the free-enthalpy change by about 300 cal./mole. Both equilibrium dialysis and ultracentrifuge sedimentation yield, under like conditions, the same binding parameters (Tables III and IV). The obtained number of binding sites averages $n_1 = 6.2 \pm 0.2$. The reason for the somewhat weaker binding tendency of human albumin may lay in the higher degree of purity of the bovine preparation employed (35).

UV difference spectra (performed with a Cary 14 recording spectrophotometer) at various degrees of binding (r_i) demonstrate marked red shifts at 21° within both the 220–235 and 260–310 mμ wavelength bands. According to Wetlaufer (36), red shifts are commonly associated with aromatic ($\pi \rightarrow \pi^*$) and peptide transitions. According to other authors (32, 37, 38), these red shifts are produced by tyrosine side-chain chromophores. This short wavelength red shift and its sign are typical of protein complexes in which the higher tryptophan-associated sites are not engaged. These spectroscopic data are also obtained by the binding to albumin of long-chain fatty acid anions (38). On the basis of the number of binding sites ($n_1 \simeq 6$), it can be assumed that tyrosine residues participate directly or indirectly in the IP–BA interaction.

Herskovits *et al.* (39–41) studied, with the aid of the solvent perturbation technique, the location of tyrosyl and tryptophyl residues in bovine serum albumin. Their results, at pH 6.8, show that about 70% of the 18 to 21 tyrosyl residues appear to be buried in the folds of the native protein, while the remaining tyrosyl groups are accessible to perturbants with molecule diameters less than 5.2 Å. The two tryptophyl residues present in the protein are found to be nearly fully exposed to perturbants with less than 4.4 A diameter, while about 50% exposure is observed with perturbants having greater than 5.2 Å diameter.

The data obtained in the case of the DMI–BA interaction indicate the involvement of a far more complicated mechanism than with IP–BA. The postulated binding models fail to explain the data. The *r versus* c_f plot of DMI (Fig. 7) yields a sigmoidal curve. In contrast to IP, UV difference spectra show blue shifts in the long wavelength and, particularly, in the short wavelength regions. The sigmoidal binding curve and the UV spectral properties allow the following interpretations for the DMI–BA complex. (*a*) Tyrosyl or tryptophyl residues are directly or indirectly involved. (*b*) A primary interaction of DMI and BA induces a change of the tertiary structure, *e.g.*, by a certain degree of unfolding, or a conformational change presumably in secondary segments of the polypeptide chain. This is suggested by the variation in reactivity of the binding sites involved. The binding mechanism is thus governed by cooperative phenomena. (*c*) The number of binding sites amounts to at least eight (Fig. 7).

Finally, it should be noted that the binding parameters obtained by different methods need not necessarily agree. Thus, the fraction calculated to be bound by gel filtration was one-third that found with equilibrium dialysis under identical experimental conditions (42). Such a disparity is determined by structural characteristics of ligands and gel phase. A combination of several methods is, therefore, advisable to avoid false conclusions. Supplementary NMR data on IP–BA and DMI–BA interactions are currently being collected and will be published.

REFERENCES

(1) W. Kauzmann, *Advan. Protein Chem.*, **14,** 1(1963).

(2) W. Kauzmann, "Quantum Chemistry," Academic, New York, N.Y., 1967.

(3) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57,** 895 (1968).

(4) H. J. Weder and M. H. Bickel, *ibid.*, **59,** 1505(1970).

(5) A. R. Peacocke and J. N. H. Skerrett, *Trans. Faraday Soc.*, **52,** 261(1956).

(6) H. A. Benesi and J. H. Hildebrand, *J. Amer. Chem. Soc.*, **71,** 2703(1949).

(7) G. G. Hammes and P. R. Schimmel, *ibid.*, **87,** 4665(1965).

(8) F. Karush and M. Sonnenberg, *ibid.*, **71,** 1369(1949).

(9) T. Higuchi, R. Kuramoto, L. Kennon, T. L. Glagon, and A. Polk, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 646(1954).

(10) G. Scatchard, I. H. Scheinberg, and S. H. Armstrong, *J. Amer. Chem. Soc.*, **72,** 535(1950).

(11) I. M. Klotz, F. M. Walker, and R. B. Pivan, *ibid.*, **68,** 1486 (1946).

(12) P. Spring, *Arzneim.-Forsch.*, **16,** 346(1966).

(13) R. M. Rosenberg and I. M. Klotz, in "A Laboratory Manual of Analytical Methods of Protein Chemistry," vol. 2, P. Alexander and R. J. Block, Eds., Pergamon, New York, N. Y., 1960, p. 131.

(14) W. Scholtan, *Makromol. Chem.*, **54,** 24(1962).

(15) G. Jürgensen, Ph. D. thesis, ETH, Zürich, Switzerland, 1966.

(16) I. Z. Steinberg and H. K. Schachman, *Biochemistry*, **5,** 3728(1966).

(17) H. K. Schachman, "Ultracentrifugation in Biochemistry," Academic, New York, N. Y., 1959, p. 169.

(18) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51,** 660(1949).

(19) J. T. Edsall and J. Wyman, "Biophysical Chemistry," vol. 1, Academic, New York, N. Y., 1960.

(20) G. A. J. van Os, *Arzneim.-Forsch.*, **16,** 1428(1966).

(21) H. E. Hard, *Bull. Math. Biophys.*, **27,** 87(1965).

(22) H. A. Rosenthal, *Anal. Biochem.*, **20,** 525(1967).

(23) C. Hansch, K. Kiens, and G. L. Lawrence, *J. Amer. Chem. Soc.*, **87,** 5770(1965).

(24) R. Franke, *Biochim. Biophys. Acta*, **160,** 378(1968).

(25) D. M. Crothers, *Biopolymers*, **6,** 575(1968).

(26) L. W. Nichol, W. J. H. Jackson, and D. J. Winzor, *Biochemistry*, **8,** 2449(1967).

(27) F. L. Rodkey, *Arch. Biochem. Biophys.*, **94,** 526(1961).

(28) *Ibid.*, **108,** 510(1964).

(29) J. F. Foster, in "The Plasma Proteins," vol. 1, F. W. Putnam, Ed., Academic, New York, N. Y., 1960.
(30) A. Wishnia and T. Pinder, *Biochemistry*, **3**, 1377(1964).
(31) D. E. Guttman and A. E. Gadzala, *J. Pharm. Sci.*, **54**, 742 (1965).
(32) A. Ray, J. A. Reynolds, H. Polet, and J. Steinhardt, *Biochemistry*, **5**, 2606(1966).
(33) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960.
(34) J. Clausen, *J. Pharmacol. Exp. Ther.*, **153**, 167(1966).
(35) H. B. Bull and K. Breese, *Arch. Biochem. Biophys.*, **120**, 303(1967).
(36) D. B. Wetlaufer, *Advan. Protein Chem.*, **17**, 303(1962).
(37) H. Polet and J. Steinhardt, *Biochemistry*, **7**, 1348(1968).
(38) J. A. Reynolds, S. Herbert, and J. Steinhardt, *ibid.*, **7**, 1357 (1968).
(39) T. T. Herskovits and M. Laskowski, *J. Biol. Chem.*, **237**, 2481(1962).
(40) T. T. Herskovits and M. Sorensen, *Biochemistry*, **7**, 2523 (1968).
(41) *Ibid.*, **7**, 2533(1968).
(42) H. J. Weder and R. Gauch, unpublished results.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 11, 1969, from the *Medizinisch-chemisches Institut, University of Berne, Berne, Switzerland.*
Accepted for publication April 14, 1970.
* Present address: Institute of Molecular Biology and Biophysics, Federal Institute of Technology, Zurich, Switzerland.

Pharmacokinetic Model for Chlordiazepoxide·HCl in the Dog

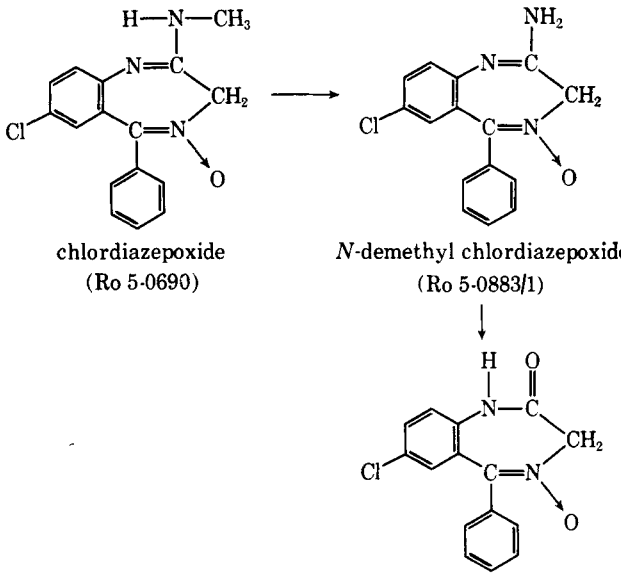
S. A. KAPLAN, M. LEWIS, M. A. SCHWARTZ, E. POSTMA, S. COTLER, C. W. ABRUZZO, T. L. LEE, and R. E. WEINFELD

Abstract □ A six-compartment open-system model is presented to elucidate the physiological disposition of chlordiazepoxide and its two pharmacologically active biotransformation products, Ro 5-0883/1 and Ro 5-2092, in the dog following the intravenous administration of 10 mg./kg. chlordiazepoxide·HCl. The pharmacokinetic parameters used in the model were obtained by administering each of the three compounds separately. Excellent agreement was obtained between the plasma levels of intact drug, Ro 5-0883/1, and Ro 5-2092 found after administration of chlordiazepoxide·HCl and the calculated levels of each generated from the model. The main features of chlordiazepoxide disposition were: (*a*) its complete biotransformation to Ro 5-0883/1; (*b*) elimination of Ro 5-0883/1 almost entirely by biotransformation with up to 50% proceeding to Ro 5-2092 by oxidative deamination; and (*c*) elimination of Ro 5-2092 by urinary excretion and further biotransformation.

Keyphrases □ Chlordiazepoxide·HCl and metabolites, disposition—pharmacokinetic model □ Biotransformation, dogs—chlordiazepoxide·HCl □ Plasma levels—chlordiazepoxide and metabolites □ Urinary excretion—chlordiazepoxide and metabolites □ TLC—separation □ Fluorometry—analysis

Chlordiazepoxide[1] (7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide) is extensively used in the treatment of anxiety states and other psychic disorders (1, 2). Chlordiazepoxide has been shown (3–5) to be biotransformed in dog and man to the *N*-demethyl chlordiazepoxide (Ro 5-0883/1), which undergoes further deamination to form the "lactam" (Ro 5-2092). The structure of chlordiazepoxide together with those of the two biotransformation products, both of which are pharmacologically active (2, 6, 7), is presented in Scheme I.

This study reports the development of a pharmacokinetic model to describe the physiological disposition of chlordiazepoxide, Ro 5-0883/1, and Ro 5-2092 following the intravenous administration of chlordiazepoxide·HCl to dogs. To elucidate an appropriate pharmacokinetic model, each of the three compounds was separately administered intravenously to two dogs. The pharmacokinetic parameters thus obtained for each compound were used to establish the model for the disposition of chlordiazepoxide·HCl.



Scheme I

EXPERIMENTAL

Protocol—Two male dogs, weighing 10.5 and 13.0 kg., each received single 10-mg./kg. i.v. doses of chlordiazepoxide·HCl, Ro

[1] Chlordiazepoxide·HCl is the active ingredient in Librium marketed by Hoffmann-La Roche Inc., Nutley, N. J.

(29) J. F. Foster, in "The Plasma Proteins," vol. 1, F. W. Putnam, Ed., Academic, New York, N. Y., 1960.
(30) A. Wishnia and T. Pinder, *Biochemistry*, **3**, 1377(1964).
(31) D. E. Guttman and A. E. Gadzala, *J. Pharm. Sci.*, **54**, 742 (1965).
(32) A. Ray, J. A. Reynolds, H. Polet, and J. Steinhardt, *Biochemistry*, **5**, 2606(1966).
(33) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960.
(34) J. Clausen, *J. Pharmacol. Exp. Ther.*, **153**, 167(1966).
(35) H. B. Bull and K. Breese, *Arch. Biochem. Biophys.*, **120**, 303(1967).
(36) D. B. Wetlaufer, *Advan. Protein Chem.*, **17**, 303(1962).
(37) H. Polet and J. Steinhardt, *Biochemistry*, **7**, 1348(1968).
(38) J. A. Reynolds, S. Herbert, and J. Steinhardt, *ibid.*, **7**, 1357 (1968).
(39) T. T. Herskovits and M. Laskowski, *J. Biol. Chem.*, **237**, 2481(1962).
(40) T. T. Herskovits and M. Sorensen, *Biochemistry*, **7**, 2523 (1968).
(41) *Ibid.*, **7**, 2533(1968).
(42) H. J. Weder and R. Gauch, unpublished results.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 11, 1969, from the *Medizinisch-chemisches Institut, University of Berne, Berne, Switzerland.*
Accepted for publication April 14, 1970.
* Present address: Institute of Molecular Biology and Biophysics, Federal Institute of Technology, Zurich, Switzerland.

Pharmacokinetic Model for Chlordiazepoxide·HCl in the Dog

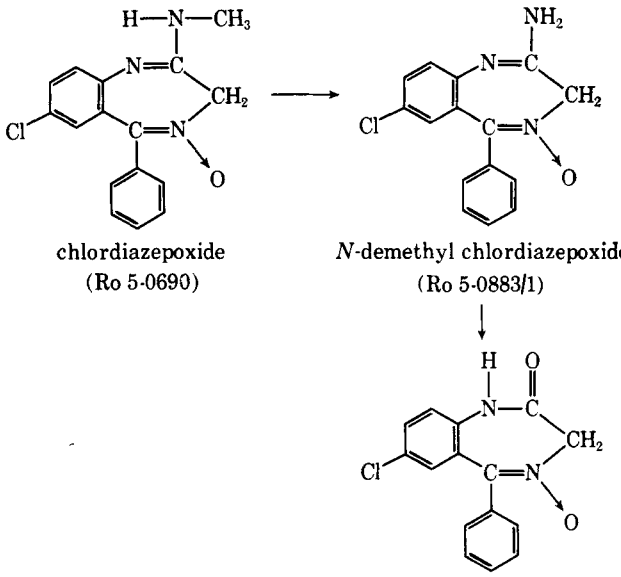
S. A. KAPLAN, M. LEWIS, M. A. SCHWARTZ, E. POSTMA, S. COTLER, C. W. ABRUZZO, T. L. LEE, and R. E. WEINFELD

Abstract □ A six-compartment open-system model is presented to elucidate the physiological disposition of chlordiazepoxide and its two pharmacologically active biotransformation products, Ro 5-0883/1 and Ro 5-2092, in the dog following the intravenous administration of 10 mg./kg. chlordiazepoxide·HCl. The pharmacokinetic parameters used in the model were obtained by administering each of the three compounds separately. Excellent agreement was obtained between the plasma levels of intact drug, Ro 5-0883/1, and Ro 5-2092 found after administration of chlordiazepoxide·HCl and the calculated levels of each generated from the model. The main features of chlordiazepoxide disposition were: (*a*) its complete biotransformation to Ro 5-0883/1; (*b*) elimination of Ro 5-0883/1 almost entirely by biotransformation with up to 50% proceeding to Ro 5-2092 by oxidative deamination; and (*c*) elimination of Ro 5-2092 by urinary excretion and further biotransformation.

Keyphrases □ Chlordiazepoxide·HCl and metabolites, disposition—pharmacokinetic model □ Biotransformation, dogs—chlordiazepoxide·HCl □ Plasma levels—chlordiazepoxide and metabolites □ Urinary excretion—chlordiazepoxide and metabolites □ TLC—separation □ Fluorometry—analysis

Chlordiazepoxide[1] (7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide) is extensively used in the treatment of anxiety states and other psychic disorders (1, 2). Chlordiazepoxide has been shown (3–5) to be biotransformed in dog and man to the *N*-demethyl chlordiazepoxide (Ro 5-0883/1), which undergoes further deamination to form the "lactam" (Ro 5-2092). The structure of chlordiazepoxide together with those of the two biotransformation products, both of which are pharmacologically active (2, 6, 7), is presented in Scheme I.

This study reports the development of a pharmacokinetic model to describe the physiological disposition of chlordiazepoxide, Ro 5-0883/1, and Ro 5-2092 following the intravenous administration of chlordiazepoxide·HCl to dogs. To elucidate an appropriate pharmacokinetic model, each of the three compounds was separately administered intravenously to two dogs. The pharmacokinetic parameters thus obtained for each compound were used to establish the model for the disposition of chlordiazepoxide·HCl.



Scheme I

EXPERIMENTAL

Protocol—Two male dogs, weighing 10.5 and 13.0 kg., each received single 10-mg./kg. i.v. doses of chlordiazepoxide·HCl, Ro

[1] Chlordiazepoxide·HCl is the active ingredient in Librium marketed by Hoffmann-La Roche Inc., Nutley, N. J.

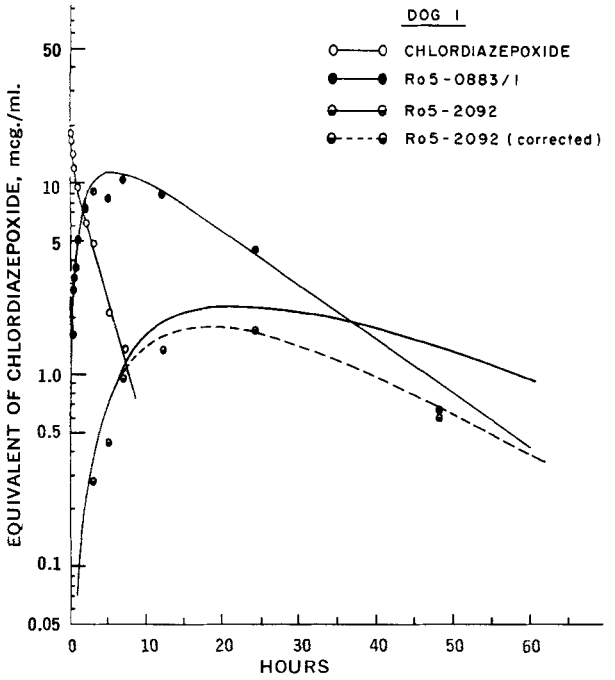


Figure 1—*Computer-simulated curves and experimental data points following the intravenous administration of 10 mg./kg. chlordiazepoxide·HCl to Dog 1.*

5-0883/1, and Ro 5-2092, with approximately 2 weeks between doses. The chlordiazepoxide and Ro 5-0883/1 were administered in aqueous solution as the hydrochloride, whereas the Ro 5-2092 was administered in propylene glycol solution. Five-milliliter blood specimens (heparinized) were obtained at various times after drug administration, and the plasma was separated. Total urine output for 3 days was collected following the administration of each drug.

Analytical Method—A differential fluorometric assay procedure (5) allowed for the specific determination of chlordiazepoxide, Ro 5-0883/1, and Ro 5-2092 when all three were present in a single plasma specimen. The sensitivity of the procedure was 0.2–0.3 mcg. of each compound per milliliter of plasma using a 1-ml. specimen.

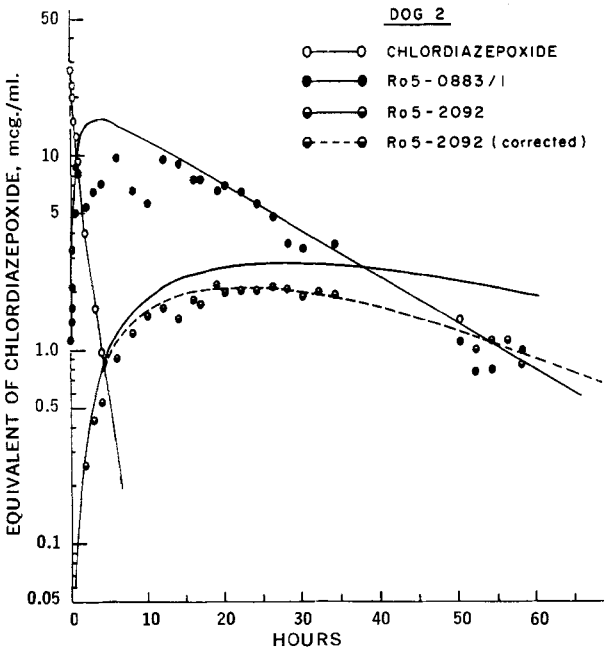


Figure 2—*Computer-simulated curves and experimental data points following the intravenous administration of 10 mg./kg. chlordiazepoxide·HCl to Dog 2.*

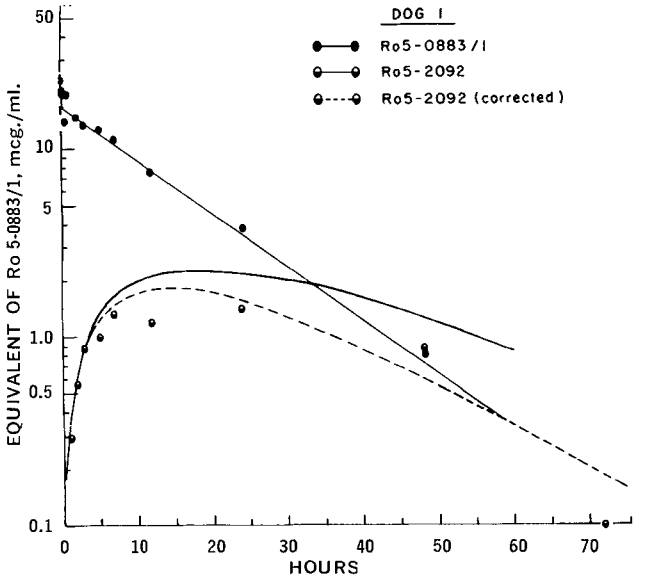


Figure 3—*Computer-simulated curves and experimental data points following the intravenous administration of 10 mg./kg. Ro 5-0883/1 to Dog 1.*

For the analyses of urine, a TLC step was added to the assay procedure to eliminate interfering urinary fluorescence. The ether extract of urine was evaporated to dryness; the residue was reconstituted in 100 μl. ethanol and spotted on Brinkmann neutral TLC plates, F-254, with appropriate standards. Following development in ethyl acetate–ethanol (90:10), the areas corresponding to chlordiazepoxide (R_f 0.25), Ro 5-0883/1 (R_f 0.16), and Ro 5-2092 (R_f 0.43) were scraped from the plate. Chlordiazepoxide was eluted from the silica gel with 0.1 *N* sulfuric acid, Ro 5-0883/1 with 7.0 *N* sulfuric acid, and Ro 5-2092 with 0.1 *N* sodium hydroxide. The analytical procedure for each compound then followed that described for plasma specimens (5). This procedure was carried out with 10-ml. urine specimens.

RESULTS AND DISCUSSION

The plasma level curves following the administration of 10-mg./kg. i.v. doses of chlordiazepoxide·HCl, Ro 5-0883/1, and Ro 5-2092 to Dogs 1 and 2 are presented in Figs. 1–6.

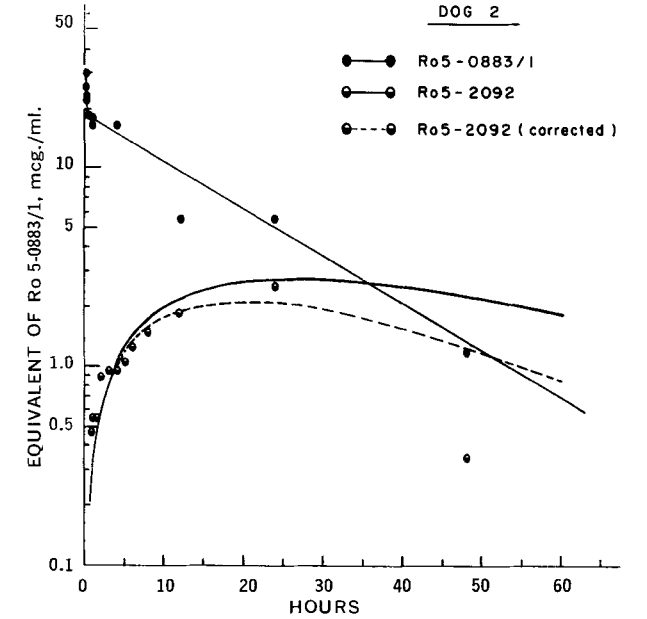


Figure 4—*Computer-simulated curves and experimental data points following the intravenous administration of 10 mg./kg. Ro 5-0883/1 to Dog 2.*

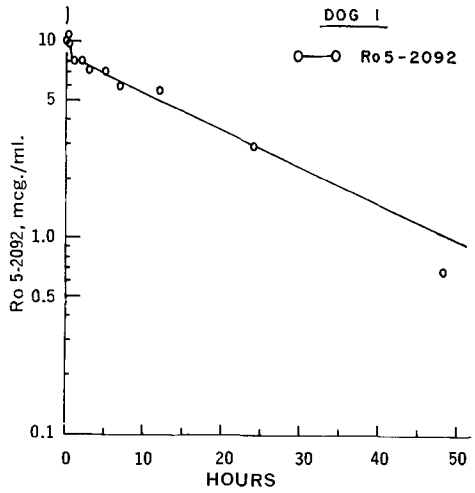


Figure 5—*Computer-simulated curve and experimental data points following the intravenous administration of 10 mg./kg. Ro 5-2092 to Dog 1.*

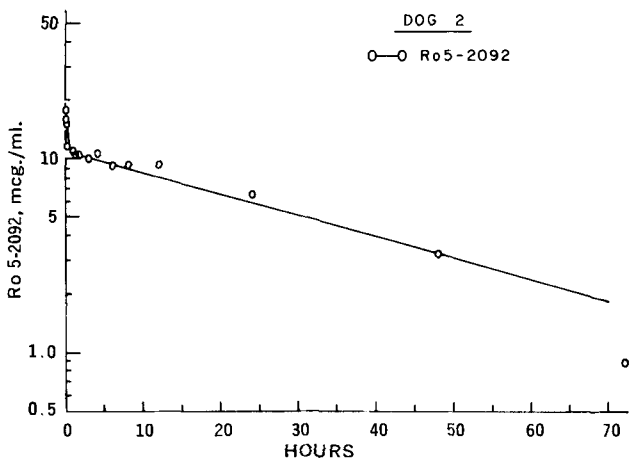


Figure 6—*Computer-simulated curve and experimental data points following the intravenous administration of 10 mg./kg. Ro 5-2092 to Dog 2.*

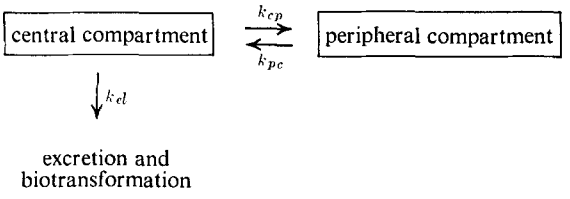
The plasma level curves of each of the three intravenously administered compounds were apparently biexponential. This suggested that the pharmacokinetic evaluation of each compound would require a minimum of a two-compartment open-system model (8). The calculated volume of the central compartment, V_p, for each of the three compounds was never less than 30% of body weight. This further suggested that the central compartment of the two-compartment open-system model consisted of plasma plus readily accessible body spaces, *e.g.*, liver and kidneys. Therefore, elimination was assumed to occur from the central compartment, as indicated in the model presented in Scheme II.

In this model, k_{cp} and k_{pc} are the first-order rate constants into and out of the peripheral compartment, and k_{el} is the sum of the simultaneous processes of biotransformation and elimination, all assumed to be first-order processes.

The experimental plasma level data of chlordiazepoxide, Ro 5-0883/1, and Ro 5-2092 obtained following the intravenous administration of each of the three compounds were fit to the biexponential equation, $C_p = Ae^{-\alpha t} + Be^{-\beta t}$, by means of a "least-squares estimation of nonlinear parameters" evaluation programmed in FORTRAN IV for use on a GE-605 digital time-sharing system (9). The parameters thus obtained (A, α, B, and β) are presented in Table I. A and B are the ordinate axis intercepts of the biexponential curve, and α and β are the hybrid rate constants reflecting the overall rate processes. The individual rate constants (k_{cp}, k_{pc}, and k_{el}) associated with the two-compartment open-system model were calculated according to Riegelman *et al.* (8) and are presented in Table I.

The 0–74-hr. urinary excretion levels in Dog 2 following the administration of chlordiazepoxide·HCl indicated no excretion of intact chlordiazepoxide, whereas 1.6% of the administered dose was recovered as Ro 5-0883/1 and 5.0% was recovered as Ro 5-2092.

Previous studies (10) indicated that following an oral 10-mg./kg. dose of chlordiazepoxide·HCl to a dog and examination of the 0–48-hr. urine, there was no recovery of intact chlordiazepoxide and that 1.1% of the dose was recovered as Ro 5-0883/1 and 7.2% was recovered as Ro 5-2092. These findings are similar to those of Dog 2. Schwartz (11) found that following the single oral administration of 4 mg./kg. ^{14}C-chlordiazepoxide·HCl to two dogs and examination of the 0–72-hr. urinary excretion levels, there was no recovery of intact chlordiazepoxide, whereas 0.2 and 0.5% of the dose were excreted as Ro 5-0883/1 and 1.3 and 3.0% were excreted as Ro 5-2092 in the two dogs, respectively.



Scheme II—Two-compartment open-system pharmacokinetic model

Further information on the biotransformation of chlordiazepoxide and of Ro 5-0883/1 was obtained by examining the areas under the Ro 5-0883/1 plasma level curves following the intravenous administration of chlordiazepoxide·HCl, *i.e.*, formed Ro 5-0883/1, and of administered Ro 5-0883/1, respectively. The (formation curve/administered curve) area ratios for Ro 5-0883/1 were 0.88 and 0.99 in Dogs 1 and 2, respectively. This would suggest virtually complete conversion of chlordiazepoxide to Ro 5-0883/1.

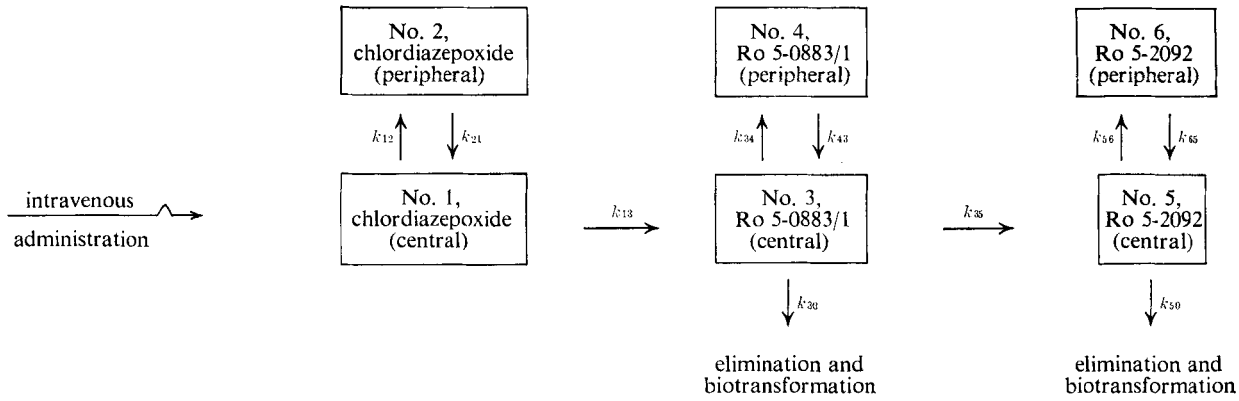
The areas under the formation curves of Ro 5-2092 were determined following the administrations of both chlordiazepoxide·HCl and Ro 5-0883/1 and compared with those following the administration of Ro 5-2092. The (formation curve/administered curve) area ratios were 0.28 and 0.28 in Dog 1 following chlordiazepoxide·HCl and Ro 5-0883/1 administration, respectively, and 0.24 and 0.22 in Dog 2. This would further indicate that chlordiazepoxide is completely biotransformed to Ro 5-0883/1 since Ro 5-0883/1 is the precursor of Ro 5-2092, and both chlordiazepoxide and Ro 5-0883/1 produce equivalent amounts of Ro 5-2092.

Therefore, inasmuch as all the chlordiazepoxide·HCl administered was biotransformed to Ro 5-0883/1, the formation constant of Ro

Table I—Pharmacokinetic Evaluation of Intravenously Administered Chlordiazepoxide·HCl, Ro 5-0883/1, and Ro 5-2092 According to a Two-Compartment Open-System Model

Parameter	Compound Administered: Chlordiazepoxide	Ro 5-0883/1	Ro 5-2092
	Dog 1		
Dose, mg./kg.	10	10	10
Dog weight, kg.	10.5	10.2	9.5
A, mcg./ml.	7.86	13.26	3.14
α hr.$^{-1}$	2.80	13.18	3.38
B, mcg./ml.	12.26	17.10	8.41
β, hr.$^{-1}$	0.33	0.066	0.04
k_{cp}, hr.$^{-1}$	0.79	5.75	0.89
k_{pc}, hr.$^{-1}$	1.84	7.45	2.47
k_{el}, hr.$^{-1}$	0.50	0.12	0.059
V_p, l.	5.22	3.36	8.21
% V_p[a]	49.7	32.9	86.6
	Dog 2		
Dose, mg./kg.	10	10	10
Dog weight, kg.	13.0	13.0	13.6
A, mcg./ml.	19.18	13.06	7.72
α, hr.$^{-1}$	1.56	11.20	11.14
B, mcg./ml.	9.19	19.04	11.26
β, hr.$^{-1}$	0.53	0.054	0.025
k_{cp}, hr.$^{-1}$	0.24	4.51	4.51
k_{pc}, hr.$^{-1}$	0.89	6.65	6.62
k_{el}, hr.$^{-1}$	1.00	0.091	0.043
V_p, l.	4.58	4.05	7.17
% V_p[a]	35.3	31.2	52.7

[a] % V_P = V_P × 100/dog wt.



Scheme III—Six-compartment open-system pharmacokinetic model for the physiological disposition of chlordiazepoxide·HCl

5-0883/1, k_{13}, was taken to be equal to that of the elimination-rate constant, k_{el}, of chlordiazepoxide. The formation constant of Ro 5-2092, k_{35}, was determined by adapting the absorption-rate equation of Loo and Riegelman (12). This constant was determined by utilizing the formation data of Ro 5-2092 following the administrations of both chlordiazepoxide·HCl and Ro 5-0883/1, and the rate constants, k_{cp}, k_{pc}, and k_{el} obtained following the intravenous administration of Ro 5-2092.

Based on these considerations, a six-compartment open-system pharmacokinetic model to describe the physiological disposition of chlordiazepoxide in the dog was developed, as indicated in Scheme III.

In the model, k_{12}, k_{21}, k_{34}, k_{43}, k_{56}, and k_{65} are the first-order rate constants of distribution of the compounds indicated in the model; k_{13} and k_{35} are the first-order rate constants of formation of Ro 5-0883/1 and Ro 5-2092, respectively; k_{30} is the sum of the simultaneous processes of elimination of Ro 5-0883/1 and biotransformation to compounds other than Ro 5-2092; and k_{50} is the sum of the simultaneous processes of elimination and biotransformation of Ro 5-2092. All the elimination and biotransformation processes are assumed to be first order.

Solution of the differential equations (13) describing the six-compartment open-system model, as presented in *Appendix A*, yields the following expressions to describe the plasma level–time curves for Compartments 1, 3, and 5, respectively, following a single intravenous administration of chlordiazepoxide·HCl:

$$C_1 = A_{13}\,e^{-a_3 t} + A_{14}\,e^{-a_4 t} \qquad \text{(Eq. 1)}$$

$$C_3 = A_{33}\,e^{-a_3 t} + A_{34}\,e^{-a_4 t} + A_{35}\,e^{-a_5 t} + A_{36}\,e^{-a_6 t} \qquad \text{(Eq. 2)}$$

$$C_5 = A_{53}\,e^{-a_3 t} + A_{54}\,e^{-a_4 t} + A_{55}\,e^{-a_5 t} + A_{56}\,e^{-a_6 t} + A_{57}\,e^{-a_7 t} + A_{58}\,e^{-a_8 t} \qquad \text{(Eq. 3)}$$

where C_1, C_3, and C_5 are the concentrations of the three compounds in the central compartment. The coefficients A_{1j}, A_{3j}, and A_{5j} and the hybrid rate constants a_3–a_8 are determined as a function of all the rate constants: k_{12}, k_{21}, k_{13}, k_{34}, k_{43}, k_{30}, k_{35}, k_{56}, k_{65}, and k_{50}. The six-compartment open-system model describing the physiological disposition of chlordiazepoxide was programmed in FORTRAN IV for use in the GE-605 digital time-sharing system (14). This program allowed for the simulation of drug quantity in the various compartments of the model as a function of time.

Table II—Pharmacokinetic Parameters for the Physiological Disposition of Chlordiazepoxide·HCl in Dogs in Terms of a Six-Compartment Open System

Rate Constant	Dog 1	Dog 2
k_{12}, hr.$^{-1}$	0.792	0.24
k_{21}, hr.$^{-1}$	1.836	0.892
k_{13}, hr.$^{-1}$	0.503	1.001
k_{34}, hr.$^{-1}$	5.754	4.510
k_{43}, hr.$^{-1}$	7.447	6.651
k_{30}, hr.$^{-1}$[a]	0.045	0.047
k_{35}, hr.$^{-1}$	0.072	0.045
k_{56}, hr.$^{-1}$	0.892	4.508
k_{65}, hr.$^{-1}$	2.473	6.619
k_{50}, hr.$^{-1}$	0.059	0.043
k_{50}, hr.$^{-1}$[b]	0.099	0.080

[a] $k_{30} = k_{el}$ (Ro 5-0883/1) − k_{35}. [b] k_{50} corrected for formation data.

Confirmation of the proposed model required coincidence of simulated and experimental plasma level curves for Compartments 1, 3, and 5 following a single intravenous administration of chlordiazepoxide·HCl. The pharmacokinetic parameters (k_{cp}, k_{pc}, and k_{el}) obtained for each of the three individually administered compounds (Table I) were utilized as computer input for the six-compartment open-system model. The constants for the six-compartment model are summarized in Table II. In addition, to allow for comparison of the experimental and simulated data of the three compounds following the administration of chlordiazepoxide·HCl, the plasma levels of Ro 5-0883/1 and Ro 5-2092 were converted to chlordiazepoxide equivalents. The three curves drawn in solid lines in Figs. 1 and 2 represent the simulated curves and corresponding experimental data points following the intravenous administration of 10 mg./kg. chlordiazepoxide·HCl to Dogs 1 and 2, respectively.

The simulated plasma curves agreed very well with the experimental data in the chlordiazepoxide and Ro 5-0883/1 compartments utilizing the pharmacokinetic parameters obtained following the intravenous administration of each of the three compounds. The simulated plasma level curves of Ro 5-2092, however, did not correlate as well since they exhibited a slower elimination rate than did the experimentally obtained plasma level curves. This small deviation appears to be reflected in differences noted in the experimentally obtained plasma level data following the intravenous administration of Ro 5-2092 as compared with the Ro 5-2092 plasma levels obtained following the administration of chlordiazepoxide or Ro 5-0883/1. The data indicate that the apparent elimination-rate constant of Ro 5-2092 when produced as a biotransformation product was greater than the apparent elimination-rate constant observed after administered Ro 5-2092. This phenomenon has been previously observed for other drugs (15, 16).

These findings indicated that k_{50} of the six-compartment open-system model would have to be calculated from the formation curve of Ro 5-2092 and not from the data following the intravenous administration of Ro 5-2092. Therefore, the elimination-rate constant of Ro 5-2092 was recalculated utilizing the formation data by rearranging Eq. A13 of *Appendix A* and solving for k_{50};

$$k_{50} = \frac{\beta \cdot k_{56} - \beta^2 + \beta \cdot k_{65}}{(k_{65} - \beta)} \qquad \text{(Eq. 4)}$$

where β is a_8 of Eq. 3. Since the data of the elimination phase of the Ro 5-2092 formation curve are utilized, the terms $A_{53}\,e^{-a_3 t}$, $A_{55}\,e^{-a_5 t}$, and $A_{57}\,e^{-a_7 t}$ of Eq. 3 rapidly approach zero. Therefore, Eq. 3 reduces to

$$C_5 = A_{54}\,e^{-a_4 t} + A_{56}\,e^{-a_6 t} + A_{58}\,e^{-a_8 t} \qquad \text{(Eq. 5)}$$

This elimination phase was fit to the triexponential equation by means of the "least-squares estimation of nonlinear parameters" (9), keeping A_{54}, A_{56}, A_{58}, a_4, and a_6 constant.

Substituting the new k_{50} into the computer program for the six-compartment open-system model resulted in an excellent simulation of the Ro 5-2092 levels in both dogs (dashed lines in Figs. 1 and 2).

CONCLUSION

A pharmacokinetic model for the disposition of chlordiazepoxide·HCl in the dog has been presented in terms of a six-compartment open system. The excellent agreement between the simulated and experimental data reflects the reliability of the assumption of first-order kinetics for all processes. The model (Scheme III) provides a basis for the elucidation and quantitation of chlordiazepoxide and its pharmacologically active biotransformation products, Ro 5-0883/1 and Ro 5-2092. The pathways in man (3–5) have been shown to be similar to those in the dog to the extent to which they are described in Scheme III. It is expected that this model will be useful for the interpretation of future human pharmacokinetic studies.

The main features of the physiological disposition of chlordiazepoxide·HCl in the dog were: (*a*) its biotransformation to Ro 5-0883/1 as the exclusive route of drug elimination; (*b*) the elimination of Ro 5-0883/1 almost entirely by biotransformation, with up to 50% going to Ro 5-2092 and the remainder going to an unidentified biotransformation product; and (*c*) the elimination of Ro 5-2092 by urinary excretion and further biotransformation.

$$\begin{bmatrix} a_3-(k_{12}+k_{13}) & k_{21} & 0 & 0 & 0 & 0 \\ k_{12} & a_4-k_{21} & 0 & 0 & 0 & 0 \\ k_{13} & 0 & a_5-(k_{34}+k_{35}+k_{30}) & k_{43} & 0 & 0 \\ 0 & 0 & k_{34} & a_6-k_{43} & 0 & 0 \\ 0 & 0 & k_{35} & 0 & a_7-(k_{56}+k_{50}) & k_{65} \\ 0 & 0 & 0 & 0 & k_{56} & a_8-k_{65} \end{bmatrix} \cdot \begin{bmatrix} A_{1j} \\ A_{2j} \\ A_{3j} \\ A_{4j} \\ A_{5j} \\ A_{6j} \end{bmatrix} = \begin{bmatrix} D_0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \quad \text{(Eq. A14)}$$

APPENDIX A

Determination of Kinetic Constants for Six-Compartment Open-System Model—The six-compartment open-system model is presented in Scheme III. The transfer and elimination process in the six-compartment open-system model may be described as follows:

$$\frac{dC_1}{dt} = C_2k_{21} - C_1(k_{12}+k_{13}) \quad \text{(Eq. A1)}$$

$$\frac{dC_2}{dt} = C_1k_{12} - C_2k_{21} \quad \text{(Eq. A2)}$$

$$\frac{dC_3}{dt} = C_1k_{13} - C_3(k_{34}+k_{55}+k_{30}) + C_4k_{43} \quad \text{(Eq. A3)}$$

$$\frac{dC_4}{dt} = C_3k_{34} - C_4k_{43} \quad \text{(Eq. A4)}$$

$$\frac{dC_5}{dt} = C_3k_{35} - C_5(k_{56}+k_{50}) + C_6k_{65} \quad \text{(Eq. A5)}$$

$$\frac{dC_6}{dt} = C_5k_{56} - C_6k_{65} \quad \text{(Eq. A6)}$$

where C_n is the amount of drug in the designated compartment, and the numerical subscripts for C define the compartment. These simultaneous differential equations were solved by: (*a*) obtaining the eigen values of the eigen vector matrix, and (*b*) evaluating the corresponding eigen vectors (9).

The eigen values, a_j's, are obtained as solutions of the characteristic equation:

$$\begin{vmatrix} a_3-(k_{12}+k_{13}) & k_{21} & 0 & 0 & 0 & 0 \\ k_{12} & a_4-k_{21} & 0 & 0 & 0 & 0 \\ k_{13} & 0 & a_5-(k_{34}+k_{35}+k_{30}) & k_{43} & 0 & 0 \\ 0 & 0 & k_{34} & a_6-k_{43} & 0 & 0 \\ 0 & 0 & k_{35} & 0 & a_7-(k_{56}+k_{50}) & k_{65} \\ 0 & 0 & 0 & 0 & k_{56} & a_8-k_{65} \end{vmatrix} = 0 \quad \text{(Eq. A7)}$$

which have the roots:

$$a_3 = \frac{(k_{12}+k_{21}+k_{13}) + \sqrt{(k_{12}+k_{21}+k_{13})^2 - 4(k_{21}k_{13})}}{2} \quad \text{(Eq. A8)}$$

$$a_4 = \frac{(k_{12}+k_{21}+k_{13}) - \sqrt{(k_{12}+k_{21}+k_{13})^2 - 4(k_{21}k_{13})}}{2} \quad \text{(Eq. A9)}$$

$$a_5 = \frac{(k_{34}+k_{43}+k_{30}+k_{35}) + \sqrt{(k_{34}+k_{43}+k_{30}+k_{35})^2 - 4(k_{43}k_{30}+k_{43}k_{35})}}{2} \quad \text{(Eq. A10)}$$

$$a_6 = \frac{(k_{34}+k_{43}+k_{30}+k_{35}) - \sqrt{(k_{34}+k_{43}+k_{30}+k_{35})^2 - 4(k_{43}k_{30}+k_{43}k_{35})}}{2} \quad \text{(Eq. A11)}$$

$$a_7 = \frac{(k_{56}+k_{65}+k_{50}) + \sqrt{(k_{56}+k_{65}+k_{50})^2 - 4(k_{65}k_{50})}}{2} \quad \text{(Eq. A12)}$$

$$a_8 = \frac{(k_{50}+k_{65}+k_{50}) - \sqrt{(k_{56}+k_{65}+k_{50})^2 - 4(k_{65}k_{50})}}{2} \quad \text{(Eq. A13)}$$

The eigen vectors, A_{ij}, are then obtained as solutions to the $6(j = 3,4, \ldots 8)$ vector equations:

where D_0 = initial dose.

The eigen vectors, A_{ij}, thus obtained are:

$$A_{13} = \frac{D_0(k_{21}-a_3)}{(a_4-a_3)} \quad \text{(Eq. A15)}$$

$$A_{14} = D_0 - A_{13} \quad \text{(Eq. A16)}$$

$$A_{2j} = \frac{k_{12}A_{1j}}{(k_{21}-a_j)} \qquad j = 3,4 \quad \text{(Eq. A17)}$$

$$A_{3j} = \frac{k_{13}(k_{43}-a_j)A_{1j}}{[(a_j-k_{34}-k_{35}-k_{30})(a_j-k_{43})-k_{43}k_{34}]} \qquad j = 3,4 \quad \text{(Eq. A18)}$$

$$A_{35} = \left[-A_{33}-A_{34}+\frac{(a_6-k_{43})}{k_{34}}(-A_{43}-A_{44})\right]\frac{(k_{43}-a_5)}{(a_6-a_5)} \quad \text{(Eq. A19)}$$

$$A_{36} = -\sum_{j=3}^{5} A_{3j} \quad \text{(Eq. A20)}$$

$$A_{4j} = \frac{A_{3j}k_{34}}{(k_{43}-a_j)} \qquad j = 3,4,5, \text{ and } 6 \quad \text{(Eq. A21)}$$

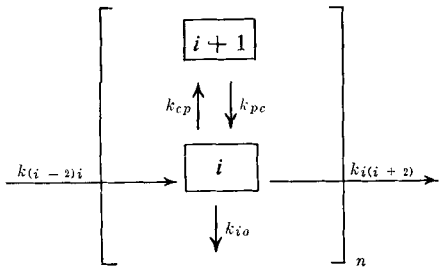
$$A_{5j} = \frac{A_{3j}k_{35}(k_{65}-a_j)}{(a_j-k_{56}-k_{50})(a_j-k_{65})-k_{56}k_{65}} \qquad j = 3,4,5, \text{ and } 6 \quad \text{(Eq. A22)}$$

$$A_{57} = \left[-\sum_{j=3}^{6} A_{5j} + \frac{(a_8-k_{65})}{k_{56}}\left(-\sum_{j=3}^{6} A_{6j}\right)\right]\frac{(k_{65}-a_7)}{(a_8-a_7)} \quad \text{(Eq. A23)}$$

$$A_{58} = -\sum_{j=3}^{7} A_{5j} \quad \text{(Eq. A24)}$$

$$A_{6j} = \frac{A_{5j}k_{56}}{k_{65}-a_j} \qquad j = 3,4,\ldots 8 \quad \text{(Eq. A25)}$$

For a general model composed of n individual two-compartment open-model units, as seen in Scheme IV, the solutions of eigen values



Scheme IV—General model composed of n *individual two-compartment open-model units*

and eigen vectors may be generalized as follows. Where:

$i = 1, 3, 5 \ldots$ $\quad i + 1$ = peripheral compartment
i = central compartment $\quad k_{io} + k_{i(i+2)} = k_{el}$

the general solution of the eigen values would therefore be:

$$a_{j-1} = \frac{(k_{cp} + k_{pc} + k_{io} + k_{i(i+2)}) + \sqrt{(k_{cp} + k_{pc} + k_{io} + k_{i(i+2)})^2 - 4\,(k_{io} + k_{i(i+2)})\,k_{pc}}}{2} \quad \text{(Eq. A26)}$$

$$a_j = \frac{(k_{cp} + k_{pc} + k_{io} + k_{i(i+2)}) - \sqrt{(k_{cp} + k_{pc} + k_{io} + k_{i(i+2)})^2 - 4\,(k_{io} + k_{i(i+2)})\,k_{pc}}}{2} \quad \text{(Eq. A27)}$$

Where $j = 3, 4, 5 \ldots i + 3$, the general solution of the eigen vector A_{ij} for the ith central compartment is:

$$A_{ij} = \frac{A_{(i-2)j}\,k_{(i-2)i}\,(k_{pc} - a_j)}{(a_j - k_{cp} - k_{el})\,(a_j - k_{pc}) - k_{cp}k_{pc}} \qquad j = 3,4,5 \cdots j - 2 \quad \text{(Eq. 28)}$$

$$A_{i(j-1)} = \left[-\sum_{j=3}^{j-2} A_{ij} + \frac{(a_{i+3} - k_{pc})}{k_{cp}} \left(-\sum_{j=3}^{j-2} A_{(i+1)j} \right) \right] \times \frac{(k_{pc} - a_{i+2})}{(a_{i+3} - a_{i+2})} \qquad j = i + 2 \quad \text{(Eq. A29)}$$

$$A_{ij} = -\sum_{j=3}^{j-1} A_{ij} \qquad j = i + 3 \quad \text{(Eq. A30)}$$

Therefore, the general solution of the eigen vectors, A_{ij}, for the $_{i+1}$th peripheral compartment is:

$$A_{(i+1)j} = \frac{A_{ij}\,K_{cp}}{k_{pc} - a_j} \qquad j = 3, 4, 5 \cdots i + 3 \quad \text{(Eq. A31)}$$

REFERENCES

(1) G. Zbinden and L. O. Randall, *Advan. Pharmacol.*, **5**, 213 (1967).

(2) L. O. Randall and W. Schallek, in "Psychopharmacology: A Review of Progress 1957–1967," D. H. Efron, Ed., Public Health Service Publication No. 1836, 1968, p. 153.

(3) B. A. Koechlin and L. D'Arconte, *Anal. Biochem.*, **5**, 195 (1963).

(4) B. A. Koechlin, M. A. Schwartz, G. Krol, and W. Oberhaensh, *J. Pharmacol. Exp. Ther.*, **148**, 339(1965).

(5) M. A. Schwartz and E. Postma, *J. Pharm. Sci.*, **55**, 1358 (1966).

(6) L. O. Randall, C. L. Scheckel, and R. F. Banziger, *Curr. Ther. Res.*, **7**, 590(1965).

(7) C. L. Scheckel and T. Smart, unpublished data.

(8) S. Riegelman, J. C. K. Loo, and M. Rowland, *J. Pharm. Sci.*, **57**, 117(1968).

(9) Pharmacokinetic Data Analyses Program No. R0717, Hoffmann-La Roche, Inc., Nutley, N. J., 1969.

(10) S. A. Kaplan, unpublished data.

(11) M. A. Schwartz, unpublished data.

(12) J. C. K. Loo and S. Riegelman, *J. Pharm. Sci.*, **57**, 918 (1968).

(13) M. Berman, M. F. Weiss, and E. Shahn, *Biophys. J.*, **2**, 289 (1962).

(14) Pharmacokinetic Computer System No. R0700, Hoffmann-La Roche, Inc., Nutley, N. J., 1969.

(15) A. H. Beckett, R. N. Boyes, and P. J. Appleton, *J. Pharm. Pharmacol.*, **18**, 765(1966).

(16) G. R. Wilkinson and A. H. Beckett, *J. Pharm. Sci.*, **57**, 1933 (1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 24, 1970, from the *Department of Clinical Pharmacology, Hoffmann-La Roche, Inc., Nutley, NJ 07110*

Accepted for publication June 10, 1970.

The authors thank Mr. A. Chelak for writing the FORTRAN program for the pharmacokinetic model.

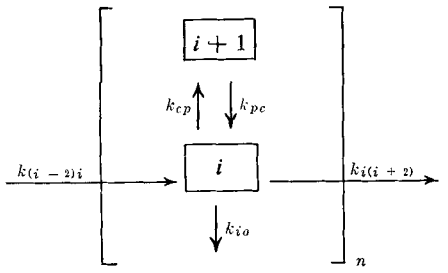
Solubility of Alkyl Benzoates I: Effect of Some Alkyl *p*-Hydroxybenzoates (Parabens) on the Solubility of Benzyl *p*-Hydroxybenzoate

F. SHIHAB, W. SHEFFIELD, J. SPROWLS, and J. NEMATOLLAHI

Abstract □ The solubility features of a homologous series of alkyl *p*-hydroxybenzoates (parabens) with alkyl groups, in an ascending order from methyl to *n*-butyl, were investigated together with benzyl paraben and methyl *p*-methoxybenzoate. A phenomenon of mutual solubilizing potential was observed to exist when the solubility of a mixture of an alkyl paraben and benzyl paraben in 60% polyethylene glycol 400–water was examined. The analysis was carried out by means of UV spectrophotometry and NMR spectroscopy. The scope of application of these esters for their antimicrobial properties, for which they are primarily employed in pharmaceutical sciences, is envisaged to be augmented by considering factors influencing solubility.

Keyphrases □ Parabens—mutual solubilizing potential □ Polyethylene glycol–water system—paraben solubility □ UV spectrophotometry—analysis □ NMR spectroscopy—analysis

A gross solubilizing effect of alkyl *p*-hydroxybenzoates (parabens) on benzyl paraben in polyethylene glycol 400–water (designated as PEG–H_2O) mixture was first observed by Sprowls (1). Owing to the interest of pharmaceutical scientists in the physicochemical properties of parabens (2–4), which are employed both as medicinal



Scheme IV—General model composed of n *individual two-compartment open-model units*

and eigen vectors may be generalized as follows. Where:

$i = 1, 3, 5 \ldots$ $\quad i + 1$ = peripheral compartment
i = central compartment $\quad k_{io} + k_{i(i+2)} = k_{el}$

the general solution of the eigen values would therefore be:

$$a_{j-1} = \frac{(k_{cp} + k_{pc} + k_{io} + k_{i(i+2)}) + \sqrt{(k_{cp} + k_{pc} + k_{io} + k_{i(i+2)})^2 - 4\,(k_{io} + k_{i(i+2)})\,k_{pc}}}{2} \quad \text{(Eq. A26)}$$

$$a_j = \frac{(k_{cp} + k_{pc} + k_{io} + k_{i(i+2)}) - \sqrt{(k_{cp} + k_{pc} + k_{io} + k_{i(i+2)})^2 - 4\,(k_{io} + k_{i(i+2)})\,k_{pc}}}{2} \quad \text{(Eq. A27)}$$

Where $j = 3, 4, 5 \ldots i + 3$, the general solution of the eigen vector A_{ij} for the ith central compartment is:

$$A_{ij} = \frac{A_{(i-2)j}\,k_{(i-2)i}\,(k_{pc} - a_j)}{(a_j - k_{cp} - k_{el})\,(a_j - k_{pc}) - k_{cp}k_{pc}} \qquad j = 3,4,5 \cdots j - 2 \quad \text{(Eq. 28)}$$

$$A_{i(j-1)} = \left[-\sum_{j=3}^{j-2} A_{ij} + \frac{(a_{i+3} - k_{pc})}{k_{cp}} \left(-\sum_{j=3}^{j-2} A_{(i+1)j} \right) \right] \times \frac{(k_{pc} - a_{i+2})}{(a_{i+3} - a_{i+2})} \qquad j = i + 2 \quad \text{(Eq. A29)}$$

$$A_{ij} = -\sum_{j=3}^{j-1} A_{ij} \qquad j = i + 3 \quad \text{(Eq. A30)}$$

Therefore, the general solution of the eigen vectors, A_{ij}, for the $_{i+1}$th peripheral compartment is:

$$A_{(i+1)j} = \frac{A_{ij}\,K_{cp}}{k_{pc} - a_j} \qquad j = 3, 4, 5 \cdots i + 3 \quad \text{(Eq. A31)}$$

REFERENCES

(1) G. Zbinden and L. O. Randall, *Advan. Pharmacol.*, **5**, 213 (1967).

(2) L. O. Randall and W. Schallek, in "Psychopharmacology: A Review of Progress 1957–1967," D. H. Efron, Ed., Public Health Service Publication No. 1836, 1968, p. 153.

(3) B. A. Koechlin and L. D'Arconte, *Anal. Biochem.*, **5**, 195 (1963).

(4) B. A. Koechlin, M. A. Schwartz, G. Krol, and W. Oberhaensh, *J. Pharmacol. Exp. Ther.*, **148**, 339(1965).

(5) M. A. Schwartz and E. Postma, *J. Pharm. Sci.*, **55**, 1358 (1966).

(6) L. O. Randall, C. L. Scheckel, and R. F. Banziger, *Curr. Ther. Res.*, **7**, 590(1965).

(7) C. L. Scheckel and T. Smart, unpublished data.

(8) S. Riegelman, J. C. K. Loo, and M. Rowland, *J. Pharm. Sci.*, **57**, 117(1968).

(9) Pharmacokinetic Data Analyses Program No. R0717, Hoffmann-La Roche, Inc., Nutley, N. J., 1969.

(10) S. A. Kaplan, unpublished data.

(11) M. A. Schwartz, unpublished data.

(12) J. C. K. Loo and S. Riegelman, *J. Pharm. Sci.*, **57**, 918 (1968).

(13) M. Berman, M. F. Weiss, and E. Shahn, *Biophys. J.*, **2**, 289 (1962).

(14) Pharmacokinetic Computer System No. R0700, Hoffmann-La Roche, Inc., Nutley, N. J., 1969.

(15) A. H. Beckett, R. N. Boyes, and P. J. Appleton, *J. Pharm. Pharmacol.*, **18**, 765(1966).

(16) G. R. Wilkinson and A. H. Beckett, *J. Pharm. Sci.*, **57**, 1933 (1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 24, 1970, from the *Department of Clinical Pharmacology, Hoffmann-La Roche, Inc., Nutley, NJ 07110*

Accepted for publication June 10, 1970.

The authors thank Mr. A. Chelak for writing the FORTRAN program for the pharmacokinetic model.

Solubility of Alkyl Benzoates I: Effect of Some Alkyl *p*-Hydroxybenzoates (Parabens) on the Solubility of Benzyl *p*-Hydroxybenzoate

F. SHIHAB, W. SHEFFIELD, J. SPROWLS, and J. NEMATOLLAHI

Abstract □ The solubility features of a homologous series of alkyl *p*-hydroxybenzoates (parabens) with alkyl groups, in an ascending order from methyl to *n*-butyl, were investigated together with benzyl paraben and methyl *p*-methoxybenzoate. A phenomenon of mutual solubilizing potential was observed to exist when the solubility of a mixture of an alkyl paraben and benzyl paraben in 60% polyethylene glycol 400–water was examined. The analysis was carried out by means of UV spectrophotometry and NMR spectroscopy. The scope of application of these esters for their antimicrobial properties, for which they are primarily employed in pharmaceutical sciences, is envisaged to be augmented by considering factors influencing solubility.

Keyphrases □ Parabens—mutual solubilizing potential □ Polyethylene glycol–water system—paraben solubility □ UV spectrophotometry—analysis □ NMR spectroscopy—analysis

A gross solubilizing effect of alkyl *p*-hydroxybenzoates (parabens) on benzyl paraben in polyethylene glycol 400–water (designated as PEG–H_2O) mixture was first observed by Sprowls (1). Owing to the interest of pharmaceutical scientists in the physicochemical properties of parabens (2–4), which are employed both as medicinal

Table I—Solubility of Alkyl Parabens in Moles/Liter (M) and in Grams/Liter (G) in PEG–H_2O Ranging from 0 to 100% at 27° (Analyzed by Using UV)

R	0%		20%		40%		60%		80%		100%	
	M	G	M	G	M	G	M	G	M	G	M	G
CH_3	0.0165	2.512	0.0601	9.150	0.2675	40.700	1.1410	174.000	2.2199	337.750	2.2594	343.750
C_2H_5	0.0062	1.030	0.0244	4.063	0.1128	18.750	0.8527	141.690	2.0461	340.000	2.1063	350.000
n-C_3H_7	0.0022	0.400	0.0101	1.825	0.0499	9.000	0.6437	116.000	2.0116	362.500	2.0255	365.000
n-C_4H_9	0.0012	0.240	0.0068	1.313	0.0399	7.750	1.0683[a]	207.500	3.1406	610.000	3.1514	612.100
CH_2-Ph	0.0002	0.040	0.0016	0.378	0.0145	3.313	0.3329	76.000	1.5992	365.000	1.9278	440.000

[a] Concentration of butyl paraben at a point just prior to separation into two phases.

agents and for inhibition of the growth of microorganisms in food, cosmetics, and pharmaceutical preparations, extensive research on the solubility property of parabens and their potential to affect the solubility of each other in a given solvent was presumed to be valuable.

Using 60% PEG–H_2O as a solvent, a preliminary observation revealed that, in contrast to the solubility of the individual parabens, combining two parabens enhances the degree of solubility of both esters. This suggested the existence of a mutual solubilizing phenomenon among parabens. After this empirical observation, a few experiments were designed in an effort to characterize some solubility properties and the solubilizing potential of each member of the homologous series of alkyl parabens (methyl, ethyl, n-propyl, and n-butyl) together with benzyl paraben and methyl p-methoxybenzoate.

Three different solubility aspects of parabens were investigated.

First, the solubility of each paraben in the series was determined in various percentages of PEG–H_2O (0–100% PEG). UV spectrophotometry was employed for quantitative measurements. The solubility information obtained, besides its usefulness *per se*, was found valuable for further solubility study, wherein a mixture of two parabens rather than a single paraben was used. Accordingly, for the solubility study of a mixture of two parabens, a solvent containing 60% PEG in H_2O was adopted. In this solvent the solubilizing affect of the parabens on each other was envisaged to be more pronounced; their individual quantitation in the mixture, using NMR (5), could be performed with adequate accuracy.

Second, four pairs of parabens (combination of benzyl paraben with each of the alkyl parabens in the homologous series: methyl, ethyl, n-propyl, and n-butyl paraben) were subjected to solubility evaluation in an effort to examine the solubilizing effect of the parabens constituting the pair on each other (mutual solubilizing potential). The procedure consisted of adding each paraben in the pair to 60% PEG–H_2O until the solids remained in equilibrium with the solution.

Third, the solubility of benzyl paraben was determined in 60% PEG–H_2O solutions of methyl, ethyl, n-propyl, and n-butyl paraben, respectively, prepared in five different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 M). Due to the limited solubility of methyl p-methoxybenzoate, only the solubilizing effect of 0.1 M of this ester on benzyl paraben was evaluated. The analysis was performed by using NMR.

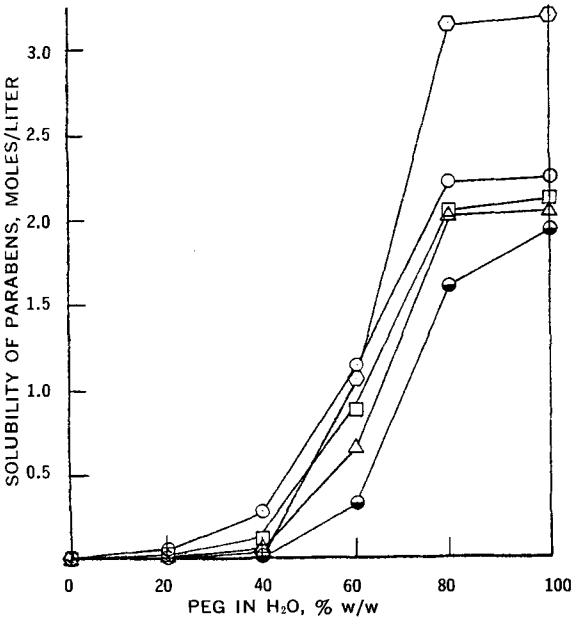


Figure 1—*Solubility of parabens in polyethylene glycol and water mixture of various proportions. Key:* O, *methyl;* □, *ethyl;* △, *n-propyl;* ○, *n-butyl; and* ◒, *benzyl parabens.*

EXPERIMENTAL

All the compounds employed in the experiments were analytical reagents: methyl p-hydroxybenzoate (Lot No. 5266),[1] ethyl p-hydroxybenzoate (Lot No. 5082),[1] n-propyl p-hydroxybenzoate (Lot No. 5294),[1] n-butyl p-hydroxybenzoate (Lot No. 5158),[1] benzyl p-hydroxybenzoate (Lot No. 1167),[1] methyl p-methoxybenzoate (methyl anisate Lot No. 1702),[2] and polyethylene glycol 400 USP YE 889.[3]

The analyses were carried out by using UV spectrophotometry (Beckman DB) and/or NMR spectrometry (Varian Associate A-60) at ambient temperature.

The solvents were prepared by mixing accurately weighed quantities of polyethylene glycol 400 and water. The percentages are expressed on a weight-to-weight basis.

The molar solution of each paraben was prepared by weighing accurately a precalculated quantity of the desired paraben in a volumetric flask and then dissolving the solid in 60% PEG–H_2O by gentle shaking.

The solubility of each paraben was determined by adding the compound beyond its saturation point to the solvent and shaking the mixture in a constant-temperature water bath (27.0 ± 0.1°) for 24 hr.

The solubility of benzyl paraben in the 0.1, 0.2, 0.3, 0.4, and 0.5 M solutions of each of the methyl, ethyl, n-propyl, and n-butyl paraben and in the 0.1 M of methyl p-methoxybenzoate was determined by

[1] Supplied by Matheson Coleman & Bell.
[2] Supplied by Eastman Organic Chemicals.
[3] Supplied by City Chemical Corp.

Table II—Quantitative Analysis of Each Phase Formed as a Result of Dissolving Butyl Paraben in 60% PEG–H_2O

	% w/v	% w/w
Upper Phase		
Butyl paraben	1.25	1.21
PEG	43.39	42.13
Water	58.36	56.66
Lower Phase		
Butyl paraben	54.31	51.94
PEG	40.53	38.66
Water	9.72	9.40

adding solid benzyl paraben, beyond its saturation point, to the solution and shaking the flask in a constant-temperature water bath (27.0 ± 0.1°) for 24 hr. The analysis of the solutions was carried out by using NMR spectrometry.

For determining the solubilizing effect of one paraben on the other, benzyl paraben and one of the alkyl parabens were added alternately and portionwise into 60% PEG–H_2O until a noticeable quantity of each paraben remained undissolved.

The samples for analysis were obtained by withdrawing the solution with a pipet on whose tip had been tied one layer of Whatman No. 1 filter paper for the purpose of ensuring the exclusion of of solid particles.

If NMR was to be used for the analysis, the samples were instilled into an NMR tube as such. For UV analysis, a known volume of the sample was diluted further with 50% ethanol using volumetric flasks. The absorbance values were recorded at $\lambda = 256$ mμ.

RESULTS AND DISCUSSION

Table I and Fig. 1 depict the results of experiments on the solubility of parabens in various proportions of PEG–H_2O. Probably due to a decrease in the polarity of the solvent, the solubility of parabens increases as a function of increase in the proportion of PEG to H_2O. In the homologous series of parabens employed in these experiments on a mole-to-mole basis up to a concentration of 40% PEG–H_2O, the solubility of each member was found to be slightly lower than its next lower homolog. If molar concentrations were converted to weight percentage, a reverse trend was observed for butyl paraben in 60% and for all the others in 80% PEG–H_2O and 100% PEG.

A sharp increase in the solubility of butyl paraben beyond 60% PEG–H_2O is probably due to the lipophilic nature of the butyl chain.

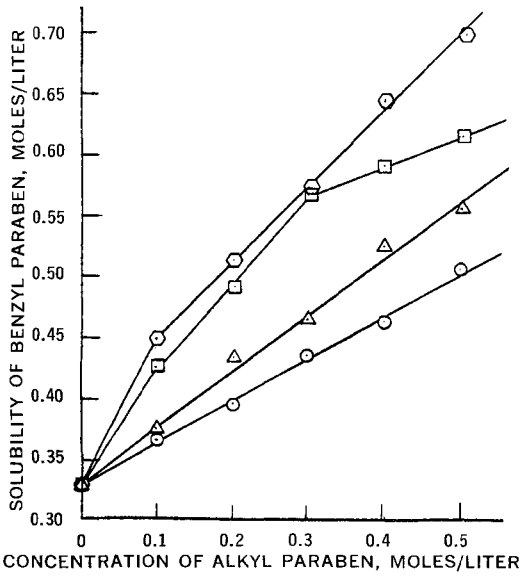


Figure 2—*Solubility of benzyl paraben in 60% PEG–H_2O solutions of a homologous series of alkyl parabens in concentrations ranging from 0.1 to 0.5* M. *Key:* O, *methyl;* △, *ethyl;* □, n-*propyl; and* ○, n-*butyl.*

Table III—Solubility and Parabens, in Moles/Liter (*M*) (60% PEG–H_2O and 27°) Determined for Each Paraben Both when Dissolved Individually and as a Combination of Two (NMR Used for Analysis)

Paraben (Single)	Solubility (*M*)	Parabens (Combined)	Solubility (*M*)
Methyl	1.1854	Methyl + Benzyl	1.3195
Benzyl	0.3301		0.6841
Ethyl	0.8346	Ethyl + Benzyl	1.0827
Benzyl	0.3301		0.7934

As indicated in Table I, the solubility of butyl paraben in 60% PEG–H_2O was recorded at the point just prior to its separation into two phases. Beyond this point the addition of more butyl paraben causes a formation of two layers. The constituent of each phase was determined quantitatively by the method described in the *Appendix*. The authors know of no previous explicitly described analysis of the two phases whose formation in alcoholic solvents also has been observed and reported (4). Nor has an unequivocally convincing explanation yet been expressed regarding the physicochemical features of each phase.

Table II depicts the quantity of each component present in each phase; details of the calculations are given in the *Appendix*.

The peaks for water and PEG in the NMR spectrum of the upper phase occur at δ 4.6 and 3.6, respectively. As shown in the calculations, correction has been made for the contribution of two protons to the water peak by the 2 OH's per molecule of PEG. There was no contribution from the phenolic OH of the butyl paraben due to its low concentration in the upper phase (no phenyl proton peak was observed at a spectrum amplitude in which the water and PEG integral values were determined).

For the lower phase, the concentration of butyl paraben was determined by comparing its NMR spectrum with that of a standard, analogous to the previously reported method (5). Similarly, the concentration of PEG was calculated, and then the quantity of H_2O was obtained by the difference (by subtraction from the weight of 1 ml. of the solution).

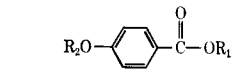
The formation of a two-phase system as a result of dissolving a high quantity of butyl paraben in 60% PEG–H_2O is probably due to the predominant occupation of etheric oxygen of polyethylene glycol by hydrogen bonding with phenolic OH of the paraben. Considering the preferred conformation of polyethylene glycol, such a hydrogen bond will restrict the free rotation of the phenyl group because of interference between phenyl hydrogens *ortho* to OH and methylene hydrogens of PEG. The butyl groups of two butyl parabens hydrogen bonded to two alternate oxygens, as becomes obvious by observing a molecular model,[4] can line up by van der Waals' force and maintain a relatively rigid structure. The net result becomes the formation of a complex, nonpolar enough to be capable of squeezing out water. The water, as it is compelled to form a separate phase, by virtue of hydrogen bonding carries with itself polyethylene glycol molecules which are not bound to butyl paraben.

A similar phase separation was also observed during the determination of mutual solubilizing potential of both benzyl paraben–propyl paraben and benzyl paraben–butyl paraben mixtures. The mechanism of formation of a two-phase system in this case is presumed to be analogous to that postulated for the butyl paraben. However, the benzyl paraben with a bulky nonpolar aralkyl moiety, like its butyl analog, is capable of participating in complex formation. The solubility expression for these two systems henceforth was thought to be meaningless and unsuitable for inclusion in a table. Table III, therefore, includes the experimental results of the mutual solubilizing potential of only two pairs of combinations (methyl paraben–benzyl paraben and ethyl paraben–benzyl paraben).

The solubilizing potential of the alkyl parabens to affect the solubility of benzyl paraben, as depicted in Fig. 2, increases in the ascending order of the alkyl chain of the homologous series and,

[4] The molecular model was constructed by using Dreiding Plastic Stereo model (Rinco Instrument Co., Inc. Greenville, Ill.).

Table IV—Solubility of Benzyl Paraben in Moles/Liter (M) in the 60% PEG–H_2O Solution of the Alkyl Parabens Ranging from 0.1 to 0.5 M at 27° (Analyzed by Using NMR)



R_1	R_2	0.0 M	0.1 M	0.2 M	0.3 M	0.4 M	0.5 M
CH_3	H	0.3301	0.3654	0.3942	0.4383	0.4627	0.5054
C_2H_5	H	0.3301	0.3753	0.4364	0.4650	0.5252	0.5574
n-C_3H_7	H	0.3301	0.4282	0.4929	0.5689	0.5917	0.6133
n-C_4H_9	H	0.3301	0.4491	0.5178	0.5742	0.6449	0.6952
CH_3	CH_3	0.3301	0.4415				

except for propyl paraben, seems to follow a linear relationship with respect to the concentration of each member in the series. Knowledge of whether or not the odd-number carbon chain of propyl paraben is an influencing factor on causing the curve to bend toward the x-axis requires additional experiments with higher homologs.

The solubilizing effect of methyl p-methoxybenzoate on benzyl paraben, as shown in Table IV, is somewhat similar to butyl paraben. The substitution of a lipophilic group, OCH_3, for OH of methyl paraben apparently enhances the solubilizing potential of the ester.

A theoretical consideration of this and the mutual solubilizing phenomenon in general is under study. The practical aspect of solubility is envisaged to possess application in pharmaceutical preparation and microbiological research and development.

APPENDIX

The following abbreviations are used in the equations: integral peak value = I, apparent = app., molar concentration = M, weight in grams = w, butyl paraben = BuPab, density of solution = d, solution = sln., and standard = st.

The PEG 400 formula is: OH—CH_2—CH_2—(OCH_2—CH_2)$_8$—OH.

Upper Phase—Both UV and NMR were used for analysis.

$$(H_2O)_{app.I} - (PEG)_I \times 1/18 = (H_2O)_I \text{ (correction for 2 OH/mole of PEG)} \quad \text{(Eq. 1)}$$

$$\frac{(PEG)_I}{(H_2O)_I} \times 1/18 = \frac{(PEG)_M}{(H_2O)_M} \text{ (molar ratio)} \quad \text{(Eq. 2)}$$

$$\frac{(PEG)_M}{(H_2O)_M} \times \frac{400}{18} = \frac{(PEG)_w}{(H_2O)_w} \text{ (weight ratio)} \quad \text{(Eq. 3)}$$

Weight of BuPab in g./ml. determined using UV = $(BuPab)_{w/ml.}$. Weight of 1 ml. of solution − $(BuPab)_w$ = $(PEG + H_2O)_{w/ml.}$.

$$\frac{(PEG + H_2O)_{w/ml.} \times (PEG)_w}{(PEG)_w + (H_2O)_w} = (PEG)_{w/ml.} \quad \text{(Eq. 4)}$$

$$\frac{(PEG + H_2O)_{w/ml.} \times (H_2O)_w}{(PEG)_w + (H_2O)_w} = (H_2O)_{w/ml.} \quad \text{(Eq. 5)}$$

$$\frac{(BuPab)_{w/ml.} \times 100}{d} = w\% \text{ BuPab} \quad \text{(Eq. 6)}$$

$$\frac{(PEG)_{w/ml.} \times 100}{d} = w\% \text{ PEG} \quad \text{(Eq. 7)}$$

$$\frac{(H_2O)_{w/ml.} \times 100}{d} = w\% \ H_2O \quad \text{(Eq. 8)}$$

Lower Phase—NMR was used for analysis.

$$\frac{(BuPab)_I \times (BuPab)_{M\cdot st.}}{(BuPab)_{I\cdot st.}} = (BuPab)_M \quad \text{(Eq. 9)}$$

$$\frac{(PEG)_I \times \frac{1}{9} \times (BuPab)_{M\cdot st.}}{(BuPab)_{I\cdot st.}} = (PEG)_M \quad \text{(Eq. 10)}$$

$$\frac{(BuPab)_M \times (BuPab)_{M\cdot w}}{1000} = (BuPab)_{w/ml.} \quad \text{(Eq. 11)}$$

$$\frac{(PEG)_M \times (PEG)_{M\cdot w}}{1000} = (PEG)_{w/ml.} \quad \text{(Eq. 12)}$$

Weight of 1 ml. of solution − $[(BuPab)_w + (PEG)_w]$ = $(H_2O)_{w/ml.}$.

$$\frac{(BuPab)_{w/ml.} \times 100}{d} = w\% \text{ BuPab} \quad \text{(Eq. 13)}$$

$$\frac{(PEG)_{w/ml.} \times 100}{d} = w\% \text{ PEG} \quad \text{(Eq. 14)}$$

$$\frac{(H_2O)_{w/ml.} \times 100}{d} = w\% \ H_2O \quad \text{(Eq. 15)}$$

REFERENCES

(1) J. B. Sprowls, unpublished data.

(2) N. K. Patel and H. B. Kostenbauder, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 289(1958).

(3) S. M. Blaug and S. S. Ahsan, *J. Pharm. Sci.*, **50**, 441(1961).

(4) A. N. Paruta, *ibid.*, **58**, 364(1969).

(5) F. Shihab, W. Sheffield, J. Sprowls, and J. Nematollahi, *ibid.*, **59**, 1182(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 7, 1970, from the *College of Pharmacy, University of Texas at Austin, Austin, TX 78712*

Accepted for publication June 3, 1970.

This investigation was supported in part by a grant from the Graduate School, University of Texas.

Molecular Scale Drug Entrapment as a Precise Method of Controlled Drug Release II: Facilitated Drug Entrapment to Polymeric Colloidal Dispersions

C. T. RHODES*, K. WAI†, and G. S. BANKER

Abstract □ Further studies have been carried out to investigate drug–polymer flocculation as a means of molecular scale drug entrapment. It has been shown that a suitable organic acid greatly increases the degree of interaction between the drug and the polymer and provides a mechanism controlling both interaction and subsequent drug-release properties. Results of tests indicate that the products prepared by the facilitated entrapment method possess excellent sustained-action characteristics. In addition, the drug entrapped as the carboxylate salt or in conjunction with the appropriate dicarboxylic acid, while demonstrating substantially complete drug-dissolution release in intestinal fluid, could be maintained in the entrapped form in aqueous suspensions during storage periods in excess of 1 month. X-ray diffraction analysis confirmed the molecular scale level of entrapment of the drug in the polymer flocculated systems.

Keyphrases □ Drug entrapment, molecular scale—controlled-release method □ Polymer–drug interaction—drug salt form effect □ Particle-size effect—drug release from polymer–drug flocculates □ X-ray diffraction—polymer–drug flocculates

Although control of the intensity and duration of drug action has been of interest to the medical and pharmaceutical profession for many years, it is only recently that controlled-action pharmaceuticals have been exploited commercially to any extent.

The advantages resulting from prolongation of drug action have been summarized by Wilson (1). Perhaps the most important is the maintenance of therapeutic effect for extended periods of time, particularly when continuous action at a therapeutic level is essential for efficient therapy. Another factor of importance is the convenience offered to the patient by this type of preparation. It has also been reported that the number of occasions when patients forget their medicine is significantly reduced when sustained-action preparations are used (2).

Optimization of drug action, including improved drug safety, may often be approached by controlling the rate of drug delivery to the absorption pool from the dosage form. A very rapid rate of drug delivery from the dosage form undoubtedly often does not represent the optimum delivery system.

It is somewhat surprising, in view of the many advantages offered by sustained-action or controlled-release dosage forms, that greater use has not been made of this approach in designing drug delivery systems. Although some drugs are inadequately absorbed along the gastrointestinal tract or have an insufficient margin of safety between therapeutic and toxic doses for sustained-action formulation to be feasible, other reasons are apparent for the still limited application of this frequently advantageous approach to the pharmacodynamic improvement of drug action. These reasons include, but are not limited to, the fact that most approaches to sustained-release formulation have involved empirical coating methods applied to nonuniform particulate drug-containing systems, which are coated at several thicknesses or with several compositions to provide a range of release characteristics. The manufacturing sequence of such products is subject to considerable variation, and control is difficult. Such processes are often costly and the resultant products are expensive; development and manufacture of such dosage forms are also complex, at least somewhat imprecise, and time consuming. Molecular scale quantitative approaches to the physicochemical binding of drugs by polymers, by ion exchange, or by complexation mechanisms offer a number of advantages over conventional coating techniques. Optimization of drug action to provide precisely controlled drug release rates will only become possible when accurate methods of drug entrapment and release, based on physicochemical phenomena, are developed.

The present paper reports studies performed during the development of a new method for the formulation of sustained- or controlled-action pharmaceuticals. The technique described involves application of stoichiometric entrapment of drugs by polymer flocculation, the products being suitable for administration in either solid or liquid dosage forms.

Earlier work by Goodman *et al.* (3) indicated that the molecular scale interaction between drugs and polymers could facilitate the quantitative development of reproducible sustained-action pharmaceuticals. The method developed by the present authors is similar to that previously described (3). However, it has been found that the properties of drug–polymer flocculates, in which the drug contains an amino functional group, can be markedly improved if a suitable organic acid is included in the formulation. The presence of such a carboxylic acid anion has been shown to increase the amount of drug which may be bound by the polymer and to provide improved control of drug release from the polymer flocculate. The inclusion of the carboxylic acid in the drug–polymer flocculate has also made possible the development of liquid and other dosage forms.

The described method of molecular scale drug entrapment by these studies is extremely simple (3) and offers many advantages over the conventional lengthy and intricate methods used in the preparation of sustained-action pharmaceuticals. The present paper describes a method of facilitated drug entrapment in which a solution of the drug and an appropriate carboxylic acid is added slowly to a suitable polymer emulsion. The resultant flocculate is washed and dried.

Table I—Effect of Carboxylic Acid Anion upon the Entrapment of Chlorpheniramine by the Acrylic Copolymer Emulsion

Chlorpheniramine Salt	Initial Concn. of Drug Soln., % w/v	% Drug Bound	% Drug in Dry Flocculant
Hydrochloride	2	54	3.3
Maleate[a]	2	91	3.7
Oxalate	2	80	3.7
Malonate	2	90	3.9
Succinate	2	98	4.5
Succinate	5	93	10.0
Succinate	10	86	17.5
Adipate	5	96	10.4

[a] Obtained from Smith Kline & French Laboratories, Philadelphia, Pa.; all other salts synthesized.

Products so obtained may be readily milled to a fine powder, tableted, encapsulated, or suspended to provide controlled-release systems.

EXPERIMENTAL

Effect of Drug Salt Form upon Binding—To determine the effect of the drug anion moiety on the polymer–drug interaction, solutions of various salts of chlorpheniramine were employed to produce polymer flocculation. In this study, 100 ml. of each drug solution was used to coagulate 100 ml. of an acrylic copolymer emulsion.[1] Each flocculated system was mixed for 30 min. and then filtered under reduced pressure, the filtrate being collected and assayed for drug content. The drug salts used were either obtained commercially or synthesized. Those that were synthesized were recrystallized one to three times from an isopropanol–ether solvent mixture and vacuum dried at 40°; the purity was checked by melting point and IR analyses. Results of the flocculation studies are shown in Table I.

Effect of Particle Size on *In Vitro* Drug Release—The dissolution release of chlorpheniramine from a chlorpheniramine maleate–acrylic copolymer entrapment product in both simulated gastric juice (without enzyme) and simulated intestinal juice (without enzyme) was determined as a function of particle size (Table II). One-half-gram samples of each polymer–drug particle-size fraction were placed in 50 ml. of gastric or intestinal fluid, rotated at 37° according to the method of Goodman *et al.* (3), and assayed at the times specified.

Release of Drug from Aqueous Solutions of Polymer–Drug Flocculates—In the initial study, 5-g. quantities of chlorpheniramine maleate–acrylic copolymer product containing 3.7% drug, as a 60-mesh undersize powder, were suspended in 100 ml. of selected vehicles in 4-oz. reagent bottles, which were shaken daily throughout the test period. The amount of drug released at room temperature into the various vehicles was determined over a period of 30 days (Table III).

A more detailed study was also performed to investigate the effect of drug concentration upon release rate. In this experiment, 5-g. quantities of polymer–drug material were placed in 60-ml. volumes of vehicle, and the resultant suspensions were rotated continuously at 37° for the time specified (Tables IV and V).

X-Ray Diffraction Studies of Drug–Polymer Flocculates—X-ray diffraction patterns of a commercially available sustained-action pharmaceutical,[2] which makes use of polymeric materials as matrices, were studied as well as drug-entrapped products made by the method described herein. All patterns were obtained with copper K-α radiation generated to 40,000 v., using a General Electric XRD-5 source and the General Electric powder diffraction camera, 143.2-mm. diameter. One-half-millimeter glass capillary tubes were used to rotate the sample in the beam. Results are shown in Fig. 1.

Table II—Release of Chlorpheniramine Maleate from a Polymer–Drug Entrapment Product[a] in Gastric and Intestinal Media as a Function of Particle Size and Time

Particle Size (Sieve Fraction)	% Drug Released in 24 hr. Gastric Juice	% Drug Released in 24 hr. Intestinal Juice	96 hr. Gastric Juice
30 mesh oversize	62	92	67
30/40 mesh	65	92	70
40/60 mesh	67	91	72
60/120 mesh	69	93	76
120/170 mesh	71	91	75
170/230 mesh	75	93	80
230 undersize	77	91	80

[a] In all cases the concentration of the drug in the polymer–drug flocculate was 4%.

Table III—Release of Chlorpheniramine Maleate from a Polymer–Drug Entrapment Product in Various Vehicles

	pH of Suspension	—% Drug Released, days— 2	7–8	14–15	30
Phosphate buffer pH 4.5	4.3	2.4	2.5	2.3	3.6
Phosphate buffer pH 6.0	5.6	0	3.8	4.2	3.6
Phosphate buffer pH 8.0	6.1	32.3	42.4	45.3	60.0
Orange syrup USP	2.4	1.6	2.0	2.4	2.7
1% Citric acid solution	2.3	11.7	23.1	27.0	35.4
Distilled water	3.3	6.4	7.1	7.5	9.6
Distilled water (stirred)[a]	3.3	4.6	6.1	6.8	7.0
Standard solution (200 mg. drug, no polymer)	4.5	100	100	100	100

[a] Stirred by a magnetic stirrer at about 30 r.p.m.

DISCUSSION

Studies with selected drug salts indicated that the addition of a stoichiometric quantity of an appropriate organic acid to the drug hydrochloride salt solutions, or the use of a drug carboxylate as the flocculant solution, greatly increased the amount of drug that could be bound by the polymer. The data shown in Table I clearly indicate the utility of the organic acid anion, since 80–90% of the drug was bound in the presence of such an anion-entrapment facilitator whereas only half the amount of drug could be bound when no facilitator was present, *i.e.*, the hydrochloride drug salt. Entrapment was facilitated equally well regardless of whether the drug carboxylate salt was prepared and entrapped or a stoichiometric quantity of the carboxylic acid was added to the drug hydrochloride salt solution. It is also significant that a 2% solution of the hydrochloride salt could only be entrapped to the extent of 54% efficiency while a 5% solution of the adipate was 96% entrapped and a 10% solution of the succinate was entrapped with 86% efficiency.

The results in Table II show several desirable features of the entrapment system from the point of view of a potential controlled-release product. Even though there is apparently a strong interaction between the drug and polymer, over 90% of the drug is available in simulated intestinal juice. Further, even though particle size has some effect upon the release in gastric juice, this dependency is not great. The release in intestinal juice appears to be substantially independent of particle size. Also, the facts that about one-third of the available drug is not released in gastric juice, even after as long as 24 hr., and as little as 5 or 10% is released in selected buffers, even on stirring for 30 days (Table III), indicate the potential of these products for controlled-release or sustained-action liquid dosage forms.

Table III describes the dissolution release of the drug from a finer than 60-mesh 3.7% chlorpheniramine maleate–polymer entrapment product. The drug, in the products made by this technique, is evenly distributed throughout the polymer matrix; the drug remains associated to the polymer without alteration

[1] Acrysol ASE-75; a 100% linear, anionically charged acrylic acid copolymer with a molecular weight in excess of 300,000; supplied in emulsion form, containing 40% solids, Rohm & Haas Co., Philadelphia, Pa.

[2] Desoxyn Gradumet, 15-mg. tablet, methamphetamine hydrochloride, Abbott Labs., North Chicago, Ill.

Table IV—Release of Chlorpheniramine from Drug–Polymer Flocculates as a Function of Carboxylic Acid Anion Type and Drug Concentration

Facilitator	% Drug Concn.	Suspension Medium[a]	% Drug Release, hr. 24	48	120	Final Suspension, pH
Succinate	4.3	1	57	62	69	1.4
Succinate	4.3	2	3	3	3	4.1
Succinate	4.3	3	99	97	—	7.1
Succinate	10.3	1	86	84	84	1.4
Succinate	10.3	2	12	12	12	4.5
Succinate	10.3	3	91	97	—	7.4
Succinate	18.9	1	81	83	85	1.4
Succinate	18.9	2	18	19	21	4.9
Succinate	18.0	3	89	94	—	8.0
Oxalate	4.0	1	50	56	56	1.4
Oxalate	4.0	2	6	6	6	3.8
Oxalate	4.0	3	86	92	—	7.0
Malonate	4.1	1	62	67	68	1.4
Malonate	4.1	2	5	5	5	3.6
Malonate	4.1	3	88	98	—	7.0
Maleate	3.9	1	55	58	67	1.4
Maleate	3.9	2	5	6	6	3.9
Maleate	3.9	3	91	100	—	7.0
Adipate	9.9	1	82	81	85	1.4
Adipate	9.9	2	10	10	10	4.4
Adipate	9.9	3	92	95	—	7.7

[a] Suspension medium: 1, simulated gastric fluid USP without enzyme; 2, phosphate USP buffer, pH 4.5; and 3, simulated intestinal fluid USP without enzyme.

after high-speed milling in a comminutor and is contained in the various particle-size fractions in the same concentration. While substantial drug release was indicated in gastric fluid and nearly complete release in intestinal fluid (Table II), minimal drug dissolution occurs at pH values of 4.5–6, and drug dissolution in vehicles which correspond to this pH was minimal (less than 3% in orange syrup in 30 days).

Results shown in Table IV further indicate that the products obtained by this flocculation technique have potential for use as controlled release-suspensions, since the amount of drug released from suspensions in vehicles at pH values between about 4 and 6 is very low.

Continuous stirring of a distilled water suspension over a 30-day period produced no substantial alteration in the release pattern as compared with a similar suspension that was only shaken daily (Table III). Likewise, samples stored at an elevated temperature demonstrated no significant difference in release rate. The results recorded in Table V show that particle size has a negligible effect upon the amount of drug release from suspensions buffered at pH 4.5; this property of the product is, of course, most advantageous for sustained-action liquid suspensions.

Figure 1 presents photographs of the X-ray diffraction patterns of a molecular scale entrapment product, a physical mixture of the drug plus polymer employed in the entrapment system, and a commercially available polymer–drug matrix system.[2]

Photo A of Fig. 1 is a photograph of the X-ray diffraction pattern of a commercial methamphetamine hydrochloride–polymer matrix system (15 mg./tablet, tablet weight 115 mg.). The diffraction lines show crystalline methamphetamine hydrochloride to be present. All the lines listed in Pattern Number 5-0246 for methamphetamine hydrochloride, American Society for Testing Materials, are found in Fig. 1A. Thus, in this product the drug is not dispersed within the

Table V—Release of Chlorpheniramine from a Chlorpheniramine Maleate–Polymer Entrapment Product (3.7% Drug) in pH 4.5 Buffer as a Function of Particle Size

Particles Size (Sieve Fraction)	% Drug Release, hr. 24	48	120
30/40 mesh	4.7	4.8	5.1
80/120 mesh	5.4	5.4	5.5
170/230 mesh	5.3	5.4	5.5

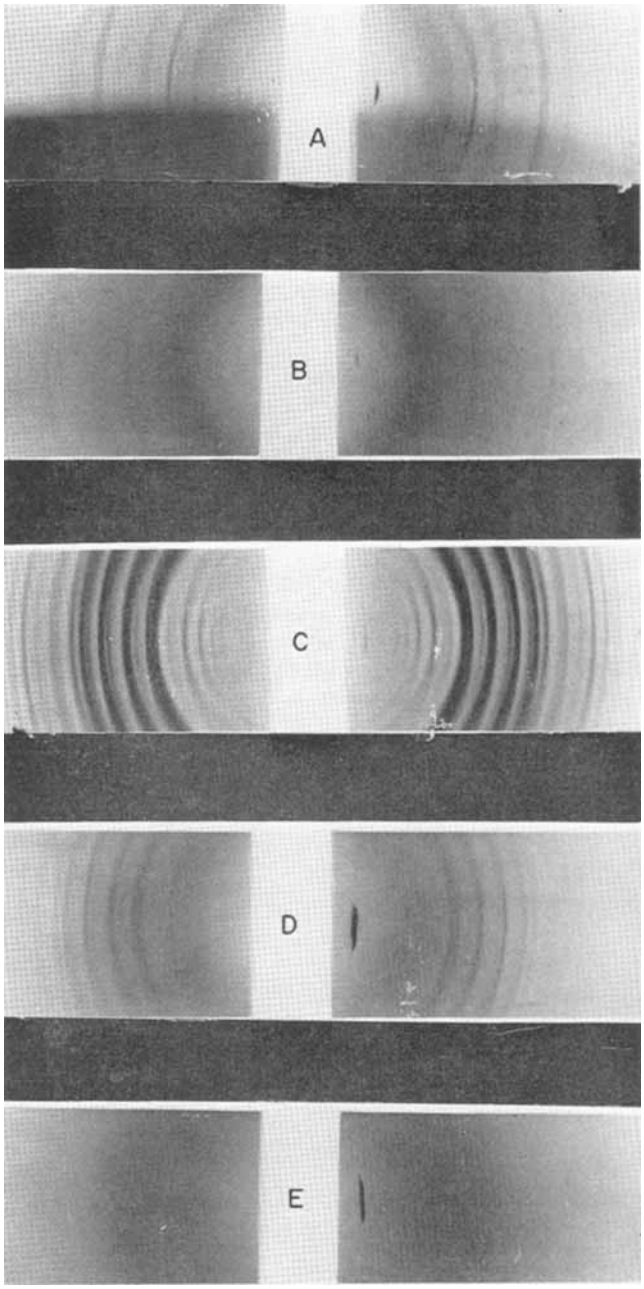


Figure 1—*X-ray diffraction patterns: A, commercial methamphetamine–polymer matrix product; B, commercial product following drug extraction; C, chlorpheniramine acid succinate; D, chlorpheniramine acid succinate, 25% as a flocculated polymer, 75% as a physical mixture; and E, chlorpheniramine acid succinate–polymer entrapment product containing 25% drug.*

product at the molecular level but merely consists of a physical compressed mixture of crystalline drug and polymer.

After leaching the commercial product with 0.1 *N* hydrochloric acid for 2 days, the X-ray diffraction pattern (Fig. 1B) shows that nearly all of the crystalline drug has been removed. The faint lines remaining may be due to talc, coloring agent, or other components of the tablet. The polymer itself shows no crystalline X-ray pattern. It is, however, responsible for the heavy, undefined, scattered radiation apparent in Fig. 1B.

Figure 1C shows the X-ray diffraction pattern of crystalline chlorpheniramine acid succinate. Figure 1D reproduces the diffraction pattern of a mechanical mixture of chlorpheniramine acid succinate (25%) and the acrylic copolymer as mechanically separated from the polymer emulsion, clearly showing the presence of crystalline drug. However, Photo E of Fig. 1, a picture of the X-ray diffraction pattern of a chlorpheniramine acid succinate–

acrylic copolymer entrapment product containing 25% drug, is quite different. The diffraction pattern indicates that crystalline drug is absent. The absence of crystalline drug in this sample demonstrates that the drug in the entrapment product is uniformly dispersed at a molecular level.

The mechanism by which the carboxylic acid anion facilitator increases the amount of drug bound by the polymer is of considerable theoretical interest and is the subject of a further study. Several modes of action appear possible. The formation of complex ions between the polymer carboxyl group, drug, and acid anions has been suggested, the resultant electrostatic forces binding the drug to the polymer. Presumably, inclusion-type complex formation may also be involved to some extent.

SUMMARY

A new method for the preparation of sustained-action pharmaceuticals by stoichiometric entrapment of drugs in polymer flocculates is described.

The advantages of the use of a suitable entrapment facilitator, an organic acid, to enhance the binding of drugs by polymers is demonstrated.

In vitro tests indicate that the products obtained by this technique could be used in either solid or liquid dosage forms.

REFERENCES

(1) A. Wilson, *Chem. Drug.*, **172**, 295(1959).

(2) R. Blythe, private communication, 1965.

(3) H. Goodman and G. S. Banker, *J. Pharm. Sci.*, **59**, 1131 (1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication May 26, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported in part by a grant from Smith Kline & French Laboratories, Philadelphia, Pa.

The authors thank Dr. W. E. Thompson and Dr. J. E. Zarembo for the X-ray diffraction studies.

* Present address: Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada.

† Present address: Biorex Laboratories, London, England.

Molecular Scale Drug Entrapment as a Precise Method of Controlled Drug Release III: *In Vitro* and *In Vivo* Studies of Drug Release

C. T. RHODES*, K. WAI†, and G. S. BANKER

Abstract □ Controlled-release drug-entrapped systems of phenylephrine and phenylpropanolamine have been prepared by the facilitated molecular scale drug-entrapment method previously described. Variables influencing the entrapment process, such as flocculation pH and rate of agitation, have been investigated. These variables are readily controllable. The influence of various polymers and carboxylic acid anions on the entrapment and release of the drugs was examined. Tests applied to the products so obtained indicate that they are well suited for pharmaceutical use. An *in vivo* study verified the sustained-release properties of a molecular scale entrapped phenylpropanolamine.

Keyphrases □ Drug entrapment, molecular scale—release-rate control □ Flocculation pH effect—drug binding, release □ Stirring rate effect—drug binding □ Release rates, *in vivo*, *in vitro*—polymer-bound drug

Parts I and II of this series described the development of a method of molecular scale drug entrapment for the physicochemical preparation of controlled-release pharmaceuticals; the advantages offered by this new technique were also discussed (1, 2). Part I described the flocculation of polymeric colloidal dispersions as a mechanism of precise drug entrapment. It was demonstrated that the resultant drug–polymer flocculates, when administered as a fine powder, exhibited very satisfactory sustained-action characteristics. However, less success was obtained when weakly basic drugs such as phenylephrine were used as flocculants.

Table I—Efficiency of Various Carboxylic Acid Anions in Binding Phenylephrine to Acrylic Copolymer Emulsion 1

Facilitator	pKa	Drug Bound by Polymer, %[a]
(Hydrochloride)		25
Adipate, H	4.43	28
Citrate, H	4.74	32
Malonate, H	2.85	32
Citrate, HH	3.06	33
Glutarate, H	4.34	35
Glutarate	5.12	36
Tartrate, H	3.01	41
Tartrate	4.54	44
Fumarate	4.47	46
Succinate, H	4.19	49
Succinate	5.57	49
Maleate, H	2.20	49
Ascorbate, H	4.17	50
Adipate	5.27	53
Citrate	5.40	58
Ascorbate	11.57	60
Maleate	6.26	60

[a] Percentage of drug entrapped from solution.

In Part II the study of drug entrapment by polymer flocculation was considerably extended. It was shown that the presence of a carboxylic acid anion greatly facilitated drug entrapment, increasing the binding of the drugs by the polymer, which substantially increased the efficiency of the drug-entrapment process and provided added control over drug release.

acrylic copolymer entrapment product containing 25% drug, is quite different. The diffraction pattern indicates that crystalline drug is absent. The absence of crystalline drug in this sample demonstrates that the drug in the entrapment product is uniformly dispersed at a molecular level.

The mechanism by which the carboxylic acid anion facilitator increases the amount of drug bound by the polymer is of considerable theoretical interest and is the subject of a further study. Several modes of action appear possible. The formation of complex ions between the polymer carboxyl group, drug, and acid anions has been suggested, the resultant electrostatic forces binding the drug to the polymer. Presumably, inclusion-type complex formation may also be involved to some extent.

SUMMARY

A new method for the preparation of sustained-action pharmaceuticals by stoichiometric entrapment of drugs in polymer flocculates is described.

The advantages of the use of a suitable entrapment facilitator, an organic acid, to enhance the binding of drugs by polymers is demonstrated.

In vitro tests indicate that the products obtained by this technique could be used in either solid or liquid dosage forms.

REFERENCES

(1) A. Wilson, *Chem. Drug.*, **172**, 295(1959).

(2) R. Blythe, private communication, 1965.

(3) H. Goodman and G. S. Banker, *J. Pharm. Sci.*, **59**, 1131 (1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication May 26, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported in part by a grant from Smith Kline & French Laboratories, Philadelphia, Pa.

The authors thank Dr. W. E. Thompson and Dr. J. E. Zarembo for the X-ray diffraction studies.

* Present address: Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada.

† Present address: Biorex Laboratories, London, England.

Molecular Scale Drug Entrapment as a Precise Method of Controlled Drug Release III: *In Vitro* and *In Vivo* Studies of Drug Release

C. T. RHODES*, K. WAI†, and G. S. BANKER

Abstract □ Controlled-release drug-entrapped systems of phenylephrine and phenylpropanolamine have been prepared by the facilitated molecular scale drug-entrapment method previously described. Variables influencing the entrapment process, such as flocculation pH and rate of agitation, have been investigated. These variables are readily controllable. The influence of various polymers and carboxylic acid anions on the entrapment and release of the drugs was examined. Tests applied to the products so obtained indicate that they are well suited for pharmaceutical use. An *in vivo* study verified the sustained-release properties of a molecular scale entrapped phenylpropanolamine.

Keyphrases □ Drug entrapment, molecular scale—release-rate control □ Flocculation pH effect—drug binding, release □ Stirring rate effect—drug binding □ Release rates, *in vivo*, *in vitro*—polymer-bound drug

Parts I and II of this series described the development of a method of molecular scale drug entrapment for the physicochemical preparation of controlled-release pharmaceuticals; the advantages offered by this new technique were also discussed (1, 2). Part I described the flocculation of polymeric colloidal dispersions as a mechanism of precise drug entrapment. It was demonstrated that the resultant drug–polymer flocculates, when administered as a fine powder, exhibited very satisfactory sustained-action characteristics. However, less success was obtained when weakly basic drugs such as phenylephrine were used as flocculants.

Table I—Efficiency of Various Carboxylic Acid Anions in Binding Phenylephrine to Acrylic Copolymer Emulsion 1

Facilitator	pKa	Drug Bound by Polymer, %[a]
(Hydrochloride)		25
Adipate, H	4.43	28
Citrate, H	4.74	32
Malonate, H	2.85	32
Citrate, HH	3.06	33
Glutarate, H	4.34	35
Glutarate	5.12	36
Tartrate, H	3.01	41
Tartrate	4.54	44
Fumarate	4.47	46
Succinate, H	4.19	49
Succinate	5.57	49
Maleate, H	2.20	49
Ascorbate, H	4.17	50
Adipate	5.27	53
Citrate	5.40	58
Ascorbate	11.57	60
Maleate	6.26	60

[a] Percentage of drug entrapped from solution.

In Part II the study of drug entrapment by polymer flocculation was considerably extended. It was shown that the presence of a carboxylic acid anion greatly facilitated drug entrapment, increasing the binding of the drugs by the polymer, which substantially increased the efficiency of the drug-entrapment process and provided added control over drug release.

Table II—Effect of pH upon Binding and Release of Phenylephrine Maleate by Acrylic Copolymer Emulsion 1

Emulsion pH	Concentration of Drug in Polymer Flocculate, % w/w	Drug Released after 30 min., %
1–1.5[a]	3.3	54
2.9	3.7	70
4.0	4.6	95
4.7	5.3	100
5.9	No flocculation	—

[a] Without pH adjustment.

Table III—Effect of Stirring Rate on Percentage of Phenylephrine Maleate Bound by Acrylic Copolymer Emulsion 1

Stirring Rate (Variac Setting)	Concentration of Drug in Flocculate, % w/w
69	1.2
80	1.3
110	1.2
140	3.0

Phenylephrine and phenylpropanolamine, two weakly basic amine drugs which initially presented considerable difficulty as to efficient entrapment and adequately retarded release utilizing the entrapment procedure, were selected for detailed study to assess the utility and applicability of the facilitated entrapment technique in a rigorous manner. Results presented in this paper show that sustained-action products of both of these drugs may be readily prepared utilizing the facilitated entrapment method. Studies made to determine the optimum conditions for facilitated entrapment were also conducted.

EXPERIMENTAL

Phenylephrine Studies—*Efficiency of Various Dicarboxylic Acid Anions in Binding Drug to Polymer*—The binding of phenylephrine[1] by an acrylic copolymer latex,[2] hereafter referred to as acrylic copolymer emulsion 1 or acrylic copolymer 1, in the presence of a variety of anions was examined in the following manner. One-fortieth of a mole quantities of drugs, as the hydrochloride, together with an equivalent amount of carboxylic acid anion and sufficient sodium hydroxide to produce the facilitator moieties of Table I, was dissolved in 50 ml. of distilled water and added over a period of 5 min. to 50 ml. of the polymer emulsion, which was stirred continuously. The flocculate was separated from the supernatant liquid by filtration, and the drug content of both product and filtrate was determined by the peak height assay method (3). Results are shown in Table I.

Effect of Flocculation pH upon Binding and Release of Drug—A stock solution of phenylephrine maleate (0.25 *M*) was prepared, and 50-ml. quantities were added to 50-ml. volumes of polymer emulsion, the pH of which had been previously adjusted by addition of suitable amounts of sodium hydroxide solution. The flocculated products, which resulted from the addition of the drug electrolyte solutions to the polymeric colloidal dispersions, were collected by filtration, dried, and reduced to a 60-mesh powder. The concentration of drug in the dry flocculates and the amount of drug released after shaking for 30 min. in simulated gastric juice (without enzyme) at 37° are recorded in Table II.

Effect of Stirring Rate upon Binding of Phenylephrine—Solutions of phenylephrine maleate were used to flocculate acrylic copolymer emulsion 1, as described in the previous section but without pH adjustment. Flocculation was effected at different stirring rates using a standard three-bladed marine propeller, driven by a laboratory mixer employing Variac speed control. The effect of stirring rate upon percentage of drug bound is shown in Table III. Attempts to effect flocculation at higher stirring rates by the use of a high-shear mixer with a rotor/stator head were unsuccessful; the flocculate blocked the narrow aperture of the mixing head.

Influence of Polymer on Drug Entrapment—Goodman *et al.* (1) preliminarily investigated the usefulness of two related polymeric dispersions as entrapment matrices. In this phase of the research the influence of the polymer on drug entrapment was more closely studied. Twenty-five polymer dispersions were screened for pH solubility properties and apparent pKa values in order to select the potentially best materials for possible use as entrapment matrices. Those which demonstrated potentially useful solubility and coagulation properties were subjected to further drug flocculation studies. Polymeric systems with reasonable flocculation values (the volumes of a stock solution of phenylephrine maleate, 0.5 *M*, required to effect flocculation) are listed in Table IV. The physical properties of some of the flocculates, in particular the ease with which they could be dried and milled, are also noted in Table IV.

The two most satisfactory products, derived from a second acrylic acid copolymer emulsion[3] (hereafter referred to as acrylic copolymer emulsion 2 or acrylic copolymer 2) and a styrene–acrylic copolymer emulsion,[4] together with the previously described acrylic copolymer emulsion 1,[2] were further examined. The percentage of drug bound

Table IV—Results of Investigations of Use of a Number of Polymers as Matrices for Molecular Scale Entrapment and Controlled Release

Polymer Emulsion	Flocculation Value, ml.[a]	Physical Properties of Product
Crosslinked acrylic copolymer 2[b]	7.60	Good
Styrene–acrylic copolymer[c]	13.10	Good
Acrylic copolymer[d]	15.30	Product very soft and elastic, could not be milled
Polyvinyl acetate copolymer[e]	20	Product very soft and elastic, could not be milled
Acrylic copolymer[f]	14.50	Incomplete flocculation product not filterable
Acrylic copolymer[g]	8.70	Incomplete flocculation product not filterable

[a] Flocculation values are for a 0.5 *M* phenylephrine maleate solution. [b] Acrysol ASE 60, Rohm & Haas Co., Philadelphia, Pa. [c] Neocryl BT-4, Polyvinyl Chemicals, Inc., Peabody, Mass. [d] Rhoplex 4530, Rohm & Haas Co., Philadelphia, Pa. [e] Neo-Vac V-26-N, Polyvinyl Chemicals, Inc., Peabody, Mass. [f] Polyco 2715, Bordon Chemical Co., New York, N. Y. [g] Rhoplex B85, Rohm & Haas Co., Philadelphia, Pa.

Table V—Data on Binding and Release of Phenylephrine by Different Polymers

Polymer	Drug Bound, %	Bound Drug Released after 30 min. in Gastric Juice, %
Acrylic copolymer emulsion 1	60	54
Acrylic copolymer emulsion 2	58	39
Styrene–acrylic copolymer emulsion	60	48

[1] Delchem, Chicago, Ill.

[2] Acrysol ASE-75; a 100% linear, anionically charged acrylic acid copolymer with a molecular weight in excess of 300,000, supplied in emulsion form, containing 40% solids; Rohm & Haas Co., Philadelphia, Pa.

[3] Acrysol ASE-60; a crosslined acrylic acid copolymer containing approximately 17% carboxyl functionality (by weight), supplied in emulsion form, containing 27.7% solids; Rohm & Haas Co., Philadelphia, Pa.

[4] Neocryl BT-4; a styrene–acrylic copolymer, existing in emulsion form; Polyvinyl Chemicals, Peabody, Mass.

Table VI—*In Vitro* Release Data for Phenylephrine Maleate–Acrylic Copolymer 1 Entrapment Products

Time, hr.	pH of Media	Cumulative Drug Release, % (1)[a]	(2)[b]	(3)[c]	(4)[d]
0.5	1.3	52	54	70	34
1.5	1.3	63	65	77	54
2	1.3	64	—	—	—
2.5	2.3	—	70	—	—
4.5	7.3	—	90	95	—
6.5	7.3	—	91	—	—
8	7.3	90	94	—	—

[a] (1) 11.3% drug, 20 mesh. [b] (2) 3.3% drug, 50 mesh. [c] (3) Phenylephrine hydrogen maleate, 1.7% drug, 50 mesh. [d] (4) Product 2 pretreated for 10 min. by washing with 0.1 *N* HCl.

by the various polymers was determined, and the amount of drug released after shaking a 60-mesh powder at 37° in simulated gastric juice (without enzyme) for 30 min. was measured (Table V).

In Vitro *Release Study*—The *in vitro* release rates of phenylephrine from phenylephrine maleate–acrylic copolymer entrapment products were examined by the method described in Part I of this series (Table VI).

Phenylpropanolamine Studies—*Efficiency of Various Carboxylic Acid Anions in Drug Binding*—Experiments similar to those performed with phenylephrine were conducted. The results are shown in Table VII.

In Vitro *Release Study*—An *in vitro* study of the release of drug from a phenylpropanolamine acetate–acrylic copolymer 1 entrapment product, as a 60-mesh powder, was performed (Table VIII) in the same manner as was employed for the phenylephrine entrapment products.

In Vivo *Release Study*—Five adult healthy male subjects were used in this test. They were given a capsule dose of 50 mg. of phenylpropanolamine: (*a*) in the form of the USP product and (*b*) as a 60-mesh powder of the phenylpropanolamine acetate–acrylic copolymer 1 entrapment product. The amount of drug appearing in the urine was determined over a 24-hr. period by the method described by Heimlich *et al.* (4). From the data so obtained, the time for half the drug to be excreted, the biological half-life, was determined (Table IX).

DISCUSSION

The results in Tables I and VII show considerable variation in the efficiency with which the various facilitators assist in the binding of these weak basic amine drugs to the acrylic polymer. For both drugs (Tables I and VII) the order of facilitator entrapment efficiency is very similar. Further, replacement of all acidic hydrogen atoms on the polybasic facilitators appears to enhance entrapment efficiency. No direct relationship between facilitator efficiency and pKa is immediately discernible. It is thought that both electronic and steric factors control the activity of the acid anions in the entrapment process.

The effect of an increase in the flocculation pH value was twofold. It increased both the amount of drug bound and the rate at which drug was released. These effects are probably due to one or

Table VII—Efficiency of Various Carboxylic Acid Anions in Binding Phenylpropanolamine to Acrylic Copolymer 1 during Flocculation Entrapment

Facilitator	Drug Bound, %	Bound Drug Released after 30 min. in Gastric Juice, %
Citrate, HH	29	85
Citrate H	30	57
Tartrate	38	88
Fumarate	43	69
Citrate	49	61
Acetate	50	49
Maleate	51	55

Table VIII—*In Vitro* Release Data for a Phenylpropanolamine Acetate–Acrylic Copolymer 1 Entrapment Product[a]

Time, hr.	pH of Media	Cumulative Drug Release, %
0.5	1.3	48
1.5	1.3	67
2.5	1.3	69
4.5	2.3	74
6	7.3	94
8	7.3	97

[a] Product contained 9.76% drug.

both of two factors. First, an increase in pH will increase the amount of phenylephrine present in the unionized form and reduce the proportion present as the ionized species. The increase in the amount of drug bound may reflect the ability of the unionized species to interact with the polymer, possibly by a different mechanism to that operating in the binding of the ionized drug. Second, an increase in flocculation pH will alter the solubility of the polymer, since it will produce an increase in the proportion of ionized polymer carboxyl groups. The results shown in Table II do not suggest that an increase in flocculation pH is advantageous in the preparation of sustained-action pharmaceuticals by the carboxylic acid facilitated entrapment method.

The increase in the amount of drug bound with an increase in stirring rate (Table III) may possibly be attributed to the effect of high shear increasing the area of polymer surface available to drug in the aqueous phase by dispersing particle flocs, thus facilitating interaction.

Examination of a number of polymer emulsions for use as molecular scale entrapment matrices for potential controlled drug-release applications resulted in the discovery of two additional polymers, acrylic copolymer emulsion 2 and the styrene–acrylic copolymer emulsion, which appeared to be well suited for this purpose (Tables IV and V). These two polymer emulsions flocculated completely; the products obtained could be easily filtered, dried, and milled. Results of preliminary *in vitro* release studies compare favorably with those obtained earlier with acrylic copolymer 1.

The results in Table VI indicate that the phenylephrine maleate–acrylic copolymer 1 product possesses very satisfactory *in vitro* release characteristics, with approximately one-half of the total drug being released in the first 30 min. for two of the products and the remainder becoming available at a steady rate during the next 6.5 hr. Similar results are shown in Table VIII for the phenylpropanolamine acetate–acrylic copolymer 1 product.

Preliminary *in vivo* tests of the phenylpropanolamine entrapment product also indicate the sustained-action properties of the phenylpropanolamine product. For all subjects the rate of excretion of drug from the polymer flocculate was lower than from the untreated drug. The mean time for half the total dose to be excreted was 4.6 hr. for the USP product, as compared with 8.0 hr. for polymer–drug entrapment product (Table IX).

SUMMARY

Further studies of the facilitated molecular scale entrapment technique for the preparation of controlled-release or sustained-action pharmaceuticals are reported. The efficiency of a number of carboxylic acid anions in facilitating drug entrapment was studied, as were pH and mechanical effects on the facilitated entrapment.

Table IX—*In Vivo* Release Data for Phenylpropanolamine–Acrylic Copolymer 1 Entrapment Sustained-Release Product

Subject Weight, lb.	Biological Half-Life of Phenylpropanolamine, hr.	Biological Half-Life of Phenylpropanolamine Polymer Product, hr.
205	5.0	7.8
175	6.0	9.8
139	5.3	7.0
139	3.0	8.5
130	3.8	6.8
Average	4.6	8.0

In vitro and *in vivo* tests of phenylephrine and phenylpropanolamine products, obtained by the facilitated molecular entrapment method, confirmed the pharmaceutical utility of this technique.

These results indicate that the drug–polymer entrapment products prepared by the facilitation method possess considerable potential for exploitation as sustained-action or controlled-release pharmaceuticals.

REFERENCES

(1) H. Goodman and G. S. Banker, *J. Pharm. Sci.*, **59,** 1131 (1970).

(2) C. T. Rhodes, K. Wai, and G. S. Banker, *ibid.*, **59,** 1578(1970).

(3) H. Goodman, C. T. Rhodes, A. M. Knevel, and G. S. Banker, *Can. J. Pharm. Sci.*, **3,** 69(1968).

(4) K. R. Heimlich and D. R. MacDonnell, *J. Pharm. Sci.*, **50,** 232(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication May 26, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported in part by a grant from Smith Kline & French Laboratories, Philadelphia, Pa.

* Present address: Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada.

† Present address: Biorex Laboratories, London, England.

Selected Pharmacological Studies of a Series of Substituted Imidazo(4,5-*d*)pyridazines

G. G. FERGUSON, H. C. HEIM, and G. D. APPELT

Abstract □ A general pharmacological screening program was applied to a series of 10 substituted imidazo(4,5-*d*)pyridazine compounds. All of the compounds produced depression of spontaneous activity in rats, and six of the compounds produced significant lengthening of hexobarbital "sleeping times." In addition, two of the compounds produced a partial reversal of reserpine hypothermia, measured by a rectal temperature monitoring apparatus, and seven of the compounds produced significant inhibition of monoamine oxidase using a Warburg apparatus. One compound produced monoamine oxidase inhibition when a spectrophotometric assay was employed.

Keyphrases □ Imidazo(4,5-*d*)pyridazines—pharmacological screening □ CNS activity—imidazo(4,5-*d*)pyridazines □ Monoamine oxidase inhibition—imidazo(4,5-*d*)pyridazines □ Reserpine-induced hypothermia—imidazo(4,5-*d*)pyridazine blocking effect

In recent years, considerable interest has arisen in the development of compounds having potential antineoplastic effects. Robins (1), for example, reported the synthesis of a series of over 1300 purine derivatives and included antineoplastic screening data on active compounds. The compounds studied in this report, the imidazo(4,5-*d*)pyridazines, were synthesized by Gerhardt *et al.* (2) as possible purine antimetabolites and are representative of 75 compounds in that series (Table I).

Little has been reported concerning the pharmacology of imidazopyridazines or similar compounds, other than antineoplastic screening data. Dimmling and Hein (3) found that certain types of imidazole derivatives decreased contractility of the frog heart and produced damage to leukocytes and macrophages. Certain pyrimidazole derivatives have shown local anesthetic effects (4), and other pyridine and pyridine–pyrrolidine imidazole compounds have been shown to have CNS depressant effects (5). Rinaldi *et al.* (6) showed some protection of mice to the effects of X-ray exposure with imidazole and benzimidazole derivatives. Certain derivatives of phenylimidazopyridine carboxylic acid have been shown to have diuretic effects (7). It was felt that a survey of the general pharmacological properties of this series of compounds might be useful to further consideration of them as possible antineoplastic agents. In this paper, studies of certain effects of these compounds on the CNS are reported.

Table I—Chemical Names of the Compounds

Compound	Chemical Name
1	1*H*-Imidazo[4,5-*d*]pyridazine-2,4,7-triol
2	1-Benzyl-2-mercapto-1*H*-imidazo[4,5-*d*]pyridazine-4,7-diol
3	1-Benzyl-2-(isopentylthio)-1*H*-imidazo[4,5-*d*]pyridazine-4,7-diol
4	1-Benzyl-2-(cyclopentylthio)-1*H*-imidazo[4,5-*d*]pyridazine-4,7-diol
5	1-Benzyl-2-[(*p*-fluorobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol
6	1-Benzyl-2-[(*m*-fluorobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol
7	1-Benzyl-2-[(2,4-dichlorobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol
8	1-Benzyl-2-[(2,6-dichlorobenzyl)thio]-1*H*-imidazo-[4,5-*d*]pyridazine-4,7-diol
9	1-Benzyl-2-[(*p*-bromobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol
10	1-Benzyl-2-[(*o*-iodobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol

EXPERIMENTAL

Evaluation of Gross CNS Effects—Adult Houston-Cheek albino rats, weighing between 200 and 350 g., were used for measurements of spontaneous activity and for determinations of hexobarbital "sleeping time." Spontaneous activity measurements were done using a "light box" similar to that proposed by Dews (8). Each animal was injected with a dose intraperitoneally of drug suspended in alkalinized distilled water (pH 9), placed in a stimulus-free chamber for 15 min. to allow for absorption of the drug, then placed in the activity box, and monitored for spontaneous activity for 1 hr.

In vitro and *in vivo* tests of phenylephrine and phenylpropanolamine products, obtained by the facilitated molecular entrapment method, confirmed the pharmaceutical utility of this technique.

These results indicate that the drug–polymer entrapment products prepared by the facilitation method possess considerable potential for exploitation as sustained-action or controlled-release pharmaceuticals.

REFERENCES

(1) H. Goodman and G. S. Banker, *J. Pharm. Sci.*, **59,** 1131 (1970).

(2) C. T. Rhodes, K. Wai, and G. S. Banker, *ibid.*, **59,** 1578(1970).

(3) H. Goodman, C. T. Rhodes, A. M. Knevel, and G. S. Banker, *Can. J. Pharm. Sci.*, **3,** 69(1968).

(4) K. R. Heimlich and D. R. MacDonnell, *J. Pharm. Sci.*, **50,** 232(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969, from the *Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication May 26, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported in part by a grant from Smith Kline & French Laboratories, Philadelphia, Pa.

* Present address: Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada.

† Present address: Biorex Laboratories, London, England.

Selected Pharmacological Studies of a Series of Substituted Imidazo(4,5-*d*)pyridazines

G. G. FERGUSON, H. C. HEIM, and G. D. APPELT

Abstract □ A general pharmacological screening program was applied to a series of 10 substituted imidazo(4,5-*d*)pyridazine compounds. All of the compounds produced depression of spontaneous activity in rats, and six of the compounds produced significant lengthening of hexobarbital "sleeping times." In addition, two of the compounds produced a partial reversal of reserpine hypothermia, measured by a rectal temperature monitoring apparatus, and seven of the compounds produced significant inhibition of monoamine oxidase using a Warburg apparatus. One compound produced monoamine oxidase inhibition when a spectrophotometric assay was employed.

Keyphrases □ Imidazo(4,5-*d*)pyridazines—pharmacological screening □ CNS activity—imidazo(4,5-*d*)pyridazines □ Monoamine oxidase inhibition—imidazo(4,5-*d*)pyridazines □ Reserpine-induced hypothermia—imidazo(4,5-*d*)pyridazine blocking effect

In recent years, considerable interest has arisen in the development of compounds having potential antineoplastic effects. Robins (1), for example, reported the synthesis of a series of over 1300 purine derivatives and included antineoplastic screening data on active compounds. The compounds studied in this report, the imidazo(4,5-*d*)pyridazines, were synthesized by Gerhardt *et al.* (2) as possible purine antimetabolites and are representative of 75 compounds in that series (Table I).

Little has been reported concerning the pharmacology of imidazopyridazines or similar compounds, other than antineoplastic screening data. Dimmling and Hein (3) found that certain types of imidazole derivatives decreased contractility of the frog heart and produced damage to leukocytes and macrophages. Certain pyrimidazole derivatives have shown local anesthetic effects (4), and other pyridine and pyridine–pyrrolidine imidazole compounds have been shown to have CNS depressant effects (5). Rinaldi *et al.* (6) showed some protection of mice to the effects of X-ray exposure with imidazole and benzimidazole derivatives. Certain derivatives of phenylimidazopyridine carboxylic acid have been shown to have diuretic effects (7). It was felt that a survey of the general pharmacological properties of this series of compounds might be useful to further consideration of them as possible antineoplastic agents. In this paper, studies of certain effects of these compounds on the CNS are reported.

Table I—Chemical Names of the Compounds

Compound	Chemical Name
1	1*H*-Imidazo[4,5-*d*]pyridazine-2,4,7-triol
2	1-Benzyl-2-mercapto-1*H*-imidazo[4,5-*d*]pyridazine-4,7-diol
3	1-Benzyl-2-(isopentylthio)-1*H*-imidazo[4,5-*d*]pyridazine-4,7-diol
4	1-Benzyl-2-(cyclopentylthio)-1*H*-imidazo[4,5-*d*]pyridazine-4,7-diol
5	1-Benzyl-2-[(*p*-fluorobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol
6	1-Benzyl-2-[(*m*-fluorobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol
7	1-Benzyl-2-[(2,4-dichlorobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol
8	1-Benzyl-2-[(2,6-dichlorobenzyl)thio]-1*H*-imidazo-[4,5-*d*]pyridazine-4,7-diol
9	1-Benzyl-2-[(*p*-bromobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol
10	1-Benzyl-2-[(*o*-iodobenzyl)thio]-1*H*-imidazo[4,5-*d*]-pyridazine-4,7-diol

EXPERIMENTAL

Evaluation of Gross CNS Effects—Adult Houston-Cheek albino rats, weighing between 200 and 350 g., were used for measurements of spontaneous activity and for determinations of hexobarbital "sleeping time." Spontaneous activity measurements were done using a "light box" similar to that proposed by Dews (8). Each animal was injected with a dose intraperitoneally of drug suspended in alkalinized distilled water (pH 9), placed in a stimulus-free chamber for 15 min. to allow for absorption of the drug, then placed in the activity box, and monitored for spontaneous activity for 1 hr.

Table II—Effect of the Compounds on Spontaneous Activity in Rats (counts/hr.)[a]

Compound	Control	5 mg./kg.	10 mg./kg.	20 mg./kg.	40 mg./kg.	80 mg./kg.
1	186 ± 14[b]	133 ± 12	261 ± 33	133 ± 33	162 ± 38	131 ± 38
2	186 ± 14	123 ± 22	125 ± 24	135 ± 16	95 ± 15	117 ± 11
3	186 ± 14	118 ± 13	94 ± 12	100 ± 15	72 ± 13	57 ± 11
4	186 ± 14	130 ± 23	123 ± 19	87 ± 18	80 ± 17	83 ± 10
5	186 ± 14	127 ± 24	73 ± 18	69 ± 19	76 ± 17	96 ± 25
6	186 ± 14	95 ± 16	134 ± 23	116 ± 13	64 ± 11	38 ± 9
7	186 ± 14	69 ± 5	85 ± 15	56 ± 8	78 ± 11	99 ± 23
8	186 ± 14	92 ± 14	84 ± 22	62 ± 8	57 ± 18	57 ± 16
9	186 ± 14	71 ± 16	95 ± 11	55 ± 7	50 ± 18	22 ± 9
10	186 ± 14	76 ± 16	64 ± 10	34 ± 6	40 ± 14	23 ± 4

[a] Each value represents the mean of five trials. [b] Standard error of the mean.

Results were compared to controls having identical treatment except for the use of the test drug.

As a corollary study, rats were tested for the effect of the compounds on the hexobarbital sleeping time. The animals were injected with 20 mg./kg., i.p., of the compound to be tested 2 hr. prior to the administration of 50 mg./kg., i.p., hexobarbital. The length of sleep between the loss and regaining of the righting reflex (sleeping time) was then determined for each animal, and the results were compared to controls (9, 10).

Evaluation of Monoamine Oxidase Inhibition—Initial screening for possible monoamine oxidase (MAO) inhibition was done using the "reserpine reversal" technique of Pletscher (11). Adult Lemberger albino mice, weighing between 25 and 40 g., were restrained in plastic holders; their rectal temperatures were monitored using an electronic thermometer equipped with temperature-sensing probes.[1]

The ability of the compounds to reverse the hypothermic effect of reserpine was measured, using four mice for each trial: two control animals received 2.5 mg./kg., i.p., reserpine and two experimental animals were pretreated with 100 mg./kg., i.p., of the compound to be tested 1 hr. prior to treatment with 2.5 mg./kg. reserpine. A small number of animals received 100 mg./kg., i.p., iproniazid to serve as a standard of MAO inhibition. The rectal temperatures were monitored for 4 hr., and the effects of the drugs on the reserpine-induced hypothermia were noted.

As a further evaluation of possible MAO inhibition, the effects of the compounds on rat brain mitochondria were investigated. Brain mitochondria were prepared, according to the method of Brody and Bain (12), and MAO activity was determined in the presence or absence of inhibitor by conventional manometric techniques (13) or spectrophotometrically (14). In the manometric determinations, Warburg flasks contained in the main compartment a final concentration of 0.01 *M* phosphate buffer (pH 7.8), 0.017 *M* compound, and the mitochondrial suspension representing 50 mg. wet weight of tissue in a total volume of 1.8 ml. The side arm of the flask contained 0.3 ml. of 0.1 *M* tyramine. After the flasks were allowed to equilibrate at 37° for 15 min., the manometer valves were closed and the side arm contents of each flask were tipped in. Oxygen uptake was recorded at 10-min. intervals for a total period of 90 min.

Those compounds which showed significant MAO inhibition using the manometric technique were then tested using a direct spectrophotometric assay for MAO activity. The 3 ml. reaction mixture contained in a final concentration 0.03% commercial emulsifier,[2] 0.09 *M* phosphate buffer (pH 7.4), 0.00016 *M* compound, and 0.017 *M* *p*-dimethylaminobenzylamine hydrochloride, with mitochondrial suspension representing 50 mg. wet weight of tissue. The conversion of *p*-dimethylaminobenzylamine hydrochloride to its aldehyde derivative by MAO was observed by determining the increase in density of the solution at 355 mμ, according to the method of Deitrich and Erwin (14). Readings were taken at 1-min. intervals for 5 min. after the reaction began.

RESULTS AND DISCUSSION

All imidazopyridazine derivatives, except Compound 1, produced significant depression of spontaneous activity at all dosage levels employed (5–80 mg./kg.). Compound 1 produced significant depression of spontaneous activity at all dosages except 10 mg./kg. (Table II). Compounds 1, 2, 4, 6, 8, and 10 produced significant prolongation of hexobarbital sleeping time (Table III).

Table III—Effects of the Compounds on the Hexobarbital "Sleeping Times" of Rats (Hexobarbital 50 mg./kg.)

Compound	Number of Animals	Dose, mg./kg.	Sleeping Time, min.	*p*
Control	28	—	44 ± 3	—
1	12	20	68 ± 6[a]	0.001
2	12	20	64 ± 4[a]	0.001
3	9	20	55 ± 5	0.10
4	10	20	65 ± 4[a]	0.001
5	12	20	34 ± 4	0.10
6	12	20	64 ± 5[a]	0.01
7	12	20	43 ± 4	0.30
8	11	20	73 ± 5[a]	0.001
9	12	20	46 ± 6	0.30
10	12	20	57 ± 4[a]	0.05

[a] Significant lengthening of sleeping time.

None of the 10 compounds produced complete reversal of reserpine-induced hypothermia in mice. Compounds 5 and 8 produced a partial reversal of the induced hypothermia, although the results were not significant at the $p = 0.05$ level (Table IV).

Table IV—Effects of the Compounds in Blocking Reserpine-Induced Hypothermia in Mice

Compound	Control[a]	Experimental
1	−9.0°[b]	−7.8°
	−8.4°	−10.0°
2	−7.6°	−7.6°
	−9.4°	−6.1°
3	−8.6°	−8.4°
	−8.7°	−12.4°
4	−10.9°	−9.4°
	−8.5°	−6.5°
5	−9.6°	−6.8°
	−10.0°	−4.5°
6	−8.1°	−11.4°
	−8.7°	−5.6°
7	−10.1°	−7.8°
	−8.9°	−10.8°
8	−6.4°	−3.4°
	−9.5°	−6.5°
9	−5.6°	−7.8°
	−6.5°	−6.2°
10	−9.4°	−7.6°
	−7.8°	−8.8°
Iproniazid	−7.2°	+1.4°
	−7.9°	−2.2°

[a] "Controls" were treated with 2.5 mg./kg., i.p., reserpine and received 0.3 ml. normal saline solution to replace the compound. [b] Values represent change in rectal temperature for 4-hr. period.

[1] TRI-R model TML, TRI-R Instruments.
[2] Lubrol 90, I.C.I./Organics/Inc., Providence, RI 02901

Table V—Effect of the Compounds on the Oxidation of Tyramine by Rat Brain Mitochondria[a]

Compound[b]	Time, min. 10 *p*	30 *p*	50 *p*	70 *p*	90 *p*
1	5[a] ± 1[b] 0.01	15[c] ± 1 0.10	25[c] ± 1 0.30	31[c] ± 1 0.20	41[c] ± 2 0.30
2	4 ± 1 0.01	13 ± 1 0.01	20 ± 1 0.01	27 ± 2 0.01	39[c] ± 2 0.20
3	2 ± 1 0.001	10 ± 1 0.01	19 ± 1 0.01	25 ± 3 0.01	37[c] ± 2 0.10
4	0 ± 1 0.001	2 ± 1 0.001	3 ± 1 0.001	7 ± 1 0.001	9 ± 2 0.001
5	2 ± 1 0.001	6 ± 1 0.001	10 ± 1 0.001	14 ± 2 0.001	19 ± 2 0.001
6	1 ± 1 0.001	5 ± 1 0.001	10 ± 1 0.001	13 ± 2 0.001	19 ± 2 0.001
7	3 ± 1 0.01	11 ± 1 0.01	18 ± 1 0.01	24 ± 2 0.01	34 ± 2 0.01
8	4 ± 1 0.01	7 ± 1 0.001	13 ± 2 0.01	19 ± 2 0.01	27 ± 2 0.01
9	5 ± 1 0.01	14 ± 1 0.01	22 ± 1 0.01	29 ± 1 0.01	36 ± 1 0.01
10	3 ± 1 0.01	9 ± 1 0.01	17 ± 1 0.01	22 ± 1 0.01	28 ± 1 0.01
Control	8 ± 1 —	18 ± 1 —	26 ± 1 —	34 ± 1 —	42 ± 1 —

[a] Values represent mean of the microliters of oxygen uptake in 12 individual flasks. [b] Standard error of the mean. [c] Nonsignificant.

In studies using the Warburg respirometer, Compounds 4–10 produced significant inhibition of monoamine oxidase at the end of 90 min. (Table V). Upon further testing of these compounds with the spectrophotometric technique, it was found that Compound 9 produced noncompetitive MAO inhibition (Fig. 1).

While Compounds 4–10 produced significant MAO inhibition in the Warburg studies, only Compound 9 produced significant inhibition of the enzyme in the spectrophotometric assays. Several factors may be involved in the explanation of these findings. The concentrations of the drugs used were quite different in the two studies. The concentration of the compounds used in the Warburg studies was approximately 100 times that of the compounds used in the spectrophotometric assays. The substrates used were different in the two studies, and thus the influence of the inhibiting drug on the reaction system might have been different in each case. Finally, if the inhibition seen was irreversible, the 15-min. preequilibration in the Warburg flasks in the absence of substrate would be quite different from the conditions in the spectrophotometric studies, in which the inhibitor and substrate were added essentially together with no preequilibration time allowed.

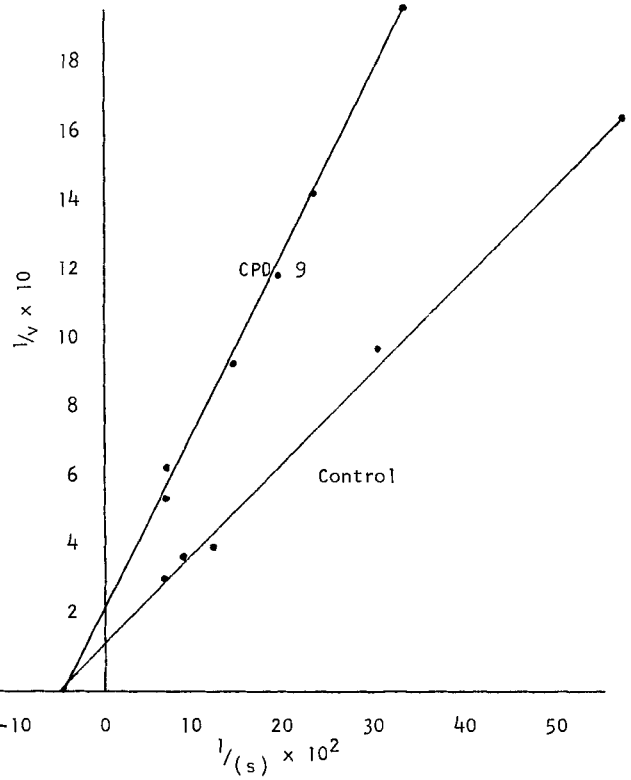


Figure 1—*MAO inhibition of Compound 9.*

SUMMARY

This study involved the evaluation of pharmacological activity of a group of 10 imidazo(4,5-*d*)pyridazine compounds which were originally synthesized as antineoplastic agents.

All of the compounds were found to exert CNS depressant effects. Compounds 1, 2, 4, 6, 8, and 10 prolonged the hexobarbital sleeping time in rats. Compounds 5 and 8 produced a partial reversal of reserpine-induced hypothermia in mice.

Compounds 4–10 inhibited MAO in Warburg studies, and Compound 9 inhibited MAO in a spectrophotometric assay technique.

REFERENCES

(1) R. K. Robins, *J. Med. Chem.*, **7**, 189(1964).

(2) G. A. Gerhardt, D. L. Aldous, and R. N. Castle, *J. Heterocycl. Chem.*, **2**, 247(1965).

(3) T. Dimmling and H. Hein, *Arzneim.-Forsch.*, **2**, 515(1952).

(4) G. A. Mednikyan, *Farmakol. Toksikol.*, **9**, 26(1946).

(5) B. L. Konson, *ibid.*, **9**, 3(1946).

(6) R. Rinaldi, Y. Bernard, and M. Guilhermet, *Compt. Rend.*, **261**, 570(1965).

(7) G. Marchetti and L. Merlo, *Farmaco (Pavia), Ed. Sci.*, **18**, 275(1963).

(8) P. B. Dews, *Brit. J. Pharmacol.*, **8**, 46(1956).

(9) B. B. Brodie, *J. Pharm. Pharmacol.*, **8**, 1(1956).

(10) J. C. Cooper and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, **114**, 409(1955).

(11) A. Pletscher, *Ann. N. Y. Acad. Sci.*, **80**, 1039(1959).

(12) T. M. Brody and J. A. Bain, *J. Biol. Chem.*, **195**, 685(1952).

(13) W. W. Umbreit, R. H. Burris, and J. F. Stauffer, "Manometric Techniques," 4th ed., Burgess, Minneapolis, Minn., **1964**, pp. 61, 76.

(14) R. A. Deitrich and V. G. Erwin, *Anal. Biochem.*, **30**, 395 (1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 21, 1970, from the *School of Pharmacy, University of Colorado, Boulder, CO 80302*

Accepted for publication June 3, 1970.

The authors thank R. N. Castle, Chairman, Department of Chemistry, University of New Mexico, who was responsible for the synthesis of the compounds studied and who supplied them to the authors.

Mathematical Optimization Techniques in Drug Product Design and Process Analysis

DALE E. FONNER, Jr.*, JAMES R. BUCK, and GILBERT S. BANKER

Abstract □ Optimization techniques represent analytical tools available to the researcher in his search for the best possible solution to a particular problem. Methodologies have been developed for structuring typical pharmaceutical development problems into a framework whereby sophisticated mathematical techniques can be employed to arrive at an optimal solution. Pharmaceutical product and process design problems were structured as constrained optimization problems and subsequently solved by the Lagrangian method of optimization. This optimization method was employed to generate optimal formulations in typical tablet design problems and to locate optimizing levels of processing variables in a typical encapsulation design problem.

Keyphrases □ Drug product design—mathematical optimization techniques □ Process analysis—mathematical optimization techniques □ Lagrangian method—optimization, drug design □ Optimum tablet design—techniques □ Tablets, optimization design—phenylpropanolamine HCl

Pharmaceutical product and process design problems are normally characterized by multiple objectives. In designing a product, for example, the pharmaceutical scientist must often meet prespecified control limits which define or influence such dosage form characteristics as the unit cost, physical stability, chemical stability, or physiological availability of the active ingredient. The parameters describing these dosage form characteristics represent response or dependent variables, and any limits or conditions placed on these response variables represent objectives. The magnitude of the observed value for each response variable generally depends upon levels of one or more of the controllable (independent) variables. Mathematically speaking, the jth response variable is measured as y_j, and the responses obtained are a function of levels of one or more of the controllable variables, X_1, X_2, ..., X_n. Thus,

$$y_j = f_j(X_i), \quad j = 1,2,\ldots,r; \quad i = 1,2,\ldots,n \quad \text{(Eq. 1)}$$

where f_j is the relationship between the one or more X_i controllable variables and each response variable y_j. The geometrical representation of y_j as a function of possible combinations of levels of the controllable variables defines a response surface (Fig. 1). The task of the pharmaceutical scientist frequently involves locating levels of the one or more X_i controllable variables that meet limits or conditions placed on each y_j response variable.

The response variables of product and process design problems are often affected differently by particular combinations of the controllable variables due to interaction effects (1–5). If the response variable effects are nonadditive, the response surface is highly complicated. Unless the program of experimentation is statistically well designed and an extensive series of experiments is conducted, accurate estimates of the response variable from a fixed set of controllable variables are virtually impossible (6).

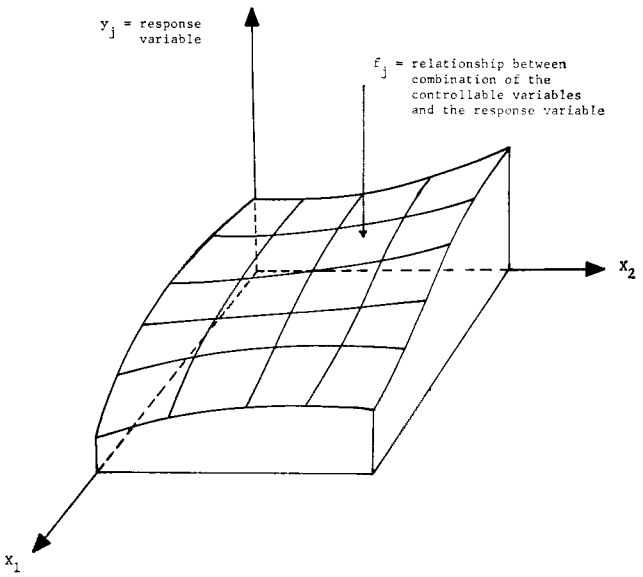


Figure 1—*Representative graph of Eq. 1 for two controllable variables,* X_1 *and* X_2.

Design problems are further complicated when objectives are competing. As a particular controllable variable is increased in most pharmaceutical design problems, one response variable tends to improve while another response variable is degraded. For example, compressional force or the concentration of granulating agent employed may produce competing effects on tablet friability and disintegration time or on tablet hardness and drug-release rate. Achievement of the best product or process design under conditions of competing objectives and interactive effects by guesswork or trial and error is time consuming, unreliable, costly, and often unsuccessful. Furthermore, the pharmaceutical researcher who employs such techniques may not recognize how close a particular solution lies to the optimal solution.

One way of dealing with complex pharmaceutical design problems is to structure them as constrained optimization problems. Problems structured in this way can be solved by any one of a number of existing techniques; one of the more versatile techniques is the Lagrangian method. This study investigated the usefulness of a systematic optimization approach in solving pharmaceutical product and process design problems.

Constrained mathematical optimization methods would appear to be broadly applicable to many pharmaceutical product design and process analysis problems. Data which are currently being generated by preformulation research programs during drug product design

should permit the use of optimization techniques in an increasing number of product design or process analysis programs.

Optimization techniques have the capability in pharmaceutical research of: (*a*) saving time and minimizing costs in achieving the desired product design; (*b*) improving the reliability of the research effort to achieve the optimal or near optimal product or process design solution; and (*c*) improving quality and assuring quality of the final drug product as affected by product and/or process design.

THEORY

Optimization problems may be broadly classified as either unconstrained or constrained. Unconstrained optimization involves the maximization or minimization of a function in which no restrictions or limits have been placed on the controllable variables or functions of the controllable variables. For example, optimization of

$$y = f(X_i), \quad i = 1,2,\ldots,n \qquad \text{(Eq. 2)}$$

represents an unconstrained optimization problem and can be solved by classical calculus techniques (7). For a function of n controllable variables, the location of relative optima requires solving a set of n simultaneous equations. The n simultaneous equations result from partially differentiating Eq. 2 with respect to the n controllable variables. The global optimum is then established by evaluating $f(X_i)$ at the relative optimum points and selecting the most extreme of all the solutions obtained.

A constrained optimization problem (7) is one in which a function is optimized subject to restrictions or limits placed on the controllable variables. Mathematically, the problem is to optimize

$$y = f(X_i), \quad i = 1,2,\ldots,n \qquad \text{(Eq. 3)}$$

such that:

$$g_j(X_i) = \alpha_j, \quad j = 1,2,\ldots,p \leq n \qquad \text{(Eq. 4)}$$

$$g_j(X_i) \geq \alpha_j, \quad j = p+1,\ldots,m \qquad \text{(Eq. 5)}$$

Equation 3 represents the function to be optimized and is generally referred to as the objective function. Equations 4 and 5 are referred to as equality and inequality constraints for the specified constants α_j. Only the greater than or equal to relationship is represented, since $h(X_i) \leq 0$ may be written as $g(X_i) = -h(X_i) \geq 0$. Thus, the constrained optimization problem involves locating levels of X_i that produce an optimal response in $f(X_i)$ such that the constraints of the problem are not violated. If the objective function and constraints are linear, the problem can be solved by linear programming techniques (8–10). If nonlinearities exist in the objective function and/or one or more of the constraints, the constrained optimization problem of Eqs. 3–5 becomes a nonlinear programming problem. Several techniques capable of dealing with nonlinear programming problems have been developed (11–13). Since systems of pharmaceutical interest are often characterized by the presence of many interactions, nonlinear models frequently result.

The Lagrangian Method—Of the techniques available for solving the constrained optimization problem in its general form (Eqs. 3–5), the most versatile method appears to be the Lagrangian method (7, 14, 15). *Appendix A* contains two numerical examples illustrating this method of optimization. The Lagrangian method has many desirable properties because this method: (*a*) locates the optimum directly and does not search infeasible points; (*b*) generates only feasible solutions; (*c*) efficiently handles inequality as well as equality constraints; and (*d*) deals with nonlinearities in the objective function and/or constraints.

Inequality constraints are converted to equality constraints by incorporating a slack variable, q_j, which must be nonnegative to assure that its value is positive in the Lagrange function (16). Thus, Eq. 5 may be written as follows:

$$g_j(X_i) - q_j^2 = \alpha_j, \quad j = p+1,\ldots,m \qquad \text{(Eq. 6)}$$

The slack variable, q_j, in effect absorbs the slack created by the original inequality relationship. For a less than or equal to constraint, the minus sign in Eq. 6 is replaced with a plus sign. Once the inequality constraints have been converted to equality constraints, the next step is to form the Lagrange function, F, which is equal to the objective function plus the products of the Lagrange multiplier, λ_j, and the constraint (7). For the general constrained optimization problem (Eqs. 3–5), the Lagrange function becomes

$$F = f(X_i) + \sum_{j=1}^{p} \lambda_j[g_j(X_i) - \alpha_j] + \sum_{j=p+1}^{m} \lambda_j[g_j(X_i) - q_j^2 - \alpha_j] \qquad \text{(Eq. 7)}$$

As shown in Eq. 7, one Lagrange multiplier is introduced for each constraint, and one slack variable is introduced for each inequality constraint.

If the task is to minimize the objective function, then the Lagrange function is minimized with respect to the controllable variables X_i and maximized with respect to the Lagrange multipliers λ_j, giving a so-called minimax solution (17, 18). In maximizing the objective function, the resulting solution is a maximin solution relative to X_i and λ_j, respectively. Either form of the resulting solution is a stationary point in which the tangent of the Lagrange function is zero with respect to each X_i and λ_j. Therefore, the values of the controllable variables which jointly satisfy each partial derivative of the Lagrange function with respect to X_i ($i = 1, 2, \ldots, n$), λ_j ($j = 1, 2, \ldots, p, \ldots, m$), and q_j ($j = p+1, \ldots, m$), each set equal to zero, provides stationary points including a constrained extreme of the objective function. These satisfying values, X_i^*, can be obtained by the simultaneous solution to the $n + m$ equations. Each set of X_i^* values obtained is a root to this set of equations and denotes a stationary point. If a set of equations gives multiple roots, then each root may be substituted into the objective function to find the one that is the desired extreme. If the equations obtained from partially differentiating the Lagrange function result in a set of simultaneous nonlinear equations, then numerical methods must be used to locate the simultaneous solution point (14).

The Lagrange multiplier has an interesting interpretation directly relating to optimization problems (19). The numerical value of λ_j represents a measure of the instantaneous rate of change of the objective function with a change of the constraint's limiting value. Thus, the value of λ_j is useful in projecting the expected gain or loss in the objective function accompanying a change in constraint of one unit.

The Lagrangian method of solving constrained optimization problems assumes that a mathematical relationship exists which relates the response variable to levels of the controllable variables. Theoretically, mathematical models can be derived from a knowledge of the natural laws governing the system. However, underlying mechanisms in pharmaceutical product and process design problems are often so complicated that the formulation of an analytical mathematical model is out of the question. If an analytical model is impossible to derive, then an empirical mathematical model may be developed by using multiple-regression techniques (4, 20). Thus, it is usually possible to fit a polynomial to the response surface which can be expected to give an adequate approximation of the response surface over the region of experimentation. The Lagrangian method has been applied to a constrained optimization problem involving polynomial models generated by multiple-regression techniques (21).

EXPERIMENTAL

A typical product design problem was studied in this investigation. It involved locating levels of the binder and disintegrant that optimized tablet physical properties and drug availability in a model tablet formulation. An optimization method was used to solve a constrained tablet design problem involving a restriction placed on the urinary elimination rate of the drug. A process design problem was also analyzed utilizing regression models describing powder encapsulation (4).

Model Tablet Design, Preparation, and Evaluation—A model tablet system was employed to demonstrate the advocated design procedure. Tablets containing various concentrations of a disintegrant and binder were prepared. The effect of the binder and dis-

integrant concentration on tablet hardness, friability, volume, *in vitro* release rate, and urinary excretion rate of drug in human subjects was recorded. Phenylpropanolamine hydrochloride[1] was the drug chosen based on its relatively low dose, safety, quantitative urinary excretion reflecting drug availability, and ease of assay from the urine. The model tablet system employed: (*a*) represented commonly used ingredients in tablet formulations; (*b*) produced tablets that did not split or fracture during the friability test; and (*c*) contained levels of binder and disintegrant that significantly affected tablet hardness, friability, volume, and *in vitro* release rate of the drug. The tablet formulas studied were all contained within the following limits:

Phenylpropanolamine hydrochloride	50 mg.
Dicalcium phosphate dihydrate[2]	*q.s.*
Corn starch[3]	1–41%
Stearic acid USP[4]	5–45%
Tablet weight =	400 mg.

To achieve reproducible friability and *in vitro* $t_{50\%}$ release rate data, it was necessary to control the moisture content of the tablets. The starch, therefore, was dried for 4 hr. at 120°, and all tablet ingredients were individually stored in a vacuum desiccator containing anhydrous calcium sulfate[5] for 24 hr. prior to compression. Tablets were individually compressed, utilizing 0.95 cm. (3/8 in.) standard cut tooling, to a load of 3000 lb. on a pneumatic press.[6] The load was maintained for 10 sec., and all tablets were stored in a vacuum desiccator for at least 24 hr. prior to use. Karl Fischer moisture determinations were conducted to ensure that the moisture content of the tablets remained below 1%. At least five replicate tablets, each based on a separate powder mixture of a particular formulation, were used to obtain a measure of tablet volume, hardness, friability, and *in vitro* $t_{50\%}$ release rate for all nine tablet formulations studied.

Tablet hardness in kilograms was determined using a Pfizer hardness tester.[7] Since tablet moisture content affected tablet friability, a special friabilation apparatus was fabricated in which humidity control was possible. This apparatus consisted of a 1.5-oz. amber, dry square bottle whose bottom was covered with a 0.25-in. layer of Wood's metal (soft solder). A 0.08-cm. (1/32-in.) thick piece of Teflon was dried over the Wood's metal to provide a smooth standard surface for tablet contact. A silica gel bag,[8] wrapped in muslin, was attached to the cap of the bottle. Attrition was provided to the tablet in the bottle by placing the entire unit in an Eberbach shaker unit[9] oscillating at 275 ± 3 c.p.m., with a stroke length of 3.8 cm. (1.5 in.). Friability was measured as the percent weight loss of an individual tablet after 40 min. of shaking. The conditions selected for the friabilation test were found to give reproducible data and to distinguish real friability differences existing among the nine different tablet formulations.

Tablet volume was computed from the cylindrical tablet volume and the spherical segment volume of the standard cup punches used. The volume of the spherical segment was calculated as (22)

$$V = 1/6\pi h(3r^2 + h^2) \qquad \text{(Eq. 8)}$$

where r is the radius of the segment and h is its depth. Punch diameter [0.94 cm. (0.3720 in.)] and punch depth [0.10 cm. (0.0410 in.)] were measured with calipers. The volume of each spherical segment was calculated to be $V = 0.037$ cm.^{3} (0.0023 in.^{3}). The crown thickness of the tablets was measured using an Ames micrometer.[10]

The *in vitro* release rate tests were conducted in a 250-ml., three-necked, round-bottom flask, utilizing 200 ml. of distilled water as the solvent, at 37 ± 1°. Agitation was provided by a model 12 Stedi-Speed adjustable stirrer[11] equipped with a two-bladed, 3.18-cm. (1.25-in.) diameter stainless steel impeller, which was operated at 130 ± 2 r.p.m. This agitation level provided solvent circulation but did not disturb the particles formed by the disintegrating tablet at the bottom of the flask. Five-milliliter aliquots were removed at selected time intervals for analysis, with each aliquot being replaced by 5 ml. of distilled water. The concentration of drug in the bulk solvent was corrected for drug lost from the previous samples. Each aliquot was filtered through a Millipore filter (0.65-μ pore opening), and its absorbance was determined at 256.4 mμ. Cumulative percent of drug released *versus* time plots were prepared for each of the replicate tablets of each formulation. The time for 50% of the drug to be released ($t_{50\%}$) was determined graphically from each plot.

Table I—Tablet Formulations Used in the Optimization Study

Formulation No.	Mg. of Ingredient per Tablet: Phenylpropanolamine HCl	Dicalcium Phosphate $\cdot 2H_2O$	Starch	Stearic Acid
1	50	326	4 (1%)	20 (5%)
2	50	246	84 (21%)	20
3	50	166	164 (41%)	20
4	50	246	4	100 (25%)
5	50	166	84	100
6	50	86	164	100
7	50	166	4	180 (45%)
8	50	86	84	180
9	50	6	164	180

The nine tablet formulations listed in Table I define a full 3^2 factorial design. Levels of stearic acid and starch were equally spaced to permit a trend analysis of the data in the framework given by Davies (6). Replication provided an estimate of the experimental error involved, and experiments were randomized to prevent systematic biasing of the estimates of experimental effects.

Urinary Excretion Rate—The urinary excretion rate of phenylpropanolamine hydrochloride was determined for four different tablet formulations (Formulations 1, 3, 4, and 7 of Table I) and for a control solution of the drug. The control solution contained 47.66 mg. of drug in 200 ml. of distilled water. Five healthy human male subjects were used. Each subject was separately administered all four of the dosage forms and the control solution of drug at time intervals of $7 \times t'_{50\%}$ or longer, where $t'_{50\%}$ represents a measure of the urinary elimination rate half-life in hours of the last formulation administered. The order of administration of the dosage forms and the order in which the individuals were tested were randomized. A modification of the assay of Heimlich *et al.* (23) was used for the drug urinalysis in which cyclohexane was employed as the extracting solvent. Three standards were run during each assay in which three known amounts of drug were added to a blank urine sample; the sample had been collected from each subject prior to administration of the dosage form. Semilog plots of cumulative percent drug remaining in the body *versus* time were made, the least-squares equation for each line was solved, and the $t'_{50\%}$ value was calculated.

RESULTS AND DISCUSSION

The effects of varying concentrations of stearic acid and starch, the controllable variables X_1 and X_2, on the tablet response variables of hardness (y_1), *in vitro* $t_{50\%}$ release rate (y_2), friability (y_3), and volume (y_4), are presented in Table II. A two-factor analysis of variance (24) for homogeneity of variances disclosed that the *in vitro* $t_{50\%}$ release rate (y_2) and friability (y_3) data appearing in Table II were not homoscedastic at the 99% confidence level. A transformation was found which, when applied to the $t_{50\%}$ and friability data, resulted in statistically equal variances at the nine different experimental conditions. An arithmetic plot was prepared showing the combination of standard deviation and mean value for each experimental condition of the *in vitro* release rate and of the friability. In both cases the standard deviations were found to be proportional to the mean values, as indicated in the least-squares proportions of Eqs. 9 and 10, with correlations of fit exceeding 0.99:

$$S_{y_2} = (0.7356 \times 10^{-1})\bar{y}_2 \qquad \text{(Eq. 9)}$$

$$S_{y_3} = (0.8274)\bar{y}_3 \qquad \text{(Eq. 10)}$$

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Monsanto Chemical Co., St. Louis, MO 63166
[3] A. E. Staley Mfg. Co., Decatur, IL 62525
[4] Ruger Chemical Co., Irvington-on-Hudson, N. Y.
[5] Drierite, W. A. Hammond Drierite Co., Xenia, OH 45385
[6] Fred S. Carver, Inc., Summit, N. J.
[7] Chas. Pfizer & Co., Inc., Brooklyn, NY 10017
[8] W. R. Grace Chemical & Co., Baltimore, MD 21203
[9] Eberbach Corp., Ann Arbor, Mich.
[10] B. C. Ames Co., Waltham, MA 46518
[11] Fisher Scientific Co., Chicago, Ill.

Table II—Effect of Stearic Acid and Starch Concentration on Certain Physical and Chemical Properties of the Tablets

Stearic Acid, X_1	Percentage of Starch (X_2): 1 — Response Variables				21 — Response Variables				41 — Response Variables			
	y_1[a]	y_2[b]	y_3[c]	y_4[d] × 10^2	y_1	y_2	y_3	$y_4 \times 10^2$	y_1	y_2	y_3	$y_4 \times 10^2$
5	11.7	52.5	4.49	2.36	5.4	15.0	13.12	2.53	1.5	5.1	40.03	2.72
	12.0	44.3	4.68	2.37	4.0	14.2	13.71	2.56	1.3	5.4	47.20	2.72
	10.8	47.9	4.43	2.37	4.4	15.7	15.07	2.56	1.3	5.9	41.21	2.69
	10.2	56.3	5.28	2.38	4.2	17.3	15.88	2.54	1.0	6.0	47.92	2.69
	9.5	51.0	4.33	2.37	5.8	15.8	14.80	2.55	2.5	5.4	42.74	2.70
Mean	10.8	50.4	4.64	2.37	4.8	15.6	14.52	2.55	1.5	5.6	41.82	2.70
SD	1.04	4.56	0.378	0.005	0.79	1.15	1.102	0.012	0.71	0.38	3.498	0.010
25	15.0	118.0	1.13	2.52	11.0	27.2	1.55	2.67	6.5	12.5	4.74	2.82
	16.2	141.9	0.99	2.53	9.5	25.2	1.69	2.66	5.4	11.8	5.03	2.85
	16.4	123.5	1.10	2.53	9.8	23.2	1.48	2.66	6.7	13.5	5.17	2.84
	14.6	116.0	1.21	2.53	10.3	28.3	1.78	2.66	6.4	12.0	4.79	2.82
	15.5	123.0	1.09	2.53	11.2	24.0	1.64	2.64	7.2	11.1	5.75	2.83
Mean	15.5	124.5	1.10	2.53	10.4	25.6	1.63	2.66	6.4	12.2	5.10	2.83
SD	0.77	10.25	0.080	0.001	0.74	2.14	0.115	0.010	0.61	0.89	0.406	0.013
45	17.1	300.0	0.08	2.73	13.5	36.2	0.49	2.86	9.8	19.6	0.88	3.00
	17.7	270.0	0.08	2.74	13.0	39.2	0.45	2.87	9.2	18.2	0.95	3.01
	17.8	324.0	0.10	2.73	13.1	43.1	0.40	2.85	9.8	20.7	0.79	2.99
	17.8	285.0	0.10	2.71	13.0	38.4	0.38	2.86	10.2	19.8	0.84	2.97
	16.1	281.0	0.08	2.73	12.8	41.7	0.38	2.86	11.0	21.7	0.74	2.97
Mean	17.3	292.0	0.09	2.73	13.1	39.7	0.42	2.86	10.0	20.0	0.84	2.99
SD	0.73	20.88	0.011	0.008	0.46	2.73	0.049	0.005	0.66	1.32	0.081	0.016

[a] The y_1 is tablet hardness in Pfizer kilogram units. [b] The y_2 is *in vitro* release rate as measured by time in minutes for 50% of the drug to be in solution ($t_{50\%}$). [c] The y_3 is tablet friability as measured by percentage weight loss. [d] The y_4 is tablet volume in cubic inches.

Brownlee (25) has shown that when $S_y = k\bar{y}$, the natural logarithmic transformation stabilizes the variances. This transformation was applied to the release rate and friability data; the results appear in Table III. Three units were added to each transformation of friability datum to eliminate negative logarithmic values. The test for homogeneity of variances was then applied to the transformed data, and the existence of homoscedasticity could not be rejected at the 99% confidence level.

Regression Models—In stepwise regression, there are two basic versions: forward and backward (26). In this study, a backward stepwise regression analysis program[12] was utilized to generate polynomial models relating the response variables to the controllable variables. The results of the regression analysis appear in Table IV. Although the multiple-correlation coefficients for the regression models were high (Table IV), the predictive power of each model required further evaluation. A valid means of extending the polynomial evaluation is to select experimental conditions or treatments not included in the original set of experimental conditions and to compute both the predicted response and its associated confidence interval. The result of a new experiment can then be compared with the prediction to determine if the new experimental result is contained within the confidence interval. Four new experimental conditions were tested, and these formulations corresponded to the possible combinations of $X_1 = 15$ and 35% and $X_2 = 11$ and 31%. The predictions from the polynomial models were determined, and the 95% confidence intervals about a single prediction were computed. Construction of confidence intervals associated with multiple regression can be found in Chew (20). Next these formulations were prepared and the responses in question experimentally determined. Results of these analyses are given in Table V. The experimental response values all fell within the 95% confidence interval about a single prediction (Table V), with one exception. Thus, acceptance of the regression models in Table IV for predictive purposes in this study appears to be reasonable.

Contour Graphs—Contour graphs illustrate combinations of the controllable variables producing the same response. For each predesignated response of y_j, X_2 was solved at values of $X_1 = 2, 4, 6, \ldots, 40$. A computer program for this purpose for use on the IBM 7090 was written, and contours were set by interpolation from the X_1 and X_2 values obtained in the program output. The generated contour graphs are presented in Figs. 2–5. Contour graphs not only give various combinations of the controllable variables which produce the same response, but they also provide other interesting and useful information. For example, from the contour graph for tablet hardness (Fig. 2), it can be seen that to maintain a tablet hardness of 6.0 kg., the ratio of starch to stearic acid must be about 2:1; for a tablet hardness of 12.0 kg., the ratio needs to be about 1:2. For the sake of illustration, assume that the requirements on the final tablet are that hardness be 8–10 kg. and *in vitro* $t_{50\%}$ be 20–33 min. The solution to this problem is readily

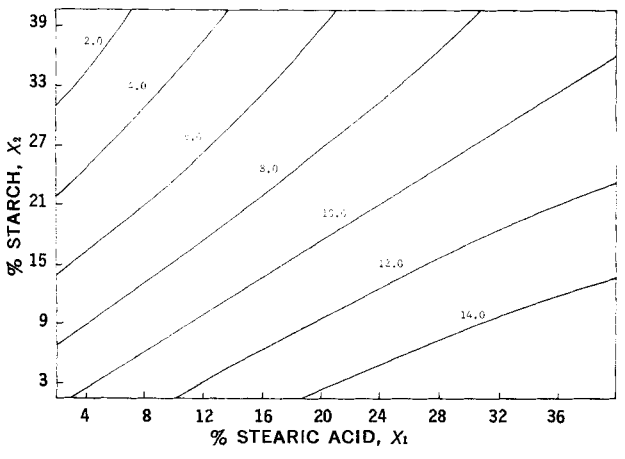


Figure 2—*Contour curves for tablet hardness (kilogram), illustrating levels of starch and stearic acid producing similar responses.*

[12] This regression analysis program was developed for use on the IBM 7090 by M. Dale Fimple of the Sandia Corp.

Table III—Stabilizing Effect of the Logarithmic Transformation on *In Vitro* Release Rate (y_2) and Friability (y_3)

% Stearic Acid (X_1)	% Starch (X_2)	—ln y_2[a]— Mean	*SD*	—ln y_3[b] + 3.0— Mean	*SD*
5	1	3.901	0.1212	4.533	0.0789
5	21	2.745	0.0729	6.674	0.0770
5	41	1.714	0.0686	6.731	0.0821
25	1	4.822	0.0796	3.100	0.0729
25	21	3.239	0.0835	3.485	0.0729
25	41	2.498	0.0776	4.626	0.0776
45	1	5.675	0.0843	0.563	0.1218
45	21	3.580	0.0690	2.127	0.1129
45	41	2.994	0.0657	2.822	0.0967

[a] The *in vitro* $t_{50\%}$ release (y_2) is measured in minutes. [b] The tablet friability (y_3) is measured by percentage weight loss.

Table IV—Results of Multiple Regression Analyses[a]

Coefficients and Trend Components	Trend Component Name	Regression Coefficient Value: Tablet Hardness, y_1	*In Vitro* $t_{50\%}$, ln y_2[b]	Tablet Friability, ln y_3 + 3.0[b]	Table Volume, $y_4 \times 10^2$
B_{i0}	y-Intercept	0.96089×10^1	0.37657×10^1	0.45164×10^0	0.23441×10^1
$B_{i1}X_1$	Linear in X_1	0.31689×10^0	0.45581×10^{-1}	0.0	0.49647×10^{-2}
$B_{i2}X_2$	Linear in X_2	-0.33759×10^0	-0.55720×10^{-1}	0.72057×10^{-1}	0.83571×10^{-2}
$B_{i3}X_1^2$	Quadratic in X_1	-0.29915×10^{-2}	0.0	-0.20254×10^{-2}	0.79259×10^{-4}
$B_{i4}X_2^2$	Quadratic in X_2	0.21832×10^{-2}	0.0	0.0	0.0
$B_{i5}X_1X_2$	Linear × linear interaction	0.12626×10^{-2}	-0.17935×10^{-2}	-0.77965×10^{-2}	-0.52103×10^{-4}
$B_{i6}X_1X_2^2$	Linear × quadratic interaction	0.0	0.45949×10^{-4}	0.11249×10^{-3}	0.11770×10^{-5}
$B_{i7}X_1^2X_2$	Quadratic × linear interaction	0.0	0.0	0.18878×10^{-3}	0.0
$B_{i8}X_1^2X_2^2$	Quadratic × quadratic interaction	0.0	-0.20698×10^{-6}	-0.30361×10^{-5}	-0.19681×10^{-7}
Multiple correlation coefficient		0.9899	0.9982	0.9975	0.9983

[a] These analyses were performed on a polynomial of the form $y_i = B_{i0} + B_{i1}X_1 + B_{i2}X_2 + B_{i3}X_1^2 + B_{i4}X_2^2 + B_{i5}X_1X_2 + B_{i6}X_1X_2^2 + B_{i7}X_1^2X_2 + B_{i8}X_1^2X_2^2$, where i = 1, 2, 3, and 4. [b] Since the logarithmic transformation was applied to *in vitro* $t_{50\%}$ and tablet friability data to stabilize the variances, trend and regression analyses were performed on the transformed data.

available by superimposing the contour graphs for tablet hardness and *in vitro* $t_{50\%}$ on each other. Figure 6 illustrates the desired solution space (shaded portion of graph). Figure 6 points out the fact that there are many combinations of X_1 and X_2 producing responses which meet these restrictions on tablet hardness and *in vitro* release rate. When this is the case, the final selection of levels of X_1 and X_2 can be based on some other criterion such as cost. The reader interested in the different types of contour graphs and their physical interpretation is referred to Box (27).

Constrained Optimization—Since a reasonably rapid release rate of drug is generally an important objective in the design of solid dosage forms, optimization of this parameter was employed in studying the applicability of constrained optimization to a pharmaceutical product design problem. The problem was thus to locate levels of stearic acid (X_1) and starch (X_2) that minimized the *in vitro* release rate, such that the average tablet volume did not exceed 0.442 cm.^{3} (0.0270 in.^{3}) and the average friability value did not exceed 2.72%. Expressed mathematically, this constrained optimization problem was to minimize

$$y_2 = f_2(X_1,X_2) \qquad \text{(Eq. 11)}$$

such that the following constraints were not violated:

$$5 \leq X_1 \leq 45 \qquad \text{(Eqs. 12–13)}$$

$$1 \leq X_2 \leq 41 \qquad \text{(Eqs. 14–15)}$$

$$y_3 = f_3(X_1,X_2) \leq 2.72 \qquad \text{(Eq. 16)}$$

$$y_4 = f_4(X_1,X_2) \leq 0.0270 \qquad \text{(Eq. 17)}$$

Equations 12–15 serve as constraints to keep the X_1 and X_2 values in the known experimental region.

Figure 7 represents a graphical analysis of the constrained optimization problem defined by Eqs. 11–17. The shaded portion of the graph defines the region of feasible solutions where none of the constraints is violated. Any pair of X_1 and X_2 values within the shaded region meets the friability and volume constraints as well as the minimum starch percentage (Eqs. 14, 16, and 17), without consideration of such nonbinding constraints as those of Eqs. 12, 13, and 15. The dashed contour lines of Fig. 7 represent *in vitro* $t_{50\%}$ responses of 12, 20, and 33 min. Since this family of contour lines is decreasing in the direction of Point A, it is also evident that the minimum starch percentage of Eq. 14 will not be binding in a minimization problem. By eliminating obviously nonbinding inequality constraints, the optimization problem of Eqs. 11–17 reduces to the following: minimize

$$\ln y_2 = B_{20} + B_{21}X_1 + B_{22}X_2 + B_{25}X_1X_2 + B_{26}X_1X_2^2 + B_{28}X_1^2X_2^2 \qquad \text{(Eq. 18)}$$

such that

$$B_{30} + B_{32}X_2 + B_{33}X_1^2 + B_{35}X_1X_2 + B_{36}X_1X_2^2 + B_{37}X_1^2X_2 + B_{38}X_1^2X_2^2 + q_1^2 - (\ln 2.72 + 3.0) = 0 \qquad \text{(Eq. 19)}$$

$$B_{40} + B_{41}X_1 + B_{42}X_2 + B_{43}X_1^2 + B_{45}X_1X_2 + B_{46}X_1X_2^2 + B_{48}X_1^2X_2^2 + q_2^2 - (0.0270 \times 10^2) = 0 \qquad \text{(Eq. 20)}$$

Table V—Verification of Generated Polynomial Models

New Experimental Conditions: % Stearic Acid, X_1	% Starch, X_2	Variable	Predicted y_i from Polynomial	95% CI about the Single Prediction	Experimental Results
15.0	11.0	Hardness (y_1), kg.	10.4	8.8 – 12.1	9.4
15.0	31.0		5.9	4.3 – 7.5	4.4
35.0	11.0		14.1	12.4 – 15.7	14.6
35.0	31.0		10.1	8.4 – 11.7	9.6
15.0	11.0	*In vitro* $t_{50\%}$ (y_2), min.	37.3	31.7 – 43.8	34.4
15.0	31.0		12.3	10.4 – 14.4	12.9
35.0	11.0		68.0	57.9 – 80.2	72.8
35.0	31.0		19.9	16.9 – 23.4	19.9
15.0	11.0	Tablet friability (y_3), % wt. loss	3.18	2.32 – 4.36	4.14
15.0	31.0		7.04	5.13 – 9.66	6.64
35.0	11.0		0.55	0.40 – 0.78	0.51
35.0	31.0		1.21	0.88 – 1.66	1.21
15.0	11.0	Table volume (y_4), in.^{3}	0.0252	0.0249 – 0.0255	0.0254
15.0	31.0		0.0268	0.0266 – 0.0271	0.0272[a]
35.0	11.0		0.0269	0.0266 – 0.0272	0.0270
35.0	31.0		0.0283	0.0281 – 0.0286	0.0284

[a] This value fell slightly outside the 95% confidence range for a single prediction.

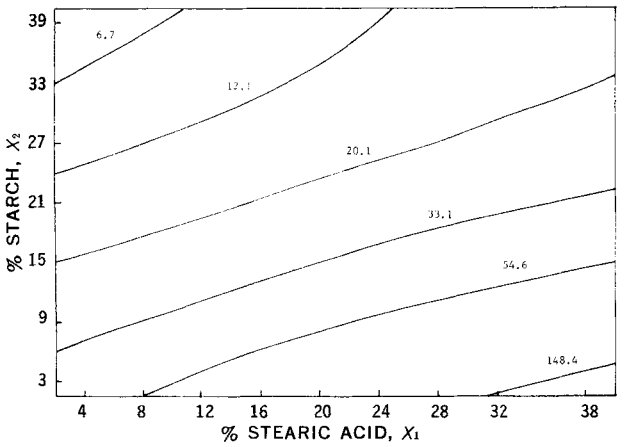


Figure 3—*Contour curves for* in vitro $t_{50\%}$ *(minute), illustrating levels of starch and stearic acid producing similar responses.*

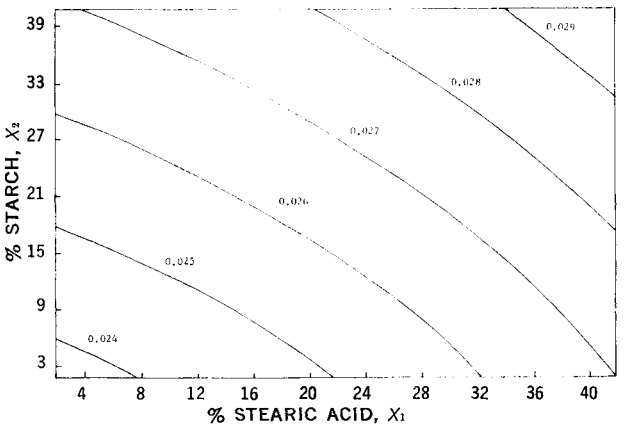


Figure 5—*Contour curves for tablet volume (cubic inch), illustrating levels of starch and stearic acid producing similar responses.*

In Eqs. 18–20, the regression models developed earlier (Table IV) have been substituted into Eqs. 11, 16, and 17. The slack variables q_1 and q_2 have been included in Eqs. 19 and 20 in order that these constraints may be written as equality constraints. The formulated Lagrange function is then

$$F = B_{20} + B_{21}X_1 + B_{22}X_2 + B_{25}X_1X_2 + B_{26}X_1X_2^2 + B_{28}X_1^2X_2^2 + \lambda_1(B_{30} + B_{32}X_2 + B_{33}X_1^2 + B_{35}X_1X_2 + B_{36}X_1X_2^2 + B_{37}X_1^2X_2 + B_{38}X_1^2X_2^2 + q_1^2 - 4.000) + \lambda_2(B_{40} + B_{41}X_1 + B_{42}X_2 + B_{43}X_1^2 + B_{45}X_1X_2 + B_{46}X_1X_2^2 + B_{48}X_1^2X_2^2 + q_2^2 - 2.70) \quad \text{(Eq. 21)}$$

where λ_1 and λ_2 are terms known as Lagrange multipliers. As can be seen from Eq. 21, one is introduced for each constraint.

The original constrained optimization problem involving three equations has now been converted into an unconstrained minimax problem involving one equation. The minimax solution of Eq. 21 defines values of X_1 and X_2 which minimize the *in vitro* $t_{50\%}$ value subject to the constraints on tablet friability and volume. When this Lagrange function is partially differentiated with respect to each variable, each differentiation is set equal to zero, and the set of six equations is solved simultaneously, the values of each variable are

$$X_1^* = 22.5\% \quad \lambda_1 = 0.50 \quad q_1 = 0.0$$
$$X_2^* = 26.8\% \quad \lambda_2 = 2.90 \quad q_2 = 0.0$$

By substituting these values of X_1 and X_2 into Eq. 18, $t^*_{50\%}$ = 17.9 min., which is the best release rate that can be achieved under the restrictions involved. Since $q_1 = q_2 = 0.0$, both previously described constraints are binding as indicated by Fig. 7, where it is evident that the contour line for $t^*_{50\%}$ = 17.9 min. would pass through Point A, where the contour lines for y_3 = 2.72% and y_4 = 0.442 cm.^{3} (0.0270 in.^{3}) cross.

Sensitivity Analysis—The solution to a constrained optimization problem may depend heavily upon the restricting values assigned to the secondary objectives or constraints. Consequently, minor modifications in these restricting values may result in a substantial improvement in the primary objective. Sensitivity analysis serves to identify the changes in the primary objective resulting from such modifications of the restricting values. Sensitivity analysis involves solving the constrained optimization problem for systematic changes of the restricting values assigned to the secondary objectives. Mathematically, the problem is to minimize ln y_2, such that

$$\ln y_3 + 3.0 \leq \ln \alpha_k + 3.0 \quad \text{(Eq. 22)}$$

$$5 \leq X_1 \leq 45, \text{ and } 1 \leq X_2 \leq 41 \quad \text{(Eqs. 23–24)}$$

The restraining values of percent friability (α_k) were allowed to assume the following values: 0.3, 0.4, 0.5, 0.6, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. These results are shown in Fig. 8, and they demonstrate that substantial improvements in $t^*_{50\%}$ can be obtained for values of α_k up to about 1–2%. Beyond 2%, the rate of decrease of $t^*_{50\%}$ is very low. Consequently, the scientist designing the drug product can evaluate the potential gains in the primary objective which accrue from modifications of restraining values on secondary objectives. An objective decision on whether or not restraining values on secondary objectives should be relaxed (or tightened), and by how much, is thus made possible. Also, the locus of X_1^* and X_2^* points may be shown as a function of α_k, as demonstrated by Fig. 9, so that any decision on the revision of α_k can immediately provide a revised solution in terms of the tablet formulation.

Frequently, it is useful to consider the sensitivity of multiple constraints which requires a Lagrangian solution for each different combination of restricting values. For example, if the friability restriction was retained as α_k and a tablet volume constraint of

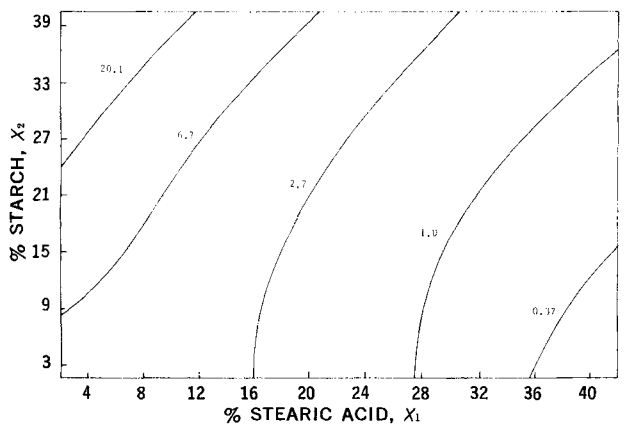


Figure 4—*Contour curves for tablet friability (percent weight loss), illustrating levels of starch and stearic acid producing similar responses.*

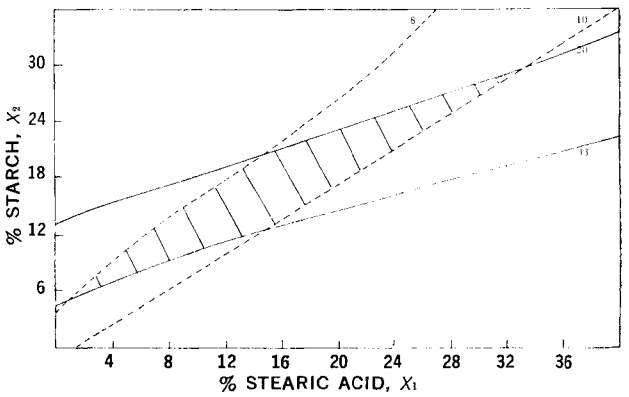


Figure 6—*Feasible solution space defined by hypothetical restrictions on tablet hardness and* in vitro $t_{50\%}$ *release rate. Key: - - -, hardness contours (kilogram); and —,* in vitro $t_{50\%}$ *contours (minute).*

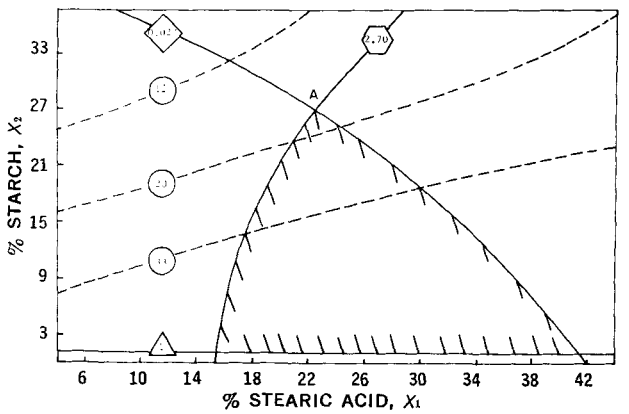


Figure 7—*Graphical analysis of constrained optimization problem defined by Eqs. 26–32. Key:* O, in vitro $t_{50\%}$ *(minute);* ◇, *volume (cubic in.);* ○, *hardness (kilogram); and* △, *percent starch.*

β_k' was added, then each α_k, β_k' combination requires a new solution that provides $t^*_{50\%}$, X_1^*, and X_2^* values. The results for nine combinations of α_k and β_k' for this example are shown in Table VI. An examination of the X_1^* and X_2^* values indicates that none of the experimental condition constraints was binding. Figure 10 provides a graphic illustration of $t^*_{50\%}$ as a function of α_k and β_k'. Figure 10 demonstrates that a sizable improvement in $t^*_{50\%}$ can be achieved by relaxing α_k and β_k' values, but the rate of improvement drops off sharply past the midvalue of $\alpha_k = 2.70$ and $\beta_k' = 0.0270$.

Constrained Optimization and *In Vivo* Studies—Clinical trials performed on a new drug in its various dosage forms or dosage form modifications may result in information on the relationship between blood levels, level of therapeutic action, duration of action, toxicity properties, and the absorption rate or possibly the consequent elimination rate of the drug. Establishment of such relationships could lead to criteria being established on the absorption rate or consequent elimination rate in order that the best therapeutic response and/or duration of effect and minimal toxicity be achieved. The results of urinary elimination-rate studies performed on certain formulas used in this investigation are presented in Table VII. The time in hours for 50% of the drug to be eliminated ($t'_{50\%}$) was used as a measure of the urinary elimination rate of the drug as modified by the availability from the respective formulation. The linear correlation coefficients for the semilog plots of drug retention *versus* time all exceeded 0.98, indicating that the excretion of phenylpropanolamine hydrochloride followed an apparent first-order process. A comparison of the mean *in vitro* ($t_{50\%}$) and *in vivo* ($t'_{50\%}$) data showed that the rank order of the formulations was the same for each criterion. Further analysis revealed that the logarithm of the mean *in vivo* $t'_{50\%}$ data correlated very highly ($r = 0.993$) with the mean *in vitro* $t_{50\%}$ data on a linear scale. Figure 11 depicts this plot and illustrates the correlation. The confidence bands about the $t'_{50\%}$ values represent 95% confidence intervals. It would appear that prediction of the *in vivo* $t'_{50\%}$ response from a knowledge of the *in vitro* $t_{50\%}$ response can be made fairly accurately for the tablet system used in this study, employing the correlation curve appearing in Fig. 11.

To illustrate the utility of the constrained optimization approach as applied to *in vivo* data, a sample constrained optimization problem was formulated and solved using the Lagrangian method.

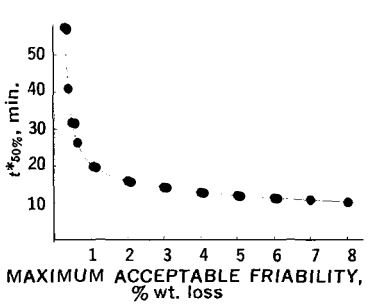


Figure 8—*Optimum* in vitro $t_{50\%}$ *release rate as a function of restrictions on tablet friability.*

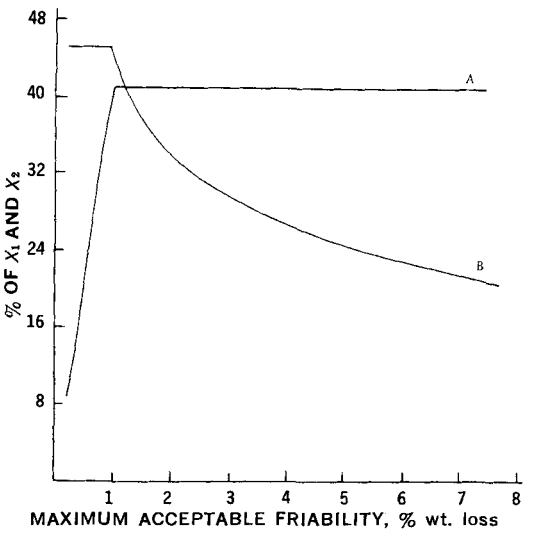


Figure 9—*Optimizing values of stearic acid and starch as a function of restrictions on tablet friability. Key: A, percent starch; and B, percent stearic acid.*

The following situation was assumed to exist in this illustrative example:

1. The primary objective was to minimize tablet friability or its equivalent ($\ln y_3 + 3$).
2. Secondary objectives were to maintain a reproducible fill in the packaging container and to avoid noticeable differences in tablet size so that the tablet volume (y_4) is held within the interval 0.0261–0.0269.
3. Another secondary objective was to keep the *in vivo* elimination half-life below 6.5 hr. for the desired therapeutic effect of this drug.

In constrained optimization form, the problem may be mathematically stated as: minimize

$$(\ln y_3 + 3.0) = f_3(X_1, X_2) \qquad \text{(Eq. 25)}$$

such that

$$2.61 \leq y_4 \times 10^2 \leq 2.69, \text{ and} \qquad \text{(Eqs. 26–27)}$$

$$0.69937 + (0.1908 \times 10^{-2})(e^{\ln y_2}) \leq \log 6.5 \qquad \text{(Eq. 28)}$$

In Eq. 28, the left-hand side of the equation represents the least-squares estimate of the curve appearing in Fig. 11, and $\ln y_2$ as a function of X_1 and X_2 can be found in Table IV.

Employing the Lagrangian method, the solution to the constrained optimization problem (Eqs. 25–28) was found to be as follows: $X_1^* = 34.1\%$, $X_2^* = 13.3\%$, and $y_3^* = 0.64\%$ wt. loss. The volume constraint of Eq. 26 was nonbinding, and the other two constraints (Eqs. 27–28) were binding. To demonstrate further the validity of this approach to tablet design, a formulation was prepared using the optimal percentages of stearic acid and starch (34.1 and 13.3%, respectively) and the empirical test results were

Table VI—Optimizing Percentages of Stearic Acid and Starch and the Corresponding Optimal *In Vitro* Release Rate as a Function of Simultaneous Restraining Values on Tablet Friability and Volume

Maximum Acceptable Constraints		Optimizing Values of Controllable Variables		Optimum *In Vitro* Release Rate
Friability, % wt. loss	Volume, cm.³ (in.³)	% Stearic Acid, X_1^*	% Starch, X_2^*	($t^*_{50\%}$), min.
1.00	0.426 (0.0260)	27.9	7.9	71.6
	0.442 (0.0270)	30.7	18.2	34.9
	0.459 (0.0280)	23.8	26.5	23.8
2.70	0.426 (0.0260)	18.7	17.7	26.0
	0.442 (0.0270)	22.5	26.8	17.9
	0.459 (0.0280)	27.1	34.8	14.8
7.40	0.426 (0.2060)	10.9	23.9	15.4
	0.442 (0.0270)	32.6	32.8	11.6
	0.459 (0.0280)	20.6	40.4	10.6

Table VII—Results of Urinary Excretion Studies for Tablets Containing Various Concentrations of Starch and Stearic Acid

Formulation No.[a]	Subject	Urinary Excretion Rate ($t'_{50\%}$), hr.	Mean ($t'_{50\%}$), hr.	*SD*
Solution of Drug	1	6.30		
	2	4.09		
	3	4.38	4.95	0.853
	4	5.09		
	5	4.88		
3	1	4.62		
	2	4.57		
	3	6.71	5.20	0.874
	4	5.05		
	5	5.07		
1	1	5.70		
	2	5.94		
	3	7.34	6.23	0.641
	4	6.07		
	5	6.09		
4	1	9.13		
	2	7.00		
	3	11.77	9.14	1.723
	4	9.17		
	5	8.62		
7	1	22.68		
	2	13.48		
	3	16.54	18.00	6.878
	4	17.66		
	5	19.65		

[a] See Table I.

compared with the theoretical values predicted. These results appear in Table VIII, and the *in vivo* urinary elimination rate curves appear in Fig. 12. The mean tablet volume of 0.442 cm.^{3} (0.0270 in.^{3}) and the mean $t'_{50\%}$ excretion of 6.4 hr. were very close to the theoretical predictions of 0.0269 and 6.5, respectively. By optimizing the tablet friability, a cohesive tablet was formed, with an average friability weight loss of about 0.58%, which is sufficiently close to the theoretical prediction of 0.64%.

Pharmaceutical Process Optimization—Reier *et al.* (4) have recently quantified the weight and weight variation of filled capsules with controllable process variables. They found the significant set

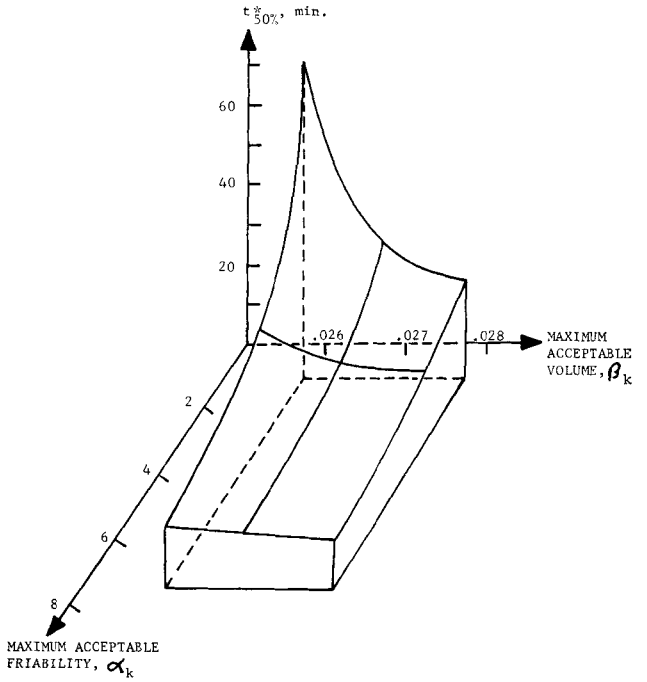


Figure 10—*Optimum* $t_{50\%}$ *as a function of restrictions on tablet friability and volume.*

Table VIII—Experimental Response Values for the Optimal Tablet Formulation of 34.1% Stearic Acid and 13.3% Starch

Volume, cm.^{3} (in.^{3})	Tablet Friability, % wt. loss	Urinary Excretion Rate ($t'_{50\%}$), hr.
0.442 (0.0270)	0.58	5.0
0.442 (0.0269)	0.55	5.9
0.442 (0.0270)	0.57	7.1
0.442 (0.0270)	0.63	7.8
0.444 (0.0271)	0.55	6.3
Mean: 0.442 (0.0270)	0.58	6.4

of controllable variables to be: (*a*) machine speed, r.p.m. (X_1); (*b*) capsule size, cm.^{3} (X_2); (*c*) specific volume, ml./g. (X_3); (*d*) flowability, in.^{2} (X_4); and (*e*) presence or absence of talc (X_5). Regression models relating these controllable variables with mean gross capsule weight (y_1), capsule weight standard deviation (y_2), and capsule weight coefficient of variation (y_3) were given. These regression models can be employed to formulate an unconstrained optimization problem for determining levels of machine speed (X_1), specific volume (X_3), and flowability (X_4), which minimize the capsule weight coefficient of variation (y_3). Assuming that talc is present in the formulation, the model for y_3, using the data of Reier *et al.* (4), becomes

$$y_3 = 4.15 + 0.04X_1^2 + 0.23X_3^2 - 1.86X_4 + 0.45X_4^2 - 0.15X_1X_3 - 0.11X_1X_4 \quad \text{(Eq. 29)}$$

Taking the first partial derivatives of Eq. 29 with respect to X_1, X_3, and X_4, setting these expressions equal to zero, and solving this set of three simultaneous linear equations produced the following: X_1^* = 13.3 r.p.m., X_3^* = 4.34 ml./g., and X_4^* = 3.75 in.^{2} At this global minimum, y_3^* = 0.78%. However, the values for X_1^* and X_3^* far exceed values used by the authors in deriving Eq. 29 and may, therefore, not be valid. An interesting point is, however, that this information would suggest using a light, free-flowing powder and a high machine speed to minimize capsule weight variation. Since the maximum machine speed used by Reier *et al.* (4) was 8.7 r.p.m., the optimization problem may be reformulated using this maximum machine speed as fixed, or:

$$y_3 = 7.18 - 1.31X_3 - 2.82X_4 + 0.23X_3^2 + 0.45X_4^2 \quad \text{(Eq. 30)}$$

The solution to Eq. 30 is a global minimum at X_3^* = 2.85 ml./g. and X_4^* = 3.13 in.^{2}, with the criterion of y_3^* = 0.90% at this minimum. Insofar as these regression models hold for various possible formulations, which appear to be reasonably general, the values X_1^* = 8.7, X_3^* = 2.85, and X_4^* = 3.13 for the controllable variables minimize the capsule weight coefficient of variation. If y_3 is independent of capsule size, which appears valid for at least moderate variations, then the solution to this optimization problem holds for various capsule sizes.

However, it may not always be practical to utilize these optimum operating conditions. Often the bulk formulation, capsule size, and weight are specified in advance. As an example, assume the following information has been prespecified: (*a*) capsule size = No. 0, or X_2 = 0.699; (*b*) capsule weight = 400 mg., or y_1 = 400; (*c*) specific volume of powder = 2.80 ml./g., or X_3 = 2.80; and (*d*) talc is present, or X_5 = +1. The objective is to locate levels of machine speed (X_1) and flowability (X_4) which minimize the capsule weight

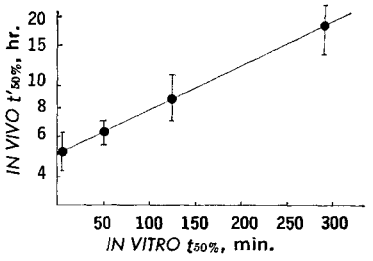


Figure 11—*Correlation of* in vivo *and* in vitro *release rate data.*

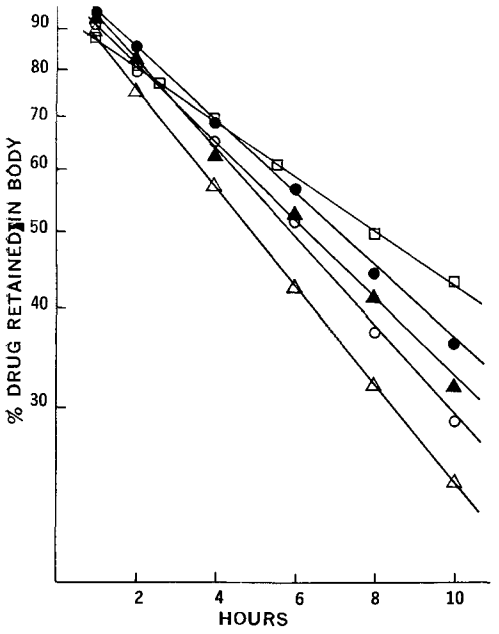


Figure 12—*Urinary excretion data from five humans for the optimal tablet formulation of 34.1% stearic acid and 13.3% starch. Key:* O, *Subject 1;* △, *Subject 2;* □, *Subject 3;* ●, *Subject 4; and* ▲, *Subject 5.*

coefficient of variation. This represents a constrained optimization problem which may be formulated as follows: minimize

$$y_3 = 6.09 - 1.86X_4 + 0.04X_1^2 - 0.45X_4^2 - 0.42X_1 - 0.11X_1X_4 \quad \text{(Eq. 31)}$$

such that

$$548.49 - 32.27X_1 - 11.20X_2 + 1.92X_1^2 = 400 \quad \text{(Eq. 32)}$$

The Lagrangian expression then becomes

$$F = 6.09 - 1.86X_4 + 0.04X_1^2 + 0.45X_4^2 - 0.42X_1 - 0.11X_1X_4 + \lambda(148.49 - 32.27X_1 - 11.20X_2 + 1.92X_1^2) \quad \text{(Eq. 33)}$$

By partially differentiating Eq. 33 and solving this set of nonlinear equations, it was found that $X_1^* = 5.63$ r.p.m., $X_4^* = 2.47$ in.^{2}, and $y_3^* = 1.62$. Thus, for a 400-mg. No. 0 capsule, whose contents have a specific volume of 2.8 ml./g., the minimum coefficient of variation possible is 1.62, and this is achieved at a machine speed setting of 5.63 r.p.m. and a flowability value of 2.47 in.^{2}

SUMMARY

The use of constrained optimization techniques, employing the Lagrangian method, has been successfully applied to complex pharmaceutical product and process design problems involving many competing objectives. Location of optimal solutions to pharmaceutical design problems by this analytical–mathematical approach has been demonstrated. In most situations the location of the theoretical optimal solution point can provide the starting reference for adjustments due to other practical considerations, thereby permitting more rapid and accurate solution of the design problem. The steps involved in solving a design problem *via* the constrained optimization approach may be summarized as follows:

Identify important response variables y_j, $j = 1, 2, \ldots, r$, the significant set of controllable variables X_i, $i = 1, 2, \ldots, n$, and measures on both classes of variables.

Determine a mathematical relationship between each y_j as a function of X_i, either analytically or empirically.

Select the response variable which is of greatest importance as the principal objective to be optimized and place control limits on the remaining response variables.

Solve the resultant constrained optimization problem by one of the several existing techniques.

In addition to finding optimal solutions to constrained pharmaceutical problems, the application of sensitivity analysis studies to such problems was also illustrated. The rate of change of the optimal response in the principal objective to changes of restrictions on the competing or lesser important objectives was analyzed for a product design problem.

The mathematical and statistical techniques described in this paper are suggested as an improvement over the trial-and-error approach widely employed today in pharmaceutical product and process design. Improved reliability of the research effort and a saving in time and money are the direct results of this sophisticated approach to pharmaceutical research problems.

APPENDIX A

Example 1—Locate the levels of X_1 and X_2 which maximize

$$y = X_1X_2 \quad \text{(Eq. A-1)}$$

such that

$$X_1 + X_2 = 4 \quad \text{(Eq. A-2)}$$

Since the constraint in Eq. A-2 is already in the form of an equality constraint, the Lagrangian function can be formed immediately:

$$F = X_1X_2 + \lambda(X_1 + X_2 - 4) \quad \text{(Eq. A-3)}$$

Taking the first partial derivatives of the Lagrangian function (Eq. A-3) and setting the resulting equations equal to zero,

$$\partial F/\partial X_1 = X_2 + \lambda = 0 \quad \text{(Eq. A-4)}$$

$$\partial F/\partial X_2 = X_1 + \lambda = 0 \quad \text{(Eq. A-5)}$$

$$\partial F/\partial \lambda = X_1 + X_2 - 4 = 0 \quad \text{(Eq. A-6)}$$

Solving these three equations simultaneously, $X_1^* = 2$, $X_2^* = 2$, and $\lambda = -2$. Substituting these values of X_1 and X_2 back into the objective function (Eq. A-1), it was found that $y^* = 4$. Thus, the maximum response in y that can be obtained subject to the constraint of $X_1 + X_2 = 4$ is 4 units. This constrained optimization problem is represented graphically in Fig. A-1. Contour curves for objective function responses of 2, 4, and 6 units are shown. The dashed, straight line in Fig. A-1 represents the constraint (Eq. A-2), and Point A depicts the optimal solution point ($X_1^* = 2$, $X_2^* = 2$).

Example 2—Locate the levels of X_1 and X_2 which minimize

$$y = X_1^2 + X_2^2 \quad \text{(Eq. A-7)}$$

such that

$$X_1 + X_2 \geq 4 \quad \text{(Eq. A-8)}$$

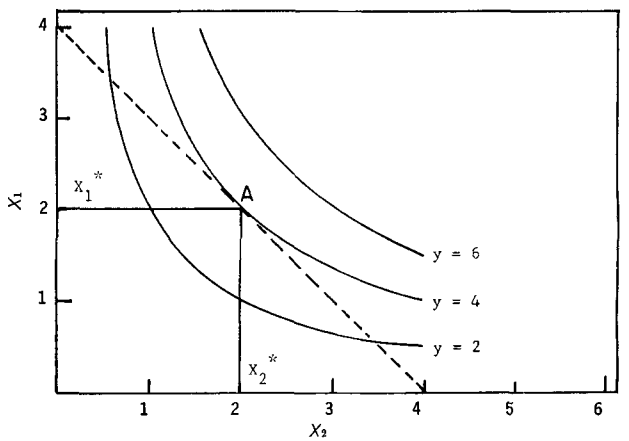


Figure A-1—*Graphical solution for constrained optimization problem of Eqs. A-1 and A-2. Key:* - - -, *contour line for* $X_1 + X_2 = 4$.

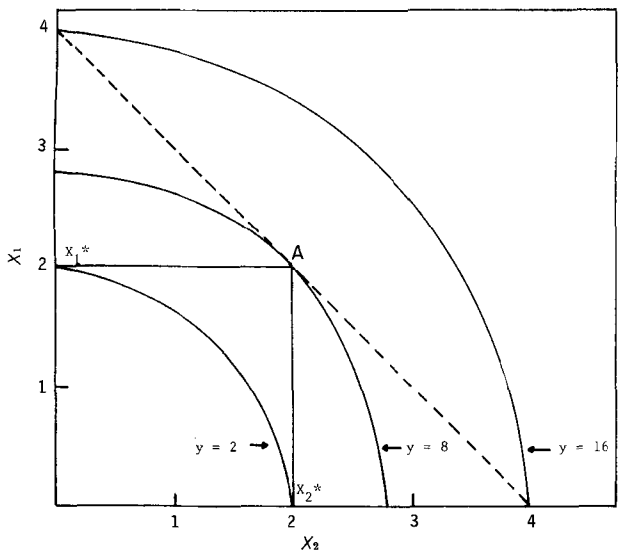


Figure A-2—*Graphical solution for constrained optimization problem of Eqs. A-7 and A-8. Key: - - - -, contour line for* $X_1 + X_2 = 4$.

The inequality constraint of Eq. A-8 must first be converted to an equality constraint by introducing a slack variable, q. Thus, Eq. A-8 may be written as

$$X_1 + X_2 - q^2 = 4 \qquad \text{(Eq. A-9)}$$

and the Lagrangian function becomes

$$F = X_1^2 + X_2^2 + \lambda(X_1 + X_2 - q^2 - 4) \qquad \text{(Eq. A-10)}$$

Taking the first partial derivatives of Eq. A-10 and setting these expressions equal to zero,

$$\partial F/\partial X_1 = 2X_1 + \lambda = 0 \qquad \text{(Eq. A-11)}$$

$$\partial F/\partial X_2 = 2X_2 + \lambda = 0 \qquad \text{(Eq. A-12)}$$

$$\partial F/\partial \lambda = X_1 + X_2 - q^2 - 4 = 0 \qquad \text{(Eq. A-13)}$$

$$\partial F/\partial q = -2\lambda q = 0 \qquad \text{(Eq. A-14)}$$

Solving these four equations simultaneously, $X_1^* = 2$, $X_2^* = 2$, $q = 0$, and $\lambda = -4$. At this optimal solution point, $y^* = 8$ units. This constrained optimization problem is represented graphically in Fig. A-2. Point A in Fig. A-2 illustrates the minimum response in y that can be achieved under the restriction that $X_1 + X_2 \geq 4$. At the simultaneous solution point for Eqs. A-11–A-14, it is noted that $q = 0$. Thus, at the optimal solution point, $X_1 + X_2 = 4$.

REFERENCES

(1) E. A. Holstius and H. G. DeKay, *J. Amer. Pharm. Ass., Sci. Ed.*, **41**, 505(1952).

(2) K. C. Kwan, F. O. Swart, and A. M. Mattocks, *ibid.*, **46**, 236(1957).

(3) E. Marlowe and R. E. Shangraw, *J. Pharm. Sci.*, **56**, 498 (1967).

(4) G. Reier, R. Cohn, S. Rock, and F. Wagenblast, *ibid.*, **57**, 660(1968).

(5) E. D. Sumner, H. O. Thompson, W. K. Poole, and J. E. Grizzle, *ibid.*, **55**, 1441(1966).

(6) O. L. Davies, "The Design and Analysis of Industrial Experiments," 2nd ed., Oliver and Boyd, Edinburgh, England, 1963, pp. 290–336.

(7) H. A. Spang, *SIAM Rev.*, **4**, 343(1962).

(8) R. W. Llewellyn, "Linear Programming," Holt, Rinehart, and Winston, New York, N. Y., 1964.

(9) G. H. Hadley, "Linear Programming," Addison-Wesley, Reading, Mass., 1963.

(10) G. B. Dantzig, "Linear Programming and Extensions," Princeton University Press, Princeton, N. J., 1963.

(11) R. L. Graves and P. Wolfe, "Recent Advances in Mathematical Programming," McGraw-Hill, New York, N. Y., 1963, pp. 67–86.

(12) D. J. Wilde, *Ind. Eng. Chem.*, **57 (No. 8)**, 19(1965).

(13) W. S. Dorn, *Mgt. Sci.*, **9**, 171(1963).

(14) A. H. Boas, *Chem. Eng.*, **70 (No. 3)**, 105(1963).

(15) H. Everett, *Oper. Res.*, **11**, 399(1963).

(16) W. S. Dorn, *ibid.*, **9**, 95(1961).

(17) A. W. Tucker, *ibid.*, **5**, 244(1957).

(18) J. Neyman, "Proceedings Third Berkeley Symposium on Mathematical Statistics and Probability," vol. 5, University of California Press, Berkeley, Calif., 1956, pp. 1–20.

(19) G. Hadley and T. M. Whittin, "Analysis of Inventory Systems," Prentice-Hall, Englewood Cliffs, N. J., 1963, pp. 433–440.

(20) V. Chew, "Experimental Designs in Industry," Wiley, New York, N. Y., 1958, pp. 108–137.

(21) A. W. Umland and W. N. Smith, *Technometrics*, **1 (No. 3)**, 289(1959).

(22) N. A. Lange, "Handbook of Chemistry," 9th ed., Handbook Publishers, Sandusky, Ohio, 1956, pp. 1878, 1879.

(23) K. R. Heimlich, D. R. MacDonnell, T. L. Flanagan, and P. D. O'Brien, *J. Pharm. Sci.*, **50**, 232(1961).

(24) M. S. Bartlett and D. G. Kendall, *J. Roy. Statist. Soc., Suppl.*, **8**, 133(1946).

(25) K. A. Brownlee, "Statistical Theory and Methodology in Science and Engineering," Wiley, New York, N. Y., 1960, pp. 113–115.

(26) H. C. Hamaker, *Statist. Neer.*, **16**, 31(1962).

(27) G. E. P. Box, *Biometrics*, **10**, 16(1954).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 19, 1970, from the *Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication May 11, 1970.

Abstracted in part from a thesis submitted by Dale E. Fonner, Jr., to the Graduate School, Purdue University, in partial fulfillment of Doctor of Philosophy degree requirements.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, November 1969, Washington, D. C.

* Present address: The Upjohn Co., Pharmaceutical Manufacturing Development Unit, Kalamazoo, Mich.; Fellow, American Foundation for Pharmaceutical Education.

Effect of Structural Similarity on Molecular Interaction in Aqueous Solution: Interaction of Phenazine and Tetramethylpyrimidopteridinetetrone with Alkylxanthines and Benzene Derivatives

KIICHIRO KAKEMI, HITOSHI SEZAKI, TAKAYOSHI MITSUNAGA, and MASAHIRO NAKANO*

Abstract □ The molecular interactions in water of phenazine and tetramethylpyrimidopteridinetetrone (TMPPT) with alkylxanthines and benzene derivatives were studied by means of the phase solubility technique. Alkylxanthines showed a greater affinity toward phenazine than toward TMPPT. Benzene derivatives, on the other hand, formed more stable complexes with TMPPT than with phenazine. These results are discussed in terms of structural similarity between interacting species. Studies concerning the effects of solvent on the extent of complex formation have revealed that water plays a unique role in these interactions. The observation that complexation between two structurally dissimilar compounds is favored over that between two similar compounds suggested that some forces other than mere hydrophobic bonding should be taken into consideration. It is postulated that the results are best rationalized by hydrophobic bonding stabilized by a type of bonding similar to polarization bonding.

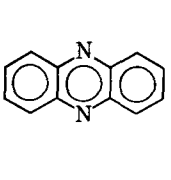
Keyphrases □ Molecular interaction, aqueous solutions—structural similarity effect □ Phenazine–alkylxanthines, benzene derivatives—interaction □ Tetramethylpyrimidopteridinetetrone–alkylxanthines, benzene derivatives—interaction □ Phase solubility—molecular interactions □ UV spectrophotometry—analysis

While molecular interaction in nonpolar solvents can best be understood on the basis of hydrogen bonding or charge transfer complexation, a mechanism of molecular interaction in aqueous solution still remains largely unsolved. Earlier workers initially believed that hydrogen bonding was responsible for complexation in aqueous solution. Considerable evidence has since been accumulated to dispute this interpretation (1). The application of the charge transfer complexation theory in nonpolar sovents (2) to aqueous solutions is not in accord with present knowledge of interactions in aqueous solution. Such an interaction has been shown to be quite weak or nonexistant in an aqueous environment (3), since a typical charge transfer band is not usually observed in water (3). It is thought that thermal electron transfer leading to the formation of radicals or reactions may be responsible for the appearance of such a band in some cases.

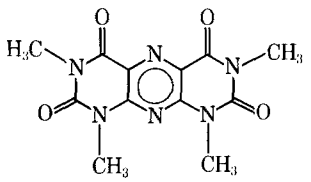
The more or less nonpolar molecules in water tend to come together, reducing the number of water-solute contacts. This phenomenon is known as hydrophobic bonding. Solvophobic bonding refers to a similar phenomenon in any solvent. The source of stability of a complex in aqueous solution has been attributed either to a favorable enthalpy term [large surface energy of water (4, 5)] or to a favorable entropy term [increased ordering of water around the hydrophobic group in a solute molecule (6, 7)]. The "squeezing-out" effect proposed by Higuchi and Lach as early as 1954 (8) is essentially the same as the theory by Sinanoğlu and Abdulnur (4, 5). Studies on the effect of solvents on the extent of complexation (9–11) generally support the hydrophobic bonding theory. Complexes stabilized in aqueous solution tend to dissociate in less polar and nonpolar solvents, except when solute molecules associate by hydrogen bonding or charge transfer interaction (12).

From a considerable amount of work concerning complex formation in aqueous solution, Higuchi (13) concluded that hydrophobic bonding alone cannot be responsible for interaction in aqueous solution. He suggested that interacting molecules may be divided into groups or classes such that compounds which belong to the same class do not interact very strongly with each other while those belonging to different classes bind strongly.

In pursuit of the elucidation of the mechanism of molecular interaction in water the present study was undertaken by selecting two model compounds, phenazine and tetramethylpyrimidopteridinetetrone (TMPPT) so that the extent of interaction can be monitored by the increase in their solubility in the presence of



phenazine



TMPPT

complexing agents. The choice of phenazine and TMPPT is based on simplicity in their spectrophotometric assay and on the polarizable nature of the former in contrast to the polar nature of the latter. The present results demonstrate that there is some discrimination in molecular interaction even in aqueous media, the extent of interaction being greater between polar and polarizable compounds than that between two polar compounds or two polarizable molecules. The results are further interpreted in terms of additional stabilization of complexes by polarization bonding (14) in the presence of hydrophobic bonding contribution due to water as solvent.

EXPERIMENTAL

Materials—Phenazine (Aldrich) was recrystallized from methanol–water, m.p. 171°. 1,3,7,9-Tetramethylpyrimido(5,4-*g*)pteridine-2,4,6,8(1*H*,3*H*,7*H*,9*H*)-tetrone (abbreviated as TMPPT; Alfred Bader Chemicals, Aldrich) was used without further purification since its NMR spectrum indicated no impurity; m.p. >340°. With the exception of *N*-substituted amides (15), complexing agents were obtained from commercial sources and purified whenever necessary. Water was purified by distillation. Organic solvents employed were of spectroscopic grade.

Methods—Phenazine (or TMPPT) in excess of the solubility was added to vials containing aqueous solutions (5 ml.) of varying concentrations of the complexing agent. Complexing agent concentrations were obtained by dilution of a stock solution. The vials were

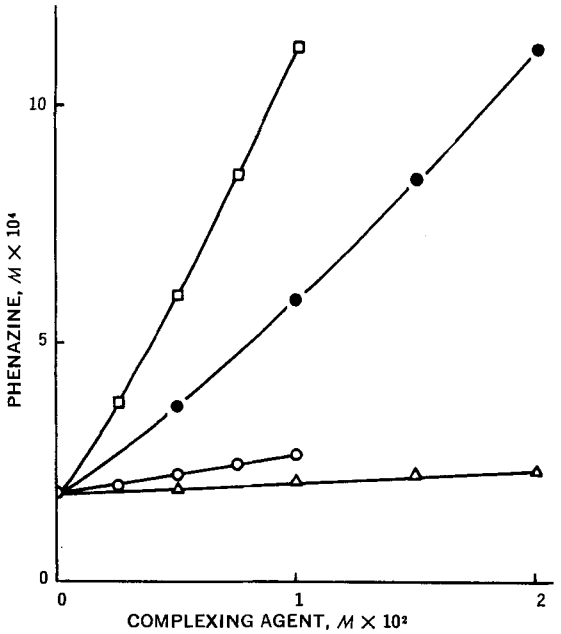


Figure 1—*Solubility diagrams of phenazine in the presence of 8-methoxycaffeine* (□), *theophylline* (●), N,N-*dimethylcinnamamide* (○), *and sodium salicylate* (△) *in water at 25°.*

closed and shaken in a constant-temperature water bath at 25° for about 40 hr. The equilibrated contents of the vials were quickly filtered through sintered-glass funnels (medium porosity) under reduced pressure. Samples (2 ml.) were then diluted with water and the total concentration of phenazine (or TMPPT) solubilized was measured spectrophotometrically at 366 mμ (or at 360 mμ for TMPPT) employing a spectrophotometer (Shimadzu QV-50). The observed solubility of phenazine (or TMPPT) was plotted against the concentration of complexing agent added. Stability constants were calculated from the solubility diagrams according to the following manner.

1:1 Complex—Where a straight line was obtained in the plot, the formation of a 1:1 complex was assumed and the stability constant was calculated from Eq. 1 (16);

$$K_{1:1} = \frac{\text{slope}}{\text{intercept}(1 - \text{slope})} \qquad \text{(Eq. 1)}$$

1:1 and 1:2 Complexes—Where an upward curve was obtained in the plot, the formation of both 1:1 and 1:2 complexes given by

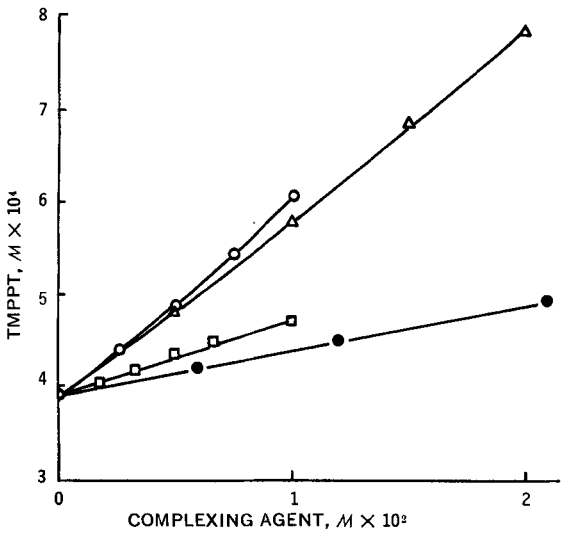


Figure 2—*Solubility diagrams of TMPPT in the presence of* N,N-*dimethylcinnamamide* (○), *sodium salicylate* (△), *8-methoxycaffeine* (□), *and theophylline* (●) *in water at 25°.*

Eqs. 2 and 3 was assumed and the stability constants defined by Eqs. 4 and 5 were computed.

$$A + B \rightleftharpoons AB \qquad \text{(Eq. 2)}$$

$$AB + B \rightleftharpoons AB_2 \qquad \text{(Eq. 3)}$$

$$K_{1:1} = \frac{C_{AB}}{C_A^\circ(C_B - C_{AB} - 2C_{AB_2})} \qquad \text{(Eq. 4)}$$

$$K_{1:2} = \frac{C_{AB_2}}{C_{AB}(C_B - C_{AB} - 2C_{AB_2})} \qquad \text{(Eq. 5)}$$

where C_A°, C_B, C_{AB}, and C_{AB_2} are the solubility of A in the absence of B, the total concentration of B, the equilibrium concentration of AB, and that of AB_2, respectively. From the material balance, the apparent solubility of A, C_A, is given by

$$C_A = C_A^\circ + C_{AB} + C_{AB_2} \qquad \text{(Eq. 6)}$$

From Eqs. 4–6 the following equation was obtained (17):

$$\frac{C_A - C_A^\circ}{C_A^\circ(C_B - C_{AB} - 2C_{AB_2})} = K_{1:1} + K_{1:1}K_{1:2}(C_B - C_{AB} - 2C_{AB_2}) \qquad \text{(Eq. 7)}$$

Since C_{AB} and C_{AB_2} were not known experimentally, they had to be calculated by an iteration (17). The initial values for $K_{1:1}$ and $K_{1:1}K_{1:2}$ were obtained from Eq. 7 by ignoring the C_{AB} and C_{AB_2} terms. The first approximate values for C_{AB} and C_{AB_2} were obtained from Eqs. 4 and 5, *i.e.*,

$$C_{AB} = K_{1:1}C_A^\circ C_B \qquad \text{(Eq. 8)}$$

$$C_{AB_2} = K_{1:1}K_{1:2}C_A^\circ C_B^2 \qquad \text{(Eq. 9)}$$

The C_{AB} and C_{AB_2} values were then put into Eq. 7 to obtain better values for $K_{1:1}$ and $K_{1:1}K_{1:2}$. These steps were repeated until a convergent straight line was obtained.

RESULTS

The solubility diagrams of phenazine in the presence of theophylline, 8-methoxycaffeine, *N,N*-dimethylcinnamamide, and sodium salicylate are shown in Fig. 1. The solubility diagrams for the TMPPT systems are shown in Fig. 2.

Similar solubility measurements were carried out for both phenazine and TMPPT in the presence of 1,3-dimethyluracil, β-hydroxyethylphthalimide, cinnamamide, sodium benzoate, sodium cinnamate, benzoic acid, phenol, benzamide, anisamide, and *N*-methylbenzamide. The solubility diagram of phenazine in the presence of caffeine is shown in Fig. 3, while that of TMPPT is presented in Fig. 4.

A downward trend in the solubility diagram of the TMPPT–caffeine system is apparent in Fig. 4. Guttman (18) earlier found that the solubility diagram for the interaction of riboflavin with caffeine was similarly convex. He has explained this tendency on the basis of the dimerization of caffeine (19). When the dimerization of caffeine was taken into consideration for the present system, a straight line was obtained giving $K_{1:1}$ of 19 M^{-1}. In this calculation, equilibria $2C \overset{K_d}{\rightleftharpoons} C_2$ and $T + C \overset{K_{1:1}}{\rightleftharpoons} TC$ were taken into consideration, while the contribution from $2T \rightleftharpoons T_2$ was neglected because of low concentrations of T present, where C = caffeine and T = TMPPT. A value of K_d = 12.7 M^{-1} (19) was used in the calculation. The upward curve in Fig. 3, on the other hand, clearly demonstrates the presence of higher order complexes as well as a 1:1 complex. Stability constants computed from such solubility data are presented in Table I for the complexes formed in each system. A typical example of the iterative procedure used, when applicable, for calculating $K_{1:1}$ and $K_{1:2}$ values is illustrated in Fig. 5 for the interaction of phenazine with 8-methoxycaffeine.

The effects of solvent on molecular interaction were examined for the interaction of TMPPT with *N,N*-dimethylcinnamamide. The results shown in Table II clearly indicate the important part played by water as a solvent. Studies with less polar solvents than those employed, such as carbon tetrachloride and cyclohexane, could not be made because of insolubility of TMPPT in such solvents.

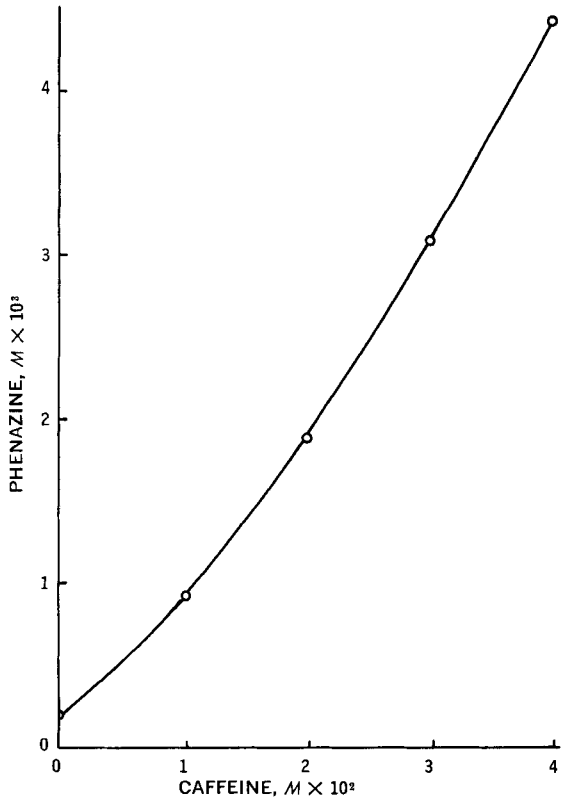
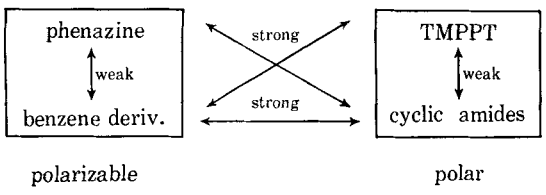


Figure 3—*Solubility diagram of phenazine in the presence of caffeine in water at 25°.*

DISCUSSION

Results shown in Figs. 1–4 and Table I clearly indicate that cyclic amides (alkylxanthines, 1,3-dimethyluracil, and β-hydroxyethylphthalimide) interacted much more strongly with phenazine than with TMPPT, while benzene derivatives (with the exception of benzamides) interacted more strongly with TMPPT than with phenazine. Although benzamides appeared to complex with both phenazine and TMPPT to the same extent, the complexing behavior of other agents toward these model compounds unequivocally supports the view that compounds belonging to the same class (benzene derivatives and phenazine on the one hand and cyclic amide and TMPPT on the other) do not interact very strongly, while those belonging to different classes (cyclic amides and phenazine on the one hand and benzene derivatives and TMPPT on the other) interact strongly in aqueous solutions (13). Phenazine and benzene derivatives may be classified as polarizable since the charge distribution in phenazine and in the benzene ring of the benzene derivatives are expected to be more or less uniform, although the benzene derivatives have net dipole moment. Cyclic amides including TMPPT, on the other hand, are classified as polar because the charge distribution in 1,3-dimethyluracil, the six membered ring of the alkylxanthines, and the maleimide group of β-hydroxyethylphthalimide, are considered to be very much irregular (20). The observed complexing behavior between these compounds may be summarized as in Scheme I.



Scheme I

Though the interaction of TMPPT with phenazine cannot be determined experimentally because of the low solubility of these compounds, the interaction of cyclic amides with benzene derivatives has been reported to be appreciable (16). It should be pointed out here that distinction between polar and polarizable molecules may become obscure when a compound carries both polar and polarizable groups.

Table I—Stability Constants of Phenazine and TMPPT Complexes in Water at 25°

Complexing Agent	—Phenazine— $K_{1:1}$ M^{-1}	$K_{1:2}$ M^{-1}	—TMPPT— $K_{1:1}$ M^{-1}	$K_{1:2}$ M^{-1}
Theophylline	166	24	13	—
Caffeine	314	20	19	—
8-Methoxycaffeine	345	42	21	—
1,3-Dimethyluracil	23	—	2.3	—
β-Hydroxyethylphthalimide	42	27	10	—
Cinnamamide	28	—	69	—
N,N-Dimethylcinnamamide	37	—	44	33
Sodium salicylate	12	—	43	8
Sodium cinnamate	17	—	46	—
Sodium benzoate	4.7	—	8.7	—
Benzoic acid	9.8	—	23	—
Phenol	5.5	—	18	—
Benzamide	11	—	13	—
Anisamide	34	—	31	—
N-Methylbenzamide	15	—	13	—

The present studies concerning the effects of solvent on complex formation (Table II) have quantitatively demonstrated the specific role played by water as a solvent. Since both TMPPT and *N,N*-dimethylcinnamamide have no hydrogen capable of forming hydrogen bonds, the possibility of direct hydrogen bonding between the interactants can be ruled out. The results do not appear to support charge transfer as the major force responsible for the interaction since there seems little tendency for stabilization of the complex in less polar solvents (21).

A valid mechanism for this associative behavior of organic molecules in aqueous media should, therefore, take into account both the selective nature of interaction and the important role of water as the environment which facilitates such interactions. The following hypothesis is proposed in order to give a rationale to the present knowledge of interactions. Water seems to have a role of bringing solute molecules together (hydrophobic bonding). When two solute molecules are brought together, a bonding similar to polarization bonding may become operative and stabilizes the complex. The detailed reasoning on which the hypothesis is based follows.

If one of the direct interactive mechanisms such as hydrogen bonding, charge transfer complexation, or interactions due to orientation forces and induction forces were the primary forces which bind two solutes together, even larger stability constants in less polar solvents than in water would be expected. The authors' results (9) as well as others' (10, 11), however, showed that the extent of interaction is greatest in water, decreasing with increasing

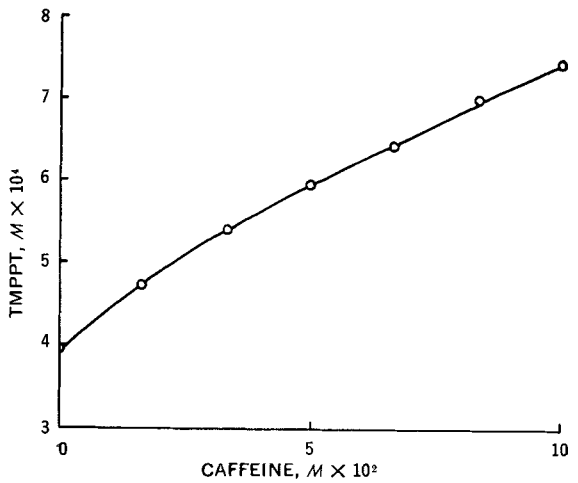


Figure 4—*Solubility diagram of TMPPT in the presence of caffeine in water at 25°.*

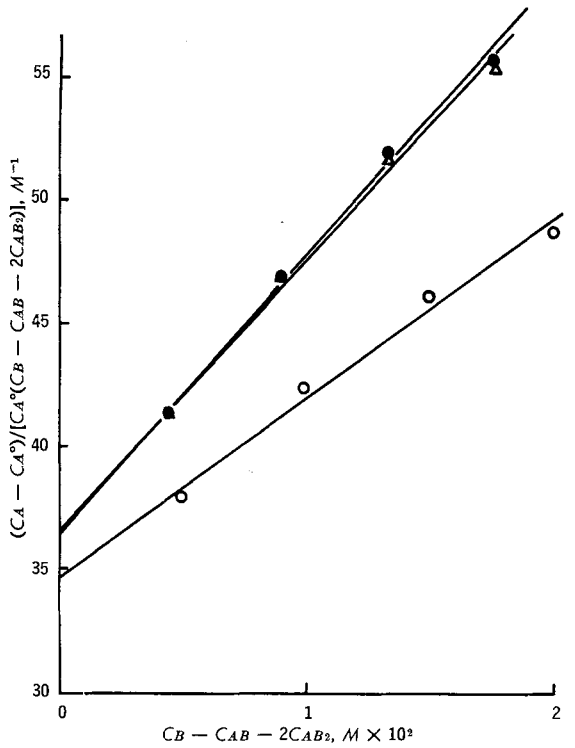


Figure 5—*Analysis of the solubility data for the interaction of phenazine* (A) *with 8-methoxycaffeine* (B) *in water at 25°. Key:* O, *first iteration;* Δ, *second iteration; and* ●, *third and further iterations.*

percentage of polar organic solvents in water–organic solvent mixtures, and it is very small in pure organic solvents. The extent of interactions in various solvents was correlated with the surface free energy of the solvent (10, 22). Sinanoğlu and Abdulnur attributed the main interactive force to the large enthalpy of water (4, 5). Observed results (Table I), however, cannot be rationalized merely by solvophobic bonding. If the bonding were merely due to the squeezing-out property of a solvent, it would not be expected to discriminate among polar and nonpolar molecules to such an extent as was observed since the solvophobic bonding is not affected very much by the nature (polar or nonpolar) of the molecule (5).

The present data cannot be explained by dispersion force alone. The dispersion interaction energy, E, is given by (23)

$$E = -\frac{3}{2r^6} \cdot \frac{I_1 I_2}{I_1 + I_2} \alpha_1 \alpha_2 \qquad \text{(Eq. 10)}$$

where α_i = polarizability of Molecule i, I_i = ionization potential of Molecule i, and r = intermolecular separation. Since the ionization potential varies but little for organic molecules (23), the dispersion energy is mainly affected by polarizability. Then a complexing agent with a large polarizability would bind with both phenazine and TMPPT strongly. This is not the case. Thus forces such as the dispersion force do not seem to be the sole source of stability of the complex in aqueous solution. It is important to note here that the present finding of favorable binding between structurally dissimilar molecules over that between structurally similar molecules is contrary to what is expected on the basis of dispersion interaction (24). The theory provides for a self-recognition of a molecule by an identical molecule. If the dispersion force is responsible for the stability of the complex, the difference in energy, E, between the interaction of Molecule A with Molecule B, A-B, and self-interactions, A-A and B-B, as depicted by Arrangements 1 and 2:

A-A	*A-B*
B-B	*A-B*
Arrangement 1	Arrangement 2

(E in Arrangement 1) − (E in Arrangement 2)

is proportional to

$$-(\alpha_A^2 + \alpha_B^2) - (-2\alpha_A\alpha_B)$$
$$= -(\alpha_A - \alpha_B)^2 \leq 0$$

Then it would be concluded that Arrangement 1 is more energetically stable than Arrangement 2 (24). Again this has been found not to be the case.

Thus a bonding with some degree of selectivity between dissimilar molecules must be sought. Polarization bonding (14) describes weak interactions between polar groups of one component and a polarizable second component in crystalline state. This kind of bonding in a crystalline xanthine complex has recently been postulated by Shefter (25). He also pointed out that in order to propose molecular models for xanthine complexes of pharmaceutical interest, one should take into account polarization interactions. The exact nature of polarization bonding is not known, although it has been proposed that polycyclic hydrocarbon–tetramethyluric acid complexes in the crystalline state (26–28) are due to this bonding. The interactive force seems to be very weak and effective only when the polar and polarizable molecules are in close proximity as in the crystalline lattice. The bonding was not observable in nonpolar solvents such as cyclohexane and benzene (29). It may be speculated, however, that molecular interactions similar to polarization bonding in crystalline complexes may become operative in water when two molecules are brought together by hydrophobic bonding. Polarization bonding, however, has to be distinguished from the classical induction interaction described by (23)

$$E = -\frac{\mu_1^2\alpha_2 + \mu_2^2\alpha_1}{\epsilon^2 r^6} \qquad \text{(Eq. 11)}$$

where μ_i = dipole moment of Molecule i and ϵ = effective value for the dielectric constant of the medium. The formula describes the energy of interaction when the size of a molecule is smaller than the intermolecular distance. Thus the formula is not applicable to the case when the size of the molecule is larger than the intermolecular distance as is the case of complexes of planar molecules in aqueous solution. The most favorable relative orientation of two flat molecules seems to be that of stacking one of the molecules on top of the other (30, 31). A new theoretical development which describes an induction interaction applicable to such a vertically stacked complex of large flat molecules seems to be essential in order to understand theoretically the observed results.

Current works in the authors' laboratories have revealed that cyclic amides employed in the present studies are salted-out by tetramethylammonium chloride while benzene derivatives are salted-in by the same salt (32). Again differences in nature of these molecules in aqueous solution seem to play a part.

Table II—Stability Constants for the TMPPT–*N*,*N*-Dimethylcinnamamide Complex in Several Solvents at 25°

Solvent	Stability Constant, $K_{1:1}$, M^{-1}
Water	44 ($K_{1:2}$ = 33 M^{-1})
Methanol	3.6
Acetone	2.5
Chloroform	2.1
Dioxane	4.6
Benzene	4.7

REFERENCES

(1) I. M. Klotz, *Fed. Proc.*, **24,** s-24 (1965).

(2) R. S. Mullinken, *J. Phys. Chem.*, **56,** 801(1952).

(3) E. M. Kosower, in "Flavins and Flavoproteins," E. C. Slater, Ed., Elsevier Publishing Co., Amsterdam, The Netherlands, 1966, pp. 1–14.

(4) O. Sinanoğlu and S. Abdulnur, *Photochem. Photobiol.*, **3,** 333(1964).

(5) O. Sinanoğlu and S. Abdulnur, *Fed. Proc.*, **24,** s-12(1965).

(6) W. Kauzmann, *Advan. Protein Chem.*, **14,** 1(1959).

(7) G. Némethy and H. A. Scheraga, *J. Phys. Chem.*, **66,** 1773 (1962).

(8) T. Higuchi and J. L. Lach, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 465(1954).

(9) M. Nakano, Ph.D. thesis, University of Wisconsin, 1967; through *Diss. Abstr.*, **28**, 867-B(1967).
(10) R. E. Moser and H. G. Cassidy, *J. Amer. Chem. Soc.*, **87**, 3463(1965).
(11) D. M. Crothers and D. I. Ratner, *Biochemistry*, **7**, 1823 (1968).
(12) K. Kakemi, H. Sezaki, E. Suzuki, and M. Nakano, *Chem. Pharm. Bull. (Tokyo)*, **17**, 242(1969).
(13) T. Higuchi, personal communication, 1967.
(14) S. C. Wallwork, *J. Chem. Soc.*, **1961**, 494.
(15) M. Nakano and T. Higuchi, *J. Pharm. Sci.*, **57**, 183(1968).
(16) T. Higuchi and K. A. Connors, *Advan. Anal. Chem. Instr.*, **4**, 117(1965).
(17) N. I. Nakano, Ph.D. thesis, University of Wisconsin, 1967; through *Diss. Abstr.*, **28**, 971-B(1967).
(18) D. E. Guttman, *J. Pharm. Sci.*, **51**, 1162(1962).
(19) D. E. Guttman and T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 4(1957).
(20) B. Pullman and A. Pullman, "Quantum Biochemistry," Interscience, New York, N. Y., 1963, pp. 678–844.
(21) R. Foster and S. L. Hammick, *J. Chem. Soc.*, **1954**, 2685.
(22) T. Halicioglu and O. Sinanoğlu, cited by O. Sinanoğlu, in "Molecular Association in Biology," B. Pullman, Ed., Academic, New York, N. Y., 1968, pp. 427–445.
(23) B. Pullman, in "Molecular Biophysics," B. Pullman and M. Weissbluth, Eds., Academic, New York, N. Y., 1965, pp. 117–189.
(24) H. Jehle, *Advan. Quantum Chem.*, **2**, 195(1965).
(25) E. Shefter, *J. Pharm. Sci.*, **57**, 350(1968).
(26) A. Damiani, P. De Santis, E. Giglio, A. M. Liquori, R. Puliti, and A. Ripamonti, *Acta Cryst.*, **19**, 340(1965).
(27) A. Damiani, E. Giglio, A. M. Liquori, R. Puliti, and A. Ripamonti, *J. Mol. Biol.*, **20**, 21(1966).
(28) *Ibid.*, **23**, 113(1967).
(29) B. L. Van Duuren, *J. Phys. Chem.*, **68**, 2544(1964).
(30) O. Jardetzky, *Biopolymers Symposia*, **1**, 501(1964).
(31) S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, *J. Amer. Chem. Soc.*, **86**, 4182(1964).
(32) K. Kakemi, H. Sezaki, T. Mitsunaga, and M. Nakano, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 4, 1969, from *Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan.*
Accepted for publication April 30, 1969.
*Present address: Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada
To whom all correspondence should be addressed.

Binding Specificity between Small Organic Solutes in Aqueous Solution: Classification of Some Solutes into Two Groups According to Binding Tendencies

TAKERU HIGUCHI* and HARALD KRISTIANSEN

Abstract □ Experimental data have been obtained which appear to show that the binding between organic species dissolved in water apparently takes place most effectively *between* members of two large, distinct classes of structures, classified arbitrarily as A and B types. Typical examples of Class A are the uncharged alkylxanthines and tetramethylpyrimidopteridinetetrone. Among the compounds in Class B are various benzene derivatives, salicylates, and *trans*-cinnamic acid anions. Many drugs may be included in the present classification system; some examples for which data are available are caffeine, theophylline, and prednisolone in Class A, and phenacetin, promethazine, and menadione in Class B. The complexing tendencies of series of systems involving pairs of interacting organic molecules in aqueous solution were investigated by the phase-solubility technique. Stability constants for some caffeine interactions were evaluated by means of partitioning studies.

Keyphrases □ Organic solute binding specificity—aqueous solution □ Solutes, small organic—binding tendency classification □ Stability constants—solute binding □ Solubility—solute interaction effect □ Spectrophotometric analysis—organic solutes

Water strongly stabilizes a large number of molecular complexes, apart from its participation in hydrophobic bonding (1). Water provides a medium which seems to be unique for the molecular binding tendencies of organic molecules (2–10), many of them of great biological and pharmacological importance. In the last few years a number of papers have reported the properties of pyrimidines, purines, and the important nucleoside and nucleotide polymers in the aqueous environment. It has conclusively been shown, for example, that the bases associate to varying degree in aqueous solution, evidently through plane-to-plane stacking (11–16). It is believed that molecular interactions between adjacent bases in nucleic acid strands to a major extent are responsible for the structural stability of nucleic acids in solution (17–19).

The exact nature of the force or balance of forces operating between the complex components in aqueous solution still is the subject of controversial discussions in the literature. The observed binding between organic molecules in water is, however, firmly believed to be strictly physical in nature. As pointed out earlier (1, 5, 6), the observed intensity of binding cannot be rationalized on the basis of simple charge-transfer-type interactions (the binding constants are extremely low in alcohol, dioxane, and purely nonpolar solvents), dispersion forces (little or no interactive tendency is evident among systems of low polarizability), hydrophobic associations (very small contributions from flexible alkyl side chains), or hydrogen bonding alone. The matter is, of course, complicated by the possible interplay of different interacting forces. The problem is obviously related to the structure of liquid water, which in itself is a very intricate one, and many safe conclusions have not been made so far (20–25).

In this article the authors present their most recent observations carried out on series of systems involving pairs of interacting molecules in aqueous solution. These results have strongly reinforced a growing belief

(9) M. Nakano, Ph.D. thesis, University of Wisconsin, 1967; through *Diss. Abstr.*, **28**, 867-B(1967).
(10) R. E. Moser and H. G. Cassidy, *J. Amer. Chem. Soc.*, **87**, 3463(1965).
(11) D. M. Crothers and D. I. Ratner, *Biochemistry*, **7**, 1823 (1968).
(12) K. Kakemi, H. Sezaki, E. Suzuki, and M. Nakano, *Chem. Pharm. Bull. (Tokyo)*, **17**, 242(1969).
(13) T. Higuchi, personal communication, 1967.
(14) S. C. Wallwork, *J. Chem. Soc.*, **1961**, 494.
(15) M. Nakano and T. Higuchi, *J. Pharm. Sci.*, **57**, 183(1968).
(16) T. Higuchi and K. A. Connors, *Advan. Anal. Chem. Instr.*, **4**, 117(1965).
(17) N. I. Nakano, Ph.D. thesis, University of Wisconsin, 1967; through *Diss. Abstr.*, **28**, 971-B(1967).
(18) D. E. Guttman, *J. Pharm. Sci.*, **51**, 1162(1962).
(19) D. E. Guttman and T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 4(1957).
(20) B. Pullman and A. Pullman, "Quantum Biochemistry," Interscience, New York, N. Y., 1963, pp. 678–844.
(21) R. Foster and S. L. Hammick, *J. Chem. Soc.*, **1954**, 2685.
(22) T. Halicioglu and O. Sinanoğlu, cited by O. Sinanoğlu, in "Molecular Association in Biology," B. Pullman, Ed., Academic, New York, N. Y., 1968, pp. 427–445.
(23) B. Pullman, in "Molecular Biophysics," B. Pullman and M. Weissbluth, Eds., Academic, New York, N. Y., 1965, pp. 117–189.
(24) H. Jehle, *Advan. Quantum Chem.*, **2**, 195(1965).
(25) E. Shefter, *J. Pharm. Sci.*, **57**, 350(1968).
(26) A. Damiani, P. De Santis, E. Giglio, A. M. Liquori, R. Puliti, and A. Ripamonti, *Acta Cryst.*, **19**, 340(1965).
(27) A. Damiani, E. Giglio, A. M. Liquori, R. Puliti, and A. Ripamonti, *J. Mol. Biol.*, **20**, 21(1966).
(28) *Ibid.*, **23**, 113(1967).
(29) B. L. Van Duuren, *J. Phys. Chem.*, **68**, 2544(1964).
(30) O. Jardetzky, *Biopolymers Symposia*, **1**, 501(1964).
(31) S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, *J. Amer. Chem. Soc.*, **86**, 4182(1964).
(32) K. Kakemi, H. Sezaki, T. Mitsunaga, and M. Nakano, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 4, 1969, from *Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan.*
Accepted for publication April 30, 1969.
*Present address: Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada
To whom all correspondence should be addressed.

Binding Specificity between Small Organic Solutes in Aqueous Solution: Classification of Some Solutes into Two Groups According to Binding Tendencies

TAKERU HIGUCHI* and HARALD KRISTIANSEN

Abstract □ Experimental data have been obtained which appear to show that the binding between organic species dissolved in water apparently takes place most effectively *between* members of two large, distinct classes of structures, classified arbitrarily as A and B types. Typical examples of Class A are the uncharged alkylxanthines and tetramethylpyrimidopteridinetetrone. Among the compounds in Class B are various benzene derivatives, salicylates, and *trans*-cinnamic acid anions. Many drugs may be included in the present classification system; some examples for which data are available are caffeine, theophylline, and prednisolone in Class A, and phenacetin, promethazine, and menadione in Class B. The complexing tendencies of series of systems involving pairs of interacting organic molecules in aqueous solution were investigated by the phase-solubility technique. Stability constants for some caffeine interactions were evaluated by means of partitioning studies.

Keyphrases □ Organic solute binding specificity—aqueous solution □ Solutes, small organic—binding tendency classification □ Stability constants—solute binding □ Solubility—solute interaction effect □ Spectrophotometric analysis—organic solutes

Water strongly stabilizes a large number of molecular complexes, apart from its participation in hydrophobic bonding (1). Water provides a medium which seems to be unique for the molecular binding tendencies of organic molecules (2–10), many of them of great biological and pharmacological importance. In the last few years a number of papers have reported the properties of pyrimidines, purines, and the important nucleoside and nucleotide polymers in the aqueous environment. It has conclusively been shown, for example, that the bases associate to varying degree in aqueous solution, evidently through plane-to-plane stacking (11–16). It is believed that molecular interactions between adjacent bases in nucleic acid strands to a major extent are responsible for the structural stability of nucleic acids in solution (17–19).

The exact nature of the force or balance of forces operating between the complex components in aqueous solution still is the subject of controversial discussions in the literature. The observed binding between organic molecules in water is, however, firmly believed to be strictly physical in nature. As pointed out earlier (1, 5, 6), the observed intensity of binding cannot be rationalized on the basis of simple charge-transfer-type interactions (the binding constants are extremely low in alcohol, dioxane, and purely nonpolar solvents), dispersion forces (little or no interactive tendency is evident among systems of low polarizability), hydrophobic associations (very small contributions from flexible alkyl side chains), or hydrogen bonding alone. The matter is, of course, complicated by the possible interplay of different interacting forces. The problem is obviously related to the structure of liquid water, which in itself is a very intricate one, and many safe conclusions have not been made so far (20–25).

In this article the authors present their most recent observations carried out on series of systems involving pairs of interacting molecules in aqueous solution. These results have strongly reinforced a growing belief

that the observed binding in water takes place most effectively *between* members of two large classes of structures. Typical examples of these two apparently natural classes, arbitrarily called Classes A and B, are:

Class A	Class B
Caffeine	Benzoates and salicylates
Theophylline	Cinnamates
Prednisolone	Cinnamamides
Tetramethylpyrimido-pteridinetetrone	Naphthoic acids
	Phenols and naphthols
	Aromatic aminoacids
	Phenacetin
	Menadione
	Tryptophan

Although members of Classes A and B bind noticeably with others within their own class, the strongest interactions seem to be between the two groups.

For the purpose of expressing the extent of interaction between the complexing pairs, in this report the authors have assumed that essentially only 1:1 and 1:2 complexes were formed. The experimental data have been analyzed to yield stability constants for formation of these species. Most of the equilibrium constants were obtained through a systematic investigation using tetramethylpyrimidopteridinetetrone (TMPPT), cinnamamide, and/or *N,N*-dimethylcinnamamide as model compounds. TMPPT has a planar molecular structure similar to the alkylxanthines. The binding behavior of this compound for organic molecules appeared to be very strong, and it was felt that it would provide a useful insight into the general phenomenon of molecular association in water.

EXPERIMENTAL

Materials—Caffeine, theophylline, and phenacetin of USP grade and antipyrine NF were used directly. Caffeine and theophylline were dried at 110° prior to use. *N,N*-Dimethylcinnamamide was synthesized from cinnamoyl chloride and dimethylamine in ether and recrystallized from water–methanol, m.p. 102–103°. 1,3,7,9-Tetramethylpyrimido(5,4-*g*)pteridine-2,4,6,8(1*H*,3*H*,7*H*,7*H*,9*H*)-tetrone (TMPPT),[1] m.p. > 320°, was used usually without further purification.[2] All other compounds used were from commercial sources and were purified by recrystallization from appropriate solvents. Their melting points were found to deviate no more than 2° from literature reports. 7-(2-Dimethylaminoethyl)-theophylline,[3] phenylbutazone (lot SN 34514), and 10-(2-dimethylaminopropyl)phenothiazine hydrochloride (promethazine hydrochloride, control no. F-663110) were used.[4]

All reagents used for buffered solutions and the organic solvents used were of analytical grade. The water was purified by redistillation.

[1] Aldrich Chemical Co.

[2] The material as received was found to contain no significant amount of interfering impurities from analysis of its saturated solution. An appropriately diluted sample of an aqueous solution in equilibrium with a large excess of unpurified material revealed the same spectral characteristics as those found in solutions of the recrystallized substance. Accurately prepared solutions from both untreated and recrystallized material showed identical, highly characteristic spectra over the range 220–400 mμ; λ_{max}. 233 mμ (ε 49,000), 264 (ε 11,875), 271 (ε 12,000), 362 (ε 25,500); λ_{min}. 303 mμ (ε 1500). The equilibrium solubility in water of unpurified and recrystallized material was found to be the same. Since this is a characteristic physical property of a pure compound, just as is the melting point, the unpurified substance was considered to be essentially free of interfering impurities.

Anal.—Untreated material: Calcd. for $C_{12}H_{12}N_8O_4$: C, 47.37; H, 3.98; N, 27.62. Found: C, 47.21; H, 3.87; N, 27.82.

[3] Dimethazan.

[4] Supplied by Endo Laboratories Inc., Geigy Pharmaceuticals, and Wyeth Laboratories Inc., respectively.

Procedure—*Solubility Studies*—The experimental method for most of the systems investigated was the phase-solubility technique, which recently was reviewed in detail (26). An equal amount of the slightly soluble material to be tested, in considerable excess of its normal solubility, was added into each of several 15-ml. screw-cap vials. Increments of a stock solution of the complexing agent (the ligand) were pipeted into the vials, and the solution in each vial was brought to a constant final volume of 10 ml. with the necessary amount of the solvent. The vials were closed, and the screws were securely sealed with parafilm and masking tape.

When the hydroxy-substituted benzoic and cinnamic acids were tested, the vials were closed and sealed under slight nitrogen pressure to prevent oxidation of the acids.

Solubility equilibrium was obtained by tumbling the vials in a water bath thermostated at 25.0 ± 0.1° for at least 48 hr. It was determined that this was sufficient time to ensure solubility equilibrium for all of the substances tested.

When the ligand was the ionized form of an acid (and the solid phase material was a neutral molecule over a wide pH range), a stock solution was prepared *in situ* upon dissolving the acid in 0.1 *M* sodium bicarbonate; pH was adjusted to 8.3 with the necessary amount of sodium hydroxide. The vials were then prepared so that the resulting solution in each also was 0.1 *M* with respect to the bicarbonate. When the ligand was the unionized form of the acid, a 0.005 *M* sulfuric acid solution was used as the solvent to depress ionization of the ligand. A phosphate buffer of pH 6.5 was used for the theophylline (pKa 8.8) systems to ensure that essentially all theophylline was in its neutral form. Stock solutions of the aromatic acids were then made up by adding an equivalent amount of base and phosphate buffer to the desired volume. For some of the acids, pH 6.7–6.8 was maintained in the theophylline systems. For 8-chlorotheophylline, apparent pKa 5.3 (27), in its neutral form, a monochloroacetate buffer of pH 2.9 was used. Theophyllinate and 8-chlorotheophyllinate systems were tested at pH 11.3 and in 0.1 *M* bicarbonate, respectively.

The pKa of phenylbutazone, determined spectrophotometrically in water (25.0°), was found to be 4.56 ± 0.02. A stock solution of the sodium salt was prepared *in situ* by dissolving the compound in a solution containing an equivalent amount of sodium hydroxide.

The pKa of TMPPT, determined by the solubility technique outlined by Albert and Serjeant (28), in hydrochloric acid solutions was found to be −0.55 ± 0.10.

Following equilibration, the content of the vials was filtered through Pyrex sintered-glass filters and analyzed for total solid solubilized.

Analysis—An aliquot of the filtered samples diluted with methanol was determined spectrophotometrically in most instances on a Cary 16 spectrophotometer. (Sometimes a Cary 15 or a Cary 14 instrument was used.) The analytical wavelength chosen for the determination of the solubilized material was one where the ligand did not absorb. In some cases, small absorbances due to the presence of the ligand were subtracted from the observed values.

The dilution of the filtered samples with methanol prior to analysis was consequently carried out for all systems, because the complex formation in this solvent was practically negligible. In this way the complexes formed in water were essentially broken down so that the measured absorbances were due to uncomplexed substances. In water, noticeable spectral perturbations occurred with some of the compounds upon complex formation. In all cases, an exactly known concentration of the analyzed compound in methanol was used as the standard. The maximum water content in the diluted samples was 2%.

When theophylline was the solid phase material, it was separated from the complexing agent by extraction with a mixture of 3 parts chloroform and 1 part isopropyl alcohol and measured spectrophotometrically in this solvent.

Partitioning Studies—Since it has been shown (29) that caffeine undergoes self-association rather markedly in water, a phase diagram with caffeine as the solid phase cannot be unambiguously interpreted. For this reason a partitioning technique was employed so that the binding of caffeine in relative low concentration with some of the ionized aromatic acids could be studied. The experimental operation was essentially the one described by Guttman and Higuchi (29), but the total caffeine concentration was kept constant. The distribution of caffeine between 10% chloroform in isooctane and a 0.1 *M* sodium bicarbonate solution containing varying amounts of the ionized form of the acid was studied at 25.0 ± 0.2°.

Calculation of Equilibrium Constants—*Solubility Method*—Phase diagrams were made by plotting the total molar concentration found in solution of the substance to be tested, S_t, *versus* the total concentration of ligand, L_t. The quantitatve description of the different types of phase diagrams was treated by Higuchi and Connors (26). In the cases where the diagrams indicated formation of only soluble complexes and appeared to be first order with respect to the ligand, the apparent $K_{1:1}$ stability constants were evaluated according to Eq. 1:

$$K_{1:1} = \frac{\text{slope}}{S_0(1 - \text{slope})} \qquad \text{(Eq. 1)}$$

where S_0 is the equilibrium solubility in the absence of L and thus equal to the intercept in the plot. The data were treated by the method of least squares to get the best fit values for slope and intercept. For this purpose, a computer program was used on an Olivetti-Underwood Programma 101.

Methods are available to obtain the individual stability constants from phase diagrams exhibiting a positive curvature (26), and some assumptions usually are made depending upon the extent of complex formation. Therefore, the calculations applied when this type of diagram was observed in the present study will be outlined.

The assumption was made that only two complexes were formed, SL and SL_2,[5] with the stability constants given by:

$$K_{1:1} = \frac{[SL]}{[S][L]} \qquad \text{(Eq. 2)}$$

$$K_{1:2} = \frac{[SL_2]}{[SL][L]} \qquad \text{(Eq. 3)}$$

Concentrations in molar units are represented by the brackets. The mass balance equations are:

$$S_t = [S] + [SL] + [SL_2] \qquad \text{(Eq. 4)}$$

$$L_t = [L] + [SL] + 2[SL_2] \qquad \text{(Eq. 5)}$$

By combining Eqs. 2–4 and since $[S] = S_0$,

$$\frac{S_t - S_0}{[L]} = K_{1:1}S_0 + K_{1:1}K_{1:2}S_0[L] \qquad \text{(Eq. 6)}$$

and a plot of the left-hand term of Eq. 6 *versus* $[L]$ gives both $K_{1:1}$ and $K_{1:2}$ from slope and intercept. However, free ligand concentration, $[L]$, is not known. To obtain the estimate of $[L]$, the first step is to assume that all complexed was in the SL form. Then Eq. 5 reduces to:

$$[L] = L_t - (S_t - S_0) \qquad \text{(Eq. 7)}$$

and Eq. 6 takes the form:

$$\frac{S_t - S_0}{L_t - (S_t - S_0)} = K_{1:1}S_0 + K_{1:1}K_{1:2}S_0\,[L_t - (S_t - S_0)] \qquad \text{(Eq. 8)}$$

The first estimate of $K_{1:1}$ and $K_{1:2}$ came from a plot of the left-hand side of Eq. 8 *versus* $L_t - (S_t - S_0)$.

Combining Eqs. 2–5 and solving for $[L]$,

$$[L] = \frac{-(K_{1:1}S_0 + 1) + \sqrt{(K_{1:1}S_0 + 1)^2 + 8K_{1:1}K_{1:2}S_0L_t}}{4K_{1:1}K_{1:2}S_0} \qquad \text{(Eq. 9)}$$

The quadratic form of Eq. 9 was used in computing the free ligand concentration from the known values of S_t and L_t and the preliminary stability constants obtained from Eq. 8. Thus, the stability constants obtained from the exact Eq. 6 should represent better values than those obtained from Eq. 8. The procedure of getting better $K_{1:1}$ and $K_{1:2}$ values involved repeated computations in using Eqs. 9 and 6 successively, after the initial $K_{1:1}$, $K_{1:2}$, and $[L]$ were calculated from Eqs. 8 and 9.

Convergent values for slope and intercept (constant values for the stability constants) were usually obtained after three to four iterations. The data for the linear plots were treated by a computer-programmed least-squares method, and the calculations of $[L]$ were also programmed.

Partitioning—It was assumed that the ligand, for all practical purposes, was present only in the aqueous phase, since the aromatic acids would be ionized at the pH used in these studies. The free caffeine was distributed between the two solvents. For the interaction of the substance S with the ligand L in the aqueous solution,

$$K_{1:1} = \frac{[SL]}{[S]_{aq.}[L]} \qquad \text{(Eq. 10)}$$

where $[SL]$, $[S]_{aq.}$, and $[L]$ are the molar concentrations of the complex, free S, and L species.

The partition coefficient (PC) was defined as: PC = molar concentration of S in aqueous phase/molar concentration of S in organic phase, or

$$PC = \frac{S_{t,aq.}}{[S]_{org.}} = \frac{S_t - [S]_{org.}}{[S]_{org.}} \qquad \text{(Eq. 11)}$$

where S_t is the total molar concentration of the substance S in the system, and $[S]_{org.}$ is the molar concentration of S in the organic phase. The mass balance equations for the aqueous phase are:

$$S_{t,aq.} = [S]_{aq.} + [SL] \qquad \text{(Eq. 12)}$$

$$L_t = [L] + [SL] \qquad \text{(Eq. 13)}$$

Defining for simplicity,

$$(PC)_0 = \frac{[S]_{aq.}}{[S]_{org.}} \qquad \text{(Eq. 14)}$$

Combining Eqs. 10, 11, and 12,

$$PC = (PC)_0 + K_{1:1}(PC)_0[L] \qquad \text{(Eq. 15)}$$

As a first approximation, the authors set $[L] = L_t$ and got a preliminary estimate of $K_{1:1}$ from slope and intercept by plotting PC against $[L]$ in Eq. 15, $K_{1:1}$ = slope/intercept.

Now, combining Eqs. 10 and 13 with 14,

$$[L] = \frac{L_t}{1 + K_{1:1}(PC)_0[S]_{org.}} \qquad \text{(Eq. 16)}$$

Free ligand concentration could then be calculated from Eq. 16 from the initial estimate of $K_{1:1}$ and the known values of $(S)_{org.}$

Equations 15 and 16 were used successively until convergent values for the slope were obtained (note that the intercept is a constant in the system), *i.e.*, constant values of $[L]$ and $K_{1:1}$. The linear plots were fitted by a programmed least-squares method. Provided only a single 1:1 complex is present in the system under investigation, the final $K_{1:1}$ value should represent the true stability constant.

RESULTS

The stability constants calculated from phase-solubility studies for the binding of a variety of compounds to TMPPT, cinnamamide, and *N,N*-dimethylcinnamamide are listed in Table I. Data for some other interacting systems are given in Table II. Typical increases observed in the solubility of the pyrimidopteridine compound in the presence of benzoate and cinnamate anions are shown in Fig. 1. The solubility of the compound is increased more than 25 times with 0.05 *M* ferulic acid anion. The upward curvature in the plot is also easily recognized, indicating the formation of higher order associations with the added material. Another apparent feature of the TMPPT complexes of this type was their yellow color, which increased in intensity with the number of substituents on the ligand. Unsubstituted *trans*-cinnamate and benzoate did not appear to form colored complexes. Spectrophotometric studies showed a shift toward longer wavelengths of the 362-mμ absorption band of TMPPT in the presence of the cinnamate anions.

In general, the solubility diagrams for TMPPT as the solid phase material were either linear over the whole concentration range of ligand used or showed a positive curvature, as in Fig. 1. In a few instances, notably for *p*-anisidine, 2,6-dihydroxybenzoic acid, 4-

[5] It is not possible to prove that the equilibria occurring in solution are restricted to the formation of only two complexes; neither is it possible to show that these equilibria are actually occurring. Nevertheless, with the assumptions made, it is possible to describe accurately the phase-solubility diagrams and thus express the extent of complexation.

Table I—Stability Constants (liters/mole) for the Interaction of Various Compounds with TMPPT and Cinnamamides in Water at 25°

Ligand	TMPPT	Cinnamamide	*N,N*-Dimethylcinnamamide
1 Caffeine	11.9	37.5	37.6
2 Theophylline	12.8	27.3	27.5
3 8-Methoxycaffeine	19.1	48.5	54.0
4 8-Chlorotheophylline	18.5	39.3	
5 Theophylline-7-acetic acid	5.0	22.7	
6 Theophylline-7-acetate	5.9	20.0	17.3
7 Phenylbutazone, sodium salt	6.0	5.0	
8 Antipyrine	1.9	3.6	
9 Imidazole	2.3	0.8	
10 Theophyllinate	56.2 (7.1)[a]	14.3	
11 8-Chlorotheophyllinate	154.1 (11.6)	25.8	
12 Phenacetin	43.3	8.0	6.8
13 Phenol	13.1 (5.7)	1.7	
14 4-Chlorophenol	29.2	2.7	
15 Sodium sorbate	4.2	1.4	
16 *p*-Anisidine	14.9[b]	2.4	
17 10-(2-Dimethylaminopropyl) phenothiazine hydrochloride	65.5 (2.7)	11.5	
18 7-(2-Dimethylaminoethyl) theophylline	10.0		31.0
19 β-Hydroxyethyltheophylline	7.8		27.3
20 Sodium salicylate	44.3 (4.7)		1.9
21 2,6-Dihydroxybenzoate	102.1 (37.1)		5.9
22 2,6-Dihydroxybenzoic acid	96.6[b]		6.1
23 Nicotinamide	5.5		3.9
24 4-Hydroxycoumarin	99.0[b]		10.8

[a] The numbers in parentheses are the 1:2 stability constants for TMPPT–ligand, also expressed in l./mole. [b] Calculated from initial increase in solubility of TMPPT. An insoluble complex is also formed.

hydroxycoumarin, and 3-methoxy-4-hydroxymandelic acid anion, there was an initial linear increase in the solubility of TMPPT with ligand concentration, followed by a plateau region where precipitation of an insoluble complex occurred. The slopes were always considerably less than one for the diagrams or portions of them exhibiting increasing linear behavior or at any point in the diagrams showing curvature. The calculation of stability constants from the diagrams where a linear increase in the solubility of TMPPT was observed was based on the assumption that a single 1:1 complex was formed. Both 1:1 and 1:2 stability constants were evaluated from the phase diagrams showing positive curvature. Studies of the partitioning of TMPPT between water and an organic phase composed of 25% chloroform in isooctane (v/v) indicated that this compound did not self-associate to a detectable extent in water. This observation was given the interpretation that higher order associations of the compound should not interfere with the evaluation of reliable stability constants from the phase-solubility diagrams.

The interactions of the unionized form of *trans*-cinnamic, caffeic, and ferulic acid with TMPPT gave insoluble complexes. These interactions were not investigated further since the authors were primarily interested in the associations taking place in solution. The formation of insoluble complexes has also been found for theophylline interactions with a series of unionized benzoic acids (5).

The 1:1 stability constants in Table II for the interaction of caffeine with some of the ionized aromatic acids require some additional explanation. The calculated constants from the partitioning data were dependent upon the total caffeine concentration. To get the most reliable 1:1 constants for these systems, several experiments were carried out with different caffeine concentrations in

Table II—Stability Constants for the Interaction of Some Organic Molecules in Water at 25°

Interacting System	$K_{1:1}$ l./mole	$K_{1:2}$ l./mole
TMPPT–sodium benzoate	9.2	
TMPPT–*trans*-cinnamic acid anion	40.7	10.4
TMPPT–*d,l*-mandelic acid anion	4.2	
TMPPT–mandelamide	2.8	
TMPPT–5-phenyl-2,4-pentadienoic acid anion	128	25.9
TMPPT–*p*-aminohippuric acid anion	29.2	
TMPPT–*p*-coumaric acid anion	106	26.1
TMPPT–3-methoxy-4-hydroxybenzoic acid anion	50.5	14.6
TMPPT–3-methoxy-4-hydroxymandelic acid anion	139[a]	
TMPPT–caffeic acid anion	202	75
TMPPT–ferulic acid anion	228	85
TMPPT–cinnamamide	70.0	
TMPPT–*N,N*-dimethylcinnamamide	57.4	
TMPPT–β-naphthaleneacetic acid anion	141	16.0
TMPPT–4-methoxyphenylacetic acid anion	10.9	1.7
TMPPT–3-(*p*-methoxyphenyl)propionic acid anion	14.1	3.6
TMPPT–*N,N*-dimethyl-*p*-anisidine	8.2	
Caffeine–3-methoxy-4-hydroxybenzoic acid anion	17.8	
Caffeine–ferulic acid anion	46.0	
Phenacetin–caffeine	17.0	
Theophylline–caffeic acid anion	39.5	
Theophylline–2,6-dihydroxybenzoic acid anion	300	
Cinnamamide–ferulic acid anion	8.0	
N,N-Dimethylcinnamamide–caffeic acid anion	7.2	

[a] See *Footnote* [b] in Table I.

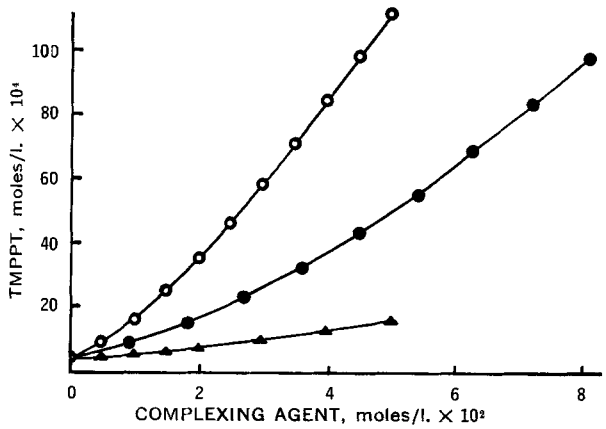


Figure 1—*Increase in solubility of TMPPT produced by addition of ferulic acid anion* (○), *2,6-dihydroxybenzoate* (●), *and trans-cinnamate* (▲) *in 0.1 M sodium bicarbonate at 25°.*

each. Figure 2 shows the data for the interaction of caffeine with ferulic acid anion with the apparent $K_{1:1}$ constants, calculated as outlined in the *Experimental* section, plotted against the caffeine concentration. The increase in stability constants with increasing caffeine concentration strongly suggests that higher order caffeine complexes are formed. This is not surprising, because caffeine has been found to exist in aqueous solution as monomer, dimer, and tetramer (29), and all the caffeine species might possibly associate with the aromatic anion. This was supported with data from the solubility technique, which showed that one molecule of the anion brought close to two molecules of caffeine into solution. When the linear plot in Fig. 2 was extrapolated to zero caffeine concentration, the value of 46.0 for the $K_{1:1}$ constant was believed to represent the best "true" constant.

When the two cinnamamides were used as solid phase material in the solubility technique, only a linear increase in the solubility was observed. The slopes of the solubility diagrams were in every instance less than one. Partitioning studies between water and 10% chloroform in isooctane (v/v) indicated that self-association of cinnamamide and *N,N*-dimethylcinnamamide in the aqueous phase did not occur. The calculation of stability constants from the phase diagrams was made assuming formation of a single 1:1 complex in the systems.

Studies were attempted with vitamin A acid anion and phenothiazine as complexing agents, but they did not lead to definitive results. The salt form of vitamin A acid apparently formed micelles at very low concentrations, and phenothiazine was unstable in solution.

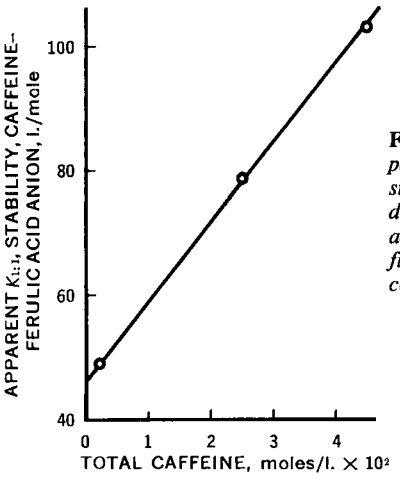


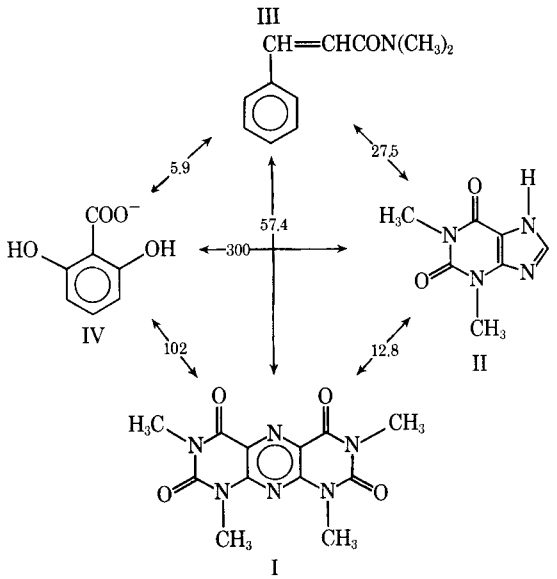
Figure 2—*Plot of apparent* $K_{1:1}$ *stability constants from partitioning data of the caffeine–ferulic acid anion system as a function of total caffeine concentration at 25°.*

DISCUSSION

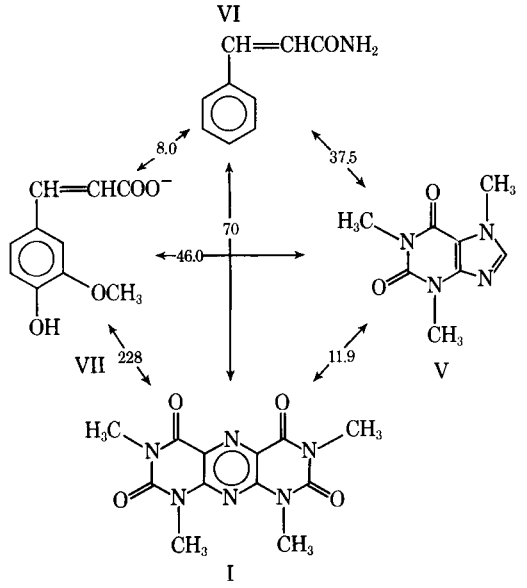
Earlier studies indicated that there is relatively little specificity in the type of interactions under present consideration. For example, if the stability constants of complexes formed by Compound 1 with a series of other species were plotted against the stability constants derived using Compound 2 for the same series of interactants, it has been shown that the points fall essentially on a straight line passing through the origin (6, 7). The present investigation strongly suggests, however, that there are at least two broad classes, labeled here as Class A and Class B. The data collected indicate that although members of Class A and Class B will interact with others within their own class, the strongest binding seems to be between the two groups.

Thus, for example, TMPPT and theophylline have been classified as being in Class A. *N,N*-Dimethylcinnamamide and γ-resorcylic acid anion are considered to be in Class B. As listed in Table I, the former two interact weakly, K equal to 12.8 l./mole, with each other; the latter pair interact even more weakly with K less than 6 l./mole. The four constants for the crossinteractions are substantially larger, being 27.5, 57.4, 102, and 300 l./mole. This is shown diagrammatically in Scheme I. The same trend is revealed by examining Scheme II, where the TMPPT–caffeine system represents the A–A interaction with K equal to 11.9 l./mole. The cinnamamide–ferulic acid anion system, with a K of 8.0 l./mole, is the B–B interaction. The four possible A–B interactions have 1:1 stability constants of 37.5, 46.0, 70, and 228 l./mole.

The possible existence of such a generic difference in the binding behaviors of these compounds is strongly supported by the plots shown in Figs. 3 and 4. Here the stability constants listed in Table I for the interactions of the various complexing agents with cinnamamide (Class B) and *N,N*-dimethylcinnamamide (Class B) have been compared with those for the interactions of the same compounds with TMPPT (Class A). Both Figs. 3 and 4 show that all the compounds fall readily in two groups, those binding the cinnamamides strongly and TMPPT weakly, and those characterized by the opposite binding tendencies. Thus, these plots demonstrate that a large number of organic compounds may be classified as belonging to either of the two categories, A or B. Such a classification is apparently valid among the compounds tested that showed relatively strong binding tendencies. However, the classification may, of course, be somewhat obscure for compounds that tended to bind rather weakly with any interactant. Examples of the latter type



Scheme I—A–B system interactions in water at 25°. The numbers on the arrows represent the 1:1 stability constants for the interacting species. I, TMPPT; II, theophylline; III, N,N-*dimethylcinnamamide; and IV,* 2,6-*dihydroxybenzoate*



Scheme II—A–B system interactions in water at 25°. The numbers on the arrows represent the 1:1 stability constants for the interacting species. I, TMPPT; V, caffeine; VI, cinnamamide; and VII, ferulic acid anion

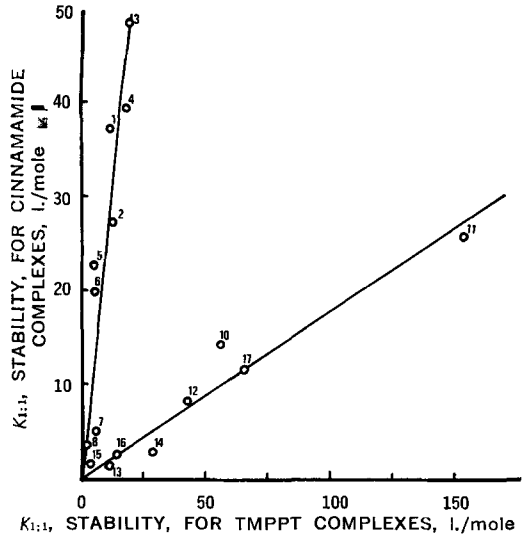


Figure 3—*A plot of the 1:1 stability constants for cinnamamide and TMPPT with each of the indicated compounds. The numbers refer to the compounds listed in Table I.*

from the present data are phenylbutazone, antipyrine, imidazole, sodium sorbate, and nicotinamide.

From Figs. 3 and 4, it is seen that the apparent two classes of compounds fall essentially on their own straight line passing through the origin in the plots, thus indicating a reasonable linear free energy relationship for the A–B interactions. The rationale for this type of plot is that the difference in the free energy ($\delta\Delta G°$) for the binding of one class of compounds to TMPPT compared to the cinnamamides is expressed by the slope. That a linear free energy relationship exists does not, of course, imply that a single binding mechanism is operating among the two categories of interactants. It has been found that simple linear free energy relationships are not necessarily limited to closely related reactions but might apply to entire groups of reactions (30). The free energy changes involved in these systems, in general, are small. From the slopes of the lines drawn in Figs. 3 and 4, it was calculated that the binding of Class A compounds to the cinnamamides (Class B) was favored by about 600 cal./mole compared to the binding of the same class to TMPPT (Class A). For the binding of Class B compounds, TMPPT was favored over the cinnamamides by 1000–1500 cal./mole.

The present data suggest that all the investigated neutral xanthines and the pyrimidopteridine compounds constitute the same class, Class A. Examination of previously reported stability constants for prednisolone and hydrocortisone interactions (5, 6) indicates that these compounds also apparently may be classified as being in the same A category. Class B consists of a large number of aromatic compounds. Apparently all benzoate derivatives, phenols, naphthols, cinnamates, and naphthoates, for which data are available, form much stronger complexing pairs with the Class A compounds than with members of their own group. In Class B the cinnamates appear to be among the strongest binders. Since molecular complexation is believed to be a possible mechanism for drug–receptor interactions *in vivo*, it is interesting to note that drugs like phenacetin and salicylate, according to the present classification system, are typical B compounds. The observed binding of the phenothiazine derivative (promethazine hydrochloride) that was tested indicated that it apparently fit into the same Class B. The strong binding to TMPPT of the biologically important metabolite 3-methoxy-4-hydroxymandelic acid anion (K equal to 139 l./mole) showed that it most likely belongs to Class B. The stability constants reported for the interaction of menadione and tryptophan with some alkylxanthines (7, 31) further suggest that both these compounds may be members of Class B.

Some insight into factors that confer characteristics of A and B compounds can be gained by correlating the data for the theophylline systems. The ionized form of theophylline and 8-chlorotheophylline bind TMPPT stronger than cinnamamide, whereas the reverse is true for the uncharged form of these xanthines. While the neutral xanthines were labeled Class A compounds, Fig. 3 shows that their ionized forms fit nicely into Class B. Recently, it was also shown that theophyllinate binds stronger to 8-methoxycaffeine than its neutral form (32), and neutral theophylline had a stronger affinity for some cinnamate esters than theophyllinate (10). For a long time it has been assumed, and in some cases confirmed by experiment (5, 8, 32), that the stronger solvation around the ions compared to the neutral molecules should decrease the complexing capability in water. The theophyllinate behavior cannot be explained in this way. Although the binding with cinnamamide is reduced, theophyllinate and 8-chlorotheophyllinate bind TMPPT markedly stronger than do the corresponding neutral forms. The 1:1 stability constants were found to be 12.8 and 18.5 l./mole for the interaction with theophylline and 8-chlorotheophylline, respectively, compared to 56 l./mole for the binding of TMPPT to theophyllinate and 154 l./mole for the interaction with 8-chlorotheophyllinate. On the other hand, data for the neutral and ionized forms of theophylline-7-acetic acid show that when the negative charge is adjacent but isolated from the imidazole ring in theophylline, there is no significant change in the binding strength with either cinnamamide or TMPPT. The neutral and ionized molecules interacted with cinnamamide to give K equal to 22.7 and 20.0 l./mole, respectively. The corresponding constants for the interaction with TMPPT were 5.0 and 5.9 l./mole.

An important factor regarding the observed behavior of the theophyllinates appears to be the great change in the resonance structures of both theophylline and 8-chlorotheophylline upon formation of their anions. In theophylline-7-acetic acid, the resonance character of the heterocyclic ring system of the molecule cannot be affected by ionization of the carboxylic group. The binding of the theophyllinates, in general, resembled the binding of the

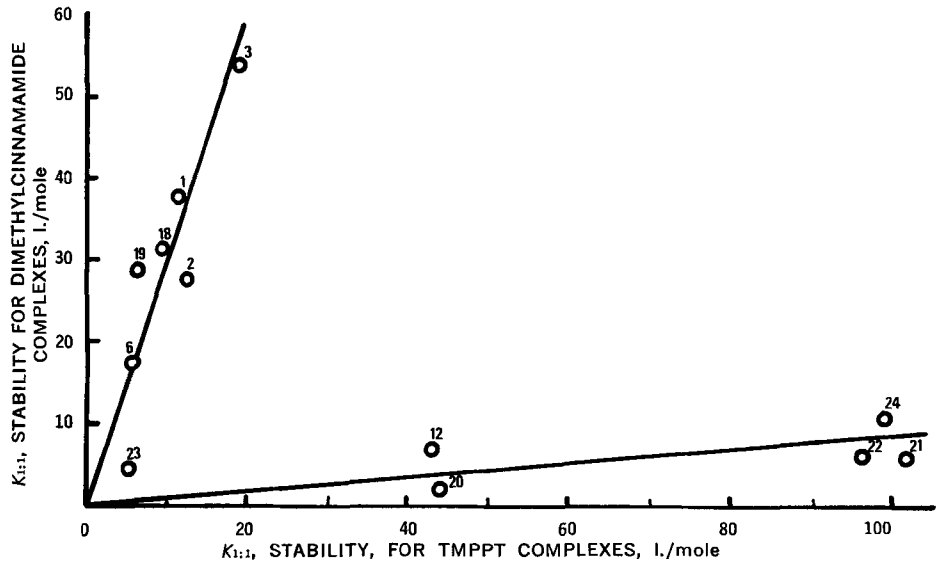


Figure 4—*A plot of the 1:1 stability constants for* N,N-*dimethylcinnamamide and TMPPT with each of the indicated compounds. The numbers refer to the compounds listed in Table I.*

benzoates and the cinnamates with TMPPT. All these ligands gave yellow, soluble complexes with TMPPT, and the solubility diagrams were all characterized by an upward curvature. The neutral alkylxanthines, on the other hand, interacted in a first-order manner with TMPPT, and no colored complexes were formed.

Since the difference in electronic character of the two forms of theophylline crudely appears to be responsible for their differences in binding with the A–B system compounds, one is tempted to speculate if this reflects any important property which would clearly distinguish an A from a B compound. Still there are contradicting opinions about the electronic properties of, for example, the purine derivatives. Although Pullman and Pullman (33) found from MO calculations that a correlation existed between the electron donating properties of the purines and their solubilizing effect on hydrocarbons (33), the influence of the medium was not taken into account. Hanna and Sandoval (34) carried out NMR studies of caffeine in complexes with benzene and mesitylene in carbon tetrachloride, and they concluded that caffeine acts as a typical acceptor in these complexes. This is, of course, contrary to the predictions made from MO theory. It is evident that the effect of the solvent environment on the extent of complex formation has to be taken into account (1). In attempts to rationalize the observed data, many problems arise because knowledge about this refinement is far from complete.

The interacting species listed in Tables I and II represent structurally a spectrum of different planar aromatic and heterocyclic molecules. Both neutral and ionized species have been tested. The substituents on the compounds are differently oriented, and some would be expected to exert an electron-withdrawing effect while others should be donating electrons and, in any case, affect the π-orbitals of the aromatic and heterocyclic ring systems. The variations in structure appear to be pronounced within each of the proposed two classes of interacting species. In general, therefore, it seems impossible to ascribe the interactions to any localized binding site. The binding of one class of compounds to a member of the other class must be mainly nonspecific in nature. The same reasoning is applicable to the observed weak binding between members of the same class. However, since the collected data clearly demonstrated that there are at least two broad classes of compounds, some selectivity must be operating among the interactants. In other words, a particular molecule must be able to recognize an A from a B compound. This property of the interactants seems to eliminate the important contribution to the stability of the complexes due to dispersion forces. The dispersion energy of attraction can be expressed in terms of a single parameter, the product of the polarizabilities of the interacting molecules (35, 36). Thus, if dispersion forces were important for the binding in water, one would not expect to see the observed selectivity since all the molecules would be more or less "blind."

At the present time, the authors believe that charge–transfer complexes of the formal type (37) are not a major factor in stabilizing these complexes in water. Experiments have shown that the binding in these systems is considerably reduced in media less polar than water (1), and this is the opposite effect of what should be expected if charge–transfer was a major factor (38). But the possibility cannot be excluded that a mechanism of nonclassical "donor–acceptor" type, peculiar to water, may be operating in the interactions being considered. It is clear that factual information about the role of water in these interactions is lacking. This may be the main reason why no satisfactory explanation has emerged as to the exact nature of the forces involved, although numerous complex interactions in aqueous media have been reported.

The complex geometry of the systems investigated cannot be described with great assurance since they exist only in solution in equilibrium with the uncomplexed components. With all the possible solute–solute, solvent–solvent, and solute–solvent interactions taking place simultaneously in aqueous solution, any detailed structural or mechanistic interpretation can only be speculative and may involve assumptions that are far from safe. However, some progress in understanding the spatial arrangement of complexes of organic molecules in aqueous solution is evident from previous studies. In general, it has been shown that planarity of the interacting molecules is important (5–7), and it has been observed that expansion of the ring system from a benzenoid to the naphthalene structure led to substantial increased binding with, for example, theophylline (5, 6). Recently, it was reported that a reasonable linear correlation existed between the standard unitary free energy change and the estimated planar area of the smaller neutral interactants for theophylline complexes with a series of cinnamate esters and related compounds, and it was postulated that the 1:1 complexes were most likely of a plane-to-plane orientation (10). NMR measurements of the tryptophan–caffeine complex have suggested that the geometry apparently corresponded to plane-to-plane stacking (7). The effect of increased planar area upon the complex stability is substantially confirmed by the present study. For example, by increasing the planar surface from that of a typical alkylxanthine like caffeine or theophylline to the three-membered heterocyclic ring system in the pyrimidopteridine compound, the more favorable is the observed binding in water. The coplanarity of the benzene ring with the ethylenic bond and the carboxylate in the series of cinnamic acid anions increases the planar surface area of these molecules compared to the benzoates and other benzene derivatives. It is evident from the present data that the cinnamic acid anions, in in general, are much stronger binders than the latter type of compounds, notably toward the Class A compounds. However, it cannot be ascertained at the present time if the strong binding tendency of the cinnamates is due to the increase in surface area or to the extended conjugation with increased π-electron delocalization in the molecular system. It may be suggested that a combination of both the effects contributes favorably to the observed binding.

Since the strongest binding, in general, is seen with compounds that are polycyclic in nature (of which there are examples of both Class A and Class B compounds in the present study) or contain extended conjugation, the view that planar area overlap of the partners in the complex takes place has considerable appeal. But it is not possible to make any conclusions, which at this time may be more than speculative, regarding the preferred mutual orientation of the components in these complexes in solution. Again it is felt that the role of water may be essential for the modes of orientation.

REFERENCES

(1) H. Kristiansen, M. Nakano, N. I. Nakano, and T. Higuchi, to be published.

(2) T. Higuchi and D. A. Zuck, *J. Amer. Pharm. Ass., Sci. Ed.*, **41**, 10(1952).

(3) *Ibid.*, **42**, 132, 138(1953).

(4) T. Higuchi and J. L. Lach, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 349, 524, 527(1954).

(5) T. Higuchi and A. Drubulis, *J. Pharm. Sci.*, **50**, 905(1961).

(6) T. Higuchi and F. D. Pisano, *ibid.*, **53**, 644(1964).

(7) M. Nakano, Ph.D. thesis, University of Wisconsin, 1967; through *Diss. Abstr.*, **28**, 867-B(1967).

(8) J. A. Mollica and K. A. Connors, *J. Amer. Chem. Soc.*, **89**, 308(1967).

(9) P. A. Kramer and K. A. Connors, *ibid.*, **91**, 2600(1969).

(10) K. A. Connors, M. H. Infeld, and B. J. Kline, *ibid.*, **91**, 3597 (1969).

(11) P. O. P. Ts'o, I. S. Melvin, and C. Olson, *ibid.*, **85**, 1289 (1963).

(12) P. O. P. Ts'o and S. I. Chan, *ibid.*, **86**, 4176(1964).

(13) S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, *ibid.*, **86**, 4182(1964).

(14) M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, *ibid.*, **87**, 5241(1965).

(15) A. D. Broom, M. P. Schweizer, and P. O. P. Ts'o, *ibid.*, **89**, 3612(1967).

(16) M. P. Schweizer, A. D. Broom, P. O. P. Ts'o, and D. P. Hollis, *ibid.*, **90**, 1042(1968).

(17) H. DeVoe and I. Tinoco, Jr., *J. Mol. Biol.*, **4**, 500(1962).

(18) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic, New York, N. Y., 1963, chap. 8.

(19) G. Felsenfeld and H. T. Miles, *Ann. Rev. Biochem.*, **36**, 407 (1967).

(20) H. S. Frank, *Fed. Proc., Suppl. 15*, **24**, S-1(1965).

(21) F. Franks, *Chem. Ind. London*, **1968**, 560.

(22) K. W. Miller and J. H. Hildebrand, *J. Amer. Chem. Soc.*, **90**, 3001(1968).

(23) G. Némethy, *Fed. Proc., Suppl. 15*, **24**, S-38(1965).

(24) J. L. Kavanau, "Water and Solute–Water Interactions," Holden-Day, San Francisco, Calif., 1964.

(25) D. Eisenberg and W. Kauzmann, "The Structure and Properties of Water," Oxford University Press, New York, N. Y., and Oxford, England, 1969.

(26) T. Higuchi and K. A. Connors, *Advan. Anal. Chem. Instr.*, **4,** 117(1965).
(27) W. E. Hall, Ph.D. thesis, University of Wisconsin, 1966.
(28) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen & Co., Ltd., London, England, 1962.
(29) D. Guttman and T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **46,** 4(1957).
(30) J. E. Leffler and E. Grunwald, "Rates and Equilibria of Organic Reactions," Wiley, New York, N. Y., 1963, chaps. 7 and 8.
(31) M. Nakano and T. Higuchi, *J. Pharm. Sci.*, **57,** 1865(1968).
(32) K. Kakemi, H. Sezaki, T. Mitsunaga, and M. Nakano, *Chem. Pharm. Bull.*, **16,** 2018(1968).
(33) B. Pullman and A. Pullman, "Quantum Biochemistry," Wiley, New York, N. Y., 1964.
(34) N. W. Hanna and A. Sandoval, *Biochim. Biophys. Acta*, **155,** 433(1968).
(35) H. Jehle, *Advan. Quant. Chem.*, **2,** 195(1965).
(36) J. O. Hirschfelder, in "Molecular Biophysics," Academic, New York, N. Y., 1965, p. 325.
(37) R. S. Mulliken and W. B. Person, *Ann. Rev. Phys. Chem.*, **13,** 107(1962).
(38) G. Briegleb, "Elektronen-Donator-Acceptor-Komplexe," Springer-Verlag, Berlin, Germany, 1961.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 13, 1970, from the *Department of Analytical Pharmaceutical Chemistry and Pharmaceutics, School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication June 2, 1970.

This investigation was supported in part by Grant GM-05830 from the National Institutes of Health.

H. K. gratefully acknowledges partial financial support from the Norwegian Research Council for Science and the Humanities.

* To whom all correspondence should be addressed.

Alkaloids of Tylophora II: Structural Studies

KOPPAKA V. RAO

Abstract □ Structural studies on the six alkaloids isolated from *Tylophora crebriflora* (N. O. Asclepiadaceae) are described here. Spectral data indicate that five of these alkaloids (A–E) possess the dibenzo[*f,h*]-pyrrolo[1,2*b*]isoquinoline skeleton known to be present in tylocrebrine. They differ in the number, nature, and distribution of the oxygen-bearing substituents and in the presence or absence of a benzylic-type hydroxyl. An oxygen substitution pattern of 3, 4, 6, and 7 is suggested for Alkaloids A, B, and C and that of 2, 3, 4, 6, and 7 for Alkaloids D and E. Alkaloid F is shown to be a seco analog of tylocrebrine with a 1,2-diphenyl *cis* stilbene skeleton.

Keyphrases □ Alkaloids—*Tylophora crebriflora* □ Structural studies—*T. crebriflora* alkaloids □ NMR spectroscopy—structure □ IR spectrophotometry—structure □ UV spectrophotometry—structure

The isolation of six new alkaloids designated as A, B, C, D, E, and F, together with the known compounds tylocrebrine and tylophorine from *Tylophora crebriflora*, S. T. Blake (N. O. Asclepiadaceae), was described in Part I (1). An examination of the analytical and spectral data indicated that Compounds A–E resemble tylocrebrine (I) in that they possess the dibenzo[*f,h*]-pyrrolo[1,2*b*]isoquinoline skeleton with four or five oxygen-bearing substituents. The studies that provided evidence for the structures of these new members are described in this paper.

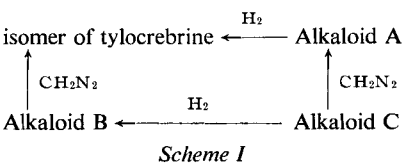
DISCUSSION

Alkaloid A, $C_{24}H_{27}NO_5$, resembles tylocrebrine in its UV and IR spectra and in having four methoxyl groups. The extra oxygen atom is in the form of a hydroxyl group, as shown by the formation of a monoacetate (bands at 1725 and 1225 cm.$^{-1}$ in IR and a sharp 3-proton peak at τ 7.85 in the NMR spectrum). Clemmensen reduction or catalytic hydrogenation converts Alkaloid A to a nonhydroxylic compound $C_{24}H_{27}NO_4$, thus indicating that the hydroxyl is benzylic. In its spectral and chromatographic behavior, the reduction product is almost indistinguishable from tylocrebrine, but the mixed melting (decomposition) point seems to indicate that the two may not be identical.

The presence of the benzylic hydroxyl in Alkaloid A is analogous to the case of tylophorinine (II, R = OH), another known member of this group (2). In this compound, the hydroxyl was placed at 14 instead of 9 (for numbering, see Structure I), because the latter structure would have represented a highly labile carbinolamine system and the stability of the compound was inconsistent with such a structure (2). This was later confirmed by synthesis (3). In an analogous manner, the stability of Alkaloid A strongly suggests the location of the hydroxyl to be 14. This is also supported by the following NMR spectral evidence: the chemical shifts of the benzylic CH(OH) protons in tylophorinine and Alkaloid A are very close: τ 3.96 and 3.87, respectively. The corresponding chemical shift of the same proton in the acetates of both compounds is τ 3.48; in both cases, it is split as a doublet, as would be expected. It is, therefore, concluded that Alkaloid A has the hydroxyl at 14.

Alkaloid B, $C_{23}H_{25}NO_4$, has three methoxyl groups. The fourth oxygen is part of a phenolic group (UV spectral shift in base and band at 3540 cm.$^{-1}$). This is supported further by the formation of a monoacetate (1750 cm.$^{-1}$ in IR and a 3-proton peak at τ 7.59). Methylation with diazomethane leads to a tetramethoxy compound, $C_{24}H_{27}NO_4$, which is almost indistinguishable from tylocrebrine on the basis of spectral and chromatographic data, but the mixed melting (decomposition) point suggests that the two may not be identical.

Alkaloid C, $C_{23}H_{25}NO_5$, shows features similar to both A and B. It has three methoxyls and forms a diacetate (1725 and 1760 cm.$^{-1}$ in IR and 3-proton peaks at τ 7.87 and 7.59 for the alcoholic acetate and phenolic acetate groups, respectively). It can be converted by methylation to Alkaloid A and by Clemmensen reduction to B. Hence, this compound is the desmethyl derivative of Alkaloid A, and the position of the phenolic hydroxyl in B and C is the same. The transformations are shown in Scheme I.



Scheme I

Alkaloids D and E have the compositions $C_{25}H_{29}NO_6$ and $C_{25}H_{29}NO_5$, respectively. They each have five methoxyl groups. Clem-

(26) T. Higuchi and K. A. Connors, *Advan. Anal. Chem. Instr.*, **4,** 117(1965).
(27) W. E. Hall, Ph.D. thesis, University of Wisconsin, 1966.
(28) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen & Co., Ltd., London, England, 1962.
(29) D. Guttman and T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **46,** 4(1957).
(30) J. E. Leffler and E. Grunwald, "Rates and Equilibria of Organic Reactions," Wiley, New York, N. Y., 1963, chaps. 7 and 8.
(31) M. Nakano and T. Higuchi, *J. Pharm. Sci.*, **57,** 1865(1968).
(32) K. Kakemi, H. Sezaki, T. Mitsunaga, and M. Nakano, *Chem. Pharm. Bull.*, **16,** 2018(1968).
(33) B. Pullman and A. Pullman, "Quantum Biochemistry," Wiley, New York, N. Y., 1964.
(34) N. W. Hanna and A. Sandoval, *Biochim. Biophys. Acta*, **155,** 433(1968).
(35) H. Jehle, *Advan. Quant. Chem.*, **2,** 195(1965).
(36) J. O. Hirschfelder, in "Molecular Biophysics," Academic, New York, N. Y., 1965, p. 325.
(37) R. S. Mulliken and W. B. Person, *Ann. Rev. Phys. Chem.*, **13,** 107(1962).
(38) G. Briegleb, "Elektronen-Donator-Acceptor-Komplexe," Springer-Verlag, Berlin, Germany, 1961.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 13, 1970, from the *Department of Analytical Pharmaceutical Chemistry and Pharmaceutics, School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication June 2, 1970.

This investigation was supported in part by Grant GM-05830 from the National Institutes of Health.

H. K. gratefully acknowledges partial financial support from the Norwegian Research Council for Science and the Humanities.

* To whom all correspondence should be addressed.

Alkaloids of Tylophora II: Structural Studies

KOPPAKA V. RAO

Abstract □ Structural studies on the six alkaloids isolated from *Tylophora crebriflora* (N. O. Asclepiadaceae) are described here. Spectral data indicate that five of these alkaloids (A–E) possess the dibenzo[*f,h*]-pyrrolo[1,2*b*]isoquinoline skeleton known to be present in tylocrebrine. They differ in the number, nature, and distribution of the oxygen-bearing substituents and in the presence or absence of a benzylic-type hydroxyl. An oxygen substitution pattern of 3, 4, 6, and 7 is suggested for Alkaloids A, B, and C and that of 2, 3, 4, 6, and 7 for Alkaloids D and E. Alkaloid F is shown to be a seco analog of tylocrebrine with a 1,2-diphenyl *cis* stilbene skeleton.

Keyphrases □ Alkaloids—*Tylophora crebriflora* □ Structural studies—*T. crebriflora* alkaloids □ NMR spectroscopy—structure □ IR spectrophotometry—structure □ UV spectrophotometry—structure

The isolation of six new alkaloids designated as A, B, C, D, E, and F, together with the known compounds tylocrebrine and tylophorine from *Tylophora crebriflora*, S. T. Blake (N. O. Asclepiadaceae), was described in Part I (1). An examination of the analytical and spectral data indicated that Compounds A–E resemble tylocrebrine (I) in that they possess the dibenzo[*f,h*]-pyrrolo[1,2*b*]isoquinoline skeleton with four or five oxygen-bearing substituents. The studies that provided evidence for the structures of these new members are described in this paper.

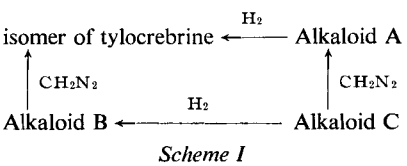
DISCUSSION

Alkaloid A, $C_{24}H_{27}NO_5$, resembles tylocrebrine in its UV and IR spectra and in having four methoxyl groups. The extra oxygen atom is in the form of a hydroxyl group, as shown by the formation of a monoacetate (bands at 1725 and 1225 cm.$^{-1}$ in IR and a sharp 3-proton peak at τ 7.85 in the NMR spectrum). Clemmensen reduction or catalytic hydrogenation converts Alkaloid A to a nonhydroxylic compound $C_{24}H_{27}NO_4$, thus indicating that the hydroxyl is benzylic. In its spectral and chromatographic behavior, the reduction product is almost indistinguishable from tylocrebrine, but the mixed melting (decomposition) point seems to indicate that the two may not be identical.

The presence of the benzylic hydroxyl in Alkaloid A is analogous to the case of tylophorinine (II, R = OH), another known member of this group (2). In this compound, the hydroxyl was placed at 14 instead of 9 (for numbering, see Structure I), because the latter structure would have represented a highly labile carbinolamine system and the stability of the compound was inconsistent with such a structure (2). This was later confirmed by synthesis (3). In an analogous manner, the stability of Alkaloid A strongly suggests the location of the hydroxyl to be 14. This is also supported by the following NMR spectral evidence: the chemical shifts of the benzylic CH(OH) protons in tylophorinine and Alkaloid A are very close: τ 3.96 and 3.87, respectively. The corresponding chemical shift of the same proton in the acetates of both compounds is τ 3.48; in both cases, it is split as a doublet, as would be expected. It is, therefore, concluded that Alkaloid A has the hydroxyl at 14.

Alkaloid B, $C_{23}H_{25}NO_4$, has three methoxyl groups. The fourth oxygen is part of a phenolic group (UV spectral shift in base and band at 3540 cm.$^{-1}$). This is supported further by the formation of a monoacetate (1750 cm.$^{-1}$ in IR and a 3-proton peak at τ 7.59). Methylation with diazomethane leads to a tetramethoxy compound, $C_{24}H_{27}NO_4$, which is almost indistinguishable from tylocrebrine on the basis of spectral and chromatographic data, but the mixed melting (decomposition) point suggests that the two may not be identical.

Alkaloid C, $C_{23}H_{25}NO_5$, shows features similar to both A and B. It has three methoxyls and forms a diacetate (1725 and 1760 cm.$^{-1}$ in IR and 3-proton peaks at τ 7.87 and 7.59 for the alcoholic acetate and phenolic acetate groups, respectively). It can be converted by methylation to Alkaloid A and by Clemmensen reduction to B. Hence, this compound is the desmethyl derivative of Alkaloid A, and the position of the phenolic hydroxyl in B and C is the same. The transformations are shown in Scheme I.

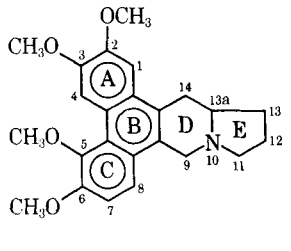


Scheme I

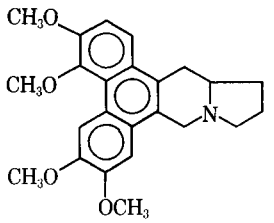
Alkaloids D and E have the compositions $C_{25}H_{29}NO_6$ and $C_{25}H_{29}NO_5$, respectively. They each have five methoxyl groups. Clem-

mensen reduction of Alkaloid D affords E. Based on arguments similar to those already advanced, Compound D is the 14-hydroxy derivative of E. The arrangement of the methoxyl groups will be discussed later in the paper.

At the time they proposed the structure of tylocrebrine, Gellert *et al.* (4) considered two possible alternatives for the arrangement of the methoxyl groups, as shown in Structures I and I*a*. To make a choice, both isomers were synthesized and compared with the natural tylocrebrine. It was observed that the spectral and physical properties of both isomers were very similar to each other, which made the comparison somewhat difficult. Hence, they subjected both synthetic compounds to Hofmann degradation and found that the product from I*a* depressed the melting point of the corresponding derivative of natural tylocrebrine. Thus, Structure I was selected as the correct one for tylocrebrine.



I



I*a*

The NMR spectrum of tylocrebrine (Fig. 1) shows resonance peaks which correspond to four aromatic protons, four methoxyls, and benzylic and methylene protons of expected chemical shifts and splitting patterns. The spectrum, however, cannot distinguish between Structures I and I*a*. The peak at τ 0.7 can be assigned to the proton at 4 (or 5), the peak at τ 2.74 to the proton at 1 (or 8), and the quartet at τ 2.33, 2.49, 2.74, and 2.89 to protons at 7 and 8 (or 1 and 2), depending on whether Structure I or I*a* is being considered.

The spectrum of Alkaloid A (Fig. 2B) shows general similarity to that of tylocrebrine, with an additional peak due to the benzylic hydroxyl and a shifted peak due to the proton at 14. In spite of the otherwise general similarity, one striking feature may be noted; that is the downfield shift (by 44 c.p.s.) of one-half of the AB quartet as compared with its position in the spectrum of tylocrebrine (Fig. 2A). The other half of the quartet also shows a slight downfield shift of 10 c.p.s. In the spectrum of the acetyl derivative of A (Fig. 2C), it can be seen that the larger downfield shift is not present and the positions of the peaks are very close to those of tylocrebrine.

A parallel situation exists in the spectra of Alkaloids B, C, and the diacetyl derivative of C (Figs. 2D, 2E, and 2F). The direction and magnitude of the downfield shift are the same as already noted. Also, in an analogous manner, the spectrum of the diacetate of C (Fig. 2F) is very close to that of Alkaloid B.

It appears that a hydroxyl function in the benzylic position has a strong deshielding effect on one of the two protons which form the AB system; protection of the hydroxyl by acetylation nullifies this effect. Since the hydroxyl group is located at 14 instead of 9 for reasons already stated, and since the 14-hydroxyl group is more likely to affect the proton at 1 than that at 8, it is apparent that the AB system is due to the protons at 1 and 2 rather than those at 7 and 8. This suggests that an oxygen substitution of the type shown in I*a* is present in Alkaloid A.

Very similar arguments have been used recently by Wiegrebe *et al.* (5) in assigning the structure for their Alkaloid A isolated from

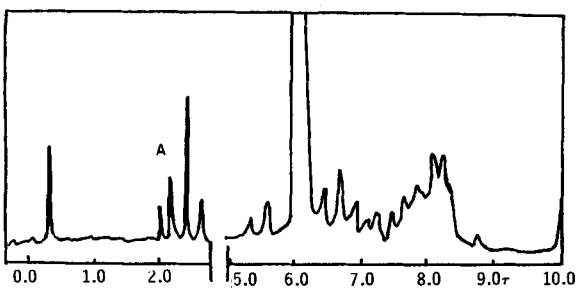


Figure 1—*NMR spectrum of tylocrebrine. Key: A, offset 200 c.p.s.*

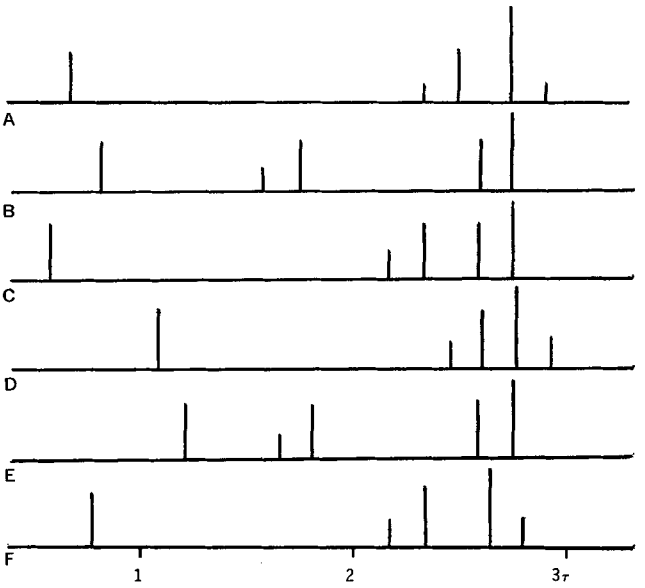
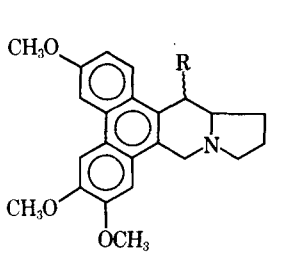


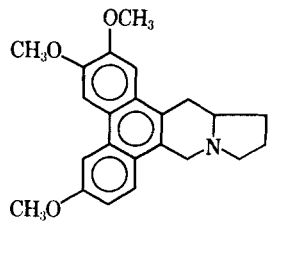
Figure 2—*NMR spectra of tylocrebrine, Alkaloid A,* O*-acetyl A, Alkaloid B, Alkaloid C, and* O*-acetyl C.*

Cyanchum vincetoxicum (L.) Pers. Because of the ambiguity of the NMR spectrum in distinguishing between the two methoxyl patterns: 3, 6, 7 (II, R = H) and 2, 3, 6 (II*a*), they introduced a substituent at 9 (a cyano group). Based on its deshielding influence on the protons at 8 and 7, the choice was made as the 2, 3, 6 isomer (II*a*).

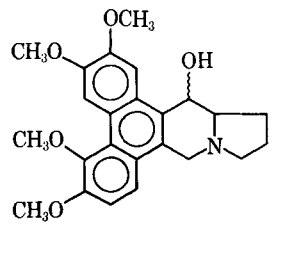
As further evidence for the structure of Alkaloid A (*T. crebriflora*), a comparison of the NMR spectra of deoxytylophorinine (II, R = H), tylophorinine, (II, R = OH), and acetyl tylophorinine (II, R = $OCOCH_3$) was undertaken. The location of the benzylic hydroxyl at 14 and the methoxyl pattern of 3, 6, 7 were established by synthesis by Govindachari *et al.* (3). Portions of the spectra pertinent to the discussion are shown in Figs. 3A, 3B, and 3C. Although the spectra are slightly more complex because of the additional proton in Ring A, one can recognize the half of the AB quartet in question. In deoxytylophorinine (Fig. 3A), these two peaks are located at τ 2.17 and τ 2.02; while in tylophorinine (Fig. 3B), which has the 14-hydroxyl group, these are shifted downfield to τ 1.75 and τ 1.60. In acetyltylophorinine (Fig. 3C), the peaks are shifted upfield to τ 2.24 and τ 2.09. Thus, introduction of the 14-hydroxyl group in deoxytylophorinine brings about a downfield shift of 25 c.p.s. of the peaks due to the proton at 1, and acetylation of the hydroxyl nearly cancels this effect. Based on these strong analogies, it appears that Alkaloid A is represented better by Struc-



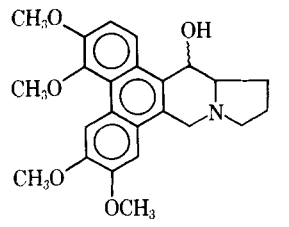
II



II*a*



III



IIIa

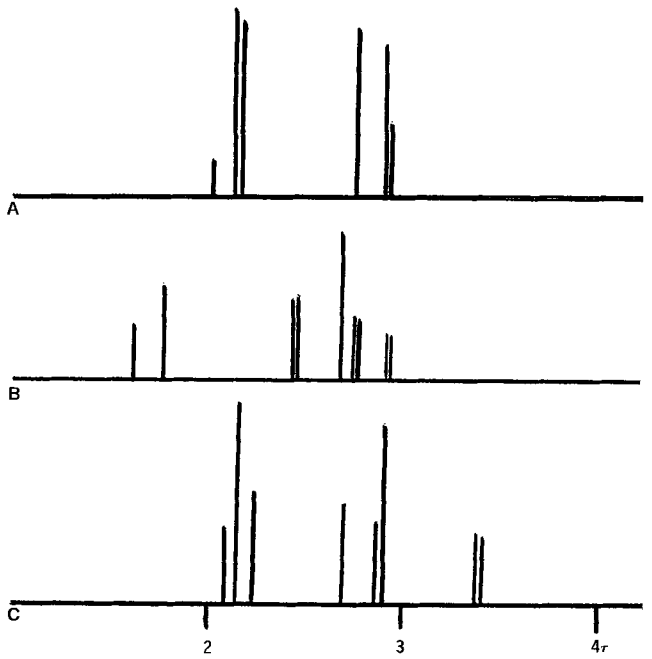
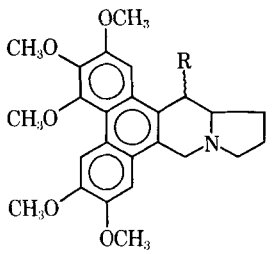


Figure 3—*NMR spectra of deoxytylophorinine, tylophorinine, and O-acetyltylophorinine.*

ture III*a* instead of III. It is thus possible that the reduction product of Alkaloid A will be the isomer of Structure I*a*.

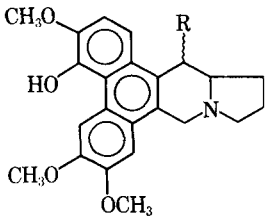
Alkaloids D and E contain five methoxyls each, and it has already been shown that D is the 14-hydroxy derivative of E. Their UV spectra bear much closer resemblance to the spectra of tylocrebrine and Alkaloid A than that of tylophorine, the 2, 3, 6, 7-tetramethoxy derivative. In the NMR spectra of both D and E (Figs. 4B and 4A), the familiar AB pattern characteristic of tylocrebrine and its derivatives is lacking. This suggests that the vicinal proton system (1, 2 or 7, 8) is absent and that the additional methoxyl is located at one of these. The 2-position was chosen for the following reasons. In the spectrum of E (Fig. 4A), the peak at τ 0.79 can be assigned to the proton at 5, and the signal at τ 2.87 (2 protons) can be assigned to those at 1 and 8. In the spectrum of D (Fig. 4B), the peak at τ 1.0 is equal to two protons (5 and 1), and the signal at τ 2.17 is equal to one proton (8). Thus the signal due to one of the protons is shifted downfield (105 c.p.s.) as a result of the introduction of the hydroxyl at 14. The most probable explanation appears to be that the additional methoxyl is located at 2 and the downfield shift (105 c.p.s.) observed is that due to the proton at 1 under the deshielding influence of the 14-hydroxyl. The NMR spectrum of the acetate of D (Fig. 4C) supports this view; the peak at τ 0.92 is again equal to one proton (5) and the other two peaks due to the protons 1 and 8 can be clearly seen upfield at τ 2.80 and 2.89, almost at the same positions as they are in E. The structures of E and D are represented in IV and V.



IV; R = H
V; R = OH

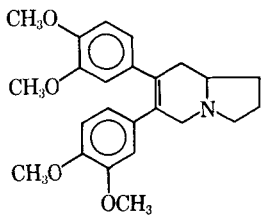
The magnitude of the downfield shift of the H-1 peak under the influence of the 14-hydroxyl appears to be roughly a function of the extent of substitution in Ring A. When there is only one methoxyl and three protons (at 1, 2, and 4), the shift is 25 c.p.s.; with two methoxyls and two protons (at 1 and 2), the shift is 44 c.p.s.; and with three methoxyls and one proton (at 1), the shift is 105 c.p.s. This suggests that the 14-hydroxyl group might be involved in some type of a hydrogen bonding with the π-electron system of Ring A. In addition to the observations of Wiegrebe *et al.* (5) already mentioned, similar effects were noted in the catechin series where they were attributed to the presence of a pseudoequatorial hydroxyl in close proximity to the aromatic system (6).

The site of demethylation in Alkaloids B and C will now be considered. They both have the 3, 4, 6, 7-substitution pattern, and one o the four groups is a phenolic hydroxyl. A comparison of the NMR spectra of A and C shows that the only significant difference between the two is the upfield shift (by 25 c.p.s.) of the signal due to the proton at 5. On acetylation, this shift becomes absent. The effect appears to be due to the increased electron density as a result of the close proximity to the phenolic hydroxyl. An examination of models indicates that the effect will be more pronounced if the hydroxyl is present at 4 than at 6. As a further support, the sharp band at 3540 cm.$^{-1}$ in IR suggests the presence of a hindered phenolic hydroxyl (7). Alkaloids B and C also give a positive Gibb's test (2,6-dibromo-*N*-chloroquinonimine), and this requires the phenolic group to be present at 4 since it is the only one with a free *para*-position. Also, the ready oxidizability to quinonoid products indicates the presence of a hindered phenolic group. Thus, B and C are represented by VI and VII.



VI; R = H
VII; R = OH

Alkaloid F differs from all the hitherto known members of the tylophora alkaloids in its physical properties. Chief among these is the UV spectrum, which shows a relatively broad maximum at 288 mμ (log ϵ, 4.03) and a shoulder at 240 mμ (log ϵ, 4.2), in contrast with the sharp maximum in the region 257–263 mμ (log ϵ, 4.8) shown by all the other members. However, the molecular formula, $C_{24}H_{29}NO_4$, differs from that of tylocrebrine by only two hydrogen atoms and they both contain four methoxyl groups. The mass spectra of both show intense peaks at M-69 mass units, which can be explained as being the result of a retro Diels-Alder type of fission with the expulsion of a neutral fragment, C_4H_7N. This result gives strong support to the presence of the indolizidine system in F. The NMR spectrum shows only two signals in the aromatic region at τ 3.39 and τ 3.43 in a ratio of 2:1 and shows general similarity to the spectrum of tylocrebrine otherwise. The absence of signals below τ 1.0 indicates alteration of the phenanthrene part of the skeleton. On oxidation with permanganate, veratric acid is isolated as the most significant product. Based on these results, Structure VIII is proposed. The physical properties and the proposed structure show that it is identical with septicine isolated earlier from *Ficus septica* (8).



VIII

Tylocrebrine and Alkaloids A, B, and C give dark-red solutions when treated with a number of oxidizing agents such as ceric sulfate, nitric acid, bromine water, or chromic acid. In contrast, tylophorine and tylophorinine do not respond to this reaction. In the cases of the two members (B and C) that possess the phenolic hydroxyl group, this oxidation can be achieved not only by the mentioned reagents but also by nitrous acid, periodate, and lead tetra-

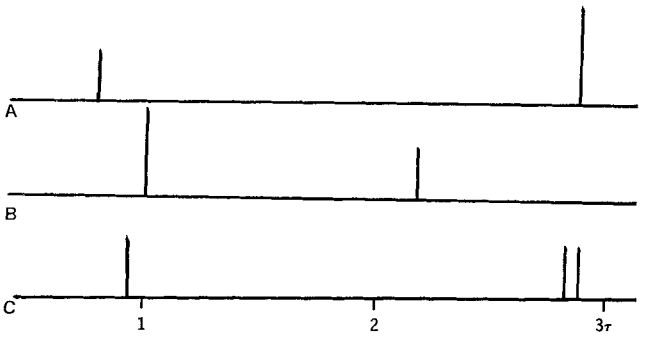
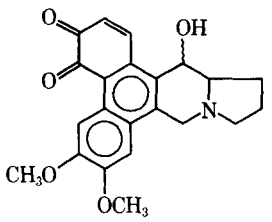


Figure 4—*NMR spectra of Alkaloid E, Alkaloid D, and* O-*acetyl D.*

acetate. The reaction product from Alkaloid A has the composition $C_{22}H_{21}NO_5$ with only two methoxyl groups. The compound appears to be an orthoquinone formed by the loss of two methoxyl groups, and the spectral evidence is in agreement with its formulation as IX. The requirement for the hindered methoxyl (or hydroxyl) group is indicated by the absence of the oxidation with tylophorine or tylophorinine.



IX

EXPERIMENTAL

General Method of Acetylation—A solution of the alkaloid (0.2–0.5 g.) in acetic anhydride (5–10 ml.) and pyridine (0.5–1 ml.) was heated at 100° for 2 hr. The cooled solution was diluted and made slightly basic. The precipitated solid was filtered and crystallized from methanol.

The acetate of Compound A is a colorless crystalline solid, m.p. 197–198°.

Anal.—Calcd. for $C_{26}H_{29}NO_6$: C, 69.16; H, 6.47; N, 3.10. Found: C, 69.13; H, 6.52; N, 3.03.

The acetate of Compound B is a colorless crystalline solid, m.p. 226–228°.

Anal.—Calcd. for $C_{25}H_{27}NO_5$: C, 71.25; H, 6.46; N, 3.32. Found: C, 70.75; H, 6.51; N, 3.36.

The diacetate of Compound C is a colorless crystalline solid, m.p. 216–218°.

Anal.—Calcd. for $C_{27}H_{29}NO_7$: C, 67.63; H, 6.10; N, 2.92. Found: C, 67.78; H, 6.17; N, 2.83.

The acetate of Alkaloid D is crystallized from ether–isopropyl ether. It is a colorless crystalline solid, m.p. 188–190°.

Anal.—Calcd. for $C_{27}H_{31}NO_7$: C, 67,34; H, 6.49; N, 2.91. Found: C, 67.27; H, 6.51; N, 2.79.

General Procedure for Clemmensen Reduction—Zinc dust (50 g.) was washed twice with acetone. It was then suspended in water (250 ml.) and treated with concentrated hydrochloric acid (25 ml.) and aqueous mercuric chloride (5 g. in 100 ml.). After standing for 10 min., the clear solution was decanted off and the zinc amalgam washed with water twice and kept under water.

A mixture of the alkaloid (0.5 g.), 2 *N* hydrochloric acid (50 ml.), and zinc amalgam (approximately 10 g.) was boiled under reflux for 24–36 hr. Aliquots were tested by paper chromatography for completion of the reaction. At the end, the clear solution was decanted off and enough sodium acetate added to pH 4–5. The solution was extracted twice with chloroform and the solvent extract concentrated to dryness. The solid was crystallized from a mixture of chloroform and methanol.

When reduced by this method, Alkaloid A gave a colorless crystalline solid, m.p. 219–220°. Mixed melting point with tylocrebrine was 210–215°.

Alkaloid C gave a colorless crystalline solid identical with Alkaloid B, and Alkaloid D gave a crystalline solid identical with Alkaloid E.

Hydrogenation of Alkaloid A—A solution of Alkaloid A (0.5 g.) glacial acetic acid (10 ml.) was hydrogenated in the presence of Adanis catalyst (0.2 g.) at 50 p.s.i. for 48 hr. The reaction mixture was filtered, diluted with water, heated with a slight excess of ammonia, and extracted with chloroform. Paper chromatography of the extract showed the presence of unchanged starting material, which was removed by countercurrent distribution in the chloroform–ethyl acetate–3% aqueous acetic acid (7:3:10) system. The hydrogenation product was recovered and crystallized twice from chloroform–methanol. It was identical with the product obtained from Clemmensen reduction.

Oxidation of Alkaloid A—A solution of Alkaloid A (1 g.) in 1 *N* HCl (10 ml.) was cooled in an ice bath and treated with 5% ceric sulfate (15 ml.). The dark-red soluton was extracted with chloroform in the presence of sodium acetate. The dark-red extract was concentrated to dryness, and the solid crystallized from methylene chloride. The quinone hydrochloride is a maroon-red crystalline solid, m.p. >300°.

Anal.—Calcd. for $C_{22}H_{21}NO_5 \cdot HCl$: C, 63.53; H, 5.33, N, 3.36, and $OCH_3(2)$, 14.91. Found: C, 63.72; H, 5.52; N, 3.26, and OCH_3, 15.32.

SUMMARY

Of the six alkaloids, A, B, C, D, E, and F, isolated from *T. crebriflora*, S. T. Blake, five (A–E) retain the dibenzo[*f,h*]-pyrrolo[1,2*b*]isoquinoline skeleton present in tylocrebrine. The distribution of the oxygen-bearing substituents in these compounds is discussed based on the NMR spectral evidence. An oxygen substitution pattern of 3, 4, 6, and 7 is suggested for Alkaloids A, B, and C and that of 2, 3, 4, 6, and 7 for Alkaloids D and E. Alkaloid F is an interesting member, and a structure based on tetramethoxy stilbene is proposed for this.

REFERENCES

(1) K. V. Rao, R. A. Wilson, and B. M. Cummings, *J. Pharm. Sci.*, **59**, 1501(1970).

(2) T. R. Govindachari, B. R. Pai, I. S. Ragade, S. Rajappa, and N. Viswanathan, *Tetrahedron*, **14**, 288(1961).

(3) T. R. Govindachari, B. R. Pai, S. Prabhakar, and T. S. Savitri, *ibid.*, **21**, 2573(1965).

(4) E. Gellert, T. R. Govindachari, M. V. Lakshmikantham, I. S. Ragade, R. Rudzats, and N. Viswanathan, *J. Chem. Soc.*, **1962**, 1008.

(5) W. Wiegrebe, L. Faber, H. Brockmann, Jr., H. Budzikiewicz, and U. Krüger, *Ann. Chem.*, **721**, 154(1969).

(6) F. M. Dean and H. D. Locksley, *J. Chem. Soc.*, **1963**, 393.

(7) S. M. Kupchan, S. Kubota, E. Fujita, S. Kobayashi, J. H. Block, and S. A. Telang, *J. Amer. Chem. Soc.*, **88**, 4212(1966).

(8) J. H. Russel, *Naturwissenschaften*, **50**, 443(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 8, 1969, from *The John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., Maywood, NJ 07607*

Accepted for publication May 11, 1970.

This investigation was supported by Contract PH43-68-45 from the Cancer Chemotherapy National Service Center, National Institutes of Health, U. S. Public Health Service.

Present address: College of Pharmacy, University of Florida, J. Hillis Miller Health Center, Gainesville, FL 32601

Allylic Phosphate Hydrolysis: Kinetics and Mechanism of Degradation of 3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-Acetate, 3-Phosphate

T. O. OESTERLING and J. H. GUSTAFSON*

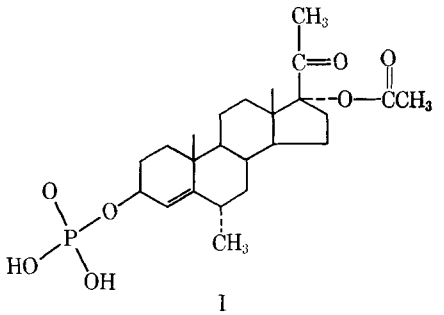
Abstract □ The kinetics and mechanism of degradation of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I) was studied as a function of pH and temperature. At low pH the least stable moiety is the allylic phosphate ester at C_3, while the most reactive function at high pH is the acyl ester at position 17. At pH 7 and 37°, the rate of phosphate ester hydrolysis is approximately 10^4 times faster than hydrolysis of the 17α-acetoxy function. Between pH 4 and 10 at 37° and between pH 2 and 6 at 4°, the rate of phosphate ester hydrolysis is first order with respect to hydrogen-ion concentration. The major products of allylic phosphate hydrolysis are an epimeric mixture of C_3 alcohols, supplying evidence that the reaction occurs principally by carbon–oxygen bond fission. The activation energy for phosphate ester hydrolysis of I is 21.5 ± 0.9 kcal./mole.

Keyphrases □ 3β,17α-Dihydoxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate degradation—kinetics, mechanism □ pH, temperature effects—allylic phosphate hydrolysis □ TLC—separation, identification □ Mass spectroscopy—identification □ NMR spectroscopy—identification

The synthesis and bioactivity of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I) have been reported by Morozowich *et al.* (1). It is a highly water-soluble progestational agent, which could be administered intravenously when rapid high levels of progestin are required such as in threatened abortion or premature labor. *In vivo*, I is enzymatically metabolized to form the corresponding C_3 alcohol (II) (1) and other potent progestational agents such as the 3-keto analog (2).

Table I—Effect of pH and Temperature on Rate of Phosphate Ester Hydrolysis of 3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-Acetate, 3-Phosphate (I)

pH	Buffer	Temperature	Ionic Strength	k, sec.$^{-1}$ × 10^5
2.42	3-Chloropropionate	4°	0.1	739
3.12	3-Chloropropionate	4°	0.1	131
4.08	Acetate	4°	0.1	12.8
4.97	Acetate	4°	0.1	1.61
6.12	Acetate	4°	0.1	0.124
6.10	Acetate	15°	0.1	0.582
6.10	Acetate	25°	0.1	1.23
4.00	Acetate	37°	0.1	1070
5.05	Acetate	37°	0.1	81.5
5.14	Acetate	37°	0.1	92.7
6.05	pH stat	37°	—	13.6
6.09	Acetate	37°	0.05	10.2
6.11	Acetate	37°	0.1	8.78
6.80	Maleate	37°	0.1	2.70
6.95	pH stat	37°	—	2.19
7.80	pH stat	37°	—	0.518
8.01	Borate	37°	0.1	0.286
8.90	Ammonia	37°	0.1	0.0700
9.00	Borate	37°	0.1	0.0301
9.04	Borate	37°	0.1	0.0308
9.95	Borate	37°	0.1	0.00375
6.20	Acetate	47°	0.1	19.0



I

The two moieties that are least stable from the standpoint of development of a chemically stable parenteral formulation of I are the acetoxy function at C_{17} and the allylic phosphate ester at C_3. Esters such as the C_{17}-acyl function of I are highly susceptible to general acid–general base catalysis. Turner (3) reported that the 17α-acetoxy function of 3β,17α-diacetoxyallopregnan-20-one was completely hydrolyzed after standing overnight in 0.25 *N* methanolic sodium hydroxide at room temperature. However, these type esters are usually much less reactive near neutral pH where, on the other hand, allylic phosphates such as the C_3 moiety of I are relatively unstable. For example, the half-life for phosphate hydrolysis of I at pH 8.78 and 37° is approximately 5 days (1).

Most previous studies of hydrolysis of allylic phosphate esters have been confined to those of acyclic monoterpene and sesquiterpene alcohols. The enzyme-catalyzed synthesis of squalene from mevalonic acid proceeds through acyclic terpenol phosphate and pyrophosphate intermediates such as farnesyl and geranyl pyrophosphate (4–6). These intermediates have been isolated and their properties studied to elucidate their role in the biochemical process. Such acyclic allylic phosphates are very unstable in acid and hydrolyze through carbonium-ion intermediates.

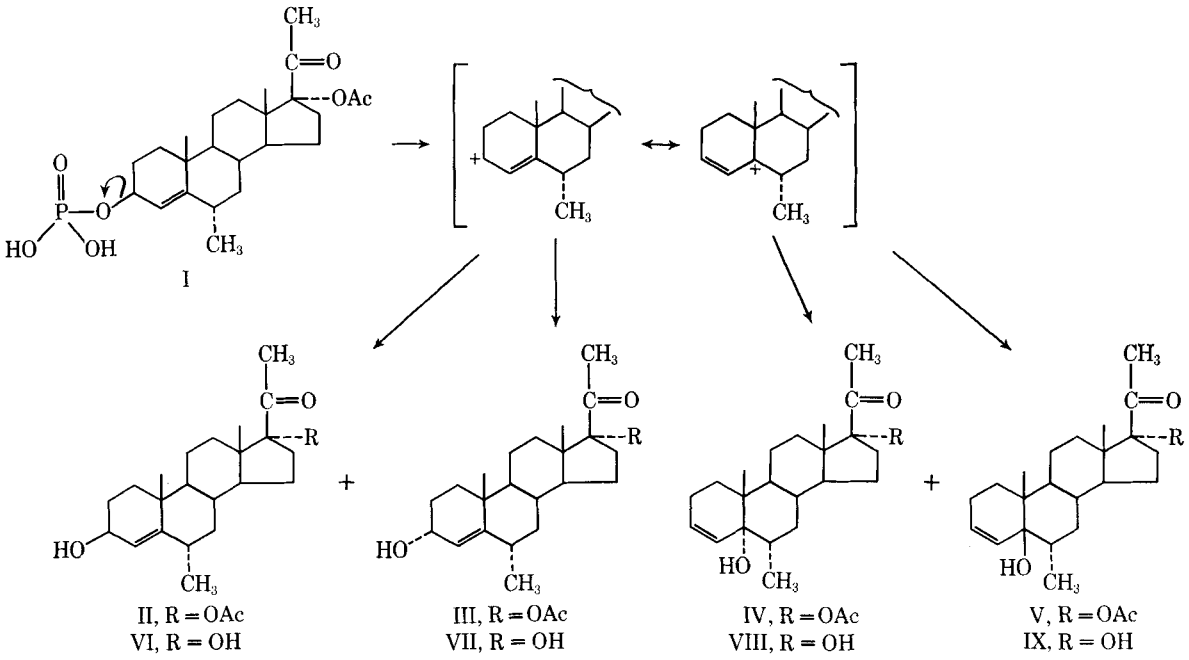
This report describes the kinetics and mechanism of degradation of I. The effects of pH and temperature on the rates of hydrolysis of the C_3-phosphate and C_{17}-acyl esters have been studied, and the major products of degradation have been identified.

EXPERIMENTAL

Materials—3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-acetate (II), 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I), and 17α-acetoxy-6-methylpregna-3,5-diene-20-one (XII) containing less than 2% impurities were used.[1] All other chemicals were reagent grade.

Kinetic Studies—*Phosphate Ester Hydrolysis*—The pH of reaction mixtures of I was maintained by a pH stat technique or by the

[1] Supplied by the research laboratories of The Upjohn Co.



Scheme I

buffers shown in Table I. Individual buffers were prepared from 3-chloropropionic acid, acetic acid, maleic acid, boric acid, ammonium hydroxide, and disodium carbonate and were adjusted to the desired pH at the temperature of the run by the addition of sodium hydroxide or hydrochloric acid. The ionic strength of buffered reaction mixtures was adjusted by the addition of potassium nitrate. Reactions were initiated by mixing temperature-equilibrated aqueous solutions of I with the buffer so that the final concentration was: I, 0.001 *M;* buffer, 0.05 *M;* and ionic strength, 0.1. After placing the mixture in a constant-temperature bath, 1.0-ml. samples were withdrawn at appropriate times, added to 1.0 ml. of 0.1 *M* NH_4OH, and refrigerated until assayed for inorganic phosphate. The pH of the reaction mixture was measured periodically during the run; if the pH varied by more than 0.2 unit from the initial pH, the run was discarded. All reaction mixtures in which phosphate ester hydrolysis was measured were prepared with freshly boiled deionized water.

For reaction mixtures where the half-life of phosphate ester hydrolysis was less than 1 hr., special techniques were employed. One-milliliter portions of an aqueous solution of I containing 2 mg./ml. were equilibrated at the temperature of the run. To obtain one time point, 1.0 ml. of equilibrated buffer was added with a syringe. At an appropriate time the reaction was quenched by the addition of 2.0 ml. of 0.1 *M* ammonium hydroxide with a syringe. Each portion of the reaction mixture was equilibrated for a different length of time until enough samples for kinetic analysis were obtained.

Kinetic runs in which pH was maintained with a pH stat were carried out as follows. Electrodes from the pH stat (Radiometer TTT1C titrator and SBR2C titragraph) and the titrant delivery tube were immersed in 39.0 ml. of freshly boiled deionized water in a stoppered jacketed vessel maintained at 37 ± 0.1°. After thermal equilibrium was attained, 40 mg. of I in 1.0 ml. of deionized water was added, and the reaction was initiated by adjusting the pH to the desired value by the addition of 1 *N* HCl or 1 *N* NaOH. Drift in pH during the run was corrected by the addition of 1 *N* HCl or 1 *N* NaOH. At appropriate times, 1.0-ml. aliquots were withdrawn and added to 1.0 ml. of deionized water; the sample was assayed immediately for inorganic phosphate content by the following procedure. Corrections for the volume of titrant added prior to sample withdrawal were incorporated into the assay results.

Table II—TLC Analysis of 3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-Acetate, 3-Phosphate (I) Reaction Mixtures

Zone Number	R_f	Percent	Molecular Ion
1	0.10	<1	388
2	0.18	5	346
3	0.31	57	388
4	0.39	3	346
5	0.48	32	388
6	0.55	<1	—[a]
7	0.63	<1	—[a]
8	0.70	<1	—[a]

[a] Insufficient sample.

The amount of inorganic phosphate in each reaction mixture aliquot was determined by Mokrasch's (7) assay. Because of the instability of the mixed reagent used in this assay, a standard was assayed after every three unknowns. Rates of phosphate ester hydrolysis were determined by least-squares analysis of log ($P_{i,\infty} - P_{i,t}$)-time data, where $P_{i,\infty}$ is the molar concentration of inorganic phosphate present at completion of hydrolysis that is equivalent to the initial molar concentration of I, and $P_{i,t}$ is the molar concentration of inorganic phosphate present at any time.

Acyl Ester Hydrolysis—Reaction mixtures were prepared by mixing 5 ml. of a temperature-equilibrated aqueous solution of I (40 mg./ml.) with a sufficient volume of 0.1 *N* NaOH to give the final desired pH. After diluting to 10 ml. with deionized water, the solution was mixed and placed at 37°. At appropriate times, 1.0-ml. samples were withdrawn and mixed with 1.0 ml. of 0.1 *M*, pH 9 borate buffer. The amount of intact 17α-acetoxy function remaining in the reaction mixture aliquot was measured by the method of Goddu *et al.* (8) as modified by Forist and Theal (9). Second-order rate constants of acyl ester hydrolysis were obtained by least-squares analysis of ln $(a-x)/(b-x)$-time data, where a is the initial concentration of I, b is the initial concentration of sodium hydroxide, and x is the amount of each reacted at any time

Identification of Products—Degradation products were identified by mass spectral and NMR studies and by comparison of TLC R_f values with authentic samples, when available. TLC studies were carried out by spotting ethyl acetate extracts of quenched reaction mixture aliquots on silica gel G and developing with methanol–acetone–water–chloroform–cyclohexane–hexane (2:10:0.2:80:20:40). Zones were visualized by heating the plates at 100–130° after spraying with 30% ammonium sulfate.

The relative amount of each zone was estimated by scanning the thin-layer plates with a Schoeffel model SD-3000 spectrodensitometer. Samples for mass spectral and NMR analyses were obtained by preparative TLC. Mass spectra were determined with an Atlas CH4 mass spectrometer in which samples were introduced by the direct inlet technique. NMR spectra were determined with a Varian HA-100 spectrometer.

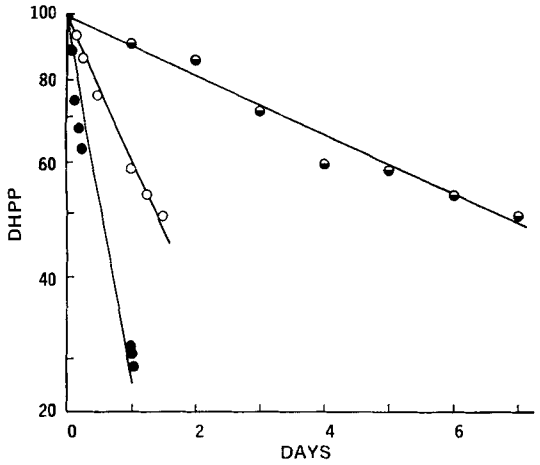


Figure 1—*Apparent first-order phosphate ester hydrolysis of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I) at pH 6.1. Key: ◒, 4°; ○, 15°; and ●, 25°.*

RESULTS AND DISCUSSION

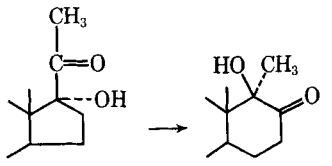
Identification of Products—Table II shows the R_f values of the products in a reaction mixture of I after about 50% phosphate hydrolysis. Also shown are the relative amounts of the zones and the number assigned as molecular ion determined by mass spectral studies of compounds isolated by preparative TLC. In general, the relative amount of each species represented in Table II was independent of pH, although at higher pH the percentages of Zones 2 and 4 tended to increase. Four of the eight degradation products of I (Table II) were present only in trace amounts; of these four, a sufficient sample for mass spectral studies could only be obtained from Zone 1.

Numerous reports have shown that allylic phosphates and pyrophosphates hydrolyze through carbonium-ion intermediates (4–6, 10). Such a mechanism is reasonable for dephosphorylation of I and is compatible with the data shown in Table II. Scheme I shows a possible mechanism by which I could hydrolyze; it involves a carbonium-ion intermediate stabilized by the allylic double bond. The three products in Table II possessing molecular weight 388, the molecular weight of the alcohol which would result from hydrolysis of the C_3-phosphate, could correspond to three of the four products shown in Scheme I (I–IV). Goodman and Popjak (4) reported that the relative amounts of secondary and tertiary alcohols resulting from pyrophosphate hydrolysis through a carbonium-ion intermediate differ from one compound to another (4). For the case of I, the secondary C_3 epimeric alcohols (II and III) are the most likely products due to the relative instability of tertiary allylic alcohols formed at C_5 and the steric hindrance involved in addition reactions at C_5. The R_f of Zone 3 is identical to the authentic sample of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate (II) which, combined with the mass spectral data in Table II, establishes the identity of the major product. The presence of a carbinol proton in the NMR spectra of Zone 5 in conjunction with a molecular weight of 388 was taken as conclusive evidence that Zone 5 represents III, the C_3 epimer of II. Mass spectra of the compounds isolated from Zones 3 and 5 provided further evidence that they are II and III. The signal at *m/e* 370 (molecular ion minus water) of Zone 3 was of lower intensity than the molecular ion signal typical of equatorial hydroxyl groups (11, 12), while the signal at *m/e* 370 of the compound from Zone 5 was of greater intensity than the molecular ion signal typical of axial hydroxyl groups (11, 12). The unsaturation in Ring A of II and III slightly distorts the configuration of the C_3 hydroxyl groups. However, probably sufficient axial (Compound III) and equatorial (Compound II) character remains in the 3-hydroxyl groups of these products to show this mass spectral response.

An epimeric mixture of C_3 alcohols could be formed in reaction mixtures of I by means other than the mechanism shown in Scheme I. For example, III could be formed by epimerization of II after phosphate hydrolysis. However, only trace amounts of III were determined by TLC when authentic II was subjected to similar conditions for comparable periods of time, providing evidence that epimerization subsequent to hydrolysis is unlikely. Since a substantial amount of III was formed in reaction mixtures of I under conditions where epimerization of II is negligible, the mechanism of hydrolysis postulated in Scheme I is quite reasonable and indicates that the reaction proceeds primarily by carbon–oxygen bond fission.

The molecular weights of the other two major products of I, Zones 2 and 4 in Table II, correspond to the alcohol that would result from hydrolysis of the acyl ester at C_{17}. Since hydrolysis of the 17α-acetoxy moiety proceeds with retention of configuration under the conditions of this study (3), Zones 2 and 4 probably represent C_3 epimers of the 17α-deacetoxy derivative of I. By analogy to the products resulting from phosphate hydrolysis, Zone 2 represents 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one (VI), and Zone 4 represents 3α,17α-dihydroxy-6α-methylpregn-4-en-20-one (VII).

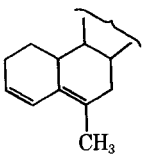
The identities of Zones 1 and 6–8 in Table II were not conclusively established. Mass spectral data suggest that Zone 1 may be IV or V, and inspection of Drieding models shows that IV is favored. However, there was an insufficient sample for further tests. Other possible products which might be represented by Zones 6–8 include those that would arise from D-homoannulation of Compounds VI–IX (Scheme II).



Scheme II

Ring expansions, such as the one depicted in Scheme II, are both acid (13) and base (14) catalyzed, and only trace amounts would be expected under the mild conditions of this study.

Secondary and tertiary allylic alcohols, such as Compounds II–V, reportedly undergo facile dehydration in acid to form conjugated dienes of the type represented by XI (15). The R_f of an authentic sample of the diene of Compounds II–V, 17α-acetoxy-6-methylpregna-3,5-dien-20-one (XII), was 0.9 in the solvent system



XI

described in the *Experimental* section, and none of this product was detected in any of the reaction mixtures in Table I. How-

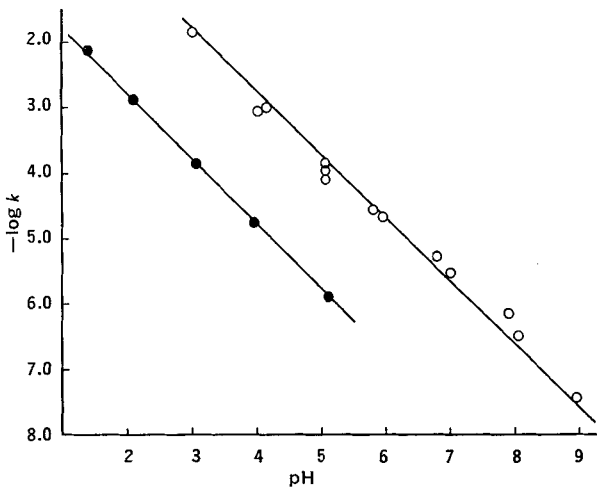


Figure 2—*pH–rate profile of phosphate hydrolysis of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I). Key: ●, 4°; and ○, 37°.*

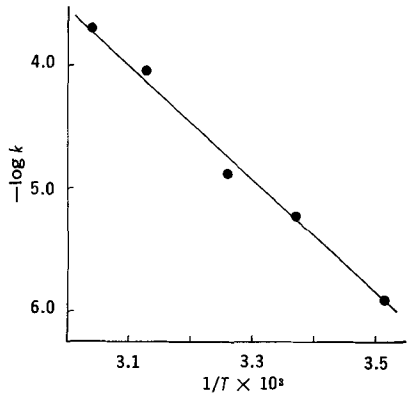


Figure 3—*Arrhenius plot of phosphate hydrolysis of 3β,17α-dihydroxy - 6α-methylpregn - 4 - en-20-one 17-acetate, 3-phosphate (I) at pH 6.1.*

ever, this diene can be detected in lower pH reaction mixtures where phosphate hydrolysis cannot be conveniently measured. For example, when I was treated with 0.25 *N* HCl at room temperature, almost complete conversion to XII occurred after 1 hr.

Rate Studies—The phosphate ester moiety of I hydrolyzed by an apparent first-order process under all experimental conditions. Some typical first-order curves of phosphate hydrolysis are shown in Fig. 1.

The effects of pH and temperature on the rate of phosphate ester hydrolysis of I are shown in Table I. In general, effects of ionic strength and buffer species on the rate constant appear minimal. However, the data at pH 6 and 37° show a slight tendency toward suppression of the rate constant with increasing ionic strength, and the pH 9 data suggest a possible buffer effect. Rate–pH profiles between pH 4 and 10 at 37° and between pH 2 and 6 at 4° are shown in Fig. 2. The slope of the curve of the 4° data is −1.00 and that of the 37° data is −0.97.

Three species of monosubstituted orthophosphate esters such as I contribute to the observed rate constant in the pH range 2–10. Although dissociation constants vary slightly with the nature of the substitution, in general the predominant species at pH 2 is the neutral or undissociated form; in the pH range 3–6, the monoanionic species predominates; and at pH 6–10, the dianion is the major species. The shape of the rate–pH profile depends on the concentration and relative reactivities of these three species. For example, in the case of 2,4-dinitrophenyl phosphate (16) where the dianion is the most reactive of the three species, the observed rate constant is greater in the pH range 6–10 where the dianion is the major species. If the monoanion is more reactive than the neutral or dianionic forms, as in the case of methyl phosphate (17), the rate–pH profile shows a maximum in the neighborhood of pH 4 where the monoanion is present at maximum concentration. The maximum in the pH–rate profile will become less pronounced as the reactivity of the neutral species increases relative to that of the monoanion. Examples of this behavior are glucose-1-phosphate (18) and lincomycin-2-phosphate (19).

Finally, the case arises where the relative reactivity of the neutral form increases to the point where the log of the rate constant shows a linear dependence on pH. The slope of the resulting line can indicate the number of species contributing to the observed rate contant; for example, a plot of log *k versus* pH for the hydrolysis of *tert*-butyl phosphate is a straight line between pH 2 and 7 (20) with a slope of −0.7. The nonunity value of the slope could be interpreted as an indication that more than one species, *i.e.*, monoanion and undissociated, of *tert*-butyl phosphate is contributing to the overall rate constant.

On the other hand, Bunton and Humeres (21) obtained a slope of −1 for the straight-line plot of pH *versus* log *k* for the hydrolysis of ribose-1-phosphate in the pH range 3.5–7.5. This behavior was attributed to the much higher reactivity of the neutral or undissociated form over the monoanionic or other negatively charged species of ribose-1-phosphate. A similar interpretation can be applied to the data shown in Fig. 2. The major reacting species for phosphate hydrolysis of I is probably the undissociated form over the entire pH range covered in this study. A rate–pH profile of allylic pyrophosphate hydrolysis based on a single-point measurement method has been reported by Goodman and Popjak (4); however, their method of measurement and the possibility of assay variation at high pH preclude direct comparison with Fig. 2.

Table III—Second-Order Rate Constants of Acyl Ester Hydrolysis of 3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-Acetate, 3-Phosphate (I) at 37°

NaOH, *N*	*k*, l. mole^{-1} sec.$^{-1}$ × 10^3
0.03	3.36
0.05	3.00
0.07	4.07
0.09	3.37

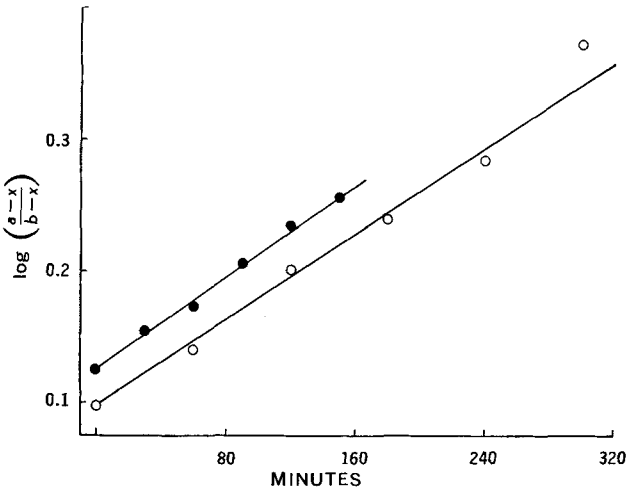


Figure 4—*Second-order plot of acyl ester hydrolysis of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I). Key:* ●, a = *0.04* M, b = *0.05* M; *and* ○, a = *0.04* M, b = *0.03* M.

The phosphate moiety of I is not sufficiently stable to allow measurement of the dissociation constant of the most acidic proton. Thus, no estimate of the specific rate constant for hydrolysis of the neutral species could be obtained. However, the data in Table I clearly show that the neutral form of I is many times more reactive than the analogous species of other types of monosubstituted-orthophosphate esters such as alkyl (17, 20), acyl (22), glycosidic (18, 21), and carbohydrate phosphates as glucose-2-phosphate (23) and lincomycin-2-phosphate (19). In fact, I is also much more reactive than acyclic allylic phosphates and pyrophosphates such as neryl, dimethylallyl, and geranyl (24, 25) phosphate and geranyl, dimethylallyl, neryl, and farnesyl pyrophosphates (4, 5). The half-lives for phosphate hydrolysis of these acyclic allylic phosphates and pyrophosphates range from 2 to 15 min. in 0.1 *N* HCl at 25°, while the extrapolated half-life (Table I) for hydrolysis of I at pH 1 and 25° is about 0.01 min.

An Arrhenius plot of phosphate ester hydrolysis (I) is shown in Fig. 3. The activation energy calculated from the slope of Fig. 3 is 21.5 ± 0.9 kcal./mole. This activation energy represents the reactivity of the neutral species, even though it was present in very low concentration in the reaction mixtures at pH 6.1.

Second-order rate constants obtained for hydrolysis of the 17α-acetoxy function of I as a function of sodium hydroxide concentration at 37° are shown in Table III. Figure 4 shows typical second-order plots of the 0.03 and 0.05 *N* NaOH data. Extrapolation of the average second-order rate constant in Table III to pH 7 (assuming minimal buffer effects) shows that phosphate ester hydrolysis is about 10^4 times faster than acyl hydrolysis at C_{17}.

REFERENCES

(1) W. Morozowich, G. W. Duncan, M. J. Taraszka, D. J. Lamb, and J. B. LaPidus, to be published.

(2) H. J. Ringold, J. Graves, M. Hayano, and H. Lawrence, Jr., *Biochem. Biophys. Res. Commun.*, **13**, 162(1963).

(3) R. B. Turner, *J. Amer. Chem. Soc.*, **75**, 3489(1953).

(4) W. S. Goodman and G. Popjak, *J. Lipid Res.*, **1**, 286(1960).

(5) P. Valenzuela and O. Cori, *Tetrahedron Lett.*, **32**, 3089(1967).

(6) J. W. Cornforth and G. Popjak, *ibid.*, **19**, 29(1959).

(7) L. C. Mokrasch, *Anal. Chem.*, **33**, 432(1961).

(8) R. F. Goddu, N. F. LeBlanc, and C. M. Wright, *ibid.*, **27**, 1251(1955).

(9) A. A. Forist and S. Theal, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 520(1958).
(10) F. Cramer and W. Rittersdorf, *Tetrahedron*, **23,** 3015(1967).
(11) V. I. Zaretskii, N. S. Wulfrom, V. G. Zaikin, L. M. Kozan, N. E. Voishvillo, and I. V. Torzov, *ibid.*, **22,** 1399(1966).
(12) M. F. Grostic and K. L. Rinehart, *J. Org. Chem.*, **33,** 1740 (1968).
(13) H. E. Stavely, *J. Amer. Chem. Soc.*, **61,** 79(1939).
(14) J. van Euw and T. Reichstein, *Helv. Chim. Acta*, **26,** 2089 (1943).
(15) H. E. Stavely and W. Bergman, *J. Org. Chem.*, **1,** 575(1937).
(16) C. A. Bunton, E. J. Fendler, and J. H. Fendler, *J. Amer. Chem. Soc.*, **89,** 1221(1967).
(17) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, **1958,** 3574.
(18) *Ibid.*, **1958,** 3588.
(19) T. O. Oesterling and E. L. Rowe, *J. Pharm. Sci.*, **59,** 175 (1970).
(20) A. Lapidot, D. Samuel, and M. W. Broday, *J. Chem. Soc.*, **1964,** 637.
(21) C. A. Bunton and E. Humeres, *J. Org. Chem.*, **34,** 572 (1969).
(22) P. W. C. Barnard, C. A. Bunton, D. Kellerman, M. M. Mhala, B. Silver, C. A. Vernon, and V. A. Welch, *J. Chem. Soc. (B)*, **1966,** 227.
(23) K. R. Farrar, *J. Chem. Soc.*, **1949,** 3131.
(24) F. Lynen, B. W. Agranoff, H. Eggerer, U. Henning, and E. M. Moslein, *Angew. Chem.*, **71,** 684(1959).
(25) W. Rittersdorf and F. Cramer, *Tetrahedron*, **24,** 43(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Research Laboratories, The Upjohn Co., Kalamazoo, MI 49001*

Accepted for publication June 10, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington D. C. meeting, April 1970.

The authors thank Dr. M. F. Grostic for obtaining and aiding in interpretation of mass spectra, Dr. G. Slomp for obtaining and aiding in intepretation of NMR spectra, and G. R. Munting for excellent technical assistance.

* 1969 Upjohn Summer Scholar in Pharmacy Research. Present address: College of Pharmacy, University of Minnesota, Minneapolis, Minn.

pH Effects on Salicylate Absorption from Hydrophilic Ointment

FRED MARCUS*, JOHN L. COLAIZZI, and HERBERT BARRY, III†

Abstract □ Hydrophilic ointment formulations containing salicylic acid were prepared at different pH levels by inclusion of phosphate buffers. Percutaneous salicylic acid absorption was studied by measuring salicylate blood levels in rabbits at 1.5-hr. intervals from 1.5 to 7.5 hr. after ointment application. The effect of 15% dimethyl sulfoxide in the various formulations was also determined. Formulations without dimethyl sulfoxide produced maximal blood concentrations at the highest pH level (10.78) and higher concentrations at the lowest pH (2.97) than at the intermediate ones (4.48, 6.80, 9.23). Proportionally higher concentration of undissociated species appeared to account for the increased absorption observed at the most acidic pH, while increased dissolution was probably responsible for the greater blood levels recorded at the most alkaline pH. Dimethyl sulfoxide produced a more rapid rate of salicylate absorption as well as greater peak blood levels at each pH. However, its influence on rate of absorption and on peak blood levels was less pronounced at the highest pH levels. This finding seems to indicate that the positive effect of dimethyl sulfoxide on percutaneous salicylate absorption is due in part to its ability to enhance the dissolution of salicylic acid.

Keyphrases □ Salicylate absorption—hydrophilic ointment □ pH effect—salicylate absorption from hydrophilic ointments □ Dimethyl sulfoxide effect—salicylate absorption, hydrophilic ointment □ Percutaneous absorption—salicylate in hydrophilic ointment

Experimental evidence indicates that percutaneous absorption of most drugs occurs by passive diffusion of an undissociated therapeutic entity across a lipoidal barrier (1). Characteristics of an ointment base, in particular its thermodynamic activity and included solvents, influence the rate of release from the base and the rate and quantity of percutaneous penetration and absorption. Knowledge of the specific influence of each of these factors can aid in the formulation and choice of ointment bases designed to elicit a specified rate and magnitude of percutaneous drug absorption for a particular drug administered topically.

Higuchi (2) indicated that the chief driving force for diffusion and penetration is thermodynamic activity, which for a weakly acidic drug is inversely proportional to the term 10^{pH}. Bhatia and Barber (3) found changes in the local anesthetic activity of ethyl aminobenzoate incorporated into hydrophilic ointment USP buffered at nine different pH values ranging from 3.5 to 10.0. They observed maximum pharmacologic activity at pH 6 and 7 and found a marked decrease when the pH was decreased or increased from that neutral region. Bhatia and Barber (3) attributed their results to the possibility that maximum penetration and pharmacologic activity occurred at or near the pH of rat skin. Stolar *et al.* (4) theorized that the concentration of free salicylic acid present at a pH of 6.2–6.5, in either an aqueous solution of 6.95% sodium salicylate or in a sodium salicylate cream having an aqueous dispersion medium, could not account for the measurable salicylate blood levels observed in rabbits. Blank and Gould (5) observed an increase in the absorption of sodium laurate solutions in contact with excised human skin at reduced pH. They attributed the increase in absorption at the lower pH to the formation of more undissociated lauric acid.

The use of solvents to enhance percutaneous absorption was suggested by the ease with which gases, such as nitrobenzene vapor (6), can penetrate the skin (7–10), and has been confirmed by the greater enhancement of percutaneous absorption produced by the volatile

(9) A. A. Forist and S. Theal, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 520(1958).
(10) F. Cramer and W. Rittersdorf, *Tetrahedron*, **23,** 3015(1967).
(11) V. I. Zaretskii, N. S. Wulfrom, V. G. Zaikin, L. M. Kozan, N. E. Voishvillo, and I. V. Torzov, *ibid.*, **22,** 1399(1966).
(12) M. F. Grostic and K. L. Rinehart, *J. Org. Chem.*, **33,** 1740 (1968).
(13) H. E. Stavely, *J. Amer. Chem. Soc.*, **61,** 79(1939).
(14) J. van Euw and T. Reichstein, *Helv. Chim. Acta*, **26,** 2089 (1943).
(15) H. E. Stavely and W. Bergman, *J. Org. Chem.*, **1,** 575(1937).
(16) C. A. Bunton, E. J. Fendler, and J. H. Fendler, *J. Amer. Chem. Soc.*, **89,** 1221(1967).
(17) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, **1958,** 3574.
(18) *Ibid.*, **1958,** 3588.
(19) T. O. Oesterling and E. L. Rowe, *J. Pharm. Sci.*, **59,** 175 (1970).
(20) A. Lapidot, D. Samuel, and M. W. Broday, *J. Chem. Soc.*, **1964,** 637.
(21) C. A. Bunton and E. Humeres, *J. Org. Chem.*, **34,** 572 (1969).
(22) P. W. C. Barnard, C. A. Bunton, D. Kellerman, M. M. Mhala, B. Silver, C. A. Vernon, and V. A. Welch, *J. Chem. Soc. (B)*, **1966,** 227.
(23) K. R. Farrar, *J. Chem. Soc.*, **1949,** 3131.
(24) F. Lynen, B. W. Agranoff, H. Eggerer, U. Henning, and E. M. Moslein, *Angew. Chem.*, **71,** 684(1959).
(25) W. Rittersdorf and F. Cramer, *Tetrahedron*, **24,** 43(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Research Laboratories, The Upjohn Co., Kalamazoo, MI 49001*

Accepted for publication June 10, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington D. C. meeting, April 1970.

The authors thank Dr. M. F. Grostic for obtaining and aiding in interpretation of mass spectra, Dr. G. Slomp for obtaining and aiding in intepretation of NMR spectra, and G. R. Munting for excellent technical assistance.

* 1969 Upjohn Summer Scholar in Pharmacy Research. Present address: College of Pharmacy, University of Minnesota, Minneapolis, Minn.

pH Effects on Salicylate Absorption from Hydrophilic Ointment

FRED MARCUS*, JOHN L. COLAIZZI, and HERBERT BARRY, III†

Abstract □ Hydrophilic ointment formulations containing salicylic acid were prepared at different pH levels by inclusion of phosphate buffers. Percutaneous salicylic acid absorption was studied by measuring salicylate blood levels in rabbits at 1.5-hr. intervals from 1.5 to 7.5 hr. after ointment application. The effect of 15% dimethyl sulfoxide in the various formulations was also determined. Formulations without dimethyl sulfoxide produced maximal blood concentrations at the highest pH level (10.78) and higher concentrations at the lowest pH (2.97) than at the intermediate ones (4.48, 6.80, 9.23). Proportionally higher concentration of undissociated species appeared to account for the increased absorption observed at the most acidic pH, while increased dissolution was probably responsible for the greater blood levels recorded at the most alkaline pH. Dimethyl sulfoxide produced a more rapid rate of salicylate absorption as well as greater peak blood levels at each pH. However, its influence on rate of absorption and on peak blood levels was less pronounced at the highest pH levels. This finding seems to indicate that the positive effect of dimethyl sulfoxide on percutaneous salicylate absorption is due in part to its ability to enhance the dissolution of salicylic acid.

Keyphrases □ Salicylate absorption—hydrophilic ointment □ pH effect—salicylate absorption from hydrophilic ointments □ Dimethyl sulfoxide effect—salicylate absorption, hydrophilic ointment □ Percutaneous absorption—salicylate in hydrophilic ointment

Experimental evidence indicates that percutaneous absorption of most drugs occurs by passive diffusion of an undissociated therapeutic entity across a lipoidal barrier (1). Characteristics of an ointment base, in particular its thermodynamic activity and included solvents, influence the rate of release from the base and the rate and quantity of percutaneous penetration and absorption. Knowledge of the specific influence of each of these factors can aid in the formulation and choice of ointment bases designed to elicit a specified rate and magnitude of percutaneous drug absorption for a particular drug administered topically.

Higuchi (2) indicated that the chief driving force for diffusion and penetration is thermodynamic activity, which for a weakly acidic drug is inversely proportional to the term 10^{pH}. Bhatia and Barber (3) found changes in the local anesthetic activity of ethyl aminobenzoate incorporated into hydrophilic ointment USP buffered at nine different pH values ranging from 3.5 to 10.0. They observed maximum pharmacologic activity at pH 6 and 7 and found a marked decrease when the pH was decreased or increased from that neutral region. Bhatia and Barber (3) attributed their results to the possibility that maximum penetration and pharmacologic activity occurred at or near the pH of rat skin. Stolar *et al.* (4) theorized that the concentration of free salicylic acid present at a pH of 6.2–6.5, in either an aqueous solution of 6.95% sodium salicylate or in a sodium salicylate cream having an aqueous dispersion medium, could not account for the measurable salicylate blood levels observed in rabbits. Blank and Gould (5) observed an increase in the absorption of sodium laurate solutions in contact with excised human skin at reduced pH. They attributed the increase in absorption at the lower pH to the formation of more undissociated lauric acid.

The use of solvents to enhance percutaneous absorption was suggested by the ease with which gases, such as nitrobenzene vapor (6), can penetrate the skin (7–10), and has been confirmed by the greater enhancement of percutaneous absorption produced by the volatile

Table I—Buffer Systems and Corresponding pH Values for Aqueous Phase

Aqueous Phase, pH	Salt or Buffer Pair	g./30 g. of Ointment	moles/30 g. of Ointment,
2.97	KH_2PO_4[a]	1.0048	0.0074
	H_3PO_4[a]	0.1096	0.0011
4.48	KH_2PO_4[a]	1.1568	0.0085
6.80	K_2HPO_4[b]	0.7403	0.0042
	KH_2PO_4[a]	0.5784	0.0042
9.23	K_2HPO_4[b]	1.4805	0.0085
10.78	$K_3PO_4 \cdot H_2O$[a]	0.4033	0.0019
	K_2HPO_4[b]	1.1496	0.0066

[a] Mallinckrodt Chemical Works, St. Louis, Mo. [b] Analytical reagent grade, Fisher Scientific Co., Pittsburgh, Pa.

Table II—Experimental Design for the Four Rabbits Tested at Each pH Level[a]

	Variables Tested among Different Animals		Variables Tested in Each Animal			
Rabbit	Replicate	Initial Condition	Trial 1	Trial 2	Trial 3	Trial 4
			Stage 1		Stage 2	
1	1	No DMSO	No	DMSO	No	DMSO
2	1	DMSO	DMSO	No	DMSO	No
3	2	No DMSO	No	DMSO	No	DMSO
4	2	DMSO	DMSO	No	DMSO	No

[a] Two variables tested among different animals (Initial Drug Condition and Replications), and, for the four trials, two variables tested in each animal (Drug Condition and Stages). "No" here refers to the absence of DMSO from the ointment base.

component of a volatile–nonvolatile topical steroid solution (11).

One nonvolatile solvent, dimethyl sulfoxide (DMSO), which has received much attention in recent years, has been found to enhance the percutaneous absorption of numerous drugs (12–18). Mechanisms proposed to explain the effect of DMSO include its reversible interaction with keratin, the extraction of lipids within the skin by disulfide interchange, the possibility of its interaction with another diffusing solute, and its inhibition of the redox polymerization of hyaluronic acid (19–22). Studies to date have indicated that DMSO is low in toxicity and causes no abnormal changes in the eyes of man, even when applied directly to the eyes (23).

The objective of this study was to determine the influence of pH on the percutaneous absorption of salicylic acid from cream-type bases, using rabbits as the test animals. In addition, the effect of DMSO, which is known to influence the absorption of salicylic acid and sodium salicylate (24), perhaps partially by altering the solubility characteristics of the drug, was investigated at different pH values. The measurement of effects of DMSO at several different pH levels may indicate the optimal combination for either maximizing or retarding the rate and magnitude of percutaneous drug absorption and can provide information on the mechanisms by which pH and DMSO both influence absorption.

MATERIALS AND METHODS

Animals—The study employed a total of 20 white, male, New Zealand rabbits with a weight range of 1.68–3.48 kg. and an average weight of 2.70 kg. They were housed in temperature- and humidity-controlled cages, and were supplied with Purina[1] rabbit chow and water *ad libitum*, except that food was withdrawn for 24 hr. prior to each ointment application.

Preparation of Ointment Base—Salicylic acid[2] particle size was reduced in a ball mill,[3] and the material was passed through an 80-mesh sieve. The powder was dried in a heated vacuum desiccator[4] at 50° for a minimum of 48 hr. Hydrophilic ointment USP XVII (25), categorized as a water-removable ointment base, was prepared and stored in tight containers in a refrigerator at a constant temperature of 9°.

Ointment bases of various pH values were prepared by substituting the combinations and amounts of salts shown in Table I for the same weight of water in the original formula. The pH was determined with a Beckman Zeromatic II pH meter[5] for the aqueous phase of each ointment base, and a calomel junction electrode[6] attachment was exchanged for the usual electrode to observe the pH of the emulsion-type bases themselves standardized against acid mantle cream[6] of pH 4.22. The values obtained with either system, base or aqueous phase, showed only very slight differences, and the aqueous phase pH value was then utilized as the more convenient value.

After each base of each pH was prepared, 10% w/w salicylic acid was incorporated into one set of bases at each pH, and 10% w/w salicylic acid plus 15% w/w DMSO[7] was incorporated into another set of bases at each pH.

Test Procedures—The rabbits were weighed, and hair was removed with an Oster animal clipper[8] from the dorsal area and the ears 24 hr. prior to the ointment application. A 5-g. sample of ointment was spread uniformly over a rectangular area, 6.35 × 12.70 cm., on the dull side of a sheet of aluminum foil. This was applied to the shaved dorsal area of the rabbit and carefully pressed down with the aid of adhesive tape and an elastic bandage. After the 7.5-hr. test period, the tape and remaining ointment were removed, and a small amount of white petrolatum was applied to prevent chapping before the rabbit was returned to its cage.

Six blood samples were taken at intervals of 1.5 hr., the first immediately before the ointment was applied (to provide the zero time value) and the last 7.5 hr. afterward. Prior to each blood collection, the animal was set into a rabbit restraining box. One ear was warmed by manual massage and the heat from a small lamp, and a clip was placed near the base of the back of the ear to block venous blood flow as soon as the marginal ear vein distended. After the vein was pierced with a lancet[9] at a point 1 cm. above the clip, 0.5 ml. of blood was withdrawn into a syringe containing 0.1 ml. of heparin,[10] 5000 u./ml. The blood and heparin mixture was added to 5.0 ml. of a bifunctional protein precipitant and color developer in a centrifuge tube and analyzed for salicylate content according to a method originally described by Trinder (26) and more recently employed by Stelzer *et al.* (24).

Experimental Design and Statistical Analysis—The 20 rabbits were divided into five groups of four each, and each group was tested with a different pH level of ointment (2.97, 4.48, 6.80, 9.23, and 10.78). The animals were tested in pairs, one rabbit with DMSO and the other with no DMSO added to the ointment. Two such pairs (replicates) were tested with each pH level. The rabbits were given four tests, with a 7-day interval between each test. Two tests were with DMSO and the other two with no DMSO added to the ointment. Table II summarizes the experimental design for the four rabbits tested with each pH level.

Since the time of peak blood level varied in different animals and under different conditions, the blood level at any one time interval or for all intervals averaged together depended partly on absorption rate. A measure of maximal blood level, independent of rate, was obtained by recording for each animal, in each test, the highest of

[1] Ralston-Purina Co., St. Louis, Mo.
[2] Analytical reagent grade, Mallinckrodt Chemical Works, St. Louis, Mo.
[3] Erweka G. m. b. H., Frankfurt-am-Main, Germany.
[4] Model No. 68351, Precision Scientific Co., Chicago, Ill.
[5] Beckman Instruments, Inc., Fullerton, Calif.
[6] Dome Laboratories, West Haven, Conn., a water-miscible, aluminum acetate-buffered cream base.
[7] Supplied by Crown Zellerbach Corp., Camas, Wash.
[8] Model A-2 with size 40 head, John Oster Manufacturing Co., Milwaukee, Wis.
[9] Medipoint, Inc., Brooklyn, N. Y.
[10] Liquaemin Sodium, Organon, Inc., West Orange, N. J.

Table III—Average of Eight Salicylate Blood Level Determinations[a]

Type of Ointment	Time, hr.		pH 2.97	pH 4.48	pH 6.80	pH 9.23	pH 10.78	Mean Values, Combined pH
No DMSO	1.5		5.75	2.47	2.58	5.49	7.03	4.66
	3.0		11.56	4.98	7.77	9.49	14.49	9.66
	4.5		12.79	7.48	8.99	10.76	16.00	11.20
	6.0		13.54	9.51	9.14	11.96	15.72	11.97
	7.5		14.07	9.98	9.34	11.01	15.66	12.01
		Mean values (Combined times)	11.54	6.88	7.56	9.74	13.78	
DMSO	1.5		13.68	8.31	10.39	8.67	11.32	10.47
	3.0		19.43	12.27	15.13	16.42	17.96	16.24
	4.5		21.38	12.58	15.24	16.70	17.37	16.65
	6.0		21.12	12.73	14.23	15.86	14.66	15.72
	7.5		18.80	11.12	12.54	14.82	12.80	14.02
		Mean values (Combined times)	18.88	11.40	13.50	14.50	14.82	

[a] Expressed as milligram percent of salicylic acid, at five time intervals after application of ointment at one of five pH values with or without DMSO.

the five blood levels in the span from 1.5 to 7.5 hr. after application of the ointment. A statistical analysis of the effects of DMSO and pH was performed for these peak blood levels as well as for blood levels as a function of time.

The milligram percent blood level of salicylic acid for each rabbit, at each of the five collection times from 1.5 to 7.5 hr., was corrected for the zero time blank. The BMD 02V Analysis of Variance Program for the IBM 7090 digital computer provided the test for statistical significance (27, 28). The analysis included three variables tested among different animals (five pH levels, two initial drug conditions, and two replications), two variables comprising different ointment applications for each animal ("no DMSO" or "DMSO" conditions, and the two stages), and one variable comprising different blood samples for each ointment application (five time periods). The residual variance for testing statistical significance, as in previous studies (29–32), consisted of the pooled interactions of all appropriate variables with the randomly selected variable (two replications). Mechanical failure of the spectrophotometer caused loss of the data on blood levels for two rabbits tested at pH 2.97 in the second of their two tests with DMSO and also in the second of their two tests with no DMSO. The scores for the first test under the same conditions were used, and the degrees of freedom for the residual variance was reduced accordingly in statistical tests for variations among different ointment applications.

For variables with more than two levels (five pH values and five time periods), the appropriate orthogonal polynomial function was used in prior studies (29–32) to test the hypothesis that increasing pH or time intervals resulted in a progressive, monotonic change in scores (linear) or monotonic change in one direction to an intermediate pH or time, followed by a monotonic change in the opposite direction (quadratic).

RESULTS

Table III summarizes the effects of the experimental treatments on blood levels of salicylic acid. Each value in the table represents an average value of blood levels obtained from the four rabbits assigned to one of the five pH levels. The value for each rabbit at each of the five time periods represents the average for two tests with and two without the addition of DMSO to the ointment.

The values in the extreme right-hand column of Table III, averages for the five pH levels, are portrayed as Fig. 1. At each time interval, the addition of DMSO resulted in higher blood levels of salicylic acid. This effect of DMSO was statistically highly reliable ($F = 76.24$, $df = 1/26$, and $p < 0.001$). More rapid absorption with DMSO is shown by a large difference at the earliest (1.5 hr.) interval. The difference in the shape of the two curves in Fig. 1 resulted in a statistically significant linear function in the interaction between DMSO treatment and time intervals ($F = 12.77$, $df = 1/136$, and $p < 0.001$).

Figures 2 and 3 portray the blood levels over the entire time span of the experiment for the tests with and without DMSO at the two extreme pH levels (2.97 and 10.78). Figure 2 shows the greater effect of DMSO on salicylic acid blood levels at the lowest pH of 2.97 than at the highest pH of 10.78 (Fig. 3) throughout the five time intervals. In spite of the large difference in magnitude of DMSO effect, both figures show that DMSO caused higher blood levels at the early time intervals and a decline in blood levels at the later intervals. This decline was greater and began at an earlier time interval at the highest pH of 10.78. Similarly, the tests with no DMSO showed maximal blood levels at 4.5 hr. at the highest pH of 10.78. The tests with no DMSO showed maximal blood levels at 4.5 hr. at the highest pH of 10.78, whereas the blood levels continued to rise throughout the 7.5 hr. at the lowest pH of 2.97.

To obtain an overall measure of the effect of pH, the blood levels at all five time intervals were averaged together, as shown in Fig. 4. Figures 5 and 6 show the average salicylic acid blood levels as a function of pH at the earliest interval of 1.5 hr. and at the latest interval of 7.5 hr., respectively. The diminished effect of DMSO at the later time interval is evident from a comparison between Figs. 5 and 6. Blood levels of salicylic acid were generally higher at the lowest and the highest pH values than at the intermediate pH levels, both with and without DMSO and at both time intervals.

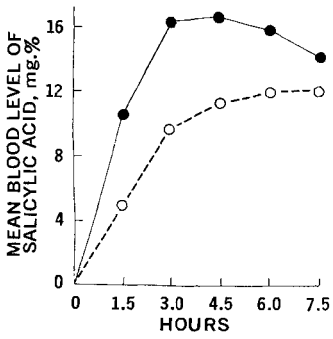


Figure 1—*Mean blood levels of salicylic acid at five time intervals after application of ointment, with all five pH values combined. Key: ●, DMSO added; and ○, no DMSO.*

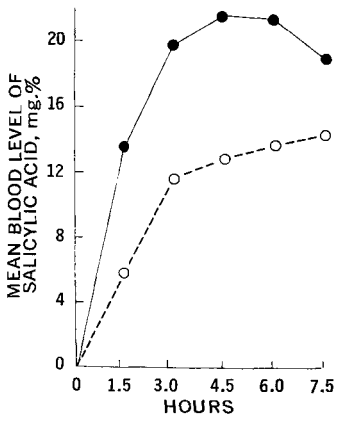


Figure 2—*Mean blood levels of salicylic acid at five time intervals after application of ointment at the lowest pH of 2.97. Key: ●, DMSO added; and ○, no DMSO.*

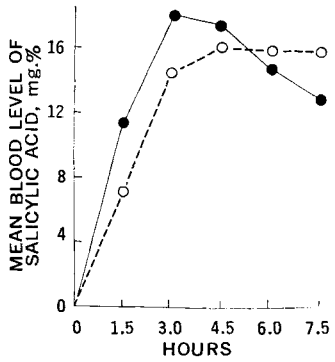


Figure 3—*Mean blood levels of salicylic acid at five time intervals after application of ointment at the highest pH of 10.78. Key:* ●, *DMSO added; and* ○, *no DMSO.*

The decrease in blood levels at the last two time intervals (6.0 and 7.5 hr.) in tests with DMSO treatment (Figs. 1–3) indicates that salicylate available for absorption decreased to the extent that the rate of metabolism and elimination exceeded the rate of absorption at these time intervals. The continuing rise in blood levels at these time intervals after application of the ointment without DMSO further indicates the more gradual rate of absorption in the absence of DMSO. The biasing effect of the decline of blood levels of salicylic acid with DMSO added, at the later time intervals, was removed by using the highest value in the series of five time intervals for each experimental run, as shown in Fig. 7. This analysis resulted in higher average blood levels than in Fig. 4, as would be expected, but otherwise the results in the two figures are rather similar. The lowest blood level occurred at the second lowest pH (4.48), with progressively higher blood levels as the pH increased to 6.80, 9.23, and 10.78. The lowest pH (2.97) also increased blood levels greatly above those obtained with pH 4.48. Statistical analysis of the peak blood levels (Fig. 7) showed a highly reliable effect of DMSO (F = 48.55, df = 1/26, and p <0.001) and a highly reliable quadratic function of pH (F = 10.78, df = 1/9, and p <0.01).

The quadratic function of pH was similar whether or not DMSO was added to the ointment. However, the highest blood levels were obtained at the lowest pH with DMSO and at the highest pH without DMSO. This indicated a tendency for the effect of DMSO to decrease with progressively increasing pH. The test of this differential effect of DMSO, shown in Fig. 7, revealed a linear function for the interaction between DMSO and pH (F = 3.26, df = 1/26, and p <0.10%).

Solubility of salicylic acid was estimated by the amount visible immediately after adding 100 g. of salicylic acid to 1000 ml. of water. Most of the salicylic acid remained in suspension, whereas solution was complete when 150 g. of DMSO was substituted for an equivalent weight of water.

DISCUSSION

The results of this investigation show that at the lowest pH the blood levels of salicylic acid obtained with the emulsion-type ointment base (without DMSO) exceed the blood levels obtainable at the intermediate pH values. As Higuchi (2) theorized, decreasing the pH of a vehicle would be expected to increase the thermodynamic activity of the undissociated form of a weakly acidic drug like salicylate. As Table IV demonstrates, the highest concentration of undissociated salicylic acid is present at the lowest pH. The much lower concentrations of the undissociated species at the three intermediate pH values probably account for the lower levels observed. These findings agree with those of Blank and Gould (5) who noted the increased percutaneous absorption of sodium laurate solutions at pH values at which undissociated lauric acid was present in relatively greater concentration.

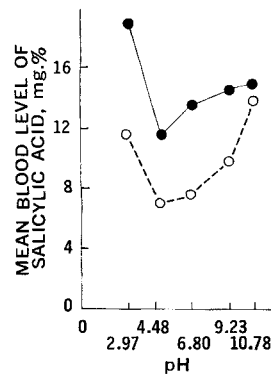


Figure 4—*Mean blood levels of salicylic acid as a function of pH, showing the average for all five time intervals. Key:* ●, *DMSO added; and* ○, *no DMSO.*

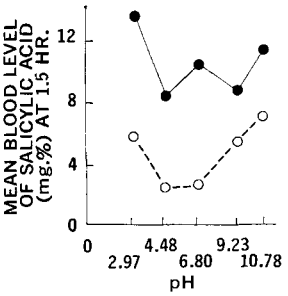


Figure 5—*Mean blood levels of salicylic acid as a function of pH at 1.5 hr. after application of ointment. Key:* ●, *DMSO added; and* ○, *no DMSO.*

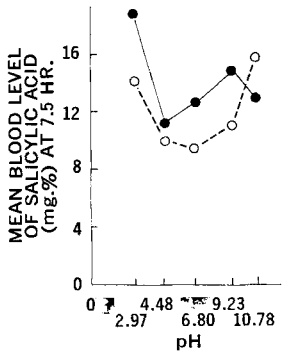


Figure 6—*Mean blood levels of salicylic acid as a function of pH at 7.5 hr. after application of ointment. Key:* ●, *DMSO added; and* ○, *no DMSO.*

Although the three intermediate pH values produced lower blood salicylate levels than the most acidic ointment, a progressive increase in blood levels was observed as the intermediate pH values rose. At the most alkaline pH value, the salicylate levels observed were higher than for any of the other ointments without DMSO, even higher than the levels observed at the most acidic pH. The apparent increase in absorption as the pH values increased might be explained by the more rapid rate of dissolution of salicylic acid that occurs with increasing pH. The rate of dissolution of salicylic acid has been shown to increase by a factor of nearly 15.9 when the pH of an aqueous buffer solution was increased from 1.5 to 6.8, and by a factor of nearly 2 when the pH was increased from 6.8 to 9.0 (33). It is well established that dissolution *in vivo* is often a rate-limiting step in the gastrointestinal absorption process; higher blood levels due to increased absorption can result from a more rapid rate of dissolution of the drug. Although a far greater proportion of salicylate is present in the dissociated form at the more alkaline pH levels, the undissociated species is probably still the absorbing species, because when one molecule of the undissociated species is absorbed (lost from the ointment), another molecule must be formed from the dissociated species to maintain the equilibrium (1).

As Fig. 4 indicates, when the DMSO was incorporated into the ointment bases, the maximum blood levels were achieved at the

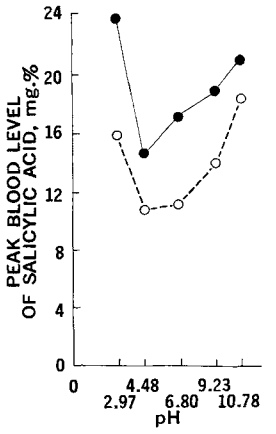


Figure 7—*Mean blood levels of salicylic acid as a function of pH at the time of peak blood level between 1.5 and 7.5 hr. after application of ointment. Key:* ●, *DMSO added; and* ○, *no DMSO.*

Table IV—Comparative Percentages of Undissociated Salicylic Acid in the Aqueous Phase at Each pH and Comparative Total Molar Solubilities of the Drug at Each pH[a]

pH of Aqueous Phase	Percent of Salicylic Acid in Undissociated Form in Aqueous Phase at 25°	Total Molar Solubility at 25°
2.97	50	3.20×10^{-2}
4.48	2.997	5.30×10^{-1}
6.80	0.014	—[c]
9.23	—[b]	—[c]
10.78	—[b]	—[c]

[a] Percentages were determined from the Henderson-Hasselbalch buffer equation (without correcting the activity coefficients). Total solubilities were determined from the equation:

$$pH = pKa + \log \frac{S - S_o}{S_o}$$

where Ka is the dissociation constant of salicylic acid, S is the total solubility (the sum of the molar concentration of salicylic acid in solution plus the molar concentration of salicylate ion), and S_0 is the molar concentration of salicylic acid in water. [b] Essentially complete dissociation. [c] Complete solubility.

lowest rather than highest pH. Therefore, DMSO appears to function as a penetration facilitator of acidic drug molecules more efficiently at the most acidic pH. DMSO completely solubilized the salicylic acid in the aqueous phase, and this increased activity of salicylic acid appears to have been an important factor in the increased percutaneous absorption of the drug. It is not surprising that DMSO enhanced overall percutaneous salicylate absorption to the greatest extent at the most acidic pH, since this is the pH at which undissociated salicylic acid is present to the greatest extent. Thus, solubilization of the less water-soluble (but more absorbable) form into the aqueous phase of the base, from which percutaneous absorption occurs more readily, could have maximal effect.

At the more alkaline pH levels, as dissolution factors become less rate limiting and as a decreasing proportion of undissociated salicylic acid is present, the degree to which the solubilizing effect of DMSO could promote absorption would be expected to diminish. The present results confirm this expectation, but DMSO enhanced absorption even at the most alkaline pH value where its solubilization effect would be minimal. This suggests that DMSO also enhances percutaneous absorption by other mechanisms, possibly by direct action on the skin and cutaneous tissue.

With no DMSO present in the ointment and with all pH values combined, as in Fig. 1, the blood levels gradually rose over the time period from 1.5 to 7.5 hr., reaching an apparent plateau at about 5.5 hr., at which time the rate of absorption apparently approximated the rate of disappearance of drug from the blood. When DMSO was incorporated into the ointments and with all pH values combined, the blood level at 1.5 hr. was more than double that obtained without DMSO. At 3.0 hr., the blood level rose to the extent that it was still almost double the value for no DMSO. The steeper initial slope observed with DMSO indicates an increased absorption rate following initial application of the drug. The subsequent decline might indicate that with DMSO the store of absorbable salicylic acid is exhausted more rapidly. Another possible reason for the decline is that the DMSO, which is itself absorbed rapidly, is no longer available to facilitate salicylate absorption. Thus, it is probable that the effect of DMSO is underestimated at later time periods, when its rapid absorption has exhausted the concentration of the solvent.

REFERENCES

(1) J. G. Wagner, *J. Pharm. Sci.*, **50,** 379(1961).

(2) T. Higuchi, *J. Soc. Cosmet. Chem.*, **11,** 85(1960).

(3) V. N. Bhatia and R. H. Barber, *J. Amer. Pharm. Ass., Sci. Ed.*, **44,** 342(1955).

(4) M. E. Stolar, G. V. Rossi, and M. Barr, *ibid.*, **49,** 144(1960).

(5) I. H. Blank and E. Gould, *J. Invest. Dermatol.*, **37,** 485 (1961).

(6) J. Piotrowski, *Brit. J. Ind. Med.*, **24,** 60(1967).

(7) T. Fredriksson, *Arzneim.-Forsch.*, **15,** 1070(1965).

(8) T. Dutkiewicz and H. Tyras, *Brit. J. Ind. Med.*, **25,** 243 (1968).

(9) R. Penneys, W. Felder, and E. Christophers, *Proc. Soc. Exp. Biol. Med.*, **127,** 1020(1968).

(10) A. C. Groom and L. E. Farhi, *J. Appl. Physiol.*, **22,** 740 (1967).

(11) M. F. Coldman, B. J. Poulsen, and T. Higuchi, *J. Pharm. Sci.*, **58,** 1098(1969).

(12) J. K. Herd, A. Cramer, and F. C. Hoak, *Pediatrics*, **40,** 905 (1967).

(13) J. E. Wahlberg and E. Skog, *Acta Dermato-Venereol.*, **47,** 209(1967).

(14) H. L. McDermot, G. W. Murray, and R. M. Heggie, *Can. J. Physiol. Pharmacol.*, **43,** 845(1965).

(15) R. B. Stoughton and W. Fritsch, *Arch. Dermatol.*, **90,** 512 (1964).

(16) A. Horita and L. J. Weber, *Life Sci.*, **3,** 1389(1964).

(17) H. Baker and A. M. Kligman, *J. Invest. Dermatol.*, **48,** 273 (1967).

(18) H. I. Maibach and R. J. Feldmann, *Ann. N. Y. Acad. Sci.*, **141,** 423(1967).

(19) S. G. Elfbaum and K. Laden, *J. Soc. Cosmet. Chem.*, **19,** 841(1968).

(20) V. H. Price and E. Menefee, *J. Invest. Dermatol.*, **49,** 297 (1967).

(21) W. D. Morain, C. A. Replogle, and P. F. Curran, *J. Pharmacol. Exp. Ther.*, **154,** 298(1966).

(22) S. A. Barker, S. J. Crews, J. B. Marsters, and M. Stacey, *Nature*, **207,** 1388(1965).

(23) I. L. Ochs, *Trans. Amer. Acad. Ophthol. Otolaryngol.*, **11,** 918(1967).

(24) J. M. Stelzer, Jr., J. L. Colaizzi, and P. J. Wurdack, *J. Pharm. Sci.*, **57,** 1732(1968).

(25) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 422.

(26) P. Trinder, *Biochem. J.*, **57,** 301(1954).

(27) "Computers in Biomedical Research," vol. I, R. W. Stacy and B. Waxman, Eds., Academic, New York, N. Y., 1965, pp. 47–64.

(28) C. A. Bennett and N. L. Franklin, "Statistical Analysis in Chemistry and the Chemical Industry," Wiley, New York, N. Y., 1954, pp. 196–200.

(29) N. Watzman, H. Barry, III, W. J. Kinnard, Jr., and J. P. Buckley, *J. Pharm. Sci.*, **55,** 518(1966).

(30) *Ibid.*, **55,** 907(1966).

(31) *Ibid.*, **57,** 1572(1968).

(32) N. Watzman, H. Barry, III, W. J. Kinnard, Jr., and J. P. Buckley, *Arch. Int. Pharmacodyn. Ther.*, **165,** 352(1967).

(33) E. Nelson, *J. Amer. Pharm. Ass., Sci. Ed.*, **47,** 297(1958).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 20, 1970, from the *Departments of Pharmaceutics and Pharmacology, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213*

Accepted for publication June 18, 1970.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This research was supported in part by Grant 5-S01-FR-05455-06 (General Research Support Grant) from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

* Fellow of the American Foundation for Pharmaceutical Education.

† Supported by a Public Health Service Research Scientist Development Award (No. K2-MH-5921) from the National Institute of Mental Health.

Comparison of Plasma Concentrations of Warfarin Measured by Both Simple Extraction and TLC Methods

P. G. WELLING, K. P. LEE, URMILLA KHANNA*, and J. G. WAGNER

Abstract □ Human volunteers received 25-mg. single oral doses of sodium warfarin. Ninety-three plasma samples were independently assayed by: (*a*) a simple extraction method, and (*b*) a TLC method using a UV instead of a fluorescent end-point. Both assays employed 7.5-cm. pathlength cells and the Cary 14 UV spectrophotometer. The assays were concluded to be equivalent and to measure only unchanged warfarin after single doses, since: (*a*) the least-squares line forced through the origin when assays by the extraction method were plotted against assays by the TLC method had a slope of 1.00; (*b*) of the differences (TLC − extraction), 44 were positive, 2 were zero, and 47 were negative; and (*c*) average elimination half-lives of warfarin estimated from terminal plasma concentrations measured by the two methods were not significantly different. The absolute value of the differences averaged 0.17 mcg./ml. with a *SD* of 0.14 mcg./ml. One may assume that each assay had an average deviation of 0.085 mcg./ml. from the "true" warfarin concentration and that the deviation was independent of the plasma concentration in the range studied (0.1–4.5 mcg./ml.).

Keyphrases □ Warfarin, plasma determination—method comparison □ Extraction procedure—warfarin determination in plasma □ TLC—warfarin determination in plasma □ UV spectrophotometry—analysis

An analytical method, developed by O'Reilly *et al.* (1), for the measurement of unchanged warfarin in plasma has been used by several investigators (2–6) to study the absorption, distribution, and metabolism of this compound and to correlate prothrombin times with plasma concentrations of the drug. Although other analytical techniques have been used (4, 7), the O'Reilly assay, involving a series of extractions and UV determination, has proved to be the simplest and most reliable procedure.

Recently, Lewis and Ilnicki (8) published, in abstract form, data suggesting that the O'Reilly assay was not specific for unchanged warfarin in plasma. TLC evidence was mentioned, indicating that two other compounds, presumably warfarin metabolites, could be detected at a stage in the assay which necessitated their being included in the final estimation. The implication of this report is clear and throws some doubt on the validity of all data using the original assay.

Interest in the pharmacokinetics of warfarin made it necessary to investigate this problem further. The data reported here are part of a more detailed study, the results of which will be published later. The objects of this particular investigation were: (*a*) to increase the sensitivity of the existing O'Reilly assay by variation of plasma and solvent volumes and measuring techniques in order to measure lower plasma drug concentrations, and (*b*) to develop a new assay, based on the abstract of Lewis and Ilnicki (8), specific for unchanged warfarin and equally as sensitive as the modified O'Reilly assay. Thus, the second assay would assess the validity of the first.

EXPERIMENTAL

Assay Methods—*Modified O'Reilly Assay*—After some experimentation, the following procedure was found to give reproducible linear absorbance-to-concentration ratios over the required plasma concentration range (0.1–4.5 mcg./ml.).

To 1,2-ethylenedichloride[1] (EDC, reagent grade, 20 ml.) in a square-sided 50-ml. bottle, fitted with a polyethylene-lined screw cap, were added plasma (4 ml.), distilled water (2 ml.), and 3 *N* hydrochloric acid (1 ml.). The bottle was tightly closed and horizontally agitated in a Kahn shaker for 10 min. The contents were then poured into a 125-ml. separator fitted with a Teflon tap. The bottle was allowed to drain into the funnel for 30 min., which was sufficient time for separation of the organic phase from the emulsion. The EDC layer was drained into a second separator, to which was then added 0.1 *M* phosphate buffer, pH 7.2 (5 ml.). After shaking for 4 min. and allowing 4 min. for phase separation, the EDC layer was run off. Exactly 15 ml. of this washed EDC extract was added to a third separator, shaken for 4 min. with 2.5 *N* sodium hydroxide (5 ml.), and allowed to stand for 4 min.

The EDC layer was discarded, and the alkaline aqueous phase containing sodium warfarin was poured into a 15-ml. centrifuge tube. This was centrifuged at 1500 r.p.m. for 5 min. to separate residual EDC; the absorbance of the clear aqueous solution was read by scanning in a 7.5-cm. pathlength cell from 380 to 280 mμ in a Cary 14 spectrophotometer.

The subject's zero-hour plasma was carried through each assay batch and was used as the reference solution in the spectrophotometer. Pooled plasma, from the hospital blood bank, was spiked with warfarin (0.5 ml. of a 40 mcg./ml. aqueous solution per 9.5 ml. of plasma) to a concentration of 2.0 mcg./ml. This was included in each assay batch and measured against unspiked blood bank plasma to determine assay reproducibility.

In this and in the TLC assay, a baseline drift was observed when scanning from 360 to 308 mμ, giving an average net absorbance (A_{308}–A_{360}) of 0.025 (*SD* = 0.004). This figure, hereafter referred to as "cell correction," was deducted from all net absorbance readings. The baseline drift is presumably due to the cell composition or the long pathlength used and not to their being unmatched, because the drift remained constant on reversing the cell positions.

Plasma concentrations of warfarin were calculated using Eq. 1:[2]

$$\text{plasma concentration} = \frac{A_{308}-A_{360} - (\text{cell correction})}{0.183} \quad \text{(Eq. 1)}$$

TLC Assay—This assay is similar to that reported by Lewis and Ilnicki (8). Estimation of warfarin by UV absorption was, however, substituted for fluorescence. The problem of fluorescence fading with exposure of acetone solutions of warfarin to UV light and the consequent difficulty in reassaying samples were therefore avoided [Corn and Berberich (7)]. At the plasma concentrations used in this study, the fluorescence technique seemed to offer no advantage over the method used.

Methodology in this assay was the same as in the modified O'Reilly assay up to the point of obtaining the 15 ml. of washed EDC extract.

Specificity was achieved in this assay by separating warfarin from other EDC extractable material, present after washing with phosphate buffer, by TLC. In all steps subsequent to obtaining 15 ml. of buffer-washed EDC extracts, contact of warfarin solutions with light was minimized. Glass containers were wrapped in aluminum foil. Volume reduction of solutions under nitrogen and plate develop-

[1] Eastman.
[2] For the derivation of these constants, see Tables I, II, and IV.

Table I—Statistics of Beer's Law Plots for Runs with Human Plasma Spiked with Warfarin[a]

Day	No. of[b] Samples	Statistics of Least-Squares Line Free to Pass through Any Intercept: Slope	*SE* of Slope	Intercept	*SE* of Intercept	Correlation Coefficient	Slope of Least-Squares Line Forced through the Origin
1	6	0.168	0.0128	−0.006[c]	0.0288	0.989	0.166
2	6	0.181	0.0029	−0.010[c]	0.0054	0.9995	0.178
3	6	0.180	0.0029	−0.007[c]	0.0054	0.9995	0.177
4	6	0.200	0.0032	−0.024	0.0072	0.9995	0.191
5	5	0.177	0.0028	−0.002[c]	0.0064	0.9996	0.185
6	5	0.183	0.0044	−0.002[c]	0.0107	0.9992	0.182
	Av.	0.182					0.180[d]
	SE	0.0043					0.0035
	95% *CI*	0.171 to 0.193					0.171 to 0.189
	Pooled data	0.182	0.0031	−0.009[c]	0.0067	0.9954	0.179

[a] Using 7.5-cm. pathlength cells in the Cary 14 recording spectrophotometer (modified O'Reilly assay method). [b] Concentrations used were 0.1, 0.2, 0.5, 1, 2, 3, and 4 mcg. warfarin/ml. [c] Not significantly different from zero. [d] Average not including Day 1 (which was inordinately low) is 0.183.

ment were done in the absence of light, and application of solutions to TLC plates was done in a minimum of light.

It has been shown in this laboratory that EDC and acetone solutions of warfarin are light sensitive. Reproducible results cannot be obtained in this assay, in which warfarin must remain in the solution for longer periods, unless these precautions are taken.

The 15-ml. washed EDC extract was reduced to dryness in a 50-ml. wide-mouthed centrifuge tube at room temperature under nitrogen. The tube was washed down with 3, 2, and 1 ml. of EDC and taken to dryness each time to concentrate material at the bottom of the tube. The contents were redissolved in a minimum volume of EDC, quantitatively transferred by capillary tube to 250-μ thick silica gel GF 254[3] chromatography plates (20 × 20 cm.), and developed in EDC–acetone (9:1).

Warfarin could be clearly detected at the lowest plasma concentration by its quenching of plate fluorescence under a wide-range UV lamp. The use of quenching on fluorescing plates is more sensitive than warfarin fluorescence on normal plates and eliminates the need to expose the plates to ammonia vapor before detection.

The spot at R_f 0.50–0.54 due to warfarin was scraped off into a 15-ml. centrifuge tube with a small spatula and eluted by agitating with acetone (3 × 1.5 ml.) on a vortex mixer, centrifuging at 2000 r.p.m., decanting, and finally washing the walls of the tube down with 1 ml. of acetone, centrifuging, and decanting again. Plate areas from blank plasmas at identical R_f values to warfarin were identically treated to yield reference solutions in the spectrophotometer.

The acetone solution was taken to dryness under nitrogen at room temperature. The warfarin was redissolved by vigorous agitation in 2.5 *N* sodium hydroxide (5 ml.) for 10 min. on a vortex mixer. After centrifuging at 2000 r.p.m. to precipitate any remaining silica gel, the clear supernatant was measured as before and the plasma warfarin concentration was calculated using Eq. 2:[2]

$$\text{plasma concentration} = \frac{A_{308}-A_{360} - (\text{cell correction})}{0.167} \quad \text{(Eq. 2)}$$

In the use of both assays, all the plasma samples from one phase of the study for a particular subject were assayed at the same time.

The developed chromatograms from dosed subjects were identical to those from warfarin-spiked plasma. Spots at R_f 0.91, 0.85, and 0.25 were identical to blank plasma in all cases. A spot at R_f 0.1 was also observed in blank plasma in 18 out of 24 cases. No other spots could be detected.

The 4′—OH, 6—OH, and 7—OH metabolites have R_f values of 0.22, 0.18, and 0.23, respectively, in this system. These R_f values are not affected when chromatographing the metabolites in combination with the parent drug.

Assay Standardization—On six different days, human plasma was spiked with warfarin at 0.1–4.0 mcg./ml. concentrations. The absorbances obtained in the modified O'Reilly assay gave rise to the statistics given in Table I. Multiple samples of plasma spiked at one concentration only were also assayed using 1-cm. cells in a Beckman DB recording spectrophotometer. The results of these assays, and also equivalent figures for a 7.5-cm. light path, are given in Table II.

As a consequence of these data, it was decided to divide the observed net absorbance of final extracts in the modified O'Reilly assay by 0.183 to obtain the plasma warfarin concentration.

Table III lists recoveries of warfarin from thin-layer plates at five different concentrations on 4 separate days. The average recovery was 91.7%. Therefore, the expected net absorbance/concentration ratio in the TLC assay is 0.183 × 0.917 = 0.168. The actual ratio obtained when pooled plasma was spiked with warfarin was 0.167 (Table IV). Therefore, the figure 0.167 was substituted for 0.183 in the TLC assay.

In both assays the sensitivity compared to the original O'Reilly assay (1) has been increased by a factor of

$$\frac{4}{2} \times \frac{7.5}{1} \times \frac{4}{5} = 12 \quad \text{(Eq. 3)}$$

where the fractions represent plasma volumes, cell pathlengths, and sodium hydroxide volumes, respectively.

Clinical Study—Six healthy adult human volunteers, four male and two female, weighing between 120 and 200 lb. and between 21 and 30 years of age, were selected. All had normal vital signs and screening laboratory values. No barbiturates or other enzyme-inducing agents were permitted to be taken for 30 days preceding initiation of the study. No medication, apart from warfarin and menadiol diphosphate (tetrasodium salt),[4] was permitted for a period of 7 days before commencement to the end of the study. The study was arranged as a two-phase crossover design. In each phase each subject ingested orally a 25-mg. dose of warfarin sodium as five 5-mg. tablets or one 25-mg. tablet. The tablets were swallowed whole. Subjects were fasted overnight and for 4 hr. postadministration of drug. Eight fluid ounces of water was taken within 1 hr. after arising and also when the tablets were ingested. No other beverage, water, or food was taken until 4 hr. after dosage. After this period, food and liquids were allowed *ad libitum*.

Twenty milliliters of blood was taken from a forearm vein just before dosing (0 hr.) and at 1, 4, 8, 12, 24, 48, 72, and 96 hr. after dosing. The blood was drawn into one or two Vacutainer tubes containing EDTA as the anticoagulant. After centrifuging, the plasma was aspirated off into a second tube and quick-frozen at −18° until required.

To reduce the possibility of hemorrhage, each subject was administered a 10-mg. dose of menadiol diphosphate (2 × 5-mg. tablets) 1 day before, on the same day, and 3 days following warfarin dosage in each phase of the crossover study. This compound and its metabolites have been shown in this laboratory not to interfere with the O'Reilly or the TLC assay for warfarin. The plasma concentrations, arranged according to the experimental design, will be reported in a forthcoming publication.

Materials—As a standard, USP reference standard warfarin acid was solubilized as the sodium salt by dissolving in 0.1 *M* phosphate buffer, pH 7.5. A stock solution of concentration 100 mcg./ml. was kept refrigerated, and aliquots were taken and brought to room temperature before use. Plasma used for assay standardization and for controls was obtained from the blood bank, University of Michigan Medical Center.

[3] Merck & Co., Inc., Rahway, NJ 07065

[4] Synkayvite, Hoffmann-La Roche, Nutley, NJ 07110

Table II—Net Absorbance–Concentration Ratios (A_N/C) Observed on Different Days with Modified O'Reilly Assay[a]

Day (No. of Samples)	Conditions				A_N/C		
	Plasma Vol., ml.	EDC Vol., ml.	NaOH Vol., ml.	Warfarin Concn., mcg./ml.	Observed Average	Coefficient of Variation, %	Expected Average A_N/C for 7.5-cm. Cell[b]
1(5)	4	20	4	5	0.0305	4.52	0.183
2(5)	4	20	4	10	0.0261	2.61	0.157
3(5)	4	20	4	10	0.0320	3.53	0.192
4(5)	4	20	4	10	0.0308	1.66	0.185
5(3)	4	10	4	5	0.0339	1.36	0.203
6(4)	4	20	2	5	0.0634	1.74	0.190
7(5)	4	20	2	5	0.0607	1.29	0.182
8(5)[c]	4	20	2	5	0.0580	1.03	0.174
						Av.	0.183

[a] Using 1-cm. pathlength cells in a Beckman DB spectrophotometer. [b] Expected average A_N/C value for the final assay adopted (4 ml. plasma, 20 ml. EDC, backextracted into 5 ml. NaOH, and absorbance read in 7.5-cm. pathlength cells) based on results obtained. [c] Assays on Day 8 were run by a different analyst than those on Days 1–7.

4′—OH, 6—OH, and 7—OH metabolites of warfarin were donated.[5] All other chemicals and solvents were reagent grade and were not further purified before use.

RESULTS

Because this report is concerned only with the sensitivity and comparison of results of two methods of assay, no specification of plasma levels of warfarin obtained from the five 5-mg. or the one 25-mg. dosage is made here.

In the modified O'Reilly assay, the average net absorbance ($A_{308} - A_{360}$ − cell correction) of final extracts from subjects' zero-hour plasmas, using water in the reference cell, was 0.216 with a *SD* of 0.056. When corrected for cell pathlength and plasma and sodium hydroxide solution volumes, this figure is equivalent to an average of 0.018 absorbance unit compared with 0.010 reported by O'Reilly *et al.* (1). The average net absorbance of final extracts from unspiked blood bank plasma was 0.261, with a *SD* of 0.024, yielding an O'Reilly equivalent of 0.022.

In the TLC assay, the average net absorbance of final extracts from subjects' zero-hour plasmas was 0.155 with a *SD* of 0.030 and an O'Reilly equivalent of 0.013. The average net absorbance of final extracts from unspiked blood bank plasma was 0.158 with a *SD* of 0.035 and an O'Reilly equivalent of 0.013.

Hence, both assays gave higher blanks than reported by O'Reilly *et al.* (1). A reduction in the net absorbance of the blanks was obtained by the TLC purification step.

In Fig. 1 the plasma concentration of warfarin measured by the modified O'Reilly method is plotted on the ordinate against the corresponding plasma concentration of warfarin measured by the TLC method for the 93 plasma samples analyzed by both methods. The slope of the least-squares line forced through the origin is 1.00; this is the exact theoretical value for equivalence of the assays.

For the differences (TLC assay − O'Reilly assay), there were 44 positive values, 2 zero values, and 47 negative values. These differences are plotted against the averages, namely (TLC assay + O'Reilly assay)/2, in Fig. 2. It appears that the differences are independent of plasma concentration in the range studied. The average absolute value of the difference was 0.17 mcg./ml. with a *SD* of 0.143 mcg./ml. If the average of the two assays on each plasma sample is taken as the true concentration of warfarin, the average deviation of an assay value from the true concentration is 0.085 mcg./ml.

From Fig. 3 it may be seen that the UV spectra of final extracts from both assays are essentially identical. However, since the metabolites of warfarin have spectra similar to the parent compound (9), small amounts of metabolite would not be detected by this method. This evidence is, therefore, of minor importance.

Elimination half-lives of warfarin were estimated from terminal plasma concentrations of warfarin from each of the 12 sets of plasma samples assayed by both methods. The method of least squares was applied to the logarithms of the plasma concentrations of each set, which appeared to be randomly distributed about a straight line when plotted against time; the slopes of the least-squares lines were divided into the logarithm of 2 to obtain the half-lives. In Fig. 4 the elimination half-life estimated from plasma samples assayed by the modified O'Reilly method is plotted on the ordinate against the elimination half-life estimated from plasma samples assayed by the TLC method on the abscissa. The least-squares line free to pass through any intercept had a slope of 0.99 and an intercept of 2.5, which was not significantly different from zero. The least-squares line forced through the origin had a slope of 1.0, and this line is drawn through the points in Fig. 4. The average half-life of 37.4 hr. obtained with the modified O'Reilly assay was not significantly different from the average half-life of 35.2 hr. obtained with the TLC assays by a paired *t*-test ($t = 0.95, p > 0.25$).

DISCUSSION

O'Reilly (10) showed that the binding strength of warfarin metabolites to crystalline human albumin was 7- to 17-fold less than that of unchanged warfarin. He suggested that the introduction of the polar hydroxyl groups by metabolism greatly decreases the hydrophobic binding of warfarin metabolites, not only to albumin but also to the receptor sites for the unchanged drug; this allows for their ready renal clearance and accounts for the loss of anticoagulant activity. This may explain why warfarin metabolites are present in urine but not in plasma in measurable amounts.

Since the TLC assay employed measures only unchanged warfarin in plasma, from the data presented it may be concluded that the original and the modified O'Reilly assays are specific for unchanged warfarin in plasma. Results reported here strongly support the countercurrent distribution studies of O'Reilly *et al.* (1), which indicated the specificity of his assay method. If warfarin metabolites are present in plasma, they must be present in minute amounts and, in such trace amounts, would not invalidate the O'Reilly assay as a

Table III—Recoveries of Warfarin from TLC Plates

No. of Samples[a]	% Recovery of Warfarin	
	Average	*SD*
5	92.7	1.1
5	92.1	0.64
5	90.2	1.8
5	91.7	2.9

[a] All five samples in each case were different concentrations of warfarin. The range of amounts spotted was 1.66–20.9 mcg.

Table IV—Standardization of the TLC Assay Using Pooled Plasma Spiked with USP Reference Standard Warfarin

Day	No. of Samples	Concentration Range, mcg./ml.	A_N/C		
			Average	*SD*	Range
1	6	0.25–3.0	0.168	0.011	0.151–0.182
2	4	2.0 only	0.166	0.005	0.162–0.174
		Av.	0.167		

[5] Dr. Karl Paul Link and Fred W. Deckert, Department of Biochemistry, University of Wisconsin.

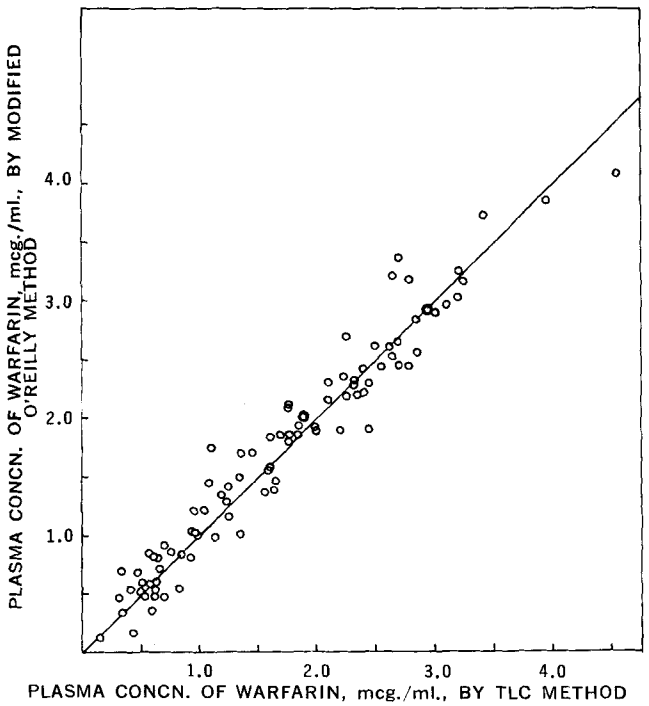


Figure 1—*Plot of plasma concentration of warfarin measured by the modified O'Reilly method against plasma concentration of warfarin measured by the TLC method.*

specific method. It is feasible that warfarinlike products detected on thin-layer plates after spotting extracts from plasma by Lewis and Ilnicki (8) were degradation products which arose from the action of light on warfarin during their TLC assay procedure. However, if these authors were using a very high dose of warfarin, then the possibility of an increase in the activity of hydroxylating enzymes at high plasma concentrations of drug, causing a disproportionate increase in plasma metabolites, would also be a feasible explanation for their results.

If a metabolite also had a long half-life in the body, its concentration in blood could build up after multiple doses of drug to levels that would invalidate the conclusions reached following administration of single doses of drug.

SUMMARY

Ninety-three plasma samples obtained from human volunteers, following ingestion of 25-mg. single oral doses of warfarin sodium, were independently assayed by a modification of the method of O'Reilly *et al.* (1) and by a TLC method. There was no detectable evidence of the presence of warfarin metabolites on any of the TLC plates developed from extracts of plasma from subjects dosed with warfarin in this study. The UV spectra of final extracts from both assays were essentially identical. Comparison of the 93 pairs of plasma concentrations arising from the application of the two assay methods on the same plasma samples and the 12 pairs of elimination half-lives of warfarin, estimated from terminal plasma concentrations, measured by the two assay methods indicated that the assays gave equivalent results. It is concluded that the original and the modified O'Reilly assay methods are specific for unchanged warfarin in plasma in the concentration range of 0.1–4.5 mcg. warfarin/ml. plasma observed in this study.

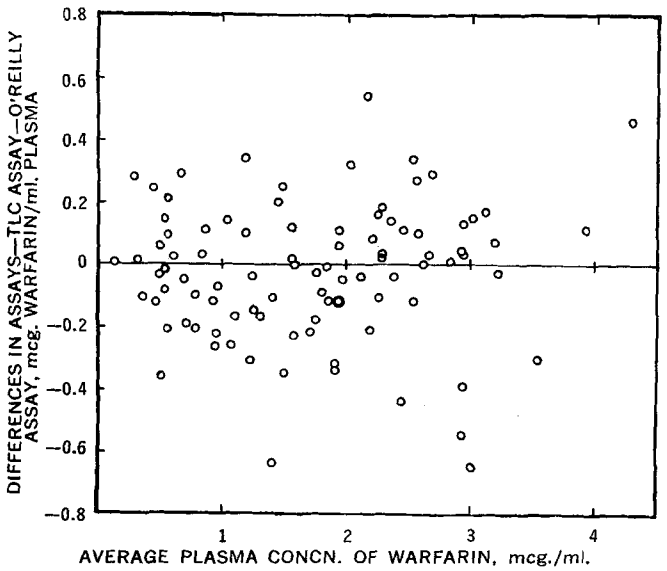


Figure 2—*Plot of difference between assays against average of assays.*

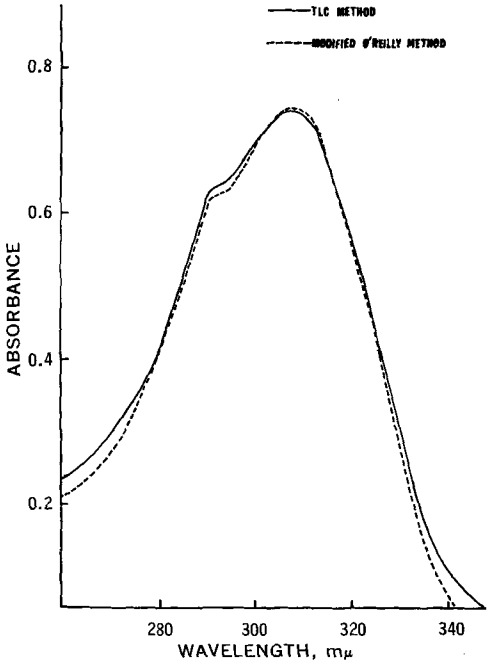


Figure 3—*UV spectra of final extracts from TLC and modified O'Reilly assays.*

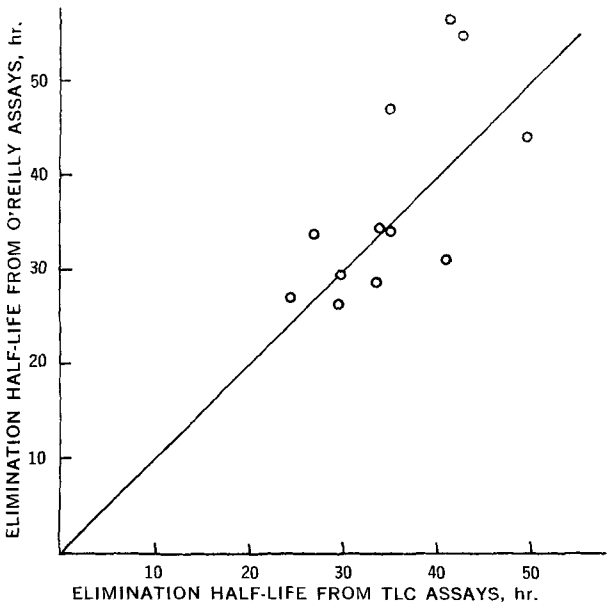


Figure 4—*Plot of the elimination half-life of warfarin estimated from modified O'Reilly assays against the elimination half-life of warfarin estimated from TLC assays.*

REFERENCES

(1) R. A. O'Reilly, P. M. Aggeler, M. S. Hoag, and L. Leong, *Thromb. Diath. Haemorrh.*, **8**, 82(1962).

(2) R. A. O'Reilly, P. M. Aggeler, and L. S. Leong, *J. Clin. Invest.*, **42**, 1542(1963).

(3) J. G. Pool, R. A. O'Reilly, L. J. Schneiderman, and M. Alexander, *Amer. J. Physiol.*, **215**, 627(1968).

(4) R. Nagashima and G. Levy, *J. Pharm. Sci.*, **58**, 845(1969).

(5) M. G. McDonald, D. S. Robinson, D. Sylwester, and J. J. Jaffe, *Clin. Pharmacol. Ther.*, **10**, 80(1969).

(6) R. A. O'Reilly and P. M. Aggeler, *Circulation*, **38**, 169 (1968).

(7) M. Corn and R. Berberich, *Clin. Med.*, **13**, 126(1967).

(8) R. J. Lewis and L. P. Ilnicki, *Clin. Res.*, **17**, 332(1969).

(9) R. A. O'Reilly, *J. Clin. Invest.*, **48**, 193(1969).

(10) R. A. O'Reilly, *Clin. Res.*, **16**, 311(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 2, 1970, from the *College of Pharmacy and Pharmacy Service, University Hospital, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication May 26, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This investigation was supported principally by Contract CPF 69-22, Food and Drug Administration, Washington, D. C., and in part by Grant No. HE-05526, National Heart Institute.

The authors thank Edward A. Carr, Jr., M.D., the medical director of the project; J. E. Walker, M.D., who performed the physical examinations; and Kathleen M. Hoffman, R.N., for withdrawal of blood and screening of volunteers.

* Present address: Department of Pharmacology (Program in Investigative Clinical Pharmacology), University of Michigan.

Salt Effects in Aqueous Solutions of Urea

RICHARD E. LINDSTROM and ALEXANDER R. GIAQUINTO

Abstract ☐ It is suggested, when working with drug–urea–water systems, that more than passing consideration be given to the effect or effects produced by addition of a fourth component, as, for example, when an acid is used to adjust pH. Data are cited which indicate that these effects are measurable and often predictable. Solubility data for methyl salicylate and methyl benzoate are given as functions of varying acid, salt, and urea concentrations. These data represent the equilibrium solubilities of the esters in the various systems at 30°. The solubilities were obtained by sampling and subsequent determination of the ester concentration using UV spectrophotometry. A mathematical model was derived which permits a quantitative evaluation of salt effects in urea solutions. The theoretical calculations, based on this model, were found to be in good agreement with the experimental values observed for neutral and pH 1 solutions of methyl salicylate and methyl benzoate in urea. Extension of these findings to an earlier investigation indicates that significant error in interpretation of solubility in urea may result if these salt effects are disregarded.

Keyphrases ☐ Urea aqueous solutions—salt effects ☐ Methyl salicylate and benzoate solubility—urea–water mixture ☐ Electrolyte, HCl concentration effects—methyl salicylate and benzoate solubility ☐ UV spectrophotometry—analysis

The introduction of a fourth component into a urea–drug–water system will produce changes in both the physical and chemical properties of the system. Understandably, the inclination is to disregard or minimize the resultant effects, especially when the rationale of the investigation appears to be unaltered by doing so. However, it is imperative that some quantitative estimate be obtained before following this premise, since the magnitude of these effects may be large enough to require reassessment of the object of the experiment.

Work on systems involving urea–water mixtures are particularly prone to questionable assumptions. For example, during their study of the solubility of salicylic acid in urea solutions, various authors (1–3) sought to preclude ionization of the salicylic acid by making the solutions pH 1 with strong acid. In two instances, the workers made the solutions 0.1 *N* in H^+. Taking note of observed irregularities in this matter, however, Feldman and Gibaldi (1) were careful to adjust the pH of each solution to 1. These two different procedures result in systems that are not equivalent, a fact that may have been responsible for the differences in observations and conclusions. The strong acid which was added in these studies is certain to exhibit a characteristic influence on the solubility of any additional solutes. Whether this influence is significant and measurable in multicomponent mixtures of urea, water, drug, and salt is typical of the problem which should concern the investigator.

Wetlaufer *et al.* (4) demonstrated that salt effects do exist in urea solutions. These workers found that the solubility of skatole in aqueous urea–sodium chloride solutions was measurably less than in solutions of the same urea concentration but containing no sodium chloride. Additionally, it was estimated that this "salting out" approximated that observed when the solubility of skatole in water and in water–sodium chloride solution was compared. Thus, it would seem that the salt effects are not only measurable but also are relatively independent of the urea concentration. In that event, one is in a favorable position to quantify similar effects in urea solutions in general.

THEORETICAL CONSIDERATIONS

The phenomena of "salting in" and "salting out" have been described empirically by the Setschenow equation (5):

$$\log S^\circ/S = KC \qquad \text{(Eq. 1)}$$

where S° and S are the molar solubilities of a nonelectrolyte in pure water and in a solution containing C moles/l. of electrolyte, respectively. The symbol, K, is an empirical salt parameter, which is characteristic of both the electrolyte and nonelectrolyte species.

REFERENCES

(1) R. A. O'Reilly, P. M. Aggeler, M. S. Hoag, and L. Leong, *Thromb. Diath. Haemorrh.*, **8**, 82(1962).

(2) R. A. O'Reilly, P. M. Aggeler, and L. S. Leong, *J. Clin. Invest.*, **42**, 1542(1963).

(3) J. G. Pool, R. A. O'Reilly, L. J. Schneiderman, and M. Alexander, *Amer. J. Physiol.*, **215**, 627(1968).

(4) R. Nagashima and G. Levy, *J. Pharm. Sci.*, **58**, 845(1969).

(5) M. G. McDonald, D. S. Robinson, D. Sylwester, and J. J. Jaffe, *Clin. Pharmacol. Ther.*, **10**, 80(1969).

(6) R. A. O'Reilly and P. M. Aggeler, *Circulation*, **38**, 169 (1968).

(7) M. Corn and R. Berberich, *Clin. Med.*, **13**, 126(1967).

(8) R. J. Lewis and L. P. Ilnicki, *Clin. Res.*, **17**, 332(1969).

(9) R. A. O'Reilly, *J. Clin. Invest.*, **48**, 193(1969).

(10) R. A. O'Reilly, *Clin. Res.*, **16**, 311(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 2, 1970, from the *College of Pharmacy and Pharmacy Service, University Hospital, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication May 26, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This investigation was supported principally by Contract CPF 69-22, Food and Drug Administration, Washington, D. C., and in part by Grant No. HE-05526, National Heart Institute.

The authors thank Edward A. Carr, Jr., M.D., the medical director of the project; J. E. Walker, M.D., who performed the physical examinations; and Kathleen M. Hoffman, R.N., for withdrawal of blood and screening of volunteers.

* Present address: Department of Pharmacology (Program in Investigative Clinical Pharmacology), University of Michigan.

Salt Effects in Aqueous Solutions of Urea

RICHARD E. LINDSTROM and ALEXANDER R. GIAQUINTO

Abstract ☐ It is suggested, when working with drug–urea–water systems, that more than passing consideration be given to the effect or effects produced by addition of a fourth component, as, for example, when an acid is used to adjust pH. Data are cited which indicate that these effects are measurable and often predictable. Solubility data for methyl salicylate and methyl benzoate are given as functions of varying acid, salt, and urea concentrations. These data represent the equilibrium solubilities of the esters in the various systems at 30°. The solubilities were obtained by sampling and subsequent determination of the ester concentration using UV spectrophotometry. A mathematical model was derived which permits a quantitative evaluation of salt effects in urea solutions. The theoretical calculations, based on this model, were found to be in good agreement with the experimental values observed for neutral and pH 1 solutions of methyl salicylate and methyl benzoate in urea. Extension of these findings to an earlier investigation indicates that significant error in interpretation of solubility in urea may result if these salt effects are disregarded.

Keyphrases ☐ Urea aqueous solutions—salt effects ☐ Methyl salicylate and benzoate solubility—urea–water mixture ☐ Electrolyte, HCl concentration effects—methyl salicylate and benzoate solubility ☐ UV spectrophotometry—analysis

The introduction of a fourth component into a urea–drug–water system will produce changes in both the physical and chemical properties of the system. Understandably, the inclination is to disregard or minimize the resultant effects, especially when the rationale of the investigation appears to be unaltered by doing so. However, it is imperative that some quantitative estimate be obtained before following this premise, since the magnitude of these effects may be large enough to require reassessment of the object of the experiment.

Work on systems involving urea–water mixtures are particularly prone to questionable assumptions. For example, during their study of the solubility of salicylic acid in urea solutions, various authors (1–3) sought to preclude ionization of the salicylic acid by making the solutions pH 1 with strong acid. In two instances, the workers made the solutions 0.1 *N* in H^+. Taking note of observed irregularities in this matter, however, Feldman and Gibaldi (1) were careful to adjust the pH of each solution to 1. These two different procedures result in systems that are not equivalent, a fact that may have been responsible for the differences in observations and conclusions. The strong acid which was added in these studies is certain to exhibit a characteristic influence on the solubility of any additional solutes. Whether this influence is significant and measurable in multicomponent mixtures of urea, water, drug, and salt is typical of the problem which should concern the investigator.

Wetlaufer *et al.* (4) demonstrated that salt effects do exist in urea solutions. These workers found that the solubility of skatole in aqueous urea–sodium chloride solutions was measurably less than in solutions of the same urea concentration but containing no sodium chloride. Additionally, it was estimated that this "salting out" approximated that observed when the solubility of skatole in water and in water–sodium chloride solution was compared. Thus, it would seem that the salt effects are not only measurable but also are relatively independent of the urea concentration. In that event, one is in a favorable position to quantify similar effects in urea solutions in general.

THEORETICAL CONSIDERATIONS

The phenomena of "salting in" and "salting out" have been described empirically by the Setschenow equation (5):

$$\log S^\circ/S = KC \qquad \text{(Eq. 1)}$$

where S° and S are the molar solubilities of a nonelectrolyte in pure water and in a solution containing C moles/l. of electrolyte, respectively. The symbol, K, is an empirical salt parameter, which is characteristic of both the electrolyte and nonelectrolyte species.

For the case of low nonelectrolyte concentration, it can be assumed that the interactions between the molecules of nonelectrolyte are minimal and may be disregarded (6). Equation 1 then becomes

$$\log S°/S = kC \qquad \text{(Eq. 2)}$$

where k is a salt parameter of considerably less complexity than K. Because of the relatively low solubilities of the drugs involved in the present study, as well as the availability of pertinent salt parameters in this form, it is convenient to use k rather than K. Mention is made of the more complex term, K, to emphasize the need for consideration of an alternative when the solubility of the drug or nonelectrolyte is proportionately greater.

The salt parameter, k, is often referred to as the "salting-out" constant. This may be misleading since the inference is that all electrolytes reduce the solubility of nonelectrolyte or drug species. Such is not the case. Referring to Eq. 2, it is seen that k is a salting-out constant if positive, but it becomes a salting-in constant if negative. Long and McDevit (6) compiled data which show that the magnitude and algebraic sign of k are determined by the nature of the electrolyte and nonelectrolyte. For solutions of benzoic acid, the salt constant is positive when the electrolyte is composed of small ions, and it becomes increasingly negative as the size of the ions increases. For example, in sodium chloride, $k = +0.18$; while in tetraethylammonium iodide, $k = -0.63$. On replacing benzoic acid with salicylic acid, the same constants decrease by approximately 0.01.

At this point it is apparent that the solubility of a sparingly soluble drug varies in a regular fashion with the concentration of added electrolyte. Furthermore, the magnitude of the solubility of the drug in electrolyte solution relative to that in pure water is indicative of the nature of the electrolyte. One might now ask whether a similar rationalization is possible when the solvent is a urea–water mixture. In this regard, findings of Wetlaufer *et al.* (4) hold some promise. On substitution into Eq. 2, their data yield a value of $k = 0.13$ for the solubility of skatole in pure water relative to that in a 0.15 M NaCl solution. This compares favorably with $k = 0.18$, obtained for the solubility of skatole in a 7 M urea solution relative to that in a solution of the same urea concentration but 0.15 M in sodium chloride as well. These results suggest that the solubilizing of the urea and the salting out of the sodium chloride may be additive effects.

PRESENT INVESTIGATION

An extension of these findings was undertaken in the present investigation. The solubility of methyl salicylate and of methyl benzoate was studied as a function of varying urea and hydrochloric acid concentrations in an attempt to identify and estimate the effect of added electrolyte on the solubility of these esters in urea–water mixtures. The work presupposes the establishment of two criteria upon which subsequent interpretations are dependent. First, the salt parameter, k, is a characteristic of a particular electrolyte, at least in the case where the solutes are electrolytes of similar structure. Second, the salt parameter is relatively independent of the urea concentration.

EXPERIMENTAL

The solubilities of methyl salicylate and methyl benzoate were studied as a function of urea concentration in both neutral solutions and solutions adjusted to pH 1. In addition, the solubility of methyl salicylate was studied as a function of urea concentration in solutions containing sodium chloride. The experimental uncertainty was estimated to be 1%.

Materials—Urea, reagent grade;[1] methyl salicylate, Eastman grade;[2] methyl benzoate, reagent grade;[3] hydrochloric acid, Baker analyzed reagent;[1] sodium chloride, reagent grade;[1] and methanol, absolute,[1] were used without further purification in this investigation.

Method—The neutral urea solutions, ranging in concentration from 0 to 5 M, were prepared in 25-ml. volumetric flasks by dissolving the appropriate amount of urea in distilled, deionized water and diluting to volume. The acidic urea solutions, also ranging in concentration from 0 to 5 M, required additional precautions. The desired amount of urea was placed in a 25-ml. beaker and dissolved in a minimum of water. Then, by using a Corning model 10 pH meter, the solution was adjusted to pH 1 with concentrated hydrochloric acid. Enough water and acid were added to bring the volume of the solution to approximately 22–23 ml. The solution was transferred to a 25-ml. volumetric flask, where the volume of the solution was adjusted to the mark using 1-ml. portions of a 0.1 N hydrochloric acid solution, each of which had previously been used to rinse the original beaker. A final check of the resulting solvent system showed no measurable change from pH 1. All urea solutions were prepared on the day of their use.

Each 25-ml. sample of urea solution was transferred to a 50-ml. glass-stoppered flask. One milliliter of the methyl ester was introduced using a pipet. This quantity assured ample excess. The flask and contents were then placed in a shaker bath, thermostated at 30 ± 0.2°, and allowed to equilibrate for 24 hr.

After the equilibration period, three 1-ml. samples of the clear supernatant were withdrawn from each flask by pipet. The pipets were equipped with a simple, cotton prefilter to exclude extraneous material and were preheated to ensure the continuous solubility of the ester within the sample. The samples were subsequently diluted to an appropriate volume, using a 1:10 water–methanol solvent mixture. The absorbance at 306 mμ for methyl salicylate or at 272 mμ for methyl benzoate was obtained with a Beckman DB-G spectrophotometer. The ester concentration was then obtained from a Beer's law plot. It had previously been determined that neither the acid nor the urea offered interference with the spectral measurements.

The work involving sodium chloride was handled in the same manner. Again, it was found that the salt offered no spectral interference.

pH of Urea–Water–HCl Mixtures—A 50-ml. sample of 8.0 M urea was transferred, by pipet, to a 100-ml. beaker which had been suspended in a bath thermostated at 30.0°. Electrodes from a Corning model 10 pH meter were then positioned so that contact was made with the urea solution in the beaker. Constant boiling hydrochloric acid, 20.248% by weight, was introduced in a stepwise fashion from a 50-ml. buret. The quantities of acid required for the mixture to reach pH values of 3.0, 2.5, 2.0, 1.5, and 1.0 were re-

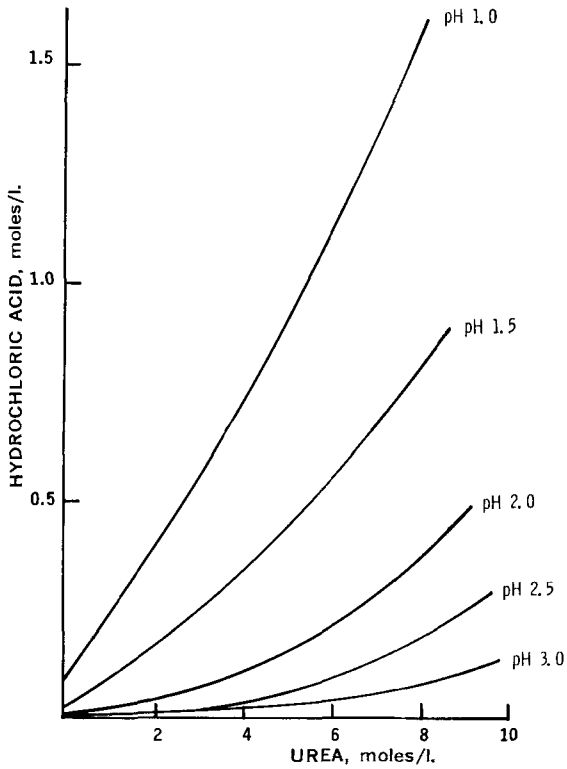


Figure 1—*pH of aqueous solutions containing both urea and hydrochloric acid.*

[1] Baker Chemical Co., Phillipsburg, N. J.
[2] Eastman Organic Chemicals, Rochester, N. Y.
[3] Fisher Scientific Co., Pittsburgh, Pa.

Table I—Solubility of Methyl Salicylate in 1.5 *M* Sodium Chloride Solutions of Varying Urea Concentration at 30°

Urea Concentration, *M*	Solubility, mg./ml.	Salt Parameter, *k*
0	0.46	0.20
3	0.90	0.18
4	1.06	0.18
5	1.29	0.16

corded. The molar concentration of urea and of HCl at each of these pH values was calculated by assuming ideal additivity of the volumes of mixture and added titrant. The entire procedure was repeated for 1.0, 4.0, and 6.0 *M* urea solutions.

RESULTS AND DISCUSSION

This work may be viewed as the result of three successive determinations, each of which was dependent upon the preceding. Initially, it was necessary to establish the fact that a characteristic salt effect was identifiable and would yield quantitative results in solutions of varying urea concentration. The solubility of methyl salicylate in solutions of urea and sodium chloride was studied for this purpose. A parameter was then derived that represented the salt effects influencing the solubility of methyl salicylate in solutions of urea and hydrochloric acid. Finally, predictions based on the proposed model were compared with the experimental values observed for the solubility of methyl benzoate, also in solutions of urea and hydrochloric acid.

Methyl Salicylate–Urea–Sodium Chloride—The existence of measurable and predictable salt effects was verified by determining the solubility of methyl salicylate in solutions that were 1.5 *M* in sodium chloride but which contained varying concentrations of urea. This high concentration of sodium chloride represents an upper limit of the ionic strength in the hydrochloric acid–urea solutions shown in Fig. 1. At the same time, it provides data which may serve as an indication of the limit to which meaningful quantitative relationships may be extended.

Table I gives the observed solubility of methyl salicylate in aqueous systems of the indicated composition, as well as the salt

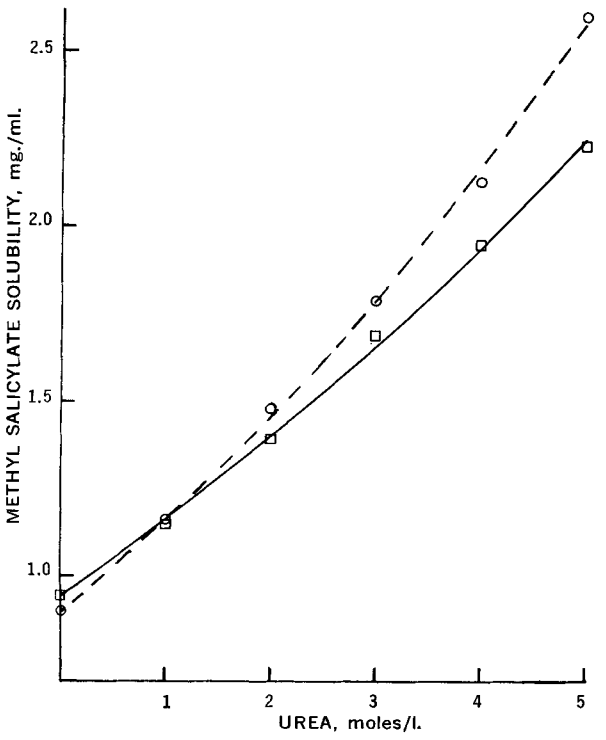


Figure 2—*Solubility of methyl salicylate in solutions of urea. Key: □, experimental points determined in neutral media; ○, experimental points determined at pH 1; ——, plot of Eq. 5; and - - -, plot of Eq. 6.*

Table II—Solubility of Methyl Salicylate and Methyl Benzoate in Solutions of Urea at 30°

Ester	Molar Concentration of Urea 0	1	2	3	4	5
	Ester Solubility, mg./ml.					
Methyl salicylate (neutral media)	0.95	1.15	1.39	1.69	1.95	2.23
Methyl salicylate (pH 1)	0.88	1.16	1.48	1.79	2.13	2.60
Methyl benzoate (neutral media)	2.42	2.90	3.34	3.75	4.30	4.88
Methyl benzoate (pH 1)	2.16	2.68	3.20	3.90	4.62	5.42

parameters for sodium chloride in each system. The latter were calculated according to Eq. 2 by comparing the solubilities of the ester in solutions of like urea concentration.

Thus, for example,

$$\log \frac{\text{solubility of drug in 5 } M \text{ urea}}{\text{solubility of drug in 5 } M \text{ urea} + 1.5 \text{ } M \text{ NaCl}} = kC \quad \text{(Eq. 3)}$$

where the denominator of the log term is the solubility of methyl salicylate in the urea–salt solutions as given in Table I. The numerator is the solubility of methyl salicylate in solutions of urea alone and may be found in Table II. The salt concentration, *C*, is 1.5 in all cases.

The results shown in Table I establish the criteria necessary for continued effort on the overall problem. The salt parameter for sodium chloride in these solutions agrees well with the value cited earlier (6) with regard to the effect of this salt on the aqueous solubility of salicylic acid. In view of the high concentration of salt used in the present work, the agreement is excellent. Additionally, the parameter is in fair agreement with the value calculated in connection with the solubility of skatole (1). This is as expected, since skatole bears some structural resemblance to methyl salicylate. Furthermore, the salt parameter appears to be only slightly dependent on the urea concentration. Presumably, even this small dependence will vanish at lower salt concentrations, where the change in sodium chloride activity with urea concentration decreases (7).

From the foregoing, it must be concluded that the solubility of a drug in urea–water systems containing electrolyte relative to that in electrolyte-free solutions may be approximated in a quantitative fashion. This, in turn, assumes prior knowledge of the sign and magnitude of the salt parameter and a realization that the predictions are less valid at high salt and high urea concentrations. The effects under consideration are not those by which urea–solute or urea–solvent interactions alter drug solubility, but are simply those which are presumed to result from the presence of electrolyte species.

Methyl Salicylate–Urea–Hydrochloric Acid—In contrast to the work already discussed, the solubility of methyl salicylate in urea solutions at pH 1 presents an interesting complication. Unlike the obviously consistent and predictable effect produced by adding sodium chloride, Fig. 2 suggests the salt effect of hydrochloric acid is strongly dependent on the urea concentration. In the absence of urea, hydrochloric acid salts out the ester. Then, as the urea concentration is increased, a salting in appears, which offsets the initial effect and soon predominates. As seen from Fig. 1, the concentration of hydrochloric acid is increasing along with that of the urea. This suggests the possiblity of a relationship between the increasing salt effect and the increasing acid concentration. However, a direct correlation seems impractical since hydrochloric acid characteristically salts out benzoic acid derivatives (6), and the addition of more acid should further decrease the solubility of the methyl salicylate. The answer to this paradox rests with observations that these solutions may contain another species in addition to the obvious components.

Consider, first, the fact that the solubility of methyl salicylate increases with additional acid and urea. In terms of the earlier discussion, this phenomenon results when the ions of the added electrolyte are large. Although one might regard the hydronium ion and its associated waters of hydration as being large, an explanation of the enhanced solubility on the basis of additional hydrochloric acid is out of the question. The results of this investigation, as well as those cited from other workers, have shown that this acid definitely salts out the ester.

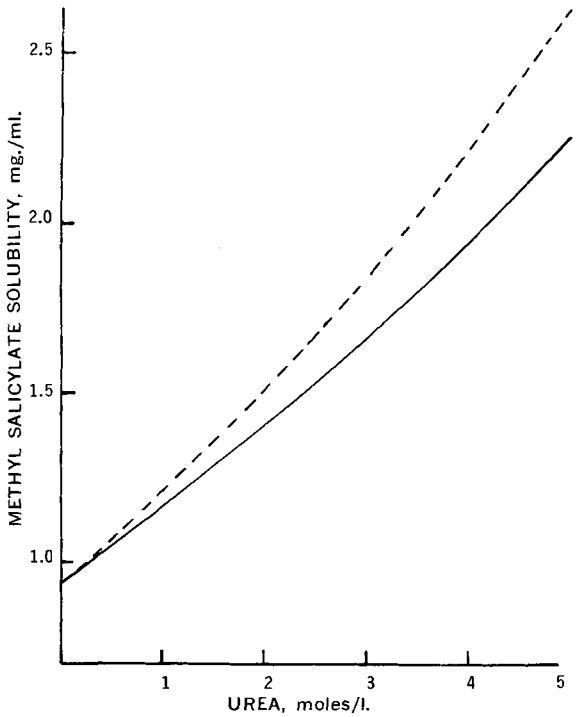


Figure 3—*Solubility of methyl salicylate in solutions of urea adjusted so as to exclude the salting out of the hydrochloric acid. Key:* ——, *plot of Eq. 5; and* - - -, *plot of Eq. 7.*

Second, the activity of the hydrochloric acid is drastically reduced in urea solutions, as implied from Fig. 1. Of the explanations for this particular phenomenon, the formation of some proton–urea complex seems the more feasible at the present time. Thus, Bull *et al.* (8) studied the conductance of hydrochloric acid in aqueous solutions of urea. Their findings suggest the existence of an electrolyte species that is considerably less mobile, and presumably bulkier, than the proton. This view has been supported by spectral (9) and dilatometric (10) evidence, as well. In essence, these workers have described an electrolyte species which is capable of salting in.

By assuming this interaction to be a fact, it becomes possible to rationalize the conditions that account for the observed ester solubilities in acid media. For example, consider an aqueous solution saturated with respect to methyl salicylate at pH 1. The concentration of ester in this solution is approximately 0.05 mg./ml. less than it would be in neutral media because of the salting out caused by the nearly 0.1 *M* concentration of hydrochloric acid. If this solution is made 5 *M* in urea, Fig. 1 shows that the hydrochloric acid concentration must be increased to 0.90 *M* to maintain the pH at 1. At the same time, the concentration of methyl salicylate increases to 2.60 mg./ml. Since the pH is 1, the activity of the hydrochloric acid must, likewise, remain at 0.1 *M*, as in the original solution. As a result, the new solution is 0.8 *M* in a species which is not contributing to the acidity *per se*. This is the quantity of hydrochloric acid that has interacted with the urea to form the larger, salting-in species. In that event, the concentration of unreacted or pure urea has decreased to 4.2 *M*. Thus, the final solution may be described as being 0.1 *M* in acid, 4.2 *M* in urea, and 0.8 *M* in the complex and containing 2.60 mg./ml. of methyl salicylate.

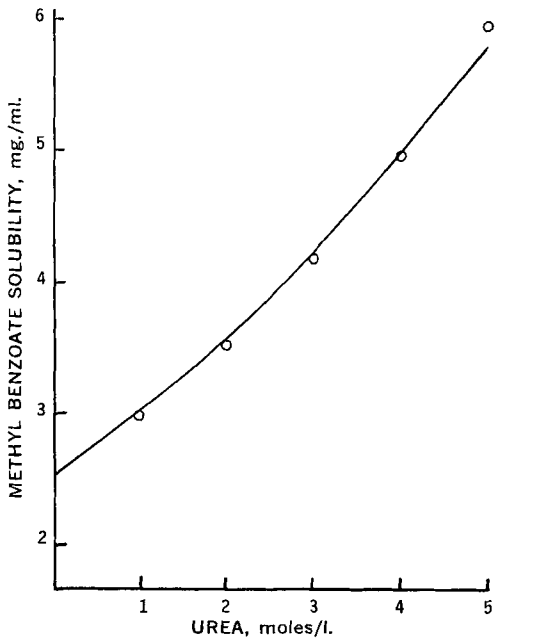


Figure 4—*Solubility of methyl benzoate in solutions of urea containing the hydrochloric acid–urea complex. Key:* O, *values calculated according to Eq. 2; and* ——, *plot of Eq. 11.*

The use of the term "complex" to define the acid–urea interaction product does not necessarily imply that the authors advocate complex formation in the customary sense. The exact nature of the interaction is uncertain and open to conjecture. Use of the term is simply an expediency, which will be employed throughout the remainder of this report. Additionally, it appears that the authors have tacitly assumed a relatively simple relationship between the observed pH values and the corresponding concentrations of the electrolytes in each solution. Unfortunately, such a relationship is inaccurate. However, since the final conclusion is unaffected by this assumption, it was decided to utilize this approach to simplify the discussion.

Salt Parameter for the Complex—A straightforward calculation of a salt parameter for the complex was precluded by the presence of a second salt, namely, the "unreacted" hydrochloric acid. This situation is simplified considerably if the data are adjusted so as to exclude the salting-out effect of the hydrochloric acid. An attempt was made to quantify this effect, using the data of both the methyl salicylate and methyl benzoate solubilities at pH 1. The adjustments proved to be unduly complicated; in view of the associated experimental uncertainties, the attempt was abandoned in favor of a more direct but approximate procedure. Since the activity of the acid is presumed to be constant by virtue of maintaining the pH at 1, and in view of the findings with regard to the initial study on the salt effects at constant sodium chloride concentration, it was assumed that the salting-out effect of the hydrochloric acid was the same over the entire range of urea concentrations studied. Thus, in equation form, the assumption was

$$\log S^\circ/S^* = \text{constant} \qquad \text{(Eq. 4)}$$

where S° is the solubility of ester in neutral media; and S^* is the solubility of ester in a hypothetical solution containing water, urea, and the acidic form of the hydrochloric acid only. The error introduced is minimized by the fact that the salting-out effect is, in addition, relatively small. Using a parameter of 0.1 (4), the constant in Eq. 4 becomes 0.01. This particular value is not derivable from the present data, but it does reflect the magnitude of the effect.

The actual adjustment is illustrated in Fig. 3. The dashed curve in Fig. 2, which represents the solubilities of methyl salicylate at pH 1, has been shifted upward so that its intercept now corresponds with that of the solid curve, representing the solubilities in neutral media. In effect, this cancels the salting out of the "acidic" hydrochloric acid.

The use of empirical equations presents a more quantitative view of the procedure. Equations 5 and 6 are the result of a least-squares treatment of the data in Table II and represent the solubilities of methyl salicylate, S, as functions of urea concentration, U, in neutral media and at pH 1, respectively.

$$S = 0.94 + 0.211\,U + 0.010\,U^2 \qquad \text{(Eq. 5)}$$

$$S = 0.89 + 0.247\,U + 0.018\,U^2 \qquad \text{(Eq. 6)}$$

If the curve is shifted upward, Eq. 6 becomes

$$S = 0.94 + 0.247\,U + 0.018\,U^2 \qquad \text{(Eq. 7)}$$

and, to a good approximation, represents the solubility of methyl salicylate in urea solutions that contain the acid–urea complex only.

The salt parameter for the complex may be determined by using the appropriate values obtained from Eqs. 5 and 7 and Fig. 1. The variables used in Eq. 2 are defined as: S = the solubility of methyl salicylate in a solution containing U moles/l. of urea and C moles/l. of the complex; C = the molar concentration of the complex, found by subtracting 0.1 M from the concentration of hydrochloric acid in a U M urea solution at pH 1; and $S°$ = the solubility of methyl salicylate in neutral media containing U' moles/l. of unreacted urea, where $U' = (U - C)$.

As an example, consider the solubility of methyl salicylate in 5 M urea at pH 1. It was shown earlier that this solution, while 5 M in total urea, is only 4.20 M in unreacted urea. Substitution of the latter value in Eq. 5 gives $S° = 2.01$ mg./ml. Using 5 M in Eq. 7, one finds $S = 2.63$ mg./ml. Since the solution is presumably 0.80 M in the complex, Eq. 2 becomes

$$\log 2.01/2.63 = 0.80\,k \qquad \text{(Eq. 8)}$$

A similar treatment also was applied to solutions 1, 2, 3, and 4 M in urea. The pertinent data are listed in Table III. Subsequently, a value of $k = -0.160$ was determined from the slope of a log $S°/S$ *versus* C plot.

Methyl Benzoate–Urea–Hydrochloric Acid—The basic premise of this paper may now be tested by using the salt parameter for the complex to predict analagous effects on the solubility of other nonelectrolyte solutes. The methyl benzoate solubility data, given in Table II, were used for this purpose. In neutral media and at pH 1, respectively, these data may be represented as

$$S = 2.52 + 0.295\,U + 0.038\,U^2 \qquad \text{(Eq. 9)}$$

$$S = 2.16 + 0.457\,U + 0.039\,U^2 \qquad \text{(Eq. 10)}$$

On shifting the intercept, as is done with the data on methyl salicylate to exclude the salting out of the acidic hydrochloric acid, Eq. 10 becomes

$$S = 2.52 + 0.457\,U + 0.039\,U^2 \qquad \text{(Eq. 11)}$$

If the proposed method is correct, it should be possible to calculate solubility data at pH 1, given data in neutral media and the necessary parameters for Eq. 2. In a like manner, the data in neutral media may be determined from those at pH 1. As an example, assume that the solubilities of methyl benzoate in neutral urea solutions are known and that the data at pH 1 are desired. The procedure is to (*a*) calculate the product k C, using the value $k = -0.160$ and the appropriate C; (*b*) determine $S°$, using the corresponding U' and Eq. 9; and (*c*) calculate S, using Eq. 2. For purposes of illustration, it is convenient to use the values for C and U' given in Table III. Thus, at $U' = 4.20$, $S°$ is found to be 4.43 mg./ml. and C is 0.80. On substitution, Eq. 2 becomes

$$\log 4.43/S = (-0.160)\,(0.80) \qquad \text{(Eq. 12)}$$

from which the solubility of methyl benzoate in a 5 M urea solution at pH 1, S, is calculated to be 5.95 mg./ml. Five such values were derived and are indicated by circles in Fig. 4. For comparison, the figure includes a curve representing Eq. 11. The agreement is excellent and attests to the validity of the proposed concepts.

An interesting situation becomes evident when these findings are extended to an earlier investigation reported by Feldman and Gibaldi (1). These workers determined the thermodynamic changes associated with the solution of salicylic and benzoic acids in urea solutions at pH 1. Their results are based on a direct application of solubility data, without regard for the salting-in effects suggested in the present study. As such, the calculated free energy changes are probably too high, and actually represent a composite of the salting-in and urea–water effects. By way of comparison, the parameters previously outlined indicate that the free energy values derived by Feldman and Gibaldi for their 3 M urea systems should be reduced by 110 cal./mole. In turn, this would require an adjustment of the observed entropy changes. On the average, these alterations would represent approximately 25% of the reported values.

pH of Urea–Water Solutions—Figure 1 is a composite of the results obtained by titrating various urea solutions with concentrated hydrochloric acid, according to the procedure outlined in the *Experimental* section. The data at pH 1 were used in the present investigation. The additional values are included for informational purposes only. It is not presumed that this figure represents other than a good approximation. The observations are similar to those described by Bull *et al.* (8), except that the current data were obtained for much higher acid concentrations.

Table III—Data Employed in Calculation of the Salt Parameter, k

Total Urea, M (U)	Complexed Urea, M (C)	Free Urea, M (U')	Solubility of Methyl Salicylate, mg./ml.: Complex-Free Solution ($S°$)	Solubility of Methyl Salicylate, mg./ml.: In Presence of Complex (S)	$-\log S°/S$
1	0.16	0.84	1.13	1.21	0.030
2	0.31	1.69	1.33	1.50	0.052
3	0.47	2.53	1.54	1.84	0.077
4	0.63	3.37	1.76	2.22	0.102
5	0.80	4.20	2.01	2.63	0.118

A problem in interpretation arises with the realization that these are not actually pH values. With a change in solvent from water to a urea–water mixture, the term pH loses its meaning, and there is no assurance that a pH meter is indicating hydrogen-ion activity. As a result, there are additional uncertainties in the assumptions made earlier with regard to the concentration of "free" or uncomplexed HCl. None of these uncertainties, however, greatly affects the findings of this investigation.

The determination of actual hydrogen-ion activities is beyond the scope of the present work. Nevertheless it is still possible to estimate the effect of changing solvents by using a modification of the Born equation (11). On substituting the appropriate dielectric constants, one finds that a 1-mv. change in potential may be expected on transferring a 1–1 electrolyte of given activity from water to a solvent having the dielectric constant of a 5 M urea solution. This is, admittedly, an oversimplification of a complex problem, but the treatment should convey the magnitude of the effect. In relation to the practice of equating pH and concentration, the effect would seem to be negligible.

While it would be desirable from the standpoint of establishing a more accurate value for the salt parameter, k, a precise knowledge of the "free" HCl concentration is unnecessary for the present purpose. In the final analysis, the term log $S°/S$ relates the ester solubilities in HCl–urea solutions. Through the relationship $S° = f(U—C)$, this term is found to be somewhat insensitive to variations in the concentration of uncomplexed acid. For example, a 25% error in "free" acid concentration produces only a 5% error in log $S°/S$ in 1 M urea. This is considerably smaller than the potential error if the salt effect is disregarded completely. In view of the results, it appears that the approximation, pH = "free" acid concentration, is reasonable. It is by no means precise. A more precise analysis will depend on a quantitative evaluation of the activity coefficients as a function of ionic strength and solvent composition.

CONCLUSION

It is well to emphasize that the model proposed in this paper is speculative. To indicate otherwise would presuppose intimate knowledge of a solvent system which is, to say the least, complex and little understood. The treatment, of necessity, is based on circumstantial evidence. However, the high degree of self-consistency within the model cannot be denied, and it would seem that any real judgment against it must await additional evidence. At this time, there appears to be little reason why a similar analysis cannot be applied to any aqueous urea solution, provided, of course, that the characteristic salt constant is known or determined.

REFERENCES

(1) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **56**, 370(1967).

(2) S. Bolton, *ibid.*, **52**, 1071(1963).

(3) D. M. Altwein, J. N. Delgado, and F. P. Cosgrove, *ibid.*, **54**, 603(1965).

(4) D. B. Wetlaufer, S. K. Malik, L. Stoller, and R. L. Coffin, *J. Amer. Chem. Soc.*, **86**, 508(1964).

(5) J. Setschenow, *Z. Physik. Chem.*, **4**, 117(1889).

(6) F. A. Long and W. F. McDevit, *Chem. Rev.*, **51**, 119(1952).

(7) V. E. Bower and R. A. Robinson, *J. Phys. Chem.*, **67**, 1524 (1963).
(8) H. B. Bull, K. Breese, G. L. Ferguson, and C. A. Swenson, *Arch. Biochem. Biophys.*, **104**, 297(1964).
(9) R. C. Paul and S. L. Chadha, *Spectrochim. Acta*, **23**, 1243 (1967).
(10) W. A. Hargraves and G. C. Kresheck, *J. Phys. Chem.*, **73**, 3249(1969).
(11) H. Harned and B. Owen, "Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold, New York, N. Y., 1958, p. 464.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 26, 1970, from the *Division of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CT 06268*
Accepted for publication June 10, 1970.
The work upon which this report is based was supported in part by funds provided by the Office of Water Resources, U. S. Department of the Interior, as authorized under the Water Resources Research Act of 1964.

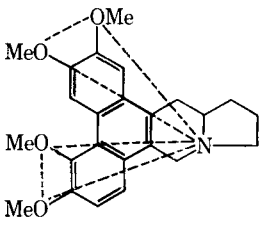
Common Receptor-Complement Feature among Some Antileukemic Compounds

KWANG-YUEN ZEE-CHENG and C. C. CHENG

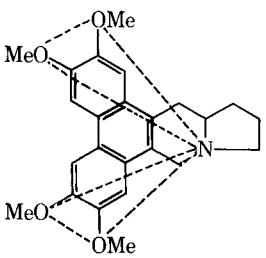
Abstract □ A common structural feature was found among some nonalkylating antileukemic agents such as aminopterin, anthramycin, 5-azacytidine, bisketopiperazines, camptothecin, cytosine arabinoside, daunomycin, demecolcine, emetine, glutarimide antibiotics, harringtonine, 6-mercaptopurine riboside, methotrexate, sangivamycin, streptonigrin, tylocrebrine, tylophorine, vinblastine, and vincristine. This structural feature consists of a triangulation composed of one nitrogen and two oxygen atoms with rather definite interatomic distances. This structural characteristic may contribute to the *in vivo* binding to one of the pertinent receptor sites involved in leukemia geneses.

Keyphrases □ Antileukemic compounds—common receptor complement □ Structural similarity, antileukemic agents—triangular pattern, nitrogen, oxygen atoms □ Oxygen, nitrogen pattern, interatomic distances—antileukemic activity

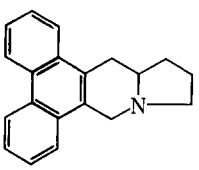
In connection with structure–activity studies of various oncolytic agents, a common structural feature was noted among a number of antileukemic agents. The purpose of this paper is to present a preliminary account of this observation.



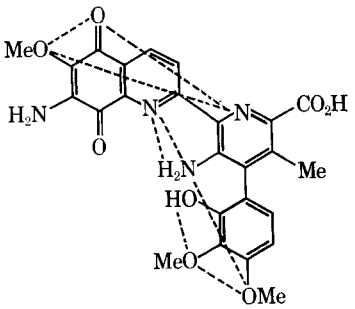
I



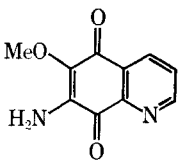
II



III



IV

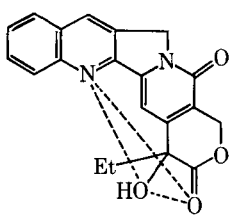


V

THEORETICAL

The tylophora alkaloids (1), tylocrebrine (I) and tylophorine (II), were found to possess antileukemic activity against leukemia L-1210 in mice (2). The nucleus of these alkaloids, phenanthro-[9,10:6′,7′]indolizidine (III) (3), however, is devoid of the activity (4) exhibited by the polysubstituted methoxy derivatives.

The antibiotic streptonigrin (IV), the structure of which contains an *o*-aminoquinone unit (5), exhibits a broad spectrum of inhibitory activity against a number of leukemias, lymphomas, carcinomas, and other tumor systems (6–13). Although the *o*-aminoquinone unit is also present (5) in two other types of antitumor antibiotics (mitomycin C and actinomycin D, for example), a synthetic compound, 7-amino-6-methoxy-5,8-quinolinedione (V) (14), which possesses a partial structure of this antibiotic in that it contains the *o*-aminoquinone unit, failed to retain the original antileukemic activity.[1]



VI

[1] Test results were provided by Dr. Harry B. Wood and Robert B. Ing of the Cancer Chemotherapy National Service Center, National Cancer Institute, U. S. Public Health Service.

(7) V. E. Bower and R. A. Robinson, *J. Phys. Chem.*, **67**, 1524 (1963).
(8) H. B. Bull, K. Breese, G. L. Ferguson, and C. A. Swenson, *Arch. Biochem. Biophys.*, **104**, 297(1964).
(9) R. C. Paul and S. L. Chadha, *Spectrochim. Acta*, **23**, 1243 (1967).
(10) W. A. Hargraves and G. C. Kresheck, *J. Phys. Chem.*, **73**, 3249(1969).
(11) H. Harned and B. Owen, "Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold, New York, N. Y., 1958, p. 464.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 26, 1970, from the *Division of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CT 06268*
Accepted for publication June 10, 1970.
The work upon which this report is based was supported in part by funds provided by the Office of Water Resources, U. S. Department of the Interior, as authorized under the Water Resources Research Act of 1964.

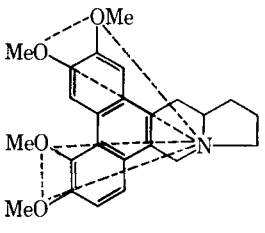
Common Receptor-Complement Feature among Some Antileukemic Compounds

KWANG-YUEN ZEE-CHENG and C. C. CHENG

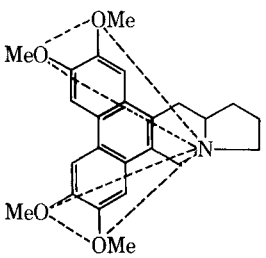
Abstract □ A common structural feature was found among some nonalkylating antileukemic agents such as aminopterin, anthramycin, 5-azacytidine, bisketopiperazines, camptothecin, cytosine arabinoside, daunomycin, demecolcine, emetine, glutarimide antibiotics, harringtonine, 6-mercaptopurine riboside, methotrexate, sangivamycin, streptonigrin, tylocrebrine, tylophorine, vinblastine, and vincristine. This structural feature consists of a triangulation composed of one nitrogen and two oxygen atoms with rather definite interatomic distances. This structural characteristic may contribute to the *in vivo* binding to one of the pertinent receptor sites involved in leukemia geneses.

Keyphrases □ Antileukemic compounds—common receptor complement □ Structural similarity, antileukemic agents—triangular pattern, nitrogen, oxygen atoms □ Oxygen, nitrogen pattern, interatomic distances—antileukemic activity

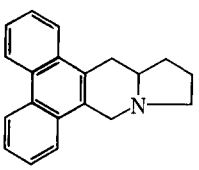
In connection with structure–activity studies of various oncolytic agents, a common structural feature was noted among a number of antileukemic agents. The purpose of this paper is to present a preliminary account of this observation.



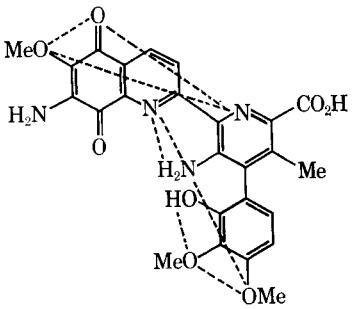
I



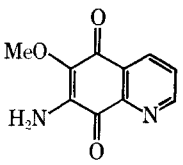
II



III



IV

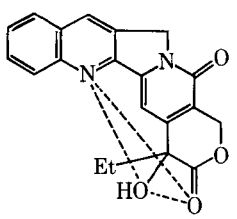


V

THEORETICAL

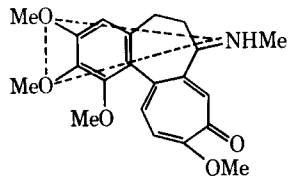
The tylophora alkaloids (1), tylocrebrine (I) and tylophorine (II), were found to possess antileukemic activity against leukemia L-1210 in mice (2). The nucleus of these alkaloids, phenanthro-[9,10:6′,7′]indolizidine (III) (3), however, is devoid of the activity (4) exhibited by the polysubstituted methoxy derivatives.

The antibiotic streptonigrin (IV), the structure of which contains an *o*-aminoquinone unit (5), exhibits a broad spectrum of inhibitory activity against a number of leukemias, lymphomas, carcinomas, and other tumor systems (6–13). Although the *o*-aminoquinone unit is also present (5) in two other types of antitumor antibiotics (mitomycin C and actinomycin D, for example), a synthetic compound, 7-amino-6-methoxy-5,8-quinolinedione (V) (14), which possesses a partial structure of this antibiotic in that it contains the *o*-aminoquinone unit, failed to retain the original antileukemic activity.[1]

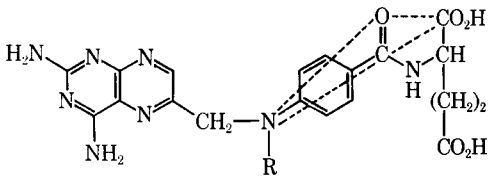


VI

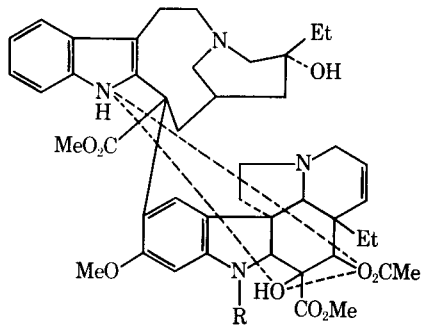
[1] Test results were provided by Dr. Harry B. Wood and Robert B. Ing of the Cancer Chemotherapy National Service Center, National Cancer Institute, U. S. Public Health Service.



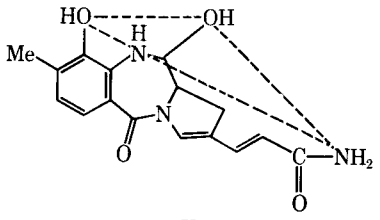
VII



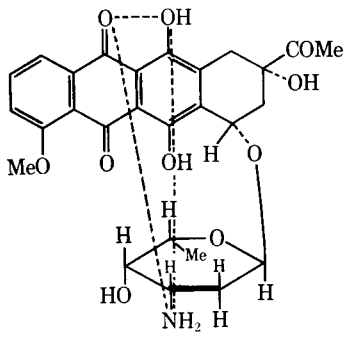
VIII*a*, R = H
VIII*b*, R = Me



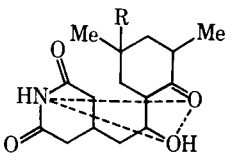
IX*a*, R = Me
IX*b*, R = CHO



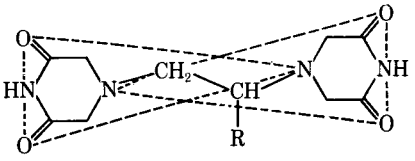
X



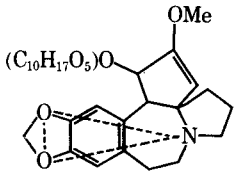
XI



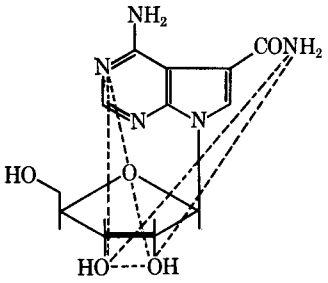
XII*a*, R = H
XII*b*, R = OAc
XII*c*, R = OH



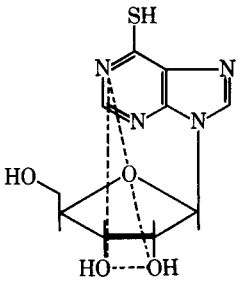
XIII



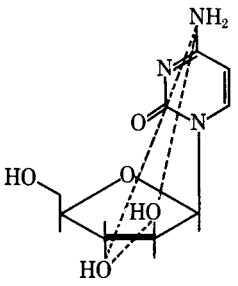
XIV



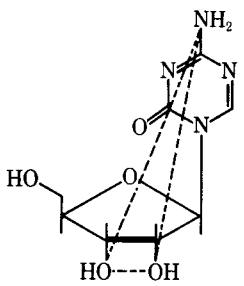
XV



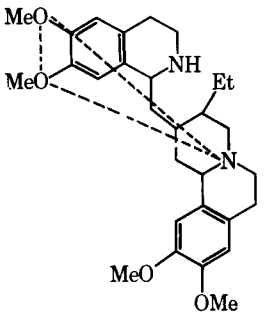
XVI



XVII



XVIII



XIX

It has been reported that the hydroxyl and carbonyl groups presented in the lactone ring portion of camptothecin (VI) (15–19) may account for the antileukemic activity displayed by this alkaloid. Modification or replacement of these functions resulted in deprivation of its oncolytic property (15, 18, 19).

A comparative study of the structure of these compounds, using the Briegleb (20)–Stuart (21) molecular models, revealed that there is a common atomic arrangement among these very differently constituted molecules. Three electronegative atoms, containing at least one lone pair of electrons—one nitrogen and two oxygen atoms—form a triangle with very distinct parameters. The interatomic distances are rather definite, as shown in Fig. 1.

The triangular arrangments in Compounds I, II, IV, and VI are indicated by dotted lines. It is obvious that no such relationship could be found in Compounds III and V.

This interesting observation immediately prompted an examination of the structure of other compounds with antileukemic activity (at least active *versus* L-1210). This included demecolcine (VII) (22–24); aminopterin (VIII*a*) (25–28); methotrexate (VIII*b*) (27) [many related compounds such as dichloromethotrexate, 2,4-diaminopyrimidine analogs of aminopterin and methotrexate, and

Table I—Interatomic Distance Measurements (Å) of Some Nonalkylating Antileukemic Compounds

Compounds	N—O_1	N—O_2	O_1—O_2
Tylocrebrine	7.50–7.71	8.75–8.87	3.01–3.35
	7.38	8.04	3.01–3.35
Tylophorine	7.50–7.71	8.75–8.87	3.01–3.35
	6.84	8.04	3.01–3.35
Streptonigrin	7.37[a]	8.71[a]	3.05–3.35
	7.37[a]	8.71[a]	3.05–3.35
Camptothecin	6.02–7.05	8.70–9.71	3.35
Demecolcine	7.03–7.71	8.38–8.71	3.03–3.36
Aminopterin	6.37–6.70	7.70–9.37	2.69–4.03
Methotrexate	6.37–6.70	8.03–9.03	2.69–4.03
Vinblastine	6.70	8.71	3.36
Vincristine	6.70	8.71	3.36
Anthramycin	8.04–8.36	8.71–9.05	3.01–3.35
Daunomycin	7.05–10.20	8.36–11.40	2.70
Glutarimide antibiotics	6.70–7.05	8.40–9.71	3.05–3.35
Bisketopiperazines	7.04[a]	7.85–8.38	4.00–4.35
Harringtonine	7.04	7.90	3.08
Sangivamycin	7.05[a]	8.70[a]	3.40
	6.70[a]	8.70[a]	3.40
6-MP riboside	7.55[a]	8.70[a]	3.05
Cytosine arabinoside	6.36–7.05	8.70–9.36	3.68–4.02
5-Azacytidine	7.05[a]	8.70[a]	3.03
Emetine	7.03[a]	8.50[a]	3.30

[a] Interatomic distance of conformed conformation.

quinazoline antifolates, which can have similar triangular arrangements, also displayed antileukemic activity (29–35)]; vinblastine (IX*a*) and vincristine (IX*b*) (36–44); the methyl ether of anthramycin (X) (45–49); daunomycin (XI) (50–56); the glutarimide antibiotics cycloheximide (XII*a*, actidione), E-72 (XII*b*), and streptovitacin A (XII*c*) (57–69); bisdiketopiperazines (XIII, R = H, CH_3) (70, 71); harringtonine (XIV) (72); sangivamycin (XV) (73–75) [toyocamycin (76), which lacks the side-chain amide group, is devoid of antileukemic activity]; 6-MP riboside (XVI) (77–80) [the active form of 6-MP riboside, as well as for 6-MP, is 6-MP ribonuclotide (80)] and related compounds (80–83); cytosine arabinoside (XVII) (84–89); 5-azacytidine (XVIII) (90–95); and emetine (XIX) (96). The interatomic distances (Table I) indicated by the dotted lines, determined from the Briegleb (20)–Stuart (21) molecular models, with minor deviations in the values of anthramycin and harringtonine, are all intriguingly within the limitations of the values shown in Fig. 1.

The relationship between the triangular pattern and antileukemic activity is further substantiated by the reports that neither daunomycinone (the aglycone of daunomycin) nor the amino sugar daunosamine possesses antileukemic activity,[1] and that the indole and indoline units of vinblastine and vincristine, when separated, are also inactive (97, 98). Triangular patterns of different sizes and shapes have been reported in explaining sites of other receptors (99–101) (the muscarinic, nicotinic, histamine, serotonin, inflammatory, *etc.*). Although some of the triangulations are also composed of one nitrogen and two oxygen atoms, the compounds were inactive against leukemia L-1210.[1] The interatomic distances in these cases differ markedly from those illustrated in Fig. 1.

DISCUSSION

It appears that the triangular pattern, which is present among the aforementioned antileukemic compounds of both synthetic and natural origin, may actually contribute in the binding to one of the pertinent receptor sites (99–107) in certain biopolymers (proteins, polysaccharides, nucleic acids, *etc.*) involved in leukemia geneses. Thus, it may result in inhibiting the active site of the enzymes, in altering the specificity of enzyme systems, in disturbing the template molecules in transcription process, in changing the permeability of certain biological membranes, or in causing other interruptions of biological functions. Admittedly, the present observation is quite empirical, and the facts as presented are oversimplified. Additional work, such as molecular orbital calculations of preferred conformations of molecules and electron-density distribution of atoms, is definitely needed to verify the finding. Furthermore, the triangular pattern cannot be used to explain the antileukemic activity of some other compounds including hydroxyurea,[2] ellipticine,[2] and biological alkylating agents. Nevertheless, perhaps the receptor-complement feature can be used to explore the *in vivo* drug interaction in greater detail. It can also be regarded, among other considerations,[3] as one of the working hypotheses in designing better and more useful antileukemic drugs.

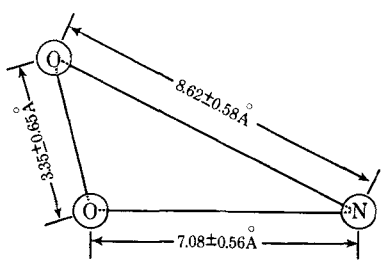


Figure 1—N—O—O triangular pattern.

REFERENCES

(1) T. R. Govindachari, in "Tylophora Alkaloids," in The Alkaloids, vol. 9, R. H. F. Manske, Ed., Academic, New York, N. Y., 1967, p. 517.

(2) E. Gellert and R. Rudzats, *J. Med. Chem.* **7**, 361(1964).

(3) T. R. Govindachari, M. V. Lakshmikantham, K. Nagarajan, and B. R. Pai, *Tetrahedron*, **4**, 311(1958).

(4) K. Y. Zee-Cheng and C. C. Cheng, *J. Med. Chem.* **12**, 157 (1969).

(5) K. V. Rao, K. Biemann, and R. B. Woodward, *J. Amer. Chem. Soc.*, **85**, 2532(1963).

(6) K. V. Rao and W. P. Cullen, in "Antibiotics Annual," H. Welch and Marti-Ibañez, Eds., Interscience, New York, N. Y., 1960, p. 950.

(7) J. J. Oleson, L. A. Calderella, K. J. Mjos, A. R. Reith, R. S. Thie, and I. Toplin, *Antibiot. Chemother.*, **11**, 158(1961).

(8) W. L. Wilson, C. Labra, and E. Barrist, *ibid.*, **11**, 147 (1961).

(9) W. S. Marsh, A. L. Garretson, and E. M. Wesel, *ibid.*, **11**, 151(1961).

(10) S. L. Rivers, R. M. Whittington, and T. J. Medrek, *Cancer Chemother. Rep.*, **46**, 17(1965).

(11) P. F. Nora, J. C. Kukral, T. Soper, and F. W. Preston, *ibid.*, **48**, 41(1965).

(12) M. N. Harris, T. J. Medrek, F. M. Golomb, S. L. Gumport, A. H. Postel, and J. C. Wright, *Cancer*, **18**, 49(1965).

(13) D. S. Miller, J. Laszlo, K. S. McCarty, W. R. Guild, and P. Hochstein, *Cancer Res.*, **27**, 632(1967).

(14) T. K. Liao, W. H. Nyberg, and C. C. Cheng, *Angew. Chem.*, **79**, 100(1967).

(15) M. E. Wall, M. C. Wani, C. E. Cook, K. H. Palmer, A. T. McPhail, and G. A. Sim, *J. Amer. Chem. Soc.*, **88**, 3888(1966).

(16) A. T. McPhail and G. A. Sim, *J. Chem. Soc. (B)*, **1968**, 923.

(17) R. E. Perdue, Jr., M. E. Wall, J. L. Hartwell, and B. J. Abbott, *Lloydia*, **31**, 229(1968).

(18) M. E. Wall and M. C. Wani, *Abstracts of Papers of 153rd Meeting of ACS*, Miami Beach, Fla., April 1967, M6; M. E. Wall and M. C. Wani, private communication.

(19) J. L. Hartwell and B. J. Abbott, *Advan. Pharmacol. Chemother.*, **7**, 117(1969).

(20) G. Briegleb, *Angew. Chem.*, **62**, 264(1950); *Fortschr. Chem. Forsch.*, **1**, 642(1950).

(21) A. Stuart, "Die Struktur des freien Molekuels," Springer-Verlag, Berlin, Germany, 1952.

(22) S. Moeschlin, H. Meyer, and A. Lightman, *Schweiz. Med. Wochenschr.*, **83**, 990(1953).

[2] Although these compounds may possibly act through different mechanisms, it is not improbable that they may undergo *in vivo* structural conversions to yield derivatives possessing the triangular characteristics.

[3] For example, geometric effects, electronic effects, lipid–aqueous partition coefficients, redox potentials, basicity and acidity, and *in vivo* stability and reaction rates.

(23) B. J. Leonard and J. F. Wilkinson, *Brit. Med. J.*, **1,** 874 (1955).
(24) L. Vercillo and S. Esposito, *Haematologica*, **43,** 345(1958).
(25) D. R. Seeger, J. M. Smith, Jr., and M. E. Hultquist, *J. Amer. Chem. Soc.*, **69,** 2567(1947).
(26) S. Farber, L. K. Diamond, R. D. Mercer, R. F. Sylvester, Jr., and J. A. Wolff, *N. Engl. J. Med.* **238,** 787(1948).
(27) D. R. Seeger, D. B. Cosulich, J. M. Smith, Jr., and M. E. Hultquist, *J. Amer. Chem. Soc.*, **71,** 1753(1949).
(28) A. Goldin, E. M. Greenspan, J. M. Venditti, and E. B. Schoenbach, *J. Nat. Cancer Inst.*, **12,** 987(1952).
(29) S. Farber, R. Toch, E. M. Sears, and D. Pinkel, *Advan. Cancer Res.*, **4,** 1(1956).
(30) L. Delmonte and T. H. Jukes, *Pharmacol. Rev.*, **14,** 91 (1962).
(31) J. R. Bertino, *Cancer Res.*, **25,** 1614(1965).
(32) L. R. Schroeder, *Proc. Amer. Ass. Cancer Res.*, **3,** 267 (1961).
(33) E. Frei, III, C. L. Spurr, C. O. Brindley, O. Selawry, J. F. Holland, D. P. Rall, L. R. Wasserman, B. Hoogstraten, B. I. Shnider, O. R. McIntyre, L. B. Matthews, Jr., and S. P. Miller, *Clin. Pharmacol. Ther.*, **6,** 160(1965).
(34) L. T. Weinstock, D. E. O'Brien, and C. C. Cheng, *J. Med. Chem.*, **11,** 1238(1968).
(35) D. J. Hutchison, *Cancer Chemother. Rep.*, **52,** 697(1968).
(36) R. L. Noble, C. T. Beer, and J. H. Cutts, *Biochem. Pharmacol.*, **1,** 347(1958).
(37) M. Gorman, N. Neuss, and G. H. Svoboda, *J. Amer. Chem. Soc.*, **81,** 4745(1959).
(38) N. Neuss, M. Gorman, G. H. Svoboda, G. Maciak, and C. T. Beer, *J. Amer. Chem. Soc.*, **81,** 4754(1959).
(39) M. E. Hodes, R. J. Rohn, and W. H. Bond, *Cancer Res.*, **20,** 1041(1960); *Can. Cancer Conf.*, **4,** 373(1962).
(40) N. Neuss, M. Gorman, H. E. Boaz, and N. J. Cone, *J. Amer. Chem. Soc.*, **84,** 1509(1962).
(41) R. A. Bohannon, D. G. Miller, and H. D. Diamond, *Cancer Res.*, **23,** 613(1963).
(42) I. S. Johnson, J. G. Armstrong, M. Gorman, and J. P. Burnett, Jr., *Cancer Res.*, **23,** 1390(1963).
(43) N. Neuss, M. Gorman, W. Hargrove, N. J. Cone, K. Biemann, G. Büchi, and R. E. Manning, *J. Amer. Chem. Soc.*, **86,** 1440(1964).
(44) J. W. Moncrief and W. N. Lipscomb, *ibid.*, **87,** 4963(1965).
(45) M. D. Tendler and S. Korman, *Nature*, **199,** 501(1963).
(46) W. Leimgruber, V. Stefanović, F. Schenker, and J. Berger, *J. Amer. Chem. Soc.*, **87,** 5791(1965).
(47) W. Leimgruber, A. D. Batcho, and F. Schenker, *J. Amer. Chem. Soc.*, **87,** 5793(1965).
(48) E. Grunberg, H. N. Prince, E. Titsworth, G. Beskid, and M. D. Tendler, *Chemotherapia*, **11,** 249(1966).
(49) R. H. Adamson, L. G. Hart, V. T. DeVita, and V. T. Oliverio, *Cancer Res.*, **28,** 343(1968).
(50) A. Green, C. Spella, A. DiMarco, and G. Canevazzi, *Gazz. Microbiol.*, **11,** 109(1963).
(51) A. DiMarco, M. Gaetani, L. Dorigotti, M. Soldati, and O. Bellini, *Tumori*, **49,** 203(1963).
(52) C. Tan, H. Tasaka, K. P. Yu, M. L. Murphy, and D. A. Karnofsky, *Cancer*, **20,** 333(1967).
(53) C. Jacquillot, M. Weil, Y. Najaen, J. Tanzer, P. Sortholary, M. Boiron, and J. Bernard, *Arzneim.-Forsch.*, **17,** 955(1967).
(54) A. DiMarco, in "Antibiotics," vol. I, D. Gottlieb and P. D. Shaw, Eds., Springer-Verlag, Berlin, Germany, 1967, p. 190.
(55) F. Arcamone, G. Franceschi, P. Orezzi, S. Penco, and R. Mondelli, *Tetrahedron Lett.*, **1968,** 3349.
(56) F. Arcamone, G. Cassinelli, G. Franceschi, P. Orezzi, and R. Mondelli, *ibid.*, **1968,** 3353.
(57) A. J. Whiffen, N. Bohonos, and R. L. Emerson, *J. Bacteriol.*, **52,** 610(1946).
(58) J. H. Ford and B. E. Leach, *J. Amer. Chem. Soc.*, **70,** 1223 (1948).
(59) A. J. Whiffen, *J. Bacteriol.*, **56,** 283(1948).
(60) E. C. Kornfeld, R. G. Jones, and T. V. Parke, *J. Amer. Chem. Soc.*, **71,** 150(1949).
(61) E. J. Eisenbraun, J. Osiecki, and C. Djerassi, *J. Amer. Chem. Soc.*, **80,** 1261(1958).
(62) R. R. Herr, *J. Amer. Chem. Soc.*, **81,** 2595(1959).
(63) F. R. White, *Cancer Chemother. Rep.*, **5,** 48(1959).
(64) L. L. Sensenbrenner, *ibid.*, **5,** 65(1959).
(65) K. V. Rao and W. P. Cullen, *J. Amer. Chem. Soc.*, **82,** 1127 (1960).
(66) F. Johnson, W. D. Gurowitz, and N. A. Starkovsky, *Tetrahedron Lett.*, **1962,** 1173.
(67) F. Johnson, N. A. Starkovsky, and A. A. Carlson, *J. Amer. Chem. Soc.*, **87,** 4612(1965).
(68) A. Goldin, A. A. Serpick, and N. Mantel, *Cancer Chemother. Rep.*, **50,** 173(1966).
(69) F. Johnson, N. A. Starkovsky, A. C. Paton, and A. A. Carlson, *J. Amer. Chem. Soc.*, **88,** 149(1966).
(70) A. M. Creighton, K. Hellmann, and S. Whitecross, *Nature*, **222,** 384(1969).
(71) A. M. Creighton and G. D. Birnie, *Biochem. J.*, **114,** 58p (1969).
(72) R. G. Powell, D. Weisleder, C. R. Smith, Jr., and I. A. Wolff, *Abstracts of 158th National ACS Meeting*, New York, N. Y. September 1969, MEDI-61.
(73) K. V. Rao and D. W. Renn, in "Antimicrobial Agents and Chemotherapy," J. C. Sylvester, Ed., Amer. Soc. Microbiology, Ann Arbor, Mich., 1963, p. 77.
(74) K. V. Rao, *Abstracts of 150th National ACS Meeting*, Atlantic City, N. J., September 1965, 24P.
(75) R. L. Tolman, R. K. Robins, and L. B. Townsend, *J. Amer. Chem. Soc.*, **90,** 524(1968).
(76) K. Anzai, G. Nakamura, and S. Suzuki, *J. Antibiot.*, **10A,** 201(1957).
(77) G. B. Elion, E. Burgi, and G. H. Hitchings, *J. Amer. Chem. Soc.*, **74,** 411(1952).
(78) J. H. Burchenal, R. R. Ellison, M. L. Murphy, D. A. Karnofsky, M. P. Sykes, C. T. C. Tan, A. C. Mermann, M. Yuceoglu, W. P. L. Meyers, I. Krakoff, and N. Alberstadt, *Ann. N. Y. Acad. Sci.*, **60,** 359(1954).
(79) B. E. Hall, M. D. Richards, F. M. Willet, and T. V. Feichtmeir, *ibid.*, **60,** 374(1954).
(80) D. A. Clarke, G. B. Elion, G. H. Hitchings, and C. C. Stock, *Cancer Res.*, **18,** 445(1958).
(81) J. J. Fox, I. Wempen, A. Hampton, and I. L. Doerr, *J. Amer. Chem. Soc.*, **80,** 1669(1958).
(82) M. L. Murphy, C. T. C. Tan, R. R. Ellison, D. A. Karnofsky, and J. H. Burchenal, *Proc. Amer. Ass. Cancer Res.*, **2,** 36(1955).
(83) J. H. Krakoff, R. R. Ellison, and C. T. C. Tan, *ibid.*, **3,** 34(1959).
(84) L. I. Pizer and S. S. Cohen, *J. Biol. Chem.*, **235,** 2387 (1960).
(85) J. S. Evans, E. A. Musser, G. D. Mengel, K. R. Forsbald, and J. H. Hunter, *Proc. Soc. Exp. Biol. Med.*, **106,** 350(1961).
(86) M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.*, **11,** 423(1962).
(87) R. W. Carey and R. E. Ellison, *Clin. Res.*, **13,** 337(1965).
(88) J. P. Howard, N. Cevik, and M. L. Murphy, *Cancer Chemother. Rep.*, **50,** 287(1966).
(89) P. J. Burke, R. E. Lenhard, and A. H. Owens, Jr., *ibid.*, **52,** 305(1968).
(90) A. Píškala and F. Šorm, *Collect, Czech. Chem. Commun.*, **29,** 2060(1964).
(91) F. Šorm, A. Píškala, A. Čihák, and J. Veselý, *Experientia*, **20,** 202(1964).
(92) J. Veselý and F. Šorm, *Neoplasma*, **12,** 3(1965).
(93) A. Čihák, J. Veselý, and F. Šorm, *Biochim. Biophys. Acta*, **108,** 516(1965).
(94) J. M. Venditti and B. J. Abbott, *Lloydia*, **30,** 332(1967).
(95) J. Veselý, A. Čihák, and F. Šorm, *Cancer Res.*, **28,** 1995 (1968).
(96) R. B. Livingston, "Phase I Studies with Emetine," unpublished work.
(97) I. S. Johnson, H. F. Wright, G. H. Svoboda, and J. Vlantis, *Cancer Res.*, **20,** 1016(1960).
(98) M. Gorman, N. Neuss, and K. Biemann, *J. Amer. Chem. Soc.*, **84,** 1058(1962).
(99) L. B. Kier, *Mol. Pharmacol.*, **3,** 487(1967).
(100) L. B. Kier, *Battelle Techn. Rev.*, **6,** 9(1968).
(101) L. B. Kier, *J. Pharm. Sci.*, **59,** 112(1970).
(102) E. W. Gill, in "Drug Receptor Interactions" in Progress in Medicinal Chemistry, vol. 4, G. P. Ellis and G. B. West, Eds., Butterworths, London, England, 1965, p. 39.
(103) N. Y. Khromov-Borrisov and M. J. Michelson, *Pharmacol.*

Rev., **18**, 1051(1966).
(104) H. G. Mautner, *ibid.*, **19**, 107(1967).
(105) D. R. Waud, *ibid.*, **20**, 49(1968).
(106) S. Ehrenpreis, J. H. Fleisch, and T. W. Mittag, *ibid.*, **21**, 131(1969).
(107) H. G. Mautner, *Ann. Rep. Med. Chem.*, 230(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Midwest Research Institute, Kansas City, MO 64110*

Accepted for publication July 1, 1970.

This investigation was supported by Contract PH 43-65-94 with Chemotherapy, National Cancer Institute, National Institutes of Health.

The authors thank Dr. Harry B. Wood, Jr., and Dr. Kenneth Fountain for their interest and encouragement, and Dr. Henry G. Mautner and Dr. Louis H. Goodson for many helpful discussions and suggestions.

Theoretical Justification of Reciprocal Rate Plots in Studies of Water Vapor Transmission through Films

DANE O. KILDSIG, RONALD L. NEDICH*, and GILBERT S. BANKER

Abstract □ A theoretical equation has been developed justifying the graphical representation of vapor permeation data by 1/rate *versus* film thickness plots. The permeability coefficient for the film may be determined from the slope of this plot and has units in square centimeters per second. The intercept at zero film thickness is dependent upon the geometry of the experimental design and the diffusion coefficient for the vapor within the diffusion cell. The derivation of the equation assumes a nonequilibrium condition for water vapor in the diffusion cell, as well as the existence of steady-state conditions.

Keyphrases □ Water vapor—transmission through films □ Film transmission—water vapor □ Reciprocal rate plots, vapor transmission—theoretical justification □ Polymeric, unplasticized films—water vapor transmission

The passage of water vapor through polymer films, with reference to the free films having potential application as tablet film coatings, has been reported in the pharmaceutical literature (1–3). In the first of these studies (1), a vacuum was created on one side of the film so that a pressure difference existed across the film. More recently (2, 3), the transfer of water vapor through films has been studied in which a water vapor pressure difference existed but in which the total pressure, atmospheric, was the same on both sides of the film. In these investigations (2, 3), a linear relationship was found between the reciprocal of the rate of water vapor permeation and film thickness. In one case (2), the authors stated: "An interesting relationship is observed when $(w/t)^{-1}$ is plotted against thickness of film; although no theoretical basis for such a plot can be proposed at the present time, we feel it is worth presenting." The research described by this study and the accompanying theory present a theoretical justification for the linear relationship between the reciprocal of rate of water vapor permeation and film thickness.

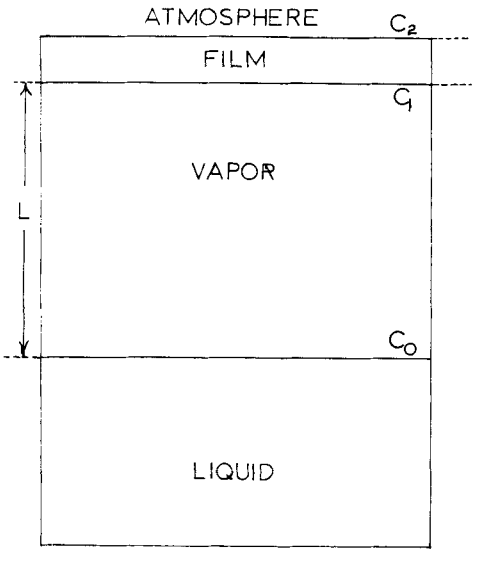


Figure 1—*Schematic diagram of water vapor transmission cell depicting water concentrations existing at various surfaces.* C_0 = *concentration of molecules above liquid surface,* C_1 = *concentration of molecules at inside film surface, and* C_2 = *concentration of molecules at outside film surface.*

THEORY

Previous investigations have used equations that relate the rate of vapor transfer to the water vapor pressure differential existing across the film. Alternatively, an expression may be used relating this rate to the concentration difference existing across the film:

$$\frac{R = PA\,\Delta C}{a} \qquad \text{(Eq. 1)}$$

where R is the rate of permeation, P is the permeability coefficient, A is the area of the film, a is its thickness, and ΔC is the water vapor concentration difference across the film. ΔC may be expressed in any suitable units such as molecules. The type of transmission cell used in previous investigations (2, 3) is shown in Fig. 1, where $\Delta C = C_1 - C_2$.

In calculating ΔC, the diffusion of water vapor from the surface of the liquid to the polymer film must first be considered. Because the distance between the liquid and the film influences the rate of water vapor transmission through the film (4), it can be assumed that the vapor pressure at the film surface is not in equilibrium with the liquid surface, and that a vapor pressure difference exists through this distance. The diffusion through this distance is given by Fick's one-dimensional equation:

$$\frac{\partial C}{\partial t} = \frac{D\partial^2 C}{\partial x^2} \qquad \text{(Eq. 2)}$$

Rev., **18**, 1051(1966).
(104) H. G. Mautner, *ibid.*, **19**, 107(1967).
(105) D. R. Waud, *ibid.*, **20**, 49(1968).
(106) S. Ehrenpreis, J. H. Fleisch, and T. W. Mittag, *ibid.*, **21**, 131(1969).
(107) H. G. Mautner, *Ann. Rep. Med. Chem.*, 230(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Midwest Research Institute, Kansas City, MO 64110*

Accepted for publication July 1, 1970.

This investigation was supported by Contract PH 43-65-94 with Chemotherapy, National Cancer Institute, National Institutes of Health.

The authors thank Dr. Harry B. Wood, Jr., and Dr. Kenneth Fountain for their interest and encouragement, and Dr. Henry G. Mautner and Dr. Louis H. Goodson for many helpful discussions and suggestions.

Theoretical Justification of Reciprocal Rate Plots in Studies of Water Vapor Transmission through Films

DANE O. KILDSIG, RONALD L. NEDICH*, and GILBERT S. BANKER

Abstract □ A theoretical equation has been developed justifying the graphical representation of vapor permeation data by 1/rate *versus* film thickness plots. The permeability coefficient for the film may be determined from the slope of this plot and has units in square centimeters per second. The intercept at zero film thickness is dependent upon the geometry of the experimental design and the diffusion coefficient for the vapor within the diffusion cell. The derivation of the equation assumes a nonequilibrium condition for water vapor in the diffusion cell, as well as the existence of steady-state conditions.

Keyphrases □ Water vapor—transmission through films □ Film transmission—water vapor □ Reciprocal rate plots, vapor transmission—theoretical justification □ Polymeric, unplasticized films—water vapor transmission

The passage of water vapor through polymer films, with reference to the free films having potential application as tablet film coatings, has been reported in the pharmaceutical literature (1–3). In the first of these studies (1), a vacuum was created on one side of the film so that a pressure difference existed across the film. More recently (2, 3), the transfer of water vapor through films has been studied in which a water vapor pressure difference existed but in which the total pressure, atmospheric, was the same on both sides of the film. In these investigations (2, 3), a linear relationship was found between the reciprocal of the rate of water vapor permeation and film thickness. In one case (2), the authors stated: "An interesting relationship is observed when $(w/t)^{-1}$ is plotted against thickness of film; although no theoretical basis for such a plot can be proposed at the present time, we feel it is worth presenting." The research described by this study and the accompanying theory present a theoretical justification for the linear relationship between the reciprocal of rate of water vapor permeation and film thickness.

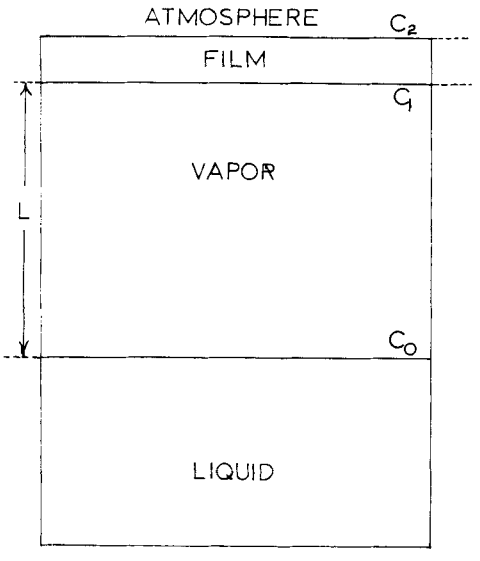


Figure 1—*Schematic diagram of water vapor transmission cell depicting water concentrations existing at various surfaces.* C_0 = *concentration of molecules above liquid surface,* C_1 = *concentration of molecules at inside film surface, and* C_2 = *concentration of molecules at outside film surface.*

THEORY

Previous investigations have used equations that relate the rate of vapor transfer to the water vapor pressure differential existing across the film. Alternatively, an expression may be used relating this rate to the concentration difference existing across the film:

$$\frac{R = PA\,\Delta C}{a} \qquad \text{(Eq. 1)}$$

where R is the rate of permeation, P is the permeability coefficient, A is the area of the film, a is its thickness, and ΔC is the water vapor concentration difference across the film. ΔC may be expressed in any suitable units such as molecules. The type of transmission cell used in previous investigations (2, 3) is shown in Fig. 1, where $\Delta C = C_1 - C_2$.

In calculating ΔC, the diffusion of water vapor from the surface of the liquid to the polymer film must first be considered. Because the distance between the liquid and the film influences the rate of water vapor transmission through the film (4), it can be assumed that the vapor pressure at the film surface is not in equilibrium with the liquid surface, and that a vapor pressure difference exists through this distance. The diffusion through this distance is given by Fick's one-dimensional equation:

$$\frac{\partial C}{\partial t} = \frac{D\partial^2 C}{\partial x^2} \qquad \text{(Eq. 2)}$$

where D is the diffusion coefficient for water vapor in air. If a steady state is assumed,

$$\frac{\partial C}{\partial t} = 0 \qquad \text{(Eq. 3)}$$

Therefore,

$$\frac{D\partial^2 C}{\partial x^2} = 0 \qquad \text{(Eq. 4)}$$

or

$$\frac{\partial C}{\partial x} = \text{constant} \qquad \text{(Eq. 5)}$$

$\partial C/\partial x$ may be related to the concentration of water vapor by the equation

$$\frac{\partial C}{\partial x} = \frac{C_1 - C_0}{L} \qquad \text{(Eq. 6)}$$

where L is the distance between the liquid surface and the film, and C_0 is the equilibrium concentration of water molecules above the liquid surface (Fig. 1). The steady-state assumption may be justified by observing the rate dependence on the liquid–film distance and the linearity of the rate of water vapor transmission.

The quantity of water that has evaporated and diffused, W, during time t is given by

$$W = \frac{-DA't(C_1 - C_0)}{L} \qquad \text{(Eq. 7)}$$

where A' is the area of the liquid surface.

Because the diffusion coefficient, D, may be dependent upon the concentration difference, $C_1 - C_0$ (5), the diffusion coefficient is more accurately represented by the mean diffusion coefficient, $\bar{D}$, of the form

$$\bar{D} = \frac{1}{C_1 - C_0}\int_{C_0}^{C_1} D(c)\,dc \qquad \text{(Eq. 7a)}$$

In the absence of data quantifying the concentration dependence of D on water vapor concentration for the water vapor–air system, only the observed mean value of the diffusion coefficient, $\bar{D}$, can be determined.

The concentration of water vapor at the inside surface of the film, C_1, may then be calculated from the equation:

$$C_1 = C_0 - \frac{WL}{\bar{D}A't} \qquad \text{(Eq. 8)}$$

If the effective film surface area is assumed to be equal to the liquid surface area, Eq. 8 may be written as

$$C_1 = C_0 \frac{WL}{-\bar{D}At} \qquad \text{(Eq. 9)}$$

Further assumption of steady-state diffusion within the diffusion cell implies that $W/t = R$, where the symbols are as previously defined. As a result, Eq. 9 becomes

$$C_1 = C_0 \frac{RL}{-\bar{D}A} \qquad \text{(Eq. 10)}$$

If C_2, the concentration of water outside the film, is maintained at zero, $\Delta C = C_1$. Therefore,

$$\Delta C = C_0 \frac{RL}{-\bar{D}A} \qquad \text{(Eq. 11)}$$

Substituting this relationship for ΔC into Eq. 1 and taking the reciprocal yield

$$\frac{1}{R} = \frac{a}{PAC_0} + \frac{L}{\bar{D}AC_0} \qquad \text{(Eq. 12)}$$

It can be seen from this equation that a plot of 1/rate of permeation *versus* film thickness should be linear with a slope of $1/PAC_0$ and an intercept of $L/\bar{D}AC_0$.

Table I—Description of the Polymers Employed

Viscosity Grade, cps.	Molecular Weight, M_n	—Percent Substitution[a]— Methoxyl	Hydroxypropoxyl
5–7	11,600	28.0	8.9
50	25,000	30.0	8.6
400	45,300	28.4	9.3

[a] Expressed on a weight basis, as supplied by Dow Chemical Co. (6).

EXPERIMENTAL

Polymer Selection and Solution Preparation—Methylhydroxypropoxyl ethers of cellulose,[1] viscosity grades 5–7, 50, and 400 cps., were selected for use in this study (Table I). Their selection was based on their ability to form free unplasticized films, their solubility in aqueous and organic solvent systems, and their widespread commercial use as pharmaceutical coating materials.

Solutions, 3% w/v, were prepared by dispersing the polymers in either deionized water or an organic solvent system consisting of methylene chloride, methyl alcohol, and isopropyl alcohol in a 40:30:30 ratio. The solvents were cooled to 5° prior to polymer addition and were stirred until the dispersions reached room temperature. The solutions were brought to volume and allowed to solvate for a minimum of 24 hr. before use.

Preparation of Free, Unplasticized Film Samples—Free films were prepared by pouring the polymer solutions onto a clean, leveled surface of aluminum foil. Film area was controlled by the use of a

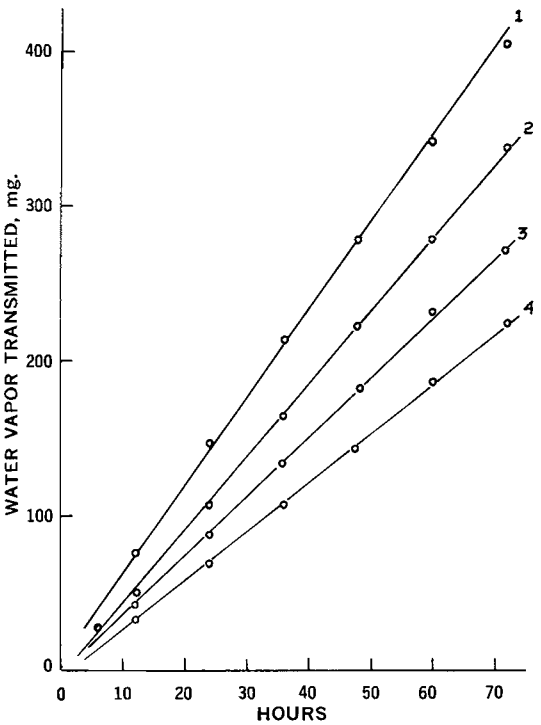


Figure 2—*Transmission of water vapor through the cellulose films as a function of time:*

Number	*Polymer Grade*	*Solvent*	*Thickness, in.*
1	*400 cps.*	*Water*	*0.00194*
2	*400 cps.*	*Organic*	*0.00355*
3	*5–7 cps.*	*Organic*	*0.0129*
4	*5–7 cps.*	*Organic*	*0.0176*

[1] Methocel 60 HG, Dow Chemical Co., Midland, Mich.

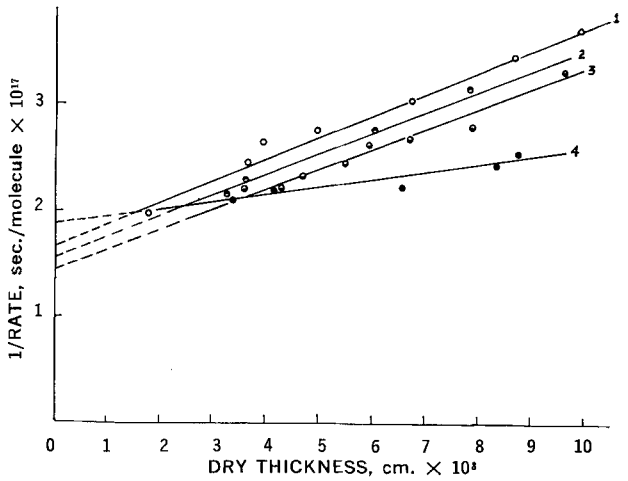


Figure 3—*Relationship between the reciprocal of the water vapor transmission rate and film thickness as predicted by Eq. 12:*

Number	*Polymer*	*Solvent*
1	*400 cps.*	*Water*
2	*5–7 cps.*	*Water*
3	*50 cps.*	*Organic*
4	*5–7 cps.*	*Organic*

rectangular 10 × 15-cm. Plexiglas frame, and film thickness was varied by adjusting the volume of the polymer solution employed. Organic cast films were allowed to evaporate for 12 hr. at room temperature before removal from the substrate. Films cast from deionized water were dried in an oven at 50° for 12 hr. before removal from the substrate. Films cast from deionized water, which were dried for 48–60 hr. at room temperature, showed no significant difference in permeation properties compared with those dried in the oven at 50°. The film was removed from the aluminum foil by peeling the substrate away in strips.

Film samples were cut from the sheet with a metal cylinder having an internal diameter of 2.35 cm. Film thickness was determined with a micrometer. The samples were stored in a desiccator over calcium sulfate for 5 days prior to use.

Transmission Cell and Environmental Chamber—The type of experimental design used in this study was similar to that used in previous investigations (2, 3).

A saturated solution of sodium tartrate was placed in each transmission cell (Fig. 1), maintaining a relative humidity of 91% at 30° equivalent to a vapor pressure of 28.96 mm. of mercury. Since the film-to-liquid surface was found to be a critical factor in the reproducibility of results, a sufficient volume of the saturated solution (approximately 5 ml.) was utilized to ensure an initial distance of 4.5 cm. between the liquid surface and the film sample. The cells were placed within a glovebox at 30° and maintained at a relative humidity below 3% using anhydrous calcium sulfate. Each cell was weighed on an analytical balance within the glovebox initially and at 12-hr. intervals for a 72-hr. period. The value of C_0, the equilibrium concentration of vapor existing above the solution having a vapor pressure of 28.96 mm. of mercury, was calculated to be 9.2 × 10^{17} molecules/cm.^{3}, using the ideal gas law.

Table II—Permeability Coefficients for Water Vapor Permeation through Methylhydroxypropoxyl Ethers of Cellulose Films

Polymer	Solvent	Permeability Coefficient, cm.^{2}/sec. × 10^4
Methylhydroxypropoxyl ethers of cellulose, 5–7 cps.	Organic	7.39
	Water	3.13
Methylhydroxypropoxyl ethers of cellulose, 50 cps.	Organic	3.30
	Water	2.54
Methylhydroxypropoxyl ethers of cellulose, 400 cps.	Organic	3.15
	Water	3.04

Table III—Mean Diffusion Coefficients for Water Vapor–Air Diffusion within the Diffusion Cell for Various Film Systems

Polymer	Solvent	Mean Diffusion Coefficient, cm.^{2}/sec.
Methylhydroxypropoxyl ethers of cellulose, 5–7 cps.	Organic	0.166
	Water	0.177
Methylhydroxypropoxyl ethers of cellulose, 50 cps.	Organic	0.189
	Water	0.224
Methylhydroxypropoxyl ethers of cellulose, 400 cps.	Organic	0.162
	Water	0.164
Semiinfinite column	Free diffusion water in air	0.258[a]

[a] From *Reference 7* corrected to 30° (8).

RESULTS AND DISCUSSION

The transmission of water vapor through the polymer films was linear with time and dependent on film thickness and composition (Fig. 2). For a given polymer the relationship between 1/rate and film thickness was linear, as predicted by Eq. 12 (Fig. 3). The permeability coefficient, P, which reflects the interaction of the vapor with the film and its diffusion through the film, can be determined from the slope in Fig. 2, since the slope is $1/PAC_0$, and C_0 and A are constants. The permeability coefficients for films of the three molecular weight grades of the hydroxypropyl methylcellulose polymer, for films cast from both an organic solvent and water, are shown in Table II. Without exception the water-cast films had a lower permeability coefficient, although this difference decreased as the molecular weight of the polymer increased and is probably not significant for the water-cast *versus* organic solvent-cast 400-cps. polymer films.

This approach, *i.e.*, the method of data expression shown in Fig. 3, provides a direct experimental method for determining permeability coefficients. When determined in this manner, the units of P are square centimeters per second, and the solubility coefficient, S, in the relationship $P = DS$ is dimensionless. The linearity of the graphical data (Fig. 3) indicates that P is independent of film thickness over the range of thickness studied.

The permeability of the film does affect the concentration gradient existing in the diffusion cell. As the permeability of the film decreases and the rate of water vapor transfer through the film decreases, the concentration gradient within the diffusion cell, $C_0 - C_1$, decreases, and equilibrium conditions are approached. Conversely, an increase in film permeability increases the concentration gradient, $C_0 - C_1$. Although the concentration gradient across the film, $C_1 - C_2$, decreases, the increased permeability of the film would result in a greater total flux through the film.

The intercept in Fig. 2, $L/\bar{D}AC_0$, is dependent on the geometry of the experimental design and the value of the mean diffusion coefficient, $\bar{D}$. Since L remained essentially constant during the experiment (a maximum change of 1% occurred), the values of $\bar{D}$ were readily calculated from the intercept values ($L/\bar{D}AC_0$) and are reported in Table III. The values determined for $\bar{D}$ are lower, although of the same order of magnitude, compared to those for water vapor–air in a semiinfinite column (Table III) and must reflect the dependence of the diffusion coefficient on water vapor concentration. The linearity of Fig. 3 also indicates that $\bar{D}$ is a function of concentration only and is not dependent on film thickness. As such, the observed variations in $\bar{D}$ do not affect the magnitude of the permeability coefficient nor the graphical method used in determining the permeability coefficient.

SUMMARY AND CONCLUSIONS

In the past, investigators have graphically represented water vapor permeation data by plotting 1/rate *versus* film thickness. A theoretical basis for this relationship was not apparent. A theoretical justification for this relationship has been derived, based on the

observation that an equilibrium does not exist between the liquid surface and the film for water vapor. The resulting equation readily leads to the calculation of a permeability coefficient for the film; the units of P are the same as the diffusion coefficient, square centimeters per second. A steady state was assumed to exist within the diffusion cell following the lag time. This was justified by the linearity of the graphical data in Fig. 1. The theory was tested with films of unplasticized methylhydroxypropoxyl ethers of cellulose cast from water and an organic solventj and the permeability coefficients were calculated from the slope of 1/rate *versus* film thickness. This provides a direct experimental method for easily determining permeability coefficients and, by maintaining constant cell geometry, relative permeability values can be assigned to any film or series of films.

REFERENCES

(1) T. Higuchi and A. Aguiar, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 574(1959).

(2) M. Patel, J. M. Patel, and A. P. Lemberger, *J. Pharm. Sci.*, **53**, 286(1964).

(3) G. Banker, A. Gore, and J. Swarbrick, *J. Pharm. Pharmacol.*, **18**, 457(1965).

(4) G. Banker, unpublished data.

(5) W. Jost, "Diffusion in Solids, Liquids, Gases," Academic, New York, N. Y., 1952, p. 9.

(6) G. H. Mercer, personal communication, 1967.

(7) W. Jost, "Diffusion in Solids, Liquids, Gases," Academic, New York, N. Y., 1952, p. 412.

(8) W. P. Boynton and W. H. Brattain, in "International Critical Tables of Numerical Data, Physics, Chemistry and Technology," vol. 5, E. Washburn, Ed., McGraw-Hill, New York, N. Y., 1929, p. 62.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 15, 1968, from the *Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication June 9, 1970.

* Present address: Baxter Laboratories, Morton Grove, IL 60053

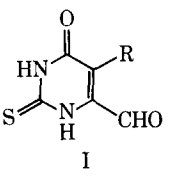
Potential Anticancer Agents VI: 5-Substituted Pyrimidine-6-carboxaldehydes

CHUNG IL HONG*, CLAUDE PIANTADOSI†, and J. LOGAN IRVIN

Abstract □ A series of 5-substituted pyrimidine-6-carboxaldehydes and their derivatives were synthesized and tested for inhibition of growth of the Ehrlich ascites carcinoma and Ehrlich carcinoma. Further studies included inhibition of incorporation of L-phenylalanine-1-^{14}C and formate-^{14}C into proteins, and orotic acid-5-^{3}H, thymidine-2-^{14}C, and formate-^{14}C into nucleic acids of the ascitic tumor cells *in vitro*. The following compounds were found to be particularly active as inhibitors: 2-mercapto-4-hydroxy-5-(3-phenylpropyl)pyrimidine-6-carboxaldehyde (VIII-2), 2-mercapto-4-hydroxy-5-(4-phenylbenzyl)pyrimidine-6-carboxaldehyde (VIII-3), and 2-mercapto-4-hydroxy-5-(α-naphthylmethyl)pyrimidine-6-carboxaldehyde (*Ic*). The best compounds of this series are equally as effective as 5-fluorouracil and 2-mercapto-4-hydroxy-5-(4-chlorobenzyl)pyrimidine-6-carboxaldehyde (*Ib*) in inhibiting formate incorporation into DNA and growth of the ascitic tumor. They are more effective than 5-fluorouracil in inhibiting incorporation of formate and orotic acid into RNA, thymidine into DNA, and phenylalanine into proteins. The active compounds also showed a strong inhibitory activity against respiration of the ascitic tumor. Compounds VIII-2 and VIII-3 also inhibited growth of the Ehrlich carcinoma as a solid tumor after subcutaneous transplantation, but in these tests the drugs were more toxic to the host when injected intraperitoneally since the drugs were not preferentially absorbed by the tumor cells in contrast to the tests *versus* the ascites form of the carcinoma.

Keyphrases □ Pyrimidine-6-carboxaldehydes, 5-substituted—synthesis □ Anticancer activity—5-substituted pyrimidine-6-carboxaldehydes □ Protein synthesis inhibition—5-substituted pyrimidine-6-carboxaldehydes □ Nucleic acid synthesis inhibition—5-substituted pyrimidine-6-carboxaldehydes □ Tumor aerobic respiration inhibition—5-substituted pyrimidine-6-carboxaldehydes

In earlier studies (1–3) on 5-substituted derivatives of Compound I, it was observed that 5-fluoro (*Ia*) and 4-(4-chlorobenzyl) (*Ib*) substituents resulted in derivatives possessing strong inhibitory activity against the Ehrlich ascites carcinoma.



I

Ia, R = F

Ib, R = CH_2—C$_6$H$_4$—Cl

Ic, R = CH_2—C$_{10}$H$_7$

Id, R = CH_2—C$_6$H$_5$

It was further observed that this series of 5-substituted pyrimidine-6-carboxaldehydes inhibited incorporation of amino acids and formate into proteins, and of orotic acid, thymidine, and formate into nucleic acids of the tumor cells *in vitro* (3). Compound *Ib* was equally as effective as 5-fluorouracil (FU) in inhibiting formate incorporation into DNA and growth of the tumor and more effective than FU in inhibiting incorporation of formate and orotic acid into RNA, thymidine into DNA, and phenylalanine and glycine into proteins. However, Compound *Ib* had only negligible inhibitory activity against the folate reductases.

The fact that an enhanced inhibitory activity was obtained by the introduction of benzyl substituents in the 5-position led to the synthesis of further derivatives of pyrimidine-6-carboxaldehydes containing bulky substituents in the 5-position in order to study the structure–activity relationship of these analogs in the test system.

RESULTS AND DISCUSSION

Chemistry—The majority of the α-substituted β-keto esters (VI) were prepared by the alkylation reaction of ethyl γ,γ-dimethoxyacetoacetate (IV) with an aralkyl (benzyl or naphthylmethyl) halide

observation that an equilibrium does not exist between the liquid surface and the film for water vapor. The resulting equation readily leads to the calculation of a permeability coefficient for the film; the units of P are the same as the diffusion coefficient, square centimeters per second. A steady state was assumed to exist within the diffusion cell following the lag time. This was justified by the linearity of the graphical data in Fig. 1. The theory was tested with films of unplasticized methylhydroxypropoxyl ethers of cellulose cast from water and an organic solventj and the permeability coefficients were calculated from the slope of 1/rate *versus* film thickness. This provides a direct experimental method for easily determining permeability coefficients and, by maintaining constant cell geometry, relative permeability values can be assigned to any film or series of films.

REFERENCES

(1) T. Higuchi and A. Aguiar, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 574(1959).

(2) M. Patel, J. M. Patel, and A. P. Lemberger, *J. Pharm. Sci.*, **53**, 286(1964).

(3) G. Banker, A. Gore, and J. Swarbrick, *J. Pharm. Pharmacol.*, **18**, 457(1965).

(4) G. Banker, unpublished data.

(5) W. Jost, "Diffusion in Solids, Liquids, Gases," Academic, New York, N. Y., 1952, p. 9.

(6) G. H. Mercer, personal communication, 1967.

(7) W. Jost, "Diffusion in Solids, Liquids, Gases," Academic, New York, N. Y., 1952, p. 412.

(8) W. P. Boynton and W. H. Brattain, in "International Critical Tables of Numerical Data, Physics, Chemistry and Technology," vol. 5, E. Washburn, Ed., McGraw-Hill, New York, N. Y., 1929, p. 62.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 15, 1968, from the *Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication June 9, 1970.

* Present address: Baxter Laboratories, Morton Grove, IL 60053

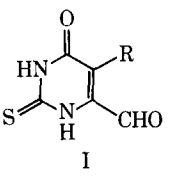
Potential Anticancer Agents VI: 5-Substituted Pyrimidine-6-carboxaldehydes

CHUNG IL HONG*, CLAUDE PIANTADOSI†, and J. LOGAN IRVIN

Abstract □ A series of 5-substituted pyrimidine-6-carboxaldehydes and their derivatives were synthesized and tested for inhibition of growth of the Ehrlich ascites carcinoma and Ehrlich carcinoma. Further studies included inhibition of incorporation of L-phenylalanine-1-^{14}C and formate-^{14}C into proteins, and orotic acid-5-^{3}H, thymidine-2-^{14}C, and formate-^{14}C into nucleic acids of the ascitic tumor cells *in vitro*. The following compounds were found to be particularly active as inhibitors: 2-mercapto-4-hydroxy-5-(3-phenylpropyl)pyrimidine-6-carboxaldehyde (VIII-2), 2-mercapto-4-hydroxy-5-(4-phenylbenzyl)pyrimidine-6-carboxaldehyde (VIII-3), and 2-mercapto-4-hydroxy-5-(α-naphthylmethyl)pyrimidine-6-carboxaldehyde (*Ic*). The best compounds of this series are equally as effective as 5-fluorouracil and 2-mercapto-4-hydroxy-5-(4-chlorobenzyl)pyrimidine-6-carboxaldehyde (*Ib*) in inhibiting formate incorporation into DNA and growth of the ascitic tumor. They are more effective than 5-fluorouracil in inhibiting incorporation of formate and orotic acid into RNA, thymidine into DNA, and phenylalanine into proteins. The active compounds also showed a strong inhibitory activity against respiration of the ascitic tumor. Compounds VIII-2 and VIII-3 also inhibited growth of the Ehrlich carcinoma as a solid tumor after subcutaneous transplantation, but in these tests the drugs were more toxic to the host when injected intraperitoneally since the drugs were not preferentially absorbed by the tumor cells in contrast to the tests *versus* the ascites form of the carcinoma.

Keyphrases □ Pyrimidine-6-carboxaldehydes, 5-substituted—synthesis □ Anticancer activity—5-substituted pyrimidine-6-carboxaldehydes □ Protein synthesis inhibition—5-substituted pyrimidine-6-carboxaldehydes □ Nucleic acid synthesis inhibition—5-substituted pyrimidine-6-carboxaldehydes □ Tumor aerobic respiration inhibition—5-substituted pyrimidine-6-carboxaldehydes

In earlier studies (1–3) on 5-substituted derivatives of Compound I, it was observed that 5-fluoro (*Ia*) and 4-(4-chlorobenzyl) (*Ib*) substituents resulted in derivatives possessing strong inhibitory activity against the Ehrlich ascites carcinoma.



I

Ia, R = F

Ib, R = CH_2—C$_6$H$_4$—Cl

Ic, R = CH_2—C$_{10}$H$_7$

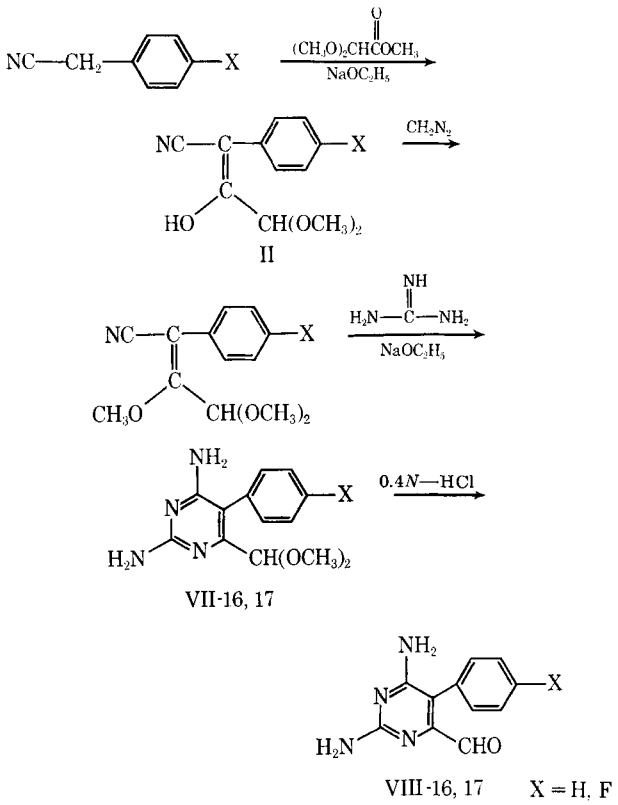
Id, R = CH_2—C$_6$H$_5$

It was further observed that this series of 5-substituted pyrimidine-6-carboxaldehydes inhibited incorporation of amino acids and formate into proteins, and of orotic acid, thymidine, and formate into nucleic acids of the tumor cells *in vitro* (3). Compound *Ib* was equally as effective as 5-fluorouracil (FU) in inhibiting formate incorporation into DNA and growth of the tumor and more effective than FU in inhibiting incorporation of formate and orotic acid into RNA, thymidine into DNA, and phenylalanine and glycine into proteins. However, Compound *Ib* had only negligible inhibitory activity against the folate reductases.

The fact that an enhanced inhibitory activity was obtained by the introduction of benzyl substituents in the 5-position led to the synthesis of further derivatives of pyrimidine-6-carboxaldehydes containing bulky substituents in the 5-position in order to study the structure–activity relationship of these analogs in the test system.

RESULTS AND DISCUSSION

Chemistry—The majority of the α-substituted β-keto esters (VI) were prepared by the alkylation reaction of ethyl γ,γ-dimethoxyacetoacetate (IV) with an aralkyl (benzyl or naphthylmethyl) halide



Scheme I

in the presence of sodium ethoxide in 70% yield. Claisen condensation of isopropyl ester (V) with methyl dimethoxyacetate (III) in benzene in the presence of sodium hydride afforded the α-alkyl (*n*-hexyl and *n*-dodecyl) and α-aralkyl (2-phenylethyl and 3-phenylpropyl) β-keto esters in 95% yield. The isopropyl esters were employed to reduce self-condensation (4). Due to extensive decomposition during fractionation, the crude esters were used without purification. The condensation of the β-keto ester with thiourea gave the pyrimidine acetal (VII) in 30% yield. With guanidine hydrochloride, the yield was 45%.

When the guanidine reaction was performed utilizing guanidine hydrochloride and sodium methoxide in boiling ethanol, the intermediate guanide (2-methoxyacetyl-2-substituted acetyl guanide) separated during the 1st hour (5). This guanide was redissolved and cyclized to the pyrimidine acetal (VII). Hydrolysis of the acetals (VII) was accomplished in dioxane with 10% H_2SO_4 or 10% HCl, resulting in a 60% yield of the pyrimidine-6-carboxaldehydes (VIII). Dioxane was used as a solvent for those acetals which did not dissolve in acid solution, and hydrolysis was completed in about 1 hr. Claisen condensation of the phenylacetonitriles with methyl dimethoxyacetate and ethanolic sodium ethoxide gave α-acylphenylacetonitriles (II) in 50% yield. Compound II was converted to the enol ether by diazomethane and then condensed with guanidine to give Compounds VII-16 and VII-17. These pyrimidine acetals were then hydrolyzed with aqueous 0.4 *N* HCl to the aldehydes (VIII-16,17) (Scheme I).

The pyrimidine aldehydes (VIII) contained a molecule of ethanol when recrystallized from ethanol or ethanol–water. This molecule of ethanol was removed by heating at 100° for several hours *in vacuo*. Although a molecule of ethanol could be removed, some compounds were found to have one or one-half molecule of water, which was difficult to remove. The Cannizzaro reaction of Compound VIII-2 with 25% KOH yielded the corresponding alcohol (XI) and acid (XII) in good yield. The alcohol was precipitated at pH 7–8, and the acid was precipitated at lower pH (1–3). The thiosemicarbazones (IX), hydrazones (X), and Schiff bases (X) were prepared in the usual manner in good yields. Compound XII was decarboxylated by heating in tetralin, resulting in Compound XIII. Scheme II shows the sequence of reactions discussed.

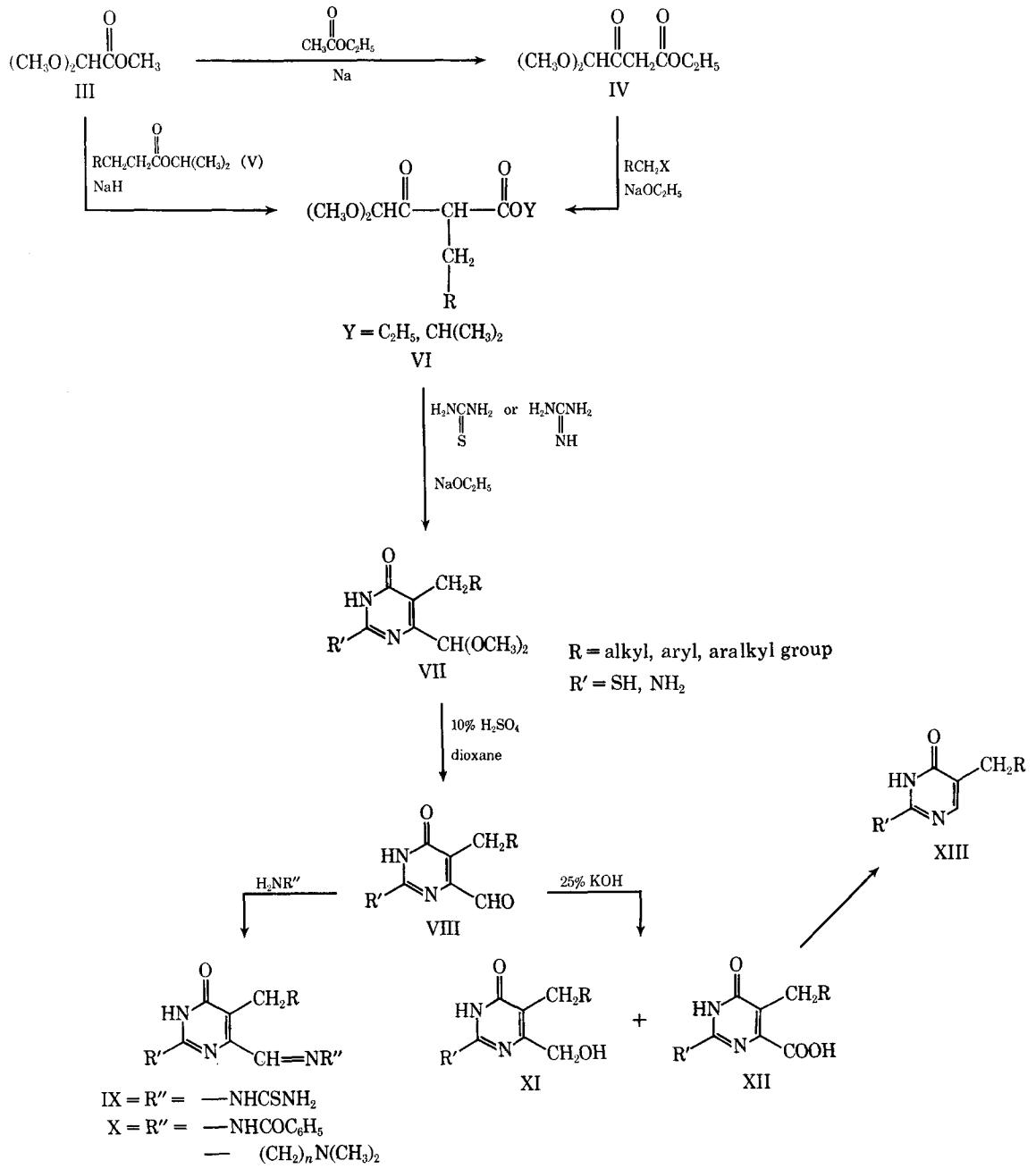
Screening Tests—*Test* versus *Ehrlich Ascites Carcinoma*—The compounds were tested *versus* the Ehrlich ascites carcinoma in Swiss-Webster white mice by a slight modification of procedures described previously (6, 7). Twenty-four hours after the inoculation, each control mouse received an intraperitoneal injection of 0.2 ml. of dimethyl sulfoxide (DMSO), and each experimental mouse received a solution of the tested compound in DMSO. The intraperitoneal injections of control and experimental mice were continued twice daily for 6 days (one injection on the last day; total of 11 injections). On the 7th day, all surviving mice in control and experimental groups were sacrificed. The volume of ascitic fluid was measured for each sample of ascitic fluid by centrifugation in heparinized capillary tubes. The total packed-cell volume (TPCV) of tumor cells was calculated in each case together with average values and standard deviations.

The results of tests of representative compounds are recorded in Table I. The results showed that almost all compounds had an inhibitory activity and low toxicity at the dosage of 50–100 mg./kg./day. Compounds Ic, VIII-2, VIII-3, VIII-10, X-4, and XII showed strong activity against the tumor growth at the dosage of 10–60 mg./kg./day. Compounds Ic, VIII-2, VIII-3, and VIII-10 were as effective as Compound I*b*, but they showed greater toxicity than I*b*. Since VIII-6 showed less activity than Ic, the α-substituted naphthalene derivative seemed to be superior to the β-substituted compound. Also, the unsubstituted naphthylmethyl derivative (Ic) was found to be a better compound than the methyl-substituted derivatives (VIII-7 and VIII-8).

The 5-dodecyl-pyrimidine-6-carboxaldehyde (VIII-10) showed greater antitumor activity than the 5-hexyl derivative (VIII-9), but both compounds were quite toxic. Since Compound Ic was found to be more active than VIII-13, the SH group at the pyrimidine 2-position seemed to be a better functional group than NH_2 for anticancer activity in this system. A similar observation was noticed for Compound I*b* in a previous study (3). The aldehyde group at the pyrimidine 6-position also seemed to be very important for activity, because Compounds I*b* and VIII-2 showed superior activity when compared with corresponding acetal (VII-2), thiosemicarbazone (IX-2), hydrazones (X-4 and X-5), and Schiff bases (X-1 and X-2). Furthermore, the corresponding alcohol and carboxylic derivatives (XI and XII) were found to be less active than the corresponding aldehyde derivative (VIII-2).

Test versus *Ehrlich Carcinoma* (*Solid Tumor*)—The compounds were also tested *versus* the Ehrlich solid tumor in Swiss-Webster white mice by a modification of a procedure described previously (8). Each mouse (initial weight approximately 30 g.) received a subcutaneous injection of 0.2 ml. of 10% ascitic fluid (containing 1.4×10^7 carcinoma cells) into the inguinal region or the back shoulder region on one side. For each assay the mice were divided into a control group of eight mice and several experimental groups of eight mice each. Seventy-two hours after the inoculation, each control mouse received an intraperitoneal or a subcutaneous (tumor area) injection of 0.2 ml. DMSO, and each experimental mouse received a solution of the tested compound in DMSO. The intraperitoneal or subcutaneous injections of control and experimental mice were continued once daily for 9 days (total of nine injections). On the 12th day after inoculation, all surviving mice in control and experimental groups were sacrificed. The solid tumors were carefully taken out and weighed. The average weight of solid tumor was calculated in each case together with standard deviations. The results of the more active pyrimidine analogs on solid tumor growth are listed in Table II.

These compounds showed some activity and high toxicity against the solid tumor as opposed to a strong activity and low toxicity against the ascites tumor. Compound VIII-2 showed 74% inhibition with no mortality at a dosage of 16.46 mg./kg./day on subcutaneous injection, while this compound showed 25% inhibition with 3/8 mortality at a similar dosage on intraperitoneal injection. A similar result was obtained with Compound VIII-3. Compounds I*b* and Ic showed 20% inhibition at a dosage of 15–20 mg./kg./day. FU showed 50% inhibition at a dosage of 16.06 mg./kg./day on intraperitoneal injection. However, subcutaneous injection did not enhance the inhibitory activity for FU and I*b*. Administration of 31.6 mg./kg./day of I*b* resulted in complete mortality. The higher toxicity of these compounds in the tests *versus* the solid tumor in comparison with the tests *versus* the ascites tumor can be explained on the basis that in the latter tests the compounds are immediately in contact with the carcinoma cells upon intraperitoneal injection, and the rapid uptake by these cells minimizes the concentrations attained in normal tissues of the host animal. On the other hand, when the compounds are injected intraperitoneally in the tests *versus* the



Scheme II

solid tumor, the tumor cells do not have a preferential opportunity to absorb the drugs, and higher concentrations are attained in normal tissues of the host. The lower toxicity upon subcutaneous injection in the region of the tumor also can be explained upon the basis of preferential uptake by tumor cells, as well as a slower rate of rise in drug concentration in the peripheral circulation.

Inhibition of Protein Synthesis—The effects of the compounds upon protein synthesis were studied by determining the inhibition of incorporation of L-phenylalanine-1-^{14}C and formate-^{14}C into the proteins of Ehrlich ascites carcinoma cells, which were incubated aerobically for 1 hr. with labeled substrate and the compound *in vitro* in Krebs-Ringer phosphate (KRP) buffer at 37 ± 1° by a procedure described previously (1). After the incubation, the acid-soluble fraction (ASF) and the total proteins were isolated and freed of lipids and nucleic acids. Each protein preparation was dissolved in 2.0 ml. of 0.3 *N* NaOH, and 0.2 ml. of the protein solution was transferred to a glass scintillation vial. To each vial, 0.2 ml. of hyamine hydroxide 10-X and 16 ml. of POPOP-PPO tyloxapol[1] scintillation fluid were added for determination of radioactivity in a Packard liquid scintillation spectrometer, model 3320.

The effects of the compounds upon incorporation of L-phenylalanine-1-^{14}C and formate-^{14}C into proteins of the carcinoma cells are recorded in Tables III and V. Compounds Ic, VIII-1, VIII-2, VIII-3, VIII-6, VIII-7, VIII-9, VIII-10, IX-2, and X-4 almost completely inhibited the incorporation of L-phenylalanine-1-^{14}C into proteins at the concentrations of 0.48–0.96 m*M*. Compounds Ic, VIII-2, and VIII-3 showed 90–95% inhibition of formate-^{14}C incorporation into proteins at 0.77 m*M*.

The effects of the homologous 5-aralkylpyrimidine-6-carboxaldehydes (Id, VIII-1, and VIII-2) are shown in Fig. 1. The phenylpropyl derivative (VIII-2) was found to be the most active compound among them, and the activity decreased in the order: $C_6H_5CH_2CH_2CH_2 > C_6H_5CH_2CH_2 > C_6H_5CH_2$. However, the difference in activity was not significant; only a 5–10% difference in inhibition existed at a concentration range of 0.24–0.48 m*M*. These compounds lost their inhibitory activity at a concentration of 0.12 m*M*. The difference in activity might be explained from the binding difference by

[1] In 1 l. of toluene, 5 g. of 2,5-diphenyloxazole (PPO) and 0.3 g. of 1,4-bis(4-methyl-5-phenyloxazolyl)benzene (dimethyl-POPOP) were dissolved. Two liters of this POPOP–PPO solution and 1 l. of tyloxapol (Triton X-100) were mixed.

Table I—Results of Screening Tests *versus* Ehrlich Ascites Carcinoma[a]

Compound	Dose,[b] mg./kg./day	Mortality C	Mortality T	Average Weight Change, T/C, g.	Average TPCV T/C, ml.	Average TPCV *SD* T ±ml.	Average TPCV T as % of C
Ic	9.5	0/8	0/8	6.3/3.7	1.1/2.6	0.58	42
	31.7	0/8	1/8	3.2/5.2	0.4/1.8	0.16	22
	59.8	0/8	1/8	−0.1/5.2	0.3/1.8	0.78	17
	115.9	3/8	3/8	6.1/1.0	0.1/1.6	0.06	6
VII-2	57.4	0/8	2/8	−0.6/3.2	0.8/2.4	0.44	35
VII-3	64.2	0/8	1/8	2.3/3.2	0.5/2.3	0.19	22
VIII-1	58.4	0/8	2/8	4.4/5.2	1.3/1.8	0.38	72
	93.1	0/8	3/8	4.0/3.2	0.5/2.3	0.30	22
VIII-2	9.3	0/8	1/8	6.6/3.7	1.3/2.6	0.81	50
	16.2	0/8	1/8	9.1/3.2	0.5/2.3	0.17	22
	30.2	0/8	1/8	4.2/5.2	0.3/1.8	0.10	17
	55.8	0/8	0/8	1.0/5.2	0.16/1.8	0.22	9
	91.7	0/8	1/8	3.1/3.2	0.17/2.3	0.15	7
VIII-3	8.6	0/8	2/8	1.9/3.7	0.6/2.6	0.05	24
	16.3	0/8	0/8	3.7/3.2	0.5/2.3	0.18	22
	31.9	0/8	1/8	0.4/3.2	0.4/2.3	0.33	17
	59.8	0/8	2/8	0.4/5.2	0.15/1.8	0.29	8
	93.3	0/8	3/8	0.3/3.2	0.3/2.3	0.16	13
VIII-4	68.5	0/8	3/8	7.0/3.2	0.67/2.3	0.29	29
VIII-6	59.8	0/8	0/8	0.8/5.2	0.7/1.8	0.79	39
	98.8	0/8	4/8	10.2/3.7	0.9/2.6	0.34	35
VIII-7	58.4	0/8	3/8	4.1/5.2	1.7/1.8	0.99	94
VIII-8	55.8	0/8	1/8	2.3/5.2	0.9/1.8	0.64	50
VIII-9	95.8	0/8	3/8	6.1/3.7	0.9/2.6	0.21	35
VIII-10	63.5	0/8	4/8	5.0/3.2	0.2/2.3	0.06	9
VIII-11	61.0	0/8	2/8	9.0/3.2	0.8/2.3	0.59	35
VIII-12	59.4	0/8	0/8	4.7/5.2	0.7/1.8	0.41	39
	88.9	0/8	0/8	4.0/3.2	0.6/2.3	0.43	26
VIII-13	64.0	0/8	0/8	0.3/3.2	0.7/2.3	0.31	30
VIII-16	60.0	0/8	0/8	6.2/5.2	0.6/1.8	0.29	33
VIII-17	63.1	0/8	1/8	3.7/5.2	0.4/1.8	0.09	22
IX-1	31.6	0/8	0/8	0.9/5.2	0.4/1.8	0.19	22
IX-2	57.4	0/8	0/8	−1.8/5.2	0.5/1.8	0.27	28
IX-5	40.5	1/8	1/8	4.1/8.2	1.4/1.9	0.51	74
X-1	58.7	0/8	0/8	−4.0/5.2	0.4/1.8	0.21	22
X-2	58.3	0/8	0/8	−1.6/5.2	0.7/1.8	0.34	39
X-4	57.2	0/8	0/8	−5.3/5.2	0.3/1.8	0.79	17
X-5	60.4	0/8	0/8	3.3/5.2	0.4/1.8	0.17	22
XI	61.6	0/8	1/8	7.5/3.2	0.5/2.3	0.34	22
XII	49.6	0/8	2/8	2.9/3.2	0.3/2.3	0.09	13
Ib[c]	8.9	0/8	0/8	1.6/3.7	0.7/2.6	0.29	28
	30.0	0/8	0/8	3.4/8.1	0.3/2.4	0.10	12
	48.2	0/8	0/8	3.1/9.1	0.1/3.0	0.23	3
FU[d]	27.5	0/8	0/8	−0.3/5.3	0.15/1.5	0.10	10

[a] T = treated group; C = controls; and TPCV = total packed-cell volume of tumor cells on final day of assay. The average standard deviation for TPCV of all control groups was ±0.44 ml. [b] The compound, 5, 10, 25, 50, 100, or 150 mg., was dissolved in 20 ml. of DMSO. Each mouse (initial weight approximately 30 g.) received two intraperitoneal injections of 0.2 ml. of the drug solution daily. [c] Data from *Reference 5*. [d] 5-Fluorouracil; data from *Reference 3*.

hydrophobic bonding with these groups to the enzyme which might be involved at the inhibitory site. This hypothesis seems to be plausible, because the 5-hydrocarbon chain alkylpyrimidine-6-carboxaldehydes (VIII-9 and VIII-10) showed 95–98% inhibition at a concentration of 0.96 m*M*, while 2-thioorotic aldehyde (TOA) showed no activity at 0.65 m*M* and only 70% inhibition at 1.96 m*M* (1). The aldehyde group at pyrimidine 6-position appears to be essential, since Compound VIII-2 showed an increased inhibitory activity

Table II—Results of Screening Tests *versus* the Ehrlich Carcinoma (Solid Tumor)[a]

Compound	Injection	Dose,[b] mg./kg./day	Mortality C	Mortality T	Average Tumor Weight T/C, g.	Average Tumor Weight *SD* T ± g.	Average Tumor Weight T as % of C
Ib	Intraperitoneal	14.16	0/8	2/8	2.86/3.95	0.73	72
	Intraperitoneal	15.5	1/8	0/8	3.31/4.02	0.14	83
	Intraperitoneal	21.4	1/8	4/8	3.05/4.02	0.07	75
	Intraperitoneal	31.6	1/8	8/8	—	—	—
	Subcutaneous	16.0	1/8	2/8	2.64/3.00	0.25	88
Ic	Intraperitoneal	15.0	1/8	4/8	3.26/4.02	0.25	81
	Intraperitoneal	16.3	1/8	4/8	3.46/4.02	0.05	86
VIII-2	Intraperitoneal	16.0	1/8	3/8	3.03/4.02	0.33	75
	Subcutaneous	16.6	0/8	0/8	0.36/1.35	0.39	26
VIII-3	Intraperitoneal	16.3	1/8	5/8	3.57/4.02	0.06	89
	Subcutaneous	16.7	0/8	0/8	0.76/1.35	0.51	56
FU[c]	Intraperitoneal	16.0	1/8	3/8	2.05/4.02	0.33	51
	Subcutaneous	15.6	1/8	2/8	3.05/3.00	0.26	100

[a] T = treated group; C = control. The average standard deviation for the control group was ±0.21 g. for the intraperitoneal injection and ±0.95 g. for the subcutaneous injection. [b] The compound, 50, 70, or 100 mg., was dissolved in 20 ml. of DMSO. The drug solution (0.2 ml.) was injected intraperitoneally or subcutaneously into the mouse (initial weight approximately 30 g.) once daily. [c] FU = 5-fluorouracil.

Table III—Effects of Compounds on Incorporation of L-Phenylalanine-1-^{14}C[a] into Proteins of Ehrlich Ascites Carcinoma Cells

Compound	Concentration of Compound, m*M*	Average Experimental Values, % of Control Protein	ASF[b]
None	0	100[c]	100[d]
Ic	0.96	6	96
VIII-1	0.12	113	124
	0.24	26	205
	0.48	10	231
	0.96	4	70
VIII-2	0.12	100	136
	0.24	17	202
	0.48	6	216
	0.96	0	18
VIII-3	0.96	3	45
VIII-6	0.96	5	30
VIII-7	0.96	3	31
VIII-9	0.96	2	30
VIII-10	0.96	5	11
VIII-13	0.96	34	123
VIII-14	0.96	30	94
IX-1	0.48	73	152
IX-2	0.96	6	157
X-2	0.48	22	209
X-4	0.48	3	48
XI	0.96	28	—
XII	0.96	127	—
Ib[e]	0.38	5	—
	0.96	5	67
FU[f]	0.68	87	83
	2.0	81	85

[a] 0.1 μmole/ml. (0.2 μCi). [b] ASF = acid-soluble fraction. [c] 1.122 × 10^3 counts/min./mg. [d] 1.751 × 10^3 counts/min. [e] Data from *Reference 5*. [f] FU = 5-fluorouracil; data from *Reference 3*.

over the alcohol (XI) and the acid (XII) derivatives. The 5-naphthylmethyl-pyrimidine-6-carboxaldehydes were also found to be very effective compounds against the incorporation of L-phenylalanine-1-^{14}C into proteins. However, the 2-amino-5-naphthylmethyl derivatives (VIII-13 and VIII-14) showed 70% inhibition at a concentration of 0.96 m*M*, while the corresponding 2-mercaptopyrimidine derivatives showed 95–98% inhibition at the same concentration.

When an amino acid is concentrated in a cell, it rapidly equilibrates with exogenous amino acids and is competitively displaced by certain structurally related analogs (9). The radioactivity of the ASF obtained after 1 hr. incubation of a suspension of Ehrlich ascites cells and L-phenylalanine-1-^{14}C is due to unchanged L-phenylalanine-1-^{14}C and other compounds produced metabolically from L-phenylalanine-1-^{14}C. Inhibition of L-phenylalanine-1-^{14}C transport into the cell interior or relatively faster biochemical reactions involving L-phenylalanine-1-^{14}C may reduce the total radioactivity in the ASF of the cell. The inhibition of L-phenylalanine-1-

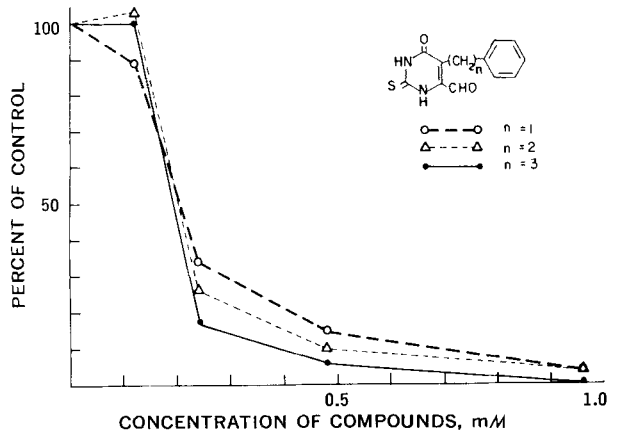


Figure 1—*Effects of phenylalkylpyrimidine-6-carboxaldehydes on incorporation of L-phenylalanine-1-^{14}C into proteins of Ehrlich ascites carcinoma cells* in vitro. *L-Phenylalanine-1-^{14}C: 0.1 μmole/ml.; 0.2 μCi.*

Table IV—Effects of Compounds on Incorporation of Thymidine-2-^{14}C and Orotic Acid-5-^{3}H into Nucleic Acids of Ehrlich Ascites Carcinoma Cells[a]

Compound	Concentration of Compound, m*M*	Average Experimental Values, % of Control DNA	RNA
None	0	100[b]	100[c]
Ic	0.96	39	30
	1.92	5	4
VIII-2	0.96	4	42
	1.92	8	3
VIII-3	0.96	21	11
	1.92	6	7
VIII-10	0.96	107	101
	1.92	74	85
XII	0.96	190	91
XIII	0.96	23	—
Ib[d]	0.96	4[e]	24
	1.92	0	13
FU[f]	0.57	—	90
	1.60	330	—

[a] Thymidine-2-^{14}C: 0.1 μmole/ml. (1 μCi); orotic acid-5-^{3}H: 0.04 μmole/ml. (10 μCi). [b] 3.26 × 10^5 c.p.m./mg. [c] 1.43 × 10^5 c.p.m./mg. [d] Data from *Reference 5*. [e] New result. [f] FU = 5-fluorouracil; data from *Reference 3*.

^{14}C incorporation into the ASF by the compounds listed in Table III may be the result of such effects. However, inhibition of incorporation into protein was always greater than inhibition of incorporation into the ASF. Compounds VIII-2, VIII-6, VIII-7, VIII-9, and VIII-10 gave 70–90% inhibition of accumulation in the ASF at a concentration of 0.96 m*M*. Compounds VIII-1 and VIII-2, however, stimulated L-phenylalanine-1-^{14}C incorporation into the ASF to 220% of controls at 0.48 m*M* concentration. This increase in accumulation of radioactivity in the ASF could result in part from the inhibition of incorporation of phenylalanine into protein, which would produce a "pile-up" of phenylalanine-1-^{14}C in the ASF.

Inhibition of Nucleic Acid Synthesis—The effects of the compounds upon nucleic acid synthesis were studied by determining the inhibition of incorporation of orotic acid-5-^{3}H and formate-^{14}C into RNA and of thymidine-2-^{14}C and formate-^{14}C into DNA of Ehrlich ascites carcinoma cells. These cells were incubated aerobically for 1 hr. with the labeled substrate and the compound *in vitro* in KRP buffer at 37 ± 1° by a procedure described previously (3).

Incorporation of Orotic Acid-5-^{3}H and Thymidine-2-^{14}C into Nucleic Acids—Each incubation flask contained 5 ml. of a 20% suspension of tumor cells in KRP buffer (pH 7.1–7.2), 1 ml. of KRP buffer containing orotic acid-5-^{3}H (0.04 μmole/ml., 10 μCi), thymidine-2-^{14}C (0.1 μmole/ml., 1 μCi), glucose (5 mg./ml.), and 0.2 ml. of the compound in DMSO.

After incubation, the acid-insoluble residue was obtained and washed as previously described (1). The acid-insoluble residue then was suspended in 5 ml. of 0.5 *N* $HClO_4$ and heated for 30 min. at 95°. At the end of heating, the tubes were centrifuged, and the supernatant was neutralized with KOH. To each glass scintillation

Table V—Effects of Compounds on Incorporation of Formate-^{14}C into Nucleic Acids and Proteins of Ehrlich Ascites Carcinoma Cells[a]

Compound	Concentration of Compound, m*M*	Average Experimental Values, % of Control RNA	DNA	Protein
None	0	100[b]	100[c]	100[d]
Ic	0.77	9	15	9
VIII-2	0.77	4	8	6
VIII-3	0.77	2	2	4
XIII	0.77	62	59	—
Ib[e]	0.77	4.4	5.3	2.2
	1.54	0.4	0.09	0.2
FU[f]	0.64	126	7	—

[a] Formate-^{14}C: 0.318 μmole/ml. (8 μCi). [b] 1.57 × 10^4 c.p.m./mg. of RNA. [c] 2.26 × 10^4 c.p.m./mg. of DNA. [d] 1.88 × 10^4 c.p.m./mg. [e] Data from *Reference 5*. [f] FU = 5-fluorouracil; data from *Reference 3*.

Table VI—Effect of Compounds on Respiration of Ehrlich Ascites Carcinoma Cells

Compound	Concentration of Compound, m*M*	Q_{O_2},[a] μl. O_2/mg. cells/hr. Control	Experiment	Inhibition, %
I*b*	1.0	3.50	1.57	55
	2.0	3.50	1.30	63
I*c*	1.0	3.50	1.31	63
	2.0	3.50	1.44	60
VIII-1	1.0	4.00	1.48	63
	2.0	4.00	1.51	63
IX-2	1.0	4.00	3.79	5
	2.0	4.00	3.31	17
X-1	1.0	4.00	2.23	44
	2.0	4.00	2.14	47
X-2	1.0	4.00	1.38	66
	2.0	4.00	1.06	72
X-4	1.0	4.00	3.79	5

[a] For calculation of Q_{O_2} value, 2 ml. of 10% cell suspension was considered as equivalent to 32 mg. dry weight.

vial, 0.2 ml. of the neutralized solution, 0.2 ml. of hyamine hydroxide 10-X, and 16 ml. of the scintillation fluid were added for determining radioactivity. The radioactivities of ^{14}C and ^{3}H were determined according to Kabara *et al.* (10), and the exact channel ratios of ^{14}C and ^{3}H were determined (1). The concentration of RNA was determined by the orcinol reaction and corrected for the interference by DNA. The amount of DNA was determined by the diphenylamine reaction (11). The specific activities were calculated as c.p.m. (^{3}H)/mg. of RNA and c.p.m. (^{14}C)/mg. of DNA.

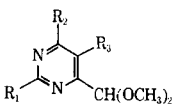
The effects of the compounds upon incorporation of orotic acid-5-^{3}H into RNA and thymidine-2-^{14}C into DNA of the carcinoma cells are recorded in Table IV. Compounds I*b* and VIII-2 showed complete inhibition of thymidine-2-^{14}C incorporation into DNA–thymine at a concentration of 0.96 m*M*. A recent study with Compound I*b* (12) showed that the inhibition of thymidine incorporation into DNA was possibly due to the inhibition of thymidine monophosphate (TMP) kinase by this compound.

Compounds I*b*, I*c*, VIII-2, and VIII-3 showed 80–95% inhibition of incorporation of orotic acid-5-^{3}H into RNA at a relatively high concentration (1.92 m*M*) of the compounds.

The previous work showed that the conversion of orotic acid to orotidylic acid was inhibited by some of the 5-fluoroorotic aldehyde derivatives (1). The 5-substituted pyrimidine derivatives might also inhibit this conversion. The possibility that Compound XII is acting as an antimetabolite of orotic acid does not seem to be valid. While this compound was quite active as a growth inhibitor in this system, it showed no inhibition against incorporation of L-phenylalanine into proteins and of orotic acid into RNA at a concentration of 0.96 m*M*.

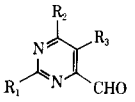
Incorporation of Formate-^{14}C into Nucleic Acids—Each incubation flask contained 5 ml. of a 40% suspension of tumor cells in KRP buffer, 1 ml. of glucose in KRP buffer (3 mg./ml.), 1 ml. of formate-^{14}C in KRP buffer (0.318 μmole/ml., 8 μCi), and 0.2 ml. of the compound in DMSO. After incubation, nucleic acids were isolated as sodium nucleates. The proteins also were isolated for evaluation of inhibition of formate incorporation into protein. RNA was separated from DNA by hydrolyzing with 0.3 *N* KOH at 37° for 16–18 hr. After purification, the radioactivities were counted for each nucleic acid by a procedure described previously (1). The effects of the compounds upon incorporation of formate-^{14}C into the nucleic acids of ascites cells are recorded in Table V. Compounds I*c*, VIII-2, and VIII-3 showed almost complete inhibition of incorporation of formate into DNA and RNA at a concentration of 0.77 m*M*, and they were as active as 2-mercapto-5-(4-chlorobenzyl) pyrimidine-6-carboxaldehyde (I*b*) as previously reported (3). These compounds are practically identical in activity with FU in inhibiting formate incorporation into DNA, but they are more active than FU in inhibiting formate incorporation into RNA. Compound XIII was less active than Compound VIII-2, which further indicates the im-

Table VII—6-(Dimethoxymethyl)-5-substituted Pyrimidines



No.	R_1	R_2	R_3	Yield, %	M.p.	Formula	Anal. Calcd.	Anal. Found
1	SH	OH	$CH_2CH_2C_6H_5$	40	175–177°	$C_{15}H_{18}N_2O_3S$	C, 58.82 H, 5.88	C, 58.96 H, 6.01
2	SH	OH	$CH_2CH_2CH_2C_6H_5$	12	118–120°	$C_{16}H_{20}N_2O_3S$	C, 60.00 H, 6.25	C, 60.06 H, 6.32
3	SH	OH	$CH_2C_6H_4$-C_6H_5(4)	33	210–212°	$C_{20}H_{20}N_2O_3S$	C, 65.25 H, 5.44	C, 65.17 H, 5.41
4	SH	OH	$CH_2C_6H_3(CH_3)_2$(2,5)	34	185–187°	$C_{16}H_{20}N_2O_3S$	C, 60.00 H, 6.25	C, 60.34 H, 6.32
5	SH	OH	$CH_2C_6H_3(OH)(2)NO_2$(5)	10	238–240° dec.	$C_{14}H_{15}N_3O_6S$	C, 47.59 H, 4.25	C, 47.53 H, 4.21
6	SH	OH	$CH_2(\beta)C_{10}H_7$	16.3	163–165°	$C_{18}H_{18}N_2O_3S$	C, 63.16 H, 5.26	C, 63.09 H, 5.18
7	SH	OH	$CH_2(\alpha)C_{10}H_6CH_3$(2)	23	262–263°	$C_{19}H_{20}N_2O_3S$	C, 64.04 H, 5.62	C, 64.28 H, 5.59
8	SH	OH	$CH_2(\alpha)C_{10}H_6CH_3$(4)	10	183–185°	$C_{19}H_{20}N_2O_3S.H_2O$	C, 61.00 H, 5.89	C, 61.11 H, 6.01
9	SH	OH	$CH_2(CH_2)_4CH_3$	28	99–100°	$C_{13}H_{22}N_2O_3S$	C, 54.54 H, 7.69	C, 54.62 H, 7.96
10	SH	OH	$CH_2(CH_2)_{10}CH_3$	36	81.5–82.5°	$C_{19}H_{34}N_2O_3S$	C, 61.62 H, 9.19	C, 61.48 H, 9.22
11	NH_2	OH	$CH_2C_6H_4Br$(4)	26.2	242–243° dec.	$C_{14}H_{15}BrN_3O_3$	C, 47.46 H, 4.52	C, 47.34 H, 4.43
12	NH_2	OH	$CH_2CH_2C_6H_5$	47	219–221°	$C_{15}H_{19}N_3O_3$	C, 62.28 H, 6.58	C, 62.26 H, 6.74
13	NH_2	OH	$CH_2(\alpha)C_{10}H_7$	19.1	243–245°	$C_{18}H_{19}N_3O_3$	C, 66.46 H, 5.85	C, 66.48 H, 6.03
14	NH_2	OH	$CH_2(\beta)C_{10}H_7$	22	222–224° dec.	$C_{18}H_{19}N_3O_3$	C, 66.46 H, 5.85	C, 66.34 H, 6.27
15	NH_2	OH	$CH_2(\alpha)C_{10}H_6CH_3$(4)	32.7	264–265° dec.	$C_{19}H_{21}N_3O_3$	C, 67.25 H, 6.19	C, 67.07 H, 5.96
16	NH_2	NH_2	C_6H_5	22.3	247–248°	$C_{13}H_{16}N_4O_2$	C, 60.00 H, 6.16	C, 59.97 H, 6.15
17	NH_2	NH_2	C_6H_4F(4)	18.7	259–260°	$C_{13}H_{15}FN_4O_2$	C, 56.11 H, 5.40	C, 56.15 H, 5.55

Table VIII—5-Substituted Pyrimidine-6-carboxaldehydes



No.	R_1	R_2	R_3	Yield, %	M.p.	Formula	Anal. Calcd.	Anal. Found
1	SH	OH	$CH_2CH_2C_6H_5$	40	201–202°	$C_{13}H_{12}N_2O_2S$	C, 60.00 H, 6.16	C, 59.97 H, 6.15
2	SH	OH	$CH_2CH_2CH_2C_6H_5$	93.5	168–170°	$C_{14}H_{14}N_2O_2S$	C, 61.31 H, 5.11	C, 60.98 H, 5.04
3	SH	OH	$CH_2C_6H_4$-C_6H_5(4)	74	234–235° dec	$C_{18}H_{14}N_2O_2S$	C, 67.10 H, 4.35	C, 67.24 H, 4.28
4	SH	OH	$CH_2C_6H_3(CH_3)_2$(2,5)	47	254–256° dec.	$C_{14}H_{14}N_2O_2S$	C, 61.31 H, 5.11	C, 62.06 H, 5.09
5	SH	OH	$CH_2C_6H_3(OH)(2),NO_2(5)$	75	263–265° dec.	$C_{12}H_{13}N_3O_5S$	C, 46.90 H, 2.92	C, 47.00 H, 3.01
6	SH	OH	$CH_2(\beta)C_{10}H_7$	75	204–205°	$C_{16}H_{12}N_2O_2S.\ ^1/_2H_2O$	C, 62.95 H, 4.26	C, 63.07 H, 4.18
7	SH	OH	$CH_2(\alpha)C_{10}H_6CH_3$(2)	69	239–240° dec.	$C_{17}H_{14}N_2O_2S$	C, 65.80 H, 4.51	C, 66.05 H, 4.57
8	SH	OH	$CH_2(\alpha)C_{10}H_6CH_3$(4)	71	247–249° dec.	$C_{17}H_{14}N_2O_2S.\ ^1/_2H_2O$	C, 63.95 H, 4.70	C, 64.07 H, 4.79
9	SH	OH	$CH_2(CH_2)_4CH_3$	100	123–125°	$C_{11}H_{16}N_2O_2S$	C, 55.00 H, 6.67	C, 55.04 H, 6.79
10	SH	OH	$CH_2(CH_2)_{10}CH_3$	100	75–77°	$C_{17}H_{28}N_2O_2S$	C, 62.96 H, 8.64	C, 62.56 H, 8.51
11	NH_2	OH	$CH_2C_6H_4Br$(4)	72.5	>300° dec.	$C_{12}H_{10}BrN_3O_2$	C, 46.75 H, 3.24	C, 47.10 H, 3.32
12	NH_2	OH	$CH_2CH_2C_6H_5$	95	>330° dec.	$C_{13}H_{13}N_3O_2$	C, 64.20 H, 5.35	C, 63.91 H, 5.40
13	NH_2	OH	$CH_2(\alpha)C_{10}H_7$	94	293–294° dec.	$C_{16}H_{13}N_3O_2.\ ^1/_2H_2O$	C, 66.66 H, 4.86	C, 66.40 H, 4.90
14	NH_2	OH	$CH_2(\beta)C_{10}H_7$	47	>300° dec	$C_{16}H_{13}N_3O_2$	C, 68.82 H, 4.66	C, 68.89 H, 4.95
15	NH_2	OH	$CH_2(\alpha)C_{10}H_6CH_3$(4)	96	>300° dec.	$C_{17}H_{15}N_3O_2.\ ^1/_2H_2O$	C, 67.55 H, 5.30	C, 67.78 H, 5.55
16	NH_2	NH_2	C_6H_5	93.5	>300° dec.	$C_{11}H_{10}N_4O.\ ^1/_2H_2O$	C, 59.19 H, 4.93	C, 59.65 H, 4.78
17	NH_2	NH_2	C_6H_4F(4)	90	>300° dec.	$C_{11}H_9FN_4O.\ ^1/_2H_2O$	C, 54.77 H, 4.14	C, 55.03 H, 4.11

portance of the aldehyde group at the 6-position.

In the initial studies by Chae (13), the effects of the 5-fluoroorotic aldehydes on the incorporation of labeled formate into DNA–purines were not investigated because of the low radioactivity in the purines of DNA; the radioactivity could be mainly accounted as DNA–thymine. In the present experiments, the total radioactivity of DNA was counted without separation of each base. Therefore, the authors did not determine whether the radioactivity was counted mainly from DNA–thymine or whether the DNA–purines made a significant contribution. However, since previous studies (13) indicated a low radioactivity from DNA–purines, the authors assumed that the radioactivity was counted mainly from DNA–thymine. This assumption indicated that probably the inhibition of incorporation of formate-^{14}C into DNA by the 5-substituted pyrimidine derivatives might be attributable to inhibition of thymidylate synthesis by these compounds.

A recent study (12) also indicated that these compounds inhibited TMP kinase, and the inhibition of formate incorporation into DNA might be due to these two inhibitions. It was also observed (12) that Compound I*b* inhibited the incorporation of formate-^{14}C into guanine of RNA 2–4 times more than the formate incorporation into adenine of RNA. Furthermore, Compound I*b* showed 75–80% inhibition of incorporation of labeled 5-amino-4-imidazolecarboxamide (AICA) and hypoxanthine (Hx) into guanine of RNA at a concentration of 0.25 m*M*, while this compound showed practically no inhibition against incorporation of AICA and Hx into adenine of RNA at the same concentration. These results would indicate that one of the possible steps inhibited by I*b* is the conversion of xanthosine monophosphate (XMP) to guanosine monophosphate (GMP). Since the incorporation of formate-^{14}C into adenine of RNA was also inhibited by I*b*, there must be other possible inhibition steps which utilize formate or THFA coenzymes. However, the 5-substituted pyrimidine derivatives were essentially inactive as folate reductase inhibitors when compared with accepted antifols such as aminopterin, pyrimethamine, and trimethoprim (3). Therefore, the inhibition of formate incorporation into DNA and RNA by these compounds could not be attributed to inhibition of the folate reductases.

Inhibition of Aerobic Respiration of the Tumor—The effects of the compounds upon aerobic respiration of the Ehrlich ascites tumor were studied by measuring oxygen consumption by means of the Warburg apparatus. To each Warburg flask was added 2 ml. of the 10% cell suspension in KRP buffer, and 0.2 ml. of 20% KOH was added in the center well which contained a small piece of filter paper. Three-tenths milliliter of drug solution in DMSO (or 0.3 ml. of DMSO for the control) and 0.3 ml. of 0.5 *M* glucose solution were then added to each sidearm. For the thermobarometer, 2.6 ml. of distilled water was added to the flask and 0.2 ml. of 20% KOH in the center well. The flasks were equilibrated in a 37° water bath for 15 min. after gassing with oxygen (10 min.). After equilibration the stopcocks were closed, and the contents of the flask were then mixed. Readings were taken every 10 min. thereafter for 1-hr. For each experiment, at least two controls and one thermobarometer were needed.

The oxygen uptake for respiration was calculated by the method described previously (14). The change in readings, corrected for thermobarometer, was multiplied by the flask constant (K_{O_2}), resulting in the oxygen uptake (μl. O_2/hr.).

Two milliliters of the 10% cell suspension contained 32 mg. dry weight of cells. Therefore, Q_{O_2} (microliter O_2 taken up per milligram dry weight of cells per hour) was calculated by dividing the oxygen uptake by 32 mg. The inhibitory activity was calculated from the mean value (Q_{O_2}) of the duplicate experiments of each sample.

The effects of the compounds upon aerobic respiration of the Ehrlich ascites tumor are recorded in Table VI.

Compounds I*b*, I*c*, VIII-1, and X-2 showed 55–80% inhibition of respiration at a concentration of 1 m*M* in the presence of added glucose. Most of these compounds did not show an enhanced inhibitory activity at an increased concentration (2 m*M*).

There may be two possible explanations for the inhibition of respiration by these compounds. Since preliminary data seem to indicate that these analogs inhibit the synthesis of AMP and GMP

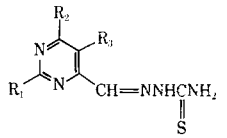


Table IX—Thiosemicarbazones of 5-Substituted Pyrimidine-6-carboxaldehydes

No.	R_1	R_2	R_3	Yield, %	M.p.	Formula	Anal. Calcd.	Anal. Found
1	SH	OH	$CH_2C_6H_5$	62.5	200–201°	$C_{13}H_{13}N_5OS_2$	C, 48.90 H, 4.07	C, 48.56 H, 4.17
2	SH	OH	$CH_2C_6H_4Cl(4)$	51	245–246° dec.	$C_{13}H_{12}ClN_5OS_2$	C, 44.13 H, 3.39	C, 44.16 H, 3.30
3	C_2H_5S	OH	$CH_2C_6H_4Cl(4)$	63	231–233° dec.	$C_{15}H_{16}ClN_5OS_2$	C, 47.18 H, 4.19	C, 46.99 H, 4.11
4	SH	OH	$CH_2C_6H_4$-$C_6H_5(4)$	51	215–217°	$C_{19}H_{17}N_2OS_2 \cdot {}^1/_2H_2O$	C, 56.43 H, 4.46	C, 56.70 H, 4.74
5	NH_2	NH_2	C_6H_4Cl-4	62	270–275° dec.	$C_{12}H_{12}ClN_7S$	C, 44.79 H, 3.73	C, 44.86 H, 4.02
6	OH	OH	Br	68.5	>300° dec.	$C_6H_6BrN_5O_2S$	C, 24.65 H, 2.05	C, 24.33 H, 2.31

(12), such inhibition could result in inhibition of oxidative phosphorylation and synthesis of the coenzymes which participate in glycolysis, the tricarboxylic acid cycle, and the electron-transport system. The second possibility may be a direct inhibition of the oxidative enzymes themselves by the compounds. However, these compounds have less inhibitory effect upon respiration than upon the synthesis of nucleic acids and proteins. Consequently, it seems likely that the inhibition of growth of the carcinoma cells by these compounds is attributable to the latter rather than to the former. The elucidation of the sites of inhibition will be the subject for further research.

EXPERIMENTAL[2]

Melting points were taken in capillary tubes in a Mel-Temp apparatus and have been corrected. The designation of a roman numeral followed by an arabic numeral indicates a specific compound number in the table indicated by the roman numeral (Tables I–VI).

Isopropyl-5-phenylvalerate (V)—This compound was prepared by a known procedure (4) from 5-phenylvaleric acid and isopropyl alcohol; yield 79% of crude product; b.p. 102–105° (0.4 mm.).

The isopropyl-4-phenylbutyrate (b.p. 100–102° at 0.4 mm.), isopropyl octanoate, and isopropyl myristate were all prepared in an analogous manner.

Isopropyl α-(3-Phenylpropyl)-γ,γ-dimethoxyacetoacetate (VI)—This compound was prepared by a known procedure (4) from isopropyl 5-phenylvalerate and methyl dimethoxyacetate; yield 99% of crude product.

The isopropyl α-(2-phenylethyl)-γ,γ-dimethoxyacetoacetate, isopropyl α-(*n*-hexyl)-γ,γ-dimethoxyacetoacetate, and isopropyl α-(*n*-dodecyl)-γ,γ-dimethoxyacetoacetate were all prepared by a similar procedure.

2-Bromomethylnaphthalene—A mixture of 56.8 g. (0.4 mole) of 2-methylnaphthalene, 71.2 g. (0.4 mole) of *N*-bromosuccinimide, and 0.2 g. of benzoyl peroxide in 500 ml. of carbon tetrachloride was refluxed with magnetic stirring for 10 hr. The hot reaction mixture was filtered, and the filtrate was evaporated *in vacuo*. The crude product was recrystallized from petroleum ether (30–60°); yield 70.7 g. (80%) of white crystals, m.p. 55–56°.

Anal.—Calcd. for $C_{11}H_9Br$: C, 59.75; H, 4.10. Found: C, 59.73; H, 4.09.

The other chloromethylnaphthanes used were commercially available.

Ethyl α-(4-Phenylbenzyl)-γ,γ-dimethoxyacetoacetate (VI)—To a sodium ethoxide solution prepared from 2.88 g. (0.125-g. atom) of sodium in 75 ml. of absolute ethanol, 23.8 g. (0.125 mole) of ethyl γ,γ-dimethoxyacetoacetate (15) and 25.34 g. (0.125 mole) of *p*-phenylbenzyl chloride were added. The brown solution was refluxed gently with magnetic stirring for 9 hr., during which time sodium chloride separated. The neutral reaction mixture was filtered, and the sodium chloride was washed with 20 ml. of absolute ethanol. The combined filtrate was dried with anhydrous magnesium sulfate for 3 hr., and the ethanol was evaporated *in vacuo* at 80–90°. A light-brown oil was obtained; yield 44 g.

The α-benzyl-β-keto esters and α-naphthylmethyl-β-keto esters were prepared in an analogous manner. Because of the extensive decomposition that occurred during distillation, the crude esters were used for the next step.

2-Mercapto-6-(dimethoxymethyl)-5-(3-phenylpropyl)-4-pyrimidol (VII-2)—To a sodium ethoxide solution prepared from 5.72 g. (0.248 g. atom) of sodium in 200 ml. of absolute ethanol, 80 g. (about 0.248 mole) of crude isopropyl α-(3-phenylpropyl)-γ,γ-dimethoxyacetoacetate and 18.9 g. (0.248 mole) of dried thiourea were added. The mixture was refluxed with magnetic stirring for 12 hr. During the refluxing, a yellowish-brown precipitate separated. The solvent was evaporated *in vacuo*, and the residue was dissolved in 600 ml. of water. The aqueous mixture was filtered, and the filtrate was extracted twice with 200 ml. portions of ether. The aqueous solution was acidified with 10% aqueous HCl, resulting in the separation of a brown oil. This oil solidified gradually in an ice bath after several hours. The brown precipitate was filtered and washed with cold water. Recrystallization from ethanol gave 9.5 g. (12%) of white crystals; m.p. 118–120°. Table VII lists the compounds prepared in an analogous manner.

2-Amino-6-(dimethoxymethyl)-5-(2-phenylethyl)-4-pyrimidol (VII-12)—A mixture of 41 g. (about 0.133 mole) of crude isopropyl α-(2-phenylethyl)-γ,γ-dimethoxyacetoacetate and a solution of guanidine [from 12.7 g. (0.133 mole) of guanidine hydrochloride and 3.06 g. (0.133 g. atom) of sodium in 100 ml. of absolute ethanol] was refluxed with stirring for 6 hr. The white precipitate, which separated during the 1st hour, gradually dissolved to yield a reddish-brown solution. The solution was evaporated to dryness under reduced pressure, and 500 ml. of water was added. The mixture was acidified with acetic acid. After cooling at 0° for several hours, the gummy precipitate was filtered and washed with cold ethanol and ether. The product was recrystallized from ethanol; yield 18 g. (47%) of white crystals, m.p. 219–221°. Table VII lists the compounds prepared by a similar procedure.

2,4-Diamino-6-(dimethoxymethyl)-5-(4-fluorophenyl)pyrimidine (VII-17)—This compound was prepared by a known procedure (3) from guanidine hydrochloride and α-(dimethoxyacetyl)-*p*-fluorophenylacetonitrile; yield 18.7% of white crystals, m.p. 259–260°.

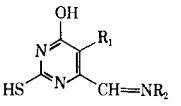
2,4-Diamino-6-(dimethoxymethyl)-5-phenylpyrimidine (VII-16) was prepared in an analogous manner.

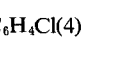
2-Mercapto-4-hydroxy-5-(3-phenylpropyl)pyrimidine-6-carboxaldehyde (VIII-2)—A mixture of 2 g. (6.25 mmoles) of VII-2, 20 ml. of 10% aqueous HCl, and 30 ml. of dioxane was refluxed for 1 hr. The cooled yellow solution was diluted with ethanol–water (1:1) to turbidity. After being cooled at 0° for several hours, the crystals were filtered and washed with water. The crude product was recrystallized from ethanol and dried at 100°; yield 1.6 g. (93.5%) of yellow crystals, m.p. 168–170°. Table VIII lists the compounds prepared in an analogous manner.

2-Amino-4-hydroxy-5-(2-phenylethyl)pyrimidine-6-carboxaldehyde (VIII-12)—A mixture of 5 g. (17.3 mmoles) of VII-12, 30 ml. of

[2] Analyses were performed by M-H-W Laboratories, Garden City, MI 48135

Table X—Schiff Base and Hydrazones of 5-Substituted Pyrimidine-6-carboxaldehydes



No.	R_1	R_2	Yield, %	M.p.	Formula	Anal. Calcd.	Anal. Found
1	$CH_2C_6H_4Cl(4)$	$CH_2CH_2N(CH_3)_2$	80.5	181–183° dec.	$C_{16}H_{19}ClN_4OS$	C, 54.78 H, 5.42	C, 54.71 H, 5.36
2	$CH_2C_6H_4Cl(4)$	$CH_2CH_2CH_2N(CH_3)_2$	62.5	161.5–162.5° dec.	$C_{17}H_{21}ClN_4OS$	C, 55.97 H, 5.76	C, 56.13 H, 5.51
3	$CH_2CH_2C_6H_5$	$CH_2CH_2CH_2N(CH_3)_2$	100	177–178° dec.	$C_{18}H_{24}N_4OS$	C, 62.79 H, 6.97	C, 63.17 H, 6.97
4	$CH_2C_6H_4Cl(4)$	$NHCOC_6H_5$	80	262–264°	$C_{19}H_{15}ClN_4O_2S$	C, 57.21 H, 3.76	C, 57.37 H, 3.92
5	$CH_2C_6H_4Cl(4)$		100	335–338° dec.	$C_{18}H_{14}ClN_5O_2S$	C, 54.03 H, 3.50	C, 54.05 H, 3.68

10% aqueous HCl, and 50 ml. of dioxane was refluxed for 1 hr. The solvent was evaporated *in vacuo*, and the residue was triturated with 25 ml. of cold water and then dissolved in warm 2-methoxyethanol. The solution was decolorized with charcoal and then poured into 100 ml. of saturated aqueous $NaHCO_3$. The pale-yellow powder was collected on a filter and washed with water. Recrystallization from ethanol–water (1:1) and drying at 100° gave 4 g. (95%) of yellow crystals, m.p. > 300° dec. Table VIII lists the compounds prepared by a similar procedure.

Thiosemicarbazone of 2-Mercapto-4-hydroxy-5-benzylpyrimidine-6-carboxaldehyde (IX-1)—To a warm solution of 0.616 g. (2.5 mmoles) of *Id* in 30 ml. of ethanol was added a warm solution of 0.23 g. (2.5 mmoles) of thiosemicarbazide in 15 ml. of 80% ethanol. The solution was refluxed for 20 min. and filtered while warm. After cooling at 0° for 1 hr., the yellow crystals were filtered and washed with cold ethanol. The crude product was recrystallized from ethanol; yield 0.5 g. (62.5%) of yellow crystals, m.p. 200–201°. Table IX lists the compounds prepared by a similar procedure.

***N*-[2-Mercapto-4-hydroxy-5-(4-chlorobenzyl)-6-pyrimidylmethylidene]-*N*,*N*-dimethylethylenediamine (X-1)**—A stirred mixture of 0.7 g. (2.5 mmoles) of *Ib* in 30 ml. of absolute ethanol and 0.22 g. (2.5 mmoles) of *N*,*N*-dimethylethylenediamine was refluxed for 30 min. White to yellow crystals separated. This was filtered, washed with cold ethanol, and recrystallized from ethanol; yield 0.7 g. (80.5%), of yellow crystals, m.p. 181–183° dec. Table X lists the compounds prepared by a similar procedure.

Hydrazones of the Pyrimidine Aldehydes (X-4 and X-5)—To a solution of 2.5 mmoles of the aldehyde *Ib* in 50 ml. of 50% aqueous acetic acid was added a solution of 2.5 mmoles of the respective hydrazide in 20 ml. of 50% aqueous acetic acid. It was heated at 80–90° for 10 min., during which time yellow crystals precipitated. After cooling at 0° for 1 hr., the crystalline precipitate was collected by filtration and recrystallized from ethanol. Table X lists these derivatives.

2-Mercapto-6-hydroxymethyl-5-(3-phenylpropyl)-4-pyrimidol (XI) and 2-Mercapto-4-hydroxy-5-(3-phenylpropyl)pyrimidine-6-carboxylic Acid (XII)—To a cold solution of 1.5 g. of KOH in 6 ml. of water was added 1.5 g. of VIII-2. The mixture was left standing at room temperature for a total of 48 hr. The yellow solution was diluted with 20 ml. of water and filtered. To the filtrate, 10% aqueous HCl was added to bring the mixture to pH 7–8, resulting in the precipitation of white crystals (XI). After cooling at 0° for 1 hr., the white crystals were filtered and washed thoroughly with cold water. The filtrate was further acidified with 10% aqueous HCl, resulting in precipitate of yellow crystals (XII). After cooling at 0° for 1 hr., the yellow crystals were filtered and washed with cold water. Both products were recrystallized from ethanol:

The alcohol (XI); yield 0.65 g. (86%), m.p. 214–215°.

Anal.—Calcd. for $C_{14}H_{16}N_2O_2S$: C, 60.87; H, 5.80. Found: C, 61.07; H, 5.84.

The acid (XII); yield 0.65 g. (82%), m.p. 271–273° dec.

Anal.—Calcd. for $C_{14}H_{14}N_2O_3S$: C, 57.93; H, 4.83. Found: C, 57.88; H, 4.98.

2-Mercapto-5-(3-phenylpropyl)-4-pyrimidol (XIII)—A mixture of 0.3 g. (1.03 mmoles) of XII and 20 ml. of tetralin was refluxed for 5 hr. The brown solution was cooled at 0° for 1 hr. The precipitate was filtered and washed with toluene. The crude product was dissolved in 10 ml. of 10% aqueous NaOH and acidified with 10% aqueous HCl to pH 7, resulting in a white precipitate. Recrystallization from ethanol gave 62 mg. (24.5%), m.p. 172–174°.

Anal.—Calcd. for $C_{13}H_{14}N_2OS$: C, 63.41; H, 5.69. Found: C, 62.96; H, 5.86.

REFERENCES

(1) C. B. Chae, J. L. Irvin, and C. Piantadosi, *J. Med. Chem.*, **11,** 506(1968).

(2) C. Piantadosi, C. S. Kim, and J. L. Irvin, *J. Pharm. Sci.*, **58,** 821(1969).

(3) C. I. Hong, C. Piantadosi, C. B. Chae, and J. L. Irvin, *J. Med. Chem.*, **11,** 1182(1968).

(4) B. R. Baker and J. H. Jordaan, *J. Heterocycl. Chem.*, **3,** 315(1966).

(5) P. B. Russell and G. H. Hitchings, *J. Amer. Chem. Soc.*, **73,** 3763(1951).

(6) J. E. Wilson, J. L. Irvin, J. E. Suggs, and K. Liu, *Cancer Res.*, **19,** 272(1959).

(7) J. E. Wilson, J. E. Suggs, and J. L. Irvin, *ibid.*, **21,** 692(1961).

(8) H. Yuki, K. Kariya, and Y. Hashimoto, *Chem. Pharm. Bull.*, **15,** 727(1967).

(9) W. Shieve and C. G. Skinner, in "Metabolic Inhibitors," Hochster and Quastel, Eds., vol. 1, Academic, New York, N. Y., 1963, pp. 1–73.

(10) J. J. Kabara, N. R. Spafford, M. A. Mekendry, and N. L. Freeman, *Advan. Trace Methodol.*, **1,** 76(1963).

(11) W. C. Schneider, *Methods Enzymol.*, **3,** 680(1957).

(12) D. A. Williams, C. B. Chae, J. L. Irvin, and C. Piantadosi, unpublished results, 1968.

(13) C. B. Chae, Ph.D. dissertation, University of North Carolina, Chapel Hill, N. C. (1967).

(14) W. W. Umbreit, R. H. Burris, and J. F. Stauffer, "Manometric Techniques," 4th ed., Burgess, Minneapolis, Minn., 1964, pp. 1–60.

(15) C. Piantadosi, V. G. Skulason, J. L. Irvin, J. M. Powell, and L. Hall, *J. Med. Chem.*, **7,** 337(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 16, 1970, from the *Department of Medicinal Chemistry, School of Pharmacy, and Department of Biochemistry, School of Medicine, University of North Carolina, Chapel Hill, NC 27514*

Accepted for publication June 25, 1970.

This investigation was supported in part by Public Health Service Research Grant CA-06364-06 from the National Cancer Institute, from the American Cancer Society Institutional Grant (I_m-15-I) to the University of North Carolina, and a University Research Council Grant.

Abstracted in part from a dissertation presented by Chung Il Hong, to the University of North Carolina, in partial fulfillment of the Doctor of Philosophy degree requirements.

The authors thank Mr. W. T. Walker, Mr. W. R. Engen, and Mr. A. M. Mohamed for assisting in the biochemical studies.

* Present address: Roswell Park Memorial Institute, Buffalo, NY 14203

† To whom inquiries should be sent.

Colorimetric Determination of Atropine, Homatropine, Scopolamine, and Their Derivatives by the Ferric Hydroxamate Method

JOSEPH A. FELDMAN and BRUCE J. ROBB*

Abstract ☐ The purpose of this study was to develop a procedure for the quantitative analysis of the salts of the solanaceous alkaloids in the presence of their hydrolysis products. The salts taken were atropine sulfate, homatropine hydrobromide, homatropine methylbromide, scopolamine hydrobromide, and scopolamine methylbromide. The ferric hydroxamate procedure was used. The conditions necessary for a reproducible reaction were established; a procedure of sufficient sensitivity was developed which will allow unit dose analysis; and an analysis was developed which will permit the salts of the solanaceous alkaloids to be quantitatively analyzed in the presence of their degradation products.

Keyphrases ☐ Atropine, homatropine, scopolamine dosage forms—analysis ☐ Solanaceous alkaloids with degradation products—analysis ☐ Ferric hydroxamate method—solanaceous alkaloid analysis ☐ Colorimetric analysis—spectrophotometer

Atropine and its synthetic homolog homatropine are, respectively, the tropic acid and mandelic acid esters of the aminoalcohol tropine, whereas scopolamine is the tropic acid ester of scopine. The official assay methods for these drugs have inadequate sensitivity; a single assay sample for Scopolamine Hydrobromide Injection requires a minimum of 40 ml. of the solution, and at least 100 tablets are needed for the USP XVII assay of Atropine Sulfate Tablets. Another limitation of the compendial assays is that they do not discriminate between the intact ester drugs and their inactive hydrolysis products. The USP XVII assays (1) for Atropine Sulfate Injection, Homatropine Hydrobromide Ophthalmic Solution, and Scopolamine Hydrobromide Injection provide for chloroform extraction of the bases from ammoniacal solution followed by evaporation of the extracts and acidimetric titration. Chafetz and Daly (2) recently observed that tropine is extracted along with homatropine base in the USP XVII assay, invalidating it as a stability-indicating method. They proposed a selective method for homatropine; however, their method is inapplicable to tropic acid esters. Separative techniques have also been applied to the solanaceous alkaloids (3). This report describes a method which: (*a*) is generally applicable to the solanaceous alkaloids and their derivatives; (*b*) is stability indicating; and (*c*) affords adequate sensitivity for unit-dose analysis.

Hydroxylaminolysis of esters and formation of colored complexes of the hydroxamic acids so derived with ferric ion afford convenient means for determination of esters in the presence of their alcohol and acid constituents. Siegel *et al.* (4) used this reaction to follow the hydrolysis rates of methylphenidate. Vincent and Schwal (5) evaluated a ferric hydroxamate colorimetric procedure for the analysis of alkaloid drugs with ester and lactone functions such as the veratrum alkaloids, cocaine, aconitine, and pilocarpine. However, they reported that atropine, homatropine, and scopolamine gave only very faint colors. Kinetic studies (6–8) have shown that these compounds are easily hydrolyzed in alkali; hydroxylaminolysis of esters is a base-catalyzed reaction. The inference derived from these observations was that conditions wherein formation of the hydroxamate was facilitated and the rate of the competing ester saponification inhibited would afford colors intense enough to be analytically useful. Such conditions were attained by use of a large excess of hydroxylamine at ice-water bath temperature. The method was applied to atropine sulfate, homatropine hydrobromide, homatropine methylbromide, scopolamine hydrobromide, and scopolamine methylbromide. The potential utility of the method in the analysis of dosage forms of these compounds is indicated.

Table I—Absorbance Concentration Relationships

mg./5 ml.	Absorbance at 540 mμ: Atropine Sulfate	Homatropine Hydrobromide	Homatropine Methylbromide	Scopolamine Hydrobromide	Scopolamine Methylbromide
0.5	0.060	0.062	0.060	0.070	0.092
1.0	0.140	0.115	0.121	0.130	0.180
2.0	0.260	0.258	0.240	0.280	0.360
3.0	0.410	0.340	0.360	0.410	0.535
4.0	0.540	0.490	0.472	0.530	0.640
5.0	0.650	0.600	0.540	0.650	0.890

EXPERIMENTAL

Reagents and Supplies—Saturated hydroxylamine HCl (83 g. in 100 ml.), 10.5 *M* potassium hydroxide, 4 *M* hydrochloric acid, and 0.37 *M* ferric chloride in 0.1 *M* hydrochloric acid were filtered before use if necessary and stored under refrigeration. Absorbance data were obtained with 11.67-mm. diameter cylindrical cells in a Bausch and Lomb Spectronic 20 spectrometer.

All chemicals used were USP, NF, or analytical reagent grade.

Standard Preparation—Dissolve an accurately weighed amount of the appropriate reference standard in water, and dilute the solution quantitatively and stepwise to obtain a concentration of about 600 mcg./ml.

Assay Preparation—*Tablets*—Weigh and finely powder not less than 20 tablets. Extract a weighed amount with water, filter the extract if necessary, and dilute it quantitatively to represent a concentration of about 600 mcg./ml.

Solutions—If the solution is preserved with chlorobutanol or parabens, extract it with ether as described by Brochmann-Hanssen

et al. (9). Transfer an appropriate volume of the injection or ophthalmic solution to a volumetric flask and dilute it to represent about 600 mcg./ml.

Procedure—Transfer 5-ml. portions of the assay preparation, the standard preparation, and water to separate 50-ml. conical flasks chilled in an ice-water bath. Successively pipet 1 ml. of hydroxylamine HCl reagent and 1 ml. of potassium hydroxide solution into each flask, and mix. After 1 hr., add 2.0 ml. of 4 *M* hydrochloric acid (pH 1.2–1.4) and 1.0 ml. of ferric chloride reagent to each flask, mixing after each addition. Remove the flasks from the ice-water bath, allow the gas evolution to subside, and determine the absorbance of the assay and standard solutions *versus* the blank at the wavelength of maximum absorbance at about 540 mμ in a suitable spectrometer. Calculate the amount of drug, in milligrams, in the sample taken from the formula: $0.001\ C(A_U/A_S)$, in which C is the concentration in micrograms per milliliter of the standard preparation, and A_U and A_S are the absorbances of the solutions from the assay preparation and from the standard preparation, respectively.

RESULTS AND DISCUSSION

Color Intensity—The absorbance values found in trials of this procedure are presented in Table I, from which conformance to Beer's law is evident. According to Aksnes (10), the color results from a 1:1 complex of ferric ion and hydroxamic acid.

Since tropohydroxamic acid is formed by three of the compounds and mandelohydroxamic acid by the homatropine derivatives, the molar absorptivities for atropine and the scopolamine derivatives, on the one hand, and for the other two compounds, on the other, should be identical if ester saponification or other side reactions do not occur. It is evident that scopolamine methylbromide, although it has the highest molecular weight, is converted to the hydroxamate most efficiently. Since reference standards are reacted concomitantly, the differences in molar absorptivities are of little practical consequence. The molar absorptivities, ε, in liters/mole/centimeter for atropine sulfate, homatropine hydrobromide, homatropine methylbromide, scopolamine hydrobromide, and scopolamine methylbromide are, respectively, 420, 350, 385, 490, and 615. The color intensity obtained in this procedure is about 10 times that reported by Vincent and Schwal (5) for atropine, homatropine, and scopolamine, from which one may calculate ε-values of 41, 35, and 92, respectively. The increase in absorptivity effected by the procedure reported here affords a useful analytical method.

Two precautions must be taken to achieve a procedure that produces absorbance values conforming to Beer's law. First, the volume taken of the assay preparation must be constant (5 ml. in the procedure proposed). Second, the pH of the solution prior to the addition of the ferric chloride reagent must be adjusted to a value of 1.2–1.4.

The main advantage of this analysis, in addition to its sensitivity, is the fact that the analysis reaction is specific for esters and will not react with the degradation products of ester hydrolysis, the acids and aminoalcohols. To verify this, the procedure was performed on several of the degradation products which might be formed by the hydrolysis of the esters involved. Tropic acid, mandelic acid, tropine, and scopoline were tested alone in solution and in combinations. These solutions did not absorb any energy at 540 mμ.

Instrumental reproducibility, according to the manufacturers, will account for a relative error of 10% at a concentration of 0.5 mg./5 ml. However, by taking proportionate quantities of reaction solutions, unit-dose solutions containing concentrations of 0.5 mg./ml. (2.5 mg./5 ml.) can be analyzed at a relative instrumental error of 4%.

The procedure was also applied to a typical scopolamine hydrobromide injection formulation which contained 0.4 mg./ml. of scopolamine hydrobromide, mannitol, acetate buffer, and a non-ester preservative. A control was run which contained everything but the scopolamine hydrobromide, and no interference from the formulation was found. The lack of absorbance by the blank, while the absorbance of the formulation containing the scopolamine hydrobromide reflected the proper concentration of the compound, signifies that the procedure is applicable to the analysis of formulations containing esters and ester salts of solanaceous alkaloids.

CONCLUSION

A procedure that allows for the quantitative analysis of several of the salts of the solanaceous alkaloids was developed. The conditions necessary for reproducible reaction were established; a procedure of sufficient sensitivity was developed which will allow for unit-dose analysis; and an analysis was developed which will permit the salts of the solanaceous alkaloids to be selectively and quantitatively analyzed in the presence of their degradation products. Official USP and NF procedures leave much to be desired as far as analyzing for these compounds in the presence of their degradation products. It is hoped that the proposed procedure will offer a better means of analysis for the salts of the solanaceous alkaloids.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 53, 284, 577.

(2) L. Chafetz and R. E. Daly, *J. Pharm. Sci.*, **57**, 1977(1968).

(3) H. A. Lloyd, H. M. Fales, P. F. Highet, W. J. A. Vandenheuver, and J. C. Wildman, *J. Amer. Chem. Soc.*, **82**, 3791(1960).

(4) S. Siegel, L. Lachman, and L. J. Malspeis, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 431(1959).

(5) D. Vincent and H. Schwal, *Ann. Pharm. Franc.*, **19**, 6(1961).

(6) E. R. Garrett, *J. Amer. Chem. Soc.*, **79**, 1071(1957).

(7) P. Zvirblis, I. Socholitsky, and A. A. Kondritzer, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 450(1956).

(8) J. L. Patel and A. P. Lemberger, *ibid.*, **47**, 878(1958).

(9) E. Brochmann-Hanssen, P. Schmid, and J. D. Benmaman, *J. Pharm. Sci.*, **54**, 783(1965).

(10) G. Aksnes, *Acta Chem. Scand.*, **11**, 710(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 27, 1970, from the *Department of Pharmaceutical Chemistry, School of Pharmacy, Duquesne University, Pittsburgh, PA 15219*

Accepted for publication May 27, 1970.

Presented in part to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This paper received Honorable Mention, Lunsford-Richardson Pharmacy Award for Undergraduate Research, 1969.

This work was supported in part by the Work-Scholarship program of Duquesne University.

* Present address: Department of Pharmacology, University of Iowa, Iowa City, IA 52240

Spectrophotofluorometric Determination of Mestranol in Oral Contraceptive Tablets

THERON JAMES

Abstract □ A rapid, reliable fluorometric method has been developed for assaying 17α-ethynylestradiol-3-methyl ether (mestranol) in some oral contraceptive tablets. The method utilizes the native fluorescence of mestranol. No separations are required and the method is applicable in the presence of norethynodrel, ethynodiol diacetate, or norethindrone. Chlormadinone acetate interferes in the procedure. The method is sensitive (0.8–1.0 mcg./ml.) and applicable to single-tablet analyses. The accuracy under the conditions studied was ±1.29%, and the precision ranged from ±0.896 to ±1.73%.

Keyphrases □ Mestranol determination—tablets □ UV spectrophotometric, fluorometric, colorimetric analyses, mestranol—comparison □ Fluorometry—analysis

The estrogenic component of oral contraceptives is usually 17α-ethynylestradiol-3-methyl ether (mestranol). Because of its low dosage in tablets, mestranol is difficult to assay. UV (1–3), GLC (4–8), and colorimetric (9–14) methods have been proposed but all lack specificity or are unsuitable for routine analysis.

Several fluorometric procedures have also been reported (13, 15–20). The procedures involved induced fluorescence where reagent preparation, temperature, and time are all very critical for reproducibility. Use of fluorescence does increase sensitivity, but the methods currently available suffer the same limitations and drawbacks as colorimetric methods.

Duggan *et al.* (21) reported that natural estrogens possess native fluorescence. Mestranol, a synthetic estrogen, also possesses native fluorescence, whereas the more common progestins do not fluoresce appreciably. To date, methods that utilize native fluorescence (22) usually require internal standards and excipient blanks which are not readily available except to the manufacturer. Conceivably, each commercial product could have different excipients which would preclude the use of a "universal excipient blank."

The proposed assay procedure overcomes these limitations and is applicable to a variety of commercial products. No internal standards or excipient blanks are required, no preliminary separations are necessary, and the method is suitable for single-tablet analysis.

EXPERIMENTAL

Apparatus—A recording spectrophotofluorometer[1] with 1-cm. cells was used. The following instrument parameters were employed: xenon lamp; meter multiplier, 0.01; sensitivity, 45–50; 1P21 photomultiplier; slit arrangement No. 4; excitation wavelength, about 284 mμ; and emission wavelength, about 327 mμ.

Standard Preparation—Prepare a solution containing 0.8–1.0 mcg. of mestranol[2] per ml. in absolute ethanol.

[1] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Inc., Silver Spring, Md.
[2] Syntex Labs, Inc., Palo Alto, Calif.

Table I—Fluorometric Assay of Commercial Tablets Containing Mestranol

Mestranol Found, mg./Tablet (Mestranol Declared, 0.1 mg./Tablet)		
Sample 1[a]	Sample 2[a]	Sample 3[b]
0.101	0.0997	0.0979 0.100
0.101	0.100	0.0986 0.100
0.105	0.0984	0.100 0.100
0.104	0.103	0.101 0.0995
0.103	0.101	0.0991 0.100
0.101	0.100	0.0985
Av. = 0.102	Av. = 0.100	Av. = 0.0995
SD = ±1.73%	*SD* = ±1.59%	*SD* = ±0.896%

[a] Samples 1 and 2 also contain norethindrone, 2 mg./tablet. [b] Sample 3 also contains norethynodrel, 2.5 mg./tablet.

Procedure—Rinse all glassware with ethanol prior to use. Grind at least 20 tablets to pass a 60-mesh sieve. Accurately weigh a portion of powder equivalent to one tablet, and transfer to a 50-ml. centrifuge tube (for single-tablet analysis, place tablet in centrifuge tube and crush with glass rod). Pipet 20 ml. of absolute ethanol into the tube, stopper securely, and shake on a mechanical shaker for 30 min. Centrifuge to clarify the solution. Pipet an aliquot of the supernatant, equivalent to 20–25 mcg. of mestranol, into a 25-ml. volumetric flask, and dilute to volume with absolute ethanol.

Fluorescence Determination—Adjust the spectrophotofluorometer to read 70% emission, at 327 mμ, with the standard solution. Determine the fluorescence spectra of the standard and sample solutions by scanning from 285 to 400 mμ. Make calculations using the maximum emission at about 327 mμ. Use absolute ethanol as a blank and correct for it.

RESULTS AND DISCUSSION

Progestins commonly associated with mestranol in contraceptive tablets exhibit negligible native fluorescence. However, because of their relatively high concentrations in tablets as compared to mestranol (as much as 25:1), concentration quenching will occur. This could affect the fluorescence of mestranol but is effectively negated by diluting samples to large volumes. Recovery samples containing norethindrone (a progestin) and mestranol gave low recoveries for mestranol when the norethindrone concentration in the final solution was 100 mcg./ml. or more. This same sample gave 100% recovery when the norethindrone concentration was 50 mcg./ml. or less.

The accuracy of the method was demonstrated by five assays of a synthetic mix. The average mestranol recovery was 99.8%, with a standard deviation of ±1.29%.

Table I gives results of the fluorometric assay for three commercial products. Samples 1 and 2 were labeled to contain 2 mg. of norethindrone and 0.1 mg. of mestranol per tablet; Sample 3 was labeled to contain 2.5 mg. of norethynodrel and 0.1 mg. of mestranol per tablet. The standard deviation ranged from ±0.869 to ±1.73%.

Table II compares the data obtained by UV (23) and colorimetric (12) methods with results by the present fluorometric method. Results by the fluorometric method compare favorably to those by the colorimetric method. The samples represent four different manufacturers.

Mestranol in combination with chlormadinone acetate could not be determined by the fluorometric procedure without prior separation, because the excitation wavelength for mestranol (about 284 mμ) is too close to the absorption peak for chlormadinone acetate (283.5 mμ).

Table II—Comparison of Assay Methods for Mestranol in Commercial Samples

Sample	Progestin Present	Mestranol Declared, mg.	% of Amount Declared by— UV	Color	Fluorometric
1	Norethynodrel	0.1	85.1[a]	99.0[a]	100
2	Ethynodiol diacetate	0.1	98.0	—[b]	104
3	Norethindrone	0.1	100	99.0	101
4	Norethindrone	0.1	95.4	100	99.7
5A[c]	—	0.08	102	102	102
5B[c]	Chlormadinone acetate	0.08	101[a]	—[b]	—[b]

[a] Corrected for interference in the spectrum. [b] Interferences prevented accurate calculations. [c] Sequential-type tablets.

The method is also applicable to single-tablet analyses. Assay values for 10 tablets ranged from 98.5 to 105% of declared values.

REFERENCES

(1) S. Klein, A. James, and M. Tuckerman, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 314(1960).

(2) A. Shroff and J. Grodsky, *J. Pharm. Sci.*, **56**, 460(1967).

(3) R. Bastow, *J. Pharm. Pharmacol.*, **19**, 41(1967).

(4) W. Vanden Heuvel, C. Sweeley, and E. Horning, *J. Amer. Chem. Soc.*, **82**, 3481(1960).

(5) J. Talmage, M. Penner, and M. Geller, *J. Pharm. Sci.*, **54**, 1194(1965).

(6) O. Boughton, R. Bryant, W. Ludwig, and D. Timma, *ibid.*, **55**, 951(1966).

(7) E. Schulz, *ibid.*, **54**, 144(1965).

(8) J. France and B. Knox, *J. Gas Chromatogr.*, **4**, 173(1966).

(9) H. Ganshirt and J. Polderman, *J. Chromatogr.*, **16**, 510 (1964).

(10) D. Heusser, *Deut. Apoth. Z.*, **106**, 411(1966).

(11) D. Tsilifonis and L. Chafetz, *J. Pharm. Sci.*, **56**, 625(1967).

(12) A. Shroff and R. Heuttemann, *ibid.*, **56**, 654(1967).

(13) P. Comer and C. Stevenson, *ibid.*, **57**, 147(1968).

(14) W. Beyer, *ibid.*, **57**, 1415(1968).

(15) R. Boscott, *Nature*, **162**, 577(1948).

(16) W. Slaunwhite, L. Engle, P. Olmsted, and D. Carter, *J. Biol. Chem.*, **191**, 627(1951).

(17) J. McAnally and E. Mausman, *J. Lab. Clin. Med.*, **44**, 647 (1954).

(18) H. Strickler, R. Grauer, and M. Caughey, *Anal. Chem.*, **28**, 1240(1956).

(19) R. Huttenraugh and I. Keiner, *Pharmazie*, **20**, 242(1965).

(20) R. Templeton, W. Arnett, and I. Jakovljevic, *J. Pharm. Sci.*, **57**, 1168(1968).

(21) D. Duggan, R. Bowman, B. Brodie, and S. Udenfriend, *Arch. Biochem. Biophys.*, **68**, 1(1957); through S. Udenfriend, "Fluorescence Assay in Biology and Medicine," Academic, New York, N. Y., 1962, p. 353.

(22) N. Gochman and R. T. Dillon, G. D. Searle & Co., private communication, 1969.

(23) F. Kunze, Div. of Pharmaceutical Sciences, Bureau of Science, Food and Drug Administration, Washington, D. C., private communication.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 5, 1970, from the *Food and Drug Administration, Department of Health, Education, and Welfare, Los Angeles, CA 90015*

Accepted for publication May 11, 1970.

Assay of Emetine Hydrochloride Injection

KATALIN ACEL and MURRAY M. TUCKERMAN

Abstract □ Data are presented for the DAB 7 assay of emetine hydrochloride and for quantitative assay of emetine hydrochloride injection by a method previously reported for injections of amin-salts. The method involves nonaqueous titration of a chloroform. eluted sample from a magnesium oxide–siliceous earth mixture Thermogravimetric data show that emetine hydrochloride forms no stable hydrates, that water can be lost even at room temperature, and that loss still continues slowly beyond the usual drying temperature so that it is difficult to render the material anhydrous. For these reasons, it is suggested that emetine hydrochloride and emetine hydrochloride injection be labeled with the content of anhydrous emetine hydrochloride. Permissible variation should be ±1% for emetine hydrochloride and ±5% for the injection.

Keyphrases □ Emetine HCl injection—analysis □ Potentiometric titration—analysis □ Titrimetry—analysis

Emetine Hydrochloride Injection USP XVII has a peculiar definition in that "it contains an amount of anhydrous emetine hydrochloride ($C_{29}H_{40}N_2O_4 \cdot 2HCl$) equivalent to not less than 84% and not more than 94% of the labeled amount of emetine hydrochloride" (1). Thus it is the only pharmaceutical preparation formulated at less than 100% of label claim. This peculiar definition is necessitated by the official definition of Emetine Hydrochloride USP XVII as a hydrate of uncertain composition, which "contains not less than 98.0% and not more than 101.5% of $C_{29}H_{40}N_2O_4 \cdot 2HCl$, calculated on the anhydrous basis" (2).

Water is determined as follows: "Dry it at 105° for 2 hr.: it loses not less than 8% and not more than 14% of its weight" (2). The average water content of the solid is thus 11%, which corresponds to the average requirement for the injection of 89% of anhydrous material.

An attempt was made to assay emetine hydrochloride injection by a previously proposed method (3). The method involves distributing the sample over a mixture of magnesium oxide and purified diatomaceous earth held on a sintered-glass filtering funnel, eluting the

Table II—Comparison of Assay Methods for Mestranol in Commercial Samples

Sample	Progestin Present	Mestranol Declared, mg.	% of Amount Declared by— UV	Color	Fluorometric
1	Norethynodrel	0.1	85.1[a]	99.0[a]	100
2	Ethynodiol diacetate	0.1	98.0	—[b]	104
3	Norethindrone	0.1	100	99.0	101
4	Norethindrone	0.1	95.4	100	99.7
5A[c]	—	0.08	102	102	102
5B[c]	Chlormadinone acetate	0.08	101[a]	—[b]	—[b]

[a] Corrected for interference in the spectrum. [b] Interferences prevented accurate calculations. [c] Sequential-type tablets.

The method is also applicable to single-tablet analyses. Assay values for 10 tablets ranged from 98.5 to 105% of declared values.

REFERENCES

(1) S. Klein, A. James, and M. Tuckerman, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 314(1960).

(2) A. Shroff and J. Grodsky, *J. Pharm. Sci.*, **56**, 460(1967).

(3) R. Bastow, *J. Pharm. Pharmacol.*, **19**, 41(1967).

(4) W. Vanden Heuvel, C. Sweeley, and E. Horning, *J. Amer. Chem. Soc.*, **82**, 3481(1960).

(5) J. Talmage, M. Penner, and M. Geller, *J. Pharm. Sci.*, **54**, 1194(1965).

(6) O. Boughton, R. Bryant, W. Ludwig, and D. Timma, *ibid.*, **55**, 951(1966).

(7) E. Schulz, *ibid.*, **54**, 144(1965).

(8) J. France and B. Knox, *J. Gas Chromatogr.*, **4**, 173(1966).

(9) H. Ganshirt and J. Polderman, *J. Chromatogr.*, **16**, 510 (1964).

(10) D. Heusser, *Deut. Apoth. Z.*, **106**, 411(1966).

(11) D. Tsilifonis and L. Chafetz, *J. Pharm. Sci.*, **56**, 625(1967).

(12) A. Shroff and R. Heuttemann, *ibid.*, **56**, 654(1967).

(13) P. Comer and C. Stevenson, *ibid.*, **57**, 147(1968).

(14) W. Beyer, *ibid.*, **57**, 1415(1968).

(15) R. Boscott, *Nature*, **162**, 577(1948).

(16) W. Slaunwhite, L. Engle, P. Olmsted, and D. Carter, *J. Biol. Chem.*, **191**, 627(1951).

(17) J. McAnally and E. Mausman, *J. Lab. Clin. Med.*, **44**, 647 (1954).

(18) H. Strickler, R. Grauer, and M. Caughey, *Anal. Chem.*, **28**, 1240(1956).

(19) R. Huttenraugh and I. Keiner, *Pharmazie*, **20**, 242(1965).

(20) R. Templeton, W. Arnett, and I. Jakovljevic, *J. Pharm. Sci.*, **57**, 1168(1968).

(21) D. Duggan, R. Bowman, B. Brodie, and S. Udenfriend, *Arch. Biochem. Biophys.*, **68**, 1(1957); through S. Udenfriend, "Fluorescence Assay in Biology and Medicine," Academic, New York, N. Y., 1962, p. 353.

(22) N. Gochman and R. T. Dillon, G. D. Searle & Co., private communication, 1969.

(23) F. Kunze, Div. of Pharmaceutical Sciences, Bureau of Science, Food and Drug Administration, Washington, D. C., private communication.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 5, 1970, from the *Food and Drug Administration, Department of Health, Education, and Welfare, Los Angeles, CA 90015*

Accepted for publication May 11, 1970.

Assay of Emetine Hydrochloride Injection

KATALIN ACEL and MURRAY M. TUCKERMAN

Abstract □ Data are presented for the DAB 7 assay of emetine hydrochloride and for quantitative assay of emetine hydrochloride injection by a method previously reported for injections of amin-salts. The method involves nonaqueous titration of a chloroform. eluted sample from a magnesium oxide–siliceous earth mixture Thermogravimetric data show that emetine hydrochloride forms no stable hydrates, that water can be lost even at room temperature, and that loss still continues slowly beyond the usual drying temperature so that it is difficult to render the material anhydrous. For these reasons, it is suggested that emetine hydrochloride and emetine hydrochloride injection be labeled with the content of anhydrous emetine hydrochloride. Permissible variation should be ±1% for emetine hydrochloride and ±5% for the injection.

Keyphrases □ Emetine HCl injection—analysis □ Potentiometric titration—analysis □ Titrimetry—analysis

Emetine Hydrochloride Injection USP XVII has a peculiar definition in that "it contains an amount of anhydrous emetine hydrochloride ($C_{29}H_{40}N_2O_4 \cdot 2HCl$) equivalent to not less than 84% and not more than 94% of the labeled amount of emetine hydrochloride" (1). Thus it is the only pharmaceutical preparation formulated at less than 100% of label claim. This peculiar definition is necessitated by the official definition of Emetine Hydrochloride USP XVII as a hydrate of uncertain composition, which "contains not less than 98.0% and not more than 101.5% of $C_{29}H_{40}N_2O_4 \cdot 2HCl$, calculated on the anhydrous basis" (2).

Water is determined as follows: "Dry it at 105° for 2 hr.: it loses not less than 8% and not more than 14% of its weight" (2). The average water content of the solid is thus 11%, which corresponds to the average requirement for the injection of 89% of anhydrous material.

An attempt was made to assay emetine hydrochloride injection by a previously proposed method (3). The method involves distributing the sample over a mixture of magnesium oxide and purified diatomaceous earth held on a sintered-glass filtering funnel, eluting the

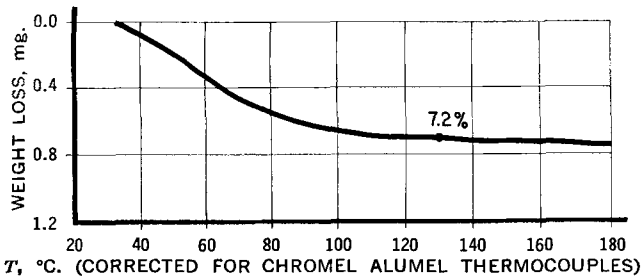


Figure 1—*Thermogravimetric study of emetine hydrochloride.*

liberated base with chloroform, and titrating the eluates with perchloric acid. However, due to the low requirement in meeting label claims, this could not be used as a guide to recovery. Therefore, a more extensive investigation was pursued.

EXPERIMENTAL

Procedure A—Weigh accurately 170 mg. of emetine hydrochloride, previously dried at 105° for 3 hr. Dissolve it in 40 ml. of glacial acetic acid; add 10 ml. of mercuric acetate T.S. and 6 drops of 0.25% *p*-naphtholbenzein in glacial acetic acid. Titrate to the green end-point. Perform a blank titration and make any necessary correction. Concomitantly determine the end-point potentiometrically. This is essentially the method of DAB 7 (4).

Weigh an amount of dried, assayed emetine hydrochloride, equivalent to 325.0 mg. of anhydrous salt, and dissolve in enough distilled water to make 5.00 ml. Assay by *Procedure B*.

Procedure B—Distribute 2.00 ml. of the solution over 3 g. of a mixture of 1 g. of chromatographic magnesium oxide and 10 g. of high flowrate, purified siliceous earth[1] held on a coarse-porosity, sintered-glass filtering funnel. Elute with five 10-ml. portions of warm (55°) chloroform, mixing each portion well with the contents of the crucible and then draining with gentle suction into 40 ml. of glacial acetic acid. Add 6 drops of *p*-naphtholbenzein indicator and titrate to the green end-point. Perform a blank titration and make any necessary correction. Concomitantly, determine the end-point potentiometrically (3).

The content of anhydrous emetine hydrochloride in the dried material found by duplicate titration by *Procedure A* was: potentiometric, 97.535 ± 0.077%; and indicator, 98.250 ± 0.084%.

Recovery from the standard solution found by duplicate titration by *Procedure B* was: potentiometric, 98.92 ± 0.25%; and indicator, 99.03 ± 0.15%.

Recovery from a commercial injection found by triplicate determination was 87.66 ± 0.76%, using the indicator end-point.

DISCUSSION

Data for recovery of assayed material show that the proposed method for the injection is accurate and reproducible enough for use as a method of control. Recovery from the commercial product is satisfactory in view of an 89 ± 5% requirement. The authors were troubled, however, by the low result obtained in the assay of the supposedly anhydrous solid, which differs significantly from 100% and barely meets the 98.0% minimum required if the indicator end-point value is taken. The sample used had been dried to constant weight, as shown by drying for an additional hour, so that the discrepancy was difficult to explain.

A sample of the original material was submitted for thermogravimetric analysis. A copy of the record is shown in Fig. 1 for a 10.8-mg. sample heated at 5°/min. in a nitrogen atmosphere. From this it can be concluded that:

1. Emetine hydrochloride forms no stable hydrates.
2. Water loss takes place even at room temperature. This usually indicates that the water content of the solid will fluctuate with the relative humidity.
3. Slow loss continues to take place at temperatures above 105°, so that the drying time specified in USP XVII would not be expected to remove all moisture. Moisture loss is so slow, however, that the sample will appear to have reached constant weight by the usual criterion. It is suggested, therefore, that emetine hydrochloride be defined in terms of its content of anhydrous salt without drying. The definition should then read: "contains not less than 99% and not more than 101% of the labeled content of anhydrous emetine hydrochloride." The injection should also be labeled in terms of content of anhydrous salt.
4. A new sample of emetine hydrochloride was obtained for moisture determination by the Karl Fischer titrimetric method (5). The reagent was standardized in triplicate against sodium tartrate dihydrate containing 15.61% water to yield a value of 6.276 ± 0.021 mg. H_2O/ml. The moisture in the emetine hydrochloride sample, determined in duplicate, was 9.40 ± 0.01%. Moisture content by drying to constant weight was 9.27%. This finding again shows that drying to constant weight does not quite remove all of the moisture. The close agreement between the oven-drying method and the Karl Fischer method suggests that the titrimetric procedure should be adopted.

[1] Celite 545, Johns-Manville.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 222.

(2) *Ibid.*, p. 221.

(3) M. M. Tuckerman and T. Bićan-Fister, *J. Pharm. Sci.*, **58**, 1014(1969).

(4) "Deutsches Arzneibuch," 7, Ausgabe, Deutscher Apotheker-Verlag, Stuttgart, West Germany, 1968, pp. 510, 511.

(5) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 924.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 17, 1969, from *Temple University, School of Pharmacy, Health Sciences Center, Philadelphia, PA 19118*

Accepted for publication May 26, 1970.

Abstracted in part from a thesis submitted by K. A. to the Graduate School, Temple University, Philadelphia, Pa., in partial fulfillment of Master of Science degree requirements.

The authors thank Smith Kline & French Laboratories, Philadelphia, Pa., for the thermogravimetric determination, and Mr. Howard Ecker for aid in the Karl Fischer determinations.

Colorimetric and Gas Chromatographic Analyses of Arecoline in Capsule Preparations

BARBARA J. KOVENSKY and CHARLES W. POOLE

Abstract □ Two procedures have been developed for the quantitative determination of arecoline hydrobromide in capsule preparations. In the colorimetric assay, the arecoline salt was converted to the base with aqueous sodium bicarbonate, extracted into chloroform, and reacted with methyl orange. The absorbance was determined at 527 mμ. With the GLC method, the arecoline salt was converted to the base in a 5:100 triethylamine–chloroform solution and directly injected onto the column, using nicotine as the internal standard. The GLC method was found to be faster and more accurate.

Keyphrases □ Arecoline capsules—analysis □ Colorimetric analysis—spectrophotometry □ GLC—analysis

Arecoline hydrobromide, methyl 1,2,5,6-tetrahydro-1-methylnicotinate hydrobromide, is a parasympathomimetic agent. Its major pharmaceutical uses are as a cathartic for horses and a teniacide for dogs.

The only officially recognized assay pertaining to this material has its major application with the raw material (1). Until 1960, when Stainier and Gloesener (2) published their somewhat laborious gas chromatographic procedure, the compound had been virtually ignored in light of advanced analytical instrumentation.

The presence of arecoline hydrobromide in many soft gelatin capsule preparations necessitated these investigations. First a colorimetric assay was developed and subsequently a faster and more convenient gas chromatographic assay.

EXPERIMENTAL

Colorimetric Assay—*Preparation of Sample and Standard*—Fifty milligrams of arecoline hydrobromide dissolved in 500 ml. of water served as the standard. Capsules sufficient to yield a sample of 10 mg. of arecoline hydrobromide were cut into a 200-ml. centrifuge bottle; 25 ml. of chloroform was added, and the sample was swirled to disperse the fill material. To the solution, 0.1 *N* H_2SO_4 (100 ml.) was added; then the sample was shaken for 10 min. and centrifuged to separate the biphase mixture.

Assay Procedure—A 5-ml. aliquot of the aqueous phase and the standard were each transferred to 200-ml. centrifuge bottles. Five milliliters of water and 1 g. of sodium bicarbonate were then added to the solutions. The samples (*i.e.*, the prepared sample and standard) were heated on a steam bath until effervescence subsided and then cooled to room temperature. Chloroform (100 ml.) was added to the samples, which were then shaken for 10 min. The samples were centrifuged and the aqueous phase was discarded.

A 40-ml. portion of each sample was transferred to a 50-ml. glass-stoppered centrifuge tube along with a 40-ml. portion of chloroform as a reagent blank. One milliliter of a saturated aqueous solution of boric acid and 1.0 ml. of a saturated aqueous solution of methyl orange were added to each sample and the reagent blank. The resulting solutions were shaken for 20 min., centrifuged, and the water layer discarded. A 15-ml. aliquot of each was then transferred to 50-ml. glass-stoppered tubes, and 1.0 ml. of a 2% H_2SO_4 solution in absolute ethanol was added. The absorbance was read at 527 mμ against the reagent blank.

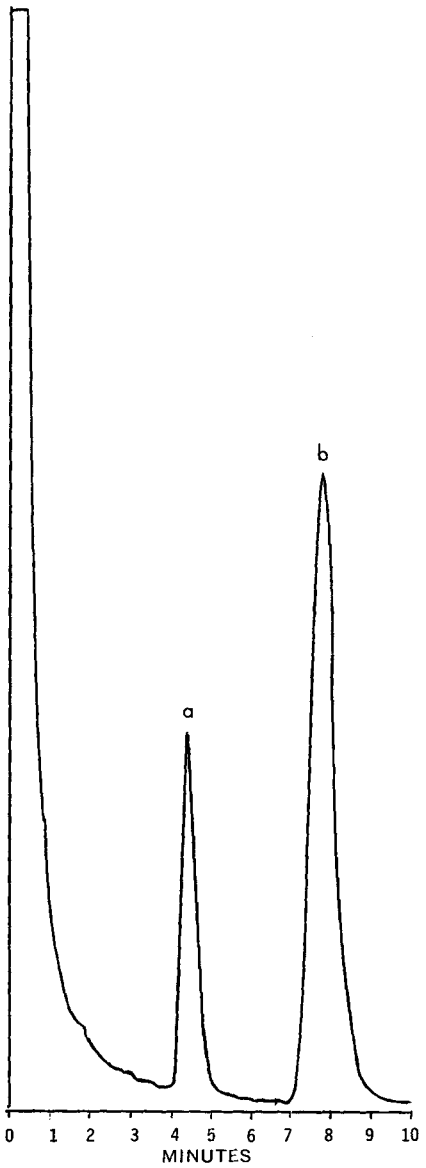


Figure 1—*Representative chromatogram: a, arecoline (4.5 min.); and b, nicotine (8 min.).*

Calculations—The potency of arecoline hydrobromide in milligrams per capsule was calculated using the following formula:

$$\frac{A_u}{A_s} \times \frac{10}{\text{number of capsules}} = \text{mg. arecoline HBr/capsule} \quad \text{(Eq. 1)}$$

where A_u is the absorbance of assay sample solution, and A_s is the absorbance of standard solution.

Gas Chromatographic Assay—*Preparation of Sample and Standard*—A 52.0-mg. sample of arecoline hydrobromide was weighed into a 250-ml. centrifuge bottle to use as the standard. Capsules comprising a 52.0-mg. sample of arecoline hydrobromide were cut open and dispersed in a 250-ml. centrifuge bottle. To both sample and standard, a 5:100 triethylamine solution in chloroform (100 ml.) was added, and the samples were shaken for 10 min. A 50-ml. portion of water was added to extract any compound

Table I—Comparison of the Two Assay Procedures

Product Lot	Capsule Claim, mg.	Methyl Orange	Recovery, %	GLC	Recovery, %
I	25.92	25.5	98.3	25.8	99.5
II	25.92	25.0	96.5	25.1	96.8
III	4.14	4.1	97.6	4.2	103.0
IV	34.56	31.8	92.0	33.4	96.8
V	2.70	2.7	100.0	3.3	121.1
VI	35.5	29.4	85.0	33.8	97.8

from the shell surfaces. The biphase mixture was shaken, centrifuged, and the water layer discarded.

The standard and sample solutions were then diluted 1:1 with a 0.5 mg./ml. solution of nicotine in chloroform, the internal standard. Two microliters of each solution was injected onto the column.

Gas Chromatograph—A Varian 2100 chromatograph with a hydrogen-flame ionization detector was used with a model 480 electronic digital integrator equipped with a Victor Digit-matic printout.

Column and Conditions—A 1.52 m. × 0.63 cm. × 2 mm. (5 ft. × 0.25 in. × 2 mm.) Pyrex column was packed with 5% polyethylene glycol 20 M[1] and 0.2% KOH on 60/80 diatomaceous earth.[2] The column was preconditioned overnight at 170° in a stream of helium. The operating conditions were as follows: column temperature, 120°; injector temperature, 235°; detector temperature, 250°; carrier gas, helium, 57 ml./min.; detector gas, hydrogen, 25 ml./min.; air, 300 ml./min.; and sensitivity, 124 × 10^{-12} amp./mv.

Identification of Constituents—The relative retention times were arecoline, 4.5 min., and nicotine, 8 min.

Calculations—The potency of arecoline hydrobromide in milligrams per capsule was calculated according to the following formula:

$$As \cdot \frac{Ca}{Ar} \cdot X = \text{mg. arecoline HBr/capsule} \qquad \text{(Eq. 2)}$$

where As is the area of the arecoline peak in sample/area of internal standard peak, Ca is the concentration of arecoline hydrobromide in mg./ml. in the standard solution, Ar is the area of arecoline peak in prepared standard/area of internal standard peak, and X is the dilution factor.

RESULTS AND DISCUSSION

Several gelatin capsule preparations were assayed by the method proposed in the "Official Methods of Analysis" (1). The method was found inadequate for capsules, because the average dosage level of arecoline hydrobromide is 1–8 mg. The number of capsules needed to procure the necessary 100.0-mg. sample created severe emulsion problems. The overabundance of gelatin made it virtually impossible to separate the two phases required for the assay. The methyl orange colorimetric method proposed here, although sensitive to any trace of amines or ammonia compounds on the glassware, did suffice as an analytical tool for assaying low concentrations of arecoline hydrobromide.

In an attempt to conserve the relatively large amount of time necessary for running the assay colorimetrically, the investigation then turned to developing a quicker, more precise instrumental method. A perusal of the literature revealed Stainier and Gloesener's (2) chromatographic assay of arecoline. Their samples of arecoline were hydrolyzed with KOH, and the corresponding alcohols were assayed quantitatively. Since the method was rather indirect, it was felt that arecoline could be chromatographed directly by merely converting the hydrobromide salt to its base in an appropriate solvent and injecting the arecoline directly onto the proper column.

A typical chromatogram is shown in Fig. 1. The peaks are well resolved and symmetrical; absorption effects appear to be absent.

Table I shows a comparison of values for each of the proposed methods, which were each run on six capsule lot preparations of varying concentrations of arecoline hydrobromide. In each case, a greater part of the theoretical claim was recovered with the gas chromatographic method, which also took less time to complete. Nine samples, each containing 54.5 mg. of arecoline hydrobromide, showed a mean percent recovery of 99.4 with a relative standard deviation from the mean of ±3.0 (Table II). The precision of the colorimetric assay was found in experimental studies to be of the magnitude of ±6.0%.

Table II—Statistical Treatment of GLC Procedure

Sample No.	Claim, mg.	Amount Recovered, mg.	Recovery, %
1A	54.5	52.0	95.4
1B	54.5	56.1	102.9
IC	54.5	52.6	96.5
IIA	54.5	55.5	101.8
IIB	54.5	54.6	100.2
IIC	54.5	52.9	97.1
IIIA	54.5	52.5	96.3
IIIB	54.5	55.5	101.9
IIIC	54.5	55.8	102.3
Mean		54.2	99.4
SD			3.0

REFERENCES

(1) "Official Methods of Analysis," 10th ed., Association of Official Agricultural Chemists, Washington, D. C., Section 32.008, 1965, p. 535.

(2) C. Stainier and E. Gloesener, *Farmaco Ed. Prat.*, **15**, 721 (1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 6, 1970, from the *Analytical Research Laboratories, R. P. Scherer Corp., Detroit, MI 48213*

Accepted for publication May 28, 1970.

[1] Carbowax, Union Carbide Corp., New York, N. Y.

[2] Gas-Chrom W, Applied Science Laboratories, Inc., State College, Pa.

Characterization of the Content Uniformity Plan

C. B. SAMPSON, H. L. BREUNIG, J. P. COMER, and D. E. BROADLICK

Abstract □ The operating characteristic of the official content uniformity plans is developed in terms of proportion defective in a lot. This proportion defective is represented as a function of the means and variances, respectively, of the drug substance weight and the tablet weight. It is then shown that the probability of acceptance of a lot can be derived for each process from knowledge of only: (*a*) the coefficient of variation, and (*b*) the proportional process bias. That is, the proportion defective in a lot is considered in terms of departures from theoretical drug substance weight and target tablet weight and their coefficients of variation which can arise in normal manufacturing operations. A completely worked example of these calculations outlines the procedure. The tables show, through computer calculation, the probability of acceptance arising through the interplay of several selected values of the coefficients of variation and of the proportional process bias.

Keyphrases □ Tablet content uniformity—determination □ Content uniformity, tablets—sampling plan □ Defective tablets, proportion—determination □ Formulas, tablet—content uniformity determination

The content uniformity sampling plans for tablets in USP XVII and NF XIII have the disadvantages that all information available to the analyst is not efficiently utilized and that the probability of acceptance of a lot cannot be evaluated in terms of departures from the theoretical drug content and target tablet weight.

A new procedure for estimating content uniformity in pharmaceutical products has been proposed by Comer *et al.* (1). Information from both tablet weights and assays may be utilized to provide more efficient estimates of mean potency and tolerance limits. In the present paper, the authors develop the operating characteristics of current official sampling plans for content uniformity tests. Formulas are presented for studying the effect of slight changes in drug content and tablet weight during manufacture upon the probability that a lot of material will meet the official content uniformity limits. These formulas include not only the coefficients of variation of both tablet weight and assay data but also what are described as proportional process biases in these variables. Similar statements concerning the USP weight variation test have been made by Roberts (2).

STRUCTURE

The primary forces which determine the amount of drug substance in the unit dose of pharmaceutical tablet forms are: (*a*) the weight of drug substance per weight of formulation material as estimated by assay, say P, and (*b*) tablet weights, say Y.

If a population is assumed to be normal, then the population parameters of interest are the mean, μ, and the variance, σ^2. Such a normal distribution is denoted by the conventional $N(\mu,\sigma^2)$. Then, restating the assumption of normality, the population of tablet weights is assumed to be normally distributed with mean μ_y and variance σ_y^2, *i.e.*, $N(\mu_y,\sigma_y^2)$. Similarly, the distribution of single-unit assay measurements is $N(\mu_p,\sigma_p^2)$. For consistency, say that Y is measured in milligrams per tablet and P in milligrams of drug substance per milligram of formulation material. It is further assumed that the concentration of drug, P, is independent of the weight of the tablet, Y. The unbiased estimates of μ_p, μ_y, σ_p^2, and σ_y^2 are denoted by $\bar{p}$, $\bar{y}$, s_p^2, and s_y^2, respectively. These estimates are based on n_p observations on the P distribution and n_y observations on the Y distribution.

The variable of interest is PY, the weight of drug substance in milligrams per tablet. Some distribution-free results concerning the mean and variance of PY are now presented. Since P is assumed to be independent of Y, the population mean, μ_{py}, is given by the product of the respective population mean, $\mu_p\mu_y$, and the population variance, σ_{py}^2, is equal to $\mu_p^2\sigma_y^2 + \mu_y^2\sigma_p^2 + \sigma_p^2\sigma_y^2$. [See, for example, Goodman (3).] The unbiased estimates of μ_{py} and σ_{py}^2 are easily shown to be

$$\hat{\mu}_{py} = \overline{py} \qquad \text{(Eq. 1}a\text{)}$$

$$\hat{\sigma}_{py}^2 = \bar{p}^2 s_y^2 + \bar{y}^2 s_p^2 + \left(1 - \frac{1}{n_p} - \frac{1}{n_y}\right) s_p^2 s_y^2 \qquad \text{(Eq. 1}b\text{)}$$

The distribution of PY is not easily determined, but some exact distribution results have been reported by Craig (4), Aroian (5), and DeZur and Donahue (6). These results are somewhat mathematically intractable and are not readily amenable to practical utilization. However, Aroian (5) has demonstrated that as the ratios σ_p/μ_p and σ_y/μ_y, singly or together, become small, the distribution of PY approaches normality. These ratios, denoted by γ_p and γ_y, respectively, are called population coefficients of variation.

Also, approximate formulas for skewness and kurtosis have been worked out for the product of two random variables. [See Burr (7).] Skewness and kurtosis are measures of shape of distribution which, along with the mean and variance, characterize most distributions fairly well. Skewness is a measure of symmetry and has a value of zero for symmetric distributions. Kurtosis measures flatness or peakedness and equals 3.0 for a normal distribution and 1.8 for the rectangular (uniform) distribution.

Sampson (8) evaluated the skewness and kurtosis formulas, performed some Monte Carlo simulations, and concluded that, for the coefficients of variation of magnitudes usually encountered and considered acceptable in tablet manufacture, the assumption of normality for the distribution of PY is reasonable, provided P and Y have normal distributions.

With this structure in mind, the probability of meeting content uniformity requirements for various population coefficients of variation for the respective populations of single-unit assays and of tablet weights is developed.

OPERATING CHARACTERISTIC FORMULAS

The current content uniformity sampling plan for tablets in USP XVII (9) and NF XIII (10) may be summarized as follows: Individually assay 10 tablets. If all 10 are within 85–115% of the mean of the tolerances specified in the official monograph, then the requirements are met. If one of 10 fails, *i.e.*, falls outside the limits, then 20 more tablets are individually assayed. If only one of the combined sample of 30 falls outside limits, the lot is passed. All other possibilities result in failure to meet requirements.

A tablet outside of limits is defined as a "defective." The probability of accepting a lot with a proportion, π, of defective units

under this sampling plan is

$$P_\pi = (1 - \pi)^{10} + 10\pi(1 - \pi)^{29} \quad \text{(Eq. 2)}$$

P_π is easily derived from the criteria for the sampling plan and delineates the operating characteristic curve for various values of the proportion defective π. Once π is evaluated in light of this structure, Eq. 2 characterizes the content uniformity plan.

The lower and upper allowable limits for a particular characteristic of a product unit are denoted by L and U, respectively. If the mean of the tolerances specified by the official monograph is μ, then $L = 0.85\mu$ and $U = 1.15\mu$ for the official plan. If label claim is μ, then the process is expected to operate such that $\mu = \mu_p\mu_y$; therefore, $1 - \pi = Pr[L < PY < U] = Pr[0.85\mu_p\mu_y < PY < 1.15\mu_p\mu_y]$ may be evaluated. It is probable that the parameter mean for the drug substance in formulation material may be running not exactly at μ_p but at $\mu_p + \Delta_p$ for a given point in time, where Δ_p is some incremental change and may be positive or negative. Similarly the process may not be making tablets that weigh on the average μ_y but rather $\mu_y + \Delta_y$. With this in mind, π is computed as a function of the coefficients of variation, $\gamma_p = \sigma_p/\mu_p$ and $\gamma_y = \sigma_y/\mu_y$, and also of what may be termed proportional process biases, $\delta_p = \Delta_p/\mu_p$ and $\delta_y = \Delta_y/\mu_y$. That is, π is a function only of γ_p, γ_y, δ_p, and δ_y; when the processes are running on target so that δ_p and δ_y both equal zero, then π is dependent only upon γ_p and γ_y, the population coefficients of variation.

From these definitions,

$$\mu_{py} = (\mu_p + \Delta_p)(\mu_y + \Delta_y) = \mu_p\mu_y(1 + \delta_p)(1 + \delta_y) \quad \text{(Eq. 3)}$$

$$\sigma_y^2 = (\mu_p + \Delta_p)^2\sigma_y^2 + (\mu_y + \Delta_y)^2\sigma_p^2 + \sigma_p^2\sigma_y^2$$
$$= \mu_p^2\sigma_y^2(1 + \delta_p)^2 + \mu_y^2\sigma_p^2(1 + \delta_y)^2 + \mu_p^2\mu_y^2\gamma_p^2\gamma_y^2 \quad \text{(Eq. 4)}$$

Furthermore:

$$1 - \pi = Pr[L < PY < U]$$
$$= Pr\left[\frac{L - \mu_{py}}{\sigma_{py}} < \frac{PY - \mu_{py}}{\sigma_{py}} < \frac{U - \mu_{py}}{\sigma_{py}}\right]$$
$$= F\left[\frac{U - \mu_{py}}{\sigma_{py}}\right] - F\left[\frac{L - \mu_{py}}{\sigma_{py}}\right] \quad \text{(Eq. 5a)}$$

where $F[\,]$ may be found in tables of the cumulative distribution of the standardized normal function. [For example, see Beyer (11).] From Eqs. 3 and 4,

$$\frac{U - \mu_{py}}{\sigma_{py}} = \frac{1.15\mu_p\mu_y - \mu_p\mu_y(1 + \delta_p)(1 + \delta_y)}{[\mu_p^2\sigma_y^2(1 + \delta_p)^2 + \mu_y^2\sigma_p^2(1 + \delta_y)^2 + \mu_p^2\mu_y^2\gamma_p^2\gamma_y^2]^{1/2}} \quad \text{(Eq. 5b)}$$

By combining and cancelling terms on the right-hand side, this becomes

$$\frac{U - \mu_{py}}{\sigma_{py}} = \frac{1.15 - (1 + \delta_p)(1 + \delta_y)}{\gamma_{py}} \quad \text{(Eq. 6)}$$

where

$$\gamma_{py} = [\gamma_y^2(1 + \delta_p)^2 + \gamma_p^2(1 + \delta_y)^2 + \gamma_p^2\gamma_y^2]^{1/2} \quad \text{(Eq. 7)}$$

Similarly,

$$\frac{L - \mu_{py}}{\sigma_{py}} = \frac{0.85 - (1 + \delta_p)(1 + \delta_y)}{\gamma_{py}} \quad \text{(Eq. 8)}$$

By carrying out the calculations of Eqs. 7, 6, 8, and 5*a* and inserting into Eq. 2, the probability of accepting a lot for given values of γ_p, γ_y, δ_p, and δ_y may be computed.

ILLUSTRATION

Suppose that for a given tablet item labeled at 10 mg. drug per tablet, the process specifications stipulate that the target weight for the granulation is 0.1 mg. of drug substance per milligram of formulation material, *i.e.*,

$$\mu_p = 0.1 \text{ mg./mg.} \quad \text{(Eq. 9)}$$

Table I—Probability of Meeting Content Uniformity Requirements when $\delta_p = \delta_y = 0$[a]

γ_p	γ_y 0.01	0.02	0.03	0.04	0.05
0.01	1.0000	1.0000	1.0000	1.0000	0.9975
0.02		1.0000	1.0000	0.9998	0.9936
0.03			1.0000	0.9983	0.9786
0.04				0.9861	0.9331
0.05					0.8322

[a] When $\delta_p = \delta_y = 0$, the tables are symmetrical.

The repeatability of assay determinations, which also reflects homogeneity, is given by a standard deviation of, say,

$$\sigma_p = 4 \times 10^{-3} \text{ mg./mg.} \quad \text{(Eq. 10)}$$

Similarly, the target weight for compression and weighing may be

$$\mu_y = 100 \text{ mg./tablet} \quad \text{(Eq. 11)}$$

and the repeatability of compression and weighing may be

$$\sigma_y = 3.0 \text{ mg./tablet} \quad \text{(Eq. 12)}$$

Assume, however, that for a particular lot the process is running low on formulation and high on compression so that

$$\mu_p + \Delta_p = 0.093 \text{ mg./mg.} \quad \text{(Eq. 13)}$$

and

$$\mu_y + \Delta_y = 101 \text{ mg./tablet} \quad \text{(Eq. 14)}$$

with the standard deviations remaining the same.

Then, by definition, $\delta_p = -0.07$, $\delta_y = 0.01$, $\gamma_p = 0.04$, and $\gamma_y = 0.03$. Calculating from Eq. 3,

$$\mu_{py} = (0.1)(100)(1 - 0.07)(1 + 0.01)$$
$$= 9.393 \text{ mg. drug/tablet} \quad \text{(Eq. 15)}$$

and from Eq. 7,

$$\gamma_{py} = [(0.04)^2(1 + 0.01)^2 + (0.03)^2(1 - 0.07)^2 + (0.04)^2(0.03)^2]^{1/2}$$
$$= 0.04911 \quad \text{(Eq. 16)}$$

From Eq. 6:

$$\frac{U - \mu_{py}}{\sigma_{py}} = \frac{1.15 - (1 - 0.07)(1 + 0.01)}{0.04911}$$
$$= 4.29 \quad \text{(Eq. 17)}$$

And, similarly, from Eq. 8,

$$\frac{L - \mu_{py}}{\sigma_{py}} = \frac{0.85 - (1 - 0.07)(1 + 0.01)}{0.04911}$$
$$= -1.818 \quad \text{(Eq. 18)}$$

By Eq. 5*a*,

$$1 - \pi = F[4.29] - F[-1.818]$$
$$= 1.00 - 0.03454 \textit{ (Reference 11)} \quad \text{(Eq. 19)}$$

and thus $\pi = 0.03454$.

By Eq. 2

$$P_{0.03454} = (0.96546)^{10} + 10(0.03454)(0.96546)^{29}$$
$$= 0.8283 \quad \text{(Eq. 20)}$$

for the values given for γ_p, γ_y, δ_p, and δ_y.

That is, if the label claim is 10 mg. of drug substance per tablet and if the processes are running at $\mu_p = 0.093$ mg./mg. and $\mu_y =$

Table II—Probability of Meeting Content Uniformity Requirements

γ_p	γ_y 0.01	0.03	0.05
	when $\delta_p = -0.04$, $\delta_y = 0.02$		
0.01	1.0000	1.0000	0.9954
0.03	1.0000	0.9997	0.9663
0.05	0.9896	0.9539	0.7871
	when $\delta_p = 0.09$, $\delta_y = -0.08$		
0.01	1.0000	1.0000	0.9986
0.03	1.0000	1.0000	0.9786
0.05	0.9836	0.9459	0.7943
	when $\delta_p = 0.07$, $\delta_y = -0.01$		
0.01	1.0000	0.9986	0.7794
0.03	0.9996	0.9560	0.6310
0.05	0.8593	0.6823	0.4063

101 mg./tablet with coefficients of variation of 3% for P and 4% for Y, then the probability of meeting the content uniformity requirements as specified in USP XVII and NF XIII is 0.8283.

Although it is not feasible to publish complete tables of $P[\pi]$ for even a representative indexing of γ_p, γ_y, δ_p, and δ_y, a few additional examples (Tables I and II) are included.

DISCUSSION

Recalling that the probabilities of meeting content uniformity requirements depend only upon the four parameters δ_p, δ_y, γ_p, and γ_y, it is convenient to note that these probabilities are symmetric with respect to the pairs (δ_p, γ_p) and (δ_y, γ_y). That is, if $(\delta_p, \gamma_p) = (0.07, 0.03)$ and $(\delta_y, \gamma_y) = (-0.01, 0.05)$, then the probability of acceptance is the same as if $(\delta_p, \gamma_p) = (-0.01, 0.05)$ and $(\delta_y, \gamma_y) = (0.07, 0.03)$. This can be easily verified from Eqs. 6 and 7.

The IBM Mathpack Subroutine NDTR (12) was used to calculate the function $F[\,]$. Since this particular subroutine has a maximum error of $7(\times 10^{-7})$, the computed probabilities are in general more accurate than if they were computed using $F[\,]$ from usual tabled sources of the cumulative normal distribution. The difference is in the fourth decimal place and of little practical significance.

Note that several probabilities in Tables I and II are given as 1.0000. This means that, even though it is not absolutely certain that a lot will meet content uniformity requirements under the indexed parameter conditions, the probabilities of acceptance are so high that rounding to four decimal places carries these values to 1.0000.

The formulas in this paper compute the probability of meeting content uniformity requirements for given population parameters γ_p, γ_y, δ_p, and δ_y. They characterize the content uniformity sampling plan and are to be used as supplemental information in making decisions before and during the production of particular products.

REFERENCES

(1) J. P. Comer, H. L. Breunig, D. E. Broadlick, and C. B. Sampson, *J. Pharm. Sci.*, **59**, 210(1970).

(2) C. Roberts, *Technometrics*, **11**, 161(1969).

(3) L. A. Goodman, *J. Amer. Statist. Ass.*, **55**, 708(1960).

(4) C. C. Craig, *Ann. Math. Statist.*, **7**, 1(1936).

(5) L. A. Aroian, *ibid.*, **18**, 265(1947).

(6) R. S. DeZur and J. D. Donahue, *Aeronaut. Res. Lab. Tech. Rep.*, **1965**, 71.

(7) I. W. Burr, Chemical Division, American Society for Quality Control, *News* (*Special Issue*), **10**, 7(1964).

(8) C. B. Sampson, "Statistical Procedures for the Estimation of Content Uniformity," presented to the APhA Academy of Pharmaceutical Sciences, Pharmaceutical Analysis and Control Section, Washington, D. C. meeting, November 1969.

(9) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 905.

(10) "National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, p. 799.

(11) "Handbook of Tables for Probability and Statistics," W. N. Beyer, Ed., The Chemical Rubber Co., Cleveland, Ohio, 1966.

(12) IBM System/360 Scientific Subroutine Package (360A-CM-03X) Version III, Program H20-0205-3, International Business Machines, Technical Publications Dept., White Plains, NY 10601

ACKNOWLEDGMENTS AND ADDRESSES

Received March 26, 1970, from the *Lilly Research Laboratories, Indianapolis, IN 46206*

Accepted for publication May 19, 1970.

Solid-Phase Synthesis and Degradation of a Model Polypeptide by an Automated Approach

ANDREW M. TOMETSKO*, JOHN TISCHIO, and JOHN GARDEN, II

Abstract □ The solid-phase synthesis and degradation of a model polypeptide, L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-alanine, was carried out using a computer-oriented automated approach. Two computer programs were used to generate control paper tapes for the total synthesis and degradation of the model peptide. Encouraging results were obtained in automating the degradation of polypeptides on the solid phase and in carrying out the degradation in a nonaqueous solvent system. The course of the synthesis and the degradation was monitored by amino acid analysis of acid hydrolyzates of the resin which were taken periodically. The automated solid-phase degradation of naturally occurring polypeptides and proteins should be possible through modifications of the described approach.

Keyphrases □ Polypeptide—automated synthesis, degradation □ Amino acid sequence, computer controlled—polypeptide synthesis □ Degradation, polypeptide, Edman—automated □ Automated system—peptide synthesis, degradation

Classical methods have been employed in the chemical synthesis (1–6) and Edman degradation (7–10) of polypeptides and proteins. Recently, a number of laboratories have automated solid-phase polypeptide synthesis (11–14); in one instance (15), the automation of classical sequential analysis of polypeptides has been reported.

Table II—Probability of Meeting Content Uniformity Requirements

γ_p	γ_y 0.01	0.03	0.05
	when $\delta_p = -0.04$, $\delta_y = 0.02$		
0.01	1.0000	1.0000	0.9954
0.03	1.0000	0.9997	0.9663
0.05	0.9896	0.9539	0.7871
	when $\delta_p = 0.09$, $\delta_y = -0.08$		
0.01	1.0000	1.0000	0.9986
0.03	1.0000	1.0000	0.9786
0.05	0.9836	0.9459	0.7943
	when $\delta_p = 0.07$, $\delta_y = -0.01$		
0.01	1.0000	0.9986	0.7794
0.03	0.9996	0.9560	0.6310
0.05	0.8593	0.6823	0.4063

101 mg./tablet with coefficients of variation of 3% for P and 4% for Y, then the probability of meeting the content uniformity requirements as specified in USP XVII and NF XIII is 0.8283.

Although it is not feasible to publish complete tables of $P[\pi]$ for even a representative indexing of γ_p, γ_y, δ_p, and δ_y, a few additional examples (Tables I and II) are included.

DISCUSSION

Recalling that the probabilities of meeting content uniformity requirements depend only upon the four parameters δ_p, δ_y, γ_p, and γ_y, it is convenient to note that these probabilities are symmetric with respect to the pairs (δ_p, γ_p) and (δ_y, γ_y). That is, if $(\delta_p, \gamma_p) = (0.07, 0.03)$ and $(\delta_y, \gamma_y) = (-0.01, 0.05)$, then the probability of acceptance is the same as if $(\delta_p, \gamma_p) = (-0.01, 0.05)$ and $(\delta_y, \gamma_y) = (0.07, 0.03)$. This can be easily verified from Eqs. 6 and 7.

The IBM Mathpack Subroutine NDTR (12) was used to calculate the function $F[\,]$. Since this particular subroutine has a maximum error of $7(\times 10^{-7})$, the computed probabilities are in general more accurate than if they were computed using $F[\,]$ from usual tabled sources of the cumulative normal distribution. The difference is in the fourth decimal place and of little practical significance.

Note that several probabilities in Tables I and II are given as 1.0000. This means that, even though it is not absolutely certain that a lot will meet content uniformity requirements under the indexed parameter conditions, the probabilities of acceptance are so high that rounding to four decimal places carries these values to 1.0000.

The formulas in this paper compute the probability of meeting content uniformity requirements for given population parameters γ_p, γ_y, δ_p, and δ_y. They characterize the content uniformity sampling plan and are to be used as supplemental information in making decisions before and during the production of particular products.

REFERENCES

(1) J. P. Comer, H. L. Breunig, D. E. Broadlick, and C. B. Sampson, *J. Pharm. Sci.*, **59**, 210(1970).

(2) C. Roberts, *Technometrics*, **11**, 161(1969).

(3) L. A. Goodman, *J. Amer. Statist. Ass.*, **55**, 708(1960).

(4) C. C. Craig, *Ann. Math. Statist.*, **7**, 1(1936).

(5) L. A. Aroian, *ibid.*, **18**, 265(1947).

(6) R. S. DeZur and J. D. Donahue, *Aeronaut. Res. Lab. Tech. Rep.*, **1965**, 71.

(7) I. W. Burr, Chemical Division, American Society for Quality Control, *News* (*Special Issue*), **10**, 7(1964).

(8) C. B. Sampson, "Statistical Procedures for the Estimation of Content Uniformity," presented to the APhA Academy of Pharmaceutical Sciences, Pharmaceutical Analysis and Control Section, Washington, D. C. meeting, November 1969.

(9) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 905.

(10) "National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, p. 799.

(11) "Handbook of Tables for Probability and Statistics," W. N. Beyer, Ed., The Chemical Rubber Co., Cleveland, Ohio, 1966.

(12) IBM System/360 Scientific Subroutine Package (360A-CM-03X) Version III, Program H20-0205-3, International Business Machines, Technical Publications Dept., White Plains, NY 10601

ACKNOWLEDGMENTS AND ADDRESSES

Received March 26, 1970, from the *Lilly Research Laboratories, Indianapolis, IN 46206*

Accepted for publication May 19, 1970.

Solid-Phase Synthesis and Degradation of a Model Polypeptide by an Automated Approach

ANDREW M. TOMETSKO*, JOHN TISCHIO, and JOHN GARDEN, II

Abstract □ The solid-phase synthesis and degradation of a model polypeptide, L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-alanine, was carried out using a computer-oriented automated approach. Two computer programs were used to generate control paper tapes for the total synthesis and degradation of the model peptide. Encouraging results were obtained in automating the degradation of polypeptides on the solid phase and in carrying out the degradation in a nonaqueous solvent system. The course of the synthesis and the degradation was monitored by amino acid analysis of acid hydrolyzates of the resin which were taken periodically. The automated solid-phase degradation of naturally occurring polypeptides and proteins should be possible through modifications of the described approach.

Keyphrases □ Polypeptide—automated synthesis, degradation □ Amino acid sequence, computer controlled—polypeptide synthesis □ Degradation, polypeptide, Edman—automated □ Automated system—peptide synthesis, degradation

Classical methods have been employed in the chemical synthesis (1–6) and Edman degradation (7–10) of polypeptides and proteins. Recently, a number of laboratories have automated solid-phase polypeptide synthesis (11–14); in one instance (15), the automation of classical sequential analysis of polypeptides has been reported.

Studies in this laboratory have been directed toward the development of automated techniques that might be applicable to solid-phase synthesis and solid-phase degradation of polypeptides. Toward this goal an Automatic Chemical Reaction System (ACRS) (16) has been designed and constructed to carry out the physical operations involved in the synthesis and/or degradation following commands punched in a paper tape. Another phase of this research program has involved the development of computer approaches for generating the paper tape containing the commands for executing the synthesis or degradation (*e.g.*, amino acid additions, filtration steps, stirring sequences, deblocking steps, wash cycles, and activation steps).

Although the synthesis of polypeptides on the solid phase has been extensively studied and discussed (17–21), reports on solid-phase degradation have been limited to studies employing a resin-bound isothiocyanate functional group (22, 23) and to studies with the peptide bound to the resin but using methylisothiocyanate (MTC) in the degradation (24, 25). Dijkstra *et al.* (25) suggested that MTC is preferable to the classical phenylisothiocyanate (PTC) due to its higher solubility in aqueous solvents. They also suggested that PTC interacts unfavorably with the resin under their reaction conditions. Since Edman and Begg (15) demonstrated nearly quantitative coupling of PTC with free amines, and since the nature of the solvent could have a considerable influence on the interactions occurring within a chemical system, a study of the degradation using PTC in nonaqueous solvent systems seemed worthwhile. Information concerning the use of PTC with solid-phase degradative systems in nonaqueous solvents has been limited (26).

In this article, the authors report the application of ACRS to the solid-phase synthesis and degradation of a model polypeptide, L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-alanine. This model peptide was chosen to study: (*a*) the use of PTC for the degradation of polypeptides in conjunction with a solid-phase (polystyrene) matrix; (*b*) the solid-phase degradation in a nonaqueous solvent that would be more compatible with both PTC and the resin; and (*c*) the feasibility of automating the synthesis and Edman degradation of polypeptides by applying a computer-oriented automated approach.

EXPERIMENTAL

Chemicals—All reagents were of analytical grade. L-Amino acids,[1] trifluoroacetic acid (TFA),[2] and chloromethylated polystyrene[3] were used. *tert*-Butyloxycarbonyl (*t*-BOC) amino acids were prepared by the method of Schwyzer *et al.* (27).

Preparation of *tert*-Butyloxycarbonyl Alanine (*t*-BOC ala.) Resin—The *t*-BOC ala. resin was prepared according to the method of Merrifield (17). Specifically, chloromethylated polystyrene (25 g., 0.7 meq./g.) was refluxed for 48 hr. with *t*-BOC ala. (9.88 g.) and triethylamine (Et_3N) (7.65 ml.) in absolute ethanol (150 ml.). The resin was filtered and washed three times with ethanol (50 ml. each) and three times with methanol (50 ml. each) and then was dried *in vacuo*. Amino acid analysis of an acid hydrolyzate of resin gave an alanine content of 0.523 meq./g.

Automatic Chemical Reaction System (ACRS)—ACRS (16) is an electromechanical device, which was designed and constructed to execute automatically the physical operations normally involved in solid-phase synthesis or degradation. Specifically, it is capable of measuring and dispensing appropriate chemicals into a reaction vessel, stirring chemicals, filtering, timing events, and, generally, controlling the synthetic or degradative processes. The information for every operation is punched onto a paper tape. The paper tape is fed into the ACRS unit, which translates the punched holes into physical operations such as turning on a stirrer or operating valve networks. Obviously, the ACRS is merely assisting by executing operations which would normally be carried out by the investigator.

Table I—Sequence of Chemical Additions for Coupling an Amino Acid

Chemical Additions	Operation	Time, min.	Total Time, min.
1. Chloroform	Wash (three times)	2.0 each	6
2. *t*-BOC leu	Amino acid addition	2.0 each	6
3. DCC	Activating agent	120.0	120
4. $CHCl_3$	Wash (three times)	2.0 each	6
5. Ethanol	Wash (three times)	2.0 each	6
6. $CHCl_3$	Wash (three times)	2.0 each	6
7. 50% TFA	Deblocking reagent	30.0	30
8. $CHCl_2$	Wash (three times)	2.0 each	6
9. 10% Et_3N	Neutralization	10.0	10
10. $CHCl_3$	Wash (three times)	2.0 each	6
		Total time/cycle =	202

The principal component of this type of reaction system is the decoder which translates the command tape. In essence, this approach divides the synthesis or degradation into two categories: (*a*) those features that are primarily physical in nature (*e.g.*, measuring and adding chemicals), and (*b*) those features that are primarily chemical in nature (*e.g.*, removal of protecting groups and coupling of amino acids). By assuming the burden of the first category, the ACRS permits the investigator to concentrate more on the chemical aspects of the synthesis or degradation.

Preparation of Control Tape with Computers—In the automated approach discussed in this report, the sequence of operations involved in the chemical synthesis or degradation is incorporated onto a paper tape. Methods for preparing the command tape quickly and accurately are extremely important. Manual methods could be used to prepare the control tape. However, such methods are very time-consuming and increasingly subject to error as the number of commands increase. These difficulties led to a search for a more rapid and reliable means for preparing control tapes. Since the linear progression of commands with time is analogous in form to a one-dimensional array, a computer appeared to be an obvious candidate for setting up a simple array of commands of any reasonable dimension (*e.g.*, 30,000 commands). Once the computer program for generating the command array is available, the computer provides a control tape with speed (60 commands/sec.) and with reliability for any number of commands.

By using this approach for the synthesis of polypeptides, it is only necessary to specify the amino acid sequence, and the computer generates a one-dimensional array of commands for the complete synthesis. To specify the desired amino acid sequence, a set of data cards is prepared with the name of each amino acid punched onto a separate card. The cards are then placed in the data deck in the order that the amino acids occur in the polypeptide chain. Thus, if the sequence gly.val.leu.phe is desired, four cards corresponding to gly, val, leu, and phe, respectively, are arranged in order and inserted at the end of the data section of the program. For a 100-amino acid protein, 100 cards in the appropriate sequence are used. The program and data are read into the computer, which generates all of the commands involved in the complete synthesis of the peptide. With a functional program,[4] an investigator (with little or no programming experience) could generate a control tape of any desired length within minutes by simply supplying the desired amino acid sequence.

Computers—Computer programs[4] were written in the FORTRAN IV language and processed by an IBM 360-44 computer and a PDP-7 computer (University of Rochester). In this process, an array of commands was generated by the IBM 360-44 and copied

[1] Nutritional Biochemical Corp.
[2] Eastman Chemical Corp.
[3] Bio-Rad Laboratories.

[4] A listing of the computer programs is available from the authors.

Table II—Amino Acids Incorporated into the Resin during Synthesis

Leucine Addition Cycles	Average mmoles of Amino Acid/g. of Resin: Alanine	Leucine	Ratio of Alanine to Leucine: Experimental	Theoretical
0	0.495 ± 0.025	0.00	1:0	1:0
1	0.273 ± 0.030	0.241 ± 0.038	1:0.88	1:1
2	0.243 ± 0.040	0.476 ± 0.080	1:1.96	1:2
3	0.281 ± 0.013	0.800 ± 0.045	1:2.85	1:3
4	0.207[a]	0.840[a]	1:4.06[a]	1:4
5	0.195 ± 0.008	0.945 ± 0.010	1:4.86	1:5

[a] Values present are from one experiment since sampling from the second experiment was taken 5 min. after the addition of the fifth leucine and activating agent. (See *Discussion.*)

onto a magnetic tape. The magnetic tape was then read by the PDP-7 computer which produced a paper tape paralleling the commands on the magnetic tape.

Chemical Synthesis—The automated chemical synthesis of the model polypeptide, L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-alanine, was carried out using the sequence of operations shown in Table I. Initially, stock solutions of the various reagents were prepared and placed in the ACRS unit. These solutions consisted of: (*a*) 0.2 *M* *t*-BOC leu in chloroform ($CHCl_3$); (*b*) 0.2 *M* dicyclohexylcarbodiimide (DCC) in $CHCl_3$; (*c*) 10% Et_3N in $CHCl_3$; (*d*) 50% TFA in $CHCl_3$; (*e*) ethanol; and (*f*) $CHCl_3$. The desired amino acid sequence was read into the computer (*i.e.*, five data cards calling for leucine) along with the FORTRAN IV program deck. A one-dimensional array of commands for the synthesis of the peptide was then set up by the computer and copied onto a magnetic tape. The magnetic tape was then read by a PDP-7 computer which produced the control paper tape. The ACRS unit (through its decoder) executed the following operations from the tape.

A sample of deblocked *t*-BOC ala. resin (3.0 g.) in the reaction vessel was treated three times with $CHCl_3$ to wash and swell the resin. A solution of *tert*-butyloxycarbonyl-L-leucine (30 ml.) was then added, the mixture was stirred for 1 min., and a solution of DCC in $CHCl_3$ (30 ml.) was added. The resulting mixture was stirred at room temperature for 2 hr. and filtered. The resin was washed three times with each of the wash reagents: $CHCl_3$, ethanol, and $CHCl_3$, respectively (60 ml. of each reagent); the deblocking reagent [50% TFA in $CHCl_3$ (60 ml.)] was added. The deblocking mixture was stirred for 30 min. and filtered. Control experiments indicated that complete cleavage of the BOC group was obtained within 15 min. under these conditions. The resin was washed with $CHCl_3$ (60 ml.) three times, and 10% Et_3N in $CHCl_3$ (60 ml.) was added. The mixture was stirred for 10 min. and was filtered and washed three times with $CHCl_3$.

The resin was then ready for the next amino acid addition in subsequent positions of the control tape. The internal timer of the ACRS unit was set to move the control tape to the next position at 1.5-min. intervals. Following the last wash (Step 10 in Table I) in each coupling cycle, a sample of the resin was removed from the reaction vessel and dried. This resin (10–15 mg.) was then transferred to a hydrolysis tube, and a solution of 6 *N* HCl in 50% dioxane (1 ml.) was added. The tube was then sealed and incubated at 110° for 48 hr. Table II shows the average amino acid analysis of an acid hydrolyzate after each amino acid addition cycle for two experiments. In Fig. 1, the leucine residues on the resin during each experiment were plotted as a function of the reaction cycle (dotted line), together with the theoretically expected values (solid line).

Edman Degradation of the Model Peptide—As in the automated synthesis described, a control tape was prepared for the series of operations involved in the degradation of the resin-bound polypeptide. A computer program was used to set up an array of commands, which were copied onto a magnetic tape and subsequently translated into punched holes on a paper tape by the PDP-7 computer. The general sequence of operation is shown in Table III. In this case, stock solutions consisted of: (*a*) PTC–Et_3N–50% pyridine in dimethyl formamide (DMF) (1:1:8); (*b*) 50% pyridine in DMF; (*c*) $CHCl_3$; (*d*) 10% Et_3N in $CHCl_3$; and (*e*) 50% TFA in $CHCl_3$. The stock solutions were placed in the ACRS unit, and the paper control tape for the degradation was fed into the ACRS. Initially, the resin with the synthetic peptide attached was washed three times with 50% pyridine in DMF (60 ml.). The PTC solution (60 ml.) was then added. The reaction mixture was stirred under nitrogen for 120 min. and was filtered. After washing the resin three times with 50% pyridine in DMF and three times with $CHCl_3$, a solution of 50% TFA in $CHCl_3$ (60 ml.) was added. The resulting mixture was stirred for 120 min. and was filtered and washed with $CHCl_3$ (60 ml.) three times. The resin was stirred for 10 min. with 10% Et_3N in $CHCl_3$ (60 ml.) and was filtered and washed with $CHCl_3$ (60 ml.) three times.

Table III—Sequence of Chemical Addition for Edman Degradations

Chemical Additions	Operation	Time, min.	Total Time, min.
1. 50% pyridine in DMF	Wash (three times)	2.0 each	6
2. PTC	Derivative formation	120.0	120
3. 50% pyridine in DMF	Wash (three times)	2.0 each	6
4. $CHCl_3$	Wash (three times)	2.0 each	6
5. TFA in $CHCl_3$	Cyclization	120.0	120
6. $CHCl_3$	Wash (three times)	2.0 each	6
7. 10% Et_3N in $CHCl_3$	Neutralization	10.0	10
8. $CHCl_3$	Wash (three times)	2.0 each	6
		Total time/cycle =	280

This sequence of operations was repeated for each cycle (amino acid removal) in the degradation. The progress of the degradative process was monitored by analyzing the residual peptide attached to the resin after the final wash in each degradative cycle (Step 8 in Table III) as described. The change in leucine to alanine ratio indicated the progress of the degradation. Table IV shows the average amino acid analysis of acid hydrolyzates after each degradation cycle for two experiments. In Fig. 2, the leucine residues on the resin during each experiment were plotted as a function of the degradation cycle (dotted lines), together with the theoretically expected values (solid line).

RESULTS AND DISCUSSION

The studies with the model polypeptide, L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-alanine, have demonstrated the feasibility of applying computer-oriented automated techniques to the chemical synthesis and/or degradation of polypeptides and proteins. The ACRS unit with the computer-generated control tape carried out

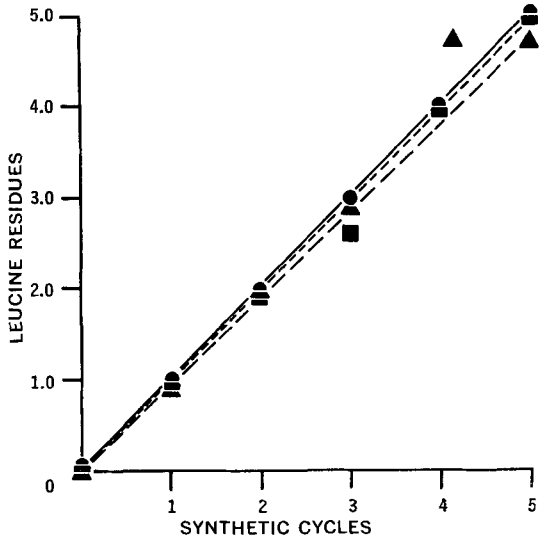


Figure 1—*The number of leucine residues present in an acid hydrolyzate of the resin is plotted as a function of the synthetic cycle. Theoretical values are indicated by the solid lines and circles. Experimental values are illustrated with dotted lines and with triangles in one case and with squares in the other. Leucine residues were determined with respect to the alanine content of the resin (Table II).*

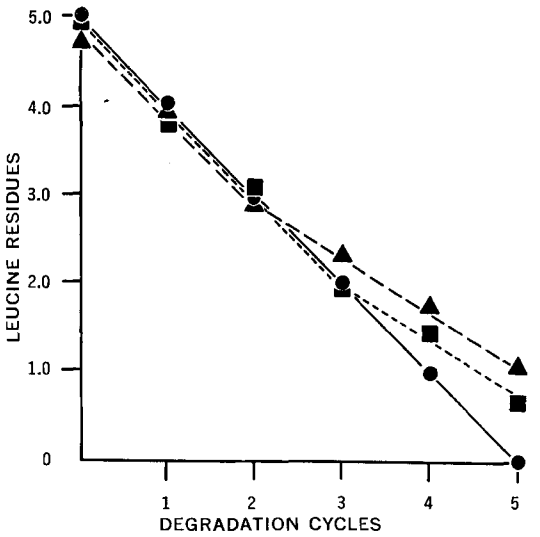


Figure 2—*The number of leucine residues present in an acid hydrolyzate of the resin is plotted as a function of the degradative cycle. Theoretical values are indicated by the solid line and circles. Experimental values are illustrated with dotted lines and with triangles in one case and with squares in the other. Leucine residues were determined with respect to the alanine content of the resin (Table IV).*

most of the physical operations involved in the synthesis and degradation of the model peptide. Progress of the amino acid incorporation into the growing chain was evidenced by the increase in leucine ratio in the amino acid analysis of an acid hydrolyzate of the resin-bound peptide.

Figure 1 compares the experimental values (dotted lines) for two different experiments with the theoretically expected values (solid line). The results indicate that the amount of leucine incorporated into the growing polypeptide chain is close to the theoretically expected amounts. Ultimately, it is desirable and necessary to drive the synthesis at each step to completion (at least 98% or higher yield at each step) to avoid a heterogeneous final product and a massive isolation and purification problem. For a synthesis to run under optimum conditions, information concerning the progress of each reaction step would be extremely important. Not only would such information provide an indication of the current status of the synthesis, it would also suggest future changes in the experimental approach which would accelerate the synthesis or optimize the yield of the reactions involved.

In Fig. 1 the triangle at Cycle 4 provides an example of the information that could be used to reevaluate the experimental approach. In this case, a sample of resin for acid hydrolysis was removed from the reaction vessel 5 min. after the addition of the new (activated) amino acid. The amino acid analysis of the resin demonstrated that leucine had been significantly incorporated into the polypeptide during this brief period. These results are consistent with those of Esko *et al.* (28) for the reaction of leucine with polypeptides on the solid phase. Other amino acids gave differences in reaction time for 90% completion extending from a few minutes to 24 hr. The rapid incorporation observed in this study would suggest a modification in reaction time in future studies and would result in the addition of more leucine residues per day. Reaction conditions [*e.g.*, Esko *et al.* (28)] that are specific for each amino acid could be readily incorporated into the computer program and the resulting paper command tape. In this case, when the amino acid sequence is specified, those conditions that are specific for a given amino acid (*e.g.*, reaction time, amount and concentration of chemicals to be added, and temperature of the reaction) would be generated by the computer in essentially the same time taken to generate the tape for reaction cycles that are identical for each amino acid. Information concerning the different chemical characteristics should be forthcoming in future studies and should permit the implementation of reaction conditions tailored to each amino acid.

The Edman degradation is an obvious choice for monitoring the current status of a synthetic operation at each major step since the free *N*-terminal amino acid is readily removed. The resulting peptide

Table IV—Amino Acids on the Resin following Edman Degradation

Degradation Cycles	Average mmoles of Amino Acid/g. of Resin		Ratio of Alanine to Leucine	
	Alanine	Leucine	Experimental	Theoretical
0	0.195 ± 0.008	0.945 ± 0.010	1:4.86	1:5
1	0.154 ± 0.012	0.600 ± 0.067	1:3.90	1:4
2	0.214 ± 0.002	0.623 ± 0.001	1:3.00	1:3
3	0.228 ± 0.008	0.483 ± 0.042	1:2.12	1:2
4	0.231 ± 0.012	0.322 ± 0.043	1:1.45	1:1
5	0.231 ± 0.037	0.212 ± 0.079	1:0.92	1:0

can then be analyzed as in this study, or the amino acid thiohydantoin derivative could be characterized and quantitated. A degradation following a coupling sequence could indicate the presence of free (uncoupled) *N*-terminal amino acid, while a degradation following a deblocking cycle would indicate the extent to which the preceding coupling cycle has gone to completion and the extent that the preceding amine protective group has been deblocked. The model peptide in this study does not lend itself to this type of interpretation because of the repeating sequence of leucine residues. In future studies, degradative methods will be used to evaluate the synthesis at each step for peptides containing different amino acids in adjacent positions.

This model peptide did indicate that the solid-phase degradation of polypeptides following computer-generated programs is indeed feasible in the nonaqueous solvent systems employed in this study. Figure 2 demonstrates the progress during the degradation of the model peptide in two different experiments (dotted lines). Beginning with the intact peptide attached to the resin, subsequent degradation steps reduced the leucine to alanine ratio by about one residue until the *N*-terminal of the residual peptide was within three amino acid residues of the resin. At this point, removal of additional leucine residues became more difficult. The nature of the decrease in cleavage is unclear and will be studied further. This lower cleavage rate has been reproduced in a number of experiments and is also in agreement with the results of Laursen (24). Attempts were made to overcome the reduction by employing different chemical preparations, solvents, and reaction conditions, but results consistent with Fig. 2 were obtained in each case. It is possible that the free amine is rendered inaccessible due to interactions between the smaller peptides and the resin. More experiments are needed to clarify this point.

Automation of the classical Edman degradation approach (15) has greatly accelerated the process of sequential analysis of proteins with the removal of 15 amino acid residues in 24 hr. The computer-oriented automated approach to solid-phase degradations discussed in this report should be equally useful in determining the amino acid sequence of proteins, with some modifications in the described method. Studies on the automated solid-phase degradation of naturally occurring polypeptides are currently being carried out in this laboratory

REFERENCES

(1) J. Rudinger, *Pure Appl. Chem.*, **7**, 385(1963).

(2) K. Hofmann and P. G. Katsoyannis, in "The Proteins," H. Neurath, Ed., Academic, New York, N. Y., 1963, p. 53.

(3) E. Schröder and K. Lubke, "The Peptides," vol. I, translated by E. Gross, Academic, New York, N. Y., 1965.

(4) H. D. Law, *Ann. Rep. Progr. Chem.*, **63**, 517(1966).

(5) M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience, New York, N. Y., 1966.

(6) P. G. Katsoyannis and J. Ginos, *Ann. Rev. Biochem.*, **38**, 89(1969).

(7) P. Edman, *Acta Chem. Scand.*, **4**, 277(1950).

(8) *Ibid.*, **10**, 761(1956).

(9) D. Ilse and P. Edman, *Aust. J. Chem.*, **16**, 411(1963).

(10) W. R. Gray, *Methods Enzymol.*, **11**, 469(1967).

(11) R. B. Merrifield, J. M. Stewart, and N. Jernberg, *Anal. Chem.*, **38**, 1905(1966).

(12) K. Brunfeldt, J. Halstom, and P. Roepstorff, in "Peptides," E. Bricas, Ed., Wiley, New York, N. Y., 1968, p. 197.

(13) A. Loffet and J. Close, *ibid.*, p. 189.

(14) G. W. H. A. Mansveld, H. Hindriks, and H. C. Beyerman, *ibid.*, p. 195.
(15) P. Edman and G. Begg, *Eur. J. Biochem.*, **1**, 80(1967).
(16) A. M. Tometsko, patent pending (1969).
(17) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149(1963).
(18) *Ibid.*, **91**, 501(1969).
(19) R. B. Merrifield, *Science*, **150**, 178(1965).
(20) R. B. Merrifield, *Advan. Enzymol.*, **32**, 221(1969).
(21) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," Freeman, San Francisco, Calif., 1969.
(22) G. R. Stark, *Fed. Proc.*, **24**, 225(1965).
(23) L. M. Dowling and G. R. Stark, *Biochemistry*, **8**, 4728 (1969).
(24) R. A. Laursen, *J. Amer. Chem. Soc.*, **88**, 5344(1966).
(25) A. Dijkstra, H. Billiet, A. H. Van Coninck, and H. Van-Velthuyzen, *Rec. Trav. Chim.*, **86**, 65(1967).
(26) A. Schellenberger, H. Jeschkeit, R. Henkel, and H. Lehmann, *Z. Chem.*, **7**, 191(1967).
(27) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622(1959).
(28) K. Esko, S. Karlsson, and J. Porath, *Acta Chem. Scand.*, **22**, 3342(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 9, 1970, from the *School of Medicine and Dentistry, University of Rochester, Rochester, NY 14620*

Accepted for publication May 26, 1970.

The development of the Automatic Chemical Reaction System was supported by the Chemtrox Corporation, Rochester, N. Y.

The authors thank George Mourtzikos for amino acid analysis and Ruth Howard for computing assistance.

* To whom inquiries concerning the Automatic Chemical Reaction System should be directed. Instrumentation aspects of the research will be reported in future publications.

NOTES

Effect of Mescaline HCl on Resistance of Male Mice to Histamine Stress

A. STANLEY WELTMAN, ARTHUR M. SACKLER, and LEROY JOHNSON

Abstract □ Single intraperitoneal injections of mescaline HCl caused significant decreases in the ability of albino mice to tolerate histamine phosphate when administered intraperitoneally 40 min. after mescaline. The dose levels of mescaline HCl ranged from 5–100 mg./kg. of body weight. Eight subcutaneous injections of mescaline (100 mg./kg. of body weight) administered during a 2-week period showed no difference between the LD_{50} values of the test *versus* saline control groups when challenged with histamine 24 hr. after the eighth and last dose of mescaline. No effects were noted in the body weights and growth patterns of the test mice in the 2-week investigation.

Keyphrases □ Mescaline HCl—effect on histamine tolerance, mice □ Histamine tolerance, mice—effect of mescaline HCl □ Hallucinogens, mescaline—acute *versus* prolonged effects on growth, histamine stress resistance

Previous investigations have demonstrated that LSD-25 stimulates adrenocortical activity and inhibits growth, metabolism, and gonadal and thyroidal function in female (1–3) and male rats (4). LD_{50} analyses of male rats given eight sequential injections of LSD-25 spaced during a 2-week period presented suggestive evidence of increased ability of the treated animals to tolerate histamine stress (5). Parallel studies with male mice receiving mescaline HCl (6) revealed identical, but somewhat smaller alterations in adrenal and thyroidal function but no effects on body weight and food consumption. Adrenal weights and adrenocortical activity [*i.e.*, thymus involution and white blood cell count (WBC) decreases] were significantly increased (6). The lesser effects produced by mescaline *versus* LSD-25 may be attributed to the lower potency of mescaline. The equivalent hallucinogenic dose of mescaline in humans is 4000 times greater than that of LSD (7). As with LSD-25 (3, 4, 8), various physiological findings have demonstrated the development of tolerance or accommodation of the treated mice to mescaline (6). The present investigation sought to determine acute *versus* prolonged effects of mescaline administration on body growth and the resistance of male mice to histamine stress.

EXPERIMENTAL

Three series of young male experimental albino mice (Carworth Farms, CFW) averaging 22 g. in body weight were selected for histamine LD_{50} analyses. Series I and II test and control mice were challenged intraperitoneally with histamine phosphate 40 min. after receiving a single intraperitoneal injection of mescaline HCl dissolved in normal saline or equivalent doses of saline. Series III mice received histamine phosphate 24 hr. after the eighth and final subcutaneous injection of mescaline HCl or normal saline. The eight injections were administered over a 2-week period on alternate days with the exception that on the 13th and 14th days, the two doses were given consecutively.

Series I consisted of 225 mice divided equally into three groups (two test groups and one saline control). The two doses of mescaline administered were 5 and 20 mg./kg. body weight. Series II consisted of an equivalent number of mice also divided equally into two test and one control groups. The test mice received a single dose of either 50 or 100 mg./kg. body weight of mescaline HCl. Series III was represented by a single test (100 mg./kg.) and normal saline group. In general, the various test groups were challenged with 5–6 dose levels of histamine phosphate and utilized 15–19 mice per dose level. The appropriate challenging doses of histamine, depending on the susceptibility of the test and control groups, ranged from 350–1450 mg. histamine base/kg. body weight. Finney's (9) method of probit analysis was used to calculate the LD_{50} values and dose–response lines.

To determine further the effects of various doses of mescaline on survival periods, the time of death following histamine phosphate

(14) G. W. H. A. Mansveld, H. Hindriks, and H. C. Beyerman, *ibid.*, p. 195.
(15) P. Edman and G. Begg, *Eur. J. Biochem.*, **1**, 80(1967).
(16) A. M. Tometsko, patent pending (1969).
(17) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149(1963).
(18) *Ibid.*, **91**, 501(1969).
(19) R. B. Merrifield, *Science*, **150**, 178(1965).
(20) R. B. Merrifield, *Advan. Enzymol.*, **32**, 221(1969).
(21) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," Freeman, San Francisco, Calif., 1969.
(22) G. R. Stark, *Fed. Proc.*, **24**, 225(1965).
(23) L. M. Dowling and G. R. Stark, *Biochemistry*, **8**, 4728 (1969).
(24) R. A. Laursen, *J. Amer. Chem. Soc.*, **88**, 5344(1966).
(25) A. Dijkstra, H. Billiet, A. H. Van Coninck, and H. Van-Velthuyzen, *Rec. Trav. Chim.*, **86**, 65(1967).
(26) A. Schellenberger, H. Jeschkeit, R. Henkel, and H. Lehmann, *Z. Chem.*, **7**, 191(1967).
(27) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622(1959).
(28) K. Esko, S. Karlsson, and J. Porath, *Acta Chem. Scand.*, **22**, 3342(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 9, 1970, from the *School of Medicine and Dentistry, University of Rochester, Rochester, NY 14620*

Accepted for publication May 26, 1970.

The development of the Automatic Chemical Reaction System was supported by the Chemtrox Corporation, Rochester, N. Y.

The authors thank George Mourtzikos for amino acid analysis and Ruth Howard for computing assistance.

* To whom inquiries concerning the Automatic Chemical Reaction System should be directed. Instrumentation aspects of the research will be reported in future publications.

NOTES

Effect of Mescaline HCl on Resistance of Male Mice to Histamine Stress

A. STANLEY WELTMAN, ARTHUR M. SACKLER, and LEROY JOHNSON

Abstract □ Single intraperitoneal injections of mescaline HCl caused significant decreases in the ability of albino mice to tolerate histamine phosphate when administered intraperitoneally 40 min. after mescaline. The dose levels of mescaline HCl ranged from 5–100 mg./kg. of body weight. Eight subcutaneous injections of mescaline (100 mg./kg. of body weight) administered during a 2-week period showed no difference between the LD_{50} values of the test *versus* saline control groups when challenged with histamine 24 hr. after the eighth and last dose of mescaline. No effects were noted in the body weights and growth patterns of the test mice in the 2-week investigation.

Keyphrases □ Mescaline HCl—effect on histamine tolerance, mice □ Histamine tolerance, mice—effect of mescaline HCl □ Hallucinogens, mescaline—acute *versus* prolonged effects on growth, histamine stress resistance

Previous investigations have demonstrated that LSD-25 stimulates adrenocortical activity and inhibits growth, metabolism, and gonadal and thyroidal function in female (1–3) and male rats (4). LD_{50} analyses of male rats given eight sequential injections of LSD-25 spaced during a 2-week period presented suggestive evidence of increased ability of the treated animals to tolerate histamine stress (5). Parallel studies with male mice receiving mescaline HCl (6) revealed identical, but somewhat smaller alterations in adrenal and thyroidal function but no effects on body weight and food consumption. Adrenal weights and adrenocortical activity [*i.e.*, thymus involution and white blood cell count (WBC) decreases] were significantly increased (6). The lesser effects produced by mescaline *versus* LSD-25 may be attributed to the lower potency of mescaline. The equivalent hallucinogenic dose of mescaline in humans is 4000 times greater than that of LSD (7). As with LSD-25 (3, 4, 8), various physiological findings have demonstrated the development of tolerance or accommodation of the treated mice to mescaline (6). The present investigation sought to determine acute *versus* prolonged effects of mescaline administration on body growth and the resistance of male mice to histamine stress.

EXPERIMENTAL

Three series of young male experimental albino mice (Carworth Farms, CFW) averaging 22 g. in body weight were selected for histamine LD_{50} analyses. Series I and II test and control mice were challenged intraperitoneally with histamine phosphate 40 min. after receiving a single intraperitoneal injection of mescaline HCl dissolved in normal saline or equivalent doses of saline. Series III mice received histamine phosphate 24 hr. after the eighth and final subcutaneous injection of mescaline HCl or normal saline. The eight injections were administered over a 2-week period on alternate days with the exception that on the 13th and 14th days, the two doses were given consecutively.

Series I consisted of 225 mice divided equally into three groups (two test groups and one saline control). The two doses of mescaline administered were 5 and 20 mg./kg. body weight. Series II consisted of an equivalent number of mice also divided equally into two test and one control groups. The test mice received a single dose of either 50 or 100 mg./kg. body weight of mescaline HCl. Series III was represented by a single test (100 mg./kg.) and normal saline group. In general, the various test groups were challenged with 5–6 dose levels of histamine phosphate and utilized 15–19 mice per dose level. The appropriate challenging doses of histamine, depending on the susceptibility of the test and control groups, ranged from 350–1450 mg. histamine base/kg. body weight. Finney's (9) method of probit analysis was used to calculate the LD_{50} values and dose–response lines.

To determine further the effects of various doses of mescaline on survival periods, the time of death following histamine phosphate

Table I—Effects of Mescaline HCl[a] on the Resistance of Male Albino Mice to Histamine Stress (Single Intraperitoneal Injection)

Group	Dose	*n*	Body Wt., g.	LD_{50}, mg./kg.
	Series I			
Group 1	5 mg./kg.	75	24.5	830.5
±*SE*			±0.2	±25.4
Group 2	20 mg./kg.	75	24.2	753.3
±*SE*			±0.2	±28.2
Group 3	Saline	75	24.1	913.4
±*SE*	control		±0.2	±22.5
% Diff. 1 *vs.* 3			+1.7	−9.1
p value			0.17	0.05
% Diff. 2 *vs.* 3			+0.4	−17.5
p value			0.76	<0.01
% Diff. 1 *vs.* 2			−1.2	−9.3
p value			0.34	0.09
	Series II			
Group 4	50 mg./kg.	115	24.5	622.3
±*SE*			±0.1	±15.2
Group 5	100 mg./kg.	115	24.7	537.9
±*SE*			±0.2	±17.2
Group 6	Saline	95	24.6	921.6
±*SE*	control		±0.1	±39.2
% Diff. 4 *vs.* 6			−0.4	−32.5
p value			0.44	<.001
% Diff. 5 *vs.* 6			+0.4	−41.6
p value			0.67	<.001
% Diff. 4 *vs.* 5			+0.8	−13.6
p value			0.39	0.01

[a] Sigma Chemical Co., St. Louis, Mo., Lot No. 76B-1460.

injections was recorded for Series II animals. Proportionate numbers of mice receiving 50 or 100 mg./kg. of mescaline or normal saline were selected for analyses from groups given 850, 1050, and 1250-mg./kg. doses of histamine phosphate.

Prior to initiation of the investigation, all animals were housed for 1 week to acclimatize the mice to laboratory conditions. The animals were housed five per cage in stainless steel cages 40.64 × 45.72 × 27.94 cm. (16 × 18 × 11 in.) in an air-conditioned laboratory maintained at 22.7° (73° F). The mice used in the acute studies were weighed immediately prior to administration of the mescaline to permit calculation of the approximate doses of mescaline HCl and histamine phosphate. Animals used in the 2-week study were weighed initially and at the end of each week. Previous studies have indicated that prolonged isolation (10) or excessive crowding (11) and temperature changes (12) can produce metabolic and endocrinological alterations. Similarly, to minimize handling (13) and auditory stress (14, 15) influences, all animals were handled alike, and care was taken to prevent harsh and extraneous loud noises. The average level of the laboratory background noise during the period of experimentation was 68 decibels at daytime and 85 decibels at nighttime due to the increased nocturnal activity of the animals in the colony room.

RESULTS AND DISCUSSION

Standard *t*-test analyses (16), as indicated in Table I, revealed no significant differences between the various body weights of the randomly selected mice in the acute Series I and II tests *versus* control group studies. All *p* values had higher probabilities than the 0.05 level used for statistical significance. Table I also presents the LD_{50} data for the Series I and II groups administered single intraperitoneal doses of mescaline 40 min. before the histamine challenge. In both series, all doses of mescaline (5, 20, 50, and 100 mg./kg.), when compared with their respective controls, showed significant decreases in the resistance of the test mice to histamine stress (*i.e.*, 5 mg./kg. *versus* saline, $p = 0.05$). No significant difference was noted between the LD_{50} values of the two saline controls of Series I and II ($p = 0.86$). Comparisons further revealed significant differences between the various doses of mescaline when each level was compared with the other (*i.e.*, 20 *versus* 50 mg./kg., $p = <0.01$; 50 *versus* 100 mg./kg., $p = 0.01$; *etc.*). An exception was observed in the comparison of the LD_{50} values of the 5 *versus* 20-mg./kg. dose. The *p* value of 0.09, although low, was not statistically significant. Analyses of the slopes of the regression equations for the mescaline and saline groups, on the other hand, showed no significant differences ($p = >0.05$) between the various slope values when the test groups were compared with their respective controls and each other. In accord with LD_{50} findings, standard *t*-test analyses (16) of the time of death following histamine injections likewise revealed that prior administration of 50 or 100 mg./kg. of mescaline significantly hastened the onset of death. Compared to saline controls (control death time: 5.2 ± 0.2 min.), 50 mg./kg. of mescaline significantly reduced the time of death by 43.8% ($p = <0.01$) and 100 mg./kg. by 49.4% ($p = <0.001$). The difference in the time of death between the two mescaline doses was not statistically significant ($p = 0.36$).

At the time of histamine challenge, comparable studies with additional test and control groups revealed that at 40 min. the test mice had recovered from the depressive influences of mescaline and were displaying heightened locomotor activity when compared to the saline controls (17). The reduction in resistance to histamine may reflect the combined effects of two nonspecific stress agents (mescaline and histamine) administered within too short a time period of each other (40 min.). The decrease in resistance may also be related to hypoglycemic influences of mescaline. Speck (18) reported significantly lower blood glucose levels in male rats given single injections of mescaline at doses of 5 mg./100 g. body weight.

Table II presents the final body weights and the LD_{50} values of the test (100 mg./kg.) *versus* control group receiving eight repeated injections of mescaline (Series III). The findings indicated no differences between the initial body weights of the test and control groups (21.9 ± 0.3 g.) and no effects on the final body weights of the test *versus* control mice at the completion of the prolonged 2-week study ($p = 0.81$). Analyses further revealed no statistically significant differences between the LD_{50} values ($p = 0.82$) or the slopes of the regression equations ($p = 0.39$) of the test *versus* saline control group. The two LD_{50} control values of the acute Series I and II were significantly higher than the control of Series III (*p* values = <0.01). This difference in LD_{50}'s may possibly reflect the influences of eight repeated saline injections, a 2-week age factor, or biological and environmental variables resulting from a year's lapse in time between the previous series and Series III. Despite evidence of increased adrenocortical activity (6), as demonstrated in related test groups (*i.e.*, increased adrenal weights, thymic involution, and WBC decreases), no marked or significant increase was noted in the ability of the test mice to withstand histamine stress. Feasibly, other than the route of administration of mescaline, the difference in the increased mortality demonstrated by the mice receiving a single *versus* repeated doses might rest in the time lapse between the successive doses of mescaline and histamine challenge. In the acute study, the time period was 40 min. *versus* 24 hr. for the prolonged 2-week investigation. Further groups are required to duplicate the 40-min. time interval in mice receiving eight injections and the 24-hr. period in mice receiving a single injection. It should be noted that contrary to hypoglycemic effects produced by a single dose, Speck (18) reported hyperglycemia in rats injected daily for 1.5 months. In an additional 2-week study of mice receiving 50- or 100-mg./kg. doses of mescaline subcutaneously, significant increases were noted in blood glucose levels of both groups of test mice (19).

In conclusion, single intraperitoneal doses of mescaline (5–100 mg./kg.) were found to decrease significantly the resistance of male mice to histamine stress when challenged 40 min. later. Repeated injection of mescaline HCl administered subcutaneously

Table II—Effects of Mescaline HCl[a] on the Resistance of Male Albino Mice to Histamine Stress (8 Subcutaneous Injections in 2 Weeks)

Group	Dose	*n*	Final Body Wt. 2nd Wk., g.	LD_{50}, mg./kg.
	Series III			
Group 7	100 mg./kg.	81	26.7	678.4
±*SE*			±0.2	±44.5
Group 8	Saline	80	26.8	691.1
±*SE*	control		±0.4	±29.1
% Diff. 7 *vs.* 8			0.4	−1.8
p value			0.81	0.82

[a] Sigma Chemical Co., St. Louis, Mo., Lot No. 78B-1790.

during 2 weeks showed no significant effects on the histamine LD_{50} values of test *versus* control groups despite findings of increased adrenocortical activity (6). This may be related to the time interval factor between mescaline and histamine challenge. Histamine in this last instance was administered 24 hr. after the last mescaline injection. No significant effects were noted on body weights and growth patterns of mice in the 2-week investigation as demonstrated by the present and previous findings (6).

REFERENCES

(1) A. M. Sackler, A. S. Weltman, M. Russakow, S. B. Sparber, and H. Owens, *Fed. Proc.*, **21,** 416(1962).

(2) A. M. Sackler, A. S. Weltman, and H. Owens, *Nature*, **198,** 1119(1963).

(3) A. M. Sackler, A. S. Weltman, and H. Owens, *Toxicol. Appl. Pharmacol.*, **9,** 324(1966).

(4) A. S. Weltman and A. M. Sackler, *J. Endocrinol.*, **34,** 81 (1966).

(5) A. S. Weltman and A. M. Sackler, *J. Pharm. Sci.*, **54,** 1382 (1965).

(6) A. S. Weltman, A. M. Sackler, and R. Schwartz, *Exp. Med. Surg.*, **26,** 187(1968).

(7) S. Cohen, *Ann. Rev. Pharmacol.*, **7,** 301(1967).

(8) D. X. Freedman and G. K. Aghajanian, *Science*, **127,** 1173 (1958).

(9) D. J. Finney, "Probit Analysis," Cambridge University Press, London, England, 1952.

(10) A. S. Weltman, A. M. Sackler, and S. B. Sparber, *Aerosp. Med.*, **37,** 804(1966).

(11) J. J. Christian, *Amer. J. Physiol.*, **182,** 292(1955).

(12) E. J. Farris, "The Care and Breeding of Laboratory Animals," Wiley, New York, N. Y., 1957.

(13) A. S. Weltman, A. M. Sackler, and J. Gennis, *J. Appl. Physiol.*, **16,** 587(1961).

(14) A. M. Sackler, A. S. Weltman, M. Bradshaw, and P. Jurtshuk, Jr., *Acta Endocrinol.*, **31,** 405(1959).

(15) A. M. Sackler, A. S. Weltman, and P. Jurtshuk, Jr., *Aerosp. Med.*, **31,** 749(1960).

(16) G. W. Snedecor, "Statistical Methods," Iowa State College Press, Ames, Iowa, 1949.

(17) A. S. Weltman, A. M. Sackler, J. Gennis, P. Steinglass, and R. Schwartz, *Fed. Proc.*, **27,** 220(1968).

(18) L. B. Speck, "The Pharmacology of Mescaline," Doctoral thesis, University of Colorado, Boulder, Colo., 1955.

(19) A. S. Weltman, A. M. Sackler, R. Schwartz, L. Johnson, and P. Steinglass, *Amer. Zool.*, **9,** 1079(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 27, 1970, from the *Laboratories for Therapeutic Research, Research Institute of The Brooklyn College of Pharmacy, Long Island University, Brooklyn, NY 11216*

Accepted for publication April 30, 1970.

Teratogenic Effects of Audiogenic Stress in Albino Mice

CHARLES O. WARD, MICHAEL A. BARLETTA, and TANIS KAYE

Abstract □ The teratogenic effects of audiogenic stress were studied in 60-day-old pregnant Swiss-Webster mice by exposing the pregnant dams to the effects of a noise generator for varying periods during pregnancy. The most severe fetal effects were noted when the pregnant dams were stressed on Days 8–17 and Days 10–15 of pregnancy. In an attempt to discern the days of greatest susceptibility to stress, a second group of mice was studied, and it was determined that Days 7–8 was the period of maximal susceptibility. The teratogenic effects produced by audiogenic stress included a reduction in fetal weight, complete or partial resorption of fetuses, cranial hematoma, dwarfed hind limbs, and tail defects.

Keyphrases □ Teratogenicity—audiogenic stress effects, albino mice □ Audiogenic stress, effects—teratogenicity, albino mice

The maternal organism has been exposed to a wide variety of noxious chemicals and environmental changes in an effort to determine the effects of such exposure on fetal development (1–4). Procedures that only affect the maternal organism have, however, received little attention until recently. Environmental stresses to which virtually all maternal organisms are subjected during pregnancy have been proven to exert real and deleterious effects on fetal development (5–8). Geber (5) recently demonstrated that decreased fetal weight as well as resorption frequency can be produced in the offspring of rats subjected to audiovisual stress for varying periods during development. The stress of handling pregnant dams (6) and severe audiogenic stress (7) have been shown either to block pregnancy completely or to reduce the chance for successful fetal development. The purposes of this investigation were to study the effects of audiogenic stress on pregnant albino mice in an attempt to determine the effects of such stress on fetal development and also to determine the period of pregnancy in which the fetus is most susceptible to the deleterious effects of stress.

METHODS

Sexually mature Swiss-Webster mice, received at 60 days of age, were used throughout this study. They were kept in a separate room in the animal quarters for 1 week prior to breeding to allow for adjustment to their surroundings. When proestrus was determined by the technique of vaginal smearing (9), two females were placed with each male for an 11-hr. period. When the sexes were separated, the presence of a vaginal plug was taken as proof of pregnancy. This was then Day 0. Between 20–35% of the mated females became pregnant using this technique. Pregnant females were then isolated and kept in separate cages for the duration of pregnancy.

The stress chamber was rectangular [25.40 × 20.32 × 60.96 cm. (10 × 8 × 24 in.)] and constructed of stainless steel. Pregnant mice as well as controls were placed in this chamber in a separate room, and the stressed dams were subjected to the effects of a noise generator which consisted of a motorcycle horn connected to a microswitch timer and set to deliver an output of 82–85 decibels[1] at a

[1] Sound Level Meter, Type 1551-A, General Radio Co., Cambridge, Mass.

during 2 weeks showed no significant effects on the histamine LD_{50} values of test *versus* control groups despite findings of increased adrenocortical activity (6). This may be related to the time interval factor between mescaline and histamine challenge. Histamine in this last instance was administered 24 hr. after the last mescaline injection. No significant effects were noted on body weights and growth patterns of mice in the 2-week investigation as demonstrated by the present and previous findings (6).

REFERENCES

(1) A. M. Sackler, A. S. Weltman, M. Russakow, S. B. Sparber, and H. Owens, *Fed. Proc.*, **21,** 416(1962).

(2) A. M. Sackler, A. S. Weltman, and H. Owens, *Nature*, **198,** 1119(1963).

(3) A. M. Sackler, A. S. Weltman, and H. Owens, *Toxicol. Appl. Pharmacol.*, **9,** 324(1966).

(4) A. S. Weltman and A. M. Sackler, *J. Endocrinol.*, **34,** 81 (1966).

(5) A. S. Weltman and A. M. Sackler, *J. Pharm. Sci.*, **54,** 1382 (1965).

(6) A. S. Weltman, A. M. Sackler, and R. Schwartz, *Exp. Med. Surg.*, **26,** 187(1968).

(7) S. Cohen, *Ann. Rev. Pharmacol.*, **7,** 301(1967).

(8) D. X. Freedman and G. K. Aghajanian, *Science*, **127,** 1173 (1958).

(9) D. J. Finney, "Probit Analysis," Cambridge University Press, London, England, 1952.

(10) A. S. Weltman, A. M. Sackler, and S. B. Sparber, *Aerosp. Med.*, **37,** 804(1966).

(11) J. J. Christian, *Amer. J. Physiol.*, **182,** 292(1955).

(12) E. J. Farris, "The Care and Breeding of Laboratory Animals," Wiley, New York, N. Y., 1957.

(13) A. S. Weltman, A. M. Sackler, and J. Gennis, *J. Appl. Physiol.*, **16,** 587(1961).

(14) A. M. Sackler, A. S. Weltman, M. Bradshaw, and P. Jurtshuk, Jr., *Acta Endocrinol.*, **31,** 405(1959).

(15) A. M. Sackler, A. S. Weltman, and P. Jurtshuk, Jr., *Aerosp. Med.*, **31,** 749(1960).

(16) G. W. Snedecor, "Statistical Methods," Iowa State College Press, Ames, Iowa, 1949.

(17) A. S. Weltman, A. M. Sackler, J. Gennis, P. Steinglass, and R. Schwartz, *Fed. Proc.*, **27,** 220(1968).

(18) L. B. Speck, "The Pharmacology of Mescaline," Doctoral thesis, University of Colorado, Boulder, Colo., 1955.

(19) A. S. Weltman, A. M. Sackler, R. Schwartz, L. Johnson, and P. Steinglass, *Amer. Zool.*, **9,** 1079(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 27, 1970, from the *Laboratories for Therapeutic Research, Research Institute of The Brooklyn College of Pharmacy, Long Island University, Brooklyn, NY 11216*

Accepted for publication April 30, 1970.

Teratogenic Effects of Audiogenic Stress in Albino Mice

CHARLES O. WARD, MICHAEL A. BARLETTA, and TANIS KAYE

Abstract □ The teratogenic effects of audiogenic stress were studied in 60-day-old pregnant Swiss-Webster mice by exposing the pregnant dams to the effects of a noise generator for varying periods during pregnancy. The most severe fetal effects were noted when the pregnant dams were stressed on Days 8–17 and Days 10–15 of pregnancy. In an attempt to discern the days of greatest susceptibility to stress, a second group of mice was studied, and it was determined that Days 7–8 was the period of maximal susceptibility. The teratogenic effects produced by audiogenic stress included a reduction in fetal weight, complete or partial resorption of fetuses, cranial hematoma, dwarfed hind limbs, and tail defects.

Keyphrases □ Teratogenicity—audiogenic stress effects, albino mice □ Audiogenic stress, effects—teratogenicity, albino mice

The maternal organism has been exposed to a wide variety of noxious chemicals and environmental changes in an effort to determine the effects of such exposure on fetal development (1–4). Procedures that only affect the maternal organism have, however, received little attention until recently. Environmental stresses to which virtually all maternal organisms are subjected during pregnancy have been proven to exert real and deleterious effects on fetal development (5–8). Geber (5) recently demonstrated that decreased fetal weight as well as resorption frequency can be produced in the offspring of rats subjected to audiovisual stress for varying periods during development. The stress of handling pregnant dams (6) and severe audiogenic stress (7) have been shown either to block pregnancy completely or to reduce the chance for successful fetal development. The purposes of this investigation were to study the effects of audiogenic stress on pregnant albino mice in an attempt to determine the effects of such stress on fetal development and also to determine the period of pregnancy in which the fetus is most susceptible to the deleterious effects of stress.

METHODS

Sexually mature Swiss-Webster mice, received at 60 days of age, were used throughout this study. They were kept in a separate room in the animal quarters for 1 week prior to breeding to allow for adjustment to their surroundings. When proestrus was determined by the technique of vaginal smearing (9), two females were placed with each male for an 11-hr. period. When the sexes were separated, the presence of a vaginal plug was taken as proof of pregnancy. This was then Day 0. Between 20–35% of the mated females became pregnant using this technique. Pregnant females were then isolated and kept in separate cages for the duration of pregnancy.

The stress chamber was rectangular [25.40 × 20.32 × 60.96 cm. (10 × 8 × 24 in.)] and constructed of stainless steel. Pregnant mice as well as controls were placed in this chamber in a separate room, and the stressed dams were subjected to the effects of a noise generator which consisted of a motorcycle horn connected to a microswitch timer and set to deliver an output of 82–85 decibels[1] at a

[1] Sound Level Meter, Type 1551-A, General Radio Co., Cambridge, Mass.

Table I—Teratogenic Effects of Audiogenic Stress in Albino Mice

No. of Pregnant Dams	Period of Stress, Days	Hours Stressed per Day	Total No. Normal *vs.* Abnormal Fetuses	Mean Litter Size	Mean Fetal Weight, g.	Mean CR/TU[a] Distance, mm.	No. Litters Delivered *vs.* No. Resorbed	Teratogenic Effects
5	Control	0	52/0	10.5	1.45	20/9	5/0	None
5	8–17	8	18/2	10.0	0.44	15/6	2/3	Hematoma kinked tail resorption
6	10–15	5	43/14	9.5	0.87	18/7	6/0	Hematoma dwarfed hind limbs straight tail resorption
4	7–8	5	All resorbed				0/5	Resorption
3	9–10	5	24/0	8.0	1.80	25/10	3/0	None
4	11–12	5	52/0	13.0	1.70	23/9	4/0	None
4	13–14	5	38/1	9.5	1.60	20/8	4/0	Hematoma
3	15–16	5	29/0	9.0	1.40	21/9	3/0	None
2	17–18	5	18/0	9.0	1.59	24/10	2/0	None

[a] CR = crown-rump distance in millimeters; TU = transumbilical distance in millimeters.

frequency of 320–580 c.p.s. The timer delivered these noise levels for 60–75% of each hour in an intermittent fashion, *i.e.*, 3 min. on, 2 min. off. Background noise was measured as 35–50 decibels at a frequency of 20–32 c.p.s.

Food and water were continuously supplied to both control and experimental groups; control mice were placed in the exposure chamber for 5-hr. periods on each day of pregnancy.

Both experimental and control females were sacrificed on Day 18 of pregnancy; a laparotomy was performed, the uterus was exposed and incised, and the fetuses were removed. Care was taken to note uterine distribution of fetuses as well as any vacant uterine sites which were indicative of fetal resorption. The fetuses were then weighed on a Class A torsion balance to the nearest 0.01 g.; transumbilical and crown-rump measurements were taken to the nearest millimeter, and the fetuses were then divided into two groups. Every third fetus was placed in 90.0 ml. of a 1.0% potassium hydroxide solution to which 6.0 mg. of Alizarin red S was added to stain the bones selectively. The remaining fetuses were placed in Bouin's solution for several days and then examined microscopically after gross dissection, according to established techniques (9). The following parameters were recorded: total number of normal and abnormal fetuses, litter size, fetal weight, crown-rump and transumbilical distances, number of litters resorbed or delivered, and any demonstrable fetal malformations.

RESULTS AND DISCUSSION

The results of this investigation, summarized in Table I, clearly demonstrate the deleterious effects of audiogenic stress on the developing fetus. The most severe fetal effects were observed when the pregnant dams were stressed 8 hr./day on Days 8–17 of pregnancy, with 40% of the litters resorbing and a mean fetal weight of 0.44 g. compared to a control value of 1.45 g. The next period of greatest susceptibility was Days 10–15 of pregnancy when, although no litters resorbed, the mean fetal weight was 0.87 g. In all cases, the crown-rump and transumbilical distances were decreased when the mean fetal weight was decreased, indicating that this parameter is also a good indication of fetal development. In none of the stressed groups was the litter size or the uterine distribution altered over control values. These observations are in agreement with Geber (5) and Sontag *et al.* (7), who reported decreased fetal weight as a result of audiovisual and audiogenic stress. But the observations contradict the findings of Geber (5) as well as those of Thompson and Sontag (10) with regard to litter size, because these investigators reported decreased litter size and no effect on either litter size or fetal weight, respectively. The latter, however, stressed their experimental groups only during the last trimester of pregnancy.

It is evident from Table I that, when murine pregnancy was studied in 2-day intervals, the period of greatest susceptibility to the effects of audiogenic stress was Days 7–8 of pregnancy, with 100% of the litters resorbing before Day 18.

The teratogenic effects observed (cranial hematoma, dwarfed hind limbs, and tail defects) compare favorably with those reported by other investigators (5). These effects have been attributed to the endocrinologic effects of stress on either the maternal or fetal organism, including the discharge of catecholamines and steroid hormones from the adrenals and decreased maternal and fetal blood flow, especially of the uterine and placental vasculature, thus causing fetal hypoxia and even possibly delaying or preventing the implantation of the fertilized ova (5). This observation correlates also with the findings of Weir and DeFries (6) who reported a decreased rate of pregnancy when fertilized dams were subjected to the stress of handling prior to implantation. The adaptation to maternal stress, suggested by Geber (5), did not appear to be a factor in this investigation, because the data demonstrate that, in general, the more severe the stress, the greater the effect on fetal development. Maternal adaptation would also not be a factor in the portion of this investigation in which the murine pregnancy cycle was studied in 2-day segments. While maternal influences in fetal development as the result of stress are the most plausible explanation for the spectrum of teratogenic effects, the direct effects of sound on the fetus itself cannot be disregarded (7).

The significance of this study is that teratogenic effects have been produced with no manipulations of either the fetus or the maternal organism other than environmental alterations of maternal and/or fetal physiological mechanisms. Also, the period of greatest susceptibility to environmental audiogenic stress appears to be during the period of implantation (Table I), which compares favorably to the period of susceptibility to chemical stress factors, *i.e.*, the administration of known teratogenic drugs (11).

REFERENCES

(1) J. B. Baker, *Pharmacol. Rev.*, **12,** 37(1960).

(2) J. M. Robson and F. M. Sullivan, *J. Physiol.*, **184,** 717(1966).

(3) H. Kalter and J. Warkany, *Physiol. Rev.*, **39,** 69(1959).

(4) H. Kalter, *Ann. N.Y. Acad. Sci.*, **123,** 287(1965).

(5) W. F. Geber, *J. Embryol. Exp. Morphol.*, **16,** 1(1966).

(6) M. W. Weir and J. C. DeFries, *Psychol. Rep.*, **13,** 365 (1963).

(7) L. W. Sontag, W. G. Steele, and M. Lewis, *Human Develop.*, **12,** 1(1969).

(8) W. F. Geber and T. A. Anderson, *Biol. Neonat.*, **11,** 209 (1967).

(9) C. O. Ward, *Amer. J. Pharm. Ed.*, **33,** 229(1969).

(10) W. D. Thompson and L. W. Sontag, *J. Comp. Physiol. Psychol.*, **49,** 454(1956).

(11) J. G. Wilson and J. Warkany, "Teratology, Principles and Techniques," University of Chicago Press, Chicago, Ill., 1965, p. 255.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 20, 1970, from the *Department of Pharmacology, College of Pharmacy, St. John's University, Jamaica, NY 11432*

Accepted for publication April 17, 1970.

The authors thank Andrew Stephens and B. Wolyniec for their technical assistance.

Thermal Dissociation of Sulfonylureas: Dissociation of Tolbutamide in a Series of Aliphatic Alcohols and in Polyethylene Glycol 400

F. BOTTARI, M. MANNELLI, and M. F. SAETTONE

Abstract □ A study on the thermal dissociation of tolbutamide (I) in a series of 12 primary aliphatic alcohols, C_1 to C_{18}, and in polyethylene glycol 400 USP at 80° is presented. It is shown that the compound dissociates in only one of two possible fashions, *i.e.*, only to give butylamine and *p*-toluenesulfonyl isocyanate. In all cases, an *N*-(*p*-toluenesulfonyl)carbamate, formed by reaction of the sulfonyl isocyanate with the alcohols, was present in the equilibrium mixture. *p*-Toluenesulfonamide and alkyl *N*-butylcarbamates, arising from the other possible dissociation, were never evidenced. The results are consistent with a mechanism involving, as the first step, a preferential conversion of I into one of two possible tautomeric forms.

Keyphrases □ Sulfonylureas—thermal dissociation in alcohols, polyethylene glycol 400 □ Tolbutamide—thermal dissociation in alcohols, polyethylene glycol 400 □ Dissociation, thermal—tolbutamide in alcohols, polyethylene glycol 400 □ TLC—separation, identification □ UV spectrophotometry—analysis □ Alkyl *N*-(*p*-tosyl)carbamates—formation, synthesis

Although 1,3-disubstituted sulfonylureas, in view of their importance as oral hypoglycemic agents, have been widely investigated from the synthetic and analytical standpoint, little is known about their chemical reactivity. The acid hydrolysis of tolbutamide (I), carbutamide, and chlorpropamide has been investigated by Vogt (1), and Haussler and Hajdù (2) have reported on the hydrolytic breakdown of I in alkaline solution. More recently, Ulrich and Sayigh (3) have described a method for the preparation of isocyanates based on the pyrolysis of some sulfonylureas in high-boiling solvents.

In a previous communication (4), it was reported that the tolbutamide, carbutamide, or chlorpropamide content of some o/w creams for topical use showed a significant decrease when the said drugs were dissolved at 70–80° in the oil phase of the emulsions. No such decrease occurred when the sulfonylureas were incorporated into the bases at room temperature. A series of tests, carried out by heating at 80° the mixtures of I with the single components of the oil phase, showed that the observed effect was due to some components containing hydroxyl groups, such as stearyl alcohol or polyethylene glycol. This led to the investigation of the behavior of I, chosen as a representative member of the series, in a number of alcohols, in an attempt to establish the reaction course both on a qualitative and quantitative basis. Indeed, although the thermal dissociation[1] of symmetrical and unsymmetrical mono- and disubstituted ureas has been investigated under various experimental conditions (5, 6), no studies could be found in the literature on the dissociation of pharmacologically active sulfonylureas at relatively low temperatures and in media such as may occur in pharmaceutical vehicles.

The results of a preliminary investigation on the dissociation of I at 80° in 12 primary alcohols (methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, *n*-octyl, *n*-decyl, *n*-dodecyl, *n*-tetradecyl, *n*-hexadecyl, and *n*-octadecyl alcohols) and in polyethylene glycol (PEG) 400, a common constituent of pharmaceutical preparations, are the subject of this paper.

Table I—Tolbutamide (I), *N*-(*p*-Toluenesulfonyl)carbamates, and Butylamine Found in the Equilibrium Mixtures after 2 hr. at 80°. Initial Concentration of I was 10% w/w (37 mmoles/100 g.)

Solvent	Tolbutamide, % w/w (mmoles/100 g.)	*N*-(*p*-Tosyl)-carbamate, % w/w (mmoles/100 g.)	Butylamine, % w/w (mmoles/100 g.)
Methyl alcohol	7.2[a] (26.6)	—	0.76 (10.4)
Ethyl alcohol	5.8[a] (21.5)	—	1.13 (15.5)
n-Propyl alcohol	6.5 (24.0)	3.1 (12.0)	0.87 (11.9)
n-Butyl alcohol	6.3 (23.3)	3.5 (12.9)	0.93 (12.7)
n-Pentyl alcohol	6.4 (23.7)	3.7 (13.0)	0.93 (12.7)
n-Hexyl alcohol	6.0 (22.2)	4.4 (14.7)	1.06 (14.5)
n-Octyl alcohol	6.1 (22.6)	4.4 (13.4)	0.96 (13.2)
n-Decyl alcohol	6.5 (24.2)	4.5 (12.7)	0.92 (12.6)
n-Dodecyl alcohol	6.0 (22.2)	5.6 (14.6)	1.05 (14.4)
n-Tetradecyl alcohol	6.0 (22.2)	5.7 (13.9)	1.01 (13.8)
n-Hexadecyl alcohol	6.3 (23.3)	6.0 (13.6)	0.98 (13.4)
n-Octadecyl alcohol	6.2 (23.0)	6.5 (14.0)	1.00 (13.7)
Polyethylene glycol 400	6.6 (24.5)	—	—

[a] Calculated from the amount of amine found in the mixture.

EXPERIMENTAL

A Beckman model DU spectrophotometer was used for all analyses. TLC separations were carried out on Merck F 254 silica gel plates, using the benzene–acetone–methanol–acetic acid (70:20:5:5) solvent system described by Kaistha and French (7). Tolbutamide (I) was the medicinal grade drug; it was crystallized from benzene to constant melting point (127–129°) and was shown by TLC to be free from contaminants or decomposition products. *p*-Toluenesulfonamide[2] was crystallized from ethanol, m.p. 136–137°. *n*-Butylamine was distilled prior to use, b.p. 77–78°. All alcohols (Table I) were anhydrous, analytical reagent grade products. Their purity was checked by gas chromatography. PEG 400 was found to contain 2% H_2O by azeotropic distillation with toluene, and it was used as such. *p*-Toluenesulfonyl isocyanate (III) was prepared from *p*-toluenesulfonamide and oxalyl chloride as indicated by Franz and Osuch (8). *N*-(*p*-Toluenesulfonyl)carbamates, Compounds 1–12, were prepared from III and the appropriate alcohols by the method of McFarland and Howard (9); analyses and physical constants are reported in Table II. Some of the compounds had been described previously (footnotes [c] and [d], Table II), but no analytical data had been given. "PEG

[1] The term "dissociation" is preferred to the terms "decomposition" and "dearrangement" used by earlier authors, since the process is the reversal of the formation reaction (6).

[2] Fluka A.G.

Table II—Alkyl *N*-(*p*-Toluenesulfonyl)carbamates

Compound No.	R	M.p.[a]	$R_f \times 100$[b]	Empirical Formula	Calcd. C	Calcd. H	Calcd. N	Calcd. S	Found C	Found H	Found N	Found S
1	$-CH_3$	106–107°[c]	69	$C_9H_{11}NO_4S$	47.15	4.84	6.11	13.98	47.00	4.65	5.94	13.70
2	$-C_2H_5$	82–84°[c]	69	$C_{10}H_{13}NO_4S$	49.37	5.38	5.76	13.18	49.21	5.42	5.63	12.89
3	$-CH_2CH_2CH_3$	64–65°[c]	72	$C_{11}H_{15}NO_4S$	51.34	5.88	5.44	12.46	51.31	5.79	5.25	12.29
4	$-CH_2(CH_2)_2CH_3$	118–120°[d]	72	$C_{12}H_{17}NO_4S$	53.12	6.32	5.16	11.81	52.98	6.27	5.12	11.71
5	$-CH_2(CH_2)_3CH_3$	45–46°[c]	73	$C_{13}H_{19}NO_4S$	54.71	6.71	4.91	11.23	54.55	6.63	4.63	11.02
6	$-CH_2(CH_2)_4CH_3$	Oil[e]	76	$C_{14}H_{20}NNaO_4S$	52.32	6.27	4.36	9.98	52.25	6.11	4.30	9.77
7	$-CH_2(CH_2)_6CH_3$	53–54°[c]	77	$C_{16}H_{25}NO_4S$	58.69	7.70	4.28	9.79	58.46	7.58	4.16	9.64
8	$-CH_2(CH_2)_8CH_3$	72–74°	79	$C_{18}H_{29}NO_4S$	60.81	8.22	3.94	9.02	60.70	8.29	3.98	9.12
9	$-CH_2(CH_2)_{10}CH_3$	87–89°	81	$C_{20}H_{33}NO_4S$	62.62	8.67	3.65	8.35	62.44	8.50	3.83	8.17
10	$-CH_2(CH_2)_{12}CH_3$	86–87°	81	$C_{22}H_{37}NO_4S$	64.19	9.06	3.40	7.79	63.94	8.51	3.08	7.71
11	$-CH_2(CH_2)_{14}CH_3$	86–87°	82	$C_{24}H_{41}NO_4S$	65.56	9.40	3.18	7.29	66.24	9.06	3.23	7.15
12	$-CH_2(CH_2)_{16}CH_3$	97–98°	86	$C_{26}H_{45}NO_4S$	66.76	9.60	3.15	7.14	66.49	9.70	3.00	6.85
13	PEG 400	—	29	—	—	—	—	—	—	—	—	—

[a] Determined on a Kofler block and uncorrected. All compounds were crystallized from petroleum ether, boiling range 60–80°. [b] Solvent system: benzene–acetone–methanol–acetic acid (70:20:5:5); see text. [c] *Cf.* L. Fishbein, *J. Chromatogr.*, **30**, 245(1967). [d] Synthesis described by A. Nováček and B. Vondráček, Czech. pat. 96,582 (Sept. 1960); through *Chem. Abstr.*, **55**, 15420g(1961). [e] Analyzed as the sodium salt.

400 *N*-(*p*-tosyl)carbamate," prepared from III and excess PEG 400, was not purified.

Procedure for Study of Dissociation of I in Anhydrous C_1–C_{18} Alcohols—Mixtures containing I (50 mg.) and an alcohol (450 mg.) were placed in 5-ml. ampuls. The sealed ampuls were heated at 80° in an electrically controlled oil bath. The reaction temperature was selected after some preliminary trials; temperatures below 80° were insufficient for a quick dissolution of the drug in some alcohols, while higher temperatures favored formation of small amounts of unidentified side products. After 2 hr. or, for the reaction in *n*-octadecyl alcohol, at the time intervals specified in Fig. 1, the ampuls were cooled and their contents were quantitatively dissolved in acetone (25 ml.). Ten 50-μl. portions of the solution were applied on a chromatoplate as a row of points. Appropriate reference solutions [I, *N*-(*p*-tosyl)carbamates and butylamine] were also applied. After development, the areas corresponding to I (R_f 0.68) and to the *N*-(*p*-tosyl)carbamates (R_f reported in Table II) were evidenced by examination under a shortwave UV light and were marked. Their identity was confirmed by comparison with the standards. The marked areas were removed from the plate and quantitatively eluted with ethanol (10 ml.). The UV absorption of the solutions at 228 mμ (I) and at 226 mμ (carbamates) was recorded, applying a correction for the blank extinction value. The amounts of I and of *N*-(*p*-tosyl)carbamate were obtained by comparison with suitable standard curves. The procedure gave errors not exceeding ±5%, as proven by separate tests carried out with standard solutions of I and of carbamates. The location of butylamine (R_f 0.04) was evidenced by spraying the "standard" portion of the plates with 0.5% ninhydrin solution and heating briefly at 60°. The corresponding "sample" area was removed and quantitatively eluted with ethanol (10 ml.). The determination of the butylamine content of the solution was carried out by the ninhydrin method outlined by Kaistha and French (7). In this case also, the error did not exceed ±5%.

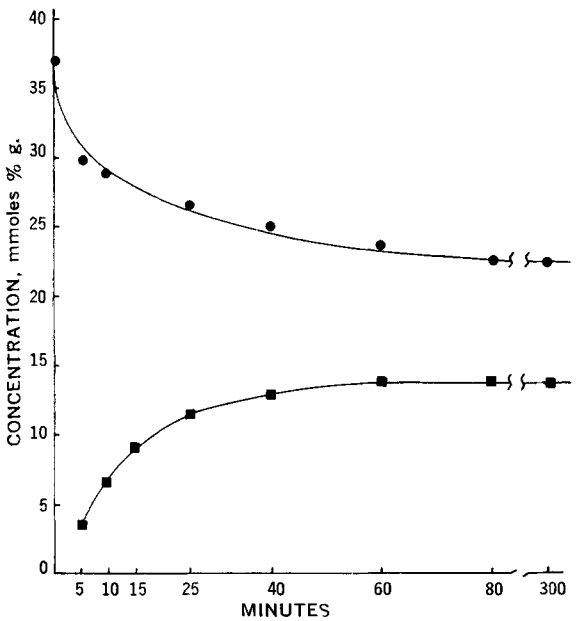


Figure 1—*Concentrations of tolbutamide (I)* (●) *and of* n-*octadecyl* N-(p-*tosyl*)*carbamate (Compound 12)* (■) versus *time at 80°. Initial concentration of I in* n-*octadecyl alcohol is 10% w/w (37 mmoles/100 g.).*

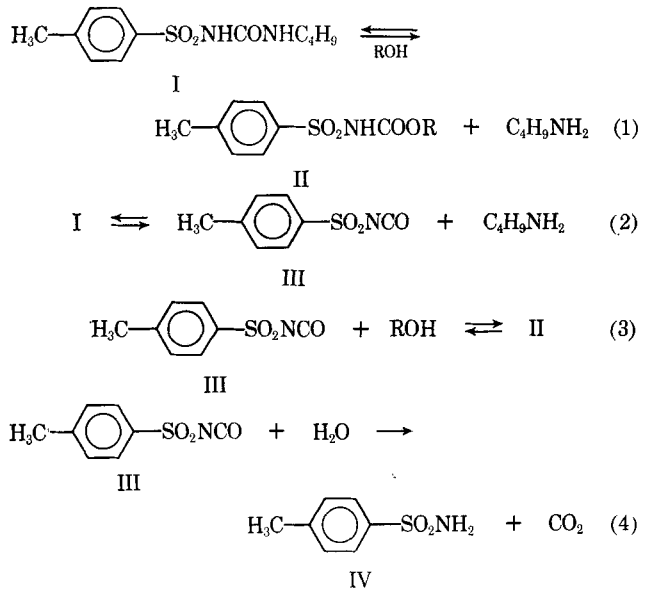
When methyl and ethyl alcohols were used as solvents, a quantitative estimation of I and of the *N*-(*p*-tosyl)carbamates was impossible, since the substances had almost identical R_f's and could not be separated. In these cases, the extent of dissociation of I was calculated from the amount of amine found in the equilibrium mixtures.

Dissociation of I in PEG 400 and in 2% Aqueous C_1–C_8 Alcohols—One-gram samples of 10% w/w solutions of I were placed in 5-ml. flasks and heated 2 hr. at reflux temperature (methyl and ethyl alcohols) or at 80° (*n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, and *n*-octyl alcohols). Each flask was connected to a small gas absorption trap containing a $Ba(OH)_2$ solution. Evolution of CO_2 was qualitatively evidenced by a white precipitate in the trap. After cooling, a sample (500 mg.) of each mixture was dissolved in acetone (25 ml.) and processed as described previously. In this case, a *p*-toluenesulfonamide reference solution (R_f 0.58) was also applied to the plates. For PEG 400, a quantitative estimation of the *N*-(*p*-tosyl)carbamate could not be effected.

Reaction of III with Aqueous Alcohols—To 1-g. samples of the alcohols mentioned in the preceding paragraph, placed in 5-ml. ampuls, 0.1 g. of III was added. The ampuls were sealed and heated 30 min. at 80°. After cooling, a sample (500 mg.) of each mixture was dissolved in acetone (25 ml.) and processed as described. The products consisted of a mixture containing approximately equal portions of *p*-toluenesulfonamide and of the *N*-(*p*-tosyl)carbamate of the alcohol used as solvent.

RESULTS AND DISCUSSION

The results obtained heating 10% solutions of I in 12 straight-chain primary alcohols and in PEG 400 are summarized in Table I. In all cases, the reaction practically reached equilibrium within 2 hr., after which time about 40% of I had disappeared and other compounds, identified as *n*-butylamine and the *N*-(*p*-tosyl)carbamate of the alcohol used as solvent, were formed in stoichiometric amounts. All tosylcarbamates (Table II) were independently synthesized; some of them (Compounds 8, 9, 10, 11, and 12) were unknown. In the case of PEG 400, the carbamate had the same chromatographic behavior of a sample obtained from *p*-toluenesulfonyl isocyanate and PEG 400. No attempts were made at purification of this "PEG 400 *N*-(*p*-tosyl)carbamate" on consideration of its probably complex composition. The overall reaction of I with alcohols is indicated as (1) in Scheme I.



Scheme I

A more detailed study of the behavior of a 10% solution of I in *n*-octadecyl alcohol at 80° is shown in Fig. 1. The tolbutamide concentration (initially 37 mmoles/100 g.) was found to diminish as carbamate (and butylamine, not shown in the figure) formation increased, until an equilibrium mixture containing about 6.2% (23.0 mmoles/100 g.) of I, 6.5% (14.0 mmoles/100 g.) of *n*-octadecyl *N*-(*p*-tosyl)carbamate (Compound 12), and 1.0% (13.7 mmoles/100 g.) of butylamine was reached after about 1.5 hr.

In every case, the amounts of *n*-butylamine and of *N*-(*p*-tosyl)-carbamate present in the mixture satisfactorily accounted for the amount of I which had disappeared. A prolonged heating (5–10 hr.) of the solutions of I in higher alcohols resulted in formation of very small amounts of a third compound, evidenced by a fluorescent spot on the chromatographic plates. The R_f of this spot (*e.g.*, 0.88 for the reaction in *n*-octadecyl alcohol) was in all cases different from those of all the possible dissociation products of I, and investigation in this direction was not pursued.

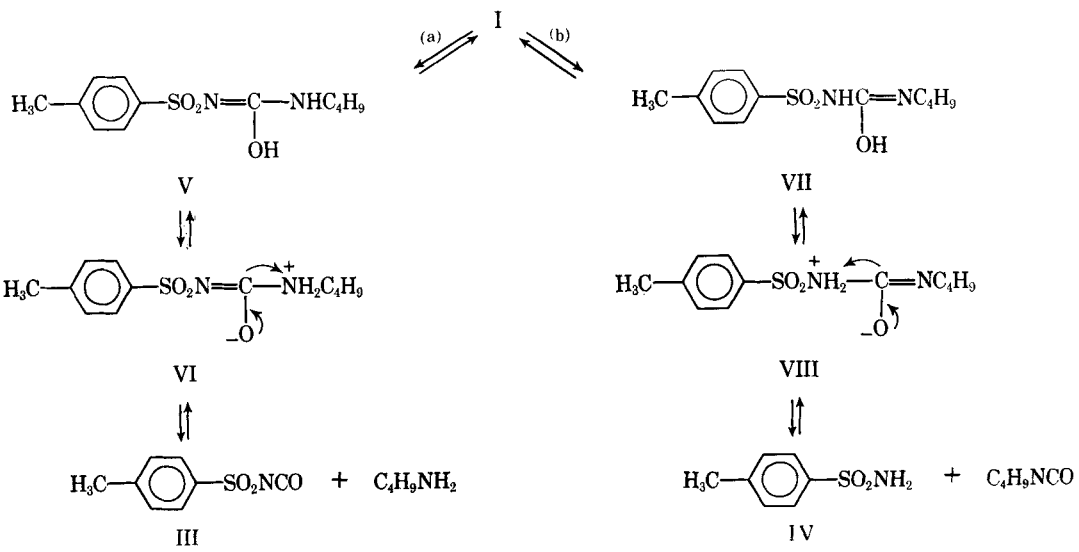
The presence of 2% water in some solvents (PEG 400 and alcohols C_1–C_8) did not influence the equilibrium concentrations of I and of *n*-butylamine. However, the equilibrium mixtures contained about 2% of *p*-toluenesulfonamide and a correspondingly reduced amount of *N*-(*p*-tosyl)carbamate. Furthermore, evolution of CO_2 during the reaction was observed when water was present.

The reported results might be rationalized as follows. Mono- and disubstituted ureas and some sulfonylureas are known to dissociate on heating to isocyanates and amines (3, 5, 6). The thermal dissociation of *p*-ethoxyphenylurea, for example, in aqueous solution leads to *p*-ethoxyphenyl isocyanate, ammonia, and, in minor amounts, phenetidine and isocyanic acid (10). The isocyanates originating from the dissociation are often evidenced indirectly, *e.g.*, through derivatives formed with suitable reagents. The reaction medium itself can function in some cases as one such reagent; *sym*-di-*p*-ethoxyphenylurea was assumed to dissociate to phenetidine and *p*-ethoxyphenyl isocyanate, since it gave phenetidine and *n*-butyl *N*-(*p*-ethoxyphenyl)carbamate on heating in *n*-butanol (11). The results seem to be in line with these data, and they suggest that tolbutamide dissociates on heating in the presence of alcohols to butylamine and *p*-toluenesulfonyl isocyanate. Interaction between the latter compound and the alcohol used as solvent would give the observed *N*-(*p*-tosyl)carbamate. The reactions are indicated as (2) and (3) in Scheme I.

The additional formation of *p*-toluenesulfonamide and carbon dioxide, when water is present in the reaction mixtures, can be explained assuming that water might compete with the alcohols for *p*-toluenesulfonyl isocyanate. The latter is known to react readily with water as indicated in (4), Scheme I (12). Support for this hypothesis came from experiments in which *p*-toluenesulfonyl isocyanate was allowed to react with a series of aqueous alcohols. In all cases, a mixture of *N*-(*p*-tosyl)carbamate and *p*-toluenesulfonamide was obtained, and CO_2 evolved during the reaction.

A significant outcome of the present study is the observation that tolbutamide, under the experimental conditions described, dissociates in only one of two possible fashions. The alternative dissociation leading to *p*-toluenesulfonamide and alkyl *N*-butylcarbamates as the end products could not be detected even to a minimal extent. A tentative rationalization of this phenomenon is presented in Scheme II, where the mechanism for the thermal dissociation of I has been patterned after that proposed by Hoshino *et al.* (6) for *sym*-diphenylurea. It can be seen that path (a), leading to the observed products, requires tautomerization of I to V, while path (b), leading to *p*-toluenesulfonamide and butyl isocyanate, requires tautomerization to VII. Tautomerization of I to V should be strongly favored by the high acidity of the NH group bound to the sulfonyl group. Furthermore, even if a small amount of VII was present at equilibrium, its subsequent conversion into the ion VIII should be rather difficult, since it would require protonation of a nitrogen atom endowed with a very low basicity. Conversely, formation of the ion VI from V should occur easily, on account of the greater degree of basicity of the nitrogen bound to the alkyl group.

Solvent effects and/or reaction temperature might play a significant role in determining the fashion and extent of the reaction. A relevant example is the formation of good yields of alkyl isocyanates and sulfonamides from 1,3-disubstituted sulfonylureas under pyrolysis conditions, where a completely different mechanism is probably operative (3). Moreover, although this proposed dissociation mechanism receives strong support from several analogous examples reported in the literature, alternative mechanisms, such as



Scheme II

alcoholysis, should not be entirely disregarded. Additional experiments are needed to obtain a satisfactory picture of the reaction. Further work, aimed at collecting kinetic data for the dissociation of I and of other pharmacologically active sulfonylureas in different media, is now in progress.

REFERENCES

(1) H. Vogt, *Pharm. Zentralh.*, **98**, 651(1959).

(2) A. Haussler and P. Hajdù, *Arch. Pharm.*, **295**, 471(1962).

(3) H. Ulrich and A. A. R. Sayigh, *Angew. Chem. Int. Ed. Engl.*, **5**, 724(1966).

(4) F. Bottari, M. F. Saettone, B. Giannaccini, and M. E. Lo Brutto, *Rass. Dermatol. Sifilogr.*, **20**, 335(1967).

(5) T. L. Davis and K. C. Blanchard, *J. Amer. Chem. Soc.*, **45**, 1816(1923).

(6) T. Hoshino, T. Mukaiyama, and N. Hoshino, *ibid.*, **74**, 3097(1952), and references therein.

(7) K. K. Kaistha and W. N. French, *J. Pharm. Sci.*, **57**, 459 (1968).

(8) J. E. Franz and C. Osuch, *J. Org. Chem.*, **29**, 2592(1964).

(9) J. W. McFarland and G. B. Howard, *ibid.*, **30**, 957(1965).

(10) K. Taufel, C. Wagner, and H. Dunwald, *Z. Electrochem.*, **34**, 115(1928).

(11) Y. Iwakura and K. Nakagubo, *Bull. Tokyo Inst. Technol.*, **18**, 25(1948).

(12) H. Ulrich, *Chem. Rev.*, **65**, 369(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 9, 1970, from the *Institute of Pharmaceutical Chemistry, University of Pisa, Via Bonanno 6, 56100 Pisa, Italy.*

Accepted for publication May 19, 1970.

This investigation was supported in part by a grant from Consiglio Nazionale delle Ricerche.

The authors thank Professor A. Marsili for many helpful discussions.

Molecular Association of Barbital and Caffeine in 2:1 Crystalline Complex

B. M. CRAVEN and G. L. GARTLAND

Abstract □ The crystal structure of the 2:1 complex of barbital with caffeine has been determined by X-ray diffraction methods. The crystals are triclinic, space group $P\bar{1}$, with $a = 14.627$, $b = 14.160$, $c = 6.902$ Å, $\alpha = 95°15'$, $\beta = 92°48'$, and $\gamma = 100°45'$, and with four barbital and two caffeine molecules in the cell. The block-diagonal least-squares refinement of 496 atomic positional and thermal parameters, based on 4665 X-ray intensity data, gave a final R factor of 0.05. The structure consists of ribbons of barbital molecules linked by NH···O=C hydrogen bonds. Caffeine molecules are bound to the ribbon by an NH···N(9) hydrogen bond and by an unusual interaction involving C(8)H with two barbital oxygen atoms. Weak interactions of nonhydrogen-bonded caffeine carbonyl groups with barbital carbonyl groups may also be important in this crystal. There is minimal overlap of the flat ring systems of the component molecules.

Keyphrases □ Molecular association—barbital–caffeine 2:1 crystalline complex □ Barbital, molecular association—caffeine in 2:1 crystalline complex □ X-ray diffraction—barbital–caffeine 2:1 crystalline structure, determination

Barbiturates (1–3) and xanthines (4, 5) form crystalline complexes with a variety of other molecular species as well as with each other (6–8). The crystal complex of barbital with caffeine was chosen for study because consideration of the structure of the component molecules (Fig. 1) shows that the nature of barbital–caffeine association must differ in two important respects from that found in previously determined crystal structures of complexes of purines and pyrimidines.

The strong association by pairs of hydrogen bonds, which occurs in complexes of barbiturates with adenine derivatives (3, 9) and which is analogous to the hydrogen bonding in the crystal structures of nucleic acid base pairing model systems (10), cannot occur between barbital and caffeine. As a hydrogen-bonding donor caffeine can at most form a weak C(8)H hydrogen bond

The stacking together of flat molecules with extensive overlap of their π-bonded ring systems has been found in complexes of tetramethyluric acid with pyrene (11) and caffeine with 5-chlorosalicylic acid (5). This type of interaction, which has been termed polarization bonding, is postulated as an important cohesive factor in xanthine complexes (12). However, in the complex of caffeine with barbital, overlap of the two flat ring systems is largely prevented by the ethyl groups, which shield each side of the barbital ring.

The crystal structure of the 2:1 barbital–caffeine complex was determined to reveal the detailed geometry of molecular association.

EXPERIMENTAL

Triclinic crystals (m.p. 142°) of the complex were obtained as described by Higuchi and Lach (6). The lattice parameters are $a = 14.627$, $b = 14.160$, $c = 6.902$ Å, $\alpha = 95°15'$, $\beta = 92°48'$, and $\gamma = 100°45'$. The space group is $P\bar{1}$, and there are four barbital and two caffeine molecules in the unit cell. The X-ray intensity data (4665 reflections) were collected on a four-circle automatic diffractometer using CuKα radiation. All 40 nonhydrogen atoms of the crystal chemical unit were found in the first E map, derived from an application of the direct method of phase determination similar to that described by Karle (13). All 34 hydrogen atoms were subsequently found in a difference Fourier synthesis. The positional and anisotropic thermal parameters for heavier atoms and positional and isotropic thermal parameters for hydrogen atoms were refined by a block-diagonal least-squares procedure to give a final R factor of 0.05.

Description of the Structure—The crystal structure consists of stacks of hydrogen-bonded ribbons, one of which is shown in Fig. 2. The backbone of the ribbon is made up of the barbital molecules,

alcoholysis, should not be entirely disregarded. Additional experiments are needed to obtain a satisfactory picture of the reaction. Further work, aimed at collecting kinetic data for the dissociation of I and of other pharmacologically active sulfonylureas in different media, is now in progress.

REFERENCES

(1) H. Vogt, *Pharm. Zentralh.*, **98**, 651(1959).

(2) A. Haussler and P. Hajdù, *Arch. Pharm.*, **295**, 471(1962).

(3) H. Ulrich and A. A. R. Sayigh, *Angew. Chem. Int. Ed. Engl.*, **5**, 724(1966).

(4) F. Bottari, M. F. Saettone, B. Giannaccini, and M. E. Lo Brutto, *Rass. Dermatol. Sifilogr.*, **20**, 335(1967).

(5) T. L. Davis and K. C. Blanchard, *J. Amer. Chem. Soc.*, **45**, 1816(1923).

(6) T. Hoshino, T. Mukaiyama, and N. Hoshino, *ibid.*, **74**, 3097(1952), and references therein.

(7) K. K. Kaistha and W. N. French, *J. Pharm. Sci.*, **57**, 459 (1968).

(8) J. E. Franz and C. Osuch, *J. Org. Chem.*, **29**, 2592(1964).

(9) J. W. McFarland and G. B. Howard, *ibid.*, **30**, 957(1965).

(10) K. Taufel, C. Wagner, and H. Dunwald, *Z. Electrochem.*, **34**, 115(1928).

(11) Y. Iwakura and K. Nakagubo, *Bull. Tokyo Inst. Technol.*, **18**, 25(1948).

(12) H. Ulrich, *Chem. Rev.*, **65**, 369(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 9, 1970, from the *Institute of Pharmaceutical Chemistry, University of Pisa, Via Bonanno 6, 56100 Pisa, Italy.*

Accepted for publication May 19, 1970.

This investigation was supported in part by a grant from Consiglio Nazionale delle Ricerche.

The authors thank Professor A. Marsili for many helpful discussions.

Molecular Association of Barbital and Caffeine in 2:1 Crystalline Complex

B. M. CRAVEN and G. L. GARTLAND

Abstract □ The crystal structure of the 2:1 complex of barbital with caffeine has been determined by X-ray diffraction methods. The crystals are triclinic, space group $P\bar{1}$, with $a = 14.627$, $b = 14.160$, $c = 6.902$ Å, $\alpha = 95°15'$, $\beta = 92°48'$, and $\gamma = 100°45'$, and with four barbital and two caffeine molecules in the cell. The block-diagonal least-squares refinement of 496 atomic positional and thermal parameters, based on 4665 X-ray intensity data, gave a final R factor of 0.05. The structure consists of ribbons of barbital molecules linked by NH···O=C hydrogen bonds. Caffeine molecules are bound to the ribbon by an NH···N(9) hydrogen bond and by an unusual interaction involving C(8)H with two barbital oxygen atoms. Weak interactions of nonhydrogen-bonded caffeine carbonyl groups with barbital carbonyl groups may also be important in this crystal. There is minimal overlap of the flat ring systems of the component molecules.

Keyphrases □ Molecular association—barbital–caffeine 2:1 crystalline complex □ Barbital, molecular association—caffeine in 2:1 crystalline complex □ X-ray diffraction—barbital–caffeine 2:1 crystalline structure, determination

Barbiturates (1–3) and xanthines (4, 5) form crystalline complexes with a variety of other molecular species as well as with each other (6–8). The crystal complex of barbital with caffeine was chosen for study because consideration of the structure of the component molecules (Fig. 1) shows that the nature of barbital–caffeine association must differ in two important respects from that found in previously determined crystal structures of complexes of purines and pyrimidines.

The strong association by pairs of hydrogen bonds, which occurs in complexes of barbiturates with adenine derivatives (3, 9) and which is analogous to the hydrogen bonding in the crystal structures of nucleic acid base pairing model systems (10), cannot occur between barbital and caffeine. As a hydrogen-bonding donor caffeine can at most form a weak C(8)H hydrogen bond

The stacking together of flat molecules with extensive overlap of their π-bonded ring systems has been found in complexes of tetramethyluric acid with pyrene (11) and caffeine with 5-chlorosalicylic acid (5). This type of interaction, which has been termed polarization bonding, is postulated as an important cohesive factor in xanthine complexes (12). However, in the complex of caffeine with barbital, overlap of the two flat ring systems is largely prevented by the ethyl groups, which shield each side of the barbital ring.

The crystal structure of the 2:1 barbital–caffeine complex was determined to reveal the detailed geometry of molecular association.

EXPERIMENTAL

Triclinic crystals (m.p. 142°) of the complex were obtained as described by Higuchi and Lach (6). The lattice parameters are $a = 14.627$, $b = 14.160$, $c = 6.902$ Å, $\alpha = 95°15'$, $\beta = 92°48'$, and $\gamma = 100°45'$. The space group is $P\bar{1}$, and there are four barbital and two caffeine molecules in the unit cell. The X-ray intensity data (4665 reflections) were collected on a four-circle automatic diffractometer using CuKα radiation. All 40 nonhydrogen atoms of the crystal chemical unit were found in the first E map, derived from an application of the direct method of phase determination similar to that described by Karle (13). All 34 hydrogen atoms were subsequently found in a difference Fourier synthesis. The positional and anisotropic thermal parameters for heavier atoms and positional and isotropic thermal parameters for hydrogen atoms were refined by a block-diagonal least-squares procedure to give a final R factor of 0.05.

Description of the Structure—The crystal structure consists of stacks of hydrogen-bonded ribbons, one of which is shown in Fig. 2. The backbone of the ribbon is made up of the barbital molecules,

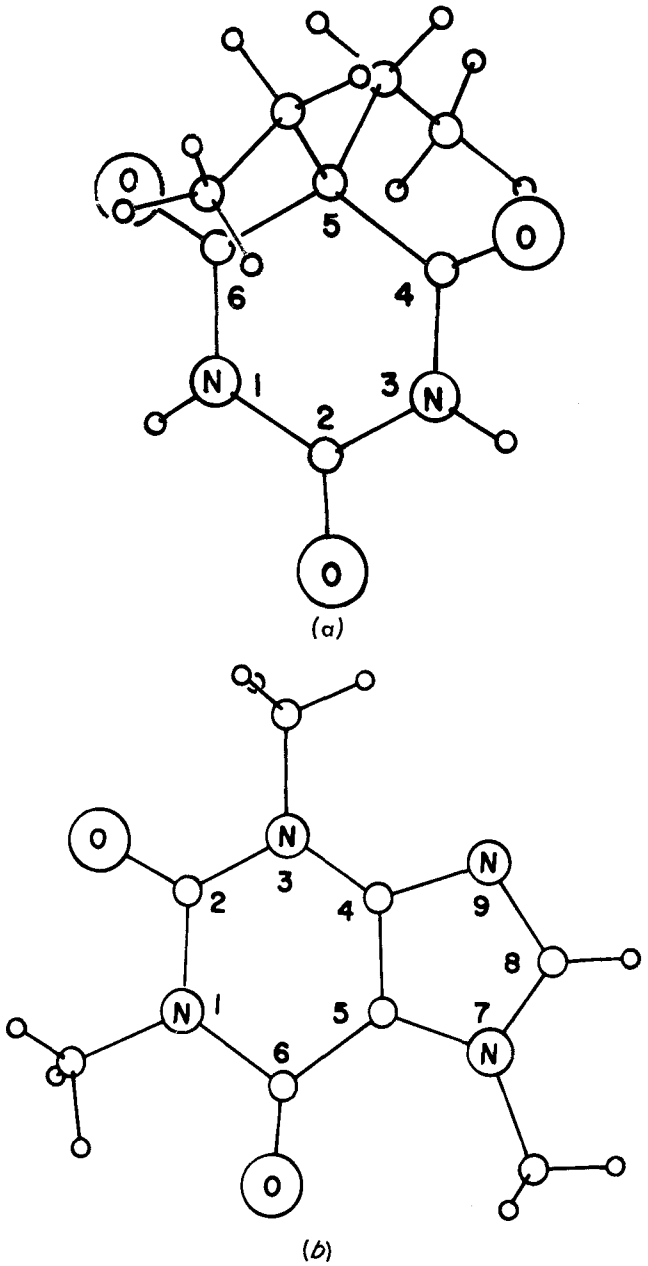


Figure 1—*Molecular structure and atomic nomenclature for* (a) *barbital and* (b) *caffeine. Unlabeled atoms are carbon and hydrogen, shown as large and small spheres, respectively. Both molecules are drawn with the stereochemistry including hydrogen atom orientation, which was determined in the crystal structure determination of the 2:1 complex. The barbital molecule is Molecule B viewed as in Fig. 2.*

which are linked by NH···O=C hydrogen bonds. The ribbons are puckered so as to avoid close contact between the nonhydrogen-bonded carbonyl oxygen atom O(6) of barbital Molecule A and the ethyl group of Molecule B′ further along the same ribbon. Each caffeine molecule is associated with a ribbon by accepting an NH··· N(9) hydrogen bond from barbital and by an unusual type of interaction C(8)H···O(2), with two barbital carbonyl groups. The angles C—H···O are 144° and 130° with e.s.d.'s of 4°, and the corresponding H···O distances are 2.32 and 2.64 Å with e.s.d.'s of 0.05 Å. Only the former distance is appreciably shorter than the sum of the appropriate van der Waals' radii (2.6 Å) as listed by Pauling (14).

Figure 2 shows that the ethyl and methyl groups of the barbital and caffeine molecules generally project outward from the border of the hydrogen-bonded ribbon. The mode of assembly of ribbons to form the three-dimensional crystal structure gives rise to close packing of these alkyl groups, as well as partial overlap of stacked flat ring systems.

There are 11 H···H distances less than 2.6 Å between alkyl hydrogen atoms of adjacent ribbons, of which the shortest is 2.32 Å. In stacking along the *z*-direction, *i.e.*, above or below the page in Figs. 2 and 3, there are five such distances between barbital ethyl groups, four between barbital ethyl and caffeine methyl groups, and one between caffeine methyl groups. There are two such distances between barbital ethyl groups of laterally adjacent ribbons.

The superposition of the flat ring systems of caffeine and barbital in the crystal structure is shown in Fig. 3. Corresponding interatomic distances are listed in Table I.

Details concerning molecular structure and stereochemistry in this crystal structure will be reported.

DISCUSSION

In barbiturate crystal structures, there are two types of intermolecular hydrogen-bonding linkage. These may be termed "cyclic" if the molecules are linked by a pair of NH···O=C hydrogen bonds, as between barbital A::::A′ and barbital B::::B′ (Fig. 2), or "noncyclic" if only one hydrogen bond is formed, as between barbital A····barbital B. Although there are both cyclic and noncyclic barbital linkages in the 2:1 complex with caffeine and also in barbital polymorph I (15), there are crystal structures in which the hydrogen bonding is exclusively cyclic, as in barbital polymorph II (15) and amobarbital polymorphs I and II (16), or exclusively noncyclic, as in γ-methylamobarbital (17).

Further possibilities for hydrogen-bonding variations arise when there are insufficient NH or other donor groups to form crystal structures in which all three barbiturate carbonyl oxygen atoms are hydrogen bonded. Under these circumstances, C(2)—O(2) and C(4)—O(4)[1] usually accept one hydrogen bond each and C(6)—O(6) is not hydrogen bonded as in the 2:1 complex of barbital with caffeine (Fig. 2), the 1:2 complex of phenobarbital with 8-bromo-9-ethyladenine (3), barbital I, γ-methylamobarbital, and amobarbital I and II. In barbital II, both C(4)—O(4) and C(6)—O(6) are hydrogen bonded and C(2)—O(2) is not. These crystal structures all contain hydrogen-bonded ribbons with different geometries. In vinbarbital I (18), heptabarbital (19), and barbital IV (20), C(4)—O(4) forms two hydrogen bonds while C(6)—O(6) and C(2)—O(2) are not hydrogen bonded. There is no case as yet in which only C(2)—O(2) is hydrogen bonded.

Thus the barbital–barbital hydrogen bonding in the 2:1 complex with caffeine represents only one of the variety of different modes that have been found in barbiturate crystal structures.

Although only three crystal structures containing caffeine have so far been determined, the hydrogen-bonding behavior of this molecule is more consistent. In caffeine hydrate (21), the 1:1 complex of caffeine with 5-chlorosalicylic acid (5) and in the presently reported structure (Fig. 2), atom N(9) is the acceptor for a normal hydrogen bond [HOH···N(9), —COOH···N(9), and >NH···N(9), respectively], while the caffeine carbonyl groups C(2)—O(2) and C(6)—O(6) are nonhydrogen bonded, or at most these oxygen atoms are acceptors in very weak CH···O interactions.

While such consistency may be fortuitous, it suggests that for caffeine the N(9) hydrogen-bonding acceptor site is favored over the carbonyl oxygen atoms.

Crystal structures containing caffeine are also similar in that the C(8)H bond of the caffeine is directed more or less toward a carbonyl oxygen atom of another molecule. The occurrence and geometry of CH···O hydrogen bonds in xanthine and other crystal structures have been reviewed by Sutor (22). More recently, Donohue (23) has doubted the inference that H···O distances between 2.2 and 2.6 Å in CH···O interactions are hydrogen bonds, at least in the sense that this term is used for O—H···O, N—H···O, and N—H···N systems. Whether or not these are called hydrogen bonds, the recurrence of H···O distances less than 2.5 Å between caffeine C(8)H and carbonyl oxygen atoms suggests that an attractive interaction is present which is stronger than the usual van der Waals' effect.

[1] In the isolated molecule (Fig. 1), C(4)—O(4) and C(6)—O(6) are symmetry-related but distinct from C(2)–O(2).

Table I—Intermolecular Distances[a]

Hydrogen Bonding Distances[b]		Other Intermolecular Distances Involving Caffeine[c]			
N(1)A···O(2)B	2.98 Å	N(1)C···O(2)B	3.40 Å	C(2)C···O(2)A	3.04 Å
N(3)A···O(4)A	2.94	N(3)C···C(8)C	3.35	O(2)C···C(2)A	3.36
N(1)B···N(9)C	2.99	C(4)C···C(8)C	3.31	O(2)C···N(3)A	3.41
N(3)B···O(4)B	2.93	C(4)C···N(9)C	3.28	N(3)C···O(2)A	3.21
		C(5)C···N(9)C	3.42	C(3)C···O(4)A	3.34
		C(6)C···N(1)B	3.53	C(5)C···C(8)C	3.44
		C(8)C···N(3)C	3.35	C(6)C···O(2)B	3.40
C(8)H···O(2) distances		N(9)C···C(4)C	3.28	O(6)C···C(2)B	3.02
		N(9)C···N(9)C	3.39	O(6)C···O(2)B	3.22
C(8)C···O(2)A	3.16 Å			O(6)C···N(3)B	3.15
C(8)C···O(2)B	3.35			N(7)C···C(8)C	3.41
				N(7)C···N(9)C	3.40
				C(7)C···N(9)C	3.43
				C(8)C···N(7)C	3.41
				C(8)C···C(5)C	3.44
				N(9)C···N(7)C	3.40
				N(9)C···C(7)C	3.43

[a] Atomic nomenclature is as in Fig. 1. The labeling A, B, or C refers to barbital molecules (A and B as shown in Fig. 2) and caffeine (C). The barbital ring systems for both Molecules A and B are numbered so that C(6)—O(6) is the nonhydrogen-bonded carbonyl group. E.s.d.'s in the interatomic distances range between 0.005 and 0.009 Å. [b] See Fig. 2 for the distances involving hydrogen-bonded hydrogen atoms. [c] For each distance, the atom listed on the left belongs to the caffeine molecule shown centrally in either Fig. 3*a* or 3*b*. The column at the left gives distances to molecules "above," *i.e.*, in the positive *z*-direction, as in Fig. 3*a*; the column at the right gives distances to molecules "below," *i.e.*, in the negative *z*-direction, as in Fig. 3*b*.

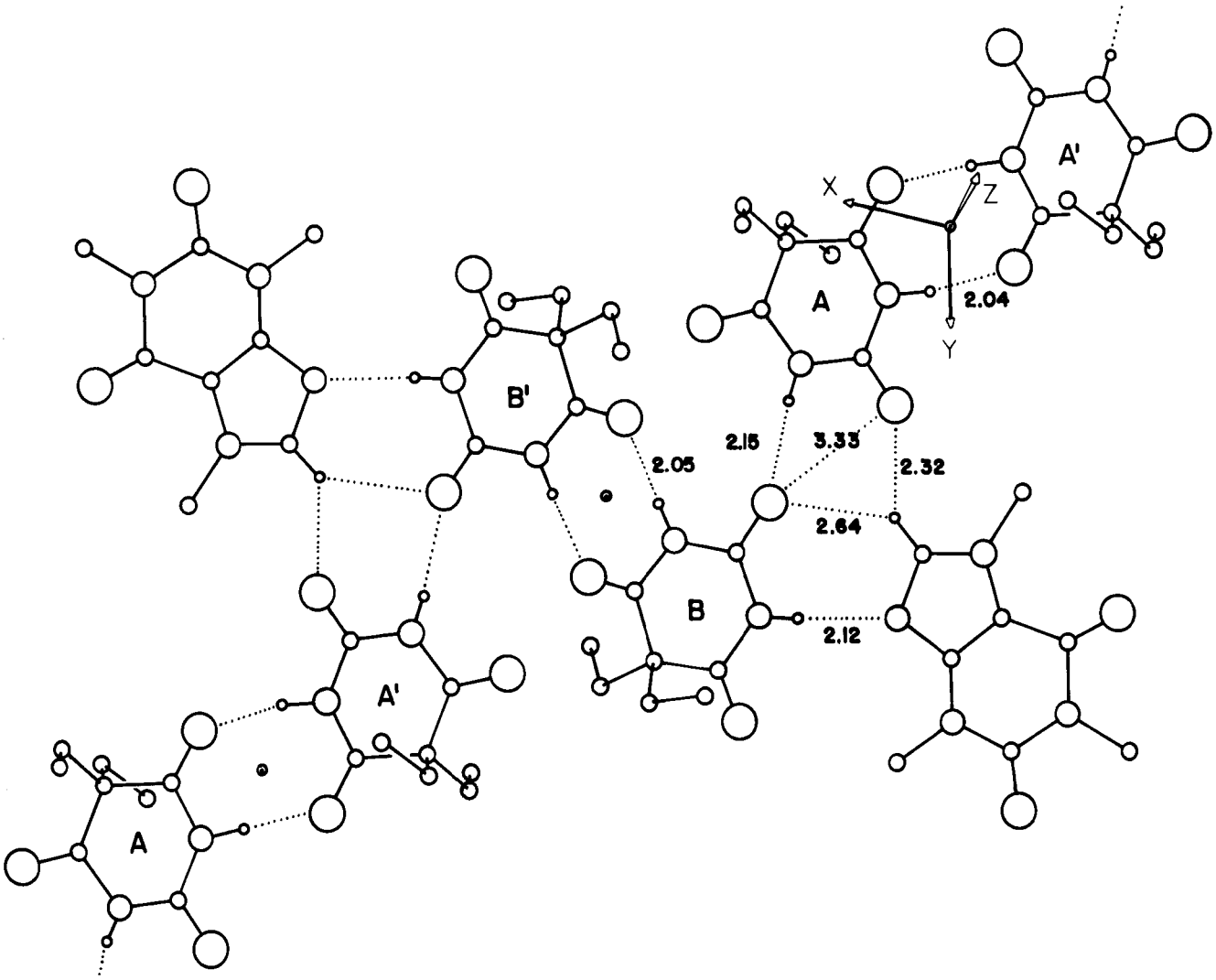


Figure 2—*The hydrogen-bonded ribbon structure observed in the crystal structure of the 2:1 complex of barbital and caffeine. The ribbon repeating unit is from barbital A (bottom left) to barbital A' (upper right). Barbital Molecules A and B are crystallographically independent. The ribbons are viewed normal to the plane of the caffeine five-membered ring. Spheres of decreasing size represent oxygen, nitrogen, carbon, and hydrogen-bonded hydrogen atoms, respectively.*

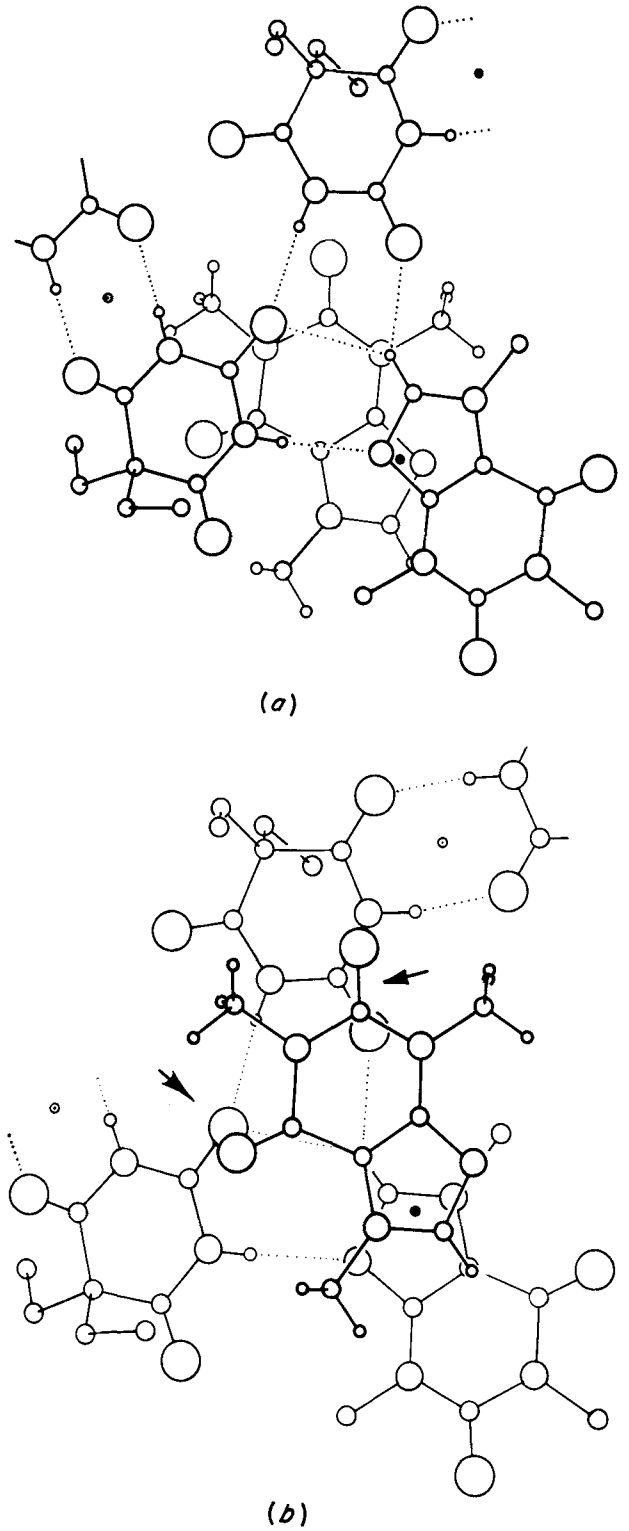


Figure 3—*Molecular overlap in the crystal structure of the 2:1 complex of barbital with caffeine. A central caffeine molecule is shown overlapped by the hydrogen-bonded ribbon "above" in* (a) *and "below" in* (b), *where "above" corresponds to the positive z-direction in the crystal. The view is the same as in Fig. 2,* i.e., *along the normal to the plane of the caffeine five-membered ring. Arrows in* (b) *indicate the overlapping carbonyl groups.*

The overlap of flat ring systems of barbital and caffeine in the 2:1 complex is restricted to the partial overlap of the five-membered rings of the caffeine molecules with each other and to the overlap of caffeine carbonyl groups with those of barbital molecules (Fig. 3). The caffeine–caffeine interatomic distances such as N(9)···N(9) (3.39 Å) and N(9)···N(7) (3.40 Å) are all longer than the usual van der Waals' distances (Table I), from which it is concluded that these caffeine–caffeine interactions are not significant as a structure-determining influence.

The caffeine carbonyl–barbital carbonyl overlap is of greater interest because the short intermolecular C···O distances (3.02 and 3.04 Å) and the approximately antiparallel C—O stacking resemble the configuration found in the crystal structures of the non-pharmacologically active barbiturates, violuric acid monohydrate (24) and dilituric acid trihydrate (25).

Bolton (26) has pointed out a number of crystal structures, including anhydrous barbituric acid and alloxan, which have intermolecular C=O···C=O distances ranging from as short as 2.77 Å, but with a different geometry such that the C=O bond of one carbonyl group is directed toward the carbon atom of a second. Bolton describes these as dipole–dipole interactions, emphasizing that the carbonyl groups in each case are flanked by electron-withdrawing groups which might be expected to enhance the polar character of the C=O bond.

Prout and Wallwork (27) consider the two different orientations for the carbonyl–carbonyl interactions as examples of a general class of intermolecular dipole–dipole or induced dipole interactions involving polar groups such as C=O and P=O and/or polarizable groups such as aromatic systems and carbon–carbon double bonds. For previously determined xanthine crystal structures, where steric factors permit more extensive molecular overlap, Shefter (12) has summarized results showing a tendency for carbonyl groups to lie over the delocalized π-bonded ring systems of adjacent molecules.

The crystal structure of the 2:1 complex of barbital with caffeine appears to be dominated by barbital–barbital and barbital–caffeine interactions, with NH···O and NH···N hydrogen bonding playing a major role. The C(8)H···O and C=O···C=O interactions may also be important, but the nature of these interactions and the extent to which they impose steric relationships in the environment of the component molecules are not yet well understood. These weaker interactions merit further study because they may enhance drug molecular association in solution and drug binding *in vivo* at sites where polar or polarizable groups are favorably oriented.

REFERENCES

(1) K. Sekiguchi, I. Himuro, I. Horikoshi, T. Tsukada, T. Okamoto, and T. Yotsuyanagi, *Chem. Pharm. Bull.*, **17,** 191(1969).

(2) F. Adamanis, *Kron. Farm.*, **35,** 93(1936); *Chem. Zentralbl.*, **1936,** II, 2405; through *Chem Abstr.*, **32,** 4528(1938).

(3) S. H. Kim and A. Rich, *Proc. Nat. Acad. Sci. (USA)*, **60,** 402(1968).

(4) T. Higuchi and D. A. Zuck, *J. Amer. Pharm. Ass., Sci. Ed.*, **42,** 132(1953).

(5) E. Shefter, *J. Pharm. Sci.*, **57,** 1163(1968).

(6) T. Higuchi and J. L. Lach, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 349(1954).

(7) W. M. Higgins and M. F. W. Dunker, *ibid.*, **33,** 310(1944).

(8) M. F. W. Dunker, *ibid.*, **39,** 311(1950).

(9) Y. Kyogoku, R. C. Lord, and A. Rich, *Nature*, **218,** 69(1968).

(10) K. Hoogsteen, in "Molecular Associations in Biology," B. Pullman, Ed., Academic, New York, N. Y., 1968, p. 21.

(11) A. Damiani, P. de Santis, E. Giglio, A. M. Liquori, R. Puliti, and A. Ripamonti, *Acta Crystallogr.*, **19,** 340(1965).

(12) E. Shefter, *J. Pharm. Sci.*, **57,** 350(1968).

(13) I. L. Karle, *Acta Crystallogr.*, **B25,** 2119(1969).

(14) L. Pauling, "The Nature of the Chemical Bond," 3rd ed., Cornell University Press, Ithaca, N.Y., 1960.

(15) B. M. Craven, E. A. Vizzini, and M. M. Rodrigues, *Acta Crystallogr.*, **B25,** 1978(1969).

(16) B. M. Craven and E. A. Vizzini, *ibid.*, **B25,** 1993(1969).

(17) G. L. Gartland and B. M. Craven, unpublished data.

(18) B. M. Craven and C. Cusatis, *Acta Crystallogr.*, **B25,** 2291 (1969).

(19) J. P. Bideau, F. Leroy, and J. Housty, *C. R. Acad. Sci. Paris*, **268C,** 1590(1969).

(20) B. M. Craven and E. A. Vizzini, unpublished data.

(21) D. J. Sutor, *Acta Crystallogr.*, **11,** 453(1958).

(22) D. J. Sutor, *Nature*, **195,** 68(1962).

(23) J. Donohue, in "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Eds., W. H. Freeman, San Francisco, Calif., 1968, p. 443.

(24) B. M. Craven and Y. Mascarenhas, *Acta Crystallogr.*, **17**, 407 (1964).
(25) B. M. Craven, S. Martinez-Carrera, and G. A. Jeffrey, *ibid.*, **17**, 891(1964).
(26) W. Bolton, *Nature*, **201**, 987(1964).
(27) C. K. Prout and S. C. Wallwork, *Acta Crystallogr.*, **21**, 449 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 20, 1970, from the *Department of Crystallography, University of Pittsburgh, Pittsburgh, PA 15213*
Accepted for publication April 30, 1970.
This work was supported by Grant No. NB-02763 from the U. S. Public Health Service, National Institutes of Health.

Influences of Heredity and Environment on Alkaloidal Phenotypes in Solanaceae

MARK J. SOLOMON* and FRANK A. CRANE

Abstract □ Modern biometrical analysis of reported data on *Atropa belladonna* L. indicates varying heritabilities at successive stages of plant ontogeny, exhibiting almost complete additivity at the early flowering stage. A discussion of the importance of this observation with accompanying consideration of more general implications is presented.

Keyphrases □ Phenotypes, alkaloidal, in Solanaceae—heredity, environmental influences □ Solanaceae biovariation—selecting for alkaloidal phenotypes, effects of heredity, environment □ Genetic variation, Solanaceae alkaloidal phenotypes—equations, phenotypic scale diagram

Research efforts involving selection for or against alkaloidal phenotypes are relatively rare in the literature. Such studies are lengthy, involved, and relatively wasteful in time and effort in comparison to the information obtained.

Upon reviewing the literature on Solanaceous alkaloidal biovariation, the authors noted that Sievers (1) had grown plants of *Atropa belladonna* L. for economic reasons during World War I and collected data on total alkaloidal content, expressed as milligrams atropine per gram dry weight of powdered leaf. These data lent themselves to heritability analysis (h^2, the potential for selection under artificial or natural conditions), utilizing modern statistical and biometric techniques (2).[1]

The authors' analysis of Sievers' data is summarized in Table I. Since Sievers analyzed parental types and their respective progeny at different stages of ontogeny, the following conclusions may be drawn:

1. Heritability (h^2) is present.
2. This heritability varies with ontogeny, ranging from high values (maximum value of 1) during vegetative and early flowering stages to lower values in early and late fruiting stages.
3. Heritability is maximum at the flowering stage of ontogeny, reaching almost complete additivity.

To the writers' knowledge, this analysis represents the first application of quantitative genetic techniques to tropane alkaloid biovariation, and it is the first example of quantitative estimation of a relationship of plant ontogenesis to a tropane alkaloid.

[1] The statistical techniques used for this analysis were not known in 1915. It is a tribute to his scientific acumen that Sievers' data could be analyzed over a half-century later to yield these results.

DISCUSSION

Without the information obtained from Sievers' original data (1) and the authors' later interpretation, four models of genetic control appear possible if workers do not postulate genetic and environmental control of alkaloidal phenotypic variation to occur, as most geneticists would assume. These models are:

1. Alkaloidal phenotypes are not inherited; *i.e.*, they are all environmentally controlled.
2. Alkaloidal phenotypes are inherited in Mendelian fashion; *i.e.*, they are monogenic with no environmental control.
3. Alkaloidal phenotypes are the product of multiple allele segregation (with no environmental control).

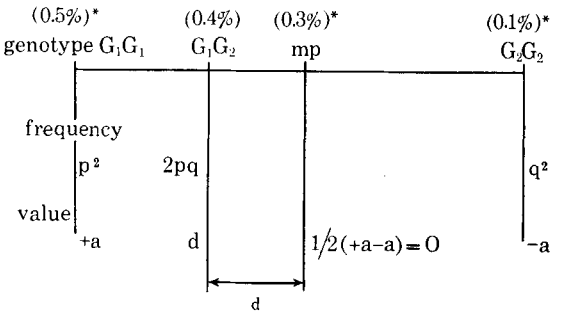


Figure 1—*Alkaloidal phenotypic scale for one gene in the biosynthetic pathway of an alkaloid (26). * A hypothetical case: presence of both dominant alleles would produce a final contribution of 0.5% alkaloidal content. The double recessive G_2G_2 would produce a concentration of 0.1%. If the alleles are additive, the heterozygote A_1A_2 would be 0.3%. If dominance is present, the G_1G_2 genotype would have the value of 0.4%.*

G_1G_1 = *homozygous dominant (maximum contribution to alkaloidal phenotype)*
G_2G_2 = *homozygous recessive (minimum contribution to alkaloidal phenotype)*
G_1G_2 = *heterozygote value (0 point or deviation if dominance is present)*
d = *dominance deviation from midparent*
mp = *midparent value (additive value of G_1G_1 + additive value of G_2G_2 divided by 2)*
α_1 = *average effect when G_1 is substituted for G_2*
α_2 = *average effect when G_2 is substituted for G_1*
α_T = *total average effects on the phenotype*
a = *additive effect due to one allele substitution*
p = *frequency of G_1 allele in population*
q = *frequency of G_2 allele in population*
$p + q$ = *1 since each gene has only two alleles*

(24) B. M. Craven and Y. Mascarenhas, *Acta Crystallogr.*, **17**, 407 (1964).
(25) B. M. Craven, S. Martinez-Carrera, and G. A. Jeffrey, *ibid.*, **17**, 891(1964).
(26) W. Bolton, *Nature*, **201**, 987(1964).
(27) C. K. Prout and S. C. Wallwork, *Acta Crystallogr.*, **21**, 449 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 20, 1970, from the *Department of Crystallography, University of Pittsburgh, Pittsburgh, PA 15213*
Accepted for publication April 30, 1970.
This work was supported by Grant No. NB-02763 from the U. S. Public Health Service, National Institutes of Health.

Influences of Heredity and Environment on Alkaloidal Phenotypes in Solanaceae

MARK J. SOLOMON* and FRANK A. CRANE

Abstract □ Modern biometrical analysis of reported data on *Atropa belladonna* L. indicates varying heritabilities at successive stages of plant ontogeny, exhibiting almost complete additivity at the early flowering stage. A discussion of the importance of this observation with accompanying consideration of more general implications is presented.

Keyphrases □ Phenotypes, alkaloidal, in Solanaceae—heredity, environmental influences □ Solanaceae biovariation—selecting for alkaloidal phenotypes, effects of heredity, environment □ Genetic variation, Solanaceae alkaloidal phenotypes—equations, phenotypic scale diagram

Research efforts involving selection for or against alkaloidal phenotypes are relatively rare in the literature. Such studies are lengthy, involved, and relatively wasteful in time and effort in comparison to the information obtained.

Upon reviewing the literature on Solanaceous alkaloidal biovariation, the authors noted that Sievers (1) had grown plants of *Atropa belladonna* L. for economic reasons during World War I and collected data on total alkaloidal content, expressed as milligrams atropine per gram dry weight of powdered leaf. These data lent themselves to heritability analysis (h^2, the potential for selection under artificial or natural conditions), utilizing modern statistical and biometric techniques (2).[1]

The authors' analysis of Sievers' data is summarized in Table I. Since Sievers analyzed parental types and their respective progeny at different stages of ontogeny, the following conclusions may be drawn:

1. Heritability (h^2) is present.
2. This heritability varies with ontogeny, ranging from high values (maximum value of 1) during vegetative and early flowering stages to lower values in early and late fruiting stages.
3. Heritability is maximum at the flowering stage of ontogeny, reaching almost complete additivity.

To the writers' knowledge, this analysis represents the first application of quantitative genetic techniques to tropane alkaloid biovariation, and it is the first example of quantitative estimation of a relationship of plant ontogenesis to a tropane alkaloid.

[1] The statistical techniques used for this analysis were not known in 1915. It is a tribute to his scientific acumen that Sievers' data could be analyzed over a half-century later to yield these results.

DISCUSSION

Without the information obtained from Sievers' original data (1) and the authors' later interpretation, four models of genetic control appear possible if workers do not postulate genetic and environmental control of alkaloidal phenotypic variation to occur, as most geneticists would assume. These models are:

1. Alkaloidal phenotypes are not inherited; *i.e.*, they are all environmentally controlled.
2. Alkaloidal phenotypes are inherited in Mendelian fashion; *i.e.*, they are monogenic with no environmental control.
3. Alkaloidal phenotypes are the product of multiple allele segregation (with no environmental control).

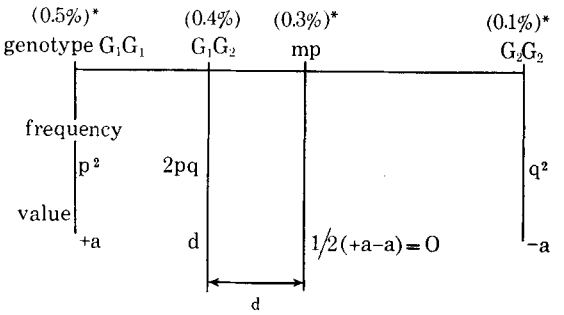


Figure 1—*Alkaloidal phenotypic scale for one gene in the biosynthetic pathway of an alkaloid (26). * A hypothetical case: presence of both dominant alleles would produce a final contribution of 0.5% alkaloidal content. The double recessive G_2G_2 would produce a concentration of 0.1%. If the alleles are additive, the heterozygote A_1A_2 would be 0.3%. If dominance is present, the G_1G_2 genotype would have the value of 0.4%.*

G_1G_1 = *homozygous dominant (maximum contribution to alkaloidal phenotype)*
G_2G_2 = *homozygous recessive (minimum contribution to alkaloidal phenotype)*
G_1G_2 = *heterozygote value (0 point or deviation if dominance is present)*
d = *dominance deviation from midparent*
mp = *midparent value (additive value of G_1G_1 + additive value of G_2G_2 divided by 2)*
α_1 = *average effect when G_1 is substituted for G_2*
α_2 = *average effect when G_2 is substituted for G_1*
α_T = *total average effects on the phenotype*
a = *additive effect due to one allele substitution*
p = *frequency of G_1 allele in population*
q = *frequency of G_2 allele in population*
$p + q$ = *1 since each gene has only two alleles*

Table I—Effect of Ontogenesis on Heritability of Total Alkaloids (Determined as Atropine) in Leaves of *A. belladonna* [Determined from the Data of Sievers (1)]

Statistic	Flowering		Fruiting		Overall
	Before	After	Early	Late	
h^2 [a]	0.8838	0.9520	0.4828	0.3097	0.7240
95% CL_h^2 [b]	0.7874–0.9801	0.8344–1.0000+	0.4007–0.5648	0.2270–0.3924	0.6158–0.8321
Regression equation [c]	$y = 0.1294 + 0.8838x$	$y = 0.0098 + 0.9520x$	$y = 0.2360 + 0.4828x$	$y = 0.4443 + 0.3097x$	$y = 0.2991 + 0.7240x$

[a] h^2 = heritability, s_a^2/s_p^2. When $s_g^2 = s_a^2$, then $h^2 = s_g^2/s_p^2$. [b] 95% CL_h^2 = in 19 of 20 cases the heritability will be between these limits. [c] Regression equation: x = parental value; y = progeny values. Five randomly selected progeny per parent were used to avoid weighting factors. See Falconer (2) for technique, p. 179.

4. Alkaloidal phenotypes are the product of a polygenic system (quantitative inheritance with some environmental control and possible genetic–environmental interaction).

Model 1—In view of Sievers' data and other corroborating evidence, this model is not feasible for *A. belladonna*. Furthermore, other genera in the Solanaceae exhibit evidence of genetic control. The increased numbers of genes in autotetraploids demonstrated by Rowson (3, 4) and recently extended by Solomon and Crane (5) indicate that increased numbers of genes cause increased alkaloid concentration. Stary (6), working with "Poinsettia aneuploids" and "Globe aneuploids," indicated that the 17·18 and 21·22 chromosomes of *Datura* regulate alkaloid production and further implicate genetic control.

The work of Hills *et al.* (7) on crosses of *Duboisia* species and of Evans *et al.* (8, 9) on *Datura* species indicates that genes may regulate the presence or absence of specific alkaloids in interspecific hybrids.

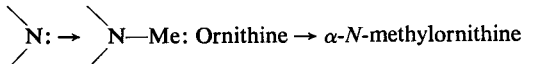
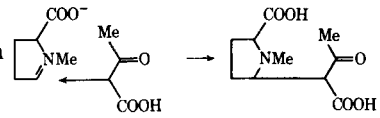
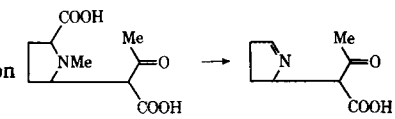
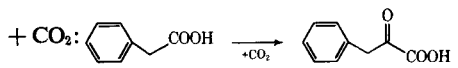
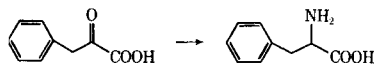
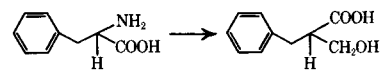
Lecat (10) demonstrated that selection for increased morphine content in *Papaver somniferum* var. *nigrum* is possible. Good chemical screening for alkaloids is based on the empirical premise that if alkaloids are found in a given species, other members of the genus and other genera in the family have a greater probability of producing alkaloids than a random sampling of the plant kingdom. Thus, it appears that genetic control of alkaloidal biovariation and all resulting implications in nautral selection and fitness considerations may be generalized to other families in higher plants.

Model 2—Although Mendelian segregation may be responsible for the presence or absence of specific alkaloids in interspecific genetic studies (7–9) in view of the complex set of biochemical reactions thought to be responsible for various alkaloidal biosyntheses, a single-gene mechanism of inheritance within a particular species should be considered highly unlikely (Table II). Such a mechanism would indicate one gene regulating more than one enzymatically controlled reaction for the biosynthesis of hyoscyamine and possibly even more steps in the biosynthesis of scopolamine. A monogenic mechanism of inheritance would also require that the one gene regulating the alkaloidal phenotype in *Datura* would have the unlikely task of being located on two different chromosomes (the 17·18 and 21·22) at the same time (6).

Model 3—The many types of biochemical transformations involved in the alkaloid biosynthesis already demonstrated for *Datura* effectively eliminate the multiple-allele model as a total explanation of alkaloid inheritance. But even with another model, the possibility of multiple alleles at any genic locus identified with regulating alkaloid inheritance must be considered. This explanation would have to be confined to individual steps in particular biosynthetic schemes rather than to the total biosynthesis of the final alkaloidal product.

Model 4—Quantitative characteristics or metrical characters such as secondary plant product concentrations, *i.e.*, alkaloid concentrations, are usually assumed to be polygenically controlled. Geneticists assume *a priori* that such characteristics have, by their

Table II—Outline of Major Steps Involved in Hyoscyamine and Hyoscine Biosynthesis

Gene [a] (Possible)	Hypothetical Function of Enzyme	Type of Reaction
I	*N*-Methylation	 N: → N—Me: Ornithine → α-*N*-methylornithine
II	Oxidation	Transamination: α-*N*-methylornithine → α-*N* methyl glutamic-γ-aldehyde
III	Ring-closure equilibrium	Ring closure: α-*N*-methylglutamic-γ-aldehyde → *N*-methylpyrrolinium salt
IV	Condensation with acetoacetic acid	Mannich reaction 
V	Oxidation	Oxidative decarboxylation 
VI	Esterification	Tropic acid + tropanone carboxylic acid → hyoscyamine
VII	Carboxylation	 + CO_2:
VIII	Amination	 Transamination:
IX	Intramolecular rearrangement	
X	Oxidation	−2H: Hyoscyamine → 6,7-dehydrohyoscyamine
XI	Oxidation	+O: 6,7-dehydrohyoscyamine → 6-hydroxyhyoscyamine
XII	Oxidation	Oxidation: 6-hydroxyhyoscyamine → hyoscine

[a] Genes are designated by Roman numerals. The dominant alleles would be I_{A1}, I_{A2}, I_{A3} .... The recessive alleles would be I_{a1}, I_{a2}, I_{a3} ....

basic nature, genetic and environmental variation. Since Beadle's (11) statement of the "one-gene–one-enzyme" hypothesis, it has been assumed that one gene regulates one biochemical step through one enzyme. Reexamination of Table II indicates 12 possible biosynthetic reactions resulting from enzymatic activity (12). Thus, there appears to be a high probability that extensive additive genetic variance, estimated by heritability, should be possible. Sievers' data (1) and the writers' analysis indicate that this is the case.

Figure 1 summarizes recognized aspects of established polygenic relationships. A hypothetical numerical example relating to alkaloidal polygenic contributions is given. Significantly, each gene in the biosynthetic pathway has an additive genetic variance which contributes to the rate of selection, either artificial or natural, for or against the final phenotype. This rate of selection, designated as h^2, is the term for heritability. Thus, the presence of heritability in a parent–offspring regression analysis, as demonstrated by Sievers' data, indicates that additive genetic variance is present and that polygenic inheritance applies.

Other types of variation than additive genetic variation are possible. These variances are summarized by the following equation:

$$s_p^2 = s_e^2 + s_s^2 + s_{ge}^2 + s_a^2 + s_d^2 + s_i^2 \qquad \text{(Eq. 1)}$$

$$\text{where } s_a^2 + s_d^2 + s_i^2 = s_g^2$$

and

s_p^2 = total observed variation (phenotypic)
s_e^2 = total environmental variation
s_s^2 = sampling error induced by not using an infinite population
s_{ge}^2 = genetic–environmental variation
s_g^2 = total genetic variation
s_a^2 = total additive genetic variation
s_d^2 = total dominant deviation variation
s_i^2 = total epistatic or intergenic variation

The total average effects of all the genes affecting the final concentration of alkaloids in the phenotype would simulate a normal distribution because of the large number of frequency classes possible. The enzymes listed in Table II need not be specific for a particular step in alkaloid biosynthesis but may be involved in general metabolic activity as well. A polygenic model requires only that a number of enzymes and their respective genes controlling production of the final product, alkaloid, additively produce the final concentration.

Therefore, since the heritability, h^2, varies with ontogeny of the plant in *A. belladonna*, the ratio of the additive genetic variance to the total phenotypic variance also appears to vary. Thus, it appears that the additivity, or the ability to select for or against total alkaloidal variation expressed as milligrams atropine per gram dry weight of powdered leaf, also varies with the ontogeny of the plant.

Most pharmacognosists have stressed the importance of environmental effects on the alkaloids of Solanaceous plants. Such environmental variables as photoperiod, temperature, inorganic nutrition, and their ratios have been reported to influence growth and alkaloid production (13–25). These environmental studies were made on uniform strains of plants, assuming genetic influence as nonvariable. In view of Sievers' data and the authors' analyses, heritability, additivity, and, therefore, the possibility of selection are present. Moreover, the high heritabilities observed in the early vegetative and early flowering phases indicate that natural selection under various environmental circumstances would most probably occur at these stages of ontogeny.

CONCLUSIONS

Modern biometric analysis of reported data on *A. belladonna* indicates that heritability, or the possible rate of selection, either natural or artificial, for the total alkaloids of this species varies with the ontogeny of the plant, reaching maximum levels (almost complete additivity) at the early flowering stage. Reexamination of existing information in pharmacognostical literature indicates that the polygenic model, rather than the three alternative models, appears most satisfactory for explanation of the biovariation of alkaloidal concentrations in specific phenotypes. The presence or absence of particular alkaloids in interspecific hybrids and their genetic segregants may be explained, however, by the usual Mendelian laws.

REFERENCES

(1) A. F. Sievers, U.S.D.A. Bull. No. 306, 1915.

(2) D. S. Falconer, "Introduction to Quantitative Genetics," Ronald Press, New York, N. Y., 1967, pp. 171–179.

(3) J. M. Rowson, *Quart. J. Pharm. Pharmacol.*, **18,** 175(1945).

(4) *Ibid.*, **17,** 185(1944).

(5) M. J. Solomon and F. A. Crane, *Trans. Ill. Acad. Sci.*, **63,** 67(1970).

(6) F. Stary, *Abh. Deut. Akad. Wiss. Berlin, Kl. Chem. Geol. Biol.*, **4,** 175(1963).

(7) L. W. Hills, W. Bottomley, and T. I. Mortimer, *Aust. J. Appl. Sci.*, **5,** 283(1954).

(8) W. C. Evans, N. A. Stevenson, and R. F. Timoney, *Planta Med.*, **17,** 120(1969).

(9) *Ibid.*, **17,** 19(1969).

(10) P. Lecat, *Ann. Pharm. Franc.*, **14,** 714(1956).

(11) G. W. Beadle, *Physiol. Rev.*, **25,** 643(1945).

(12) "Biogenesis of Natural Compounds," 2nd ed., P. Bernfeld, Ed., Pergamon Press, London, England, 1967, pp. 959–961.

(13) "Oxford Medicinal Plants Scheme," Annual Reports, 1942–1945.

(14) G. M. James, *Econ. Bot.*, **1,** 230(1947).

(15) W. R. Brewer and L. D. Hiner, *J. Amer. Pharm. Ass., Sci. Ed.*, **39,** 586(1950).

(16) E. N. Stillings and A. Laurie, *Proc. Amer. Soc. Hort. Sci.*, **42,** 590(1943).

(17) W. O. James, in "The Alkaloids," vol. I, R. H. F. Manske and H. L. Holmes, Eds., Academic, New York, N. Y., 1952, pp. 16–90.

(18) A. Stutzer and S. Goy, *Biochem. Z.*, **56,** 220(1913).

(19) H. W. Conner, *Plant Physiol.*, **12,** 79(1937).

(20) E. S. Mika, *Bot. Gaz. (Chicago)*, **116,** 333(1955).

(21) L. J. Schermeister, F. A. Crane, and R. F. Voigt, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 694(1960).

(22) *Ibid.*, **49,** 698(1960).

(23) S. J. Smolenski, F. A. Crane, and R. F. Voigt, *J. Pharm. Sci.*, **56,** 599(1967).

(24) R. A Uphaus, F. A. Crane, M. I. Blake, and J. J. Katz, *ibid.*, **54,** 202(1965).

(25) F. A. Crane, K. Mohammed, M. I. Blake, R. A. Uphaus, and J. J. Katz, *Lloydia*, **33,** 11(1970).

(26) K. Mather, "Biometrical Genetics," Dover, New York, N. Y., 1949.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 11, 1969, from the *Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680*

Accepted for publication June 9, 1970.

Presented at the Annual Meeting of the American Society of Pharmacognosy, Corvallis, Ore., August 1969.

This work was supported by a grant-in-aid by the Society of the Sigma Xi.

* American Foundation for Pharmaceutical Education Fellow, 1969–1970; A.F.P.E. Pharmaceutical Manufacturers Association Fellow, 1970–1971.

Complexes of Ergot Alkaloids and Derivatives IV: Investigations into the Nature of Ergot Alkaloid–Xanthine Complexes

H. V. MAULDING and M. A. ZOGLIO*

Abstract □ Studies of the effect of xanthines on the solubility of molecules related to the proteinaceous ergot alkaloids indicate the indole moiety to be a primary site for complexation. The tendency of indole and several analogs to form intermolecular complexes with caffeine was surveyed. This property leads to considerable solubility elevation for many of the compounds examined. Apparent equilibrium or association constants are calculated based on an assumed 1:1 complex between the indole substrate and xanthine ligand. Experimental evidence with several simple indoles and caffeine indicates general adherence to this principle. These relatively uncomplex compounds and even larger more complicated molecules such as methysergide exhibit this property of increased solubility through interaction. These substances may be treated in a somewhat rigorous manner contrary to the intricate proteinaceous alkaloids previously examined.

Keyphrases □ Ergot alkaloid–xanthine complexes—solubility effect □ Xanthine–ergot alkaloid complexes—solubility effect □ Spectrophotometry—analysis

The oral administration of ergotamine tartrate with caffeine has been reported to bring about an enhancement of the clinical activity of the alkaloid against migraine headaches (1, 2).

In the course of investigating the physicochemical nature of the combination, several ergot alkaloids and their analogs, such as ergotamine, 9,10-dihydroergocristine, and methysergide, have been observed to possess the capacity for interaction with caffeine and other xanthines in aqueous solution (3–6). This mutual attraction is exemplified by: (*a*) increased solubility; (*b*) increased dissolution-rate constant, and (*c*) altered partitioning-rate constants depending on pH. These changes are noted on inclusion of xanthines as compared to the alkaloids alone (3–6). Recent biological results (7) were found to be in good agreement with the postulation that complex formation leads to increased enteral absorption of ergotamine and ergot alkaloids.

A series of papers in 1957 reported that caffeine and related compounds complex with a wide variety of organic compounds (8–10) as well as with themselves (11). This behavior of caffeine and other xanthines has been studied by means of solubility (8, 11, 12), spectral (13), and kinetic (9, 10, 14) techniques.

It was believed that an investigation of simpler indoles might give some clue regarding the nature of the complex present in complicated ergot alkaloids. A project was initiated to examine indole and several congeners to determine structural factors affecting interaction and delineation of structure–activity relationships. It was hoped this approach would help in the eventual unraveling of the nature and stoichiometry of the species comprising the ergot–xanthine complex, the ultimate aim being application of these and other data to formulation of more suitable medicinal agents.

EXPERIMENTAL

Watertight, screw-capped vials (18-ml. capacity) containing exactly 10 ml. of solvent, 100 mg. of substrate (indole compound), and varying amounts of caffeine were clamped onto the edge of metal disks, 15.24 cm. (6 in.) in diameter, mounted on a motor-driven shaft and rotated vertically at 6 r.p.m. in a constant-temperature bath at 30 ± 0.2°. After exactly 24 hr., samples were withdrawn using pipets with filters attached and analyzed for the indole compound by the Van Urk method (3). Each sample was run at minimum in triplicate.

RESULTS AND DISCUSSION

The absence of observable spectral changes precluded utilization of this technique in these experiments. The kinetic method was not employed because of the absence of a suitable reaction for study, thus leaving solubility analysis. This mode has previously been found to be practicable in the study of proteinaceous ergot derivatives, although certain problems have arisen from their erratic solubility properties (3–6).

The usual experimental operation involves addition of an equal weight (in excess of its normal solubility) of the slightly soluble substrate into each of several vials containing a fixed amount of solvent. Varying amounts of xanthine were placed in the vials, which were rotated 24 hr. in a constant-temperature bath. The solution phase was withdrawn by means of a filter pipet with care taken not to include undissolved indole. Assays were performed by the Van Urk (15) method, which lends itself to most molecules containing the indole nucleus. The colors formed absorbed at various wavelengths, depending on the compound in question (Table I).

Data obtained by the solubility analysis scheme may be treated in the following manner, as summarized by Connors and Mollica (16) and using their symbolism. The term S in Eq. 1 represents substrate, indole compound, and L represents ligand, xanthine:

$$mS + nL \rightleftarrows S_mL_n \qquad \text{(Eq. 1)}$$

The strength of the complex is given in terms of K_{mn}, which is the stability or association constant:

$$K_{mn} = \frac{[S_mL_n]}{[S]^m[L]^n} \qquad \text{(Eq. 2)}$$

Table I—Absorptivity and Wavelengths of Maximum Absorbance of Indole Compounds

Compound	ϵ^a	λ, mμ^b
1. Indole	4217	400
2. 1-Methylindole	5245	400
3. 2-Methylindole	4810	380
4. 3-Methylindole	3142	410
5. 5-Methylindole	4810	410
6. 7-Methylindole	4198	425
7. 5-Methoxyindole	5151	400
8. 6-Methoxyindole	3287	400
9. 7-Methoxyindole	2362	450
10. 5-Fluoroindole	5575	400
11. 5-Chloroindole	6216	400
12. 5-Bromoindole	6240	400
13. Methysergide[c]	6905	322

[a] Molar absorptivity. [b] Compound 13, methysergide, measured in the UV region; the remainder in the visible, using Van Urk reagent (13). [c] Measured as bimaleate salt, pH 6.65, 25°.

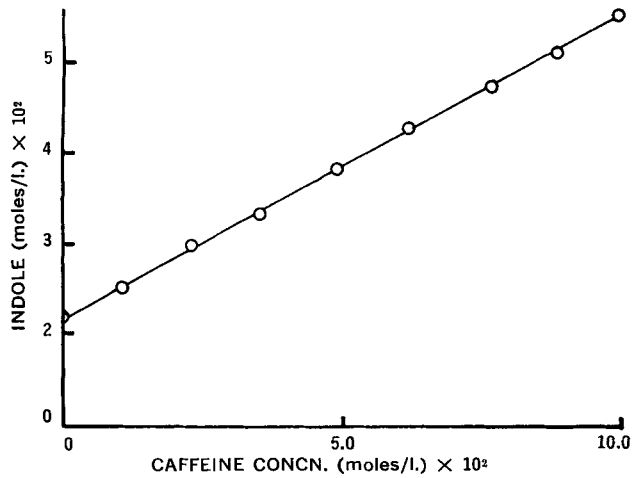


Figure 1—*Solubility phase diagram for the indole–caffeine system at 30° in pH 6.65 phosphate buffer.*

The parsimonious approach stipulates assumption, in lieu of conflicting information, of a 1:1 stoichiometry brought about by a single complex to be responsible for the results. Thus the values for m and n (Eqs. 1 and 2) are 1. Making proper substitutions in these equations, one obtains the following (16):

$$S_t = \frac{K_{11}S_0L_t}{1 + K_{11}S_0} + S_0 \qquad \text{(Eq. 3)}$$

In Eq. 3 the quantities S_t and S_0 are the total concentrations (moles/l.) of indole or alkaloid in solution at various finite and zero xanthine concentrations, respectively. K_{11} is the association constant as previously stated, and L_t is xanthine concentration in moles/liter.

A plot of L_t against S_t gives a straight line with a slope of

$$\frac{K_{11}S_0}{1 + K_{11}S_0}$$

and a y-intercept of S_0 or the solubility at zero ligand concentration. If a single 1:1 complex is present, the apparent K_{11}, written K'_{11} may be written (16):

$$K'_{11} = \frac{\text{slope}}{y\text{-intercept}\ (1 - \text{slope})} \qquad \text{(Eq. 4)}$$

The compounds considered in this work were run at pH 6.65 in phosphate buffer. The simpler substances such as indole, 1-methylindole, and 5-methoxyindole all have pKa values of 2 or less. Therefore, they are essentially present in only the neutral species at the hydrogen-ion concentrations employed. The same is true for caffeine. As a result of negligible protonation of both molecules (substrate and ligand) at these pH values, the result should be binding between two neutral species. Methysergide possesses a pKa of 6.62 ± 0.02 (6), which indicates any study concerning the molecule at pH 6.65 is at the point of half-ionization, *i.e.*, pH = pKa.

Caffeine was chosen as the best candidate for the ligand for several reasons. It is more soluble than the naturally occurring xanthines such as theophylline and theobromine and offers the best basis for comparison. Much of the earlier work was done with this compound, even though it does complex to an appreciable extent with itself in aqueous solutions (8–14). Caffeine exhibits about 2% solubility at 30°. For this reason, caffeine (ligand) concentrations of greater than 20 mg./ml. were not generally examined.

When the concentration of proteinaceous alkaloid is plotted *versus* caffeine concentration, a strictly linear function is not generated (3). Positive and negative deviations from this linearity take place more or less at random, depending on the alkaloid, xanthine, and solvent in some unknown manner (4, 5). With simple

Table II—Values Obtained from Phase Diagrams of Indole and Analogs in the Presence of Caffeine[a]

Compound[b]	Slope[c]	$S_0 \times 10^{2}$[d]	K'_{11}[e]
1. Indole	0.34	2.11	24.1
2. 1-Methylindole	0.13	0.30	50.0
3. 2-Methylindole	0.19	0.41	59.4
4. 3-Methylindole	0.31	0.55	83.8
5. 5-Methylindole	0.35	0.48	112.0
6. 7-Methylindole	0.23	0.50	59.7
7. 5-Methoxyindole	0.47	1.30	64.1
8. 6-Methoxyindole	0.16	0.41	47.1
9. 7-Methoxyindole	0.30	0.85	52.9
10. 5-Fluoroindole	0.19	1.52	15.8
11. 5-Chloroindole	0.06	0.28	23.0
12. 5-Bromoindole	0.07	0.13	58.3
13. Methysergide	0.26	1.60	22.0

[a] Measurements made at 30 ± 0.2° in pH 6.65 phosphate buffer. [b] Compound 2 secured from K & K, Inc., Plainview, N. Y.; Compounds 6 and 8 from Chemical Procurement, College Point, N. Y.; and the remainder from Aldrich Chemical Co., Milwaukee, Wis. [c] For calculations, see text and figures. [d] Intrinsic solubility of substrate, 30°, and zero xanthine concentration (moles/l.). [e] K'_{11} = slope/(1 − slope)S_0.

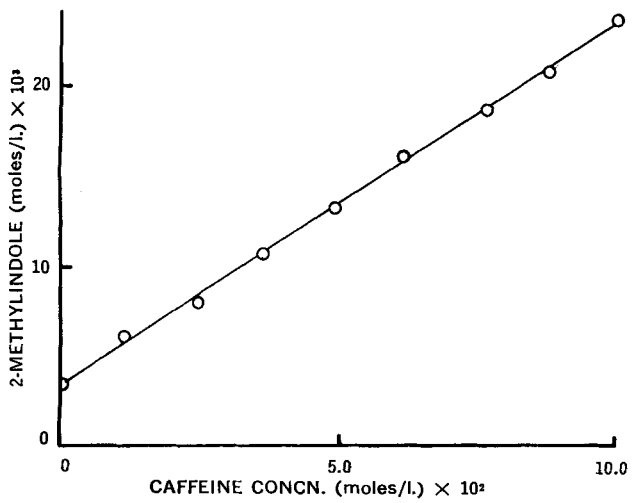


Figure 2—*Phase solubility diagram for the 2-methylindole–caffeine system at 30° in pH 6.65 phosphate buffer.*

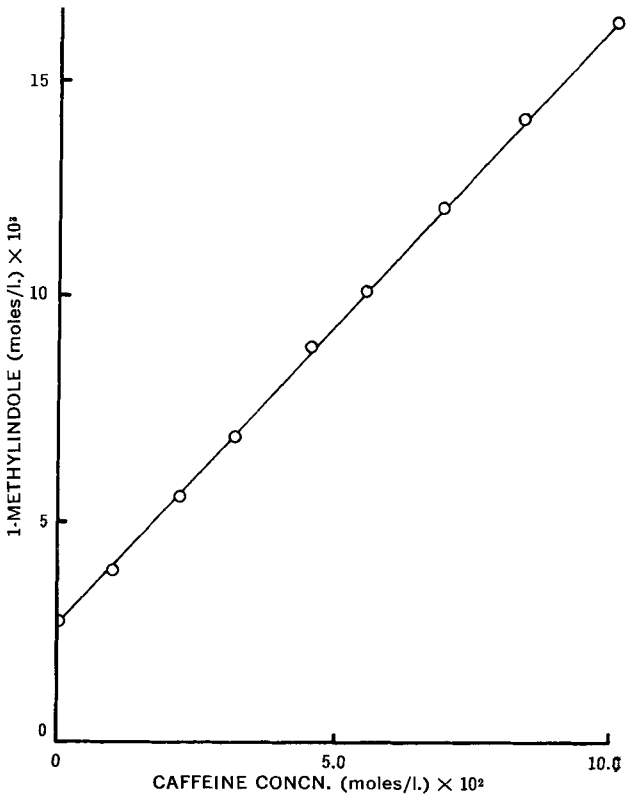


Figure 3—*Phase solubility diagram for the 1-methylindole–f system at 30° in pH 6.65 phosphate buffer.*

indoles, this is not the situation, as may be seen in Figs. 1–3. These substances have K'_{11} values; other pertinent information is listed in Table II.

CONCLUSION

The stoichiometry of the complex has not been delineated, but slopes of less than one as encountered in Figs. 1–3 imply a 1:1 complex. This does not prove the absence of other complex species (16).

Table II illustrates that all K'_{11} values, including that of methysergide, are of approximately the same magnitude. This indicates that a good portion of the capacity for complexation of the high molecular weight ergot alkaloids lies within the indole moiety of the molecule. The role the cyclic tripeptide portion plays in the mutual attraction of caffeine and related xanthines for these proteinaceous alkaloids has yet to be spelled out; however, it is likely that these protein substituents may alter binding in some way.

REFERENCES

(1) N. A. Bercel, *Calif. Med.*, **72**, 234(1950).

(2) A. P. Friedman, *New Engl. J. Med.*, **250**, 600(1954).

(3) M. A. Zoglio, H. V. Maulding, and J. J. Windheuser, *J. Pharm. Sci.*, **58**, 222(1969).

(4) M. A. Zoglio and H. V. Maulding, *ibid.*, **59**, 215(1970).

(5) H. V. Maulding and M. A. Zoglio, *ibid.*, **59**, 398(1970).

(6) *Ibid.*, **59**, 700(1970).

(7) B. Berde, A. Cerletti, M. J. Dengler, and M. A. Zoglio, presented to Third Migraine Trust Symposium, London, England, April 24–25, 1969, vol. III, to be published.

(8) S. Bolton, D. Guttman, and T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 38(1957).

(9) L. Lachman and T. Higuchi, *ibid.*, **46**, 32(1957).

(10) L. Lachman, D. Guttman, and T. Higuchi, *ibid.*, **46**, 36 (1957).

(11) D. Guttman and T. Higuchi, *ibid.*, **46**, 4(1957).

(12) J. L. Cohen and K. A. Connors, *J. Pharm. Educ.*, **31**, 476 (1967).

(13) P. A. Kramer and K. A. Connors, *ibid.*, **33**, 193(1969).

(14) K. A. Connors, M. H. Infeld, and B. J. Kline, *J. Amer. Chem. Soc.*, **91**, 3597(1969).

(15) N. L. Allport and T. T. Cocking, *Quart. J. Pharm. Pharmacol.*, **5**, 341(1932).

(16) K. A. Connors and J. A. Mollica, *J. Pharm. Sci.*, **55**, 772 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *Pharmacy Research & Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication May 23, 1970.

The authors would like to acknowledge the technical assistance of J. Nazareno.

* Present address: American Höechst Corp., Cincinnati, Ohio.

Absolute Configuration of (+)-*trans*-2-*o*-Tolylcyclohexanol by X-Ray Crystallography

ARTHUR CAMERMAN*, LYLE H. JENSEN, TODD G. COCHRAN†, and ALAIN C. HUITRIC

Abstract □ The determination of the absolute configuration of the 3-nitro-4-bromobenzoate ester of (+)-*trans*-2-*o*-tolylcyclohexanol by X-ray crystallographic analysis is reported. The results give unequivocal proof that the original assignments of (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol, (1R,2R)-(−)-*cis*-2-*o*-tolylcyclohexanol, and (2R)-(+)-2-*o*-tolylcyclohexanone are correct.

Keyphrases □ X-ray crystallography—configuration, (+)-*trans*-2-*o*-tolylcyclohexanol □ (+)-*trans*-2-*o*-Tolylcyclohexanol—configuration confirmation, X-ray crystallography

The absolute configurations of (+)-*trans*-2-*o*-tolylcyclohexanol (I) and (−)-*cis*-2-*o*-tolylcyclohexanol (II) were reported in an earlier communication (1) on the basis of the positive Cotton effect of the carbonyl chromophore of the (+)-2-*o*-tolylcyclohexanone (III) obtained from the oxidation of I and II. Compounds I and II are two of several key reference compounds currently used in this laboratory in a study associated with Cotton effects of aromatic chromophores. Although the original assignment of absolute configurations of I and II was considered reliable, an unquestionable proof was desired. Unequivocal proof is now given from X-ray crystallographic analysis of the 3-nitro-4-bromobenzoate ester of I that the initial assignments of (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol for I, (1R,2R)-(−)-*cis*-3-*o*-tolylcyclohexanol for II, and (2R)-(+)-2-*o*-tolylcyclohexanone for III are correct.

Figure 1 shows perspective drawings of the three-dimensional structure and correct absolute configuration of the 3-nitro-4-bromobenzoate ester of (+)-*trans*-2-*o*-tolylcyclohexanol.

EXPERIMENTAL

(1S,2R)-(+)-*trans*-2-*o*-Tolylcyclohexyl 3-nitro-4-bromobenzoate was prepared by reaction of the known (+)-*trans*-2-*o*-tolylcyclohexanol (I) with 3-nitro-4-bromobenzoyl chloride in pyridine. The ester was purified by chromatography on silica gel, using a 50:50 benzene–hexane mixture, and recrystallized from hexane, m.p. 64.5–65.5°, IR (KBr) 1720 cm.$^{-1}$ (C=O), 1536, 1352 (NO_2), $[\alpha]_D^{27}$ + 100° (c 1.0, methanol).

The compound crystallizes in space group $P2_12_12$ with the following crystal data:

a = 7.922 ± 0.002 $\alpha = \beta = \gamma = 90°$
b = 26.342 ± 0.008 Dm = 1.4 (flotation in CsCl solution)
c = 18.694 ± 0.006 $D_{calc.}$ 1.426 Z = 8 molecules/unit cell

X-ray intensities were measured on a crystal approximately 0.13 × 0.38 × 0.53 mm. to $2\theta = 45°$, corresponding to an interplanar spacing of 0.93 Å, on a computer-controlled, four-circle diffractometer. The $\omega/2\theta$ scan technique using Nb-filtered Mo radiation was employed. A total of 2928 independent reflections were measured, of which 1852 had intensities greater than twice the standard deviation of their measurement. Absorption corrections were applied using the method of De Meulenaer and Tompa (2), and struc-

indoles, this is not the situation, as may be seen in Figs. 1–3. These substances have K'_{11} values; other pertinent information is listed in Table II.

CONCLUSION

The stoichiometry of the complex has not been delineated, but slopes of less than one as encountered in Figs. 1–3 imply a 1:1 complex. This does not prove the absence of other complex species (16).

Table II illustrates that all K'_{11} values, including that of methysergide, are of approximately the same magnitude. This indicates that a good portion of the capacity for complexation of the high molecular weight ergot alkaloids lies within the indole moiety of the molecule. The role the cyclic tripeptide portion plays in the mutual attraction of caffeine and related xanthines for these proteinaceous alkaloids has yet to be spelled out; however, it is likely that these protein substituents may alter binding in some way.

REFERENCES

(1) N. A. Bercel, *Calif. Med.*, **72**, 234(1950).

(2) A. P. Friedman, *New Engl. J. Med.*, **250**, 600(1954).

(3) M. A. Zoglio, H. V. Maulding, and J. J. Windheuser, *J. Pharm. Sci.*, **58**, 222(1969).

(4) M. A. Zoglio and H. V. Maulding, *ibid.*, **59**, 215(1970).

(5) H. V. Maulding and M. A. Zoglio, *ibid.*, **59**, 398(1970).

(6) *Ibid.*, **59**, 700(1970).

(7) B. Berde, A. Cerletti, M. J. Dengler, and M. A. Zoglio, presented to Third Migraine Trust Symposium, London, England, April 24–25, 1969, vol. III, to be published.

(8) S. Bolton, D. Guttman, and T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 38(1957).

(9) L. Lachman and T. Higuchi, *ibid.*, **46**, 32(1957).

(10) L. Lachman, D. Guttman, and T. Higuchi, *ibid.*, **46**, 36 (1957).

(11) D. Guttman and T. Higuchi, *ibid.*, **46**, 4(1957).

(12) J. L. Cohen and K. A. Connors, *J. Pharm. Educ.*, **31**, 476 (1967).

(13) P. A. Kramer and K. A. Connors, *ibid.*, **33**, 193(1969).

(14) K. A. Connors, M. H. Infeld, and B. J. Kline, *J. Amer. Chem. Soc.*, **91**, 3597(1969).

(15) N. L. Allport and T. T. Cocking, *Quart. J. Pharm. Pharmacol.*, **5**, 341(1932).

(16) K. A. Connors and J. A. Mollica, *J. Pharm. Sci.*, **55**, 772 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *Pharmacy Research & Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication May 23, 1970.

The authors would like to acknowledge the technical assistance of J. Nazareno.

* Present address: American Höechst Corp., Cincinnati, Ohio.

Absolute Configuration of (+)-*trans*-2-*o*-Tolylcyclohexanol by X-Ray Crystallography

ARTHUR CAMERMAN*, LYLE H. JENSEN, TODD G. COCHRAN†, and ALAIN C. HUITRIC

Abstract □ The determination of the absolute configuration of the 3-nitro-4-bromobenzoate ester of (+)-*trans*-2-*o*-tolylcyclohexanol by X-ray crystallographic analysis is reported. The results give unequivocal proof that the original assignments of (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol, (1R,2R)-(−)-*cis*-2-*o*-tolylcyclohexanol, and (2R)-(+)-2-*o*-tolylcyclohexanone are correct.

Keyphrases □ X-ray crystallography—configuration, (+)-*trans*-2-*o*-tolylcyclohexanol □ (+)-*trans*-2-*o*-Tolylcyclohexanol—configuration confirmation, X-ray crystallography

The absolute configurations of (+)-*trans*-2-*o*-tolylcyclohexanol (I) and (−)-*cis*-2-*o*-tolylcyclohexanol (II) were reported in an earlier communication (1) on the basis of the positive Cotton effect of the carbonyl chromophore of the (+)-2-*o*-tolylcyclohexanone (III) obtained from the oxidation of I and II. Compounds I and II are two of several key reference compounds currently used in this laboratory in a study associated with Cotton effects of aromatic chromophores. Although the original assignment of absolute configurations of I and II was considered reliable, an unquestionable proof was desired. Unequivocal proof is now given from X-ray crystallographic analysis of the 3-nitro-4-bromobenzoate ester of I that the initial assignments of (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol for I, (1R,2R)-(−)-*cis*-3-*o*-tolylcyclohexanol for II, and (2R)-(+)-2-*o*-tolylcyclohexanone for III are correct.

Figure 1 shows perspective drawings of the three-dimensional structure and correct absolute configuration of the 3-nitro-4-bromobenzoate ester of (+)-*trans*-2-*o*-tolylcyclohexanol.

EXPERIMENTAL

(1S,2R)-(+)-*trans*-2-*o*-Tolylcyclohexyl 3-nitro-4-bromobenzoate was prepared by reaction of the known (+)-*trans*-2-*o*-tolylcyclohexanol (I) with 3-nitro-4-bromobenzoyl chloride in pyridine. The ester was purified by chromatography on silica gel, using a 50:50 benzene–hexane mixture, and recrystallized from hexane, m.p. 64.5–65.5°, IR (KBr) 1720 cm.$^{-1}$ (C=O), 1536, 1352 (NO_2), $[\alpha]_D^{27}$ + 100° (c 1.0, methanol).

The compound crystallizes in space group $P2_12_12$ with the following crystal data:

a = 7.922 ± 0.002 $\alpha = \beta = \gamma = 90°$
b = 26.342 ± 0.008 Dm = 1.4 (flotation in CsCl solution)
c = 18.694 ± 0.006 $D_{calc.}$ 1.426 Z = 8 molecules/unit cell

X-ray intensities were measured on a crystal approximately 0.13 × 0.38 × 0.53 mm. to $2\theta = 45°$, corresponding to an interplanar spacing of 0.93 Å, on a computer-controlled, four-circle diffractometer. The $\omega/2\theta$ scan technique using Nb-filtered Mo radiation was employed. A total of 2928 independent reflections were measured, of which 1852 had intensities greater than twice the standard deviation of their measurement. Absorption corrections were applied using the method of De Meulenaer and Tompa (2), and struc-

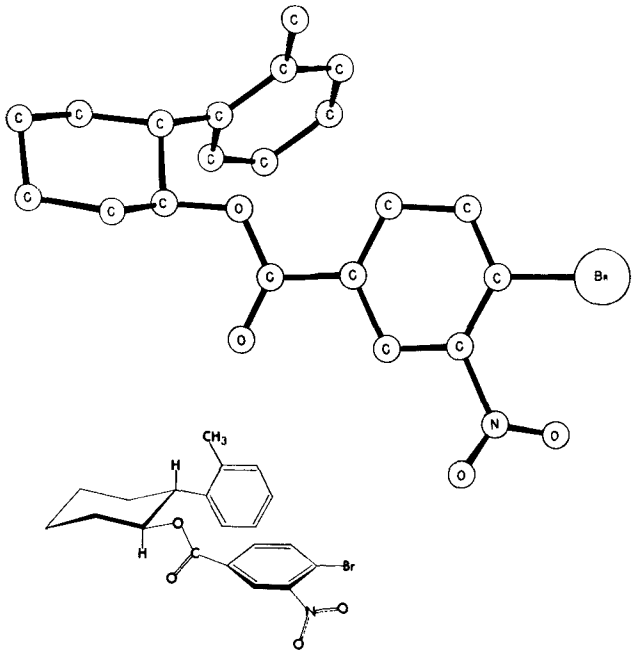


Figure 1—*(1S,2R)*−(+)-trans-*2*-o-*Tolylcyclohexyl 3-nitro-4-bromobenzoate.*

ture amplitudes were obtained from the intensities in the usual fashion. A sharpened, origin-removed, three-dimensional Patterson synthesis enabled the positions of the two unique bromine atoms to be found; the coordinates of the 50 other nonhydrogen atoms comprising the two molecules in the asymmetric unit were determined in subsequent electron-density maps based on phases calculated from the bromine positions. The positional and thermal parameters of all the atoms were refined using full matrix least squares until a discrepancy factor, R, of 0.089 was achieved.

Up to this point the normal bromine scattering curve, f_{Br}^0, was used with no correction for anomolous scattering of the X-rays by the bromine atoms. At this stage, the absolute configuration of the molecule was determined by the following method. The true bromine scattering curve, including anomolous scattering effects, $f_{Br} = f_{Br}^0 + \Delta f_{Br}' + i\Delta f_{Br}''$, was then applied; structure factors were calculated for molecules with atom coordinates x, y, and z and for molecules with atom coordinates $-x$, $-y$, and $-z$. That is, structure factors were calculated for both possible optical isomers. The discrepancy factor, $R = (\Sigma||Fo| - |Fc||)/\Sigma|Fo|$, was 0.098 for one structure and 0.086 for the other enantiomer. The difference is highly significant (3), and the absolute configuration for this structure is thus unambiguously established as the one giving the lower R. One last cycle of least-squares refinement, using the atom coordinates corresponding to the correct optical isomer but neglecting anomalous corrections to the scattering curve, brought R down to 0.079. Although hydrogen atoms could be located from a difference Fourier map, they were not included in the calculations since the additional significance does not compensate for the added cost of refinement for a structure of this size.

REFERENCES

(1) D. R. Galpin and A. C. Huitric, *J. Pharm. Sci.*, **57**, 447(1968).

(2) J. De Meulenaer and H. Tompa, *Acta Crystallogr.*, **19**, 1014 (1965).

(3) W. C. Hamilton, *ibid.*, **18**, 502(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 16, 1970, from the *College of Pharmacy and the Department of Biological Structure, University of Washington, Seattle, WA 98105*

Accepted for publication May 26, 1970.

This investigation was supported in part by Grants 5 R01 NS 08329 and GM-13366 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

* To whom inquiries may be addressed.

† Public Health Service Predoctoral Fellow 5 F01 GM 41752.

Muscarinic Receptors: 4-Substituted-3-trimethylammoniumtetrahydrofuran Halides

WENDEL L. NELSON, JOHNNY K. WONG*, FRANK F. VINCENZI, PETER H. BLAKE†, and DONALD L. SMITH

Abstract □ Preparation of the *cis*- and *trans*-4-hydroxy-3-trimethylammoniumtetrahydrofuran and 4-acetoxy-3-trimethylammoniumtetrahydrofuran halides and 3-trimethylammoniumtetrahydrofuran iodide is described. Weak muscarinic activity was noted for the unsubstituted 3-trimethylammoniumtetrahydrofuran salt, being about 1000-fold less potent than acetylcholine. The *trans*-hydroxy and *trans*-acetoxy compounds showed even less activity, and the *cis*-compounds were inactive.

Keyphrases □ 3-Trimethylammoniumtetrahydrofuran halides, 4-substituted—muscarinic receptors, synthesis, pharmacologic testing □ Muscarinic receptors—3-trimethylammoniumtetrahydrofuran halides, 4-substituted, synthesis, pharmacologic testing

The fundamental problem of relating molecular structure to biological activity at various drug receptors becomes extremely difficult when considering small conformationally mobile molecules such as acetylcholine. Regardless of the existence of preferred conformations in the solid state and in solution (1–5), it should not be assumed that these conformations are those in the drug–receptor surface complex (6).

Various conformationally rigid or semirigid cholinergic agents have been prepared to aid in determination of the architectural features of the drug–receptor complex on various cholinergic sites, *e.g.*, muscarinic, nicotinic, and acetylcholinesterase (7–12). Attempts which incorporate the least number of additional atoms have generally been most successful, although comparisons of closely related compounds in higher series also seem valid (12).

In this study, various analogs of 3-trimethylammoniumtetrahydrofuran iodide were prepared. This system

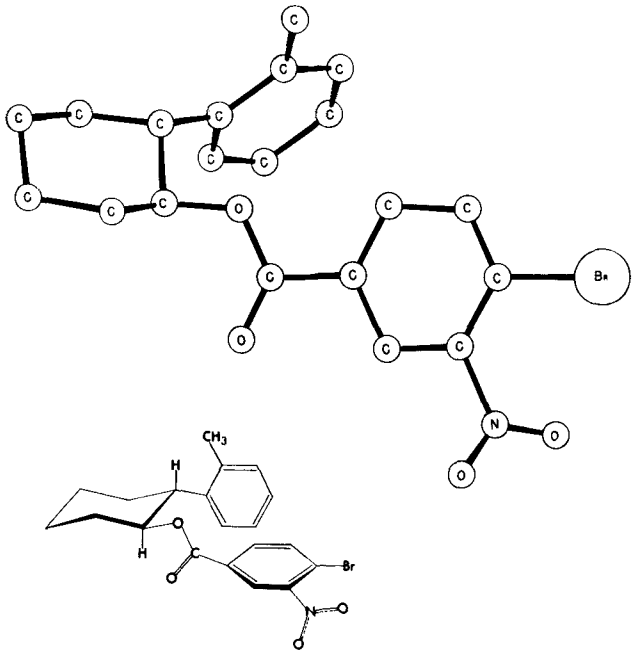


Figure 1—*(1S,2R)*−(+)-trans-*2*-o-*Tolylcyclohexyl 3-nitro-4-bromobenzoate.*

ture amplitudes were obtained from the intensities in the usual fashion. A sharpened, origin-removed, three-dimensional Patterson synthesis enabled the positions of the two unique bromine atoms to be found; the coordinates of the 50 other nonhydrogen atoms comprising the two molecules in the asymmetric unit were determined in subsequent electron-density maps based on phases calculated from the bromine positions. The positional and thermal parameters of all the atoms were refined using full matrix least squares until a discrepancy factor, R, of 0.089 was achieved.

Up to this point the normal bromine scattering curve, f_{Br}^0, was used with no correction for anomolous scattering of the X-rays by the bromine atoms. At this stage, the absolute configuration of the molecule was determined by the following method. The true bromine scattering curve, including anomolous scattering effects, $f_{Br} = f_{Br}^0 + \Delta f_{Br}' + i\Delta f_{Br}''$, was then applied; structure factors were calculated for molecules with atom coordinates x, y, and z and for molecules with atom coordinates $-x$, $-y$, and $-z$. That is, structure factors were calculated for both possible optical isomers. The discrepancy factor, $R = (\Sigma||Fo| - |Fc||)/\Sigma|Fo|$, was 0.098 for one structure and 0.086 for the other enantiomer. The difference is highly significant (3), and the absolute configuration for this structure is thus unambiguously established as the one giving the lower R. One last cycle of least-squares refinement, using the atom coordinates corresponding to the correct optical isomer but neglecting anomalous corrections to the scattering curve, brought R down to 0.079. Although hydrogen atoms could be located from a difference Fourier map, they were not included in the calculations since the additional significance does not compensate for the added cost of refinement for a structure of this size.

REFERENCES

(1) D. R. Galpin and A. C. Huitric, *J. Pharm. Sci.*, **57**, 447(1968).

(2) J. De Meulenaer and H. Tompa, *Acta Crystallogr.*, **19**, 1014 (1965).

(3) W. C. Hamilton, *ibid.*, **18**, 502(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 16, 1970, from the *College of Pharmacy and the Department of Biological Structure, University of Washington, Seattle, WA 98105*

Accepted for publication May 26, 1970.

This investigation was supported in part by Grants 5 R01 NS 08329 and GM-13366 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

* To whom inquiries may be addressed.

† Public Health Service Predoctoral Fellow 5 F01 GM 41752.

Muscarinic Receptors: 4-Substituted-3-trimethylammoniumtetrahydrofuran Halides

WENDEL L. NELSON, JOHNNY K. WONG*, FRANK F. VINCENZI, PETER H. BLAKE†, and DONALD L. SMITH

Abstract □ Preparation of the *cis*- and *trans*-4-hydroxy-3-trimethylammoniumtetrahydrofuran and 4-acetoxy-3-trimethylammoniumtetrahydrofuran halides and 3-trimethylammoniumtetrahydrofuran iodide is described. Weak muscarinic activity was noted for the unsubstituted 3-trimethylammoniumtetrahydrofuran salt, being about 1000-fold less potent than acetylcholine. The *trans*-hydroxy and *trans*-acetoxy compounds showed even less activity, and the *cis*-compounds were inactive.

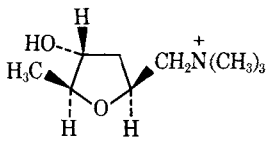
Keyphrases □ 3-Trimethylammoniumtetrahydrofuran halides, 4-substituted—muscarinic receptors, synthesis, pharmacologic testing □ Muscarinic receptors—3-trimethylammoniumtetrahydrofuran halides, 4-substituted, synthesis, pharmacologic testing

The fundamental problem of relating molecular structure to biological activity at various drug receptors becomes extremely difficult when considering small conformationally mobile molecules such as acetylcholine. Regardless of the existence of preferred conformations in the solid state and in solution (1–5), it should not be assumed that these conformations are those in the drug–receptor surface complex (6).

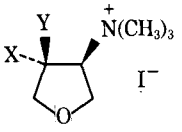
Various conformationally rigid or semirigid cholinergic agents have been prepared to aid in determination of the architectural features of the drug–receptor complex on various cholinergic sites, *e.g.*, muscarinic, nicotinic, and acetylcholinesterase (7–12). Attempts which incorporate the least number of additional atoms have generally been most successful, although comparisons of closely related compounds in higher series also seem valid (12).

In this study, various analogs of 3-trimethylammoniumtetrahydrofuran iodide were prepared. This system

offered the opportunity to look at possible analogs of muscarine, in which a conformation similar to the one in which the trimethylammonium head is directed toward C-4 of muscarine is represented by attaching this radical at C-3 in tetrahydrofuran (Structure I). In these com-



muscarine



I

1. X = Y = H
2. X = OH Y = H
3. X = OAc Y = H
4. X = H Y = OH
5. X = H Y = OAc

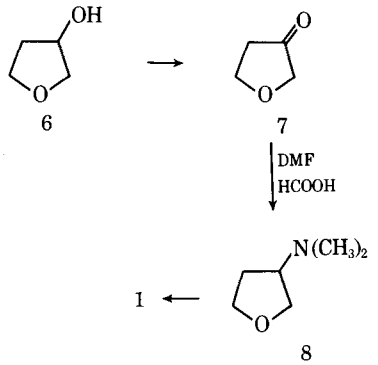
pounds, the distance of the two-carbon separation of oxygen from nitrogen is similar to that in the *transoid*-conformation of acetylcholine (ACh).

In addition, the *cis*- and *trans*-3-acetoxy (and hydroxy) analogs were prepared as conformational analogs of ACh in which the acetoxy oxygen and trimethylammonium nitrogen are fixed distances apart, similar to totally eclipsed and eclipsed conformations of ACh. These compounds can also be related to the acetyl-α,β-dimethylcholines held in fixed conformations, with the methyl groups attached to each other through an ether bridge.

Also, the 4-hydroxy compounds could show some structural analogy to muscarine, if the other speculation concerning the position of the trimethylammonium groups proved to be true.

SYNTHESIS

Preparation of Compound 1 (Scheme I) was accomplished from

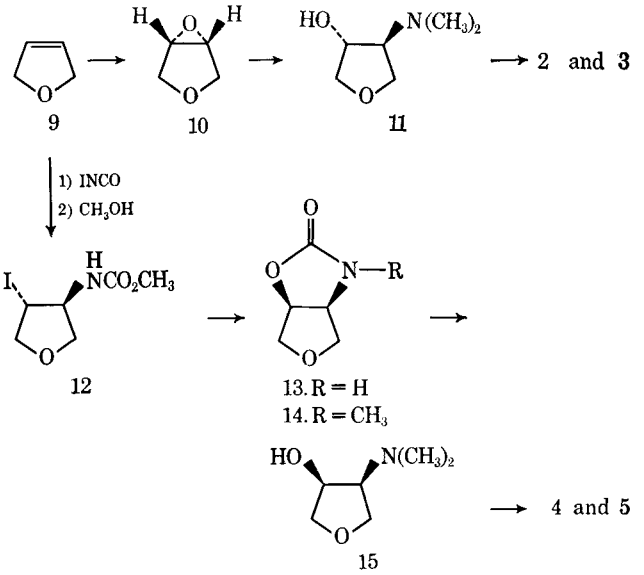


Scheme I

3-tetrahydrofuranone (Compound 7) which was readily prepared from 1,2,4-butanetriol (13). Dehydration afforded Compound 6, which was oxidized to Compound 7 (14).

Initially, it was planned to convert the oxime of ketone 7 to 3-aminotetrahydrofuran, as reported by Korobitsyna *et al.* (15). In the present study, the reported solid oxime could not be isolated, and the sodium amalgam reduction of the crude oxime failed to produce material that could be proven to be the desired amine. However, direct conversion of ketone 7 to Compound 8 was accomplished using Bach's modification (16) of the Leuckart reductive alkylation procedure, using dimethylformamide and 90% formic acid (17).

The 3,4-disubstituted analogs of tetrahydrofuran were prepared by stereospecific routes from 2,5-dihydrofuran (Scheme II). Epoxi-

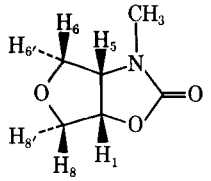


Scheme II

dation of Compound 9 at 0° provided 3,6-dioxabicyclo[3.1.0]heptane (Compound 10), which was treated with dimethylamine to yield *trans*-3-hydroxy-4-dimethylaminotetrahydrofuran (Compound 11). Compound 2 was prepared from Compound 11 by quaternization with iodomethane, and Compound 3 by acetylation followed by quaternization.

Formation of Compounds 4 and 5 was accomplished from *trans*-3-carbomethoxyamino-4-iodotetrahydrofuran (Compound 12), which was prepared by addition of iodine isocyanate to Compound 9, followed by methanolysis of the intermediate isocyanate. Pyrolysis (130°) of Compound 12 produced 2,7-dioxa-4-azabicyclo[3.3.0]-octan-2-one (Compound 13), which was alkylated by formation of the sodium salt of Compound 13 and dimethyl sulfate. Other alkylation procedures, including sodium hydride–iodomethane, and utilization of other bases, *e.g.*, sodium hydroxide and sodium carbonate, failed.

The stereochemistry of these tetrahydrofuran-fused oxazolidinones, although not in question from the synthetic scheme, is readily assigned from their NMR spectra. This is most apparent in the spectrum of the *N*-methylated derivative (Compound 14) (Structure II).



II

The signals for H-1 and H-5 are quartets at 5.05 and 4.28 δ, respectively, with $J_{1,5} = 7$ c.p.s., $J_{5,6} = J_{1,8} = 4$ c.p.s. Signals for protons H-6 and H-8 were observed as overlapping quartets at 3.42 and 3.58 δ, $J_{gem} = 10$ c.p.s. The downfield signal was assigned to H-8 on the basis of decoupling experiments. Upon irradiation at the frequency of H-1, H-8 collapsed into a doublet, showing J_{gem} only. Protons H-6′ and H-8′ apparently have almost identical chemical shifts. A slightly broadened doublet at 4.10 δ, J_{gem} = 10 c.p.s., was observed integrating for two protons.

Lithium aluminum hydride reduction of Compound 14 produced *cis*-amino alcohol (Compound 15), which was converted to Compounds 4 and 5 in steps analogous to conversion of Compound 11 to 2 and 3.

PHARMACOLOGY

Muscarinic assays were determined using strips of guinea pig ileum in oxygenated Tyrode's solution. 3-Trimethylammonium iodide (Compound 1) showed significant muscarinic activity, being

active at 1×10^{-4} *M*, equivalent to 1×10^{-7} *M* acetylcholine, or 1000-fold less potent, but $\alpha = 1.0$. The *trans*-compounds, 2 and 3, showed activity at 5×10^{-3} *M* ($\alpha = 1.0$). The *cis*-compounds, 4 and 5, were inactive at concentrations up to 10^{-2} *M*. The agonist effects were not blocked by hexamethonium, indicating the observed effects are postganglionic parasympathetic in origin. No atropinelike effects were observed for the compounds tested.

The marginal activity of the former compounds, 1, 2, and 3, can be rationalized in terms of a maximal O→N$^+$ distance, similar to the 1,3-dioxolanes (18) and calculations on muscarine (19), or the extended conformation of acetylcholine (7, 11, 12). However, the low level of activity precludes worthwhile speculation.

EXPERIMENTAL

Melting points were obtained with a calibrated Thomas-Hoover Unimelt apparatus and are corrected. IR data were recorded on Beckman IR-5A and IR-8 spectrophotometers. NMR spectra were determined with Varian A-60 and T-60 spectrometers, using tetramethylsilane (TMS) or 3-trimethylsilyl-1-propanesulfonic acid sodium salt as the internal standard. Microanalyses were conducted by Drs. G. Weiler and F. B. Strauss, Oxford, England.

3-Tetrahydrofuranone (Compound 7)—A mixture of 100 g. (0.94 mole) of 1,2,4-butanetriol and 2.0 g. (0.01 mole) of *p*-toluenesulfonic acid monohydrate was distilled, using a water aspirator at 100–120°, to afford about 100 g. of distillate in 2–3 hr. (13). The product, a mixture of water and 3-hydroxytetrahydrofuran, was not separated but subjected to oxidation as described by Yurev *et al.* (14).

To a cold solution (−10°) of 100 g. of the crude distillate of Compound 6 in 150 ml. of ether was added dropwise a cold (0°) solution containing 100 g. (0.33 mole) of sodium dichromate dihydrate, 120 g. (0.12 mole) of concentrated sulfuric acid, and 150 ml. of water. The stirred solution was kept cold (−10 to 0°) for 5–6 hr. and then overnight at room temperature. The mixture was partitioned between ether and water, and the aqueous layer was extracted four additional times with ether. The combined ether layers were dried ($MgSO_4$), and solvent was removed, affording 42.1 g. (52% in two steps) of a slightly yellow oil; IR, 3.37, 3.47 (C—H stretching), 5.72 μ (C=O stretching); NMR ($CDCl_3$) δ: 4.23 (triplet, $J = 7$ c.p.s., C-5 protons), 3.83 (singlet, C-2 protons), 2.47 (triplet, $J = 7$ c.p.s., C-4 protons).

3-Dimethylaminotetrahydrofuran (Compound 8)—In a glass-lined autoclave were placed 6.00 g. (70 mmoles) of Compound 7, 10.0 g. (210 mmoles) of 90.9% formic acid, and 15.3 g. (210 mmoles) of dimethylformamide. The autoclave was sealed and heated at 185° for 16 hr., cooled to room temperature, and opened; the contents were then removed. The crude reaction mixture was acidified with 40 ml. of aqueous 10% HCl and washed with several portions of ether. The aqueous acid extract was made alkaline with aqueous 2 *N* NaOH and extracted with ether. The combined organic extracts were dried ($MgSO_4$) and evaporated, affording a colorless oil which was converted to the quaternary ammonium salt without further purification.

3-Trimethylammoniumtetrahydrofuran Iodide (Compound 1)—To a cold (0°) solution of 2.88 g. (25 mmoles) of crude Compound 8 in 10 ml. of acetone was added 9.90 g. (70 mmoles) of iodomethane. After 12 hr., the slightly yellow crystalline solid was collected and recrystallized from methanol–ether, affording 1.50 g. (23% of theory) of Compound 1, m.p. 235–236°, NMR (CD_3OD) δ: 3.06 [singlet, $\overset{+}{N}(CH_3)_3$ protons], overlapping multiplets centered at 3.65, 4.20, and 4.37 integrating for the five aliphatic protons, 2.43 (multiplet, $W_h = 14$ c.p.s., C-4 methine protons).

Anal.—Calcd. for $C_7H_{16}INO$: C, 32.70; H, 6.27; N, 5.45. Found: C, 32.86; H, 6.33; N, 5.66.

3,6-Dioxabicyclo[3.1.0]heptane (Compound 10)—To a cold (0°) solution of 34.0 g. (0.186 mole) of *m*-chloroperbenzoic acid in 400 ml. of chloroform was added carefully 12.6 g. (0.18 mole) of 2,5-dihydrofuran (Compound 9) in 100 ml. of chloroform. The solution was maintained at 0° for 18 hr. with constant stirring; it was then extracted with cold aqueous 5% NaOH, washed with H_2O, and dried ($MgSO_4$). The solvent was removed utilizing a rotary evaporator, affording 10.0 g. (65%) of a slightly yellow, thin oil which was used without further purification.

***trans*-4-Hydroxy-3-dimethylaminotetrahydrofuran (Compound 11)**—To a cold (0°) 150-ml. capacity, steel autoclave chamber were added 5.0 g. (0.058 mole) of Compound 10 and 26.2 g. (0.58 mole) of anhydrous dimethylamine; 50 ml. of benzene was also added as solvent. The autoclave was sealed and heated at 100° overnight. Initial pressure on the gauge was 100 p.s.i. After cooling to 0°, the autoclave was opened and the contents were removed. The solvent was evaporated and the residual oil distilled, affording 6.08 g. (80%) of a slightly yellow oil, b.p. 82° (1.2 mm.). The product was carried to the next reaction without further purification.

***trans*-4-Hydroxy-3-trimethylammoniumtetrahydrofuran Iodide (Compound 2)**—This compound was prepared by a method similar to that used with Compound 1, using ethyl acetate as solvent. An analytical sample was prepared by crystallization from methanol–ethyl acetate, m.p. 194–195°; NMR (D_2O) δ: 3.22 [singlet, $\overset{+}{N}(CH_3)_3$], 3.60 (quartet, $J = 6$ c.p.s., C-3 methine proton), 4.25 (multiplet, W_h about 18 c.p.s., five protons).

Anal.—Calcd. for $C_7H_{16}INO_2$: C, 30.78; H, 5.90; N, 5.13. Found: C, 30.79; H, 5.86; N, 5.15.

***trans*-4-Acetoxy-3-trimethylammoniumtetrahydrofuran Iodide (Compound 3)**—To 2.00 g. (0.015 mole) of *trans*-4-hydroxy-3-dimethylaminotetrahydrofuran (Compound 11) was added 20 ml. of acetic anhydride. After heating the mixture at reflux for 1 hr., excess acetic anhydride and the by-product acetic acid were removed. Aqueous 3% HCl, 100 ml., was added to the residual oil and allowed to stand for 1 hr. The aqueous mixture was washed with ether, made alkaline with aqueous 10% NaOH, and extracted four times with ether. The combined ether extracts were dried ($MgSO_4$) and the solvent removed, affording 1.95 g. (75%) of a light-yellow oil.

A solution of 1.00 g. (5.7 mmoles) of the crude amino ester in 75 ml. of ethyl acetate and 5 ml. of iodomethane was stoppered and shaken occasionally for 1 hr. A white precipitate formed and was removed by filtration, affording 1.82 g. (100%) slightly yellow solid. An analytical sample was prepared by crystallization from methanol–ethyl acetate, m.p. 231°.

Anal.—Calcd. for $C_9H_{18}INO_3$: C, 34.29; H, 5.71. Found: C, 34.73; H, 5.71.

The chloride salt of Compound 3 was prepared from the quaternary ammonium salt, Compound 2, which was first converted to the chloride salt by ion exchange, followed by acetylation, m.p. 185°.

Anal.—Calcd. for $C_9H_{18}ClNO_3$: C, 48.78; H, 8.11. Found: C, 48.49; H, 8.17.

***trans*-3-Iodo-4-carbomethoxyaminotetrahydrofuran (Compound 12)**—To a cold (−15°) solution of 12.6 g. (0.18 mole) of 2,5-dihydrofuran in 540 ml. of anhydrous ether were added 59.9 g. (0.40 mole) of freshly prepared silver cyanate and 45.7 g. (0.18 mole) of iodine. The stirred mixture was allowed to warm to room temperature over a period of 6 hr. After this time, the initially red solution had become a yellow slurry. The inorganic salts (silver iodide and excess silver cyanate) were removed by filtration. Anhydrous methanol, 50 ml., was added to the filtrate, and the resulting mixture was refluxed overnight. After removing the organic solvents utilizing a rotary evaporator, the residual dark-brown oil was dissolved in ether, washed with 10% aqueous sodium bisulfite, with water, dried ($MgSO_4$), and evaporated, affording 24.4 g. (50% in two steps) of a yellow solid. An analytical sample was prepared by crystallization from ether, m.p. 107–108°; IR (potassium bromide), 3.08 (N—H), 3.40, 3.50 (C—H stretching), 5.98 (C=O stretching).

Anal.—Calcd. for $C_6H_{10}INO_3$: C, 26.59; H, 3.72; N, 5.17. Found: C, 26.65; H, 3.75; N, 5.14.

2,7-Dioxa-4-azabicyclo[3.3.0]octan-3-one (Compound 13)—A stream of dry nitrogen was passed over 5.0 g. (18.4 mmoles) of *trans*-3-iodo-4-carbomethoxyaminotetrahydrofuran and bubbled through a mixture of benzene and pyridine while heating the solid in an oil bath at a temperature of 130°. When no additional pyridine methiodide formed, the reaction was stopped (about 45 min.), affording 2.0 g. (83.3%) of a brown oil which solidified at room temperature. An analytical sample was prepared by crystallization from methanol–ethyl acetate, m.p. 116–117°, IR (potassium bromide), 3.08 (N—H), 3.35, 3.50 (C—H stretching), 5.77 (C=O). NMR (CD_3OD) δ: 5.17 (quartet, $J = 4$ and 7 c.p.s., methine proton at C-1), 4.33 (quartet, $J = 4$ and 7 c.p.s., methine proton at C-5), 4.40 (broad multiplet, N—H proton), 3.93 (doublet, $J_{gem} = 11$ c.p.s., one proton at C-6 and at C-8), 3.53 and 3.47 (overlapping quartets giving the appearance of a doublet of triplets, $J = 4$ and 11 c.p.s. in each case, one proton at C-6 and at C-8).

Anal.—Calcd. for $C_5H_7NO_3$: C, 46.51; H, 5.47; N, 10.86. Found: C, 46.61; H, 5.64; N, 11.10.

4-Methyl-2,7-dioxa-4-azabicyclo[3.3.0]octan-3-one (Compound 14)—A solution of sodium methoxide was prepared from 2.0 g. (0.087 g. atom) of sodium and 150 ml. of anhydrous methanol. After the addition of 5.0 g. (0.04 mole) of Compound 13, the mixture was evaporated to dryness *in vacuo*. The residue was suspended in 200 ml. of dry toluene, and 10 ml. of freshly distilled dimethyl sulfate was added dropwise with stirring and cooling. The reaction was then heated at 100° for 1 hr. An equal amount of H_2O was added to the toluene solution to destroy any excess dimethyl sulfate, and the mixture was partitioned. The aqueous solution was then extracted with five portions of chloroform. The combined chloroform and toluene extracts were dried ($MgSO_4$) and evaporated, affording 4.67 g. (85%) of a slightly yellow oil which solidified on cooling. An analytical sample was prepared by crystallization from chloroform–hexane, m.p. 80°. NMR ($CDCl_3$); δ 2.88 (N—CH_3, singlet), 3.42 and 3.58 (two overlapping quartets, J = 4 and 10 c.p.s. integrating for two protons tentatively assigned to one proton at C-8 and one proton at C-6). The downfield quartet was assigned to the C-8 proton, because upon irradiation of the C-5 proton the 3.58 δ signal collapsed into a doublet, J_{gem} = 10 c.p.s. Similarly upon irradiation in the 3.4–3.5 region, the C-1 proton collapsed into a singlet $J_{1,5}$ = 7 c.p.s., 4.10 (broadened doublet, J_{gem} = about 10 c.p.s., assigned to one proton at C-8 and one at C-6 which show coupling constants with protons at C-1 and C-5, respectively, approaching zero), 4.28 (quartet partially obscured by doublet, J = 4 and 7 c.p.s. assigned to other protons at C-6 and C-8), 5.05 (quartet, J = 4 and 7 c.p.s., assigned to proton at C-1).

Anal.—Calcd. for $C_6H_9NO_3$: C, 50.34; H, 6.34; N, 9.78. Found: C, 49.97; H, 6.36; N, 9.99.

***cis*-4-Hydroxy-3-dimethylaminotetrahydrofuran (Compound 15)**—All equipment was dried at 120° overnight. A stream of dry nitrogen was passed over a suspension of 2.0 g. (52 mmoles) of lithium aluminum hydride in 200 ml. of anhydrous tetrahydrofuran. Two grams (14 mmoles) of Compound 14 in 50 ml. of tetrahydrofuran was added dropwise over a period of 10 min., and the resulting slurry was refluxed overnight. Excess hydride was destroyed by addition of aqueous 40% Rochelle salt, and the mixture was filtered with suction through a diatomaceous earth[1] pad. The filter cake was extracted four times with ethyl acetate. The combined tetrahydrofuran and ethyl acetate portions were evaporated, affording 1.18 g. (65%) of a slightly yellow oil which turned into a solid upon cooling. An analytical sample was prepared by crystallization from ethyl acetate, m.p. 51°.

Anal.—Calcd. for $C_6H_{13}NO_2$: C, 54.94; H, 9.90; N, 10.68. Found: C, 54.87; H, 9.68; N, 10.92.

***cis*-4-Hydroxy-3-trimethylammoniumtetrahydrofuran Iodide (Compound 4)**—This compound was prepared by a method similar to that used with Compound 1 using ethyl acetate as solvent. An analytical sample was prepared by crystallization from methanol–ethyl acetate, m.p. 162°.

Anal.—Calcd. for $C_7H_{16}INO_2$: C, 30.78; H, 5.90; N, 5.13. Found: C, 31.00; H, 5.78; N, 5.19.

[1] Celite, Johns-Manville, N. Y.

***cis*-4-Acetoxy-3-trimethylammoniumtetrahydrofuran Iodide (Compound 5)**—This compound was prepared from Compound 15 by a method similar to the conversion of Compound 11 to Compound 3. An analytical sample was prepared by crystallization from methanol–ethyl acetate, m.p. 193°.

Anal.—Calcd. for $C_9H_{18}INO_3$: C, 34.29; N, 4.44; H, 5.71. Found: C, 34.48; N, 4.38; H, 5.71.

REFERENCES

(1) H. Sörum, *Acta Chem. Scand.*, **13**, 345(1959).

(2) J. F. Dunitz, *ibid.*, **17**, 1471(1963).

(3) F. G. Canepa, *Nature*, **207**, 1152(1965).

(4) F. G. Canepa, P. Pauling, and H. Sörum, *ibid.*, **210**, 907 (1966).

(5) C. C. J. Culvenor and N. S. Ham, *Chem. Commun.*, **1966**, 537.

(6) P. Pauling, in "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Eds., W. H. Freeman, San Francisco, Calif., 1968, pp. 555–565.

(7) M. Martin-Smith, G. A. Smail, and J. B. Stenlake, *J. Pharm. Pharmacol.*, **19**, 565(1967).

(8) B. R. Barlow, "Introduction to Chemical Pharmacology," 2nd ed., Wiley, New York, N. Y., 1964.

(9) D. R. Garrison, M. May, H. F. Ridley, and D. J. Triggle, *J. Med. Chem.*, **12**, 130(1969).

(10) M. Friedman, in "Drugs Affecting the Peripheral Nervous System," A. Burger, Ed., Marcel-Dekker, New York, N. Y., 1967, pp. 79–132.

(11) C. Y. Chiou, J. P. Long, J. G. Cannon, and P. D. Armstrong, *J. Pharmacol. Exp. Ther.*, **166**, 243(1969).

(12) E. E. Smissman, W. L. Nelson, J. B. Lapidus, and J. L. Day, *J. Med. Chem.*, **9**, 456(1966).

(13) H. Wynberg, *J. Amer. Chem. Soc.*, **80**, 364(1958).

(14) Y. K. Yurev, E. M. Lukina, and I. K. Korobitsyna, *J. Gen. Chem. USSR*, **24**, 1225(1954).

(15) I. K. Korobitsyna, Y. K. Yurev, and E. M. Lukina, *ibid.*, **25**, 531(1955).

(16) R. D. Bach, *J. Org. Chem.*, **33**, 1647(1968).

(17) M. L. Moore, *Org. React.*, **5**, 301(1949).

(18) H. F. Ridley, S. S. Chatterjee, J. F. Moran, and D. J. Triggle, *J. Med. Chem.*, **12**, 931(1969).

(19) L. B. Kier, *Mol. Pharmacol.*, **3**, 487(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 1, 1970, from the *College of Pharmacy and Department of Pharmacology, University of Washington, Seattle, WA 98105*

Accepted for publication May 28, 1970.

This investigation was supported in part by Grant NB-08121 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

* NSF Undergraduate Research Participant, 1967–1968.

† NSF Undergraduate Research Participant, 1968–1969.

Critical Evaluation of Extraction Procedures in Determination of Atropine and Scopolamine from *Datura* Powder

MARK J. SOLOMON* and FRANK A. CRANE

Abstract □ The simultaneous quantitative extraction and determination of atropine and scopolamine from *Datura* powder has been performed. This combination procedure afforded relative precisions of 5.11 and 3.85% for atropine and scopolamine with relative accuracies of −3.02 and −1.47%, respectively. Duplicate determinations of duplicate extractions over a 2-month period indicated that for atropine, 23.92% of the variation was attributable to the determination procedure and 76.08% was attributable to the extraction method. For scopolamine, 29.09% of the variation was due to the determination procedure and 70.91% to the extraction method. Comparisons of the USP percolation, continuous extraction procedure, and differential solvent extraction procedures are given. A theoretical discussion of total variation in quantitative methods in relation to extraction and determination is developed. Attempts to identify an unknown peak related to scopolamine are reported.

Keyphrases □ Atropine, scopolamine, extraction from *Datura* powder—comparison, evaluation of various procedures □ Scopolamine, atropine, extraction from *Datura* powder—comparison, evaluation of various procedures □ *Datura* powder—extraction of atropine, scopolamine, comparison, evaluation of methods

The work of Brochmann-Hanssen and Svendsen (1) led Solomon *et al.* (2) to attempt the GLC determination of atropine and scopolamine from solutions and plant powders. This method provided good accuracy and precision, although dehydration products of atropine and scopolamine were formed. A minor GLC peak attributable to scopolamine was not determined. Wu Chu and Mika (3) are presently studying several substances as internal standards for this partition chromatographic method. Recently, Zimmerer and Grady (4, 5) reported a GLC and spectrophotometric assay for hyoscyamine sulfate, atropine sulfate, scopolamine bromide, and phenobarbital tablets and elixir. This method utilized homatropine as an internal standard.

Wu Chu *et al.* (6) reported an extraction procedure for atropine and scopolamine, utilizing a photodensitometric determination method adapted to the quantitation of plant powders. No attempt was made, however, to combine the extraction method with the GLC method of Solomon *et al.* (2).

Some initial considerations of the problems of combining an extraction and determination procedure indicated that a good quantitative method must not only have good accuracy and precision, but the extraction procedure must have a variation within the range of that of the determination method. Upon closer examination, if: s_q^2 = sampling variation in the total quantitation method, s_d^2 = sampling variation in the determination method, s_e^2 = sampling variation in the extraction method, and

$$s^2 = \sum \frac{(\bar{x} - x)^2}{N} = \sum \frac{(\text{expectancy of } x - \text{value of } x)^2}{\text{number of samples studied}} \quad \text{(Eq. 1)}$$

Table I—Overall Precision and Accuracy of Combined Extraction and Determination of a Standard Powder

Statistic	Alkaloid	
	Atropine	Scopolamine
$\bar{x}$, mg. %	21.87	6.81
95% $CL_{\bar{x}}$, mg. %[a]	21.60–22.14	6.78–6.87
$RSD \times 100\%$[b]	5.12	3.85
Absolute accuracy,[c] mg.	−0.66	−0.10
Relative accuracy[d]	−3.02	−1.47
N	64	64

[a] The mean will occur within these limits in 19 of 20 determinations. [b] Relative standard deviation = $SD/\bar{x} \times 100\%$. [c] Calculated as deviations from the median. [d] Relative accuracy = *absolute accuracy* $\times 100\%/\bar{x}$.

where $\bar{x}_q$ = expectancy of the total quantitation method, $\bar{x}_d$ = expectancy of the determination method, and $\bar{x}_e$ = expectancy of the extraction method, it can be shown that

$$s_q^2 = s_d^2 + s_e^2 \quad \text{(Eq. 2)}$$

Thus,

$$s_e^2 = s_q^2 - s_d^2 \quad \text{(Eq. 3)}$$

For n samples, where n is small enough to be corrected for the small sample bias, after expansion and subsequent simplification, Eq. 3 can be written as

$$\sum_{n=2}^{n=n} \frac{(\bar{x}_q + \bar{x}_d - 2x_n)(\bar{x}_q - \bar{x}_d)}{n-1} = \sum_{n=2}^{n=n} \frac{(\bar{x}_e - x_n)^2}{n-1} \quad \text{(Eq. 4)}$$

If the determination method is vastly improved without improving the extraction procedure,

$$\lim_{n \to N} (\bar{x}_d - x_n) = 0, \qquad \text{where } N \gg n \quad \text{(Eq. 5)}$$

By simplifying Eq. 4,

$$\sum_{2}^{N} \frac{(\bar{x}_q - x_n)^2}{N} = \sum_{2}^{N} \frac{(\bar{x}_e - x_n)^2}{N} \quad \text{(Eq. 6)}$$

Thus, an excellent determination procedure coupled with a relatively coarse extraction procedure will produce a quantitative method in which the variation of the extraction procedure is measured with excellent precision and accuracy. Consideration of this problem along with a study of the variation in extraction of *Datura* alkaloids in relation to an established determination method is the purpose of this paper.

MATERIALS AND METHODS

Standard Powder—A standard powder was prepared from the leaf laminae (petiole removed) of 144 DS 2 (7) field-grown plants of

Table II—Contribution of Extraction and Determination to Total Variation[a] ($s_q^2 = s_d^2 + s_e^2$)

Source of Variation	DF	Atropine[b] F	Atropine[b] P	Atropine[b] % Contribution to Total MS[d]	Scopolamine[c] F	Scopolamine[c] P	Scopolamine[c] % Contribution to Total MS
Extraction							
Between months	1	1.6630	n.s.	48.52	0.4249	n.s.	12.36
Between weeks within months	2	0.2450	n.s.	7.15	1.6617	n.s.	48.34
Between days within weeks	4	0.1272	n.s.	3.71	0.2382	n.s.	6.93
Between extractions within days	8	0.5720	n.s.	16.69	0.1130	n.s.	3.29
Determination							
Within	16	Error term	—	23.92	Error term	—	29.09
Total	31	—	—	99.99	—	—	100.01

[a] Modified analysis of variance table. [b] Calculated as $y = (0.1784x + 1.8600)/w \times 100\%$, where y = milligrams percent of atropine, x = integrator counts adjusted to 200 atten., and w = weight (grams) of powder extracted. [c] Calculated as $y = (0.2472x + 0.1200)/w \times 100\%$, where y = milligrams percent of scopolamine, x = integrator counts adjusted to 200 atten., and w = weight (grams) of powder extracted. [d] Mean square of variable/sum of all mean squares × 100%.

Datura stramonium, forced-air dried at 50°, and ground to a 40-mesh powder.

Extraction Method—Ten grams of the standard powder was extracted by the method of Wu Chu *et al.* (6), scaled up from 4 g., and finally washed to volume in a 5-ml. rather than 10-ml. volumetric flask. Unless otherwise stated, this extraction method was referred to as the "differential solvent extraction method." The USP XVII (8) methods of percolation and continuous extraction procedure were utilized as stated, except for the addition of washed sand as a bulking agent. All continuous extraction procedures were run for 8 hr. to ensure exhaustive extraction.

Determination Procedures—Two-microliter samples of the final extract, brought to volume in a 5-ml. volumetric flask, were quantitated according to the GLC procedure of Solomon *et al.* (2).

Materials—All chemicals used for the chromatographic portion of this paper were analytical reagent grade. Atropine and scopolamine[1] and scopoline[2] produced a single spot on TLC. Silane-treated glass wool[3] was used (9).

Aposcopolamine Synthesis—Aposcopolamine was unavailable commercially, so it was synthesized according to the method of Willstätter and Hug (10). The resulting product was recovered as long needles (m.p. 96.5–97.0°), and the identity was confirmed by NMR and mass spectrometry.

Experimental Designs—The precision and accuracy study (Table I) was run on 64 independent extractions. The study of variation with time (Table II) represents the effect of heirarchal or nested time levels on the variation of the total quantitative method. The USP XVII percolation and continuous extraction procedures, as stated in the Belladonna Leaf Monograph, were compared (Table III), and these procedures were then compared to the differential solvent extraction method in a 3 × 3 Latin square design (11). All precautions for randomization were taken.

RESULTS AND DISCUSSION

The use of silane-treated glass wool to hold column contents in place decreased the formation of dehydration products but did not eliminate them entirely.

Attempts to identify the minor peak associated with scopolamine occurring at retention time 14.5 min. (236°), as previously reported (2), were not successful. Injection of pure scopoline, the compound considered as the most likely degradation product, afforded a single peak with a retention time 3 min. 24 sec. (170°). Mass spectral analysis of a collection of the effluent at 14.5 min. (236°) retention time for a combined running time of 20 hr. was inconclusive, because only two peaks were obtained (*m/e* 263 and 240). The placement of the scopoline GLC peak and the fact that fragments of at least *m/e* 263 could be obtained from the unknown compound did indicate, however, that the compound was probably not scopoline.

Since collection of the compound was not possible with the system available, it was hypothesized that dehydration of scopolamine was occurring similar to the formation of apoatropine from atropine. Synthetic apoatropine was prepared (parent ion *m/e* 285 representing 72% of the base peak of *m/e* 94 in the mass spectrum), but upon injection it afforded a single peak at retention time 16 min. 54 sec. The mass spectral studies of aposcopolamine indicated no fragments between *m/e* 285 (parent ion) and *m/e* 154. Thus, the partial spectrum of the unknown compound and the spectrum of the aposcopolamine definitely appeared dissimilar. The partition pattern of scopolamine and its dehydration product aposcopolamine was parallel to the partition pattern of atropine and apoatropine. Both dehydration products were detected about 1 min. before the parent compound on the methylsilicone gum on a silanized diatomite system. The unknown compound, however, had a retention time of 3.5 min. less than the parent scopolamine.

The differential extraction method was very satisfactory for combination with GLC analysis. The photodensitometric determination method (6) originally reported with this extraction procedure was not as accurate or precise as the GLC method. However, it had the advantage that when the alkaloid was in low concentration, more of the final chloroform extract could be applied to the TLC plate without too much danger of overloading. The GLC determination method was more accurate and precise but dilute solutions required injection of more than 5 μl., which produced tremendous tailing and even occasional extinction of the flame.

Examination of Table I indicates that the overall relative precision of the combined extraction and determination of the standard powder was 5.12 and 3.85% with relative accuracies of −3.02 and −1.47% for atropine and scopolamine, respectively. The 95% confidence limits of the mean (95% $CL_{\bar{x}}$) indicated that these data were repeatable.

The contribution of the combined extraction and determination components over a 2-month period was examined (Table II). The variation between months or between weeks within months represented the largest component of variation for both atropine and scopolamine, although the components of variation between months, between weeks within months, between extraction sets within weeks (between days within weeks), and between extractions within days were all nonsignificant for both alkaloids quantitated using the determination variance component as the error term. Further examination indicated that 23.92% of the variation for atropine and 29.09% of the variation for scopolamine were attributable to the determination method, while 76.08% of the variation for atropine and 70.91% of the variation for scopolamine were functions of the extraction method. These analyses indicated that the extraction and determination methods were comparable; even though the extraction method was more variable than the determination method, the difference was not so extensive that the

[1] Aldrich Chemical Co.
[2] K & K Laboratories.
[3] Applied Science Laboratories.

Table III—Comparison of Continuous Extraction, Percolation, and Differential Solvent Extraction Procedures[a]

Source of Variation	DF	Atropine[b] F	Atropine[b] P	Scopolamine[c] F	Scopolamine[c] P
Between treatments	2				
Continuous extraction *vs.* percolation	1	17.174	<0.001	30.96	<0.001
Continuous extraction and percolation *vs.* differential solvent	1	191.450	<0.001	39.85	<0.001
Between days	2	2.553	<0.01	<1	n.s.
Between replicates	2	1.256	n.s.	<1	n.s.
Treatment *vs.* days	4				
Continuous extraction *vs.* days	1	2.86	<0.05	<1	n.s.
Percolation *vs.* days	1	12.69	<0.001	4.57	<0.05
Differential solvent *vs.* days	1	<1	n.s.	3.12	n.s.
Continuous extraction and percolation *vs.* days	1	27.65	<0.001	2.38	n.s.
Pooled error	43	Error term	Error term	Error term	Error term
Total	53	—	—	—	—

[a] 3 × 3 Latin square design with one structural restriction (11). [b] Milligram percent per gram dry weight of powder. [c] Milligram percent per gram dry weight of powder.

total variation could be considered a total function of either procedure. A comparison, however, of the USP XVII extraction methods with the differential solvent method was indicated.

Statistical analysis of the secondary and tertiary interactions for atropine and scopolamine (Table III) indicated that only the differential solvent method was nonsignificant over the days tested in this experiment. The continuous extraction ($p < 0.05$), percolation ($p < 0.001$), and continuous extraction and percolation combination ($p < 0.001$) methods varied over the period studied (3 randomized days). However, only the percolation method had a significant contribution for days when scopolamine was analyzed ($p < 0.05$). All the methods did not vary significantly between replicates (three) within days as would be expected. These data were interpreted to indicate that although the USP extraction methods may be satisfactory for total alkaloidal quantitation, they may be too variable for the present determination needs. Therefore, this would be an experimental example of the conclusion reached in Eq. 6.

When the orthogonal comparison of the combination of continuous and percolation extraction methods *versus* the differential solvent extraction method was considered, the $F_{1,43}$ value was highly significant for both alkaloids studied ($p < 0.001$). When the continuous extraction and percolation methods were compared, they were highly significantly different ($p < 0.001$) in this study, although USP XVII lists these methods as alternate extraction procedures.

SUMMARY

The combination of the differential solvent extraction and GLC determination methods produced an analytical procedure which was both precise and accurate. Analyses over a 2-month period indicated that time did not produce a significant variation in results. Comparison with USP XVII extraction methods indicated that the differential solvent extraction method was less variable. Comparison of the continuous extraction and percolation methods, listed as alternate quantitative extraction procedures in the belladonna leaf assay, demonstrated that these methods were not as satisfactory extraction procedures in this study as the differential solvent method.

REFERENCES

(1) E. Brochmann-Hanssen and A. B. Svendsen, *J. Pharm. Sci.*, **51,** 1095(1962).

(2) M. J. Solomon, F. A. Crane, B. L. Wu Chu, and E. S. Mika, *ibid.*, **58,** 264(1969).

(3) B. L. Wu Chu and E. S. Mika, unpublished data.

(4) R. O. Zimmerer and L. T. Grady, *J. Pharm. Sci.*, **59,** 87 (1970).

(5) "Gas-Chrom Newsletter," Applied Science Laboratories, 10/5(1969).

(6) B. L. Wu Chu, E. S. Mika, M. J. Solomon, and F. A. Crane, *J. Pharm. Sci.*, **58,** 1073(1969).

(7) M. J. Solomon and F. A. Crane, *Trans. Ill. Acad. Sci.*, **63,** 67(1970).

(8) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 62.

(9) F. E. Gainer and W. A. Arnett, *J. Pharm. Sci.*, **58,** 1548 (1969).

(10) R. Willstätter and E. Hug, *Physiol. Chem.*, **79,** 146(1912).

(11) G. W. Snedecor and W. G. Cochran, "Statistical Methods," 6th ed., Iowa State Press, Ames, Iowa, 1967.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Department of Pharmacognosy and Pharmacology, University of Illinois College of Pharmacy, Chicago, IL 60612*

Accepted for publication June 17, 1970.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This work was supported by a grant-in-aid from the Society of the Sigma Xi.

The authors thank Dr. Ludwig Bauer for his verification of their interpretation of the instrumental spectra utilized in this paper. The technical assistance of Kenneth King and Arthur Falk on the instrumental analyses was also appreciated.

* American Foundation for Pharmaceutical Education Fellow, 1969–1970. A.F.P.E. Pharmaceutical Manufacturers Association Fellow, 1970–1971.

First pKa Values of Some Acid-Base Indicators

V. DAS GUPTA and JOHN B. REED, Jr.*

Abstract □ An attempt was made to estimate the first pKa values of bromocresol green, bromocresol purple, bromophenol blue, cresol red, and phenol red using a method similar to that of Reilley and Sawyer. Values of −0.75 (−2.13 on the Hammett acidity scale) for bromocresol purple, +1.05 for cresol red, and +1.03 for phenol red were estimated. pKa values of cresol red and phenol red were not estimated on the Hammett acidity scale, since this scale is good only for acidic solutions stronger than 0.1 *M*. Trials to estimate the first pKa values of bromocresol green and bromophenol blue were not successful due to the upper limitation on the strength of commercially available hydrochloric acid. Values of −0.85 (approximately −2.7 on the Hammett acidity scale) or less for bromocresol green and −0.95 (−3.2 on the Hammett acidity scale) or less for bromophenol blue have been predicted.

Keyphrases □ Indicators, acid-base—first pKa determination □ pKa determination, first—acid-base indicators □ Dyes, organic—first pKa determination

Many organic dyes are used as acid-base indicators by scientists in a variety of analytical and industrial applications. Some of these dyes have also found use as diagnostic agents in medicine (1–4). Some of them are used in the quantitative determination of many pharmaceutical amines, since they form stable salts with this group of chemicals (5–8). These amine dye salts can be extracted with organic solvents and the dye component measured spectrophotometrically (9).

Preliminary investigations on bromothymol blue, reported by Gupta and Cadwallader (10), indicated that these dyes may have more than one pKa value. For example, the literature value of about 6.3 for bromocresol purple (11) may be the pKa_2 rather than the pKa_1 value.

The purpose of this research was to determine the first pKa values for: (*a*) bromocresol green (BCG); (*b*) bromocresol purple (BCP); (*c*) bromophenol blue (BPB); (*d*) cresol red (CR); and (*e*) phenol red (PR). The usefulness of this information is well documented. For example, without knowing the first pKa value, it is not possible to determine the true partition coefficients of these dyes.

EXPERIMENTAL

Reagents—All chemicals and reagents used were either USP, NF, or A.C.S. grade. All of the dyes were used without further purification.[1]

Preparation of Solutions—Each dye (50.0 mg.) was dissolved in 10 ml. of ethyl alcohol in a 100-ml. glass-stoppered volumetric flask, and sufficient distilled water was added to make a total volume of 100 ml. Solutions of lower dye concentration were prepared by diluting this stock solution with hydrochloric acid of various concentrations. The concentrations of the various dyes are reported in Table I. Hydrochloric acid USP was diluted with distilled water to obtain the dilutions needed.

Determination of pKa Values—The pKa values were determined according to the procedure of Reilley and Sawyer (12). Solutions of each dye in hydrochloric acid solutions of various concentrations were scanned between 360 and 600 mμ, using a Beckman DK2 spectrophotometer. Distilled water was used as the blank. Two wavelengths were chosen where the (H_2I) and (HI^-) forms of each dye showed a maximum difference in their absorbance. Plots of absorbance *versus* the theoretical pH value ($-\log H^+$) were prepared for each of the two wavelengths selected. Two horizontal lines across each of the plots were drawn, one corresponding to the absorbance of the dye solution having a theoretical pH value of −1.0 and the other corresponding to the absorbance of the dye solution having a theoretical pH value of −0.398 or higher.

Line A represents the absorbance of the dye, *i.e.*, the indicator dye represented as "I," when present entirely in the (H_2I) form. Line B represents the absorbance of the dye when present entirely in the (HI^-) form. The midpoint of each curve, between Lines A and B,

Table I—Concentrations of Various Dyes Used

Dye	Concentration, % w/v
BCG	0.001
BCP	0.001
BPB	0.001
CR	0.001[a]
	0.0005[a]
PR	0.001[a]
	0.0005[a]
	0.00025[a]

[a] More than one concentration was used to obtain readings within the scale of the spectrophotometer.

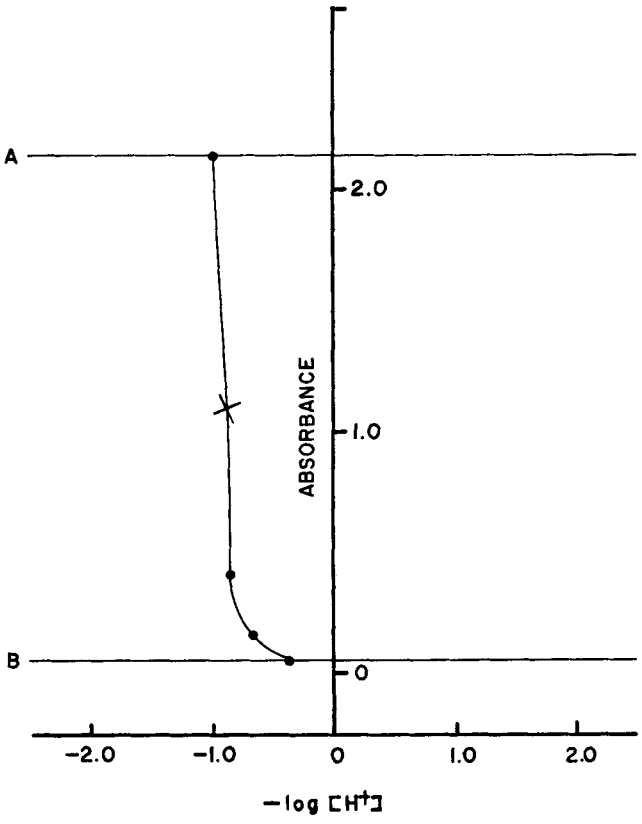


Figure 1—*Determination of the first pKa value of BCG using absorbance values at 564 mμ.*

[1] CR was manufactured by Allied Chemicals, and all other dyes were from Eastman Organic Chemicals.

Table II—First pKa Values of Various Dyes

Dye	First pKa Value	Average	First pKa Value Using Hammett Acidity Function	Average
BCP	−0.78		−2.15	
BCP	−0.73		−2.11	
BCP	−0.75		−2.12	
BCP	−0.75		−2.13	
		−0.75[a]		−2.13[a]
CR	1.08			
CR	0.97			
CR	1.10			
CR	1.05			
		1.05[a]		
PR	1.03			
PR	0.95			
PR	1.05			
PR	1.07			
		1.03[a]		

[a] Average of the four values.

represents the equal concentration of the (H_2I) and (HI^-) forms of each dye. This was marked as the midpoint on each curve. The results are presented in Table II. Figures 1–3 are presented as samples. In the case of BCP, the pKa values were also estimated using H_0 (Hammett acidity function) instead of theoretical pH. Results are presented in Table II. From these figures, the ratio of (HI^-) to (H_2I) at various pH values was obtained by measuring the relative distances of the point from the (HI^-) and (H_2I) lines. Plots of log (HI^-/H_2I) (vertically) *versus* theoretical pH were prepared for each of the two wavelengths selected. The point where the line crossed the pH axis corresponded to the pKa_1 value, since (H_2I) = (HI^-) + (H^+). The dissociation of (HI^-) to (I^{--}) and (H^+) can be neglected in the pH range of −1.0 to approximately 2.0, since K_2 values of these dyes range from 1×10^{-4} to 5×10^{-9}. The results are presented in Table II. Figure 4 is presented as a sample. In the case of BCP, the pKa values were also estimated using H_0 instead of pH. Results are presented in Table II.

DISCUSSION AND CONCLUSIONS

Bromocresol Green—Trials to estimate the first pKa value of this dye were not successful, since the difference between the absorbance readings at the theoretical pH values of −0.875 and −1.0 was so high (Fig. 1) that it was not possible to assume that the whole dye existed in the (H_2I) form at the lowest theoretical pH value of −1.0. It was not possible to prepare dye solutions of much lower theoretical pH values due to the upper limit on the strength of commercially available hydrochloric acid. From Fig. 1, it appears that the first pKa value of this dye is −0.85 (approximately −2.7 on the Hammett acidity scale) or less.

Bromocresol Purple—The first pKa value of this dye was estimated to be −0.75 or −2.13 on the Hammett acidity scale (Figs. 2 and 4 and Table II). The true pKa value may be somewhat lower, since the dye might not be totally in the (H_2I) form at the theoretical pH value of −1.0. This is due to the fact that the difference between the absorbance values at the theoretical pH values of −0.875 and −1.0 (Fig. 2) is not negligible.

Bromophenol Blue—Trials to estimate the first pKa value of this dye were not successful due to the reasons reported for BCG. From Fig. 3, it appears that the first pKa value of this dye is −0.95 (approximately −3.2 on the Hammett acidity scale) or less.

Cresol Red—The first pKa value of this dye was estimated to be +1.05 (Table II). This value appears to be correct, since a transition interval of +0.2 to +1.8 (red to yellow) for this dye has been reported (13). The pKa value of this dye was not estimated on the Hammett acidity scale, since this scale is good only for acidic solutions stronger than 0.1 *M*.

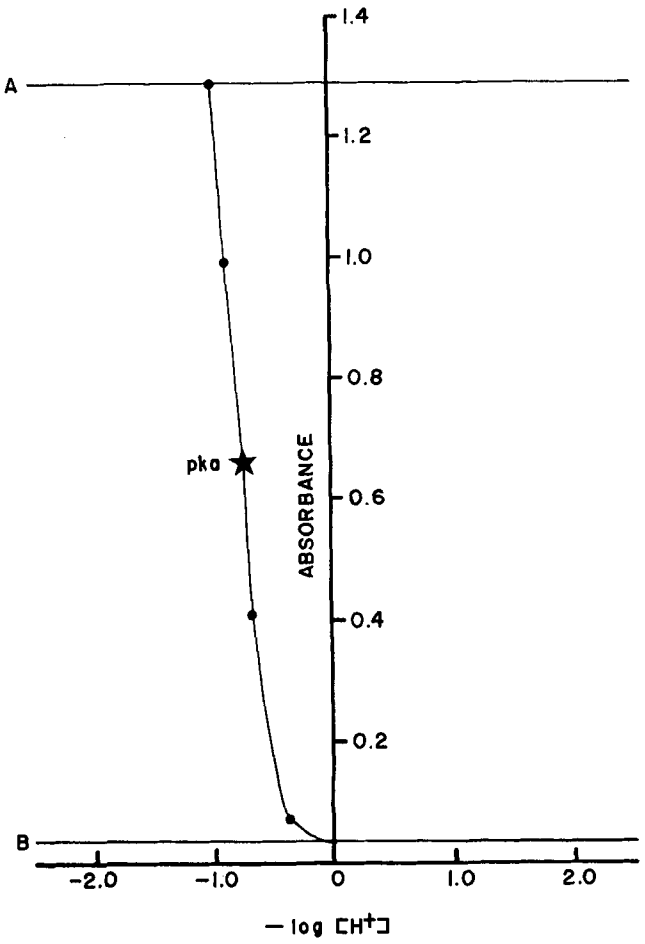


Figure 2—*Determination of the first pKa value of BCP using absorbance values at 530 mμ.*

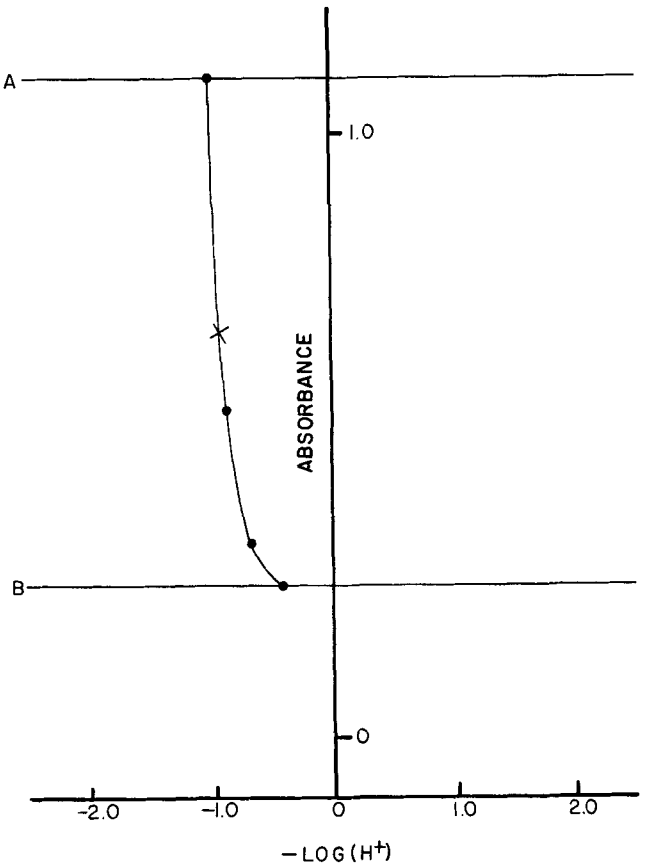


Figure 3—*Determination of the first pKa value of BPB using absorbance values at 530 mμ.*

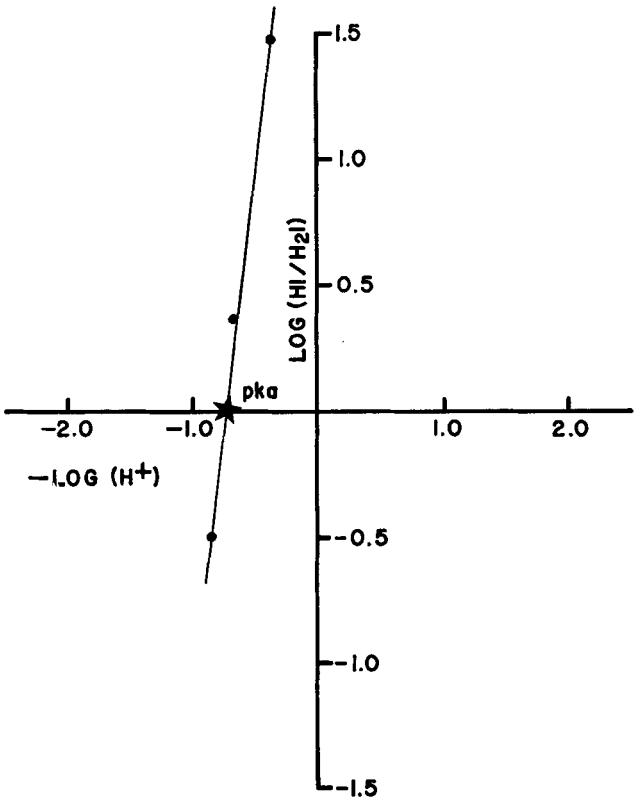


Figure 4—*Determination of the first pKa value of BCP using log* (HI^-/H_2I) *(from Fig. 2)* versus $-\log(H^+)$ *plot.*

Phenol Red—The first pKa value of this dye was estimated to be +1.03 (Table II). The Hammett acidity scale was not used due to reasons previously explained.

It was not possible to take into account the activity coefficients at these higher concentrations of hydrochloric acid. This will, no doubt, cause some error in the determination of the first pKa values of these dyes. Wherever possible, the Hammett acidity scale was also used as explained previously.

Analytical Applications—The information provided is very useful for the determination of the true partition coefficients as reported by Gupta and Cadwallader (10). This information could also be useful to correlate the structural formulas of the dyes.

REFERENCES

(1) J. T. Geraghty and L. G. Rountree, *J. Amer. Med. Ass.*, **57,** 811(1911).

(2) G. Speck, *ibid.*, **143,** 357(1950).

(3) B. A. Thomas and J. C. Birdsall, *ibid.*, **69,** 1747(1917).

(4) S. M. Rosenthal and E. C. White, *ibid.*, **84,** 1112(1925).

(5) V. D. Gupta and D. E. Cadwallader, *J. Pharm. Sci.*, **57,** 112(1968).

(6) V. D. Gupta, D. E. Cadwallader, H. B. Herman, and I. L. Honigberg, *ibid.*, **57,** 1199(1968).

(7) V. D. Gupta and L. A. Luzzi, *Amer. J. Hosp. Pharm.*, **25,** 360(1968).

(8) T. Higuchi and J. I. Bodin, in "Pharmaceutical Analysis," T. Higuchi and E. Brochmann-Hanssen, Eds., Interscience, New York, N. Y., 1961, pp. 414, 415.

(9) G. Schill, *Acta Pharm. Suecica*, **2,** 99(1965).

(10) V. D. Gupta and D. E. Cadwallader, *J. Pharm. Sci.*, **57,** 2140(1968).

(11) "The Merck Index," 8th ed., Merck and Co., Inc., Rahway, N. J., 1968, p. 166.

(12) C. N. Reilley and D. T. Sawyer, "Experiments for Instrumental Methods," McGraw-Hill, New York, N. Y., 1961, pp. 153–155.

(13) I. M. Kolthoff, "Acid-Base Indicators," Macmillan, New York, N. Y., 1937, p. 108.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 16, 1970, from the *College of Pharmacy, University of Houston, Houston, TX 77004*

Accepted for publication June 3, 1970.

Abstracted from a thesis submitted by John B. Reed, Jr., to the Graduate School, University of Houston, in partial fulfillment of Master of Science degree requirements.

*Present address: Ben Taub Hospital, Houston, TX 77025

Dipole Moment and Structure of Thiophene Derivatives and Benzene Analogs

E. J. LIEN* and W. D. KUMLER

Abstract □ The dipole moments of 2-halothiophene, 2,2′-bithiophene, 2-thiophenecarboxylic acid derivatives, and the corresponding benzene analogs have been measured in benzene and in dioxane. Evidence is presented that the conformation in 2-thiophenecarboxylic acid derivatives has the thiophene dipole (which has the sulfur negative) opposed to the resultant dipole of the carboxyl or ester group. The conformation in 2,2′-bisthiophene is shown to be *cis*. In dioxane, there is more resonance interaction between the thiophene ring and the functional groups than between the benzene ring and these groups. The chemical shift and the coupling constant of the NMR spectra of the 2-halothiophenes and 5,5′-dihalo-2,2′-bithiophenes have been assigned.

Keyphrases □ Thiophene derivatives, benzene analogs—dipole moment, structure determination □ NMR spectroscopy—structure

The dipole moment has been shown to be a useful electronic parameter in some structure–activity correlation studies (1), *e.g.*, the insecticidal activity of chlorphenothane (DDT) isomers (2), the cholinesterase inhibitory activity of *N*-alkylsubstituted amides (3), the respiratory stimulation activity of cyclic ureas and thioureas (4), and the inhibition of viral neuraminidase by dihydroisoquinoline derivatives (5).

The usefulness of dipole moments in structure–activity correlation is more restricted than other physico-

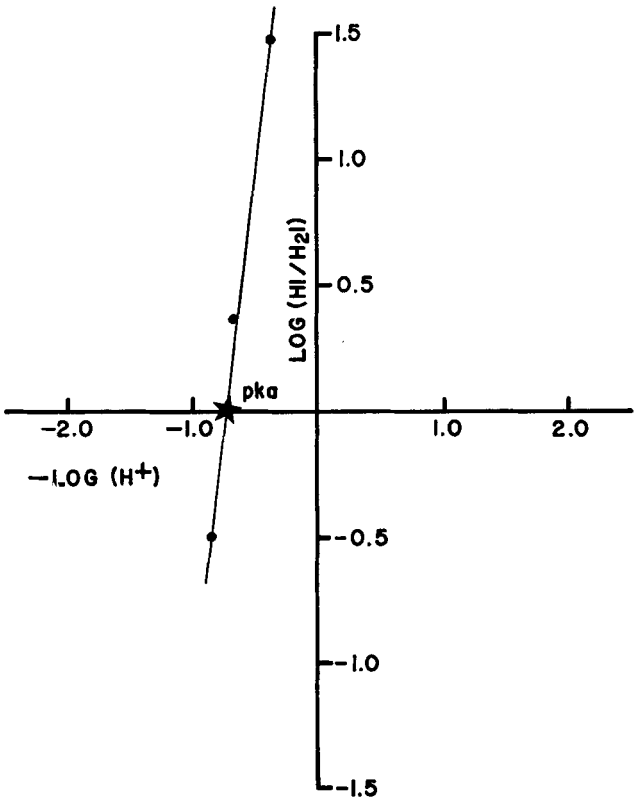


Figure 4—*Determination of the first pKa value of BCP using log* (HI^-/H_2I) *(from Fig. 2)* versus $-\log(H^+)$ *plot.*

Phenol Red—The first pKa value of this dye was estimated to be +1.03 (Table II). The Hammett acidity scale was not used due to reasons previously explained.

It was not possible to take into account the activity coefficients at these higher concentrations of hydrochloric acid. This will, no doubt, cause some error in the determination of the first pKa values of these dyes. Wherever possible, the Hammett acidity scale was also used as explained previously.

Analytical Applications—The information provided is very useful for the determination of the true partition coefficients as reported by Gupta and Cadwallader (10). This information could also be useful to correlate the structural formulas of the dyes.

REFERENCES

(1) J. T. Geraghty and L. G. Rountree, *J. Amer. Med. Ass.*, **57,** 811(1911).

(2) G. Speck, *ibid.*, **143,** 357(1950).

(3) B. A. Thomas and J. C. Birdsall, *ibid.*, **69,** 1747(1917).

(4) S. M. Rosenthal and E. C. White, *ibid.*, **84,** 1112(1925).

(5) V. D. Gupta and D. E. Cadwallader, *J. Pharm. Sci.*, **57,** 112(1968).

(6) V. D. Gupta, D. E. Cadwallader, H. B. Herman, and I. L. Honigberg, *ibid.*, **57,** 1199(1968).

(7) V. D. Gupta and L. A. Luzzi, *Amer. J. Hosp. Pharm.*, **25,** 360(1968).

(8) T. Higuchi and J. I. Bodin, in "Pharmaceutical Analysis," T. Higuchi and E. Brochmann-Hanssen, Eds., Interscience, New York, N. Y., 1961, pp. 414, 415.

(9) G. Schill, *Acta Pharm. Suecica*, **2,** 99(1965).

(10) V. D. Gupta and D. E. Cadwallader, *J. Pharm. Sci.*, **57,** 2140(1968).

(11) "The Merck Index," 8th ed., Merck and Co., Inc., Rahway, N. J., 1968, p. 166.

(12) C. N. Reilley and D. T. Sawyer, "Experiments for Instrumental Methods," McGraw-Hill, New York, N. Y., 1961, pp. 153–155.

(13) I. M. Kolthoff, "Acid-Base Indicators," Macmillan, New York, N. Y., 1937, p. 108.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 16, 1970, from the *College of Pharmacy, University of Houston, Houston, TX 77004*

Accepted for publication June 3, 1970.

Abstracted from a thesis submitted by John B. Reed, Jr., to the Graduate School, University of Houston, in partial fulfillment of Master of Science degree requirements.

*Present address: Ben Taub Hospital, Houston, TX 77025

Dipole Moment and Structure of Thiophene Derivatives and Benzene Analogs

E. J. LIEN* and W. D. KUMLER

Abstract □ The dipole moments of 2-halothiophene, 2,2′-bithiophene, 2-thiophenecarboxylic acid derivatives, and the corresponding benzene analogs have been measured in benzene and in dioxane. Evidence is presented that the conformation in 2-thiophenecarboxylic acid derivatives has the thiophene dipole (which has the sulfur negative) opposed to the resultant dipole of the carboxyl or ester group. The conformation in 2,2′-bisthiophene is shown to be *cis*. In dioxane, there is more resonance interaction between the thiophene ring and the functional groups than between the benzene ring and these groups. The chemical shift and the coupling constant of the NMR spectra of the 2-halothiophenes and 5,5′-dihalo-2,2′-bithiophenes have been assigned.

Keyphrases □ Thiophene derivatives, benzene analogs—dipole moment, structure determination □ NMR spectroscopy—structure

The dipole moment has been shown to be a useful electronic parameter in some structure–activity correlation studies (1), *e.g.*, the insecticidal activity of chlorphenothane (DDT) isomers (2), the cholinesterase inhibitory activity of *N*-alkylsubstituted amides (3), the respiratory stimulation activity of cyclic ureas and thioureas (4), and the inhibition of viral neuraminidase by dihydroisoquinoline derivatives (5).

The usefulness of dipole moments in structure–activity correlation is more restricted than other physico-

Table I—Dipole Moment and Melting Point or Boiling Point of Some Thiophene and Benzene Derivatives

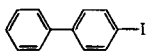
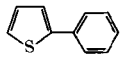
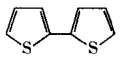
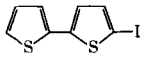
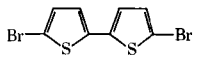
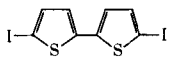
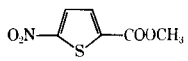
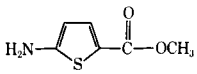
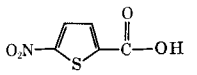
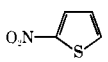
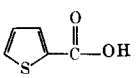
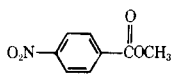
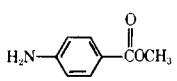
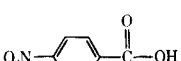
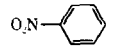
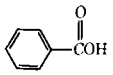
Compound	Dipole Moment, μ (Debye, 25°) Benzene	Dioxane	$\mu_D - \mu_B$	M.p. (corrected)	B.p./(mm. Hg)
 Thiophene	0.52[a]				
 2-Chlorothiophene	1.48 ± 0.01	1.60 ± 0.01	0.12		30°/(5)
 2-Bromothiophene	1.35 ± 0.01	1.41 ± 0.03	0.06		31–32°/(3)
 2-Iodothiophene	1.20 ± 0.01	0.83 ± 0.03	−0.37		57–58°/(3)
 4-Iodobiphenyl	1.55 ± 0.04	0.99 ± 0.02	−0.56	112–114° (113–114°)[b]	
 2-Phenylthiophene	1.04 ± 0.01	—[c]	—	33–35°	
 2,2′-Bithiophene	0.96 ± 0.02	1.15 ± 0.02	0.19	32–33° (32–33°)[b]	
 5-Iodo-2,2′-bithiophene	1.28 ± 0.04	1.79 ± 0.04	0.51		150°/(3.5)
 5,5′-Dibromo-2,2′-bithiophene	1.73 ± 0.09	—[c]	—	144°	
 5,5′-Diiodo-2,2′-bithiophene	0.85 ± 0.04	1.87 ± 0.08	1.02	167–168°	
 Methyl 5-nitro-2-thiophenecarboxylate	3.74 ± 0.01	3.96 ± 0.01	0.22	75–76° (75°)[d]	
 Methyl 5-amino-2-thiophenecarboxylate	3.35 ± 0.01	3.94 ± 0.01	0.58	86–89° (81°)[d]	
 5-Nitro-2-thiophenecarboxylic acid	3.18 ± 0.02	4.43 ± 0.01	1.25	154–157° (157°)[d]	
 2-Nitrothiophene	4.27[a]				
 2-Thiophenecarboxylic acid	1.30[a]				
 Methyl *p*-nitrobenzoate	3.48 ± 0.02	3.74 ± 0.01	0.26	95–96° (96°)[b]	
 Methyl *p*-aminobenzoate	3.41 ± 0.01	3.70 ± 0.01	0.29	112° (114°)[b]	
 *p*-Nitrobenzoic acid	3.42 ± 0.12 (3.5)[a]	4.13 ± 0.03	0.71	240–242° (242°)[b]	

Table I—(*Continued*)

Compound	Dipole Moment, μ (Debye, 25°) Benzene	Dioxane	$\mu_D - \mu_B$	M.p. (corrected)	B.p./(mm. Hg)
 Nitrobenzene	4.01[a]				
 Benzoic acid	1.80[a]				

[a] From A. L. McClellan, "Tables of Experimental Dipole Moments," W. H. Freeman, 1963. [b] From "Handbook of Chemistry and Physics," 45th ed., R. C. Weast, Ed., The Chemical Rubber Co., Cleveland, Ohio. [c] No measurement because of the limited amount of sample available. [d] From P. Fournari and J. P. Chane, *Bull. Soc. Chim. France*, **1963**, 479.

chemical constants such as Hammett's σ constant or Taft's σ^* constant (6), because the resultant moment of a molecule has to be calculated by vector addition, not by simple addition.

That substitution of a thiophene ring for the benzene ring produces compounds with similar physical and biological properties has long been recognized (7, 8). Based on the concept of "bioisosterism," many thiophene analogs of biologically active compounds containing the benzene ring have been investigated (8).

In considering some of the differences between the two sets of compounds, a knowledge of the conformation of the thiophene compounds and how thiophene transmits resonance effects compared with benzene appeared desirable. It seemed likely that a study of the dipole moments of suitable compounds could give this information.

EXPERIMENTAL

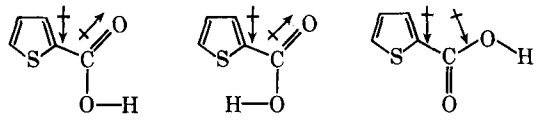
The simple halothiophenes[1] were further purified by vacuum distillation. The other thiophene and benzene derivatives were used as provided.[2] The melting points and the boiling points are given in Table I.

All dipole moments were measured at 25° using a WTW-Dipole Meter model DM01 and a DFL-2 cell. The method of Halverstadt and Kumler (9), programmed for an IBM 1401 by Simpson (10) and converted for an IBM 360/40 computer by the authors, was used to calculate the dipole moments. The solute molar electronic polarization (P_E) was obtained from refractive index measurements. The dipole moments are assembled in Table I.

The NMR spectra of the five compounds examined indicated the samples were of high purity. The NMR spectra were measured on a Varian A-60A spectrometer. The position of the peaks are with reference to that of trimethylsilane (TMS). A complete analysis of the second-order splitting of the ABC pattern for the 2-halothiophenes and the first-order splitting of the AB pattern for the 5,5′-dihalo-2,2′-bithiophenes are summarized in Table II.

RESULTS AND DISCUSSION

The analysis of the dipole moment data is dependent on whether the dipole moment in thiophene has the negative end toward sulfur  or toward the carbons  . The latter might result if there were appreciable contributions from forms with a separation of charges such as  . The literature contains conflicting opinions in regard to the direction of this dipole (8, 11). A consideration of the dipole moments of 2- and 3-methylthiophenes and 2,5-dichlorothiophenes and 2,5-dibromothiophenes (10, 12) demonstrates, however, that the negative end of the dipole is toward sulfur. This, coupled with the evidence that 2-thiophenecarboxylic acid is essentially flat (13) and that its dipole moment is considerably less than that of benzoic acid, establishes the conformation of 2-thiophene carboxylic acid as being I or II or a combination of the two but not III:



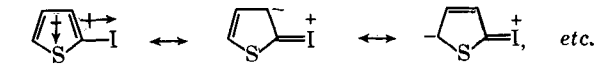
I II III

Only in I or II is the dipole in the thiophene ring in a position to reduce the resultant moment of the carboxyl group. There are conflicting reports on the conformation of 2,2′-bithiophene (12, 14). Thiophene itself has a dipole moment of 0.52 *D*. The dipole moment of 2,2′-bithiophene (0.96 *D* in benzene, 1.15 *D* in dioxane) unequivocally indicates that the two thiophene rings are in a *cis*-conformation rather than in a *trans*-conformation, because the latter conformer should have a moment much lower than 0.52 *D*:

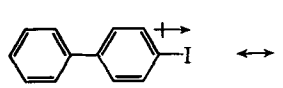
cis *trans*

IV V

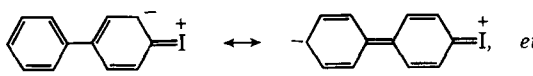
Generally speaking, dipole moments measured in dioxane are higher than in benzene (positive $\mu_D - \mu_B$), because the local dipoles of dioxane tend to augment the dipole moment of the solute molecules. However, for 2-iodothiophene and 4-iodobiphenyl, negative $\mu_D - \mu_B$ values were obtained. This may be due to the lower electronegativity of iodine as compared with that of bromine or chlorine and a greater contribution of the resonance structures in dioxane, opposing the dipole of the C—I bond:



VI*a* VI*b* VI*c*



VII*a*



VII*b* VII*c*

The resonance structure like VII*c* is impossible for 5,5′-dihalo-2,2′-

[1] Eastman Organic Chemicals.

[2] By Dr. J. C. Craig and Dr. A. R. Naik of the University of California, School of Pharmacy.

Table II—NMR Data of 2-Halothiophenes and 5,5′-Dihalo-2,2′-bithiophenes (Measured in $CDCl_3$)

	Chemical Shift (δ) p.p.m. Relative to TMS				Coupling Constant, c.p.s.		
X	H_4	H_3	H_5	No. of Lines	$J_{3,4}$	$J_{3,5}$	$J_{4,5}$
Cl	6.88	6.96	7.07	8	2.5	1.3	5.0
Br	6.84	7.09	7.21	12	4.0	1.5	5.5
I	6.81	7.28	7.42	11	4.0	1.0	5.8

X	$H_3=H_3'$	$H_4=H_4'$		$J_{3,4} = J_{3',4'}$
Br	6.76	6.88	4	4.0
I	6.73	7.10	4	3.7

bithiophene; therefore, positive $\mu_D - \mu_B$ values would be expected. The experimental data fulfill this expectation, since a $\mu_D - \mu_B$ of 1.02 *D* is obtained for 5,5′-diiodo-2,2′-bithiophene.

For 5-iodo-2,2′-bithiophene, 0.51 *D* is obtained for $\mu_D - \mu_B$; this may be due to the noncoplanarity of the bithiophene system and the decreased contribution from dipolar forms like VIIc. It has been shown by electron-diffraction study that in biphenyl two benzene rings form an angle of 45° (15).

For the 2-thiophenecarboxylic acid derivatives, the dipole moments measured in dioxane are higher than those of the corresponding benzene ring compounds. This may be attributed to the greater resonance-transmitting character of the thiophene ring, since dioxane tends to stabilize the dipolar forms. In benzene, no such general trend is observed because benzene does not have local dipoles. For the purpose of structure–activity correlation studies, the dipole moments obtained from dioxane may be better than the moments obtained from benzene, since water in the biological system is a highly polar solvent.

For the NMR data in Table II, the assignment of the coupling constant of 2-halothiophenes is simplified by the study of 5,5′-dihalo-2,2′-bithiophenes, since in the latter case only one coupling constant exists ($J_{3,4}$) which is about 4.0 c.p.s. in both cases where the halogen is Br or I. For 2-chlorothiophene, $J_{3,4}$ is somewhat lower (2.5 c.p.s.), probably due to the higher electronegativity of the chlorine atom and lower electron density in the C_3—C_4 bond. The order of the coupling constants varies as $J_{4,5} > J_{3,4} > J_{3,5}$. This is explainable since there is more double-bond character in the C_4—C_5 bond than in the C_3—C_4 bond. $J_{3,5}$ is the smallest because there are four bonds between H_3 and H_5, while only three bonds are between H_3 and H_4.

For the 2-halothiophenes, the chemical shift of the proton at the 4-position appears to be determined by the inductive effect of the halogen, *i.e.*, the higher the electronegativity of the halogen, the lower the field for the resonance of the proton. This is as expected, since the lower the electron density, the less will be the shielding effect due to the diamagnetism of the circulating electrons (16). On the other hand, for the chemical shifts of the protons at the 3- and 5-positions, an opposite order was obtained. Namely, H_3 and H_5 of 2-iodothiophene (least electronegativity of I) appear at a lower field than those of 2-bromothiophene and 2-chlorothiophene. This is probably due to the contribution from the paramagnetic effect of the C—X bond, since this effect would be the greatest for iodine (17). Free halide ion, having a spherically symmetrical charge distribution, will be magnetically isotropic. The C—I bond has less ionic character than the C—Cl bond, and the magnetic anisotropy of the former would be greater than the latter.

REFERENCES

(1) E. J. Lien, *Amer. J. Pharm. Ed.*, **33,** 368(1969).

(2) R. Riemschneider, in "Advances in Pest Control Research," vol. II, R. L. Metcalf, Ed., Interscience, New York, N. Y., 1958, p. 339.

(3) W. P. Purcell, J. G. Beasley, R. R. Quintana, and J. A. Singer, *J. Med. Chem.*, **9,** 297(1965).

(4) E. J. Lien and W. D. Kumler, *ibid.*, **11,** 214(1968).

(5) M. S. Tute, *ibid.*, **13,** 48(1970).

(6) J. E. Leffler and E. Grundwald, "Rates and Equilibria of Organic Reactions," Wiley, New York, N. Y., 1963, p. 222.

(7) O. Hinsberg, *J. Prakt. Chem.*, **93,** 302(1916).

(8) M. Martin-Smith and S. T. Reid, *J. Med. Pharm. Chem.*, **1,** 507(1959).

(9) I. F. Halverstadt and W. D. Kumler, *J. Amer. Chem. Soc.*, **64,** 2988(1942).

(10) T. R. Simpson, Jr., Ph.D. thesis, University of California Medical Center, San Francisco, Calif., 1964.

(11) L. Felloni and F. Pulidori, *Ann. Chim.*, **51,** 1027(1961).

(12) A. J. H. Wachters and D. W. Davies, *Tetrahedron*, **20,** 2841 (1964).

(13) M. Nardelli, G. Fava, and L. Armellini, *Ric. Sci.*, **28,** 383 (1958).

(14) H. Lumbroso and C. Carpanelli, *Bull. Soc. Chim. France*, **1964,** 3198.

(15) S. Gronowitz and R. A. Hoffman, *Arkiv Kemi*, **13,** 279(1958).

(16) R. M. Silverstein and G. C. Bassler, "Spectrometric Identification of Organic Compounds," Wiley, New York, N. Y., 1964, p. 75.

(17) L. M. Jackman, "Application of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," 4th ed., Pergamon, New York, N. Y., 1964, p. 54.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 29, 1970, from the *School of Pharmacy, University of California at San Francisco, San Francisco, CA 94122*

Accepted for publication May 26, 1970.

The dipole moment data of 2-thiophenecarboxylic acid derivatives were abstracted from the thesis of E. J. L. submitted in 1966 to the Graduate Division, University of California, San Francisco, Calif., in partial fulfillment of the Doctor of Philosophy degree requirements.

This study was supported in part by U. S. Public Health Service Grant FR-00122 for computing services at the University of California, San Francisco.

The authors express their thanks to Dr. J. C. Craig and Dr. A. R. Naik for synthesizing some of the compounds studied.

* Present address: School of Pharmacy, University of Southern California, University Park, Los Angeles, CA 90007

Latentiation of Dihydrostreptomycin by Pamoate Formation

H. C. CALDWELL, A. B. REDNICK, G. C. SCOTT, G. J. YAKATAN*, and D. ZIV

Abstract □ Dihydrostreptomycin was formulated as its pamoate salt as part of a search for a long-acting product. In dogs, an intramuscular injection of a cottonseed oil suspension of dihydrostreptomycin pamoate was absorbed more slowly than equivalent doses of dihydrostreptomycin sulfate suspended in cottonseed oil or dissolved in water. Pharmacokinetic studies suggest that plasma data can be described by the one-compartment open model, and that both salts are equally available.

Keyphrases □ Latentiation, dihydrostreptomycin—by pamoate formation □ Pamoate formation—role in dihydrostreptomycin latentiation □ Dihydrostreptomycin pamoic acid salt—synthesis

Dihydrostreptomycin, first reported in 1946 (1), is made by chemical reduction of streptomycin. Both antibacterial agents possess similar activity against Gram-negative and a few Gram-positive bacteria. For example, they are used to treat bovine actinomycosis, calf pneumonia, newborn foal septicemia, horse cystitis, dog acute nephritis, infectious feline panleukopenia, turkey infectious sinsitis, *etc.* (2). But these drugs must be administered often to ensure high blood levels because they are rapidly removed from the blood. For example, after intramuscular injection, dihydrostreptomycin serum concentrations are reduced to about half in several instances, as follows: man, 3 hr. (3); rabbit, 1.5 hr. (4); cow, 3 hr. (5); chicken, 2 hr. (6, 7); goat, 1–2 hr. (8); and cat, 3 hr. (3).

The objective of the present study was to make a long-acting dihydrostreptomycin injectable by making the drug slowly available from the injection site, thus delaying its transit to the bloodstream. Several examples of delayed transport have been reported. Zini (9) reported that dihydrostreptomycin in pectin–procaine solution markedly prolonged significant serum levels after injection in man, but Hammond (5) tested the solution in cattle and found no prolonged serum levels. Malék *et al.* (10) showed that, on injection, antibiotic salts of high molecular weight acids were transported differently and provided more prolonged blood levels than antibiotic salts of inorganic acids. Several potentially useful dihydrostreptomycin salts are known (11–24); they include naphthalenesulfonate (11), chaulmoograte (14), cholate (15), caprylate (17), and laurylsulfonate (20), but the unknown pamoic acid salt[1] was made for this study.

EXPERIMENTAL

Chemical Synthesis—Sodium pamoate (64.8 g.—0.3 equivalent) was dissolved in 600 ml. of distilled water and filtered. This solution was added with mechanical stirring to a solution of dihydrostreptomycin sulfate (73.2 g.—0.3 equivalent) in 400 ml. of distilled water. The gummy precipitate of dihydrostreptomycin pamoate was collected and washed several times with distilled water. It was then dried at 60° in a vacuum oven for 12 hr. to yield 91.5 g. (78%) of tan solid that decomposed at about 230°. In the analysis of pamoic acid, a theoretical value of 50% was calculated.

[1] Pamoic acid is 4,4′-methylenebis(3-hydroxy-2-naphthoic acid).

Table I—Values of k_a, k_e, and Areas Obtained from Data on Administration of Dihydrostreptomycin to Dogs

Salt	Dose,[a] mg./kg.	$10^5\ k_a$, sec.$^{-1}$	$t_{1/2a}$, hr.	$10^4\ k_e$, sec.$^{-1}$	$t_{1/2e}$, hr.	Area,[b] %
Sulfate[c]	4	58.3	0.33	1.76	1.09	100
Sulfate[d]	4	36.8	0.52	1.76	1.09	100
Pamoate[d]	4	5.56	3.46	1.76	1.09	100

[a] Calculated as dihydrostreptomycin base. [b] Based on comparison to the actual area of the dihydrostreptomycin sulfate formulation in water. [c] Dosed in water. [d] Dosed in oil suspension.

The values were found to be 50.5% by UV and 49.6% by nonaqueous titration.

Formulation Studies—A 10% suspension of dihydrostreptomycin pamoate was readily prepared by rotating 6 g. of dihydrostreptomycin pamoate (passed through a 100-mesh screen) in 15 ml. of cottonseed oil[2] in a bottle containing glass beads for 6 hr. The suspension was poured out, and the beads were washed several times with cottonseed oil to make a final 60 ml. of suspension. A 10% suspension of dihydrostreptomycin sulfate in cottonseed oil was prepared in the same way. Dihydrostreptomycin pamoate could not be formulated as an aqueous suspension because it formed a gum.

Plasma Microbiological Assays—Dihydrostreptomycin plasma levels were determined by the *Bacillus subtilis* cylinder plate assay (25) with human serum as the diluent.

Dosing Dogs—Crossover studies were done with three male dogs. First, they were injected intramuscularly with 4 mg./kg. of dihydrostreptomycin (as sulfate) in water and bled at 0, 0.5, 1, 3, 4, and 5 hr. After a rest period of 1 week, the dogs were dosed with 4 mg./kg. of dihydrostreptomycin (as pamoate) in cottonseed oil and bled at 0, 1, 3, 4, 5, 6, 8, 10, 12, and 26 hr. Finally, they were dosed with 4 mg./kg. of dihydrostreptomycin (as sulfate) in cottonseed oil and bled at 1, 3, 4, 5, and 6 hr.

RESULTS AND DISCUSSION

Three injectable dosage formulations of dihydrostreptomycin were compared in dogs. For these studies, the performances of the formulations were judged by their ability to delay absorption. Figure 1 shows that dihydrostreptomycin absorption from the pamoate formulation is slower and more prolonged than from the sulfate formulations. For example, dihydrostreptomycin pamoate in cottonseed oil provides dihydrostreptomycin levels above 3 mcg./ml. for about 7 hr., while dihydrostreptomycin sulfate in water and in cottonseed oil provides plasma levels above 3 mcg./ml. for about 4 and 4.5 hr., respectively. This does not imply that 3 mcg./ml. is a therapeutic plasma level for infections in dogs; it is merely a cutoff point for this comparative study.

Determination of Pharmacokinetic Constants—Plasma data can be described by the $A \xrightarrow{k_a} B \xrightarrow{k_e} C$ consecutive first-order model, where A represents the amount of drug at the injection site, B the amount of drug in the central body compartment (blood and rapidly equilibrating tissues), and C the amount of drug eliminated from the central compartment at any time. The absorption rate constant, k_a, and the elimination rate constant, k_e, govern the drug-transfer rate from the depot and central compartment, respectively.

Semilogarithmic plots of plasma concentration *versus* time were made. An estimate of the elimination rate constant, k_e, was made from the terminal log linear segment of the data given for the administration of dihydrostreptomycin sulfate in water. The assumption was then made that the different formulations should in no way alter the elimination rate constant. The only changes that would normally be expected would be variations in the time and magnitude of the peak blood levels, and these factors are the result of changes

[2] Wesson Oil, Hunt-Wesson Foods, Fullerton, Calif.

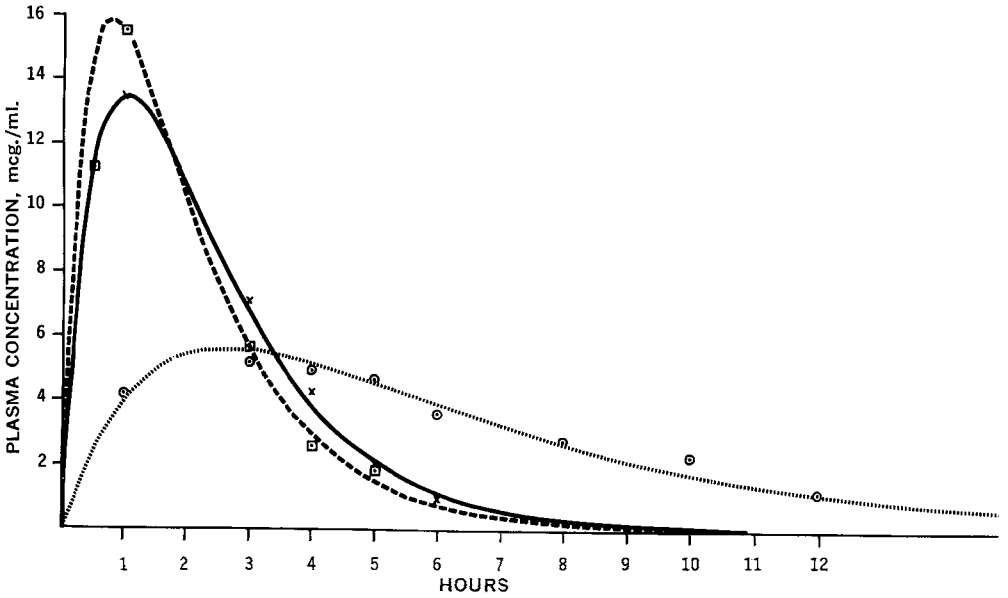


Figure 1—*Concentration of dihydrostreptomycin in the plasma of dogs as a function of time after intramuscular administration of 4 mg./kg. of dihydrostreptomycin (as sulfate) in water (⊡) and in cottonseed oil (×) and of 4 mg./kg. of dihydrostreptomycin (as pamoate) in cottonseed oil (⊙). Dotted lines give one-compartment open-model fits.*

in the magnitude of the absorption rate constant, k_a, for the different formulations.

Using this approach of maintaining k_e constant, k_a values for the sulfate data could be obtained graphically by the "feathering" technique, but the pamoate data could not be treated this way because dihydrostreptomycin was more slowly absorbed from this form. Therefore, the k_a and k_e values obtained by this graphical technique were used in an analog computer simulation of the $A \rightarrow B \rightarrow C$ model. This permitted estimation of k_a for the pamoate form. The values of k_a and k_e are given in Table I. These values are only estimates of the true values because there was a lack of data points to characterize completely the blood level curve prior to and after attainment of peak levels. Nevertheless, the values obtained seem to be reasonably good estimates and give both a magnitude and direction to the characterization of the effect of formulation on the blood levels.

Table I also gives the area under the blood level *versus* time curves following administration of dihydrostreptomycin. The area under these curves can be taken as a measure of the availability of drug from its dosage form, especially in the present case where elimination of the drug occurs by a first-order process, and the dosage forms are given by the same route of administration. All areas are expressed as a percentage of the actual area under the curve for dihydrostreptomycin sulfate in water, which was assumed to be the most "available" formulation.

The areas under the blood level *versus* time curves were obtained by plotting the plasma concentration *versus* time on cartesian coordinate graph paper, connecting each successive two points by a straight line using dark ink and using a line follower to trace the line and generate a voltage which was fed into an analog computer and integrated as a function of time.[3]

If elimination is assumed to be a first-order process, the areas under the blood level *versus* time curves should be proportional to dose if all formulations are equally available (26). The data in Table I indicate that this is the situation with the dihydrostreptomycin formulations, because the same dose of dihydrostreptomycin given as pamoate or sulfate salt gives the same area under the blood level *versus* time plot; this shows that the pamoate salt is just as "available" as the sulfate salt. However, the pamoate formulation differs from the others because it is more slowly absorbed.

SUMMARY

A dihydrostreptomycin pamoate suspension in cottonseed oil (dosed intramuscularly) maintained dog plasma levels above 3 mcg./ml. longer than equivalent doses of a dihydrostreptomycin sulfate suspension in cottonseed oil or a solution in water.

Plasma data can be described by the $A \xrightarrow{k_a} B \xrightarrow{k_e} C$ model.

The pamoate salt was as available as the sulfate salt, but it was absorbed more slowly.

[3] The line follower was a Moseley type F3B used in conjunction with a Moseley 2D-2 X-Y recorder and a Pace TR-10 analog computer, Electronic Associates, Inc., West Long Branch, N. J.

REFERENCES

(1) Q. R. Bartz, J. Controulis, H. M. Crooks, Jr., and M. C. Rebstock, *J. Amer. Chem. Soc.*, **68**, 2163(1946).

(2) "The Merck Veterinary Manual," 2nd ed., Merck & Co., Inc., Rahway, N. J., 1961, pp. 515–517.

(3) L. B. Hobson, *Amer. Rev. Tuberc.*, **58**, 501(1948).

(4) F. J. Murray, B. Taylor, and M. J. Foter, *Ann. Allergy*, **8**, 652(1950).

(5) P. B. Hammond, *J. Amer. Vet. Med. Ass.*, **122**, 203(1953).

(6) S. Barnstein and Y. Samberg, *Amer. J. Vet. Res.*, **15**, 607 (1954).

(7) A. W. Smith, *J. Comp. Pathol. Ther.*, **64**, 225(1954).

(8) S. J. Edwards and M. D. Haskins, *ibid.*, **63**, 53(1953).

(9) F. Zini, *Acta Med. Scand.*, **136**, 209(1950).

(10) P. Malék, J. Kolc, M. Herold, and J. Hoffman, *Antibiot. Ann.*, **1957–1958**, 546.

(11) R. A. Carboni and P. P. Regna, U. S. pat. 2,650,216(1953).

(12) T. Kobari, Japanese pat. 94/55(1955).

(13) W. A. Lott, J. Bernstein, and M. A. Dolliver, U. S. pat. 2,509,191(1950).

(14) H. Mückter and E. Jansen, German pat. 937,049(1955).

(15) H. Mückter and E. Jansen, German pat. 929,665(1955).

(16) R. L. Peck, C. E. Hoffhine, Jr., and K. Folkers, *J. Amer. Chem. Soc.*, **68**, 1390(1946).

(17) Sharp & Dohme, Inc., British pat. 667,751(1952).

(18) E. R. Squibb & Sons, British pat. 642,249(1950).

(19) Takeda Chem. Inds. Ltd., Japanese pat. 11839/62(1962).

(20) Takeda Pharm., Japanese pat. 235/62(1962).

(21) Takeda Pharm., Japanese pat. 8947/61(1961).

(22) M. Wolf, U. S. pat. 2,783,220(1957).

(23) F. Ziegler, U. S. pat. 2,857,375(1958).

(24) F. Ziegler, U. S. pat. 2,857,376(1958).

(25) D. C. Grove and W. A. Randall, "A Laboratory Manual," Antibiotics Monograph No. 2, Medical Encyclopedia, Inc., New York, N. Y., 1955, p. 46.

(26) F. H. Dost, *Klin. Wochenschr.*, **36**, 655(1958).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 13, 1970, from *Smith Kline & French Laboratories, Philadelphia, PA 19101*

Accepted for publication June 3, 1970.

* Present address: College of Pharmacy, University of Florida, Gainesville, FL 32601

New Compounds: Potential Antineoplastics VI: 2,4-Diamino-6-hydroxy-5-arylazopyrimidines and 1,3-Dimethyl-5-arylhydrazonoalloxans

H. G. GARG and R. A. SHARMA

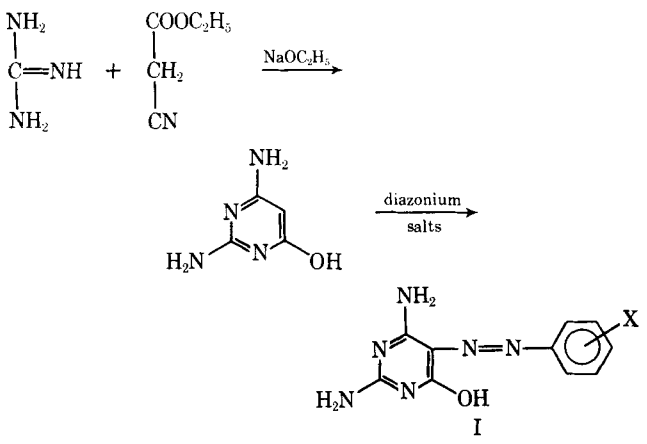
Abstract □ A series of 2,4-diamino-6-hydroxy-5-arylazopyrimidines and 1,3-dimethyl-5-arylhydrazonoalloxans has been synthesized by the coupling of aryldiazonium salts with 2,4-diamino-6-hydroxypyrimidine and 1,3-dimethylbarbituric acid, respectively. The former are also obtained by the cyclization of ethyl cyanoglyoxalate arylhydrazones and guanidine hydrochloride in the presence of sodium ethoxide.

Keyphrases □ 2,4 - Diamino - 6 - hydroxy - 5 - arylazopyrimidines—synthesis, potential antineoplastics □ 1,3-Dimethyl-5-arylhydrazonoalloxans—synthesis, potential antineoplastics □ Antineoplastics—2,4-diamino-6-hydroxy-5-arylazopyrimidines, 1,3-dimethyl-5-arylhydrazonoalloxans, synthesis

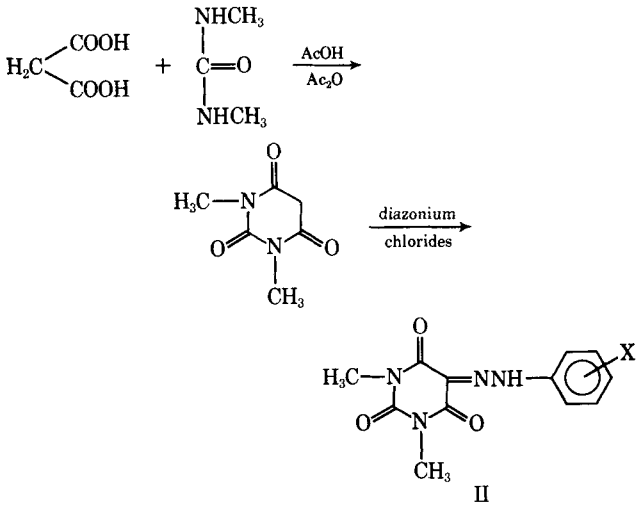
Several arylazopyrimidine analogs are known to possess considerable antitumor activity (1–3). Recently, the synthesis of a few 2-amino-4,6-dimethyl-5-arylazopyrimidines and related compounds has been reported from the authors' laboratories (4–8). Screening results encouraged the present authors to explore the anticancer activity of more congeners of pyrimidines. Interest in pyrimidines led to the extension of this study to include derivatives of 1,3-dimethylalloxan, because this ring constitutes one of the rings of the interesting antibiotic, fervenulin (9).

This paper describes the synthesis of 2,4-diamino-6-hydroxy-5-arylazopyrimidines (I, Scheme I) and 1,3-dimethyl-5-arylhydrazonoalloxans (II, Scheme II). These were prepared by the coupling of aryldiazonium salts with 2,4-diamino-6-hydroxypyrimidine (10) and 1,3-dimethylbarbituric acid (11), respectively.

The coupling reactions were carried out in different media: water, sodium hydroxide, sodium hydrogen carbonate, and ethanol containing sodium acetate. However, the reaction was found to proceed satisfactorily in the basic medium.



Scheme I



Scheme II

EXPERIMENTAL

Melting points were taken on a Kofler hot-stage apparatus and are uncorrected.

Ethyl Cyanoglyoxalate 4-Chlorophenylhydrazone—A solution of 4-chloroaniline (1.27 g., 0.01 mole) in concentrated hydrochloric acid (6 ml.) was cooled to 0°. Sodium nitrite (0.70 g., 0.01 mole) was gradually added. The diazonium salt solution was filtered into a well-cooled, stirred mixture of sodium acetate (6.0 g.) and ethyl cyanoacetate (1.13 g., 0.01 mole) in ethanol (30 ml.). After 1 hr., the precipitate was filtered off and washed with water; it gave pale-yellow needles (2.26 g., 90%), m.p. 145–146° (from ethanol).

Anal.—Calcd.: .... Found: Cl, 14.0; $C_{11}H_{10}ClN_3O_2$ requires Cl, 14.2%.

The details of other ethyl cyanoglyoxalate arylhydrazones which were prepared are given in Table I.

2,4-Diamino-6-hydroxypyrimidine—Ethyl cyanoacetate (22.6 g., 0.2 mole) was mixed with a solution of sodium ethoxide prepared from sodium (4.6 g., 0.2 g.-atom) and anhydrous ethanol (50 ml.). This mixture was allowed to stand while a second solution of sodium ethoxide of the same volume and concentration was prepared. To this solution was added guanidine hydrochloride (19.2 g., 0.2 mole). The sodium chloride was separated by filtration, and the clear filtrate containing guanidine was added to the solution of sodiocyanoacetate. The mixture was heated for 2 hr. under reflux and was then evaporated to dryness at ordinary pressure. The solid product thus obtained was dissolved in water (65 ml.) and acidified with glacial acetic acid (15 ml.). Upon cooling of the solution, 2,4-diamino-6-hydroxypyrimidine was obtained as light-yellow needles (22.16 g., 80%), m.p. 260–265° dec. [lit. (10) m.p. 260–270° dec.].

1,3-Dimethylbarbituric Acid—This was prepared by the procedure of Pfleiderer and Schundehutte (11) from *N,N'*-dimethylurea (17.6 g., 0.2 mole) and malonic acid (17.6 g., 0.2 mole). The product was obtained as colorless needles (21.84 g., 70%), m.p. 118–119° (from ethanol) [lit. (11) m.p. 124°].

2,4 - Diamino - 6 - hydroxy - 5 - (4 - chlorophenylazo)pyrimidine—*Method A*—A solution of 4-chlorobenzenediazonium chloride from

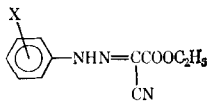


Table I—Characteristics of Ethyl Cyanoglyoxalate Arylhydrazones

No.	X	Yield, %	M.p.	Color[a]	Formula	Analysis, % Calcd.	Found
1	H	90	89–90°	PeYN	$C_{11}H_{11}N_3O_2$	N 19.4	N 19.2
2	2—Me	85	121–122°	BYN	$C_{12}H_{13}N_3O_2$	N 18.2	N 18.0
3	2—NO_2	85	114–115°	BYN	$C_{11}H_{10}N_4O_4$	N 21.4	N 21.3
4	3—NO_2	80	156–157°	OP	$C_{11}H_{10}N_4O_4$	N 21.4	N 21.1
5	4—NO_2	85	193–194°	PeYN	$C_{11}H_{10}N_4O_4$	N 21.4	N 21.2
6	2—Br	75	129–130°	PeYN	$C_{11}H_{10}BrN_3O_2$	Br 27.0	Br 26.8
7	2—Et	80	109–110°	YN	$C_{13}H_{15}N_3O_2$	N 17.1	N 17.3
8	2—MeO	75	135–136°	YN	$C_{12}H_{13}N_3O_3$	N 17.0	N 17.2
9	3—MeO	75	95–96°	BYN	$C_{12}H_{13}N_3O_3$	N 17.0	N 16.7
10	4—EtO	78	81–82°	BYN	$C_{13}H_{15}N_3O_3$	N 16.1	N 16.0
11	2,3—Me_2	80	100–101°	YN	$C_{13}H_{15}N_3O_2$	N 17.1	N 17.2
12	2,4—Me_2	85	157–158°	BYN	$C_{13}H_{15}N_3O_2$	N 17.1	N 16.8
13	2,5—Me_2	85	115–116°	YN	$C_{13}H_{15}N_3O_2$	N 17.1	N 17.0
14	2,6—Me_2	78	100–101°	PeYN	$C_{13}H_{15}N_3O_2$	N 17.1	N 17.0
15	3,4—Me_2	75	84–85°	PeYN	$C_{13}H_{15}N_5O_2$	N 17.1	N 16.9
16	3,5—Me_2	80	129–130°	OYN	$C_{13}H_{15}N_3O_2$	N 17.1	N 17.0
17	2,6—Et_2	85	101–102°	YP	$C_{15}H_{19}N_3O_2$	N 15.4	N 15.2
18	2,3—Cl_2	86	119–120°	OYN	$C_{11}H_9Cl_2N_3O_2$	Cl 24.8	Cl 24.6
19	2,4—Cl_2	85	168–169°	BYN	$C_{11}H_9Cl_2N_3O_2$	Cl 24.5	Cl 24.5
20	2,5—Br_2	70	165–166°	OYN	$C_{11}H_9Br_2N_3O_2$	Br 42.7	Br 42.6
21	2,5—$(MeO)_2$	75	102–103°	OP	$C_{13}H_{15}N_3O_4$	N 15.2	N 15.0
22	2—Cl—6—Me	90	107–108°	BYN	$C_{12}H_{12}ClN_3O_2$	Cl 13.4	Cl 13.2
23	2,5—Cl_2—4—NO_2	65	143–144°	BYN	$C_{11}H_8Cl_2N_4O_4$	Cl 21.5	Cl 21.1
24	4—Cl—2,5—$(MeO)_2$	80	164–165°	GYN	$C_{13}H_{14}ClN_3O_4$	Cl 11.4	Cl 11.2
25	5—Cl—2,4—$(MeO)_2$	85	176–177°	GYN	$C_{13}H_{14}ClN_2O_4$	Cl 11.4	Cl 11.1

[a] B, bright; Bn, brown; D, deep; Da, dark; F, fibers; G, golden; Gr, green; N, needles; O, orange; P, plates; Pe, pale; R, red; and Y, yellow.

4-chloroaniline (1.27 g., 0.01 mole) in concentrated hydrochloric acid (5 ml.) was slowly added to a well-cooled, stirred mixture of 2,4-diamino-6-hydroxypyrimidine (1.26 g., 0.01 mole) in 2 *N* sodium hydroxide (10 ml.) containing sodium acetate (5.0 g.). The precipitated solid was filtered off and washed with water; it gave bright-yellow needles (2.25 g., 85%), m.p. >300° [from ethanol–dimethylformamide (DMF)].

Anal.—Calcd.: .... Found: Cl, 13.2; $C_{10}H_9ClN_6O$ requires Cl, 13.5%.

Method B—Guanidine hydrochloride (1.92 g., 0.02 mole) was added to ethyl cyanoglyoxalate 4-chlorophenylhydrazone (5.02 g., 0.02 mole) dissolved in alcoholic sodium ethoxide (1.2 g. of sodium in 50 ml. anhydrous ethanol). After keeping for 1 hr., the mixture was refluxed for 2 hr. and left overnight. The solid, which separated, was collected and washed with water. It was obtained as bright-yellow needles (1.90 g., 70%), m.p. >300° (from ethanol–DMF). The melting point was not depressed upon admixture with the sample prepared by Method A.

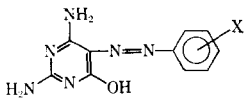


Table II—Characteristics of 2,4-Diamino-6-hydroxy-5-arylazopyrimidines

No.	X	Yield, %	M.p.	Color[a]	Formula	Analysis, % Calcd.	Found
1	H	85	>300°	OP	$C_{10}H_{10}N_6O$	N 36.5	N 36.3
2	2—Me	87	>300°	ORN	$C_{11}H_{12}N_6O$	N 34.4	N 34.2
3	2—NO_2	80	>300°	ORN	$C_{10}H_9N_7O_3$	N 35.6	N 35.5
4	3—NO_2	73	295° dec.	PeYN	$C_{10}H_9N_7O_3$	N 35.6	N 35.4
5	4—NO_2	75	>300°	DRN	$C_{10}H_9N_7O_3$	N 35.6	N 35.4
6	2—Br	75	>300°	ORN	$C_{10}H_9BrN_6O$	Br 26.0	Br 26.3
7	2—Et	80	>300°	ON	$C_{12}H_{14}N_6O$	N 32.6	N 32.3
8	2—MeO	77	270° dec.	DRN	$C_{11}H_{12}N_6O_2$	N 32.3	N 32.1
9	3—MeO	75	170° dec.	BnN	$C_{11}H_{12}N_6O_2$	N 32.3	N 32.4
10	4—EtO	70	>300°	RN	$C_{12}H_{14}N_6O_2$	N 30.6	N 30.4
11	2,3—Me_2	85	>300°	RN	$C_{12}H_{14}N_6O_2$	N 32.6	N 32.3
12	2,4—Me_2	80	>300°	RN	$C_{12}H_{14}N_6O$	N 32.6	N 32.5
13	2,5—Me_2	75	>300°	ORN	$C_{12}H_{14}N_6O$	N 32.6	N 32.4
14	2,6—Me_2	83	290° dec.	OYN	$C_{12}H_{14}N_6O$	N 32.6	N 32.5
15	3,4—Me_2	75	280° dec.	RN	$C_{12}H_{14}N_6O$	N 32.6	N 32.3
16	3,5—Me_2	77	295° dec.	YN	$C_{12}H_{14}N_6O$	N 32.6	N 32.7
17	2,6—Et_2	81	>300°	ON	$C_{14}H_{18}N_6O$	N 29.4	N 29.2
18	2,3—Cl_2	73	>300°	ON	$C_{10}H_8Cl_2N_6O$	Cl 23.7	Cl 23.5
19	2,4—Cl_2	75	>300°	DaBnN	$C_{10}H_8Cl_2N_6O$	Cl 23.7	Cl 23.4
20	3,5—Cl_2	85	>300°	BYN	$C_{10}H_8Cl_2N_6O$	Cl 23.7	Cl 23.5
21	2,5—Br_2	87	240° dec.	DRN	$C_{10}H_8Br_2N_6O$	Br 41.2	Br 41.0
22	2,5—$(MeO)_2$	76	>300°	RBnN	$C_{12}H_{14}N_6O_3$	N 29.0	N 29.2
23	2—Cl—6—Me	80	>300°	YN	$C_{11}H_{11}ClN_6O$	Cl 12.7	Cl 12.6
24	2,5—Cl_2—4—NO_2	85	285° dec.	YN	$C_{10}H_7Cl_2N_7O_3$	Cl 20.6	Cl 20.3
25	4—Cl—2,5—$(MeO)_2$	70	>300°	DRN	$C_{12}H_{13}ClN_6O_3$	Cl 10.9	Cl 10.7
26	5—Cl—2,4—$(MeO)_2$	75	277° dec.	DRN	$C_{12}H_{13}ClN_6O_3$	Cl 10.9	Cl 10.6

[a] See footnote [a] of Table I.

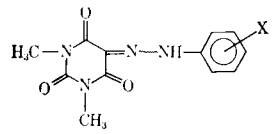


Table III—Characteristics of 1,3-Dimethyl-5-arylhydrazonoalloxan Derivatives

No.	X	Yield, %	M.p.	Color[a]	Formula	Analysis, % Calcd.	Found
1	H	75	255–256°	YN	$C_{12}H_{12}N_4O_3$	N 21.5	N 21.2
2	2—NO_2	65	244–245°	ON	$C_{12}H_{11}N_5O_5$	N 23.0	N 23.2
3	3—NO_2	75	232–233°	ON	$C_{12}H_{11}N_5O_5$	N 23.0	N 23.3
4	4—Cl	75	245–246°	YN	$C_{12}H_{11}ClN_4O_3$	Cl 12.1	Cl 12.0
5	2—Me	70	238–239°	PeYN	$C_{13}H_{14}N_4O_3$	N 20.4	N 20.2
6	2—Et	80	200–201°	BYN	$C_{14}H_{16}N_4O_3$	N 19.4	N 19.5
7	3—MeO	78	151–152°	BGrN	$C_{13}H_{14}N_4O_4$	N 19.3	N 19.1
8	4—MeO	85	195–196°	YP	$C_{13}H_{14}N_4O_4$	N 19.3	N 19.0
9	2,3—Cl_2	70	260–265° dec.	PeYN	$C_{12}H_{10}Cl_2N_4O_3$	Cl 21.6	Cl 21.3
10	2,4—Cl_2	90	244–245°	YN	$C_{12}H_{10}Cl_2N_4O_3$	Cl 21.6	Cl 21.5
11	3,5—Cl_2	85	254–255° dec.	ON	$C_{12}H_{10}Cl_2N_4O_3$	Cl 21.6	Cl 21.2
12	2,4—Br_2	75	247–248°	YGrN	$C_{12}H_{10}Br_2N_4O_3$	Br 38.3	Br 38.0
13	2,3—Me_2	80	203–204°	PeYN	$C_{14}H_{16}N_4O_3$	N 19.4	N 19.2
14	2,5—Me_2	80	246–247°	YN	$C_{14}H_{16}N_4O_3$	N 19.4	N 19.1
15	2,6—Me_2	75	225–226°	PeYP	$C_{14}H_{16}N_4O_3$	N 19.4	N 19.0
16	3,4—Me_2	85	240–241°	GrN	$C_{14}H_{16}N_4O_3$	N 19.4	N 19.6
17	2,5—Cl_2—4—NO_2	65	278–279°	PeYF	$C_{12}H_9Cl_2N_5O_5$	Cl 19.0	Cl 19.2
18	4—Cl—2,5—$(MeO)_2$	75	288–289°	ORN	$C_{14}H_{15}ClN_4O_5$	Cl 10.0	Cl 9.7
19	5—Cl—2,4—$(MeO)_2$	80	245–246°	ORN	$C_{14}H_{15}ClN_4O_5$	Cl 10.0	Cl 9.8

[a] See footnote [a] of Table I.

The details of other 2,4-diamino-6-hydroxy-5-arylazopyrimidines are given in Table II.

1,3 - Dimethyl - 5 - (4 - nitrophenylhydrazono)alloxan—1,3 - Dimethylbarbituric acid (1.56 g., 0.01 mole) was dissolved in water (20 ml.) and cooled to 0°. This was then treated with a stirred solution of 4-nitrobenzenediazonium chloride obtained from 4-nitroaniline (1.38 g., 0.01 mole) in concentrated hydrochloric acid (5 ml.) containing sodium acetate (5.0 g.). The solid, which separated, was collected, washed well with water, and obtained as yellow needles (2.75 g., 90%), m.p. 303–304° (from ethanol–DMF).

Anal.—Calcd.: .... Found: N, 22.8; $C_{12}H_{11}N_5O_5$ requires N, 23.0%.

The details of other 1,3-dimethyl-5-arylhydrazonoalloxans are given in Table III.

REFERENCES

(1) J. Hampshire, P. Hebborn, A. M. Triggle, D. J. Triggle, and S. Vickers, *J. Med. Chem.*, **8**, 745(1965).

(2) E. J. Modest, H. N. Schlein, and G. E. Foley, *J. Pharm. Pharmacol.*, **9**, 68(1957).

(3) P. Roy-Burman and D. Sen, *Biochem. Pharmacol.*, **13**, 1437(1964).

(4) H. G. Garg and R. A. Sharma, *J. Med. Chem.*, **12**, 1122 (1969).

(5) *Ibid.*, **13**, 579(1970).

(6) H. G. Garg and R. A. Sharma, *J. Pharm. Sci.*, **59**, 348 (1970).

(7) H. G. Garg and R. A. Sharma, *J. Med. Chem.*, **13**, 763(1970).

(8) *Ibid.*, **13** (1970).

(9) C. W. Noell and C. C. Cheng, *J. Med. Chem.*, **12**, 545(1969).

(10) J. A. Van Allan, "Organic Syntheses," coll. vol. IV, Wiley, New York, N. Y., 1963, p. 245.

(11) W. Pfleiderer and K. H. Schundehutte, *Ann.*, **612**, 158 (1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 3, 1970, from the *Department of Chemistry, University of Roorkee, Roorkee, India*

Accepted for publication May 19, 1970.

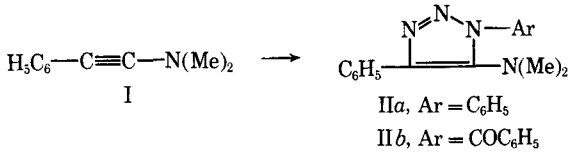
The authors thank Professor W. U. Malik, Head of the Chemistry Department, University of Roorkee, Roorkee, India, for providing the necessary facilities for carrying out the work; Dr. Maxwell Gordon, Research Director, Smith Kline & French Laboratories, Philadelphia, Pa., for the supply of some rare chemicals; and the C.S.I.R., New Delhi, India, for a Junior Research Assistantship (held by R.A.S.).

Antimetabolite Activity of Some *N,N*-Dialkylamino-1,2,3-triazoles and α-Diazoamidines

Keyphrases □ *N,N*-Dialkylamino-1,2,3-triazoles—synthesis □ α-Diazoamidines—synthesis □ Antibacterial activity—*N,N*-dialkylamino-1,2,3-triazoles, α-diazoamidines

Sir:

A number of 1,3-dipolar additions to ynamines or *N,N*-disubstituted-1-amino-1-alkynes have been reported to produce a series of five-membered heterocycles (1–3). For instance, the additions of both aryl and aroyl azides to *N,N*-dimethylaminophenylacetylene (I) gave the corresponding 1,2,3-triazoles II*a* and II*b*, respectively (Scheme I). From the 1,3-dipolar additions of a number of substituted benzenesulfonyl azides to the ynamine (I) and *N,N*-diethylaminoprop-1-yne, we have isolated crystalline *N,N*-dialkylamino-1,2,3-triazoles (X and XI) and α-diazoamidines (III–IX). Details about the preparation and characterization of Compounds III–XI (Table I) will be published (4).



Scheme I

Compounds III–XI were subjected to *in vitro* screening for antimetabolites (5). In this method, the detection system utilizes the Gram-positive *Bacillus subtilis* (UC-564) and Gram-negative *Escherichia coli* (ATCC 26). Both of these organisms were grown in two types of agar: (*a*) nutrient agar—a complex medium containing 0.3% beef extract, 0.5% peptone, and 1.5% agar; and (*b*) a completely synthetic medium with glucose as the only source of carbon. Acetone solutions of Compounds III–XI at concentrations of 1 mg./ml. were applied onto 13-mm. paper disks, and they were placed on the surface of the seeded agar. The trays containing these paper disks, seeded with *E. coli* and *B. subtilis*, were incubated at 37° for 18 hr., and the zones of inhibition were measured. The results are given in Table I. None of the compounds inhibited the growth of *E. coli*. All the compounds inhibited the growth of *B. subtilis* in synthetic agar but not (or inhibited much less) in nutrient agar. This indicated that some of them could be potential antimetabolites. Furthermore, most of the α-diazoamidines (Compounds III–IX) showed more pronounced activity than the triazoles (X and XI). Compounds IV–VI and IX were further tested by the technique of specific reversal against *B. subtilis*, and the results of these experiments are included in Table II. Once again, all the samples inhibited *B. subtilis* more strongly on synthetic agar than on nutrient agar. However, none of the samples was reversed by amino acids, vitamins, purines, and pyrimidines. Therefore,

Table I—Inhibition of *B. subtilis* Grown in Two Different Media (Zones of Inhibition in Millimeters)

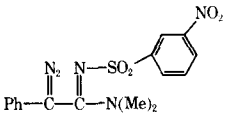
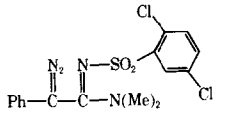
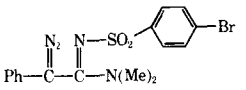
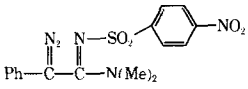
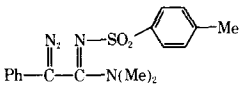
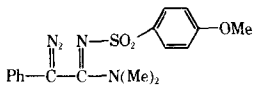
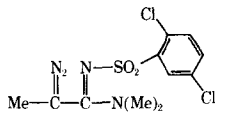
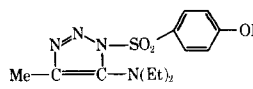
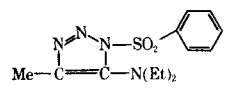
Compound Number	Compound Structure	Nutrient Agar	Synthetic Agar
III		Trace	25
IV		0	41
V		0	25
VI		22	39
VII		0	18
VIII		0	17
IX		Trace	38
X		0	15
XI		0	16

Table II—Specific Reversal Studies against *B. subtilis*

Compound	Nutrient	Synthetic Agar Further Implemented with: No Supplements (Control)	Amino Acids	Combined Vitamins	Combined Purines and Pyrimidines
III	0	35	25	26	30
IV	0	59	41	51	50
V	0	25	37	Trace	21
VI	22	40	38	36	21
IX	15	32	28	28	35

these compounds do possess significant antibacterial activity, but this activity is noncompetitive in nature.

(1) R. Huisgen, *Angew. Chem.*, **75**, 604(1963); *Angew. Chem. Int. Ed.*, **2**, 565(1963).
(2) R. Fuks, R. Buijle, and H. G. Viehe, *Angew. Chem.*, **78**, 594 (1966); *Angew. Chem. Int. Ed.*, **5**, 585(1966).
(3) H. G. Viehe, "Chemistry of Acetylenes," Marcel Dekker, New York, N. Y., 1969, p. 901.
(4) R. E. Harmon, F. Stanley, Jr., S. K. Gupta, and J. Johnson, to be published.
(5) L. J. Hanka, *Abstr., Int. Congr. Chemother., 5th*, **1967**, B. g/2, 351.

R. E. Harmon
S. K. Gupta
J. Johnson
F. Stanley, Jr.
Department of Chemistry
Western Michigan University
Kalamazoo, MI 49001

L. J. Hanka
Research Laboratories
The Upjohn Co.
Kalamazoo, MI 49001

Received June 9, 1970.
Accepted for publication August 5, 1970.
The work at Western Michigan University was supported by Grant CA-06140 from the National Cancer Institute. The work at The Upjohn Co. was in part supported by Contract PH 43-68-1023 with Chemotherapy, National Cancer Institute, National Institutes of Health, Bethesda, Md.

Solubility Relationships in Urea–Water Systems

Keyphrases □ Urea–water systems—solubilization mechanism □ Solute–urea interaction effect—solubility □ Transfer, standard free energy—solute in urea solution, water

Sir:

Interpretations regarding the mechanisms of solution in cosolvent systems are facilitated if one can rely on various simplifying assumptions. In urea–water mixtures, for example, it would be convenient to assume that the interactions of the cosolvent with a given solute moiety are specific and predictable. Further, it would be of immeasurable aid if explanations of observed solubility phenomena were possible in terms of purely basic theoretical concepts. Therefore, as part of a larger study of solution properties, we investigated the potential usefulness of certain of these assumptions relative to compounds of intrinsic pharmaceutical interest.

The solubilities of compounds having various moieties in common were determined in water and in 5 *M* urea solutions at 30°. After equilibrating excess drug in both solvent systems, samples of the resulting solutions were withdrawn, and solubilities were determined spectrophotometrically. The standard free energies of transfer, $\Delta G_t°$, were then calculated according to the equation used by Wetlaufer *et al.* (1):

$$\Delta G_t° = -RT \ln C_u/C_w + RT \ln N_u/N_w \qquad \text{(Eq. 1)}$$

where C_u and C_w are the molar concentrations of drug in the urea and water solutions, respectively; and N_u and N_w represent the moles/liter summed over all components of solvent and solute for the urea and water solutions, respectively. In the present investigation, the free energy change is that which accompanies the transfer of 1 mole of drug from water to a 5 *M* urea solution. These data, along with the aqueous solubilities, are shown in Table I.

Table I—Standard Free Energy of Transfer, $\Delta G_t°$, from Water to 5 *M* Urea Solutions at 30°

Solute	Aqueous Solubility, moles/l. × 10^3	$\Delta G_t°$, cal./mole
Methyl *o*-methoxybenzoate	41.6	+621
Methyl paraben	19.2	−690
Methyl benzoate	17.8	−480
Methyl salicylate	6.27	−612
Ethyl paraben	5.85	−781
n-Propyl paraben	2.78	−645
n-Butyl paraben	1.34	−792

The results strongly suggest the following:

1. Moieties having the same chemical structure may not be expected to exhibit parallel interactions in solvent systems of similar composition. In all likelihood, these interactions are also a function of the chemical entity to which a given moiety is attached. This is in contrast to the interpretation by Nozaki and Tanford (2), who reported additive solubility effects for the hydrocarbon groups attached to various amino acids. The data in Table I show that this is not the case. For example, the difference in the standard free energy of transfer between methyl benzoate and methyl paraben is 210 cal./mole. The difference between phenylalanine and tyrosine is only 85 cal./mole for the same conditions of transfer (2).

2. The use of a simple homologous series gives no assurance of success in obtaining consistent resolution of solute–solvent interactions. As might be expected, the interactions observed on transferring alkanes from water to urea solutions vary as methane, ethane, propane, and butane (1). In contrast, the interactions of the parabens vary as propyl, methyl, ethyl, and butyl.

3. Relative hydrophobicity, as determined by a comparison of aqueous solubilities, may not be used to explain differences in enhanced solubilities in urea solutions. The data in Table I illustrate this point. For example, the aqueous solubility of methyl paraben is three times greater than methyl salicylate and seven times greater than propyl paraben, yet the transfer of methyl paraben to a 5 *M* urea solution is thermodynamically favored over either of these compounds.

4. Direct urea–solute interaction is a significant factor in altering the solubility of drug species. Although this has been shown (3, 4), reports persist in which attempts are made to explain observations solely on the basis of solvent structuring. The latter approach was taken by Feldman and Gibaldi (5) in rationalizing the increased solubility of benzoic and salicylic acids in

these compounds do possess significant antibacterial activity, but this activity is noncompetitive in nature.

(1) R. Huisgen, *Angew. Chem.*, **75**, 604(1963); *Angew. Chem. Int. Ed.*, **2**, 565(1963).
(2) R. Fuks, R. Buijle, and H. G. Viehe, *Angew. Chem.*, **78**, 594 (1966); *Angew. Chem. Int. Ed.*, **5**, 585(1966).
(3) H. G. Viehe, "Chemistry of Acetylenes," Marcel Dekker, New York, N. Y., 1969, p. 901.
(4) R. E. Harmon, F. Stanley, Jr., S. K. Gupta, and J. Johnson, to be published.
(5) L. J. Hanka, *Abstr., Int. Congr. Chemother., 5th*, **1967**, B. g/2, 351.

R. E. Harmon
S. K. Gupta
J. Johnson
F. Stanley, Jr.
Department of Chemistry
Western Michigan University
Kalamazoo, MI 49001

L. J. Hanka
Research Laboratories
The Upjohn Co.
Kalamazoo, MI 49001

Received June 9, 1970.
Accepted for publication August 5, 1970.
The work at Western Michigan University was supported by Grant CA-06140 from the National Cancer Institute. The work at The Upjohn Co. was in part supported by Contract PH 43-68-1023 with Chemotherapy, National Cancer Institute, National Institutes of Health, Bethesda, Md.

Solubility Relationships in Urea–Water Systems

Keyphrases □ Urea–water systems—solubilization mechanism □ Solute–urea interaction effect—solubility □ Transfer, standard free energy—solute in urea solution, water

Sir:

Interpretations regarding the mechanisms of solution in cosolvent systems are facilitated if one can rely on various simplifying assumptions. In urea–water mixtures, for example, it would be convenient to assume that the interactions of the cosolvent with a given solute moiety are specific and predictable. Further, it would be of immeasurable aid if explanations of observed solubility phenomena were possible in terms of purely basic theoretical concepts. Therefore, as part of a larger study of solution properties, we investigated the potential usefulness of certain of these assumptions relative to compounds of intrinsic pharmaceutical interest.

The solubilities of compounds having various moieties in common were determined in water and in 5 *M* urea solutions at 30°. After equilibrating excess drug in both solvent systems, samples of the resulting solutions were withdrawn, and solubilities were determined spectrophotometrically. The standard free energies of transfer, $\Delta G_t°$, were then calculated according to the equation used by Wetlaufer *et al.* (1):

$$\Delta G_t° = -RT \ln C_u/C_w + RT \ln N_u/N_w \qquad \text{(Eq. 1)}$$

where C_u and C_w are the molar concentrations of drug in the urea and water solutions, respectively; and N_u and N_w represent the moles/liter summed over all components of solvent and solute for the urea and water solutions, respectively. In the present investigation, the free energy change is that which accompanies the transfer of 1 mole of drug from water to a 5 *M* urea solution. These data, along with the aqueous solubilities, are shown in Table I.

Table I—Standard Free Energy of Transfer, $\Delta G_t°$, from Water to 5 *M* Urea Solutions at 30°

Solute	Aqueous Solubility, moles/l. × 10^3	$\Delta G_t°$, cal./mole
Methyl *o*-methoxybenzoate	41.6	+621
Methyl paraben	19.2	−690
Methyl benzoate	17.8	−480
Methyl salicylate	6.27	−612
Ethyl paraben	5.85	−781
n-Propyl paraben	2.78	−645
n-Butyl paraben	1.34	−792

The results strongly suggest the following:

1. Moieties having the same chemical structure may not be expected to exhibit parallel interactions in solvent systems of similar composition. In all likelihood, these interactions are also a function of the chemical entity to which a given moiety is attached. This is in contrast to the interpretation by Nozaki and Tanford (2), who reported additive solubility effects for the hydrocarbon groups attached to various amino acids. The data in Table I show that this is not the case. For example, the difference in the standard free energy of transfer between methyl benzoate and methyl paraben is 210 cal./mole. The difference between phenylalanine and tyrosine is only 85 cal./mole for the same conditions of transfer (2).

2. The use of a simple homologous series gives no assurance of success in obtaining consistent resolution of solute–solvent interactions. As might be expected, the interactions observed on transferring alkanes from water to urea solutions vary as methane, ethane, propane, and butane (1). In contrast, the interactions of the parabens vary as propyl, methyl, ethyl, and butyl.

3. Relative hydrophobicity, as determined by a comparison of aqueous solubilities, may not be used to explain differences in enhanced solubilities in urea solutions. The data in Table I illustrate this point. For example, the aqueous solubility of methyl paraben is three times greater than methyl salicylate and seven times greater than propyl paraben, yet the transfer of methyl paraben to a 5 *M* urea solution is thermodynamically favored over either of these compounds.

4. Direct urea–solute interaction is a significant factor in altering the solubility of drug species. Although this has been shown (3, 4), reports persist in which attempts are made to explain observations solely on the basis of solvent structuring. The latter approach was taken by Feldman and Gibaldi (5) in rationalizing the increased solubility of benzoic and salicylic acids in

urea solutions. In turn, Nogami *et al.* (6) analyzed the adsorption of tryptophan from urea solutions in much the same manner, using the Feldman and Gibaldi paper as a reference. There are implications of direct interaction in each of the results of the present investigation. Perhaps the most striking evidence of this interaction is that the introduction of methyl *o*-methoxybenzoate, a liquid, into a 5 *M* urea solution results immediately in the formation of a flocculent white precipitate, the composition of which is currently unknown.

These findings demonstrate the need for caution in interpreting solubility phenomena involving cosolvent systems, in general, and urea–water mixtures, in particular. At present, few, if any, simplifying assumptions appear to be valid for this purpose.

(1) D. B. Wetlaufer, S. K. Malik, L. Stoller, and R. L. Coffin, *J. Amer. Chem. Soc.*, **86**, 508(1964).
(2) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **238**, 4074(1963).
(3) T. Higuchi and S. Bolton, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 557(1959).
(4) T. Higuchi and K. A. Connors, *Advan. Anal. Chem. Instr.*, **4**, 117(1965).
(5) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **56**, 370(1967).
(6) H. Nogami, T. Nagai, E. Fukuoka, and H. Uchida, *Chem. Pharm. Bull.*, **16**, 2248(1968).

RICHARD E. LINDSTROM
Division of Pharmaceutics
School of Pharmacy
University of Connecticut
Storrs, CT 06268

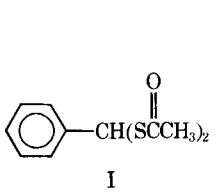
Received June 5, 1970.
Accepted for publication July 20, 1970.
This investigation was supported in part by Grant 093 from the University of Connecticut Research Foundation, Storrs, CT 06268

Preparation of Bis(acetylthiobenzyl)sulfide

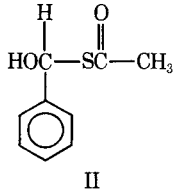
Keyphrases ☐ Bis(acetylthiobenzyl)sulfide—synthesis ☐ NMR spectroscopy—structure ☐ Mass spectroscopy—structure

Sir:

Bongartz (1), in 1886, obtained a product from the reaction of benzaldehyde and thioacetic acid, m.p. 147–148°, which he believed to be phenylmethanedithiol diacetate (I). In 1952, Cairns *et al.* (2) synthesized this compound from phenylmethanedithiol and found the melting point to be 37–38°.



I

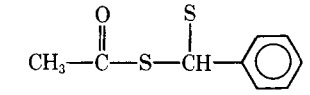


II

By utilizing the procedure of Böhme *et al.* (3) in the reaction of benzaldehyde and thioacetic acid to obtain the hydroxymethyl thioester (II), a small amount of a compound, m.p. 150–151°, which displayed the same properties as the material isolated by Bongartz (1), was obtained. The NMR spectrum showed absorption at 7.32δ, a singlet at 5.85δ, and a singlet at 2.29δ, having an integration ratio of 5:1:3. Mass spectral analysis indicated that the compound contained the following groups:

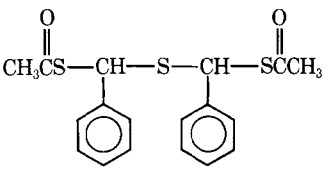
m/e		*m/e*	
15	CH_3	77	C_6H_5
28	CO	78	C_6H_6
32	S	90	C_6H_5CH
43	CH_3CO	105	OCSCHS
58	CH_2CS	121	C_6H_5CHS
60	COS	154	$SCH(C_6H_5)S$
75	CH_3COS	165	$CH_3COSCH(C_6H_5)$

From the NMR and mass spectral data, the following partial structure was assigned:



III

The elemental analysis and molecular weight indicate the compound to be $C_{18}H_{18}O_2S_3$. Thus, we propose the structure of the compound reported by Bongartz (1) as phenylmethanedithiol diacetate to be bis(acetylthiobenzyl)sulfide (IV).



IV

The reaction of benzaldehyde with thioacetic acid was conducted as follows. Benzaldehyde, 21.2 g. (0.2 mole), and thioacetic acid, 15.2 g. (0.2 mole), were mixed together and heated at 100° for 18 hr. After cooling, 1.6 g. of a white crystalline solid was obtained. The solid was recrystallized from methanol and then from petroleum ether (63–68°), and it was identified as bis(acetylthiobenzyl)sulfide (IV), m.p. 150–151°. The NMR and IR are in agreement with the assigned structure.

Anal.—Calcd. for $C_{18}H_{18}O_2S_3$: C, 59.63; H, 5.00; S, 26.54; mol. wt., 362. Found: C, 59.43; H, 5.02; S, 27.10; mol. wt., 361 (osmometer).

Mass spectral data were obtained from a Nuclide mass spectrometer.

(1) J. Bongartz, *Chem. Ber.*, **1886**, 1934.
(2) T. L. Cairns, G. L. Evans, A. W. Larchar, and B. C. McKusick, *J. Amer. Chem. Soc.*, **74**, 3982(1952).
(3) H. Böhme, H. Bezzenberger, M. Clement, A. Dick, E. Nürnberg, and W. Schlephack, *Ann. Chem.*, **623**, 92(1958).

E. E. SMISSMAN
W. L. ALBRECHT
Department of Medicinal Chemistry
School of Pharmacy
University of Kansas
Lawrence, KS 66044

Received April 10, 1970.
Accepted for publication June 29, 1970.
The authors gratefully acknowledge the support of this project by the National Institutes of Health, Grant GM-09254.

urea solutions. In turn, Nogami *et al.* (6) analyzed the adsorption of tryptophan from urea solutions in much the same manner, using the Feldman and Gibaldi paper as a reference. There are implications of direct interaction in each of the results of the present investigation. Perhaps the most striking evidence of this interaction is that the introduction of methyl *o*-methoxybenzoate, a liquid, into a 5 *M* urea solution results immediately in the formation of a flocculent white precipitate, the composition of which is currently unknown.

These findings demonstrate the need for caution in interpreting solubility phenomena involving cosolvent systems, in general, and urea–water mixtures, in particular. At present, few, if any, simplifying assumptions appear to be valid for this purpose.

(1) D. B. Wetlaufer, S. K. Malik, L. Stoller, and R. L. Coffin, *J. Amer. Chem. Soc.*, **86**, 508(1964).
(2) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **238**, 4074(1963).
(3) T. Higuchi and S. Bolton, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 557(1959).
(4) T. Higuchi and K. A. Connors, *Advan. Anal. Chem. Instr.*, **4**, 117(1965).
(5) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **56**, 370(1967).
(6) H. Nogami, T. Nagai, E. Fukuoka, and H. Uchida, *Chem. Pharm. Bull.*, **16**, 2248(1968).

RICHARD E. LINDSTROM
Division of Pharmaceutics
School of Pharmacy
University of Connecticut
Storrs, CT 06268

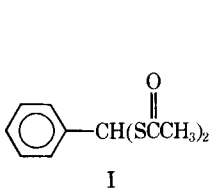
Received June 5, 1970.
Accepted for publication July 20, 1970.
This investigation was supported in part by Grant 093 from the University of Connecticut Research Foundation, Storrs, CT 06268

Preparation of Bis(acetylthiobenzyl)sulfide

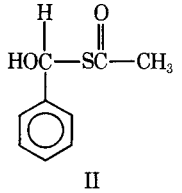
Keyphrases ☐ Bis(acetylthiobenzyl)sulfide—synthesis ☐ NMR spectroscopy—structure ☐ Mass spectroscopy—structure

Sir:

Bongartz (1), in 1886, obtained a product from the reaction of benzaldehyde and thioacetic acid, m.p. 147–148°, which he believed to be phenylmethanedithiol diacetate (I). In 1952, Cairns *et al.* (2) synthesized this compound from phenylmethanedithiol and found the melting point to be 37–38°.



I

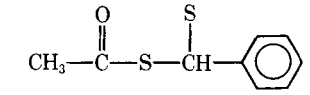


II

By utilizing the procedure of Böhme *et al.* (3) in the reaction of benzaldehyde and thioacetic acid to obtain the hydroxymethyl thioester (II), a small amount of a compound, m.p. 150–151°, which displayed the same properties as the material isolated by Bongartz (1), was obtained. The NMR spectrum showed absorption at 7.32δ, a singlet at 5.85δ, and a singlet at 2.29δ, having an integration ratio of 5:1:3. Mass spectral analysis indicated that the compound contained the following groups:

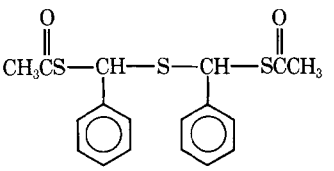
m/e		*m/e*	
15	CH_3	77	C_6H_5
28	CO	78	C_6H_6
32	S	90	C_6H_5CH
43	CH_3CO	105	OCSCHS
58	CH_2CS	121	C_6H_5CHS
60	COS	154	$SCH(C_6H_5)S$
75	CH_3COS	165	$CH_3COSCH(C_6H_5)$

From the NMR and mass spectral data, the following partial structure was assigned:



III

The elemental analysis and molecular weight indicate the compound to be $C_{18}H_{18}O_2S_3$. Thus, we propose the structure of the compound reported by Bongartz (1) as phenylmethanedithiol diacetate to be bis(acetylthiobenzyl)sulfide (IV).



IV

The reaction of benzaldehyde with thioacetic acid was conducted as follows. Benzaldehyde, 21.2 g. (0.2 mole), and thioacetic acid, 15.2 g. (0.2 mole), were mixed together and heated at 100° for 18 hr. After cooling, 1.6 g. of a white crystalline solid was obtained. The solid was recrystallized from methanol and then from petroleum ether (63–68°), and it was identified as bis(acetylthiobenzyl)sulfide (IV), m.p. 150–151°. The NMR and IR are in agreement with the assigned structure.

Anal.—Calcd. for $C_{18}H_{18}O_2S_3$: C, 59.63; H, 5.00; S, 26.54; mol. wt., 362. Found: C, 59.43; H, 5.02; S, 27.10; mol. wt., 361 (osmometer).

Mass spectral data were obtained from a Nuclide mass spectrometer.

(1) J. Bongartz, *Chem. Ber.*, **1886**, 1934.
(2) T. L. Cairns, G. L. Evans, A. W. Larchar, and B. C. McKusick, *J. Amer. Chem. Soc.*, **74**, 3982(1952).
(3) H. Böhme, H. Bezzenberger, M. Clement, A. Dick, E. Nürnberg, and W. Schlephack, *Ann. Chem.*, **623**, 92(1958).

E. E. SMISSMAN
W. L. ALBRECHT
Department of Medicinal Chemistry
School of Pharmacy
University of Kansas
Lawrence, KS 66044

Received April 10, 1970.
Accepted for publication June 29, 1970.
The authors gratefully acknowledge the support of this project by the National Institutes of Health, Grant GM-09254.

Gas Chromatography in Biology and Medicine. *A Ciba Foundation Symposium.* Edited by RUTH PORTER. J. & A. Churchill, 104 Gloucester Place, London, England. 1969. 224 pp. Price $9.00.

This is another excellent monograph published under the auspices of the Ciba Foundation. This foundation is well known to those in the chemical and biological sciences for the numerous and excellent symposia it presents annually at which internationally known scientists participate in presenting papers dealing not only with original research but also in presenting lucid reviews of the "State of the Science" in which they are participating. The proceedings of these symposia are published as monographs in their entirety, thus presenting not only the formal papers but also the usually lively discussions which take place following each presentation. These following discussions are often the more interesting part of the proceedings, which are usually omitted in monographs of most other symposia. Once again the high standards of excellence of this Ciba Foundation Symposium have been preserved, resulting in a publication that makes a substantial contribution to the literature available on the subject.

Gas Chromatography in Biology and Medicine is a very appropriate title because this is the area of scientific endeavor where the technique is put to the ultimate tests of sensitivity, specificity, and usefulness. The formal papers published herein were presented at a symposium of the same title held in London, England, February 1969.

The material covered is divided into four main sections. The *Introduction* contains a survey of the historical background of chromatographic separation by A. J. P. Martin and an account of the many significant developments in gas chromatography, especially when used with ancillary techniques such as mass spectrometry and NMR, by S. R. Lipsky.

The second section is devoted to the *design* of the chromatographic system. J. H. Parnell presents a good argument for the use of the shortest column length possible for achieving the desired separation and also recommends that more emphasis be placed in this regard on improving the selectivity factor of a column rather than the number of theoretical plates generated. The subject of detectors is well covered by D. W. Hill, especially with respect to ionization detectors such as electron-capture detectors which are being extensively used in drug analysis at the nanogram to picogram level of detection from biological specimens. The use of the pulsed D. C. mode of E. C. detection produces greater linearity and reproducibility with these detectors. The effects of water vapor on argon ionization detectors is discussed by B. C. H. Warren and M. G. Dalzell, while the use of the W-value detector in the determination of oxygen and anesthetic vapors in expired air is discussed by B. C. H. Warren and J. E. Lovelock—a pioneer worker in the design of ionization detectors.

The *Biological and Medical Application section* covers the bulk of the monograph with several very interesting and informative chapters—*viz.*,

- H. L. Lowe and K. Hagler—Determination of volatile organic anesthetics in blood, gases, tissues, and lipids using their partition coefficients for differential extraction, and represents an interesting thermodynamic approach to the determination of the tissue to gas partition coefficients.
- M. J. Pumes—Measurement of the gas content of blood samples using gas chromatography.
- A. S. Curry—Recent developments in the use of gas chromatography in forensic toxicology.
- D. J. Blackmore—The use of gas liquid chromatography in aircraft accident toxicology. These two chapters give a very informative account of the problems facing the toxicologist and the pathologist in determining whether the cause of death is accidental or intentional. This is an area which tests the analytical competence of the practicing forensic toxicologist in his ability to come up with the right answer to trace amounts of foreign materials recovered from the subject or the scene of event where the sensitivity and specificity of a gas chromatographic procedure are the only means of obtaining reliable and foolproof evidence.
- S. Garattini, F. Marcucci, and E. Mussini—Gas chromatographic analysis of benzodiazepines. This is an interesting account of the use of GLC with different detectors (flame and electron capture) in the determination of members of a class of drugs (tranquilizers) and their major metabolites in animal species and in man. The significance of the blood and tissue levels of the drug in animal species, its influence on the pharmacological responses obtained, and the interpretation and extrapolation of these findings to clinical observations seen in man are presented. This chapter highlights the results from several other publications by the same authors.

The final section covers future trends in development where the use of complementary techniques in filtering the information obtained from GLC alone is presented.

R. P. W. Scott—Gas chromatographic and spectrometric techniques, covers the problems of interphasing a mass spectrometer with a gas chromatograph with computerized data handling facilities, a technique which is of great use in the isolation and characterization of drugs and their metabolites in biological extracts. The use of complementary techniques such as IR and NMR using micro cells to enhance sensitivity of detection of the fractions trapped from the effluent of a gas chromatographic column is discussed.

- G. B. Marson—Digital computers and the analysis of chromatographic data, presents some of the hardware used in automating the data handling and interpretation of GLC data.

All the papers presented are concise, well illustrated, and documented with up-to-date literature references making this volume as current as such a monograph can possibly be. The discussions following each presentation are very thought-stimulating and make the reader wish he were participating actively in it rather than reading about it second hand.

This monograph is in keeping, with respect to its content and presentation, with the philosophy of the Ciba Foundation: "Consocient Gentes"—let the peoples come together. It is a worthy addition to the library of the practicing chromatographer.

Reviewed by J. A. F. deSilva
Hoffmann-La Roche Inc.
Nutley, NJ 07110 ■

The History of Penicillin Production. Chemical Engineering Progress Symposium Series, No. 100, Vol. 66. Edited by ALBERT L. ELDER. American Institute of Chemical Engineers, 345 East 47th St., New York, NY 10017, 1970. vi + 100 pp. 21.5 × 28 cm.

This volume of the *Chemical Engineering Progress Symposium Series* deals with the history and problems of developing mass-produced penicillin, necessitated by the Second World War. The Institute hopes, by historically documenting particular projects, this knowledge might be used to facilitate and expedite future projects of a similar or related nature.

Staff Review ■

Gas Chromatography in Biology and Medicine. *A Ciba Foundation Symposium.* Edited by RUTH PORTER. J. & A. Churchill, 104 Gloucester Place, London, England. 1969. 224 pp. Price $9.00.

This is another excellent monograph published under the auspices of the Ciba Foundation. This foundation is well known to those in the chemical and biological sciences for the numerous and excellent symposia it presents annually at which internationally known scientists participate in presenting papers dealing not only with original research but also in presenting lucid reviews of the "State of the Science" in which they are participating. The proceedings of these symposia are published as monographs in their entirety, thus presenting not only the formal papers but also the usually lively discussions which take place following each presentation. These following discussions are often the more interesting part of the proceedings, which are usually omitted in monographs of most other symposia. Once again the high standards of excellence of this Ciba Foundation Symposium have been preserved, resulting in a publication that makes a substantial contribution to the literature available on the subject.

Gas Chromatography in Biology and Medicine is a very appropriate title because this is the area of scientific endeavor where the technique is put to the ultimate tests of sensitivity, specificity, and usefulness. The formal papers published herein were presented at a symposium of the same title held in London, England, February 1969.

The material covered is divided into four main sections. The *Introduction* contains a survey of the historical background of chromatographic separation by A. J. P. Martin and an account of the many significant developments in gas chromatography, especially when used with ancillary techniques such as mass spectrometry and NMR, by S. R. Lipsky.

The second section is devoted to the *design* of the chromatographic system. J. H. Parnell presents a good argument for the use of the shortest column length possible for achieving the desired separation and also recommends that more emphasis be placed in this regard on improving the selectivity factor of a column rather than the number of theoretical plates generated. The subject of detectors is well covered by D. W. Hill, especially with respect to ionization detectors such as electron-capture detectors which are being extensively used in drug analysis at the nanogram to picogram level of detection from biological specimens. The use of the pulsed D. C. mode of E. C. detection produces greater linearity and reproducibility with these detectors. The effects of water vapor on argon ionization detectors is discussed by B. C. H. Warren and M. G. Dalzell, while the use of the W-value detector in the determination of oxygen and anesthetic vapors in expired air is discussed by B. C. H. Warren and J. E. Lovelock—a pioneer worker in the design of ionization detectors.

The *Biological and Medical Application section* covers the bulk of the monograph with several very interesting and informative chapters—*viz.*,

- H. L. Lowe and K. Hagler—Determination of volatile organic anesthetics in blood, gases, tissues, and lipids using their partition coefficients for differential extraction, and represents an interesting thermodynamic approach to the determination of the tissue to gas partition coefficients.
- M. J. Pumes—Measurement of the gas content of blood samples using gas chromatography.
- A. S. Curry—Recent developments in the use of gas chromatography in forensic toxicology.
- D. J. Blackmore—The use of gas liquid chromatography in aircraft accident toxicology. These two chapters give a very informative account of the problems facing the toxicologist and the pathologist in determining whether the cause of death is accidental or intentional. This is an area which tests the analytical competence of the practicing forensic toxicologist in his ability to come up with the right answer to trace amounts of foreign materials recovered from the subject or the scene of event where the sensitivity and specificity of a gas chromatographic procedure are the only means of obtaining reliable and foolproof evidence.
- S. Garattini, F. Marcucci, and E. Mussini—Gas chromatographic analysis of benzodiazepines. This is an interesting account of the use of GLC with different detectors (flame and electron capture) in the determination of members of a class of drugs (tranquilizers) and their major metabolites in animal species and in man. The significance of the blood and tissue levels of the drug in animal species, its influence on the pharmacological responses obtained, and the interpretation and extrapolation of these findings to clinical observations seen in man are presented. This chapter highlights the results from several other publications by the same authors.

The final section covers future trends in development where the use of complementary techniques in filtering the information obtained from GLC alone is presented.

R. P. W. Scott—Gas chromatographic and spectrometric techniques, covers the problems of interphasing a mass spectrometer with a gas chromatograph with computerized data handling facilities, a technique which is of great use in the isolation and characterization of drugs and their metabolites in biological extracts. The use of complementary techniques such as IR and NMR using micro cells to enhance sensitivity of detection of the fractions trapped from the effluent of a gas chromatographic column is discussed.

- G. B. Marson—Digital computers and the analysis of chromatographic data, presents some of the hardware used in automating the data handling and interpretation of GLC data.

All the papers presented are concise, well illustrated, and documented with up-to-date literature references making this volume as current as such a monograph can possibly be. The discussions following each presentation are very thought-stimulating and make the reader wish he were participating actively in it rather than reading about it second hand.

This monograph is in keeping, with respect to its content and presentation, with the philosophy of the Ciba Foundation: "Consocient Gentes"—let the peoples come together. It is a worthy addition to the library of the practicing chromatographer.

Reviewed by J. A. F. deSilva
Hoffmann-La Roche Inc.
Nutley, NJ 07110 ■

The History of Penicillin Production. Chemical Engineering Progress Symposium Series, No. 100, Vol. 66. Edited by ALBERT L. ELDER. American Institute of Chemical Engineers, 345 East 47th St., New York, NY 10017, 1970. vi + 100 pp. 21.5 × 28 cm.

This volume of the *Chemical Engineering Progress Symposium Series* deals with the history and problems of developing mass-produced penicillin, necessitated by the Second World War. The Institute hopes, by historically documenting particular projects, this knowledge might be used to facilitate and expedite future projects of a similar or related nature.

Staff Review ■

Clinical Pharmacology Scope, Organization, Training. By WHO Study Group, World Health Organization, Geneva, Switzerland, 1970. i + 21 pp. 23.5 × 16 cm. Price: Annual Subscription $16.00. (*English*)

The role of the clinical pharmacologist has recently received considerable attention. The World Health Organization Study Group on Clinical Pharmacology was convened to suggest means of eliminating the shortage of clinical pharmacologists, to demarcate the scope of clinical pharmacology, and to suggest ways for carrying out drug studies in all parts of the world in a scientific and coordinated manner.

The WHO report provides considerable information relative to clinical pharmacology and could be used as a focus for future discussions concerning this emerging discipline.

Staff Review ■

Modern Microcrystal Tests for Drugs, The Identification of Organic Compounds by Microcrystalloscopic Chemistry. By CHARLES W. FULTON. Wiley, New York, NY 10016, 1969. xviii + 466 pp. 17.5 × 26 cm. Price $29.95.

Early in the book a table is presented to illustrate what the author considers the best aqueous tests with the 28 best reagents of the past for identifying alkaloids. The author has added also a number of nonalkaloidal but related substances to the list of materials tested and points out that although many of the tests are old, some of them being over a hundred years old, they yield reliable results with each of the 159 substances tested.

The author then discusses some new procedures that he has developed in microcrystal techniques. He describes tests conducted in acid media and procedures for testing the volatility of various chemical classes of substances. As a toxicologist he extends volatility tests to the detection of putrefactive bases. He also includes chromatography in general procedures for testing unknowns and indicates the usefulness of spectrophotometry.

In separate chapters, tests for barbiturates, sympathomimetics and central stimulants, phenothiazines, and steroids are presented. The author also discusses oxonium and carbonium compounds and iodine–iodide complexes; he describes general coverage tests, specific tests, and color tests.

The author explains crystal shapes in unique descriptive terms and he presents a system of classification of microcrystals. In the last of the 31 chapters are included 192 very good photomicrographs to illustrate his classification of crystal forms and also to illustrate important tests and significant reactions. Accompanying the photomicrographs are word descriptions of the crystal forms.

The index lists all of the substances for which tests are described in the book and includes a directory of essential information for each of these materials. It should be pointed out also that this book contains a very complete bibliography of literature in the form of appropriate references at the conclusion of each chapter.

The advent of many new, potent drugs has complicated the task of the toxicologist in using microcrystal tests for identification purposes. The author has included a number of new synthetic drugs in his tests and he points out that at least two or three different tests must yield positive results with both the unknown and with known material in order to be sure of the unknown tested. This book is recommended as a useful reference to those working with microcrystal identification techniques.

Reviewed by Elmer M. Plein
College of Pharmacy
University of Washington
Seattle, WA 98105 ■

The Control of Chemotherapy. Edited by P. J. WATT. E. & S. Livingstone Ltd., London, England, 1970. i + 109 pp. 14 × 22 cm. Price $6.25.

The papers in this volume were presented at a symposium held in March 1969, in the Wright-Fleming Institute, St. Mary's Hospital Medical School, London, England. Starting from the premise that the treatment of infections with chemotherapeutic drugs can only be effective if the drug that is used is one to which the bacteria are sensitive and which can reach the site of infection, the symposium considered areas in which progress may be expected and areas in which inadequate techniques are still in use.

Staff Review ■

NOTICES

Chemical Control of Fibrinolysis-Thrombolysis. Edited by JOSEPH M. SCHOR. Wiley, 605 Third Ave., New York, NY 10016, 1970. xi + 328 pp. 15.5 × 23.5 cm. Price $17.95.

Programmed Thermodynamics. Vol. 1. By CHARLES E. WALES. McGraw-Hill, 330 West 42nd St., New York, NY 10036, 1970. ix + 316 pp. 21 × 28 cm. Price $5.95.

The Dictocrats. Our Unelected Rulers. By OMAR V. GARRISON. Specialty Features Syndicate, 17261 Reuford Ave., Detroit, MI 48219, 1970. xi + 333 pp. 10.5 × 18 cm. Price $1.25.

Arzneimittelwirkungen. Ein Lehrbuch der Pharmakologie fur Pharmazeuten, Chemiker und Biologen. By ERNST MUTSCHLER. Wissenschaftliche Verlagsgesellschaft mbh Stuttgart, Germany, 1970. xvi + 477 pp. 17 × 24.5 cm. Price DM 48. (*German*)

Parathyroid Hormone, Thyrocalcitonin and Related Drugs. Section 51, Vol. 1, International Encyclopedia of Pharmacology and Therapeutics. Edited by G. PETERS. Lausanne, Pergamon Press Ltd., Headington Hill Hall, Oxford, England, 1970. vii + 364 pp. 15.5 × 23.5 cm. Price $16.00.

Antibiotics in Clinical Practice. By HILLAS SMITH. Williams & Wilkins, 428 E. Preston St., Baltimore, MD 21202, 1969. ix + 346 pp. 15 × 23 cm. Price $11.75.

Clinical Pharmacology Scope, Organization, Training. By WHO Study Group, World Health Organization, Geneva, Switzerland, 1970. i + 21 pp. 23.5 × 16 cm. Price: Annual Subscription $16.00. (*English*)

The role of the clinical pharmacologist has recently received considerable attention. The World Health Organization Study Group on Clinical Pharmacology was convened to suggest means of eliminating the shortage of clinical pharmacologists, to demarcate the scope of clinical pharmacology, and to suggest ways for carrying out drug studies in all parts of the world in a scientific and coordinated manner.

The WHO report provides considerable information relative to clinical pharmacology and could be used as a focus for future discussions concerning this emerging discipline.

Staff Review ■

Modern Microcrystal Tests for Drugs, The Identification of Organic Compounds by Microcrystalloscopic Chemistry. By CHARLES W. FULTON. Wiley, New York, NY 10016, 1969. xviii + 466 pp. 17.5 × 26 cm. Price $29.95.

Early in the book a table is presented to illustrate what the author considers the best aqueous tests with the 28 best reagents of the past for identifying alkaloids. The author has added also a number of nonalkaloidal but related substances to the list of materials tested and points out that although many of the tests are old, some of them being over a hundred years old, they yield reliable results with each of the 159 substances tested.

The author then discusses some new procedures that he has developed in microcrystal techniques. He describes tests conducted in acid media and procedures for testing the volatility of various chemical classes of substances. As a toxicologist he extends volatility tests to the detection of putrefactive bases. He also includes chromatography in general procedures for testing unknowns and indicates the usefulness of spectrophotometry.

In separate chapters, tests for barbiturates, sympathomimetics and central stimulants, phenothiazines, and steroids are presented. The author also discusses oxonium and carbonium compounds and iodine–iodide complexes; he describes general coverage tests, specific tests, and color tests.

The author explains crystal shapes in unique descriptive terms and he presents a system of classification of microcrystals. In the last of the 31 chapters are included 192 very good photomicrographs to illustrate his classification of crystal forms and also to illustrate important tests and significant reactions. Accompanying the photomicrographs are word descriptions of the crystal forms.

The index lists all of the substances for which tests are described in the book and includes a directory of essential information for each of these materials. It should be pointed out also that this book contains a very complete bibliography of literature in the form of appropriate references at the conclusion of each chapter.

The advent of many new, potent drugs has complicated the task of the toxicologist in using microcrystal tests for identification purposes. The author has included a number of new synthetic drugs in his tests and he points out that at least two or three different tests must yield positive results with both the unknown and with known material in order to be sure of the unknown tested. This book is recommended as a useful reference to those working with microcrystal identification techniques.

Reviewed by Elmer M. Plein
College of Pharmacy
University of Washington
Seattle, WA 98105 ■

The Control of Chemotherapy. Edited by P. J. WATT. E. & S. Livingstone Ltd., London, England, 1970. i + 109 pp. 14 × 22 cm. Price $6.25.

The papers in this volume were presented at a symposium held in March 1969, in the Wright-Fleming Institute, St. Mary's Hospital Medical School, London, England. Starting from the premise that the treatment of infections with chemotherapeutic drugs can only be effective if the drug that is used is one to which the bacteria are sensitive and which can reach the site of infection, the symposium considered areas in which progress may be expected and areas in which inadequate techniques are still in use.

Staff Review ■

NOTICES

Chemical Control of Fibrinolysis-Thrombolysis. Edited by JOSEPH M. SCHOR. Wiley, 605 Third Ave., New York, NY 10016, 1970. xi + 328 pp. 15.5 × 23.5 cm. Price $17.95.

Programmed Thermodynamics. Vol. 1. By CHARLES E. WALES. McGraw-Hill, 330 West 42nd St., New York, NY 10036, 1970. ix + 316 pp. 21 × 28 cm. Price $5.95.

The Dictocrats. Our Unelected Rulers. By OMAR V. GARRISON. Specialty Features Syndicate, 17261 Reuford Ave., Detroit, MI 48219, 1970. xi + 333 pp. 10.5 × 18 cm. Price $1.25.

Arzneimittelwirkungen. Ein Lehrbuch der Pharmakologie fur Pharmazeuten, Chemiker und Biologen. By ERNST MUTSCHLER. Wissenschaftliche Verlagsgesellschaft mbh Stuttgart, Germany, 1970. xvi + 477 pp. 17 × 24.5 cm. Price DM 48. (*German*)

Parathyroid Hormone, Thyrocalcitonin and Related Drugs. Section 51, Vol. 1, International Encyclopedia of Pharmacology and Therapeutics. Edited by G. PETERS. Lausanne, Pergamon Press Ltd., Headington Hill Hall, Oxford, England, 1970. vii + 364 pp. 15.5 × 23.5 cm. Price $16.00.

Antibiotics in Clinical Practice. By HILLAS SMITH. Williams & Wilkins, 428 E. Preston St., Baltimore, MD 21202, 1969. ix + 346 pp. 15 × 23 cm. Price $11.75.

Clinical Pharmacology Scope, Organization, Training. By WHO Study Group, World Health Organization, Geneva, Switzerland, 1970. i + 21 pp. 23.5 × 16 cm. Price: Annual Subscription $16.00. (*English*)

The role of the clinical pharmacologist has recently received considerable attention. The World Health Organization Study Group on Clinical Pharmacology was convened to suggest means of eliminating the shortage of clinical pharmacologists, to demarcate the scope of clinical pharmacology, and to suggest ways for carrying out drug studies in all parts of the world in a scientific and coordinated manner.

The WHO report provides considerable information relative to clinical pharmacology and could be used as a focus for future discussions concerning this emerging discipline.

Staff Review ■

Modern Microcrystal Tests for Drugs, The Identification of Organic Compounds by Microcrystalloscopic Chemistry. By CHARLES W. FULTON. Wiley, New York, NY 10016, 1969. xviii + 466 pp. 17.5 × 26 cm. Price $29.95.

Early in the book a table is presented to illustrate what the author considers the best aqueous tests with the 28 best reagents of the past for identifying alkaloids. The author has added also a number of nonalkaloidal but related substances to the list of materials tested and points out that although many of the tests are old, some of them being over a hundred years old, they yield reliable results with each of the 159 substances tested.

The author then discusses some new procedures that he has developed in microcrystal techniques. He describes tests conducted in acid media and procedures for testing the volatility of various chemical classes of substances. As a toxicologist he extends volatility tests to the detection of putrefactive bases. He also includes chromatography in general procedures for testing unknowns and indicates the usefulness of spectrophotometry.

In separate chapters, tests for barbiturates, sympathomimetics and central stimulants, phenothiazines, and steroids are presented. The author also discusses oxonium and carbonium compounds and iodine–iodide complexes; he describes general coverage tests, specific tests, and color tests.

The author explains crystal shapes in unique descriptive terms and he presents a system of classification of microcrystals. In the last of the 31 chapters are included 192 very good photomicrographs to illustrate his classification of crystal forms and also to illustrate important tests and significant reactions. Accompanying the photomicrographs are word descriptions of the crystal forms.

The index lists all of the substances for which tests are described in the book and includes a directory of essential information for each of these materials. It should be pointed out also that this book contains a very complete bibliography of literature in the form of appropriate references at the conclusion of each chapter.

The advent of many new, potent drugs has complicated the task of the toxicologist in using microcrystal tests for identification purposes. The author has included a number of new synthetic drugs in his tests and he points out that at least two or three different tests must yield positive results with both the unknown and with known material in order to be sure of the unknown tested. This book is recommended as a useful reference to those working with microcrystal identification techniques.

Reviewed by Elmer M. Plein
College of Pharmacy
University of Washington
Seattle, WA 98105 ■

The Control of Chemotherapy. Edited by P. J. WATT. E. & S. Livingstone Ltd., London, England, 1970. i + 109 pp. 14 × 22 cm. Price $6.25.

The papers in this volume were presented at a symposium held in March 1969, in the Wright-Fleming Institute, St. Mary's Hospital Medical School, London, England. Starting from the premise that the treatment of infections with chemotherapeutic drugs can only be effective if the drug that is used is one to which the bacteria are sensitive and which can reach the site of infection, the symposium considered areas in which progress may be expected and areas in which inadequate techniques are still in use.

Staff Review ■

NOTICES

Chemical Control of Fibrinolysis-Thrombolysis. Edited by JOSEPH M. SCHOR. Wiley, 605 Third Ave., New York, NY 10016, 1970. xi + 328 pp. 15.5 × 23.5 cm. Price $17.95.

Programmed Thermodynamics. Vol. 1. By CHARLES E. WALES. McGraw-Hill, 330 West 42nd St., New York, NY 10036, 1970. ix + 316 pp. 21 × 28 cm. Price $5.95.

The Dictocrats. Our Unelected Rulers. By OMAR V. GARRISON. Specialty Features Syndicate, 17261 Reuford Ave., Detroit, MI 48219, 1970. xi + 333 pp. 10.5 × 18 cm. Price $1.25.

Arzneimittelwirkungen. Ein Lehrbuch der Pharmakologie fur Pharmazeuten, Chemiker und Biologen. By ERNST MUTSCHLER. Wissenschaftliche Verlagsgesellschaft mbh Stuttgart, Germany, 1970. xvi + 477 pp. 17 × 24.5 cm. Price DM 48. (*German*)

Parathyroid Hormone, Thyrocalcitonin and Related Drugs. Section 51, Vol. 1, International Encyclopedia of Pharmacology and Therapeutics. Edited by G. PETERS. Lausanne, Pergamon Press Ltd., Headington Hill Hall, Oxford, England, 1970. vii + 364 pp. 15.5 × 23.5 cm. Price $16.00.

Antibiotics in Clinical Practice. By HILLAS SMITH. Williams & Wilkins, 428 E. Preston St., Baltimore, MD 21202, 1969. ix + 346 pp. 15 × 23 cm. Price $11.75.

Clinical Pharmacology Scope, Organization, Training. By WHO Study Group, World Health Organization, Geneva, Switzerland, 1970. i + 21 pp. 23.5 × 16 cm. Price: Annual Subscription $16.00. (*English*)

The role of the clinical pharmacologist has recently received considerable attention. The World Health Organization Study Group on Clinical Pharmacology was convened to suggest means of eliminating the shortage of clinical pharmacologists, to demarcate the scope of clinical pharmacology, and to suggest ways for carrying out drug studies in all parts of the world in a scientific and coordinated manner.

The WHO report provides considerable information relative to clinical pharmacology and could be used as a focus for future discussions concerning this emerging discipline.

Staff Review ■

Modern Microcrystal Tests for Drugs, The Identification of Organic Compounds by Microcrystalloscopic Chemistry. By CHARLES W. FULTON. Wiley, New York, NY 10016, 1969. xviii + 466 pp. 17.5 × 26 cm. Price $29.95.

Early in the book a table is presented to illustrate what the author considers the best aqueous tests with the 28 best reagents of the past for identifying alkaloids. The author has added also a number of nonalkaloidal but related substances to the list of materials tested and points out that although many of the tests are old, some of them being over a hundred years old, they yield reliable results with each of the 159 substances tested.

The author then discusses some new procedures that he has developed in microcrystal techniques. He describes tests conducted in acid media and procedures for testing the volatility of various chemical classes of substances. As a toxicologist he extends volatility tests to the detection of putrefactive bases. He also includes chromatography in general procedures for testing unknowns and indicates the usefulness of spectrophotometry.

In separate chapters, tests for barbiturates, sympathomimetics and central stimulants, phenothiazines, and steroids are presented. The author also discusses oxonium and carbonium compounds and iodine–iodide complexes; he describes general coverage tests, specific tests, and color tests.

The author explains crystal shapes in unique descriptive terms and he presents a system of classification of microcrystals. In the last of the 31 chapters are included 192 very good photomicrographs to illustrate his classification of crystal forms and also to illustrate important tests and significant reactions. Accompanying the photomicrographs are word descriptions of the crystal forms.

The index lists all of the substances for which tests are described in the book and includes a directory of essential information for each of these materials. It should be pointed out also that this book contains a very complete bibliography of literature in the form of appropriate references at the conclusion of each chapter.

The advent of many new, potent drugs has complicated the task of the toxicologist in using microcrystal tests for identification purposes. The author has included a number of new synthetic drugs in his tests and he points out that at least two or three different tests must yield positive results with both the unknown and with known material in order to be sure of the unknown tested. This book is recommended as a useful reference to those working with microcrystal identification techniques.

Reviewed by Elmer M. Plein
College of Pharmacy
University of Washington
Seattle, WA 98105 ■

The Control of Chemotherapy. Edited by P. J. WATT. E. & S. Livingstone Ltd., London, England, 1970. i + 109 pp. 14 × 22 cm. Price $6.25.

The papers in this volume were presented at a symposium held in March 1969, in the Wright-Fleming Institute, St. Mary's Hospital Medical School, London, England. Starting from the premise that the treatment of infections with chemotherapeutic drugs can only be effective if the drug that is used is one to which the bacteria are sensitive and which can reach the site of infection, the symposium considered areas in which progress may be expected and areas in which inadequate techniques are still in use.

Staff Review ■

NOTICES

Chemical Control of Fibrinolysis-Thrombolysis. Edited by JOSEPH M. SCHOR. Wiley, 605 Third Ave., New York, NY 10016, 1970. xi + 328 pp. 15.5 × 23.5 cm. Price $17.95.

Programmed Thermodynamics. Vol. 1. By CHARLES E. WALES. McGraw-Hill, 330 West 42nd St., New York, NY 10036, 1970. ix + 316 pp. 21 × 28 cm. Price $5.95.

The Dictocrats. Our Unelected Rulers. By OMAR V. GARRISON. Specialty Features Syndicate, 17261 Reuford Ave., Detroit, MI 48219, 1970. xi + 333 pp. 10.5 × 18 cm. Price $1.25.

Arzneimittelwirkungen. Ein Lehrbuch der Pharmakologie fur Pharmazeuten, Chemiker und Biologen. By ERNST MUTSCHLER. Wissenschaftliche Verlagsgesellschaft mbh Stuttgart, Germany, 1970. xvi + 477 pp. 17 × 24.5 cm. Price DM 48. (*German*)

Parathyroid Hormone, Thyrocalcitonin and Related Drugs. Section 51, Vol. 1, International Encyclopedia of Pharmacology and Therapeutics. Edited by G. PETERS. Lausanne, Pergamon Press Ltd., Headington Hill Hall, Oxford, England, 1970. vii + 364 pp. 15.5 × 23.5 cm. Price $16.00.

Antibiotics in Clinical Practice. By HILLAS SMITH. Williams & Wilkins, 428 E. Preston St., Baltimore, MD 21202, 1969. ix + 346 pp. 15 × 23 cm. Price $11.75.

Journal of Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

INDEX TO AUTHORS
INDEX TO SUBJECTS

VOLUME 59
JANUARY TO DECEMBER, 1970

Published monthly under the supervision of the Committee on Publications with the approval of the Board of Trustees

Journal of Pharmaceutical Sciences

DECEMBER 1970
VOLUME 59 NUMBER 12

REVIEW ARTICLE

Peyote Constituents: Chemistry, Biogenesis, and Biological Effects

GOVIND J. KAPADIA and M. B. E. FAYEZ

Keyphrases ☐ Peyote constituents—chemistry ☐ Biogenesis—peyote alkaloids ☐ Biological effects—peyote constituents ☐ Mescaline—behavioral effects ☐ Addiction, habituation, tolerance—mescaline ☐ Metabolism—mescaline ☐ Structure-activity relationships—mescaline derivatives

CONTENTS

BACKGROUND

Peyote represents one of the earliest known hallucinogenic drugs, the use of which was exclusively limited to the New World. Today much more potent hallucinogens, of natural and synthetic origin, are known, yet investigation into the chemistry and pharmacology of peyote constituents and their synthetic analogs does not seem to have relented. The literature contains several early surveys covering various aspects of peyote and its constituents. These include articles and monographs dealing with cactus alkaloids (1–7) and natural hallucinogens (8–27) in general and more specialized ones dealing mainly with peyote and its active principle, mescaline (28–40). Several of the earlier articles also cover, more or less adequately, such diverse aspects of peyote as history, cultic and modern uses, chemical composition, synthesis of its constituents—as phenethylamines (41) and tetrahydroisoquinolines (42)—and the pharmacology of these products and their synthetic analogs along with their possible medicinal applications (40).

In the past decade or so, there has been no such comprehensive coverage in one review. The need for one is accentuated by the fact that during the past 5 years alone, more than 31 new constituents of peyote were discovered (compared to six before 1900 and five during the 1930's), several synthetic methods were developed, and an important part of the pharmacological and clinical (as well as social) studies and practically all the biogenetic studies of the major peyote constituents were conducted, with impressive revelations made.

Names—Peyote, a member of the family Cactaceae, has received varied taxonomical treatments with time by different botanists. The first "scientific" name given to it was *Peyotl zacatensis* by Hernandez in 1638. The designation *Echinocactus williamsii* was used by Lemaire in 1840, *Anhalonium williamsii* by Engelman, *Lophophora williamsii* by Coulter in 1894, *Anhalonium lewinii* by Lewin, and *A. jourdanianum* by Rebut. Although the name *Echinocactus williamsii* appears in the *Kew Index* (vol. II, p. 813), it does not seem to be as commonly used as *A. lewinii* or *L. williamsii* (7, 37). The latter name is the one more commonly used in the current chemical literature. The introduction of these names, and there seems to be more, appears to be the result of confusion between the different types of peyote which actually belong to a single species (37, 39).* The confu-

* According to E. F. Anderson [*Brittonia*, 21, 299(1969)], the genus *Lophophora*, which has an extensive range within the Chihuahuan Desert of Texas and Mexico, consists of two species, *L. williamsii* and *L. diffusa*. The former is both wide ranging and highly variable morphologically, whereas the latter consists of a single, fairly small population restricted to an area in the state of Querétaro.

sion arises from the differences between the young peyote individual specimens, with eight straight ribs, and the mature ones, with about 12 sinuous ribs, and a third form with irregular ribs, in addition to slight morphological variations according to locality.

Peyote is a popular name given to the cactus under discussion; other less common names are peyotl, pellote, challote, and devil's root. The name peyote derives from peyotl which appears to be of Aztec origin. The sun-dried slices of the plant constitute the "mescal buttons" of commerce. It is this material, which can be preserved for a very long time, that is usually consumed[1] in ritual ceremonies or for nonreligious purposes to produce euphoric effects. The name mescal buttons[2] is incorrect, since the material is not related to the noncactus succulent, the mescal proper, from which fermented beverages are prepared. Another incorrect name is "mescal beans," which is also given to the buttons but in fact is the Red Bean, *Sophora secundiflora* (Leguminosae).

Habitat—The cactus (*L. williamsii*) grows wild on the Mexican plateau and in the southwestern United States in dry places, on cliffs, or on rocky slopes. These regions are very hot and the vegetation is subtropical. Peyote is encountered sometimes singly but more often in clusters. It is barely visible, except when in flower, since it is mostly covered with earth and looks like a pebble. Peyote, like most cacti, can be grown practically anywhere under glass or in a room. The plant seems to be capable of standing adverse conditions of drought, heat, and cold.

Description—The peyote plant is a small (rarely exceeding 15–20 cm. in total length), fleshy, spineless cactus, simple or cespitose, proliferous, turbinate with a thick napiform root. The cylindrical stalk, which has horizontal wrinkles, is suberized and grayish-fawn in color. At the top it becomes more chlorophyllose, globular, flattened, and sage-green in color and appears divided into 5–13 thick ribs, only slightly salient, rounded, and separated from each other by well-marked longitudinal grooves. In the center of the spherical portion (2–50 mm. in diameter) of the plant, there is a tuft of silky hairs from which the flowers (usually one or two) emerge; the latter are usually pink but are sometimes white or yellow. The fruit is a flesh-colored, or sometimes grayish-yellow, berry which contains a few small black seeds.

History—Peyote was used and revered—as a panacea, an amulet, and a hallucinogen—in the mountainous regions of northern Mexico centuries before the settlers arrived; it was probably known to the Chichimec tribe long before the Christian era (43). Bernardino de Sahagun (43), a Franciscan missionary and chronicler of the Spanish conquest of Mexico, wrote about peyote, which was eaten by local natives because "it gives them strength and incites them to battle, alleviates fear, and they feel neither hunger nor thirst, and they say it protects them from every kind of danger." At that time, missionaries and administrators tried to suppress peyote consumption for various social, political, and religious reasons, apparently without much success.

During the 17th and 18th centuries, peyote was used in vast areas stretching northward up to the Arkansas River (44), and it was consumed by many Indian tribes including the Apaches, Comanches, and Kiowas. Although a true peyote cult did not seem to have existed at that time, the drug was taken mainly: (*a*) by individuals as a medicine and to induce visions leading to prophetic utterances, and (*b*) collectively to obtain the desired state of trance for ritual dances.

During the 19th century, the ritual use of peyote (peyotism) extended among Indian tribes with their migrations northward and southward and gradually became more fully organized into isolated cult groups (44–46). This led, toward the end of the century, to the establishment of the "Peyote Church." With time, peyote votaries increased in number, variety, and distribution; the Church, founded in Oklahoma, gradually joined in a strange synthesis of old Mexican, Christian, and local religious rites (47). The advocates of this Church remained predominantly Indians, although they were joined by some Negroes, and the cult remained essentially an ethnographical curiosity rather than a serious movement.[3] The present believers worship God as the great spirit who, as a master of the universe, infused some of his being into peyote; Christ is regarded as the man who made the cactus available to them when they needed it. Usually the ceremony is conducted at Saturday night gatherings in a large Indian tepee. Participants then sit around a fire and spend the evening in ritual singing, prayer, and contemplation (39).

In the United States, peyote is not regarded as a narcotic drug, although its use is controlled by the federal government except in *bona fide* religious ceremonies of the Native American Church.

The history, economic importance, cultic use, and psychic effects of peyote are described at length by several authors (9–11, 31–33, 35, 38, 45, 49–58).

Constituents of Peyote—The first chemical investigations of peyote were made by Lewin in 1888 (52). While traveling in America, he collected peyote plants; these were identified by Hennings as a new *Anhalonium* species that was named *A. lewinii*. He was able to isolate the first crystalline constituent, an alkaloid which was called anhalonine and shown to be devoid of any hallucinatory action. This initial finding, however, aroused considerable interest in the drug and the possible constituents that would account for the sensory excitations produced in those who took it. Up to that time, the family Cactaceae was regarded as being free of alkaloids; as a result of Lewin's findings, more attention was directed to the study of several other cacti.

In 1894, Heffter (59–64), working with fresh plants, isolated another alkaloid which received the name pello-

[1] The drug is usually taken orally. The buttons, whole or chopped, are brewed with tea or chewed while drinking tea, coffee, wine, or milk (to conceal an unpleasant taste). Less commonly, a liquid concoction is injected intravenously.

[2] Other slang names include: tops, moon, full moon (a large button) and bad seed.

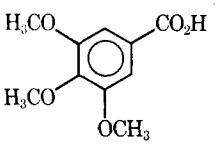
[3] In the United States, about 1950, some ethnologists protested strongly the idea that the use of peyote should be prohibited on the pretext that the Native American Church (practicing peyotism) is a legitimate religious organization with the same right to religious freedom as other churches (48).

tine. Shortly afterward he isolated three additional alkaloids. One, mescaline, was recognized—from pharmacological tests and personal experience—as the hallucinatory principle of peyote; the other two were named anhalonidine and lophophorine (61). In 1899, Kauder (65) discovered an additional alkaloid, anhalamine, besides the previously known pellotine in the mescal buttons. During or about the same period, other cacti yielded related alkaloids (nonhallucinogenic): anhaline (≡hordenine) from *Anahalonium fissuratum* and pectenine (≡carnegine) from *Cereus pecten-aboriginum* (66) and later from *Carnegia gigantea* (67).

Lewin (52, 53) also made the first pharmacological studies of peyote. His reports were followed by more elaborate and expanded studies made by Prentiss and Morgan (68), Mitchell (69), Ellis (70), Dixon (71), and Mogilewa (72), who described the physiological and special euphoric effects of peyote and its constituent alkaloids.

The elucidation of the structure and the synthesis of all these alkaloids had to wait until Späth made his contributions. He published the first of a series of papers on "Anhalonium alkaloids" in 1919 and continued his work up to 1939 (3, 73–90); his work also included studies on the alkaloids obtained from other Cactaceae. In addition to the previously mentioned alkaloids, Späth isolated from peyote five other products: anhalinine and anhalidine in 1935 (80, 81), *N*-methylmescaline in 1937 (83), *N*-acetylmescaline in 1938 (84), and *O*-methyl-*d*-anhalonidine in 1939 (85).

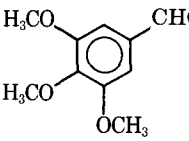
Interest in peyote, at least in research laboratories, remained dormant after the last contributions of Späth until very recent years, presumably with the resurge or increase of interest in the psychotomimetic agents and their sources which has become a hallmark of our days. Three tyramine derivatives were identified in peyote during 1965–1966; they are tyramine and its *N*-methyl and *N,N*-dimethyl (hordenine) derivatives (91, 92). The presence of candicine was suggested (92) on the basis of TLC evidence but could not be confirmed in later work (93). All these products were previously encountered in several cacti of other plant species. From 1967 onward, Kapadia and his coworkers examined the relatively minor alkaloidal and related nonbasic (quaternary, acid, and amide) constituents of peyote and identified 26 additional compounds. These include the alkaloids peyophorine (94) and 3-demethylmescaline (95); the quaternary bases anhalotine, lophotine, and peyotine (93); the amides *N*-acetyl-3-demethylmescaline, *N*-acetylanhalamine, and *N*-acetylanhalonine; *N*-formylmescaline, *N*-formyl-3-demethylmescaline, *N*-formylanhalamine, *N*-formylanhalonine, *N*-formylanhalinine, *N*-formylanhalonidine, and *N*-formyl-*O*-



$\longrightarrow$ ArCOCl $\longrightarrow$ ArCHO $\longrightarrow$

$$ArCH{=}CHNO_2 \xrightarrow{Zn/AcOH} ArCH_2CH{=}NOH \xrightarrow{Na{-}Hg} Ar(CH_2)_2NH_2 \quad (I)$$

Scheme I



$\xrightarrow{CH_2(CO_2H)_2}$ ArCH=CHCOOH $\longrightarrow$

$$Ar(CH_2)_2COOH \longrightarrow Ar(CH_2)_2CONH_2 \longrightarrow Ar(CH_2)_2NH_2 \ (I)$$

Scheme II

methylanhalonidine (96); six cyclic derivatives of mescaline [succinimide, malimide, maleimide (96), citrimide, isocitrimide lactone, and pyrrole (97)] in addition to two other cyclic amides, mescalotam and peyoglutam (96); and the acids peyonine (98), peyoruvic acid, and peyoxylic acid (99, 100). Agurell and Lundström also identified 3,4-dimethoxyphenethylamine (101) in addition to 3-demethylmescaline (102). Table I contains a list of the hitherto identified constituents of peyote.

CHEMISTRY OF PEYOTE CONSTITUENTS

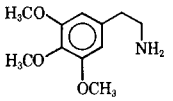
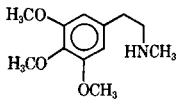
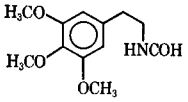
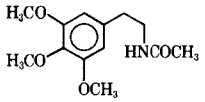
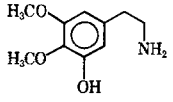
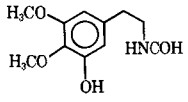
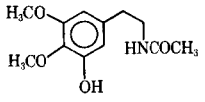
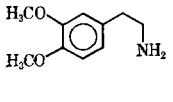
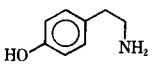
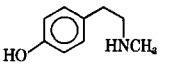
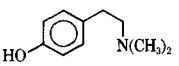
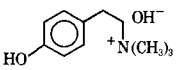
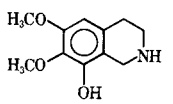
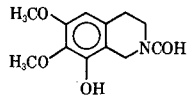
Phenethylamines—*Mescaline and Its Congeners*—Mescaline, 3,4,5-trimethoxy-β-phenethylamine (I), is the active hallucinatory principle of peyote (*L. williamsii*); it occurs in the drug to the extent of 6% and is the main constituent.

The principal synthetic approaches for mescaline and related β-phenethylamine derivatives are basically methods for the construction of the ethylamine side chain onto the appropriately substituted aromatic system.

The first synthesis of mescaline was realized by Späth in 1919 (73). 3,4,5-Trimethoxybenzoic acid was transformed into the corresponding aldehyde and subsequently, by reaction with nitromethane in ethanol solution containing alkali, into the ω-nitrostyrene, which was finally reduced to mescaline in two steps (Scheme I). 3-Demethylmescaline (V) was also prepared as an intermediate toward anhalamine (XIII) by Späth and Röder (90), using essentially the same method after temporary protection of the phenol group. This fundamental sequence was used by many later workers (114–129) in the preparation of mescaline and several of its isomers and analogs. Improvisations have essentially been concerned with the conditions of reaction in the individual steps. The reaction of the benzaldehyde with nitromethane can afford better yields of the ω-nitrostyrene derivative when performed in acetic acid containing ammonium acetate (121, 123, 126, 129, 130), as originally found by Raiford and Fox (131) and Gairaud and Lappin (132), or when an aliphatic primary amine is used as a condensation catalyst (122, 133), as suggested by Worrall and Cohen (134). Erne and Ramirez (118) and Ramirez and Burger (135) found that reduction of the nitrostyrene side chain can be performed with high yield in one step using lithium aluminum hydride[4] (119, 121, 130, 133). This reduction may also be achieved

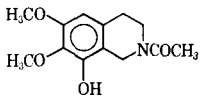
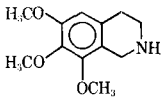
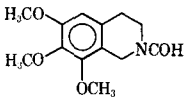
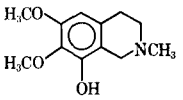
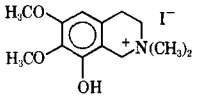
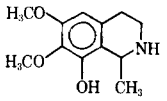
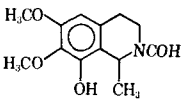
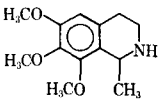
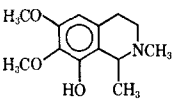
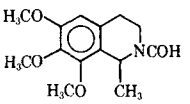
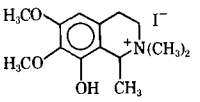
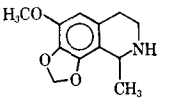
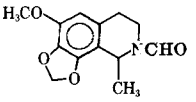
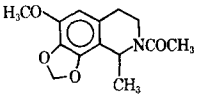
[4] Recently, Kapadia and Shah (136) found that this reaction can also lead to partial demethylation. Thus 4-demethylmescaline resulted, in low yields, from the lithium aluminum hydride treatment of the nitrostyrene discussed, the corresponding phenylacetonitrile, or mescaline itself; similar treatment of pyrogallol trimethyl ether and some of its derivatives also gave the corresponding syringyl phenols.

Table I—Peyote Constituents

Number and Name	Structure	Molecular Formula	M.p., b.p./mm., $[\alpha]_D$	References	Other Natural Sources (References)
I Mescaline		$C_{11}H_{17}O_3N$	30–32° 180°/12	7,40,139	*Trichocereus terscheckii* (103, 105), *T. pachanoi* (104, 105, 188), *T. werdermannianus* (105, 188), *T. bridgesii* (105, 188), *T. macrogonus* (105), *Gymnocalycium gibbosum* (106), *Opuntia cylindrica* (107, *cf.* 105)
II *N*-Methylmescaline		$C_{12}H_{19}O_3N$	177.5–178° (As picrate)	7, 83	
III *N*-Formylmescaline		$C_{12}H_{17}O_4N$	68–69°	96,108	
IV *N*-Acetylmescaline		$C_{13}H_{19}O_4N$	93–94°	7,84,96	
V 3-Demethylmescaline		$C_{10}H_{15}O_3N$	178–179° (As HCl)	95,102	*T. pachanoi* (105)
VI *N*-Formyl-3-demethylmescaline		$C_{11}H_{15}O_4N$	–[a]	96	
VII *N*-Acetyl-3-demethylmescaline		$C_{12}H_{17}O_4N$	–[a]	96	
VIII 3,4-Dimethoxyphenethylamine		$C_{10}H_{15}O_2N$	188°/15	101	*T. bridgesii* (105), *T. camarguensis* (105), *T. macrogonus* (105), *T. pachanoi* (105, 188), *T. werdermannianus* (105, 188), *Echinocereus merkeri* (113)
IX Tyramine		$C_8H_{11}ON$	161°	7,92	Several Cactaceae, *cf.* ref. 105, for other sources, *cf.* refs. 41, 109
X *N*-Methyltyramine		$C_9H_{13}ON$	127–128°	92	*T. camarguensis* (105), *T. schickendantzii* (105), also *cf.* refs. 41, 109
XI Hordenine		$C_{10}H_{15}ON$	117–118°	7,91,92	Several Cactaceae, *cf.* ref. 105; also *cf.* refs. 41, 109
XII Candicine[b]		$C_{11}H_{19}O_2N$	230–231° (As iodide)	7,92,93	*T. candicans* (110), *T. lamprochlorus* (111), *T. spachianus* (112)
XIII Anhalamine		$C_{11}H_{15}O_3N$	189–191°	7,64,65	
XIV *N*-Formylanhalamine		$C_{12}H_{15}O_4N$	–[a]	96	

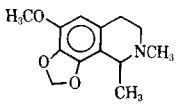
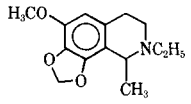
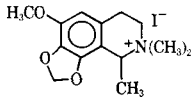
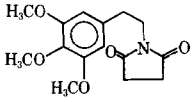
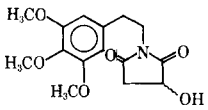
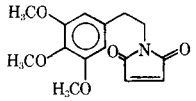
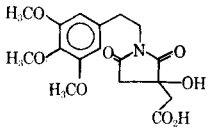
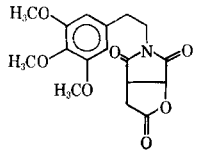
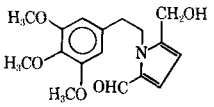
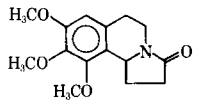
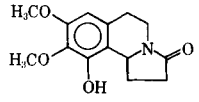
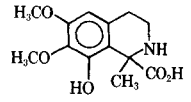
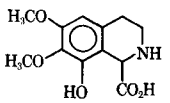
(*Continued*)

Table I—*Continued*

Number and Name	Structure	Molecular Formula	M.p., b.p./mm., $[\alpha]_D$	References	Other Natural Sources (References)
XV *N*-Acetylanhalamine		$C_{13}H_{17}O_4N$	–[a]	96	
XVI Anhalinine		$C_{12}H_{17}O_3N$	61–63°	7,80	
XVII *N*-Formylanhalinine		$C_{13}H_{17}O_4N$	–[a]	96	
XVIII Anhalidine		$C_{12}H_{17}O_3N$	131–133°	7,81	
XIX Anhalotine (iodide)		$C_{13}H_{20}O_3NI$	219–220°	93	
XX Anhalonidine		$C_{12}H_{17}O_3N$	160–161°	7,61	*T. pachanoi* (105)
XXI *N*-Formylanhalonidine		$C_{13}H_{17}O_4N$	–[a]	96	
XXII *O*-Methyl-*d*-anhalonidine		$C_{13}H_{19}O_3N$	140°/0.05 +20.7° (MeOH)	7,85	
XXIII Pellotine		$C_{13}H_{19}O_3N$	111–112°	7,60,65	
XXIV *N*-Formyl-*O*-methylanhalonidine		$C_{14}H_{19}O_4N$	–[a]	96	
XXV Peyotine (iodide)		$C_{14}H_{22}O_3NI$	185–186°	93	
XXVI Anhalonine		$C_{12}H_{15}O_3N$	85.5° –56.3° ($CHCl_3$)	7,52–54, 61	*T. terscheckii* (168)
XXVII *N*-Formylanhalonine		$C_{13}H_{15}O_4N$	–[a]	96	
XXVIII *N*-Acetylanhalonine		$C_{14}H_{17}O_4N$	–[a]	96	

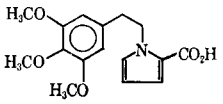
(*Continued*)

Table I—*Continued*

Number and Name	Structure	Molecular Formula	M.p., b.p./mm., $[\alpha]_D$	References	Other Natural Sources (References)
XXIX Lophophorine		$C_{13}H_{17}O_3N$	−47° ($CHCl_3$)	7,61	
XXX Peyophorine		$C_{14}H_{19}O_3N$	155–156° (As picrate)	94	
XXXI Lophotine (iodide)		$C_{14}H_{20}O_3NI$	240–242°	93	
XXXII Mescaline succinimide		$C_{15}H_{19}O_5N$	125–126°	96	
XXXIII Mescaline maleimide		$C_{15}H_{19}O_6N$	–[a]	96	
XXXIV Mescaline maleimide		$C_{15}H_{17}O_5N$	–[a]	96	
XXXV Mescaline citrimide		$C_{17}H_{21}O_8N$	–[a]	97	
XXXVI Mescaline isocitrimide lactone		$C_{17}H_{19}O_7N$	–[a]	97	
XXXVII Peyoglunal		$C_{17}H_{21}O_5N$	–[a]	97	
XXXVIII Mescalotam		$C_{15}H_{19}O_4N$	–[a]	96	
XXXIX Peyoglutam		$C_{14}H_{17}O_4N$	217–219°	96	
XL Peyoruvic acid		$C_{13}H_{17}O_5N$	233–234°	100	
XLI Peyoxylic acid		$C_{12}H_{15}O_5N$	237–238°	100	

(*Continued*)

Table I—*Continued*

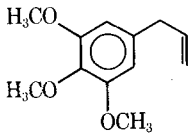
Number and Name	Structure	Molecular Formula	M.p., b.p./mm., $[\alpha]_D$	References	Other Natural Sources (References)
XLII Peyonine		$C_{16}H_{19}O_5N$	131–133.5°	98	

[a] Products identified by GLC–mass spectrometry in comparison with authentic preparations. [b] The presence of candicine was suggested (92) on the basis of TLC evidence but could not be substantiated in a later study (93); additional work is evidently needed to resolve this question.

electrolytically (115, 137) and by modified catalytic hydrogenation (117, 138–140).

Slotta (141) and Slotta and Heller (142) followed a different course for the synthesis of mescaline, which does not, however, seem to have attracted the attention of later workers. The appropriately substituted phenylpropionamide was prepared as indicated in Scheme II and finally subjected to Hoffmann degradation to give mescaline.

In another synthesis, Hahn and Wassmuth (116) and Hahn (143) obtained mescaline by reduction of the trimethoxyphenylacetaldehyde oxime which was prepared from the ozonization product of elemicine (Scheme III).



$\xrightarrow{O_3}$ $ArCH_2CHO$ $\longrightarrow$

$ArCH_2CH{=}NOH \longrightarrow Ar(CH_2)_2NH_2$ (I)

Scheme III

A successful and versatile method for the construction of the ethylamine side chain (144, 145) involves transformation of the substituted benzoic acid into the corresponding phenylacetamide *via* the Arndt–Eistert synthesis, followed by reduction with lithium aluminum hydride (Scheme IV). The use of appropriate amines in

$$ArCOOH \longrightarrow ArCOCl \longrightarrow ArCOCHN_2 \xrightarrow[(AgNO_3)]{NH_4OH}$$

$$ArCH_2CONH_2 \xrightarrow{LAH} Ar(CH_2)_2NH_2 \quad (I)$$

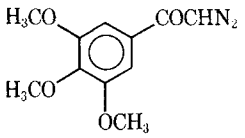
Scheme IV

place of ammonia, in the treatment of the diazoketone, affords *N*-substituted phenethylamines (144). This approach was recently used by Kapadia *et al.* (95) in the synthesis of 3-demethylmescaline (V), identified as a minor companion of mescaline in peyote. The suitably substituted phenylacetamide may also be obtained from the corresponding acetophenone *via* the Kindler modification of the Willgerodt reaction (Scheme V), which gives the intermediate phenylacetic acid derivative (146).

$$ArCOCH_3 \rightarrow ArCH_2COOH \rightarrow ArCH_2CONH_2 \rightarrow Ar(CH_2)_2NH_2$$

Scheme V

Perhaps of only historical interest is the mescaline synthesis of Hadáček *et al.* (147) in which the diazoketone group was transformed by a relatively long route to the ethylamine side chain (Scheme VI).



$\longrightarrow ArCH_2COOCH_3 \longrightarrow$

$ArCH_2COOH \longrightarrow ArCH_2COCHN_2 \longrightarrow Ar(CH_2)_2CONH_2 \longrightarrow$

$Ar(CH_2)_2NH_2$ (I)

Scheme VI

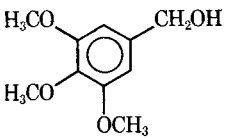
Mescaline was also synthesized, by Kindler and Peschke (139) and later by Amos (148), from the trimethoxybenzaldehyde through condensation with potassium cyanide, followed by acetylation of the formed mandelonitrile and catalytic hydrogenation (Scheme VII).

$ArCHO \rightarrow ArCH(OH)CN \rightarrow ArCH(OAc)CN \rightarrow$

$Ar(CH_2)_2NH_2$ (I)

Scheme VII

A convenient synthetic pathway of mescaline (Scheme VIII), which seems to be of general utility in the prepara-



$\longrightarrow ArCH_2Cl \longrightarrow$

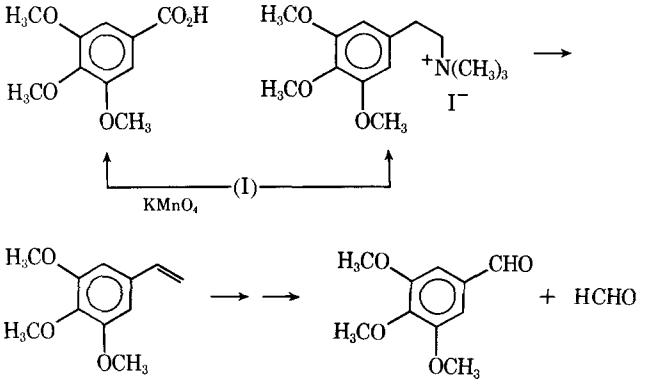
$ArCH_2CN \xrightarrow{LAH} Ar(CH_2)_2NH_2$ (I)

Scheme VIII

tion of analogous phenethylamines, involves use of the substituted benzyl alcohol which is transformed into the phenylacetonitrile, *via* the benzyl chloride, with final reduction using lithium aluminum hydride (149–154). Recently, Abdel-Rahman *et al.* (155) obtained excellent yields of mescaline by this route using thionyl chloride in the benzyl chloride preparation and potassium cyanide in aqueous formic acid or dimethyl sulfoxide in the subsequent stage. Benington *et al.* (123, 130, 145, 156) obtained the desirable benzyl chloride derivatives by chloromethylation (using aqueous formaldehyde and concentrated hydrochloric acid or chloromethyl ether in acetic acid) (130) of the appropriately substituted benzene derivative.

The recent review of Patel (40) on mescaline and its analogs gives a comprehensive listing of the various aromatic and *N*-substituted β-phenethylamines reported in the literature which have been obtained essentially by the foregoing methods (115, 137, 142, 157–162).

The most important degradative reactions of mescaline are those used in recent times in biosynthetic studies to determine the location of an introduced label. Cleavage of the ethylamine side chain (Scheme IX) may be



Scheme IX

effected by oxidation with potassium permanganate to give, for example, 3,4,5-trimethoxybenzoic acid from mescaline (163–167) and 4-acetoxybenzoic acid from *O*-acetylhordenine (166). Products comprising both carbon atoms of the side chain may also be obtained, as illustrated by Leete's (164) systematic degradation of mescaline. In the latter, the *N*,*N*-dimethyl methiodide derivative was subjected to Hoffmann degradation; the resulting styrene was oxidized to a diol with osmium tetroxide followed by cleavage with sodium metaperiodate, yielding 3,4,5-trimethoxybenzaldehyde and formaldehyde.

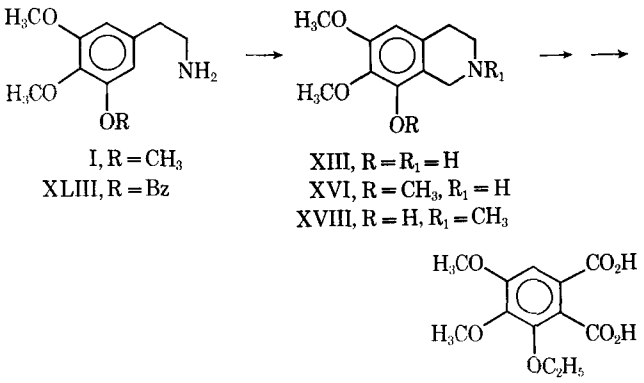
Several simple *N*-substituted derivatives of mescaline and 3-demethylmescaline are also known to exist in peyote. *N*-Methylmescaline (II) and *N*-acetylmescaline (IV) were identified by Späth and Bruck (83, 84) in 1937–1938; the first was synthesized (83) from mescaline by condensation with benzaldehyde followed by quaternarization with methyl iodide and hydrolysis. By using the technique of combined GLC–mass spectrometry, Kapadia and Fales (96) recently identified the *N*-formyl and *N*-acetyl derivatives of both mescaline (III and IV, respectively) and 3-demethylmescaline (VI and VII, respectively) in the nonbasic fractions of peyote. 3-Demethylmescaline (V) itself was more recently found to be a constituent of peyote, and its synthesis was realized as indicated before (95). 3,4-Dimethoxyphenethylamine (VIII) (≡homoveratyrlamine) was detected in trace amounts in peyote by Lundström and Agurell (101) using GLC.

Tyramine and Its Derivatives—The presence of tyramine (IX) and *N*-methyltyramine (X) in Cactaceae is an extremely rare event (7, 92). The first was detected by TLC and the second isolated by McLaughlin and Paul (92) from the phenolic fractions of peyote. Hordenine (XI) was isolated (91) from the phenolic fractions, and candicine (hordenine quaternary metho derivative) (XII) was detected by TLC (92) in the quaternary base fractions of peyote by the same workers (*cf.* 93). Both products are known to be constituents of other cacti (7, 168). In a recent report, Todd (169) showed that hordenine, unlike several other phenethylamine and tetrahydroisoquinoline constituents of peyote, occurs only in the roots.

Tetrahydroisoquinolines—The principal reported approaches for the preparation of the peyote tetrahydroisoquinoline alkaloids seem to have depended basically on the general Pictet–Spengler (170), Bischler–Napieralski (171), and Pomeranz–Fritsch (172) isoquinoline syntheses. In the first two types, the appropriately substituted (aromatic 3,4,5-trioxygenated) phenethylamine derivative is the starting material to be transferred into the desirable tetrahydroisoquinoline carrying a methylene or a $CH \cdot CH_3$ group at C-1. This additional unit is provided by reaction with an aldehyde in the first type of synthesis and by cyclization of the *N*-acyl derivative in the second type. In both, the unsymmetrically 3,4,5-trisubstituted phenethylamines are likely to follow either (or both) of two possible directions of cyclization. It remains, therefore, as an additional task, to ascertain the exact disposition of the resulting tetrahydroisoquinoline. This problem is not encountered in the syntheses depending on the Pomeranz–Fritsch method and its modifications (173), since the starting material, an *N*-benzylaminoacetaldehyde diethylacetal, can be so substituted as to give only one possible cyclization product. It is a fact that the structure elucidation of the principal tetrahydroisoquinolines of peyote—for which Späth must be credited—was realized largely by synthetic approaches, inspired by biogenetic considerations, rather than by degradative ones.

Anhalamine, Anhalidine, Anhalinine, and Anhalotine—Anhalamine (XIII) was first isolated from peyote by Kauder (65) and is estimated to exist in the cactus to the extent of 0.1% (64). Anhalidine (*N*-methylanhalamine) (XVIII) and anhalinine (*O*-methylanhalamine) (XVI) were discovered by Späth and Becke (80, 81) in peyote, where they exist as minor alkaloidal constituents; their yields are estimated to be 0.001 and 0.01%, respectively.

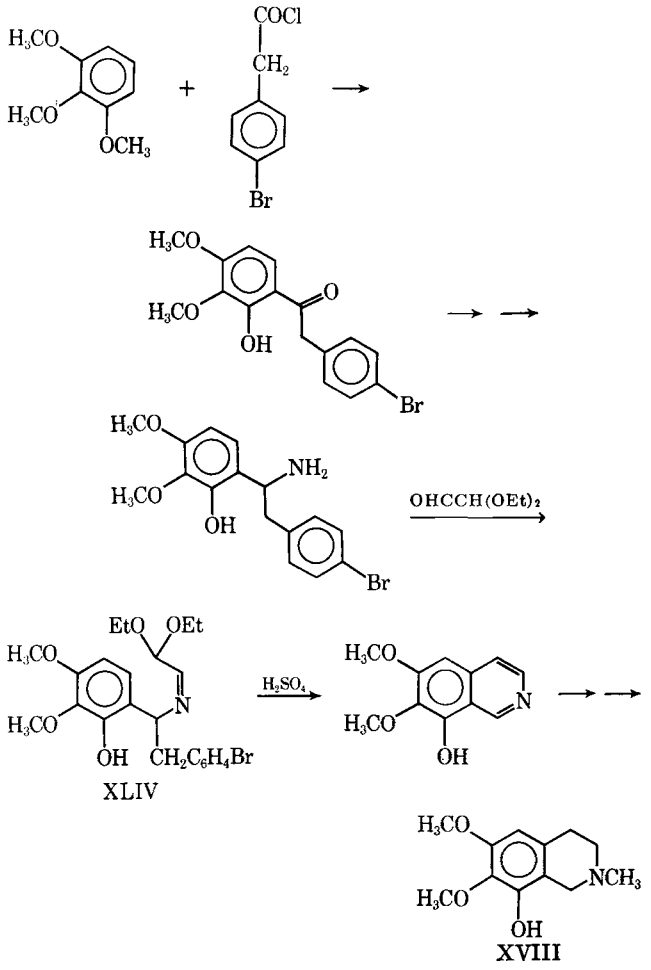
The first synthesis of anhalamine (XIII) was realized by Späth and Röder (90) through Pictet–Spengler condensation of 3,4-dimethoxy-5-benzyloxyphenethylamine (XLIII) with formaldehyde (Scheme X). The direction



Scheme X

of cyclization and, hence, location of the phenol group were determined by permanganate oxidation of the *O*,*N*-diethyl derivative which gave 4,5-dimethoxy-3-ethoxyphthalic acid (79). Anhalinine was also obtained by Späth and Becke (80) by a similar treatment of mescaline (I); anhalidine (XVIII) was obtained simply by treatment of anhalamine with methyl iodide and isolation from the complex reaction mixture (81). During a

Pomeranz–Fritsch reaction of the Schiff base XLIV, obtained as shown in Scheme XI, Inubushi and Fujitani

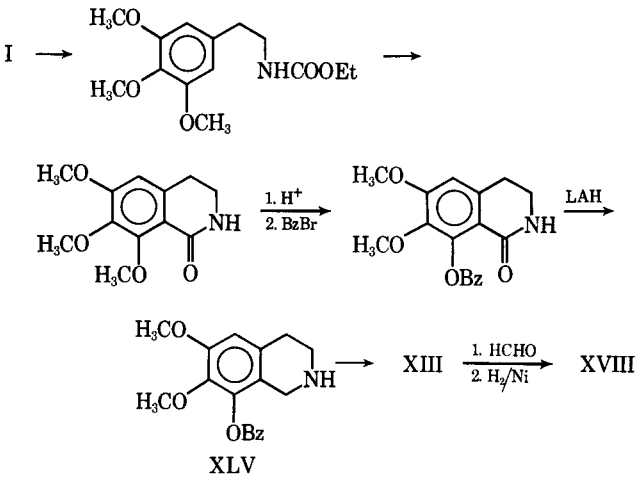


Scheme XI

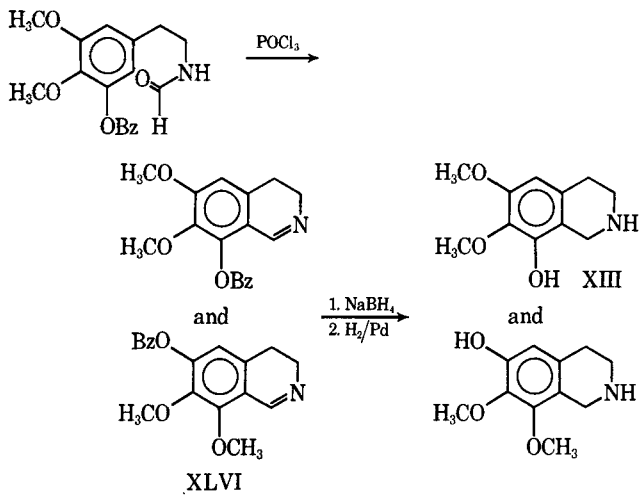
(125) found, unexpectedly, that the isoquinoline product had lost the C-1 substituent; they obtained anhalidine (XVIII) by subsequent quaternarization and reduction.

An interesting adaptation of the Pomeranz–Fritsch isoquinoline synthesis, originally developed by Bobbitt *et al.* (173) for the construction of tetrahydroisoquinolines by acid-catalyzed cyclization of the requisite *N*-benzylaminoacetaldehyde diethylacetal followed by hydrogenation, was recently used by Bobbitt and Dutta (174) in a novel synthesis of anhalamine, anhalinine, and anhalidine (Scheme XII).

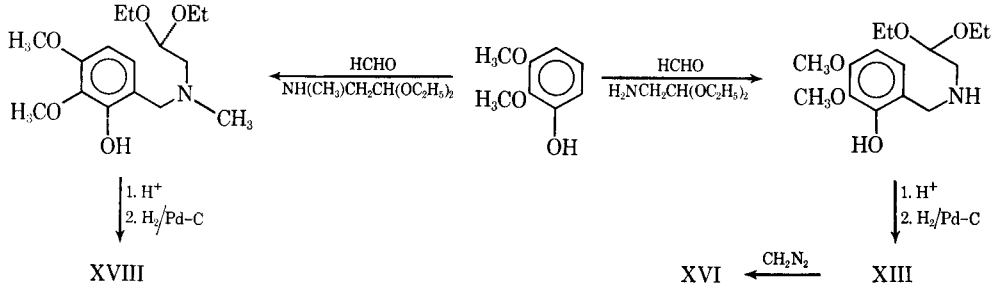
A relatively simple but less familiar method was earlier developed by Brossi *et al.* (175) for the construction of the heterocyclic ring by polyphosphoric acid cyclization of the urethan derivative obtained from the appropriate phenethylamine, followed by stages of reduction of the formed lactam system and introduction of the required substituents. Scheme XIII outlines the application of this route in the synthesis of anhalamine (XIII) and anhalidine (XVIII). The same sequence of reactions was also used by Kametani *et al.* (154) for the synthesis of the same compounds. In a later report, Brossi *et al.* (176) showed that the Bischler–Napieralski cyclization of *N*-formyl-3,4-dimethoxy-5-benzyloxyphenethylamine (Scheme XIV) occurs in both possible directions—unlike the 5-acetoxy analog (XLVII)—to give a mixture of two dihydroisoquinolines (XLVI).



Scheme XIII

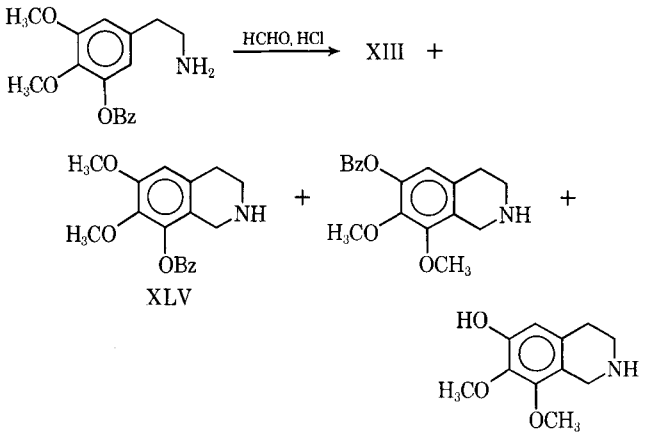


Scheme XIV



Scheme XII

Reduction and hydrogenolysis finally afforded a binary mixture from which anhalamine (XIII) was isolated. A Pictet–Spengler reaction of 3,4-dimethoxy-5-benzyloxyphenethylamine with formaldehyde was found by Brossi *et al.* (176) also to afford products, resulting from cyclization in both directions, including anhalamine (Scheme XV).

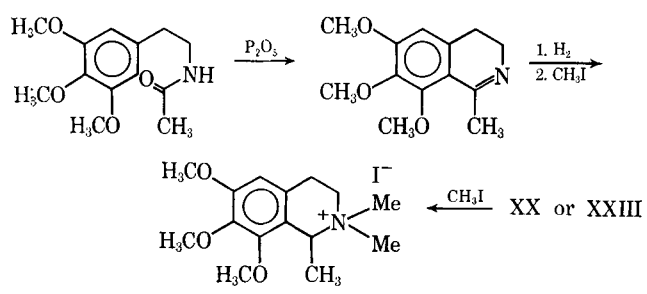


Scheme XV

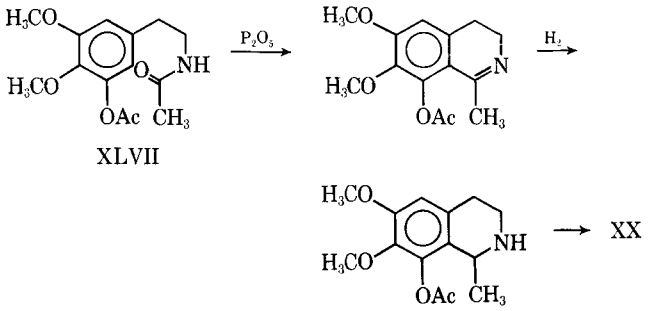
Anhalotine (XIX) was isolated from the fraction of quaternary bases of peyote by Kapadia *et al.* (93) and, as the iodide, was shown to be identical with anhalidine methiodide. *N*-Formylanhalamine (XIV), *N*-acetylanhalamine (XV), and *N*-formylanhalinine (XVII) were detected by combined GLC–mass spectrometry in the nonbasic fractions of peyote (96). Synthesis of the formyl derivatives (XIV and XVII) was achieved by treatment of the corresponding base with triethylamine and formic acid, a method recommended by Durand *et al.* (177).

Degradation of the heterocyclic system in tetrahydroisoquinolines was previously used in structure determination work and is currently a means to locate the label in biosynthetic studies. An example is the relatively recent degradation of anhalamine (178), as the *O,N*-dimethyl derivative methiodide, to 3,4,5-trimethoxyphthalic anhydride (loss of C-3 of anhalamine), using the conditions described much earlier by Späth and Becke (179).

Anhalonidine, Pellotine, and Peyotine—Anhalonidine (XX) was first isolated by Heffter (61) who found as much as 5% of it in peyote. *O*-Methyl-*d*-anhalonidine (XXII), an optically active alkaloid, was discovered in very small amounts by Späth and Bruck (85). Pellotine (XXIII) was discovered also by Heffter (60) in 0.74% yield and later by Kauder (65). Späth and Kesztler (89) prepared the optically active forms of pellotine



Scheme XVI

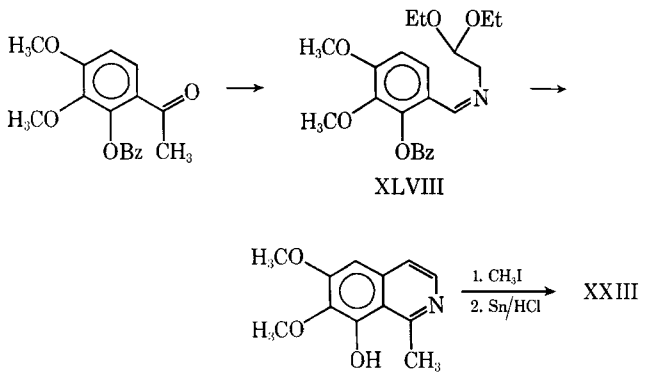


Scheme XVII

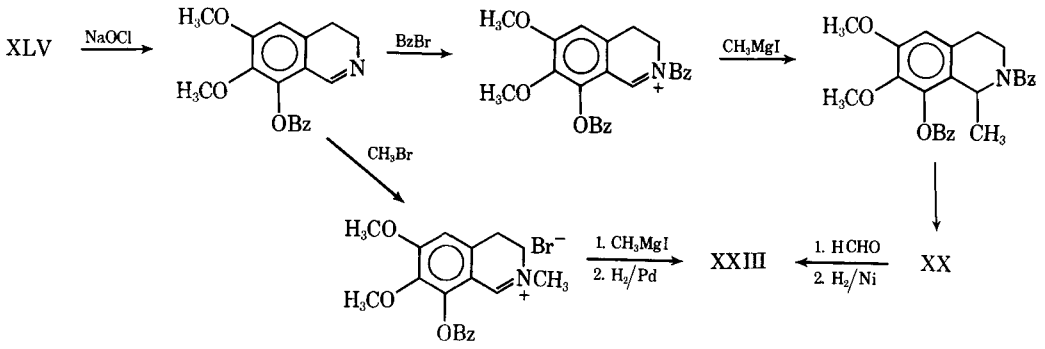
and, considering the ease with which they racemize, these authors thought that the natural product might be optically active and that racemization occurs by aging of the drug or during the process of isolation.

Anhalonidine and pellotine were the first peyote tetrahydroisoquinoline alkaloids to be synthesized—as early as 1921 by Späth (74). The synthesis was realized employing a Bischler–Napieralski condensation of *N*-acetylmescaline and leading, as indicated in Scheme XVI, to a quaternary iodide which was identical with *O*-methylpellotine methiodide. Anhalonidine (XX) was correlated to pellotine (XXIII) by complete methylation of both to the same product. In an independent synthesis of *rac.* anhalonidine, Späth (76) followed the same route using *N*-acetyl-3,4-dimethoxy-5-hydroxyphenethylamine after a temporary protection of the phenol group by acetylation (XLVII) as shown in Scheme XVII. The direction of cyclization and, hence, location of the phenol group were determined (86) by permanganate oxidation of the *O*-ethyl derivative of pellotine, which gave the known 4,5-dimethoxy-3-ethoxyphthalic acid; this was also confirmed by an analytical approach (82).

In another synthesis of pellotine (XXIII), Späth and Becke (78) subjected the Schiff base (XLVIII), obtained by condensation of 2-benzyloxy-3,4-dimethoxyacetophenone with aminoacetaldehyde diethylacetal, to Pomeranz–Fritsch cyclization using sulfuric acid (Scheme XVIII). The resulting isoquinoline was quaternarized and finally reduced to *rac.* pellotine. More recently, Brossi *et al.* (175) used the urethan derivative of mescaline (Scheme XIII) in a new synthesis of *rac.* anhalonidine and *rac.* pellotine *via* the 6,7-dimethoxy-8-benzyloxytetrahydroisoquinoline intermediate (XLV) as outlined in Scheme XIX. In a later report, Brossi *et al.* (176) obtained, in the Bischler–Napieralski cyclodehydration of *N*-formyl-3,4-dimethoxy-5-benzyloxyphenethyla-

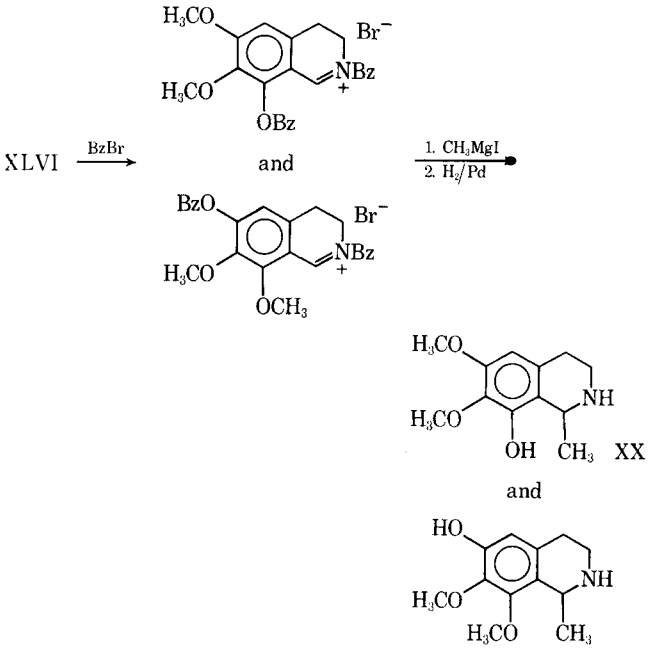


Scheme XVIII



Scheme XIX

mine, a mixture of the two possible dihydroisoquinolines (XLVI)—unlike the acetoxy counterpart (XLVII) (76, 86)—which was quaternarized and treated with methyl Grignard reagent to lead eventually to a mixture from which *rac.* anhalonidine (XX) was separated (Scheme XX).

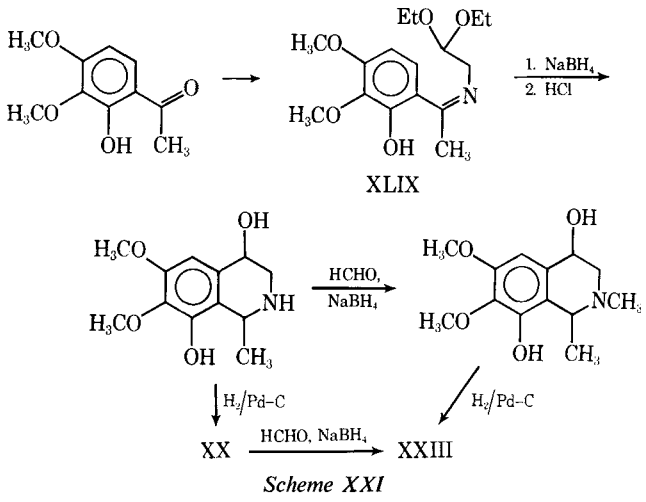


Scheme XX

Very recently, Takido *et al.* (180) made use of Bobbitt's modification (173) of the Pomeranz–Fritsch isoquinoline synthesis. The requisite Schiff base (XLIX), obtained from reaction of the appropriate acetophenone with aminoacetaldehyde diethylacetal, was cyclized to give *rac.* anhalonidine and *rac.* pellotine (Scheme XXI).

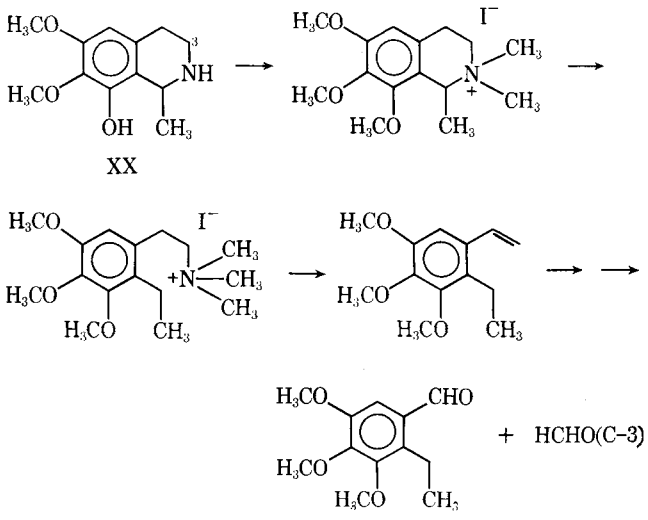
Peyotine (XXV) was recently isolated by Kapadia *et al.* (93) by fractionation of the quaternary bases obtained from peyote; it was shown, as the iodide, to be identical with pellotine methiodide. By combined GLC–mass spectrometry technique, Kapadia and Fales (96) proved the presence of *N*-formylanhalonidine (XXI) and *N*-formyl-*O*-methylanhalonidine (XXIV) in the non-basic fractions of peyote.

A useful degradative sequence of anhalonidine, reported by Leete (164), consists in reductive cleavage of the heterocyclic system in the methiodide of *O*-methyl derivative followed by quaternarization and Hoffmann degradation of the product (Scheme XXII). The resulting styrene derivative was oxidized to a glycol with osmium tetroxide and finally cleaved with sodium metaperiodate, giving 2-ethyl-3,4,5-trimethoxybenzaldehyde and formaldehyde. By use of this systematic degradation, it was possible to ascertain the location of the label



Scheme XXI

at C-3 in anhalonidine resulting from feeding of tyrosine-2-^{14}C to peyote (164). The Kuhn–Roth oxidative degradation of such alkaloids carrying a methyl group on C-1 is also often resorted to as a diagnosis of the label present

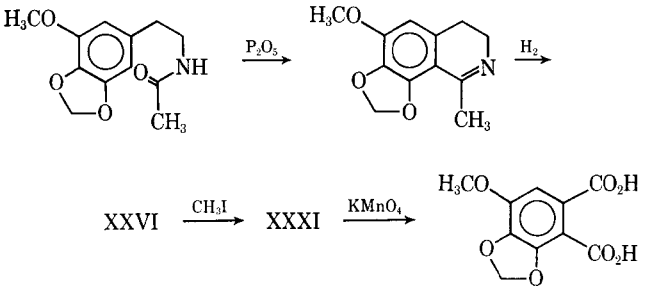


Scheme XXII

in this part of the molecule giving rise to acetic acid (100, 181, 182).

Anhalonine, Lophophorine, Peyophorine, and Lophotine—Anhalonine (XXVI) (as the *l*-form) was first isolated by Lewin (52–54) and was estimated (61) to occur to the extent of about 3% in peyote. Lophophorine (XXIX), as the *l*-form, was found by Heffter (61) to occur in peyote in 0.5% yield. Späth and Kesztler (88) resolved the synthetic racemic mixture of anhalonine (87) and showed that natural lophophorine results by *N*-methylation of *l*-anhalonine. Peyophorine (XXX) was discovered fairly recently by Kapadia and Fales (94) as a minor constituent of peyote. It was prepared (94) by *N*-ethylation of anhalonine with diethyl sulfate and by lithium aluminum hydride reduction of *N*-acetylanhalonine (XXVIII); the latter is itself a natural constituent of peyote (94). This is the only *N*-ethylated alkaloid so far found in peyote, and its presence is remarkable in view of the extreme rarity of such moiety in nature (183). *N*-Formylanhalonine (XXVII) is another minor constituent of peyote; it was identified by combined GLC–mass spectrometry in the nonbasic fractions (96).

The synthesis of *rac.* anhalonine (XXVI) and *rac.* lophophorine (XXIX) was realized by Späth and Gangl (87) by a Bischler–Napieralski cyclodehydration of *N*-acetylhomomyristicylamine followed by reduction and quaternarization (Scheme XXIII). The structure was

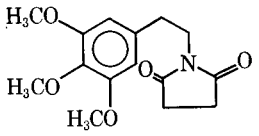


Scheme XXIII

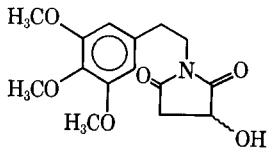
later confirmed by oxidation to 3,4-methylenedioxy-5-methoxyphthalic acid (80). The quaternary base (XXXI), corresponding to anhalonine and lophophorine and synthesized in 1923 (87), was quite recently shown by Kapadia *et al.* (93) to be itself a constituent of peyote; it was named lophotine.

Alkaloidal Amides—Very little attention has been given to the study of the nonbasic fractions of the alkaloid-bearing Cactaceae (and, in fact, most other alkaloid-bearing plants). This is testified to by the fact that only peyote has been found to contain *N*-formyl derivatives of its own alkaloids. It is also unlikely that *N*-acetyl alkaloidal derivatives have been found in cacti other than peyote. A discussion of these simple amide derivatives of the phenethylamine and tetrahydroisoquinoline constituents of peyote has already been given under the appropriate titles. The probable biogenetic implications of such one- and two-carbon units are discussed later.

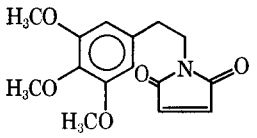
The recent GLC–mass spectrometric studies by Kapadia and Fales (96) showed that the nonbasic fractions of peyote contain more complex amide derivatives, the nature of which has been elucidated from mass spectral considerations and by comparison with synthetic materials. These include the succinimide (XXXII), malimide (XXXIII), and maleimide (XXXIV) derivatives of mescaline, which were synthesized simply by sublimation of the mescaline salts of the corresponding acids. Very recently, Kapadia *et al.* (97) characterized three additional products in the same nonbasic fractions.



XXXII

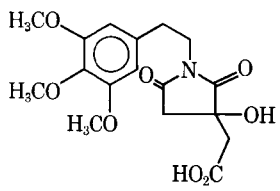


XXXIII

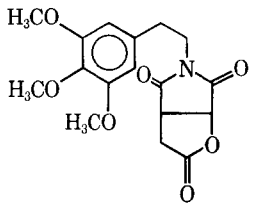


XXXIV

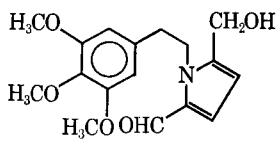
These were the citrimide (XXXV), the isocitrimide lactone (XXXVI), and the 2-formyl-5-hydroxymethylpyrrole derivative (XXXVII) related to mescaline. The structures of the first two products were evidenced by mass spectral data and supported by synthesis from mescaline through treatment with citric acid anhydride and isocitric acid lactone, respectively. The constitution of the isolated XXXVII, designated peyoglunal, was postulated from IR, NMR, and mass spectral evidence; its synthesis was attempted.[5]



XXXV



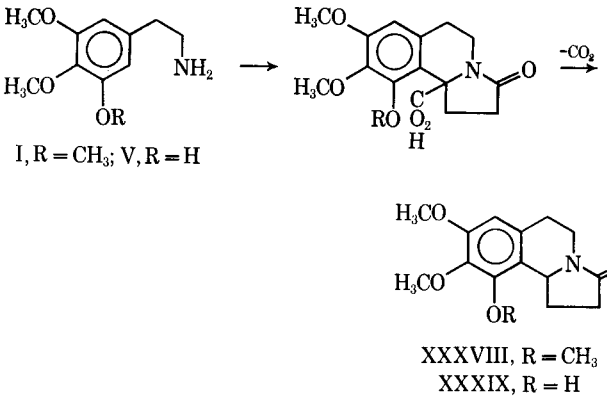
XXXVI



XXXVII

The nonbasic fractions of peyote were also found (96) to contain two structurally interesting lactams, XXXVIII and XXXIX, related to mescaline (I) and 3-demethylmescaline (V), which were named mescalotam and peyoglutam, respectively. Their constitutions, prompted by biogenetic considerations, were proven by synthesis from the corresponding bases by treatment with α-ketoglutaric acid followed by decarboxylation (Scheme XXIV). The direction of cyclization in

[5] While this manuscript was in preparation, a product containing XXXVII (GLC–mass spectrometry and other spectral evidence) was obtained by condensation of 2,5-dimethoxy-5-hydroxymethyltetrahydrofurfuraldehyde dimethyl acetal with mescaline. Treatment of desformyl-XXXVII under Riemer-Tiemann conditions also furnished XXXVII (97).



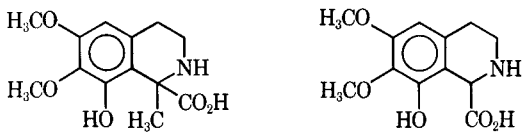
I, R = CH_3; V, R = H

XXXVIII, R = CH_3
XXXIX, R = H

Scheme XXIV

XXXIX was established by NMR evidence (96, 184).

Amino Acids—There does not seem to be any published data regarding the general proteinic amino acid content of peyote, significant and relevant to the biosynthetic processes as they may be. The presence of proline in the amino acid fraction was, however, demonstrated lately by Kapadia *et al.* (185). Among the complex composition of this fraction, containing at least 12 products, two nonproteinic amino acids, named peyoruvic and peyoxylic acids, were characterized by GLC–mass spectrometry and shown to have Structures XL and XLI, respectively (99, 100). Their presence was anticipated on biogenetic grounds, and their synthesis was realized by condensation of 3-demethylmescaline (V) with pyruvic acid and glyoxylic acid, respectively, under physiological conditions.

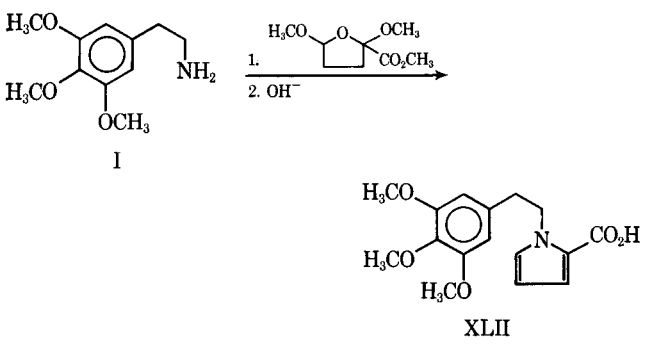


XL XLI

Peyonine (XLII) is another minor constituent of peyote which was recently isolated (186) from the nonbasic fractions. Its structure was determined (98) from spectral evidence and through synthesis by treatment of mescaline with methyl 2,5-dimethoxytetrahydro-2-furoate followed by saponification (Scheme XXV). Peyonine appears to be the first simple pyrrole-2-carboxylic acid derivative isolated from a natural source.

ANALYTICAL METHODS

Several reagents have been used in the detection and identification of mescaline and related compounds (187–192). Some are particularly useful in working with peyote alkaloid mixtures, for example, on thin-layer chromatoplates, such as dansyl chloride for nonphenolic products and tetrazotized benzidine (92) and tetrazotized di-*O*-anisidine (93) for detection and identification of phenolic ones. Also, a number of paper (193–198), thin-layer (92, 105, 169, 199–203), and gas (101, 105, 204–207) chromatographic methods have been reported for the separation and identification of mescaline and other cactus alkaloids. Kapadia and Rao (206) observed a relationship between the retention time and structure of peyote alkaloids and related bases.



I

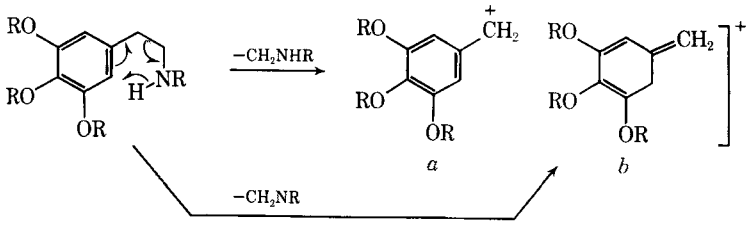
XLII

Scheme XXV

Several methods have been recommended for the quantitation of mescaline in biological fluids. They include colorimetric methods depending on measurement of the color formed by interaction with picric acid (208, 209), bromocresol purple (210), and *p*-nitrophenyldiazonium chloride (211, 212). In a fluorometric method (213), mescaline is transformed into a fluorescent isoquinoline derivative; another method (214) is based on the characteristic oscillographic behavior of mescaline in acid and alkaline solutions.

Mass Spectra of Peyote Alkaloids—The spectra of the phenethylamines and tetrahydroisoquinolines invariably exhibit molecular ions with very low intensities. The principal fragmentations result by cleavage of the β-bonds relative to the aromatic ring. The phenethylamines [such as I (215), V (95), and VIII (205)] and their *N*-alkyl-substituted derivatives thus give the benzyl ions *a* (which may also have tropylium structures) and, through transfer of a hydrogen atom from the departing fragment (arrows in Scheme XXVI), the equally important (and often stronger) ions *b*. In tertiary amines, such as peyonine (XLII), the latter type of breakdown obviously does not take place, and the predominant fragment ion is due to Species *a* (98). The spectra are complicated further only by combinations of losses from the aromatic substituent groups. The *N*-formylphenethylamines [III (216)] and *N*-acetylphenethylamines [VII (216)], as well as the related cyclic imide derivatives (XXXII–XXXIV) (96), give as principal fragmentation products ions of Species *a* in addition to styrene ions (*c*), resulting by scission of the C—N bond of the side chain with transfer of hydrogen (as depicted by the arrows in Scheme XXVII) in a McLafferty rearrangement.

The primary reactions exhibited by the tetrahydroisoquinolines [typified by XIII, XV, XVII, XVIII, XXIV, XXIX (216), and XXX (94)] involve expulsion of the C-1 substituent (217), giving the highly stabilized dihydroisoquinolinium ion Species *d* (Scheme XXVIII). The strongest peaks in the spectra are due to ions (Species *e*) formed by collapse of the heterocyclic system through the retro-Diels–Alder reaction. This type of breakdown does not seem to take place appreciably in those products carrying a methyl group on C-1, probably because the elimination of this substituent is more favored and becomes by far the most important reaction. Mescalotam (XXXVIII) and peyoglutam (XXXIX) (96) exhibit relatively strong M^+ ions and ones resulting by loss of the hydrogen atom on C-1 of the tetrahydroisoquinoline system. Fragmentation of the lactam

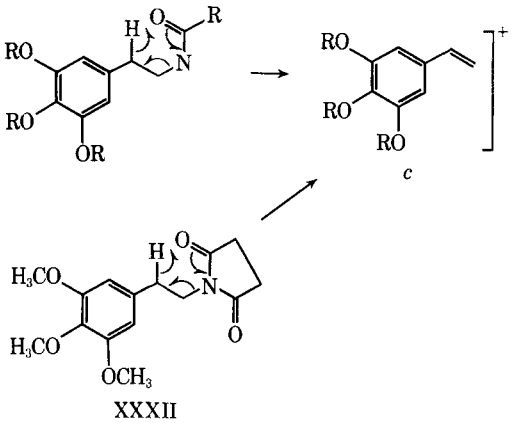


Scheme XXVI

ring by loss of CH_2CH_2CO gives ions with appreciable abundance which may have Structure *f* (Scheme XXIX).

BIOGENESIS OF PEYOTE ALKALOIDS

Phenethylamines—As with the general interest in peyote and its constituents, studies on the biogenetic origin of mescaline (I) and its companion alkaloids, using labeled substrates, were initiated during the late 1950's. Prior to this, Reti (7) expressed certain hypothetical views—fairly reasonable as they later proved—which were unsubstantiated by experimental evidence



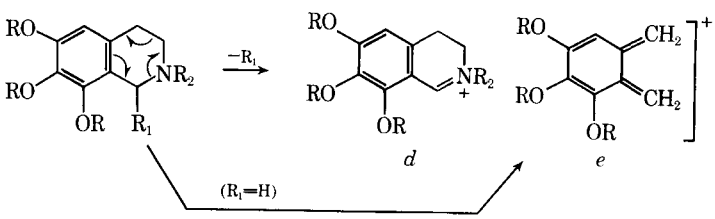
XXXII

Scheme XXVII

and largely based on Guggenheim's fundamental views regarding natural amines (218). It was already a matter of general agreement that the natural aromatic amino acids, such as phenylalanine (L), tyrosine (LI), and dopa (LII) must be biogenetically related to the phenethylamine and tetrahydroisoquinoline alkaloids. Reti visualized the transformations as "simple" processes of decarboxylation, oxidation, *O*- and *N*-methylation, and ring closure with formaldehyde or acetaldehyde equivalents. He thought that tyrosine, which "must" be present in peyote,[6] undergoes oxidation by tyrosinase to give dopa, which may then successively suffer decarboxylation to dopamine (LIII), oxygenation to 3,4,5-trihydroxy-β-phenethylamine (LIV), and *O*-methylation to mescaline (I). Scheme XXX shows these transformations; the postulated (7) stages, which in recent years became established by convincing experimental evidence (*vide infra*), are indicated by heavy arrows.

Reti (7) also offered a possible pathway for hordenine (XI) and candicine (XII) (of which the former is now proven to be a natural constituent of peyote), depending on the initial decarboxylation of tyrosine to tyramine (IX) followed—or perhaps preceded—by methylation. The source of the *O*- and *N*-methyl groups as well as the CH_2 in the methylenedioxy groups was assumed to be formaldehyde. It was shown shortly later, by other workers using tracer techniques, that the *N*-methyl groups of *N*-methyltyramine (X) and hordenine, found in germinating barley, originate from the *S*-methyl group of methionine (219–221) and also from formate (220, 222). In fact, several studies carried out predominantly during the 1950's showed that the methyl group of methionine serves as the source of *O*- and *N*-methyl groups of many alkaloids and that it may also be the origin of the carbon bridge in methylenedioxy groups (219). The extensive studies of the Marion group (220–226) on the biogenesis of hordenine and *N*-methyltyramine in barley rootlets showed that phenylalanine, tyrosine, and tyramine[7] are direct precursors.

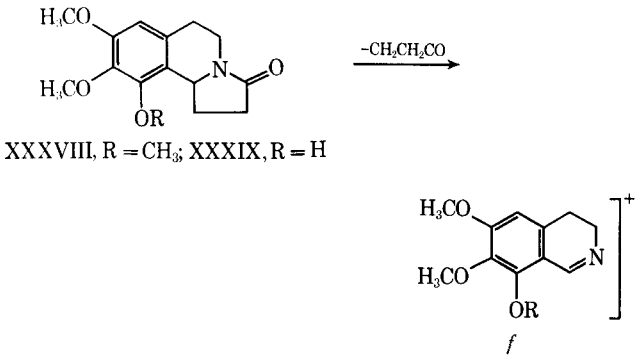
Working with peyote, Leete reported in 1959 (163) that the feeding of 2-^{14}C-DL-tyrosine gave mescaline in which all the activity was located on the α-carbon atom; this constituted the first experimental evidence that tyrosine is a precursor in the pathway to mescaline (I). It was only in 1966 that the next report, concerning the biogenesis of mescaline, was made by Leete (164) and later by McLaughlin and Paul (166) who had anticipated the existence of simple *N*-methylated tyramines on biogenetic grounds and actually isolated hordenine (XI) and *N*-methyltyramine (X) and detected tyramine (IX) itself (91, 92). Tyrosine (LI), which may result



Scheme XXVIII

[6] Reti (7) also assumed the presence of tyrosine in all cacti since the darkening of cut stems, preceded by a red phase, is characteristic for the whole family, probably due to advanced oxidations involving tyrosinase. Tyrosine and tyrosinase have been found in some cacti.

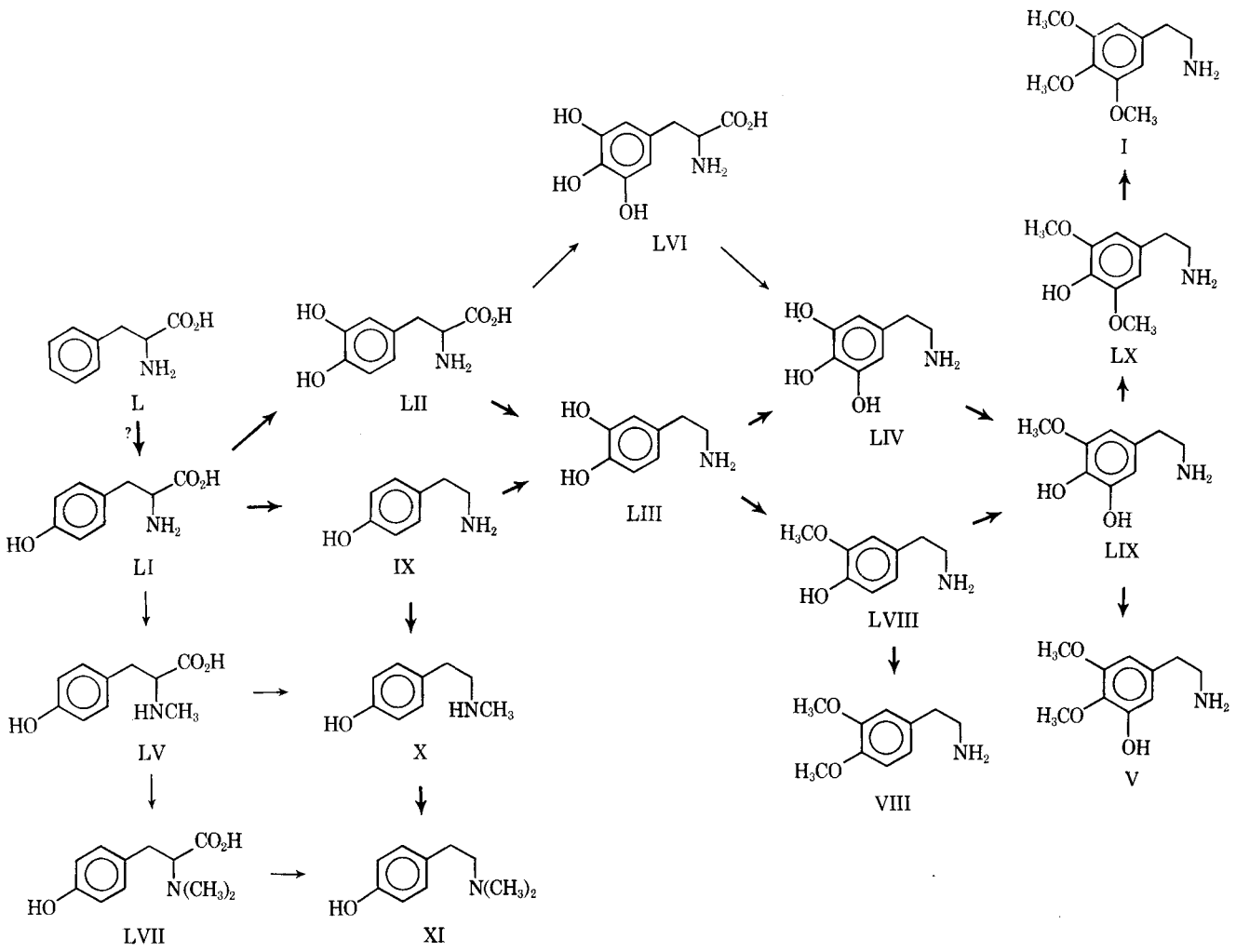
[7] The enzyme-controlled *N*-methylations of tyramine in barley were studied by Mudd (227, 228) who isolated a methionine-activating enzyme which converts methionine to *S*-adenosylmethionine, the actual methylating agent; he also purified the enzyme tyramine methylpherase (229).



Scheme XXIX

from phenylalanine (L), was regarded as the common intermediate leading to the production of tyramine and its *N*-methyl analogs by reactions involving decarboxylation and *N*-methylation, and also to mescaline by a different pathway involving decarboxylation and nuclear oxygenation. In these transformations, one may envisage two possible routes in each case: (*a*) decarboxylation occurs early in the biogenetic sequence, and (*b*) this process is preceded by *N*-methylations or ring oxygenation (Scheme XXX).

In his classical treatise, Guggenheim (218) held a view which was more in favor of the second alternative. In this proposal, he depended on the fact that 3,4-dihydroxyphenylalanine (dopa) (LII) and *N*-methyltyrosine (LV) are frequently encountered in nature; by extrapolation, 3,4,5-trihydroxyphenylalanine (LVI) and *N*,*N*-dimethyltyrosine (LVII) might also be expected to exist. However, McLaughlin and Paul (166), after administering α-^{14}C-tyrosine and α-^{14}C-tyramine to peyote, obtained incorporations which indicated that tyramine is a more direct precursor of mescaline and hordenine than tyrosine and that tyrosine is converted to tyramine. These findings seemed to offer more support to the alternative pathway (*a*). Rosenberg *et al.* (167) subsequently showed by similar feeding experiments that both dopa and dopamine are excellently incorporated in the biosynthesis of mescaline. Although quite expectable, they showed that also phenylalanine serves as a precursor for mescaline[8] and that it is converted in peyote into tyrosine. The latter conclusion, however, was not agreeable to Leete (164) who observed negligible incorporation of phenylalanine and suggested that peyote lacks the enzymes to convert it to tyrosine. However, the overall results clearly supported the earlier



Scheme XXX

[8] The low incorporation which they observed (167) is reasonable in view of the involvement of this amino acid in other biological roles; however, see *Reference 164*.

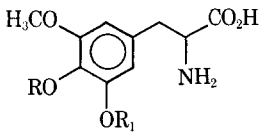
postulations of Reti (7) but indicated that two pathways are open from tyrosine to mescaline *via* dopamine,[9] with one proceeding through dopa and the other through tyramine. In fact, quantitative comparisons made by Rosenberg *et al.* (167) showed that tyramine and dopa appear to be about equally efficient as precursors to mescaline. Similar findings were obtained almost at the same time by Agurell *et al.* (165). However, they reported somewhat different incorporation values and demonstrated that the *O*-methyl groups of mescaline may be derived from methionine. The enzymatic transformations of tyrosine to dopa and then to dopamine are well established in plant systems (172), and the specific enzymes involved (a tyrosinase and a dopa decarboxylase) may be quite different from those that transform tyramine into dopamine and tyrosine into tyramine, respectively (167).

In more recent studies, Lundström and Agurell (178) showed that tyramine, dopamine, and 3,4,5-trihydroxyphenethylamine (LIV) (the latter so far has not been encountered in nature) are all efficient progenitors of mescaline on account of the observed high incorporations of the α-^{14}C-labeled materials. They also showed that phenethylamine and its 4-methoxy and 3,4-dimethoxy[10] (VIII)—a natural constituent of peyote (101)—derivatives are not significant precursors. These findings extend the previously established sequences a step further by showing that 3,4,5-trihydroxyphenethylamine[11] (LIV) may be a stage that follows dopamine *en route* to mescaline. 3,4-Dimethoxyphenethylamine (VIII) seems, however, to be a deadend product resulting from dopamine without being further metabolized to mescaline or to the tetrahydroisoquinoline congeners (*vide infra*).

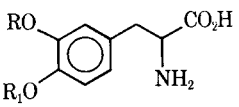
An alternative pathway from dopamine, namely that proceeding *via* the partially *O*-methylated derivative, was considered by both the Agurell (102, 233) and Paul (232, 234, 235) groups. Indeed, 3-methoxy-4-hydroxyphenethylamine (LVIII) was shown to be an important contributor to the mescaline of peyote; the 3-hydroxy-4-methoxy isomer, in contrast, was not significantly incorporated. Of the several possible pathways from 3-methoxy-4-hydroxyphenethylamine (LVIII) and 3,4,5-trihydroxyphenethylamine (LIV), the work of these two groups, using [α-^{14}C] (232–235) labeled compounds, afforded a definite choice. Whereas 3,5-dihydroxy-4-methoxyphenethylamine was hardly incorporated (233), the 3-methoxy-4,5-dihydroxy[12] analog (LIX) served as an efficient precursor (232, 233).

The subsequent metabolic stage beyond intermediate LIX *en route* to mescaline was shown to result by methylation of the *meta-* rather than the *para*-hydroxyl group. Thus, 3,5-dimethoxy-4-hydroxyphenethylamine (LX) was utilized to a higher degree, suggesting it as the immediate precursor to mescaline (232, 233, 235). 3,4-Dimethoxy-5-hydroxyphenethylamine (V), which is a constituent of peyote (95, 102), was, however, not significantly incorporated (233, 234). A complete biogenetic scheme is thus available from phenylalanine to mescaline (Scheme XXX, the heavy arrows indicate verified transformations). It will be noted that *meta*-methylations occur along the mescaline pathway from LIII, LIV and from LIX. *para*-Methylations, which also occur to give the natural constituents VIII and V, seem, however, to be the reactions involved in the pathways leading to the tetrahydroisoquinolines.

Kapadia *et al.* (185) carried out a special search by combined GLC–mass spectrometry in the amino acid fraction of peyote for products constituted as LXI–LXV, and they obtained no evidence[13] for their presence. This is significant since it may indicate that the carboxyl group probably does not survive beyond the stage of dopa in the sequence leading to the more complex alkaloids of the cactus.



LXI, $R = R_1 = CH_3$
LXII, $R = CH_3$, $R_1 = H$
LXIII, $R = H$, $R_1 = CH_3$



LXIV, $R = H$, $R_1 = CH_3$
LXV, $R = CH_3$, $R_1 = H$

Recent studies conducted with another mescaline-producing cactus, *Trichocereus pachanoi* Br. & R., revealed the presence of LVIII and LX (102); these products have so far not been encountered in peyote. Feeding experiments with labeled models (233) suggested that mescaline may be formed in this plant by the same biogenetic sequence operative in peyote (Scheme XXX). The comparatively much higher incorporations of the intermediates IX and LIV observed in *T. pachanoi* may tend to indicate that the LI → IX → LIII → LIV → LIX pathway is a better contributor to mescaline in this source than the alternative pathway(s).

Tetrahydroisoquinolines—In his review on cactus alkaloids, Reti (7) noted that the tetrahydroisoquinoline and phenethylamine alkaloids may be biogenetically related to each other on account of structural similarities and co-occurrence in plant sources. The same was suggested much earlier by Pictet and Spengler (238) who synthesized some simple tetrahydroisoquinolines by reacting the corresponding phenethylamines with formaldehyde; they suggested that a similar type of reaction may be operative in plant tissue. Späth (74) also expressed the same opinion during his earliest studies on peyote constituents. Specifically, formaldehyde and acetaldehyde were regarded as the possible biological sources of the one- and two-carbon units at C-1 of the natural tetrahydroisoquinolines of peyote. Schöpf (239, 240) and Schöpf and Bayerle (241) thought

[9] Dopamine (LIII) has not been encountered, as yet, in peyote; it is, however, a constituent of another cactus, *Carnegia gigantea* (236).

[10] 3,4-Dimethoxyphenethylamine (VIII) was contemporarily suggested by Paul *et al.* (231) as a direct precursor on account of the high incorporation percentage; later, Rosenberg *et al.* (232) revised this judgment and dismissed this product as an actual participant.

[11] The incorporation percentage of this compound reported by Paul *et al.* (231) was comparable to that given by Lundström and Agurell (178, 233), although both groups differed on its significance.

[12] In this connection, Benington and Morin (237) showed that enzymatic oxidation of LVIII in fact yielded LIX while VIII served as a poor substrate.

[13] It would still, however, be desirable to find out if amino acids corresponding to mono-*O*-methylated analogs of LIV (such as LIX) and LVI are present in the natural mixture.

that only the phenethylamines carrying free hydroxyl groups, which would activate the hydrogen atom involved in the ring closure, are capable of such condensation—a Mannich-type reaction which was demonstrated *in vitro* and shown to proceed at room temperature and in nearly neutral media. Hahn and Schales (242, 243), however, proved that other methoxy and methylenedioxyphenethylamines are also capable of condensation, although to a lesser extent. On the basis of other *in vitro* experiments, Hahn and his coworkers (244–247) suggested that similar condensation with α-keto acids, followed by decarboxylation, may lead in nature to the various tetrahydroisoquinolines.

Battersby and Garratt (182) were the first to show, by feeding experiments, that the tetrahydroisoquinolines of peyote, exemplified by pellotine, may originate from tyrosine. The finding was subsequently confirmed by feeding tyrosine-α-^{14}C to peyote and isolation of both radioactive anhalonidine (XX), in which all the activity was shown by degradation to reside in C-3 (164, 181), and lophophorine (XXIX) (181). It seemed reasonable that the phenethylamine portion of the tetrahydroisoquinolines would be biosynthesized by routes similar (or, in some stages, identical) to those that lead to mescaline. Lundström and Agurell (178, 233) found that tyramine and dopamine are efficiently incorporated into anhalamine (XIII), and Battersby *et al.* (181) showed the latter precursor to be a step on the pathway toward pellotine (XXIII). Available evidence (up to the time of writing this review) seems to indicate that beyond the stage of dopamine, different pathways exist for the different tetrahydroisoquinoline alkaloids. This may be the case since 3,4,5-trihydroxyphenethylamine (LIV), for example, was well incorporated into anhalamine (178, 233) but hardly in pellotine and anhalonidine (235). 3-Methoxy-4-hydroxyphenethylamine (LVIII) was an insignificant contributor for anhalonine (XXVI) (233) but a better one for pellotine[14] (248), anhalonidine (235), and anhalamine (233, 235). 3-Methoxy-4,5-dihydroxyphenethylamine (LIX), which served as an efficient precursor for anhalamine (233), was poorly incorporated into pellotine (248) and anhalonine (233). Further, 3,4-dimethoxy-5-hydroxyphenethylamine (V) was found to be well incorporated into anhalamine (233, 235) and anhalonidine (235) but insignificantly into pellotine (235, 248) and anhalonine (233). Mescaline itself was also considered as a possible precursor but found to be a poor contributor for anhalamine (178) and pellotine (248); the slight incorporation found may be due to previous partial demethylation.

It would appear, therefore, that the incorporations observed for the intermediates following the stage of dopamine are fragmentary and even inconsistent. Battersby *et al.* (248) suggested that cyclization of 3,4,5-trihydroxyphenethylamine (LIV) to the isoquinoline system precedes *O*-methylation. Lundström and Agurell (233), however, were of the opinion that partial *O*-methylation precedes cyclization and that 3-methoxy-4,5-dihydroxyphenethylamine (LIX) is a common precursor which, by further methylation on the *para*-hydroxy group, gives 3,4-dimethoxy-5-hydroxyphenethylamine (V); the latter may be the immediate progenitor of the tetrahydroisoquinolines or at least anhalamine. However, the situation, obviously confusing and difficult to explain, certainly calls for additional work to establish more rigorously the pathways followed, probably independently, in the elaboration of the phenethylamine portion of the different tetrahydroisoquinolines. Also, the possibility that they may evolve from each other by transformations involving *O*- and/or *N*-methylations or demethylations should not be overlooked. In fact, Battersby *et al.* (248) demonstrated that *N*-methylation of anhalonidine occurs *in vivo* to give pellotine and that the reverse also occurs to an even greater extent.

There seems to be general agreement that methionine is the sole or main source of the *O*- and *N*-methyl groups in the tetrahydroisoquinoline alkaloids. This was demonstrated by the high incorporations observed into pellotine (181, 248), anhalidine (XVIII) (248), anhalamine, and anhalonidine (178) following the feeding of [methyl-^{14}C]methionine to peyote.[15] Battersby *et al.* (248) concluded that the *S*-methyl group of methionine is transferred intact, which is in agreement with previous work (249) on nicotine.

The origin of the one-carbon (C-1) and two-carbon (C-1 and C-9) units, present in tetrahydroisoquinolines of the anhalamine (XIII) and anhalonidine (XX) types, respectively, would still have to be accounted for. From feeding studies with [1-^{14}C]- and [2-^{14}C]acetate, Battersby *et al.* showed that the *S*-methyl group of methionine cannot be a contributor (181) and that the acetate carboxyl (181, 248) and methyl (248) groups may contribute—but not through direct incorporation[16]—equally to each position in the two-carbon unit of pellotine. This unit in anhalonidine was also shown by Lundström and Agurell (178) not to originate from methionine.[17] Assuming that acetate suffers degradation prior to incorporation into the two-carbon unit (of pellotine, for example), Battersby *et al.* (248) studied the metabolism of bicarbonate and formate and found that only the latter could be incorporated into C-1 and C-9 with equal distribution. It became clear, therefore, that the two-carbon unit originates from a precursor to which both atoms of acetate and that of formate can donate and that other contributors must be involved. Battersby *et al.* (248) suggested that glycine, serine, and pyruvate may be considered as likely precursors and even that a C-5 side chain derived from mevalonate, in a suitable precursor akin to lophocerine (LXVI) (251, 252), may be involved by degradation.

[14] The incorporation value (1.5%) given by Battersby *et al.* (248) for this transformation, which those authors regarded as low, is higher than that (0.37%) found by Paul *et al.* (235).

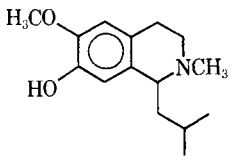
[15] The feeding of sodium ^{14}C-formate showed limited incorporation of this source into the *O*-methyl groups of pellotine (248).

[16] In fact, an appreciable proportion of the activity of the sodium [1-^{14}C]acetate fed to the peyote plant was scattered over the *O*- and *N*-methyl groups and the eight carbon atoms in the rest of the molecule (181). The acetate carboxyl group is known to contribute to the *O*-methyl groups and residues derived from the shikimic acid pathway (250).

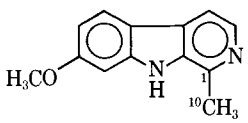
[17] With anhalamine, however, the same workers (178) found that the methyl group of methionine can be incorporated into C-1; this may be construed as evidence of a pathway involving the oxidative cyclization of an *N*-methylated derivative of phenethylamine.

Kapadia and Fales (96) recently suggested that the alkaloid amides mescalotam (XXXVIII) and peyoglutam (XXXIX) discovered in peyote may result *in vivo* by condensation of the appropriate phenethylamines with α-ketoglutarate[18] followed by decarboxylation of the resulting acids, reactions that were also realized *in vitro* (*vide supra*). These authors (96) extended their view, which was based on the original biosynthetic proposals of Hahn and his coworkers (244–247) to account for the origin of the one- and two-carbon units at C-1 in tetrahydroisoquinolines and suggested that they also arise from analogous condensations with the appropriate α-keto acids, namely, glyoxylate and pyruvate, respectively, rather than acetate. More recently, Leete and Braunstein (253) presented experimental evidence indicating that pyruvate can be involved since feeding sodium pyruvate-3-^{14}C to peyote cactus gave relatively high specific incorporation of the labeled atom into C-9 of anhalonidine. The two-carbon unit (C-1 and C-10) of harmine (LXVII) was similarly reported by Stolle and Gröger (254) to result from C-2 and C-3 of pyruvate; unspecific labeling was, however, obtained after feeding acetate-^{14}C to the *Peganum harmala* plant. In contrast, O'Donovan and Kenneally (255) found that the feeding of acetate-1-^{14}C to *Eleagnus angustifolia* plant yielded eleagnine (LXVIII) having predominantly all the activity at C-1.

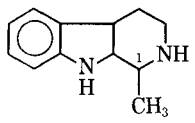
These facts, along with the recent report of Slaytor and McFarlane (256) that *N*-acetyltryptamine serves as a direct precursor of harman (LXIX) in *Passiflora edulis*, led Leete and Braunstein (253) to suggest that the two-carbon unit in the tetrahydroisoquinoline alkaloids (such as anhalonidine, XX) results by cycliza-



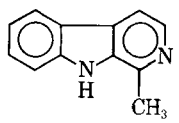
LXVI



LXVII



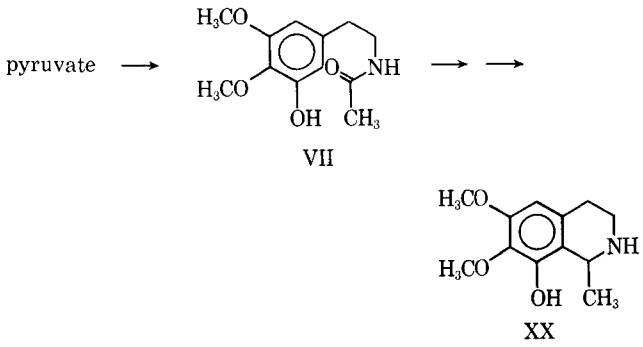
LXVIII



LXIX

tion from the *N*-acetyl moiety in the corresponding phenethylamine derivatives (such as VII) (Scheme XXXI).

For the origin of such *N*-acetyl groups, acetate must not be considered as the exclusive source since it failed to be incorporated directly into the tetrahydroisoquinolines of peyote (*vide supra*), a fact that may be rationalized by suggesting (253) that peyote contains no enzymes capable of utilizing acetic acid directly for the formation of such an *N*-acetyl derivative (as VII). A biosynthetic scheme was, therefore, proposed (253)

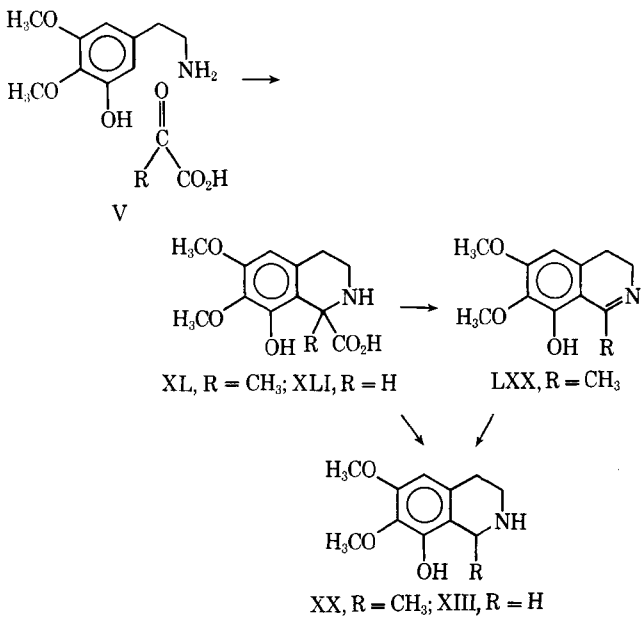


VII

XX

Scheme XXXI

whereby pyruvate acts as the primary source of the two-carbon unit and proceeds to acetylcoenzyme A, which then affords VII by contribution to the phenethylamine precursor.[19] The scheme centainly appears attractive, but its validity should be tested by the administration of *N*-acetyl- (and also *N*-formyl-) phenethylamines to peyote and determination of the degree of incorporation into the corresponding tetrahydroisoquinolines.[20] This pathway is a marked departure from the views of Hahn *et al.* (244–247) and Kapadia and Fales (96) who proposed that α-keto acids, as likely sources of the one- and two-carbon units, undergo Mannich condensation with the hydroxylated phenethylamines followed by decarboxylation of the intermediate acids (as XL and XLI) to yield the natural alkaloids; several *in vitro* simulations of such reactions were reported (Scheme XXXII).



V

XL, R = CH$_3$; XLI, R = H

LXX, R = CH$_3$

XX, R = CH$_3$; XIII, R = H

Scheme XXXII

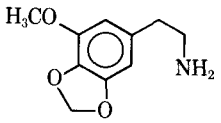
[18] It seems that the Krebs cycle acids conjugate favorably with mescaline (or a suitable precursor) in peyote, as evidenced by the proven (96) presence of the succinimide (XXXII), malimide (XXXIII), and maleimide (XXXIV) derivatives (see above).

[19] If taken literally, this would imply that glyoxylate is a source of the one-carbon unit in other tetrahydroisoquinolines (such as anhalamine) and that the *N*-formylphenethylamines, formed by a similar sequence, may be true precursors.

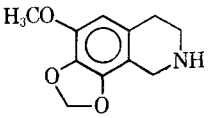
[20] It is pertinent in this connection to remember that peyote does actually contain several *N*-acetyl- (IV and VII) and *N*-formyl- (III and VI) phenethylamines (Table I) (96). While this manuscript was in preparation, Professor E. Leete (University of Minnesota, Minneapolis, Minn.) kindly informed the authors of his latest results (100) obtained from feeding *N*-[1-^{14}C]-acetyl-[α-^{14}C]-3,4-dimethoxy-5-hydroxyphenethylamine (VII) to peyote. These showed that the *N*-acetyl group was not involved significantly in the elaboration of the C-1 moiety of anhalonidine (XX) or anhalamine (XIII).

The actual presence of such nonproteinic amino acids, named peyoruvic acid (XL) and peyoxylic acid (XLI), in peyote was recently demonstrated by Kapadia *et al.* (99, 100, 185). Both were synthesized by condensation of 3,4-dimethoxy-5-hydroxyphenethylamine (V) with pyruvic acid and glyoxylic acid, respectively, at room temperature and physiological pH and resulted very rapidly in excellent yields. This intensified the belief that similar condensations may likewise operate in the plant tissue. Since normal physiological conditions were found (100) incapable of inducing subsequent decarboxylation, the transformation of both acids (XL and XLI) into the optically active anhalonidine and anahalamine, respectively, evidently necessitates the action of stereospecific decarboxylases. Experimentally, it was found (100, 185) that this could be true since facile decarboxylation of carboxyl-labeled *rac.* peyoruvic and peyoxylic acids (XL and XLI, respectively) could be brought about by short-term incubation with fresh slices of the peyote cactus, nearly half the activity was recovered as [^{14}C]-carbon dioxide. In a similar experiment in which [9-^{14}C]-peyoruvic acid was used, the decarboxylation product was isolated and found to be the Schiff base LXX, accounting for nearly half the activity. Moreover, feeding of [1-^{14}C]-peyoruvic[21] and [1-^{14}C]-peyoxylic acids to live peyote plants resulted in the incorporation of 6.0 and 6.8% of radioactivity in anhalonidine (XX) and anhalamine (XIII), respectively (100). The joint occurrence of 3-demethylmescaline (V), peyoruvic acid, and peyoxylic acid in peyote—in addition to the tracer experiments—would seem to suggest that the pathway indicated in Scheme XXXII can account for the C-1 moiety in the tetrahydroisoquinolines in addition or preference to that proposed by Leete and Braunstein (253). The isolation of the dihydroisoquinoline LXIII in the relatively short-term slice experiment may suggest that this product is a true intermediate, since it might be stereospecifically reduced to optically active anhalonidine (XX) with NADPH.

More work is certainly needed to establish rigorously the precise biogenetic route followed in the construction of the C-1 moiety in the various tetrahydroisoquinolines. Equally important is the work leading to the elucidation of the biogenetic interrelationships of the various tetrahydroisoquinoline alkaloids of peyote as well as the probable pathways that connect them with their phenethylamine precursors. In the latter, the biogenetic changes in the benzenoid oxygen substituents seem to be of special interest since a convenient explanation for the origin of the methylenedioxy system is still lacking. Kapadia *et al.* (95) recently anticipated the occurrence of homomyristicylamine (LXXI) in peyote and its probable biogenetic role. Whether the methylenedioxy group,[22] present in anhalonine (XXVI) and lophophorine (XXIX), is formed prior to or after cyclization remains to be established. The C-1 methylene counterpart (LXXII) of the latter compounds has not yet been found in peyote, although its presence is not unlikely from biogenetic reasoning.



LXXI



LXXII

BIOLOGICAL EFFECTS OF PEYOTE ALKALOIDS

General Pharmacodynamic Effects—*Mescaline (I)*—The effects of mescaline on the cardiovascular and respiratory systems were studied by several investigators (259–266). In general, low doses (~4 mg./kg.) of mescaline have no remarkable effects on blood pressure. Large doses (20–60 mg./kg.), however, produce a drop in blood pressure, bradycardia, respiratory depression, and vasodilation (259, 261–264, 266); the pressor and depressor effects persist after section of the vagi, greater and lesser splanchnic nerves, adrenalectomy, or atropinization. Moderate doses markedly inhibit the pressor effect of epinephrine without altering its accelerating effect on the cardiac rate. Grace (261), however, observed that the fall in blood pressure produced by mescaline in anesthetized cats could be prevented by vagotomy, decapitation, or atropine. Strong solutions of mescaline arrest the perfused frog heart in diastole due to a direct action on the cardiac muscle (261). Mescaline produces some sensitization to the cardiodepressant action of sodium EDTA (267). Its action on the cardiovascular system and on the nictitating membrane in cats and dogs is potentiated by pretreatment with reserpine (268). A threshold dose (152 mcg./kg. i.v.) causes slight potentiation of the effect of epinephrine on the nictitating membrane of anesthetized cats (269). Mescaline is slightly antagonistic to the vasoconstricting effect of serotonin perfused through the isolated rabbit leg (270).

In concentrations over 0.2%, mescaline causes gradual paralysis and shortening of the isolated gastrocnemius muscle of the frog. It stimulates the contractions of intestine and uterus *in situ* but not when excised (261). The intestinal muscle initially shows contraction but later is paralyzed (262). Other reports, however, show that mescaline causes respiratory stimulation, increases the tone of excised intestine, and slightly anesthetizes the cornea (260) and that it decreases the tonus of the smooth muscle at a concentration of 20 p.p.m. (264). The curarelike blockade of the dog peroneal tibialis anticus nerve-muscle preparation caused by mescaline is antagonized by epinephrine, prostigmine, and potassium chloride (271, 272). In rabbits and cats, it has no direct effect but it potentiates the inhibition of muscular contractions due to *d*-tubocurarine, gallamine, and succinyldicholine (273). Mescaline also stimulates the escape reflexes in mice in low doses and inhibits them in high doses (274).

Mescaline facilitates serotonin-induced contractions of the isolated rat uterus at lower concentrations and contracts the uterus at higher doses; atropine has no

[21] From the [1-^{14}C]-peyoruvic acid-fed plant, an unknown water-soluble basic substance, accounting for about 90% of the administered radioactivity, was isolated which from spectral (NMR and mass) data appears to be anhalonidine (or a derivative) attached to a polar moiety (100).

[22] It does not appear too unlikely that this group may arise from neighboring methoxy and hydroxy groups in the appropriate nucleus, since such transformation of aromatic substituents is a proven (257, 258) biosynthetic event.

effect but chlorpromazine inhibits these uterine contractions (275–278). Mescaline causes vasoconstriction of the umbilical vessels of the human placenta (279). In small doses, it enhances the potentiating effect of serotonin but blocks the prolongation action of reserpine on hexobarbital hypnosis in mice (280). It also augments cerebral serotonin levels and suppresses the protective effect of reserpine against toxicity of amphetamine in mice grouped in small cages (281). The favorable results obtained after administration of mescaline in a few cases of amenorrhea were attributed to an antagonism to serotonin (282).

Mescaline produces hyperthermia, and this effect in rabbits can be potentiated by pretreatment with iproniazid or reserpine (283–285). Jacob *et al.* (286, 287) observed a correlation between the mescaline-induced hyperthermia in rabbits, antianalgesia in mice, and hallucinogenic action in man. In rats, it lowers rectal temperature and is an effective antipyretic agent against tetrahydro-2-naphthylamine-induced fever (288).

A detailed discussion of the clinical pharmacology of mescaline and other related phenethylamines is given by Jacobsen (289).

The LD_{50}'s of mescaline in the rat are 330–410 mg./kg. i.p., 157 mg./kg. i.v., and 534 mg./kg. s.c. (259, 260). These values fall within the ranges given by other investigators for mice, frogs, and guinea pigs (290, 291). Flexor convulsions and respiratory arrest are the terminal events. Dogs fed with mescaline were reported (292) to develop capillary damage in the liver and nitrogen retention. Administration of sodium succinate to mice (0.5 g./kg. i.p.) was found to afford complete protection against mescaline (0.2 g./kg.) (290).

Hordenine (*XI*)—Heffter (59) gave the earliest description of the pharmacological properties of this compound; it was found to cause paralysis of the CNS in frogs without previous excitation. It was also reported to be highly antiseptic and to have an inhibitory effect on some soluble ferments (293, 294). Small doses of hordenine have no effect on blood circulation, but larger ones produce appreciable hypertension and accelerated pulsation; very large doses cause death by arrest of respiration. Rietschel (295, 296) found that the pressure effect of hordenine is not of central origin but results by stimulation of the heart muscle. Although much less active than adrenalin, it is analogous in its action, resembling ephedrine rather than adrenalin. Other workers reported that hordenine displays a nicotine-like action (297–299) and that in large doses it decreases or reverses the hypertensive effect of adrenalin (300).

Anhalonine (*XXVI*)—Heffter (62) described some aspects of the activity of this compound and the following ones after their first isolation from peyote. In the frog, injected doses of 5–10 mg. produced an increase in the reflex excitability after a phase of paresis. In the rabbit, similar symptoms resulted but general hyperexcitability predominated.

Anhalonidine (*XX*)—According to Heffter (62), doses of 20–25 mg. of the hydrochloride produced narcosis in the frog followed by increased excitability. Larger doses caused complete paralysis. Doses of 30–50 mg. provoked a curarizing effect. No significant symptoms have been observed in mammals.

Pellotine (*XXIII*)—Doses of 5–10 mg. caused temporary convulsions in frogs, dogs, and cats (62). Several authors, cited by Joachimoglu and Keeser (301), believe that pellotine could be used in man as a relatively safe narcotic.

Lophophorine (*XXIX*)—This base was shown by Heffter (62) to be the most toxic one in peyote; 0.25–1 mg. of injected hydrochloride provoked a long-lasting tetany in the frog. Although the animal recovers, the increased excitability may last for several days. There is no apparent action on the isolated frog heart. A dose of 7 mg./kg. in rabbits causes hyperexcitability and accelerated respiration; progressively higher doses induce tetany (12.5 mg./kg.) and lead to death (15–20 mg./kg.). Intravenous injection of 2.5 mg. causes an increase in blood pressure, but higher doses are hypotensive, without specific action on the heart.

More recently, Brossi *et al.* (176) examined anhalamine, anhalidine, anhalonidine, and pellotine and found all to be hardly active as anticonvulsants, tranquilizers, or muscle relaxants and to have no significant hallucinogenic effects.

Behavioral Effects of Mescaline—*On Animals*—In rats, mescaline (I) initially depresses the conditioned-avoidance response and then gives rise to a prolonged excitatory phase; the latter effect is dominant when lower doses are used (302). Harmine was found to augment the effect of mescaline (260). Maffii (303,304) observed the effect of mescaline on the secondary conditional response in an experimental avoidance situation and found the ED_{50} for blocking the response to be 32.5 mg./kg. i.p. Following an injection of 50 mg./kg., only 10% of the animals showed a loss of conditioned response. Dogs under the influence of large doses of mescaline reacted to conditioned stimulus as if it was the unconditioned one (305). Mescaline gave some protection to albino rats bred to have a high incidence of seizures when exposed to a standard sound (306).

Mescaline produced experimental catatonia in mice, guinea pigs, cats, monkeys, pigeons, and other animals (260, 307–314); the catatonic manifestations, however, could be inhibited by chlorpromazine, reserpine, and azacyclonol (315, 316). It caused reduction in the behavior patterns of contentment and sociability but increased excitement, aggressiveness, and defensive hostility (317). It was observed, however, that mescaline inhibited the isolation-induced attack behavior of mice (318). It produced a scratching response in mice, which was antagonized by tetrahydroberberine (319), various tranquilizers, serotonin, *d*-amphetamine, *etc.*, but not by barbiturates, meprobamate, azacyclonol, and mephenesin (320–322). Mescaline, given by intracerebral injection, produced aggressive tendencies and paroxysms of ear scratching in mice by doses that were ineffective by other routes (323). It also prolonged and potentiated the analgesic effect of morphine in mice (324).

In Humans—The onset, intensity, duration, and quality of the hallucinations produced in humans upon ingestion of mescaline or peyote depend both on the quantity of the drug and on the individual's cultural background. With mescaline, a latency period, de-

pending upon the route of administration, is followed by a number of disagreeable autonomic symptoms which are felt prior to the phase of inebriation. After an intravenous dose of 0.5 g. of mescaline sulfate, nausea, vomiting, sweating, generalized discomfort, dizziness, headache, palpitation, feeling of hot or cold, pupillary dilatation, and chest, neck, or abdominal cramps occur within 10 min. (325). A small rise in body temperature and systolic blood pressure are also experienced (326). Usually, the heart rate is accelerated somewhat, but sometimes a slight bradycardia is observed instead, as a reflex compensation of the hypertension. In general, the subjective vegetative symptoms like nausea and vomiting subside before the development of the psychic symptoms; the objective vegetative symptoms, such as pupillary dilatation, follow the time course of the psychic effects.

During the phase of mescaline-induced inebriation, the subject goes through abnormal mental states characterized by one or more of the following effects (37):

1. Visual hallucinations: Colors are at first intensified. Then, gradually, the subject shuts his eyes and entoptic phenomena appear, followed by true hallucinations with forms, shades, and movements. Flashing lights and complete scenes are seen, ordinary objects appear to be marvelous, and familiar sounds and music are "seen" in vivid colors.

2. Auditory hallucinations: These are fairly rare but frequently correspond to color and form visions. Hypersensitivity and pain at the slightest noise are also experienced.

3. Abnormal touch, taste, and smell: Paresthesias and alteration in perception of heat and cold occur.

4. Disturbances of personal judgement: The subject's perception of his own body, for example, is largely that it is changing shape, and he becomes unable to locate some parts of it. The perception of space and time becomes altered and both appear to be immensely stretched.

5. Emotional disturbances: An ecstatic euphoria with laughter is usually observed, especially at the beginning of the intoxication. This state is sometimes followed by anxiety and, less frequently, depression. The subject may then become suspicious and hostile, especially toward the person conducting the experiment.

6. Disturbances of the will and thinking: The subject utters words and makes gestures which he simultaneously does not wish to speak or make. He cannot pursue an idea; in tests of concentration and reasoning, failure is almost sure (327).

7. Personality disturbances: These, being the most important effects of mescaline intoxication, are manifested by symptoms similar to those of schizophrenia (328–330). The effect has catatonic features (329, 331). An experienced clinical observer can easily distinguish between schizophrenic reactions and drug-induced psychoses (328). The disorganization of psychic integration was much more apparent in schizophrenics than in normal subjects given mescaline (332). The drug precipitated schizophrenic psychoses in persons suffering from latent schizophrenia and also reactivated the psychosis in patients who improved after psychosurgery (333).

Detailed descriptions of the mescaline and peyote inebriation are given by various authors (1, 31–33, 35, 38, 58, 63, 71, 292, 325, 328, 334–341).

The reactions induced by LSD, psilocybin, and mescaline are qualitatively similar (326, 342), but mescaline is the least potent. It was noted that mescaline and LSD and their combination produced an enhancement in primary suggestibility (343). In a comparison of the effects of taraxein, a protein fraction obtained from the serum of schizophrenics, with those of mescaline, psilocybin, and LSD, Silva *et al.* (344) found that taraxein produced more dysphoria, blocking, and thought deprivation than did the three psychotomimetics. Secondary symptoms evoked by the protein fraction were more typical of those seen in schizophrenics than those induced by other substances.

The mescaline-induced state of intoxication has been used as an aid in psychotherapy (345). It has been recommended that mescaline be used when it is desirable to shorten a course of therapy, reactivate a stalled treatment of neurosis, and break down memory blocks (345). The studies of Turns and Denber (346) indicate that psychotherapy with mescaline can be effective in carefully selected cases where other methods have failed.

Tolerance, Habituation, and Addiction—Several studies show that human subjects and animals receiving mescaline daily develop tolerance to mescaline (302, 326, 347–351) and crosstolerance to LSD (326, 350, 351). The tolerance, however, regresses rapidly, and initial responsiveness is attained 3–4 days after the drug is withdrawn. Generally, the tolerance for the vegetative phase does not develop to the same degree as for the psychic phase (352). Speck (259) reported that no tolerance to the hypoglycemia and bradycardia induced by mescaline is observed. Dogs given 5 g./kg. of mescal buttons acquired tolerance to the emetic effects during 1 year of daily ingestion (353). Doses of mescaline producing acute tolerance have no effect on development of the chronic type (354). There seems to be no evidence that peyote and mescaline cause habituation and addiction (37); very few investigators hold a different view (355). Slotkin (44, 49) found that "the habitual use of peyote does not seem to produce tolerance or accrued dependence," an opinion that is also endorsed by many specialists (37) who affirm that peyote should not be regarded as a narcotic drug.

Antagonism and Synergism—The systemic administration of the phenothiazine tranquilizers (356–358), reserpine (356, 358, 359), asarone (360), trioxazine (361), lithium carbonate (362), lysergic acid derivatives (320), benactyzine, methylnonyldioxolane, chlorphenoxamine, *etc.* (358), inhibit various components of the mescaline-induced effects in different animals. Model psychosis produced by mescaline in man can also be inhibited by application of chlorpromazine and meretran (363, 364). Reserpine, injected 24 hr. before the mescaline, was found, however, to potentiate the action of the latter in mice (365).

Distribution of Mescaline in Animal Body—Mescaline taken orally is readily absorbed from the intestinal tract. The studies of various workers seem to agree

about the distribution of mescaline taken by injection. Tarsitano (366), working on dogs injected subcutaneously, found the highest concentration in the liver and kidneys but much less in the brain and no appreciable amount in the blood. However, the plasma levels of mescaline were found (367) highest immediately following intravenous injection, and the drug disappeared from the bloodstream after 6–8 hr. The concentrations of mescaline in the liver, spleen, and kidneys were 3–6 times those in the plasma; the brain and blood levels were about the same. These findings were supported by Block and coworkers (368–372) who studied the distribution of mescaline-α-^{14}C in mice. They found that, shortly after intraperitoneal injection, the highest radioactivity resided in the liver and kidneys with almost none in the brain and spinal cord. It was thus evident that mescaline was rapidly incoporated in the liver proteins. The maximum tissue concentration of mescaline coincided with the period of marked autonomic stimulation, while the highest concentration in liver protein corresponded to the period of hallucinations (372).

From his studies on the incorporation of radioactive mescaline into isolated tissues, Block (373, 374) found that the drug was incorporated in liver homogenates only if the latter were kept under oxygen first and then under nitrogen, and that the reaction rate increased 10-fold by brief heating of the mixture to 55° or by addition of tyramine. These observations indicated that there was an inhibitor, probably amine oxidase, whose action could be blocked by conjugation with tyramine or destroyed by heat, thereby facilitating the incorporation of mescaline. The inhibitor was, however, shown not to be amine oxidase, since several known inhibitors of this enzyme failed to increase mescaline incorporation (373). Moreover, Block (375), working with isolated cell components, found that nuclei could incorporate mescaline readily regardless of the action of tyramine or heat, while mitochondria and microsomes behaved similar to the total homogenate. It was, therefore, concluded that the incorporation-inhibiting factor resides within mitochondria and microsomes but not in the cell nuclei. Block (374) concluded, in general, that mescaline may be incorporated into proteins by a different mechanism from that involved in the case of amino acids; he postulated the following pattern. Oxidation of mescaline to an aldehyde is followed by condensation of the latter with the free amino groups of protein. The resulting Schiff base may subsequently either be hydrolyzed to an aldehyde, which undergoes further oxidation to an acid, or dissociated to mescaline and the oxidatively deaminated protein.

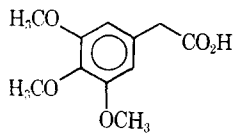
Fischer (376–378) used wool protein as a model surface to simulate the neuroreceptors involved in drug-induced psychoses. He demonstrated that the affinities (expressed in terms of absorption) of mescaline and other psychotomimetic agents, including LSD, for wool protein were parallel with their biological potency. Sympathetic overtones and subsequent adrenergic blockade seemed to contribute toward the precipitation of model psychosis. Neff *et al.* (379) found after administering radioactive mescaline intravenously into cats, which showed peak intoxication after 30 min., that the highest concentration of radioactivity was localized in the hypophysis, relatively high levels were in the cortical and subcortical gray matter, and very little was in the areas composed largely of myelinated fibers. The maximal mescaline concentration in the brain attained between 0.5 and 2 hr. roughly paralleled the period of maximal intoxication. The biological half-life of mescaline in plasma and cerebrospinal fluid was 1.5–2 hr. In human cerebrospinal fluid, however, Charalampous *et al.* (380) found significant concentrations of radioactivity throughout 9.5 hr. after administration of ^{14}C-mescaline.

Metabolism of Mescaline (I)—About one-half of the mescaline fed to rabbits was found to be excreted as 3,4,5-trimethoxyphenylacetic acid (LXXIII), a pharmacologically inactive metabolite (381, 382). Lesser amounts of the administered mescaline were recovered from dog urine (367). The studies of Block *et al.* (194) showed that rats and mice excreted intraperitoneally injected mescaline-α-^{14}C in the urine in the form of unchanged mescaline (18.4 and 79.4%), LXXIII (72.4 and 16.2%), and an unidentified product (9.1 and 4.4%), respectively. Spector (383) obtained qualitatively similar results by administering radioactive mescaline to dogs. Later, however, Neff *et al.* (379) found LXXIII to be the sole metabolite identified in the cat brain, plasma, cerebrospinal fluid, and urine. 2-(3,4,5-Trimethoxyphenyl)ethanol (LXXIV) was also found (384, 385) to be a metabolite in urine of rats fed with mescaline-α-^{14}C; its formation increases, at the expense of LXXIII, by calcium carbimide pretreatment, presumably because of the inhibition of aldehyde dehydrogenase. Pretreatment with iproniazid, however, markedly inhibits the formation of the acid (LXXIII), presumably by blockade of amine oxidase or a specific mescaline oxidase. Rabbits, estimated to be about 70 times as tolerant to mescaline as man, developed severe reactions by very small doses of mescaline when pretreated with calcium carbimide, implying that aldehyde dehydrogenase inhibition markedly enhances the pharmacological effects of mescaline. The intravenous administration of LXXIV to rabbits produced a mild mescalinelike effect, which was potentiated considerably when the alcohol was taken in combination with calcium carbimide. Based on these observations, it was suggested (385) that 3,4,5-trimethoxyphenylacetaldehyde (LXXV) is responsible for the pronounced pharmacological effects of mescaline.

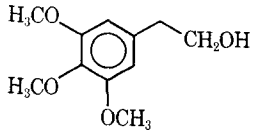
Musacchio *et al.* (386) and Musacchio and Goldstein (387) identified other mescaline metabolites, in the urine of rats, as *N*-acetyl-3,5-dimethoxy-4-hydroxyphenethylamine (LXXVI), *N*-acetyl-3,4-dimethoxy-5-hydroxyphenethylamine (VII), and *N*-acetylmescaline (IV), in addition to probably *N*-acetyl-3,4-dihydroxy-5-methoxyphenethylamine (LXXVII). They concluded that *N*-acetylation of mescaline may precede *O*-demethylation *in vivo*.

The early studies on metabolism of mescaline in man showed high recovery of the ingested drug in the urine of normal subjects and lesser amounts in psychopathic patients (388), with no evidence for the production of 3,4,5-trimethoxyphenylacetic acid (LXXIII) (382). Different figures for the recovery of mescaline in

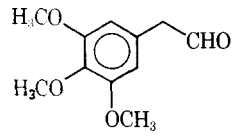
the urine were given by various workers (208, 389), depending on the route of administration and duration of the drug. Later studies (381, 390), however, showed that LXXIII was a metabolite which could be detected in human urine. 3,4-Dihydroxy-5-methoxyphenacetylglutamine (LXXVIII) (391) and 3-hydroxy-4,5-dimethoxyphenethylamine (V) (127) were other metabolites of mescaline found in small amounts in human urine. In more recent reports (40), orally taken mescaline was recovered substantially unchanged, in addition to LXXIII, VII, and IV, found in decreasing amounts



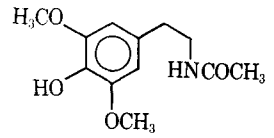
LXXIII



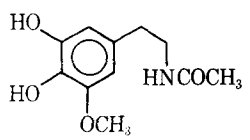
LXXIV



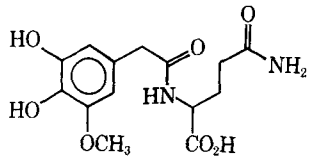
LXXV



LXXVI



LXXVII



LXXVIII

in both urine and cerebrospinal fluid. The appearance of LXXIII in human urine also was recently confirmed (392). A discussion of the metabolic fate of mescaline in man is given by Charalampous *et al.* (380).

Several *in vitro* studies (393, 394) showed that rabbit liver contains an enzyme system which oxidizes mescaline readily and that some factor other than oxidase might be involved. Amine oxidase was shown (394, 395) to have little or no effect on mescaline, and this was assumed (396) to be the reason for the high tolerance of rabbits to mescaline; a specific mescaline oxidase was claimed to have been isolated from the rabbit liver which was different from monoamine oxidase (397). Some authors (398) postulated that mescaline oxidase is identical with diamine oxidase, while others (399–401) believe that the oxidative deamination of mescaline can be effected by monoamine oxidase or diamine oxidase or both. However, mescaline was found to be a poor substrate for highly purified human plasma monoamine oxidase (402) and for dopamine-β-oxidase (403). According to Seiler (404), who treated mescaline with mouse brain homogenates, the oxidation is not caused by diamine oxidase but by a monoamine oxidase leading to 3,4,5-trimethoxyphenylacetic acid (LXXIII). Enzyme preparations obtained from rabbit lung produced *N*-methylation of mescaline (405, 406), while rabbit liver preparations caused *O*-demethylation to VII and LX along with oxidation to LXXIII (407). The formation of this acid (LXXIII) was inhibited by iproniazid, semicarbazide, nicotinamide, and triphosphopyridine nucleotide.

The excellent review of Patel (40) gives examples of the numerous studies made on various aspects of biochemical effects of mescaline with ample documentation.

Mode of Action of Mescaline—The following observations were enumerated by Patel (40) as reasons for doubting that mescaline *per se* is psychotomimetic and in support of the assumption that its action is caused by some metabolite.

1. Mescaline inhibited the oxidation of glucose, lactate, or pyruvate only if it was preincubated with brain homogenates for 2–3 hr. (408, 409).
2. In mice it was rapidly incorporated into liver proteins; during the phase of active CNS effects, the brain was almost devoid of it (371).
3. It stimulated the contractions of intestine and uterus *in situ* but not when excised (261).
4. No correlations were observed between the degree or type of behavioral responses and blood levels or rates of excretion of mescaline in man. The period of maximal behavioral changes followed the period of maximal blood level and excretion after 1–2 hr. (390).
5. The effective dose (400 mg.) was much higher than that of other hallucinogens; the effects took 1–2 hr. to develop and 5–6 hr. to reach a maximum (391).
6. The combined effect of mescaline and iproniazid could not be distinguished from that of an equivalent dose of mescaline (385).

Various explanations have been offered by different workers. Quastel and Wheatley (408) assumed that malfunction of the liver may give rise to abnormal amine metabolites which would affect brain respiration and subsequently produce CNS disturbances. It is not very likely that the hallucinogenic effect of mescaline is directly related to its sympathomimetic action on humans, since some substances possessing both the CNS and sympathomimetic properties are not hallucinogenic. The competition for adrenergic receptors leading to the disturbance of adrenergic mechanism was suggested by Speck (259). Quite recently, Clemente and Lynch (410) gave evidence suggesting that mescaline does not act *via* a cholinergic mechanism but rather through catecholamine mechanisms. The disturbance of histamine catabolism in the brain was suggested by Carlini *et al.* (411). Marrazzi and Hart (412) hold the view that "cerebral synaptic inhibition plays a part in the action of hallucinogens either by the direct disruption of normal patterns of synaptic activity as a result of alteration in the normal balance between cholinergic excitation and adrenergic inhibition at susceptible cerebral synapses."

Fischer (413) hypothesized the mode of action of mescaline, which he regarded as inactive *per se*, by suggesting that a metabolite produced *in vivo* by transformation into an indole derivative[23] resembling LSD is actually the active species. The hypothetical LSD-like compound results in very small amounts, perhaps from partially demethylated mescaline or through the condensation of mescaline with norepinephrine or serotonin. Other authors (9, 416) also postulated the *in vivo*

[23] Working on this assumption, Morin *et al.* synthesized 5,6,7-trimethoxyindole (414) and its 2,3-dihydro derivative (415) but found them to be devoid of mescalinelike activity.

Table II—Biological Data for the Isopropylamines

Compound	LD_{50} in Mice, mg./kg.	Effective Dose in Man, mg./kg.	Mescaline Units in Humans
LXXIX	260	1700	2.2
LXXX	150	1400	2.7
LXXXI	120	220	17
LXXXII	120	>1900	<2
LXXXIII	130	180	21
LXXXIV	40	210	18

formation of an indole derivative as the active species from mescaline. Snyder and Merril (417) made molecular orbital calculations for a variety of hallucinogenic and structurally similar nonhallucinogenic analogs in the phenethylamine, amphetamine, and tryptamine series and for LSD. They observed a close correlation between the energy of the highest filled molecular orbital of compounds, an index of electron donation, and their hallucinogenic potency.

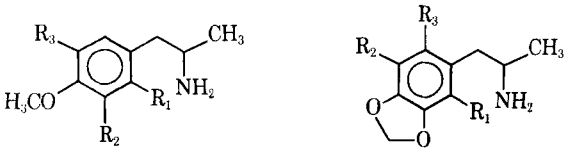
Although it appears attractive to suspect an intimate structural relationship among the active species that produce the same type of psychotomimetic effects, further and more thorough inquest should be made.

Structure–Activity Relationships—Several studies showed that alterations in the aromatic ring substituents (418–420) and the side chain can bring about substantial changes in the psychopharmacological properties of mescaline. Smythies and Levy (421), using the rope-climbing test for comparison, found that removal of the 5-methoxy group from mescaline caused about a 50% loss of activity, while demethylation of the 4-methoxy group resulted in a complete loss of activity. Substitution of the benzyloxy group for the 4-methoxyl was found to increase the activity. Ernst (422) confirmed that the presence of the 4-methoxyl group is essential for the manifestation of the hypokinetic rigid syndrome in cats. *N*-Methylation of mescaline seems to result in loss of the psychopharmacological properties of mescaline. Trichocereine (*N*,*N*-dimethylmescaline) was found (423) to produce no mental disturbances in man. However, it was observed to act on the CNS of normal cats to cause convulsions but to have no effect on decerebrated cats. This compound was shown (424, 425) to cause negligible inhibition of the conditioned-avoidance response but produced marked excitation similar to that induced by amphetamine; increase of the dosage delayed the onset of the excitation.

The introduction of a methyl group on the α-carbon of the phenethylamine system seems to maintain the hallucinogenic properties. In man, α-methylmescaline (LXXIX) (162) was found (426) to produce visual hallucinations in doses lower than that required for mescaline, without having significant effect on blood pressure or respiration. The peak urinary excretion occurred in 2–5 hr., with 20–35% of the ingested dose recovered unchanged in the urine. Several compounds analogous to LXXIX were also prepared and tested by Shulgin (427–429). The results showed that the presence of a 3,4-methylenedioxy group, as in LXXX,[24] does not result in loss of activity, while enlargement of this heterocyclic ring or increase in length of the aliphatic side chain in LXXIX results in decreased effect in humans. On the other hand, repositioning of the *meta*-methoxyl, in either LXXIX or LXXX, to an *ortho*-position produces an increase in potency (429).

Table II lists the effective dose (mg./kg.) of these isopropylamines in man, the LD_{50} (mg./kg.) in mice, and the mescaline units in humans (potency compared to mescaline = 1). The intoxication syndrome produced by these compounds in humans is qualitatively similar to that resulting from mescaline, except that the color effects and nausea are absent. In general, the methylenedioxy derivatives (LXXX, LXXXIII, and LXXXIV) gave more emphatic and pleasant responses. 2,5-Dimethoxy-3,4-methylenedioxyphenethylamine was found (431) to exhibit pharmacodynamic properties similar to those of mescaline. Schwachhofer *et al.* (432) and Schwachhofer and Chopin (433, 434) prepared several α,α-disubstituted derivatives of mescaline and found that the pharmacological properties (435) of only α,α-dimethylmescaline and α-methyl-α-veratrylmescaline are similar to those of mescaline.

A study of behavioral effects of mescaline, β-hydroxymescaline, and *N*-methyl-β-hydroxymescaline in mice performed by Friedman *et al.* (291) revealed that substitutions on mescaline did not substantially alter the biological activity of the parent compound.



LXXIX, $R_2 = R_3 = OCH_3$, $R_1 = H$ LXXX, $R_2 = OCH_3$, $R_1 = R_3 = H$
LXXXI, $R_1 = R_3 = OCH_3$, $R_2 = H$ LXXXIII, $R_3 = OCH_3$, $R_1 = R_2 = H$
LXXXII, $R_1 = R_2 = OCH_3$, $R_3 = H$ LXXXIV, $R_1 = OCH_3$, $R_2 = R_3 = H$

[24] Homomyristicylamine (LXXI) has recently been found by Shulgin (430) to be as potent as mescaline as a psychotomimetic in humans.

REFERENCES

(1) A. Hobschette, "Les Cactacées Médicinales," Gaston Doin & Co., Paris, France, 1929.
(2) L. Reti, *An. Asoc. Quim. Argent.*, **23**, 26(1935).
(3) E. Späth and F. Becke, *Monatsh. Chem.*, **66**, 327(1935).
(4) L. Reti, *Ciencia Invest.*, **3**, 405(1947).
(5) L. Reti, *Proc. Conf. Cultiv. Drug Ass. Econ. Plants Calif., 3rd*, **1947**, 110.
(6) T. A. Henry, in "The Plant Alkaloids," 4th ed., Blakiston, Philadelphia, Pa., 1949.
(7) L. Reti, *Fortsch. Chem. Org. Naturst.*, **6**, 242(1950).
(8) P. Rajotte, *Can. Med. Ass. J.*, **91**, 335(1964).
(9) A. Hofmann, *J. Exp. Med. Sci.*, **5**, 31(1961).
(10) A. Hofmann, *Indian J. Pharm.*, **25**, 245(1963).
(11) F. Barron, M. E. Jarvik, and S. Bunnell, Jr., *Sci. Amer.*, **210**, 29(1964).
(12) A. M. Ludwig and J. Levine, *Clin. Med.*, **73**, 21(1966).
(13) G. Hole, *Med. Monatsschr.*, **21**, 550(1967).
(14) G. Hole, *Muenchen. Med. Wochenschr.*, **109**, 1389(1967).
(15) S. Szara, *Amer. J. Psychiat.*, **123**, 1513(1967).
(16) Anonymous, *Med. Monatsschr.*, **21**, 214(1967).
(17) J. Delay, *Bull. Stupefiants*, **19**, 1(1967).
(18) L. Pelner, *N. Y. State J. Med.*, **67**, 2838(1967).
(19) A. Hofmann, *Therapiewoche*, **17**, 1199, 1739(1967).
(20) L. P. Solursh and W. R. Clement, *Can. Med. Ass. J.*, **98**, 407(1968).
(21) J. L. Goddard, *Chem. Eng. News*, **46**, 32(1968).
(22) Anonymous, *Anesth. Analg. (Cleveland)*, **47**, 72(1968).
(23) J. R. Unwin, *Can. Med. Ass. J.*, **98**, 402(1968).
(24) R. A. Sandison, *Practitioner*, **200**, 244(1968).
(25) R. Rossini, *Minerva Med.*, **59**, 2149(1968).

(26) N. R. Farnsworth, *Science*, **162,** 1086(1968).
(27) R. E. Schultes, *ibid.*, **163,** 245(1969).
(28) E. E. Ewell, *J. Amer. Chem. Soc.*, **18,** 624(1896).
(29) W. E. Safford, *J. Amer. Med. Ass.*, **77,** 1278(1921).
(30) L. Reutter, *Schweiz. Apoth.-Ztg.*, **62,** 441(1924).
(31) H. Klüver, *Amer. J. Psychol.*, **37,** 502(1926).
(32) K. Beringer, "Der Mezcalinrausch, seine Geschichte und Erscheinungsweise," Springer, Berlin, Germany, 1927.
(33) L. Lewin, "Phantastica," Dutton, New York, N. Y., 1931.
(34) E. Beccari, *Arch. Farmacol. Sper. Sci. Affini*, **61,** 161(1936).
(35) W. La Barre, "The Peyote Cult," Yale University Press, New Haven, Conn., 1938.
(36) M. Amorosa, *Ann. Chim. Farm.*, **1938,** 77.
(37) Anonymous, "Bulletin on Narcotics," **11** (2), 16(1959), and references cited therein, United Nations, Geneva, Switzerland.
(38) W. La Barre, *Curr. Anthropol.*, **1,** 45(1960).
(39) A. D. Marderosian, *Amer. J. Pharm.*, **138,** 204(1966).
(40) A. R. Patel, *Fortsch. Arzneim.-Forsch.*, **11,** 11(1968).
(41) L. Reti, in "The Alkaloids, Chemistry and Physiology," vol. III, R. H. F. Manske and H. L. Holmes, Eds., 1953, p. 313.
(42) L. Reti, in "The Alkaloids, Chemistry and Physiology," vol. IV, R. H. F. Manske and H. L. Holmes, Eds., 1954, p. 7.
(43) B. de Sahagun, "Historia General de las Cosas de Nueva España," 1560, Bustamente, Ed., Paris, France, 1880.
(44) J. S. Slotkin, *Amer. Anthropologist*, **57,** 202(1955).
(45) J. Mooney, *Ther. Gaz.*, **20,** 7(1896).
(46) A. Rouhier, *Paris Med.*, No. 18 (1923); cited as ref. 5 in (37).
(47) V. Petrullo, "The Diabolic Root," Philadelphia, Pa., 1934; cited as ref. 8 in (37).
(48) *Sci. News Lett.*, December 8 (1951); cited as ref. 13 in (37).
(49) J. S. Slotkin, *Trans. Amer. Phil. Soc.*, **42,** 565(1952).
(50) R. E. Schultes, "Peyote and Plants Used in the Peyote Ceremony," *Harvard Univ. Mus. Leafl.*, **4,** 129(1937).
(51) R. E. Schultes, *Planta Med.*, **13,** 125(1965).
(52) L. Lewin, *Arch. Exp. Pathol. Pharmakol.*, **24,** 401(1888).
(53) *Ibid.*, **34,** 374(1894).
(54) L. Lewin, *Ber. Deut. Bot. Ges.*, **12,** 283(1894).
(55) L. Diguet, *J. Soc. Americanistes Paris*, **4,** 21(1907).
(56) C. Lumholtz, "Unknown Mexico," C. Scribner's Sons, New York, N. Y., 1902.
(57) R. Newberne and C. Burke, "Peyotl," an abridged compilation from the files of the Bureau of Indian Affairs, Chilocco, Okla., 1923.
(58) A. Rouhier, "La Plante qui fait les yeux émerveillés," Gaston Doin & Co., Paris, France, 1927.
(59) A. Heffter, *Arch. Exp. Pathol. Pharmakol.*, **34,** 65(1894).
(60) A. Heffter, *Chem. Ber.*, **27,** 2975(1894).
(61) *Ibid.*, **29,** 216(1896).
(62) A. Heffter, *Arch. Exp. Pathol. Pharmakol.*, **40,** 385(1898).
(63) A. Heffter, *Chem. Ber.*, **31,** 1193(1898).
(64) *Ibid.*, **34,** 3004(1901).
(65) E. Kauder, *Arch. Pharm.*, **237,** 190(1899).
(66) G. Heyl, *ibid.*, **239,** 451(1901).
(67) *Ibid.*, **266,** 668(1928).
(68) D. W. Prentiss and F. P. Morgan, *Ther. Gaz.*, **19,** 577(1895).
(69) S. W. Mitchell, *Brit. Med. J.*, **2,** 1625(1896).
(70) H. Ellis, *Lancet*, **1,** 1540(1897).
(71) W. E. Dixon, *J. Physiol.*, **25,** 69(1899).
(72) A. Mogilewa, *Arch. Exp. Pathol. Pharmakol.*, **49,** 137(1903).
(73) E. Späth, *Monatsh. Chem.*, **40,** 129(1919).
(74) *Ibid.*, **42,** 97(1921).
(75) *Ibid.*, **42,** 263(1921).
(76) *Ibid.*, **43,** 477(1922).
(77) E. Späth, *Chem. Ber.*, **62,** 1021(1929).
(78) E. Späth and F. Becke, *ibid.*, **67,** 266(1934).
(79) *Ibid.*, **67,** 2100(1934).
(80) *Ibid.*, **68,** 501(1935).
(81) *Ibid.*, **68,** 944(1935).
(82) E. Späth and F. Boschan, *Monatsh. Chem.*, **63,** 141(1933).
(83) E. Späth and J. Bruck, *Chem. Ber.*, **70,** 2446(1937).
(84) *Ibid.*, **71,** 1275(1938).
(85) *Ibid.*, **72,** 334(1939).
(86) E. Späth and J. Passl, *Chem. Ber.*, **65,** 1778(1932).
(87) E. Späth and J. Gangl, *Monatsh. Chem.*, **44,** 103(1923).
(88) E. Späth and F. Kesztler, *Chem. Ber.*, **68,** 1663(1935).
(89) *Ibid.*, **69,** 755(1936).
(90) E. Späth and H. Röder, *Monatsh. Chem.*, **43,** 93(1922).
(91) J. L. McLaughlin and A. G. Paul, *J. Pharm. Sci.*, **54,** 661 (1965).
(92) J. L. McLaughlin and A. G. Paul, *Lloydia*, **29,** 315(1966).
(93) G. J. Kapadia, N. J. Shah, and T. B. Zalucky, *J. Pharm. Sci.*, **57,** 254(1968).
(94) G. J. Kapadia and H. M. Fales, *ibid.*, **57,** 2017(1968).
(95) G. J. Kapadia, Y. N. Vaishnav, and M. B. E. Fayez, *ibid.*, **58,** 1157(1969).
(96) G. J. Kapadia and H. M. Fales, *Chem. Commun.*, **1968,** 1688.
(97) G. J. Kapadia, M. B. E. Fayez, B. K. Chowdbury, and H. M. Fales, *11th Ann. Meet. Amer. Soc. Pharmacognosy* (Vienna, Austria), July 1970; to be published.
(98) G. J. Kapadia and R. J. Highet, *J. Pharm. Sci.*, **57,** 191 (1968).
(99) G. J. Kapadia, Y. N. Vaishnav, H. M. Fales, and M. B. E. Fayez, *116th Annu. Meet. Amer. Pharm. Ass.* (Montreal, Canada), 1969.
(100) G. J. Kapadia, G. S. Rao, E. Leete, M. B. E. Fayez, Y. N. Vaishnav, and H. M. Fales, to be published.
(101) J. Lundström and S. Agurell, *J. Chromatogr.*, **36,** 105 (1968).
(102) S. Agurell and J. Lundström, *Chem. Commun.*, **1968,** 1638
(103) L. Reti and J. A. Castrillón, *J. Amer. Chem. Soc.*, **73,** 1767 (1951).
(104) M. J. Poisson, *Ann. Pharm. Franc.*, **18,** 764(1960).
(105) S. Agurell, *Lloydia*, **32,** 206(1969).
(106) E. H. Ducloux, *Rev. Fac. Cienc. Quim. Univ. Nac. La Plata*, **6,** 75(1930); through *Chem. Abstr.*, **24,** 4077(1930).
(107) W. J. Turner and J. J. Heyman, *J. Org. Chem.*, **25,** 2250 (1960).
(108) E. P. Taylor, *J. Chem. Soc.*, **1951,** 1150.
(109) H. G. Boit, "Ergebnisse der Alkaloidchemie bis 1960," Akademie-Verlag, Berlin, Germany, 1961.
(110) L. Reti, *C. R. Soc. Biol.*, **114,** 811(1933).
(111) L. Reti and R. I. Arnolt, *Actas Trab. V Congr. Nac. Med., Rosario*, **3,** 39(1935).
(112) A. J. Haagen-Smit and M. Oliver, private communication (cited as Ref. 49 in 7).
(113) S. Agurell, J. Lundström, and A. Masoud, *J. Pharm. Sci.*, **58,** 1413(1969).
(114) Soc. Anon. Pour Lind. Chim. A Bâle, British pat. 360,266, Jan. 8, 1930; through *Chem. Abstr.*, **27,** 513(1933).
(115) K. H. Slotta and G. Szyska, *J. Prakt. Chem.*, **137,** 339 (1933).
(116) G. Hahn and H. Wassmuth, *Chem. Ber.*, **67,** 696(1934).
(117) G. Hahn and F. Rumpf, *ibid.*, **71,** 2141(1938).
(118) M. Erne and F. Ramirez, *Helv. Chim. Acta*, **33,** 912(1950).
(119) F. Benington and R. D. Morin, *J. Amer. Chem. Soc.*, **73,** 1353(1951).
(120) A. Dornow and G. Petsch, *Arch. Pharm.*, **284,** 160(1951).
(121) F. Benington, R. D. Morin, and L. C. Clark, Jr., *J. Org. Chem.*, **19,** 11(1954).
(122) F. Benington, R. D. Morin, and L. C. Clark, Jr., *J. Amer. Chem. Soc.*, **76,** 5555(1954).
(123) F. Benington, R. D. Morin, and L. C. Clark, Jr., *J. Org. Chem.*, **20,** 1292(1955).
(124) J. R. Merchant and A. J. Mountwala, *Curr. Sci. (India)*, **26,** 211(1957).
(125) Y. Inubushi and K. Fujitani, *Yakugaku Zasshi*, **78,** 486 (1958).
(126) J. R. Merchant and A. J. Mountwala, *J. Org. Chem.*, **23,** 1774(1958).
(127) J. Ratcliffe and P. Smith, *Chem. Ind. (London)*, **1959,** 925.
(128) K. M. Dyumaev and I. S. Belostotskaya, *Zh. Obshch Khim.*, **32,** 2661(1962).
(129) S. Kubota, T. Masui, E. Fujita, and S. M. Kupchan, *J. Org. Chem.*, **31,** 516(1966).
(130) F. Benington, R. D. Morin, and L. C. Clark, Jr., *ibid.*, **20,** 102(1955).
(131) L. C. Raiford and D. E. Fox, *ibid.*, **9,** 170(1944).
(132) C. B. Gairaud and G. R. Lappin, *ibid.*, **18,** 1(1953).
(133) F. Benington, R. D. Morin, and L. C. Clark, Jr., *ibid.*, **21,** 1470(1956).

(134) D. E. Worrall and L. Cohen, *J. Amer. Chem. Soc.*, **66,** 842(1944).
(135) F. A. Ramirez and A. Burger, *ibid.*, **72,** 2781(1950).
(136) G. J. Kapadia and N. J. Shah, *115th Annu. Meet. Amer. Pharm. Ass.* (Miami Beach, Fla.), 1968; Abstract No. 16, p. 104.
(137) K. H. Slotta and G. Szyzka, *Chem. Ber.*, **67,** 1106(1934).
(138) K. Kindler and W. Peschke, *Arch. Pharm.*, **270,** 340(1932).
(139) *Ibid.*, **270,** 410(1932).
(140) K. Kindler, *Arch. Pharm.*, **265,** 389(1927).
(141) K. H. Slotta, *J. Prakt. Chem.*, **133,** 129(1932).
(142) K. H. Slotta and H. Heller, *Chem. Ber.*, **63,** 3029(1930).
(143) G. Hahn, *ibid.*, **67,** 1210(1934).
(144) K. Banholzer, T. W. Campbell, and H. Schmid, *Helv. Chim. Acta*, **35,** 1577(1952).
(145) F. Benington, R. D. Morin, L. C. Clark, Jr., and R. P. Fox, *J. Org. Chem.*, **23,** 1979(1958).
(146) F. Benington, R. D. Morin, and L. C. Clark, Jr., *ibid.*, **22,** 332(1957).
(147) J. Hadáček, J. Michalský, and L. Macholán, *Chem. Listy*, **49,** 271(1955).
(148) D. Amos, *Aust. J. Pharm.*, **45,** 58(1964).
(149) H. Jensch, German pat. 526,172, April 7, 1929; through *Chem. Abstr.*, **25,** 4284(1931).
(150) M. U. Tsao, *J. Amer. Chem. Soc.*, **73,** 5495(1951).
(151) A. Dornow and G. Petsch, *Arch. Pharm.*, **285,** 323(1952).
(152) A. Brossi, M. Baumann, and R. Borer, *Monatsh. Chem.*, **96,** 25(1965).
(153) W. Block and K. Block, *Chem. Ber.*, **85,** 1009(1952).
(154) T. Kametani, N. Wagatsuma, and F. Sasaki, *J. Pharm. Soc. Jap.*, **86,** 913(1966).
(155) M. O. Abdel-Rahman, M. N. Aboul-Enein, and R. M. Taha, *J. Chem. UAR*, **11,** 401(1968).
(156) F. Benington, R. D. Morin, and L. C. Clark, Jr., *J. Org. Chem.*, **23,** 2034(1958).
(157) M. Jansen, *Chem. Weekbl.*, **26,** 421(1929).
(158) M. Jansen, *Rec. Trav. Chim. Pays-Bas*, **50,** 291(1931).
(159) K. H. Slotta and J. Müller, *Z. Physiol. Chem.*, **238,** 14 (1936).
(160) G. S. Grace, *J. Pharmacol. Exp. Ther.*, **50,** 359(1934).
(161) H. K. Iwamoto and W. H. Hartung, *J. Org. Chem.*, **9,** 513 (1944).
(162) P. Hey, *Quart. J. Pharm. Pharmacol.*, **20,** 129(1947).
(163) E. Leete, *Chem. Ind.*, **1959,** 604.
(164) E. Leete, *J. Amer. Chem. Soc.*, **88,** 4218(1966).
(165) S. Agurell, J. Lundström, and F. Sandberg, *Tetrahedron Lett.*, **1967,** 2433.
(166) J. L. McLaughlin and A. G. Paul, *Lloydia*, **30,** 91(1967).
(167) H. Rosenberg, J. L. McLaughlin, and A. G. Paul, *ibid.*, **30,** 100(1967).
(168) J. J. Willaman and B. G. Schubert, "Alkaloid Bearing Plants and Their Contained Alkaloids," U. S. Dept. of Agr. Tech. Bull. No. 1234, U. S. Government Printing Office, Washington, D. C.
(169) J. S. Todd, *Lloydia*, **32,** 395(1969).
(170) W. M. Whaley and T. R. Govindachari, in "Organic Reactions," R. Adams, Ed., Wiley, New York, N. Y., 1960, p. 151.
(171) *Ibid.*, p. 74.
(172) W. J. Gensler, in "Organic Reactions," R. Adams, Ed., Wiley, New York, N. Y., 1960, p. 191.
(173) J. M. Bobbitt, J. M. Kiely, K. L. Khanna, and R. Ebermann, *J. Org. Chem.*, **30,** 2247(1965).
(174) J. M. Bobbitt and C. P. Dutta, *ibid.*, **34,** 2001(1969).
(175) A. Brossi, F. Schenker, and W. Leimgruber, *Helv. Chim. Acta*, **47,** 2089(1964).
(176) A. Brossi, F. Schenker, R. Schmidt, R. Banziger, and W. Leimgruber, *ibid.*, **49,** 403(1966).
(177) S. Durand, X. Lusinchi, and R. C. Moreau, *Bull. Soc. Chim. France*, **1961,** 270.
(178) J. Lundström and S. Agurell, *Tetrahedron Lett.*, **1968,** 4437.
(179) E. Späth and F. Becke, *Chem. Ber.*, **67,** 2400(1934).
(180) M. Takido, K. L. Khanna, and A. G. Paul, *J. Pharm. Sci.*, 59, 271(1970).
(181) A. R. Battersby, R. Binks, and R. Huxtable, *Tetrahedron Lett.*, **1967,** 563.
(182) A. R. Battersby and S. Garratt, *Quart. Rev.*, **15,** 272(1961).
(183) L. Small, in "Organic Chemistry," H. Gilman, Ed., Wiley, New York, N. Y., 1943, p. 1171.
(184) R. J. Highet and P. F. Highet, *J. Org. Chem.*, **30,** 902(1965).
(185) G. J. Kapadia, M. B. E. Fayez, Y. N. Vaishnav, G. S. Rao, and H. M. Fales, *Lloydia*, **32,** 525(1969).
(186) G. J. Kapadia and N. J. Shah, *ibid.*, **30,** 287(1967).
(187) L. Rosenthaler, *Pharm. Ztg.*, **76,** 653(1931).
(188) E. H. Ducloux, *Rev. Farm. (Buenos Aires)*, **74,** 87(1931).
(189) G. Vanags and A. Dombrowski, *Chem. Ber.*, **75B,** 82 (1942).
(190) E. Kinska, *Chem. Obz.*, **22,** 77(1947).
(191) E. G. C. Clarke, *J. Pharm. Pharmacol.*, **9,** 187(1957).
(192) M. L. Bastos, *Bol. Inst. Quim. Agr. Rio de Janeiro*, **45,** 7(1956); through *Chem. Abstr.*, **52,** 156(1958).
(193) I. R. Munier and M. Macheboeuf, *Bull. Soc. Chim. Biol.*, **31,** 1144(1949).
(194) W. Block, K. Block, and B. Patzig, *Z. Phys. Chem.*, **290,** 160(1952).
(195) V. M. Palmieri and C. Romano, *Arch. Ital. Sci. Farmacol.*, **2,** 345(1952); through *Chem. Abstr.*, **50,** 1531(1956).
(196) L. Reio, *J. Chromatogr.*, **4,** 458(1960).
(197) A. Resplandy, *Mem. Inst. Sci. Madagascar*, **10,** 1(1961); through *Chem. Abstr.*, **60,** 13885(1964).
(198) R. Fischer, *Rev. Can. Biol.*, **17,** 389(1958).
(199) J. Cochin and J. W. Daly, *Experientia*, **18,** 294(1962).
(200) J. A. Steele, *J. Chromatogr.*, **19,** 300(1965).
(201) A. Jart and A. J. Bigler, *ibid.*, **29,** 255(1967).
(202) E. G. C. Clarke, *Analyst*, **92,** 175(1967).
(203) J. Lundström and S. Agurell, *J. Chromatogr.*, **30,** 271 (1967).
(204) K. D. Parker, C. R. Fontan, and P. L. Kirk, *Anal. Chem.*, **35,** 356(1963).
(205) S. Agurell, *Lloydia*, **32,** 40(1969).
(206) G. J. Kapadia and G. S. Rao, *J. Pharm. Sci.*, **54,** 1817 (1965).
(207) S. D. Brown, J. L. Massingill, Jr., and J. E. Hodgkins, *Phytochemistry*, **7,** 2031(1968).
(208) D. Richter, *Biochem. J.*, **32,** 1763(1938).
(209) P. Dessi and C. Rizzoki, *Boll. Soc. Ital. Biol. Sper.*, **24,** 1250(1948).
(210) L. A. Woods, J. Cochin, E. J. Fornefeld, F. G. McMahon, and M. H. Seevers, *J. Pharmacol. Exp. Ther.*, **101,** 188(1951).
(211) G. Vistoli, *Boll. Chim. Farm.*, **94,** 133(1955).
(212) P. Dessi and T. Franco, *Boll. Soc. Ital. Biol. Sper.*, **25,** 1368(1949); through *Chem. Abstr.*, **46,** 6325(1952).
(213) N. Seiler and M. Wiechmann, *Z. Physiol. Chem.*, **337,** 229 (1964).
(214) D. Kaláb, *Pharmazie*, **11,** 268(1956).
(215) S. W. Bellman, *J. Ass. Offic. Anal. Chem.*, **51,** 164(1968).
(216) G. J. Kapadia and H. M. Fales, *Lloydia*, **31,** 430(1968).
(217) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," vol. I, Holden–Day, San Francisco, Calif., 1964, 173.
(218) M. Guggenheim, "Die Biogenen Amine," 3rd ed., S. Karger, Basel, Switzerland, 1940; *cf.* p. 524.
(219) E. Leete, in "Biogenesis of Natural Compounds," P. Bernfeld, Ed., Macmillan, New York, N. Y., 1963, p. 739.
(220) E. Leete and L. Marion, *Can. J. Chem.*, **32,** 646(1954).
(221) T. J. Matchette, L. Marion, and S. Kirkwood, *ibid.*, **31,** 488(1953).
(222) S. Kirkwood and L. Marion, *ibid.*, **29,** 30(1951).
(223) E. Leete, S. Kirkwood, and L. Marion, *ibid.*, **30,** 749(1952).
(224) E. Leete and L. Marion, *ibid.*, **31,** 126(1953).
(225) J. Massicot and L. Marion, *ibid.*, **35,** 1(1957).
(226) S. Scribney and S. Kirkwood, *ibid.*, **32,** 918(1954).
(227) S. H. Mudd, *Biochem. Biophys. Acta*, **37,** 164(1960).
(228) *Ibid.*, **38,** 354(1960).
(229) J. D. Mann and S. H. Mudd, *J. Biol. Chem.*, **238,** 381(1963).
(230) P. Kovacs and A. Jindra, *Experientia*, **21,** 18(1965).
(231) A. G. Paul, H. Rosenberg, and K. L. Khanna, *Lloydia*, **32,** 36(1969).
(232) H. Rosenberg, K. L. Khanna, M. Takido, and A. G. Paul, *ibid.*, **32,** 334(1969).
(233) J. Lundström and S. Agurell, *Tetrahedron Lett.*, **1969,** 3371.
(234) K. L. Khanna, H. Rosenberg, and A. G. Paul, *Chem. Commun.*, **1969,** 315.

(235) A. G. Paul, K. L. Khanna, H. Rosenberg, and M. Takido, *ibid.*, **1969,** 838.
(236) C. Steelink, M. Yeung, and R. L. Caldwell, *Phytochemistry*, **6,** 1435(1967).
(237) F. Benington and R. D. Morin, *Experientia*, **24,** 33(1968); personal communication cited as ref. 8 in (233).
(238) A. Pictet and T. Spengler, *Chem. Ber.*, **44,** 2030(1911).
(239) C. Schöpf, *Int. Congr. Pure Appl. Chem., 9th*, **4,** 189(1934).
(240) C. Schöpf, *Angew. Chem.*, **50,** 779, 797(1937).
(241) C. Schöpf and H. Bayerle, *Liebig Ann.*, **513,** 190(1934).
(242) G. Hahn and O. Schales, *Chem. Ber.*, **67,** 1486(1934).
(243) *Ibid.*, **68,** 24(1935).
(244) G. Hahn and K. Stiehl, *Chem. Ber.*, **69,** 2627(1936).
(245) G. Hahn and F. Rumpf, *Chem. Ber.*, **71,** 2141(1938).
(246) G. Hahn, L. Bärward, O. Schales, and H. Werner, *Liebig Ann.*, **520,** 107(1935).
(247) G. Hahn and H. Werner, *ibid.*, **520,** 123(1935).
(248) A. R. Battersby, R. Binks, and R. Huxtable, *Tetrahedron Lett.*, **1968,** 6111.
(249) L. J. Dewey, R. U. Byerrum, and C. D. Ball, *J. Amer. Chem. Soc.*, **76,** 3997(1954).
(250) A. R. Battersby, R. Binks, W. Lawrie, G. V. Parry, and B. R. Webster, *J. Chem. Soc.*, **1965,** 7459.
(251) C. Djerassi, H. W. Brewer, C. Clarke, and L. J. Durham, *J. Amer. Chem. Soc.*, **84,** 3210(1962).
(252) M. Tomita, T. Kikuchi, K. Bessho, and Y. Inubushi, *Tetrahedron Lett.*, **1963,** 127.
(253) E. Leete and J. D. Braunstein, *ibid.*, **1969,** 451.
(254) K. Stolle and D. Gröger, *Arch. Pharm.*, **301,** 561(1968).
(255) D. G. O'Donovan and M. F. Kenneally, *J. Chem. Soc. (C)*, **1967,** 1110.
(256) M. Slaytor and I. J. McFarlane, *Phytochemistry*, **7,** 605 (1968).
(257) A. J. Birch, in "Chemical Plant Taxonomy," T. Swain, Ed., Academic, New York, N. Y., 1963, p. 141.
(258) M. Scribney and S. Kirkwood, *Nature*, **171,** 931(1953).
(259) L. B. Speck, *J. Pharmacol. Exp. Ther.*, **119,** 78(1957).
(260) H. Hoshikawa, *Nippon Yakurigaku Zasshi*, **58,** 241, 261 (1962); through *Chem. Abstr.*, **60,** 7338(1964).
(261) G. S. Grace, *J. Pharmacol. Exp. Ther.*, **50,** 359(1934).
(262) M. Raymond-Hamet, *Bull. Acad. Méd. (Paris)*, **105,** 46 (1931); through *Chem. Abstr.*, **26,** 1347(1932).
(263) M. Raymond-Hamet, *Arch. Exp. Pathol. Pharmakol.*, **169,** 97(1933).
(264) G. de Nito, *Rass. Ter. Patol. Clin.*, **6,** 577(1934); through *Chem. Abstr.*, **31,** 3994(1937).
(265) A. Geesink and W. A. den Hartog-Jager, *Arch. Neer. Physiol.*, **24,** 79(1939); through *Chem. Abstr.*, **33,** 7901(1939).
(266) Chaumerliac and Roche, *Bull. Soc. Ophthalmol. Paris*, **1948,** 800; through *Chem. Abstr.*, **46,** 2191(1952).
(267) C. H. Ellis, *Arch. Int. Pharmacodyn. Ther.*, **154,** 26(1965).
(268) H. Schmitt and H. Schmitt, *ibid.*, **125,** 30(1960).
(269) E. Costa and G. Zetler, *J. Pharmacol. Exp. Ther.*, **125,** 230 (1959).
(270) R. Meier, J. Tripod, and E. Wirz, *Arch. Int. Pharmacodyn. Ther.*, **109,** 55(1957).
(271) R. T. Schopp, W. F. Kreutter, and S. V. Guzak, *Amer. J. Physiol.*, **200,** 1226(1961).
(272) R. T. Schopp and R. R. Walsh, *Arch. Int. Pharmacodyn. Ther.*, **147,** 463(1964).
(273) J. Cheymol, J. van den Driessche, and R. Lebars, *Therapie*, **19,** 219(1964).
(274) C. Hantz, G. Prunus, and G. Trouiller, *C. R. Soc. Biol.*, **159,** 1118(1965).
(275) E. Costa, *Proc. Soc. Exp. Biol. Med.*, **91,** 39(1956).
(276) E. Costa, *Psychiat. Res. Rep. Amer. Psychiat. Ass.*, **4,** 11 (1956); through *Chem. Abstr.*, **50,** 14120(1956).
(277) J. Delay and J. Thuillier, *Comp. Rend.*, **242,** 3138(1956).
(278) J. Thuillier, *C. R. Soc. Biol.*, **150,** 1150(1956).
(279) A. Aström and U. Samelius, *Brit. J. Pharmacol. Chemother.*, **12,** 410(1957).
(280) G. C. Salmoiraghi and I. H. Page, *J. Pharmacol. Exp. Ther.*, **120,** 20(1957).
(281) Y. Ruckebusch, M. Roche, and D. Schurch, *C. R. Soc. Biol.*, **159,** 1745(1965).
(282) E. V. Salerno and A. Tallaferro, *Sem. Med.*, **110,** 47(1957); through *Chem. Abstr.*, **51,** 8997(1957).
(283) H. Bächtold and A. Pletscher, *Experientia*, **13,** 163(1957).
(284) J. Jacob and C. Lafille, *Arch. Int. Pharmacodyn. Ther.*, **145,** 528(1963).
(285) Y. Ruckebusch, M. L. Grivel, and M. Roche, *C. R. Soc. Biol.*, **159,** 911(1965).
(286) J. Jacob, C. Lafille, G. Loiseau, P. E. Garin, and C. Barthelemy, *Med. Exp.*, **7,** 296(1962).
(287) J. Jacob, G. Loiseau, P. E. Garin, C. Barthelemy, and C. Lafille, *Arch. Int. Pharmacodyn. Ther.*, **148,** 14(1964).
(288) J. Delphant and M. Lanza, *J. Physiol. (Paris)*, **52,** 70(1960).
(289) E. Jacobsen, *Clin. Pharmacol. Ther.*, **4,** 480(1963).
(290) J. Delay, H. P. Gerard, and J. Thuillier, *C. R. Soc. Biol.*, **144,** 163(1950).
(291) O. M. Friedman, K. N. Parameswaren, and S. Burstein, *J. Med. Chem.*, **6,** 227(1963).
(292) W. Mayer-Gross, *Brit. Med. J.*, **1951** (ii), 317.
(293) L. Camus, *C. R. Acad. Sci.*, **142,** 110, 237, 350(1906).
(294) L. Camus, *Arch. Int. Pharmacodyn. Ther.*, **16,** 43(1906).
(295) H. G. Rietschel, *Arch. Exp. Pathol. Pharmakol.*, **186,** 387 (1937).
(296) H. G. Rietschel, *Klin. Wochenschr.*, **16,** 714(1937).
(297) M. Raymond-Hamet, *C. R. Soc. Biol.*, **113,** 386, 875(1933).
(298) M. Raymond-Hamet, *C. R. Acad. Sci.*, **209,** 67(1939).
(299) F. P. Ludueña, cited as ref. 116 in L. Reti (7).
(300) M. Raymond-Hamet, *C. R. Soc. Biol.*, **121,** 112(1936).
(301) G. Joachimoglu and E. Keeser, in "Handbuch der Experimentellen Pharmakologie," vol. II, A. Heffter, Ed., Springer-Verlag, Berlin, West Germany, 1924, p. 1104.
(302) J. R. Smythies and E. A. Sykes, *Psychopharmacologia*, **6,** 163(1964).
(303) G. Maffii, *J. Pharm. Pharmacol.*, **11,** 129(1959).
(304) G. Maffii, *Farmaco Ed. Sci.*, **14,** 503(1959).
(305) W. H. Bridger and W. H. Gantt, *Amer. J. Psychiat.*, **113,** 352(1956).
(306) I. Mercier, *Colloq. Int. Centre Nat. Rech. Sci.*, **112,** 331 (1963); through *Chem. Abstr.*, **61,** 2356(1964).
(307) L. Noteboom, *Ned. Tijdschr. Geneesk.*, **76, I,** 512 and **II,** 2860(1932); through *Chem. Abstr.*, **27,** 1399(1933).
(308) L. Noteboom, *Proc. Acad. Exp. Amsterdam*, **37,** 562(1934).
(309) P. Divry and H. Evrard, *Psychiat. Bl.*, **39,** 58(1935); through *Chem. Abstr.*, **31,** 8025(1937).
(310) H. Baruk, S. Launay, and J. Berges, *Rev. Neurol.*, **95,** 62 (1956).
(311) R. Michaux and W. G. Verly, *C. R. Soc. Biol.*, **157,** 211 (1963).
(312) R. Michaux and W. G. Verly, *Life Sci.*, **2,** 175(1963).
(313) R. Michaux and A. C. Fossion, *C. R. Soc. Biol.*, **158,** 1188 (1964).
(314) S. Z. Kramer, E. Seifter, and J. Seifter, *Fed. Proc.*, **24,** 390 (1965).
(315) F. M. Sturtevant and V. A. Drill, *Proc. Soc. Exp. Biol. Med.*, **92,** 383(1956).
(316) A. Poloni, *Cervello*, **32,** 400(1956).
(317) S. Norton and J. Tamburro, *J. Pharmacol. Exp. Ther.*, **122,** 57A(1958).
(318) E. T. Uyeno, *J. Pharm. Sci.*, **55,** 215(1966).
(319) K. C. Chin, H. Y. Chu, H. T. T'ang, and P. Hsu, *Sheng Li Hsueh Pao*, **25,** 182(1962); through *Chem. Abstr.*, **59,** 13249(1963).
(320) J. Borsy, Z. Huszti, and M. Fekete, *Int. J. Neuropharmacol.*, **2,** 273(1964).
(321) J. F. Deegan and L. Cook, *J. Pharmacol. Exp. Ther.*, **122,** 17A(1958).
(322) G. Maffii and E. Soncin, *J. Pharm. Pharmacol.*, **10,** 541 (1958).
(323) T. J. Haley, *Acta Pharmacol. Toxicol.*, **13,** 107(1957).
(324) E. B. Sigg, G. Caprio, and J. A. Schneider, *Proc. Soc. Exp. Biol. Med.*, **97,** 97(1958).
(325) H. C. B. Denber, in "Chemical Concepts of Psychoses," M. Rinkel and H. C. B. Denber, Eds., McDowell-Obolonsky, New York, N. Y., 1958, p. 120.
(326) A. B. Wolbach, Jr., H. Isbell, and E. J. Miner, *Psychopharmacologia*, **3,** 1(1962).
(327) E. Guttmann, *J. Ment. Sci.*, **82,** 203(1936).
(328) L. E. Hollister, *Ann. N. Y. Acad. Sci.*, **96,** 80(1962).
(329) R. Fischer, F. Georgi, and R. Weber, *Schweiz. Med. Wochenschr.*, **81,** 817(1951).

(330) V. M. Buscaino, *Gazz. Sanit.*, **20,** 417(1949); *Excerpta Med., Sect. VIII*, **3,** 635(1950); through *Chem. Abstr.*, **49,** 14193 (1955).
(331) L. Mátéfi, *Confin. Neurol.*, **12,** 146(1952).
(332) P. H. Hoch, J. P. Cattell, and H. H. Pennes, *Amer. J. Psychiat.*, **108,** 579(1952).
(333) P. H. Hoch, *ibid.*, **107,** 607(1951).
(334) M. Weir, *Brit. Med. J.*, **II,** 1625(1896).
(335) E. Havelock, *Ann. Rep. Smithsonian Inst.*, **1898,** 537.
(336) D. N. Buchanan, *Med. Ann.*, **1931,** 12.
(337) G. Marinesco, *Presse Med.*, **41,** 1433, 1864(1933).
(338) H. Claude and H. Ey, *C. R. Soc. Biol.*, **115,** 838(1934).
(339) P. N. Vargas, *Rev. Conf. Med. Panamer.*, **6,** 1(1959); through *Chem. Abstr.*, **53,** 19151(1959).
(340) H. C. B. Denber and S. Merlis, *Psychiat. Quart.*, **28,** 635 (1954).
(341) A. Huxley, "The Doors of Perception," 1954; "Heaven and Hell," 1956, Chatto and Windus, London, England.
(342) A. B. Wolbach, Jr., E. J. Miner, and H. Isbell, *Psychopharmacologia*, **3,** 219(1962).
(343) B. M. Sjoberg, Jr., and L. E. Hollister, *ibid.*, **8,** 251(1965).
(344) F. Silva, R. G. Heath, T. Rafferty, R. Johnson, and W. Robinson, *Compr. Psychiat.*, **1,** 370(1960).
(345) W. Frederking, *J. Nerv. Ment. Dis.*, **121,** 262(1955).
(346) D. Turns and H. C. B. Denber, *Arzneim.-Forsch.*, **16,** 251 (1966).
(347) J. Mercier and S. Dessaigne, *Ann. Pharm. Fr.*, **17,** 606(1959).
(348) A. Saxena, B. K. Bhattacharya, and B. Mukerji, *Arch. Int. Pharmacodyn. Ther.*, **140,** 327(1962).
(349) M. J. Hosko, Jr., and R. Tislow, *Fed. Proc. Fed. Amer. Soc. Exp. Biol.*, **15,** 440(1956).
(350) A. Balestrieri and D. Fontanari, *Arch. Gen. Psychiat.*, **1,** 279(1959).
(351) A. Balestrieri, in "Psychotropic Drugs," S. Garattini and V. Ghetti, Eds., Elsevier, New York, N. Y., 1957, p. 581.
(352) D. X. Freedman, G. K. Aghajanian, and E. M. Ornitz, *Science*, **127,** 1173(1958).
(353) V. A. Drill, "Pharmacology in Medicine," McGraw-Hill, New York, N. Y., 1965, chap. 19, p. 14.
(354) D. X. Freedman and G. K. Aghajanian, *Fed. Proc.*, **18,** 390(1959).
(355) M. Critchley, *Brit. J. Inebriety*, **28,** 99(1931).
(356) N. P. Plotnikoff and H. Washington, *Proc. Soc. Exp. Biol. Med.*, **98,** 660(1958).
(357) J. Delay, P. Deniker, M. Ropert, and J. Thuillier, *C. R. Soc. Biol.*, **150,** 512(1956).
(358) W. B. Rice and J. D. McColl, *Arch. Int. Pharmacodyn. Ther.*, **127,** 249(1960).
(359) S. Sailer and C. Stumpf, *Arch. Exp. Pathol. Pharmakol.*, **230,** 378(1957).
(360) P. C. Dandiya and M. K. Menon, *Life Sci.*, **4,** 1635(1965).
(361) J. Borsy, M. Fakete, and Z. Csizmadia, *Acta Physiol.*, **19,** 27(1961).
(362) P. Stern, D. Lekovic, R. Przic, and I. Gasparovic, *Arch. Int. Pharmacodyn. Ther.*, **133,** 58(1961).
(363) H. D. Fabing, *Science*, **121,** 208(1955).
(364) B. E. Schwartz, R. G. Bickford, and H. P. Rome, *Proc. Staff Meet. Mayo Clin.*, **30,** 407(1955).
(365) J. Bost, Y. Ruckebusch, and M. Roche, *C. R. Soc. Biol.*, **159,** 403(1965).
(366) F. Tarsitano, *Boll. Soc. Ital. Biol. Sper.*, **20,** 762(1945); through *Chem. Abstr.*, **40,** 6664(1946).
(367) J. Cochin, L. A. Woods, and M. H. Seevers, *J. Pharmacol. Exp. Ther.*, **101,** 205(1951).
(368) W. Block and K. Block, *Congr. Int. Biochim., Resumes Commun., 2e Congr., Paris*, **1952,** 429; through *Chem. Abstr.*, **48,** 8966(1954).
(369) W. Block, *Z. Naturforsch.*, **8B,** 440(1953).
(370) W. Block and K. Block, *Angew. Chem.*, **64,** 166(1952).
(371) W. Block, K. Block, and B. Patzig, *Z. Phys. Chem.*, **290,** 230(1952).
(372) *Ibid.*, **291,** 119(1952).
(373) W. Block, *ibid.*, **294,** 149(1953).
(374) *Ibid.*, **296,** 1(1954).
(375) *Ibid.*, **296,** 108(1954).
(376) R. Fischer, *Experientia*, **10,** 435(1954).
(377) R. Fischer and N. Agnew, *Naturwissenschaften*, **41,** 431 (1954).
(378) R. Fischer, *J. Ment. Sci.*, **100,** 623(1954).
(379) N. Neff, G. V. Rossi, G. D. Chase, and J. L. Rabinowitz, *J. Pharmacol. Exp. Ther.*, **144,** 1(1964).
(380) K. D. Charalampous, K. E. Walker, and J. K. Wright, *Psychopharmacologia*, **9,** 48(1966).
(381) K. D. Charalampous, A. Orengo, K. E. Walker, J. K. Wright, *J. Pharmacol. Exp. Ther.*, **145,** 242(1964).
(382) K. H. Slotta and J. Müller, *Z. Physiol. Chem.*, **238,** 14 (1936).
(383) E. Spector, *Nature*, **189,** 751(1961).
(384) M. Goldstein, A. J. Friedhoff, S. Pomerantz, C. Simmons, and J. F. Contrera, *J. Neurochem.*, **6,** 253(1961).
(385) A. J. Friedhoff and M. Goldstein, *Ann. N. Y. Acad. Sci.*, **96,** 5(1962).
(386) A. Musacchio, A. Dudowitz, H. Gerber, and M. Goldstein, *Fed. Proc.*, **22,** 481(1963).
(387) J. M. Musacchio and M. Goldstein, *Biochem. Pharmacol.*, **16,** 963(1967).
(388) A. Möller, *Acta Psychiat. Neurol.*, **10,** 405(1935).
(389) K. Salomon, B. W. Gabrio, and T. Thale, *J. Pharmacol. Exp. Ther.*, **95,** 455(1949).
(390) L. C. Mokrasch and I. Stevenson, *J. Nerv. Ment. Dis.*, **129,** 177(1959).
(391) J. Harley-Mason, A. H. Laird, and J. R. Smythies, *Confin. Neurol.*, **18,** 152(1958).
(392) A. J. Friedhoff and L. E. Hollister, *Biochem. Pharmacol.*, **15,** 269(1966).
(393) F. Bernheim and M. L. C. Bernheim, *J. Biol. Chem.*, **123,** 317(1938).
(394) C. E. M. Pugh and J. H. Quastel, *Biochem. J.*, **31,** 2306 (1937).
(395) G. A. Alles and E. Heegaard, *J. Biol. Chem.*, **124,** 487(1943).
(396) H. Blaschko, *J. Physiol.*, **103,** 13P(1944).
(397) G. Steensholt, *Acta Physiol. Scand.*, **14,** 356(1947).
(398) T. L. Sourkes, *Rev. Can. Biol.*, **17,** 328(1958).
(399) E. A. Zeller, J. Barsky, E. R. Berman, M. S. Cherkas, and J. R. Fouts, *J. Pharmacol. Exp. Ther.*, **124,** 282(1958).
(400) L. C. Clark, Jr., F. Benington, and R. D. Morin, *J. Med. Chem.*, **8,** 353(1965).
(401) L. C. Clark, Jr., F. Benington, and R. D. Morin, *Ala. Med. J. Sci.*, **1,** 417(1964).
(402) C. M. McEwen, Jr., *J. Biol. Chem.*, **240,** 2003(1965).
(403) C. R. Creveling, J. W. Daly, B. Witkop, and S. Udenfriend, *Biochim. Biophys. Acta*, **64,** 125(1962).
(404) N. Seiler, *Z. Phys. Chem.*, **341,** 105(1965).
(405) J. Axelrod, *Science*, **134,** 343(1961).
(406) J. Axelrod, *J. Pharmacol. Exp. Ther.*, **138,** 28(1962).
(407) J. Daly, J. Axelrod, and B. Witkop, *Ann. N. Y. Acad. Sci.*, **96,** 37(1962).
(408) J. H. Quastel and A. H. M. Wheatley, *Biochem. J.*, **27,** 1609 (1933).
(409) F. W. Schueler, *J. Lab. Clin. Med.*, **33,** 1297(1948).
(410) E. Clemente and V. D. Lynch, *J. Pharm. Sci.*, **57,** 72(1968).
(411) E. A. Carlini, M. Santos, and M. R. P. Sampaio, *Experientia*, **21,** 72(1965).
(412) A. S. Marrazzi and E. R. Hart, *J. Nerv. Ment. Dis.*, **122,** 453(1955).
(413) R. Fischer, *Experientia*, **11,** 162(1955).
(414) R. D. Morin, F. Benington, and L. C. Clark, Jr., *J. Org. Chem.*, **22,** 331(1957).
(415) F. Benington, R. D. Morin, and L. C. Clark, Jr., *ibid.*, **24,** 917(1959).
(416) M. Gordon, in "Medical Chemistry," A. Burger, Ed., Interscience, New York, N. Y., 1960, p. 397.
(417) S. H. Snyder and C. R. Merril, *Proc. Natl. Acad. Sci. U. S.*, **54,** 258(1965).
(418) J. R. Smythies, R. J. Bradley, V. S. Johnston, F. Benington, R. D. Morin, and L. C. Clarke, Jr., *Psychopharmacologia*, **10,** 379 (1967).
(419) Y. Takeo and H. E. Himwich, *Arch. Int. Pharmacodyn. Ther.*, **166,** 47(1967).
(420) J. R. Smythies, R. J. Bradley, and V. S. Johnston, *Life Sci.*, **6,** 1887(1967).
(421) J. R. Smythies and C. K. Levy, *J. Ment. Sci.*, **106,** 531(1960).
(422) A. M. Ernst, *Psychopharmacologia*, **7,** 383(1965).

(423) F. P. Ludueña, *C. R. Soc. Biol.*, **121,** 368(1936).
(424) J. R. Smythies and E. A. Sykes, *Psychopharmacologia*, **8,** 325(1966).
(425) J. R. Smythies and E. A. Sykes, *Fed. Proc.*, **24,** 196(1965).
(426) D. I. Peretz, J. R. Smythies, and W. C. Gibson, *J. Ment. Sci.*, **101,** 317(1955).
(427) A. T. Shulgin, *Experientia*, **19,** 127(1963).
(428) *Ibid.*, **20,** 366(1964).
(429) A. T. Shulgin, *Nature*, **201,** 1120(1964).
(430) A. T. Shulgin, private communication; D. Efron, "Psychotomimetic Drugs," Raven Press, Hewlett, N. Y., 1970.
(431) J. V. Supniewski, *Acta Biol. Exp. (Warsaw)*, **7,** 49(1932); through *Chem. Abstr.*, **28,** 7368(1934).
(432) D. G. Schwachhofer, J. Chopin, and C. Mentzer, *C. R. Soc. Biol.*, **247,** 2006(1958).
(433) G. Schwachhofer and J. Chopin, *ibid.*, **252,** 2244(1961).
(434) G. Schwachhofer and J. Chopin, *Bull. Soc. Chim. Fr.*, **1962,** 835.
(435) O. F. Blanpin, J. Bretaudeau, J. Chenieux, G. Schwachhofer, and J. Chopin, *Therapie*, **18,** 1441(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Pharmacognosy and Natural Products, College of Pharmacy, Howard University, Washington, DC 20001*

Acknowledgment is made of Grant MH 155 75, National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and of a Senior Foreign Scientist Fellowship awarded (to M.B.E.F., National Research Centre, Dokki, Cairo, Egypt) by the National Science Foundation. The authors are thankful to Dr. H. M. Fales and Dr. G. S. Rao (National Heart and Lung Institute, National Institutes of Health, Bethesda, Md.) for valuable discussions and kind cooperation.

RESEARCH ARTICLES

Synthesis and Biological Activity of Deuteriobenzyl-d_7-penicillin

PAUL A. LASKAR and ROBERT G. MRTEK

Abstract □ To investigate the deuterium isotope effect on the biological activity of penicillin G, this compound was chemically synthesized by first obtaining phenylacetyl-d_7-chloride from phenylacetic-d_7-acid and thionyl chloride, followed by condensation of the acid chloride with 6-aminopenicillanic acid. The penicillin was isolated as the 1-ethylpiperidine salt. Yield of the deuteriopenicillin was approximately 60% when a 2:1 (6-aminopenicillanic acid–acid chloride) molar ratio was used. The identity of the deuteriated penicillin was confirmed using several physical constants. From NMR data, the presence of deuterium in the benzyl moiety was found to be greater than 95 atom %. The biological activity of the deuteriopenicillin was compared to that of the protioanalog by a turbidimetric assay procedure using *Staphylococcus aureus*. The results of the biological assay indicate that a significant deuterium isotope effect operates in the antistaphylococcal action of benzylpenicillin. With the test organism chosen, the ratio of the antibiotic potencies was 125% H/D.

Keyphrases □ Deuteriopenicillin—synthesis □ Biological activity—deuteriopenicillin □ Turbidimetric assay—deuteriopenicillin antimicrobial activity □ NMR spectroscopy—identification □ IR spectrophotometry—identification

Deuterium (^{2}H), a rare stable isotope of hydrogen, was discovered by Urey *et al.* (1) in 1932. Extensive work by several investigators has felled the long-standing opinion that deuterium oxide (D_2O) is incompatible with life. The successful mass culture of algae, yeasts, and certain bacteria under conditions of full deuteriation has provided a useful source of fully deuteriated sugars, amino acids, proteins, and certain drugs (2–5). Substitution of deuterium for ordinary hydrogen and deuteriated substrates for protio metabolites has been shown to produce profound changes in biosystems. Isotope effects have been categorized as being either primary or secondary. Measurement of the magnitude of the effect is usually useful in distinguishing between the larger primary mass isotope effects and the smaller secondary effects.

The substitution of deuterium in several drug molecules has been the impetus for many recent research contributions. Isotopically altered drugs have shown widely divergent pharmacologic effects. Elison *et al.* (6) demonstrated a reduced analgesic potency of morphine deuteriated in the *N*-methyl group. Nona *et al.* (7) found an increased antifungal activity with fully deuteriated griseofulvin. Foreman *et al.* (8) investigated the *in vitro* Dutch rabbit liver metabolism of selectively deuteriated amphetamines. Their studies show that the ratio of apparent rate constants (k_H/k_D) is 1.9, indicating that the deuterioamphetamine is metabolized more slowly than the protioanalog. One major effect of deuterium substitution in drug-producing organisms has been a general suppression of most nonessential metabolism. Mrtek *et al.* (9) demonstrated an inverse effect between the level of deuteriation in *Claviceps* and the amount of clavine alkaloids produced in saprophytic culture. Multimilligram quantities of highly deuteriated elymoclavine were obtained only through the use of replacement culture techniques (10). Mohammed *et al.* (11) demonstrated a similar effect with several strains of *Penicillium*. Carlstedt (12) obtained small amounts of highly deuteriated penicillin from large numbers of shake

(423) F. P. Ludueña, *C. R. Soc. Biol.*, **121,** 368(1936).
(424) J. R. Smythies and E. A. Sykes, *Psychopharmacologia*, **8,** 325(1966).
(425) J. R. Smythies and E. A. Sykes, *Fed. Proc.*, **24,** 196(1965).
(426) D. I. Peretz, J. R. Smythies, and W. C. Gibson, *J. Ment. Sci.*, **101,** 317(1955).
(427) A. T. Shulgin, *Experientia*, **19,** 127(1963).
(428) *Ibid.*, **20,** 366(1964).
(429) A. T. Shulgin, *Nature*, **201,** 1120(1964).
(430) A. T. Shulgin, private communication; D. Efron, "Psychotomimetic Drugs," Raven Press, Hewlett, N. Y., 1970.
(431) J. V. Supniewski, *Acta Biol. Exp. (Warsaw)*, **7,** 49(1932); through *Chem. Abstr.*, **28,** 7368(1934).
(432) D. G. Schwachhofer, J. Chopin, and C. Mentzer, *C. R. Soc. Biol.*, **247,** 2006(1958).
(433) G. Schwachhofer and J. Chopin, *ibid.*, **252,** 2244(1961).
(434) G. Schwachhofer and J. Chopin, *Bull. Soc. Chim. Fr.*, **1962,** 835.
(435) O. F. Blanpin, J. Bretaudeau, J. Chenieux, G. Schwachhofer, and J. Chopin, *Therapie*, **18,** 1441(1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Pharmacognosy and Natural Products, College of Pharmacy, Howard University, Washington, DC 20001*

Acknowledgment is made of Grant MH 155 75, National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and of a Senior Foreign Scientist Fellowship awarded (to M.B.E.F., National Research Centre, Dokki, Cairo, Egypt) by the National Science Foundation. The authors are thankful to Dr. H. M. Fales and Dr. G. S. Rao (National Heart and Lung Institute, National Institutes of Health, Bethesda, Md.) for valuable discussions and kind cooperation.

RESEARCH ARTICLES

Synthesis and Biological Activity of Deuteriobenzyl-d_7-penicillin

PAUL A. LASKAR and ROBERT G. MRTEK

Abstract □ To investigate the deuterium isotope effect on the biological activity of penicillin G, this compound was chemically synthesized by first obtaining phenylacetyl-d_7-chloride from phenylacetic-d_7-acid and thionyl chloride, followed by condensation of the acid chloride with 6-aminopenicillanic acid. The penicillin was isolated as the 1-ethylpiperidine salt. Yield of the deuteriopenicillin was approximately 60% when a 2:1 (6-aminopenicillanic acid–acid chloride) molar ratio was used. The identity of the deuteriated penicillin was confirmed using several physical constants. From NMR data, the presence of deuterium in the benzyl moiety was found to be greater than 95 atom %. The biological activity of the deuteriopenicillin was compared to that of the protioanalog by a turbidimetric assay procedure using *Staphylococcus aureus*. The results of the biological assay indicate that a significant deuterium isotope effect operates in the antistaphylococcal action of benzylpenicillin. With the test organism chosen, the ratio of the antibiotic potencies was 125% H/D.

Keyphrases □ Deuteriopenicillin—synthesis □ Biological activity—deuteriopenicillin □ Turbidimetric assay—deuteriopenicillin antimicrobial activity □ NMR spectroscopy—identification □ IR spectrophotometry—identification

Deuterium (^{2}H), a rare stable isotope of hydrogen, was discovered by Urey *et al.* (1) in 1932. Extensive work by several investigators has felled the long-standing opinion that deuterium oxide (D_2O) is incompatible with life. The successful mass culture of algae, yeasts, and certain bacteria under conditions of full deuteriation has provided a useful source of fully deuteriated sugars, amino acids, proteins, and certain drugs (2–5). Substitution of deuterium for ordinary hydrogen and deuteriated substrates for protio metabolites has been shown to produce profound changes in biosystems. Isotope effects have been categorized as being either primary or secondary. Measurement of the magnitude of the effect is usually useful in distinguishing between the larger primary mass isotope effects and the smaller secondary effects.

The substitution of deuterium in several drug molecules has been the impetus for many recent research contributions. Isotopically altered drugs have shown widely divergent pharmacologic effects. Elison *et al.* (6) demonstrated a reduced analgesic potency of morphine deuteriated in the *N*-methyl group. Nona *et al.* (7) found an increased antifungal activity with fully deuteriated griseofulvin. Foreman *et al.* (8) investigated the *in vitro* Dutch rabbit liver metabolism of selectively deuteriated amphetamines. Their studies show that the ratio of apparent rate constants (k_H/k_D) is 1.9, indicating that the deuterioamphetamine is metabolized more slowly than the protioanalog. One major effect of deuterium substitution in drug-producing organisms has been a general suppression of most nonessential metabolism. Mrtek *et al.* (9) demonstrated an inverse effect between the level of deuteriation in *Claviceps* and the amount of clavine alkaloids produced in saprophytic culture. Multimilligram quantities of highly deuteriated elymoclavine were obtained only through the use of replacement culture techniques (10). Mohammed *et al.* (11) demonstrated a similar effect with several strains of *Penicillium*. Carlstedt (12) obtained small amounts of highly deuteriated penicillin from large numbers of shake

cultures of *Penicillium chrysogenum*. Investigation of the deuterium isotope effect on the antibiotic potency of penicillin may provide important additional information concerning benzylpenicillin, the member of the penicillin group to which all others are compared.

Considerable research has substantiated the hypothesis that one mechanism of antibiotic activity is common to all penicillins. Several workers showed that the presence of a free amino group in the side chain increases Gram-negative activity (13), while Doyle *et al.* (14) noticed that electron-attracting groups in the α-position of the side chain increased acid stability. Similarly, hindering rotation about the single bond in the acyl grouping increases the stability of the molecule toward penicillinase (15). Changes in activity, either in spectrum or relative potency, may be attributed to modifications in the acyl group of the basic penicillin molecule. Repeated attempts to alter the penicillin nucleus have resulted in reduced biological activity when compared to the intact penicillanic derivatives. With respect to benzylpenicillin, it would seem reasonable that deuteriation of the benzyl group would alter measurably the antibiotic potency if a significant isotope effect is operating.

The objectives of the present work were to synthesize deuteriobenzyl-d_7-penicillin and to compare its antibiotic potency to that of protiobenzyl penicillin.

EXPERIMENTAL

Deuteriobenzyl-d_7-penicillin—The semisynthetic procedure of Sheehan and Henery-Logan (16) was utilized to prepare the isotopically altered penicillin. Phenylacetyl-d_7-chloride was prepared by reacting 1.36 g. (9.15 mmoles) deuteriophenylacetic-d_7-acid[1] (98 atom % D) with 4.42 g. (37.2 mmoles) thionyl chloride in a 15-ml. flask with an attached calcium chloride drying tube. The reaction was allowed to proceed at room temperature for 24 hr. with occasional agitation. The excess thionyl chloride, as well as other volatile components, was removed by aspiration. No further purification of the acid chloride was attempted.

Deuteriobenzyl-d_7-penicillin was prepared by condensing 0.176 g. (approximately 1.0 mmole) of the partially purified deuteriophenylacetyl-d_7-chloride with 0.430 g. (2.0 mmoles) 6-aminopenicillanic acid (6-APA).[2] The phenylacetyl chloride was added dropwise over a 10-min. period to the stirred chilled solution of 6-APA dissolved in 10 ml. 4% sodium bicarbonate solution and 8 ml. reagent grade acetone. The reaction was conducted at 0–4° with stirring in an open 50-ml. conical flask for 30 min. following completion of addition of the acid chloride. Then the mixture was aspirated to remove excess acetone, and the aqueous mixture was washed once with 5 ml. cold ether A.R. which was discarded. The aqueous portion was layered with 10 ml. cold ether and acidified with 10% cold phosphoric acid to pH 2 as determined by short-range pH paper. The acidified mixture was extracted with two additional 10-ml. portions of cold ether in a small Squibb funnel. The combined ether extracts were washed once with 5 ml. cold water and dried with anhydrous magnesium sulfate. The ether solution was separated from the magnesium sulfate by filtration, and 0.122 g. (1.1 mmoles) 1-ethylpiperidine in 3 ml. dry ether was added to precipitate the deuteriobenzylpenicillin as the 1-ethylpiperidine salt. Twelve milliliters of dry reagent grade acetone was added, and the crystalline precipitate was allowed to stand at approximately −5° overnight. The precipitate was filtered and washed with several portions of cold dry ether. The crystals were dried in a vacuum desiccator at room temperature to constant weight and stored in the desiccator until used. Yield: 296 mg.; 0.65 mmole; 59% of theoretical calculated on phenylacetic-d_7-acid.

Melting points were obtained simultaneously by placing equal size samples of the 1-ethylpiperidine salts of protiobenzylpenicillin and deuteriobenzylpenicillin and a mixture of equal parts of each in separate capillary melting tubes. These tubes were inserted in a Mel-Temp melting point apparatus previously heated to 100°. The temperature was increased (3–4°/min.) to 140°. The temperature was then increased slowly (approximately 1°/min.) until melting occurred. NMR spectra of the compounds were determined by using a Varian A-60 Mc.p.s. spectrometer. Each sample (approximately 0.05 mmole) was dissolved in approximately 1 ml. deuterium oxide, using an external TMS standard. All spectra were determined at probe temperature. Integration of the spectra were performed electronically. IR spectra of samples of protiobenzylpenicillin and deuteriobenzylpenicillin were obtained using a mineral oil mull in a Perkin-Elmer model 257 grating IR spectrophotometer.

Organisms—A lyophilized pellet of *Staphylococcus aureus* ATCC 6538P was added to 10 ml. Difco Penassay Broth and incubated at 35° for 24 hr. Subcultures were prepared by transferring a loopful of the culture to slants of Difco Penassay Seed Agar and incubating for 18 hr. at 35° prior to storage at 4°. Subcultures were prepared at 7–14 day intervals.

A culture of a clinically isolated penicillinase producing *S. aureus* was obtained.[3] This organism was maintained by subculture on Difco Penassay Seed Agar slants at 14-day intervals. Incubation and storage conditions were the same as for *S. aureus* ATCC 6538P.

Solutions—Accurately weighed quantities of authentic samples of the 1-ethylpiperidine salts of protiobenzylpenicillin and synthetic deuteriobenzyl-d_7-penicillin were used to prepare stock solutions. The stock solutions were prepared by dissolving nearly equal quantities (approximately 0.05 mmole) separately in 0.06 *M* phosphate buffer, pH 6, and diluting to approximately 1×10^{-9} mole/ml. concentration. Working solutions of desired concentration were prepared by dilution of these stock solutions. All solutions were stored in the dark at 4° for up to 1 week. Solutions for use with the penicillinase organism were prepared identically, except they were diluted to a concentration of 2×10^{-7} mole/ml.

Inocula—A loopful of *S. aureus* ATCC 6538P was transferred to 25 ml. Penassay Broth and incubated at 35° for 18 hr. The full quantity for overnight culture was transferred to 950 ml. Penassay Broth and incubated for 1 hr. at 37.5° prior to use. A loopful of the clinically isolated penicillinase producing *S. aureus* was transferred to 20 ml. Penassay Broth to which yeast extract (1.5 g./l.) had been added, and it was incubated at 35° for 18 hr. The full quantity of inoculum was added to 400 ml. Penassay Broth with added yeast extract (1.5 g./l.) and incubated at 37.5° for 1 hr. prior to use.

Procedure—Assay tubes were prepared by addition of 0.5 ml. of the desired working solution and 9.0 ml. of the inoculated (preincubated) broth to 16 × 125-mm. tubes. Two to four different concentrations were used for each determination, and four tubes were prepared at each concentration. A total of 8 and 10 different concentrations (ranging from 70 to 1200×10^{-12} mole/tube) of deuteriobenzylpenicillin and protiobenzylpenicillin, respectively, were used to establish the regression equation. Control tubes were prepared identically, except that 0.5 ml. of 0.06 *M* phosphate buffer pH 6 (0.0 *M* benzylpenicillin) was used. Blanks were prepared by incorporating buffer and 0.5 ml. 1:3 formaldehyde USP prior to inoculation with the organism. Both control and blanks were incubated with the test dilutions. The tubes (assay, control, and blank) were arranged randomly in a 48-position rack and incubated in a 37.5 ± 0.1° stirred water bath[4] for 3 hr. At the end of the assay period, growth was stopped by addition of 0.5 ml. 1:3 dilution of formaldehyde USP. The turbidity was determined by measuring percent transmittancy, using matched round cuvettes (19 × 150 mm.) in a Coleman Jr. spectrophotometer at 650 mμ.

Assay tubes for use with the penicillinase producing *S. aureus* were prepared by placing 0.5 ml. of the desired test solution and 9.0 ml. of inoculated preincubated broth in 16 × 125-mm. tubes.

[1] Mallinckrodt-Nuclear, Orlando, Fla., lot L-2248.
[2] Sigma Chemical Co., St. Louis, Mo., lot 44B-1550.
[3] Bacteriological Laboratory, University of Illinois Hospital.
[4] Precision Scientific, Chicago, Ill., model 6580.

Eight concentrations were used in each determination. Control and blank tubes were prepared as described. The tubes were incubated in a 48-tube rack in a stirred 37.5 ± 0.1° water bath for 4 hr. Growth was stopped, and turbidity was measured in the same way as before.

RESULTS AND DISCUSSION

Preliminary experiments with protiophenylacetyl chloride and 6-APA were performed to determine a ratio that would optimize the yield of benzylpenicillin based upon a conservation of the acid chloride. Table I summarizes the results and indicates an optimum ratio of 2:1 (6-APA–phenylacetyl chloride). Yields at the optimum ratio were similar when deuteriophenylacetyl-d_7-chloride was used.

Table II presents a summary of the physical constants for the two isotopic penicillins. The absence of proton signals corresponding to the two α-methylene (δ = 3.59 p.p.m.) and five phenyl protons (δ = 7.31 p.p.m.) in the acyl group indicate the substitution of deuterium in these positions to be greater than 95 atom %. Confirmation of the identity of the synthetic penicillins is assumed on the basis of melting points, elemental analysis, chemical shift of the remaining proton signals in the NMR spectrum, and several IR absorption bands.

Inocula broths were preincubated with overnight cultures of *S. aureus* to obtain organisms in the log phase of growth for the assay, since penicillin exerts its antimicrobial action only on rapidly dividing organisms, *i.e.*, those synthesizing new cell walls. Penicillin has little effect on microbial clones that are metabolizing but not growing or dividing.

Most linear biological assays are commonly based upon a graphical presentation of a transformed measure of some effect that is proportional to the microorganism number, such as turbidity, *versus* log concentration of the test compound (17). Plots of turbidity *versus* dose result in sigmoid curves which may be linearized by suitable transformations. One convenient method is the logarithmic transformation.

In the determination of an isotope effect, the units selected for expression of concentration of penicillin per tube as the independent variable were log (moles × 10^{-12}) rather than a more common expression of weight (microgram or unit) per tube. When dealing with antibiotics of different molecular weights, the toxic effect of drugs on bacterial growth is proportional to the number of moles present but not to the particular weight of the substance used. When concentrations of two isotopically related drugs are expressed as weight per volume, a factor relating the relative change in molecular weight is confounded with the slope of the dose–response relationship. If the molecular weights are not identical as in the case of isotope hybrids, the slope of the regressions for analogs would be anticipated to be different from each other, at least by a factor proportional to the relative molecular weights. Thus, the slopes of the dose–response lines for the two isotopic benzylpenicillins in which log weight is used as an independent variable would not be truly comparable since they differ in their components. A totally linear regression may be expressed as

$$\log \%T = \theta \log \text{moles} + K \qquad \text{(Eq. 1)}$$

when the independent variable is expressed as moles. In the converse situation, it may be expressed as

$$\log \%T = \theta' \log \text{amt.} + K' \qquad \text{(Eq. 2)}$$

when the independent variable is expressed as micrograms or units, θ,θ' = slopes of regressions, and K,K' = intercepts. Since the turbidity term is proportional to the moles of substance present, the units of slope relating the concentration term to a measure of the turbidity term may be shown as

$$\theta = \frac{\log \%T}{\log \text{moles}} \qquad \text{(Eq. 3)}$$

The slope, θ, contains no molecular weight factor. Conversely, if the data are presented as weight of substance, then the components of the slope may be seen as

$$\theta' = \frac{\log \%T}{(\log \text{moles})(\log \text{MW})} \qquad \text{(Eq. 4)}$$

Table I—Effect of Ratio of Reactants on Yield of Benzylpenicillin

Molar Ratio of 6-APA–Phenylacetyl Chloride	Yield of Benzylpenicillin,[a] %
1.0:1.0	60
1.5:1.0	63
2.0:1.0	69
5.0:1.0	65

[a] Calculated on phenylacetyl chloride.

where MW is the mole weight of the substance. Thus, comparing slopes for the isotopic penicillins, from Eq. 3,

$$\frac{\theta_H}{\theta_D} = \frac{\log \%T/\log \text{moles}_H}{\log \%T/\log \text{moles}_D} = \frac{\log \text{moles}_D}{\log \text{moles}_H} \qquad \text{(Eq. 5)}$$

and since the units are the same,

$$\frac{\theta_H}{\theta_D} = 1 \qquad \text{(Eq. 6)}$$

Conversely, from Eq. 4,

$$\frac{\theta'_H}{\theta'_D} = \frac{\log \%T/(\log \text{moles}_H)(\log \text{MW}_H)}{\log \%T/(\log \text{moles}_D)(\log \text{MW}_D)} = \frac{(\log \text{moles}_D)(\log \text{MW}_D)}{(\log \text{moles}_H)(\log \text{MW}_H)} = \frac{\theta_H \log \text{MW}_D}{\theta_D \log \text{MW}_H} \qquad \text{(Eq. 7)}$$

and

$$\frac{\theta'_H}{\theta'_D} = \frac{\log \text{MW}_D}{\log \text{MW}_H} > 1 \qquad \text{(Eq. 8)}$$

From these equations, using a drug concentration term expressed in moles rather than amount as the independent variable, confounding of the isotope mass differences is avoided. In testing for parallelism between the regression lines for the two antibiotics, if the null hypothesis is accepted, then only random sampling error remains to account for the observed differences between the two slopes, θ_H and θ_D.

The distribution of log %T has been established to be normal (18). The model developed for this work is univariate, where Y equals log %T as the random variable. The variance of Y throughout the range of X equals log moles (concentrations of penicillins) was established as homogeneous. Table III summarizes the calculated parameters used in the statistical analysis.

The observed difference between the regression coefficients for the two isotopic benzylpenicillins was tested for level of significance by a t test. The null hypothesis was that there is no difference between the slopes. (See the *Appendix* for the equations used.) No evidence for a difference other than zero was found, and

Table II—Summary of Physical Constants for Isotopic Benzylpenicillin Salts

	Protio	Deuterio
Molecular formula	$C_{23}H_{33}N_3O_3S_1$	$C_{23}H_{26}D_7N_3O_3S_1$
Elemental analysis, %	—	Calcd. C, 60.76; H, 7.44; N, 9.24 Found C, 60.66; H, 7.37; N, 9.04
Melting point	150–152° (dec.)	150–152° (dec.)
IR spectra	1370,[a] 1390,[a] 1570,[b] 3190,[c] 3240,[c] cm.$^{-1}$	1365,[a] 1390,[a] 1575[b] 3180,[c] 3230,[c] cm.$^{-1}$
NMR spectra[d]	1.51,[e] 1.42[e]	1.51,[e] 1.42[e]
	3.59[f]	—[g]
	7.31[h]	—[g]

[a] C—H bend in 2-dimethyl. [b] Carbonyl bend in β-lactam. [c] N—H stretch in amine salt. [d] Chemical shift, p.p.m. (δ) (TMS = 0). [e] 2-Dimethyl. [f] Acyl methylene. [g] Indicates lack of signal corresponding to deuterium substitution. [h] Phenyl.

Table III—Summarized Calculations for Regression Analysis of Isotopic Benzylpenicillin Analogs

	Deuterio	Protio
N	44	60
Σx^2	4.46598	6.05798
Σy^2	0.14444	0.16299
Σxy	0.65940	0.91238
b_{YX} (regression coefficient)	0.14765	0.15061
F ratio (nonzero slope)	82.5($p < 0.001$)	259.8($p < 0.001$)
$\bar{X}$ [log (moles $\times 10^{-12}$)]	2.65763	2.64677
$\bar{Y}$ [log (%T)]	1.85164	1.86463
$\hat{Y}_{bYX}$ (regression line)	$1.45471 + 0.14935X$	$1.46933 + 0.14935X$
$SS_{\text{deviations from regression}}$	0.04705	0.02649

parallelism of the regression equations is established on the basis of probability. Furthermore, these data corroborate the suggestion that both deuteriobenzylpenicillin and protiobenzylpenicillin inhibit bacterial growth by the same mechanism. The regression coefficient is a quantitative expression of the association between variables. Since the regression coefficients for protiobenzylpenicillin and deuteriobenzylpenicillin are not significantly different, it is reasonable to conclude that the presence of deuterium in the acyl group of benzylpenicillin does not alter the mechanism of action of the drug with respect to the test organism.

To obtain a valid estimate of a real difference in the antimicrobial potency of the two compounds, the assumption that the two lines are not identical must be tested. Establishment of parallelism between regression lines does not preclude the possibility of identical intercepts. The null hypothesis for the determination of the identity of the two lines tests the significance of the difference between the intercepts of two parallel lines. If the null hypothesis is accepted on the basis of a t test, a statement can be offered in terms of probability that the two regression lines are estimates of the same locus. (See the *Appendix* for summarized calculations.) Application of the test to the data for the two benzylpenicillins indicates that the two intercepts are significantly different ($p < 0.001$). This information, along with the identity of the slopes, indicates that the regression lines for the two benzylpenicillins, although parallel, describe different loci. Satisfaction of the requirement for parallelism and establishment of significantly different intercepts permit the direct comparison of the two lines in terms of horizontal displacement.

The horizontal displacement as a measure of the relative potency of the two drugs may be calculated and confidence limits generated (*Appendix*). Table IV summarizes the results of these calculations. The introduction of deuterium into the acyl group of penicillin has no effect on the mechanism of antimicrobial action. However, a deuterium isotope effect is present and is reflected in a 25% reduction in potency of the deuterium compound compared with its protioanalog.

Comparative assays were performed on several days and grouped together to increase reliability of the relative potency estimate. The absolute antistaphylococcal activity of the antibiotics varied widely from day to day, as indicated by the overlap of data points throughout the concentration range in Fig. 1. Therefore, day-to-day predictions of absolute activity for each antibiotic could not be made. However, day-to-day effects should not be expected to operate on the relative position of the two regression lines with respect to each other (relative potency estimate). On each day the protioanalog regression line was always displaced above the regression line for the deuterioanalog. The variation in displacement as a function of days was assessed for the presence of a significant trend. The log relative potency was graphed as a function of the day on which the experiment was performed (Fig. 2). No significant regression of the displacement on days was found.

Table IV—Relative Potency and Confidence Limits for Isotopic Benzylpenicillin Salt

	Percent
Relative potency (H/D)	125
95% Confidence interval	114–138
99% Confidence interval	106–148

The regression equation calculated for the data in Fig. 2 does not possess a slope that varies significantly from zero ($p \cong 0.70$). As expected, there is no correlation between the log relative potency and the day on which the experiment was completed. If a difference in the rate of decomposition of the two isotopic antibiotics did exist, a significant regression of log relative potency on days could be determined. This information would contribute toward the quantization of the difference in decomposition rate of the two compounds.

Results of preliminary studies obtained with a clinically isolated penicillinase producing *S. aureus* indicate reduced but nearly equal biological activity for the two isotopic penicillins. Direct comparison of the results obtained for *S. aureus* ATCC 6538P and the penicillinase-producing organism may not be appropriate because of the more fastidious nature of the clinically isolated test organism. Further investigation is underway to permit a complete analysis of the deuterium isotope effect on a penicillinase-producing organism (21).

The introduction of deuterium into the benzyl moiety of penicillin G has reduced the antimicrobial activity against a strain of *S. aureus* with respect to the protioanalog. This decrease may result from an altered rate of penetration by penicillin to the site of action in the cell wall or rate of association of penicillin with a crosslinking enzyme. Deuterium introduced into other pharmacologically active compounds has resulted in a decreased rate of binding to the enzyme receptor, with a concomitant increase in the strength of the bond. Since the binding of protiobenzylpenicillin to albumins is mediated by the benzyl moiety and not the β-lactam-thiazolidine nucleus (19), changes in the rate and strength of binding may account for the decreased biological activity of the deuterioanalog. Further work is in progress which will help elucidate the effect of deuterium in the acyl group on the kinetics and extent of binding of benzylpenicillin to macromolecules.

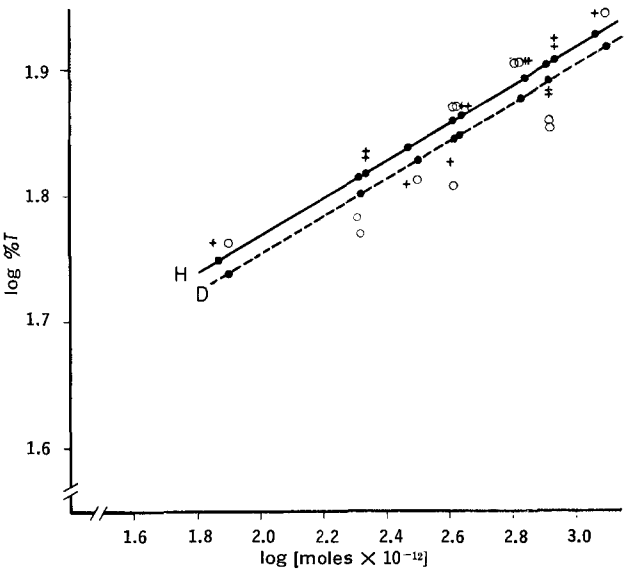


Figure 1—*Regression lines calculated for isotopic penicillins. Key:* $\hat{Y}_H = 1.46933 + 0.149352X$, *and* $\hat{Y}_D = 1.454718 + 0.149352X$.

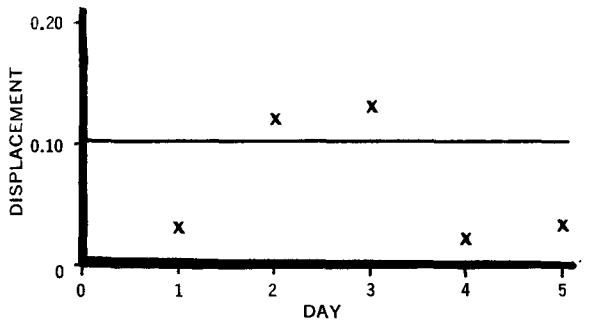


Figure 2—*Effect of days on displacement of regression lines (log relative potency). Key: —, displacement calculated for combined data; and ×, displacement for day. (See text for explanation.)*

SUMMARY

1. Deuteriobenzyl-d_7-penicillin has been chemically synthesized. Phenylacetyl-d_7-chloride was first prepared from phenylacetic-d_7-acid. Subsequently, the acid chloride was condensed with 6-APA, and the penicillin G was isolated as the 1-ethylpiperidine salt.

2. The presence of deuterium in the benzyl moiety was confirmed using several physical constants.

3. The antimicrobial activity of the deuteriopenicillin was compared to its protioanalog by a turbidimetric assay procedure using *S. aureus* as the test organism.

4. Identity of the regression coefficients for the isotopic penicillins was established by statistical analysis. This identity indicates that the regression lines are parallel. Parallelism indicates a similar mechanism of antistaphylococcal action for the two isotopic penicillins.

5. Nonidentity of the regression lines was determined using statistical analysis, permitting calculation of a valid estimate of relative potency.

6. The relative potency (H/D), as a quantitative estimate of the deuterium isotope effect, was calculated to be 125%. Neither the 95% nor the 99% confidence interval generated for the estimate includes a relative potency estimate of 100%.

APPENDIX (20)

Slope Test—

$$t = \frac{b_H - b_D}{s_{Db}} \qquad \text{(Eq. 1A)}$$

where

$$s_{Db} = \left[\bar{\sigma}^2_{YX}\left(\frac{1}{SS_{\text{dev}.H}} + \frac{1}{SS_{\text{dev}.D}}\right)\right]^{1/2} \qquad \text{(Eq. 2A)}$$

and

$$\bar{\sigma}^2_{YX} = \frac{SS_{\text{dev}.H} + SS_{\text{dev}.D}}{n_H + n_D - 4} \qquad \text{(Eq. 3A)}$$

$$t = \frac{2.96}{2.08} \times 10^{-2} = 0.014\ (100\,df)\ \text{(n.s.)} \qquad \text{(Eq. 4A)}$$

Identity of Lines—

$$t = \frac{\hat{b} - \bar{b}}{s_{(\hat{b}-\bar{b})}} \qquad \text{(Eq. 5A)}$$

where

$$s_{(\hat{b}-\bar{b})} = \left\{\bar{s}^2_{YX}\left[\frac{1}{(\bar{X}_H - \bar{X}_D)^2}\left(\frac{1}{n_H} + \frac{1}{n_D}\right) + \frac{1}{\sum x^2_H + \sum x^2_D}\right]\right\}^{1/2} \qquad \text{(Eq. 6A)}$$

and

$$\hat{b} = \frac{\bar{Y}_H - \bar{Y}_D}{\bar{X}_H - \bar{X}_D} \qquad \text{(Eq. 7A)}$$

and

$$\bar{s}^2_{YX} = \frac{SS_{\text{dev}.H} + SS_{\text{dev}.D} + \left[\dfrac{(b_H - b_D)^2}{1/(SS_{\text{dev}.H}) + 1/(SS_{\text{dev}.D})}\right]}{n_H + n_D - 3} \qquad \text{(Eq. 8A)}$$

$$t = 8.86(101\ df)(p < 0.001) \qquad \text{(Eq. 9A)}$$

Horizontal Displacement—

$$p_x = \left|\bar{X}_D - \bar{X}_H - \frac{(\bar{Y}_H - \bar{Y}_D)}{\bar{b}_{YX}}\right| \qquad \text{(Eq. 10A)}$$

Confidence Limits of P_x—

$$CL = \left|\bar{X}_D - \bar{X}_H - \frac{(\bar{Y}_D - \bar{Y}_H)}{\bar{b}_{YX}(1 - K^2)}\right| \pm \frac{K}{\bar{b}_{YX}(1 - K^2)}\left[\left(\frac{1}{n_H} + \frac{1}{n_D}\right)\bar{b}_{YX}(1 - K^2)\left(\sum x^2_H + \sum x^2_D\right) + (\bar{Y}_D - \bar{Y}_H)^2\right]^{1/2} \qquad \text{(Eq. 11A)}$$

where

$$K = \frac{t^2\, s_{\bar{b}YX}{}^2}{\bar{b}_{YX}{}^2} \qquad \text{(Eq. 12A)}$$

and

$$s_{bYX}{}^2 = \frac{\bar{s}^2_{YX}}{\sum x^2_H + \sum x^2_D} \qquad \text{(Eq. 13A)}$$

REFERENCES

(1) H. C. Urey, F. G. Brickwedde, and G. M. Murphy, *Phys. Rev.*, **39**, 164(1932).

(2) H. L. Crespi, S. M. Archer, and J. J. Katz, *Nature*, **189**, 729 (1960).

(3) J. J. Katz, *Amer. Sci.*, **48**, 544(1960).

(4) H. L. Crespi, S. M. Conrad, R. A. Uphaus, and J. J. Katz, *Ann. N. Y. Acad. Sci.*, **84**, 648(1960).

(5) M. I. Blake, H. L. Crespi, V. Mohan, and J. J. Katz, *J. Pharm. Sci.*, **50**, 425(1961).

(6) E. Elison, H. Rapoport, R. Laursone, and H. W. Elliot, *Science*, **134**, 1079(1961).

(7) D. A. Nona, M. I. Blake, H. L. Crespi, and J. J. Katz, *J. Pharm. Sci.*, **57**, 1993(1968).

(8) R. L. Foreman, F. P. Siegel, and R. G. Mrtek, *ibid.*, **58**, 189 (1969).

(9) R. G. Mrtek, H. L. Crespi, M. I. Blake, and J. J. Katz, *ibid.*, **56**, 1234(1967).

(10) R. G. Mrtek, H. L. Crespi, G. D. Norman, M. I. Blake, and J. J. Katz, *Phytochemistry*, **7**, 1535(1968).

(11) K. Mohammed, D. A. Nona, and M. I. Blake, *J. Pharm. Sci.*, **59**, 419(1970).

(12 B. Carlstedt, personal communication.

(13) F. P. Doyle and J. H. C. Nayler, *Advan. Drug Res.*, **1**, 1 (1964).

(14) F. P. Doyle, J. H. C. Nayler, H. Smith, and E. R. Stone, *Nature*, **191**, 1091(1961).

(15) F. P. Doyle and J. H. C. Nayler, *Advan. Drug Res.*, **1**, 1 (1964).

(16) J. C. Sheehan and K. R. Henery-Logan, *J. Amer. Chem. Soc.*, **84**, 2983(1962).

(17) F. Kavanaugh, in "Analytical Microbiology," F. Kavanaugh, Ed., Academic, New York, N. Y., 1963, p. 180.

(18) G. W. Snedecor and W. G. Cochran, "Statistical Methods," 6th ed., Iowa State University Press, Ames, Iowa, 1967, p. 449.

(19) J. J. Fischer and O. Jardetzky, *J. Amer. Chem. Soc.*, **87**, 3237(1965).

(20) "Documenta Geigy-Scientific Tables," 6th ed., K. Diem, Ed., Geigy Pharmaceuticals, Ardsley, N. Y., 1962, p. 177.
(21) P. A. Laskar, M.S. thesis, University of Illinois Medical Center, Chicago, Ill., 1971.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680*

Accepted for publication July 17, 1970.

Abstracted in part from a dissertation presented by Paul A. Laskar to the Graduate College, University of Illinois at the Medical Center, Chicago, Ill., in partial fulfillment of Master of Science degree requirements.

This investigation was supported in part by Research Grant I/C 2-41-35-50-3-04-4 from the Graduate College, University of Illinois.

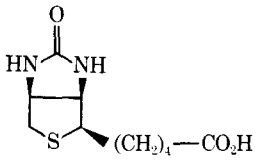
Synthesis of Azabiotin Analogs as Potential Cofactors for Biotin-Dependent Enzymes

HENRY C. WORMSER

Abstract □ As part of a program to synthesize azabiotin analogs and homologs as potential substitutes for the natural coenzyme, several 4- and 5-substituted derivatives of *cis*-hexahydropyrrolo-[3,4-*d*]imidazole-2-one have been prepared. Spectral data and certain side reactions in the synthetic scheme used point to the stereochemistry of the 4-substituted compounds.

Keyphrases □ Azabiotin analogs—synthesis □ Biotin-dependent enzymes—potential cofactor, azabiotin analogs □ NMR spectroscopy—structure □ UV spectrophotometry—structure □ IR spectrophotometry—structure □ Mass spectroscopy—structure

Biotin (I) is a cofactor required for several enzyme-catalyzed carboxylation reactions and, as such, plays a significant role in carbon dioxide fixation reactions. Although a wide variety of compounds closely related to the vitamin have been prepared, very few have been found to possess significant biochemical and growth-promoting activity in microorganisms and animals.



I

Recently (1–3), controversy has arisen about the significance of the sulfur atom of biotin with regard to interactions between the enzyme protein and the cofactor. NMR data have indicated that both the ureido oxygen and the sulfur atom should be considered as

Table I—NMR Spectra at 60 Mc. in $CDCl_3$ Using TMS Reference (Pyrrolidino Compounds)[a]

Compound	Data	*N*-Acetyl	Ester CH_2	Ester CH_3	N—H	5—CH_3	Miscellaneous
III	δ p.p.m.		4.17	1.28	6.60		C-2 and C-5
		—				—	4.25
	J, c.p.s.		q, 7.0	t, 7.0	s		s
XV	δ p.p.m.	—	4.26	1.41	6.02	1.22	C-2 4.61 (1H)
	J, c.p.s.		q, 7.0	t, 7.0	s	d, 6.0	C-5 4.25 (2H)
IV	δ p.p.m.	2.20	4.25	1.30	4.90		
				t, 7.1		—	
	J, c.p.s.	s	q, 7.0	1.33	d, 7.4		
				t, 7.1			
XVI	δ p.p.m.	2.19	4.22	1.30	5.54	2.18	C-5 4.25 (2H)
	J, c.p.s.	s	q, 7.0	m	s		
XVII	δ p.p.m.	2.37	4.43	1.36	7.95	2.20	C-2 7.81 (1H)
				t, 7.1			
	J, c.p.s.	s	q, 7.0	1.46	s	s	
				t, 7.1			
V	δ p.p.m.	1.94	4.20	1.25	7.03		
						—	
	J, c.p.s.	s	q, 7.0	t, 7.0	d, 7.0		
XVIII	δ p.p.m.	2.01	4.17	1.25	7.1	1.20	
	J, c.p.s.	s	q, 7.1	t, 7.2	d, 9.5	d, 6.0	
VI	δ p.p.m.	1.99	4.16	1.26	7.36		
	J, c.p.s.	s	q, 7.1	t, 7.0	d, 7.0	—	
XXI	δ p.p.m.	2.01	4.17	1.25	7.29	1.10	
	J, c.p.s.	s	q, 7.1	t, 7.1	d, 7.0	d, 6.0	

[a] s = singlet, d = doublet, m = multiplet, t = triplet, and q = quartet.

(20) "Documenta Geigy-Scientific Tables," 6th ed., K. Diem, Ed., Geigy Pharmaceuticals, Ardsley, N. Y., 1962, p. 177.
(21) P. A. Laskar, M.S. thesis, University of Illinois Medical Center, Chicago, Ill., 1971.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680*

Accepted for publication July 17, 1970.

Abstracted in part from a dissertation presented by Paul A. Laskar to the Graduate College, University of Illinois at the Medical Center, Chicago, Ill., in partial fulfillment of Master of Science degree requirements.

This investigation was supported in part by Research Grant I/C 2-41-35-50-3-04-4 from the Graduate College, University of Illinois.

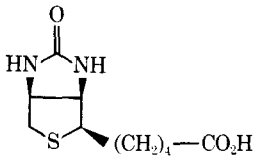
Synthesis of Azabiotin Analogs as Potential Cofactors for Biotin-Dependent Enzymes

HENRY C. WORMSER

Abstract □ As part of a program to synthesize azabiotin analogs and homologs as potential substitutes for the natural coenzyme, several 4- and 5-substituted derivatives of *cis*-hexahydropyrrolo-[3,4-*d*]imidazole-2-one have been prepared. Spectral data and certain side reactions in the synthetic scheme used point to the stereochemistry of the 4-substituted compounds.

Keyphrases □ Azabiotin analogs—synthesis □ Biotin-dependent enzymes—potential cofactor, azabiotin analogs □ NMR spectroscopy—structure □ UV spectrophotometry—structure □ IR spectrophotometry—structure □ Mass spectroscopy—structure

Biotin (I) is a cofactor required for several enzyme-catalyzed carboxylation reactions and, as such, plays a significant role in carbon dioxide fixation reactions. Although a wide variety of compounds closely related to the vitamin have been prepared, very few have been found to possess significant biochemical and growth-promoting activity in microorganisms and animals.



I

Recently (1–3), controversy has arisen about the significance of the sulfur atom of biotin with regard to interactions between the enzyme protein and the cofactor. NMR data have indicated that both the ureido oxygen and the sulfur atom should be considered as

Table I—NMR Spectra at 60 Mc. in $CDCl_3$ Using TMS Reference (Pyrrolidino Compounds)[a]

Compound	Data	*N*-Acetyl	Ester CH_2	Ester CH_3	N—H	5—CH_3	Miscellaneous
III	δ p.p.m.		4.17	1.28	6.60		C-2 and C-5
		—				—	4.25
	J, c.p.s.		q, 7.0	t, 7.0	s		s
XV	δ p.p.m.	—	4.26	1.41	6.02	1.22	C-2 4.61 (1H)
	J, c.p.s.		q, 7.0	t, 7.0	s	d, 6.0	C-5 4.25 (2H)
IV	δ p.p.m.	2.20	4.25	1.30	4.90		
				t, 7.1		—	
	J, c.p.s.	s	q, 7.0	1.33	d, 7.4		
				t, 7.1			
XVI	δ p.p.m.	2.19	4.22	1.30	5.54	2.18	C-5 4.25 (2H)
	J, c.p.s.	s	q, 7.0	m	s		
XVII	δ p.p.m.	2.37	4.43	1.36	7.95	2.20	C-2 7.81 (1H)
				t, 7.1			
	J, c.p.s.	s	q, 7.0	1.46	s	s	
				t, 7.1			
V	δ p.p.m.	1.94	4.20	1.25	7.03		
						—	
	J, c.p.s.	s	q, 7.0	t, 7.0	d, 7.0		
XVIII	δ p.p.m.	2.01	4.17	1.25	7.1	1.20	
	J, c.p.s.	s	q, 7.1	t, 7.2	d, 9.5	d, 6.0	
VI	δ p.p.m.	1.99	4.16	1.26	7.36		
	J, c.p.s.	s	q, 7.1	t, 7.0	d, 7.0	—	
XXI	δ p.p.m.	2.01	4.17	1.25	7.29	1.10	
	J, c.p.s.	s	q, 7.1	t, 7.1	d, 7.0	d, 6.0	

[a] s = singlet, d = doublet, m = multiplet, t = triplet, and q = quartet.

Table II—NMR Spectra at 60 Mc. in $CDCl_3$ Using TMS Reference (Pyrrolo[3,4-*d*]imidazolone Compounds)[a]

Compound	Data	3-Acetyl	Carbamate CH_2	Carbamate CH_3	N—H	4—CH_3
IX	δ p.p.m.	2.51	4.20	1.29	7.1	—
	J, c.p.s.	s	q, 7.0	t, 7.0	s	
XXIV	δ p.p.m.	2.51	4.16	1.28	6.92	1.05
	J, c.p.s.	s	q, 7.0	t, 7.0	s	d, 6.0
XXV	δ p.p.m.	—	4.16	1.28	6.77, s	1.28
	J, c.p.s.		q, 7.0	t, 7.0	6.54, s	d, 6.0

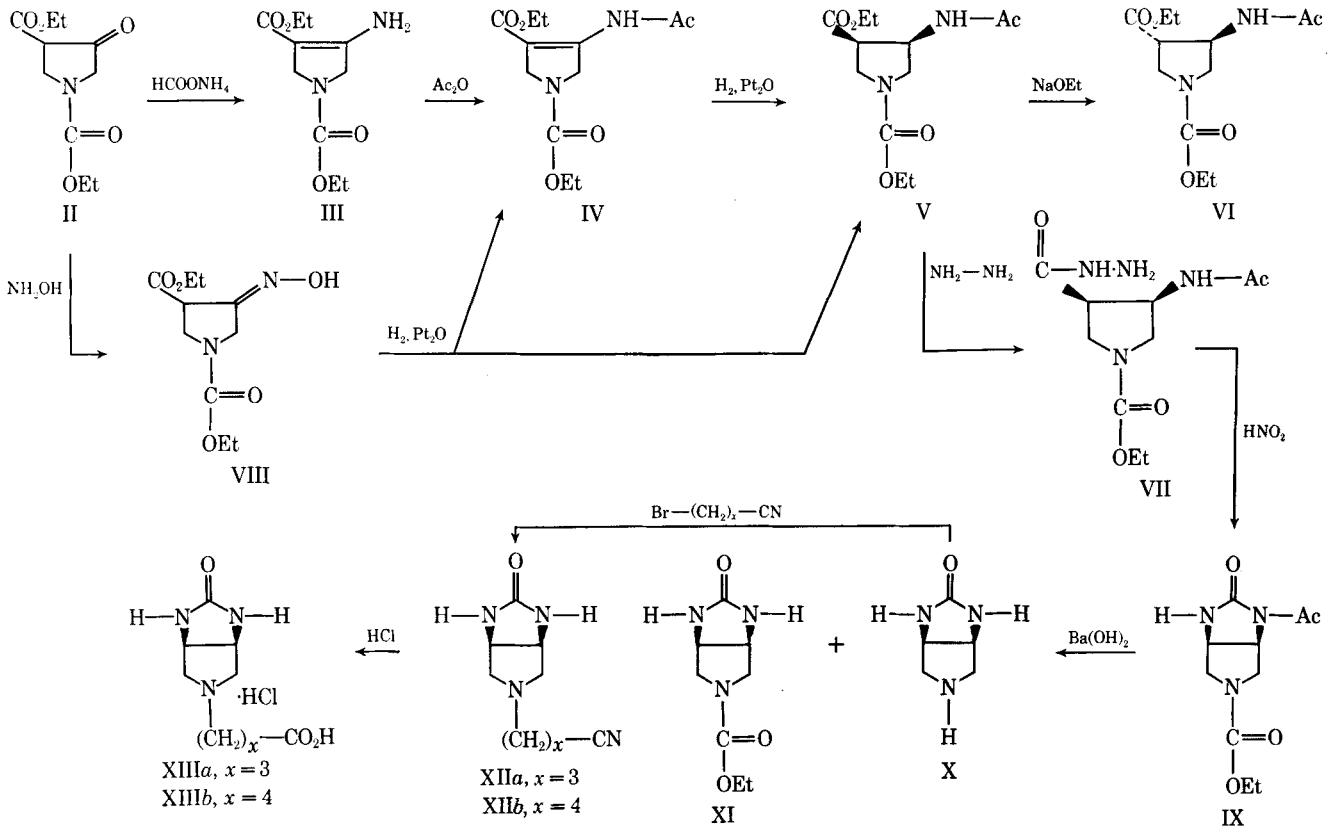
[a] s = singlet, d = doublet, m = multiplet, t = triplet, and q = quartet.

potential hydrogen-bond formers with polar groups on a protein to which biotin binds (3, 4) (Tables I and II).

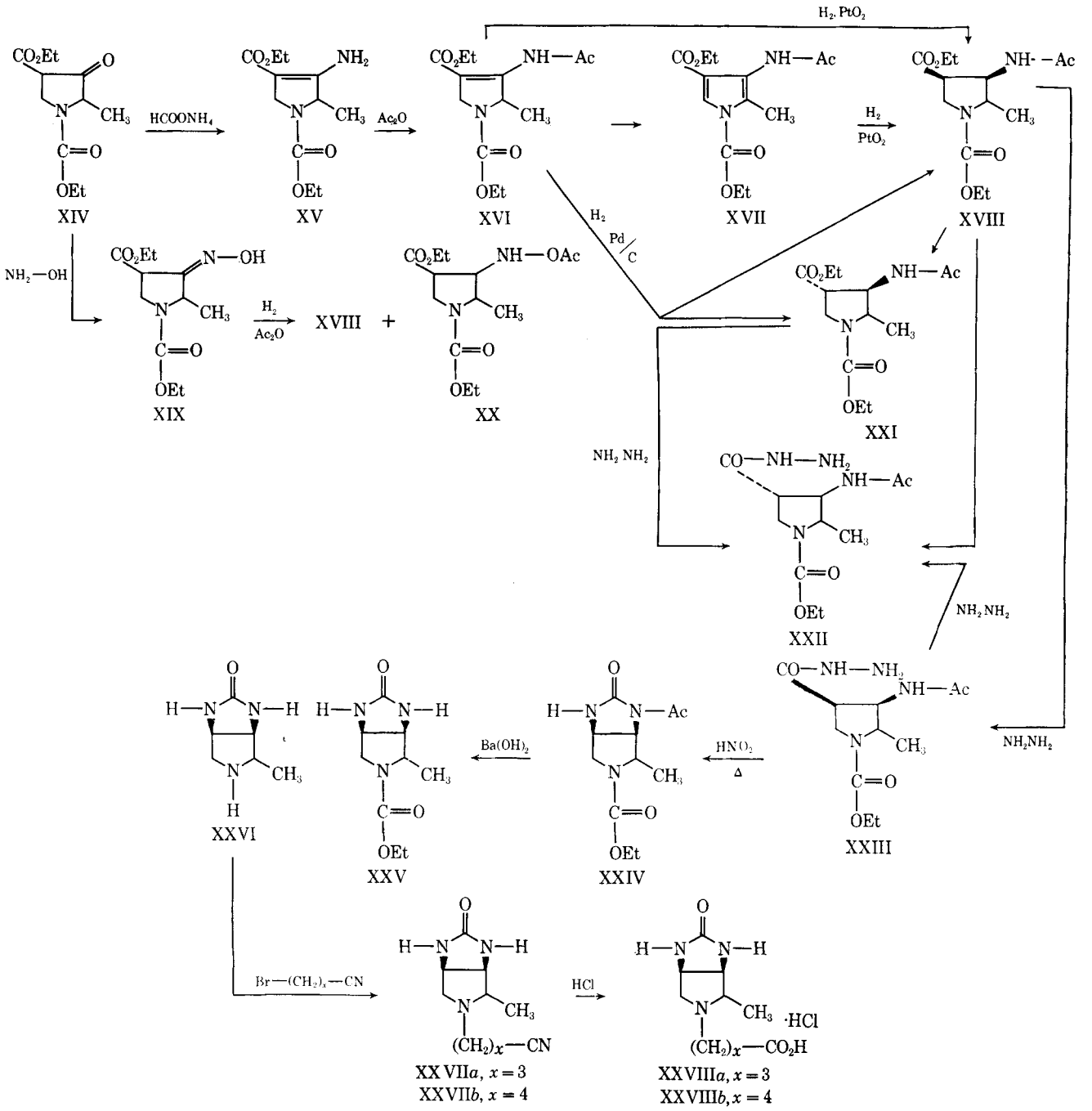
In a preceding paper (5), an approach to the pyrrolo[3,4-*d*]imidazole nucleus was presented. It was hoped that the method described would be applicable to the preparation of 4-substituted derivatives, ultimately leading to the synthesis of azabiotin. The method, in fact, offers an unequivocal synthetic route to the desired heterobicyclic system. A somewhat modified route had to be adopted to allow the introduction of a side chain on the carbon atom adjacent to the nitrogen of the pyrrolidine ring.

1,3-Dicarbethoxy-4-amino-3-pyrrolines (III and XV in Schemes I and II) had not been employed previously as intermediates for the synthesis of the pyrrolo[3,4-*d*]-imidazole system. Such compounds are readily formed by heating the extensively enolized 1,3-dicarbethoxy-4-pyrrolidones (II and XIV) with ammonium formate in ethanol solution (6, 7). The pyrrolidones were obtained by cyclizations of the Dieckmann type carried out as described previously (8). Initially, several attempts were made to obtain 1,3-dicarbethoxy-4-ureido-5-methyl-3-pyrroline *via* the condensation of urea with the corresponding pyrrolidone, but the starting materials were invariably recovered unchanged. This failure suggests that the occurrence of this type of reaction is hampered, on the one hand, by a high degree of crowding of the carbonyl group and, on the other, by the poor nucleophilic character of the attacking species. The aminopyrrolines were acetylated in good yields in the presence of acetic anhydride. *N*-Acetyl (IV) proved quite stable. However, XVI, on standing at room temperature, oxidized to the corresponding pyrrole (XVII).

Catalytic reduction with Adams' catalyst of IV, XVI, and XVII afforded stereospecifically in each case the corresponding *cis*-pyrrolidines. However, reduction of the pyrroline XVI with 10% palladium on charcoal gave a 2:1 mixture of *cis*-product XVIII and *trans*-product XXI. The *trans*-products, XXI and VI, were



Scheme I



Scheme II

independently obtained by base-catalyzed epimerization reactions. The conversion to XXI was rather facile, whereas the formation of VI required more drastic conditions. The acetamido pyrrolidines, V and XVIII, were also sought from oximes VIII and XIX, respectively, by catalytic hydrogenation in acetic anhydride. This latter method offered no advantage over the previously outlined procedure and, in both cases, led to the production of mixtures. Hydrazinolysis of ester XVIII under reflux condition afforded exclusively *trans*-hydrazide XXII, whereas milder conditions afforded only *cis*-product XXIII. This latter can be converted to hydrazide XXII by prolonged treatment with hydrazine hydrate under reflux condition or by the addition of sodium ethoxide in ethanol at room temperature. Hydrazide XXII can also be obtained in nearly quantitative yield from the *trans*-ester XXI and hydrazine hydrate in boiling ethanol. These rather facile epimerizations at carbon 3 are significant, because they point to a rather unstable situation arising from steric compression brought about by the *cis* relationships at C-3, C-4, and C-5 of the pyrrolidine ring system and, consequently, the need for relief from this strain. These findings are coupled with similar observations found in the literature (9, 10).

Conversion of hydrazides VII and XXIII to their respective azides and Curtius rearrangement of these intermediates afforded the pyrrolo[3,4-*d*]imidazole derivatives IX and XXIV, respectively. Barium hydrolysis of these two compounds afforded two bicyclic moieties,

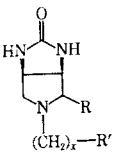


Table III—Azabiotin Analogs

Compound	R	x	R′	M.p.	Yield, %	Formula	Anal., % Calcd.	Found
XII*a*	H	3	CN[a]	127–130°	93	$C_9H_{14}N_4O \cdot HCl \cdot H_2O$	C, 43.46 H, 6.89 N, 22.52	C, 43.69 H, 6.20 N, 22.42
XIII*a*	H	3	CO_2H[a]	247–255° dec.	71	$C_9H_{15}N_3O_3 \cdot HCl$	C, 43.29 H, 6.46 N, 16.83	C, 43.06 H, 6.46 N, 16.80
XII*b*	H	4	CN	129–130°	77	$C_{10}H_{16}N_4O$	C, 57.65 H, 7.74 N, 26.90	C, 57.64 H, 7.67 N, 26.77
XIII*b*	H	4	CO_2H[a]	241–243° dec.	79	$C_{10}H_{17}N_3O_3 \cdot HCl$	C, 45.54 H, 6.88 N, 15.94	C, 45.55 H, 6.84 N, 15.89
XXVII*a*	CH_3	3	CN	174–175°	75	$C_{10}H_{16}N_4O$	C, 57.65 H, 7.74 N, 26.90	C, 57.59 H, 7.73 N, 26.95
XXVIII*a*	CH_3	3	CO_2H[a]	253–257° dec.	64	$C_{10}H_{17}N_3O_3 \cdot HCl$	C, 45.54 H, 6.88 N, 15.94	C, 45.59 H, 6.87 N, 15.90
XXVII*b*	CH_3	4	CN	201–202°	75	$C_{11}H_{18}N_4O$	C, 59.70 H, 8.20 N, 25.22	C, 59.68 H, 8.14 N, 25.15
XXVIII*b*	CH_3	4	CO_2H[a]	223–225° dec.	68	$C_{11}H_{19}N_3O_3 \cdot HCl \cdot H_2O$	C, 44.83 H, 7.70 N, 14.26	C, 44.83 H, 7.44 N, 14.17

[a] Hydrochloride salt.

which were functionalized at position 5 to yield the azabiotin analogs listed in Table III.

EXPERIMENTAL[1]

1,3-Dicarbethoxy-4-acetamido-3-pyrroline (IV)—A solution of 80.9 g. (0.35 mole) of 1,3-dicarbethoxy-4-amino-3-pyrroline (III) (11) in 250 ml. of acetic anhydride was refluxed for 5 hr. The excess acetic anhydride was removed under reduced pressure, and the crystalline product was collected to yield 89.4 g. (94%), m.p. 123–125°. Several recrystallizations from 95% ethanol gave colorless crystals of IV, m.p. 127–129°. UV spectrum showed $\lambda_{max.}^{EtOH}$ 274 mμ (ε 21,850). IR spectrum (mineral oil) showed bands at 3.01, 5.90, and 6.10 μ.

Anal.—Calcd. for $C_{12}H_{18}N_2O_5$: C, 53.31; H, 6.71; N, 10.37. Found: C, 53.31; H, 6.64; N, 10.33.

***cis*-1,3-Dicarbethoxy-4-acetamidopyrrolidine (V)**—A solution of 2.0 g. (7.4 mmoles) of IV in 100 ml. of 95% ethanol was hydrogenated in a Parr shaker over 0.2 g. (84.7%) of platinum oxide for 24 hr. Evaporation of the filtered solution under reduced pressure gave a thick, colorless, oily residue, which showed a single spot on TLC (20% methanol in ether). Treatment of the oily product with ether afforded a colorless crystalline material, 1.34 g. (66.5%), m.p. 123–125°. Two recrystallizations from ether gave the analytical sample, m.p. 127–129°. IR spectrum showed bands at 2.90, 3.00, 5.80, and 5.97 μ.

Anal.—Calcd. for $C_{12}H_{20}N_2O_5$: C, 52.91; H, 7.40; N, 10.29. Found: C, 52.93; H, 7.36; N, 10.20.

[1] Melting points were determined on a Fisher-Johns melting-point stage and a Thomas-Hoover melting-point apparatus which had been calibrated with standard samples. UV absorption spectra were determined in 95% ethanol on a Beckman (model DK2A) recording spectrophotometer. IR absorption spectra were recorded in chloroform (unless otherwise specified) on a Beckman (model 8) recording spectrophotometer. NMR spectra were determined in deuterochloroform using TMS as reference standard on a Varian A-60A spectrometer. Microanalyses were carried out by Spang Microanalytical Laboratory, Ann Arbor, Mich. Mass spectra were determined on an Atlas CH-4 mass spectrometer with TO-4 ion source. TLC was carried out with silica gel G and silica gel $HF_{254+366}$ (Brinkmann Instruments).

Catalytic Reduction of 1,3-Dicarbethoxy-4-pyrrolidone Oxime (VIII)—A solution of 0.67 g. (2.7 mmoles) of 1,3-dicarbethoxy-4-pyrrolidone oxime (VIII) (12) in 50 ml. of acetic anhydride was hydrogenated at 3 atm. over 0.2 g. (84.7%) of platinum oxide for 24 hr. Evaporation of the excess acetic anhydride afforded a tan crystalline residue, 0.64 g., m.p. 112–115°. Several recrystallizations from ether gave colorless needles, m.p. 122–124°. TLC of this product (15% methanol in chloroform) showed two spots of nearly equal size. Preparative TLC on silica gel $HF_{254+366}$ using the same solvent system gave two crystalline compounds following extraction of the respective bands. The band with the higher R_f afforded a substance that was characterized as 1,3-dicarbethoxy-4-acetamido-3-pyrroline (IV), m.p. 126–129°; mixed melting point was not depressed upon admixture with an authentic sample. The band with the lower R_f gave *cis*-1,3-dicarbethoxy-4-acetamidopyrrolidine (V), also identical in all respects to the authentic sample prepared by a previous method.

***trans*-1,3-Dicarbethoxy-4-acetamidopyrrolidine (VI)**—A solution of V (0.20 g., 0.74 mmole) and freshly prepared sodium ethoxide (0.05 g., 0.74 mmole) in 15 ml. of absolute ethanol was refluxed for 15 hr. The alcohol was removed under reduced pressure, cold water was added to the residue, and the reaction product was extracted with chloroform. The chloroform extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. TLC of the residual oil (5% methanol in chloroform) showed two spots: a major spot and a smaller spot corresponding to the starting material (V). Preparative TLC on silica gel $HF_{254+366}$ afforded a colorless oil which crystallized on standing. Recrystallization from isopropyl ether gave 0.13 g. (65%) of colorless needles, m.p. 107–108°. IR spectrum showed bands at 2.91, 3.01, 5.81, and 6.00 μ.

Anal.—Calcd. for $C_{12}H_{20}N_2O_5$: C, 52.91; H, 7.40; N, 10.29. Found: C, 52.90; H, 7.31; N, 10.19.

***cis*-1-Carbethoxy-3-hydrazino-4-acetamidopyrrolidine (VII)**—A mixture of V (1.0 g., 3.7 mmoles) and hydrazine hydrate (3.0 ml.) was stirred at room temperature for 5 hr. Excess hydrazine hydrate was removed by distillation under reduced pressure, leaving a very viscous oil. Although the product was homogeneous on TLC (20% methanol in ethyl acetate), all attempts to crystallize it failed. IR spectrum (film) showed bands at 3.01, 5.97, and 6.05 μ.

3-Acetyl-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazole-2-one (IX)—*cis*-Hydrazide (VII) (0.60 g., 2.33 mmoles) was dissolved in 3 ml. of 2 *N* hydrochloric acid, and the cooled solution was treated dropwise with a cold solution of 0.15 g. (2.17 mmoles) of sodium nitrite in 2 ml. of water. The oily, yellow azide was extracted with five 10-ml. portions of ethyl acetate, and the combined extracts were dried over anhydrous sodium sulfate. The filtered solution was then refluxed on a steam bath for 2 hr., and the solvent was evaporated to dryness. On standing, the oily residue crystallized to give 0.46 g. (82%) of IX, m.p. 115–120°. Recrystallization from ether afforded colorless needles, m.p. 119–121°. IR spectrum showed bands at 2.91, 3.05, 5.80, and 5.99 μ.

Anal.—Calcd. for $C_{10}H_{15}N_3O_4$: C, 49.80; H, 6.27; N, 17.43. Found: C, 49.63; H, 6.26; N, 17.55.

***cis*-Hexahydropyrrolo[3,4-*d*]imidazole-2-one (X)**—Bicyclic ureide (IX) (5.0 g., 20.8 mmoles) was hydrolyzed by the method previously described (5). Following workup, 2.49 g. (94%) of a colorless crystalline product was obtained, m.p. 210–214°. Recrystallization from methanol afforded X, m.p. 213–215°; mixed melting point was not depressed by admixture with an authentic sample obtained previously. In addition, 0.042 g. of 5-carbethoxyhexahydropyrrolo-[3,4-*d*]imidazole-2-one (XI) was obtained from the reaction mixture, m.p. 221–222°; mixed melting point was not depressed by admixture with an authentic sample.

1,3-Dicarbethoxy-4-amino-5-methyl-3-pyrroline (XV)—A solution of 121.7 g. (0.5 mole) of 1,3-dicarbethoxy-5-methyl-4-pyrrolidone (XIV) (13) and 78.6 g. (1.25 moles) of ammonium formate in 750 ml. of absolute ethanol was heated under reflux for 48 hr. Evaporation of the solvent under reduced pressure afforded a tan crystalline residue. The solid product was treated with 250 ml. of water to dissolve unreacted ammonium formate, and the reaction product was extracted with chloroform. The chloroform extract was washed with water and dried over anhydrous sodium sulfate. Evaporation of the solvent afforded 118.4 g. of crystalline product, m.p. 126–127°. Recrystallization from 95% ethanol gave colorless crystals of XV, 112 g. (93%), m.p. 127–129°. UV spectrum showed $\lambda_{max.}^{EtOH}$ 274 mμ (ε 21,800). IR spectrum showed bands at 2.85, 2.96, 5.98, and 6.10 μ.

Anal.—Calcd. for $C_{11}H_{18}N_2O_4$: C, 54.52; H, 7.49; N, 11.56. Found: C, 54.63; H, 7.57; N, 11.54.

1,3-Dicarbethoxy-4-acetamido-5-methyl-3-pyrroline (XVI)—A solution of 112 g. (0.46 mole) of XV in 350 ml. of acetic anhydride was refluxed for 12 hr. The excess reagent was distilled under reduced pressure, and the remaining yellow oil crystallized from petroleum ether (b.p. 30–60°), 129.3 g. (98.4%), m.p. 69–70°. UV spectrum showed $\lambda_{max.}^{EtOH}$ 274 mμ (ε 14,050). IR spectrum showed bands at 3.01, 5.95, and 6.12 μ.

Anal.—Calcd. for $C_{13}H_{20}N_2O_5$: C, 54.91; H, 7.09; N, 9.85. Found: C, 54.77; H, 7.07; N, 9.81.

1,3-Dicarbethoxy-4-acetamido-5-methylpyrrole (XVII)—On standing at room temperature for several weeks, crystalline product XVI turned pale yellow and became quite sticky. TLC revealed two spots. A small portion of the mixture was separated by preparative TLC on silica gel $HF_{254+366}$ (15% methanol in ethyl acetate). The higher R_f band gave the starting material (XVI), m.p. 68–71°; mixed m.p. 68–71°. The lower R_f band gave product XVII, which crystallized from diethyl ether as colorless needles having m.p. 132–133°. This latter product gave no significant UV absorption at 274 mμ but showed absorption at 216 mμ. IR spectrum showed bands at 2.91, 5.70, 5.90, and 6.20 μ.

Anal.—Calcd. for $C_{13}H_{18}N_2O_5$: C, 55.31; H, 6.43; N, 9.92. Found: C, 55.24; H, 6.24; N, 9.81.

***cis*-1,3-Dicarbethoxy-4-acetamido-5-methylpyrrolidine (XVIII)**—*Method A*—A solution of XVI (3.0 g., 10.6 mmoles) in 100 ml. of 95% ethanol was hydrogenated in a Parr shaker over 0.3 g. (84.7%) of platinum oxide for 24 hr. Evaporation of the filtered solution gave a thick, colorless oil which showed a single spot on TLC (8% methanol in ethyl acetate). Crystallization from isopropyl ether afforded 2.87 g. (95%) of colorless crystals, m.p. 101–103°. The IR spectrum showed bands at 2.91, 5.82, and 5.99 μ.

Anal.—Calcd. for $C_{13}H_{22}N_2O_5$: C, 54.52; H, 7.75; N, 9.78. Found: C, 54.72; H, 7.77; N, 9.73.

Method B—A solution of XVII (1.0 g., 3.5 mmoles) in 50 ml. of 95% ethanol was hydrogenated as in Method A over 0.2 g. (84.7%) of platinum oxide. Following the usual workup, 0.94 g. (93%) of a crystalline product was obtained, m.p. 101–103°. The IR and TLC mobility of the product were identical to those obtained for the product of Method A.

***trans*-1,3-Dicarbethoxy-4-acetamido-5-methylpyrrolidine (XXI)**—*Method A*—A solution of XVI (0.3 g., 1.06 mmole) in 50 ml. of 95% ethanol was hydrogenated over 0.1 g. of 10% palladium on charcoal for 12 hr. Evaporation of the filtered solution gave a colorless oil which showed two spots on TLC (10% methanol in ethyl acetate). Preparative TLC on silica gel $HF_{254+366}$ afforded 0.19 g. of product XVIII, m.p. 100–102°; mixed melting point was not depressed by admixture with Compound XVIII obtained previously. The lower R_f band gave 0.09 g. of product XXI, which crystallized from isopropyl ether as colorless needles, m.p. 128–129°. IR spectrum showed bands at 2.90, 3.00, 5.80, and 5.95 μ.

Anal.—Calcd. for $C_{13}H_{22}N_2O_5$: C, 54.52; H, 7.75; N, 9.78. Found: C, 54.66; H, 7.74; N, 9.80.

Method B—A solution of XVIII (0.10 g., 0.35 mmole) and freshly prepared sodium ethoxide (0.005 g., 0.07 mmole) in 5 ml. of absolute ethanol was stirred at room temperature for 12 hr. The solvent was removed under reduced pressure, ice was added to the residue, and the product was extracted with chloroform. The chloroform extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The tan solid residue (0.082 g., 82%) was recrystallized from isopropyl ether, affording colorless needles, m.p. 128–129°; mixed melting point was not depressed by admixture with the product obtained under Method A.

1,3-Dicarbethoxy-5-methyl-4-pyrrolidone Oxime (XIX)—A mixture of hydroxylamine hydrochloride (3.15 g., 0.045 mole) and sodium acetate trihydrate (6.30 g., 0.046 mole) in 10 ml. of 90% methanol was triturated in a glass mortar. The white suspension was filtered through diatomaceous earth,[2] and the clear filtrate was added to XIV (4.86 g., 0.02 mole). The solution, which immediately became turbid, was stirred at room temperature for 2 hr., followed by a period of heating at 60° until the reaction mixture failed to give a positive ferric chloride test. Following evaporation of the methanol, the product was dissolved in chloroform, washed with water, and dried over anhydrous sodium sulfate. Evaporation of the chloroform gave a colorless, viscous oil (5.01 g., 97%) which resisted crystallization. IR spectrum showed bands at 2.79, 3.02, 5.79, and 5.94 μ.

Catalytic Reduction of Oxime (XIX)—A solution of oxime (XIX) (4.0 g., 15.5 mmoles) in 75 ml. of acetic anhydride was hydrogenated over 0.2 g. (84.7%) of platinum oxide at 3 atm. for 48 hr. Evaporation of the filtered solution *in vacuo* gave a thick, yellow, oily residue which showed three spots on TLC. The mixture was separated by preparative TLC on silica gel $HF_{254+366}$ (5% methanol in chloroform). The higher R_f band gave 2.4 g. of the starting material (XIX) as an oil (superimposable IR spectra). The intermediate R_f band gave 0.78 g. of a crystalline product (XX), m.p. 186–187°. IR spectrum (mineral oil) showed bands at 3.02, 5.70, and 5.95 μ. An analytical sample was obtained by recrystallization from ethyl acetate, m.p. 188–190°.[3]

Anal.—Calcd. for $C_{13}H_{22}N_2O_6$: C, 51.66; H, 7.34; N, 9.27. Found: C, 51.60; H, 7.27; N, 9.35.

The lower R_f band gave 0.64 g. of *cis*-ester (XVIII), m.p. 101–102°; mixed melting point was not depressed by admixture with the compound obtained previously.

***trans*-1-Carbethoxy-3-hydrazino-4-acetamido-5-methylpyrrolidine (XXII)**—A solution of 0.14 g. (0.5 mmole) of XVIII in 3 ml. of absolute ethanol was treated with 0.2 ml. of hydrazine hydrate, and the mixture was refluxed for 5 hr. Evaporation of the solvent afforded 0.13 g. of crystalline product, m.p. 195–204°. Recrystallization from ethanol–ether mixture gave 0.11 g. (79.4%) of colorless crystals. The analytical sample had m.p. 211–212°. IR spectrum (mineral oil) showed bands at 3.04, 3.19. 5.96, and 6.05 μ. Mass spectrum (70 ev.) gave a molecular ion at *m/e* 272.

Anal.—Calcd. for $C_{11}H_{20}N_4O_4$: C, 48.52; H, 7.40; N, 20.57. Found: C, 48.60; H, 7.40; N, 20.58.

***cis*-1-Carbethoxy-3-hydrazino-4-acetamido-5-methylpyrrolidine (XXIII)**—A suspension of 1.99 g. (6.95 mmoles) of XVIII in 6 ml. of hydrazine hydrate was stirred at room temperature for 12 hr. The white suspension was filtered, affording 1.17 g. of a micro-

[2] Celite, Johns-Manville, New York, N. Y.

[3] Compound XX was characterized as an *O*-acetyl pyrrolidinohydroxylamine derivative, since the reduction of oximes to amines presumably involves the intermediacy of hydroxylamines [G. Vavon and Krajcinovic, *Bull. Soc. Chem. France*, **43**, 231(1928)].

crystalline substance, m.p. 146–152°. Evaporation of the excess hydrazine hydrate *in vacuo* afforded an additional 0.34 g. of product for an overall yield of 79.8%. Recrystallization from ethyl acetate gave 1.24 g. of a crystalline colorless product, m.p. 159–160°. IR spectrum (mineral oil) showed bands at 3.02, 5.85, 5.95, and 6.05 μ. Mass spectrum (70 ev.) gave a molecular ion at *m/e* 272.

Anal.—Calcd. for $C_{11}H_{20}N_4O_4$: C, 48.52; H, 7.40; N, 20.57. Found: C, 48.65; H, 7.46; N, 20.44.

3-Acetyl-4-methyl-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazole-2-one (XXIV)—One gram (3.67 mmoles) of hydrazide XXIII dissolved in 4 ml of 2 *N* hydrochloric acid and cooled to −5° was treated dropwise with a cold solution of 0.5 g. (7.2 mmoles) of sodium nitrite in 2 ml. of water. The white precipitate which formed was filtered, m.p. 100–104° dec. The azide was dissolved in 75 ml. of ethyl acetate and heated to reflux on a steam bath for 2 hr. Evaporation of the solvent afforded a yellowish oil which crystallized on standing, 0.62 g. (66%), m.p. 101–105°. Recrystallization from isopropyl ether gave XXIV, m.p. 108–110°. IR spectrum showed bands at 2.89, 3.02, 5.75, and 5.95 μ. Mass spectrum (70 ev.) gave a molecular ion at *m/e* 255.

Anal.—Calcd. for $C_{11}H_{17}N_3O_4$: C, 51.75; H, 6.71; N, 16.46. Found: C, 51.80; H, 6.81; N, 16.59.

4-Methyl-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazole-2-one (XXV) and 4-Methyl-*cis*-hexahydropyrrolo[3,4-*d*]imidazole-2-one (XXVI)—A mixture of 0.60 g. (2.35 mmoles) of XXIV and 3.0 g. (9.51 mmoles) of barium hydroxide octahydrate in 30 ml. of 50% aqueous methanol was refluxed for 12 hr. The creamy suspension was filtered while hot, and the filtrate was evaporated to dryness *in vacuo*. The tan residue was chromatographed over 50 g. of basic alumina, eluting successively with chloroform and 5, 10, 25, and 50% methanol in chloroform. The first three fractions (250 ml. each) afforded 0.40 g. (80%) of a crystalline product, m.p. 145–146°. Recrystallization from ethyl acetate gave an analytical sample, m.p. 145–146°. IR spectrum showed bands at 2.89, 3.09, and 5.82–5.98 (w) μ. Mass spectrum (70 ev.) gave a molecular ion at *m/e* 213.

Anal.—Calcd. for $C_9H_{15}N_3O_3$: C, 50.70; H, 7.09; N, 19.70. Found: C, 50.66; H, 6.99; N, 19.58.

The last two fractions (150 ml. each) gave a white product (0.06 g., 16%), m.p. 206–210°. Recrystallization from ethyl acetate gave XXVI, m.p. 209–210°. IR (mineral oil) showed bands at 3.10 and 5.90 μ. Mass spectrum (70 ev.) gave a molecular ion at *m/e* 141.

Anal.—Calcd. for $C_6H_{11}N_3O$: C, 51.03; H, 7.85; N, 29.76. Found: C, 51.11; H, 7.84; N, 29.69.

***N*-Alkylation of Pyrrolo[3,4-*d*]imidazolones (X and XXVI)**—One part of the secondary amine was refluxed for 12 hr. in the presence of a threefold excess of a nitriloalkyl bromide in the presence of anhydrous potassium carbonate in absolute ethanol. The salts were filtered, and the filtrate was evaporated to dryness. Recrystallization of the tertiary amines (XII*a*, XII*b*, XXVII*a*, and XXVII*b*) was achieved using ethyl acetate.

Hydrolysis to Amino Acid Hydrochlorides (XIII*a*, XIII*b*, XXVIII*a*, and XXVIII*b*)—The respective alkylated pyrrolo[3,4-*d*]imidazolone products were heated at 100° for 8 hr. in the presence of concentrated hydrochloric acid. The reaction mixture was evaporated to dryness under reduced pressure, and the crystalline residue was recrystallized from ethanol or ethanol–ether mixtures.

REFERENCES

(1) J. A. Glasel, *Biochemistry*, **5**, 1851(1966).

(2) A. C. Midvan and M. C. Scrutton, *ibid.*, **6**, 2978(1967).

(3) M. Caplow, *ibid.*, **8**, 2656(1969).

(4) G. Olah and A. M. White, *J. Amer. Chem. Soc.*, **90**, 6087 (1968).

(5) H. C. Wormser, *J. Pharm. Sci.*, **58**, 1038(1969).

(6) P. L. Southwick and G. H. Hofmann, *J. Org. Chem.*, **28**, 3058(1965).

(7) T. Sheradsky and P. L. Southwick, *ibid.*, **30**, 194(1965).

(8) M. Miyamoto, *J. Pharm. Soc. Jap.*, **77**, 568(1957).

(9) J. H. Muller, M. N. Donin, W. E. Behnke, and K. Hofmann, *J. Amer. Chem. Soc.*, **73**, 2487(1951).

(10) G. B. Brown, B. R. Baker, S. Bernstein, and S. R. Safir, *J. Org. Chem.*, **12**, 155(1947).

(11) J. Blake, C. D. Willson, and H. Rapoport, *J. Amer. Chem. Soc.*, **86**, 5293(1964).

(12) S. T. Fang, L. C. Li, C. Niu, and K. F. Ts'eng, *Sci. Sinica*, **10**, 845(1961).

(13) Y. H. Wu, W. A. Gould, W. G. Lobeck, Jr., H. R. Roth, and R. F. Feldkamp, *J. Med. Pharm. Chem.*, **5**, 752(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *College of Pharmacy, Wayne State University, Detroit, MI 48202*

Accepted for publication June 18, 1970.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

This work was supported in part by a grant-in-aid from Wayne State University, Detroit, MI 48202

The author thanks Dr. Don C. DeJongh, Associate Professor of Chemistry, and Mr. David Brent, Wayne State University, for the determination of the mass spectra.

Acetaminophen Prodrugs III: Hydrolysis of Carbonate and Carboxylic Acid Esters in Aqueous Buffers

ELISABETH S. RATTIE, ELIE G. SHAMI*, LEWIS W. DITTERT†, and JOSEPH V. SWINTOSKY†

Abstract □ The hydrolysis rates of four carbonate and five carboxylic acid ester prodrugs of acetaminophen were determined in aqueous buffers at various pH's. The hydrolysis reactions of all the compounds except the hemisuccinate were first order in ester and in hydroxyl ion over the relatively alkaline pH ranges studied. The apparent enthalpies of activation were between 18 and 23 kcal./mole. The results suggest that it should be possible to formulate pharmaceutically stable suspensions of this type of acetaminophen prodrugs.

Keyphrases □ Acetaminophen prodrugs—hydrolysis □ Carbonate, carboxylic acid esters of prodrugs—hydrolysis, aqueous buffers □ Hydrolysis rates—acetaminophen prodrug esters □ UV spectrophotometry—analysis

The synthesis, physicochemical properties, and analgesic activities in rats of several prodrug esters of acetaminophen have been previously reported (1–3). These studies showed that significant differences in analgesic potency and duration of action might be expected following the oral administration of carbonate and carboxylic acid esters of various structures. The expected differences were attributable primarily to differences in the rates of dissolution and absorption of the esters following oral administration. In the case of 4-acetamidophenyl 2,2,2-trichloroethyl carbonate, the peak heights and rates of decline of blood levels of acetaminophen in humans depended upon the particle size of the administered powder (4).

Thus, the dose–time–action profiles of drugs that can be converted into carbonate or carboxylic acid esters might be modified at will by: (*a*) selecting appropriate ester structures, and (*b*) controlling the prodrug's particle size in pharmaceutical formulations. The selection of ester structures on the basis of hydrolysis catalyzed by various body enzymes has been discussed (3), but relatively little information on the chemical stability of drugs of this type has been reported. Therefore, the base-catalyzed hydrolysis of several acetaminophen prodrugs was studied to gain information on their relative hydrolytic stability, which would be useful in the formulation of pharmaceutical dosage forms, and to further elucidate their hydrolysis by esterolytic enzymes.

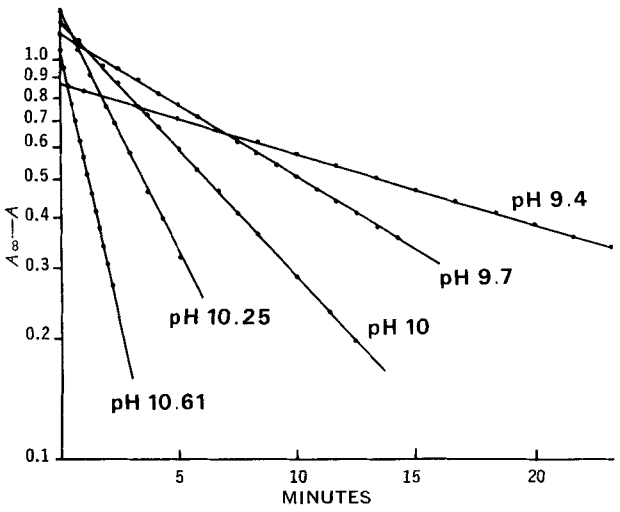


Figure 1—*Plots showing the pseudo-first-order nature of the hydrolysis of 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (II) over the pH range 9.40–10.61 at 25°. The half-lives are shown in Table I.*

EXPERIMENTAL

Materials—The synthesis and physical properties of the prodrug esters have been described (2). All other chemicals were reagent grade. A Leeds & Northrup model 7401 pH meter and a Cary model 15 spectrophotometer were used.

Buffer Solutions—*Carbonate (pH 10.61, 10.25, 10.0, 9.7, and 9.4)*—These buffers were 0.1 *M* with respect to carbonate ion and were adjusted to ionic strength 0.5 with KCl. The buffers were prepared by dissolving sodium bicarbonate and potassium chloride in distilled water and by adjusting the pH by the dropwise addition of sodium hydroxide.

Phosphate (pH 7.40 and 6.81) and Succinate (pH 5.85 and 5.4)—The method of preparation was similar to that described for the carbonate buffer.

Procedure for Hydrolysis Studies—The hydrolysis rates of the acetaminophen prodrugs in suitable buffers at constant ionic

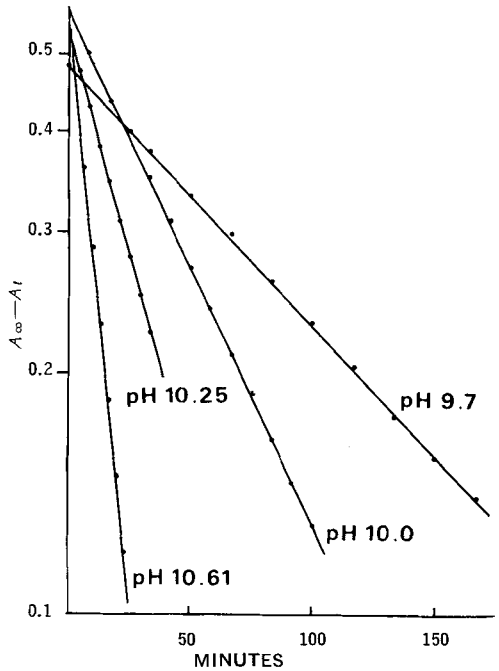


Figure 2—*Plots showing the pseudo-first-order nature of the hydrolysis of 4-acetamidophenyl butyrate (VI) over the pH range 9.70–10.61 at 25°. The half-lives are shown in Table II.*

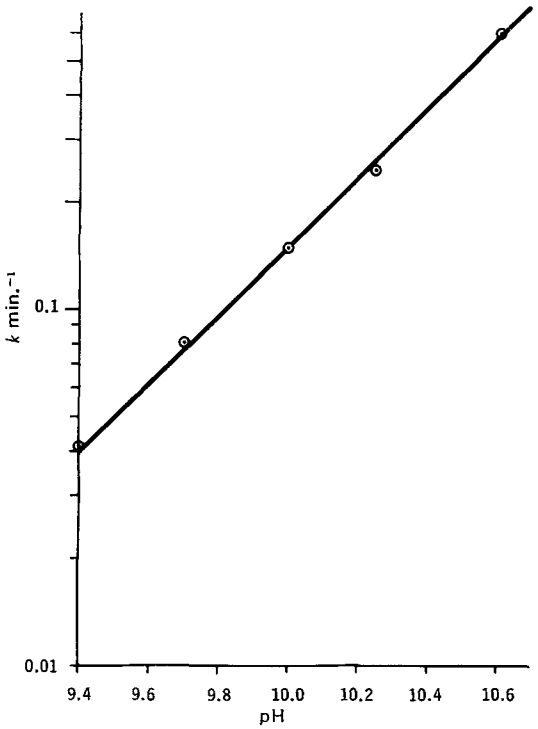


Figure 3—*Plot of pseudo-first-order rate constants* versus *pH for 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (II) over the pH range 9.4–10.61 at 25°.*

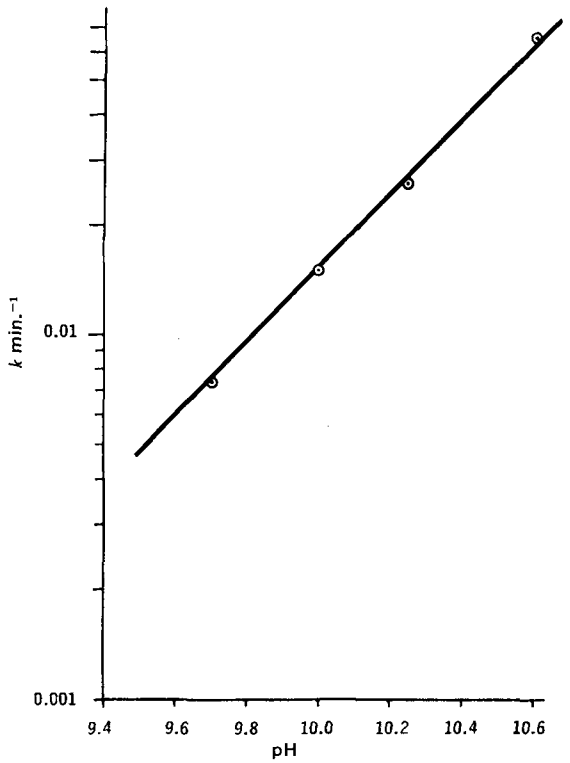
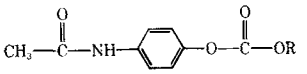


Figure 4—*Plot of pseudo-first-order rate constants* versus *pH for 4-acetamidophenyl butyrate (VI) over the pH range 9.7–10.61 at 25°.*

strength were determined spectrophotometrically by direct UV analysis in the thermostated cell compartment. Fifty milliliters of buffer solution was equilibrated at the approximate temperature in a 50-ml. mixing cylinder. One-half milliliter of anhydrous methanol, containing approximately 0.25 mg. of prodrug, was pipeted into the cylinder, which was then shaken thoroughly. A portion of this mixture was transferred to a 10-cm. sample cell of the spectrophotometer, and the absorbance at 300 mμ (the absorbance maximum of acetaminophen) was followed until no further change in absorbance could be observed (3). All hydrolysis reactions followed pseudo-first-order kinetics, and plots of log $(A_\infty - A_t)$ *versus* time were used to determine the first-order rate constants.

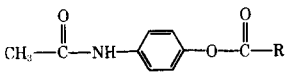
Table I—Hydrolysis Data for Carbonate Ester Prodrugs of Acetaminophen, 25°



R	pH	Buffer System[a]	$t_{1/2}$, min.	$k_{obs.}$ min.$^{-1}$	k_{OH} l. mole^{-1} min.$^{-1}$[b]
I					
$-CH_2-CH_3$	10.61	Carbonate	28	0.025	61
	10.25	Carbonate	56	0.012	68
	10.00	Carbonate	103	0.0067	67
	9.70	Carbonate	183	0.0038	76
					Av. 68
II					
$-CH_2-C-Cl_3$	10.61	Carbonate	1.1	0.62	1.5×10^3
	10.25	Carbonate	2.7	0.25	1.4×10^3
	10.00	Carbonate	4.6	0.15	1.5×10^3
	9.70	Carbonate	8.3	0.082	1.6×10^3
	9.40	Carbonate	17	0.041	1.6×10^3
					Av. 1.5×10^3
III					
$-CH_2-CH(CH_3)_2$	10.59	Carbonate	26	0.027	69
	10.31	Carbonate	48	0.015	74
	9.98	Carbonate	108	0.0064	67
	9.76	Carbonate	168	0.0041	71
					Av. 70
IV					
$-(CH_2)_2-N(CH_3)_2 \cdot HCl$	7.40	Phosphate	1.7	0.40	1.6×10^6
	6.81	Phosphate	5.8	0.12	1.9×10^6
	5.85	Succinate	55	0.013	1.8×10^6
					Av. 1.7×10^6

[a] Buffers were 0.1 *M* and adjusted to ionic strength 0.5 with KCl. [b] Calculated fom $k_{obs.}/[OH^-]$, where $[OH^-] = 10^-(pk_w - pH)$ and the pk_w = 13.9965 at 25°.

Table II—Hydrolysis Data for Carboxylic Acid Ester Prodrugs of Acetaminophen, 25°



R	pH	Buffer System[a]	$t_{1/2}$, min.	$k_{obs.}$ min.$^{-1}$	k_{OH} l.mole^{-1} min.$^{-1}$[b]
V					
$-CH_3$	10.59	Carbonate	6	0.12	320
	10.31	Carbonate	12	0.059	300
	9.98	Carbonate	26	0.026	290
	9.76	Carbonate	42	0.016	320
					Av. 310
VI					
$-(CH_2)_2CH_3$	10.61	Carbonate	11	0.066	170
	10.25	Carbonate	27	0.026	150
	10.00	Carbonate	47	0.015	150
	9.70	Carbonate	93	0.0074	150
					Av. 155
VII					
	10.61	Carbonate	17	0.041	100
	10.31	Carbonate	35	0.020	100
	9.98	Carbonate	78	0.0089	100
	9.76	Carbonate	130	0.0053	110
					Av. 103
VIII					
$-C(CH_3)_3$	10.59	Carbonate	72	0.0097	25
	10.31	Carbonate	148	0.0047	24
	9.98	Carbonate	380	0.0018	20
					Av. 23

[a] Buffers were 0.1 *M* and adjusted to ionic strength 0.5 with KCl. [b] Calculated from $k_{obs.}/[OH^-]$, where $[OH^-] = 10^{-(pk_w - pH)}$ and the pk_w = 13.9965 at 25°.

RESULTS AND DISCUSSION

Log ($A_\infty - A_t$) *versus* time plots for 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (II) and 4-acetamidophenyl butyrate (VI) at various pH's are shown in Figs. 1 and 2, respectively. These figures are typical of the hydrolysis behavior of all the prodrug esters and show that the reactions followed pseudo-first-order kinetics at constant pH. Plots of log *k* (the pseudo-first-order rate constant) *versus* pH were straight lines with slopes essentially equal to 1, showing that the hydrolysis reactions were first order in hydroxyl ion as well as in ester (Figs. 3 and 4). The results of the hydrolysis studies at 25° for the carbonate ester prodrugs are summarized in Table I; those for the carboxylic acid ester prodrugs are summarized in Table II. Essentially the same half-lives as those shown in Tables I and II were obtained in buffers ranging from 0.03 to 0.5 *M* and in solutions of ionic strength ranging from 0.1 to 0.5. Thus, specific buffer catalysis and salt effects are apparently negligible for these hydrolytic reactions.

The results in Table I show that chlorine substitution in the aliphatic alcohol portion of the carbonate ester (II) markedly

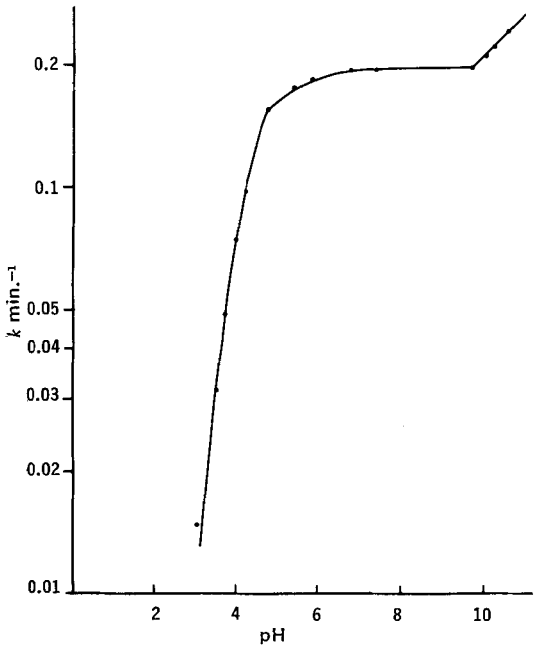


Figure 5—*Plot of pseudo-first-order rate constants* versus *pH for 4-acetamidophenyl hemisuccinate (IX) over the pH range 3.08–10.60 at 25°. The half-lives are shown in Table III.*

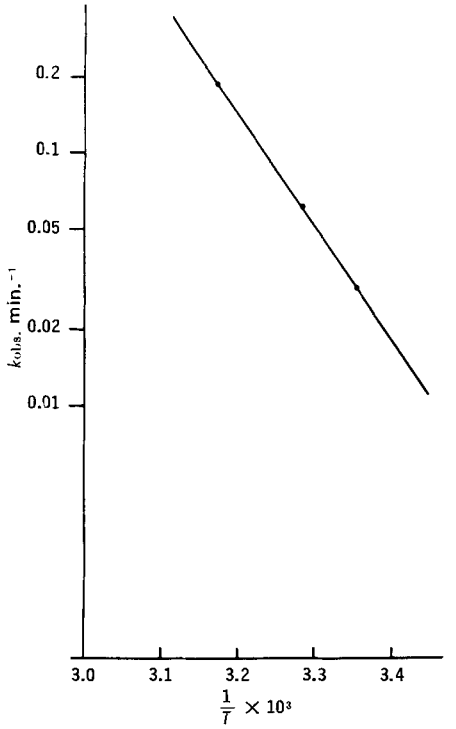
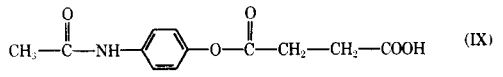


Figure 6—*Arrhenius plot for the hydrolysis of 4-acetamidophenyl ethyl carbonate (I) at pH 10.61 over the temperature range 25–42°. Apparent energy of activation* (ΔH) = *21 kcal./mole (Table IV).*

Table III—Hydrolysis Data for Hemisuccinate Ester of Acetaminophen, 25°



(IX)

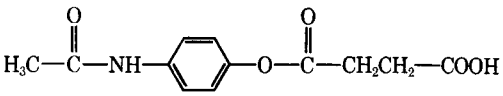
pH	Buffer System[a]	$t_{1/2}$, min.	$k_{obs.}$ min.$^{-1}$
3.08	Citrate	47	0.015
3.5	Citrate	22	0.032
3.7	Citrate	14	0.049
4.0	Acetate	9	0.074
4.25	Acetate	7	0.098
4.75	Acetate	4.5	0.16
5.4	Succinate	3.9	0.18
5.85	Succinate	3.8	0.18
6.81	Phosphate	3.6	0.19
7.41	Phosphate	3.6	0.19
9.72	Carbonate	3.5	0.19
10.05	Carbonate	3.3	0.21
10.27	Carbonate	3.1	0.22
10.60	Carbonate	2.9	0.24

[a] Buffers were 0.1 *M* and adjusted to constant ionic strength 0.5 with KCl.

increased the lability of the ester group to base-catalyzed hydrolysis. The effect was probably due to the electron-withdrawing properties of the chlorine atoms. Chain branching in this alcohol moiety (III) had relatively little influence on the rate of the hydrolysis reaction. Addition of a β-dimethylamino group to the alcohol moiety (IV) caused a dramatic increase in the lability of the ester to base attack. The hydrolysis of IV was first order in hydroxyl ion within the pH region studied (Table I) and can be assigned to an hydroxyl-ion attack on the protonated species.

The results in Table II show that the acetate ester (V) was more labile to base-catalyzed hydrolysis than the ethyl carbonate ester (I). Lengthening the aliphatic chain (VI) or substituting an aromatic hydrocarbon (VII) in the carboxylic acid moiety slightly decreased the lability of the ester group to hydrolysis. Branching of the aliphatic chain near the carboxyl group (VIII) significantly slowed the reaction.

The hydrolysis behavior of the hemisuccinate ester of acetaminophen (IX) was studied extensively because it represents a type of



IX

compound that might display a higher aqueous solubility, especially at higher pH's, than some other prodrug esters. The results shown in Table III and Fig. 5 are very similar to those reported by Gaetjens and Morawetz (5) for phenyl acid succinates. As might be expected, this compound was more stable at low pH's where the free carboxyl group is essentially completely unionized, but it is too labile to be formulated into a suitable pharmaceutical solution under any conditions.

A brief study of the temperature dependency of the hydrolysis reactions showed that, in all cases, the Arrhenius law was obeyed at the pH's studied. A typical Arrhenius plot for Compound I is shown in Fig. 6, and the results for all compounds are summarized in Table IV. The apparent energies of activation are consistent with values previously reported for base-catalyzed ester hydrolyses (5). The conclusion might be drawn from this brief study that stable pharmaceutical suspensions might be formulated from aliphatic carbonate and aliphatic and aromatic carboxylic acid prodrug esters of acetaminophen. A rough extrapolation from pH 10 to pH 5.5, the pH of maximum stability of the acetaminophen amide group

Table IV—Temperature Dependency of the Hydrolysis Reactions of Carbonate and Carboxylic Acid Prodrugs of Acetaminophen

R	pH	$k_{obs.}$ min.$^{-1}$	Temperature	ΔH (kcal./mole)
I	10.61	0.029	25°	21
		0.062	31.5°	
		0.19	42°	
II	9.35	0.044	25°	19
		0.091	31.5°	
		0.022	42°	
III	10.61	0.027	25°	23
		0.055	31°	
		0.024	43°	
IV	5.85	0.013	25°	22
		0.028	31°	
		0.11	43°	
V	10.00	0.026	25°	20
		0.051	31°	
		0.23	45°	
VI	10.61	0.063	25°	19
		0.12	31.5°	
		0.33	42°	
VII	10.25	0.019	25°	18
		0.033	31°	
		0.14	45°	
VIII	10.61	0.0097	25°	20
		0.018	31°	
		0.060	42°	

toward hydrolysis (6), yields t_{90} values of approximately 1, 1, 0.2, 0.4, 0.7, and 3.5 years for Compounds I, III, V, VI, VII, and VIII, respectively. These relatively high t_{90} values for the compounds *in solution*, coupled with their relatively low aqueous solubilities (2) and high doses, suggest that it might be possible to formulate pharmaceutical suspensions with some of the prodrug esters that would retain 90% of their potencies for at least 2 years at room temperature.

The more water-soluble prodrugs, *i.e.*, the *N,N*-dimethylethanolamine carbonate (IV) and the hemisuccinate (IX), are too labile to be formulated in liquid dosage forms under any conditions since they hydrolyze very rapidly at both slightly acidic and slightly basic pH's.

REFERENCES

(1) J. V. Swintosky, H. C. Caldwell, C. W. Chong, G. M. Irwin, and L. W. Dittert, *J. Pharm. Sci.*, **57**, 752(1968).

(2) L. W. Dittert, H. C. Caldwell, H. J. Adams, G. M. Irwin, and J. V. Swintosky, *ibid.*, **57**, 774(1968).

(3) L. W. Dittert, G. M. Irwin, C. W. Chong, and J. V. Swintosky, *ibid.*, **57**, 780(1968).

(4) L. W. Dittert, H. J. Adams, F. Alexander, C. W. Chong, T. Ellison, and J. V. Swintosky, *ibid.*, **57**, 1146(1968).

(5) E. Gaetjens and H. Morawetz, *J. Amer. Chem. Soc.*, **82**, 5328(1960).

(6) T. K. Koshy and J. L. Lach, *J. Pharm. Sci.*, **50**, 113(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 16, 1970, from *Smith Kline and French Laboratories, Philadelphia, PA 19101* and the †*College of Pharmacy, University of Kentucky, Lexington, KY 40506*

Accepted for publication June 16, 1970.

* Present address: Endo Laboratories, Garden City, Long Island, N. Y.

Biopharmaceutical Studies on Guaiacol Glycerol Ether and Related Compounds IV: Drug in Blood and Bile

SHUN-ICHI NAITO, MITSUO MIZUTANI, SEIMEI OSUMI, KAZUKO UMETSU, TADAHIRO MIKAWA, YOSHIKO NISHIMURA, and NOBUYOSHI YAMAMOTO

Abstract □ Blood levels of guaiacol glycerol ether mononicotinate in animals were determined, and it appears that the chemical is absorbed with pseudo-first-order reaction by passive transport. Guaiacol glycerol ether mononicotinate does not affect blood pressure and respiration. The compound has no effect on bile flow and the cholesterol value in bile, but it increases the amount of cholesterol excreted in the bile of hypercholesteremic rats receiving polyoxyethylene ethers intravenously. The effect of the metabolites of the compound on cholesterol content in bile was also investigated.

Keyphrases □ Guaiacol glycerol ether mononicotinate—biopharmaceutical studies □ Cholesterol excretion—guaiacol glycerol ether mononicotinate effect □ Colorimetric analysis—spectrophotometer

The hypocholesteremic effects of guaiacol glycerol ether (GGE) and guaiacol glycerol ether mononicotinate (GGE-MN) in animals have been reported (1). In a previous report (2), metabolites of GGE and GGE-MN in rabbit urine were discussed.

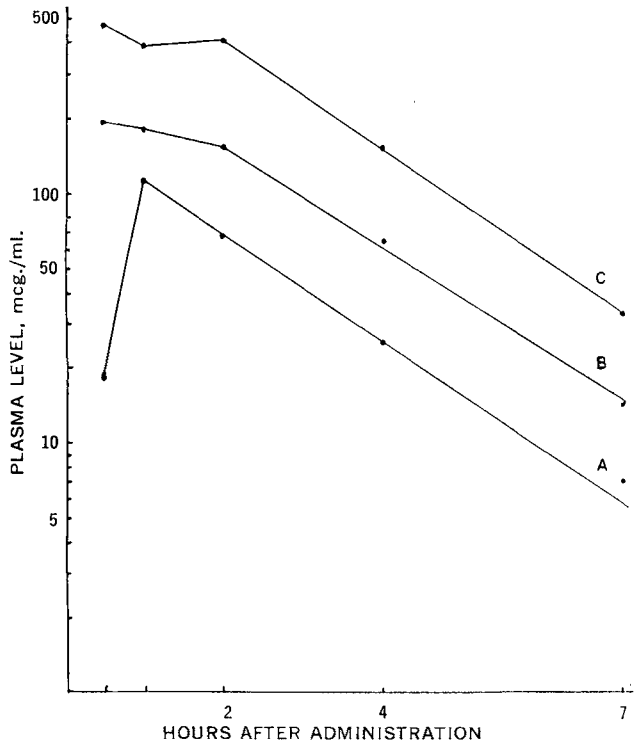


Figure 1—*Mean plasma level of GGE-MN after oral administration to mice at different doses. Key: A, 100 mg./kg.; B, 200 mg./kg.; and C, 300 mg./kg. GGE-MN was screened through 100 mesh and prepared in suspension form with water. Groups consisted of 10 male mice (dd strain); animals were killed according to the sampling schedule. At time of sacrifice, animals were bled completely, and the equivoluminal mixture of the blood from each group was reserved for analysis. All data concerned with mice show mean values of each group.*

The present study was designed to determine the blood levels of GGE-MN in animals and the effects of it and related compounds on bile in animals and the mechanism of hypocholesteremic action.

EXPERIMENTAL

Determination of GGE-MN in Blood (3)—To 1 ml. of plasma in an ice-cold centrifuge tube, 2 ml. of ethanol was added, and the mixture was stirred thoroughly. After the mixture stood for 15 min. in an ice-water bath, 0.5 ml. of the supernatant from centrifugation was mixed with 1 ml of ethanol, 2 ml. of ammonia buffer solution, and 3 ml. of 10% cyanogen bromide solution. The solution was held at room temperature for 15 min., and absorbance was determined at 400 mμ. A mixture of 0.9 ml. of normal animal plasma and 0.1 ml. of ethanolic GGE-MN solution of known concentration was treated as just described to prepare a calibration curve.

Ammonia buffer solution was prepared to make the equivoluminal mixture of 0.1 *M* NH_4OH, 0.5 *M* K_2HPO_4, and 2.0 *M* NH_4Cl.

Male mice (dd strain, mean body weight 20 g.), female rats (Wistar strain, mean body weight 200 g.), or male rabbits (mean body weight 2 kg.) were used in the study.

Collection of Rabbit and Rat Bile—Rabbits varying in weight from 3.0 to 3.5 kg. were anesthetized with urethan, 1 g./kg. s.c., following a fasting period of approximately 24 hr.

Blood pressure was recorded by a mercury manometer connected

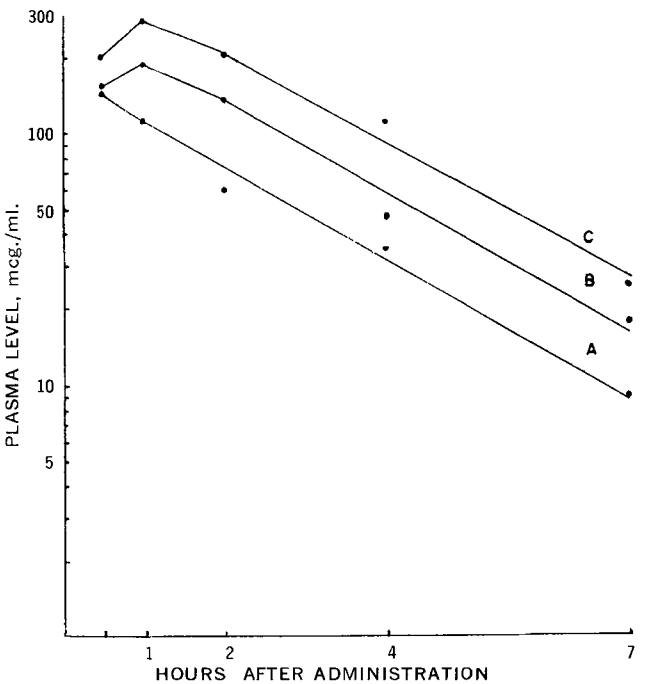


Figure 2—*Mean plasma level of GGE-MN after oral administration to rats at different doses. Key: A, 100 mg./kg.; B, 200 mg./kg.; and C, 300 mg./kg. GGE-MN was screened through 100 mesh and prepared in suspension form with water. Each female rat, five rats per group (Wistar), was killed according to the sampling schedule. At the time of sacrifice, each animal was bled completely and the blood was reserved for analysis.*

Table I—Pharmaceutical Formulas of GGE-MN Administered to Rabbits

Sample No.	Form	Composition
1	Capsule	GGE-MN,[a] 100 mg.; lactose, 125 mg.; and silicic acid anhydrous, 5 mg.
2	Capsule	GGE-MN, 100 mg.; microcrystalline cellulose,[b] 75 mg.; and silicic acid anhydrous, 5 mg.
3	Tablet	GGE-MN, 100 mg.; microcrystalline cellulose, 84 mg.; CMC-Na, 6 mg.; and talc, 10 mg.
4	Tablet	Film coated on the tablet of Sample 3 by cellulose acetate phthalate solution

[a] GGE-MN was screened through 100 mesh. [b] Avicel, Asahi Kasei Kogyo Co. Ltd., Japan.

to a cannula in a carotid artery, and respiration was recorded on a kymograph.

A polyethylene tube was inserted into a bile duct, and the bile was collected for 7.5 hr. following administration of the compound under continuous anesthesia using urethan. At the end of the 1st (control) hr., the compound was administered orally by a stomach tube.

Rats (Wistar strain), varying in body weight from 160 to 190 g., were treated by the same method as the rabbits.

Determination of GGE-MN in Rabbit and Rat Biles—One milliliter of bile instead of plasma was treated according to the method for determination of GGE-MN in blood after oral administration of GGE-MN by a stomach tube.

Collection of Rat Bile at Polyoxyethylene Ethers Hypercholesteremia—Male rats of Wistar strain, weighing from 160 to 190 g., were injected intravenously with 400 mg./kg. of tyloxapol[1] in distilled water, adjusting the volume to 0.2 ml./100 g. of body weight. The test sample was administered intraperitoneally immediately after injection of polyoxyethylene ethers. Collection

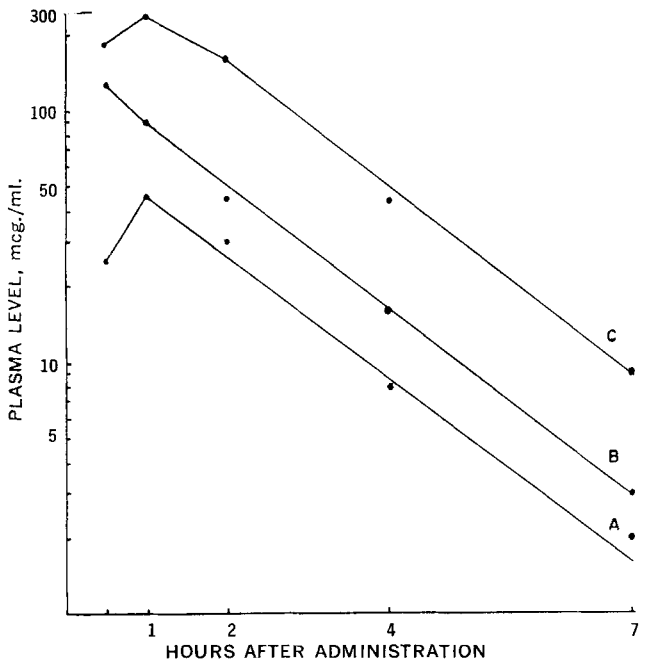


Figure 3—*Mean plasma level of GGE-MN after oral administration of water suspension to rabbits at different doses. Key: A, 50 mg./kg.; B, 150 mg./kg.; and C, 250 mg./kg. GGE-MN was screened through 100 mesh and prepared in suspension form with water. Four male rabbits weighing about 2 kg. each were used.*

[1] Triton WR-1339, Rohm & Haas Co., Philadelphia, Pa., supplied by Winthrop Laboratories, New York, N. Y.

Table II—Effect of GGE-MN and Its Related Compounds or Mixtures on Cholesterol Content in Bile of Rats[a] Receiving Polyoxyethylene Ethers (400 mg./kg.) Intravenously

Sample	Dose	Total Bile Volume for 7.5 hr. (ml./kg.), Mean Value ±SE[b]	Total Cholesterol in Bile (mcg./kg.), Mean Value ±SE[b]
No Polyoxyethylene Ethers Given			
No drug		21.3 ± 3.1	1730 ± 445
GGE-MN	300 mg./kg. (i.p.)	28.8 ± 6.4	2020 ± 421
GGE-MN	300 mg./kg. (orally)	19.4 ± 4.3	1578 ± 312
Polyoxyethylene Ethers, Given i.p.			
Control (no drug)		29.8 ± 4.8	5398 ± 536
GGE-MN	300 mg./kg.	26.7 ± 3.9	6083 ± 191
GGE-MN	200 mg./kg.	28.5 ± 8.4	5939 ± 1513
GGE	300 mg./kg.	27.2 ± 2.9	7479 ± 2780
GGE	1 mmole/kg.	25.8 ± 4.0	5043 ± 231
GGE	0.7 mmole/kg.	27.9 ± 6.5	5097 ± 770
Nicotinic acid	1 mmole/kg.	24.6 ± 6.8	6366 ± 964
Nicotinic acid	0.7 mmole/kg.	26.7 ± 8.1	6642 ± 1034
Equimolar mixture of GGE and nicotinic acid	1 mmole/kg.	23.0 ± 3.4	5385 ± 647
Equimolar mixture of GGE and nicotinic acid	0.7 mmole/kg.	22.6 ± 9.1	5658 ± 1130
β-(4-Hydroxy-2-methoxyphenoxy) lactic acid	1 mmole/kg.	26.3 ± 4.2	6998 ± 2889
(4-Hydroxy-2-methoxyphenoxy) acetic acid	1 mmole/kg.	27.2 ± 3.0	7186 ± 1666
Mesoinositol hexanicotinate	0.3 mmole/kg.	21.6 ± 6.6	5717 ± 804
2,6-Pyridine dimethanol bis-(*N*-methylcarbamate)	0.8 mmole/kg.	21.8 ± 6.6	7605 ± 1560

[a] Male rats (Wistar strain), weighing 160–200 g., were used. Each group consisted of five rats. [b] *SE* = standard error.

of bile in each group (five rats per group) was started 18 hr. after injection of the polyoxyethylene ethers (4, 5).

Assay of Cholesterol—Cholesterol in bile was determined quantitatively by the method of Zak (6).

Bilirubin in Rabbit Bile—Bilirubin concentration in bile was determined by the method of Jendrassik and Cleghorn (7).

Nicotinic Acid, Mesoinositol Hexanicotinate, and 2,6-Pyridine Dimethanol Bis(*N*-methylcarbamate)—These compounds were commercially available.

β-(4-Hydroxy-2-methoxyphenoxy)lactic Acid and (4-Hydroxy-2-methoxyphenoxy)acetic Acid—The compounds were described in the previous paper (2).

RESULTS AND DISCUSSION

It was described previously (3) that unchanged GGE-MN in blood can be determined when barbituric acid buffer solution instead of ammonia buffer solution is used (8, 9).

When GGE-MN is ingested orally, unchanged GGE-MN and free nicotinic acid as the hydrolysis product of GGE-MN were detected in rabbit plasma (3). In the present work, the blood level of GGE-MN was determined as total drug including unchanged GGE-MN and nicotinic acid liberated from GGE-MN by using an ammonia buffer for the determination of GGE-MN.

GGE-MN seems to be absorbed with pseudo-first-order reaction by passive transport in mice and rats, since the blood level peak is almost proportional to the dose when the logarithm of the blood level is plotted against time from plasma data of GGE-MN (Figs. 1 and 2).

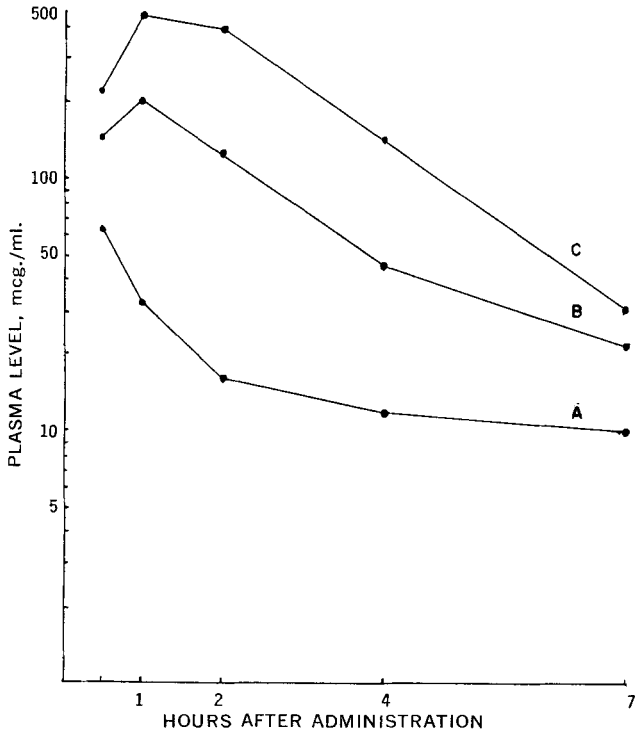


Figure 4—*Mean plasma level of GGE-MN after oral administration of Sample 1 to rabbits at different doses. Key: A, 50 mg./kg.; B, 150 mg./kg.; and C, 250 mg./kg. GGE-MN was screened through 100 mesh and prepared in suspension form with water. Four male rabbits weighing about 2 kg. each were used. Capsule contents were removed and used in water suspension form for ingestion.*

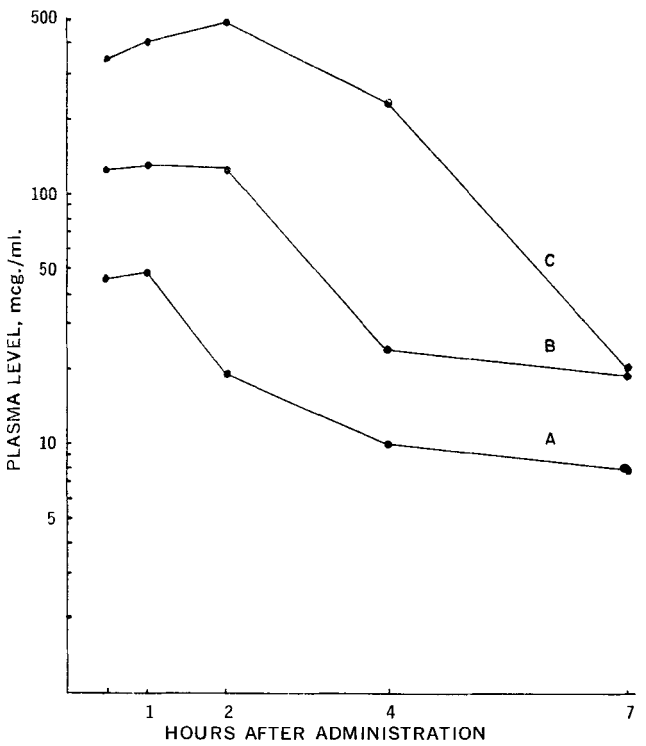


Figure 5—*Mean plasma level of GGE-MN after oral administration of Sample 2 to rabbits at different doses. Key: A, 50 mg./kg.; B, 150 mg./kg.; and C, 250 mg./kg. GGE-MN was screened through 100 mesh and prepared in suspension form with water. Four male rabbits weighing about 2 kg. each were used. Capsule contents were removed and used in water suspension form for ingestion.*

To select a proper pharmaceutical formulation of GGE-MN for clinical trials, several formulations were prepared as shown in Table I. Blood levels in rabbits when these pharmaceuticals were administered orally were compared with the blood level when a water suspension of GGE-MN was given, and the results are shown in Figs. 3–6. Formulations of Samples 3 and 4 seemed to be unsuitable, as compared to the water suspension or capsule form. Sample 1 appeared better than Sample 2, because there was less fluctuation in blood level of GGE-MN.

The bile from rabbits was collected under continuous urethan anesthesia, and arterial blood pressure and respiration were recorded on a kymograph. It was ascertained that GGE-MN and GGE have no effect upon arterial blood pressure, respiration, bile flow, and total bile volume excreted for 7.5 hr., as compared to untreated control rabbits.

When 300 mg./kg. of GGE-MN was administered orally to rabbits and rats under continuous anesthesia for 7.5 hr., no GGE-MN was observed in the bile within experimental error, and no variation of bilirubin concentration was recognized compared to control animals.

The effect of GGE-MN, GGE, or the equimolar mixture of GGE and nicotinic acid on cholesterol content in rabbit bile was determined, but it was observed that these three samples had no effect on the cholesterol content in bile.

In previous reports (1, 2), GGE-MN and the metabolites, namely, β-(4-hydroxy-2-methoxyphenoxy)lactic acid and (4-hydroxy-2-methoxyphenoxy)acetic acid, showed hypocholesteremic effect on hypercholesteremia caused by intravenous injection of polyoxyethylene ethers to rats.

In the present work, the effect of GGE-MN and its related compounds or the mixtures on cholesterol content in the bile of rats receiving polyoxyethylene ethers was investigated; the results are shown in Table II. No effect on the cholesterol value in bile was observed when GGE-MN was given orally or intraperitoneally to normal rats.

Compounds that clearly increased the amount of cholesterol excreted in the bile of hypercholesteremic rats receiving polyoxyethylene ethers intravenously were 300 mg. (1 mmole)/kg. of GGE-MN, 0.7 mmole/kg. of nicotinic acid, 0.8 mmole/kg. of 2,6-pyridine dimethanol bis(*N*-methylcarbamate),[2] and 1 mmole/kg. of (4-hydroxy-2-methoxyphenoxy)acetic acid, at 95% confidence limit.

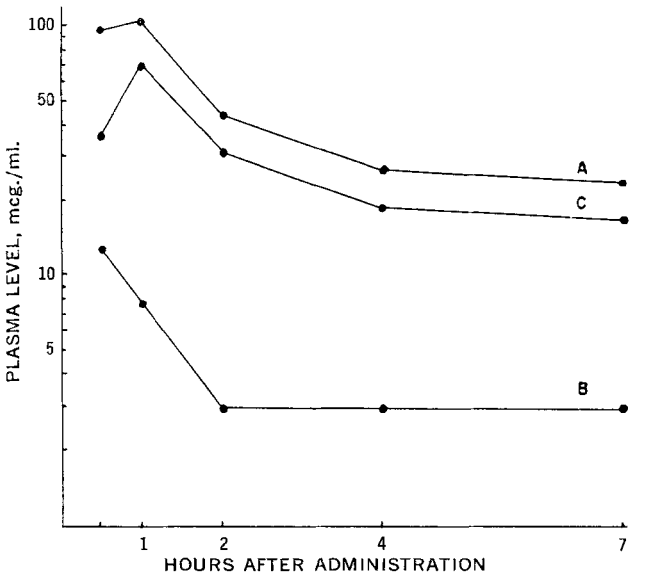


Figure 6—*Mean plasma level of GGE-MN after oral administration of Samples 3 and 4 to rabbits. Key: A, Sample 3, corresponded to 150 mg./kg. of GGE-MN; B, Sample 4, corresponded to 50 mg./kg. of GGE-MN; and C, Sample 4, corresponded to 150 mg./kg. of GGE-MN. GGE-MN was screened through 100 mesh and prepared in suspension form with water. Four male rabbits weighing about 2 kg. each were used. A mixture of 20 ml. of water and tablets after disintegration was used for ingestion.*

[2] Anginin, Banyu Pharmaceutical Co. Ltd., Japan.

On the contrary, 0.7 mmole/kg. and 1 mmole/kg. of GGE did not increase the amount of cholesterol excreted in bile.

Other compounds such as 1 mmole/kg. of β-(4-hydroxy-2-methoxyphenoxy)lactic acid, 200 mg./kg. of GGE-MN, 300 mg./kg. of GGE, 1 mmole/kg. of nicotinic acid, 0.3 mmole/kg. of meso-inositol hexanicotinate,[3] and the equimolar mixture of GGE and nicotinic acid at 0.7- and 1-mmole/kg. doses increased the amount of cholesterol excreted in bile for some rats but did not alter that for other rats in the same group, compared with that of the control group.

On the other hand, all compounds used showed no effect on the total volume of bile excreted, compared with the control group, at 95% confidence limit.

It is interesting that (4-hydroxy-2-methoxyphenoxy)acetic acid, one of the metabolites of GGE and GGE-MN, has the effect of increasing cholesterol in bile in hypercholesteremia caused by intravenous injection of polyoxyethylene ethers. GGE-MN increased the excretion of cholesterol in bile at 1 mmole/kg., but nicotinic acid, one of the metabolites of GGE-MN, also has similar action at the same dose. Therefore, some question still remains whether the increasing effect of GGE-MN on excretion of cholesterol in bile is due to that of GGE-MN itself and/or to the metabolites of GGE-MN *in vivo*. The data concerned with mesoinositol hexanicotinate and 2,6-pyridine dimethanol bis(*N*-methylcarbamate) were determined for comparison purposes, since these compounds are now widely being used as hypocholesteremic agents.

[3] Hexanicite, Yoshitomi Pharmaceutical Co. Ltd., Japan.

REFERENCES

(1) S. Naito and M. Mizutani, *Chem. Pharm. Bull.*, **17**, 822(1969).

(2) S. Naito, M. Mizutani, K. Kitao, and S. Nishimura, *ibid.*, **17**, 1794(1969).

(3) S. Naito and J. Sakai, *Yakuzaigaku*, **26**, 134(1966).

(4) S. O. Byers, M. Friedman, and T. Sugiyama, *Amer. J. Physiol.*, **204**, 1100(1963).

(5) T. Kobayashi, *Yakugaku Zasshi*, **81**, 195(1961).

(6) B. Zak, *Amer. J. Clin. Pathol.*, **27**, 583(1957).

(7) L. Jendrassik and R. A. Cleghorn, *Biochem. Z.*, **289**, 1(1937).

(8) O. Pelletier and I. A. Campbell, *J. Pharm. Sci.*, **50**, 926(1961).

(9) A. Muller and S. H. Fox, *J. Biol. Chem.*, **167**, 291(1947).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 26, 1970, from the *Kyoto College of Pharmacy, Misasagi Yamashina, Higashiyama-ku, Kyoto, Japan.*

Accepted for publication June 18, 1970.

This constitutes Part XXXIX of a series entitled "Studies on Absorption and Excretion of Drugs" by S. Naito.

The authors thank Dr. Kihachiro Okamoto, Kyoto Pharmaceutical Ind. Ltd., for his gift of GGE-MN and his suggestions.

Chlorpromazine Metabolism I: Quantitative Fluorometric Method for 11 Chlorpromazine Metabolites

PUSHKAR N. KAUL, MICHAEL W. CONWAY*, MERVIN L. CLARK, and JAMES HUFFINE*

Abstract □ Eleven chlorpromazine metabolites have been reacted with dimethylaminonaphthyl sulfonyl chloride to obtain fluorescent products. The reaction has been standardized and adapted to quantitative determination of nanogram amounts of the metabolites. A procedure for application to biological fluids has been developed. Addition and recovery experiments on urine and plasma indicate that the method is applicable to the study of the pharmacokinetic aspects of chlorpromazine metabolites in human subjects.

Keyphrases □ Chlorpromazine—metabolism □ Metabolites, chlorpromazine—quantitative determination □ 5-Dimethylaminonaphthalene-1-sulfonyl Cl–chlorpromazine metabolites reaction—fluorescence □ TLC—separation, identification □ Fluorometry—analysis

Despite a great deal of work done with chlorpromazine (CPZ), the significance of its biotransformation relative to its therapeutic response is not clear. The pharmacokinetics of CPZ and its metabolites in humans remain largely unexplored, possibly because of the limitations in sensitivity and precision of the analytical methods applied to the study of CPZ metabolism. Only recently (1–5) have the analytical methods been developed that offer desirable sensitivity for quantitating CPZ and its metabolites. However, their adaptation to routine determinations in clinical research is limited because these methods can quantitate only a few of the known metabolites (6).

The metabolites listed in Table I possess primary and secondary amino groups and/or hydroxy groups, all of which are capable of reacting with 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNS, dansyl) to yield fluorescent products. Dansyl has been routinely used as an end-group detector in the study of protein structure; its application for quantitation has, to date, remained limited only to amino acids (7). It has also been reported useful as a reagent for detection of some alkaloids (8).

A preliminary account of the dansylation reaction and separation of the dansylated metabolites of CPZ has already been reported (9). This paper deals with the development and standardization of the reaction between 11 CPZ metabolites and DNS. The method has been adapted to quantitative determinations of nanogram quantities of the metabolites. Addition and recovery experiments on aqueous solutions, urine, and plasma are also included.

EXPERIMENTAL

Materials and Equipment—The following were used: 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl, DNS), purity grade[1]; acetone, benzene, dichloromethane, dimethyl sulfoxide, di-

[1] Fluke-Buchs, Switzerland.

On the contrary, 0.7 mmole/kg. and 1 mmole/kg. of GGE did not increase the amount of cholesterol excreted in bile.

Other compounds such as 1 mmole/kg. of β-(4-hydroxy-2-methoxyphenoxy)lactic acid, 200 mg./kg. of GGE-MN, 300 mg./kg. of GGE, 1 mmole/kg. of nicotinic acid, 0.3 mmole/kg. of meso-inositol hexanicotinate,[3] and the equimolar mixture of GGE and nicotinic acid at 0.7- and 1-mmole/kg. doses increased the amount of cholesterol excreted in bile for some rats but did not alter that for other rats in the same group, compared with that of the control group.

On the other hand, all compounds used showed no effect on the total volume of bile excreted, compared with the control group, at 95% confidence limit.

It is interesting that (4-hydroxy-2-methoxyphenoxy)acetic acid, one of the metabolites of GGE and GGE-MN, has the effect of increasing cholesterol in bile in hypercholesteremia caused by intravenous injection of polyoxyethylene ethers. GGE-MN increased the excretion of cholesterol in bile at 1 mmole/kg., but nicotinic acid, one of the metabolites of GGE-MN, also has similar action at the same dose. Therefore, some question still remains whether the increasing effect of GGE-MN on excretion of cholesterol in bile is due to that of GGE-MN itself and/or to the metabolites of GGE-MN *in vivo*. The data concerned with mesoinositol hexanicotinate and 2,6-pyridine dimethanol bis(*N*-methylcarbamate) were determined for comparison purposes, since these compounds are now widely being used as hypocholesteremic agents.

[3] Hexanicite, Yoshitomi Pharmaceutical Co. Ltd., Japan.

REFERENCES

(1) S. Naito and M. Mizutani, *Chem. Pharm. Bull.*, **17**, 822(1969).

(2) S. Naito, M. Mizutani, K. Kitao, and S. Nishimura, *ibid.*, **17**, 1794(1969).

(3) S. Naito and J. Sakai, *Yakuzaigaku*, **26**, 134(1966).

(4) S. O. Byers, M. Friedman, and T. Sugiyama, *Amer. J. Physiol.*, **204**, 1100(1963).

(5) T. Kobayashi, *Yakugaku Zasshi*, **81**, 195(1961).

(6) B. Zak, *Amer. J. Clin. Pathol.*, **27**, 583(1957).

(7) L. Jendrassik and R. A. Cleghorn, *Biochem. Z.*, **289**, 1(1937).

(8) O. Pelletier and I. A. Campbell, *J. Pharm. Sci.*, **50**, 926(1961).

(9) A. Muller and S. H. Fox, *J. Biol. Chem.*, **167**, 291(1947).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 26, 1970, from the *Kyoto College of Pharmacy, Misasagi Yamashina, Higashiyama-ku, Kyoto, Japan.*

Accepted for publication June 18, 1970.

This constitutes Part XXXIX of a series entitled "Studies on Absorption and Excretion of Drugs" by S. Naito.

The authors thank Dr. Kihachiro Okamoto, Kyoto Pharmaceutical Ind. Ltd., for his gift of GGE-MN and his suggestions.

Chlorpromazine Metabolism I: Quantitative Fluorometric Method for 11 Chlorpromazine Metabolites

PUSHKAR N. KAUL, MICHAEL W. CONWAY*, MERVIN L. CLARK, and JAMES HUFFINE*

Abstract □ Eleven chlorpromazine metabolites have been reacted with dimethylaminonaphthyl sulfonyl chloride to obtain fluorescent products. The reaction has been standardized and adapted to quantitative determination of nanogram amounts of the metabolites. A procedure for application to biological fluids has been developed. Addition and recovery experiments on urine and plasma indicate that the method is applicable to the study of the pharmacokinetic aspects of chlorpromazine metabolites in human subjects.

Keyphrases □ Chlorpromazine—metabolism □ Metabolites, chlorpromazine—quantitative determination □ 5-Dimethylaminonaphthalene-1-sulfonyl Cl–chlorpromazine metabolites reaction—fluorescence □ TLC—separation, identification □ Fluorometry—analysis

Despite a great deal of work done with chlorpromazine (CPZ), the significance of its biotransformation relative to its therapeutic response is not clear. The pharmacokinetics of CPZ and its metabolites in humans remain largely unexplored, possibly because of the limitations in sensitivity and precision of the analytical methods applied to the study of CPZ metabolism. Only recently (1–5) have the analytical methods been developed that offer desirable sensitivity for quantitating CPZ and its metabolites. However, their adaptation to routine determinations in clinical research is limited because these methods can quantitate only a few of the known metabolites (6).

The metabolites listed in Table I possess primary and secondary amino groups and/or hydroxy groups, all of which are capable of reacting with 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNS, dansyl) to yield fluorescent products. Dansyl has been routinely used as an end-group detector in the study of protein structure; its application for quantitation has, to date, remained limited only to amino acids (7). It has also been reported useful as a reagent for detection of some alkaloids (8).

A preliminary account of the dansylation reaction and separation of the dansylated metabolites of CPZ has already been reported (9). This paper deals with the development and standardization of the reaction between 11 CPZ metabolites and DNS. The method has been adapted to quantitative determinations of nanogram quantities of the metabolites. Addition and recovery experiments on aqueous solutions, urine, and plasma are also included.

EXPERIMENTAL

Materials and Equipment—The following were used: 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl, DNS), purity grade[1]; acetone, benzene, dichloromethane, dimethyl sulfoxide, di-

[1] Fluke-Buchs, Switzerland.

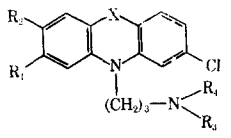


Table I—Various Chlorpromazine Metabolites Reactive with Dansyl[a]

Metabolite Compounds	Abbreviation	R_1	R_2	R_3	R_4	X
7-Hydroxychlorpromazine	7-OH CPZ	H	OH	CH_3	CH_3	S
8-Hydroxychlorpromazine	8-OH CPZ	OH	H	CH_3	CH_3	S
Monodesmethylchlorpromazine	Nor_1 CPZ	H	H	H	CH_3	S
Didesmethylchlorpromazine	Nor_2 CPZ	H	H	H	H	S
7-Hydroxychlorpromazine sulfoxide	7-OH CPZ-SO	H	OH	CH_3	CH_3	S → O
Monodesmethylchlorpromazine sulfoxide	Nor_1 CPZ-SO	H	H	H	CH_3	S → O
Didesmethylchlorpromazine sulfoxide	Nor_2 CPZ-SO	H	H	H	H	S → O
7-Hydroxymonodesmethylchlorpromazine	7-OH Nor_1 CPZ	H	OH	H	CH_3	S
7-Hydroxydidesmethylchlorpromazine	7-OH Nor_2 CPZ	H	OH	H	H	S
7-Hydroxymonodesmethylchlorpromazine sulfoxide	7-OH Nor_1 CPZ-SO	H	OH	H	CH_3	S → O
7-Hydroxydidesmethylchlorpromazine sulfoxide	7-OH Nor_2 CPZ-SO	H	OH	H	H	S → O

[a] The test samples of CPZ metabolites were supplied by the National Institute of Mental Health, Bethesda, Md.

oxane, ethanol, and pyridine, all nanograde; various phosphate and carbonate buffer salts, A.R. grade; Eastman 100-μ polyethylene terephthalate TLC plates coated with silica gel without fluorescent indicator; Eastman TLC chambers; and Vortex shakers. A recording Aminco-Bowman spectrofluorometer was used to record the fluorescence spectra, while a Turner model 110 filter fluorometer, fitted with a high sensitivity door, was used to quantitate the solutions. A Turner model 330 spectrophotometer was used for predetermination of the metabolite concentration range in the biological fluids prior to quantitation *via* the dansylation procedure.

Analytical Method—To 0.003–0.3 μmole of CPZ metabolites in acetone in a small screw-capped graduated glass centrifuge tube were added 0.015 μmole of DNS in acetone and 0.05–5 μmoles of phosphate buffer at pH 12, the total reaction volume being made up with acetone to 1 ml. The tube was capped and incubated at 45° for 2 hr. After cooling to room temperature, 10 μl. of the reaction mixture was spotted on a TLC plate and the chromatogram was developed in the appropriate solvent system (Table II). The individual DNS–metabolite spot was cut out, transferred to a 50-ml. centrifuge tube, and eluted with 10 ml. of ethanol by shaking for 1 min. on a Vortex shaker. The tube was centrifuged at 2000 r.p.m. for 2 min., and the supernatant was decanted for subsequent fluorescence analysis.

Table II—Solvent Systems for TLC Fractionation of DNS–CPZ Metabolite Products and the Corresponding R_f Values

System	Products	R_f Values
Benzene–acetone (1:1)	DNS-8-OH CPZ	0.67
	DNS-7-OH CPZ	0.50
	DNS-7-OH CPZ-SO	0.39
	DNS-Cl	0.95
	DNS-NH_2	0.84
	DNS-OH	0.07
Benzene–acetone (9:1)	DNS-Nor_1 CPZ	0.84
	DNS-Nor_1 CPZ-SO	0.37
	DNS-Nor_2 CPZ	0.74
	DNS-Nor_2 CPZ-SO	0.25
	DNS-Cl	0.89
	DNS-NH_2	0.46
	DNS-OH	0.00
Benzene–acetone (12:1)	DNS-7-OH Nor_1 CPZ	
	N-reacted	0.73
	OH-reacted	0.47
	DNS-7-OH Nor_2 CPZ	
	N-reacted	0.59
	OH-reacted	0.27
	DNS-7-OH Nor_1 CPZ-SO	
	N-reacted	0.18
	OH-reacted	0.02
	DNS-7-OH Nor_2 CPZ-SO	
	N-reacted	0.10
	OH-reacted	0.01
	DNS-Cl	0.92
	DNS-NH_2	0.26
	DNS-OH	0.00

Proof of Dansylation—A brilliantly fluorescent spot appeared on the developed chromatogram, with a R_f value different from that of the DNS and those of the reaction controls. On spraying the chromatogram with the Forrest reagent (10), the DNS–metabolite spot became purple, confirming the presence of a phenothiazine nucleus in the product. Figure 1 shows a typical chromatogram for a few of the DNS–metabolite reaction products.

Characteristics of the Dansylated CPZ Metabolites—Table II includes the solvent systems used to fractionate various groups of the dansylated metabolites and also their corresponding R_f values. The spectrofluorescent characteristics are given in Table III. Figure 2 represents the excitation and fluorescence spectra of a few representative metabolite products.

Optimum Conditions of Reaction—The optimum conditions were determined by studying only a few metabolites representing a primary amine, a secondary amine, and a phenol. The dansylation reactions were carried out under different conditions, varying one parameter at a time.

Effect of Temperature and Time—Nor_2 CPZ was reacted in the presence of phosphate buffer at pH 12 with an excess of DNS in acetone at 0, 25, and 45°. The reaction product formed was assayed at varying time intervals. Figure 3 shows the time course of the reaction at the different temperatures. Most of the reaction occurred within the first 30 min. Two hours at 45° or 6 hr. at room tempera-

Table III—Fluorescence Characteristics of the Dansylated Chlorpromazine Metabolites in Ethanol

CPZ Metabolites	Wavelength Maxima, mμ[a] Excitation	Emission
Nor_1 CPZ	345	520
Nor_1 CPZ-SO	345 (300)[b]	528
Nor_2 CPZ	340	510
Nor_2 CPZ-SO	340 (305)	515
7-OH Nor_1 CPZ		
N-reacted	344	520
OH-reacted	344	516
7-OH Nor_1 CPZ-SO		
N-reacted	344	520
OH-reacted	356 (288, 320)	520
7-OH Nor_2 CPZ		
N-reacted	342	512
OH-reacted	340	510
7-OH Nor_2 CPZ-SO		
N-reacted	346	510
OH-reacted	358 (282, 316)	512
7-OH CPZ	350	530
7-OH CPZ-SO	348 (302)	535
8-OH CPZ	346	530
DNS-Cl	340	520
DNS-OH	320	460
DNS-NH_2	340	500

[a] From uncorrected spectra recorded on Aminco-Bowman spectrofluorometer. [b] Numbers in parentheses represent secondary peaks.

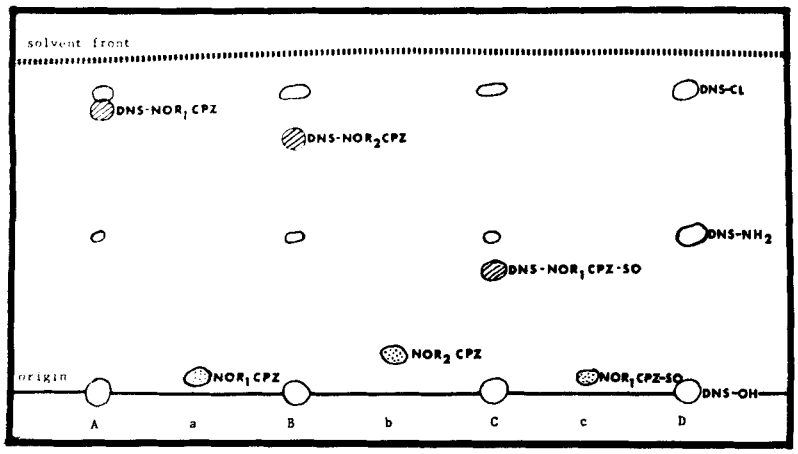


Figure 1—*TLC on a 100-μ Eastman plate developed with benzene-acetone (9,1), showing separation of some of the DNS-reacted CPZ metabolites. Dansylation reaction mixtures of the metabolites were spotted at A, B, and C, whereas at a, b, and c only the metabolites were spotted. D is the DNS-Cl reagent blank without any metabolite. Key: ○, fluorescent, nonreactive to Forrest reagent (F.R.); ◍, fluorescent, becoming purple when sprayed with F.R.; and ⊛, nonfluorescent but purple when sprayed with F.R.*

ture seems to be the practical optimum time–temperature combinations.

Effect of DNS Concentration—Figure 4 shows the formation of the dansylated product of Nor_2 CPZ under varying concentrations of DNS at three different reaction times. It appears that the optimal concentration of DNS on a molar basis is at least 5 times the concentration of the metabolite. In the actual procedure, the amount of DNS used was usually in excess of 6–8 times the molar concentration of the CPZ metabolite(s).

Effect of Hydrogen Ion, Buffer Salt, and Water Concentrations—The compounds Nor_1 CPZ, Nor_2 CPZ, and 7-OH CPZ, selected as representatives of three reactive sites in various groups of CPZ metabolites, were reacted with 6–8 times their concentration of DNS at room temperature for 6 hr. but in the presence of varying hydrogen-ion concentrations. Figure 5 shows the pH-dependent profile of the reaction products. In the optimal reaction procedure, pH 12 was used for the dansylation reaction. Figure 6 represents the influence of the buffer concentration on the dansylation, indicating that at least 0.05 μmole is required for the reaction and that higher concentrations are without any significant effect. In the absence of any buffer, the reaction is extremely slow. Figure 7 shows that the presence of water in the reaction mixture is critical.

Effect of Organic Solvents—Of several organic solvents used, the reaction proceeded only in relatively polar solvents. Of these, acetone seemed to show an optimum effect on the reaction (Fig. 8). Ethanol and methanol, when used as the reaction medium, gave their own dansylated products which interfered in the TLC fractionation. Alcohols were, therefore, avoided in all procedural steps until after the dansylation reaction. Dimethyl sulfoxide also yielded several additional fluorescent spots.

Effects of Atmosphere and Light—Dansylation of Nor_2 CPZ was carried out in nitrogen, oxygen, and air atmospheres, but no effect was observed on the formation of the reaction products. Reactions run in the dark, in ordinary fluorescent room light, and in UV light also did not show any difference in the product concentration.

Effect of Metabolite Concentration—Figure 9 shows the linear correlation of the fluorescence with the metabolite concentration. The linearity holds over a wide range of concentration.

Stability of Reaction Product—DNS–metabolite products appear to be stable with time for at least 24 hr. in solution. The atmosphere and light conditions do not seem to affect the amine-dansylated metabolites on the TLC, but the phenol-dansylated metabolites appear to be somewhat photosensitive.

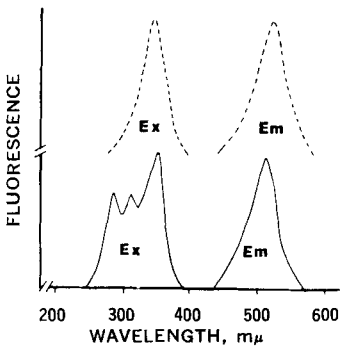


Figure 2—*Excitation (Ex) and emission (Em) curves of DNS–metabolite products. Key: - -, Nor_1 CPZ; and ——, 7-OH Nor_1 CPZ-SO (N-reacted).*

Adaptation to Studies on CPZ Metabolism—To apply the dansylation assay to the CPZ metabolites in the biological fluids of humans, it was necessary to develop an isolation and purification procedure for the metabolites prior to the dansylation reaction. For fractionation purposes, most of the CPZ metabolites may be classified into unconjugated and conjugated groups. The existing procedures (11–13) for isolation of these metabolite groups were extensively studied, modified, and adapted to quantitative determination *via* dansylation.

Procedure for Biological Materials—One-tenth to one milliliter of the biological fluid in a screw-capped centrifuge tube was extracted at pH 10 with 3 ml. of dichloromethane (DCM) for 3 min. and centrifuged. A 2-ml. aliquot of the organic layer was trans-

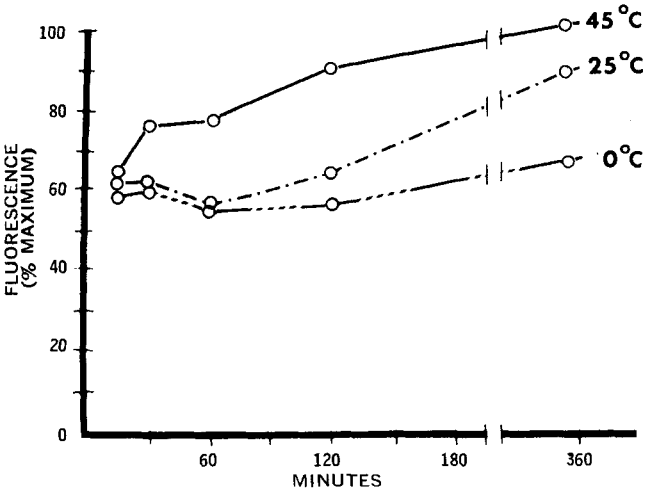


Figure 3—*Time course of the Nor_2 CPZ dansylation at various temperatures.*

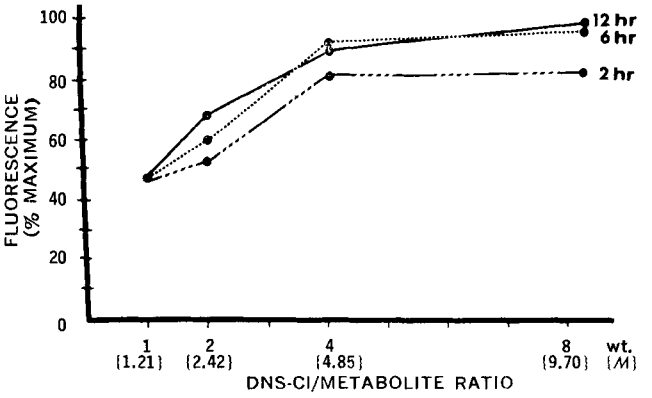


Figure 4—*Influence of DNS concentration on the dansylation of Nor_2 CPZ at varying time intervals.*

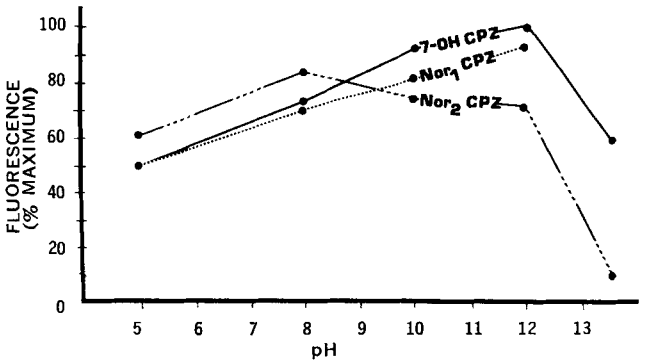


Figure 5—*Effect of pH on the course of dansylation reaction of various CPZ metabolites at room temperature in 6 hr.*

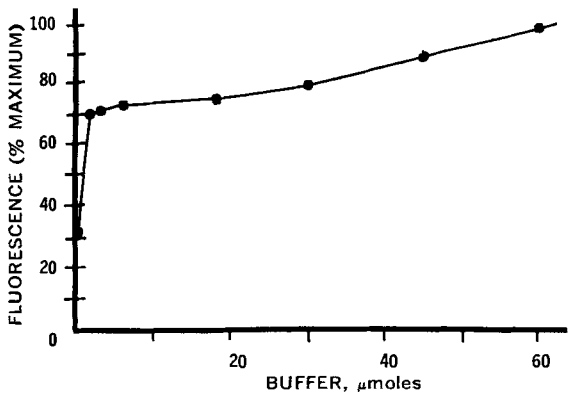


Figure 6—*Influence of pH 12 phosphate buffer concentration on the formation of dansylated Nor₂ CPZ at 1 hr.*

ferred into another centrifuge tube containing 5 ml. of modified Forrest reagent (5% ferric chloride in 50% sulfuric acid). The mixture was shaken for 1 min. and centrifuged. The lower acid layer was read on a spectrophotometer at 528 mμ, and the concentration of unconjugated metabolites extractable at pH 10 was computed from a predetermined standard curve based on the estimations of known CPZ concentrations by the same method. Although this represents only a few of the determinable metabolites, in any individual subject there should be a definite ratio between these and other CPZ metabolites. It should, therefore, serve as an index of the gross concentration of metabolites in the biological material.

After this predetermination, the biological material was diluted to contain 0.2–20 mcg./ml. of the index group metabolites. A 5-ml. sample in a 50-ml. centrifuge tube was adjusted to pH 10 with an excess addition of solid sodium bicarbonate and a dropwise addition of 6 *N* sodium hydroxide. It was extracted twice with 10 ml. of DCM for 3 min. each, 9 ml. of the organic phase being removed each time and combined. The combined organic extracts were evaporated to dryness under reduced pressure at low temperature on a rotary vacuum evaporator. The residue containing unconjugated metabolites was dansylated and assayed as described under *Analytical Method.*

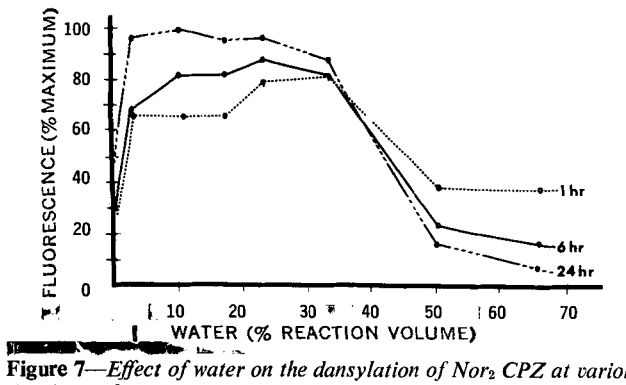


Figure 7—*Effect of water on the dansylation of Nor₂ CPZ at various time intervals.*

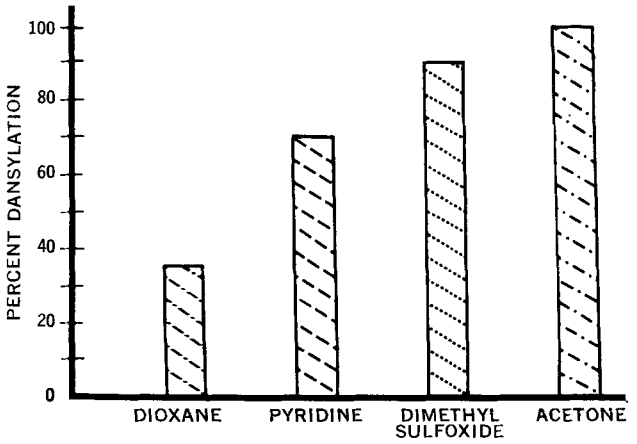


Figure 8—*Effect of solvents present in the reaction mixture at 45° on the dansylation of Nor₂ CPZ in 2 hr.*

The aqueous phase containing the polar conjugated metabolites was quantitatively transferred to a 50-ml. round-bottom flask. Sodium hydroxide solution was added to make the contents 2.5 *N* with respect to the base. The mixture was refluxed for 1 hr., cooled, and neutralized with HCl to pH 9. It was quantitatively transferred to a 50-ml. centrifuge tube and extracted for 2 min. with 20 ml. of DCM. After centrifugation, an 18-ml. aliquot of the organic phase was evaporated to dryness under reduced pressure and low temperature and was subjected to the dansylation assay.

Addition and Recovery—Known amounts of various representative metabolites of CPZ were added to water and to fresh urine and plasma samples. Aliquots of 5 ml. were subjected to fractionation, described under *Procedure for Biological Materials*, and then assayed by the *Analytical Method.* The addition and recovery data are included in Table IV.

Precision, Blanks, and Specificity—Replicate analyses varied less than ±5% in relative fluorescence whereas the precision of the Turner fluorometer was ±3–5%. A general 10% blank was contributed largely by the TLC plates, but this can be avoided by using self-made plates instead of commercial ones. The urine blanks contributed about ±10%, whereas plasma blanks varied from 0–10%. The blanks depend on the position of the DNS–metabolite on the chromatogram, since biological blank controls, particularly of urine, fractionate into several dull spots, some of which may occur at the R_f value of some of the DNS–metabolites. However, the high precision of the method, as revealed by negligible variation between the replicates, makes it feasible to subtract the blanks for concentra-

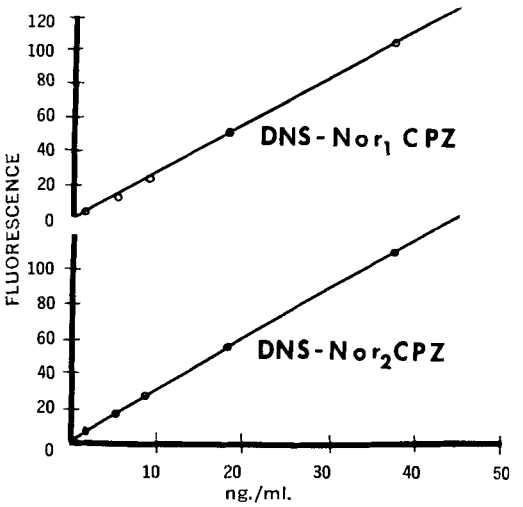


Figure 9—*Plots showing a linear correlation between the concentration of metabolites and fluorescence of their corresponding dansylated products in ethanol.*

Table IV—Addition of Various CPZ Metabolites to and Their Recovery from Biological Fluids

Metabolite	μmoles Added	Percent Recovery[a] Theoretical[b]	Water	Plasma	Urine
Nor_1 CPZ	0.318	100.00	85.81	75.88	61.70
		±1.78	±4.34	±0.71	±1.78
		Cor.[c]	100.00	88.42	71.90
Nor_2 CPZ	0.284	100.00	92.06	78.58	66.66
		±0.91	±7.77	±5.99	±1.52
		Cor.	100.00	84.34	72.41
Nor_2 CPZ-SO	0.277	100.00	78.16	82.39	76.40
		±0.91	±0.70	±0.40	±4.44
		Cor.	100.00	105.40	97.74
7-OH CPZ	0.808	100.00	83.61	77.96	116.94
		±4.26	±1.95	±3.14	±7.02
		Cor.	100.00	93.24	139.96

[a] Average of four samples ± *SE*. [b] Calculated from fluorescence values when processed directly without going through the *Procedure for Biological Materials*. [c] Corrected, basing recoveries from water as 100%

tion computation purposes. The selective extraction processes and chromatographic fractionations make the dansylation method specific to the CPZ metabolites studied.

DISCUSSION

The reaction between dansyl and the primary or secondary amine metabolites of CPZ may be regarded as a nucleophilic substitution of the S_N2 type, wherein the electron-rich amine nitrogen attacks the partially positive sulfur atom of dansyl chloride, the chloride ion departing as the nitrogen-sulfur bond is being formed. A hydrogen attached to the nitrogen may subsequently come off as a proton to form hydrochloric acid.

The dansylation reaction in absence of any buffer proceeds very slowly. This is understandable, since the acid produced during the reaction, if not buffered, will tend to protonate the amine reactant and thus reduce the nucleophile concentration in the reaction mixture. Buffer concentrations above a certain threshold level did not seem to affect the dansylation rate.

Water appears to be necessary for the optimum reaction rate, possibly because it serves as a medium for the buffer to operate. However, its concentration is critical. In quantities greater than 30% of the reaction mixture volume, water tends to hydrolyze the formed dansylated product. Also, excess water would compete with the nucleophilic metabolite for DNS molecules, decreasing the probability for DNS–metabolite formation.

As a solvent for the reaction medium, acetone appears to be the most suitable among those tested. Alcohols, in general, react with dansyl chloride, yielding highly fluorescent products which interfere in the subsequent chromatographic separation of the reaction mixture. Any alcohol should, therefore, be avoided during the dansylation process.

The dansylation assay method shows a high precision in replicates. The various blanks are within the acceptable range. It is encouraging that plasma gave negligible blanks, and the recoveries from it were better than 80%. A linear correlation graph between the concentration of the metabolites and their dansylated fluorescent products extends over a fairly large concentration range. By decreasing the dansylation reaction volume and by increasing the volume spotted on the TLC plates, the sensitivity of the method can be increased 10–50-fold. This should not only make it possible to quantitate minute amounts of the metabolites but also make feasible the detection of yet unknown trace metabolites of CPZ if any exist. The method is currently being applied to studying the pharmacokinetic aspects of CPZ metabolites in schizophrenic patients.

REFERENCES

(1) D. E. Johnson, C. F. Rodriguez, and H. P. Burchfield, *Biochem. Pharmacol.*, **14,** 1453(1965).

(2) I. J. Mellinger and C. E. Keeler, *Anal. Chem.*, **36,** 1840(1964).

(3) S. H. Curry and B. B. Brodie, *Fed. Proc.*, **26,** 761(1967).

(4) S. H. Curry, *Agressologie*, **IX-I,** 115(1968).

(5) D. H. Efron, L. E. Gaudette, and R. S. Harris, *ibid.*, **IX-I,** 103(1968).

(6) S. H. Curry, J. M. Davis, D. S. Janowsky, and J. S. H. Marshall, *Arch. Gen. Psychiat.*, **22,** 209(1970).

(7) W. R. Gray, "Methods in Enzymology," vol. XI, Academic, New York, N. Y., 1967, p. 139.

(8) H. Wachsmuth and R. Denissen, *J. Pharm. Belg.*, **21,** 290 (1966).

(9) P. N. Kaul, M. W. Conway, and M. L. Clark, *Nature*, **226,** 372(1970).

(10) F. M. Forrest and I. S. Forrest, *Amer. J. Psychiat.*, **113,** 931 (1957).

(11) I. S. Forrest, M. B. Weschsler, and J. E. Sperco, *ibid.*, **120,** 44(1963).

(12) D. E. Johnson and C. F. Rodriguez, SwRI Progress Report No. 12, prepared for Psychopharmacology Service Center, NIMH, Southwest Research Institute, San Antonio, Tex., 1965.

(13) D. E. Johnson and C. F. Rodriguez, unpublished data.

ACKNOWLEDGMENTS AND ADDRESSES

Received May 8, 1970, from the *College of Pharmacy and Experimental Therapeutics Unit, University of Oklahoma,* and *Central State Griffin Memorial Hospital, Norman, OK 73069*

Accepted for publication June 22, 1970.

Authors' Note: While this paper was in press, an article entitled "Fluorescent Labeling of Psychoactive Drugs," by I. S. Forrest, S. D. Rose, L. G. Brookes, B. Halpern, V. A. Bacon, and I. A. Silberg appeared in *Agressologie*, 11(2), 127(1970).

* Recipients of the 1970 Lunsford Richardson Award for the Southwestern region on an aspect of this work.

Effect of pH on Monolayers of Cellulose Acetate Phthalate

JOEL L. ZATZ and BEVERLY KNOWLES

Abstract □ At pH values of 2–4, cellulose acetate phthalate monolayers are uncharged and are arranged in a compact, coherent form. An increase in pH brings about partial ionization and marked changes in monolayer organization. The effects of charge repulsion and increased solvation of charged groups cause marked expansion and decreased stability of cellulose acetate phthalate monolayers. The greatest effect of pH on monolayer properties appears to occur in the vicinity of pH 6.

Keyphrases □ Cellulose acetate phthalate monolayers—pH effect □ pH effects—cellulose acetate phthalate monolayers □ Surface pressure—cellulose acetate phthalate monolayers □ Films, cellulose acetate phthalate—compression effect, area

Many properties of interest of polymer coatings depend strongly on the chemical constitution and structural arrangement of the film-forming agent. A better understanding of the molecular properties of polymers would help in interpreting the behavior of pharmaceutical coatings and in predicting the properties of untested formulations. Studies of monolayers of polymers have provided information on polymer structure (1, 2) and interactions (3). The properties of polymer monolayers have been shown to be related to those of free films and enteric coatings (3, 4). Zatz and Knowles (4) examined monolayers of several cellulose esters on subphases of pH 3 and 6.5. The force–area (π–A) isotherms of cellulose acetate butyrate and cellulose acetate stearate were virtually independent of pH. The π–A curves of cellulose acetate phthalate (CAP), on the other hand, were significantly different at the two pH values. In this communication, further experiments on pH effects in CAP monolayers are described.

EXPERIMENTAL

CAP[1] was purified by precipitation from solution in benzene–ethanol (1:1), using *n*-hexane as the nonsolvent. The polymer mass was washed with *n*-hexane and dried *in vacuo* at 30° to constant weight. The material thus purified contained 18% by weight of combined acetyl and 32% by weight of combined phthalyl. It was free of monomers and moisture. Water was deionized and then distilled in an all-glass still. Organic liquids were of reagent grade and were found free of surface-active contaminants (5). Inorganic materials employed were of reagent grade and were not further purified. The trough of the surface balance was made of Teflon and was cleaned using live steam. Surface pressure was determined by the Wilhelmy plate method, using a roughened platinum plate.

The pH of the subphase was adjusted to the desired value, using HCl for low pH values (2–5) and a mixture of K_2HPO_4 and KH_2PO_4 for high pH values (5–8). Sufficient KCl was then dissolved in the subphase to bring the ionic strength to 0.1, except as otherwise indicated. The results at pH 5 were independent of whether the subphase contained HCl or the phosphate buffers, indicating that the buffering agents did not interact with the monolayer. CAP, dissolved in isopropanol–benzene (1:1), was applied dropwise to the surface by means of an Agla micrometer syringe. Essentially the same results were obtained when isopropanol–methylene chloride was employed as the spreading solvent (6). After spreading the polymer, 10 or 15 min. was allowed for equilibration. The rate of compression in all experiments was about 0.02 m.²/mg./min. Compression was continued until surface pressure no longer varied with available surface area. This limiting surface pressure was reproducible within 0.5 dyne and was taken to be the collapse pressure of the monolayer. All studies were carried out at room temperature (25.5 ± 0.7°).

[1] Eastman Organic Chemicals.

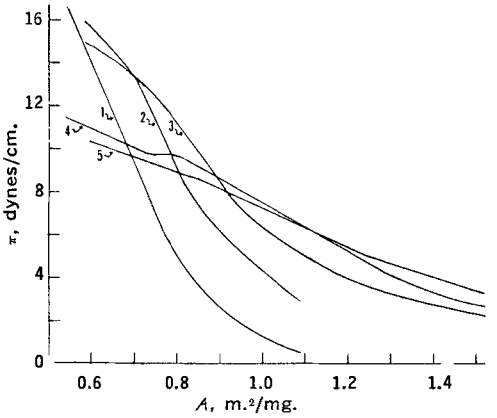


Figure 1—π–A *curves of monolayers of CAP at various subphase pH values: (1) 2, 3, and 4; (2) 5.1; (3) 5.6; (4) 6.1; and (5) 7.0.*

RESULTS AND DISCUSSION

The π–A curves for monolayers of CAP as a function of subphase pH are presented in Fig. 1. At subphase pH values of 2–4, the identical π–A curve is obtained, indicating that the film is essentially uncharged over this pH range. The polymer molecules would be expected to have the pyranose rings lying flat at the surface to allow the contact of subphase water molecules with all hydrophilic groups in each ring (7). However, even at low values of surface pressure, the repeating groups are not horizontally oriented. Based on the chemical composition of the polymer, the average formula weight per repeating group was calculated to be 317. Each milligram of polymer contained, therefore, 1.9×10^{18} repeating groups. From the amount of material spread and the surface area, one may calculate the area occupied by each group. At a surface area of

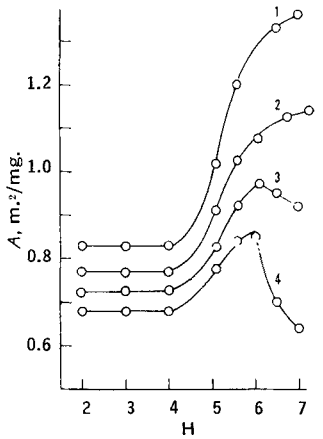


Figure 2—*Surface area of CAP as a function of pH at various values of surface pressure: (1) 4 dynes/cm.; (2) 6 dynes/cm.; (3) 8 dynes/cm.; and (4) 10 dynes/cm.*

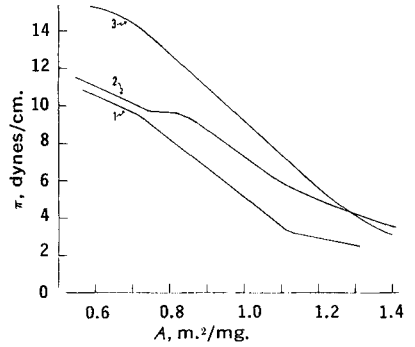


Figure 3 — π – A *curves of monolayers of CAP at various subphase ionic strengths with pH = 6.5: (1) 0.03; (2) 0.1; and (3) 1.0.*

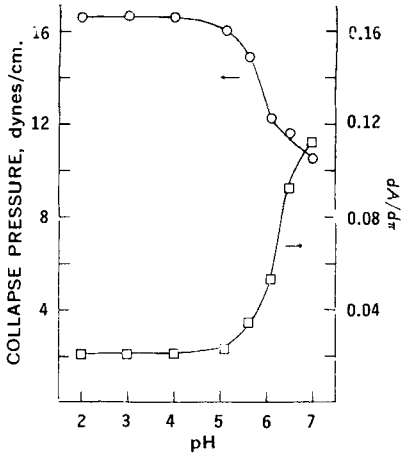


Figure 4—*Monolayer collapse pressure and* dA/dπ *as a function of subphase pH.*

2 dynes/cm., the surface area available to CAP molecules is 0.96 m.²/mg., which is equivalent to 51 Å² per pyranose unit. This is very nearly equal to the area occupied by the unsubstituted, horizontally oriented pyranose ring of cellulose (7) and is clearly too small to allow the more bulky repeating groups of CAP to lie flat at the interface. The rings of CAP are, therefore, tilted from the horizontal. In addition, some ester substituents may be folded over or under the main polymer chain. Examination of Dreiding molecular models shows that, in this way, the effective surface area occupied by each pyranose group may be reduced. These considerations, coupled with the relatively high collapse pressure of CAP compared to monolayers of other cellulose esters (4), suggest that uncharged films of this polymer are rather compact and coherent.

An increase in subphase pH (above 4) causes monolayer properties to become pH dependent. In the expanded region (1) (at surface areas greater than about 0.9 m.²/mg.), surface pressure generally increases with a rise in pH. Collapse pressure decreases over the same pH range. In general, the π–A curves tend to become flatter as pH is increased, so that the monolayers appear to be more expanded.

Above a subphase pH of 4, the monolayers are negatively charged due to ionization of the carboxyl group of the phthalate moiety. Repulsion of like charges would be expected to cause an increase in surface pressure according to the relation:

$$\pi = \pi_0 + \pi_r \qquad \text{(Eq. 1)}$$

where π is the observed surface pressure, π_0 is the surface pressure of an unionized film, and π_r is the surface pressure component resulting from charge repulsion (8). This theory predicts that, at a given surface pressure, a charged film will always occupy a larger area than an uncharged one. Figure 2 shows the effect of subphase pH on the surface area available to CAP molecules at various values of surface pressure. Up to a surface pressure of about 6 dynes/cm., the expected relation appears to hold (Curves 1 and 2, Fig. 2). At higher surface pressures (Curves 3 and 4, Fig. 2), it does not, indicating that charge repulsion is not the only factor involved.

Changes in the adhesion of the monolayer to the subphase must also be considered (2, 9). Charged groups are strongly attracted to the underlying water molecules as a result of ion–dipole forces. Thus, above a pH of 4, as a result of both solvation of charged groups and repulsion of like charges in the film, the polymer structure is rearranged from the compact, coherent form assumed in the unionized state to a more open, extended conformation. The degree of expansion is dependent upon the degree of ionization and, therefore, upon pH. Compression of the charged monolayers causes a fairly rapid increase in surface pressure at large areas because of the contribution of π_r. As the area available to polymer molecules is reduced, solvation of charges may cause the molecules to assume an "accordion" conformation in which ionized groups are immersed in the subphase as completely as possible. The increase in adhesion reduces film stability and causes the charged films to collapse at lower surface pressures.

Support for this mechanism comes from experiments in which the ionic strength of the subphase was varied. The π–A isotherm of uncharged monolayers of CAP (subphase pH = 3) was independent of ionic strength when the latter was varied over a range of 0.001–1.0. On the other hand, changes in salt concentration had a decided influence on the properties of monolayers of CAP at pH values above 4. The π–A results at a pH of 6.5, under conditions of varying ionic strength, are presented in Fig. 3. As the salt concentration is raised, the thickness of the electrical double layer around charges on the monolayer is reduced, thus weakening the effects of charge repulsion. Salting out effects are probably also involved, so that solvation and attraction of the polymer for the subphase are reduced. Therefore, increasing ionic strength should cause the charged CAP monolayer to become more condensed and to collapse at a higher surface pressure. These tendencies are observed in Fig. 4.

Inspection of Fig. 1 reveals that changes in monolayer properties are not a smooth function of pH. Instead, there appears to be a fairly narrow range of pH over which substantial changes in collapse pressure and slope occur. Figure 4 shows that the sharpest decrease in collapse pressure takes place at a pH of about 5.8. In the same figure, the reciprocal of the slope of the condensed portion of each curve, $dA/d\pi$, is plotted *versus* pH; $dA/d\pi$ is related to the compressibility of the film, which is a measure of its compressional elasticity. The sharpest increase in $dA/d\pi$ occurs at a pH of about 6.2 (Fig. 4). Plots of the area occupied in CAP molecules as a function of pH, at surface pressures at which the monolayers are condensed (Curves 3 and 4, Fig. 2), exhibit breaks around pH 6.0. The greatest effect of pH on monolayers of CAP appears, therefore, to occur in the vicinity of pH 6.

The results of this investigation suggest that complete dissolution of coating material may not be necessary for a drug to be released from a dosage form coated with CAP. In the highly acid environment of the stomach, CAP is unionized and probably quite coherent. In the duodenum, where the pH is approximately 6, ionization of accessible phthalate groups will occur, probably causing expansion and allowing penetration of water and ions. Although the coating may not be completely dissolved, sufficient penetration of water molecules through the coating may bring about disintegration or leaching.

REFERENCES

(1) J. L. Zatz, N. D. Weiner, and M. Gibaldi, *J. Pharm. Sci.*, **57,** 1440(1968).

(2) J. L. Zatz, N. D. Weiner, and M. Gibaldi, *J. Colloid Interface Sci.*, **33,** 1(1970).

(3) J. L. Zatz, N. D. Weiner, and M. Gibaldi, *J. Pharm. Sci.*, **58,** 1493(1969).

(4) J. L. Zatz and B. Knowles, *ibid.*, **59,** 1188(1970).

(5) J. L. Zatz, *ibid.*, **59,** 117(1970).

(6) J. L. Zatz, *J. Colloid Interface Sci.*, **33,** 465(1970).

(7) N. K. Adam, *Trans. Faraday Soc.*, **29,** 90(1933).

(8) J. T. Davies and E. K. Rideal, "Interfacial Phenomena," 2nd ed., Academic, New York, N. Y., 1963, p. 231.

(9) I. R. Miller, *J. Colloid Sci.*, **9,** 579(1954).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 13, 1970, from the *College of Pharmacy, Rutgers University, Newark, NJ 07104*

Accepted for publication June 17, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington D. C. meeting, April 1970.

Benzyloxyamines as Possible Inhibitors of Histamine Biosynthesis

GLENN H. HAMOR, DAVID M. BRESLOW*, and GERALD W. FISCH†

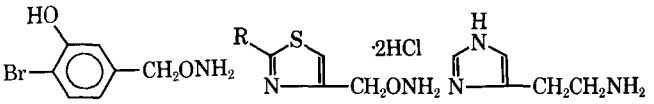
Abstract □ The synthesis of some 26 *O*-substituted hydroxylamine derivatives is described. Potent anti-inflammatory activity in the carrageenin-induced rat paw edema test is shown by *m*-nitrobenzyloxyamine hydrochloride and by *p*-nitrobenzyloxyamine hydrochloride. Moreover, *p*-nitrobenzyloxyamine hydrochloride also possesses potent inhibitory activity when tested *in vitro* against the histamine-forming enzyme, specific histidine decarboxylase, thus lending additional support to the theory linking histamine to the inflammatory process. Further evidence is given for the bioisosterism of the nitrobenzene and pyridine moieties. The necessity of a free aminooxy group for anti-inflammatory activity is demonstrated.

Keyphrases □ Benzyloxyamines—synthesis □ Anti-inflammatory activity—benzyloxyamines □ Anorexigenic activity—benzyloxyamines □ Antimalarial activity—benzyloxyamines □ Histidine decarboxylase inhibition—*p*-nitrobenzyloxyamine HCl □ IR spectrophotometry—identification

Histamine, a naturally occurring amine possessing a wide range of potent pharmacologic effects, has been suggested as playing an important part in the mediation of various physiologic functions (1). Histamine has been implicated in the cause of such human diseases as asthma and other allergic disorders, peptic ulcers, and vascular headaches (2). Histamine also has been proposed as a mediator of the inflammatory process (3–5).

Therefore, a drug that effectively inhibits the biosynthesis of histamine in man should be of interest, both as a tool for physiologic investigations and as a potential therapeutic agent.

Histamine is synthesized in mammalian tissues by enzymatic decarboxylation of the precursor amino acid, histidine. Decarboxylation, being the only enzymic process involved, is rate limiting. In the rat, at least, biosynthesis of histamine is catalyzed by a specific histidine decarboxylase enzyme (6). Therefore, inhibition of histidine decarboxylase activity may result in a depletion of histamine from tissues. Levine and other investigators have reported that the histidine decarboxylase inhibitor, 4-bromo-3-hydroxybenzyloxyamine (I), inhibits histamine synthesis in rats (6) and in man (7). Previous work has shown 4-thiazolylmethoxyamine (II) and 2-amino-4-thiazolylmethoxyamine (III)



I II, R = H III, R = NH_2 IV histamine

to be potent inhibitors, both *in vitro* and *in vivo*, of histidine decarboxylase (8). In addition, these two thiazole compounds possess preliminary anti-inflammatory activity when tested intragastrically against carrageenin-induced rat paw edema (9). Whitehouse and Skidmore

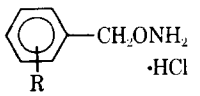


Table I—Benzyloxyamines

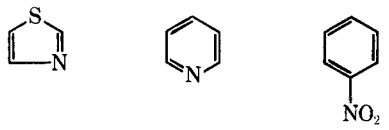
Number	R	Formula	M.p.	Recrystallizing Solvent	Yield, %	Anal., % Calcd.	Found
1	*m*-NO_2	$C_7H_9ClN_2O_3$	164–166°	1-Butanol	80	C, 41.09 H, 4.43 N, 13.49	C, 41.28 H, 4.66 N, 13.08
2	*p*-NO_2	$C_7H_9ClN_2O_3$	209–211°[a]	Methanol	80	C, 41.09 H, 4.43 N, 13.49	C, 41.02 H, 4.55 N, 13.13
3	*m*-Cl	$C_7H_9Cl_2NO$	210–211°	Ethanol	93	C, 43.35 H, 4.68 N, 7.22	C, 43.47 H, 4.64 N, 7.03
4	*p*-Cl	$C_7H_9Cl_2NO$	243–244°[b]	Ethanol	75	—	—
5	*m*-CF_3	$C_8H_9ClF_3NO$	160–170°[c]	Ethyl acetate	50	C, 42.21 H, 3.99 N, 6.15	C, 42.05 H, 4.17 N, 5.98
6	*o*-CH_2ONH_2 ·HCl	$C_8H_{14}Cl_2N_2O_2$	222–224° dec.	Chlorobenzene–methanol	90	C, 39.87 H, 5.81 N, 11.63	C, 40.78 H, 6.14 N, 11.22
7	*m*-CH_2ONH_2 ·HCl	$C_8H_{14}Cl_2N_2O_2$	235–237° dec.	Pyridine	90	C, 39.87 H, 5.81 N, 11.63	C, 40.16 H, 5.91 N, 11.41
8	*p*-CH_2ONH_2 ·HCl	$C_8H_{14}Cl_2N_2O_2$	247–249°[d] dec.	Pyridine	90	C, 39.87 H, 5.81 N, 11.63	C, 41.03 H, 6.09 N, 11.48
9	2-OCH_3, 5-NO_2	$C_8H_{11}ClN_2O_4$	188–190°	Ethanol	77	C, 40.90 H, 4.74 N, 11.95	C, 41.04 H, 4.66 N, 11.69

[a] Reported m.p. 217° dec. (17). [b] Reported m.p. 245° dec. (15). [c] Sublimes. [d] Reported m.p. 263–264° dec. (15).

(10) have shown that a number of anti-inflammatory drugs, such as salicylate, indomethacin, and flufenamic acid, are potent inhibitors of histamine formation through their inhibition of the histidine decarboxylase activity of rat pyloric stomach and fetal rat.

The aims of the present work are the synthesis and structure–activity correlation of various alkoxyamines as histidine decarboxylase inhibitors in relationship to histaminergic mechanisms in inflammation.

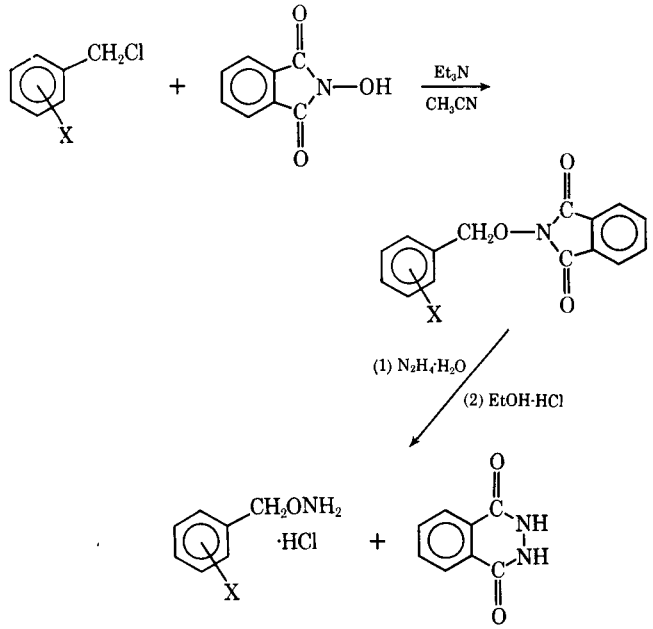
4-Thiazolylmethoxyamine (II) structurally resembles histamine (IV); the methoxyamine side chain is isosteric with the ethylamine group (11) of histamine, while the thiazole ring is isosteric with the imidazole ring. The thiazole ring (V) is also isosteric with the pyridine moiety (VI) as a result of replacement of the sulfur atom, —S—, by the vinylene grouping —CH=CH— (12). However, pyridine, while showing similari-



V VI VII

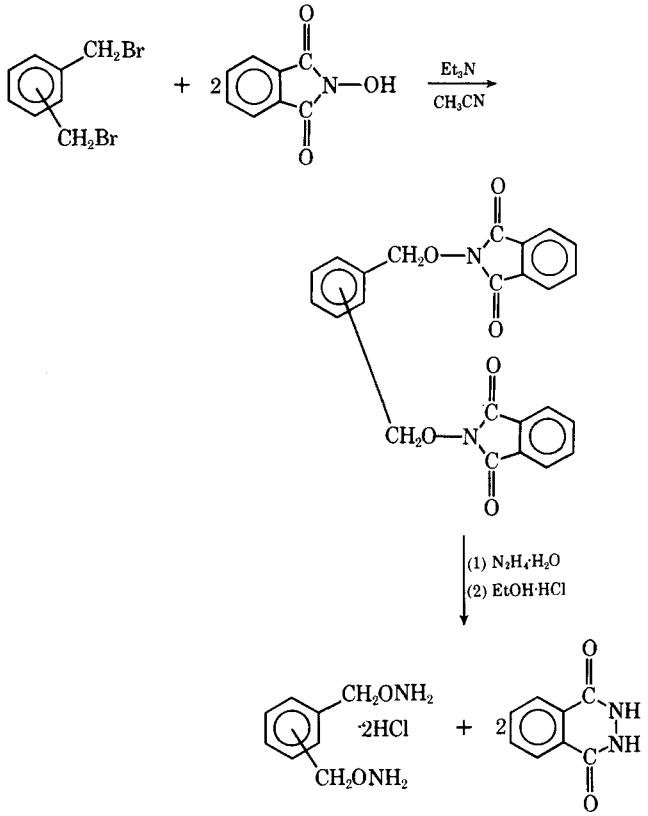
ties to thiazole and benzene (replacement of —CH= by —N=), may also resemble nitrobenzene (VII), but now by virtue of similar polar characteristics (13).

Therefore, the nitrobenzyloxyamines described herein are isosteric with both pyridylmethoxyamines and thiazolylmethoxyamine. This work, as well as reporting the synthesis of the benzyloxyamines containing a nitro substituent, describes the preparation of a number of additional benzene derivatives containing different functional group substituents. These substituted benzyloxyamines may provide data toward a correlation of chemical structure and anti-inflammatory activity, along with any possible relationships to histidine decarboxylase inhibitory potency.



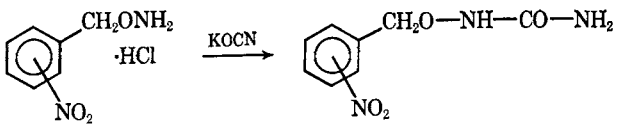
X = Cl, NO_2, CF_3, *etc.*

Scheme I



Scheme II

The nine benzyloxyamines synthesized are listed and their physical properties given in Table I. These benzyloxyamines were prepared by the method of McKay *et al.* (14) and Martin *et al.* (15), as modified by Drain *et al.* (16); it consists of the alkylation of *N*-hydroxyphthalimide, followed by hydrazinolysis as shown in Schemes I and II. The 13 *N*-benzyloxyphthalimide intermediate compounds thus synthesized are listed in Table II. *N*-(3-Pyridylmethoxy)phthalimide and *N*-(2-pyridylmethoxy)phthalimide are shown in Table III. Table IV lists 1-(*m*-nitrobenzyloxy)urea and 1-(*p*-nitrobenzyloxy)urea, synthesized by reacting the appropriate nitrobenzyloxyamine hydrochloride with potassium cyanate (Scheme III) according to the method



Scheme III

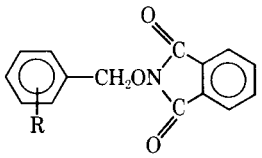
used by Bauer and Dalalian (18) for the preparation of aryloxyureas (19).

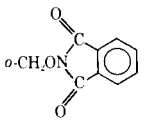
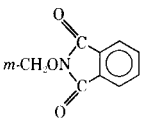
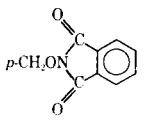
BIOLOGICAL RESULTS

Anti-Inflammatory Screening—Selected compounds have been submitted for pharmacological testing; the preliminary results[1] are shown in Table V. Two of the compounds (Compounds 1 and 2 in Table V) have displayed marked anti-inflammatory activity when

[1] The authors thank Riker Laboratories, Northridge, CA 91324, for performing the pharmacological testing.

Table II—*N*-Benzyloxyphthalimides



Number	R	Formula	M.p.	Recrystallizing Solvent	Yield, %	Anal., % Calcd.	Found
10	*o*-NO_2	$C_{15}H_{10}N_2O_5$	160–161°	Acetone–water	90	C, 60.41 H, 3.38 N, 9.39	C, 60.50 H, 3.50 N, 9.13
11	*m*-NO_2	$C_{15}H_{10}N_2O_5$	186–187°	Acetone–water	75	C, 60.41 H, 3.38 N, 9.39	C, 60.35 H, 3.53 N, 9.67
12	*p*-NO_2	$C_{15}H_{10}N_2O_5$	197–198°	Ethyl acetate	90	C, 60.41 H, 3.38 N, 9.39	C, 60.53 H, 3.47 N, 8.95
13	*m*-Cl	$C_{15}H_{10}ClNO_3$	139–140°	Ethanol	85	C, 62.80 H, 3.53 N, 4.88	C, 62.84 H, 3.69 N, 4.92
14	*p*-Cl	$C_{15}H_{10}ClNO_3$	137–138°[a]	Ethanol	72	—	—
15	*m*-F	$C_{15}H_{10}FNO_3$	130–131°	Ethanol	81	C, 66.50 H, 3.69 N, 5.17	C, 66.58 H, 3.93 N, 5.15
16	*m*-CF_3	$C_{16}H_{10}F_3NO_3$	109–110°	Ethanol	60	C, 59.82 H, 3.14 N, 4.36	C, 59.77 H, 3.26 N, 4.32
17	2-OCH_3, 5-NO_2	$C_{16}H_{12}N_2O_6$	184–185°	Acetone–water	83	C, 58.60 H, 3.99 N, 8.54	C, 58.34 H, 3.79 N, 8.27
18	2-OH, 5-NO_2	$C_{15}H_{10}N_2O_6$	216–218°	1-Butanol	65	C, 57.33 H, 3.21 N, 8.92	C, 58.10 H, 3.57 N, 8.83
19	2-Cl, 4,5-Methylenedioxy	$C_{16}H_{10}ClNO_5$	242–243°	Acetone–water	90	C, 58.10 H, 3.06 N, 4.23	C, 58.03 H, 3.15 N, 4.06
20		$C_{24}H_{16}N_2O_6$	284–285°	Pyridine	80	C, 67.28 H, 3.76 N, 6.54	C, 67.43 H, 3.98 N, 6.38
21		$C_{24}H_{16}N_2O_6$	238–240°	Chlorobenzene	80	C, 67.28 H, 3.76 N, 6.54	C, 67.31 H, 3.94 N, 6.43
22		$C_{24}H_{16}N_2O_6$	306–307° dec.	Pyridine	75	C, 67.28 H, 3.76 N, 6.54	C, 67.19 H, 3.92 N, 6.70

[a] Reported m.p. 137–138° (14).

tested intragastrically against carrageenin-induced rat paw edema (20). The ED_{50} of 230 mg./kg. for *m*-nitrobenzyloxyamine hydrochloride (Compound 1) may be compared with an ED_{50} of approximately 180 for aspirin (9). Furthermore, preliminary testing has shown that *p*-nitrobenzyloxyamine (Compound 2), as well as showing anti-inflammatory activity, possesses potent inhibitor activity of a competitive nature when tested against the histamine-forming enzyme, specific histidine decarboxylase, isolated from fetal rat (21).

The differences in toxicity of the disubstituted aminooxymethyl compounds as related to positions of the two groups on the benzene ring should be noted. The *o*-disubstituted compound (Compound 6) is almost three times as toxic (LD_{50}, mg./kg. i.p. mice) as the *meta*-compound and over four times as toxic as the *p*-di(aminooxymethyl)benzene. The anti-inflammatory activity somewhat parallels the LD_{50}; that is, the *ortho*-compound is the most active, followed by the *meta*- and then the *para*-disubstituted compounds.

Anorexigenic Screening—Several compounds have shown anorexigenic activity in reducing the food intake in rats (Table V) (9). The *o*-di(aminooxymethyl)benzene (Compound 6) is the most active, with an ED_{50} of 64 mg./kg.

Anticancer Screening—The 10 compounds shown in Table V have been submitted to the Cancer Chemotherapy National Service Center (NSC), National Cancer Institute, for screening against the L-1210 lymphoid leukemia test system (22), and they have the following assigned NSC numbers: Compound 1, Table V, NSC 121170; No. 2, NSC 79411; No. 3, NSC 131325; No. 5, NSC 129221; No. No. 6, 123485; No. 7, NSC 123486; No. 8, NSC 123487; No. 9, NSC 132330; No. 11, NSC 130679; and No. 25, NSC 125363. Preliminary results show these compounds lack any significant antitumor activity. However, *m*-nitrobenzyloxyamine hydrochloride (Compound 1) did prolong the life of L-1210-infected mice 124% (compared with controls at 100%). In general, an increase in survival value of 125% is necessary for further experimental work by NSC.

Antimalarial Screening—The 10 compounds shown in Table V have been submitted to Walter Reed Army Institute of Research for testing for antimalarial activity. Preliminary results indicate a lack of potency, with the exception of *m*-nitrobenzyloxyurea (Compound 25) which is active against the parasite (*Plasmodium gallinaceum*) in the mosquito (*Aedes aegypti*), giving 100% abnormal oocysts and 100% complete sporozoite suppression at 0.1% con-

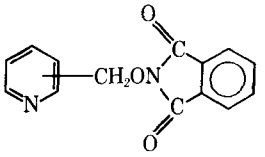


Table III—*N*-Pyridylmethoxyphthalimides

Number	Name	Formula	M.p.	Recrystallizing Solvent	Yield, %	Anal., % Calcd.	Found
23	*N*-(3-Pyridylmethoxy)-phthalimide	$C_{14}H_{10}N_2O_3$	153–154°	Acetone–water	71	C, 66.14 H, 3.96 N, 11.02	C, 66.43 H, 4.19 N, 10.95
24	*N*-(2-Pyridylmethoxy)-phthalimide	$C_{14}H_{10}N_2O_3$	129–130°	Acetone–water	50	C, 66.14 H, 3.96 N, 11.02	C, 65.97 H, 4.06 N, 9.94

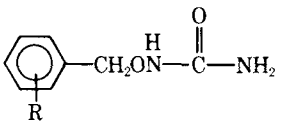


Table IV—Benzyloxyureas

Number	R	Formula	M.p.	Recrystallizing Solvent	Yield, %	Anal., % Calcd.	Found
25	*m*-NO_2	$C_8H_9N_3O_4$	130–131°	Acetone	78	C, 45.50 H, 4.30 N, 19.90	C, 45.59 H, 4.54 N, 20.08
26	*p*-NO_2	$C_8H_9N_3O_4$	209–210°[a]	Acetone–water	89	C, 45.50 H, 4.30 N, 19.90	C, 45.67 H, 4.44 N, 19.56

[a] Reported m.p. 206° (17).

centration (but inactive at 0.01% concentration). However, *m*-nitrobenzyloxyurea was inactive against malaria in chicks (*P. gallinaceum*) and in mice (*P. berghei*).

DISCUSSION

The lack of, or greatly reduced, anti-inflammatory activity of the *m*-chlorobenzyloxyamine hydrochloride (Compound 3) and the other nonnitro-substituted benzyloxyamines gives further evidence for the bioisosterism of the nitrobenzene and pyridine (and thiazole) ring systems. In addition, the lessened activity of *m*-trifluoromethylbenzyloxyamine hydrochloride (Compound 5) as compared to *m*-nitrobenzyloxyamine hydrochloride (Compound 1) is in contrast to reports of the biological equivalence of replacement of NO_2 with CF_3 (23, 24).

The necessity of a free aminooxy group for anti-inflammatory activity may be demonstrated by the essential inactivity of *N*-(*m*-nitrobenzyloxy)phthalimide (Compound 11, Table V). In addition, Schiff base derivatives of *m*-nitrobenzyloxyamine are devoid of anti-inflammatory activity (25). Also, whereas 4-thiazolylmethoxyamine dihydrochloride (II) possesses activity *versus* carrageenin-induced rat paw edema, several nitrogen-substituted Schiff base derivatives are inactive (9). Furthermore, 4-thiazolylmethoxyurea and 1-phenyl-3-(4-thiazolylmethoxy)urea show no *in vitro* inhibition of histidine decarboxylase (8, 19, 26). However, *m*-nitrobenzyloxyurea (Compound 25) and 4-thiazolylmethoxyurea (19) displayed moderate anti-inflammatory activity.

These preliminary results showing histidine decarboxylase inhibitory activity for *p*-nitrobenzyloxyamine hydrochloride (Compound 2) and marked anti-inflammatory activity for *m*-nitrobenzyloxyamine hydrochloride (Compound 1) and for the *para*-isomer lend additional support to the theory linking histamine to the inflammatory process.

Further work will be directed toward synthesis of additional alkoxy amines as possible active site-directed irreversible inhibitors according to the hypothesis of Baker (27, 28). These new inhibitors may be highly specific for the histidine decarboxylase involved in the inflammatory process by virtue of the rigid positioning requirements imposed by: (*a*) the necessity of a reversible fit within the enzyme activity site (formation of an aldimine bond between the aminooxy group and pyridoxal phosphate displacing the lysyl epsilon-amino group of the enzyme); (*b*) fitting an adjacent hydrophobic area; and (*c*) exoalkylation outside the active site.

As additional data are accumulated, it may be possible to apply regression analysis (29) to clarify the exact hydrophobic, steric, and electronic requirements to accommodate the enzyme active site. Successful elucidation of the enzyme active site may enable the design and synthesis of extremely potent, and highly specific, histidine decarboxylase inhibitors which, hopefully, may find application in the treatment of inflammatory diseases.

EXPERIMENTAL

The syntheses of representative compounds reported in Tables I–IV are described here. All melting points were taken on a Fisher-Johns hot-stage and are uncorrected. Elemental microanalyses were performed by Elek Microanalytical Laboratories, Torrance, Calif. The IR spectra of selected compounds were determined on a Perkin-Elmer infracord apparatus in mineral oil mulls and are in agreement with the assigned structures.

***N*-(*m*-Nitrobenzyloxy)phthalimide (Compound 11)**—In a 500-ml. round-bottom flask, fitted with a reflux condenser and heating mantle, were placed 75 ml. of acetonitrile, 10.5 ml. (0.075 mole) of triethylamine, and 12.4 g. (0.075 mole) of *N*-hydroxyphthalimide.[2] After heating to effect solution, 13.0 g. (0.075 mole) of α-chloro-*m*-nitrotoluene[3] was added, and the solution was refluxed for 2 hr. Upon cooling, filtering the resultant solid, adding excess water to the filtrate to obtain a second crop, and recrystallizing the total product from acetone–water, 16.4 g. (75% yield) of a white crystalline solid, m.p. 186–187°, was obtained.

***m*-Nitrobenzyloxyamine Hydrochloride (Compound 1)**[4]—To a solution of 29.8 g. (0.1 mole) *N*-(*m*-nitrobenzyloxy)phthalimide (Compound 11) in 250 ml. warm anhydrous ethanol was added 5 ml. (0.1 mole) hydrazine hydrate (99%), and the solution was refluxed for 2 hr. The reaction mixture was cooled and the precipitated phthalhydrazide removed by filtration. Addition of excess ethanolic HCl to the filtrate, followed by reduction in volume by evaporation,

[2] Aldrich Chemical Co.
[3] Eastman Organic Chemicals.
[4] *Chemical Abstracts* nomenclature; *O*-(*m*-nitrobenzyl)hydroxylamine hydrochloride.

Table V—Pharmacology[a]

Compound No.	LD_{50}, mg./kg. i.p., mice	Carrageenin-Induced Rat Paw Edema, % Reduction, 250 mg./kg.	Anorexigenic, % Inhibition, mg./kg., Rat
1	>800	64	48, 150 mg./kg.
2	>800	50	50, 130 mg./kg.
3	275	(15) increase 176 mg./kg.	22, 75 mg./kg.
5	588	23	9, 100 mg./kg.
6	236	35	50, 64 mg./kg.
7	659	16	24, 100 mg./kg.
8	>800	13	36, 100 mg./kg.
9	—	(4) increase	—
11	>800	13	43, 100 mg./kg.
25	>800	43	34, 100 mg./kg.

[a] The pharmacological testing was performed by Riker Laboratories, Northridge, Calif.

gave 16 g. (80%) white solid, m.p. 160–165°. An analytical sample, m.p. 164–166°, was obtained by three recrystallizations from 1-butanol.

***O,O'*-(*o*-Phenylenedimethylene)bishydroxylamine Dihydrochloride (Compound 6)**—To 74 g. (0.45 mole) of *N*-hydroxyphthalimide, dissolved in 175 ml. acetonitrile and 50 ml. (0.36 mole) triethylamine by warming, was added 40 g. (0.15 mole) α,α'-dibromo-*o*-xylene,[3] followed by 4 hr. of refluxing. The chilled mixture was filtered and the precipitate washed with cold water to give 64 g. (80%) of the bisphthalimido intermediate (Compound 20), m.p. 282–284°. An analytical sample, m.p. 284–285°, was obtained by two recrystallizations from pyridine.

To 42.8 g. (0.1 mole) of Compound 20 in 300 ml. anhydrous ethanol was added 10 ml. (0.2 mole) hydrazine hydrate (99%), and the mixture was refluxed for 3 hr. The precipitated phthalhydrazide was removed by filtration of the cooled reaction mixture. Addition of excess ethanolic HCl to the filtrate gave 21.6 g. (90% yield) of the desired bisaminooxy compound (Compound 6) as a white solid, m.p. 211–214° dec. An analytical sample, m.p. 222–224° dec., was obtained by four recrystallizations from chlorobenzene–methanol.

***m*-Nitrobenzyloxyurea (Compound 25)**—To 20.5 g. (0.1 mole) *m*-nitrobenzyloxyamine hydrochloride (Compound 1) dissolved in 150 ml. H_2O was added 8.1 g. (0.1 mole) potassium cyanate. The desired urea derivative precipitated out almost immediately and was filtered, washed with cold H_2O, and recrystallized from acetone to give 16.5 g. (78%) of analytically pure, white solid, m.p. 130–131°.

REFERENCES

(1) G. Ungar, *Fed. Proc.*, **24,** 1293(1965).

(2) C. F. Code, M. M. Hurn, and R. G. Mitchell, *Mayo Clin. Proc.*, **39,** 715(1964).

(3) R. W. Schayer, *Ann. N. Y. Acad. Sci.*, **116,** 891(1964).

(4) R. W. Schayer, *Pharmacologist*, **9,** 247(1967).

(5) M. A. Reilly and R. W. Schayer, *Brit. J. Pharmacol.*, **37,** 489(1969).

(6) R. J. Levine, T. L. Sato, and A. Sjoerdsma, *Biochem. Pharmacol.*, **14,** 139(1965).

(7) R. J. Levine, *Science*, **154,** 1017(1966).

(8) D. Aures, G. H. Hamor, W. G. Clark, and S. S. Laws, *Abstr. Int. Congr. Pharmacol., 4th*, **1969**, 179.

(9) D. McClure, personal communication.

(10) M. W. Whitehouse and I. F. Skidmore, *J. Pharm. Pharmacol.*, **17,** 668(1965).

(11) E. L. Schumann, R. V. Heinzelman, M. E. Greig, and W. Veldkamp, *J. Med. Chem.*, **7,** 329(1964).

(12) V. B. Bogert, in "Medicinal Chemistry," A. Burger, Ed., Interscience, New York, N. Y., 1960, p. 80.

(13) *Ibid.*, p. 84.

(14) A. F. McKay, D. L. Garmaise, G. Y. Paris, and S. Gelblum, *Can. J. Chem.*, **38,** 343(1960).

(15) D. G. Martin, E. L. Schumann, W. Veldkamp, and H. Keasling, *J. Med. Chem.*, **8,** 456(1965).

(16) D. J. Drain, J. G. Howes, and H. W. R. Williams, British pat. 984,305, Feb. 24, 1965; through *Chem. Abstr.*, **62,** 14572c(1965).

(17) O. L. Brady and L. Klein, *J. Chem. Soc.*, **1927,** 874.

(18) V. J. Bauer and H. P. Dalalian, *J. Med. Chem.*, **8,** 886(1965).

(19) G. H. Hamor and A. Hudaverdi, *J. Pharm. Sci.*, **59,** 276 (1970).

(20) C. A. Winter, E. A. Risley, and G. W. Nuss, *Proc. Soc. Exp. Biol. Med.*, **111,** 544(1962).

(21) C. Elison and J. Breeding, personal communication.

(22) *Cancer Chemother. Rep.*, No. 25, CCNSC, U. S. Department of Health, Education, and Welfare, Washington, D. C., 1962.

(23) D. E. Welch, R. R. Baron, and B. A. Burton, *J. Med. Chem.*, **12,** 299(1969).

(24) D. E. Welch, H. S. Nakaue, and R. D. Vatne, *ibid.*, **13,** 297(1970).

(25) G. H. Hamor and L. D. Watson, unpublished data.

(26) D. Aures, personal communication.

(27) B. R. Baker, *J. Pharm. Sci.*, **53,** 347(1964).

(28) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967.

(29) C. Hansch and T. Fujita, *J. Amer. Chem. Soc.*, **86,** 1616 (1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *School of Pharmacy, University of Southern California, Los Angeles, CA 90007*

Accepted for publication June 17, 1970.

Supported in part by Grant AM13552 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

* 1969 Lunsford Richardson Pharmacy Awards Regional Winner and National Science Foundation Undergraduate Research Participant, GY-4456.

† National Science Foundation Undergraduate Research Participant, GY-5829.

New Method for Assaying Antiapomorphine Activity in Pigeons

W. K. VAN TYLE and A. M. BURKMAN

Abstract □ An electronic monitoring system, which was capable of estimating and recording the intensity of the apomorphine-induced pecking syndrome, was evaluated with regard to its ability to function as an attendant-free assay instrument. Instrument responses were compared with responses derived from a visual recording technique which has been routinely used since 1960. The new monitoring system provides accurate and highly reliable estimates of the desired biological parameters.

Keyphrases □ Apomorphine-induced pecking syndromes, pigeons—assay method □ Pecking syndrome, pigeons—chlorpromazine HCl, methdilazine HCl inhibition □ Monitoring system, electronic—pecking syndrome, pigeons □ Inhibitor potency assay—apomorphine-induced pecking syndrome

Without doubt, the most familiar pharmacological property of apomorphine is its ability to evoke emesis in a variety of animal species. From pharmacodynamic, therapeutic, and toxicological standpoints, the emetic characteristics of the compound have been the subject of greatest attention through the years. On the other hand, interest in the nonemetic activities of apomorphine, particularly in animals refractory to its emetic propensities, has been sporadic, desultory, and largely uninspired. Some of these nonemetic responses are enumerated by Krueger *et al.* in their monumental "The Pharmacology of the Opium Alkaloids" (1).

Among the nonemetic responses to apomorphine is a curious "Zwangspicken" or compulsive pecking in pigeons, which was described by Amsler in 1923 (2). Interest in Amsler's "Zwangspicken" response in pigeons (for whom sublethal doses of apomorphine are *not* notably emetic) remained virtually nonexistent until 1957 when Burkman *et al.* (3) and Koster (4) described, in somewhat greater detail, this apomorphine-induced effect. The response was referred to by Koster as a "feeding hallucination" and by Burkman *et al.*, less imaginatively, as a "pecking syndrome."

Quantitative data describing the characteristics of the syndrome were published, simultaneously and independently, by Burkman (5) and by Dhawan and Saxena (6). Since then the syndrome has attracted increased attention as an example of drug-induced stereotypical behavior. The pecking syndrome is best characterized by a single symptom: a continuous, forceful, repetitive pecking by the pigeon on the floor, walls, and roof of the cage in which it is placed. Although the syndrome has been quantified in terms of intensity (7) and in terms of an all-or-none response (8), the intensity technique is regarded as both more reliable and more efficient than the quantal method. Therefore, the authors have directed most of their efforts toward improving their ability to monitor response magnitude.

The authors have successfully automated this method of quantifying the pecking syndrome and thereby have made what they believe to be a major technical improvement. Details relating to the construction of the instrument have been published (9).

This article describes and evaluates the automated instrument method for assessing the potency of apomorphine-inhibiting phenothiazines.

EXPERIMENTAL

Animals—Adult birds (1–2 years of age) of both sexes were housed in individual cages at a room temperature of 20–25° and conditioned to a 12-hr. light cycle. Birds retained for assay were those whose responsiveness to apomorphine HCl had been previously evaluated. Those that did not respond to intramuscularly administered doses of 0.5 mg./kg. were rejected as unsuitable. Thus, birds used in these experiments were *not* randomly selected from a general population, but rather were representative of a stock colony of birds having a high sensitivity to apomorphine.

All drugs used in the assays were dissolved in sterile 0.9% saline and injected intramuscularly.

Determination of Syndrome Intensity: Visual Technique—Each pigeon received the requisite dose of apomorphine HCl and was immediately placed in a wire mesh observation cage 22.9 × 38.1 × 22.9 cm. (9 × 15 × 9 in.). At 5–10-min. intervals, an observer made 1-min. counts of the bird's responses. The duration of observation was determined by the length of the period during which the animal exhibited the pecking syndrome. Since the syndrome was easily interrupted by sudden noises that distracted the bird, it was necessary to conduct the experiments in a sound-attenuated environment. The number of animals

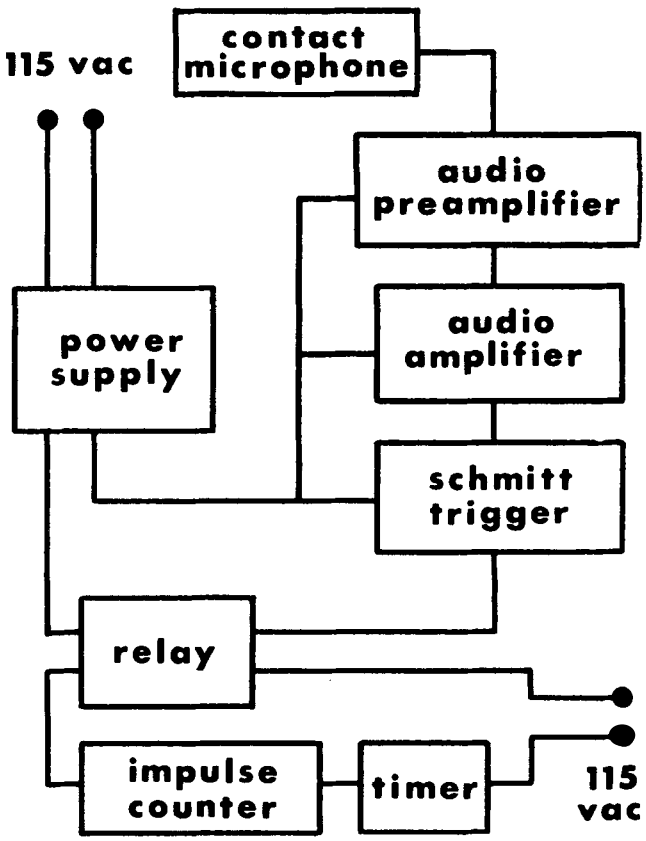


Figure 1—*Block diagram of one channel of the pecking syndrome monitor.*

Table I—Intensity of Apomorphine-Induced Pecking Syndrome as Determined by Two Monitoring Methods

	Method	
	Visual	Instrument
Number of pigeons	32	32
Mean cumulative response[a]	4395 ± 395	4440 ± 407
Coefficient of variation[b]	0.509	0.519

[a] Mean CPR ± standard error. Responses induced by apomorphine HCl, 0.5 mg./kg., i.m. No significant difference could be detected between methods. [b] Ratio of the standard deviation of a CPR sample to the mean CPR of that sample. No significant difference could be detected between methods.

that could be monitored simultaneously depended upon the observer's experience and his ability to make minute counts reliably. Generally, no more than six birds could be effectively monitored by a single person.

Response rate (pecks per minute) plotted against time from injection yielded a curve, whose parameters, suitably computed, provided such information as onset time, duration, peak rate, time-dependent rate changes, and the cumulative pecking response (CPR). The CPR, which has proved to be the most useful expression of response intensity, was determined by measuring the area under the rate–time curve with the aid of a compensating polar planimeter. Using a suitably calibrated instrument, the resulting total area was translated into total pecks, *i.e.*, the CPR.

Determination of Syndrome Intensity: Automated Instrument Technique—The design of the electromechanical CPR monitor was based upon the results of preliminary efforts to adapt an audio-pickup system for continuously recording the sharp, microphonically distinct clicks of the bird's beak as it struck the metal cage (9). The sounds that accompanied the compulsive pecking behavior provided a quantifiable variable which readily lent itself to machine processing.

To monitor apomorphine-induced pecking effectively, each channel of the system required: (*a*) a sensitive detector to pick up and transmit signals generated by sounds or vibrations developed as a result of the bird's pecking movements, and (*b*) a means for amplifying the detector signal so that (*c*) an electromechanical relay and impulse counter could be activated by each discrete pulse. Also essential was the incorporation of a device to improve the instrument's capacity to discriminate between pecking activity and fortuitous noises and movements unrelated to pecking.

The monitor design adopted for use is diagrammed in Fig. 1. The use of a simple contact microphone, bolted to the rear panel of a stainless steel observation cage 22.9 × 38.1 × 22.9 cm. (9 × 15 × 9 in.), served as a transducer. Environmental noises, bird vocalizations, and other air-transmitted sounds were incapable of activating the microphone. The unit, however, was sufficiently sensitive to pick up virtually all desired responses. Signals generated by the microphone were transmitted to a solid-state audiopreamplifier and audioamplifier. A Schmitt trigger, incorporated in the system, is a modified bistable multivibrator, which is widely used as a voltage discriminator in such devices as nuclear radiation pulse height analyzers. It served much the same function here. Input signals above a preset level provided an output signal sufficient always to activate the relay which, in turn, operated the impulse counter. This ensured that clean, artifact-free counts could be obtained. A 12- and 30-v. d.c. regulated power supply provided the energy for operation.

Although the monitor was incapable of being influenced by air-transmitted sounds, loud noises and strong visual stimuli easily distracted the animals under observation. This distraction was reflected in a distortion of the pecking response parameters. Thus, it was still necessary to control the environmental conditions under which responses were monitored. This was most easily accomplished by placing the caged pigeons in a sound-attenuating chamber constructed for that purpose. In addition, the monitor itself was placed in an adjoining room where it could be conveniently examined without disturbing the birds. This precaution further decreased the opportunity for the intrusion of environmental distortions.

Paired-Comparison Experiment—Evaluation of the utility of the pecking syndrome monitor was based on results of two experiments designed to determine the accuracy and reliability of the data supplied by the instrument.

The first experiment involved estimating syndrome intensity for a group of pigeons by recording their responses, using both the visual and instrument methods simultaneously. Thirty-two birds of mixed sex were injected intramuscularly with apomorphine HCl, 0.5 mg./kg., and placed in observation cages. The cages were placed, in turn, in the sound-attenuating chamber, and the monitors were activated. Observation ports allowed the experimenter to see the birds and to make the necessary visual counts. The CPR over its entire duration (about 2 hr.) served as the index of syndrome intensity, and this was determined for each bird by both methods. The conditions of the experiment satisfied the requirements of a "paired-comparison" design; evaluation of the visual and instrument methods was made by examining the magnitude of observed within-animal (between-methods) differences.

Inhibitor Potency Assay—The second experiment was a model assay similar to those repeatedly employed in studies of psycholeptic phenothiazines (10, 11). The apomorphine-inhibiting activities of two compounds, chlorpromazine HCl (CPZ) and methdilazine HCl (MDZ), were determined in terms of their ability to suppress the syndrome evoked by a standard dose of apomorphine HCl. The experiment was designed as a 4-point relative potency assay, utilizing 24 pigeons. Four groups of six birds received, initially, intramuscular injections of 0.5 mg./kg. of apomorphine HCl (1.6 μmole of base/kg.) 8 days prior to the start of the assay. The CPR values obtained served as controls against which the inhibiting influence of the phenothiazines was measured. On the assay day, each group of animals was premedicated with a single high or low dose of either CPZ or MDZ. All doses were expressed in terms of moles of base per kilogram body weight. Fifteen minutes after phenothiazine administration, apomorphine HCl, 0.5 mg./kg., was injected and the CPR was redetermined. Inhibition at each dose level was recorded as percent reduction of the control CPR. For each inhibitor, a log dose–probit response line was constructed, and the relative potency of MDZ was assessed using CPZ as the reference standard.

All activity data were collected by simultaneous use of visual and automated instrument techniques so that comparison could be made between methods.

RESULTS

Paired-Comparison Experiment—The characteristics of syndrome intensity are summarized in Table I. Thirty-two birds supplied two samples of CPR values; a comparison of these samples, in terms of sample means and dispersions about sample means, revealed that no significant difference existed. The computed t statistic had an associated $p > 0.6$. The visual and instrument techniques, therefore, provide equivalent estimates of syndrome intensity.

Inhibitor Potency Assay—The results are summarized in Table II. Both methods provided virtually identical estimates of median inhibitory doses (ID_{50}'s), slopes, and relative potency. The parameter estimates were so strikingly similar that a reliable conclusion could be drawn without recourse to further statistical manipulation and testing. No significant differences could be detected between the activity parameters derived from visual and instrument methods.

DISCUSSION

Although the visual technique for quantifying pecking syndrome intensity employed in earlier studies has proved to be highly reliable, its use requires an experienced observer, one who has been trained to make selective, unbiased, and reproducible counts. Even under the best conditions, an observer can usually program the simultaneous visual monitoring of no more than six birds. Under standardized conditions of the phenothiazine assays, the period of observation lasts approximately 2 hr., and the preliminary animal weighings and premedication increase the period to about 2.5 hr. Thus, the number of man-hours required to satisfy the needs of even a comparatively simple 4- or 6-point crossover relative potency assay becomes enormous, and the assay technique has been relatively inefficient in terms of the utilization of an investigator's time.

This kind of inefficiency now seems unnecessary, since the syndrome can be readily quantified by an instrument method which does not require the full and constant attention of the investigator during the recording session. Furthermore, use of the instrument that automatically accumulates and records compulsive pecking

Table II—Suppression of Apomorphine-Induced Pecking Syndrome by Chlorpromazine (CPZ) and Methdilazine (MDZ) Assayed by Two Methods

Statistic	Method	
	Visual	Instrument
ID_{50} (CPZ)[a]	2.05 (1.10–3.80)	1.65 (0.90–2.95)
ID_{50} (MDZ)	29.8 (24.0–37.0)	27.1 (20.9–34.6)
Slope (CPZ)[b]	84.9 ± 67.5	92.9 ± 29.1
Slope (MDZ)	71.6 ± 47.4	82.6 ± 30.7
Common slope (CPZ) + (MDZ)	90.11	78.43
Relative potency[c]	0.072 (0.046–0.100)	0.063 (0.043–0.96)

[a] Median inhibiting dose (95% confidence limits); in unit of micromoles of base per kilogram body weight. [b] Slope of the dose–response line ± 95% confidence limits; in units of percent/log dose. [c] Potency of MDZ (95% confidence limits) relative to CPZ.

activity obviates deriving CPR values by curve plotting and area computing manipulations. And, finally, the comparatively unsophisticated device can be fabricated with standard and readily available components.

It seems clear from the results summarized in Tables I and II that the instrument monitor provides data as accurate and as reliable as those provided by the more tedious visual method. The results of the inhibitor potency assay, particularly, demonstrate the utility and applicability of the instrument method for such experiments.

REFERENCES

(1) H. Krueger, N. B. Eddy, and M. Sumwalt, *U. S. Pub. Health Rep., Suppl. No. 165*, part 2, 919(1943).

(2) C. Amsler, *Arch. Exp. Pathol. Pharmakol.*, **97**, 1(1923).

(3) A. M. Burkman, A. Tye, and J. W. Nelson, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 140(1957).

(4) R. Koster, *J. Pharmacol. Exp. Ther.*, **119**, 406(1957).

(5) A. M. Burkman, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 558 (1960).

(6) B. N. Dhawan and P. N. Saxena, *Brit. J. Pharmacol. Chemother.*, **15**, 285(1960).

(7) A. M. Burkman, *J. Pharm. Sci.*, **50**, 156(1961).

(8) V. R. Deshpande, M. L. Sharma, P. R. Kherdikar, and R. S. Grewal, *Brit. J. Pharmacol. Chemother.*, **17**, 7(1961).

(9) A. M. Burkman, *J. Exp. Anal. Behav.*, **13**, 349(1970).

(10) A. M. Burkman, *J. Pharm. Sci.*, **50**, 771(1961).

(11) A. M. Burkman, *Arch. Int. Pharmacodyn. Ther.*, **137**, 396 (1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 3, 1970, from the *Division of Pharmacology, College of Pharmacy, Ohio State University, Columbus, OH 43210*

Accepted for publication June 17, 1970.

The authors are indebted to Dr. W. R. Williams, Mead Johnson Research Center, Evansville, Ind., for the supply of methdilazine hydrochloride (Tacaryl).

This investigation was supported by Grant MH 11733 from the U. S. Public Health Service.

Drug Permeation through Thin Model Membranes I: Development of a Polymeric Model Biomembrane

KARL A. HERZOG and JAMES SWARBRICK

Abstract □ The development of a polymeric nonporous model membrane, containing natural membrane components, and its use in a two-compartment transport cell are reported. Consideration was given to the polymer used to form the polymer matrix, membrane thickness, the amount and type of biological material incorporated, and the effect of nonbiological additives. The effect of these changes on the transport properties of the various membranes were monitored in terms of k_d, the rate of disappearance constant of salicylic acid, from the pH 2.0 compartment of the transport cell. As a result of these studies, a standard model biomembrane was designed, containing 44% ethylcellulose, 44% biological materials, and 12% mineral oil, dry weight of the membrane. From the lack of solvent flux under experimental conditions and the first-order disappearance of salicylic acid, it appears that the polymer membrane mimics the functionality of natural membranes insofar as passive diffusion is concerned.

Keyphrases □ Biomembrane model, polymeric—drug permeation □ Membrane, nonporous—natural membrane components □ Drug absorption, passive—model membranes □ Transport rates—drugs through model membranes

Model membranes have been employed in attempts to develop *in vitro* model systems whose transport characteristics correlate with *in vivo* passive drug absorption. In addition to permitting systematic study of the many variables affecting the *in vivo* process, these model systems also act as a potential tool for assessing the ability of new medicinal agents to cross the gastrointestinal membranes and similar barriers. Although considerable work has been reported concerning a number of models, significant accomplishments are yet to be made in elucidating the transport process occurring *in vivo* and in producing *in vitro* models that more closely reflect the functionality of biological membranes.

The following properties may be considered desirable in any model system for passive drug absorption:

1. The membrane should be thin, with a low volume ratio of membrane to surrounding aqueous media. This elimination of the membrane "volume" significantly reduces drug retention within the membrane phase.

2. Transport selectivity of the drug(s) in question should be based on solubility in a homogeneous barrier rather than on dialysis through a microporous structure.

3. The membrane should be sufficiently durable to withstand extended experimental procedures without loss of integrity which might lead to changes in the transport characteristics of the membrane.

4. It should be possible to demonstrate a correlation between *in vitro* transport rates and *in vivo* absorption rates.

Model membranes can be conveniently classified into two groups. First, some membranes are essentially bio-

Table II—Suppression of Apomorphine-Induced Pecking Syndrome by Chlorpromazine (CPZ) and Methdilazine (MDZ) Assayed by Two Methods

Statistic	Method	
	Visual	Instrument
ID_{50} (CPZ)[a]	2.05 (1.10–3.80)	1.65 (0.90–2.95)
ID_{50} (MDZ)	29.8 (24.0–37.0)	27.1 (20.9–34.6)
Slope (CPZ)[b]	84.9 ± 67.5	92.9 ± 29.1
Slope (MDZ)	71.6 ± 47.4	82.6 ± 30.7
Common slope (CPZ) + (MDZ)	90.11	78.43
Relative potency[c]	0.072 (0.046–0.100)	0.063 (0.043–0.96)

[a] Median inhibiting dose (95% confidence limits); in unit of micromoles of base per kilogram body weight. [b] Slope of the dose–response line ± 95% confidence limits; in units of percent/log dose. [c] Potency of MDZ (95% confidence limits) relative to CPZ.

activity obviates deriving CPR values by curve plotting and area computing manipulations. And, finally, the comparatively unsophisticated device can be fabricated with standard and readily available components.

It seems clear from the results summarized in Tables I and II that the instrument monitor provides data as accurate and as reliable as those provided by the more tedious visual method. The results of the inhibitor potency assay, particularly, demonstrate the utility and applicability of the instrument method for such experiments.

REFERENCES

(1) H. Krueger, N. B. Eddy, and M. Sumwalt, *U. S. Pub. Health Rep., Suppl. No. 165*, part 2, 919(1943).

(2) C. Amsler, *Arch. Exp. Pathol. Pharmakol.*, **97**, 1(1923).

(3) A. M. Burkman, A. Tye, and J. W. Nelson, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 140(1957).

(4) R. Koster, *J. Pharmacol. Exp. Ther.*, **119**, 406(1957).

(5) A. M. Burkman, *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 558 (1960).

(6) B. N. Dhawan and P. N. Saxena, *Brit. J. Pharmacol. Chemother.*, **15**, 285(1960).

(7) A. M. Burkman, *J. Pharm. Sci.*, **50**, 156(1961).

(8) V. R. Deshpande, M. L. Sharma, P. R. Kherdikar, and R. S. Grewal, *Brit. J. Pharmacol. Chemother.*, **17**, 7(1961).

(9) A. M. Burkman, *J. Exp. Anal. Behav.*, **13**, 349(1970).

(10) A. M. Burkman, *J. Pharm. Sci.*, **50**, 771(1961).

(11) A. M. Burkman, *Arch. Int. Pharmacodyn. Ther.*, **137**, 396 (1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 3, 1970, from the *Division of Pharmacology, College of Pharmacy, Ohio State University, Columbus, OH 43210*

Accepted for publication June 17, 1970.

The authors are indebted to Dr. W. R. Williams, Mead Johnson Research Center, Evansville, Ind., for the supply of methdilazine hydrochloride (Tacaryl).

This investigation was supported by Grant MH 11733 from the U. S. Public Health Service.

Drug Permeation through Thin Model Membranes I: Development of a Polymeric Model Biomembrane

KARL A. HERZOG and JAMES SWARBRICK

Abstract □ The development of a polymeric nonporous model membrane, containing natural membrane components, and its use in a two-compartment transport cell are reported. Consideration was given to the polymer used to form the polymer matrix, membrane thickness, the amount and type of biological material incorporated, and the effect of nonbiological additives. The effect of these changes on the transport properties of the various membranes were monitored in terms of k_d, the rate of disappearance constant of salicylic acid, from the pH 2.0 compartment of the transport cell. As a result of these studies, a standard model biomembrane was designed, containing 44% ethylcellulose, 44% biological materials, and 12% mineral oil, dry weight of the membrane. From the lack of solvent flux under experimental conditions and the first-order disappearance of salicylic acid, it appears that the polymer membrane mimics the functionality of natural membranes insofar as passive diffusion is concerned.

Keyphrases □ Biomembrane model, polymeric—drug permeation □ Membrane, nonporous—natural membrane components □ Drug absorption, passive—model membranes □ Transport rates—drugs through model membranes

Model membranes have been employed in attempts to develop *in vitro* model systems whose transport characteristics correlate with *in vivo* passive drug absorption. In addition to permitting systematic study of the many variables affecting the *in vivo* process, these model systems also act as a potential tool for assessing the ability of new medicinal agents to cross the gastrointestinal membranes and similar barriers. Although considerable work has been reported concerning a number of models, significant accomplishments are yet to be made in elucidating the transport process occurring *in vivo* and in producing *in vitro* models that more closely reflect the functionality of biological membranes.

The following properties may be considered desirable in any model system for passive drug absorption:

1. The membrane should be thin, with a low volume ratio of membrane to surrounding aqueous media. This elimination of the membrane "volume" significantly reduces drug retention within the membrane phase.

2. Transport selectivity of the drug(s) in question should be based on solubility in a homogeneous barrier rather than on dialysis through a microporous structure.

3. The membrane should be sufficiently durable to withstand extended experimental procedures without loss of integrity which might lead to changes in the transport characteristics of the membrane.

4. It should be possible to demonstrate a correlation between *in vitro* transport rates and *in vivo* absorption rates.

Model membranes can be conveniently classified into two groups. First, some membranes are essentially bio-

experimental models, whose principal research function has been elucidation of some of the biochemical and biophysical aspects of the ultrastructure of the biological membrane and determination of the role of the constituents in conducting the vital physiological functions accorded to the biomembrane. Such systems are invariably "constructed" on a molecular level, prime examples being monolayers (1), black (bimolecular) lipid membranes (2), and liposomes (3). The second group of model membrane systems are those employed primarily to study transport phenomena. Such systems are of particular relevance to *in vivo* drug-absorption processes. These membrane systems, employed to study this phenomenon on an *in vitro* basis, are termed biotransport models. Many variations of these models were utilized in the preceding decade to study the kinetics of drug transport and the many variables which are held to influence the *in vivo* process.

Biotransport model membrane systems may be divided into natural and artificial (nonliving) systems. The former include goldfish (4) or the everted sac technique (5), which has received recent attention in biopharmaceutical studies (6, 7). Skin sections stripped from hairless mice have been used as model systems to study chloramphenicol transfer (8). There are limitations to all of these approaches, although, understandably, a living system is to be preferred when experimental conditions permit.

Artificial (nonliving) membrane systems fall into two basic categories: those utilizing a liquid barrier and those employing a solid barrier. The liquid membrane system was one of the earliest model membrane systems applied to pharmaceutical research (9–11). The underlying principle of the model is the separation of the two aqueous phases, representing the plasma and the gastrointestinal lumen, by a liquid oil phase which simulates the lipid nature of the biological membrane. Much of the kinetic groundwork was carried out using these models, and their use continues today (12–14).

Procedures applying a solid barrier to separate two aqueous phases are currently being explored, not only by pharmaceutical and medical scientists but also in a wide variety of other disciplines. Whatever the application, the problem of mechanical strength of the membrane becomes significant when a certain cross-sectional dimension is exceeded. When extreme conditions are required to produce the requisite flux, such as in the case of pressurized reverse osmosis in desalination (15) or in pharmaceutical transport studies where the inherent mechanical strength of the particular polymer employed is absent (16), supports of various types are often required to maintain the physical integrity of the barrier. Nonsupported membranes appear to be better suited as biotransport model systems and, consequently, film strength is a necessary criterion upon which to evaluate potential membrane-forming materials.

Based on the mechanism of transport involved, it is possible to distinguish two types of solid barriers. In the first, the permeating molecule can penetrate through pores in the membrane, without actually being in solution within the membrane. The ability to be transported is largely dependent on the relative size of the barrier pore and the penetrant. An example of this system is dialysis, which has been used in the treatment of drug overdosage and poisoning (17) and in earlier artificial kidneys (18).

The second mechanism is that involving actual solution of the penetrant within the solid membrane. This requisite solubility, when coupled with diffusion, allows permeation across the barrier. Subsequently the penetrant is desorbed from the distal surface. Considering that prior to the solution process, sorption at the proximal membrane surface must occur, these processes parallel a model for liquid permeation proposed by Tuwiner (19). The entire mechanism depends primarily on the ability of the penetrant to dissolve in the membrane. As such, this type of solid barrier better resembles the functionality of the biological membrane with respect to passive diffusion of the uncharged drug species. Included in this category are "solid" barriers formed by the Millipore membrane dip technique, originally conceived by Tobias *et al.* (20, 21) and subsequently used by pharmaceutical investigators (8, 22). The inclusion of this type of barrier is based on the assumptions that the oil-impregnated "channels" of the porous pad constitute the effective membrane with regard to transport and that the size of this channel is much greater than the dimensions of the penetrating molecule. It is through these channels that the intramembrane flux occurs; as a result this model can be considered, along with nonporous polymer barriers, as a partition model system.

When the Millipore model is compared to the three-phase liquid partition models, it is seen that the volume ratio of the liquid phase to the aqueous phases employed has been drastically reduced. Although this diminution is significant, drug retention may still be considerable within the membrane during the time course of transport. Ideally, a model should exhibit negligible capacity for the permeating species. This requirement appears to be most adequately fulfilled by the use of polymeric film-forming agents from which solid, nonporous barriers of quite thin cross section can be made. Two sources exist: commercially prepared films or those extemporaneously formed by the experimenter. An advantage of the latter is that it permits experimental flexibility, especially in terms of membrane composition.

MEMBRANE COMPOSITION

Monoconstituent Membranes—Membranes that consist entirely of film-forming materials, alone or in combination, are termed monoconstituent or pure membranes. Regardless of the method used to produce these membranes, this type of barrier has received the most attention. Water-vapor transmission through polymeric films has been studied both in terms of the free film (23) and when the film is applied to tablets (24). Moisture uptake increased with increasing hydrophobicity of the barrier, and the transmission characteristics of the free film differed from that of the applied film. Permeation studies using polyethylene allowed the computation of apparent diffusion coefficients, permeability constants, and solubility coefficients of six aromatic compounds, the majority of which were nonelectrolytes (25). As an alternative to conventional separation methods of azeotropic binary mixtures of organic liquids, selective diffusion through polymer membranes has been employed by Carter and Jagannadhaswamy (26) as the rationale behind their extraction procedure. Termed pervaporation, the permeating component of the mixture vaporizes on the receiving side of the membrane and is subsequently collected in a cold trap.

Biomedical applications of pure films also have received attention. Extracorporeal hemodialysis efficiency has heretofore been extremely limited for large molecular volume solutes such as urea when employing cellophane-type membranes. Recently, attempts to fabricate a membrane applicable to "hemodialysis," whose mechanism of selectivity is based *not* upon dialysis but upon partition phenomena, have centered around a block copolymer system (27). Furthermore, with the upsurge in plastic packaging of a wide variety of pharmaceutical products, investigations of drug–polymer interactions continue (28), as workers attempt to elucidate the mechanisms of interaction based upon the study of kinetic and thermodynamic parameters.

There have been limited reports concerning drug transfer from one aqueous environment across a polymeric barrier into a second receiving compartment, also aqueous in nature. Johnson (29) used crosslinked thiolated gelatin films to study ionic and nonionic drug diffusion. Rummel *et al.* (30) separated an acid medium from an alkaline medium with several different polymeric membranes and noted that transport was unidirectional, favoring the unionized species. Garrett and Chemburkar (31–33) reported extensive experimentation utilizing dimethylsiloxane polymer sheeting. The silastic membranes proved to be impermeable to ions, and Fick's law was shown to apply for the diffusing species.

Composite Membranes—Flexibility of membrane composition, as an experimental variable, provides considerable potential for investigation. As a consequence, several researchers have attempted to alter the permeation properties of polymer membranes by the addition of compatible materials. Experimentally, this is most often accomplished by incorporating the additives into the membrane solution prior to casting, resulting in a composite membrane. Recently (34) a patent for a paraffin oil additive to polyethylene was awarded. The resultant film was said to have good vapor permeability and was suitable as a packaging material for fresh fruits and vegetables. Dupeyrat and Schreiber (35) studied the electrical properties of an oleic acid–collodion membrane which separated aqueous solutions of electrolytes. These properties were altered when the additive was omitted from the collodion matrix. A lipid-permeable collodion membrane, in which mineral oil was incorporated, was employed to study cholesterol transfer which was then used as a measure of serum lipoprotein–cholesterol complex dissociation (36).

Weatherby (37, 38) was one of the first to construct polymeric membranes comprised of materials distinctly biochemical in nature. He qualitatively demonstrated a relationship between pH of the bathing solution (distilled water was used in the receiving chamber) and the transport of some organic electrolytes. Ionic impermeability was attributed to like-charge repulsion arising from the exposed ionizable groups of the incorporated biochemicals on the membrane surface. Later, Weatherby (39) expanded this original work while employing phospholipids of determined composition. At about the same time, Goldman (40) prepared and studied the electrical characteristics of parlodion (purified collodion) and polystyrene membranes, some of which contained added phospholipid material. More recently, Lakshminarayanaiah (41) studied the resistance and capacitance of parlodion membranes containing stearic acid, phosphatidyl-L-serine, and cholesterol.

The literature does not appear to contain any reports of investigations utilizing a nonporous polymeric barrier, in which are incorporated additives of a biological nature, used expressly for the purpose of studying passive drug transport. Misra *et al.* (16), using collodion as the matrix material, combined lecithin into membranes cast on a mercury substrate and quantitated the permeation of several pharmaceutical substances. Analysis of the transport kinetics using such a model membrane system indicated that salicylic acid did not permeate the membrane in a fashion predicted by the pH-partition hypothesis. Moreover, nonlinearity of a semilogarithmic plot of concentration *versus* time depicting salicylic acid transport in the model system was observed and attributed to adsorption of the acid on the membrane.

It was, therefore, decided to investigate a composite model membrane system for its potential as a model biomembrane which would mimic some of the distinctive qualities of the biological membrane with regard to passive drug absorption. A prime consideration was the design of a membrane that would better fulfill the aforementioned criteria than model systems heretofore utilized. This preliminary report details the various parameters investigated in constructing such a model biomembrane.

EXPERIMENTAL

Materials—Solutions of lecithin (90% pure, bovine)[1] and cephalin (animal)[1] in chloroform were filtered through Whatman No. 2 filter paper to remove insoluble contaminants. The solvent was evaporated under ambient conditions, and the material collected was stored over a desiccant. The composition of the cephalin was taken to be 15% phosphatidylethanolamine and 85% phosphatidylserine (20). Cholesterol was similarly stored over a desiccant. The polymeric film-forming agents tested included ethylcellulose,[2] *n*-butyl methacrylate,[3] and a vinyl chloride copolymer.[4] Buffer components (sodium hydroxide, potassium phosphate monobasic, potassium chloride, and hydrochloric acid), salicylic acid,[5] and the polymer solvents employed were all of reagent grade. Light mineral oil NF[6] and sodium carboxymethylcellulose[7] were also used in this study. All chemicals, except where specified, were used as received.

Membrane Formation—Solutions of the chosen polymer (percent weight in volume ratio) and liquid paraffin (percent volume in volume ratio) in chloroform were made to contain twice the concentration desired for each component in the final membrane solution. To 5 ml. of this stock solution were added the requisite amounts of lecithin, cephalin, and cholesterol in chloroform, and the volume was brought to 10 ml. with chloroform.

The membrane was formed by a casting technique. Two milliliters of the solution containing the biological materials and the polymer was introduced into a glass ring (5.5 cm. i.d.) clamped onto a leveled glass plate approximately 12.5 cm. square. No seepage occurred at the ring-plate joint. The solvent was allowed to evaporate under controlled conditions at room temperature. This took 6–7 hr. The resulting polymer film containing the dispersed biochemicals was removed by soaking for 10 min. in distilled water at room temperature. The membrane was allowed to air dry and was stored in a glass culture dish until used.

Transport-Rate Studies—A 50-ml. Plexiglas dialysis cell, similar to that described by Patel and Foss (42) and consisting of two half-cells separated by the model membrane, was employed in the transport-rate studies. The cell was modified by the addition of two sampling ports, each having a 4.0-cm. extension of glass tubing (0.9 cm. i.d.) which permitted sampling while the cell remained submerged in a constant-temperature water bath. The sampling extensions were closed during agitation. The maximum area of contact between the membrane and solution was 11.34 cm.^{2}.

Twenty-five milliliters of a pH 7.4 buffer (43), used as the receiving medium, was introduced into one side of the cell. The unexposed surface of the membrane was inspected for leaks. The drug-containing pH 2.0 buffer solution (43) was then added to the other chamber. All solutions were previously equilibrated to 37° except where noted. The cells were immersed in the temperature-controlled water bath at 37° and shaken at 73 strokes/min. At appropriate time intervals, 1-ml. samples were withdrawn from both chambers and diluted with the respective buffer solutions; the absorbance was determined spectrophotometrically at 303 and 298 mμ, the wavelengths of maximum absorbance of salicylic acid in pH 2.0 and pH 7.4 buffer, respectively. The corresponding buffer was employed as the reference in each case. From a predetermined calibration curve, the unknown concentration of the test drug was determined. In most cases, the initial concentration of drug was 300 mg./l.

The apparent rate constant, k_a, for the disappearance of salicylic acid from the chamber buffered at pH 2.0 was obtained from a semilogarithmic plot of the concentration of drug remaining on the pH 2.0 side of the membrane *versus* time. In all cases, the reported rate constant was derived from the statistically determined slope (method of least squares) of such a plot, using the first sample period (not zero time) in the computation. Correlation coefficients were not less than 0.97. Except where specified, triplicate runs were made.

[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Ethocel, supplied by the Dow Chemical Co., Midland, Mich.
[3] Elvacite 2044, supplied by E. I. du Pont De Nemours and Co., Wilmington, Del.
[4] Exon 400 XR-77, supplied by Firestone Plastics Co., Pottstown, Pa.
[5] Crystal, Baker and Adamson, Allied Chemical Corp., Morristown, N. J.
[6] Atlas Drug and Chemical Co., New York, N. Y.
[7] CMC 7HP, Hercules Powder Co., Wilmington, Del.

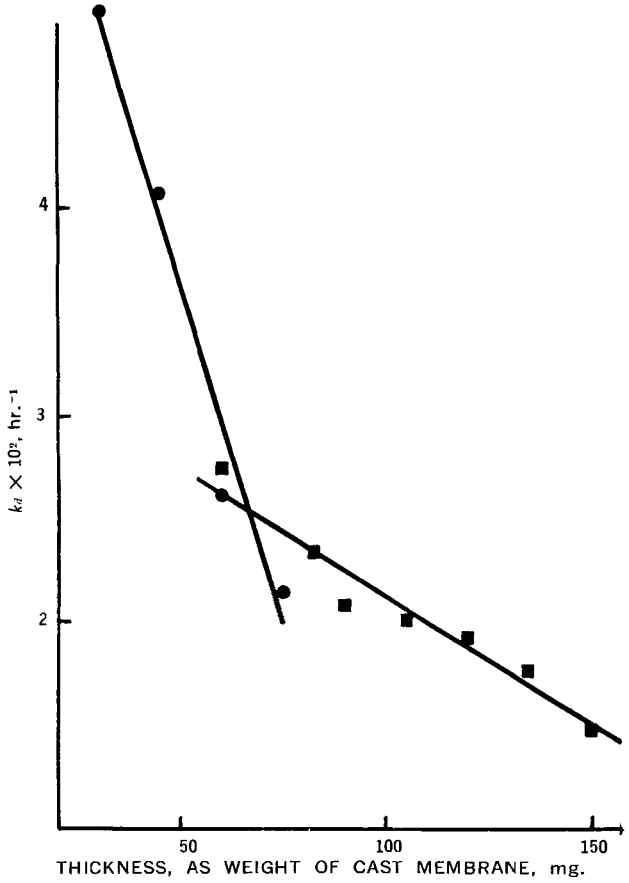


Figure 1—*Effect of the polymer forming the matrix and membrane thickness on the rate of salicylic acid disappearance. All membranes were prewashed for 4 hr. Key: ■, BMA; and ●, EC.*

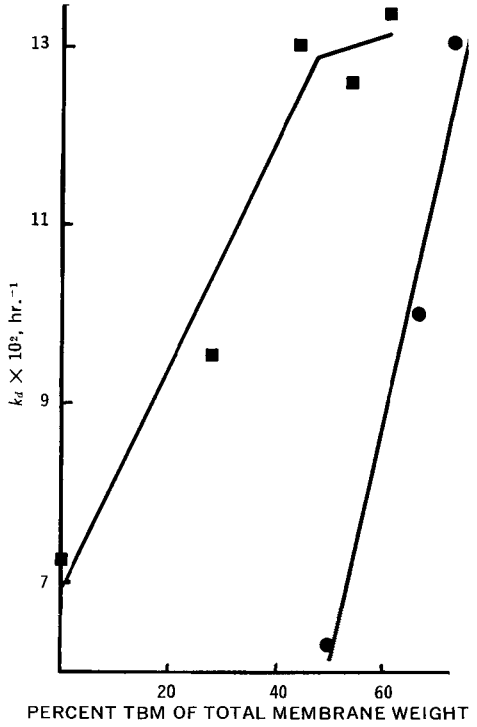


Figure 2—*Effect of TBM on the rate of salicylic acid disappearance. Key: ■, matrix solution was EC (1.5%) and MO (0.5%); and ●, matrix solution was EC (1.5%).*

RESULTS AND DISCUSSION

To represent the major biological components of membranes of animal origin (44, 45), two phospholipids, lecithin (phosphatidylcholine) and cephalin (phosphatidylethanolamine and phosphatidylserine), and the neutral lipid cholesterol were chosen. In subsequent discussions, this combination is referred to as the total biological mixture or TBM. The 1:1:2 molar ratio of cephalin, lecithin, and cholesterol, respectively, used in the majority of these initial studies represents a 1:1 molar ratio of *total* phospholipid to cholesterol and is based upon a comparable ratio found in the plasma membrane (46). A recent report (47) demonstrated that these materials are major components of the intestinal membrane and exist therein in this approximate ratio. As will be discussed, consideration was first given to the polymer used to form the supporting matrix. Attention was then directed toward the effect of increasing the TBM, both with and without additives of a nonbiological nature. Finally, the effect on transport rate of varying the ratio of the individual biological membrane components was studied.

Choice of Supporting Matrix—Several film-forming agents were chosen for their potential to create a lipophilic matrix. Only data associated with two of these materials [*n*-butyl methacrylate (BMA) and ethylcellulose (EC), 50 cps.] will be presented here. With BMA, membranes of constant-surface area were cast from a series of dilutions of a stock solution containing BMA (6.0%) and TBM (1.5%) in chloroform. In this manner, the weight of the membrane and hence the thickness varied while holding constant the ratio of polymer to TBM. A similar series of membranes was cast from dilutions of a stock solution containing EC (3.0%) and TBM (0.75%) in chloroform. The rate constants, k_d, for the transport of salicylic acid through these membranes are shown in Fig. 1 as a function of the weight per unit area (equivalent to thickness) of the cast membrane. It is apparent that the change in k_d with thickness was greater with the EC membrane than with the BMA membrane system, the rate constant increasing rapidly with the thinner membranes. The EC films possessed certain other advantages over the BMA membranes. The pliability of the BMA film limited its handling qualities and mechanical strength and these, in turn, limited the minimum thickness to which the membrane could be cast. Mechanical strength of the BMA membrane was also sensitive to small changes in TBM concentration. On this basis and because of the higher transport rates of thin EC membranes, all subsequent studies were conducted using this polymer as the film-forming agent.

Effect of TBM on k_d—Increasing amounts of TBM were added to solutions of 1.5% EC in chloroform in an attempt to potentiate transport across the resultant membrane. It was found, however, that above a certain concentration of TBM the aqueous phases in the transport system became turbid due to excessive washout of biological material from the membrane. To ensure that all such material had been removed, it was necessary to prewash the membrane for prolonged periods of time (up to 116 hr.).

To overcome this disadvantage, 0.5% mineral oil (MO) was added to the chloroform solution of EC and TBM prior to casting. The membranes were then prewashed with pH 2.0 and pH 7.4 buffers on their respective sides of the membrane mounted within the transport cell for 3 hr., this now being sufficient time to remove the excess material. The effect of increasing TBM on k_d, for membranes with and without MO, is shown in Fig. 2. Several points are apparent. First, the addition of 0.5% MO to the casting solution acts to potentiate the transport rate of salicylic acid. Second, there is a certain capacity of the polymer matrix for TBM in the presence of MO. This is evidenced by the break in the left-hand curve in Fig. 2, indicating the failure of additional TBM to enhance k_d. The breakpoint is equivalent to a membrane containing 44% TBM, expressed as a percentage of the total weight of the dry membrane. It would appear that any TBM in excess of this concentration is removed in the prewash; as a result, k_d remains constant.

As a result of this study, the standard membrane used in all subsequent investigations consisted of 44% EC, 44% TBM, and 12% MO, expressed in terms of the dry weight of the membrane.

Effect of Biochemical Constituent Variation—To study the effect of variations in the three biological components of the membrane on k_d, a series of membranes was studied in which the percentage of lecithin was progressively increased. The remaining TBM requirement was divided equally between cephalin and cholesterol. The TBM was held constant at 44% of the membrane weight, which also contained 44% EC and 12% MO. The data are shown in Fig.

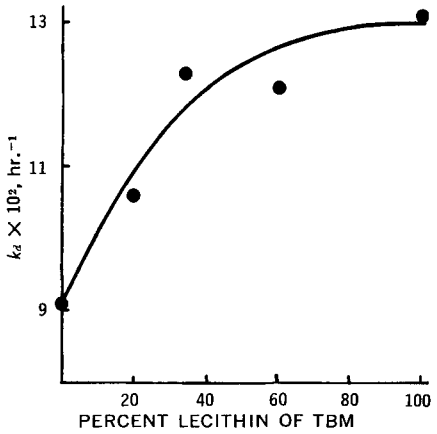


Figure 3—*Effect of lecithin as a percentage of TBM on the rate of salicylic acid disappearance. Membrane composition was 44% EC, 44% TBM, and 12% MO.*

3, where it is apparent that lecithin has a potentiating effect on the rate of salicylic acid transport. This rate increase may be attributed to the contribution of lecithin to the nonpolar character of the model biomembrane, hence increasing the passage of the lipid-soluble drug species. In contrast to both phosphatidylserine and phosphatidylethanolamine, phosphatidylchlorine has a greater alkyl moiety esterified to the common phosphatidic acid backbone. This is consistent with the effect observed with the incorporation of MO into the EC membrane (Fig. 2).

Determination of Solvent Flux—Employing the standard membrane, the effect of hydrostatic and osmotic pressure on solvent flux was investigated. Distilled water was placed on one side of the membrane and dialyzed against an equal volume of a 5% aqueous solution of sodium carboxymethylcellulose (CMC). After 6 days, there was no volume increase in the chamber containing the CMC. In another study, 25 ml. of distilled water was added to one chamber, the second chamber remaining empty. Although the membranes appeared slightly distended after 6 days, there was no evidence of leakage or the appearance of moisture on the distal side of the membrane. These observations lend support to the proposed mechanism for the transport of unionized drug molecules across polymeric barriers, wherein the permeating species moves across the membrane without the aid of the aqueous solvent.

SUMMARY

In summary, a polymeric membrane that incorporates biochemicals representing natural membrane constituents was fabricated by casting. The transport of salicylic acid through such a model followed apparent first-order kinetics and is represented by the disappearance-rate constant, k_a. Additionally, the lack of solvent flux indicates that this solid barrier is nonporous in nature. This property, coupled with approximately 2% salicylic acid retention (48), allows the model biomembrane to mimic the passive diffusion functionality of natural membranes. Lecithin potentiates transport, as does MO, both apparently contributing to the lipophilicity of the barrier.

As a result of these studies, a standard model biomembrane was prepared. The composition of this membrane is 44% EC, 44% TBM, and 12% MO, expressed as dry weight before prewashing. The transport characteristics of the standard membrane are presently being investigated and will be the subject of a future communication.

REFERENCES

(1) L. L. M. Van Deenen, U. M. T. Houtsmuller, G. H. de Haas, and E. Mulder, *J. Pharm. Pharmacol.*, **14,** 429(1962).

(2) P. Mueller, D. O. Rudin, H. T. Tien, and W. C. Wescott, *Nature*, **194,** 979(1962).

(3) G. Sessa and G. Weissmann, *J. Lipid Res.*, **9,** 310(1968).

(4) G. Levy and S. P. Gucinski, *J. Pharmacol. Exp. Ther.*, **146,** 801(1964).

(5) R. K. Crane and T. H. Wilson, *J. Appl. Physiol.*, **12,** 145 (1958).

(6) A. J. Aguiar and R. J. Fifelski, *J. Pharm. Sci.*, **55,** 1387 (1966).

(7) R. H. Reuning and G. Levy, *ibid.*, **58,** 79(1969).

(8) A. J. Aguiar and M. A. Weiner, *ibid.*, **58,** 210(1969).

(9) J. T. Doluisio and J. V. Swintosky, *ibid.*, **53,** 597(1964).

(10) *Ibid.*, **54,** 1594(1965).

(11) H. L. Rosano, J. H. Schulman, and J. Weisbuck, *Ann. N. Y. Acad. Sci.*, **92,** 457(1961).

(12) S. A. Khalil and A. N. Martin, *J. Pharm. Sci.*, **56,** 1225(1967).

(13) J. Perrin, *J. Pharm. Pharmacol.*, **19,** 25(1967).

(14) P. P. Lamy, *Pharm. Acta Helv.*, **44,** 79(1969).

(15) S. Wood, *Mod. Plast.*, **46,** 50(1969).

(16) A. L. Misra, A. Hunger, and H. Keberle, *J. Pharm. Pharmacol.*, **18,** 531(1966).

(17) N. H. Kowal, A. I. Sandler, and A. J. Davis, *Drug Intel.*, **3,** 186(1969).

(18) J. A. Maher and G. E. Schreiner, *Trans. Amer. Soc. Artif. Intern. Organs*, **14,** 440(1968).

(19) S. B. Tuwiner, "Diffusion and Membrane Technology," Reinhold, New York, N. Y., 1962, p. 215.

(20) J. M. Tobias, D. P. Agin, and R. Pawlowski, *J. Gen. Physiol.*, **45,** 989(1962).

(21) J. M. Tobias, D. P. Agin, and R. Pawlowski, *Circulation*, **26,** 1145(1962).

(22) G. Levy and E. J. Mroszczak, *J. Pharm. Sci.*, **57,** 235(1968).

(23) G. S. Banker, A. Y. Gore, and J. Swarbrick, *J. Pharm. Pharmacol.*, **18,** 457(1966).

(24) *Ibid.*, **18,** Suppl., 205S(1966).

(25) M. A. Gonzales, J. Nematollahi, W. I. Guess, and J. Autian, *J. Pharm. Sci.*, **56,** 1288(1967).

(26) J. W. Carter and B. Jagannadhaswamy, *Brit. Chem. Eng.*, **9,** 523(1964).

(27) D. J. Lyman, B. H. Loo, and W. M. Muir, *Trans. Amer. Soc. Artif. Intern. Organs*, **11,** 91(1965).

(28) M. B. Rodell, W. I. Guess, and J. Autian, *J. Pharm. Sci.*, **55,** 1429(1966).

(29) R. H. Johnson, *ibid.*, **54,** 327(1965).

(30) W. Rummel, H. Buch, W. Buzello, and O. Neurohr, *Arch. Pharmakol. Exp. Pathol.*, **262,** 366(1969).

(31) E. R. Garrett and P. Chemburkar, *J. Pharm. Sci.*, **57,** 944 (1968).

(32) *Ibid.*, **57,** 949(1968).

(33) *Ibid.*, **57,** 1410(1968).

(34) *Chem. Abstr.*, **70,** 38587e(1969).

(35) M. Dupeyrat and B. Schreiber, *Bull. Soc. Chim. Biol.*, **50,** 1513(1968); through *Chem. Abstr.*, **70,** 93249w(1969).

(36) L. J. Banaszak and H. J. McDonald, *Biochim. Biophys. Acta*, **53,** 404(1961).

(37) J. H. Weatherby, *J. Cell. Comp. Physiol.*, **21,** 1(1943).

(38) J. H. Weatherby, *J. Lab. Clin. Med.*, **28,** 1817(1943).

(39) J. H. Weatherby, *J. Cell. Comp. Physiol.*, **33,** 333(1949).

(40) D. E. Goldman, *J. Gen. Physiol.*, **27,** 37(1943).

(41) N. Lakshminarayanaiah, *J. Appl. Polym. Sci.*, **10,** 689(1966).

(42) N. K. Patel and N. E. Foss, *J. Pharm. Sci.*, **53,** 94(1964).

(43) "Remington's Pharmaceutical Sciences," 13th ed., Mack Publishing Co., Easton, Pa., 1965, p. 245.

(44) E. D. Korn, *Science*, **153,** 1491(1964).

(45) P. Ways and D. J. Hanahan, *J. Lipid Res.*, **5,** 318(1964).

(46) L. L. M. Van Deenen and J. de Gier, in "The Red Blood Cell," C. Bishop, Ed., Academic, New York, N. Y., 1964, p. 266.

(47) G. G. Forstner, K. Tanaka, and K. J. Isselbacher, *Biochem. J.*, **109,** 51(1968).

(48) K. A. Herzog and J. Swarbrick, to be published.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 24, 1970, from the *Division of Pharmaceutics, School of Pharmacy, University of Connecticut, Storrs, CT 06268*

Accepted for publication June 25, 1970.

This investigation was supported in part by Grant 085 from the University of Connecticut Research Foundation, Storrs, CT 06268, and in part by a Public Health Service Fellowship 1-F01-GM-41, 243-01 (K.A.H.) from the National Institute of General Medical Sciences, Bethesda, Md.

Chemical Balance as a Rheometer for Biological Fluids

S. S. DAVIS*, B. WARBURTON*, and J. E. DIPPY†

Abstract □ The chemical balance provides a simple, cheap, and readily available method of assessing the consistency of biological fluids such as sputum. Scale reading at an arbitrary time provides a useful empirical parameter that can be employed in routine testing of clinical samples and in the assessment of mucolytic agents. It also correlates well with data obtained using a conventional cone and plate viscometer. The change of scale reading with time can be analyzed in a fundamental rheological manner using the linear viscoelastic model. The method of measurement is nondestructive and allows repeated measurements to be made on the same sample.

Keyphrases □ Chemical balance—used as rheometer □ Rheometer—chemical balance use □ Sputum—rheological measurements □ Viscosity measurements—viscometer, chemical balance comparison □ Mucolytic agent effect—sputum viscosity

In the past, many attempts have been made to obtain some form of "viscosity" or consistency measurement for complex biological fluids such as sputum and to correlate this with variables such as state of disease, biochemistry, and the action of mucolytic agents. Unfortunately, sputum is not a simple Newtonian fluid, and its rheological evaluation is beset with many difficulties. These can be summarized as follows (1, 2): (*a*) limited sample size; (*b*) highly shear-sensitive structure which is easily destroyed in sample collection or in its preparation for examination; (*c*) inhomogeneity; (*d*) material cannot be frozen or homogenized without greatly altering its structure; (*e*) complex nature of material makes it difficult to obtain fundamental rheological parameters; (*f*) *in vitro* rheological measurements must be representative of the conditions that exist *in vivo;* and (*g*) material changes rapidly in consistency after collection due to loss of water and biodegradation.

Using sputum as the representative biological fluid, the authors examined some of the previous attempts to measure consistency (Table I). It is clear that many of the difficulties listed have not been given proper consideration; it is, therefore, not surprising that few reasonable consistency correlations have been obtained. Only in the more recent studies of Hwang *et al.* (1), Denton *et al.* (22), Davis and Dippy (24), and Sturgess *et al.* (25) have fundamental rheological param-

Table I—Rheological Evaluation of Sputum

Method and References	Advantages	Disadvantages
1. U-tube viscometer (3–9)	Simple and inexpensive	Variable shear rate; single-point method; destructive; data of doubtful significance; often impossible to obtain meaningful results
2. Falling-sphere viscometer (10)	Same as for No. 1	Same as for No. 1
3. Concentric-cylinder viscometer (5, 6, 8, 11–14)	Different shear rates can be studied (hysteresis effects); non-Newtonian behavior; can be used effectively as comparative method	Destructive; high shear rates that are almost impossible to correlate with *in vivo* conditions; some instruments only single point; automatic recording instruments are expensive; often impossible to obtain meaningful results
4. Cone and plate viscometer (15–19)	Small sample size; same as for No. 3	Material can be expelled from measuring surface; evaporation; same as for No. 3
5. Perforated disk (2, 8, 20, 21)	Simple and inexpensive; yield effects can be measured	Destructive; measured parameter difficult to interpret in fundamental manner; poorly reproducible
6. Magnetic rheometer (1, 22, 23)	Provides fundamental viscoelastic data, elasticities, viscosities, *etc.*, which can be correlated with *in vivo* conditions and molecular structure	Complex experimentally; not suitable for routine testing; complex mathematical analysis requiring digital computer
7. Rheogoniometer (oscillation) (24, 25)	Wide frequency range; same as for No. 6	Very expensive, especially when using automatic data-collection method; same as for No. 6
8. Rheogoniometer (creep testing) (24)	Same as for No. 6	Not suitable for samples of low consistency; same as for No. 6

eters been obtained that can be correlated sensibly with mucus structure and cilia transport. In these cases, the linear viscoelastic model was employed as a convenient starting point and the experimental results were interpreted in terms of viscosities, elasticities, and relaxation or retardation times.

In some cases, such as the assessment of mucolytic agents or the routine examination of clinical samples in the clinical laboratory, a detailed viscoelastic treatment is time consuming and probably unnecessary (16). It would rarely warrant the considerable capital outlay needed for setting up the experimental and data analysis procedures. Therefore, an instrument is needed that will satisfy the necessary conditions without being unduly expensive. The basic requirements for this instrument are:

1. It must be capable of dealing with a small sample size, a maximum of a few milliliters.
2. It must be robust and cheap.
3. It must be simple to operate in routine tests by unskilled technical staff.
4. The measurement should be reasonably quick to perform so that large numbers of samples can be examined without delay.
5. It should provide some easily calculated, empirical parameter that will characterize consistency in a rheologically sound manner.
6. The data obtained should also be amenable to further treatment to obtain fundamental quantities such as viscosities and elasticities, should these be required.
7. The test should be nondestructive to enable repetition with the same sample in storage or kinetic experiments.
8. The conditions of test should be such that the derived data describes as well as possible the conditions *in vivo*.

Fortunately, a minor adaptation of the modern chemical balance is suitable for this purpose. This, of course, is standard equipment in nearly all laboratories.

APPARATUS

A conventional chemical balance (Stanton A.D.3) forms the main part of the apparatus (Fig. 1). The essential requirement is some type of illuminated scale that gives readings over the range 0–100 mg. A small glass plate (microscope cover slip) is suspended from one side of the balance and is immersed in the test fluid, which can be conveniently contained in a spectrophotometer cell. The latter is adjusted using a rack and pinion movement, and the whole assembly is enclosed in a thermostated glove-box at required temperature. The final setup is similar in a number of ways to the rising sphere viscometer described by McVean and Mattocks (26) and the commercial Haake Viskowaage viscometer (27).

Operation of the Balance—The glass plate is counterbalanced so that the reading on the illuminated scale is at the 100-mg. mark. The material under test is placed in the cell, and the plate is carefully immersed so that it is in the center of the cell and well below the surface. The vertical position of the cell is then adjusted, if necessary, to correct for buoyancy and to ensure that the balance is once again "zeroed" at the 100-mg. position. A 100-mg. weight is added carefully to the right-hand scale pan, and the change in scale reading with time is followed with a stopwatch.

Theoretical Considerations—From an analysis of the principle of the chemical balance, one can easily show that in the absence of a sample in the cell the balance system can be represented by a mechanical model (Fig. 2A), consisting of a spring (elastic) element and a dashpot (viscous) element arranged in parallel. The spring G_b represents the movement of the balance in response to a weight

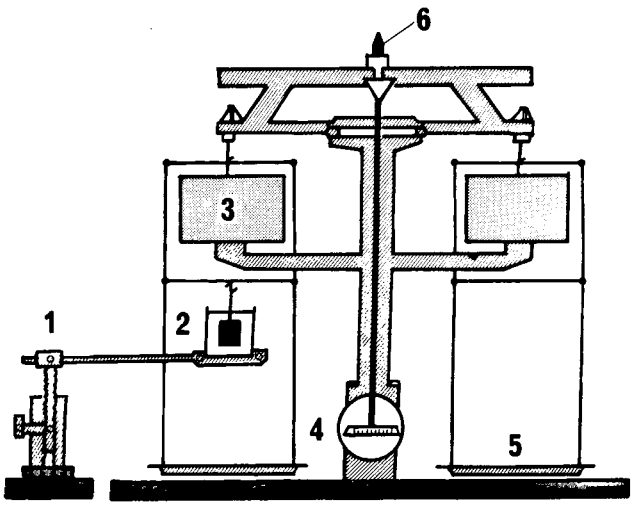


Figure 1—*Diagram of apparatus. Key: 1, rack and pinion stand; 2, cell and glass plate; 3, mechanical damper of balance; 4, illuminated scale; 5, weight added to this scale pan; and 6, counterbalance arrangement.*

placed on the scale pan, and the dashpot η_b represents the mechanical damping of the balance together with friction in the knife edges, *etc.* G_b will be directly proportional to the sensitivity of the balance. The ratio η_b/G_b is called the retardation time (τ_b).

In rheological terms, this representation is equivalent to a Voigt (Kelvin) solid (28). Material placed in the cell will naturally change the situation, as shown in Fig. 2B–E. The Newtonian fluid (Fig. 2B) and the Voigt solid (Fig. 2D) are trivial cases since similar models in parallel are additive (29); however, the Maxwell

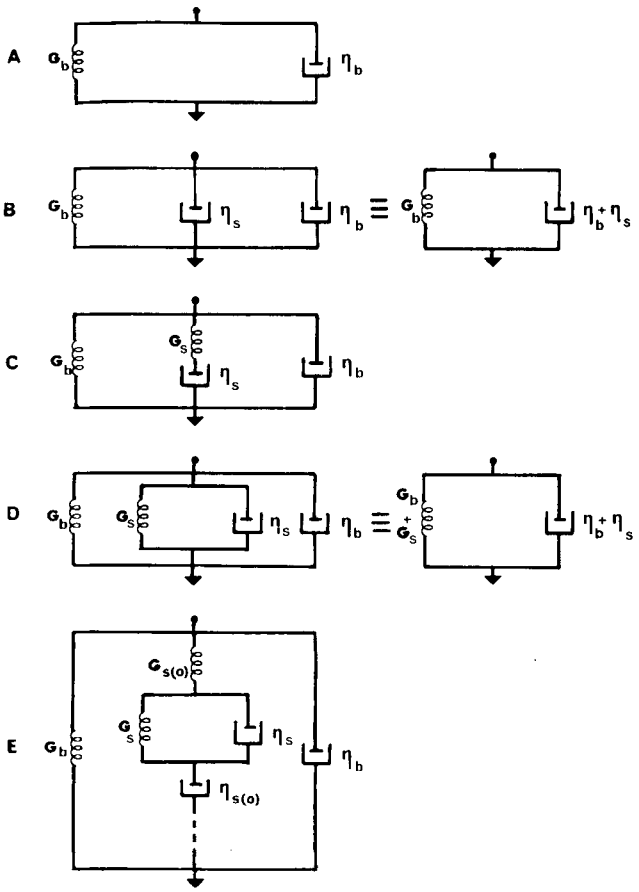


Figure 2—*Model representation. Key: A, balance alone; B, Newtonian fluid in cell; C, Maxwell fluid in cell; D, Voigt material in cell; and E, generalized viscoelastic material in cell.*

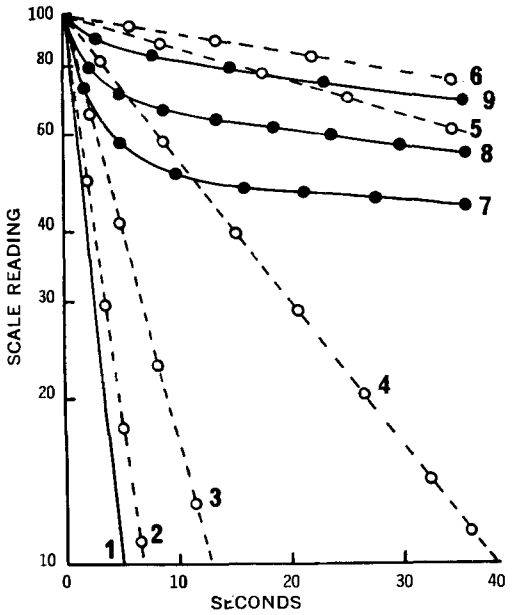


Figure 3—*Change in balance scale reading with time for different materials. Key: balance without sample (——), 1. Newtonian fluids (- - O - -): caster oil (η_s = 8.0 P), 2; silicone oils—(η_s = 1250cS), 3; (η_s = 3000cS), 4; (η_s = 60,000cS), 5; and (η_s = 100,000cS), 6. Complex materials (—●—●): egg white, 7; sputum, 8; and tragacanth mucilage, 9.*

liquid (Fig. 2C) and the generalized viscoelastic material (Fig. 2E) provide rather complex models. Nevertheless, it is possible to analyze these, subtract out the inherent contribution of the balance, and thereby represent the behavior of the material in the conventional viscoelastic manner (see *Appendix*). However, as neither stress nor strain is held constant during an experiment, this is not a simple process and, in routine testing, little is to be gained. The change in scale reading (equivalent to a change of strain) with time is an extremely useful, albeit empirical, parameter for studying rheological behavior.

The Newtonian Fluid—The Newtonian fluid (Fig. 2B) provides the simplest possible condition for rheological testing, and it is instructive to examine this in detail. When the 100-mg. weight is placed on the right-hand scale pan, the plate will be moved upward through the test fluid, and the change in strain will be proportional to the reading on the illuminated scale. The strain–time response for a Voigt model can be represented by Eq. 1 (30):

$$\gamma_t = \gamma^{\infty}(1 - e^{-t/\tau}) \qquad \text{(Eq. 1)}$$

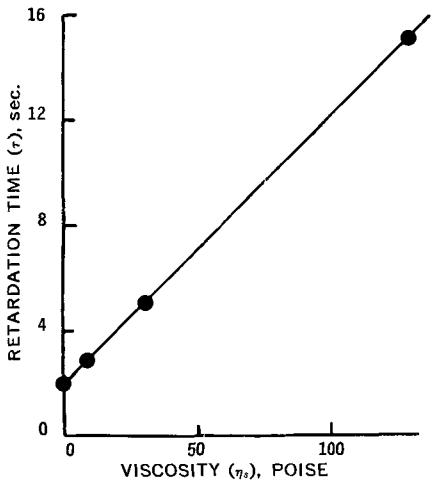


Figure 4—*Calibration of balance viscometer with Newtonian fluids (25°). Viscosity standards: mineral oil (η_s = 14.5 P); and silicone oils (η_s = 30.9 P) and (η_s = 129 P).*

where γ_t is the strain at time t, and γ^{∞} is the total strain at infinite time. Or, in terms of scale readings, S, and 100 mg. as the zero:

$$100 - S_t = 100(1 - e^{-t/\tau}) \qquad \text{(Eq. 2)}$$

which, on rearranging:

$$S_t/100 = e^{-t/\tau} \qquad \text{(Eq. 3)}$$

Taking logs:

$$2.303 \log S_t - 4.606 = -t/\tau \qquad \text{(Eq. 4)}$$

$$\log S_t = 2 - t/2.303\tau \qquad \text{(Eq. 5)}$$

The retardation time will be made up from two contributions, a retardation time associated with the balance (τ_b) and one with the Newtonian sample (τ_s):

$$\tau = \tau_b + \tau_s \qquad \text{(Eq. 6)}$$

A graph of scale reading against time, in semilog form (Fig. 3), for a Newtonian fluid will be linear with gradient $-1/(2.303\tau)$ and the intercept at the 100-mg. unstressed position. The combined retardation time can easily be calculated.

The τ is defined as the ratio of viscosity to spring modulus for the Voigt model. For the Newtonian fluid in the cell,

$$\tau_s = K_1\eta_s/G_b \qquad \text{(Eq. 7)}$$

where K_1 is an apparatus constant that will depend on the geometry of the plate and the cell. Substituting in Eq. 6:

$$\tau = \tau_b + K_2\eta_s \qquad \text{(Eq. 8)}$$

where $K_2 = K_1/G_b$. A graph of τ *versus* η_s will be linear with gradient K_2 and intercept τ_b. The latter can be obtained by following the movement of the balance when there is no sample in the cell. A calibration curve can thus be obtained using Newtonian fluids of known η_s (Fig. 4).

The viscosity term (η_s) is defined from Newton's law as the ratio of shear stress to shear rate. Shear stress will be directly related to the apparatus geometry. Changing the size of the plate will have a direct effect on the shear stress through an area relationship, and one can write

$$\tau = \tau_b + AK_3 \qquad \text{(Eq. 9)}$$

where A is the surface area of the plate and $K_3 = K_2\eta_s$.

For a given Newtonian oil, τ will be linearly related to plate area with intercept τ_b and gradient K_3. However, experimental data plotted in this manner do not pass through the expected intercept, and an end-correction term is evident such that

$$\tau = \tau_b + (A + Ae)K_3 \qquad \text{(Eq. 10)}$$

It is to be expected that this end-correction value will vary to some extent with the viscosity of the test fluid (31).

Similar experiments with polystyrene spheres also give a linear

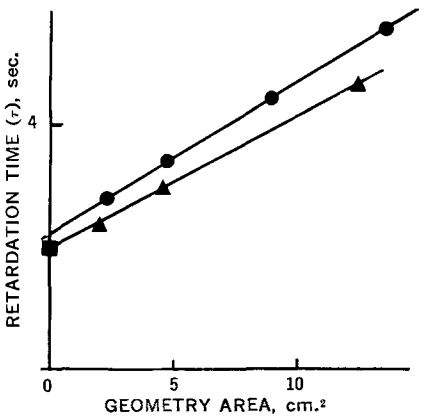


Figure 5—*Change in retardation time with apparatus geometry. Key: —●—, glass plates; and —▲—, polystyrene spheres.*

Table II—Comparison of Balance Method and Ferranti-Shirley Viscometer for the Effect of Water and Mucolytic Agent[a] on the Consistency of Sputum (Contact Time = 20 min.)

Viscometer	Measured Parameter	Percent Reduction in Parameter Compared to Untreated Sputum (Mean & *SD*)		No. Experiments
Balance	Scale reading at 60 sec.	Water	22.5 ± 12.9	20
		Mucolytic agent	54.3 ± 11.1	20
Ferranti-Shirley	Static yield value	Water	26.2 ± 14.8	10
		Mucolytic agent	55.6 ± 11.1	10
	Dynamic yield value	Water	25.0 ± 12.6	10
		Mucolytic agent	56.1 ± 16.5	10
	Apparent viscosity	Water	22.1 ± 12.4	10
		Mucolytic agent	61.0 ± 14.6	10
	Limiting viscosity	Water	22.8 ± 12.6	10
		Mucolytic agent	60.9 ± 13.7	10

[a] Ascoxal–ascorbic acid–hydrogen peroxide–cupric-ion system.

plot between τ and A but without an end correction. This is as expected from theoretical considerations (32, 33) (Fig. 5).

SOME PRACTICAL SYSTEMS

Experiments with a range of Newtonian fluids (Figs. 3 and 4) show that the instrument can be used without modification for viscosities in the range 1–1000 P. Complex non-Newtonian fluids such as sputum do not give linear relations between log S and time. Instead, the scale reading changes rapidly at short times and then more slowly at long times until it reaches an almost constant value. The same sample of biological fluid can be subjected to repeated measurements with little or no change in the measured log S *versus* t curve. The movement of the plate (*i.e.*, the strain) is small and, therefore, the method is essentially a nondestructive test. In practice the sample is usually examined a number of times, and a mean or self-consistent curve is taken as being representative. In repeated measurements the variation in measured scale readings is in the region of 10%, provided that care is taken in centering the plate in the cell well below the surface.

The sensitivity of the balance can be controlled by the counterbalance arrangement above the fulcrum of the balance. Theoretically, there is no reason why the counterbalance should not be set so that the fulcrum and center of gravity are coincident to provide the experimental conditions for pure creep (constant stress). However, experimentally the system would be mechanically unstable. The retardation time of the balance is directly related to the sensitivity, and the range on the illuminated scale can be changed if required using the counterbalance arrangement.

As an alternative to the full viscoelastic treatment for complex materials (see *Appendix*), the scale reading at an arbitrary time or the limiting scale reading as t becomes large is a suitable parameter for use in comparative experiments. For linear viscoelastic materials, the former is very similar to a compliance at arbitrary time measured from a creep curve. This is a popular approach in studies on polymer solutions and pharmaceutical systems (34, 35). In many cases, little difference exists between the two suggested parameters at times greater than 40 sec.

COMPARISON OF BALANCE METHOD WITH FERRANTI-SHIRLEY VISCOMETER

The results obtained with the balance were compared with those from the conventional Ferranti-Shirley cone and plate viscometer (28) for measurements on the consistency of sputum (Table II). The scale readings at an arbitrary time of 60 sec. were chosen for the balance experiments and compared with four rheological parameters obtained from the sputum rheogram off the Ferranti-Shirley viscometer. The rheological parameters were: (*a*) static yield value—minimum shear stress necessary to cause the unsheared material to flow (dyne cm.$^{-2}$); (*b*) dynamic yield value—minimum shear stress required to keep the sample in flow once it has been sheared (dyne cm.$^{-2}$); (*c*) apparent viscosity—ratio of shear stress to shear rate at the highest shear rate (poise); and (*d*) limiting viscosity—reciprocal of the gradient of the down-curve of the hysteresis loop (poise).

The comparison was made on the basis of percent reduction in rheological parameter upon addition of water or a mucolytic agent[1] as compared to an untreated sample. In all cases, the standard deviations about the mean are large due to considerable biological variation. Nevertheless, statistical analysis (16) shows that the results are valid and can be used in a comparative manner. The agreement between the two methods, balance and Ferranti-Shirley viscometer, is extremely satisfactory, especially when one considers that two different measurement principles are used. There is a slightly better correlation between the balance method and the two yield values from rheograms than with viscosities. This is as expected, because yield values can be considered as an estimate of solidlike structure in a material and will, therefore, be closer in physical nature to scale readings obtained in a nondestructive test than viscosity values calculated after a material has been broken down by shear.

KINETIC EXPERIMENTS

Besides having the great advantage of inexpensiveness, the balance method is also almost nondestructive and the same sample can be examined a number of times. Therefore, kinetic experiments can be performed, provided that the kinetic process proceeds at a rate whereby little change occurs in the consistency of the sample during the period of measurement. Figure 6 shows the effect of contact time for the mucolytic agent on the log S *versus* t curves of sputum. To obtain similar information with the Ferranti-Shirley viscometer, fresh samples have to be loaded for each measurement. This introduces sampling errors. The percentage reduction in consistency with mucolytic contact time is shown in Fig. 7. Once again the agreement between the two different methods of evaluation is satisfactory.

APPENDIX

Calculation of Viscoelastic Parameters—The rheological behavior of biological fluids can often be examined in a fundamental manner, using the linear viscoelastic model as a convenient starting point (1, 24). It can be used effectively in the present case, provided the strain response of the material is small and it is behaving in a linear viscoelastic manner (37). The behavior of the balance system will be superimposed upon the model and will contribute G_b and η_b, respectively, to the elasticity and viscosity of the total system (Fig. 8, System I). The material under examination can be represented by a subsystem consisting of a series of Voigt elements (G_{sr}, η_{sr}) with or without a final series viscosity ($\eta_{s(0)}$). Moreover, the total combined system can be represented by the series Voigt system in Fig. 8, System II; in general, Systems I and II are entirely equivalent over a wide range of time (in creep testing) or frequency (in oscillatory testing) (29).

The problem is to evaluate the material subsystem in System I. This may be achieved by employing an intermediate hypothetical

[1] Ascoxal, Astra-Hewlett Ltd., Watford, England.

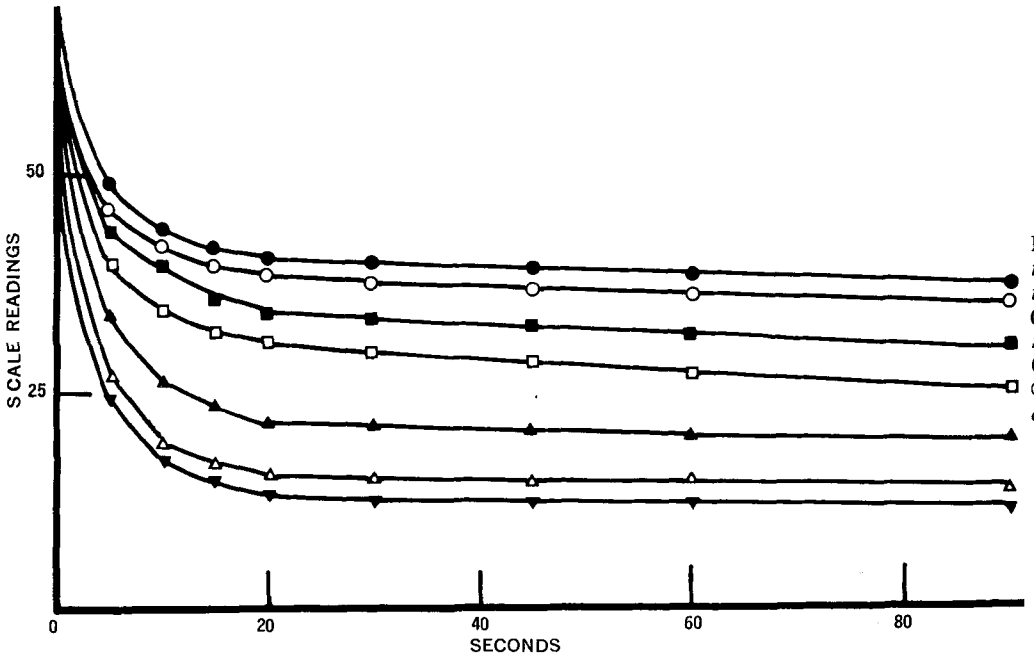


Figure 6—*Effect of contact time of the mucolytic agent on the consistency curves for sputum (repeated measurements on same sample). Mucolytic contact time (min.): ●, 0 (control with water); ○, 5; ■, 10; □, 15; ▲, 20; △, 30; and ▼, 40.*

oscillatory experiment of wide frequency range. The steps in the analysis are as follows:

1. Conventional creep curve analysis is used to evaluate the viscoelastic models in System II (30, 38).

2. From the models produced by this procedure, the frequency response over a wide range of frequency is then evaluated using the following equations (29):

$$J'(\omega) = \sum_{r=1}^{n} J_r\left(\frac{1}{1 + \omega^2\tau_r^2}\right) \qquad \text{(Eq. A1)}$$

$$J''(\omega) = \sum_{r=1}^{n} J_r\left(\frac{\omega\tau_r}{1 + \omega^2\tau_r^2}\right) \qquad \text{(Eq. A2)}$$

3. The complex modulus is then computed from the complex compliance over a similarly wide frequency range. Since

$$G^*(\omega) = 1/J^*(\omega) \qquad \text{(Eq. A3)}$$

$$G^*(\omega) = G'(\omega) + iG''(\omega) = 1/[J'(\omega) + iJ''(\omega)] = \frac{J'(\omega) - iJ''(\omega)}{[J'(\omega)]^2 + [J''(\omega)]^2} \qquad \text{(Eq. A4)}$$

4. After Step 3 is completed, the total arrangement can be considered equivalent to System I. The contribution of the balance $G_b' + iG_b''$ is known:

$$G_b' = G_b \qquad \text{(Eq. A5)}$$

$$G_b'' = \omega\eta_b \qquad \text{(Eq. A6)}$$

and, therefore, can be subtracted from the total complex modulus to give that of the subsystem in System I, using Eqs. A5 and A6:

$$G_s'(\omega) = G'(\omega) - G_b'(\omega) \qquad \text{(Eq. A7)}$$

$$G_s''(\omega) = G''(\omega) - G_b''(\omega) \qquad \text{(Eq. A8)}$$

5. The complex compliance of the subsystem can then be calculated:

$$J_s'(\omega) + iJ_s''(\omega) = \frac{G_s'(\omega) - iG_s''(\omega)}{[G_s'(\omega)]^2 + [G_s''(\omega)]^2} \qquad \text{(Eq. A9)}$$

6. The values of $J_s'(\omega)$ and $J_s''(\omega)$ can finally be used to calculate either a line spectrum or continuous spectrum of viscoelastic behavior for the material (37).

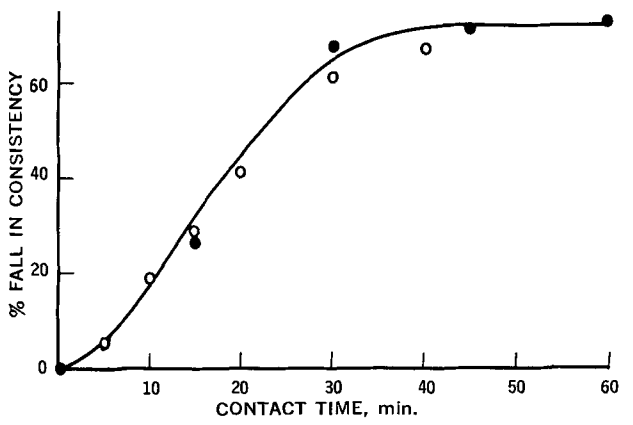


Figure 7—*Change in consistency with contact time for the action of the mucolytic agent on sputum. Comparison of balance rheometer and Ferranti-Shirley viscometer. Key: ○, balance (calculated from scale reading at 60 sec.); and ●, Ferranti-Shirley viscometer [mean value from change in four rheological parameters (Table II)].*

SYMBOLS

A = surface area, cm.^{2}
Ae = end correction, cm.^{2}
G_b = elastic contribution (shear modulus) associated with balance, dyne cm.$^{-2}$

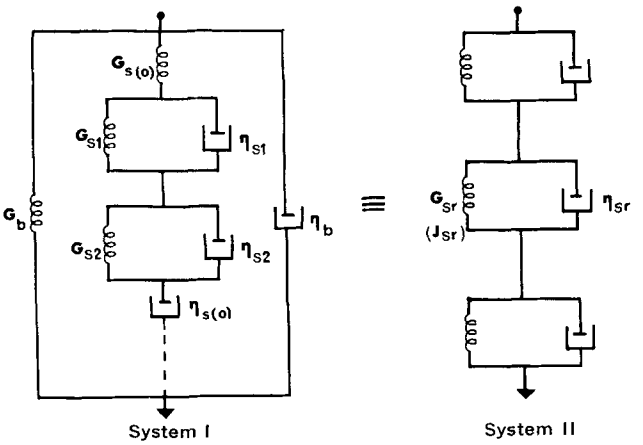


Figure 8—*Models for calculating viscoelastic data.*

G_{sr} = shear modulus of test sample, Voigt unit r, dyne cm.$^{-2}$
$G'(\omega)$ = real part of total complex modulus, at frequency ω rad. sec.$^{-1}$
$G''(\omega)$ = imaginary part of total complex modulus, at frequency ω rad. sec.$^{-1}$
$G^*(\omega)$ = complex modulus [$= G'(\omega) + iG''(\omega)$]
J_{sr} = compliance of test sample, Voigt unit r, cm.^{2} dyne^{-1}
$J'(\omega)$ = real part of complex compliance, at frequency ω rad. sec.$^{-1}$
$J''(\omega)$ = imaginary part of complex compliance, at frequency ω rad. sec.$^{-1}$
$J^*(\omega)$ = complex compliance [$= J'(\omega) + iJ''(\omega)$]
K_n = apparatus constants
S = scale reading
i = $\sqrt{-1}$
t = time
γ = strain
η_s = viscosity of Newtonian fluid, poise
η_b = viscous contribution associated with balance, poise
η_{sr} = viscosity of test sample, Voigt unit r
$\eta_{s(0)}$ = viscosity of uncoupled Newtonian dashpot in viscoelastic model
τ = total retardation time, sec.
τ_b = retardation time of balance, sec.
τ_s = retardation time for Newtonian fluid in apparatus
τ_r = retardation time for Voigt unit r ($= \eta_r/G_r$)
ω = frequency, rad. sec.$^{-1}$

REFERENCES

(1) S. H. Hwang, M. Litt, and W. C. Forsman, *Rheol. Acta*, **8,** 438(1969).
(2) P. C. Elmes and J. C. White, *Proc. Intern. Congr. Rheol., 2nd, 1953*, Butterworths, London, England, 1954, p. 382.
(3) W. B. Robertson, M. W. Ropes, and W. Bauer, *J. Biol. Chem.*, **133,** 261(1940).
(4) D. McClean and C. W. Hale, *Nature*, **145,** 867(1940).
(5) A. L. Sheffner, *Ann. N.Y. Acad. Sci.*, **106,** 298(1963).
(6) A. L. Sheffner, E. M. Medler, L. W. Jacobs, and M. P. Sarett, *Amer. Rev. Respirat. Diseases*, **90,** 721(1964).
(7) R. Munies, T. C. Grubb, and R. E. Caliari, *J. Pharm. Sci.*, **57,** 824(1968).
(8) P. E. Baldry and S. E. Josse, *Amer. Rev. Respirat. Diseases*, **98,** 392(1968).
(9) S. W. Simon and G. A. Harmon, *J. Allergy*, **32,** 493(1961).
(10) J. B. Armstrong and J. C. White, *Lancet*, **II,** 739(1950).
(11) C. Gernez-Rieux, G. Biserte, R. Havez, C. Voisin, P. Rousell, and P. Degand, *Acta Tuberc. Pneumol. Belg.*, **55,** 138(1964).
(12) J. Lieberman, *Amer. Rev. Respirat. Diseases*, **97,** 654(1968).
(13) R. Denton, *Pediatrics*, **25,** 611(1960).
(14) R. A. Bruce and K. C. Quinton, *Brit. Med. J.*, **1,** 282(1962).
(15) M. Tekeres and I. Geddes, to be published.
(16) J. E. Dippy and S. S. Davis, *Thorax*, **24,** 707(1969).
(17) C. E. Miller and H. Goldfarb, *Trans. Soc. Rheol.*, **9,** 135 (1965).
(18) R. E. Wells, R. Denton, and E. W. Merrill, *Lab. Clin. Med.*, **57,** 646(1961).
(19) G. Blanchard, *Arch. Middlesex Hosp.*, **5,** 222(1955).
(20) S. R. Hirsch and R. C. Kory, *J. Allergy*, **39,** 265(1967).
(21) S. R. Hirsch and L. H. Hamilton, *Amer. Rev. Respirat. Diseases*, **94,** 784(1966).
(22) R. Denton, W. Forsman, S. H. Hwang, M. Litt, and C. E. Miller, *ibid.*, **98,** 380(1968).
(23) S. Carson, R. Goldhamer, and M. S. Weinberg, *Ann. N.Y. Acad. Sci.*, **130,** 935(1966).
(24) S. S. Davis and J. E. Dippy, *Biorheology*, **6,** 11(1969).
(25) J. Sturgess, L. Reid, and J. Palfrey, to be published.
(26) D. E. McVean and A. M. Mattocks, *J. Pharm. Sci.*, **50,** 787 (1961).
(27) K. Berneis and K. Munzell, *Pharm. Acta Helv.*, **39,** 88(1964).
(28) J. R. Van Wazer, J. W. Lyons, K. Y. Kim, and R. E. Colwell, "Viscosity and Its Measurement," Interscience, London, England, 1963.
(29) B. Gross, "Mathematical Structure of the Theories of Viscoelasticity," Herman et Cie, Paris, France, 1953.
(30) B. Warburton and B. W. Barry, *J. Pharm. Pharmacol.*, **20,** 255(1968).
(31) C. H. Lindsley and E. K. Fischer, *J. Appl. Phys.*, **18,** 988 (1947).
(32) G. G. Stokes, *Trans. Cambridge Phil. Soc.*, **1,** 104(1850).
(33) H. Rouse and J. W. Howe, "Basic Mechanics of Fluids," Wiley, New York, N. Y., 1953.
(34) S. S. Davis, *J. Pharm. Sci.*, **58,** 418(1969).
(35) B. Warburton, thesis, London University, London, England, 1971.
(36) G. Levy, *J. Pharm. Sci.*, **51,** 947(1962).
(37) J. D. Ferry, "The Viscoelastic Properties of Polymers," Wiley, New York, N. Y., 1961.
(38) S. S. Davis and B. Warburton, *J. Pharm. Pharmacol.*, **20,** 836(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 14, 1970, from the * *Pharmaceutics Department, School of Pharmacy, University of London, London, England,* and the † *Pharmacology Department, Guy's Hospital Medical School, London, England.*

Accepted for publication July 14, 1970.

Kinetics of the Decomposition of a Mannich Base

J. A. MOLLICA, J. B. SMITH, I. M. NUNES, and H. K. GOVAN

Abstract □ The elimination reaction of 3-(4-*o*-methoxyphenyl-1-piperazinyl)-4′-morpholinopropiophenone dihydrochloride (Su 17595A) was studied over the pH range of 1–10. It appears that only the unprotonated form of the Mannich base undergoes elimination in the pH range of 3–10. The observed rate constant for elimination as a function of pH corresponds well with the dissociation curve for the compound. The value $k_1 = 7.15 \times 10^{-2}$ min.$^{-1}$ (60°) was obtained for the specific rate constant for elimination. The elimination reaction is reversible, and the extent of reaction is also a function of pH because the reverse reaction, the addition of 1-(*o*-methoxyphenyl)-piperazine to 4′-morpholinoacrylophenone, proceeds only with the unprotonated amine. The reaction does not remain at equilibrium, because competitive (*e.g.*, desaminomethylation) and/or consecutive (*e.g.*, hydration and dimerization) reactions also can occur. In strong acid solution, the elimination of the protonated form is acid catalyzed.

Keyphrases □ Mannich base—decomposition kinetics □ 3-(4-*o*-Methoxyphenyl-1-piperazinyl)-4′-morpholinopropiophenone dihydrochloride—elimination reaction □ TLC—separation □ NMR spectroscopy—structure

An increasing number of publications have appeared on the synthesis of Mannich bases with potential therapeutic application, and a recent review in this journal discussed the mechanistic and technological considerations of the Mannich reaction (1). Although it has been long recognized that Mannich bases can be decomposed by steam distillation to yield an amine and an α,β-unsaturated ketone, few studies investigating this reaction in detail have been reported. Some workers have studied the elimination of β-morpholinopropiophenone over the pH range of 5.6–9 in their studies on the application of polarography to reaction kinetics (2–4).

Riviere (5), in his studies on the reversibility of the Mannich reaction, found that three processes may occur, depending upon the reaction conditions: (*a*) desaminomethylation or reverse Mannich reaction; (*b*) deamination or elimination; and (*c*) if a free amine is added to the reaction, amine exchange between the β-aminoketone and the amine.

Angeloni and Tramontini (6) and Andrisano *et al.* (7), in their studies on Mannich bases, investigated the decomposition of β-aminoketone hydrochlorides in boiling water and reported that the reaction follows pseudo-first-order kinetics under these conditions.

Koshy and Mitchner (8) reported on the acid-catalyzed hydrolysis of 2-(4-phenyl-1-piperazinyl methyl)-cyclohexanone hydrochloride (MA 1050). The reaction was studied over the pH range 1.1–5.5 and was found to be pseudo-first-order in nature and specific acid and general base catalyzed.

In this article the authors report on the decomposition reaction over the pH range of 1–10 for the Mannich base 3-(4-*o*-methoxyphenyl-1-piperazinyl)-4′-morpholinopropiophenone dihydrochloride (Su 17595A). This reaction is novel and interesting. Although simple pseudo-first-order kinetics were expected according to several reports (2–4, 6, 8), (at pH values less than the pKa), the authors did not observe them.

EXPERIMENTAL

Materials—Su 17595A, m.p. 208–211° dec., was used.[1] Purity of the compound was determined by phase-solubility analysis and by nonaqueous titration with perchloric acid in the presence of mercuric acetate. All material used in the kinetic studies had a minimum purity of 99% by both methods. 4′-Morpholinoacrylophenone, m.p. 88°, was prepared by elimination from 3-dimethylamino-4′-morpholinopropiophenone. The NMR spectrum had bands at 3.30 and 3.84 (8H, 2 multiplets); 6.90 and 7.90 (4H, 2 doublets); and the acrylic hydrogens at 5.8, 6.4, and 7.2.

Anal.—Calcd. for $C_{13}H_{15}NO_2$: C, 71.92; H, 6.95; N, 6.44. Found: C, 71.72; H, 6.93; N, 6.42. This material was used to confirm the reaction product found in solution and to check the specificity of the assay procedure.[2] The 1-(*o*-methoxyphenyl)-piperazine dihydrochloride, m.p. 220° dec., had a single trace impurity by TLC. All other chemicals used were of reagent grade.

Buffers—Reagent grade chemicals were used in the preparation of all buffer solutions. Solutions were prepared at an ionic strength of 0.1 or were adjusted to an ionic strength of 0.1 with potassium chloride. The following buffers were used: pH 1–2, hydrochloric acid; pH 3, formate; pH 4–6, acetate; pH 6–8, phosphate; and pH 9–10, borate.

Ionization Constants—The pKa values of Su 17595A were determined spectrophotometrically and potentiometrically. Spectrophotometric determination of the dissociation constants was conducted according to the procedure of Albert and Serjeant (9).

The values obtained spectrophotometrically at 25° are $pKa_2 = 1.04$ and $pKa_1 = 6.54$. A pKa_1 of 6.7 was determined at 25° by potentiometric titration with NaOH in water. A pKa of 9.0 was determined for 1-(*o*-methoxyphenyl)-piperazine at 25° by potentiometric titration with NaOH in water.

Equipment—The pH values were measured with a Radiometer model 25 SE meter using a K401 calomel electrode and a G202B glass electrode. The meter–electrode system was standardized against the standard buffers recommended by Bates (10). Standardization of the meter and determination of pH values were carried out at 60°. Potentiometric titrations to determine substance purity and ionization constants were performed with a Radiometer titrator type TTT1a and titragraph type SBR2c. Spectrophotometric measurements were made on a Beckman model DU, and spectra were obtained with a Cary model 11 or 14 spectrophotometer. Constant-temperature baths were maintained at 60 ± 0.05°.

Assay Development and Kinetic Procedure—Su 17595A can be determined by UV absorbance at 330 mμ. The 1-(*o*-methoxyphenyl)-piperazine has no absorbance at this wavelength; however, the 4′-morpholinoacrylophenone does absorb at 330 mμ and it is necessary to separate it from Su 17595A. An extraction procedure utilizing CCl_4–$CHCl_3$ (4:1) and 0.1 *N* H_2SO_4 was employed; the 4′-morpholinoacrylophenone is quantitatively extracted into the organic phase and the Su 17595A remains in the aqueous phase. Recovery studies of Su 17595A in mixtures containing up to a twofold excess of 1-(*o*-methoxyphenyl)-piperazine show that this decomposition product does not interfere in the extraction procedure. Recovery tests using up to a twofold excess of 4′-morpholinoacrylophenone show the unsaturated ketone to be completely extracted into the organic phase.

[1] The synthesis of compounds of this class has been reported by Dr. G. de Stevens, South African patent 6705,794(1968).

[2] The authors thank Dr. J. Bishop for providing this compound.

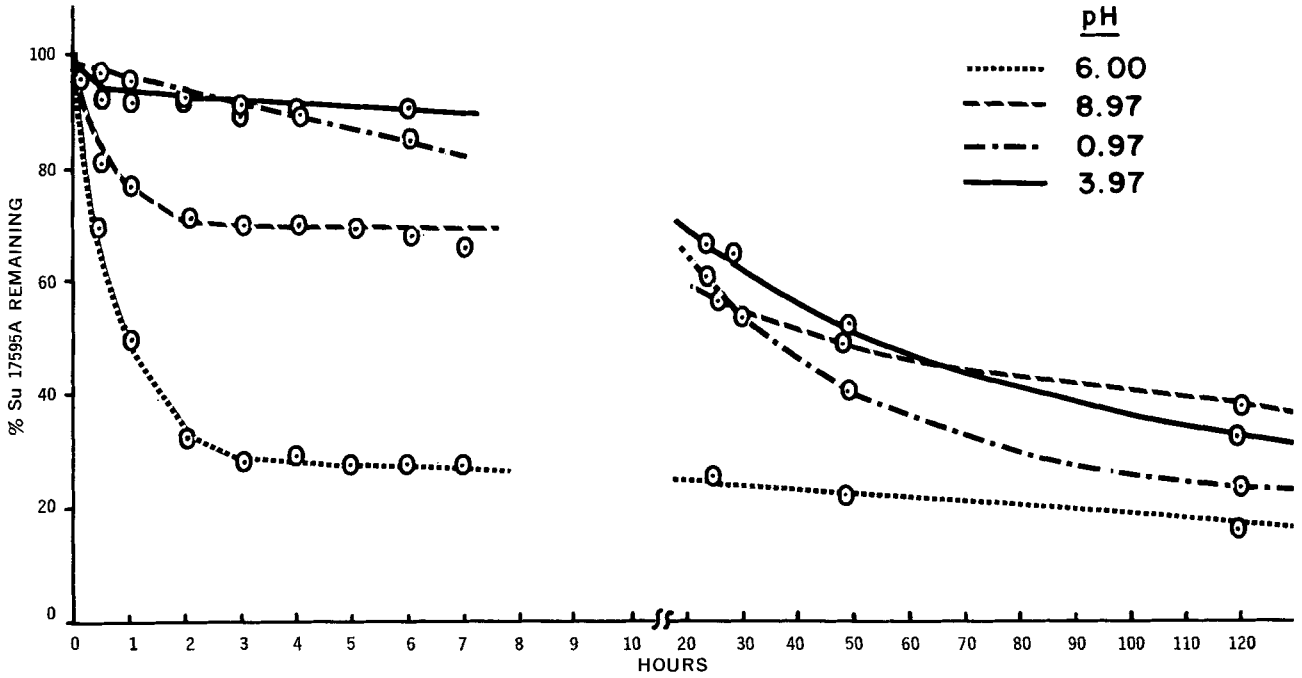


Figure 1—*Concentration of Su 17595A as a function of time at various pH values.* T = 60°.

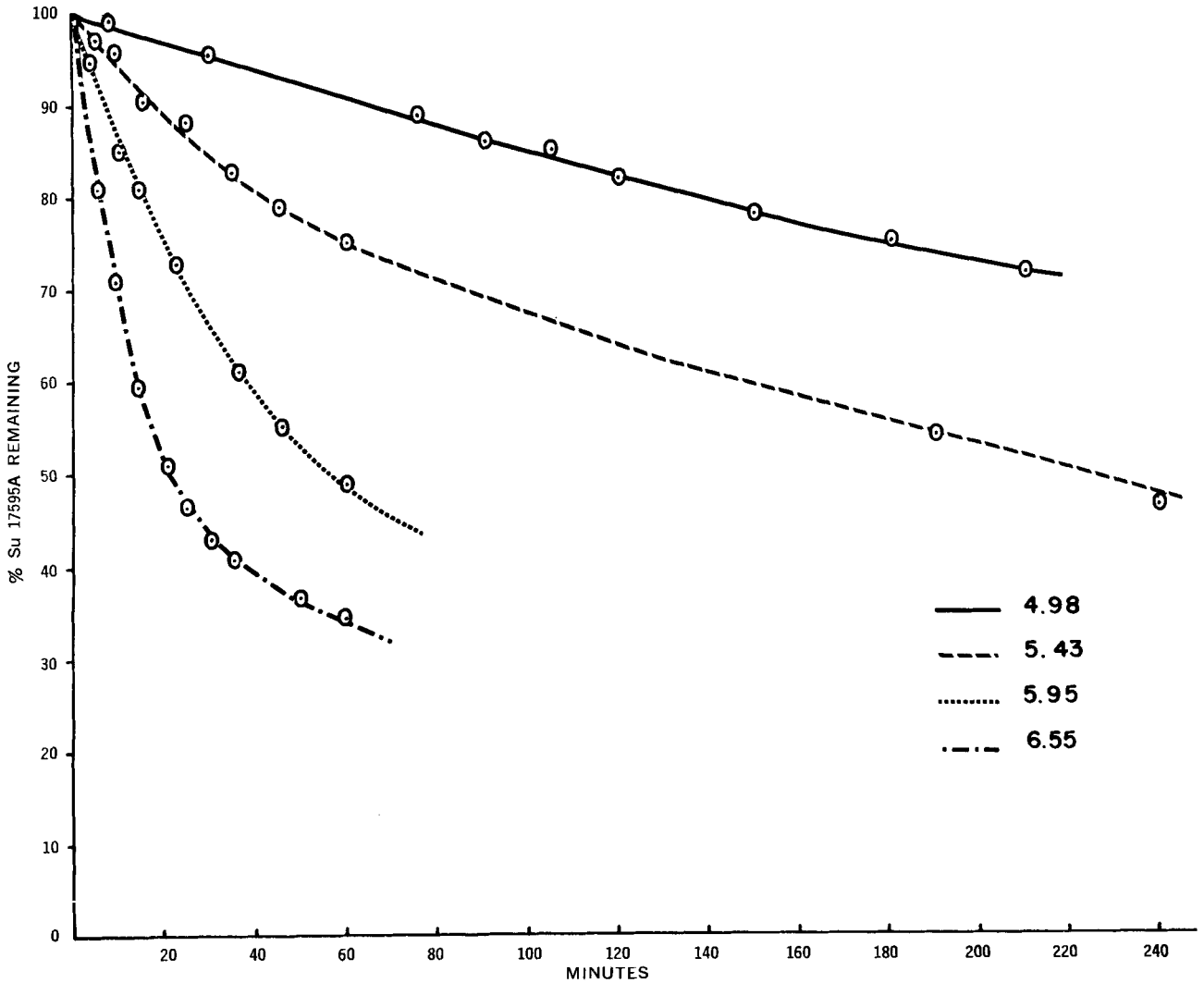


Figure 2—*Concentration* versus *time plots for the initial loss of Su 17595A at several pH values.* T = 60°; $C_0 = 4.15 \times 10^{-4}$ M.

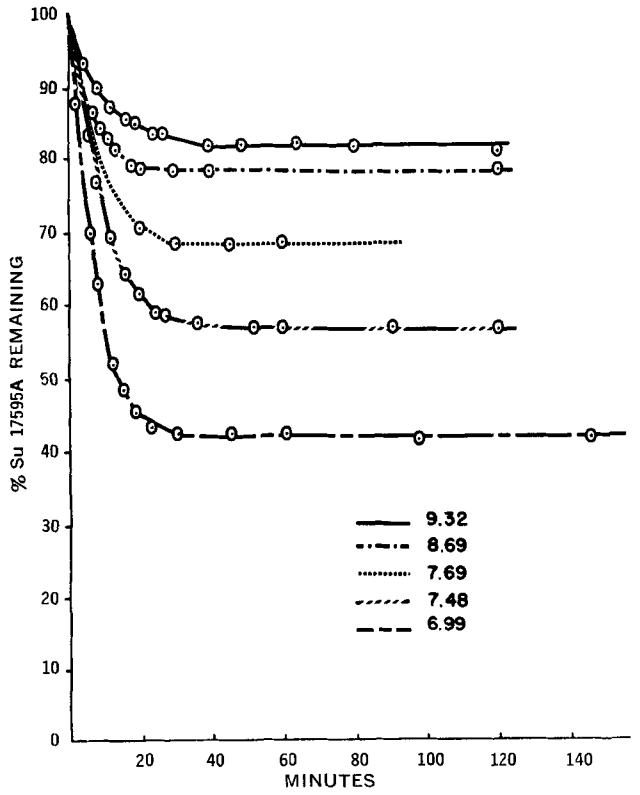
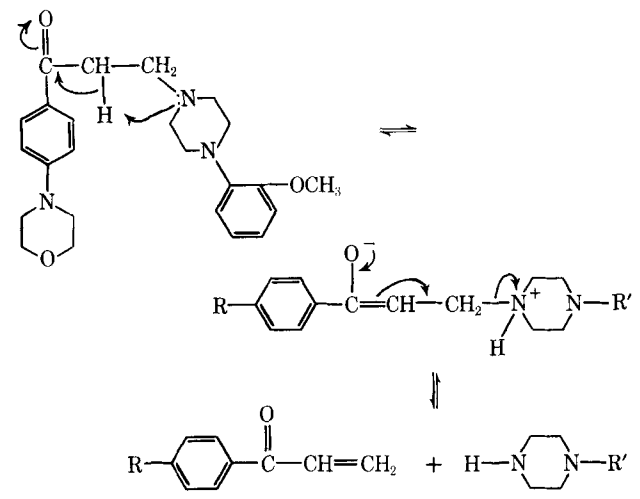


Figure 3—*Concentration* versus *time plots for the initial loss of Su 17595A and the attainment of equilibrium at several pH values.* T = 60°; $C_0 = 4.15 \times 10^{-4}$ M.

The following procedure was utilized throughout the study: a 20-mg. sample of Su 17595A was accurately weighed and transferred to a 200-ml. volumetric flask. To this was added 100 ml. of a buffer which had been equilibrated at 60°. The solution was rapidly mixed and placed in the 60° bath. A 5.0-ml. aliquot was transferred into a 125-ml. separator containing 15 ml. of the CCl_4–$CHCl_3$ solvent, 25 ml. of 0.1 *N* H_2SO_4, and sufficient 1 *N* H_2SO_4 to adjust the pH of the aqueous phase to a value of 1. The solution was cooled to room temperature to stop the reaction. The solution was shaken and the organic phase discarded. The extraction was repeated with three additional 15-ml. portions of CCl_4–$CHCl_3$; the aqueous layer was then filtered through cotton into a 50-ml. volumetric flask. The funnel was washed with two 5-ml. portions of 0.1 *N* H_2SO_4, and these washings were added to the volumetric flask. The solution was diluted to volume with 0.1 *N* H_2SO_4, and the absorbance was determined at 330 mμ against a 0.1 *N* H_2SO_4 blank.



Scheme I

Rate constants ($k_1^{obsd.}$) were obtained from the initial slopes of plots of log [Su 17595A] *versus* time and, in those instances where "equilibrium" was obtained, also from plots of

$$\log \left[\frac{1 - (1 - f_e)(1 - f)}{(1 - f) - (1 - f_e)} \right] \text{ versus } t$$

where f is the fraction reacted at the time t, and f_e is the fraction reacted at equilibrium.

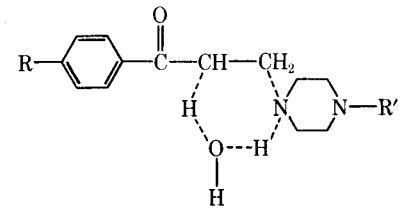
RESULTS AND DISCUSSION

The concentration *versus* time plots obtained were not amenable to simple interpretation. Figure 1 depicts the type of data obtained over a 5-day period. The reaction, in at least the intermediate pH region, appeared to approach a state of "quasiequilibrium," and the extent of reaction appeared to be a function of pH. Figures 2 and 3 show the initial loss of Su 17595A in the pH range of 4–9.

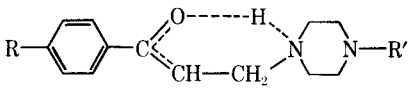
A mechanism consistent with such data indicates that only the unprotonated form of the Mannich base undergoes elimination and that only the unprotonated amine undergoes conjugate addition to the α,β-unsaturated ketone in the reverse reaction. It has been suggested (4) that in intermediate pH ranges, the elimination proceeds through intramolecular catalysis (Scheme I).

Analogous pathways, one of which incorporates a molecule of water, have been formulated using the two transition states, I and II.

Figure 4—*Plot of log* $k_1^{obsd.}$ versus *pH for elimination of Su 17595A. The points are experimental, and the solid line is a theoretical fit using the values in the text.*



I



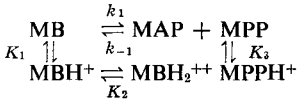
II

These two transition states would also give rise to the same products, and all three are kinetically indistinguishable.

The rate of loss of Su 17595A in accord with Scheme I can be expressed as follows:

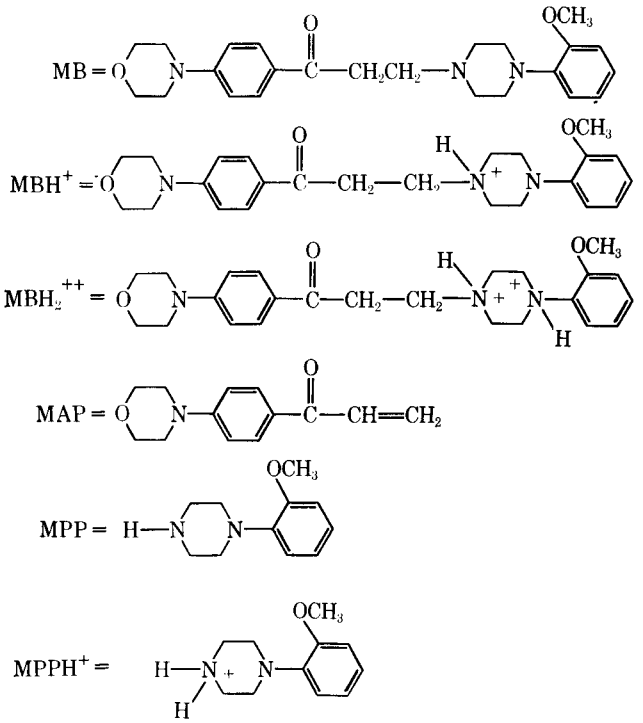
$$-d[MB_t]/dt = \frac{k_1[MB_t]}{1 + [H^+]/K_1} - \frac{k_{-1}[MAP][MPP]}{1 + [H^+]/K_3} \quad \text{(Eq. 1)}$$

where $[MB_t]$ = stoichiometric concentration of Su 17595A, [MPP] = stoichiometric concentration of 1-(*o*-methoxyphenyl)-piperazine, [MAP] = concentration of 4′-morpholinoacrylophenone, and the constants are as defined in Scheme II:



Scheme II

where



and K_1, K_2, and K_3 are the acid-dissociation constants; and k_1 and k_{-1} are rate constants for the forward reaction and reverse reaction, respectively.

The rate constant for the forward reaction (elimination reaction), $k_1^{obsd.}$, is given by the following equation:

$$k_1^{obsd.} = \frac{k_1}{1 + [H^+]/K_1} \quad \text{(Eq. 2)}$$

Figure 4 gives a plot of log $k_1^{obsd.}$ *versus* pH for the forward reaction.

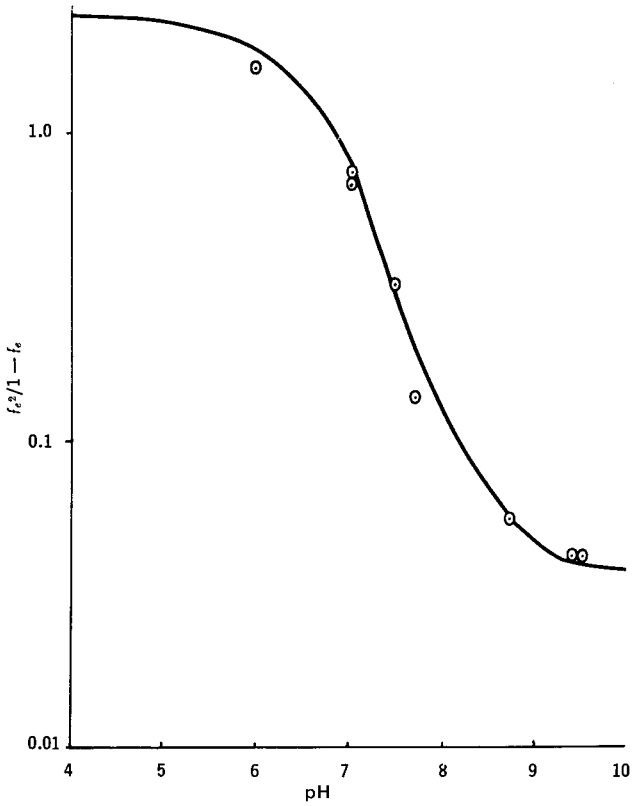


Figure 5—*Plot relating the fraction decomposed* (f_e) *to pH. The points are experimental, and the solid line is the theoretical line according to Eq. 3, using the following values:* $K_1 = 2.6 \times 10^{-7}$, $K = 1.5 \times 10^{-5}$, $K_3 = 4 \times 10^{-9}$, *and* $C_0 = 4.15 \times 10^{-4}$ M.

The solid line is drawn according to Eq. 2 using the values $k_1 = 7.15 \times 10^{-2}$ min.$^{-1}$, which is the average value calculated from the observed rate constants, and $K_1 = 2.6 \times 10^{-7}$, the dissociation constant, determined as described in the *Experimental* section. The experimental points, as can be seen, are in excellent agreement with the theoretical line.

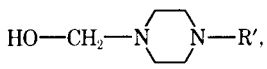
If Eq. 1 is operative and the predominant pathway for the reaction is as outlined, then at equilibrium one could write:

$$\frac{f_e^2}{1 - f_e} = \frac{K}{C_0} \cdot \frac{K_1}{K_3} \cdot \frac{[K_3 + [H^+]]}{[K_1 + [H^+]]} \quad \text{(Eq. 3)}$$

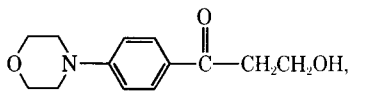
where f_e = fraction of Su 17595A decomposed at equilibrium, $K = k_1/k_{-1}$, K_1 and K_3 are as described previously, and C_0 = initial concentration of Su 17595A [*M*].

The extent of reaction was estimated from plots such as are given in Figs. 2 and 3, and the "equilibrium" values were plotted as a function of pH. As can be seen in Fig. 5, the values obtained from the initial plateau are in excellent agreement with the theoretical line according to Eq. 3. This lends further support to the hypothesis that at least the initial loss is primarily by this pathway.

The authors are not able to identify or isolate the amino alcohol,



in the reaction mixture. This would be the expected product of desaminomethylation or reverse Mannich reaction and has been reported by Riviere (5). The β-keto alcohol, which can arise from



the hydration of the α,β-unsaturated ketone, was detected in the reaction mixtures. The hydration and/or polymerization of the α,β-unsaturated ketone are the most likely driving forces which shift the equilibrium of the reaction.

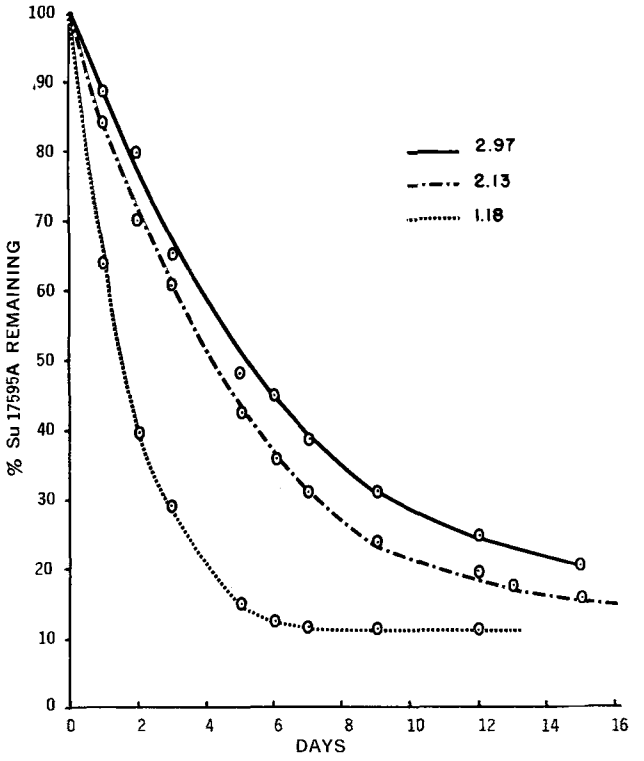


Figure 6—*Concentration* versus *time plots for the loss of Su 17595A in acidic solution.* T = 60°; $C_0 = 4.15 \times 10^{-4}$ M.

A kinetically equivalent scheme would be a bimolecular reaction involving hydroxide-ion catalysis of the protonated Mannich base. However, this would require a specific rate constant greater than 10^{-5} M^{-1} min.$^{-1}$, and one would expect significant buffer catalysis which was not observed.

In stronger acid solution (pH < 2), a mechanism consistent with the data could be as shown in Scheme III.

If there are no competitive or consecutive reactions, all equilibria shown in Scheme II would have to be satisfied. According to Eq. 1, even in solutions at pH 6 where the amine, 1-(*o*-methoxyphenyl)-piperazine, is completely protonated, the reaction does not go to completion. This can be seen in Fig. 6 where even at pH 1–2, 10–20% is still remaining at 10 days. (Equation 3 predicts a maximum of 80% degradation if only elimination is considered.)

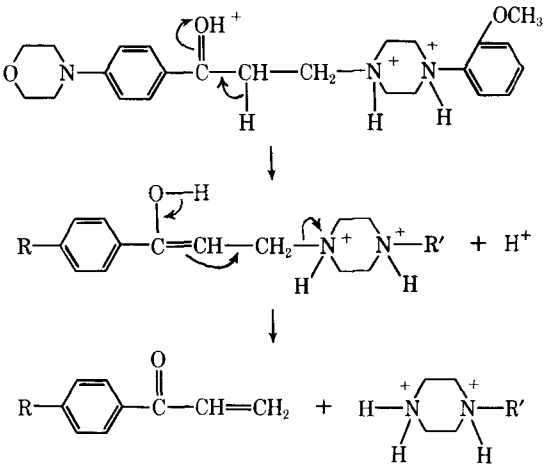
SUMMARY

1. Over a wide range of pH values, the principle route of decomposition appears to be elimination from the unprotonated Mannich base. These data are in excellent agreement with a theoretical curve constructed using the dissociation constant of the Mannich base.

2. This hypothesis is given further support in that the reversibility of the reaction is pH dependent and the extent of reaction can be correlated with the dissociation constant of the Mannich base. The reverse reaction, addition of the amine to the α,β-unsaturated ketone, can be correlated with the dissociation constant of 1-(*o*-methoxyphenyl)-piperazine.

3. Other pathways, such as desaminomethylation, are undoubtedly operative to a small extent as well as a bimolecular pathway for the elimination. The reaction is driven past equilibrium due to consecutive reactions of the α,β-unsaturated ketone such as hydration and dimerization.

4. In strong acid solution, hydrogen-ion catalysis of both the singly and doubly protonated forms (pk_2 = 1.04) is undoubtedly the major pathway.



Scheme III

REFERENCES

(1) B. B. Blackburn, *J. Pharm. Sci.*, **57**, 715(1968).

(2) P. Zuman and V. Horak, *Coll. Czech. Chem. Commun.*, **27**, 187(1962).

(3) P. Carsky, P. Zuman, and V. Horak, *ibid.*, **29**, 3044(1964).

(4) P. Zuman, in "Advances in Physical Organic Chemistry," vol. 5, Academic, New York, N. Y., 1967, pp. 1–50.

(5) H. Riviere, *Ann. Chim. (Paris)*, **5**, 1273(1960).

(6) A. S. Angeloni and M. Tramontini, *Ann. Chim. (Rome)*, **54**, 745(1964).

(7) R. Andrisano, A. S. Angeloni, and M. Tramontini, *ibid.*, **55**, 652(1965); *ibid.*, **55**, 143(1965).

(8) K. T. Koshy and H. Mitchner, *J. Pharm. Sci.*, **53**, 1381 (1964).

(9) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Wiley, New York, N. Y., 1962.

(10) R. G. Bates, *J. Res. Nat. Bur. Std.*, **66A**, 179(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 23, 1970, from the *Development and Control Department, Ciba Pharmaceutical Company, Summit, NJ 07901*

Accepted for publication June 23, 1970.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

Kinetics of Elimination of Optical Isomers of Mandelic Acid and Effect of Probenecid on Their Elimination Kinetics in Humans

JANARDAN B. NAGWEKAR* and HARRY B. KOSTENBAUDER†

Abstract □ Kinetics of metabolism and urinary excretion of the optical isomers of mandelic acid have been studied in three human subjects, and the rate constants have been determined for a model consisting of parallel apparent first-order processes for excretion of intact mandelic acid and metabolism to benzoylformic acid. The kinetic studies revealed no significant difference in the rate of urinary excretion of L-(+)- and D-(−)-mandelic acid, but there was a difference in the rate of metabolism of these two isomers in the subjects. The rate constant for metabolism of L-(+)-mandelic acid was approximately twice that for the metabolism of D-(−)-mandelic acid. The inhibitory effect of probenecid on urinary excretion of the optical isomers of mandelic acid suggests that both isomers are involved in active renal tubular secretion.

Keyphrases □ Mandelic acid optical isomers—elimination kinetics □ Probenecid effect—mandelic acid optical isomers elimination □ Metabolism—mandelic acid optical isomers □ GLC—analysis

Although metabolism and urinary excretion of mandelic acid have been the subjects of considerable investigation in both humans and lower animals for more than 50 years, there has been no previous study of the kinetics of these processes. Schotten (1), Knoop (2), Neubauer and Fisher (3), and Quick (4) showed that mandelic acid fed to dogs was excreted unchanged in the urine. Similar observations were made in humans by Rosenheim (5). However, Wrede (6), Scholz (7), and Montenbruck (8) claimed that mandelic acid undergoes significant biotransformation both in man and dogs. Prior to 1938 the urinary excretion of only the racemic mandelic acid was studied, but Wrede (6), Scholz (7), and Montenbruck (8) also investigated the excretion of the individual optical isomers of mandelic acid. Montenbruck (9) extensively investigated the fate of individual optical isomers of mandelic acid in man. He found that 65% of the administered dose of L-(+)-mandelic acid was excreted in the urine as intact dextrorotatory mandelic acid, 14% as benzoylformic acid, and 7% as levorotatory phenylaminoacetic acid. From his studies with D-(−)-mandelic acid, Montenbruck observed that 73% of the administered dose was excreted as intact levorotatory mandelic acid, 7% as benzoylformic acid, and only traces as levorotatory phenylaminoacetic acid. Montenbruck also suggested that benzoylformic acid formed in the body by oxidation of either optical isomer of mandelic acid was oxidized to yield about 2% benzoic acid.

Gary and Smith (10) investigated the urinary excretion of racemic mandelic acid in decerebrate cats, and they reported that the initial urine samples showed a preponderance of levorotatory mandelic acid and that the later urine samples contained mainly the dextrorotatory mandelic acid. Similar differing excretion of optical isomers of mandelic acid was also observed in humans by Klingmüller and Brune (11), who reported that 74% of the administered dose of mandelic acid appeared in the urine in 24 hr., regardless whether the drug was administered orally or intravenously. Neish (12) reported that 13% of the administered dose of racemic mandelic acid was excreted in the urine as benzoylformic acid within 24 hr.

All these investigators used a polarimetric method to determine mandelic acid excreted in the urine. This method, however, was shown to exhibit an error as high as 15% (13). Other analytical methods reported for mandelic acid include gravimetric (13), iodometric (14), polarographic (15), and cerimetric (16). With the exception of the gravimetric method, these procedures involve conversion of mandelic acid to benzaldehyde and are not convenient for estimation of intact acid excreted in the urine. Williams (17) demonstrated that mandelic acid can be separated from urinary aromatic acids by gas chromatography on an 8% ethylene glycol adipate column.

The isomers of many optically active drugs are known to show differences in the extent of their metabolism and in the pharmacological responses they elicit upon administration to humans. These differences could be attributed to their different rates of absorption, metabolism, and urinary excretion. Since a significant fraction of an oral dose of mandelic acid is excreted in the urine as intact drug, this compound provides a convenient model for the investigation of possible differences in the rates of metabolism and urinary excretion. Also, many organic acids are actively secreted (18) by the renal

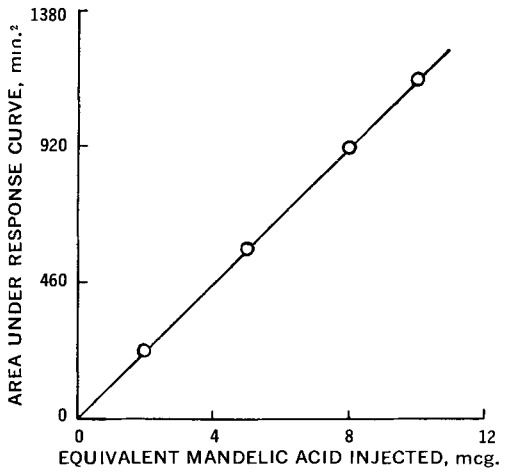


Figure 1—*Calibration curve for methyl ester of mandelic acid. Conditions: range, 10; attenuation, 64×; injection port temperature, 220°; column temperature, 180°; detector temperature, 250°; and helium flow, 80 ml./min.*

tubules; therefore, it is conceivable that this active transport might also show specificity with respect to the optical isomers of this model compound.

The specific objectives of the present study were to: (*a*) develop a gas chromatographic procedure that allowed the determination of both intact mandelic acid and its metabolites; (*b*) utilize the urinary excretion data to study the kinetics of, and determine the rate constants for, metabolism and urinary excretion of individual optical isomers of mandelic acid for the purpose of determining any differences in the metabolism and urinary excretion of the respective isomers in humans; and (*c*) determine the effect of probenecid on the excretion kinetics of the individual isomers of mandelic acid as a test for difference in active renal tubular transport for the two isomers.

EXPERIMENTAL

Materials—The following were used: L-(+)-mandelic acid,[1] recrystallized, m.p. 132–133°, $[\alpha]_D^{25}$ + 153°; D-(−)-mandelic acid,[1] recrystallized, m.p. 132–133°, $[\alpha]_D^{25}$ − 154°; benzoylformic acid,[1] m.p. 64°; benzoic acid USP,[1] m.p. 122°; and probenecid (500 mg).[2]

Apparatus—The gas chromatograph (F & M model 810) was equipped with a hydrogen-flame detector. The column used was a copper tube, 1.83 m. (6 ft.) long and 0.63 cm. (0.25 in.) outside diameter, packed with diatomaceous earth[3] coated with 8% ethylene glycol adipate. Helium was used as the carrier gas. A Beckman model G pH meter was used for all pH determinations.

Subjects and Test Procedures—Three, apparently healthy, male adult volunteers participated in this study. Each subject, after overnight fasting, ingested a given dose of an appropriate isomer of mandelic acid, dispensed in hard gelatin capsules, with about 250 ml. water. The subject was advised against eating anything for at least 2 hr. after ingesting the drug, but no dietary restrictions were imposed. Each subject collected a blank urine sample prior to ingestion of the drug. Following the ingestion of mandelic acid, urine was collected quantitatively at an interval of 1 hr. for 6 hr., at 2-hr. intervals up to 12 hr., and at longer intervals up to 48 hr. The urine samples were allowed to attain room temperature; then volume and pH were measured, and the samples were stored in the refrigerator until the time they were analyzed. No attempt was made to control the pH of urine during excretion studies. The pH of urine generally remained about 6 ± 0.5 throughout the study. Each subject ingested each of the isomers of mandelic acid in separate studies, generally after an interval of 1 week.

When the studies were carried out with probenecid, the subject ingested 1 g. of an appropriate isomer of mandelic acid after overnight fasting and collected urine quantitatively every hour for 5 or 6 hr. At the end of this period, immediately after collecting the urine sample, the subject ingested a hard gelatin capsule containing a powdered, 250 or 500 mg., probenecid tablet. The subject was advised to eat a light breakfast 2 hr. after ingestion of the dose of mandelic acid and was also advised against eating anything for at least 1 hr. after ingesting the dose of probenecid. Hourly urine samples were collected for another 5 or 6 hr. after ingestion of the probenecid, and subsequent urine samples were collected at longer intervals of time for 36–48 hr. The volume and pH of urine samples were recorded in the usual manner.

Analytical Method—Prior to analysis, urine samples were removed from the refrigerator and allowed to attain room temperature. An appropriate volume of each urine sample was pipeted into a suitable separator, 2.5 ml. of 5 *N* HCl was added to it, and mandelic acid and its metabolites were completely extracted with ether. The volume of the ether used for each of four extractions was about twice the volume of the aqueous phase. Each of the ether extracts was transferred to a 50-ml. beaker, and the ether was evaporated on a water bath at 55°. The residue was dissolved in 2 ml. methanolic sulfuric acid reagent (30 ml. concentrated sulfuric acid diluted to 200 ml. with anhydrous methanol) and transferred quantitatively to a 25-ml. glass-stoppered volumetric flask. The beaker was rinsed with two additional 2-ml. volumes of the reagent to transfer quantitatively the residue from the beaker to the flask. The flask was then stoppered, and the bulb of the flask was half-immersed in an oil bath at 75°, leaving the rest of the flask exposed to the atmosphere to serve as an air condenser for the methanol. After refluxing for 2 hr., the contents of the flask were cooled to room temperature, diluted with 5 ml. of distilled water, and transferred quantitatively to a 60-ml. separator with the aid of 20 ml. ether. After shaking and equilibration, the ether layer was transferred to a 50-ml. beaker and evaporated on a water bath at 55°. The aqueous alcoholic layer was further extracted three times with 20-ml. portions of ether, and the ether extracts were treated as described previously. The evaporation of ether was discontinued when approximately 0.3 ml. solution remained in the beaker. The residue was quantitatively transferred to a graduated test tube by rinsing the beaker with small portions of anhydrous methanol, and volume was adjusted to 5 ml. One microliter of this solution was injected onto the gas chromatographic column. The conditions employed for the gas chromatographic analysis were similar to those described elsewhere (19).

Blank urine samples of all participants in these excretion studies were treated in exactly the same manner as the other samples, and 1 μl. of the methanol extract was injected onto the column.

Fifty-milliliter solutions, each containing 10, 25, 40, or 50 mg. of mandelic acid, were prepared in distilled water; the solution was adjusted to about pH 2 with hydrochloric acid. These samples were extracted with ether, the acid esterified with the methanolic sulfuric acid reagent, and prepared for gas chromatographic analysis in the same manner as the urine samples. These samples were prepared to construct a calibration curve for mandelic acid (Fig. 1).

RESULTS AND DISCUSSION

Gas Chromatographic Method for Quantitative Determination of Mandelic Acid and Its Metabolites—To render them suitable for gas chromatographic analysis, carboxylic acids are routinely converted to their methyl esters. Various agents reported for methylating carboxylic acids include diazomethane (17, 19, 20), BF_3-methanol (21–23), and dry methanolic HCl (24). Prior to the use of diazomethane as an esterifying agent of mandelic acid and its metabolites for their gas chromatographic analysis by Kamienny *et al.* (19) and Randinitis *et al.* (20), the method described here was used to esterify the acids. Considering the simplicity of preparation and use of dry methanolic HCl, this method was employed to esterify mandelic acid, but the low yield of the ester under the conditions employed necessitated development of another suitable reagent, namely, dry methanolic sulfuric acid. As evidenced in Fig. 1, 2 hr. of refluxing mandelic acid with this reagent offered a reproducible yield of the ester.

The gas chromatograms obtained for blank urine samples of all the participants of these excretion studies revealed complete absence of a peak at that retention time (12.2 min.) where the peak for methyl mandelate appears. Small peaks appeared at retention times between 4 and 6 min. This situation afforded an effective means of estimating benzoylformic acid, the principal metabolite of mandelic acid, which was previously identified by forming a 2,4-dinitrophenylhydrazone derivative in the urine samples voided after ingesting mandelic acid (12). Benzoylformic acid was prepared for gas chromatographic analysis in the same manner as mandelic acid, and a standard calibration curve was constructed. The retention observed for methyl benzoylformate was 8.2 min.

The blank urine sample treated with methanolic sulfuric acid rarely showed a peak at a retention time of 2.2 min., but the urine samples voided after ingesting mandelic acid and treated with the reagent often showed a peak at this retention time. Since methyl benzoate[4] exhibits a peak at this retention time, such a peak in a chromatogram was attributed to benzoic acid, which appears in the urine as the ultimate oxidation product of mandelic acid. A standard curve was also constructed for methyl benzoate.

Mandelic acid and its metabolites are thus extracted together from a urine sample and are esterified in a single esterification step with the methanolic H_2SO_4 reagent. Finally, when the ester extract

[1] Aldrich Chemical Co., Inc., Milwaukee, WI 53210
[2] Benemid tablets, Merck Sharpe and Dohme.
[3] Diatoport S, 80–100 mesh.
[4] Eastman Organic Chemicals, Rochester, N. Y.

Table I—Summary of Urinary Excretion Recovery Data Obtained in Human Subjects after Oral Administration of Each Optical Isomer of Mandelic Acid in the Absence and in the Presence of Orally Administered Probenecid

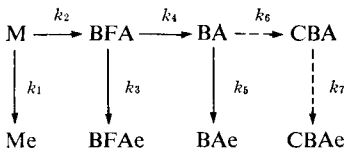
Subject	Oral Dose, mg.		Equivalent Mandelic Acid Excreted as—				Total Mandelic Acid Excreted, mg.
	Mandelic Acid	Probenecid	Intact Mandelic Acid, mg.	Benzoylformic Acid, mg.	Benzoic Acid, mg.	Percent Metabolized[a]	
			D-(−)-Mandelic Acid Alone				
A	1000	—	922	79	9	8.7	1010
B	990	—	878	65	16	8.4	959
C[b]	995	—	721	85	21	12.9	827
			L-(+)-Mandelic Acid Alone				
A	1000	—	801	208	—	20.6	1009
B	1000	—	848	147	23	16.7	1018
C[b]	1005	—	756	159	22	19.6	937
			D-(−)-Mandelic Acid + Probenecid[c]				
B	1015	250	978	66	—	6.3	1044
B	1420	500	1270	123	4	9.0	1397
			L-(+)-Mandelic Acid + Probenecid[c]				
B	1000	250	842	174	16	18.4	1032
B	972	250	701	174	5	20.2	880
B	1545	500	1270	304	27	20.7	1601
C[b]	1000	250	712	180	11	21.0	903

[a] The percentage is based on the total amount of mandelic acid recovered in the urine. [b] The subject inadvertently drank milk along with mandelic acid, which may account for incomplete recovery of mandelic acid. [c] Probenecid was administered 4.5 or 6 hr. after the ingestion of mandelic acid (Fig. 2).

is injected onto the polar column, the esters are separated on the column due to the difference in their polarity. Thus, a single injection of the sample makes the quantitative determination of intact mandelic acid and its metabolites possible, as previously shown (19). The area under the response curve for each of the respective acids was directly proportional to its concentration. The area under the response curve was calculated by the trapezoidal rule (25).

To determine accurately the amount of mandelic acid and its metabolites present in the urine samples, calibration curves of concentration *versus* area under the response curve for the respective acids were constructed each time the urine samples were analyzed. This analytical procedure produced results accurate to ±5%.

Model for Excretion Kinetics of Mandelic Acid and Determination of Rate Constants—The recovery of the administered dose of each optical isomer of mandelic acid was complete in the majority of the studies (Table I). Preliminary data obtained for excretion of intact mandelic acid following the oral administration of the respective isomer indicated that the elimination of the acid proceeded by an apparent first-order process. From the information thus obtained about the fate of mandelic acid in the human subjects, Scheme I was formulated to describe a kinetic scheme for metabolism of mandelic acid and its metabolites after absorption of drug and attainment of apparent equilibrium between the drug in blood and the drug in other fluids of distribution. In this scheme, M, BFA,



Scheme I

BA, and CBA are the respective amounts of mandelic acid, benzoylformic acid, benzoic acid, and conjugates of benzoic acid in the body at any time; Me, BFAe, BAe, and CBAe are the amounts of the respective compounds excreted in the urine at any time; and k_i (i = 1...7) represents apparent first-order rate constants for the respective processes indicated in the model. From 0 to 2% of the administered dose of D-(−)-mandelic acid and from 1 to 2% of the administered dose of L-(+)-mandelic acid were recovered in the urine as benzoic acid. Although glycine or glucuronic acid conjugates of benzoic acid are likely to be formed in the body (26), the extent of conjugation is considered negligible, since the amount of benzoic acid formed in the body is very small. Therefore, the conjugation step in the model is indicated by a broken line.

One primary purpose of the present studies was to determine the rate constants for metabolism and urinary excretion of the optical isomers of mandelic acid from the urinary excretion data. Therefore, the following differential equations were considered:

$$d\mathrm{Me}/dt = k_1\mathrm{M} = k_1\mathrm{M}_T e^{-k(t-\mathrm{T})} \quad \text{(Eq. 1)}$$

$$\log \Delta\mathrm{Me}/\Delta t = \log k_1\mathrm{M}_T - k(t - T)/2.303 \quad \text{(Eq. 2)}$$

where $\Delta\mathrm{Me}/\Delta t$ is the rate of urinary excretion of intact mandelic acid (mg./hr.); k, which is equal to $k_1 + k_2$, is the overall rate constant for disappearance of the drug from the body [referred to in recent literature as the disposition-rate constant (26)]; k_1 is the fraction of k attributable to processes leading to urinary excretion of intact drug; k_2 is the fraction of k attributable to processes leading to conversion of mandelic acid to the primary metabolite, benzoylformic acid; M_T is the amount of intact mandelic acid in the body at time T after oral administration of the drug; and T is the time period (hours) during which apparent complete absorption of drug occurred from the gastrointestinal tract and at the time, T, excretion of the drug became apparent first-order. The log $\Delta\mathrm{Me}/\Delta t$ was plotted against t and the rate constant, k, determined from the resulting straight line (least squares) obtained for the data after T hr. The T was generally less than 2 hr. in Subjects A and B, but it was 3–4 hr. in Subject C, who inadvertently drank milk along with the drug (Fig. 2). The time t in Figs. 2 and 3 represent the midpoints of the urinary collection intervals. The values of k_1 and k_2 were determined as follows:

$$k_1 = k \cdot f \quad \text{(Eq. 3)}$$

$$k_2 = k - k_1 \quad \text{(Eq. 4)}$$

$$f = \frac{\mathrm{Me}_\infty}{\mathrm{Me}_\infty + \mathrm{BFAe}_\infty + \mathrm{BAe}_\infty} \quad \text{(Eq. 5)}$$

where Me_∞, BFAe_∞, and BAe_∞ are the equivalent amounts of mandelic acid excreted in the urine as intact mandelic acid, benzoylformic acid, and benzoic acid during 48 hr., the experimental infinite time.

Comparison of Rate Constants for Metabolism and Urinary Excretion of Optical Isomers of Mandelic Acid—Comparison of data for the isomers of mandelic acid, as illustrated in Table II, indicates no significant differences in values of k and k_1. However, the value of k_2 in all subjects is found to be greater for L-(+)-mandelic acid than for D-(−)-mandelic acid. Table I also indicates that in all subjects the recovery of the metabolites for L-(+)-mandelic acid was about twice the recovery of metabolites observed for D-(−)-mandelic acid. Clearly, k_2 is a hybrid rate constant, involving processes of distribution as well as a specific rate of conversion of intact drug to benzoylformic acid at the metabolic site. However,

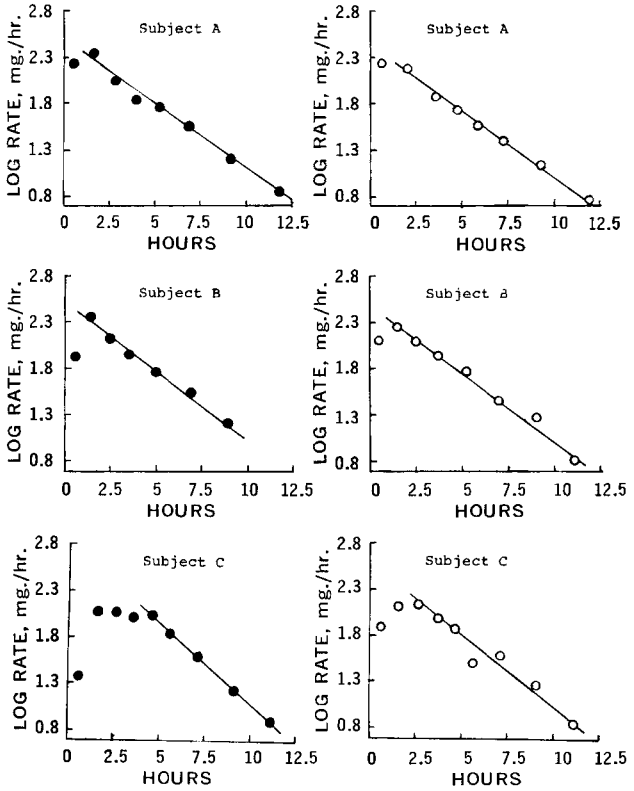


Figure 2—*Apparent first-order urinary excretion of* D-(−)-*mandelic acid* (●) *and* L-(+)-*mandelic acid* (○) *in Subjects A, B, and C. The prolonged periods for absorption of mandelic acids reflected in the plots of Subject C are probably caused by milk inadvertently drunk by the subject along with mandelic acid.*

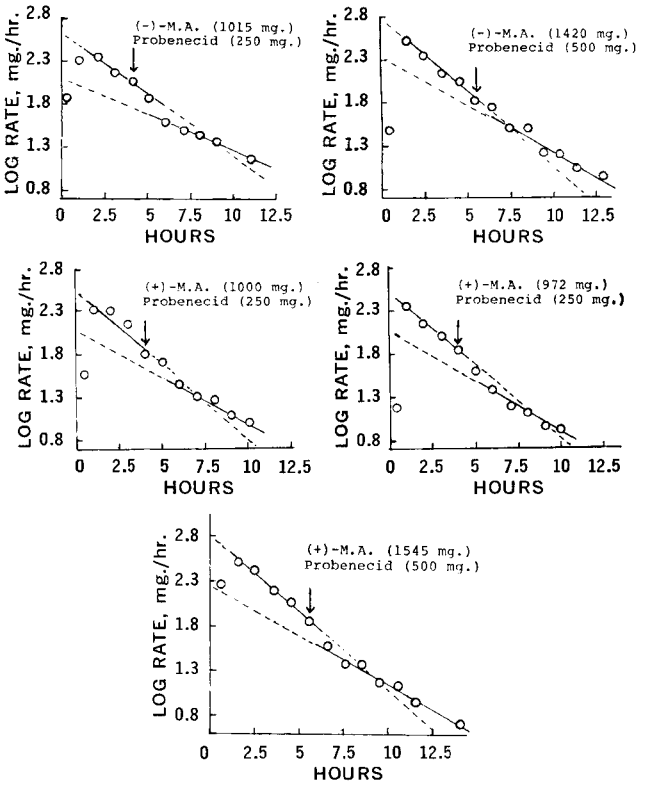


Figure 3—*Effect of probenecid on the urinary excretion of the optical isomers of mandelic acid (M.A.) in Subject B. The arrow indicates the time at which probenecid was ingested by the subject. The numbers in parentheses represent the doses of the corresponding compounds. The isomer of mandelic acid was ingested at zero time.*

since L-(+)- and D-(−)-mandelic acid have similar physical properties, if it is assumed that distribution within the body is accomplished primarily by processes other than active transport, it is probable that differences in k_2 for the two isomers do, in fact, represent differences in the rate at which intact drug is transformed at the metabolic site. It may then be concluded that, while the oxidative enzyme(s) show apparent differential affinity for the optical isomers of mandelic acid, the tubular "carrier" system does not show similar differential affinity for renal tubular secretion of these isomers in humans. The evidence indicating that both isomers of mandelic acid are involved in renal tubular secretion is presented in a later section.

Comparison of Quantitative Aspects of the Fate of Mandelic Acid Observed in Present Studies with Those Reported by Others—After the oral ingestion of L-(+)-mandelic acid, since the urine, following its exhaustive extraction with ether, exhibited levorotatory activity upon polarimetric examination, Montenbruck (9) assumed that the levorotation was caused by 7% (−)-phenylaminoacetic acid formed from mandelic acid in the human subject and eventually excreted in the urine. Furthermore, he reported that only traces of phenylaminoacetic acid were formed from D-(−)-mandelic acid, since he did not encounter similar observation in the urine collected following the oral ingestion of mandelic acid. In these studies the equivalent amount of either isomer of mandelic acid recovered from the urine as intact mandelic acid and benzoylformic acid was about 80% of the administered dose. However, the data presented in Table I indicate that the urinary recovery of either isomer of mandelic acid is practically complete (97–103% of the ingested dose) in most studies in Subjects A and B. Similar observation was also made by Kamienny *et al.* (19) in their studies with D-(−)-mandelic acid. Therefore, if (−)-phenylaminoacetic acid was formed from L-(+)-mandelic acid in the body, as suggested by Montenbruck (9), it was assumed to be negligible and no attempt was made to identify it.

Although the total urinary recovery of the administered dose of each mandelic acid isomer reported by Montenbruck (9) was less than that observed in the present study, a fairly good agreement is noted in these studies between the fractions of the dose of mandelic acid obtained as its metabolites (benzoylformic acid and benzoic acid). The extent of metabolism is indeed found to be greater for L-(+)-mandelic acid than for D-(−)-mandelic acid, and the urinary recovery of the former in the intact form is less than that of the latter. As shown by Montenbruck (9), not more than 2.3% of the administered dose of L-(+)-mandelic acid was obtained as benzoic acid in the present studies.

In their studies, Kamienny *et al.* (19) noted that the extent of metabolism of the administered dose of D-(−)-mandelic acid to benzoylformic acid was 10–15% in two of the three human subjects and 14–22% in the third subject. Subject B, who participated in those studies, was the same Subject B who participated in the present studies, and yet the subject was found to metabolize 6–8% of the administered dose of D-(−)-mandelic acid to benzoylformic acid in the present study (Table I) in contrast to 11–13% in the Kamienny *et al.* studies (19). The overall elimination rate constants observed for D-(−)-mandelic acid in Subject B prior to the administration of probenecid in the inhibitory studies were $33-36 \times 10^{-2}$ hr.$^{-1}$ (Table III), while in the Kamienny *et al.* studies (19) the overall elimination rate constants observed for D-(−)-mandelic acid in Subject B prior to the administration of sulfonamides were 44–46 $\times 10^{-2}$ hr.$^{-1}$. Furthermore, the two studies with D-(−)-mandelic acid (without probenecid and with 250 mg. probenecid) in Subject B were carried out 5 years ago (Table I); the studies with the same compound in the same Subject B were carried out by Kamienny *et al.* (19) 2 years ago, and the study with the same compound (with 500 mg. probenecid) in the same Subject B was carried out recently (Table I).

In retrospect, it is correctly recollected that, while Subject B was generally resting during the first 10–12 hr. of urine collection for the studies reported in Table I, he was ambulatory during the similar urine-collection period reported in the Kamienny *et al.* studies (19). Recently, Schmidt and Roholt (27) demonstrated that the initial plasma levels of benzylpenicillin following the intramuscular administration to the human subjects were higher when they were ambulatory than when they were resting in bed. Subjecting these data reported by Schmidt and Roholt to pharmacokinetic analysis, Levy (28) observed that, although the increase in the average rate

Table II—Apparent First-Order Rate Constants for Overall Elimination (k), for Processes Leading to Urinary Excretion (k_1), and for Processes Leading to Metabolism (k_2) of Optical Isomers of Mandelic Acid in Humans

Subject	k, hr.$^{-1}$ ($\times 10^2$)	k_1, hr.$^{-1}$ ($\times 10^2$)	k_2, hr.$^{-1}$ ($\times 10^2$)
	D-(−)-Mandelic Acid		
A	31.78	29.02	2.76
B	31.68	29.02	2.66
B[a]	33.39	31.29	2.10
B[a]	38.46	34.98	3.48
C	40.16	34.98	5.18
	L-(+)-Mandelic Acid		
A	32.31	25.66	6.65
B	33.57	27.97	5.60
B[a]	39.84	24.87	7.33
B[a]	38.13	30.43	7.70
B[a]	37.53	29.76	7.77
C	37.24	29.94	7.30
C[a]	37.90	29.94	7.96

[a] The rate constants presented for these subjects were determined from the urinary excretion data obtained prior to the administration of probenecid (Fig. 3). The reason for utilizing these data is explained in the text.

constant for overall elimination of the drug was only 1.1-fold, such an increase in the average rate constant for metabolism of the drug was 2-fold in the subjects when they were ambulatory. Therefore, the greater extent of the metabolism of D-(−)-mandelic acid noted by Kamienny *et al.* (19) for Subject B and others possibly was due to their ambulatory state during the study. Subject C (Tables I and II) was generally ambulatory during the studies, and this probably was a significant factor for the considerably higher rate of metabolism in Subject C than in Subjects A and B, especially in the study of D-(−)-mandelic acid. Further studies will be carried out for L-(+)-mandelic acid to determine the effect of an ambulatory state of the subjects during such studies.

Effect of Probenecid on Urinary Excretion of Optical Isomers of Mandelic Acid—Since probenecid is known to inhibit the renal tubular secretion of a large number of organic anions (18), studies were carried out to determine if probenecid is able to decrease the rate of urinary excretion of the optical isomers of mandelic acid. It was thought that such studies would reveal whether or not these compounds are involved in renal tubular secretion. In the event the optical isomers of mandelic acid are involved in renal tubular secretion, and their tubular secretion is suppressed by probenecid, it was further thought that the extent of inhibitory effect of probenecid on the urinary excretion of the compounds might indicate if the renal tubular transport carrier system shows differing affinity for the compounds.

As shown in Fig. 3 and Table III, the inhibitory effect of probenecid on the urinary excretion of each isomer of mandelic acid becomes evident. The rate constant (k) for overall elimination of an optical isomer in the absence of probenecid was calculated from the slope of the straight line (least squares) for the data obtained after the postabsorptive and postequilibrative period, but prior to the administration of probenecid. The modified rate constant (kp) for overall elimination of the optical isomer in the presence of probenecid was calculated from the slope of the straight line (least squares) of its excretion data obtained following the oral administration of probenecid. However, since the rate of excretion of the isomer at the 1st hr. after the oral administration of probenecid (Fig. 2) is unlikely to represent the excretion rate of the isomer in the period of postabsorption and postequilibration of probenecid in the body, it is excluded when constructing the least-squares straight line for the subsequent urinary excretion data to calculate the modified rate constant kp. The values of R obtained from the ratio k/kp are listed in Table III. The values of R are used as the index of the inhibitory effect of probenecid on the urinary excretion of the optical isomers of mandelic acid for the following reason.

It is noted from the summary of urinary excretion recovery data (Table I) that the recovery of the administered dose of the optical isomers of mandelic acid in the studies involving probenecid was generally complete and that the extent of metabolism of the isomers of mandelic acid in these studies was comparable to that observed

Table III—Rate Constants for Overall Elimination of Optical Isomers of Mandelic Acid Determined in Humans before (k) and after (kp) Oral Administration of Probenecid, and the Index of Inhibitory Effect ($R = k/kp$) of Probenecid on the Urinary Excretion of Optical Isomers of Mandelic Acid

Subject	Mandelic Acid Dose, mg.	Probenecid Dose, mg.	k, hr.$^{-1}$ ($\times 10^2$)	kp, hr.$^{-1}$ ($\times 10^2$)	R
		D-(−)-Mandelic Acid			
B	1015	250	33.39	20.22	1.65
B	1420	500	38.46	25.27	1.52
		L-(+)-Mandelic Acid			
B	1000	250	39.84	24.87	1.60
B	972	250	38.13	26.48	1.44
B	1545	500	37.53	25.81	1.45
C	1000	250	37.90	27.42	1.38

in absence of probenecid. The extent of metabolism of the isomers of mandelic acid appears unaffected in the presence of probenecid, since probenecid was administered to the subject 4.5 or 6 hr. after the administration of mandelic acid, during which period 80–85% of the administered dose of mandelic acid was eliminated. Therefore, even if the extent of metabolism of mandelic acid had probably increased in the presence of probenecid, the slight increase in the amount of metabolite due to the remaining dose (15–20% of the administered dose) of mandelic acid in the body is unlikely to become apparent when the total amount of metabolite due to the entire dose of mandelic acid is considered. Differences in the overall elimination rate constants (k and kp) observed in the inhibitory studies appear to be primarily attributable to the differences in the urinary excretion rate.

It is further concluded that the decrease in the rate of urinary excretion of the isomers of mandelic acid in the presence of probenecid is due to the decrease in the rate of secretion of the compounds caused by probenecid. The inhibitory studies described here were carried out primarily in Subject B; only one study was carried out in Subject C. Judging from the values of kp or R (Table III) determined for each isomer of mandelic acid, it does not appear that probenecid decreases the excretion rate of one isomer to a significantly greater extent than that of the other isomer at the dosage levels of the substrate compounds (mandelic acid isomers) and the inhibitor (probenecid) employed in the present study.

As already pointed out, the extent of metabolism of the optical isomers of mandelic acid remained apparently unaffected in the studies involving probenecid (Table I). Furthermore, the rate constants (k) determined for overall elimination of each optical isomer of mandelic acid in the studies involving probenecid (Table III) are found to be similar to those obtained for the isomers in the studies that did not involve probenecid (Table II). Therefore, the urinary excretion data obtained for each optical isomer of mandelic acid in the inhibitory studies (prior to the administration of probenecid) are utilized to determine the apparent rate constants, k_1 and k_2, of the respective isomers. The values of the rate constants thus determined are included in Table II, because these results may be considered as the results of the additional studies performed by the subjects in support of the results obtained in the studies involving only the respective isomer of mandelic acid.

In conclusion, it appears that, in humans, the "carrier" system responsible for renal tubular secretion of mandelic acid shows no apparent stereospecificity for the optical isomers of mandelic acid, as does the oxidative enzyme system for their metabolism.

REFERENCES

(1) C. Schotten, *Z. Physiol. Chem.*, **8,** 60(1883); through G. H. Schneller, *Drug Cosmet. Ind.*, **65,** 34(1949).

(2) F. Knoop, *Beitr. Chem. Physiol. Pathol.*, **6,** 150(1905).

(3) O. Neubauer and H. Fisher, *Z. Physiol. Chem.*, **67,** 130 (1910).

(4) A. J. Quick, *J. Biol. Chem.*, **80,** 515(1928).

(5) M. L. Rosenheim, *Lancet*, **1,** 1032(1935).

(6) F. Wrede, *Pharm. Ind.*, **1938,** 401.

(7) A. Scholz, *Inaug. Diss. Univ. Keil*, 1938; through R. C.

Gary and I. A. Smith, *Biochem. J.*, **34,** 490(1940).
(8) D. Montenbruck, *ibid.*, 1938; through R. C. Gary and I. A. Smith, *Biochem. J.*, **34,** 490(1940).
(9) D. Montenbruck, *Arch. Exp. Pathol. Pharmacol.*, **195,** 164 (1940).
(10) R. C. Gary and I. A. Smith, *Biochem. J.*, **34,** 490(1940).
(11) V. Klingmüller and G. Brune, *Biochem. Z.*, **328,** 352(1956).
(12) W. J. P. Neish, *Arch. Int. Pharmacodyn. Ther.*, **107,** 315 (1956).
(13) C. Grayson and C. Reese, *J. Urol.*, **50,** 258(1943).
(14) F. Bister and J. H. Wolff, *Arzneim.-Forsch.*, **2,** 423(1952).
(15) *Ibid.*, **3,** 481(1953).
(16) M. R. Verma and S. D. Paul, *J. Sci. Ind. Res.*, **13B,** 346 (1954).
(17) C. M. Williams, *Anal. Biochem.*, **4,** 423(1962).
(18) M. Weiner and G. H. Mudge, *Amer. J. Med.*, **36,** 743(1964).
(19) F. M. Kamienny, M. Barr, and J. B. Nagwekar, *J. Pharm. Sci.*, **58,** 1318(1969).
(20) E. R. Randinitis, M. Barr, and J. B. Nagwekar, *ibid.*, **59,** 813 (1970).
(21) L. D. Metcalfe and A. A. Schmitz, *Anal. Chem.*, **33,** 363 (1961).
(22) J. Mitchell, Jr. and D. M. Smith, "Aquametry Chemical Analysis," vol. 5, Interscience, New York, N. Y., 1948, pp. 297–309.
(23) J. Mitchell, Jr., D. M. Smith, and W. M. D. Bryant, *J. Amer. Chem. Soc.*, **62,** 4(1940).
(24) A. T. James, *J. Chromatogr.*, **2,** 552(1959).
(25) L. L. Smail, "Analytic Geometry and Calculus," Appleton Century Crofts, New York, N. Y., 1953, chap. XX, p. 426.
(26) S. Riegelman, J. C. K. Loo, and M. Rowland, *J. Pharm. Sci.*, **57,** 117(1968).
(27) H. Schmidt and K. Roholt, *Acta Pathol. Microbiol. Scand.*, **68,** 396(1966).
(28) G. Levy, *J. Pharm. Sci.*, **56,** 928(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 13, 1970, from the *School of Pharmacy, Temple University, Philadelphia, PA 19140,* and the *College of Pharmacy, Wayne State University, Detroit, MI 48202*

Accepted for publication July 1, 1970.

* Present address: College of Pharmacy, Wayne State University, Detroit, MI 48202

† Present address: College of Pharmacy, University of Kentucky, Lexington, KY 40506

Effect of Vehicles on Metabolism of Serotonin and Imipramine

BENG T. HO, VICENTE ESTEVEZ, and WILLIAM M. McISAAC

Abstract □ Effects of different vehicles on excretion and metabolism of serotonin and imipramine were studied. Rats receiving serotonin-^{14}C in dimethyl sulfoxide or propylene glycol excreted radioactivity in the urine slower and in lesser amounts than when water was the vehicle. However, the urinary excretion of radioactivity by rats administered a single dose of imipramine-^{14}C was the same for the three vehicles over a period of 72 hr. Dimethyl sulfoxide and propylene glycol caused not only an increase in the amount of urinary 5-hydroxytryptophol and *N*-acetylserotonin, but also an increase in the conjugated form of these two metabolites as well as that of 5-hydroxyindoleacetic acid. These two nonaqueous vehicles caused a diminished demethylation of imipramine when compared to the water vehicle.

Keyphrases □ Serotonin-^{14}C metabolism—vehicle effect □ Imipramine-^{14}C metabolism—vehicle effect □ Metabolism—serotonin-^{14}C, imipramine-^{14}C □ Paper chromatography—autoradiographic analysis □ Scintillometry—analysis

An aqueous solvent is the most common vehicle for the administration of drugs for pharmacological studies in animals. However, other solvents are also frequently used, such as propylene glycol and dimethyl sulfoxide (DMSO). DMSO is a powerful solvent with a remarkable ability to alter membrane permeability, and its function as a carrier for many substances has been demonstrated by a number of investigators.

Recently, effects of DMSO on the uptake of ^{14}C-pemoline (1, 2), ^{14}C-urea, and ^{14}C-sucrose (3) by the rat brain have been reported. This study is to determine the difference in effects of water and these two organic solvents as vehicles on the metabolism of two ^{14}C-labeled centrally acting agents, serotonin and imipramine.

MATERIALS AND METHODS

Labeled Compounds—Serotonin-2-^{14}C creatinine sulfate[1] and imipramine-^{14}C hydrochloride[2] were used.

Animals and Doses—Male Sprague-Dawley rats, weighing between 180 and 220 g., were used. The compounds were dissolved separately in water, DMSO, and propylene glycol and were administered intraperitoneally to groups of three animals in a dose of 20 mg./kg. (0.5 ml./rat; 1 μc./rat).

Collection of Urine and Fecal Samples—After the administration of the compound, animals were kept in metabolism cages. Urine samples were collected at intervals of 2, 3, 4, 8, 12, and 24 hr. and then at every 24-hr. interval up to 6 days. In some cases the collection was extended beyond 6 days, as indicated in the results. Toluene was added to the collection tube to prevent any bacterial growth. Fecal samples were collected every 24 hr. for several days. All samples were immediately frozen until assayed.

Determination of Radioactivity—Radioactivities were measured with a Nuclear Chicago liquid scintillation spectrometer, model 725. The scintillation fluid was composed of 4 g. of PPO, 50 mg. of POPOP, and 70 g. of naphthalene/l. of toluene. All determinations were performed in duplicate and were corrected to 100% efficiency by the channels ratio method (4) and for recovery of radioactivity.

The urine sample (0.1 ml.) was mixed with 3 ml. of methanol in a counting vial, and the radioactivity was then measured with 15 ml. of the scintillation fluid..

For measuring the radioactivity in feces, 0.1 ml. of 20% water homogenate in a counting vial was treated with 1.0 ml. of 10% hydrogen peroxide in methanol. The mixture was heated at 45° to al-

[1] New England Nuclear Corp.
[2] Nuclear Chicago Corp.

Gary and I. A. Smith, *Biochem. J.*, **34,** 490(1940).
(8) D. Montenbruck, *ibid.*, 1938; through R. C. Gary and I. A. Smith, *Biochem. J.*, **34,** 490(1940).
(9) D. Montenbruck, *Arch. Exp. Pathol. Pharmacol.*, **195,** 164 (1940).
(10) R. C. Gary and I. A. Smith, *Biochem. J.*, **34,** 490(1940).
(11) V. Klingmüller and G. Brune, *Biochem. Z.*, **328,** 352(1956).
(12) W. J. P. Neish, *Arch. Int. Pharmacodyn. Ther.*, **107,** 315 (1956).
(13) C. Grayson and C. Reese, *J. Urol.*, **50,** 258(1943).
(14) F. Bister and J. H. Wolff, *Arzneim.-Forsch.*, **2,** 423(1952).
(15) *Ibid.*, **3,** 481(1953).
(16) M. R. Verma and S. D. Paul, *J. Sci. Ind. Res.*, **13B,** 346 (1954).
(17) C. M. Williams, *Anal. Biochem.*, **4,** 423(1962).
(18) M. Weiner and G. H. Mudge, *Amer. J. Med.*, **36,** 743(1964).
(19) F. M. Kamienny, M. Barr, and J. B. Nagwekar, *J. Pharm. Sci.*, **58,** 1318(1969).
(20) E. R. Randinitis, M. Barr, and J. B. Nagwekar, *ibid.*, **59,** 813 (1970).
(21) L. D. Metcalfe and A. A. Schmitz, *Anal. Chem.*, **33,** 363 (1961).
(22) J. Mitchell, Jr. and D. M. Smith, "Aquametry Chemical Analysis," vol. 5, Interscience, New York, N. Y., 1948, pp. 297–309.
(23) J. Mitchell, Jr., D. M. Smith, and W. M. D. Bryant, *J. Amer. Chem. Soc.*, **62,** 4(1940).
(24) A. T. James, *J. Chromatogr.*, **2,** 552(1959).
(25) L. L. Smail, "Analytic Geometry and Calculus," Appleton Century Crofts, New York, N. Y., 1953, chap. XX, p. 426.
(26) S. Riegelman, J. C. K. Loo, and M. Rowland, *J. Pharm. Sci.*, **57,** 117(1968).
(27) H. Schmidt and K. Roholt, *Acta Pathol. Microbiol. Scand.*, **68,** 396(1966).
(28) G. Levy, *J. Pharm. Sci.*, **56,** 928(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 13, 1970, from the *School of Pharmacy, Temple University, Philadelphia, PA 19140,* and the *College of Pharmacy, Wayne State University, Detroit, MI 48202*

Accepted for publication July 1, 1970.

* Present address: College of Pharmacy, Wayne State University, Detroit, MI 48202

† Present address: College of Pharmacy, University of Kentucky, Lexington, KY 40506

Effect of Vehicles on Metabolism of Serotonin and Imipramine

BENG T. HO, VICENTE ESTEVEZ, and WILLIAM M. McISAAC

Abstract □ Effects of different vehicles on excretion and metabolism of serotonin and imipramine were studied. Rats receiving serotonin-^{14}C in dimethyl sulfoxide or propylene glycol excreted radioactivity in the urine slower and in lesser amounts than when water was the vehicle. However, the urinary excretion of radioactivity by rats administered a single dose of imipramine-^{14}C was the same for the three vehicles over a period of 72 hr. Dimethyl sulfoxide and propylene glycol caused not only an increase in the amount of urinary 5-hydroxytryptophol and *N*-acetylserotonin, but also an increase in the conjugated form of these two metabolites as well as that of 5-hydroxyindoleacetic acid. These two nonaqueous vehicles caused a diminished demethylation of imipramine when compared to the water vehicle.

Keyphrases □ Serotonin-^{14}C metabolism—vehicle effect □ Imipramine-^{14}C metabolism—vehicle effect □ Metabolism—serotonin-^{14}C, imipramine-^{14}C □ Paper chromatography—autoradiographic analysis □ Scintillometry—analysis

An aqueous solvent is the most common vehicle for the administration of drugs for pharmacological studies in animals. However, other solvents are also frequently used, such as propylene glycol and dimethyl sulfoxide (DMSO). DMSO is a powerful solvent with a remarkable ability to alter membrane permeability, and its function as a carrier for many substances has been demonstrated by a number of investigators.

Recently, effects of DMSO on the uptake of ^{14}C-pemoline (1, 2), ^{14}C-urea, and ^{14}C-sucrose (3) by the rat brain have been reported. This study is to determine the difference in effects of water and these two organic solvents as vehicles on the metabolism of two ^{14}C-labeled centrally acting agents, serotonin and imipramine.

MATERIALS AND METHODS

Labeled Compounds—Serotonin-2-^{14}C creatinine sulfate[1] and imipramine-^{14}C hydrochloride[2] were used.

Animals and Doses—Male Sprague-Dawley rats, weighing between 180 and 220 g., were used. The compounds were dissolved separately in water, DMSO, and propylene glycol and were administered intraperitoneally to groups of three animals in a dose of 20 mg./kg. (0.5 ml./rat; 1 μc./rat).

Collection of Urine and Fecal Samples—After the administration of the compound, animals were kept in metabolism cages. Urine samples were collected at intervals of 2, 3, 4, 8, 12, and 24 hr. and then at every 24-hr. interval up to 6 days. In some cases the collection was extended beyond 6 days, as indicated in the results. Toluene was added to the collection tube to prevent any bacterial growth. Fecal samples were collected every 24 hr. for several days. All samples were immediately frozen until assayed.

Determination of Radioactivity—Radioactivities were measured with a Nuclear Chicago liquid scintillation spectrometer, model 725. The scintillation fluid was composed of 4 g. of PPO, 50 mg. of POPOP, and 70 g. of naphthalene/l. of toluene. All determinations were performed in duplicate and were corrected to 100% efficiency by the channels ratio method (4) and for recovery of radioactivity.

The urine sample (0.1 ml.) was mixed with 3 ml. of methanol in a counting vial, and the radioactivity was then measured with 15 ml. of the scintillation fluid..

For measuring the radioactivity in feces, 0.1 ml. of 20% water homogenate in a counting vial was treated with 1.0 ml. of 10% hydrogen peroxide in methanol. The mixture was heated at 45° to al-

[1] New England Nuclear Corp.
[2] Nuclear Chicago Corp.

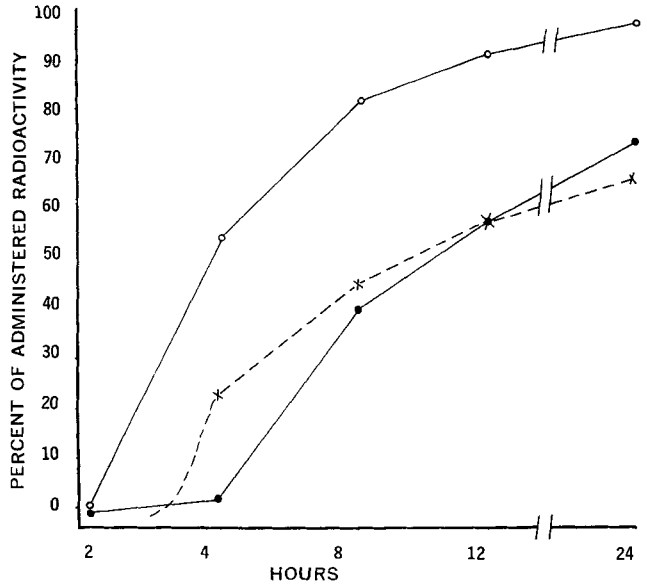


Figure 1—*Cumulative excretion of radioactivity in rat urine following intraperitoneal injection of serotonin-^{14}C. Key: O—O, in water; ●—●, in DMSO; and X--X, in propylene glycol.*

most dryness, treated with 3 ml. of methanol, and 1 hr. later again treated with 15 ml. of the scintillation fluid.

Enzymatic Hydrolysis of Conjugates—Urine was adjusted to pH 5.5 and incubated overnight with β-glucuronidase and sulfatase[3] in sodium acetate buffer, pH 5.5, at 37°.

Chromatography of Urine Sample—Hydrolyzed and unhydrolyzed urine samples from the ^{14}C-serotonin experiment were applied on Whatman 3MM paper and developed in the system of *n*-butanol–acetic acid–water (120:30:50) using the descending technique. The radioactive spots were located by autoradiography of the paper chromatograms with Kodak no-screen X-ray film and eluted by methanol. The eluates were rechromatographed on silica gel G precoated glass plates[4] in the solvent ethyl acetate (5). Serotonin and its metabolites were identified against standards and by their R_f values, UV fluorescence, and color reactions with Ehrlich's reagent. For quantitation, radioactive spots on paper or glass plate were cut out or scraped off and transferred to counting vials; the radioactivity was measured after addition of 3 ml. of methanol and 15 ml. of the scintillation fluid.

Urine samples from the imipramine-^{14}C experiment were chromatographed on silica gel G precoated TLC plates in the solvent system of chloroform–methanol (90:10) (6). Imipramine and desimipramine were detected by spraying the plates with iodoplatinate reagent.[5]

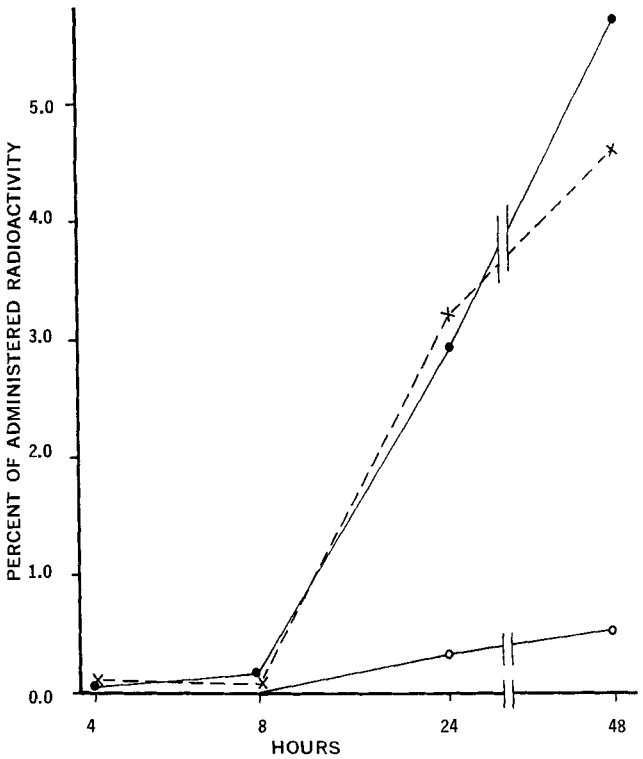


Figure 3—*Cumulative excretion of radioactivity in rat feces following intraperitoneal injection of serotonin-^{14}C. Key: O—O, in water; ●—●, in DMSO; and X--X, in propylene glycol.*

RESULTS AND DISCUSSION

Excretion—Urine levels of radioactivity after the intraperitoneal administration of serotonin-^{14}C were less when the compound was administered in DMSO or propylene glycol than when administered in water. After 24 hr., 98% of the given dose was excreted when the aqueous solution was used, whereas only 75 and 67% of the injected radioactivity were recovered respectively when DMSO and propylene glycol were the vehicles (Fig. 1). However, in rats injected

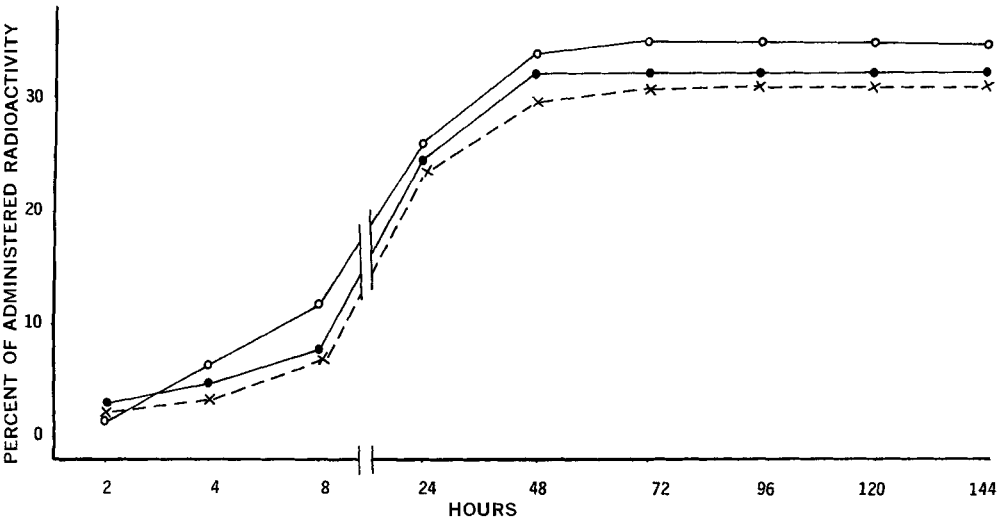


Figure 2—*Cumulative excretion of radioactivity in rat urine following intraperitoneal injection of imipramine-^{14}C. Key: O—O, in water; ●—●, in DMSO; and X--X, in propylene glycol.*

[3] Glusulase, Endo Laboratories, Inc.
[4] Brinkmann Co.

[5] This reagent was found to be useful in detecting imipramine and desimipramine in less than 1-mcg. quantities, giving a pinkish color.

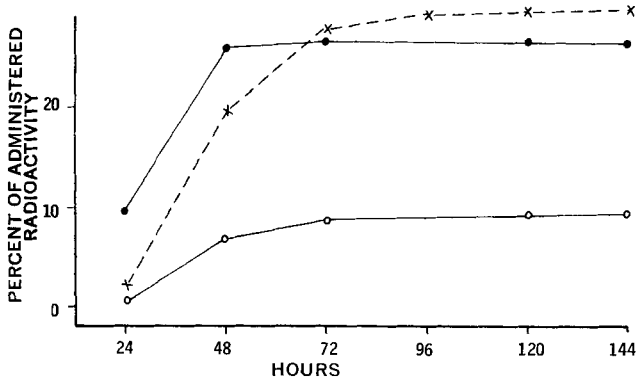


Figure 4—*Cumulative excretion of radioactivity in rat feces following intraperitoneal injection of imipramine-^{14}C. Key: O—O, in water; ●—●, in DMSO; and ×--×, in propylene glycol.*

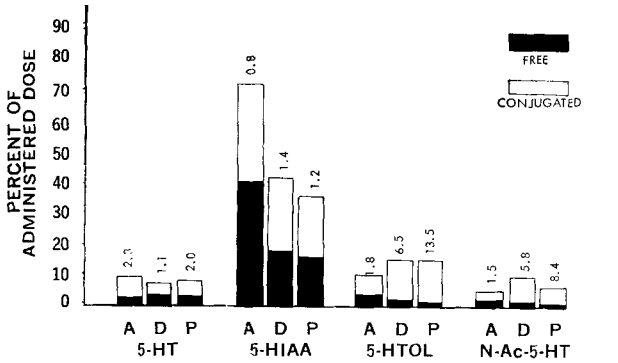


Figure 5—*Distribution of metabolites in 24-hr. rat urine following intraperitoneal injection of serotonin-^{14}C. Key: A, in water; D, in DMSO; P, in propylene glycol; 5-HT, unchanged serotonin recovered; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HTOL, 5-hydroxytryptophol; and N-Ac-5-HT, N-acetylserotonin. Values shown at the top of each column represent ratio of the conjugated form to the free form for each metabolite.*

with imipramine-^{14}C, urinary excretion of radioactivity was the same (approximately 33%) over a period of 72 hr., regardless of which of the three vehicles was used (Fig. 2).

Rats receiving serotonin or imipramine in the two nonaqueous solvents showed higher excretion of radioactivity in feces than when the aqueous solution was used (Figs. 3 and 4).

Metabolism—The urinary metabolites of serotonin-^{14}C recovered in the first 24 hr. after administration are shown in Fig. 5. Both DMSO and propylene glycol doubled the urinary excretion of 5-hydroxytryptophol and *N*-acetylserotonin over that with the aqueous vehicle (Fig. 5 and Table I). A similar alternation of metabolic pathways of serotonin by ingestion of ethanol has been reported (7).

In this study, the cause for the increased urinary 5-hydroxytryptophol and *N*-acetylserotonin remains to be determined. The latter could have resulted from a blockage of the oxidative pathway by DMSO and propylene glycol; these two solvents were found to inhibit monoamine oxidase *in vitro*.[6] It has been postulated that DMSO could alter secondary and tertiary structures of proteins by substituting the protein-bound water, and this could cause a partial loss of enzyme activity (8, 9).

[6] During the assay for inhibitors of monoamine oxidase, the solvents DMSO and propylene glycol were found to be weak inhibitors of bovine liver monoamine oxidase with I_{55} equal to 1.12 and 0.41 *M*, respectively.

Table I—Effects of Vehicles on Percent[a] Distribution of Metabolites in 24-hr. Urine of Rats Administered Intraperitoneal Dose (20 mg./kg.) of Serotonin-^{14}C

Vehicle	Percentage[a] Urinary Metabolites of Serotonin			
	5-HT[b]	5-HIAA[b]	5-HTOL[b]	N-Ac-5-HT[b]
Water	12.6	70.8	10.6	5.9
DMSO	8.3	63.6	18.0	10.0
Propylene glycol	13.7	53.1	23.0	10.1

[a] For a direct comparison of the distribution of serotonin metabolites in the 24-hr. urine with the three vehicles, the total excreted radioactivity in the individual case was corrected to 100%. [b] Key: 5-HT, unchanged serotonin recovered; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HTOL, 5-hydroxytryptophol; and N-Ac-5-HT, *N*-acetylserotonin.

Table II—Effect of Vehicles on the Ratio of Desimipramine to Imipramine in Urine of Rats Administered Imipramine-^{14}C (20 mg./kg. i.p.)

Urine Collection, hr.	Ratio of Desimipramine to Imipramine		
	Water	DMSO	Propylene Glycol
0–4	11.33	2.73	5.19
4–8	9.57	6.66	6.57
8–24	10.08	9.25	11.96

An increase in the conjugated form of the three metabolites, especially 5-hydroxytryptophol and *N*-acetylserotonin, was also observed with both DMSO and propylene glycol (Fig. 5). In this aspect, the latter solvent was more effective than the former.

Table II lists the ratio of desimipramine to imipramine in the urinary excretion. When water was the vehicle, this ratio was held constant throughout the intervals of 4, 8, and 24 hr. With DMSO and propylene glycol as the two vehicles, the low ratios in the first 4 hr. indicated a diminished demethylation of imipramine, particularly for DMSO. It is possible that the two nonaqueous solvents inhibit the demethylation enzyme, and this is currently under study.

REFERENCES

(1) J. J. Brink and D. G. Stein, *Science*, **158,** 1479(1967).

(2) D. G. Stein, J. J. Brink, and A. Patterson, *Life Sci.*, **7,** 147 (1968).

(3) A. M. Thompson and E. J. Hart, *Fed. Proc.*, **27,** 333(1968).

(4) K. A. Baillie, *Int. J. Appl. Radiat. Isotop.*, **8,** 1(1960).

(5) V. E. Davis, J. L. Cashaw, J. A. Huff, and H. Brown, *Proc. Soc. Exp. Biol. Med.*, **122,** 890(1966).

(6) I. Zingales, *J. Chromatogr.*, **31,** 405(1967).

(7) V. E. Davis, H. Brown, J. A. Huff, and J. A. Cashaw, *J. Lab. Clin. Med.*, **69,** 132(1967).

(8) T. Inagami and J. M. Stutervant, *J. Biol. Chem.*, **235,** 1019 (1960).

(9) D. H. Rammler, *Ann. N. Y. Acad. Sci.*, **141,** 291(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 1, 1970, from the *Texas Research Institute of Mental Sciences, Houston, TX 77025*

Accepted for publication June 23, 1970.

This work was supported in part by Grants MH-11168 and MH-12959, U. S. Public Health Service, Bethesda, Md., and the Britton Fund.

The authors thank Mrs. Mary Helen Moreland for her technical assistance.

Extraction of Morphine Enhanced by Addition of *N*-Methyl-3-piperidyl-(*N'N'*)-diphenylcarbamate

RAMSES HAKIM and JAMES M. FUJIMOTO

Abstract □ The extractibility of morphine (N-$^{14}CH_3$) into chloroform from aqueous solution was enhanced to completeness by adding *N*-methyl-3-piperidyl-(*N'N'*)-diphenylcarbamate under the following conditions: ratio of *N*-methyl-3-piperidyl-(*N'N'*)-diphenylcarbamate–morphine between 40:1 and 200:1, with morphine concentration no greater than 30 mcg./ml. and the pH between 6.8 and 10. This high extractibility was decreased by concentration of morphine greater than 30 mcg./ml. and by addition of dihydromorphinone, apomorphine, nalorphine, cyclazocine, and naloxone. Thebaine, codeine, methadone, meperidine, levorphanol, dextrophan, and levallorphan did not affect the extractibility. The same qualitative relationships were obtained by using carbon tetrachloride instead of chloroform as the solvent.

Keyphrases □ Morphine (N-$^{14}CH_3$) extraction enhancement—*N*-methyl-3-piperidyl-(*N'N'*)-diphenylcarbamate □ *N*-Methyl-3-piperidyl-(*N'N'*)-diphenylcarbamate—morphine (N-$^{14}CH_3$) interaction □ Countercurrent distribution—analysis □ TLC—analysis □ Scintillometry—analysis

The effect of SK&F 525A and *N*-methyl-3-piperidyl-(*N'N'*)-diphenylcarbamate (MPDC) on the renal metabolism and tubular transport of morphine in the chicken has been recently reported (1). The present investigation concerns the unusual chemical interaction that occurs between morphine and MPDC. The rationale for embarking on this study is found in the preliminary observation that when MPDC was infused along with radioactive morphine into the saphenous vein of the chicken, the free morphine excreted in the urine was more extractible into chloroform than when no MPDC was given (1). Since addition of MPDC directly to urine containing radioactive morphine produced the same increase in extractibility, it was evident that a chemical interaction was occurring between MPDC and morphine. In the present study, the authors examined and characterized the factors involved in producing this unusual interaction between the two bases. Other narcotic analgesics and antagonists also were tested. A preliminary report on these studies has appeared (2).

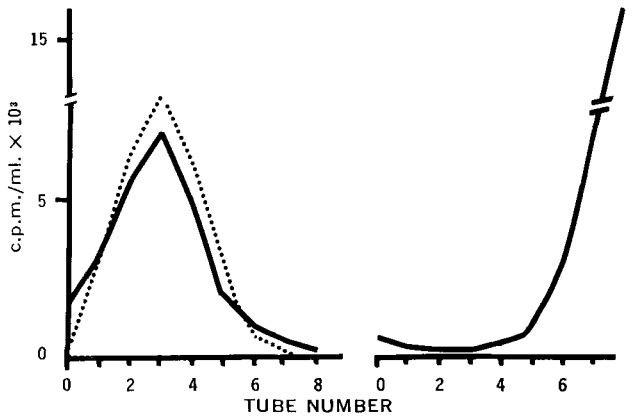


Figure 1—*Left panel is the countercurrent distribution analysis of ^{14}C-morphine. Key: ——, experimental; and . . ., theoretical. Right panel is the countercurrent distribution analysis of ^{14}C-morphine with 10 mcg./ml. MPDC added. Stationary phase, pH 8.5 buffer; mobile phase, chloroform; volume of each phase, 3 ml.*

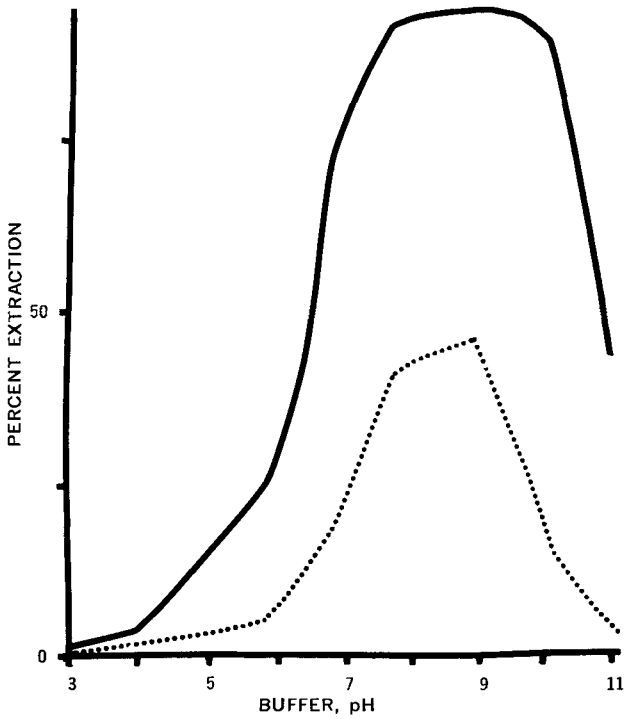


Figure 2—*Extraction of ^{14}C-morphine into chloroform from buffers at different pH's. Key: . . . ., ^{14}C-morphine extraction in absence of MPDC; and ——, ^{14}C-morphine extraction in presence of 5 mcg./ml. of MPDC. Volume of each phase was 3 ml.*

EXPERIMENTAL

Materials—Morphine-N-$^{14}CH_3$ hydrochloride,[1] specific activity 17 mc./mmole, was dissolved in distilled water to make a 0.01 μc./ml. solution. MPDC HCl[2] was used. The solvents used, chloroform, carbon tetrachloride, methylene chloride, ethylene dichloride, ethyl acetate, *n*-butanol, and ethyl ether, were reagent grade (Baker analyzed).

Countercurrent Distribution Analysis—Characterization of ^{14}C-morphine and separation of the ^{14}C-morphine MPDC reaction products were accomplished by using the solvent system previously used for separation of morphine from morphine metabolites in urine samples (1, 3, 4). The solvent system consisted of a sodium bicarbonate (2 g./l.) buffer, pH 8.3–8.5, equilibrated with chloroform. The ^{14}C-morphine, along with MPDC, was added to the system in the buffer phase. Equal volumes of buffer-to-chloroform phases were used; chloroform, the mobile phase, was transferred manually with a syringe equipped with a long needle. One milliliter of each phase was plated and counted in a Tracerlab low background counting system (efficiency of counting was 10% at infinite thinness). Countercurrent analyses were also done with buffers at pH 3; in these instances, sodium phosphate (3 g./l.) buffers were used.

[1] Purchased from Amersham/Searle.
[2] Lakeside Laboratories.

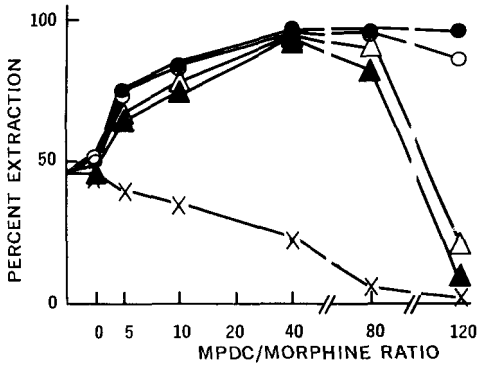


Figure 3—*The extraction of ^{14}C-morphine into chloroform from pH 8.3–8.8 buffer to which 6 (●), 12 (○), 18 (△), 30 (▲), and 80 (×) mcg./ml. morphine and 0–120 times these concentrations of MPDC were added.*

Chromatographic Studies—TLC on Gelman silica gel fiberglass sheets (TLC type SG) was performed with a solvent system consisting of benzene–dioxane–ammonium hydroxide, 5:5:0.1 (v/v/v). The nonradioactive compounds were visualized either by spraying the chromatogram with iodoplatinate reagent (5) or by spraying with sulfuric acid followed by charring the TLC on a hot plate. Radioactive components on the TLC were detected by passing the chromatogram through a 4-pi scanner (Tracerlab).

RESULTS AND DISCUSSION

Countercurrent Distribution of ^{14}C-Morphine with and without MPDC Added—In Fig. 1 the countercurrent analyses performed at pH 8.5 for ^{14}C-morphine are shown to be markedly affected when 10 mcg./ml. of MPDC was added. This addition of MPDC shifted the peak for ^{14}C-morphine from Tube 3 to Tube 8, demonstrating that an increase in the extractibility of ^{14}C-morphine occurred. Although the preliminary observation was that MPDC added to urine containing ^{14}C-morphine altered the extraction of the morphine (1), the present experiment demonstrated that the effect could be obtained in solutions without the presence of urine.

Extractibility of ^{14}C-Morphine with and without Addition of MPDC—*Effect of pH*—A single-extraction procedure was used to expedite the study of several parameters. The extraction of ^{14}C-morphine into chloroform (Fig. 2) was maximal at pH 8.3–9; outside this pH range, extraction decreased. Chloroform in the absence of MPDC, even under optimal pH conditions, did not extract over 50% of the ^{14}C-morphine. These data on extraction of morphine with chloroform agree with expectations from the literature (6). Addition of MPDC increased the extraction of ^{14}C-morphine to beyond 90% in the pH range 7.5–10.

Effect of Concentration of MPDC and Morphine—To study the stoichiometric relationship, a series of experiments was done by adding different concentrations of MPDC and carrier morphine to a constant amount of ^{14}C-morphine. For example, in Fig. 3 if the curve corresponding to 6 mcg./ml. of morphine is examined, the ^{14}C-morphine extraction was less than 50% initially in the absence of MPDC. When the MPDC concentration was increased to between 40 and 120 times the morphine concentration of 6 mcg./ml., the ^{14}C-morphine extraction was greater than 96%. Each curve in the figures should be examined in this manner, since the figures contain a large mass of data. In the range of 6–30 mcg. of carrier morphine, addition of up to 40 times as much MPDC generally increased the extraction. Increasing the relative concentration of MPDC beyond 40 had a detrimental effect on extraction of ^{14}C-morphine, especially at the 18- and 30-mcg. concentrations of morphine. At 80 mcg./ml. of carrier morphine, the extraction decreased from a control value of 46% (with no MPDC) down to 2% when MPDC was 120 times this morphine concentration. At no time was the extraction better than the control value in this situation. This decrease in extraction, as well as the increase in extraction effected by MPDC at lower concentrations of carrier morphine, indicated that chemical interaction was occurring between MPDC and morphine.

Effect of Different Solvents—As shown in Table I, a number of solvents were tested for ability to extract ^{14}C-morphine in the presence and absence of MPDC and carrier morphine. For these situations, no change in extraction of ^{14}C-morphine was seen when the solvents were ethyl acetate, hexane, methylene chloride, ethylene chloride, and ethyl ether. Butanol appeared to be a special case in which extraction was decreased by adding MPDC and carrier morphine. This case was not studied any further. On the other hand, carbon tetrachloride showed an increase in extraction of ^{14}C-mor-

Table I—Extraction of ^{14}C-Morphine from a pH 8.5 Buffer (5 ml.) with Different Organic Solvents (5 ml.)

Organic Solvent	% Extraction of ^{14}C-Morphine into Solvent: No MPDC	MPDC, 400 mcg./ml.	Morphine, 10 mcg./ml.; MPDC, 400 mcg./ml.
Ethyl acetate	53	52	52
Carbon tetrachloride	8	87	35
Hexane	10	10	10
n-Butanol	26	20	13
Methylene chloride	36	37	39
Ethylene dichloride	26	28	30
Ethyl ether	2	2	2
Ethylene dichloride, chloroform[a]	60	97	
Ethylene dichloride, carbon tetrachloride[a]	19	66	

[a] A mixture containing 2.5 ml. of each organic solvent.

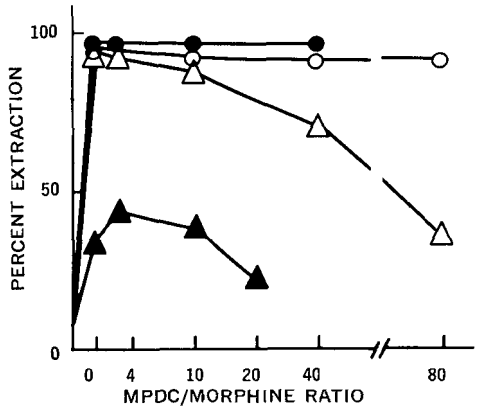


Figure 4—*The extraction of ^{14}C-morphine into carbon tetrachloride from pH 8.3–8.8 buffer to which 6 (●), 12 (○), 18 (△), and 30 (▲) mcg./ml. morphine and 0–80 times these concentrations of MPDC were added.*

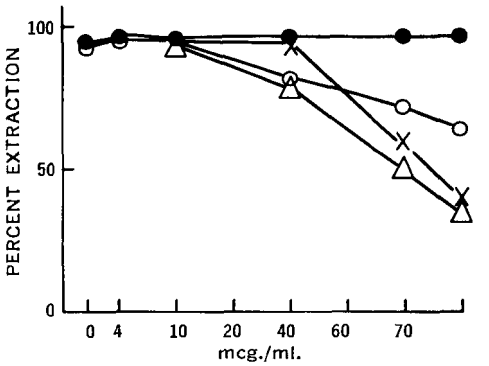


Figure 5—*The effect of various narcotics and antagonists on the extraction of ^{14}C-morphine into 5 ml. chloroform from 5 ml. of pH 8.5 buffer in the presence of MPDC. The concentration of morphine (○), dihydromorphinone (×), nalorphine (△), and thebaine (●) is read from the abscissa. The concentration of MPDC was always 40 times the drug concentrations. Codeine, methadone, meperidine, dextrophan, and levallorphan gave the same results as thebaine.*

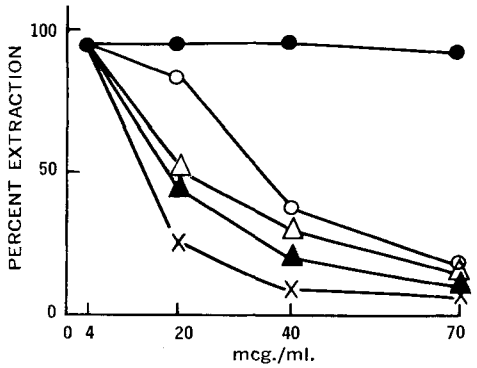


Figure 6—*The effect of various narcotics and antagonists on the extraction of ^{14}C-morphine into 5 ml. carbon tetrachloride from 5 ml. of pH 8.5 buffer in the presence of MPDC. The MPDC concentration was always 20 times that of the drugs. The other designations were as in Fig. 5:* ○, *morphine;* ×, *dihydromorphinone;* △, *nalorphine;* ▲, *naloxone; and* ●, *thebaine, codeine, methadone, meperidine, dextrophan, and levallorphan.*

phine from 8 to 87% by addition of MPDC. But, addition of 10 mcg./ml. of morphine caused the extraction to drop to 35%. Thus, carbon tetrachloride was similar in a qualitative sense to the studies with chloroform as the solvent. Chloroform or carbon tetrachloride added to an equal volume of ethylene dichloride, a solvent that showed no effect to addition of MPDC and morphine, still produced the same phenomenon. Considerable increases in extraction occurred beyond that of ethylene dichloride alone.

The extraction of ^{14}C-morphine from sodium bicarbonate buffer, pH 8.5, was examined (Fig. 4) with carbon tetrachloride used in place of chloroform (Fig. 3). The extraction of ^{14}C-morphine into carbon tetrachloride was almost complete (over 90%) for solutions containing 6 and 12 mcg./ml. of carrier morphine and up to 80 times these respective concentrations of MPDC. Beyond these concentrations of morphine and MPDC, extraction of ^{14}C-morphine decreased. The general conclusions were the same as for the results given in Fig. 3 with chloroform as the solvent. Namely, MPDC was interacting with morphine.

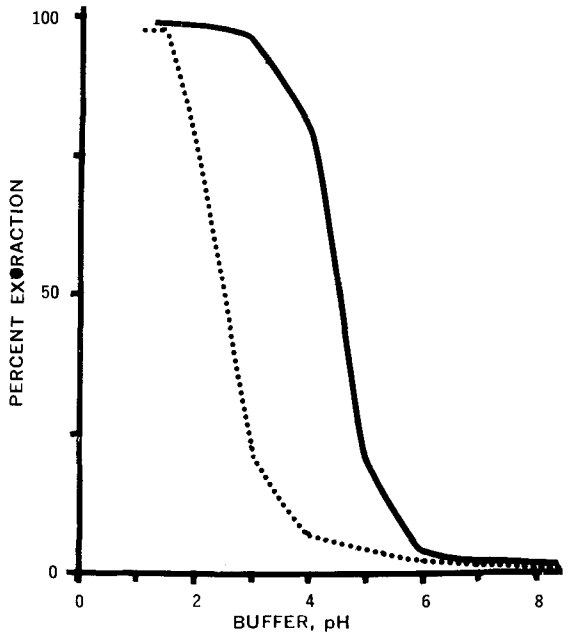


Figure 7—*Reextraction of radioactive product (—) and MPDC (. . .) from chloroform into buffers of different pH's. ^{14}C-Morphine and MPDC (in 1:40 molar ratio) were extracted from pH 8.5 buffer into chloroform. This chloroform was then shaken with an equal volume (5 ml.) of buffer. The percent extraction was calculated for this last step.*

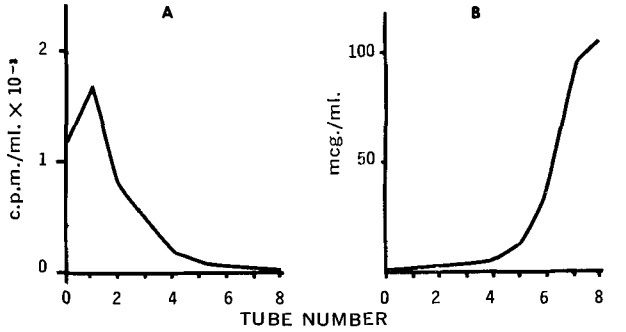


Figure 8—*Countercurrent distribution at pH 3 of material reextracted from chloroform and analyzed for (A) ^{14}C-activity and (B) MPDC. MPDC was determined by UV absorption spectrometry.*

Effect of Other Drugs in Extraction of ^{14}C-Morphine—Investigations of the effects of other narcotic analgesics and antagonists on this interaction of morphine with MPDC are shown in Figs. 5 and 6. There were two types of effects. One group of drugs had no effect; this group included thebaine, codeine, methadone, meperidine, dextrophan, and levallorphan. The other group depressed ^{14}C-morphine extraction and included morphine itself, nalorphine, and dihydromorphinone. Apomorphine at 50 mcg./ml. also belonged in the latter group, but only this one concentration was tested. The same kind of grouping into two types is seen in Fig. 6, where carbon tetrachloride was used as the solvent instead of chloroform. The first group consisted of the same drugs in which no effect was found. The second group consisted again of morphine, nalorphine, dihydromorphinone, apomorphine (one concentration of 20 mcg./ml., not shown), and, in addition, naloxone. It is evident from the experiments (Figs. 5 and 6) that certain drugs affect the interaction of MPDC with ^{14}C-morphine, and some structural specificity exists in this interaction. Dihydromorphinone, morphine, nalorphine, and naloxone all possess the phenolic OH and either an alcoholic OH or keto group at position 6. It is interesting to note that two were agonists (morphine and dihydromorphinone) and two were antagonists (nalorphine and naloxone), but there appeared to be little relationship to pharmacologic potency. At first, it appeared that the morphine–MPDC system would provide an *in vitro* approach for preliminary screening for narcotic analgesics and antagonists. However, some important compounds known to have pharmacologic activity would have been missed.

Reextraction of ^{14}C-Morphine and MPDC from Chloroform into Buffers of Different pH's—The chloroform phase containing the ^{14}C and MPDC was extracted with buffers of different pH's. Figure 7 shows that the radioactive material presumably present as a complex with MPDC was extractible back into acid buffers. This curve, which might be thought of as an inverse plot of the extraction of ^{14}C-

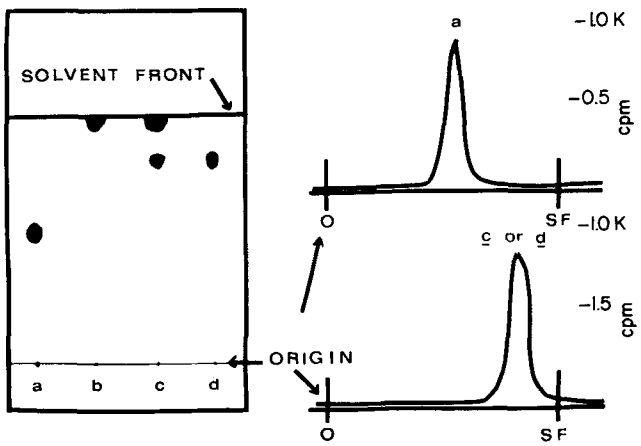


Figure 9—*TLC on Gelman silica gel fiberglass sheets. On the left the chromatogram shows (a) morphine, (b) MPDC, (c) chloroform extract at pH 8.5 containing morphine and MPDC, and (d) Tube 0 pH 3 buffer phase of countercurrent distribution analysis performed in Fig. 8.*

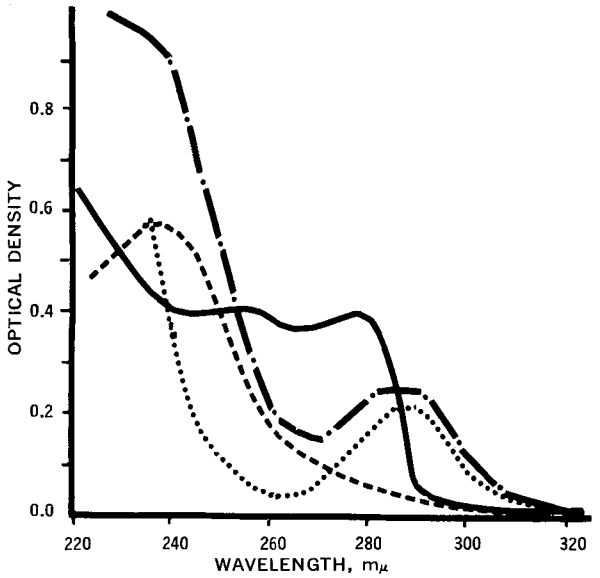


Figure 10—*UV spectrum of the ^{14}C-morphine–MPDC product (—) from Tube 0 of the countercurrent distribution at pH 3.0 (Fig. 8 and d in Fig. 9); morphine (. . .) at pH 3, MPDC (- - -) at pH 3, and morphine and MPDC together (–·–) in pH 3 buffer where no chloroform extraction was involved.*

morphine in the presence of MPDC from buffer into chloroform, was not identical to the results derivable from Fig. 2. For the experiment shown in Fig. 7, transfer of radioactive material from chloroform back to buffer required lower pH's than the transfer of ^{14}C-morphine from buffer to chloroform (Fig. 2). Extraction of ^{14}C-morphine in the absence of MPDC into chloroform and reextraction of this morphine from chloroform into the buffer showed the same type of hysteresis. Occurrence of such hysteresis militated against a clearcut interpretation of these results from the reextraction experiments. Figure 7 includes a further experiment, in which the reextraction into the buffer of the MPDC portion from the morphine–MPDC chloroform solution was measured. Since the amount of MPDC was large relative to morphine (40:1 molar ratio), it was possible to measure MPDC by UV spectrometry. The data show that at a 2–6 pH range, MPDC was not extracted from chloroform into buffer to the same proportion that the radioactive material was extracted.

The practical consequence of the information derived from Fig. 7 was that it was possible to separate the radioactive material from the excess MPDC in the chloroform by adjusting the aqueous phase to pH 3. These conditions were employed in performing the countercurrent distribution analysis given in Fig. 8. As expected, the radioactive material that peaked in Tube 1 was clearly separated from the MPDC that peaked in Tube 8. Since free ^{14}C-morphine itself would have peaked in Tube 0, the peak for the radioactive material in Tube 1 of the present experiment indicated that the radioactive material was not free ^{14}C-morphine. Therefore, attempts were made to characterize this material further.

TLC of the material from the aqueous phase of Tubes 0 and 1 of the countercurrent fractionation is shown in Fig. 9. The results indicated that this material was a single component with an R_f of 0.83, both by iodoplatinate reaction and by radioactivity. It was different from MPDC or morphine. The TLC for *c* is for a 40:1 mixture of MPDC–morphine in pH 8.5 buffer extracted with chloroform and the chloroform spotted. In this case, both the material corresponding to *d* and excess MPDC were present as expected.

UV absorption spectra were obtained from these same materials (Fig. 10). The spectrum of the material in Tube 0 of the countercurrent distribution analysis was not characteristic of either morphine or MPDC. Also, this spectrum was different from a spectrum obtained by simple addition of morphine and MPDC spectra. A spectrum obtained experimentally by having both MPDC and morphine together in a buffer at pH 3 given in the figure was also different from that for the material in Tube 0. Thus, the results from the TLC and the UV spectral studies supported the idea that some sort of "complex" was formed between morphine and MPDC. This complex acted as a single entity with its own characteristics. Efforts to crystallize this material have not been successful.

REFERENCES

(1) R. Hakim and J. M. Fujimoto, *Pharmacologist*, **12,** 292(1970).

(2) *Ibid.*, **10,** 166(1968).

(3) D. G. May, J. M. Fujimoto, and C. E. Inturrisi, *J. Pharmacol. Exp. Ther.*, **157,** 626(1967).

(4) W. M. Watrous, D. G. May, and J. M. Fujimoto, *ibid.*, **172,** 224(1970).

(5) J. Cochin and J. W. Daly, *Experientia*, **18,** 294(1962).

(6) J. M. Fujimoto, E. L. Way, and C. H. Hine, *J. Lab. Clin. Med.*, **44,** 627(1954).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 21, 1970, from the *Department of Pharmacology, Marquette School of Medicine, Milwaukee, WI 53233*

Accepted for publication July 2, 1970.

This investigation was supported in part by Grant 2R01 GM 16503 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Characterization of Tablet Surfaces by Their Critical Surface-Tension Values

S. W. HARDER, D. A. ZUCK, and J. A. WOOD

Abstract □ Experiments were conducted to determine the critical surface-tension (γ_c) values of a range of acetylsalicylic acid tablets in combination with various adjuvants. A telemicroscope was used to measure the contact angle (θ) as test liquids with known surface tensions (γ_L) advanced across the tablet surface. The cosine of the contact angles ($\cos\theta$) were plotted as a function of the γ_L of the test liquids. Straight-line extrapolation to $\cos\theta = 1$ resulted in γ_c values for each particular type of tablet. These values were determined for each group of tablets, using different sets of test liquids. The γ_c values obtained by this method indicated that pure acetylsalicylic acid tablets presented a surface with intermediate activity, having a γ_c value of about 31 dynes/cm. The addition of a lubricant like magnesium stearate presented a surface richer in $-CH_3$ and $-CH_2-$ groups, resulting in a lower value, whereas the addition of adjuvants such as starch, cellulose, and talc resulted in surfaces richer in =O and —OH, causing an increase in the γ_c values. Increased γ_c values result in increased wetting by the coating solution and in an increased bonding force between the tablet surface and the polymer film coating after the solvent has evaporated. Characterization of the tablet surface should eliminate some of the guesswork involved in developing an adequate film coating for a particular type of medicinal tablet.

Keyphrases □ Tablet surfaces—characterization □ Critical surface-tension values—tablet surface characterization □ Telemicroscopy—liquid, tablet surface contact-angle measurement □ Adjuvant effect—liquid, tablet surface contact angle

The strength of the adhesive bond between a film coating and a solid surface, as well as the uniformity of the coating, is influenced by surface energy forces acting at and across an interface (1–4). It is, therefore, important to understand the energy forces present at the surface of a tablet and to be able to characterize a tablet according to the cohesional energy density (CED) of its surface. Knowledge of the forces operative at the tablet surface will assist in prediction of the degree of bonding and permit the design of coating solutions, resulting in optimum utilization of these forces to obtain adequately bonded and uniform film coatings. Preliminary tests indicated that there is a certain amount of correlation between the critical surface-tension values and film-coating characteristics such as degree of adhesion and film texture (5).

Although no direct method of measuring solid surface energy has yet been found, critical surface-tension (γ_c) values do represent an indirect approach to the characterization of solid surfaces according to their CED.

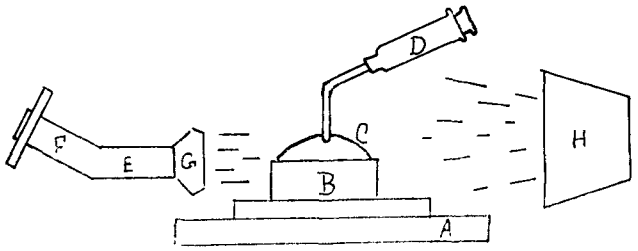


Figure 1—*Schematic arrangement of apparatus. Key: A, moveable microscope stage; B, tablet resting on glass microscope slide; C, test liquid being applied to tablet surface; D, glass syringe with bent needle used to deliver test liquid to tablet surface; E, telemicroscope; G, ring light; F, erecting eye prism and protractor eyepiece; and H, diffuse light source for backlighting the drop of liquid on the tablet surface.*

In the articles cited in the previous paper (5), γ_c values were determined using a selection of pure liquids with surface tensions in the range necessary for the determination of these values. Some investigators did use liquids of a homologous series in an effort to overcome variations in contact angles due to specific interactions. Since it is not always possible to find a homologous series with surface tensions in the required range, a decision was made to use series of high and low surface-tension liquid mixtures. These could be prepared as series of solutions with similar physical-chemical properties and with a gradual gradation of surface-tension values. Other investigators (6–11) have also used such mixtures of high and low surface-tension liquids to determine the γ_c of a variety of surfaces.

In the present study, two series of mixtures of solvents with high and low surface tensions were used to characterize the surfaces of various tablets. The methanol–water series and the 1-butanol–formamide series represent two such systems, with both polar and dispersion forces operative and a range of surface-tension values suitable for determining the γ_c values of the solid surfaces under investigation. This study consists of observations on the validity of such characterizations

Table I—Percent w/w Composition of the Tablets for which Critical Surface-Tension Values Were Determined

Tablet Number	Acetylsalicylic Acid[a]	Magnesium Stearate[b]	Talc[c]	Cornstarch[d]	Starch USP[e]	Microcrystalline Cellulose[f]
1	100	—	—	—	—	—
2	99	1	—	—	—	—
3	99	—	1	—	—	—
4	90	—	—	10	—	—
5	90	—	—	—	10	—
6	95	—	—	—	—	5

[a] Asagran, Monsanto Can. Ltd., Ville La Salle, P.Q., Canada. [b] Magnesium stearate USP, Chemical Manufacturing Division, Fisher Scientific Co., Fair Lawn, N. J. [c] Talc USP, Anachemia Chemicals Ltd., Montreal, Canada. [d] Starch, cornstarch, Canada Corn Starch, Best Foods Division, Canada Corn Starch Co. Ltd., Montreal, Canada. [e] Starch USP, Sta-Rx Starch 1500, A. E. Staley Mfg. Co., Decatur, Ill. [f] Microcrystalline cellulose, Avicel, FMC Corp., American Viscose Division, Newark, Del.

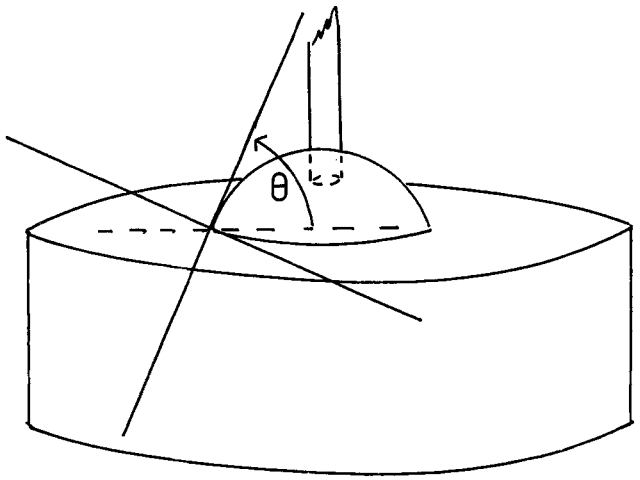


Figure 2—*View through telemicroscope showing cross hairs aligned with the advancing edge of the liquid to determine θ, the contact angle a particular test solution makes with a particular tablet surface.*

and on possible reasons for differences in γ_c values when different series of test liquids are used.

Modifications have been made in the procedure previously used in an effort to get more reproducible results. In the previous work, the contact angles reported were measurements made as soon as possible after a single drop was placed on the tablet surface. Because of the porous nature of the tablet, considerable difficulty was experienced in obtaining true initial contact-angle readings that actually reflected the surface energy of a particular tablet surface. In this paper, the contact-angle data reported are the results of successive contact-angle readings as the test liquid was being continuously applied and advanced across the surface of the tablet. This method ensured that the angles of contact measured were advancing contact angles. Also, the mean contact-angle value for a tablet surface obtained from successive measurements as the liquid advances across the tablet surface is a more accurate measure of the surface energy of that tablet. It is not as prone to distortion due to heterogeneity and tablet imperfections, as is a single measurement on one discreet drop made some time after the drop is placed on the tablet surface.

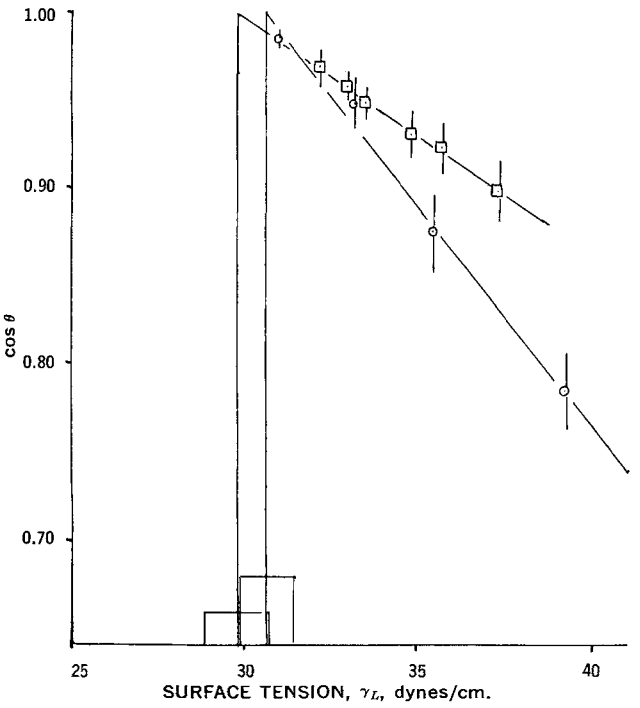


Figure 3—*Tablets of pure acetylsalicylic acid; cos θ* versus γ_L *plots of two liquid series. Key: ⊡, 1-butanol–formamide; and ⊙, methanol–water.*

Table II—Composition and Surface Tension of Test Liquids

Methanol–Water		1-Butanol–Formamide	
Concentration, %w/w	Surface Tension, dynes/cm.	Concentration, %w/w	Surface Tension, dynes/cm.
26	43.5	4	43.7
30.7	41.6	5	41.8
34	39.2	6	40.3
37.5	37.9	6.5	39.6
40	37.4	7	39.1
46	35.2	8.5	37.3
54.2	33.2	10	35.6
63.8	31.0	11.5	34.8
72.9	29.1	13.75	33.5
80.6	27.4	15	33.0
87.7	25.8	17	32.2
94.2	24.3	30	29.2
100	23.1	35	28.6
		50	27.6
		60	27.1
		75	26.2
		90	25.4
		100	24.9

EXPERIMENTAL

Materials—Tablets were prepared using a pure granular amorphous acetylsalicylic acid (ASA) with adjuvants as indicated (Table I). The tablets were prepared on a single-punch tablet machine using 1.27-cm. (0.5-in.) flat faced punches.

The two series of test liquids were mixtures of absolute methanol[1]–water[2] and 1-butanol[3]–formamide.[3] Table II indicates the surface tensions and compositions of the test liquids.

Su face-Tension Measurements—The surface tensions of the test liquids were determined with a Fisher Tensiomat.[4] Each recorded measurement is the mean of at least 10 trials, corrected for ring dimension and density of the liquid.

Contact-Angle Measurements—The apparatus used to measure the contact angles consisted of a horizontal telemicroscope[5] fitted with an erecting adapter, a protractor eyepiece (eyepiece 10X), and an objective lens (objective 2.8X) with a ring light. A further diffuse light source in the form of a microscope light was mounted behind the sample.

The sample holder consisted of a glass microscope slide mounted horizontally on a mechanical microscope stage fitted with a micrometer.

The test liquids were delivered onto the tablet surface by means of a 1-ml. glass syringe mounted in a horizontal position, fitted with a 21-gauge stainless steel needle bent in a 90° curve and containing a polyethylene tubing[6] insert protruding from the end. The syringe piston was driven by a syringe pump[7] set to deliver between 0.0355

[1] Analar, Analytical reagent, British Drug Houses (Can.) Ltd., Toronto, Canada.
[2] Doubly distilled water.
[3] Reagent grade, Chemical Manufacturing Division, Fisher Scientific Co., Fair Lawn, N. J.
[4] Fisher Surface Tensiomat, Fisher Scientific Co., Fair Lawn, N. J.
[5] Telemicroscope model M101T, Gaertner Scientific Corp., Chicago, Ill.
[6] Intramedic polyethylene tubing, i.d. 0.028 cm. (0.011 in.) × o.d. 0.061 cm. (0.024 in.), Clay-Adams, Inc., New York. N. Y.
[7] Sage Syringe Pump, model 255-1, Sage Instruments, Inc., Subsidiary of Orion Research Inc., White Plains, N. Y.

Table III—Contact Angles for the Methanol–Water Solution Series

Surface Tension of Liquid, dynes/cm.	Mean Contact Angle $\theta \pm SD$					
	Pure Acetylsalicylic Acid	Acetylsalicylic Acid–Magnesium Stearate	Acetylsalicylic Acid–Talc	Acetylsalicylic Acid–Cellulose	Acetylsalicylic Acid–Starch	Acetylsalicylic Acid–Starch USP
43.5					35.2 ± 2.6	41.2 ± 2.3
41.6			45.0 ± 1.6		33.5 ± 4.3	37.6 ± 2.1
39.2	38.1 ± 2.1			38.5 ± 1.9		34.9 ± 1.6
37.9				34.2 ± 2.1		27.9 ± 2.1
37.4			35.7 ± 1.9	33.7 ± 2.2	24.6 ± 3.1	28.1 ± 2.0
35.2	28.8 ± 2.7		29.4 ± 1.2	24.6 ± 2.9	13.2 ± 2.2	17.1 ± 1.4
33.2	18.5 ± 2.7		21.9 ± 1.9	14.3 ± 2.3	7.0 ± 1.5	8.6 ± 1.4
31.0	9.4 ± 2.0		10.4 ± 2.5			
29.1		57.1 ± 2.5				
27.4		48.8 ± 2.1				
25.8		41.7 ± 2.5				
24.3		32.7 ± 1.6				
23.1		25.3 ± 1.7				

and 0.0450 ml./min. Figure 1 shows a schematic arrangement of the apparatus.

The contact angles were measured at approximately 10-sec. intervals as the test liquid advanced across the tablet surface. Prior to testing, the tablet surface was wiped on a clean paper towel and brushed with a camel hair brush to remove any materials or loose powder which might have contaminated the surface and could affect the contact angle.

Figure 2 shows a view of the contact angle through the telemicroscope.

All experimental work was carried out in a controlled environment at 20° and 45% relative humidity.

RESULTS

Mean contact angles were determined for the six sets of tablets indicated in Table I, using the relevant members of the series of test liquids indicated in Table II. Each contact angle reported is the mean of approximately 100 readings. Ten readings were made on each of 10 tablets. The mean contact angle and standard deviation ($\theta \pm SD$) of each set were calculated. Tables III and IV are a tabulation of the contact-angle data obtained.

Figures 3–8 show graphically the results when $\cos \theta \pm SD$ is plotted against the surface tension of the test liquid applied to the surface. A linear regression analysis was used to determine the best line through the points, the slope of the line, the degree of correlation, an estimate of critical surface tension (extrapolate to $\cos \theta = 1$), and the error of the estimate. Table V shows a summary of these results.

DISCUSSION

The degree of correlation between surface tension of the test liquids and contact angle is especially good for the tests carried out with the methanol–water solutions. Between 95 and 99% of the variation in contact angle can be attributed to the variation in surface tension of the test liquid.

Although the γ_c values obtained by the use of one test liquid fall within the error of the estimate of the γ_c value obtained by using the

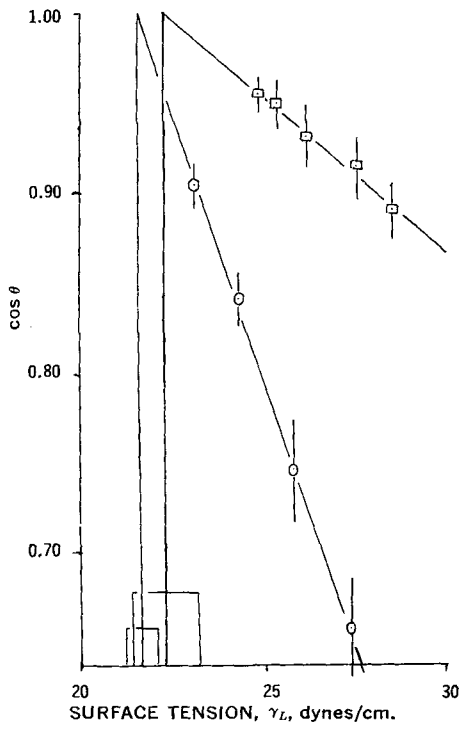


Figure 4—*Tablets of acetylsalicylic acid with 1% magnesium stearate; cos θ versus γ_L plots of two liquid series. Key: ⊡, 1-butanol–formamide; and ⊙, methanol–water.*

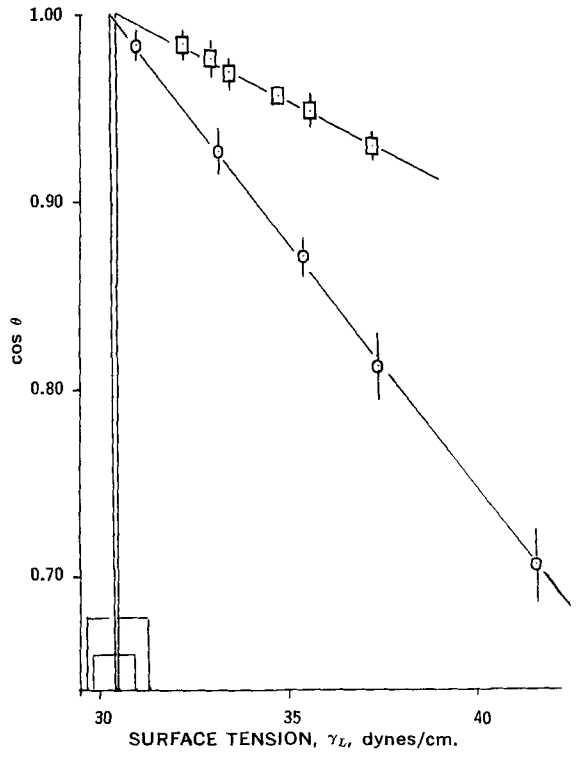


Figure 5—*Tablets of acetylsalicylic acid with 1% talc; cos θ versus γ_L plots of two liquid series. Key: ⊡, 1-butanol–formamide; and ⊙, methanol–water.*

Table IV—Contact Angles for the 1-Butanol–Formamide Solution Series

Surface Tension of Liquid, dynes/cm.	Mean Contact Angle $\theta \pm SD$					
	Pure Acetylsalicylic Acid	Acetylsalicylic Acid–Magnesium Stearate	Acetylsalicylic Acid–Talc	Acetylsalicylic Acid–Cellulose	Acetylsalicylic Acid–Starch	Acetylsalicylic Acid–Starch USP
43.7					19.4 ± 1.3	
41.8					18.3 ± 1.5	
40.3					17.2 ± 1.0	20.1 ± 2.4
39.6				21.8 ± 1.3	15.7 ± 1.4	19.2 ± 1.4
39.1				20.7 ± 1.4	15.2 ± 1.3	18.7 ± 2.5
37.3	25.8 ± 2.3		21.6 ± 1.2	18.2 ± 2.1	14.2 ± 1.2	16.0 ± 1.5
35.6	25.5 ± 2.1		18.5 ± 1.7	15.9 ± 1.9	11.0 ± 1.6	13.4 ± 1.3
34.8	21.3 ± 2.2		17.1 ± 1.8	15.0 ± 1.8	9.4 ± 1.9	11.3 ± 1.2
33.5	18.5 ± 1.7		14.4 ± 1.9	10.5 ± 2.1		
33.0	16.5 ± 1.7		12.6 ± 2.5			
32.2	14.2 ± 2.5		10.5 ± 3.0			
29.2						
28.6		27.1 ± 2.1				
27.6		23.9 ± 2.6				
27.1						
26.2		21.3 ± 2.8				
25.4		18.4 ± 2.6				
24.9		17.4 ± 1.8				

other test liquid, there is an indication that for the tablets with a higher surface energy, a higher γ_c value is obtained when methanol–water contact-angle data are used. It would appear that the γ_c value obtained for a particular tablet is dependent on the type of test solution employed.

The slopes of the curves for methanol–water in all cases are greater than the slopes of the curves for the 1-butanol–formamide solutions. This indicates that changing the concentration of the methanol–water solution so as to increase its surface tension by a fixed amount would cause a greater deterioration in the wetting properties than would be caused by a similar increase in the surface tension of the 1-butanol–formamide solution.

A straight-line relationship between cos θ and the surface tension of the liquid as cos $\theta \rightarrow 1$ has been assumed. One is aware that the overall relationship is not linear, but the part of the curve cos θ between 0.7 and 1.0 so closely approximates a straight line that it is felt that this approach is valid. The high degree of correlation, despite the considerable variation in cos θ values across a tablet surface and between tablets with the same composition, seems to justify this approximation. The results published by Dann (12) for ethanol–water mixtures on a pure hydrocarbon surface such as paraffin and on surfaces with both polar and nonpolar forces such as poly(ethylene terephthalate) and poly(methylmethacrylate) yield curves that very nearly approximate straight lines between cos θ values of 0.7–1. The error introduced by this assumption would be well within the error due to variation in contact-angle measurements, which he puts at $\pm 3°$ in his work on polar polymers.

The surface tension of an organic compound can be estimated from its chemical structure using parachor values (13). The estimated surface tension of acetylsalicylic acid using this method is 34.0 ± 1.7 dynes/cm. This value is fairly close to the estimates obtained from γ_c value data, 30.7 ± 0.8 dynes/cm. and 29.8 ± 0.9 dynes/cm. The discrepancy may well be due to an imbalance of polar and dispersion forces between the tablet surface and the test liquids used.

The addition of magnesium stearate considerably reduces the γ_c of the tablet surface. The slopes of both lines, cos θ *versus* γ_L for methanol–water and 1-butanol–formamide, become steeper (more negative) by the addition of magnesium stearate to the acetylsalicylic acid. This signifies increasingly poorer wetting properties as the surface tension of the test liquids in contact with the tablet surface is increased.

The addition of talc does not significantly affect the γ_c of the tablet surfaces, although it does alter the slopes of the curves so that the curves do not intersect as they do for pure acetylsalicylic acid. The significance, if any, of these changes has not yet been established.

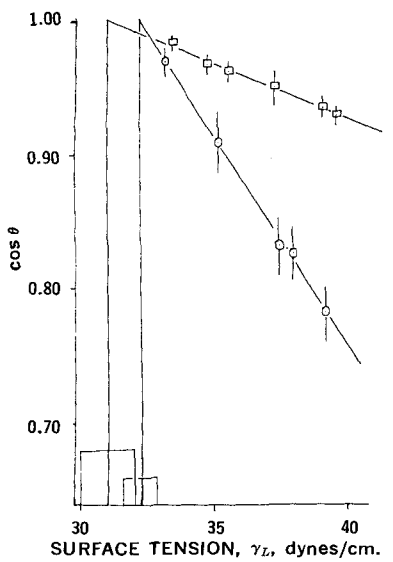


Figure 6—*Tablets of acetylsalicylic acid with 5% microcrystalline cellulose; cos θ versus γ_L plots of two liquid series. Key: ⊡, 1-butanol–formamide; and ⊙, methanol–water.*

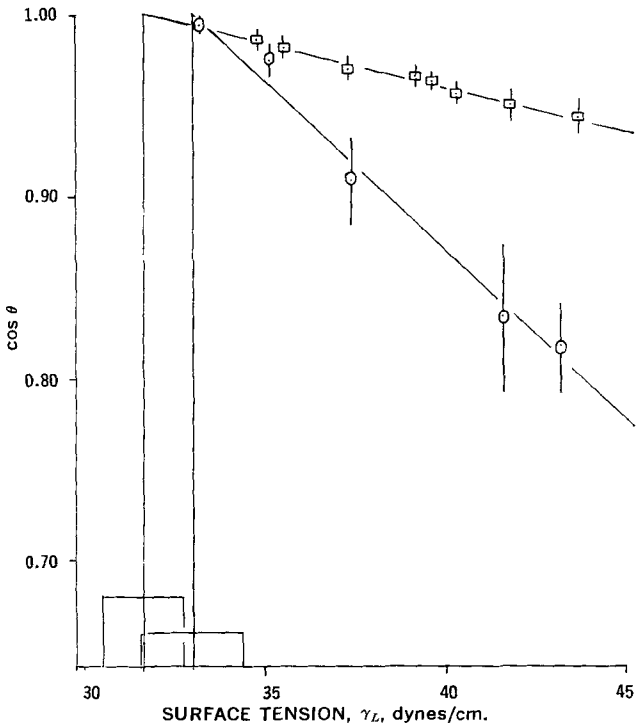


Figure 7—*Tablets of acetylsalicylic acid with 10% starch; cos θ versus γ_L plots of two liquid series. Key: ⊡, 1-butanol–formamide; and ⊙, methanol–water.*

Table V—Summary of Data Analysis from Plots, cos θ *versus* γ_L

Tablet Composition	Methanol–Water Data: Estimate γ_c, dynes/cm.	Methanol–Water Data: Slope	Methanol–Water Data: Correlation	1-Butanol–Formamide Data: Estimate γ_c, dynes/cm.	1-Butanol–Formamide Data: Slope	1-Butanol–Formamide Data: Correlation
Acetylsalicylic acid–1% magnesium stearate	21.6 ± 0.4	−0.060	0.96	22.2 ± 0.9	−0.017	0.96
Acetylsalicylic acid pure	30.7 ± 0.8	−0.025	0.94	29.8 ± 0.9	−0.013	0.77
Acetylsalicylic acid–1% talc	30.4 ± 0.6	−0.026	0.98	30.5 ± 0.8	−0.010	0.89
Acetylsalicylic acid–5% cellulose	32.2 ± 0.6	−0.031	0.96	31.0 ± 1.0	−0.008	0.96
Acetylsalicylic acid–10% starch	33.0 ± 1.4	−0.019	0.94	31.6 ± 1.1	−0.005	0.92
Acetylsalicylic acid–10% starch USP	32.9 ± 0.8	−0.025	0.97	32.1 ± 1.3	−0.008	0.85

The addition of microcrystalline cellulose and starches results in surfaces somewhat richer in hydroxyl groups, a consequent higher surface energy, and a surface more prone to polar bonding with polar materials. The experimental γ_c values are increased by the addition of these materials. The fact that for all three tablets the γ_c values obtained with the more polar methanol–water test liquids are greater than that obtained with the 1-butanol–formamide test liquids tends to indicate greater interaction of polar forces.

The attractive forces present in the surface layers and the extent and manner of interaction of these intermolecular forces have been discussed by Dann (12). When a liquid (L), with a surface tension (γ_L) made up of components (γ_L^W) due to dispersion forces and (γ_L^P) due to polar forces, is in contact with a surface made up of saturated hydrocarbons (S) where the surface tension (γ_S) is due to dispersion forces only ($\gamma_S = \gamma_S^W$), the relationship between the contact angle (D) the liquid makes with the hydrocarbon surface is given by the Good–Girifalco–Fowkes–Young equation (14):

$$\cos\theta = 2\sqrt{\gamma_S^W}\left(\frac{\sqrt{\gamma_L^W}}{\gamma_L}\right) - 1 \qquad \text{(Eq. 1)}$$

If the surface has both dispersion forces and polar forces present and the polar forces are mainly due to hydrogen bonding whose interaction across the interface can also be expressed by a geometric mean relationship, then Eq. 1 becomes:

$$\cos\theta = 2\sqrt{\gamma_S^W}\left(\frac{\sqrt{\gamma_L^W}}{\gamma_L}\right) + 2\sqrt{\gamma_S^P}\left(\frac{\sqrt{\gamma_L^P}}{\gamma_L}\right) - 1 \qquad \text{(Eq. 2)}$$

Since γ_L and cos θ are measurable, γ_S^W for paraffin is known to be 25.5 dynes/cm. (12). Moreover, since $\gamma_L = \gamma_L^W + \gamma_L^P$, the polar and dispersion forces operative in a liquid with mixed attractive forces can be determined (14). The same method could be applied to solid surfaces with mixed attractive forces to determine the individual polar and dispersion forces operative. A knowledge of the breakdown of these forces would permit adjustment of either or both the solid surface or the liquid applied to yield a balanced interface with optimum interaction and, therefore, optimum adhesion. It would also permit the intelligent application of the Zisman technique (15) in determining γ_c values of solid surfaces with mixed attractive forces.

By examining Eq. 2, one sees that if γ_S and γ_L are constant and $\gamma_S^W > \gamma_S^P$, an increase in the γ_L^P will cause a comparable decrease in the γ_L^W and an overall decrease in cos θ, *i.e.*, a larger contact angle. This would account for the difference in slopes of the plots of cos θ *versus* γ_L for the two different test liquid series.

The extent to which the γ_c values obtained represent the surface energies or attractive forces in the tablet surfaces depends on the extent to which the polar and dispersion forces in the test liquid balance the polar and dispersion forces in the solid surface, since cos $\theta \rightarrow 1$ when the test liquid only just wets the surface. Figure 9, taken from Dann (12), illustrates the effects of an unbalanced system. A solid surface, which yields a γ_c^W of 39 dynes/cm. when determined using a nonpolar liquid with only dispersion forces interacting, will yield a critical surface tension with a polar series of liquids (γ_c^{PL}), similar to the 1-butanol–formamide, of 31 dynes/cm. When an even more polar series of liquids, ethanol–water, similar to the methanol–water, is used, an even lower γ_c^{PL} value of 26 dynes/cm. is obtained. The use of highly polar liquid series to determine the

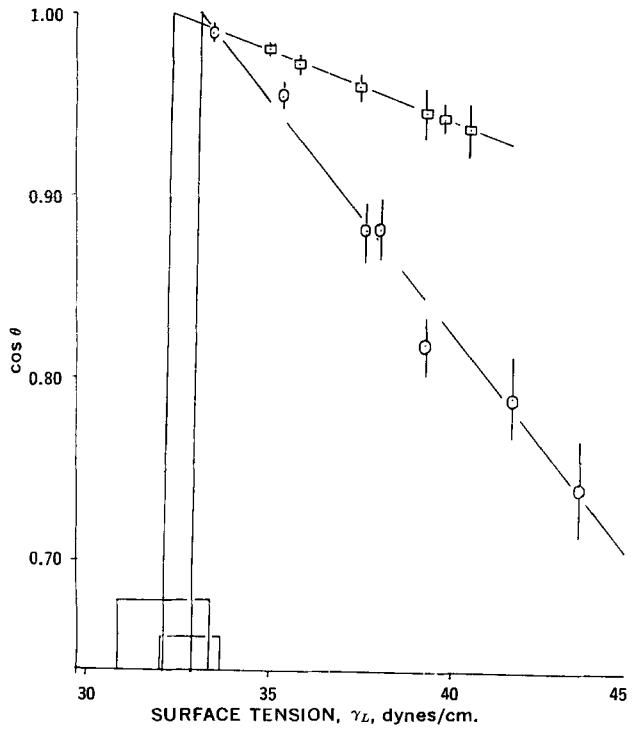


Figure 8—*Tablets of acetylsalicylic acid with 10% starch USP; cos θ versus γ_L plots of two liquid series. Key: ⊡, 1-butanol–formamide; and ⊙, methanol–water.*

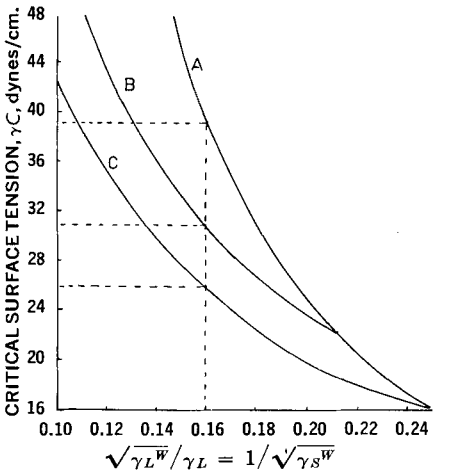


Figure 9—*Critical surface-tension conversion curves. Key: A, hydrocarbon liquid series with dispersion forces only; B, liquid series with moderate polar forces; and C, ethanol–water series with greater polar forces.*

surface energy level of surfaces with only moderate polar forces would result in a very misleading estimate of at least the dispersion forces present in that surface.

CONCLUSIONS

It is possible to characterize tablet surfaces according to their CED by using contact-angle data and applying a modification of the Zisman (15) technique. However, caution must be exercised in the interpretation of the γ_c values obtained. Particular attention should be paid to the possible effects of unbalanced polar and dispersion forces between the test liquids and surfaces investigated.

The surface energy of a tablet, as well as the types of forces present, can to some extent be predicted by studying the types of chemical components that would be present at the surface.

More work needs to be done in determining the presence and relative roles of polar and dispersion forces as they influence wetting, contact-angle data, adsorption, and adhesion.

A knowledge of the types of forces acting across the interface between the tablet surface and a film coating, their relative intensities, and the degree of interaction should greatly advance the understanding of processes involved in the formation of an adequate film coating.

REFERENCES

(1) J. N. Anand and R. Z. Balwinski, *J. Colloid Interface Sci.*, **31**, 196(1969).

(2) J. N. Anand, *ibid.*, **31**, 203(1969).

(3) J. N. Anand and H. J. Karam, *ibid.*, **31**, 208(1969).

(4) S. Orchon, *Tappi*, **41**, 33(1958).

(5) J. A. Wood and S. W. Harder, *Can. J. Pharm. Sci.*, **5**, 18(1970).

(6) M. E. Ginn, C. M. Noyes, and E. Jungerman, *J. Colloid Interface Sci.*, **26**, 146(1968).

(7) A. W. Adamson, K. Kunichika, F. Skirley, and M. Orem, *J. Chem. Educ.*, **45**, 702(1968).

(8) A. J. G. Allan, *J. Polym. Sci.*, **38**, 297(1959).

(9) F. M. Fowkes and W. D. Harkins, *J. Amer. Chem. Soc.*, **62**, 3377(1940).

(10) A. G. Pittman, D. L. Sharp, and B. A. Lugwig, *J. Polym. Sci., A-1*, **6**, 1729(1968).

(11) W. H. Smarook and S. Bonotto, *Polym. Eng. Sci.*, **8**, 41 (1968).

(12) J. R. Dann, *J. Colloid Interface Sci.*, **32**, 302(1970).

(13) R. H. Perry, C. H. Chilton, and S. D. Kirkpatrick, "Chemical Engineer's Handbook," 4th ed., McGraw-Hill, New York, N. Y., 1963, pp. 3-223.

(14) F. M. Fowkes, *J. Phys. Chem.*, **66**, 382(1962).

(15) W. A. Zisman, *Advan. Chem. Ser.*, **43**, 1(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Department of Pharmaceutics, College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.*

Accepted for publication July 17, 1970.

This work was supported by Medical Research Council of Canada Operating Grant MA3629.

Rotating-Flask Method for Dissolution-Rate Determinations of Aspirin from Various Dosage Forms

HOWARD WEINTRAUB* and MILO GIBALDI

Abstract □ The dissolution of aspirin from different commercial dosage forms was evaluated by the rotating-flask method, and the data were correlated with previously reported absorption data. Regardless of agitation intensity, over a range from 0.9 to 2.4 r.p.m., dissolution rate was found to decrease in the following order: buffered tablets > plain tablets > timed-release tablets. The data were linearized by means of log-normal probability plots and interpreted accordingly. Aspirin dissolves from the buffered tablet about twice as rapidly as from the plain tablet and about eight times as rapidly as from the timed-release tablets. Once disintegration and deaggregation take place, the dissolution of aspirin from the capsule formulation proceeds as rapidly as from the buffered tablet. Surfactant decreased the dissolution rate of aspirin from certain formulations, in contrast to the enhanced dissolution effects observed using the beaker method where mound formation occurred. Excellent single- and multiple-quantitative correlations were observed between the dissolution data and absorption data in man.

Keyphrases □ Aspirin in dosage forms—dissolution rates □ Dissolution rates—aspirin dosage forms □ Surfactant effect—aspirin dissolution from dosage forms □ Rotating-flask method—dissolution-rate determination

A number of methods designed to measure the dissolution rate of drugs from solid dosage forms are presently available (1, 2). Interest has been particularly focused on the beaker method (3) and its variants, including the rotating-basket assembly (4) and the flask and stirrer method (5), since these methods have consistently provided data which permit some correlation with *in vivo* data (5–8).

A shortcoming of the beaker method is observed at relatively low agitation intensities, *i.e.*, <40–50 r.p.m., where the geometry of the system combined with the nature of the agitation forces the granules or particles into a mound at the bottom of the flask or beaker. The mound is more or less compact, depending upon the formulation of the dosage form, and may or may not present a markedly reduced surface area to the dissolution medium. This problem was exemplified by Levy *et al.* (6), who were concerned with the quantitative correlation of dissolution data with the gastrointestinal absorption in man of aspirin from different types of dosage forms. Clinical studies indicated that aspirin was absorbed about three times more rapidly from "plain tablets" of the drug than from a timed-release preparation. Dissolution data obtained at 50 r.p.m. gave virtually perfect correlation with respect to differences between the two dosage forms observed *in vivo*, but the dissolution rate of the drug from the plain tablet was about twice the absorption rate of the drug from the same dosage form. At 45 r.p.m., where the dissolution rate of aspirin from the plain tablet was comparable to the absorption rate of drug from this dosage form, there was only a 50% difference between the timed-

surface energy level of surfaces with only moderate polar forces would result in a very misleading estimate of at least the dispersion forces present in that surface.

CONCLUSIONS

It is possible to characterize tablet surfaces according to their CED by using contact-angle data and applying a modification of the Zisman (15) technique. However, caution must be exercised in the interpretation of the γ_c values obtained. Particular attention should be paid to the possible effects of unbalanced polar and dispersion forces between the test liquids and surfaces investigated.

The surface energy of a tablet, as well as the types of forces present, can to some extent be predicted by studying the types of chemical components that would be present at the surface.

More work needs to be done in determining the presence and relative roles of polar and dispersion forces as they influence wetting, contact-angle data, adsorption, and adhesion.

A knowledge of the types of forces acting across the interface between the tablet surface and a film coating, their relative intensities, and the degree of interaction should greatly advance the understanding of processes involved in the formation of an adequate film coating.

REFERENCES

(1) J. N. Anand and R. Z. Balwinski, *J. Colloid Interface Sci.*, **31**, 196(1969).

(2) J. N. Anand, *ibid.*, **31**, 203(1969).

(3) J. N. Anand and H. J. Karam, *ibid.*, **31**, 208(1969).

(4) S. Orchon, *Tappi*, **41**, 33(1958).

(5) J. A. Wood and S. W. Harder, *Can. J. Pharm. Sci.*, **5**, 18(1970).

(6) M. E. Ginn, C. M. Noyes, and E. Jungerman, *J. Colloid Interface Sci.*, **26**, 146(1968).

(7) A. W. Adamson, K. Kunichika, F. Skirley, and M. Orem, *J. Chem. Educ.*, **45**, 702(1968).

(8) A. J. G. Allan, *J. Polym. Sci.*, **38**, 297(1959).

(9) F. M. Fowkes and W. D. Harkins, *J. Amer. Chem. Soc.*, **62**, 3377(1940).

(10) A. G. Pittman, D. L. Sharp, and B. A. Lugwig, *J. Polym. Sci., A-1*, **6**, 1729(1968).

(11) W. H. Smarook and S. Bonotto, *Polym. Eng. Sci.*, **8**, 41 (1968).

(12) J. R. Dann, *J. Colloid Interface Sci.*, **32**, 302(1970).

(13) R. H. Perry, C. H. Chilton, and S. D. Kirkpatrick, "Chemical Engineer's Handbook," 4th ed., McGraw-Hill, New York, N. Y., 1963, pp. 3-223.

(14) F. M. Fowkes, *J. Phys. Chem.*, **66**, 382(1962).

(15) W. A. Zisman, *Advan. Chem. Ser.*, **43**, 1(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Department of Pharmaceutics, College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.*

Accepted for publication July 17, 1970.

This work was supported by Medical Research Council of Canada Operating Grant MA3629.

Rotating-Flask Method for Dissolution-Rate Determinations of Aspirin from Various Dosage Forms

HOWARD WEINTRAUB* and MILO GIBALDI

Abstract □ The dissolution of aspirin from different commercial dosage forms was evaluated by the rotating-flask method, and the data were correlated with previously reported absorption data. Regardless of agitation intensity, over a range from 0.9 to 2.4 r.p.m., dissolution rate was found to decrease in the following order: buffered tablets > plain tablets > timed-release tablets. The data were linearized by means of log-normal probability plots and interpreted accordingly. Aspirin dissolves from the buffered tablet about twice as rapidly as from the plain tablet and about eight times as rapidly as from the timed-release tablets. Once disintegration and deaggregation take place, the dissolution of aspirin from the capsule formulation proceeds as rapidly as from the buffered tablet. Surfactant decreased the dissolution rate of aspirin from certain formulations, in contrast to the enhanced dissolution effects observed using the beaker method where mound formation occurred. Excellent single- and multiple-quantitative correlations were observed between the dissolution data and absorption data in man.

Keyphrases □ Aspirin in dosage forms—dissolution rates □ Dissolution rates—aspirin dosage forms □ Surfactant effect—aspirin dissolution from dosage forms □ Rotating-flask method—dissolution-rate determination

A number of methods designed to measure the dissolution rate of drugs from solid dosage forms are presently available (1, 2). Interest has been particularly focused on the beaker method (3) and its variants, including the rotating-basket assembly (4) and the flask and stirrer method (5), since these methods have consistently provided data which permit some correlation with *in vivo* data (5–8).

A shortcoming of the beaker method is observed at relatively low agitation intensities, *i.e.*, <40–50 r.p.m., where the geometry of the system combined with the nature of the agitation forces the granules or particles into a mound at the bottom of the flask or beaker. The mound is more or less compact, depending upon the formulation of the dosage form, and may or may not present a markedly reduced surface area to the dissolution medium. This problem was exemplified by Levy *et al.* (6), who were concerned with the quantitative correlation of dissolution data with the gastrointestinal absorption in man of aspirin from different types of dosage forms. Clinical studies indicated that aspirin was absorbed about three times more rapidly from "plain tablets" of the drug than from a timed-release preparation. Dissolution data obtained at 50 r.p.m. gave virtually perfect correlation with respect to differences between the two dosage forms observed *in vivo*, but the dissolution rate of the drug from the plain tablet was about twice the absorption rate of the drug from the same dosage form. At 45 r.p.m., where the dissolution rate of aspirin from the plain tablet was comparable to the absorption rate of drug from this dosage form, there was only a 50% difference between the timed-

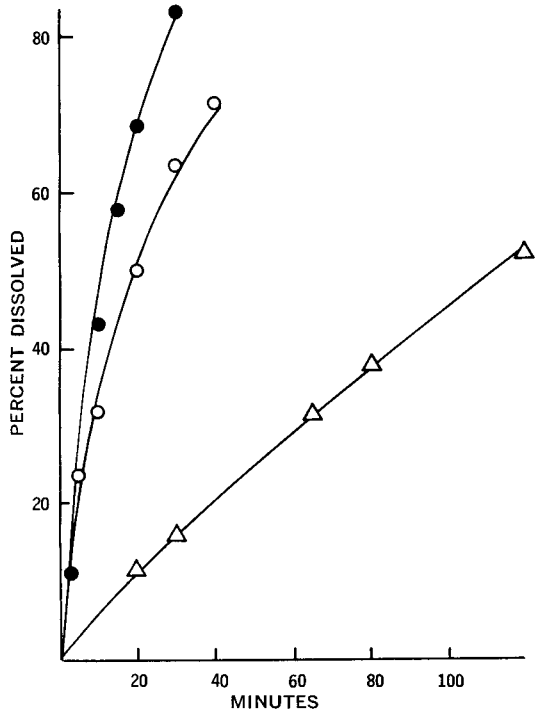


Figure 1—*Cumulative percent dissolved from aspirin dosage forms in 0.1* N *HCl at 0.9 r.p.m. Key:* ●, *buffered tablets;* ○, *plain tablets; and* △, *timed-release tablets.*

release preparation and the plain tablet, in contrast to the threefold difference observed *in vivo*. At still lower agitation rates, aspirin dissolved more rapidly from the timed-release tablets than from the plain tablets. In an attempt to overcome this problem, the rotating-flask method (9) was devised.

The philosophy underlying the development of the method is in keeping with that embodied in the development of the beaker method (3). "For tablets and other solid oral dosage forms, low intensities of agitation are highly desirable and more likely to allow distinguishing formulations and products and correlating results with *in vivo* data" (2). But at the same time, the hydrodynamics of the rotating-flask method effectively preclude mound formation, which is considered to be physiologically unrealistic. The present report concerns the evaluation of aspirin dissolution from different types of commercial dosage forms by means of this method and the correlation of these data with previously reported (6, 10) absorption data.

EXPERIMENTAL

Dosage Forms—The following were used: (*a*) rapidly disintegrating tablets, each containing 650 mg. aspirin as microencapsulated particles (timed-release tablets); (*b*) rapidly disintegrating tablets, each containing 325 mg. aspirin (plain tablets); (*c*) rapidly disintegrating tablets, each containing 325 mg. aspirin and alkaline additives (buffered tablets); and (*d*) capsules, each containing 325 mg. aspirin (capsules). Each dosage form was purchased from retail outlets.

Dissolution-Rate Determination—*In vitro* dissolution rates were determined at 37° by the rotating-flask method (9). The dissolution medium consisted of 400 ml. of 0.1 *N* HCl. In certain experiments, 0.01% polyoxyethylene (23) lauryl ether[1] was added to the medium.

[1] Brij 35 SP, Atlas Chemical Industries, Wilmington, Del.

Table I—Comparison of Aspirin Dissolution from Commercial Dosage Forms at Various Experimental Conditions

Experimental Conditions and Dosage Forms	Median Time,[a] min.	Dissolution[b] Interval, min.	*SD*[c]
0.1 *N* HCl, 0.9 r.p.m.			
Buffered tablets	12	25	0.39
Plain tablets	18	46	0.47
Capsules	19	28	0.28
Timed-release tablets	104	212	0.39
0.1 *N* HCl, 1.2 r.p.m.			
Buffered tablets	9	24	0.48
Plain tablets	10	42	0.59
Capsules	16	18	0.24
Timed-release tablets	83	211	0.45
0.1 *N* HCl, 2.4 r.p.m.			
Buffered tablets	4	15	0.60
Plain tablets	7	38	0.80
Timed-release tablets	67	186	0.48
0.1 *N* HCl + SAA,[d] 0.9 r.p.m.			
Buffered tablets	19	44	0.43
Plain tablets	30	79	0.47
Capsules	18	33	0.35
Timed-release tablets	105	209	0.38

[a] Time required to dissolve 50% of the dose. [b] Time required to dissolve 84% of the dose minus the time required to dissolve 16% of the dose. [c] Log (time required to dissolve 50% of the dose) − log (time required to dissolve 16% of the dose), *or* log (time required to dissolve 84% of the dose) − log (time required to dissolve 50% of the dose). [d] 0.01% Polyoxyethylene (23) lauryl ether.

Rotation was provided by a constant-speed motor[2] coupled to a series of gears and was varied from 0.9 to 2.4 r.p.m. A minimum of five determinations was made with each dosage form at a given rate of rotation, with the exception of the timed-release tablets where three determinations were made.

At frequent intervals after the introduction of the dosage form into the flask, rotation was briefly halted, a 1-ml. sample was taken by means of a filter pipet, and rotation was initiated once again. Elapsed time was considered to be the time during which the flask was actually rotating. Preliminary studies with the most rapidly dissolving aspirin dosage form indicated that intermittent sampling had no significant effect on the dissolution profile.[3]

Each sample was made alkaline by addition of 1 ml. 1 *N* NaOH and hydrolyzed for 1 hr. at 100°. Following hydrolysis, the pH of each sample was adjusted to pH 1 with concentrated HCl, and the samples were assayed spectrophotometrically[4] at 302.5 mμ for salicylic acid.

RESULTS AND DISCUSSION

Figure 1 is a plot of the cumulative amount (expressed as percent of dose) of aspirin dissolved as a function of time from three of the dosage forms at an agitation intensity of 0.9 r.p.m. The ranking observed—*viz.*, buffered tablet > plain tablet > timed-release tablet, agrees with the findings of Levy *et al.* (6). The same rank order was observed at 1.2 and 2.4 r.p.m. The *initial* dissolution profile of aspirin from the capsule was comparable to that observed with the plain tablet, but the time required to dissolve 80–90% of the capsule dose was considerably less than that observed with the plain tablet and, in fact, compared favorably with findings from the buffered tablet. Therefore, it was difficult to compare the dissolution of aspirin from the capsule with the data from the other dosage forms.

The interpretation of the percent dissolved–time plots adopted in this study was based on the graphical methods suggested by Wagner (11). He demonstrated that under sink conditions, the per-

[2] Dayton Electric, Chicago, Ill. Motor No. 3M095, nominally rated at 1 r.p.m., 150 in.-lb. torque.

[3] For example, the amount of drug dissolved from the buffered tablet, after 15 revolutions, at an agitation rate of 2.4 r.p.m., during sequential sampling at 5 revolution intervals was 180 ± 20 mg. (*SD*, 5 determinations), in contrast to a total amount of 154 ± 21 mg. (*SD*, 5 determinations) dissolved after 15 continuous revolutions. The difference was not statistically significant ($p > 0.2$).

[4] Hitachi-Perkin-Elmer model 139 spectrophotometer.

Table II—Regression Analysis of *In Vitro–In Vivo* Correlation According to the Equation: (% dissolved to time T) = b (% absorbed to time T) + a

Experimental Conditions and Dosage Forms	Slope (b)	Intercept (a)	Correlation Coefficient
0.1 *N* HCl, 0.9 r.p.m.			
Buffered tablets	1.32	−9.0	>0.99
Plain tablets	1.21	6.3	0.98
Timed-release tablets	0.71	5.3	0.98
All dosage forms	1.14	0.3	0.91
0.1 *N* HCl, 1.2 r.p.m.			
Buffered tablets	1.27	1.9	>0.99
Plain tablets	1.06	25.0	0.98
Timed-release tablets	0.87	4.2	0.99
All dosage forms	0.90	16.5	0.80
0.1 *N* HCl, 2.4 r.p.m.			
Buffered tablets	1.87	17.6	0.99
Plain tablets	1.44	18.4	0.99
Timed-release tablets	1.01	8.0	0.99
All dosage forms	0.85	27.3	0.78
0.1 *N* HCl + SAA,[a] 0.9 r.p.m.			
Buffered tablets	0.96	−1.7	>0.99
Plain tablets	1.20	−11.2	0.99
Timed-release tablets	0.97	0.4	0.98
All dosage forms	0.99	−1.8	0.98

[a] 0.01% Polyoxyethylene (23) lauryl ether.

cent dissolved to time T from a solid is equal to the percent of surface area generated to time T of total surface generated. Wagner proposed that the properties of the distribution of surface area available for dissolution from a capsule or tablet dosage form are similar to those of a log normal and log logistic distribution. Hence, one could plot the cumulative percent dissolved values on the probability scale (ordinate) *versus* the corresponding time values on the logarithmic scale (abscissa). If the data are described by this particular distribution function, then the points should describe a straight line. An estimate of the median dissolution time may be obtained by reading the time corresponding to the 50% point, and an estimate of the standard deviation (SD) may be obtained from the 16, 50, and 84% points by appropriate conversion of the time values to their logarithms. In the present report the difference, in units of time, between the 16 and 84% points is termed the dissolution interval.

The percent dissolved–time data obtained with the various dosage forms, with and without surfactant in the dissolution medium, at various agitation intensities were well described in each

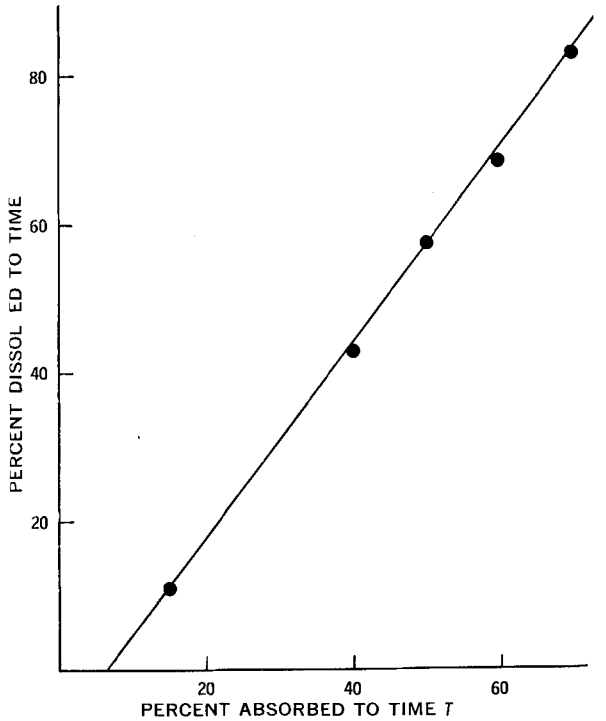


Figure 3—*Relationship between percent aspirin dissolved from buffered tablets and percent aspirin absorbed from this dosage form to the same time* T. *Dissolution data obtained in 0.1* N *HCl at 0.9 r.p.m.*

case by log–normal probability plots. Examples are shown in Fig. 2, using dissolution data obtained at 2.4 r.p.m. The critical parameters used to describe the dissolution data for all dosage forms under various experimental conditions are summarized in Table I.

As expected, for a given dosage form both the median time and dissolution interval decrease with an increase in agitation intensity; with the exception of the capsule data, the standard deviation increases with increasing agitation. Regardless of agitation intensity, the median time increases in the following order: buffered tablet < plain tablet < capsule < timed-release tablet. Comparison of the dissolution interval data suggests that aspirin dissolves from the buffered tablet about twice as rapidly as from the plain tablet and

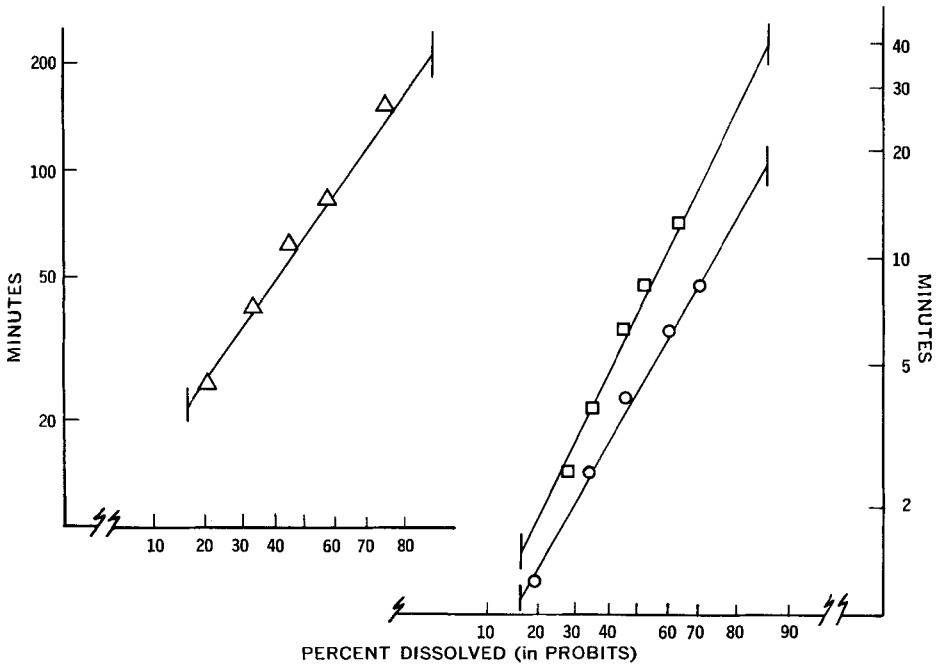


Figure 2—*Log–normal probability plots of dissolution data from aspirin dosage forms in 0.1* N *HCl at 2.4 r.p.m. Key:* ○, *buffered tablets;* □, *plain tablets; and* △, *timed-release tablets.*

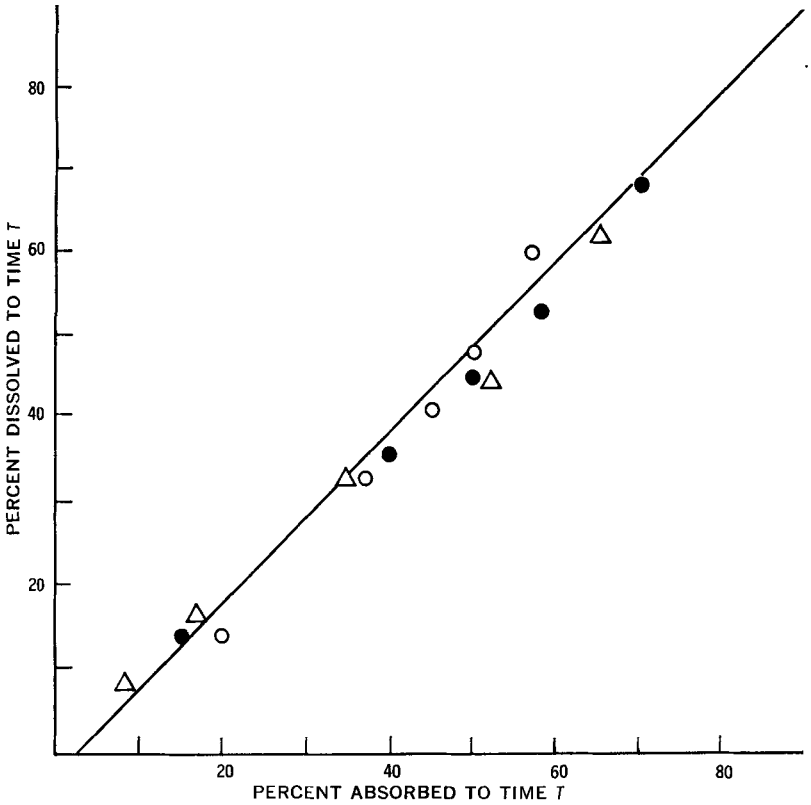


Figure 4—*Relationship between percent aspirin dissolved and percent aspirin absorbed to the same time* T. *Dissolution data obtained in 0.1* N *HCl with 0.01% polyoxyethylene (23) lauryl ether at 0.9 r.p.m. Key:* ●, *buffered tablets;* ○, *plain tablets; and* △, *timed-release tablets.*

about eight times as rapidly as from the timed-release tablet. Ratios of dissolution intervals are reasonably constant over the entire range of agitation intensities. The dissolution interval for the capsule was comparable to that of the buffered tablet. These observations suggest that disintegration and deaggregation of the capsule contents require a greater time than for similar events to occur with the plain and buffered tablets; but once these processes take place, the dissolution of aspirin from the capsule formulation proceeds as rapidly as from the disintegrated buffered tablet formulation.

The influence of 0.01% nonionic surfactant on the dissolution rate of aspirin from the various dosage forms at 0.9 r.p.m. is also summarized in Table I. Addition of the surfactant results in a considerable increase in the median time and dissolution interval for both the plain and buffered tablets but is without effect on the dissolution of aspirin from the capsule and timed-release formulations. In a previous report (12) concerned with dissolution of aspirin from the capsule and buffered tablet dosage forms using the flask and stirrer method, it was observed that 0.01% polyoxyethylene (23) lauryl ether had no effect on the dissolution of aspirin from the capsule but markedly *decreased* the median dissolution time for the buffered tablet. In the judgement of the present authors, the qualitatively different effects of the surfactant on the dissolution of aspirin from the buffered tablet, using the beaker method and rotating-flask method, are attributable to mound formation in the former and its absence in the latter.

In the beaker method, the disintegrated buffered tablet granules apparently form a rather compact mound at the bottom of the flask and give rise to a "porous plug" which the dissolution medium finds difficult to penetrate. The surfactant enhances pore penetration ostensibly by reducing the contact angle and thereby increases the effective surface area and, in turn, the dissolution rate. Since the rotating-flask method does not give rise to a mound or porous plug, one anticipates that the surfactant would not enhance the dissolution rate. This was indeed the case. The decreased dissolution rate of aspirin from the plain and buffered tablets which was observed in the presence of the surfactant may be due to a dewetting phenomenon and subsequent aggregation of the particles (13).

In a preliminary communication (9), the authors reported that the aspirin-dissolution data for the plain, buffered, and timed-release tablets obtained with the rotating-flask method provided an excellent quantitative correlation with previously reported *in vivo* absorption data (6, 10) on the same dosage forms. This type of analysis was extended, and comparisons of *in vivo* and *in vitro* data were made for the individual dosage forms as well as for the three dosage forms simultaneously under various experimental conditions. Figure 3 is an example of a single correlation. Excellent linearity is observed when the percent of dose dissolved in time T from a buffered tablet in 0.1 N HCl at 0.9 r.p.m. is plotted as a function of the percent absorbed to time T in man. The slope of the line is 1.32, and the correlation coefficient is > 0.99. Figure 4 shows the best example of a multiple correlation. Dissolution data were obtained in 0.1 N HCl with surfactant at 0.9 r.p.m. The slope of the least-squares line is 0.99, and the correlation coefficient is 0.98.

Table II summarizes the analysis for *in vivo–in vitro* correlation and includes the least-squares slope and intercept for the equation:

$$(\%\text{ dissolved to time } T) = b(\%\text{ absorbed to time } T) + a \quad \text{(Eq. 1)}$$

as well as the correlation coefficient. The latter values were computed according to Mather (14) for interclass correlation where both variables are normally distributed. In all but two cases, the correlation coefficient was greater than 0.90 and in all cases the correlation coefficient was highly significant ($p < 0.005$).

Based on the *in vivo–in vitro* correlations obtained with the aspirin dosage forms, it would appear that the rotating-flask method offers certain advantages over the beaker methods currently in use. However, before any *in vitro* dissolution method can be relied upon as an index of physiologic availability or *in vivo* absorption rate, more retrospective testing is required.

REFERENCES

(1) J. A. Hersey, *Mfg. Chem. Aerosol News*, **40**, 32(1969).

(2) J. G. Wagner, *Drug Intel. Clin. Pharm.*, **4**, 92(1970).

(3) G. Levy and B. A. Hayes, *New Engl. J. Med.*, **262**, 1053 (1960).

(4) M. Pernarowski, W. Woo, and R. O. Searl, *J. Pharm. Sci.*, **57**, 1419(1968).

(5) J. W. Poole, *Drug Inform. Bull.*, **3**, 8(1969).

(6) G. Levy, J. R. Leonards, and J. A. Procknal, *J. Pharm. Sci.*, **54**, 1719(1965).

(7) R. O. Searl and M. Pernarowski, *Can. Med. Ass. J.*, **96**, 1513(1967).
(8) R. A. O'Reilly, E. Nelson, and G. Levy, *J. Pharm. Sci.*, **55**, 435(1966).
(9) M. Gibaldi and H. Weintraub, *ibid.*, **59**, 725(1970).
(10) G. Levy, *Arch. Int. Pharmacodyn. Ther.*, **152**, 59(1964).
(11) J. G. Wagner, *J. Pharm. Sci.*, **58**, 1253(1969).
(12) H. Weintraub and M. Gibaldi, *ibid.*, **58**, 1368(1969).
(13) G. Zografi, Compilation of Symposia Papers Presented to the APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, Nov. 1968, p. 190.
(14) K. Mather, "Statistical Analysis in Biology," 4th ed., Methuen, London, England, 1964, pp. 160, 161.

ACKNOWLEDGMENTS AND ADDRESSES

Received May 25, 1970, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication July 22, 1970.

The technical assistance of Mr. James Mecca of the Health Sciences Instrument Shop, State University of New York at Buffalo, in the construction of the gear assembly used in this study is acknowledged.

* Fellow of the American Foundation for Pharmaceutical Education. Present address: Geigy Pharmaceutical, Suffern, NY 10901

Theoretical Approach to Sustained-Release Multiple-Dose Therapy: Noncumulative Attainment of Desired Blood Level

JOSEPH R. ROBINSON* and STUART P. ERIKSEN†

Abstract □ Equations are presented to allow calculation of doses and dosing interval for multiple-dose therapy of sustained-release dosage forms. Both zero- and first-order release of drug from the dosage form are considered in developing these equations. Although special problems are associated with multiple dosing of sustained-release dosage forms because of their unique design, application of the appropriate equations yields relatively uniform blood levels of drug.

Keyphrases □ Sustained-release products—multiple-dose therapy □ Equations—doses, dosing intervals, calculation □ Blood levels—noncumulative multiple-dose therapy □ Theoretical approach—noncumulative blood levels, sustained-release therapy

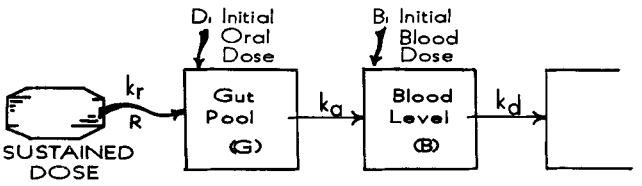
Considerable effort has been expended in the mathematical development of dosage regimens for multiple dosing of nonsustained-release dosage forms (1–7). In recent work (7), the authors developed equations to allow calculation of doses and dosing intervals to produce and maintain a desired blood level (noncumulative approach). Surprisingly, the problem of multiple dosing with sustained-release dosage forms has not been considered. In the present work, equations are presented which will allow a rational (and hopefully pragmatic) approach to such therapy.

MODEL USED

A simple four-compartment model was used throughout this study (Scheme I). All of the customary assumptions of pharmacokinetics with respect to exponential rate processes, constants, *etc.*, as well as the validity of the model are assumed in this study (see *References Ia* and *7* for a discussion of these points).

ELEMENTARY REGIMEN

The elementary regimen design, using two units initially followed by one unit each elimination half-life later, was found to be only very approximately valid for oral nonsustained-release products, as reported in another paper (7). Simple extension of this elementary concept from nonsustained- to sustained-release dosage forms suggests that for a sustained-release dosage form, which is designed to provide flat blood levels over a full treatment period, the dosing frequency, $\tau_{sust.}$, should be



Scheme I—Model used in development and evaluation of sustained-action equations

$$\tau_{sust.} = h + t_{1/2\ elimination} \qquad \text{(Eq. 1)}$$

where h is the number of hours of desired sustained effect for which one dose of the sustained-release dosage form has been designed, and $t_{1/2\ elimination}$ is the elimination half-life of the drug. Similar, but more severe, problems than those encountered with nonsustained medications are encountered here. The solution to these problems depends somewhat upon the mathematics to describe the release of drug from the dosage form, *i.e.*, zero- or first-order release, but in general involves corrections for the same difficulties present in nonsustained multiple-dose therapy, *i.e.*, accumulation of drug in the body.

As in any therapy, the interest of both the patient and the physician is safe, but rapid, and maintained relief. A therapy dependent upon accumulation does not achieve this goal. The desired sustained-action product should rapidly attain and maintain the desired blood or tissue level of drug. All subsequent doses must be designed and taken to reachieve the plateau blood level established by the first dose.

The theoretical concepts behind the design of a single sustained-action dosage form have been reported (8–10). This earlier work was designed to produce the optimum blood picture for one administered dose. While identical concepts are involved when multiple doses are to be administered, slight alterations must be made to eliminate the undesired accumulation effect.

SATISFACTORY SUSTAINED-ACTION PATTERN

The nonsustained-action definition of "satisfactory therapeutic blood level patterns" (7), *i.e.*, a rapid rise to a peak suitable to produce the desired biological action followed by a regular rise and fall between constant values, must be altered slightly for sustained-action dosage forms. Satisfactory blood level patterns, for

(7) R. O. Searl and M. Pernarowski, *Can. Med. Ass. J.*, **96**, 1513(1967).
(8) R. A. O'Reilly, E. Nelson, and G. Levy, *J. Pharm. Sci.*, **55**, 435(1966).
(9) M. Gibaldi and H. Weintraub, *ibid.*, **59**, 725(1970).
(10) G. Levy, *Arch. Int. Pharmacodyn. Ther.*, **152**, 59(1964).
(11) J. G. Wagner, *J. Pharm. Sci.*, **58**, 1253(1969).
(12) H. Weintraub and M. Gibaldi, *ibid.*, **58**, 1368(1969).
(13) G. Zografi, Compilation of Symposia Papers Presented to the APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, Nov. 1968, p. 190.
(14) K. Mather, "Statistical Analysis in Biology," 4th ed., Methuen, London, England, 1964, pp. 160, 161.

ACKNOWLEDGMENTS AND ADDRESSES

Received May 25, 1970, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication July 22, 1970.

The technical assistance of Mr. James Mecca of the Health Sciences Instrument Shop, State University of New York at Buffalo, in the construction of the gear assembly used in this study is acknowledged.

* Fellow of the American Foundation for Pharmaceutical Education. Present address: Geigy Pharmaceutical, Suffern, NY 10901

Theoretical Approach to Sustained-Release Multiple-Dose Therapy: Noncumulative Attainment of Desired Blood Level

JOSEPH R. ROBINSON* and STUART P. ERIKSEN†

Abstract □ Equations are presented to allow calculation of doses and dosing interval for multiple-dose therapy of sustained-release dosage forms. Both zero- and first-order release of drug from the dosage form are considered in developing these equations. Although special problems are associated with multiple dosing of sustained-release dosage forms because of their unique design, application of the appropriate equations yields relatively uniform blood levels of drug.

Keyphrases □ Sustained-release products—multiple-dose therapy □ Equations—doses, dosing intervals, calculation □ Blood levels—noncumulative multiple-dose therapy □ Theoretical approach—noncumulative blood levels, sustained-release therapy

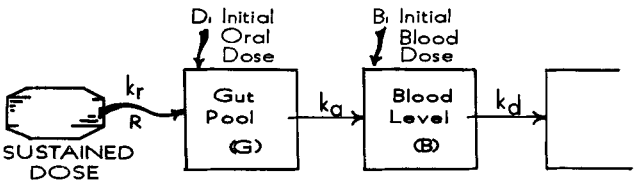
Considerable effort has been expended in the mathematical development of dosage regimens for multiple dosing of nonsustained-release dosage forms (1–7). In recent work (7), the authors developed equations to allow calculation of doses and dosing intervals to produce and maintain a desired blood level (noncumulative approach). Surprisingly, the problem of multiple dosing with sustained-release dosage forms has not been considered. In the present work, equations are presented which will allow a rational (and hopefully pragmatic) approach to such therapy.

MODEL USED

A simple four-compartment model was used throughout this study (Scheme I). All of the customary assumptions of pharmacokinetics with respect to exponential rate processes, constants, *etc.*, as well as the validity of the model are assumed in this study (see *References Ia* and *7* for a discussion of these points).

ELEMENTARY REGIMEN

The elementary regimen design, using two units initially followed by one unit each elimination half-life later, was found to be only very approximately valid for oral nonsustained-release products, as reported in another paper (7). Simple extension of this elementary concept from nonsustained- to sustained-release dosage forms suggests that for a sustained-release dosage form, which is designed to provide flat blood levels over a full treatment period, the dosing frequency, $\tau_{sust.}$, should be



Scheme I—Model used in development and evaluation of sustained-action equations

$$\tau_{sust.} = h + t_{1/2\ elimination} \qquad \text{(Eq. 1)}$$

where h is the number of hours of desired sustained effect for which one dose of the sustained-release dosage form has been designed, and $t_{1/2\ elimination}$ is the elimination half-life of the drug. Similar, but more severe, problems than those encountered with nonsustained medications are encountered here. The solution to these problems depends somewhat upon the mathematics to describe the release of drug from the dosage form, *i.e.*, zero- or first-order release, but in general involves corrections for the same difficulties present in nonsustained multiple-dose therapy, *i.e.*, accumulation of drug in the body.

As in any therapy, the interest of both the patient and the physician is safe, but rapid, and maintained relief. A therapy dependent upon accumulation does not achieve this goal. The desired sustained-action product should rapidly attain and maintain the desired blood or tissue level of drug. All subsequent doses must be designed and taken to reachieve the plateau blood level established by the first dose.

The theoretical concepts behind the design of a single sustained-action dosage form have been reported (8–10). This earlier work was designed to produce the optimum blood picture for one administered dose. While identical concepts are involved when multiple doses are to be administered, slight alterations must be made to eliminate the undesired accumulation effect.

SATISFACTORY SUSTAINED-ACTION PATTERN

The nonsustained-action definition of "satisfactory therapeutic blood level patterns" (7), *i.e.*, a rapid rise to a peak suitable to produce the desired biological action followed by a regular rise and fall between constant values, must be altered slightly for sustained-action dosage forms. Satisfactory blood level patterns, for

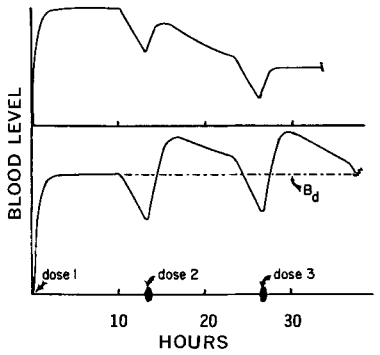


Figure 1—*Simulated blood level patterns produced with an adequately designed "zero - order released" sustained-action dosage form. Curve A (lower curve) is one dosage unit initially followed by one dosage unit every* $\tau_{sust.}$ *hr.; Curve B (upper curve) is two dosage units initially followed by one every* $\tau_{sust.}$ *hr. Both curves utilize* $\tau_{sust.}$ *as calculated from Eq. 1.*

this purpose, will be produced by dosages and regimens yielding a rapid rise to blood levels suitable to produce the therapeutic effect, maintained at that level for a desired length of time, and followed at regular intervals by suitable doses to reestablish and maintain the same blood levels. The equations required will be discussed according to the type of release produced by the dosage form used, *i.e.*, zero- or first-order release.

DRUGS RELEASED BY ZERO-ORDER PROCESSES

Calculation of Doses Required—The second dose of a sustained-release dosage form,[1] administered at time $\tau_{sust.}$ (using Eq. 1) after the start of therapy, produces blood levels similar to those shown in Fig. 1. Because of their unique design, sustained-release dosage forms are unable to maintain any blood level but the one for which they were designed (B_d). The dosage regimen, where a single sustained-release dose is taken after two initial ones, produces a slow drop of the blood level down to that value for which the single dose was designed. Single doses administered one after the other produce accumulation.

The immediately available portion of the second and subsequent sustained-action doses must be corrected to obtain the optimum flat blood level pattern for which these dosage forms are designed. For the initial dose, the maintenance portion (Dm) is prepared according to standard equations (8):

$$Dm = R \times h \qquad \text{(Eq. 2)}$$

where $R = k_d B_d$ and is the zero-order availability rate of drug from the dosage form, h is the selected time of sustained action for one dose, k_d is the elimination constant,[2] and B_d is the desired amount of drug in the blood, all ascertained from the pharmacokinetics of single, nonsustained doses. The immediately available portion (D_1), when both the immediate and maintenance portions begin release of drug from time zero, is normally calculated to be (8):

$$D_1^{corr.} = D_1 - Rt_p \qquad \text{(Eq. 3)}$$

where D_1 is the nonsustained dose required to obtain the desired peak blood level of B_d, and Rt_p is the correction on the initial dose necessary due to supply of drug from the maintenance portion over the time period zero to peak time (8). For any subsequent sustained-action dose, however, the immediately available portion must be decreased to take into account that the desired blood level (B_d) has decreased to NB_d by the time of the next dose [the fraction N is defined as the fraction of the peak height (B_d) to which the blood level is allowed to drop before the next dose is administered]. Thus, the optimum dose for the subsequent doses, $D_2^{corr.}$, can be estimated to be

$$D_2^{corr.} \ldots n \cong (1 - N)D_1 - Rt_p \qquad \text{(Eq. 4)}$$

using the approximations developed in earlier work (7, 8). It is apparent that if the half-life time is used

$$D_2^{corr.} \cong \frac{1}{2} D_1 - Rt_p \qquad \text{(Eq. 4a)}$$

To offer a complete picture, if a dosage form involving a delayed start of maintenance dose is used (8):

$$D_2 \ldots n \cong (1 - N)D_1 \qquad \text{(Eq. 5)}$$

Calculation of Dosing Interval—The calculation of $\tau^0_{sust.}$, the treatment time for the zero-order release dosage form, involves a sum of the time to empty the dosage form (h) and the time for the blood level to drop to one Nth of the plateau value:

$$\tau^0_{sust.} = h + \frac{2.3}{k_d} \log \left(\frac{1}{1 - N} \right) + \Delta t \qquad \text{(Eq. 6)}$$

where the first two terms are the general expression for which Eq. 1 is the example at $N = 1/2$. The Δt term is a correction for drug being absorbed from the gut after the moment (h) where the dosage form has ceased to yield drug (7).

The correctness of Eq. 1 assumes that the dosage form is completely empty (probably true) and that all absorption ceases at the moment h. Under these circumstances, the drop of the blood level to one Nth of the plateau value requires a time equal to $2.3/k_d \log [1/(1-N)]$ time units. The time error involved in making the assumption that all absorption ceases at the moment h for oral dosage forms can be estimated to be Δt, where Δt is the horizontal distance between that "no absorption line" and the "eventual elimination line," as shown in Fig. 2. It can be shown mathematically that the time error is identical to that previously derived for nonsustained forms (7).

At the moment that the dosage form is empty, h, the concentration in the gut is G_h, where $G_h = k_d B_d / k_a$. The equality of the rate of absorption, $k_a G_h$, and the rate of loss from the blood, $k_d B_d$, is a requirement for sustained action. From that time on, the blood level is

$$B = B_d e^{-k_d t} + \frac{G_h k_a}{k_a - k_d} (e^{-k_d t} - e^{-k_a t}) \qquad \text{(Eq. 7)}$$

Substituting $k_d B_d$ for $G_h k_a$,

$$B = B_d \left[e^{-k_d t} + \frac{k_d}{k_a - k_d} (e^{-k_d t} - e^{-k_a t}) \right] \qquad \text{(Eq. 8)}$$

The eventual elimination line for such a dosage (letting $e^{-k_a t} \to 0$) is described by the equation:

$$B = B_d e^{-k_d t} \left(\frac{k_a}{k_a - k_d} \right) \qquad \text{(Eq. 9)}$$

This equation is identical to the similar eventual elimination line described for a nonsustained dose (7). The time error, Δt, is therefore again described by the equation previously reported (7):[3]

$$\Delta t = \frac{2.3}{k_d} \log \left(\frac{k_a}{k_a - k_d} \right) \qquad \text{(Eq. 10)}$$

One precisely designed sustained-action dose, followed at intervals of $\tau^0_{sust.}$ by corrected sustained-action follow-up doses, will produce

[1] Sustained-action dosage forms, as used here, mean a system composed of an immediately available portion plus a sustaining or maintenance portion.

[2] The terms elimination and absorption refer specifically to the mathematical model considered and are so termed only for purposes of comprehension. From the standpoint of dosage form design, the only constants of concern are those describing the rise of drug concentration (or amount) in the tissue measured (blood in these discussions) and the decrease of that concentration (or amount). For a simple model, these may be called absorption and elimination constants, k_a and k_d, but they are only related to the actual constants for the physiologic processes of absorption and excretion or metabolism.

[3] The derivation of this equation involves setting the equations describing the blood level of an intravenous injection and an oral dose (Eq. 9) equal and solving for the time difference between them, *i.e.*,

$$Be^{-k_d t_1} = \frac{Bk_a}{k_d - k_d} e^{-k_d t_2}$$

$$t_2 - t_1 = \Delta t = \frac{2.3}{k_d} \log \left(\frac{k_a}{k_a - k_d} \right)$$

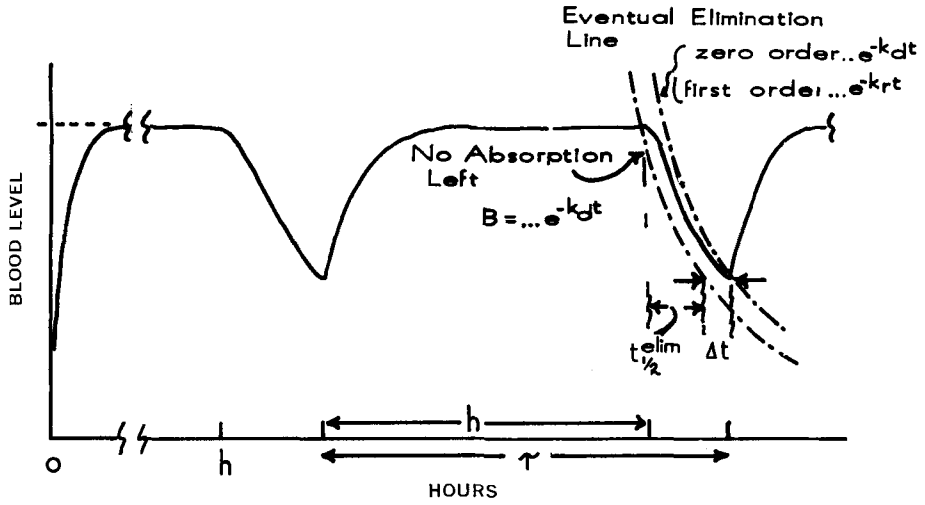


Figure 2—*Computer-drawn construction showing the development of the* Δt *correction for sustained-action dosage forms.*

the blood level patterns shown in Figs. 3 and 4. While the impractical method of administering a different dosage unit initially ($D_1^{corr.}$ and then $D_2^{corr.}$) produces the fastest rise to B_d, the use of the same corrected second dose at each interval of $\tau^0_{sust.}$ hr. achieves essentially the same result. Figure 4 shows the computer-drawn curves for a sustained-release penicillin product using zero-order release procedures and the constants of Juncher and Rasschou (11); quite acceptable sustained action is demonstrated.

DRUGS RELEASED BY FIRST-ORDER PROCESSES

Calculation of Doses Required—For the initial dose, the maintenance portion (Dm) is prepared according to standard procedures (8):

$$Dm = \frac{k_d}{k_r} B_d \qquad \text{(Eq. 11)}$$

where $k_r = k_d e^{-k_d h}$ and is the first-order availability rate of drug from the dosage form. The immediately available portion (D_1), when both the immediate and maintenance portion begin release at time zero, can be calculated using Eq. 12:

$$D_1^{corr.} = D_1 - k_r D_m t_p \qquad \text{(Eq. 12)}$$

As with the zero-order case, a correction is applied for the amount of drug contributed by the maintenance portion from time zero to the peak time (8).

Subsequent doses require correction for the immediately available portion:

$$D_2^{corr.} \ldots n = (1 - N)D_1 - k_r D_m t_p \qquad \text{(Eq. 13)}$$

If the maintenance dose has a delayed start, the equation is

$$D_2 \ldots n = (1 - N)D_1 \qquad \text{(Eq. 14)}$$

Calculation of Dosing Interval—The second and subsequent doses of a dosage form whose maintenance portion releases drug by a first-order process must be corrected in a manner similar to zero-order dosages to prevent excessive peaking. The frequency of therapy, however, plays a larger part in first-order release therapy, because after the designed sustained-release time, h, has passed, considerable drug is still available and is being released from the dosage form. First-order release forms achieve their action through small release constants, k_r, and relatively large maintenance doses, D_m (8). The blood level very rapidly becomes controlled by the dosage form release constant, so that

$$B = \frac{D_m k_r}{(k_d - k_r)} e^{-k_r t} \qquad \text{(Eq. 15)}$$

after the immediately available portion of the dosage form is no longer contributing to the blood picture. This is the equation for the "eventual elimination line" of such a dosage form (Fig. 2).

The time error (Δt_1), resulting from assuming immediate cessation of absorption and that the blood level decreases in a manner based solely on k_d, is then

$$\Delta t_1 = \frac{2.3}{k_r} \log \left(\frac{k_d}{k_d - k_r} \right) \qquad \text{(Eq. 16)}$$

making the dosing interval for first-order release dosage forms

$$\tau^1_{sust.} = h + \frac{2.3}{k_r} \log \left(\frac{1}{1 - N} \right) + \Delta t_1 \qquad \text{(Eq. 17)}$$

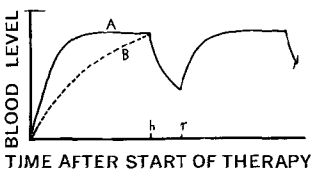


Figure 3—*Simulated blood level patterns for multiple sustained-release therapy. Curve A is initial dose unit of* ($D_1 + D_m$) *and subsequent dose units of* [$(1 - N)D_1 + D_m$] *every* $\tau_{sust.}$ *hr.; Curve B is repeated dosings with* [$(1 - N)D_1 + D_m$] *every* $\tau_{sust.}$ *hr.*

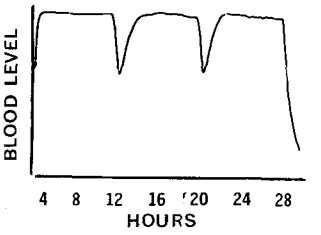


Figure 4—*Computer-drawn blood level patterns for a zero-order sustained-release penicillin product, using the pharmacokinetic constants of Juncher and Rasschou (11), calculated for a blood level of 47.65 units,* $k_a = 4.3\ hr.^{-1}$, $k_d = 0.852\ hr.^{-1}$, $R = 40.5$, $D_1^{corr.} = 51.2$, *and* $D_2^{corr.} = 25.6$.

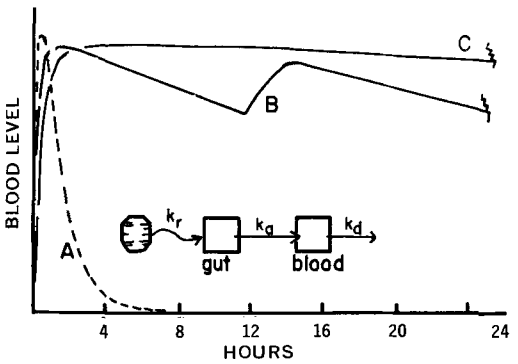


Figure 5—*A "practical" solution to sustained-action for penicillin* ($k_a = 4.3\ hr.^{-1}$, $k_d = 0.852\ hr.^{-1}$) *with a first-order release dosage form. Curve A is a computer-simulated blood level diagram for a 0.024-g. dose. Curve C is the diagram for a single sustained-action dose, the parameters calculated as described in earlier work* ($D_1^{corr.} = 0.024$ g., $D_m = 81$ g., $k_r = 0.00174\ hr.^{-1}$). *Curve B is the "practical, multiple sustained-release dosage method" described here* ($D_1^{corr.} = 0.013$ g., $D_m = 0.488$ g., $k_r = 0.028\ hr.^{-1}$).

Table I—Required Equations for Design of Sustained- and Nonsustained-Release Dosage Forms

Term	Nonsustained	Zero-Order Sustained	First-Order Sustained
D_1	D	D	D
$D_1^{corr.}$	—	$D_1 - Rt_p$	$D_1 - k_r D_m t_p$
$D_{2 \ldots n}$	$(1 - N)D$	$(1 - N)(D_1)$	$(1 - N)D_1$
$D_{2 \ldots n}^{corr.}$	—	$(1 - N)D_1 - Rt_p$	$(1 - N)D_1 - k_r D_m t_p$
D_m	—	$R \cdot h$	$\frac{k_d}{k_r} B_d$
R or k_r	—	$R = k_d B_d$	$k_r = k_d e^{-k_d h}$
τ or $\tau_{sust.}$	$t_p + \Delta t + 2.3 \log\left(\frac{1}{1-N}\right)\left(\frac{1}{k_d} - \frac{1}{k_a}\right)$	$h + \Delta t + \frac{2.3}{k_d} \log\left(\frac{1}{1-N}\right)$	$h + \Delta t_1 + \frac{2.3}{k_r} \log\left(\frac{1}{1-N}\right)$
Δt or Δt_1	$\frac{2.3}{k_d} \log\left(\frac{k_a}{k_a - k_d}\right)$	$\frac{2.3}{k_d} \log\left(\frac{k_a}{k_a - k_d}\right)$	$\frac{2.3}{k_r} \log\left(\frac{k_d}{k_d - k_r}\right)$

For the design of sustained-release dosage forms intended to be administered in more than one dose, a summary of the required equations is shown in Table I and a glossary of terms is given in Table II. For comparison, the equations required for nonsustained-release dosage forms are also shown.

PRACTICAL DESIGN OF DOSAGE FORMS

Because therapy with sustained-action dosage forms is for the benefit of the patient, not every treatment frequency is equally convenient, nor are varying dosage schedules. From a pragmatic point of view, the dosage units must be all the same or, at most, simple fractions of the first dose. In addition, treatment frequency must be constant and, by convention, be held to a very few values.

Nonsustained-Action Forms—Nonsustained-action forms are commonly administered at intervals of one, two, three, or four per day. The dosage multiples represent the only variable available to the formulator for maintaining an even series of blood level peaks and valleys.

The frequency of treatment equation (τ, see Table I) may be solved for N using τ = 6, 8, 12, and 24 hr. (7). Any simple, even fraction approximating N will enable the initial and subsequent dose ratios to be calculated (if N = 0.75 or $^3/_4$, the initial dose represents three units and the subsequent doses one unit).

Sustained-Action Forms—*Zero-Order Release*—The dosing interval contains only the sustained-action time, h, as an adjustable variable. Since $\tau_{sust.}$ for sustained-action forms is normally limited to 12 or 24 hr., h can have but two possible values for any drug. Using a selected value for $\tau_{sust.}$, the resulting value of h may be used to calculate the remaining factors.

The size of the total dose is customarily limited to 0.5 g. for one dosage unit (an administered dose could, of course, be two units).

Table II—Glossary of Terms Used

Symbol	Definition
D	Equals the 100% available oral dose required to produce the desired blood level peak, B_d
R or k_r	Zero- and first-order dosage form availability constants
t_p	Time after administration for peak blood level to occur
N	Fraction of peak or sustained blood level present at the time when the next dose is administered
h	Designated sustained action for one dose, or the time to "empty" the dosage forms
D or $D^{corr.}$	Immediately available dose (D) and corrected dose ($D^{corr.}$), with the subscript referring to the dose number
D_m	Maintenance dose
$\tau_{sust.}$	Dosing interval for sustained-action dosage forms: $\tau^0_{sust.}$ (zero order), $\tau^1_{sust.}$ (first order)
k_a, k_d	Absorption and elimination constants for the drug involved

Thus, because $D_m = R \times h$ where R is fixed by the desired blood level and the elimination constant,

$$D_m = 0.5 - (1 - N)D_1 \quad \text{(Eq. 18)}$$

$$h^* = (\text{desired } h) = D_m/R \quad \text{(Eq. 19)}$$

$\tau^0_{sust.}$ = selected value that produces a calculated value of h closest to h^* (Eq. 20)

subsequent $D_{2 \ldots n} = (1 - N)D_1$ (Eq. 21)

Note that several values of N may be tried; larger values decrease the valleys and decrease the subsequent $D_{2 \ldots n}$, smaller ones increase them.

First-Order Release—Since the total dose is again limited to approximately 0.5 g. and $\tau_{sust.}$ is again constrained to 12 or 24 hr., the required parameters are calculated from these points:

$$D_m = 0.5 - (1 - N)D_1 \quad \text{(Eq. 22)}$$

$$k_r = k_d \frac{B_d}{D_m} \quad \text{(Eq. 23)}$$

$$h^* = (\text{desired } h) = \frac{2.3}{k_d - k_r} \log\left(\frac{k_d}{k_r}\right) \quad \text{(Eq. 24)}$$

$$h = \tau^1_{sust.} - \frac{2.3}{k_r} \log\left(\frac{1}{1-N}\right) - \Delta t \quad \text{(Eq. 25)}$$

and $\tau^1_{sust.}$ is selected as 12 or 24 to produce a calculated value of h as close as possible to h^*. Several values of N should be tried. Then,

$$D_1^{corr.} = D_1 - k_r D_m t_p \quad \text{(Eq. 26)}$$

Calculation of sustained-action dosage forms, based on this pragmatic approach, will not produce the optimum in blood level patterns. Unfortunately, however, to obtain the optimum blood level pattern with these nonideal availability kinetics, very large maintenance doses may be required (especially if k_d is large). Within the limits of practical oral dosages, the "practical approach" produces the only satisfactory dosage form. The difference between the blood level pattern for the optimum and the practical design is shown in Fig. 5 for a drug with a large k_d.

Satisfactory sustained- and nonsustained-release dosage forms can be designed with relatively simple equations. The equations provided in this report are based on simple models and are intuitively correct. They have been derived completely so that the assumptions involved are apparent.

REFERENCES

(1) (*a*) E. Kruger-Thiemer, *J. Theoret. Biol.*, **13**, 212(1966); (*b*) *Ibid.*, **23**, 169(1969).

(2) F. H. Dost, "Der Blutspiegel," George Thieme, Leipzig, East Germany, 1953.

(3) R. G. Wiegand, J. D. Buddenhagen, and C. J. Endicott, *J. Pharm. Sci.*, **52**, 268(1963).

(4) G. E. Boxer, V. C. Jelinek, R. Dubois, R. Tompsett, and A. O. Edison, *J. Pharmacol. Exp. Ther.*, **92**, 226(1948).

(5) J. M. Van Rossum, *J. Pharm. Sci.*, **57**, 2162(1968).
(6) J. D. Wagner and C. D. Almay, *Nature*, **201**, 1101(1964).
(7) J. R. Robinson and S. P. Eriksen, to be published.
(8) J. R. Robinson and S. P. Eriksen, *J. Pharm. Sci.*, **55**, 1254 (1966).
(9) M. Rowland and A. H. Beckett, *J. Pharm. Pharmacol. Suppl.*, **16**, 156T(1964).
(10) M. Soliva and P. Speiser, *Pharm. Acta Helv.*, **41**, 176 (1966).
(11) H. Juncher and F. Rasschou, *Antibiotic Med. Clin. Ther.*, **4**, 497(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 25, 1970, from the *School of Pharmacy, University of Wisconsin, Madison, Wis., and †Allergan Pharmaceuticals, Santa Ana, Calif.

Accepted for publication June 24, 1970.

Metabolism of Flurazepam, a Benzodiazepine, in Man and Dog

MORTON A. SCHWARTZ and EDWARD POSTMA

Abstract □ The metabolism of ^{14}C-flurazepam hydrochloride, 7-chloro-1-(2-diethylaminoethyl)-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one-5-^{14}C dihydrochloride, was studied in a dog administered oral and intravenous 2-mg./kg. doses and in two human subjects who each received a 28-mg. oral dose. In both species, evidence was obtained for rapid and essentially complete absorption followed by a rapid elimination of plasma flurazepam. Biotransformation of the drug was rapid and virtually complete in man and dog. Pathways of biotransformation were similar in both species. All the metabolites identified either showed some alteration in the N_1-diethylaminoethyl moiety or lacked the N_1-substituent altogether. The major metabolite in the dog was a carboxylic acid, the N_1-acetic acid analog of flurazepam. In man, the analogous alcohol (the N_1-ethanol analog) predominated.

Keyphrases □ ^{14}C-Flurazepam HCl metabolism—humans, dogs □ Metabolites, flurazepam HCl—isolation, identification □ Urinary, fecal excretion—^{14}C-flurazepam □ Plasma levels—^{14}C-flurazepam □ TLC—separation □ UV spectrophotometry—analysis □ Scintillometry—analysis

The synthesis (1) and the pharmacology (2) of flurazepam hydrochloride,[1] 7-chloro-1-(2-diethylaminoethyl)-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one dihydrochloride, have been reported as well as the clinical use of this compound (also designated Ro 5-6901) as a hypnotic (3, 4).

In a previous report (5), the isolation by TLC and the characterization by high-resolution mass spectrometry of five urinary metabolites from the dog and one human urinary metabolite were described. The structures of these metabolites are shown in Table I, together with those of flurazepam, the 3-hydroxy derivative of flurazepam (F-3-OH), and Compound III. While neither of the latter two compounds had been detected as metabolites, F-3-OH was of interest as a reference compound to test for 3-hydroxylation of intact drug, and Compound III was a likely metabolic precursor of metabolites III-OH and IV. All the compounds of Table I, except the incompletely characterized III-OH, have been synthesized (6).

The synthesis of ^{14}C-labeled flurazepam hydrochloride allowed for a study of the disposition of this drug in terms of absorption, distribution, biotransformation, and excretion.

EXPERIMENTAL

Labeled Compound and Counting of ^{14}C—Flurazepam-5-^{14}C hydrochloride[2] was diluted with unlabeled compound to a specific activity of 3.12 μc./mg. flurazepam base for the dog studies and 2.10 μc./mg. base for the human studies. Before administration to the human subjects and the dog, the labeled drug was shown to be radiochemically pure by TLC with System A (described later).

All samples were counted in a Nuclear-Chicago Corp. Mark I liquid scintillation spectrometer equipped with a ^{133}Ba external standard; the external standard-channels ratio technique was used to determine counting efficiency. Aliquots of urine and extracts of both urine and plasma were counted in Phosphor I (7), while plasma (0.2–1.0 ml.) and silica gel segments from chromatoplates were counted as suspensions in Phosphor II (7) which contained a thixotropic gel. The ^{14}C in aliquots of 50% ethanol homogenates of feces was determined by the Schoniger combustion, carbon dioxide trapping, and counting procedures of Kelly *et al.* (8).

Dog Studies—A nonfasted 13-kg. male dog (beagle) was first given an oral dose of 2 mg./kg. of ^{14}C-flurazepam HCl in a gelatin capsule. Two months later, it was given the same dose as a solution in 2.6 ml. of physiological saline by rapid intravenous injection. Urine and feces were collected until the excretion of radioactivity reached the limits of detection. Blood (10 ml., heparinized) was drawn at 0, 1, 2, 3, 4, 7, 12, and 24 hr. after oral dosing and at 0, 5, 15, and 30 min., and 1, 2, 3, 4, 7, 11, and 24 hr. after intravenous dosing of the dog.

For fractionation of the plasma radioactivity, aliquots (0.2–0.5 ml.) of plasma were brought to 1 ml. with water, mixed with 1 ml. of 0.5 *M* borate buffer, pH 9.0, and extracted twice with 5 ml. of ether. This procedure quantitatively removed flurazepam (9). The aqueous phases were adjusted to pH 7.0 and were extracted twice with 5 ml. of ether and then twice with 5 ml. of ethyl acetate. Finally, the aqueous phases were brought to pH 3.0 and again extracted twice with 5 ml. of ethyl acetate. These extracts were concentrated and counted.

The urinary radioactivity was fractionated in a similar manner. Aliquots (10 ml.) of urine were extracted twice with equal volumes of ethyl acetate at various pH's before incubation at pH 5.5 and 37° for 3 hr. with a commercial preparation[3] containing β-glucuronidase

[1] Flurazepam hydrochloride is the active ingredient in the trademarked product Dalmane of Hoffmann-La Roche Inc., Nutley, N. J.

[2] The labeled flurazepam hydrochloride synthesized by H. H. Kaegi and G. Bader, Isotope Synthesis Laboratory, Hoffmann-La Roche Inc., was labeled with ^{14}C at the C-5 position. The synthesis has not been published but is available from these chemists.

[3] Glusulase, Endo Laboratories, Garden City, N. Y.

(5) J. M. Van Rossum, *J. Pharm. Sci.*, **57**, 2162(1968).
(6) J. D. Wagner and C. D. Almay, *Nature*, **201**, 1101(1964).
(7) J. R. Robinson and S. P. Eriksen, to be published.
(8) J. R. Robinson and S. P. Eriksen, *J. Pharm. Sci.*, **55**, 1254 (1966).
(9) M. Rowland and A. H. Beckett, *J. Pharm. Pharmacol. Suppl.*, **16**, 156T(1964).
(10) M. Soliva and P. Speiser, *Pharm. Acta Helv.*, **41**, 176 (1966).
(11) H. Juncher and F. Rasschou, *Antibiotic Med. Clin. Ther.*, **4**, 497(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 25, 1970, from the *School of Pharmacy, University of Wisconsin, Madison, Wis., and †Allergan Pharmaceuticals, Santa Ana, Calif.

Accepted for publication June 24, 1970.

Metabolism of Flurazepam, a Benzodiazepine, in Man and Dog

MORTON A. SCHWARTZ and EDWARD POSTMA

Abstract □ The metabolism of ^{14}C-flurazepam hydrochloride, 7-chloro-1-(2-diethylaminoethyl)-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one-5-^{14}C dihydrochloride, was studied in a dog administered oral and intravenous 2-mg./kg. doses and in two human subjects who each received a 28-mg. oral dose. In both species, evidence was obtained for rapid and essentially complete absorption followed by a rapid elimination of plasma flurazepam. Biotransformation of the drug was rapid and virtually complete in man and dog. Pathways of biotransformation were similar in both species. All the metabolites identified either showed some alteration in the N_1-diethylaminoethyl moiety or lacked the N_1-substituent altogether. The major metabolite in the dog was a carboxylic acid, the N_1-acetic acid analog of flurazepam. In man, the analogous alcohol (the N_1-ethanol analog) predominated.

Keyphrases □ ^{14}C-Flurazepam HCl metabolism—humans, dogs □ Metabolites, flurazepam HCl—isolation, identification □ Urinary, fecal excretion—^{14}C-flurazepam □ Plasma levels—^{14}C-flurazepam □ TLC—separation □ UV spectrophotometry—analysis □ Scintillometry—analysis

The synthesis (1) and the pharmacology (2) of flurazepam hydrochloride,[1] 7-chloro-1-(2-diethylaminoethyl)-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one dihydrochloride, have been reported as well as the clinical use of this compound (also designated Ro 5-6901) as a hypnotic (3, 4).

In a previous report (5), the isolation by TLC and the characterization by high-resolution mass spectrometry of five urinary metabolites from the dog and one human urinary metabolite were described. The structures of these metabolites are shown in Table I, together with those of flurazepam, the 3-hydroxy derivative of flurazepam (F-3-OH), and Compound III. While neither of the latter two compounds had been detected as metabolites, F-3-OH was of interest as a reference compound to test for 3-hydroxylation of intact drug, and Compound III was a likely metabolic precursor of metabolites III-OH and IV. All the compounds of Table I, except the incompletely characterized III-OH, have been synthesized (6).

The synthesis of ^{14}C-labeled flurazepam hydrochloride allowed for a study of the disposition of this drug in terms of absorption, distribution, biotransformation, and excretion.

EXPERIMENTAL

Labeled Compound and Counting of ^{14}C—Flurazepam-5-^{14}C hydrochloride[2] was diluted with unlabeled compound to a specific activity of 3.12 μc./mg. flurazepam base for the dog studies and 2.10 μc./mg. base for the human studies. Before administration to the human subjects and the dog, the labeled drug was shown to be radiochemically pure by TLC with System A (described later).

All samples were counted in a Nuclear-Chicago Corp. Mark I liquid scintillation spectrometer equipped with a ^{133}Ba external standard; the external standard-channels ratio technique was used to determine counting efficiency. Aliquots of urine and extracts of both urine and plasma were counted in Phosphor I (7), while plasma (0.2–1.0 ml.) and silica gel segments from chromatoplates were counted as suspensions in Phosphor II (7) which contained a thixotropic gel. The ^{14}C in aliquots of 50% ethanol homogenates of feces was determined by the Schoniger combustion, carbon dioxide trapping, and counting procedures of Kelly *et al.* (8).

Dog Studies—A nonfasted 13-kg. male dog (beagle) was first given an oral dose of 2 mg./kg. of ^{14}C-flurazepam HCl in a gelatin capsule. Two months later, it was given the same dose as a solution in 2.6 ml. of physiological saline by rapid intravenous injection. Urine and feces were collected until the excretion of radioactivity reached the limits of detection. Blood (10 ml., heparinized) was drawn at 0, 1, 2, 3, 4, 7, 12, and 24 hr. after oral dosing and at 0, 5, 15, and 30 min., and 1, 2, 3, 4, 7, 11, and 24 hr. after intravenous dosing of the dog.

For fractionation of the plasma radioactivity, aliquots (0.2–0.5 ml.) of plasma were brought to 1 ml. with water, mixed with 1 ml. of 0.5 *M* borate buffer, pH 9.0, and extracted twice with 5 ml. of ether. This procedure quantitatively removed flurazepam (9). The aqueous phases were adjusted to pH 7.0 and were extracted twice with 5 ml. of ether and then twice with 5 ml. of ethyl acetate. Finally, the aqueous phases were brought to pH 3.0 and again extracted twice with 5 ml. of ethyl acetate. These extracts were concentrated and counted.

The urinary radioactivity was fractionated in a similar manner. Aliquots (10 ml.) of urine were extracted twice with equal volumes of ethyl acetate at various pH's before incubation at pH 5.5 and 37° for 3 hr. with a commercial preparation[3] containing β-glucuronidase

[1] Flurazepam hydrochloride is the active ingredient in the trademarked product Dalmane of Hoffmann-La Roche Inc., Nutley, N. J.

[2] The labeled flurazepam hydrochloride synthesized by H. H. Kaegi and G. Bader, Isotope Synthesis Laboratory, Hoffmann-La Roche Inc., was labeled with ^{14}C at the C-5 position. The synthesis has not been published but is available from these chemists.

[3] Glusulase, Endo Laboratories, Garden City, N. Y.

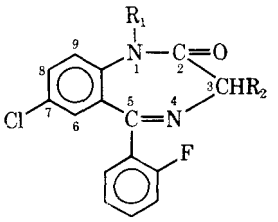


Table I—Flurazepam and Metabolites

Compound Designation	R_1	R_2	Metabolic Status from Previous Work (5)
Flurazepam	$-CH_2CH_2N(C_2H_5)_2$	H	Intact drug
F-3-OH	$-CH_2CH_2N(C_2H_5)_2$ H	OH	Not found in urine
I	$-CH_2CH_2NC_2H_5$	H	Urinary metabolite in dog
I-DE[a]	$-CH_2CH_2NH_2$	H	Urinary metabolite in dog
II	CH_2CH_2OH	H	Urinary metabolite in man and dog
III	H	H	Not found in urine
III-OH[b]	H	H	Urinary metabolite in dog
IV	H	OH	Urinary metabolite in dog

[a] DE signifies that this compound is the desethyl derivative of I. [b] Compound III-OH was not completely characterized; mass spectral analysis indicated the addition of a phenolic group to Compound III.

and sulfatase; they were reextracted at pH 7.0 after incubation. This incubation previously yielded maximum hydrolysis of benzodiazepine conjugates (7). Further details of these extractions are included with the results in Table II. Fecal homogenates in 50% ethanol were first evaporated to remove the ethanol before two extractions at pH 9.0 with an equal volume of ether.

Human Study—Two women, H-17 and H-18, were each given 28.0 mg. of labeled flurazepam hydrochloride (specific activity of 3.90×10^6 d.p.m./mg.) in a gelatin capsule. H-17, a 57-year-old hypertensive weighing 91 kg., was treated with 50 mg. of hydrochlorothiazide per day before and during the study. H-18, 61 years old and weighing 73 kg., suffered from labile hypertension but was normotensive during the study and required no therapy.

Both subjects were given a glass of water every 2 hr. during the 1st day to promote diuresis; intervals of urine collection were 0–2, 2–4, 4–6, 6–9, 9–12, and 12–24 hr. and daily thereafter until the end of the study. Feces were collected as daily pools. Oxalated blood (10 ml.) was drawn at 0, 1, 2, 4, 7, 12, and 24 hr. and at 24-hr. intervals thereafter. Aliquots of plasma were extracted at pH 9.0 with ether, as described for dog plasma, and the extracts were counted. Aliquots of urine were serially extracted at pH 9.0 with ether, at pH 7.0 with ethyl acetate before and after treatment with the β-glucuronidase–sulfatase preparation, and finally at pH 3.0 with ethyl acetate; equal volumes of solvent were used twice for each extraction. The extracts were evaporated to dryness, and the residues were dissolved in 5 ml. of ethanol; aliquots were counted and, if sufficient ^{14}C was present, were examined by TLC.

Separation and Estimation of Drug and Metabolites—The technique used previously (7) involves the determination of the amount of chromatographed radioactivity that migrates on two-dimensional TLC as an authentic reference compound. Internal reference compounds chromatographed with extracts of plasma, urine, and feces included flurazepam, F-3-OH HCl, I 2HCl, I-DE 2HCl, II HCl, III, and IV. Also used as a reference compound was the dehydrated derivative of I-DE which was shown (5) to be an artifact arising on TLC of I-DE; any ^{14}C separated as this compound was considered to have been present in the biological media as I-DE. The recovery of plasma ^{14}C-flurazepam on extraction and TLC was estimated at roughly 90%.

Silica gel containing a fluorescent indicator[4] was used for TLC, together with the following solvent systems: System A, ethyl acetate–ethanol–concentrated ammonia (95:5:0.5); System B, isopropanol–ethanol–concentrated ammonia (90:10:1); System C, benzene–ethyl acetate–ethanol–concentrated ammonia (80:20:10:0.2); System D, chloroform–acetone–concentrated ammonia (80:20:1); System D′, chloroform–acetone–concentrated ammonia (95:5:0.1); System E, benzene–*n*-butanol–methanol–water (1:1:2:1); System F, benzene–methanol–acetic acid (90:10:1); System G, heptane–chloroform–ethanol (10:10:2.5); System G′, heptane–chloroform–ethanol–concentrated ammonia (50:50:25:1); System H, benzene–*n*-butanol (90:10); System I, methylene chloride–*n*-butanol (90:10); System J, heptane–ethyl acetate–ethanol–concentrated ammonia (50:50:5:0.2); and System K, heptane–benzene–ethanol (5:5:2).

In many instances, these solvent systems were used as pairs for two-dimensional TLC. For example, System AC signifies that the chromatoplate was developed first in A and then, after being turned 90°, in C. Compounds were located on the plates under shortwave UV light.

Metabolites of II HCl in the Dog—The biotransformation in the dog of unlabeled metabolite II was studied to elucidate further the pathways of flurazepam metabolism. Three milliliters of plasma separated from blood drawn 1, 2, and 3 hr. following oral administration of 22 mg./kg. of II HCl in a gelatin capsule was combined and extracted at pH 9.0 with ethyl acetate. The extracted metabolites were separated by TLC. Urine excreted the day before and the day after the intravenous administration in propylene glycol–water (1:1) of 6 mg./kg. of II HCl was separately extracted by the Series 2 procedure of Table II. The extracts were dried over anhydrous sodium sulfate, concentrated, and examined by TLC. Identification was accomplished by the demonstration that the metabolite and an authentic reference compound migrated as a single compound.

RESULTS

Metabolism in the Dog—*Excretion of Labeled Drug and Metabolites*—The radioactivity excreted after the oral and intravenous administration of ^{14}C-flurazepam hydrochloride is shown in Table III. After each route of administration, roughly the same amount of ^{14}C was excreted in the feces as in the urine during the 1st day. Fecal excretion of ^{14}C was significant for the first 3 days, and the total amount excreted exceeded that found in the urine. These findings suggest that a considerable secretion of labeled material into the gastrointestinal tract occurred. The feces excreted in the first 2 days after intravenous drug administration were analyzed by ether extraction at pH 9.0 and TLC of the extracts with System DB. No flurazepam was detected in this extract. While I-DE and IV were found, the fecal excretion of each was less than 1% of the dose. The remaining fecal radioactivity remained unidentified.

The composition of the urinary radioactivity was first studied by solvent extraction. The results of three separate series of extractions of urine from the orally treated dog (Table II) demonstrated the following: (*a*) no more than 5% of the urinary ^{14}C, that extracted at pH 9.0, could be intact drug; (*b*) roughly 60% of the urinary metabolites was acidic (the Series 3 extraction ruled out the possibility that the ^{14}C removed at pH 1.0 and 3.0 in the first two extraction

[4] Mallinckrodt Silicar 7-GF-5.

Table II—Extractability of the Radioactivity Excreted by a Dog in the 0–36-hr. Urine after Oral and Intravenous Doses of ^{14}C-Flurazepam HCl[a]

Serial Extraction with Ethyl Acetate[b]	—Percent of Urinary ^{14}C Extracted— After Oral Dose— Series 1	Series 2	Series 3	After Intravenous Dose Series 2
pH 1.0	58.1	—[c]	—	—
pH 9.0	—	4.8	—	5.2
pH 7.0	3.5	2.6	6.1	2.0
pH 3.0	—	60.6	[d]	45.4
pH 7.0	—	0.3	4.4	0.4
After β-glucuronidase-sulfatase treatment				
pH 7.0	11.0	10.2	—	11.6
Remaining aqueous	25.7	20.0		30.2
	98.3	98.5		94.8

[a] After the oral dose, 33.0% of the ^{14}C was excreted in the urine in 36 hr.; after the intravenous dose, 24.3% was so excreted. [b] An aliquot (10 ml.) of the 0–36-hr. pooled urine was subjected to consecutive ethyl acetate extraction at the pH values shown. [c] A dash indicates that the urine was not adjusted to, nor extracted at, the designated pH. [d] The sample was adjusted to pH 3.0, allowed to stand for 15 min., and then adjusted to the next pH (pH 7.0) before being extracted.

series represented nonacidic artifacts formed on exposure to these acidic conditions); (*c*) only 10–11% of the metabolites were excreted as conjugates susceptible to hydrolysis by the β-glucuronidase–sulfatase preparation; and (*d*) 20–26% of the metabolites were of such a polar nature that they were not extractable with ethyl

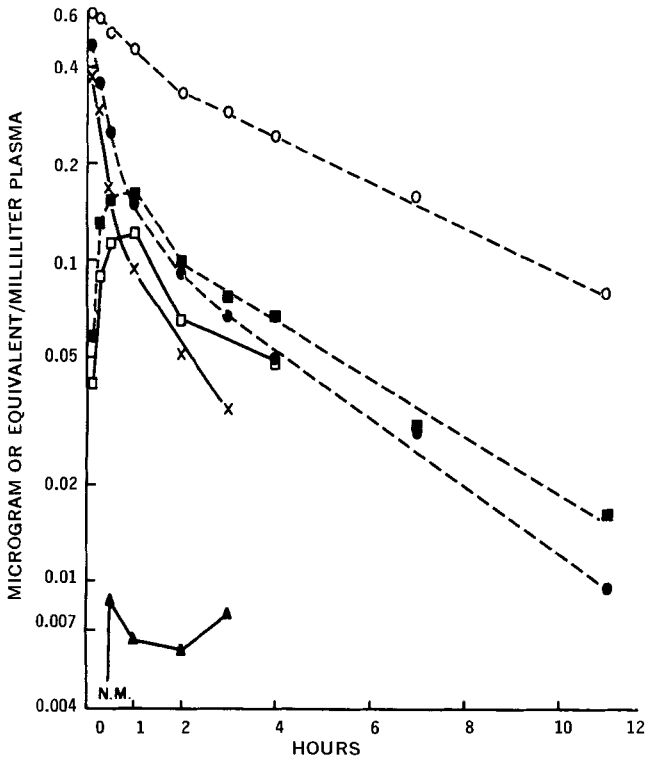


Figure 1—*Plasma levels of flurazepam-derived ^{14}C and labeled metabolites in a dog given an intravenous dose of 2 mg./kg. of ^{14}C-flurazepam hydrochloride. The concentrations of plasma total ^{14}C (○- - -○), the ^{14}C extracted at pH 9.0 into ether (●- - -●), and the ^{14}C extracted at pH 3.0 into ethyl acetate (■- -■) are expressed as microgram equivalents flurazepam per milliliter of plasma; the concentrations of flurazepam (× — ×), III (▲ — ▲), and V (□ — □) are expressed as micrograms per milliliter of plasma. The basic metabolites were quantitated by TLC of the pH 9.0 ether extract in System DB; the acidic metabolite V was quantitated by TLC of the pH 3.0 ethyl acetate extract in System EF. N.M. = not measurable.*

Table III—Excretion of Radioactivity by a Dog after Oral and Intravenous 2-mg./kg. Doses of ^{14}C-Flurazepam Hydrochloride

Time Interval after Dose, Days	—Excretion of ^{14}C, % of Dose— Urine	Feces	Total
	Oral 2-mg./kg. Dose		
1	30.4	29.4	59.8
2	4.1	14.1	18.2
3	1.1	4.0	5.1
4	0.3	0.6	0.9
5	0.2	0.2	0.4
6	0.1	0.2	0.3
7	0.04	—[a]	0.04
8	0.08	—	0.08
	36.3	48.5	84.8
	Intravenous 2-mg./kg. Dose		
1	22.8	19.9	42.7
2	2.3	28.4	30.7
3	1.1	3.9	5.0
4	0.5	0.8	1.3
5	0.3	0.4	0.7
6	0.2	0.3	0.5
7	0.07	0.2	0.27
8	0.1	—	0.1
9	0.05	—	0.05
	27.4	53.9	81.3

[a] The dash indicates that the feces were not collected.

acetate under any condition listed in Table II. It is also evident from Table II that the urine from the intravenously treated dog contained metabolites similar in extractability to those excreted after oral drug administration.

To identify the acidic metabolites, 180 ml. of the 0–36-hr. urine from the orally treated dog was extracted according to Series 2, Table II. At pH 3.0, 57.4% of the urinary ^{14}C was extracted. TLC of the concentrated extract with System H yielded a UV-absorbing band at R_f 0.3–0.4, which was associated with roughly 80% of the chromatographed radioactivity. This labeled component was eluted from the silica gel, and an aliquot of the concentrated ethanol eluate was rechromatographed with System I alongside authentic 2-[7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2-oxo-2*H*-1,4-benzodiazepin-1-yl]acetic acid (Compound V). Compound V was chosen as the reference compound because the analogous alcohol, II, was a known urinary metabolite of flurazepam hydrochloride in the dog (5). The labeled metabolite migrated as a single band (R_f 0.75), which contained 94% of the ^{14}C chromatographed with System I and did not separate from authentic V. Although TLC with System I of greater amounts of labeled material yielded a diffuse band, this diffuse component, after elution from the silica gel, and authentic V were shown by high-resolution mass spectral analysis[5] to be identical. It was estimated that roughly 45% of the urinary ^{14}C was present as V.

Although this new metabolite was quantitated successfully in plasma extracts by two-dimensional TLC, this technique could not be used with extracts of urine because of excessive tailing. It was, therefore, necessary to convert metabolite V into its methyl ester prior to TLC. Freshly prepared diazomethane (10) was added in excess to the urinary extract and, after standing 15 min. at room temperature, the solution was evaporated to dryness and the residue dissolved in ethanol. Authentic V was found to be completely esterified under these conditions.

The urinary excretion of drug and metabolites, quantitated by solvent extraction and TLC, is summarized in Table IV. After oral administration, no flurazepam was detected in the urine; the major metabolite was V, while lesser amounts of conjugated IV and free I–DE were excreted. The same three metabolites were excreted after intravenous drug administration in amounts similar to those found after oral dosing. In addition, the excretion of small amounts of intact drug (0.2% of the dose), Compound I, and conjugated II was

[5] Performed by F. M. Vane, Physical Chemistry Department, Hoffmann-La Roche Inc., Nutley, NJ 07110

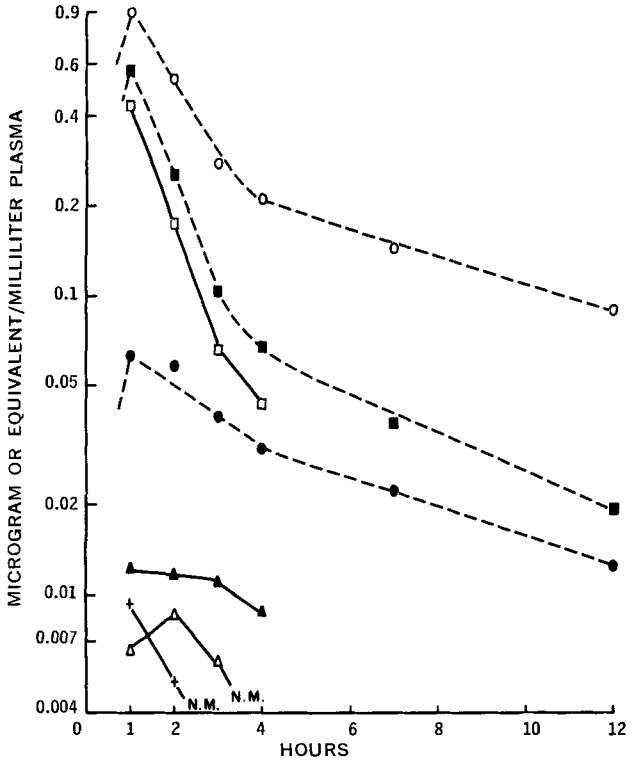


Figure 2—*Plasma levels of flurazepam-derived ^{14}C and labeled metabolites in a dog given an oral dose of 2 mg./kg. of ^{14}C-flurazepam hydrochloride. The concentrations of plasma total ^{14}C (O- - -O), the ^{14}C extracted at pH 9.0 into ether (●- - -●), and the ^{14}C extracted at pH 3.0 into ethyl acetate (■- -■) are expressed as microgram equivalents flurazepam per milliliter of plasma; the concentrations of III (▲ − ▲), I-DE (△ − △), IV (+ − +), and V (□ − □) are expressed as micrograms per milliliter of plasma. The metabolites were quantitated by TLC as described in the legend of Fig. 1. N.M. = not measurable.*

seen only after intravenous drug administration. Although not shown in this table, no excretion of F-3-OH, either as a free or conjugated metabolite, was found.

Plasma Levels of Drug and Metabolites—The plasma levels of undifferentiated ^{14}C and of specific labeled compounds following the intravenous administration of ^{14}C-flurazepam hydrochloride are shown in Fig. 1. Intact drug was the major labeled compound extracted at pH 9.0, and its rapid biexponential elimination was characterized by half-lives of 11 min. and 1.4 hr. In addition, its apparent volume of distribution in terms of the central compartment of a two-compartment open system was calculated (11) to be 44.2 l. or 340% of body weight, a value indicating extensive tissue uptake of drug. Compound III was found in the same extract as flurazepam, but its plasma levels remained below 0.01 mcg./ml. At 5 min., the

Table IV—Urinary Excretion of Drug and Metabolites by a Dog after Oral and Intravenous ^{14}C-Flurazepam Hydrochloride

Urinary Components	Amount Excreted: After Oral Drug[a] (% of Urinary ^{14}C)	After Oral Drug[a] (% of Dose)	After Intravenous Drug[b] (% of Urinary ^{14}C)	After Intravenous Drug[b] (% of Dose)
Basic				
Flurazepam	<0.17	Nil	0.57	0.2
I	<0.2	Nil	0.15	<0.1
I-DE	1.2	0.4	0.82	0.2
Unknown	3.6	1.2	2.68	0.7
Acidic				
V	45	15	41.5	11.4
Unknown	15	5.0	9.3	2.5
Conjugates				
IV	3.8	1.3	6.9	1.9
II	<0.3	Nil	0.32	0.1
Unknown	6.4	2.1	8.5	2.3
Polar nonconjugates				
Unknown	20	6.6	31.0	8.5

[a] The urinary excretion of metabolites in 36 hr. is shown; over 90% of the total urinary ^{14}C excreted was excreted in this time interval. The appropriate extracts of Series 2, Table II, were used for the quantitation of the basic and conjugated metabolites. Basic metabolites were separated by TLC with System AC, while both Systems AC and BD were used to separate the deconjugated metabolites. The amount of acidic metabolite was estimated during its isolation. The "polar nonconjugates" represent the nonextractable ^{14}C of Series 2, Table II. [b] The total urinary excretion of drug and metabolites is shown. Each urine collected during the first 3 days was analyzed by solvent extraction (Series 2, Table II) and TLC. Basic and deconjugated metabolites were separated by TLC with System DB, while the acidic metabolite was esterified with diazomethane and separated in two one-dimensional systems, G and D′.

acidic metabolite (V) was present at a plasma level of 0.041 mcg./ml.; at 1 hr., it reached a peak level of 0.12 mcg./ml. Since no appreciable amounts of intact drug were found in the urine and feces, it appears that the rapid elimination of plasma flurazepam resulted from tissue uptake and rapid biotransformation.

These two processes were also apparently responsible for the absence of detectable plasma levels of drug after oral administration. As shown in Fig. 2, the highest level of plasma ^{14}C was observed at 1 hr., suggesting that rapid absorption had occurred. But even at this early time the major portion of the plasma radioactivity resided in acidic metabolites. Metabolite V accounted for over 70% of the acidic metabolite fraction of 1, 2, 3, and 4-hr. plasma, and its levels fell rapidly from 0.43 mcg./ml. with a half-life of 0.76 hr. Also found in the plasma were relatively low concentrations of I-DE, III, and IV.

Metabolism of II HCl—Intact II and Compound III were both identified in the ethyl acetate extract of dog plasma by TLC with Systems D and J. In the urine, both free and conjugated II were found (System DB), while the presence of conjugated IV was demonstrated with Systems AD and G′B. The acidic Compound V was also shown to be a urinary metabolite by formation of its methyl ester and subsequent TLC with System GK.

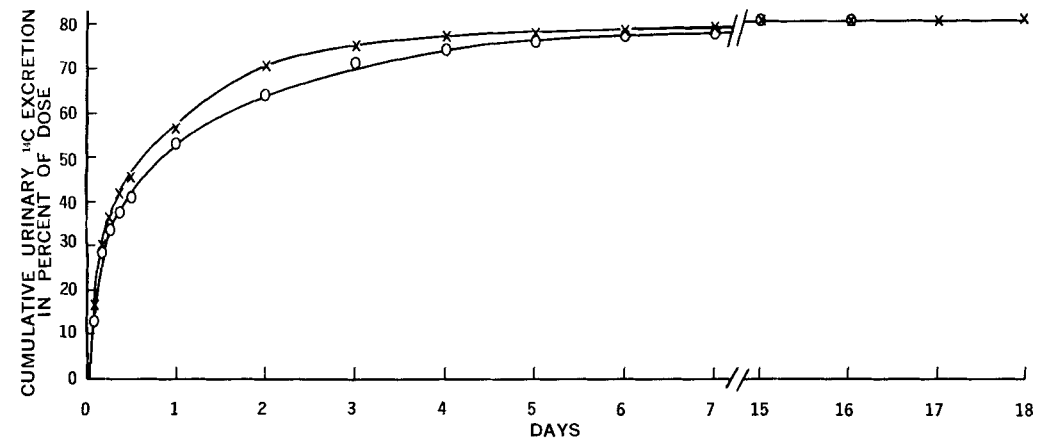


Figure 3—*Cumulative urinary excretion of radioactivity by Subject H-17 (O − O) and Subject H-18 (× − ×) following an oral dose of 28 mg. of ^{14}C-flurazepam hydrochloride.*

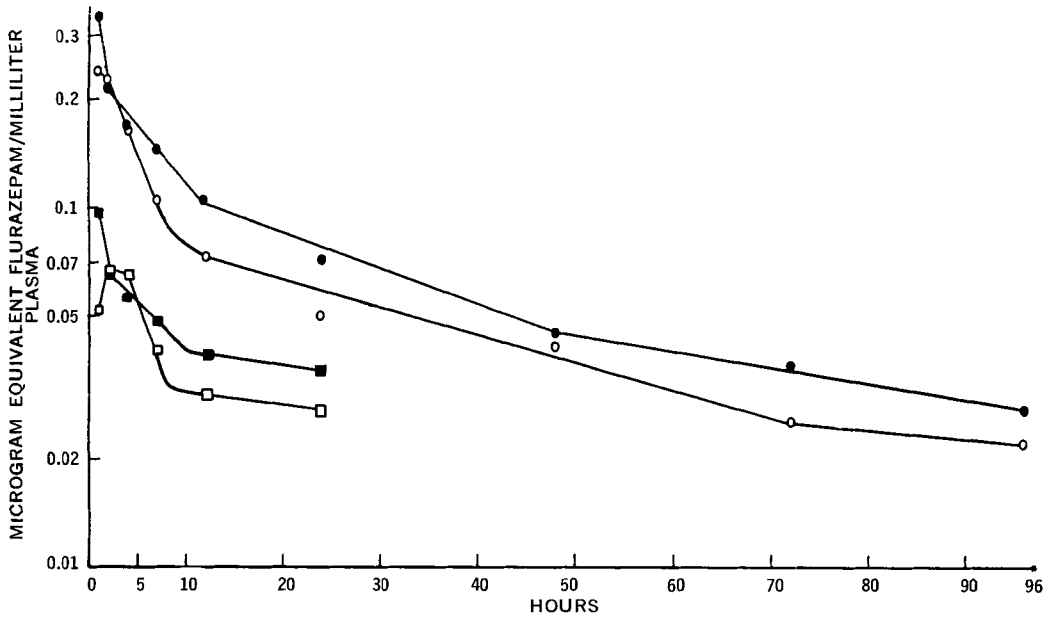


Figure 4—*Falloff of ^{14}C levels expressed as microgram equivalent flurazepam per milliliter in plasma (○) and ether extracts of plasma (□) of Subject H-17 and in plasma (●) and ether extracts of plasma (■) of Subject H-18.*

Administered II was apparently biotransformed *via* two pathways. One involved the removal of the *N*-ethanol moiety to give plasma III; this was subsequently hydroxylated at C-3 to form IV which, as a conjugate, was excreted in the urine. The second pathway involved oxidation of the alcohol moiety of II to form the carboxylic acid, V, which was found in the urine.

Metabolism in Man—The cumulative excretion of radioactivity in urine and feces was almost the same for both subjects. More than half of the administered ^{14}C was excreted by each subject in the urine during the 1st day; each excreted a total of 81% of the dose in the urine, with 9% of the dose excreted in the feces by H-17 and 8% of the dose so excreted by H-18. The similarity of the excretion of urinary radioactivity for both subjects is clear from Fig. 3, which shows the cumulative excretion of urinary ^{14}C as a function of time.

In the plasma, the highest levels of total radioactivity, 0.24 mcg. equivalents of flurazepam per ml. for H-17 and 0.34 mcg. equivalents/ml. for H-18, were seen at 1 hr. (Fig. 4). These early peak levels indicated that absorption of labeled drug had been rapid. While the levels of ether-extracted radioactivity were markedly lower than those of total ^{14}C, the falloff of this extracted radioactivity closely paralleled that of total plasma ^{14}C in each subject.

Only in the plasma samples containing the highest levels of ^{14}C was it feasible to search for drug and metabolites. As seen in Table V, intact flurazepam was detected only in the 1-hr. plasma of H-17. Furthermore, intact drug in these early samples accounted for roughly 1% or less of the total plasma ^{14}C shown in Fig. 4. In view of the evidence for rapid absorption of labeled drug, this finding indicates that the absorbed drug was rapidly biotransformed into metabolites. With respect to the plasma metabolites identified (Table V), II was the major metabolite in both subjects at 1 hr. while roughly equivalent levels of II, III, and I were found at 2 hr. In addition, trace levels of I-DE and IV were found in the plasma, while F-3-OH was not detected and 33–47% of the extracted ^{14}C was not identified.

The nature of the urinary radioactivity was investigated with pooled urine (0–4 days) representing over 90% of the total urinary ^{14}C eliminated by each subject. Only 15% (H-17) and 11% (H-18) of the urinary ^{14}C was extractable as intact drug and/or basic metabolites (Table VI). The major portion, 49% in each subject, was extracted at pH 7.0 after β-glucuronidase–sulfatase treatment; *i.e.*, it was apparently excreted as glucuronide and/or sulfate conjugates. Less than 7% of the excreted radioactivity represented acidic metabolites extractable at pH 3.0, and roughly 20% was comprised of polar nonextractable compounds.

The composition of the urinary radioactivity extractable directly at pH 9.0 and, following β-glucuronidase–sulfatase treatment, at pH 7.0 is shown in Table VII. While no intact drug was found, both subjects excreted I and I-DE as unconjugated metabolites. The major urinary metabolite was conjugated II, which accounted for 25% of the dose in H-17 and 22% of the dose in H-18. A small amount of conjugated IV was also excreted by both subjects.

The rate of excretion of metabolites was studied in H-18. The unconjugated and conjugated metabolites of each urine specimen collected during the first 4 days were extracted as described previously, and the metabolites of each extract were separated and quantitated by TLC with System DB. The total urinary excretion of each metabolite in milligrams was readily estimated (I, 0.21; I-DE, 0.76; II, 4.69; and IV, 0.30), and the amount of each compound remaining to be excreted was plotted as a logarithmic function of time (Fig. 5) to

Table V—Composition of the Plasma Radioactivity Extractable at pH 9.0 into Ether following Oral ^{14}C-Flurazepam Hydrochloride Administration to Two Subjects

Compound	Plasma Concentration, mcg./ml.[a] H-17 1 hr.	H-17 2 hr.	H-18 1 hr.	H-18 2 hr.
Flurazepam	0.004	N.S.	N.S.	N.S.
II	0.014	0.013	0.020	0.009
III	0.005	0.008	0.010	0.010
I	0.003	0.008	0.012	0.010
I-DE	N.S.	0.004	0.004	0.004
IV	0.003	N.S.	N.S.	0.004
F-3-OH	N.S.	N.S.	N.S.	N.S.
Unaccounted[b]	0.014	0.027	0.041	0.028

[a] The distribution of the ether-extracted ^{14}C was determined by two-dimensional TLC (System DB), using the compounds listed in the table as internal standards. Limit of sensitivity for each compound was roughly 0.003 mcg./ml. plasma; N.S. indicates that levels of ^{14}C were below 10 c.p.m. above background. [b] The ^{14}C unaccounted for is expressed as microgram equivalent flurazepam/milliliter.

Table VI—Extraction of the Radioactivity in 0–4-Day Pooled Urine[a] from Subjects H-17 and H-18

Serial Extraction	Radioactivity Extracted—Subject H-17 (% of Urinary ^{14}C)	Subject H-17 (% of Dose)	Subject H-18 (% of Urinary ^{14}C)	Subject H-18 (% of Dose)
pH 9.0, ether	15.0	11.1	10.9	8.4
pH 7.0, ethyl acetate	2.6	1.9	2.5	1.9
After β-glucuronidase–sulfatase treatment				
pH 7.0, ethyl acetate	49.0	36.3	49.2	37.9
pH 3.0, ethyl acetate	4.1	3.0	6.1	4.7
Remaining aqueous	21.1	15.6	17.7	13.6
Recovery	91.8		86.4	

[a] The 0–4-day urine pool represented the excretion of 74% of the administered ^{14}C dose by H-17 and 77% of the dose by H-18.

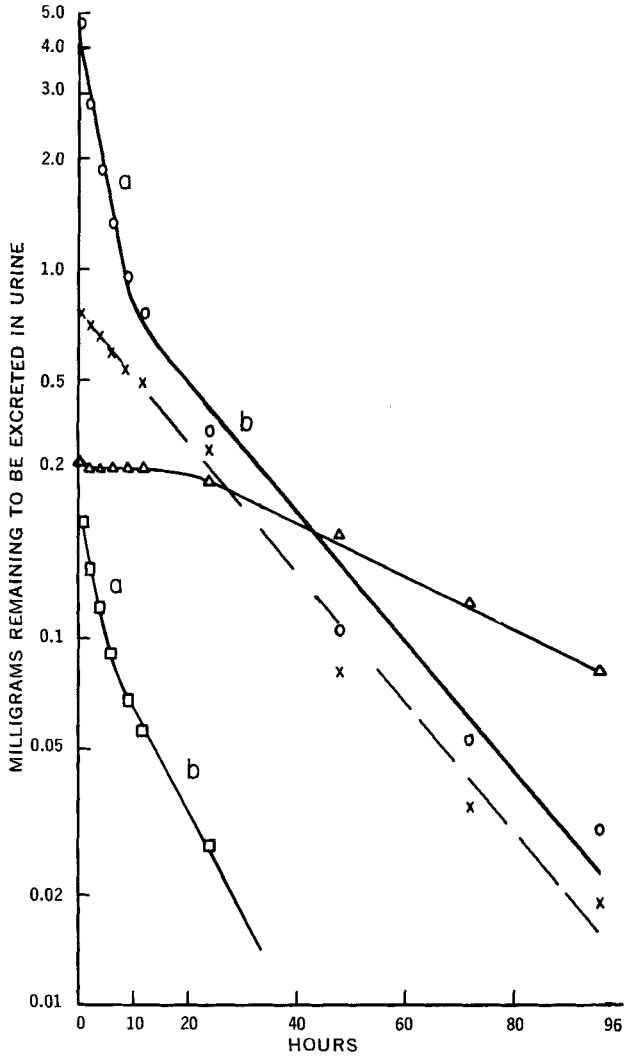


Figure 5—*Urinary excretion of metabolites by Subject H-18. The milligrams of I* (□ — □), *I-DE* (×- -×), *II* (○ — ○), *and IV* (△ — △) *remaining to be excreted are plotted logarithmically as a function of time. (Metabolites II and IV were excreted as conjugates.) The half-life in hours for each rate [with rate (a) obtained by the "residuals" method] was:*

I		*I-DE*	*II*		*IV*
(*a*)	(*b*)		(*a*)	(*b*)	
2.0	*11*	*17*	*2.5*	*17*	*42*

give the pseudo-first-order elimination rates expressed as half-lives in the legend. The major urinary metabolite, conjugated II, was eliminated biexponentially at rates characterized by half-lives of 2.5 and 17 hr. Metabolite I was also eliminated biexponentially at similar rates. Both I-DE and conjugated IV were eliminated monoexponentially; the latter metabolite exhibited a long lag period before appreciable excretion occurred and had the longest half-life.

DISCUSSION

The evidence presented indicated that in both man and dog the rate of labeled drug absorption was relatively fast. Furthermore, the dog excreted more of the labeled dose in the urine after oral ^{14}C-flurazepam administration than after intravenous administration, and the human subjects excreted over 80% of the oral dose in the urine. These findings suggest that absorption was fairly complete in both species.

Since orally administered flurazepam hydrochloride was not excreted intact by dog or man, it is clear that the drug was eliminated solely by biotransformation. Evidence for the elimination being a rapid process was provided by the fact that, in the intravenously injected dog, drug disappeared rapidly from the plasma exhibiting a biexponential decline with half-lives of 11 min. and 1.4 hr. Furthermore, in both species, metabolites predominated even in the earliest plasma samples. The rapid biotransformation, together with tissue uptake of intact drug, appeared to be responsible for keeping plasma flurazepam below measurable levels after oral administration to man or dog.

The postulated pathways of flurazepam biotransformation are shown in Scheme I. Flurazepam was metabolized by successive *N*-dealkylation to yield I and I-DE, both of which were excreted in the urine by man and dog. The dog produced V as a major metaboli e of plasma and urine and II as a minor urinary metabolite. It is likely that both arose from the same precursor, an aldehyde formed by oxidative deamination of I-DE. In the dog, oxidation of the aldehyde was preferred and V was the dominant metabolite; in man the reduction to II, which was excreted as the major urinary metabolite in the form of a conjugate, was the preferred pathway.

The study of the metabolism of II in the dog revealed that III was formed by removal of the N–CH_2CH_2OH group, a pathway shown in Scheme I. Since III was also a metabolite of flurazepam in man and dog and has not been shown to be formed exclusively *via* II as the intermediate, possible pathways (broken arrows) leading from flurazepam, I, and I-DE are also shown.

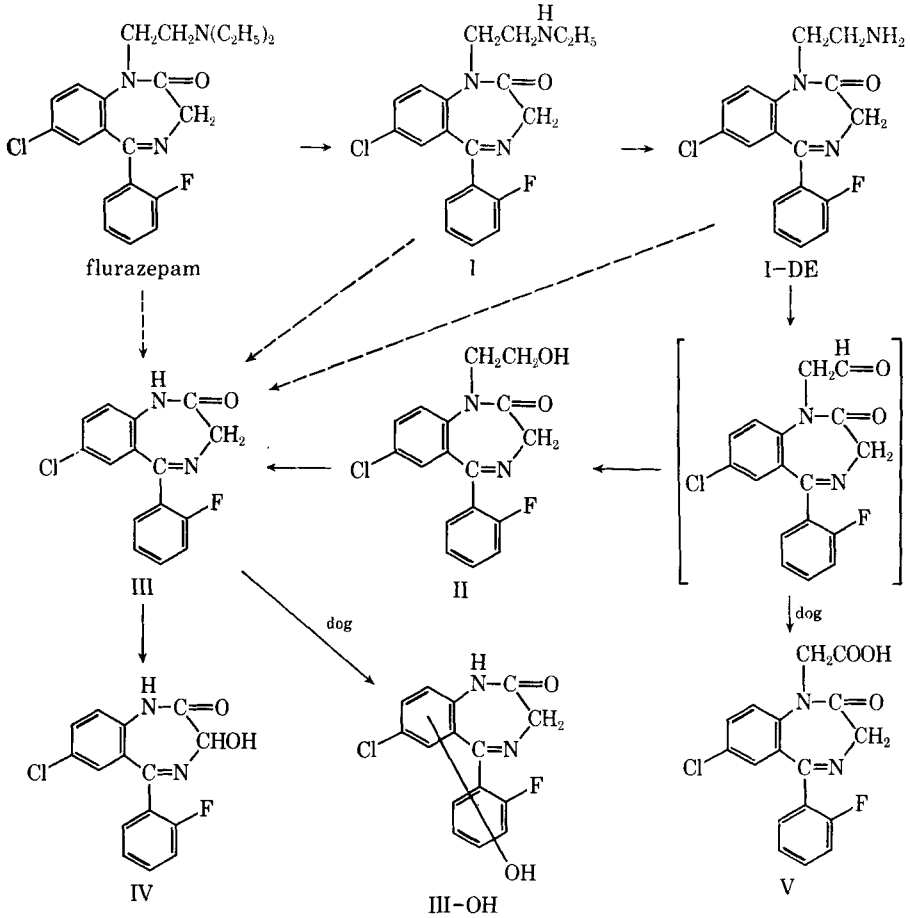
The 3-hydroxy derivative of flurazepam, F-3-OH, was not found as a metabolite in either man or dog, suggesting that metabolic attack at the N-1 side chain was preferred over 3-hydroxylation of intact drug. Therefore, the conjugated IV found in the urine of both species probably was formed *via* a pathway in which *N*-dealkylation preceded 3-hydroxylation, thus indicating that III was the immediate precursor of IV. Scheme I also shows III-OH, the incompletely characterized phenolic metabolite previously found (5) as a urinary conjugate in a dog chronically treated with flurazepam, arising from III. Although this metabolite may have been excreted in the present experiments in very small amounts, it definitely was not a significant metabolite in man or dog following single doses of flurazepam.

The three ether-extractable metabolites present in human plasma in greatest amounts were I, II, and III. I and conjugated II were excreted fairly rapidly in the urine, while III was not excreted *per se* but was biotransformed to IV. Conjugated IV did not appear in the urine in any appreciable amounts until the 2nd day, and it was excreted relatively slowly. This suggests that the conversion of III to IV may be slow and that III, on repeated administration of flurazepam, may tend

Table VII—Estimation by Two-Dimensional TLC of the Conjugated and Unconjugated Urinary Metabolites of Subjects H-17 and H-18

Source of 0–4-Day Urine	Metabolite Form	Urinary Excretion of Drug and Metabolites in Percent of Administered Dose[a]						Unaccounted for
		Flurazepam	I	I-DE	II	III	IV	
Subject H-17	Unconjugated	N.D.[b]	0.4	1.3	N.D.	0.5	N.D.	9.0
	Conjugated	N.D.	N.D.	0.8	25.0	N.D.	0.9	9.6
Subject H-18	Unconjugated	N.D.	1.2	4.1	N.D.	N.D.	N.D.	3.1
	Conjugated	N.D.	N.D.	N.D.	22.0	N.D.	1.5	14.4

[a] The values presented were based on the average distribution of ^{14}C on two-dimensional TLC in at least two systems. The "pH 9.0, ether" extract of H-18 was chromatographed in Systems DB and AD; the other extracts were each chromatographed in Systems DB, AD, and DC. [b] N.D. indicates that the compound was not detectable. The detection limits were 0.2% of the dose for unconjugated metabolites and 0.8% of the dose for conjugated metabolites. Compound F-3-OH was also used as an internal reference compound but was not detected as a metabolite in any of the extracts.



Scheme I—Postulated pathways of flurazepam metabolism in man and dog. The compound shown in parenthesis was not detected but is a presumed intermediate which would result from oxidative deamination of I-DE. The pathways labeled "dog" denote that the resulting metabolites were definitely established solely in the dog

toward higher plasma concentrations. Further studies are required to test this supposition.

REFERENCES

(1) L. H. Sternbach, G. A. Archer, J. V. Earley, R. I. Fryer, E. Reeder, N. Wasyliw, L. O. Randall, and R. Banziger, *J. Med. Chem.*, **8**, 815(1965).

(2) L. O. Randall, W. Schallek, C. L. Scheckel, P. L. Stefko, R. F. Banziger, W. Pool, and R. A. Moe, *Arch. Int. Pharmacodyn. Ther.*, **178**, 216(1969).

(3) H. Jick, *Curr. Ther. Res.*, **9**, 355(1967).

(4) A. Kales, T. Ling Tan, M. Scharf, J. Kales, and E. Malmstrom, *Psychophysiology*, **6**, 260(1969).

(5) M. A. Schwartz, F. M. Vane, and E. Postma, *J. Med. Chem.*, **11**, 770(1968).

(6) J. V. Earley, R. I. Fryer, D. Winter, and L. H. Sternbach, *ibid.*, **11**, 774(1968).

(7) M. A. Schwartz, B. A. Koechlin, E. Postma, S. Palmer, and G. Krol, *J. Pharmacol. Exp. Ther.*, **149**, 423(1965).

(8) R. G. Kelly, E. A. Peets, S. Gordon, and D. A. Buyske, *Anal. Biochem.*, **2**, 267(1961).

(9) J. A. F. de Silva and N. Strojny, data on file, Hoffmann-La Roche Inc., Nutley, N. J., 1968.

(10) "Organic Synthesis," collective vol. 2, A. H. Blatt, Ed., Wiley, New York, N. Y., 1943, p. 166.

(11) S. Riegelman, J. Loo, and M. Rowland, *J. Pharm. Sci.*, **57**, 128(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 2, 1970, from *Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication July 17, 1970.

The authors are grateful to Dr. F. M. Vane for the interpretation of the high-resolution mass spectra and to Dr. A. S. Leon and Dr. E. D. Fram for supervision of the human subjects at the Beth Israel Medical Center, Newark, N. J.

Interaction of Nitrogen Dioxide with Cholesterol Monomolecular Films: Effect of Initial Surface Pressure, Time of Exposure, and Concentration of Nitrogen Dioxide

AIDA M. KAMEL, ALVIN FELMEISTER, and NORMAN D. WEINER*

Abstract □ The interaction of NO_2 with cholesterol monomolecular films was studied as a function of the initial surface pressure, time of exposure, and concentration of NO_2. A condensation of the cholesterol films was observed that increased with the time of exposure and concentration of the NO_2. This effect apparently was due to the loss of cholesterol from the interface. A percent apparent loss of cholesterol was calculated. Films exposed at an initial surface pressure of 7 dynes/cm. exhibited a greater percent apparent loss of cholesterol than did those exposed at 0 dyne/cm. An explanation for these results based on the orientation of the cholesterol at the interface is offered, and the possible biological relevance of this work is considered.

Keyphrases □ Cholesterol monomolecular films—nitrogen dioxide interaction □ Films, cholesterol—initial pressure, exposure time effects □ Nitrogen dioxide, concentration effect—cholesterol film interaction □ Colorimetric analysis—spectrophotometer

Recently, the authors reported the interaction of a series of phospholipid films with the air pollutant nitrogen dioxide (1, 2). The data demonstrated that the unsaturated fatty acid moieties of phospholipids are sensitive to attack by NO_2. Similar results were reported *in vivo* by Thomas *et al.* (3), who postulated that some observed effects of air pollutants may be directly attributable to the interaction of NO_2 with unsaturated lipids.

Since cholesterol also is an important component of cell membranes, it was of interest to examine the interaction of NO_2 with cholesterol monomolecular films. In this study the effect of time of exposure, NO_2 concentration, and the physical state of the cholesterol film, as influenced by the initial surface pressure, were investigated.

EXPERIMENTAL

Materials and Equipment—Chromatographically pure cholesterol was obtained,[1] and spectroscopic grade hexane was used to prepare the cholesterol solutions. All other chemicals were of reagent grade. Previously deionized water was distilled from an all-glass still prior to use.

A mixture of 0.5% NO_2 (99.5% pure)[2] and 99.5% research grade nitrogen was used. The gas mixture was allowed to pass through a flow meter at a fixed rate and directed into a short length of perforated Teflon tubing which was affixed to the underside of an acrylic resin[3] trough cover as previously described (1). This served to maintain the desired gaseous atmosphere over the film.

The film balance used to study the surface pressure–surface area (π–A) characteristics of the film has been described previously (1). Surface pressures were measured by the Wilhelmy plate method (4).

Methods—Solutions of cholesterol in hexane were spread on a 0.065 M phosphate buffer at pH 7.0, and the gas was allowed to flow over the film for a fixed period of time. At the end of the time period, the flow of gas was discontinued; any NO_2 remaining over the surface was removed by use of the exhaust fan in the hood, in which the film-balance unit was set. Manual compression of the film was then initiated, and surface pressure readings were obtained at various film areas.

The concentration of the NO_2 over the subphase during exposure was determined as follows. At periodic intervals during each experiment, 10-ml. samples of the atmosphere under the acrylic resin cover were drawn into a gastight Hamilton syringe containing 10 ml. of NO_2-absorbing solution. The color of the absorbing solution was permitted to develop for 15 min., and the absorbance was measured at 550 mμ using a Spectronic-20 spectrophotometer. The concentration of NO_2 was determined from a standard nitrite curve (5).

RESULTS AND DISCUSSION

Effect of Concentration and Time of Exposure to NO_2—Figure 1 shows the π–A curves obtained after exposure of a cholesterol film (at an initial pressure of 0 dyne/cm.) to 175 ± 25 p.p.m. of NO_2 for various time periods. Under these conditions the condensation effects increased with the time of exposure up to 60 min. Exposure for periods longer than 60 min. had no further effect on the shape or position of the π–A curve. A similar limiting effect was observed also when the film was exposed at an initial surface pressure of 7 dynes/cm. Additional experiments were conducted at both

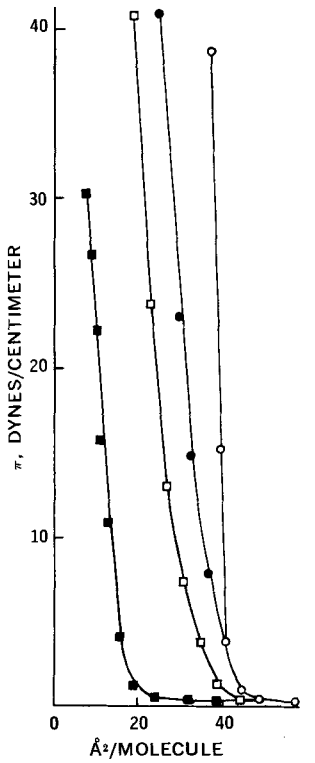


Figure 1—π–A *curves of cholesterol films exposed to 175 ± 25 p.p.m. of NO_2 at an initial surface pressure of 0 dyne/cm. for various time periods. Key:* ○, *control;* ●, *20 min.;* □, *35 min.; and* ■, *60 and 90 min.*

[1] Mann Research Laboratories, N. Y.
[2] Matheson Co., East Rutherford, N. J.
[3] Lucite, E. I. du Pont de Nemours, Wilmington, Del.

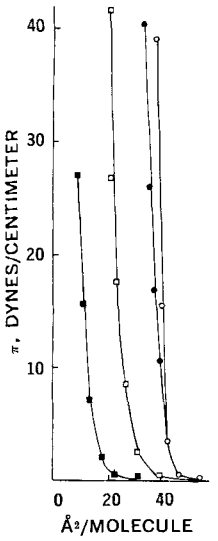


Figure 2—π–A *curves of cholesterol films at an initial surface pressure of 0 dyne/cm. exposed to various concentrations of* NO_2 *for 60 min. Key:* ○, *control;* ●, *25 ± 10 p.p.m.;* □, *95 ± 20 p.p.m.; and* ■, *175 ± 25 p.p.m.*

initial pressures using a concentration of 95 ± 20 p.p.m. of NO_2. In these cases, maximum condensation was observed after a 90-min. exposure.

The reason for this limiting effect could not be explained on the basis of the data obtained in this study. However, Bergstrom and Wintersteiner (6) previously reported such a limiting effect during the autoxidation of aqueous dispersions of cholesterol, which they attributed to an inhibitory effect exerted by the oxidation products. A maximum loss of 70% of the cholesterol was reported by these workers, which corresponds to the maximum percentage losses observed under varying conditions in the present study.

The effect on the π–A curves of films exposed at zero initial pressure for 60 min. to different concentrations of NO_2 is shown in Fig. 2. The condensation effect clearly increases with increasing concentration of NO_2. When films were exposed at an initial pressure of 7 dynes/cm. for the same period of time, the results were essentially the same.

The apparent condensation effect observed in these experiments appears to be due, at least in part, to an NO_2–cholesterol interaction. This reaction, in turn, results in the formation of relatively polar products and in their subsequent desorption or dissolution. This effect can be represented by the percent apparent loss (PAL) of cholesterol calculated from the following equation:

$$PAL = (1 - A'/A)\,100 \qquad \text{(Eq. 1)}$$

where A and A' are the area/molecule of cholesterol at 30 dynes/cm. before and after exposure to NO_2, respectively. PAL is not the actual percent loss of cholesterol from the surface, but rather a complex function which takes into account such factors as desorption, dissolution, changes in molecular orientation, and chemical modification of the cholesterol molecules. However, PAL is a useful means of comparing the effects of NO_2 concentration and initial surface pressure on the stability of NO_2-exposed cholesterol monomolecular films.

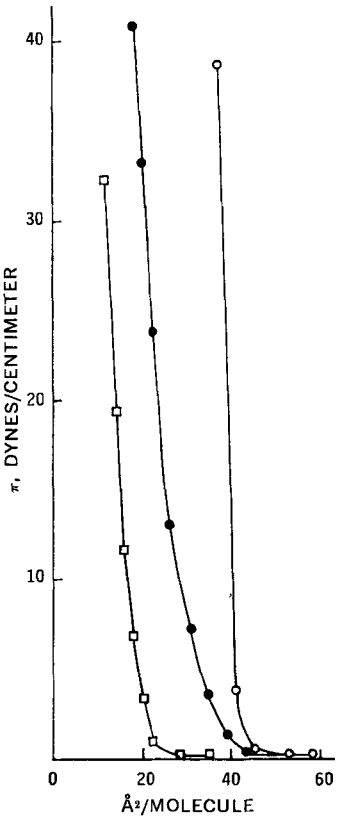


Figure 4—π–A *curves of cholesterol films at various initial surface pressures exposed to 175 ± 25 p.p.m.* NO_2 *for 35 min. Key:* ○, *control;* ●, *0 dyne/cm.; and* □, *7 dynes/cm.*

The curves shown in Fig. 3 were constructed from the calculated values of PAL of cholesterol from films exposed at an initial pressure of 0 dyne/cm. to 25 ± 10, 95 ± 20, and 175 ± 25 p.p.m. of NO_2 for periods of time ranging from 20 to 90 min.

The rate of loss of cholesterol appears to be dependent on the concentration of NO_2 and independent of the amount of cholesterol remaining in the films up to the time at which the limiting effect is observed.

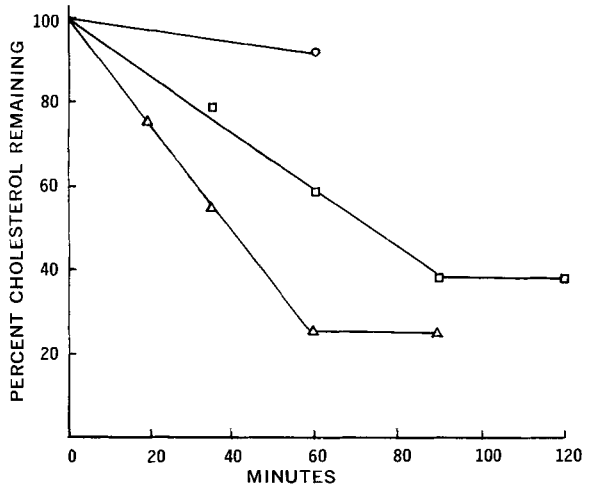


Figure 3—*Rate of loss of cholesterol from films at an initial surface pressure of 0 dyne/cm. exposed to various concentrations of* NO_2. *Key:* ○, *25 ± 10 p.p.m.;* □, *95 ± 20 p.p.m.; and* △, *175 ± 25 p.p.m.*

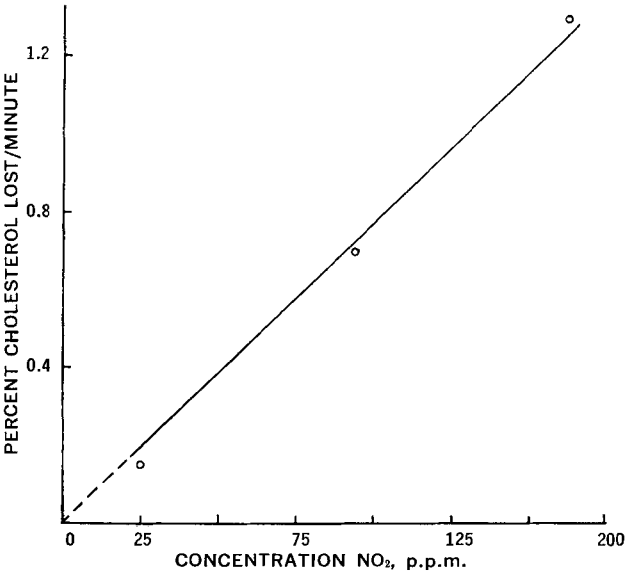


Figure 5—*Rate of loss of cholesterol as a function of* NO_2 *concentration for films at an initial surface pressure of 0 dyne/cm.*

Effect of Initial Surface Pressure—Figure 4 shows the π–A curves of cholesterol films at initial surface pressures of 0 and 7 dynes/cm. prior to and following a 35-min. exposure to 175 ± 25 p.p.m. nitrogen dioxide.

Exposure to NO_2, in the case of the film adjusted to an initial surface pressure of 7 dynes/cm., resulted in a steady decrease in pressure, reaching a value of zero in about 20 min. Furthermore, following a 35-min. exposure to NO_2, the film exposed at this higher surface pressure exhibited a greater PAL of cholesterol.

The orientation of cholesterol molecules in a monomolecular film is markedly influenced by the surface pressure of the film. At zero pressure the molecules would be expected to be essentially flat on the surface, occupying relatively large areas. At 7 dynes/cm., the cholesterol molecules are closely packed and occupy an area that indicates they are in a vertical position with the 3-hydroxy groups anchored in the subphase. Thus, at zero pressure, both polar sites of the cholesterol molecule (*i.e.*, the 3-hydroxy group and the 5–6 double bond) would likely be associated with the aqueous subphase. At 7 dynes/cm., only the 3-hydroxy group would be associated with the subphase, while the double bond would be in the gas phase.

Since the PAL of cholesterol upon exposure to NO_2 is greater when the molecules are in the vertical position, it appears that association of both polar sites with the subphase inhibits the oxidation of cholesterol by NO_2. Such a postulation is supported by Altshuller and Cohen (7), who reported that the oxidation of olefins by NO_2 occurred in the gas phase along with some nitration, while little or no oxidation was observed (though nitration did occur) in the aqueous phase reaction.

CONCLUSIONS

While it appears that the rate of loss of cholesterol from a monomolecular film is dependent on the concentration of NO_2, the rate of loss at levels of NO_2 normally found in polluted air ($\approx$0.5 p.p.m.) would be very slow. Thus, only at relatively high levels of NO_2 would the loss of cholesterol be significant, even over several hours. This can be seen from the extrapolated portion of the plot in Fig. 5 of the rate of loss of cholesterol *versus* the concentration of NO_2.

Whether these results can be related to the effect of NO_2 on cell membranes or to the *in vivo* exposure of humans or animals to NO_2 has not been established. However, the work of Steadman *et al.* (8), in which animals were exposed to wide ranges of concentrations of NO_2 for varying periods of time, does show some correlation. These workers noted that animals exposed to NO_2 concentrations of 70 p.p.m. for 8 hr. suffered pulmonary edema and vascular congestion with high mortality rates. On the other hand, exposure to a concentration of 0.5 p.p.m. even for as long as 90 consecutive days produced no apparent untoward effects. Thus, it may be that the loss of cholesterol from surface films, which would be very slow at a NO_2 concentration of 0.5 p.p.m. and relatively fast at a concentration of 70 p.p.m., gives some indication of the loss of this essential lipid from cell membranes and of the observed clinical effects of NO_2.

REFERENCES

(1) A. Felmeister, M. Amanat, and N. D. Weiner, *Environ. Sci. Technol.*, **2**, 40(1968).

(2) N. D. Weiner, M. Amanat, and A. Felmeister, *Arch. Environ. Health*, **18**, 636(1969).

(3) H. V. Thomas, P. K. Mueller, and R. L. Lyman, *Science*, **159**, 532(1968).

(4) H. W. Adamson, "Physical Chemistry of Surfaces," 2nd ed., Interscience, New York, N. Y., 1967, p. 26.

(5) B. E. Saltzman, *Anal. Chem.*, **26**, 1949(1954).

(6) S. Bergstrom and O. J. Wintersteiner, *J. Biol. Chem.*, **141**, 597(1941).

(7) A. P. Altshuller and I. Cohen, *Int. J. Air Water Pollut.*, **4**, 55(1961).

(8) B. L. Steadman, R. A. Jones, D. E. Rector, and J. Siegel, *J. Tox. Appl. Pharmacol.*, **9**, 160(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 7, 1970, from the *College of Pharmacy, Rutgers University, Newark, NJ 07104*

Accepted for publication June 29, 1970.

This study was supported by Research Grant AP 788, National Air Pollution Control Administration, Consumer Protection and Environmental Health Service, U. S. Public Health Service.

* Present address: College of Pharmaceutical Sciences, Columbia University, New York, NY 10023

Crystal Pseudopolymorphism of Cephaloglycin and Cephalexin

RALPH R. PFEIFFER, K. S. YANG, and MARY ANN TUCKER

Abstract □ The systematic investigation of the crystallization of cephaloglycin and cephalexin leads to a better understanding of the part that pseudopolymorphic crystal transitions play in the analysis, processing, and formulation of these and many other pharmaceutical compounds. The employment of solubility *versus* solvent composition diagrams to detect various crystal forms of compounds is discussed. This appears to be a convenient and sensitive method for detecting new crystalline phases. It should find application whenever crystallizations are performed with more than one solvent, and particularly when instability of the compound at elevated temperatures prevents the use of conventional thermal methods or when poor crystal development limits the use of microscopic methods. Interpretation of vapor pressure–composition relationships for various crystal forms of these compounds points to the advisability of obtaining such data for all pharmaceutical solids.

Keyphrases □ Crystal pseudopolymorphism—cephaloglycin, cephalexin □ Cephaloglycin, cephalexin—pseudomorphic crystallization □ UV spectrophotometry—analysis □ X-ray powder diffraction—analysis □ NMR spectroscopy—analysis

It is the responsibility of the pharmaceutical chemist to become familiar with the crystallizing properties of drugs in order to control the crystal form, habit, size, size distribution, degree of crystallinity, and state of aggregation of the drug particles. These parameters often determine the acceptability of bulk properties (mixing, tableting, filling, dusting, *etc.*) and pharmaceutical performance (dissolution, biological availability, chemical and physical stability, suspendibility, rheology, *etc.*). Haleblian and McCrone (1) adequately documented the broad aspects of this argument in a recent review article on polymorphism, wherein they

Effect of Initial Surface Pressure—Figure 4 shows the π–A curves of cholesterol films at initial surface pressures of 0 and 7 dynes/cm. prior to and following a 35-min. exposure to 175 ± 25 p.p.m. nitrogen dioxide.

Exposure to NO_2, in the case of the film adjusted to an initial surface pressure of 7 dynes/cm., resulted in a steady decrease in pressure, reaching a value of zero in about 20 min. Furthermore, following a 35-min. exposure to NO_2, the film exposed at this higher surface pressure exhibited a greater PAL of cholesterol.

The orientation of cholesterol molecules in a monomolecular film is markedly influenced by the surface pressure of the film. At zero pressure the molecules would be expected to be essentially flat on the surface, occupying relatively large areas. At 7 dynes/cm., the cholesterol molecules are closely packed and occupy an area that indicates they are in a vertical position with the 3-hydroxy groups anchored in the subphase. Thus, at zero pressure, both polar sites of the cholesterol molecule (*i.e.*, the 3-hydroxy group and the 5–6 double bond) would likely be associated with the aqueous subphase. At 7 dynes/cm., only the 3-hydroxy group would be associated with the subphase, while the double bond would be in the gas phase.

Since the PAL of cholesterol upon exposure to NO_2 is greater when the molecules are in the vertical position, it appears that association of both polar sites with the subphase inhibits the oxidation of cholesterol by NO_2. Such a postulation is supported by Altshuller and Cohen (7), who reported that the oxidation of olefins by NO_2 occurred in the gas phase along with some nitration, while little or no oxidation was observed (though nitration did occur) in the aqueous phase reaction.

CONCLUSIONS

While it appears that the rate of loss of cholesterol from a monomolecular film is dependent on the concentration of NO_2, the rate of loss at levels of NO_2 normally found in polluted air ($\approx$0.5 p.p.m.) would be very slow. Thus, only at relatively high levels of NO_2 would the loss of cholesterol be significant, even over several hours. This can be seen from the extrapolated portion of the plot in Fig. 5 of the rate of loss of cholesterol *versus* the concentration of NO_2.

Whether these results can be related to the effect of NO_2 on cell membranes or to the *in vivo* exposure of humans or animals to NO_2 has not been established. However, the work of Steadman *et al.* (8), in which animals were exposed to wide ranges of concentrations of NO_2 for varying periods of time, does show some correlation. These workers noted that animals exposed to NO_2 concentrations of 70 p.p.m. for 8 hr. suffered pulmonary edema and vascular congestion with high mortality rates. On the other hand, exposure to a concentration of 0.5 p.p.m. even for as long as 90 consecutive days produced no apparent untoward effects. Thus, it may be that the loss of cholesterol from surface films, which would be very slow at a NO_2 concentration of 0.5 p.p.m. and relatively fast at a concentration of 70 p.p.m., gives some indication of the loss of this essential lipid from cell membranes and of the observed clinical effects of NO_2.

REFERENCES

(1) A. Felmeister, M. Amanat, and N. D. Weiner, *Environ. Sci. Technol.*, **2**, 40(1968).

(2) N. D. Weiner, M. Amanat, and A. Felmeister, *Arch. Environ. Health*, **18**, 636(1969).

(3) H. V. Thomas, P. K. Mueller, and R. L. Lyman, *Science*, **159**, 532(1968).

(4) H. W. Adamson, "Physical Chemistry of Surfaces," 2nd ed., Interscience, New York, N. Y., 1967, p. 26.

(5) B. E. Saltzman, *Anal. Chem.*, **26**, 1949(1954).

(6) S. Bergstrom and O. J. Wintersteiner, *J. Biol. Chem.*, **141**, 597(1941).

(7) A. P. Altshuller and I. Cohen, *Int. J. Air Water Pollut.*, **4**, 55(1961).

(8) B. L. Steadman, R. A. Jones, D. E. Rector, and J. Siegel, *J. Tox. Appl. Pharmacol.*, **9**, 160(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 7, 1970, from the *College of Pharmacy, Rutgers University, Newark, NJ 07104*

Accepted for publication June 29, 1970.

This study was supported by Research Grant AP 788, National Air Pollution Control Administration, Consumer Protection and Environmental Health Service, U. S. Public Health Service.

* Present address: College of Pharmaceutical Sciences, Columbia University, New York, NY 10023

Crystal Pseudopolymorphism of Cephaloglycin and Cephalexin

RALPH R. PFEIFFER, K. S. YANG, and MARY ANN TUCKER

Abstract □ The systematic investigation of the crystallization of cephaloglycin and cephalexin leads to a better understanding of the part that pseudopolymorphic crystal transitions play in the analysis, processing, and formulation of these and many other pharmaceutical compounds. The employment of solubility *versus* solvent composition diagrams to detect various crystal forms of compounds is discussed. This appears to be a convenient and sensitive method for detecting new crystalline phases. It should find application whenever crystallizations are performed with more than one solvent, and particularly when instability of the compound at elevated temperatures prevents the use of conventional thermal methods or when poor crystal development limits the use of microscopic methods. Interpretation of vapor pressure–composition relationships for various crystal forms of these compounds points to the advisability of obtaining such data for all pharmaceutical solids.

Keyphrases □ Crystal pseudopolymorphism—cephaloglycin, cephalexin □ Cephaloglycin, cephalexin—pseudomorphic crystallization □ UV spectrophotometry—analysis □ X-ray powder diffraction—analysis □ NMR spectroscopy—analysis

It is the responsibility of the pharmaceutical chemist to become familiar with the crystallizing properties of drugs in order to control the crystal form, habit, size, size distribution, degree of crystallinity, and state of aggregation of the drug particles. These parameters often determine the acceptability of bulk properties (mixing, tableting, filling, dusting, *etc.*) and pharmaceutical performance (dissolution, biological availability, chemical and physical stability, suspendibility, rheology, *etc.*). Haleblian and McCrone (1) adequately documented the broad aspects of this argument in a recent review article on polymorphism, wherein they

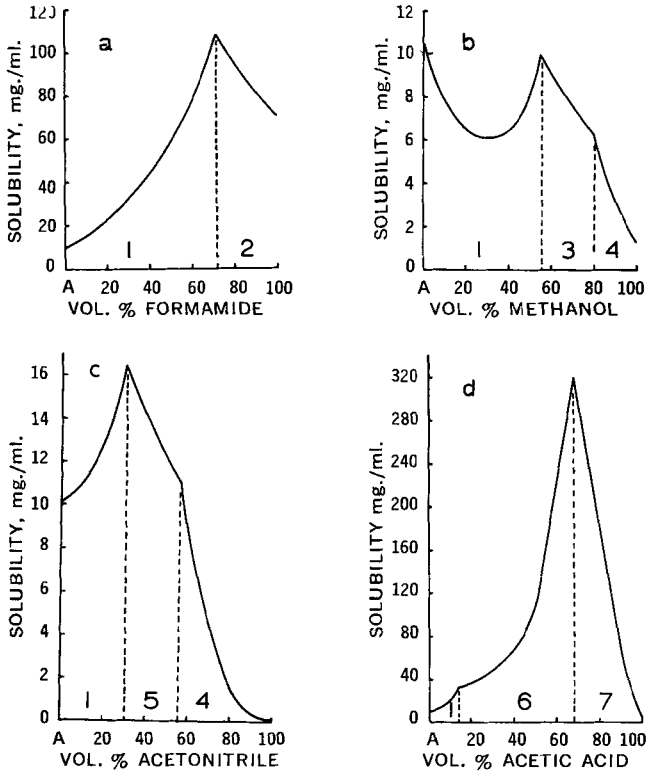


Figure 1—*Solubility of cephaloglycin in binary solvent mixtures at 25°. A = water in all cases. Arabic numerals refer to solid phases in Table I.*

also discussed thoroughly the preparation and characterization of true polymorphic[1] crystal forms by thermal methods. The related and equally pertinent phenomenon of pseudopolymorphism, *i.e.*, modification caused by inclusion of solvent in the crystal structure, apparently has received no such general treatment in the pharmaceutical literature, although many specific cases of pseudopolymorphism in drugs have been reported (2–4).

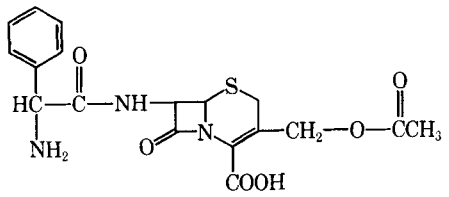
This article suggests some new approaches to the study of pseudopolymorphic crystal transformations as they apply to pharmaceutical manufacturing practice. As a case in point, it reports the crystallizing properties of the antibiotics cephaloglycin (I) (5) and cephalexin (II) (6), both of which occur in a wide variety of solvated crystal forms.

EXPERIMENTAL

Reagents—The cephaloglycin and cephalexin each had a microbiological potency (5, 6) of >970 mcg./mg.; phase-solubility analysis in water indicated a purity of 97 ± 1%. (Both analyses were corrected for volatile components.)

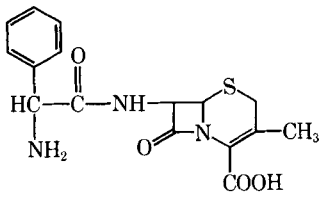
Solvents and other chemicals were reagent grade and were taken from freshly opened packages without further purification.

Equilibration with Solvents—Finely ground cephaloglycin (dihydrate) or cephalexin (monohydrate) was suspended and equilibrated at 25° in a variety of solvents and solvent mixtures. In a typical experiment, 11 tubes, containing a total of 5 ml. of two miscible solvents in ratios ranging from 1:0 to 0:1, were prepared. A quantity of the compound, sufficient to ensure the presence of a



cephaloglycin

I



cephalexin

II

substantial amount of solid phase throughout the equilibration period, was added to each tube. Three hours of shaking on a vibrating mixer,[2] with occasional manual stirring to disperse any lumps, proved satisfactory for the present purpose. The tubes were then centrifuged, and the clear supernates were analyzed spectrophotometrically at 260 mμ for dissolved cephaloglycin or cephalexin. A preliminary solubility *versus* solvent composition diagram was constructed from the data. The crystals, wet with mother liquor, were examined under a polarizing microscope and by X-ray powder diffraction to detect changes in the solid phase.

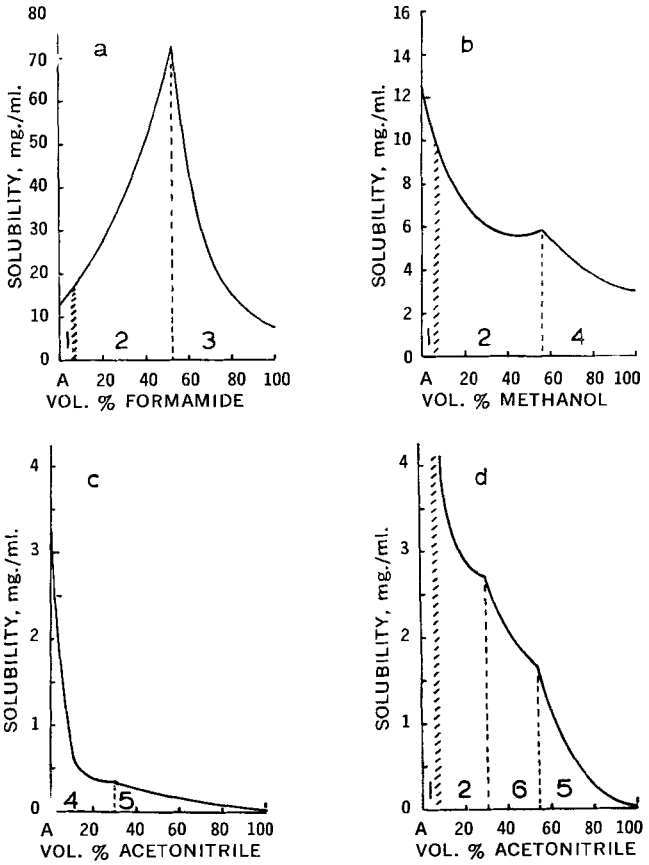


Figure 2—*Solubility of cephalexin in binary solvent mixtures at 25°. A = water in Sections a, b, and d. A = methanol in Section c. Arabic numerals refer to solid phases in Table II.*

[1] True crystal polymorphism involves forms of identical chemical composition. Thus, a compound may exhibit pseudopolymorphism by forming variously solvated crystals; each of these solvates (including the unsolvated form) may exhibit polymorphism.

[2] Vibromixer, model El, AG. für Chemie-Apparatebau, Zurich, Switzerland.

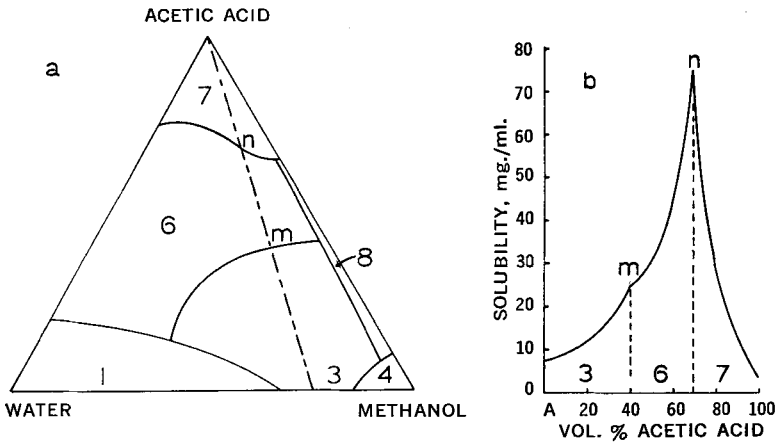


Figure 3—*(a) Stability zones of cephaloglycin crystal forms in mixtures of water–methanol–glacial acetic acid. The broken line indicates the compositions used in Section b. (b) Typical solubility diagram (also Fig. 1, Section d) used to locate zone boundaries in Section a. A = 75% methanol–water. Points m and n correspond in the two sections. Arabic numerals refer to solid phases in Table I.*

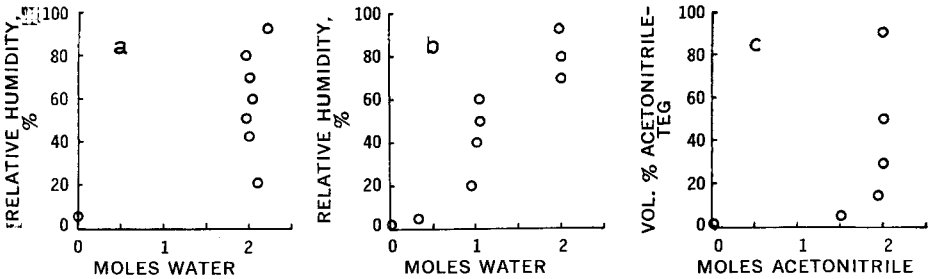


Figure 4—*Crystal–vapor equilibria: a, cephaloglycin–water; b, cephalexin–water; and c, cephalexin–acetonitrile. The ordinate in Section c describes the volume composition of acetonitrile–triethylene glycol (TEG) used to provide a range of acetonitrile vapor pressures whose exact magnitude was not determined.*

Preparation of Pure Reference Phases—To obtain the various crystalline phases in a homogeneous form, each phase was crystallized from the totally dissolved state. A solution (about 10% w/v) of the antibiotic in the appropriate solvent mixture, *i.e.*, a composition that had yielded a new solid phase in the equilibration, was prepared by acidification with an amount of concentrated hydrochloric acid[3] equivalent to the amount of solute. Precipitation of the zwitterion in the desired crystal form was then effected by slow addition of an equivalent amount of concentrated ammonium hydroxide.[3] The resulting crystals were removed by filtration and dried under conditions that would not disrupt the crystal stoichiometry (see *Discussion*). Analysis by selected methods followed. These methods always included X-ray powder diffraction and some of the following methods: elemental analysis, thermogravimetry, titration, spectrophotometry (NMR and UV), and specific analysis for water (Karl Fischer).

Determination of Solubility *versus* Solvent Composition Diagrams—The solubility determinations were repeated under the earlier conditions but using the proper equilibrium solid phases at the start of the individual equilibrations. In this way the solubility *versus* composition curve for each solid phase could be obtained over its entire stability range without relying on phase transformations. The equilibrium diagrams in Figs. 1 and 2 were constructed using the nonoverlapping parts of these curves. It was found that the same solubility information (agreement within 5%) could be obtained by using admixtures of two appropriately chosen solid phases for equilibrations near transition points. This procedure was used to obtain the information in Fig. 3. It was assumed that the stable (least soluble) phase was essentially controlling the solubility, even in the presence of unconverted metastable crystals.

Equilibration with Vapor—Vapor equilibration chambers were made from 5-lb. ointment jars with tight fitting lids. Each jar contained about 500 ml. of a constant vapor pressure slurry. Water vapor pressures were determined with electric hygrometer[4] sensors. In one study, the jars contained 200 ml. of acetonitrile and triethylene glycol mixed in various ratios, thus providing atmospheres covering a wide range of acetonitrile vapor pressures. Previously analyzed samples of about 1 g., spread thinly in open weighing dishes, were set on trivets within the jars. The dishes were covered and weighed daily for 1 week, although constant weight was achieved after 2 or 3 days. Samples showing significant gain or loss in weight were checked for phase changes by X-ray diffraction. All changes in solvent content or phase identity were found to be reversible upon subsequent exposure of the samples to the appropriate atmospheres.

[3] Possible effects caused by the water and salt thus introduced were ignored.

[4] Hygrodynamics, Inc., Silver Spring, Md.

RESULTS AND DISCUSSION

Because cephaloglycin and cephalexin were each observed to occur in many solvated crystal forms (Tables I–IV), and often in widely varying mixtures of these forms, a systematic study of the crystallizing properties of these compounds was undertaken. The properties of the pure crystalline components under equilibrium conditions were first explored. Mixtures of these components resulting from kinetic factors are subsequently considered.

Crystal–Solution Equilibria—The solubility diagrams resulting from this study (Figs. 1 and 2) provide a clear indication of not only the existence of multiple crystalline forms of cephaloglycin and cephalexin but also the ranges of solvent compositions in which the various forms are stable.

When considering crystal transformations that occur with changes

Table I—Some Solvated Crystal Forms of Cephaloglycin

Moles Solvent/Moles Cephaloglycin[a]	Method[b]
1. $2H_2O$	a, b, c, d, e, f, g
2. Formamide	a, b, e, f
3. Methanol · H_2O	a, b, c, e, f, g
4. Unsolvated (anhydrate)	a, b, c, e, f, g
5. Acetonitrile · H_2O	a, b, d, e, f
6. Acetic acid · H_2O	a, b, c, e, f, g, h
7. 2 Acetic acid	a, b, e, f, g, h
8. Acetic acid · methanol	a, b, e, f
9. Ethanol · H_2O	a, b, e
10. *N*-Methylformamide	a, b, i

[a] Arabic numerals correspond to phases in Figs. 1 and 3. [b] Compositions were determined by: a, uniqueness of X-ray powder diffraction pattern; b, Karl Fischer water; c, thermogravimetric analysis; d, vapor pressure–composition diagram; e, UV spectrophotometry; f, NMR spectrometry; g, elemental analysis; h, titration; and i, qualitative stoichiometry only. The quantitative results confirmed the stated compositions to ±0.1 mole solvent. All forms, when recrystallized from water, had retained the X-ray diffraction properties of the starting material, indicating that no chemical modification of the antibiotic had occurred.

Table II—Some Solvated Crystal Forms of Cephalexin[a]

Moles Solvent/Moles Cephalexin	Method
1. $2H_2O$	a, b, d, e
2. H_2O	a, b, d, e, f, g
3. Formamide	a, i
4. Methanol	a, b, g
5. 2 Acetonitrile	a, d, e
6. Acetonitrile·H_2O	a, b, e
7. *N*-Methylformamide	a, i
8. *N*-Ethylformamide	a, i

[a] Arabic numerals correspond to phases in Fig. 2. See Table I for explanation of other symbols.

in solvent composition, it is helpful to recall that the changing solubility values along a smooth curve refer to an equilibrium between a solid phase of definite, unchanging composition and a solution of changing properties. The proportions of the variously solvated, aggregated, and charged solute species in solution vary with and are determined by the composition of the solvent. At equilibrium, the *concentration* of solute in solution, *i.e.*, the solubility, may vary as the solvent changes, but the *activity of the crystallizing molecular species* remains constant, *i.e.*, equals that of the crystalline form present. When individual solubility *versus* solvent composition curves of several available crystal forms cross one another to give a peak or cusp, a phase transition point is revealed. At such points the two solid phases represented by the separate curves have the same solubility and may therefore coexist at equilibrium. At other compositions, the respectively more soluble phase is necessarily metastable with respect to the less soluble one.

Phase Relationships in Ternary and Quaternary Solvent Mixtures—The compositions of 3-solvent mixtures in which the various crystal forms of a compound are stable can be conveniently represented by a triangular diagram such as the one presented in Fig 3*a* for cephaloglycin in water–methanol–glacial acetic acid. Each edge of the triangle was marked with the zone boundaries determined earlier in the respective binary solvent mixtures (Figs. 1*b* and 1*d*), and the positions of boundaries within the triangle were located by determining solubilities in series of appropriately chosen ternary solvent mixtures and interpreting the resulting curves in the manner described earlier (Fig. 3*b*).

Occasionally, a ternary mixture of solvents serves as a recrystallizing solvent, with a fourth miscible liquid acting as the antisolvent. The phase relationships, or stability zones, of solute crystals in such a system can be analogously represented by a triangular pyramid.

Doubly Solvated Crystals—Both cephaloglycin and cephalexin display the ability to crystallize with simultaneous stoichiometric inclusion of two solvents in the crystal lattice. The existence and identity of these phases are evident from their unique and uniform microscopic appearance, their X-ray pattern, the solubility diagram, and the various analyses of the crystals (Tables I–IV).

Crystal–Vapor Equilibria—One cause of erratic changes in the properties of powders is their physical interaction with solvent vapors. In the interests of crystal stability, therefore, the vapor composition of the environment, especially with regard to water vapor, must be given due consideration throughout the history of a powder, and the quantitative equilibrium relationships between crystal and vapor phases must be determined.

Vapor pressure *versus* composition diagrams of the kind presented for cephaloglycin–water, cephalexin–water, and cephalexin–acetonitrile (Fig. 4) delimit the vapor pressure range over which a solvated crystal is stable after isolation from the mother liquor. These diagrams also reveal the true stoichiometry of the solvated crystals.

Multiple Hydrated Forms—When a compound exists in several hydrated crystal forms at one temperature, as is clearly the case

Table III—X-Ray Powder Diffraction Data for Some Crystal Forms of Cephaloglycin

Cephaloglycin —Dihydrate—		Cephaloglycin —Anhydrate—		Cephaloglycin Methanolate —Hydrate—		Cephaloglycin —Formamidate—		Cephaloglycin Acetonitrile —Hydrate—		Cephaloglycin Acetic Acid —Hydrate—		Cephaloglycin 2 Acetic Acid —Solvate—	
d[a]	I/I_1[b]	d	I/I_1	d	I/I_1	d	I/I_1	d	I/I_1	d	I/I_1	d	I/I_1
16.8	1.00	9.40	1.00	10.64	1.00	10.9	1.00	20.0	1.00	10.6	1.00	11.5	1.00
11.0	0.04	8.58	0.50	9.40	0.60	9.55	0.50	16.0	0.40	9.25	0.80	9.00	0.30
9.95	0.30	7.13	0.30	7.56	0.50	8.50	0.20	11.9	1.00	7.60	0.50	7.50	0.30
8.35	0.40	6.70	0.50	6.62	0.40	7.65	0.40	9.30	0.50	6.60	0.60	6.70	0.50
7.15	0.50	5.71	0.40	5.86	0.40	6.80	0.40	8.50	0.20	5.75	0.65	5.70	0.40
6.15	0.20	5.18	0.15	5.50	0.05	5.05	0.30	7.30	0.30	5.42	0.20	5.40	0.40
5.80	0.10	4.69	1.00	5.00	0.15	5.40	0.30	6.90	0.30	4.90	0.30	4.97	0.30
5.40	0.70	4.37	0.70	4.69	1.00	4.90	0.30	5.80	0.50	4.65	1.00	4.75	0.30
4.90	0.10	4.28	0.90	4.52	0.20	4.60	1.00	5.40	0.10	4.48	0.40	4.60	0.70
4.55	0.10	3.98	0.60	4.23	0.10	4.35	0.40	5.20	0.30	4.22	0.30	4.42	0.10
4.32	0.30	3.75	0.04	4.09	0.10	4.16	0.30	4.78	0.60	4.08	0.30	4.12	0.60
4.10	0.10	3.58	0.70	3.43	0.10	3.96	0.40	4.48	0.70	3.89	0.30	3.90	0.05
4.00	0.40	3.36	0.40	3.75	0.60	3.70	0.30	4.40	0.20	3.70	0.70	3.78	0.70
3.72	0.40	3.14	0.20	3.50	0.90	3.56	0.90	4.15	0.20	3.50	1.00	3.70	0.70
3.45	0.40	3.03	0.30	3.42	0.20	3.35	0.30	3.97	0.15	3.40	0.40	3.00	0.05
3.32	0.20	2.87	0.08	3.31	0.20	3.25	0.15	3.74	0.60	3.30	0.35	3.35	0.60
3.20	0.10	2.73	0.70	3.23	0.30	3.18	0.30	3.55	0.30	3.20	0.30	3.24	0.05
3.06	0.10	2.60	0.40	3.14	0.30	2.90	0.20	3.40	0.10	3.08	0.30	3.18	0.05
2.92	0.30	2.52	0.15	3.09	0.05	2.80	0.05	3.16	0.10	2.96	0.15	3.07	0.50
2.79	0.20	2.39	0.10	2.95	0.15	2.70	0.20	2.90	0.50	2.94	0.15	2.91	0.40
2.61	0.20	2.28	0.10	2.82	0.05	2.61	0.30	2.84	0.50	2.86	0.20	2.74	0.15
2.54	0.10	2.23	0.10	2.76	0.05	2.55	0.15	2.65	0.05	2.80	0.20	2.66	0.15
2.42	0.15	2.12	0.02	2.69	0.05	2.40	0.15	2.55	0.10	2.66	0.20	2.61	0.05
2.31	0.20	2.02	0.02	2.64	0.05	2.35	0.15	2.45	0.05	2.00	0.25	2.55	0.10
2.24	0.10	1.93	0.02	2.54	0.10	2.28	0.15	2.35	0.10	2.55	0.15	2.47	0.20
2.19	0.08	1.88	0.02	2.47	0.05	2.24	0.15	2.20	0.05	2.48	0.15	2.44	0.20
2.13	0.08	1.68	0.02	2.42	0.10	2.15	0.10	2.12	0.15	2.45	0.15	2.40	0.05
2.08	0.20			2.30	0.30	2.07	0.05	1.80	0.10	2.34	0.10	2.30	0.30
1.97	0.06			2.23	0.20	1.90	0.05			2.26	0.20	2.24	0.30
1.94	0.06			2.18	0.20					2.22	0.20	2.16	0.10
1.92	0.04			2.11	0.15					2.17	0.30	2.11	0.20
				1.99	0.15					2.08	0.30	2.04	0.05
				1.95	0.05					1.98	0.10	1.90	0.05
				1.88	0.10					1.96	0.10	1.85	0.20
				1.81	0.05					1.86	0.20		
				1.67	0.05					1.54	0.10		
				1.59	0.05					1.52	0.10		

[a] Interplanar spacing in A. U. [b] Relative intensity, visual estimation. Radiation; Cu/Ni. Norelco DeBye-Scherrer camera.

Table IV—X-Ray Powder Diffraction Data[a] for Some Crystal Forms of Cephalexin

Cephalexin Dihydrate		Cephalexin Monohydrate		Cephalexin Diacetonitrilate		Cephalexin Formamidate		Cephalexin Methanolate		Cephalexin Acetonitrile Hydrate	
d	I/I_1	*d*	I/I_1	*d*	I/I_1	*d*	I/I_1	*d*	I/I_1	*d*	I/I_1
14.3	0.20	15.15	0.40	11.18	0.05	10.4	1.00	10.8	0.80	9.50	1.00
13.0	1.00	11.85	1.00	9.81	1.00	9.80	0.40	8.55	0.50	8.60	0.05
11.1	0.15	11.00	0.30	8.84	0.50	9.00	0.05	7.60	0.50	7.20	0.10
9.65	0.20	9.36	0.20	6.96	0.40	8.00	0.05	7.05	0.50	6.70	0.40
7.43	0.50	8.55	0.50	6.55	0.30	6.90	0.50	6.55	0.50	5.76	0.60
7.28	0.10	7.86	0.50	5.64	0.60	6.15	0.20	6.00	0.60	5.25	0.40
6.68	0.50	6.89	0.20	5.40	0.10	5.61	0.15	5.35	0.40	4.76	0.70
5.80	0.80	5.98	0.40	4.79	0.50	5.25	0.60	5.00	0.05	4.60	0.30
5.44	0.20	5.39	1.00	4.64	0.60	5.00	0.10	4.85	1.00	4.35	0.60
5.29	0.40	4.97	0.50	4.39	0.30	4.75	0.70	4.65	1.00	4.25	0.30
4.94	0.60	4.76	0.40	4.19	1.00	4.38	0.90	4.11	1.00	3.96	0.60
4.47	0.15	4.57	0.40	4.03	0.30	4.08	0.70	3.98	1.00	3.80	0.10
4.21	0.90	4.39	0.60	3.66	0.15	3.80	0.50	3.89	0.30	3.55	0.50
3.97	0.70	4.22	0.60	3.61	0.10	3.00	0.60	3.76	0.40	3.35	0.50
3.79	0.60	4.00	0.70	3.41	0.40	3.42	0.50	3.53	0.30	3.18	0.10
3.54	0.50	3.86	0.70	3.28	0.30	3.26	0.50	3.28	0.55	3.12	0.20
3.39	0.15	3.60	0.80	3.12	0.30	3.10	0.30	3.08	0.15	2.99	0.30
3.27	0.30	3.46	0.30	3.06	0.05	2.95	0.40	2.95	0.30	2.90	0.20
3.15	0.40	3.24	0.60	2.90	0.15	2.23	0.40	2.87	0.20	2.75	0.30
3.02	0.40	3.10	0.60	2.80	0.20	2.75	0.30	2.76	0.20	2.63	0.45
2.92	0.50	2.98	0.40	2.77	0.15	2.68	0.20	2.05	0.40	2.55	0.20
2.78	0.30	2.90	0.60	2.66	0.15	2.60	0.30	2.57	0.20	2.36	0.20
2.64	0.20	2.81	0.40	2.59	0.20	2.50	0.20	2.54	0.20	2.30	0.45
2.55	0.15	2.73	0.20	2.50	0.05	2.37	0.30	2.45	0.20	2.24	0.10
2.45	0.10	2.68	0.40	2.46	0.10	2.28	0.40	2.36	0.20	2.12	0.50
2.34	0.15	2.63	0.10	2.37	0.20	2.22	0.15	2.32	0.20	1.90	0.05
2.26	0.10	2.47	0.30	2.31	0.05	2.15	0.15	2.17	0.15	1.81	0.10
2.15	0.05	2.41	0.15	2.25	0.15	2.11	0.15	1.99	0.05	1.68	0.15
2.09	0.10	2.31	0.30	2.16	0.15	2.02	0.05	1.89	0.05		
2.01	0.05	2.25	0.30	2.10	0.05	1.97	0.02				
1.98	0.05	2.12	0.10	1.97	0.02	1.93	0.02				
1.94	0.02	2.09	0.05	1.89	0.10	1.87	0.10				
1.86	0.02	2.01	0.02	1.85	0.10	1.72	0.05				
1.81	0.02	1.93	0.05	1.80	0.10	1.70	0.05				
1.77	0.02	1.87	0.05	1.67	0.02	1.67	0.05				
		1.85	0.05	1.53	0.02						
		1.82	0.10								
		1.72	0.05								
		1.66	0.02								
		1.62	0.02								

[a] See Table III for explanation of symbols and experimental conditions.

with cephalexin (Fig. 4*b*), the relative humidity *versus* composition diagram indicates the exact relative humidity that marks the transition from one form to another. Thus, cephalexin crystallized from water at room temperature is separated from solution as the dihydrate but converts to the monohydrate when the relative humidity is below 70%.

The Desolvated Crystal—Cephalexin·2 acetonitrile (Tables II and IV) serves as an example of what the authors believe to be a widely occurring but often undetected or improperly described case in powder technology, *viz.*—the "desolvated" crystal. This crystal is solvated stoichiometrically while in equilibrium with saturated solution; but when isolated and dried under ordinary conditions, it loses all except small mole fractions of solvent from the crystal lattice without thereby converting to another form (Fig. 4*c*). It is in this condition that the compound is commonly analyzed for the first time. When subsequently exposed to ambient atmospheres, the desolvated crystal takes up limited amounts of moisture, the water molecules presumably occupying lattice vacancies left by the original solvent. This water can be removed by drying or it can be displaced by exposing the crystal to vapor of the original solvent. In the latter case, the crystal returns to its native stoichiometric composition. All these changes may occur reversibly and without significant changes in the lattice, as evidenced by the X-ray powder diffraction pattern.[5] It is, therefore, proper in such cases to refer to a "desolvated" crystal, *e.g.*, "desolvated acetonitrilate crystal," "desolvated methanolate crystal," *etc.* This is necessary to differentiate it from a truly unsolvated unique structure (anhydrate), which would have different physicochemical properties despite having a chemical composition virtually identical to that of the desolvated crystal.

The authors have also observed the partial desolvation of the doubly solvated crystals listed in Tables I and II, but have not studied these more complex vapor–composition relationships.

The proper handling of solvated crystals to assure the maintenance of their stoichiometry for purposes of analysis should be evident from this discussion. Such crystals should always be isolated from the mother liquor and blotted free of liquid. To avoid exposure to atmospheres differing from that above the saturated mother liquor, well-sealed containers with a minimum of void space should be used for storage. Temperature changes should also be avoided.

Mixtures and Achievement of Equilibrium—While the results in this study lend themselves particularly well to interpretation by equilibrium principles, the authors realize that in studying other systems, kinetic barriers may be encountered that prevent the practical achievement of equilibrium, leading to highly variable mixtures.

Failure to observe equilibrium in laboratory experiments should, therefore, be regarded as highly pertinent to everyday industrial product variability. To be sure, it is sometimes desirable to deliberately make nonequilibrium forms, or even to reproducibly halt crystal transformations at a given stage. In any event, it is necessary to characterize all the available crystal forms to evaluate properly the role of crystal transformations in a pharmaceutical process.

[5] In some cases of crystal desolvation, the diffraction intensities of one or two planes are particularly sensitive to the solvent content of the crystals; in other cases, the loss of solvent is accompanied by a general loss of diffraction sharpness or by slight changes in the unit cell constants (2). However, the X-ray powder diffraction pattern retains its value in identifying the unique origin of the solid phase.

REFERENCES

(1) J. Haleblian and W. McCrone, *J. Pharm. Sci.*, **58**, 911(1969).
(2) J. H. Chapman, J. E. Page, A. C. Parker, D. Rogers, C. J. Sharp, and S. E. Stainforth, *J. Pharm. Pharmacol.*, **20**, 418(1968).
(3) M. Kuhnert-Brandstätter and H. Grimm, *Microchim. Acta*, **1968**, 115.
(4) *Ibid.*, **1968**, 127.
(5) J. L. Spencer, E. H. Flynn, R. W. Roeske, F. Y. Siu, and R. R. Chauvette, *J. Med. Chem.*, **9**, 746(1966).
(6) C. W. Ryan, R. L. Simon, and E. M. Van Heyningen, *ibid.*, **12**, 310(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 25, 1970, from *Eli Lilly and Company, Indianapolis, IN 46206*

Accepted for publication June 19, 1970.

The authors thank Dale Spurgeon and Herbert Smith for extensive X-ray diffraction work.

Influence of Several Autonomic Drugs on Sodium Nitroprusside and Oxotremorine-Induced Hypothermia in Immature and Mature Mice

DAVID H. BURKE* and DAVID E. MANN, Jr.

Abstract □ Sodium nitroprusside and oxotremorine each produced body temperature depression that was independent of age. Atropine inhibited oxotremorine hypothermia in both age groups, but was ineffective in modifying thermal responses to nitroprusside in both age categories. Pilocarpine administration did not alter oxotremorine activity at either age level, while nitroprusside hypothermia was enhanced and partially reversed, respectively, in immature and mature mice. Nicotine and tetraethylammonium chloride were unable to modify hypothermia produced by oxotremorine and nitroferricyanide in adult mice. Nicotine enhanced nitroprusside hypothermia in 10-day-old mice, while temperature depression due to oxotremorine was unaffected in the same age group. Administration of tetraethylammonium chloride to immature animals treated with oxotremorine and nitroprusside resulted in greater temperature depression. Chlorpromazine, which produced no change in oxotremorine or nitroprusside hypothermia in 10-day-old mice, partially blocked oxotremorine-induced hypothermia in mature animals; the weak parasympatholytic phenothiazine produced no significant difference in hypothermia when given prior to nitroprusside in the adult group.

Keyphrases □ Hypothermia, mice—sodium nitroprusside, oxotremorine induced □ Oxotremorine, sodium nitroprusside-induced hypothermia—autonomic drugs effect □ Autonomic drugs effect—oxotremorine, sodium nitroprusside-induced hypothermia □ Age of mice, effect—induced hypothermia

The mammalian body "thermostat," because of its vast complexity, is susceptible to the action of various drugs and agents, particularly those that mimic or interfere with neurotransmitter substances. Oxotremorine, the active metabolite of tremorine, was shown to produce hypothermia in rodents through a central cholinergic mechanism (1, 2).

Age has been shown to modify the effects of certain centrally acting drugs in rats (3, 4). These animals are born functionally immature with poorly developed nervous systems. Bagdon and Mann (5) demonstrated the age factor in mice with the drug chlorpromazine. The hypothermia normally seen in mature mice in response to chlorpromazine administration was reversed in immature animals to marked hyperthermia.

During routine screening procedures in this laboratory, it was discovered that sodium nitroprusside induces a pronounced fall in the body temperature of mice. It was the purpose of this investigation, therefore, to compare the hypothermia caused by oxotremorine with that induced by sodium nitroprusside with respect to alteration by several autonomic drugs in order to ascertain the mechanism of action and to observe the effects of age on the thermic response.

EXPERIMENTAL

Young (10-day-old) and adult male mice (1398) of the Huntingdon Farms (HTF) strain were utilized in this investigation.

The drugs employed were as follows: oxotremorine,[1] sodium nitroprusside,[2] atropine sulfate,[3] pilocarpine nitrate,[3] nicotine,[4] tetraethylammonium chloride,[5] and chlorpromazine hydrochloride.[6] All solutions were freshly prepared with sterile water distilled in the laboratory.

A model 43 Telethermometer equipped with a No. 402 probe[7] was used for obtaining oral and rectal temperatures. The animals were kept at a constant environmental temperature of 23–24° for 24 hr. prior to and including the time of the experimental course.

All injections were given as a fixed dose in a volume of 0.05 ml. for immature mice and 0.25 ml. for mature mice. Calculation of doses for the immature mice was based upon an average weight of 4.5 g. obtained from preliminary experiments in this laboratory. Mature male mice, weighing from 20 to 25 g., received doses calculated on the basis of 22.5 g./mouse.

The agonists with their concentration and dose were: oxotremorine (0.0045%; 0.5 mg./kg.) and sodium nitroprusside (0.045%; 5 mg./kg.).

[1] Nutritional Biochemicals Corp.
[2] Baker Chemical Co., Phillipsburg, N. J.
[3] Merck & Co., Inc., Rahway, N. J.
[4] Eastman Organic Chemicals, Rochester, N. Y.
[5] Etamon, Parke, Davis & Company, Detroit, Mich.
[6] Thorazine, Smith Kline & French Laboratories, Philadelphia, Pa.
[7] Yellow Springs Instrument Co.

REFERENCES

(1) J. Haleblian and W. McCrone, *J. Pharm. Sci.*, **58**, 911(1969).
(2) J. H. Chapman, J. E. Page, A. C. Parker, D. Rogers, C. J. Sharp, and S. E. Stainforth, *J. Pharm. Pharmacol.*, **20**, 418(1968).
(3) M. Kuhnert-Brandstätter and H. Grimm, *Microchim. Acta*, **1968**, 115.
(4) *Ibid.*, **1968**, 127.
(5) J. L. Spencer, E. H. Flynn, R. W. Roeske, F. Y. Siu, and R. R. Chauvette, *J. Med. Chem.*, **9**, 746(1966).
(6) C. W. Ryan, R. L. Simon, and E. M. Van Heyningen, *ibid.*, **12**, 310(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 25, 1970, from *Eli Lilly and Company, Indianapolis, IN 46206*

Accepted for publication June 19, 1970.

The authors thank Dale Spurgeon and Herbert Smith for extensive X-ray diffraction work.

Influence of Several Autonomic Drugs on Sodium Nitroprusside and Oxotremorine-Induced Hypothermia in Immature and Mature Mice

DAVID H. BURKE* and DAVID E. MANN, Jr.

Abstract □ Sodium nitroprusside and oxotremorine each produced body temperature depression that was independent of age. Atropine inhibited oxotremorine hypothermia in both age groups, but was ineffective in modifying thermal responses to nitroprusside in both age categories. Pilocarpine administration did not alter oxotremorine activity at either age level, while nitroprusside hypothermia was enhanced and partially reversed, respectively, in immature and mature mice. Nicotine and tetraethylammonium chloride were unable to modify hypothermia produced by oxotremorine and nitroferricyanide in adult mice. Nicotine enhanced nitroprusside hypothermia in 10-day-old mice, while temperature depression due to oxotremorine was unaffected in the same age group. Administration of tetraethylammonium chloride to immature animals treated with oxotremorine and nitroprusside resulted in greater temperature depression. Chlorpromazine, which produced no change in oxotremorine or nitroprusside hypothermia in 10-day-old mice, partially blocked oxotremorine-induced hypothermia in mature animals; the weak parasympatholytic phenothiazine produced no significant difference in hypothermia when given prior to nitroprusside in the adult group.

Keyphrases □ Hypothermia, mice—sodium nitroprusside, oxotremorine induced □ Oxotremorine, sodium nitroprusside-induced hypothermia—autonomic drugs effect □ Autonomic drugs effect—oxotremorine, sodium nitroprusside-induced hypothermia □ Age of mice, effect—induced hypothermia

The mammalian body "thermostat," because of its vast complexity, is susceptible to the action of various drugs and agents, particularly those that mimic or interfere with neurotransmitter substances. Oxotremorine, the active metabolite of tremorine, was shown to produce hypothermia in rodents through a central cholinergic mechanism (1, 2).

Age has been shown to modify the effects of certain centrally acting drugs in rats (3, 4). These animals are born functionally immature with poorly developed nervous systems. Bagdon and Mann (5) demonstrated the age factor in mice with the drug chlorpromazine. The hypothermia normally seen in mature mice in response to chlorpromazine administration was reversed in immature animals to marked hyperthermia.

During routine screening procedures in this laboratory, it was discovered that sodium nitroprusside induces a pronounced fall in the body temperature of mice. It was the purpose of this investigation, therefore, to compare the hypothermia caused by oxotremorine with that induced by sodium nitroprusside with respect to alteration by several autonomic drugs in order to ascertain the mechanism of action and to observe the effects of age on the thermic response.

EXPERIMENTAL

Young (10-day-old) and adult male mice (1398) of the Huntingdon Farms (HTF) strain were utilized in this investigation.

The drugs employed were as follows: oxotremorine,[1] sodium nitroprusside,[2] atropine sulfate,[3] pilocarpine nitrate,[3] nicotine,[4] tetraethylammonium chloride,[5] and chlorpromazine hydrochloride.[6] All solutions were freshly prepared with sterile water distilled in the laboratory.

A model 43 Telethermometer equipped with a No. 402 probe[7] was used for obtaining oral and rectal temperatures. The animals were kept at a constant environmental temperature of 23–24° for 24 hr. prior to and including the time of the experimental course.

All injections were given as a fixed dose in a volume of 0.05 ml. for immature mice and 0.25 ml. for mature mice. Calculation of doses for the immature mice was based upon an average weight of 4.5 g. obtained from preliminary experiments in this laboratory. Mature male mice, weighing from 20 to 25 g., received doses calculated on the basis of 22.5 g./mouse.

The agonists with their concentration and dose were: oxotremorine (0.0045%; 0.5 mg./kg.) and sodium nitroprusside (0.045%; 5 mg./kg.).

[1] Nutritional Biochemicals Corp.
[2] Baker Chemical Co., Phillipsburg, N. J.
[3] Merck & Co., Inc., Rahway, N. J.
[4] Eastman Organic Chemicals, Rochester, N. Y.
[5] Etamon, Parke, Davis & Company, Detroit, Mich.
[6] Thorazine, Smith Kline & French Laboratories, Philadelphia, Pa.
[7] Yellow Springs Instrument Co.

Table I—Effect of Sodium Nitroprusside or Oxotremorine (Agonists) Given 15 Min. after Saline (Antagonist) on the Body Temperature of Mature Mice

Treatment	Mice, No.	Weight, g.	Initial Temperature	Antagonist Temperature[a]	Agonist Temperature[b]	Temperature Change[c]	Compared to Saline: Drug-[d] Induced Change	Compared to Saline: t	Compared to Saline: P
Saline–saline	30	22.0	37.53°	37.77°	37.61°	−0.16°			
Saline–NTPR[e]	107	23.1	37.43°	37.91°	34.37°	−3.54°	−3.38°	26.06	0.001
Saline–OTMN[f]	114	22.5	37.85°	37.99°	34.28°	−3.71°	−3.55°	22.65	0.001

[a] Temperature 15 min. after antagonist injection. [b] Temperature 15 min. after agonist injection. [c] Antagonist–agonist temperature difference. [d] Sodium nitroprusside or oxotremorine. [e] Sodium nitroprusside. [f] Oxotremorine.

Table II—Effect of Sodium Nitroprusside or Oxotremorine (Agonists) Given 15 Min. after Saline (Antagonist) on the Body Temperature of Immature Mice

Treatment	Mice, No.	Weight, g.	Initial Temperature	Antagonist Temperature[a]	Agonist Temperature[b]	Temperature Change[c]	Compared to Saline: Drug-[d] Induced Change	Compared to Saline: t	Compared to Saline: P
Saline–saline	40	4.71	26.70°	27.57°	27.79°	+0.22°			
Saline–NTPR[e]	91	4.67	26.48°	27.33°	26.46°	−0.87°	−1.09°	9.09	0.001
Saline–OTMN[f]	103	4.85	26.74°	27.51°	26.21°	−1.30°	−1.52°	13.15	0.001

[a] Temperature 15 min. after antagonist injection. [b] Temperature 15 min. after agonist injection. [c] Antagonist–agonist temperature difference. [d] Sodium nitroprusside or oxotremorine. [e] Sodium nitroprusside. [f] Oxotremorine.

Concentrations and doses of the antagonists used in the experiment were: atropine sulfate (0.045%; 5 mg./kg.), pilocarpine nitrate (0.0009%; 0.1 mg./kg.), nicotine (0.0023%; 0.25 mg./kg.), tetraethylammonium chloride (0.0023%; 0.25 mg./kg.), and chlorpromazine hydrochloride (0.009%; 1 mg./kg.).

The animals were grouped according to age and treatment regimen. Experiments were performed at the same time each day to minimize temperature variation.

Groups of 12 animals were weighed individually on a triple-beam Ohaus balance, and their weights were recorded at the start of the experiment. Immediately following weight determination, the animals were confined singly in beakers for a 2-hr. period, during which food and water deprivation was accomplished to preserve metabolic stability. Following the 2-hr. conditioning period, oral (immature) and rectal (mature) temperatures were recorded. In the younger mice, oral temperatures were noted because their small size did not permit taking rectal temperatures without extreme discomfort to the animal. Subcutaneous administration of the antagonist occurred immediately after initial temperature recordings, whereupon the mice were returned to their containers for 15 min. At the end of this period, temperatures were again noted and the agonist was injected intraperitoneally. Fifteen minutes after the latter challenge, final temperatures were recorded. Control animals were subjected to an identical treatment procedure with the exception that either antagonist or agonist was replaced with saline.

In the comparison of mean temperature changes (*i.e.*, temperature before and after administration of agonist), statistical significance was determined by the use of Student's t distribution. Temperature differences were considered to be significant at the probability level of 5% or less. Temperature alterations attributed to antagonist or agonist were ascertained through a comparison with their saline counterparts. Antagonist effect on agonist hypothermia was determined through comparison of saline–agonist with antagonist–agonist treatments.

RESULTS AND DISCUSSION

The effects of sodium nitroprusside on the body temperature of mature male and immature (mixed sex) albino mice at a dosage level of 5 mg./kg. are outlined in Tables I and II. Temperature changes attributed to the agonist were significant for both age groups, with probability levels reaching 99.9%. In 107 mature mice, sodium nitroprusside caused an average temperature depression

Table III—Effect of Various Autonomic Drugs Given 15 Min. before Sodium Nitroprusside on the Body Temperature of Mature Mice

Treatment	Mice, No.	Weight, g.	Initial Temperature	Antagonist Temperature[b]	Agonist Temperature[c]	Temperature Change[d]	NTPR[e] Temperature Change	Compared to Table I Saline and NTPR Controls: Temperature Change	Compared to Table I Saline and NTPR Controls: t	Compared to Table I Saline and NTPR Controls: P
Atropine–saline	30	24.4	37.76°	38.10°	37.73°	−0.37°		−0.21°	2.19	0.05
							−3.45°			
Atropine–NTPR	30	22.2	37.64°	37.87°	34.05°	−3.82°		−0.28°	1.98	0.05
Pilocarpine–saline	30	21.5	37.48°	37.98°	37.80°	−0.18°		−0.02°	0.21	–[a]
							−2.92°			
Pilocarpine–NTPR	30	24.2	36.94°	37.61°	34.51°	−3.10°		+0.44°	3.05	0.01
Nicotine–saline	30	22.0	37.74°	38.17°	38.03°	−0.14°		+0.02°	0.10	–[a]
							−3.50°			
Nicotine–NTPR	30	23.3	37.71°	38.10°	34.46°	−3.64°		−0.10°	0.64	–[a]
TEA[f]–saline	30	24.0	37.45°	37.56°	37.40°	−0.16°		0.0°	0.10	–[a]
							−3.10°			
TEA–NTPR	30	23.2	38.17°	38.37°	35.11°	−3.26°		+0.28°	1.95	0.10
CPZ[g]–saline	30	23.6	37.05°	37.13°	35.69°	−1.44°		−1.28°	11.35	0.001
							−2.03°			
CPZ–NTPR	30	24.0	37.42°	37.70°	34.23°	−3.47°		+0.07°	0.53	–[a]

[a] No significance. [b] Temperature 15 min. after antagonist injection. [c] Temperature 15 min. after agonist injection. [d] Antagonist–agonist temperature difference. [e] Sodium nitroprusside. [f] Tetraethylammonium chloride. [g] Chlorpromazine HCl.

Table IV—Effect of Various Autonomic Drugs Given 15 Min. before Sodium Nitroprusside on the Body Temperature of Immature Mice

Treatment	Mice, No.	Weight, g.	Initial Temperature	Antagonist Temperature[b]	Agonist Temperature[c]	Temperature Change[d]	NTPR[e] Temperature Change	Compared to Table II Saline and NTPR Controls Temperature Change	*t*	*P*
Atropine–saline	30	4.88	26.79°	27.54°	27.44°	−0.10°		−0.32°	2.81	0.05
							−0.74°			
Atropine–NTPR	30	4.93	26.27°	27.28°	26.44°	−0.84°		+0.03°	0.18	–[a]
Pilocarpine–saline	30	4.84	26.94°	27.60°	27.70°	+0.10°		−0.12°	0.90	–[a]
							−1.35°			
Pilocarpine–NTPR	30	4.65	27.13°	28.08°	26.83°	−1.25°		−0.38°	2.73	0.05
Nicotine–saline	30	4.59	26.68°	27.70°	28.42°	+0.72°		+0.50°	5.08	0.001
							−2.50°			
Nicotine–NTPR	34	4.90	26.79°	28.32°	26.54°	−1.78°		−0.91°	6.82	0.001
TEA[f]–saline	38	4.97	26.02°	26.95°	27.64°	+0.69°		+0.47°	4.17	0.001
							−1.96°			
TEA–NTPR	30	4.74	26.62°	27.57°	26.30°	−1.27°		−0.40°	2.89	0.05
CPZ[g]–saline	30	4.71	26.12°	27.57°	27.84°	+0.27°		+0.05°	0.52	–[a]
							−1.37°			
CPZ–NTPR	27	4.97	27.16°	27.94°	26.84°	−1.10°		−0.23°	1.60	–[a]

[a] No significance. [b] Temperature 15 min. after antagonist injection. [c] Temperature 15 min. after agonist injection. [d] Antagonist–agonist temperature difference. [e] Sodium nitroprusside [f] Tetraethylammonium chloride. [g] Chlorpromazine HCl.

of 3.38°, while 91 immature animals exhibited a mean decrease in body temperature of 1.09°.

At the dosage level of 0.5 mg./kg., oxotremorine gave mean drops of 3.55 and 1.52°, respectively, in 114 mature and 103 immature albino mice. Again, these temperature changes were significantly different from the controls at the probability level of 99.9%.

A paucity of information exists in the literature with respect to age variation for oxotremorine. Bowman and Osuide (6) have shown the hypothermic action of tremorine to be independent of age in 4-day-old chicks. It has been shown in this investigation that the intraperitoneal administration of oxotremorine elicited body temperature depression for both age groups.

Subcutaneous injections of atropine sulfate (5 mg./kg.) produced a significant decrease from control values in the body temperature of mature animals (Table III). This 0.21° drop was probably mediated through the ganglionic blocking ability of the alkaloid (7). When this agent was administered prior to sodium nitroprusside, a significant increase in hypothermia (0.28°) was realized. These results indicate that the increased hypothermia was representative of positive summation (agonist plus antagonist).

Ten-day-old mice responded to the action of atropine in a manner similar to that of the mature group (Table IV). A temperature depression, which was 0.32° lower than the saline control value of +0.22°, was significant at the 95% probability level. Temperature depression brought about by the combination of atropine and sodium nitroprusside in this age group was not significantly different from saline–sodium nitroprusside treatment. The lack of positive summation for atropine and this agonist indicates that nitroprusside may block the gangliolytic action of atropine. On the other hand, atropine may produce a partial blockade of nitroprusside hypothermia.

From the results in Table III, it can be seen that pilocarpine, when injected subcutaneously at a dosage level of 0.1 mg./kg., was not significantly different from saline on the body temperature of the older animals. In mature mice, this cholinomimetic agent was able to reverse partially the hypothermic action of sodium nitroprusside at the probability level of 99% (Table III). It is possible that this antagonism was carried out through the capacity of pilocarpine to stimulate sympathetic ganglia and to release catecholamines from the adrenal medulla (8).

As observed in mature animals, oral temperature changes in the pilocarpine-treated juveniles were not different from the saline-treated control group, yet this diaphoretic agent was able to augment the hypothermic action of sodium nitroprusside (Table IV). It is, therefore, concluded from the results of this investigation that the effect of pilocarpine on sodium nitroprusside hypothermia varies according to age; slight antagonism occurs in the mature group, while the immature animals show hypothermic enhancement.

In adult mice treated with nicotine (0.25 mg./kg.), no significant effect was noted. As shown in Table III, nicotine was not able to modify hypothermia produced by sodium nitroprusside.

Table V—Effect of Various Autonomic Drugs Given 15 Min. before Oxotremorine on the Body Temperature of Mature Mice

Treatment	Mice, No.	Weight, g.	Initial Temperature	Antagonist Temperature[b]	Agonist Temperature[c]	Temperature Change[d]	OTMN[e] Temperature Change	Compared to Table I Saline and OTMN Controls Temperature Change	*t*	*P*
Atropine–saline	30	24.4	37.76°	38.10°	37.73°	−0.37°		−0.21°	2.19	0.05
							−0.20°			
Atropine–OTMN	30	24.5	37.02°	37.13°	36.56°	−0.57°		+3.14°	18.04	0.001
Pilocarpine–saline	30	21.5	37.48°	37.98°	37.80°	−0.18°		−0.02°	0.21	–[a]
							−3.86°			
Pilocarpine–OTMN	30	22.3	37.94°	37.97°	33.93°	−4.04°		−0.33°	1.86	0.1
Nicotine–saline	30	22.0	37.74°	38.17°	38.03°	−0.14°		+0.02°	0.10	–[a]
							−3.41°			
Nicotine–OTMN	30	23.9	38.14°	38.45°	34.90°	−3.55°		+0.16°	0.95	–[a]
TEA[f]–saline	30	24.0	37.45°	37.56°	37.40°	−0.16°		0.0°	0.10	–[a]
							−3.55°			
TEA–OTMN	30	23.1	38.18°	38.49°	34.78°	−3.71°		0.0°	0.04	–[a]
CPZ[g]–saline	30	23.6	37.05°	37.13°	35.69°	−1.44°		−1.28°	11.35	0.001
							−1.60°			
CPZ–OTMN	30	22.4	37.35°	37.51°	34.47°	−3.04°		+0.67°	3.94	0.001

[a] No significance. [b] Temperature 15 min. after antagonist injection. [c] Temperature 15 min. after agonist injection. [d] Antagonist–agonist temperature difference. [e] Oxotremorine. [f] Tetraethylammonium chloride. [g] Chlorpromazine HCl.

Table VI—Effect of Various Autonomic Drugs Given 15 Min. before Oxotremorine on the Body Temperature of Immature Mice

Treatment	Mice, No.	Weight, g.	Initial Temperature	Antagonist Temperature[b]	Agonist Temperature[c]	Temperature Change[d]	OTMN[e] Temperature Change	Compared to Table II Saline and OTMN Controls Temperature Change	*t*	*P*
Atropine–saline	30	4.88	26.79°	27.54°	27.44°	−0.10°		−0.32°	2.81	0.05
							+0.17°			
Atropine–OTMN	38	5.09	27.46°	27.94°	28.01°	+0.07°		+1.37°	10.54	0.001
Pilocarpine–saline	30	4.84	26.94°	27.60°	27.70°	+0.10°		−0.12°	0.90	–[a]
							−1.62°			
Pilocarpine–OTMN	30	4.70	26.59°	27.65°	26.13°	−1.52°		−0.22°	1.74	0.1
Nicotine–saline	30	4.59	26.68°	27.70°	28.42°	+0.72°		+0.50°	5.08	0.001
							−2.17°			
Nicotine–OTMN	30	4.90	25.35°	27.03°	25.58°	−1.45°		−0.15°	1.16	–[a]
TEA[f]–saline	38	4.97	26.02°	26.95°	27.64°	+0.69°		+0.47°	4.17	0.001
							−2.25°			
TEA–OTMN	30	4.84	26.67°	27.48°	25.92°	−1.56°		−0.26°	2.04	0.05
CPZ[g]–saline	30	4.71	26.12°	27.57°	27.84°	+0.27°		+0.05°	0.52	–[a]
							−1.44°			
CPZ–OTMN	30	4.95	26.46°	27.73°	26.56°	−1.17°		+0.13°	0.95	–[a]

[a] No significance. [b] Temperature 15 min. after antagonist injection. [c] Temperature 15 min. after agonist injection. [d] Antagonist–agonist temperature difference. [e] Oxotremorine. [f] Tetraethylammonium chloride. [g] Chlorpromazine HCl.

In Table IV, nicotine has been shown to produce a rise in the body temperature of 10-day-old mice at the dosage level of 0.25 mg./kg. In 30 animals treated with this compound, a mean elevation of 0.5° was observed, which was significant at the 99.9% probability level. When administered 15 min. before sodium nitroprusside, this agent approximately doubled agonist-induced temperature depression. The possibility exists that the agonist drug may have inhibited nicotine pyresis in these animals. It is proposed that the immature mammalian thermoregulatory system modifies the effect of nicotine on sodium nitroprusside temperature reduction.

Tetraethylammonium chloride administration, at a dosage level of 0.25 mg./kg., resulted in no alteration of the thermoregulatory ability of adult mice. When administered prior to sodium nitroprusside, a very slight temperature inhibition of borderline significance was observed. In young animals of this species, tetraethylammonium chloride produced a significant rise in body temperature, amounting to 0.47°. As observed with nicotine, this ganglionic blocking agent further enhanced the temperature depression of sodium nitroprusside; again, the possibility exists that the agonist drug may have inhibited the hyperthermia caused by tetraethylammonium chloride.

It is probable that nicotine and tetraethylammonium chloride act in the same fashion because they both produced qualitatively similar responses when administered alone or prior to sodium nitroprusside. It was reported that these two compounds have in common an ability to stimulate the release of epinephrine from the adrenal medulla (9). It is possible that these agents may produce hyperthermia in young animals through this mechanism and that the administration of sodium nitroprusside inhibits this response. Potentiation of nitroprusside hypothermia may result from inhibition of sympathetic ganglia in the presence of a poorly developed thermoregulatory system.

Chlorpromazine hydrochloride has been shown to achieve a temperature deficit of 1.28° in the mature age group. These results agree with those of Kopera and Armitage (10), who demonstrated the ability of chlorpromazine to cause hypothermia in mice. From the results given in Table III, it can be seen that either nitroprusside blocked the temperature effects due to chlorpromazine or the latter agent caused a partial reversal of nitroprusside hypothermia. Chlorpromazine did not alter the body temperature in young animals and failed to alter the action of nitroprusside.

Tables V and VI show that the effect of atropine sulfate on oxotremorine temperature depression is similar for both age groups. In both cases, a complete inhibition of oxotremorine hypothermia occurred and was significant at the probability level of 99.9%. Lomax and Jenden (11) blocked the hypothermic response of oxotremorine in rats with atropine. The observations of these investigators have been confirmed by this study.

The administration of pilocarpine nitrate (0.1 mg./kg.) 15 min. prior to oxotremorine caused a very slight enhancement of agonist hypothermia in both age groups; it was of borderline significance in each case (Tables V and VI). Therefore, the effect of age was not demonstrated for pilocarpine modification of oxotremorine hypothermia.

Oxotremorine-induced temperature drops were not modified by the prior administration of nicotine in mature mice. The immature animals showed no significant change in agonist hypothermic response when treated with oxotremorine in combination with nicotine. Because nicotine caused hyperthermia in immature mice (Table VI), it is possible that oxotremorine inhibited this rise in temperature. Conversely, nicotine could have enhanced oxotremorine hypothermia (negative summation).

Age has been shown to modify effects of tetraethylammonium chloride on oxotremorine-induced temperature depression. In the older group of mice, the administration of tetraethylammonium chloride did not affect the hypothermia caused by oxotremorine. Everett *et al.* (12) demonstrated that this antagonist was ineffective in blocking the temperature-lowering effect of tremorine in mice. These results lend support to their claim that the hypothermic action of tremorine is central in origin. A slight increase in the agonist hypothermic response was noted in immature mice after the administration of tetraethylammonium chloride (Table VI). A significant difference was present at the probability level of 95%. Oxotremorine may also inhibit the thermogenic effect of tetraethylammonium chloride in this age group.

A partial blockade of oxotremorine activity by chlorpromazine was observed in the mature group, which was probably caused by the weak parasympatholytic effect of the phenothiazine (13, 14). Conversely, it is also possible that oxotremorine may have interfered with the hypothermia elicited by chlorpromazine. Preadministration of chlorpromazine in the younger animals produced no change in the hypothermia due to oxotremorine. The effect of chlorpromazine on oxotremorine-induced hypothermia has thus been shown to be modified by age.

Although the means by which sodium nitroprusside causes a fall in body temperature has not been elucidated as a result of this study, a comparison of its activity with that of the centrally acting oxotremorine in the presence of various autonomic drugs in mature mice has provided strong evidence for a different mechanism of action. While the administration of atropine resulted in a complete blockage of oxotremorine-induced depression of body temperature, no such inhibition was observed with sodium nitroprusside following the parasympatholytic agent. On the other hand, pilocarpine failed to modify oxotremorine hypothermia, whereas this parasympathomimetic partially inhibited nitroprusside hypothermia.

SUMMARY AND CONCLUSIONS

1. Sodium nitroprusside and oxotremorine each produced a hypothermic response that was independent of age in the mouse.

2. The effect of atropine on oxotremorine-induced hypothermia appeared to be uninfluenced by age. In both cases, a complete inhibition of agonist hypothermia occurred.

3. The prior administration of atropine enhanced sodium nitroprusside-induced hypothermia, indicating the existence of positive summation in the mature group. This antagonist was unable to alter nitroprusside hypothermia in immature animals. Nitroprusside may block atropine-induced changes.

4. Pilocarpine effects on oxotremorine-induced hypothermia were unaffected by age.

5. The action of pilocarpine on sodium nitroprusside-induced hypothermia was age related, because slight antagonism occurred in the mature group while immature animals showed hypothermic enhancement.

6. The influence of nicotine on sodium nitroprusside-induced hypothermia was affected by age. Agonist hypothermia was not modified in the mature group; hypothermia was enhanced in the immature group. Nicotine pyresis may be antagonized in the younger group.

7. Oxotremorine temperature depression was not modified by nicotine in mature mice. In the immature animals, this agonist may have inhibited nicotine hyperthermia; conversely, nicotine may have enhanced oxotremorine hypothermia (negative summation).

8. Tetraethylammonium chloride produced effects on sodium nitroprusside hypothermia which were qualitatively similar to those of nicotine. It has been suggested that these antagonistic drugs act in a comparable manner.

9. Age was shown to modify the effect of tetraethylammonium chloride on oxotremorine-induced temperature depression. Agonist hypothermia was not affected by tetraethylammonium chloride in the mature group. Slight enhancement was shown in the immature animals. Oxotremorine may have inhibited tetraethylammonium chloride hyperthermia.

10. Sodium nitroprusside-induced hypothermia was not affected by chlorpromazine in the immature age group. In the mature animals, either nitroprusside blocked chlorpromazine hypothermia or the latter agent partially reversed nitroprusside hypothermia.

11. Chlorpromazine demonstrated no effect on oxotremorine-induced hypothermia in the immature animals. Partial antagonism was noted in the mature group. Oxotremorine may have inhibited chlorpromazine-induced hypothermia.

12. Evidence has been provided that oxotremorine and sodium nitroprusside each produce hypothermia in mature mice *via* a different mechanism. The parasympathomimetic agent, pilocarpine, did not alter oxotremorine-induced hypothermia, while this drug partially inhibited that of sodium nitroprusside. Conversely, atropine inhibited hypothermia attributed to oxotremorine but failed to modify that of nitroprusside.

REFERENCES

(1) P. Spencer, *Life Sci.*, **5**, 1015(1966).

(2) W. E. Kirpatrick, *Proc. West. Pharmacol. Soc.*, **10**, 51(1967).

(3) T. S. Watts, J. Overpeck, J. Reilly, and S. Krop, *Toxicol. Appl. Pharmacol.*, **9**, 463(1966).

(4) S. M. Wolf, R. L. Simmons, and W. L. Nastuk, *Proc. Soc. Exp. Biol. Med.*, **117**, 1(1964).

(5) W. J. Bagdon and D. E. Mann, Jr., *J. Pharm. Sci.*, **51**, 753 (1962).

(6) W. C. Bowman and G. Osuide, *Eur. J. Pharmacol.*, **1**, 71 (1967).

(7) M. L. Tainter and W. C. Cutting, *J. Pharmacol. Exp. Ther.*, **48**, 410(1933).

(8) L. S. Goodman and P. Gilman, "The Pharmacological Basis of Therapeutics," 3rd ed., Macmillan, New York, N. Y., 1966, p. 474.

(9) T. Sollmann, "A Manual of Pharmacology," 8th ed., W. B. Saunders, Philadelphia, Pa., 1957, p. 455.

(10) J. Kopera and A. K. Armitage, *Brit. J. Pharmacol.*, **9**, 392(1954).

(11) P. Lomax and D. J. Jenden, *Int. J. Neuropharmacol.*, **5**, 353(1966).

(12) G. M. Everett, L. E. Blockus, and I. M. Sheppard, *Science*, **124**, 79(1956).

(13) F. Huidobro, *Arch. Int. Pharmacodyn. Ther.*, **98**, 308(1954).

(14) T. J. Haley, A. M. Flesher, and K. Raymond, *ibid.*, **124**, 455(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *School of Pharmacy, Temple University, Philadelphia, PA 19104*

Accepted for publication July 14, 1970.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

Abstracted from a thesis submitted by David H. Burke to the Graduate School, Temple University, Philadelphia, Pa., in partial fulfillment of Master of Science degree requirements.

The authors express their thanks to Dr. W. J. Bagdon, Dr. R. F. Gautieri, and Dr. R. H. Golder for their suggestions and discussions and to Mr. J. C. Tatnall for his technical assistance.

* National Defense Education Act Fellow.

Potentiometric Titration of Allantoin in Cream Formulations

D. J. WEBER* and J. W. HIGGINS†

Abstract □ An analytical procedure is described which uses a rapid partition chromatographic separation of intact allantoin from cream formulations. The allantoin content is measured by potentiometric titration in a mixed solvent system. Accuracy and precision data are presented, and good results are obtained with commercial preparations.

Keyphrases □ Allantoin cream formulation—analysis □ Column chromatography—separation □ Potentiometric titration—analysis

The current methods of analysis of allantoin involve hydrolysis and quantification of the resulting glyoxylic acid (1–3) or urea (3–5). These procedures are deficient for the analysis of residual allantoin, since they measure urea or glyoxylic acid which are the products of normal allantoin degradation (6). Using these methods of analysis after preliminary isolation of allantoin from its degradation products requires a difficult separation step. Other procedures use paper chromatography (7) or paper electrophoresis (5, 7), which are rather slow and have been applied only to urine samples. A TLC separation and UV assay of allantoin at 220 mμ was reported for creams and lotions (8), but data on samples were not included and the molar absorptivity is only about 1200.

This report describes an assay for allantoin in cream formulations based on a partition chromatographic separation of allantoin from interfering substances and potentiometric titration of the allantoin with alkali. Those substances, which are eluted with the allantoin, are either nontitratable or have acid-dissociation constants sufficiently different from allantoin to permit its analysis.

EXPERIMENTAL

Apparatus—The following were used: recording pH meter[1] equipped with a glass-reference combination electrode[2] and a magnetic stirrer;[3] a motorized, constant-speed syringe buret[4] to deliver the titrant; and a glass chromatographic column, 30 × 2 cm.

Reagents—The following were used: sodium hydroxide, 0.1 *N* solution standardized potentiometrically against primary standard potassium biphthalate; acetone, A.R.; acid-washed diatomaceous earth;[5] ethyl acetate, A.R.; ethyl ether, A.R.; glass wool; and *p*-dioxane.[6] Allantoin[7] was used as received.

Preparation of Solvent Mixtures—Ethyl acetate (200 ml.) and water (20 ml.) were combined in a separator and shaken to equilibrate. The layers were allowed to separate. The water phase (lower) serves as the stationary phase in the column and for sample preparation. The ethyl acetate layer serves as the organic mobile phase for column elution.

Ether saturated with water was prepared by adding 25 ml. of water to 100 ml. of ethyl ether and separating after equilibration. The aqueous phase was discarded.

Column Preparation—To 4 g. of acid-washed diatomaceous earth, 3.5 ml. of stationary phase is added and mixed thoroughly. This material is added in portions to a 2-cm. i.d. chromatography column containing a pledget of glass wool at the bottom. The column is tapped on a wooden block after each addition of support until it is compact. The column is then tamped semifirmly with a column-packing rod to a length of 5.6 cm. The column is washed with 50 ml. of water-saturated ethyl acetate.

Preparation of Artificial Sample—A placebo batch of Cream E, containing all ingredients except allantoin, was prepared. Allantoin was added to samples of this placebo cream, lightly heated, mixed to give a homogeneous mix, and then rapidly cooled. The samples were put into beakers, sealed with Parafilm to avoid evaporation, and stored at room temperature for 4 days before being analyzed.

Procedure—Accurately weigh a quantity of sample equivalent to about 25 mg. of allantoin into a 50-ml. beaker, and add 1.5 g. of acid-washed diatomaceous earth. Mix thoroughly with a spatula and then add 0.5 ml. of stationary phase and mix thoroughly again. Add the sample preparation to the prepared column and tamp semifirmly. Place a pledget of cotton on top of the sample, and elute the column with 100 ml. of mobile phase. Allow the ethyl acetate mobile phase to run just below the surface of the diatomaceous earth, add 5 ml. of ethyl ether (water saturated), and again allow this to run just below the surface. Add an additional 25 ml. of ethyl ether (water saturated) and allow the column to run dry. Discard all solvent eluted. Strip the allantoin from the column by eluting with 40 ml. of deionized water. Only the water is collected into a 150-ml. beaker. The boundary between the residual ethyl ether on the column and the water eluate containing the allantoin is easily determined by the initially cloudy nature of the water eluate.

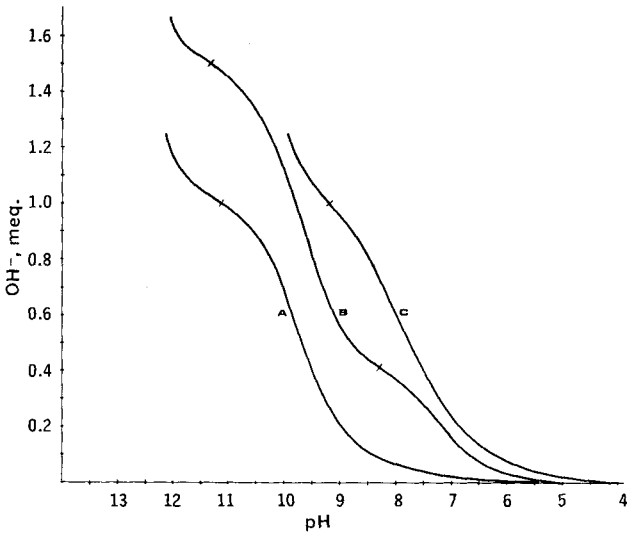


Figure 1—*Potentiometric titration curves of allantoin in 66% acetone–water (A); allantoin + triethanolammonium ion in 66% acetone–water (B); and allantoin in water (C).*

[1] Heath Recording.
[2] Sargent-Welsh.
[3] Sargent-Welsh.
[4] Micro-Metric Instrument Co., Model MIP-2.
[5] Celite 545.
[6] Matheson Coleman and Bell.
[7] Aceto Chemical Co.

Table I—Solvent Effect of the pKa′ of Allantoin, Triethanolamine, and Glyoxylic Acid

Substance	pKa′ H_2O	Dioxane–H_2O 1:2	Dioxane–H_2O 2:1	Acetone–H_2O 1:2	Acetone–H_2O 2:1
Allantoin	8.01	9.13	10.38	8.75	9.91
Triethanolamine	7.66	7.52	7.33	7.25	7.10
Glyoxylic acid	3.50	Reaction	Reaction		4.98

Collect all eluent until the column runs dry. Add 70 ml. of acetone to the 30–35 ml. of eluate collected and potentiometrically titrate the sample with standard 0.1 *N* NaOH to an apparent pH of 11.5–12.0. Usually, two inflections are observed, one near pH 8 and the other near pH 11. Calculate the milligrams of allantoin per gram of sample by substituting the observed values into the following equation:

$$\frac{V \times N \times 158.12}{\text{(sample wt., g.)}} = \text{mg. allantoin/g. sample} \qquad \text{(Eq. 1)}$$

where V is the milliliters of titrant consumed between the two inflections, N is the normality of the titrant, and 158.12 is the equivalent weight of allantoin.

DISCUSSION

Potentiometric Titration of Allantoin—The potentiometric titration curves of allantoin in water and 66% acetone–water are shown in Fig. 1. The equivalence points, although short, are distinct and analytically useful. Many cream samples were found to contain a basic emulsifying agent such as triethanolamine, which was eluted from the column with the allantoin and interfered with its titration in a purely aqueous solvent system. The addition of acetone or dioxane to the aqueous solution caused a large increase in the pKa′ of allantoin and a smaller decrease in the pKa′ of the amine. These shifts are due to the combined effects of a lower solvent dielectric constant and changes in the solvation sphere of the molecules (9). The effects of different solvents on the pKa′ of allantoin, triethanolamine, and glyoxylic acid are given in Table I. Dioxane has a lower dielectric constant than acetone and, therefore, produced a greater separation in pKa′ of allantoin and triethanolamine than was found for acetone. The titration curve of allantoin plus triethanolammonium ion in 66% acetone–water is given in Fig. 1. Glyoxylic acid, a natural degradation product of allantoin, reacted very rapidly with a substance in the *p*-dioxane to give a product which interfered with the titration of allantoin. This problem was avoided by using acetone as the solvent. Dioxane, besides containing material reactive to glyoxylic acid, also contains small amounts (9.6×10^{-4} meq./ml.) of an acid substance which also interferes with the titration of allantoin.

Partition Column Separation of Allantoin—The partition coefficient of allantoin estimated from the ratio of its solubility in ethyl acetate to its solubility in water is 3×10^{-3}. Therefore, in an aqueous stationary phase the allantoin is essentially immobile and is easily separated from nearly all excipients after sufficient ethyl acetate mobile phase has been passed through the column. Those excipients that are not separated are either not titratable or have a sufficiently different pKa′ to allow differentiation using a mixed solvent system.

Table II—Elution of Allantoin[a] from Partition Column with Water

Fraction	Total Milliliters Eluted	Allantoin Observed in Fraction, meq.
1	4	0.0400
2	8	0.0995
3	12	0.0529
4	16	0.0231
5	20	0.0074
6	24	Negligible
		Sum 0.2229[b]

[a] The sample analyzed was Product E. [b] The sum represents 101.18% of theory.

Table III—Accuracy Data for the Assay of Allantoin in Creams[a]

Sample	Placebo Weight, g.	Allantoin Added, mg.	Theoretical Allantoin, meq.	Observed Allantoin, meq.	Theory, %[b]
1	1.5253	28.20	0.1783	0.1792	100.50
2	1.4829	30.25	0.1912	0.1897	99.21
3	1.5842	29.82	0.1885	0.1885	100.00
4	1.4417	32.50	0.2055	0.2001	97.37
5	1.4045	29.46	0.1850	0.1862	100.64
6	1.3810	36.20	0.2289	0.2280	99.60
7	1.6279	31.14	0.1969	0.1941	98.57
8	1.6254	30.13	0.1905	0.1908	100.15

[a] Samples were prepared by the addition of allantoin to a placebo cream. [b] The mean percent recovery is 99.51%, and the 95% confidence interval is ±1.07%.

The apparent partition coefficient on a chromatographic column has been shown (10) to be a function of the stationary phase volume. The present assay uses a column with a ratio of milliliters of stationary phase to grams of support of 0.875. To maintain equilibrium conditions and produce a satisfactory elution pattern, it was found useful to add water to the sample preparation sufficient to give a ratio of aqueous phase to grams of support close to 0.875. This produced a satisfactory elution pattern (Table II) Excellent analytical results were obtained with flow rates as high as 5 ml./min.

The function of the ethyl ether wash is to remove residual ethyl acetate from the column. Any ethyl acetate present during the titration of allantoin interferes due to its rapid hydrolysis. This rapid hydrolysis was indicated by the fact that when the titration was stopped at high pH values (greater than 8), the pH began to drift toward lower values.

Accuracy and Precision—Accuracy data for the assay of allantoin added to placebo samples of Cream E are given in Table III. The data indicate that both the accuracy and precision of the analysis are excellent. The results of repeated analysis of a commercial sample of Cream E are given in Table IV. The 95% confidence interval is nearly the same as was calculated for the data of Table III. This suggests that the samples prepared for the accuracy study are truly representative of commercial creams and that the data in Table III are valid estimates of the accuracy of the assay.

The results of the analysis of a variety of commercial creams are given in Table V. The assay gave acceptable results in every case, and repeat assays of Products A and D showed good precision.

It has been shown (6) that under conditions of temperature and pH that would be expected in cream formulations, the products of allantoin hydrolysis are allantoic acid and then urea and glyoxylic acid. Potentiometric titration of mixtures of allantoin, glyoxylic acid, and triethanolammonium chloride and allantoin plus allantoic acid in 66% acetone–water gave allantoin recoveries of 100.3 and 100.0%, respectively. These results demonstrate that no interference is caused by these compounds.

Alkaline hydrolysis of allantoin (1.5×10^{-3} *M* NaOH at 80° for 30 min.), acidification, and then analysis showed a loss of allantoin of 10.5%. If ammonium ion had been produced, the recovery of allantoin would have been greater than 100% since urea, an initial hydrolysis product of allantoin, gives two molecules of ammonium ion when it hydrolyzes. The pKa′ of the allantoin from the hydrolyzed sample was 9.95, in excellent agreement with the value 9.91 previously determined.

Table IV—Precision Data for Assay of Allantoin

Sample[a]	Sample Weight, g.	Observed Concentration, mg./g.	Formula Strength, %[b]
1	1.2765	20.28	96.57
2	1.4072	20.53	98.10
3	1.4026	20.42	97.24
4	1.3113	19.97	95.10
5	1.5625	20.28	96.57
6	1.2270	20.54	98.10

[a] All samples are from a single tube of Cream E. [b] The mean percent formula strength is 96.9% and the 95% confidence interval is ±1.18%.

Table V—Assay Results for Allantoin in Commercial Creams

Product	Sample Weight, g.	Weight Allantoin/g. Cream, mg.	Label Strength, %
A	1.1495	18.6	93.0
A	1.0523	18.4	92.0
B	1.0670	20.9	104.5
C	0.9141	20.2	101.0
D	3.2041	2.44	97.6
D	3.3492	2.47	98.8
E	1.2765	20.28	96.57

Chromatography and potentiometric titration of a mixture of allantoin and ammonium chloride showed that the ammonium ion is eluted with the allantoin and interferes with the quantification of allantoin in the titration. However, since no significant production of ammonia from allantoin in creams occurs, this causes no problem in the assay. Precipitation of ammonium ion from equimolar allantoin–ammonium-ion mixtures using sodium tetraphenyl borate was 96% effective in removing the ammonium-ion interference and resulted in 103% apparent recovery of allantoin. Smaller amounts of ammonium ion gave correspondingly smaller interferences.

REFERENCES

(1) G. Young and C. Conway, *J. Biol. Chem.*, **142,** 839(1942).

(2) S. A. Katz, R. T. Turse, and S. B. Mecca, *J. Soc. Cos. Chem.*, **15,** 303(1964).

(3) R. Crokaert, *Bull. Soc. Chim. Bio.*, **41,** 1001(1959).

(4) G. Siest, *Bull. Soc. Pharm. Nancy*, **64,** 55(1965).

(5) R. Zimmermann, *Naturwissenschaften*, **43,** 399(1956).

(6) G. D. Vogels, F. E. De Windt, and W. Bassie, *Recueil*, **88,** 940(1969).

(7) J. Wagner and E. Franzen, *Arch. Tierenahrung*, **9,** 11(1959).

(8) I. Bonadeo and G. Bottezzi, *Ital. Essenzy Profumi*, **50,** 78 (1968).

(9) H. S. Harned and B. B. Owen, "Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold, New York, N. Y., 1958, p. 683.

(10) A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **35,** 1358 (1941).

(11) "The Merck Index," 8th ed., Merck and Co., Rahway, N. J., 1968, p. 33.

(12) "Handbook of Chemistry and Physics," 45th ed., The Chemical Rubber Co., Cleveland, Ohio, 1964, p. D77.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Applications Research Laboratory, Quality Control Division, Syntex Laboratories, Palo Alto, CA 94304*

Accepted for publication June 23, 1970.

Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

The motorized, constant-speed syringe buret was constructed by Mr. J. W. Higgins.

* Present address: Department of Physical and Analytical Chemistry, The Upjohn Co., Kalamazoo, Mich. To whom requests for reprints should be directed.

† Present address: 16405 Marine View Dr., S. W., Seattle, WA 98166

NMR Analysis of Mestranol Bulk Drug

HAJRO W. AVDOVICH, MARTHA BOWRON, and BRUCE A. LODGE

Abstract □ A double assay procedure for mestranol is described. The method is based upon measurement of the NMR spectrum of mestranol in pyridine, using diphenylacetic acid as an internal standard. The signals chosen are those from the methoxyl and ethinyl groups. Three commercial lots of the steroid were studied, and a TLC study of each lot is described. The impurities are tentatively identified.

Keyphrases □ Mestranol bulk drug—analysis □ TLC—separation □ Potentiometric titration—analysis □ NMR spectroscopy—analysis

The synthetic oral estrogen mestranol (17α-ethinyl-3-methoxyestra-1,3,5(10)-trien-17β-ol) is now in widespread use, chiefly as a component of oral contraceptive preparations. At present, the only official assay (1) for the raw material is a potentiometric titration. Other methods reported in the literature are colorimetric (2–4), UV (5–7), GLC (4, 6, 8, 9), TLC (7), and fluorometric (4, 10–12).

Mestranol lends itself very well to quantitative analysis by means of NMR spectroscopy, since the signals from the protons of both the ethinyl and methoxyl groups are single sharp peaks. With a suitable choice of solvent, these peaks appear in a region of the spectrum that is unaffected by signals from other protons.

The effect of solvents on the chemical shift of acetylenic protons is well documented (13). In particular, addition of pyridine to a dilute solution of a monosubstituted acetylene in carbon tetrachloride can result in deshielding of up to 1 p.p.m. of the acetylenic proton (14). Such significant deshielding is due to the fact that acetylenic compounds can form weak hydrogen bonds with molecules containing electronegative centers, such as acetone, acetonitrile, and pyridine (14–16).

Since the impurities present in mestranol bulk drug might interfere with either the signal from the ethinyl group or that from the methoxyl group, a study of the assayed samples was made by means of TLC.

EXPERIMENTAL

Spectra were obtained at 60 Mc.p.s., using a Varian A-60A analytical NMR spectrometer. A sweep time of 50 sec. for a chart width of 500 c.p.s. was used for all integrals. A r.f. power of 0.25 mG. (nominal dial setting) gave the maximum integral amplitude (17) and was used for the integrations. Tetramethylsilane in chloroform was used as an external reference to measure chemical shifts.

Assay Procedure—*NMR*—Approximately 200 mg. of mestranol and 150 mg. of pure diphenylacetic acid (DPAA) were accurately weighed and dissolved in the minimum amount of pure pyridine (approximately 0.5 ml.). The NMR spectrum was obtained in the usual manner and integrated five times in each direction through the region of interest.

Potentiometric Titration—Approximately 200 mg. of mestranol,

Table V—Assay Results for Allantoin in Commercial Creams

Product	Sample Weight, g.	Weight Allantoin/g. Cream, mg.	Label Strength, %
A	1.1495	18.6	93.0
A	1.0523	18.4	92.0
B	1.0670	20.9	104.5
C	0.9141	20.2	101.0
D	3.2041	2.44	97.6
D	3.3492	2.47	98.8
E	1.2765	20.28	96.57

Chromatography and potentiometric titration of a mixture of allantoin and ammonium chloride showed that the ammonium ion is eluted with the allantoin and interferes with the quantification of allantoin in the titration. However, since no significant production of ammonia from allantoin in creams occurs, this causes no problem in the assay. Precipitation of ammonium ion from equimolar allantoin–ammonium-ion mixtures using sodium tetraphenyl borate was 96% effective in removing the ammonium-ion interference and resulted in 103% apparent recovery of allantoin. Smaller amounts of ammonium ion gave correspondingly smaller interferences.

REFERENCES

(1) G. Young and C. Conway, *J. Biol. Chem.*, **142,** 839(1942).

(2) S. A. Katz, R. T. Turse, and S. B. Mecca, *J. Soc. Cos. Chem.*, **15,** 303(1964).

(3) R. Crokaert, *Bull. Soc. Chim. Bio.*, **41,** 1001(1959).

(4) G. Siest, *Bull. Soc. Pharm. Nancy*, **64,** 55(1965).

(5) R. Zimmermann, *Naturwissenschaften*, **43,** 399(1956).

(6) G. D. Vogels, F. E. De Windt, and W. Bassie, *Recueil*, **88,** 940(1969).

(7) J. Wagner and E. Franzen, *Arch. Tierenahrung*, **9,** 11(1959).

(8) I. Bonadeo and G. Bottezzi, *Ital. Essenzy Profumi*, **50,** 78 (1968).

(9) H. S. Harned and B. B. Owen, "Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold, New York, N. Y., 1958, p. 683.

(10) A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **35,** 1358 (1941).

(11) "The Merck Index," 8th ed., Merck and Co., Rahway, N. J., 1968, p. 33.

(12) "Handbook of Chemistry and Physics," 45th ed., The Chemical Rubber Co., Cleveland, Ohio, 1964, p. D77.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Applications Research Laboratory, Quality Control Division, Syntex Laboratories, Palo Alto, CA 94304*

Accepted for publication June 23, 1970.

Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

The motorized, constant-speed syringe buret was constructed by Mr. J. W. Higgins.

* Present address: Department of Physical and Analytical Chemistry, The Upjohn Co., Kalamazoo, Mich. To whom requests for reprints should be directed.

† Present address: 16405 Marine View Dr., S. W., Seattle, WA 98166

NMR Analysis of Mestranol Bulk Drug

HAJRO W. AVDOVICH, MARTHA BOWRON, and BRUCE A. LODGE

Abstract □ A double assay procedure for mestranol is described. The method is based upon measurement of the NMR spectrum of mestranol in pyridine, using diphenylacetic acid as an internal standard. The signals chosen are those from the methoxyl and ethinyl groups. Three commercial lots of the steroid were studied, and a TLC study of each lot is described. The impurities are tentatively identified.

Keyphrases □ Mestranol bulk drug—analysis □ TLC—separation □ Potentiometric titration—analysis □ NMR spectroscopy—analysis

The synthetic oral estrogen mestranol (17α-ethinyl-3-methoxyestra-1,3,5(10)-trien-17β-ol) is now in widespread use, chiefly as a component of oral contraceptive preparations. At present, the only official assay (1) for the raw material is a potentiometric titration. Other methods reported in the literature are colorimetric (2–4), UV (5–7), GLC (4, 6, 8, 9), TLC (7), and fluorometric (4, 10–12).

Mestranol lends itself very well to quantitative analysis by means of NMR spectroscopy, since the signals from the protons of both the ethinyl and methoxyl groups are single sharp peaks. With a suitable choice of solvent, these peaks appear in a region of the spectrum that is unaffected by signals from other protons.

The effect of solvents on the chemical shift of acetylenic protons is well documented (13). In particular, addition of pyridine to a dilute solution of a monosubstituted acetylene in carbon tetrachloride can result in deshielding of up to 1 p.p.m. of the acetylenic proton (14). Such significant deshielding is due to the fact that acetylenic compounds can form weak hydrogen bonds with molecules containing electronegative centers, such as acetone, acetonitrile, and pyridine (14–16).

Since the impurities present in mestranol bulk drug might interfere with either the signal from the ethinyl group or that from the methoxyl group, a study of the assayed samples was made by means of TLC.

EXPERIMENTAL

Spectra were obtained at 60 Mc.p.s., using a Varian A-60A analytical NMR spectrometer. A sweep time of 50 sec. for a chart width of 500 c.p.s. was used for all integrals. A r.f. power of 0.25 mG. (nominal dial setting) gave the maximum integral amplitude (17) and was used for the integrations. Tetramethylsilane in chloroform was used as an external reference to measure chemical shifts.

Assay Procedure—*NMR*—Approximately 200 mg. of mestranol and 150 mg. of pure diphenylacetic acid (DPAA) were accurately weighed and dissolved in the minimum amount of pure pyridine (approximately 0.5 ml.). The NMR spectrum was obtained in the usual manner and integrated five times in each direction through the region of interest.

Potentiometric Titration—Approximately 200 mg. of mestranol,

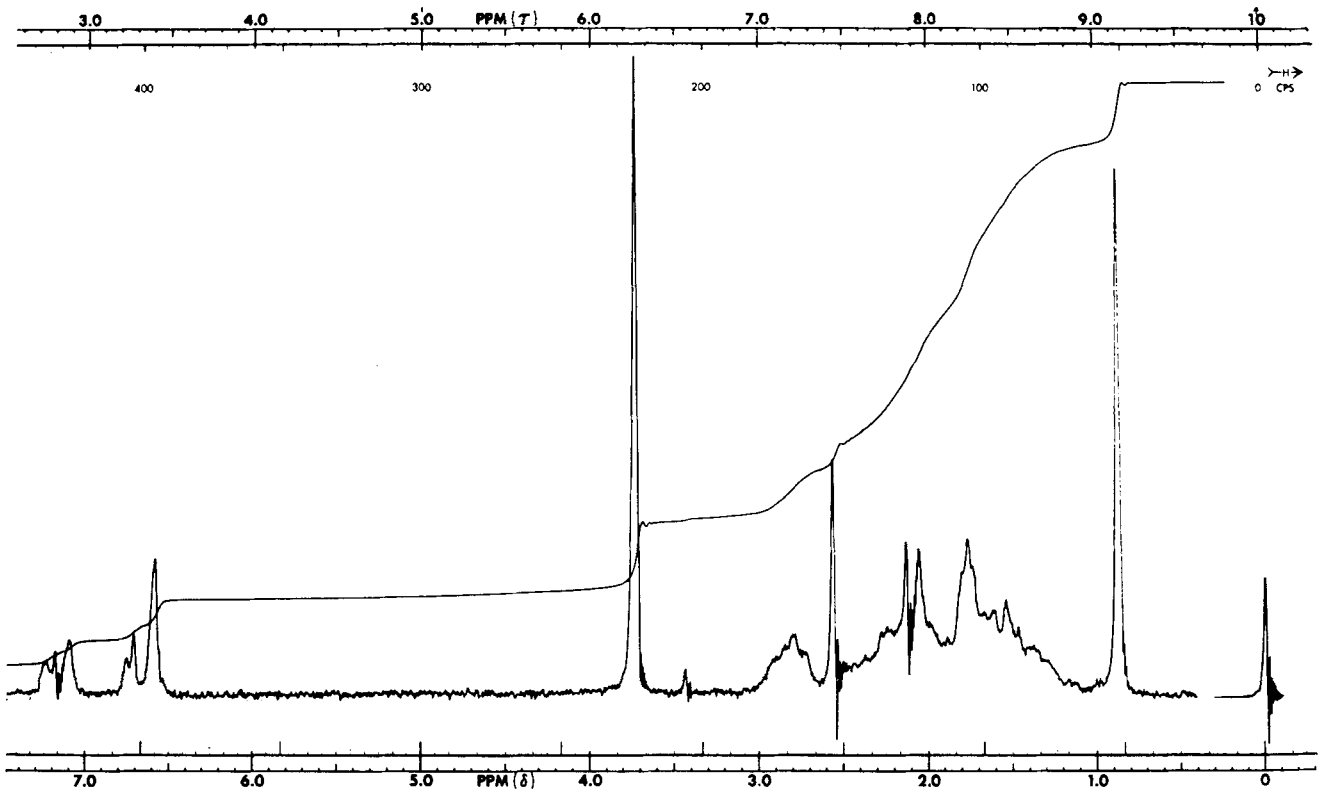


Figure 1—*NMR spectrum of mestranol in deuteriochloroform.*

accurately weighed, was dissolved in 40 ml. of tetrahydrofuran. To the solution was added 10 ml. of 5% silver nitrate solution, and the mixture was titrated with 0.1 *N* sodium hydroxide, the end-point being determined potentiometrically.

TLC was carried out on silica gel GF plates of 0.25-mm. thickness. The developing mixture was benzene–methanol (19:1), and spots were detected by spraying with concentrated sulfuric acid and heating for 20 min.

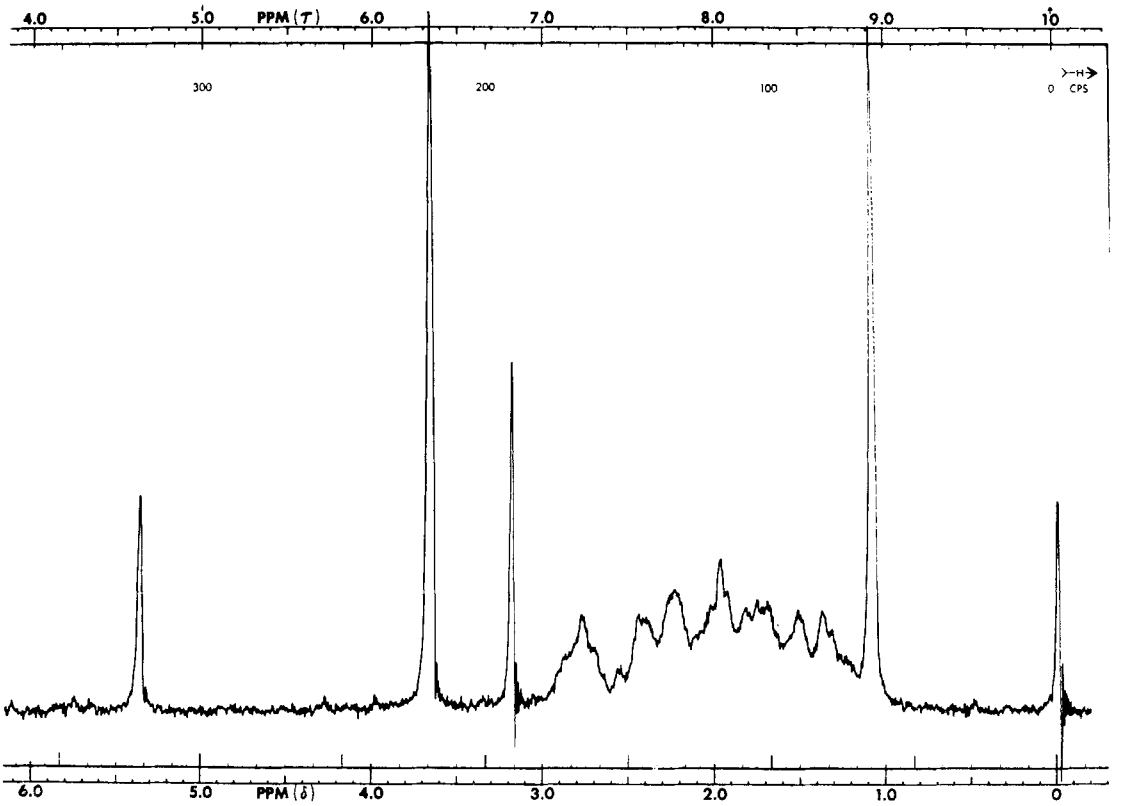


Figure 2—*Partial NMR spectrum of mestranol plus DPAA in pyridine.*

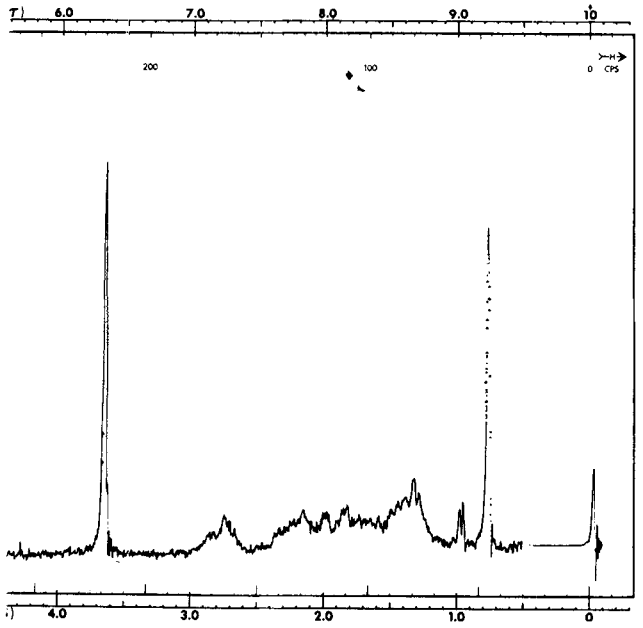


Figure 3—*Partial NMR spectrum of estrone-3-methyl ether in pyridine.*

Calculation—*NMR*—

$$\text{Wt. of mestranol (mg.)} = \frac{\text{E.W. mestranol}}{\text{E.W. DPAA}} \times \frac{\text{I mestranol}}{\text{I DPAA}} \times \text{wt. DPAA (mg.)} \quad \text{(Eq. 1)}$$

where E.W. is the molecular weight of the substance divided by the number of protons corresponding to the signal chosen for the assay, and I is the integral height.

Potentiometric Titration—

$$\text{Wt. of mestranol (mg.)} = 31.04 \times \text{number of ml. 0.1 } N \text{ sodium hydroxide} \quad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

NMR spectroscopy, based on the 60-Mc.p.s. spectrum, has been shown (18) to be of value in the analysis of meprobamate and chemically related substances. Information has been presented (19) demonstrating the use of NMR in the quantitative analysis of synthetic corticosteroids of the 1,4-dien-3-one type.

The NMR spectrum of mestranol in deuteriochloroform (Fig. 1) possesses signals at 6.27 τ (3 protons, due to the 3-methoxy group) and at 7.44 τ (1 proton, due to 17α-ethinyl group). The signal from the methoxyl protons is in an isolated region of the spectrum and is thus available for use in quantitative analysis. The ethinyl proton signal is not suitable, however, since it is surrounded by a large number of other signals. On the other hand, in the NMR spectrum of mestranol in pyridine (Fig. 2), the methoxyl proton signal is very slightly shielded (6.33 τ), but the ethinyl proton signal is significantly deshielded (6.80 τ) and is isolated from all other signals, thus making it suitable for use in quantitative analysis. Therefore, using pyridine as the solvent, two simultaneous independent determinations can be carried out on the same molecule. The signal at 4.62 τ (Fig. 2) is due to the methine proton of the internal standard, DPAA.

Table I—Analysis of Mestranol Bulk Drug by NMR and Potentiometric Titration

	NMR				Potentiometric Titration	
	Percent Found, Based on Ethinyl Protons	Mean	Percent Found, Based on Methoxyl Protons	Mean	Percent Found	Mean
Lot A	99.0	98.9	101.8	101.2	99.6	99.4
	98.8		100.5		99.2	
Lot B	99.6	100.1	101.1	101.0	101.4	101.4
	100.4		101.0		100.6	
	100.3		100.9		102.2	
Lot C	99.9	99.6	100.1	100.3	98.6	98.4
	99.2		100.6		98.4	
	99.7		101.3		98.2	

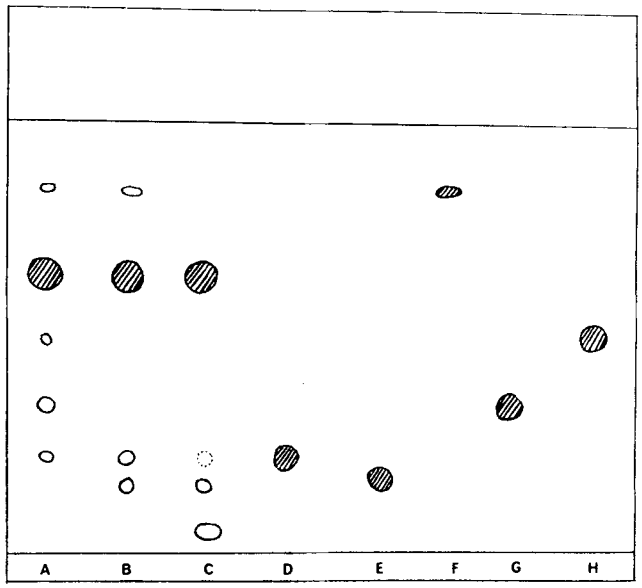


Figure 4—*Schematic TLC chromatogram of three commercial lots of mestranol and likely impurities. Key: A–C = samples, D = 17β-ethinylestradiol, E = estradiol, F = estrone-3-methyl ether, G = estrone, and H = estradiol-3-methyl ether. The adsorbent is silica gel GF, 0.25 mm.; the solvent is benzene–methanol (19:1).*

Mestranol is synthesized from estrone *via* estrone-3-methyl ether (20); both of these are, therefore, likely to occur as impurities. Other possible foreign related steroids in the final raw material are estradiol, estradiol-3-methyl ether, and 17α-ethinylestradiol. Of these, two (estrone-3-methyl ether and estradiol-3-methyl ether) interfere with the signal from the methoxyl protons of mestranol, and one (17α-ethinylestradiol) interferes with the signal from the ethinyl proton. Figure 3 shows a partial NMR spectrum of estrone-3-methyl ether in pyridine, with the signal from the methoxyl protons, at 6.3 τ, being in exactly the same position as the corresponding signal in mestranol (Fig. 2).

Figure 4 shows a schematic representation of the results of a TLC study of the three commercial lots investigated, together with the most likely impurities. Lot A contained significantly more methoxyl than ethinyl impurities, together with some estrone, which would not be detected by either the NMR or the BP potentiometric method (1). Lot B contained rather more methoxyl than ethinyl impurities, together with some estradiol, which again would not be detected. Lot C contained a trace of 17α-ethinylestradiol but no methoxyl impurities; in addition, there was a very faint but diffuse spot, close to the origin, which was not identified.

Results obtained from the analysis of the samples are shown in Table I. The potentiometric titration (1) is essentially an estimation of ethinyl protons, since the alkyne is treated with silver nitrate, liberating nitric acid which is determined by titration with standard alkali. Therefore, the results obtained from the NMR ethinyl proton signal should agree with those obtained by titration. The results from Lot A are in good agreement; the results from Lots B and C differ by 1.3 and 1.2%, respectively. The NMR method gives an average deviation of ±0.6% (19); the accuracy of the titration method was not determined, but if it is no worse than the NMR procedure, the results obtained from the ethinyl protons by the two techniques are not significantly different.

Of immediate interest in the NMR method is a comparison of the pairs of results. For pure mestranol, the results should be identical (within the experimental limits ±0.6%). In the case of Lots B and C, the results are in good agreement. The results obtained from Lot

A, however, show a difference of 2.3%, the methoxyl signal giving the higher answer. This is probably because this sample contained both estrone-3-methyl ether and estradiol-3-methyl ether as impurities in amounts which appeared, from TLC, to be significantly greater than the amount of 17α-ethinylestradiol present. Therefore, the result would be expected to be biased by an increase in the methoxyl signal.

The NMR procedure as described offers an attractive alternative assay for mestranol bulk drug. Simultaneous independent answers, from two different and isolated functional groups in the same molecule, act as a built-in check for the procedure.

REFERENCES

(1) "British Pharmacopoeia," General Medical Council, London, England, 1968, p. 595.

(2) A. P. Shroff and R. E. Huetteman, *J. Pharm. Sci.*, **56,** 654 (1967).

(3) D. C. Tsilifonis and L. Chafetz, *ibid.*, **56,** 625(1967).

(4) R. J. Templeton, W. A. Arnett, and I. M. Jakovljevic, *ibid.*, **57,** 1168(1968).

(5) R. A. Bartow, *J. Pharm. Pharmacol.*, **19,** 41(1967).

(6) A. P. Shroff and J. Grodsky, *J. Pharm. Sci.*, **56,** 460(1967).

(7) E. P. Schulz, *ibid.*, **54,** 144(1965).

(8) J. T. France and B. S. Knox, *J. Gas Chromatogr.*, **4,** 173 (1966).

(9) A. R. Umbreit and J. V. Wisniewski, *Facts Methods*, **5,** 9(1964).

(10) R. Hüttenrauch and I. Keiner, *Pharmazie*, **20,** 242(1965).

(11) J. P. Comer, P. E. Hartsaw, and C. E. Stevenson, *J. Pharm. Sci.*, **57,** 147(1968).

(12) L. J. Cali and A. J. Khoury, *Automat. Anal. Chem., Technicon Symp.*, **1,** 196(1966).

(13) J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," vol. 2, Pergamon Press, Oxford, England, 1966, p. 745.

(14) M. M. Kreevoy, M. B. Charman, and D. R. Vinard, *J. Amer. Chem. Soc.*, **83,** 1978(1961).

(15) E. B. Whipple, J. H. Goldstein, L. Mandell, G. S. Reddy, and G. R. McClure, *ibid.*, **81,** 1321(1959).

(16) R. E. Richards and J. V. Matton, *Trans. Faraday Soc.*, **57,** 28(1961).

(17) J. L. Jungnickel and J. W. Forbes, *Anal. Chem.*, **35,** 939 (1965).

(18) J. W. Turczan and T. C. Kram, *J. Pharm. Sci.*, **56,** 1643 (1967).

(19) H. W. Avdovich and B. A. Lodge, APhA, Montreal meeting, 1969.

(20) F. B. Colton, L. N. Nysted, B. Riegel, and A. L. Raymond, *J. Amer. Chem. Soc.*, **79,** 1123(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 30, 1970, from the *Research Laboratories, Food and Drug Directorate, Tunney's Pasture, Ottawa, Canada.*

Accepted for publication June 26, 1970.

Fluorometric Determination of Reserpine and Related Compounds by Reaction with Vanadium Pentoxide

TIBOR URBÁNYI and HENRY STOBER*

Abstract □ A rapid and fairly specific fluorometric procedure has been developed for the routine quantitative determination of reserpine and its derivatives alone and in tablet formulations. The method is based on the formation of fluorescence induced by the oxidation of reserpine with a reagent containing vanadium pentoxide in phosphoric acid. The oxidation product exhibits a greenish-yellow fluorescence, with the maximum around 500 mμ in an acidic alcoholic solution. The dependence of the intensity of fluorescence upon the nature of the solvent, reagent concentration, and other parameters is discussed. The fluorogen developed follows Beer's law over a very wide range, from 0.004 to 2 mcg./ml. of sample solution. The advantages and disadvantages of the proposed method are discussed, and the applicability in different formulations is demonstrated.

Keyphrases □ Reserpine and derivatives in tablets—determination □ Vanadium pentoxide–reserpine reaction—fluorescence □ Phosphoric acid effect—reserpine–vanadium pentoxide fluorogen □ Fluorometry—analysis

Since the isolation of reserpine[1] from the various Rauwolfia roots was completed, its double therapeutic effect as an antihypertensive and a tranquilizer has been recognized. When the usefulness of the reserpine as an effective human medicine was realized, Szalkowski and Mader (1) developed a quantitative method for its determination. This method underwent many modifications (2, 3) which, however, did not alter the methodology of the original procedure significantly.

In recent years, photometric methods (4) have been introduced for the quantitative determination of reserpine. These methods are based on reactions with suitable reagents resulting in the formation of chromophores or fluorogens, which can be measured by colorimetric or fluorometric techniques. Colorimetric measurements (5) are applied mostly for pharmaceutical formulations where the sensitivity of the determination is not critical. Fluorescence determinations are used for reserpine in feeds and biological materials (6), where extremely sensitive and selective methods are required. A direct UV method (7) is frequently used for the determination of reserpine and has the advantage of speed of assay where the concentration of reserpine is high enough for UV absorption. Column chromatographic methods (8) are highly specific and fairly sensitive, but they are time consuming. TLC methods (9) are often used for the separation of the active alkaloids, but these methods are primarily qualitative rather than quantitative.

The development of a specific method for the determination of reserpine is difficult because of its structural similarity to other active alkaloids isolated from the Rauwolfia root. The greenish-yellow color produced

[1] Serpasil, Ciba Pharmaceutical Co.

A, however, show a difference of 2.3%, the methoxyl signal giving the higher answer. This is probably because this sample contained both estrone-3-methyl ether and estradiol-3-methyl ether as impurities in amounts which appeared, from TLC, to be significantly greater than the amount of 17α-ethinylestradiol present. Therefore, the result would be expected to be biased by an increase in the methoxyl signal.

The NMR procedure as described offers an attractive alternative assay for mestranol bulk drug. Simultaneous independent answers, from two different and isolated functional groups in the same molecule, act as a built-in check for the procedure.

REFERENCES

(1) "British Pharmacopoeia," General Medical Council, London, England, 1968, p. 595.

(2) A. P. Shroff and R. E. Huetteman, *J. Pharm. Sci.*, **56,** 654 (1967).

(3) D. C. Tsilifonis and L. Chafetz, *ibid.*, **56,** 625(1967).

(4) R. J. Templeton, W. A. Arnett, and I. M. Jakovljevic, *ibid.*, **57,** 1168(1968).

(5) R. A. Bartow, *J. Pharm. Pharmacol.*, **19,** 41(1967).

(6) A. P. Shroff and J. Grodsky, *J. Pharm. Sci.*, **56,** 460(1967).

(7) E. P. Schulz, *ibid.*, **54,** 144(1965).

(8) J. T. France and B. S. Knox, *J. Gas Chromatogr.*, **4,** 173 (1966).

(9) A. R. Umbreit and J. V. Wisniewski, *Facts Methods*, **5,** 9(1964).

(10) R. Hüttenrauch and I. Keiner, *Pharmazie*, **20,** 242(1965).

(11) J. P. Comer, P. E. Hartsaw, and C. E. Stevenson, *J. Pharm. Sci.*, **57,** 147(1968).

(12) L. J. Cali and A. J. Khoury, *Automat. Anal. Chem., Technicon Symp.*, **1,** 196(1966).

(13) J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," vol. 2, Pergamon Press, Oxford, England, 1966, p. 745.

(14) M. M. Kreevoy, M. B. Charman, and D. R. Vinard, *J. Amer. Chem. Soc.*, **83,** 1978(1961).

(15) E. B. Whipple, J. H. Goldstein, L. Mandell, G. S. Reddy, and G. R. McClure, *ibid.*, **81,** 1321(1959).

(16) R. E. Richards and J. V. Matton, *Trans. Faraday Soc.*, **57,** 28(1961).

(17) J. L. Jungnickel and J. W. Forbes, *Anal. Chem.*, **35,** 939 (1965).

(18) J. W. Turczan and T. C. Kram, *J. Pharm. Sci.*, **56,** 1643 (1967).

(19) H. W. Avdovich and B. A. Lodge, APhA, Montreal meeting, 1969.

(20) F. B. Colton, L. N. Nysted, B. Riegel, and A. L. Raymond, *J. Amer. Chem. Soc.*, **79,** 1123(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 30, 1970, from the *Research Laboratories, Food and Drug Directorate, Tunney's Pasture, Ottawa, Canada.*

Accepted for publication June 26, 1970.

Fluorometric Determination of Reserpine and Related Compounds by Reaction with Vanadium Pentoxide

TIBOR URBÁNYI and HENRY STOBER*

Abstract □ A rapid and fairly specific fluorometric procedure has been developed for the routine quantitative determination of reserpine and its derivatives alone and in tablet formulations. The method is based on the formation of fluorescence induced by the oxidation of reserpine with a reagent containing vanadium pentoxide in phosphoric acid. The oxidation product exhibits a greenish-yellow fluorescence, with the maximum around 500 mμ in an acidic alcoholic solution. The dependence of the intensity of fluorescence upon the nature of the solvent, reagent concentration, and other parameters is discussed. The fluorogen developed follows Beer's law over a very wide range, from 0.004 to 2 mcg./ml. of sample solution. The advantages and disadvantages of the proposed method are discussed, and the applicability in different formulations is demonstrated.

Keyphrases □ Reserpine and derivatives in tablets—determination □ Vanadium pentoxide–reserpine reaction—fluorescence □ Phosphoric acid effect—reserpine–vanadium pentoxide fluorogen □ Fluorometry—analysis

Since the isolation of reserpine[1] from the various Rauwolfia roots was completed, its double therapeutic effect as an antihypertensive and a tranquilizer has been recognized. When the usefulness of the reserpine as an effective human medicine was realized, Szalkowski and Mader (1) developed a quantitative method for its determination. This method underwent many modifications (2, 3) which, however, did not alter the methodology of the original procedure significantly.

In recent years, photometric methods (4) have been introduced for the quantitative determination of reserpine. These methods are based on reactions with suitable reagents resulting in the formation of chromophores or fluorogens, which can be measured by colorimetric or fluorometric techniques. Colorimetric measurements (5) are applied mostly for pharmaceutical formulations where the sensitivity of the determination is not critical. Fluorescence determinations are used for reserpine in feeds and biological materials (6), where extremely sensitive and selective methods are required. A direct UV method (7) is frequently used for the determination of reserpine and has the advantage of speed of assay where the concentration of reserpine is high enough for UV absorption. Column chromatographic methods (8) are highly specific and fairly sensitive, but they are time consuming. TLC methods (9) are often used for the separation of the active alkaloids, but these methods are primarily qualitative rather than quantitative.

The development of a specific method for the determination of reserpine is difficult because of its structural similarity to other active alkaloids isolated from the Rauwolfia root. The greenish-yellow color produced

[1] Serpasil, Ciba Pharmaceutical Co.

Table I—Effect of Acids Saturated with Vanadium Pentoxide on Reserpine-Induced Fluorescence[a]

Acid	Reagent Added, ml.	Fluorescence, %
Phosphoric	0.5	85
Hydrochloric	0.5	1
Sulfuric	0.5	45
Perchloric	0.5	4
Acetic	0.5	1

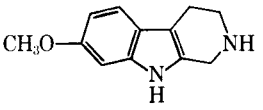
[a] The total volume was 10.5 ml.; concentration 10 mcg.

Table II—Variation of Vanadium Pentoxide Reagent on the Fluorescence of Reserpine in Ethanolic Solution[a]

Reagent, ml.	Fluorescence, %
0.25	83
0.50	82
0.75	76
1.00	72

[a] The total volume of solution was 11.0 ml.

using the general nitrite procedure for reserpine is specific for alkaloids having a 2,3,4,9-tetrahydro-7-methoxy-1*H*-pyrido[3,4-*b*]indole group (10) with the following structural formula (I):



I

It was observed that when reserpine was exposed to intense light, air, or oxidizing agents, a greenish-yellow colored compound was produced (11). This compound is identical with the reaction product obtained from the nitrite procedure and was identified by several authors (11, 12) as 3-dehydroreserpine, a first-step oxidation product of reserpine. Among many oxidative reagents, the official method in Pharmacopeia Belgique IV (13) is the sulfovanadic acid, which produces a greenish-yellow color with reserpine in acetone solution.

In the USP and NF, the official method is the nitrite procedure in the presence of hydrochloric acid. The chromophores produced by these different reagents are alike and are measured colorimetrically at the absorption maximum at about 390 mμ. The greenish-yellow color recommended by the official procedure permits the determination of reserpine by fluorescence measurements, since the fluorescent product increases the sensitivity for the determination of reserpine.

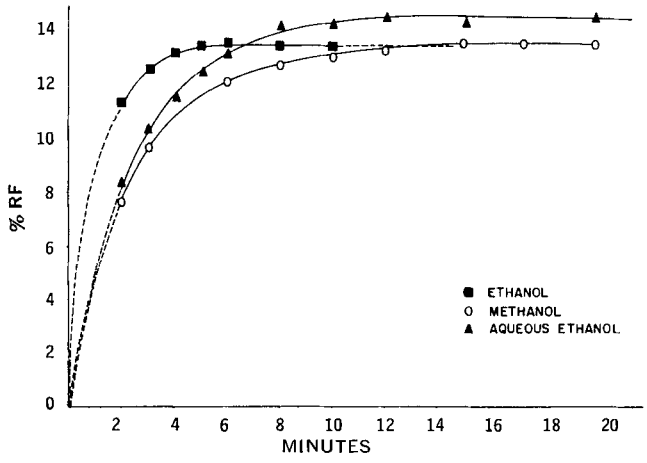


Figure 1—*Rate of formation of fluorogen with vanadium pentoxide in different solvents.*

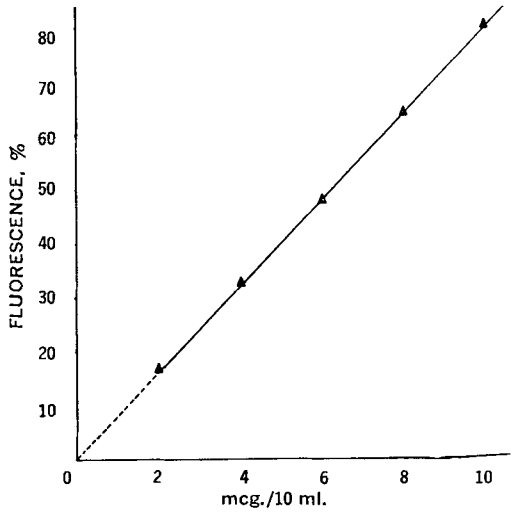


Figure 2—*Plot of fluorescence of alcoholic solutions against concentrations.*

The goal of this study was to develop a fluorescence method which would be as sensitive or better than that of the nitrite procedure and, with its simplicity, would improve the precision of the official method. The proposed method is based on the formation of a greenish-yellow fluorogen with vanadium pentoxide in phosphoric acid in alcohol or with an aqueous ethanolic solution of reserpine. On the basis of these observations, the method described can be adopted for the reserpine determination of the single-tablet assay, where extremely high sensitivities are desired. The optimal reaction conditions and the characteristics of the fluorescent species are presented.

EXPERIMENTAL

Reagents and Solutions—Concentrated phosphoric acid was saturated with reagent grade vanadium pentoxide by mechanical

Table III—Influence on Fluorescence by the Amount of Phosphoric Acid Added to Alcoholic Solution of Reserpine[a]

Phosphoric Acid Added, ml.	Reagent, ml.	Fluorescence, %
1.5	0.5	77
0.5	0.5	75
	0.5	64

[a] The total volume of solution was 12.0 ml.

Table IV—Effect of Water Content on Fluorescence in Ethanolic Solution of Reserpine[a]

Water in Ethanol, %	Reagent, ml.	Fluorescence, %
10	0.5	83
25	0.5	83
50	0.5	75

[a] The total volume of solution was 10.5 ml.

shaking for approximately 1 or 2 hr. The solution was filtered through a medium-porosity sintered-glass funnel. The saturated solution contained about 0.8 mg. of vanadium pentoxide/ml. This reagent was quite stable and could be stored for a long period without any changes in composition; alcohol USP was used as solvent.

Apparatus—An Aminco SPF-125 spectrophotofluorometer, equipped with variable slit controls and a mercury lamp for the light source, and an Aminco-Bowman recording spectrophotofluorometer with xenon lamp for the light source were used for the measurements.

Preparation of Standard Solution—A solution containing 1 mcg./ml. of USP reserpine reference standard in alcohol USP was prepared.

Preparation of Assay Solution—A reserpine solution of 1 mcg./ml. in alcohol USP was prepared. To facilitate the dissolution of reserpine from the tablet mass, a sonifier bath was employed.

Procedure—Into a 25-ml. volumetric flask, pipet 10 ml. of assay solution and 0.5 ml. of reagent. Mix well and let the solution stand about 10–15 min. At the same time, pipet standard solutions into separate 25-ml. volumetric flasks containing 6, 8, and 10 mcg. of reserpine in 10 ml.; add 0.5 ml. of reagent solution to each flask, and mix well. After the standing time, set the activation slit control at 2 mm. and the emission slit control at 4 mm. at full sensitivity, and set the percent full scale setting at 100 of the instrument. Measure the fluorescence of the standard and sample solutions at 495 mμ with the activation wavelength of 365 mμ, using a 1-cm. fluorescence cell.

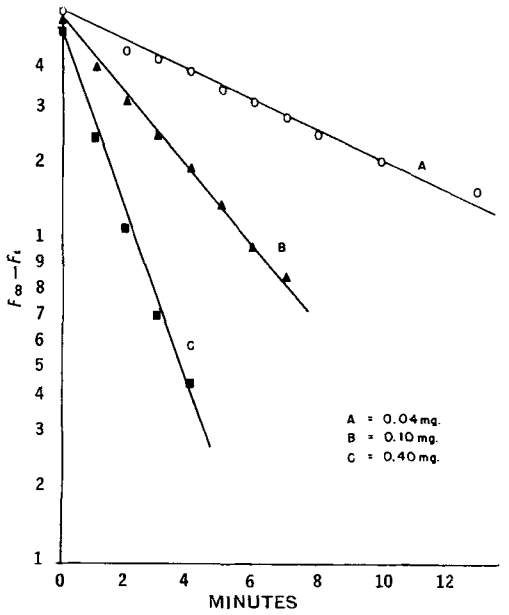


Figure 3—*Plot of the log* $F_\infty - F_t$ *of reserpine treated with varying amounts of vanadium pentoxide per 0.5 ml.* H_3PO_4 *against time. Reserpine concentration = 0.01 mg./10 ml.*

DISCUSSION

The most frequently reported quantitative method for the determination of reserpine and its related compounds, alone or in formulations, is still the nitrite procedure. The use of colorimetric or fluorometric determinations depends on the concentration of reserpine in the product to be analyzed, since the species assayed in both instances are identical. The colorimetric or fluorometric procedures currently used are fairly complicated (using several reagents and extractions). A simpler procedure requiring a single reagent and fewer associated steps with possibilities of automation would be desirable.

It is well known that reserpine can be fairly easily oxidized to a fluorescent product in acid solution using suitable oxidants. Several oxidizing reagents such as ceric compounds, peroxides, selenium dioxide, permanganates, periodates, and vanadium salts and its oxides were tested in these laboratories. Because the oxidation of

Table V—Comparison Data on Tablets, Using the New and Official Methods

Tablets	V_2O_5, mg./Tablet	Nitrite Assay, mg./Tablet*[a]
Reserpine (0.10)	0.101*	0.100
	0.099	
	0.258*	0.253
Reserpine (0.25)	0.244	0.245
	0.255	
Syrosingopine (1.0)	0.995	0.982
	0.982*	
	0.994	

[a] These assays were performed on chloroform extracts obtained by the official nitrite procedure. An aliquot of the chloroform extract was evaporated to dryness, the residue dissolved in ethanol, and the resultant solution assayed by the vanadium pentoxide procedure. The nonstarred results are based on analysis by vanadium pentoxide without prior extraction.

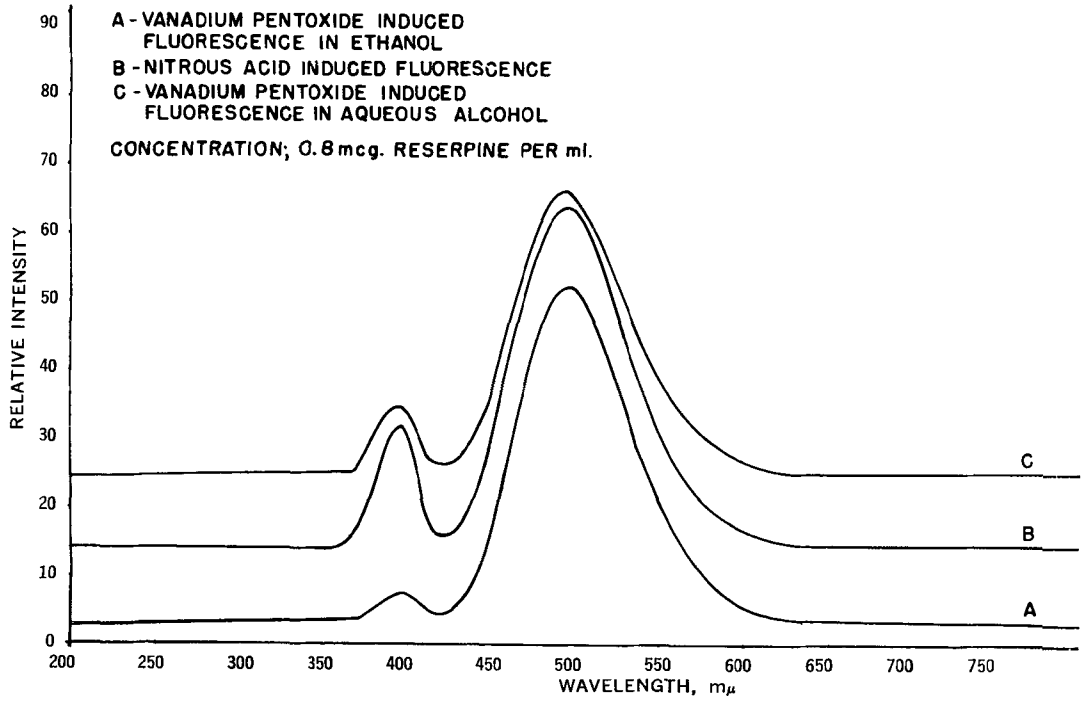


Figure 4 — *Fluorescence emission spectra of reserpine. (The base lines for Spectra B and C were displaced for visual convenience.)*

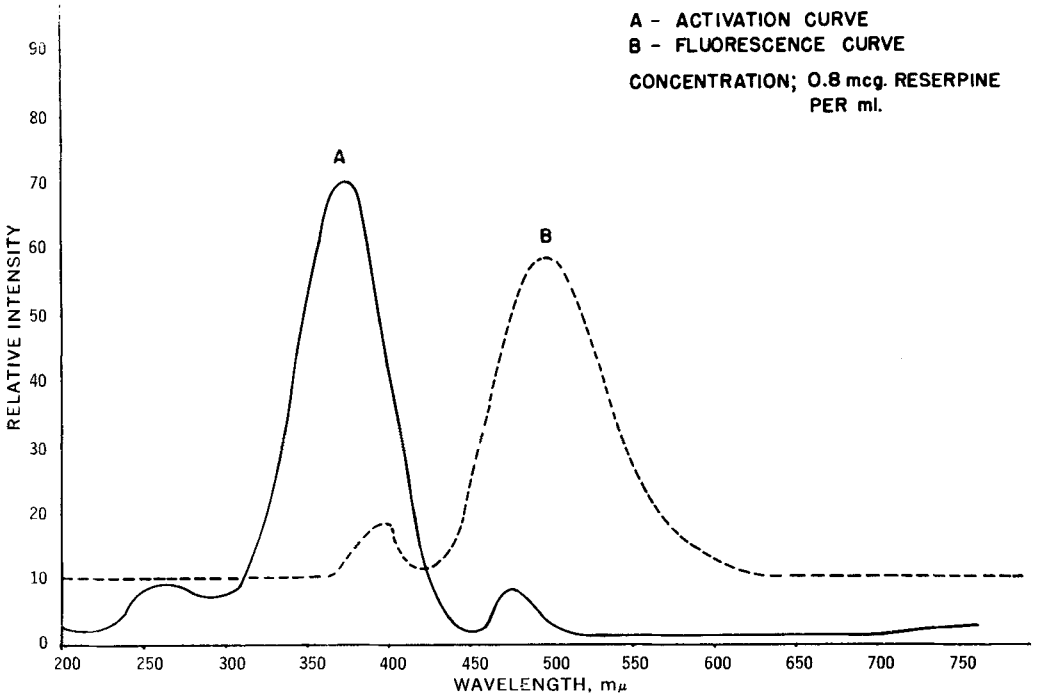


Figure 5—*3-Dehydroreserpine treated with vanadium pentoxide reagent. (The base line for Spectrum B was displaced for visual convenience.)*

reserpine occurs in acid solution, the effect of different inorganic and organic acids on the oxidants was tested. Ceric compounds dissolved in mineral acids produced fluorescence with reserpine in alcoholic solution; however, the usefulness of this reagent was impaired by a rapidly forming precipitate in the solution. The other oxidants mentioned failed to produce significant fluorescence with reserpine, regardless of the acid or solvent used for the dissolution.

The dissolution of the pure substance or the tablet mass was carried out in alcoholic solutions. Appreciable fluorescence was observed when an alcoholic solution of the reserpine was treated with a saturated solution of vanadium pentoxide in concentrated phosphoric acid. The fluorescence yields of reserpine with vanadium pentoxide were considerably inhibited when the vanadium pentoxide was dissolved in mineral acids other than phosphoric acid. Table I illustrates the fluorescent intensity of reserpine obtained with saturated vanadium pentoxide in different acids. It is evident from these data that the maximum fluorescent intensity was achieved with phosphoric acid. The phosphoric acid reagent also proved to be more stable. From these observations it was obvious that the optimum concentration of the reagent should be investigated. The volume and the concentration of the sample solution were kept constant. The optimal fluorescent intensity was achieved using 0.25–0.5 ml. of reagent, and the data for these observations are presented in Table II. The effect of phosphoric acid upon the production of fluorescence was investigated by maintaining the reserpine and the reagent concentration constant. These data are presented in Table III and show that the variation of the phosphoric acid concentration insignificantly influenced the fluorescent intensity of the recommended assay.

During this investigation it also was observed that the fluorescence measurements were not only affected by the oxidation reagent but that the rate of oxidation was substantially affected by the solvents used in dissolution. From Fig. 1 it is apparent that the reaction proceeds most rapidly in alcohol USP but with less fluorescent intensity than in ethanolic water solutions that were tested for suitability with respect to automated procedures. This difference can be explained by the quenching effect, since the reagent imparts a slight yellow color in alcohol USP. However, this yellow color can be significantly decreased upon the addition of water. Ethanolic solutions of reserpine containing various amounts of water were analyzed by this procedure. The data presented in Table IV indicate that with 25% water, maximum fluorescence was produced. With aqueous ethanolic solution, a very faint straw-yellow color was observed; however, the fluorescence for reserpine in this solution was approximately 10–15% higher than in alcohol USP alone. Since the rate of fluorescence development is faster in alcohol USP, the slight decrease due to quenching can be overcome if one allows the fluorescence to develop in alcohol USP and then, after a suitable time, dilutes the solution to a desired volume with water.

Almost the same quenching effect as in the case of alcohol was observed when a large excess of phosphoric acid was employed for the assay. A maximum fluorescence was obtained upon addition of 0.5 ml. reagent and 0.5–1.5 ml. of phosphoric acid to 12 ml. of an ethanolic solution containing 1 mcg. reserpine/ml. These data indicate the optimum reaction conditions and the most stable fluorescence species. To determine the validity of the reaction using optimum conditions, the fluorescent intensity was plotted against the concentration of reserpine (Fig. 2). The linear plots obtained by this procedure indicate that the method is reliable. The fluorescence species appears to be stable in solution for a period of several hours. This procedure would be suitable for the determination of reserpine, alone and in tablet formulations.

Reserpine and syrosingopine[2] are fairly soluble in alcohols; tablets containing these active ingredients were extracted with alcoholic solutions. The results obtained for reserpine and syrosingopine tablets, using the new vanadium pentoxide procedure, are presented in Table V. The data were compared with the results obtained by the official nitrite procedure. The proposed new vanadium pentoxide method without an extraction procedure fails to distinguish the possible degradation products, such as reserpic acid, from the parent compound. To determine the amount of reserpic acid, the citric acid extracts remaining from the analysis of syrosingopine tablets by the nitrite procedure were subjected to the vanadium pentoxide method. Approximately 0.4% of fluorescent material based on syrosingopine was found in the citric acid extract. The differences between the extracted and nonextracted analyses indicate, therefore, that the reserpic acid concentration is negligible in comparison with the experimental error of the method. The reproducibility of the method according to the new procedure is presented in Table VI.

After a practical determination of reserpine by fluorescence measurements, an attempt was made to determine if the reaction products from the nitrite and vanadium pentoxide methods were identical. Haycock *et al.* (12) studied the kinetics of nitrous acid-induced fluorescence and suggested that the fluorescent product in reserpine by nitrous acid is a two-step reaction which can be explained by Scheme I:

$$\underset{\text{reserpine}}{\text{protonated}} + \text{HONO} \underset{}{\overset{k_1}{\rightleftharpoons}} \underset{\text{reserpine}}{\text{protonated}} \cdot\text{HONO} \xrightarrow{k_2} \text{H}^+ \text{ 3-dehydroreserpine fluorescent product}$$

Scheme I

[2] Singoserp, Ciba Pharmaceutical Co.

Table VI—Reproducibility of Vanadium Pentoxide Method[a]

Sample	Assay, mg./Tablet
A	0.2580
B	0.2540
C	0.2580
D	0.2500
E	0.2570
Average	0.2554
	$SD \pm 0.0017$

[a] Analysis of 0.25-mg. reserpine tablets.

A linear relationship was obtained by plotting the logarithm of unreacted reserpine expressed as $F_\infty - F_t$, where F_∞ is the final fluorescence of the solution and F_t the fluorescence at time t, *versus* the time required for fluorescence development with nitrous acid. A plot of this type is linear for several different concentrations of nitrite reagent and indicates first-order kinetics with respect to reserpine concentration. Preliminary data of this type have been obtained for the fluorescence produced by the reaction of reserpine with vanadium pentoxide reagent. A plot of the logarithm of $F_\infty - F_t$ *versus* time for different concentrations of vanadium pentoxide produces straight lines, indicating an apparent first-order reaction with respect to reserpine concentration as in the nitrite case (Fig. 3).

To establish that the fluorescent species is the same, 3-dehydroreserpine was synthesized from reserpine, and the synthesized product was compared with the fluorescent species produced by vanadium pentoxide reagent. Satisfactory combustion analysis was obtained for the synthesized product, and the TLC data indicated only a few trace impurities. The activation and fluorescence spectra of 3-dehydroreserpine in the presence and absence of vanadium pentoxide reagent were essentially identical to that of reserpine treated with vanadium pentoxide reagent (Figs. 4 and 5). Since the spectra were generated by an Aminco-Bowman spectrophotofluorometer, the activation and fluorescence maxima show slight differences in wavelengths from those obtained on SPF-125. This difference between the Aminco-Bowman and SPF-125 is most likely due to the difference in sources for the two instruments. The spectra were generated in ethanol and aqueous alcoholic solutions. The fluorescence yields of reserpine treated with vanadium pentoxide reagent were 106% based on the fluorescence obtained from an equimolar amount of 3-dehydroreserpine treated similarly. Some fluorescence quenching is observed for 3-dehydroreserpine treated with reagent compared to a solution of 3-dehydroreserpine treated with phosphoric acid only.

An attempt was made to isolate the fluorescent species of reserpine generated by vanadium pentoxide reagent by making the reaction mixture basic with sodium hydroxide and extracting with chloroform. Approximately 250 mcg. of reserpine was allowed to react with vanadium pentoxide reagent for several hours. The reaction mixture was made basic with sodium hydroxide, diluted to 100 ml. with water, and extracted with chloroform. The chloroform extract was evaporated to dryness, and the residue was taken up in ethanol. The UV spectrum of this solution did not compare well to the UV spectrum of 3-dehydroreserpine in ethanol, but it was practically identical to that obtained for 3-dehydroreserpine treated with vanadium pentoxide reagent and extracted as described. Rather than indicating a fluorescent species other than 3-dehydroreserpine, the different UV spectra obtained for 3-dehydroreserpine in the absence of vanadium pentoxide and after extraction from a basic solution containing vanadium pentoxide might possibly be explained by the formation of an extractable complex between 3-dehydroreserpine and a vanadium species.

The authors feel that the proposed method, with its simplicity and efficiency, would provide a suitable procedure for the routine analysis of reserpine and may contribute to the progress of an automated technique for reserpine.

CONCLUSION

A new fluorometric method has been developed for the determination of reserpine and related compounds in bulk and tablet formulations. The proposed method is based on the formation of a fluorogen with vanadium pentoxide. The intensity of the fluorogen was significantly increased when the reagent was saturated in phosphoric acid. This method, with its simplicity and rapidity, can be used for the quantitative determination of reserpine. The possible formation of 3-dehydroreserpine as the fluorescent product was postulated, and the optimum reaction conditions, were evaluated. Since the mechanism of the reaction is not completely evident and is strongly dependent on the reagent and the acid used for the reaction, these parameters will be the subject of a further investigation.

REFERENCES

(1) C. R. Szalkowski and W. J. Mader, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 613(1956).

(2) D. Banes, J. Wolf, H. O. Fallscheer, and J. Carol, *ibid.*, **45**, 708(1956).

(3) D. Banes, *ibid.*, **46**, 601(1957).

(4) R. E. Booth, *ibid.*, **44**, 568(1955).

(5) D. Banes, *ibid.*, **44**, 640(1955).

(6) W. J. Mader, R. P. Haycock, P. B. Sheth, and R. J. Connolly, *J. Ass. Office. Agr. Chem.*, **43**, 291(1960).

(7) M. M. Dhar and S. Bhattacharji, *J. Sci. Ind. Res.*, **14B**, 276(1955).

(8) R. J. Boscott and A. B. Kar, *Nature*, **176**, 1077(1955).

(9) K. Teichert, E. Mutschler, and H. Rochelmeyer, *Deut. Apoth. Ztg.*, **100**, 477(1960).

(10) R. P. Haycock and W. J. Mader, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 744(1957).

(11) K. G. Krebs and N. Futscher, *Arzneim.-Forsch.*, **10**, 2, 75 (1959).

(12) R. P. Haycock, P. B. Sheth, T. Higuchi, W. J. Mader, and G. J. Papariello, *J. Pharm. Sci.*, **55**, 826(1966).

(13) R. Stainier, *Farmaco*, **24**, 167(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Analytical Research and Development Division, Ciba Pharmaceutical Co., Summit, NJ 07901*

Accepted for publication July 14, 1970.

* Present address: Seton Hall University, South Orange, N. J.

Quantitative Analysis and Stability of 5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide in Acidic Aqueous Solutions

DAVID S. DRESBACK and JOSEPH F. GALLELLI

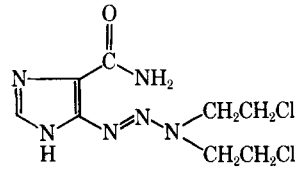
Abstract □ Methods of quantitative analysis by UV spectrophotometric, colorimetric, and ionic chloride determination are reported for 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (NSC-82196) in the presence of its degradation products. With the exception of the ionic chloride method of analysis of samples exposed to light, all three methods showed good agreement for stability studies of NSC-82196 in acidic aqueous solutions exposed to light and dark and stored at 25°. A sterile lyophilized hydrochloride salt of NSC-82196 for parenteral use, reconstituted with water for injection, was found to have a $t_{1/2}$ = 150 min. at 25° and $t_{1/2}$ = 65 hr. at 4°.

Keyphrases □ 5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide—quantitative analysis, stability □ TLC—separation □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer □ Ionic chloride determination—analysis

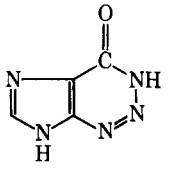
5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (NSC-82196) is a promising antileukemic agent. It has been shown to be superior, in the murine leukemia L-1210 system, to 5(or 4)-[3,3-dimethyl-1-triazeno]imidazole-4(or 5)-carboxamide (NSC-45388), an analog of similar structure presently in clinical trials (1).

A sterile lyophilized hydrochloride salt of NSC-82196, for parenteral use, was prepared by the Pharmaceutical Development Service. Since the drug is extremely unstable in aqueous solutions, adequate assay procedures were needed to determine the potency and stability of the formulated dosage form.

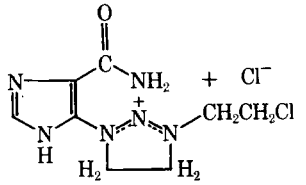
NSC-82196 is an analog belonging to a series of dialkyltriazenoimidazoles that undergoes rapid decomposition in aqueous acidic solutions. The resulting degradation products have been reported to be "transformation" product (NSC-112970) (II) and 2-azahypoxanthine (NSC-22419) (III) (2–4). The formation of NSC-22419 is preceded by the photodegradation of NSC-82196 to an intermediate 5-diazoimidazole-4-carboxamide (IV), which cyclizes by intramolecular coupling to NSC-22419 (3–5).



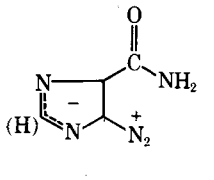
NSC-82196
I



NSC-22419
III



NSC-112970
II



5-diazoimidazole-4-carboxamide
IV

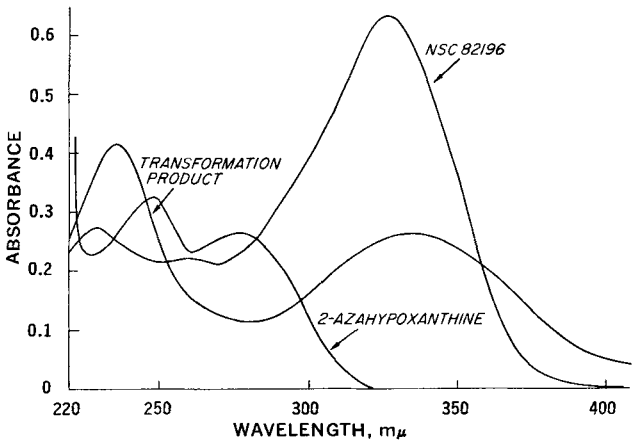


Figure 1—*UV spectra of NSC-82196, NSC-112970, and NSC-22419 in pH 2.31 phosphate buffer.*

Loo and Stasswender (6) reported a colorimetric method of assay suitable for intact NSC-82196. A UV spectrophotometric assay has also been reported which is specific for the degradation products, NSC-112970 and NSC-22419, as well as NSC-82196 (2). Adaptations of these two methods were used in this study to assay intact NSC-82196. Since one chloride ion is liberated for each molecule of transformation product formed, an ionic chloride method of assay was also used to follow the degradation of NSC-82196. Stability studies were performed to correlate all three methods of assay and to outline limitations, if any. The stability of a reconstituted, lyophilized, parenteral dosage form was determined under various conditions of light and dark at 25 and 4°.

EXPERIMENTAL

Reagents—The following were used: 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide,[1] transformation product[2] (m.p. 209° dec.), and 2-azahypoxanthine monohydrate[2] [m.p. 208–209° (explosive), darkens at 150°]. 5-Diazoimidazole-4-carboxamide[3] was synthesized according to the method of Shealy

[1] Prepared by Parke, Davis & Co., Detroit, Mich., and obtained from the Drug Development Branch, Cancer Chemotherapy, National Service Center, NCI, Bethesda, Md.; m.p. 208° dec., darkens at 150°, purity determined from IR spectrum and ionic chloride determination.

[2] Prepared by Southern Research Institute, Birmingham, Ala.

[3] *Elemental analysis*—Calcd.: C = 35.04%, H = 2.21%, N = 51.09%; Found: C = 35.01%; H = 2.17%, N = 50.74%. Elemental analysis was performed by Microanalytical Laboratory, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.

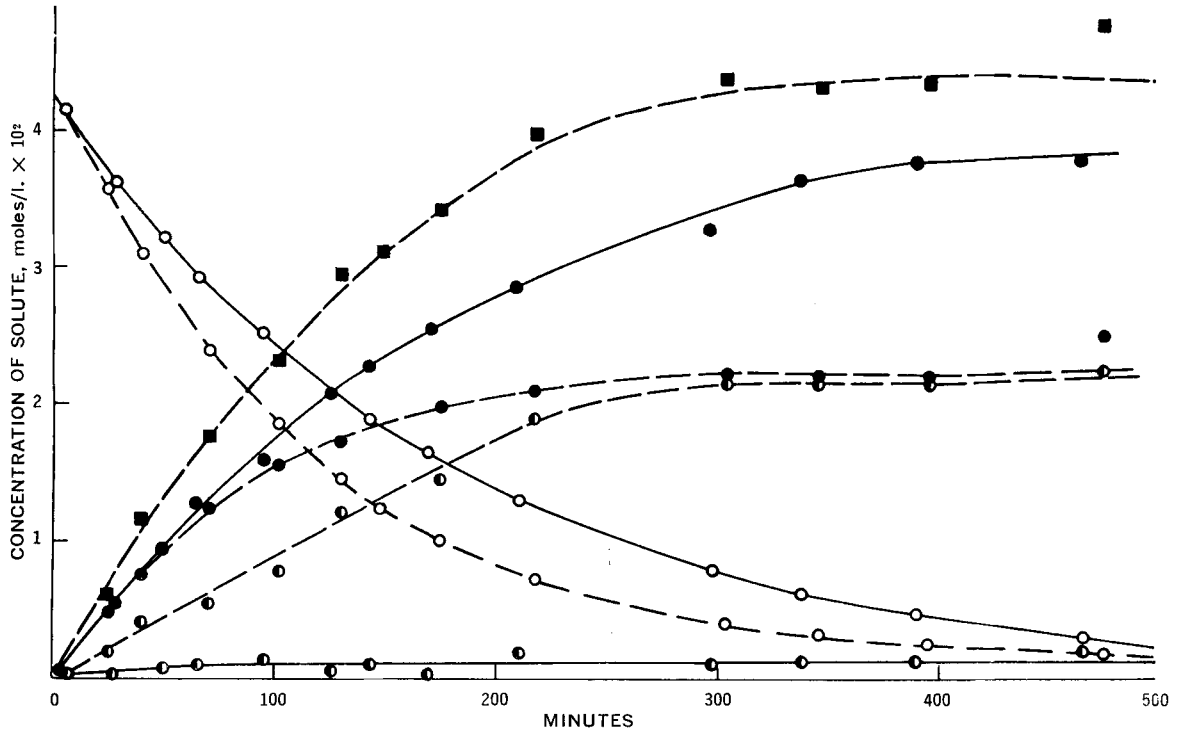


Figure 2—*Verification of stoichiometry of NSC-82196 hydrolysis at pH 2.31 phosphate buffer and 25° in light* (- - -) *and dark* (——). *Key:* ○, *NSC-82196;* ●, *NSC-112970;* ◐, *NSC-22419; and* ■, *NSC-112970 plus NSC-22419.*

et al. (5) [m.p. 199° (explosive), darkens at 149–150°]. Sterile lyophilized hydrochloride salt of NSC-82196 for injection, 100 mg./vial;[4] *N*-(1-naphthyl)-ethylenediamine dihydrochloride (Bratton-Marshall reagent), 3% in distilled water; 0.2 *M* phosphate buffer, pH 2.31; gelatin reagent;[5] and silica gel GF thin-layer plates[6] were also used. All other chemicals were of reagent grade.

Apparatus—The following were used: Cary model 11 recording spectrophotometer; UV lamp (longwave),[7] maximum intensity at 366 mμ; Aminco chloride titrator, model 4–4433;[8] IBM 1620 computer;[9] Beckman zeromatic pH meter; Desaga TLC apparatus;[10] Beckman IR-5A spectrophotometer; constant-temperature water bath (±0.1°); and Thomas-Hoover capillary melting-point apparatus.

Colorimetric Assay—To a 10-ml. volumetric flask was added 0.2 ml. of freshly prepared 3% Bratton-Marshall reagent followed by 20–110-mcg. portions of NSC-82196. The flask was rapidly brought to volume with pH 2.31 phosphate buffer and mixed in subdued light. Approximately one-half of the solution was removed and placed in the dark. The other half was irradiated with a longwave UV lamp for 30 min. The lamp was positioned approximately 8 cm. from the center of the solution in the 10-ml. volumetric flask. The absorbance of both fractions was measured on a Cary 11 recording spectrophotometer at 520 mμ, using a phosphate buffer as the blank. A blank of the reagents alone, without NSC-82196, was also treated in the same manner, and its absorbance was recorded at 520 mμ.

Ionic Chloride Assay—A sample portion was taken, diluted if necessary, and titrated on an Aminco chloride titrator at one of the three titration speeds. The titrator can accurately determine a minimum of 9 mcg. of chloride ion in a sample. Accuracy was better than 0.1% and reproducibility was ±1%, with the entire determination taking less than 2 min. to perform.

UV Spectrophotometric Assay—Sample solutions from 1 to 2 mg./100 ml. in pH 2.31 phosphate buffer were read on the Cary 11 recording spectrophotometer from 400 to 225 mμ. Absorbances at 326, 278, and 236 mμ were recorded. Calculations were made with an IBM 1620 computer, using simultaneous equations with three equations and three unknowns.

TLC—TLC for qualitative studies was carried out on activated layers of silica gel GF_{254}, 250 μ thick, in two systems: water (saturated with *n*-butanol)–glacial acetic acid (4:1) and *n*-butanol–glacial acetic acid–water (4:3:3). All TLC was done at room temperature. The quantity of the compounds applied on TLC varied from 5 to 60 mcg. The limits of detection for each of the compounds were: NSC-82196, 0.5 mcg.; NSC-112970, 2 mcg.; and NSC-22419, 0.5 mcg. Spots were detected by UV quenching, I_2 vapor, aqueous 0.2% Bratton-Marshall reagent spray, and concentrated H_2SO_4 spray followed by heating.

Kinetic Studies—The degradation of NSC-82196 was monitored by the UV, colorimetric, and ionic chloride methods of assay. Various portions of NSC-82196 (depending upon the assay method used) were dissolved in pH 2.31 phosphate buffer[11] in 50-ml. volumetric flasks. The solutions were brought to volume at the temperature of the bath. The flasks were then immersed to 90% of their length in the thermostatically controlled bath at 25 ± 0.1° and exposed to the light and dark. The light samples were exposed to two fluorescent bulbs,[12] placed 15 cm. from the center of the volumetric flasks in the bath. At appropriate time intervals, portions were withdrawn for assay.

Stability of Parenteral Product—Amber glass vials of a lyophilized product were each reconstituted with 20 ml. of water for injection and stored in light and dark at 25° and in dark at 4°. All vials were reconstituted with water for injection at 25°. Sample portions

[4] John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., Maywood, N. J.
[5] American Instrument Co., Silver Spring, Md.
[6] Analtech, Inc., Wilmington, Del.
[7] Burton model 1910, obtained from Cavitron Corp., Van-Nuys, Calif., equipped with two Burton 1911 bulbs.
[8] Obtained from American Instrument Co., Inc., Silver Spring, Md.
[9] Courtesy of Laboratory of Physiology, National Cancer Institute, NIH, Bethesda, Md.
[10] Distributed by Brinkmann Instruments, Inc., Westbury, Long Island, N. Y.

[11] A 0.2 *M*, pH 2.31 phosphate buffer was chosen for the kinetic and stability studies because the parenteral formulation used in clinical trials, when reconstituted with water for injection, is pH 2.3. Also, samples below pH 2.5 were found to be in an optimum stability range as seen with the data on the effect of pH on stability reported by the University of Michigan (2). Since the authors were trying to mimic conditions of the actual formulation, pH 2.3 was chosen for the study.
[12] Westinghouse, 15W No. F15T8ID.

Table I—Results of UV Analyses of Three Mixtures of NSC-82196, NSC-112970, and NSC-22419 in pH 2.31 Phosphate Buffer

Mixtures	Added, mg./100 ml.	Found, mg./100 ml.	Error, %
NSC-82196	1.7380	1.7318	0.8
NSC-112970	1.0450	1.0678	2.2
NSC-22419	1.1360	1.0877	4.2
NSC-82196	0.6440	0.6574	2.0
NSC-112970	0.9280	0.9250	0.3
NSC-22419	0.8180	0.7711	5.7
NSC-82196	0.9111	0.9010	1.1
NSC-112970	0.7651	0.7530	1.6
NSC-22419	0.9121	0.9311	2.1

were withdrawn at appropriate time intervals and assayed by the UV method.

RESULTS AND DISCUSSION

TLC Studies—TLC confirmed the presence of NSC-22419 and NSC-112970 as degradation products of NSC-82196 in acidic aqueous media. The decomposition products had R_f values identical to authentic NSC-22419 and NSC-112970. However, only trace amounts of NSC-22419 were found in samples degraded in the dark, while large amounts of NSC-22419 were found in samples degraded with longwave UV light. No attempt was made to confirm the presence of the photodecomposition product, 5-diazoimidazole-4-carboxamide, because of its small buildup and concentration.

UV Method of Assay—The UV method of assay was specific for NSC-82196 and its decomposition products NSC-22419 and NSC-112970. Table I shows the results of three separate trials assaying mixtures of all three authentic materials. Figure 1 shows the UV spectrum of all three components in pH 2.31 phosphate buffer.

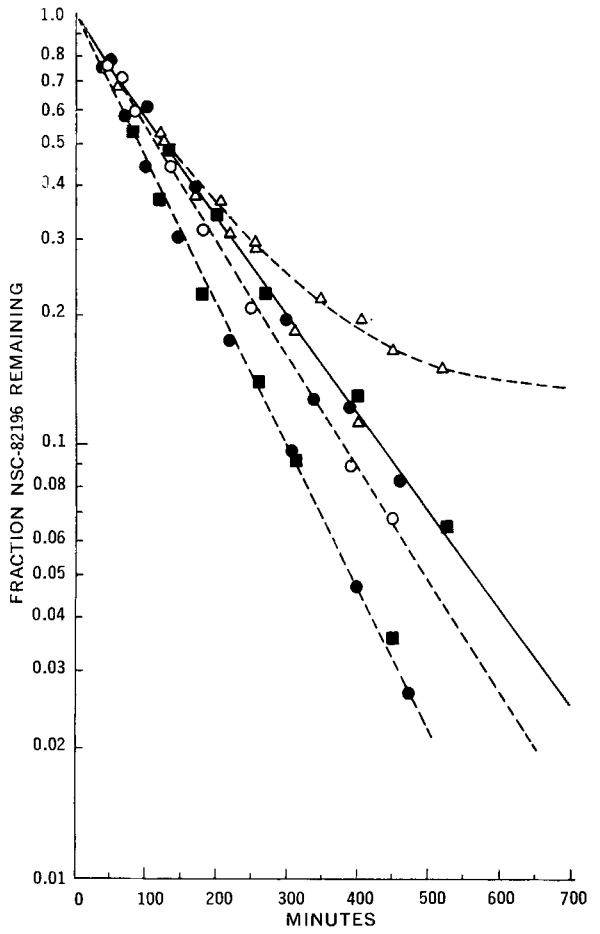


Figure 3—*Semilogarithmic plot of fraction NSC-82196 remaining* versus *time at 25.0°, pH 2.31 phosphate buffer, in light and dark. Key: dark, ——; light, - - -; UV, ●; colorimetric corrected, ■; colorimetric uncorrected, ○; and chloride, △.*

Table II—a Values (at pH 2.31) for NSC-82196, NSC-112970, and NSC-22419 at Three Wavelengths Used in the UV Assay

Mixtures	326 mμ	278 mμ	236 mμ
NSC-82196	635.2	230.3	252.0
NSC-112970	250.2	112.2	412.5
NSC-22419	000.0	263.8	255.0

The $\lambda_{max.}$ used for the calculations were 278 mμ (NSC-22419), 236 mμ (NSC-112970), and 326 mμ (NSC-82196).[13] The a values at pH 2.31, used to calculate the final formulas using simultaneous equations, are seen in Table II. All calculations were based on three components only.

Colorimetric Assay—Intact NSC-82196 follows Beer's law over the concentration range 1–12 mcg./ml. The a value was found to be 937.5 ± 1.4%. At low concentrations, less than 3 mcg./ml., the $\lambda_{max.}$ of the solution was seen to shift from 520 to 525–526 mμ. Samples of intact NSC-82196 developed for 30 min. in the dark

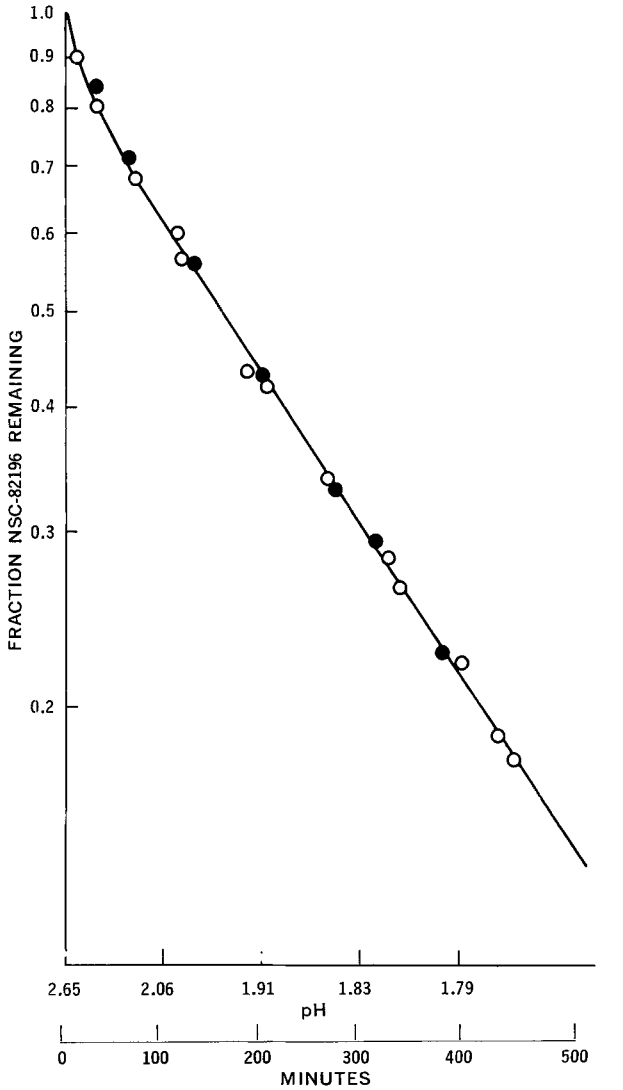


Figure 4—*Semilogarithmic plot of fraction NSC-82196 remaining* versus *time for reconstituted parenteral product at 25° in light* (○) *and dark* (●).

[13] The wavelength of 209 mμ for NSC-22419 recommended by the University of Michigan group (2) for quantitation could not be used in this laboratory due to limitations of the Cary 11 recording spectrophotometer.

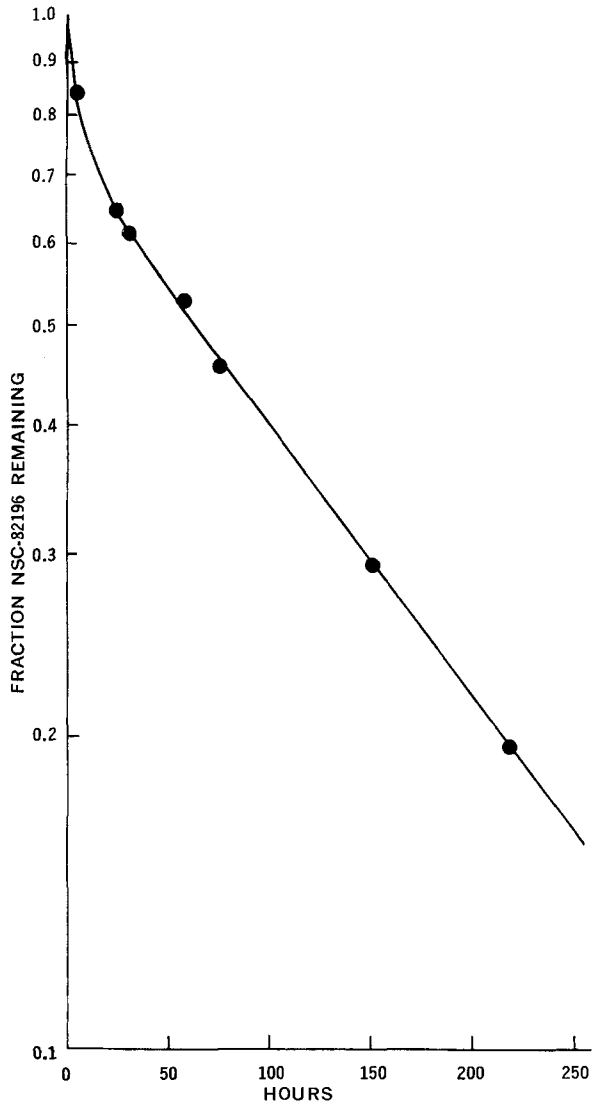


Figure 5—*Semilogarithmic plot of fraction NSC-82196 remaining* versus *time for reconstituted parenteral product at 4° in dark.*

demonstrated zero absorbance, thus indicating that no 5-diazoimidazole-4-carboxamide was formed in the absence of light. If diazoimidazole carboxamide was found, it would be indicative of what was already present at the time the sample was taken. Therefore, subtraction of the sample's absorbance from the irradiated sample's absorbance is necessary to obtain the corrected absorbance of the remaining intact NSC-82196 in the sample. This procedure is necessary since in a stability study with rigorous lighting conditions, a buildup of 5-diazoimidazole-4-carboxamide is expected.

Kinetic Studies—Apparent adherence to stoichiometry during the course of the reaction of NSC-82196 in acidic aqueous solution with time using the UV assay method is seen in Fig. 2. Samples exposed to light showed a significant amount of NSC-22419 formation. Table III shows the rate constants and half-lives obtained by the assay methods used. Figure 3 is a semilogarithmic plot of fraction NSC-82196 remaining *versus* time for all assay methods. With the exception of the ionic chloride method for light-stored samples, all assay methods used indicated that the degradation of NSC-82196 followed apparent first-order kinetics. All chloride calculations were based on the premise that NSC-82196 was degrading solely to NSC-112970; thus the chloride method would be expected to be invalid if anything other than NSC-112970 was being formed.

It can be seen in Fig. 3 that an error can be introduced if one does not account for existing 5-diazoimidazole-4-carboxamide at any given time when using the colorimetric method. The presence of a significant amount of the diazo compound also would be expected to introduce error into the UV results, since the UV calculations

Table III—First-Order Rate Constants and $t_{1/2}$ Values for Degradation of NSC-82196 in Phosphate Buffer at pH 2.31, 25.0°, in Light and Dark

Assay Method Used	Light		Dark	
	k min.$^{-1}$ × 10^3	$t_{1/2}$, min.	k min.$^{-1}$ × 10^3	$t_{1/2}$, min.
Colorimetric corrected	7.90 ± 3.13%	87.8	5.46 ± 4.3%	126.9
Colorimetric noncorrected	6.14 ± 3.12%	112.9	5.46 ± 4.3%	126.9
Chloride	Invalid		5.59 ± 2.8%	123.9
UV	7.75 ± 3.9%	89.5	5.30 ± 5.1%	130.7

allow for three components only in the system. In this study the amount of diazo compound existing at a given time in light samples was small (there was a maximum amount of 10% at $t_{1/2}$) and was calculated to result in less than 3% error in the concentration of NSC-82196 in a solution at $t_{1/2}$ by the UV method of assay.

Stability of Parenteral Product—Figures 4 and 5 are the semilogarithmic plots for the reconstituted parenteral product at 25 and 4°, respectively. No difference in stability of NSC-82196 was noted between those samples stored in light or dark at 25°, presumably due to the drug being stored in the amber glass vials.

The pH was found to change considerably in this unbuffered system. It is likely that the pH lowering causes apparent variance from first-order kinetics in both cases, since the drug in general has been reported to be more stable in acidic aqueous media (2). Also, water at 25° was used to reconstitute the 4° sample; thus a faster degradation rate would be expected in the first few minutes (Fig. 5) until the system actually comes to 4°.

The purpose of this experiment was to mimic conditions the product would actually encounter in clinical use. Therefore, the results are different than would be predicted from a controlled stability study carried out in a buffer system at constant pH. The apparent half-life of the parenteral product is approximately 150 min. at 25° and 65 hr. at 4°

CONCLUSIONS

Good agreement was found between the UV and colorimetric methods of assay. The University of Michigan's UV method of assay was found to be quite specific for NSC-82196 and its decomposition products, NSC-22419 and NSC-112970, but does not include the decomposition product 5-diazoimidazole-4-carboxamide. The University of Michigan group did not conduct their studies of the drug in light. Since the diazo compound is a photodegradation product of the drug, no allowances were made for it in the UV method of assay. However, as mentioned previously, even when there was a buildup of diazo compound to a maximum of 10% at $t_{1/2}$ in the light, this resulted in an error of 3% in the concentration of NSC-82196.

Values obtained from the colorimetric method would also be in error if any 5-diazoimidazole-4-carboxamide was present as a degradation product and not accounted for, as seen in Table III. Therefore, correction of the colorimetric method, as reported by Loo and Stasswender (6), is definitely needed in a study with rigorous lighting conditions where the buildup of diazo compound, although small, does occur.

The chloride method was found in agreement with the other two methods of assay when used for stability studies in which NSC-112970 was the only degradation product (samples protected from light). The chloride method is of no value in determining the absolute amount of NSC-82196 remaining in the sample because it is not specific for intact NSC-82196. Also, obvious problems arise if one attempts to use the chloride method to follow the stability of a hydrochloride salt of NSC-82196. Thus the chloride method is not recommended due to these serious limitations. By protecting the solutions from light, it appears that the 5-diazoimidazole-4-carboxamide formation can be inhibited. The degradation of the reconstituted parenteral product did not appear to follow first-order kinetics, possibly due to the pH decrease which accompanies the degradation. The apparent $t_{1/2}$ was 150 min. for amber glass vials

of the reconstituted hydrochloride salt of NSC-82196 stored at 25° in dark or light. The apparent $t_{1/2}$ was 65 hr. for the same vials stored at 4° in the dark.

REFERENCES

(1) G. Hoffman, I. Kline, M. Gang, D. Tyrer, J. Vendetti, and A. Goldin, *Cancer Chemother. Rep.*, **52**, 715(1968).

(2) *University of Michigan College of Pharmacy Interim Progress Report*, January 14, 1969.

(3) Y. F. Shealy, C. A. Krauth, L. B. Holum, and W. E. Fitzgibbon, *J. Pharm. Sci.*, **57**, 83(1968).

(4) Y. F. Shealy, C. A. Krauth, and J. A. Montgomery, *J. Org. Chem.*, **27**, 2150(1962).

(5) Y. F. Shealy, R. F. Struck, L. B. Holum, and J. A. Montgomery, *ibid.*, **26**, 2396(1961).

(6) T. L. Loo and E. A. Stasswender, *J. Pharm. Sci.*, **56**, 1016 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 23, 1970, from the *Pharmaceutical Development Service, Pharmacy Department, Clinical Center, National Institutes of Health, Bethesda, MD 20014*

Accepted for publication June 26, 1970.

TECHNICAL ARTICLES

Automated Dual Extraction Procedure for Analysis of Phenmetrazine Hydrochloride Tablets

S. AHUJA, C. SPITZER, and F. R. BROFAZI

Abstract □ An automated method of analysis, based on dual extraction of phenmetrazine hydrochloride, has been developed for analysis of phenmetrazine hydrochloride tablets. The method requires use of a continuous digestor along with other more familiar modules of an automatic analyzer. A total of 520 individual tablets was analyzed by this method. Of all the tablets analyzed, 99.8% were within ±10% of the indicated dosage and 100% were within ±15% of the indicated dosage.

Keyphrases □ Phenmetrazine HCl tablets—analysis □ Automated extraction procedure—phenmetrazine HCl analysis □ Diagram—automated system, phenmetrazine HCl analysis □ UV spectrophotometry—analysis

Automated analytical methods have been limited generally to dissolution and/or extraction of active ingredients from the formulation, filtration, and dilution to suitable concentration for analytical determinations. Manual methods involving evaporation of the organic solvent, followed by dissolution of the residue in suitable aqueous solvents, have been considered difficult to automate. One semiautomated approach to the solution of this problem has been reported by Feller *et al.* (1) for analysis of ethopabate in poultry feeds. This approach should provide a valuable means of automating pharmaceutical methods of analysis, where such extractions are frequently used.

A variety of manual methods (2–9) has been used for analysis of phenmetrazine hydrochloride.[1] For several years, a manual dual-extraction procedure (9) has been used in the authors' laboratories for analysis of 25-mg. tablets of this compound. The tedious and time-consuming manual procedure was automated by the use of the continuous digestor[2] along with other more familiar modules of the automatic analyzer.[2]

Table I—Recoveries and Precision with the Automated Method

Number	Percent Phenmetrazine Hydrochloride Recovered
1	99.2
2	101.2
3	100.4
4	103.2
5	101.2
6	98.0
7	101.2
8	103.2
9	99.2
10	100.4
Average	100.7
Relative *SD*	±1.7

EXPERIMENTAL

Materials and Methods—The flow diagram of the analytical system, including tubing sizes, is shown in Fig. 1. The Solidprep sampler is programmed to operate at a rate of 13 samples/hr. The sample (tablet) is deposited in a cup placed on the turntable of the sampler. In turn, each sample is dumped into the homogenizer and homogenized with 100 ml. of water; a 0.9-ml./min. sample (segmented with air) is pumped from the sampler. The sample is made alkaline with 1% w/v NaOH and filtered through a continuous filter. About 50% is resampled and extracted with chloroform, and the chloroform extract is washed with water and fed into the continuous digestor. Simultaneously, dilute hydrochloric acid (4 in 100)

[1] Preludin, Geigy Pharmaceuticals, Ardsley, N. Y.

[2] Technicon Corp., Tarrytown, N. Y.

of the reconstituted hydrochloride salt of NSC-82196 stored at 25° in dark or light. The apparent $t_{1/2}$ was 65 hr. for the same vials stored at 4° in the dark.

REFERENCES

(1) G. Hoffman, I. Kline, M. Gang, D. Tyrer, J. Vendetti, and A. Goldin, *Cancer Chemother. Rep.*, **52**, 715(1968).

(2) *University of Michigan College of Pharmacy Interim Progress Report*, January 14, 1969.

(3) Y. F. Shealy, C. A. Krauth, L. B. Holum, and W. E. Fitzgibbon, *J. Pharm. Sci.*, **57**, 83(1968).

(4) Y. F. Shealy, C. A. Krauth, and J. A. Montgomery, *J. Org. Chem.*, **27**, 2150(1962).

(5) Y. F. Shealy, R. F. Struck, L. B. Holum, and J. A. Montgomery, *ibid.*, **26**, 2396(1961).

(6) T. L. Loo and E. A. Stasswender, *J. Pharm. Sci.*, **56**, 1016 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 23, 1970, from the *Pharmaceutical Development Service, Pharmacy Department, Clinical Center, National Institutes of Health, Bethesda, MD 20014*

Accepted for publication June 26, 1970.

TECHNICAL ARTICLES

Automated Dual Extraction Procedure for Analysis of Phenmetrazine Hydrochloride Tablets

S. AHUJA, C. SPITZER, and F. R. BROFAZI

Abstract □ An automated method of analysis, based on dual extraction of phenmetrazine hydrochloride, has been developed for analysis of phenmetrazine hydrochloride tablets. The method requires use of a continuous digestor along with other more familiar modules of an automatic analyzer. A total of 520 individual tablets was analyzed by this method. Of all the tablets analyzed, 99.8% were within ±10% of the indicated dosage and 100% were within ±15% of the indicated dosage.

Keyphrases □ Phenmetrazine HCl tablets—analysis □ Automated extraction procedure—phenmetrazine HCl analysis □ Diagram—automated system, phenmetrazine HCl analysis □ UV spectrophotometry—analysis

Automated analytical methods have been limited generally to dissolution and/or extraction of active ingredients from the formulation, filtration, and dilution to suitable concentration for analytical determinations. Manual methods involving evaporation of the organic solvent, followed by dissolution of the residue in suitable aqueous solvents, have been considered difficult to automate. One semiautomated approach to the solution of this problem has been reported by Feller *et al.* (1) for analysis of ethopabate in poultry feeds. This approach should provide a valuable means of automating pharmaceutical methods of analysis, where such extractions are frequently used.

A variety of manual methods (2–9) has been used for analysis of phenmetrazine hydrochloride.[1] For several years, a manual dual-extraction procedure (9) has been used in the authors' laboratories for analysis of 25-mg. tablets of this compound. The tedious and time-consuming manual procedure was automated by the use of the continuous digestor[2] along with other more familiar modules of the automatic analyzer.[2]

Table I—Recoveries and Precision with the Automated Method

Number	Percent Phenmetrazine Hydrochloride Recovered
1	99.2
2	101.2
3	100.4
4	103.2
5	101.2
6	98.0
7	101.2
8	103.2
9	99.2
10	100.4
Average	100.7
Relative *SD*	±1.7

EXPERIMENTAL

Materials and Methods—The flow diagram of the analytical system, including tubing sizes, is shown in Fig. 1. The Solidprep sampler is programmed to operate at a rate of 13 samples/hr. The sample (tablet) is deposited in a cup placed on the turntable of the sampler. In turn, each sample is dumped into the homogenizer and homogenized with 100 ml. of water; a 0.9-ml./min. sample (segmented with air) is pumped from the sampler. The sample is made alkaline with 1% w/v NaOH and filtered through a continuous filter. About 50% is resampled and extracted with chloroform, and the chloroform extract is washed with water and fed into the continuous digestor. Simultaneously, dilute hydrochloric acid (4 in 100)

[1] Preludin, Geigy Pharmaceuticals, Ardsley, N. Y.

[2] Technicon Corp., Tarrytown, N. Y.

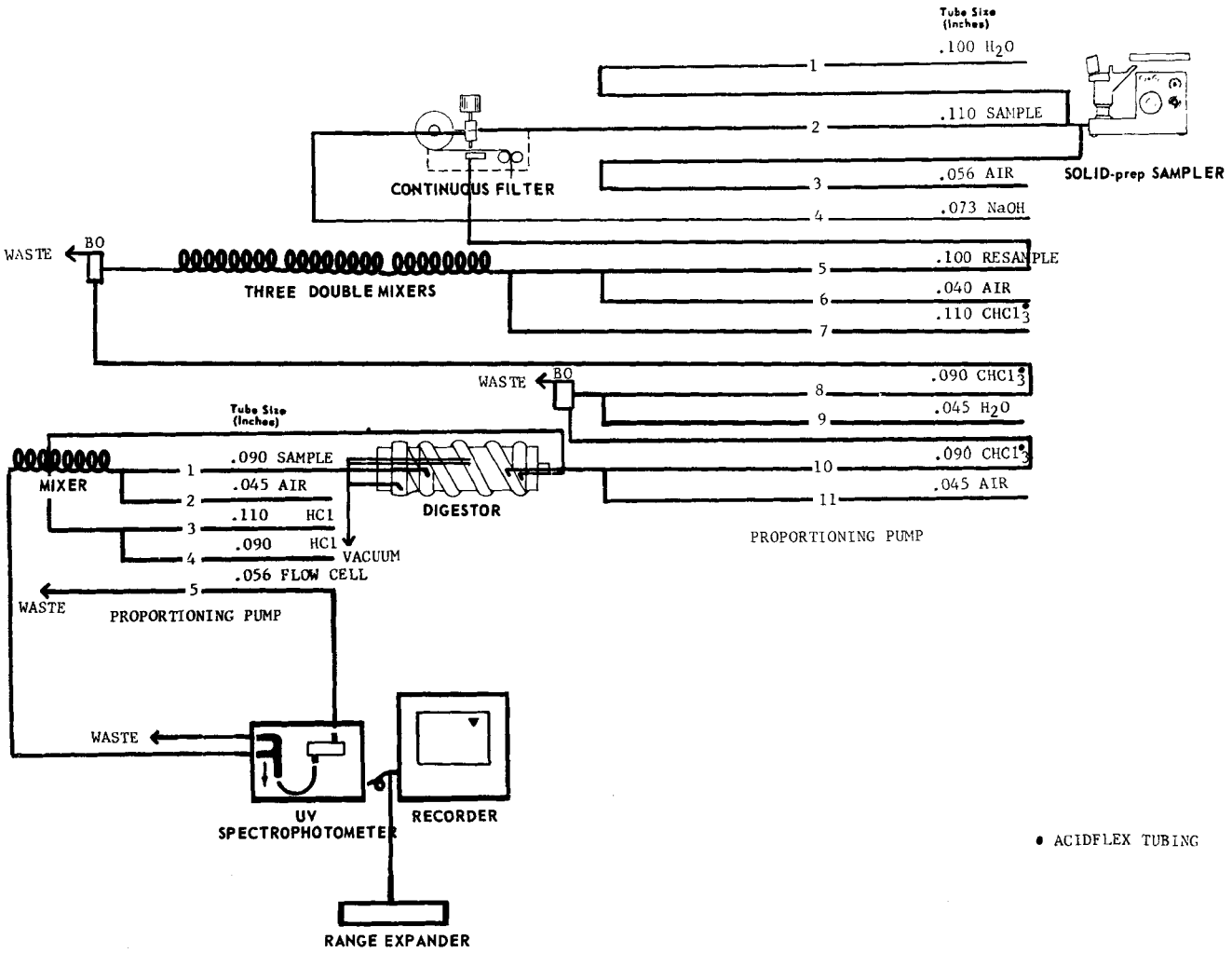


Figure 1—*Flow diagram of the analytical system.*

is fed into the continuous digestor where chloroform is evaporated in a heated rotating helix, with subsequent solution of the residue in the dilute hydrochloric acid. A portion of the dilute hydrochloric acid solution is sampled on the other end of the rotating helix (the rest of the sample and chloroform are sent to waste) and passed through a mixing coil; its absorbance is determined at 256 mμ in a 15-mm. tubular flow cell with the range expander set at the 2× setting. Standards are run at various intervals to provide a means for calculation of sample concentration and to check any instrumental fluctuations.

RESULTS AND DISCUSSION

Tablets are initially extracted with water instead of the dilute hydrochloric acid (4 in 100) used in the manual method. The latter solvent was undesirable for use with the automatic analyzer. After investigating various alternate solvents, it was found that water was the solvent of choice because of its greater suitability for further extractions.

In the manual method, 50% NaOH w/v solution is used for alkalinization of dilute hydrochloric acid (4 in 100) prior to chloroform extraction. The use of such a strong alkaline solution was considered neither desirable nor necessary in the automated method. A much lower concentration, such as a 1% w/v NaOH solution, was found very suitable for alkalinization in the automated method.

Beaded mixing coils are generally used for liquid/liquid extraction in automated methods. For this work, these coils were found unusable because they yielded very heavy emulsions which could not be resolved. This problem was solved by using three mixing coils in a row along with two separators. The first separator provides the separation of chloroform from the aqueous phase. In the second separator, chloroform is washed with water, whereby emulsion problems are completely eliminated.

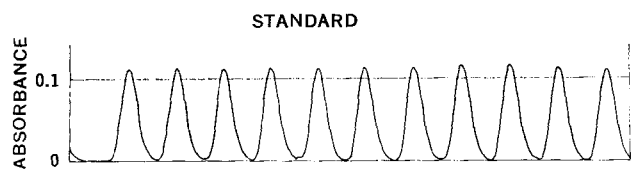


Figure 2—*Typical recording of phenmetrazine tablets, 25 mg.*

Table II—Comparison of Results Obtained by Automated and Manual Methods

Sample	Phenmetrazine Hydrochloride, mg. Automated Method	Manual Method	Deviation, mg.
A	25.0	25.3	−0.3
B	24.8	24.8	0.0
C	24.3	24.0	+0.3
D	23.8	23.9	−0.1
E	24.4	25.1	−0.7
F	24.7	24.3	+0.4
G	24.3	24.6	−0.3
H	25.1	24.7	+0.4
I	24.5	24.6	−0.1
J	24.6	24.5	+0.1
Average	24.6	24.6	±0.3

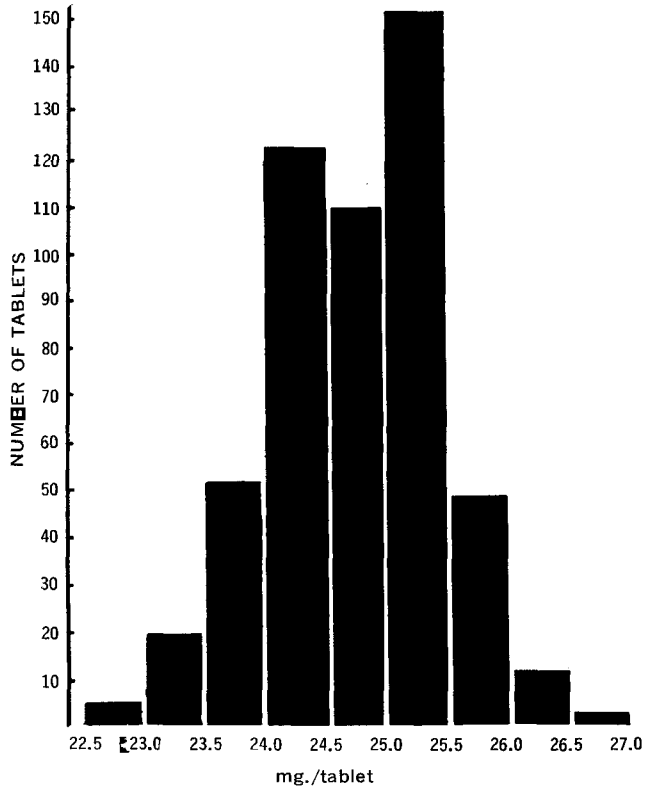


Figure 3—*Histogram of phenmetrazine tablets, 25 mg.*

The second extraction step, involving reextraction of phenmetrazine into dilute hydrochloric acid (4 in 100) from chloroform, is automated by the use of the continuous digestor. This is accomplished in a heated rotating helix. The chloroform and the dilute hydrochloric acid are introduced in such a proportion as to create thin layers of both of these phases. The organic phase is volatilized by heat and vacuum (which is applied on the other end), and the aqueous acid phase is aspirated into the automated system and processed as shown in Fig. 1. The use of the continuous digestor provides increased sensitivity (1) and eliminates the emulsification problems commonly encountered in manual liquid/liquid extraction methods.

A linear relationship was observed between the concentration of phenmetrazine hydrochloride and absorbance in the range of concentration studied. The precision and recoveries obtained by the method were checked by running "synthetic" formulations (prepared in a manner similar to the commercial formulation). The percentage recovery results on the synthetic formulation are shown in Table I. No significant interference from the excipients was observed when placebos of this formulation were run by this method.

A typical recording is shown in Fig. 2. The average of 10 individual tablets compared favorably with the results obtained manually (Table II), thus suggesting that the automated method provides an acceptable alternate means of assaying phenmetrazine hydrochloride in phenmetrazine hydrochloride tablets.

To study tablet-to-tablet variation, a very large number of individual tablets was analyzed by these methods. The tablets used were selected randomly from several production batches to obtain a good overall picture of tablet-to-tablet variation. The distribution of dosage in these tablets is presented in the form of a histogram (Fig. 3). Of all the tablets analyzed, 99.8% were within ±10% of the indicated dosage and 100% were within ±15% of the indicated dosage (±15% is the present limit in the NF for content uniformity of tablets).

REFERENCES

(1) B. Feller, W. A. Boyd, B. E. DiDario, and A. Ferrari, "Automation in Analytical Chemistry," Mediad Inc., White Plains, N. Y., 1967, p. 206.

(2) E. Vidic and J. Schuette, *Arch. Pharm.*, **295,** 342(1962).

(3) E. Smith, L. F. Worrell, and J. E. Sinsheimer, *Anal. Chem.*, **35,** 58(1963).

(4) J. Kracmar and J. Pokarny, *Cesk. Farm.*, **11,** 7(1962).

(5) W. N. French and J. R. Truelove, *J. Pharm. Sci.*, **54,** 306 (1965).

(6) H. Kolb and P. W. Patt, *Arzneim.-Forsch.*, **15,** 924(1965).

(7) A. Heyndrickse and A. de Leenheer, *J. Pharm. Belg.*, **22,** 109 (1967).

(8) G. P. Quinn, M. M. Cohn, M. B. Reid, P. Greengard, and M. Weiner, *Clin. Pharmacol. Ther.*, **8,** 369(1967).

(9) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, p. 542.

ACKNOWLEDGMENTS AND ADDRESSES

Received May 1, 1970, from the *Analytical Research and Development Section, Quality Control Department, Geigy Pharmaceuticals Ardsley, NY 10502*

Accepted for publication July 2, 1970.

Complexes of Ergot Alkaloids and Derivatives V: Interaction of Methysergide Maleate and Caffeine in Aqueous Solution

M. A. ZOGLIO* and H. V. MAULDING

Abstract □ Evidence of complex formation between methysergide maleate and caffeine is presented, utilizing solubility and vapor pressure osmometer information. The effect of this interaction on the rate of dissolution and partitioning-rate behavior of methysergide maleate has been measured. The absorption consequences of this interaction as implied from the *in vitro* studies are presented.

Keyphrases □ Methysergide maleate, caffeine complexes—interactions in aqueous solutions, effect on dissolution, partition rates □ Vapor pressure osmometry—complex formation data, methysergide maleate, caffeine □ Ergot alkaloids, derivatives—complexes, methysergide, caffeine

The successful utilization of complexing phenomena to enhance the absorption of proteinaceous ergot alkaloids (1, 2) prompted an investigation into the possible interaction of a synthetic nonproteinaceous ergot alkaloid with caffeine. The drug, methysergide maleate,[1] (+)-9,10-didehydro-*N*-[1-(hydroxymethyl)propyl]-1,6-dimethylergoline-8β-carboxamide maleate, was studied with caffeine in aqueous media, utilizing changes in apparent solubility and vapor pressure osmometry to detect complexation. After establishing an interaction, the pH dependency of the interaction was examined. Studies of the consequence of the interaction on *in vitro* dissolution and partition rates were undertaken to gain some insight into potential *in vivo* absorption effects.

EXPERIMENTAL

Materials—Methysergide maleate[2] (mol. wt. 469.54) showed only trace contaminants when subjected to TLC. Anhydrous caffeine powder USP,[3] m.p. 238°, was utilized in this study.

Reagent grade chloroform[4] was employed in the partitioning-rate studies. A pH 6.65 buffer was made by dissolving 13.6 g. KH_2PO_4 in 500 ml. water, adjusting the pH with concentrated KOH, and diluting to 1 l. (ionic strength 0.2).

The pH measurements were made on a Metrohm pH meter, and spectrophotometric data were obtained from a Cary model 14 spectrophotometer.

Solubility Studies—Methysergide maleate, 1.5 g., was placed in watertight, amber, screw-capped vials (50 ml.) containing 10 ml. of either distilled water, 0.1 *M* pH 6.65 phosphate buffer, or 0.1 *N* HCl, and varying quantities of anhydrous powdered caffeine were added. The vials were clamped onto the edge of metal disks (15-cm. diameter) mounted on a motor-driven shaft and rotated vertically at 6 r.p.m. in a constant-temperature bath, 30 ± 0.1°. After 24 hr., samples were taken using pipets with filters attached and were analyzed for methysergide maleate by UV spectrophotometry (325 mμ).

[1] Sansert, Sandoz Pharmaceuticals, Hanover, N. J.
[2] Sandoz A.-G., Basel, Switzerland.
[3] Chas. Pfizer Co.
[4] Mallinckrodt.

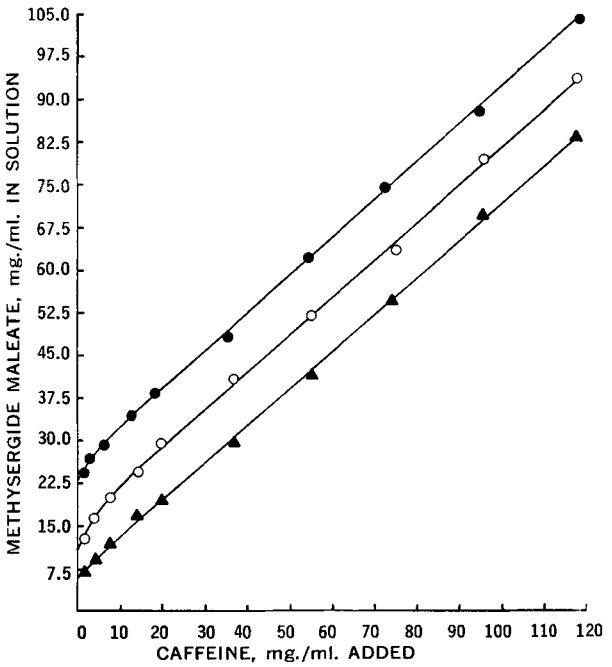


Figure 1—*Apparent solubility changes for methysergide maleate as a function of added caffeine. Key:* ●, *0.1* N *HCl, pH = 1.25;* ○, *distilled water; and* ▲, *0.1* M *phosphate buffer, pH = 6.65.*

Vapor Pressure Osmometry—A model 301A Mecrolab (Hewlett-Packard) vapor pressure osmometer was employed in these studies. The instrument was calibrated using solutions of raffinose in distilled water. Forty-eight hours was allowed for thermal equilibration before measurements were taken. Samples were inserted into the chamber containing the thermistor beads 3 hr. prior to reading. Solutions were prepared from the same lot of deionized distilled water. The reservoir of water used in the measuring chamber was also taken from this lot and changed after each series of five readings with subsequent reequilibration of the instrument.

Partitioning Studies—A solution was prepared by placing methysergide maleate, 100 mg., in 950 ml. of pH 6.65 phosphate buffer, stirring magnetically for 30 min. to 1 hr., and filtering (Whatman No. 1 filter paper) into a flask immersed in a water bath maintained at 30°. Finally, pH 6.65 buffer was added to make 1 l. of solution. Five hundred milliliters of this solution was kept, and 500 ml. had caffeine (5 g.) added to it.

Fifteen milliliters of the freshly prepared aqueous phase—either with or without xanthine—was added carefully to 15 ml. chloroform in screw-capped vials (50 ml.). The vials were sealed and rotated at 6 r.p.m. in a 30 ± 0.1° water bath. Five-milliliter samples were taken at 3, 5, 7, 9, 11, 13, and 15 min. from the aqueous phase and analyzed for methysergide maleate by UV spectrophotometry.

Dissolution Rates—A 6- or 25-r.p.m. stirrer motor, fitted with a 2.54-cm. propeller blade placed 4 cm. from the bottom of an 800-ml. beaker containing 500 ml. 0.1 *N* HCl solution, was employed for these determinations. Methysergide maleate, 50 mg., or the alkaloid in combination with caffeine, prepared by mixing 50 mg.

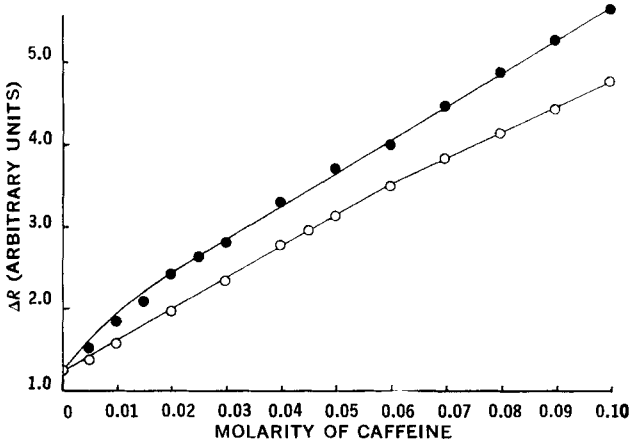


Figure 2—*Vapor pressure osmometer measurements for aqueous solutions of caffeine and combined caffeine–0.01* M *methysergide maleate. Key:* ●, *caffeine solution measurements added to measurement for a 0.01* M *methysergide maleate solution; and* ○, *measurements for combined caffeine–0.01* M *methysergide maleate solutions.*

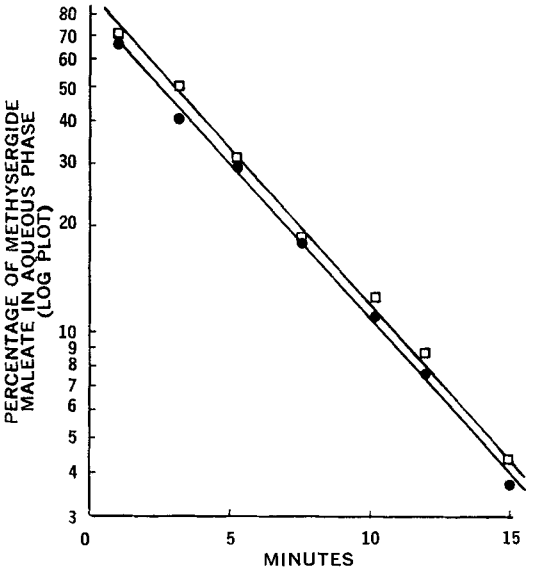


Figure 3—*Methysergide maleate–caffeine (1:100 w/w ratio). Partitioning-rate experiment in pH 6.65 0.1* M *phosphate buffer. Key:* ●, *methysergide maleate in the presence of caffeine; and* □, *methysergide maleate.*

alkaloid with 5 g. caffeine in a mortar, was placed into the stirred solution from a height of about 1.5 cm. The temperature was maintained at 37 ± 0.1° by immersing the beaker in a constant-temperature bath. Samples were withdrawn periodically for UV analysis of methysergide maleate.

RESULTS AND DISCUSSION

Methysergide maleate showed some unexpected changes in apparent solubility when combined with caffeine. The intact proteinaceous alkaloids previously studied (1–3) had all required high ratios of xanthines to effect solubilization, demonstrated a pH dependency for complexation, and gave nonlinear changes in solubility as a function of added caffeine. Methysergide maleate (Fig. 1) shows a linear dependency of apparent solubility upon the amount of caffeine added. This has been suggested to imply a 1:1 relationship between the two compounds (4, 5). These changes are apparently pH-independent because the slopes are parallel. Their displacement only reflects the variation of inherent methysergide solubility at different pH values. The difference in behavior of this ergot alkaloid derivative indicates that the cyclic polypeptide moiety present in the naturally occurring alkaloids plays an important role in their complexing behavior.

As further evidence of interaction, vapor pressure osmometry studies were performed in a manner similar to that described by Goyan and Borazan (6). These measurements differ from those of Goyan and Borazan in that the units are not expressed in ohms but rather in arbitrary units of resistance change. Since the studies were used solely to determine interaction, the units read from the instrument, which are proportional to the resistance change in ohms, were found to be sufficient.

In Fig. 2, there are two noteworthy observations. First, note the evidence of interaction from the decreased values of ΔR when solutions of caffeine in 0.01 *M* methysergide are compared to expected values. These lower values of ΔR reflect a change in the colligative properties indicative of association of molecules. Second, the breaks in the continuity of the curves appear to support the suggestion that these inflections relate in some manner to the nature of the interaction. The complexing capacity number, as indicated by Fig. 2, is approximately 3.0, which is close to the value found for sodium salicylate–caffeine interaction (6). Although the significance of complexing capacity numbers has not yet been well established, the indication here is that the methysergide maleate–caffeine and sodium salicylate–caffeine interactions are related in some manner.

After establishing an interaction, the effect of this interaction on parameters related to drug absorption was studied. The effect of the interaction on dissolution rate, which had been quite dramatic in previous studies (1–3), was negligible for methysergide maleate. Since the drug has a greater inherent solubility than the ergot alkaloids, an attempt to demonstrate dissolution enhancement by slowing the stirrer speed to 6 r.p.m. was tried but was unsuccessful. The partitioning behavior, which had paralleled *in vivo* absorption effects (2, 3), also was negligible for caffeine–methysergide maleate combinations (Fig. 3). The *in vitro* studies, therefore, suggest that the stronger interaction demonstrated in this study is possibly a deleterious factor when attempting absorption enhancement through the use of complexing agents.

REFERENCES

(1) M. A. Zoglio, H. V. Maulding, and J. J. Windheuser, *J. Pharm. Sci.*, **58**, 222(1969).

(2) M. A. Zoglio and H. V. Maulding, *ibid.*, **59**, 215(1970).

(3) H. V. Maulding and M. A. Zoglio, *ibid.*, **59**, 384(1970).

(4) *Ibid.*, to be published.

(5) K. A. Connors and J. A. Mollica, *J. Pharm. Sci.*, **55**, 772 (1966).

(6) F. M. Goyan and H. N. Borazan, *ibid.*, **57**, 861(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Pharmacy Research and Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication June 12, 1970.

The technical assistance of David Murdock, Josephine Nazareno, Alison Bossert, and Virginia Nieman is gratefully acknowledged.

* Present address: American Hoechst Corp., Cincinnati, Ohio.

Substituted Benzamides with Potential CNS-Depressant and Hypotensive Activity

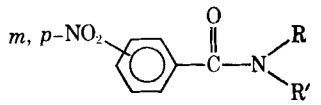
W. D. ROLL

Abstract □ A series of 14 *N*-alkyl, *N*-aryl, and *N*-aralkyl analogs of *N*-cyclohexyl nitrobenzamide has been synthesized and characterized. The compounds have been studied for their ability to depress the spontaneous motor activity of mice and for their hypotensive activity in rats. Four of the compounds, which are structurally characterized by small alkyl groups attached to the amide nitrogen and a *p*-nitro group, showed the greatest CNS-depressant activity. The hypotensive action of these compounds paralleled their depressant action.

Keyphrases □ Nitrobenzamide, *N*-cyclohexyl—synthesis, *N*-alkyl, *N*-aryl, *N*-aralkyl analogs; evaluation, CNS-depressant activity □ CNS-depressant activity—synthesis, pharmacological evaluation of *N*-cyclohexyl nitrobenzamide analogs.

In previous publications (1, 2) the synthesis and pharmacological evaluation of two series of ring-substituted benzamides of *N*-(β-cyanoethyl)- and *N*-(β-hydroxyethyl)-*N*-cyclohexylamine were reported. The biological activity of these compounds was studied in mice and rats for their effect on spontaneous motor activity and blood pressure. They were found to exert a depressant action on the spontaneous activity of mice at a 4–5-mg./kg. dosage level and to effect a prolonged reduction in blood pressure of normotensive rats at this dosage. Of the β-cyanoethyl analogs, the most active compounds were the *p*-chlorobenzamides and *p*-methoxybenzamides.

This paper reports a convenient synthesis and preliminary pharmacological evaluation for a series of amides of *m*-nitro- and *p*-nitro-substituted benzoic acids represented by the formula (I):



R = cyclohexyl; R′ = alkyl, aryl, or aralkyl

I

To study the *in vivo* effects of the test compounds, C_3H mice weighing between 20–25 g. were used. All test compounds were dissolved in propylene glycol and administered orally and intraperitoneally. Dose–response curves were obtained using eight mice (rats) at each of four dosage levels, and the median effective dose (ED_{50}) was calculated.

Concurrent with the toxicity range studies in mice and rats, careful gross observation of the intact animals was made at several dosage levels, and signs of CNS depression (decreased spontaneous motor activity, *etc.*) were noted. The general type of pharmacological activity encountered in these compounds was predominantly evinced by their effects on the CNS.

At a dose of 5 mg./kg., i.p., the test compounds caused sedation without sleep in mice and rats. The animals developed a persistent state of tranquility within 10 min. following administration, and this activity persisted for several hours. The animals could be handled with essentially no resistance, although they were otherwise seemingly unaltered. They were calm but

Table I—Effects on Spontaneous Activity in Mice

Compound Number	Dose, mg./kg.	Reduction in Spontaneous Activity, %
Chlorpromazine	1.0	2.5
	3.0	42.4
	5.0	60.0
	7.0	75.2
	1.0	32.4
	3.0	72.0
	5.0	91.1
	7.0	—
2	1.0	23.1
	3.0	62.9
	5.0	80.4
	7.0	95.2
3	1.0	30.8
	3.0	66.4
	5.0	86.0
	7.0	—
4	1.0	4.5
	3.0	44.1
	5.0	61.9
	7.0	76.5
5	1.0	32.0
	3.0	73.9
	5.0	93.4
	7.0	—
6	1.0	0.5
	3.0	39.0
	5.0	57.2
	7.0	72.0
7	1.0	32.5
	3.0	71.8
	5.0	89.6
	7.0	—
8	1.0	18.5
	3.0	57.6
	5.0	75.8
	7.0	90.4
9	1.0	18.5
	3.0	58.4
	5.0	76.0
	7.0	91.0
10	1.0	6.0
	3.0	45.2
	5.0	63.1
	7.0	76.8
11	1.0	18.8
	3.0	58.1
	5.0	76.5
	7.0	90.9
12	1.0	20.2
	3.0	60.0
	5.0	77.4
	7.0	92.5
13	1.0	—
	3.0	36.1
	5.0	54.2
	7.0	69.0
14	1.0	23.8
	3.0	63.4
	5.0	80.4
	7.0	95.2

Table II—Hypotensive Activity

Compound Number	Minutes following Administration 15	30	60	90	120
	Blood Pressure, % Reduction of Control				
1	50.0	28.6	20.5	15.0	0.0
3	49.4	32.0	22.6	14.2	2.5
5	50.4	31.6	20.8	12.5	3.1
7	48.3	30.2	20.0	5.1	0.0

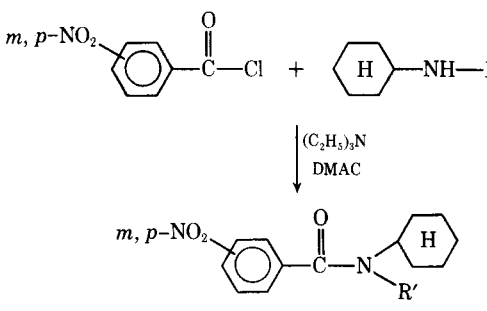
alert to attention. The animals walked normally and their movements were coordinated. Respiration was reduced and somewhat irregular during the 1st hr. following administration. In comparison to control animals, urination and defecation seem to be suppressed, which may be indicative of antispasmodic activity. Side reactions such as tremors were not noted in this study. The test compounds potentiated the sedative activity of barbiturates (amobarbital, pentobarbital, and secobarbital) and reserpine.

The most active compounds were Compounds 1, 3, 5, and 7 (Tables I and II). The *p*-nitro analogs were generally more potent depressants than the *m*-nitro derivatives, except those compounds where R′ (Table III) contained an aromatic ring (Compounds 11–14). Analogs where R′ was small (C_1–C_3) had the greatest CNS-depressant and blood pressure-depressor activity. Compounds that contained an OH functional group at R′ (Compounds 7, 8, 13, and 14) had a shorter duration of action. In all cases, the compounds' relative depressor activity paralleled their depressant activity (Table II).

PHARMACOLOGICAL RESULTS

Spontaneous Activity—The depressant activity of the test compounds was determined in mice with actophotometers.[1] The total body movements of single animals were measured at 15-min. intervals for a period of 1 hr. The mice were placed in the photocell unit immediately after intraperitoneal administration of the test compounds. Eight animals were used to study the effect of each compound at each dosage level, and the mean activity of each series of test animals was compared with the mean activity of a comparable number of control animals to ascertain the percent reduction in activity.

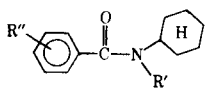
Hypotensive Activity—Indirect blood pressure measurements were performed using a photoelectric tensometer.[1] The test compounds were administered intraperitoneally to normotensive Wistar rats, and the systolic blood pressure was determined for a period of 2 hr. The mean response of eight test animals was used to determine the percent reduction in blood pressure produced by each test compound.



Scheme I

[1] Metro Industries, Inc., New York, N. Y.

Table III—Substituted Nitrobenzamides



Compound No.	R″ R′	Yield, %	M.p.	Anal., % Calcd.	Found
1	*p*-NO_2—CH_3	76.5	102.5°	C, 64.11 H, 6.92	C, 64.40 H, 6.95
2	*m*-NO_2—CH_3	72.2	94.5°	C, 64.11 H, 6.92	C, 64.35 H, 6.91
3	*p*-NO_2—C_2H_5	70.5	72.3°	C, 65.44 H, 6.96	C, 65.40 H, 6.97
4	*m*-NO_2—C_2H_5	72.3	45.0°	C, 65.44 H, 6.96	C, 65.46 H, 6.94
5	*p*-NO_2—$CH(CH_3)_2$	69.5	107.8°	C, 66.18 H, 7.64	C, 65.99 H, 7.67
6	*m*-NO_2—$CH(CH_3)_2$	74.8	35.6°	C, 66.18 H, 7.64	C, 66.10 H, 7.61
7	*p*-NO_2—CH_2CH_2—OH	75.0	210.5°	C, 61.63 H, 6.90	C, 61.59 H, 6.93
8	*m*-NO_2—CH_2OH—OH	74.2	Oil[a]	C, 61.63 H, 6.90	C, 61.66 H, 6.91
9	*p*-NO_2—CH_2CH_2CN	72.5	120.1°	C, 63.77 H, 6.35	C, 63.82 H, 6.30
10	*m*-NO_2—CH_2CH_2CN	69.6	93.1°	C, 63.77 H, 6.35	C, 63.90 H, 6.38
11	*p*-NO_2—C_6H_5	71.3	132.4°	C, 70.37 H, 6.21	C, 70.39 H, 6.20
12	*m*-NO_2—C_6H_5	75.0	105.1°	C, 70.37 H, 6.21	C, 70.31 H, 6.19
13	*p*-NO_2 —CH_2—CH(OH)—C_6H_5	65.8	126.1°	C, 68.46 H, 6.57	C, 68.40 H, 6.55
14	*m*-NO_2 —CH_2—CH(OH)—C_6H_5	66.9	25.2°	C, 68.46 H, 6.57	C, 68.55 H, 6.60

[a] Purified by chromatography (eluted by petroleum ether) on silica gel. TLC showed single spot.

EXPERIMENTAL

Melting points were determined using a Mettler FP-1 melting- and boiling-point apparatus. Analyses for carbon and hydrogen were obtained with a Coleman C-H analyzer; IR spectra were obtained with a Perkin-Elmer spectrophotometer model 137-B in KBr, and UV spectra were obtained with a Coleman model 124 Hitachi double-beam grating spectrophotometer. IR and UV spectra were as expected.

General Method of Preparation of Amides—Equimolecular amounts of *m*- and *p*- nitrobenzoyl chloride and *N*-substituted cyclohexylamine (Abbott Laboratories, North Chicago, Ill.) were dissolved in purified dimethylacetamide. The nitrobenzoyl chloride solution was then added to a cooled, well-stirred mixture of the substituted cyclohexylamine solution and triethylamine (Scheme I). When the addition of the acyl chloride solution was completed, the crude substituted benzamide was precipitated by the addition of ice water and collected on a filter. The crude product was recrystallized from EtOH/H_2O or Skelly Solve C/Skelly Solve B to give the pure compounds listed in Table III.

REFERENCES

(1) W. D. Roll, *J. Pharm. Sci.*, **57**, 1671(1968).
(2) W. D. Roll, *J. Med. Chem.*, **13**, 303(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Department of Medicinal Chemistry, College of Pharmacy, University of Toledo, Toledo, OH 43606*

Accepted for publication June 15, 1970.

Cactus Alkaloids VIII: Isolation of *N*-Methyl-3,4-dimethoxy-β-phenethylamine from *Ariocarpus fissuratus* var. *fissuratus*

D. G. NORQUIST and J. L. McLAUGHLIN

Abstract □ *Ariocarpus fissuratus* var. *fissuratus* (Engelmann) K. Schumann, commonly known as living rock, chaute, peyote, sunami, or dry whiskey, has an interesting history of folkloric medicinal uses. In previous phytochemical studies the alkaloids, hordenine and *N*-methyltyramine, have been identified in both the *fissuratus* and *lloydii* varieties of the species. In the present investigation, a new alkaloid was crystallized from the nonphenolic alkaloid fractions and identified as *N*-methyl-3,4-dimethoxy-β-phenethylamine HCl. This compound has previously been reported in three other cactus species.

Keyphrases □ *N* - Methyl - 3,4-dimethoxy - β - phenethylamine—isolation from *Ariocarpus fissuratus* □ *Ariocarpus fissuratus*—*N*-methyl-3,4-dimethoxy-β-phenethylamine isolation □ Alkaloids, cactus—isolation, identification of constituents

Several authors have reported that the cactus, *Ariocarpus fissuratus* (Engelmann) K. Schumann, has had various folkloric medicinal uses among the Indians of Mexico and the southwestern United States (1–4). Sometimes known as living rock, chaute, sunami, and pezuña de venado, the additional common names of peyote, peyote cimarron, and dry whiskey suggest a use similar to that of *Lophophora* (5, 6). References to the intoxicating and stimulating attributes of the plant are especially interesting (2–4). Heffter (1) in 1894 isolated hordenine from *A. fissuratus* var. *fissuratus*, and McLaughlin (7) recently identified hordenine and *N*-methyltyramine in both the *fissuratus* and *lloydii* varieties of the species.

In the present investigation the nonphenolic alkaloids were resolved from the phenolics, hordenine and *N*-methyltyramine, by use of an anion-exchange resin. TLC analysis of the nonphenolic fraction identified the major constituent as *N*-methyl-3,4-dimethoxy-β-phenethylamine. After acid–base partitioning, this alkaloid was easily crystallized from the nonphenolic fraction as the hydrochloride. Several recrystallizations resulted in a product which gave a comparable melting point and no mixed melting-point depression with synthesized *N*-methyl-3,4-dimethoxy-β-phenethylamine HCl. IR spectra of the isolated and the synthetic compounds were indistinguishable.

Agurell (8) has recently identified traces of this new cactus alkaloid in *Coryphantha macromeris* (Engelmann) Britton and Rose var. *runyonii* L. Benson and has crystallized the compound from extracts of *Echinocereus merkeri* Hildmann (9). Speir *et al.* (10) have also recently crystallized this alkaloid from *Ariocarpus trigonus* (Weber) K. Schumann. A single pharmacological study (11) has indicated that *N*-methyl-3,4-dimethoxy-β-phenethylamine has slight activity in the depletion of cardiac norepinephrine, but the reputed stimulating or intoxicating activities of the plant are not likely attributable to this compound, especially in view of its low concentration in the plant.

EXPERIMENTAL

Living plants of *A. fissuratus* var. *fissuratus* were purchased[1] and matched the latest taxonomic descriptions (12). Sample plants are being maintained as greenhouse specimens.[2] The fresh plants were sliced, dried in a forced-air oven at 48°, and ground through a 6-mm. screen in a Wiley mill.

In a large continuous-extraction apparatus, 5.28 kg. of the plant material was defatted, basified, and extracted with chloroform; the residues from the chloroform extracts were purified, essentially as previously described (13), to produce a mixture of crude alkaloids. This mixture was separated into phenolic and nonphenolic fractions by the use of an anion-exchange resin (14). TLC of the nonphenolic fraction, using methods and solvent systems previously described (10), identified *N*-methyl-3,4-dimethoxy-β-phenethylamine as the major constituent.

The residue of the nonphenolic alkaloid fraction was dissolved in about 20 ml. of 1 *N* HCl, filtered, and extracted twice with equivalent volumes of both chloroform and ethyl ether. After basification of the aqueous solution to pH 9.5 with concentrated ammonium hydroxide, the alkaloids were extracted into chloroform through several partitionings. The chloroform residue was dissolved in absolute ethanol and acidified with 5% HCl (w/w) in absolute ethanol. The acidified solution was condensed under a stream of nitrogen to about 2 ml., ethyl ether was added to produce cloudiness, and the solution was placed in a freezer (−12°) to induce crystallization. The crystals were recovered by vacuum filtration and recrystallized 5 times from absolute ethanol–ethyl ether to a constant melting point (m.p. 139–140°; yield 186 mg., 0.004% of the dried material). A mixed melting point with synthetic *N*-methyl-3,4-dimethoxy-β-phenethylamine HCl (10) was not depressed, and the IR spectra (KBr pellets) of these compounds were essentially identical.

REFERENCES

(1) A. Heffter, *Arch. Exp. Pathol. Pharmakol.*, **34,** 65(1894).
(2) L. G. Kloesel, *Amer. J. Pharm.*, **130,** 307(1958).
(3) C. W. Pennington, "The Tarahumar of Mexico," University of Utah Press, Salt Lake City, Utah, 1963, pp. 152, 166, 171, 186.
(4) R. E. Schultes, *Bot. Mus. Leafl. Harvard Univ.*, **5,** 61(1937).
(5) R. E. Schultes, *Science*, **163,** 345(1969).
(6) R. E. Schultes, in "Ethnopharmacologic Search for Pyschoactive Drugs," D. Efron, Ed., U. S. Public Health Service Publication No. 1645, Washington, D. C., 1967, pp. 37, 38.
(7) J. L. McLaughlin, *Lloydia*, **32,** 392(1969).
(8) S. Agurell, *ibid.*, **32,** 206(1969).
(9) S. Agurell, J. Lundström, and A. Masoud, *J. Pharm. Sci.*, **58,** 1413(1969).
(10) W. W. Speir, V. Mihranian, and J. L. McLaughlin, *Lloydia*, **33,** 15(1970).
(11) J. W. Daly, C. R. Creveling, and B. Witkop, *J. Med. Chem.*, **9,** 273(1966).
(12) E. F. Anderson, *Cactus Succulent J.*, **37,** 39(1965).

[1] Southwest Cactus Co., Alpine, TX 79830
[2] Identification was confirmed by Dr. E. F. Anderson, Whitman College, Walla Walla, Wash.

(13) D. L. Braga and J. L. McLaughlin, *Planta Med.*, **17**, 87 (1969).

(14) J. L. McLaughlin and A. G. Paul, *Lloydia*, **29**, 315(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 1, 1970, from the *Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle, WA 98105*

Accepted for publication May 19, 1970.

D. G. Norquist is grateful for support as a National Science Foundation undergraduate research participant, 1969. The investigation was further supported by U. S. Public Health Service Research Grant MH-17128-01 from the National Institute of Mental Health.

The authors thank E. F. Anderson of Whitman College for confirming the plant identification.

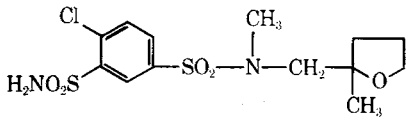
Effects of Mefruside on Renal Hemodynamics

JAMES H. LUDENS* and HAROLD E. WILLIAMSON†

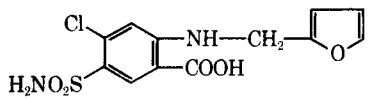
Abstract □ Mefruside, a natriuretic drug similar in structure to furosemide, was examined for activity on renal hemodynamics in the dog. This agent, when administered at 10 mg./kg. i.v., increased renal vascular resistance and decreased renal blood flow. Blood pressure and the rate of glomerular filtration were not affected. Mefruside differs from furosemide in its action on renal hemodynamics, inasmuch as furosemide decreases renal vascular resistance and increases renal blood flow.

Keyphrases □ Mefruside—effects on renal hemodynamics □ Renal dynamics—mefruside, effects

Mefruside {4-chloro-3-sulfonamido-1-[*N*-methyl-*N*-(2′-methyl-2′-tetrahydrofurylmethyl)]-benzenesulfonamide} (I) is a new orally active natriuretic (1). Structurally, this agent is similar to furosemide (II). In



I mefruside



II furosemide

addition to natriuretic activity, furosemide also affects renal hemodynamics to enhance renal blood flow (2–5). The purpose of this study was to determine if mefruside also enhances renal blood flow.

EXPERIMENTAL

Mongrel dogs, weighing 13–16 kg., were anesthetized with sodium pentobarbital, 30 mg./kg. A tracheal cannula was inserted to ensure free passage of air. The kidney to be utilized was exposed by a flank incision, and the renal artery was cleared of the surrounding tissue. A flow transducer (Carolina Medical Electronics model EMP-411, lumen size, 11-mm. circumference) was placed around the exposed renal artery, and renal blood flow was monitored with a square-wave electromagnetic flowmeter (Carolina Medical Electronics model 321). The flowmeter was adjusted to zero flow by briefly occluding the renal artery distal to the flow transducer. Blood pressure was monitored from the carotid artery. Both blood flow and blood pressure were recorded on a Beckman recorder. A solution containing 0.9% NaCl and 0.4% inulin was infused into the right femoral vein at a rate of 0.25 ml./kg./min. for at least 30 min. before and throughout the entire experiment. Mefruside was dissolved in saline using sodium bicarbonate. The drug was given intravenously *via* the right femoral vein.

Urine samples from the exposed kidney were obtained from a renal pelvic catheter introduced into the ureter by way of the retroperitoneal incision. Although urine samples were collected only from the kidney to which blood flow was measured, the other ureter was cannulated *via* a midline incision to ensure free urine flow from the contralateral kidney. Blood samples were obtained from the right femoral artery.

Urine and plasma inulin concentrations were determined by the method of Shreiner (6). Sodium concentrations were determined with a Coleman flame photometer. All data were analyzed with Student's *t* test paired comparisons (7). A *p* value less than 0.05 was used as the level of significance.

Table I—Effect of Mefruside (10 mg./kg.) on Renal Hemodynamics

	Control[a]	Mefruside[b]	Difference[c]
Renal blood flow, ml./min.	174	158	−16 ± 4[d]
Blood pressure, mm. Hg	142	140	−2 ± 2
Renal vascular resistance, mm. Hg/ml./min.	0.84	0.92	0.08 ± 0.02[d]
Sodium excretion, μeq./min.	124	424	300 ± 53[d]
Inulin clearance, ml./min.	30	30	0 ± 3

[a] Control values represent values taken immediately prior to mefruside administration. [b] Mefruside values represent values taken 10 min. after drug administration. [c] Difference from control ± *SE*. [d] Indicates a significant difference, $p < 0.05$, $n = 4$.

RESULTS AND DISCUSSION

Mefruside (10 mg./kg. i.v.) produced a significant decrease in renal blood flow of 16 ml./min. as renal vascular resistance was increased significantly (Table I). These effects were transient. They were maximal about 10 min. after drug administration and returned to control levels by 20 min. after drug administration. Blood pressure was not altered by the drug. Mefruside had no effect on inulin clearance. Sodium excretion was increased from 124 to 424 μeq./min. upon drug administration. The natriuretic action was still present 60 min. after drug administration when the experiments were ended. A lower dose of the drug (2 mg./kg. i.v.) did not produce a significant change in renal vascular resistance or renal blood flow.

(13) D. L. Braga and J. L. McLaughlin, *Planta Med.*, **17**, 87 (1969).

(14) J. L. McLaughlin and A. G. Paul, *Lloydia*, **29**, 315(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 1, 1970, from the *Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle, WA 98105*

Accepted for publication May 19, 1970.

D. G. Norquist is grateful for support as a National Science Foundation undergraduate research participant, 1969. The investigation was further supported by U. S. Public Health Service Research Grant MH-17128-01 from the National Institute of Mental Health.

The authors thank E. F. Anderson of Whitman College for confirming the plant identification.

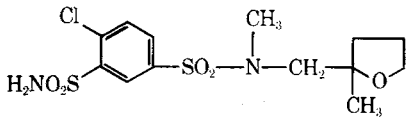
Effects of Mefruside on Renal Hemodynamics

JAMES H. LUDENS* and HAROLD E. WILLIAMSON†

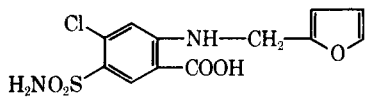
Abstract □ Mefruside, a natriuretic drug similar in structure to furosemide, was examined for activity on renal hemodynamics in the dog. This agent, when administered at 10 mg./kg. i.v., increased renal vascular resistance and decreased renal blood flow. Blood pressure and the rate of glomerular filtration were not affected. Mefruside differs from furosemide in its action on renal hemodynamics, inasmuch as furosemide decreases renal vascular resistance and increases renal blood flow.

Keyphrases □ Mefruside—effects on renal hemodynamics □ Renal dynamics—mefruside, effects

Mefruside {4-chloro-3-sulfonamido-1-[*N*-methyl-*N*-(2′-methyl-2′-tetrahydrofurylmethyl)]-benzenesulfonamide} (I) is a new orally active natriuretic (1). Structurally, this agent is similar to furosemide (II). In



I mefruside



II furosemide

addition to natriuretic activity, furosemide also affects renal hemodynamics to enhance renal blood flow (2–5). The purpose of this study was to determine if mefruside also enhances renal blood flow.

EXPERIMENTAL

Mongrel dogs, weighing 13–16 kg., were anesthetized with sodium pentobarbital, 30 mg./kg. A tracheal cannula was inserted to ensure free passage of air. The kidney to be utilized was exposed by a flank incision, and the renal artery was cleared of the surrounding tissue. A flow transducer (Carolina Medical Electronics model EMP-411, lumen size, 11-mm. circumference) was placed around the exposed renal artery, and renal blood flow was monitored with a square-wave electromagnetic flowmeter (Carolina Medical Electronics model 321). The flowmeter was adjusted to zero flow by briefly occluding the renal artery distal to the flow transducer. Blood pressure was monitored from the carotid artery. Both blood flow and blood pressure were recorded on a Beckman recorder. A solution containing 0.9% NaCl and 0.4% inulin was infused into the right femoral vein at a rate of 0.25 ml./kg./min. for at least 30 min. before and throughout the entire experiment. Mefruside was dissolved in saline using sodium bicarbonate. The drug was given intravenously *via* the right femoral vein.

Urine samples from the exposed kidney were obtained from a renal pelvic catheter introduced into the ureter by way of the retroperitoneal incision. Although urine samples were collected only from the kidney to which blood flow was measured, the other ureter was cannulated *via* a midline incision to ensure free urine flow from the contralateral kidney. Blood samples were obtained from the right femoral artery.

Urine and plasma inulin concentrations were determined by the method of Shreiner (6). Sodium concentrations were determined with a Coleman flame photometer. All data were analyzed with Student's *t* test paired comparisons (7). A *p* value less than 0.05 was used as the level of significance.

Table I—Effect of Mefruside (10 mg./kg.) on Renal Hemodynamics

	Control[a]	Mefruside[b]	Difference[c]
Renal blood flow, ml./min.	174	158	−16 ± 4[d]
Blood pressure, mm. Hg	142	140	−2 ± 2
Renal vascular resistance, mm. Hg/ml./min.	0.84	0.92	0.08 ± 0.02[d]
Sodium excretion, μeq./min.	124	424	300 ± 53[d]
Inulin clearance, ml./min.	30	30	0 ± 3

[a] Control values represent values taken immediately prior to mefruside administration. [b] Mefruside values represent values taken 10 min. after drug administration. [c] Difference from control ± *SE*. [d] Indicates a significant difference, $p < 0.05$, $n = 4$.

RESULTS AND DISCUSSION

Mefruside (10 mg./kg. i.v.) produced a significant decrease in renal blood flow of 16 ml./min. as renal vascular resistance was increased significantly (Table I). These effects were transient. They were maximal about 10 min. after drug administration and returned to control levels by 20 min. after drug administration. Blood pressure was not altered by the drug. Mefruside had no effect on inulin clearance. Sodium excretion was increased from 124 to 424 μeq./min. upon drug administration. The natriuretic action was still present 60 min. after drug administration when the experiments were ended. A lower dose of the drug (2 mg./kg. i.v.) did not produce a significant change in renal vascular resistance or renal blood flow.

The results show that mefruside does possess renal hemodynamic properties but that they are not similar to the renal hemodynamic properties of furosemide. Furosemide decreases renal vascular resistance and thereby enhances renal blood flow (2–5). In contrast, mefruside (10 mg./kg. i.v.) increases renal vascular resistance and thereby decreases renal blood flow. The effect of mefruside on renal blood flow is more like that of the thiazide agents which also increase renal vascular resistance and decrease renal blood flow (2, 8).

REFERENCES

(1) K. Meng and G. Kroneberg, *Arzneim.-Forsch.*, **17**, 659(1967).

(2) J. B. Hook, A. H. Blatt, M. J. Brody, and H. E. Williamson, *J. Pharmacol. Exp. Ther.*, **154**, 667(1966).

(3) J. H. Ludens, J. B. Hook, M. J. Brody, and H. E. Williamson, *ibid.*, **163**, 456(1968).

(4) C. Vorburger, A. M. Harvey, and R. L. Malvin, *Arch. Pharmakol. Exp. Pathol.*, **261**, 346(1968).

(5) J. H. Ludens and H. E. Williamson, *Fed. Proc.*, **28**, 739(1969).

(6) G. E. Schreiner, *Proc. Soc. Exp. Biol. Med.*, **74**, 117(1950).

(7) G. W. Snedecor, "Statistical Methods," 5th ed., Iowa State College Press, Ames, Iowa, 1956, p. 49.

(8) S. Cassin and B. Vogh, *Proc. Soc. Exp. Biol. Med.*, **122**, 970 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 22, 1970, from the *Department of Pharmacology, University of Iowa, Iowa City, IA 52240*

Accepted for publication July 20, 1970.

This investigation was supported in part by Public Health Service Grants AM05298 and GM00141.

* Present address: University of Kansas Medical Center, Kansas City, Kan.

† To whom inquiries should be addressed.

1-(3,4-Dimethoxyphenyl)-2-propanol Effect on Conditioned Avoidance Response in the Rat

C. F. BARFKNECHT, J. M. MILES, and J. L. LESENEY

Abstract □ The compound, 1-(3,4-dimethoxyphenyl)-2-propanol, was found to prolong latency times initially in a conditioned avoidance response test in rats. It was found to be a CNS depressant in mice. The structural implications of this action, plus the relationship between 1-(3,4-dimethoxyphenyl)-2-propanol and psychotomimetic amphetamines, are discussed.

Keyphrases □ 1-(3,4-Dimethoxyphenyl)-2-propanol—effect on conditioned avoidance response, rat □ Psychotomimetic agents—3,4-dimethoxyamphetamine oxygen analog, pharmacological screening

The literature contains a report indicating that the oxygen analog of mescaline, namely, 3,4,5-trimethoxyphenylethanol (TE), may be psychotomimetic (1). TE was isolated as a product of mescaline metabolism. When it was injected intravenously into rabbits, a mild mescalinelike action was observed.

In structure–activity relationship studies on mescaline, certain methoxylated amphetamines were found to be more potent than mescaline (2). If conversion from β-phenylethylamine to phenylisopropylamine can enhance the potency of psychotomimetics, then a similar logic possibly could be applied to the oxygen analogs of these psychotomimetics. One could anticipate that any mescalinelike actions would be more pronounced in 1-phenyl-2-propanols than in 2-phenylethanols. This paper reports the results of a study on the effect of 1-(3,4-dimethoxyphenyl)-2-propanol (DP), which is the oxygen analog of the psychotomimetic agent 3,4-dimethoxyamphetamine, on conditioned avoidance response (CAR) in the rat.

RESULTS AND DISCUSSION

Although the synthesis of DP was reported in the literature (3), no pharmacological information on the compound could be found. Initially the effect of DP on CAR in rats was studied. The evaluation of drug action on CAR has been exploited widely. This technique has been especially valuable for the evaluation of psychotomimetics (4).

To determine whether DP is producing an effect similar to mescaline or 3,4-dimethoxyamphetamine (3,4-DMA), the effects of these drugs at various dosages on a running response in conditioned male rats were determined. Each drug was given in three dosage levels ("effective dose," one quarter the effective dose, and four times the effective dose) to groups of six rats. Figures 1 and 2 show the mean reaction time for each trial during the drug sessions for each dosage level of mescaline and 3,4-DMA. That the profile of 3,4-DMA is not completely analogous to mescaline may be due to the amphetamine structure present in 3,4-DMA. CNS stimulation caused by amphetamine would be expected to counteract the increased reaction times. Figure 3 shows the enhanced performance caused by amphetamine sulfate relative to placebo. The lower dose was more effective.

Since 3,4-DMA is expected to be a poorer stimulant than amphetamine itself (*i.e.*, require a much larger dose to cause the same effect), the greatest variation from the mescaline profile would be expected at the higher doses. The large variation between reaction times of adjacent trials is observed in both mescaline and 3,4-DMA, which suggests that this is a characteristic feature of the behavior disruption caused by these agents.

Figure 4 shows the effects of DP. The overall profile of the drug session is substantially different from any of the other drugs tested. When it became clear that DP was different, it was subjected to a more conventional pharmacological screening. In mice intraperitoneally it was a CNS depressant [ED_{50}(sleep) = 150 mg./kg; LD_{50} = 650 mg./kg.] (5). This pharmacological action of DP is not unexpected in light of the report that acetophenones and other phenones and their corresponding alcohols exhibit depressant effects (6). Thus, DP may be viewed as a structural isomer of these phenones and alcohols.

The data presented here suggest that a reevaluation of the pharmacological effects of TE is required.[1] A detailed study of the structure–activity relationships and the mechanism of action of ring-substituted phenyl-2-propanols, phenyl-2-propanones, and chemi-

[1] Preliminary, unpublished studies indicate that TE has a CNS-depressant effect, ED_{50}(sleep) > 650 mg./kg. i.p. in mice.

The results show that mefruside does possess renal hemodynamic properties but that they are not similar to the renal hemodynamic properties of furosemide. Furosemide decreases renal vascular resistance and thereby enhances renal blood flow (2–5). In contrast, mefruside (10 mg./kg. i.v.) increases renal vascular resistance and thereby decreases renal blood flow. The effect of mefruside on renal blood flow is more like that of the thiazide agents which also increase renal vascular resistance and decrease renal blood flow (2, 8).

REFERENCES

(1) K. Meng and G. Kroneberg, *Arzneim.-Forsch.*, **17**, 659(1967).

(2) J. B. Hook, A. H. Blatt, M. J. Brody, and H. E. Williamson, *J. Pharmacol. Exp. Ther.*, **154**, 667(1966).

(3) J. H. Ludens, J. B. Hook, M. J. Brody, and H. E. Williamson, *ibid.*, **163**, 456(1968).

(4) C. Vorburger, A. M. Harvey, and R. L. Malvin, *Arch. Pharmakol. Exp. Pathol.*, **261**, 346(1968).

(5) J. H. Ludens and H. E. Williamson, *Fed. Proc.*, **28**, 739(1969).

(6) G. E. Schreiner, *Proc. Soc. Exp. Biol. Med.*, **74**, 117(1950).

(7) G. W. Snedecor, "Statistical Methods," 5th ed., Iowa State College Press, Ames, Iowa, 1956, p. 49.

(8) S. Cassin and B. Vogh, *Proc. Soc. Exp. Biol. Med.*, **122**, 970 (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 22, 1970, from the *Department of Pharmacology, University of Iowa, Iowa City, IA 52240*

Accepted for publication July 20, 1970.

This investigation was supported in part by Public Health Service Grants AM05298 and GM00141.

* Present address: University of Kansas Medical Center, Kansas City, Kan.

† To whom inquiries should be addressed.

1-(3,4-Dimethoxyphenyl)-2-propanol Effect on Conditioned Avoidance Response in the Rat

C. F. BARFKNECHT, J. M. MILES, and J. L. LESENEY

Abstract □ The compound, 1-(3,4-dimethoxyphenyl)-2-propanol, was found to prolong latency times initially in a conditioned avoidance response test in rats. It was found to be a CNS depressant in mice. The structural implications of this action, plus the relationship between 1-(3,4-dimethoxyphenyl)-2-propanol and psychotomimetic amphetamines, are discussed.

Keyphrases □ 1-(3,4-Dimethoxyphenyl)-2-propanol—effect on conditioned avoidance response, rat □ Psychotomimetic agents—3,4-dimethoxyamphetamine oxygen analog, pharmacological screening

The literature contains a report indicating that the oxygen analog of mescaline, namely, 3,4,5-trimethoxyphenylethanol (TE), may be psychotomimetic (1). TE was isolated as a product of mescaline metabolism. When it was injected intravenously into rabbits, a mild mescalinelike action was observed.

In structure–activity relationship studies on mescaline, certain methoxylated amphetamines were found to be more potent than mescaline (2). If conversion from β-phenylethylamine to phenylisopropylamine can enhance the potency of psychotomimetics, then a similar logic possibly could be applied to the oxygen analogs of these psychotomimetics. One could anticipate that any mescalinelike actions would be more pronounced in 1-phenyl-2-propanols than in 2-phenylethanols. This paper reports the results of a study on the effect of 1-(3,4-dimethoxyphenyl)-2-propanol (DP), which is the oxygen analog of the psychotomimetic agent 3,4-dimethoxyamphetamine, on conditioned avoidance response (CAR) in the rat.

RESULTS AND DISCUSSION

Although the synthesis of DP was reported in the literature (3), no pharmacological information on the compound could be found. Initially the effect of DP on CAR in rats was studied. The evaluation of drug action on CAR has been exploited widely. This technique has been especially valuable for the evaluation of psychotomimetics (4).

To determine whether DP is producing an effect similar to mescaline or 3,4-dimethoxyamphetamine (3,4-DMA), the effects of these drugs at various dosages on a running response in conditioned male rats were determined. Each drug was given in three dosage levels ("effective dose," one quarter the effective dose, and four times the effective dose) to groups of six rats. Figures 1 and 2 show the mean reaction time for each trial during the drug sessions for each dosage level of mescaline and 3,4-DMA. That the profile of 3,4-DMA is not completely analogous to mescaline may be due to the amphetamine structure present in 3,4-DMA. CNS stimulation caused by amphetamine would be expected to counteract the increased reaction times. Figure 3 shows the enhanced performance caused by amphetamine sulfate relative to placebo. The lower dose was more effective.

Since 3,4-DMA is expected to be a poorer stimulant than amphetamine itself (*i.e.*, require a much larger dose to cause the same effect), the greatest variation from the mescaline profile would be expected at the higher doses. The large variation between reaction times of adjacent trials is observed in both mescaline and 3,4-DMA, which suggests that this is a characteristic feature of the behavior disruption caused by these agents.

Figure 4 shows the effects of DP. The overall profile of the drug session is substantially different from any of the other drugs tested. When it became clear that DP was different, it was subjected to a more conventional pharmacological screening. In mice intraperitoneally it was a CNS depressant [ED_{50}(sleep) = 150 mg./kg; LD_{50} = 650 mg./kg.] (5). This pharmacological action of DP is not unexpected in light of the report that acetophenones and other phenones and their corresponding alcohols exhibit depressant effects (6). Thus, DP may be viewed as a structural isomer of these phenones and alcohols.

The data presented here suggest that a reevaluation of the pharmacological effects of TE is required.[1] A detailed study of the structure–activity relationships and the mechanism of action of ring-substituted phenyl-2-propanols, phenyl-2-propanones, and chemi-

[1] Preliminary, unpublished studies indicate that TE has a CNS-depressant effect, ED_{50}(sleep) > 650 mg./kg. i.p. in mice.

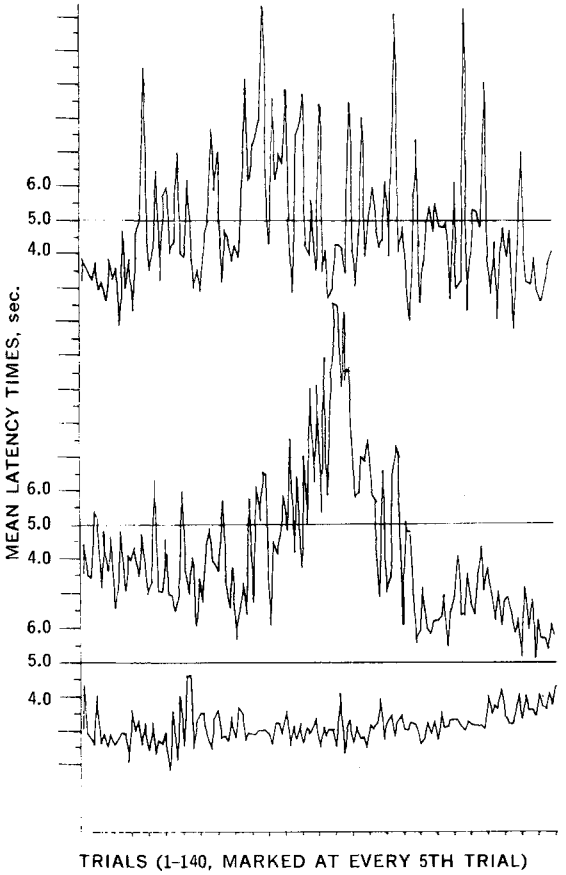


Figure 1—*Effect of mescaline hydrochloride on CAR. Doses (bottom to top) were 6.3, 25, and 100 mg./kg., respectively. The horizontal lines at 5.0 sec. for each dose separate avoidance (<5 sec.) and non-avoidance (>5 sec.) of shock.*

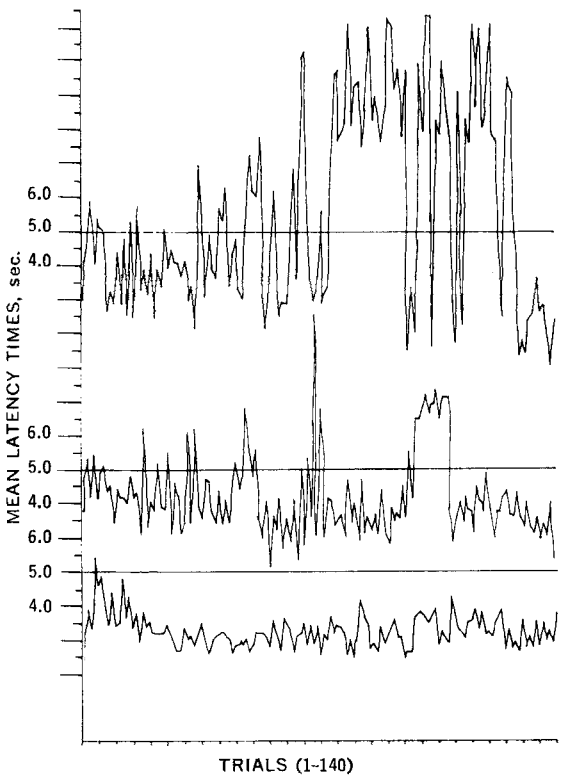


Figure 2—*Effect of 3,4-dimethoxyamphetamine hydrochloride on CAR. Doses (bottom to top) were 3.1, 12.5, and 50 mg./kg., respectively.*

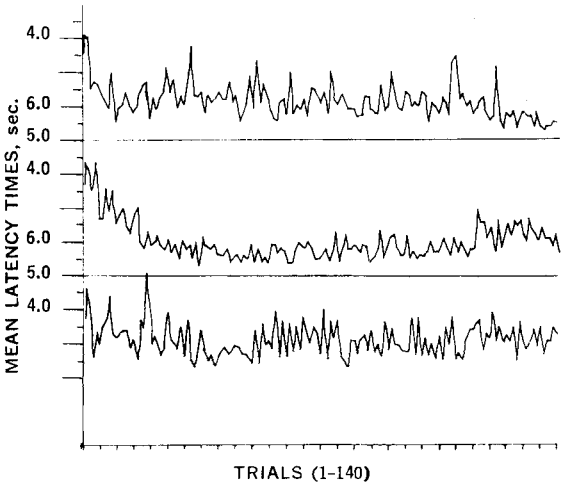


Figure 3—*Effect of placebo (0.9% saline solution, bottom) and dextroamphetamine sulfate at 1 and 4 mg./kg., respectively, on CAR.*

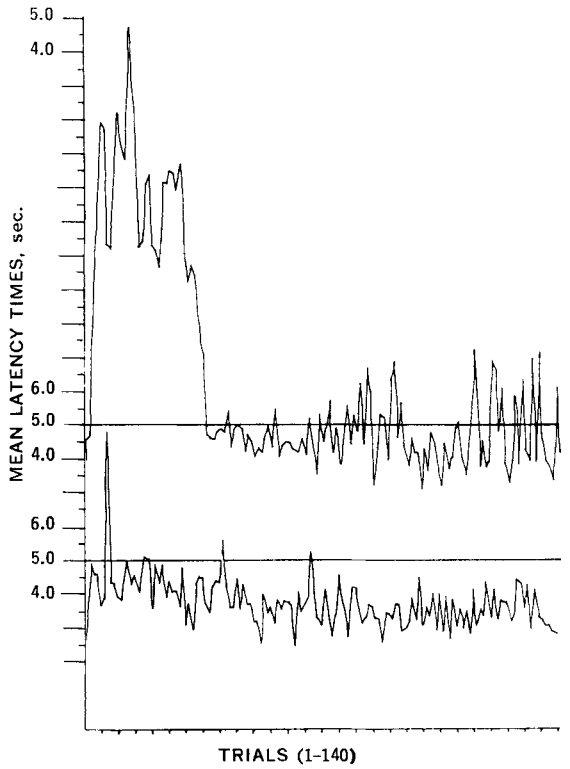


Figure 4—*Effect of 1-(3,4-dimethoxyphenyl)-2-propanol at 3.1 and 12.5 mg./kg., respectively, on CAR.*

cally related compounds is underway and will be the subject of a future communication.

EXPERIMENTAL

An A-584 automatic shuttlebox system[2] was used to measure a running response in male, 70–100-day-old, hooded rats.

Training—Each animal was required to cross from one side to the other in response to a conditioned stimulus (a buzzer, 5 sec.). At the end of this time, the unconditioned stimulus (electric shock, 1.0 mamp., maximum time = 15 sec.) was administered if the animal had failed to cross. The trial ended when the animal made the cross or when a total of 20 sec. had elapsed. After a 40-sec. intertrial interval, the sequence was repeated. Each rat received 20 trials per day until

[2] Lafayette Instrument Co., Lafayette, Ind.

it attained 90% avoidance of unconditioned stimulus on that day or until it had received 200 trials. Rats failing to reach the criterion were discarded. Those attaining 90% avoidance were dosed with drugs (randomly selected) the following day.

Drug Session—The day following attainment of criterion the rat was injected with a saline solution of a drug intraperitoneally and immediately placed in the shuttlebox. A series of 140 trials was given, and the data (escape or avoidance and reaction time) were automatically recorded. With the exception of extremely unruly subjects, the rats were completely isolated during the entire drug session. After the drug day, the rats were discarded.

Some animals died during the drug sessions with mescaline hydrochloride at 25 mg./kg. (after Trials 77, 87, and 97) and at 100 mg./kg. (after Trials 60, 61, and 68), and with 3,4-DMA hydrochloride at 12.5 mg./kg. (after Trial 53) and at 50 mg./kg. (after Trials 66, 67, and 73). The animals were not considered in the computation of mean reaction times after death.

REFERENCES

(1) M. Goldstein, A. L. Friedhoff, S. Pomerantz, C. Simmons, and J. F. Contrera, *J. Neurochem.*, **6**, 253(1961); A. L. Friedhoff and M. Goldstein, *Ann. N. Y. Acad. Sci.*, **96**, 5(1962).

(2) J. R. Smythies, V. S. Johnston, R. J. Bradley, F. Benington, R. D. Morin, and L. C. Clark, Jr., *Nature*, **216**, 128(1967); A. T. Shulgin, T. Sargent, and C. Naranjo, *ibid.*, **221**, 537(1969).

(3) A. A. Chuksarrova, L. L. Serguva, and N. N. Sharygina, *Izv. Akad. Nauk SSSR*, **1959**, 2219; E. Adler and J. Morton, *Acta Chem. Scand.*, **15**, 357(1961).

(4) J. R. Smythies, E. A. Sykes, and C. P. Lord, *Psychopharmacologia*, **9**, 434(1966); J. R. Smythies, R. J. Bradley, V. S. Johnston, F. Benington, R. D. Morin, and L. C. Clark, Jr., *ibid.*, **10**, 379 (1967).

(5) J. P. Long, personal communication.

(6) R. B. Moffett, A. R. Hanze, and P. H. Seay, *J. Med. Chem.*, **7**, 178(1964) and previous references cited therein.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 26, 1970, from the *Division of Medicinal Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication June 19, 1970.

The authors thank Dr. Eugene Gauron, Department of Psychiatry, University of Iowa, for his advice and counsel on the CAR procedure, and Dr. John P. Long, Department of Pharmacology, University of Iowa, for the preliminary pharmacological screening data. Funds for the purchase of the automatic shuttlebox were provided by the Mental Health Research Fund of Iowa.

COMMUNICATIONS

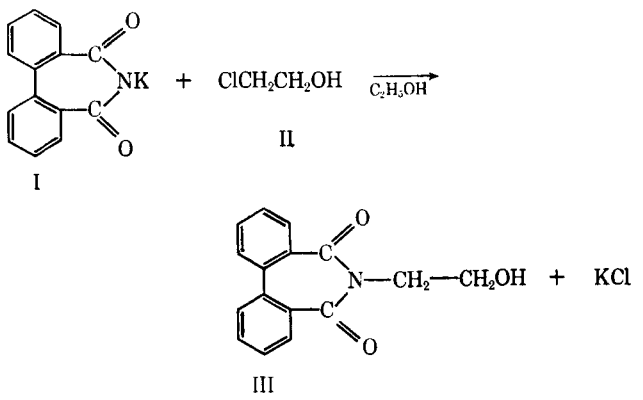
Preparation of Ethyl Diphenamate in Ethanol Using Potassium Diphenimide and 2-Chloroethanol

Keyphrases ☐ Ethyl diphenamate—synthesis ☐ *N*-(2-Hydroxyethyl)-diphenimide synthesis—literature correction ☐ NMR spectroscopy—structure

Sir:

The preparation of *N*-(2-hydroxyethyl)-diphenimide (III, Scheme I) was reported in 1952 by Demers and Jenkins (1). The approach taken in preparing this compound involved the reaction of potassium diphenimide (I) with 2-chloroethanol (II) in ethanol (1). Since this route of synthesis had been used successfully by Moore and Rapala (2) in the preparation of a series of dialkylaminoalkyl phthalimide derivatives, it appeared unlikely that a product other than that reported (III) would be obtained. Elemental nitrogen analysis of Compound III reported by these workers was well within experimental error.

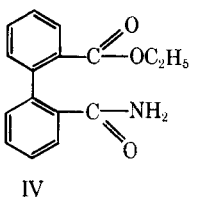
In 1963, Jenkins *et al.* (3) reported that during the attempted synthesis of a series of dialkylaminoalkyl diphenimide hydrochloride salts, using a similar procedure to that reported by Demers and Jenkins (1), the corresponding hydrochloride salts of ethyl diphenamate were formed rather than the diphenimide derivatives. The similar chemical nature of the alkylating species in these reactions prompted us to repeat



Scheme I

the work of Demers and Jenkins to determine if Compound III had actually been obtained.

The results of our work, which we report at this time, shows that Demers and Jenkins did not prepare *N*-(2-hydroxyethyl)-diphenimide (III) as reported but instead obtained ethyl diphenamate (IV).



IV

Identification of Compound IV was established as follows: (*a*) an NMR spectrum showed peaks at 6.83–8.00 δ (m, 8, Ar), 6.08 δ (b.s., NH_2), 4.09 δ (q, CH_2),

it attained 90% avoidance of unconditioned stimulus on that day or until it had received 200 trials. Rats failing to reach the criterion were discarded. Those attaining 90% avoidance were dosed with drugs (randomly selected) the following day.

Drug Session—The day following attainment of criterion the rat was injected with a saline solution of a drug intraperitoneally and immediately placed in the shuttlebox. A series of 140 trials was given, and the data (escape or avoidance and reaction time) were automatically recorded. With the exception of extremely unruly subjects, the rats were completely isolated during the entire drug session. After the drug day, the rats were discarded.

Some animals died during the drug sessions with mescaline hydrochloride at 25 mg./kg. (after Trials 77, 87, and 97) and at 100 mg./kg. (after Trials 60, 61, and 68), and with 3,4-DMA hydrochloride at 12.5 mg./kg. (after Trial 53) and at 50 mg./kg. (after Trials 66, 67, and 73). The animals were not considered in the computation of mean reaction times after death.

REFERENCES

(1) M. Goldstein, A. L. Friedhoff, S. Pomerantz, C. Simmons, and J. F. Contrera, *J. Neurochem.*, **6**, 253(1961); A. L. Friedhoff and M. Goldstein, *Ann. N. Y. Acad. Sci.*, **96**, 5(1962).

(2) J. R. Smythies, V. S. Johnston, R. J. Bradley, F. Benington, R. D. Morin, and L. C. Clark, Jr., *Nature*, **216**, 128(1967); A. T. Shulgin, T. Sargent, and C. Naranjo, *ibid.*, **221**, 537(1969).

(3) A. A. Chuksarrova, L. L. Serguva, and N. N. Sharygina, *Izv. Akad. Nauk SSSR*, **1959**, 2219; E. Adler and J. Morton, *Acta Chem. Scand.*, **15**, 357(1961).

(4) J. R. Smythies, E. A. Sykes, and C. P. Lord, *Psychopharmacologia*, **9**, 434(1966); J. R. Smythies, R. J. Bradley, V. S. Johnston, F. Benington, R. D. Morin, and L. C. Clark, Jr., *ibid.*, **10**, 379 (1967).

(5) J. P. Long, personal communication.

(6) R. B. Moffett, A. R. Hanze, and P. H. Seay, *J. Med. Chem.*, **7**, 178(1964) and previous references cited therein.

ACKNOWLEDGMENTS AND ADDRESSES

Received January 26, 1970, from the *Division of Medicinal Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52240*

Accepted for publication June 19, 1970.

The authors thank Dr. Eugene Gauron, Department of Psychiatry, University of Iowa, for his advice and counsel on the CAR procedure, and Dr. John P. Long, Department of Pharmacology, University of Iowa, for the preliminary pharmacological screening data. Funds for the purchase of the automatic shuttlebox were provided by the Mental Health Research Fund of Iowa.

COMMUNICATIONS

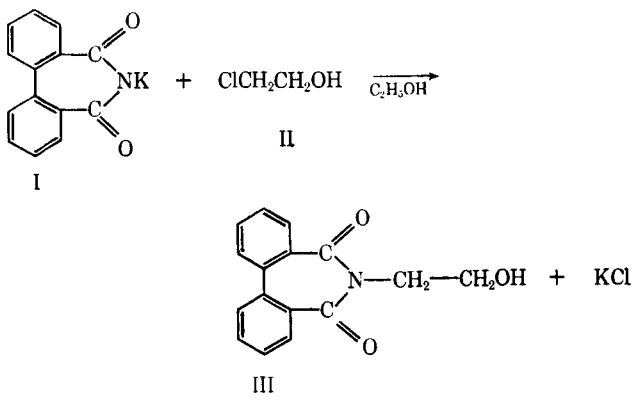
Preparation of Ethyl Diphenamate in Ethanol Using Potassium Diphenimide and 2-Chloroethanol

Keyphrases ☐ Ethyl diphenamate—synthesis ☐ *N*-(2-Hydroxyethyl)-diphenimide synthesis—literature correction ☐ NMR spectroscopy—structure

Sir:

The preparation of *N*-(2-hydroxyethyl)-diphenimide (III, Scheme I) was reported in 1952 by Demers and Jenkins (1). The approach taken in preparing this compound involved the reaction of potassium diphenimide (I) with 2-chloroethanol (II) in ethanol (1). Since this route of synthesis had been used successfully by Moore and Rapala (2) in the preparation of a series of dialkylaminoalkyl phthalimide derivatives, it appeared unlikely that a product other than that reported (III) would be obtained. Elemental nitrogen analysis of Compound III reported by these workers was well within experimental error.

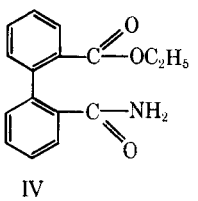
In 1963, Jenkins *et al.* (3) reported that during the attempted synthesis of a series of dialkylaminoalkyl diphenimide hydrochloride salts, using a similar procedure to that reported by Demers and Jenkins (1), the corresponding hydrochloride salts of ethyl diphenamate were formed rather than the diphenimide derivatives. The similar chemical nature of the alkylating species in these reactions prompted us to repeat



Scheme I

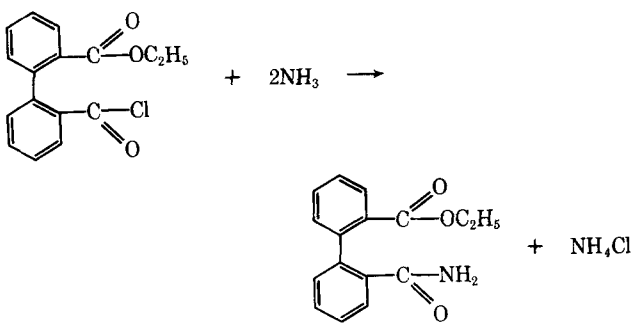
the work of Demers and Jenkins to determine if Compound III had actually been obtained.

The results of our work, which we report at this time, shows that Demers and Jenkins did not prepare *N*-(2-hydroxyethyl)-diphenimide (III) as reported but instead obtained ethyl diphenamate (IV).



IV

Identification of Compound IV was established as follows: (*a*) an NMR spectrum showed peaks at 6.83–8.00 δ (m, 8, Ar), 6.08 δ (b.s., NH_2), 4.09 δ (q, CH_2),



Scheme II

and 1.02 δ (t, CH_3); and (*b*) a mixed melting-point determination of the product prepared by the method of Demers and Jenkins (1) with a sample of ethyl diphenamate prepared by reacting ethyl diphenoyl chloride with ammonia showed no depression, and single melting-point determinations and NMR spectra were identical within experimental error.

Ethyl Diphenamate (IV)—(*a*) This was prepared following the procedure of Demers and Jenkins (1), m.p. 93° (lit 93°).

Anal.—Calcd. for N[1]: 5.20. Found: 5.19.

(*b*) Items prepared by reacting ethyl diphenoyl chloride with ammonia as follows. Approximately 74 mmoles of ethyl diphenoyl chloride was prepared in a manner reported by Demers and Jenkins (1), except that the final product was not isolated following the 8-hr. reflux period because of its hygroscopic nature. Instead the solvent was first removed *in vacuo*, and three successive 10-ml. portions of benzene were then added to the residue and each stripped off in a like manner to remove unreacted thionyl chloride, solvent, and HCl. The residue was dissolved in 200 ml. of anhydrous ether and cooled in a dry ice bath. Ammonia was then bubbled slowly into the mixture for several minutes (Scheme II). A white precipitate (NH_4Cl and ethyl diphenamate) formed immediately and was collected. The solid was triturated with water to remove the NH_4Cl. The remaining solid (ethyl diphenamate) was collected and recrystallized from alcohol and water. Yield 95%, m.p. 89–90°, NMR spectrum identical to that obtained in (*a*).

(1) F. X. Demers, Jr., and G. L. Jenkins, *J. Amer. Pharm. Ass., Sci. Ed.*, **41**, 61(1952).

(2) M. B. Moore and R. T. Rapala, *J. Amer. Chem. Soc.*, **68**, 1657(1946).

(3) G. L. Jenkins, C. S. Davis, A. M. Knevel, and D. S. Yoder, *J. Pharm. Sci.*, **52**, 902(1963).

A. M. Knevel
J. N. Wells

Department of Medicinal Chemistry
and Pharmacognosy
School of Pharmacy and Pharmacal Sciences
Purdue University
Lafayette, IN 47907

Received June 9, 1970.
Accepted for publication August 5, 1970.

[1] Nitrogen determination by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

Evidence for Capacity-Limited Biotransformation of Sulfanilamide

Keyphrases ☐ Sulfanilamide—capacity-limited biotransformation ☐ Biotransformation, sulfanilamide—rate limited ☐ Dose dependence—sulfanilamide half-life

Sir:

Blood as well as lymph concentrations of various agents were determined following intravenous administration in recent studies concerned with the influence of plasma protein binding on rate of drug distribution to the body (1). Among these agents were several sulfonamides. The data obtained with sulfanilamide appeared to indicate that the fraction of unmetabolized drug increased with increasing dose. Subsequent to the recently presented evidence that the biotransformation of salicylate, in the analgesic dose range usually used in man, is rate limited (2), a significant number of other agents were shown also to be metabolized in man and animals by various enzyme systems which are capacity limited (3). Since clear evidence for the capacity-limited nature of acetylation has been obtained only for *p*-aminobenzoic acid (4), we carried out additional studies to determine whether the biotransformation of low doses of sulfanilamide was, indeed, a rate-limited phenomenon.

These studies were carried out using nonfasted male rats of the Sprague-Dawley strain, weighing between 250 and 300 g. Sulfanilamide was injected into the femoral vein in doses of 2.5, 5.0, or 10.0 mg. Blood was sampled by cardiac puncture at 12, 18, 30, 42, and, in two instances, 5 min. after administration. Since lymph was also collected in some of the experiments, the animals were kept under light ether anesthesia. How-

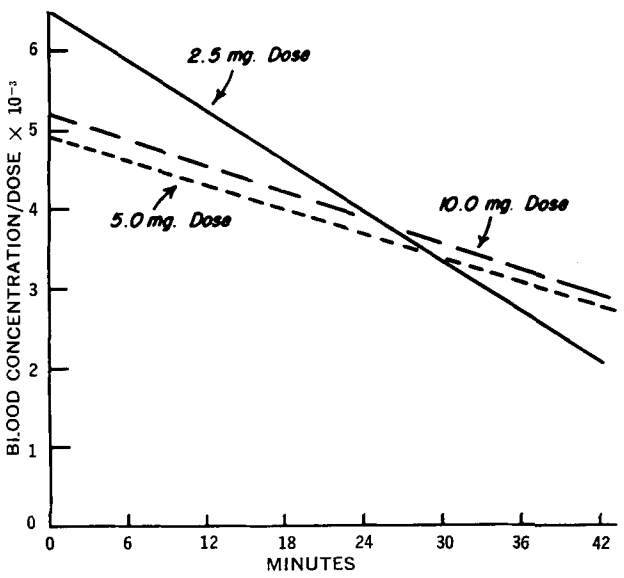


Figure 1—*Comparison of blood levels of unchanged drug after intravenous administration of several doses of sulfanilamide. Straight lines are regression lines calculated from the individual values determined at each time level (same number of animals as indicated in Table I).*

Table I—Effect of Dose on Percent of Total Sulfanilamide Present in Blood as Unmetabolized Drug

Minutes	Percent Unmetabolized[a] Dose, mg. 2.5	5.0	10.0
5	72.7 (2)	—	—
12	61.5 ± 12.3 (4)	81.3 ± 4.3 (4)	85.6 ± 3.8 (3)
18	65.8 ± 15.6 (4)	78.4 ± 2.8 (4)	87.1 ± 2.4 (3)
30	55.6 ± 16.9 (6)	72.6 ± 12.4 (4)	85.3 ± 4.5 (3)
42	56.5 ± 11.6 (7)	68.9 ± 11.1 (4)	79.0 ± 4.3 (5)

[a] Mean ± *SD*. Numbers in parentheses indicate number of animals.

ever, several experiments carried out in unanesthetized animals verified the blood level data collected in the anesthetized rats. The volume of all but the final sample of blood was 0.2 ml. The final sample, taken at 42 min., was sufficiently large to permit the determination of the hematocrit as well as the drug content in whole blood, plasma, and red cells. These data were then used to calculate the plasma concentrations for the earlier blood samples from the same animal. The distribution of sulfanilamide between red blood cells and plasma was also determined in another group of animals at 12, 18, and 30 min. after intravenous administration. For all samples the amounts of free (unmetabolized) and total (unmetabolized plus metabolized) sulfanilamide were determined by the method of Bratton and Marshall (5).

The results of the studies of distribution between plasma and red blood cells indicated that the interpretation of the data would be the same whether plasma concentrations or whole blood levels were used. The analyses of the data, therefore, were carried out using the actual values obtained for blood rather than the calculated plasma values. These analyses clearly indicate that the biotransformation of sulfanilamide has the characteristics of a capacity-limited process (3).

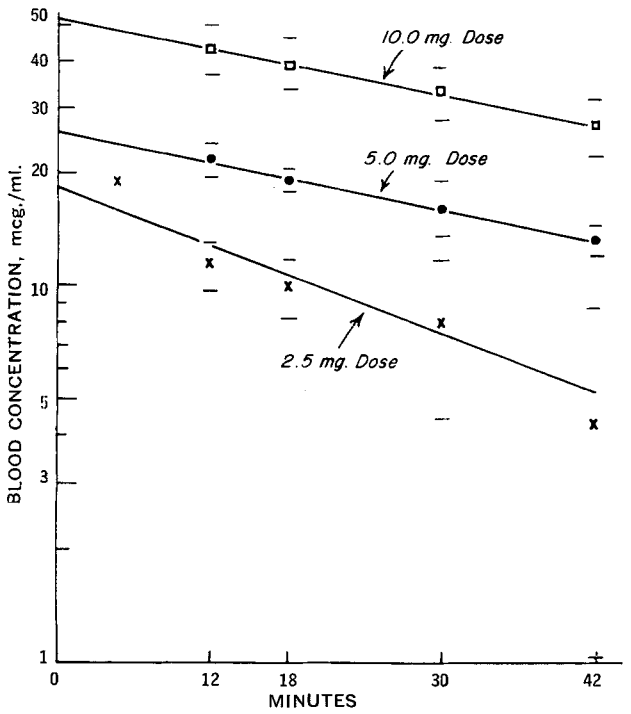


Figure 2—*Semilogarithmic plots of blood levels of unchanged drug after intravenous administration of several doses of sulfanilamide. Straight lines are regression lines calculated from the individual values;* ×, ●, *and* □ *are means at each time for doses of 2.5, 5.0, and 10.0 mg., respectively. Bars surrounding points represent standard deviations (same number of animals at each point as indicated in Table I).*

First of all, the percentage of the unchanged drug in the blood increases with dose (Table I). When the regression functions of the individual values of percent free sulfanilamide on time were estimated and tested for the three doses, it was found that the regressions were linear only for the 5.0 and 10.0-mg. doses and that the negative slopes of only these two regression lines were significantly different from zero. This indicates that above a dose of 2.5 mg., the rate of metabolism increases relatively with time. Thus, the fractional composition of sulfanilamide in blood is dependent on dose—a characteristic that is a good index of deviation from apparent first-order kinetics.

Second, deviation from first-order kinetics also can be demonstrated when the data are treated as in Fig. 1. For first-order kinetics the principle of superimposition should apply when blood concentration/dose is plotted against time (3). However, it can be readily seen that the regression line for the 2.5-mg. dose is not superimposable on the other two lines, and statistical analysis verified this conclusion. The slope of the line for the 2.5-mg. dose was found to be significantly different from those of 5.0 and 10.0 mg., and the slopes of the latter two lines were found to be insignificantly different from each other.

Finally, deviation from apparent first-order kinetics can be shown by the lack of linearity of semilogarithmic plots of blood concentration as a function of time (Fig. 2). Although each of the regression lines is insignificantly different from a straight line and the corresponding slopes are significantly different from zero, the slope of the line for the 2.5-mg. dose is again significantly different from those for the other two doses. Therefore, the drug half-life becomes dose dependent at a dose between 2.5 and 5.0 mg. Alinearity of the plots for the 5.0 and 10.0-mg. doses would be demonstrable only if the time of sampling was extended beyond 42 min.

The demonstration that a capacity-limited system for acetylation of sulfanilamide exists in the rat is important for drug metabolism studies in this species but may not be necessarily relevant to man. However, the saturation phenomenon occurred at doses in the rat that, when extrapolated to man, lie well within the therapeutic dose range. Moreover, Levy (3) pointed out that several capacity-limited drug-metabolizing systems observed in man have been demonstrated also in rats. These include a limited capacity for the conjugation of salicylate (6) and benzoate (3) with glycine and for sulfate and glucuronide conjugation with *N*-acetyl-*p*-aminophenol (7). Whether capacity-limited acetylation of sulfanilamide can be added to the list of similarities between man and the rat has to await additional evidence. Certainly it becomes increasingly apparent that drug metabolism studies must be carried out at multiple-dose levels and that linearity of the semilogarithmic plots of drug elimination as a function of time is frequently insufficient to discriminate between dose-independent or dose-dependent biotransformations.

(1) R. R. Levine, T. J. De Marco, and C. M. Dizon, *Proc. Int. Congr. Pharmacol., 4th*, **2**, 79(1970).
(2) G. Levy, *J. Pharm. Sci.*, **54**, 959(1965).
(3) G. Levy, in "Importance of Fundamental Principles in Drug Evaluation," D. H. Tedeschi and R. E. Tedeschi, Eds., Raven Press, New York, N. Y., 1968, pp. 141–172.
(4) M. M. Drucker, S. H. Blondheim, and I. Wislicki, *Clin. Sci.*, **27**, 133(1964).
(5) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).
(6) E. Nelson, M. Hanano, and G. Levy, *J. Pharmacol. Exp. Ther.*, **153**, 159(1966).
(7) H. Büch, K. Pfleger, and W. Rüdiger, *Z. Klin. Chem. Klin. Biochem.*, **5**, 110(1967).

RUTH R. LEVINE
CARMELITA M. DIZON
Department of Pharmacology and Experimental Therapeutics
Boston University Medical Center
Boston, MA 02118

Received July 16, 1970.
Accepted for publication August 21, 1970.
This investigation was supported in part by Grant AFOSR-914 from the U. S. Air Force Office of Scientific Research and Grant NB-01966 from the National Institute of Neurological Diseases and Stroke of the National Institutes of Health.

Apparent Dose-Dependent Elimination Kinetics as an Experimental Artifact

Keyphrases ☐ Dose-dependent elimination kinetics, apparent—experimental artifact consideration ☐ Plasma concentration—experimental blank errors

Sir:

It is now known that the kinetics of elimination of several drugs are dose dependent. The decline in the plasma concentrations of some drugs is exponential throughout, but the apparent first-order rate constant for this process descreases with increasing dose (1). The elimination of other drugs involves one or more saturable processes, and semilogarithmic plots of plasma concentrations as a function of time curve downward until they attain an exponential phase which is reached at the same concentration irrespective of the dose (2). In view of the great interest and investigative activity in the area of dose-dependent pharmacokinetics, it is appropriate to point out that errors in blank corrections can artifactually lead to the conclusion that an entirely linear, dose-independent system is in fact dose dependent and nonlinear.

Figure 1 shows hypothetical plasma concentrations obtained after intravenous injection of 1, 3, and 10 weight units of a drug which is actually eliminated by apparent first-order kinetics ($t_{1/2}$ = 2.0 hr.), with the plasma concentration data describable by means of a one-compartment open model. However, the data points in the figure are, in each case, 2 mg./l. lower than the "real" concentrations. This would be so if, for a number of possible reasons, a 2 mg./l. error in the blank value determination would have occurred. The data points thus obtained can be fitted readily to straight lines which yield a decreasing half-life with increasing dose. In the example shown, there is an almost 50% change in the apparent half-life.

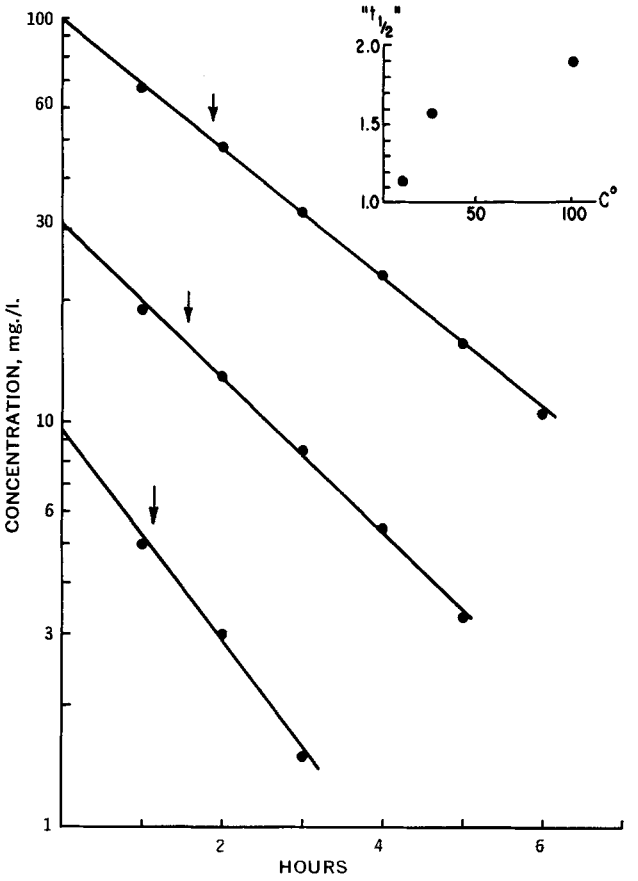
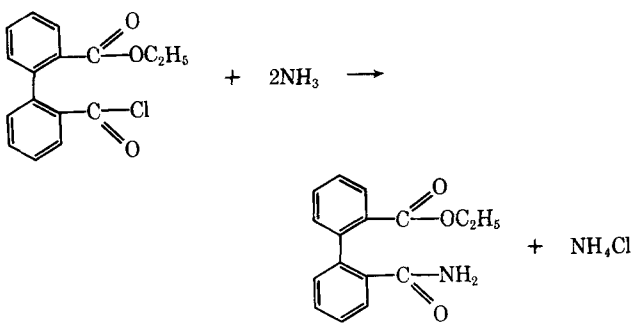


Figure 1—*Hypothetical plasma concentration data obtained after intravenous injection of 1, 3, and 10 weight units of a drug which is eliminated by apparent first-order kinetics* ($t_{1/2}$ = 2 hr.), *if the blank correction is 2 mg./l. too large. Arrows indicate apparent half-life. Inset: Relationship between initial plasma concentration* (C_0) *and apparent half-life* ("$t_{1/2}$").

If plasma concentrations are determined over a wide concentration range, an error in the blank correction can lead to the erroneous conclusion that elimination involves a combination of parallel linear and saturable (*i.e.*, capacity-limited) processes. Such systems may show an initial exponential concentration decline phase at high concentrations, a subsequent downward curvature, and finally another exponential phase which is steeper than the initial exponential phase (2). This pattern is evident in Fig. 2; the figure shows hypothetical plasma concentration data at two doses (differing 10-fold) of a drug, which is actually eliminated by apparent first-order kinetics but where an error of 3 mg./l. in the blank correction causes appreciable deviations from linearity. In this example, parallel straight lines may be fitted erroneously to the two sets of terminal data points, suggesting that the elimination kinetics above a plasma concentration of about 10 mg./l. are capacity limited.

The potential artifacts outlined in this article necessitate that considerable attention be directed to the correct determination of blank values. The magnitude



Scheme II

and 1.02 δ (t, CH_3); and (*b*) a mixed melting-point determination of the product prepared by the method of Demers and Jenkins (1) with a sample of ethyl diphenamate prepared by reacting ethyl diphenoyl chloride with ammonia showed no depression, and single melting-point determinations and NMR spectra were identical within experimental error.

Ethyl Diphenamate (IV)—(*a*) This was prepared following the procedure of Demers and Jenkins (1), m.p. 93° (lit 93°).

Anal.—Calcd. for N[1]: 5.20. Found: 5.19.

(*b*) Items prepared by reacting ethyl diphenoyl chloride with ammonia as follows. Approximately 74 mmoles of ethyl diphenoyl chloride was prepared in a manner reported by Demers and Jenkins (1), except that the final product was not isolated following the 8-hr. reflux period because of its hygroscopic nature. Instead the solvent was first removed *in vacuo*, and three successive 10-ml. portions of benzene were then added to the residue and each stripped off in a like manner to remove unreacted thionyl chloride, solvent, and HCl. The residue was dissolved in 200 ml. of anhydrous ether and cooled in a dry ice bath. Ammonia was then bubbled slowly into the mixture for several minutes (Scheme II). A white precipitate (NH_4Cl and ethyl diphenamate) formed immediately and was collected. The solid was triturated with water to remove the NH_4Cl. The remaining solid (ethyl diphenamate) was collected and recrystallized from alcohol and water. Yield 95%, m.p. 89–90°, NMR spectrum identical to that obtained in (*a*).

(1) F. X. Demers, Jr., and G. L. Jenkins, *J. Amer. Pharm. Ass., Sci. Ed.*, **41**, 61(1952).

(2) M. B. Moore and R. T. Rapala, *J. Amer. Chem. Soc.*, **68**, 1657(1946).

(3) G. L. Jenkins, C. S. Davis, A. M. Knevel, and D. S. Yoder, *J. Pharm. Sci.*, **52**, 902(1963).

A. M. Knevel
J. N. Wells

Department of Medicinal Chemistry
and Pharmacognosy
School of Pharmacy and Pharmacal Sciences
Purdue University
Lafayette, IN 47907

Received June 9, 1970.
Accepted for publication August 5, 1970.

[1] Nitrogen determination by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

Evidence for Capacity-Limited Biotransformation of Sulfanilamide

Keyphrases ☐ Sulfanilamide—capacity-limited biotransformation ☐ Biotransformation, sulfanilamide—rate limited ☐ Dose dependence—sulfanilamide half-life

Sir:

Blood as well as lymph concentrations of various agents were determined following intravenous administration in recent studies concerned with the influence of plasma protein binding on rate of drug distribution to the body (1). Among these agents were several sulfonamides. The data obtained with sulfanilamide appeared to indicate that the fraction of unmetabolized drug increased with increasing dose. Subsequent to the recently presented evidence that the biotransformation of salicylate, in the analgesic dose range usually used in man, is rate limited (2), a significant number of other agents were shown also to be metabolized in man and animals by various enzyme systems which are capacity limited (3). Since clear evidence for the capacity-limited nature of acetylation has been obtained only for *p*-aminobenzoic acid (4), we carried out additional studies to determine whether the biotransformation of low doses of sulfanilamide was, indeed, a rate-limited phenomenon.

These studies were carried out using nonfasted male rats of the Sprague-Dawley strain, weighing between 250 and 300 g. Sulfanilamide was injected into the femoral vein in doses of 2.5, 5.0, or 10.0 mg. Blood was sampled by cardiac puncture at 12, 18, 30, 42, and, in two instances, 5 min. after administration. Since lymph was also collected in some of the experiments, the animals were kept under light ether anesthesia. How-

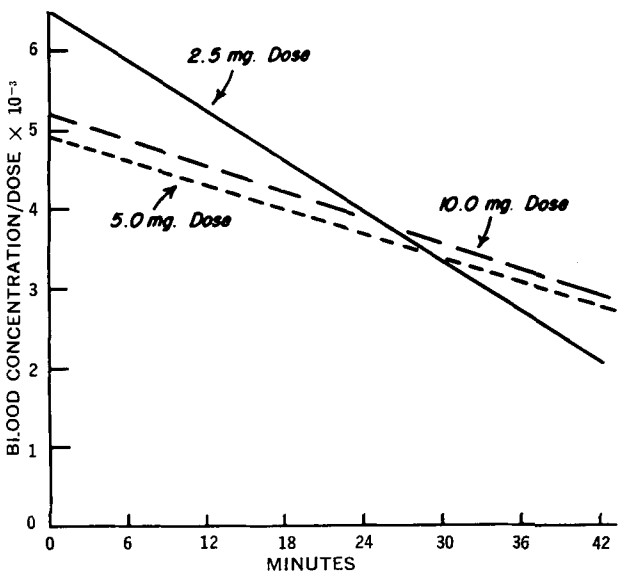


Figure 1—*Comparison of blood levels of unchanged drug after intravenous administration of several doses of sulfanilamide. Straight lines are regression lines calculated from the individual values determined at each time level (same number of animals as indicated in Table I).*

Table I—Effect of Dose on Percent of Total Sulfanilamide Present in Blood as Unmetabolized Drug

Minutes	Percent Unmetabolized[a] Dose, mg. 2.5	5.0	10.0
5	72.7 (2)	—	—
12	61.5 ± 12.3 (4)	81.3 ± 4.3 (4)	85.6 ± 3.8 (3)
18	65.8 ± 15.6 (4)	78.4 ± 2.8 (4)	87.1 ± 2.4 (3)
30	55.6 ± 16.9 (6)	72.6 ± 12.4 (4)	85.3 ± 4.5 (3)
42	56.5 ± 11.6 (7)	68.9 ± 11.1 (4)	79.0 ± 4.3 (5)

[a] Mean ± *SD*. Numbers in parentheses indicate number of animals.

ever, several experiments carried out in unanesthetized animals verified the blood level data collected in the anesthetized rats. The volume of all but the final sample of blood was 0.2 ml. The final sample, taken at 42 min., was sufficiently large to permit the determination of the hematocrit as well as the drug content in whole blood, plasma, and red cells. These data were then used to calculate the plasma concentrations for the earlier blood samples from the same animal. The distribution of sulfanilamide between red blood cells and plasma was also determined in another group of animals at 12, 18, and 30 min. after intravenous administration. For all samples the amounts of free (unmetabolized) and total (unmetabolized plus metabolized) sulfanilamide were determined by the method of Bratton and Marshall (5).

The results of the studies of distribution between plasma and red blood cells indicated that the interpretation of the data would be the same whether plasma concentrations or whole blood levels were used. The analyses of the data, therefore, were carried out using the actual values obtained for blood rather than the calculated plasma values. These analyses clearly indicate that the biotransformation of sulfanilamide has the characteristics of a capacity-limited process (3).

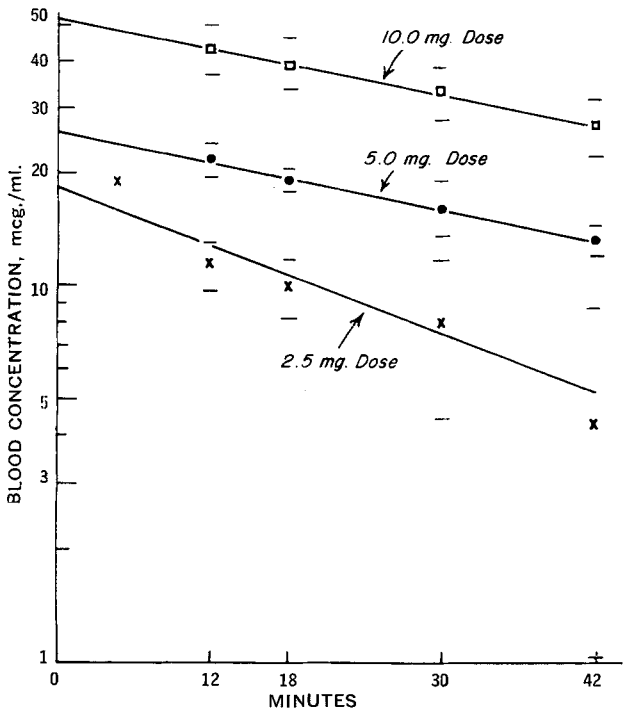


Figure 2—*Semilogarithmic plots of blood levels of unchanged drug after intravenous administration of several doses of sulfanilamide. Straight lines are regression lines calculated from the individual values;* ×, ●, *and* □ *are means at each time for doses of 2.5, 5.0, and 10.0 mg., respectively. Bars surrounding points represent standard deviations (same number of animals at each point as indicated in Table I).*

First of all, the percentage of the unchanged drug in the blood increases with dose (Table I). When the regression functions of the individual values of percent free sulfanilamide on time were estimated and tested for the three doses, it was found that the regressions were linear only for the 5.0 and 10.0-mg. doses and that the negative slopes of only these two regression lines were significantly different from zero. This indicates that above a dose of 2.5 mg., the rate of metabolism increases relatively with time. Thus, the fractional composition of sulfanilamide in blood is dependent on dose—a characteristic that is a good index of deviation from apparent first-order kinetics.

Second, deviation from first-order kinetics also can be demonstrated when the data are treated as in Fig. 1. For first-order kinetics the principle of superimposition should apply when blood concentration/dose is plotted against time (3). However, it can be readily seen that the regression line for the 2.5-mg. dose is not superimposable on the other two lines, and statistical analysis verified this conclusion. The slope of the line for the 2.5-mg. dose was found to be significantly different from those of 5.0 and 10.0 mg., and the slopes of the latter two lines were found to be insignificantly different from each other.

Finally, deviation from apparent first-order kinetics can be shown by the lack of linearity of semilogarithmic plots of blood concentration as a function of time (Fig. 2). Although each of the regression lines is insignificantly different from a straight line and the corresponding slopes are significantly different from zero, the slope of the line for the 2.5-mg. dose is again significantly different from those for the other two doses. Therefore, the drug half-life becomes dose dependent at a dose between 2.5 and 5.0 mg. Alinearity of the plots for the 5.0 and 10.0-mg. doses would be demonstrable only if the time of sampling was extended beyond 42 min.

The demonstration that a capacity-limited system for acetylation of sulfanilamide exists in the rat is important for drug metabolism studies in this species but may not be necessarily relevant to man. However, the saturation phenomenon occurred at doses in the rat that, when extrapolated to man, lie well within the therapeutic dose range. Moreover, Levy (3) pointed out that several capacity-limited drug-metabolizing systems observed in man have been demonstrated also in rats. These include a limited capacity for the conjugation of salicylate (6) and benzoate (3) with glycine and for sulfate and glucuronide conjugation with *N*-acetyl-*p*-aminophenol (7). Whether capacity-limited acetylation of sulfanilamide can be added to the list of similarities between man and the rat has to await additional evidence. Certainly it becomes increasingly apparent that drug metabolism studies must be carried out at multiple-dose levels and that linearity of the semilogarithmic plots of drug elimination as a function of time is frequently insufficient to discriminate between dose-independent or dose-dependent biotransformations.

(1) R. R. Levine, T. J. De Marco, and C. M. Dizon, *Proc. Int. Congr. Pharmacol., 4th*, **2**, 79(1970).
(2) G. Levy, *J. Pharm. Sci.*, **54**, 959(1965).
(3) G. Levy, in "Importance of Fundamental Principles in Drug Evaluation," D. H. Tedeschi and R. E. Tedeschi, Eds., Raven Press, New York, N. Y., 1968, pp. 141–172.
(4) M. M. Drucker, S. H. Blondheim, and I. Wislicki, *Clin. Sci.*, **27**, 133(1964).
(5) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).
(6) E. Nelson, M. Hanano, and G. Levy, *J. Pharmacol. Exp. Ther.*, **153**, 159(1966).
(7) H. Büch, K. Pfleger, and W. Rüdiger, *Z. Klin. Chem. Klin. Biochem.*, **5**, 110(1967).

RUTH R. LEVINE
CARMELITA M. DIZON
Department of Pharmacology and Experimental Therapeutics
Boston University Medical Center
Boston, MA 02118

Received July 16, 1970.
Accepted for publication August 21, 1970.
This investigation was supported in part by Grant AFOSR-914 from the U. S. Air Force Office of Scientific Research and Grant NB-01966 from the National Institute of Neurological Diseases and Stroke of the National Institutes of Health.

Apparent Dose-Dependent Elimination Kinetics as an Experimental Artifact

Keyphrases ☐ Dose-dependent elimination kinetics, apparent—experimental artifact consideration ☐ Plasma concentration—experimental blank errors

Sir:

It is now known that the kinetics of elimination of several drugs are dose dependent. The decline in the plasma concentrations of some drugs is exponential throughout, but the apparent first-order rate constant for this process descreases with increasing dose (1). The elimination of other drugs involves one or more saturable processes, and semilogarithmic plots of plasma concentrations as a function of time curve downward until they attain an exponential phase which is reached at the same concentration irrespective of the dose (2). In view of the great interest and investigative activity in the area of dose-dependent pharmacokinetics, it is appropriate to point out that errors in blank corrections can artifactually lead to the conclusion that an entirely linear, dose-independent system is in fact dose dependent and nonlinear.

Figure 1 shows hypothetical plasma concentrations obtained after intravenous injection of 1, 3, and 10 weight units of a drug which is actually eliminated by apparent first-order kinetics ($t_{1/2}$ = 2.0 hr.), with the plasma concentration data describable by means of a one-compartment open model. However, the data points in the figure are, in each case, 2 mg./l. lower than the "real" concentrations. This would be so if, for a number of possible reasons, a 2 mg./l. error in the blank value determination would have occurred. The data points thus obtained can be fitted readily to straight lines which yield a decreasing half-life with increasing dose. In the example shown, there is an almost 50% change in the apparent half-life.

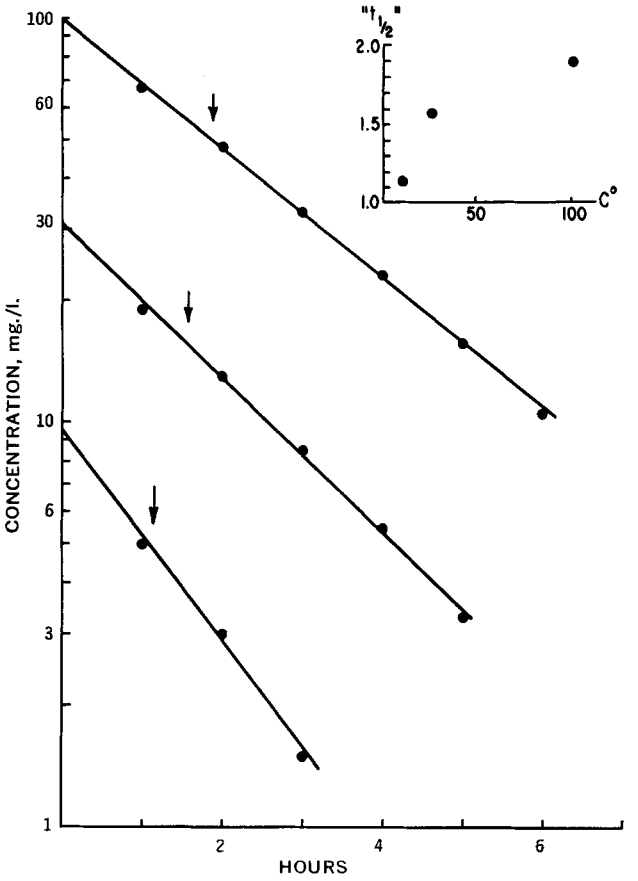


Figure 1—*Hypothetical plasma concentration data obtained after intravenous injection of 1, 3, and 10 weight units of a drug which is eliminated by apparent first-order kinetics* ($t_{1/2}$ = 2 hr.), *if the blank correction is 2 mg./l. too large. Arrows indicate apparent half-life. Inset: Relationship between initial plasma concentration* (C_0) *and apparent half-life* ("$t_{1/2}$").

If plasma concentrations are determined over a wide concentration range, an error in the blank correction can lead to the erroneous conclusion that elimination involves a combination of parallel linear and saturable (*i.e.*, capacity-limited) processes. Such systems may show an initial exponential concentration decline phase at high concentrations, a subsequent downward curvature, and finally another exponential phase which is steeper than the initial exponential phase (2). This pattern is evident in Fig. 2; the figure shows hypothetical plasma concentration data at two doses (differing 10-fold) of a drug, which is actually eliminated by apparent first-order kinetics but where an error of 3 mg./l. in the blank correction causes appreciable deviations from linearity. In this example, parallel straight lines may be fitted erroneously to the two sets of terminal data points, suggesting that the elimination kinetics above a plasma concentration of about 10 mg./l. are capacity limited.

The potential artifacts outlined in this article necessitate that considerable attention be directed to the correct determination of blank values. The magnitude

(1) R. R. Levine, T. J. De Marco, and C. M. Dizon, *Proc. Int. Congr. Pharmacol., 4th*, **2**, 79(1970).
(2) G. Levy, *J. Pharm. Sci.*, **54**, 959(1965).
(3) G. Levy, in "Importance of Fundamental Principles in Drug Evaluation," D. H. Tedeschi and R. E. Tedeschi, Eds., Raven Press, New York, N. Y., 1968, pp. 141–172.
(4) M. M. Drucker, S. H. Blondheim, and I. Wislicki, *Clin. Sci.*, **27**, 133(1964).
(5) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537(1939).
(6) E. Nelson, M. Hanano, and G. Levy, *J. Pharmacol. Exp. Ther.*, **153**, 159(1966).
(7) H. Büch, K. Pfleger, and W. Rüdiger, *Z. Klin. Chem. Klin. Biochem.*, **5**, 110(1967).

RUTH R. LEVINE
CARMELITA M. DIZON
Department of Pharmacology and Experimental Therapeutics
Boston University Medical Center
Boston, MA 02118

Received July 16, 1970.
Accepted for publication August 21, 1970.
This investigation was supported in part by Grant AFOSR-914 from the U. S. Air Force Office of Scientific Research and Grant NB-01966 from the National Institute of Neurological Diseases and Stroke of the National Institutes of Health.

Apparent Dose-Dependent Elimination Kinetics as an Experimental Artifact

Keyphrases ☐ Dose-dependent elimination kinetics, apparent—experimental artifact consideration ☐ Plasma concentration—experimental blank errors

Sir:

It is now known that the kinetics of elimination of several drugs are dose dependent. The decline in the plasma concentrations of some drugs is exponential throughout, but the apparent first-order rate constant for this process descreases with increasing dose (1). The elimination of other drugs involves one or more saturable processes, and semilogarithmic plots of plasma concentrations as a function of time curve downward until they attain an exponential phase which is reached at the same concentration irrespective of the dose (2). In view of the great interest and investigative activity in the area of dose-dependent pharmacokinetics, it is appropriate to point out that errors in blank corrections can artifactually lead to the conclusion that an entirely linear, dose-independent system is in fact dose dependent and nonlinear.

Figure 1 shows hypothetical plasma concentrations obtained after intravenous injection of 1, 3, and 10 weight units of a drug which is actually eliminated by apparent first-order kinetics ($t_{1/2}$ = 2.0 hr.), with the plasma concentration data describable by means of a one-compartment open model. However, the data points in the figure are, in each case, 2 mg./l. lower than the "real" concentrations. This would be so if, for a number of possible reasons, a 2 mg./l. error in the blank value determination would have occurred. The data points thus obtained can be fitted readily to straight lines which yield a decreasing half-life with increasing dose. In the example shown, there is an almost 50% change in the apparent half-life.

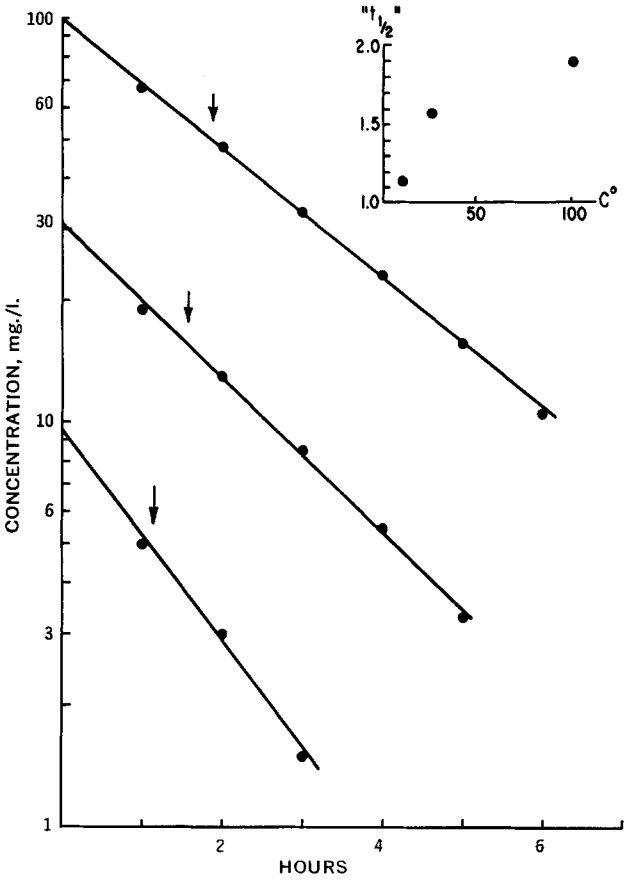


Figure 1—*Hypothetical plasma concentration data obtained after intravenous injection of 1, 3, and 10 weight units of a drug which is eliminated by apparent first-order kinetics* ($t_{1/2}$ = 2 hr.), *if the blank correction is 2 mg./l. too large. Arrows indicate apparent half-life. Inset: Relationship between initial plasma concentration* (C_0) *and apparent half-life* ("$t_{1/2}$").

If plasma concentrations are determined over a wide concentration range, an error in the blank correction can lead to the erroneous conclusion that elimination involves a combination of parallel linear and saturable (*i.e.*, capacity-limited) processes. Such systems may show an initial exponential concentration decline phase at high concentrations, a subsequent downward curvature, and finally another exponential phase which is steeper than the initial exponential phase (2). This pattern is evident in Fig. 2; the figure shows hypothetical plasma concentration data at two doses (differing 10-fold) of a drug, which is actually eliminated by apparent first-order kinetics but where an error of 3 mg./l. in the blank correction causes appreciable deviations from linearity. In this example, parallel straight lines may be fitted erroneously to the two sets of terminal data points, suggesting that the elimination kinetics above a plasma concentration of about 10 mg./l. are capacity limited.

The potential artifacts outlined in this article necessitate that considerable attention be directed to the correct determination of blank values. The magnitude

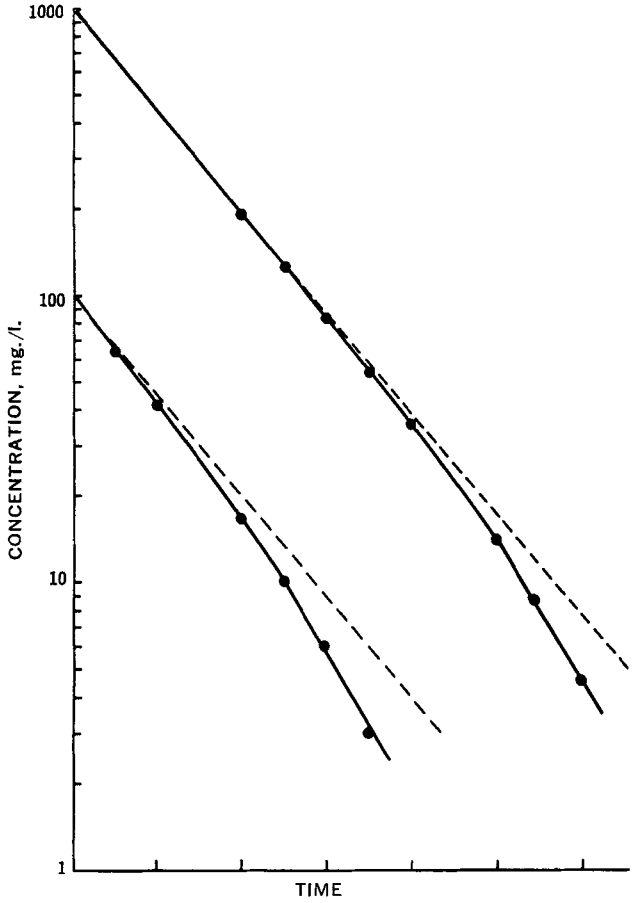


Figure 2—*Hypothetical plasma concentration data* (●) *obtained after intravenous injection of two different doses of a drug which is eliminated by apparent first-order kinetics, if the blank correction is 3 mg./l. too large. The dashed lines represent the correct curve. Note the apparent parallelism of the straight lines fitted to the last three points of each set of data.*

and variability of the blank, relative to the lower range of drug concentrations encountered in the investigation, must be carefully considered in the pharmacokinetic analysis of the data. It is recommended that apparent dose-dependent changes in elimination-rate constants, as shown in Fig. 1, be tested statistically for lack of parallelism of the respective log concentration *versus* time curves *in the same concentration range.* Where plasma concentration data show the pattern presented in Fig. 2, it is best to focus attention on the plasma concentration and/or urinary excretion pattern of the metabolite that is presumed to be subject to capacity-limited formation.

An underestimation of blank values, resulting in higher than correct drug concentration data, has exactly the opposite effects as those described here. Apparent first-order elimination-rate constants may be mistakenly assumed to increase with increasing dose [a type of kinetics that can actually occur due to dose-dependent distributional effects (3)], and a decrease in the slope of log drug concentration *versus* time curves with decreasing concentration might be treated as a linear multicompartment model or be interpreted as suggesting saturation of a renal tubular reabsorption process [a type of kinetics that can, in fact, occur (4)]. Thus, one must be concerned not only with the specificity and sensitivity of an analytical method but also with the possibility of systematic errors in the blank correction.

(1) P. G. Dayton, S. A. Cucinell, M. Weiss, and J. M. Perel, *J. Pharmacol. Exp. Ther.*, **158,** 305(1967).
(2) G. Levy, in "Importance of Fundamental Principles in Drug Evaluation," D. H. Tedeschi and R. E. Tedeschi, Eds., Raven Press, New York, N. Y., 1968, pp. 141–172.
(3) G. Levy, *Proc. Int. Congr. Pharmacol., 4th,* **4,** 134(1970).
(4) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **59,** 765(1970).

GERHARD LEVY
Department of Pharmaceutics
School of Pharmacy
State University of New York at Buffalo
Buffalo, NY 14214

Received July 15, 1970.
Accepted for publication August 20, 1970.
Supported in part by Grant 5 RO1 FD00015 from the National Institutes of Health.

Flocculation Theory and Polysorbate 80–Sulfaguanidine Suspensions

Keyphrases □ Flocculation theory—sulfaguanidine–polysorbate 80 suspension □ Sedimentation height—sulfaguanidine–polysorbate 80 suspension

Sir:

Flocculation has been defined as an open network structure formed by aggregated suspension particles (1). Three possible mechanisms by which such a structure can occur are: (*a*) aggregation in the secondary minimum which can theoretically result when the forces of attraction exceed the forces of repulsion (2, 3); (*b*) adsorption bridging—the aggregation of particles whose surface sites are occupied by segments of extended macromolecules; the extended molecules act as bridges between particles (4); and (*c*) chemical bridging—the aggregation by chemical reaction between adsorbed ions extending from the particle surface and media precipitation ions (5, 6).

In a study of the aggregation of a sulfaguanidine suspension with particles wetted by polysorbate 80, it was reported that the addition of increasing amounts of aluminum chloride produced a "flocculated system" which showed a steady increase in sedimentation height (7). A maximum volume was reached, and further additions of salt produced no change in sedimentation height.

Aluminum chloride at the concentrations used in the report could not react with the nonionic surfactant in a manner similar to those interactions that cause flocculation by chemical bridging.

Polysorbate 80 has never, in our experience, shown the characteristics exhibited by macromolecules that produce floccules in suspensions; therefore, it seemed

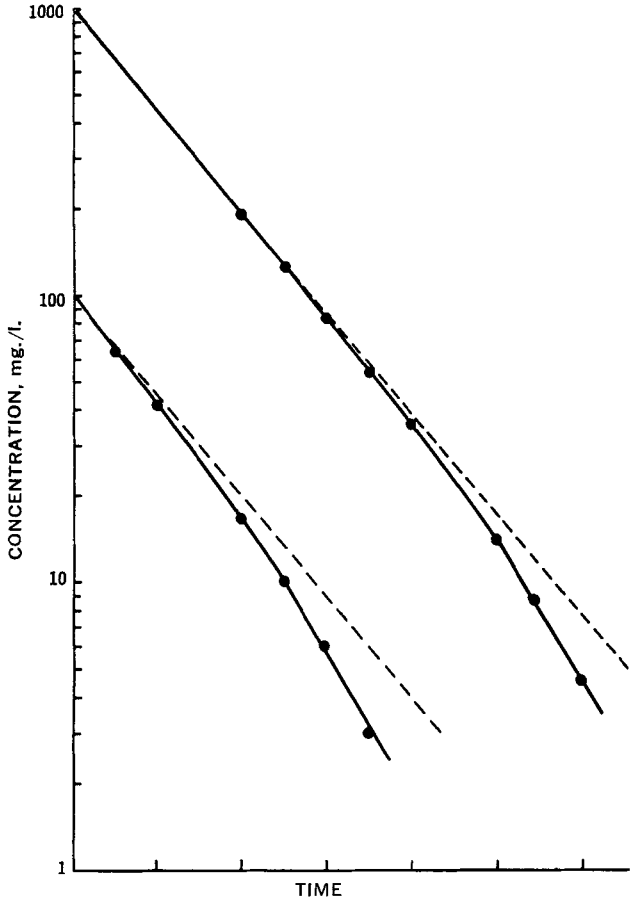


Figure 2—*Hypothetical plasma concentration data* (●) *obtained after intravenous injection of two different doses of a drug which is eliminated by apparent first-order kinetics, if the blank correction is 3 mg./l. too large. The dashed lines represent the correct curve. Note the apparent parallelism of the straight lines fitted to the last three points of each set of data.*

and variability of the blank, relative to the lower range of drug concentrations encountered in the investigation, must be carefully considered in the pharmacokinetic analysis of the data. It is recommended that apparent dose-dependent changes in elimination-rate constants, as shown in Fig. 1, be tested statistically for lack of parallelism of the respective log concentration *versus* time curves *in the same concentration range.* Where plasma concentration data show the pattern presented in Fig. 2, it is best to focus attention on the plasma concentration and/or urinary excretion pattern of the metabolite that is presumed to be subject to capacity-limited formation.

An underestimation of blank values, resulting in higher than correct drug concentration data, has exactly the opposite effects as those described here. Apparent first-order elimination-rate constants may be mistakenly assumed to increase with increasing dose [a type of kinetics that can actually occur due to dose-dependent distributional effects (3)], and a decrease in the slope of log drug concentration *versus* time curves with decreasing concentration might be treated as a linear multicompartment model or be interpreted as suggesting saturation of a renal tubular reabsorption process [a type of kinetics that can, in fact, occur (4)]. Thus, one must be concerned not only with the specificity and sensitivity of an analytical method but also with the possibility of systematic errors in the blank correction.

(1) P. G. Dayton, S. A. Cucinell, M. Weiss, and J. M. Perel, *J. Pharmacol. Exp. Ther.*, **158,** 305(1967).
(2) G. Levy, in "Importance of Fundamental Principles in Drug Evaluation," D. H. Tedeschi and R. E. Tedeschi, Eds., Raven Press, New York, N. Y., 1968, pp. 141–172.
(3) G. Levy, *Proc. Int. Congr. Pharmacol., 4th,* **4,** 134(1970).
(4) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **59,** 765(1970).

GERHARD LEVY
Department of Pharmaceutics
School of Pharmacy
State University of New York at Buffalo
Buffalo, NY 14214

Received July 15, 1970.
Accepted for publication August 20, 1970.
Supported in part by Grant 5 RO1 FD00015 from the National Institutes of Health.

Flocculation Theory and Polysorbate 80–Sulfaguanidine Suspensions

Keyphrases □ Flocculation theory—sulfaguanidine–polysorbate 80 suspension □ Sedimentation height—sulfaguanidine–polysorbate 80 suspension

Sir:

Flocculation has been defined as an open network structure formed by aggregated suspension particles (1). Three possible mechanisms by which such a structure can occur are: (*a*) aggregation in the secondary minimum which can theoretically result when the forces of attraction exceed the forces of repulsion (2, 3); (*b*) adsorption bridging—the aggregation of particles whose surface sites are occupied by segments of extended macromolecules; the extended molecules act as bridges between particles (4); and (*c*) chemical bridging—the aggregation by chemical reaction between adsorbed ions extending from the particle surface and media precipitation ions (5, 6).

In a study of the aggregation of a sulfaguanidine suspension with particles wetted by polysorbate 80, it was reported that the addition of increasing amounts of aluminum chloride produced a "flocculated system" which showed a steady increase in sedimentation height (7). A maximum volume was reached, and further additions of salt produced no change in sedimentation height.

Aluminum chloride at the concentrations used in the report could not react with the nonionic surfactant in a manner similar to those interactions that cause flocculation by chemical bridging.

Polysorbate 80 has never, in our experience, shown the characteristics exhibited by macromolecules that produce floccules in suspensions; therefore, it seemed

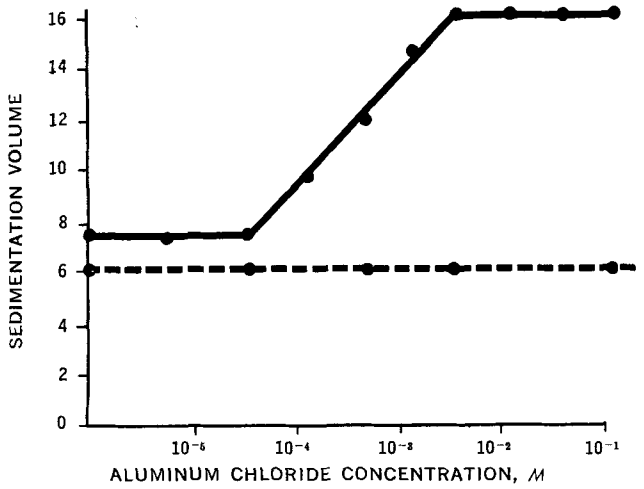


Figure 1—*Aggregation of sulfaguanidine in a suspension containing polysorbate 80 and aluminum chloride. Key:* ●—●, *from Jones* et al. (7); *and* ●- - -●, *this report.*

doubtful that in this experiment aggregation by adsorption bridging would occur.

We do not believe that the theory of long-range forces of attraction, classically believed to be responsible for aggregation in the secondary minimum, applies to the aggregation of hydrophilic particles (5). Since the sulfaguanidine covered with surfactant is a hydrophilic system, no flocculation will occur by mechanism *a*.

What we believed likely to happen is that the dispersed sulfa particles settle as individual entities and/or aggregate by surfactant–water film to surfactant–water film interactions to settle as close packed coagula (1). In either or both cases, the final height could not vary in the manner described in the report.

Since the properties of the system used did not appear to us to be capable of producing a flocculated structure on the basis of any of the three mechanisms enumerated, we studied these same systems for clarification.

Sulfaguanidine NF suspensions in water with polysorbate 80 and aluminum chloride were prepared in the exact manner described in the report (7), with the exception that mixing was done by a magnetic stirrer. The results of our experiments were always a sedimentation to a small volume (Fig. 1). The height did not change over the range of aluminum chloride concentrations shown. The suspensions sedimented in about 3 hr. and the final (*Hu*/*Ho*) was measured after 24 hr. The experimental results reported here indicate that the particles are either in the dispersed state and/or the coagulated state (1).

The system reported to be flocculated, *i.e.*, to increase to approximately twice its minimum (*Hu*/*Ho*) height was found in this laboratory to give only a contant height under all conditions stated in the report. These results support our *a priori* assumption that flocculation cannot take place in this system.

(1) B. Ecanow, B. Gold, and C. Ecanow, *Amer. Perfum. Cosmet.*, **84**, 27(1969).

(2) B. Derjaguin and L. Landau, *Acta Physiochim.*, **14**, 633 (1941).

(3) E. Verwey and J. Overbeek, "Theory of Stability of Lyophobic Colloids," Elsevier, New York, N. Y., 1948.

(4) V. LaMer and T. Healy, *J. Phys. Chem.*, **67**, 2417(1963).

(5) R. Wilson and B. Ecanow, *J. Pharm. Sci.*, **52**, 757(1963).

(6) V. LaMer, *J. Colloid Sci.*, **19**, 291(1964).

(7) R. Jones, B. Matthews, and C. Rhodes, *J. Pharm. Sci.*, **59**, 518(1970).

BERNARD ECANOW
HARUN TAKRURI
Department of Pharmacy
University of Illinois
at the Medical Center
Chicago, IL 60612

Received June 1, 1970.
Accepted for publication August 18, 1970.

Carboxyl Protection Using Salt Formation for the Synthesis of Linear Sequential Polypeptides: Synthesis of Poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C Ethyl Ester

Keyphrases □ Polypeptides, linear sequential—synthesis □ Poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester— synthesis □ Carboxyl protection—peptide synthesis □ Immunochemical properties—linear sequential polypeptide

Sir:

The least elaborate approach to carboxyl protection in peptide synthesis is the use of salt formation with such bases as triethylamine, tributylamine, or dicyclohexylamine. This method of protection seems to work best when an activated ester of an *N*-protected amino acid is used for the coupling reaction to amino acids and peptides which were carboxyl protected by salt formation (1, 2).

We have extended this method of protection to the synthesis of high molecular weight linear polypeptides. This is illustrated by a new synthesis of the antigenic polymer poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (Scheme I).

The previously reported tetrapeptide (3), *N*-carbobenzoxy-*O*-*tert*-butyl-L-tyrosyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine methyl ester (I), was saponified with 1 equivalent of *N* NaOH to yield the tetrapeptide free acid, *N*-carbobenzoxy-*O*-*tert*-butyl-L-tyrosyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine (II), m.p. 159–160°, $[\alpha]_D^{25}$ − 10.3° (c 4.23 in dimethylformamide).

Anal.—Calcd. for $C_{35}H_{48}N_4O_{10}$: C, 61.4; H, 7.1; N, 8.2. Found: C, 61.5; H, 7.15; N, 8.1.

Coupling II with pentachlorophenol, using dicyclohexylcarbodiimide, yielded the tetrapeptide activated ester, *N*-carbobenzoxy-*O*-*tert*-butyl-L-tyrosyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester (III), m.p. 185°, $[\alpha]_D^{28}$ − 17.0° (c 1.06 in dimethylformamide).

Anal.—Calcd. for $C_{41}H_{47}Cl_5N_4O_{10}$: C, 52.8; H, 5.1; N, 6.0. Found: C, 52.6; H, 4.9; N, 6.0.

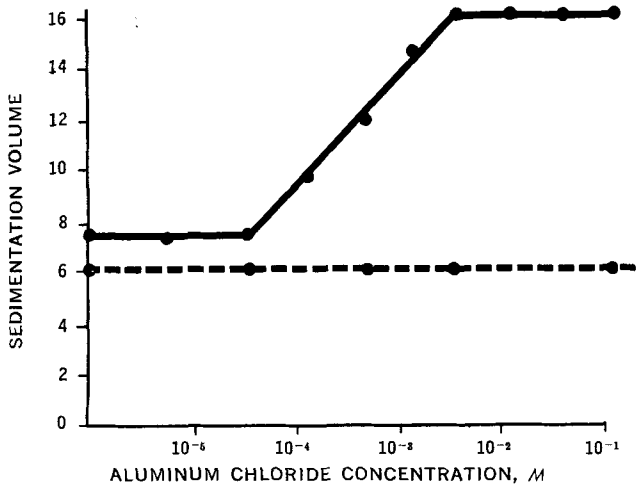


Figure 1—*Aggregation of sulfaguanidine in a suspension containing polysorbate 80 and aluminum chloride. Key:* ●—●, *from Jones* et al. (7); *and* ●- - -●, *this report.*

doubtful that in this experiment aggregation by adsorption bridging would occur.

We do not believe that the theory of long-range forces of attraction, classically believed to be responsible for aggregation in the secondary minimum, applies to the aggregation of hydrophilic particles (5). Since the sulfaguanidine covered with surfactant is a hydrophilic system, no flocculation will occur by mechanism *a*.

What we believed likely to happen is that the dispersed sulfa particles settle as individual entities and/or aggregate by surfactant–water film to surfactant–water film interactions to settle as close packed coagula (1). In either or both cases, the final height could not vary in the manner described in the report.

Since the properties of the system used did not appear to us to be capable of producing a flocculated structure on the basis of any of the three mechanisms enumerated, we studied these same systems for clarification.

Sulfaguanidine NF suspensions in water with polysorbate 80 and aluminum chloride were prepared in the exact manner described in the report (7), with the exception that mixing was done by a magnetic stirrer. The results of our experiments were always a sedimentation to a small volume (Fig. 1). The height did not change over the range of aluminum chloride concentrations shown. The suspensions sedimented in about 3 hr. and the final (*Hu*/*Ho*) was measured after 24 hr. The experimental results reported here indicate that the particles are either in the dispersed state and/or the coagulated state (1).

The system reported to be flocculated, *i.e.*, to increase to approximately twice its minimum (*Hu*/*Ho*) height was found in this laboratory to give only a contant height under all conditions stated in the report. These results support our *a priori* assumption that flocculation cannot take place in this system.

(1) B. Ecanow, B. Gold, and C. Ecanow, *Amer. Perfum. Cosmet.*, **84**, 27(1969).

(2) B. Derjaguin and L. Landau, *Acta Physiochim.*, **14**, 633 (1941).

(3) E. Verwey and J. Overbeek, "Theory of Stability of Lyophobic Colloids," Elsevier, New York, N. Y., 1948.

(4) V. LaMer and T. Healy, *J. Phys. Chem.*, **67**, 2417(1963).

(5) R. Wilson and B. Ecanow, *J. Pharm. Sci.*, **52**, 757(1963).

(6) V. LaMer, *J. Colloid Sci.*, **19**, 291(1964).

(7) R. Jones, B. Matthews, and C. Rhodes, *J. Pharm. Sci.*, **59**, 518(1970).

BERNARD ECANOW
HARUN TAKRURI
Department of Pharmacy
University of Illinois
at the Medical Center
Chicago, IL 60612

Received June 1, 1970.
Accepted for publication August 18, 1970.

Carboxyl Protection Using Salt Formation for the Synthesis of Linear Sequential Polypeptides: Synthesis of Poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C Ethyl Ester

Keyphrases □ Polypeptides, linear sequential—synthesis □ Poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester— synthesis □ Carboxyl protection—peptide synthesis □ Immunochemical properties—linear sequential polypeptide

Sir:

The least elaborate approach to carboxyl protection in peptide synthesis is the use of salt formation with such bases as triethylamine, tributylamine, or dicyclohexylamine. This method of protection seems to work best when an activated ester of an *N*-protected amino acid is used for the coupling reaction to amino acids and peptides which were carboxyl protected by salt formation (1, 2).

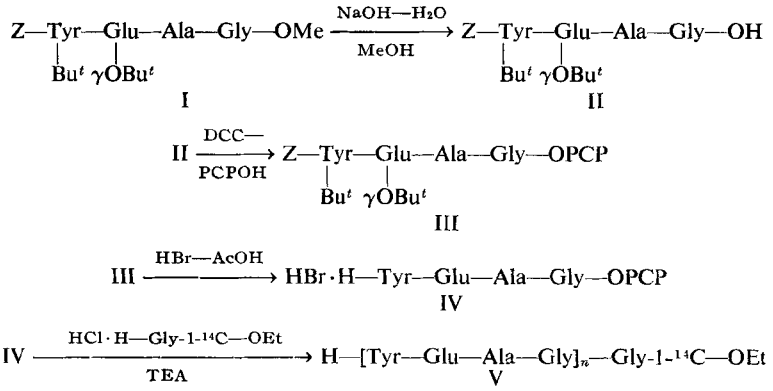
We have extended this method of protection to the synthesis of high molecular weight linear polypeptides. This is illustrated by a new synthesis of the antigenic polymer poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (Scheme I).

The previously reported tetrapeptide (3), *N*-carbobenzoxy-*O*-*tert*-butyl-L-tyrosyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine methyl ester (I), was saponified with 1 equivalent of *N* NaOH to yield the tetrapeptide free acid, *N*-carbobenzoxy-*O*-*tert*-butyl-L-tyrosyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine (II), m.p. 159–160°, $[\alpha]_D^{25}$ − 10.3° (c 4.23 in dimethylformamide).

Anal.—Calcd. for $C_{35}H_{48}N_4O_{10}$: C, 61.4; H, 7.1; N, 8.2. Found: C, 61.5; H, 7.15; N, 8.1.

Coupling II with pentachlorophenol, using dicyclohexylcarbodiimide, yielded the tetrapeptide activated ester, *N*-carbobenzoxy-*O*-*tert*-butyl-L-tyrosyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester (III), m.p. 185°, $[\alpha]_D^{28}$ − 17.0° (c 1.06 in dimethylformamide).

Anal.—Calcd. for $C_{41}H_{47}Cl_5N_4O_{10}$: C, 52.8; H, 5.1; N, 6.0. Found: C, 52.6; H, 4.9; N, 6.0.



where DCC = dicyclohexylcarbodiimide, PCP = pentachlorophenyl, and TEA = triethylamine

Scheme I

Treatment of III with anhydrous hydrogen bromide in glacial acetic acid removed the *N*-carbobenzoxy and the *tert*-butyl protecting groups to yield the polymerizing unit, L-tyrosyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrobromide (IV), m.p. 180°, $[\alpha]_D^{28}$ − 3.3° (c 1.83 in dimethylformamide).

Anal.—Calcd. for $C_{25}H_{26}BrCl_5N_4O_8$: C, 39.1; H, 3.4; N, 7.3 Found: C, 39.1; H, 3.6; N, 7.6.

The polymerization of IV was conducted under dilute conditions in the presence of a preformed monomer glycine-1-^{14}C ethyl ester hydrochloride. This established polymerizing procedure has been shown to yield linear high molecular weight polypeptides (3–6) when the side groups are protected. In this case, the polymerizing unit (IV), dissolved in dimethyl sulfoxide, was added dropwise to a solution of glycine-1-^{14}C ethyl ester hydrochloride containing the total amount (3.5 equivalents) of triethylamine such that the final concentration of reactants was never more than 70 mmole/l. The polymerization was allowed to proceed for a week, after which the mixture was acidified and dialyzed extensively for 3 days. The precipitated polypeptide, poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (V), was collected by centrifugation, converted to its sodium salt, and dialyzed extensively for a week to remove all low molecular weight materials. This dialyzed polymer was lyophilized, converted to the free acid form by acidification, and again dialyzed to remove all traces of salt. Radioactive assay indicated 85% incorporation of the starting preformed monomer.

Anal.—Calcd. for $C_{19}H_{24}N_4O_7 \cdot \frac{1}{2}\ H_2O$: C, 53.15; H, 5.85; N, 13.05. Found: C, 52.8; H, 5.9; N, 13.0.

Filtration of the polymer through a calibrated column (7) of synthetic polysaccharide[1] (2.5 × 45 cm.), using a solution of 0.1 *M* NaCl–0.05 *M* $NaHCO_3$ buffer as eluent, indicated a molecular weight of at least 1 × 10^5.

To evaluate this method of preparing polypeptides with one that uses conventional protecting groups (3–6), a comparison was made of this polypeptide with that prepared using the *tert*-butyl ester for carboxyl protection (3). It was found that both polymers eluted from a column of the polysaccharide (2.5 × 45 cm.) in the same fractionation pattern, using a 0.1 *M* NaCl–0.05 *M* $NaHCO_3$ buffer as eluent. The two polymers were considered to be structurally identical since both materials were similarly antigenic in rabbits; each polypeptide crossreacted with the antibodies produced by the other, giving the same precipitin curve which was similar to that previously reported (8). From this evidence it was concluded that this method of carboxyl protection is compatible with the synthesis of linear high molecular weight polypeptides.

[1] Sephadex G-100.

(1) D. S. Kemp and S. W. Chien, *J. Amer. Chem. Soc.*, **89,** 2743 (1967).
(2) A. Kapoor, E. J. Davis, and M. J. Graetzer, *J. Pharm. Sci.*, **57,** 1514(1968).
(3) B. J. Johnson and E. G. Trask, *J. Chem. Soc., C,* **1969,** 2644.
(4) B. J. Johnson, *ibid.*, **1967,** 2638.
(5) *Ibid.*, **1968,** 3008.
(6) *Ibid.*, **1969,** 1412.
(7) P. Andrews, *Biochem. J.*, **91,** 222(1964).
(8) B. J. Johnson and E. G. Trask, *J. Pharm. Sci.*, **59,** 724(1970).

BRIAN J. JOHNSON
Department of Chemistry
Tufts University
Medford, MA 02155

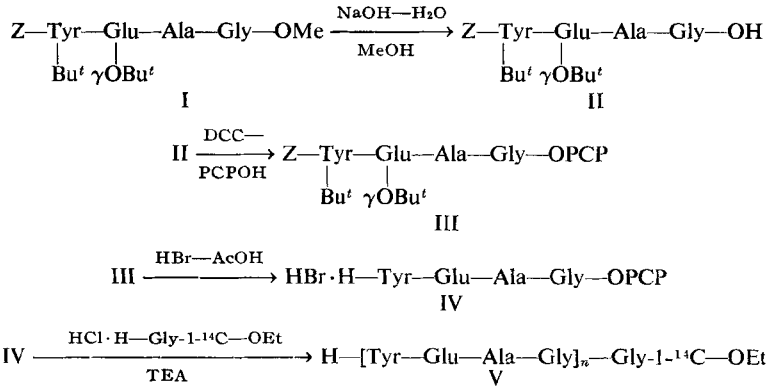
Received June 15, 1970.
Accepted for publication July 31, 1970.
This work was supported by a grant from the National Science Foundation.

Aporphines V: Total Synthesis of (±)-Apomorphine

Keyphrases □ (±)-Apomorphine—total synthesis □ IR spectrophotometry—structure □ UV spectrophotometry—structure

Sir:

(−)-Apomorphine [(−)-I], the semisynthetic alkaloid obtained by vigorous treatment of morphine with strong mineral acids, has found medicinal application



where DCC = dicyclohexylcarbodiimide, PCP = pentachlorophenyl, and TEA = triethylamine

Scheme I

Treatment of III with anhydrous hydrogen bromide in glacial acetic acid removed the *N*-carbobenzoxy and the *tert*-butyl protecting groups to yield the polymerizing unit, L-tyrosyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrobromide (IV), m.p. 180°, $[\alpha]_D^{28}$ − 3.3° (c 1.83 in dimethylformamide).

Anal.—Calcd. for $C_{25}H_{26}BrCl_5N_4O_8$: C, 39.1; H, 3.4; N, 7.3 Found: C, 39.1; H, 3.6; N, 7.6.

The polymerization of IV was conducted under dilute conditions in the presence of a preformed monomer glycine-1-^{14}C ethyl ester hydrochloride. This established polymerizing procedure has been shown to yield linear high molecular weight polypeptides (3–6) when the side groups are protected. In this case, the polymerizing unit (IV), dissolved in dimethyl sulfoxide, was added dropwise to a solution of glycine-1-^{14}C ethyl ester hydrochloride containing the total amount (3.5 equivalents) of triethylamine such that the final concentration of reactants was never more than 70 mmole/l. The polymerization was allowed to proceed for a week, after which the mixture was acidified and dialyzed extensively for 3 days. The precipitated polypeptide, poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (V), was collected by centrifugation, converted to its sodium salt, and dialyzed extensively for a week to remove all low molecular weight materials. This dialyzed polymer was lyophilized, converted to the free acid form by acidification, and again dialyzed to remove all traces of salt. Radioactive assay indicated 85% incorporation of the starting preformed monomer.

Anal.—Calcd. for $C_{19}H_{24}N_4O_7 \cdot \frac{1}{2}\ H_2O$: C, 53.15; H, 5.85; N, 13.05. Found: C, 52.8; H, 5.9; N, 13.0.

Filtration of the polymer through a calibrated column (7) of synthetic polysaccharide[1] (2.5 × 45 cm.), using a solution of 0.1 *M* NaCl–0.05 *M* $NaHCO_3$ buffer as eluent, indicated a molecular weight of at least 1 × 10^5.

To evaluate this method of preparing polypeptides with one that uses conventional protecting groups (3–6), a comparison was made of this polypeptide with that prepared using the *tert*-butyl ester for carboxyl protection (3). It was found that both polymers eluted from a column of the polysaccharide (2.5 × 45 cm.) in the same fractionation pattern, using a 0.1 *M* NaCl–0.05 *M* $NaHCO_3$ buffer as eluent. The two polymers were considered to be structurally identical since both materials were similarly antigenic in rabbits; each polypeptide crossreacted with the antibodies produced by the other, giving the same precipitin curve which was similar to that previously reported (8). From this evidence it was concluded that this method of carboxyl protection is compatible with the synthesis of linear high molecular weight polypeptides.

[1] Sephadex G-100.

(1) D. S. Kemp and S. W. Chien, *J. Amer. Chem. Soc.*, **89,** 2743 (1967).
(2) A. Kapoor, E. J. Davis, and M. J. Graetzer, *J. Pharm. Sci.*, **57,** 1514(1968).
(3) B. J. Johnson and E. G. Trask, *J. Chem. Soc., C,* **1969,** 2644.
(4) B. J. Johnson, *ibid.*, **1967,** 2638.
(5) *Ibid.*, **1968,** 3008.
(6) *Ibid.*, **1969,** 1412.
(7) P. Andrews, *Biochem. J.*, **91,** 222(1964).
(8) B. J. Johnson and E. G. Trask, *J. Pharm. Sci.*, **59,** 724(1970).

BRIAN J. JOHNSON
Department of Chemistry
Tufts University
Medford, MA 02155

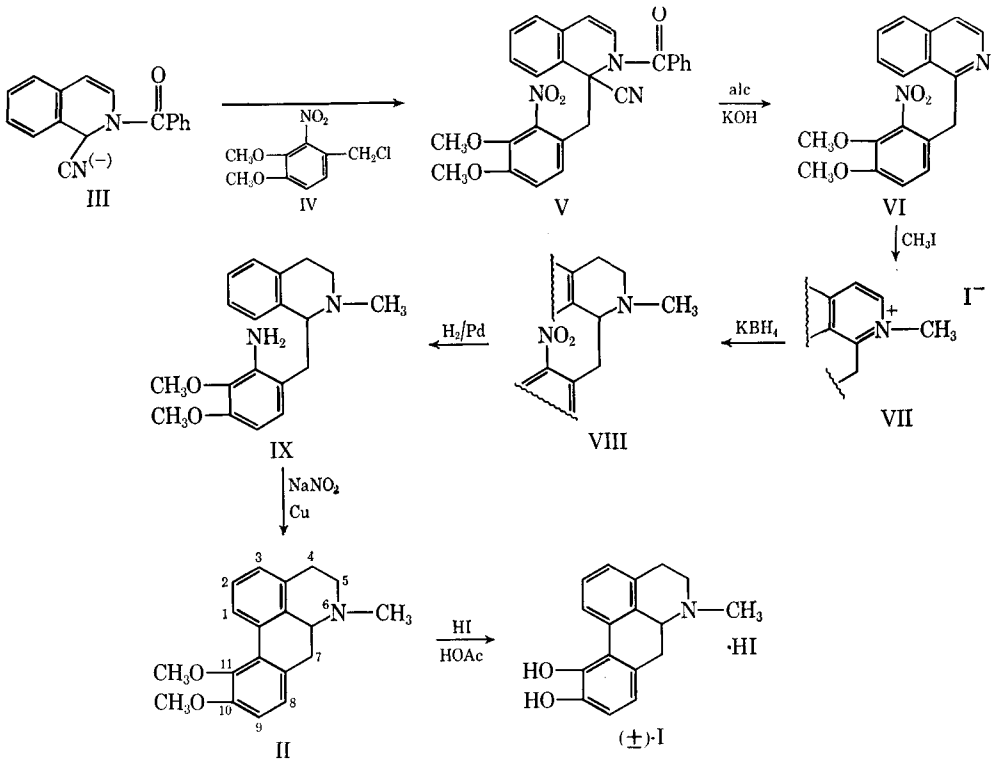
Received June 15, 1970.
Accepted for publication July 31, 1970.
This work was supported by a grant from the National Science Foundation.

Aporphines V: Total Synthesis of (±)-Apomorphine

Keyphrases □ (±)-Apomorphine—total synthesis □ IR spectrophotometry—structure □ UV spectrophotometry—structure

Sir:

(−)-Apomorphine [(−)-I], the semisynthetic alkaloid obtained by vigorous treatment of morphine with strong mineral acids, has found medicinal application



Scheme I

as a powerful, centrally acting emetic and has been included in the official drug compendia of the United States (1) and is regulated by Federal Narcotic Laws (2). Apomorphine exerts a direct stimulating action on the vomiting center in the brain and thus initiates emesis (3). The biochemical mechanism of its action is not yet clearly understood.

The finding that subcutaneous administration of apomorphine to patients with selected neurologic disorders temporarily changed the neurologic manifestations in the same direction as with the administration of oral L-dopa has stimulated renewed interest in these catecholaminelike compounds (4).

We wish to report the first total synthesis of (±)-apomorphine [(±)-I] and to describe a versatile pathway with potential for the elaboration of structurally similar alkaloids. Although (±)-apomorphine has not been synthesized previously, the preparation of (±)-apomorphine dimethyl ether [(±)-II] has been the subject of considerable interest and was first successfully carried out by Späth and Hromatka (5) and subsequently by Hey and Palluel (6) and Sugasawa and Tachikawa (7). Their general methods were based on a Bischler–Napieralski cyclization of an *o*-nitrophenyl-*N*-phenylethylacetamide to a 3,4-dihydroisoquinoline derivative, which was subsequently transformed *via* the Pschorr procedure to apomorphine dimethyl ether. Several workers have found this sequence impracticable, since the Bischler–Napieralski cyclization is generally unsatisfactory (8) when the amide used lacks an activating substituent on the aromatic ring of the *N*-phenylethyl moiety. Our scheme (Scheme I) was based on the alkylation of the Reissert anion (III) with 2-nitro-3,4-dimethoxybenzyl chloride (IV) (9). A variety of 1-alkylisoquinolines has been prepared by this method (10). The standard procedure (11), in which phenyllithium is used to generate the anion in ether at −20°, gave a 35% yield of V, m.p. 208–209°, IR (KBr): $\nu_{max.}$ 2245 cm.$^{-1}$ (w), 1675 (s), 1640 (s), 1530 (s), 1375 (m); $\lambda_{max.}^{MeOH}$: 282 mμ (ε, 6100), 297 (ε, 6300), 316 (ε, 6100).

Anal.—Calcd. for $C_{26}H_{21}N_3O_5$: C, 68.56; H, 4.65; N, 9.23. Found: C, 68.57, H, 4.73, N, 9.22.

A procedure for the generation of the anion III employing sodium hydride in *N*,*N*-dimethylformamide at room temperature has recently been reported (12). Applying this modification to the synthesis of aporphines (13, 14), we obtained excellent yields of the desired isoquinoline (VI) without the isolation of V. The crude product (V) was hydrolyzed to furnish a 90% yield of VI (from III), m.p. 129–130°; $\lambda_{max.}^{MeOH}$: 261 mμ (ε, 6800), 272 (ε, 7200), 281 (ε, 5900), 308 (ε, 4400), 322 (ε, 5200).

Anal.—Calcd. for $C_{18}H_{16}N_2O_4$: C, 66.66; H, 4.97; N, 8.64. Found: C, 66.48; H, 4.88; N, 8.56,.

Treatment of the base VI with methyl iodide gave the ammonium salt, m.p. 190–193°, in a quantitative yield (15). The methiodide VII was reduced with potassium borohydride to the tetrahydroisoquinoline VIII, m.p. 97–98.5°, in 80% yield (15). Reduction of the nitro compound VIII with palladium-on-charcoal and hydrogen gave 1-(2-amino-3,4-dimethoxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinoline (IX), m.p. 85–87.5°, in 80% yield; $\lambda_{max.}^{MeOH}$: 212 mμ (ε, 39,000), 265 (ε, 11,000), 272 (ε, 1400), 285 (ε, 1600). This compound was previously reported as a brown oil (5–7). It furnished a dipicrolonate, m.p. 191–192°, which did not depress the melting point of an authentic sample of the dipicrolonate, m.p. 189–190° (6). The amine IX was converted to (±)-apomorphine dimethyl ether [(±)-II] by modifications of procedures of Späth and Hromatka (5), copper powder being used as the coupling agent for the diazonium salt. (±)-II was isolated as an oil in 41%

yield after column chromatography. TLC (silica gel, chloroform) of this product showed only one component, and the R_f was identical with that of a sample of (−)-II prepared in 74% yield from (−)-I hydrochloride and diazomethane. The oily (±)-II was converted into a crystalline picrate, m.p. 187–189° dec., and a crystalline perchlorate, m.p. 263° dec.

The two preparations of apomorphine dimethyl ether [(−)-II and (±)-II] showed identical IR (film), UV, and NMR spectra; $\lambda_{max.}^{MeOH}$: 216 mμ (ε, 47,000), 269 (ε, 19,000), 306 s (ε, 2300); NMR ($CDCl_3$): 2.57 (3H, singlet), 2.66–3.37 (7H, multiplet), 3.73 (3H, singlet), 3.91 (3H, singlet), 6.91–7.18 (4H, multiplet), 8.28 (1H, doublet, J = 2 c.p.s.). (±)-Apomorphine dimethyl ether was converted to its hydroiodide salt with 57% hydriodic acid. Recrystallization from acetone–water yielded white needles, m.p. 279° dec.[1]

Anal.—Calcd. for $C_{19}H_{22}INO_2$: C, 53.91; H, 5.24; I, 29.98; N, 3.31. Found: C, 54.08; H, 5.40; I, 30.17; N, 3.13.

The demethylation of this hydroiodide proved an exceptionally facile reaction, considering the sensitivity of apomorphine to oxidizing agents as well as to acylating agents. The hydroiodide was heated with an equimolar mixture of 57% hydriodic acid and acetic anhydride at reflux for 1 hr. When the reaction mixture was diluted with ether, pure (±)-apomorphine hydroiodide was precipitated and isolated as a white crystalline powder, m.p. 282° dec.,[1] in 93% yield. This compound oxidized only slowly when stored in the cold under nitrogen. $\lambda_{max.}^{MeOH}$: 217 mμ (ε, 41,000), 273 (ε, 17,000), 309 (ε, 3300).

Anal.—Calcd. for $C_{17}H_{18}INO_2$: C, 51.66, H, 4.59, I, 32.11; N, 3.54. Found: C, 51.48; H, 4.69; I, 32.00; N, 3.40.

[1] Determined on a duPont differential thermal analyzer under nitrogen.

(1) "The United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 63; "The National Formulary," XIIth ed., Mack Publishing Co., Easton, Pa., 1965, p. 37.

(2) The Harrison Narcotic Law of 1914 as amended by the Narcotics Manufacturing Act of 1960 (controls the importation, manufacture, production, compounding, and dispensing of opiates; classifies apomorphine as a class B narcotic drug).

(3) J. C. Krantz and C. J. Carr, "Pharmacologic Principles of Medical Practice," 7th ed., William & Wilkins, Baltimore, Md., 1969, p. 143.

(4) G. C. Cotzias, P. S. Papavasiliou, C. Fehling, B. Kaufman, and E. Mena, *New Engl. J. Med.*, **282,** 31(1970) and references cited therein.

(5) E. Späth and O. Hromatka, *Chem. Ber.*, **62,** 326(1929).

(6) D. H. Hey and A. L. Palluel, *J. Chem. Soc.*, **1956,** 4123.

(7) S. Sugasawa and R. Tachikawa, *Tetrahedron*, **4,** 205(1958).

(8) W. M. Whaley and T. R. Govindachari, "Organic Reactions," vol. 6, Wiley, New York, N. Y., 1951, p. 98.

(9) K. H. Slotta and F. Laurersen, *J. Prakt. Chem.*, **139,** 220 (1934).

(10) W. E. McEwen and R. L. Cobb, *Chem. Rev.*, **55,** 511(1955).

(11) J. Weinstock and V. Boekelheide, "Organic Synthesis," coll. vol. 4, p. 641.

(12) F. D. Popp and J. M. Wefer, *Chem. Commun.*, **1966,** 207.

(13) J. L. Neumeyer, K. H. Oh, K. K. Weinhardt, and B. R. Neustadt, *J. Org. Chem.*, **34,** 3786(1969).

(14) J. L. Neumeyer, B. B. Neustadt, and J. W. Weintraub, *Tetrahedron Lett.*, **32,** 3107(1967).

(15) J. L. Neumeyer, M. McCarthy, K. K. Weinhardt, and P. L. Levins, *J. Org. Chem.*, **33,** 2890(1968).

JOHN L. NEUMEYER*
BERNARD R. NEUSTADT
KLAUS K. WEINHARDT
Arthur D. Little, Inc.
Acorn Park
Cambridge, MA 02140

Received June 1, 1970.

Accepted for publication August 7, 1970.

The authors thank Dr. P. L. Levins and Dr. J. T. Funkhouser for the spectral data and the differential thermal analyses, and Professor Hey, Kings College, London, for supplying a sample of the dipicrolonate of IX for comparison with our compound.

* To whom inquiries should be addressed. Present address: Department of Medicinal Chemistry, School of Pharmacy, Northeastern University, Boston, MA 02115

BOOKS

REVIEWS

International Encyclopedia of Pharmacology and Therapeutics. Section 13, Volume 1-Anticholinesterase Agents. Edited by C. RADOUCO-THOMAS, Pergamon Press Ltd., Oxford, England, 1970. ix + 508 pp. 15.5 × 23.5 cm. Price $24.00.

The number of compounds that inhibit the class of enzymes known as cholinesterases has increased markedly in recent years. These compounds, which have had only limited applications in medical therapy and have few equals as investigational tools in pharmacology, are now important industrially as insecticides and militarily as the highly toxic "nerve gases," *e.g.*, sarin, soman, and tabun. Concurrently, large numbers of papers have been published dealing with chemical and biological aspects of these inhibitors and of the enzymes with which they react. Many reviews have subsequently appeared, of which the one edited by Koelle in 1963, *Cholinesterases and Anticholinesterase Agents*, may be the most extensive and most widely used.

The present volume of anticholinesterase agents for the most part covers the same literature as that of the review of Koelle and attempts to include most papers published through late 1968. The material is presented in two major subsections, with an introduction, titled "History of the Research With Anticholinesterase Agents," by Professor A. G. Karczmar, the Section Editor. This part considers in brief, historic detail the pharmacology of physostigmine, the concept of neurohumoral transmission, some novel aspects of the cholinergic system, organophosphorous inhibitors, early work on cholinesterases, the therapeutic uses of inhibitors of these enzymes, and some interesting points of disagreement among investigators of these areas.

Subsection I, titled "Reactions of Cholinesterases With Substrates, Inhibitors and Reactivators," compiled by Dr. Earl Usdin, comprises the major portion of the volume. The author states that a somewhat different point of view has guided this review: an intention not only to describe the reactions of cholinesterases with substrates, inhibitors, and reactivators, but to make known the differences and similarities among the reactions of these three classes of compounds. In this effort, reference has been made to the active

yield after column chromatography. TLC (silica gel, chloroform) of this product showed only one component, and the R_f was identical with that of a sample of (−)-II prepared in 74% yield from (−)-I hydrochloride and diazomethane. The oily (±)-II was converted into a crystalline picrate, m.p. 187–189° dec., and a crystalline perchlorate, m.p. 263° dec.

The two preparations of apomorphine dimethyl ether [(−)-II and (±)-II] showed identical IR (film), UV, and NMR spectra; $\lambda_{max.}^{MeOH}$: 216 mμ (ε, 47,000), 269 (ε, 19,000), 306 s (ε, 2300); NMR ($CDCl_3$): 2.57 (3H, singlet), 2.66–3.37 (7H, multiplet), 3.73 (3H, singlet), 3.91 (3H, singlet), 6.91–7.18 (4H, multiplet), 8.28 (1H, doublet, J = 2 c.p.s.). (±)-Apomorphine dimethyl ether was converted to its hydroiodide salt with 57% hydriodic acid. Recrystallization from acetone–water yielded white needles, m.p. 279° dec.[1]

Anal.—Calcd. for $C_{19}H_{22}INO_2$: C, 53.91; H, 5.24; I, 29.98; N, 3.31. Found: C, 54.08; H, 5.40; I, 30.17; N, 3.13.

The demethylation of this hydroiodide proved an exceptionally facile reaction, considering the sensitivity of apomorphine to oxidizing agents as well as to acylating agents. The hydroiodide was heated with an equimolar mixture of 57% hydriodic acid and acetic anhydride at reflux for 1 hr. When the reaction mixture was diluted with ether, pure (±)-apomorphine hydroiodide was precipitated and isolated as a white crystalline powder, m.p. 282° dec.,[1] in 93% yield. This compound oxidized only slowly when stored in the cold under nitrogen. $\lambda_{max.}^{MeOH}$: 217 mμ (ε, 41,000), 273 (ε, 17,000), 309 (ε, 3300).

Anal.—Calcd. for $C_{17}H_{18}INO_2$: C, 51.66, H, 4.59, I, 32.11; N, 3.54. Found: C, 51.48; H, 4.69; I, 32.00; N, 3.40.

[1] Determined on a duPont differential thermal analyzer under nitrogen.

(1) "The United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 63; "The National Formulary," XIIth ed., Mack Publishing Co., Easton, Pa., 1965, p. 37.

(2) The Harrison Narcotic Law of 1914 as amended by the Narcotics Manufacturing Act of 1960 (controls the importation, manufacture, production, compounding, and dispensing of opiates; classifies apomorphine as a class B narcotic drug).

(3) J. C. Krantz and C. J. Carr, "Pharmacologic Principles of Medical Practice," 7th ed., William & Wilkins, Baltimore, Md., 1969, p. 143.

(4) G. C. Cotzias, P. S. Papavasiliou, C. Fehling, B. Kaufman, and E. Mena, *New Engl. J. Med.*, **282,** 31(1970) and references cited therein.

(5) E. Späth and O. Hromatka, *Chem. Ber.*, **62,** 326(1929).

(6) D. H. Hey and A. L. Palluel, *J. Chem. Soc.*, **1956,** 4123.

(7) S. Sugasawa and R. Tachikawa, *Tetrahedron*, **4,** 205(1958).

(8) W. M. Whaley and T. R. Govindachari, "Organic Reactions," vol. 6, Wiley, New York, N. Y., 1951, p. 98.

(9) K. H. Slotta and F. Laurersen, *J. Prakt. Chem.*, **139,** 220 (1934).

(10) W. E. McEwen and R. L. Cobb, *Chem. Rev.*, **55,** 511(1955).

(11) J. Weinstock and V. Boekelheide, "Organic Synthesis," coll. vol. 4, p. 641.

(12) F. D. Popp and J. M. Wefer, *Chem. Commun.*, **1966,** 207.

(13) J. L. Neumeyer, K. H. Oh, K. K. Weinhardt, and B. R. Neustadt, *J. Org. Chem.*, **34,** 3786(1969).

(14) J. L. Neumeyer, B. B. Neustadt, and J. W. Weintraub, *Tetrahedron Lett.*, **32,** 3107(1967).

(15) J. L. Neumeyer, M. McCarthy, K. K. Weinhardt, and P. L. Levins, *J. Org. Chem.*, **33,** 2890(1968).

JOHN L. NEUMEYER*
BERNARD R. NEUSTADT
KLAUS K. WEINHARDT
Arthur D. Little, Inc.
Acorn Park
Cambridge, MA 02140

Received June 1, 1970.

Accepted for publication August 7, 1970.

The authors thank Dr. P. L. Levins and Dr. J. T. Funkhouser for the spectral data and the differential thermal analyses, and Professor Hey, Kings College, London, for supplying a sample of the dipicrolonate of IX for comparison with our compound.

* To whom inquiries should be addressed. Present address: Department of Medicinal Chemistry, School of Pharmacy, Northeastern University, Boston, MA 02115

BOOKS

REVIEWS

International Encyclopedia of Pharmacology and Therapeutics. Section 13, Volume 1-Anticholinesterase Agents. Edited by C. RADOUCO-THOMAS, Pergamon Press Ltd., Oxford, England, 1970. ix + 508 pp. 15.5 × 23.5 cm. Price $24.00.

The number of compounds that inhibit the class of enzymes known as cholinesterases has increased markedly in recent years. These compounds, which have had only limited applications in medical therapy and have few equals as investigational tools in pharmacology, are now important industrially as insecticides and militarily as the highly toxic "nerve gases," *e.g.*, sarin, soman, and tabun. Concurrently, large numbers of papers have been published dealing with chemical and biological aspects of these inhibitors and of the enzymes with which they react. Many reviews have subsequently appeared, of which the one edited by Koelle in 1963, *Cholinesterases and Anticholinesterase Agents*, may be the most extensive and most widely used.

The present volume of anticholinesterase agents for the most part covers the same literature as that of the review of Koelle and attempts to include most papers published through late 1968. The material is presented in two major subsections, with an introduction, titled "History of the Research With Anticholinesterase Agents," by Professor A. G. Karczmar, the Section Editor. This part considers in brief, historic detail the pharmacology of physostigmine, the concept of neurohumoral transmission, some novel aspects of the cholinergic system, organophosphorous inhibitors, early work on cholinesterases, the therapeutic uses of inhibitors of these enzymes, and some interesting points of disagreement among investigators of these areas.

Subsection I, titled "Reactions of Cholinesterases With Substrates, Inhibitors and Reactivators," compiled by Dr. Earl Usdin, comprises the major portion of the volume. The author states that a somewhat different point of view has guided this review: an intention not only to describe the reactions of cholinesterases with substrates, inhibitors, and reactivators, but to make known the differences and similarities among the reactions of these three classes of compounds. In this effort, reference has been made to the active

sites of the enzymes, to the mechanisms and kinetics of the reactions, and to structure–activity relationships; stereoisomeric effects have been emphasized. Separate sections are included that are concerned with studies of the active sites of cholinesterases and on the "aging" of the organophosphate–enzyme product, since the latter phenomenon has particular significance in the therapy of poisoning by organophosphate inhibitors.

Subsection II, titled "Toxicity of Anticholinesterases and Treatment of Poisoning," by Dr. J. H. Wills, has two goals: (*a*) to complement and update reviews concerned with the pharmacology and toxicology of anti-ChE compounds, and (*b*) to describe and place on as firm a foundation as possible the treatment of poisoning by ChE inhibitors. Dr. Wills has assembled a large amount of basic data and information about the toxicology of a variety of inhibitors of ChE; and, more importantly, he has laid a foundation for therapy by a highly competent and thorough pharmacological analysis of the relevant material. The medical literature should still be consulted for clinical applications, to which the discussion of Dr. Wills is a prerequisite.

This review will be helpful to the student, investigator, and teacher interested in the various phases of ChE enzymes and their inhibitors, especially those for whom the historiographic approach is important. The subsection by Dr. Usdin, in addition to almost 300 pages of data and discussion, includes over 50 pages of references, a testimony to literature searches aided by computers. The computer, however, cannot be faulted for the fact that in an important table listing inhibitor constants the references are identified by number, whereas they are listed alphabetically. More rigorous editing here and throughout the text would have added to the readability and importance of this very useful review.

Reviewed by A. A. Kondritzer
Medical Research Laboratory
Edgewood Arsenal, MD 21010 ■

The Chemistry and Biochemistry of Steroids. Edited by N. KHARASCH, Intra-Science Research Foundation, Santa Monica, Calif., 1969. iv + 166 pp. 21.5 × 28.5 cm. Price $12.00.

This book represents a compilation of a series of lectures presented by Drs. Leland J. Chinn, John S. Baran, Paul D. Klimstra, and Raphael Pappo at the University of Southern California during the fall of 1968. The authors are all research chemists with G. D. Searle and Company and considerable discussion is given to research conducted in the Searle laboratories over the past two decades. Taken in this light, the book represents informative reading for students in medicinal chemistry interested in an industrial career.

The book is not, however, a complete text describing "The Chemistry and Biochemistry of Steroids" as represented by the publishers. Only selected areas of steroid chemistry are emphasized, and often times repeated (*e.g.*, synthesis of 19-norsteroids), and the biochemical aspects are even more limited in scope. For example, the initial chapter by Dr. Chinn represents an excellent discussion of mineralocorticoids, but little mention is made of the equally important glucocorticoids. Moreover, the chapter on conformational analysis is lucidly presented, but the section on structure–receptor interaction fails to evaluate critically the various hypotheses associated with this nebulous subject.

The chapters on biosyntheses of cholesterol and hypocholesterolemic agents by Dr. Baran are an up-to-date summary of the research in these fields. To confuse the matter, however, these chapters are followed by another given the same title as the text itself, but which deals largely with the metabolism of cholesterol. Similarly, a chapter entitled "Biological and Clinical Aspects of Estrogenic Hormones" is four pages in length and describes mainly Dr. Baran's own research with 11β-methyl-19-norsteroids.

The chapters by Dr. Klimstra on oral contraceptives and androgenic and anabolic steroids are well-documented reviews. As pointed out by the author, however, similar reviews recently have been published in other texts.

The final two chapters are written by Dr. Raphael Pappo and pertain to a discussion of the chemistry and biology of 2-oxasteroids and total and partial synthesis of steroids. The author has contributed significantly to the former and describes in detail the chemical development of the 2-oxa isostere of methyltestosterone which is marketed as an anabolic agent under the name of Anavar. A considerable portion of the chemistry presented in this chapter describes the author's own work and has not been published elsewhere.

Thus, this book represents an interesting and easy-to-read series of lectures representing selected areas of steroid chemistry and biology. It is not, however, a text on "The Chemistry and Biochemistry of Steroids" as the title implies.

Reviewed by Raymond E. Counsell
Laboratory of Medicinal Chemistry
College of Pharmacy
University of Michigan
Ann Arbor, MI 48104 ■

International Encyclopedia of Pharmacology and Therapeutics. Pharmacology of the Endocrine System and Related Drugs: The Neurohypophysis. Executive Editor, G. PETERS; Section Editors, H. HELLER and B. T. PICKERING. Pergamon Press Ltd., Oxford, England, 1970. x + 486 pp. 15 × 23.5 cm. Price $16.00.

This volume I of section 41 of the *International Encyclopedia of Pharmacology and Therapeutics* is composed of 12 chapters by 19 distinguished contributors. The subject matter includes the history of neurohypophysial research, the chemistry of the natural and synthetic neurohypophysial hormones, their distribution and evolution in vertebrates, structural–activity relationships of the natural and synthetic hormones, and the latest theories on their storage, release, biosynthesis, and analysis in body fluids. There is also an expanded chapter on the physiological and pharmacological effects of posterior pituitary peptides and their derivatives on the kidney, uterus, and mammary gland. In addition, there are chapters on the effects of posterior pituitary hormones and their derivatives in lower vertebrates, their cellular mode of action and fate, as well as a concluding discourse on the clinical pharmacology of oxytocin and vasopressin.

Through this text which deservedly carries the term encyclopedia are extensive references to books, reviews, monographs, and original papers which easily bring the researcher up to date on the most recent data available concerning every conceivable aspect of the neurohypophysis. Appended is a complete author and subject index.

The reader is urged to spend some time studying a series of electron-micrographs which very clearly demonstrate the ultrastructural changes and the effects on hormone release of ether anesthesia, and ether-and-hemorrhage on rat and rabbit neural lobes.

One major and obvious oversight is the unintentional absence of pages 264–265, 268–269, 272–273, 276, and 277. It is hoped that the publisher can, at this time, still rectify this before the book begins to circulate widely. It is too fine a reference source to be known for its missing pages and accompanying facts. This work should be included in all medical research libraries and is recommended to all investigators interested in the most up-to-date information concerning the pharmacology and endocrinology of the posterior pituitary.

Reviewed by Ronald F. Gautieri
Department of Pharmacology
School of Pharmacy
Temple University
Philadelphia, PA 19140 ■

sites of the enzymes, to the mechanisms and kinetics of the reactions, and to structure–activity relationships; stereoisomeric effects have been emphasized. Separate sections are included that are concerned with studies of the active sites of cholinesterases and on the "aging" of the organophosphate–enzyme product, since the latter phenomenon has particular significance in the therapy of poisoning by organophosphate inhibitors.

Subsection II, titled "Toxicity of Anticholinesterases and Treatment of Poisoning," by Dr. J. H. Wills, has two goals: (*a*) to complement and update reviews concerned with the pharmacology and toxicology of anti-ChE compounds, and (*b*) to describe and place on as firm a foundation as possible the treatment of poisoning by ChE inhibitors. Dr. Wills has assembled a large amount of basic data and information about the toxicology of a variety of inhibitors of ChE; and, more importantly, he has laid a foundation for therapy by a highly competent and thorough pharmacological analysis of the relevant material. The medical literature should still be consulted for clinical applications, to which the discussion of Dr. Wills is a prerequisite.

This review will be helpful to the student, investigator, and teacher interested in the various phases of ChE enzymes and their inhibitors, especially those for whom the historiographic approach is important. The subsection by Dr. Usdin, in addition to almost 300 pages of data and discussion, includes over 50 pages of references, a testimony to literature searches aided by computers. The computer, however, cannot be faulted for the fact that in an important table listing inhibitor constants the references are identified by number, whereas they are listed alphabetically. More rigorous editing here and throughout the text would have added to the readability and importance of this very useful review.

Reviewed by A. A. Kondritzer
Medical Research Laboratory
Edgewood Arsenal, MD 21010 ■

The Chemistry and Biochemistry of Steroids. Edited by N. KHARASCH, Intra-Science Research Foundation, Santa Monica, Calif., 1969. iv + 166 pp. 21.5 × 28.5 cm. Price $12.00.

This book represents a compilation of a series of lectures presented by Drs. Leland J. Chinn, John S. Baran, Paul D. Klimstra, and Raphael Pappo at the University of Southern California during the fall of 1968. The authors are all research chemists with G. D. Searle and Company and considerable discussion is given to research conducted in the Searle laboratories over the past two decades. Taken in this light, the book represents informative reading for students in medicinal chemistry interested in an industrial career.

The book is not, however, a complete text describing "The Chemistry and Biochemistry of Steroids" as represented by the publishers. Only selected areas of steroid chemistry are emphasized, and often times repeated (*e.g.*, synthesis of 19-norsteroids), and the biochemical aspects are even more limited in scope. For example, the initial chapter by Dr. Chinn represents an excellent discussion of mineralocorticoids, but little mention is made of the equally important glucocorticoids. Moreover, the chapter on conformational analysis is lucidly presented, but the section on structure–receptor interaction fails to evaluate critically the various hypotheses associated with this nebulous subject.

The chapters on biosyntheses of cholesterol and hypocholesterolemic agents by Dr. Baran are an up-to-date summary of the research in these fields. To confuse the matter, however, these chapters are followed by another given the same title as the text itself, but which deals largely with the metabolism of cholesterol. Similarly, a chapter entitled "Biological and Clinical Aspects of Estrogenic Hormones" is four pages in length and describes mainly Dr. Baran's own research with 11β-methyl-19-norsteroids.

The chapters by Dr. Klimstra on oral contraceptives and androgenic and anabolic steroids are well-documented reviews. As pointed out by the author, however, similar reviews recently have been published in other texts.

The final two chapters are written by Dr. Raphael Pappo and pertain to a discussion of the chemistry and biology of 2-oxasteroids and total and partial synthesis of steroids. The author has contributed significantly to the former and describes in detail the chemical development of the 2-oxa isostere of methyltestosterone which is marketed as an anabolic agent under the name of Anavar. A considerable portion of the chemistry presented in this chapter describes the author's own work and has not been published elsewhere.

Thus, this book represents an interesting and easy-to-read series of lectures representing selected areas of steroid chemistry and biology. It is not, however, a text on "The Chemistry and Biochemistry of Steroids" as the title implies.

Reviewed by Raymond E. Counsell
Laboratory of Medicinal Chemistry
College of Pharmacy
University of Michigan
Ann Arbor, MI 48104 ■

International Encyclopedia of Pharmacology and Therapeutics. Pharmacology of the Endocrine System and Related Drugs: The Neurohypophysis. Executive Editor, G. PETERS; Section Editors, H. HELLER and B. T. PICKERING. Pergamon Press Ltd., Oxford, England, 1970. x + 486 pp. 15 × 23.5 cm. Price $16.00.

This volume I of section 41 of the *International Encyclopedia of Pharmacology and Therapeutics* is composed of 12 chapters by 19 distinguished contributors. The subject matter includes the history of neurohypophysial research, the chemistry of the natural and synthetic neurohypophysial hormones, their distribution and evolution in vertebrates, structural–activity relationships of the natural and synthetic hormones, and the latest theories on their storage, release, biosynthesis, and analysis in body fluids. There is also an expanded chapter on the physiological and pharmacological effects of posterior pituitary peptides and their derivatives on the kidney, uterus, and mammary gland. In addition, there are chapters on the effects of posterior pituitary hormones and their derivatives in lower vertebrates, their cellular mode of action and fate, as well as a concluding discourse on the clinical pharmacology of oxytocin and vasopressin.

Through this text which deservedly carries the term encyclopedia are extensive references to books, reviews, monographs, and original papers which easily bring the researcher up to date on the most recent data available concerning every conceivable aspect of the neurohypophysis. Appended is a complete author and subject index.

The reader is urged to spend some time studying a series of electron-micrographs which very clearly demonstrate the ultrastructural changes and the effects on hormone release of ether anesthesia, and ether-and-hemorrhage on rat and rabbit neural lobes.

One major and obvious oversight is the unintentional absence of pages 264–265, 268–269, 272–273, 276, and 277. It is hoped that the publisher can, at this time, still rectify this before the book begins to circulate widely. It is too fine a reference source to be known for its missing pages and accompanying facts. This work should be included in all medical research libraries and is recommended to all investigators interested in the most up-to-date information concerning the pharmacology and endocrinology of the posterior pituitary.

Reviewed by Ronald F. Gautieri
Department of Pharmacology
School of Pharmacy
Temple University
Philadelphia, PA 19140 ■

sites of the enzymes, to the mechanisms and kinetics of the reactions, and to structure–activity relationships; stereoisomeric effects have been emphasized. Separate sections are included that are concerned with studies of the active sites of cholinesterases and on the "aging" of the organophosphate–enzyme product, since the latter phenomenon has particular significance in the therapy of poisoning by organophosphate inhibitors.

Subsection II, titled "Toxicity of Anticholinesterases and Treatment of Poisoning," by Dr. J. H. Wills, has two goals: (*a*) to complement and update reviews concerned with the pharmacology and toxicology of anti-ChE compounds, and (*b*) to describe and place on as firm a foundation as possible the treatment of poisoning by ChE inhibitors. Dr. Wills has assembled a large amount of basic data and information about the toxicology of a variety of inhibitors of ChE; and, more importantly, he has laid a foundation for therapy by a highly competent and thorough pharmacological analysis of the relevant material. The medical literature should still be consulted for clinical applications, to which the discussion of Dr. Wills is a prerequisite.

This review will be helpful to the student, investigator, and teacher interested in the various phases of ChE enzymes and their inhibitors, especially those for whom the historiographic approach is important. The subsection by Dr. Usdin, in addition to almost 300 pages of data and discussion, includes over 50 pages of references, a testimony to literature searches aided by computers. The computer, however, cannot be faulted for the fact that in an important table listing inhibitor constants the references are identified by number, whereas they are listed alphabetically. More rigorous editing here and throughout the text would have added to the readability and importance of this very useful review.

Reviewed by A. A. Kondritzer
Medical Research Laboratory
Edgewood Arsenal, MD 21010 ■

The Chemistry and Biochemistry of Steroids. Edited by N. KHARASCH, Intra-Science Research Foundation, Santa Monica, Calif., 1969. iv + 166 pp. 21.5 × 28.5 cm. Price $12.00.

This book represents a compilation of a series of lectures presented by Drs. Leland J. Chinn, John S. Baran, Paul D. Klimstra, and Raphael Pappo at the University of Southern California during the fall of 1968. The authors are all research chemists with G. D. Searle and Company and considerable discussion is given to research conducted in the Searle laboratories over the past two decades. Taken in this light, the book represents informative reading for students in medicinal chemistry interested in an industrial career.

The book is not, however, a complete text describing "The Chemistry and Biochemistry of Steroids" as represented by the publishers. Only selected areas of steroid chemistry are emphasized, and often times repeated (*e.g.*, synthesis of 19-norsteroids), and the biochemical aspects are even more limited in scope. For example, the initial chapter by Dr. Chinn represents an excellent discussion of mineralocorticoids, but little mention is made of the equally important glucocorticoids. Moreover, the chapter on conformational analysis is lucidly presented, but the section on structure–receptor interaction fails to evaluate critically the various hypotheses associated with this nebulous subject.

The chapters on biosyntheses of cholesterol and hypocholesterolemic agents by Dr. Baran are an up-to-date summary of the research in these fields. To confuse the matter, however, these chapters are followed by another given the same title as the text itself, but which deals largely with the metabolism of cholesterol. Similarly, a chapter entitled "Biological and Clinical Aspects of Estrogenic Hormones" is four pages in length and describes mainly Dr. Baran's own research with 11β-methyl-19-norsteroids.

The chapters by Dr. Klimstra on oral contraceptives and androgenic and anabolic steroids are well-documented reviews. As pointed out by the author, however, similar reviews recently have been published in other texts.

The final two chapters are written by Dr. Raphael Pappo and pertain to a discussion of the chemistry and biology of 2-oxasteroids and total and partial synthesis of steroids. The author has contributed significantly to the former and describes in detail the chemical development of the 2-oxa isostere of methyltestosterone which is marketed as an anabolic agent under the name of Anavar. A considerable portion of the chemistry presented in this chapter describes the author's own work and has not been published elsewhere.

Thus, this book represents an interesting and easy-to-read series of lectures representing selected areas of steroid chemistry and biology. It is not, however, a text on "The Chemistry and Biochemistry of Steroids" as the title implies.

Reviewed by Raymond E. Counsell
Laboratory of Medicinal Chemistry
College of Pharmacy
University of Michigan
Ann Arbor, MI 48104 ■

International Encyclopedia of Pharmacology and Therapeutics. Pharmacology of the Endocrine System and Related Drugs: The Neurohypophysis. Executive Editor, G. PETERS; Section Editors, H. HELLER and B. T. PICKERING. Pergamon Press Ltd., Oxford, England, 1970. x + 486 pp. 15 × 23.5 cm. Price $16.00.

This volume I of section 41 of the *International Encyclopedia of Pharmacology and Therapeutics* is composed of 12 chapters by 19 distinguished contributors. The subject matter includes the history of neurohypophysial research, the chemistry of the natural and synthetic neurohypophysial hormones, their distribution and evolution in vertebrates, structural–activity relationships of the natural and synthetic hormones, and the latest theories on their storage, release, biosynthesis, and analysis in body fluids. There is also an expanded chapter on the physiological and pharmacological effects of posterior pituitary peptides and their derivatives on the kidney, uterus, and mammary gland. In addition, there are chapters on the effects of posterior pituitary hormones and their derivatives in lower vertebrates, their cellular mode of action and fate, as well as a concluding discourse on the clinical pharmacology of oxytocin and vasopressin.

Through this text which deservedly carries the term encyclopedia are extensive references to books, reviews, monographs, and original papers which easily bring the researcher up to date on the most recent data available concerning every conceivable aspect of the neurohypophysis. Appended is a complete author and subject index.

The reader is urged to spend some time studying a series of electron-micrographs which very clearly demonstrate the ultrastructural changes and the effects on hormone release of ether anesthesia, and ether-and-hemorrhage on rat and rabbit neural lobes.

One major and obvious oversight is the unintentional absence of pages 264–265, 268–269, 272–273, 276, and 277. It is hoped that the publisher can, at this time, still rectify this before the book begins to circulate widely. It is too fine a reference source to be known for its missing pages and accompanying facts. This work should be included in all medical research libraries and is recommended to all investigators interested in the most up-to-date information concerning the pharmacology and endocrinology of the posterior pituitary.

Reviewed by Ronald F. Gautieri
Department of Pharmacology
School of Pharmacy
Temple University
Philadelphia, PA 19140 ■

Cytotoxic Drugs in the Treatment of Cancer. Edited by EVELYN BOESEN and WALTER DAVIS. Williams & Wilkins Co., Baltimore, MD 21202, 1969. ix + 208 pp. 15 × 23 cm. Price $12.75.

This book is aimed primarily at the physician using cytotoxic agents to control cancer; it is intended to provide a basic understanding of the biochemistry of these agents. Alkylating agents, antimetabolites, plant products such as vincristine and vinblastine, and antibiotics are first discussed historically, and nucleic acid synthesis is reviewed. A chapter about structure–activity relations in the nitrogen mustard and antimetabolite classes of compounds describes briefly the rationale behind these drugs. This chapter is followed by a review of methods of testing for anticancer activity, and an excellent discussion of the advantages and disadvantages of the methods in use today.

The remainder of the book is devoted to consideration of the clinical application of the various classes of cytotoxic agents and of individual compounds in each class. The alkylating agents (nitrogen mustards, dimethane-sulfonates, polyethyleneimines, and diepoxides) are extensively discussed, especially from a clinical standpoint. Throughout this discussion and others, rational drug therapy and the uses and limitations of cytotoxic agents are stressed. A brief chapter at the end of the book covers the use of drugs in conjunction with other methods of treatment and special techniques of administration.

This reviewer feels that the book has accomplished its stated objective of discussing the use and limitations of cytotoxic drug therapy, and that the book will be of value to the physician concerned with the clinical use of such agents. Although some areas of current research interest are omitted, notably the work being done on camptothecin, the book would be a valuable addition to a medically oriented library.

Reviewed by Gerald Carlson
Department of Medicinal Chemistry
University of North Carolina
Chapel Hill, NC 27514 ■

Neuropsychopharmacology and the Affective Disorders. By JOSEPH J. SCHILDKRAUT. Little, Brown and Co., Boston, MA 02106, 1970. xiv + 111 pp. 14.5 × 21.5 cm. Price $10.50.

This book, the contents of which were originally published in the *New England Journal of Medicine*, briefly reviews the biochemical pharmacology of psychoactive drugs. The emphasis is on the findings of the past decade concerning the changes in biogenic amine metabolism produced by various psychoactive drugs.

Staff Review ■

Nursing Home Standards: A Tragic Dilemma in American Health. By JORDAN BRAVERMAN. American Pharmaceutical Association, Washington, DC 20037, 1970. i + 75 pp. 21.5 × 27.5 cm. Price $2.50.

This study of nursing home standards examines both state and Federal regulations relative to the construction and operation of nursing homes. Also covered are the standards which American nursing homes must meet to qualify for certification and operation under Federal Medicare and state licensure programs as well as the apparent absence of even minimum standards in some areas. Summary tables of Federal and state standards are given.

Staff Review ■

NOTICES

Aktuelle Probleme der Toxikologie und Angrenzender Gebiete. (Festschrift Franz Borbely) Swiss Toxicological Information Center, Zurichbergstrasse 8, CH-8028 Zurich, Switzerland, 1970. xii + 249 pp. 17.5 × 24 cm. Price S.fr. 68. (*English summaries*)

Subsidia Pharmaceutica. Edited and published by Societe Suisse de Pharmacie Centre Scientifique, Zurich, Switzerland, 1969. 17.5 × 25 cm. Page 1447–1660. (*German and French*)

Introduction to the Chemistry of Enzyme Action. By A. WILLIAMS. Daniel Davey & Co., Inc., 964 Asylum Ave., Hartford, CT 06105, 1970. viii + 130 pp. 14 × 22 cm. Price $7.25.

Chemical Engineering Kinetics. 2nd edition. By J. M. SMITH. McGraw-Hill, 330 West 42nd St., New York, NY 10036, 1970. xviii + 612 pp. 15.5 × 23.5 cm. Price $15.50.

Clinical Endocrinology. By R. HALL, J. ANDERSON, and G. A. SMART. Lippincott, East Washington Square, Philadelphia, PA 19105, 1969. vii + 418 pp. 19 × 25.5 cm. Price $15.00.

Primer of Immunoelectrophoresis. By PIERRE C. ARQUEMBOURG, JOHN E. SALVAGGIO, and JOHN N. BICKERS. Ann Arbor-Humphrey Science Publishers, Ann Arbor, MI 48106, 1970. viii + 83 pp. 19 × 27 cm. Price $8.25.

Cytologie Moleculaire Des Membranes Et Compartiments De La Cellule Animale. By A. POLICARD. Masson et Cie, Editeurs, 120 Boulevard Saint-Germain, Paris, France, 1970. vii + 122 pp. 16 × 24 cm. (*French*)

Cytotoxic Drugs in the Treatment of Cancer. Edited by EVELYN BOESEN and WALTER DAVIS. Williams & Wilkins Co., Baltimore, MD 21202, 1969. ix + 208 pp. 15 × 23 cm. Price $12.75.

This book is aimed primarily at the physician using cytotoxic agents to control cancer; it is intended to provide a basic understanding of the biochemistry of these agents. Alkylating agents, antimetabolites, plant products such as vincristine and vinblastine, and antibiotics are first discussed historically, and nucleic acid synthesis is reviewed. A chapter about structure–activity relations in the nitrogen mustard and antimetabolite classes of compounds describes briefly the rationale behind these drugs. This chapter is followed by a review of methods of testing for anticancer activity, and an excellent discussion of the advantages and disadvantages of the methods in use today.

The remainder of the book is devoted to consideration of the clinical application of the various classes of cytotoxic agents and of individual compounds in each class. The alkylating agents (nitrogen mustards, dimethane-sulfonates, polyethyleneimines, and diepoxides) are extensively discussed, especially from a clinical standpoint. Throughout this discussion and others, rational drug therapy and the uses and limitations of cytotoxic agents are stressed. A brief chapter at the end of the book covers the use of drugs in conjunction with other methods of treatment and special techniques of administration.

This reviewer feels that the book has accomplished its stated objective of discussing the use and limitations of cytotoxic drug therapy, and that the book will be of value to the physician concerned with the clinical use of such agents. Although some areas of current research interest are omitted, notably the work being done on camptothecin, the book would be a valuable addition to a medically oriented library.

Reviewed by Gerald Carlson
Department of Medicinal Chemistry
University of North Carolina
Chapel Hill, NC 27514 ■

Neuropsychopharmacology and the Affective Disorders. By JOSEPH J. SCHILDKRAUT. Little, Brown and Co., Boston, MA 02106, 1970. xiv + 111 pp. 14.5 × 21.5 cm. Price $10.50.

This book, the contents of which were originally published in the *New England Journal of Medicine*, briefly reviews the biochemical pharmacology of psychoactive drugs. The emphasis is on the findings of the past decade concerning the changes in biogenic amine metabolism produced by various psychoactive drugs.

Staff Review ■

Nursing Home Standards: A Tragic Dilemma in American Health. By JORDAN BRAVERMAN. American Pharmaceutical Association, Washington, DC 20037, 1970. i + 75 pp. 21.5 × 27.5 cm. Price $2.50.

This study of nursing home standards examines both state and Federal regulations relative to the construction and operation of nursing homes. Also covered are the standards which American nursing homes must meet to qualify for certification and operation under Federal Medicare and state licensure programs as well as the apparent absence of even minimum standards in some areas. Summary tables of Federal and state standards are given.

Staff Review ■

NOTICES

Aktuelle Probleme der Toxikologie und Angrenzender Gebiete. (Festschrift Franz Borbely) Swiss Toxicological Information Center, Zurichbergstrasse 8, CH-8028 Zurich, Switzerland, 1970. xii + 249 pp. 17.5 × 24 cm. Price S.fr. 68. (*English summaries*)

Subsidia Pharmaceutica. Edited and published by Societe Suisse de Pharmacie Centre Scientifique, Zurich, Switzerland, 1969. 17.5 × 25 cm. Page 1447–1660. (*German and French*)

Introduction to the Chemistry of Enzyme Action. By A. WILLIAMS. Daniel Davey & Co., Inc., 964 Asylum Ave., Hartford, CT 06105, 1970. viii + 130 pp. 14 × 22 cm. Price $7.25.

Chemical Engineering Kinetics. 2nd edition. By J. M. SMITH. McGraw-Hill, 330 West 42nd St., New York, NY 10036, 1970. xviii + 612 pp. 15.5 × 23.5 cm. Price $15.50.

Clinical Endocrinology. By R. HALL, J. ANDERSON, and G. A. SMART. Lippincott, East Washington Square, Philadelphia, PA 19105, 1969. vii + 418 pp. 19 × 25.5 cm. Price $15.00.

Primer of Immunoelectrophoresis. By PIERRE C. ARQUEMBOURG, JOHN E. SALVAGGIO, and JOHN N. BICKERS. Ann Arbor-Humphrey Science Publishers, Ann Arbor, MI 48106, 1970. viii + 83 pp. 19 × 27 cm. Price $8.25.

Cytologie Moleculaire Des Membranes Et Compartiments De La Cellule Animale. By A. POLICARD. Masson et Cie, Editeurs, 120 Boulevard Saint-Germain, Paris, France, 1970. vii + 122 pp. 16 × 24 cm. (*French*)

Cytotoxic Drugs in the Treatment of Cancer. Edited by EVELYN BOESEN and WALTER DAVIS. Williams & Wilkins Co., Baltimore, MD 21202, 1969. ix + 208 pp. 15 × 23 cm. Price $12.75.

This book is aimed primarily at the physician using cytotoxic agents to control cancer; it is intended to provide a basic understanding of the biochemistry of these agents. Alkylating agents, antimetabolites, plant products such as vincristine and vinblastine, and antibiotics are first discussed historically, and nucleic acid synthesis is reviewed. A chapter about structure–activity relations in the nitrogen mustard and antimetabolite classes of compounds describes briefly the rationale behind these drugs. This chapter is followed by a review of methods of testing for anticancer activity, and an excellent discussion of the advantages and disadvantages of the methods in use today.

The remainder of the book is devoted to consideration of the clinical application of the various classes of cytotoxic agents and of individual compounds in each class. The alkylating agents (nitrogen mustards, dimethane-sulfonates, polyethyleneimines, and diepoxides) are extensively discussed, especially from a clinical standpoint. Throughout this discussion and others, rational drug therapy and the uses and limitations of cytotoxic agents are stressed. A brief chapter at the end of the book covers the use of drugs in conjunction with other methods of treatment and special techniques of administration.

This reviewer feels that the book has accomplished its stated objective of discussing the use and limitations of cytotoxic drug therapy, and that the book will be of value to the physician concerned with the clinical use of such agents. Although some areas of current research interest are omitted, notably the work being done on camptothecin, the book would be a valuable addition to a medically oriented library.

Reviewed by Gerald Carlson
Department of Medicinal Chemistry
University of North Carolina
Chapel Hill, NC 27514 ■

Neuropsychopharmacology and the Affective Disorders. By JOSEPH J. SCHILDKRAUT. Little, Brown and Co., Boston, MA 02106, 1970. xiv + 111 pp. 14.5 × 21.5 cm. Price $10.50.

This book, the contents of which were originally published in the *New England Journal of Medicine*, briefly reviews the biochemical pharmacology of psychoactive drugs. The emphasis is on the findings of the past decade concerning the changes in biogenic amine metabolism produced by various psychoactive drugs.

Staff Review ■

Nursing Home Standards: A Tragic Dilemma in American Health. By JORDAN BRAVERMAN. American Pharmaceutical Association, Washington, DC 20037, 1970. i + 75 pp. 21.5 × 27.5 cm. Price $2.50.

This study of nursing home standards examines both state and Federal regulations relative to the construction and operation of nursing homes. Also covered are the standards which American nursing homes must meet to qualify for certification and operation under Federal Medicare and state licensure programs as well as the apparent absence of even minimum standards in some areas. Summary tables of Federal and state standards are given.

Staff Review ■

NOTICES

Aktuelle Probleme der Toxikologie und Angrenzender Gebiete. (Festschrift Franz Borbely) Swiss Toxicological Information Center, Zurichbergstrasse 8, CH-8028 Zurich, Switzerland, 1970. xii + 249 pp. 17.5 × 24 cm. Price S.fr. 68. (*English summaries*)

Subsidia Pharmaceutica. Edited and published by Societe Suisse de Pharmacie Centre Scientifique, Zurich, Switzerland, 1969. 17.5 × 25 cm. Page 1447–1660. (*German and French*)

Introduction to the Chemistry of Enzyme Action. By A. WILLIAMS. Daniel Davey & Co., Inc., 964 Asylum Ave., Hartford, CT 06105, 1970. viii + 130 pp. 14 × 22 cm. Price $7.25.

Chemical Engineering Kinetics. 2nd edition. By J. M. SMITH. McGraw-Hill, 330 West 42nd St., New York, NY 10036, 1970. xviii + 612 pp. 15.5 × 23.5 cm. Price $15.50.

Clinical Endocrinology. By R. HALL, J. ANDERSON, and G. A. SMART. Lippincott, East Washington Square, Philadelphia, PA 19105, 1969. vii + 418 pp. 19 × 25.5 cm. Price $15.00.

Primer of Immunoelectrophoresis. By PIERRE C. ARQUEMBOURG, JOHN E. SALVAGGIO, and JOHN N. BICKERS. Ann Arbor-Humphrey Science Publishers, Ann Arbor, MI 48106, 1970. viii + 83 pp. 19 × 27 cm. Price $8.25.

Cytologie Moleculaire Des Membranes Et Compartiments De La Cellule Animale. By A. POLICARD. Masson et Cie, Editeurs, 120 Boulevard Saint-Germain, Paris, France, 1970. vii + 122 pp. 16 × 24 cm. (*French*)

Cytotoxic Drugs in the Treatment of Cancer. Edited by EVELYN BOESEN and WALTER DAVIS. Williams & Wilkins Co., Baltimore, MD 21202, 1969. ix + 208 pp. 15 × 23 cm. Price $12.75.

This book is aimed primarily at the physician using cytotoxic agents to control cancer; it is intended to provide a basic understanding of the biochemistry of these agents. Alkylating agents, antimetabolites, plant products such as vincristine and vinblastine, and antibiotics are first discussed historically, and nucleic acid synthesis is reviewed. A chapter about structure–activity relations in the nitrogen mustard and antimetabolite classes of compounds describes briefly the rationale behind these drugs. This chapter is followed by a review of methods of testing for anticancer activity, and an excellent discussion of the advantages and disadvantages of the methods in use today.

The remainder of the book is devoted to consideration of the clinical application of the various classes of cytotoxic agents and of individual compounds in each class. The alkylating agents (nitrogen mustards, dimethane-sulfonates, polyethyleneimines, and diepoxides) are extensively discussed, especially from a clinical standpoint. Throughout this discussion and others, rational drug therapy and the uses and limitations of cytotoxic agents are stressed. A brief chapter at the end of the book covers the use of drugs in conjunction with other methods of treatment and special techniques of administration.

This reviewer feels that the book has accomplished its stated objective of discussing the use and limitations of cytotoxic drug therapy, and that the book will be of value to the physician concerned with the clinical use of such agents. Although some areas of current research interest are omitted, notably the work being done on camptothecin, the book would be a valuable addition to a medically oriented library.

Reviewed by Gerald Carlson
Department of Medicinal Chemistry
University of North Carolina
Chapel Hill, NC 27514 ■

Neuropsychopharmacology and the Affective Disorders. By JOSEPH J. SCHILDKRAUT. Little, Brown and Co., Boston, MA 02106, 1970. xiv + 111 pp. 14.5 × 21.5 cm. Price $10.50.

This book, the contents of which were originally published in the *New England Journal of Medicine*, briefly reviews the biochemical pharmacology of psychoactive drugs. The emphasis is on the findings of the past decade concerning the changes in biogenic amine metabolism produced by various psychoactive drugs.

Staff Review ■

Nursing Home Standards: A Tragic Dilemma in American Health. By JORDAN BRAVERMAN. American Pharmaceutical Association, Washington, DC 20037, 1970. i + 75 pp. 21.5 × 27.5 cm. Price $2.50.

This study of nursing home standards examines both state and Federal regulations relative to the construction and operation of nursing homes. Also covered are the standards which American nursing homes must meet to qualify for certification and operation under Federal Medicare and state licensure programs as well as the apparent absence of even minimum standards in some areas. Summary tables of Federal and state standards are given.

Staff Review ■

NOTICES

Aktuelle Probleme der Toxikologie und Angrenzender Gebiete. (Festschrift Franz Borbely) Swiss Toxicological Information Center, Zurichbergstrasse 8, CH-8028 Zurich, Switzerland, 1970. xii + 249 pp. 17.5 × 24 cm. Price S.fr. 68. (*English summaries*)

Subsidia Pharmaceutica. Edited and published by Societe Suisse de Pharmacie Centre Scientifique, Zurich, Switzerland, 1969. 17.5 × 25 cm. Page 1447–1660. (*German and French*)

Introduction to the Chemistry of Enzyme Action. By A. WILLIAMS. Daniel Davey & Co., Inc., 964 Asylum Ave., Hartford, CT 06105, 1970. viii + 130 pp. 14 × 22 cm. Price $7.25.

Chemical Engineering Kinetics. 2nd edition. By J. M. SMITH. McGraw-Hill, 330 West 42nd St., New York, NY 10036, 1970. xviii + 612 pp. 15.5 × 23.5 cm. Price $15.50.

Clinical Endocrinology. By R. HALL, J. ANDERSON, and G. A. SMART. Lippincott, East Washington Square, Philadelphia, PA 19105, 1969. vii + 418 pp. 19 × 25.5 cm. Price $15.00.

Primer of Immunoelectrophoresis. By PIERRE C. ARQUEMBOURG, JOHN E. SALVAGGIO, and JOHN N. BICKERS. Ann Arbor-Humphrey Science Publishers, Ann Arbor, MI 48106, 1970. viii + 83 pp. 19 × 27 cm. Price $8.25.

Cytologie Moleculaire Des Membranes Et Compartiments De La Cellule Animale. By A. POLICARD. Masson et Cie, Editeurs, 120 Boulevard Saint-Germain, Paris, France, 1970. vii + 122 pp. 16 × 24 cm. (*French*)

ERRATA

In the article titled "Essential Oil of *Anemopsis californica* Part II: Minor Constituents" (1), the following correction should be made:

On page 1215, Table II, the value under Polyester Vinyl Plasticizer, 160°, RRT, for Peak No. 2, Methyleugenol, should read 18.4 instead of 26.3.

(1) D. R. Sanvordeker and M. G. Chaubal, *J. Pharm. Sci.*, **58,** 1213(1969).

In the article titled "Two-Phase Method for the Investigation of Interphase Transport II: Experimental Aspects" (1), the following correction should be made:

On page 1332, column 2, the last line should read 10^{-5} cm.^{2}/sec. for dinitrotoluene instead of 10^{-6} cm.^{2}/sec.

(1) S. A. Howard, A. Suzuki, M. A. Farvar, and W. I. Higuchi, *J. Pharm. Sci.*, **58,** 1330(1969).

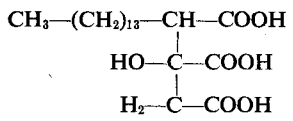
In the article titled "Study of Density Gradients in Certain Oil-in-Water Emulsions Using Multichannel Gamma Ray Analysis" (1), the following correction should be made:

On page 1452, under "Summary," the third line should read o/w emulsions instead of w/o emulsions.

(1) W. H. Parsons and D. L. Dondero, *J. Pharm. Sci.*, **58,** 1449 (1969).

In the article titled "Phytochemical Evaluation of Some Cantharelloid Fungi" (1), the following correction should be made:

On page 1497, Structure I should be



(1) E. D. Henry and G. Sullivan, *J. Pharm. Sci.*, **58,** 1497(1969).

In the article titled "Aminosteroids" (1), the following correction should be made:

The nomenclature for Compound V of Scheme I should be: 3-aza-17β-acetamido-6α,16α-dimethyl-A-homoandrost-4a-en-4-one.

(1) A. P. Shroff, *J. Pharm. Sci.*, **59,** 110(1970).

In the article titled "Estimation of Mean Potency and Content Uniformity of Tablets: A New Approach" (1), the following correction should be made:

On page 214, References 9 and 11, *J. Acoust. Soc. Am.* should read *J. Am. Statist. Assoc.*

(1) J. P. Comer, H. L. Breunig, D. E. Broadlick, and C. B. Sampson, *J. Pharm. Sci.*, **59,** 210(1970).

In the article titled "Constituents from *Gymnema sylvestre* Leaves V: Isolation and Preliminary Characterization of the Gymnemic Acids" (1), the following correction should be made:

On page 627, column 2, under "Comparison of Gymnemic Acids Isolated by Various Investigators," line 23, "their acids A_2 and A_3 being" should read "their acids A_2, A_3, and A_4 being."

(1) J. E. Sinsheimer, G. S. Rao, and H. M. McIlhenny, *J. Pharm. Sci.*, **59,** 622(1970).

In the article titled "Constituents from *Gymnema sylvestre* Leaves VI: Acylated Genins of the Gymnemic Acids—Isolation and Preliminary Characterization" (1), the following correction should be made:

On page 630, column 1, under *Genin G*, line 12, Calcd. for $C_{43}H_{66}O_{11}$ should read Calcd. for $C_{43}H_{66}O_{10}$.

(1) J. E. Sinsheimer and G. S. Rao, *J. Pharm. Sci.*, **59,** 629(1970).

In the article titled "Theoretical Model Studies of Drug Absorption and Transport in the Gastrointestinal Tract I" (1), the following corrections should be made:

On page 645, Eq. 16 should read:

$$(H^+)^4_h + \{K_{a,R} + K_{a,HB} + (TR)_{-h} + (Na^+)_{-h}\}(H^+)^3_h + \{[(TR)_{-h} + K_{a,R} - (TB)_{-h}]K_{a,HB} + (K_{a,R} + K_{a,HB})(Na^+)_{-h} - K_w\}(H^+)^2_h - \{K_{a,R}K_{a,HB}(TB)_{-h} + (K_{a,R} + K_{a,HB})K_w - K_{a,R}K_{a,HB}(Na^+)_{-h}\}(H^+)_{-h} - K_{a,R}K_{a,HB}K_w = 0$$

On page 646, Eq. 17 should read:

$$\gamma(H^+)^4_0 + [\beta + \gamma\eta + \alpha + \gamma(Na^+)_{-0}](H^+)^3_0 + [(\alpha + \beta)\eta - \gamma K_w - \gamma\delta + (\gamma\eta + \beta)(Na^+)_{-0}](H^+)^2_0 - [(\beta + \gamma\eta)K_w + \beta\delta - \beta\eta(Na^+)_{-0}](H^+)_{-0} - \beta\eta K_w = 0$$

and

$$\delta = \frac{K_{a,HB}}{D_{HB}}[D_B(B^-)_{-h} + D_{HB}(HB)_{-h}]$$

On page 647, in Fig. 5, the ordinate should read *F* and *f*.

On page 650, in the caption of Fig. 10, the diffusion efficiency coefficient *T* should be replaced by T^2.

Although the equations were incorrectly stated in the article, the calculations are correct and, therefore, the conclusions drawn are unaltered.

(1) A. Suzuki, W. I. Higuchi, and N. F. H. Ho, *J. Pharm. Sci.*, **59,** 644(1970).

In the article titled "Theoretical Model Studies of Drug Absorption and Transport in the Gastrointestinal Tract II" (1), the following corrections should be made:

On page 653, Eq. 19 should read:

$$K_u = \frac{AD_{eff(1)}}{VL_1}.$$

On page 655, in Fig. 4 the ordinate should read in the following ascending order: 0.85, 0.90, 0.95, 1.00.

(1) A. Suzuki, W. I. Higuchi, and N. F. H. Ho, *J. Pharm. Sci.*, **59,** 651(1970).

In the article titled "Dissolution of Slightly Soluble Powders under Sink Conditions I: Development of an Apparatus and Dissolution Studies of Salicylic Acid Powders" (1), the following corrections should be made:

On page 980, column 1, paragraphs 5 and 6 under "Apparatus," the propeller dimensions of 1.9 cm., 2.6 cm., and 3.0 cm. should read 3.8 cm., 5.2 cm., and 6.0 cm., respectively.

(1) I. Ullah and D. E. Cadwallader, *J. Pharm. Sci.*, **59,** 979(1970).

In the article titled "Steric Aspects of Adrenergic Drugs" (1), the following correction should be made:

Nordefrin should be substituted for levonordefrin throughout this article.

(1) P. N. Patil, J. B. LaPidus, and A. Tye, *J. Pharm. Sci.*, **59,** 1205(1970).

In the article titled "Physical–Chemical Evaluation of 3-(3-Hydroxy-3-methylbutylamino)-5-methyl-*as*, Triazino [5,6-*b*] Indole (SK&F 30097)" (1), the following correction should be made:

On page 1290, Footnote 1 should read: Polyoxyethylene polyoxypropylene glycol; Pluronic F-68, Wyandotte Chemical Co., Wyandotte, MI 48193

(1) L. J. Ravin, E. G. Shami, and E. Rattie, *J. Pharm. Sci.*, **59,** 1290(1970).

In the article titled "Determination of Total Iron in Hematinics by Atomic Absorption Spectrophotometry" (1), the following corrections should be made:

On page 1329, column 1, line 12 should read "where A_u is the absorbance of the sample."

On page 1329, column 2, line 2, "with 75 ml. of 6 *N* HCl" should read "with two 50-ml. portions of 6 *N* HCl."

On page 1329, column 2, under *d* = density of the sample, should be added: *W* = weight of sample, g.

(1) H. I. Tarlin and M. Batchelder, *J. Pharm. Sci.*, **59,** 1328(1970).